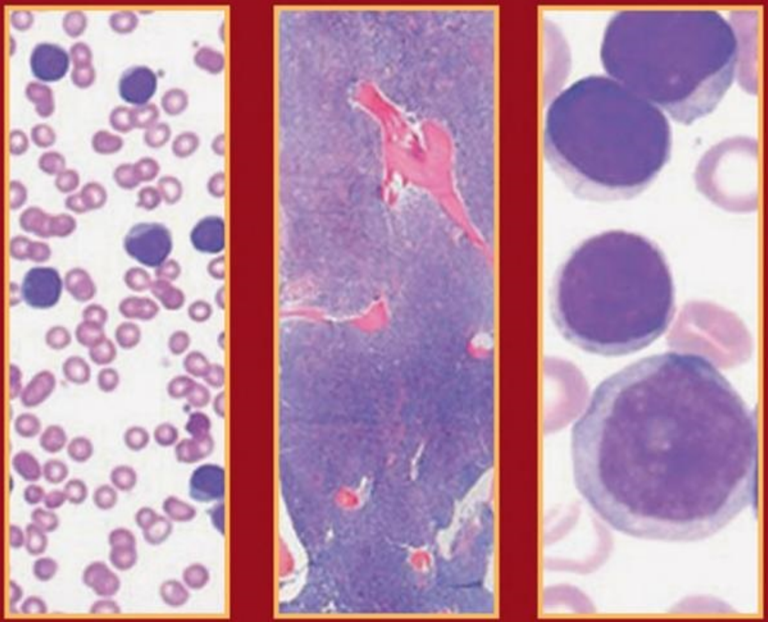
Enhanced
DIGITAL
VERSION
Included

EIGHTH EDITION

HEMATOLOGY

BASIC PRINCIPLES AND PRACTICE



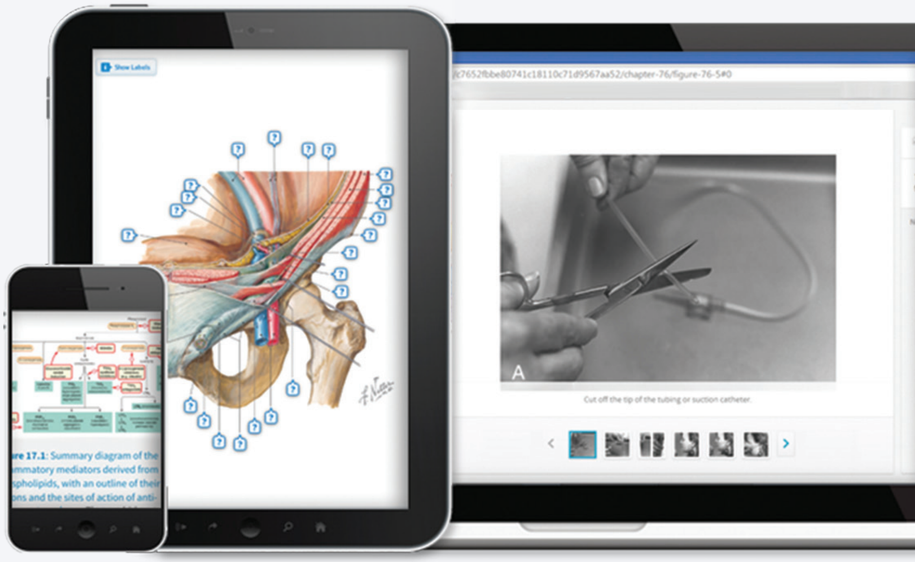
Ronald Hoffman
Edward J. Benz, Jr.
Leslie E. Silberstein
Helen E. Heslop
Jeffrey I. Weitz
Mohamed E. Salama
Syed Ali Abutalib

ELSEVIER

eBooks+
ELSEVIER

Any screen.
Any time.
Anywhere.

Activate the eBook version
of this title at no additional charge.



Elsevier eBooks+ gives you the power to browse, search, and customize your content, make notes and highlights, and have content read aloud.

Unlock your eBook today.

1. Visit **http://ebooks.health.elsevier.com/**
2. Log in or Sign up
3. Scratch box below to reveal your code
4. Type your access code into the “Redeem Access Code” box
5. Click “Redeem”

It’s that easy!

Place Peel Off
Sticker Here

For technical assistance:
email textbookscom.support@elsevier.com
call 1-800-545-2522 (inside the US)
call +44 1 865 844 640 (outside the US)

Use of the current edition of the electronic version of this book (eBook) is subject to the terms of the nontransferable, limited license granted on http://ebooks.health.elsevier.com/. Access to the eBook is limited to the first individual who redeems the PIN, located on the inside cover of this book, at http://ebooks.health.elsevier.com/ and may not be transferred to another party by resale, lending, or other means.

2022v1.0

HEMATOLOGY

BASIC PRINCIPLES AND PRACTICE

EIGHTH EDITION

HEMATOLOGY

BASIC PRINCIPLES AND PRACTICE

Ronald Hoffman, MD
Albert A. and Vera G. List Professor of Medicine
Tisch Cancer Institute
Division of Hematology and Medical Oncology
Department of Medicine
Icahn School of Medicine at Mount Sinai
New York, New York

Edward J. Benz, Jr., MD
President and CEO Emeritus, Dana-Farber Cancer Institute
Director and Principal Investigator Emeritus, Dana-Farber/Harvard Cancer Center
Richard and Susan Smith Distinguished Professor of Medicine
Professor of Pediatrics and Genetics
Harvard Medical School
Boston, Massachusetts

Leslie E. Silberstein, MD
Professor of Pathology (Pediatrics)
Harvard Medical School
Director, Joint Program in Transfusion Medicine
Boston Children's Hospital
Brigham and Women's Hospital
Boston, Massachusetts

Helen E. Heslop, MD, DSc (Hon)
Dan L. Duncan Chair
Professor of Medicine and Pediatrics
Director, Center for Cell and Gene Therapy
Baylor College of Medicine
Houston Methodist Hospital and Texas Children's Hospital
Houston, Texas

Jeffrey I. Weitz, MD, FRCP(C), FACP, FRSC
Professor of Medicine and Biochemistry and Biomedical Sciences
McMaster University
Research Chair in Thrombosis
Heart and Stroke Foundation J. F. Mustard Chair in Cardiovascular Research
Executive Director
Thrombosis and Atherosclerosis Research Institute
Hamilton, Ontario, Canada

Mohamed E. Salama, MD
Chief Medical Officer
Sonic Healthcare USA
Austin, Texas

Syed A. Abutalib, MD
Co-Director, Hematology and Cellular Therapy
Director, Clinical Apheresis Programs of Midwest NMDP and Cancer Treatment Centers of America
Part of City of Hope
Zion, Illinois

ELSEVIER

Elsevier
1600 John F. Kennedy Blvd.
Ste 1800
Philadelphia, PA 19103-2899

HEMATOLOGY, Basic Principles and Practice, EIGHTH EDITION ISBN: 978-0-323-73388-5

Copyright © 2023 by Elsevier Inc. All rights reserved

Chapter 78: "The Pathologic Basis for the Classification of Non-Hodgkin and Hodgkin Lymphomas" is in the Public Domain.

No part of this publication may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, recording, or any information storage and retrieval system, without permission in writing from the publisher. Details on how to seek permission, further information about the Publisher's permissions policies and our arrangements with organizations such as the Copyright Clearance Center and the Copyright Licensing Agency, can be found at our website: www.elsevier.com/permissions.

This book and the individual contributions contained in it are protected under copyright by the Publisher (other than as may be noted herein).

Notices

Knowledge and best practice in this field are constantly changing. As new research and experience broaden our understanding, changes in research methods, professional practices, or medical treatment may become necessary.

Practitioners and researchers must always rely on their own experience and knowledge in evaluating and using any information, methods, compounds, or experiments described herein. In using such information or methods they should be mindful of their own safety and the safety of others, including parties for whom they have a professional responsibility.

With respect to any drug or pharmaceutical products identified, readers are advised to check the most current information provided (i) on procedures featured or (ii) by the manufacturer of each product to be administered, to verify the recommended dose or formula, the method and duration of administration, and contraindications. It is the responsibility of practitioners, relying on their own experience and knowledge of their patients, to make diagnoses, to determine dosages and the best treatment for each individual patient, and to take all appropriate safety precautions.

To the fullest extent of the law, neither the Publisher nor the authors, contributors, or editors, assume any liability for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions, or ideas contained in the material herein.

ISBN: 978-0-323-73388-5

Content Strategist: Nancy Duffy
Content Development Specialist: Anne Snyder
Publishing Services Manager: Deena Burgess
Senior Project Manager: Anne Collett
Book Designer: Ryan Cook
Marketing Manager: Kate Bresnahan

Printed in India

Last digit is the print number: 9 8 7 6 5 4 3 2 1

ELSEVIER Book Aid International Working together to grow libraries in developing countries
www.elsevier.com • www.bookaid.org

CONTENTS

PART I
MOLECULAR AND CELLULAR BASIS OF HEMATOLOGY 1

1. **Anatomy and Physiology of the Gene 1**
Andrew J. Wagner, Nancy Berliner, and Edward J. Benz, Jr.

2. **Epigenomics in Hematology 16**
Myles Brown and Alok Tewari

3. **Genomic Approaches to Hematology 24**
Gareth J. Morgan and Eileen M. Boyle

4. **Regulation of Gene Expression in Hematology 33**
Stephanie Halene, Toma Tebaldi, and Gabriella Viero

5. **Genome Editing 50**
Matthew Porteus

6. **Signaling Transduction and Metabolomics 59**
Pere Puigserver

7. **Protein Architecture: Relationship of Form and Function 71**
Jia-huai Wang and Michael J. Eck

8. **Pharmacogenomics and Hematologic Diseases 79**
Leo Kager and William E. Evans

PART II
CELLULAR BASIS OF HEMATOLOGY 95

9. **Hematopoietic Stem Cell Biology 95**
Marlies P. Rossmann and John P. Chute

10. **Mitochondria and Hematopoiesis 115**
Luena Papa

11. **Cytokines, Chemokines, Other Growth Factors, and Their Receptors 123**
Hal E. Broxmeyer[†] and Maegan L. Capitano

12. **Role of Chemokines in Leukocyte Trafficking 137**
Antal Rot, Elin Hub, Steffen Massberg, Alexander G. Khandoga, and Ulrich H. von Andrian

13. **Stem Cell Model of Hematologic Diseases 149**
Omar Abdel-Wahab

14. **Hematopoietic Microenvironment 157**
David Scadden and Lev Silberstein

15. **Cell Adhesion 165**
Rodger P. McEver, Pilar Alcaide, and Francis W. Luscinskas

16. **Current Biology of Stem Cell Homing and Mobilization: Dynamic Interactions Between Hematopoietic Stem and Progenitor Cells and Their Surrounding Bone Marrow Microenvironment 174**
Orit Kollet, Montaser Haddad, Priyasmita Chakrabarti, Alejandra Ordonez-Moreno, and Tsvee Lapidot

17. **Control of Cell Division 181**
Martin Fischer and James A. DeCaprio

18. **Cell Death 191**
Paolo Strati, Marina Konopleva, and William Wierda

19. **Aging and Hematopoiesis 201**
Daozheng Yang, Arthur Flohr Svendsen, and Gerald de Haan

PART III
IMMUNOLOGIC BASIS OF HEMATOLOGY 207

20. **Dendritic Cell Biology 207**
Cansu Cimen Bozkus and Nina Bhardwaj

21. **Natural Killer Cell Immunity and Therapy 218**
William E. Carson III

22. **B-Cell Development 231**
Kenneth Dorshkind, Dinesh S. Rao, and David J. Rawlings

23. **Complement and Immunoglobulin Biology Leading to Clinical Translation 242**
David J. Araten, David E. Isenman, and Michael C. Carroll

24. **T-Cell Immunity 271**
Shannon A. Carty, Matthew J. Riese[†], and Gary A. Koretzky

25. **Unmodified Ex Vivo Expanded T Cells 289**
Ifigeneia Tzannou, Wingchi Leung, and Premal Lulla

26. **Treatment of Hematologic Malignancies with Genetically Modified T Cells 295**
Eben I. Lichtman, Malcolm K. Brenner, and Gianpietro Dotti

PART IV
DISORDERS OF HEMATOPOIETIC CELL DEVELOPMENT 303

27. **Biology of Erythropoiesis, Erythroid Differentiation, and Maturation 303**
Thalia Papayannopoulou and Anna Rita Migliaccio

28. **Granulocytopoiesis and Monocytopoiesis 322**
Frederick D. Tsai, Arati Khanna-Gupta, and Nancy Berliner

29. **Thrombocytopoiesis 334**
Camelia Iancu-Rubin and Alan B. Cantor

30. **Inherited Bone Marrow Failure Syndromes 350**
Yigal Dror

31. **Aplastic Anemia 396**
Neal S. Young and Jaroslaw P. Maciejewski

32. **Paroxysmal Nocturnal Hemoglobinuria 416**
David J. Araten and Robert A. Brodsky

33. **Acquired Disorders of Red Cell, White Cell, and Platelet Production 431**
Francis R. LeBlanc, Jaroslaw P. Maciejewski, and Thomas P. Loughran, Jr.

PART V
RED BLOOD CELLS 451

34. **Pathobiology of the Human Erythrocyte and Its Hemoglobins 451**
Martin H. Steinberg, Edward J. Benz, Jr., and Benjamin L. Ebert

35. **Approach to Anemia in the Adult and Child 463**
Judith C. Lin and Edward J. Benz, Jr.

36. **Iron Homeostasis and Its Disorders 473**
Tomas Ganz

37. **Disorders of Iron Homeostasis: Iron Deficiency and Overload 483**
Clara Camaschella

38. **Anemia of Chronic Inflammation 498**
Yelena Z. Ginzburg

39. **Heme Biosynthesis and Its Disorders: Porphyrias and Sideroblastic Anemias 507**
Stephen J. Fuller and James S. Wiley

40. **Megaloblastic Anemias 524**
Aśok C. Antony

41. **Thalassemia Syndromes 555**
Sujit Sheth

42. **Pathobiology of Sickle Cell Disease 585**
Robert P. Hebbel and Gregory M. Vercellotti

43. **Clinical Aspects of Sickle Cell Disease 599**
Laurel A. Menapace and Swee Lay Thein

44. **Hemoglobin Variants Associated with Hemolytic Anemia, Altered Oxygen Affinity, and Methemoglobinemias 630**
Edward J. Benz, Jr. and Benjamin L. Ebert

45. **Red Blood Cell Enzymopathies 638**
Xylina T. Gregg and Josef T. Prchal

46. **Red Blood Cell Membrane Disorders 650**
Patrick G. Gallagher

47. **Autoimmune Hemolytic Anemia 672**
Marc Michel and Ulrich Jäger

48. **Extrinsic Nonimmune Hemolytic Anemias 688**
William C. Mentzer and Stanley L. Schrier†

PART VI
NON-MALIGNANT LEUKOCYTES 698

49. **Neutrophilic Leukocytosis, Neutropenia, Monocytosis, and Monocytopenia 698**
Lawrence Rice, Arthur W. Zieske, and Moonjung Jung

50. **Lymphocytosis, Lymphocytopenia, Hypergammaglobulinemia, and Hypogammaglobulinemia 708**
Sravanti P. Teegavarapu and Martha P. Mims

51. **Disorders of Phagocyte Function 717**
Mary C. Dinauer and Thomas D. Coates

52. **Congenital Disorders of Lymphocyte Function 736**
Sung-Yun Pai and Luigi D. Notarangelo

53. **Pediatric and Adult Histiocytic Disorders 750**
Adi Zoref Lorenz, Olive S. Eckstein, Nitya Gulati, Michael B. Jordan, and Carl E. Allen

54. **Lysosomal Storage Diseases, Focusing on Gaucher Disease: Perspectives and Principles 769**
Atul Mehta, Mia Horowitz, Joaquin Carrillo-Farga, and Ari Zimran

55. **Epstein-Barr Virus and Associated Lymphoproliferative Conditions 782**
Nader Kim El-Mallawany, Lisa R. Forbes, Rayne H. Rouce, and Carl E. Allen

PART VII
HEMATOLOGIC MALIGNANCIES 800

56. **Progress in the Classification of Hematopoietic and Lymphoid Neoplasms: Clinical Implications 800**
Mohamed E. Salama and Ronald Hoffman

57. **Conventional and Molecular Cytogenomic Basis of Hematologic Malignancies 813**
Vesna Najfeld

58. **Pharmacology and Molecular Mechanisms of Antineoplastic Agents for Hematologic Malignancies 900**
Stanton L. Gerson, Paolo F. Caimi, Ehsan Malek, and Benjamin Tomlinson

59. **Pathobiology of Acute Myeloid Leukemia 937**
Andrew M. Brunner and Timothy A. Graubert

60. **Clinical Manifestations and Treatment of Acute Myeloid Leukemia 950**
Harry P. Erba

61. **Myelodysplastic Syndromes 977**
Christopher J. Gibson and David P. Steensma

62. **Allogeneic Hematopoietic Stem Cell Transplantation for Acute Myeloid Leukemia and Myelodysplastic Syndrome in Adults 1001**
John Koreth, Joseph H. Antin, and Corey Cutler

63. **Acute Myeloid Leukemia in Children 1013**
C. Michel Zwaan, Olaf Heidenreich, and E. Anders Kolb

64. **Blastic Plasmacytoid Dendritic Cell Neoplasm 1029**
Andrew A. Lane

65. **Myelodysplastic Syndromes and Myeloproliferative Neoplasms in Children 1036**
Elliot Stieglitz, Christopher C. Dvorak, and Benjamin S. Braun

66. **Pathobiology of Acute Lymphoblastic Leukemia 1049**
Melissa A. Burns, Alejandro Gutierrez, and Lewis B. Silverman

67. **Clinical Manifestations and Treatment of Childhood Acute Lymphoblastic Leukemia 1066**
Rayne H. Rouce and Rachel E. Rau

68. **Acute Lymphoblastic Leukemia in Adults 1078**
Shira Dinner, Sandeep Gurbuxani, Alexandra E. Rojek, Nitin Jain, and Wendy Stock

69. **Chronic Myeloid Leukemia 1103**
Michael W. Deininger

70. **The Polycythemias 1129**
Marina Kremyanskaya, Vesna Najfeld, John Mascarenhas, and Ronald Hoffman

71. **Essential Thrombocythemia 1169**
Bridget K. Marcellino, John Mascarenhas, Camelia Iancu-Rubin, Marina Kremyanskaya, Vesna Najfeld, and Ronald Hoffman

72. **Primary Myelofibrosis and Chronic Neutrophilic Leukemia 1193**
Sangeetha Venugopal, Vesna Najfeld, Alla Keyzner, Siraj M. El Jamal, Ronald Hoffman, and John Mascarenhas

73. **Myelodysplastic Syndrome/Myeloproliferative Neoplasm Overlap Syndromes 1225**
Douglas Tremblay, Jonathan Feld, Nicole Kucine, Noa Rippel, Siraj M. El Jamal, and John Mascarenhas

74. **Eosinophilia, Eosinophilic Neoplasms, and the Hypereosinophilic Syndromes 1243**
Peter Valent, Andreas Reiter, and Jason Gotlib

75. **Mast Cells and Mastocytosis 1263**
Jason Gotlib, Hans-Peter Horny, and Peter Valent

76. **Chronic Lymphocytic Leukemia 1282**
Farrukh T. Awan and John C. Byrd

77. **Hairy Cell Leukemia 1301**
Farhad Ravandi

78. **The Pathologic Basis for the Classification of Non-Hodgkin and Hodgkin Lymphomas 1314**
Girish Venkataraman, Elaine S. Jaffe, and Stefania Pittaluga

79. **Origin of Hodgkin Lymphoma and Therapeutic Targets 1331**
Ralf Küppers

80. **Hodgkin Lymphoma 1339**
Anas Younes, Ann S. LaCasce, Graham Collins, Bouthaina Dabaja, and Ahmet Dogan

81. **Origin of Non-Hodgkin Lymphoma and Therapeutic Targets 1352**
Matthew S. McKinney and Sandeep S. Dave

82. **Clinical Manifestations, Staging, and Treatment of Follicular Lymphoma 1367**
Lucy Pickard and John G. Gribben

83. **Marginal Zone Lymphomas (Extranodal/MALT, Splenic, and Nodal) 1378**
Samer Al Hadidi and Carlos A. Ramos

84. **Diffuse Large B-Cell Lymphoma of the Central Nervous System 1390**
Syed A. Abutalib, Nilanjan Ghosh, Alexander Feldman†, Karan S. Dixit, and Rimas V. Lukas

85. **High-Grade B-Cell Lymphomas 1420**
Kieron Dunleavy and Stephen Douglas Smith

86. **Mantle Cell Lymphoma 1430**
Julie M. Vose

87. **Virus-Associated Lymphoma 1439**
Katherine C. Rappazzo, Jennifer A. Kanakry, and Richard F. Ambinder

88. **Malignant Lymphomas in Childhood 1448**
Kara M. Kelly, Birgit Burkhardt, and Catherine M. Bollard

89. **T-Cell Lymphomas 1462**
Alessandro Broccoli and Pier Luigi Zinzani

90. **Monoclonal Gammopathy of Undetermined Significance and Smoldering Multiple Myeloma 1492**
S. Vincent Rajkumar and Shaji Kumar

91. **Multiple Myeloma 1506**
Sydney X. Lu, Even H. Rustad, Saad Z. Usmani, and C. Ola Landgren

92. **Waldenström Macroglobulinemia/Lymphoplasmacytic Lymphoma 1539**
Jorge J. Castillo and Steven P. Treon

93. **Immunoglobulin Light-Chain Amyloidosis (Primary Amyloidosis) 1553**
Morie A. Gertz, Francis K. Buadi, Martha Q. Lacy, and Suzanne R. Hayman

PART VIII

COMPREHENSIVE CARE OF PATIENTS WITH HEMATOLOGIC MALIGNANCIES 1567

94. **Key Considerations for Managing Infections in the Compromised Host 1567**
Samuel A Shelburne, Russell E. Lewis, and Dimitrios P. Kontoyiannis

95. **Principles of Radiation Therapy for Hematologic Disease 1583**
Idalid Franco, Daphne Haas-Kogan, and Andrea K. Ng

96. **Grading and Toxicity Management after Immune Effector Therapy 1594**
Emily C. Ayers, Noelle V. Frey, and Daniel W. Lee

97. **Identification and Management of Checkpoint Inhibition Toxicity 1599**
Evgeniya Kharchenko and John W. Sweetenham

98. **Psychosocial Aspects of Hematologic Disorders 1605**
Hermioni L. Amonoo, Cynthia S. Peng, Rebecca M. Hammond, and Roxanne Sholevar

99. **Pain Management and Antiemetic Therapy in Hematologic Disorders 1616**
Thomas W. LeBlanc

100. **Palliative Care 1631**
Kathleen A. Lee, Hilary McGuire, Barbara Reville, and Janet L. Abrahm

101. **Therapy-related Late Effects of Hematologic Malignancies 1638**
Wendy Landier and Smita Bhatia

PART IX

TRANSPLANTATION AND OTHER CELL-BASED THERAPIES 1653

102. **Practical Aspects of Hematopoietic Stem Cell Harvesting and Mobilization 1653**
Abba C. Zubair and Scott D. Rowley

103. **Graft Engineering and Cell Processing 1667**
Adrian P. Gee

104. **Principles of Cell-Based Genetic Therapies 1679**
David A. Williams

105. **Indications, Outcomes, and Donor Selection for Allogeneic Hematopoietic Cell Transplantation for Hematologic Malignancies in Adults 1689**
Saurabh Chhabra, Mehdi Hamadani, and Parameswaran N. Hari

106. **Unrelated Donor Hematopoietic Cell Transplantation 1703**
Effie Wang Petersdorf and Katharine Hsu

107. **Haploidentical Hematopoietic Stem Cell Transplantation 1713**
Stefan O. Ciurea

108. **Cord Blood Transplantation 1732**
Joseph E. Maakaron, Najla El-Jurdi, and Claudio G. Brunstein

109. **Graft-versus-Host Disease and Graft-versus-Leukemia Responses 1749**
Mary Riwes, James L. Ferrara, Pavan Reddy, and John M. Magenau

110. **Supportive Care for the Transplant Patient 1770**
Abraham S. Kanate and Navneet S. Majhail

PART X

TRANSFUSION MEDICINE 1785

111. **Human Blood Group Antigens and Antibodies 1785**
William J. Lane, Connie M. Westhoff, Jill R. Storry, and Beth H. Shaz

112. **Principles of Red Blood Cell Transfusion 1801**
Robert A. DeSimone, Paul M. Ness, and Melissa M. Cushing

113. **Clinical Considerations in Platelet Transfusion Therapy 1814**
Richard M. Kaufman

114. **Human Leukocyte Antigen and Human Neutrophil Antigen Systems 1820**
Ena Wang, Sharon Adams, David F. Stroncek, and Francesco M. Marincola

115. **Principles of Plasma and Plasma Derivatives 1837**
Alexandra Jimenez, Christopher D. Hillyer, and Beth H. Shaz

116. **Hemapheresis 1852**
Kamille A. West and Harvey G. Klein

117. **Transfusion Reactions to Blood and Hematopoietic Stem Cell Therapy Products 1864**
Martin R. Schipperus and Johanna C. Wiersum-Osselton

118. **Transfusion-Transmitted Diseases 1874**
Lauren A. Crowder and Susan L. Stramer

119. **Pediatric Transfusion Medicine 1892**
Bentley B. Rodrigue and Steven R. Sloan

120. **Transfusion and Apheresis Support for Sickle Cell Disease Patients 1900**
John P. Manis

PART XI

HEMOSTASIS AND THROMBOSIS 1906

121. **Overview of Hemostasis and Thrombosis 1906**
James C. Fredenburgh and Jeffrey I. Weitz

122. **Blood Vessels 1919**
Aly Karsan and Janusz Rak

123. **Megakaryocyte and Platelet Structure 1937**
Kellie R. Machlus and Joseph E. Italiano, Jr.

124. **Molecular Basis of Platelet Function 1950**
Margaret L. Rand and Sara J. Israels

125. **Molecular Basis of Blood Coagulation 1968**
Kathleen Brummel-Ziedins, Kenneth G. Mann, James C. Fredenburgh, and Jeffrey I. Weitz

126. **Evaluation of the Patient with Suspected Bleeding Disorders 1988**
Catherine P. M. Hayward and Alice D. Ma

127. **Laboratory Evaluation of Hemostatic and Thrombotic Disorders 1996**
Menaka Pai and Karen A. Moffat

128. **Acquired Disorders of Platelet Function 2007**
Peter L. Gross and José A. López

129. **Diseases of Platelet Number: Immune Thrombocytopenia, Neonatal Alloimmune Thrombocytopenia, and Posttransfusion Purpura 2020**
Michelle P. Zeller, Shuoyan Ning, Donald M. Arnold, and Caroline Gabe

130. **Thrombocytopenia Caused by Hypersplenism, Platelet Destruction, or Surgery/Hemodilution 2033**
Theodore E. Warkentin

131. **Heparin-Induced Thrombocytopenia 2049**
Theodore E. Warkentin

132. **Thrombotic Thrombocytopenic Purpura and the Hemolytic Uremic Syndromes 2063**
Gemlyn George and Kenneth D. Friedman

133. **Structure, Biology, and Genetics of von Willebrand Factor 2081**
Paula James, Orla Rawley, and Mackenzie Bowman

134. **Hemophilia A and B 2095**
Manuel Carcao, Keith Gomez, Davide Matino, and Glenn F. Pierce

135. **Rare Coagulation Factor Deficiencies 2125**
David Gailani, Benjamin F. Tillman, and Allison P. Wheeler

136. **Transfusion Therapy for Coagulation Factor Deficiencies 2144**
Elizabeth Roman and Catherine S. Manno

137. **Disseminated Intravascular Coagulation 2156**
Marcel Levi

138. **Hypercoagulable States 2167**
Julia A. M. Anderson and Jeffrey I. Weitz

139. **Antiphospholipid Syndrome 2179**
Lucia R. Wolgast and Jacob H. Rand

140. **Venous Thromboembolism 2196**
Noel C. Chan and Jeffrey I. Weitz

141. **Prevention and Treatment of Venous Thromboembolism in Pregnancy 2205**
Leslie Skeith and Shannon M. Bates

142. **Atherothrombosis 2212**
Daisy Sahoo, Moua Yang, and Roy L. Silverstein

143. **Antithrombotic Drugs 2223**
Iqbal H. Jaffer and Jeffrey I. Weitz

144. **Stroke 2241**
Emer Mcgrath, Michelle Canavan, and Martin O'Donnell

145. **Acute Coronary Syndromes 2251**
John W. Eikelboom and Jeffrey I. Weitz

146. **Peripheral Artery Disease 2261**
Stanislav Henkin and Mark A. Creager

147. **Atrial Fibrillation 2270**
Monika Kozieł Siołkowska, Tatjana S. Potpara, and Gregory Y. H. Lip

148. **Bleeding and Clotting Disorders in Pediatrics 2278**
Nasrin Samji, Anthony K. C. Chan, and Mihir D. Bhatt

PART XII
CONSULTATIVE HEMATOLOGY 2292

149. **Hematologic Changes in Pregnancy 2292**
Arielle L. Langer, Michael Paidas, and Caroline Cromwell

150. **Hematologic Manifestations of End-Organ Failure 2305**
Marissa Laureano and Christopher Hillis

151. **Hematologic Manifestations of Solid Tumors 2312**
Kathryn DeCarli, Peter Barth, Andrew M. Brunner, and Fred J. Schiffman

152. **Hematologic Manifestations of HIV/AIDS 2319**
Maryam Own and James B. Bussel

153. **Hematologic Findings and Consequences of Novel Coronavirus (SARS-CoV-2) Infection 2335**
Leonard Naymagon and Douglas Tremblay

154. **Hematologic Aspects of Parasitic Diseases 2342**
David J. Roberts

155. **Hematologic Problems in the Surgical Patient: Bleeding and Thrombosis 2369**
Iqbal H. Jaffer and Jeffrey I. Weitz

156. **The Spleen and Its Disorders 2378**
Thomas A. Ollila, Adam S. Zayac, and Fred J. Schiffman

157. **Aging and Hematologic Disorders 2394**
Kah Poh Loh, Mazie Tsang, Shakira J. Grant, Richard J. Lin, and Heidi D. Klepin

158. **Onco-cardiology: Focus on Cardiac Complications of Hematologic Treatments 2400**
Andrea Gallardo-Grajedau and Gagan Sahni

159. **Resources for the Hematologist: Interpretive Comments and Selected Reference Values for Neonatal, Pediatric, and Adult Populations 2408.e1**
Andrea N. Marcogliese and Lisa Hensch

Chapter 159 can be found online at Elsevier eBooks for Practicing Clinicians

Index 2409

PART I MOLECULAR AND CELLULAR BASIS OF HEMATOLOGY

CHAPTER 1

ANATOMY AND PHYSIOLOGY OF THE GENE

Andrew J. Wagner, Nancy Berliner, and Edward J. Benz, Jr.

Normal blood cells have limited life spans; they must be replenished in precise numbers by a continuously renewing population of progenitor cells. Homeostasis of the blood requires that proliferation of these cells be efficient yet strictly constrained. Many distinctive types of mature blood cells must arise from these progenitors by a controlled process of commitment to, and execution of, complex programs of differentiation. Thus developing red blood cells must produce large quantities of hemoglobin but not the myeloperoxidase characteristic of granulocytes, the immunoglobulins characteristic of lymphocytes, or the fibrinogen receptors characteristic of platelets. Similarly, the maintenance of normal amounts of procoagulant and anticoagulant proteins in the circulation requires an exquisitely regulated production, destruction, and interaction of the components. Understanding the basic biologic principles underlying cell growth, differentiation, death, and the homeostasis of critical proteins requires a thorough knowledge of the structure and regulated expression of genes because the gene is now known to be the fundamental unit by which biologic information is stored, transmitted, and expressed in this regulated fashion.

Genes were originally characterized as mathematic units of inheritance. They are now known to consist of molecules of deoxyribonucleic acid (DNA). By virtue of their ability to store information in the form of nucleotide sequences, to transmit it by means of semiconservative replication to daughter cells during mitosis and meiosis, and to express it by directing the incorporation of amino acids into proteins, DNA molecules are the chemical transducers of genetic information flow. Efforts to understand the biochemical means by which this transduction is accomplished have given rise to the disciplines of molecular biology and molecular genetics.

THE GENETIC VIEW OF THE BIOSPHERE: THE CENTRAL DOGMA OF MOLECULAR BIOLOGY

The fundamental premise of the molecular biologist is that the magnificent diversity encountered in nature is ultimately governed by genes. The capacity of genes to exert this control is in turn determined by relatively simple stereochemical rules, first appreciated by Watson and Crick in the 1950s. These rules govern the types of interactions that can occur between two molecules of DNA or ribonucleic acid (RNA).

DNA and RNA are linear unbranched polymers consisting of four types of nucleotide subunits. Each nucleotide is distinguished from the others by a unique purine or pyrimidine "base" projecting from the chain. Proteins are linear unbranched polymers consisting of 21 types of amino acid subunits. Each amino acid is distinguished from the others by the chemical nature of its side chain, the moiety not involved in forming the peptide bond links of the chain. The properties of cells, tissues, and organisms depend largely on the aggregate structures, properties and biochemical activities of their proteins, and the interactions occurring among them. The central dogma of molecular biology states that genes control these properties by encoding the structures of proteins, controlling the timing and amount of their production, and coordinating their synthesis with that of other proteins. The information needed to achieve these ends is transmitted (expressed) from DNA and translated into proteins by a class of nucleic acid molecules called RNA. Genetic information thus flows in the direction DNA → RNA → protein. This central dogma provides, in principle, a universal approach for investigating the biologic properties and behavior of any given cell, tissue, or organism by study of the controlling genes. Methods permitting direct manipulation of DNA and RNA sequences should then be universally applicable to the study of all living entities. Indeed, the power of the methodologies of molecular genetics lie in the universality of their utility.

One exception to the central dogma of molecular biology that is especially relevant to hematologists is the storage of genetic information in RNA molecules in certain viruses, notably the retroviruses associated with T-cell leukemia and lymphoma, and the human immunodeficiency virus. When retroviruses enter the cell, the RNA genome (the term "genome" refers to the totality of DNA or RNA sequences encoding the genetic information of a cell, tissue, or organism) is copied into a DNA replica (cDNA). This is accomplished with RNA-dependent DNA polymerases, enzymes also called *reverse transcriptases*. This DNA representation of the viral genome is then expressed according to the pathway specified by the central dogma. Retroviruses thus represent a variation on the theme rather than a true exception to or violation of the dogma. There are also some RNA viruses (coronaviruses being the most universally known example) that carry an RNA-dependent RNA polymerase capable of replicating many copies of its own RNA genome. These messenger RNAs (mRNAs) then encode proteins essential to their life cycle.

THE ANATOMY AND PHYSIOLOGY OF THE GENE

DNA and RNA Structure

DNA molecules are extremely long, unbranched polymers of nucleotide subunits. Each nucleotide contains a sugar moiety called deoxyribose, a phosphate group attached to the 5′ carbon position, and a purine or pyrimidine base attached to the 1′ position (Fig. 1.1). The linkages in the chain are formed by phosphodiester bonds between the 5′ position of each sugar residue and the 3′ position of the adjacent residue in the chain (see Fig. 1.1). The sugar-phosphate links

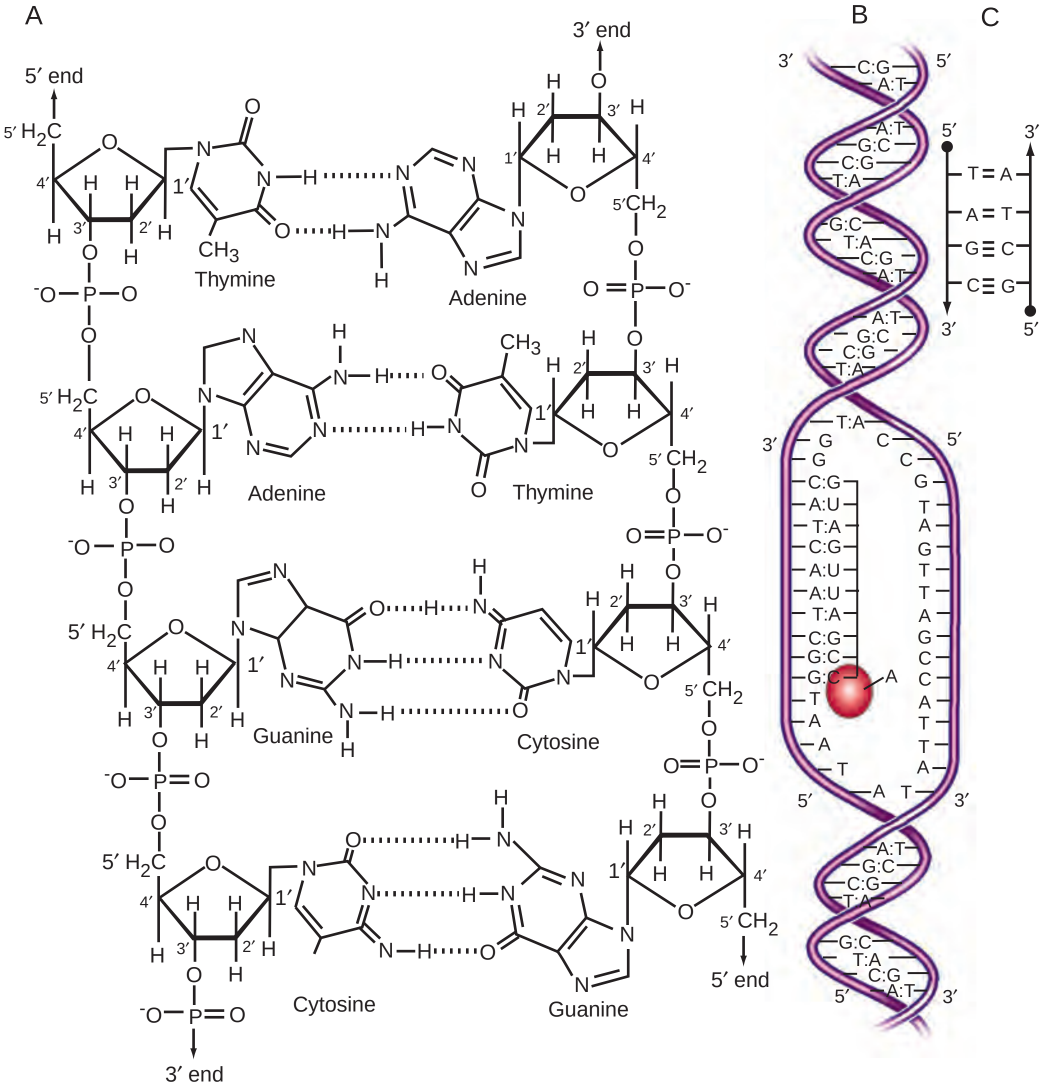


Figure 1.1 STRUCTURE, BASE PAIRING, POLARITY, AND TEMPLATE PROPERTIES OF DNA. (A) Structures of the four nitrogenous bases projecting from sugar phosphate backbones. The hydrogen bonds between them form base pairs holding complementary strands of DNA together. Note that A–T and T–A base pairs have only two hydrogen bonds, whereas C–G and G–C pairs have three. (B) The double helical structure of DNA results from base pairing of strands to form a double-stranded molecule with the backbones on the outside and the hydrogen-bonded bases stacked in the middle. Also shown schematically is the separation (unwinding) of a region of the helix by mRNA polymerase, which is shown using one of the strands as a template for the synthesis of an mRNA precursor molecule. Note that new bases added to the growing RNA strand obey the rules of Watson-Crick base pairing (see text). Uracil (U) in RNA replaces T in DNA and, like T, forms base pairs with A. (C) Diagram of the antiparallel nature of the strands, based on the stereochemical 3′ → 5′ polarity of the strands. The chemical differences between reading along the backbone in the 5′ → 3′ and 3′ → 5′ directions can be appreciated by reference to (A). *A*, Adenosine; *C*, cytosine; *G*, guanosine; *T*, thymine; *U*, uracil.

form the backbone of the polymer, from which the purine or pyrimidine bases project perpendicularly.

The haploid human genome consists of 23 long, double-stranded DNA molecules tightly complexed with histones and other nuclear proteins to form compact linear structures called *chromosomes*. The genome contains approximately 3 billion nucleotides; the individual chromosomes range from 50 to 200 million bases in length. By convention they are numbered from the longest (chromosome 1) to the shortest (chromosome 22), with the sex chromosomes getting the special designation X and Y. Females inherit the XX genotype and males, XY. The individual genes are aligned along each chromosome. The human genome contains about 2000 to 30,000 genes. Blood cells, like most somatic cells, are diploid. That is, each chromosome is present in two copies, so there are 46 chromosomes consisting of approximately 6 billion base pairs (bp) of DNA.

The four nucleotide bases in DNA are two purines (adenosine and guanosine) and two pyrimidines (thymine and cytosine). The basic chemical configuration of the other nucleic acid found in cells, RNA, is quite similar, except that the sugar is ribose (having a hydroxyl group attached to the 2′ carbon rather than the hydrogen found in deoxyribose) and the pyrimidine base uracil is used in place of thymine. The bases are commonly referred to by a shorthand notation: the letters A, C, G, T, and U are used to refer to adenosine, cytosine, guanosine, thymine, and uracil, respectively.

The ends of DNA and RNA strands are chemically distinct because of the 3′ → 5′ phosphodiester bond linkage that ties adjacent bases together (see Fig. 1.1). One end of the strand (the 3′ end) has an unlinked (free at the 3′ carbon) sugar position, and the other (the 5′ end) has a free 5′ position. There is thus a directionality (polarity) to the sequence of bases in a DNA strand: the same sequence of bases read in a 3′ → 5′ direction carries a different meaning than if read in a 5′ → 3′ direction. Cellular enzymes can thus distinguish one end of a nucleic acid from the other and one strand from its paired mate; most enzymes that "read" the DNA sequence tend to do so only in one direction (3′ → 5′ or 5′ → 3′ but not both). For instance, most nucleic acid–synthesizing enzymes read the template strand in 3′ → 5′ direction, thus adding new bases to the strand in a 5′ → 3′ direction.

Storage of Genetic Information in the Nucleotide Sequences of DNA

The ability of DNA molecules to store information resides in the sequence of nucleotide bases arrayed along the polymer chain. Under the physiologic conditions in living cells, DNA is thermodynamically most stable when two strands coil around each other to form a double-stranded helix. The strands are aligned in an "antiparallel" direction, having opposite 3′ → 5′ polarities (see Fig. 1.1). The DNA strands are held together by hydrogen bonds between the bases on one strand and the bases on the opposite (complementary) strand. The stereochemistry of these interactions allows bonds to form between the two strands only when adenine on one strand pairs with thymine at the same position of the opposite strand, or guanine with cytosine. These are the "Watson-Crick" rules of base pairing. Two strands joined together in compliance with these rules are said to have "complementary" base sequences. Similar rules apply to the formation of DNA-RNA or RNA-RNA double-stranded hybrids, except that A-U base pairs replace A-T pairs.

These thermodynamic rules imply that the sequence of bases along one DNA strand immediately dictates the sequence of bases that must be present along the complementary strand in the double helix. For example, whenever an A occurs along one strand, a T must be present at that exact position on the opposite strand; a G must always be paired with a C, a T with an A, and a C with a G.

Single-stranded nucleic acids can also fold back on themselves if two complementary sequences exist at different points along the molecule, thus forming "hairpin loops." Hairpin loop structures create secondary structures that affect the accessibility of sequences and the interaction of the molecule with proteins or other nucleic acids.

Transmission of Genetic Information to the Next Generation

Enzymes that replicate (polymerize) DNA and RNA molecules obey the base-pairing rules. By using an existing strand of DNA or RNA as the template, a new (daughter) strand is copied (transcribed) by reading processively along the base sequence of the template strand, adding to the growing strand at each position only that base that is complementary to the corresponding base in the template according to the Watson-Crick rules. Thus a DNA strand having the base sequence 5′-GGCTATG-3′ could be copied by DNA polymerase only into a daughter strand having the sequence 3′-CCGATAC-5′. Note that the sequence of the template strand provides all the information needed to predict the nucleotide sequence of the complementary daughter strand. Genetic information is thus stored in the form of base-paired nucleotide sequences.

If a double-stranded DNA molecule is separated into its two component strands and each strand is then used as a template to synthesize a new daughter strand, the product will be two double-stranded daughter DNA molecules, each identical to the original parent molecule. This semiconservative replication process is exactly what occurs during mitosis and meiosis as cell division proceeds (Fig. 1.2). The rules of Watson-Crick base pairing thus provide for the faithful transmission of exact copies of the cellular genome to subsequent generations.

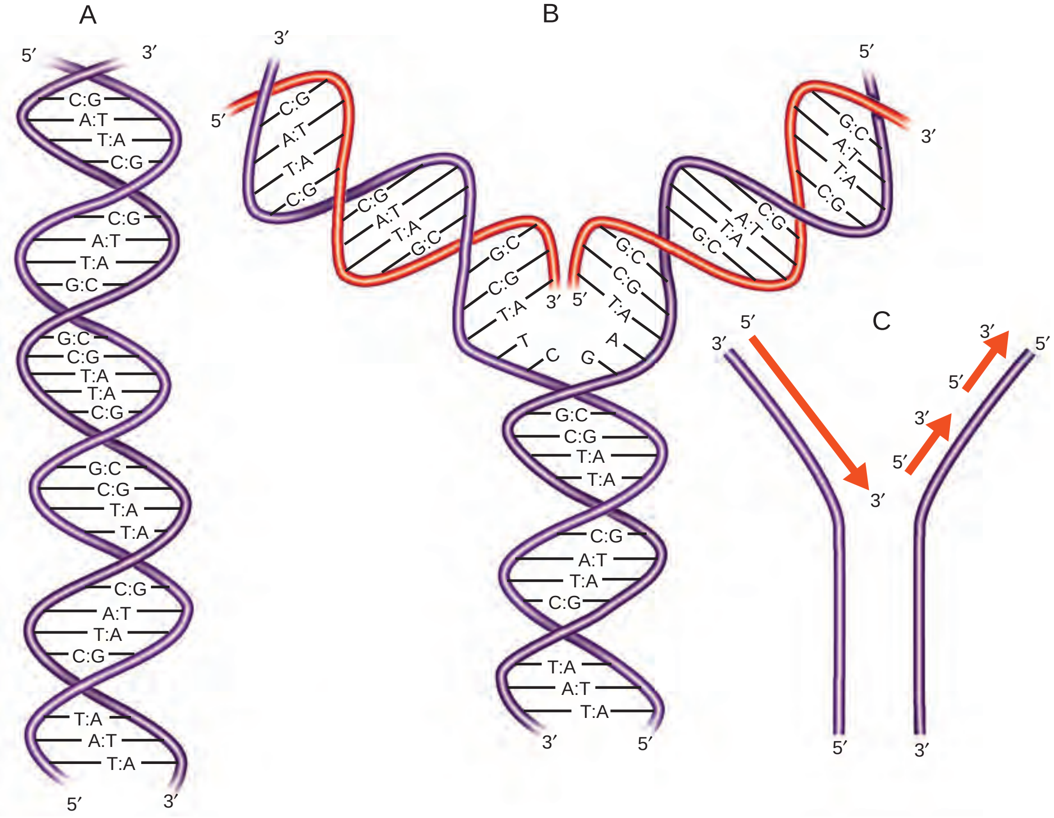


Figure 1.2 SEMICONSERVATIVE REPLICATION OF DNA. (A) The process by which the DNA molecule on the left is replicated into two daughter molecules, as occurs during cell division. Replication occurs by separation of the parent molecule into the single-stranded form at one end, reading of each of the daughter strands in the 3′ → 5′ direction by DNA polymerase, and addition of new bases to growing daughter strands in the 5′ → 3′ direction. (B) The replicated portions of the daughter molecules are identical to each other *(red)*. Each carries one of the two strands of the parent molecule, accounting for the term *semiconservative replication*. Note the presence of the replication fork, the point at which the parent DNA is being unwound. (C) The antiparallel nature of the DNA strands demands that replication proceed toward the fork in one direction and away from the fork in the other *(red)*. This means that replication is actually accomplished by reading of short stretches of DNA followed by ligation of the short daughter strand regions to form an intact daughter strand.

The Expression of Genetic Information Via Translation Into Proteins Using the Genetic Code

The information stored in the DNA base sequence of genes achieves its impact on the structure, function, and behavior of organisms by governing the structures, timing, and amounts of proteins and certain RNAs synthesized in the cells. The primary structure (i.e., the amino acid sequence) of each protein determines its three-dimensional conformation and therefore its properties (e.g., shape, enzymatic activity, ability to interact with other molecules, localization, and stability). In the aggregate, these proteins control cell structure and metabolism. The process by which DNA achieves its control of cells through protein synthesis is called *gene expression*.

An outline of the basic pathway of gene expression in eukaryotic cells is shown in Fig. 1.3. The DNA base sequence of the "minus," "anticoding" strand is first copied into an RNA molecule with a complementary base sequence, called *premessenger RNA* (pre-mRNA), by mRNA polymerase. Pre-mRNA thus has a base sequence identical to

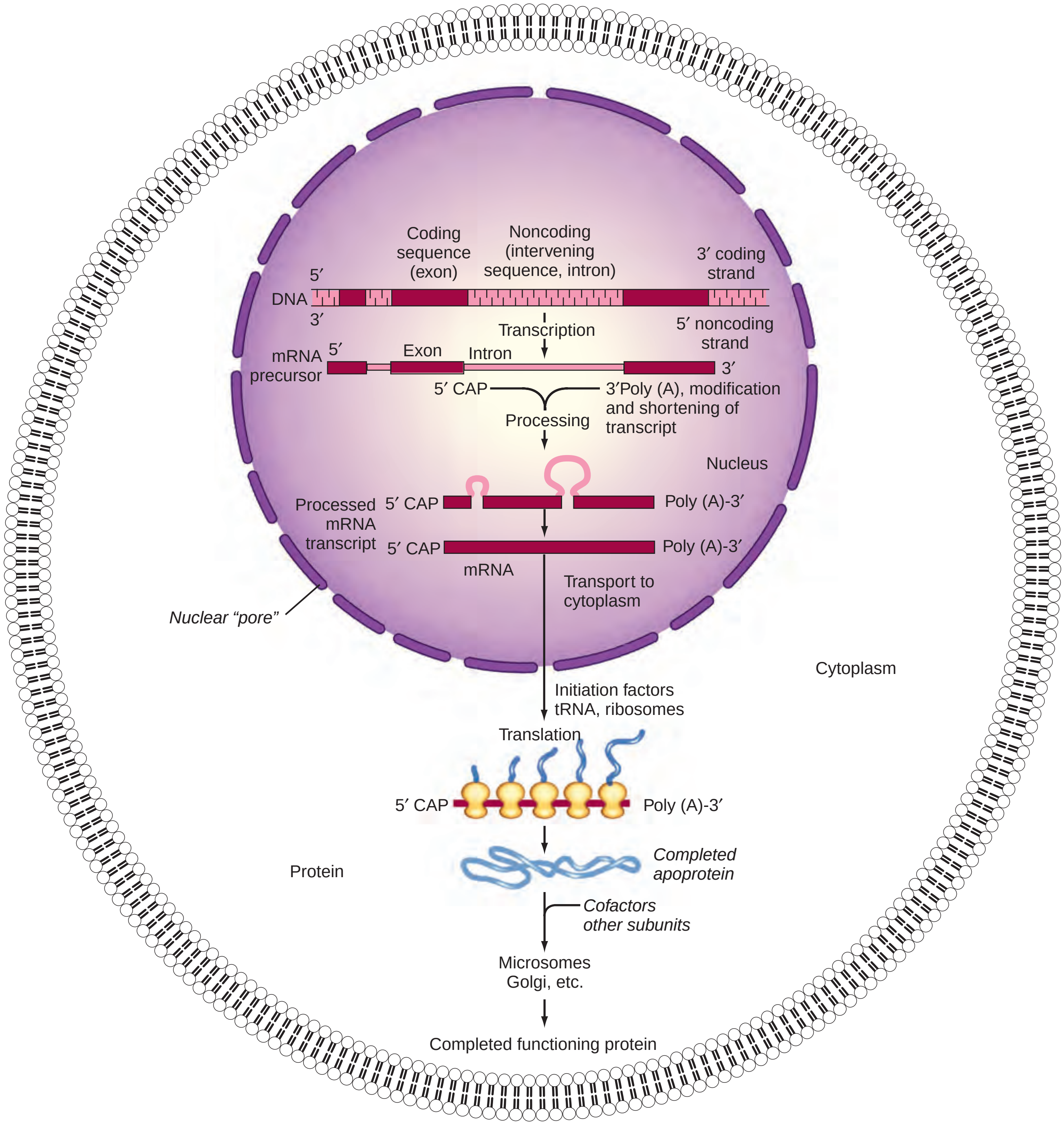

Coding sequence (exon)
Noncoding (intervening sequence, intron)
5′
3′ coding strand
DNA
3′
Transcription
5′ noncoding strand
mRNA precursor
5′
Exon
Intron
3′
5′ CAP
3′Poly (A), modification and shortening of transcript
Processing
Nucleus
Processed mRNA transcript
5′ CAP
Poly (A)-3′
5′ CAP
Poly (A)-3′
mRNA
Transport to cytoplasm
Nuclear "pore"
Cytoplasm
Initiation factors tRNA, ribosomes
Translation
5′ CAP
Poly (A)-3′
Protein
Completed apoprotein
Cofactors other subunits
Microsomes Golgi, etc.
Completed functioning protein

Figure 1.3 SYNTHESIS OF mRNA AND PROTEIN—THE PATHWAY OF GENE EXPRESSION. The diagram of the DNA gene shows the alternating array of exons *(red)* and introns *(shaded color)* typical of most eukaryotic genes. Transcription of the mRNA precursor, addition of the 5′-CAP and 3′-poly (A) tail, splicing and excision of introns, transport to the cytoplasm through the nuclear pores, translation into the amino acid sequence of the apoprotein, and posttranslational processing of the protein are described in the text. Translation proceeds from the initiator methionine codon near the 5′ end of the mRNA, with incorporation of the amino terminal end of the protein. As the mRNA is read in a 5′ → 3′ direction, the nascent polypeptide is assembled in an amino → carboxyl terminal direction.

the DNA "plus" or "coding" strand. Genes in eukaryotic species consist of tandem arrays of sequences encoding mature mRNA (exons) alternating with sequences (introns) present in the initial mRNA transcript (pre-mRNA) but absent from the mature mRNA. The entire gene is transcribed into the larger precursor, which is then further processed (spliced) in the nucleus. The introns are excised from the final mature mRNA molecule, which is then further processed, as discussed later, and exported to the cytoplasm to be decoded (translated) into the amino acid sequence of the protein by association with a biochemically complex group of ribonucleoprotein structures called *ribosomes*. Ribosomes contain two subunits: the 60 S subunit contains a single, large (28 S) ribosomal RNA (rRNA) molecule complexed with multiple proteins, and the 40 S subunit. The RNA component of the 40 S subunit is a smaller (18 S) rRNA.

Ribosomes read an mRNA sequence in a ticker tape fashion three bases at a time, inserting the appropriate amino acid encoded by each three-base code word or codon into the appropriate position of the growing protein chain. This process is called *mRNA translation*. The glossary used by cells to know which amino acids are encoded by each DNA codon is called the *genetic code* (Table 1.1). Each amino acid is encoded by a sequence of three successive bases. Because there are four code letters (A, C, G, and U) and because sequences read in the 5′ → 3′ direction have a different biologic meaning than sequences read in the 3′ → 5′ direction, there are 4^3, or 64, possible codons consisting of three bases.

There are 21 naturally occurring amino acids found in proteins. Thus more codons are available than amino acids to be encoded. As noted in Table 1.1, a consequence of this redundancy is that some amino acids are encoded by more than one codon. For example, six distinct codons can specify incorporation of arginine into a growing amino acid chain, four codons can specify valine, two can specify glutamic acid, and only one each methionine or tryptophan. However, in no case does a single codon encode more than one amino acid. Codons thus predict unambiguously the amino acid sequence they encode. In contrast, one cannot easily read backward from the amino acid sequence to decipher the *exact* encoding DNA sequence. These facts are summarized by saying that the code is degenerate but not ambiguous.

Some specialized codons serve as punctuation points during translation. The methionine codon (AUG), when surrounded by a consensus nucleotide sequence motif (the Kozak box) near the beginning (5′ end) of the mRNA, serves as the initiator codon signaling the first amino acid to be incorporated. All proteins initially begin with a methionine residue, but this is often removed later in the translational process. Three codons, UAG, UAA, and UGA, serve as translation terminators, signaling the end of translation.

The adaptor molecules mediating individual decoding events during mRNA translation are small (40 bases long) RNA molecules called *transfer RNAs* (tRNAs). When bound into a ribosome, each tRNA exposes a three-base segment within its sequence called the *anticodon*. These three bases attempt to pair with the three-base codon exposed on the mRNA. If the anticodon is complementary in sequence to the codon, a stable interaction among the mRNA, the ribosome, and the tRNA molecule results. Each tRNA also contains a separate region that is adapted for covalent binding to an amino acid. The enzymes that catalyze the binding of each amino acid are constrained in such a way that each tRNA species can bind only to a single amino acid. For example, tRNA molecules containing the anticodon 3′-AAA-5′, which is complementary to a 5′-UUU-3′ (phenylalanine) codon in mRNA, can be bound to or charged with only phenylalanine; tRNA containing the anticodon 3′-UAG-5′ can be charged with only isoleucine, and so forth.

tRNAs and their amino acyl tRNAs transduce nucleic acid information into the amino acid sequence that determines it physiologic properties. Ribosomes provide the structural matrix on which tRNA anticodons and mRNA codons become properly exposed and aligned in an orderly, linear, and sequential fashion. As each new codon is exposed, the appropriate charged tRNA species is bound. A peptide bond is then formed between the amino acid carried by this tRNA and the C-terminal residue on the existing nascent protein chain. The growing chain is transferred to the new tRNA in the process, so that it is held in place as the next tRNA is brought in. This cycle is repeated until completion of translation. The completed polypeptide is then transferred to other organelles for further processing (e.g., to the endoplasmic reticulum and the Golgi apparatus) or released into the cytosol for association with other subunits to form complex multimeric proteins (e.g., hemoglobin) and so forth, as discussed in Chapters 4 and 7.

TABLE 1.11 **The Genetic Code[a] Messenger RNA Codons for the Amino Acids**

Alanine	Arginine	Asparagine	Aspartic Acid	Cysteine
5′-GCU-3′	CGU	AAU	GAU	UGU
GCC	CGC	AAC	GAC	UGC
GCA	CGA			
GCG	AGA			
	AGG			

Glutamic Acid	Glutamine	Glycine	Histidine	Isoleucine
GAA	CAA	GGU	CAU	AUU
GAG	CAG	GGC	CAC	AUC
		GGA		AUA
		GGG		

Leucine	Lysine	Methionine	Phenylalanine	Proline[b]
UUA	AAA	AUG[c]	UUU	CCU
UUG	AAG		UUC	CCC
CUU				CCA
CUC				CCG
CUA				
CUG				

Serine	Threonine	Tryptophan	Tyrosine	Valine
UCU	ACU	UGG	UAU	GUU
UCC	ACC		UAC	GUC
UCA	ACA			GUA
UCG	ACG			GUG
AGU				
AGC				
Chain Termination[d]				
UAA				
UAG				
UGA				

[a] Note that most of the degeneracy in the code is in the third base position (e.g., lysine, AA [G or C]; asparagine, AA [C or U]; valine, GUN [where N is any base]).

[b] Hydroxyproline, the 21st amino acid, is generated by posttranslational modification of proline. It is almost exclusively confined to collagen subunits.

[c] AUG is also used as the chain-initiation codon when surrounded by the Kozak consensus sequence.

[d] The codons that signal the end of translation, also called nonsense or termination codons, are described by their nicknames *amber* (UAG), *ochre* (UAA), and *opal* (UGA).

A, Adenosine; *C*, cytosine; *G*, guanosine; *T*, thymine; *U*, uracil.

REGULATION OF GENE EXPRESSION

Virtually all cells of an organism receive a complete copy of the DNA genome inherited at the time of conception. The diversity of distinct cell types and tissues found in any complex organism is possible only because different portions of the genome are selectively expressed or repressed in each cell type. Each cell must "know" which genes to express, how actively to express them, and when to express them. This biologic necessity has come to be known as *gene regulation* or *regulated gene expression*. Understanding gene regulation provides insight into how pluripotent stem cells determine that they will express the proper sets of genes in daughter progenitor cells that differentiate along each lineage. Major hematologic disorders (e.g., the leukemias and lymphomas), immunodeficiency states, and myeloproliferative syndromes result from derangements in the system of gene regulation. An understanding of the ways that genes are selected for expression thus remains one of the major frontiers of biology and medicine. Chapters 2, 4, and 6 offer a more thorough coverage of these topics. The following sections provide brief introductions.

Chromatin and the Epigenetic Regulation of Gene Expression

Only a small fraction of the 6 billion base pairs of DNA present in a diploid human cell codes for proteins or for the ribosomal, transfer, and spliceosome RNAs, even including the nearby DNA sequences (promoters, repressors, enhancers, silencers, and insulator sequences) that are needed to support regulated protein synthesis. As discussed later and in Chapter 4, many additional species of RNA molecules exhibiting important regulatory effects on gene expression have been and still are being discovered. Yet, less than 10% of the genome accounts for all DNA sequences having a known function in gene expression. The remainder is called "DNA dark matter." It is being intensively investigated, but its purpose and impact on homeostasis remain unknown. A major challenge for cells, then, is how to find the genes and how to identify and activate only those genes whose expression it needs for its vital functions. The field of study that has arisen to address these questions is called epigenetics. This section provides only a brief introduction to epigenetics; Chapter 2 offers a thorough review and documents the increasing importance of epigenetics to hematology.

Most of the DNA in living cells is inactivated by formation of a nucleoprotein complex called *chromatin*. The histone and nonhistone proteins in chromatin effectively sequester genes from enzymes needed for expression. The most tightly compacted chromatin regions are called *heterochromatin*. *Euchromatin*, less tightly packed, contains actively transcribed genes. Activation of a gene for expression (i.e., transcription) requires that it become less compacted and more accessible to the transcription apparatus. These processes involve both *cis*-acting and *trans*-acting factors. *Cis*-acting elements are regulatory DNA sequences within or flanking the genes. They are recognized by *trans*-acting factors, which are nuclear DNA–binding proteins needed for transcriptional regulation.

DNA sequence regions flanking genes are called *cis*-acting because they influence expression of nearby genes only on the same chromosome. These sequences do not usually encode mRNA or protein molecules. They alter the conformation of the gene within chromatin twisting or kinking the surrounding DNA in ways that facilitate or inhibit access to the factors that modulate transcription. When exogenous nucleases (DNAses) are added experimentally in small amounts to nuclei, these exposed regions are especially sensitive to their DNA-cutting action. Thus DNAse hypersensitive sites in chromatin have come to be useful as markers for regions in or near genes that are accessible for transcription (Chapter 2).

DNA methylation is an epigenetic structural feature that also marks differences between actively transcribed and inactive genes. Most eukaryotic DNA is heavily methylated; that is, the DNA is modified by the addition of a methyl group to the 5 position of the cytosine pyrimidine ring (5-methyl-C). In general, heavily methylated genes are inactive; active genes are relatively hypomethylated, especially in the 5′ and 3′ flanking regions containing the promoter and other regulatory elements (see "Enhancers, Promoters, and Silencers"). These flanking regions frequently include DNA sequences with a high content of Cs and Gs (CpG islands). Hypomethylated CpG islands serve as markers of actively transcribed genes. For example, a search for undermethylated CpG islands on chromosome 7 facilitated the search for the gene for cystic fibrosis.

DNA methylation is facilitated by DNA methyltransferases (DMTs). DNA replication incorporates unmethylated nucleotides into each nascent strand, thus leading to demethylated DNA. For cytosines to become methylated, the methyltransferases must act after each round of replication. After an initial wave of demethylation early in embryonic development, regulatory elements are methylated during various stages of development and differentiation (Chapter 2). Aberrant DNA methylation also occurs as an early step during tumorigenesis, leading to silencing of tumor suppressor genes and of genes related to differentiation. This finding has led to induction of DNA demethylation as a target in cancer therapy. Indeed, 5-azacytidine, a cytidine analog that inhibits DMT, and the related compound decitabine, are approved by the US Food and Drug Administration (FDA) for use in myelodysplastic syndromes, and their use in cases of other malignancies is being investigated.

The mechanisms by which particular regions of DNA are targeted for methylation are under intense investigation. It is becoming increasingly apparent that this modification begets further alterations in chromatin proteins that in turn influence gene expression.

The "opening" of chromatin is necessary but not sufficient for genes to be expressed. The sequences within the now-accessible regions of DNA that are intended for transcription, and no others, must be identified and configured for binding by the intranuclear factors and mRNA polymerase that will execute the transcription program. This is accomplished by the presence of sequences embedded near or within the gene that are recognized by specific proteins that activate or inactivate transcription depending on which stimulatory or inhibitory proteins the sequences attract. These are discussed in the next section.

The major protein components of chromatin are histones, which are a small, highly basic protein family that binds tightly to the acidic residues in DNA. Histones can be acetylated, reducing their affinity for DNA, or methylated, which stabilizes their binding. Histone acetylation, phosphorylation, and methylation of the N-terminal tail are the focus of intense study for their potential roles in opening or closing access to regions of DNA for expression. For example, acetylation of histone lysine residues (catalyzed by histone acetyltransferases) is associated with transcriptional activation. Conversely, histone deacetylation (catalyzed by histone deacetylase) leads to gene silencing. Histone deacetylases are recruited to areas of DNA methylation by DMT and by methyl–DNA-binding proteins, thus linking DNA methylation to histone deacetylation. Drugs inhibiting these enzymes have been demonstrated to be active anticancer agents and continue to be the focus of ongoing studies. The regulation of histone acetylation and deacetylation appears to be linked to gene expression, but the roles of histone phosphorylation and methylation are less well understood. Current research suggests that in addition to gene regulation, histone modifications contribute to the "epigenetic code" and are thus a means by which information regarding chromatin structure is passed to daughter cells after DNA replication occurs.

Regulatory Sequence Motifs in or Near Genes: Enhancers, Promoters, and Silencers

Several types of *cis*-active DNA sequence elements have been defined according to the presumed consequences of their interaction with nuclear proteins (see Fig. 1.5). *Promoters* are found just upstream (to the 5′ side) of the start of mRNA transcription (the CAP). mRNA polymerases appear to bind first to the promoter region and thereby gain access to the structural gene sequences downstream. Promoters thus serve a dual function of being binding sites for mRNA polymerase and marking for the polymerase the downstream point at which transcription should start.

Enhancers are more complicated DNA sequence elements. Enhancers can lie on either side of a gene or even within the gene. Enhancers are bound by enhancer binding proteins, thereby stimulating expression of genes nearby. The domain of influence of enhancers (i.e., the number of genes to either side whose expression is stimulated) varies. Some enhancers influence only the adjacent gene; others seem to mark the boundaries of large multigene clusters (gene domains) whose coordinated expression is appropriate to a particular tissue type or a particular time. For example, the very high levels of globin gene expression in erythroid cells depend on the function of an enhancer that seems to activate the entire gene cluster and is thus called a *locus-activating region* (see Fig. 1.5). The nuclear factors interacting with enhancers are probably induced into synthesis or activation as part of the process of differentiation. Chromosomal rearrangements that place a gene that is usually tightly regulated under the control of a highly active enhancer can lead to overexpression of that gene. This commonly occurs in Burkitt lymphoma, for example, in which the MYC proto-oncogene is juxtaposed and dysregulated by an immunoglobulin enhancer.

Silencer sequences serve a function that is the obverse of enhancers. When bound by the appropriate nuclear proteins, silencer sequences cause repression of gene expression. Some evidence indicates that the same sequence elements can act as enhancers or silencers under different conditions, presumably by being bound by different sets of proteins having opposite effects on transcription. *Insulators* are sequence domains that mark the "boundaries" of multigene clusters, thereby preventing activation of one set of genes from "leaking" into nearby genes. The concerted actions of enhancers, silencers, and insulators delineate the specific DNA sequences to be transcribed or prevented from transcription within an opened region of chromatin.

One way that activation of transcription of a genomic DNA segment is accomplished is by a "looping" out phenomenon whereby some DNA binding proteins first bind to each end of a potentially expressed segment of open chromatin; those proteins then bind to one other, pulling the ends together and forming a looped-out segment of chromatin. Additional factors then bind to enhancers, silences, promotors, and enhancers, thereby demarcating those parts meant for transcription or silencing. Loops, in other words, may be a secondary structure that identifies areas primed for transcription (see Fig. 2.1).

Transcription Factors

Transcription factors are nuclear proteins that exhibit gene-specific DNA binding. Considerable information is now available about these nuclear proteins and their biochemical properties, but their physiologic behavior remains incompletely understood. Common structural features have become apparent. Most transcription factors have DNA-binding domains sharing homologous structural motifs (cytosine-rich regions called zinc fingers, leucine-rich regions called leucine zippers, and so on), but other regions appear to be unique. Some factors recognize specific DNA sequence motifs within promoters, enhancers, silencers, or insulators and bind directly to them, whereas others bind to these factors, forming complexes that promote or inhibit transcription. Many factors implicated in the regulation of growth, differentiation, and development (e.g., homeobox genes, proto-oncogenes, antioncogenes) appear to be DNA-binding proteins and may be involved in the steps needed for activation of a gene within chromatin. These factors are discussed in more detail in several other chapters (see Chapters 2, 4, and 6); when mutated, many are involved in the pathogenesis of blood dyscrasias, such as c-myc and c-myb.

Regulation at the Level of Pre-mRNA and mRNA Metabolism

In eukaryotic cells, mRNA is initially synthesized in the nucleus (see Figs. 1.3 and 1.4). Before the initial transcript becomes suitable for translation in the cytoplasm, mRNA processing and transport occur by a complex series of events including excision of the portions of the mRNA corresponding to the introns of the gene (mRNA splicing), modification of the 5′ and 3′ ends of the mRNA to render them more stable and translatable, and transport to the cytoplasm. Moreover, the amount of any particular mRNA moiety in both prokaryotic and eukaryotic cells is governed not only by the composite rate of mRNA synthesis (transcription, processing, and transport) but also by its degradation by cytoplasmic ribonucleases (RNA degradation). Many mRNA species of special importance in hematology (e.g., mRNAs for growth factors and their receptors, proto-oncogene mRNAs, acute phase reactants) are exquisitely regulated by control of their stability (half-life) in the cytoplasm.

Posttranscriptional mRNA metabolism is complex. Only a few relevant aspects are considered in this section. Chapter 4 provides more detail.

Pre-mRNA Splicing

The initial transcript of eukaryotic genes contains several subregions (see Fig. 1.4). Most striking is the tandem alignment of exons and introns. Precise excision of intron sequences and ligation of exons is critical for production of mature mRNA. This process is called mRNA splicing, and it occurs on complexes of small nuclear RNAs and proteins called snRNPs; the term *spliceosome* is also used to

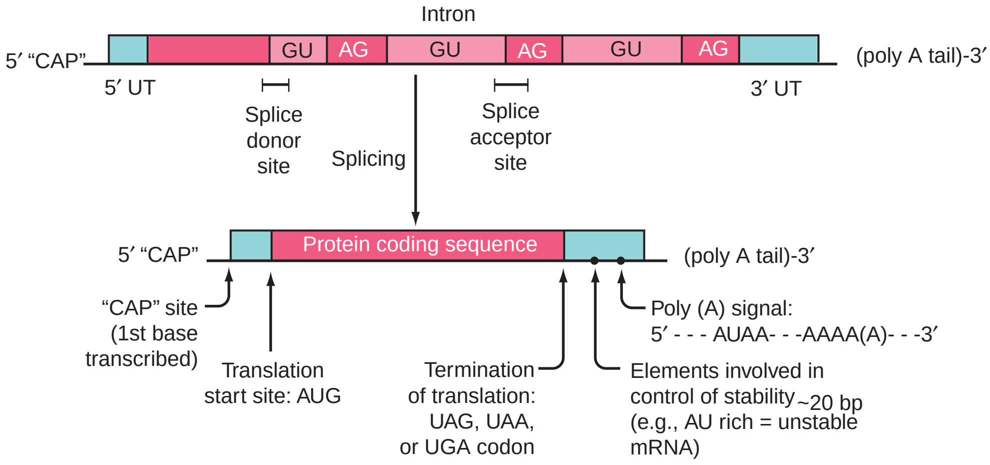

Intron
5′ "CAP"
GU
AG
GU
AG
GU
AG
(poly A tail)-3′
5′ UT
3′ UT
Splice donor site
Splicing
Splice acceptor site
5′ "CAP"
Protein coding sequence
(poly A tail)-3′
"CAP" site (1st base transcribed)
Translation start site: AUG
Termination of translation: UAG, UAA, or UGA codon
Elements involved in control of stability (e.g., AU rich = unstable mRNA)
~20 bp
Poly (A) signal:
5′ - - - AUAA- - -AAAA(A)- - -3′

Figure 1.4 ANATOMY OF THE PRODUCTS OF THE STRUCTURAL GENE (mRNA PRECURSOR AND mRNA). This schematic shows the configuration of the critical anatomic elements of an mRNA precursor, which represents the primary copy of the structural portion of the gene. The sequences GU and AG indicate, respectively, the invariant dinucleotides present in the donor and acceptor sites at which introns are spliced out of the precursor. Not shown are the less stringently conserved consensus sequences that must precede and succeed each of these sites for a short distance.

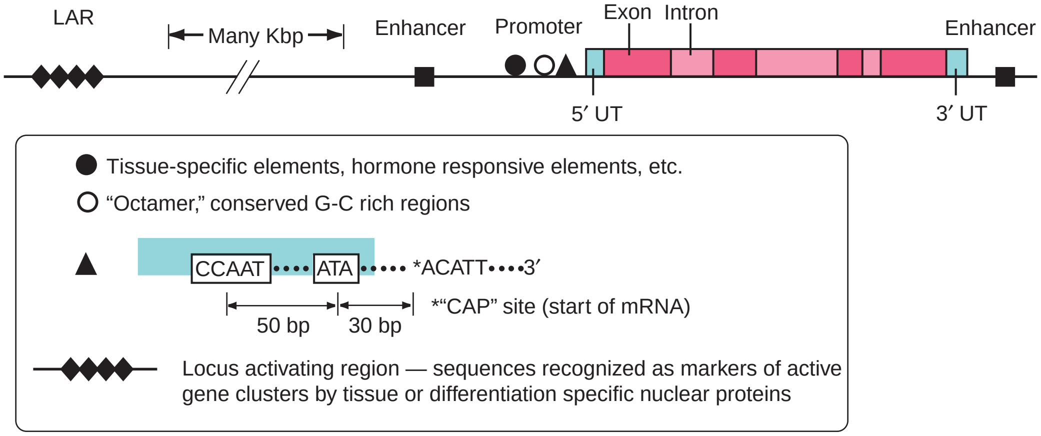

LAR
Many Kbp
Enhancer
Promoter
Exon
Intron
Enhancer
5′ UT
3′ UT
Tissue-specific elements, hormone responsive elements, etc.
"Octamer," conserved G-C rich regions
CCAAT •••• ATA ••••• *ACATT••••3′
50 bp
30 bp
*"CAP" site (start of mRNA)
Locus activating region — sequences recognized as markers of active gene clusters by tissue or differentiation specific nuclear proteins

Figure 1.5 REGULATORY ELEMENTS FLANKING THE STRUCTURAL GENE. (*For more information refer to suggested readings from Jones B; Kumar A, et al; Waddington S, et al.)

describe the intranuclear organelle that mediates mRNA splicing reactions. The biochemical mechanism for splicing is complex. A consensus sequence, which includes the dinucleotide GU, is recognized as the donor site at the 5′ end of the intron (5′ end refers to the polarity of the mRNA strand coding for protein); a second consensus sequence ending in the dinucleotide AG is recognized as the acceptor site, which marks the distal end of the intron (see Figs. 1.4 and 1.5). The spliceosome recognizes the donor and acceptor and forms an intermediate lariat structure that provides for both excision of the intron and proper alignment of the cut ends of the two exons for ligation in precise register.

mRNA splicing has proven to be an important mechanism for greatly increasing the versatility and diversity of expression of a single gene. Several different mRNA and protein products can arise from a single gene by selective inclusion or exclusion of individual exons from the mature mRNA products. This phenomenon is called *alternative mRNA splicing*. It permits a single gene to code for multiple mRNA and protein products with related but distinct structures and functions. The mechanisms by which individual exons are selected or rejected are complex and highly context-specific, varying among different cell types, differentiation stages, and physiologic states. Chapter 4 provides additional details. For present purposes, it is sufficient to note that important physiologic changes in cells can be regulated by altering the patterns of mRNA splicing products arising from single genes.

Many inherited hematologic diseases arise from mutations that derange mRNA splicing. For example, some of the most common forms of the thalassemia syndromes and hemophilias (see Chapters 41 and 134) arise by mutations that alter normal splicing signals or create splicing signals where they normally do not exist (activation of cryptic splice sites). Conversely, mutations altering key protein factors that modulate alternative splicing pathways are known to contribute to the pathogenesis of bone marrow dyscrasias (see Chapters 59, 61, and 66).

Modification of the Ends of the mRNA Molecule

Most eukaryotic mRNA species are polyadenylated at their 3′ ends. Polyadenylation results in the addition of stretches of 100 to 150 "A" residues at the 3′ end. Such an addition is often called the *poly-A tail* and is of variable length. Polyadenylation facilitates rapid early cleavage of the unwanted 3′ sequences from the transcript and is also important for stability or transport of the mRNA out of the nucleus. Signals near the 3′ extremity of the mature mRNA mark positions at which polyadenylation occurs. The consensus signal is AUAAA (see Fig. 1.4). Mutations in the poly-A signal sequence have been shown to cause thalassemia (see Chapter 41).

At the 5′ end of the mRNA, a complex oligonucleotide having unusual phosphodiester bonds is added. This structure contains the nucleotide 7-methyl-guanosine and is called *CAP* (see Fig. 1.4). The 5′-CAP enhances both mRNA stability and the ability of the mRNA to interact with protein translation factors and ribosomes.

5′ and 3′ Untranslated Sequences Within mRNAs That Modulate Stability and Translatability

Most mature mRNAs contain sequence motifs at the 5′and 3′ ends of the molecule extending beyond the initiator and terminator codons that mark the beginning and the end of the sequences actually translated into proteins (see Figs. 1.4 and 1.5). These so-called 5′ and 3′ untranslated regions (5′ UTRs and 3′ UTRs) influence both mRNA stability and the efficiency with which mRNA species can be translated. For example, if the 3′ UTR of a very stable mRNA (e.g., globin mRNA) is swapped with the 3′ UTR of a highly unstable mRNA (e.g., the c-myc gene), the c-myc mRNA becomes more stable. Conversely, attachment of the 3′ UTR of c-myc to a globin molecule renders it unstable. Instability is often associated with repeated sequences rich in A and U in the 3′ UTR (see Fig. 1.4). The UTRs in mRNAs coding for proteins involved in iron metabolism mediate altered mRNA stability or translatability by binding iron-laden proteins and thus govern iron storage and turnover (see Chapter 36).

Transport of mRNA From Nucleus to Cytoplasm: mRNP Particles

An additional potential step for regulation or disruption of mRNA metabolism occurs during the transport from nucleus to cytoplasm. mRNA transport is an active, energy-consuming process (Chapter 4). Moreover, at least some mRNAs appear to enter the cytoplasm in the form of complexes bound to proteins (mRNPs). mRNPs may regulate stability of the mRNAs and their access to translational apparatus. Some evidence indicates that certain mRNPs are present in the cytoplasm but are not translated (masked message) until proper physiologic signals are received.

Regulation of mRNA Processing and Stability

As mentioned earlier, cells can regulate the relative amounts of different protein isoforms arising from a given gene by altering the relative amounts of an mRNA precursor that are spliced along one pathway or another (alternative mRNA splicing). Many striking examples of this type of regulation are known—for example, the ability of B lymphocytes to make both immunoglobulin M (IgM) and IgD at the same developmental stage, changes in the particular isoforms of cytoskeletal proteins produced during red blood cell differentiation, and a switch from one isoform of the *c-myb* protooncogene product to another during red blood cell differentiation. Abnormalities of mRNA splicing due to mutations at the splice sites can lead to defective protein synthesis, as can occur in β-globin pre-mRNA, leading to some forms of β-thalassemia. The effect of controlling the pathway of mRNA processing used in a cell is to include or exclude portions of the mRNA sequence. These portions encode peptide sequences that influence the ultimate physiologic behavior of the protein, or the RNA sequences that alter stability or translatability.

The importance of the control of mRNA stability for gene regulation is being increasingly appreciated. The steady-state level of any given mRNA species ultimately depends on the balance between the rate of its production (transcription and mRNA processing) and its destruction. One means by which stability is regulated is the inherent structure of the mRNA sequence, especially the 3′ and 5′ UTRs. As already noted, these sequences appear to affect mRNA secondary structure, recognition by nucleases, or both. Different mRNAs thus have inherently longer or shorter half-lives, almost regardless of the cell type in which they are expressed. Some mRNAs tend to be highly unstable. In response to appropriate physiologic needs, they can thus be produced quickly and removed from the cell quickly when a need for them no longer exists. In contrast, globin mRNA is inherently quite stable, with a half-life measured in the range of 15 to 50 hours. This is appropriate for the need of reticulocytes to continue to synthesize globin for 24 to 48 hours after the ability to synthesize new mRNA has been lost by the terminally mature erythroblasts.

The stability of mRNA can also be altered in response to changes in the intracellular milieu. This phenomenon usually involves nucleases capable of destroying one or more broad classes of mRNA defined on the basis of their 3′ or 5′ UTR sequences. Thus, for example, histone mRNAs are destabilized after the S-phase of the cell cycle is complete. Presumably this occurs because histone synthesis is no longer needed. Induction of cell activation, mitogenesis, or terminal differentiation events often results in the induction of nucleases that destabilize specific subsets of mRNAs. Selective stabilization of mRNAs probably also occurs; for example, α-globin mRNA is stabilized by the protective binding of a specific stabilizing protein to a nuclease target sequence in its 3′ UTR.

Another critical mechanism that ensures the efficiency and fidelity of gene expression is nonsense-mediated decay (NMD). NMD has evolved to deal with the fact that common classes of mutations (either germ line or somatic, and including point mutations, “frame shifts” due to small deletions or insertions, and mutations causing mis-splicing; see Chapters 3 and 4) result in the creation of a premature translation termination codon in the translation reading frame (also stop codons or nonsense mutations). Nonsense codons can also be created by transcription or processing errors occurring during expression of normal genes. Indeed, as many as 5% to 30% of mature mRNA transcripts may carry nonsense codons in some cells under certain conditions. These mRNAs can be translated only into fragments of the intended protein and are thus physiologically useless. This impairs the efficiency of gene expression, expending the considerable energy required for even partial translation while serving no functional purpose. Moreover, those fragments fold abnormally and can trigger stress responses such as the unfolded protein response (Chapter 4) that can trigger other undesired cellular reactions. These fragments can also contain some of the functional domains of the intended complete protein. These can interact deleteriously with other cellular components, deranging cellular homeostasis.

NMD addresses these issues by recognizing nonsense codons and destroying the affected mRNA, thus avoiding its translation. The process exists across evolution from yeast to mammals. It is mediated by complex protein and RNA components functioning and supporting at least two recognition and destruction pathways. It is becoming clear that the integrity of these pathways is likely relevant to multiple disease states, including neoplasia.

Regulation at the Level of mRNA Translation

The amount of a given protein accumulating in a cell depends not only on the amount of the mRNA present but also on the rate at which it is translated into the protein and the stability of the protein. Translational efficiency depends in part on the structural features of any given mRNA, including polyadenylation, secondary structure of the 5′ and 3′ UTRs, and presence of the 5′ cap. The amounts and state of activation of protein factors needed for translation are also crucial. The secondary structure of the mRNA, particularly in the 5′ UTR, greatly influences the intrinsic translatability of an mRNA molecule by constraining the access of translation factors and ribosomes to the translation initiation signal in the mRNA. Secondary structures along the coding sequence of the mRNA may also have some impact on the rate of elongation of the peptide.

Changes in capping, polyadenylation, and translation factor efficiency affect the overall rate of protein synthesis within each cell. These effects tend to be global rather than specific to a particular gene product. However, these effects influence the relative amounts of different proteins made. mRNAs whose structures inherently lend themselves to more efficient translation tend to compete better for rate-limiting components of the translational apparatus, but mRNAs that are inherently less translatable tend to be translated less efficiently in the face of limited access to other translational components. For example, the translation factor eIF-4 tends to be produced in higher amounts when cells encounter transforming or mitogenic events. This causes an increase in overall rates of protein synthesis but also leads to a selective increase in the synthesis of some proteins that were underproduced before mitogenesis because they competed less well when the supply of active eIF-4 was limiting. It is also now being increasingly recognized that several classes of low-molecular-weight RNAs (micro-RNAs [miRNAs]) can have profound effects on the output of proteins from individual mRNAs or related groups of mRNAs by recognizing specific sequences in them and thereby altering stability or translatability.

Translational regulation of individual mRNA species is critical for some events important to blood cell homeostasis. For example, as discussed in Chapter 36, the amount of iron entering a cell is an exquisite regulator of the rate of ferritin mRNA translation. An mRNA sequence called the *iron response element* is recognized by a specific mRNA-binding protein but only when the protein lacks iron. mRNA bound to the protein is translationally inactive. As iron accumulates in the cell, the protein becomes iron bound and loses its affinity for the mRNA, resulting in translation into apoferritin molecules that bind the iron.

Tubulin synthesis involves coordinated regulation of translation and mRNA stability. Tubulin regulates the stability of its own mRNA by a feedback loop. As tubulin concentrations rise in the cell, it interacts with its own mRNA through the intermediary of an mRNA-binding protein. This results in the formation of an mRNA-protein complex and nucleolytic cleavage of the mRNA. The mRNA is destroyed, and further tubulin production is halted.

Heterogeneity of rRNAs and tRNAs

The 18 S and 28 S rRNAs, the many ribosomal proteins needed to assemble a ribosome, and tRNAs are encoded by many genes and are actually quite heterogeneous. The heterogeneity also varies among cell types and under varied cellular states such as the nutritional stress found in cancer cells. These variations appear to create significant alterations in the translatability of specific mRNAs. These effects can be blunted or accentuated by the tendency of different ribosome classes to favor or disfavor certain patterns of codon use. Disease states have been associated with mutations in these proteins and RNAs (ribosomeopathies), and manipulation of this complexity for therapeutic purposes is under intense investigation.

These few examples of posttranscriptional regulation emphasize that cells tend to use every step in the complex pathway of gene expression as points at which exquisite control over the amounts of a particular protein or RNA species can be regulated. In other chapters, additional levels of regulation are described (e.g., regulation of the production, stability, activity, localization, and access to other cellular components of the proteins that are present in a cell [see Chapters 6 and 7]).

Roles of Small Interfering RNAs, Micro RNAs, Short Hairpin RNAs, and Long Noncoding RNAs in Regulating Gene Expression

Cells were once thought to possess only three basic classes of RNA molecules: mRNA, rRNAs (5 S, 18 S, and 28 S), and tRNA. Moreover, the physiologic capacity of these RNA species was thought to be only informational, their nucleic acid sequences serving as codons, anticodons, or binding sites for ribosomal proteins, splicing and translation factors, mRNA transport factors, etc. Two fundamental discoveries have profoundly changed our view of the biologic role of RNAs. First was the recognition that some RNA molecules have catalytic activity that sustain key steps in gene expression such as pre-mRNA splicing. In cells, these activities are often carried out within ribonucleic acid (RNP) complexes. The second was the discovery that cells contain a potpourri of small RNA species in both the nucleus and the cytoplasm. Collectively these RNA moieties provide another layer of complex posttranscriptional mechanisms modulating gene expression. Some of these small RNAs might modulate transcription and processing as well.

One such process is carried out *by small interfering RNAs (siRNAs)*: short, double-stranded fragments of RNA containing 21 to 23 bp (Fig. 1.6). The process is triggered by perfectly complementary double-stranded RNA, which is cleaved by Dicer, a member of the RNase III family, into siRNA fragments. These small fragments of double-stranded RNA are unwound by a helicase in the RNA-induced silencing complex (RISC). The antisense strand anneals to mRNA transcripts in a sequence-specific manner and in doing so brings the endonuclease activity within the RISC to the targeted transcript. An RNA-dependent RNA polymerase in the RISC may then create new siRNAs to processively degrade the mRNA, ultimately leading to complete degradation of the mRNA transcript and abrogation of protein expression.

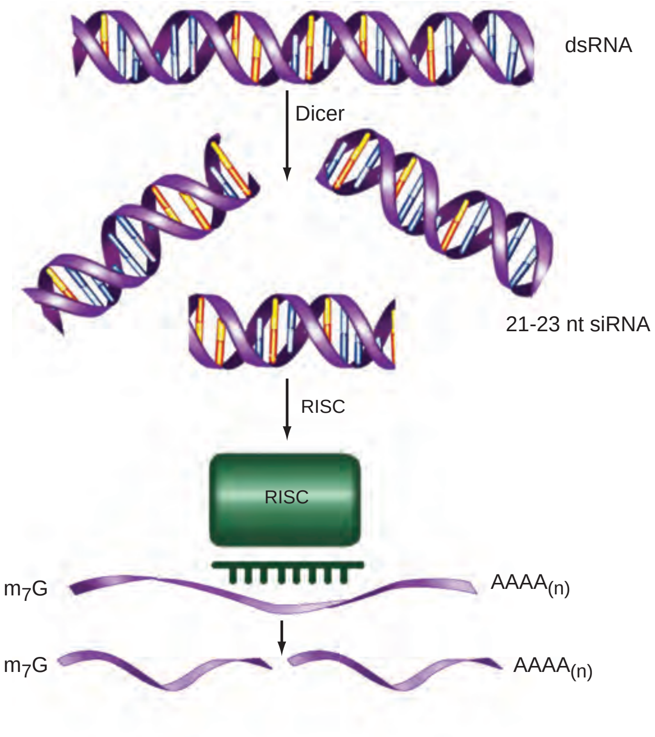


Figure 1.6 mRNA DEGRADATION BY siRNA. dsRNA is digested into 21- to 23-bp (base pair) small interfering RNAs by the Dicer RNase. These RNA fragments are unwound by RISC and bring the endonucleolytic activity of RISC to mRNA transcripts in a sequence-specific manner, leading to degradation of the mRNA. *dsRNA*, double-stranded RNA; *RISC*, RNA-induced silencing complex; *siRNA*, small interfering RNA.

Although this endogenous process likely evolved to destroy invading viral RNA, the use of siRNA has become a commonly used tool for evaluation of gene function. Sequence-specific synthetic siRNA may be directly introduced into cells or introduced via gene transfection methods and targeted to an mRNA of a gene of interest. The siRNA will lead to degradation of the mRNA transcript and accordingly prevent new protein translation. This technique is a relatively simple, efficient, and inexpensive means to investigate cellular phenotypes after directed elimination of expression of a single gene. Experimentally, engineered short hairpin RNAs (shRNAs) are used extensively to degrade or block the translation of a gene's mRNA product in a highly specific fashion, thus allowing one to target or "knock down" the expression of any gene or collection of genes at will and allowing assessment of a cell's behavior in the absence of expression of the targeted genes.

miRNAs, or MIRs, are 22-nucleotide small RNAs encoded by the cellular genome that alter mRNA stability and protein translation. These genes are transcribed by RNA polymerase II and capped and polyadenylated similar to other RNA polymerase II transcripts. The precursor transcript of approximately 70 nucleotides is cleaved into mature miRNAs by the enzymes Drosha and Dicer. One strand of the resulting duplex forms a complex with the RISC that together binds the target mRNA with imperfect complementarity. Through mechanisms that are still incompletely understood, miRNA suppresses gene expression, likely either through inhibition of protein translation or through destabilization of mRNA. miRNAs appear to have essential roles in development and differentiation and are aberrantly regulated in many types of cancer cells. The identification of miRNA sequences, their regulation, and their target genes are areas of intense study.

Other classes of small RNA molecules, such as circular or ringed RNAs and glycosylated RNAs, are under active study. Discussion of these is beyond the scope of this chapter. Moreover, a class of extraordinarily long RNA transcripts (long noncoding RNA [lncRNA]) has been known to exist for decades, but its functions are just beginning to be uncovered. lncRNA may be support an important mechanism for "opening" large domains of chromatin to access by mRNA polymerase (RNA polymerase II), transcription factors, enhancer- and silencer-binding proteins, etc., so the genes within that domain can be expressed. This might also provide clues into the role played by DNA "dark matter" in gene regulation, if the signals for the production, start points, and end points of lncRNAs are encoded in the regions "opened" by lncRNA transcription.

Some Illustrative Structural Features of the Genome Relevant to Hematology

Structural genes are separated from one another by as few as 1 to 5 kilobases or as many as several thousand kilobases of DNA. Almost nothing is known about the reason for the erratic clustering and spacing of genes along chromosomes. It is clear that intergenic DNA contains a variegated landscape of structural features that provide useful tools to localize genes, identify individual human beings as unique from every other human being (DNA fingerprinting), and diagnose human diseases by linkage. Only a brief introduction is provided here.

Polymorphism and Single Nucleotide Polymorphisms

The genomic landscape of each of our genomes is dotted with scattered sequence differences that distinguish us from any other living creature. These are a consequence of the nonzero error rate of base copying during normal DNA replication; under normal circumstances it is approximately $1/10^6$. In other words, one of 1 million bases of DNA will be miscopied (mutated) during each round of DNA replication. A set of enzymes called *DNA proofreading enzymes* corrects most of these mutations so that the rate of mutation following a normal cell division is closer to $1/10^9$. When these enzymes are themselves altered by mutation, the rates of mutation (and therefore the odds of neoplastic transformation) increase considerably. If these mutations occur in bases critical to the structure or function of a protein or gene, altered function, disease, or a lethal condition can result. Most pathologic mutations tend not to be preserved throughout many generations because of their unfavorable phenotypes. Exceptions, such as the hemoglobinopathies, occur when the heterozygous state for these mutations confers selective advantage in the face of unusual environmental conditions, such as malaria epidemics. These "adaptive" mutations drive the dynamic change in the genome with time (evolution).

Because these copying errors occur randomly most will occur in either the vast stretches of intergenic DNA or the "silent" bases of gene DNA, such as the degenerate third bases of codons. They thus do not pathologically alter the function of the gene or its products. These clinically harmless mutations are called *DNA polymorphisms*. DNA polymorphisms can be regarded in exactly the same way as other types of polymorphisms that have been widely recognized for years (e.g., eye and hair color, blood groups). They are variations in the population that occur without apparent clinical impact. Each of us differs from other humans in the precise number and type of DNA polymorphisms that we possess. Most polymorphisms represent single-nucleotide changes and are called single-nucleotide polymorphisms (SNPs).

DNA polymorphisms breed true. In other words, if an individual's DNA contains a G 1200 bases upstream from the α-globin gene, instead of the C most commonly found in the population, that G will be transmitted to that individual's offspring. Note that if one had a means for distinguishing the G at that position from a C, one would have a linked marker for that individual's α-globin gene. Before the completion of the human genome project, only limited regions of the genome could be analyzed by direct DNA sequencing and SNPs were detectable only if they altered the recognition site for one or more restriction endonucleases, enzymes that cut DNA only at sites possessing a specific recognition sequence (Fig. 1.7). SNPs not altering such sites were not readily detectable. Contemporary DNA sequencing methods now allow for routine comprehensive cataloging of SNPs in a population or individual. However, the principles of choosing the right comparison populations and of the "breeding true" through generations remain important principles in interpreting the results.

The importance of polymorphic variations in each is that they can be used to identify individuals uniquely and to compare two individuals at the genomic level. For example, the severity of sickle cell anemia varies greatly, even within families, even though the disease is always caused by a specific point mutation in the β-globin gene. This suggests that the products of other genes exert a modifying effect on clinical phenotype. By scanning the genomes of many sickle cell patients of varying severity, an SNP was identified in less severely affected individuals near the Bcl11a gene, which was then shown to participate in the perinatal shutdown of fetal hemoglobin synthesis. Less severely affected individuals turned out to express a less active variant of Bcl11a. Similarly, the pattern of variations in the polymorphisms strung along the HLA gene cluster on chromosome 6 (i.e., the "haplotype") can be measured to compare the HLA "match" between two individuals and assess the compatibility of a potential bone marrow donor and recipient. The term haploidentical transplant is derived form a donor-recipient pair who have matching HLA cluster haplotypes.

Repeated Sequence Motifs

A related important feature of the DNA landscape is the existence of highly repeated DNA sequence motifs. A DNA sequence is said to be repeated if it or a sequence very similar (homologous) to it occurs more than once in a genome. Some multicopy genes, such as the histone genes and the rRNA genes, are repeated DNA sequences. However, most repeated DNA occurs outside genes, or within introns. Indeed, 30% to 45% of the human genome appears to consist of repeated DNA sequences.

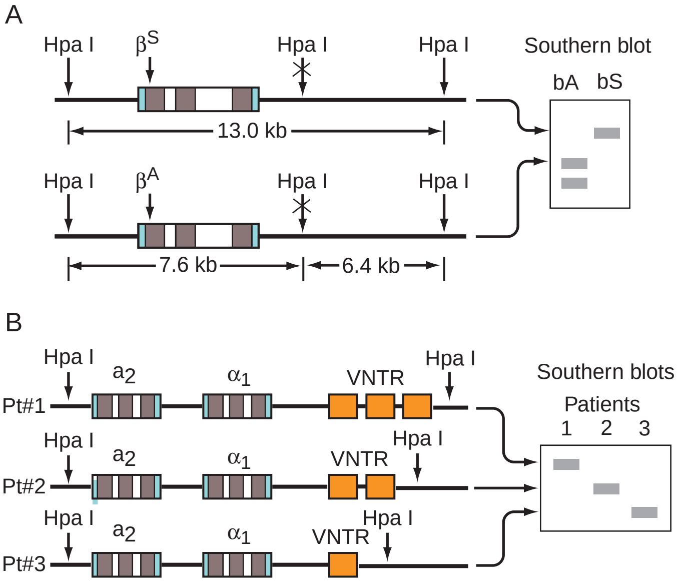


Figure 1.7 TWO USEFUL FORMS OF SEQUENCE VARIATION AMONG THE GENOMES OF NORMAL INDIVIDUALS. (A) Presence of a DNA sequence polymorphism that falls within a restriction endonuclease site, thus altering the pattern of restriction endonuclease digests obtained from this region of DNA on Southern blot analysis. (Readers not familiar with Southern blot analysis should return to examine this figure after reading later sections of this chapter.) (B) A variable-number tandem repeat (VNTR) region (defined and discussed in the text). Note that individuals can vary from one to another in many ways according to how many repeated units of the VNTR are located on their genomes, but restriction fragment length polymorphism differences are in effect all-or-none differences, allowing for only two variables (restriction site presence or absence).

The function of repeated sequences remains unknown, but their presence has inspired useful strategies for detecting and characterizing individual genomes. For example, a pattern of short repeated DNA sequences, characterized by the presence of flanking sites recognized by the restriction endonuclease Alu-1 (called "Alu repeats") occurs approximately 300,000 times in a human genome. These sequences are not present in the mouse genome. If one wishes to infect mouse cells with human DNA and then identify the human DNA sequences in the infected mouse cells, one simply probes for the presence of Alu repeats. The Alu repeat thus serves as a signature of human DNA.

Classes of highly repeated DNA sequences (tandem repeats) have proven to be useful for distinguishing genomes of each human individual. These short DNA sequences, usually less than a few hundred bases long, tend to occur in clusters, with the number of repeats varying among individuals (see Fig. 1.6). Alleles of a given gene can therefore be associated with a variable number of tandem repeats (VNTRs) in different individuals or populations. For example, there is a VNTR near the insulin gene. In some individuals or populations, it is present in only a few tandem copies, but in others, it is present in many more. When the population as a whole is examined, there is a wide degree of variability from individual to individual as to the number of these repeats residing near the insulin gene. It can readily be imagined that if probes were available to detect a dozen or so distinct VNTR regions, each human individual would differ from virtually all others with respect to the aggregate pattern of these VNTRs. Indeed, it can be shown mathematically that the probability of any two human beings sharing exactly the same pattern of VNTRs is exceedingly small if approximately 10 to 12 different VNTR elements are mapped for each person. A technique called *DNA fingerprinting* that is based on VNTR analysis has become widely publicized because of its forensic applications.

There are many other classes of repeated sequences in human DNA. For example, human DNA has been invaded many times in its history by retroviruses. Retroviruses tend to integrate into human DNA and then "jump out" of the genome when they are reactivated, to complete their life cycle. The proviral genomes often carry with them nearby bits of the genomic DNA in which they sat. If the retrovirus infects the DNA of another individual at another site, it will insert this genomic bit. Through many cycles of infection, the virus will act as a transposon, scattering its attached sequence throughout the genome. These types of sequences are called *long interspersed elements*. They represent footprints of ancient viral infections.

MOLECULAR GENETIC METHODOLOGIES ALLOWING THE ISOLATION, ANALYSIS, AND MANIPULATION OF GENES

The application of molecular genetics to the understanding, diagnosis, treatment, and prevention of hematologic diseases became possible in limited ways during the 1970s and 1980s, when a variety of experimental methods, both biochemical and genetic, made it possible to isolate any desired DNA fragment from chromosomes, or from DNA copies of cellular RNA (cDNAs). These methodologies, such as "Southern" blotting analysis of DNA, "Northern" blotting of RNA, and initial DNA sequencing techniques, although elegant, were laborious and required sophisticated personnel and equipment. They are now largely of historical interest, although still useful for some purposes. Four methodologies that made widespread routine use of DNA- and RNA-based disease-oriented research, diagnostics, and therapeutics feasible are the polymerase chain reaction (PCR), gene cloning, high-throughput DNA sequencing, and gene transfer techniques. The latter allows one to insert of the genetic material of choice into almost any desired cells, tissues, or organisms. All of these capabilities have been greatly enhanced by advances in computational methods, computerization, and automation. These four merit a brief introductory discussion because they are alluded to in many chapters in this book.

The Polymerase Chain Reaction

The development of the PCR revolutionized DNA-based strategies for diagnosis and treatment. It permits the detection, synthesis, and isolation of specific genes and allows one to discriminate among the alleles of a gene differing by as little as one base. It requires only readily available equipment and basic technical skills. A specimen consisting of only minute amounts of material will suffice; in most circumstances, no special preparation of the tissue is necessary. PCR made direct genetic and genomic analyses readily accessible to clinical, epidemiologic, and forensic laboratories. This single advance fueled quantum increases in the use of direct gene analysis for diagnosis of human diseases. Indeed, PCR analysis combined with direct DNA sequencing technologies have largely supplanted older strategies, such as restriction enzyme mapping and DNA/RNA blotting strategies for many research and diagnostic applications, although these older methods remain useful for some niche applications. PCR coupled with now-routinely available gene cloning methodologies allows one to synthesize in microgram quantities naturally occurring or engineered genes at will. These can then readily be inserted into cells, tissues, or organisms where they will be expressed and their physiologic or pathologic effects investigated. Similarly, industrial scale production of novel therapeutics based on the PCR-designed DNA itself or its expressed RNA or protein products is now routine. Hematopoietic growth factors and monoclonal antibody therapeutics are just two examples of widely used hematologic therapies that depended on these strategies.

PCR is based on the prerequisites for copying an existing DNA strand by DNA polymerase: an existing denatured strand of DNA to be used as the template and primers. Primers are short oligonucleotides, 12 to 100 bases in length, having a base sequence complementary to the desired region of the existing DNA strand. Oligonucleotide primers are now easily designed and produced using biochemical techniques developed in the 1970s and 1980s. The primer allows the polymerase to "know" where to begin copying. If the base sequence of the DNA of the gene under study is known (see DNA sequencing), two synthetic oligonucleotides complementary to sequences flanking the region of interest can be prepared. If these are the only oligonucleotides present in the reaction mixture, then the DNA polymerase can copy only daughter strands of DNA downstream from those oligonucleotides. In other words, it can copy only that gene. Recall that DNA is double stranded, that the strands are held together by the rules of Watson-Crick base pairing, and that they are aligned in antiparallel fashion. This implies that the effect of incorporation of both oligonucleotides into the reaction mix will be to synthesize two daughter strands of DNA, one originating upstream of the gene and the other originating downstream. The net effect is synthesis of only the DNA between the two primers, thus doubling only the DNA containing the region of interest. If the DNA is now heat denatured and then cooled again, allowing hybridization of the daughter strands to the primers, and the polymerization is repeated, then the region of DNA through the gene of interest is doubled again. Thus two cycles of denaturation, annealing, and elongation result in a selective quadrupling of the gene of interest. The cycle can be repeated 30 to 50 times, resulting in a selective and geometric amplification of the sequence of interest to the order of 2^{30} to 2^{50} times. The result is a millionfold or higher selective amplification of the gene of interest, yielding microgram quantities of that DNA sequence.

PCR achieved practical utility when DNA polymerases from thermophilic bacteria were discovered; when synthetic oligonucleotides of any desired sequence could be produced efficiently, reproducibly, and cheaply by automated instrumentation; and when DNA thermocycling machines were developed. Thermophilic bacteria live in hot springs and other exceedingly warm environments, and their DNA polymerases can tolerate 100°C (212°F) incubations without substantial loss of activity. The advantage of these thermostable polymerases is that they retain activity in a reaction mix that is repeatedly heated to the high temperature needed to denature the DNA strands into the single-stranded form. Microprocessor-driven DNA thermocycler machines can be programmed to increase temperatures to 95°C to 100°C (203°F to 212°F) (denaturation), to cool the mix to 50°C (122°F) rapidly (a temperature that favors oligonucleotide annealing), and then to raise the temperature to 70°C to 75°C (158°F to 167°F) (the temperature for optimal activity of the thermophilic DNA polymerases). In a reaction containing the test specimen, the thermophilic polymerase, a sufficient supply of primers to support the amplification, and the chemical components needed to sustain the multiple rounds of copying (e.g., nucleotide triphosphate precursors, reaction buffer, an adenosine triphosphate [ATP]-generating system to support the endothermic polymerase reaction), the thermocycler can conduct many cycles of denaturation, annealing, and polymerization in a completely automated fashion. The gene of interest can thus be amplified more than a millionfold in a matter of a few hours. The DNA product is readily identified and isolated by routine agarose gel electrophoresis. The DNA can then be analyzed by restriction endonuclease, digestion, hybridization to specific probes, sequencing, further amplification by cloning, and so forth.

Reverse transcriptases (RNA-dependent DNA polymerases) derived from retroviruses greatly extend the utility of PCR. By copying all the RNAs into their cDNAs, reverse transcriptase allows RNA sequences in a specimen to be amplified much like DNA sequences. This procedure, called reverse transcription (RT)-PCR, inserts a reverse transcriptase step into the beginning of the procedure, which then proceeds exactly like PCR. RT-PCR permits one to amplify all of the mRNAs expressed in a cell for high-throughput nucleotide sequence analysis, to detect just one or a few mRNAs to analyze their expression patterns, or to clone them (see later) to isolate their encoding genes.

High-Throughput DNA and RNA Sequencing

Knowing the nucleotide base sequence of a gene, its RNA products, its flanking regulatory elements, and its variation in a disease state is essential to understanding its normal or pathologic behavior. Techniques for sequencing (i.e., deciphering the nucleotide base sequence) DNA that emerged in the 1970s were valuable but limited. Only short stretches of a few hundred bases could be read during a single “run.” The methods required the use of radioactive tracers, sophisticated electrophoretic steps, and/or toxic chemicals. Nonetheless, the coding sequences of many genes relevant to hematologic disorders were obtained in this way. Fortunately, the human genome project inspired major technologic innovations (e.g., in the application of physicochemical and chromatographic principles to nucleic acid chemistry, the development of novel nonradioactive tracers, and the creation of software and firmware that allowed one to assemble the sequences of multiple independent sequencing “runs” of shorter fragments into a coherent sequence of the whole length of a gene). Sequencing of millions of nucleotides in a single sitting became feasible.

Modern sequencing techniques are commonly described as high-throughput sequencing or “next-gen” (i.e., next-generation) sequencing. Their efficiency and cost-effectiveness are such that whole genome sequences can now be gotten from a clinical specimen within a few days for a direct cost of less than a thousand dollars. The profound effect that these advances have had on the practical utility of DNA analysis in medicine is evident in the routine application of high-throughput sequencing to tumor specimens to identify therapeutic targets or infer prognostic information or the many thousands of SARS-CoV-2 genomes sequenced every day to track variants.

Next-gen sequencing has inspired the discipline of genomics, which attempts to understand the anatomy and functioning of any gene in the context of all of the DNA in the entire genome of a cell. Indeed, the technology has advanced to the point that one can sequence the genome of a single cell. Similarly, one can obtain the sequences of all of the mRNAs expressed in a specimen or even a single cell (the transcriptome) by first copying the cellular RNA into cDNA. This is called RNA sequencing or RNAseq.

Chapter 3 discusses genomics and the uses of sequencing technologies in hematology in greater detail.

Gene Cloning

PCR allows one to generate microgram amounts of pure DNA fragments up to a few kilobases in length. Most genes are considerably longer than that. To study their function or pathology, one needs to isolate the entire gene and its flanking sequences and insert it into cells for expression. Moreover, for any applications, such as manufacturing DNA reagents for diagnostic kits, the capability to generate much larger amounts is desirable. Gene cloning, or recombinant DNA technology, is a collection of methods that meets these goals. Basically, an amplified PCR fragment, or a mixture of all of the DNA fragments from a cell up to megabase lengths (1 megabase =1 million bp) generated by sonication or limited nuclease digestion, is modified at the ends with oligonucleotide “adaptors” that allow them to be ligated into a “vector.” In this context, a vector is an engineered microbial DNA element that can be inserted into a host cell, where it will coexist with the host genome and be able to be expressed. The most common vectors are viral genomes that were engineered to retain infectiousness but have had their pathogenic properties removed from their genomes.

If the “recombinant” genome has been placed in a bacteriophage genome and exposed to an excess of host bacterial cells, each cell acquires a single recombinant molecule. When cultured at low density on petri plates, each colony that grows out is a clone derived from a single transfected bacterium that in turn contains and expresses a single recombinant molecule. Many screening techniques have been devised by which one can identify and purify the clone(s) containing the desired DNA fragment among the thousands of clones on the plates. The clone can then be grown in bulk culture to generate large amounts of that DNA fragment for analysis, used as a diagnostic or experimental probe, or refined for use as a therapeutic, for transfer into cells, tissue, or whole organisms for studies of its biologic function. “Gene cloning” is thus named for the fact that the method allows one to capture, purify, and mass produce any single desired DNA fragment (e.g., a whole gene) in a single bacterial clone. This clone can also be preserved in a manner that sustains viability and be used repeatedly to generate additional DNA. Much of our contemporary molecular understanding of hematologic pathobiology has been gleaned by application of gene cloning approaches. Important therapeutics, such as erythropoietin, granulocyte-macrophage colony-stimulating factor (GM-CSF), monoclonal antibody therapeutics, CAR-T cells, and many more, are derived from recombinant DNA molecule purified by gene cloning methods.

Extensions and variations of techniques of gene cloning into bacteria have made possible the cloning of genes into cells of a wide variety of species, including human tissue culture cells. This adds great versatility to the methodology for expressing large quantities of the RNAs or proteins encoded by the cloned genes with all the appropriate posttranslation modifications present in their natural state.

Use of Transgenic and Knockout/Knockin Organisms to Model Gene Function

Recombinant DNA technology has resulted in the identification of many disease-related genes. To advance the understanding of the disease related to a previously unknown gene, the function of the protein encoded by that gene must be verified or identified, and the way changes in the gene’s expression influence the disease phenotype must be characterized. Analysis of the role of these genes and their encoded proteins was made possible by the development of recombinant DNA technology that allowed the production of mice that are genetically altered at the cloned locus. Mice can be produced that express an exogenous gene and thereby provide an in vivo model of its function. Linearized DNA is injected into a fertilized mouse oocyte pronucleus and reimplanted in a pseudopregnant mouse. The resultant transgenic mice can then be analyzed for the phenotype induced by the injected transgene. Placing the gene under the control of a strong promoter that stimulates expression of the exogenous gene in all tissues allows the assessment of the effect of widespread overexpression of the gene. Alternatively, placing the gene under the control of a regulatory sequence that can function only in certain tissues (a tissue-specific promoter) elucidates the function of that gene in a particular tissue or cell type. A third approach is to study control elements of the gene by testing their capacity to drive expression of a “marker” gene that can be detected by chemical, immunologic, or functional means. For example, the promoter region of a gene of interest can be joined to the cDNA encoding green jellyfish protein and activity of the gene assessed in various tissues of the resultant transgenic mouse by fluorescence microscopy. Use of such a reporter gene demonstrates the normal distribution and timing of expression of the gene from which the promoter elements are derived. Transgenic mice contain exogenous genes that insert randomly into the genome of the recipient. Expression can thus depend as much on the location of the insertion as it does on the properties of the injected DNA.

In contrast, any defined genetic locus can be specifically altered by targeted recombination between the locus and a plasmid carrying an altered version of that gene (Fig. 1.8). If a plasmid contains that altered gene with enough flanking DNA identical to that of the normal gene locus, homologous recombination can occur, and the altered gene in the plasmid will replace the gene in the recipient cell. Using a mutation that inactivates the gene allows the production of a null mutation, in which the function of that gene is completely lost. To induce such a mutation, the plasmid is introduced into an embryonic stem cell, and the rare cells that undergo homologous recombination

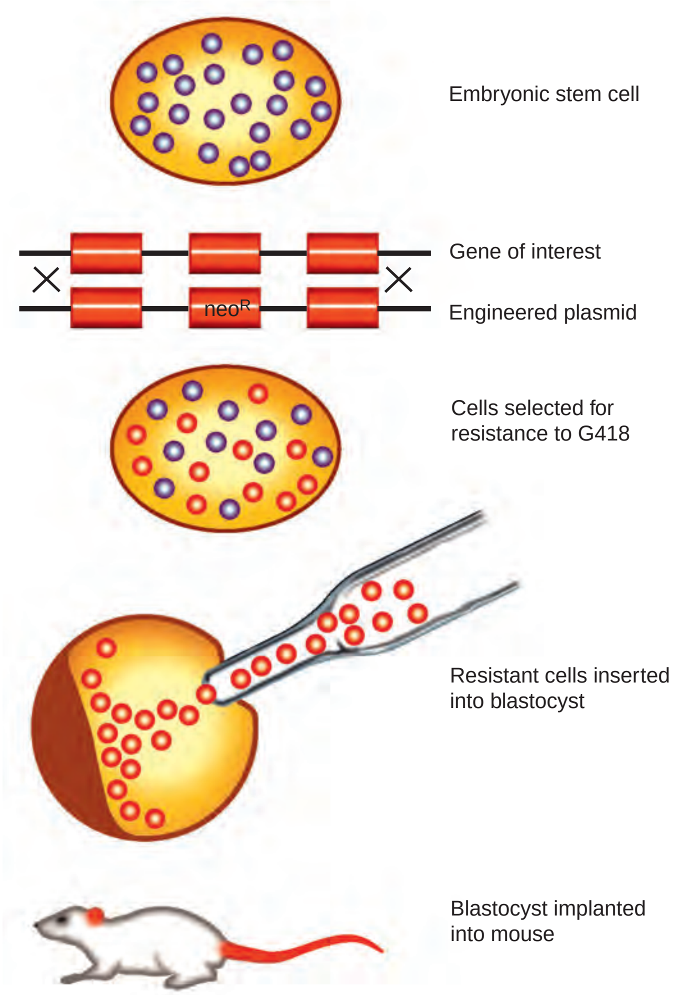

Embryonic stem cell
Gene of interest
Engineered plasmid
neo^R
Cells selected for resistance to G418
Resistant cells inserted into blastocyst
Blastocyst implanted into mouse

Figure 1.8 GENE "KNOCKOUT" BY HOMOLOGOUS RECOMBINATION. A plasmid containing genomic DNA homologous to the gene of interest is engineered to contain a selectable marker positioned so as to disrupt expression of the native gene. The DNA is introduced into embryonic stem cells, and cells resistant to the selectable marker are isolated and injected into a mouse blastocyst, which is then implanted into a mouse. Offspring mice that contain the knockout construct in their germ cells are then propagated, yielding mice with heterozygous or homozygous inactivation of the gene of interest.

are selected. The "knockout" embryonic stem cell is then introduced into the blastocyst of a developing embryo. The resultant animals are chimeric; only a fraction of the cells in the animal contain the targeted gene. If the new gene is introduced into some of the germline cells of the chimeric mouse, then some of the offspring of that mouse will carry the mutation as a gene in all of their cells. These heterozygous mice can be further bred to produce mice homozygous for the null allele.

Knockout mice reveal the function of the targeted gene by the phenotype induced by its absence. Methods for "knocking in" a gene have been developed to allow one to assess the functional consequences of replacing the function of the knocked-out gene with a modified version of that gene or an alternative gene with a related function. Genetically altered mice have been essential for discerning the biologic and pathologic roles of large numbers of genes implicated in the pathogenesis of human disease. These methods were originally developed in mice, but they have been extended to many animal species. The methods are now refined enough to generate recombinant organisms in which multiple endogenous genes are replaced by human genes, generating model organisms "humanized" for certain key functions (e.g., hemoglobin synthesis in mouse erythroid cells, certain immune functions); these humanized models are proving useful for preclinical testing of novel therapeutics.

DNA- AND RNA-BASED THERAPEUTICS

Gene Therapy and Gene Editing

The application of gene therapy to genetic hematologic disorders is an appealing idea. In some cases, this would involve isolating hematopoietic stem cells from patients with diseases with defined genetic lesions, inserting normal genes into those cells, and reintroducing the genetically engineered stem cells back into the patient. A few candidate diseases for such therapy include sickle cell disease, thalassemia, hemophilia, and adenosine deaminase–deficient severe combined immunodeficiency. The technology for separating hematopoietic stem cells and for performing gene transfer into those cells has advanced rapidly, and clinical trials are actively testing the applicability of these techniques. Indeed, the use of this "ex vivo" approach has led to the approval in Europe of a therapeutic gene for β-thalassemia. In other cases, such as treatments for hemophilia, the therapeutic gene is injected directly into a target tissue or infused. In both cases the gene must be packaged in a vector, usually a virus engineered to infect a particular cell type and to have lost any potential to cause a viral disease pathogenic. Presently, there are only few (but increasing, such as severe combined immunodeficiency syndromes, Wiskott–Aldrich disease, and thalassemia) proven therapeutic successes from gene therapy.

Progress in this field continues rapidly and is likely to accelerate as a consequence of the development of "gene editing" technologies (see Chapter 5). Among these, "CRISPR" is the most prominent current example. It is based on the discovery of enzyme systems used by microorganisms to excise foreign DNA sequences (e.g., integrated viral genomes) from the host genome. These systems can be adapted to insert, replace, or delete, in principle, any desired DNA sequence at its naturally occurring position in the host genome. For example, one could excise the mutation causing sickle cell anemia and replace it with the normal DNA sequence in the β-globin gene of a patient's hematopoietic stem cells and then reintroduce them into the patient's bone marrow without introducing any foreign DNA. This exciting technology is in clinical trials for a number of hematologic conditions, including hemoglobinopathies.

RNA Therapeutics

The recognition that abnormal expression of oncogenes plays a role in malignancy has stimulated attempts to suppress oncogene expression to reverse the neoplastic phenotype. One early attempt blocking mRNA expression is with antisense oligonucleotides. These are single-stranded DNA sequences 17 to 20 bases long, having a sequence complementary to the transcription or translation start of the mRNA. These relatively small molecules can be engineered with modified nucleotides that resist nucleotide destruction and freely enter the cell, where they complex to the targeted mRNA by Watson-Crick base pairing. Alternatively, one can use a modified gene therapy approach by transfecting the cells with a DNA segment encoding the antisense RNA. The binding of the oligonucleotide may directly block translation and clearly enhances the rate of mRNA degradation, thus downregulating the expression of the desired gene. The discovery, mentioned earlier, of naturally occurring small inhibitory RNAs has stimulated the development of RNA therapeutics that have largely superseded the original antisense approach.

RNA therapeutics is a burgeoning field of early drug development. Synthetic small hairpin RNAs containing modified nucleotides that stabilize them in the circulation and tissue spaces can be readily manufactured and engineered to contain any desired nucleotide sequences needed to identify and bind to only the targeted gene or RNA gene product, form metabolically active complexes with other intracellular

RNAs or proteins, and thereby achieve the desired therapeutic effect. RNA therapeutics are promising to be extremely versatile. In addition to binding to the target mRNA to block its translation and enhance its destruction, engineered shRNAs have been successfully designed to interact with the translational apparatus to "read through" or "skip over" nonsense codons, permitting completion of translation of the mutated protein, and to interact with the pre-mRNA splicing apparatus to alter the pattern of alternative mRNA splicing of the desired pre-mRNA in a physiologically favorable way. The latter strategy has been elegantly deployed to develop an FDA-approved therapy for spinal muscular atrophy. Using more conventional gene therapy methods to employ an shRNA targeting the binding of Bcl11a to its erythroid specific enhancer, thereby blocking the postnatal shutdown of fetal hemoglobin, is also being tested in clinical trials for treating sickle cell anemia and β-thalassemia.

FUTURE DIRECTIONS

The elegance of recombinant DNA technology and its successor technologies of genomics, epigenomics, proteomics, genetic therapies, gene editing, and RNA therapeutics resides in the capacity they confer on investigators to examine each gene as a discrete physical entity that can be purified, reduced to its basic building blocks for decoding of its primary structure, analyzed for its patterns of expression, and perturbed by alterations in sequence or molecular environment so that the effects of changes in each region of the gene can be assessed. Purified genes can be deliberately modified or mutated to create novel genes not available in nature. These provide the potential to generate useful new biologic entities, such as modified live virus or purified peptide vaccines, modified proteins customized for specific therapeutic purposes, and altered combinations of regulatory and structural genes that allow for the assumption of new functions by specific gene systems.

The most important impact of the genetic approach to the analysis of biologic phenomena is the most indirect. Diligent and repeated application of the methods outlined in this chapter to the study of many genes from diverse groups of organisms is beginning to reveal the basic strategies used by nature for the regulation of cell and tissue behavior. As our knowledge of these rules of regulation grows, our ability to understand, detect, and correct pathologic phenomena will increase substantially. So too will the complexity of ethical and policy issues about what comprises the appropriate and inappropriate uses of technologies capable of altering the nature of what it means to be human. For all of these reasons, it is incumbent on students of hematology to be as conversant with this discipline.

SUGGESTED READINGS

Bentley D. The mRNA assembly line: transcription and processing machines in the same factory. *Curr Opin Cell Biol*. 2002;14:336.

Collins FS, Doudna JA, Lander ES, Routimi CN. Human molecular genetics and genomics—important advances and exciting possibilities. *N Engl J Med*. 2021;384:1–4.

Dykxhoorn DM, Novina CD, Sharp PA. Killing the messenger: short RNAs that silence gene expression. *Nat Rev Mol Cell Biol*. 2003;4:457.

Fischle W, Wang Y, Allis CD. Histone and chromatin cross-talk. *Curr Opin Cell Biol.*. 2003;15:172.

Grewal SI, Moazed D. Heterochromatin and epigenetic control of gene expression. *Science*. 2003;301:798.

Jones B. Layers of gene regulation. *Nat Rev Genet*. 2015;16:128–129.

Jongbloed JDH, Lekanne Deprez RH, Vatta M. Introduction to molecular genetics. In: Baars HF, Doevendans PAFM, Houweling A, van Tintelen J, eds. *Clinical Cardiogenetics*. Cham: Springer; 2016.

Kloosterman WP, Plasterk RHA. The diverse functions of microRNAs in animal development and disease. *Dev Cell*. 2006;11:441.

Klose RJ, Bird AP. Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci*. 2006;31:89.

Kumar A, Garg S, Garg N. Regulation of gene expression: RNA regulation. In: Meyers RA, ed. *Synthetic Biology,* Vol. 1. Weinheim: Wiley-VCH Verlag; 2014:61–121.

Lee TI, Young RA. Transcription of eukaryotic protein-coding genes. *Ann Rev Genet*. 2000;34:77.

Tefferi A, Wieben ED, Dewald GW, et al. Primer on medical genomics, part II: background principles and methods in molecular genetics. *Mayo Clin Proc*. 2002;77:785.

Waddington S, Privolizzi R, Karda R, et al. A broad overview and review of CRISPR-CAS technology and stem cells. *Curr Stem Cell Rep*. 2016;2:9–20.

Wilusz CJ, Wormington M, Peltz SW. The cap-to-tail guide to mRNA turnover. *Nat Rev Mol Cell Biol*. 2001;2:237.

CHAPTER 2

EPIGENOMICS IN HEMATOLOGY

Myles Brown and Alok Tewari

Epigenetics can be defined as inheritance of variation, above and beyond changes in the DNA sequence. In other words, epigenetics comprises the study of how cells sharing the same exhaustive DNA blueprint can appear and function so distinctly as white blood cells, hepatocytes, neurons, etc. Whereas the genome contains all of the vital information to direct the development of an organism, the epigenome dynamically filters and organizes that information into highly coordinated programs of gene expression.

Within the nucleus, DNA interacts with histone and non-histone proteins to form chromatin, which can be broadly classified as highly compacted and transcriptionally silent (heterochromatin) versus loosely compacted and transcriptionally active (euchromatin). Heterochromatin comprises two distinct classes of DNA: (1) noncoding, often repetitive, "structural," DNA of centromeres and telomeres (constitutive heterochromatin), and (2) gene-encoding and gene-regulatory "functional," DNA that is selectively rendered inactive in different cell types (facultative heterochromatin). When euchromatin is described as loosely compacted, the information content of its DNA is readily accessible to binding the protein and RNA machinery that regulate gene expression. Therefore, the study of epigenetics and chromatin aims to describe and understand the chromatin dynamics that orchestrate the four-dimensional symphony of molecular and cellular biology, from the (seemingly) one-dimensional score that is the genome.

The information contained within chromatin can be grossly divided into two main categories: (1) the structural genes themselves, which are transcribed and translated into proteins or act as functional RNAs, and (2) gene-regulatory regions, which control the timing and amount of transcription (Fig. 2.1A). The information contained in transcribed and translated regions can be interpreted using the "genetic code," wherein the DNA sequence of the gene specifies, through a messenger RNA intermediate, the amino acid sequences of resulting proteins. While there is no universal genetic code to decipher the function of RNAs that are not translated into proteins, some such as ribosomal-RNA and transfer-RNA genes have well understood functions. In addition, several other classes of non-protein coding RNA genes with known functions exist, including small nuclear RNA (snRNA) involved in RNA splicing, Piwi-interacting RNA (piRNA) involved in silencing of transposable elements, small nucleolar RNA (sno-RNA) involved in directing the chemical modification of other RNA, and micro-RNA (miRNA) involved in translational silencing. A growing class of long noncoding RNA (lncRNA) have been identified, with a variety of proposed functions. Interestingly, these lncRNA genes appear to be regulated in much the same way as protein-coding genes. Protein-coding regions comprise approximately 1% to 2% of the genome. In contrast, the information contained in gene-regulatory regions is the "epigenetic code," which has yet to be fully deciphered and is based on the accessibility of those regions to dynamic protein-DNA interactions, the identity of those interacting proteins, and the identity of the gene(s) whose expression is being modulated.

The most dramatic example of chromatin compaction is the condensation that occurs during mitosis, making individual chromosomes visible by light microscopy and allowing segregation of replicates equally among daughter cells. A condensed or compacted chromosome is folded many times upon itself and is highly protein-bound, affording little or no access to genomic information and remaining transcriptionally silent (see Fig. 2.1B). Contrast this with the "decondensed," chromatin state that is necessary for DNA replication, during the synthesis phase of the cell cycle. DNA replication requires unfolding of chromatin, disruption of its protein-DNA interactions, and "unzipping," the double helix to allow every base in the genome to be copied. When not dividing, cells maintain their chromatin in intermediate states of compaction. Actively transcribed genes and their associated regulatory chromatin regions are "open," and "accessible," insofar as the underlying protein-DNA interactions are readily modified and disrupted to accommodate binding of transcription factors, cofactors, RNA polymerases, and the totality of functional components underlying gene expression.

It is important to remember some key differences between genomic and epigenomic research. Whereas the genome is essentially an unvarying feature of every cell in an organism (with the important exception of T and B cells that rearrange and mutate their antigen receptor genes), the epigenome of each cell within that organism is unique. Moreover, epigenomes are fluid throughout a cell's life span, integrating intrinsic cellular "identity," with contextual signals to specify a program of gene expression. Finally, the mechanics of DNA replication and cell division necessarily disrupt the protein-DNA interactions that comprise the epigenome. How cells re-establish their epigenetic identity, after cell division, is not well understood.

FUNCTIONAL CHROMATIN DOMAINS

Regulatory, noncoding DNA regions can have a variety of different functions, illustrated in Fig. 2.1A and variously classified as promoters, enhancers/silencers, super-enhancers, and insulators. Promoters are typically located within 1 to 2 kb of the transcriptional start site (TSS) of a gene. At a minimum, RNA-polymerase-II-dependent promoters contain binding sites for general transcription factors TBP and TFIIB, which form the core of the transcriptional complex. Within the promoter, transcription factor binding sites (TFBS) modulate gene expression by recruiting histone modifying enzymes and transcriptional coactivators or corepressors.

An enhancer/silencer is a short (50 to 1500 bp) region of DNA that can be bound by transcription factors to increase/decrease the likelihood that transcription of a particular gene will occur. Enhancers/silencers can act both in *cis* (within a chromosome) and rarely in *trans* (between chromosomes), can be located up to 1 Mb away from the gene, and can be upstream or downstream from the TSS. Promoters physically interact with their associated enhancers or silencers via three-dimensional chromatin "looping," facilitated by Mediator and Cohesin protein complexes (see Fig. 2.1D). Genes may be regulated by several enhancers/silencers, and each enhancer/silencer may modulate expression of one or more genes. A super-enhancer is a cluster of physically and functionally associated enhancers that regulates genes critical for cell identity. Super-enhancers are marked by high levels of enhancer-associated histone modification and bind high levels of cell-type specific and lineage-defining transcription factors (known as "master" transcription factors).

By blocking the physical interactions between enhancers and promoters, insulators help to restrict the set of genes that can be modulated by an enhancer. Insulators are bound by cohesin and CTCF proteins and form boundaries between silenced and active genes. Clusters of insulators separate heterochromatin from euchromatin, and the segments of active chromatin bounded by these clusters are known as topological domains-genomic regions, within which regulation occurs.

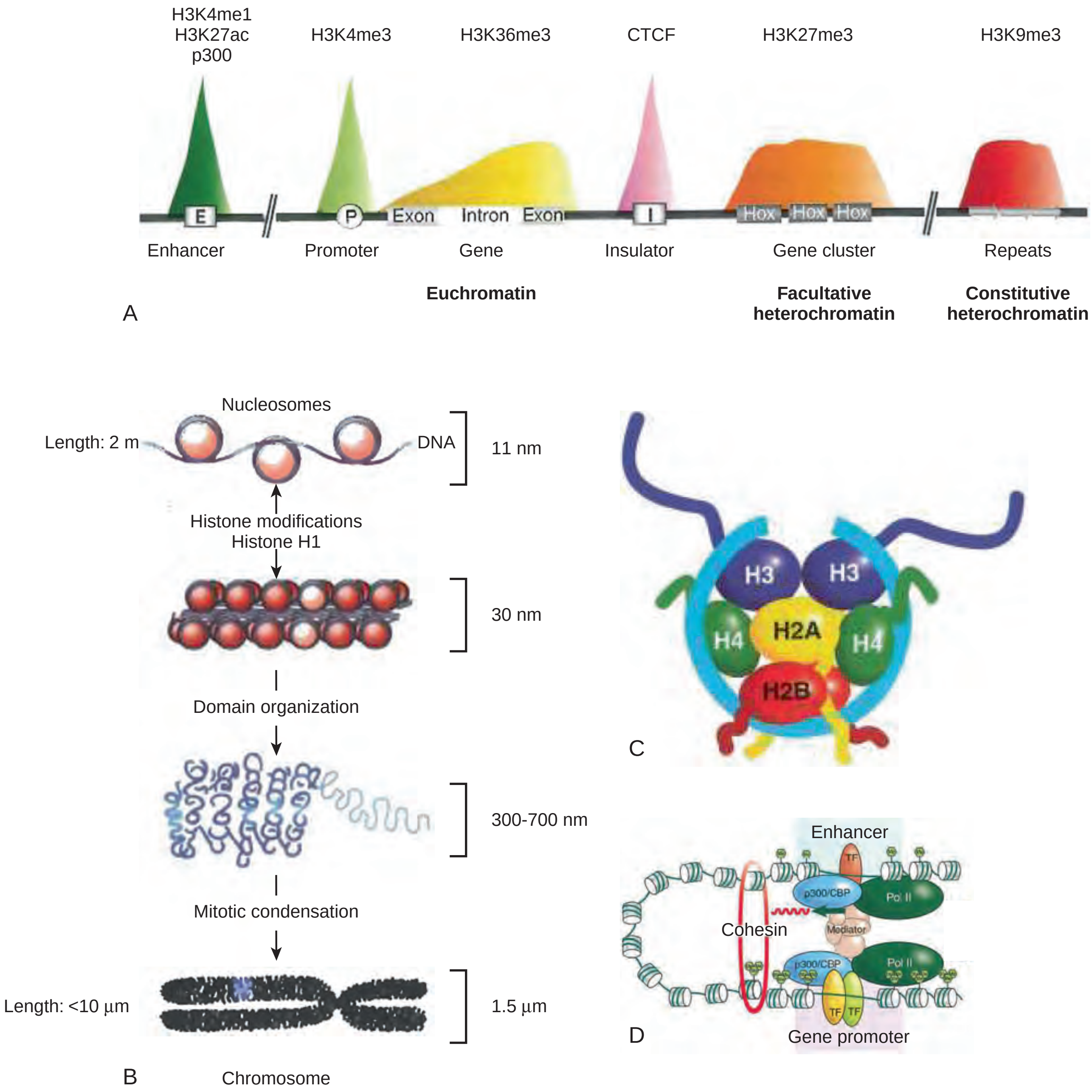

H3K4me1
H3K27ac
p300
H3K4me3
H3K36me3
CTCF
H3K27me3
H3K9me3
E
P
Exon
Intron
Exon
I
Hox
Hox
Hox
Enhancer
Promoter
Gene
Insulator
Gene cluster
Repeats
Euchromatin
Facultative heterochromatin
Constitutive heterochromatin
A
Nucleosomes
Length: 2 m
DNA
11 nm
Histone modifications
Histone H1
30 nm
Domain organization
300-700 nm
Mitotic condensation
Length: <10 µm
1.5 µm
Chromosome
B
H3
H3
H4
H2A
H4
H2B
C
Enhancer
TF
p300/CBP
Pol II
Cohesin
Mediator
p300/CBP
Pol II
TF
TF
Gene promoter
D

Figure 2.1 CHROMATIN STRUCTURE. (A) Functional chromatin domains and their characteristic histone modifications and protein-binding features. (B) Higher-order chromatin structure, from least condensed *(top)* to most condensed *(bottom)*. (C) Schematic of nucleosome with DNA *(light blue)* wrapped around histone octamer (H2A, H2B, H3, H4) having protruding histone tails. (D) Three-dimensional chromatin looping brings enhancers into close proximity with promoters via interactions with Cohesin and Mediator protein complexes.

DNA METHYLATION

Methylation of cytosine by DNA methyltransferases (DNMTs) occurs at 60% to 90% of CpG dinucleotides, in the mammalian genome. Methylated DNA is bound by methyl-CpG-binding domain proteins (MBDs) that recruit histone-modifying enzymes and chromatin-remodeling proteins, resulting in highly condensed heterochromatin. Methylation of promoter regions thereby represses transcription. Patterns of DNA methylation are replicated during DNA synthesis, and cell division and can be used to distinguish cell types and stages of differentiation.

The genome-wide pattern of DNA methylation, known as the methylome, has been characterized for a wide variety of tissues. Approximately 75% of the methylome is consistent across all cell types. The remaining 25% is differentially hypo- or hypermethylated in a cell type-specific manner. Cell type-specific hypomethylated regions are enriched for nucleosomes with modifications associated with active regions and TFBSs, while cell type-specific hypermethylation is associated with transcription factor silencing during differentiation. Aberrant DNA methylation is an extremely common feature of cancers, where hypermethylation of tumor-suppressor genes and hypomethylation of oncogenes may play important roles in oncogenesis and tumor progression.

HISTONES AND HISTONE VARIANTS

Histones H2A, H2B, H3, and H4 are known as the core histones, while histones H1 and H5 are known as the linker histones. The core histones all exist as dimers, and the four dimers come together to form one octameric nucleosome core. The smallest unit of chromatin structure is the nucleosome, consisting of 147 base pairs of DNA double helix wrapped around the core histone octamer (see

Fig. 2.1C). Linker histones, primarily H1, bind the nucleosome at the entry and exit sites of the DNA and allow the formation of higher order structure. Histone N-terminal domains are rich in lysine and arginine residues that are subject to a variety of post-translational modifications (see below).

In addition to these major histones, dozens of minor histone variants have been identified and are highly evolutionarily conserved. Some minor variants have very specific roles in chromatin regulation. For example, histone H3-like CENPA is associated with centromeres. H2A.Z is associated with the promoters and enhancers of actively transcribed genes. Histone H3.3 is associated with the body of actively transcribed genes. Phosphorylated H2A.X is found in regions around double-stranded DNA breaks and recruits DNA-repair machinery.

COVALENT HISTONE MODIFICATIONS

Histones undergo a variety of post-translational modifications (including methylation, acetylation, phosphorylation, SUMOylation, citrullination, ubiquitination, and ADP-ribosylation) that alter their interactions with DNA and nuclear proteins (Fig. 2.2). Histone-modifying enzymes are broadly classified as "writers," such as histone methyltransferases (HMTs) and histone acetyltransferases (HATs) that add functional groups, or "erasers," such as histone demethylases (HDMs) and histone deacetylases (HDACs). DNA-binding proteins contain a variety of "reader," protein domains (including bromodomains, chromodomains, Tudor domains, SANT domains, etc.) that have increased affinity for modified histones. In this way, covalently-modified histones constitute a "histone code," that is a defining feature of the dynamic epigenome. Each of the eight histones in a nucleosome can harbor multiple covalent modifications, giving the histone code tremendous combinatorial complexity.

Trimethylation of H3 lysine 4 (H3K4me3) and of H3 lysine 36 (H3K36me3) are both associated with transcriptional activation. H3K4me3 occurs at the promoter of active genes, and the degree of trimethylation is broadly correlated with transcriptional activity of the gene. H3K36me3 is deposited by lysine methyltransferase KMT2A (also known as MLL1) component of the Mediator complex and occurs in the body of active genes. H3K36me3 associates with elongating RNA polymerase II, thus marking actively transcribed genes. Mono- and dimethylation of H3 lysine 4 (H3K4me1/2) and acetylation of H3 lysine 27 (H3K27ac) are marks of active enhancers, and the degree of H3K27ac is broadly correlated with enhancer activation. H3K27ac is the enhancer mark most used to define super-enhancers.

Several histone modifications are particularly associated with repressed genes: trimethylation of H3 lysine 27 (H3K27me3), di- and tri-methylation of H3 lysine 9 (H3K9me2/3), and trimethylation of H4 lysine 20 (H4K20me3). H3K27me3 is deposited at both promoters and enhancers by the PRC2 Polycomb complex and mediates recruitment of PRC1, resulting in chromatin condensation

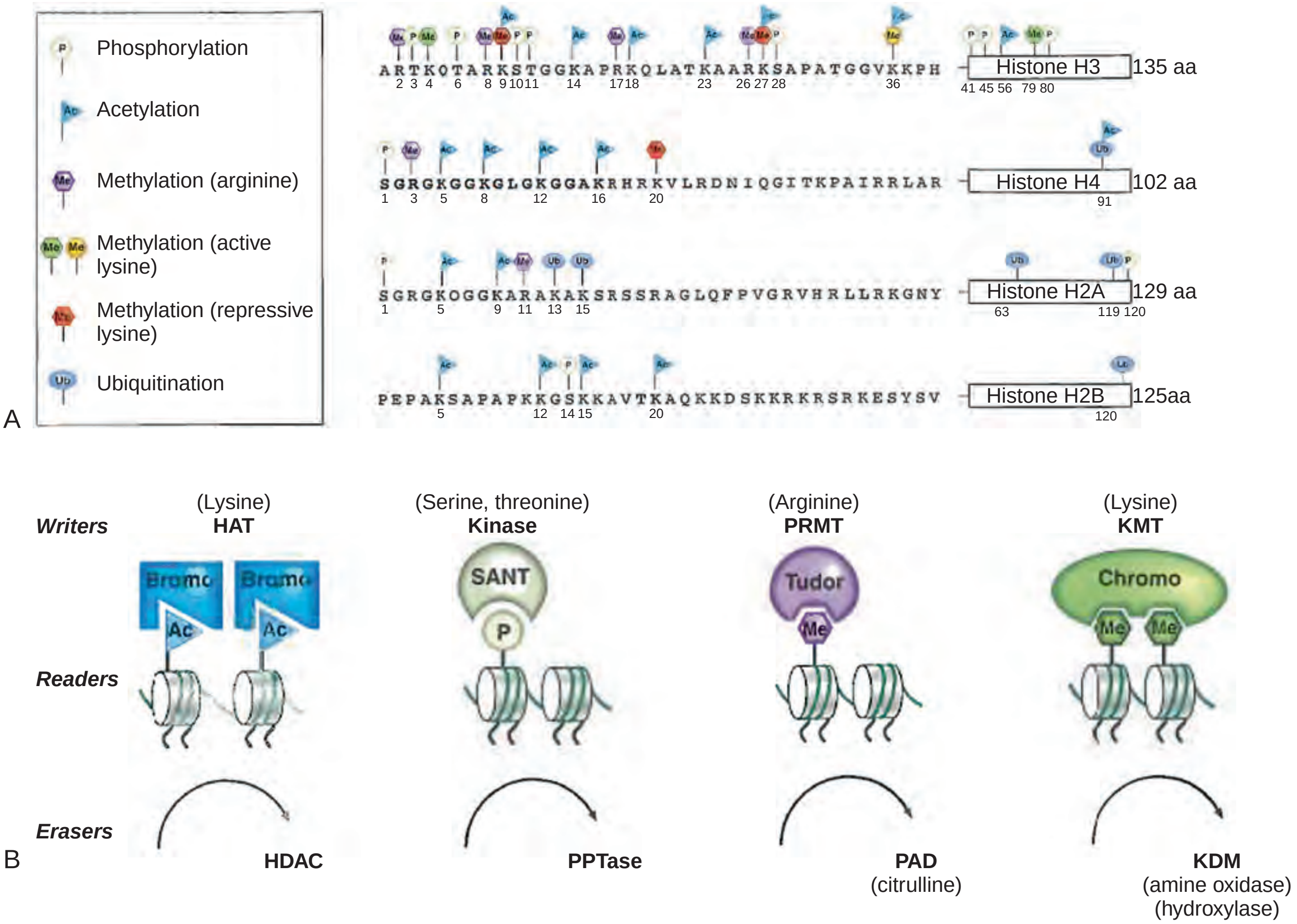

A
Phosphorylation
Acetylation
Methylation (arginine)
Methylation (active lysine)
Methylation (repressive lysine)
Ubiquitination
ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPH
2 3 4 6 8 9 10 11 14 17 18 23 26 27 28 36
Histone H3 135 aa
41 45 56 79 80
SGRGKGGKGLGKGGAKRHRKVLRDNIQGITKPAIRRLAR
1 3 5 8 12 16 20
Histone H4 102 aa
91
SGRGKQGGKARAKAKSRSSRAGLQFPVGRVHRLLRKGNY
1 5 9 11 13 15
Histone H2A 129 aa
63 119 120
PEPAKSAPAPKKGSKKAVTKAQKKDSKKRKRSRKESYSV
5 12 14 15 20
Histone H2B 125aa
120
P
Ac
Me
Ub
B
Writers
Readers
Erasers
(Lysine)
HAT
(Serine, threonine)
Kinase
(Arginine)
PRMT
(Lysine)
KMT
Bromo
Bromo
Ac
Ac
SANT
P
Tudor
Me
Chromo
Me
Me
HDAC
PPTase
PAD
(citrulline)
KDM
(amine oxidase)
(hydroxylase)

Figure 2.2 HISTONE MODIFICATIONS AND HISTONE-MODIFYING ENZYMES. (A) The N-terminal tails of core histones contain lysine (K), arginine (R), serine (S), and threonine (T) residues that are common targets for a variety of post-translational modfications, including methylation (Me), acetylation (Ac), phosphorylation (P), and ubiquitination (Ub). (B) Histone-modifying enzymes can be broadly classified as either "writers" or "erasers" based upon addition or removal of functional groups, respectively. Moreover, many DNA-binding proteins contain "reader" protein domains (Bromodomains, SANT domains, Tudor domains, or Chromodomains) having increased affinity for acetylated, phosphorylated, methyl-arginine, and methyl-lysine modified nucleosomes, respectively. *HAT*, Histone acetyltransferase; *HDAC*, histone deacetylase; *KDM*, lysine demethylase; *KMT*, lysine methyltransferase; *PAD*, peptidylarginine deiminase; *PPTase*, protein phosphatase; *PRMT*, protein arginine methyltransferase.

and transcriptional repression. H3K9me2/3 and H4K20me3 are both highly associated with heterochromatin. H3K9me2/3 serves as a binding site for heterochromatin protein 1 (HP1). HP1 recruits additional histone-modifying enzymes, including the lysine methyltransferases, KMT5B and KMT5C, that produce H4K20me3.

Stem cells harbor promoters, marked by both activating H3K4me3 and repressive H3K27me3. Upon cellular differentiation, these "bivalent," or "poised," promoters are rapidly converted to either an activated or repressed state.

The Aurora B kinase phosphorylates histone H3 at serine 10 (phospho-H3S10), triggering the chromosome condensation during mitosis. Phosphorylation of H2B at serine 14 (phospho-H2BS14) mediates chromatin condensation during apoptosis.

TRANSCRIPTION FACTORS

Transcription factors are proteins that bind to specific DNA sequences, contribute to modulation of gene expression, and are the key determinants of the epigenetic state of the cell. Transcription factors are modular in structure and contain the following domains:

- DNA-binding domain (DBD), having high affinity for specific sequences of DNA,
- trans-activating domain (TAD) or trans-repressive domain (TRD), mediating protein-protein interactions with transcriptional coregulators, and
- an optional signal-sensing domain (SSD) (e.g., a ligand-binding domain), which can modulate DNA-binding and/or protein-binding activity, in response to cellular cues.

DNA sequences, having high affinity for transcription factor binding, are often referred to as response elements. Transcription factor binding to accessible promoters and enhancers recruits additional proteins, such as coactivators/corepressors, chromatin remodelers, histone-modifying enzymes, and RNA polymerases to modulate gene expression.

Although sequence-specific DNA binding is a defining feature of transcription factors, chromatin accessibility is a key determinant of transcription factor binding. Most transcription factors preferentially bind nucleosome-free DNA. In many cases, a transcription factor needs to compete for DNA binding with other transcription factors, histones, and non-histone chromatin proteins. The competitive balance between nucleosome and transcription factor binding is critically affected by chromatin remodeling complexes (see below). In practice, only a small fraction of potential response elements are actually bound, and many experimentally detected TFBS lack canonical response elements. The genome-wide pattern of transcription-factor binding can be experimentally determined using chromatin immunoprecipitation and next-generation sequencing (ChIP-seq, see below) and is known as the transcription-factor cistrome.

Different cell types typically express both common and distinct transcription factors. Moreover, the cistrome of a transcription factor differs among cell types, reflecting differences in chromatin accessibility and helping to define active promoters and enhancers. Master transcription factors are a special subset of lineage-defining transcription factors, having expression restricted to specific cell types and demonstrate very high binding at super-enhancers.

CHROMATIN REMODELERS

Chromatin remodeling alters the position, occupancy, or histone composition of a nucleosome within chromatin. ATP-dependent changes in nucleosome position and occupancy are mediated by the multisubunit, chromatin remodeling complexes, which fall into four families: SWI/SNF, ISWI, CHD, and INO80. ATP-independent changes in nucleosome position and occupancy can occur in response to transcription factor binding, or through the action of histone chaperones that can deposit, remove, or exchange histones. Each of these activities alters the accessibility of DNA to transcription factors and other DNA-binding proteins.

Complexes in the SWI/SNF family include the BAF, PBAF, and WINAC complexes they and contribute to transcriptional regulation and DNA repair. In addition to nucleosome sliding, SWI/SNF complexes have been implicated in chromatin looping, as well as eviction of H2A/H2B dimers from the nucleosome. Members of the INO80 family of complexes participate in transcription and DNA repair but can also catalyze the exchange of histones from the nucleosome structure. For example, SRCAP can exchange the H2A/H2B histone dimer for a variant H2A.Z/H2B dimer, which is associated with actively transcribed promoters. The CHD nucleosome remodeling family is the largest, and its best-characterized member is the NURD complex. A subset of NURD complexes incorporates the MBD2 subunit, which preferentially binds methylated DNA and promotes the repression of genes through its remodeling and HDAC activities. Many alternative NURD complexes incorporate different DNA-binding proteins and can contribute to transcriptional activation. ISWI family chromatin remodeling complexes catalyze the sliding of nucleosomes in short increments and participate in nucleosome spacing after DNA replication, RNA polymerase elongation, transcriptional regulation, and DNA damage repair.

Remarkably, cancer genome sequencing studies have identified frequent inactivating mutations in chromatin remodelers in a variety of human cancers. The SWI/SNF complex has particularly emerged as a powerful tumor suppressor whose disruption occurs in nearly 20% of primary human tumors.

EXPERIMENTAL APPROACHES IN EPIGENETICS

As dramatically as high-throughput sequencing has impacted our ability to understand the genome, its facilitation of epigenomic research has been equally profound. A wide variety of experimental approaches are in use and in development for epigenomic research, but most are predicated on detecting (1) DNA methylation, (2) protein-DNA interactions, (3) chromatin accessibility, and (4) three-dimensional chromatin structure/looping (Fig. 2.3).

A key feature of all these techniques is the ability to isolate a subset of DNA sequences from the larger genome, based upon a specific chromatin feature. This has several practical implications for experiments. First, many techniques rely on cross-linking agents, such as formaldehyde, to covalently link proteins to each other and to the DNA they bind. Cross-linking rapidly kills cells and "freezes" chromatin. Second, all these experimental techniques involve fragmenting chromosomes into much smaller pieces, either by physical disruption (sonication) or endonuclease treatment. Third, the chromatin subset of interest is extracted and enriched by immunoprecipitation, isolation of chromatin fragments of specific sizes, and/or sequence-specific amplification via polymerase chain reaction (PCR). Finally, DNA is isolated from this chromatin subset and subjected to next-generation sequencing.

A common technique for determining the genome-wide methylome is Bisulfite-seq. Treatment of DNA with bisulfite converts cytosine residues to uracil but leaves 5-methylcytosine (5mC) residues unaffected. Comparing results of bisulfite-treated and -untreated DNA sequencing permits genome-wide differentiation of methylated and un-methylated cytosines. Alternatively, methylated DNA immunoprecipitation (MeDIP-seq) utilizes an antibody, recognizing 5mC to enrich for methylated segments of the genome, prior to next-generation sequencing.

DNA binding by transcription factors, transcriptional machinery, structural proteins, and covalently modified histones can all be mapped in a genome-wide fashion using ChIP-seq. ChIP-seq typically requires cross-linking of proteins to DNA, using formaldehyde or other chemical fixation techniques. Antibodies are then used to enrich for a protein of interest, and the associated DNA fragments

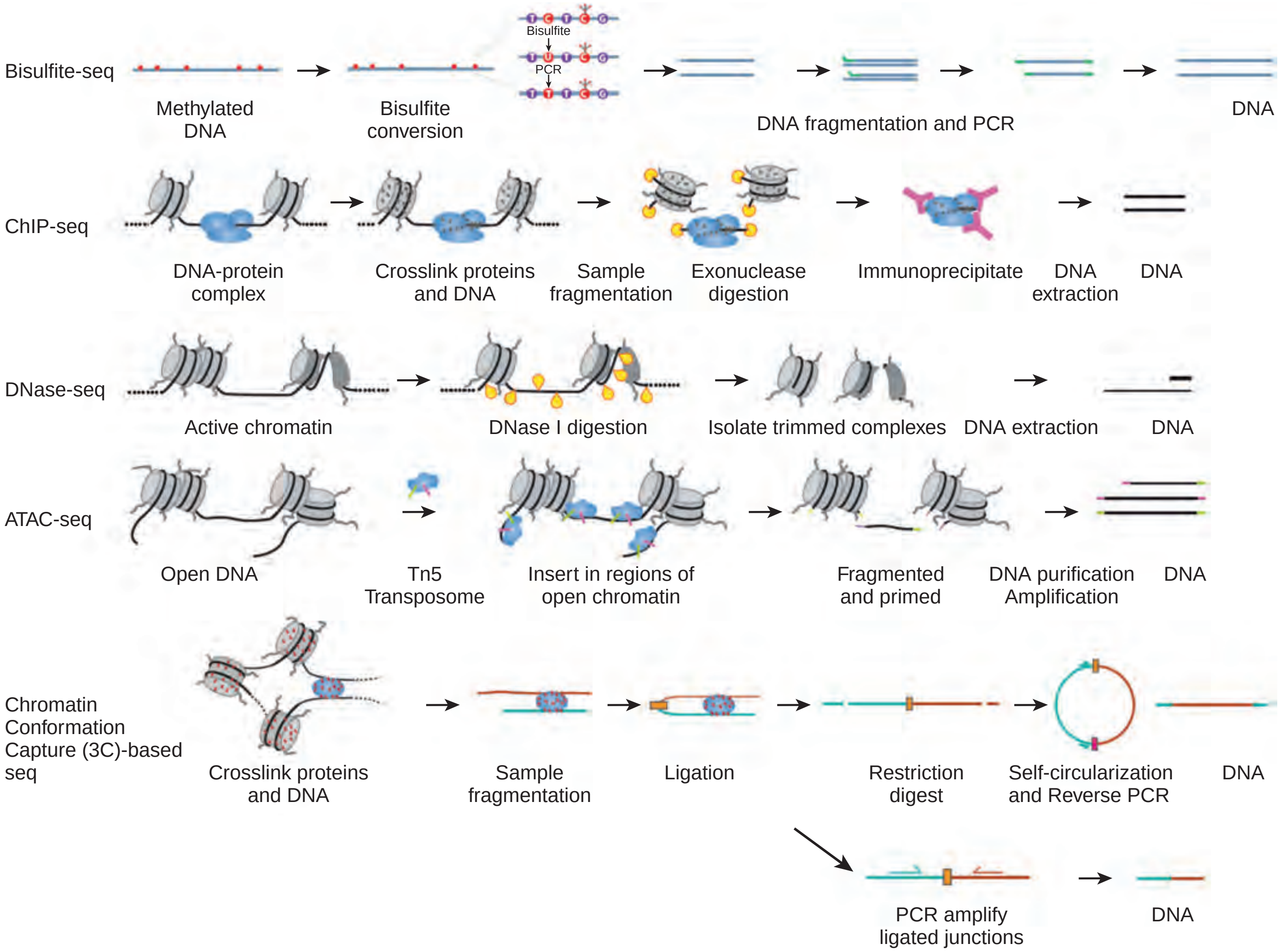

Bisulfite-seq
Methylated DNA
Bisulfite conversion
Bisulfite
PCR
DNA fragmentation and PCR
DNA
ChIP-seq
DNA-protein complex
Crosslink proteins and DNA
Sample fragmentation
Exonuclease digestion
Immunoprecipitate
DNA extraction
DNA
DNase-seq
Active chromatin
DNase I digestion
Isolate trimmed complexes
DNA extraction
DNA
ATAC-seq
Open DNA
Tn5 Transposome
Insert in regions of open chromatin
Fragmented and primed
DNA purification Amplification
DNA
Chromatin Conformation Capture (3C)-based seq
Crosslink proteins and DNA
Sample fragmentation
Ligation
Restriction digest
Self-circularization and Reverse PCR
DNA
PCR amplify ligated junctions
DNA

Figure 2.3 EXPERIMENTAL TECHNIQUES IN EPIGENOMICS. Schematic representations of Bisulfite-seq, ChIP-seq, DNase-seq, assay for transposase-accessible chromatin, and chromatin conformation capture-based (3C-based) experimental techniques. *PCR*, Polymerase chain reaction.

identified by next-generation sequencing. ChIP-seq is the most versatile technique in epigenomic research. For example, genome-wide maps of histone modifications (such as H3K27ac or H3K36me3), active RNA polymerase II, insulator protein CTCF, superenhancer-associated Mediator complex, and transcription factors can all be accomplished via ChIP-seq, using different antibodies.

Assay for transposase-accessible chromatin (ATAC-seq) and DNase-seq are two techniques used to assess genome-wide chromatin accessibility. DNase-seq exposes native chromatin to cleavage by the DNase I endonuclease, the activity of which is inversely related to protein binding by DNA. Chromatin regions most sensitive to DNase I cleavage are termed DNase hypersensitive sites (DHSs) and are highly enriched for transcriptionally active and gene-regulatory segments of the genome. ATAC-seq is an alternative measure of chromatin accessibility, based upon susceptibility of chromatin regions to the activity of a hyperactive transposase. Transposase activity is highest in nucleosome-free regions, and ATAC-seq typically identifies transcriptionally active and gene-regulatory regions, largely similar to DNase-seq. Importantly, these two assays provide genome-wide snapshots of active chromatin regions, irrespective of the involved transcription factors or chromatin regulators.

Chromosome conformation capture (3C) techniques aim to identify three-dimensional chromatin loops, such as those bringing promoters near enhancers. All 3C-based methods begin with chromatin cross-linking. Following DNA fragmentation, a random DNA ligation step is performed to generate circular DNA molecules. Sequencing these DNA loops yields fragment pairs that, although separated by many kb in linearly organized DNA, are physically approximated in functional chromatin. 3C-based methods have tremendous potential to map enhancers to the genes whose activity they modulate. Beyond the original 3C method, which requires *a priori* selection of two potentially interacting genomic regions to allow for proper PCR primer design, several higher throughput techniques have been developed. These allow for the detection of interactions between a single genomic locus and other regions (4C), all genomic interactions within a given chromosomal region (5C), and all DNA-DNA interactions using high-throughput sequencing (Hi-C). Hi-C allows for the identification of topologically-associating domains (TADs), which are spans of the genome whose boundaries are marked by CTCF and cohesion binding; they associate more often with each other than other genomic regions. Disruption of TADs has been linked to multiple disease processes, including cancer.

Improved technical capabilities have led to the application of many of the above techniques to human tissue at both the bulk and single-cell level. For example, it is now feasible to profile both chromatin accessibility using ATAC-seq and transcription factor binding profiles using ChIP-seq in freshly acquired and frozen tissue. Though these assays are not currently feasible in formalin-fixed, paraffin-embedded (FFPE) tissue, it is possible to identify active enhancers in these samples, using ChIP-seq for acetylation at the histone H3K27 position. Single-cell analysis of chromatin accessibility, termed scATAC-seq, is also possible in fresh and frozen tissue. This approach allows for unprecedented analysis of cellular heterogeneity within tissues and can be combined with sequencing of transcribed RNAs from the same cells.

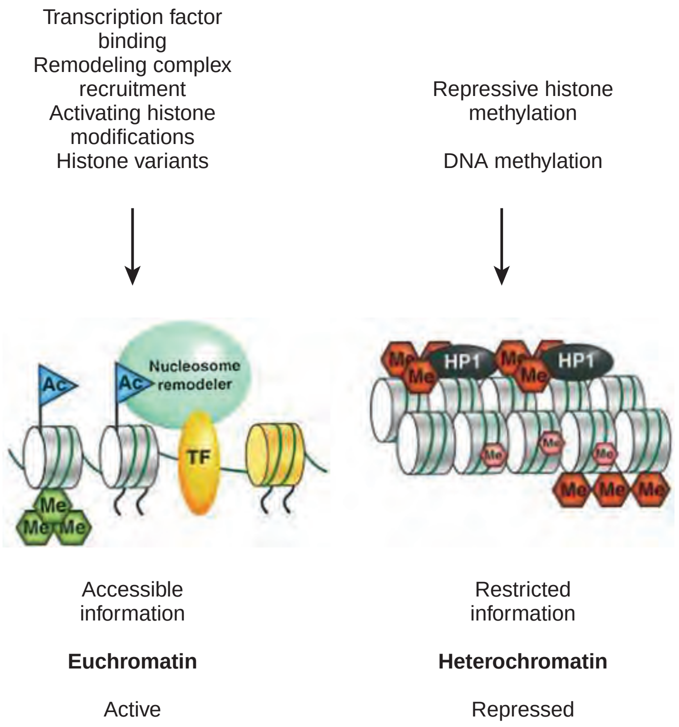

Transcription factor binding
Remodeling complex recruitment
Activating histone modifications
Histone variants
Repressive histone methylation
DNA methylation
Ac
Ac
Nucleosome remodeler
TF
Me
Me
Me
HP1
HP1
Me
Accessible information
Euchromatin
Active
Restricted information
Heterochromatin
Repressed

Figure 2.4 DNA-PROTEIN INTERACTIONS IN EUCHROMATIN AND HETEROCHROMATIN.

The fundamental challenge in epigenomic research is integrating the results of many different experiments to understand how the myriad chromatin features interact in regulating transcription and cellular behavior (Fig. 2.4). Thus, interpreting the epigenetic code requires measuring transcriptional activity in addition to chromatin features. Measurement of global transcript levels by mRNA sequencing (RNA-seq) is now the most common technique used to study gene expression, but interest is growing in the related genomic run-on sequencing (GRO-seq) and precision run-on sequencing (PRO-seq) techniques. These approaches measure active transcription, rather than total cellular transcript level and therefore holds promise for improved correlation with epigenomic data.

Several collaborative research consortia are dedicated to generating and curating genome-wide epigenetic data for public use, including the National Human Genome Research Institute's (NHGRI) ENCODE and Roadmap Epigenomics Projects. These resources include results of histone modifications and transcription factor ChIP-seq, DNase-seq, DNA Methyl-seq, and RNA-seq experiments for hundreds of human cancer cell lines and primary human tissues, respectively. The most versatile and widely available tool for visualizing epigenomic data is the UCSC Genome Browser, which incorporates easy access to ENCODE, Roadmap, and other data sources for integrative analysis of epigenomic and gene expression data (Fig. 2.5).

MECHANISMS OF DISEASE

The mechanisms of disease we describe here are not "strictly" epigenetic, insofar as they are all predicated on changes in genome sequence or structure (genetic mutations). Nonetheless, our insights into disease pathogenesis and development of novel therapeutic targets have been vastly informed by understanding the ways in which these genetic changes drive aberrant chromatin regulation and gene expression. The examples given below represent only a subset of the known epigenetic drivers of disease.

Sickle cell anemia has long been known to result from a point mutation in the hemoglobin beta gene. The severity of this often life-threatening hemoglobinopathy is attenuated in patients having increased expression of the fetal gamma hemoglobin variant, a trait known as hereditary persistence of fetal hemoglobin (HFPH). Genome-wide association studies in patients with HFPH identified frequent single nucleotide polymorphisms (SNPs) in a small number of noncoding regions, near the BCL11A gene on chromosome 2. Subsequent studies have elegantly demonstrated that these SNPs are in erythroid-specific enhancers, modulating BCL11A expression. The HFPH-associated SNPs diminish binding of transcription factors GATA1 and TAL1, which results in decreased expression of BCL11A. Because BCL11A is required for efficient silencing of fetal hemoglobin expression, sickle cell anemia patients having these common variant SNPs demonstrate elevated fetal hemoglobin throughout adulthood and are often protected from the most severe manifestations of the disease. Just as sickle cell anemia is among the most striking examples of disease, caused by a point mutation in the coding region of a gene, these BCL11A enhancer SNPs demonstrate the power of gene-regulatory elements to modulate the sickle cell disease phenotype.

Chromosomal translocations that result in aberrant expression of oncogenes or leukemogenic transcription factors are another common mechanism of disease. The classical example of this is Burkitt's lymphoma, in which t(8;13) translocations juxtapose the highly active immunoglobulin heavy chain enhancers and the c-myc oncogene, driving myc overexpression and oncogenic transformation of mature B cells. Similarly, many different translocations have been identified in T acute lymphoblastic leukemia (T-ALL), whereby overexpression

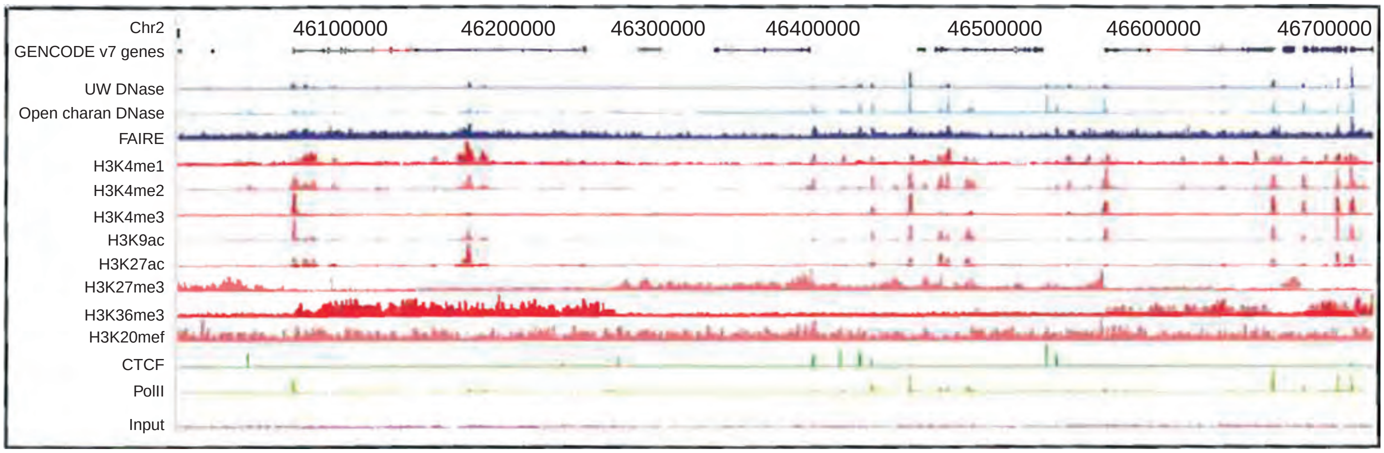

Chr2
46100000
46200000
46300000
46400000
46500000
46600000
46700000
GENCODE v7 genes
UW DNase
Open charan DNase
FAIRE
H3K4me1
H3K4me2
H3K4me3
H3K9ac
H3K27ac
H3K27me3
H3K36me3
H3K20mef
CTCF
PolII
Input

Figure 2.5 VISUALIZING THE EPIGENOMIC LANDSCAPE. Sample of a UCSC Genome Browser representation of a 700-kb segment of chromosome 2 in the lymphoblastoid human cell line GM12878. Integrating publicly-available, genome-wide data for a variety of epigenomic experiments is the cornerstone of efforts to decode the epigenome.

of master transcription factors such as TAL1, LMO1, LMO2, and HOX11 is driven by chromosomal rearrangements involving the T-cell receptor loci.

An alternate mechanism driving TAL1 overexpression in T-ALL has recently been described, in which small genomic insertions (2 to 18 bp) upstream of the TAL1-coding region introduce novel binding sites for the MYB transcription factor. This aberrant MYB binding recruits additional transcription factors RUNX1, GATA-3, and TAL1, as well as the HAT CBP and forms a super-enhancer driving leukemogenic TAL1 overexpression.

Many different translocations resulting in fusion of the mixed-lineage leukemia (MLL1/KMT2A) gene, located on chromosome 11q23, with over 70 different partner proteins have been identified in infant ALL and therapy-associate acute myeloid leukemia (AML). Only recently have the mechanisms underlying the leukemogenic nature of these translocations been elucidated. Leukemogenic MLL1 fusion proteins fuse the N-terminal targeting domain with a transcription elongation factor, such as ENL or AF9. The resulting fusion protein drives overexpression of common MLL1 targets by recruiting the DOT1L complex (having H3K79 methyltransferase activity) and the positive transcription elongation factor b (P-TEFb) complex (containing CDK9 and phosphorylating RNA PolII). Moreover, a subset of leukemogenic MLL1 fusions can inhibit the transcriptional repressive activity of PRC1. In summary, MLL translocations in ALL and AML define a paradigm of leukemia development, based upon transcriptional dysregulation through aberrant targeting and control of transcription elongation activity.

As noted earlier, inactivating mutations in components of chromatin remodeling complexes, such as SWI/SNF, have been identified in a wide variety of human cancers. For example, a recent study found mutations in the ARID1A subunit of SWI/SNF in 17% of Waldenström's macroglobulinemia cases, and patients with ARID1A mutations had more aggressive disease features. In addition to their nucleosome remodeling activities, chromatin remodeling complexes contribute to three-dimensional chromatin structure, participate in DNA damage repair, modulate transcription factor binding, and recruit histone-modifying enzymes. Precisely how disruption of these many chromatin regulatory activities contributes to disease is an extremely active area of research.

In addition to these epigenetic contributions to disease development, much interest has evolved in potential epigenetic mechanisms of resistance to existing cancer therapies. One example of this is resistance of T-ALL to γ-secretase inhibitors (GSIs), used to target abnormal NOTCH1 activation. In vitro, treatment of T-ALL cell lines with GSIs kills a large proportion of cells but leaves behind a "persister" population of GSI-resistant cells. If GSI treatment is removed, these persister cells revert to their prior GSI-sensitive state, suggesting an epigenetic mechanism of drug resistance. A screen of chromatin regulators, required for persister cell viability, identified the bromodomain containing 4 protein (BRD4), a key factor in activating transcriptional elongation. This study, and many others, has ignited broad interest in other potential epigenetic mechanisms of therapy resistance, as well as BRD4 as a specific therapeutic target.

EPIGENETIC THERAPIES

Epigenetic therapies are among the most active areas of pre-clinical and clinical cancer research, due to their potential to specifically target chromatin-mediated disease mechanisms and the expectation that these therapies will have fewer side effects than conventional cytotoxic chemotherapies. As seen in Table 2.1, several classes of drugs have emerged, and we will briefly discuss the rationales for their ongoing development.

The first class of epigenetic drugs to show significant clinical benefit is the DNMT inhibitors, particularly 5-azacytidine and its analog decitabine. As discussed earlier, abnormal DNA methylation is a common feature of many cancers. However, azacytidine is primarily beneficial in treating myelodysplastic syndromes (MDS) and AML. The theoretical basis for this therapeutic effect is re-activation of key tumor suppressor genes, by disruption of DNA methylation at their promoters. However, this mechanism has not yet been confirmed in azacytidine-treated patients, and alternate mechanisms of action are under investigation.

TABLE 2.1 Emerging Epigenetic Therapies

Class	Target	Disease
DNA methylation inhibitors	DNMTs	MDS, AML
Histone-modifying enzymes		
HMT inhibitors	DOT1L, EZH2, non-specific	MLL-rearranged leukemias, NHL, MDS, AML
HDAC inhibitors	HDAC6, non-specific	MM, CLL, lymphoma
HMT activators	SIRT1, SIRT5	MM
HDM inhibitors	KDM1A	AML
Bromodomain inhibitors	BRD4, p300/CBP, non-specific	Hematologic malignancies

AML, Acute myeloid leukemia; *BRD4*, bromodomain containing 4 protein; *CLL*, chronic lymphocytic leukemia; *DNMTs*, DNA methyltransferases; *HDAC*, histone deacetylase; *HDM*, histone demethylase; *HMT*, histone methyltransferase; *MDS*, myelodysplastic syndrome; *MM*, multiple myeloma; *NHL*, non-Hodgkin's lymphoma.

By far the largest class of epigenetic therapies is inhibitors of histone-modifying enzymes. Drugs inhibiting HMTs and HDACs are most prevalent, though several compounds that activate HDACs or inhibit HDMs are also being developed. For example, inhibitors of the H3K79 methyltransferase DOT1L are in clinical trials for MLL-rearranged leukemias. Alternately, inhibitors of the H3K27 methyltransferase EZH2 (the catalytic component of the PRC2 complex) are being tested in multiple hematologic malignancies. Specific inhibitors of HDAC6 are being used in trials for multiple myeloma, while drugs having broad HDAC-inhibitory activity are in ongoing trials for a wide variety of hematologic malignancies.

Newer classes of epigenetic therapies include bromodomain inhibitors that target the BET proteins and others such as p300. As discussed briefly above, bromodomains are an extremely common feature of DNA-binding proteins and preferentially recognize acetylated chromatin. The abundance of bromodomain-containing, DNA-binding proteins makes development of substrate-specific drugs extremely challenging. However, initial clinical trials using BET bromodomain inhibitors, having broad binding specificity, have been very promising in a wide variety of advanced hematologic and non-hematologic malignancies. The likely therapeutic targets of these drugs are the transcriptional machinery itself, though many additional mechanisms plausibly contribute.

FUTURE DIRECTIONS

Interpreting the "epigenetic code" holds great potential for bridging the gaps between the molecular biology of the genome, cellular biology, and physiology of health and disease. The application of next-generation sequencing technology and development of novel techniques to interrogate chromatin have produced a profusion of new epigenetic data. Collaborative epigenomics projects such as ENCODE and the Epigenome Roadmap, as well as genomics efforts such as the 1000 Genomes Project and the Cancer Genome Atlas, make these vast data widely available to researchers. The substantial challenge remains integrating and interpreting these data to generate novel insights into human health and disease. Substantial collaboration between biomedical scientists, computational biologists, and physicians will be necessary to design, execute, and analyze projects with high relevance to medical progress.

SUGGESTED READINGS

Allis CD, Jenuwein T, Reinberg D, eds. *Epigenetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2007.

Bauer DE, Kamran SC, Lessard S, et al. An erythroid enhancer of *BCL11A* subject to genetic variation determines fetal hemoglobin level. *Science*. 2013;342:253.

Chadwick LH. The NIH roadmap epigenomics program data resource. *Epigenomics*. 2012;4:317–324.

Chaidos A, Caputo V, Karadimitris A. Inhibition of bromodomain and extra-terminal proteins (BET) as a potential therapeutic approach in haematological malignancies: emerging preclinical and clinical evidence. *Ther Adv Hematol*. 2015;6:128–141.

Clarke L, Zheng-Bradley X, Smith R, et al. The 1000 Genomes Project: data management and community access. *Nat Methods*. 2012;9:459–462.

Consortium EP. A user's guide to the encyclopedia of DNA elements (ENCODE). *PLoS Biol*. 2011;9:e1001046.

Karolchik D, Barber GP, Casper J, et al. The UCSC Genome Browser database: 2014 update. *Nucleic Acids Res*. 2014;42:D764.

Knoechel B, Roderick JE, Williamson KE, et al. An epigenetic mechanism of resistance to targeted therapy in T cell acute lymphoblastic leukemia. *Nat Genet*. 2014;46:364–370.

Mansour MR, Abraham BJ, Anders L, et al. Oncogene regulation. An oncogenic super-enhancer formed through somatic mutation of a noncoding intergenic element. *Science*. 2014;346:1373–1377.

National Cancer Institute The cancer genome atlas data portal. *Nature*. 2013;458:719–724.

Slany RK. The molecular mechanics of mixed lineage leukemia. *Oncogene*. 2016;35:5215–5223.

Treon SP, Xu L, Yang G, et al. MYD88 L265P Somatic mutation in Waldenström's macroglobulinemia. *N Engl J Med*. 2012;367:826–833.

Weinstein JN, Collisson EA, Mills GB, et al. The cancer genome atlas pan-cancer analysis project. *Nat Genet*. 2013;45:1113–1120.

CHAPTER 3

GENOMIC APPROACHES TO HEMATOLOGY

Gareth J. Morgan and Eileen M. Boyle

INTRODUCTION

The publication of the sequence of the human genome in 2001[1] heralded a new era in biomedical research and delivered a novel perspective on the biologic basis of the leukemias and lymphomas. A major tenet of these new approaches was their emphasis on the generation of large unbiased datasets as a means of discovery. The rapid application of this methodology combined with ready access to tissue for analysis pushed hematology into an era of "*precision medicine*" based on the use of molecular diagnostics and targeted interventions. The speed with which this approach was taken up was enhanced by the reduction in sequencing costs, which made testing generally available. In the background, large-scale efforts led to the establishment of repositories of genomic data (e.g., The Cancer Genome Atlas[2]), which allowed the development of effective diagnostic, prognostic, and predictive biomarkers. The extension of this information by integrating molecular markers into the World Health Organization (WHO) classification enhanced disease definitions, making their behavior easier to understand and predict.[3] Risk-stratified therapy based on the integration of molecular markers is now a reality for many hematologic cancers, and the development of predictive markers is a particular aim based on targeting specific genomic variants (e.g., BRAF inhibitors targeting *BRAF* V600E mutations). This chapter describes the approaches and progress that have been made for the integration of genomic approaches into the clinic to improve the management of hematologic diseases.

GENERAL PRINCIPLES OF GENOMIC TESTING

Genomic Analysis

Analysis of the genome (Table 3.1) aims to identify, quantify, or compare genomic features such as DNA sequence, structural variation (SV), gene expression, or regulatory and functional annotation at a genomic scale. Methods for genomic analysis typically require high-throughput sequencing and computational analysis.

DNA-based whole genome high-throughput sequencing approaches for the detection of genetic variants have been used to identify differences between individuals or pathologic conditions. Typically, this approach aims at identifying single-nucleotide variants (SNVs), small insertions and deletions (indels), and SVs. SVs are diverse, ranging from approximately 50 base pairs (bp) to more than megabases in size, and affect more of the genome than any other class of sequence variant. They comprise a number of subclasses of unbalanced copy number abnormalities (CNAs), which include deletions, duplications, and insertions, as well as balanced rearrangements, such as inversions and interchromosomal and intrachromosomal translocations. In addition, SVs include mobile element insertions, multiallelic copy number variants of highly variable copy number, segmental duplications, and complex rearrangements that consist of multiple combinations of these events.

Genome-wide analysis of gene expression, also referred to as transcriptomics, is the study of transcription at the genomic scale. These analyses use RNA and analysis results from microarrays or high-throughput sequencing. The results can be used to determine the range of genes expressed and their isoforms within a particular cell or tissue type, for a disease, or associated with a clinical phenotype such as risk status.

The epigenome refers to the changes made to the DNA that govern its function and include methylation and acetylation of DNA, histones, nonhistone chromatin proteins, and nuclear RNA. The tools for studying epigenetic phenomena are focused on the global analysis of epigenetic status of the cells and tissues. With improvements in epigenomic profiling, new opportunities are available to understand normal epigenomes and their perturbations in cancer.

The Importance of Sample Quality

The acquisition of the appropriate samples for a genomic analysis is one of the most crucial steps for the generation of an accurate result. This is particularly true for gene expression analysis based on samples of RNA. Gene expression is a dynamic process that can be affected by cellular manipulation, RNA abundance and stability, isolation methodology, and the time between when the sample was obtained and subsequently isolated. The highest-quality RNA is obtained if, as soon as possible after harvesting a sample, cells are dissolved in a solution that inactivates RNase enzymes. It is also possible to measure gene expression from stored tissue such as formalin-fixed, paraffin-embedded (FFPE) tissues, but the variability in quality of these data makes routine interpretation difficult. The cellular makeup of samples for RNA analysis is also important (e.g., tumor cells, normal cells, stromal cells, and immune cells) if tumor-specific expression patterns are important; if this is the case, then methods for cell separation are required. This requirement may be less of an issue in tissues such as bone marrow samples taken from patients with acute myeloid leukemia (AML), where the number of blasts cells is high; however, if the percentage of blast cells is low, a selection approach is required. Methods used for this purpose include: flow cytometry, immunomagnetic bead sorting, and laser-capture microdissection. A good example of where cell selection is important is in multiple myeloma, where CD138 selection is crucial for gene expression analysis and is mandated in guidelines for interphase fluorescence in situ hybridization (iFISH) analysis.[4]

At the DNA level the admixture of nonmalignant cells within a tumor may obscure the presence of mutations in the tumor cells, especially if they constitute a minority population. However, to be sure of detecting mutations in a tumor cell population, a greater depth of sequencing is required. Therefore it is critical to have a rough estimate of the purity of the sample so that the appropriate genomic approach can be used. Furthermore, the clonal fraction of tumor cells carrying a specific mutation is important if it is considered to be "actionable." In this respect, knowing the subclonal percentage is important not only for detecting the mutation but also to be sure that a therapeutic benefit could be expected.

Analytical Considerations

It is important to distinguish approaches used for discovery from those required for a routine diagnosis. For discovery, unsupervised learning approaches are used in which samples are grouped on the basis of data obtained without regard to any prior knowledge of either the samples or the disease. Unsupervised learning methods that have been used include hierarchical clustering, principal component analysis, nonnegative matrix factorization, *k*-means clustering,

TABLE 3.1 **Definition of the Different Genomes**

Nuclear genome represents the DNA that may be found within the nucleus of a cell that encodes the majority of DNA in eukaryote cells. In humans, it comprises approximately 3,200,000,000 nucleotides, divided into 24 linear sections, each contained in a different chromosome. These 24 chromosomes consist of 22 autosomes and the two sex chromosomes. The vast majority of cells, somatic cells, are diploid (in contrast to gametes, which are haploid). The spontaneous mutation rate of nuclear DNA is low (0.3%).

Mitochondrial genome: is a closed circular DNA molecule of approximately 16,500 nucleotides, present within the mitochondria. It contains 37 genes, all of which are crucial for normal mitochondrial function. Thirteen of these genes encode enzymes involved in oxidative phosphorylation; the remaining genes code for transfer RNA (tRNA) and ribosomal RNA (rRNA). Haploid, the mitochondrial genome is inherited through the mother. It has a higher mutation rate than nuclear DNA, that is overcome by the multiple copies (100–10,000) present in one cell.

Epigenome: The epigenome comprises specific covalent modifications of the chromatin that ensure the somatic inheritance of differentiated cell states. Not only does it act during the differentiation of somatic cells but also in response to environmental cues and stresses. The passing on of these modulations to the descendants constitutes epigenetic inheritance. The structure and function of the epigenome are controlled by covalent marks applied to components of a nucleosome (DNA and histones) by enzymes (“writers”). These marks instruct the proteins that recognize them (“readers”) to identify and remodel particular regions of the genome in order to modulate expression. The plasticity of the epigenome comes from the “erasers”, enzymes capable of removing active and repressive marks. Disturbed in cancers, epigenetic changes modulate both the structure and the function of the chromatin.

Microbiome: is genetic material of all bacteria, fungi, protozoa, and viruses that live on and within the human body. Its role in disease susceptibility is largely unknown. Recent technologic advances in DNA sequencing and the development of metagenomics have made it feasible to analyze the entire human microbiome and to gain insight into its composition.

and *t*-distributed stochastic neighbor embedding. Great care must be taken in the interpretation of clustering results because clusters may be caused by irrelevant factors such as sample processing, termed batch effect. Adjustments and normalization processes may be required to account for batch effects to minimize the effect of sample processing and to ensure comparability. Unsupervised learning approaches have been used to cluster leukemia, lymphoma, or myeloma, based on their gene expression profiles, with the goal of uncovering the most robust classification schemes. In myeloma, this approach led to the description of the Translocation Cyclin D classification, which described the major molecular subtypes of disease.[5]

In contrast to unsupervised approaches, supervised learning approaches are best suited for comparing data between classes of samples that can be distinguished by a known property, such as biologic subtype or clinical outcome. For example, to determine the gene expression differences between different leukemia subtypes with distinct genetic abnormalities, one would use a supervised approach.

For routine diagnostic testing the optimum analysis approach is to have a clear idea of the information required and to report the data in a yes/no binary fashion to ensure clinical interpretability.

NEXT-GENERATION SEQUENCING TECHNOLOGY

Next-generation sequencing (NGS) has transformed the field of molecular diagnostics, leading to its routine uptake in clinical care. A single lane on a modern sequencer generates a huge amount of data, allowing for multiplexing and testing of samples.

RNA Profiling

In the late 1990s, profiling was done using an array format in which sequence-specific probes were immobilized onto a solid surface mRNA. These probes were hybridized to RNA from a sample of interest that was labeled with a fluorescent tag, and the array was captured by a laser-scanning device.[6] However, more recently sequencing-based approaches (RNA sequencing [RNA-seq]) have come to dominate because they allow for the profiling of previously unknown genes, alternative splice forms of known mRNAs, and gene fusions.[7]

Expression profiling of FFPE tissues deserves consideration because formalin fixation causes the degradation of mRNAs into fragments of only approximately 80 nucleotides in length. Array-based profiling approaches do not work well, particularly those that involve labeling of the mRNAs by priming of the 3′ polyadenylation tail. However, approaches have been developed that allow for the profiling of FFPE-derived tissues such that even degraded mRNAs can be profiled. Although it is likely that any method applied to FFPE samples will yield “noisier” data than frozen samples, the ability to analyze archived material, particularly those samples with long-term clinical outcome data, is sometimes invaluable. An example of this type of utility is the Lymphoma/Leukemia Molecular Profiling Project (LLMPP) Lymph2Cx assay, which is a parsimonious digital gene expression–based test (NanoString) for cell of origin (COO) assignment in FFPE tissue routinely produced from standard diagnostic processes.[8] The NanoString’s nCounter technology is a variation on the DNA microarray and uses molecular barcodes and microscopic imaging to detect and count-up to several hundred unique transcripts in one hybridization reaction.[9]

Noncoding RNA

Two major classes of noncoding RNAs are short RNAs, known as *microRNAs* (miRNAs), and *large intergenic noncoding RNAs* (lincRNAs). miRNAs are small (approximately 22-nucleotide) RNAs that do not encode for proteins but bind to mRNA transcripts to regulate translation and mRNA stability. Several hundred miRNAs are thought to exist in the human genome. In mammalian cells, a role for miRNAs has been recognized in the regulation of cellular differentiation through regulation of translation of key proteins. Not only are many miRNAs differentially expressed across hematopoietic lineages, but several miRNAs have also been demonstrated to play key functional roles in hematopoietic lineage specification and differentiation. The expression and/or function of several miRNAs is altered by chromosomal translocations, deletions, or mutations in leukemia and other hemopoietic disease. In addition, members of the protein complex, including the protein DICER, that process the maturation of miRNAs from longer RNA forms, have been implicated in malignancy.[10] Long noncoding (lnc)RNAs are approximately 1000 nucleotides in length and number approximately 5000 in the human genome. Recent evidence suggests that they may play important roles in establishing and maintaining cell fate and may play key roles in regulation of the epigenome. Interestingly, lncRNAs appear to have exquisite tissue-specific patterns of expression, suggesting that they may have diagnostic potential.

Mutation Detection Strategies

NGS can yield near-perfect fidelity for the detection of a mutation at a specific site, but at the same time, the error rates for any given sequencing read can be as high as 1%. This paradox reflects the fact that most sequencing errors are idiosyncratic, and, by simply resequencing the same region multiple times, developing a consensus results in such errors being lost. Thus, for normal, diploid genomes, sequencing is typically done to at least 30-fold coverage, meaning 30 reads for any given locus (referred to as *30× coverage*). The coverage is influenced by copy number changes (e.g., aneuploidy or regions or gene deletion or amplification) or when there is admixture of normal cells within the tumor sample. To compensate for these copy number variations and normal cell contamination, typical cancer sequencing projects aim for a depth of coverage of at least 100×.

The US Food and Drug Administration (FDA) has issued guidelines[11] for test design, performance characteristics, run quality metrics, performance evaluation, variant annotation, and filtering. The guidance identifies six key aspects of test design: the indications for use statement, user needs for the tests, specimen type, the region of the genome being interrogated, performance needs, and components and methods. The guidance further identifies four key aspects of test performance: accuracy, precision, limit of detection, and analytical specificity. They also specify six test run quality metrics: coverage, specimen quality, DNA quality and processing, sequence generation base calling, mapping or assembly metrics, and variant calling metrics. Several minimum standards for test performance and quality metrics are suggested, including the following:

- a point estimate of 99.9% accuracy (e.g., positive predictive agreement, negative predictive agreement, and technical positive predictive value) with a lower bound of the 95% confidence interval of 99.0% for all variant types reported;
- reproducibility and repeatability of at least 95% of the lower bound of the 95% confidence interval;
- a minimum coverage (i.e., depth and completeness threshold) of 20× for targeted panels and 30× average coverage depth at 100% of bases targeted in the panel or 97% of bases for whole exome sequencing;
- base calling with a base quality score of at least 30.

Germline Versus Somatic Variants

Mutations present in tumors but absent in the normal cells are referred to as *somatic*. Somatic mutations are major drivers of cancer behavior, but not all are causal. Indeed, the majority of somatic mutations observed in any individual tumor are most likely to be *passenger* mutations that play no functional role in the pathogenesis of the tumor but rather were expanded by an association with a driver mutation. The proportion of passengers to drivers differs dramatically from tumor type to tumor type. For example, tumors associated with tobacco (e.g., lung cancer) or sunlight exposure (e.g., melanoma) have very high mutation frequencies, with the majority of the observed mutations being "passengers." In contrast, many hematologic malignancies (e.g., acute lymphoblastic leukemia [ALL]) have relatively low mutation rates, and some cancers such as infant leukemias have extra ordinarily low rates.

It is important to recognize the difference between *germline* variants and acquired *somatic* variants in the cancer genome. Germline variants are present in all cells of the body and may contribute to disease risk. Germline risk variants can be common (i.e., seen in ≥5% of the human population), or they can be a rare observation, which has given rise to the concept of "common disease common variant" that is associated with low penetrance alleles in contrast to rare variants associated with disease that are likely to be highly penetrant.

Point Mutations or Single Nucleotide Variants

The most common type of genetic variants is *single-nucleotide variants*, also known as *point mutations*. Although not as common as point mutations, small somatic insertions or deletions *(indels)* that consist of the loss or gain of one or a few nucleotides that results in translational frameshifts, generally yielding loss-of-function alleles, are also seen. In the human population, it is estimated that every individual will harbor 50 to 100 unique coding mutations, which implies, if we are to prevent the miss-assignment of "private" germline variants as cancer-acquired somatic mutations, that we should compare the somatic genome of the tumor with its matched normal germline sequence.

Recently, computational models have been applied to large series of solid cancers, and signatures have been derived.[12] These signatures associated with mutations reflect their causative factors and have been termed *mutographs*. For example, G>T/C>A transversions are characteristic of tobacco-associated lung cancer, and C>T/G>A transitions are characteristic of ultraviolet radiation–associated skin cancers. The scientific rationale for mutographs is based on the preferential induction of a given nucleotide change within a 5′ and 3′ context, which is identified as a specific "signature."[13] Considering six possible substitutions in pyrimidine context, and four possible bases each at the neighboring 5′ and 3′ positions, there are 96 possible combinations of substitutions in a trinucleotide context. In addition to point mutations, many other molecular events participate in shaping the pathogenesis of cancer; whole-genome sequencing (WGS) techniques can interrogate the full repertoire of SNVs, CNAs, and SVs, and these can also provide information on the mutational processes operative in the early pathogenesis of multiple myeloma (MM) (Fig. 3.1).[14]

Structural Variation

Copy Number Abnormalities

Gains (amplifications) or losses (deletions) of chromosomal material at specific loci are recognized as playing an important role in the pathophysiology of cancer by either amplifying oncogene expression or decreasing tumor suppressor gene activity. In the germline, trisomy 21, for example, predisposes individuals to transient myeloproliferative disorders and acute megakaryoblastic leukemia.[15] Deletions at the *RB1* locus encoding the retinoblastoma gene or deletions of the *TP53* gene encoding the p53 tumor suppressor both predispose to the development of cancer.[16] In a landmark set of studies, it was shown that tumors from patients who inherit a mutant copy of the retinoblastoma tumor suppressor gene often have deletions of the remaining allele. This process has been termed *loss of heterozygosity*, and searching for such events in tumor samples has been used as a tool to identify genes involved in cancer progression. Similarly, amplification of genomic locus can play an important role in cancer progression.[17] For example, in multiple myeloma, amplification of a small amplicon at chromosome 1q23 is associated with adverse prognosis.[18]

Identifying CNAs has been done using a number of techniques. The original method was with metaphase *cytogenetic analysis*, which can identify abnormalities affecting large regions of the genome and was the basis for many important initial insights in hematologic malignancies. More recently, methods for assessing CNAs have advanced to include comparative genomic hybridization (CGH) and high-density single nucleotide polymorphism mapping arrays. However, these are being replaced by massively parallel genome sequencing. Although cytogenetic analysis remains a part of the diagnostic work-up for new cases of leukemia, it is likely that it will be replaced by NGS methods that also have the ability to detect point mutations, deletions/insertions, copy number changes, and chromosomal translocations, all at high resolution.

To identify statistically significant regions of CNAs, algorithms such as the genomic identification of significant targets in cancer (GISTIC) have been developed which can plot regions of amplification and deletion.[19]

Chromosomal Rearrangements

Translocations were among the very first genomic defects to be discovered in cancer because cytogenetic analysis of metaphase chromosome spreads was feasible on cell lines, especially for the acute leukemias. Chromosomal rearrangements include balanced and unbalanced translocations, inversions, and complex aberrations. Two basic types of translocations are common: those that result in fusion proteins involving two distinct genes and those that result in overexpression of an otherwise structurally normal gene. Translocations resulting in fusion transcripts (e.g., *ETV6/RUNX1* in ALL) generally involve chromosomal breakage within intronic regions of the two genes, with

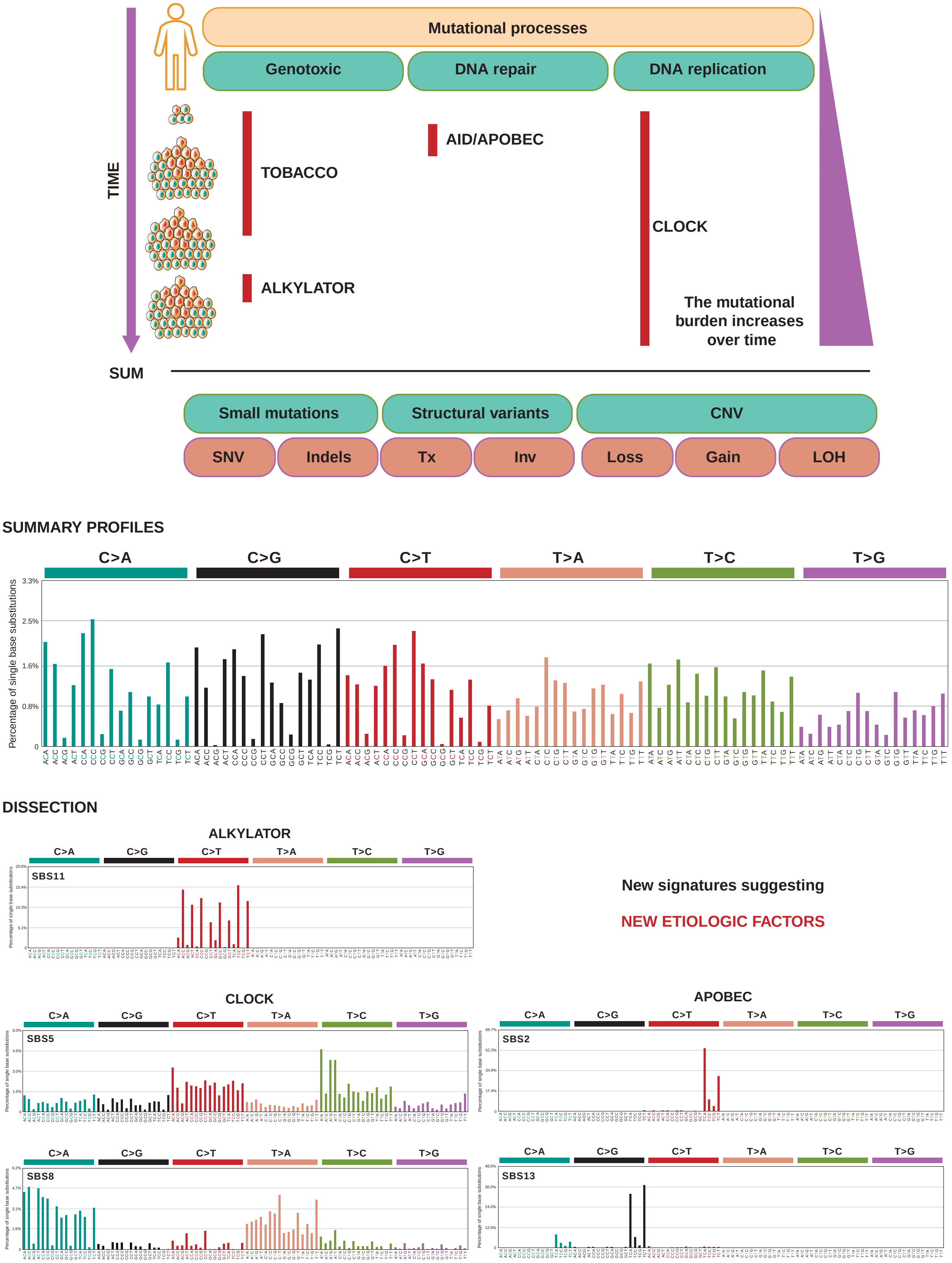


Figure 3.1 THE COMPLEXITY OF MUTATIONAL SIGNATURES. From the initiating, self-propagating cell to the relapsed and refractory stage, patients will acquire mutations secondary to different events that can be either tumor specific (e.g., AID or APOBEC), related to treatment or exposures (e.g., melphalan, cisplatinum, or even chemical exposure), or simply related to the aging process (e.g., Clock mutations). Tumors will represent a combination of these different signatures that can be teased out bioinformatically, with some remaining unexplained, suggesting they could be used to seek for novel etiologies.

in-frame fusion producing a new protein with a novel function being a result of the normal process of RNA splicing.[20]

In contrast, translocations resulting in overexpression typically involve the juxtaposition of a coding region next to a highly active promoter or enhancer region, such as an immunoglobulin region in B cells. For example, in follicular lymphoma, translocations frequently involve juxtaposition of the antiapoptotic gene *BCL2* to the immunoglobulin heavy chain enhancer region, leading to massive overexpression of *BCL2* RNA and protein.[21]

Complex chained rearrangements termed chromoplexy and regions of massive chromosomal rearrangement termed *chromothripsis* are more frequent than previously thought (Fig. 3.2).[22]

For the discovery of novel translocations, either whole genome sequencing or RNA-seq are the optimum methods. However, when a distinct fusion characterizes a specific disease (e.g., chronic myeloid leukemia [CML] and the BCR/ABL fusion), specific PCR reactions to detect it can be used for both diagnosis and response following therapy.[23]

Epigenomics

Epigenetic gene regulatory mechanisms play a critical role in the regulation of transcription, DNA repair, and replication. Several large-scale profiling efforts (e.g., through the National Institutes of Health ENCODE [Encyclopedia of DNA Elements] project) have used these technologies to annotate cancer cell lines and normal human and murine tissues, including hematopoietic subsets. Sequencing approaches to identify epigenomic changes include chromatin immunoprecipitation followed by sequencing (ChIP-seq), micrococcal nuclease (MNase) sequencing, DNAse sequencing (DNAse-seq), bisulfite sequencing and assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq), and a range of chromatin capture techniques including HiC (high-throughput chromatin conformation capture). Massively parallel sequencing, coupled with bisulfite sequencing approaches, allows for genome-wide assessment of DNA methylation in development and disease.

Modifications to histones are orchestrated and tightly regulated by a group of enzymes called *chromatin regulators*. Perhaps one of the most striking results derived from genome-wide sequencing analyses in cancer is the frequency of somatic mutations in chromatin regulators, which account for up to 25% of all cancer drivers. With the use of NGS techniques combined with chromatin immunoprecipitation, it is now possible to comprehensively investigate the molecular mechanisms of epigenetic alterations and define their disease relevance. ChIP-seq can be used to map histone modifications that are associated with actively transcribed regions, repressed regions, or regions found at distal regulatory elements. Single-cell sequencing–NGS applications have been developed that allow DNA and RNA-seq of *single* cells derived from the tumor as well from the tumor microenvironment. A widely used approach takes advantage of packaging single cells into an emulsion droplet; when combined with a molecular barcoding of every RNA molecule from each single cell and then RNA-seq of the entire population, it is possible to precisely assign each RNA molecule to each cell, making it possible to determine the gene expression profile of each single cell. This approach allows in-depth dissection of the tumor and its subclonal structure. Perhaps the major use of this approach will be to identify the nature of the cells of the microenvironment and how it is altered by infiltrating tumor cells.

THE CLINICAL UTILITY OF GENOMICS IN HEMATOLOGIC MALIGNANCIES

Diagnosis

The use of genomics to enhance hematologic diagnosis was introduced following the identification of the disease-defining genetic event, the t(9;22) characteristic of CML. The use of this genetic

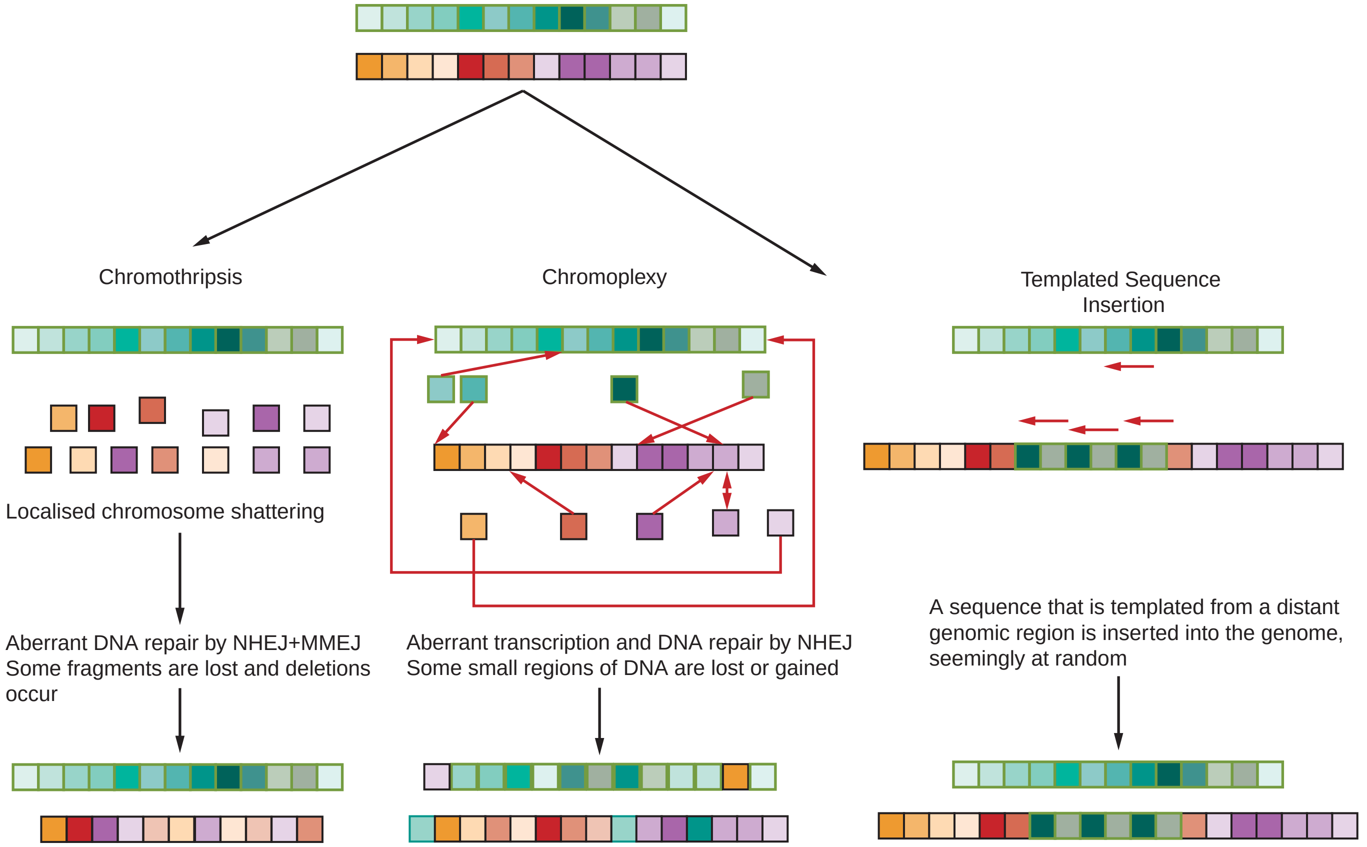

Chromothripsis
Chromoplexy
Templated Sequence Insertion
Localised chromosome shattering
Aberrant DNA repair by NHEJ+MMEJ
Some fragments are lost and deletions occur
Aberrant transcription and DNA repair by NHEJ
Some small regions of DNA are lost or gained
A sequence that is templated from a distant genomic region is inserted into the genome, seemingly at random

Figure 3.2 CARTOONS DEPICTING THE MAJOR COMPLEX STRUCTURAL VARIANTS. Chromothripsis, templated insertion, and chromoplexy.

diagnosis was expanded by the WHO classification of tumors of hematopoietic tissue,[3] which built diagnostic classifiers encompassing both histopathologic and genetic features (e.g., *JAK2* mutations in polycythemia vera, the t(15;17) in acute promyelocytic leukemia, 5q-syndrome in myelodysplasia) and gene expression profiles in lymphoproliferative malignancies (e.g., germinal center versus nongerminal center subtype of diffuse large B-cell lymphoma).

Beyond the refinement of diagnostic approaches, the application of genomic analysis can allow the early detection of hematologic malignancies using blood samples. Blood draws are considered safe and are less complicated and less expensive than a tissue biopsy; because they can easily be done at multiple time points, they can allow repeated assessment of the tumor over time.[24] This approach used *blood biopsies* that are based on the analysis of circulating tumor cells (CTCs), circulating tumor DNA, cell-free DNA (cfDNA), and circulating microvesicles/exosomes/apoptotic bodies in the blood. This material can provide an accurate representation of the tumor-acquired genetic changes simply by analyzing a vial of blood. An example is in angioimmunoblastic T-cell lymphoma where the G17V *RHOA* mutation in circulating DNA has been shown to be a useful diagnostic marker.[25] Genetics may also have a role in the generic work-up of cytopenia. Indeed, identifying genetic markers may help to discriminate various disease entities, some malignant or premalignant and some generally considered as benign (Fig. 3.3).

Precision Medicine and Molecularly Targeted Therapies

The application of genomics has allowed us to subcategorize blood diseases based on their molecular features and as such to develop novel precision treatment strategies. These strategies may rely upon using a therapy that directly targets the mutation (e.g., a BRAF inhibitor in a patient with a *BRAF* V600E-mutated neoplasm in hairy cell leukemia) or inform therapeutic decisions that are less directly related (e.g., not using ibrutinib in the germinal center subtype of diffuse large B-cell lymphoma).

There are numerous examples of precision medicine in hematologic malignancies. In ***myeloid malignancies***, the core-binding factor AML is defined by the presence of t(8;21)(q22;q22) or inv(16)(p13q22)/t(16;16)(p13;q22) that disrupts *RUNX1* (previously *CBFA/AML1*) or *CBFB* transcription factor functions. These variants are associated with a favorable outcome with chemotherapy and therefore are generally not assigned to allotransplant in first complete remission. Nonetheless, they may co-occur with activating *KIT* mutations, in which case they are associated with an adverse prognosis and may potentially be treated with tyrosine kinase inhibitors (TKIs) such as dasatinib[26] in an attempt to overcome the adverse prognosis. In ***diffuse large B-cell lymphoma***, building on the work of the Staudt group,[27] it has been possible to add COO subtypes to the mutation and refine the application of Bruton tyrosine kinase (BTK) inhibition (TKIs) (Fig. 3.4). The application of genomics in the clinic has led to a greater understanding of the complexities of multiple gene modifiers of outcome, including if an individual carries several driver mutations and which inhibitors should be targeted, as well as an appreciation of the statistical challenges of understanding such data.

Risk-Stratified Therapy

It has been more than a decade since the first proof-of-principle studies were published demonstrating the possibility of using gene expression profiling to subclassify cancer. These studies raised the possibility that gene expression signatures might be implemented in the routine clinical setting. In ***myeloma***, risk stratification has relied on iFISH analysis,[29] but it has been shown that risk scores based on gene expression signatures can outperform this strategy.[5] Currently, the gene expression based MyPRS test, based on a 70-gene signature, is approved for use in New York State but has not been widely taken up.[30] In ***chronic lymphocytic leukemia***, risk stratification and appropriate selection of treatment rely upon the identification of the mutation status at the immunoglobulin genes, cytogenetic factors (del(13q), del(11q), trisomy 12, del(17p)), and mutations (*TP53* mutation). Cases with loss of 17p and, more recently, mutation of TP53 are known to be chemoresistant and are treated differently with first line ibrutinib.[31] In ***acute myeloid leukemia***, the identification of cytogenetic subgroups derived from metaphase cytogenetic analysis has been used for many years to determine risk status and to assign

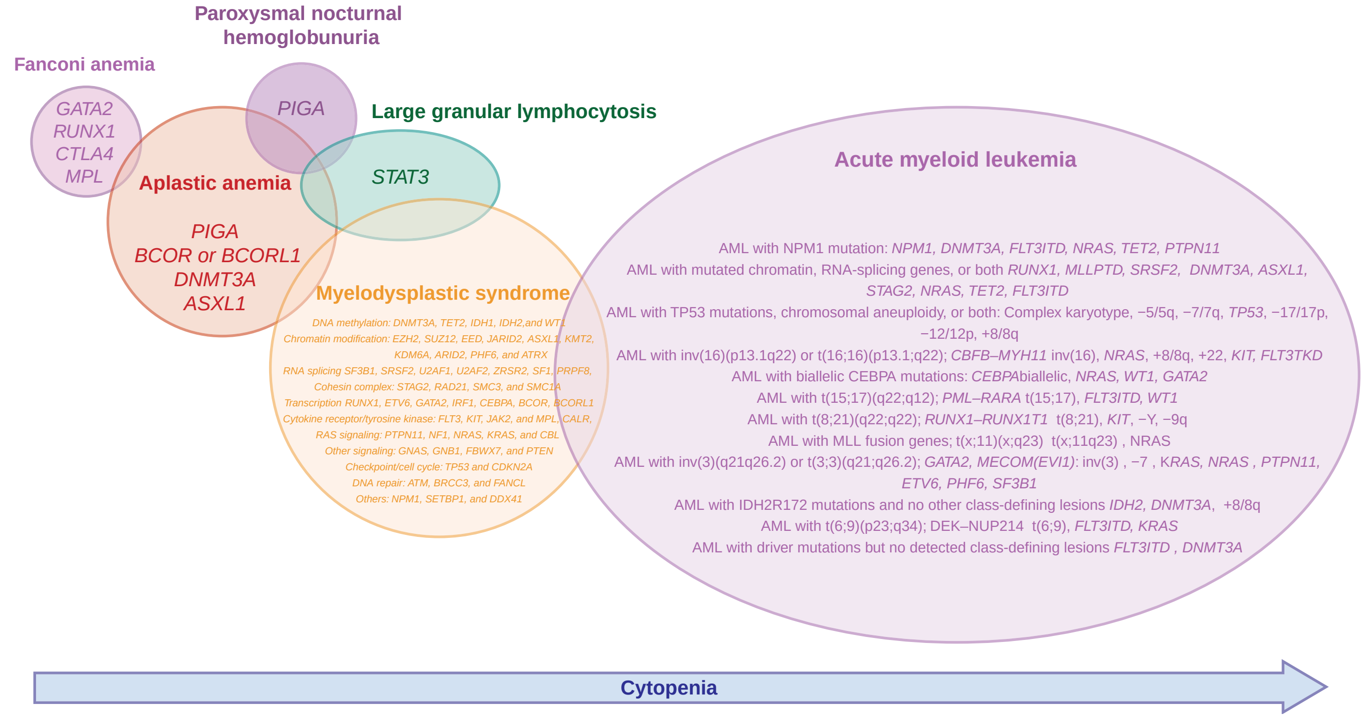

Paroxysmal nocturnal hemoglobunuria
Fanconi anemia
GATA2
RUNX1
CTLA4
MPL
PIGA
Large granular lymphocytosis
STAT3
Aplastic anemia
PIGA
BCOR or BCORL1
DNMT3A
ASXL1
Myelodysplastic syndrome
DNA methylation: DNMT3A, TET2, IDH1, IDH2,and WT1
Chromatin modification: EZH2, SUZ12, EED, JARID2, ASXL1, KMT2, KDM6A, ARID2, PHF6, and ATRX
RNA splicing SF3B1, SRSF2, U2AF1, U2AF2, ZRSR2, SF1, PRPF8,
Cohesin complex: STAG2, RAD21, SMC3, and SMC1A
Transcription RUNX1, ETV6, GATA2, IRF1, CEBPA, BCOR, BCORL1
Cytokine receptor/tyrosine kinase: FLT3, KIT, JAK2, and MPL, CALR,
RAS signaling: PTPN11, NF1, NRAS, KRAS, and CBL
Other signaling: GNAS, GNB1, FBWX7, and PTEN
Checkpoint/cell cycle: TP53 and CDKN2A
DNA repair: ATM, BRCC3, and FANCL
Others: NPM1, SETBP1, and DDX41
Acute myeloid leukemia
AML with NPM1 mutation: NPM1, DNMT3A, FLT3ITD, NRAS, TET2, PTPN11
AML with mutated chromatin, RNA-splicing genes, or both RUNX1, MLLPTD, SRSF2, DNMT3A, ASXL1, STAG2, NRAS, TET2, FLT3ITD
AML with TP53 mutations, chromosomal aneuploidy, or both: Complex karyotype, −5/5q, −7/7q, TP53, −17/17p, −12/12p, +8/8q
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB–MYH11 inv(16), NRAS, +8/8q, +22, KIT, FLT3TKD
AML with biallelic CEBPA mutations: CEBPAbiallelic, NRAS, WT1, GATA2
AML with t(15;17)(q22;q12); PML–RARA t(15;17), FLT3ITD, WT1
AML with t(8;21)(q22;q22); RUNX1–RUNX1T1 t(8;21), KIT, −Y, −9q
AML with MLL fusion genes; t(x;11)(x;q23) t(x;11q23) , NRAS
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); GATA2, MECOM(EVI1): inv(3) , −7 , KRAS, NRAS , PTPN11, ETV6, PHF6, SF3B1
AML with IDH2R172 mutations and no other class-defining lesions IDH2, DNMT3A, +8/8q
AML with t(6;9)(p23;q34); DEK–NUP214 t(6;9), FLT3ITD, KRAS
AML with driver mutations but no detected class-defining lesions FLT3ITD , DNMT3A
Cytopenia

Figure 3.3 EXAMPLE OF THE ROLE OF GENOMICS IN THE WORK-UP OF CYTOPENIA. Among the major causes of cytopenia, several disease entities can be identified. Despite clinical (usually depth of cytopenia, age), morphologic, and flow differences, molecular studies can help to differentiate between these similar entities. *(Modified from Young NS. Aplastic anemia. N Engl J Med. 2018; 379:1643–1656.)*

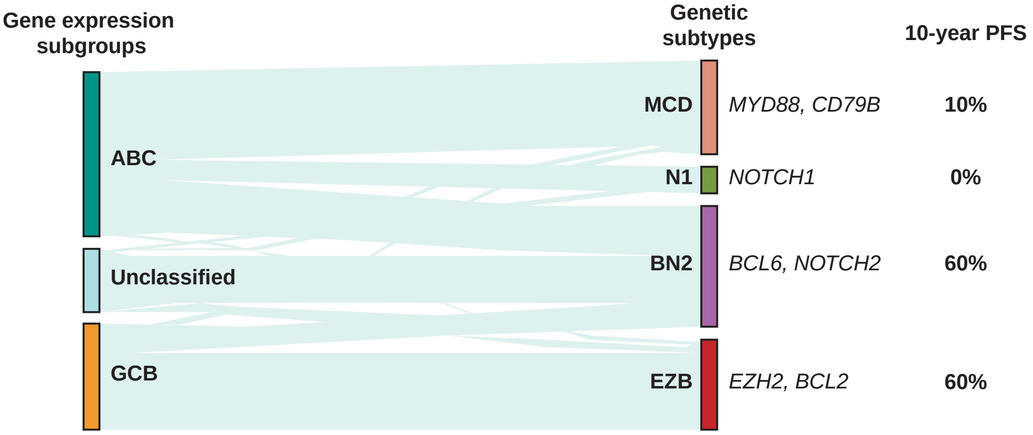


Figure 3.4 THE MOLECULAR DIAGNOSIS OF DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL). Gene expression subgroups first stratified DLBCL patients based on their cell of origin, whether germinal center B cell, activated B cell, or unclassified. By combining genetic events, this classification can be refined and four subgroups identified, characterized by mutational patterns and prognostic features termed N1 (for *NOTCH1*), MCD (for *MYD88* and *CD79B*), BN2 (for *BCL6* and *NOTCH2*), and EZB (for *EZH2* and *BCL2*) (adapted from Schmitz et al.[28]); it can guide personalized treatment strategies with agents such as lenalidomide, ibrutinib, and tazemetostat.

patients to receive allogeneic transplantation or not. This approach in acute leukemia has been further refined by the European Leukemia Network (ELN), who introduced the use of mutations such as biallelic *CEBPA*, monoallelic *NPM1, RUNX1, ASXL1, or TP53* and internal tandem repeats at the *FLT3* locus.[32]

Response-Adapted Therapy and Minimal Residual Disease Monitoring

Combination chemotherapeutic regimens have been a great success in the management of hematologic malignancies, leading to deep and durable responses, including cures, in some settings. The ability to monitor response and to adjust therapy based upon the level of response opens the potential for response-adapted therapeutic approaches. This response-adapted approach relies upon the development of sensitive testing strategies able to detect and monitor tumor cells below the level of clinical detection and has been termed minimal residual disease (MRD) monitoring. Classically, flow cytometry has been used, but it is restricted by sample requirements, disease type, and technical limitations. Other approaches have been developed based on molecular approaches based either on PCR or NGS.

Response-adapted therapy was developed initially in CML. The initial approach to detect response was cytogenetics but lacked sensitivity, as did iFISH. Quantitative reverse transcription PCR (QRT-PCR) was able to detect the Bcr-Abl RNA fusion gene down to a level of 1 tumor cell in 10^6 normal cells and provided an excellent tool to monitor therapy in patients undergoing treatment with TKIs. In this setting the achievement of MRD negativity is one of the critical clinical end points. More recently, this end point has been used to design MRD-driven TKI discontinuation trials (e.g., the STIM study).[33] In this trial, 38% of patients remained in treatment-free remission at 60 months, without molecular recurrence. Patients eligible for discontinuation had to achieve MRD negative as measured by QRT-PCR that was maintained for at least 2 years. Across TKI discontinuation trials, treatment-free remission rates after maintaining deep molecular response for at least 1 year ranged from 40% to 60%.[34]

At around the same time as monitoring of CML was being developed, in childhood acute lymphoblastic leukemia high remission rates and cures were being achieved. Despite this high cure rate, a substantial proportion of cases relapsed, which was addressed by the application of MRD monitoring. A sensitive clonality-based test using rearrangement of the immunoglobulin gene Ig loci was developed for application in lymphoid tumors. Applying this approach in ALL showed that the failure to fully eradicate the disease to a sensitivity level of one tumor cell in a million normal cells at a prespecified time point during treatment was associated with high rates of relapse, allowing the potential to modify the therapy early on in therapy.

The early technical approach to clonality detection relied on Southern blotting and was very time consuming but has now been replaced by NGS of T-cell and B-cell receptor genes. This sequencing approach targets a limited number of genomic regions that are involved in V(D)J recombination of the T-cell and B-cell receptors, thus allowing identification of monoclonal B and T cells, which define the malignant tumor cells. Because these regions are sequenced to great "depth," malignant clones can be detected even if they occur with a frequency of only 1 in 10^5 to 10^6.

One of the approved indications for this MRD detection with NGS-based clonality testing is multiple myeloma, the therapy of which has been transformed over the past 15 years with the advent of many new therapeutic agents. In younger patients after autologous stem cell transplantation, a meta-analysis has provided strong evidence for improved outcomes in patients achieving MRD-negative responses. However, there remains debate around the optimum level of sensitivity, with the optimum level being one tumor cell in 10^6 normal cells. There is also debate about the optimum testing strategy to be used, either flow cytometry or DNA-based clonality assays based on NGS.[35] These debates will be resolved as the approach goes through evaluation by the FDA for application as a legitimate trial end point.

Pharmacogenomics

Pharmacogenomics aims to apply genome variants that reflect drug behavior, typically via alterations in drugs' pharmacokinetics (absorption, distribution, elimination, metabolism) or via accentuation of drugs' pharmacodynamics (modifying the pharmacologic effects of a drug target). Classical examples of pharmacogenomics approaches in hematology include methylene tetrahydrofolate reductase *(MTHFR)* genotypes that affect the safety and efficacy of 6-mercaptopurine and methotrexate therapies[36] in leukemia and lymphoma. Similarly, a nonsynonymous SNP in the *OCT2* gene (rs316019), the organic cation transporter, in lymphoma or myeloma has been associated with reduced cisplatin-induced nephrotoxicity.[37,38]

To understand interpatient responses to drugs is pressing in oncology, where anticancer agents have narrow therapeutic indices and severe side effects. Pharmacogenomic approaches are also being used to determine the safety and efficacy of novel, targeted treatments, not only by analyzing the presence of a target tumor biomarker such as *ALK* fusions for crizotinib or *IDH2* mutations for enasidenib but also by determining their safety profile. For instance, belinostat, a histone deacetylase inhibitor drug approved in T-cell lymphoma, is predominantly metabolized by UGT1A1, which is polymorphic and requires genotype-based dose adjustment to normalize belinostat exposure, allowing for a better, more tolerable therapeutic experience.[39]

THE CLINICAL UTILITY OF GENOMICS IN BENIGN HEMATOLOGY

The diagnosis of inherited disorders in the early years of life and later in life can be very complex. The increasing knowledge of the genetic basis for many of the inherited disorders affecting the blood, together with the power of genomic approaches, has opened the way for the relatively simple screening for such disorders. The optimum approach for this is not fully established as yet but can be done by either the identification of single gene variants or multiple variants in a specific disease area, or by sequencing the entire genome. Sequencing the entire genome is the most comprehensive approach but at this stage brings with it issues of data handling, analysis, and ethics associated with the potential to sequence everybody in their early life. However, it is likely that such approaches will come into widespread use over time.

Some examples of the potential approaches in hematologic disorders are given as follows:

The Hemoglobinopathies

The genetic basis for the hemoglobinopathies and thalassemias is well known, and many causative genetic variants can be detected using simple polymerase chain reaction (PCR) approaches. Some uncommon mutations (Hb Q-India, HbNedlands, Hb Queens Park) require specific primers, but the approach to detecting such disorders is readily applied during prenatal screening. Deletions and mutations can readily be detected using allele-specific PCR for mutations or Gap-PCR for deletions, which use primers that bind to both sides of a deletion and can be used to successfully diagnose α-thalassemias, resulting from variable-sized deletions of α-globin gene.

Clotting Disorders

Genomic techniques can be helpful to refine thrombotic risk prediction. Current approaches focus on five common genetic risk factors for venous thromboembolism, including antithrombin, protein C, and protein S deficiency; factor V Leiden; and the G20210A prothrombin gene variant. Although the diagnosis of these thrombophilias is routinely based on functional assays of the coagulation cascades, the use of genetic testing to augment this approach can be useful. To date, genotyping has not replaced plasma-based assays for diagnostic purposes, with the exception of the prothrombin gene variant. Testing for activated protein C resistance remains controversial, even with the second-generation plasma assays using factor V–deficient plasmas. Some institutions simply do factor V Leiden DNA testing, whereas others use a less expensive plasma-based PCR assay and do DNA testing only for validation.

Disease with Rare Penetrant Variants Involving Multiple Loci

The ability of NGS to capture and analyze multiple gene loci has given it the ability to screen multiple loci in a single test. This approach relies upon a knowledge of the genetic basis of the disorder and the development of a specific testing panel. Thus genome-wide targeted exon capture followed by high-throughput DNA sequencing can provide an unbiased analysis of coding exons and is applicable to diseases associated with significant genotypic variability caused by mutations in numerous genes that result in the same clinical phenotype. One example of such a disease is Fanconi anemia, a heterogeneous bone marrow failure syndrome associated with defective DNA repair associated with cancer predisposition and congenital anomalies. It is inherited primarily as an autosomal recessive fashion, with more than a dozen Fanconi genes having been described. Application of exome sequencing to Fanconi patients has identified a variety of mutations in Fanconi-associated genes, several of which are novel, such as the *XRCC2*, one of five *RAD51* paralogs that act nonredundantly in the pathway of homologous recombination repair.[40] The increasing knowledge of the genetic basis for such disorders will allow the design and application of increasingly refined panels in a clinical setting. Currently the approach is readily applicable and easier to apply than sequencing the entire genome; however, as technology improves, it is likely that whole genome sequencing will replace looking for variants already described.

Common Low Penetrance Risk Variants

Inherited variants can modify disease response by the inheritance of common genetic variants with low penetrance. These inherited variants have been investigated by genome-wide association studies (GWASs), which often require thousands of patient samples to have sufficient power to detect statistically significant associations. Many GWASs have been performed, attempting to identify common variants contributing to complex disease. An example is the sequencing of candidate genes near loci implicated in fetal hemoglobin (HbF) level variation, which showed that rare variants in *MYB* to be associated with HbF levels.[41]

The approach of identifying common variants that modify responses of specific pathways has been extensively explored in the coagulation cascades. Numerous clinical studies have addressed genetic variation at *VCORC1* and *CYP2C9* to identify risk in the use of vitamin K antagonists for anticoagulation.[42] These approaches have been extended further to define genetic risk scores associated with venous thromboembolism (VTE) bAe with the goal of personalizing anticoagulation therapy for prevention of recurrent VTE, but much more development is required before such approaches are clinically useful. Similar GWAS approaches have been evaluated for antiplatelet agents. Clopidogrel, a P2Y12 inhibitor, is activated by the cytochrome P450 system. Patients carrying the CYP2C19*2 allele metabolize clopidogrel poorly and are good candidates for alternative P2Y12 inhibitors due to their higher risk of arterial thrombosis.[43]

APPROACHES TO THE DEVELOPMENT OF MOLECULAR TESTING

Important considerations for the application of genomic testing strategies in the clinic are the models by which they will be applied and how to store and analyze the data generated. The use of DNA-based testing has advantages over RNA-based approaches. In comparison with RNA-based analysis, DNA-based diagnostics have the advantage of being more definitive in detecting target variants (e.g., the presence of a mutation [A, G, C, or T]) as opposed to the detection of the relative abundance of a particular transcript.

The model by which testing is done is also relevant, with centralized testing approaches where all samples are sent to a set of national laboratories where testing and quality control are managed or whether it is done locally, taking advantage of infrastructure in pathology departments. The latter approach requires the use of defined machinery and diagnostic kits providing the means of maintaining quality control. Perhaps the most important of all is whether whole genome sequencing approaches that are agnostic to the clinical question being asked are used or whether it is optimum to use panels designed for a specific clinical question. Clearly, data handling and analysis requirements influence the approach used, as do statistical analysis and the generation of false-positive results.

The uptake of molecular diagnostics has been slow, which can be explained by a number of features. Financial reimbursement by health insurance payers has been difficult, making it important to demonstrate the utility, and measurable patient benefit is critical. Validation and regulatory approval are required to develop valid diagnostic tests, and for this to be done successfully the test must be applied to large numbers of patients; in many cases, such series of patients simply do not exist. Furthermore, the academic publishing system tends to reward *initial* discoveries, but the essential follow-up *validation* studies tend to be valued less and therefore are more difficult to fund. The economics of reimbursement for molecular diagnostics have in general not been favorable, thus discouraging companies from making major investments in the validation and commercialization of promising diagnostic tests. It is likely that diagnostic tests will command more of a premium in the future as a mechanism to use expensive therapeutics only in patients likely to benefit, but the time required for this to evolve is uncertain.

DNA sequencing is now routine at many academic centers and is increasingly being used to drive precision medicine by suggesting potential therapies based on an individual patients' genetic profile. The development of precision medicine will drive the application of genomic testing. With genomic, transcriptomic, and epigenetic data already available for the most common hematologic and malignant diseases and with new data being generated at an ever-increasing rate, there will be great opportunity for diagnostic and therapeutic development. The integration of genomic and other high-throughput sequencing approaches will continue to be one of the greatest challenges and opportunities in medicine in the decade ahead.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Alexandrov LB, Nik-Zainal S, Wedge DC, et al. Signatures of mutational processes in human cancer. *Nature*. 2013;500(7463):415–421

Della Starza I, Chiaretti S, De Propris MS, et al. Minimal residual disease in acute lymphoblastic leukemia: technical and clinical advances. *Front Oncol*. 2019;9:726.

Forment JV, Kaidi A, Jackson SP. Chromothripsis and cancer: causes and consequences of chromosome shattering. *Nat Rev Cancer*. 2012;12(10):663–670.

Galarneau G, Palmer CD, Sankaran VG, Orkin SH, Hirschhorn JN, Lettre G. Fine-mapping at three loci known to affect fetal hemoglobin levels explains additional genetic variation. *Nat Genet*. 2010;42(12):1049–1051.

Geiss GK, Bumgarner RE, Birditt B, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol*. 2008;26(3):317–325.

Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. *Nature*. 2001;409(6822):860–921.

Nik-Zainal S, Davies H, Staaf J, et al. Landscape of somatic mutations in 560 breast cancer whole-genome sequences. *Nature*. 2016;534(7605):47–54.

Phelan JD, Young RM, Webster DE, et al. A multiprotein supercomplex controlling oncogenic signalling in lymphoma. *Nature*. 2018;560(7718):387–391

Scott DW, Wright GW, Williams PM, et al. Determining cell-of-origin subtypes of diffuse large B-cell lymphoma using gene expression in formalin-fixed paraffin-embedded tissue. *Blood*. 2014;123(8):1214–1217.

Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127(20):2375–2390.

Weinstein JN, Collisson EA, Mills GB, et al. The Cancer Genome Atlas Pan-Cancer Analysis Project. *Nat Genet*. 2013;45(10):1113–1120.

REFERENCES

1. Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. *Nature*. 2001;409(6822):860–921. https://doi.org/10.1038/35057062.
2. Weinstein JN, Collisson EA, Mills GB, et al. The Cancer Genome Atlas Pan-Cancer Analysis Project. *Nat Genet*. 2013;45(10):1113–1120. https://doi.org/10.1038/ng.2764.
3. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127(20):2375–2390. https://doi.org/10.1182/blood-2016-01-643569.
4. Ross FM, Avet-Loiseau H, Ameye G, et al. Report from the European Myeloma Network on interphase FISH in multiple myeloma and related disorders. *Haematologica*. 2012;97(8):1272–1277. https://doi.org/10.3324/haematol.2011.056176.
5. Shaughnessy JD, Zhan F, Burington BE, et al. A validated gene expression model of high-risk multiple myeloma is defined by deregulated expression of genes mapping to chromosome 1. *Blood*. 2007;109(6):2276–2284. https://doi.org/10.1182/blood-2006-07-038430.
6. Szalat R, Avet-Loiseau H, Munshi NC. Gene expression profile in clinical practice. *Clin Cancer Res*. 2016;22(22):5434–5442. https://doi.org/10.1158/1078-0432.CCR-16-0867.
7. Stark R, Grzelak M, Hadfield J. RNA sequencing: the teenage years. *Nat Rev Genetics*. 2019;20(11):631–656. https://doi.org/10.1038/s41576-019-0150-2.
8. Scott DW, Wright GW, Williams PM, et al. Determining cell-of-origin subtypes of diffuse large B-cell lymphoma using gene expression in formalin-fixed paraffin-embedded tissue. *Blood*. 2014;123(8):1214–1217. https://doi.org/10.1182/blood-2013-11-536433.
9. Geiss GK, Bumgarner RE, Birditt B, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol*. 2008;26(3):317–325. https://doi.org/10.1038/nbt1385.
10. Swahari V, Nakamura A, Deshmukh M. The paradox of dicer in cancer. *Mol Cell Oncol*. 2016;3(3):e1155006. https://doi.org/10.1080/23723556.2016.1155006.
11. Luh F, Yen Y. FDA guidance for next generation sequencing-based testing: balancing regulation and innovation in precision medicine. *NPJ Genom Med*. 2018;3(1):1–3. https://doi.org/10.1038/s41525-018-0067-2.
12. Nik-Zainal S, Davies H, Staaf J, et al. Landscape of somatic mutations in 560 breast cancer whole-genome sequences. *Nature*. 2016;534(7605):47–54. https://doi.org/10.1038/nature17676.
13. Alexandrov LB, Nik-Zainal S, Wedge DC, et al. Signatures of mutational processes in human cancer. *Nature*. 2013;500(7463):415–421. https://doi.org/10.1038/nature12477.
14. Stratton MR, Campbell PJ, Futreal PA. The cancer genome. *Nature*. 2009;458(7239):719–724. https://doi.org/10.1038/nature07943.
15. Rabin KR, Whitlock JA. Malignancy in children with trisomy 21. *Oncologist*. 2009;14(2):164–173. https://doi.org/10.1634/theoncologist.2008-0217.
16. Malkin D. Li-Fraumeni syndrome. *Genes Cancer*. 2011;2(4):475–484. https://doi.org/10.1177/1947601911413466.
17. Ryland GL, Doyle MA, Goode D, et al. Loss of heterozygosity: what is it good for? *BMC Med Genomics*. 2015;8:45. https://doi.org/10.1186/s12920-015-0123-z.
18. Walker BA, Mavrommatis K, Wardell CP, et al. A high-risk, Double-Hit, group of newly diagnosed myeloma identified by genomic analysis. *Leukemia*. 2019;33(1):159–170. https://doi.org/10.1038/s41375-018-0196-8.
19. Beroukhim R, Getz G, Nghiemphu L, et al. Assessing the significance of chromosomal aberrations in cancer: methodology and application to glioma. *PNAS*. 2007;104(50):20007–20012. https://doi.org/10.1073/pnas.0710052104.
20. Raynaud S, Cave H, Baens M, et al. The 12;21 translocation involving TEL and deletion of the other TEL allele: two frequently associated alterations found in childhood acute lymphoblastic leukemia. *Blood*. 1996;87(7):2891–2899.
21. Godon A, Moreau A, Talmant P, et al. Is t(14;18)(q32;q21) a constant finding in follicular lymphoma? An interphase FISH study on 63 patients. *Leukemia*. 2003;17(1):255–259. https://doi.org/10.1038/sj.leu.2402739.
22. Forment JV, Kaidi A, Jackson SP. Chromothripsis and cancer: causes and consequences of chromosome shattering. *Nat Rev Cancer*. 2012;12(10):663–670. https://doi.org/10.1038/nrc3352.
23. Kantarjian H, Schiffer C, Jones D, Cortes J. Monitoring the response and course of chronic myeloid leukemia in the modern era of BCR-ABL tyrosine kinase inhibitors: practical advice on the use and interpretation of monitoring methods. *Blood*. 2008;111(4):1774–1780. https://doi.org/10.1182/blood-2007-09-110189.
24. Crowley E, Di Nicolantonio F, Loupakis F, Bardelli A. Liquid biopsy: monitoring cancer-genetics in the blood. *Nat Rev Clin Oncol*. 2013;10(8):472–484. https://doi.org/10.1038/nrclinonc.2013.110.
25. Nakamoto-Matsubara R, Sakata-Yanagimoto M, Nguyen T, et al. G17V Rhoa mutation in circulating DNA is a useful marker for diagnosis of AITL and AITL-related lymphoma. *Blood*. 2015;126(23). https://doi.org/10.1182/blood.V126.23.1447.1447. 1447–1447.
26. Boissel N, Renneville A, Leguay T, et al. Dasatinib in high-risk core binding factor acute myeloid leukemia in first complete remission: a French Acute Myeloid Leukemia Intergroup trial. *Haematologica*. 2015;100(6):780–785. https://doi.org/10.3324/haematol.2014.114884.
27. Phelan JD, Young RM, Webster DE, et al. A multiprotein supercomplex controlling oncogenic signalling in lymphoma. *Nature*. 2018;560(7718):387–391. https://doi.org/10.1038/s41586-018-0290-0.
28. Schmitz R, Wright GW, Huang DW, et al. Genetics and pathogenesis of diffuse large B-cell lymphoma. *N Engl J Med. 2018;*378:1396–1407.
29. Boyd KD, Ross FM, Chiecchio L, et al. A novel prognostic model in myeloma based on co-segregating adverse FISH lesions and the ISS: analysis of patients treated in the MRC Myeloma IX trial. *Leukemia*. 2012;26(2):349–355. https://doi.org/10.1038/leu.2011.204.
30. van Laar R, Flinchum R, Brown N, et al. Translating a gene expression signature for multiple myeloma prognosis into a robust high-throughput assay for clinical use. *BMC Med Genomics*. 2014;7:25. https://doi.org/10.1186/1755-8794-7-25.
31. Edelmann J, Gribben JG. Managing patients with TP53-deficient chronic lymphocytic leukemia. *J Oncol Pract*. 2017;13(6):371–377. https://doi.org/10.1200/JOP.2017.023291.
32. Döhner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*. 2017;129(4):424–447. https://doi.org/10.1182/blood-2016-08-733196.
33. Etienne G, Guilhot J, Rea D, et al. Long-term follow-up of the French Stop Imatinib (STIM1) study in patients with chronic myeloid leukemia. *J Clin Oncol*. 2017;35(3):298–305. https://doi.org/10.1200/JCO.2016.68.2914.
34. Saussele S, Richter J, Guilhot J, et al. Discontinuation of tyrosine kinase inhibitor therapy in chronic myeloid leukaemia (EURO-SKI): a prespecified interim analysis of a prospective, multicentre, non-randomised, trial. *Lancet Oncol*. 2018;19(6):747–757. https://doi.org/10.1016/S1470-2045(18)30192-X.
35. Della Starza I, Chiaretti S, De Propris MS, et al. Minimal residual disease in acute lymphoblastic leukemia: technical and clinical advances. *Front Oncol*. 2019;9:726. https://doi.org/10.3389/fonc.2019.00726.
36. Gervasini G, Vagace JM. Impact of genetic polymorphisms on chemotherapy toxicity in childhood acute lymphoblastic leukemia. *Front Genet*. 2012;3:249. https://doi.org/10.3389/fgene.2012.00249.
37. Filipski KK, Mathijssen RH, Mikkelsen TS, Schinkel AH, Sparreboom A. Contribution of organic cation transporter 2 (OCT2) to cisplatin-induced nephrotoxicity. *Clin Pharmacol Ther*. 2009;86(4):396–402. https://doi.org/10.1038/clpt.2009.139.
38. Ciarimboli G, Deuster D, Knief A, et al. Organic cation transporter 2 mediates cisplatin-induced oto- and nephrotoxicity and is a target for protective interventions. *Am J Pathol*. 2010;176(3):1169–1180. https://doi.org/10.2353/ajpath.2010.090610.
39. Peer CJ, Goey AKL, Sissung TM, et al. UGT1A1 genotype-dependent dose adjustment of belinostat in patients with advanced cancers using population pharmacokinetic modeling and simulation. *J Clin Pharmacol*. 2016;56(4):450–460. https://doi.org/10.1002/jcph.627.
40. Shamseldin HE, Elfaki M, Alkuraya FS. Exome sequencing reveals a novel Fanconi group defined by XRCC2 mutation. *J Med Genet*. 2012;49(3):184–186. https://doi.org/10.1136/jmedgenet-2011-100585.
41. Galarneau G, Palmer CD, Sankaran VG, Orkin SH, Hirschhorn JN, Lettre G. Fine-mapping at three loci known to affect fetal hemoglobin levels explains additional genetic variation. *Nat Genet*. 2010;42(12):1049–1051. https://doi.org/10.1038/ng.707.
42. Misasi S, Martini G, Paoletti O, et al. VKORC1 and CYP2C9 polymorphisms related to adverse events in case-control cohort of anticoagulated patients. *Medicine (Baltimore)*. 2016;95(52):e5451. https://doi.org/10.1097/MD.0000000000005451.
43. Dean L. Clopidogrel therapy and CYP2C19 genotype. In: Pratt VM, McLeod HL, Rubinstein WS, eds. *Medical Genetics Summaries*. National Center for Biotechnology Information (US); 2012. Accessed March 18, 2020. http://www.ncbi.nlm.nih.gov/books/NBK84114/.

CHAPTER 4

REGULATION OF GENE EXPRESSION IN HEMATOLOGY

Stephanie Halene, Toma Tebaldi, and Gabriella Viero

INTRODUCTION

The function of a cell is not only determined by the sum of the specific RNAs and proteins expressed but also by their metabolism, modification, and localization. To understand how a cell behaves, one must understand how the expression of genes, translation of transcripts, and processing of proteins are regulated.

Through concerted regulation of these processes, hematopoietic stem cells (HSCs) maintain a balance between quiescence and differentiation to mature blood cell types; erythroid progenitors produce vast quantities of hemoglobin; myeloid cells generate granules of immune responses; lymphocytes control immunoglobulin levels; and platelets regulate levels of thrombotic receptors.

Aberrant gene expression and RNA metabolism can result in hematologic disorders such as lymphomas, leukemias, and myelodysplastic and myeloproliferative syndromes. Furthermore, mutations in elements of the ribosomal machinery result in bone marrow failure syndromes. Understanding the process behind RNA and protein synthesis, trafficking, and degradation is crucial for the diagnosis and treatment of hematologic disorders.

This chapter will present the foundation necessary to understand the process of gene expression through RNA synthesis and processing, including transcription, splicing, modification, nuclear export, localization, stability and translation as well as posttranslational protein modification, targeting, and localization.

The first step of gene expression is transcription, where RNA polymerases decode the DNA using specific start and stop signals to synthesize RNA (Fig. 4.1). In the subsequent step, splicing removes introns, portions of the RNA that do not code for protein. The RNA is "capped" at the 5′ end and supplied with a poly-A tail at the 3′ end; RNA is also modified cotranscriptionally providing an additional layer for regulation of its stability and localization. RNA modifications define the *epitranscriptome* in analogy to DNA and histone modifications (called the *epigenome*). Next, the spliced RNA is targeted for export out of the nucleus and into the cytoplasm, where ribosomes translate the RNA into protein products (see Fig. 4.1). Protein synthesis occurs in the cytoplasm and generates a great variety of products endowed with a wide spectrum of functions. The complete set of proteins produced by a cell is called the proteome and is responsible for the remarkable diversity in cell specialization that is typical of metazoan organisms. To be functional, proteins need to be properly folded, assembled, often modified, and transported to their specific destination. The cells' interior harbors several membrane-bound organelles, such as the mitochondria, peroxisomes, nucleus, and endoplasmic reticulum (ER), to which the proteins may be targeted. In addition, membraneless organelles have been identified both in the nucleus and in the cytoplasm, including nucleoli, Cajal bodies, P-bodies, and stress granules. These organelles exist as liquid droplets within the cell and arise from the condensation of cellular material in a process termed liquid-liquid phase separation (LLPS).

This chapter briefly describes gene expression from start to end, exploring both classic and emergent regulatory mechanisms and connecting them with hematologic disorders.

REGULATION OF TRANSCRIPTION

Each cell in the human body contains approximately 60,000 genes (approximately 20,000 protein coding, 18,000 long noncoding, 7500 small noncoding and 14,500 pseudogenes). Only 1% to 2% of human DNA actually serves to code for proteins; the remaining part is prevalently involved in regulating DNA replication and gene expression across cell types and developmental stages. Together these regulatory sequences determine in which cell, at what time, and in what amount the gene is converted into the corresponding protein.

RNA Polymerase Binding and Regulation by Transcription Factors

RNA polymerase synthesizes RNA from a DNA template. For transcription to begin, RNA polymerase must attach to a specific DNA region at the beginning of a gene, known as *promoter*. Transcription factors control access of and frequently recruit RNA polymerases to promoter regions. Promoters can additionally function together with other more distant regulatory DNA regions, such as *enhancers* or *repressors* to further control the level of transcription of a given gene. *Insulator* regions in the genome protect genes from influences from regulation of neighboring genes. Multiple enhancer sites may tune the transcription of one gene, and each enhancer may be bound by more than one transcription factor, increasing the complexity of transcriptional regulation. Enhancers are often the major determinant of transcription of developmental genes in the differing lineages and stages of hematopoiesis. Genes can have more than one transcription start site, giving rise to RNA molecules starting with distinct sequences.

RNA is heterogeneous and stretches of genomic DNA may encode for more than one RNA or more than one type of RNA. Most eukaryotic RNA genes, especially messenger RNAs (mRNAs), contain a basic structure consisting of alternating coding exons and noncoding introns, subsequently dealt with in the splicing process.

While most RNAs in the cell are encoded by chromosomes in the nucleus, several mitochondrial proteins are encoded by the mitochondrial genome, often referred to as mtDNA. Transcription of the different classes of RNAs in eukaryotes is carried out by three different RNA polymerase enzymes. RNA polymerase I synthesizes the ribosomal RNAs (rRNAs), except for the 5 S species. RNA polymerase II synthesizes the mRNAs and some small nuclear RNAs (snRNAs) involved in RNA splicing. RNA polymerase III synthesizes 5 S rRNA and transfer RNAs (tRNAs). Transcription levels are finely tuned by the binding strength of the RNA polymerase to the promoter region at the beginning of a given gene, the interaction between activating and inhibiting transcription factors that bind to the given promoter, and transcriptional regulatory domains such as the enhancers or silencers mentioned previously.

Gene-specific transcription factors are sequence-specific DNA binding proteins that can be modified by cell signals. Numerous genetic diseases are associated with mutations in a gene's coding

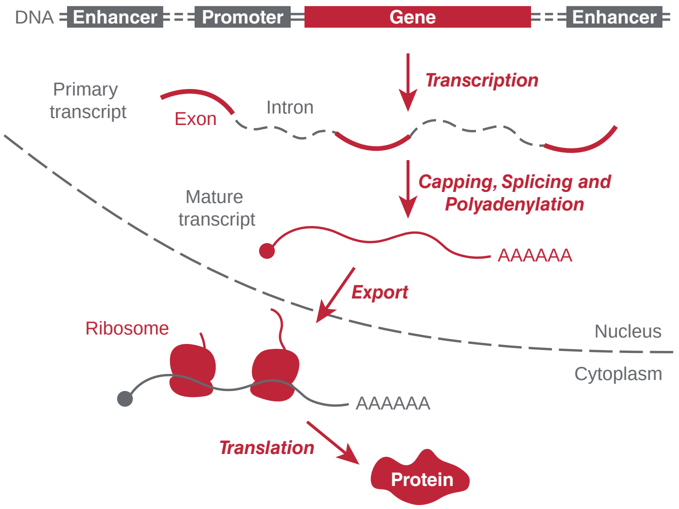


Figure 4.1 OVERVIEW OF GENE EXPRESSION FROM DNA TO PROTEIN VIA RNA. Gene expression is a complex process requiring multiple and strictly regulated steps: transcription of the primary transcript, RNA maturation through capping, splicing and polyadenylation, export to the cytoplasm, and translation into protein.

region, promoter, or enhancers. In β-thalassemia, mutations can occur in the promoter region, the enhancer region, or the coding region of the gene. Mutations can involve single nucleotide substitutions, small deletions, or insertions and can heavily affect transcription, RNA splicing or stability, translation, and ultimately protein availability or functionality. Regulation of transcription is fundamental during T-lymphocyte differentiation, which requires binding of multiple activating transcription factors, such as lymphocyte enhancer factor (LEF)-1, GATA binding protein 3 (GATA)-3, and ETS proto-oncogene (ETS)-1, to the T-cell receptor alpha (TCRA) gene enhancer.

Mutations in promoter sequences that result in decreased transcription factor binding, and therefore less RNA polymerase binding, ultimately lead to decreased gene expression. One of the best examples of a mutation in a transcription factor binding site associated with a human disease is in the factor IX gene. The transcription factor hepatocyte nuclear factor 4 alpha (HNF4α) is required to bind to the factor IX promoter before this gene can be transcribed.[1] Patients with a mutation in the HNF4α binding site can develop hemophilia B, an X-linked recessive bleeding disorder primarily affecting males (Fig. 4.2).

Many transcription factors, such as signal transducer and activator of transcription (STAT) proteins, require phosphorylation to bind DNA. Since transcription factors can be targeted by kinases and phosphatases, phosphorylation can effectively integrate information carried by multiple signal transduction pathways, thus providing versatility and flexibility in gene regulation. For example, the Janus kinase (JAK)-STAT pathway is widely used by members of the cytokine receptor superfamily, including those for granulocyte colony-stimulating factor (G-CSF), erythropoietin, thrombopoietin, interferons, and interleukins. Normally, ligand-bound growth factor receptors lead to JAK2 phosphorylation, which then activates STAT, also by phosphorylation. Activated STAT then dimerizes, translocates to the hematopoietic cell nucleus, binds DNA, and promotes transcription of genes for hematopoiesis. Alteration of JAK2, such as a V617F mutation, results in a constitutively active kinase capable of driving STAT activation. This leads to constitutive transcription of STAT target genes and results in myeloproliferative disorders such as polycythemia vera.

Regulation of Transcription by Chromatin

The ability of transcription factors and RNA polymerases to access specific promoters and transcribe genes is also regulated by the packaging of DNA by proteins and RNA, together forming the *chromatin*. Chromatin can package DNA tightly *(heterochromatin)* or loosely *(euchromatin)*. In *euchromatin*, RNA polymerases can freely bind to DNA and genes are actively transcribed. In *heterochromatin*, DNA is tightly packaged, protected from the transcription machinery, sequestering genes away from transcription. The basic unit of chromatin is the nucleosome, which contains eight histone proteins packaging 146 base pairs of DNA. Histones can be extensively modified to regulate the accessibility of the DNA to the transcriptional apparatus (see Chapter 3). Histones can be chemically modified by acetylation, methylation, phosphorylation, or ubiquitination. In general, acetylation opens the nucleosome to increase transcription, whereas phosphorylation marks damaged DNA. Histone methylation can either open chromatin to increase transcription or close it to repress transcription, depending on where the histone is methylated. Transcription factors can themselves recruit histone-modifying enzymes that further regulate transcription. In hematopoiesis, transcription factors, including GATA-1, EKLF, NF-E2, and PU.1, recruit histone acetyltransferases (HATs) and histone deacetylases (HDACs) to promoters of their respective target genes, leading to addition or subtraction of acetyl groups from histones, that in turn alters chromatin structure and accessibility for transcription.[2] GATA-1, a gene essential to erythroid maturation and survival, directly recruits HAT complexes to the β-globin locus to stimulate transcription activation.

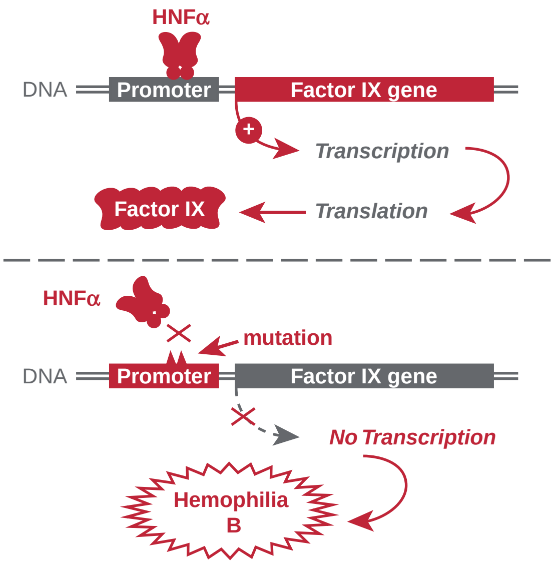


Figure 4.2 ROLE OF TRANSCRIPTION FACTORS IN THE REGULATION OF EUKARYOTIC GENE EXPRESSION. Upper panel: schematic diagram of the DNA region containing the locus of the coagulation factor IX gene and its promoter, containing a binding site for the HNFα transcription factor. Lower panel: mutations in either the promoter region or in the HNFα transcription factor reduce the expression of factor IX, leading to bleeding disorders such as hemophilia B.

Chromatin remodeling is mediated by a family of proteins with switch/sucrose nonfermentable (SWI/SNF) domains. These proteins use adenosine triphosphate (ATP) hydrolysis to shift the nucleosome core along the length of the DNA, a process also known as *nucleosome sliding*. By sliding nucleosomes away from a gene sequence, SWI/SNF complexes can activate gene transcription. SWI/SNF proteins also contain helicase enzyme activity, which unwinds the DNA by breaking hydrogen bonds between the complementary nucleotides on opposite strands. By unwinding the DNA into two single strands, the DNA can then be read by RNA polymerases in the direction 3′ to 5′, allowing RNA polymerase to produce an antiparallel RNA strand. The SWI/SNF complex has been shown to be active in the DNA damage response and is also responsible for tumor suppression. These processes are described in further detail in Chapter 2.

Regulation of Transcription by DNA Modification

DNA can also itself be chemically modified to amplify or suppress transcription. CpG sites within gene promoter regions can be chemically modified by methylation enzymes called DNA methyltransferases (DNMTs), which decrease DNA binding of RNA polymerase and associated transcription factors. Hypermethylation has been observed in bone marrow cells of patients with myelodysplastic syndromes (MDSs), and the degree of DNA hypermethylation correlates with disease stage. In MDSs, the promoters of genes that are important for myeloid differentiation are hypermethylated, repressing their transcription and inhibiting proper maturation of the myeloid lineages. Hypomethylating agents such as azacitidine and decitabine can induce remission and may prolong survival in some MDS patients.

The regulation of gene expression by modification of chromatin conformation or DNA itself is termed *epigenetic* because it tunes cell function without altering the nucleotide sequence of the DNA. Regulation and function of the epigenome and their role in hematopoiesis and diseases thereof are described in Chapter 3 and reviewed in Cullen et al.[3] Examples of hematologic malignancies driven by disordered epigenetic regulation include MDSs and acute myeloid leukemia (AML), with mutations in the *DNMT3A* gene observed in approximately 5% of MDSs and approximately 20% of AML cases. DNMT3A mutations confer a worse prognosis in AML. The ten-eleven-translocation methylcytosine dioxygenase member, TET2, catalyzes hydroxymethylation of cytosines in DNA and results in demethylation of DNA; TET2 is mutated in AML, MDSs, chronic myelomonocytic leukemia (CMML), and other myeloproliferative neoplasms (MPNs), and all mutations represent loss-of-function mutations. Both DNTM3A and TET2 mutations, together with mutations in other chromatin modifiers such as ASXL1, can widely regulate gene expression and are often present in clonal hematopoiesis, preceding onset of frank malignancy.

RNA Proofreading

Before a final mRNA product is made that can be translated, several proofreading regulatory steps must take place. The RNA polymerase may not even clear the promoter and slip off, producing truncated transcripts. Once the nascent transcript reaches approximately 23 nucleotides, the RNA polymerase no longer slips off, and full transcript elongation can occur. RNA polymerase then continues to traverse the template DNA strand, using ATP while complementarily pairing bases and forming the phosphodiester-ribose backbone. Many RNA transcripts may be rapidly produced from a single copy of a gene, as multiple RNA polymerases can transcribe a gene simultaneously, spaced out from one another. An important proofreading mechanism during elongation allows the substitution of incorrectly incorporated bases or editing of bases for other purposes, usually by permitting short pauses during which the appropriate RNA editing factors can bind. RNA editing mechanisms in mRNAs include nucleoside modifications of cytidine to uridine (C-U) and adenosine to inosine (A-I) by deamination, as well as nucleotide insertions and additions without a DNA template by protein complexes called *editosomes*. Adenosine-to-inosine (A-to-I) modifications make up nearly 90% of all editing events in RNA. The deamination of adenosine is catalyzed by the double-stranded RNA(dsRNA)-specific adenosine deaminase (ADAR). The deamination of adenosine to inosine disrupts and destabilizes dsRNA base pairing with multiple possible outcomes, such as reduced formation of small interfering RNAs (siRNAs) but also labeling of RNA as self and prevention of activation of an innate immune response. Studies in hematopoiesis and leukemia have elucidated the critical roles of A-to-I editing.[4]

Another important repair mechanism is transcription-coupled nucleotide excision repair, where RNA polymerase stops transcribing when it comes to a bulky lesion in one of the nucleotides in the gene. A large protein complex excises the DNA segment containing the bulky lesion, and a new DNA segment is synthesized to replace it, using the opposite strand as a template. The RNA polymerase then resumes transcribing the gene. However, in general, RNA proofreading mechanisms are not as effective as in DNA replication, and transcription fidelity is generally lower.

REGULATION OF RNA PROCESSING: CAPPING, SPLICING, AND POLYADENYLATION

After a eukaryotic gene is transcribed, the primary transcript is modified to protect it from degradation and target it for export into the cytoplasm and eventually translation to protein (see Fig. 4.1). These modifications generate the mature transcript and include capping, splicing, and polyadenylation. Capping occurs shortly after the start of transcription, when a modified guanine nucleotide is added to the 5′ end of the mRNA. This terminal 7-methylguanosine residue is necessary for proper attachment to the 40 S ribosome subunit during translation initiation. It also protects the RNA from endogenous ribonucleases that degrade uncapped RNA, which is often viral in origin. RNA polymerases do not terminate transcription in an orderly manner. They tend to be processive, yet the cell cannot tolerate a population of mRNAs that are enormous in size. Therefore mRNAs have a signal, the sequence AAUAA, that defines the end of the transcript. In general, ribonucleases cut mRNAs shortly after that signal, and a chain of several hundred adenosine residues, the poly(A), is added to the free 3′ transcript end. RNA cleavage and synthesis of the poly(A) tail require binding of specific proteins, including cleavage/polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), polyadenylate polymerase (PAP), polyadenylate binding protein 2 (PAB2), cleavage factor I (CFI), and CFII, that function to catalyze cleavage and protect the mRNA from exoribonucleases. The poly(A) tail increases RNA stability and assists in RNA export to the cytoplasm and translation. Mutations in the poly(A) signal can result in hematologic disease. For example, there are thrombophilic patients with a mutation in the polyadenylation signal in the prothrombin gene that increases the stabilization of this mRNA, resulting in higher prothrombin protein levels and increased thrombosis.

Before the mRNA is exported from the nucleus to be translated into protein, introns must be removed and the exons reconnected (see Fig. 4.1). In complex multicellular organisms such as vertebrates, introns are approximately 10-fold longer than the exons. The sequence and length of introns varied rapidly over evolutionary time. The process of removing introns, termed *splicing*, requires a series of reactions mediated by the spliceosome, a dynamic complex formed anew on its substrate from 5 snRNAs and approximately 100 proteins to form small nuclear ribonucleoproteins (snRNPs).[5] Recent critical advances in cryoelectron microscopy have shed light on the dynamics of assembly and disassembly of the spliceosome and the splicing process.[6] Canonical splicing uses the major spliceosome and accounts for more than 99% of splicing. The major spliceosome is composed of the nuclear active snRNPs U1, U2, U4, U5, and U6 along with multiple specific accessory proteins, such as U2AF and SF1. The spliceosome complex recognizes the dinucleotide GU at the 5′ end of an intron and an AG at the 3′ end (Fig. 4.3). Splicing can also occur cotranscriptionally and is regulated by chromatin factors that regulate transcription.[7] As transcription proceeds, an RNA lariat structure forms as intermediate, connecting intron ends, providing for both excision of the intron and proper alignment of the ends of the two bordering exons to allow precise ligation. When the intronic flanking sequences do not follow the GU-AG rule, noncanonical splicing removes these rarer introns with different splice site sequences using the minor spliceosome.[8] The same U5 snRNP is found in the minor spliceosome, in addition to the unique yet functionally similar U11, U12, U4atac, and U6atac. Furthermore, there are splicing mechanisms, including tRNA splicing and self-splicing, that function without use of the spliceosomal machinery. tRNA intron excision and exon ligation are catalyzed by protein-only complexes. tRNA introns typically interrupt the anticodon loop and must be removed so that the mature tRNA can properly function in protein translation.

Splicing is central to the output of a diverse transcriptome and then proteome. Alternative splicing (AS) can enhance the versatility and diversity of a single gene. By alternatively excising different introns along with the intervening exons, a wide range of unique transcripts and proteins of differing sizes can be generated. These alternatives, termed *isoforms*, come from one gene that generates a variety of mRNAs with varying exon composition. AS is widespread in complex

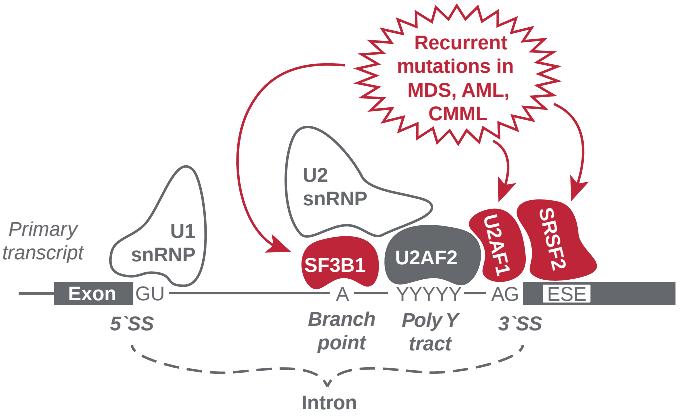


Figure 4.3 DESCRIPTION OF THE CANONICAL SPLICING JUNCTION, HIGHLIGHTING SPLICING FACTORS RECURRENTLY MUTATED IN HEMATOLOGIC MALIGNANCIES. Recognition of splicing sites is mediated by small conserved motifs: a GU dinucleotide at the beginning of the intron and an AG dinucleotide at the end, preceded by a polypyrimidine tract and a branch point A. Exons also contain functional sequences that promote splicing. All these motifs are bound by splicing factors that mediate the splicing process. Splicing factors highlighted in red are associated with hematologic malignancies. *3′SS*, 3′ splice site; *5′SS*, 5′ splice site; *AML*, acute myeloid leukemia; *CMML*, chronic myelomonocytic leukemia; *ESE*, exonic splicing enhancer; *MDSs*, myelodysplastic syndromes; *poly Y tract*, poly-pyrimidine tract; *snRNP*, small nuclear ribonucleoprotein.

multicellular organisms: the average human gene has seven different isoforms, and the number of known isoforms is rapidly increasing thanks to technologies such as long-read next-generation sequencing. AS is common and essential for the proper function of almost all hematopoietic cells. For example, B cells can produce both immunoglobulin M (IgM) and IgD at the same developmental stage using AS. Erythrocytes use AS to produce differing isoforms of cytoskeletal proteins. However, AS does not always give beneficial results.

One of the best examples of inappropriate splicing leading to hematologic disease is β-thalassemia, where several mutations that occur in the GU-AG splicing signals result in aberrant β-globin mRNAs. Abnormal splicing can also lead to AML, MDSs, and other hematologic disorders. Translocated in liposarcoma (TLS) is a protein that recruits splicing complexes to mRNAs and is involved in the TLS-ETS transcription factor ERG fusion oncogene in t(16;21) in AML. This fusion of TLS with the transcription factor ERG alters the splicing profile of immature myeloid cells, blocking the expression of genes required for proper differentiation. Trans-splicing is a form of splicing that joins two exons that are not within the same mRNA transcript. Some trans-splicing events occur when the intron splice sites are not filled by spliceosomes. Trans-splicing can lead to mRNAs displaying exon repetitions or chimeric fusion RNAs, which can mimic the presence of a chromosomal translocation in normal cells. For example, specific chimeric fusion mRNAs seen in acute leukemias, such as MLL-AF4, BCR-ABL, TEL-AML1, AML1-ETO, PML-RAR, NPM-ALK, and ATIC-ALK, have been found in blood cells of healthy individuals with normal chromosome karyotype. Interestingly, these individuals generally do not develop leukemia. In addition, in patients with chronic myelogenous leukemia (CML) resistance to tyrosine kinase inhibitor therapy has been linked to AS of the BCR-ABL transcript.

Splicing mutations in genes can occur *in cis* within the RNA itself as is the case for the aforementioned β-thalassemia, or *in trans* such as mutations in splicing factors or members of the spliceosome. Recurring mutations in several factors of the spliceosome result in MDSs and other hematologic malignancies (see Fig. 4.3).[9] Mutations in the splicing factor 3b, subunit 1 (SF3B1) have been observed in 68% to 75% and 81% of refractory anemia with ring sideroblasts (RARS) and RARS with thrombocytosis (T) patients, respectively, and result in alternative choice of the branchpoint site within the intron. Mutations in the U2 small nuclear RNA auxiliary factor I (U2AF1) and the serine/arginine-rich splicing factor 2 (SRSF2) result in sequence-specific splicing aberrations. U2AF1, a subunit of the U2AF heterodimer that also contains the polypyrimidine tract binding subunit U2AF2, carries distinct point mutations in its two zinc-fingers that contact the AG dinucleotide in the 3′ splice site. Mutations of U2AF1 S34 or Q157 residues create de novo 3′ splice site contacts that alter RNA splicing and result in preferential exon inclusion or exclusion, depending on the −3 or +1 nucleotide sequence at the 3′ splice site, respectively.[10] Mutations in U2AF1 are associated with a number of myeloid malignancies and occur in 8.7% to 11.6% of de novo cases of MDS. U2AF1-mutant MDS/AML cells exhibit enhanced stress granule response, pointing to a novel role for biomolecular condensates in adaptive oncogenic strategies.[10] SRSF2 is a member of the serine/arginine-rich pre-mRNA splicing factors. SRSF2 recognizes so-called splicing enhancer sequences (ESE) within exons (see Fig. 4.3), with a 5′-SSNG-3′ consensus motif where S = C/G and N = C/G/T/U. Mutations of P95 in the RNA-binding domain of SRSF2 alter RNA binding and splicing, reflected in higher affinity for 5′-CCNG-3′ than 5′-GGNG-3′ containing exons and resulting in preferential inclusion of alternative exons containing CCNG-rich motifs and exclusion of exons containing GGNG-rich motifs.[11,12] Mutations in the SRSF2 gene are associated with MDS and related diseases, particularly CMML, with SRSF2 mutations reported in up to 47% of patients. Recent studies have identified numerous critical targets aberrantly spliced, such as the member of the PRC2 complex EZH2, involved in epigenetic regulation.[12] In addition, it has been found that splicing factor mutations result in enhanced formation of DNA:RNA hybrids, so called *R-loops*, that induce DNA damage that promises to be exploitable in the treatment of these diseases.[13,14]

NUCLEAR EXPORT OF RNA

The nuclear envelope (NE) serves as a major regulator of gene expression, by controlling the movement of mature RNA from the nucleus to the cytoplasm for translation. The NE is made up of a double membrane. The outer nuclear membrane is continuous with the ER and has a composition distinct from that of the inner membrane. Nuclear pore complexes (NPCs) inserted within the NE regulate the transport of molecules in and out of the nucleus.[15] Ions, small metabolites, and proteins smaller than 40 kDa passively diffuse across NPC channels. However, larger proteins and mRNAs are transported through NPCs via energy-dependent (guanosine triphosphate [GTP]) and signal-mediated processes that require chaperoning transport proteins. Approximately 3000 NPCs perforate the NE in animal cells. NPCs are approximately 120 nm in external diameter and composed of three major parts: (1) a central core containing a 10-nm channel, (2) a nuclear basket that can dilate in response to large cargoes, and (3) flexible fibrils that extend from the central core into the cytoplasm. NPCs contain approximately 50 different proteins (nucleoporins), arranged in a complex cylindrical structure with an octagonal symmetry.[16] Nucleoporins constitute the scaffold of the NPC and are arranged in rings. In the inner ring, nucleoporins containing repeats of two hydrophobic amino acids, phenylalanine and glycine (FG-repeats), are essential for the movement of the cargo-carrier complexes and for creating a selectivity barrier against the diffusion of nonnuclear proteins. The FG-nucleoporin filaments protrude toward the inner core of the NPC and the weak hydrophobic interactions between the FG-repeats and the cargo-carrier complexes mediate the passage of molecules.

One nucleoporin, Nup98, is involved in numerous translocations and resulting fusion proteins that cause MDS and leukemia. The N-terminal (Nt) domain that contains the FG-repeats is fused to more than 28 partners in MDS and AML and has shown leukemogenicity in cell lines and mouse models. Interestingly, it is likely Nup98's transcriptional activation function rather than its role in nuclear pore formation that is critical in the function of these fusion proteins.[17]

Naked RNA cannot be exported through NPC channels. Rather, RNA export from the nucleus requires that newly synthesized RNAs undergo the previously described processing steps: 5′ capping, splicing, and 3′ polyadenylation. RNA-binding proteins, such as NXF1 that mediates the export of most mRNAs or XPO1, are required to fold and shuttle the modified RNA through NPCs. The eukaryotic translation initiation factor 4E (eIF4E) enhances nuclear export of a subset of RNA transcripts and is critical for proper granulocyte differentiation. Overexpression of eIF4E impedes myeloid maturation and can result in AML.[18] The cellular RNA export machinery is coopted by gammaretroviruses for the export of viral transcripts, such as binding of murine leukemia virus transcripts by the host cell's NXF1.

RNA HETEROGENEITY

The transcriptome of a cell is represented by a myriad of different RNA molecules with and without protein-coding capacities. Before recognition of the versatility of RNA, DNA was considered the sole conveyor of information while RNA was thought to solely function as an intermediate in protein synthesis (mRNA) or as effector molecules (tRNA, rRNA, snRNA). In the past couple of decades, RNA research has shed light on the pliability of RNA and its many regulatory functions in gene expression (Fig. 4.4). Several classes of noncoding RNAs (ncRNAs) such as *micro*RNAs (miRNAs), *piwi-interacting* RNAs (piRNAs), and *long noncoding* RNAs (lncRNAs) have been identified and associated with regulatory functions and diseases.[19] According to current genome annotations, the number of human ncRNAs is much higher than the number of protein-coding genes. The RNA landscape is further enriched by the presence of retrotransposons, a broad class of transposable elements, or "jumping genes," that duplicate through RNA intermediates that are reverse transcribed and inserted at new genomic locations. Due to their "copy and paste" mechanism, retrotransposons amplify in number quickly, composing 40% of the entire human genome.

LncRNAs are defined by their length, greater than 200 nucleotides (nt). They have gene regulatory roles in the nucleus or function in the cytoplasm via epigenetic, transcriptional, posttranscriptional, translational, and protein location effects. With almost 18,000 genes in the human genome, lncRNAs are apparently the most numerous and functionally diverse class of ncRNAs. Depending on their genomic location, they can be further divided in antisense (asRNAs), overlapping protein coding genes, or intergenic (lincRNAs) (see Fig. 4.4). Several lncRNAs undergo maturation processes such as capping, splicing, and polyadenylation. Despite their designation as ncRNAs, a considerable number of these transcripts tend to contain short open reading frames (sORFs) and bind with ribosomes, suggesting that the coding potential of lncRNAs has been vastly underestimated. Although the function of most lncRNAs is still unknown, multiple lincRNAs such as MALAT1, NEAT1, or XIST have already been implicated in human diseases such as cancer. An inherited form of α-thalassemia is caused by the translocation of an antisense lncRNA near the α-globin gene, resulting in the epigenetic silencing of the HBA2 gene and causing the disease.

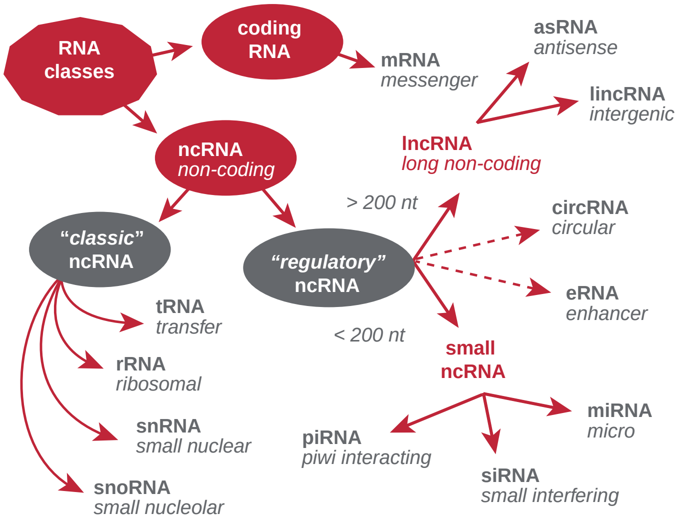


Figure 4.4 HIERARCHY OF RNA CLASSES DEFINED BY FUNCTION, BIOGENESIS, AND SIZE. The human genome contains more noncoding RNAs than messenger RNAs. Noncoding RNAs perform multiple functions, essential for both gene expression and its regulation.

Short ncRNAs, with a length less than 200 nucleotides, account for approximately 8500 genes in the human genome. They include well-known RNA classes such as tRNAs and rRNAs, essential for translation and described below in the section Protein Synthesis. snRNAs are associated with specific proteins in snRNPs and part of the spliceosomal complexes described in the section Regulation of RNA Processing: Capping, Splicing and Polyadenylation. Small nucleolar RNAs (snoRNAs) are a class of small RNA molecules that primarily guide chemical modifications of other RNAs, mainly rRNAs, tRNAs, and snRNAs.

More recently discovered classes of short RNAs include miRNAs, siRNAs, and piRNAs. The role of miRNAs and siRNAs are described in detail later (section RNA Interference). piRNAs are approximately 26- to 31-nt-long transcripts and function in suppression of transposable elements and maintenance of germline integrity.

Among emerging classes of RNAs, *circular RNAs* (circRNAs) are primarily produced via backsplicing of the 3′ end to the 5′ end of exons within the same transcript of a coding gene, giving these RNAs the circular shape. Compared with linear RNA, circRNAs form a covalently closed circular continuous loop and are highly conserved, stable, and tissue specific. CircRNAs can result in alternative protein isoforms and function as decoys for miRNAs and RNA-binding proteins, thereby regulating RNA stability and translation.

Enhancer RNAs (eRNAs) are generally relatively short ncRNAs transcribed from the genomic DNA at enhancer regions and represent a diverse class of molecules. They were originally described as nonpolyadenylated, bidirectionally transcribed RNA transcripts (<2000 nt) that originate from active enhancers. Subsequently some eRNAs were shown to be longer (>4000 nt), polyadenylated, and unidirectionally transcribed from higher-activity enhancers. eRNAs can regulate transcription in *cis* and in *trans*. Studies to identify their specific functions and mechanisms of action are ongoing.

ncRNAs, that once were mostly considered "junk" because they were diverging from the "central dogma," are proving to be an increasingly important part of our genome, an intricate layer of signals that control gene expression in physiology and disease.

REGULATION OF RNA METABOLISM: STABILITY AND LOCALIZATION

In mammalian cells, RNA lifetimes range from several minutes to days and represent a tightly regulated balance of transcription (detailed earlier) and degradation. The limited lifetime of mRNAs enables a cell to alter protein synthesis in response to its changing needs. The stability of mRNA is regulated by several mechanisms, including via sequences within the *untranslated regions* (UTRs) of mRNA, via nonsense-mediated decay (NMD) and via RNA modifications. Within the mature mRNA, the coding sequence (CDS) contains the sequence that is translated into protein, from the start codon to the stop codon. UTRs before the start codon (5′UTR) and after the stop codon (3′UTR) are not translated but govern mRNA half-life, localization, and translational efficiency. In human mRNAs, UTR regions are on average as long as the CDS (5′UTR: 236 nt, CDS:1121 nt, 3′UTR: 1047 nt). Both proteins and small RNA species can bind to either the 5′ or 3′ UTRs to regulate translation or influence survival of the transcript. NMD is a surveillance pathway present in all eukaryotes whose main function is to reduce errors in gene expression by eliminating mRNA transcripts that contain premature termination codons (PTCs). In addition to quality control, NMD can also serve to regulate expression in conjunction with AS. More recently, RNA modifications have emerged as a powerful mechanism of regulation of RNA stability and possibly translation. miRNAs, small RNA molecules that bind to complementary sequences on target mRNA transcripts, provide additional regulation of mRNA stability and translation.

Regulation of RNA Stability by 5' and 3' UTR Sequences and Structures

UTR sequence regulation of mRNA survival is essential for proper hematopoietic differentiation. The best example of this is globin synthesis, where the mRNA is very stable because of its UTR sequences. This long half-life meets the needs of reticulocytes to synthesize globin for up to 2 days after terminally mature erythroblasts lose the nucleus and the ability to make new mRNA.

Some of the elements contained in UTRs form a characteristic secondary structure that alters the survival of the mRNA transcript, exemplified by the prothrombin 3′ UTR. This mRNA is constitutively polyadenylated at seven or more alternative positions, and the 3′ UTR folds into at least two distinct stem-loop conformations.[20] These alternate structures expose a consensus binding site for trans-acting factors, such as heterogeneous nuclear ribonucleoprotein 1 (hnRNP-I), polypyrimidine tract-binding protein-1 (PTB-1), and nucleolin, with translational regulatory properties. Another type of 3′ UTR regulatory sequence involves selenocysteine insertion sequence (SECIS) elements. These represent another stem-loop RNA structure found in mRNA transcripts and serve as protein-binding sites on UTR segments that direct the ribosome to translate the codon UGA as selenocysteine rather than as a stop codon. An example of this regulation can be found in selenoprotein P in plasma. Bacteria contain another class of these mRNA elements, the *riboswitches*, that directly bind the small molecules that their mRNAs encode thereby directly regulating their own activity in response to the concentrations of their effector molecules. Bacterial riboswitches are relevant to hematopoiesis since the mRNAs for several enzymes in the cobalamine pathway in intestinal bacteria have riboswitches; these bind adenosylcobalamine that in turn regulates the survival and translation of these mRNAs and finetunes cobalamine synthesis.[21] Riboswitches are promising new drug targets, for now against bacteria, exemplifying the opportunities in RNA therapeutics.

Another class of UTR functional sequences affecting the stability of the mRNA is the *AU-rich element* (ARE). AREs are stretches of mRNA consisting mostly of adenine and uracil nucleotides. These sequences destabilize their transcripts through the action of riboendonucleases that stimulate poly(A) tail removal. Loss of the poly(A) tail is thought to promote mRNA degradation by facilitating attack by both the exosome complex and the decapping complex. Rapid mRNA degradation via AREs is a critical mechanism for preventing the overproduction of potent cytokines such as tumor necrosis factor (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). AREs also regulate the synthesis of mRNA for proto-oncogenic transcription factors such as c-Jun and c-Fos. The AU elements in the mRNA of these genes mediate destruction of their transcripts in quiescent cells, preventing inappropriate cell proliferation that would occur if Fos/Jun were still active.

Besides transcript stability, the efficiency of translation can be regulated by cellular factors that bind mRNA in a sequence-specific manner. Iron metabolism is an excellent example of how cells coordinate uptake and sequestration of an essential metabolite in response to availability. Transferrin is a plasma protein that carries iron. Receptors for transferrin (TfR) are expressed on cells requiring iron for maturation, such as erythroid progenitor cells. They mediate internalization of transferrin loaded with iron into the cytoplasm through receptor-mediated endocytosis. When a cell becomes iron deficient, iron-responsive element–binding proteins (IRE-BPs) can bind to iron-responsive elements (IREs) in the UTR of transferrin receptor (TfR) mRNA (Fig. 4.5). UTR binding leads to stabilization of the TfR mRNA transcript and in increased protein expression. However, when a cell has sufficient iron, as iron binds to more and more IRE-BPs, they change shape and unbind the TfR mRNA. The TfR mRNA becomes unstable and is rapidly degraded (see Fig. 4.5). Therefore, in that situation, TfR receptor expression is low and the fewer receptors import lessiron.

Nonsense-Mediated Decay

Eukaryotic mRNA messages are subject to surveillance for accuracy by a mechanism termed *nonsense-mediated decay* (NMD). The NMD complex surveys the transcript for the presence of PTCs (nonsense codons) in the message. These PTCs can arise via either a mutation in the gene from a sense codon to a termination codon (seen in thalassemias), incomplete splicing due to mutations in DNA, transcription errors, or leaky scanning by the ribosome, causing frame shifts. Detection of a PTC by NMD triggers mRNA degradation by 5′ decapping, 3′ poly(A) tail removal, or endonucleolytic cleavage. NMD does not only function to safeguard the cell against transcripts with PTCs generated in error, but it also serves gene expression regulation in a negative feedback loop, in which, for example, RNA-binding proteins bind their own pre-mRNAs and cause AS to introduce a PTC. Prominent examples in hematopoiesis that use NMD for regulation of transcript abundance include erythropoiesis[22] and granulopoiesis,[23] where AS coupled to NMD regulates transcript abundance at the posttranscriptional level. Examples of NMD in disease include splicing factor mutants, and specifically SRSF2 mutant MDS, in which preferential inclusion of an alternatively spliced exon, called *poison exon* for its deleterious effects on the transcript, results in a PTC and NMD of the EZH2 transcript.[12]

RNA Modifications: The Epitranscriptome

The past half century has witnessed numerous discoveries in the mechanisms regulating gene expression. Direct modifications of RNAs, abundant in tRNAs, rRNAs, and mRNAs, serve to regulate the function of the RNA molecules themselves. Posttranscriptional RNA modifications encompass more than 160 different chemical variations on the four canonical ribonucleotides, with diverse impacts on transcript fate and function. mRNAs can be modified by more than 10 different chemical marks, including m^{6}A, N^6,2′-O-dimethyladenosine (m^{6}A$_m$), 5-methylcytosine (m^{5}C),

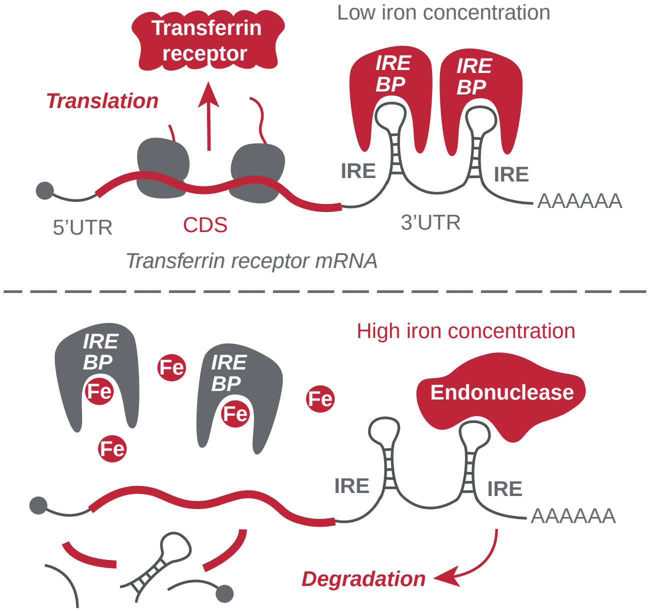


Figure 4.5 CONTROL OF TRANSFERRIN RECEPTOR EXPRESSION BY RNA STABILITY. The transferrin receptor messenger RNA *(mRNA)* has five RNA iron-responsive elements *(IRE)* in the 3′ untranslated region *(3′ UTR)*. Upper panel: when the iron concentration is low, the IRE-binding proteins *(IRE-BPs)* bind to the IRE elements and stabilize the mRNA, reading to increased protein expression. When the iron concentration is high *(Fe)*, IRE-BPs are inactive and the transferrin receptor transcript is susceptible to degradation by endonucleases, leading to decreased protein expression. *CDS*, Coding sequence.

and rarely N^1-methyladenosine (m^{1}A). The modified nucleotide N^6-methyladenosine (m^{6}A) is the most prevalent mRNA internal modification in mammalian cells and is placed on average on one to three adenosines per mRNA transcript; the m^{6}A modification was first identified as an abundant internal mRNA modification as early as the mid-1970s, and its principal methyltransferase, METTL3, was discovered shortly thereafter (Fig. 4.6). However, the field of "RNA epigenetics" or "epitranscriptomics" was not unlocked until publication of the first transcriptome-wide m^{6}A maps, derived by coupling RNA immunoprecipitation with an m^{6}A-specific antibody with next-generation sequencing (MeRIP-seq). The m^{6}A mark is placed within the DRACH RNA consensus motif (where D = G, A, or U; R = G or A; and H = C, A, or U) by the methyltransferase complex with the METTL3-METTL14 heterodimer at the core (m^{6}A "writers"). m^{6}A modification can be removed by m^{6}A "erasers" (ALKBH5) (see Fig. 4.6). The function of m^{6}A is executed by m^{6}A "readers" that bind to m^{6}A directly (YTH domain-containing proteins, eIF3, IGF2BPs) or indirectly (HNRNPA2B1). The m^{6}A RNA modification plays key roles in RNA metabolism, in particular RNA stability (reviewed in Zaccara et al.[24] and Shi et al.[25]). Deletion of the m^{6}A methyltransferase METTL3 or its binding partner METTL14 in HSCs results in bone marrow failure in adult as well as fetal hematopoiesis. One of the mechanisms at the basis of hematopoietic failure is loss of MYC, whose transcript is highly m^{6}A modified[26,27]; in addition, loss of m^{6}A results in aberrant endogenous dsRNA formation and activation of a deleterious innate immune response.[28] Reexpression of MYC or suppression of the innate immune response in *Mettl3*-deleted HSPCs partially rescues hematopoiesis. Perturbation of m^{6}A RNA writers, readers, and erasers (see Fig. 4.6) further supports the critical role of the m^{6}A modification in hematopoiesis. In addition, writers, erasers, and readers are emerging as possible targets in the treatment of leukemia and development and studies of small molecule inhibitors are in their early stages[29] (reviewed in Vasic et al.[30]).

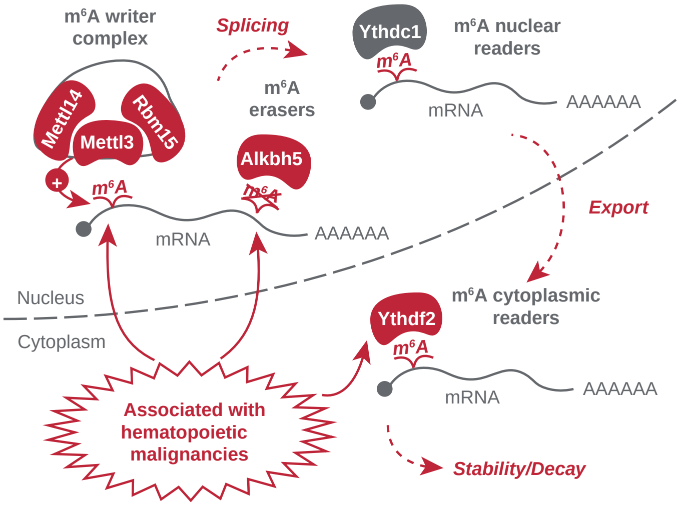


Figure 4.6 OVERVIEW OF M^{6}A MODIFICATION AND ITS RELEVANCE IN HEMATOLOGY. The m^{6}A writer complex, including the core methyltransferase METTL3 and its adaptors, adds m^{6}A to mRNAs prevalently during transcription. The m^{6}A erasers are largely localized in the nucleus as well. m^{6}A deposition can be reversed in the nucleus by the m^{6}A eraser ALKBH5. m^{6}A can be recognized by specific nuclear reader proteins such as YTHDC1, affecting nuclear processes such as splicing or export. In the cytoplasm, m^{6}A can be bound by specific reader proteins, such as YTHDF2, that affect the stability and possibly the localization or translation of the mRNA. m^{6}A factors highlighted in red are associated with hematologic malignancies.

RNA Interference

Another powerful mechanism of regulation of gene expression at the RNA level is RNA interference (RNAi), mediated by *miRNAs*; miRNAs are small RNAs (21 to 24 nucleotides in length) that bind to complementary sequences on target mRNA transcripts, prevalently in the 3′UTR. This binding results in transcript degradation and inhibition of translation and consequent silencing of gene expression (i.e., lack of protein output). There are now more than 2000 miRNA molecules coded in the human genome that regulate approximately one third of human genes. Each miRNA has the potential to target hundreds of genes. Conversely, an estimated 60% of all mRNAs have one or more sequences that are predicted to interact with miRNAs. RNAi has been very useful in the laboratory, allowing investigators to repress the expression of specific genes to study artificially induced phenotypes. In these studies, siRNAs are synthesized to bind to homologous sequences within specific mRNAs. The siRNAs and respective nontargeting controls are then transfected into cells, where they mediate destruction of their target mRNA through endogenous ribonucleases. Repression of gene expression in this manner has become known as "gene knockdown" and continues to be widely used to define the function of genes, by assessing what function the cell lacks in the absence of the expression of the target gene. The siRNA-mediated knockdown strategy is now being partially replaced by so-called CRISPR/Cas9-mediated gene deletion, which is covered in other chapters of this book.

To perform their function, both miRNAs and siRNAs are bound by a multiprotein complex called the RNA-induced silencing complex (RISC). Members of the Ago family of proteins are central to RISC function. Argonautes contain two conserved RNA-binding domains: a PAZ domain that can bind the single-stranded 3′ end of the mature miRNA and a PIWI domain that functions to interact with the 5′ end of the guide strand. Ago proteins bind the mature miRNA, orient it for interaction with a target mRNA, and may also directly cleave target transcripts (Ago2). The specificity of miRNA and siRNA interactions with their target mRNAs mediates how they regulate gene expression. For example, the specificity of miRNA targeting is ruled by Watson-Crick complementarities between positions 2 to 7 at the 5′ end of the miRNA, the so-called seed sequence, and the 3′ UTR of their target mRNAs.

Two models have been proposed to explain how miRNAs and siRNAs interfere with the expression of target genes: directed degradation of the target mRNA, performed as aforementioned by the RISC complex, and interference with the translation of a target mRNA (Fig. 4.7). In the case of *directed mRNA degradation*, the proposed model involves miRNA-mRNA binding and recruitment of RISC, which ultimately leads to degradation of the target mRNA. In the *interference model* the interaction of miRNA, RISC, and mRNA blocks the ribosomal machinery along the mRNA transcript, preventing translation. In reality, both models could contribute to gene expression downregulation. Most of the proteins necessary for miRNA-mediated gene silencing are located in cytoplasmic organelles, so called *P-bodies*, that are formed via phase separation. The specific circumstance of P-body involvement in miRNA-mediated RNA degradation is still an area of active research.

Various diseases, including many cancers, exhibit aberrant expression of miRNAs.[31] Regulation of the Tet2 mRNA by miR-22, dysregulated in MDSs and AML, is shown in Fig. 4.7. Another example is the *miR-15a/miR-16-1* cluster (located on chromosome 13q) chronic lymphocytic leukemia (CLL). When this cluster is deleted in B lymphocytes, there are higher levels of antiapoptotic proteins such as BCL2 and MCL1 but also higher levels of the tumor suppressor protein TP53. High levels of antiapoptosis yet with an intact TP53 tumor suppressor pathway could explain why 13q deletions in CLL are associated with an indolent form of the disease.[32] Several miRNAs have been identified that regulate hematopoiesis and in particular lineage choice and differentiation. One such miRNA is *miR-155* that retains HSCs and progenitor cells in an undifferentiated state and is downregulated with lineage differentiation. *MiR-155* is regulated by the homeobox gene *HOXA9*.

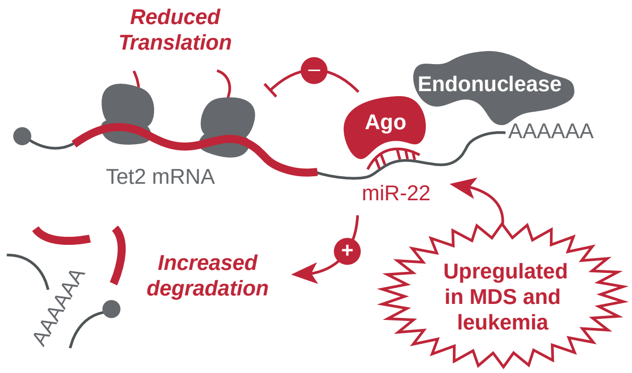


Figure 4.7 RNA INTERFERENCE AND THE CONTROL OF TET2 GENE EXPRESSION. The fully processed miR-22 microRNA associates with Ago proteins and binds target messenger RNAs (mRNAs), such as the 3′ untranslated region of Tet2, by sequence complementarity. RNA interference results in decreased translation and increased degradation of the target mRNA. The expression of several miRNAs, such as miR-22, is altered in hematologic malignancies, leading to dysregulation of all their targets. *MDSs*, Myelodysplastic syndromes.

RNA Granules and Membraneless Organelles

In the nucleus and in the cytoplasm, RNA availability and fate are additionally controlled by their localization in so-called RNA granules, liquid droplets, or membraneless organelles; within the same organelles, RNA may exert regulatory control on the function and availability of proteins and RNA-protein complexes.[33] Within the cell such organelles exist in the nucleus, such as nucleoli, Cajal bodies, nuclear speckles, and paraspeckles. In the cytoplasm, they exist in the form of processing (P) bodies, stress granules, and germ granules (Table 4.1). Liquid droplet organelles are formed by LLPS similar to the separation of oil and vinegar in vinaigrette. They function as organizational tools by concentrating cellular components in membraneless structures that allow rapid exchange of their components unhindered by membrane barriers. Their "fluidity," for most components' residence times, ranges from seconds to tens of seconds and allows for dynamic finetuning of biochemical reactions and rapid response to cellular stress. In general, liquid droplet organelles are made up of RNA and RNA-binding proteins. As an example, Cajal bodies in the nucleus form on active snRNA loci and are the site of snRNA processing and snRNP assembly and surveillance. Nucleoli regulate rRNA synthesis and are known to capture and detain proteins via lncRNA binding. Nuclear speckles and paraspeckles form on the two long ncRNAs MALAT1 and NEAT1, respectively, and contain mRNAs and their RNA-binding proteins. Nuclear speckles are the sites of RNA splicing. Paraspeckles sequester proteins and RNAs in response to cellular stress. Stress granules and P-bodies in the cytoplasm regulate RNA stability and protein translation. RNA is sequestered in stress granules in response to stress signals, hypothesized to regulate translation, RNA stability, and cell survival; in P-bodies, translation is stalled and transcripts are targeted for degradation by exonucleases.

TABLE 4.1 **RNA Granules**

Name	Localization	Composition	Function
P-bodies	Cytoplasm	RNA & protein	mRNA decapping, degradation, nonsense mediated decay, storage, translational repression by miRNAs
Stress granules	Cytoplasm	RNA & protein	Storage of RNAs and translation components under stress
Germ granules	Cytoplasm	RNA & protein	Regulation of proteins and RNAs required for germ cell development
Nucleoli	Nucleus	DNA, RNA, & protein	Ribosome biogenesis
Cajal bodies	Nucleus	RNA & protein	snRNP biogenesis
Nuclear speckles	Nucleus	DNA, RNA, & protein	Splicing regulation
Paraspeckles	Nucleus	RNA & protein	Transcription regulation, retention of edited RNAs under stress, mediated by lncRNAs (NEAT1)

lncRNA, Long noncoding RNA; *miRNA*, microRNA; *mRNA*, messenger RNA; *snRNP*, small nuclear ribonucleoproteins.

The study of LLPS and the function of liquid droplet organelles in vivo and their role in physiologic responses and disease are an ongoing subject of intense studies.[34] Aberrant phase transitions from a liquid state to a more aggregated state have been recognized as the causal mechanism in neurodegenerative disorders (e.g. via mutations in the RNA-binding proteins usually part of stress granules that render the proteins more likely to aggregate) and cancers.

PROTEIN SYNTHESIS

Translation is a biologic process performed by ribosomes, which use an mRNA as template for protein synthesis. Translation is a highly conserved, energy-consuming process. For this reason, it is tightly controlled.[35] In cells, multiple ribosomes can bind the same mRNA, forming complex structures, called polyribosomes, or polysomes. They mediate the formation of a polypeptide chain using the noncoding tRNA bound to amino acids to catalyze the synthesis of proteins. Amino acids, each with unique physicochemical properties (see Table 4.2 for single letter designations) are joined to tRNAs by amino-acyl-tRNA synthetases. The amino-acylated tRNAs enter the ribosome, which catalyzes the formation of peptide bonds by the condensation of the α-carboxyl group (COOH) of one amino acid with the α-amino group (NH_2) of another. The free NH_2 and COOH groups of the terminal amino acids define the amino- or Nt and the carboxyl- or C-terminal end of the resulting polypeptide chain, respectively. In many cases multiple polypeptide chains assemble into a functional protein complex. For example, hemoglobin is formed by four polypeptide chains, two α-globin and two β-globin chains that assemble with heme, an iron-containing prosthetic group, to yield the functional protein that delivers molecular oxygen to all cells and tissues.

The Process of Translation

The *ribosome* is a large, macromolecular machine that orchestrates the entire process of protein synthesis. Each human ribosome comprises four rRNAs and approximately 80 proteins, called ribosomal proteins (RPs), assembling into a 4.5-MDa structure. The ribosome is formed by two subunits; the large 60 S (S stands for Svedberg unit and refers to the sedimentation coefficient) and the small 40 S subunit join and constitute the 80 S ribosome in eukaryotic cells. rRNAs are the catalytic elements, and RPs are classically considered structural "scaffolding" units. The process of translation of an mRNA is typically divided into four phases: initiation, elongation, termination, and ribosome recycling. During the initiation phase the 40 S subunit binds to the 5′ cap of the mRNA and scans the mRNA toward its 3′-end searching for the translation start codon. The start codon is a trinucleotide sequence, usually an AUG, located within a Kozak consensus

TABLE 4.2 Examples of Sorting Signals

Organelle	Signal Location[a]	Example
Posttranslational Uptake		
Nucleus	Internal	SP**KKKRK**V*E* (import; NLS of SV40 large T antigen)
		KR-spacer **(**PAATKKAGQ)**-KKKK** (import; bipartite NLS of nucleoplasmin)
		LQLPPL*E***R**LTL*D* (export; NES of HIV-1 rev)
Mitochondrion	N-terminal	MLGI**R**SSV**K**TCF**K**PMSLTS**KR**L (iron-sulfur protein of complex III)
Peroxisomes	C-terminal	**K**ANL (PTS1, human catalase)
	N-terminal	**R**LQVVLG**H**L (PTS2, human 3-ketoacyl-CoA thiolase)
Cotranslational Uptake		
ER	N-terminal	MMSFVSLLLVGILFWAT*EAE*
		QLT**K**C*E*VFQ (ovine lactalbumin)

[a]Acidic residues (negatively charged) are in italic type; basic residues (positively charged) are in bold type. Amino acids: *A*, alanine; *C*, cysteine; *D*, aspartic acid; *E*, glutamic acid; *F*, phenylalanine; *G*, glycine; ***H***, histidine; *I*, isoleucine; ***K***, lysine; *L*, leucine; *M*, methionine; *N*, asparagine; *P*, proline; *Q*, glutamine; ***R***, arginine; *S*, serine; *T*, threonine; *V*, valine; *W*, tryptophan; *Y*, tyrosine.
ER, Endoplasmic reticulum; *HIV*, human immunodeficiency virus; *NES*, nuclear export signal; *NLS*, nuclear localization signal; *PTS1*, peroxisomal targeting signal-1; *PTS2*, peroxisomal targeting signal-2; *SV40*, simian virus 40.

sequence (A/GNNAUGG), which is the optimal nucleotide context for translation initiation. The initiator tRNA specific for methionine, called the initiator $tRNA_i^{Met}$, binds the 40 S subunit and helps to recruit the larger 60 S subunit once the AUG is found. Next, the mRNA sequence is read in triplets by additional aminoacyl-tRNAs which mediate the elongation of the polypeptide chain (elongation phase). Translation is terminated as soon as the ribosomes reach a stop codon (termination phase). The polypeptide is then released, and ribosomal subunits detach from the mRNAs and are used for a new cycle of translation (ribosome recycling phase). Typically, multiple ribosomes are simultaneously engaged in translating a single mRNA molecule forming a polyribosome, or polysome. The phases of protein synthesis are regulated by eukaryotic initiation, elongation, and termination (or release) factors, termed eIFs, eEFs, and eRFs, respectively. eIFs are members of the guanine nucleotide-binding proteins (G protein) superfamily that function as molecular switches and promote unidirectionality of cellular processes. eIF2a also functions as a key mediator of the *integrated stress response* (ISR) limiting translation initiation and protein synthesis in response to stress conditions, such as amino acid or glucose starvation and viral infection.

As an example, in reticulocytes, heme starvation inhibits the synthesis of α- and β-globin chains via regulation of eIF2. Activated protein kinase hemin-regulated inhibitor (HRI) phosphorylates the α subunit of eIF2; phosphorylated eIF2 sequesters the guanine-nucleotide exchange factor (GEF) eIF2B, limiting its availability for the exchange reaction of eIF2-GDP to eIF2-GTP, halting globin translation. Heme availability thus becomes the rate-limiting step and prevents toxic accumulation of globin chains in the absence of heme.

A second major control point of general protein synthesis is mediated by the eIF4F heterotrimeric protein complex that makes the mRNA accessible to binding by the 40 S ribosome subunit: its cap-binding protein eIF4E binds the mRNA cap, the ATP-dependent RNA helicase eIF4A unwinds structural elements in the 5′-end of mRNA, and eIF4G serves as scaffold protein in the complex. As the lowest abundance subunit, eIF4E represents the rate-limiting step in protein synthesis. In addition, inhibition of the cap-binding activity of eIF4E by eIF4E-binding proteins (eIF4EBPs) prevents assembly of the eIF4F complex.

Additional modes of regulation take advantage of the mRNA sequence itself. In the control of iron metabolism, IREs, hairpin structures formed in the UTRs of mRNAs, are bound by IRE-BPs. In iron-starved cells, IREs in the 3′UTR and 5′UTR of TfR and ferritin mRNAs, respectively, are bound by IRE-BPs that stabilize the TfR transcript while at the same time inhibiting translation initiation of ferritin (see Fig. 4.5). Conversely, when iron is abundant, IRE-BPs have a lower affinity to IREs, and as a result TfR mRNA is degraded whereas ferritin mRNA translation is stimulated. In this manner, cells can coordinately regulate iron uptake and iron sequestration in response to changes in iron availability.

Ribosome Disorders

Given the importance of translation in regulating protein expression in all fundamental physiologic processes, gene expression is largely controlled at the translational on top of the transcriptional level.[36] Translation is the most energy-consuming process in cells, and its dysregulation is implicated in an ever-increasing number of diseases. Evidence accrued over the past decades demonstrates that cancer[37] and neurodegenerative diseases[38,39] share defects in RNA processing or translation. Hematologic disorders are no exception.

Ribosomes have been put in the spotlight as putative direct players in finely tuning translation by acting as mRNA filters.[40] Recent papers suggest that ribosome composition is not fixed and uniform but rather heterogeneous and modulated at the level of RP composition[41] or rRNA variants[42,43] and further modified posttranslationally or posttranscriptionally.[44,45] This ribosome heterogeneity, known as "specialized ribosome" hypothesis, could exert a direct role in mRNA selection.[46] Moreover, ribosome-associated factors[45] have been shown to possibly control translation. In addition, the number of ribosomes can play a crucial role in controlling the subset of mRNAs undergoing translation,[47] thus shaping the cellular proteome.

Recent years have led to the discovery of an increasing number of germline and somatic mutations affecting translation at multiple levels. These defects include mutations in structural constituents of the ribosomes, such as RPs, or in translation-related ncRNAs such as tRNAs (Table 4.3). Interestingly, in very recent years, alterations in posttranscriptional modifications of rRNA have been observed as well as deregulation of the cellular ISR and the mTOR pathways (reviewed in Tahmasebi et al.[54]).

Classically, the best-known hematologic disorders associated with genetic mutations in genes encoding RPs or assembly factors required for ribosome biogenesis and function are called *ribosomopathies*. Ribosomopathies represent a range of disorders characterized by genetic abnormalities impacting on ribosome biogenesis and function. These defects cause specific clinical phenotypes and are well-known risk factors for myelodysplastic disorders and AML. Ribosomopathies can be divided into subgroups depending on where the mutation affects the ribosome.

Ribosomopathies caused by mutations in RPs most often have in common the development of bone marrow failure or anemia and/or craniofacial or other skeletal defects with an increased cancer risk later in life of the affected individual.

Commonly inherited mutations in as many as 18 diverse RP genes cause Diamond-Blackfan anemia (DBA) in children. The clinical features are erythroid hypoplasia in an otherwise normocellular marrow that manifests with a macrocytic anemia. Up to 50% of patients with DBA also have short stature, craniofacial defects, thumb abnormalities, and congenital heart malformations. Mutations in RPs (see Table 4.3) are identified in up to 50% of DBA patients and in general function in an autosomal dominant manner. Mutations in these genes lead to haploinsufficiency of the corresponding RPs which causes direct defects in proper ribobiogenesis and decrease in ribosome number. Interestingly, also mutations in GATA1, a transcription factor essential in erythropoiesis, cause DBA, and, as seen in DBA with RP defects, GATA1 mRNA is more sensitive to downregulation of RPs.[55,56]

TABLE 4.3 Disorders of Translation

Translational Defect	Gene	Defective Process	Genetic	Disorder/ Syndrome	Incidence/ Onset	Clinical Feature	Cancer Risk	References
Ribosomal proteins	RPS7; RPS10; RPS15; RPS17; RPS19; RPS24; RPS26; RPS27; RPS28; RPS29; RPL5; RPL11; RPL18; RPL26; RPL27; RPL35; RPL35A; RPL36	Ribiobiogenesis	Inherited and Somatic (50%)	Diamond-Black fan anemia (DBA)	5–10 in 1 million Childhood	Bone marrow failure; Anemia; macrocytosis; reticulocytopenia; decrease erythroid precursors	MDS AML	Mirabello et al., 2017[48] Narla et al., 2010 [49]
	RPS14	Ribiobiogenesis of 40 S subunit	Inherited	5-q syndrome	1:20,000 Adulthood	Macrocytic anemia; elevated platelets; hypoglobulated megakaryocytes	AML	Narla et al., 2010 [49]
	Indirect/ SBDS	Ribiobiogenesis	Inherited	Schachman-Diamond syndrome	1:50,000	Ineffective hematopoiesis	Leukemia	Narla et al., 2010[49]
rRNA modifications	DKC1 (dyskerin)	rRNA pseudo-uridylation	X-linked	Dyskeratosis congenita	Difficult to assess	Periferal cytopenia; bone marrow failure	AML	Narla et al., 2010[49]
	SNORD50	rRNA 2′O-methylations	Somatic	None	Not determined	B-cell lymphoma	B-cell lymphoma	Tanaka et al., 2000[50]
	SNORD14D SNORD35A	rRNA 2′O-methylations	Not known	None	Not determined	Self-renewal of leukemia cells	Not known	Zhou et al., 2017[51]
	NAT10	rRNA- acetylation	Not known	None	Not detemined	AML	AML	Liang et al., 2020[52]
tRNA defects	RMRP	tRNA precursor maturation	Inherited	Cartilage hair hypoplasia	1:1300 (Amish) 1:20,000 (Finnish descent)	Macrocytic anemia Lymphopenia	Non-Hodgkin lymphoma	Narla et al., 2010[49]
	QTRT	Queuosine tRNA modification	Not known	None	Not determined	Carcinogenesis	Leukemia	Ishiwata et al., 2001[53]

Another remarkable example of hematologic disorders connected to mutations in an RP is the $5q^{-}$-syndrome, a subtype of MDS. Haploinsufficiency of RPS14, resulting from acquisition of an interstitial deletion on chromosome 5q,[57] causes a refractory anemia very similar to DBA.

Another inherited hematologic disorder indirectly connected to translation is the Schwachman-Diamond syndrome (SDS). This syndrome is characterized by impaired hematopoiesis and neutropenia, exocrine pancreas dysfunction, and bone defects. SDS is caused by mutation in the *SBDS* gene, which does not encode for an RP as in the case of DBA and 5-q syndrome but which has critical function in ribosome maturation.

In addition to mutations in RPs, defects in proper posttranscriptional modifications of rRNA exist. Examples include dyskeratosis congenital (DKC), an x-linked disorder, caused by mutations in dyskerin (DKC1) which mediates pseudouridylation of rRNA. In addition to this genetic disorder, defects in other types of posttranscriptional modifications of rRNA have been observed. Changes in the expression level of ncRNA involved in highly specific 2′O-methylations have been connected to B-cell lymphomas and self-renewal control of leukemia cells. Similarly, changes in expression levels of NAT10, involved in rRNA acetylation, were linked to AML (reviewed in Janin et al.[58]), further suggesting that finely tuned translational control plays a fundamental role in the hematologic pathophysiology.

In the past years, defects in tRNA processing and posttranscriptional modification have been observed both in genetic and somatic hematologic conditions. Cartilage hair hypoplasia (CHH) was first described in Amish families as a form of short-limbed dwarfism caused by skeletal dysplasia. The patients show immune dysfunction and an increased risk of acquiring non-Hodgkin lymphoma in adulthood. In addition, defects in uncommon modifications of tRNA, such as queuosine, have been found to be associated with carcinogenesis and leukemia.

Several RPs have extraribosomal functions important for cell proliferation and differentiation, DNA repair, apoptosis, and others resulting in defects independent of protein translation. Mutations affecting members of the ISR that senses cellular stresses and adapts gene expression to stress predominantly cause disorders of cells with high secretory demands, such as pancreatic cells or in neuronal cells. The mTOR pathway coordinates anabolic and catabolic processes in response to internal and external stimuli; rapamycin, an inhibitor of mTOR, inhibits translation initiation. Defects in the mTOR pathway predominantly result in neurologic disorders. Both activating and loss-of-function mutations in the class IA PI3K PIK3CD or its regulatory subunit PIK3R1 cause immunodeficiency.

All ribosomopathies connected to hematologic outcomes have increased cancer risk with development of MDSs or AML, or head and neck tumors in DKC and lymphomas and basal cell carcinomas in CHH.[59] Studies suggest that translational output of the misassembled, structurally distinct ribosomes may favor translation of growth-promoting, oncogenic proteins or translation-independent regulation of oncogenes, such as MYC. Changes in ribosome composition may result in "onco-ribosomes." In addition, ribosome defects may alter cellular protein and energy balance and create cellular stress conditions conducive to acquisition of secondary mutations. Elevated levels of reactive oxygen species (ROS) in ribosomopathies are associated with high oxidative stress and result in DNA damage and genomic instability and acquisition of cooperating mutations. Preventing the switch from hypoproliferation to hyperproliferation offers a chance to prevent cancer in patients affected by these disorders.

Despite the universal expression of ribosomal genes or ribosome-related genes and ribosomal assembly factors in all tissues, the clinical presentation is dictated by the function of the affected genes and tissue-specific reliance on affected pathways. It is clear that mutations in RP genes or other proteins involved in ribosome biogenesis are causing highly specific phenotypes in specific cell types and tissues. The question as to why changes in the ubiquitous expression of these proteins and ncRNAs as well as the global requirement for protein synthesis drive specific phenotypes remains to be fully answered.

Three models prevail to explain the phenotypic variability of ribosomopathies: (1) defects in ribosome biogenesis and function result in reduced effective *ribosome concentration* that leads to diminished translation of a subset of mRNAs, as recently proposed for DBA[60] in certain tissues that are most sensitive to changes in ribosomal function; (2) the composition of ribosomes varies, imparting *specialized ribosome* function to different cell types resulting in disruption of translation of a subset of mRNAs; (3) impaired ribosome biogenesis activates the tumor suppressor p53 pathway via accumulation of unassembled RPs that bind the E3 ubiquitin-protein ligase MDM2, suppress its activity, and stabilize p53, resulting in cell cycle arrest and apoptosis.[47]

PROTEIN SORTING

Most proteins are synthesized on free polysomes and remain in the cytosol, where they are further modified. These proteins, which should reside in the cytoplasm, include enzymes involved in many metabolic and signal transduction pathways, proteins required for mRNA translation or assembly of the cytoskeleton. Other proteins are targeted toward organelles while being translated on ribosomes and are imported cotranslationally. Other proteins are produced in the cytoplasm and transported posttranslationally from the cytosol into organelles, including the nucleus, mitochondrion, and peroxisome (Fig. 4.8). These protein-trafficking events are governed by sorting signals (i.e., short amino-acid sequences or three-dimensional patches of particular amino acids) and by cognate receptors (see some examples in Table 4.2). The first sorting decision occurs after approximately 30 amino acids of the nascent polypeptide are extruded from the ribosome. If the nascent polypeptide lacks a "signal sequence," the translation of the polypeptide is completed in the cytosol. The protein either remains in the cytosol or is posttranslationally translocated into an organelle. If the protein contains an amino-terminal signal sequence, it is imported cotranslationally into the ER. From the ER, proteins are trafficked to the Golgi compartment and lysosomes via the vesicle pathway.

Targeting of Nuclear Proteins

For a detailed description of the NPCs, refer to the previous section on Nuclear Export of RNA.

NPCs are capable of importing and exporting molecules or complexes, provided that the molecules have an exposed nuclear localization signal (NLS) or a nuclear export signal (NES). In Table 4.2, some of the best-known signals are listed. The NLSs are not cleaved off as occurs for other signals (see later) and thus can function repetitively. Candidates exposing signals for nuclear import (i.e., transcription factors, coactivators or corepressors, DNA repair enzymes, RPs and mRNA processing factors, etc.) or export (ribosomal subunits, mRNA-containing particles, tRNAs, etc.) are transported through the NPC in association with soluble carrier proteins, called karyopherins (also called importins, exportins, or transportins), which function as shuttling receptors for different protein cargos. Ran is a small Ras-like GTPase, belonging to the G protein superfamily, that controls both the docking of carrier proteins with their cargo and the directionality of transport through cycles of GTP binding and hydrolysis.

NF-κB, a nuclear factor for the transcriptional enhancer of the κ light chain in B lymphocytes and a key element of the stress response, is normally retained in the cytoplasm by interaction with IκB. The TNF-α–dependent phosphorylation of IκB releases NF-κB, thereby exposing an NLS and that allows NF-κB to migrate into the nucleus, where it activates transcription of its target genes. For the glucocorticoid receptor (GR), which is localized in the cytoplasm, binding to its ligand exposes an NLS recognized by an importin and allows translocation into the nucleus, where GR activates genes by binding to GR-responsive elements in their promoter regions. The karyopherin export factor XPO1 is overexpressed in a variety of cancer cell types, including multiple myeloma and leukemia. A small molecule inhibitor of XPO1 inhibits the export of leucine-rich NES-dependent proteins, including that of tumor suppressors, such as TP53, TP21, BRCA1/2, pRB, and FOXO, that detect DNA damage in the nucleus and limit cancer cell survival.[61]

Cotranslational Protein Translocation Into the Endoplasmic Reticulum

The ER is an extensive membranous network that is continuous with the outer nuclear membrane and is the site for the synthesis of the massive amounts of lipids and proteins used to build the plasma membrane and the membranes of most cellular organelles. The ER comprises three interconnected domains: rough ER, smooth ER, and ER exit sites. The rough ER, abundant in cells specialized in protein secretion, such as cells of exocrine glands or plasma cells, is studded with bound ribosomes that are actively synthesizing proteins. The smooth ER lacks ribosomes, is not very abundant in most cells (except hepatocytes), and is thought to be the site of lipid biosynthesis and of cytochrome P450–mediated detoxification reactions. ER exit sites are specialized areas of the ER membrane where transport cargo is packaged into transport vesicles en route to the Golgi apparatus.

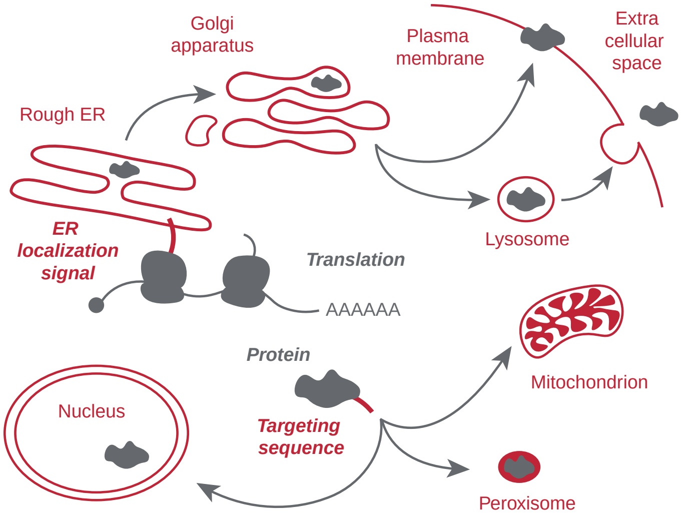


Figure 4.8 FOLLOWING TRANSLATION, PROTEINS ARE SORTED TO DIFFERENT DESTINATIONS. Depending on the presence of targeting sequences, proteins can be targeted to the nucleus, mitochondrion, or peroxisome. The endoplasmic reticulum (ER) signal sequence leads the ribosome to the ER, where proteins are destined to organelles of the secretory pathway: Golgi, plasma membrane, lysosome, or extracellular space.

Nascent secretory proteins are marked for cotranslational translocation into the ER by the presence of an amino-terminal ER signal sequence (see Table 4.2) that contains one or more positively charged amino acids followed by a continuous stretch of 6 to 12 hydrophobic residues. As the signal sequence emerges from the ribosome, it is recognized by the signal recognition particle (SRP), a ribonucleoprotein, that docks the ribosome to the ER membrane by interaction with the SRP receptor. Upon binding of GTP to the SRP and its receptor, the ribosome and the nascent chain are transferred in close proximity to the Sec61 translocon complex on the cytosolic face of the ER membrane. As the nascent polypeptide emerges from the luminal side of the translocon in an N-to-C direction, its signal sequence is cleaved by signal peptidase, translation is completed, and the protein assumes its folded configuration. Proteins without specific targeting sequences fully enter the ER lumen. For proteins destined to membranes, transmembrane domains consisting of approximately 20 largely apolar amino acids anchor the protein to the ER membrane lipid bilayer.

From the ER, proteins are transported to the plasma membrane and secreted or integrated into the membrane or targeted to endosomes; alternatively they are transported to the Golgi, lysosomes, and other organelles or retained in the ER as their final destination.[62] Transport through the secretory pathway is mediated by vesicles, and sorting motifs within proteins dictate the selective incorporation of cargo proteins into vesicles and their delivery to the intended destination.

On achieving transport competence, proteins are granted access to higher-ordered membrane domains termed ER exit sites. At ER exit sites, membrane-bound and soluble proteins are concentrated into transport vesicles for trafficking to a network of smooth membranes called the *ER-Golgi intermediate compartment* (ERGIC). COPII complex, composed of coat proteins, concentrates and packages the protein cargo into vesicles. COPII binds to cargo molecules either directly, if they span the membrane, or through intermediate cargo receptors and then provides some of the force that causes vesicle budding, thereby linking cargo acquisition to vesiculation.

ER-resident proteins are selectively sequestered in the ER, both for the absence of export signals and to the presence of ER retention signals. Soluble luminal ER resident proteins are retained through a C-terminal ER tetrapeptide retention motif KDEL. Frequently, transmembrane proteins have either a C-terminal dilysine motif KKXX or an Nt diarginine motif XXRR, or variants thereof for transmembrane proteins. However, it is more accurate to indicate ER localization signals as "retrieval motifs" because proteins bearing these signals can transiently escape from the ER into the ERGIC, from which they are returned to the ER through the retrograde vesicular transport.

For the KDEL motif of luminal ER proteins, a specific retrieval receptor has been identified, first in yeast and then in mammals. The KKXX motif has been shown to interact directly with the COPI coat protein complex that is involved in retrograde transport from the ER to the Golgi. Retrograde transport also serves to replenish the vesicle components lost as a result of anterograde (forward) transport. In conclusion, selective protein exit from the ER is achieved by monitoring/regulating (1) transport competence of nascent proteins, (2) capture of cargo in transport vesicles, and (3) protein retention/retrieval for ER-localized proteins.

Intra-Golgi Transport and Protein Processing

The Golgi complex comprises a stack of flattened, membrane-bound cisternae that is highly dependent on microtubules for structural integrity. The stack of cisternae can be subdivided into three parts referred to as *cis, medial*, and *trans*, with the *cis* and *trans* sides facing the ER and the plasma membrane, respectively. Both the *cis* and *trans* faces are associated with tubulovesicular bundles of membranes. The ERGIC comprises the bundle on the *cis* side of the Golgi stack and is the site where incoming proteins from the ER are sorted into those directed for anterograde or for retrograde transport. The tubulovesicular bundle at the *trans* side is the *trans*-Golgi network (TGN).

A major feature of the Golgi is polarity. The processing events are temporally and spatially ordered because the processing enzymes have a characteristic distribution across the Golgi stack. In the Golgi, different types of modifications take place (e.g., proteolytic processing, protein *O*-glycosylation and elaboration of N-linked chains, phosphorylation or sulfation of oligosaccharides, and sulfation of tyrosines as described later). The importance of protein glycosylation for human biology is underlined by the identification of many inherited human disorders that are caused by defects in these processes and cause clinical manifestations as described in Box 4.1.

Extensive analysis has failed to reveal a clear retention motif enabling subdomain-specific retention of resident Golgi proteins. Two possible models have been proposed. One model is *retention by preferential interaction with membranes of optimal thickness*. It is based on the finding that the transmembrane domains of Golgi proteins are shorter than transmembrane domains of plasma membrane proteins. These differences should allow a preferential interaction with the Golgi membrane lipid bilayer that is thinner than that of plasma membrane. The other model is *kin-recognition/oligomerization*. It postulates that proteins of a given subdomain of the Golgi membrane can aggregate into large detergent-insoluble oligomers as a way of minimizing lipid-protein contact. This would prevent the entry of proteins into the vesicles and thus their traffic to more distal cisternae. There is evidence in support of both models.

Cargo proteins exit the ER in COPII-coated vesicles that enter the ERGIC and are ultimately delivered to the *cis*-Golgi either in vesicles or along extended tubules. However, the mechanism whereby cargo proteins move across the Golgi complex from *cis* to *trans* remains controversial. Two models have been proposed. The vesicular transport model contends that anterograde transport occurs in vesicles or tubules that traffic cargo in an anterograde direction. The second suggests that there is a cisternal progression and maturation. This alternative model proposes that Golgi cisternae are not fixed structures but move forward from the *cis* side to the *trans* side generating an anterograde movement. As cisternae mature, resident Golgi proteins that belong to more *cis*-like cisternae must be selectively pinched off in vesicles and trafficked back to the *cis* side of the Golgi stack. This would occur by COPI-mediated retrograde vesicular transport. Although which of

BOX 4.1 Human Glycosylation Disorders

In humans, the three main glycosylation pathways are the *N*- and the *O*-glycosylation and the glycosylphosphatidylinositol (GPI) anchoring. Approximately 2% of the human genome encodes enzymes critical in glycosylation reaction, such as glycosyltransferases (GTs), glycoside hydrolases (GHs), or glycosidases, nucleotide sugar transporters.[63]

Glycosylation pathways intersect with glucose, lipid, and isoprenoid metabolism, expanding the number of players involved and the effects of these key protein modifications. Nearly 70 inherited glycosylation disorders have been identified so far, and this number is steadily increasing because of the progress in the technology of DNA sequencing and in mapping mutations. The characterized mutations combined with the biochemical lesion and with the clinical manifestations are classified in the congenital disorders of glycosylation (CDG) database (https://rarediseases.org/rare-diseases/congenital-disorders-of-glycosylation/). Abnormalities in *N*-glycosylation cause severe myasthenic syndromes caused by hypoglycosylation of the acetylcholine receptor that affects the signal transmission at the neuromuscular plaque. Others cause neurologic disorders. Hematologic disorders of glycosylation in particular are exemplified by hemolytic transfusion reactions due to differences in α-Gal(NAc) transferase alleles between individuals determining the ABO subtypes as well as differences in less prominent glycan antigens. In liver disorders, patients can manifest with bleeding due to aberrations in N-glycosylation of fibrinogen associated with a prolonged prothrombin time. Paroxysmal nocturnal hemoglobinuria (PNH) is a well-known, rare acquired hemolytic anemia arising from a somatic mutation in hematopoietic stem cells in the *PIGA* gene essential in the first step of biosynthesis of the GPI anchor for numerous cell surface proteins. The effects in red blood cells are the most prominent, resulting in hemolysis due to aberrant complement activation; patients are also at high risk for thrombosis due to loss of GPI-anchored proteins on monocytes and platelets (glycosylation disorders are reviewed in Freeze et al.[64]).

these models is correct is currently unclear, a majority of the experimental data support the cisternal maturation model. In particular, technical progress in live-cell imaging provided evidence supporting a very dynamic nature of this organelle as expected by the progression/maturation model.

The TGN is an important site of intracellular sorting, where proteins bound for lysosomes or regulated secretory vesicles are separated from those entering the constitutive pathway leading to the plasma membrane. The secretion process is called exocytosis. The molecular basis for diversion of proteins into lysosomes and regulated secretory granules are described later.

Sorting Into Lysosomes

Lysosomes are acidic (pH of approximately 5.0 to 5.5), membrane-bound organelles containing numerous hydrolytic enzymes designed to degrade proteins, carbohydrates, and lipids. Soluble hydrolases are selectively marked for sorting into lysosomes by phosphorylation of their *N*-linked oligosaccharides that creates the mannose-6-phosphate (M6P) sorting signal. On arrival at the TGN, the M6P-modified hydrolase is bound by a cargo receptor, the M6P-receptor (M6P-R), which delivers it first to a "late endosomal compartment," where the low pH releases the hydrolase from the M6P-R. Subsequently, the hydrolase is delivered to the lysosome, and the M6P-R is recycled from the endosomes through retromer-coated vesicles to the TGN to be reused.

The motif responsible for targeting M6P-R to lysosomes is YSKV and is recognized by all three distinct adaptor protein (AP) complexes (AP-l, -2, and -3) that contribute to delivery of cargo to lysosomes by linking cargo acquisition to vesiculation. Cargo recruitment occurs in a manner similar to that described for the COPI- and COPII-dependent vesicles, except that the cytosolic coat complex is clathrin. In addition to luminal hydrolases, lysosomes also contain a wide array of membrane proteins that are targeted to lysosomes via one of two consensus motifs: (1) YXXe, where X is any amino acid and e is any amino acid with a bulky hydrophobic side chain, and (2) a leucine-based motif (LL or LI). Trafficking of these membrane-bound proteins to lysosomes is indirect, proceeding first to late endosomes or the plasma membrane before their retrieval to lysosomes. Failure to accurately target lysosomal hydrolases underlies two well-known human diseases, Hurler syndrome, and I-cell disease. *Hurler syndrome* is caused by a mutation in a hydrolase responsible for breakdown of glycosaminoglycans that prevents the hydrolase from acquiring the M6P modification, consequently preventing targeting to lysosomes. Similarly, in *I-cell diseases*, undigested material accumulates in lysosomes because of a mutation in the enzymes that create the M6P modification, causing missorting of lysosomal hydrolases.

Targeting of Mitochondrial Proteins

The mitochondrion is an essential cellular compartment in eukaryotes. Although it contains its own genome organized in a circular DNA molecule and an independent transcriptional/translational machinery, 98% of the approximately 1500 proteins that constitute the mitochondrial proteome are encoded by nuclear DNA and are imported from the cytosol after their synthesis. A small number of highly hydrophobic proteins is encoded by mtDNA and transcribed and synthesized inside the organelle.

Like nuclei, mitochondria have two membranes: the outer membrane (MOM) contacts the cytosol, whereas the inner membrane (mitochondrial import machinery [MIM]) forms numerous invaginations named *cristae*, where the enzymes reside that synthesize ATP through reactions of the electron transport chain and oxidative phosphorylation. The MOM is permeable to small molecules (less than 5 kDa) and ions, whereas the inner membrane is highly impermeable, a property essential to create an electrochemical gradient necessary to drive the synthesis of ATP. Protein transport into mitochondria appears to be unidirectional because no proteins are known to be exported from mitochondria. Posttranslational translocation and sorting of proteins into the various mitochondrial subcompartments are achieved by the concerted action of translocases. Precursor proteins usually have one of two targeting signals: (1) an amino-terminal presequence that is generally between 10 and 80 amino acids long and forms an amphipathic α-helix, which is rich in positively charged, hydrophobic, and hydroxylated amino acids (see Table 4.2); or (2) a less well-defined, hydrophobic targeting sequence distributed throughout the protein. The translocase of the outer membrane (TOM) complex functions as a single entry point of incoming precursors into the mitochondria and is crucial for the biogenesis of the organelle and for the viability of eukaryotic cells. After crossing the outer membrane, proteins segregate according to their signals and recognize two distinct translocases of the inner membrane (TIM23 and TIM22). The intermembrane space import and assembly machinery (MIA) drives the import of proteins with cysteine-rich motifs, the sorting/assembly machinery (SAM) inserts precursors of proteins with a transmembrane β-barrel domains in the MOM, and the MIM imports outer membrane proteins with α-helical transmembrane segments after their recognition by the TOM complex.[65] Interestingly, cryoelectron microscopy images in cells show that translation-arrested cytosolic ribosomes are in close proximity of the surface of mitochondria. These ribosomes are organized in the clusters with the TOM complex, suggesting localized translation of mitochondrial proteins.[66]

In the context of cell biology, mitochondria play relevant roles in apoptosis, in the communication with the ER, and in oxidative stress. Among the proteins associated with the cytosolic side of MOM, those of the Bcl-2 family have both proapoptotic or antiapoptotic functions. BCL-2 family proteins function through direct protein-protein interactions. When BH3-only proteins are released by antiapoptotic BCL-2-like family members, they promote homo-oligomerization of the effector proteins BAX and BAK and induce mitochondrial outer membrane permeabilization, release of cytochrome c, and activation of the caspase cascade. BCL-2–mediated antiapoptotic effects are critical to the survival of CLL cells and inhibition of BCL-2 in CLL has led to complete responses even in relapsed, refractory CLL[67]; expansion of inhibition of BCL-2 and other antiapoptotic proteins in CLL and other hematologic malignancies is focus of intense studies.

Targeting of Peroxisomal Proteins

Peroxisomes are membrane-bound compartments that perform key roles in lipid metabolism and convert ROS. In this organelle, hydrogen peroxide is rapidly degraded by catalase to prevent oxidative reactions that have potential damaging effects on cellular structures. A single membrane surrounds the peroxisome that lacks a genetic system and transcriptional/translational machinery. All peroxisomal proteins are nuclear encoded. Their mRNAs are translated on cytosolic-free ribosomes, and proteins are imported posttranslationally by proteins called peroxins (Pex) that recognize peroxisomal targeting signals (PTSs) (see Table 4.2). Pexs function as cargo receptors; they bind cargo proteins in the cytosol, release them into the matrix, and cycle back to the cytosol. Experimental evidence suggests the existence of two routes by which peroxisomal matrix proteins can reach peroxisomes, one direct route and one via the ER.[68,69]

Sorting into Regulated Secretory Granules

In regulated secretion, proteins are condensed into stored secretory granules that require an appropriate stimulus for release from the cell. After budding from TGN, the granule proteins are concentrated (up to 200-fold in some cases) by selective removal of extraneous contents from clathrin-coated vesicles. Mature secretory granules are thought to be stored in association with microtubules until the stimulation of a surface receptor triggers their exocytosis. One example of stimulus-induced exocytosis is the binding of a ligand to the T-cell antigen receptor (TCR) complex on a cytotoxic T lymphocyte. Conjugation of a cytotoxic T cell with its target causes its microtubules and associated secretory granules to reorient toward the target cell. Subsequently, the granules are delivered along microtubules until they fuse with the plasma membrane, releasing their contents for lysis of the target cell. Following release of the granule contents, the granule membrane components are internalized and transported back to the TGN, where the granule can be refilled with cargo proteins.

PROTEIN FOLDING AND POSTTRANSLATIONAL MODIFICATIONS

Protein Folding

As the polypeptide emerges from the ribosome, it must fold to become a mature functional protein. The conformation of a protein is dictated primarily by the primary structure. Some proteins can spontaneously acquire their mature three-dimensional conformation as they are synthesized in the cell. However, most polypeptides require assistance from other proteins for proper folding, so-called molecular chaperones that either directly assist folding reactions and/or prevent aberrant interactions, such as aggregation that can occur in a densely packed environment such as the cytosol of eukaryotic cells (protein concentration of 200 to 300 mg/mL). Most molecular chaperones are heat-shock proteins (Hsps) and in particular members of the Hsp70 family. Chaperones bind to short sequence protein motifs, in many cases containing hydrophobic amino acids. By undergoing cycles of binding and release (linked to ATP hydrolysis), chaperones help the nascent polypeptide to find its native conformation. Examples are hiding of hydrophobic sequence motifs in the protein interior so that they no longer contact the hydrophilic environment of the cytosol or assembly into multisubunit complexes with other proteins. The population of chaperones that assist folding and assembly in the cytosol is distinct from those that operate within the ER or mitochondria.

Protein Modifications

Modifications of the polypeptide chains introduce new functional groups to proteins and extend the repertoire of the standard amino acid residues. More than a hundred modifications have been identified, and modifications occur during (cotranslationally) or after synthesis of the polypeptide (posttranslationally). Most of the reversible modifications are carried out by enzymes that catalyze the transfer of a chemical group from a donor molecule to the target amino acid. In contrast, proteolytic cleavage of precursor proteins to generate mature products is generally irreversible. The most common protein modifications include acetylation, methylation, myristoylation, prenylation, phosphorylation, sulfation, γ-carboxylation, ubiquitination, sumoylation, and proteolysis. Protein modifications are critical in numerous physiologic processes and central to inherited and acquired diseases. Histone proteins undergo posttranslational modifications (PTMs), such as methylation, acetylation, and others, that modulate the epigenetic code; their specific function is reviewed in Chapter 2. Here are some features of the most common protein modifications:

- *Acetylation*: approximately 80% to 90% of human proteins are cotranslationally, irreversibly N-acetylated (acetyl group: $COCH_3$). Ribosome-associated Met-aminopeptidases and acetylases cotranslationally cleave off the Nt methionine and acetylate the second residue, respectively. Consequences of Nt-acetylation are manifold, ranging from determining subcellular localization, to regulating protein-protein interactions, to protein folding and other functions. Reversible acetylation can occur posttranslationally on the ε-amino group of lysines, abolishing its positive charge. Besides phosphorylation, lysine acetylation is perhaps the most abundant and important PTM in cell signaling and metabolism.[70]
- *Methylation*: is the reversible addition of a methyl group (CH_3) to lysine or arginine residues. This modification increases the hydrophobicity of the protein-altering protein-protein interactions. Protein arginine methylases are explored as therapeutic targets in leukemia and other hematologic malignancies.[71,72]
- *Phosphorylation*: introduces a charged and hydrophilic phosphate group in the side chains of serine, threonine, or tyrosine with a ratio of occurrence of 1000:100:1 and represents a flexible mechanism for cells to respond to external signals and environmental conditions. Phosphorylation is reversed by the action of protein phosphatases that remove the phosphate group. It has been estimated that the human genome encodes approximately 500 protein kinases and 100 protein phosphatases. The catalytic or biologic activity of many enzymes is transiently regulated by reversible phosphorylation (e.g., switched "on" or "off" by phosphorylation or dephosphorylation, respectively). There are several prominent examples of mutations in tyrosine kinases or their involvement in translocations that result in loss of response and regulation in hematologic diseases, such as mutations in FLT3 in myeloid leukemia, in JAK2 in polycythemia vera, and translocations involving the ABL tyrosine kinase in t(9;22) in chronic myeloid leukemia. All result in constitutive activation providing proliferative and survival advantage to malignant clones.
- *Sulfation:* this modification occurs at tyrosine residues and is catalyzed by tyrosyl-protein sulfotransferases (TPSTs), which are membrane-associated enzymes of the *trans*-Golgi network. An unusual nucleotide, a 3′-phosphoadenosine 5′-phosphosulfate (PAPS), is the universal sulfate donor for TPST-catalyzed reactions. Addition of sulfate occurs almost exclusively on secreted or membrane proteins and is believed to play a role in the modulation of protein-protein interactions. Sulfation is critical in blood coagulation (coagulation factors V and VIII), various immune functions, intracellular trafficking, and ligand recognition by several G protein–coupled receptors.
- *γ-Carboxylation:* vitamin K is an essential cofactor for the enzyme γ-glutamyl carboxylase (GGCX) that converts glutamate into γ-carboxyglutamate in numerous factors of the clotting cascade, specifically factors II, VII, IX, and X. γ-Glutamyl carboxylation introduces affinity for calcium ions, and its role in coagulation is described in more detail in Box 4.2.

BOX 4.2 Protein γ-Carboxylation: A Rare Posttranslational Modification Crucial for Life

γ-Carboxylation of a cluster of glutamic acid residues near the amino termini of factors involved in blood coagulation are essential for these proteins to bind calcium ions and for their proper biologic activity. These factors are prothrombin (factor II) and factors VII, IX, and X, which are involved in the coagulant response, and protein C and S with critical roles in the antithrombotic pathway that limits coagulation. In addition, protein Z and GAS6 are also γ-carboxylated. Protein Z functions as cofactor in inhibition of factor Xa and GAS6 contributes to αIIbβ3 integrin activation on platelets and release of tissue factor by endothelial cells.[73]

This PTM is catalyzed by γ-glutamyl carboxylase, an endoplasmic reticulum (ER) membrane protein, that binds the substrate protein via a sequence motif on the amino terminal side of the glutamate residues to be carboxylated and releases the protein after all glutamates in the cluster have been carboxylated. Its obligate cofactor, reduced vitamin K, is produced by the action of vitamin K-epoxide reductase (VKOR), which converts oxidized vitamin K to the reduced form. The activity of VKOR is inhibited by warfarin, a potent anticoagulant compound. γ-Carboxylase homozygous null mutants manifested dramatic effects on development with partial midembryonic loss and postnatal hemorrhage. Similar effects were observed in prothrombin or factor V–deficient mice. Thus the results of these studies have suggested that the functionally critical substrates for γ-carboxylation are primarily restricted to components of the blood coagulation cascade, although bone proteins osteocalcin and matrix Gla- (γ-carboxyglutamate) protein also require processing by γ-carboxylation for full activity. These results highlight the importance of this rare protein modification for blood coagulation.

- *Ubiquitination:* the role of ubiquitination is manifold. It marks proteins for degradation (explained in detail later) and alters their function, their location, and protein-protein interaction. Ubiquitination is achieved by three main steps involving ubiquitin activating and conjugating enzymes, and ubiquitin ligases. Single (monoubiquitination) or multiple (polyubiquitination) ubiquitins can be attached to lysines in target proteins with differing consequences.
- *Sumoylation:* **s**mall **u**biquitin-related **mo**difier (SUMO) proteins are similar to ubiquitin and hence called ubiquitin-like (Ubl). The small peptide SUMO is among the best characterized Ubl, and more than 50 proteins are modified by SUMO addition.

Additional modifications form anchors for membrane association. Among the most important of these modifications are:

- *Myristoylation:* irreversible attachment of a 14-carbon myristoyl group to the Nt glycine residue of a protein allows association of the target protein with membranes, other proteins, or lipids.
- *Prenylation:* involves attachment of the 15-carbon farnesyl group or 20-carbon geranyl group to acceptor proteins that harbor a CAAX consensus sequence (C = cysteine, AA = any aliphatic amino acid except alanine, X = any amino acid) at the C-terminus. Examples of prenylation occur on RAS and RAB protein family members and control their intracellular membrane traffic (see later). Lack of prenylation generates severe phenotypes.

The aforementioned modifications are in general reversible, whereas proteolysis is generally considered an irreversible PTM:

- *Proteolysis:* results in cleavage of the polypeptide chain at a specific site during its maturation (proinsulin to insulin) or occurs upon secretion of the polypeptide's inactive form (zymogen). The need for and specific timing of proteolysis prevent unwanted toxic effects of the processed protein in the cell, as is the case for digestive proteases, or direct activation of proteins to specific sites and contexts, as is the case for activation of coagulation factors at sites of damaged blood vessels. Proteolytic processing of propolypeptides into their mature forms occurs after the Arg-Xxx-Lys-Arg motif and is mediated by the protease FURIN. FURIN is ubiquitous and activates clotting factors, growth factors, and other proteases. Other members of the proprotein convertase (PC) family cleave after pairs of dibasic amino acids (e.g., Lys-Arg) and are expressed in specific cell types, such as neuroendocrine cells.

Additional enzymatic modifications (*N*-glycosylation, *O*-glycosylation, and attachment of a glycosylphosphatidylinositol [GPI] anchor) are discussed later and in Box 4.2.

In addition to enzymatic reactions, proteins can also undergo nonenzymatic modifications, such as during oxidative stress or in the presence of excess glucose in the blood, as in the case of hemoglobin glycation.

Protein Folding and Modifications in the Endoplasmic Reticulum

Protein folding in the ER is facilitated by protein chaperones, such as the Hsp70 family member BiP, and PTMs, such as asparagine(*N*)-linked-glycosylation and disulfide bond formation.[74] Folding begins cotranslationally and is enhanced by folding catalysts. For example, the proper pairing and formation of disulfide bonds is catalyzed by oxidoreductases, such as protein disulfide isomerase (PDI), that also shuffle nonnative disulfide bonds. In contrast to the highly reducing environment of the cytosol where disulfide bonds do not typically form, the lumen of the ER is highly oxidizing so that disulfide bond formation is favored.

Most proteins that enter the secretory pathway are modified by *N-glycosylation* which starts with the transfer of a core oligosaccharide from a lipid-linked dolichol donor to an asparagine residue within the consensus sequence N-X-S/T of a nascent polypeptide (X can be any amino acid except for proline). The N-linked oligosaccharide is composed of a glucose$_3$-mannose$_9$-*N*-acetylglucosamine$_2$ unit (Glc$_3$Man$_9$GlcNac$_2$) and is transferred to the asparagine residue by an ER oligosaccharyltransferase (OST). Further processing of the terminal sugars occurs in the ER and after the polypeptide transits the Golgi compartment. Many blood proteins (e.g., immunoglobulins, antiproteases, and coagulation factors) and many membrane proteins are glycosylated. The human A, B, and O blood-group system is defined by distinct glycosyltransferases. Both the A and B antigens are similar to the O antigen but contain additional residues: the A antigen contains an additional *N*-acetylgalactosamine, and the B antigen an extra galactose residue attached to the outer galactose. People with the O antigen carry antibodies against A and B, whereas people with the A or B antigen carry anti-B or anti-A antibodies, respectively, determining blood transfusion compatibility. The hormone erythropoietin requires a particular, complex type of *N*-glycan chains for its biologic function to stimulate erythropoiesis in vivo: recombinant erythropoietin that lacks its normal *N*-linked oligosaccharides when injected into humans is much less potent than the normal protein because it is degraded much faster than the endogenous, glycosylated protein. Thus glycans protect proteins from protease digestion and heat denaturation, confer hydrophilicity and adhesive properties, mediate protein-protein interactions, and promote protein folding and trafficking.

The ER is also the site of γ-carboxylation of glutamic acid residues (e.g., in the coagulation factors II, VII, IX, and X as described earlier and as detailed in Box 4.1). Another modification is the attachment of a GPI anchor to the C-terminal end of proteins with concomitant release of the GPI signal peptide. The GPI attachment promotes membrane association.

PROTEIN DEGRADATION

Endoplasmic Reticulum-Associated Degradation and the Unfolded Protein Response

In the ER, proteins undergo quality control, which ensures that only correctly folded proteins exit the ER. Misfolded proteins are extracted from the ER for *endoplasmic reticulum-associated degradation* (ERAD) (Fig. 4.9). ERAD occurs in three major steps: (1) detection by the ER quality control machinery and targeting for ERAD, (2) transport

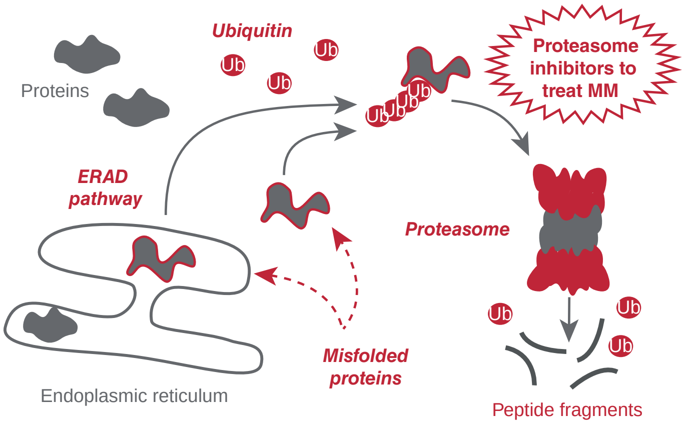


Figure 4.9 PROTEIN DEGRADATION PATHWAYS AND UNFOLDED PROTEIN RESPONSE. The major pathway of selective protein degradation in eukaryotic cells uses ubiquitin as a marker that targets proteins for rapid proteolysis thanks to the proteasome complex. This pathway deals also with misfolded proteins that could accumulate in the endoplasmic reticulum (ER) during stress or disease. Misfolded proteins are extracted from the ER for *endoplasmic reticulum-associated degradation (ERAD)*. Proteasome inhibitors are the therapeutic backbone for the treatment of several hematologic diseases, such as multiple myeloma *(MM)*.

across the ER membrane into the cytosol, and (3) ubiquitylation and degradation by the proteasome.

The ER monitors the amount of unfolded protein in its lumen. When that number exceeds a certain threshold, ER sensors activate the so-called unfolded protein response (UPR). A number of cellular insults can disrupt protein folding and cause accumulation of unfolded proteins in the ER lumen. The UPR is an adaptive response signaled through three ER-localized transmembrane proteins PERK, IRE1, and ATF6. These proteins function as sensors through the properties of their ER-luminal domains and trigger a concerted response through the function of their cytosolic domains aimed to (i) limit accumulation of unfolded protein through reduction of protein synthesis, to (ii) increase the degradation of unfolded protein, and to (iii) increase the ER protein-folding capacity. PERK-mediated phosphorylation of eIF2α attenuates general mRNA translation while increasing translation of the transcription factor ATF4 that induces transcription of UPR genes. IRE1 carries protein kinase and endoribonuclease activities that, upon activation, mediate unconventional splicing of a 26-base intron from the basic leucine zipper (bZip) transcription factor XBP1 mRNA. ATF6 is transported to the Golgi compartment upon accumulation of unfolded protein in the ER lumen, where it is cleaved by two proteases to release its cytosolic fragment also containing a bZip-transcription factor. Both XBP1 and ATF6 upregulate transcription of ER "stress elements" to correct protein folding. If the UPR adaptive response is not sufficient to correct the protein folding defect, the cells enter apoptotic death. The ER in this role is regarded as a sensor of perturbations of cell homeostasis and both activation and defects of the UPR are central to many disease processes, ranging from metabolic, neurologic, and infectious diseases to cancer.[75] In hematologic malignancies, such as multiple myeloma, the UPR response mediated by the IRE1 axis is highly activated and essential for cell survival. The role of the UPR in cancer survival and cancer immunogenicity is reviewed in Vanacker et al.[76]

Protein Degradation by the Proteasome

Protein degradation serves to regulate protein levels and to eliminate mutated proteins that would otherwise result in accumulation of misfolded proteins. As described earlier, proteins targeted for degradation are tagged via polyubiquitination and ferried to the 26 S *proteasome*, an ATP-dependent protease complex and multisubunit molecular machinery specialized in protein destruction (see Fig. 4.9). Proteasomes are located both in the nucleus and in the cytoplasm, and peptides and amino acids derived from protein disposal are recycled. The ubiquitin-proteasome system regulates a wide array of cellular processes, including differentiation, tissue development, induction of inflammatory responses, antigen presentation, cell cycle progression, and programmed cell death.[77] In addition, according to the pattern of modification of the target proteins (monoubiquitination, polyubiquitination) different proteins can trigger DNA repair (monoubiquitination of Nt tails in histones) or be subjected to endocytosis (monoubiquitination of surface receptors). Discoveries over the past several years have highlighted the central role of the ubiquitin-proteasome system in the treatment of hematologic malignancies. Targeting of the transcription factors IKZF1 and IKZF3 in multiple myeloma to the proteasome via enhanced docking to the cereblon E3 ubiquitin ligase complex by the small molecule thalidomide,[78,79] inhibition of the proteasome in multiple myeloma,[80] and most recently the development of proteolysis-targeting chimeras (PROTACs) and other technologies to therapeutically degrade proteins[81] and take advantage of the proteasome's role in cellular function.

Several pathways as described earlier serve to degrade RNA, and the proteasome serves to degrade proteins.

Autophagy

In a process called *autophagy*, cellular components and organelles are captured and degraded. Cellular material is sequestered inside double-membrane vesicles, called autophagosomes, and degraded upon fusion with lysosomal compartments. Raw precursors are subsequently recycled for new biosynthesis. Autophagy at baseline serves to demolish damaged organelles or cytosolic components; autophagy is also stress responsive to catabolize cellular components to maintain adequate energy supply and cell homeostasis. An increasing body of evidence suggests that autophagy plays an important role in development and cell differentiation by facilitating cell and tissue remodeling. Remarkably, the basis for terminal erythrocyte maturation, which involves elimination of mitochondria, ribosomes, and other organelles, is now known to be partly dependent on autophagy and in particular mitophagy, the elimination of mitochondria. Defective autophagy compromises fitness of an organism. Defective autophagy increases susceptibility to tumorigenesis, neurodegenerative disorders, liver disease, aging, inflammatory diseases, and defective host defense against pathogens.

CONCLUSIONS

Control of gene expression is a highly regulated process with several steps, including: (1) DNA transcription into RNA, (2) splicing and maturation of RNA, (3) modification of RNAs affecting their stability and localization, (4) export of RNAs from the nucleus to the cytoplasm, (5) regulation or RNA degradation and storage in RNA granules and membraneless organelles, (6) translation of mRNAs into proteins by polyribosomes, and (7) protein folding, sorting, modification, and degradation. Perturbations in any of these steps, or in their combinations, can result in hematologic disease but also possess the potential of therapeutic intervention. RNA metabolism and RNA biologics have entered drug development as targets or as actual therapeutics with great promise, respectively. The deciphering of RNA modifications and the ability to perturb the RNA methylase, eraser, and reader proteins represent novel therapeutic opportunities in MDSs and leukemia.

The mechanisms regulating protein synthesis, processing, degradation, and transport have undergone intensive investigation. Protein motifs and their cognate receptors have been identified for many intracellular sorting and processing reactions. Advanced technologies have identified numerous players of the proteasome and the mechanistic basis of protein degradation, resulting in numerous therapeutics exploiting this pathway in hematologic malignancies.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Bernabò P, Tebaldi T, Groen EJN, et al. In vivo translatome profiling in spinal muscular atrophy reveals a role for SMN protein in ribosome biology. *Cell Rep*. 2017;21(4):953–965.

Burslem GM, Crews CM. Proteolysis-targeting chimeras as therapeutics and tools for biological discovery. *Cell*. 2020;181(1):102–114.

Conboy JG. RNA splicing during terminal erythropoiesis. *Curr Opin Hematol*. 2017;24(3):215–221.

Guccione E, Richard S. The regulation, functions and clinical relevance of arginine methylation. *Nat Rev Mol Cell Biol*. 2019;20(10):642–657.

Heraud-Farlow JE, Chalk AM, Walkley CR. Defining the functions of adenosine-to-inosine RNA editing through hematology. *Curr Opin Hematol*. 2019;26(4):241–248.

Naftelberg S, Schor IE, Ast G, Kornblihtt AR. Regulation of alternative splicing through coupling with transcription and chromatin structure. *Annu Rev Biochem*. 2015;84:165–198.

Kim E, Ilagan JO, Liang Y, et al. SRSF2 mutations contribute to myelodysplasia by mutant-specific effects on exon recognition. *Cancer Cell*. 2015;27(5):617–630.

Hombach S, Kretz M. Non-coding RNAs: classification, biology and functioning. *Adv Exp Med Biol*. 2016;937:3–17.

Lee H, Bao S, Qian Y, et al. Stage-specific requirement for Mettl$_3$-dependent m^{6}A mRNA methylation during haematopoietic stem cell differentiation. *Nat Cell Biol*. 2019;21(6):700–709.

Chari A, Vogl DT, Gavriatopoulou M, et al. Oral selinexor-dexamethasone for triple-class refractory multiple myeloma. *N Engl J Med*. 2019;381(8):727–738.

Cheng Y, Luo H, Izzo F, et al. m^{6}A RNA methylation maintains hematopoietic stem cell identity and symmetric commitment. *Cell Rep*. 2019;28(7): 1703–1716. e6.

Gao Y, Vasic R, Song Y, et al. m^{6}A modification prevents formation of endogenous double-stranded RNAs and deleterious innate immune responses during hematopoietic development. *Immunity*. 2020;52(6):1007–1021. e8.

Hershey JWB, Sonenberg N, Mathews MB. Principles of translational control. *Cold Spring Harb Perspect Biol*. 2019;11(9):a032607.

Kampen KR, Sulima SO, Vereecke S, et al. Hallmarks of ribosomopathies. *Nucleic Acids Res*. 2020;48(3):1013–1028.

Khajuria RK, Munschauer M, Ulirsch JC, et al. Ribosome levels selectively regulate translation and lineage commitment in human hematopoiesis. *Cell*. 2018;173(1):90–103. e19.

Ludwig LS, Gazda HT, Eng JC, et al. Altered translation of GATA1 in Diamond–Blackfan anemia. *Nat Med*. 2014;20(7):748–753.

Mills EW, Green R. Ribosomopathies: there's strength in numbers. *Science*. 2017;358(6363):eaan2755.

Shi H, Wei J, He C. Where, when, and how: context-dependent functions of RNA methylation writers, readers, and erasers. *Mol Cell*. 2019;74(4):640–650.

Simsek D, Barna M. An emerging role for the ribosome as a nexus for post-translational modifications. *Curr Opin Cell Biol*. 2017;45:92–101.

Simsek D, Tiu GC, Flynn RA, et al. The mammalian ribo-interactome reveals ribosome functional diversity and heterogeneity. *Cell*. 2017;169(6):1051–1065. e18.

Tahmasebi S, Khoutorsky A, Mathews MB, Sonenberg N. Translation deregulation in human disease. *Nat Rev Mol Cell Biol*. 2018;19(12):791–807.

Tian S, Curnutte HA, Trcek T. RNA granules: a view from the RNA perspective. *Molecules*. 2020;25(14):3130.

Vasic R, Gao Y, Liu C, Halene S. The role of RNA epigenetic modification in normal and malignant hematopoiesis. *Curr Stem Cell Rep*. 2020;6(4):144–155.

Wilkinson ME, Charenton C, Nagai K. RNA splicing by the spliceosome. *Annu Rev Biochem*. 2020;89:359–388.

Xiang G, Keller CA, Heuston E, et al. An integrative view of the regulatory and transcriptional landscapes in mouse hematopoiesis. *Genome Res*. 2020;30(3):472–484.

Zaccara S, Ries RJ, Jaffrey SR. Reading, writing and erasing mRNA methylation. *Nat Rev Mol Cell Biol*. 2019;20(10):608–624.

REFERENCES

1. Funnell APW, Crossley M. Hemophilia B Leyden and once mysterious cis-regulatory mutations. *Trends Genet*. 2014;30(1):18–23.
2. Xiang G, Keller CA, Heuston E, et al. An integrative view of the regulatory and transcriptional landscapes in mouse hematopoiesis. *Genome Res*. 2020;30(3):472–484.
3. Cullen SM, Mayle A, Rossi L, Goodell MA. Hematopoietic stem cell development: an epigenetic journey. *Curr Top Dev Biol*. 2014;107:39–75.
4. Heraud-Farlow JE, Chalk AM, Walkley CR. Defining the functions of adenosine-to-inosine RNA editing through hematology. *Curr Opin Hematol*. 2019;26(4):241–248.
5. Wahl MC, Will CL, Lührmann R. The spliceosome: design principles of a dynamic RNP machine. *Cell*. 2009;136(4):701–718.
6. Wilkinson ME, Charenton C, Nagai K. RNA splicing by the spliceosome. *Annu Rev Biochem*. 2020;89:359–388.
7. Naftelberg S, Schor IE, Ast G, Kornblihtt AR. Regulation of alternative splicing through coupling with transcription and chromatin structure. *Annu Rev Biochem*. 2015;84:165–198.
8. Patel AA, Steitz JA. Splicing double: insights from the second spliceosome. *Nat Rev Mol Cell Biol*. 2003;4(12):960–970.
9. Yoshida K, Sanada M, Shiraishi Y, et al. Frequent pathway mutations of splicing machinery in myelodysplasia. *Nature*. 2011;478(7367):64–69.
10. Biancon G, Joshi P, Zimmer JT, et al. Precision analysis of mutant U2AF1 activity reveals deployment of stress granules in myeloid malignancies. *Mol Cell*. 2022;82(6):1107–1122.
11. Liang Y, Tebaldi T, Rejeski K, et al. SRSF2 mutations drive oncogenesis by activating a global program of aberrant alternative splicing in hematopoietic cells. *Leukemia*. 2018;32:2659–2671.
12. Kim E, Ilagan JO, Liang Y, et al. SRSF2 mutations contribute to myelodysplasia by mutant-specific effects on exon recognition. *Cancer Cell*. 2015;27(5):617–630.
13. Chen L, Chen JY, Huang YJ, et al. The augmented R-loop is a unifying mechanism for myelodysplastic syndromes induced by high-risk splicing factor mutations. *Mol Cell*. 2018;69(3):412–25.
14. Nguyen HD, Leong WY, Li W, et al. Spliceosome mutations induce R loop-associated sensitivity to ATR inhibition in myelodysplastic syndromes. *Cancer Res*. 2018;78(18):5363–74.
15. Raices M, D'Angelo MA. Nuclear pore complex composition: a new regulator of tissue-specific and developmental functions. *Nat Rev Mol Cell Biol*. 2012;13(11):687–699.
16. Hampoelz B, Andres-Pons A, Kastritis P, Beck M. Structure and assembly of the nuclear pore complex. *Annu Rev Biophys*. 2019;48:515–536.
17. Gough SM, Slape CI, Aplan PD. NUP98 gene fusions and hematopoietic malignancies: common themes and new biologic insights. *Blood*. 2011;118(24):6247–6257.
18. Osborne MJ, Borden KLB. The eukaryotic translation initiation factor eIF4E in the nucleus: taking the road less traveled. *Immunol Rev*. 2015;263(1):210–223.
19. Hombach S, Kretz M. Non-coding RNAs: classification, biology and functioning. *Adv Exp Med Biol*. 2016;937:3–17.
20. Liu X, Jiang Y, Russell JE. A potential regulatory role for mRNA secondary structures within the prothrombin 3′UTR. *Thromb Res*. 2010;126(2):130–136.
21. Polaski JT, Holmstrom ED, Nesbitt DJ, Batey RT. Mechanistic insights into cofactor-dependent coupling of RNA folding and mRNA transcription/translation by a cobalamin riboswitch. *Cell Rep*. 2016;15(5):1100–1110.
22. Conboy JG. RNA splicing during terminal erythropoiesis. *Curr Opin Hematol*. 2017;24(3):215–221.
23. Wong JJ-L, Ritchie W, Ebner OA, et al. Orchestrated intron retention regulates normal granulocyte differentiation. *Cell*. 2013;154(3):583–595.
24. Zaccara S, Ries RJ, Jaffrey SR. Reading, writing and erasing mRNA methylation. *Nat Rev Mol Cell Biol*. 2019;20(10):608–624.
25. Shi H, Wei J, He C. Where, when, and how: context-dependent functions of RNA methylation writers, readers, and erasers. *Mol Cell*. 2019;74(4):640–650.
26. Lee H, Bao S, Qian Y, et al. Stage-specific requirement for Mettl$_3$-dependent m^{6}A mRNA methylation during haematopoietic stem cell differentiation. *Nat Cell Biol*. 2019;21(6):700–709.
27. Cheng Y, Luo H, Izzo F, et al. m^{6}A RNA methylation maintains hematopoietic stem cell identity and symmetric commitment. *Cell Rep*. 2019;28(7):1703–1716. e6.
28. Gao Y, Vasic R, Song Y, et al. m^{6}A modification prevents formation of endogenous double-stranded RNAs and deleterious innate immune responses during hematopoietic development. *Immunity*. 2020;52(6):1007–1021. e8.
29. Yankova E, Blackaby W, Albertella M, et al. Small-molecule inhibition of METTL3 as a strategy against myeloid leukaemia. *Nature* 2021;593, 597–601.
30. Vasic R, Gao Y, Liu C, Halene S. The role of RNA epigenetic modification in normal and malignant hematopoiesis. *Curr Stem Cell Rep*. 2020;6(4):144–155.
31. Shah MY, Calin GA. MicroRNAs as therapeutic targets in human cancers. *Wiley Interdiscip Rev RNA*. 2014;5(4):537–548.
32. Calin GA, Pekarsky Y, Croce CM. The role of microRNA and other non-coding RNA in the pathogenesis of chronic lymphocytic leukemia. *Best Pract Res Clin Haematol*. 2007;20(3):425–437.
33. Tian S, Curnutte HA, Trcek T. RNA granules: a view from the RNA perspective. *Molecules*. 2020;25(14):3130.
34. Corbet GA, Parker R. RNP granule formation: lessons from P-bodies and stress granules. *Cold Spring Harb Symp Quant Biol*. 2019;84:203–215.
35. Hershey JWB, Sonenberg N, Mathews MB. Principles of translational control. *Cold Spring Harb Perspect Biol*. 2019;11(9):a032607.
36. Schwanhäusser B, Busse D, Li N, et al. Global quantification of mammalian gene expression control. *Nature*. 2011;473(7347):337–342.
37. Topisirovic I, Sonenberg N. Translation and cancer. *Biochim Biophys Acta*. 2015;1849(7):751–752.
38. Patel A, Lee HO, Jawerth L, et al. A liquid-to-solid phase transition of the ALS protein FUS accelerated by disease mutation. *Cell*. 2015;162(5):1066–1077.
39. Bernabò P, Tebaldi T, Groen EJN, et al. In vivo translatome profiling in spinal muscular atrophy reveals a role for SMN protein in ribosome biology. *Cell Rep*. 2017;21(4):953–965.
40. Mauro VP, Edelman GM. The ribosome filter hypothesis. *Proc Natl Acad Sci U S A*. 2002;99(19):12031–12036.
41. Kondrashov N, Pusic A, Stumpf CR, et al. Ribosome-mediated specificity in Hox mRNA translation and vertebrate tissue patterning. *Cell*. 2011;145(3):383–397.
42. Parks MM, Kurylo CM, Dass RA, et al. Variant ribosomal RNA alleles are conserved and exhibit tissue-specific expression. *Sci Adv*. 2018;4(2):eaao0665.
43. Simsek D, Barna M. An emerging role for the ribosome as a nexus for post-translational modifications. *Curr Opin Cell Biol*. 2017;45:92–101.
44. Walczak CP, Leto DE, Zhang L, et al. Ribosomal protein RPL26 is the principal target of UFMylation. *Proc Natl Acad Sci U S A*. 2019;116(4):1299–1308.
45. Simsek D, Tiu GC, Flynn RA, et al. The mammalian ribo-interactome reveals ribosome functional diversity and heterogeneity. *Cell*. 2017;169(6):1051–1065. e18.
46. Shi Z, Fujii K, Kovary KM, et al. Heterogeneous ribosomes preferentially translate distinct subpools of mRNAs genome-wide. *Mol Cell*. 2017;67(1):71–83. e7.
47. Mills EW, Green R. Ribosomopathies: there's strength in numbers. *Science*. 2017;358(6363):eaan2755.
48. Mirabello L, Khincha PP, Ellis SR, et al. Novel and known ribosomal causes of Diamond–-Blackfan anaemia identified through comprehensive genomic characterisation. *J Med Genet*. 2017;54(6):417–425.
49. Narla A, Ebert BL. Ribosomopathies: human disorders of ribosome dysfunction. *Blood*. 2010;115(16):3196–3205.
50. Tanaka R, Satoh H, Moriyama M, et al. Intronic U50 small-nucleolar-RNA (snoRNA) host gene of no protein-coding potential is mapped at the chromosome breakpoint t(3;6)(q27;q15) of human B-cell lymphoma. *Genes Cells*. 2000;5(4):277–287.
51. Zhou F, Liu Y, Rohde C, et al. AML1-ETO requires enhanced C/D box snoRNA/RNP formation to induce self-renewal and leukaemia. *Nat Cell Biol*. 2017;19(7):844–855.
52. Liang P, Hu R, Liu Z, Miao M, Jiang H, Li C. NAT10 upregulation indicates a poor prognosis in acute myeloid leukemia. *Curr Probl Cancer*. 2020;44(2):100491.
53. Ishiwata S, Katayama J, Shindo H, Ozawa Y, Itoh K, Mizugaki M. Increased expression of queuosine synthesizing enzyme, tRNA-guanine transglycosylase, and queuosine levels in tRNA of leukemic cells. *J Biochem*. 2001;129(1):13–17.
54. Tahmasebi S, Khoutorsky A, Mathews MB, Sonenberg N. Translation deregulation in human disease. *Nat Rev Mol Cell Biol*. 2018;19(12):791–807.
55. Sankaran VG, Ghazvinian R, Do R, et al. Exome sequencing identifies GATA1 mutations resulting in Diamond–Blackfan anemia. *J Clin Invest*. 2012;122(7):2439–2443.
56. Ludwig LS, Gazda HT, Eng JC, et al. Altered translation of GATA1 in Diamond–Blackfan anemia. *Nat Med*. 2014;20(7):748–753.
57. Ebert BL, Pretz J, Bosco J, et al. Identification of RPS14 as a 5q- syndrome gene by RNA interference screen. *Nature*. 2008;451(7176):335–339.

58. Janin M, Coll-SanMartin L, Esteller M. Disruption of the RNA modifications that target the ribosome translation machinery in human cancer. *Mol Cancer*. 2020;19(1):70.
59. Kampen KR, Sulima SO, Vereecke S, De Keersmaecker K. Hallmarks of ribosomopathies. *Nucleic Acids Res*. 2020;48(3):1013–1028.
60. Khajuria RK, Munschauer M, Ulirsch JC, et al. Ribosome levels selectively regulate translation and lineage commitment in human hematopoiesis. *Cell*. 2018;173(1):90–103. e19.
61. Chari A, Vogl DT, Gavriatopoulou M, et al. Oral selinexor-dexamethasone for triple-class refractory multiple myeloma. *N Engl J Med*. 2019;381(8):727–738.
62. Dancourt J, Barlowe C. Protein sorting receptors in the early secretory pathway. *Annu Rev Biochem*. 2010;79:777–802.
63. Henrissat B, Surolia A, Stanley P. A Genomic view of glycobiology Copyright 2015–2017 by The Consortium of Glycobiology Editors, La Jolla, California. All rights reserved. In: Varki A, Cummings RD, Esko JD, eds. *Essentials of Glycobiology*. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2015:89–97.
64. Freeze HH, Kinoshita T, Varki A. Glycans in acquired human diseases In: Varki A, Cummings RD, Esko JD, eds. *Essentials of Glycobiology*. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2015:583–595. Copyright 2015–2017 by The Consortium of Glycobiology Editors, La Jolla, California. All rights reserved.
65. Wiedemann N, Pfanner N. Mitochondrial machineries for protein import and assembly. *Annu Rev Biochem*. 2017;86:685–714.
66. Gold VA, Chroscicki P, Bragoszewski P, Chacinska A. Visualization of cytosolic ribosomes on the surface of mitochondria by electron cryo-tomography. *EMBO Rep*. 2017;18(10):1786–1800.
67. Roberts AW, Davids MS, Pagel JM, et al. Targeting BCL2 with venetoclax in relapsed chronic lymphocytic leukemia. *N Engl J Med*. 2016;374(4):311–322.
68. Kim PK, Hettema EH. Multiple pathways for protein transport to peroxisomes. *J Mol Biol*. 2015;427(6 Pt A):1176–1190.
69. Mayerhofer PU. Targeting and insertion of peroxisomal membrane proteins: ER trafficking versus direct delivery to peroxisomes. *Biochim Biophys Acta*. 2016;1863(5):870–880.
70. Drazic A, Myklebust LM, Ree R, Arnesen T. The world of protein acetylation. *Biochim Biophys Acta*. 2016;1864(10):1372–1401.
71. Guccione E, Richard S. The regulation, functions and clinical relevance of arginine methylation. *Nat Rev Mol Cell Biol*. 2019;20(10):642–657.
72. Fong JY, Pignata L, Goy PA, et al. Therapeutic targeting of RNA splicing catalysis through inhibition of protein arginine methylation. *Cancer Cell*. 2019;36(2):194–209. e199.
73. Robins RS, Lemarié CA, Laurance S, et al. Vascular Gas6 contributes to thrombogenesis and promotes tissue factor up-regulation after vessel injury in mice. *Blood*. 2013;121(4):692–699.
74. Shrimal S, Cherepanova NA, Gilmore R. Cotranslational and posttranslocational N-glycosylation of proteins in the endoplasmic reticulum. *Semin Cell Dev Biol*. 2015;41:71–78.
75. Cao SS, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease. *Antioxid Redox Signal*. 2014;21(3):396–413.
76. Vanacker H, Vetters J, Moudombi L, et al. Emerging role of the unfolded protein response in tumor immunosurveillance. *Trends Cancer*. 2017;3(7):491–505.
77. Amm I, Sommer T, Wolf DH. Protein quality control and elimination of protein waste: the role of the ubiquitin-proteasome system. *Biochim Biophys Acta*. 2014;1843(1):182–196.
78. Krönke J, Udeshi ND, Narla A, et al. Lenalidomide causes selective degradation of IKZF1 and IKZF3 in multiple myeloma cells. *Science*. 2014;343(6168):301–305.
79. Lu G, Middleton RE, Sun H, et al. The myeloma drug lenalidomide promotes the cereblon-dependent destruction of Ikaros proteins. *Science*. 2014;343(6168):305–309.
80. Hideshima T, Richardson P, Chauhan D, et al. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Res*. 2001;61(7):3071–3076.
81. Burslem GM, Crews CM. Proteolysis-targeting chimeras as therapeutics and tools for biological discovery. *Cell*. 2020;181(1):102–114.

CHAPTER 5

GENOME EDITING

Matthew Porteus

Genome editing is a rapidly developing field in which the genome of cells is modified with single nucleotide precision.[1,2] This degree of precision is not achievable by other forms of genetic engineering, including contemporary lentiviral vectors, recombinases, or transposases. Not only is genome editing precise in the location of the changes made, but it is highly flexible and robust in the types of changes that can be made, including creating new mutations at specific genomic sites, the conversion of a single nucleotide to another nucleotide, and the insertion of cassettes of genes at specific genomic sites. Genome editing is also known as "gene editing," but because the entire genome can be modified, not just genes, a more precise and comprehensive term is genome editing. Genome editing has become a powerful research tool and has generated tremendous excitement as a therapeutic approach to treat or cure disease. When genome editing is used therapeutically, it is a specific and precise approach that falls under the broader gene therapy umbrella.[3] This chapter will describe the process of genome editing, explain different types of edits that can be made to the genome, use specific hematologic diseases as examples of how genome editing is being developed therapeutically, and ends with a brief discussion of technical and ethical issues of applying genome editing outside of the ex vivo manipulation of somatic cells.

DOUBLE-STRANDED DNA BREAKS: THE BASIS OF NUCLEASE-BASED GENOME EDITING

The spontaneous frequency of a mutation occurring at a specific site in a nontransformed human somatic cell is on the order of 10^{-8} to 10^{-9}. Thus the ability to create specific mutations at a specific site in a reproducible fashion at high frequency is not generally possible. Moreover, the frequency of spontaneous targeted integration (the insertion of a DNA fragment at a specific location in the genome [also called gene targeting]) is on the order of 10^{-6} (only one in a million cells will undergo this process).[4] The identification of cells having undergone gene targeting can be increased by several orders of magnitude by using positive and negative selection strategies.[5–7]

The frequencies of targeted mutation and targeted integration change dramatically if a DNA double-strand break (DSB) is created at the target site.[8–10] Using contemporary methods to create DSBs, the frequency of targeted mutations, even in primary human cells, can now exceed greater than 90% of alleles and the frequency of targeted integration can routinely exceed greater than 50% of alleles. Therefore a site-specific DSB increases the frequency of targeted genome sequence changes (genome editing) by 6 to 9 orders of magnitude and is a key foundational principle of genome editing. This natural process can be manipulated experimentally or therapeutically to modify the cell's endogenous genome, making genome editing both a powerful research tool and potential therapeutic modality.

The basis on which a DSB increases genome editing by this enormous magnitude is that a genomic DNA DSB is a dangerous event to the cell. It causes the loss of chromosomal integrity (it separates the telomere from the centromere). Dozens of DSBs occur spontaneously in all living cells per day, caused by variety of different genomic insults. Cells have developed redundant mechanisms to respond to and repair DSBs. The two most prominent are nonhomologous end-joining (NHEJ) and homologous recombination (HR).[11,12]

In NHEJ, a complex of proteins holds the broken ends together while the ends are processed to allow ligases to seal the two ends back together. Conceptually, the ends are stitched back together. In general, the NHEJ process is quite accurate, with the two ends being joined precisely 70% of the time, but in the remaining instances the ends are joined after the loss or gain of nucleotides at the site of the break (insertions and deletions [indels]). The NHEJ repair pathway is active throughout the cell cycle.[13] Inactivation of genes needed for NHEJ leads to genomic instability but not to immediate cell death.[14,15]

HR is a biochemically more complex process in which a much larger number of proteins are involved. In mitotic mammalian cells, the mechanism by which cells repair DSBs by HR is through the "synthesis-dependent strand annealing" (SDSA) mechanism (Fig. 5.1).[16,17] Conceptually, the cell uses an undamaged homologous piece of DNA as a template to synthesize new DNA and then uses the newly synthesized DNA to paste into the damaged DNA to fix the DSB. It can be thought of as a "copy and paste" mechanism. An important feature of the SDSA mechanism of repair is that the template DNA is not physically transferred into the break. Instead, it is the information in the template DNA that the recombination machinery copies and transfers into the site of the break. The normal and natural template DNA is the sister chromatid. The HR repair pathway is thought to be active only in S/G_2 of the cell cycle (when a sister chromatid is present). Mutations in important HR genes can cause cell lethality within a single round of replication.[18]

BASE EDITING

The most well-developed genome editing system that does not require the creation of a DSB in the genome is base editing.[19–23] In base editing, single nucleotide changes are made by deamination of either adenine (adenine base editors [ABEs]) and cytosine (cytosine base editors [CBEs]). The natural repair mechanisms that exist to convert deaminated adenine and cytosine back to adenine and cytosine are inhibited as part of the base editing system. In ABEs the adenine is thus converted to a guanine, whereas with CBEs the cytosine is converted to a thymidine.

The base editing systems consist of a Cas9 (Cas9 is an enzyme that cuts DNA at specific sites to which it is guided by an attached "guide RNA" [gRNA] sequence—discussed in more detail later), which has been engineered such that it makes a break (nick) in only one strand of the DNA and is fused to a deamination enzyme and an inhibitor of a deglycosylase. The inhibitor of the deglycosylase blocks the natural repair of deaminated bases. The large Cas9-deaminase-inhibitor complex is directed to the site of base editing by complexing with its gRNA. It is the engineered sequence of the gRNA that targets the complex to the correct site in the genome.

Since the first reports of the development of the CBE and ABE systems in cancer cell lines, both have been applied therapeutically as well. These applications include making new stop codons in genes to create null versions of the gene. Because base editors do not generate a DSB, they can be applied to make multiplex edits

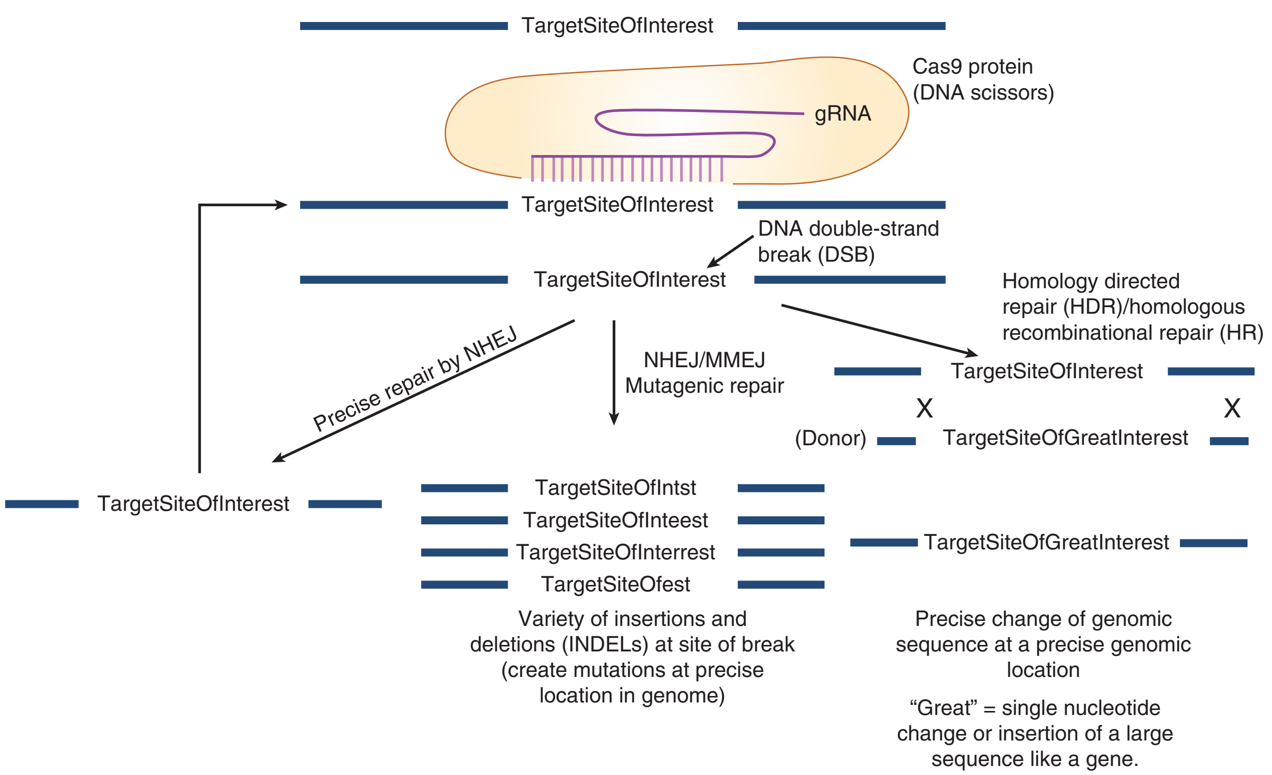

TargetSiteOfInterest
Cas9 protein (DNA scissors)
gRNA
TargetSiteOfInterest
DNA double-strand break (DSB)
TargetSiteOfInterest
Homology directed repair (HDR)/homologous recombinational repair (HR)
Precise repair by NHEJ
NHEJ/MMEJ Mutagenic repair
TargetSiteOfInterest
X
X
(Donor)
TargetSiteOfGreatInterest
TargetSiteOfInterest
TargetSiteOfIntst
TargetSiteOfInteest
TargetSiteOfInterrest
TargetSiteOfest
TargetSiteOfGreatInterest
Variety of insertions and deletions (INDELs) at site of break (create mutations at precise location in genome)
Precise change of genomic sequence at a precise genomic location
"Great" = single nucleotide change or insertion of a large sequence like a gene.

Figure 5.1 OVERVIEW OF NUCLEASE-BASED GENOME EDITING

without a high risk of causing translocations such as can occur when using multiplexing nuclease-based editing.[21] In addition to making new stop codons, base editors have also now been applied ex vivo to genetically convert the mutation that causes sickle cell disease to a hemoglobin (Hb) variant called Hb Makaser (HbM). In contrast to HbS in which a valine is at position 6 of the HBB protein, in HbM an alanine is at position 6, a harmless change.[22,23] Finally, base editing has been applied in vivo in a mouse model of a Hutchinson-Gilford progeria (a genetic premature aging disease in humans) and been demonstrated to increase the longevity and improve the symptoms.[24]

The limit of base editing is that only single nucleotide changes can be made, although simultaneous single nucleotide changes can be made at multiple sites, and that the ABE and CBEs still do not allow every possible single nucleotide substitution to be made. Although the avoidance of a DSB has been described as a possible way to reduce genotoxicity compared with nuclease-based genome editing, the full scope of off-target effects of base editors on both genomic DNA and RNA are still being investigated.

OTHER GENOME EDITING SYSTEMS

In addition to nuclease-based genome editing and base editing systems, there are multiple other genome editing systems being developed, but they have not reached the same broad utility. These include non–nuclease-based adeno-associated virus (AAV) targeted integration systems,[25,26] peptide nucleic acid–based systems,[27] PRIME-editing systems,[28] targeted transposase systems,[29] and targeted recombinase systems.[30] It is likely that other systems will continue to be discovered. The range of genomic changes that can be made using these other systems can all be made at least as efficiently nuclease-based systems, but there are several types of genomic changes that can only be made using nuclease-based genome editing. The targeted insertion of large genes in primary cells, such as hematopoietic stem cells or T cells, can only be efficiently done using engineered nucleases, for example.

USING ENGINEERED NUCLEASES TO INITIATE THE GENOME EDITING PROCESS

For genome editing to become a practical methodology, one needed to develop nucleases that could be engineered to specifically recognize any site in the genome needed to be discovered.[31–33] There are now multiple engineered nuclease platforms. Each fundamentally achieves the same effect by creating a site-specific DSB. There are three major nuclease platforms that achieve specificity by protein-DNA recognition. These platforms include engineered meganucleases, zinc finger nucleases (ZFNs), and TAL effector nucleases (TALENs).[2,34] Although there is a relatively well-defined protein-DNA recognition code for TALENs, the recognition code for meganucleases and ZFNs is more obscure. These systems are workable but complex and less readily adapted to widespread use.

Fortunately, the field of genome editing was revolutionized by the discovery of a nuclease platform that achieved specificity through Watson-Crick base pairing rather than protein-DNA recognition—the CRISPR/Cas9 nuclease system.[35,36] In the CRISPR/Cas9 system, the multifunctional Cas9 protein is complexed to a 99-nucleotide gRNA. The gRNA has two parts—a 3′ scaffold that allows it to complex with Cas9 and a 5′ recognition sequence that determines the target site that Cas9 will cut. By designing the 5′ recognition sequence to bind to the desired genomic target (by Watson-Crick base pairing), the gRNA can be used to bring the Cas9 nuclease to the correct location. This activates its nuclease activity and cuts the DNA. The CRISPR/Cas9 nuclease system has transformed the field of genome editing not just because of the ease of designing gRNAs to desired genomic targets but also, for remarkable reasons still not completely understood, because CRISPR/Cas9 has exhibited high on-target activity with low off-target activity in essentially every cellular/animal system it has been tested in. Refinements have been made to increase activity and specificity, but the CRISPR/Cas9 nuclease worked extremely well "right out of the box."

The transformational importance of the CRISPR/Cas9 system was codified by the awarding of the Nobel Prize in Chemistry in 2020 to Emmanuelle Charpentier and Jennifer Doudna for their discovery that the nuclease could be reduced to the two-component single-gRNA/

Cas9 protein complex.[36] That achievement depended on vast amounts of earlier work that went into the discovery and understanding of the CRISPR system by many others who should also be recognized.[37,38]

GENOME EDITING USING ONLY A NUCLEASE AND NONHOMOLOGOUS END-JOINING REPAIR

There are a wide variety of changes to the genome that can be made by simply delivering the nuclease like CRISPR/Cas9 into a cell and creating a break. When a single nuclease is delivered, high frequencies of indels can be created at a specific site. These mutations can be used to: (1) inactivate the coding region of a gene; (2) inactivate a promoter region of a gene; and (3) inactivate an enhancer or silencer element modulating a gene, among many other things (Fig. 5.2).

While high frequencies of indels (>90%) can be generated using a nuclease targeting a single site, some of those indels may not result in complete inactivation of the target. The repaired gene remnant may exhibit some residual activity. By delivering nucleases targeting two sites in the same genomic region, very high frequencies of defined deletions can be created (>95% when optimized).[39,40] Using a two-nuclease system, defined segments of the genome can be removed, thus creating more definitive inactivation of a gene or genetic element (e.g., an enhancer or aberrant splice site). The frequency of deletions is highest when the region of the deletion is relatively short but can still be high for deletions of several thousand base pairs in some cases and can even create megabase deletions at reasonable frequency as well.

The two-nuclease approach for targeting sites on different chromosomes can generate high frequencies of translocations. For research this can be a useful method to interrogate the role of specific translocations in development and oncogenesis.[41,42] For therapeutic purposes, such translocations need to be evaluated as a potential safety issue.[43]

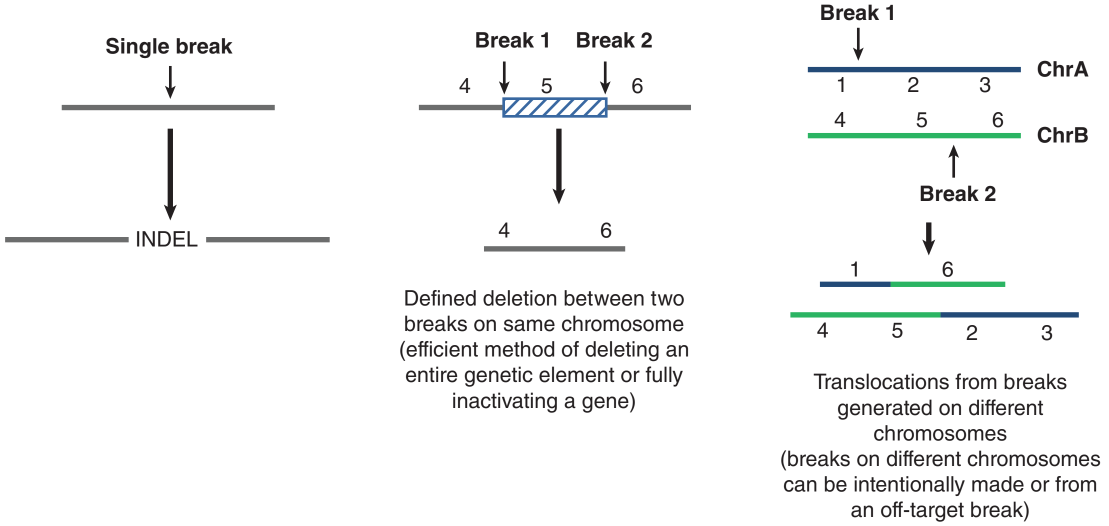
A BROAD RANGE OF GENOMIC CHANGES BY HOMOLOGOUS RECOMBINATION–MEDIATED GENOME EDITING

HR-based genome editing requires that both a nuclease and a donor DNA molecule be provided to the cell. In HR-based editing, the cell's natural HR machinery uses the engineered undamaged donor as the template for the repair of the nuclease-induced break. To differentiate HR that repairs a DSB using the sister chromatid as a natural form of repair from genome editing HR, the term "homology-directed repair" (HDR) has been coined to describe the application for genome editing (Fig. 5.3). In nuclease-based gene targeting, the donor vector needs to have flanking homology arms of greater than 400 bp (although shorter can work).[44] These relatively short homology arms are in contrast to the several thousand base pair homology arms needed for mouse ES cell gene targeting.

There are a wide variety of changes that can be made using HDR-based genome editing that can be used for both research and therapeutic purposes. These include:

1. Making single nucleotide changes to the genome, such as to revert a disease-causing genetic single-nucleotide variant;
2. Inserting a short stretch of nucleotides at specific location, such as to add an epitope tag to a protein;
3. Inserting a full complementary DNA (cDNA) or partial super-exon cDNA back into a gene to functionally correct downstream mutations;
4. Inserting one gene into another gene or replacing one gene with another gene. In this form of synthetic biology, the genome is being reprogramed to express the inserted gene under the control of the target gene's regulatory apparatus.
5. Inserting one gene into another gene without disrupting the target gene. Thus the knockin gene is now expressed along with the target gene in the cell.
6. Inserting promoter-gene cassette(s) into a safe harbor locus. A safe harbor is defined as a genomic location that if DNA is inserted into it, there would be no deleterious consequence. A safe harbor can be universal, in which it would safe to insert in all cell types or can be cell type specific, where insertion is safe in that cell type but not in another. There are many possible safe harbor options, but each needs to be carefully evaluated.

A version of HDR is to use a single-stranded oligonucleotide (ssODN) as a template rather than a gene targeting donor vector. The mechanism by which ssODNs are used by the cell to repair a DSB is not through the standard recombination pathway but instead through a pathway called "single-stranded template repair" (SSTR). SSTR does not require canonical HR genes such as Rad51 but instead requires genes in the Fanconi anemia family.[45,46] The nonnuclease targeted integration of single-stranded AAV vectors also requires genes from the Fanconi anemia family.[47] Presumably SSTR-based editing, like HR based editing, is harnessing a natural pathway that cells use to repair DSBs, but the natural function of SSTR, unlike HR, has not been well established.



Defined deletion between two breaks on same chromosome (efficient method of deleting an entire genetic element or fully inactivating a gene)

Translocations from breaks generated on different chromosomes (breaks on different chromosomes can be intentionally made or from an off-target break)

Figure 5.2 VARIATIONS ON EDITING USING NONHOMOLOGOUS END-JOINING.

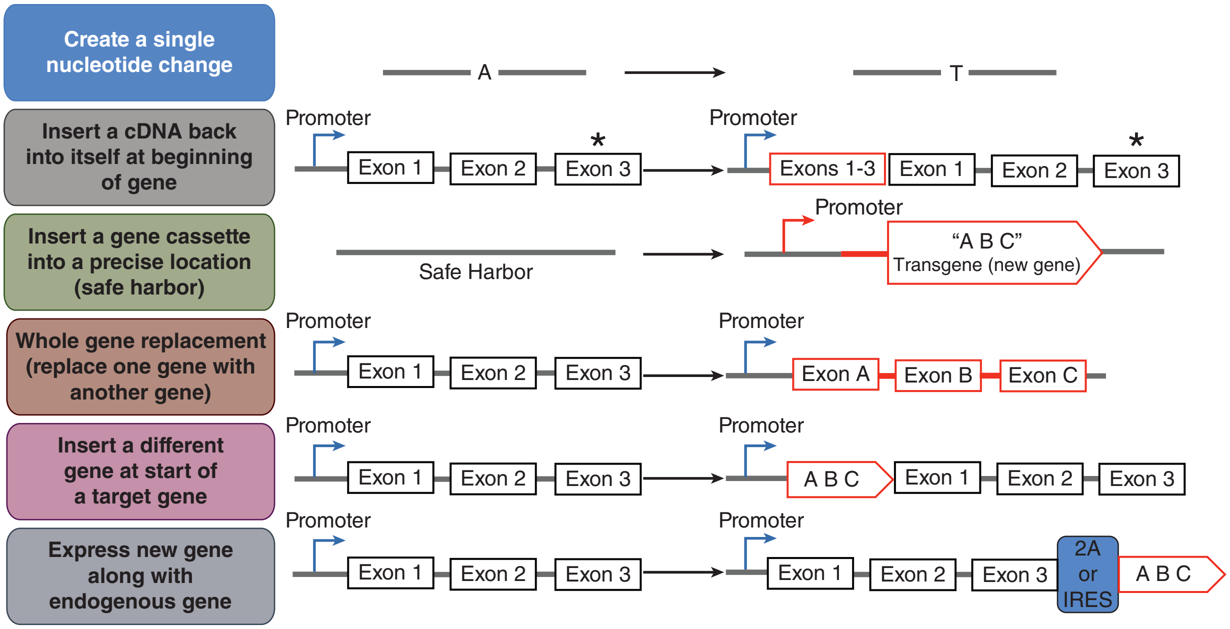

Create a single nucleotide change
A
T
Insert a cDNA back into itself at beginning of gene
Promoter
Exon 1
Exon 2
*
Exon 3
Promoter
Exons 1-3
Exon 1
Exon 2
*
Exon 3
Insert a gene cassette into a precise location (safe harbor)
Safe Harbor
Promoter
"A B C" Transgene (new gene)
Whole gene replacement (replace one gene with another gene)
Promoter
Exon 1
Exon 2
Exon 3
Promoter
Exon A
Exon B
Exon C
Insert a different gene at start of a target gene
Promoter
Exon 1
Exon 2
Exon 3
Promoter
A B C
Exon 1
Exon 2
Exon 3
Express new gene along with endogenous gene
Promoter
Exon 1
Exon 2
Exon 3
Promoter
Exon 1
Exon 2
Exon 3
2A or IRES
A B C

Figure 5.3 VARIATIONS ON EDITING USING HOMOLOGY-DIRECTED REPAIR/HOMOLOGOUS RECOMBINATION.

GENOTOXICITY THROUGH POTENTIAL OFF-TARGET NUCLEASE-INDUCED DOUBLE-STRAND BREAKS

Although all engineered nucleases are designed to have single site specificity, as proteins with biochemical properties, there is the possibility that because off-target binding (determined by standard on- and off-rates), they might create DSBs at unintended genomic sites. Because DSBs have the potential to create genotoxicity, the measurement and minimization of potential off-target breaks have been an important part of the genome editing field.[48]

There are multiple different methods to identify potential off-target sites, including bioinformatic methods, in vitro biochemical methods, and cellular-based methods. Each of these methods has its own strengths and weaknesses, and currently the approach to evaluating the specificity of nucleases used for translational purposes is to use a combination of approaches. In contrast to knockdown approaches using short-hairpin RNAs (shRNAs), it is remarkable that there have been no reports of off-target effects confounding an experimental result, and thus, for research purposes, using bioinformatics to identify guides with low probability of off-target cutting based on sequence homology has been adequate. The gold standard for measuring potential off-target effects is to quantify the frequency of indels using amplicon deep sequencing from the cell population of interest. Currently the sensitivity of this approach is reliable only down to the 0.1% level.

Because of the importance of specificity, there have been several engineered variants of the Cas9 derived from *Streptococcus pyogenes* (the most used source for Cas9 for ex vivo editing) with increased specificity.[49–53] The increased specificity has been obtained by targeting different biochemical properties of the Cas9 protein. However, some of these variants may have been overengineered and have lost on-target activity in the process of creating increased specificity. Care must then be used to identify which Cas9 is best designed for the specific project. Using these engineered specificity variants, there are now multiple examples of no measurable off-target indels being created using a given gRNA.

The potential for off-target breaks induced by a nuclease should also be put into context of the cell's robust ability to repair the wide variety and large number of DNA insults it is challenged with each day. These include tens of DSBs at random sites and thousands if not tens of thousands of other lesions, most of which occur randomly in the genome, including in tumor suppressors and oncogenes. In contrast to lentiviral vectors, there has been no description of a well-designed engineered nuclease causing lesions in a tumor suppressor or oncogene as an off-target event.

GENOME EDITING AS A RESEARCH TOOL

Genome editing, especially with the discovery and development of the CRISPR/Cas9 system, has become a powerful research tool in hematology. It is a genetic tool that now gives researchers the opportunity to genetically modify blood cells, both by NHEJ and HR, at frequencies that were once only available to yeast researchers. It has become the new standard to create genetically engineered models of human disease because zygote editing in animals has become so efficient. Animal models of human disease can now be more rapidly made in mice (including in different genetic backgrounds) and, even more importantly, beyond mice to other species. These nonmice animal models are likely to be more informative to the human condition than what was possible using in-bred laboratory mouse models.

The CRISPR/Cas9 system has also given researchers a tool to do whole genome genetic screens using libraries of gRNAs that target different parts of the genome. These screens were first focused on the coding regions of protein-coding genes but are now being applied to other parts of the genome as well. These screens allow mutations to be created simultaneously in a wide variety of genes and then the population of cells to be screened for changes in phenotype. The specific genes identified by CRISPR/Cas9 knockout screens are different than those identified by shRNA knockdown screens but usually identify the same pathways—thus these two types of genetic screens are complementary to each other.

Although most of the broad screens have been done in transformed cell lines, it is also now possible to perform such screens in primary cells, especially human T cells, by limiting the breadth of the screen.[54] Although there is some compromise in breadth, by avoiding the artifacts of screens in transformed cells that have been adapted to grow in a laboratory environment, often for decades, it is possible that understanding of the functioning of primary human blood cells will proceed more rapidly.

GENOME EDITING AS A THERAPEUTIC STRATEGY

Hematopoietic stem cell transplantation (HSCT), whether allogeneic or autologous, has been a validated curative approach to a wide variety of malignant and nonmalignant diseases since the late 1960s and early 1970s. In the past decade, the utility of hematopoietic cell therapy (HCT) to deliver genetically engineered T cells to fight cancer (notably chimeric antigen receptor [CAR] T cells) has also been established. Delivering ex vivo genome-edited hematopoietic stem and progenitor cells (HSPCs) or HCT (e.g., CAR-T cells) via HCT is building on this well-established therapeutic foundation and has the potential to make HSCT and HCT both safer and more effective.

However, two key principles need to be considered for ex vivo genome editing for HSCT- and HCT-based therapies. The first is that there is still a need to create niche space for the cell product prior to infusion. Currently for HSCT, this requires the use of myeloablative, genotoxic chemotherapy and, for HCT, pharmacologic depletion of T cells. The requirement for niche clearance creates toxicity and is presently needed for genome-edited cell therapies. It is possible in the future that genome editing could lead to approaches to make niche clearance safer and more effective. The second is the need to preserve potency and safety of the cell product after ex vivo manufacturing. Currently, genome editing requires cells to be maintained outside the body in an unnatural environment, and care must be taken to ensure that in the process of genome editing, the target cell population does not lose its biologic potency. For HSCs, for example, that would be the ability maintain the hematopoietic system for the lifetime of the patient by undergoing self-renewal and the ability to differentiate into all of the hematopoietic lineages.

EX VIVO MANUFACTURING OF THERAPEUTIC CELLS

Genome editing of cells is a quantitative process in that it is not enough simply to deliver a nuclease or a donor template, but instead they have to be delivered in sufficient quantities to achieve the desired on-target efficacy without delivering so much that it causes toxicity.[44] The relative indel formation is directly related to the amount of nuclease delivered to the cell until a plateau is reached. Similarly, the frequency of HDR is directly proportional to the amount of donor that is delivered until a plateau is reached. The primary counterforce in delivering sufficient quantities of genome editing reagents is that primary human cells have redundant and robust mechanisms to detect and inhibit the delivery of foreign nucleic acids (e.g., through the intracellular type I interferon innate immune response).[55] A secondary counterforce to efficient genome editing while maintaining cell potency is the cell's DNA damage response (DDR) to the creation of genomic DSBs and to the donor molecules themselves (in the case of HDR).[56] Thus, for successful therapeutic editing, sufficient quantities of genome editing reagents must be delivered to cells while minimizing the inhibitory responses to the process. Specifically, the delivery of genome editing reagents as naked DNA plasmids, a process that works well in transformed cell lines with damaged cytoplasmic DNA sensing systems, is not tolerated well by primary human cell such as HSPCs and T cells.

In addition to delivering the reagents without causing toxicity, for HDR-based editing, in which the cell needs to be in S/G_2 for HDR to occur, a manufacturing key is to develop ex vivo cell culture conditions that put the cells into cycle without compromising their long-term potency.

For meganucleases, ZFNs, and TALENs, the solution to ex vivo manufacturing is to deliver the nuclease as a highly purified messenger RNA (mRNA) with modifications to minimize detection of foreign RNA molecules, including the use pseudo-uridine, 5-methylcytosine, and HPLC purification to reduce double-stranded structures.

For CRISPR/Cas9, the current optimal delivery system is to deliver the nuclease as a ribonucleoprotein (RNP) complex. In this complex, purified Cas9 (which is easy to produce and stable) is mixed with a gRNA that is made synthetically with end-modifications to protect against degradation.[39] The gRNA is then complexed with Cas9 protein prior to delivery.

For both mRNA and RNP delivery, electroporation of cells to create pores through which the macromolecule enters is highly effective. Electroporation without contaminating DNA in the mix is surprisingly nontoxic to cells following optimization. Various electroporation devices that allow cell manufacturing of billions of cells are now available. Other methods that allow the macromolecules to enter cells may also be developed in the future. In all methods, the amount of nuclease macromolecule introduced needs to be titrated to achieve the desired ratio of efficacy to toxicity. One advantage of both mRNA and especially RNP delivery is the nuclease is present in the cell for only a short period (for Cas9 RNP for ~48 hours), which minimizes the possibility of creating breaks at off-target sites and decreases the cellular DDR. Using these nuclease delivery strategies, greater than 90% indels can be routinely achieved at the target site in primary human hematopoietic cells.

For HDR, the donor template must also be delivered in sufficient quantities. For HDR based on ssODNs, the purity of the ssODN is essential to enhance efficacy and minimize toxicity. Careful titrations of the amount of ssODN included are an important aspect of optimizing the manufacturing process. After optimization 30% to 50% HDR can be achieved in HSPCs using ssODN-based genome editing.[57]

For HDR using classic gene targeting donors and HR, recombinant adeno-associated virus serotype 6 (AAV6) has become the most effective method of delivering the donor molecule.[58–60] AAV6 has evolved to deliver its single-stranded DNA cargo to the nucleus of cells without being easily detected by cytoplasmic sensors of viral transduction.[55] The efficiency of AAV6 transduction of primary human HSPCs and T cells is enhanced by electroporation and is maximal if the AAV6 is applied to cells within 15 minutes of the electroporation.[61] As with the nuclease, ssODN, each AAV6 prep needs to be titered to achieve the desired efficacy and toxicity ratio. Using CRISPR/Cas9 RNP and AAV6, greater than 40% HDR can routinely achieved in HSPCs and up to 80% in T cells and certain loci in HSPCs. These frequencies of HDR are on allele basis, so a 40% HDR allele frequency results in between 55% to 70% HDR cell frequency depending on the ratio of monoallelic versus biallelic HDR.

Small molecules are increasingly being incorporated into the cell manufacturing process to improve efficacy and potency. These include small molecules to maintain stem cell potency,[62] inhibitors of the p53 response to minimize the DDR,[56] and small molecules that bias toward HDR rather than NHEJ for cells in situations where HDR is the desired editing event.[63,64]

THERAPEUTIC GENOME EDITING FOR HEMATOLOGIC DISEASES

Genome editing is being applied to a variety of diseases related to hematology and the blood system. The following are selected brief examples:

Sickle Cell Disease

Sickle cell disease is an autosomal recessive disease caused by a single point mutation in the *HBB* gene (see Chapters 42, 43). It is among the best understood genetic diseases, but currently the only curative therapy is allogeneic HSCT. However, the deep understanding of the pathophysiology of the disease has led to multiple exciting genome editing strategies to cure the disease. All currently involve the ex vivo manipulation of the patient's own HSPCs which are then transplanted back into the patient following myeloablative conditioning.

Fetal Hemoglobin Derepression

It is well documented that the severity of sickle cell disease is ameliorated by increasing the level of fetal Hb (HbF) in red blood cells because HbF acts as an antisickling Hb.[65] Upregulation of HbF is one of the major mechanisms of action for hydroxyurea, a disease-modifying small molecule.[66] Decades of research have uncovered the pathways by which HbF is downregulated at birth. One of the central proteins in this downregulation pathway is BCL11A, which is a transcription factor that suppresses gamma-globin expression.[67] There are several different genome editing approaches to inhibit the BCL11A repression (Fig. 5.4). The first is to inactivate an erythroid-specific enhancer for the BCL11A gene such that the protein is not expressed in the red cell lineage.[68,69] The inactivation is done by delivering a

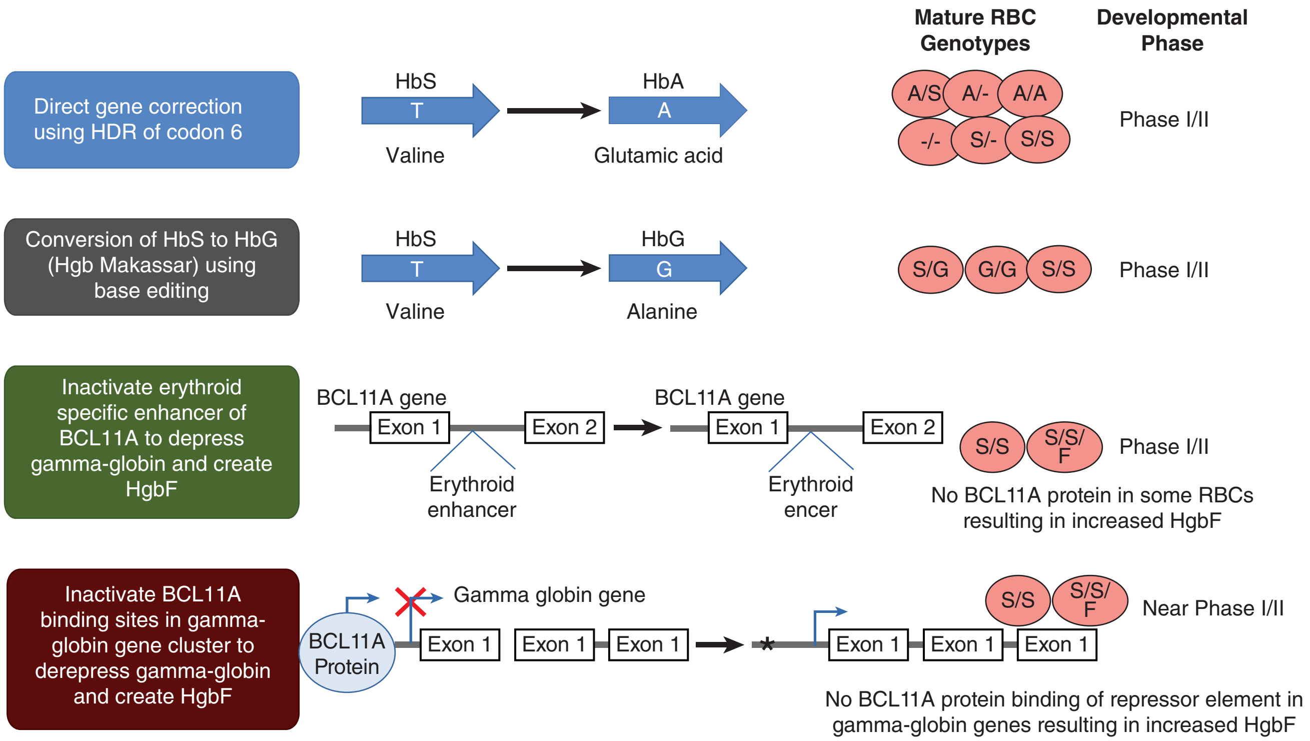

Mature RBC Genotypes
Developmental Phase
Direct gene correction using HDR of codon 6
HbS
T
Valine
HbA
A
Glutamic acid
A/S
A/-
A/A
-/-
S/-
S/S
Phase I/II
Conversion of HbS to HbG (Hgb Makassar) using base editing
HbS
T
Valine
HbG
G
Alanine
S/G
G/G
S/S
Phase I/II
Inactivate erythroid specific enhancer of BCL11A to depress gamma-globin and create HgbF
BCL11A gene
Exon 1
Exon 2
Erythroid enhancer
BCL11A gene
Exon 1
Exon 2
Erythroid encer
S/S
S/S/F
Phase I/II
No BCL11A protein in some RBCs resulting in increased HgbF
Inactivate BCL11A binding sites in gamma-globin gene cluster to derepress gamma-globin and create HgbF
BCL11A Protein
Gamma globin gene
Exon 1
Exon 1
Exon 1
*
Exon 1
Exon 1
Exon 1
S/S
S/S/F
Near Phase I/II
No BCL11A protein binding of repressor element in gamma-globin genes resulting in increased HgbF

Figure 5.4 DIFFERENT GENOME EDITING STRATEGIES FOR SICKLE CELL DISEASE.

CRISPR/Cas9 RNP complex to HSPCs that makes a break in the enhancer resulting in indels that inactivate the genetic element. Early clinical trial results show exciting promise in this approach. The small number of patients who have been treated are showing an elimination of vaso-occlusive pain crises and 50% to 60% HbF with 40% to 50% HbS in the red cell lineage in the first months following treatment.[70] This amount of HbF is predicted to prevent almost all sickling in the pancellular state, as the early results suggest it is. The long-term safety and efficacy are being evaluated.

The second is to inactivate BCL11A-binding sites in the gamma-globin gene cluster (mimicking naturally occurring hereditary persistence of fetal hemoglobin [HPFH] variants). In this strategy the CRISPR/Cas9 RNP is targeted to the BCL11A-binding sites in the gamma-globin cluster, thereby creating indels that prevent BCL11A from binding and repressing the gamma-globin genes.[71–73] Preclinical results show encouraging results in depepressing HbF using this direct globin cluster editing.

Direct Gene Correction

An alternative to the indirect approach of HbF derepression is to use HDR to directly convert the pathologic HbS allele to the nonpathologic HbA allele. Preclinical success using CRISPR/Cas9 RNP combined with either ssODN or AAV6 to template the correction has been achieved.[57,58,74] The frequency of allele correction has been 30% to 50% with ssODN and 40% to 80% with AAV6, resulting in 50% to 90% of cells having at least one allele corrected. The predicted threshold for clinical benefit based on mixed chimerism following allogeneic HSCT is that 5% to 20% cellular correction would have substantial, if not curative, clinical benefit. This increase in effect is because of the selective advantage of nonsickling red blood cells both in red blood cell development within the bone marrow and in the periphery.[75,76] Because CRISPR/Cas9 RNP is so active, the predominant genotype following the gene correction process is β thalassemiac trait. Thus patients with homozygous sickle cell disease (SS disease) may be converted to β thalassemia trait, a condition of mild anemia that millions of people around the world live healthy lives with and a condition that may even be more healthy than sickle cell trait. Clinical trials using both ssODNs and AAV6 began in 2022.

β-Thalassemia

β-Thalassemia is an autosomal recessive disease caused by mutations in the *HBB* gene that result in the production of insufficient HBB protein (see Chapter 41). Unlike sickle cell disease, the mutations that cause β-thalassemia are scattered throughout the *HBB* gene, including in the regulatory and pre-mRNA splicing elements. NHEJ and HDR approaches to β-thalassemia are being developed.

Fetal Hemoglobin Derepression

HbF upregulation, as described for sickle cell disease, is also an approach to treating β-thalassemia. By upregulating gamma-globin, the missing beta-globin is compensated for and unpaired alpha-globin chain precipitates would be prevented. The genome editing approaches for β-thalassemia to upregulate HbF are identical to those being used for sickle cell disease. The early clinical results for inactivating the erythroid specific enhancer of *BCL11A* show great promise with patients becoming transfusion independent within months of treatment and stable total Hb levels greater than 12, with essentially all of the Hb being HbF.[70] The long-term safety and efficacy of having such high levels of HbF are being evaluated, although patients with HPFH suggest that it is a relatively healthy condition.

Knocking HBB into HBA1

An HR approach to β-thalassemia has been described in which the *HBB* gene was knocked-into the *HBA1* gene without disrupting the *HBA2* gene (called “whole gene replacement”).[77] In this way, more HBB protein and less HBA protein is made, thus correcting the chain imbalance that is a key pathology in the disease. This can be considered a form of synthetic biology because the genome is being engineered such that one gene (HBB) is now being expressed instead of another gene (HBA1) through the HBA1 natural regulatory elements. In preclinical work, the ratio of beta-globin protein to alpha-globin protein was increased to the β-thalassemia trait level of 0.5 when red blood cells were evaluated from genome-edited HSPCs derived from β-thalassemia major patients. This expression level should mimic β-thalassemia trait.

Primary Immunodeficiency Diseases

There are hundreds of primary immunodeficiency diseases (PIDs) that affect the hematologic system. Unlike sickle cell disease, these are diseases in which disease-causing mutations are scattered throughout the gene. Unlike sickle cell disease and β-thalassemia, there are not indirect knockout approaches that can be used to compensate for the defective gene. A general "one size fits all" genome editing approach using HR has been developed for multiple PIDs (SCID-X1, WAS, IPEX, HIGM, CGD…).[78–83] In this strategy, a full wild-type cDNA is inserted into the early part of the gene (exon 1, intron 1, or exon 2, depending on the biology of the target gene regulation and the specifics of the strategy), thereby correcting downstream mutations. Using this strategy, more than 95% of disease-causing mutations can be addressed. In contrast to lentiviral-based gene therapy approaches, the cDNA is inserted into the natural locus and remains under the control of the endogenous regulatory elements. Because the strategy requires the targeted insertion of a full cDNA, the ssODN method will not work; gene targeting AAV6 donors have thus been used. The robustness of the CRISPR/Cas9 RNP and AAV6 donor platform is demonstrated by 30% to 50% targeted cDNA integration in HSPCs, including patient-derived HSPCs. This frequency of targeted integration would be predicted to be curative if achieved following transplantation because of the selective advantage of genetically corrected cells over mutated cells and because of the clinical potency of action of even a small percentage of functionally wild-type cells.

The improved effectiveness of the cDNA integration into the natural locus compared with lentiviral semirandom integration has been demonstrated for two genes. There was improved lineage-specific development (equivalent to wild-type development) in the natural killer (NK) lineage for severe combined immunodeficiency (SCID)-X1 and the platelet lineage for Wiskott-Aldrich syndrome using targeted integration compared with lentiviral insertion.[83] Moreover, for IPEX and HIGM (and other PIDs), lineage specific expression from endogenous regulation is essential for both safety and efficacy reasons.

Using the Blood System to Treat Nonhematologic Diseases (Metabolic Diseases)

The pharmacokinetic and pharmacodynamic properties of the hematologic system are powerful ways to deliver proteins to cure nonhematologic diseases. The system creates 200 billion new red blood cells, platelets, and neutrophils per day. Moreover, the cells from the hematologic compartment, especially macrophages, migrate into all of the tissues of the body, including the brain under stress. These properties give the blood system the potential to deliver therapeutic proteins to all tissues of the body.

The potential of the hematologic system to cure nonhematologic diseases is demonstrated by the efficacy of allo-HSCT in treating metabolic diseases such as metachromatic leukodystrophy (MLD), mucopolysaccharidosis type I (MPS I), and Gaucher disease (among others). The limitation of allo-HSCT is the inability to quickly find immunologically matched donors to use, the immunologic complications of allo-HSCT including graft-versus-host disease, poor/slow immune reconstitution, and the sometimes-slow pharmacokinetics/pharmacodynamics that are not sufficient to halt a rapidly progressive disease. Genome editing autologous HSCs to express potent amounts of enzyme has the potential to solve many of the problems of allo-HSCT and expand applications for treating nonhematologic diseases. Successful proof-of-concept preclinical studies of MPS I, Gaucher, hemophilia B, and lysosomal acid lipase deficiency (causing Wolman disease) have all been published.[84–86]

Genome Editing of Immune Effector Cells for Safer and More Potent Cancer Therapies (CAR-T Cells)

CAR-T cells have shown significant efficacy and safety at treating hematologic malignancies, especially those expressing CD19 on the surface but also BCMA-expressing malignancies. The US Food and Drug Administration (FDA)–approved CAR-T-cell products are autologous T cells genetically engineered by lentiviral or retroviral vectors to express an anti-CD19 CAR. Genome editing is being applied to CAR-T-cell therapies in numerous different ways, but the two most advanced are: (1) to generate off-the-shelf, chemotherapy-resistant CAR-T cells and (2) to make CAR-T cells more potent by using targeted integration rather than retroviral or lentiviral semirandom insertion or by knocking out checkpoint inhibitors.

Off-the-Shelf CAR-T Cells

Autologous CAR-T-cell therapies, although effective, have challenges, including the inability to manufacture a potent cell product from patients who have been exposed to large amounts of prior lympholytic chemotherapy; the inability to manufacture a product in a timely fashion before the patient's clinical condition deteriorates; and the cost of making personalized cellular products. An "off-the-shelf" allogeneic product would solve many of these problems because they would be manufactured from people with healthy T cells; the products would be ready to go and premade for each patient, and multiple doses (tens to hundreds) could be manufactured at once, thereby decreasing cost. However, allogeneic CAR-T cells would cause graft-versus-host disease without modification, but genome editing, in a nuclease-agnostic manner, can create a population of allogeneic CAR-T cells by creating inactivating indels in the T-cell receptor alpha *(TRAC)* gene.[87] Moreover, genome editing can be used to create an allogeneic product that is resistant to lymphodepletion (e.g., creating anti-CD52 antibody–resistant cells by genome editing the *CD52* gene) (Fig. 5.5).[43] Early clinical trials have demonstrated that

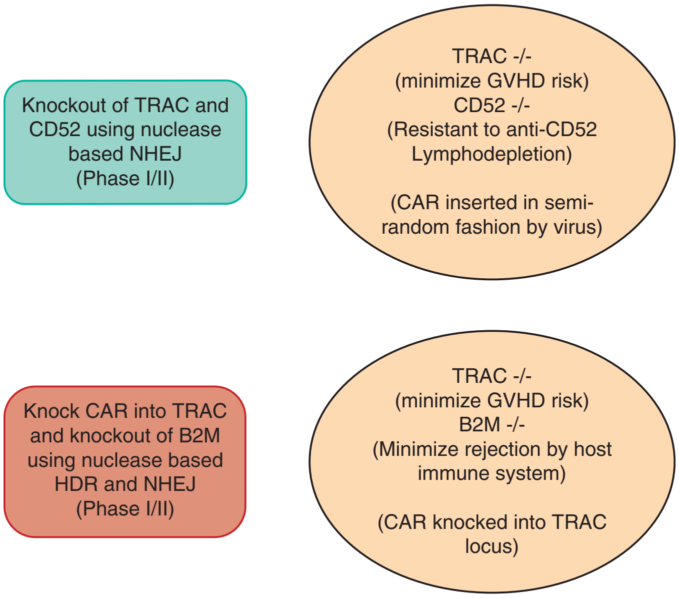

Knockout of TRAC and CD52 using nuclease based NHEJ (Phase I/II)
TRAC -/- (minimize GVHD risk) CD52 -/- (Resistant to anti-CD52 Lymphodepletion)
(CAR inserted in semi-random fashion by virus)
Knock CAR into TRAC and knockout of B2M using nuclease based HDR and NHEJ (Phase I/II)
TRAC -/- (minimize GVHD risk) B2M -/- (Minimize rejection by host immune system)
(CAR knocked into TRAC locus)

Figure 5.5 GENOME EDITING TO CREATE CHIMERIC ANTIGEN RECEPTOR (CAR)-T CELLS. GVHD, graft-versus-host disease; *HDR*, homology-directed repair; *NHEJ*, nonhomologous end-joining; *TRAC*, T-cell receptor alpha.

genomes edited off-the-shelf do have efficacy but durability seems to be an issue.[88] More patients, as part of different trials, with longer follow-up will be informative. Even if the durability issue is not readily solved, if off-the-shelf CAR-T cells can create significant remissions, it would create the opportunity to build such cells into an HSCT program in which it is known that the efficacy of HSCT is higher when the patients undergo transplant with minimal residual disease.

Increasing Potency

The long-term remission rates following CAR-T-cell therapy remain in the 30% to 40% range for the FDA-approved products. Thus a goal is to improve the potency of these therapies to increase the long-term remission rate. Several genome editing approaches have been reported to increase the potency. One approach is to knock the CAR into the *TRAC* locus such that it is regulated as if it were a T-cell receptor. The proof-of-concept publication demonstrated that the knock-in CAR-T cells had decreased features of exhaustion, including in functional assays.[89] A second approach to improve potency is to use genome editing to knockout out PD-1 to mimic what is achieved pharmacologically by checkpoint blockade using inhibitors of the PD-1 pathway. Preliminary evidence of PD-1 knockout safety has been demonstrated, but the broader safety and efficacy of this genetic manipulation need to be determined.[90]

OPPORTUNITIES FOR GENOME EDITING IN VIVO

Although there are current partial solutions to the need for substantial room for improvement in ex vivo genome editing/autologous HSC transplantation, including in the patient conditioning process, the harvesting of the target cells, the manufacturing the cells, and the subsequent reintroduction of those cells into a patient, the need for all of these steps in and of itself suggests that if one could replace the ex vivo process with a "one-shot" in vivo genome editing drug, it could be ideal for many diseases. Absent simpler delivery platforms, widespread deployment of these therapies will be severely limited by the need to access sophisticated highly expert healthcare facilities capable of managing these complex processes safely.

In 2021, the first evidence of human in vivo genome editing was reported. In this work, a Cas9 mRNA and gRNA were complexed as part of a lipid nanoparticle (LNP) designed to be taken up by liver cells.[91] The target gene was *TTR*, a gene mutated in hereditary amyloidosis—a progressive multisystemic disease. The LNPs were infused intravenously into six patients without evidence of toxicity. Excitingly, there was short-term evidence of efficacy with reduction of protein levels of 86% in the blood at the highest LNP dose tested. Although the target gene was in the liver, the effect was to alter the concentration of a protein in the blood. Data beyond day 28 were not reported. This first safe use of in vivo Cas9-based editing is an important milestone in the field.

CHALLENGES TO IN VIVO EDITING OF HEMATOPOIETIC STEM CELLS

The challenges for in vivo editing of the hematologic system are currently even more difficult than those outlined for ex vivo editing (which is built on the established HSCT and HCT platforms). These challenges fall into two broad categories: (1) delivery and (2) immunogenicity.

Delivery

In vivo genome editing requires the development of a novel vector that would specifically target the cell of interest (whether HSCs or T cells or other blood cell) while not being taken up by the trillions of other cells (red blood cells, platelets, endothelial cells…) that the target cells live and coexist with in close proximity. In contrast to ex vivo editing where the target cell can be purified way from the competitor cells before editing, in an in vivo setting that would not possible. Furthermore, as discussed earlier, to achieve high frequencies of genome editing, whether through indels or HDR, requires a sufficient quantity of the genome editing reagent to enter the cell. A little gets only a little. Thus the new vector would not only have to selectively deliver to the target cell of interest but would also have to deliver the editing reagents in sufficient quantities to achieve therapeutic frequencies of editing. Finally, it should be noted that in terms of HDR, which requires cells to be cycling to be efficient, most HSCs are quiescent and so to harness HDR in vivo would require novel ways to put HSCs into cycle without compromising their long-term hematopoietic stem cell function.

Immunogenicity

The human immune system has been one of the barriers to safe and effective in vivo gene therapy, and one would anticipate the same to be true for in vivo gene editing. The first barrier is to avoid activating the intracellular innate immune response (type I interferon response) in all cells to which the novel vector delivered its cargo in vivo. The second barrier is to design a vector that does not profoundly activate the cellular innate immune response, such as macrophages and the reticuloendothelial system. The third barrier is the adaptive immune response to the genome editing reagents and vector. All of the nuclease systems involve parts that are foreign. For CRISPR/Cas9, the Cas9 protein has been demonstrated to generate an adaptive immune response, even in naïve animals.[92,93] Therefore, for in vivo editing, one would need enough nuclease to get therapeutic levels of editing but have it disappear before the adaptive immune response recognized the foreign protein and eliminated the cells still expressing it (like a virus). Moreover, most healthy adults already have a preexisting immune response to both *Staphylococcus aureus* and *S. pyogenes* Cas9.[94–97] For in vivo AAV gene therapy, patients who have even very low titers of preexisting antibodies to the AAV serotype are excluded from treatment because the preexisting immunity diminishes efficacy and might create systemic inflammation, toxicity, and death.

Proponents of in vivo editing also argue that it would be cheaper than an ex vivo process, but this assumption has not been pressure tested. A simple counter example is that the current most expensive drug in the world is an in vivo AAV-based gene therapy for spinal muscular atrophy, not an ex vivo HSPC gene therapy. In addition, the amount of AAV (the current best vector) for systemic in vivo delivery of nucleic acids needed for one patient for in vivo editing would be sufficient for thousands, if not tens of thousands, of patients using an ex vivo approach.

Nonetheless, ex vivo editing of HSCs at levels that might cure hematologic diseases was also considered an unrealistic dream not even two decades ago, so the creativity, ambition, and motivation of a large number of researchers might, over the next decades, discover solutions to the known challenges described earlier. We can anticipate that solving currently unknown barriers to in vivo editing will have to, and will, occur.

HERITABLE GENOME EDITING

The concept of genetically engineering the genome to pass along changes to future generations has been a serious topic of study over decades for a wide range of people, including the subject of movies and books. However, until the development of the CRISPR/Cas9 system, the specificity and efficacy of any genetic engineering system was never even close to reaching levels to take the discussion beyond theory and speculation. The efficiency of the CRISPR/Cas9 system in nonhuman zygotes to create genetically engineered animal models took the possibility of doing the same in humans beyond speculation into the realm of possible. It has prompted a global discussion of the technical, ethical, societal, and governance aspects of the potential of heritable human editing. These discussions are ongoing and international in scope. The broad consensus is that, because there remains a lack of clarity around technical, ethical, societal, and governance issues, creating a human pregnancy from genome-edited zygotes, genome-edited gametes, or genome-edited germ cell precursors should not be permitted. Anyone proceeding with such an activity at this time demonstrates profound hubris and pathologic disrespect for humanity and should be considered a rogue actor and treated accordingly as a criminal.

Even if a broad positive consensus on technical, ethical, societal, and governance issues were one day attained, the need for heritable genome editing to prevent a child born with a devastating disease is remarkably small. The need is small because of the already available alternatives, especially the use of in vitro fertilization, preimplantation genetic diagnosis to identify zygotes who wound not carry the disease, and subsequent implantation of only those zygotes not at risk in vitro fertilization [IVF]/pre-implantation genetic diagnosis [PGD]. The number of couples for whom in vitro fertilization [IVF]/pre-implantation genetic diagnosis [PGD] would not be a way to have a healthy child is nonzero but continues to be almost academic in nature. The need will become even smaller as effective curative somatic cell therapies are developed over the coming years—therapies that the FDA predicts will be approved in the near future at tens or more per year. The paradox that by curing more patients while maintaining fertility we might actually increase the number of patients who would then need to undergo the same postnatal curative therapy. This then becomes an argument about economics as well as ethics.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Barrangou R, Marraffini LA. CRISPR-Cas systems: prokaryotes upgrade to adaptive immunity. *Mol Cell*. 2014;54(2):234–244.

Bauer DE, Orkin SH. Hemoglobin switching's surprise: the versatile transcription factor BCL11A is a master repressor of fetal hemoglobin. *Curr Opin Genet Dev*. 2015;33:62–70.

Canny MD, et al. Inhibition of 53BP1 favors homology-dependent DNA repair and increases CRISPR-Cas9 genome-editing efficiency. *Nat Biotechnol*. 2018;36(1):95–102.

Chu SH, et al. Rationally designed base editors for precise editing of the sickle cell disease mutation. *CRISPR J*. 2021;4(2):169–177.

DeWitt MA, et al. Selection-free genome editing of the sickle mutation in human adult hematopoietic stem/progenitor cells. *Sci Transl Med*. 2016;8(360):360ra134.

Dever DP, et al. CRISPR/Cas9 beta-globin gene targeting in human haematopoietic stem cells. *Nature*. 2016;539(7629):384–389.

Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science*. 2014;346(6213):1258096.

Eyquem J, et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature*. 2017;543(7643):113–117.

Fares I, et al. Small molecule regulation of normal and leukemic stem cells. *Curr Opin Hematol*. 2015;22(4):309–316.

Frangoul H, et al. CRISPR-Cas9 gene editing for sickle cell disease and beta-thalassemia. *N Engl J Med*. 2021;384(3):252–260.

Gaudelli NM, et al. Programmable base editing of AT to GC in genomic DNA without DNA cleavage. *Nature*. 2017;551(7681):464–471.

Gillmore JD, et al. CRISPR-Cas9 in vivo gene editing for transthyretin amyloidosis. *N Engl J Med*. 2021;385(6):493–502.

Horvath P, Barrangou R. CRISPR/Cas, the immune system of bacteria and archaea. *Science*. 2010;327(5962):167–170.

Jinek M, et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. 2012;337(6096):816–821.

Komor AC, et al. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature*. 2016;533(7603):420–424.

Lattanzi A, et al. Development of β-globin gene correction in human hematopoietic stem cells as a potential durable treatment for sickle cell disease. *Sci Transl Med*. 2021;13(598):eabf2444.

Naldini L. Gene therapy returns to centre stage. *Nature*. 2015;526(7573):351–360.

Newby GA, et al. Base editing of haematopoietic stem cells rescues sickle cell disease in mice. *Nature*. 2021;595(7866):295–302.

Porteus M. Genome editing: a new approach to human therapeutics. *Annu Rev Pharmacol Toxicol*. 2016;56:163–190.

Porteus MH. A new class of medicines through DNA editing. *N Engl J Med*. 2019;380(10):947–959.

Porteus MH, Carroll D. Gene targeting using zinc finger nucleases. *Nat Biotechnol*. 2005;23(8):967–973.Qasim W, et al. Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. *Sci Transl Med*. 2017;9(374):eaaj2013.

Rouet P, Smih F, Jasin M. Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. *Mol Cell Biol*. 1994;14(12):8096–8106.

Schiroli G, et al. Precise gene editing preserves hematopoietic stem cell function following transient p53-mediated DNA damage response. *Cell Stem Cell*. 2019;24(4):551–565. e8.

Smithies O, et al. Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. *Nature*. 1985;317(6034):230–234.

Stadtmauer EA, et al. CRISPR-engineered T cells in patients with refractory cancer. *Science*. 2020;367(6481):eaba7365.

Wang J, et al. Homology-driven genome editing in hematopoietic stem and progenitor cells using ZFN mRNA and AAV6 donors. *Nat Biotechnol*. 2015;33(12):1256–1263.

Wyman C, Kanaar R. DNA double-strand break repair: all's well that ends well. *Annu Rev Genet*. 2006;40:363–383.

REFERENCES

1. Porteus M. Genome editing: a new approach to human therapeutics. *Annu Rev Pharmacol Toxicol*. 2016;56:163–190.
2. Porteus MH. A new class of medicines through DNA editing. *N Engl J Med*. 2019;380(10):947–959.
3. Naldini L. Gene therapy returns to centre stage. *Nature*. 2015;526(7573):351–360.
4. Smithies O, et al. Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. *Nature*. 1985;317(6034):230–234.
5. Sedivy JM, Sharp PA. Positive genetic selection for gene disruption in mammalian cells by homologous recombination. *Proc Natl Acad Sci USA*. 1989;86:227–231.
6. Doetschman T, Maeda N, Smithies O. Targeted mutation of the Hprt gene in mouse embryonic stem cells. *Proc Natl Acad Sci USA*. 1988;85(22):8583–8587.
7. Thomas KR, Capecchi MR. Introduction of homologous DNA sequences into mammalian cells induces mutations in the cognate gene. *Nature*. 1986;324(6092):34–38.
8. Rouet P, Smih F,M. Jasin, Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. *Mol Cell Biol*. 1994;14(12):8096–8106.
9. Choulika A, et al. Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of Saccharomyces cerevisiae. *Mol Cell Biol*. 1995;15(4):1968–1973.
10. Sargent RG, Brenneman MA, Wilson JH. Repair of site-specific double-strand breaks in a mammalian chromosome by homologous and illegitimate recombination. *Mol Cell Biol*. 1997;17(1):267–277.
11. Wyman C, Kanaar R. DNA double-strand break repair: all's well that ends well. *Annu Rev Genet*. 2006;40:363–383.
12. West SC, et al. Double-strand break repair in human cells. *Cold Spring Harb Symp Quant Biol*. 2000;65:315–321.
13. Davis AJ, Chen BP, Chen DJ. DNA-PK: a dynamic enzyme in a versatile DSB repair pathway. *DNA Repair (Amst)*. 2014;17:21–29.
14. Burma S, Chen BP, Chen DJ. Role of non-homologous end joining (NHEJ) in maintaining genomic integrity. *DNA Repair (Amst)*. 2006;5(9-10):1042–1048.
15. Mills KD, Ferguson DO, Alt FW. The role of DNA breaks in genomic instability and tumorigenesis. *Immunol Rev*. 2003;194:77–95.
16. Xue C, Greene EC. DNA repair pathway choices in CRISPR-Cas9-mediated genome editing. *Trends Genet*. 2021;37(7):639–656.
17. Paques F, Haber JE. Multiple pathways of recombination induced by double-strand breaks in Saccharomyces cerevisiae. *Microbiol Mol Biol Rev*. 1999;63(2):349–404.
18. Lim DS, Hasty P. A mutation in mouse rad51 results in an early embryonic lethal that is suppressed by a mutation in p53. *Mol Cell Biol*. 1996;16(12):7133–7143.
19. Komor AC, et al. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature*. 2016;533(7603):420–424.
20. Gaudelli NM, et al. Programmable base editing of AT to GC in genomic DNA without DNA cleavage. *Nature*. 2017;551(7681):464–471.
21. Webber BR, et al. Highly efficient multiplex human T cell engineering without double-strand breaks using Cas9 base editors. *Nat Commun*. 2019;10(1):5222.
22. Chu SH, et al. Rationally Designed Base Editors for Precise Editing of the Sickle Cell Disease Mutation. *CRISPR J*. 2021;4(2):169–177.
23. Newby GA, et al. Base editing of haematopoietic stem cells rescues sickle cell disease in mice. *Nature*. 2021;595(7866):295–302.
24. Koblan LW, et al. In vivo base editing rescues Hutchinson-Gilford progeria syndrome in mice. *Nature*. 2021;589(7843):608–614.
25. Hirata R, et al. Targeted transgene insertion into human chromosomes by adeno-associated virus vectors. *Nat Biotechnol*. 2002;20(7):735–738.
26. Barzel A, et al. Promoterless gene targeting without nucleases ameliorates haemophilia B in mice. *Nature*. 2015;517(7534):360–364.
27. Ricciardi AS, et al. Peptide nucleic acids as a tool for site-specific gene editing. *Molecules*. 2018;23(3):632.
28. Anzalone AV, Koblan LW, Liu DR. Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors. *Nat Biotechnol*. 2020;38(7):824–844.
29. Klompe SE, et al. Transposon-encoded CRISPR-Cas systems direct RNA-guided DNA integration. *Nature*. 2019;571(7764):219–225.
30. Gaj T, Gersbach CA, Barbas CF 3rd. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol*. 2013;31(7):397–405.
31. Porteus MH, Baltimore D. Chimeric nucleases stimulate gene targeting in human cells. *Science*. 2003;300(5620):763.
32. Bibikova M, et al. Enhancing gene targeting with designed zinc finger nucleases. *Science*. 2003;300(5620):764.
33. Porteus MH, Carroll D. Gene targeting using zinc finger nucleases. *Nat Biotechnol*. 2005;23(8):967–973.
34. Porteus MH. Towards a new era in medicine: therapeutic genome editing. *Genome Biol*. 2015;16:286.
35. Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science*. 2014;346(6213):1258096.
36. Jinek M, et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. 2012;337(6096):816–821.
37. Horvath P, Barrangou R. CRISPR/Cas, the immune system of bacteria and archaea. *Science*. 2010;327(5962):167–170.
38. Barrangou R, Marraffini LA. CRISPR-Cas systems: Prokaryotes upgrade to adaptive immunity. *Mol Cell*. 2014;54(2):234–244.
39. Hendel A, et al. Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. *Nat Biotechnol*. 2015;33(9):985–989.
40. Kim HJ, et al. Targeted genome editing in human cells with zinc finger nucleases constructed via modular assembly. *Genome Res*. 2009;19(7):1279–1288.
41. Buechele C, et al. MLL leukemia induction by genome editing of human CD34+ hematopoietic cells. *Blood*. 2015;126(14):1683–1694.
42. Piganeau M, et al. Cancer translocations in human cells induced by zinc finger and TALE nucleases. *Genome Res*. 2013;23(7):1182–1193.
43. Poirot L, et al. Multiplex genome-edited T-cell manufacturing platform for "off-the-shelf|" adoptive T-cell immunotherapies. *Cancer Res*. 2015;75(18):3853–3864.
44. Hendel A, et al. Quantifying on- and off-target genome editing. *Trends Biotechnol*. 2015;33(2):132–140.
45. Gallagher DN, et al. A Rad51-independent pathway promotes single-strand template repair in gene editing. *PLoS Genet*. 2020;16(10):e1008689.
46. Richardson CD, et al. CRISPR-Cas9 genome editing in human cells occurs via the Fanconi anemia pathway. *Nat Genet*. 2018;50(8):1132–1139.
47. de Alencastro G, et al. Improved Genome Editing through Inhibition of FANCM and Members of the BTR Dissolvase Complex. *Mol Ther*. 2021;29(3):1016–1027.
48. Tsai SQ, Joung JK. Defining and improving the genome-wide specificities of CRISPR-Cas9 nucleases. *Nat Rev Genet*. 2016;17(5):300–312.
49. Vakulskas CA, et al. A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells. *Nat Med*. 2018;24(8):1216–1224.
50. Slaymaker IM, et al. Rationally engineered Cas9 nucleases with improved specificity. *Science*. 2016;351(6268):84–88.
51. Kleinstiver BP, et al. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature*. 2016;529(7587):490–495.
52. Casini A, et al. A highly specific SpCas9 variant is identified by in vivo screening in yeast. *Nat Biotechnol*. 2018;36(3):265–271.
53. Chen JS, et al. Enhanced proofreading governs CRISPR-Cas9 targeting accuracy. *Nature*. 2017;550(7676):407–410.
54. Shifrut E, et al. Genome-wide CRISPR screens in primary human T cells reveal key regulators of immune function. *Cell*. 2018;175(7):1958–1971. e15.
55. Cromer MK, et al. Global transcriptional response to CRISPR/Cas9-AAV6-based genome editing in CD34(+) Hematopoietic stem and progenitor cells. *Mol Ther*. 2018;26(10):2431–2442.
56. Schiroli G, et al. Precise gene editing preserves hematopoietic stem cell function following transient p53-mediated DNA damage response. *Cell Stem Cell*. 2019;24(4):551–565. e8.
57. DeWitt MA, et al. Selection-free genome editing of the sickle mutation in human adult hematopoietic stem/progenitor cells. *Sci Transl Med*. 2016;8(360):360ra134.
58. Dever DP, et al. CRISPR/Cas9 beta-globin gene targeting in human haematopoietic stem cells. *Nature*. 2016;539(7629):384–389.
59. Wang J, et al. Homology-driven genome editing in hematopoietic stem and progenitor cells using ZFN mRNA and AAV6 donors. *Nat Biotechnol*. 2015;33(12):1256–1263.
60. De Ravin SS, et al. Targeted gene addition in human CD34(+) hematopoietic cells for correction of X-linked chronic granulomatous disease. *Nat Biotechnol*. 2016;34(4):424–429.
61. Charlesworth C, et al. Priming human repopulating hematopoietic stem and progenitor cells for Cas9/sgRNA gene targeting. *Mol Ther Nucleic Acids*. 2018;12:89–104. in press.
62. Fares I, et al. Small molecule regulation of normal and leukemic stem cells. *Curr Opin Hematol*. 2015;22(4):309–316.
63. Canny MD, et al. Inhibition of 53BP1 favors homology-dependent DNA repair and increases CRISPR-Cas9 genome-editing efficiency. *Nat Biotechnol*. 2018;36(1):95–102.
64. Certo MT, et al. Tracking genome engineering outcome at individual DNA breakpoints. *Nat Methods*. 2011;8(8):671–676.
65. Platt OS, et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. *N Engl J Med*. 1994;330(23):1639–1644.
66. Charache S, et al. Hydroxyurea: effects on hemoglobin F production in patients with sickle cell anemia. *Blood*. 1992;79(10):2555–2565.

67. Bauer DE, Orkin SH. Hemoglobin switching's surprise: the versatile transcription factor BCL11A is a master repressor of fetal hemoglobin. *Curr Opin Genet Dev*. 2015;33:62–70.
68. Canver MC, et al. BCL11A enhancer dissection by Cas9-mediated in situ saturating mutagenesis. *Nature*. 2015;527(7577):192–197.
69. Wu Y, et al. Highly efficient therapeutic gene editing of human hematopoietic stem cells. *Nat Med*. 2019;25(5):776–783.
70. Frangoul H, et al. CRISPR-Cas9 Gene Editing for Sickle Cell Disease and beta-Thalassemia. *N Engl J Med*. 2021;384(3):252–260.
71. Traxler EA, et al. A genome-editing strategy to treat beta-hemoglobinopathies that recapitulates a mutation associated with a benign genetic condition. *Nat Med*. 2016;22(9):987–990.
72. Ikawa Y, et al. Gene therapy of hemoglobinopathies: progress and future challenges. *Hum Mol Genet*. 2019;28(R1):R24–R30.
73. Hoban MD, Bauer DE. A genome editing primer for the hematologist. *Blood*. 2016;127(21):2525–2535.
74. Lattanzi A, et al. Development of β-globin gene correction in human hematopoietic stem cells as a potential durable treatment for sickle cell disease. *Sci Transl Med*. 2021;13(598):eabf2444.
75. Iannone R, et al. Results of minimally toxic nonmyeloablative transplantation in patients with sickle cell anemia and beta-thalassemia. *Biol Blood Marrow Transplant*. 2003;9(8):519–528.
76. Fitzhugh CD, et al. At least 20% donor myeloid chimerism is necessary to reverse the sickle phenotype after allogeneic HSCT. *Blood*. 2017;130(17):1946–1948.
77. Cromer MK, et al. Gene replacement of alpha-globin with beta-globin restores hemoglobin balance in beta-thalassemia-derived hematopoietic stem and progenitor cells. *Nat Med*. 2021;27(4):677–687.
78. Pavel-Dinu M, et al. Gene correction for SCID-X1 in long-term hematopoietic stem cells. *Nat Commun*. 2019;10(1):1634.
79. Schiroli G, et al. Preclinical modeling highlights the therapeutic potential of hematopoietic stem cell gene editing for correction of SCID-X1. *Sci Transl Med*. 2017;9(411).
80. Goodwin M, et al. CRISPR-based gene editing enables FOXP3 gene repair in IPEX patient cells. *Sci Adv*. 2020;6(19). eaaz0571.
81. Hubbard N, et al. Targeted gene editing restores regulated CD40L expression and function in X-HIGM T cells. *Blood*. 2016;127(21):2513–2522.
82. Sweeney CL, et al. Correction of X-CGD patient HSPCs by targeted CYBB cDNA insertion using CRISPR/Cas9 with 53BP1 inhibition for enhanced homology-directed repair. *Gene Ther*. 2021;28(6):373–390.
83. Rai R, et al. Targeted gene correction of human hematopoietic stem cells for the treatment of Wiskott-Aldrich Syndrome. *Nat Commun*. 2020;11(1):4034.
84. Pavani G, et al. Correction of beta-thalassemia by CRISPR/Cas9 editing of the alpha-globin locus in human hematopoietic stem cells. *Blood Adv*. 2021;5(5):1137–1153.
85. Gomez-Ospina N, et al. Human genome-edited hematopoietic stem cells phenotypically correct Mucopolysaccharidosis type I. *Nat Commun*. 2019;10(1):4045.
86. Scharenberg SG, et al. Engineering monocyte/macrophage-specific glucocerebrosidase expression in human hematopoietic stem cells using genome editing. *Nat Commun*. 2020;11(1):3327.
87. Osborn MJ, et al. Evaluation of TCR gene editing achieved by TALENs, CRISPR/Cas9, and megaTAL nucleases. *Mol Ther*. 2016;24(3):570–581.
88. Qasim W, et al. Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. *Sci Transl Med*. 2017;9(374):eaaj2013.
89. Eyquem J, et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature*. 2017;543(7643):113–117.
90. Stadtmauer EA, et al. CRISPR-engineered T cells in patients with refractory cancer. *Science*. 2020;367(6481):eaba7365.
91. Gillmore JD, et al. CRISPR-Cas9 In vivo gene editing for transthyretin amyloidosis. *N Engl J Med*. 2021;385(6):493–502.
92. Nelson CE, et al. Long-term evaluation of AAV-CRISPR genome editing for Duchenne muscular dystrophy. *Nat Med*. 2019;25(3):427–432.
93. Li A, et al. AAV-CRISPR gene editing is negated by pre-existing immunity to Cas9. *Mol Ther*. 2020;28(6):1432–1441.
94. Charlesworth CT, et al. Identification of pre-existing adaptive immunity to Cas9 proteins in humans. *BioRxiv*. 2018.
95. Wagner DL, et al. High prevalence of *S. pyogenes* Cas9-specific T cell sensitization within the adult human population – A balanced effector/regulatory T cell response. *BioRxiv*. 2018.
96. Simhadri VL, et al. Cas9-derived peptides presented by MHC Class II that elicit proliferation of CD4(+) T-cells. *Nat Commun*. 2021;12(1):5090.
97. Simhadri VL, et al. Prevalence of pre-existing antibodies to CRISPR-associated nuclease Cas9 in the USA population. *Mol Ther Methods Clin Dev*. 2018;10:105–112.

CHAPTER 6

SIGNALING TRANSDUCTION AND METABOLOMICS

Pere Puigserver

Hematopoiesis is a cellular process in which self-renewing stem progenitor cells differentiate into mature blood cells, which carry out specific biologic functions. These functions include oxygen delivery, clot formation, and immune responses, including defense of the host from infection and inflammation. Homeostasis of the whole hematopoietic system in vivo requires a tight control of systems and networks governing proliferation, cell fate, cell death, differentiation, cell–cell interaction, and migration. Imbalance in or dysregulation of these processes results in pathologic alterations. For example, uncontrolled cell proliferation is a signature of leukemias, and defective lymphocyte differentiation can lead to immunodeficiency, or tumor immune responses will determine cancer progression. A better understanding at the molecular level of these biologic events will help to identify new therapeutic targets for the design of better drugs to treat hematologic diseases.

Because of the diversity in cellular types and their respective, specific biologic functions, hematopoietic cells respond to a broad array of extrinsic and intrinsic signals transduced through signaling and metabolic pathways. It is therefore important to recognize that these pathways serve to ultimately define a specific functional response and activity in each cell type. These regulatory signals (Table 6.1) can be general, such as growth factors (e.g., insulin growth factor [IGF], fibroblast growth factor [FGF]) or amino acids that control proliferation, or highly specific, such as the antigen signaling response in immune cells or 2,3-diphosphoglycerate in erythrocytes. Importantly, the action of these signals, as well as their integration inside the cell, is needed to accomplish a specific cellular task (either a physiologic or cellular fate decision). Moreover, as will be discussed later in this chapter, these signals also serve to tightly control metabolites in hematopoietic cells, defining a metabolomic profiles involved in processes such as anaerobic glycolysis for energy generation in red blood cells (RBCs) or immune and inflammatory responses in tumors.

Extrinsic cellular signals, often polypeptides, are recognized by plasma membrane receptors that trigger a phosphorylation cascade (using tyrosine and/or serine/threonine residues) that propagates through the cytoplasm and cellular organelles, including the nucleus. Thus the sequential activation of this cascade occurs in a temporal and spatial manner to define the specific biologic response. In general, there are two types of signals (Fig. 6.1): (1) signals that transduce immediate- or short-term biologic outputs without changes in gene expression, and (2) signals that transduce medium- and long-term biologic outputs with changes in gene expression. In the first case, for example, chemoattractants induce the phosphatidylinositol 3-kinase (PI3K) and Cdc42 pathways to rapidly establish neutrophil polarity. One example in the second case is the signaling transduced through Frizzled (Fz) receptors and the transcription factor T cell–specific transcription factor (TCF)-1 necessary for T-cell development. Another example is the programmed death-1 (PD-1) ligand in tumor cells that binds to the PD-1 receptor in T cells controlling the immune response. In these cases, the signals transduced are amplified through a series of physical interactions and chemical modifications on proteins, the most common being phosphorylation, but others such as ubiquitination, acetylation, and sumoylation also play important roles.

In this chapter, a general survey of the different key signaling and changes in energy metabolism that operate in hematopoietic cells will be reviewed. The goal is to provide the molecular basis by which signals are transduced and control fundamental cellular processes, including energy metabolism, in different lineages of the hematopoietic system.

SIGNALING TRANSDUCTION

Hematopoietic cells use general signaling transduction pathways that are common to most human cell types. The specificity in these signaling transduction pathways is often established at the beginning of the pathway's activation (e.g., by specific antigen-binding or ligand–membrane receptor complexes) (Table 6.2) and at downstream targets, including transcription of the specific genes that will serve to define a particular biologic process and response (see Fig. 6.1). Here we will review these general signaling transduction pathways, illustrating some of the specific components of hematopoietic cells.

Receptor Tyrosine Kinases, Phosphoinosite-3-Kinase, and Mitogen-Activated Protein Kinase Pathways

Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) are enzyme-linked receptors localized at the plasma membrane containing an extracellular ligand-binding domain, a transmembrane domain, and an intracellular protein–tyrosine kinase domain. In general, the ligands for RTKs are proteins such as IGF, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and FGF. Ephrins that bind to Eph receptors also form a large subset of RTK ligands. Colony-stimulating factor 1 (CSF-1), which is important for macrophage function, is another example of an RTK ligand. RTKs can function as monomers or multimeric subunits assembled at the plasma membrane that, upon ligand binding, cause oligomerization or conformational changes followed by tyrosine (trans)-phosphorylation in the kinase activation loop. Activation of RTKs results in phosphorylation of additional sites in the cytoplasmic part of the receptor, leading to docking of protein substrates, which initiates the intracellular signaling cascade. These substrates bind to RTK-phosphorylated tyrosines through Src homology-2 (SH2) domain or phosphotyrosine-binding (PTB) domains. Examples of these types of proteins are insulin receptor substrates or the p85 regulatory subunit of PI3K. RTKs recruit, assemble, and phosphorylate different proteins, including adaptors and enzymes.

There are mechanisms to terminate ligand-induced RTK activity through cellular processes including receptor-mediated endocytosis and/or through a family of regulated protein tyrosine phosphatases (PTPs), some of which are transmembrane and have extracellular domains, suggesting the possibility of ligand-mediated regulation. Interestingly, there is also intracellular regulation of PTPs through negative-feedback loops to attenuate the signal or direct control through reactive oxygen species (ROS) (see later discussion).

Phosphatidylinositol 3-Kinase Pathway

One of the key signaling components associated with RTKs is the PI3K signaling transduction pathway. This pathway is also activated by cytokine receptors and G protein–coupled receptors (GPCRs). Among the many functions of this pathway in hematopoietic cells, the interleukin-3 (IL-3)-dependent survival of these cells largely depends on activation of the PI3K pathway. PI3K is a heterodimeric complex formed of a regulatory and a catalytic subunit. The regulatory protein subunits are encoded by isoforms (which include p85α and p85β) that contain SH3-binding domains that mediate binding to activated RTKs. This binding allows additional recruitment and activation of the PI3K catalytic subunits (p110α, p110β, and p110*). At the plasma membrane, activated PI3K phosphorylates phosphoinosite-2 (PIP2) at position 3 of the inositol to produce PIP3. In addition, Ras, a small GTP-binding protein and potent oncogene, also activates PI3K. PTEN, an important lipid phosphatase and tumor suppressor, dephosphorylates PIP3, counteracting PI3K

TABLE 6.1 **Signals in the Hematopoietic System**

Types of Ligands	Examples
Peptide or Protein	
Soluble	Growth factors or cytokine
ECM	Fibronectin, collagen
Cell surface bound	ICAM, Kit ligand
Small organics	Thyroid hormone
Nucleotides	
Soluble	ADP
DNA	Double-strand breaks
Lipids	Eicosanoids, LPS
Gases	H_2O_2, nitric oxide[a]

[a] Function in hematopoietic system not well defined.

ADP, Adenosine diphosphate; *ECM*, extracellular matrix; *ICAM*, intercellular adhesion molecule; *LPA*, lipopolysaccharide.

TABLE 6.2 **Receptors in the Hematopoietic System**

Types of Receptors	Examples	Types of Ligands
RTK	Insulin, Kit, Fms	Kit ligand, M-CSF
RSK	TGF-β receptors	Activin, BMPs, TGF-β
GPCR	Thrombin receptor, CXC, CC receptors	Thrombin chemokines
PTK-associated	Cytokine receptors	Epo, interleukins,
MIRR	BCR/TCR/FcR	IFN peptide/MHC, Fc domains
TNF family	Fas, TNFR, CD40	Fas, TNF, CD40L
Notch	Notch	Delta-serrate-LAG-2
Frizzled family	Wnt receptors	Wnts
Toll receptors	TLR1-10	Bacterial DNA, LPS
RPTP	CD45	Unknown
Nuclear receptors	AR, RAR	Testosterone, retinoids
Adhesion receptors	Integrins	Fibronectin, collagen

AR, Androgen receptor; *BCR*, B-cell antigen receptor; *BMP*, bone morphogenetic protein; *CC, CXC*, types of chemokine receptors; *CD40L*, ligand for CD40; *Epo*, erythropoietin; *FcR*, receptors for Fc portion of antibodies; *GPCR*, G protein–coupled receptor; *IFN*, interferon; *LPS*, lipopolysaccharide; *M-CSF*, macrophage colony-stimulating factor; *MHC*, major histocompatibility complex; *MIRR*, multichain immune recognition receptor; *RAR*, retinoic acid receptor; *RPTP*, receptor protein-tyrosine phosphatase; *RSK*, receptor serine kinase; *RTK*, receptor tyrosine kinase; *TCR*, T-cell antigen receptor; *TGF-β*, transforming growth factor β; *TNF*, tumor necrosis factor; *TNFR*, tumor necrosis factor receptor.

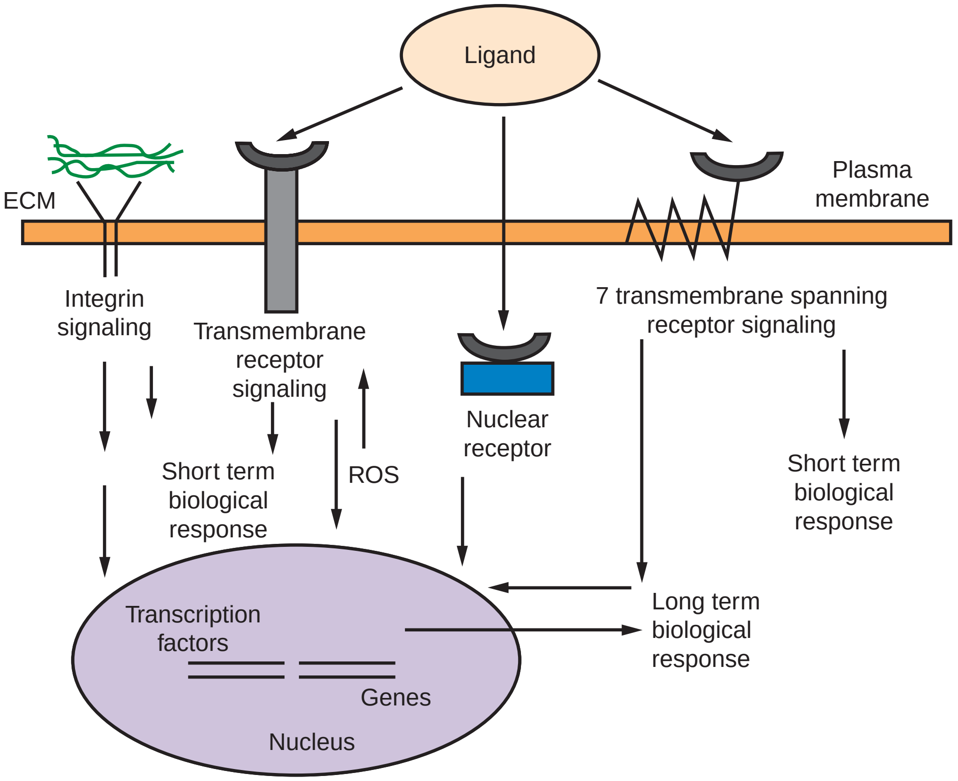

Ligand
ECM
Plasma membrane
Integrin signaling
Transmembrane receptor signaling
7 transmembrane spanning receptor signaling
Nuclear receptor
ROS
Short term biological response
Short term biological response
Long term biological response
Transcription factors
Genes
Nucleus

Figure 6.1 EXAMPLES OF LIGANDS AND RECEPTORS THAT TRANSDUCE BIOLOGIC RESPONSES. Signals can originate from fixed ligands (e.g., the extracellular matrix [ECM]) or soluble ligands that are not membrane permeable and bind to extracellular regions of transmembrane receptors. Membrane-permeable ligands bind to intracellular receptors, such as the nuclear receptor family. Signals can also originate from within the cell, such as increases in ROS levels. These signals cause short-term biologic outputs without changes in gene expression, or transduce medium- and long-term biologic outputs with changes in gene expression. *ECM*, Extracellular matrix; *ROS*, reactive oxygen species.

and decreasing the intensity of the pathway. Accumulation of PIP3 at the plasma membrane recruits several pleckstrin homology domain (PHD)-containing proteins, among them PDK and AKT serine/threonine kinases, which are key components in transducing PI3K signaling. Activated AKTs target different protein substrates for initiation of a biologic response. For example, the Bad protein, phospho-Bad, does not bind Bcl-2 and functions as an antiapoptotic mechanism, promoting cell survival. Other key targets of AKTs are the Forkhead transcription factors (FoxOs) (Fig. 6.2). When phosphorylated by AKT, phospho-FoxOs are sequestered and inactive in the cytoplasm through direct binding to 14-3-3 proteins. In contrast, dephosphorylated FoxOs activate gene expression associated with stress resistance and cell growth arrest. Another major component downstream of Akt is mammalian target of rapamycin (mTOR, a kinase that belongs to the PI3K-related protein kinase family), which is involved in metabolism, growth, and proliferation. Akt phosphorylates TSC2, which forms a complex with TSC1, decreasing its GTPase-activating protein (GAP) activity for the small GTPase Rheb; as a consequence, the increases in GTP-Rheb activate mTORC1 (one of the mTOR complexes). Among the key downstream targets of mTOR are S6K and 4EBP1, which control protein translation. mTOR can also be activated independently of RTKs through nutrients including branched chain amino acids. mTORC1 forms an amino acid–sensing complex at the lysosomal membranes called the pentameric Ragulator complex that contains Rags, small GTPases that are controlled by the GATOR complex. Interestingly, mTORC1 inhibitors such as rapamycin are used as immunosuppressors in organ transplantation.

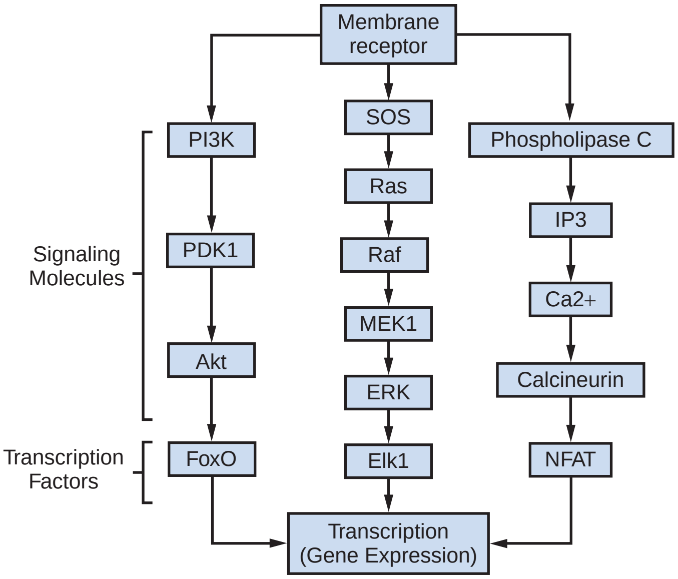


Figure 6.2 EXAMPLES OF SIGNALING/TRANSCRIPTIONAL PATHWAYS PROGRAMMING GENE EXPRESSION. Proteins involved in gene expression are a common target of many signaling pathways, and receptors often stimulate multiple pathways that can regulate common and distinct transcription factors. In the examples shown here, production of PtdIns-3,4,5-P3 by PI3K leads to the activation of the serine/threonine kinase Akt. Akt phosphorylates and inactivates FoxO transcription factors. Ras is activated by the guanine nucleotide exchange factor SOS. Ras activation initiates a cascade of serine/threonine kinases activity: Ras activates Raf, Raf phosphorylates and activates Mek1, and Mek1 phosphorylates and activates ERK. Phosphorylation of the transcription factor Elk1 by ERK activates gene expression. Increased intracellular calcium is also a common signaling event. Activation of phospholipase C leads to hydrolysis of PtdIns-4,5-P2 and production of IP3. IP3 binds to its receptor, leading to intracellular calcium release and then extracellular calcium influx. Calcium activates the serine phosphatase calcineurin, which dephosphorylates NFAT proteins, allowing them to enter the nucleus and stimulate transcription. *FoxO*, Forkhead transcription factors; *IP3*, inositol triphosphate; *NFAT*, nuclear factor of activated T cells; *PI3K*, phosphatidylinositol 3-kinase; *SOS*, Son of Sevenless.

MAPK/ERK Pathway

Activated RTKs recruit docking proteins, such as Grb2 and SOS, that allow binding of GTP to Ras to become active and trigger a kinase signaling cascade. Ras activates RAF kinase that, in turn, triggers a series of MEKs, which finally activate MAPK or EEK kinases. ERK phosphorylates many proteins involved in cell growth, including ribosomal S6K, which is involved in protein translation, and AP-1 and c-myc transcription factors, which increase many different cell cycle and antiapoptotic-related genes (see Fig. 6.2). Other MAPKs include the stress-activated kinases JNK and p38. Constitutive MAPK in hematopoietic stem cells is known to induce myeloproliferative disorders.

The Transforming Growth Factor-β Pathway

The transforming growth factor (TGF)-β family of cytokines contains two subfamilies: the TGF-β/Activin/Nodal and the bone morphogenetic protein (BMP)/growth and differentiation factor (GDF)/müllerian-inhibiting substance (MIS) subfamilies. At the plasma membrane, TGF-β ligands bind with high affinity to the ectodomain of type II receptors, which then recruit type I receptors. This forms a large ligand-receptor complex involving a ligand dimer and four receptor subunits. Upon ligand binding, the type II receptor phosphorylates multiple serine and threonine residues in the cytoplasmic GS-rich region of the type I receptor, leading to its activation. The phosphorylated TGF-β type I receptor binds to and phosphorylates Smad2 and Smad3 transcription factors, which are critical mediators of TGF-β signaling and function. Upon phosphorylation, Smad proteins translocate to the nucleus to activate gene expression through binding to specific DNA-binding sites. There are several mechanisms to terminate Smad activation, which include proteasomal degradation and dephosphorylation. TGF-β-1 has been shown to be associated with active centers of hematopoiesis and lymphopoiesis in the developing fetus.

Signaling Through Receptors Associated With Protein-Tyrosine Kinases

Here, three different types of receptors and their signaling are included: (1) cytokine receptors, (2) multichain immune recognition receptors, and (3) integrin receptors.

Cytokine Receptors and Janus-Activated Kinase Signaling

The cytokine receptor superfamily mediates many of the central specific responses in hematopoietic cells. Ligands for these receptors include interleukins, thrombopoietin, erythropoietin, and so on. Cytokine receptors possess a conserved extracellular region (cytokine receptor homology domain [CDH]) and several structural modules, including extracellular immunoglobulin or fibronectin type III–like domains, transmembrane domains, and intracellular homology regions. Based on the divergence of the CHD, cytokine receptors are classified into two classes: class I and class II receptors. Class I receptors contain two pairs of cysteines linked through a disulfide bond and a C-terminal WSXWS motif within the CHD. This class is further subdivided into three families: IL-2R, IL-3R, and IL-6R. All three receptor families share similar receptor chains. The class I cytokine receptors are formed by one chain containing two motifs (Box 1 and Box 2), which transduce signaling through binding to Janus-activated kinase (JAK; see later discussion). Also included in this class are the homomeric receptors that form homodimers upon ligand binding. Examples of these receptors include the erythropoietin, thrombopoietin, prolactin, and growth hormone receptors. Class II receptors also have two pairs of cysteines but lack the WSXWS motif found in class I receptors. There are pools of 12 class II receptor chains that are capable of forming a total of 10 receptor complexes. This class is functionally divided into antiviral receptors (three receptor complexes that bind interferons) and non-antiviral receptors, which bind to several interleukins such as IL-10 and IL-20.

The oligomeric structures of cytokine receptors are complex and cannot be generalized. Cytokine binding often induces oligomerization, which activates protein tyrosine kinases in the JAK family that are constitutively associated with the Box 1 and 2 motifs of the cytokine receptor. Oligomerization brings JAKs in close enough proximity to trans phosphorylate on Tyr residues. This activates JAK, which results in the phosphorylation of other cytokine receptors as well as other substrate proteins. Among these substrates, the signal transducer and activator of transcription (STAT) family of transcription factors are pivotal to JAK-mediated cytokine signaling. STATs are phosphorylated on Tyr residues by JAKs upon cytokine binding to the receptor. Phospho-STATs homodimerize or heterodimerize and translocate to the nucleus to activate gene expression. STATs are also phosphorylated on a serine residue via MAPK, which serves to strengthen the intensity of the signal. As part of the cytokine signaling attenuation, STATs induce genes encoding for suppressors of cytokine signaling (SOCS) proteins, which bind to phosphotyrosine residues of the cytokine receptor and JAK through SH2-binding domains.

JAK inhibitors, based on their ability to block cytokine signaling, are used in allergic and rheumatoid arthritis disease therapy.

Multichain Immune Recognition Receptors

This family of receptors includes antigen receptors in B and T lymphocytes, activating receptors in natural killer (NK) cells, and immunoglobulin E (IgE) and Fc receptors. This class of receptors contains different integral membrane subunits that bind the ligand at the cell surface and transduce the signal. Ligand binding induces oligomerization of receptor subunits that contain immunoreceptor tyrosine-based activation motifs (ITAMs) within their cytoplasmic domains. These domains become phosphorylated on tyrosine residues upon receptor activation. These phosphotyrosines are involved in activation of a series of protein tyrosine kinases containing SH2 domains that include Src (Src family kinase [SFK]), Syk (Syk or ZAP-70), and Tec (Btk, Itk, Rlk), which mediate immune signaling through downstream pathways that include MAPK, calcium signaling, and nuclear factor (NF)-κB, among others. In Tec kinases, additional downstream targets include enzymes such as phospholipase C γ (PLCγ). The precise mechanism of this activation is not completely understood, and in some cases, such as T-cell receptors, a PTP (CD45, which counteracts the action of SFKs) is regulated upon ligand binding.

The activities of some of these receptors are the basis of immunotherapy in cancer. For example, PD-1 mediates tumor-induced immunosuppression. Cancer cells express the PD-1 ligand, which activates the PD-1 receptor present in tumor-infiltrated lymphocytes, suppressing the immune response. Signaling transduction through the PD-1 receptor involves tyrosine-depending binding to SHIP1/2 phosphatases. Blockade of PD-1 activation with monoclonal antibodies has been successful in treating several human tumors such as melanoma. Mechanistically, T cells are activated through the T-cell receptor upon binding of major histocompatibility complex (MHC) plus peptides on an antigen-presenting cell (APC; in this case in the tumor cell), and binding of APC CD80/86 to T cell CD28. Activation of the T-cell receptor increases expression of PD-1 to suppress the immune/inflammatory response. Cancer cells activate this pathway, upregulating the PD-1 ligand to promote survival and suppress the immune-mediated death of tumor cells.

Integrin Signaling

Integrin receptors are involved in cell adhesion, migration, survival, and growth. This signaling is central in hematopoietic cell function (e.g., at places of inflammation or infection, where integrins trigger a cascade by which leukocytes exit the vasculature). Interestingly, these receptors signal bidirectionally through the plasma membrane in pathways referred to as *inside-out* and *outside-in signaling*. Integrins are a class of receptors that comprise heterodimeric type I transmembrane proteins consisting of α and β subunits. These subunits contain a large extracellular domain, a single transmembrane domain, and a short cytoplasmic tail. There are 18 α and 8 β subunits that are associated and form 24 different integrins with different affinities for ligands. Most of the ligands are extracellular matrix (ECM) proteins containing one of the two motifs: arginine-glycine-aspartate (RGD) or leucine-aspartate-valine (LDV). Examples of integrin ligands are ICAM-1, which is present at the plasma membrane of APCs and binds to the integrin receptor LFA-1 to promote cell-cell adhesion.

Ligand binding to the extracellular domain induces clustering of integrins, allowing separation of the different subunits cytoplasmic portions forming interactions with cytoskeleton proteins involved in actin polymerization (outside-in signaling). Signals arising from the cellular interior, including phosphorylation, can also separate these cytoplasmic domains and can affect ligand binding (inside-out). Ligand binding to integrin receptors also signals to protein tyrosine kinases such as the SFKs and focal adhesion kinase (Fak). This part of the signaling is not completely understood but appears to involve a domain in the β-integrin tail (NPXY motif) that binds talin, which in turn recruits paxillin that binds Fak, which, once activated, phosphorylates SFKs to mediate integrin response. Paxilin-independent integrin signaling that also mediates survival and migration have also been observed in intracellular endosomal compartments.

Tumor Necrosis Factor Receptors and Signaling

Tumor necrosis factor receptors (TNFRs) influence inflammation, innate immunity, lymphoid organization, and T-cell responses. There are approximately 19 different ligands for TNFRs that mediate cellular responses through 29 TNFRs. TNFRs are a family of single membrane–spanning proteins that contain an extracellular tumor necrosis factor (TNF)-binding region and a cytoplasmic tail. As in the case of other cytokine receptors, ligand binding causes oligomerization and the formation of a mature receptor complex that is required to transduce the signal. TNFRs fall into three classes: (1) death domain (DD)-containing receptors (fatty acid synthase [FAS], TNFR1, and DR3), which activate the caspase cascade via the DD-initiating extrinsic apoptotic pathway; (2) decoy receptors, which lack a cytoplasmic tail and therefore cannot transmit the signal, making these receptors ligand sequesters; and (3) TNFR-associated factor (TRAF) receptors such as TNFR2, which lack the DD-recruiting TRAF proteins. In general, TRAFs are associated with either proapoptotic or survival pathways through activation of the NF-κB family of transcription factors and MAPK signaling (ERK, JNK, and p38). TRAFs activate NF-κB through ubiquitin-mediated degradation of their inhibitor IκBα, which retains NF-κB inactive in the cytoplasm. This process is initiated by phosphorylation of IκBα by the IκBα kinase (IKK) complex, mainly by the IKK-β catalytic subunit, and requires a regulatory subunit (also known as NEMO). Upstream of IKKs are other kinases including NF-κB-inducing kinase (NIK), which binds to TRAFs. Nuclear-activated NF-κB modulates gene expression, which mediates TNF biologic responses.

Toll-Like Receptors and Signaling

Toll-like receptors (TLRs) play essential roles in the innate immune response. Ten TLRs have been identified and can be grouped into two classes based on their extracellular domain: (1) TLRs with leucine-rich repeats and (2) TLRs with immunoglobulin domains. The ligands for TLRs are diverse and include the different constituent components of the microorganism, such as lipopolysaccharides (LPSs) and heat shock proteins (which bind to TLR2 and TLR4). Host defense against microorganisms mainly relies on signals originating from the TIR (Toll/IL-1) intracellular domain (a domain present in TLRs and IL-1Rs). The TLR signaling pathway is similar to the one triggered by the IL-1R. Ligand binding induces TLR multimeric receptor complexes, recruiting adaptor proteins such as MyD88, which contains a TIR domain and a DD, that in turn binds to the IL-1R–associated kinase (IRAK). IRAK is activated by phosphorylation and then associates with TRAF6, leading to activation of mainly two different pathways, JNK and NF-κB to activate the innate immune response, including release of inflammatory cytokines.

Wnt Signaling

Wnt proteins are lipid-modified, secreted proteins of approximately 400 amino acids that bind to Wnt cell surface transmembrane receptors, Fz, to initiate the canonical Wnt signaling transduction pathway. At the plasma membrane, binding of Wnt ligands to Fz receptors connect through direct binding to several intracellular proteins including Disheveled (Dsh), glycogen synthase kinase-3β (GSK3β), Axin, and adenomatous polyposis coli tumor suppressor (APC), inhibiting proteasome-mediated degradation of the transcriptional protein β-catenin. This degradation is regulated through GSK3β-mediated phosphorylation of β-catenin. As a consequence, β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it interacts with transcription factors such as lymphoid enhancer-binding factor 1 (LEF)/TCF to modulate gene expression.

Notch Signaling

Notch ligands are plasma single-pass transmembrane proteins named Delta-like and Jagged. Thus cells expressing the ligands are adjacent to cells expressing the Notch receptors, which are also transmembrane proteins. The Notch receptor interacts with a Notch ligand on a contacting cell; this interaction produces Notch receptor cleavage, which releases the Notch intracellular domain (NICD). The NICD translocates to the nucleus, where it binds to several DNA-binding proteins including CBF1/Suppressor of Hairless/LAG-1 (CSL). As a result of this interaction between NICD and CSL, changes in Notch target genes occur. In contrast to the other signaling pathways discussed in this chapter that mainly function through phosphorylation, there is no amplification from the initial Notch ligand binding to the receptor. Moreover, this core pathway is modulated through auxiliary proteins that influence the response to the Notch ligand. Among these proteins are acute myeloid leukemia 1 (AML1), discoidin domain receptor family (DDR1), NECD, Notch extracellular domain, and CBF1-interacting protein.

Hedgehog Signaling

Hedgehog (Hh) signaling is a ligand-dependent signaling pathway. There are three different protein ligands—Sonic, Desert, and Indian—that are secreted and produce an N-terminal active fragment. Indian appears to be highly expressed in hematopoietic tissue. These ligands bind to Patch transmembrane receptors and are internalized, and Smoothened (a GPCR member) translocates to the plasma membrane of the primary cilium and promotes activation of the Gli family of zinc finger transcription factors. Hh targets include genes involved in differentiation, apoptosis, and the cell cycle. Abnormal activation of Hh signaling occurs in hematologic malignancies and maintains stem cell expansion. Because these cells are resistant to conventional chemotherapy, Hh antagonism is considered a plausible target in these malignancies.

Nuclear Hormone Receptor Superfamily

Nuclear hormones include steroid hormones (sex hormones, glucocorticoids, and mineralocorticoids), sterol hormones (vitamin D and its derivatives), thyroid hormones, and retinoids. These hormones are lipophilic and need carrier proteins to be transported in the blood. Due to this hydrophobicity, they can diffuse across the plasma membrane to reach the receptor proteins inside the cells, either in the cytoplasm or in the nucleus. These receptors are called the nuclear hormone receptor (NHR) superfamily. What distinguishes this receptor family from those discussed previously is their ability to directly bind to DNA and coordinate gene expression, which effectively makes them a form of transcription factor. NHRs contain a central DNA-binding domain, which targets the receptor to DNA sequences known as hormone response elements. In addition, the C-terminal part of the receptor contains a ligand-binding domain where the ligand or hormone binds. Upon ligand binding, NHRs control the expression of diverse sets of genes related to the hormonal response. Based on the types of ligands that they can bind, NHRs can be grouped into four classes: (1) steroid receptors, which include receptors for glucocorticoids, mineralocorticoids, progesterone, androgen, and estrogen; (2) retinoid X receptor heterodimers, such as thyroid receptor, retinoic acid receptor, vitamin D receptor, and peroxisome proliferator-activated receptors; (3) dimeric orphan receptors, such as COUPTF or HNF4; and (4) monomeric orphan receptors, such as NGFI. The cognate ligands for orphan receptors have yet to be identified.

G Protein–Coupled Receptor and Chemokine Signaling

G Protein–Coupled Receptor Signaling

The GPCR superfamily comprises a large collection of proteins, with approximately 2000 annotated genes in the human genome (~10% of the entire genome). GPCRs are involved in a large array of physiologic functions, including platelet aggregation and leukocyte chemotaxis. GPCRs are single polypeptides with seven-pass transmembrane domains containing both cytoplasmic and extracellular regions. Ligands for GPCRs are very diverse and include proteins or peptides, amino acids, lipids, and nucleotides that bind at the cell surface where GPCRs are localized. In spite of its vast size and variety of activational ligands, the GPCR superfamily relies upon three main intracellular signaling cascades for communicating receptor activation: the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA), the phosphatidylinositol/phospholipase C, and the Rho GTPase-based cascades.

GPCRs are coupled to a heterotrimeric G protein formed from three unique subunits (α, β, and γ) that are membrane bound. The G-α subunit contains a GTPase domain, which is capable of hydrolyzing GTP to GDP. When bound to GDP, the complex is functionally inactive, with the G-α subunit remaining tightly associated with the other subunits of the GPCR complex. Upon ligand binding to the GPCR, structural conformational changes produce the release of GDP from the heterotrimeric complex, allowing GTP to bind to the G-α subunit. In this GTP-bound form, the G-α subunit dissociates from the G-β and G-γ subunits with which it interacts. The G-α subunit then proceeds to interact with its downstream cognate targets to affect a particular signal response, depending upon the GPCR and the specific G-α subunit isoform. Among these second-messenger effectors are the cAMP/PKA pathway, ion channels, Rho GTPase, MAPK, PI3K, and inositol-3-phosphate/diacylglycerol (InsP3/DAG) pathways. In the case of the cAMP pathway, adenylate cyclase is downstream of different GPCRs (e.g., adrenergic receptors) and is activated by GTP-bound G-α. Adenylate cyclase converts ATP to cAMP, a freely diffusible second-messenger molecule. A key effector of intracellular cAMP is PKA, an inactive tetrameric protein complex consisting of two regulatory and two catalytic subunits. Binding of cAMP to the regulatory subunits causes release and activation of the catalytic subunits, which phosphorylate different cellular targets. Among them are the transcription factor cAMP-responsive element (CREB) and several ion channels. In addition to adenylate cyclase, there are other common effectors downstream of GPCRs, such as phospholipase C, a plasma membrane–bound enzyme that cleaves phosphatidyl inositol (PIP2) into two products and messengers: inositol triphosphate (IP3) and DAG. IP3 can diffuse through the cytoplasm and bind receptors in the endoplasmic reticulum (ER), resulting in calcium release to the cytoplasm. Importantly, calcium propagates the signaling cascade through different proteins such as calcineurin and nuclear factor of activated T cells (NFAT) transcription factors (see Fig. 6.2), which are involved in, for example, IL-2 gene expression. DAG at the plasma membrane binds and activates, in conjunction with calcium, protein kinase C (PKC), which will phosphorylate other downstream targets. Rho guanine nucleotide exchange factor (RhoGEF) is also a target for some G-α subunits. Binding of the G-α subunit to Rho allosterically activates it, causing GTP to be preferentially bound. This, in turn, allows RhoGEFs to activate Rho kinase, which is involved in the cytoskeletal reorganization necessary for changes in cell shape and motility.

Chemokine Signaling

Chemokines mediate cell migration in immune surveillance, inflammation, and development. There are nearly 50 human chemokines divided into four families (CXC, CC, C, and CX3C) on the basis of the pattern of internal cysteine residues; thus C stands for cysteine and X/X3, one or three noncysteine amino acids. Expression of some of these chemokines is induced by inflammatory signals such as TNF-α, interferon-γ, trauma, or microbial infection. There are approximately 20 signaling chemokine receptors, and they are all GPCR receptors; thus the chemokine acts as a ligand, and activation of the chemokine receptor follows the principles described previously. The major downstream effectors are cAMP and calcium messengers. Interestingly, some of the chemokine receptors also bind human immunodeficiency virus (HIV) viral proteins.

cGAS–cGAMP–STING Signaling Pathway

The presence of cytoplasmic DNA, through infection or DNA damage, activates innate immune responses. A mechanism of sensing this misplaced DNA is through the cGAS–cGAMP–STING signaling pathway. Cytosolic DNA binds and activates the cGAS enzyme (cGAMP synthase), which produces a second messenger, the cyclic dinucleotide 2′,3′-cGAMP, using ATP and GDP as substrates. 2′,3′-cGAMP is a high-affinity ligand for STING, an ER membrane protein that undergoes several structural conformations. 2′,3′-cGAMP bound to STING binds to the protein kinase TBK1, which phosphorylates IRF3; in addition, activated STING signals to IKK to phosphorylate and degrade IκBα, which sequesters NF-κB in the cytoplasm. Both phosphorylated and dimerized IRF3 and NFκB translocate to the nucleus to activate expression of type I interferons and other cytokines.

Endoplasmic Reticulum Stress Signaling Pathways

Most of the proteins that are secreted or delivered to the cell surface are folded and assembled in the ER. Cells monitor this protein-folding capacity in the ER lumen, and when this capacity is exceeded, it triggers an intracellular signaling pathway termed the unfolded protein response (UPR). Excess of unfolded proteins in the ER is delivered for proteasomal degradation after retrotranslocation through a process known as ERAD (ER-associated degradation). There are three UPR signal transducers initiated by transmembrane ER resident proteins: IRE1 (inositol-requiring enzyme 1), PERK (double-stranded RNA-activated protein kinase [PKR-like ER kinase]), and ATF6 (activating transcription factor 6). Each branch activates a transcription factor: for IRE1, XBP1 (X-box binding protein 1), for PERK ATF4 (activated transcription factor 4), and for ATF6, a cleaved form ATF6(N). These transcription factors will reestablish protein folding; however, in case it is not resolved, cells will undergo apoptosis. IRE1 is a transmembrane protein with two enzymatic activities, kinase and endoribonuclease (RNase). IRE1 RNAse activity is triggered by its own oligomerization and cleaves XBP1 mRNA, excising an intron that is translated and translocated to the nucleus. Interestingly, immunoglobulin-secreting plasma cells need a larger ER network to cope with high rates of immunoglobulin synthesis. IRE1 and XBP1 are necessary in these cells to maintain protein folding and support immunoglobulin production and secretion. The other branch of the UPR is PERK, an ER transmembrane kinase that senses ER stress. When activated, PERK oligomerizes and autophosphorylates itself and the translation initiation factor eIF2a, inhibiting large part of mRNA translation. Some exceptions are mRNAs involved in the stress response that contain short open reading frames, such as ATF4 that drives expression of genes such as CHOP and others involved in the ER stress response. The third branch of the UPR is ATF6, a transcription factor localized at the ER membrane. Upon excess of unfolded proteins, ATF6 traffics to the Golgi apparatus, where it is cleaved by two proteases and translocated to the nucleus and activate genes involved in the ER response including chaperones.

Heme Signaling Transduction

Heme (iron protoporphyrin IX) is a prosthetic group in numerous proteins, such as globins, functioning in oxygen transport or electron transfer. Heme is mainly synthesized in a series of reactions in the cytoplasm and mitochondria of the bone marrow and liver. Heme degradation starts in spleen macrophages involving heme oxygenase that produces biliverdin, that is converted to bilirubin by biliverdin reductase that is transported to the liver. Heme levels are tightly controlled in cells through sensing and signaling mechanisms that include kinases and transcription factors that regulate heme synthesis and/or degradation. In reticulocytes, heme signaling is controlled through HRI (heme-regulated inhibitor) kinase that phosphorylates eIF2a (eukaryotic initiation factor a), coordinating protein synthesis with heme availability. Incorporation of heme into globin chains avoids the aggregation and proteotoxicity of unassembled heme-free globin chains. HRI has several structural domains that include two kinases domains with two binding sites for heme, one reversible in a kinase domain that senses heme levels, and one stable heme binding domain in the N-terminus. Under low intracellular heme levels (e.g., dietary iron deficiency), reduced heme binding to the HRI reversible site activates its kinase activity and phosphorylates eIF2a, reducing global protein synthesis, including globin synthesis. HRI activation also increases peIF2a-dependent translation of ATF4, a transcription factor involved in integrated stress responses. HRI and ATF4 are necessary for erythroid differentiation. Other heme-sensing proteins include the transcriptional repressor Bach1 (BTB domain and CNC homolog 1) that is negatively regulated by heme and controls globin gene expression. Bach1 heterodimerizes with Maf DNA-binding proteins that bind to globin and oxidative stress promoters, such as heme oxygenase 1 gene promoters. Bach1 heme-binding domains are near the C-terminus that contains cysteine-proline motifs. High intracellular heme concentrations bind to Bach1 and promote translocation from the nucleus to the cytoplasm; thus Bach1 targets increases in these conditions, promoting hemoglobin synthesis.

Hypoxia Signaling Transduction

Human cells adapt to oxygen concentrations through signaling mechanisms that involve changes in gene expression, including the erythropoietin and glycolytic enzymes. Oxygen is sensed through a family of enzymes called EGLN or PHD prolyl hydroxylase enzymes that are 2-oxoglutarate-dependent dioxygenases whose activity depends on oxygen levels. A major substrate of these enzymes is the hypoxia-induced factor HIF1a that, once hydroxylated on two prolines, binds to VHL, causing ubiquitination and subsequent degradation. Physiologic hypoxia occurs in the hematopoietic stem cell niche and facilitates survival.

METABOLOMICS AND CONTROL OF HEMATOPOIETIC CELL METABOLISM

There are three important general pathways by which metabolism impacts cellular function and the metabolomic state contributing to cell, tissue, and human body physiology or pathology (Fig. 6.3): (1) activity of catabolic routes that supply energy in the form of ATP, such as glycolysis or oxidative phosphorylation; (2) activity of anabolic routes that synthesize molecules that are used for cellular growth or a specific cellular function; and (3) changes in metabolites that control intrinsic and extrinsic cellular activities. This regulation is intimately connected to signaling transduction, because most of the pathways described in the previous section directly control cellular metabolism and metabolite levels. Here, this part of the review will cover the main metabolic pathways and new metabolomic research, taking into consideration their implications in hematopoietic cells.

Glucose Metabolism

Hematopoietic cells have different types of glucose transporters; for example, activation of T cells causes dramatic increases in Glut1 expression to maintain immune homeostasis. Once transported into the cell, glucose is metabolized through different biochemical pathways to provide energy and building blocks for macromolecules that constitute the cell or regulatory metabolites. Glucose can be stored in cells in the form of glycogen, which constitutes a rapid source of energy through its breakdown to free glucose (glycogenolysis), although this pathway is limited to a certain number of hematopoietic cells. Chemotaxins (FMLP, C5ades arg, arachidonic acid) activate granulocytes to catabolize significant amounts of endogenous glycogen.

Glycolysis

Glycolysis is a series of chemical reactions by which six-carbon glucose is converted into two three-carbon keto-acids (pyruvate). Importantly, these oxidative reactions generate energetic molecules such as ATP and NADH and can occur in the absence of oxygen and mitochondria. In some cells, such as erythrocytes, anaerobic glycolysis produces lactate, but in most cell types pyruvate is completely oxidized to acetyl coenzyme-A (acetyl-CoA) and carbon dioxide by the mitochondrial pyruvate dehydrogenase complex and the tricarboxylic acid (TCA) cycle coupled to oxidative phosphorylation. In general, hematopoietic stem cells are thought to largely depend on glycolysis, whereas more differentiated cells, except for erythrocytes, use mitochondrial oxidative metabolism. Glycolytic fluxes are under intrinsically tight control through intermediate metabolites in the pathway. The most powerful control is exerted by fructose 2,6-bisphosphate (F-2,6-BP), which is generated by phosphofructokinase 2. F-2,6-BP allosterically activates phosphofructokinase, providing a "feed-forward" mechanism of stimulation. Activation of growth factor signaling pathways potently stimulates glycolysis at different points, including phosphorylation of phosphofructokinase 2 and pyruvate kinase cell-specific isoforms. The PI3K pathway is a major signaling pathway that controls glycolysis.

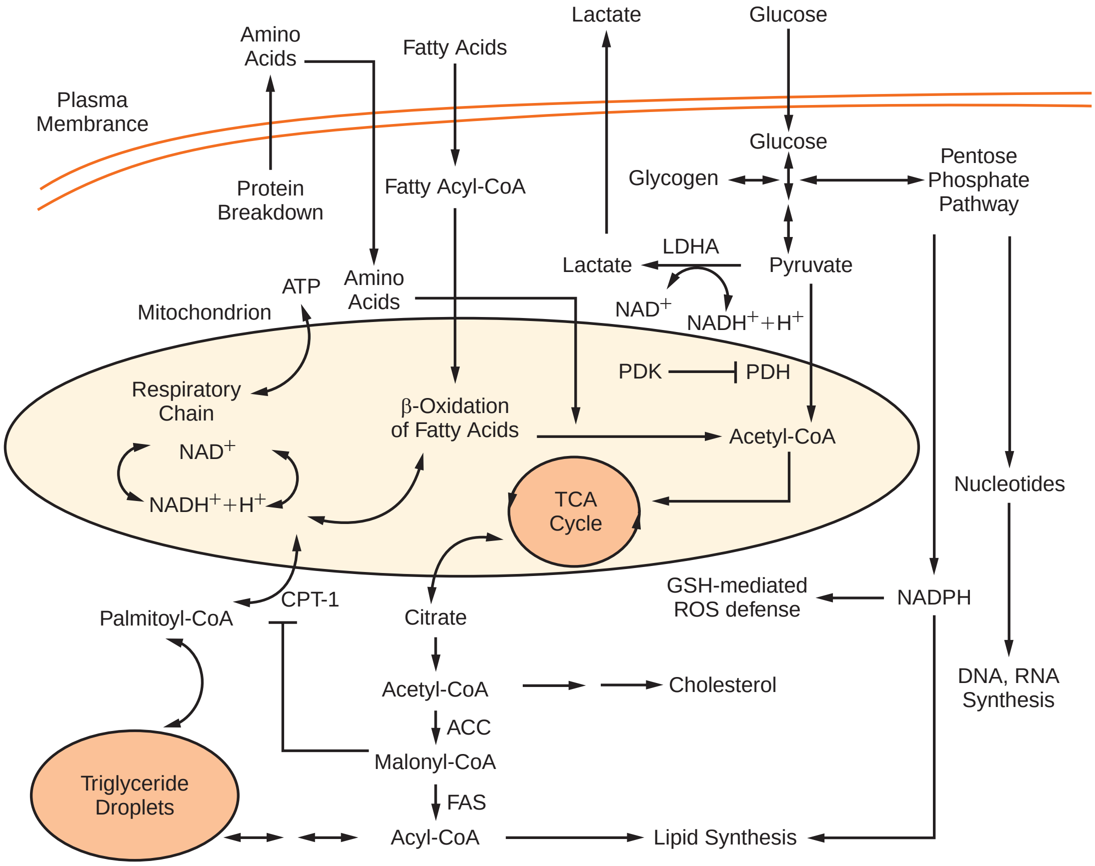

Amino Acids
Fatty Acids
Lactate
Glucose
Plasma Membrance
Glucose
Glycogen
Pentose Phosphate Pathway
Protein Breakdown
Fatty Acyl-CoA
LDHA
Lactate
Pyruvate
ATP
Amino Acids
Mitochondrion
NAD+
NADH++H+
PDK
PDH
Respiratory Chain
β-Oxidation of Fatty Acids
Acetyl-CoA
NAD+
NADH++H+
TCA Cycle
Nucleotides
Palmitoyl-CoA
CPT-1
Citrate
GSH-mediated ROS defense
NADPH
Acetyl-CoA
Cholesterol
DNA, RNA Synthesis
ACC
Malonyl-CoA
Triglyceride Droplets
FAS
Acyl-CoA
Lipid Synthesis

Figure 6.3 INTEGRATION OF CENTRAL METABOLIC PATHWAYS. The metabolic fluxes within anabolic and catabolic routes are controlled by different signals including metabolite concentrations. These metabolic pathways are localized in different cellular compartments to adequately provide cellular energetic and nutrient homeostasis necessary for growth and survival. See text for further details. *ACC*, Acetyl-CoA carboxylase; *CoA*, coenzyme A; *FAS*, fatty acid synthase; *LDHA*, lactate dehydrogenase A; *PDH*, pyruvate dehydrogenase; *PDK*, pyruvate dehydrogenase kinase; *ROS*, reactive oxygen species; *TCA*, tricarboxylic acid.

Interestingly, in erythrocytes, 1,3-diphosphoglycerate can be diverted from glycolysis to synthesize 2,3-diphosphoglycerate (2,3-DPG) via the enzyme diphosphoglycerate (Rapoport-Laubering shunt). 2,3-DPG is an important metabolite that regulates oxygen binding to hemoglobin; thus increased levels of 2,3-DPG (e.g., under hypoxic conditions) allow hemoglobin to release oxygen under low partial oxygen tensions.

Pentose Phosphate Pathway

The pentose phosphate pathway (PPP) derives from glycolysis in the cytoplasm. The first enzyme in this pathway is glucose-6-phosphate dehydrogenase (G6PDH) and produces NADPH, a substrate used for lipogenesis and glutathione regeneration by glutathione reductase. The regulation of NADPH production through G6PDH is through NADPH-mediated product inhibition. The PPP is also important in generating ribose-5 phosphate, which is a precursor for nucleotide synthesis in proliferating cells. Interestingly, G6PDH deficiency leads to low levels of NADPH, which is essential for controlling ROS through glutathione reductase. It is one of the most common erythrocyte enzymopathies, and these cells cannot prevent oxidative damage in critical molecules such as heme, causing overall irreparable damage to the cell at a much higher rate than normal, particularly in response to certain environmental triggers such as drugs and stress. The damaged erythrocytes are removed from circulation in the spleen and destroyed by macrophages at an elevated rate, leading to anemia. This enzymopathy occurs in areas with high malarial burden, in part because the mutated recessive allele confers malarial resistance. This resistance is because RBCs with low G6PDH activity, when infected with the parasite, are continuously removed from the circulation.

Tricarboxylic Acid or Krebs Cycle

A major route for pyruvate oxidation is conversion to acetyl-CoA, a reaction catalyzed by the mitochondrial pyruvate dehydrogenase enzymatic complex. Acetyl-CoA is a high-energy intermediate that can be further oxidized by the TCA cycle or used for fatty acid synthesis. The TCA cycle is initiated by the condensation of oxaloacetic acid with acetyl-CoA, forming citrate. In reactions involving decarboxylation and oxidation, CO_2 is produced and NADH and flavin adenine dinucleotide (FADH) are produced for use in the mitochondrial respiratory chain. The flux of the TCA cycle is regulated by the levels of acetyl-CoA and oxaloacetic acid, which are entry points in the cycle, and by the availability of NAD^+ and FAD^+ substrates. The rate of oxidation through the TCA cycle depends on mitochondrial electron transport activity, which is governed in part by NADH and ADP levels. The TCA cycle also produces metabolites for biosynthetic processes (anaplerotic reactions). For example, citrate is converted to fatty acids and sterols, oxaloacetate is used to produce aspartate to be used in biosynthesis of nucleotides, aconitate generates itaconate that attenuates inflammation in LPS-activated macrophages, and succinyl CoA is an intermediate in heme and porphyrin synthesis. Aside from the bioenergetic and anaplerotic aspect of this cycle, several reactions have important clinical implications.

Oxidative Phosphorylation

In most cell types, oxidative phosphorylation is dominant on ATP generation. Exceptions include RBCs, which lack mitochondria. Oxidative phosphorylation complexes are located at the inner mitochondrial membrane and receive high-energy electrons from NADH (produced from the oxidation of acetyl-CoA). These electrons are passed through the different oxidative phosphorylation complexes (which contain heme, copper iron–sulfur groups, and flavins as electron carriers) until they reach the final electron acceptor, molecular oxygen. Because of electron transfer, protons are pumped into the mitochondrial intermembrane space, generating an electrochemical gradient used to synthesize ATP. There are five oxidative phosphorylation complexes: complex I (NADH–CoQ reductase complex), complex II (succinate–CoQ reductase complex), complex III ($CoQH_2$–cytochrome c reductase complex), complex IV (cytochrome C oxidase complex), and complex V (ATP synthase complex). In general, hematopoietic stem cells are in low-oxygen niches and largely depend on glycolysis instead of oxidative phosphorylation to maintain ATP levels. The differentiation process is associated with increases in mitochondria, which allow for the generation of ATP through the respiratory chain. For example, this occurs in quiescent T cells that are in a catabolic phase, producing ATP mainly through oxidative phosphorylation. Upon stimulation, activated T cells shift towards an anabolic phase, relying upon a high rate of glycolysis for ATP generation. In addition, oxidative phosphorylation plays an important role in immune antitumor responses. Thus the tumor microenvironment is known to repress T-cell mitochondrial biogenesis to facilitate tumor growth. Similar oxidative phosphorylation repression is caused by the inhibitory receptor PD-1 driving CD8(+) T-cell exhaustion. Another example relates to cellular metabolism in M1 and M2 macrophages. M1 macrophages are largely glycolytic to function during acute inflammation. In contrast, M2 macrophages mainly use oxidative phosphorylation for lengthy processes to resolve tissue inflammation and repair or to promote antiparasite immunity. Mitochondrial DNA encodes for several oxidative phosphorylation subunits, and mutations in this DNA produce mitochondrial diseases. Interestingly, anemia, a symptom associated with patients having Pearson syndrome, is caused by accumulation of mutated mitochondrial DNA in sideroblasts. This suggests that hematopoietic cell–specific respiration defects can be responsible for anemia by inducing abnormalities in erythropoiesis during development.

Reactive Oxygen Species Metabolism

ROS are chemically reactive small molecules with oxygen in different oxidation states, such as partially reduced oxygen ions and peroxides. The three major species are superoxide, hydrogen peroxide, and hydroxyl radicals. The major cellular sites for ROS production are the mitochondria and NADPH oxidase, a plasma membrane or phagosome-bound enzyme. Approximately 85% of cellular ROS is a subproduct of normal oxidative phosphorylation. Superoxide is the initial ROS produced in the electron transport chain, and it is transformed to hydrogen peroxide by the enzyme superoxide dismutase. Hydrogen peroxide is the substrate of catalase or glutathione peroxidase, which reduces it to water. However, hydrogen peroxide is also converted to hydroxyl radicals, the most ROS, in a Fenton reaction with ferrous iron. NADPH oxidase catalyzes the NADPH-dependent reduction of oxygen into the superoxide anion.

ROS cause cellular damage through oxidation and chemical modifications of proteins, lipids, and DNA. Nuclear and mitochondrial DNA can be oxidized, producing strand breaks. Intracellular levels of ROS are regulated through different signaling transduction pathways. Growth factor–mediated signaling increases ROS levels, for instance. Conversely, ROS also affect this signaling through modulation of PTPs that contain cysteine-sensitive residues that modulate their enzymatic activity and regulate the biologic responses associated with this signaling.

ROS are particularly deleterious to hematopoietic stem cells because of their effect on genomic stability and survival. In phagocytic cells (neutrophils, macrophages, or eosinophils), NADPH oxidase is responsible for the oxidative burst that is triggered upon phagocytosis of pathogens. Superoxide generated by NADPH oxidase is rapidly converted to other ROS, which, in cooperation with pH-sensitive proteases, are responsible for killing the microorganisms in the phagosome vacuole.

Cell death via ferroptosis occurs with extensive phospholipid-containing polyunsaturated fatty acid chains peroxidation caused by transition metal iron-dependent ROS production. Certain blood tumors such as diffuse larger B-cell lymphomas are very susceptible to ferroptosis, which is often associated with mesenchymal and metastatic states. An important regulator in this process is GPX4 (glutathione peroxidase 4), a selenium-dependent enzyme that uses reduced glutathione to inhibit phospholipid peroxidation. Excessive iron-dependent phospholipid peroxidation disrupts membrane integrity causing cell death that can be inhibited by GPX4 inhibitors, antioxidants, or iron quelators.

Recently, gain-of-function mutations of isocitrate dehydrogenase 1 and 2 (IDH1 is cytoplasmic and is unrelated to the TCA cycle; IDH2 is the TCA mitochondrial form) have been found in 20% of acute leukemia patients. IDH1 and IDH2 are highly homologous but distinct (in structure and function) from the NAD$^+$-dependent heterotrimeric IDH3 enzyme that is part of the TCA cycle producing NADH to the respiratory chain. The cellular function of the NADP-dependent IDH1/2 enzymes is not clear, but they are part of glucose, fatty acids, and glutamine metabolism and contribute to the maintenance of cellular reduction-oxidation balance. In three identified mutations, the enzyme undergoes a change in its normal physiologic catalytic reaction (i.e., oxidative decarboxylation of isocitrate to produce α-ketoglutarate and CO_2 while converting NAD[P] to NAD[P]H) and instead produces 2-hydroxyglutarate, which is now considered to be a proto-oncometabolite. The mechanism appears to be linked to competition with α-ketoglutarate for the active site of ketoglutarate-dependent dioxygenases, such as TET2, which functions as a cytosine demethylase.

Lipid Metabolism

Fatty acids and triglycerides (the storage form of fatty acids) constitute an energetic reserve in the body. Most of the cells can synthesize fatty acids, but there are essential fatty acids such as linoleic acid, α-linoleic, and arachidonic acid that cannot be synthesized. Arachidonic acid is made from linoleic acid and is the precursor for prostaglandins, thromboxanes, and leukotrienes that participate in different pathways such as the inflammatory response. Drugs that block the enzyme cyclo-oxygenase and prostaglandin synthesis such as acetaminophen, ibuprofen, and acetylsalicylate provide pain relief. Fatty acids can directly mediate transcriptional responses, acting as ligands for peroxisome proliferator-activated receptors, a family of NHRs. In addition, there are specific GPCR receptors such as GPR40 and GPR120 activated by medium- or long-chain fatty acids. GPR43 is activated by short-chain fatty acids and is highly abundant in leukocytes.

Fatty Acid Synthesis

In the mitochondrial matrix, acetyl-CoA is generated from pyruvate and is the precursor for fatty acid synthesis. Acetyl-CoA cannot cross the mitochondrial membrane; thus acetyl-CoA condenses with oxaloacetate (first reaction in the TCA cycle) to form citrate that is exchanged into the cytoplasm through TCA translocases. Once in the cytoplasm, citrate is converted to acetyl-CoA by ATP citrate lyase. The rate-limiting reaction of fatty acid synthesis is the carboxylation of acetyl-CoA to form malonyl CoA, which is catalyzed by acetyl-CoA carboxylase (ACC). Malonyl CoA is a potent inhibitor of fatty acid oxidation. ACC is allosterically regulated by citrate to form active enzyme polymers, which are depolymerized by the end product of fatty acid synthesis: long-chain fatty acids. Growth factors positively control ACC dephosphorylation. In contrast, catecholamines result in the phosphorylation and inhibition of ACC via PKA. Fatty acids are synthesized in the cytoplasm by a multifunctional enzyme, FAS. Two of these functional domains are the acyl carrier protein and the condensing enzyme (CE). After completion of the different rounds of synthesis, the palmityl group is transferred to CoASH. In macrophages, LPS activates lipogenesis through activation of sterol regulatory element–binding protein (SREBP), a key transcriptional mediator of cholesterol and fatty acid synthesis.

Fatty Acid Oxidation

Fatty acids are "charged" before oxidation to form acyl-SCoA, a cytoplasmic reaction catalyzed by the enzyme fatty acyl-CoA synthetase. However, fatty acid β-oxidation occurs in the mitochondrial matrix, and charged fatty acids must first be conjugated to carnitine to cross the mitochondrial membrane. This transport is carried out by the carnitine acyltransferases I and II. These enzymes constitute a rate-limiting step for β-oxidation of fatty acids and are allosterically regulated by malonyl CoA, allowing the cell to avoid a futile cycle of fatty acid synthesis and breakdown. Inside the mitochondria, acyl-CoA undergoes a cycle of reactions removing acetyl-CoA from the main chain. This acetyl-CoA is then processed through the TCA cycle.

Cholesterol

Cholesterol is an important component of cellular membranes and a substrate to produce steroid hormones. Free cholesterol is tightly controlled in cells through synthesis, storage, and transport. Excess cholesterol in cells is secreted through reverse cholesterol transport or stored in the cytoplasm as cholesterol ester, produced by Acy-CoA:cholesterol acyltransferase located in the ER. Cholesterol is transported in the plasma by lipoproteins, including chylomicrons and very-low-density lipoprotein (VLDL). The main sources of cellular cholesterol for hematopoietic cells are the cholesterol-rich lipoprotein, low-density lipoprotein (LDL), and de novo synthesis from acetyl-CoA. The rate-limiting step for cholesterol synthesis is catalyzed by hydroxymethylglutaryl (HMG)-CoA reductase, the direct target of cholesterol-lowering statin drugs, and converts HMG-CoA to mevalonic acid. Cellular cholesterol levels are sensed in the ER through the SREBP transcription factor, which directly controls most the enzymes in cholesterol synthesis as well as LDL transport. Excess of LDL becomes oxidized and taken by macrophages, a main cause of atherosclerosis. The SREBP pathway is also important for T-cell activation under antigenic challenge, because its activation favors cholesterol synthesis and transport, which is used for membrane biogenesis and cell proliferation in the activated T cell.

Phospholipids

Phospholipids contain a hydrophilic head with a glycerol phosphate group that is esterified to a hydroxyl group of serine, choline, or ethanolamine, and two hydrophobic tails that are fatty acids esterified to two of the carbons of glycerol phosphate. They are main constituents of cellular membranes and form the lipid bilayer. Phospholipid synthesis takes place in the ER membrane, and they are delivered to cellular membranes. Some types of phospholipids also form part of signaling transduction as substrates for phospholipase C. Phospholipids are unequally distributed among organelle membranes and are also asymmetrically localized between the two sides of the bilayer. For example, phosphatidylcholine (PS) is enriched in the inner part of the plasma membrane and becomes exposed during cell death and targeted through the macrophage's PS receptor. The synthesis of phospholipids starts with DAG or cytidine diphosphate (CDP)-DAG that is formed from phosphatidic acid, which is made from the glycolytic intermediate glycerol-3-phosphate or dihydroxyacetone phosphate. Specific enzymes synthesize PS, phosphatidylethanolamine, and phosphatidylserine. PS is made via the Kennedy pathway via CDP-choline. A potent phospholipid that plays important roles in leukocyte chemotaxis, macrophage oxidative burst, and platelet aggregation is the platelet-activating factor (acetyl-glyceryl-ether-phosphorylcholine).

Amino Acid Metabolism

The major sources of amino acids derive from the diet or protein breakdown. Nonessential amino acids are synthesized from carbon skeletons using different metabolic pathways. Amino acids conjugated to transfer RNA (tRNA) are used in protein synthesis; however, in excess, they can be used for energy production. In addition, amino acids are necessary for the synthesis of other compounds. For example, tryptophan catabolism constitutes a route for de novo NAD^+ synthesis in a pathway that is important in leukocytes for the replenishment of NAD^+ levels after oxidative stress. Interestingly, different metabolites derived from tryptophan catabolism via the kynurenine pathway play a role in immune tolerance. Plasma amino acids are transported in cells against a concentration gradient. Amino acid transporters are specific for neutral (small and larger), basic, and acidic amino acids. Depending on the cell type and specific state (growth, hypoxia, fasting, and so on), intracellular amino acids are used in anabolic or catabolic pathways.

Most of the regulation of amino acid metabolism is achieved through substrate fluxes affecting specific enzyme kinetics. However, there are two major regulatory pathways that involve amino acid–sensing mechanisms and metabolic control. (1) General control nonrepressed 2 (GCN2) is a protein kinase that senses amino acid deficiency through direct binding to uncharged tRNA. GCN2 controls the transcription factor ATF4, affecting different enzymes of amino acid metabolism. The high asparagine requirement of certain acute lymphoblastic leukemias has resulted in the use of asparaginase to deplete circulating levels of asparagine. Limited amounts of asparagine result in activation of GCN2 in leukemic cells and reduce their proliferation and viability rates. (2) mTOR is a protein kinase activated in response to increased amino acid concentrations (particularly branch chain amino acids). mTOR controls many aspects involved in protein synthesis, inhibition of protein degradation, and amino acid biosynthetic enzymes.

Biosynthesis of Nonessential Amino Acids

Nonessential amino acids are synthesized by most of the cells, including hematopoietic lineages. Nonessential amino acids are mainly synthesized from glucose (alanine, arginine [from the urea cycle in hepatic cells], asparagine, aspartate, cysteine, glutamate, glutamine, glycine, proline, and serine), except for tyrosine, which is synthesized from phenylalanine. The rest of the nine amino acids are essential, and the body needs to obtain these from the diet. Serine, glycine, and cysteine are synthesized from glycolytic intermediates. Serine synthesis has recently been found to be increased and necessary in stem cells. For some hematopoietic cells, the synthesis of cysteine and glycine is of elevated importance owing to their use in the synthesis of the tripeptide glutathione. Aspartate and asparagines are synthesized by transamination of oxaloacetate by glutamate and amide transfer from glutamine, respectively. Glutamate, glutamine, proline, and arginine are formed from the TCA cycle intermediate α-ketoglutarate.

Amino Acid Catabolism

Two central reactions in amino acid catabolism are the generation of ammonia through transamination (catalyzed by amino transferases) and oxidative deamination (catalyzed by glutamate dehydrogenase) in which the α-amino group of the different amino acids is transferred to α-ketoglutarate to form glutamate, which undergoes the release of free NH_3. Free ammonium is added to glutamate to generate glutamine, which is then exported into the circulation to the liver, where it then enters the urea cycle. The urea cycle mainly occurs in the liver and has two purposes: (1) to get rid of free ammonium and (2) to supply arginine. Interestingly, one of the enzymes of the urea cycle, arginase (which converts arginine to ornithine), is expressed in immune cells. Myeloid cell arginase depletes arginine and suppresses T-cell immune responses and is an important mechanism of inflammation associated with immunosuppression. Arginase is viewed as a promising strategy in the treatment of cancer and autoimmunity. Arginine is also essential for the differentiation and proliferation of erythrocytes.

Nucleotide Metabolism

Nucleotides are involved in a diverse array of cellular functions, including (1) energy metabolism (ATP, NAD^+, $NADP^+$, and FAD^+ and their corresponding reduced forms), (2) units of nucleic acids (NTPs are substrates for RNA and DNA polymerases), and (3) physiologic mediators such as adenosine, ADP (which is critical in platelet aggregation), cAMP and cGMP (second messenger molecules), and GTP (which participates in signal transduction via GTP-binding proteins).

Most of the regulatory pathways that are associated with nucleotide synthesis and degradation are strictly controlled by regulatory components of the cell cycle machinery. The amount of intracellular nucleotides has to reach certain levels for the cell to proceed through the S-phase checkpoint. In addition, several of the key cell cycle regulators, including the c-myc oncogene (which is translocated in certain myelomas), directly increase the expression of most of the key enzymes associated with nucleotide synthesis.

Nucleotide Synthesis

There are two pathways for the synthesis of nucleotides, salvage and de novo. The salvage pathway uses free bases via a reaction with phosphoribosyl pyrophosphate (PRPP) and generation of nucleotides. De novo pathways synthesize pyrimidines and purine nucleotides from amino acids, carbon dioxide, folate derivatives, and PRPP. Importantly, both salvage and de novo pathways depend on PRPP, which is produced from ATP and ribose-5-phosphate (generated in the PPP) by PRPP synthetase, an enzyme that is inhibited by metabolic markers of low-energy AMP, ADP, and GDP to avoid nucleotide synthesis in these conditions. In general, PRPP levels are low in postmitotic cells but high in proliferating cells. Folate is essential in nucleotide biosynthesis, and lack of folate in the diet can lead to anemia due to inhibition of proliferation of RBC precursors.

Nucleotide Degradation

Nucleotidases and nucleosidases initially participate in purine nucleotide degradation. For example, adenosine is deaminated to produce inosine, which, after ribose is removed, generates hypoxantine, which is used by xanthine oxidase to form uric acid. Immune cells have potent nucleotide salvage pathways, and a lack of adenosine deaminase causes severe combined immune deficiency (SCID) syndrome. SCID is associated with a large accumulation of dATP in immune cells, which, through a negative-feedback mechanism on ribonucleotide reductase, blocks production of dNTPs and results in a failure to replicate DNA.

Introduction to Metabolomics

Analytical measurements of blood metabolites such as glucose, urea, and cholesterol are part of clinical biochemistry to track diseases. Along these lines and facing the new era of personalized medicine emerges metabolomics, which evaluates metabolism with a comprehensive and quantitative analysis of all metabolites, as well as its impact on cell biology, and aims to discover novel biomarkers or targets for therapy. Recent technical innovations in mass spectrometry and nuclear magnetic resonance (NMR) have allowed the measurement of many metabolites simultaneously. These advances, in combination with metabolite flux analysis with isotopic tracers, have provided new information on many metabolic processes. The use of metabolomics also offers a tool to identify metabolic enzymes as drug targets, because they are poised for inhibition with small-molecule drugs and possess allosteric sites that can be used to alter catalytic activity.

A major effort in metabolomics has been the identification of biomarkers for diseases and therapeutic targets. As an example, metabolomics was used to analyze plasma from diabetic patients showing increases in branch chain amino acids before hyperglycemia. Another example comes from the combination of genome-wide sequencing analysis and metabolomics: sequence analysis of AMLs was able to identify IDH1 or IDH2 mutations in 20% of patients. Metabolomics analysis revealed accumulation of a noncanonical metabolite, 2-hydroxyglutarate, which promotes the tumorigenic process (see following discussion). In addition, recent blood metabolomics profiles in critically ill COVID-19 patients identified increased kynurenine and decreased arginine, sarcosine, and lysophosphatidylcholines. In fact, the arginine/kynurenine ratio provided a classification accuracy between patients and healthy controls. In general, there are two different metabolomic approaches: targeted, which measures known and specific metabolites; and nontargeted, which includes analytical measurements of unknown metabolites.

Metabolomics of Glucose Metabolism

Systematic and simultaneous quantitative targeted polar metabolite analysis of glucose metabolic pathways using metabolomic and flux measurement techniques has introduced new basic and clinically relevant information in hematology (Fig. 6.4). For example, increases in glucose metabolism signatures measuring serum metabolite linked to glycolysis and the TCA cycle have been correlated with a prognostic risk score in AML patients.

Metabolic reprogramming or switches are crucial for T-cell activation. Quiescent naive T cells obtain most of their ATP from mitochondrial oxidative phosphorylation for energy, whereas activated T cells switch to glycolytic, glutaminolysis, and anabolic metabolism to promote clonal expansion that appears to be dependent on the transcription factor Myc. Moreover, metabolomic signatures have revealed that T helper (Th)1, Th2, and Th17 cell lineages and T effectors exhibit an increased glycolytic metabolism. However, regulatory T cells and CD8^{+} memory T cells depend more on mitochondrial oxidative phosphorylation. In addition, T cells are exposed to different nutrient environments, from high nutrient levels in the lymphoid organs to a more restricted nutrient availability in the effector sites such as tumors or infection. Under these conditions, metabolic reprogramming through mTOR and AMP kinases controls and maintains survival and immune function of T cells. Based on untargeted metabolomic studies, RBCs from G6PDH donors do not meet the US Food and Drug Administration (FDA) guidelines for storage quality because of pyruvate/lactate ratios and glycolytic and PPP intermediates involved in NADPH production.

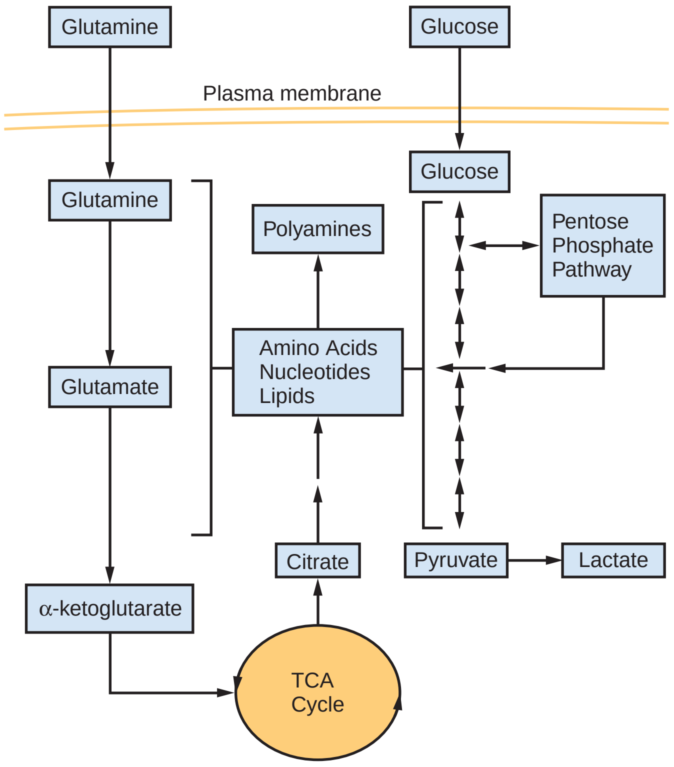


Figure 6.4 METABOLITE PROFILING UPON T-CELL ACTIVATION. Metabolomic analysis has revealed that glycolytic fluxes and glutaminolysis are increased during activation of T cells. Polyamines that are required for T-cell proliferation are synthesized from glutamine. See text for further details. *TCA*, Tricarboxylic acid.

Metabolomics of Lipid Metabolism

Nonpolar metabolomics has provided metabolite profiles linked to lipid metabolism. In the case of fatty acids, de novo fatty acid synthesis is necessary for differentiation of Th17. Bioactive lipids have also been profiled in different types of blood cells. For example, sphingosine-1-phosphate is stored in erythrocytes and is found highly elevated in the blood of sickle cell disease patients, owing to increased erythrocyte sphingosine kinase 1. Quantitative lipidomics platforms show that 20% of the platelet lipidomes is remodeled upon activation, including mainly arachidonic acid–containing lipids.

Metabolomics of Nucleotide Metabolism

Measurements of the different polar metabolites in nucleotide metabolism are linked to stages of cell growth. For example, unbiased metabolomics has identified that pyrimidine starvation is a mechanism for specific types of cell death in multiple myeloma cells. Hypoxanthine is one of the metabolite markers that is increased in plasma in different subtypes of lymphomas.

Metabolomics of Amino Acid Metabolism

Metabolomic studies have revealed that activated T cells reprogram their metabolism from fatty acid and pyruvate oxidation, and the TCA cycle, to aerobic glycolysis, the PPP, and glutaminolysis, and metabolic fingerprint-like tumor cells. Glutamine is used to increase polyamine biosynthesis, which is essential for T-cell proliferation, a process controlled by the transcription factor Myc. Metabolomic analysis of two cohorts of hematopoietic stem cell transplantation recipients and donors shows a significant change of host and microbiota indole metabolites related to tryptophan metabolism.

SUMMARY AND PERSPECTIVES

This short review summarizes the central signaling and metabolic pathways that play a pivotal role in all the processes executed by hematopoietic cellular systems. In normal physiologic conditions these pathways are regulated and operating to achieve homeostatic cellular functions in healthy individuals. However, in pathologic conditions, dysregulation or failure of these pathways leads to diseases of lymphohematopoietic tissues. To a large extent, the main components and regulatory circuitries of these pathways have been elucidated, but the challenge for the future is to fully integrate them and identify novel therapeutic targets that will enable the development of effective treatments for these diseases. New technologies in metabolomics are promising for the identification of biomarkers that can be used in personalized medicine, as well as new therapeutic targets including metabolic enzymes.

SUGGESTED READINGS

Abram CL, Lowell CA. The ins and outs of leukocyte integrin signaling. *Annu Rev Immunol*. 2009;27:339.

Aggarwal BB. Signaling pathways of the TNF superfamily: a double- edged sword. *Nat Rev Immunol*. 2003;3:745.

Bolanos JP, Almeida A, Moncada S. Glycolysis: a bioenergetic or a survival pathway? *Trends Biochem Sci*. 2010;35:145.

Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell*. 1997;89:331.

Buck MD, O'Sullivan D, Pearce EL. T cell metabolism drives immunity. *J Exp Med*. 2015;212:1345.

Cai X, Chiu YH, Chen ZJ. The cGAS-cGAMP-STING pathway of cytosolic DNA sensing and signaling. *Mol Cell*. 2014;54:289.

Cairns RA, Mak TW. Oncogenic isocitrate dehydrogenase mutations: mechanisms, models and clinical opportunities. *Cancer Discov*. 2013;3:730.

Chan DI, Vogel HJ. Current understanding of fatty acid biosynthesis and the acyl carrier protein. *Biochem J*. 2010;430:1.

Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet*. 2006;7:606.

Evans DR, Guy HI. Mammalian pyrimidine biosynthesis: fresh insights into an ancient pathway. *J Biol Chem*. 2004;279:33035.

Fritsche K. Fatty acids as modulators of the immune response. *Annu Rev Nutr*. 2006;26:45.

Gordon MD, Nusse R. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. *J Biol Chem*. 2006;281:22429.

Hamanaka RB, Chandel NS. Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. *Trends Biochem Sci*. 2010;35:505.

Hurlbut GD, Kankel MW, Lake RJ, et al. Crossing paths with Notch in the hyper-network. *Curr Opin Cell Biol*. 2007;19:166.

Irvine DA, Copland M. Targeting hedgehog in hematologic malignancy. *Blood*. 2012;119:2196.

Ivan M, Kaelin WG. The EGLN-HIF O2-sensing system: multiple inputs and feedbacks. *Mol Cell*. 2017;66:772.

Jiang X, Stockwell BR, Conrad M. Ferroptosis: mechanisms, biology and role in disease. *Nat Rev Mol Cell Biol*. Jan 25, 2021.;22:266.

Kim C, Ye F, Ginsberg MH. Regulation of integrin activation. *Annu Rev Cell Dev Biol*. 2011;27:321.

Kolch W. Coordinating ERK/MAPK signaling through scaffolds and inhibitors. *Nat Rev Mol Cell Biol*. 2005;6:827.

Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell*. 2010;141:1117.

Levine AJ, Puzio-Kuter AM. The control of the metabolic switch in cancer by oncogenes and tumor suppressor genes. *Science*. 2010;330:1340.

Liu GY, Sabatini DM. mTOR at the nexus of nutrition, growth, ageing and diseases. *Nat Rev Mol Cell Biol*. 2020;21:183.

Lunt SY, Vander-Heiden MG. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu Rev Cell Dev Biol*. 2011;27:441.

Mangelsdorf DJ, Thummel C, Beato M, et al. The nuclear receptor superfamily: the second decade. *Cell*. 1995;83:835.

McDermott DF, Atkins MB. PD1 as a potential target in cancer therapy. *Cancer Med*. 2013;2:662.

Mitin N, Rossman KL, Der CJ. Signaling interplay in Ras superfamily function. *Curr Biol*. 2005;15:R563.

Munder M. Arginase: an emerging player in the mammalian immune system. *Br J Pharmacol*. 2009;15:638.

Norlund P, Reichard P. Ribonucleotide reductases. *Annu Rev Biochem*. 2006;75:681.

Owen OE, Kalhan SC, Hanson RW. The key role of anaplerosis and cataplerosis for citric acid cycle function. *J Biol Chem*. 2002;277:30409.

Patel CH, Leone RD, Horton MR, Powell JD. Targeting metabolism to regulate immune responses in autoimmunity and cancer. *Nat Rev Drug Discov*. 2019;18(9):669–688.

Patsouikis N, Wang Q, Strauss L, Boussiotis VA. Revisiting the PD1 pathway. *Sci Adv*. 2020;6(38):2712.

Rabinowitz JD, Purdy JG, Vastag L, et al. Metabolomics in drug target discovery. *Cold Spring Harb Symp Quant Biol*. 2011;76:235.

Robertson DG, Watkins PB, Reily MD. Metabolomics in toxicology: preclinical and clinical applications. *Toxicol Sci*. 2011;1:S146.

Saggerson D. Malonyl-CoA, a key signaling molecule in mammalian cells. *Annu Rev Nutr*. 2008;28:253.

Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell*. 2003;113:685.

Sigalov A. Multi-chain immune recognition receptors: spatial organization and signal transduction. *Semin Immunol*. 2005;17:51.

Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol*. 2003;21:335.

Vance JE. Phospholipid synthesis and transport in mammalian cells. *Traffic*. 2015;16:1.

Wallace DC, Fan W, Procaccio V. Mitochondrial energetics and therapeutics. *Annu Rev Pathol*. 2010;5:297.

Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science*. 2011;334:1081.

Waters C, Pyne S, Pyne NJ. The role of G-protein coupled receptors and associated proteins in receptor tyrosine kinase signal transduction. *Semin Cell Dev Biol*. 2004;15:309.

Watowich SS, Wu H, Socolovsky M, et al. Cytokine receptor signal transduction and the control of hematopoietic cell development. *Annu Rev Cell Dev Biol*. 1996;12:91.

Watts TH. TNF/TNFR family members in costimulation of T cell responses. *Annu Rev Immunol*. 2005;23:23.

Zhang S, Macias-Garcia A, Ulirsch JC, et al. HRI coordinates translation necessary for protein homeostasis and mitochondrial function in erythropoiesis. *Elife*. 2019;8:e46976.

Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes, and ageing. *Nat Rev Mol Cell Biol*. 2011;12:21.

CHAPTER 7

PROTEIN ARCHITECTURE: RELATIONSHIP OF FORM AND FUNCTION

Jia-huai Wang and Michael J. Eck

Previous chapters outline the central dogma of mlecular biology: the storage of genetic information in DNA and its regulated transcription into messenger RNA and eventual translation into proteins. In this chapter, we briefly outline the chemical structure of proteins and their posttranslational modifications (PTMs). We explain how the properties of the 20 amino acids of which proteins are composed allow these polymers to fold into compact, functional domains and how particular domains and motifs have been assembled, modified, and reused in the course of evolution. Finally, we describe a sampling of proteins and domains of relevance to the hematologist and explore briefly how point mutations, chromosomal translocations, and other genetic alterations may modify protein structure and function to cause disease.

AMINO ACIDS AND THE PEPTIDE BOND

Proteins are linear polymers of the 20 naturally occurring amino acids, linked together by the peptide bond. All of the amino acids share a common core or backbone structure and differ only in the "sidechain" emanating from the central "α-carbon" (Cα) of this core. The common backbone elements include an amino group, the central Cα, and a carboxylic acid group. Peptide bonds are formed by reaction of the carboxylic acid of one amino acid with the amino group of the next amino acid in the chain. This reaction is templated and catalyzed by the ribosome. Coupling of multiple amino acids together via the peptide bond produces the repeating mainchain structure of the polypeptide chain, composed of the amide (NH) nitrogen, Cα, and carbonyl carbon (CO), followed by the amide nitrogen of the next amino acid in the chain (Fig. 7.1A). The resonant, partial double bond character of the peptide bond prevents rotation about this bond; thus the five mainchain carbon, nitrogen, and oxygen atoms of each peptide unit lie in a plane. The conformational flexibility in the polypeptide chain is conferred by rotation about the bonds on either side of the Cα atom; these bond angles are referred to as phi and psi angles. The angle of the N–Cα bond is the phi angle (Φ), and that of the Cα–CO bond is the psi angle (ψ).

The *primary structure* or primary sequence of a protein refers to the order in which various residues of the 20 amino acids are assembled into the polypeptide chain, and this sequence is critically important for determining the three-dimensional fold and thus function of the protein. The diverse chemical structure and physicochemical properties of the 20 amino acid sidechains guide the three-dimensional fold of proteins and also provide for the enormous repertoire of protein function, from catalysis of myriad chemical reactions to immune recognition to establishment of muscle and skeletal structure.

The amino acids can be divided into general classes based on the physicochemical properties of their side chains and in particular their propensity to interact with water. *Hydrophobic* amino acids have aliphatic or aromatic side chains and include alanine, valine, leucine, isoleucine, proline, methionine, and phenylalanine. The hydrophobic amino acids predominate in the interior of proteins, where they are sequestered from water. They tend to pack against each other via *van der Waals* interactions, which contribute to the overall stability of folded protein domains. By contrast, *hydrophilic* or *polar* amino acids (including serine, threonine, tyrosine, asparagine, glutamine, cysteine, and tryptophan) are often exposed on the surface of proteins, where they can form *hydrogen bonds* with each other, with the protein mainchain, and with water or ligand molecules. Hydrogen bonding refers to the attractive interaction of a proton covalently bonded to one electronegative atom (usually a nitrogen or oxygen in proteins) with another electronegative atom. Hydrogen bonds are an important contributor to the stability of proteins and to the specificity of protein-protein and protein-ligand interactions. *Charged* amino acids are also polar and are important participants in hydrogen bonding. Hydrogen bonds between negatively charged (acidic) and positively charged (basic) amino acids are also termed *salt bridges* and are also important components of protein stability and protein-protein interactions. The acidic amino acids are aspartate and glutamate, and the basic amino acids are lysine, arginine, and histidine. Histidine merits special mention because it is the only amino acid whose side chain can be protonated or unprotonated, and therefore charged or uncharged, around physiologic ranges of pH. For this reason, histidine is part of many enzyme active sites. For example, in the serine proteases of the coagulation cascade, an active site histidine acts as a general base, accepting and then releasing a proton in sequential steps of the enzymatic reaction. It is also important to note that some of the polar amino acids are *amphipathic* (i.e., they have both polar and hydrophobic character). This dual nature of threonine, lysine, tyrosine, arginine, and tryptophan makes them well suited for participating in protein-protein interactions, where they may be alternately exposed to solvent or buried upon formation of a complex.

The amino acid cysteine is unique in that its side chain contains a relatively reactive thiol group. The structure of cell-surface and extracellular proteins is often stabilized by *disulfide bonds*, covalent bonds formed between the thiol groups of spatially juxtaposed cysteine residues. In general, disulfide bonds are not found in intracellular proteins, where the reducing environment disfavors their formation. Disulfide bonds can form between cysteines within the same polypeptide chain, stabilizing the fold of the polypeptide backbone, or they may covalently join two different polypeptide chains, for example, the heavy and light chains of an immunoglobulin (Ig). In addition to their role in disulfide bond formation, cysteine residues often contribute to protein stability via their participation in metal ion coordination, in particular zinc, which is often bound by conserved sets of cysteine and histidine residues in small protein domains.

Protein Secondary Structure

The alternating pattern of hydrogen bond–donating amide groups and hydrogen bond–accepting carbonyl groups gives rise to repeating elements of protein structure that are stabilized by hydrogen bonds between these mainchain groups. These *secondary structure* elements include α-helices and β-sheets. In an α-helix, the mainchain adopts a right-handed helical conformation in which the carbonyl oxygen of the i^{th} residue in the polypeptide chain accepts a hydrogen bond from the amide nitrogen of the $(i + 4)^{th}$ residue (see Fig. 7.1B). The pattern may repeat for only a few residues, forming a single turn of α-helix, or for more than 100 residues, forming dozens of turns of helix. There are 3.6 residues per turn of helix, and the pitch or rise of the helix is

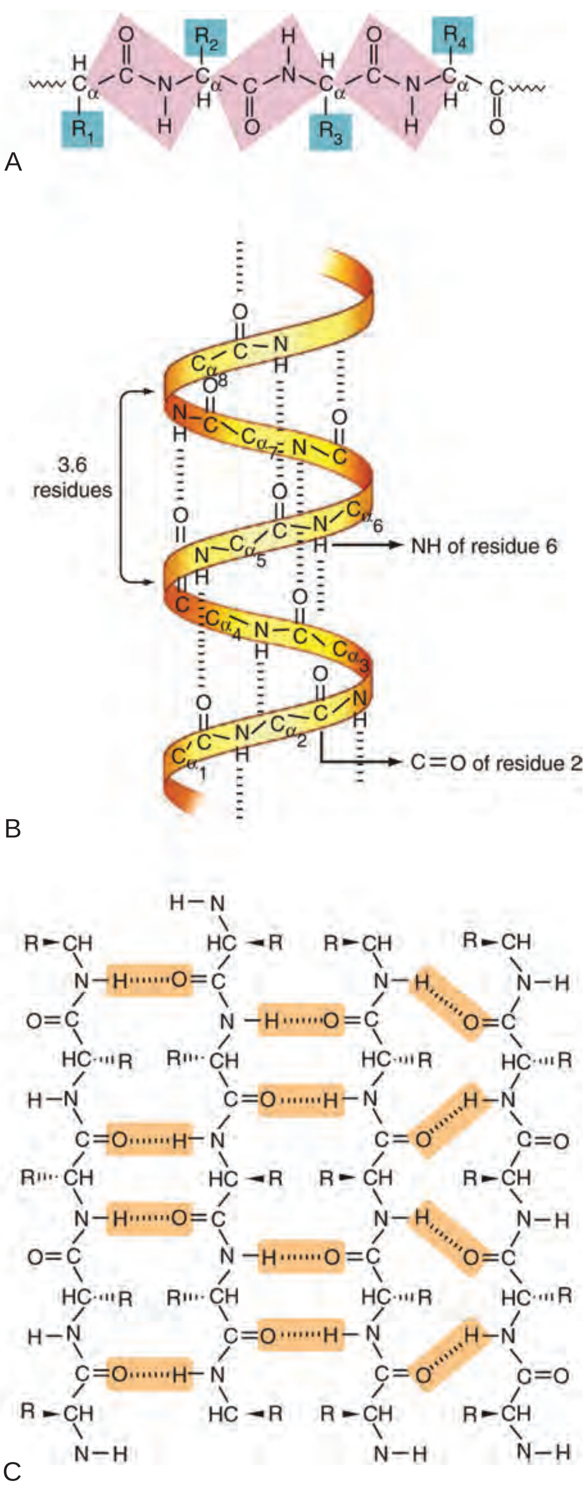


Figure 7.1 (A) Diagram showing a polypeptide chain where the mainchain atoms are represented as peptide units, linked through the α-carbon (Cα) atoms. Each peptide unit is a planar, rigid group *(shaded pink)* and has two degrees of freedom; it can rotate around the Cα-CO bond and the N-Cα bond. The peptide bonds are depicted in the trans conformation; adjacent Cα carbons and their side chains *(highlighted blue)* on opposite sides of the N–Cα bond. This is the preferred configuration for most amino acids because it minimizes steric hindrance. (B) The α helix. The hydrogen bonds between residue *n* and residue *n* + 4, which stabilizes the helix, are shown as dashed lines. (C) Schematic drawing of a mixed β-sheet. The three β-strands on the left are antiparallel to one another, while the two rightmost β-strands are parallel. The hydrogen bonds that stabilize the sheet are shaded.

1.5 Å per residue or 5.4 Å per turn. The sidechains of residues in an α-helix project outward, away from the central axis of the helix. Often a polar sidechain will "cap" the end of a helix by forming a hydrogen bond with the otherwise unpartnered amide or carbonyl group at the N- or C-terminal end of the helix.

In β-sheet secondary structure, the protein backbone adopts an extended conformation and two or more strands are arranged side by side, with hydrogen bonds between the strands. The strands can run in the same direction (parallel β sheet) or antiparallel to one another, and both parallel and antiparallel strands are often found together in mixed sheets (Fig. 7.2C). In β-sheets, the sidechains of a given strand extend alternately above and below the plane defined by the hydrogen-bonded mainchains. Other common types of secondary structure include a variant of the helix with an $i + 3$ hydrogen bonding pattern (the 3_{10} helix) and specific types of β-turns, short segments connecting other elements of secondary structure that are stabilized by β-sheet–like hydrogen bonds. Although any of the amino acids can be found within α-helices or β-sheets, the special characteristics of proline and glycine merit mention. The cyclic structure of proline means that it lacks an amide proton; thus it introduces an irregularity in hydrogen bonding. For this reason, it is infrequently found in α-helices, but if present, it will introduce a "kink" stemming from its constrained structure. Glycine lacks a sidechain—it has only a second hydrogen atom on its Cα—and therefore has less steric restriction and can adopt a wider range of backbone phi and psi angles. This added flexibility means that it tends to disfavor regular secondary structure.

Because proteins are large and complicated structures, they are typically illustrated with "ribbon" diagrams that trace the path of the polypeptide backbone. In such representations, helices are drawn as helical coils or cylinders, and β-strands as elongated rectangles with an arrow as a guide to the direction of the protein chain from its amino- to carboxy-terminal end.

Posttranslational Modifications

The covalent structure of proteins is commonly modified in structurally and functionally important ways beyond the linear coupling of amino acids via the peptide bond. Regulated proteolysis can be considered a PTM and can serve an important regulatory role, as in the cleavage of prothrombin in the blood clotting cascade.

A number of functional groups are appended to proteins to regulate their function, localization, protein interactions, and degradation. Examples of these PTMs include phosphorylation, glycosylation, ubiquitination, methylation, acetylation, and lipidation.[1] PTMs occur at distinct amino acid side chains or peptide linkages and are most often mediated by enzymatic activity and can occur at any step in the "life cycle" of a protein. As discussed later, a number of protein domains have evolved to recognize and bind specifically to proteins labeled by a particular PTM. Protein *phosphorylation*, most commonly on serine, threonine, or tyrosine residues, is one of the most important and well-studied PTMs. Phosphorylation is mediated by protein kinases and can activate or deactivate many enzymes through conformational changes and as such plays a critical role in the regulation of many cellular processes, including cell cycle, growth, apoptosis, and signal transduction pathways. Protein *glycosylation* encompasses a diverse selection of sugar-moiety additions to proteins that ranges from simple monosaccharide modifications to highly complex branched polysaccharides. Glycosylation has significant effects on protein folding, conformation, distribution, stability, and activity. Carbohydrates in the form of asparagine-linked (N-linked) or serine/threonine-linked (O-linked) oligosaccharides are major structural components of many cell surface and secreted proteins and also many viral proteins. Protein *methylation* on arginine or lysine residues is carried out by methyltransferases with S-adenosyl methionine (SAM) as the primary methyl group donor. Methylation is an important mechanism of epigenetic regulation because histone methylation and demethylation influence the availability of DNA for transcription. N-*acetylation*, the transfer of an

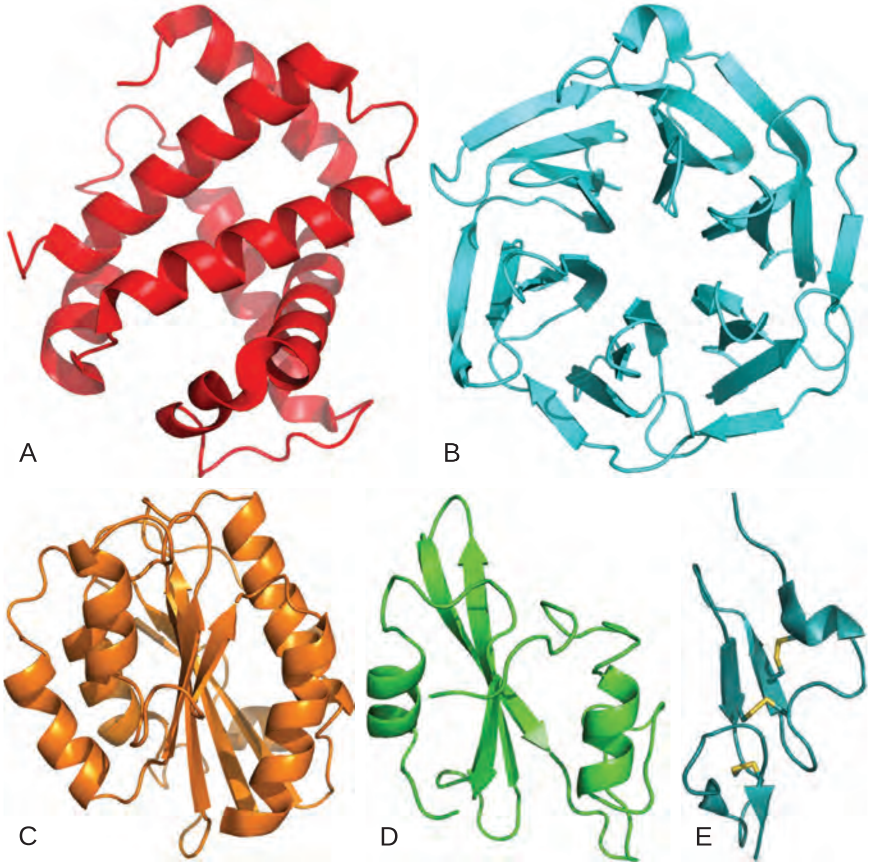


Figure 7.2 SEVERAL COMMON DOMAIN STRUCTURES. (A) The α-globin domain of hemoglobin is all α-helical (PDB entry 2MHB). (B) The β-propeller domain is an all β-strand structure found in many extracellular matrix and cell surface proteins (PDB entry 1NPE). (C) The integrin "I" domain is composed of alternate β-strands and α-helices (PDB entry 1ID0). (D) The SH2 (Src homology-2) domain is found in proteins involved in tyrosine kinase signaling and is also a mixed α/β-fold (PDB entry 1FMK). (E) The EGF (epidermal growth factor) domain is found in many extracellular matrix proteins and cell adhesion molecules. Its structure is stabilized by 3 to 4 disulfide bonds (PDB entry 1UZJ). *PDB*, Protein Data Bank.

acetyl group to the amine nitrogen at the N-terminus of the polypeptide chain, occurs in a majority of eukaryotic proteins. Lysine acetylation and deacetylation is an important regulatory mechanism in a number of proteins. It is best characterized in histones, where histone acetyl transferases (HATs) and histone deacetylases (HDACs) regulate gene expression via modification of histone tails. Many cytoplasmic proteins are also acetylated, and therefore acetylation seems to play a greater role in cell biology than simply transcriptional regulation.[2] *Lipidation* is a modification that targets proteins to membranes in organelles, vesicles, and the plasma membrane. Examples of lipidation include *myristoylation, palmitoylation, and prenylation*. Each type of modification gives proteins distinct membrane affinities, although all types of lipidation increase the hydrophobicity of a protein and thus its affinity for membranes. In N-myrisoylation, the myristoyl group (14-carbon saturated fatty acid) is transferred to an N-terminal glycine by N-myristoyltransferase. The myristoyl group does not always permanently anchor the protein in the membrane; in a number of proteins the N-terminal myristoyl group has been observed to pack into the protein core. N-myristoylation can therefore act as a conformational localization switch in which protein conformational changes influence the availability of the handle for membrane attachment.

Finally, ubiquitination (or equivalently, *ubiquitylation*) involves modification of a target protein with the protein ubiquitin, which is typically attached to a lysine residue via an isopeptide bond with the C-terminus of ubiquitin. Modification with ubiquitin, either singly (monoubiquitination) or in chains (polyubiquitination), is used to regulate diverse processes from protein degradation to gene expression to the cell cycle.[3]

The Domain Structure of Proteins

In general, the minimal biologically functional unit of protein three-dimensional structure is the protein *domain*. Domains are locally compact and semi-independent units of usually contiguous polypeptide chain. The common size of a domain is between 100 and 200 amino acid residues, although much larger and smaller domains are also frequently observed. Protein domains are composed of closely packed secondary structure elements—α-helices, β-sheets, or a combination of both—and the loops that connect them. Domains are stabilized by hydrophobic interactions among these elements and typically have very hydrophobic central cores, with more hydrophilic amino acids extending from their surface. Alternating patterns of hydrophobic residues in secondary structure elements are a reflection of the role of hydrophobicity in driving protein folding and stability. Helices are often amphipathic and pack in a folded domain such that their hydrophobic face is buried in the domain interior and their hydrophilic face is exposed on the surface. Likewise, β-sheets often have a buried hydrophobic face and an exposed hydrophilic face. The importance of the hydrophobic core to the stability of protein domains is highlighted by the fact that point mutations that introduce polar or charged residues into the protein interior often cause misfolding and thus a loss of function. Although these general characteristics are shared by protein domains that are found in an aqueous environment, such as those in the cytosol or on the cell surface, membrane-embedded proteins have very different properties reflective of their residence in the lipid bilayer. Several common domain structures representing different categories with regard to their secondary structure composition are shown in Fig. 7.2.

Deciphering this basic protein building block is key for understanding the structure and evolution of proteins. Kinetically, the domain structure of a protein may simplify the folding process into a step-wise course.[4] Thus a long amino acid sequence may fold into multiple domains rapidly and correctly. For many proteins, individual domains fold in a cotranslational manner; from the N-terminal region, a growing nascent polypeptide chain immediately begins to fold domain-by-domain during translation from the ribosome in a very efficient manner.[5] Genetically, it was long suspected that the exon structure of genes was correlated with the domains structure of proteins.[6] Recent multigenome analysis does find a strong correlation between domain organization and exon-intron arrangement in genomic DNA. The exon-domain correlation facilitates extensive exon shuffling events during evolution,[7] although it is not necessarily always one-exon/one-domain. This mechanism ensures that a stable and functionally efficient domain can be repeatedly used as a module assembled into many proteins with shared functions.

Not all protein sequences fold into a compact domain. Depending on computational methods used, 35% to 50% of the human proteome is estimated to lack a folded three-dimensional structure. Nevertheless, these *intrinsically disordered* proteins (or more often, intrinsically disordered regions within proteins) can perform critically important biologic functions that complement those of structured proteins.[8] Many disordered regions mediate protein-protein interactions and may undergo a disorder-to-order transition upon binding to an interacting partner, a process dubbed as folding-upon-binding. They function in crucial areas such as transcriptional regulation, translation, and cellular signal transduction.[9] Unstructured segments are well suited for protein interactions controlled by PTMs. For example, sites of tyrosine phosphorylation are typically unstructured and therefore accessible for modification, but after phosphorylation they become ordered upon phosphorylation-dependent binding to a partner protein.

Genomic analysis indicates that a large majority of known protein sequences arise from new combinations of various domains, and consequently more than 70% of all sequences can be at least partially modeled from known structures with homologous domains,[10] thanks to a large and growing database of experimentally determined macromolecular structures available in the *Protein Data Bank*.[11] This database of known structures has also fueled advances in our ability to computationally predict unknown protein folds.[12]

Although most new proteins arise from gene duplications or reshuffling of existing protein-encoding elements, this is not always the case; at least one-tenth may have arisen de novo from noncoding region of the genome.[13] Only a few percent of the human genome actually encodes functional genes. Vast regions of DNA that do not encode genes or regulate their expression may constitute a reservoir for creation of new protein-encoding genes.

Most proteins are composed of multiple domains, which may confer multiple functions, couple a targeting function to a catalytic function, or provide for allosteric regulation. In the following sections we highlight the structure of a few proteins and domains that are of central and recurring importance in hematology, to illustrate the relationship between domain architecture and function. We discuss representative examples from the extracellular space (the Ig domain), intracellular signaling (protein kinase domain), and the cell membrane (G protein–coupled receptors [GPCRs] and the vitamin K receptor).

The Immunoglobulin Domain and Variations

As implied by its name, the Ig domain was first recognized in antibodies.[14] A detailed discussion on antibody biology can be found in the Chapters 22 and 23. The human genome project has identified the Ig superfamily (IgSF) as the largest superfamily in the human genome, due to its extensive usage in the immune system.[15] Although Ig-like domains also exist in a few intracellular proteins, they are found predominately in the extracellular space and are the most abundant structural unit found in cell surface receptors, serving key recognition functions in both the immune and nervous systems. Along with a handful of other modular domains such as fibronectin type III domains and epidermal growth factor (EGF) domains, they form modular structures of most receptor molecules on the cell surface.

An Ig domain is composed of approximately 100 residues, folding into two β-sheets packing face-to-face, forming a β-barrel. This distinctively folded structure is commonly known as the Ig fold (Fig. 7.3A). An intact IgG antibody consists of two heavy chains and two light chains. Each heavy chain contains four Ig domains, one "variable" domain and three "constant" domains, and each light chain contains two Ig domains, one variable and one constant. The variable and constant Ig domains differ somewhat in structure and

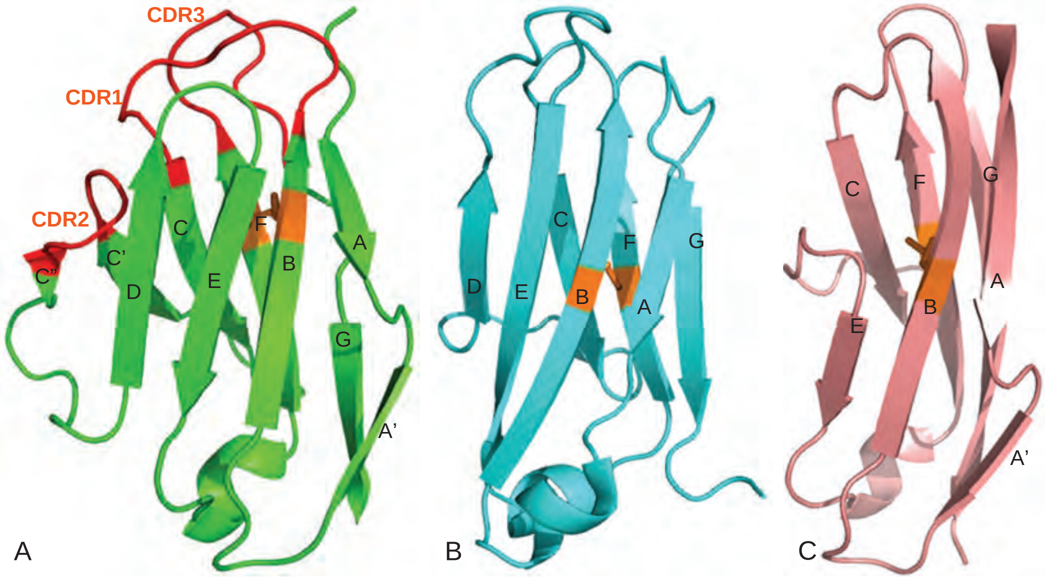


Figure 7.3 IMMUNOGLOBULIN (IG) DOMAIN ARCHITECTURE. (A) V-set Ig domain (PDB entry 3IDG). The complementarity determining regions *(CDR)* are colored *red*. (B) C-set Ig domain (PDB entry 3IDG). (C) I-set Ig domain, which can be described as a truncated V-set domain (PDB entry 2V5M). Intersheet disulfide bonds are highlighted *orange*. *PDB*, Protein Data Bank.

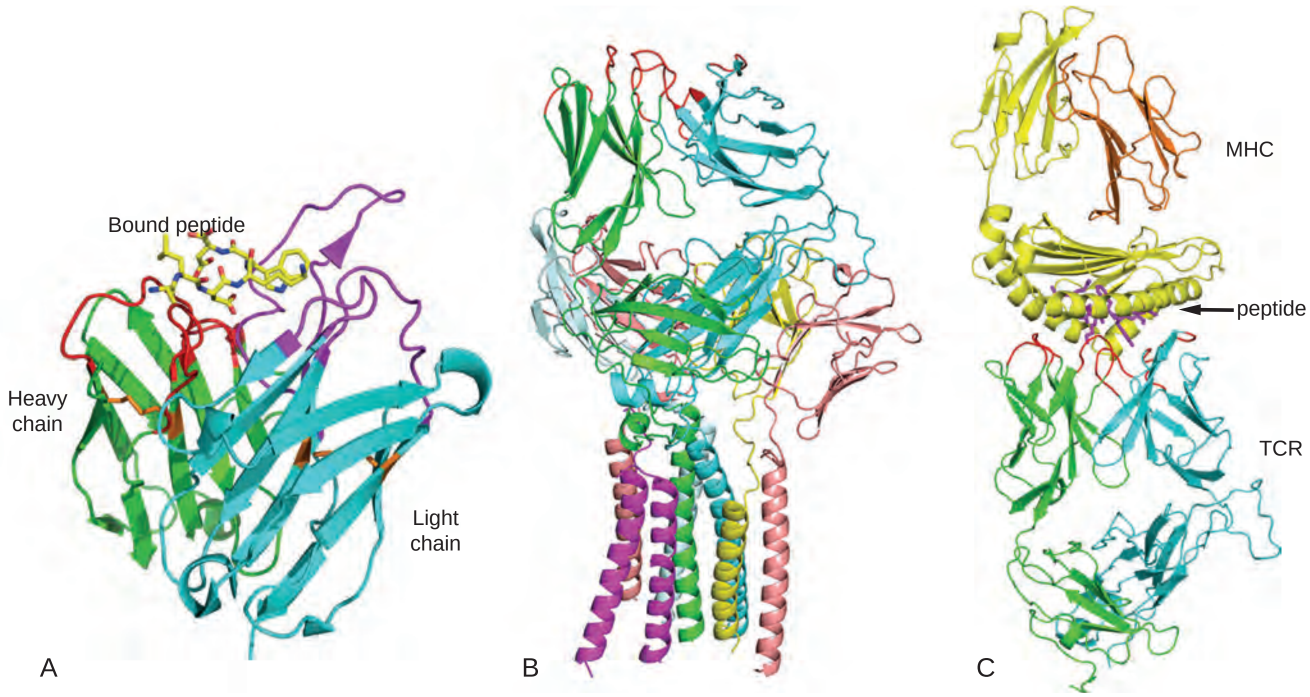


Figure 7.4 (A) Structure of a human immunodeficiency virus–neutralizing antibody in complex with an antigenic peptide. Complementarity determining regions (CDRs) of the heavy and light chains are shown in *red* and *magenta*, respectively. Only the two variable domains of the antibody are shown (drawn from PDB entry 3IDG). (B) Cryoelectron microscopy structure of a complete T-cell receptor (TCR)-CD3 complex. The TCR's α- and β-chains are shown in *green* and *cyan*, respectively, with their CDR loops colored *red*. The CD3ε chains are *pink*, CD3δ *light blue*, CD3γ *yellow*, and CD3ζ *magenta*. (C) Structure of an antigenic peptide bound to a major histocompatibility complex (MHC) molecule in complex with TCR (drawn from PDB entry 2CKB). The TCR is shown in a similar orientation and with the same coloring as in panel **B**. MHC heavy chain is in *yellow* and β_2-microglobulin domain in *orange*. The peptide antigen is shown in *magenta*. *PDB*, Protein Data Bank.

are correspondingly classified as V-set and C-set Ig folds. A V-set Ig domain has β-strands A, B, E, and D on one sheet and A′, G, F, C, C′ and C″ strands on the other (see Fig. 7.3A), whereas a C-set Ig domain lacks A′, C′ and C″ strands on either edge (Fig. 7.3B). In both, the two sheets are linked together by a conserved disulfide bond between the B and F strands (reviewed in Williams et al.[16]). Within variable domains, hypervariable sequences are found in three connecting loops at one end of the domain. These loops are termed complementarity determining regions (CDRs) (see Fig. 7.3A). In the intact antibody, the CDRs of the heavy and light chains combine to make up the antigen-binding site. Fig. 7.4A depicts how the CDRs of a human immunodeficiency virus (HIV)-neutralizing antibody form an antigen-binding pocket that recognizes an antigenic peptide from an HIV surface protein.[17]

A similar structural platform is used in cellular immunity by T-cell receptors (TCRs), which, distinct from antibodies, recognize an antigenic peptide along with the major histocompatibility complex (MHC) molecule that presents the peptide on surface of an infected or cancerous cell. The complete TCR is a membrane-spanning complex with CD3 that contains a total of eight Ig domains—two in each of the TCR α- and β-chains, and one in each of the dimeric CD3γε and CD3δε subunits. The CD3ζζ subunit, which contains only transmembrane helices and cytoplasmic tails, completes the receptor. Recent advances in cryoelectron microscopy (cryo-EM) have allowed elucidation of the structure of the intact TCR-CD3 complex,[18] revealing an overall architecture in which the extracellular "bouquet" of Ig domains is arranged on "stems" of eight transmembrane helices (Fig. 7.4B). Within this complex the CDR3 loops of the TCR α and β variable domains play a key role in antigen recognition, whereas germline-encoded CDR1 and CDR2 loops of the TCR are responsible for contacting the polymorphic region of the MHC molecule, with CDR1 also taking part in peptide binding.[19,20] Fig. 7.4C illustrates a typical crystal structure of a TCR in complex with an antigenic peptide bound to the MHC molecule. An extensive discussion on the role of these proteins in cellular immunity can be found in Chapter 24.

A number of variations on the Ig fold are found in other cell surface receptors. These Ig-like domains include the topologically similar fibronectin type III domains and the domains of cadherins, both of which lack the disulfide bridge found in the canonical Ig domain. Further variations are found in modular cell surface receptors, which often have a V-set Ig-like domain at their N-terminus, positioned to extend from the plasma membrane for ligand-binding, serving a role analogous to antigen-recognition. By contrast, "I-set" Ig-like domains (see Fig. 7.3C) usually function as one of the building blocks lined up in tandem to present the ligand-binding V-set domain on the cell surface. This can be seen in many immune receptors such as CD2, CD58,[21] and CD4.[22] There are also many receptors that are exclusively composed of I-set domains, including immune receptor intercellular adhesion molecule-1 (ICAM-1)[23] and neuroreceptors NCAM[24] and Dscam.[25] Thus the I-set variant is the most abundant Ig-like domain and plays a critical biologic role in cell surface receptors.

The Protein Kinase Domain

Protein kinases catalyze the transfer of a phosphate group from adenosine triphosphate (ATP) to specific sites on target proteins. More than 500 protein kinases have been identified in the human genome; approximately 90 of these are tyrosine kinases, the remainder specifically phosphorylate serine or threonine residues. Both serine/threonine and tyrosine kinases share a conserved bilobed protein fold, composed of a smaller N-terminal subdomain (N-lobe) and larger C-terminal subdomain (C-lobe).[26] The active site cleft, including the site for binding the substrate ATP, is found at the interface between the N- and C-lobes. The phosphate-coordinating "P-loop"

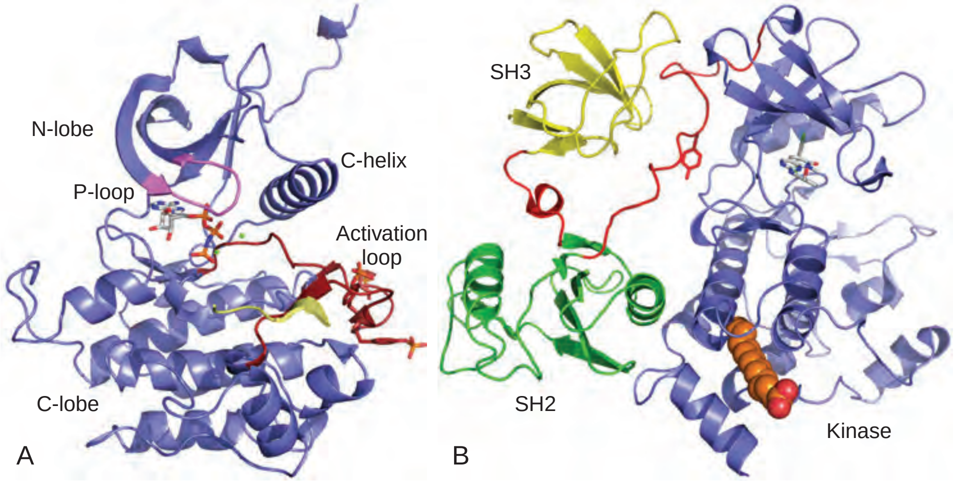


Figure 7.5 (A) A kinase domain in complex with an adenosine triphosphate (ATP) analog and peptide substrate (PDB entry 1IR3). The phosphate-binding loop is highlighted *purple*, the activation loop is *red*, the substrate peptide is *yellow*, and the ATP analog is shown in *gray*. (B) The autoinhibited structure of Abelson tyrosine kinase (c-ABL) in complex with the kinase inhibitor PD166326 (PDB entry 1OPK). The Src homology-3 (SH3), SH2, and kinase domains are shown in *yellow*, *green*, and *blue*, respectively. The SH2–kinase domain linker and the SH3-SH2 connector are shown in *red*. The myristate is shown in *orange* spheres in the C-lobe of the kinase. *PDB*, Protein Data Bank.

is a portion of the β-sheet in the N-lobe that coordinates the triphosphate moiety of ATP. The activity of protein kinases is often regulated by phosphorylation on a loop in the C-lobe termed the activation loop or A-loop. In the absence of phosphorylation, the A-loop may play an inhibitory role, sometimes blocking binding of ATP in the active site, or it may be disordered altogether. Upon autophosphorylation, or phosphorylation in trans by an upstream activating kinase, the activation loop rearranges to adopt a characteristic hairpin conformation that creates the site for docking of the polypeptide segment that will become phosphorylated. Activation loop phosphorylation may also induce other structural rearrangements required for catalytic activation, in particular a reorientation of a helix within the N-lobe (known as the C-helix) that brings a glutamic acid residue into proper position within the active site (Fig. 7.5A).

Deregulated tyrosine kinases are the cause of a number of hematologic malignancies. Two general classes of tyrosine kinases can be defined: receptor and non-receptor tyrosine kinases. Receptor tyrosine kinases are transmembrane proteins with an extracellular ligand–binding domain, a single transmembrane domain, and the cytoplasmic tyrosine kinase domain. They are typically activated by dimerization upon binding of ligands to their extracellular region, which induces autophosphorylation and activation of their catalytic domains inside the cell.[27] Chromosomal translocations that underlie a number of human leukemias fuse a tyrosine kinase domain to an oligomerization domain from an otherwise unrelated protein, often the dimerization domain of a transcription factor, to generate a constitutively dimeric, and therefore constitutively active kinase. Examples of such oncogenic translocations include the fusion of the dimerization domain of an ETS-family transcription factor ETV6 (also called Tel) to a Jak-family tyrosine kinase in the leukemogenic ETV6-Jak2 fusion,[28] and the fusion of the oligomerization domain of nucleophosmin with the tyrosine kinase domain of ALK in the NPM-ALK fusion in anaplastic large cell lymphoma.[29] These translocations are further described in Chapters 57 and 78, respectively.

Perhaps the best characterized kinase translocation is the BCR-ABL fusion protein produced by the (9:22) chromosomal translocation in chronic myelogenous leukemia (see also Chapter 69). Treatment of this disease with imatinib, a specific inhibitor of ABL, has established a paradigm for targeted therapy in cancer.[30] ABL is a non–receptor tyrosine kinase which contains Src-homology 3 and 2 (SH3 and SH2) domains in addition to its tyrosine kinase domain. In addition, the normal ABL protein is myristoylated at its N-terminus. In the normal protein, the N-terminal region including the myristoyl-group and adjacent sequences and the SH3 and SH2 domains assemble with the kinase domain to lock it in an inactive conformation (Fig. 7.5B).[31] These interactions are released to activate the kinase when the phosphotyrosine-binding SH2 domain and proline motif-binding SH3 domains bind their cognate ligands in a target protein.[32] The myristoyl group may also be released from its docking site in the C-lobe of the kinase upon activation to promote membrane localization of the protein. Thus in its normal state, the various domains of ABL comprise an exquisite signaling switch that is regulated by appropriate binding interactions; in the absence of the proper targeting interactions, the kinase is maintained in an inactive state by the intramolecular associations of its domains. In the oncogenic BCR-ABL fusion protein, this regulatory control is lost because the N-terminal regulatory region including the myristoylation site is truncated and replaced with unrelated sequences from the BCR protein. Interestingly, the vacant myristate pocket in BCR-ABL is the target of recently developed allosteric inhibitors of BCR-ABL, which may synergize with ATP-site inhibitors.[33]

High-resolution structural information has facilitated development of selective inhibitors for many tyrosine kinases in addition to BCR-ABL. Prominent examples include ruxolitinib, a Jak1/Jak2 inhibitor that is used to treat myelofibrosis driven by JAK2^{V617F}, and ibrutinib, a Bruton tyrosine kinase (BTK) inhibitor that is used in the treatment of chronic lymphocytic leukemia, mantle cell lymphoma, and Waldenström macroglobulinemia.[34,35] These and other kinase inhibitors are discussed in the chapters covering specific hematologic malignancies.

Membrane Proteins

Membrane proteins account for 20% to 30% of all gene products in most genomes, and they are the targets of 50% of modern drugs.[36] Proteins embedded in or traversing the lipid bilayer mediate exchange of information and materials across membrane barriers. They are architecturally and functionally diverse. Single-pass transmembrane proteins have functional extracellular and/or intracellular domains connected by a single membrane-spanning helix. By contrast, integral membrane proteins typically have much of their mass embedded within the lipid bilayer, with multiple membrane-spanning segments connected by cytoplasmic and extracellular loops. Historically, integral membrane proteins have been difficult to study at a structural level. However, innovations in membrane protein crystallization and protein engineering have made such studies more tractable, allowing elucidation of many important structures at near-atomic resolution.

Most membrane-embedded proteins are predominately helical, although β-strand membrane proteins also occur. Diverse ion channels and GPCRs are integral membrane proteins. One of the largest and most complicated membrane protein complexes characterized to date is that of mitochondrial complex I. This huge proton-pumping machine features 82 transmembrane helices, accounting for approximately half of its molecular mass.[37]

GPCRs are the largest family of membrane proteins—more than 800 have been identified in the human genome. GPCRs mediate fundamental signal transduction processes, touching virtually every aspect of human physiology, from vision, taste, and smell to cardiovascular, endocrine, immunologic, and reproductive functions. Not surprisingly, they represent an important class of drug target. The conserved domain structure of GPCRs includes seven transmembrane helices that pack together across the lipid bilayer. They form a ligand-binding cleft that opens to the extracellular space. The cleft can vary dramatically in size and shape in different GPCRs because some receptors recognize small molecules (e.g., the β_2-adrenergic receptor) whereas others have protein ligands (e.g., chemokine receptors).

Structural studies of the β_2-adrenergic receptor have revealed its mechanism of transmembrane signal transduction via the heterotrimeric G protein $G\alpha_s\beta\gamma$ (Fig. 7.6A).[38] Binding of agonist in the extracellular-facing cleft induces key conformational changes in the cytoplasmic region, in particular a large movement of the sixth transmembrane helix and an extension of the cytoplasmic end of the fifth transmembrane helix (TM5). These alterations promote binding to the Gα-subunit of $G\alpha_s\beta\gamma$. In binding Gα, the agonist-bound receptor functions as a guanine-nucleotide exchange factor (GEF), inducing exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP). GTP-bound Gα dissociates from the βγ heterodimer to activate adenyl cyclase, while the free βγ component signals to Ca^{2+} channels. The inactive and active β_2-adrenergic receptor structures are illustrated in Fig. 7.6A. Currently, at least 70 unique GPCR structures are available, providing substantial impact in drug development.[39]

Membrane proteins can also fulfill catalytic roles, and they operate not only in the plasma membrane but in every lipid membrane in the cell. One example of interest is vitamin K epoxide reductase (VKOR), the target of the anticoagulant drug warfarin (see Chapters 125 and 143). VKOR resides in the endoplasmic reticulum (ER) membrane and catalyzes a key step in the vitamin K cycle—regeneration of vitamin K hydroquinone. This compound is a cofactor for the enzyme that converts glutamic acid residues in the N-termini of vitamin K–dependent clotting factors to γ-carboxy glutamate (Gla) residues. This PTM is required for interaction of these proteins with Ca^{2+} and thus for their function in coagulation. Although the structure of the human enzyme has not been elucidated, the structure of a homologous bacterial protein has been described.[40] In this crystal structure (Fig. 7.6B), the bacterial homolog of VKOR is naturally fused to a thioredoxin (Trx)-like domain, which supplies reducing equivalents. The core of VKOR structure is a four-helix bundle embedded in the membrane (transmembrane helices TM1-TM4, shown in green) with a TM5 linked to the Trx-like domain on the extracellular surface (topologically equivalent to the luminal side of ER-resident mammalian VKOR). The ubiquinone compound has its quinone ring located near the membrane surface with its isoprenyl tail intercalated into the V-shaped cleft between TM2 and TM3. All the enzymatically important residues are on or close to the extracellular side of

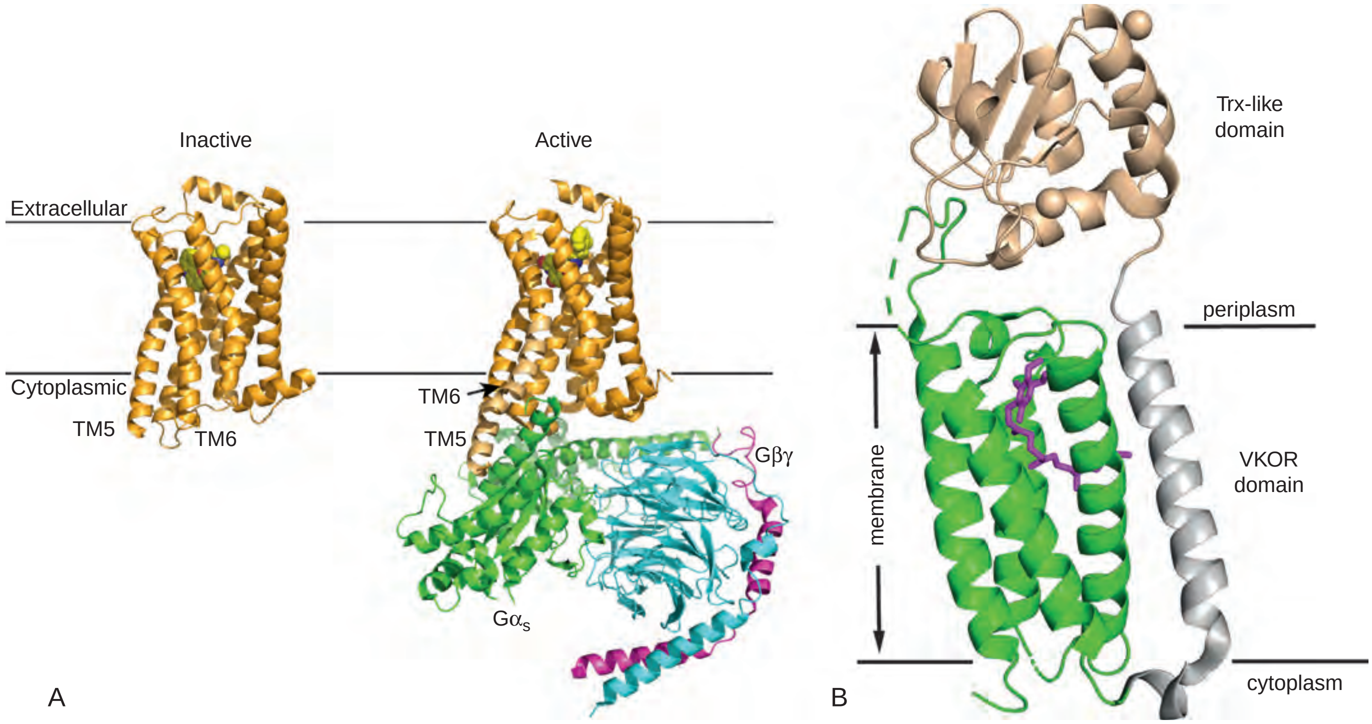


Figure 7.6 MEMBRANE PROTEIN STRUCTURES. (A) Structures of the β_2 adrenergic receptor (β_2AR) in the inactive *(left)* and active *(right)* conformations. Like all G protein–coupled receptors, β_2AR contains seven transmembrane helices. The inactive structure was determined with the antagonist carazolol bound in the ligand-binding site, while the active structure was determined in complex with the tight-binding agonist BI-167107 (ligand are shown as *yellow spheres*). Agonist binding induces conformational changes on the cytoplasmic face of the receptor, including reorientation of the sixth transmembrane helix (TM6) and lengthening of TM5, which in turn promote binding of the heterotrimeric Gαβγ complex. Interactions with the receptor are mediated by the $G\alpha_s$ subunit *(green)*. This interaction induces exchange of guanosine diphosphate for guanosine triphosphate in the $G\alpha_s$ subunit, and promotes dissociation of βγ complex (shown in *cyan* and *magenta* for β and γ, respectively). Illustration is drawn from PDB entries 2RH1 and 3SN6 for the inactive and active structures, respectively. T4 lysozyme fusion partners and a nanobody that was engineered to facilitate crystallization are not illustrated. (B) Structure of a bacterial homolog of the human vitamin K epoxide reductase (VKOR). VKOR is expected to bind vitamin K in a pocket homologous to that formed by four conserved transmembrane helices *(green)* that is occupied by a ubiquinone *(magenta)* in this structure. Warfarin inhibits VKOR by displacing vitamin K from this pocket. See text for further details. Illustration drawn from PDB entry 3KP9. *PDB*, Protein Data Bank.

the membrane, adjacent to the Trx-like domain, providing a plausible path for electron transfer.[40] Interestingly, mapping of mutations that confer resistance to warfarin onto the bacterial structure reveals striking clustering around the ligand-binding pocket, confirming that it is also the site of warfarin binding and that warfarin exerts its inhibitory effect by displacing vitamin K.[40]

FUTURE DIRECTIONS

In this chapter, we have reviewed basic principles of protein structure and introduced a few representative protein folds that recur in proteins of key importance in hematology. Further examples are found throughout the text, reflecting the value of understanding the structural foundations of biologic processes. Structure is key for understanding macromolecular function at a mechanistic level and, by extension, for understanding pathogenic mutations and mechanisms of drug action. Advances in cryo-EM over the past decade promise to bring ever-larger and more complicated proteins and macromolecular complexes into focus.[41] In addition to the TCR complex discussed earlier,[18] single particle cryo-EM has already revealed the molecular architecture of many macromolecular assemblies including the splicesome[42] and the inflammasome.[43] A remarkable time-resolved cryo-EM study of the elongating ribosome yielded 33 distinct states that map the aminoacyl–transfer RNA delivery and proofreading process.[44] Although single-particle studies such as these are carried out with purified macromolecular complexes, cryoelectron *tomography* can provide snapshots of cellular structures in situ at sub-nanometer resolution.[45] A recent application of this technique has revealed the architecture of mammalian primary cilia.[46] In the coming years, we can look forward to an avalanche of new structural and mechanistic insights fueled by these approaches.

Recent advances in computational protein structure prediction promise to provide actionable insights into the structure of proteins for which experimentally determined structures are not available. A remarkably powerful and accurate protein folding engine has been created by combining genetic information (evolutionary covariation derived from multiple sequence alignments of a target of interest) with machine learning tools.[47] Finally, these and other computational tools[48] have enabled de novo protein design.[49] It is now possible to design an entirely novel protein domain with atomic level accuracy and to engineer it to have specific functional properties, such as self-assembly into a higher-order complex or ligand recognition. In the not-too-distant future, we can expect these approaches to open new therapeutic modalities.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Barreca A, Lasorsa E, Riera L, et al. Anaplastic lymphoma kinase in human cancer. *J Mol Endocrinol*. 2011;47:R11–23.
Burley SK, Berman HM, Christie C, et al. RCSB Protein Data Bank: sustaining a living digital data resource that enables breakthroughs in scientific research and biomedical education. *Protein Sci*. 2018;27:316–330.
Cheng Y. Single-particle cryo-EM—how did it get here and where will it go. *Science*. 2018;361:876–880.
Congreve M, de Graaf C, Swain NA, Tate CG. Impact of GPCR structures on drug discovery. *Cell*. 2020;181:81–91.
Cunningham BA, Hemperly JJ, Murray BA, et al. Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science*. 1987;236:799–806.
Das R, Baker D. Macromolecular modeling with rosetta. *Annu Rev Biochem*. 2008;77:363–382.
Dong D, Zheng L, Lin J, et al. Structural basis of assembly of the human T cell receptor-CD3 complex. *Nature*. 2019;573:546–552.
Druker BJ. Translation of the Philadelphia chromosome into therapy for CML. *Blood*. 2008;112:4808–4817.
Dyson HJ, Wright PE. Intrinsically unstructured proteins and their functions. *Nat Rev Mol Cell Biol*. 2005;6:197–208.
Golub TR, McLean T, Stegmaier K, et al. The TEL gene and human leukemia. *Biochim Biophys Acta*. 1996;1288:M7–10.
Huang PS, Boyken SE, Baker D. The coming of age of de novo protein design. *Nature*. 2016;537:320–327.
Kiesel P, Alvarez Viar G, Tsoy N, et al. The molecular structure of mammalian primary cilia revealed by cryo-electron tomography. *Nat Struct Mol Biol*. 2020;27:1115–1124.
Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. *Nature*. 2001;409:860–921.
Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell*. 2010;141:1117–1134.
Levitt M. Nature of the protein universe. *Proc Natl Acad Sci U S A*. 2009;106:11079–11084.
Li W, Schulman S, Dutton RJ, et al. Structure of a bacterial homologue of vitamin K epoxide reductase. *Nature*. 2010;463:507–512.
Loveland AB, Demo G, Korostelev AA. Cryo-EM of elongating ribosome with EF-Tu•GTP elucidates tRNA proofreading. *Nature*. 2020;584:640–645.
Meijers R, Puettmann-Holgado R, Skiniotis G, et al. Structural basis of Dscam isoform specificity. *Nature*. 2007;449:487–491.
Nagar B, Hantschel O, Young MA, et al. Structural basis for the autoinhibition of c-Abl tyrosine kinase. *Cell*. 2003;112:859–871.
Oh E, Akopian D, Rape M. Principles of Ubiquitin-Dependent Signaling. *Annu Rev Cell Dev Biol*. 2018;34:137–162.
Oldfield CJ, Dunker AK. Intrinsically disordered proteins and intrinsically disordered protein regions. *Annu Rev Biochem*. 2014;83:553–584.
Rasmussen SG, DeVree BT, Zou Y, et al. Crystal structure of the β2 adrenergic receptor-Gs protein complex. *Nature*. 2011;477:549–555.
Rudolph MG, Stanfield RL, Wilson IA. How TCRs bind MHCs, peptides, and coreceptors. *Annu Rev Immunol*. 2006;24:419–466.
Senior AW, Evans R, Jumper J, et al. Improved protein structure prediction using potentials from deep learning. *Nature*. 2020;577:706–710.
Sharif H, Wang L, Wang WL, et al. Structural mechanism for NEK7-licensed activation of NLRP3 inflammasome. *Nature*. 2019;570:338–343.
Turk M, Baumeister W. The promise and the challenges of cryo-electron tomography. *FEBS Lett*. 2020;594:3243–3261.
Wang JH, Reinherz EL. The structural basis of αβ T-lineage immune recognition: TCR docking topologies, mechanotransduction, and co-receptor function. *Immunol Rev*. 2012;250:102–119.
Wylie AA, Schoepfer J, Jahnke W, et al. The allosteric inhibitor ABL001 enables dual targeting of BCR-ABL1. *Nature*. 2017;543:733–737.
Yan C, Hang J, Wan R, et al. Structure of a yeast spliceosome at 3.6-angstrom resolution. *Science*. 2015;349:1182–1191.
Zickermann V, Wirth C, Nasiri H, et al. Structural biology. Mechanistic insight from the crystal structure of mitochondrial complex I. *Science*. 2015;347:44–49.

REFERENCES

1. Walsh C. *Posttranslational Modification of Proteins: Expanding Nature's Inventory*. Englewood, CO: Roberts and Co. Publishers; 2006.
2. Glozak MA, Sengupta N, Zhang X, Seto E. Acetylation and deacetylation of non-histone proteins. *Gene*. 2005;363:15–23. https://doi.org/10.1016/j.gene.2005.09.010.
3. Oh E, Akopian D, Rape M. Principles of ubiquitin-dependent signaling. *Annu Rev Cell Dev Biol*. 2018;34:137–162. https://doi.org/10.1146/annurev-cellbio-100617-062802.
4. Richardson JS. The anatomy and taxonomy of protein structure. *Adv Protein Chem*. 1981;34:167–339.
5. Kolb VA, Makeyev EV, Spirin AS. Co-translational folding of an eukaryotic multidomain protein in a prokaryotic translation system. *J Biol Chem*. 2000;275:16597–16601.
6. Gilbert W. Why genes in pieces? *Nature*. 1978;271:501.
7. Liu M, Grigoriev A. Protein domains correlate strongly with exons in multiple eukaryotic genomes—evidence of exon shuffling? *Trends Genet*. 2004;20:399–403.
8. Oldfield CJ, Dunker AK. Intrinsically disordered proteins and intrinsically disordered protein regions. *Annu Rev Biochem*. 2014;83:553–584. https://doi.org/10.1146/annurev-biochem-072711-164947.
9. Dyson HJ, Wright PE. Intrinsically unstructured proteins and their functions. *Nat Rev Molec Cell Biol*. 2005;6:197–208. https://doi.org/10.1038/nrm1589.
10. Levitt M. Nature of the protein universe. *Proc Natl Acad Sci U S A*. 2009;106:11079–11084.
11. Burley SK, Berman HM, Christie C, et al. RCSB Protein Data Bank: sustaining a living digital data resource that enables breakthroughs in scientific research and biomedical education. *Protein Sci*. 2018;27:316–330. https://doi.org/10.1002/pro.3331.
12. Das R, Baker D. Macromolecular modeling with Rosetta. *Annu Rev Biochem*. 2008;77:363–382. https://doi.org/10.1146/annurev.biochem.77.062906.171838.
13. Levy A. How evolution builds genes from scratch. *Nature*. 2019;574:314–316. https://doi.org/10.1038/d41586-019-03061-x.
14. Bork P, Holm L, Sander C. The immunoglobulin fold. Structural classification, sequence patterns and common core. *J Mol Biol*. 1994;242:309–320.
15. Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. *Nature*. 2001;409:860–921. https://doi.org/10.1038/35057062.
16. Williams AF, Davis SJ, He Q, Barclay AN. Structural diversity in domains of the immunoglobulin superfamily. *Cold Spring Harb Symp Quant Biol*. 1989;54(Pt 2):637–647.
17. Zwick MB, Kiyomi Komori H, Stanfield RL, et al. The long third complementarity-determining region of the heavy chain is important in the activity of the broadly neutralizing anti-human immunodeficiency virus type 1 antibody 2F5. *J Virol*. 2004;78:3155–3161.
18. Dong D, Zheng L, Lin J, et al. Structural basis of assembly of the human T cell receptor-CD3 complex. *Nature*. 2019;573:546–552. https://doi.org/10.1038/s41586-019-1537-0.
19. Wang JH, Reinherz EL. The structural basis of αβ T-lineage immune recognition: TCR docking topologies, mechanotransduction, and co-receptor function. *Immunol Rev*. 2012;250:102–119. https://doi.org/10.1111/j.1600-065X.2012.01161.x.
20. Rudolph MG, Stanfield RL, Wilson IA. How TCRs bind MHCs, peptides, and coreceptors. *Annu Rev Immunol*. 2006;24:419–466.
21. Wang J-H, Smolyar A, Tan K, et al. Structure of a heterophilic adhesion complex between human CD2 and CD58 (LFA-3) counter-receptors. *Cell*. 1999;97:791–803.
22. Wu H, Kwong PD, Hendrickson WA. Dimeric association and segmental variability in the structure of human CD4. *Nature*. 1997;387:527–530.
23. Yang Y, Chang-Duk J, Liu J-H, et al. Structural basis for dimerization of ICAM-1 on the cell surface. *Mol Cell*. 2004;14:269–276.
24. Cunningham BA, Hemperly JJ, Murray BA, et al. Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science*. 1987;236:799–806.
25. Meijers R, Puettmann-Holgado R, Skiniotis G, et al. Structural basis of Dscam isoform specificity. *Nature*. 2007;449:487–491.
26. Taylor SS, Kornev AP. Protein kinases: evolution of dynamic regulatory proteins. *Trends Biochem Sci*. 2011;36:65–77.
27. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell*. 2010;141:1117–1134.
28. Golub TR, McLean T, Stegmaier K, et al. The TEL gene and human leukemia. *Biochim Biophys Acta*. 1996;1288:M7–10.
29. Barreca A, Lasorsa E, Riera L, et al. Anaplastic lymphoma kinase in human cancer. *J Mol Endocrinol*. 2011;47:R11–23.
30. Druker BJ. Translation of the Philadelphia chromosome into therapy for CML. *Blood*. 2008;112:4808–4817.
31. Nagar B, Hantschel O, Young MA, et al. Structural basis for the autoinhibition of c-Abl tyrosine kinase. *Cell*. 2003;112:859–871.
32. Nagar B, Hantschel O, Seeliger M, et al. Organization of the SH3-SH2 unit in active and inactive forms of the c-Abl tyrosine kinase. *Mol Cell*. 2006;21:787–798.
33. Wylie AA, Schoepfer J, Jahnke W, et al. The allosteric inhibitor ABL001 enables dual targeting of BCR-ABL1. *Nature*. 2017;543:733–737. https://doi.org/10.1038/nature21702.
34. Hobbs GS, Rozelle S, Mullally A. The development and use of Janus kinase 2 inhibitors for the treatment of myeloproliferative neoplasms. *Hematol Oncol Clin North Am*. 2017;31:613–626. https://doi.org/10.1016/j.hoc.2017.04.002.
35. Wen T, Wang J, Shi Y, Qian H, Liu P. Inhibitors targeting Bruton's tyrosine kinase in cancers: drug development advances. *Leukemia*. 2020;35:312–332. https://doi.org/10.1038/s41375-020-01072-6.
36. Overington JP, Al-Lazikani B, Hopkins AL. How many drug targets are there? *Nat Rev Drug Discov*. 2006;5:993–996. https://doi.org/10.1038/nrd2199.
37. Zickermann V, Wirth C, Nasiri H, et al. Structural biology. Mechanistic insight from the crystal structure of mitochondrial complex I. *Science*. 2015;347:44–49. https://doi.org/10.1126/science.1259859.
38. Rasmussen SG, DeVree BT, Zou Y, et al. Crystal structure of the β2 adrenergic receptor-Gs protein complex. *Nature*. 2011;477:549–555. https://doi.org/10.1038/nature10361.
39. Congreve M, de Graaf C, Swain NA, Tate CG. Impact of GPCR structures on drug discovery. *Cell*. 2020;181:81–91. https://doi.org/10.1016/j.cell.2020.03.003.
40. Li W, Schulman S, Dutton RJ, et al. Structure of a bacterial homologue of vitamin K epoxide reductase. *Nature*. 2010;463:507–512. https://doi.org/10.1038/nature08720.
41. Cheng Y. Single-particle cryo-EM—how did it get here and where will it go. *Science*. 2018;361:876–880. https://doi.org/10.1126/science.aat4346.
42. Yan C, Hang J, Wan R, et al. Structure of a yeast spliceosome at 3.6-angstrom resolution. *Science*. 2015;349:1182–1191. https://doi.org/10.1126/science.aac7629.
43. Sharif H, Wang L, Wang WL, et al. Structural mechanism for NEK7-licensed activation of NLRP3 inflammasome. *Nature*. 2019;570:338–343. https://doi.org/10.1038/s41586-019-1295-z.
44. Loveland AB, Demo G, Korostelev AA. Cryo-EM of elongating ribosome with EF-Tu•GTP elucidates tRNA proofreading. *Nature*. 2020;584:640–645. https://doi.org/10.1038/s41586-020-2447-x.
45. Turk M, Baumeister W. The promise and the challenges of cryo-electron tomography. *FEBS Lett*. 2020;594:3243–3261. https://doi.org/10.1002/1873-3468.13948.
46. Kiesel P, Alvarez Viar G, Tsoy N, et al. The molecular structure of mammalian primary cilia revealed by cryo-electron tomography. *Nat Struct Mol Biol*. 2020;27:1115–1124. https://doi.org/10.1038/s41594-020-0507-4.
47. Senior AW, Evans R, Jumper J, et al. Improved protein structure prediction using potentials from deep learning. *Nature*. 2020;577:706–710. https://doi.org/10.1038/s41586-019-1923-7.
48. Leman JK, Weitzner BD, Bonneau R, et al. Macromolecular modeling and design in Rosetta: recent methods and frameworks. *Nat Methods*. 2020;17:665–680. https://doi.org/10.1038/s41592-020-0848-2.
49. Huang PS, Boyken SE, Baker D. The coming of age of de novo protein design. *Nature*. 2016;537:320–327. https://doi.org/10.1038/nature19946.

CHAPTER 8

PHARMACOGENOMICS AND HEMATOLOGIC DISEASES

Leo Kager and William E. Evans

It is widely recognized that heritable genetic variation (i.e., genotypes or haplotypes) can translate into inherited phenotypes, some of which predispose to or cause diseases and others alter response to treatment. One aim of medical genetics and pharmacogenomics (PGx) is to understand the myriad associations between inherited genotypes and specific phenotypes of disease or drug response, with the ultimate goal of better defining the risk for, or outcome of, diseases and the response to specific medications. In cancer, disease prognosis and treatment response can be affected by both inherited (germline) and acquired (somatic) genome variation, and both types of genome variation have been shown to alter the effects of certain medications. Many seminal discoveries in medical genetics were made in the course of investigating hematologic disorders, with hemoglobinopathies among the most prevalent monogenic disorders, affecting approximately 7% of the world's population. PGx also has a long tradition in hematology; one of the first documented clinical observations of inherited differences in drug effects was the relationship between hemolysis after antimalarial therapy and the inherited glucose-6-phosphate dehydrogenase (G6PD) activity in erythrocytes.[1]

In the pregenomic era, efforts concentrated on mapping highly penetrant monogenic (mendelian) loci for both specific diseases and drug-metabolizing pathways that influence the effects of medications. Completion of the Human Genome Project and the development of arrays for genome-wide single-nucleotide polymorphism (SNP) and DNA methylation analyses, "next-generation" DNA (whole-exome sequencing [WES; coding regions only] and whole-genome sequencing [WGS; coding and noncoding regions]), and RNA sequencing (RNA-seq) technologies have enabled relatively inexpensive and essentially agnostic genome-wide approaches to identify genomic variants that predispose to diseases and/or modify drug responses and/or contribute to heterogeneity of monogenetic disorders and complex diseases that are polygenetic in nature.[2] More recently, single cell sequencing has allowed further resolution of the heterogeneity in genome variation in diseases such as hematologic malignancies. In addition to genome sequence variation, epigenetic differences are increasingly recognized as important for the development of diseases and contribute to differences in the pharmacologic effects of many medications, referred to as *pharmacoepigenomics*.[3] This chapter provides a brief overview of PGx and pharmacoepigenomics, using selected examples to illustrate its current and potential impact on the treatment of hematologic diseases.

VARIATION IN THE HUMAN GENOME

The genome-wide systematic identification of heritable (i.e., germline) and acquired (i.e., somatic) variants, and the functional analysis of genes, their variants, their expression, and their related products (i.e., proteins) have revolutionized the study of many diseases, the development of new medications, and the optimization of drug therapy. Genomics, transcriptomics, methylomics, and metabolomics increasingly enable more comprehensive assessments of a person's risk for acquiring a particular disease, to identify drug targets, and to explain interindividual differences in the effectiveness and toxicity of medications.[1, 2]

The Human Genome Project and subsequent projects such as the International Genome Sample Resource (IGSR) and 1000 Genomes Project (1KGP), and the WGS/Trans-Omics-Precision Medicine (TOPMed) have unveiled many types of variations within the 3.27 billion base pairs (bp) of the human haploid genome (Genome Reference Consortium Human Build 38 patch release 13 [GRCh38.p13]) (Table 8.1); the spectrum ranges from single base-pair differences to large chromosome events, such as insertions or deletions of chromosomal DNA, or structural variants (translocations and other genomic rearrangements that affect more than 50 bp of sequence). For practical purposes, the term *sequence variation* is mainly used herein. *Polymorphisms* are defined as common inherited variations in DNA sequence that are typically, although somewhat arbitrarily, defined as the least common allele having a frequency of 1% or more in the population.

SINGLE-NUCLEOTIDE POLYMORPHISMS

The most common and important inherited sequence variations are SNPs, positions in the genome where individuals have inherited a nucleotide that differs from the most common sequence ("wild-type") at the position in the genome. Many efforts are underway to catalog these variants, because a comprehensive SNP catalog offers the possibility to pinpoint important variants in which nucleotide changes alter the function or expression of a gene that influences diseases or response to medications. The main public database is the "Database of Short Genetic Variations" (dbSNP; a repository of genetic variations less than 50 bp in length) and a growing number of SNPs (currently approximately 135 million validated; dbSNP Build 150) has recently been driven largely by the 1KGP and TOPMed project (see Table 8.1).

SINGLE-NUCLEOTIDE POLYMORPHISMS AND PHENOTYPES

SNPs are present throughout the genome, in exons, introns, promoters, enhancers, and intergenic regions. To elucidate the relationship between SNPs and phenotypes of interest, initial efforts have concentrated mainly on SNPs that are likely to alter the function or expression of a gene. However, only a small portion of the identified SNPs lies within coding regions; only about half of those SNPs cause amino acid changes in expressed proteins, and only a subset of those alters the function of the encoded protein ("damaging SNPs"). SNPs that cause amino acid changes are referred to as *nonsynonymous SNPs* (nsSNPs) and are the main sequence variants underlying most of the highly penetrant inherited monogenic diseases currently known, such as hemoglobinopathies. The likelihood that nsSNPs will result in disease or functional changes in drug metabolism or transport depends on the localization and nature of the amino acid change within the encoded protein; multiple software algorithms have been developed to "predict" whether a certain amino acid change is likely to have a major or minor effect on protein function (i.e., "damaging" versus "nondamaging").

Although it is intuitively obvious that amino acid substitutions have the potential to change the function of a protein, gene expression also can be affected by SNPs positioned in regulatory sequences or intronic regions. For example, a "silent" or synonymous SNP has been identified that affects protein folding and function of an

TABLE 8.1 A Selection of Relevant Websites

Genomic Variants	**Description**	**Address**
National Human Genome Research Institute (NHGRI)	Website of the NHGRI with the aim to improve human health by genome research	http://www.genome.gov/
GenBank	NIH genetic sequence database—an annotated collection of all publicly available DNA sequences	http://www.ncbi.nlm.nih.gov/genbank/
Genome Reference Consortium (GRC)	Produce a single consensus representation of the human genome	https://www.ncbi.nlm.nih.gov/grc/human
The International Genome Sample Resource (IGSR) and 1000 Genomes Project (1KGP)	A deep catalog of human genetic variation	https://www.internationalgenome.org/
Database of Short Genomic Variations (dbSNP)	Repository of all types of short genetic variations <50 bp in length	http://www.ncbi.nlm.nih.gov/SNP/
Database of Human Genomic Structural Variation (dbVar)	Archive of large-scale genomic variants (generally >50 bp) such as insertions, deletions, translocations and inversions	http://www.ncbi.nlm.nih.gov/dbvar/
ClinVar	Archive of reports of the relationships among human variations and phenotypes	http://www.ncbi.nlm.nih.gov/clinvar/
Database of Genomic Variants (DGV)	Catalog of human genomic structural variation	http://dgv.tcag.ca/dgv/app/home
Encyclopedia of DNA Elements (ENCODE)	Project to identify all functional elements in the human genome sequence	http://www.genome.gov/10005107
Roadmap Epigenomics Project	Public resource of human epigenomic data	http://www.roadmapepigenomics.org/
GWAS Catalog NHGRI-EBI	Catalog of human GWASs	https://www.ebi.ac.uk/gwas/
Pediatric Cancer Genome Project	Decoded the complete and normal genomes of ~800 pediatric cancer patients	http://www.pediatriccancergenomeproject.org/site/
TOPmed Program	Short-read WGS data from 53,831 individuals	https://www.nhlbiwgs.org/
Pharmacology and PGx	**Description**	**Address**
Pharmacogenomics (PGx) Knowledge Base	Most comprehensive website on PGx	http://pharmgkb.org
Clinical Pharmacogenetics Implementation Consortium (CPIC)	Provides guidelines that enable the translation of genetic laboratory test results into actionable prescribing decisions for specific drugs (see Table 8.2)	https://www.pharmgkb.org/page/cpic
US Food and Drug Administration (FDA)—PGx Biomarkers	Contains a list of FDA-approved drugs with PGx information in their labeling	http://www.fda.gov/drugs/scienceresearch/researchareas/pharmacogenetics/ucm083378.htm
The miRNA PGx Database	Identifies associations of miRNAs, genes they regulate, and the drugs annotated in literature as dependent on these genes.	http://www.pharmaco-mir.org/about
Pharmacogene Variation Consortium (PharmVar)	Repository for pharmacogene variation that focuses on haplotype structure and allelic variation	https://www.pharmvar.org/
PGRN-RIKEN	Data from published GWAS of PGx research	https://www.pgrn.org/riken-gwas-statistics.html
Genetic Testing Registry (GTR)	Central location for voluntary submission of genetic test information by providers	https://www.ncbi.nlm.nih.gov/gtr/
The UCSF-FDA TransPortal	A public drug transporter database	http://dbts.ucsf.edu/fdatransportal/
The IUPHAR/BPS Guide to PHARMACOLOGY	Database with quantitative information on drug targets and their ligands	https://www.guidetopharmacology.org/about.jsp
Clinical Interpretation of Variants in Cancer (CIViC)	Community-driven web resource for clinical interpretation of variants in cancer	https://civicdb.org/home

EBI, European Bioinformatics Institute; *GWAS*, genome-wide association study; *miRNA*, microRNA; *NIH*, National Institutes of Health; *IUPHAR/BPS*, International Union of Basic and Clinical Pharmacology/British Pharmacological Society; *PGRN*, Pharmacogenomics Research Network; *TOPMed*, Trans-Omics-Precision Medicine; *UCSF*, University of California, San Francisco; *WGS*, whole-genome sequencing.

important drug transporter, namely adenosine triphosphate (ATP)–binding cassette (ABC) transporter ABCB1, and this variant has the potential to influence the intracellular accumulation of drugs that are substrates for ABCB1 (transports some drugs out of cells).[4] Moreover, SNPs in the promoter region can alter the regulatory promoter function and the gene's expression, thereby influencing drug effects. Using a genome-wide association study (GWAS), children and adults who were homozygous for the rs924607 T allele polymorphism in the promoter region of the gene encoding a centrosomal protein involved in microtubule formation *(CEP72)* were found to be significantly predisposed to vincristine-induced peripheral neuropathy, and in vitro experiments have shown that the *CEP72* promoter rs924607 T allele creates a binding site for a transcriptional repressor leading to lower expression of CEP72 messenger RNA (mRNA) and increased sensitivity of neurons and acute lymphoblastic leukemia (ALL) cells to vincristine.[5]

In addition, diverse classes of small to long noncoding RNAs (ncRNAs) have emerged as important regulators of gene expression and genome stability. For example, micro-RNAs (miRNAs) are small (19 to 22 nucleotides), single-stranded RNA molecules that can influence cellular mRNA levels or impair translation after binding to miRNA binding sites at the target gene's 3′-untranslated region. SNPs in miRNA binding sites or in the sequence encoding miRNAs have the potential to alter binding and function of miRNAs, respectively. Indeed, a so-called miRSNP, which is defined as a functional SNP that can interfere with miRNA function, had been reported to affect the expression of the antifolate target dihydrofolate reductase, thereby influencing antifolate pharmacodynamics. The miRNA PGx Database (Pharmaco-miR) is a helpful research resource to identify associations of miRNAs, genes they regulate, and the drugs annotated as being dependent on these genes (see Table 8.1).[6]

Collectively, these examples demonstrate that SNPs in functionally different genomic regions can influence drug disposition and response.

STRUCTURAL GENOMIC VARIANTS

Structural variations are balanced or unbalanced changes in DNA content and encompass alterations ranging from submicroscopic sequence variants greater than 50 bp to larger, sometimes cytogenetically visible, variants. Unbalanced DNA alterations that change the number of base pairs in comparison with a reference genome are as frequent as or even more common than SNPs and include copy number variants (CNVs) or smaller insertions/deletions (indels). Balanced variations such as inversions and translocations are less common in germline DNA but are often found as somatic genome variants in hematologic malignancies (e.g., ALL). Many efforts focus on the identification, validation, and mapping of these variants, and the major catalogs are the Database of Genomic Variants (DGV) and the Database of Human Genomic Structural Variation (dbVAR; see Table 8.1). CNVs are found in a wide spectrum of genomic regions; therefore many pharmacologically relevant genes can be affected by these variants. Indeed, CNVs have been described to influence activity of some of the most important drug-metabolizing enzymes, such as cytochrome P450 enzymes and glutathione *S*-transferases.[7]

SOMATIC GENOMIC VARIANTS

Genomic instability is a hallmark of cancer cells. Nonrandom genetic abnormalities, including aneuploidy (gains and losses of whole chromosomes) and structural rearrangements that often result in the expression of chimeric fusion genes (e.g., *BCR-ABL1*), can be found in the majority of hematologic malignancies. These acquired (somatic) genomic variations can differ significantly from inherited (germline) genomic variations and can, for example, create allele-specific copy number differences between normal host cells and cancer cells. Such differences can have pharmacologically relevant consequences. Indeed, it was shown that the cellular acquisition of additional chromosomes in leukemia cells—for example, the gain of additional chromosomes 21 in hyperdiploid ALL (more than 50 chromosomes)—can cause discordance between germline genotypes and leukemia cell phenotypes, which are important when these discordant genotypes/phenotypes influence the disposition of antileukemic agents. Moreover, somatic deletions of genes encoding proteins that regulate the stability of the DNA mismatch repair enzyme mutS homolog 2 (MSH2) have been identified in approximately 11% of children with newly diagnosed ALL. These deletions in ALL cells have been shown to cause DNA mismatch repair deficiency and increased resistance to thiopurines (TPs), representing another genomic mechanism by which leukemia cells can acquire MSH2 deficiency and mercaptopurine (MP) resistance.[8] In addition, recent comprehensive genomic analyses comparing paired diagnostic and relapsed ALL samples provide evidence for a darwinian selection process of relapse-associated somatic mutations in genes encoding key enzymes involved in antileukemic agent activation/inactivation (e.g., TP resistance due to variants in genes involved in TP inactivation [e.g., *NT5C2* or *PRPS1*], methotrexate [MTX] resistance due to variants in *FPGS* which encodes the cellular MTX activating enzyme folyl-polyglutamyl synthetase, and glucocorticoid (GC) resistance due to variants in the glucocorticoid receptors (GCRs) *NR3C1/2*, and variants in genes encoding epigenetic regulators which affect GC response [e.g., *CREBBP*, and *WHSC1*]) during disease progression.[9]

CATALOGS OF GENOMIC VARIANTS, GENOTYPING PLATFORMS, AND GENOME-WIDE ASSOCIATION STUDIES

Cataloging the pattern of genome variation in diverse populations is fundamental in understanding areas of human phenotypic diversity such as interindividual and interethnic differences in drug responses; increasingly detailed maps of human genomic variation are provided in public databases (see Table 8.1). Information from these maps has been used to design high-throughput genotyping platforms (e.g., SNP chips), thereby providing tools to interrogate the relationship between genetic variation across the human genome and important phenotypes such as disease or response to medications in a relatively unbiased (agnostic) fashion.[2]

SNP catalogs have been used in GWASs to pinpoint genes important to diseases and drug responses, and approximately 10% of published GWAS have focused on PGx. International consortia like the Pharmacogenomics Research Network–RIKEN Global Alliance (PGRN-RIKEN) were established to facilitate GWAS PGx studies,[10] and a publicly available resource—the National Human Genome Research Institute–European Bioinformatics Institute (NHGRI-EBI) GWAS Catalog—provides an updated summary of published GWASs (see Table 8.1).

Variation in the Human Epigenome

Epigenetics encompasses inherited and acquired changes in gene function that cannot be explained by alterations in sequence of nucleic acids. The epigenome is a complex layer of regulatory information that is superimposed on the genome (*epigenetics* literally means "above genetics"), with major mechanisms that contribute to epigenetic variation including DNA methylation, DNA hydroxymethylation, and various histone modifications such as histone acetylation and methylation. As in medical genetics, many seminal discoveries in medical epigenetics were made during investigations of hematologic diseases, and the myelodysplastic syndrome is considered a prototypical example of an epigenetic disease. In contrast to stable sequence variants, the epigenetic cellular state is principally malleable and can be influenced by environmental factors such as diet and toxin exposure. Of note, the expression of genes that encode important drug-metabolizing enzymes (e.g., cytochrome P450) and drug transporters (e.g., solute carrier [SLC] family) have been shown to be altered via intrinsic and extrinsic factors that modify the epigenetic signature, thereby influencing the disposition and effects of drugs.[3] Moreover, the dynamic nature of epigenetics provides a mechanism to modulate the expression of genes that influence drug sensitivity, and so-called epidrugs (i.e., drugs that influence gene expression via epigenetic mechanisms) have already been successfully incorporated into the treatment of hematologic diseases (e.g., hypomethylating agents such as decitabine and azacitidine for myelodysplastic syndrome, and histone deacetylase inhibitors (HDACis) such as vorinostat for acute myeloid leukemia [AML], belinostat for T-cell lymphoma and romidepsin for cutaneous T-cell lymphomas).[3]

Efforts are ongoing to generate detailed epigenomic maps to provide a basis for understanding cellular processes, the pathogenesis of diseases, and alterations in drug responses, such as the Encyclopedia of DNA Elements (ENCODE) and the Epigenomics Roadmap (see Table 8.1).

GENETIC VARIATIONS INFLUENCING DRUG RESPONSE: PHARMACOGENETICS–PHARMACOGENOMICS–PHARMACOEPIGENOMICS

PGx is a major element of the Precision Medicine Initiative announced by US President Barak Obama in 2015. Currently, empiric approaches are typically used to select drug therapy for most patients and most diseases, despite the fact that there is great heterogeneity in the way people respond to medications, in terms of both host toxicity and treatment efficacy. Unfortunately, for almost all medications, interindividual differences are the rule, not the exception, and these differences result from the interplay of many variables, including genetics and environment. Variables influencing drug response include pathogenesis and severity of the underlying disease being treated; drug interactions; the patient's age, sex, nutritional status, and renal and liver function; the presence of concomitant illnesses; and other components of treatment. In addition to these clinical variables, both inherited and acquired (e.g., somatic mutations in cancers) genome variation can influence the disposition and effects of medications, including many used to treat hematologic diseases. Clinical observations of inherited differences in drug effects (based on family studies and twin studies) were first documented in the 1950s, and the concept of pharmacogenetics was defined initially in 1959 by Friedrich Vogel as "the study of the role of genetics in drug response." The number of recognized clinically important pharmacogenetic traits grew steadily in the 1970s; the elucidation of the molecular genetics underlying these traits began in the late 1980s and 1990s, with their translation to molecular diagnostics to guide drug therapy being well underway in the 2000s. The study of pharmacogenetics began with the analysis of genetic variations in drug-metabolizing enzymes and how those variations translate into inherited differences in drug effects. Subsequently, the field has incorporated genome-wide approaches to identify networks of genes that govern the clinical response to drug therapy (i.e., PGx). However, the terms *pharmacogenetics* and *PGx* are generally considered to be synonymous for all practical purposes. With the recognition that epigenetic modification affects gene expression and can contribute to variability in drug effects, the field of pharmacoepigenomics has gained additional attention and importance.

Overall, PGx can be viewed as a broad strategy to establish models of drug disposition and effects by integrating information from genome sequencing, functional genomics, high-throughput molecular analyses, pharmacokinetics (e.g., drug metabolism and disposition), and pharmacodynamics (treatment response, adverse drug reactions [ADRs]). Approaches to establish PGx models include candidate gene analyses (which focus on the analysis of single genes or sets of functionally related genes in pathways thought to be important for the medicine under study) and more agnostic genome-wide analyses. PGx models can be used to maximize efficacy and reduce toxicity of existing medications and to identify novel therapeutic targets.

Comprehensive reviews on PGx and pharmacoepigenomics are available elsewhere.[1–3] Herein, clinically relevant examples are provided to illustrate the potential of PGx and pharmacoepigenomics to improve current drug therapy for hematologic disorders, to prevent hematologic toxicity, and perhaps to identify novel targets for developing new therapeutic approaches in hematology.

OPTIMIZATION OF DRUG THERAPY

Drug effects are typically determined by the interplay of several gene products that influence the pharmacokinetics and pharmacodynamics of medications. Pharmacokinetics entails characterization of the absorption, distribution, metabolism, and excretion (ADME) of medications. Pharmacodynamics is the relationship between the pharmacokinetic properties of drugs and their pharmacologic effects, either desired or adverse. The ultimate goals of PGx and pharmacoepigenomics in this context are to elucidate the inherited determinants for drug disposition and response to select medications and dosages on the basis of each patient's inherited ability to metabolize, eliminate, and respond to specific drugs. A model of how polygenic variables can determine drug response is illustrated in Fig. 8.1.

GENETIC VARIATIONS THAT INFLUENCE DRUG DISPOSITION AND EFFECTS

Drug Metabolism and Effects

There are many enzymes involved in drug metabolism, which are often categorized into phase I reactions that involve oxidation, reduction, or hydrolysis of medications, and phase II enzymes that conjugate drugs via acetylation, glucuronidation, sulfation, or methylation. Although phase I metabolism often inactivates medications, this is not always the case, as exemplified by codeine's activation by cytochrome P450 CYP2D6 and clopidogrel's activation via CYP2C19. Phase II conjugation generally makes medications more water soluble and therefore more readily excreted in the urine, but some phase II conjugates have pharmacologic effects. Although the liver is generally considered the central organ for drug metabolism involved in the clearance of approximately 70% of drugs, phase I and II metabolic enzymes are found in many other tissues, including the kidney, intestinal tract, lung, brain, spleen, erythrocytes, and lymphocytes.

Essentially all genes encoding drug-metabolizing enzymes with more than 30 families of enzymes in humans exhibit genetic variation, many of which translate into functional changes in the proteins encoded. Inheritance of genes containing sequence variations that alter the function of enzymes they encode, as well as CNVs or epigenetic signatures that alter the expression of functionally relevant genes, can influence either drug activation or inactivation and ultimately determine the extent of drug effects. This is most evident when polymorphic genes encode enzymes that are involved in crucial pathways of elimination or activation of the administered medication. It should also be recognized that genetic polymorphism in genes that encode the protein targets of medications (e.g., VKORC1, the target of warfarin) can also have a significant influence on drug effects.

The focus of this chapter is to provide examples that are relevant to hematologists to illustrate the potential impact of genome variation on the effects of medications. We discuss PGx mechanisms involved in toxicity and/or drug resistance of the antileukemic and immunosuppressive TPs and GCs as well as variants in the gene encoding the enzyme (CYP2C9) that metabolizes (inactivates) warfarin and variants in the gene that encodes warfarin's target (VKORC1). These examples therefore involve both phase I (CYP2C9) and phase II (TP *S*-methyltransferase [TPMT]) drug-metabolizing enzymes and the target of a still widely prescribed anticoagulant. Our examples include both inherited genome variations (*CYP2C9, TPMT, NUDT15*, and *VKORC1*) and somatically acquired genomic and epigenomic variants (*NT5C2, PRPS1, CASP1, NLRP3*, and *CELSR2*) that have been shown to alter drug effects in humans. This is a rapidly evolving component of "precision medicine," thus providing an understanding of their relevance and potential is of greater value than attempting a current and comprehensive literature review.

Pharmacogenomics of Thiopurines in Childhood Leukemia

As prodrugs, the TPs MP (major treatment component in lymphoid malignancies) and thioguanine (TG; treatment component

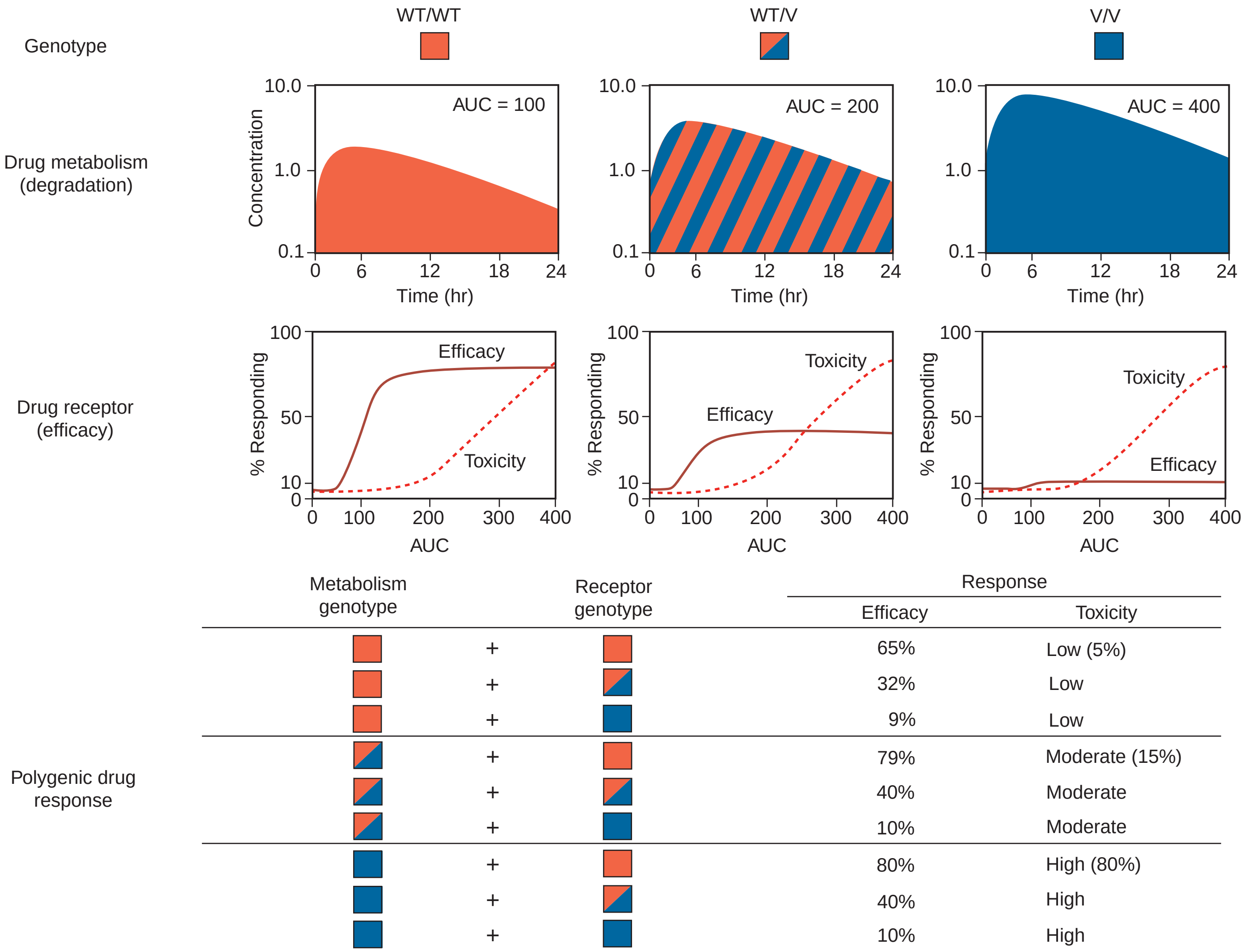


Figure 8.1 POLYGENIC DETERMINANTS OF DRUG RESPONSE. The potential effects of two genetic variants are illustrated. One genetic variant involves a drug-metabolizing enzyme *(top)*, and the second involves a drug receptor *(middle)*. Differences in drug clearance (or the AUC) and receptor sensitivity are depicted in patients who are either homozygous for the wild-type allele (WT/WT) or heterozygous for one wild-type and one variant allele (WT/V) or have two variant alleles (V/V) for the two genetic variants. At the bottom are shown the nine potential combinations of drug metabolism, drug-receptor genotypes, and the corresponding drug-response phenotypes, which were calculated with data from the top. The therapeutic indexes (efficacy-to-toxicity ratios) ranged from 13 (65%:5%) to 0.125 (10%:80%). *AUC*, Area under the plasma concentration–time curve; *V*, variant; *WT*, wild-type. *(Courtesy Evans WE, McLeod HL. Pharmacogenomics: drug disposition, drug targets, and side effects. N Engl J Med. 2003;348:538. Copyright 2003 Massachusetts Medical Society. All rights reserved.)*

in myeloid malignancies) are metabolized by numerous enzymes, either to activate TPs to thioguanine nucleotides (TGNs) or to inactivate TPs via methylation or dephosphorylation of TGNs. Although there is genetic polymorphism in enzymes involved in TP activation (e.g., hypoxanthine phosphoribosyltransferase 1 [HPRT1]), there is little evidence that genetic polymorphisms in these enzymes play an important role in controlling the pharmacologic effects of TPs, with the exception of patients who inherit HPRT1 deficiency, an X-linked disease that occurs in approximately 1 in 350,000 males of European ancestry (Lesch–Nyhan syndrome), because these patients cannot activate TPs. In contrast, genetic polymorphisms in two enzymes involved in the inactivation of TPs increase the accumulation of their active TGNs, thereby increasing the risk of hematopoietic toxicity; TPMT and NUDT15 (nucleoside diphosphate linked moiety X-type motif 15 or nucleotide triphosphate diphosphatase). Inherited variants in TPMT were first discovered in the 1990s, with two major inactive variant alleles accounting for the majority of inherited TPMT deficiency in major world populations studied to date (*TPMT*3C* is the most common variant allele in persons of Asian and African ancestry, and *TPMT*3A* in persons of European ancestry). *TPMT*3A*, *TPMT*3C*, and a third variant *TPMT*2* account for more than 95% of the clinically actionable TPMT variants. Variant *TPMT* alleles encode unstable proteins, and each named star *(*)* allele is defined by a certain genotype (haplotype) at one (e.g., *TPMT*2* [rs1800462] and *TPMT*3C* [rs1800460]) or more (e.g., *TPMT*3A* [rs1142345 and rs1800469]) specific loci and is associated with a lower level of enzyme activity (the star *[*]* nomenclature system provides a “common language” to name a growing number of variants being discovered; however, attempts are being made to implement the conventional “rs” designation). The *TPMT*1* allele encodes the wild-type normal function allele, and individuals who carry *TPMT*1/*1* alleles are named “normal metabolizers” (for details see the website of the Clinical Pharmacogenetics Implementation Consortium [CPIC])[11] (see Table 8.1). Patients who are heterozygous (e.g., *TPMT*1/*2*,

TABLE 8.2 **CPIC Recommendations on Medications Whose Adverse Effects Have Been Associated with Variability in Candidate Genes and Manifest Predominantly as Hematologic Abnormalities**

Adverse Drug Reaction	Drug(s) That Cause ADR	Important Genetic Variant(s)	CPIC Recommendation
Myelosuppression	6-Mercaptopurine 6-Thioguanine azathioprine	*TPMT*2* (rs1800426), **3A* (rs1800460 + rs1142345), **3C* (rs1142345) *NUDT15*2* (rs116855232+rs869320766), *NUDT15*3* (rs116855232)	*Poor metabolizers:* Start with drastically reduced doses (reduce daily dose by 10-fold and dose thrice weekly instead of daily) and adjust doses of thiopurines based on degree of myelosuppression and disease-specific guidelines. Allow 4–6 weeks to reach steady-state after each dose adjustment. If myelosuppression occurs, emphasis should be on reducing thiopurines over other agents. For nonmalignant conditions, consider alternative immune-suppressants. *Intermediate metabolizers:* see Ref. 11
Bleeding risk	Clopidogrel	*CYP2C19*17* (rs12248560)	Recommends an alternative antiplatelet therapy (e.g., prasugrel, ticagrelor) for poor or intermediate CYP2C19 metabolizers if there is no contraindication
Myelosuppression (mucositis, neurotoxicity)	5-Fluorouracil (5-FU)	Dihydropyrimidine dehydrogenase: *DPYD*2A* (rs3918290), **13* (rs55886062), *DPYD* rs67376798 A (on the positive chromosomal strand)	For fluoropyrimidines (i.e., 5-FU, capecitabine, or tegafur) recommends an alternative drug for patients who are homozygous for *DPYD* nonfunctional variants, as these patients are typically DPD deficient. Consider a 50% reduction in starting dose for heterozygous patients (intermediate activity)
Bleeding risk	Warfarin and other coumarin derivatives	*CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910), *VCORC1* (rs9923231)	The best way to estimate the anticipated stable dose of warfarin is to use the algorithms available on http://www.warfarindosing.org
Acute hemolytic anemia	Rasburicase and other drugs (see Refs. 31 and 33 for a full list of drugs)	Deficient or deficient with chronic nonspherocytic hemolytic anemia (CNSHA). A male carrying a class I, II, or III allele, a female carrying two deficient class I–III alleles (see text for more details)	Rasburicase is contraindicated in G6PD-deficient patients with or without CNSHA. In patients with a negative or inconclusive genetic test results an enzyme activity test is recommended prior to rasburicase treatment to determine whether a patient is G6PD deficient; alternatives include allopurinol

CPIC, Clinical Pharmacogenetics Implementation Consortium. For CPIC website, see Table 8.1.

**1/*3A*, or **1/*3C* alleles, so-called intermediate metabolizers, 5% to 10% of Europeans or Africans) are approximately 5 times more likely to develop hematologic toxicity, whereas patients who inherit two variant alleles (e.g., the alleles *TPMT*3A/*3A, *2/*3A, *3A/*3C*, or **2/*3C*, so-called poor metabolizers, 1 in 300 persons of European or African ancestry) will all develop hematologic toxicity if treated with conventional doses of TPs.[11]

It has been recognized for many years that patients of Asian ancestry develop more hematologic toxicity than patients of European or African ancestry, yet the frequency of nonfunctional *TPMT* alleles is lower in Asians. Important new insights were initially provided in 2014 by the identification of a missense variant in *NUDT15* (nsSNP rs116855232, c.415C>T causing p.R139C) in South Korean patients with inflammatory bowel disease who developed hematologic toxicity while receiving azathioprine therapy.[12] *NUDT15* encodes a nucleoside diphosphatase which catalyzes the conversion of cytotoxic thioguanine triphosphate (TGTP) metabolites to the less toxic thioguanine monophosphates (TGMP). In vitro studies showed that the p.R139C change leads to nearly complete loss of NUDT15 enzymatic activity due largely to protein instability.[13] Loss-of-function variants in *NUDT15* result in higher levels of cytotoxic TGTPs, which are incorporated into DNA (i.e., DNA-TGs), leading to apoptosis, resulting in antileukemic and myelotoxic effects. Like for *TPMT*, other rare variants have been discovered and functionally characterized in *NUDT15*.[14] However, the p.R139C variant is the most common and clinically actionable and can be observed either alone (*NUDT15*3* allele) or together with the p.V18_V19dup (rs869320766; c.50_55dup) variant as a distinctive haplotype (*NUDT15*2* allele).[11] In a GWAS of children with ALL from diverse ethnic backgrounds receiving MP maintenance therapy, both *TPMT* (20%) and *NUDT15* (22%) were estimated to account for 42% of interpatient variability in MP tolerance; 80% of patients with severe myelosuppression during MP therapy had risk alleles of either one of these genes.[15] Together, these studies show that genetic polymorphisms in both of these genes influence TP tolerance and that *TPMT* variants are more common in patients of European and African ancestry, whereas *NUDT15* variants are much more common in East Asians and Hispanics with high levels of Native American ancestry.[15] The CPIC guidelines for dosing TPs now incorporate both *TPMT* and *NUDT15* (see box on MP Dosage Adjustment and Table 8.2).[11]

In addition, it was recently discovered that acquired somatic mutations in *NT5C2* (encoding 5′-nucleotidase, cytosolic II) and in *PRPS1* (encoding ribose-phosphate pyrophosphokinase 1) are relatively common in ALL cells at the time of disease relapse, conferring resistance to TP.[16, 17] The mechanisms by which each of these genetic polymorphisms or somatic variants influence the pharmacologic effects of TP medications in children with ALL and the appropriate dosage adjustments for such patients are discussed in greater detail in the box on MP Dosage Adjustment and in the following section.

Relapse of Acute Lymphoblastic Leukemia, Thiopurine Resistance, and Somatic Variants in *NT5C2* and *PRPS1*

In 1988 Pieters and Veerman published a “breakdown hypothesis” to explain TP resistance in leukemia; that is, leukemic cells are resistant to MP and TG because of the breakdown of the toxic trinucleotide form of TPs (i.e., TGNs) into the nontoxic nucleoside form by cytosolic 5′ nucleotidase. This hypothesis was verified 25 years later, when two study groups explored molecular mechanisms for drug resistance in childhood ALL, by sequencing

MP DOSAGE ADJUSTMENT ACCORDING TO THE CPIC GUIDELINES BASED ON *TPMT* AND *NUDT15* GENOTYPES IN ACUTE LYMPHOBLASTIC LEUKEMIA

MP is a mainstay of treatment of childhood ALL. However, the conventional starting dose of 75 mg/m^2/day of this prodrug can induce severe myelotoxicity in patients who have impaired TP metabolism in hematopoietic tissues, owing to less stable TPMT enzyme variants or loss of activity NUDT15 enzyme variants. The three major variant alleles in *TPMT* (*TPMT*2*, *TPMT*3C*, and *TPMT*3A*) and the two major variant haplotypes in *NUDT15* (*NUDT15*2* and *NUDT15*3*) encoding the variant proteins can quickly be determined by certified clinical laboratories (Genetic Testing Registry [GTR]; see Table 8.1) using samples obtained from peripheral blood before MP therapy. In TPMT poor metabolizers (TPMT-PMs; ~1 out of 300 Europeans and Africans), starting with drastically reduced MP doses (i.e., 10-fold reduction of conventional dose and reduction of frequency to thrice weekly instead of daily) is recommended. In TPMT intermediate metabolizers (TPMT-IMs; 5% to 10% of Europeans and Africans), the MP starting dose should be reduced to 30% to 80% of normal dose if the starting dose is 75 mg/m^2/day or greater. If the starting dose is already less than 75 mg/m^2/day, upfront dose reduction may not be necessary in TPMT-IMs.

An MP starting dose of 10 mg/m^2/day is recommended for NUDT15 poor metabolizers (NUDT15-PMs; 1 in 50 patients from East Asian descent). For NUDT15 intermediate metabolizers, MP dosing recommendations are the same as for TPMT-IMs.[11]

the transcriptomes and whole exomes of diagnostic, remission, and relapse samples from ALL patients.[16,18] In B-cell ALL up to 10% and in T-cell ALL up to 19% of the relapse samples had mutations in the *NT5C2* nucleotidase. The NT5C2 nucleotidase physiologically regulates the cellular purine nucleotide pool and can inactivate the cytotoxic forms of MP and TG (i.e., TGNs). To date, 32 *NT5C* mutant alleles have been described with *NT5C2* R367Q being the most common relapse-associated variant, accounting for 90% of mutant causes.[19] Of note, the relapse-associated variants in *NT5C2* increased the enzyme activity of the variant proteins (up to 48-fold), thereby protecting ALL cells against TP-induced apoptosis, by cleaving the phosphate molecules, preventing incorporation into DNA. Maintenance therapy with the antimetabolites MP and MTX is an essential component of successful ALL therapy, and one can speculate that resistance to MP can give rise to early relapse of ALL. Indeed, a significant association of activating *NT5C2* variants and early ALL relapse was found.[16, 18] Moreover, ALL clones harboring *NT5C2* mutations seem not to be present at diagnosis of ALL but rather are emerging and selected for in a darwinian process (i.e., cellular competition and chemotherapy may act as dynamic evolutionary forces shaping the clonal architecture of ALL relapse) during antimetabolite-based maintenance therapy.[19] Novel strategies are necessary to overcome this drug resistance phenotype in the subset of patients with *NT5C2* activating mutations, and such strategies may help to further improve outcome in ALL by avoiding inappropriate drug levels at the target site (e.g., by using drugs that are not inactivated via NT5C2 or by design of small molecules that inhibit NT5C2 function). However, caution must be noted in the context of small molecule NT5C2 inhibition, because germline homozygous loss-of-function variants in *NT5C2* cause a severe neurologic syndrome (i.e., spastic paraplegia 45), and thereby such inhibitors may have neurotoxic adverse effects.

Likewise to the emergence of *NT5C2* mutant clones, also the emergence of clones with mutations in another important mediator of TP metabolism, namely variants in *PRPS1* (encoding phosphoribosyl pyrophosphate synthetase 1), were more recently identified in children with relapsed ALL.[17] Activating *NT5C2* mutations directly affect the degradation of active TGNs, whereas mutations in *PRPS1* can indirectly impair metabolism and activation of TGs by causing defects in the purine salvage pathway.[17] Like activating variants in *NT5C2*, *PRPS1* mutations seem not to originate from a preexisting clone but rather arise during ALL therapy. The drug resistance phenotype deregulating de novo purine synthesis in relapsed ALL harboring *PRPS1* variants may be overcome by inhibition of this upregulated pathway. Indeed, lometrexol, an inhibitor of de novo purine synthesis, effectively abrogated PRPS1 mutant-driven TP resistance in vitro and may be a therapeutic option in this subset of patients with ALL relapse.

Glucocorticoid Pharmacogenomics in Childhood Acute Lymphoblastic Leukemia

GCs are a major component of successful ALL therapies, and leukemia cell resistance to GCs (assessed either in vitro or in vivo) confers a poor prognosis. As steroid hormones, GCs bind to the GCR (encoded by the nuclear receptor subfamily 3 gene [*NR3C1*]), and this leads to translocation of the receptor into the nucleus, where it acts as a transcription factor and binds to GC response elements triggering the activation or repression of gene transcription, which ultimately results in diminished proliferative capacity and apoptosis of ALL cells.

GC therapy can be associated with severe ADRs with sometimes debilitating consequences. For example, adolescent females are at highest risk for an epiphyseal arteriopathy leading to avascular osteonecrosis (AVN), which often necessitates early joint replacement. Although several PGx investigations were designed to identify predictors of AVN, besides sex and age, no clinically actionable predictors have been identified to date. Unlike the situation in TP myelotoxicity where *TPMT* and *NUDT15* have been proven as clinically actionable common genetic variants with large effect sizes, GC-induced AVN might be mediated by numerous rarer variants with small effect sizes, necessitating more complex models.

In parallel to the situation in ADRs, GC resistance of leukemia cells has also been identified to be rather polygenic in nature. In integrated genomic and epigenomic investigations in primary ALL cells and in vitro model systems, dysregulation of key components (i.e., caspase 1 encoded by *CASP1*, and its activator NLR family pyrin domain containing 3 encoded by *NLRP3*) of the so-called NALP3 inflammasome pathway—which cleaves and inactivates the GCR NR3C1—were identified as a novel GC resistance mechanism.[20] Reduced somatic promotor methylation in *CASP1* and *NLRP2* was found in ALL cells resistant to GCs, leading to an increase of transcription and translation of *CASP1* and *NLRP3*, resulting in higher mRNA and protein levels of CASP1 and NLRP3 and enhanced caspase1 cleavage and inactivation of the GCR.[20] This discovery opens several avenues for further action (e.g., prospectively screen childhood ALL samples for *CASP1* and *NLRP3* expression to alter treatment in patients whose leukemia cells carry these variants and to search for small molecule inhibitors of CASP1), although there are no known caspase1 inhibitors approved for clinical use (e.g., VX-765 or VRT-0431198 are preclinical candidates).

Moreover, in a GWAS on diagnostic and relapsed ALL samples, variants in *NR3C1*/*NR3C2* (encoding the GCRs), in *CREBBP* (encoding the histone lysine acetyltransferase CREB binding protein), and in *NSD2* (encoding the nuclear receptor binding SET domain protein 2) were found to be significantly enriched or exclusively present in relapse; variants in these genes have been identified previously to be implicated in GC resistance.[9] Monitoring for these and other drug resistance/relapse-associated mutations (e.g., variants in *NT5C2*, *PRPS1*, or *MSH2*) may help to early identify emergence of a TP- or GC-resistant ALL cell clone; such patients may then benefit from the introduction of other therapies, such as the proteasome inhibitor bortezomib or, if the ALL is of B-cell origin, from CD19-targeting immunotherapies such as chimeric antigen receptor (CAR) T cells or a bispecific antibody therapy with blinatumumab, or CD22-targeting therapy with inotuzumab ozogamicin (InO).

More recently, it was discovered that approximately 50% of GC-resistant ALL have low expression of the G protein–coupled receptor CELSR2. Downregulation of CELSR2 was associated with reduced expression of the GCR and with increased expression of the antiapoptotic protein BCL2 after GC treatment, and preclinical studies showed that the BCL2 inhibitor venetoclax can mitigate this form of GC resistance in ALL.[21]

Inherited Genome Variants in Cytochrome P450 Enzymes

The cytochrome P450 (CYP) superfamily is a system of phase I enzymes involved in the metabolism of endogenous substances and exogenous compounds (e.g., drugs, environmental chemicals). In humans the CYP enzymes are encoded by more than 57 genes, and the majority of these genes are polymorphic. Updated information regarding the nomenclature and properties of the variant alleles with links to the rs numbers/dbSNP identifiers is available at the website of the Pharmacogene Variation Consortium (PharmVar.org) (see Table 8.1).

On the basis of patient genotype (diplotype) CYP variant alleles, individuals are often categorized into one of four major predicted drug metabolism phenotypes: poor metabolizers (having two loss-of-function alleles), intermediate metabolizers (being deficient in one allele), extensive metabolizers (having two copies of functional alleles), and ultrarapid metabolizers (having three or more functional gene copies due to gene duplications, or two increased-activity alleles, or one functional allele plus one increased-activity allele).

Different populations of metabolizers have been linked to different types of variants in the coding region of CYP genes (i.e., SNPs that alter the amino acid encoded, thereby altering protein function or stability); SNPs in intronic regions, which can alter CYP gene mRNA expression; CNVs (e.g., gene deletions, gene duplications) of CYP genes; or differences in the methylation at CpG islands in promoter and 5′ regions, which alter expression of CYP genes.

Many pharmacologically relevant variants in CYP genes have been identified. The focus here is on the variants in *CYP2C9*, which have been shown to influence the metabolism of an extensively prescribed medication: warfarin.

Pharmacogenomics of Warfarin

The oral vitamin K antagonist warfarin is still used to prevent thromboembolic events in patients with chronic conditions such as atrial fibrillation (AF). A narrow therapeutic index with a risk for serious hemorrhage and interindividual variability in response to warfarin necessitate individualization of treatment, which has been based primarily on monitoring prothrombin time via international normalized ratio (INR) testing. Compared with the therapeutic INR range (i.e., 2 to 3), an INR greater than 4 is associated with a 25-fold higher risk of bleeding in elderly patients treated with warfarin, and the percentage time in therapeutic range (PTTR) is a widely accepted read-out for treatment effect. Complications from inappropriate warfarin dosing remain among common reasons for hospitalization due to ADRs.

Pharmacologically, warfarin is a racemic mixture of *R*- and *S*-enantiomers that differ in their patterns of metabolism and in their potency of pharmacologic effects, with *S*-warfarin being more potent. Warfarin dose requirements can be influenced by both modifiable (e.g., compliance, dietary vitamin K intake, therapeutic level surveillance) and nonmodifiable factors (e.g., age, gender, genetics). Candidate gene studies initially demonstrated that the *CYP2C9* genotype influences warfarin clearance and alters oral anticoagulant dose requirements and bleeding risks. CYP2C9 is the principal CYP2C isoenzyme in the human liver, and it is involved in the oxidative metabolism and inactivation of *S*-warfarin.[22]

The two most common *CYP2C9* variants with diminished enzyme activities are *CYP2C9*2* (c.430 C>T, p.Arg144Cys, rs1799853) and *CYP2C9*3* (c.1075 A>C, p.Ile359Leu, rs1057910). Approximately 35% of Caucasians have one or two of these variant alleles; the **2* and **3* variants are virtually nonexistent in Africans and Asians (95% express the wild-type genotype [i.e., extensive metabolizers]).

Compared with the wild-type genotype (*CYP2C9*1/*1*), patients with two nonfunctional variants have a reduction of enzyme activity to approximately 12% for *CYP2C9*2/*2* and approximately 5% for *CYP2C9*3/*3*. Therefore the required dose of warfarin is lowest in homozygous carriers of the *CYP2C9*3* variant and intermediate in homozygote carriers of the *CYP2C9*2* variant.[22]

An important finding was the identification of an additional mechanism underlying warfarin resistance in 2004—the discovery of sequence variants in the warfarin target gene *VKORC1*, which encodes the vitamin K epoxide reductase complex 1. This complex regenerates reduced vitamin K for another cycle of catalysis, which is essential for the posttranslational γ-carboxylation of vitamin K–dependent clotting factors. A common noncoding variant (−1639G>A, rs9923231) was shown to be significantly associated with warfarin dose requirements. Patients with the −1639 AA genotype require lower initial warfarin doses when compared with individuals with the −1639 GG variant. As the −1639G>A polymorphism affects a VKORC1 transcription factor binding site, the functional effect of the variant is thought to be related to decreased VKORC1 transcription, leading to lower protein expression. There are major differences in the distribution of VKOCR1 haplotypes among ethnic groups, and this may explain interethnic differences in coumarin requirement.[22] GWAS in patients treated with warfarin showed two major signals in and around *VKORC1* and *CYP2C9* and identified a much weaker association with *CYP4F2*. The CYP2F4 enzyme catalyzes vitamin K oxidation and the *CYP4F2*3* variant (c.1297 G>A, p.Val433Met, rs2108622) was identified to require increased warfarin dosing. Overall, *VKORC1* explains approximately 25% of the variance in coumarin dose requirement, *CYP2C9* explains approximately 15%, and *CYP4F2* explains approximately 2%.[22]

In 2010 the US Food and Drug Administration (FDA) updated the label on warfarin, providing *VKORC1* and *CYP2C9* genotype-specific ranges of doses, and suggested that *VKORC1* and *CYP2C9* genotypes be taken into consideration when the drug is prescribed. In addition, dosing algorithms are available online, including genetic and nongenetic information that can help to optimize the warfarin starting dose (see CPIC Guidelines and Table 8.2).[22]

Three large randomized controlled trials (RCTs) have prospectively evaluated the benefit of genotype-guided warfarin dosing. The European Pharmacogenetics of Anticoagulant Therapy (EU-PACT; ClinicalTrials.gov number, NCT01119300) trial demonstrated that PGx-guided dosing is superior to a fixed-dosing regimen for achieving therapeutic INRs, whereas the US Clarification of Optimal Anticoagulation through Genetics (COAG; NCT00839657) study failed to demonstrate an improvement in PTTR with genotype-guided dosing compared with the algorithm-guided dosing control arm. Potential reasons for the differences include differences in the algorithmic strategies and control arms, as well as ancestry-related differences in the nature of variant VKORC1 alleles. The Genetic Informatics Trial (GIFT; NCT01006733) of warfarin to prevent deep venous thrombosis included 1597 patients after knee or hip arthroplasty; and the rate of a composite risk of major bleeding, INR of 4 or greater, venous thromboembolism (VTE), or death was reduced in the genotype-guided warfarin dosing arm compared with clinically guided dosing arm.

Alternative anticoagulants (e.g., the four direct oral FXa inhibitors dabigatran, apixaban, rivaroxaban, and edoxaban which are not influenced by the same polymorphisms as warfarin) have been developed, and there was a major shift toward the use of these drugs (e.g., apixaban was the second highest selling drug with 9.8 billion dollars in 2018) because they are at least as effective as warfarin for stroke prevention in patients with AF or for the treatment of VTE but are associated with a significant reduced risk of intracranial bleeding and are more convenient to administer.[23] However, because the most common ADR in all currently approved anticoagulants is bleeding, precision pharmacotherapy can help hematologists to cost-effectively guide oral anticoagulant therapy to avoid this ADR with both the availability of already identified reliable genomic markers and with the choice of different approved drugs. Efforts are underway to identify genomic markers for ADRs and efficacy of the novel, now widely used direct oral FXa inhibitors and to further expand the therapeutic armamentarium (e.g., develop drugs that do not target components of the common pathway—FXa and thrombin).

DRUG TRANSPORTERS

Although passive diffusion is thought to account for tissue distribution of some drugs and their metabolites, there is a growing body of evidence that membrane transporters play an important role in drug disposition and effects. Membrane transporters are highly expressed in epithelial cells and move drugs and other xenobiotics across the gastrointestinal (GI) tract into systemic circulation and across hepatic and renal tissue into the bile and urine, respectively, for excretion. They also distribute drugs into and out of "therapeutic sanctuaries" such as the brain and testes and transport them into and out of sites of action, such as leukemia cells.

The two multispecific drug transporter superfamilies encompass the ABC and the SLC transporters.[24] SLC transporters largely, but not exclusively, mediate cellular uptake, whereas the ABC drug transporters mainly efflux substrates from cells. The function, substrate specificity, and organ distribution among different transporters vary, and comprehensive drug transporter databases are available elsewhere (see Table 8.1). There is a growing body of data on the endogenous functions (e.g., transport of metabolites, antioxidants, signaling molecules, and hormones) of ATP and SLC transporters, and a number of hematologic diseases were identified to be caused by damaging variants in these transporters (e.g., variants in *SLC19A2* in thiamine-responsive megaloblastic anemia, variants in *SLC11A2* in sideroblastic anemia, variants in *SLC4A1* in hereditary spherocytosis type 4 and southeast Asian ovalocytosis, variants in *ABCG5* and *ABCG8* in mitochondriopathies with large platelets and ovalocytes, and variants in *ABCD4* in inborn error of vitamin B_{12} metabolism). Variants in membrane transporter genes were also identified as major determinants of variation in drug disposition, response, and toxicity. For example, pharmacologically "highly important polymorphisms" were discovered and validated in the genes *ABCG2* (variant rs2231142), *SLCO1B1* (variant rs4149056), and *SLC22A1* (variants rs12208357, rs55918055, rs34130495, rs202220802, rs34059508) (International Transporter Consortium, see Table 8.1).[25] Herein we provide one selected example on how variants in *SLCO1B1* have been identified to influence the disposition and toxicity of the antileukemic drug MTX.

SLCO1B1 and Methotrexate

The SLC organic anion-transporter family member 1B1 (*SLCO1B1*) gene, for example, encodes an organic anion transporter 1B1 (OATP1B1) that is located primarily on the sinusoidal face of human hepatocytes. OATP1B1 mediates the hepatic uptake of many endogenous compounds (e.g., bilirubin) and xenobiotics such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (e.g., simvastatin) from sinusoidal blood, resulting in their net excretion from blood, likely via biliary excretion. A common sequence variant in the coding region of *SLCO1B1* (rs4149056) decreases the transport activity of the encoded protein and results in markedly increased plasma concentrations of drugs that are eliminated from the blood via hepatic uptake. Using GWAS, correlations have been established between variants in *SLCO1B1* and myopathy after treatment with the HMG-CoA reductase inhibitor simvastin.[2]

In the field of hematology, a GWAS identified the rs4149056 and other variants in *SLCO1B1* to be significantly associated with MTX clearance; this association was robustly confirmed with five different MTX treatment regimens in more than 1000 pediatric ALL patients.[26] MTX is mainly excreted in the kidney, and *SLCO1B1* is expressed in the liver and associated with hepatobiliary excretion, which is a relatively minor path for MTX elimination (less than 30%). However, results of targeted urinary metabolome analyses suggest that dysfunction of OATP1B1 in the liver may affect the excretion profile of endogenous and exogenous substrates through metabolite-mediated interactions, thereby influencing other transport systems in distant organs (e.g., MTX excretion in the kidney).[27] Clearly, further investigations are necessary to better characterize such a comprehensive concept of complex interactions between transporters and their substrates.

GENETIC VARIATIONS INFLUENCING DRUG TARGETS

To exert their pharmacologic effects, most drugs interact with specific target proteins, such as receptors, enzymes, or proteins involved in signal transduction, cell cycle control, or other cellular events. A comprehensive, regularly updated list of drug targets and their ligands is provided at the website of the International Union of Basic and Clinical Pharmacology (IUPHAR)/British Pharmacological Society (BPS) Guide to Pharmacology (see Table 8.1). Molecular studies have revealed that many of the genes encoding these drug targets exhibit genetic variations, which can alter the sensitivity of these targets to specific medications (e.g., *VKORC1* and warfarin effects).

The following section illustrates this, focusing on somatic genetic variants in chronic myeloid leukemia (CML) cells that alter the targets of tyrosine kinase inhibitors (TKIs).

BCR-ABL1 and Tyrosine Kinase Inhibitors in Chronic Myeloid Leukemia

Somatic genome variants caused by major structural variants, such as the t(9;22) chromosomal translocation producing the *BCR-ABL1* fusion gene, are major mechanisms underlying many forms of hematopoietic malignancies. The increased tyrosine kinase activity of the BCR-ABL1 protein (encoded by the chimeric BCR-ABL1 or "Philadelphia [Ph] chromosome") is the driving oncogenic event in the majority of patients with CML and in a subset of patients with poor-prognosis ALL. This realization resulted in the development of specific TKIs. The treatment of CML was revolutionized with the introduction of the first TKI STI-571 (imatinib), a small-molecular-weight drug that binds to the kinase domain of ABL1, thereby leading to inhibition of tyrosine phosphorylation of proteins involved in downstream signal transduction. In the landmark phase III International Randomized Study of Interferon alpha (IFN-α) and STI-571 (IRIS) trial, imatinib monotherapy was established as the standard-of-care for CML patients, which led to a paradigm shift in cancer treatment (i.e., a more targeted therapy instead of the nonspecific inhibition of rapidly dividing cells).

Although long-term outcome of IRIS participants was excellent in the imatinib arm (with 83.3% survival at 10 years and infrequent serious side effects), up to 17% of patients had been identified to have developed resistance to imatinab within 5 years of therapy. Both, BCR-ABL1 kinase–dependent (i.e., mutations in or near to the imatinib binding site of the target kinase domain of ABL1, found in 60% of the patients who relapsed after achieving an initial response) and BCR-ABL1–independent drug resistance mechanisms were subsequently identified. The latter include reactivation of downstream signaling pathways (e.g., Janus kinase/signal transducer and activator of transcription proteins [JAK/STAT], mitogen-activated protein kinase [MAPK], or phosphatidylinositol 3-kinase [PI3K]) despite effective BCR-ABL1 inhibition; mutations in epigenetic regulators such as DNA methyltransferase 3 alpha (DNMT3A) and isocitrate dehydrogenase 1 (IDH1); or microenvironmental factors such as cytokines that influence, for example, STAT3 phosphorylation independent of BCR-ABL1 kinase activity. BCR-ABL1 kinase–independent resistance mechanisms predominate in primary resistant CML clones.[28] Sequence variants governing BCR-ABL1 kinase–dependent drug resistance are localized either around the phosphate binding loop (e.g., variants M244V, G250E, Q252H, Y253F, E255V), the SH2 contact and C-lobe (e.g., variants M351T, F359V), the activation loop (e.g., variant H396P, H396R), or the gatekeeper residue (variants T315I and F317L) of the imatinib binding site.[28] The identification of these variants led to the design of second- and third-generation ABL1 TKIs. Of these, currently four are FDA approved for the therapy of CML, namely nilotinib (binding to the same pocket as imatinib but with higher affinity; strong resistance: T315I), dasatinib (binds to the ATP-binding site with stronger activity against the ABL1 kinase compared with imatinib and dasatinib; strong resistance: T315I), bosutinib (similar binding site as dasatinib but lower potency; strong resistance: T315I, V299L), and ponatinib (high-affinity binding to ABL1 and efficacy also against T315I; compound mutations affecting E255V and T315I do result in drug resistance)[28] (see also the website Clinical Interpretation of Variants in Cancer [CIViC]). The relatively common T315I or "gatekeeper" variant confers resistance to imatinib, nilotinib, dasatinib, and bosutinib; therefore the third-generation TKI ponatinib was designed. Ponatinib was successfully tested in clinical trials, and its use was first approved by the FDA in 2012 for patients with CML resistant to other TKIs. However, ponatinib was temporarily suspended in 2013 due to serious vascular adverse events (VAEs; i.e., arterial and venous thromboembolic events, arterial hypertension), and VAEs are now recognized as also limiting the use of second-generation TKIs. Moreover, the strong selective pressure of ponatinib has led to the emergence of TKI resistance due to so-called compound mutations in ABL1. Compound mutations are multiple-point variants occurring in the same *BCR-ABL1* allele, and this drug-resistance mechanism is different to the emergence of multiple clones with different mutations. Computed modeling and in vitro proliferation studies were used to analyze the impact of compound mutations in *BCR-ABL1* on TKI resistance and showed, for instance, that the compound mutation Y253H/E255V induced a shift in the P-loop of the ABL1 kinase, obstructing the ponatinib binding site, resulting in resistance to ponatinib.[29] Moreover, it was found that additional acquisition of an E255V variant in T315I-positive CML confers also resistance to ponatinib.

To avoid off-target toxicities of ponatenib and to retain the ability to target the T315I variant, the allosteric inhibitor asciminib (ABL001), which binds to the myristoylation pocket of *BCR-ABL1*, was designed. Asciminib showed promising results in a phase I clinical trial in heavily pretreated CML patients (NCT02081378); that its, a major molecular response (MMR; which is a *BCR-ABL1* PCR level of 0.01% on the Internationale Scale [IS]) was achieved in 48% of patients at 12 months of therapy.[28] However, like for ATP-binding site TKIs, mutations at the binding site of asciminib can also cause resistance to this drug, and a combination of asciminib with an ATP-binding site TKI may overcome the emergence of resistant CML clones because these drugs bind at different sites of *BCR-ABL1* and have therefore different drug resistance mechanisms.

As second- and third-generation TKIs have a risk for VAEs, especially in older patients with preexisting vascular disease, TKI selection based on cardiovascular risk factors and mutational *BCR-ABL1* status is of utmost importance to guide CML therapy. Upfront and repeated monitoring of the mutational status of patients with BCR-ABL1–positive leukemias can help to select appropriate TKIs and tailor TKI treatment. Recent treatment trials also focus on combining TKIs with other drugs such as IFN-α (which can augment immune reaction against TKI-resistant residual CML clones; NCT01933906, NCT02001818) or with drugs that target signaling pathways downstream of the BCR-ABL1 kinase (e.g., the JAK inhibitor ruxolitinib; NCT01702064, NCT03610971) to overcome ABL1 kinase domain mutation–independent resistance mechanisms in order to promote deep molecular responses and to allow discontinuation of TKI therapy.[28]

ADVERSE DRUG EFFECTS PRESENTING AS HEMATOLOGIC DISORDERS

ADRs constitute a major clinical problem, and strong evidence indicates that ADRs account for approximately 5% of all hospital admissions. Moreover, two large surveys in the United States in 1998 and 2010 suggested that ADRs occurring in hospitals were the fourth to sixth leading cause of in-hospital mortality.[2] Although the factors that determine susceptibility to ADRs are unclear in most cases, there is increasing interest in the role of genetic predisposition to these ADRs; the possibility of a genetic test to identify patients at risk for rare but serious adverse effects would be of great clinical value.[30] Based on the clinical relevance of ADRs, the FDA has provided advice on the use of certain pharmacogenomic diagnostics (e.g., variants in *TPMT*, *NUTD15*, *DPYD*, *UGT1A1*, *CYP2C9*, *CYP2C19*) to avoid serious adverse drug effects; a full list of these genetic test is available on the FDA website, although contents of this site have not all been subjected to rigorous evidence-based assessment comparable with the CPIC (see Table 8.1).

CPIC recommendations on medications whose adverse effects have been associated with variability in candidate genes and manifest predominantly as hematologic abnormalities are listed in Table 8.2. In the following section, we provide information on genetic variants in G6PD that can cause acute hemolytic anemia (AHA) after administration of certain drugs.

Glucose-6-Phosphate Dehydrogenase Deficiency and Rasburicase

Occurrence of AHA after mass administration of the antimalaria drug primaquine (PQ) was first documented in some US soldiers in Korea. The so-called PQ sensitivity syndrome was more common among African Americans and clinically identical to "favism" (i.e., AHA after ingestion of fava beans). The underlying biochemical (Fig. 8.2) and genetic (variants in *G6PD*) causes of the clinical phenotype (i.e., AHA after PQ and fava beans, as well as for neonatal jaundice) were identified, and the disease was named G6PD deficiency.[31] The severity of AHA in individuals with G6PD deficiency after treatment with drugs that induce oxidative stress is also influenced by host and environmental factors (see Fig. 8.2).

The *G6PD* gene is localized on Xq28, and currently more than 160 pathogenic variants have been identified. The biochemical characterization of these variants has revealed a range of effects, such as changes in kinetic activity, thermostability, and protein folding.[32] The clinical phenotypes of G6PD variants are largely determined by a trade-off between protein stability and catalytic activity.

G6PD is an "essential gene" (i.e., complete loss of G6PD is lethal). Very rare, more complex variants, for instance, in-frame deletions in exon 10, which affect important regions within the enzyme such as the substrate binding site, can cause severe transfusion-dependent chronic nonspherocytic hemolytic anemia (CNSHA). Variants have been divided into five classes based on enzyme activity in red blood cells (RBCs) and clinical presentation: class I (CNSHA, activity less than 10%), class II (no CNSHA, activity less than 10%), class III (no CNSHA, greater than 10% to 60%), class IV (normal activity; variants G6PD B and G6PD A), and class V (higher activity). It is estimated that approximately 5% of the world's population has G6PD deficiency, and almost all of these individuals have class II or III variants.[33]

Drugs that have the potential to cause oxidative stress in erythrocytes, which results in AHA in G6PD-deficient patients, have been recently classified into two groups: (1) predictable hemolysis (i.e., AHA can be expected in a G6PD-deficient patient after administration of the drug) and (2) possible hemolysis (i.e., AHA may or may not occur, related to dosage and administration of the drug, comorbidities).[31] Drugs with predictable hemolysis include, for instance, the antimalaria drug PQ and the recombinant urate oxidase rasburicase. A comprehensive list of drugs is provided elsewhere,[31, 33] and we focus on rasburicase herein.

Rasburicase is used in the prophylaxis and treatment of hyperuricemia, and the most important indications are tumor lysis (e.g., in patients with hyperleukocytosis leukemia or lymphoma) or after acute renal failure in infants. Rasburicase catalyzes the cleavage of uric acid, thereby producing hydrogen peroxide. Normally, hydrogen

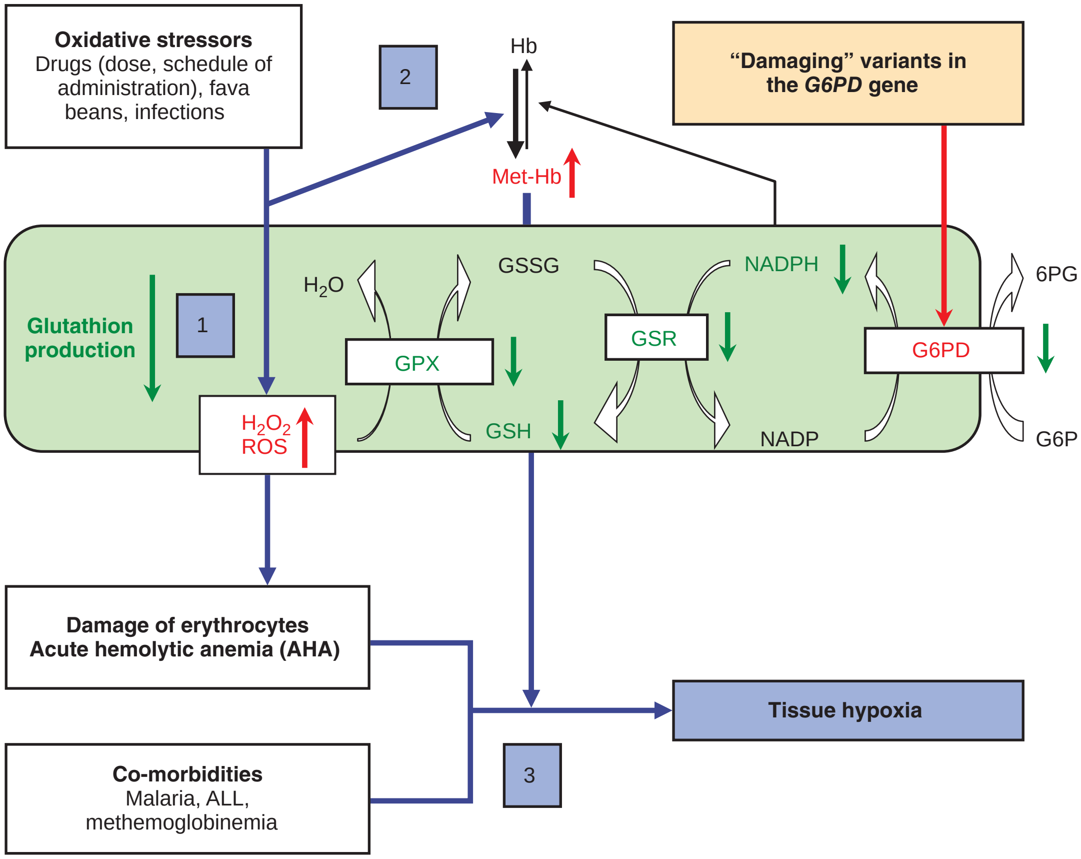

Oxidative stressors
Drugs (dose, schedule of administration), fava beans, infections
2
Hb
Met-Hb
"Damaging" variants in the G6PD gene
Glutathion production
1
H2O
GSSG
NADPH
6PG
GPX
GSR
G6PD
H2O2 ROS
GSH
NADP
G6P
Damage of erythrocytes
Acute hemolytic anemia (AHA)
Tissue hypoxia
Co-morbidities
Malaria, ALL, methemoglobinemia
3

Figure 8.2 FACTORS INFLUENCING THE PHENOTYPE HEMOLYSIS IN INDIVIDUALS WITH G6PD DEFICIENCY. Damaging variants in the *G6PD* gene lead to G6PD deficiency in red blood cells, which, under oxidative stress, (1) leads to depletion of glutathione (with low cellular NADPH and GSH pools causing low activity of GPX [the enzyme that detoxifies H_2O_2]) and subsequent loss of protection against oxidation of proteins, with precipitation of denaturized hemoglobin leading to AHA, and (2) oxidation of hemoglobin (Hb) iron with formation of methemoglobin (MetHb is converted back to Hb via an NADPH-dependent reaction; as the cellular NADPH pool is low, MetHb accumulates), resulting in methemoglobinemia. The severity of AHA and tissue hypoxia in individuals with G6PD deficiency after treatment with drugs that induce oxidative stress is influenced by (3) host factors such as genetic variants in *G6PD* and comorbidities such as malaria, methemoglobinemia, and ALL, as well as environmental factors (i.e., drugs, their dose, and schedule of administration). *6PG*, 6-Phosphogluconolactone; *AHA*, acute hemolytic anemia; *ALL*, acute lymphoblastic leukemia; *G6P*, glucose 6-phosphate; *G6PD*, glucose-6-phosphate dehydrogenase; *GPX*, glutathione peroxidase; *GSH*, glutathione; *GSR*, glutathione reductase; *GSSG*, glutathione disulfide; H_2O_2, hydrogen peroxide; *NADP(H)*, nicotinamide adenine dinucleotide phosphate; *ROS*, reactive oxygen species.

peroxide is promptly inactivated via glutathione peroxidase (GPX). However, in individuals with G6PD deficiency, the activity of GPX is markedly reduced due to impaired glutathione metabolism, and rasburicase induces AHA and often significant methemoglobinemia. This can cause severe tissue hypoxia, especially in patients with leukemia who have already reduced RBC counts, and fatalities have been reported after rasburicase administration in ALL patients with G6PD deficiency (see Fig. 8.2).[33]

The FDA and the European Medicines Agency (EMA) have contraindicated the use of rasburicase in individuals with G6PD deficiency. However, tumor lysis syndrome (TLS) is also a life-threatening condition, and one must carefully balance the risk of reversible severe AHA and methemoglobinemia, which can be treated via RBC transfusions, and the risk of renal failure and hyperkalemia in TLS. Online enzyme activity testing before the use of rasburicase (i.e., providing results within 1 hour) is an ideal but not commonly achievable scenario. More information on available genetic and activity test options is provided in the updated CPIC guidelines (see Table 8.2).[33]

Of interest are the results of a recent pharmacoepigenetic investigation in which HDACis selectively reinstated enzyme activity in G6PD-deficient erythroid precursors in vitro by boosting *G6PD* gene transcription.[34] Whether administration of the epidrug HDACi sodium butyrate can also increase G6PD activity in patients with G6PD deficiency to levels that protect their erythrocytes from oxidative damage by rasburicase or other oxidative stressors and if such an approach is not associated with severe side effects remain to be proven. However, the ability of HDACi to increase the transcription of a subset of active genes, such as *G6PD*, would offer a novel and appealing therapeutic approach, especially for the subset of patients with severe forms of enzymopathies, such as CNSAH class I or II G6PD deficiency.

A different PGx approach to establish drug therapy for patients with severe G6PD deficiency was recently reported. Using crystallographic studies and mutagenesis analyses, researchers identified the structural and functional defects of one common variant in *G6PD* (i.e., "Canton variant [R459L], which is the most common causative variant in patients with G6PD deficiency in China and Southeast Asia). The Canton variant is associated with severe G6PD deficiency (class II, 18% activity). Using high-throughput screening, a small molecule—AG1—was identified, which increases the activity of wild-type G6PD, the Canton variant, and several other common G6PD variants in vitro. Clearly, further studies are necessary to investigate if treatment with AG1 can result in clinical benefit for a subset of patients with severe forms of G6PD deficiency.[32]

DRUG DEVELOPMENT

Optimizing the selection and dosage of medications is a principal goal of PGx. Another important application is in drug development, which is evolving in parallel with improved insights into the pathobiology of diseases (i.e., target identification) and into the mechanisms by which medications exert their pharmacologic effects as well as mechanisms of drug resistance. Improved insights into the mechanism(s) of diseases and drug actions in target cells can help to identify novel therapeutic targets (e.g., targetable key molecules in signaling pathways that are dysregulated in a certain disease) or to elucidate mechanisms that confer drug resistance (e.g., inactivation of TPs via NT5C2 or PRPS1), and this knowledge can be used to engineer drugs that induce or amplify treatment effects or bypass resistance mechanisms, or both.

Here we focus on examples to show how recent novel insights from ALL biology coupled with PGx investigations have helped to develop strategies to further improve outcome in so-called high-risk subgroups in young patients with B-cell ALL (HR B-cell ALL) who still have poor outcomes (i.e., less than 70% overall survival [OS] estimates) despite intensive contemporary therapies.

Drug Development in Pediatric High-Risk B-Cell Acute Lymphoblastic Leukemia

Currently more than 20 subtypes of B-cell ALL have been defined by constellation of genomic alterations, and these subtypes can also be grouped according to outcomes achieved with current therapies.[35] Children with the “low-risk” B-cell ALL subtypes ETV6-RUNX1 (occurring in 20% to 25% of patients) and high hyperdiploid ALL (51 to 67 chromosomes; 20% to 30% of patients) have excellent outcomes, with greater than 90% OS estimates. “Intermediate-risk” B-cell ALL subtypes include ETV6-RUNX1–like (3%), TCF3-PBX1 (5%), DUX4-ERG (5%), PAX5alt and PAX5 P80R (4% to 5%), ZNF384-r, and iAMP21 (2.5%) ALL; patients with this subtypes have OS estimates of 70% to 90% in more developed countries with current personalized therapies.[36] Despite the high prevalence of this prognostic favorable B-cell ALL subtypes in children in high-income countries, ALL remains a leading cause of death from disease in children older than 1 year, and treatment of children with HR-ALL (approximately 30% of patients) remains one of the greatest challenges in pediatric oncology. HR-ALL features include the resistance of leukemia cells to steroids and multidrug therapy (clearance of leukemia blasts in the peripheral blood, bone marrow, and sanctuary sites), and the presence of certain genetic alterations in leukemia cells—for instance, lysine methyltransferase 2 A gene rearrangements (KMT2A-r ALL, formerly MLL-r ALL; 5% of patients), the BCR-ABL1 fusion gene (BCR-ABL1 ALL; 3%), hypodiploidy (less than 44 chromosomes; 2.5%), myocyte enhancer factor 2D rearrangements (MEF2D-r ALL; 2.5%), and the “BCR-ABL1–like ALL” (14%).[36]

The introduction of TKIs in the treatment of BCR-ABL1 ALL has led to a significant improvement in outcome in this HR B-cell ALL subtype, as demonstrated by results from the Children’s Oncology Group (COG) AALL0031 trial. The following sections provide examples on the development of novel approaches to treat infants, children, as well as adolescents and young adults (AYAs), with other HR B-cell ALL subtypes.

Identification of Novel Therapies for KMT2A-Rearranged Infant ALL

Infants (age less than 1 year) with ALL (nearly all have B-cell ALL, with only 5% T-cell ALL) have long been recognized to have very poor outcomes when treated with ALL standard therapy. The identification that transcriptome profiles separate infant ALL from ALL and AML and that infant ALL blast cells are highly sensitive to cytarabine in vitro (similar to AML) provided the rationale to establish an ALL/AML hybrid treatment concept.[37] Indeed, Interfant-99 hybrid therapy (NCT00015873) resulted in better but still poor outcomes (5-year event-free survival [EFS] for infants with KMT2A-r ALL remains less than 40%), and further improvements of infant ALL therapy are needed. Of note, the pharmacokinetics of many conventional antileukemia drugs differ between infants and older children, and an intensive induction therapy concept (with intensive dosing of standard ALL medications) in the COG AALL0631 trial had to be modified because of a high rate of fatal toxicities during this treatment phase. Therefore the unique drug metabolism profile in infants (i.e., developmental pharmacology) needs to be considered when planning future infant ALL trials, and novel, less toxic therapies are needed.

Approximately 80% of infants with ALL have rearrangements of the *KMT2A* gene (KMT2A-r) at 11q23 and KMT2A translocations. Next-generation sequencing approaches identified that infant KMT2A-r ALL has one of the lowest frequencies of somatic mutations of any as-yet sequenced cancer, with a mean of 1.3 nonsilent mutations.[38] Wild-type *KMT2A* encodes a histone methyltransferase that targets lysine 4 on histone 3 (H3K4), and leukemias carrying KMT2A-r can be considered as prototypical cancers driven by dysregulated epigenetic mechanisms. *KMT2A* rearrangements lead to the loss of the catalytic methyltransferase domain, with subsequent in-frame fusion to one of greater than 100 known translocation partners. The prognostic significance of *KMT2A* rearrangement varies according to the fusion partner and the disease; for example, 50% of infants with AML have a KMT2A-r, but this is not associated with a poorer prognosis. Interestingly, KMTA2-r ALLs in older children have more somatic mutations, with a mean of 6.5 per patient versus 1.3 mutations in infants. In infant ALL the most common partner gene (49% of patients) is *AFF1*, which encodes AF4/FMR2 family member 1 (formerly *AF4*) on chromosome 4. The resulting chimeric oncoproteins interact with another methyltransferase—DOT1-like histone H3K79 methyltransferase (DOT1L)—which then methylates lysin 79 in the globular region of histone H3 (H3K79) at KMT2A target genes, causing aberrant gene expression and leukemogenesis. Inhibition of DOT1L has emerged as an attractive concept for therapeutic intervention in KMT2A-r leukemias, and in vitro and animal studies have shown that the small molecular inhibitors of DOT1L selectively target KMT2A-r leukemia cells.[39] In a phase I trial for adults with KMT2A-r leukemias (NCT01684150) monotherapy with the epidrug pinometostat (EPZ-5676) demonstrated evidence of DOT1L target inhibition but limited clinical responses (only 2 of 51 had complete responses). In a pediatric phase I trial (NCT02141828), transient decreases in peripheral or marrow leukemic blasts were reported in 7 of 18 children with relapsed/refractory KMT2A-r leukemia treated with pinometostat, but these reductions did not meet formal thresholds for objective response. However, the observed safety profile of pinometostat provides potential for exploration of a combination with standard chemotherapy, and a phase Ib/II trial (NCT03724084) in adults with KMT2A-r AML is currently enrolling patients; if this approach proves to be successful, it remains primed for testing in infants with KMT2A-r ALL. Because KMT2A-r infant ALL can be considered as an epigenetic disease, other epidrugs are being incorporated into clinical trials based on preclinical efficacy data; for example, the demethylating agent azacitidine is currently being investigated in combination with the Interfant chemotherapy backbone (NCT02828358).[37]

A different approach focuses on targeting the class III receptor tyrosine kinase (RTK) Fms-related receptor tyrosine kinase 3 (FLT3). Using genome-wide gene expression analyses, the *FLT3* wild-type gene was identified as being overexpressed in KMT2A-r ALL. FLT3 inhibitors have been shown to inhibit growth in cells that overexpress FLT3.[40] The COG AALL0631 trial investigated the combination of the FLT3 inhibitor lestaurtinib given after induction therapy in combination with an intensive chemotherapy backbone in children with KMT2A-r ALL. However, the trial failed to demonstrate a benefit from the addition of lestaurtinib.

In addition, novel CD19-directed immunotherapies, such as the treatment with the bispecific antibody blinatumomab or with CAR T cells, are being explored in infant KMT2A-r ALL. For example, a pilot trial of blinatumumab in combination with the Interfant chemotherapy backbone has been initiated (trialregister.nl NTR6359). However, potential limitations of such approaches are that the CD19 antigen is not uniformly expressed in KMT2A-r leukemias and that "class switching" from lymphoid to myeloid phenotypes has been reported in infants with KMT2A-r ALL during CD19-targeted immunotherapies. Moreover, manufacturing CAR-T cells from autologous infant T cells is challenging. To overcome this problem, novel gene-editing techniques were developed to simultaneously introduce the CD19 CAR construct and disrupt mediators of allogeneic rejection via transcription activator–like effector nuclease (TALEN)-mediated gene editing of T-cell receptor α chain and CD52 gene loci in healthy donor T cells, creating allogeneic universal CAR-T (UCART19) products.[37] Of note, two infants with relapsed KMT2A-r ALL were treated and achieved molecular remissions via such UCART19 cells, and these cells persisted until conditioning ahead of successful subsequent allogeneic stem cell transplantation.

Although infants with KMT2A-r ALL still fare worse than older children with ALL, the aforementioned approaches hold promise to improve outcomes in these patients via novel personalized therapies that will be tested in international cooperative treatment trials.

Identification of Novel Therapies for "BCR-ABL1–Like" ALL

In contrast to the low- and intermediate-risk B-cell ALL subtypes (which account for approximately 70% of childhood ALL cases), B-cell ALL with the *BCR-ABL1* fusion gene responds poorly to conventional ALL therapy, and patients with BCR-ABL1 ALL belong to the HR group, with less than 70% OS estimates. BCR-ABL1 ALL is rare in children (1 to 15 years, 4.2%) but is more common in adolescents (16 to 20 years, 5.9%) and young adults (21 to 39 years, 22%); this is a contributor to the overall poorer prognosis of AYAs with ALL.[36]

In 2009, genome-wide transcriptome analyses identified a subtype of HR B-cell ALL that has a gene expression profile similar to that of BCR-ABL1 ALL.[41, 42] In contrast to BCR-ABL1 ALL, leukemia cells in the identified subtype did not harbor the *BCR-ABL1* fusion gene; therefore this subtype has been named BCR-ABL1–like ALL. BCR-ABL1–like ALL is more common but has the same age distribution pattern as BCR-ABL1 ALL (i.e., 1 to 15 years, 11.9%; 16 to 20 years, 20.6%; 21 to 39 years, 27.4%). Because BCR-ABL1–like ALL has an activated kinase gene expression profile resembling BCR-ABL1 ALL, it was speculated that genetic alterations that can influence tyrosine kinase signaling pathways similar to those downstream of BCR-ABL1 might be involved in the pathogenesis of BCR-ABL1–like ALL. Indeed, kinase-activating alterations (rearrangements, sequence variants, and CNVs) were subsequently identified in comprehensive analyses, which included transcriptome, WGS, and WES.[43]

Subsequent large-scale investigations corroborated and extended early findings in BCR-ABL1–like ALL and identified this subtype to be a heterogeneous disease with a wide range of genetic alterations (i.e., more than 70 discrete alterations reported as of January 2020).[43, 44] However, two major subgroups, distinguished by the type of cytokine receptor or kinase alterations, emerged. For example, 60% of adolescents with BCR-ABL1–like ALL had rearrangements in the lymphoid signaling receptor gene *CRLF2* (encoding cytokine receptor–like factor 2) and concomitant mutations in *JAK1* or *JAK2* (which encode the JAKs). These rearrangements (e.g., the frequently observed *PAX5-JAK2* fusion), when ectopically expressed in cell lines, activated JAK-STAT signaling and conferred cytokine-independent proliferation that can be suppressed by the JAK2 inhibitor (JAKi) ruxolitinib in vitro. Preclinical studies have shown activity of JAK in vivo in CRLF2-r and other JAK pathway mutant BCR-ABL1–like ALL patient-derived xenograft models. In a phase I/II trial (NCT02420717), low-dose ruxolitinib with chemotherapy was tested for adolescents and adults with relapsed/refractory CRLF2-r/JAK-mutant ALL; this combination therapy was well tolerated, but only limited efficacy was observed. In a phase II trial (AALL1521, NCT02723994), the COG is testing the safety and efficacy of ruxolitinib addition to chemotherapy in children and AYAs with newly diagnosed BCR-ABL1–like ALL and CRLF2 rearrangements or other JAK pathway alterations.[44]

The second major subgroup within the BCR-ABL1–like ALL cohort was identified to have so-called ABL class-alterations (ABL class-mutant ALL), namely, fusions that involve the Abelson-related (ABL)-class kinase genes (e.g., *ABL1* and *ABL2*), platelet-derived growth factors A and B (*PDGFRA*, *PDGFRB*), or colony-stimulating factor 1 receptor (*CSF1R*). The use of ABL TKIs, such as imatinib and dasatinib, has markedly improved survival for patients with BCR-ABL1 B-cell ALL, and it can be anticipated that patients with BCR-ABL1–like B-cell ALL might also benefit from such a therapy. Indeed, preclinical studies have shown promising results in treating ABL class-mutant Ph-like ALL with imatinib or dasatinib; in addition, anecdotal case reports have shown impressive activity in refractory patients with the combination of imatinib or dasatinib with chemotherapy, especially for PDGFRB-r (e.g., *EBF1-PDGFRB*) BCR-ABL1–like ALL. The combination of dasatinib with chemotherapy is tested in patients with newly diagnosed ABL class-mutant BCR-ABL1–like ALL in ongoing trials of the COG (AALL1131, NCT01406756) and the SJCRH Total XVII (NCT03117751).[44]

Other less frequently observed targetable alterations affect the *NTRK3* gene, which encodes the neurotrophic tyrosine kinase receptor type 3 and *ETV6* (ets variant 6). Malignancies with *NTRK3* fusions (e.g., infantile fibrosarcomas) have been shown to respond well to TRK inhibitors such as crizotinib and larotrectinib; larotrectinib had clinical activity in an adult patient with ETV6-NTRK3-r BCR-ABL1–like ALL.[45]

In summary, this provides evidence that kinase-activating genetic alterations are biologically relevant drivers in BCR-ABL1–like ALL and that the genetic signature can help to guide the use of additional drugs such as TKIs, JAK2, or TRK inhibitors. However, rapid molecular profiling is needed to identify potentially actionable mutations in patients with BCR-ABL1–like ALL, and additional prospective trials are needed to establish their benefit in the clinical setting. In contrast to children, outcomes in ALL have not improved significantly during recent decades in the AYA cohort; because BCR-ABL1–like ALL is the most common subtype in AYAs, the introduction of signaling pathway inhibitors may be an attractive strategy to improve outcome in these patients who have been therapeutically neglected for too long.

FUTURE DIRECTIONS

PGx was perhaps the earliest application of precision medicine and has already been proven to be an important approach to personalize drug therapy, especially in situations where common variants in key pharmacogenes, encoding essential proteins for drug transport, metabolism, or targets, have large effects on drug disposition and/or action. Examples are variants in *TPMT* and *NUDT15* and their influence on TP toxicity and activity, variants in *G6PD* and their influence on rasburicase toxicity, variants in *VKORC1* and *CYP2C9* and their effects on warfarin pharmacology, damaging variants in cytochrome P450, family 2, subfamily C, and polypeptide 19 *(CYP2C19)* and their influence on clopidogrel (antiplatelet medication) activation resulting in reduced drug effect and increased risk of major adverse cardiovascular events, or variants in dihydropyrimidine dehydrogenase *(DPYD)* and toxicity of fluoropyrimidine-based anticancer therapy with capecitabine or fluorouracil. However, even in the case of the aforementioned examples, there is a relatively slow pace of translating PGx knowledge into clinical practice. Laboratory tests (e.g., liver and kidney function tests) are widely used to adjust drug dosages. Although technology for testing relevant PGx biomarkers is widely available, simple, robust, and inexpensive, genotyping tests are yet rarely used to optimize drug therapy. Because genotyping at the time of prescribing is compromised by the relatively long delay until genotype results are available, preemptive genotyping early in a person's life or at the time of initial illness is a more efficient and cost-effective approach. In addition, because a person's germline genotype does not change over a lifetime, it needs to be done only once. Recently, preemptive panel-based strategies were implemented, including the so-called PGx-Passport, which contains a set of 58 germline-variant alleles located within 14 genes considered to be pharmacogenomically actionable according to the Dutch Pharmacogenetics Working Group (DPWG),[46] and a panel testing within the PREemptive PGx Testing for Preventing Adverse Drug Reactions (PREPARE; NCT03093818) trial from the European Union (EU) Ubiquitous-PGx (U-PGx) Study group. The future likely holds that WGS or WES data will be the most comprehensive and cost-effective approach for diagnosing patients with inherited or acquired (somatic) genome variants that can guide the selection and dosing of a growing number of medications.

To implement a change in clinical practice, mainly RCTs are currently used to prove an approach to be superior to standard practice. The number of completed RCTs studying PGx-informed preemptive dosing compared with conventional dosing is rather low, and it is not clear that RCTs are essential (RCTs have not been done to show superiority of many treatments, nor the benefits of parachutes for those jumping from airplanes).[47, 48] However, most reported results from such RCTs are in favor for the PGx-guided drug dosing regimens (e.g., *CYP2C9*, *VKORC* and warfarin dosing, NCT01119300; NCT01006733; *DPYD* and fluoropyrimidine toxicity, NCT02324452; *TPMT* and TP toxicity, NCT00521950). Currently recruiting prospective trials include the PREPARE (NCT03093818) trial from the EU U-PGx Study group, the Tailored Antiplatelet Initiation to Lessen Outcomes due to Decreased Clopidogrel Response After Percutaneous Coronary Intervention (TAILOR-PCI; NCT01742117), and the Patient Outcome After Primary PCI (POPular; NCT01761786) trial. These RTCs will further inform the field of precision medicine on the utility of PGx-based drug dosing.

Another instrument for clinical implementation of PGx are PGx drug labels in the Summary of Product Characteristics (SmPC), and in 2019 approximately 15% of prescribed drugs were reported to carry PGx information in their SMPCs in the United States. PGx guidelines and SmPC labels for the use of genomic biomarkers in clinical practice are provided, for example, by the CPIC, FDA, DPWG, and EMA. However, the consensus of actionable PGx labels of 184 different gene–drug interactions between the FDA and EMA was only 54%; further efforts are necessary to harmonize the PGx drug labels in the SmPCs.[49]

Sophisticated electronic health record (EHR) systems, in combination with clinical decision support systems (CDSSs) that include PGx information as one variable, create a solid basis for implementation of PGx and other diagnostics; initiatives such as the "Clinical Implementation of PGx—PG4KDS protocol" at St. Jude Children's Research Hospital provide proof-of-principle on the feasibility to use genetic information to prescribe medications as a routine part of clinical care.[50, 51]

The recent unprecedented gain of insights into the human genome and genomic variations among individuals has already changed the practice of medicine. High-throughput technologies, such as hybridization-based microarray approaches and next-generation sequencing technologies, are available for genome-wide analyses of genomic variants, gene expression patterns, epigenetic patterns, and proteomic and metabolomic profiles. Moreover, sophisticated methods have been developed to integrate these data to uncover genotype–phenotype interactions, and some of the most important methods are metadimensional and multistaged analyses. The application of these genome-wide tools has already yielded significant insights into the biology of diseases and complex drug actions and led to interesting novel PGx discoveries. For example, by moving beyond candidate gene approaches, a genetic risk score derived from 61 individual variants was identified to separate cases from controls for drug-induced QT prolongation in a recent GWAS.[2] However, the extent to which such multigene markers can explain the complex genomic architecture of diseases or drug responses remains to be further explored.

Novel genomic testing tools are also being used to elucidate differences between genomes of normal cells and cancer cells (e.g., the Pediatric Cancer Genome Project; see Table 8.1), and this knowledge has proven potential to illuminate paths toward novel prognostic markers (those that can be used for risk stratification in clinical trials) and/or novel therapeutic targets (those that can be used to discover new medications). For example, novel comprehensive genomic approaches (including RNA sequencing [RNA-seq], WES, WGS, and gene expression profiling) have helped to identify novel subtypes of B-cell leukemia (e.g., DUX4-r ALL, PAX5 P80R ALL, PAX5-altered [PAX5alt] ALL, ETV6-RUNX1–like ALL, ZNF384-r ALL, and MEF2D-r ALL) and to gain insights in how leukemia cells become resistant to TPs during antimetabolite-based maintenance therapy via darwinian selection of ALL clones which harbor variants in *PRPS1* or *NT5C2*. This knowledge from precision oncology studies can be used to stratify patients based on their recently identified novel risk profiles into different treatments or to develop strategies to overcome their drug resistance mechanisms. Many efforts are underway around the globe to identify molecularly informed therapeutic targets in precision oncology; in pediatric oncology; these trials include the US National Cancer Institute (NCI) Pediatric Molecular Analysis for Therapy Choice (MATCH; NCT03155620) trial, the Precision Oncology for Young People (PROFYLE) trial in Canada, the European Proof-of-Concept Therapeutic Stratification Trial of Molecular Anomalies in Relapsed or Refractory Tumors (ESMART; NCT02813135), and the German INdividualized Therapy FOr Relapsed Malignancies in Childhood 2 (INFORM2; NCT03838042) trial.

One important consideration in modern medicine is that clinically useful approaches must also be cost effective. It was recently reported that the costs of developing new molecular entities (NMEs) into approved drugs continue to increase and range from US$3 billion to $10 billion per launched NME. However, only 5% to 10% of candidate NMEs in early clinical stage trials will become approved drugs. Of note, there is evidence that drug targets with genetic support are twice as likely to become approved, which supports investments into PGx investigations for pharmaceutical companies being beneficial.[52] In addition, the cost for the first full human genome sequence was approximately US$3 billion; this cost is now (as of 2019) approximately US$1000. The markedly lower cost for robust genotyping points to an exciting future for genomics and PGx research and translation, suggesting that the current approach to selecting medications (often "trial and error") will continue to evolve into more scientific-based methods for selecting the optimal medications and doses for individual patients—with genomics playing an increasing role in such therapeutic decisions (Fig. 8.3). As PGx research expands the number of robust associations between genome variation and drug response, the challenges of successful clinical translation and implementation will be a hurdle that will need to be overcome for precision medicine to become a common reality.[53]

SUGGESTED READING

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Abul-Husn NS, Kenny EE. Personalized medicine and the power of electronic health records. *Cell*. 2019;177(1):58–69.

Autry RJ, Paugh SW, Carter R, et al. Integrative genomic analyses reveal mechanisms of glucocorticoid resistance in acute lymphoblastic leukemia. *Nat Cancer*. 2020;1:329–344.

Braun TP, Eide CA, Druker BJ. Response and resistance to BCR-ABL1-targeted therapies. *Cancer Cell*. 2020;37(4):530–542.

Brown P, Pieters R, Biondi A. How I treat infant leukemia. *Blood*. 2019;133(3):205–214.

Dieck CL, Ferrando A. Genetics and mechanisms of NT5C2-driven chemotherapy resistance in relapsed ALL. *Blood*. 2019;133(21):2263–2268.

Diouf B, Cheng Q, Krynetskaia N, et al. Somatic deletions of genes regulating MSH2 protein stability cause DNA mismatch repair deficiency and drug resistance in human leukemia cells. *Nat Med*. 2011;17(10):1298–1303.

Diouf B, Evans WE. Pharmacogenomics of vincristine-induced peripheral neuropathy: progress continues. *Clin Pharmacol Ther*. 2019;105(2):315–317.

Evans WE, Pui CH, Yang JJ. The promise and the reality of genomics to guide precision medicine in pediatric oncology: the decade ahead. *Clin Pharmacol Ther*. 2020;107(1):176–180.

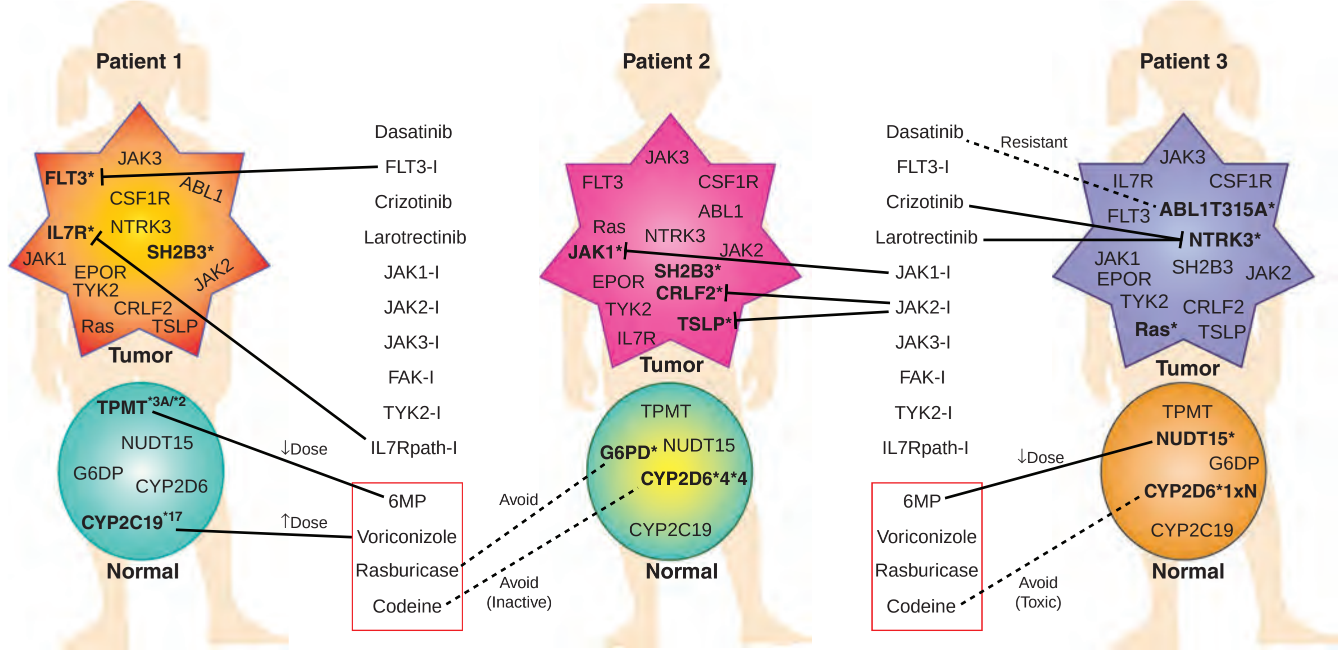

Patient 1
JAK3
FLT3*
ABL1
CSF1R
IL7R*
NTRK3
SH2B3*
JAK1
EPOR
JAK2
TYK2
CRLF2
Ras
TSLP
Tumor
TPMT*3A/*2
NUDT15
G6DP
CYP2D6
CYP2C19*17
Normal
↓Dose
↑Dose
Dasatinib
FLT3-I
Crizotinib
Larotrectinib
JAK1-I
JAK2-I
JAK3-I
FAK-I
TYK2-I
IL7Rpath-I
6MP
Voriconizole
Rasburicase
Codeine
Avoid
Avoid (Inactive)
Patient 2
JAK3
FLT3
CSF1R
ABL1
Ras
NTRK3
JAK1*
JAK2
EPOR
SH2B3*
CRLF2*
TYK2
TSLP*
IL7R
Tumor
TPMT
G6PD*
NUDT15
CYP2D6*4*4
CYP2C19
Normal
Dasatinib
FLT3-I
Crizotinib
Larotrectinib
JAK1-I
JAK2-I
JAK3-I
FAK-I
TYK2-I
IL7Rpath-I
Resistant
6MP
Voriconizole
Rasburicase
Codeine
↓Dose
Avoid (Toxic)
Patient 3
JAK3
IL7R
CSF1R
FLT3
ABL1T315A*
NTRK3*
JAK1
EPOR
SH2B3
JAK2
TYK2
CRLF2
Ras*
TSLP
Tumor
TPMT
NUDT15*
G6DP
CYP2D6*1xN
CYP2C19
Normal

Figure 8.3 COMPLEXITY IN SELECTING OPTIMAL MEDICATIONS BASED ON COMBINATIONS OF GERMLINE AND SOMATIC GENOMIC VARIATION IN CANCER. Somatic (tumor) and germline (normal) genome variation is reflected for three hypothetical patients with Ph-like acute lymphoblastic leukemia (ALL), based on actual genome variants documented from sequencing patients with this disease. For each hypothetical patient, genes indicated have already been shown to have functional alterations in ALL (mutations or structural alterations in leukemia cells, inherited variants altering function in germline DNA), and those with mutations in each patient are indicated in bold font with an asterisk. Somatic variants are often activating, whereas germline genes are typically loss-of-function (*TPMT*, *NUDT15*, and *CYP2D6*) or more rarely gain-of function (*CYP2C19* and *CYP2D6* duplication alleles). Potential selection of medications targeting somatic mutations is based on in vitro or in vivo activity of each medication against target proteins. Multiple variants often occur in the same leukemia cell, as documented in prior sequencing studies, and often only a subset is treated, as depicted for each patient. All inherited germline variants are essentially always present in the tumor (not depicted). Selection of optimal therapy using inherited germline variants follows evidence-based Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines (for medications within the *red box*). A substantial number of additional somatic and inherited genome variants are known to exist in this disease, adding further complexity to evidenced-based selection of optimal treatment. *ABL1*, ABL proto-oncogene 1; *CRLF2*, cytokine receptor–like factor 2; *CSF1R*, colony-stimulating factor 1 receptor; *CYP2C19*, cytochrome P450 family 2 subfamily C member 19; *CYP2D6*, cytochrome P450 family 2 subfamily D member 6; *EPOR*, erythropoietin receptor; *FAK-I*, focal adhesion kinase inhibitor; *FLT3*, Fms-related receptor tyrosine kinase 3; *FLT3-I*, Fms-related receptor tyrosine kinase 3 inhibitor; *G6PD*, glucose 6-phosphate dehydrogenase; *IL7R*, interleukin-7 receptor; *IL7Rpath-I*, interleukin-7 pathway inhibitor; *JAK 1–3*, Janus kinase 1–3; *JAK-I*, Janus kinase inhibitor; *NTRK3*, neurotrophic receptor tyrosine kinase 3; *NUDT15*, nudix hydrolase 15; *6MP*, 6-mercaptopurine; *RAS*, Ras proto-oncogene, GTPase; *SH2B3*, SH2B adaptor protein 3; *TPMT*, thiopurine S-methyltransferase; *TSLP*, thymic stromal lymphopoietin; *TYK2*, tyrosine kinase 2; *TYK2-I*, tyrosine kinase 2 inhibitor. *(Courtesy Evans WE, Pui CH, Yang JJ. The promise and the reality of genomics to guide precision medicine in pediatric oncology: The decade ahead. Clin Pharmacol Ther. 2020;107(1):176–180. All rights reserved.)*

Gu Z, Churchman ML, Roberts KG, et al. PAX5-driven subtypes of B-progenitor acute lymphoblastic leukemia. *Nat Genet*. 2019;51(2):296–307.

Harvey RC, Tasian SK. Clinical diagnostics and treatment strategies for Philadelphia chromosome–like acute lymphoblastic leukemia. *Blood Adv*. 2020;4(1):218–228.

Hunt RC, Simhadri VL, Indoli M, et al. Exposing synonymous mutations. *Trends Genet*. 2014;30(7):308–321.

Hwang S, Mruk K, Rahighi S, et al. Correcting glucose-6-phosphate dehydrogenase deficiency with a small-molecule activator. *Nat Commun*. 2018;9(1):4045.

Johnson JA, Caudle KE, Gong L, et al. Clinical Pharmacogenetics Implementation Consortium (CPIC) guideline for pharmacogenetics-guided warfarin dosing: 2017 update. *Clin Pharmacol Ther*. 2017;102(3):397–404.

King EA, Davis JW, Degner JF. Are drug targets with genetic support twice as likely to be approved? Revised estimates of the impact of genetic support for drug mechanisms on the probability of drug approval. *PLoS Genet*. 2019;15(12):e1008489.

Lauschke VM, Barragan I, Inglman-Sundberg M. Pharmacoepigenetics and toxicoepigenetics: novel mechanistic insights and therapeutic opportunities. *Annu Rev Pharmacol Toxicol*. 2018;58:161–185.

Li B, Brady SW, Ma X, et al. Therapy-induced mutations drive the genomic landscape of relapsed acute lymphoblastic leukemia. *Blood*. 2020;135(1):41–55.

Li B, Li H, Bai Y, et al. Negative feedback-defective PRPS1 mutants drive thiopurine resistance in relapsed childhood ALL. *Nat Med*. 2015;21(6):563–571.

Mackman N, Bergmeier W, Stouffer GA, et al. Therapeutic strategies for thrombosis: new targets and approaches. *Nat Rev Drug Discov*. 2020;19(5):333–352.

Moriyama T, Nishii R, Perez-Andreu V, et al. NUDT15 polymorphisms alter thiopurine metabolism and hematopoietic toxicity. *Nat Genet*. 2016;48(4):367–373.

Paugh SW, Bonten EJ, Savic D, et al. NALP3 inflammasome upregulation and CASP1 cleavage of the glucocorticoid receptor cause glucocorticoid resistance in leukemia cells. *Nat Genet*. 2015;47(6):607–614.

Ramsey LB, Panetta JC, Smith C, et al. Genome-wide study of methotrexate clearance replicates SLCO1B1. *Blood*. 2013;121(6):898–904.

Relling MV, Evans WE. Pharmacogenomics in the clinic. *Nature*. 2015;526: 343–350.

Relling MV, McDonagh EM, Chang T, et al. Clinical pharmacogenetics implementation consortium (CPIC) guidelines for rasburicase therapy in the context of G6PD deficiency genotype. *Clin Pharmacol Ther*. 2014;96(2):169–174.

Relling MV, Schwab M, Whirl-Carrillo M, et al. Clinical Pharmacogenetics Implementation Consortium guideline for thiopurine dosing based on *TPMT* and *NUDT15* genotypes: 2018 update. *Clin Pharmacol Ther*. 2019;105(5):1095–1105.

Roberts KG, Li Y, Payne-Turner D, et al. Targetable kinase-activating lesions in Ph-like acute lymphoblastic leukemia. *N Engl J Med*. 2014;371:1005–1015.

Roden DM, McLeod HL, Relling MV, et al. Pharmacogenomics. *Lancet*. 2019;394(10197):521–532.

Teachy D, Pui CH. Comparative features and outcomes between paediatric T-cell and B-cell acute lymphoblastic leukaemia. *Lancet Oncol*. 2019;20:e142–54.

Tzoneva G, Perez-Garcia A, Carpenter Z, et al. Activating mutations in the NT5C2 nucleotidase gene drive chemotherapy resistance in relapsed ALL. *Nat Med*. 2013;19(3):368–371.

Yang SK, Hong M, Baek J, et al. A common missense variant in NUDT15 confers susceptibility to thiopurine-induced leucopenia. *Nat Genet*. 2014;46(9):1017–1020.

Yee SW, Brackman DJ, Ennis EA, et al. Influence of transporter polymorphisms on drug disposition and response: a perspective from the International Transporter Consortium. *Clin Pharmacol Ther*. 2018;104(5):803–817.

REFERENCES

1. Relling MV, Evans WE. Pharmacogenomics in the clinic. *Nature*. 2015;526:343–350.
2. Roden DM, McLeod HL, Relling MV, et al. Pharmacogenomics. *Lancet*. 2019;394(10197):521–532.
3. Lauschke VM, Barragan I, Inglman-Sundberg M. Pharmacoepigenetics and toxicoepigenetics: novel mechanistic insights and therapeutic opportunities. *Annu Rev Pharmacol Toxicol*. 2018;58:161–185.
4. Hunt RC, Simhadri VL, Indoli M, et al. Exposing synonymous mutations. *Trends Genet*. 2014;30(7):308–321.
5. Diouf B, Evans WE. Pharmacogenomics of vincristine-induced peripheral neuropathy: progress continues. *Clin Pharmacol Ther*. 2019;105(2):315–317.
6. Rukov JL, Wilentzik R, Jaffe I, et al. Pharmaco-miR: linking microRNAs and drug effects. *Brief Bioinform*. 2014;15(4):648–659.
7. Tremmel R, Klein K, Battke F, et al. Copy number variation profiling in pharmacogenes using panel-based exome resequencing and correlation to human liver expression. *Hum Genet*. 2020;139(2):137–149.
8. Diouf B, Cheng Q, Krynetskaia N, et al. Somatic deletions of genes regulating MSH2 protein stability cause DNA mismatch repair deficiency and drug resistance in human leukemia cells. *Nat Med*. 2011;17(10):1298–1303.
9. Li B, Brady SW, Ma X, et al. Therapy-induced mutations drive the genomic landscape of relapsed acute lymphoblastic leukemia. *Blood*. 2020;135(1):41–55.
10. Relling MV, Krauss RM, Roden DM, et al. New Pharmacogenomics Research Network: An open community catalyzing research and translation in precision medicine. *Clin Pharmacol Ther*. 2017;102(6):897–902.
11. Relling MV, Schwab M, Whirl-Carrillo M, et al. Clinical Pharmacogenetics Implementation Consortium guideline for thiopurine dosing based on *TPMT* and *NUDT15* genotypes: 2018 update. *Clin Pharmacol Ther*. 2019;105(5):1095–1105.
12. Yang SK, Hong M, Baek J, et al. A common missense variant in NUDT15 confers susceptibility to thiopurine-induced leucopenia. *Nat Genet*. 2014;46(9):1017–1020.
13. Moriyama T, Nishii R, Perez-Andreu V, et al. NUDT15 polymorphisms alter thiopurine metabolism and hematopoietic toxicity. *Nat Genet*. 2016;48(4):367–373.
14. Suiter CC, Moriyama T, Matreyek KA, et al. Massively parallel variant characterization identifies NUDT15 alleles associated with thiopurine toxicity. *Proc Natl Acad Sci U S A*. 2020;117(10):5394–5401.
15. Yang JJ, Landier W, Yang W, et al. Inherited NUDT15 variant is a genetic determinant of mercaptopurine intolerance in children with acute lymphoblastic leukemia. *J Clin Oncol*. 2015;33:1235–1242.
16. Tzoneva G, Perez-Garcia A, Carpenter Z, et al. Activating mutations in the NT5C2 nucleotidase gene drive chemotherapy resistance in relapsed ALL. *Nat Med*. 2013;19(3):368–371.
17. Li B, Li H, Bai Y, et al. Negative feedback-defective PRPS1 mutants drive thiopurine resistance in relapsed childhood ALL. *Nat Med*. 2015;21(6):563–571.
18. Meyer JA, Wang J, Hogan LE, et al. Relapse specific mutations in NT5C2 in childhood acute lymphoblastic leukemia. *Nat Genet*. 2013;45(3):290–294.
19. Dieck CL, Ferrando A. Genetics and mechanisms of NT5C2-driven chemotherapy resistance in relapsed ALL. *Blood*. 2019;133(21):2263–2268.
20. Paugh SW, Bonten EJ, Savic D, et al. NALP3 inflammasome upregulation and CASP1 cleavage of the glucocorticoid receptor cause glucocorticoid resistance in leukemia cells. *Nat Genet*. 2015;47(6):607–614.
21. Autry RJ, Paugh SW, Carter R, et al. Integrative genomic analyses reveal mechanisms of glucocorticoid resistance in acute lymphoblastic leukemia. *Nat Cancer*. 2020;1:329–344.
22. Johnson JA, Caudle KE, Gong L, et al. Clinical Pharmacogenetics Implementation Consortium (CPIC) guideline for pharmacogenetics-guided warfarin dosing: 2017 update. *Clin Pharmacol Ther*. 2017;102(3):397–404.
23. Mackman N, Bergmeier W, Stouffer GA, et al. Therapeutic strategies for thrombosis: New targets and approaches. *Nat Rev Drug Discov*. 2020;19(5):333–352.
24. Nigam SK. What do drug transporters really do? *Nat Rev Drug Discov*. 2015;14:29–44.
25. Yee SW, Brackman DJ, Ennis EA, et al. Influence of transporter polymorphisms on drug disposition and response: A perspective from the International Transporter Consortium. *Clin Pharmacol Ther*. 2018;104(5):803–817.
26. Ramsey LB, Panetta JC, Smith C, et al. Genome-wide study of methotrexate clearance replicates SLCO1B1. *Blood*. 2013;121(6):898–904.
27. Martinez D, Muhrez K, Woillard JB, et al. Endogenous metabolites-mediated communication between OAT1/OAT3 and OATP1B1 may explain the association between SLCO1B1 SNPs and methotrexate toxicity. *Clin Pharmacol Ther*. 2018;104(4):687–698.
28. Braun TP, Eide CA, Druker BJ. Response and resistance to BCR-ABL1-targeted therapies. *Cancer Cell*. 2020;37(4):530–542.
29. Zabriskie MS, Eide CA, Tantravahi SK, et al. BCR–ABL1 compound mutations combining key domain positions confer clinical resistance to ponatinib in Ph chromosome-positive leukemia. *Cancer Cell*. 2014;26:428–442.
30. Lauschke V, Ingelman-Sundberg M. Prediction of drug response and adverse drug reactions: from twin studies to next generation sequencing. *Eur J Pharm Sci*. 2019;130:65–77.
31. Luzzatto L, Seneca E. G6PD deficiency: a classic example of pharmacogenomics with on-going clinical implications. *Br J Haematol*. 2014;164:469–480.
32. Hwang S, Mruk K, Rahighi S, et al. Correcting glucose-6-phosphate dehydrogenase deficiency with a small-molecule activator. *Nat Commun*. 2018;9(1):4045.
33. Relling MV, McDonagh EM, Chang T, et al. Clinical pharmacogenetics implementation consortium (CPIC) guidelines for rasburicase therapy in the context of G6PD deficiency genotype. *Clin Pharmacol Ther*. 2014;96(2):169–174.
34. Makarona K, Caputo VS, Costa JR, et al. Transcriptional and epigenetic basis for restoration of G6PD enzymatic activity in human G6PD-deficient cells. *Blood*. 2014;124(1):134–141.
35. Gu Z, Churchman ML, Roberts KG, et al. PAX5-driven subtypes of B-progenitor acute lymphoblastic leukemia. *Nat Genet*. 2019;51(2):296–307.
36. Teachy D, Pui CH. Comparative features and outcomes between paediatric T-cell and B-cell acute lymphoblastic leukaemia. *Lancet Oncol*. 2019;20:e142–e154.
37. Brown P, Pieters R, Biondi A. How I treat infant leukemia. *Blood*. 2019;133(3):205–214.
38. Andersson AK, Ma J, Wang J, et al. The landscape of somatic mutations in infant MLL-rearranged acute lymphoblastic leukemias. *Nat Genet*. 2015;47(4):330–337.
39. Neff T, Armstrong SA. Recent progress toward epigenetic therapies: the example of mixed lineage leukemia. *Blood*. 2013;121(24):4847–4853.
40. Annesley CE, Brown P. The biology and targeting of FLT3 in pediatric leukemia. *Front Oncol*. 2014;4:263 https://doi.org/10.3389/fonc.2014.00263. eCollection 2014.
41. Den Boer ML, van Slegtenhorst M, De Menezes RX, et al. A subtype of childhood acute lymphoblastic leukemia with poor treatment outcome: a genome-wide classification study. *Lancet Oncol*. 2009;10:125–134.
42. Mullighan CG, Su X, Zhang J, et al. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. *N Engl J Med*. 2009;360:470–480.
43. Roberts KG, Li Y, Payne-Turner D, et al. Targetable kinase-activating lesions in Ph-like acute lymphoblastic leukemia. *N Engl J Med*. 2014;371:1005–1015.
44. Harvey RC, Tasian SK. Clinical diagnostics and treatment strategies for Philadelphia chromosome–like acute lymphoblastic leukemia. *Blood Adv*. 2020;4(1):218–228.
45. Nardi V, Ku N, Frigault MJ, et al. Clinical response to larotrectinib in adult Philadelphia chromosome-like ALL with cryptic ETV6-NTRK3 rearrangement. *Blood Adv*. 2020;4(1):106–111.
46. van der Wouden CH, van Rhenen MH, Jama WOM, et al. Development of the PGx-passport: a panel of actionable germline genetic variants for pre-emptive pharmacogenetic testing. *Clin Pharmacol Ther*. 2019;106(4):866–873.
47. Smith GC, Pell JP. Parachute use to prevent death and major trauma related to gravitational challenge: systematic review of randomised controlled trials. *BMJ*. 2003;327(7429):1459–1461.
48. Yeh RW, Valsdottir LR, Yeh MW. Parachute use to prevent death and major trauma when jumping from aircraft: randomized controlled trial. *BMJ*. 2018;363:k5094.
49. Shekhani R, Steinacher L, Swen JJ, et al. Evaluation of current regulation and guidelines of pharmacogenomic drug labels: opportunities for improvements. *Clin Pharmacol Ther*. 2020;107(5):1240–1255.
50. Abul-Husn NS, Kenny EE. Personalized medicine and the power of electronic health records. *Cell*. 2019;177(1):58–69.
51. Hoffman JM, Haidar CE, Wilkinson MR, et al. PG4KDS: a model for the pre-emptive implementation of pharmacogenetics. *Am J Med Genet C Semin Med Genet*. 2014;166C:45–55.
52. King EA, Davis JW, Degner JF. Are drug targets with genetic support twice as likely to be approved? Revised estimates of the impact of genetic support for drug mechanisms on the probability of drug approval. *PLoS Genet*. 2019;15(12):e1008489.
53. Evans WE, Pui CH, Yang JJ. The promise and the reality of genomics to guide precision medicine in pediatric oncology: the decade ahead. *Clin Pharmacol Ther*. 2020;107(1):176–180.

PART II CELLULAR BASIS OF HEMATOLOGY

CHAPTER 9

HEMATOPOIETIC STEM CELL BIOLOGY

Marlies P. Rossmann and John P. Chute

Hematopoietic stem cells (HSCs) are characterized by their unique ability to self-renew and give rise to the entirety of the blood and immune system throughout the lifetime of an individual.[1–3] HSCs are very rare cells, representing approximately one in 100,000 bone marrow (BM) cells in the adult.[4] The concept of the existence of an HSC that is capable of reconstituting hematopoiesis in vivo was first introduced more than 70 years ago, when Jacobson et al.[5] demonstrated that lead shielding of the spleen protected mice from otherwise lethal γ-irradiation.[5] Subsequently, Jacobson and colleagues[6] reported that similar radioprotection of mice could be achieved via shielding of one femur. Shortly thereafter, it was demonstrated that intravenous injection of BM cells also provided radioprotection of lethally irradiated mice.[7] Interestingly, investigators initially hypothesized that the radioprotected spleen or BM provided soluble factors that mediated radiation protection.[8,9] However, subsequent experiments by Nowell et al.[10] and Ford et al.[11] critically demonstrated that transplanted BM cells provided radioprotection directly via cellular reconstitution of the blood system. The historical significance of these studies cannot be overestimated because they provided the basis for not only the ultimate isolation and characterization of HSCs but also for the field of hematopoietic stem cell transplantation.

Subsequent landmark studies by Till and McCulloch[12] demonstrated that transplantation of limiting doses of BM cells gave rise to myeloid and erythroid colonies in the spleens of irradiated recipient mice. Importantly, Till and McCulloch showed that the numbers of colonies detected in recipient mice was proportional to the numbers of BM cells injected into the irradiated mice, suggesting that a particular population of hematopoietic cells was capable of reconstituting hematopoiesis in vivo.[12–14] The clonogenic nature of a subset of BM cells was definitively shown when these investigators irradiated BM cells and then transplanted the cells into lethally irradiated mice. Persistent chromosomal aberrations were demonstrated in spleen colonies in recipient mice.[15] It was subsequently shown that cells within the spleen colonies were radioprotective of lethally irradiated mice and contained myeloid, erythroid, and lymphoid cells.[12,16] Taken together, these data strongly suggested the presence of hematopoietic stem or progenitor cells that were capable of in vivo engraftment and generation of multilineage progeny from a small number of parent cells.[17]

EMBRYONIC ORIGIN OF HEMATOPOIETIC STEM CELLS

Mammalian hematopoiesis occurs in several waves, which are separated temporally and spatially and produce different cell types: a transient first "primitive" is followed by a "prodefinitive" and then a "definitive" wave, which persists through life.[18–20] While most of the evidence is derived from the mouse, data from humans, albeit limited, point to a very comparable hematopoietic program.[21,22]

During embryogenesis, the hematopoietic cells of the first, primitive wave are formed when cells from the epiblast that constitute the prospective mesoderm ingress and migrate through the primitive streak between the endoderm and ectoderm, both in the embryo proper and in the extraembryonic yolk sac (YS). In the latter, mesodermal cells aggregate to form blood islands surrounded by visceral endodermal cells on mouse embryonic day (E) 7 to 7.5. The close proximity of erythroid cells and vascular endothelium in YS blood islands, their origin from mesoderm, and their simultaneous differentiation led to the proposal of a common precursor, the hemangioblast, over a century ago.[23,24] In support of this hypothesis, a spontaneous zebrafish mutant, *cloche* (named for its bell-shaped heart because of the loss of endothelium), lacks both vasculature and hematopoietic cells but no other mesodermal lineages such as cardiac progenitors.[25,26] The gene mutated in *cloche* was recently cloned and encodes a PAS (PER-ARNT-SIM) domain–containing basic helix-loop-helix (bHLH) transcription factor (*npas4l*), which belongs to the same class that also includes the aryl hydrocarbon receptor and hypoxia-induced factor (HIF)-1α.[26a] Also, mice lacking FLK1 (VEGFR2, a receptor for vascular endothelial growth factor), expressed on endothelial (progenitor) cells, fail to develop both vascular endothelium and blood islands during embryogenesis.[27,28] Indeed, gene tracing studies in mouse and human embryonic stem cell cultures have identified a progenitor with both hematopoietic and endothelial potential.[29–31]

Primitive hematopoiesis encompasses the generation of primarily large erythroid cells and primitive macrophages.[32–34] Following this initial wave, beginning at mouse E8.25, erythromyeloid progenitors (EMPs) are generated as prodefinitive progenitors.[35,36] Both waves arise transiently in the YS during a time comparable to the first trimester in humans,[37] but the cells lack the capacity for self-renewal and multilineage differentiation present in definitive HSCs. The emergence of EMPs during development is presaged by ckit+ EMP precursors that are characterized by the expression of toll-like receptor 2 (TLR2) at E7.5.[38] Three lineage-tracing studies recently demonstrated that embryonic erythropoiesis is sustained completely by YS EMPs, rather than HSC-derived progenitors.[39] Remarkably, Hoxa neg/low Kit+CD41+CD16/32+ HSC-independent EMPs from the YS have also been shown to give rise to NK cells with cytotoxic capability,[40] and YS EMPs produce osteoclast precursor cells that create space for post-natal BM hematopoiesis.[41,42] Interestingly, epidermal γδ T cells, also known as dendritic epidermal T cells, function in the adult epidermis, and are also derived from YS progenitors.[43] Utilizing single cell transcriptomic analysis and single cell cultures of YS-derived myeloid progenitors, Bian et al. recently provided a comprehensive characterization of the spatiotemporal dynamics of early macrophage development from YS progenitors during embryogenesis.[44] This study provides unprecedented insight into the distinct biology of tissue resident macrophages that are derived from YS progenitors. Importantly, Stremmel et al.[45] showed that CX3DR1+ YS-derived pre-macrophages migrate between embryonic day 8.5

through day 12.5 via the bloodstream into embryonic tissues where they take up long-term residence.

Definitive HSCs capable of long-term (LT), multilineage reconstitution of irradiated adult recipient mice appear at E10.5 in the intraembryonic region encompassing the aorta, gonads, and mesonephros (AGM), in particular in hematopoietic intra-aortic clusters in the ventral wall of the dorsal aorta.[20,46–48] Then, within a remarkably short period of 1.5 days during embryonic development, virtually all HSCs are generated that will replenish the hematopoietic system throughout fetal and adult life.[49,50] Studies by Ganuza et al.[51] utilizing cultured 2 to 7 somite pairs (sp) murine embryonic explants and 2 to 7 sp YS explants confirmed that the embryo, not the YS, is the source of definitive HSCs. Several complementary studies using lineage tracing experiments in both mice and zebrafish have demonstrated that within the dorsal aorta, hemogenic endothelial cells (ECs) are the direct precursors of definitive HSCs.[50,52–56] In a process known as endothelial-to-hematopoietic transition (EHT), HSCs bud off the hemogenic endothelium to form intra-aortic hematopoietic clusters from which they are released into circulation. Interestingly, while the AGM gives rise to HSCs, it is not the site of hematopoietic differentiation.[57] Rather, HSCs colonize the fetal liver where they expand and then differentiate (Ref. 58 and references therein).

Recent advances in single cell labeling and single cell transcriptomic analysis has allowed the isolation and characterization of a population of "pre-HSCs" during murine development.[59] CD45+ pre-HSCs in the AGM were shown to have a unique molecular signature and activation of mechanistic target of rapamycin (mTOR) was reported to be indispensable for the emergence of HSCs.[59] Separate studies which evaluated ex vivo maturation of HSCs from pre-HSCs showed that ex vivo matured HSCs and fetal liver HSCs express programmed death ligand 1 (PDL1), among other immune response genes, although PDL1 expression was not required for engraftment of embryonic HSCs.[60]

Evidence from studies in mice suggests that some definitive hematopoiesis also occurs at sites other than the AGM. By E12 the fetal liver contains more HSCs than can be accounted for by HSCs generated in the AGM alone.[61] Quantitative analysis of HSC distribution showed that both YS[61] and placenta[62,63] generate definitive HSCs that migrate to the liver and other hematopoietic sites.[64] Lastly, a c-Myb- and thus HSC-independent cell lineage that emerges between E8.5 and E9.5 in the YS has recently been shown to give rise to YS macrophages and later on to tissue macrophages in brain (microglia), liver (Kupffer cells), and skin (Langerhans cells).[65]

Human induced pluripotent stem cell models and studies of zebrafish have suggested that a population of immune precursors may originate directly from hemogenic endothelium rather than HSCs.[66,67] Utilizing a RAG1:GFP human reporter system, Motazedian et al.[67] showed that early RAG1+ cells could differentiate into CD4+CD8+ T cells, also possessed B-cell, myeloid, and erythroid potential, while also expressing endothelial markers, and resided within CD31+ endothelial structures. The authors concluded that a wave of T-cell development might originate directly from hemogenic endothelium via a RAG1+ intermediate population.[67] Utilizing a novel technique called "ScarTrace," a single cell sequencing strategy to quantify the clonal history and cell type of thousands of cells in different organs of the developing zebrafish, Alemany et al.[68] identified a novel population of immune cells in the zebrafish fin that had a distinct clonal origin from other hematopoietic cells. In keeping with these studies, Tian et al.[66] utilized temporal-spatial fate mapping analysis and time-lapse imaging to show that a wave of T lymphopoiesis could be detected in the developing zebrafish, arising from ventral endothelium in the AGM and posterior blood islands. This generation of CD4+ T cells is transient and occurs only in the early larval stage, and is later replaced by HSC-dependent T cells of all subtypes.[66] Taken together, these studies provide the impetus for further exploration of evidence for HSC-independent immune cell generation during mammalian development.[69]

DEFINITION AND CHARACTERIZATION OF HEMATOPOIETIC STEM CELLS

Phenotype

Murine Hematopoietic Stem Cells

The HSC is the most well-defined somatic, multipotent stem cell in the body. With the emergence of monoclonal antibody technology and flow cytometry[17,70,71] coupled with in vitro and in vivo functional assays,[72–77] biologists have developed reproducible methods to analyze and isolate murine and human HSCs with a high level of enrichment. In mice, Weissman and colleagues were able to show that antibody-based depletion of BM cells expressing myeloid, B-cell, T-cell, and erythroid cells along with positive selection for cells expressing c-Kit, Sca-1, and Thy-1.1^{lo} ("KTLS" cells) allowed for enrichment of HSCs to approximately one of 10 to 30 cells as measured by the capacity to provide LT, multilineage hematopoietic reconstitution in a competitively transplanted, lethally irradiated congenic mouse.[73,78–81] Because Thy-1.1 is not expressed in many strains of mice,[79] additional markers were developed, including FLK2 (FLT3), the absence of which was shown to substantially enrich for murine LT-HSCs.[82,83] Similarly, it has been demonstrated that the isolation of murine BM c-Kit$^+$Sca-1$^+$Lin$^-$ (KSL) cells based upon the lack of expression of CD34 (34$^-$KSL) enriches for HSCs with LT reconstituting capability at the level of one of 5 to 10 cells (Fig. 9.1).[84]

An alternative and effective method for isolating BM HSCs involves the use of intravital dyes, Hoechst 33342 (Ho33342) and Rhodamine 123 (Rh123).[85–89] HSCs, unlike more committed progenitor cells, efficiently efflux these dyes such that HSCs display low-intensity staining for these dyes.[89,90] Li and Johnson[88] demonstrated that HSCs capable of LT, multilineage repopulation in lethally irradiated mice were significantly enriched in the Rh123^{lo}Sca-1$^+$Lin$^-$ cells, but Rh123^{hi}Sca-1$^+$Lin$^-$ cells possessed little repopulating activity. Similarly, McAlister et al.[87] showed that isolation of Ho33342^{lo} BM mononuclear cells significantly enriched for both the potential to produce colony-forming units in the spleen (CFU-S) on day 14 and cells capable of radioprotection and multilineage reconstitution in lethally irradiated mice. A subsequent and important refinement in the use of Ho33342 to isolate HSCs was made by Goodell et al.,[89] who showed that a Ho33342 side population (SP) can be identified via the emission of Ho33342 at two wavelengths, which yields a tail profile on flow cytometric analysis. Importantly, isolation of Ho33342 SP cells has been shown to yield variable enrichment for HSCs compared with CD34$^-$FLT3$^-$KSL cells, which may be caused by the sensitivity of the assay to variations in staining techniques and batch-to-batch differences in Ho33342 dye.[91–93] However, Matsuzaki et al.[94] demonstrated that transplantation of single Ho33342 SP 34$^-$KSL cells into lethally irradiated C57BL/6 mice yielded donor cell multilineage engraftment greater than 1% in more than 95% of transplanted mice. Therefore, the combination of Ho33342 SP cells with CD34$^-$KSL markers provides a basis for isolation of highly enriched LT-HSCs from mice.[70,91–93]

A major advance in this field involved the discovery by Kiel et al.[95] that the surface expression of CD150, a member of the signaling lymphocyte activation molecule (SLAM) family, significantly enriched for murine BM HSCs. It was also shown that the absence of CD41 and CD48 on CD150$^+$ cells enriches further for the HSC population and that approximately half of CD150$^+$CD41$^-$CD48$^-$ or CD150$^+$CD48$^-$KSL cells reconstitute lethally irradiated mice competitively transplanted with limiting numbers of cells.[95] Taken together, combined isolation of SLAM- and KSL-enriched BM cells has become a reproducible and efficient strategy to isolate murine LT-HSCs with maximal enrichment (see Fig. 9.1).[96]

Expression of the gene Ctnnal1, which encodes alpha-catulin, coupled with ckit expression enriches at a very high level for murine HSCs.[97] Anatomic imaging of alpha-catulin-GFP+ckit+ cells revealed that the vast majority of these HSCs reside in the central marrow and diaphysis, in contact with leptin receptor+ and CXLC12+

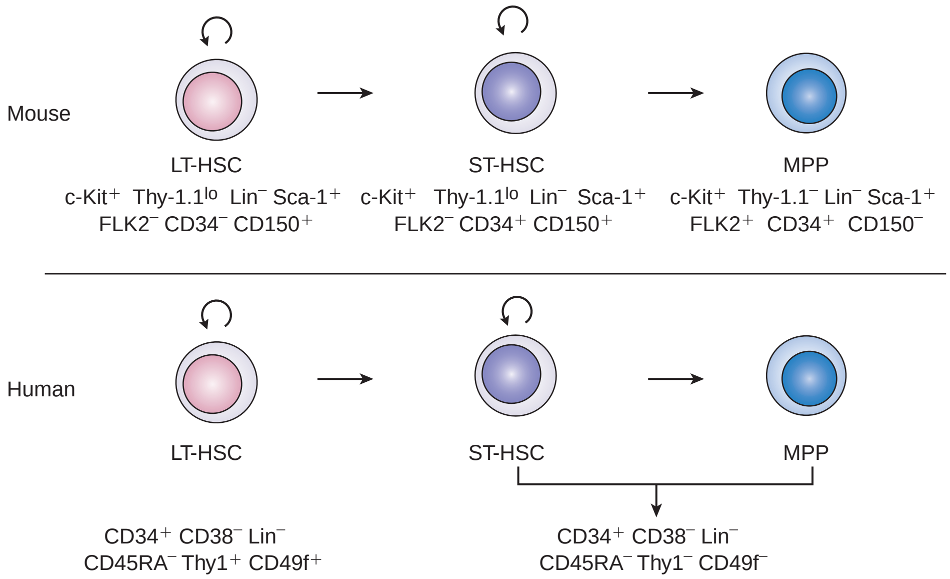

Mouse
LT-HSC
c-Kit$^+$ Thy-1.1^{lo} Lin$^-$ Sca-1$^+$
FLK2$^-$ CD34$^-$ CD150$^+$
ST-HSC
c-Kit$^+$ Thy-1.1^{lo} Lin$^-$ Sca-1$^+$
FLK2$^-$ CD34$^+$ CD150$^+$
MPP
c-Kit$^+$ Thy-1.1$^-$ Lin$^-$ Sca-1$^+$
FLK2$^+$ CD34$^+$ CD150$^-$
Human
LT-HSC
ST-HSC
MPP
CD34$^+$ CD38$^-$ Lin$^-$
CD45RA$^-$ Thy1$^+$ CD49f$^+$
CD34$^+$ CD38$^-$ Lin$^-$
CD45RA$^-$ Thy1$^-$ CD49f$^-$

Figure 9.1 PHENOTYPE OF MURINE AND HUMAN HEMATOPOIETIC STEM CELLS (HSCS). Long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs), and multipotent progenitor cells (MPPs) have precise cell surface markers that discriminate them from more committed progenitor cells. *(Adapted from Prohaska SS, Weissman I. Biology of hematopoietic stem and progenitor cells. In: Appelbaum F, Forman SJ, Negrin RS, Blume K, eds. Thomas' Hematopoietic Cell Transplantation. Chichester: Wiley-Blackwell; 2008:36–63.)*

perivascular stromal cells, and within 10 μm of sinusoidal blood vessels, not arterioles.[97] Similarly, HoxB5 expression was shown to mark HSCs with long-term repopulating capacity in mice and greater than 94% of HoxB5+ long-term HSCs were found to reside in contact with VEcadherin+ ECs, suggesting a BM vascular/perivascular niche for adult HSCs.[98] Conversely, combined analysis of three different HSC reporter lines and nine distinct niche populations in mice suggested that while HSCs were found in close association with leptin receptor+ stromal cells, sinusoidal ECs, and megakaryocytes, this distribution was not different than that of random dots.[99] The authors concluded that the distribution of HSCs in the adult BM may simply reflect the frequency of different cell types in the BM niche, rather than true enrichment in association with a particular niche cell. A more detailed description of the niches for HSCs is presented in Chapters 11 and 14.

Through a combination of single cell gene expression analysis, flow cytometric index sorting, and single cell transplantation assays, Wilson et al.[100] demonstrated that murine hematopoietic cells with high expression of endothelial protein C receptor (EPCR) combined with CD150+CD48-Sca-1^{hi} (EPCR^{hi}CD48-CD150+Sca-1^{hi} HSCs) were the most highly enriched for long-term self-renewal capacity. Additional studies by Cohen et al.[101] revealed that EPCR also regulates HSC retention in the BM via modulation of nitrous oxide production in coordination with protease activated receptor 1. Interestingly, EPCR surface expression on human cord blood (CB) HSCs has been shown to mark human cells with in vivo repopulating capacity following culture with the HSC expansion reagent, UM171.[102] Ito et al. separately demonstrated that murine CD34-HSCs displayed high expression of the angiopoietin 1 (ANGPT1) receptor gene, Tie2, and that 68% of Tie2-GFP+CD34-CD150+CD38low/-Flt3-KSL cells from Tie2 reporter mice were capable of long-term reconstitution in single cell transplantation assays.[103] Other markers recently described to enrich murine HSCs include leptin receptor, which is expressed on BM stromal cells, but has been used to isolate lepR+SLAM+HSCs which display enhanced repopulating capacity in vivo compared to lepR-SLAM+HSCs and an embryonic-like transcriptome.[104] Gulati et al.[105] reported that Hoxb5+ HSCs can be further purified using Neogenin surface expression such that Neogenin-Hoxb5+ HSCs are lineage balanced and provide long-term hematopoietic reconstitution, while Neogenin+Hoxb5+ HSCs are myeloid biased and display reduced reconstituting capacity. Finally, endothelial cell selective adhesion molecule (ESAM) is also expressed by murine HSCs and identifies a proliferative population of HSCs that resides near BM vasculature and retains in vivo repopulating capacity.[106]

Although this chapter focuses on the phenotypic and functional characterization of HSCs, increasing evidence suggests that a subset of adult T-cell progenitors may possess myeloid potential.[107–109] Using various methodologies, Bell et al.[108] and Wada et al.[107] first reported that adult T-cell thymic progenitors possessed myeloid differentiation potential. However, a subsequent study using in vivo transplantation models did not confirm the myeloid potential of adult T cells.[109] De Obaldia et al. reported that the majority of resident granulocytes in the mouse thymus were derived from early thymic progenitors.[110] Mechanistically, it has also been shown that the transcription factor, HES1, constrains myeloid gene expression in T-cell progenitors via repression of C/EPB-α.[111] Taken together, these data suggest that a population of common lymphoid progenitors (CLPs) may indeed possess myeloid differentiation potential.[110,111] Recent studies have also clarified the nature of CLPs and have dissected this population further into an all-lymphoid progenitor cell (ALP), which retains full lymphoid potential and thymic seeding capability, and B lymphoid progenitor cells (BLPs), which are restricted to the B-cell lineage.[112] Whereas ALPs are characterized by the lack of surface expression of LY6D, BLPs express LY6D and upregulate the B-cell-specific factors, EBF1 and PAX5.[112] The phenotypic markers of the hematopoietic hierarchy through myeloid and lymphoid differentiation are shown in Fig. 9.2.

Human Hematopoietic Stem Cells

Significant progress has also been made in the phenotypic characterization of human HSCs via flow cytometric analysis combined with in vivo transplantation assays in immune-deficient mice.[113,114] Of particular note, although murine HSCs can be characterized by the absence of CD34 expression on the cell surface, human HSCs are primarily enriched using CD34 surface expression, and this provides the basis for confirming sufficient HSC content to allow for successful hematopoietic cell transplantation in patients.[17,115,116] There is also some controversy in this area because some investigations have suggested that LT-HSCs can be isolated from CD34$^-$ human hematopoietic cells.[117–120] Of note, only a small percentage (less than 0.1%) of CD34$^+$ human hematopoietic cells possess

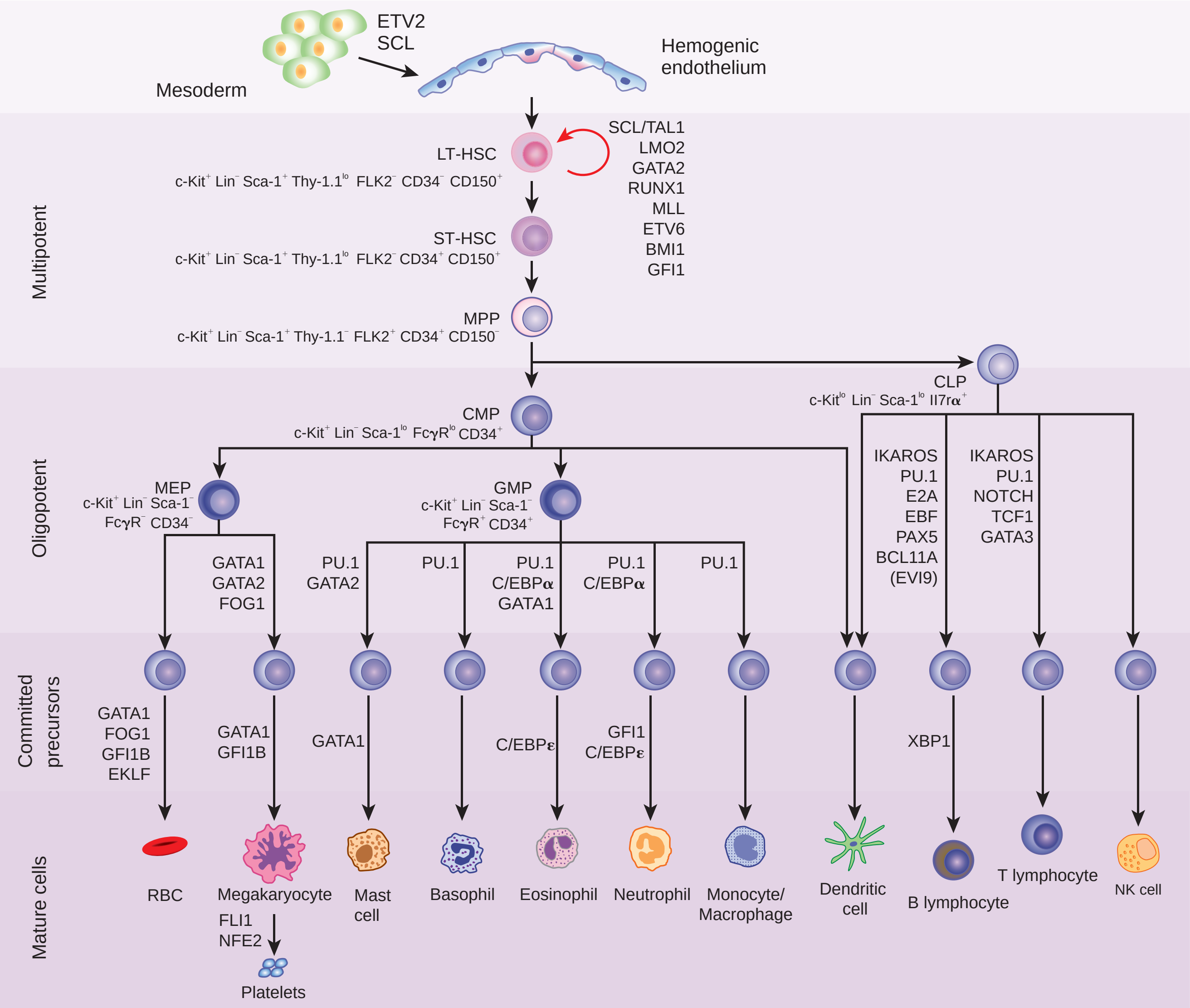

ETV2
SCL
Mesoderm
Hemogenic endothelium
Multipotent
LT-HSC
c-Kit$^+$ Lin$^-$ Sca-1$^+$ Thy-1.1^{lo} FLK2$^-$ CD34$^-$ CD150$^+$
SCL/TAL1
LMO2
GATA2
RUNX1
MLL
ETV6
BMI1
GFI1
ST-HSC
c-Kit$^+$ Lin$^-$ Sca-1$^+$ Thy-1.1^{lo} FLK2$^-$ CD34$^+$ CD150$^+$
MPP
c-Kit$^+$ Lin$^-$ Sca-1$^+$ Thy-1.1$^-$ FLK2$^+$ CD34$^+$ CD150$^-$
Oligopotent
CMP
c-Kit$^+$ Lin$^-$ Sca-1^{lo} FcγR^{lo} CD34$^+$
CLP
c-Kit^{lo} Lin$^-$ Sca-1^{lo} Il7rα$^+$
MEP
c-Kit$^+$ Lin$^-$ Sca-1$^-$
FcγR$^-$ CD34$^-$
GMP
c-Kit$^+$ Lin$^-$ Sca-1$^-$
FcγR$^+$ CD34$^+$
IKAROS PU.1 E2A EBF PAX5 BCL11A (EVI9)
IKAROS PU.1 NOTCH TCF1 GATA3
GATA1 GATA2 FOG1
PU.1 GATA2
PU.1
PU.1 C/EBPα GATA1
PU.1 C/EBPα
PU.1
Committed precursors
GATA1 FOG1 GFI1B EKLF
GATA1 GFI1B
GATA1
C/EBPε
GFI1 C/EBPε
XBP1
Mature cells
RBC
Megakaryocyte
FLI1
NFE2
Platelets
Mast cell
Basophil
Eosinophil
Neutrophil
Monocyte/ Macrophage
Dendritic cell
B lymphocyte
T lymphocyte
NK cell

Figure 9.2 HEMATOPOIETIC CELLULAR HIERARCHY. The phenotypes of murine hematopoietic stem cells (HSCs) and oligopotent progenitor cells, as well as transcription and epigenetic factors critical for specification and differentiation of HSCs are shown. *CLP*, Common lymphoid progenitor; *CMP*, common myeloid progenitor; *GMP*, granulocyte-macrophage progenitor; *LT-HSC*, long-term HSC; *MEP*, megakaryocytic-erythroid progenitor; *NK*, natural killer; *RBC*, red blood cell; *ST-HSC*, short-term HSC. *(Adapted from Prohaska SS, Weissman I. Biology of hematopoietic stem and progenitor cells. In: Appelbaum F, Forman SJ, Negrin RS, Blume K, eds. Thomas' Hematopoietic Cell Transplantation. Hoboken, NJ: Wiley-Blackwell; 2009:36–63; Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. Cell. 2008;132:631–644; Voehringer D. Protective and pathological roles of mast cells and basophils. Nat Rev Immunol. 2013;13:362–375.)*

the capacity to engraft following intravenous injection into non-obese diabetic/severe combined immune deficient (NOD/SCID) mice.[4,114] Further enrichment of human HSCs has been demonstrated via negative selection for surface expression of CD38 and depletion of lineage surface markers.[114,121,122] Thy-1 (CD90) surface expression also enriches for multilineage colony-forming ability and in vivo reconstituting capacity of human hematopoietic cells.[17,123] Majeti et al. showed that the Lin$^-$CD34$^+$CD38$^-$CD45RA$^-$Thy-1$^+$ population in human CB was enriched at the level of one in 10 cells for LT-HSCs.[123] The authors also showed that candidate multipotent progenitor cells (MPPs) were demarcated by the Lin$^-$CD34$^+$CD38$^-$CD45RA$^-$Thy-1$^-$ population, suggesting that the loss of Thy-1 reflects the transition of LT-HSCs to short-term (ST)-HSCs/MPPs.[17,123]

CD49f$^+$ Human Hematopoietic Stem Cells

Although it is possible to enrich murine BM HSCs to the level of nearly single-cell purity using various combinations of cell surface markers, isolation of human BM HSCs to the same level of purity has not been readily achieved.[89,95,124] However, Notta et al. demonstrated that intrafemoral injection of a fluorescence-activated cell sorting (FACS)-purified population of human CB CD34$^+$CD38$^-$CD45RA$^-$Thy-1$^+$ cells that were additionally purified based on surface expression of the integrin α6 (CD49f) yielded 6.7-fold increased human donor chimerism at 20 weeks in NOD/SCID IL2R$\gamma^{-/-}$ (NSG) mice compared with injection with the identical dose of CD34$^+$CD38$^-$CD45RA$^-$Thy-1$^+$CD49f$^-$ cells.[125] Only the Thy-1$^+$CD49f$^+$ cells could be serially transplanted in this study, and the enrichment for LT-HSCs via limiting dilution analysis was estimated to be approximately one in 11 CD34$^+$CD38$^-$CD45RA$^-$Thy-1$^+$CD49f$^+$ cells.[125] Further purification of this population of cells using Rh123 dye demonstrated that single-cell transplantation of Thy-1$^+$Rh123^{lo}CD49f$^+$ cells yielded LT, multilineage engraftment in five of 18 transplanted recipients. Serial transplantation was also successful in two of four secondary mice, suggesting that at least some of the Thy-1$^+$Rh123^{lo}CD49f$^+$ cells undergo self-renewal.[125] Of note, because mice were transplanted via intrafemoral injection in these studies, it remained unknown whether this panel of markers equally identified human HSCs capable of homing properly to the BM after intravenous injection. Nonetheless, these studies revealed that the addition of CD49f$^+$ to the panel of human LT-HSC markers provided an improved capability to isolate human HSCs at a level of purity that was comparable to that applied to murine HSC isolation.

Two additional novel cell surface markers for human HSCs are CD166 (activated leukocyte adhesion molecule) and protein tyrosine phosphatase-sigma (PTPσ).[126,127] Human Lin$^-$CD34$^+$CD38$^-$CD49f+CD166$^+$ cells engrafted in primary and secondary NSG mice at a significantly higher level than Lin$^-$CD34$^+$CD38$^-$CD49f$^+$CD166$^-$ cells.[126] Interestingly, CD166 is also expressed by BM osteoblasts and it was postulated that CD166 mediated HSC maintenance in vivo via homophilic interactions between CD166 expressed on HSCs and osteoblasts.[126] Quarmyne et al. reported that NSG mice transplanted with human CB Lin$^-$CD34$^+$CD38$^-$CD45RA$^-$PTPσ^- cells displayed 15-fold higher human hematopoietic cell engraftment at 16 weeks compared to mice transplanted with Lin$^-$CD34$^+$CD38$^-$CD45RA$^-$ cells or Lin$^-$CD34$^+$CD38$^-$CD45RA$^-$PTPσ^+ cells.[127] Protein tyrosine phosphatase–sigma (PTPσ) was shown to negatively regulate both murine and human HSC repopulation following transplantation, via inhibition of the RhoGTPase, RAC1.[127] Subsequent studies showed that systemic administration of a small molecule, an allosteric inhibitor of PTPσ to irradiated- or chemotherapy-treated mice promoted the early regeneration of HSCs, white blood cells, and neutrophils in vivo, while ex vivo treatment of irradiated human CD34+ hematopoietic stem and progenitor cells (HSPCs) similarly promoted the rescue of human NSG mice repopulating cells.[128]

Advances in methods to perform single cell transcriptomic, genomic, and proteomic analysis have led to several studies characterizing the molecular profile of single human HSCs.[129–135] These studies have revealed remarkable heterogeneity within phenotypically identical human HSCs in steady state and in response to growth factor treatment and have shown that HSC fate determinations can be related directly to expression levels of lineage-specific transcription factors.[136,137] Single cell analysis of HSCs from aging donors revealed age-associated epigenetic reprogramming within cancer and developmental pathways, suggesting the basis for increased incidence of acute myeloid leukemia (AML) with aging (see Chapters 13 and 19).[138] Continued progress in the molecular and functional characterization of single human HSCs will undoubtedly lead to much improved definition of the human hematopoietic hierarchy and the more optimized selection of purified human HSCs for transplantation.

Functional Characterization

In Vitro Assays

The colony-forming cell (CFC) assay does not measure HSC content but rather committed myeloid progenitor cell content via a 14-day assay for colonies within methylcellulose media that is supplemented with specific growth factors.[93] The CFC assay measures colony-forming unit-granulocyte/macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E), and CFU-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM). The CFU-GEMM, or CFU-mix colonies, represent a more immature progenitor cell population. B- and T-cell progenitor cell content can also be measured via in vitro assays but requires specialized coculture conditions, which are described elsewhere.[93,139,140]

The LT culture-initiating cell (LTC-IC) assay is a 6-week in vitro assay in which BM cells are cocultured with murine stromal cells for four weeks followed by replating of the entire culture system into methylcellulose and additional two-week assay for colony formation.[93,141] The LTC-IC, unlike the CFC, measures a more immature stem/progenitor cell population, although the results of the LTC-IC are inherently dependent and limited by technical variabilities in stromal coculture experiments.[93] Importantly, the LTC-IC population lacks LT repopulating cells because transplantation of LTC-ICs into mice in a competitive transplantation assay does not result in any LT reconstitution.[93,113,114]

The cobblestone area-forming cell (CAFC) assay also involves coculture of HSCs with pre-established stromal cell monolayers and relies on microscopic quantification of cobblestone-forming cells embedded underneath the stromal layer.[141,142] It has been shown that CAFC content correlates well with CFU-S content on day 12 and marrow repopulating capacity.[93,141] However, similarly to the LTC-IC, the CAFC assay does not measure LT-HSCs. An advantage of the CAFC and LTC-IC assays is that the estimate of stem/progenitor cell content is not confounded by the homing capacity of the cell population being tested. However, competitive transplantation assays provide a more physiologically relevant measure of functional HSC content and allow quantification of LT-HSC content as well as homing efficiency.[79,93,143]

In Vivo Assays

Colony-Forming Unit–Spleen Assays

The first reproducible in vivo assay for hematopoietic progenitor cells (HPCs) was the CFU-S assay, which was developed by Till and McCullough.[12,113] In this assay, BM cells are injected into lethally irradiated mice, and macroscopic spleen colonies are measured from one to three weeks after injection.[93] These colonies represent ST repopulating cell and MPP activity but do not measure LT-HSC content.[4,93]

Competitive Repopulation Assays

A significant advance in the study of hematopoiesis was the development of the competitive repopulating assay (Fig. 9.3).[93,144] In this assay, an unknown population of hematopoietic cells is transplanted via intravenous injection into lethally irradiated syngeneic mice along with a competing dose of host-derived BM cells.[93,145,146] This assay has been refined over time such that it is typically performed using a limiting dilution method in which several cell doses (typically more than three to five doses; $n = 10$ mice/dose level) of BM cells or purified HSCs (e.g., CD34$^-$KSL cells) are injected into lethally irradiated mice along with a fixed dose of host competitor BM cells, such that a fraction of the recipient mice can be predicted to have nonengraftment.[93,147,148] This approach allows the application of Poisson statistical analysis to provide an estimate of competitive repopulating units (CRUs) within the donor hematopoietic cell population.[93,147–149] An important feature of the CRU assay is the potential to estimate the frequency of LT-HSCs in a given hematopoietic cell population. Donor cell engraftment that is detected within the first 8 to 12 weeks after transplantation reflects the contribution of ST-HSCs, which extinguish at or beyond 12 weeks posttransplant. Therefore, the number of LT-HSCs cannot be convincingly estimated until more than 12 to 20 weeks posttransplantation.[93,150] Dykstra et al.[151] showed that competitive transplantation of single, phenotypic HSCs results in stable donor cell engraftment in lethally irradiated mice beyond 16 weeks, and retroviral marking of HSCs revealed that stable donor-derived hematopoiesis was not observed in recipient mice until six months posttransplant.[150]

A commonly used and rigorous approach to estimate the presence of LT-HSCs is the performance of secondary, tertiary, and quaternary HSC transplants.[93] This approach is based on the principle that a singular feature of primitive LT-HSCs is the capacity to serially reconstitute multilineage hematopoiesis in vivo without exhaustion.[93,152–154] In this method, whole BM is typically collected from primary recipient mice and then injected, along with host competitor BM cells, into lethally irradiated syngeneic mice. Donor cell repopulation is then measured at 12 to 20 weeks post transplantation. Serial transplantation assays have the limitation of being potentially confounded by variables such as homing efficiency of the donor cells.[93,155,156] Therefore, as pointed out by Purton and Scadden[93] in an excellent review of this subject, serial transplantation assays may be better suited to studies of wild type hematopoietic cell populations as opposed to mutant mice-derived hematopoietic cells, which may have alterations in homing or engraftment mechanisms independent of HSC content.[93] Utilization of whole BM avoids issues regarding the fidelity of phenotypic markers of HSCs in mutant mice and is perhaps more broadly feasible than FACS-isolated HSC populations at

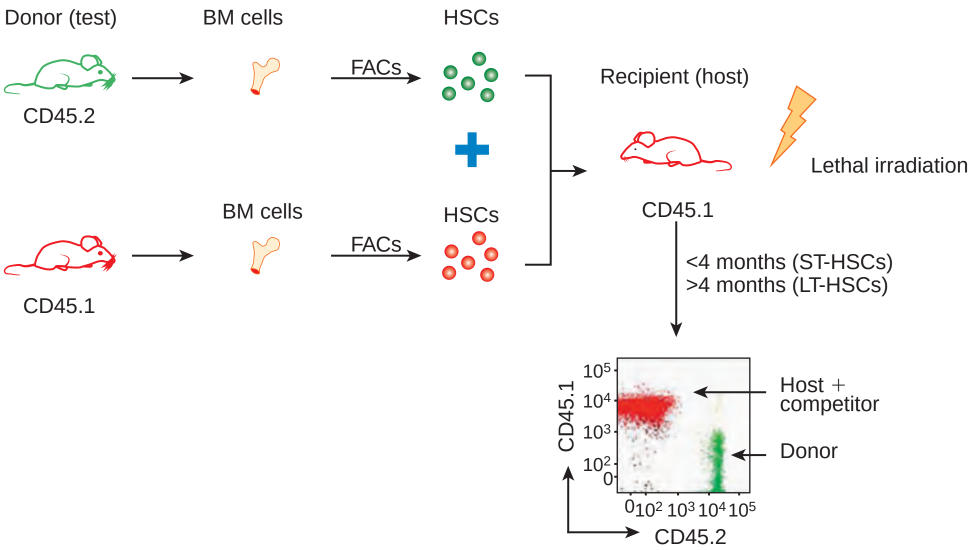


Figure 9.3 COMPETITIVE REPOPULATION ASSAY. Bone marrow (BM) cells from donor mice carrying the CD45.2 allele are sorted by fluorescence-activated cell sorting (FACS) and transplanted with an excess of BM cells from CD45.1 mice into lethally irradiated CD45.1 recipient mice. In general, three to four months posttransplantation, peripheral blood cells are analyzed by flow cytometry to identify the fraction of donor CD45.2 BM cells that, if present, must have homed to and engrafted the myeloablated recipient mouse. While short-term hematopoietic stem cells (ST-HSCs) do not persist in the recipient mouse after four months, long-term (LT)-HSCs are defined by their presence in the recipient mouse after four months and the ability to repopulate secondary, tertiary, and quaternary recipients. *(Adapted from http://stemcellassays.com/2011/11/experimental-bone-marrow-transplantation-101-%E2%80%93-part-2-congenic-mouse-model/.)*

some centers.[93,157–160] However, the use of purified HSCs avoids the potential confounding effects of accessory cells contained within the BM graft on donor cell repopulation and allows for precise determination of effects of growth factors on HSC content in vitro compared with un-manipulated BM.[139,161] Lastly, Poisson statistical analysis and estimation of CRU frequency is based on particular criteria for "positive" donor engraftment in recipient mice, typically 0.1% to 1% multilineage donor engraftment.[139,162] Therefore, the estimation of CRU frequency can be substantially altered depending on what criteria for engraftment are utilized. Given the limitations of flow cytometric analysis for accurate multilineage engraftment of hematopoietic cells, it is recommended that greater than 1% multilineage engraftment is used as a criterion for evidence of donor cell repopulation using the competitive repopulating assay.[93]

Clonal Dynamics of Hematopoietic Stem Cells

Historically, the transplantation assay, in which prospectively purified cell populations are transplanted into myeloablated recipients, has served as the "gold standard" for testing BM compartments for HSC potential. Under optimal conditions, a single HSC can reconstitute the entire hematopoietic system of the host.[78,84,95,151,163] Based on initial transplantation studies, HSCs have been fitted into a simple linear branching hierarchy.[164] Such a hierarchical model assumes that all HSCs have similar developmental potential and, when committed to differentiate, can give rise to both a myeloid and lymphoid progenitor with equal probability. However, tracking experiments with individual retrovirally marked HSCs have revealed extensive heterogeneity within the HSC pool,[150,152,165,166] which was subsequently confirmed by limiting dilution transplantation.[78,163,167–171] Several models have been proposed to explain how diversity in HSC functionality is generated. These are broadly separated into instructive and intrinsic regulation models. According to the instructive models, each HSC is provided with slightly different cues from the microenvironment in which it resides.[172–174] On the other hand, intrinsic regulation of HSC heterogeneity is either completely unpredictable (stochastic)[175–178] or "programmed" (deterministic).[171,179]

The development of methods to obtain highly purified HSCs by FACS has permitted single-cell transplants that address the basis of heterogeneity.[151,163,180–183] Some of these studies demonstrate the existence of myeloid and lymphoid "biased" HSCs and that LT repopulation is dependent on sustained myeloid reconstitution, irrespective of a contribution to the lymphoid compartment. Other studies using various combinations of phenotypic markers and FACS with functional single-cell in vitro or transplantation assays and single-cell expression profiling identified stem-like megakaryocyte-committed progenitors in the HSC compartment in mice.[183–185] In addition, single-cell gene expression profiling of index-sorted mouse or human HSPCs confirmed that unipotent progenitors directly emerge from a continuum of immunophenotypically highly interconnected HSPCs.[186,187] HSCs are also heterogeneous in their response to extrinsic signals such as transforming growth factor-β1 (TGF-β1).[181,188] Importantly, it has been observed that HSC "heterogeneity" is stably propagated to recipients upon secondary transplantation.[151,189,190] This finding argues for some intrinsic regulation in which all HSCs in a clone follow a predetermined fate that is preset earlier in development.

Hematopoiesis as analyzed by transplantation is generally oligoclonal, that is, only few of the transplanted HSC clones contribute to multilineage repopulation.[150,152,191,192] These results have argued for a "clonal succession" model of stem cell activation which posits that a small number of HSCs are sequentially activated from a pool of otherwise noncycling quiescent cells, but that these HSCs exhaust and are replaced over time.[150,193,194] In contrast, the "clonal stability" model states that many or all HSCs have a low but constant cell cycle activity allowing them to continuously contribute to an organism's blood life-long. Support for this model is derived from experiments showing that after transplantation a few HSC clones persist for a long time.[177,195,196] HSCs could also follow the behavior predicted by both models as other studies have shown that HSCs may reversibly switch between the quiescent and a self-renewal state in a homeostatic environment or when challenged by injury, respectively.[197–199]

A general limitation to transplantation approaches is their dependence on HSCs that home to and engraft a niche, proliferate rapidly, and tolerate the stress imposed by the engraftment and an unbalanced cytokine milieu in myeloablated niches. Intriguing studies employing novel genetic labeling strategies in the mouse have explored native, unperturbed hematopoiesis and have demonstrated that, in this setting, steady-state hematopoiesis appears to rely predominantly on long-lived progenitors rather than HSCs.[200–202] While these results have been debated, they argue for the dominant contribution of MPPs or ST-HSCs to hematopoiesis in the untransplanted mouse. Thus, in contrast to the transplantation setting, native hematopoiesis could be highly polyclonal, supported by the successive recruitment of thousands of clones and as such fits the clonal succession model. Why more restricted progenitors, such as ST-HSCs or MPPs, cannot repopulate the hematopoietic system after transplantation is unknown but presumably relates to the ex vivo handling of cells for transplantation or the stress of increased proliferation following transplantation. Furthermore, while it is commonly thought that in myeloid malignancies the "cell of origin," which acquires the first cancer-promoting mutation,[203] is an HSC because of their extended lifespan, these recent studies suggest that long-lived progenitors might be equally suitable candidates for tumor-initiating cells.[201]

Simultaneous tracing of lineage fates and gene expression profiling of single HSPCs has confirmed the existence of uni-lineage clones and revealed that the megakaryocyte can be the primary product of LT-HSCs.[204] More recently, these efforts have been taken a step further in the form of genetically engineered mouse models that can also track cell ancestry information.[205,206] This strategy has revealed four classes of HSC clones operational during native hematopoiesis: differentiation inactive, myeloerythroid restricted, myeloerythroid biased, and multilineage clones.[206] This study also for the first time investigated the relationship between HSC dormancy and cell cycle activity in unperturbed hematopoiesis.[206] Interestingly, differentiation-active HSCs were found with a transcriptional signature characteristic of dormant HSCs. At the same time, differentiation-inactive HSC clones were comprised of cycling cells, suggesting that they contribute to HSC maintenance by self-renewal, which is consistent with other data showing that even the most quiescent HSCs still display low levels of proliferation.[207,208] The approach of studying unperturbed clonal hematopoiesis is rapidly evolving, and future studies will be needed to comprehensively interrogate all HSC fates at steady-state and under perturbation.

REGULATION OF HEMATOPOIETIC STEM CELL FATE

HSCs have the capacity to generate more stem cells, a process called self-renewal, and to produce cells that differentiate into the entire spectrum of mature hematopoietic cells. The balance between these fate choices is thought to be regulated by the type of cell division that HSCs undergo.[209] Asymmetrical cell divisions result in one HSC (self-renewal) and one committed HPC, allowing the maintenance of the stem cell pool while concomitantly ensuring the supply of differentiated cells. However, during development and regeneration the stem cell pool must have the capacity to expand. This can be achieved by symmetrical cell divisions, which will lead to two daughter HSCs capable of self-renewing. Another outcome of symmetric division would be two HPCs, diminishing and ultimately exhausting the HSC pool. The fate decision governing this balance between self-renewal and differentiation is regulated by cell-intrinsic mechanisms including transcriptional and epigenetic mechanisms that are intertwined with cell-extrinsic mechanisms from the microenvironment or by the action of systemic factors.

Extrinsic Regulation

In the adult, HSCs reside in a complex microenvironment or niche, in close association with vascular ECs, perivascular stromal cells, osteoblasts and bone progenitor cells, sympathetic neurons, and other mature hematopoietic cells, such as megakaryocytes and macrophages.[210,211] HSC maintenance, self-renewal, regeneration, and malignant transformation are all necessarily impacted by extrinsic regulation by niche elements.[210,211] The precise contributions of BM niches in regulating HSC fate are described in detail separately in Chapters 11 and 14. Here, we will focus on select extrinsic mechanisms that have been validated to have fundamental roles in regulating HSC fate.

NOTCH Signaling

The NOTCH signaling pathway has been shown to have an important role in regulating the development of the central nervous system, eye, muscle, hematopoietic system, and germline, among others.[212,213] To date, four NOTCH receptors have been identified (NOTCH1–4) as well as five ligands for Notch receptors (JAGGED1 and 2 and DELTA1, 3, and 4).[214] Ligand receptor interaction on HSCs induces two NOTCH cleavage steps, the last of which is mediated by γ-secretase and releases the constitutively active NOTCH-intracellular domain (NICD). The NICD then translocates to the nucleus, interacts with the DNA-binding protein RBPJ (recombination signal binding protein for immunoglobulin kappa J region), and initiates transcription of target genes such as the transcription factors HES1 and HES5 (mammalian homologues of Drosophila hairy and Enhancer of split).[215–218] Depending on the specific NOTCH ligand, different NOTCH receptors and thus different NOTCH target genes are activated, leading to diverse cellular outcomes. For example, NOTCH1 activation by Delta1 and 4 is required for T-cell differentiation while it inhibits differentiation of the B-cell lineage. Jagged2- or Delta1-mediated activation of NOTCH2 inhibits myeloid differentiation and induces the generation of LT-HSCs and MPPs.[216,219]

Activation of NOTCH signaling is sufficient to induce ex vivo HSC expansion. Retroviral expression of the *Notch1* ICD in murine HSCs leads to the generation of an immortal, cytokine-dependent cell line with multilineage in vivo repopulating capacity,[220] and immobilized Delta1 promotes a several-log expansion of murine HSC cultures.[221] Furthermore, Jagged2-mediated activation of NOTCH signaling inhibits the differentiation of human CB CD34$^+$ cells,[222] and culture of human CB HSCs with soluble human Jagged1 induces HSC expansion ex vivo.[223] NOTCH ligands that are expressed in the surrounding BM niche are thought to be critical in promoting HSC maintenance through the activation of NOTCH receptors expressed on HSCs. For example, BM osteoblasts express *Jagged1* and blocking NOTCH activation with a γ-secretase inhibitor significantly decreases murine HSC expansion in BM osteoblast cocultures.[224] Similarly, sinusoidal ECs that express NOTCH ligands stimulate expansion of wild-type but not *Notch1*$^{-/-}$*Notch2*$^{-/-}$ LT-HSCs in vitro.[225] However, although deletion of *Jagged1*, *Notch1*, or *Rbpj* results in impaired embryonic hematopoiesis in the mouse, the physiologic role of NOTCH signaling in the maintenance of the adult HSC pool in vivo is controversial.[224] Deletion of *Jagged1* was shown to have no effect on HSC content in mice,[226] and deletion of *Rbpj* did not cause a defect in HSC repopulating capacity.[227] Interestingly, while neither *Notch1* nor *Notch2* were found to be required for HSC function under homeostatic conditions in vivo,[219,226] challenging the BM with chemotherapy or radiation in the presence of a conditional *Notch2* deletion resulted in more rapid myeloid differentiation at the expense of HSC self-renewal.[219]

In keeping with the evidence obtained from mouse studies that activation of NOTCH signaling can induce HSC expansion, Delaney et al.[228] showed that serum-free culture of human CB progenitor cells with immobilized Delta1 plus cytokines for three weeks yielded a 5.3-fold increase in human hematopoietic cell engraftment in transplanted NOD/SCID mice. This group subsequently completed a phase I clinical trial showing that transplantation of CB cells, which had been expanded with immobilized Delta1 along with an unmanipulated CB unit shortened the time interval to neutrophil recovery (median, 16 days) compared with a cohort that received two un-manipulated CB units (median, 26 days).[229] Of note, in this phase I study, the un-manipulated CB cells demonstrated dominant engraftment by day 80 after transplant and in seven of eight reported recipients, ex vivo expanded CB cells were not detectable in recipients by day 40 posttransplant.[229] The extinction of the Delta1-expanded CB units might be explained by T-cell depletion as donor CB CD8$^+$ T cells of a successful graft have been shown to mediate the rejection of the other CB unit in the setting of double CB transplantation.[230]

WNT Signaling

Wingless-related integration site (WNT) signaling is initiated by the interaction of WNT ligands with the so-called Frizzled/ lipoprotein receptor-related protein (LRP) cell surface receptor complex. In the absence of ligand binding, the WNT signal transducer β-catenin is phosphorylated by kinases such as glycogen synthase kinase-3 beta (GSK-3β), leading to its rapid degradation. Upon activation of the canonical WNT pathway, β-catenin is no longer phosphorylated and thereby stabilized, translocates into the nucleus and interacts with transcription factors of the T-cell factor/lymphoid enhancer factor (LEF/TCF) family to regulate expression of target genes.[231] Several lines of evidence implicate WNT signaling in the regulation of HSC self-renewal and differentiation. First, WNT proteins have been shown to be expressed at sites of embryonic and fetal hematopoiesis, and WNT ligands, receptors, and LEF/TCF transcription factors are expressed by adult HSCs as well as components of the BM microenvironment.[232,233] Second, ample evidence suggests that activation of WNT signaling is capable of promoting HSC expansion, at least in vitro.[234–238] Using mice transgenic for human *BCL2* enabling HSCs to survive in the presence of stem cell factor (SCF) alone,[239,240] treatment of BMKTLS cells with purified WNT3A protein or transduced with active β-catenin ex vivo resulted in their expansion and multilineage reconstitution of competitively transplanted recipients.[234,238] In a related study, treating immune-deficient recipient mice with a GSK-3β inhibitor after transplantation of lineage-depleted human CB HSCs increased their engraftment and repopulating capacity, which was accompanied by faster recovery from post-transplant cytopenias.[241] Importantly, WNT-mediated maintenance of the HSC pool was demonstrated to depend on intact NOTCH signaling,[242] suggesting a deterministic role for the NOTCH pathway in controlling the effects of WNT signaling on the undifferentiated HSC pool.[243] On the other hand, HSCs from mice engineered to conditionally express a stable form of β-catenin had a block in differentiation and failed to self-renew leading to their exhaustion.[244,245] These results raise the possibility that the prior report of HSC expansion in response to β-catenin overexpression may have been affected by the use of *Bcl2* transgenic mice.[234] Alternatively, WNT signaling might have a more pronounced role in vitro than in the more complex in vivo setting.[237]

Some WNT ligands, for example, WNT5A, are able to activate pathways other than the canonical WNT pathway, depending on the particular WNT receptor context.[246] In vivo activation of non-canonical WNT signaling via systemic administration of WNT5A was shown to induce a greater than threefold increase in human CB HPC repopulation in NOD/SCID mice.[235] More recent studies have demonstrated noncanonical WNT signaling in the BM niche is required for HSC maintenance in vitro and in vivo.[247,248] Interestingly, it was shown that *Wnt5a* expression is increased in aging LT-HSCs. Induction of *Wnt5a* in young mice induced aging-associated HSC phenotypes, including apolarity, decreased repopulation capacity, and myeloid bias. Conversely, knocking down *Wnt5a* in old mice attenuated HSC aging.[249]

Although activation of WNT signaling can induce HSC expansion, it is uncertain whether WNT signaling is indispensable for normal hematopoiesis to occur. Conditional deletion of β-catenin in adult BM progenitors did not impair their ability for multi-lineage reconstitution.[250] In support of these results, hematopoietic cell ablation of porcupine, a membrane-bound *O*-acyl transferase essential for WNT ligand secretion and receptor interaction, had no effect on proliferation, differentiation, and self-renewal of adult HSCs in vivo.[251] Conversely, embryonic conditional knockout of β-catenin caused a deficiency in self-renewal of murine LT-HSCs,[252] and HSCs derived from *Wnt3a*$^{-/-}$ mice failed to repopulate secondary recipients,[253] suggesting that WNT signaling might have different roles in embryonic versus adult HSCs.

Transforming Growth Factor-β and Hedgehog Signaling

The TGF-β pathway represents a signaling mechanism that can be activated by members of the TGF-β superfamily including TGF-β, activins, and bone morphogenetic proteins (BMPs).[254] Each of these ligands binds to a specific receptor heterodimer composed of a type I and II receptor, leading to the phosphorylation of a subset of the receptor-regulated SMAD proteins (R-SMADs: SMAD1, 2, 3, 5, and 8). Thus activated R-SMADs then form a complex with the common SMAD SMAD4, and translocate into the nucleus to co-regulate target gene transcription. Another class of SMADs, inhibitory SMADs (SMAD6 and 7) block TGF-β family signaling by binding to R-SMADs.

TGF-β is one of the most potent inhibitors of HSC proliferation in vitro, and neutralization of TGF-β releases HSCs from quiescence.[255–257] It has been suggested that TGF-β mediates cell cycle inhibition in HSCs via upregulation of cyclin-dependent kinase inhibitors, p21, p27, and p57, as well as downregulation of cytokine receptors.[258–262] The role of TGF-β in vivo appears to be more complex. TGF-β likely functions as a negative hematopoietic regulator in vivo as supported by the observation that deletion of TGF-β1 results in extensive myelopoiesis in mice[263] as well as defective homing of HSCs.[264] Moreover, HSCs from mice with a conditional deletion of the TGF-β type II receptor show increased cell cycling and impaired repopulation capacity.[265] Conversely, TGF-β type I receptor null mice display normal HSC self-renewal and regeneration in vivo, although these HSCs exhibited increased proliferation in vitro.[266,267] The discrepancies between different knockout phenotypes could be caused by differences in expression levels of the TGF-β receptors in HSCs and thus their different in vivo importance.[258] Still, TGF-β is considered a critical signal for HSC quiescence in vivo.[258]

BMP signaling is required for mesoderm formation and patterning, and BMPs are key regulators for the hematopoietic specification from mesoderm across different species (reviewed in Ref. 254). BMP4 has been shown to modulate adult human HSC maintenance and proliferation in a concentration-dependent manner, with high BMP4 levels extending the survival of hematopoietic repopulating cells in ex vivo cultures.[268] However, in vivo, BMP signaling does not seem to be required for adult HSC function as determined in mouse knockouts for its signal transducers, SMAD1 and 5.[254] On the other hand, complete inhibition of the SMAD network has demonstrated the importance of SMAD proteins in regulating HSC self-renewal in vivo. Conditional deletion of *Smad4* in mice led to a significantly reduced ability of HSCs to repopulate primary and secondary recipients.[269] Also, retrovirus-mediated overexpression of the inhibitory *Smad7* promoted HSC self-renewal in vivo.[270] Taken together, these results have been interpreted to indicate that SMAD4 positively regulates HSC self-renewal independently from its role as a mediator of SMAD pathway signaling. This hypothesis is supported by evidence demonstrating that SMAD proteins can activate WNT signaling,[271,272] which has been shown to promote HSC expansion as discussed earlier.

As in other species and developmental contexts,[273–275] an intersection between BMP4 and Hedgehog signaling has been described in the human hematopoietic system. Hedgehog proteins play an essential role in the embryonic development of a wide variety of organs, and, like BMPs, they are required for mesoderm patterning.[276,277] Culturing of human CB progenitor cells with Sonic Hedgehog (SHH), one of three human Hedgehog proteins,[278,279] promoted the expansion of cells capable of multi-lineage repopulation in NOD/SCID mice.[280] The addition of Noggin, an endogenous inhibitor of BMP4, blocked the effect of SHH on CB stem cell proliferation in vitro, whereas Hedgehog inhibition did not block BMP4-induced HSPC proliferation, suggesting that BMP4 acts downstream of *SHH* in the regulation of human HSC growth.[280] Several other studies suggest that hedgehog signaling regulates HSC growth. Mice heterozygous for the Hedgehog antagonist Patched1 (*Ptc1*) were shown to have an expanded HSC compartment[281] and their fetal

HSCs exhibited increased colony-forming potential in serial plating assays.[282] Interestingly, while conditional deletion of the Hedgehog effector Smoothened (*Smo*) resulted in a profound loss of LT-HSCs in the embryo,[283] conditional deletion of *Smo* or pharmacologic inhibition of Hedgehog had no effect on HSC content or hematopoiesis in adult mice.[284,285] Thus, Hedgehog signaling during embryogenesis might be required for certain aspects of HSC function important in adult life.[286]

CXCL12–CXCR4 Signaling

CXCL12 is a chemokine that is expressed by BM osteoblasts, ECs, and perivascular stromal cells in the BM microenvironment and regulates the homing and retention of HSCs.[287–289] Expression of CXCL12 or its receptor, CXCR4, is necessary for HSC maintenance in vivo.[288–290] Ding et al. reported that deletion of *Cxcl12* from perivascular stromal cells or vascular ECs depleted HSCs in mice, whereas depletion of *Cxcl12* from nestin$^+$ mesenchymal cells or osteoblasts had no effect on HSC numbers.[288] Greenbaum et al. also showed that deletion of *Cxcl12* in *Prx1*-expressing perivascular stromal cells led to HSC depletion,[291] confirming the importance of CXCL12 signaling in the perivascular niche for HSC maintenance. A more detailed review of CXCL12-CXCR4 signaling in the context of the BM microenvironment, as well as a comprehensive review of HSC-HSC niche signaling interactions,[292] are presented in Chapters 11, 12, 14, and 16.

Intrinsic Pathways

Transcription Factors

Transcription factors are proteins that bind specific sequences of DNA within promoter or enhancer regions to regulate the process of transcribing DNA into RNA.[293] Because the intrinsic phenotype or state of a cell is the result of its gene expression, it is governed by the concerted action of transcription factors (guided by the epigenetic landscape discussed further below). The balance between self-renewal and differentiation of HSCs is intricately regulated by transcription factors of many different classes.[294] Several general principles have emerged. First, given the relative limited number of transcription factors, they are used at multiple stages in development, such that they may be required in HSCs and also subsequently in lineage differentiation.[18] Second, the balance between self-renewal and lineage commitment is thought to be regulated, at least in part, by the antagonism of lineage-specific transcription factors. To promote a given lineage, transcription factors need to actively counteract a factor or factors supporting other cell fates. Third, and most relevant to clinical situations, most hematopoietic transcription factors are subject to somatic mutation and/or chromosomal translocation in one or more hematopoietic malignancies. Thus, malignancy can be viewed as a disruption of normal development. Fig. 9.2 depicts key transcription factors within the hematopoietic hierarchy, and Table 9.1 summarizes main roles of critical transcription factors in HSPCs and hematologic malignancies.

Transcription Factors Required for the Specification of Hematopoietic Stem Cells

As one of the few transcription factors known to be essential for the mesoderm-hemangioblast transition, the ETS class protein ETV2 is expressed in a subset of FLK1$^+$ cells with enhanced endothelial and hematopoietic potential and downregulated thereafter.[295,296] Together with the forkhead transcription factor FOXC2, it cooperatively induces the FLK1$^+$ mesoderm by stimulating the expression of key endothelial and hematopoietic genes such as *Flk1*, *vascular endothelial* (*VE*)*-cadherin*, *Tie2*, *Scl*, and *Notch4*.[297]

Before the emergence of HSCs, the bHLH protein SCL is required for the specification of the bipotent hemogenic endothelium within the hemangioblast during embryonic development.[298] Its knockout in mice, as well as that of its binding partner LIM domain protein *Lmo2*,[299–301] results in the absence of hematopoiesis resulting in early embryonic lethality,[302–305] and an absence of adult HSCs.[306,307] SCL expression is regulated by three hematopoiesis-specific enhancers, one of which comprises an ETS/ETS/GATA motif that binds ETS transcription factors FLI1 and ELF1 as well as GATA2, revealing the transcriptional cascade at the top of the hematopoietic hierarchy.[308] Interestingly, once emerged, HSCs require either SCL or the closely related bHLH factor LYL1 for function and survival.[309–311] Elevated expression of *SCL* and/or *LMO2*, as well as *LYL1*, is found in 35% to 65% of T-cell acute lymphoblastic leukemias (see Chapter 66) (T-ALL), mostly because of chromosome translocations but also, in the case of *SCL*, intragenic deletions.[312]

GATA2, a zinc finger transcription factor, is expressed before HSC emergence in the paraaortic splanchnopleura and later in the AGM.[313] GATA2 has been shown to be essential for the production of cells belonging to all lineages in definitive (or adult) hematopoiesis.[314,315] Interestingly, it functions to preserve the pool of immature HSPCs by preventing the differentiation of hematopoietic precursor cells.[316] Thus, reduction of *GATA2* expression or activity is a prerequisite for HSC commitment. Heterozygous germline mutations in *GATA2* are the cause of several clinical syndromes (see Chapter 49): MonoMAC (monocytopenia and *Mycobacterium avium* complex infections),[317,318] which is also described as dendritic cell, monocyte, and lymphoid deficiency (DCML).[319,320] Individuals with MonoMAC almost invariably progress to a distinct form of myelodysplastic syndrome (MDS) and in 14% and 8% to AML or chronic myelomonocytic leukemia (CMML), respectively.[321] Familial myelodysplastic and AML syndrome[322] as well as Emberger syndrome (primary lymphedema with predisposition to AML)[323] also belong to this group, and in some cases are caused by the identical *GATA2* mutation.

The core-binding protein RUNX1 and its obligate binding partner CBF-β are both required for the transition from hemogenic endothelium to definitive HSCs.[324–327] *Runx1* expression is upregulated during the EHT in the hemogenic endothelium, probably by GATA2, the ETS transcription factors FLI1 and PU.1, and the SCL complex (SCL, LMO2, LDB1).[328] RUNX1 then induces expression of other critical transcription factors such as GFI1 and GFIB which in turn downregulate the endothelial markers TIE2, VE-cadherin, and KIT.[329] In addition, RUNX1 causes rapid global reorganization of transcription factors such as SCL, FLI1, and C/EBPβ which is critical for the EHT and hematopoietic fate.[330] RUNX1 is also required to antagonize the effects of the homeobox transcription factor HOXA3, a negative regulator of specification of the hemogenic endothelium. HOXA3 represses a cascade of transcription factors that promote hemogenesis while at the same time inducing a set of genes critical for maintaining endothelial character.[331] *RUNX1* and *CBF-β* are the most common target of chromosomal translocations in acute leukemia. In particular, translocations resulting in *RUNX1-ETO* and *CBF-β-MYH11* cumulatively account for 15% of AML.[332] Consistent with the fact that neither *RUNX1-ETO*[333] nor *CBF-β-MYH1*[334] induce leukemia by themselves in mouse models but rather require additional mutations, next-generation sequencing studies have identified both as preleukemic lesions.[335,336] In addition, mutations in *RUNX1* are observed in patients with various hematologic diseases including MDS, CMML, ALL, de novo and therapy-related AML, and the autosomal dominant preleukemic syndrome familial platelet disorder with predisposition to AML (FPD/AML).[337]

Transcription Factors Required for Hematopoietic Stem Cell Homeostasis

Many transcription factors from different protein classes that play a role in setting up the hematopoietic hierarchy are necessary for HSC self-renewal in competitive transplantation assays.[1] For example, SCL is expressed much higher in LT-HSCs compared to ST-HSCs, and promotes their quiescence,[338] which preserves the LT-HSC pool.[339] In HSCs as well as differentiating progenitors, SCL exists in different complexes most of which include GATA2 (or GATA1 in the erythroid lineage), E2A, and the non-DNA binding adaptor proteins LMO2 and LDB1.[328,340] Loss of any of these SCL partners leads to

TABLE 9.1 **Roles of Critical Transcription and Epigenetic Factors Involving Hematopoietic Stem and Progenitor Cells in the Development of Hematologic Malignancies**

	Requirement in HSPCs[a]	Type of Alteration	Disease	Reference
Transcription Factor				
SCL (TAL-1)	Mesoderm-endothelial/hematopoietic lineage transition; MegE lineage differentiation	Chromosomal translocations involving *TCR* genes; *SIL-TAL1* fusion gene; aberrant expression for other reasons	T-ALL	245, 263, 533
LMO2	Primitive erythropoiesis; generation and maintenance of definitive HSCs	Chromosomal translocations involving *TCR* genes; interstitial deletion; aberrant expression for other reasons	T-ALL (B-cell lymphoma; B-ALL; X-SCID gene therapy associated T-cell leukemia)	533, 534
GATA2	EHT; HSC survival and self-renewal; MegE, mast cell, monocyte lineage differentiation	Mutations	MonoMAC, MDS, AML, CMML, Emberger syndrome	535
RUNX1 (AML1, CBF-α)	Formation of intra-aortic clusters and HSCs during EHT; lymphopoiesis	*RUNX1-ETO* chromosomal translocations; mutations	AML	288, 299, 536
CBF-β (CBFB)	Emergence of HSCs from HE (in complex with RUNX1)	*CBF-β-MYH11* chromosomal translocations	AML	288, 537, 538
E2A (TCF3)	Maintenance of adult LT-HSC; LMPP, CLP, early thymocyte progenitor, pro-B-cell differentiation	*E2A-PBX1* chromosomal translocations; mutations	Pre-B-cell ALL; T-cell lymphoma	245, 539
ETV6 (TEL)	Maintenance/survival of adult LT-HSCs	*ETV6-RUNX1* chromosomal translocations; mutations	Pre-B-cell ALL; immature adult T-ALL	297, 299, 539, 540
MYB (C-MYB)	Self-renewal and multilineage differentiation of adult LT-HSCs	Chromosomal translocation involving TCRβ; duplication	T-ALL	541–543
EVI1	Generation of definitive HSCs; Self-renewal of adult LT-HSCs	Aberrant expression; Chromosomal translocations involving *RUNX1* and *ETV6*	AML; MDS, CML blast crisis	246, 544
Epigenetic Factor				
MLL	Generation of definitive HSCs; adult HSC quiescence and self-renewal	Chromosomal translocations involving *AF4*, AF9, *ENL*, *AF10*, *ELL*, *AF6*, etc. (79)	ALL, AML	545, 546
DNMT3A	HSC differentiation	Mutations	AML, T-cell leukemia and lymphoma, MDS	348
TET2	HSC differentiation	Mutations, *IDH1/2* mutations	CMML, AML, MDS, T-cell lymphoma, DLBCL	547, 548
ASXL1	HSC differentiation	Mutations	CMML, AML, MDS	359, 549, 550

[a]Data from complete or conditional knockout studies in mice.

AML, Acute myeloid leukemia; *B-ALL*, B-cell acute lymphoblastic leukemia; *CML*, chronic myeloid leukemia; *CMML*, chronic myelomonocytic leukemia; *DLBCL*, diffuse large B-cell lymphoma; *EHT*, endothelial-to-hematopoietic transition; *HE*, hemogenic endothelium; *HSC*, hematopoietic stem cell; *HSPCs*, hematopoietic stem and progenitor cells; *IDH1/2*, isocitrate dehydrogenase 1 and 2; *LMPP*, lymphoid primed multipotent progenitors; *LT*, long-term; *MDS*, myelodysplastic syndrome; *MegE*, megakaryocyte/erythrocyte; *MLL*, mixed-lineage leukemia; *MonoMAC*, monocytopenia and *Mycobacterium avium* complex infections; *SIL*, *SCL* interrupting locus; *T-ALL*, T-cell acute lymphoblastic leukemia; *X-SCID*, X-linked severe combined immunodeficiency.

defects in HSC maintenance.[304,311,341–343] Apart from GATA2 and LMO2, deregulation of E2A also causes hematologic malignancies. Six percent of all pediatric ALLs, in particular pre-B-cell ALL (23%), are caused by a chromosomal translocation that fuses the *E2A* gene with *PBX1* (pre-B-cell leukemia homeobox 1),[344,345] which encodes an important HOX interacting factor discussed later.

The ETS-related transcriptional repressor TEL/ETV6 is specifically required for HSC survival but not their emergence.[346] Like E2A, ETV6 is also involved in chromosomal translocations; the *ETV6-RUNX1* is the most common fusion gene in pediatric cancers, found in 22% of childhood ALL (of the pre-B-cell subtype).[347] In contrast to the translocation generating the *E2A-PBX1* fusion gene most of the *ETV6-RUNX1* fusions seem to originate in utero.[348]

Another transcription factor associated with human malignancies, *EVI1*, is specifically expressed in LT-HSCs. When overexpressed in mice *EVI1* boosts LT-HSC self-renewal, whereas its heterozygous loss leads to marked reduction of their self-renewal capacity.[349] Aberrant expression of *EVI1* isoforms is observed in 8% to 11% of AML,[350,351] and 28% of mixed-lineage leukemia (MLL)-rearranged AML,[352] and usually associated with a poor prognosis.[351] Furthermore, its translocation with *ETV6* or *RUNX1* is associated with progression to blast crisis in chronic myeloid leukemia (CML).[353,354]

The Homeobox Cluster in Hematopoietic Stem Cell Self-Renewal

Homeobox (HOX) genes encode homeodomain-containing transcription factors critical for embryonic patterning, organized into four paralogous clusters (A, B, C, and D) on four chromosomes.[355] Because of limited DNA sequence specificity and selectivity, HOX proteins function through interaction with DNA-binding cofactors, in particular, PBX and/or myeloid ectopic insertion site (MEIS) family members.[356,357] At least 22 of the 52 HOX genes (none from the HOXD cluster) are expressed in mouse and human HSPCs and are subsequently downregulated to permit lineage commitment.[358] Therefore, continuous HOX expression generally blocks differentiation and leads to rapid expansion of preleukemic HSPCs. In mice, overexpression of *HOXA10* has been shown to block myeloid and lymphoid differentiation leading to AML.[359] This is also the case for *Hoxa9*, in particular conjunction with *Meis1* or the *E2A-Pbx1* fusion gene.[360–362] *HOXA9* overexpression belongs to a gene signature that distinguishes AML from ALL, and in AML patients which highly correlates with treatment failure.[363]

A number of leukemic chromosomal translocations, either directly or indirectly, lead to the overexpression of HOX genes (see Chapter 58). The nuclear pore complex protein NUP98 was first implicated in hematologic malignancies by the discovery of *NUP98-HOXA9* fusions in AML.[364,365] Approximately half of all *NUP98* translocations involve HOX genes, most commonly *HOXA9* in AML, MDS, CML, and CMML, but also *HOXA11* and *HOXA13* as well as their paralogues in the B and C cluster.[366,367] While the overall prevalence of these fusions is low, they are associated with a poor prognosis.[368]

In normal HSPCs, the expression of HOX genes is regulated by MLL1,[369] as part of a multiprotein complex that regulates the chromatin structure at HOX clusters.[370,371] MLL1 fusion proteins in *MLL1*-rearranged leukemias further induce the transcription of specific HOX genes including *HOXA5*, *HOXA9*, and *HOXA10*.[372,373] Indeed, HOX gene overexpression is essential for MLL1-fusion induced leukemogenesis as demonstrated by the dependence of transplanted AMLs induced by both *MLL1-ENL* and *MLL1-AF4* rearrangements on *HOXA9*.[374,375]

Transcription Factor Networks

Transcription factors act within larger multiprotein complexes. In the setting of hematopoiesis, transcription factors often act positively to sustain their own expression, while simultaneously acting to cross-regulate other transcription factors, thereby establishing complex transcriptional networks. With the advent of genome-wide molecular studies, networks can be constructed computationally. Examples include a core heptad regulatory network consisting of SCL, LYL1, LMO2, ERG, FLI1, GATA2, and RUNX1 which binds to over 1000 genes in HSPCs[376,377] or a regulatory module composed of GATA1, GFI1, and GFI1B with a potentially important role in specifying early lymphoid cells.[378] Also, profiling of transcription factors in single HSPCs combined with computational lineage progression analysis suggests a role for GATA2 in driving a network that specifies megakaryocytic and erythroid from lymphomyeloid lineage cells.[379] As these constructed networks become more mature, in silico methods will predict developmental outcomes that can be tested experimentally by modulation of one or multiple transcription factors in a direct manner.[380] A comprehensive understanding of how gene regulatory networks are perturbed in hematologic malignancies may lead to new therapeutic approaches based on restoring normal regulatory patterns.

Epigenetic Regulation of Hematopoietic Stem Cell Self-Renewal

Epigenetic regulation (see Chapter 2) leads to a "stably heritable phenotype resulting from changes in a chromosome without alteration in the DNA sequence."[381] Epigenetic modifications include DNA methylation, covalent histone modification, chromatin remodeling, and mechanisms involving noncoding RNAs (ncRNAs) which will be discussed further later. By modifying chromatin structure and accessibility, epigenetic mechanisms regulate the expression of genes involved in determining the balance between self-renewal and lineage commitment of HSCs, ultimately leading to all hematopoietic cell types. Epigenetic dysregulation has been implicated in the pathogenesis of virtually all hematologic malignancies.[382,383] In contrast to genetic mutations, epigenetic alterations are in principle reversible, making epigenetic modifiers attractive targets for the treatment of hematologic malignancies. Fig. 9.2 depicts key epigenetic factors within the hematopoietic hierarchy, and Table 9.1 summarizes the main roles of critical epigenetic factors in HSPCs and hematologic malignancies.

DNA methylation plays a critical role in HSC self-renewal and commitment.[384–387] Through recruitment of multiprotein complexes, methylated DNA results in transcriptional repression of nearby genes. In mammalian cells, DNA methylation occurs at cytidines, mostly in the context of CpG dinucleotides, dispersed throughout the genome and clustered (as CpG islands) within gene promoters. In a number of hematologic cancers, for example, the progression from MDS to AML, promoter CpG islands become hypermethylated and the affected genes, including tumor suppressor genes, silenced.[388] Furthermore, subgroups of AML can be defined by unique DNA methylation profiles, which can be used to stratify AML patients with respect to their overall survival.[389] The family of DNA methyltransferases includes the de novo DNA methyltransferases DNMT3A and DNMT3B that establish methylation whereas another family member, DNMT1, maintains DNA methylation.[390,391] DNA methylation is required for HSCs to differentiate. Conditional knockout of *Dnmt3a* in mice, further pronounced by ablation of *Dnmt3b*, leads to an accumulation of self-renewing HSCs in the BM which lose their differentiation capacity upon serial transplantation.[392,393] Next-generation sequencing studies have identified *DNMT3A* mutations in most hematologic malignancies, in particular in patients with cytogenetically normal AML (over 30%),[394] where they can be detected as preleukemic lesions,[335,395] and T-ALL (16%[396]; for a complete list see[397]). In contrast, *DNMT1* mutations are rarely found in AML.[383] In agreement with the high frequency of *DNMT3A* mutations in these malignancies and the persistence of *DNMT3A*-mutant clones after chemotherapy, a recent study found that mouse HSCs depleted of *Dnmt3a* can regenerate over 12 rounds of transplantation, with the transplanted HSC pool becoming more homogenous, that is, harboring fewer somatic variants, over time.[398]

Recently it was discovered that DNA methylation is, in fact, reversible,[399,400] owing to the activity of ten-eleven translocation (TET) proteins (TET1, TET2, and TET3), which iteratively oxidize 5-methylcytosine present in methylated CpG dinucleotides.[401] Mice deficient for *Tet2*, the only TET expressed in the BM, showed enhanced HSC self-renewal and developed a CMML-like disease.[402–404] *TET2* is mutated in 49% of CMML[405] and up to 23% of AML patients (see Chapter 59 and 73).[406–408] In a mouse model of *Tet2* deficiency, vitamin C treatment mimicked *Tet2* restoration by enhancing TET function, and prevented the progression of CMML and AML.[409] Conversely, depleting vitamin C levels in mice increased self-renewal of HSCs, in part by reducing TET2 function, and vitamin C depletion cooperated with leukemic $Flt3^{ITD}$ mutations to promote leukemogenesis.[410] Interestingly, gain-of-function mutations in isocitrate dehydrogenase 1 and 2 (*IDH1/2*) that indirectly impair TET2 function are mutually exclusive with *TET2* mutations in AML (see Chapter 59). *IDH1* and *2* are found mutated in 13% to 33% of AML cases and lead to similar methylation profiles as *TET2* mutations.[411–415] IDH1/2 enzymes catalyze the conversion of isocitrate to α-ketoglutarate (α-KG), but mutated IDH1/2 converts α-KG further to 2-hydroxyglutarate, which inhibits the α-KG dependent TET2 enzyme.[416,417] In contrast, both in AML and T-cell lymphoma cases, DNMT3A and TET2 mutations have been found to co-occur,[418,419] and mutations in both genes cooperate to promote leukemogenesis in mice.[419]

The effects of histone modifications (see Chapter 2) are combinatorial in nature, and there is crosstalk between DNA methylation and histone modifications.[420] Histones have protruding flexible and charged NH_2-termini ("tails") that can be post-translationally modified in various ways, by methylation, acetylation, phosphorylation, ubiquitination, and SUMOylation, to name but a few.[421] Many histone modifications have been implicated in HSC self-renewal by knockout or overexpression of chromatin regulators that function as their "writers," "readers," or "erasers" (for a recent list, see Ref. 422). Recurrent mutations of CREBBP and/or EP300, members of the histone acetyltransferase family that activate transcription, have been detected in several lymphoma types,[423] and 18% of relapsed pediatric ALL patients see Chapters 66 and 78 exhibit mutations in the enzymatic domain of CREBBP, which are thought to contribute to drug resistance.[424]

Unlike histone acetylation, methylation of histones can have activating or repressive effects, dependent on the context and the targeted histone residue. With respect to active marks, most epigenetic studies have focused on lysine methylation carried out by lysine methyltransferases (KMTs) and removed by lysine demethylases (KDMs). The large multidomain KMT *MLL1* is essential for the generation of definitive HSCs in the mouse,[425] likely through its regulation of HOX cluster genes.[369] The *MLL1* locus has been found to be involved in chromosomal translocations in up to 70% of infant and childhood AML and ALL and in 5% to 10% of adult leukemia (see Chapter 56),[426] which is generally associated with a poor prognosis.[427] 85% of *MLL1* translocations involve six proteins (AF4, AF9, ENL, AF10, ELL, and AF6), of which MLL1-AF4 in ALL and MLL1-AF9 in AML are most common.[428] Many MLL1 fusion partners belong to the super elongation complex (SEC); when fused to MLL1 the aberrantly recruited SEC bypasses the normal transcription initiation-to-elongation checkpoints leading to high expression of MLL1-regulated genes such as HOX, WNT, and leukemic stem cell (LSC) target genes.[429] Furthermore, almost all fusion proteins aberrantly recruit another KMT, DOT1L, which by methylating H3K79 induces the expression of MLL1 target genes sufficient for leukemogenesis such as *HOXA9* and *MEIS1*.[360] In addition, MLL1 fusion proteins provoke a global loss of DNA methylation in AMLs.[430] Still, *Dnmt1* haploinsufficiency, while not perturbing normal HSC function, significantly delays leukemia progression in the *MLL-AF9* AML mouse model.[431]

At many lineage-specifying promoters in HSCs, epigenetic activators are balanced with repressive complexes, namely the polycomb repressive complex 1 (PRC1) and PRC2. The latter typically comprises the catalytically active EZH2, as well as EED and SUZ12, and is responsible for di- and trimethylation of H3K27, a repressive histone mark that is then "read" and maintained by PRC1 through histone H2A ubiquitination.[432,433] EZH2 also recruits DNA methyltransferases to its target genes, thereby enhancing its repressive effect.[434] Overexpression of *Ezh2* in mice enhances LT-HSC self-renewal by silencing differentiation genes[435,436] and causing a shift to the expression of proliferation genes.[437] EZH2 has been described with either tumor suppressive or oncogenic roles, depending on the cellular context. *EZH2* gain-of-function mutations, resulting in overall "hyper-trimethylation," occur in 7.2% and 21.7% of follicular lymphoma and diffuse large B-cell lymphoma, respectively (see Chapter 78). Loss-of-function mutations have been observed in 12% of myelodysplastic/myeloproliferative neoplasms and 18% of T-ALL cases but are rare in de novo AML (1% to 2%) (see Chapters 59, 66 and 73). PRCs do not bind to DNA directly but require the interaction with other proteins such as ASXL1. *ASXL1* is mutated in 6% to 30% of AML and 43% of CMML.[411] These mutations hinder ASXL1 from recruiting PRC2 to and thus repressing target genes such as *HOXA9*.[438]

Histone positioning or sliding, ejection and the incorporation of histone variants is achieved by ATP-dependent chromatin remodeling complexes which can be classified into four families, imitation switch (ISWI), chromodomain helicase DNA-binding (CHD), switch/sucrose non-fermentable (SWI/SNF), and INO80.[439] Multiple subtypes exist within each family, so that the composition of remodeling complexes varies across tissues and differentiation stages. Several subunits of the mammalian BAF subtype of SWI/SNF remodelers have been found to be essential for HSC function.[440,441] On the other hand, the BAF complex ATPase subunit BRG1 (SMARCA4) is required for leukemia maintenance,[442,443] in particular, by remodeling chromatin at Myc enhancers in mouse AML cells.[443]

Recurrent loss-of-function mutations in ARID1A (BAF250a) occur in acute promyelocytic leukemia,[444] and conditional *Arid1a* knockout resulted in an accumulation of LT-HSCs that, however, retained markedly reduced multilineage reconstitution capability.[445] Molecularly, Arid1a loss leads to a globally repressed chromatin landscape, including at several genes critical for hematopoietic differentiation.[445] BPTF, a member of the NURF subtype of the ISWI remodelers, has been shown to promote chromatin accessibility at genes involved in HSC "stemness" such as *Meis1*, *Pbx1*, *Mn1*, and *Lmo2*, and, indeed, maintenance of adult HSCs including LT-HSCs requires BPTF.[446]

Beyond studying the importance of epigenetic modifications, recently, attention has been directed toward higher chromatin organization in HSC function (see Chapter 4). Within nuclei, chromosomes occupy territories, and, within those, transcriptionally active euchromatin and inactive heterochromatin segregates into so-called A and B compartments, respectively. Compartments in turn are subdivided into regions called topologically associating domains (TADs), which are anchored by the CCCTC-binding zinc finger protein CTCF and the cohesin complex and harbor enhancer-promoter loops that dynamically form in a tissue-specific manner and are required to activate gene transcription.[447] The cohesin complex member *STAG2* is mutated in 5% to 20% and 2% to 12% in MDS and AML (see Chapter 59), respectively.[448–450] In mice, *Stag2*-deficient HSCs display increased self-renewal and reduced differentiation potential, leading to a myeloid dysplasia.[451] Interestingly, while STAG1 can maintain TADs in the absence of STAG2, enhancer-promoter loops at PU.1 target genes are not formed properly, leading to the block in myeloid differentiation.[451] A recent study found that in HSPCs, but not differentiated erythroid or T cells, large genomic regions with low DNA methylation and high levels of trimethylated H3K27 form chromatin loops that connect distant chromatin regions, including regions on different chromosomes.[452] The three-dimensional chromatin organization also might contribute to the differences in phenotypes and functions between fetal liver and adult HSCs because, despite TADs being largely conserved between the two HSC types, the enhancer-promoter loops forming within them were dependent on the developmental stage.[453]

The recent development of an assay to map accessible chromatin regions (see Chapter 2) including enhancers (assay for transposase-accessible chromatin using sequencing or ATAC-seq) that operates with small numbers of cells has allowed the systematic characterization of the chromatin landscape of both HSCs and leukemic clones or single cells.[454,455] Of note, the landscape of accessible and thus "open" enhancers seems to better reflect cell identity than its transcriptional profile.[455] Furthermore, ATAC-seq profiles of cells at distinct stages of AML evolution have revealed substantial regulatory heterogeneity with individual clones exhibiting mixed chromatin profiles reminiscent of multiple healthy and normally distinct hematopoietic cell states. These findings lead to the intriguing hypothesis that leukemic cells might exist as stable intermediate cell states, which differs from healthy hematopoiesis, where stable intermediate cell states have not been observed.[455] Future large-scale single-cell studies of leukemic cells will be needed to address whether stable intermediate leukemic cell states indeed exist or whether such cells just represent clonal dominance of a rare cell type.

Regulation of Hematopoietic Stem Cells by Noncoding RNAs

Sequencing the human genome revealed that less than 1.5% of the DNA encodes proteins[456] but, remarkably, subsequent analyses discovered that still 76% of the genome is transcribed into RNA.[457] Many ncRNAs are enriched in HSCs compared to hematopoietic progenitors.[458] ncRNAs other than ribosomal RNAs and transfer RNAs are arbitrarily categorized into long ncRNAs (lncRNAs) and short ncRNAs if greater than 200 or less than 200 nucleotides long, respectively. Among the short ncRNAs, microRNAs (miRNAs) are ~22-nucleotide regulatory ncRNAs that repress gene expression predominantly by binding to the 3′ untranslated region of protein-encoding mRNAs leading to their destabilization.[459] A miRNA can target large numbers of mRNAs, and a single mRNA can be regulated by multiple miRNAs.[460] HSCs depend on miRNA function for their survival as demonstrated by the finding that deletion of *Dicer* or *Ars2*, both essential factors for miRNA biogenesis, results in HSC apoptosis and BM failure.[461,462] More than 100 different miRNAs are specifically expressed during hematopoiesis, preferentially targeting and fine-tuning expression of transcription factors and their upstream activators.[463] Hematologic malignancies show characteristic changes in their miRNA expression profiles. For example, ALL samples can be classified into subtypes according to their miRNA expression patterns.[464]

Numerous miRNAs are specifically enriched in HSPCs, including miR-155,[465] miR-125a,[462] miR-125b,[466–468] miR-29a,[469] miR-126, and miR-130a.[470] Overexpression of the miRNA cluster miR-99b/let7e/125a or miR-125a alone is capable of expanding the HSC pool in mice,[71,462,466] potentially by inhibiting apoptosis of HSCs mediated by the proapoptotic gene *Bak1*, which is a miR-125a target.[462] In addition, several miRNAs have been implicated in regulating HPC differentiation, including miR-155 (lymphoid and myeloid development),[465,472] miR-223 (myeloid development),[473,474] and the miR-181/miR-150/miR-17–92 cluster (lymphoid development).[475–478] The example of miR-155 shows that, like transcription factors, some miRNAs are repurposed during hematopoietic ontogeny.[479]

Most of the miRNAs conferring a competitive advantage to the engrafted BM have been implicated in malignant transformation, and are therefore called oncomiRs.[480] Overexpression of miR-125 family members causes myeloid and lymphoid malignancies in mice[466,468,471,481,482] and they are upregulated in chromosomal translocations leading to MDS/AML and B-cell ALL.[483–485] oncomiRs contribute to leukemic phenotypes by different mechanisms such as expediting cell cycle transitions,[469] targeting tumor suppressors such as TET2,[486] or dysregulating the balance between lineage-specific transcription factors.[472] miR-155, the first miRNA shown to be sufficient to cause lymphoblastic leukemia or high-grade lymphoma in a transgenic mouse model,[487] is overexpressed in B-cell lymphomas[488] and AML.[489,490] miR-196, which is upregulated specifically during the transition from quiescent LT-HSCs to ST-HSCs, targets several of its neighboring HOX genes important for self-renewal such as *HOXA9*.[479,491–494] Like HOX genes, miR-196b is transcriptionally regulated by MLL and highly induced in MLL-rearranged leukemias, contributing to an unfavorable prognosis.[493–495] In this context, miR-196 has been shown to be necessary for *MLL-AF9* dependent immortalization of BM cells.[495] miRNAs can also function as tumor suppressors, exerting their function by targeting oncogenes. For example, the miRNA cluster miR-15a/miR16-1 is deleted or epigenetically silenced in 68% of chronic lymphoid leukemia patients (see Chapter 76),[496] resulting in the upregulation of its target oncogene *BLC2*.[497]

In contrast to miRNAs, little is known about the expression of lncRNAs in HSPCs and only a few lncRNAs have been further functionally characterized. Several recent studies have compiled lists of lncRNAs expressed in HSCs, some of which are downregulated in more differentiated progeny[387,458] and in leukemic cells.[498] Knockdown of a lncRNA specifically expressed in mouse BM LT-HSCs increased self-renewal of HSCs in vivo and abolished recruitment of the transcription factor E2A, essential for the development of multilineage HPCs,[341] to chromatin.[499] HSCs from female mice depleted for *Xist* lncRNA (responsible for X chromosome inactivation) were shown to have maturation defects and to be compromised in their repopulation capacity.[500] Furthermore, all *Xist* mutant female mice developed highly aggressive mixed myeloproliferative neoplasms/MDS.[500] With those relatively few studies the overall relevance of lncRNAs for the pathogenesis of hematopoietic diseases is not yet well understood.

HEMATOPOIETIC STEM CELL METABOLISM

In their quiescent state, HSCs mainly rely on anaerobic glycolysis rather than oxidative phosphorylation (OxPhos) to produce ATP, in contrast to progenitors that have higher OxPhos activity (see Chapter 10).[501,502] Indeed, PPARγ antagonists were shown to enhance *ex-vivo* expansion of CB HSCs by promoting glycolysis.[503] Interestingly, Wang et al. reported that deficiencies in either the M2 isoform of pyruvate kinase (PKM2) or lactate dehydrogenase A (LDHA), two enzymes responsible for the production of pyruvate and lactate during glycolysis, respectively, result in differential effects on HSC and progenitor cell function.[504] Deletion of *Ldha* inhibited normal HSC and progenitor cell function, whereas deficiency in *Pkm2* impaired progenitor cell function without affecting HSCs.[504] The authors further showed that deficiency of either *Ldha* or *Pkm2* inhibited leukemia initiation, suggesting that these metabolic enzymes may be therapeutically useful in targeting leukemia.[504]

Due to their reliance on glycolysis, HSCs were long thought to have a low mitochondrial mass, which has been traditionally assayed using dyes, such as MitoTracker Green. However, at least adult HSCs[505] express two efflux pumps (Bcrp1 and Mdr1a/b) that prevent dye accumulation in mitochondria which has led to an underestimation of mitochondrial mass in these HSCs.[506–508] In agreement, essential roles have been found for mitochondrial metabolism in HSC maintenance. Ito et al. demonstrated that the loss of peroxisome proliferator-activated receptor δ (PPAR-δ) and inhibition of mitochondrial fatty acid oxidation (FAO) induced the loss of HSC reconstitution capacity.[509] Interestingly, this PPAR-δ-FAO axis was found to serve as a "metabolic switch" which regulated HSC symmetric versus asymmetric division.[509] In a subsequent study, the same group showed that activation of the PPAR-δ-FAO axis promotes expansion of Tie2$^+$ HSCs through Pink/Parkin-mediated mitochondrial autophagy (mitophagy).[103] Similarly, when culturing LT-HSCs under differentiation-inducing conditions, treatment with the uncoupler FCCP to lower mitochondrial activity increased their self-renewal, which was accompanied by mitophagy.[510] In contrast, Ho et al. found that young, but not old HSCs clear their healthy mitochondria in a Parkin-independent manner involving the autophagy protein Atg12.[511] Further studies will be needed to reconcile these findings with the high mitochondrial mass that now has been shown for HSCs (discussed above), and also to address the functional relevance of HSCs' heterogeneity regarding their numbers of mitochondria.[506,507,512]

The mammalian target of rapamycin (mTOR) pathway regulates cellular proliferation through metabolism and plays a critical role in hematopoiesis.[513] mTOR complex 1 (mTORC1) is a key sensor of the cellular energetic state and an important stimulator of cellular anabolism (process by which the body utilizes metabolic pathways to synthesize complex molecules from smaller units), including mitochondrial biogenesis via activation of PPARγ coactivator-1α (PGC-1α).[514] While mTORC1 activation causes HSC exhaustion,[515,516] it is required cell-autonomously for HSC regeneration, [517] indicating that mTORC1 activity must be tightly regulated in HSCs. The tumor suppressor gene *Lkb1* encodes a serine/threonine kinase that activates AMP-activated protein kinase (AMPK), which leads to inhibition of mTORC1 and promotes the function of FoxO family of transcription factors.[518] Lkb1 inactivation in mice led to an immediate loss of quiescent HSCs associated with depletion of all hematopoietic subpopulations in a cell-autonomous manner, but, interestingly, through a mechanism only partially dependent on AMPK, but rather involving mitochondrial defects, depletion of ATP and alterations in lipid and nucleotide metabolism.[518–520]

The benefit from relying on glycolysis for HSCs might be a limited exposure to reactive oxygen species (ROS) produced by several complexes of the electron transport chain responsible for OxPhos. Indeed, numbers and LT repopulating potential of HSCs are rapidly declining upon their exposure to ambient air.[521] The microenvironment in which HSCs reside has been found to be hypoxic, especially in deeper peri-sinusoidal regions.[522] In support of these results, long term HSCs express high levels of HIF-1α,[523] which is known to transcriptionally induce glycolysis.[524] However, a recent study using a newly generated dual genetic reporter line that enables better enrichment of LT-HSCs and imaging them in their native niche did not find these LT-HSCs in the BM niches with the highest level of hypoxia but rather in regions of similar oxygen tension as MPPs.[525] Thus, hypoxia does not seem to be sufficient to instruct LT-HSC potential. These observations are in agreement with other studies that suggest that glycolysis in HSCs might in fact not be driven by the induction of the HIF pathway in response to hypoxia.[526]

In addition to glycolysis and FAO, other metabolic pathways including glutamine metabolism[527] and branched-chain amino acid catabolism[528] have recently been shown to be important for HSC activation and LSC function, respectively. Furthermore, Taka et al. reported that depriving recipient mice of the essential amino acid valine depleted their BM of HSCs such that a 2-week course of a valine-free diet and subsequent transplantation of healthy donor HSCs into these recipient mice resulted in LT donor chimerism in almost all mice without the need for irradiation conditioning.[529] Even though the achieved repopulation levels by donor HSCs was still low, these results suggest that valine starvation could be further explored as a strategy to replace myeloablative conditioning.

The therapeutic importance of understanding the cellular metabolic state of HSCs was recently highlighted by Liu et al.,[530] who showed that treatment of HSCs with alexidine dihydrochloride, a selective inhibitor of the mitochondrial phosphatase PTPMT1, reprogrammed HSC metabolism from mitochondrial respiration to glycolysis, increasing HSC maintenance in culture. Alterations in HSC metabolism have also been reported to impact HSC function with aging. Mohrin et al. reported that SIRT7, a histone deacetylase which regulates the expression of the mitochondrial master regulator, nuclear respiratory factor 1, is reduced in aged HSCs.[531] SIRT7 loss of function led to increased mitochondrial protein folding stress (PFS^{mt}) that was accompanied by an induction of the mitochondrial unfolded protein response (UPR^{mt}), and a compromised regenerative capacity of HSCs.[531] Intriguingly, reintroduction of SIRT7 into aged HSCs reduced the PFS^{mt} and multilineage reconstitution capacity of aged HSCs,[531] indicating that dysregulated cell-intrinsic protective programs such as the UPR^{mt} might be targetable to reverse HSC aging. Improved methods that allow the accurate metabolic profiling of small numbers of sorted HSCs will be instrumental in better understanding HSC function.[532]

TABLE 9.2 **Soluble Proteins and Small Molecules Suggested to Regulate Hematopoietic Stem Cell Self-Renewal or Expansion**

Growth Factor	Function in HSC Self-Renewal	Reference[a]
NOTCH ligands	Sufficient, not necessary	216, 220–222, 226, 227
WNT proteins	Sufficient, ? necessary	234, 238, 250, 252
BMPs	? Sufficient, *SMAD4* necessary	269
SCF	Necessary, not sufficient	533
TPO	Necessary, not sufficient	534
CXCL12	Necessary	288, 291
RAR-γ	Necessary	153
ANGPTL	Sufficient	535, 536
PGE_2	Sufficient	539, 542
PTN	Necessary, sufficient	143, 548
AHR antagonist	Sufficient	550
UM171	Sufficient	556
PPAR-γ antagonist	Sufficient	570
Zwitterionic Hydrogel	Sufficient	574
Polyvinyl alcohol	Sufficient	565
Valproic acid	Sufficient	569
Nicotinamide	Sufficient	564

[a] References are representative, not all-inclusive.

AHR, Aryl hydrocarbon receptor; *ANGPTL*, angiopoietin-like protein; *BMP*, bone morphogenetic protein; *CXCL12*, C-X-C motif chemokine 12; *HSC*, hematopoietic stem cell; *PGE_2*, prostaglandin E_2; *PTN*, pleiotrophin; *RAR-γ*, retinoic acid receptor γ; *SCF*, stem cell factor; *TPO*, thrombopoietin.

Adapted from Zon L: Intrinsic and extrinsic control of haematopoietic stem cell self-renewal. *Nature*. 453:306, 2008, with permission.

NOVEL GROWTH FACTORS FOR EXPANDING HEMATOPOIETIC STEM CELLS AND CLINICAL TESTING

Several novel proteins and small molecules have been reported to promote potent expansion of murine or human HSCs in culture (Table 9.2).[143,533,534] Moreover, leveraging insights into the mechanisms which regulate HSC self-renewal and differentiation, several different approaches to expand human CB HSCs have been tested in early clinical trials. Zhang et al.[535] reported the discovery of the proteins angiopoietin-like 2 (ANGPTL2) and ANGPTL3 in a fetal liver stromal cell line and demonstrated that the addition of ANGPTL2 or ANGPTL3 to cytokine cultures supported a 24- to 30-fold expansion of human BM cells capable of LT repopulation in NOD/SCID mice. Subsequently, Zhang et al.[536] demonstrated that the addition of ANGPTL5 and insulin-like growth factor binding protein 2 (IGFBP2) to the combination of SCF, TPO, and FGF1 supported up to a 20-fold increase in human CB cells capable of 8-week engraftment in NOD/SCID mice. Of note, because the addition of ANGPTL5 and IGFBP2 did not substantially increase total cell expansion compared with SCF, TPO, and FGF1 alone, it remains possible that treatment with ANGPTL proteins or IGFBP2 may enhance the homing of HSCs in immune-deficient transplant models.[536] Because of their potency in expanding human CB HSCs in preclinical models, ANGPTL proteins represent attractive targets for translation into the clinic. Several ANGPTL proteins, including ANGPTL2 and ANGPTL5, have been found to bind and activate the immune-inhibitory receptor human leukocyte immunoglobulin–like receptor B2 (LILRB2).[537] Interestingly, studies in zebrafish and human cells indicate that ANGPTL2, through its interaction with LILRB2, leads to cleavage and activation of the NOTCH receptor, ultimately inducing MYC target genes stimulating HSPC formation and expansion.[538]

North et al.[539] reported that prostaglandin E_2 (PGE_2) positively regulates HSC formation in the zebrafish model. These authors also demonstrated that ST (2-hour) treatment of murine HSCs with PGE_2 produced a two- to threefold increase in donor cell repopulation in transplanted mice compared with mice transplanted with untreated cells.[539] Subsequently, Goessling et al.[540] showed that PGE_2 modulates WNT signaling via regulation of β-catenin degradation and PGE_2/WNT activation regulated both hematopoietic regeneration in the zebrafish and LT HSC repopulation in mice. Hoggatt et al.[541] also showed that ST exposure to PGE_2 promoted the enhanced homing and repopulation of human CB HSCs in immune-deficient mice caused by increased CXCR4 expression on PGE_2-treated CB HSCs. Ex vivo treatment with PGE_2 was subsequently shown to increase human CB CFC content and engraftment capacity after transplant into immune-deficient mice, and PGE_2-treated BM cells were also found to provide more than one year of multilineage reconstitution in a nonhuman primate model.[542] Based on these encouraging results, a phase I clinical trial was undertaken, in which one unmanipulated CB unit and a second CB unit that was cultured for two hours in the presence of 16,16-dimethyl PGE_2 ($dmPGE_2$) were transplanted into adult patients after nonmyeloablative conditioning. $dmPGE_2$-treated CB cells resulted in accelerated neutrophil recovery (17.5 days vs. 21 days) and LT engraftment in 10 of 12 patients.[543]

Recently, screening strategies in human cells have been successfully used to identify novel growth factors, developmental factors, and chemical compounds for HSCs. Himburg et al. identified pleiotrophin (PTN), a heparin binding growth factor, from a gene expression analysis of human brain-derived ECs that support human HSC expansion in vitro.[544–547] Treatment of murine BM HSCs with PTN produced a 10-fold expansion of LT repopulating HSCs in culture, and systemic administration of PTN to irradiated mice caused a 20-fold increase in the recovery of BM LTC-ICs in vivo.[143] Mechanistically, PTN signaling caused the upregulation of PI3K/AKT signaling and *Hes1* expression in HSCs, suggesting that activation of these signaling cascades may contribute to PTN-mediated HSC expansion.[143] Mice lacking *Ptn* had 11-fold less BM HSC content than their wild-type littermates.[548] Interestingly, LT-HSC content, as measured in tertiary and quaternary transplants, was increased in chimeric mice with *Ptn* deletion in the BM microenvironment compared with wild-type mice.[549] Taken together, these results suggest that PTN regulates BM HSC expansion and regeneration, and in the context of constitutive *Ptn* knockout, *Ptn* loss in hematopoietic cells may dominate over effects of *Ptn* loss in the niche.[549] Further studies utilizing Cre-loxP technology to delete PTN specifically in VEcadherin+ BM ECs and lepR+ stromal cells revealed that PTN produced by lepR+ stromal cells is important for HSC maintenance in vivo, whereas HSC regeneration following total-body irradiation (TBI) is differentially dependent on PTN production by BM ECs.[550]

Boitano et al.[551] described a screening approach of more than 100,000 heterocyclic compounds for the capacity to maintain human $CD34^+$ cells in culture for five days. This screen yielded the discovery of the purine derivative StemRegenin 1 (SR1), which was shown to promote the expansion of human CB repopulating cells in vitro.[551] Three-week cultures of human CB $CD34^+$ cells with thrombopoietin, SCF, FLT3 ligand, interleukin-6 (IL-6), and SR1 promoted a 17-fold increase in SCID-repopulating cells compared with the progeny of cultures containing thrombopoietin, SCF, FLT3 ligand, and IL-6 alone.[551] SR1 appears to mediate its effects via inhibition of the aryl hydrocarbon receptor. Aryl hydrocarbon receptors are expressed by HSCs, but the downstream signaling mechanism through which SR1 mediates HSC expansion remains unknown.[551] Dahlberg et al. reported that the combination of SR1 with the NOTCH ligand, $Delta^{Ext\text{-}IgG}$, caused a threefold increase in human CB-derived myeloid repopulation in NSG mice at two weeks compared to CB cells cultured with $Delta^{Ext\text{-}IgG}$ or SR1 alone.[552] Results from a phase I trial of transplantation with SR1-treated CB cells have demonstrated the feasibility and safety of this approach.[553]

In a recent analysis of growth factors elaborated by an AGM-derived stromal cell line, Wohrer et al. reported that nerve growth factor and collagen 1, when added to a defined serum-free medium containing SCF and IL-11, produced fourfold expansion of murine LT repopulating HSCs in seven-day cultures compared to SCF and IL-11 alone.[554,555] Separately, treatment of human CB HSCs with a pyrimidoindole derivative, UM171, was shown to promote a 13-fold expansion of CB cells capable of repopulating NSG mice at 20 weeks posttransplantation.[556] Although the mechanism of action of UM171 has not been elucidated, preliminary analyses suggested that UM171 inhibited erythroid and megakaryocytic differentiation of human HSCs in culture. Jaroscak et al.[557] tested the combination of FLT3 ligand, a GM-CSF/IL-3 fusion protein, and erythropoietin in a continuous perfusion culture system as a means to expand human CB cells before transplant. Similarly, Shpall et al.[558] tested the capacity of SCF, granulocyte colony-stimulating factor (G-CSF), and megakaryocyte growth and differentiation factor to expand human CB cells that were then transplanted into adult CB transplant recipients. An alternative approach to cytokine-based expansion of human CB cells was suggested by Peled et al.,[559–561] who demonstrated a 159-fold increase in human $CD34^+$ cells after seven-week culture with a copper chelator, tetraethylenepentamine (TEPA), and cytokines. Subsequently, de Lima et al.[562] reported the safety and feasibility of culturing human CB cells with TEPA and SCF, FLT3 ligand, IL-6, and thrombopoietin followed by transplantation into patients in a phase I/II clinical trial. Although each of these clinical trials has shown the feasibility of transplanting ex vivo–cultured CB cells, none demonstrated substantial acceleration in hematopoietic cell engraftment in CB transplant recipients compared to historical controls.

Several other clinical trials have recently indicated progress toward the clinical expansion of human CB HSCs for therapeutic purposes. De Lima et al. reported a median time to neutrophil engraftment of 15 days in recipients of one unmanipulated CB unit plus CB cells cultured with mesenchymal stromal cells, compared to 24 days in historical controls, although LT donor hematopoiesis derived almost exclusively from the unmanipulated CB unit.[563] Lastly, Horwitz et al. reported that transplantation of one unmanipulated CB unit and the progeny of 21-day culture of human CB cells with nicotinamide produced earlier neutrophil recovery (13 versus 25 days for historical controls) and dominant engraftment from the nicotinamide-treated CB unit in eight of 11 treated patients.[564] The approach with nicotinamide to expand human HSCs for use as grafts for allogeneic transplantation has proceeded along the pathway to possible clinical approval. When administered to a transplant recipient in phase II clinical trial a CB singled unit expanded ex vivo with nicotinamide shortened the median time to neutrophil recovery by 9.5 days (95% CI, 7 to 12 days) and median platelet recovery by 12 days (95% CI, 3 to 16.5 days), establishing the feasibility, safety, and efficacy of an ex vivo expanded UCB unit as a stand-alone graft. The efficacy of nicotinamide expanded CB stem cells has been further evaluated in an open-label controlled, multicenter, international, Phase III, randomized study comparing the reconstitution kinetics following transplantation of a single CB unit expanded with nicotinamide versus transplantation of one or two un-manipulated, unrelated CB units in patients with ALL AML, MDS, CML, or lymphoma, with required disease features rendering them eligible for allogeneic stem cell transplantation. The final results of this trial are eagerly awaited. At the more basic level, recent studies have shown that treatment with Nicotinamide riboside attenuates age-associated loss of hematopoietic stem cell function in mice, suggesting additional therapeutic potential for this approach.[565]

In addition to these approaches that are being tested in clinical trials of human CB expansion, several novel mechanisms have been identified that may provide the basis for additional clinical trials of the products of ex vivo human HSC expansion. Wilkinson et al.[566] reported culture methods including polyvinyl alcohol as a replacement for albumin, coupled with high concentrations of thrombopoietin, that produced between 236-fold and 899-fold expansion of murine HSCs capable of in vivo repopulation over 1 month. Several additional methods have recently been described to expand human CB HSCs, including by Shao et al.,[567] who demonstrated that HSCs express the neurotransmitter receptor Gabbr1, and that treatment of human CB HSPCs with a GABBR1 agonist caused the expansion of cells capable of engraftment in immune-deficient mice. Similarly, Hua et al.[568] reported that treatment of human CB HSCs with a bromodomain and extra-terminal motif inhibitor, CP1203, caused the expansion of CB cells capable of long-term engraftment in-immune deficient mice. Interestingly, inhibition of the epigenetic regular lysine-specific histone demethylase 1A (LSD1) promotes the expansion of CB CD34+ cells in culture and long-term CB HSCs via blockade of differentiation.[569] The authors also showed that LSD1 is a mechanistic target of UM171, which expands human CB HSCs and is currently being evaluated in clinical trials. Separately, Zimran et al. demonstrated that treatment of human CB CD34+ cells or adult human BM or mobilized peripheral blood CD34+ with the histone deacetylase inhibitor, valproic acid, produced a significant expansion of human cells capable of engraftment in immune deficient mice.[570] Treatment of human CB HSCs with a peroxisome proliferator–activated receptor (PPAR)-γ antagonist, GW9662, was also shown to significantly increase cytokine-mediated expansion of CB HSCs with repopulating capacity.[571] These authors further showed that treatment with the PPAR-γ antagonist promoted CB HSC expansion via enhancement of HSC glycolysis, suggesting that the metabolic state of human HSCs directly impacts in vivo repopulating capacity. Other candidate mechanisms that have been described to expand human HSCs include treatment with a recombinant form of the DNA-binding chromatin factor, DEK, which acts via CXCR2 and heparan sulfate proteoglycans on HSCs,[572] and Nov (CCN3), a matricellular regulator, that was shown to increase the frequency of human CB HSCs with serial repopulating capacity after only 8 hours of incubation.[573] Remarkably, Nov incubation increases HSC repopulating capacity and clonogenicity without increasing HSC numbers through cell division, suggesting that Nov incubation harnesses latent stem cell activity in CB HSCs.[573] Calvanese et al.[574] performed a transcriptomic analysis of self-renewing human fetal liver HSCs versus cultured HSPCs to identify the transcription factor MLLT3 as a candidate regulator of human HSC self-renewal. These authors showed that forced expression of MLLT3 in human HSCs promoted greater than 12-fold expansion of human HSCs capable of engrafting NSG mice and sustained levels of H3K79me2, while maintaining the HSC transcriptional program in culture.[574] Bai et al.[575] demonstrated an expansion of human CB cells capable of long-term reconstitution in immune-deficient mice using a zwitterionic hydrogel 3D culture system. Mechanistically, the authors showed that this novel culture system promoted HSC self-renewal due to inhibition of ROS generation in culture. Finally, the translational potential of methods to expand human HSCs was validated recently by the successful completion of a clinical trial of autologous ex vivo lentiviral gene therapy for patients with adenosine deaminase deficiency.[576] In this study, the authors cultured human BM CD34+ cells with SCF, flt-3 ligand, thrombopoietin, and IL-3 for 40 hours to facilitate lentiviral integration and showed LT normalization of ADA activity in transplanted patients. Taken together, these studies suggest that the greater understanding of mechanisms that regulate human HSC self-renewal and methods to sustain human HSCs in culture will continue to increase, raising hope for the realization of a translatable, reproducible method to expand human HSCs for therapeutic purposes, including gene therapy.

GENERATING HEMATOPOIETIC STEM CELLS FROM PLURIPOTENT STEM CELLS AND BY REPROGRAMMING OF SOMATIC CELLS

Globally more than 50,000 patients per year receive allogeneic and autologous HSC transplantations as treatments for congenital and acquired hematopoietic diseases and other malignancies.[577] At present, the only cell sources for HSC transplantations are BM, CB, or mobilized peripheral blood. However, insufficient numbers, shelf-life concerns as well as immunologic incompatibility leading to graft-versus-host disease, even in human leukocyte antigen–matched grafts, limits their availability.[577,578] One option to generate more HSCs is by expanding existing HSCs in vitro as described earlier. Despite substantial efforts, this has proven difficult; because of the tendency of HSCs to differentiate in culture, the expansion of HSCs is not very efficient and does not lead often to fully functional HSCs in terms of their migratory behavior and LT multilineage reconstitution potential.[579,580]

The observation in 1981 that embryonic stem cells (ESCs) could be derived from mouse or, later, human blastocysts[581–583] fueled efforts to differentiate HSCs from ESCs. In 2006, the discovery that mouse or human fibroblasts could be reprogrammed to induced pluripotent stem cells (iPSCs) by retroviral transduction with the same four factors, OCT3/4, SOX2, KLF4, and c-Myc,[584,585] opened the door to the possibility of autologous stem cell–based therapies in the clinic.[586,587] Since its first report, this technology has been constantly modified[588] and is allowing the generation of iPSC lines from patients with a variety of blood disorders (references in 589). This approach presents a new opportunity for disease modeling and drug screening.

Numerous methods for directed differentiation of HSCs from ESCs or more recently iPSCs have been developed, but so far none yield long-lived cells with full HSC functionality. In one study, inducible expression of five transcription factors, *HOXA9*, *RORA*, *ERG*, *SOX4*, and *MYB*, imparted human ESC- and iPSC-derived progenitors with ST myeloid and erythroid engraftment potential.[589] This and similar approaches are characterized by the lack of robust lymphoid potential, likely because these progenitors are developmentally still too immature.[590]

In light of the obstacles in generating HSCs from ESCs or iPSCs, approaches to directly reprogram somatic cells into HSCs, or transdifferentiate them to hematopoietic cells are being explored as alternative strategies. The concept of direct reprogramming was first demonstrated with the conversion of embryonic fibroblasts into contracting myocytes by just the transcription factor MyoD.[591] Pluripotency-related factors are upregulated during endogenous reconstitution of mouse hematopoiesis after irradiation.[592] Thus, studies successfully reprogramming human skin fibroblasts directly into HSPC-like cells used the pluripotency factors OCT4 or SOX2 together with a specific cytokine cocktail.[592,593] While these cells engrafted, they again lacked lymphoid potential. In contrast to these examples of indirect lineage conversion via a less differentiated state, direct lineage conversion (or transdifferentiation) attempts have included the enforced expression of transcription factors critical for normal hematopoiesis.[594] In analogy to the HSC-producing hemogenic endothelium, human umbilical vein ECs cultured on an artificial vascular niche and overexpressing four transcription factors (*FOSB*, *GFI1*, *RUNX1*, and *PU.1*) yielded serially transplantable hematopoietic colonies.[595] However, these cells did not differentiate into T cells. In another approach demonstrating the benefit of the niche, lymphoid or myeloid progenitors were transduced with a transcription factor cocktail (*HLF*, *LMO2*, *PBX1*, *PRDM5*, *RUNX1T1*, and *ZFP37*) and matured in irradiated mice to yield serially transplantable HSCs producing all lineages.[596] Despite this progress in mice, fully functional human HSCs have not yet been generated in vitro using this technology. In addition, future studies will have to focus on strategies to avoid the risk of malignant transformation inherent in any directed differentiation or cellular reprogramming method.[597]

HEMATOPOIETIC STEM CELL REGENERATION

Although much is now known about the intrinsic and extrinsic mechanisms that regulate adult HSC self-renewal and differentiation,[1,215,237] the process through which HSCs regenerate after injury (e.g., chemotherapy or radiation) remains less well understood. Successful delineation of the mechanisms that control HSC regeneration could have significant therapeutic implications because a large proportion of cancer patients receive myelosuppressive or myeloablative therapy during the course of their disease. Signaling through the BMP and WNT signaling pathways has been shown to be necessary for hematopoietic regeneration to occur in zebrafish after sublethal irradiation.[598] These authors further demonstrated that SMAD and TCF, the downstream effectors of BMP and WNT signaling, respectively, couple with master regulators of myeloid and erythroid differentiation (C/EBPα and GATA1) to drive lineage-specific regeneration.[598] In a murine model of hematopoietic injury, Congdon et al.[599] showed that *Wnt10b* expression is increased in BM stromal cells in response to irradiation, and WNT signaling is activated in BM HSCs after irradiation. As discussed earlier, in a zebrafish model, activation of WNT signaling during hematopoietic regeneration is modulated by PGE_2.[540] WNT reporter activity was responsive to PGE_2 treatment, and the effect of Wnt8 toward enhancing hematopoietic recovery after sublethal irradiation was inhibited by administration of indomethacin, a PGE_2 antagonist.[540] NOTCH signaling has also been implicated in the regulation of hematopoietic regeneration after stem cell transplantation.[328] Deletion of *Notch2*, but not *Notch1*, was shown to delay myeloid reconstitution in mice after stem cell transplantation.[219] These data suggest that the BMP, WNT, and NOTCH pathways are attractive mechanistic targets for strategies to augment hematopoietic regeneration after myelosuppressive therapy.

Additional signaling pathways have been implicated in regulating hematopoietic regeneration. Deletion of plasminogen (*Plg*), a fibrinolytic factor, was shown to prevent HPC proliferation and recovery after 5-fluorouracil (5-FU)-induced myelosuppression in mice.[600] Conversely, activation of PLG by administration of tissue *Plg* activator promoted HPC proliferation and differentiation after myelosuppression, and this effect was dependent on matrix metallopeptidase 9–mediated release of c-Kit ligand.[600] Similarly, Trowbridge et al.[281] reported that mice that were heterozygous for the hedgehog receptor *Ptc1* displayed earlier recovery of hematopoiesis after 5-FU-induced myelosuppression compared with wild-type littermate mice. Hedgehog binding blocks PTC1-mediated inhibition of SMO, thereby promoting downstream Hedgehog signaling. Therefore, heterozygous *Ptc1* mice have enhanced Hedgehog signaling, and these results implicate Hedgehog signaling as positively regulating ST hematopoietic regeneration after injury. However, this acceleration in hematopoietic recovery in mice heterozygous for *Ptc1* occurred at the expense of LT-HSCs, which were exhausted in these mice.[281] Genetic studies have similarly demonstrated that the homozygous deletion of *Ship* in mice (SH2-containing inositol phosphatase) is associated with increased loss of HSCs after 5-FU exposure compared with heterozygous *Ship* deletion.[601] In a similar model of 5-FU-mediated myelosuppression, Nemeth et al.[602] reported that mice deficient in the high-mobility group box 3 (*Hmg3b*) DNA binding protein exhibited more rapid recovery of phenotypic HSCs compared with wild-type mice. The enhanced recovery of the stem/progenitor pool in *Hmgb3*-deficient mice was associated with activation of WNT signaling, again suggesting that activation of the WNT pathway may accelerate HSC recovery after myelosuppression. Of note, expression of a constitutively active form of the signal transducer and activator of transcription 3 (*Stat3*) in HSCs increases their regenerative capacity after transplant into lethally irradiated mice.[603] In this study, it was not determined whether alteration in *Stat3* expression affected HSC regeneration after myelosuppression (e.g., 5-FU or irradiation).[603]

At the cellular level, increasing evidence suggests an important role for BM ECs in promoting hematopoietic regeneration after myelotoxic stress.[604–606] Genetic deletion or antibody-based inhibition of VEGFR2, which is expressed by sinusoidal BM ECs, was shown to delay both BM vascular and hematopoietic recovery after TBI.[605] Systemic infusion of syngeneic or allogeneic ECs has also been shown to significantly accelerate the recovery of both the HSC pool and overall hematopoiesis in mice after high-dose TBI.[607,608] Salter et al.[609] and Butler et al.[225] further demonstrated that hematopoietic regeneration after irradiation is dependent on VE-cadherin-mediated vascular reorganization because administration of a neutralizing anti-VE-cadherin antibody caused significant delay in hematologic recovery in mice after TBI. While the precise mechanisms through which BM ECs regulate HSC regeneration in vivo remain unclear, it was shown that systemic administration of PTN, a heparin binding growth factor that is secreted by both BM and brain ECs, causes a rapid increase in recovery of the HSC pool in mice after high-dose TBI.[143] Taken together, these studies suggested that the BM vascular niche may be an important reservoir for the discovery of growth factors and membrane-bound proteins that mediate HSC regeneration. Additional studies have further validated the important role of the BM vascular niche in regulating HSC regeneration following myelosuppressive injury. Deletion of the proapoptotic proteins, BAK and BAX, from *Tie2*-expressing BM ECs was shown to protect HSCs from radiation-induced depletion in mice, independent of HSC-autonomous effects.[609] Furthermore, Doan et al. reported that epidermal growth factor (EGF) is expressed by BM ECs after TBI and that systemic administration of EGF improved HSC regeneration and survival after TBI.[609] EC-specific deletion of the NOTCH ligand, Jagged1, has also been shown to cause delayed white blood cell recovery and decreased survival in mice following sublethal TBI.[610] Additional studies have suggested several novel mechanisms through which HSC regeneration can be augmented following radiation-induced myelotoxicity, including augmentation of the thrombomodulin-activated protein C pathway,[611] administration of the bactericidal/permeability-increasing protein ($rBPI_{21}$),[612] activation of nuclear factor erythroid-2-related factor 2,[613] or Ras/MEK/ERK signaling in HSCs.[614] Interestingly, it has also been shown that both HSCs and leptin receptor-expressing BM stromal cells secrete ANGPT1 and that deletion of *Angpt1* in these cell populations accelerated vascular and hematopoietic recovery in mice after irradiation.[615] Furthermore Vannini and coworkers reported that nicotinamide riboside reduced mitochondrial activity within HSCs by increasing mitochondrial clearance, leading to increased asymmetric HSC divisions.[616] They reported that dietary supplementation of mice after they received lethal doses of irradiation with nicotinamide riboside resulted in a larger pool of HPC, without HSC exhaustion, improving survival by 80% and accelerating blood cell recovery. This report established a link between HSC mitochondrial stress, mitophagy, and stem-cell fate decision, and unveiled the potential for the use of nicotinamide riboside to accelerate the recovery of patients suffering from cytopenias following chemotherapy and radiation therapy. Taken together, these studies reveal the remarkable complexity and orchestration of molecular responses to myelotoxicity and also suggest several potential pathways that can potentially be exploited for the therapeutic regeneration of HSCs.

Several additional mechanisms have been discovered through which distinct components of the BM microenvironment regulate HSC regeneration. Using single cell gene expression analysis of mesenchymal osteolineage cells, Silberstein et al.[617] identified a secreted RNase, Angiogenin, as a candidate HSPC growth factor. Angiogenin was subsequently shown to decrease the proliferation of HSPCs, provide radioprotection to irradiated mice, and augment hematopoietic reconstitution of mouse and human HSPCs following transplantation.[618] Subtractive gene expression analysis of BM osterix+ mesenchymal osteoprogenitor cells in mice prior to and following TBI revealed increased expression of the Wnt antagonist, Dickkopf 1 (Dkk1), by BM osterix+ cells following irradiation. Himburg et al. showed that Dkk1 promoted early HSC regeneration in mice following TBI via direct suppression of ROS generation and HSC senescence, and indirectly via action on BM ECs, causing BM EC secretion of EGF.[619] Importantly, Zhou et al.[620] demonstrated that BM adipocytes secrete SCF in mice following TBI and adipocyte-derived SCF is essential for HSC regeneration and mouse survival following TBI. Adrenergic nerve function in the BM is also important for hematopoietic regeneration in mice following chemotherapy,[621] and adrenergic nerves regulate hematopoietic regeneration via stabilization of the BM mesenchymal stromal cell niche. Utilizing techniques to isolate sinusoidal BM ECs from arterial BM ECs, Xu et al.[622] suggested that arterial BM ECs are the primary source of EC–SCF that contributes to HSC maintenance and recovery following injury. Chen et al.[623] described a rare population of BM apelin+ ECs that critically contribute to BM vascular regeneration following myelosuppressive injury. Apelin+ BM ECs were shown to be essential for hematopoietic regeneration following BM transplantation. Separately, Himburg et al.[550] and Guo et al.[624] illustrated that EC-derived PTN and EC-derived Jagged 2 have essential roles in promoting hematopoietic regeneration following myelosuppression.

Interestingly, donor-derived granulocytes have been shown to promote recipient BM vascular regeneration and hematopoietic recovery following myeloablation in mice and this granulocyte-mediated BM vascular regeneration occurs via TNFα secretion by donor granulocytes, acting on TNFR1 expressed on recipient BM ECs.[625] In keeping with the concept of donor hematopoietic cell contribution to BM restoration following myeloablation, Golan et al.[626] showed that donor HSPCs transfer healthy mitochondria to damaged recipient BM stromal cells in manner dependent upon transfer through HSPC Connexin-43 channels. Global strategies to augment host BM stromal cell function following myelosuppressive injury have potential applications since Severe et al.[627] demonstrated that many BM stromal cell subsets are ablated by TBI.

Other novel molecules reported to regulate hematopoietic regeneration include luteinizing hormone (LH).[628] Velardi et al. demonstrated that administration of an LH-releasing hormone (LHRH) antagonist promoted HSC regeneration and survival in irradiated mice.[628] The authors showed that LH inhibition promoted HSC quiescence after injury, thereby preventing HSC exhaustion. Fang et al. reported that EGF promotes HSC regeneration in irradiated mice via augmentation of NHEJ repair in HSCs,[629] while Zhang et al.[128] demonstrated that small molecule allosteric inhibitors of the receptor PTPσ potently drove murine HSC regeneration in vivo and human HSC regeneration ex vivo. Yamashita et al.[630] demonstrated that TNFα promotes HSC survival and primes HSCs for myeloid differentiation following inflammatory stress, while simultaneously inducing myeloid progenitor cell apoptosis. Most recently, Sudo et al. reported that group 2 innate lymphoid cells promote hematopoietic regeneration following 5FU chemotherapy through secretion of GM-CSF.[631] At the molecular level, Yi et al. demonstrated an essential role for S-nitrosoglutathione reductase in controlling protein aggregation and unfolded protein response to allow HSC regeneration following 5FU chemotherapy.[632] Similarly, it has been shown that the E3 ubiquitin ligase, HectD1, has an essential role in supporting HSC regeneration by regulating ribosome assembly and proper protein translation.[633] Chavez et al. also reported that LT-HSCs upregulate expression of the transcription factor, PU.1, which represses cell cycle entry and protein synthesis to maintain the homeostatic HSC pool during stress.[634] Importantly, Kaufmann et al. recently discovered a latent subset of human HSCs that display resistance to regeneration-mediated stress.[635] Taken together, these studies provide new avenues for additional exploration of stress-resistant HSC populations and new understanding of the intrinsic and extrinsic machinery that regulates HSC survival and regeneration following injury.

Lastly, the effect of age on the capacity for HSCs to regenerate after a myelosuppressive challenge remains an important question.[636] Clinical studies have confirmed the impaired reconstitutive capacity of HSCs from older patients in autologous stem cell transplant settings.[637] Not surprisingly, older mice with defects in DNA damage repair mechanisms (nucleotide excision repair, nonhomologous end-joining) and telomere maintenance displayed severe defects in their capacity to reconstitute hematopoiesis after transplantation into lethally irradiated recipient mice compared with age-matched control subjects that retained the DNA repair and telomerase genes.[638] Furthermore, Flach et al. showed that aging HSCs display heightened levels of replication stress while cycling as a result of decreased expression of mini-chromosome maintenance replicative helicase components and altered DNA replication forks.[639] Therefore, therapeutic targeting to accentuate these DNA repair and replication mechanisms may facilitate the recovery of the functional HSC pool after myelosuppression and may lessen the oncogenic risk incurred via repeated exposure to DNA-damaging therapies (e.g., alkylators and irradiation).[631,638] Interestingly, prolonged fasting has been shown to ameliorate chemotherapy-induced HSC damage and age-dependent myeloid bias in mice, associated with reduction in IGF1 levels.[640] In keeping with these findings, long-term dietary restriction has subsequently been shown to improve hematopoietic reconstitution capacity of aged BM HSCs.[641] Further research into the HSC-autonomous and extrinsic mechanisms which regulate HSC aging and HSC regeneration during aging should be prioritized going forward and will hopefully yield therapeutic avenues to reverse some aspects of hematopoietic aging.

HEMATOPOIETIC STEM CELLS AND MALIGNANCY (SEE CHAPTERS 13 AND 59)

Similar to the HSC at the apex of the hematopoietic hierarchy, an entity termed a LSC has been proposed to drive tumorigenesis because of its ability to self-renew and reinitiate leukemia upon transplantation in an experimental setting (e.g., mouse transplant; Fig. 9.4).[642–644] A clonal origin of a hematopoietic malignancy was first demonstrated for CML where the presence of the characteristic Philadelphia chromosome in myeloid, erythroid, megakaryocytic, and B-lymphoid cells suggested a common origin,[645–647] which was later proven by molecular analyses.[648] Genetic analyses in a case of CML also provided the first proof for another important concept in cancer, that of clonal evolution (see Fig. 9.4),[649] which had already been hypothesized for solid tumors.[643] This model posits that a subclone within the initial LSC-derived clone acquires additional genetic or epigenetic alterations that convey a growth advantage and lead to heterogeneity within the tumor.[642] Whereas the HSC pool itself does not expand during progression of chronic phase CML to blast crisis, granulocyte-macrophage progenitors (GMPs) with increased expression of the continuously active tyrosine kinase fusion protein BCR-ABL and increased self-renewal capacity driven by activation of nuclear β-catenin are amplified.[650] Thus, the LSC may differ from the tumor-initiating "cell of origin."[203,650] While in CML the tumor-initiating HSC maintains the chronic phase of the disease, subsequent genetic events arising in the GMPs give rise to LSCs sustaining the blast crisis.

The first cancer stem cell to be identified was the LSC in AML.[644,651] $CD34^+CD38^-$ cells but not $CD34^+CD38^+$ cells derived from all known AML subtypes (except for the AML subtype M3)

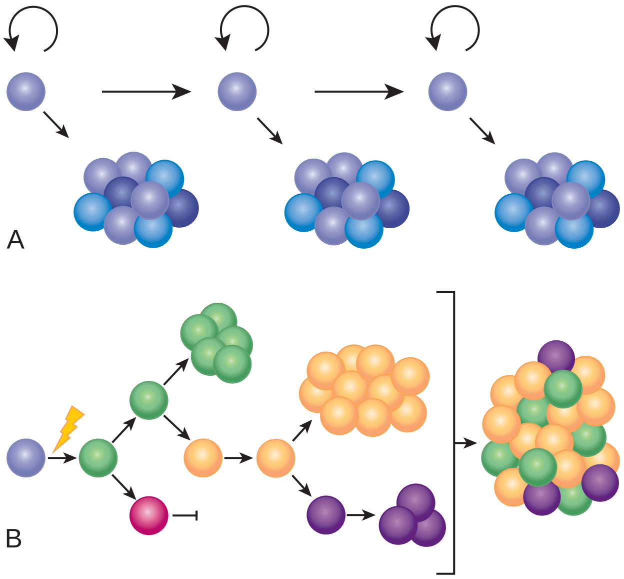


Figure 9.4 CLONAL DYNAMICS IN HEMATOLOGIC MALIGNANCIES. (A) The cancer stem cell (CSC) model assumes a hierarchical structure akin the normal hematopoietic hierarchy. The CSC can give rise to the hematopoietic lineage but usually results in a differentiation block leading to the accumulation of immature progenitor (blast) cells. (B) The clonal evolution model suggests that a carcinogen-induced change in a normal cell confers a growth advantage to that cell permitting its clonal expansion. Selective pressures are responsible for some mutant subclones to expand while others become extinct or dormant. A combination of both models is also possible. *(A, Adapted from Wang J, Ma Y, Cooper MK. Cancer stem cells in glioma: challenges and opportunities. Transl Cancer Res. 2013;2:429. B, Adapted from Nowell PC. The clonal evolution of tumor cell populations. Science. 1976;194:23–28.)*

repopulated secondary NOD/SCID recipient mice and fully reproduced AML.[651,652] Next-generation sequencing efforts have revealed the clonal evolution in primary and relapsed AML.[336,383,394,653–656] While healthy and AML genomes contain hundreds of exonic mutations,[336] as few as two key somatic "driver" mutations enable clonal expansion of a cell that takes along all the background "passenger" mutations. An additional driver mutation occurring in this clone then gives rise to the founding LSC.[336] Mutations in either the LSC itself or again its subclones can evolve into the dominating clone in relapsed AML.[653] Genomic profiling of AML patients has yielded strong evidence for the presence of nonmalignant preleukemic HSPCs[335,395,657] that survive chemotherapy and produce mature lymphoid cells while at the same time generating new waves of mutant clones as the disease progresses. Mutations in epigenetic genes, for instance *DNMT3A*, *IDH2*, and *ASXL1*, are often identified in the ancestral clones, suggesting their early role in AML pathogenesis, whereas mutations in *NPM1* and signaling genes appear to be acquired later.[335,395] Of note, *DNMT3A* and *ASXL1* are also among the three most recurrently mutated genes in clonal hematopoiesis (along with *TET2*) and present with a frequency of about 10% of healthy people over 65 years.[658,659] Furthermore, these findings indicate that the LSC crucial for driving the progression to AML can be a downstream progenitor (in this case, a GMP and/or multilymphoid progenitor). Further support comes from mouse models: LSCs from leukemias initiated by forced expression of an *MLL-AF9* fusion gene (common in AML patients) in GMPs retain the identity of the progenitor cells, despite having acquired self-renewal potential.[373] The identification of both tumor-initiating ancestral HSPCs and LSCs has important implications for the treatment of hematologic malignancies, as relapse-causing clones may be unrelated to the predominant clones at diagnosis in ~50% of patients with B-cell acute lymphoblastic leukemia (B-ALL), T-ALL, and AML.[395]

The combination of advanced mouse models and genetics have yielded a refined understanding of the hematopoietic cells of origin for specific subsets of leukemia. Furthermore, LSCs have been shown to leverage HSC properties to support their survival and expansion.[660] Specifically, Luo et al.[661] demonstrated that aberrant activation of the HOX gene-associated long non-coding RNA, HOTTIP, increased HSC self-renewal, leading to an AML-like disease in mice. Activation of this long non-coding RNA transformed HSCs into LSCs by reprogramming leukemic-associated chromatin and gene transcription.[661] Expression of the fusion oncogenes, ETO2-GLIS2, in fetal HSCs was shown to induce acute megakaryoblastic leukemia, while expression of the same oncogenes in adult HSCs caused a myeloid shift but a delayed leukemogenic transformation.[662] In an analysis of 434 primary human AML samples, Boileau et al.[663] reported the increased frequency of H3 histone mutations and the presence of such mutations in "pre-leukemic HSC," suggesting that such mutations preceded the evolution to secondary AML in these patients. Vu et al.[664] developed a conditionally inducible mouse model in which the caudal-related homeobox transcription factor, Cdx2, was overexpressed in HSPCs. This led to features of MDS with subsequent progression to acute leukemia associated with additional driver mutations.[664] The stage of hematopoietic cell differentiation at which oncogene expression occurs also appears to impact sensitivity to therapy. Cai et al.[665] discovered that expression of oncogenic transcription factor, EVI1, in HSCs produced leukemic cells with decreased apoptotic priming, attenuated p53 response, and resistance to LSD1 inhibitors. These results suggest that the cell of origin for acute leukemias may dictate disease responsiveness to antileukemic therapies. Other studies have demonstrated that myeloid progenitor cells are the cells of origin for common types of leukemia, including MLL-AF9 leukemia.[666] Chen et al. showed that the rapid cell cycle of myeloid progenitors makes them permissive for transformation by MLL-AF9 oncogene.[666] Basilico et al. further characterized the transcriptional profile of multipotent HPCs transformed by expression of MLL-ENL and the molecular relationship between parental HPCs and leukemic cells post–oncogene expression.[667] Di Genua et al. also demonstrated that acute erythroid leukemia arises from combined mutations in CEBPA and Gata2 zinc finger 1 (Znf1) in a neutrophil-monocyte progenitor cell population.[668] Taken together, these studies confirm the heterogeneous nature of the cellular origins of myeloid leukemias and suggest new targets for therapeutic interventions.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Acar M, Kocherlakota K, Murphy M, et al. Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. *Nature*. 2015;526:126–130.

Adams PD, Jasper H, Rudolph KL. Aging-induced stem cell mutations as drivers for disease and cancer. *Cell Stem Cell*. 2015;16:601.

Agathocleous M, Meacham CE, Burgess RJ, et al. Ascorbate regulates haematopoietic stem cell function and leukaemogenesis. *Nature*. 2017;549:476–481.

Aifantis I, Raetz E, Buonamici S. Molecular pathogenesis of T-cell leukaemia and lymphoma. *Nat Rev Immunol*. 2008;8:380.

Bai T, Li J, Sinclair A, et al. Expansion of primitive human hematopoietic stem cells by culture in a zwitterionic hydrogel. *Nat Med*. 2019;25:1566–1575.

Balounova J, Splichalova I, Dobesova M, et al. Toll-like receptor 2 expression on c-kit $^{+}$ cells tracks the emergence of embryonic definitive hematopoietic progenitors. *Nature*. 2019;10:5176.

Baylin SB, Jones PA. A decade of exploring the cancer epigenome—biological and translational implications. *Nat Rev Cancer*. 2011;11:726–734.

Beerman I, Rossi DJ. Epigenetic control of stem cell potential during homeostasis, aging, and disease. *Cell Stem Cell*. 2015;16:613–625.

Bertrand JY, Chi NC, Santoso B, et al. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature*. 2010;464:108–111.

Bian Z, Gong Y, Huang T, et al. Deciphering human macrophage development at single-cell resolution. *Nature*. 2020;582:571–576.

Blank U, Karlsson G, Karlsson S. Signaling pathways governing stem-cell fate. *Blood*. 2008;111:492–503.

Buenrostro J, Corces MR, Lareau C, et al. Integrated single-cell analysis maps the continuous regulatory landscape of human hematopoietic differentiation. *Cell*. 2018;173:1535–1548.

Cai S, Chu S, Goldberg A, et al. Leukemia cell of origin influences apoptotic priming and sensitivity to LSD1 inhibition. *Cancer Discov*. 2020;10:1500–1513.

Calvanese V, Nguyen A, Bolan T, et al. MLLT3 governs human haematopoietic stem cell self-renewal and engraftment. *Nature*. 2019;576:281–286.

Chen MJ, Yokomizo T, Zeigler BM, et al. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature*. 2009;457:887–891.

Cheng CW, Adams GB, Perin L, et al. Prolonged fasting reduces IGF-1/PKA to promote hematopoietic-stem-cell-based regeneration and reverse immunosuppression. *Cell Stem Cell*. 2014;14:810–823.

Delaney C, Heimfeld S, Brashem-Stein C, et al. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. *Nat Med*. 2010;16:232–236.

Doan PL, Himburg HA, Helms K, et al. Epidermal growth factor regulates hematopoietic regeneration after radiation injury. *Nat Med*. 2013;19:295–304.

Doulatov S, Vo LT, Chou SS, et al. Induction of multipotential hematopoietic progenitors from human pluripotent stem cells via respecification of lineage-restricted precursors. *Cell Stem Cell*. 2013;13:459–470.

Dykstra B, Kent D, Bowie M, et al. Long-term propagation of distinct hematopoietic differentiation programs in vivo. *Cell Stem Cell*. 2007;1:218–229.

Dzierzak E, Speck NA. Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nat Immunol*. 2008;9:129–136.

Eaves CJ. Hematopoietic stem cells: concepts, definitions, and the new reality. *Blood*. 2015;125:2605–2613.

Fares I, Chagraoui J, Gareau Y, et al. Cord blood expansion. Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. *Science*. 2014;345:1509–1512.

Fatica A, Bozzoni I. Long non-coding RNAs: new players in cell differentiation and development. *Nat Rev Genet*. 2014;15:7–21.

Golub R, Cumano A. Embryonic hematopoiesis. *Blood Cells Mol Dis*. 2013;51:226–231.

Goncalves K, Silberstein L, Li S, et al. Angiogenin promotes hematopoietic regeneration by dichotomously regulating quiescence of stem and progenitor cells. *Cell*. 2016;166:894–906.

Greaves M, Maley CC. Clonal evolution in cancer. *Nature*. 2012;481:306–313.

Greenblatt SM, Nimer SD. Chromatin modifiers and the promise of epigenetic therapy in acute leukemia. *Leukemia*. 2014;28:1396–1406.

Guo G, Luc S, Marco E, et al. Mapping cellular hierarchy by single-cell analysis of the cell surface repertoire. *Cell Stem Cell*. 2013;13:492–505.

Gurumurthy S, Xie SZ, Alagesan B, et al. The Lkb1 metabolic sensor maintains haematopoietic stem cell survival. *Nature*. 2010;468:659–663.

Himburg H, Termini C, Schlussel L, et al. Distinct bone marrow sources of pleiotrophin control hematopoietic stem cell maintenance and regeneration. *Cell Stem Cell*. 2018;23:370–381.

Ho TT, Warr MR, Adelman ER, et al. Autophagy maintains the metabolism and function of young and old stem cells. *Nature*. 2017;543:205–210.

Ito K, Carracedo A, Weiss D, et al. A PML-PPAR-delta pathway for fatty acid oxidation regulates hematopoietic stem cell maintenance. *Nat Med*. 2012;18:1350–1358.

Jamieson CH, Ailles LE, Dylla SJ, et al. Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N Engl J Med*. 2004;351:657–667.

Keller G. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev*. 2005;19:1129–1155.

Kiel MJ, Yilmaz OH, Iwashita T, et al. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell*. 2005;121:1109–1121.

Ling H, Fabbri M, Calin GA. MicroRNAs and other non-coding RNAs as targets for anticancer drug development. *Nat Rev Drug Discov*. 2013;12:847–865.

Lucas D, Scheiermann C, Chow A, et al. Chemotherapy-induced bone marrow nerve injury impairs hematopoietic regeneration. *Nat Med*. 2013;19:695–703.

Mikkola HK, Klintman J, Yang H, et al. Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukaemia SCL/tal-1 gene. *Nature*. 2003;421:547–551.

Miller PH, Knapp DJ, Eaves CJ. Heterogeneity in hematopoietic stem cell populations: implications for transplantation. *Curr Opin Hematol*. 2013;20:257–264.

Mohrin M, Shin J, Liu Y, et al. Stem cell aging. A mitochondrial UPR-mediated metabolic checkpoint regulates hematopoietic stem cell aging. *Science*. 2015;347:1374–1377.

Morita Y, Ema H, Nakauchi H. Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. *J Exp Med*. 2010;207:1173–1182.

Nguyen LV, Vanner R, Dirks P, et al. Cancer stem cells: an evolving concept. *Nat Rev Cancer*. 2012;12:133–143.

North TE, Goessling W, Walkley CR, et al. Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. *Nature*. 2007;447:1007–1011.

Notta F, Doulatov S, Laurenti E, et al. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science*. 2011;333:218–221.

O'Connell RM, Baltimore D. microRNAs and hematopoietic cell development. *Curr Top Dev Biol*. 2012;99:145–174.

Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell*. 2008;132:631–644.

Pearson JC, Lemons D, McGinnis W. Modulating Hox gene functions during animal body patterning. *Nat Rev Genet*. 2005;6:893–904.

Pei W, Feyerabend T, Rossler J, et al. Polylox barcoding reveals haematopoietic stem cell fates realized in vivo. *Nature*. 2017;548:456–460.

Purton LE, Scadden DT. Limiting factors in murine hematopoietic stem cell assays. *Cell Stem Cell*. 2007;1:263–270.

Reya T, Duncan AW, Ailles L, et al. A role for WNT signalling in self-renewal of haematopoietic stem cells. *Nature*. 2003;423:409–414.

Riddell J, Gazit R, Garrison BS, et al. Reprogramming committed murine blood cells to induced hematopoietic stem cells with defined factors. *Cell*. 2014;157:549–564.

Severe N, Karabacak N, Gustafsson K, et al. Stress-induced changes in bone marrow stromal cell populations revealed through single cell protein expression mapping. *Cell Stem Cell*. 2019;25:570–583.

Shlush LI, Zandi S, Mitchell A, et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature*. 2014;506:328–333.

Spencer JA, Ferraro F, Roussakis E, et al. Direct measurement of local oxygen concentration in the bone marrow of live animals. *Nature*. 2014;508:269–273.

Sun J, Ramos A, Chapman B, et al. Clonal dynamics of native haematopoiesis. *Nature*. 2014;514:322–327.

Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;126:663–676.

Trowbridge JJ, Snow JW, Kim J, et al. DNA methyltransferase 1 is essential for and uniquely regulates hematopoietic stem and progenitor cells. *Cell Stem Cell*. 2009;5:442–449.

Visvader JE. Cells of origin in cancer. *Nature*. 2011;469:314–322.

Vo LT, Daley GQ. De novo generation of HSCs from somatic and pluripotent stem cell sources. *Blood*. 2015;125:2641–2648.

Wang YH, Israelsen WJ, Lee D, et al. Cell-state-specific metabolic dependency in hematopoiesis and leukemogenesis. *Cell*. 2014;158:1309–1323.

Watcham S, Kucinski I, Gottgens B. New insights into hematopoietic differentiation landscaptes from single-cell RNA sequencing. *Blood*. 2019;133:1415–1426.

Wilson NK, Foster SD, Wang X, et al. Combinatorial transcriptional control in blood stem/progenitor cells: genome-wide analysis of ten major transcriptional regulators. *Cell Stem Cell*. 2010;7:532–544.

Yamashita M, Dellorusso P, Olson O, et al. Dysregulated haematopoietic stem cell behaviour in myeloid leukemogenesis. *Nat Rev Cancer*. 2020;20:365–382.

Zang Y, Roos M, Himburg H, et al. PTPσ inhibitors promote hematopoietic stem cell regeneration. *Nat Commun*. 2019;10:3667.

Zhou B, Yu H, Yue R, et al. Bone marrow adipocytes promote the regeneration of stem cells and haematopoiesis by secreting SCF. *Nat Cell Biol*. 2017;19:891–902.

Zhou F, Li Z, Wang W, et al. Tracing haematopoietic stem cell formation at single cell resolution. *Nature*. 2016;533:487–492.

Zon LI. Intrinsic and extrinsic control of haematopoietic stem-cell self-renewal. *Nature*. 2008;453:306–313.

REFERENCES

1. Zon LI. Intrinsic and extrinsic control of haematopoietic stem-cell self-renewal. *Nature*. 2008;453:306–313. https://doi.org/10.1038/nature07038.
2. Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. *Cell*. 2000;100:157–168.
3. Orkin SH, Zon LI. SnapShot: hematopoiesis. *Cell*. 2008;132:712. https://doi.org/10.1016/j.cell.2008.02.013.
4. Schoemans H, Verfaillie C. Cellular biology of hematopoiesis. In: Hoffman R, ed. *Hematology*. Philadelphia, PA: Churchill Livingstone; 2009:200–212.
5. Jacobson LO, Simmons EL, Marks EK, et al. The role of the spleen in radiation injury and recovery. *J Lab Clin Med*. 1950;35:746–770.
6. Jacobson LO, Simmons EL, Marks EK, et al. Recovery from radiation injury. *Science*. 1951;113:510–511.
7. Lorenz E, Uphoff D, Reid TR, et al. Modification of irradiation injury in mice and guinea pigs by bone marrow injections. *J Natl Cancer Inst*. 1951;12:197–201.
8. Little MT, Storb R. History of haematopoietic stem-cell transplantation. *Nat Rev Cancer*. 2002;2:231–238. https://doi.org/10.1038/nrc748.
9. Jacobson LO, Marks EK, Gaston EO. Effect of protection of the spleen during total body irradiation on the blood in rabbit. *Rev Hematol*. 1953;8:515–532.
10. Nowell PC, Cole LJ, Habermeyer JG, et al. Growth and continued function of rat marrow cells in X-radiated mice. *Cancer Res*. 1956;16:258–261.
11. Ford CE, Hamerton JL, Barnes DW, et al. Cytological identification of radiation-chimaeras. *Nature*. 1956;177:452–454.
12. Till JE, McCulloch CE. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res*. 1961;14:213–222.
13. McCulloch EA, Siminovitch L, Till JE. Spleen-colony formation in anemic mice of genotype WW. *Science*. 1964;144:844–846.
14. Siminovitch L, McCulloch EA, Till JE. The distribution of colony-forming cells among spleen colonies. *J Cell Physiol*. 1963;62:327–336.
15. Becker AJ, Mc CE, Till JE. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature*. 1963;197:452–454.
16. Abramson S, Miller RG, Phillips RA. The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems. *J Exp Med*. 1977;145:1567–1579.
17. Prohaska SS, Weissman I. Biology of hematopoietic stem and progenitor cells. In: Appelbaum F, Forman S, Negrin MD, eds. *Thomas' Hematopoietic Cell Transplantation*. Chichester: Wiley-Blackwell; 2008:36–63.
18. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell*. 2008;132:631–644. https://doi.org/10.1016/j.cell.2008.01.025.
19. Golub R, Cumano A. Embryonic hematopoiesis. *Blood Cells Mol Dis*. 2013;51:226–231. https://doi.org/10.1016/j.bcmd.2013.08.004.
20. Dzierzak E, Speck NA. Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nat Immunol*. 2008;9:129–136. https://doi.org/10.1038/ni1560.
21. Galloway JL, Zon LI. Ontogeny of hematopoiesis: examining the emergence of hematopoietic cells in the vertebrate embryo. *Curr Top Dev Biol*. 2003;53:139–158.
22. Ivanovs A, Rybtsov S, Welch L, et al. Highly potent human hematopoietic stem cells first emerge in the intraembryonic aorta-gonad-mesonephros region. *J Exp Med*. 2011;208:2417–2427. https://doi.org/10.1084/jem.20111688.
23. His W. Lecithoblast und Angioblast der Welbeltiere. *Abhandl Math-Phys Ges Wiss*. 1900;26:171–328.
24. Murray PDF. The development in vitro of the blood of the early chick embryo. *Proc R Soc Lond [Biol]*. 1932;111:497–521. https://doi.org/10.1098/rspb.1932.0070.
25. Stainier DY, Weinstein BM, Detrich HW, et al. Cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. *Development*. 1995;121:3141–3150. https://doi.org/10.1242/dev.121.10.3141.
26. Vogeli KM, Jin SW, Martin GR, et al. A common progenitor for haematopoietic and endothelial lineages in the zebrafish gastrula. *Nature*. 2006;443:337–339. https://doi.org/10.1038/nature05045.

26*a*. Reischauer S, Stone OA, Villasenor A, et al. Cloche is a bHLH-PAS transcription factor that drives haemato-vascular specification. *Nature*. 2016;535:294–298. https://doi.org/10.1038/nature18614.

27. Shalaby F, Ho J, Stanford WL, et al. A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. *Cell*. 1997;89:981–990. https://doi.org/10.1016/s0092-8674(00)80283-4.
28. Shalaby F, Rossant J, Yamaguchi TP, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature*. 1995;376:62–66. https://doi.org/10.1038/376062a0.
29. Choi K, Kennedy M, Kazarov A, et al. A common precursor for hematopoietic and endothelial cells. *Development*. 1998;125:725–732. https://doi.org/10.1242/dev.125.4.725.
30. Kennedy M, Firpo M, Choi K, et al. A common precursor for primitive erythropoiesis and definitive haematopoiesis. *Nature*. 1997;386:488–493. https://doi.org/10.1038/386488a0.
31. Zambidis ET, Peault B, Park TS, et al. Hematopoietic differentiation of human embryonic stem cells progresses through sequential hematoendothelial, primitive, and definitive stages resembling human yolk sac development. *Blood*. 2005;106:860–870. https://doi.org/10.1182/blood-2004-11-4522.
32. Kingsley PD, Malik J, Fantauzzo KA, et al. Yolk sac-derived primitive erythroblasts enucleate during mammalian embryogenesis. *Blood*. 2004;104:19–25. https://doi.org/10.1182/blood-2003-12-4162.
33. Tober J, Koniski A, McGrath KE, et al. The megakaryocyte lineage originates from hemangioblast precursors and is an integral component both of primitive and of definitive hematopoiesis. *Blood*. 2007;109:1433–1441. https://doi.org/10.1182/blood-2006-06-031898.
34. Wong PM, Chung SW, Chui DH, et al. Properties of the earliest clonogenic hemopoietic precursors to appear in the developing murine yolk sac. *Proc Natl Acad Sci USA*. 1986;83:3851–3854.
35. Palis J, Robertson S, Kennedy M, et al. Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development*. 1999;126:5073–5084.
36. McGrath KE, Frame JM, Fegan KH, et al. Distinct sources of hematopoietic progenitors emerge before hscs and provide functional blood cells in the mammalian embryo. *Cell Rep*. 2015;11:1892–1904. https://doi.org/10.1016/j.celrep.2015.05.036.
37. Van Handel B, Prashad SL, Hassanzadeh-Kiabi N, et al. The first trimester human placenta is a site for terminal maturation of primitive erythroid cells. *Blood*. 2010;116:3321–3330. https://doi.org/10.1182/blood-2010-04-279489.
38. Balounova J, Splichalova I, Dobesova M, et al. Toll-like receptor 2 expression on c-kit $^{+}$ cells tracks the emergence of embryonic definitive hematopoietic progenitors. *Nature*. 2019;10:5176. https://doi.org/10.1038/s41467-019-13150-0.
39. Soares-da-Silva F, Freyer L, Elsaid R, et al. Yolk sac, but not hematopoietic stem cell-derived progenitors, sustain erythropoiesis throughout murine embryonic life. *J Exp Med*. 2021;218:20201729. https://doi.org/10.1084/jem.20201729.
40. Dege C, Fegan K, Creamer J, et al. Potently cytotoxic natural killer cells initially emerge from erythro-myeloid progenitors during mammalian development. *Dev Cell*. 2020;53:229–239. https://doi.org/10.1016/j.devcel.2020.02.016.
41. Yahara Y, Barrientos T, Tang Y, et al. Erythromyeloid progenitors give rise to a population of osteoclasts that contribute to bone homeostasis and repair. *Nat Cell Biol*. 2020;22:49–59. https://doi.org/10.1038/s41556-019-0437-8.
42. Jacome-Galarza C, Percin G, Muller J, et al. Developmental origin, functional maintenance and genetic rescue of osteoclasts. *Nature*. 2019;568:541–545. https://doi.org/10.1038/s41586-019-1105-7.
43. Gentek R, Ghigo C, Hoeffel G, et al. Epidermal γδ T cells originate from yolk sac hematopoiesis and clonally self-renew in the adult. *J Exp Med*. 2018;215:2994–3005. https://doi.org/10.1084/jem.20181206.
44. Bian Z, Gong Y, Huang T, et al. Deciphering human macrophage development at single-cell resolution. *Nature*. 2020;582:571–576. https://doi.org/10.1038/s41586-020-2316-7.
45. Stremmel C, Schuchert R, Wagner F, et al. Yolk sac macrophage progenitors traffic to the embryo during defined stages of development. *Nat Comm*. 2018;9:75. https://doi.org/10.1038/s41467-017-02492-2.
46. Medvinsky A, Dzierzak E. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell*. 1996;86:897–906.
47. Medvinsky AL, Samoylina NL, Muller AM, et al. An early pre-liver intraembryonic source of CFU-S in the developing mouse. *Nature*. 1993;364:64–67. https://doi.org/10.1038/364064a0.
48. Muller AM, Medvinsky A, Strouboulis J, et al. Development of hematopoietic stem cell activity in the mouse embryo. *Immunity*. 1994;1:291–301. https://doi.org/10.1016/1074-7613(94)90081-7.
49. Samokhvalov IM, Samokhvalova NI, Nishikawa S. Cell tracing shows the contribution of the yolk sac to adult haematopoiesis. *Nature*. 2007;446:1056–1061. https://doi.org/10.1038/nature05725.
50. Zovein AC, Hofmann JJ, Lynch M, et al. Fate tracing reveals the endothelial origin of hematopoietic stem cells. *Cell Stem Cell*. 2008;3:625–636. https://doi.org/10.1016/j.stem.2008.09.018.
51. Ganuza M, Chabot A, Tang X, et al. Murine hematopoietic stem cell activity is derived from pre-circulation embryos but not yolk sacs. *Nat Commun*. 2018;9:5405.https://doi.org/10.1038/s41467-018-07769-8.

52. Bertrand JY, Chi NC, Santoso B, et al. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature*. 2010;464:108–111. https://doi.org/10.1038/nature08738.
53. Boisset JC, van Cappellen W, Andrieu-Soler C, et al. In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature*. 2010;464:116–120. https://doi.org/10.1038/nature08764.
54. Kissa K, Herbomel P. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature*. 2010;464:112–115. https://doi.org/10.1038/nature08761.
55. Jaffredo T, Gautier R, Eichmann A, et al. Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. *Development*. 1998;125:4575–4583. https://doi.org/10.1242/dev.125.22.4575.
56. Eilken HM, Nishikawa S, Schroeder T. Continuous single-cell imaging of blood generation from haemogenic endothelium. *Nature*. 2009;457:896–900. https://doi.org/10.1038/nature07760.
57. Godin I, Garcia-Porrero JA, Dieterlen-Lievre F, et al. Stem cell emergence and hemopoietic activity are incompatible in mouse intraembryonic sites. *J Exp Med*. 1999;190:43–52. https://doi.org/10.1084/jem.190.1.43.
58. Delassus S, Cumano A. Circulation of hematopoietic progenitors in the mouse embryo. *Immunity*. 1996;4:97–106. https://doi.org/10.1016/s1074-7613(00)80302-7.
59. Zhou F, Li Z, Wang W, et al. Tracing haematopoietic stem cell formation at single cell resolution. *Nature*. 2016;533:487–492. https://doi.org/10.1038/nature17997.
60. Tober J, Maijenburg M, Li Y, et al. Maturation of hematopoietic stem cells from prehematopoietic stem cells is accompanied by up-regulation of PD-L1. *J Exp Med*. 2018;215:645–659. https://doi.org/10.1084/jem.20161594.
61. Kumaravelu P, Hook L, Morrison AM, et al. Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. *Development*. 2002;129:4891–4899. https://doi.org/10.1242/dev.129.21.4891.
62. Gekas C, Dieterlen-Lievre F, Orkin SH, et al. The placenta is a niche for hematopoietic stem cells. *Dev Cell*. 2005;8:365–375. https://doi.org/10.1016/j.devcel.2004.12.016.
63. Ottersbach K, Dzierzak E. The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Dev Cell*. 2005;8:377–387. https://doi.org/10.1016/j.devcel.2005.02.001.
64. Li Z, Lan Y, He W, et al. Mouse embryonic head as a site for hematopoietic stem cell development. *Cell Stem Cell*. 2012;11:663–675. https://doi.org/10.1016/j.stem.2012.07.004.
65. Schulz C, Gomez Perdiguero E, Chorro L, et al. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science*. 2012;336:86–90. https://doi.org/10.1126/science.1219179.
66. Tian Y, Xu J, Feng S, et al. The first wave of T lymphopoiesis in zebrafish arises from aorta endotherlium independent of hematopoietic stem cells. *J Exp Med*. 2017;214:3347–3360. https://doi.org/10.1084/jem.20170488.
67. Motazedian A, Bruveris F, Kumar S, et al. Multipotent RAG1+ progenitors emerge directly from haemogenic endothelium in human pluripotent stem cell-derived haematopoietic organoids. *Nat Cell Biol*. 2020;22:60–73. https://doi.org/10.1038/s41556-019-0445-8.
68. Alemany A, Florescu M, Baron C, et al. Whole-organism clone tracing using single-cell sequencing. *Nature*. 2018;556:108–112. https://doi.org/10.1038/nature25989.
69. Park M, Kumar A, Jung HS, et al. Activation of the arterial program drives development of definitive hemogenic endothelium with lymphoid potential. *Cell Rep*. 2018;23:2467–2481. https://doi.org/10.1016/j.celrep.2018.04.092.
70. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*. 1975;256:495–497. https://doi.org/10.1038/256495a0.
71. Hulett HR, Bonner WA, Barrett J, et al. Cell sorting: automated separation of mammalian cells as a function of intracellular fluorescence. *Science*. 1969;166:747–749. https://doi.org/10.1126/science.166.3906.747.
72. Ezine S, Weissman IL, Rouse RV. Bone marrow cells give rise to distinct cell clones within the thymus. *Nature*. 1984;309:629–631. https://doi.org/10.1038/309629a0.
73. Muller-Sieburg CE, Whitlock CA, Weissman IL. Isolation of two early B lymphocyte progenitors from mouse marrow: a committed pre-pre-B cell and a clonogenic Thy-1-lo hematopoietic stem cell. *Cell*. 1986;44:653–662. https://doi.org/10.1016/0092-8674(86)90274-6.
74. Wu AM, Till JE, Siminovitch L, et al. A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. *J Cell Physiol*. 1967;69:177–184. https://doi.org/10.1002/jcp.1040690208.
75. Dexter TM, Allen TD, Lajtha LG. Conditions controlling the proliferation of haemopoietic stem cells in vitro. *J Cell Physiol*. 1977;91:335–344. https://doi.org/10.1002/jcp.1040910303.
76. Weilbaecher K, Weissman I, Blume K, et al. Culture of phenotypically defined hematopoietic stem cells and other progenitors at limiting dilution on Dexter monolayers. *Blood*. 1991;78:945–952. https://doi.org/10.1182/blood.V78.4.945.945.
77. Eaves CJ, Sutherland HJ, Cashman JD, et al. Regulation of primitive human hematopoietic cells in long-term marrow culture. *Sem Hematol*. 1991;28:126–131.
78. Smith LG, Weissman IL, Heimfeld S. Clonal analysis of hematopoietic stem-cell differentiation in vivo. *Proc Natl Acad Sci USA*. 1991;88:2788–2792. https://doi.org/10.1073/pnas.88.7.2788.
79. Morrison SJ, Weissman IL. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity*. 1994;1:661–673. https://doi.org/10.1016/1074-7613(94)90037-x.
80. Morrison SJ, Wandycz AM, Hemmati HD, et al. Identification of a lineage of multipotent hematopoietic progenitors. *Development*. 1997;124:1929–1939. https://doi.org/10.1242/dev.124.10.1929.
81. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. *Science*. 1988;241:58–62. https://doi.org/10.1126/science.2898810.
82. Adolfsson J, Borge OJ, Bryder D, et al. Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. *Immunity*. 2001;15:659–669. https://doi.org/10.1016/s1074-7613(01)00220-5.
83. Christensen JL, Weissman IL. Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. *Proc Natl Acad Sci USA*. 2001;98:14541–14546. https://doi.org/10.1073/pnas.261562798.
84. Osawa M, Hanada K, Hamada H, et al. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science*. 1996;273:242–245. https://doi.org/10.1126/science.273.5272.242.
85. Wolf NS, Kone A, Priestley GV, et al. In vivo and in vitro characterization of long-term repopulating primitive hematopoietic cells isolated by sequential Hoechst 33342-rhodamine 123 FACS selection. *Exp Hematol*. 1993;21:614–622.
86. Phillips RL, Reinhart AJ, Van Zant G. Genetic control of murine hematopoietic stem cell pool sizes and cycling kinetics. *Proc Natl Acad Sci USA*. 1992;89:11607–11611. https://doi.org/10.1073/pnas.89.23.11607.
87. McAlister I, Wolf NS, Pietrzyk ME, et al. Transplantation of hematopoietic stem cells obtained by a combined dye method fractionation of murine bone marrow. *Blood*. 1990;75:1240–1246. https://doi.org/10.1182/blood.V75.6.1240.1240.
88. Li CL, Johnson GR. Murine hematopoietic stem and progenitor cells: I. Enrichment and biologic characterization. *Blood*. 1995;85:1472–1479. https://doi.org/10.1182/blood.V85.6.1472.bloodjournal8561472.
89. Goodell MA, Brose K, Paradis G, et al. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med*. 1996;183:1797–1806. https://doi.org/10.1084/jem.183.4.1797.
90. Goodell MA. Stem cell identification and sorting using the Hoechst 33342 side population (SP). *Curr Protoc Cytom*. 2005 Chapter 9, Unit 9 18. https://doi.org/10.1002/0471142956.cy0918s34.
91. Lin KK, Goodell MA. Purification of hematopoietic stem cells using the side population. *Methods Enzymol*. 2006;420:255–264. https://doi.org/10.1016/S0076-6879(06)20011-9.
92. Lin KK, Goodell MA. Detection of hematopoietic stem cells by flow cytometry. *Methods Cell Biol*. 2011;103:21–30. https://doi.org/10.1016/B978-0-12-385493-3.00002-4.
93. Purton LE, Scadden DT. Limiting factors in murine hematopoietic stem cell assays. *Cell Stem Cell*. 2007;1:263–270. https://doi.org/10.1016/j.stem.2007.08.016.
94. Matsuzaki Y, Kinjo K, Mulligan RC, et al. Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells. *Immunity*. 2004;20:87–93. https://doi.org/10.1016/s1074-7613(03)00354-6.
95. Kiel MJ, Yilmaz OH, Iwashita T, et al. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell*. 2005;121:1109–1121. https://doi.org/10.1016/j.cell.2005.05.026.
96. Yilmaz OH, Kiel MJ, Morrison SJ. SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. *Blood*. 2006;107:924–930. https://doi.org/10.1182/blood-2005-05-2140.
97. Acar M, Kocherlakota K, Murphy M, et al. Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. *Nature*. 2015;526:126–130. https://doi.org/10.1038/nature15250.
98. Chen J, Miyanishi M, Wang S, et al. Hoxb5 marks long-term haematopoietic stem cells and reveals a homogeneous perivascular niche. *Nature*. 2016;530:223–227. https://doi.org/10.1038/nature16943.

99. Kokkaliaris K, Kunz L, Cabezas-Wallscheid N, et al. Adult blood stem cell localization reflects the abundance of reported bone marrow niche cell types and their combinations. *Blood* 136;2296–2307. https://doi.org/10.1182/blood.2020006574.
100. Wilson N, Kent D, Buettner F, et al. Combined single-cell functional and gene expression analysis resolves heterogeneity within stem cell populations. *Cell Stem Cell*. 2015;16:712–724. https://doi.org/10.1016/j.stem.2015.04.004.
101. Gur-Cohen S, Itkin T, Chakrabarty S, et al. PAR1 signaling regulates the retention and recruitment of EPCR-expressing bone marrow hematopoietic stem cells. *Nat Med*. 2015;21:1307–1317. https://doi.org/10.1038/nm.3960.
102. Fares I, Chagraoui J, Lehnertz B, et al. EPCR expression marks UM171-expanded CD34 $^{+}$ cord blood stem cells. *Blood*. 2017;129:3344–3351. https://doi.org/10.1182/blood-2016-11-750729.
103. Ito K, Turcotte R, Cui J, et al. Self-renewal of a purified Tie2+ hematopoietic stem cell population relies on mitochondrial clearance. *Science*. 2016;354:1156–1160. https://doi.org/10.1126/science.aaf5530.
104. Trinh T, Ropa J, Aljoufi A, et al. Leptin receptor, a surface marker for a subset of highly engrafting long-term functional hematopoietic stem cells. *Leukemia*. Nov 7, 2020 https://doi.org/10.1038/s41375-020-01079-z.
105. Gulati G, Zukowska M, Noh J, et al. Neogenin-1 distinguishes between myeloid-biased and balanced *Hoxb5*$^{+}$ mouse long-term hematopoietic stem cells. *Proc Natl Acad Sci USA*. 2019;116:25115–25125. https://doi.org/10.1073/pnas.1911024116.
106. Sudo T, Yokota T, Oritani K, et al. The endothelial antigen ESAM monitors hematopoietic stem cell status between quiescence and self-renewal. *J Immunol*. 2012;189:200–210. https://doi.org/10.4049/jimmunol.120056.
107. Wada H, Masuda K, Satoh R, et al. Adult T-cell progenitors retain myeloid potential. *Nature*. 2008;452:768–772. https://doi.org/10.1038/nature06839.
108. Bell JJ, Bhandoola A. The earliest thymic progenitors for T cells possess myeloid lineage potential. *Nature*. 2008;452:764–767. https://doi.org/10.1038/nature06840.
109. Richie Ehrlich LI, Serwold T, Weissman IL. In vitro assays misrepresent in vivo lineage potentials of murine lymphoid progenitors. *Blood*. 2011;117:2618–2624. https://doi.org/10.1182/blood-2010-05-287102.
110. De Obaldia ME, Bell JJ, Bhandoola A, et al. Cell progenitors are the major granulocyte precursors in the adult mouse thymus. *Blood*. 2013;121:64–71. https://doi.org/10.1182/blood-2012-08-451773.
111. De Obaldia ME, Bell JJ, Wang X, et al. T cell development requires constraint of the myeloid regulator C/EBP-alpha by the Notch target and transcriptional repressor Hes1. *Nat Immunol*. 2013;14:1277–1284. https://doi.org/10.1038/ni.2760.
112. Inlay MA, Bhattacharya D, Sahoo D, et al. Ly6d marks the earliest stage of B-cell specification and identifies the branchpoint between B-cell and T-cell development. *Genes Dev*. 2009;23:2376–2381. https://doi.org/10.1101/gad.1836009.
113. Larochelle A, Vormoor J, Hanenberg H, et al. Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. *Nat Med*. 1996;2:1329–1337. https://doi.org/10.1038/nm1296-1329.
114. Bhatia M, Wang JC, Kapp U, et al. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci USA*. 1997;94:5320–5325. https://doi.org/10.1073/pnas.94.10.5320.
115. Terakura S, Azuma E, Murata M, et al. Hematopoietic engraftment in recipients of unrelated donor umbilical cord blood is affected by the CD34+ and CD8+ cell doses. *Biol Blood Marrow Transplant*. 2007;13:822–830. https://doi.org/10.1016/j.bbmt.2007.03.006.
116. Civin CI, Strauss LC, Brovall C, et al. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J Immunol*. 1984;133:157–165.
117. Bhatia M, Bonnet D, Murdoch B, et al. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. *Nat Med*. 1998;4:1038–1045. https://doi.org/10.1038/2023.
118. Goodell MA, Rosenzweig M, Kim H, et al. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat Med*. 1997;3:1337–1345. https://doi.org/10.1038/nm1297-1337.
119. Zanjani ED, Almeida-Porada G, Livingston AG, et al. Human bone marrow CD34- cells engraft in vivo and undergo multilineage expression that includes giving rise to CD34+ cells. *Exp Hematol*. 1998;26:353–360.
120. Gao Z, Fackler MJ, Leung W, et al. Human CD34+ cell preparations contain over 100-fold greater NOD/SCID mouse engrafting capacity than do CD34- cell preparations. *Exp Hematol*. 2001;29:910–921. https://doi.org/10.1016/s0301-472x(01)00654-3.
121. Baum CM, Weissman IL, Tsukamoto AS, et al. Isolation of a candidate human hematopoietic stem-cell population. *Proc Natl Acad Sci USA*. 1992;89:2804–2808. https://doi.org/10.1073/pnas.89.7.2804.
122. Petzer AL, Zandstra PW, Piret JM, et al. Differential cytokine effects on primitive (CD34+CD38-) human hematopoietic cells: novel responses to Flt3-ligand and thrombopoietin. *J Exp Med*. 1996;183:2551–2558. https://doi.org/10.1084/jem.183.6.2551.
123. Majeti R, Park CY, Weissman IL. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. *Cell Stem Cell*. 2007;1:635–645. https://doi.org/10.1016/j.stem.2007.10.001.
124. McKenzie JL, Takenaka K, Gan OI, et al. Low rhodamine 123 retention identifies long-term human hematopoietic stem cells within the Lin-CD34+CD38- population. *Blood*. 2007;109:543–545. https://doi.org/10.1182/blood-2006-06-030270.
125. Notta F, Doulatov S, Laurenti E, et al. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science*. 2011;333:218–221. https://doi.org/10.1126/science.1201219.
126. Chitteti BR, Kobayashi M, Cheng Y, et al. CD166 regulates human and murine hematopoietic stem cells and the hematopoietic niche. *Blood*. 2014;124:519–529. https://doi.org/10.1182/blood-2014-03-565721.
127. Quarmyne M, Doan PL, Himburg HA, et al. Protein tyrosine phosphatase-sigma regulates hematopoietic stem cell-repopulating capacity. *J Clin Invest*. 2015;125:177–182. https://doi.org/10.1172/JCI77866.
128. Zang Y, Roos M, Himburg H, et al. PTPσ inhibitors promote hematopoietic stem cell regeneration. *Nat Commun*. 2019;10:3667.https://doi.org/10.1038/s41467-019-11490-5.
129. Buenrostro J, Corces MR, Lareau C, et al. Integrated single-cell analysis maps the continuous regulatory landscape of human hematopoietic differentiation. *Cell*. 2018;173:1535–1548. https://doi.org/10.1016/j.cell.2018.03.074.
130. Knapp D, Hammond C, Wang F, et al. A topological view of human CD34^{+} cell state trajectories from integrated single-cell output and proteomic data. *Blood*. 2019;133:927–939. https://doi.org/10.1182/blood-2018-10-878025.
131. Moussy A, Cosette J, Parmentier R, et al. Integrated time-lapse and single-cell transcription studies highlight the variable and dynamic nature of human hematopoietic cell fate commitment. *PLoS Biol*. 2017;15:e2001867. https://doi.org/10.1371/journal.pbio.2001867.
132. Knapp D, Hammon C, Aghaeepour N, et al. Distinct signaling programs control human hematopoietic stem cell survival and proliferation. *Blood*. 2017;129:307–318. https://doi.org/10.1182/blood-2016-09-740654.
133. Han X, Zhou Z, Fei L, et al. Construction of a human cell landscape at single-cell level. *Nature*. 2020;581:303–309. https://doi.org/10.1038/s41586-020-2157-4.
134. Pellin D, Loperfido M, Baricordi C, et al. A comprehensive single cell transcriptional landscape of human hematopoietic progenitors. *Nat Commun*. 2019;10:2395. https://doi.org/10.1038/s41467-019-10291-0.
135. Rodriguez-Fraticelli A, Camargo F. Systems analysis of hematopoiesis using single-cell lineage tracing. *Curr Opin Hematol*. 2021;28:18–27. https://doi.org/10.1097/MOH.0000000000000624.
136. Pali C, Cheng Q, Gillespie M, et al. Single-cell proteomics reveal that quantitative changes in co-expressed lineage-specific transcription factors determine cell fate. *Cell Stem Cell*. 2019;24:812–820. https://doi.org/10.1016/j.stem.2019.02.006.
137. Watcham S, Kucinski I, Gottgens B. New insights into hematopoietic differentiation landscaptes from single-cell RNA sequencing. *Blood*. 2019;133:1415–1426. https://doi.org/10.1182/blood-2018-08-835355.
138. Adelman E, Huang HT, Roisman A, et al. Aging human hematopoietic stem cells manifest profound epigenetic reprogramming of enhancers that may predispose to leukemia. *Cancer Discovery*. 2019;9:1080–1101. https://doi.org/10.1158/2159-8290.CD-18-1474.
139. Schmitt TM, Zuniga-Pflucker JC. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity*. 2002;17:749–756. https://doi.org/10.1016/s1074-7613(02)00474-0.
140. Whitlock CA, Witte ON. Long-term culture of B lymphocytes and their precursors from murine bone marrow. *Proc Natl Acad Sci USA*. 1982;79:3608–3612. https://doi.org/10.1073/pnas.79.11.3608.
141. van Os R, Kamminga LM, de Haan G. Stem cell assays: something old, something new, something borrowed. *Stem Cells*. 2004;22:1181–1190. https://doi.org/10.1634/stemcells.2004-0095.
142. Brandt JE, Bartholomew AM, Fortman JD, et al. Ex vivo expansion autologous bone marrow CD34(+) cells with porcine microvascular endothelial cells results in a graft capable of rescuing lethally irradiated baboons. *Blood*. 1999;94:106–113. https://doi.org/10.1182/blood.V94.1.106.413k01_106_113.

143. Himburg HA, Muramoto GG, Daher P, et al. Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells. *Nat Med*. 2010;16:475–482. https://doi.org/10.1038/nm.2119.
144. Harrison DE. Competitive repopulation: a new assay for long-term stem cell functional capacity. *Blood*. 1980;55:77–81.https://doi.org/10.1182/blood.V55.1.77.77.
145. Harrison DE, Jordan CT, Zhong RK, et al. Primitive hemopoietic stem cells: direct assay of most productive populations by competitive repopulation with simple binomial, correlation and covariance calculations. *Exp Hematol*. 1993;21:206–219.
146. Yuan R, Astle CM, Chen J, et al. Genetic regulation of hematopoietic stem cell exhaustion during development and growth. *Exp Hematol*. 2005;33:243–250. https://doi.org/10.1016/j.exphem.2004.10.014.
147. Szilvassy SJ, Humphries RK, Lansdorp PM, et al. Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. *Proc Natl Acad Sci USA*. 1990;87:8736–8740. https://doi.org/10.1073/pnas.87.22.8736.
148. Szilvassy SJ, Lansdorp PM, Humphries RK, et al. Isolation in a single step of a highly enriched murine hematopoietic stem cell population with competitive long-term repopulating ability. *Blood*. 1989;74:930–939. https://doi.org/10.1182/blood.V74.3.930.930.
149. Taswell C. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. *J Immunol*. 1981;126:1614–1619.
150. Jordan CT, Lemischka IR. Clonal and systemic analysis of long-term hematopoiesis in the mouse. *Genes Dev*. 1990;4:220–232. https://doi.org/10.1101/gad.4.2.220.
151. Dykstra B, Kent D, Bowie M, et al. Long-term propagation of distinct hematopoietic differentiation programs in vivo. *Cell Stem Cell*. 2007;1:218–229. https:// doi.org/10.1016/j.stem.2007.05.015.
152. Lemischka IR, Raulet DH, Mulligan RC. Developmental potential and dynamic behavior of hematopoietic stem cells. *Cell*. 1986;45:917–927. https://doi.org/10.1016/0092-8674(86)90566-0.
153. Purton LE, Dworkin S, Olsen GH, et al. RARgamma is critical for maintaining a balance between hematopoietic stem cell self-renewal and differentiation. *J Exp Med*. 2006;203:1283–1293. https://doi.org/10.1084/jem.20052105.
154. Rosendaal M, Hodgson GS, Bradley TR. Organization of haemopoietic stem cells: the generation-age hypothesis. *Cell Tissue Kinet*. 1979;12:17–29.https://doi.org/10.1111/j.1365-2184.1979.tb00110.x.
155. Adams GB, Chabner KT, Alley IR, et al. Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. *Nature*. 2006;439:599–603. https://doi.org/10.1038/nature04247.
156. Cancelas JA, Lee AW, Prabhakar R, et al. Rac GTPases differentially integrate signals regulating hematopoietic stem cell localization. *Nat Med*. 2005;11:886–891. https://doi.org/10.1038/nm1274.
157. Ema H, Morita Y, Yamazaki S, et al. Adult mouse hematopoietic stem cells: purification and single-cell assays. *Nat Protoc*. 2006;1:2979–2987. https://doi.org/10.1038/nprot.2006.447.
158. Ema H, Sudo K, Seita J, et al. Quantification of self-renewal capacity in single hematopoietic stem cells from normal and Lnk-deficient mice. *Dev Cell*. 2005;8:907–914. https://doi.org/10.1016/j.devcel.2005.03.019.
159. Janzen V, Forkert R, Fleming HE, et al. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature*. 2006;443:421–426. https://doi.org/10.1038/nature05159.
160. Walkley CR, Fero ML, Chien WM, et al. Negative cell-cycle regulators cooperatively control self-renewal and differentiation of haematopoietic stem cells. *Nat Cell Biol*. 2005;7:172–178. https://doi.org/10.1038/ncb1214.
161. Muramoto GG, Russell JL, Safi R, et al. Inhibition of aldehyde dehydrogenase expands hematopoietic stem cells with radioprotective capacity. *Stem Cells*. 2010;28:523–534. https://doi.org/10.1002/stem.299.
162. Yang L, Bryder D, Adolfsson J, et al. Identification of Lin(-)Sca1(+)kit(+)CD34(+)Flt3- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. *Blood*. 2005;105:2717–2723. https://doi.org/10.1182/blood-2004-06-2159.
163. Sieburg HB, Cho RH, Dykstra B, et al. The hematopoietic stem compartment consists of a limited number of discrete stem cell subsets. *Blood*. 2006;107:2311–2316. https://doi.org/10.1182/blood-2005-07-2970.
164. Bryder D, Rossi DJ, Weissman IL. Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. *Am J Pathol*. 2006;169:338–346. https://doi.org/10.2353/ajpath.2006.060312.
165. Dick JE, Magli MC, Huszar D, et al. Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hemopoietic system of W/Wv mice. *Cell*. 1985;42:71–79. https://doi.org/10.1016/s0092-8674(85)80102-1.
166. Keller G, Snodgrass R. Life span of multipotential hematopoietic stem cells in vivo. *J Exp Med*. 1990;171:1407–1418. https://doi.org/10.1084/jem.171.5.1407.
167. Spangrude GJ, Brooks DM, Tumas DB. Long-term repopulation of irradiated mice with limiting numbers of purified hematopoietic stem cells: in vivo expansion of stem cell phenotype but not function. *Blood*. 1995;85:1006–1016. https://doi.org/10.1182/blood.V85.4.1006.bloodjournal8541006.
168. Zhong RK, Astle CM, Harrison DE. Distinct developmental patterns of short-term and long-term functioning lymphoid and myeloid precursors defined by competitive limiting dilution analysis in vivo. *J Immunol*. 1996;157:138–145.
169. Randall TD, Weissman IL. Phenotypic and functional changes induced at the clonal level in hematopoietic stem cells after 5-fluorouracil treatment. *Blood*. 1997;89:3596–3606. https://doi.org/10.1182/blood.V89.10.3596.
170. Szilvassy SJ, Ragland PL, Miller CL, et al. The marrow homing efficiency of murine hematopoietic stem cells remains constant during ontogeny. *Exp Hematol*. 2003;31:331–338. https://doi.org/10.1016/s0301-472x(03)00005-5.
171. Muller-Sieburg CE, Sieburg HB. Clonal diversity of the stem cell compartment. *Curr Opin Hematol*. 2006;13:243–248. https://doi.org/10.1097/01.moh.0000231421.00407.65.
172. Trentin JJ. Determination of bone marrow stem cell differentiation by stromal hemopoietic inductive microenvironments (HIM). *Am J Pathol*. 1971;65:621–628.
173. Metcalf D. Lineage commitment and maturation in hematopoietic cells: the case for extrinsic regulation. *Blood*. 1998;92:345–347. discussion 352. https://doi.org/10.1182/blood.V92.2.345b.
174. Moore KA, Lemischka IR. Stem cells and their niches. *Science*. 2006;311:1880–1885. https://doi.org/10.1126/science.1110542.
175. Abkowitz JL, Persik MT, Shelton GH, et al. Behavior of hematopoietic stem cells in a large animal. *Proc Natl Acad Sci USA*. 1995;92:2031–2035. https://doi.org/10.1073/pnas.92.6.2031.
176. Till JE, McCulloch EA, Siminovitch L. A stochastic model of stem cell proliferation, based on the growth of spleen colony-forming cells. *Proc Natl Acad Sci USA*. 1964;51:29–36. https://doi.org/10.1073/pnas.51.1.29.
177. Kirkland MA. A phase space model of hemopoiesis and the concept of stem cell renewal. *Exp Hematol*. 2004;32:511–519. https://doi.org/10.1016/j.exphem.2004.02.013.
178. Roeder I, Kamminga LM, Braesel K, et al. Competitive clonal hematopoiesis in mouse chimeras explained by a stochastic model of stem cell organization. *Blood*. 2005;105:609–616. https://doi.org/10.1182/blood-2004-01-0282.
179. Muller-Sieburg CE, Sieburg HB. The GOD of hematopoietic stem cells: a clonal diversity model of the stem cell compartment. *Cell Cycle*. 2006;5:394–398. https://doi.org/10.4161/cc.5.4.2487.
180. Morita Y, Ema H, Nakauchi H. Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. *J Exp Med*. 2010;207:1173–1182. https://doi.org/10.1084/jem.20091318.
181. Challen GA, Boles NC, Chambers SM, et al. Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. *Cell Stem Cell*. 2010;6:265–278. https://doi.org/10.1016/j.stem.2010.02.002.
182. Muller-Sieburg CE, Cho RH, Karlsson L, et al. Myeloid-biased hematopoietic stem cells have extensive self-renewal capacity but generate diminished lymphoid progeny with impaired IL-7 responsiveness. *Blood*. 2004;103:4111–4118. https://doi.org/10.1182/blood-2003-10-3448.
183. Yamamoto R, Morita Y, Ooehara J, et al. Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell*. 2013;154:1112–1126. https://doi.org/10.1016/j.cell.2013.08.007.
184. Haas S, Hansson J, Klimmeck D, et al. Inflammation-induced emergency megakaryopoiesis driven by hematopoietic stem cell-like megakaryocyte progenitors. *Cell Stem Cell*. 2015;17:422–434. https://doi.org/10.1016/j.stem.2015.07.007.
185. Notta F, Zandi S, Takayama N, et al. Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. *Science*. 2016;351:aab2116. https://doi.org/10.1126/science.aab2116.
186. Paul F, Arkin Y, Giladi A, et al. Transcriptional heterogeneity and lineage commitment in myeloid progenitors. *Cell*. 2015;163:1663–1677. https://doi.org/10.1016/j.cell.2015.11.013.
187. Velten L, Haas SF, Raffel S, et al. Human haematopoietic stem cell lineage commitment is a continuous process. *Nat Cell Biol*. 2017;19:271–281. https://doi.org/10.1038/ncb3493.
188. Mallaney C, Kothari A, Martens A, et al. Clonal-level responses of functionally distinct hematopoietic stem cells to trophic factors. *Exp Hematol*. 2014;42:317–327 e312. https://doi.org/10.1016/j.exphem.2013.11.015.

189. Muller-Sieburg CE, Cho RH, Thoman M, et al. Deterministic regulation of hematopoietic stem cell self-renewal and differentiation. *Blood*. 2002;100:1302–1309. https://doi.org/10.1182/blood.V100.4.1302.h81602001302_1302_1309.
190. Benz C, Copley MR, Kent DG, et al. Hematopoietic stem cell subtypes expand differentially during development and display distinct lymphopoietic programs. *Cell Stem Cell*. 2012;10:273–283. https://doi.org/10.1016/j.stem.2012.02.007.
191. Gerrits A, Dykstra B, Kalmykowa OJ, et al. Cellular barcoding tool for clonal analysis in the hematopoietic system. *Blood*. 2010;115:2610–2618. https://doi.org/10.1182/blood-2009-06-229757.
192. Naik SH, Perié L, Swart E, et al. Diverse and heritable lineage imprinting of early haematopoietic progenitors. *Nature*. 2013;496:229–232. https://doi.org/10.1038/nature12013.
193. Kay HE. How many cell-generations? *Lancet*. 1965;2:418–419. https://doi.org/10.1016/s0140-6736(65)90763-4.
194. Drize NJ, Keller JR, Chertkov JL. Local clonal analysis of the hematopoietic system shows that multiple small short-living clones maintain life-long hematopoiesis in reconstituted mice. *Blood*. 1996;88:2927–2938. https://doi.org/10.1182/blood.V88.8.2927.bloodjournal8882927.
195. McKenzie JL, Gan OI, Doedens M, et al. Individual stem cells with highly variable proliferation and self-renewal properties comprise the human hematopoietic stem cell compartment. *Nat Immunol*. 2006;7:1225–1233. https://doi.org/10.1038/ni1393.
196. Prchal JT, Prchal JF, Belickova M, et al. Clonal stability of blood cell lineages indicated by X-chromosomal transcriptional polymorphism. *J Exp Med*. 1996;183:561–567. https://doi.org/10.1084/jem.183.2.561.
197. Wilson A, Laurenti E, Oser G, et al. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell*. 2008;135:1118–1129. https://doi.org/10.1016/j.cell.2008.10.048.
198. Glauche I, Moore K, Thielecke L, et al. Stem cell proliferation and quiescence—two sides of the same coin. *PLoS Comput Biol*. 2009;5:e1000447. https://doi.org/10.1371/journal.pcbi.1000447.
199. Takizawa H, Regoes RR, Boddupalli CS, et al. Dynamic variation in cycling of hematopoietic stem cells in steady state and inflammation. *J Exp Med*. 2011;208:273–284. https://doi.org/10.1084/jem.20101643.
200. Busch K, Klapproth K, Barile M, et al. Fundamental properties of unperturbed haematopoiesis from stem cells in vivo. *Nature*. 2015;518:542–546. https://doi.org/10.1038/nature14242.
201. Sun J, Ramos A, Chapman B, et al. Clonal dynamics of native haematopoiesis. *Nature*. 2014;514:322–327. https://doi.org/10.1038/nature13824.
202. Sawai CM, Babovic S, Upadhaya S, et al. Hematopoietic stem cells are the major source of multilineage hematopoiesis in adult animals. *Immunity*. 2016;45:597–609. https://doi.org/10.1016/j.immuni.2016.08.007.
203. Visvader JE. Cells of origin in cancer. *Nature*. 2011;469:314–322. https://doi.org/10.1038/nature09781.
204. Rodriguez- Fraticelli AE, Wolock SL, Weinreb CS, et al. Clonal analysis of lineage fate in native haematopoiesis. *Nature*. 2018;553:212–216. https://doi.org/10.1038/nature25168.
205. Pei W, Feyerabend T, Rossler J, et al. Polylox barcoding reveals haematopoietic stem cell fates realized in vivo. *Nature*. 2017;548:456–460. https://doi.org/10.1038/nature23653.
206. Pei W, Shang F, Wang X, et al. Resolving fates and single-cell transcriptomes of hematopoietic stem cell clones by polyloxexpress barcoding. *Cell Stem Cell*. 2020;27:383–395. https://doi.org/10.1016/j.stem.2020.07.018.
207. Morcos MNF, Zerjatke T, Glauche I, et al. Continuous mitotic activity of primitive hematopoietic stem cells in adult mice. *J Exp Med*. 2020;217 e20191284. https://doi/org/10.1084/jem.20191284.
208. Oguro H, Ding L, Morrison SJ. SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. *Cell Stem Cell*. 2013;13:102–116. https://doi.org/10.1016/j.stem.2013.05.014.
209. Morrison SJ, Kimble J. Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature*. 2006;441:1068–1074. https://doi.org/10.1038/nature04956.
210. Pinho S, Frenette P. Haematopoietic stem cell activity and interactions with the niche. *Nat Rev Mol Cell Biol*. 2019;20:303–320. https://doi.org/10.1038/s41580-019-0103-9.
211. Crane G, Jeffery E, Morrison S. Adult haematopoietic stem cell niches. *Nat Rev Immunol*. 2017;17:573–590. https://doi.org/10.1038/nri.2017.53.
212. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science*. 1999;284:770–776. https://doi.org/10.1126/science.284.5415.770.
213. Hori K, Sen A, Artavanis-Tsakonas S. Notch signaling at a glance. *J Cell Sci*. 2013;126:2135–2140. https://doi.org/10.1242/jcs.127308.
214. Bray SJ. Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol*. 2006;7:678–689. https://doi.org/10.1038/nrm2009.
215. Dahlberg A, Delaney C, Bernstein ID. Ex vivo expansion of human hematopoietic stem and progenitor cells. *Blood*. 2011;117:6083–6090. https://doi.org/10.1182/blood-2011-01-283606.
216. Radtke F, Fasnacht N, Macdonald HR. Notch signaling in the immune system. *Immunity*. 2010;32:14–27. https://doi.org/10.1016/j.immuni.2010.01.004.
217. Varnum-Finney B, Dallas MH, Kato K, et al. Notch target Hes5 ensures appropriate Notch induced T- versus B-cell choices in the thymus. *Blood*. 2008;111:2615–2620. https://doi.org/10.1182/blood-2007-03-079855.
218. Wendorff AA, Koch U, Wunderlich FT, et al. Hes1 is a critical but context-dependent mediator of canonical Notch signaling in lymphocyte development and transformation. *Immunity*. 2010;33:671–684. https://doi.org/10.1016/j.immuni.2010.11.014.
219. Varnum-Finney B, Halasz LM, Sun M, et al. Notch2 governs the rate of generation of mouse long- and short-term repopulating stem cells. *J Clin Invest*. 2011;121:1207–1216. https://doi.org/10.1172/JCI43868.
220. Varnum-Finney B, Xu L, Brashem-Stein C, et al. Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nat Med*. 2000;6:1278–1281. https://doi.org/10.1038/81390.
221. Varnum-Finney B, Brashem-Stein C, Bernstein ID. Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability. *Blood*. 2003;101:1784–1789. https://doi.org/10.1182/blood-2002-06-1862.
222. Carlesso N, Aster JC, Sklar J, et al. Notch1-induced delay of human hematopoietic progenitor cell differentiation is associated with altered cell cycle kinetics. *Blood*. 1999;93:838–848. https://doi.org/10.1182/blood.V93.3.838.
223. Karanu FN, Murdoch B, Gallacher L, et al. The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells. *J Exp Med*. 2000;192:1365–1372. https://doi.org/10.1084/jem.192.9.1365.
224. Calvi LM, Adams GB, Weibrecht KW, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature*. 2003;425:841–846. https://doi.org/10.1038/nature02040.
225. Butler JM, Nolan DJ, Vertes EL, et al. Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell Stem Cell*. 2010;6:251–264. https://doi.org/10.1016/j.stem.2010.02.001.
226. Mancini SJ, Mantei N, Dumortier A, et al. Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. *Blood*. 2005;105:2340–2342. https://doi.org/10.1182/blood-2004-08-3207.
227. Maillard I, Koch U, Dumortier A, et al. Canonical notch signaling is dispensable for the maintenance of adult hematopoietic stem cells. *Cell Stem Cell*. 2008;2:356–366. https://doi.org/10.1016/j.stem.2008.02.011.
228. Delaney C, Varnum-Finney B, Aoyama K, et al. Dose-dependent effects of the Notch ligand Delta1 on ex vivo differentiation and in vivo marrow repopulating ability of cord blood cells. *Blood*. 2005;106:2693–2699. https://doi.org/10.1182/blood-2005-03-1131.
229. Delaney C, Heimfeld S, Brashem-Stein C, et al. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. *Nat Med*. 2010;16:232–236. https://doi.org/10.1038/nm.2080.
230. Gutman JA, Turtle CJ, Manley TJ, et al. Single-unit dominance after double-unit umbilical cord blood transplantation coincides with a specific CD8+ T-cell response against the nonengrafted unit. *Blood*. 2010;115:757–765. https://doi.org/10.1182/blood-2009-07-228999.
231. Clevers H. Wnt/beta-catenin signaling in development and disease. *Cell*. 2006;127:469–480. https://doi.org/10.1016/j.cell.2006.10.018.
232. Austin TW, Solar GP, Ziegler FC, et al. A role for the Wnt gene family in hematopoiesis: expansion of multilineage progenitor cells. *Blood*. 1997;89:3624–3635. https://doi.org/10.1182/blood.V89.10.3624.
233. Lento W, Congdon K, Voermans C, et al. WNT signaling in normal and malignant hematopoiesis. *Cold Spring Harb Perspect Biol*. 2013;5 https://doi.org/10.1101/cshperspect.a008011.
234. Reya T, Duncan AW, Ailles L, et al. A role for WNT signalling in self-renewal of haematopoietic stem cells. *Nature*. 2003;423:409–414. https://doi.org/10.1038/nature01593.
235. Murdoch B, Chadwick K, Martin M, et al. WNT-5A augments repopulating capacity and primitive hematopoietic development of human blood stem cells in vivo. *Proc Natl Acad Sci USA*. 2003;100:3422–3427. https://doi.org/10.1073/pnas.0130233100.
236. Nemeth MJ, Topol L, Anderson SM, et al. WNT5a inhibits canonical Wnt signaling in hematopoietic stem cells and enhances repopulation. *Proc Natl Acad Sci USA*. 2007;104:15436–15441. https://doi.org/10.1073/pnas.0704747104.
237. Blank U, Karlsson G, Karlsson S. Signaling pathways governing stem-cell fate. *Blood*. 2008;111:492–503. https://doi.org/10.1182/blood-2007-07-075168.

238. Willert K, Brown JD, Danenberg E, et al. WNT proteins are lipid-modified and can act as stem cell growth factors. *Nature*. 2003;423:448–452. https://doi.org/10.1038/nature01611.
239. Domen J, Gandy KL, Weissman IL. Systemic overexpression of BCL-2 in the hematopoietic system protects transgenic mice from the consequences of lethal irradiation. *Blood*. 1998;91:2272–2282. https://doi.org/10.1182/blood.V91.7.2272.
240. Domen J, Weissman IL. Hematopoietic stem cells need two signals to prevent apoptosis; BCL-2 can provide one of these, Kitl/c-Kit signaling the other. *J Exp Med*. 2000;192:1707–1718. https://doi.org/10.1084/jem.192.12.1707.
241. Trowbridge JJ, Xenocostas A, Moon RT, et al. Glycogen synthase kinase-3 is an in vivo regulator of hematopoietic stem cell repopulation. *Nat Med*. 2006;12:89–98. https://doi.org/10.1038/nm1339.
242. Duncan AW, Rattis FM, DiMascio LN, et al. Integration of Notch and WNT signaling in hematopoietic stem cell maintenance. *Nat Immunol*. 2005;6:314–322. https://doi.org/10.1038/ni1164.
243. Nie Y, Han YC, Zou YR. CXCR4 is required for the quiescence of primitive hematopoietic cells. *J Exp Med*. 2008;205:777–783. https://doi.org/10.1084/jem.20072513.
244. Kirstetter P, Anderson K, Porse BT, et al. Activation of the canonical WNT pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. *Nat Immunol*. 2006;7:1048–1056. https://doi.org/10.1038/ni1381.
245. Scheller M, Huelsken J, Rosenbauer F, et al. Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. *Nat Immunol*. 2006;7:1037–1047. https://doi.org/10.1038/ni1387.
246. Mikels AJ, Nusse R. Purified WNT5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. *PLoS Biol*. 2006;4:e115. https://doi.org/10.1371/journal.pbio.0040115.
247. Buckley SM, Ulloa-Montoya F, Abts D, et al. Maintenance of HSC by WNT5a secreting AGM-derived stromal cell line. *Exp Hematol*. 2011;39:114–123.e1-5. https://doi.org/10.1016/j.exphem.2010.09.010.
248. Sugimura R, He XC, Venkatraman A, et al. Noncanonical WNT signaling maintains hematopoietic stem cells in the niche. *Cell*. 2012;150:351–365. https://doi.org/10.1016/j.cell.2012.05.041.
249. Florian MC, Nattamai KJ, Dörr K, et al. A canonical to non-canonical WNT signalling switch in haematopoietic stem-cell ageing. *Nature*. 2013;503:392–396. https://doi.org/10.1038/nature12631.
250. Cobas M, Wilson A, Ernst B, et al. Beta-catenin is dispensable for hematopoiesis and lymphopoiesis. *J Exp Med*. 2004;199:221–229. https://doi.org/10.1084/jem.20031615.
251. Kabiri Z, Numata A, Kawasaki A, et al. WNTs are dispensable for differentiation and self-renewal of adult murine hematopoietic stem cells. *Blood*. 2015;126:1086–1094. https://doi.org/10.1182/blood-2014-09-598540.
252. Zhao C, Blum J, Chen A, et al. Loss of beta-catenin impairs the renewal of normal and CML stem cells in vivo. *Cancer Cell*. 2007;12:528–541. https://doi.org/10.1016/j.ccr.2007.11.003.
253. Luis TC, Weerkamp F, Naber BAE, et al. WNT3a deficiency irreversibly impairs hematopoietic stem cell self-renewal and leads to defects in progenitor cell differentiation. *Blood*. 2009;113:546–554. https://doi.org/10.1182/blood-2008-06-163774.
254. Blank U, Karlsson S. The role of SMAD signaling in hematopoiesis and translational hematology. *Leukemia*. 2011;25:1379–1388. https://doi.org/10.1038/leu.2011.95.
255. Hatzfeld J, Li ML, Brown EL, et al. Release of early human hematopoietic progenitors from quiescence by antisense transforming growth factor beta 1 or Rb oligonucleotides. *J Exp Med*. 1991;174:925–929. https://doi.org/10.1084/jem.174.4.925.
256. Sitnicka E, Ruscetti FW, Priestley GV, et al. Transforming growth factor beta 1 directly and reversibly inhibits the initial cell divisions of long-term repopulating hematopoietic stem cells. *Blood*. 1996;88:82–88.
257. Soma T, Yu JM, Dunbar CE. Maintenance of murine long-term repopulating stem cells in ex vivo culture is affected by modulation of transforming growth factor-beta but not macrophage inflammatory protein-1 alpha activities. *Blood*. 1996;87:4561–4567. https://doi.org/10.1182/blood.V87.11.4561.bloodjournal87114561.
258. Blank U, Karlsson S. TGF-beta signaling in the control of hematopoietic stem cells. *Blood*. 2015;125:3542–3550. https://doi.org/10.1182/blood-2014-12-618090.
259. Dao MA, Taylor N, Nolta JA. Reduction in levels of the cyclin-dependent kinase inhibitor p27(kip-1) coupled with transforming growth factor beta neutralization induces cell-cycle entry and increases retroviral transduction of primitive human hematopoietic cells. *Proc Natl Acad Sci USA*. 1998;95:13006–13011. https://doi.org/10.1073/pnas.95.22.13006.
260. Dubois CM, Ruscetti FW, Palaszynski EW, et al. Transforming growth factor beta is a potent inhibitor of interleukin 1 (IL-1) receptor expression: proposed mechanism of inhibition of IL-1 action. *J Exp Med*. 1990;172:737–744. https://doi.org/10.1084/jem.172.3.737.
261. Scandura JM, Boccuni P, Massague J, et al. Transforming growth factor beta-induced cell cycle arrest of human hematopoietic cells requires p57KIP2 up-regulation. *Proc Natl Acad Sci USA*. 2004;101:15231–15236. https://doi.org/10.1073/pnas.0406771101.
262. Yamazaki S, Iwama A, Takayanagi S, et al. TGF-beta as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation. *Blood*. 2009;113:1250–1256. https://doi.org/10.1182/blood-2008-04-146480.
263. Letterio JJ, Geiser AG, Kulkarni AB, et al. Autoimmunity associated with TGF-beta1-deficiency in mice is dependent on MHC class II antigen expression. *J Clin Invest*. 1996;98:2109–2119. https://doi.org/10.1172/JCI119017.
264. Capron C, Lacout C, Lécluse Y, et al. A major role of TGF-beta1 in the homing capacities of murine hematopoietic stem cell/progenitors. *Blood*. 2010;116:1244–1253. https://doi.org/10.1182/blood-2009-05-221093.
265. Yamazaki S, Ema H, Karlsson G, et al. Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. *Cell*. 2011;147:1146–1158. https://doi.org/10.1016/j.cell.2011.09.053.
266. Larsson J, Blank U, Helgadottir H, et al. TGF-beta signaling-deficient hematopoietic stem cells have normal self-renewal and regenerative ability in vivo despite increased proliferative capacity in vitro. *Blood*. 2003;102:3129–3135. https://doi.org/10.1182/blood-2003-04-1300.
267. Larsson J, Blank U, Klintman J, et al. Quiescence of hematopoietic stem cells and maintenance of the stem cell pool is not dependent on TGF-beta signaling in vivo. *Exp Hematol*. 2005;33:592–596. https://doi.org/10.1016/j.exphem.2005.02.003.
268. Bhatia M, Bonnet D, Wu D, et al. Bone morphogenetic proteins regulate the developmental program of human hematopoietic stem cells. *J Exp Med*. 1999;189:1139–1148. https://doi.org/10.1084/jem.189.7.1139.
269. Karlsson G, Blank U, Moody JL, et al. SMAD4 is critical for self-renewal of hematopoietic stem cells. *J Exp Med*. 2007;204:467–474. https://doi.org/10.1084/jem.20060465.
270. Blank U, Karlsson G, Moody JL, et al. SMAD7 promotes self-renewal of hematopoietic stem cells. *Blood*. 2006;108:4246–4254. https://doi.org/10.1182/blood-2006-02-005611.
271. Labbe E, Letamendia A, Attisano L. Association of SMADs with lymphoid enhancer binding factor 1/T cell-specific factor mediates cooperative signaling by the transforming growth factor-beta and WNT pathways. *Proc Natl Acad Sci USA*. 2000;97:8358–8363. https://doi.org/10.1073/pnas.150152697.
272. Nishita M, Hashimoto MK, Ogata S, et al. Interaction between Wnt and TGF-beta signalling pathways during formation of Spemann's organizer. *Nature*. 2000;403:781–785. https://doi.org/10.1038/35001602.
273. Murtaugh LC, Chyung JH, Lassar AB. Sonic hedgehog promotes somitic chondrogenesis by altering the cellular response to BMP signaling. *Genes Dev*. 1999;13:225–237. https://doi.org/10.1101/gad.13.2.225.
274. Roberts DJ, Johnson RL, Burke AC, et al. Sonic hedgehog is an endodermal signal inducing Bmp-4 and Hox genes during induction and regionalization of the chick hindgut. *Development*. 1995;121:3163–3174. https://doi.org/10.1242/dev.121.10.3163.
275. Astorga J, Carlsson P. Hedgehog induction of murine vasculogenesis is mediated by Foxf1 and Bmp4. *Development*. 2007;134:3753–3761. https://doi.org/10.1242/dev.004432.
276. Farrington SM, Belaoussoff M, Baron MH. Winged-helix, Hedgehog and Bmp genes are differentially expressed in distinct cell layers of the murine yolk sac. *Mech Dev*. 1997;62:197–211. https://doi.org/10.1016/s0925-4773(97)00664-3.
277. Zhang XM, Ramalho-Santos M, McMahon AP. Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R asymmetry by the mouse node. *Cell*. 2001;105:781–792. https://doi.org/10.1016/S0092-8674(01)00385-3.
278. Briscoe J, Therond PP. The mechanisms of Hedgehog signalling and its roles in development and disease. *Nat Rev Mol Cell Biol*. 2013;14:416–429. https://doi.org/10.1038/nrm3598.
279. Mar BG, Amakye D, Aifantis I, et al. The controversial role of the Hedgehog pathway in normal and malignant hematopoiesis. *Leukemia*. 2011;25:1665–1673. https://doi.org/10.1038/leu.2011.143.
280. Bhardwaj G, Murdoch B, Wu D, et al. Sonic Hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nat Immunol*. 2001;2:172–180. https://doi.org/10.1038/84282.
281. Trowbridge JJ, Scott MP, Bhatia M. Hedgehog modulates cell cycle regulators in stem cells to control hematopoietic regeneration. *Proc Natl Acad Sci USA*. 2006;103:14134–14139. https://doi.org/10.1073/pnas.0604568103.

282. Dierks C, Beigi R, Guo G, et al. Expansion of Bcr-Abl-positive leukemic stem cells is dependent on Hedgehog pathway activation. *Cancer Cell*. 2008;14:238–249. https://doi.org/10.1016/j.ccr.2008.08.003.
283. Zhao C, Chen A, Jamieson CH, et al. Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. *Nature*. 2009;458:776–779. https://doi.org/10.1038/nature07737.
284. Gao J, Graves S, Koch U, et al. Hedgehog signaling is dispensable for adult hematopoietic stem cell function. *Cell Stem Cell*. 2009;4:548–558. https://doi.org/10.1016/j.stem.2009.03.015.
285. Hofmann I, Stover EH, Cullen DE, et al. Hedgehog signaling is dispensable for adult murine hematopoietic stem cell function and hematopoiesis. *Cell Stem Cell*. 2009;4:559–567. https://doi.org/10.1016/j.stem.2009.03.016.
286. Merchant AA, Matsui W. Smoothening the controversial role of hedgehog in hematopoiesis. *Cell Stem Cell*. 2009;4:470–471. https://doi.org/10.1016/j.stem.2009.05.006.
287. Dar A, Goichberg P, Shinder V, et al. Chemokine receptor CXCR4-dependent internalization and resecretion of functional chemokine SDF-1 by bone marrow endothelial and stromal cells. *Nat Immunol*. 2005;6:1038–1046. https://doi.org/10.1038/ni1251.
288. Ding L, Morrison SJ. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature*. 2013;495:231–235. https://doi.org/10.1038/nature11885.
289. Sugiyama T, Kohara H, Noda M, et al. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity*. 2006;25:977–988. https://doi.org/10.1016/j.immuni.2006.10.016.
290. Tzeng YS, Li H, Kang Y, et al. Loss of Cxcl12/Sdf-1 in adult mice decreases the quiescent state of hematopoietic stem/progenitor cells and alters the pattern of hematopoietic regeneration after myelosuppression. *Blood*. 2011;117:429–439. https://doi.org/10.1182/blood-2010-01-266833.
291. Greenbaum A, Hsu YS, Day RB, et al. CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature*. 2013;495:227–230. https://doi.org/10.1038/nature11926.
292. Ding L, Saunders TL, Enikolopov G, et al. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature*. 2012;481:457–462. https://doi.org/10.1038/nature10783.
293. Spitz F, Furlong EE. Transcription factors: from enhancer binding to developmental control. *Nat Rev Genet*. 2012;13:613–626. https://doi.org/10.1038/nrg3207.
294. Wilkinson AC, Gottgens B. Transcriptional regulation of haematopoietic stem cells. *Adv Exp Med Biol*. 2013;786:187–212. https://doi.org/10.1007/978-94-007-6621-1_11.
295. Kataoka H, Hayashi M, Nakagawa R, et al. Etv2/ER71 induces vascular mesoderm from Flk1+PDGFRalpha+ primitive mesoderm. *Blood*. 2011;118:6975–6986. https://doi.org/10.1182/blood-2011-05-352658.
296. Lee D, Park C, Lee H, et al. ER71 acts downstream of BMP, Notch, and WNT signaling in blood and vessel progenitor specification. *Cell Stem Cell*. 2008;2:497–507. https://doi.org/10.1016/j.stem.2008.03.008.
297. De Val S, Chi NC, Meadows SM, et al. Combinatorial regulation of endothelial gene expression by ets and forkhead transcription factors. *Cell*. 2008;135:1053–1064. https://doi.org/10.1016/j.cell.2008.10.049.
298. Lancrin C, Sroczynska P, Stephenson C, et al. The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. *Nature*. 2009;457:892–895. https://doi.org/10.1038/nature07679.
299. Osada H, Grutz G, Axelson H, et al. Association of erythroid transcription factors: complexes involving the LIM protein RBTN2 and the zinc-finger protein GATA1. *Proc Natl Acad Sci USA*. 1995;92:9585–9589.https://doi.org/10.1073/pnas.92.21.9585.
300. Valge-Archer VE, Osada H, Warren AJ, et al. The LIM protein RBTN2 and the basic helix-loop-helix protein TAL1 are present in a complex in erythroid cells. *Proc Natl Acad Sci USA*. 1994;91:8617–8621. https://doi.org/10.1073/pnas.91.18.8617.
301. Wadman I, Li J, Bash RO, et al. Specific in vivo association between the bHLH and LIM proteins implicated in human T cell leukemia. *EMBO J*. 1994;13:4831–4839. https://doi.org/10.1002/j.1460-2075.1994.tb06809.x.
302. Robb L, Lyons I, Li R, et al. Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene. *Proc Natl Acad Sci USA*. 1995;92:7075–7079. https://doi.org/10.1073/pnas.92.15.7075.
303. Shivdasani RA, Mayer EL, Orkin SH. Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature*. 1995;373:432–434. https://doi.org/10.1038/373432a0.
304. Warren AJ, Colledge WH, Carlton MB, et al. The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development. *Cell*. 1994;78:45–57. https://doi.org/10.1016/0092-8674(94)90571-1.
305. Porcher C, Swat W, Rockwell K, et al. The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell*. 1996;86:47–57. https://doi.org/10.1016/s0092-8674(00)80076-8.
306. Yamada Y, Warren AJ, Dobson C, et al. The T cell leukemia LIM protein Lmo2 is necessary for adult mouse hematopoiesis. *Proc Natl Acad Sci USA*. 1998;95:3890–3895. https://doi.org/10.1073/pnas.95.7.3890.
307. Robb L, Elwood NJ, Elefanty AG, et al. The scl gene product is required for the generation of all hematopoietic lineages in the adult mouse. *EMBO J*. 1996;15:4123–4129.https://doi.org/10.1002/j.1460-2075.1996.tb00787.x.
308. Gottgens B, Nastos A, Kinston S, et al. Establishing the transcriptional programme for blood: the SCL stem cell enhancer is regulated by a multiprotein complex containing Ets and GATA factors. *EMBO J*. 2002;21:3039–3050. https://doi.org/10.1093/emboj/cdf286.
309. Hall MA, Curtis DJ, Metcalf D, et al. The critical regulator of embryonic hematopoiesis, SCL, is vital in the adult for megakaryopoiesis, erythropoiesis, and lineage choice in CFU-S12. *Proc Natl Acad Sci USA*. 2003;100:992–997. https://doi.org/10.1073/pnas.0237324100.
310. Mikkola HK, Klintman J, Yang H, et al. Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukaemia SCL/tal-1 gene. *Nature*. 2003;421:547–551. https://doi.org/10.1038/nature01345.
311. Souroullas GP, Salmon JM, Sablitzky F, et al. Adult hematopoietic stem and progenitor cells require either Lyl1 or Scl for survival. *Cell Stem Cell*. 2009;4:180–186. https://doi.org/10.1016/j.stem.2009.01.001.
312. Aifantis I, Raetz E, Buonamici S. Molecular pathogenesis of T-cell leukaemia and lymphoma. *Nat Rev Immunol*. 2008;8:380–390. https://doi.org/10.1038/nri2304.
313. Minegishi N, Ohta J, Yamagiwa H, et al. The mouse GATA-2 gene is expressed in the para-aortic splanchnopleura and aorta-gonads and mesonephros region. *Blood*. 1999;93:4196–4207. https://doi.org/10.1182/blood.V93.12.4196.
314. Tsai FY, Keller G, Kuo FC, et al. An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature*. 1994;371:221–226. https://doi.org/10.1038/371221a0.
315. Tsai FY, Orkin SH. Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. *Blood*. 1997;89:3636–3643. https://doi.org/10.1182/blood.V89.10.3636.
316. Minegishi N, Suzuki N, Yokomizo T, et al. Expression and domain-specific function of GATA-2 during differentiation of the hematopoietic precursor cells in midgestation mouse embryos. *Blood*. 2003;102:896–905. https://doi.org/10.1182/blood-2002-12-3809.
317. Hsu AP, Sampaio EP, Khan J, et al. Mutations in GATA2 are associated with the autosomal dominant and sporadic monocytopenia and mycobacterial infection (MonoMAC) syndrome. *Blood*. 2011;118:2653–2655. https://doi.org/10.1182/blood-2011-05-356352.
318. Vinh DC, Patel SY, Uzel G, et al. Autosomal dominant and sporadic monocytopenia with susceptibility to mycobacteria, fungi, papillomaviruses, and myelodysplasia. *Blood*. 2010;115:1519–1529. https://doi.org/10.1182/blood-2009-03-208629.
319. Bigley V, Haniffa M, Doulatov S, et al. The human syndrome of dendritic cell, monocyte, B and NK lymphoid deficiency. *J Exp Med*. 2011;208:227–234. https://doi.org/10.1084/jem.20101459.
320. Dickinson RE, Griffin H, Bigley V, et al. Exome sequencing identifies GATA-2 mutation as the cause of dendritic cell, monocyte, B and NK lymphoid deficiency. *Blood*. 2011;118:2656–2658. https://doi.org/10.1182/blood-2011-06-360313.
321. Spinner MA, Sanchez LA, Hsu AP, et al. GATA2 deficiency: a protean disorder of hematopoiesis, lymphatics, and immunity. *Blood*. 2014;123:809–821. https://doi.org/10.1182/blood-2013-07-515528.
322. Hahn CN, Chong C, Carmichael CL, et al. Heritable GATA2 mutations associated with familial myelodysplastic syndrome and acute myeloid leukemia. *Nat Genet*. 2011;43:1012–1017. https://doi.org/10.1038/ng.913.
323. Ostergaard P, Simpson MA, Connell FC, et al. Mutations in GATA2 cause primary lymphedema associated with a predisposition to acute myeloid leukemia (Emberger syndrome). *Nat Genet*. 2011;43:929–931. https://doi.org/10.1038/ng.923.
324. Chen MJ, Yokomizo T, Zeigler BM, et al. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature*. 2009;457:887–891. https://doi.org/10.1038/nature07619.
325. Okuda T, van Deursen J, Hiebert SW, et al. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell*. 1996;84:321–330. https://doi.org/10.1016/s0092-8674(00)80986-1.
326. Sasaki K, Yagi H, Bronson RT, et al. Absence of fetal liver hematopoiesis in mice deficient in transcriptional coactivator core binding factor beta.

Proc Natl Acad Sci USA. 1996;93:12359–12363.https://doi.org/10.1073/pnas.93.22.12359.

327. Wang Q, Stacy T, Miller JD, et al. The CBFbeta subunit is essential for CBFalpha2 (AML1) function in vivo. *Cell*. 1996;87:697–708. https://doi.org/10.1016/s0092-8674(00)81389-6.
328. Nottingham WT, Jarratt A, Burgess M, et al. Runx1-mediated hematopoietic stem-cell emergence is controlled by a Gata/Ets/SCL-regulated enhancer. *Blood*. 2007;110:4188–4197. https://doi.org/10.1182/blood-2007-07-100883.
329. Lancrin C, Mazan M, Stefanska M, et al. GFI1 and GFI1B control the loss of endothelial identity of hemogenic endothelium during hematopoietic commitment. *Blood*. 2012;120:314–322. https://doi.org/10.1182/blood-2011-10-386094.
330. Lichtinger M, Ingram R, Hannah R, et al. RUNX1 reshapes the epigenetic landscape at the onset of haematopoiesis. *EMBO J*. 2012;31:4318–4333. https://doi.org/10.1038/emboj.2012.275.
331. Iacovino M, Chong D, Szatmari I, et al. HoxA3 is an apical regulator of haemogenic endothelium. *Nat Cell Biol*. 2011;13:72–78. https://doi.org/10.1038/ncb2137.
332. Byrd JC, Mrózek K, Dodge RK, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). *Blood*. 2002;100:4325–4336. https://doi.org/10.1182/blood-2002-03-0772.
333. Yuan Y, Zhou L, Miyamoto T, et al. AML1-ETO expression is directly involved in the development of acute myeloid leukemia in the presence of additional mutations. *Proc Natl Acad Sci USA*. 2001;98:10398–10403. https://doi.org/10.1073/pnas.171321298.
334. Castilla LH, Garrett L, Adya N, et al. The fusion gene Cbfb-MYH11 blocks myeloid differentiation and predisposes mice to acute myelomonocytic leukaemia. *Nat Genet*. 1999;23:144–146. https://doi.org/10.1038/13776.
335. Corces-Zimmerman MR, Hong WJ, Weissman IL, et al. Preleukemic mutations in human acute myeloid leukemia affect epigenetic regulators and persist in remission. *Proc Natl Acad Sci USA*. 2014;111:2548–2553. https://doi.org/10.1073/pnas.1324297111.
336. Welch JS, Ley TJ, Link DC, et al. The origin and evolution of mutations in acute myeloid leukemia. *Cell*. 2012;150:264–278. https://doi.org/10.1016/j.cell.2012.06.023.
337. Mangan JK, Speck NA. RUNX1 mutations in clonal myeloid disorders: from conventional cytogenetics to next generation sequencing, a story 40 years in the making. *Crit Rev Oncog*. 2011;16:77–91. https://doi.org/10.1615/critrevoncog.v16.i1-2.80.
338. Lacombe J, Herblot S, Rojas-Sutterlin S, et al. Scl regulates the quiescence and the long-term competence of hematopoietic stem cells. *Blood*. 2010;115:792–803. https://doi.org/10.1182/blood-2009-01-201384.
339. Laurenti E, Frelin C, Xie S, et al. CDK6 levels regulate quiescence exit in human hematopoietic stem cells. *Cell Stem Cell*. 2015;16:302–313. https://doi.org/10.1016/j.stem.2015.01.017.
340. Wadman IA, Osada H, Grütz GG, et al. The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. *EMBO J*. 1997;16:3145–3157. https://doi.org/10.1093/emboj/16.11.3145.
341. Semerad CL, Mercer EM, Inlay MA, et al. E2A proteins maintain the hematopoietic stem cell pool and promote the maturation of myelolymphoid and myeloerythroid progenitors. *Proc Natl Acad Sci USA*. 2009;106:1930–1935. https://doi.org/10.1073/pnas.0808866106.
342. Li L, Jothi R, Cui K, et al. Nuclear adaptor Ldb1 regulates a transcriptional program essential for the maintenance of hematopoietic stem cells. *Nat Immunol*. 2011;12:129–136. https://doi.org/10.1038/ni.1978.
343. Lim KC, Hosoya T, Brandt W, et al. Conditional Gata2 inactivation results in HSC loss and lymphatic mispatterning. *J Clin Invest*. 2012;122:3705–3717. https://doi.org/10.1172/JCI61619.
344. Burmeister T, Gökbuget N, Schwartz S, et al. Clinical features and prognostic implications of TCF3-PBX1 and ETV6-RUNX1 in adult acute lymphoblastic leukemia. *Haematologica*. 2010;95:241–246. https://doi.org/10.3324/haematol.2009.011346.
345. Nourse J, Mellentin JD, Galili N, et al. Chromosomal translocation t(1;19) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. *Cell*. 1990;60:535–545.
346. Hock H, Meade E, Medeiros S, et al. Tel/Etv6 is an essential and selective regulator of adult hematopoietic stem cell survival. *Genes Dev*. 2004;18:2336–2341. https://doi.org/10.1101/gad.1239604.
347. Shurtleff SA, Buijs A, Behm FG, et al. TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with an excellent prognosis. *Leukemia*. 1995;9:1985–1989.
348. Greaves MF, Wiemels J. Origins of chromosome translocations in childhood leukaemia. *Nat Rev Cancer*. 2003;3:639–649. https://doi.org/10.1038/nrc1164.
349. Kataoka K, Sato T, Yoshimi A, et al. Evi1 is essential for hematopoietic stem cell self-renewal, and its expression marks hematopoietic cells with long-term multilineage repopulating activity. *J Exp Med*. 2011;208:2403–2416. https://doi.org/10.1084/jem.20110447.
350. Groschel S, Lugthart S, Schlenk RF, et al. High EVI1 expression predicts outcome in younger adult patients with acute myeloid leukemia and is associated with distinct cytogenetic abnormalities. *J Clin Oncol*. 2010;28:2101–2107. https://doi.org/10.1200/JCO.2009.26.0646.
351. Lugthart S, van Drunen E, van Norden Y, et al. High EVI1 levels predict adverse outcome in acute myeloid leukemia: prevalence of EVI1 overexpression and chromosome 3q26 abnormalities underestimated. *Blood*. 2008;111:4329–4337. https://doi.org/10.1182/blood-2007-10-119230.
352. Balgobind BV, Lugthart S, Hollink IH, et al. EVI1 overexpression in distinct subtypes of pediatric acute myeloid leukemia. *Leukemia*. 2010;24:942–949. https://doi.org/10.1038/leu.2010.47.
353. Helbling D, Mueller BU, Timchenko NA, et al. The leukemic fusion gene AML1-MDS1-EVI1 suppresses CEBPA in acute myeloid leukemia by activation of Calreticulin. *Proc Natl Acad Sci USA*. 2004;101:13312–13317. https://doi.org/10.1073/pnas.0404731101.
354. Raynaud SD, Baens M, Grosgeorge J, et al. Fluorescence in situ hybridization analysis of t(3; 12)(q26; p13): a recurring chromosomal abnormality involving the TEL gene (ETV6) in myelodysplastic syndromes. *Blood*. 1996;88:682–689.
355. Pearson JC, Lemons D, McGinnis W. Modulating Hox gene functions during animal body patterning. *Nat Rev Genet*. 2005;6:893–904. https://doi.org/10.1038/nrg1726.
356. Shen WF, Chang CP, Rozenfeld S, et al. Hox homeodomain proteins exhibit selective complex stabilities with Pbx and DNA. *Nucleic Acids Res*. 1996;24:898–906.
357. Shen WF, Montgomery JC, Rozenfeld S, et al. AbdB-like Hox proteins stabilize DNA binding by the Meis1 homeodomain proteins. *Mol Cell Biol*. 1997;17:6448–6458.
358. Alharbi RA, Pettengell R, Pandha HS, et al. The role of HOX genes in normal hematopoiesis and acute leukemia. *Leukemia*. 2013;27:1000–1008. https://doi.org/10.1038/leu.2012.356.
359. Thorsteinsdottir U, Sauvageau G, Hough MR, et al. Overexpression of HOXA10 in murine hematopoietic cells perturbs both myeloid and lymphoid differentiation and leads to acute myeloid leukemia. *Mol Cell Biol*. 1997;17:495–505.
360. Kroon E, Krosl J, Thorsteinsdottir U, et al. Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1a but not Pbx1b. *EMBO J*. 1998;17:3714–3725. https://doi.org/10.1093/emboj/17.13.3714.
361. Thorsteinsdottir U, Krosl J, Kroon E, et al. The oncoprotein E2A-Pbx1a collaborates with Hoxa9 to acutely transform primary bone marrow cells. *Mol Cell Biol*. 1999;19:6355–6366.
362. Nakamura T, Largaespada DA, Shaughnessy Jr JD, et al. Cooperative activation of Hoxa and Pbx1-related genes in murine myeloid leukaemias. *Nat Genet*. 1996;12:149–153. https://doi.org/10.1038/ng0296-149.
363. Golub TR, Slonim DK, Tamayo P, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science*. 1999;286:531–537.
364. Borrow J, Shearman AM, Stanton VP Jr, et al. The t(7;11)(p15;p15) translocation in acute myeloid leukaemia fuses the genes for nucleoporin NUP98 and class I homeoprotein HOXA9. *Nat Genet*. 1996;12:159–167. https://doi.org/10.1038/ng0296-159.
365. Nakamura T, Largaespada DA, Lee MP, et al. Fusion of the nucleoporin gene NUP98 to HOXA9 by the chromosome translocation t(7;11)(p15;p15) in human myeloid leukaemia. *Nat Genet*. 1996;12:154–158. https://doi.org/10.1038/ng0296-154.
366. Gough SM, Slape CI, Aplan PD. NUP98 gene fusions and hematopoietic malignancies: common themes and new biologic insights. *Blood*. 2011;118:6247–6257. https://doi.org/10.1182/blood-2011-07-328880.
367. Romana SP, Radford-Weiss I, Ben Abdelali R, et al. NUP98 rearrangements in hematopoietic malignancies: a study of the Groupe Francophone de Cytogenetique Hematologique. *Leukemia*. 2006;20:696–706. https://doi.org/10.1038/sj.leu.2404130.
368. Andreeff M, Ruvolo V, Gadgil S, et al. HOX expression patterns identify a common signature for favorable AML. *Leukemia*. 2008;22:2041–2047. https://doi.org/10.1038/leu.2008.198.
369. Yu BD, Hess JL, Horning SE, et al. Altered Hox expression and segmental identity in Mll-mutant mice. *Nature*. 1995;378:505–508. https://doi.org/10.1038/378505a0.

370. Milne TA, Briggs SD, Brock HW, et al. MLL targets SET domain methyltransferase activity to Hox gene promoters. *Mol Cell*. 2002;10:1107–1117.
371. Nakamura T, Mori T, Tada S, et al. ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. *Mol Cell*. 2002;10:1119–1128.
372. Armstrong SA, Staunton JE, Silverman LB, et al. MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. *Nat Genet*. 2002;30:41–47. https://doi.org/10.1038/ng765.
373. Krivtsov AV, Twomey D, Feng Z, et al. Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature*. 2006;442:818–822. https://doi.org/10.1038/nature04980.
374. Ayton PM, Cleary ML. Transformation of myeloid progenitors by MLL oncoproteins is dependent on Hoxa7 and Hoxa9. *Genes Dev*. 2003;17:2298–2307. https://doi.org/10.1101/gad.1111603.
375. Faber J, Krivtsov AV, Stubbs MC, et al. HOXA9 is required for survival in human MLL-rearranged acute leukemias. *Blood*. 2009;113:2375–2385. https://doi.org/10.1182/blood-2007-09-113597.
376. Beck D, Thoms JA, Perera D, et al. Genome-wide analysis of transcriptional regulators in human HSPCs reveals a densely interconnected network of coding and noncoding genes. *Blood*. 2013;122:e12–e22. https://doi.org/10.1182/blood-2013-03-490425.
377. Wilson NK, Foster SD, Wang X, et al. Combinatorial transcriptional control in blood stem/progenitor cells: genome-wide analysis of ten major transcriptional regulators. *Cell Stem Cell*. 2010;7:532–544. https://doi.org/10.1016/j.stem.2010.07.016.
378. Moignard V, Macaulay IC, Swiers G, et al. Characterization of transcriptional networks in blood stem and progenitor cells using high-throughput single-cell gene expression analysis. *Nat Cell Biol*. 2013;15:363–372. https://doi.org/10.1038/ncb2709.
379. Guo G, Luc S, Marco E, et al. Mapping cellular hierarchy by single-cell analysis of the cell surface repertoire. *Cell Stem Cell*. 2013;13:492–505. https://doi.org/10.1016/j.stem.2013.07.017.
380. Novershtern N, Subramanian A, Lawton LN, et al. Densely interconnected transcriptional circuits control cell states in human hematopoiesis. *Cell*. 2011;144:296–309. https://doi.org/10.1016/j.cell.2011.01.004.
381. Berger SL, Kouzarides T, Shiekhattar R, et al. An operational definition of epigenetics. *Genes Dev*. 2009;23:781–783. https://doi.org/10.1101/gad.1787609.
382. Goodell MA. Epigenetics in hematology: introducing a collection of reviews. *Blood*. 2013;121:3059–3060. https://doi.org/10.1182/blood-2012-12-475467.
383. The Cancer Genome Atlas Research Network. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med*. 2013;368:2059–2074. https://doi.org/10.1056/NEJMoa1301689.
384. Bock C, Beerman I, Lien WH, et al. DNA methylation dynamics during in vivo differentiation of blood and skin stem cells. *Mol Cell*. 2012;47:633–647. https://doi.org/10.1016/j.molcel.2012.06.019.
385. Hodges E, Molaro A, Dos Santos CO, et al. Directional DNA methylation changes and complex intermediate states accompany lineage specificity in the adult hematopoietic compartment. *Mol Cell*. 2011;44:17–28. https://doi.org/10.1016/j.molcel.2011.08.026.
386. Ji H, Ehrlich LI, Seita J, et al. Comprehensive methylome map of lineage commitment from haematopoietic progenitors. *Nature*. 2010;467:338–342. https://doi.org/10.1038/nature09367.
387. Cabezas-Wallscheid N, Klimmeck D, Hansson J, et al. Identification of regulatory networks in HSCs and their immediate progeny via integrated proteome, transcriptome, and DNA methylome analysis. *Cell Stem Cell*. 2014;15:507–522. https://doi.org/10.1016/j.stem.2014.07.005.
388. Jiang Y, Dunbar A, Gondek LP, et al. Aberrant DNA methylation is a dominant mechanism in MDS progression to AML. *Blood*. 2009;113:1315–1325. https://doi.org/10.1182/blood-2008-06-163246.
389. Figueroa ME, Lugthart S, Li Y, et al. DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. *Cancer Cell*. 2010;17:13–27. https://doi.org/10.1016/j.ccr.2009.11.020.
390. Bestor TH. Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain. *EMBO J*. 1992;11:2611–2617.
391. Gruenbaum Y, Cedar H, Razin A. Substrate and sequence specificity of a eukaryotic DNA methylase. *Nature*. 1982;295:620–622.
392. Challen GA, Sun D, Jeong M, et al. Dnmt3a is essential for hematopoietic stem cell differentiation. *Nat Genet*. 2011 Dec 4;44(1):23–31. https://doi.org/10.1038/ng.1009.
393. Challen GA, Sun D, Mayle A, et al. Dnmt3a and Dnmt3b have overlapping and distinct functions in hematopoietic stem cells. *Cell Stem Cell*. 2014;15:350–364. https://doi.org/10.1016/j.stem.2014.06.018.
394. Ley TJ, Ding L, Walter MJ, et al. DNMT3A mutations in acute myeloid leukemia. *N Engl J Med*. 2010;363:2424–2433. https://doi.org/10.1056/NEJMoa1005143.
395. Shlush LI, Zandi S, Mitchell A, et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature*. 2014;506:328–333. https://doi.org/10.1038/nature13038.
396. Grossmann V, Haferlach C, Weissmann S, et al. The molecular profile of adult T-cell acute lymphoblastic leukemia: mutations in RUNX1 and DNMT3A are associated with poor prognosis in T-ALL. *Genes Chromosomes Cancer*. 2013;52:410–422. https://doi.org/10.1002/gcc.22039.
397. Yang L, Rau R, Goodell MA. DNMT3A in haematological malignancies. *Nat Rev Cancer*. 2015;15:152–165. https://doi.org/10.1038/nrc3895.
398. Jeong M, Park HJ, Celik H, et al. Loss of dnmt3a immortalizes hematopoietic stem cells in vivo. *Cell Rep*. 2018;23:1–10.
399. Kriaucionis S, Heintz N. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science*. 2009;324:929–930. https://doi.org/10.1126/science.1169786.
400. Tahiliani M, Koh KP, Shen Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science*. 2009;324:930–935. https://doi.org/10.1126/science.1170116.
401. Kohli RM, Zhang Y. TET enzymes, TDG and the dynamics of DNA demethylation. *Nature*. 2013;502:472–479. https://doi.org/10.1038/nature12750.
402. Cimmino L, Dawlaty MM, Ndiaye-Lobry D, et al. TET1 is a tumor suppressor of hematopoietic malignancy. *Nat Immunol*. 2015;16:653–662. https://doi.org/10.1038/ni.3148.
403. Moran-Crusio K, Reavie L, Shih A, et al. Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. *Cancer Cell*. 2011;20:11–24. https://doi.org/10.1016/j.ccr.2011.06.001.
404. Shide K, Kameda T, Shimoda H, et al. TET2 is essential for survival and hematopoietic stem cell homeostasis. *Leukemia*. 2012;26:2216–2223. https://doi.org/10.1038/leu.2012.94.
405. Jankowska AM, Makishima H, Tiu RV, et al. Mutational spectrum analysis of chronic myelomonocytic leukemia includes genes associated with epigenetic regulation: UTX, EZH2, and DNMT3A. *Blood*. 2011;118:3932–3941. https://doi.org/10.1182/blood-2010-10-311019.
406. Chou WC, Chou SC, Liu CY, et al. TET2 mutation is an unfavorable prognostic factor in acute myeloid leukemia patients with intermediate-risk cytogenetics. *Blood*. 2011;118:3803–3810. https://doi.org/10.1182/blood-2011-02-339747.
407. Delhommeau F, Dupont S, Della Valle V, et al. Mutation in TET2 in myeloid cancers. *N Engl J Med*. 2009;360:2289–2301. https://doi.org/10.1056/NEJMoa0810069.
408. Metzeler KH, Maharry K, Radmacher MD, et al. TET2 mutations improve the new European LeukemiaNet risk classification of acute myeloid leukemia: a Cancer and Leukemia Group B study. *J Clin Oncol*. 2011;29:1373–1381. https://doi.org/10.1200/JCO.2010.32.7742.
409. Cimmino L, Dolgalev I, Wang Y, et al. Restoration of TET2 function blocks aberrant self-renewal and leukemia progression. *Cell*. 2017;170:1079–1095.
410. Agathocleous M, Meacham CE, Burgess RJ, et al. Ascorbate regulates haematopoietic stem cell function and leukaemogenesis. *Nature*. 2017;549:476–481.
411. Greenblatt SM, Nimer SD. Chromatin modifiers and the promise of epigenetic therapy in acute leukemia. *Leukemia*. 2014;28:1396–1406. https://doi.org/10.1038/leu.2014.94.
412. Abbas S, Lugthart S, Kavelaars FG, et al. Acquired mutations in the genes encoding IDH1 and IDH2 both are recurrent aberrations in acute myeloid leukemia: prevalence and prognostic value. *Blood*. 2010;116:2122–2126. https://doi.org/10.1182/blood-2009-11-250878.
413. Boissel N, Nibourel O, Renneville A, et al. Prognostic impact of isocitrate dehydrogenase enzyme isoforms 1 and 2 mutations in acute myeloid leukemia: a study by the Acute Leukemia French Association group. *J Clin Oncol*. 2010;28:3717–3723. https://doi.org/10.1200/JCO.2010.28.2285.
414. Marcucci G, Maharry K, Wu YZ, et al. IDH1 and IDH2 gene mutations identify novel molecular subsets within de novo cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. *J Clin Oncol*. 2010;28:2348–2355. https://doi.org/10.1200/JCO.2009.27.3730.
415. Paschka P, Schlenk RF, Gaidzik VI, et al. IDH1 and IDH2 mutations are frequent genetic alterations in acute myeloid leukemia and confer adverse prognosis in cytogenetically normal acute myeloid leukemia with NPM1 mutation without FLT3 internal tandem duplication. *J Clin Oncol*. 2010;28:3636–3643. https://doi.org/10.1200/JCO.2010.28.3762.
416. Figueroa ME, Abdel-Wahab O, Lu C, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell*. 2010;18:553–567. https://doi.org/10.1016/j.ccr.2010.11.015.
417. Xu W, Yang H, Liu Y, et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. *Cancer Cell*. 2011;19:17–30. https://doi.org/10.1016/j.ccr.2010.12.014.
418. Couronne L, Bastard C, Bernard OA. TET2 and DNMT3A mutations in human T-cell lymphoma. *N Engl J Med*. 2012;366:95–96.

419. Zhang X, Su J, Jeong M, et al. DNMT3A and TET2 compete and cooperate to repress lineage-specific transcription factors in hematopoietic stem cells. *Nat Genet*. 2016;48:1014–1023.
420. Rose NR, Klose RJ. Understanding the relationship between DNA methylation and histone lysine methylation. *Biochim Biophys Acta*. 2014;1839:1362–1372. https://doi.org/10.1016/j.bbagrm.2014.02.007.
421. Kouzarides T. Chromatin modifications and their function. *Cell*. 2007;128:693–705. https://doi.org/10.1016/j.cell.2007.02.005.
422. Cullen SM, Mayle A, Rossi L, et al. Hematopoietic stem cell development: an epigenetic journey. *Curr Top Dev Biol*. 2014;107:39–75. https://doi.org/10.1016/B978-0-12-416022-4.00002-0.
423. Jiang Y, Hatzi K, Shaknovich R. Mechanisms of epigenetic deregulation in lymphoid neoplasms. *Blood*. 2013;121:4271–4279. https://doi.org/10.1182/blood-2012-12-451799.
424. Mullighan CG, Zhang J, Kasper LH, et al. CREBBP mutations in relapsed acute lymphoblastic leukaemia. *Nature*. 2011;471:235–239. https://doi.org/10.1038/nature09727.
425. Ernst P, Fisher JK, Avery W, et al. Definitive hematopoiesis requires the mixed-lineage leukemia gene. *Dev Cell*. 2004;6:437–443.
426. De Braekeleer M, Morel F, Le Bris MJ, et al. The MLL gene and translocations involving chromosomal band 11q23 in acute leukemia. *Anticancer Res*. 2005;25:1931–1944.
427. Garrido SM, Bryant E, Appelbaum FR. Allogeneic stem cell transplantation for relapsed and refractory acute myeloid leukemia patients with 11q23 abnormalities. *Leuk Res*. 2000;24:481–486.
428. Meyer C, Hofmann J, Burmeister T. et al. The MLL recombinome of acute leukemias in 2013. *Leukemia*. 2013;27:2165–2176. https://doi.org/10.1038/leu.2013.135.
429. Mohan M, Lin C, Guest E, et al. Licensed to elongate: a molecular mechanism for MLL-based leukaemogenesis. *Nat Rev Cancer*. 2010;10:721–728. https://doi.org/10.1038/nrc2915.
430. Akalin A, Garrett-Bakelman FE, Kormaksson M, et al. Base-pair resolution DNA methylation sequencing reveals profoundly divergent epigenetic landscapes in acute myeloid leukemia. *PLoS Genet*. 2012;8:e1002781. https://doi.org/10.1371/journal.pgen.1002781.
431. Trowbridge JJ, Sinha AU, Zhu N, et al. Haploinsufficiency of Dnmt1 impairs leukemia stem cell function through derepression of bivalent chromatin domains. *Genes Dev*. 2012;26:344–349. https://doi.org/10.1101/gad.184341.111.
432. Radulovic V, de Haan G, Klauke K. Polycomb-group proteins in hematopoietic stem cell regulation and hematopoietic neoplasms. *Leukemia*. 2013;27:523–533. https://doi.org/10.1038/leu.2012.368.
433. Stock JK, Giadrossi S, Casanova M, et al. Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. *Nat Cell Biol*. 2007;9:1428–1435. https://doi.org/10.1038/ncb1663.
434. Vire E, Brenner C, Deplus R, et al. The Polycomb group protein EZH2 directly controls DNA methylation. *Nature*. 2006;439:871–874. https://doi.org/10.1038/nature04431.
435. Kamminga LM, Bystrykh LV, de Boer A, et al. The Polycomb group gene Ezh2 prevents hematopoietic stem cell exhaustion. *Blood*. 2006;107:2170–2179. https://doi.org/10.1182/blood-2005-09-3585.
436. Ezhkova E, Pasolli HA, Parker JS, et al. Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. *Cell*. 2009;136:1122–1135. https://doi.org/10.1016/j.cell.2008.12.043.
437. Bracken AP, Pasini D, Capra M, et al. EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. *EMBO J*. 2003;22:5323–5335. https://doi.org/10.1093/emboj/cdg542.
438. Abdel-Wahab O, Pardanani A, Patel J, et al. Concomitant analysis of EZH2 and ASXL1 mutations in myelofibrosis, chronic myelomonocytic leukemia and blast-phase myeloproliferative neoplasms. *Leukemia*. 2011;25:1200–1202. https://doi.org/10.1038/leu.2011.58.
439. Clapier CR, Iwasa J, Cairns BR, et al. Mechanisms of action and regulation of ATP-dependent chromatin-remodelling complexes. *Nat Rev Mol Cell Biol*. 2017;18:407–422.
440. Krasteva V, Buscarlet M, Diaz-Tellez A, et al. The BAF53a subunit of SWI/SNF-like BAF complexes is essential for hemopoietic stem cell function. *Blood*. 2012;120:4720–4732.
441. Krasteva V, Crabtree GR, Lesser JA. The BAF45a/PHF10 subunit of SWI/SNF-like chromatin remodeling complexes is essential for hematopoietic stem cell maintenance. *Exp Hematol*. 2017;48:58–71.
442. Buscarlet M, Krasteva V, Ho L, et al. Essential role of BRG, the ATPase subunit of BAF chromatin remodeling complexes, in leukemia maintenance. *Blood*. 2014;123:1720–1728.
443. Shi J, Whyte WA, Zepeda-Mendoza CJ, et al. Role of SWI/SNF in acute leukemia maintenance and enhancer-mediated Myc regulation. *Genes Dev*. 2013;27:2648–2662.
444. Madan V, Shyamsunder P, Han L, et al. Comprehensive mutational analysis of primary and relapse acute promyelocytic leukemia. *Leukemia*. 2016;30:1672–1681.
445. Han L, Madan V, Mayakonda A, et al. Chromatin remodeling mediated by ARID1A is indispensable for normal hematopoiesis in mice. *Leukemia*. 2019;33:2291–2305.
446. Xu B, Cai L, Butler JM, et al. The chromatin remodeler BPTF activates a stemness gene-expression program essential for the maintenance of adult hematopoietic stem cells. *Stem Cell Reports*. 2018;10:675–683.
447. Spielmann M, Lupianez DG, Mundlos S. Structural variation in the 3D genome. *Nat Rev Genet*. 2018;19:453–467.
448. Cancer Genome Atlas Research N Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med*. 2013;368: 2089–2074.
449. Kon A, Shih LY, Minamino M, et al. Recurrent mutations in multiple components of the cohesion complex in myeloid neoplasms. *Nat Genet*. 2013;45:1232–1237.
450. Thota S, Viny AD, Makishima H, et al. Genetic alterations of the cohesin complex genes in myeloid malignancies. *Blood*. 2014;124:1790–1798.
451. Viny AD, Bowman RL, Liu Y, et al. Cohesin Members Stag1 and Stag2 display distinct roles in chromatin accessibility and topological control of HSC self-renewal and differentiation. *Cell Stem Cell*. 2019;25:682–696.
452. Zhang X, Jeong M, Huang X, et al. Large DNA methylation nadirs anchor chromatin loops maintaining hematopoietic stem cell identity. *Mol Cell*. 2020;78:506–521.
453. Chen C, Yu W, Tober J, et al. Spatial genome re-organization between fetal and adult hematopoietic stem cells. *Cell Rep*. 2019;29:4200–4211.
454. Buenrostro JD, Giresi PG, Zaba LC, et al. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat Methods*. 2013;10:1213–1218.
455. Corces MR, Buenrostro J, Wu B, et al. Lineage-specific and single-cell chromatin accessibility charts human hematopoiesis and leukemia evolution. *Nat Genet*. 2016;48:1193–1203.
456. International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. *Nature*. 2001;409:860–921. https://doi.org/10.1038/35057062.
457. Pennisi E. Genomics. ENCODE project writes eulogy for junk DNA. *Science*. 2012;337(1159):1161. https://doi.org/10.1126/science.337.6099.1159.
458. Klimmeck D, Cabezas-Wallscheid N, Reyes A, et al. Transcriptome-wide profiling and posttranscriptional analysis of hematopoietic stem/progenitor cell differentiation toward myeloid commitment. *Stem Cell Reports*. 2014;3:858–875. https://doi.org/10.1016/j.stemcr.2014.08.012.
459. Guo H, Ingolia NT, Weissman JS, et al. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature*. 2010;466:835–840. https://doi.org/10.1038/nature09267.
460. Lim LP, Lau NC, Garrett-Engele P, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*. 2005;433:769–773. https://doi.org/10.1038/nature03315.
461. Gruber JJ, Zatechka DS, Sabin LR, et al. Ars2 links the nuclear cap-binding complex to RNA interference and cell proliferation. *Cell*. 2009;138:328–339. https://doi.org/10.1016/j.cell.2009.04.046.
462. Guo S, Lu J, Schlanger R, et al. MicroRNA miR-125a controls hematopoietic stem cell number. *Proc Natl Acad Sci USA*. 2010;107:14229–14234. https://doi.org/10.1073/pnas.0913574107.
463. O'Connell RM, Baltimore D. MicroRNAs and hematopoietic cell development. *Curr Top Dev Biol*. 2012;99:145–174. https://doi.org/10.1016/B978-0-12-387038-4.00006-9.
464. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature*. 2005;435:834–838. https://doi.org/10.1038/nature03702.
465. Georgantas RW 3rd, HildrethR, Morisot S, et al. CD34+ hematopoietic stem-progenitor cell microRNA expression and function: a circuit diagram of differentiation control. *Proc Natl Acad Sci USA*. 2007;104:2750–2755. https://doi.org/10.1073/pnas.0610983104.
466. O'Connell RM, Chaudhuri AA, Rao DS, et al. MicroRNAs enriched in hematopoietic stem cells differentially regulate long-term hematopoietic output. *Proc Natl Acad Sci USA*. 2010;107:14235–14240. https://doi.org/10.1073/pnas.1009798107.
467. Petriv OI, Kuchenbauer F, Delaney AD, et al. Comprehensive microRNA expression profiling of the hematopoietic hierarchy. *Proc Natl Acad Sci USA*. 2010;107:15443–15448. https://doi.org/10.1073/pnas.1009320107.
468. Ooi AG, Sahoo D, Adorno M, et al. MicroRNA-125b expands hematopoietic stem cells and enriches for the lymphoid-balanced and lymphoid-biased subsets. *Proc Natl Acad Sci USA*. 2010;107:21505–21510. https://doi.org/10.1073/pnas.1016218107.

469. Han YC, Park CY, Bhagat G, et al. MicroRNA-29a induces aberrant self-renewal capacity in hematopoietic progenitors, biased myeloid development, and acute myeloid leukemia. *J Exp Med*. 2010;207:475–489. https://doi.org/10.1084/jem.20090831.
470. Gentner B, Visigalli I, Hiramatsu H, et al. Identification of hematopoietic stem cell-specific miRNAs enables gene therapy of globoid cell leukodystrophy. *Sci Transl Med*. 2010;2:58ra84. https://doi.org/10.1126/scitranslmed.3001522.
471. Gerrits A, Walasek MA, Olthof S, et al. Genetic screen identifies microRNA cluster 99b/let-7e/125a as a regulator of primitive hematopoietic cells. *Blood*. 2012;119:377–387. https://doi.org/10.1182/blood-2011-01-331686.
472. O'Connell RM, Rao DS, Chaudhuri AA, et al. Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J Exp Med*. 2008;205:585–594. https://doi.org/10.1084/jem.20072108.
473. Fazi F, Rosa A, Fatica A, et al. A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell*. 2005;123:819–831. https://doi.org/10.1016/j.cell.2005.09.023.
474. Johnnidis JB, Harris MH, Wheeler RT, et al. Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. *Nature*. 2008;451:1125–1129. https://doi.org/10.1038/nature06607.
475. Chen CZ, Li L, Lodish HF, et al. MicroRNAs modulate hematopoietic lineage differentiation. *Science*. 2004;303:83–86. https://doi.org/10.1126/science.1091903.
476. Xiao C, Calado DP, Galler G, et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell*. 2007;131:146–159. https://doi.org/10.1016/j.cell.2007.07.021.
477. Xiao C, Srinivasan L, Calado DP, et al. Lymphoproliferative disease and autoimmunity in mice with increased miR-17–92 expression in lymphocytes. *Nat Immunol*. 2008;9:405–414. https://doi.org/10.1038/ni1575.
478. Zhou B, Wang S, Mayr C, et al. miR-150, a microRNA expressed in mature B and T cells, blocks early B cell development when expressed prematurely. *Proc Natl Acad Sci USA*. 2007;104:7080–7085. https://doi.org/10.1073/pnas.0702409104.
479. Arnold CP, Tan R, Zhou B, et al. MicroRNA programs in normal and aberrant stem and progenitor cells. *Genome Res*. 2011;21:798–810. https://doi.org/10.1101/gr.111385.110.
480. Lazare SS, Wojtowicz EE, Bystrykh LV, et al. microRNAs in hematopoiesis. *Exp Cell Res*. 2014;329:234–238. https://doi.org/10.1016/j.yexcr.2014.08.033.
481. Chaudhuri AA, So AY, Mehta A, et al. Oncomir miR-125b regulates hematopoiesis by targeting the gene Lin28A. *Proc Natl Acad Sci USA*. 2012;109:4233–4238. https://doi.org/10.1073/pnas.1200677109.
482. Bousquet M, Harris MH, Zhou B, et al. MicroRNA miR-125b causes leukemia. *Proc Natl Acad Sci USA*. 2010;107:21558–21563. https://doi.org/10.1073/pnas.1016611107.
483. Bousquet M, Quelen C, Rosati R, et al. Myeloid cell differentiation arrest by miR-125b-1 in myelodysplastic syndrome and acute myeloid leukemia with the t(2;11)(p21;q23) translocation. *J Exp Med*. 2008;205:2499–2506. https://doi.org/10.1084/jem.20080285.
484. Chapiro E, Russell LJ, Struski S, et al. A new recurrent translocation t(11;14)(q24;q32) involving IGH@ and miR-125b-1 in B-cell progenitor acute lymphoblastic. *Leukemia*. 2010;24:1362–1364. https://doi.org/10.1038/leu.2010.93.
485. Sonoki T, Iwanaga E, Mitsuya H, et al. Insertion of microRNA-125b-1, a human homologue of lin-4, into a rearranged immunoglobulin heavy chain gene locus in a patient with precursor B-cell acute lymphoblastic leukemia. *Leukemia*. 2005;19:2009–2010. https://doi.org/10.1038/sj.leu.2403938.
486. Song SJ, Ito K, Ala U, et al. The oncogenic microRNA miR-22 targets the TET2 tumor suppressor to promote hematopoietic stem cell self-renewal and transformation. *Cell Stem Cell*. 2013;13:87–101. https://doi.org/10.1016/j.stem.2013.06.003.
487. Costinean S, Zanesi N, Pekarsky Y, et al. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proc Natl Acad Sci USA*. 2006;103:7024–7029. https://doi.org/10.1073/pnas.0602266103.
488. Kluiver J, Poppema S, de Jong D, et al. BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas. *J Pathol*. 2005;207:243–249. https://doi.org/10.1002/path.1825.
489. Garzon R, Volinia S, Liu CG, et al. MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. *Blood*. 2008;111:3183–3189. https://doi.org/10.1182/blood-2007-07-098749.
490. Whitman SP, Maharry K, Radmacher MD, et al. FLT3 internal tandem duplication associates with adverse outcome and gene- and microRNA-expression signatures in patients 60 years of age or older with primary cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. *Blood*. 2010;116:3622–3626. https://doi.org/10.1182/blood-2010-05-283648.
491. Lawrence HJ, Christensen J, Fong S, et al. Loss of expression of the Hoxa-9 homeobox gene impairs the proliferation and repopulating ability of hematopoietic stem cells. *Blood*. 2005;106:3988–3994. https://doi.org/10.1182/blood-2005-05-2003.
492. Thorsteinsdottir U, Mamo A, Kroon E, et al. Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. *Blood*. 2002;99:121–129.
493. Li Z, Huang H, Chen P, et al. miR-196b directly targets both HOXA9/MEIS1 oncogenes and FAS tumour suppressor in MLL-rearranged leukaemia. *Nat Commun*. 2012;3:688. https://doi.org/10.1038/ncomms1681.
494. Yekta S, Shih IH, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. *Science*. 2004;304:594–596. https://doi.org/10.1126/science.1097434.
495. Popovic R, Riesbeck LE, Velu CS, et al. Regulation of mir-196b by MLL and its overexpression by MLL fusions contributes to immortalization. *Blood*. 2009;113:3314–3322. https://doi.org/10.1182/blood-2008-04-154310.
496. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA*. 2002;99:15524–15529. https://doi.org/10.1073/pnas.242606799.
497. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci USA*. 2005;102:13944–13949. https://doi.org/10.1073/pnas.0506654102.
498. Yan X, Hu Z, Feng Y, et al. Comprehensive genomic characterization of long non-coding RNAs across human cancers. *Cancer Cell*. 2015;28:529–540. https://doi.org/10.1016/j.ccell.2015.09.006.
499. Luo M, Jeong M, Sun D, et al. Long non-coding RNAs control hematopoietic stem cell function. *Cell Stem Cell*. 2015;16:426–438. https://doi.org/10.1016/j.stem.2015.02.002.
500. Yildirim E, Kirby JE, Brown DE, et al. Xist RNA is a potent suppressor of hematologic cancer in mice. *Cell*. 2013;152:727–742. https://doi.org/10.1016/j.cell.2013.01.034.
501. Nakamura-Ishizu A, Ito K, Suda T. Hematopoietic stem cell metabolism during development and aging. *Dev Cell*. 2020;54:239–255.
502. Simsek T, Kocabas F, Zheng J, et al. The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. *Cell Stem Cell*. 2010;7:380–390.
503. Guo B, Huang X, Lee MR, et al. Antagonism of PPAR-gamma signaling expands human hematopoietic stem and progenitor cells by enhancing glycolysis. *Nat Med*. 2018;24:360–367.
504. Wang YH, Israelsen WJ, Lee D, et al. Cell-state-specific metabolic dependency in hematopoiesis and leukemogenesis. *Cell*. 2014;158:1309–1323. https://doi.org/10.1016/j.cell.2014.07.048.
505. Uchida N, Dykstra B, Lyons K, et al. ABC transporter activities of murine hematopoietic stem cells vary according to their developmental and activation status. *Blood*. 2004;103:4487–4495.
506. Bonora M, Ito K, Morganti C, et al. Membrane-potential compensation reveals mitochondrial volume expansion during HSC commitment. *Experimental Hematology*. 2018;68:30–37.
507. de Almeida MJ, Luchsinger LL, Corrigan DJ, et al. Dye-independent methods reveal elevated mitochondrial mass in hematopoietic stem cells. *Cell Stem Cell*. 2017;21:725–729.
508. Takihara Y, Nakamura-Ishizu A, Tan DQ, et al. High mitochondrial mass is associated with reconstitution capacity and quiescence of hematopoietic stem cells. *Blood Adv*. 2019;3:2323–2327.
509. Ito K, Carracedo A, Weiss D, et al. A PML-PPAR-delta pathway for fatty acid oxidation regulates hematopoietic stem cell maintenance. *Nat Med*. 2012;18:1350–1358. https://doi.org/10.1038/nm.2882.
510. Vanini N, Girotra M, Naveiras O, et al. Specification of haematopoietic stem cell fate via modulation of mitochondrial activity. *Nat Commun*. 2016;7:13125.
511. Ho TT, Warr MR, Adelman ER, et al. Autophagy maintains the metabolism and function of young and old stem cells. *Nature*. 2017;543:205–210.
512. Nakamura-Ishizu A, Matsumura T, Stumpf PS, et al. Thrombopoietin metabolically primes hematopoietic stem cells to megakaryocyte-lineage differentiation. *Cell Rep*. 2018;25:1772–1785.
513. Fernandes H, Moura J, Carvalho E. mTOR signaling as a regulator of hematopoietic stem cell fate. *Stem Cell Rev Rep*. 2021

514. Cunningham JT, Rodgers JT, Arlow DH, et al. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. *Nature*. 2007;450:736–740.
515. Lee JY, Nakada D, Yilmaz OH, et al. mTOR activation induces tumor suppressors that inhibit leukemogenesis and deplete hematopoietic stem cells after Pten deletion. *Cell Stem Cell*. 2010;7:593–605.
516. Yilmaz OH, Valdez R, Theisen BK, et al. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature*. 2006;441:475–482.
517. Kalaitzidis D, Sykes SM, Wang Z, et al. mTOR complex 1 plays critical roles in hematopoiesis and Pten-loss-evoked leukemogenesis. *Cell Stem Cell*. 2012;11:429–439.
518. Gurumurthy S, Xie SZ, Alagesan B, et al. The Lkb1 metabolic sensor maintains haematopoietic stem cell survival. *Nature*. 2010;468:659–663.
519. Gan B, Hu J, Jiang S, Liu Y, et al. Lkb1 regulates quiescence and metabolic homeostasis of haematopoietic stem cells. *Nature*. 2010;468:701–704.
520. Nakada D, Saunders TL, Morrison SJ. Lkb1 regulates cell cycle and energy metabolism in haematopoietic stem cells. *Nature*. 2010;468:653–658.
521. Mantel CR, O'Leary HA, Chitteti BR, et al. Enhancing hematopoietic stem cell transplantation efficacy by mitigating oxygen shock. *Cell*. 2015;161:1553–1565.
522. Spencer JA, Ferraro F, Roussakis E, et al. Direct measurement of local oxygen concentration in the bone marrow of live animals. *Nature*. 2014;508:269–273. https://doi.org/10.1038/nature13034.
523. Simsek T, Kocabas F, Zheng J, et al. The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. *Cell Stem Cell*. 2010;7:380–390.
524. Kierans SJ, Taylor CT. Regulation of glycolysis by the hypoxia-inducible factor (HIF): implications for cellular physiology. *J Physiol*. 2021;599: 23–37.
525. Christodoulou C, Spencer JA, Yeh SA, et al. Live-animal imaging of native haematopoietic stem and progenitor cells. *Nature*. 2020;578:278–283.
526. Snoeck HW. Mitochondrial regulation of hematopoietic stem cells. *Curr Opin Cell Biol*. 2017;49:91–98.
527. Sommerkamp P, Altamura S, Renders S, et al. Differential alternative polyadenylation landscapes mediate hematopoietic stem cell activation and regulate glutamine metabolism. *Cell Stem Cell*. 2020;26:722–738.
528. Raffel S, Falcone M, Kneisel N, et al. BCAT1 restricts alphaKG levels in AML stem cells leading to IDHmut-like DNA hypermethylation. *Nature*. 2017;551:384–388.
529. Taya Y, Ota Y, Wilkinson AC, et al. Depleting dietary valine permits nonmyeloablative mouse hematopoietic stem cell transplantation. *Science*. 2016;354:1152–1155.
530. Liu X, Zheng H, Yu W-M, et al. Maintenance of mouse hematopoietic stem cells ex vivo by reprogramming cellular metabolism. *Blood*. 2015;125:1562–1565. https://doi.org/10.1182/blood-2014-04-568949.
531. Mohrin M, Shin J, Liu Y, et al. Stem cell aging. A mitochondrial UPR-mediated metabolic checkpoint regulates hematopoietic stem cell aging. *Science*. 2015;347:1374–1377. https://doi.org/10.1126/science.aaa2361.
532. DeVilbiss W, Zhao Z, Martin-Sandoval MS, et al. Metabolomic profiling of rare cell populations isolated by flow cytometry from tissues. *Elife*. 2021;10
533. Bowie MB, Kent DG, Copley MR, et al. Steel factor responsiveness regulates the high self-renewal phenotype of fetal hematopoietic stem cells. *Blood*. 2007;109:5043–5048. https://doi.org/10.1182/blood-2006-08-037770.
534. Petit-Cocault L, Volle-Challier C, Fleury M, et al. Dual role of Mpl receptor during the establishment of definitive hematopoiesis. *Development*. 2007;134:3031–3040. https://doi.org/10.1242/dev.001818.
535. Zhang CC, Kaba M, Ge G, et al. Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. *Nat Med*. 2006;12:240–245. https://doi.org/10.1038/nm1342.
536. Zhang CC, Kaba M, Iizuka S, et al. Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation. *Blood*. 2008;111:3415–3423. https://doi.org/10.1182/blood-2007-11-122119.
537. Zheng J, Umikawa M, Cui C, et al. Inhibitory receptors bind ANGPTLs and support blood stem cells and leukaemia development. *Nature*. 2012;485:656–660. https://doi.org/10.1038/nature11095.
538. Lin MI, Price EN, Boatman S, et al. Angiopoietin-like proteins stimulate HSPC development through interaction with notch receptor signaling. *Elife*. 2015;4 https://doi.org/10.7554/eLife.05544.
539. North TE, Goessling W, Walkley CR, et al. Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. *Nature*. 2007;447:1007–1011. https://doi.org/10.1038/nature05883.
540. Goessling W, North TE, Loewer S, et al. Genetic interaction of PGE2 and WNT signaling regulates developmental specification of stem cells and regeneration. *Cell*. 2009;136:1136–1147. https://doi.org/10.1016/j.cell.2009.01.015.
541. Hoggatt J, Singh P, Sampath J, et al. Prostaglandin E2 enhances hematopoietic stem cell homing, survival, and proliferation. *Blood*. 2009;113:5444–5455. https://doi.org/10.1182/blood-2009-01-201335.
542. Goessling W, Robyn RS, Guan X, et al. Prostaglandin E2 enhances human cord blood stem cell xenotransplants and shows long-term safety in preclinical nonhuman primate transplant models. *Cell Stem Cell*. 2011;8:445–458. https://doi.org/10.1016/j.stem.2011.02.003.
543. Cutler C, Multani P, Robbins D, et al. Prostaglandin-modulated umbilical cord blood hematopoietic stem cell transplantation. *Blood*. 2013;122:3074–3081. https://doi.org/10.1182/blood-2013-05-503177.
544. Chute JP, Muramoto G, Fung J, et al. Quantitative analysis demonstrates expansion of SCID-repopulating cells and increased engraftment capacity in human cord blood following ex vivo culture with human brain endothelial cells. *Stem Cells*. 2004;22:202–215. https://doi.org/10.1634/stemcells.22-2-202.
545. Chute JP, Muramoto GG, Dressman HK, et al. Molecular profile and partial functional analysis of novel endothelial cell-derived growth factors that regulate hematopoiesis. *Stem Cells*. 2006;24:1315–1327. https://doi.org/10.1634/stemcells.2005-0029.
546. Chute JP, Muramoto GG, Fung J, et al. Soluble factors elaborated by human brain endothelial cells induce the concomitant expansion of purified human BM CD34+CD38- cells and SCID-repopulating cells. *Blood*. 2005;105:576–583. https://doi.org/10.1182/blood-2004-04-1467.
547. Chute JP, Saini AA, Chute DL, et al. Ex vivo culture with human brain endothelial cells increases the SCID-repopulating capacity of adult human bone marrow. *Blood*. 2002;100:4433–4439. https://doi.org/10.1182/blood-2002-04-1238.
548. Himburg HA, Harris JR, Ito T, et al. Pleiotrophin regulates the retention and self-renewal of hematopoietic stem cells in the bone marrow vascular niche. *Cell Rep*. 2012;2:964–975. https://doi.org/10.1016/j.celrep.2012.09.002.
549. Istvanffy R, Kröger M, Eckl C, et al. Stromal pleiotrophin regulates repopulation behavior of hematopoietic stem cells. *Blood*. 2011;118:2712–2722. https://doi.org/10.1182/blood-2010-05-287235.
550. Himburg H, Termini C, Schlussel L, et al. Distinct bone marrow sources of pleiotrophin control hematopoietic stem cell maintenance and regeneration. *Cell Stem Cell*. 2018;23:370–381. https://doi.org/10.1016/j.stem.2018.07.003.
551. Boitano AE, Wang J, Romeo R, et al. Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. *Science*. 2010;329:1345–1348. https://doi.org/10.1126/science.1191536.
552. Dahlberg A, Brashem-Stein C, Delaney C, et al. Enhanced generation of cord blood hematopoietic stem and progenitor cells by culture with StemRegenin1 and Delta1(Ext-IgG. *Leukemia*. 2014;28:2097–2101. https://doi.org/10.1038/leu.2014.181.
553. Wagner JE, Brunstein CG, McKenna D, et al. Safety and exploratory efficacy of ex vivo expanded umbilical cord blood (ucb) hematopoietic stem and progenitor cells (HSPC) using cytokines and stem-regenin 1 (SR1): interim results of a phase 1/2 dose escalation clinical study. *Blood*. 2013;122
554. Oostendorp RA, Harvey KN, Kusadasi N, et al. Stromal cell lines from mouse aorta-gonads-mesonephros subregions are potent supporters of hematopoietic stem cell activity. *Blood*. 2002;99:1183–1189.
555. Wohrer S, Knapp DJHF, Copley MR, et al. Distinct stromal cell factor combinations can separately control hematopoietic stem cell survival, proliferation, and self-renewal. *Cell Rep*. 2014;7:1956–1967. https://doi.org/10.1016/j.celrep.2014.05.014.
556. Fares I, Chagraoul J, Gareau Y, et al. Cord blood expansion. Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. *Science*. 2014;345:1509–1512. https://doi.org/10.1126/science.1256337.
557. Jaroscak J, Goltry K, Smith A, et al. Augmentation of umbilical cord blood (UCB) transplantation with ex vivo-expanded UCB cells: results of a phase 1 trial using the AastromReplicell System. *Blood*. 2003;101:5061–5067. https://doi.org/10.1182/blood-2001-12-0290.
558. Shpall EJ, Quinones R, Giller R, et al. Transplantation of ex vivo expanded cord blood. *Biol Blood Marrow Transplant*. 2002;8:368–376.
559. Peled T, Landau E, Prus E, et al. Cellular copper content modulates differentiation and self-renewal in cultures of cord blood-derived CD34+ cells. *Br J Haematol*. 2002;116:655–661.
560. Peled T, Landau E, Mandel J, et al. Linear polyamine copper chelator tetraethylenepentamine augments long-term ex vivo expansion of cord blood-derived CD34+ cells and increases their engraftment potential in NOD/SCID mice. *Exp Hematol*. 2004;32:547–555. https://doi.org/10.1016/j.exphem.2004.03.002.
561. Peled T, Glukhman E, Hasson N, et al. Chelatable cellular copper modulates differentiation and self-renewal of cord blood-derived

hematopoietic progenitor cells. *Exp Hematol*. 2005;33:1092–1100. https://doi.org/10.1016/j.exphem.2005.06.015.

562. de Lima M, McMannis J, Gee A, et al. Transplantation of ex vivo expanded cord blood cells using the copper chelator tetraethylenepentamine: a phase I/II clinical trial. *Bone Marrow Transpl*. 2008;41:771–778. https://doi.org/10.1038/sj.bmt.1705979.
563. de Lima M, McNiece I, Robinson SN, et al. Cord-blood engraftment with ex vivo mesenchymal-cell coculture. *N Engl J Med*. 2012;367:2305–2315. https://doi.org/10.1056/NEJMoa1207285.
564. Horwitz ME, Chao NJ, Rizzieri DA, et al. Umbilical cord blood expansion with nicotinamide provides long-term multilineage engraftment. *J Clin Invest*. 2014;124:3121–3128. https://doi.org/10.1172/JCI74556.
565. Sun X, Cao B, Naval-Sanchez M, et al. Nicotinamide riboside attenuates age-associated metabolic and functional changes in hematopoietic stem cells. *Nat Commun*. 2021;12:2665.
566. Wilkinson A, Ishida R, Kikuchi M, et al. Long-term ex vivo hematopoietic stem cell expansion allows nonconditioned transplantation. *Nature*. 2019;571:117–121.
567. Shao L, Elujoba-Bridenstine A, Zink K, et al. The neurotransmitter receptor Gabbr1 regulates proliferation and function of hematopoietic stem and progenitor cells. *Blood*. 2021;137:775–787.
568. Hua P, Hester J, Adigbli G, et al. The BET inhibitor CPI203 promotes ex vivo expansion of cord blood long-term repopulating HSCs and megakaryocytes. *Blood*. 2020;136:2410–2415.
569. Subramaniam A, Zemaitis K, Talkhoncheh M, et al. Lysine-specific demethylase 1A restricts ex vivo propagation of human HSCs and is a target of UM171. *Blood*. 2020;136:2151–2161.
570. Zimran E, Papa L, Djedaini M, et al. Expansion and preservation of the functional activity of adult hematopoietic stem cells cultured ex vivo with a histone deacetylase inhibitor. *Stem Cells Transl Med*. 2020;9:531–542.
571. Buo B, Huang X, Lee M, et al. Antagonism of PPAR-gamma signaling expands human hematopoietic stem and progenitor cells by enhancing glycolysis. *Nat Med*. 2018;24:360–367.
572. Capitano M, Mor-Vaknin N, Saha A, et al. Secreted nuclear protein DEK regulates hematopoiesis through CXCR2 signaling. *J Clin Invest*. 2019;129:2555–2570.
573. Gupta R, Turati V, Brian D, et al. Nov/CCN3 enhances cord blood engraftment by rapidly recruiting latent human stem cell activity. *Cell Stem Cell*. 2020;26:527–541.
574. Calvanese V, Nguyen A, Bolan T, et al. MLLT3 governs human haematopoietic stem cell self-renewal and engraftment. *Nature*. 2019;576:281–286.
575. Bai T, Li J, Sinclair A, et al. Expansion of primitive human hematopoietic stem cells by culture in a zwitterionic hydrogel. *Nat Med*. 2019;25:1566–1575.
576. Kohn D, Booth C, Shaw K, et al. Autologous ex vivo lentiviral gene therapy for adenosine deaminase deficiency. *N Engl J Med*. 2021;384:2002–2013.
577. Gratwohl A, Baldomero H, Aljurf M, et al. Hematopoietic stem cell transplantation: a global perspective. *JAMA*. 2010;303:1617–1624. https://doi.org/10.1001/jama.2010.491.
578. Copelan EA. Hematopoietic stem-cell transplantation. *N Engl J Med*. 2006;354:1813–1826. https://doi.org/10.1056/NEJMra052638.
579. Walasek MA, van Os R, de Haan G. Hematopoietic stem cell expansion: challenges and opportunities. *Ann N Y Acad Sci*. 2012;1266:138–150. https://doi.org/10.1111/j.1749-6632.2012.06549.x.
580. Gao Y, Yang P, Shen H, et al. Small-molecule inhibitors targeting INK4 protein p18(INK4C) enhance ex vivo expansion of haematopoietic stem cells. *Nat Commun*. 2015;6:6328. https://doi.org/10.1038/ncomms7328.
581. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature*. 1981;292:154–156.
582. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA*. 1981;78:7634–7638.
583. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. *Science*. 1998;282:1145–1147.
584. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007;131:861–872. https://doi.org/10.1016/j.cell.2007.11.019.
585. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;126:663–676. https://doi.org/10.1016/j.cell.2006.07.024.
586. Kimbrel EA, Lanza R. Current status of pluripotent stem cells: moving the first therapies to the clinic. *Nat Rev Drug Discov*. 2015;14(10):681–692. https://doi.org/10.1038/nrd4738.
587. Hotta A, Yamanaka S. From genomics to gene therapy: induced pluripotent stem cells meet genome editing. *Annu Rev Genet*. 2015;49:47–70. https://doi.org/10.1146/annurev-genet-112414-054926.
588. Hussein SM, Nagy AA. Progress made in the reprogramming field: new factors, new strategies and a new outlook. *Curr Opin Genet Dev*. 2012;22:435–443. https://doi.org/10.1016/j.gde.2012.08.007.
589. Doulatov S, Vo LT, Chou S, et al. Induction of multipotential hematopoietic progenitors from human pluripotent stem cells via respecification of lineage-restricted precursors. *Cell Stem Cell*. 2013;13:459–470. https://doi.org/10.1016/j.stem.2013.09.002.
590. Keller G. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev*. 2005;19:1129–1155. https://doi.org/10.1101/gad.1303605.
591. Davis RL, Weintraub H, Lassar AB. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell*. 1987;51:987–1000.
592. Pulecio J, Nivet E, Sancho-Martinez I, et al. Conversion of human fibroblasts into monocyte-like progenitor cells. *Stem Cells*. 2014;32:2923–2938. https://doi.org/10.1002/stem.1800.
593. Szabo E, Rampalli S, Risueno RM, et al. Direct conversion of human fibroblasts to multilineage blood progenitors. *Nature*. 2010;468:521–526. https://doi.org/10.1038/nature09591.
594. Pereira CF, Chang B, Qiu Jiajing, et al. Induction of a hemogenic program in mouse fibroblasts. *Cell Stem Cell*. 2013;13:205–218. https://doi.org/10.1016/j.stem.2013.05.024.
595. Sandler VM, Lis R, Liu Y, et al. Reprogramming human endothelial cells to haematopoietic cells requires vascular induction. *Nature*. 2014;511:312–318. https://doi.org/10.1038/nature13547.
596. Riddell J, Gazit R, Garrison BS, et al. Reprogramming committed murine blood cells to induced hematopoietic stem cells with defined factors. *Cell*. 2014;157:549–564. https://doi.org/10.1016/j.cell.2014.04.006.
597. Lucas D, Frenette PS. Stem cells: reprogramming finds its niche. *Nature*. 2014;511:301–302. https://doi.org/10.1038/nature13516.
598. Trompouki E, Bowman TV, Lawton LN, et al. Lineage regulators direct BMP and Wnt pathways to cell-specific programs during differentiation and regeneration. *Cell*. 2011;147:577–589. https://doi.org/10.1016/j.cell.2011.09.044.
599. Congdon KL, Voermans C, Ferguson EC, et al. Activation of WNT signaling in hematopoietic regeneration. *Stem Cells*. 2008;26:1202–1210. https://doi.org/10.1634/stemcells.2007-0768.
600. Heissig B, Lund LR, Akiyama H, et al. The plasminogen fibrinolytic pathway is required for hematopoietic regeneration. *Cell Stem Cell*. 2007;1:658–670. https://doi.org/10.1016/j.stem.2007.10.012.
601. Helgason CD, Antonchuk J, Bodner C, et al. Homeostasis and regeneration of the hematopoietic stem cell pool are altered in SHIP-deficient mice. *Blood*. 2003;102:3541–3547. https://doi.org/10.1182/blood-2002-12-3939.
602. Nemeth MJ, Kirby MR, Bodine DM. Hmgb3 regulates the balance between hematopoietic stem cell self-renewal and differentiation. *Proc Natl Acad Sci USA*. 2006;103:13783–13788. https://doi.org/10.1073/pnas.0604006103.
603. Chung YJ, Park B-B, Kang Y-J, et al. Unique effects of Stat3 on the early phase of hematopoietic stem cell regeneration. *Blood*. 2006;108:1208–1215. https://doi.org/10.1182/blood-2006-01-010199.
604. Avecilla ST, Hattori K, Heissig B, et al. Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nat Med*. 2004;10:64–71. https://doi.org/10.1038/nm973.
605. Hooper AT, Butler JM, Nolan DJ, et al. Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. *Cell Stem Cell*. 2009;4:263–274. https://doi.org/10.1016/j.stem.2009.01.006.
606. Kopp HG, Avecilla ST, Hooper AT, et al. Tie2 activation contributes to hemangiogenic regeneration after myelosuppression. *Blood*. 2005;106:505–513. https://doi.org/10.1182/blood-2004-11-4269.
607. Salter AB, Meadows SK, Muramoto GG, et al. Endothelial progenitor cell infusion induces hematopoietic stem cell reconstitution in vivo. *Blood*. 2009;113:2104–2107. https://doi.org/10.1182/blood-2008-06-162941.
608. Chute JP, Muramoto GG, Salter AB, et al. Transplantation of vascular endothelial cells mediates the hematopoietic recovery and survival of lethally irradiated mice. *Blood*. 2007;109:2365–2372. https://doi.org/10.1182/blood-2006-05-022640.
609. Doan PL, Himburg HA, Heims K, et al. Epidermal growth factor regulates hematopoietic regeneration after radiation injury. *Nat Med*. 2013;19:295–304. https://doi.org/10.1038/nm.3070.
610. Poulos MG, Guo P, Kofler NM, et al. Endothelial Jagged-1 is necessary for homeostatic and regenerative hematopoiesis. *Cell Rep*. 2013;4:1022–1034. https://doi.org/10.1016/j.celrep.2013.07.048.
611. Geiger H, Pawar SA, Kerschen EJ, et al. Pharmacological targeting of the thrombomodulin-activated protein C pathway mitigates radiation toxicity. *Nat Med*. 2012;18:1123–1129. https://doi.org/10.1038/nm.2813.

612. Guinan EC, Barbon CM, Kalish LA, et al. Bactericidal/permeability-increasing protein (rBPI21) and fluoroquinolone mitigate radiation-induced bone marrow aplasia and death. *Sci Transl Med*. 2011;3:110ra118. https://doi.org/10.1126/scitranslmed.3003126.
613. Kim JH, Thimmulappa RK, Kumar V, et al. NRF2-mediated Notch pathway activation enhances hematopoietic reconstitution following myelosuppressive radiation. *J Clin Invest*. 2014;124:730–741. https://doi.org/10.1172/JCI70812.
614. Himburg HA, Yan X, Doan PL, et al. Pleiotrophin mediates hematopoietic regeneration via activation of RAS. *J Clin Invest*. 2014;124:4753–4758. https://doi.org/10.1172/JCI76838.
615. Zhou BO, Ding L, Morrison SJ. Hematopoietic stem and progenitor cells regulate the regeneration of their niche by secreting Angiopoietin-1. *Elife*. 2015;4:e05521. https://doi.org/10.7554/eLife.05521.
616. Vannini N, Campos V, Girotra M, et al. The NAD-booster nicotinamide riboside potently stimulates hematopoiesis through increased mitochondrial clearance. *Cell Stem Cell*. 2019;24:405–418.
617. Silberstein L, Goncalves K, Kharchenko P, et al. Proximity-based differential single cell analysis of the niche to identify stem/progenitor cell regulators. *Cell Stem Cell*. 2016;19:530–543.
618. Goncalves K, Silberstein L, Li S, et al. Angiogenin promotes hematopoietic regeneration by dichotomously regulating quiescence of stem and progenitor cells. *Cell*. 2016;166:894–906.
619. Himburg HA, Doan P, Quarmyne M, et al. Dickkopf-1 promotes hematopoietic regeneration via direct and niche-mediated mechanisms. *Nat Med*. 2017;23:91–99.
620. Zhou B, Yu H, Yue R, et al. Bone marrow adipocytes promote the regeneration of stem cells and haematopoiesis by secreting SCF. *Nat Cell Biol*. 2017;19:891–902.
621. Lucas D, Scheiermann C, Chow A, et al. Chemotherapy-induced bone marrow nerve injury impairs hematopoietic regeneration. *Nat Med*. 2013;19:695–703.
622. Xu C, Gao X, Wei Q, et al. Stem cell factor is selectively secreted by arterial endothelial cells in bone marrow. *Nat Commun*. 2018;9:2449.
623. Chen Q, Liu Y, Jeong H, et al. Apelin+ endothelial niche cells control hematopoiesis and mediate vascular regeneration after myeloablative injury. *Cell Stem Cell*. 2019;25:768–783.
624. Guo P, Poulos M, Palikuqi B, et al. Endothelial jagged-2 sustains hematopoietic stem and progenitor reconstitution after myelosuppression. *J Clin Invest*. 2017;127:4242–4256.
625. Bowers E, Slaughter A, Frenette P, et al. Granulocyte-derived TNF alpha promotes vascular and hematopoietic regeneration in the bone marrow. *Nat Med*. 2018;24:95–102.
626. Golan K, Singh A, Kollet O, et al. Bone marrow regeneration requires mitochondrial transfer from donor Cx43-expressing hematopoietic progenitors to stroma. *Blood*. 2020;136:2607–2619.
627. Severe N, Karabacak N, Gustafsson K, et al. Stress-induced changes in bone marrow stromal cell populations revealed through single cell protein expression mapping. *Cell Stem Cell*. 2019;25:570–583.
628. Velardi E, Tsai J, Radtke S, et al. Suppression of luteinizing hormone enhances HSC recovery after hematopoietic injury. *Nat Med*. 2018;24:239–246.
629. Fang T, Zhang Y, Chang V, et al. Epidermal growth factor receptor-dependent DNA repair promotes murine and human hematopoietic regeneration. *Blood*. 2020;136:441–454.
630. Yamashita M, Passegue E. TNA alpha coordinates hematopoietic stem cell survival and myeloid regeneration. *Cell Stem Cell*. 2019;25:357–372.
631. Sudo T, Motomura Y, Okuzaki D, et al. Group 2 innate lymphoid cells support hematopoietic recovery under stress conditions. *J Exp Med*. 2021;3:218.
632. Yi W, Zhang Y, Liu B, et al. Protein S-nitrosylation regulates proteostasis and viability of hematopoietic stem cells during regeneration. *Cell Rep*. 2021;34:108922.
633. Lv K, Gong C, Antony C, et al. HectD1 controls hematopoietic stem cell regeneration by coordinating ribosome assembly and protein synthesis. *Cell Stem Cell*. 2021;28:1–16.
634. Chavez J, Rabe J, Loeffler D, et al. PU.1 enforces quiescence and limits hematopoietic stem cell expansion during inflammatory stress. *J Exp Med*. 2021;218:e20201169.
635. Kaufmann K, Zeng A, Coyaud E, et al. A latent subset of human hematopoietic stem cells resists regenerative stress to preserve stemness. *Nat Immunol*. 2021;22:723–734.
636. Rossi DJ, Bryder D, Zahn JM, et al. Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proc Natl Acad Sci USA*. 2005;102:9194–9199. https://doi.org/10.1073/pnas.0503280102.
637. Woolthuis CM, Mariani N, Varkaik-Schakel RN, et al. Aging impairs long-term hematopoietic regeneration after autologous stem cell transplantation. *Biol Blood Marrow Transplant*. 2014;20:865–871. https://doi.org/10.1016/j.bbmt.2014.03.001.
638. Rossi DJ, Bryder D, Seita J, et al. Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature*. 2007;447:725–729. https://doi.org/10.1038/nature05862.
639. Flach J, Bakker ST, Mohrin M, et al. Replication stress is a potent driver of functional decline in ageing haematopoietic stem cells. *Nature*. 2014;512:198–202. https://doi.org/10.1038/nature13619.
640. Cheng CW, Adams GB, Perin L, et al. Prolonged fasting reduces IGF-1/PKA to promote hematopoietic-stem-cell-based regeneration and reverse immunosuppression. *Cell Stem Cell*. 2014;14:810–823. https://doi.org/10.1016/j.stem.2014.04.014.
641. Tao S, Wang Y, Wu J, et al. Long-term mid-onset dietary restriction rejuvenates hematopoietic stem cells and improves regeneration capacity of total bone marrow from aged mice. *Aging Cell*. 2020;19:e13241.
642. O'Connor ML, Xiang D, Shigdar S, et al. Cancer stem cells: a contentious hypothesis now moving forward. *Cancer Lett*. 2014;344:180–187. https://doi.org/10.1016/j.canlet.2013.11.012.
643. Nguyen LV, Vanner R, Dirks P, et al. Cancer stem cells: an evolving concept. *Nat Rev Cancer*. 2012;12:133–143. https://doi.org/10.1038/nrc3184.
644. Lapidot T, Sirard C, Vormoor J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature*. 1994;367:645–648. https://doi.org/10.1038/367645a0.
645. Tough IM, Jacobs PA, Court Brown WM, et al. Cytogenetic studies on bone-marrow in chronic myeloid leukaemia. *Lancet*. 1963;1:844–846.
646. Whang J, Frei 3rd E, Tjio JH, et al. The distribution of the Philadelphia chromosome in patients with chronic myelogenous leukemia. *Blood*. 1963;22:664–673.
647. Martin PJ, Najfeld V, Hansen JA, et al. Involvement of the B-lymphoid system in chronic myelogenous leukaemia. *Nature*. 1980;287:49–50.
648. Fialkow PJ, Gartler SM, Yoshida A. Clonal origin of chronic myelocytic leukemia in man. *Proc Natl Acad Sci USA*. 1967;58:1468–1471.
649. Levan A, Norden A, Nichols WW. A case of chronic myeloid leukemia with 2 leukemic stemlines in blood. *Hereditas*. 1963;49:433.
650. Jamieson CH, Ailles LE, Dylla SJ, et al. Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N Engl J Med*. 2004;351:657–667. https://doi.org/10.1056/NEJMoa040258.
651. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med*. 1997;3:730–737.
652. Huntly BJ, Gilliland DG. Leukaemia stem cells and the evolution of cancer-stem-cell research. *Nat Rev Cancer*. 2005;5:311–321. https://doi.org/10.1038/nrc1592.
653. Ding L, Ley TJ, Larson DE, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature*. 2012;481:506–510. https://doi.org/10.1038/nature10738.
654. Ley TJ, Mardis ER, Ding L, et al. DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. *Nature*. 2008;456:66–72. https://doi.org/10.1038/nature07485.
655. Mardis ER, Ding L, Dooling DJ, et al. Recurring mutations found by sequencing an acute myeloid leukemia genome. *N Engl J Med*. 2009;361:1058–1066. https://doi.org/10.1056/NEJMoa0903840.
656. Yan XJ, Xu J, Gu Z-H, et al. Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nat Genet*. 2011;43:309–315. https://doi.org/10.1038/ng.788.
657. Kronke J, Bullinger L, Teleanu V, et al. Clonal evolution in relapsed NPM1-mutated acute myeloid leukemia. *Blood*. 2013;122:100–108. https://doi.org/10.1182/blood-2013-01-479188.
658. Genovese G, Kähler A, Handsaker RE, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med*. 2014;371:2477–2487. https://doi.org/10.1056/NEJMoa1409405.
659. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med*. 2014;371:2488–2498. https://doi.org/10.1056/NEJMoa1408617.
660. Yamashita M, Dellorusso P, Olson O, Passegue E. Dysregulated haematopoietic stem cell behaviour in myeloid leukemogenesis. *Nat Rev Cancer*. 2020;20:365–382.
661. Luo H, Zhu G, Xu J, et al. HOTTIP lncRNA promotes hematopoietic stem cell self-renewal leading to AML-like disease in mice. *Cancer Cell*. 2019;36:645–659.
662. Lopez C, Noguera E, Stavropoulou V, et al. Ontogenic changes in hematopoietic hierarchy determine pediatric specificity and disease phenotype in fusion oncogene-driven myeloid leukemia. *Cancer Discov*. 2019;9:1736–1753.
663. Boileau M, Shirinian M, Gayden T, et al. Mutant H3 histones drive human pre-leukemic hematopoietic stem cell expansion and promote leukemic aggressiveness. *Nat Commun*. 2019;10:2891.

664. Vu T, Straube J, Porter A, et al. Hematopoietic stem and progenitor cell-restricted Cdx2 expression induces transformation to myelodysplasia and acute leukemia. *Nat Commun*. 2020;11:3021.
665. Cai S, Chu S, Goldberg A, et al. Leukemia cell of origin influences apoptotic priming and sensitivity to LSD1 inhibition. *Cancer Discov*. 2020;10:1500–1513.
666. Chen X, Burkhardt D, Hartman A, et al. MLL-AF9 initiates transformation from fast-proliferating myeloid progenitors. *Nat Commun*. 2019;10:5767.
667. Basilico S, Wang X, Kennedy A, et al. Dissecting the early steps of MLL induced leukaemogenic transformation using a mouse model of AML. *Nat Commun*. 2020;11:1407.
668. Di Genua C, Valletta S, Buono M, et al. C/EBP alpha and GATA-2 mutations induce bilineage acute erythroid leukemia through transformation of neomorphic neutrophil-erythroid progenitor. *Cancer Cell*. 2020;37:690–704.

CHAPTER 10

MITOCHONDRIA AND HEMATOPOIESIS

Luena Papa

> *Without mitochondria, nothing would exist of the world we know and love*
>
> Nick Lane.

MITOCHONDRIAL STRUCTURE AND FUNCTION

Mitochondria are very complex and highly dynamic organelles. Although responsible for only 10% of the cellular proteome, mitochondria serve not only as powerhouse of the cells but also as critical regulators of essential cellular processes including iron-sulfur cluster biosynthesis, calcium homeostasis, and cell death; hence they contribute to health and disease.

Mitochondria are surrounded by a double-membrane system, consisting of an inner mitochondrial membrane (IMM) and outer mitochondrial membrane (OMM) separated by an intermembrane space (IMS). The inner membrane forms numerous folds (*cristae*), which extend into the interior or the matrix of the organelle (Fig. 10.1). Each of these subcompartments plays distinct functional roles.

Mitochondria use almost 90% of O_2 consumed by the cell to generate adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS). During this process, pyruvate that is generated by conversion of glucose in the cytosol is transported into the mitochondria, where it enters the tricarboxylic acid (TCA) cycle and becomes oxidized to yield acetyl coenzyme-A (CoA). Acetyl CoA is also generated by oxidation of fatty acids via the TCA cycle. The series of reactions implicated in the TCA cycle, which occur in the mitochondrial matrix, result in generation of critical metabolites that are further transported into the cytoplasm, where they provide the building blocks for biosynthesis of macromolecules such as nucleotides, lipids, and proteins.[1]

In addition to metabolites, the completion of the TCA cycle is also coupled to reduction of NAD^+ and FAD to NADH (nicotinamide adenine dinucleotide, reduced) and $FADH_2$ (flavin adenine dinucleotide, reduced), respectively. Both of these molecules are then transferred along the "respiratory chain," also known as the "electron transport chain" (ETC). Specially, NADH and $FADH_2$ feed the ETC complex I and II, respectively, which then further transfer electrons to complex III and IV of the ETC. Such electron transfer through the four multisubunit protein complexes of ETC embedded in the IMM generates a proton gradient across the inner membrane that is used by complex V to synthesize ATP through OXPHOS (see Fig. 10.1).[1]

Mitochondrial proteins and hence their functions are originated and controlled by two distinct genetic systems: nuclear deoxyribonucleic acid (nDNA) and mitochondrial (mt)DNA. The majority of the mitochondrial proteins (~1158) are nuclear-encoded proteins. They are imported as unfolded polypeptide chains into the mitochondria through complex mechanisms that mainly rely on the translocase TIM/TOM (translocase of the inner membrane/translocase of the outer membrane) system. These newly synthesized polypeptides contain specific signaling sequences, which direct and target them into distinct mitochondrial subcompartments (mitochondrial matrix, IMM, or IMS) where they undergo proper folding. Unlike the nDNA, mtDNA is simple and lacks both introns and histones. According to the endosymbiotic theory, the simplicity of mtDNA is a result of engulfment of a proteobacterium by another archeabacteria. The resulting double-membrane organelle was capable of producing ATP and providing the host with energy. Over time, however, most of the genetic material was either transferred to the host's genome or lost. These events resulted in the double-stranded, circular mtDNA molecule, which consists of 16,569 base pairs. The main DNA coding sequence is the 1.1 kb loop and consists of two promoters for mtDNA transcription and an origin for replication. mtDNA encodes for only 37 genes of which 13 are structural subunits required for OXPHOS, 2 mitochondrial ribosomal (r)RNAs, and 22 mitochondrial transfer (t)RNAS, essential for synthesis of these subunits.

MITOCHONDRIAL DISEASES

The pathophysiology of mitochondrial diseases is complex and is characterized by defects in OXPHOS. These defects can be triggered by mutations in either nDNA or mtDNA and hence in genes responsible for both the structure and function of mitochondria. As such, complex genetics that underlie mitochondrial diseases can display several patterns of inheritance, including autosomal and X-linked inheritance due to nDNA mutations and maternal inheritance for mtDNA mutations. Because multiple copies of mtDNA are present in each cell, mutations can affect all (known as homoplasmy) or a portion of mtDNA content (termed heteroplasmy). The levels of heteroplasmy vary between cells of the same tissue or organ and from organ to organ. Interestingly, 1 in 200 healthy individuals carry the 10 most common pathogenic mtDNA mutations, although at low levels of heteroplasmy. However, rare, de novo mutations have also been observed. Originally thought to be extremely rare, mitochondrial diseases have now been reported to affect 1 in 4300 people. Mitochondrial diseases are characterized by a high morbidity and mortality because they often involve multiple systems, particularly those that heavily rely on mitochondrial metabolism (Fig. 10.2). However, in a relatively small number of cases, single organ involvement such as isolated ocular involvement in Leber hereditary optic neuropathy (LHON) has been also observed. The heterogeneity of the clinical manifestations makes both the diagnosis and treatment extremely challenging.

The onset of mitochondrial diseases has been reported to have a bimodal distribution reaching a peak in the first 3 years of life followed by a second peak that begins by the end of teenage years and extends to the fourth decade of life. Childhood-onset mitochondrial diseases are usually severe, although not always fatal. These diseases are often caused by recessive nDNA mutations or mtDNA mutations, which are present with very high levels of mtDNA heteroplasmy. Several syndromes that are due to mitochondrial abnormalities, including Leigh syndrome characterized by symmetric spongiform degeneration of the corpus striatum and brainstem with demyelination and vascular proliferation; Alpers-Huttenlocher characterized by epilepsy, psychomotor regression, and liver disease; Pearson syndrome and congenital lactic acidosis; and progressive pure myopathy or a spinal muscular atrophy-like phenotype, arise during childhood. In addition, some forms of renal cystic disorders, proximal tubulopathy, and hypertrophic cardiomyopathy that occur in children have also been attributed to mitochondrial defects.

In this context, Pearson syndrome that was first described in 1979 by Howard Pearson at Yale University (see Chapter 30) is of special interest to hematologists. It is characterized by a mutation in mitochondrial DNA that leads to transfusion-dependent sideroblastic

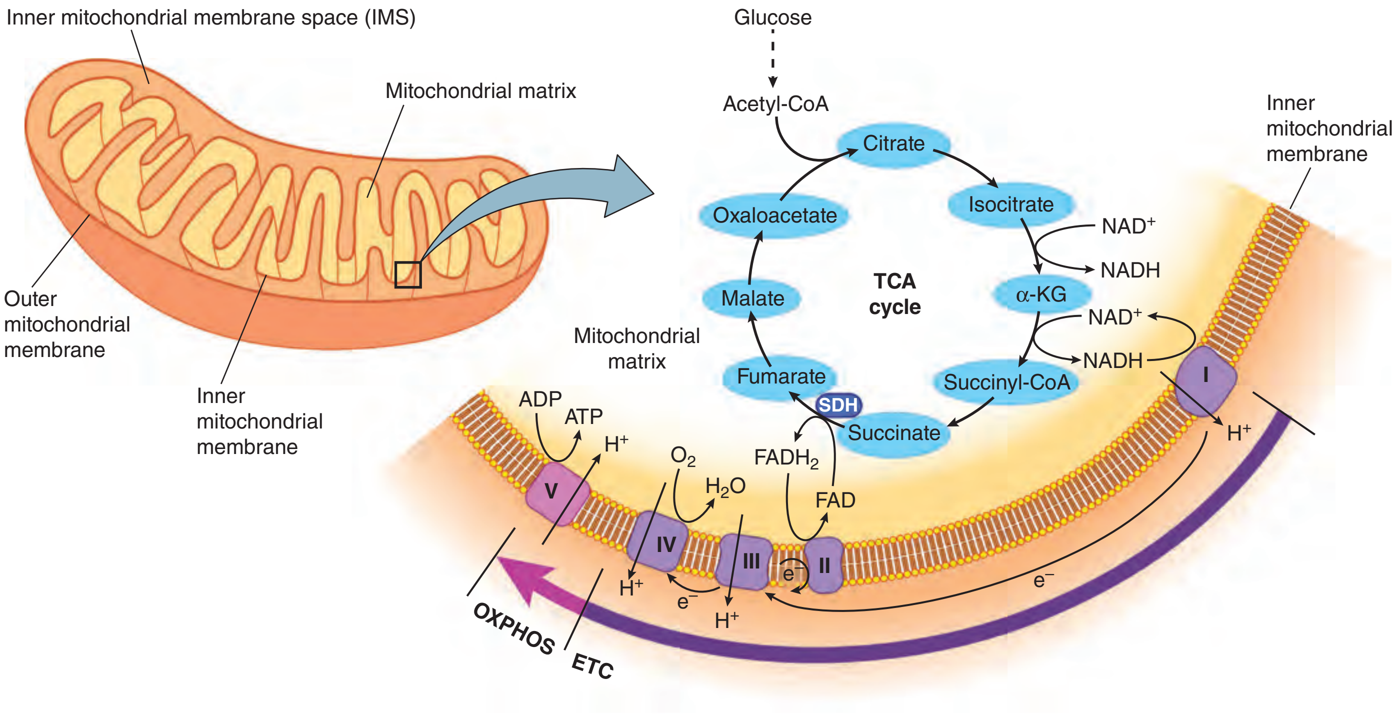


Figure 10.1 MITOCHONDRIAL SUBCOMPARTMENTS, ETC, AND TCA CYCLE. A mitochondrion is composed of an outer (OMM) and inner membrane (IMM), which folds into the interior of matrix to form cristae. The four multisubunit protein complexes (complexes I–IV) of the electron transport chain (ETC) as well as complex V that synthesizes adenosine triphosphate (ATP) are embedded in the IMM *(black square)*. Pyruvate that is generated by conversion of glucose in the cytosol is transported into the mitochondria where it enters the citric acid (TCA) cycle and becomes oxidized to yield acetyl coenzyme-A (CoA). The series of enzymatic reactions of the TCA cycle, which occurs in the mitochondrial matrix, results in the generation of indicated metabolites. The completion of the TCA cycle is also coupled to reduction of NAD^+ and FAD to NADH (nicotinamide adenine dinucleotide, reduced) and $FADH_2$ (flavin adenine dinucleotide, reduced), respectively. Both are then transferred along the ETC. Specially, NADH and $FADH_2$ feed the ETC complex I and II, respectively, which then further transfer electrons to complex III and IV of the ETC. Such electron transfer results in generation of a proton gradient across the IMM known as mitochondrial membrane potential that is then used by complex V to synthesize ATP. This process requires the presence of oxygen and it is known as oxidative phosphorylation.

anemia, vacuolization of marrow precursor cells, and exocrine pancreas dysfunction. Affected individuals experience failure to thrive, pancreatic fibrosis with insulin-dependent diabetes and exocrine pancreatic deficiency, muscle and neurologic impairment, and frequently, early death during infancy. The few patients who survive into adulthood often develop symptoms of Kearns-Sayre syndrome. Less than 100 cases of Pearson syndrome have been reported. In infants, Pearson syndrome is associated with large mtDNA deletions ranging in size from 1.1 to 10 kb. Such mtDNA deletions may occur de novo or be transmitted in a maternal inheritance pattern. The mother of the child, although clinically unaffected, may potentially harbor a great degree of mtDNA deletions in a population of her oocytes. If the mother is clinically unaffected, the risk for the other siblings is estimated to be 1% to 4% because of the possibility of maternal germline mosaicism. Because mitochondria are maternally inherited, offsprings of a male with an mtDNA pathogenic variant are not at risk of inheriting the condition. Prenatal testing for pregnancies at increased risk is available, but results are not reliably predictive of disease severity. Intriguingly, several isogenic immortalized pluripotent stem cell (iPSCs) lines derived from cells of a patient with Pearson lacked detectable levels of the deleted mtDNA, whereas other lines had a great degree of deletions. The iPSCs carrying a high burden of deleted mtDNA displayed differences in proliferation, mitochondrial function, and hematopoietic phenotype when differentiated in vitro, as compared with isogenic iPSC which lacked the deleted mtDNA.[2]

Interestingly, a study of a cohort of 178 infants with Diamond-Blackfan anemia showed that 4.8% of them actually had Pearson syndrome. The identification of Pearson syndrome is not insignificant because patients with Diamond-Blackfan anemia are empirically treated with corticosteroids, which do not benefit patients with Pearson syndrome and may lead to infectious and metabolic complications.[3]

Mitochondrial diseases can also present and progress in several ways during adult life. The abnormalities in an affected adult patient often do not fit into a specific syndrome because mitochondrial disease can encompass multiple systems of the body. Patients with Kearns-Sayre syndrome, a progressive cradioencephalomyopathy, in infancy can be clinically manifested as Pearson syndrome, whereas in middle age the patients develop chronic progressive external ophthalmoplegia (CPEO). Multiple endocrinopathies involving the adrenal gland, pancreas, thyroid, and parathyroid gland frequently develop in patients with more severe disease.

Neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) syndrome, a progressive neurologic disorder, forms a clinical continuum with maternally inherited Leigh syndrome. NARP is caused by a mutation in mtDNA involving specifically the *MT-ATP6* gene. Individuals with heteroplasmy levels of greater than 70% to 90% clinically manifest NARP, whereas those with greater than 90% present Leigh syndrome. Leigh syndrome becomes apparent during the first year of life, whereas the first symptoms of NARP syndrome begin in childhood or early adulthood. Together, this pattern of disease indicates the complexity and the marked clinical variation of disease manifestations observed in patients, which frequently delays both diagnosis and appropriate clinical care. Next-generation sequencing

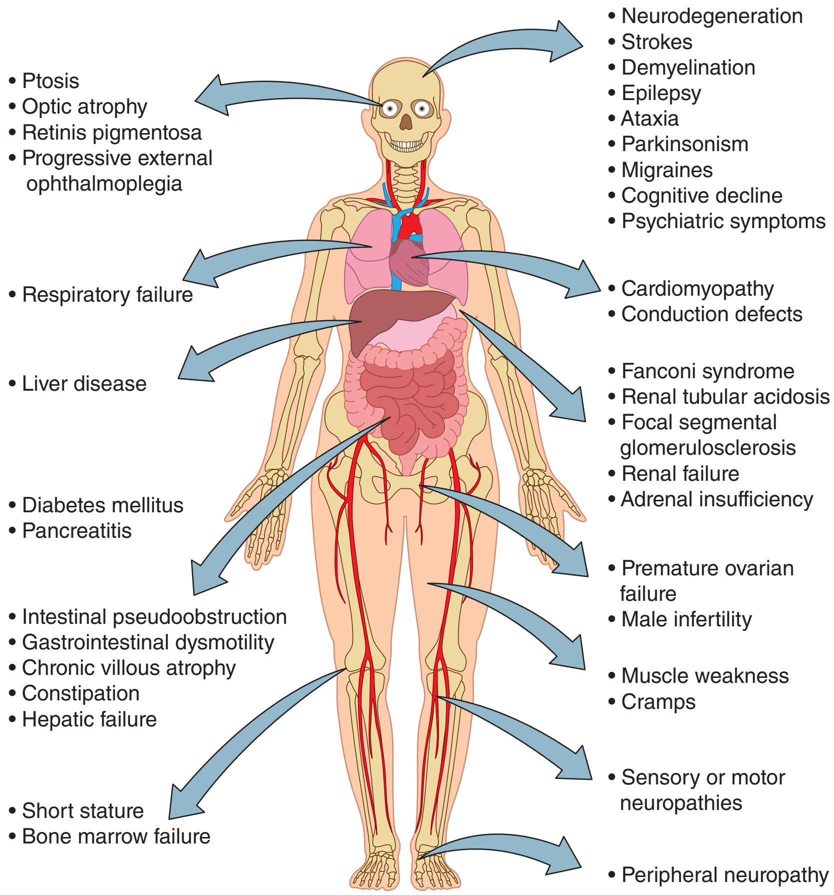

• Ptosis
• Optic atrophy
• Retinis pigmentosa
• Progressive external ophthalmoplegia
• Respiratory failure
• Liver disease
• Diabetes mellitus
• Pancreatitis
• Intestinal pseudoobstruction
• Gastrointestinal dysmotility
• Chronic villous atrophy
• Constipation
• Hepatic failure
• Short stature
• Bone marrow failure
• Neurodegeneration
• Strokes
• Demyelination
• Epilepsy
• Ataxia
• Parkinsonism
• Migraines
• Cognitive decline
• Psychiatric symptoms
• Cardiomyopathy
• Conduction defects
• Fanconi syndrome
• Renal tubular acidosis
• Focal segmental glomerulosclerosis
• Renal failure
• Adrenal insufficiency
• Premature ovarian failure
• Male infertility
• Muscle weakness
• Cramps
• Sensory or motor neuropathies
• Peripheral neuropathy

Figure 10.2 CLINICAL FEATURES OF MITOCHONDRIAL DISEASES. Mitochondrial disease may present within single organ (myopathy or cardiomyopathy) or multisystem.

has dramatically advanced the diagnosis and increased our understanding of the molecular landscape and the genetic basis of these disorders. Many novel therapeutic approaches are being explored in preclinical models, and several new therapies are being evaluated in ongoing clinical trials. Despite this success, many challenges remain, including understanding of the mechanisms implicated in the phenotypic expression of a particular genetic defect as well as the optimal care for patients with mitochondrial diseases. In this chapter, we will review the role of mitochondria in both normal and abnormal hematopoiesis, as well as in aging.

MITOCHONDRIAL REGULATION OF HEMATOPOIETIC STEM CELL FATES

Hematopoiesis relies on the integrity of a unique pool of hematopoietic stem cells (HSCs). HSCs and specifically those with long-term (LT) hematopoietic reconstitution capabilities occupy the apex of the hematopoietic system. They sustain LT hematopoiesis throughout the lifespan of an individual by constantly replenishing the hematopoietic system with committed progenitors and differentiated blood cells. Vast numbers of all types of adult mature hematopoietic cells (10^{11} to 10^{12}) are continuously generated each day through a series of lineage-committed progenitor cells.[4] The LT regeneration capacity is due to HSCs' ability to balance the self-renewal with differentiation. Such balance is central to blood cell homeostasis and is regulated by the complex interplay of cell-intrinsic and cell-extrinsic signaling networks.[5] This interplay is orchestrated and controlled by the mitochondrial network, which recently has emerged as a critical determinant of HSC homeostasis, commitment, and differentiation.

During homeostasis, HSCs are predominantly quiescent. To meet the physiologic demands for mature cells during steady-state condition, HSCs exit the quiescent state and become activated into slowly dividing cells.[6] Upon hematopoietic stress that occurs after acute depletion of progenitors and mature hematopoietic cells, HSCs rapidly enter the cell cycle and proliferate to replace the depleted cells. The exit of HSCs from quiescence, and their proliferation and commitment to multiple different cell fates, are intrinsically coupled with altered cellular metabolism and mitochondrial activity (Fig. 10.3).[7] It has been postulated that a metabolic switch from glycolysis to mitochondrial OXPHOS precedes the transition of HSCs from quiescence to proliferation. However, evidence has demonstrated that priming of quiescent HSCs and their entry into the cell cycle appears to be accompanied by upregulation of glycolysis.[8] Because glycolysis is linked to mitochondrial metabolism, such an increase in glycolysis rate might provide the energy required for entrance of quiescent HSCs into the cell cycle and activation of the mitochondrial network. Increased mitochondrial activity is essential for actively dividing HSCs and their commitment into each type of lineage-differentiated hematopoietic cells. In fact, compromised mitochondrial function and perturbation of metabolic pathways result in impaired differentiation and leukemia.[9]

Metabolic cues and mitochondrial content and activity vary drastically within different stages of hematopoiesis. LT HSCs are characterized by low mitochondrial mass, mitochondrial membrane potential (MMP), and reactive oxygen species (ROS) as opposed to committed progenitors and differentiated mature cells. The increase in the mitochondrial activity upon cell commitment allows the differentiating cells to meet their altered high metabolic and bioenergetic demands. Such metabolic rewiring is accompanied by profound alterations in the mitochondrial ultrastructure and dynamics, both of

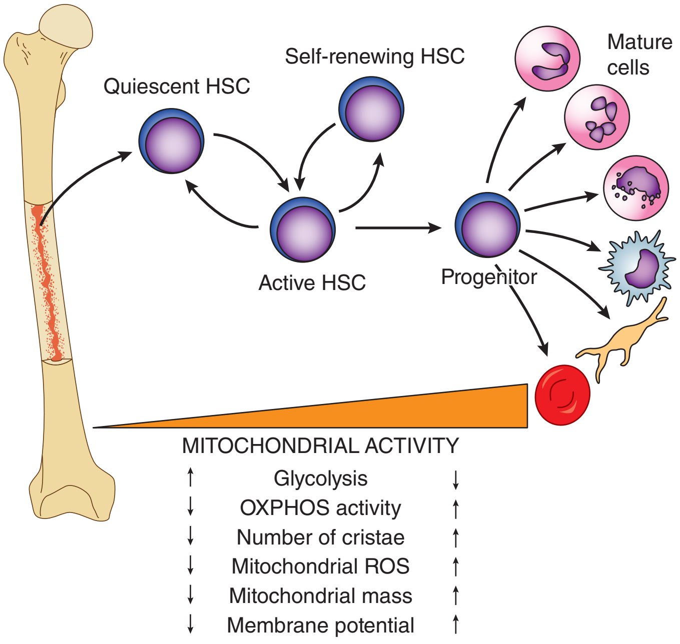


Figure 10.3 MODEL OF HEMATOPOIESIS. Quiescent hematopoietic stem cells (HSCs) reside in the bone marrow (BM) niches and rely mainly on glycolysis for their energy production. Upon hematopoietic stress, HSCs exit the quiescent state and become activated into slowly dividing HSCs, giving rise to a daughter cell that may remain in the cell cycle or give rise to a daughter cell that may return into a quiescent state to maintain the pool of primitive HSCs. Frequent and rapid cell divisions lead to transiently amplifying progenitors, which in turn give rise to more differentiated effector hematopoietic cells. A metabolic switch associated with an increased level of mitochondrial oxidative phosphorylation activity, reactive oxygen species generation, membrane potential, and mass occurs during the course of differentiation. *(Adapted from Papa L, et al. Ex vivo HSC expansion challenges the paradigm of unidirectional human hematopoiesis.* Ann NY Acad Sci. *2020;1466[1]:39–50.)*

which determine HSC fates.[6] Indeed, mitochondrial dynamics, morphology, and metabolism reciprocally influence each other to maintain the balance between self-renewal and proper differentiation of HSCs.[10] Impairment of mitochondrial metabolism affects mitochondrial dynamics and morphology, resulting in loss of HSC quiescence and self-renewal potential.[11] Conversely, aberrations in mitochondrial dynamics compromise the capacity of HSCs to differentiate into lymphoid cells.[6,12]

Beyond OXPHOS and ATP generation, mitochondria drive HSC fate decisions by regulating biosynthesis and the epigenome. Mitochondrial metabolites generated by OXPHOS and TCA cycles control DNA methylation, chromatin remodeling, and posttranslational modifications of various proteins, including histones and other epigenetic factors. In turn, such changes shape the epigenetic landscape and affect gene repertoires, ultimately compromising HSC function and cell fates.[1]

Mitochondria are signaling organelles that govern HSC fate decisions by also regulating calcium homeostasis and pathways involved in inflammation and cell death. In fact, each of these pathways is differently impacted by various levels of mitochondrial ROS.

Although mitochondria are the major source of ROS generation, they are also the main targets of ROS. Mitochondrial DNA and the mitochondrial proteome (mitochondrial and nuclear encoded proteins) are particularly vulnerable to ROS-mediated damages. This is due in part to their close proximity to the ETC, which is the primary site of ROS generation. A subtle elevation in ROS levels, in part, triggers HSC commitment and differentiation. Alternatively, excessive ROS levels impair HSC multilineage differentiation and induce uncontrolled proliferation and sustained cumulative damages, leading to HSC exhaustion, loss of self-renewal potential, and cell death.[6]

Unlike other cellular compartments, mitochondria are poorly equipped with quality control elements such as chaperones and proteases, which monitor proper protein folding and eliminate misfolded and oxidative damaged proteins. Accumulation of such damaged proteins further impacts the ETC activity and electron leakage from ETC, leading ultimately to increased ROS levels. The increased ROS levels, in turn, can trigger vicious cycles of mitochondrial damage and sustained energetic catastrophe. To reduce oxidative and metabolic stress that can be detrimental for HSC fates and integrity, HSCs use complex regulatory pathways and defense mechanisms[6] that are summarized in Fig. 10.4.

In the following sections we will discuss each of these topics and review the latest advances in our understanding of the multiple mechanisms by which the mitochondrial network controls stemness and differentiation of HSCs during normal hematopoiesis, leukemic transformation, and regeneration.

The Mitochondrial Unfolded Protein Response Mechanism

HSCs have a low protein synthesis rate and a limited folding capacity in comparison with committed progenitors and differentiated cells.[13] To achieve rapid and effective blood cell replacement with various hematopoietic progenitor and differentiated cells, HSCs face a burst in mitochondrial biogenesis. This burst is accompanied by an increased need for protein synthesis.[14] Due to the poor folding capacity, the increase in protein synthesis can exceed and overwhelm the capacity of the protein quality control system, leading to mitochondrial proteostasis stress, increased oncogenic transformation, and impaired HSC regenerative potential. To cope with the mitochondrial proteostatic stress, mitochondria signal the stress to the nucleus. Such events establish the mitochondria-to-nucleus signaling crosstalk, which result in the activation of the mitochondrial unfolded protein response (UPRmt). The UPRmt acts by coordinating the function of several pathways, all of which culminate in induction of proteases and chaperones that are required for proper protein folding and their complex assembly. Thus UPRmt monitors transcriptional programs and critical pathways that ensure overall mitochondrial fitness and integrity. A recent study identified a novel arm of UPRmt in HSCs, which is regulated by the coordinated interplay of Sirtuin (SIRT7), a histone deacetylase, and nuclear respiratory factor 1 (NRF1), a master regulator of mitochondrial biogenesis.[15] In response to bioenergetic stress, HSCs activate SIRT7, which represses the activity of NRF1. In turn, this suppression reduces mitochondrial translation and biogenesis and alleviates stress. Remarkably, SIRT7 expression, which is downregulated with aging, is

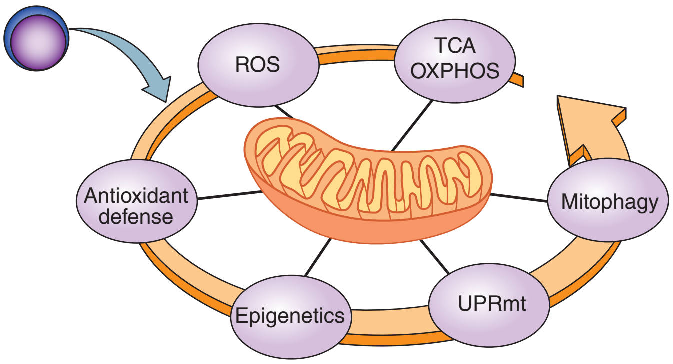


Figure 10.4 MECHANISMS OF MITOCHONDRIAL CONTROL OF HEMATOPOIETIC STEM CELLS (HSCS). Mitochondria activate and coordinate multiple and complex mechanisms to regulate the self-renewal potential of HSCs and their cell fate decisions. Under homeostasis and stress conditions, mitochondria controls HSC fate by regulating the OXPHOS activity and reactive oxygen species (ROS) production, activating mitophagy, mitochondrial unfolded protein response, antioxidant defense, as well as shaping the epigenomic landscape of HSCs.

associated with reduced HSC regenerative capacity.[15] In fact, in aged HSCs, mitochondrial protein folding stress is also linked to activation of NLR family pyrin domain containing 3 (NLRP3) inflammasome.[16] Repression of the NLRP3 inflammatory pathway by activated SIRT2, a cytoplasmic deacetylase, regulates the functional deterioration of aged HSCs.[16] Failure to engage UPRmt can irreversibly damage HSC cellular and metabolic fitness, leading to increased ROS generation, uncontrolled HSC proliferation and differentiation, and loss of HSC integrity and cell death.[15]

The Mitochondrial Oxidative Self-Defense Response Mechanism

Stringent regulation of ROS levels in HSCs is a complex process and relies on activation of the antioxidant defense mechanisms. As discussed earlier, ROS regulates HSC fate decisions by acting as rheostats. To maintain low ROS levels, HSCs engage multiple antioxidant pathways. Activation of such pathways are tightly coupled to redox sensors, including thioredoxin-interacting protein (TXNIP) and magnesium superoxide dismutase (MnSOD).[6,7] Intriguingly, upon transient and low levels of oxidative stress and ROS, TXNIP alters the function of p53 from acting as a prooxidant to an antioxidant. A limited activation of p53 mediates activation of ROS scavengers, including MnSOD and Sestrins. In addition to p53, several other molecular pathways have been reported to act as central hubs that control both metabolism and the redox state of HSCs. Of great interest in this regard are both Forkhead box O3 (FOXO3) and p38 mitogen-activated protein kinase (MAPK) cascade. Loss of FOXO3 resulting in ROS-dependent activation of p38 compromises the mitochondrial activity, redox state, and metabolism and hence leads to a decline of the LT repopulating capacity of HSCs.[17]

Remarkably, both FOXO3 and p53 activities are controlled by the family of Sirtuins, in particular SIRT1, a deacetylase that requires NAD⁺ for its activity. By deacetylating both histone and nonhistone proteins, including key transcription factors, SIRT1 modulates metabolism, cellular signaling pathways, and the epigenome in HSCs. Following metabolic stress, SIRT1 mitigates high ROS levels in part by activating SIRT3, a mitochondrial deacetylase that regulates the global acetylation of mitochondrial proteins.[18] Induced SIRT3 expression can rejuvenate aged HSCs due to enhanced mitochondrial antioxidant defenses.[6] Together, this evidence underscores the ability of HSCs to integrate multiple and complex pathways, all of which are required to activate antioxidant defense pathways and thereby preserve their LT self-renewal potential.

Mitochondrial Dynamics Controls the Self-Renewing Potential of Hematopoietic Stem Cells

HSC fate decisions are also regulated by dynamic changes in mitochondrial networks, which are highly dynamic and undergo continued remodeling and changes in shape and architecture. This dynamic remodeling, which contributes to mitochondrial quality control and mitochondrial fitness, is orchestrated by balanced fusion and fission of mitochondria. Human LT HSCs contain mitochondria that are small and globular with few deformed and poorly developed cristae, which reflects their low bioenergetic state (Fig. 10.5). In fact, immature cristae, which are the folded sections of the IMM and house the ETC complexes, are tightly linked to the low degree of respiration, OXPHOS activity, and thus low MMP. Upon HSC commitment and differentiation, mitochondrial network maturation results in the appearance of elongated and filamentous mitochondria with well-developed cristae accompanied by increased MMP and activity. Consistent with this notion is the evidence that loss of imprinting at the DLK1-GLT2 locus, which is associated with increased mature cristae folds, results in enhanced mitochondrial activity, ROS generation, and impairment of HSC LT reconstitution potential.[19] Moreover, increased mitochondrial size and volume due to loss of mitochondrial carrier homolog 2 (MITCH2), which primes mitochondrial OXPHOS activity, result in accelerated differentiation and exhaustion of HSCs.[11]

The fusion and fission machinery relies on the balanced activities of several proteins, including mitofusin 1 (MNF1), MNF2, and dynamin-related protein 1 (DRP1). Disruption of this balance is accompanied by a shift towards fusion and increased MFN2 levels favoring generation of abundant and highly filamentous mitochondrial networks in metabolically activated cells. MNF2 levels are particularly upregulated during commitment and differentiation of HSCs, whereas reduced MNF2 levels result in the maintenance of the HSC pool with retention of lymphoid potential.[12] Changes in mitochondrial shape can be also triggered by the decreased mitochondrial

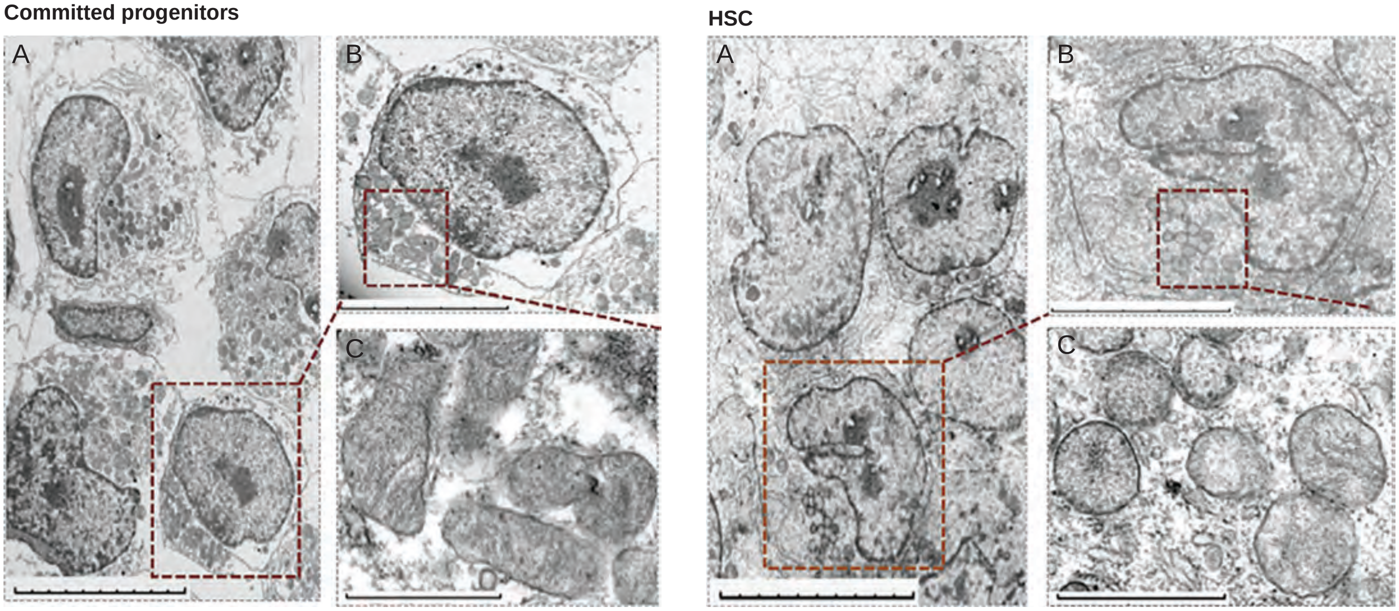


Figure 10.5 MITOCHONDRIAL MORPHOLOGY IN COMMITTED PROGENITORS AND HEMATOPOIETIC STEM CELLS (HSCs). Electromicrographs of committed progenitors *(left panel)* and HSCs *(right panel)*. Committed progenitors contain tubular, elongated-shaped mitochondria filled with regular and high number of cristae. HSCs contain globular, round-shaped mitochondria with low number and deformed cristae. A, 1000 magnification; scale bar: 10 µm; B, 2500 magnification; scale bar: 5 µm; C, 10,000 magnification; scale bar: 1 µm. *(From Papa L, et al. Ex vivo human HSC expansion requires coordination of cellular reprogramming with mitochondrial remodeling and p53 activation. Blood Adv. 2018 Oct 23;2(20):2766–2779.)*

translocation of DRP1, which is a master regulator of mitochondrial fragmentation. Interestingly, loss of MITCH2 and increased differentiation of HSCs is accompanied by reduced levels of DRP1.[11] Collectively, these findings highlight the importance of mitochondrial dynamics and shape in the retention of the pool of LT HSCs and their differentiation capacities.

Mitophagy as a Gatekeeper of Hematopoietic Stem Cells Fate Decisions

The fitness of the mitochondrial network in HSCs is largely controlled by activation of a selective form of autophagy termed mitophagy. Mitophagy acts as a major gatekeeper of metabolic activity in HSCs by selectively removing damaged mitochondria that represent a great threat to both HSC self-renewal potential and integrity. Mitophagy also limits the number of active mitochondria contributing in a reduction of both mitochondrial mass and activity. Although this view has been recently challenged due to the heterogeneity of HSCs, a recent report has reinforced the role of mitophagy in maintaining the pool of LT HSCs.[6] Activation of mitophagy is required for purified HSCs marked by angiopoietin-1 receptor (Tie2) expression to retain their self-renewal potential without lineage bias.[20] In addition, impaired autophagy caused by loss of the autophagy gene, *ATG12*, drives accelerated myeloid differentiation and compromises HSC self-renewal capacity. Specifically, ATG12$^{-/-}$ HSCs contain increased numbers of filamentous mitochondria with enhanced membrane potential, elevated ATP generation, and OXPHOS activity. ATG12$^{-/-}$ HSCs exhibit a skewed ratio of circulating myeloid versus lymphoid cells that resembles the biased HSC differentiation, a characteristic of aged HSCs. Remarkably, aged HSCs with high levels of mitophagy activation display an enhanced repopulating capacity as opposed to aged HSCs.

To rapidly reconstitute the hematopoietic system with differentiated cells upon stress, transplanted HSCs require a boost in their mitochondrial activity. Under stress conditions, retention of the self-renewing HSC pool is tightly controlled by two metabolic checkpoints that involve UPRmt and FOXO3-mediated autophagy.[15,21] This activation results in reduced mitochondrial mass and activity and preservation of the HSC pool. Recent evidence has pinpointed that activation of these mechanisms might depend on SIRT1. Activation of the SIRT1 axis via use of the NAD$^+$-boosting agent nicotinamide riboside (NR) increases HSC regeneration capacity by increasing mitochondrial clearance. This NR-induced mitophagy is required to reduce both MMP and metabolism of HSCs.[4] Collectively, this evidence clearly highlights the role of mitophagy as another critical checkpoint that controls mitochondrial activity and metabolic programs under homeostasis, stress, and HSC transplantation and thus controls and regulates HSC fate decisions.

Mitochondrial Metabolic Regulation of the Epigenetic Landscape of Hematopoietic Stem Cells

HSC fate and function are controlled by mitochondrial metabolic regulation of their epigenetic landscape, which relies on mitochondrial-generated metabolites of the TCA and OXPHOS cycles. Such metabolites act not only as critical regulators of cellular homeostasis but also as cofactors for histone modifiers and other key elements that influence the epigenetic state of HSCs. One of the most prominent metabolites is acetyl-CoA that fuels the TCA cycle to generate ATP. Acetyl CoA also provides the acetyl groups for acetylation of histone tails, resulting in dynamic alterations of the epigenetic state of chromatin. Such alterations also influence the epigenetic control of transcriptional programs and thus impact HSC fate decisions.[1] The epigenomic state of HSCs can also be influenced by the alpha ketoglutarate (αKG), which promotes DNA demethylation. Aberrant DNA demethylation associated with altered αKG levels results in HSC differentiation and aberrant HSC self-renewal.[1]

In addition to the TCA cycle, the epigenetic landscape of HSCs is also controlled by mitochondrial OXPHOS activity. Inhibition of mitochondrial OXPHOS activity decreases histone acetylation and blocks differentiation of HSCs. Impaired HSC differentiation was also observed upon loss of Rieske iron-sulfur protein, an element of the complex III subunit of ETC, that impairs OXPHOS respiration.[9] Notably, repressed respiration was accompanied by accumulation of TCA-derived metabolites such as 2-hydroxyglutarate, fumarate, and succinate, resulting in an increase in both DNA hypermethylation and histone hypoacetylation.[9] In turn, this metabolic imbalance alters both the epigenomic and transcriptomal profiles, resulting in impaired differentiation and depletion of myeloid progenitors. Collectively, these findings point to the regulation of OXPHOS activity and its constant feedback with the TCA cycle, both of which shape the epigenome and determine HSC fates.

Remarkably, the interplay between mitochondrial metabolism and epigenome in HSCs is tightly regulated by SIRTs. Specifically, activated SIRT1 by NAD$^+$ or acetyl CoA acts as a histone deacetylase to regulate chromatin remodeling and achieve silencing of transcriptional programs in HSCs. Loss of SIRT1 results in skewed differentiation of HSCs favoring the myeloid cell lineage. HSCs that lack SIRT1 exhibit a premature aging phenotype and loss of quiescence. Similar effects were observed in HSCs with ablated SIRT6, which epigenetically suppress the transcription of Wnt target genes through deacetylation of histone H3K56.[22]

Thus, in summary, by regulating the flux and proper balance of metabolites and their epigenetic activities, the mitochondrial network serves as a central hub that controls the HSC epigenetic landscape and therefore HSC fate decisions.

INTERACTION OF MITOCHONDRIAL NETWORK WITH THE BONE MARROW MILIEU REGULATES HEMATOPOIETIC STEM CELL FATES

The profound role of mitochondria as critical determinants of HSC self-renewal potential cannot be envisioned without the extrinsic signals imposed on HSC by their bone marrow (BM) niches. To maintain a low metabolic state, mitochondria in HSCs act in close interaction with HSC niches. Primitive HSCs reside not only in extremely hypoxic areas, but also in periarteriolar and highly vascular areas of the BM where oxygen tension is high. Thus, in contrast to previous beliefs, hypoxia does not appear to be the sole mechanism by which the BM niche contributes to retention of LT HSCs. The arterial BM endothelial cells, which are exposed to the highest oxygen tension, create a barrier for quiescent HSCs. The integrity of this barrier is attributed to the ability of endothelial cells to scavenge excessive ROS levels. In addition, endothelial cells of the periarteriolar regions rely mainly on glycolysis to avoid ROS generation. Accordingly, this barrier provides an ROS-low microenvironment to protect the integrity of HSCs.

Mesenchymal stem cells (MSCs), another component of HSC niches, have been also reported to scavenge mitochondria and ROS from HSCs under high oxidative stress conditions. Connexin 43 (CX43), a component of gap junctional channels, mediates the transfer of ROS from HSCs to MSCs, resulting in reduced ROS levels.[6] Alternatively, CX43 facilitates the transfer of mitochondria from HSCs to MSCs. This dynamic mitochondrial transfer inversely regulates low levels of ROS generation and thus contributes to the retention of HSC self-renewal potential.[6] Conversely, transfer of mitochondria from MSCs to HSCs drives the HSC response to acute bacterial infection, resulting in rapid HSC differentiation and leukocyte expansion.[23] Remarkably, such mitochondrial transfer occurs prior to activation of the transcriptional programs that control the burst in mitochondrial biogenesis.[23] The rapid increase in mitochondrial mass and subsequent metabolic changes in HSCs enable their prompt differentiation, resulting in increased leukocyte numbers that are required to successfully fight infection. Thus overall it appears that the mutual transfer of mitochondria between HSCs and BM niche cells acts as an early physiologic response to regulate either HSC self-renewal potential or facilitate the rapid onset of emergency hematopoiesis through HSC differentiation. Together, this evidence highlights the dynamic relationship between extrinsic signals imposed by the BM and intrinsic mechanisms regulated by the mitochondrial network that dictate HSC fate decisions.

MITOCHONDRIAL AND BONE MARROW ROLES IN LEUKEMIC STEM CELLS AND AGED HEMATOPOIETIC STEM CELLS

As discussed earlier, great progress has been reached in our understanding of the plasticity of both BM niches and mitochondrial network, and their coordinated control of HSC fates. Despite major developments, our ability to trace and understand the role of mitochondria in regulating normal HSC fates within the BM microenvironment under stress is limited. In this regard, studies of aged HSCs and leukemogenesis have provided critical insights. With age, HSCs lose their regenerative potential, exhibit unbalanced myeloid differentiation, and frequently become transformed, leading to the development of various hematologic malignancies. Mutations in mtDNA alone cannot account for malignant transformation of HSCs. Aberrant mitochondrial activity and metabolism in concert with an inflamed microenvironment accompanied by a burst of inflammatory chemokines are also critical for HSC aging and their malignant transformation into leukemic stem cells (LSCs). In fact, changes in the BM stroma with age create a permissive environment that supports both leukemic transformation and propagation of their clones. Alternatively, LSCs can directly remodel BM stroma to preferentially permit their survival and uncontrolled proliferation at the expense of normal HSCs. Such remodeling allows for changes in niche structure and a burst of inflammatory cytokines. Together, these events exhaust normal HSCs and enhance LSC survival. Remodeling of the BM microenvironment has been reported in many types of leukemia, including chronic myeloid leukemia (CML), acute myeloid leukemia (AML), and B-cell acute lymphoblastic leukemia. Evidence indicates that the remodeled BM fuels mitochondrial fatty acid oxidation (FAO) metabolism by LSCs, thereby shaping their epigenetic and metabolic landscape. In fact, resistance to several treatment approaches, including the combination of the Bcl2 inhibitor venetoclax with the hypomethylating agent 5-azacitidine, is mediated by the upregulation of mitochondrial FAO. FAO activation reverses the inhibitory effect of this chemotherapy combination on mitochondrial OXPHOS activity of LSCs, allowing them to survive.[24] Notably, LSCs rely both on glycolysis and OXPHOS to meet their bioenergetic demands. Enhanced mitochondrial OXPHOS activity triggered by the mitochondrial transfer from BM MSCs to AML LSC blasts provides them with energy and thus with survival advantages.[25] Intriguingly, mitochondrial transfer to BM MSCs rescues acute lymphoblastic leukemia cells from ROS-inducing chemotherapy and cell death.[26] Therefore targeting the reciprocal dependence of LSCs on perturbed BM niche cells and vice versa might open novel therapeutic treatment avenues to perturb both the epigenome and metabolome of LSCs and overcome chemotherapy resistance. LSC resistance to tyrosine kinase inhibitor (TKI) treatment for CML (see Chapter 69) has been attributed to the role of SIRT1. Specifically, SIRT1 activity enhances mitochondrial OXPHOS and metabolic regulation of LSC maintenance, growth, and resistance due to upregulation of PGC-1α.[27] In fact, these mitochondrial alterations occur independently of kinase activation, and TKI treatment enhanced inhibition of CML hematopoiesis in SIRT1-deleted mice. Collectively, these findings point to the close interplay of the transformed mitochondrial network and the altered BM microenvironment in promoting transformation and resistance of LSCs to various treatments.

MITOCHONDRIAL AND EPIGENOME CONTROL REPROGRAMMING OF HEMATOPOIETIC STEM CELLS

Understanding of the dynamic signaling networks that shape the epigenome and mitochondrial metabolism in HSCs and govern their LT self-renewing potential can undoubtedly lead to advances in regenerative medicine and stem cell therapy. HSC transplantation has been used as therapy for patients with blood disorders and refractory hematologic malignancies. Different sources of HSCs, including mobilized peripheral stem cells and BM, can be used for HSC transplantation. Unfortunately, difficulties in human leukocyte antigen matching and graft-versus-host disease or other forms of immune rejection limit the use of HSCs for stem cell therapy. In this context, umbilical cord blood (UCB) can be used as an alternative source. However, the inadequate number of HSCs within a single UCB unit and the inability of many cytokine cocktails to expand the number of UCB LT HSCs in ex vivo cultures represent a major limitation for their use in clinical settings. Currently, various ex vivo expansion strategies, including stemreginin 1 (SR1), pyrimidole derivative UM171, and valproic acid (VPA), have been successfully implemented in ongoing clinical trials. Although most of these ex vivo strategies expand the number of existing HSCs present within UCBs, the VPA strategy has been reported to also trigger cellular reprogramming of committed progenitors into HSCs (Fig. 10.6). In fact, VPA is clearly an excellent example that tightly links shaping of the epigenome with altered transcriptome and mitochondrial metabolism in LT HSC. VPA, which is a deacetylase inhibitor, alters the epigenome and regulates the transcriptional programs of UCB $CD34^+$ cells. This altered transcriptome greatly resembles the transcriptomic profiles that characterize unmanipulated LT HSCs.[7] Intriguingly, cellular reprogramming by VPA is accompanied by remodeling of the mitochondrial network characterized by low mass, MMP, and activity. Such a restructured mitochondrial network is reminiscent of mitochondria of unmanipulated LT HSCs. Both acquisition and maintenance of LT HSCs were influenced by the antioxidant activity of the p53-MnSOD axis. Likewise, activation of autophagy in cultures of UCB HSCs expanded with SR1 or UM71 may further improve expansion of LT HSCs.[28] Moreover,

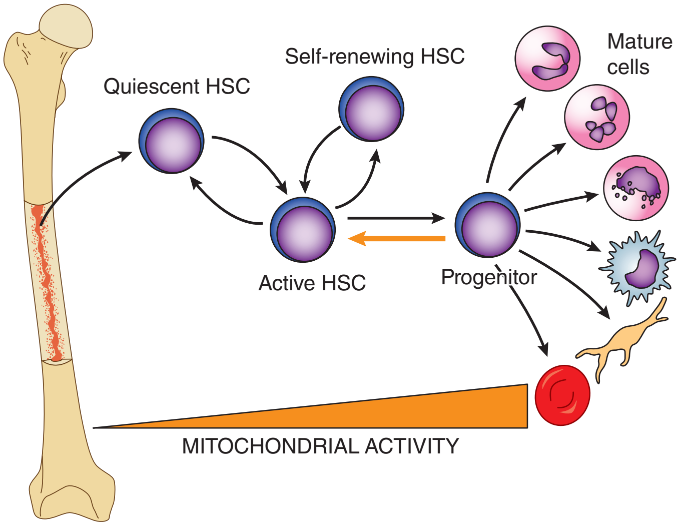


Figure 10.6 CELLULAR REPROGRAMMING OF COMMITTED PROGENITORS INTO HEMATOPOIETIC STEM CELLS (HSCs). Valproic acid (VPA) triggers rapid reprogramming of committed progenitors into functional HSCs by altering their epigenome, transcriptome, and mitochondrial profile. The reprogrammed HSCs exhibit a reduced mitochondrial activity and profile that closely resembles that of the unmanipulated HSCs. *(Adapted from Papa L, et al. Ex vivo HSC expansion challenges the paradigm of unidirectional human hematopoiesis.* Ann NY Acad Sci. *2020;1466(1):39–50.)*

NR treatment that regulates metabolic and mitochondrial fitness by inducing mitophagy might prove useful as a potential booster of hematopoiesis, not only following ex vivo HSC expansion cultures but also in vivo in patients with BM aplasia and myelodysplastic syndromes where age-dependent mitochondrial defects have been observed.[4]

CONCLUDING REMARKS

Clearly, the ability of the mitochondrial network to integrate intrinsic signals with cues from the microenvironment and control the epigenome and metabolism of young HSCs, aged HSCs, and LSCs is emerging as a key aspect of stem cell biology. A further comprehensive characterization of how the coordinated interplay of these signaling networks governs HSC fates and their plasticity under both homeostasis and hematopoietic stress will undoubtedly lead to potential novel strategies for promoting self-renewal of healthy HSCs in aging and during ex vivo HSC expansion. On the other hand, identifying the key metabolic cues and mitochondrial mechanisms that control LSC fate decisions will likely lead to new pathways and targets to deplete their numbers.

REFERENCES

1. Martinez-Reyes I, Chandel NS. Mitochondrial TCA cycle metabolites control physiology and disease. *Nat Commun*. 2020;11(1):102.
2. Cherry ABC, Gagne KE, Mcloughlin EM, et al. Induced pluripotent stem cells with a mitochondrial DNA deletion. *Stem Cells*. 2013;31(7):1287–1297.
3. Gagne KE, Ghazvinian R, Yuan D, et al. Pearson marrow pancreas syndrome in patients suspected to have Diamond-Blackfan anemia. *Blood*. 2014;124(3):437–440.
4. Vannini N, Campos V, Girotra M, et al. The NAD-booster nicotinamide riboside potently stimulates hematopoiesis through increased mitochondrial clearance. *Cell Stem Cell*. 2019;24(3):405–418 e407.
5. Seita J, Weissman IL. Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med*. 2010;2(6):640–653.
6. Papa L, Djedaini M, Hoffman R. Mitochondrial role in stemness and differentiation of hematopoietic stem cells. *Stem Cells Int*. 2019;2019:4067162.
7. Papa L, Djedaini M, Hoffman R. Ex vivo HSC expansion challenges the paradigm of unidirectional human hematopoiesis. *Ann N Y Acad Sci*. 2020;1466(1):39–50.
8. Umemoto T, Hashimoto M, Matsumura T, Nakamura-Ishizu A, Suda T. Ca(2+)-mitochondria axis drives cell division in hematopoietic stem cells. *J Exp Med*. 2018;215(8):2097–2113.
9. Anso E, Weinberg SE, Diebold LP, et al. The mitochondrial respiratory chain is essential for haematopoietic stem cell function. *Nat Cell Biol*. 2017;19(6):614–625.
10. Bahat A, Gross A. Mitochondrial plasticity in cell fate regulation. *J Biol Chem*. 2019;294(38):13852–13863.
11. Maryanovich M, Zaltsman Y, Ruggiero A, et al. An MTCH2 pathway repressing mitochondria metabolism regulates haematopoietic stem cell fate. *Nat Commun*. 2015;6:7901.
12. Luchsinger LL, de Almeida MJ, Corrigan DJ, Mumau M, Snoeck HW. Mitofusin 2 maintains haematopoietic stem cells with extensive lymphoid potential. *Nature*. 2016;529(7587):528–531.
13. Signer RA, Magee JA, Salic A, Morrison SJ. Haematopoietic stem cells require a highly regulated protein synthesis rate. *Nature*. 2014;509(7498):49–54.
14. Sigurdsson V, Miharada K. Regulation of unfolded protein response in hematopoietic stem cells. *Int J Hematol*. 2018;107(6):627–633.
15. Mohrin M, Shin J, Liu Y, et al. Stem cell aging. A mitochondrial UPR-mediated metabolic checkpoint regulates hematopoietic stem cell aging. *Science*. 2015;347(6228):1374–1377.
16. Luo H, Mu WC, Karki R, et al. Mitochondrial stress-initiated aberrant activation of the NLRP3 inflammasome regulates the functional deterioration of hematopoietic stem cell aging. *Cell Rep*. 2019;26(4):945–954 e944.
17. Miyamoto K, Araki KY, Naka K, et al. Foxo3a is essential for maintenance of the hematopoietic stem cell pool. *Cell Stem Cell*. 2007;1(1):101–112.
18. Brown K, Xie S, Qiu X, et al. SIRT3 reverses aging-associated degeneration. *Cell Rep*. 2013;3(2):319–327.
19. Qian P, He XC, Paulson A, et al. The Dlk1-Gtl2 Locus preserves LT-HSC function by inhibiting the PI3K-mTOR pathway to restrict mitochondrial metabolism. *Cell Stem Cell*. 2016;18(2):214–228.
20. Ito K, Turcotte R, Cui J, et al. Self-renewal of a purified Tie2+ hematopoietic stem cell population relies on mitochondrial clearance. *Science*. 2016;354(6316):1156–1160.
21. Ho TT, Warr MR, Adelman ER, et al. Autophagy maintains the metabolism and function of young and old stem cells. *Nature*. 2017;543(7644):205–210.
22. Wang H, Diao D, Shi Z, et al. SIRT6 Controls hematopoietic stem cell homeostasis through epigenetic regulation of wnt signaling. *Cell Stem Cell*. 2016;18(4):495–507.
23. Mistry JJ, Marlein CR, Moore JA, et al. ROS-mediated PI3K activation drives mitochondrial transfer from stromal cells to hematopoietic stem cells in response to infection. *Proc Natl Acad Sci U S A*. 2019;116(49):24610–24619.
24. Jones CL, Stevens BM, D'Alessandro A, et al. Inhibition of amino acid metabolism selectively targets human leukemia stem cells. *Cancer Cell*. 2018;34(5):724–740 e724.
25. Marlein CR, Zaitseva L, Piddock RE, et al. NADPH oxidase-2 derived superoxide drives mitochondrial transfer from bone marrow stromal cells to leukemic blasts. *Blood*. 2017;130(14):1649–1660.
26. Burt R, Dey A, Aref S, et al. Activated stromal cells transfer mitochondria to rescue acute lymphoblastic leukemia cells from oxidative stress. *Blood*. 2019;134(17):1415–1429.
27. Abraham A, Qiu S, Chacko BK, et al. SIRT1 regulates metabolism and leukemogenic potential in CML stem cells. *J Clin Invest*. 2019;129(7):2685–2701.
28. Xie SZ, Garcia-Prat L, Voisin V, et al. Sphingolipid modulation activates proteostasis programs to govern human hematopoietic stem cell self-renewal. *Cell Stem Cell*. 2019;25(5):639–653 e637.

CHAPTER 11

CYTOKINES, CHEMOKINES, OTHER GROWTH FACTORS, AND THEIR RECEPTORS

Hal E. Broxmeyer[†] and Maegan L. Capitano

INTRODUCTION

Hematopoiesis is regulated by cytokine-cell and cell-cell interactions.[1] Cytokines are polypeptides secreted by many cell types either constitutively or after induction and are usually classified based on primary structures associated with their receptor extracellular domains.[2,3] They regulate a large range of biologic functions, including hematopoiesis, immunity, and inflammation. In context of hematopoiesis, they have effects on proliferation, differentiation, survival/cell death (apoptosis), and cell movement/migration/induced mobilization. They can act in a paracrine fashion on closely adjacent cells, cells at a distance in the same tissue area, or other tissue areas sometimes by their movement from one tissue/organ to another tissue/organ. Cytokines can also work in an autocrine fashion. A rigorous definition of autocrine regulation is that the cytokine works within the cell that produces it, and either maintains the cytokine for internal cellular signaling/activity or can release it, upon which it can then act on the same cell that produced and released it. Autocrine regulation is a much-discussed mode of action, especially in leukemia and other cancers, but in reality it is very difficult to prove this concept of a cell. Many papers that have referred to autocrine production/action have not rigorously proven this concept, even in the case of leukemia or other cancers. Paracrine modes of action refer to the situation when proteins are secreted/released by a cell and then act on another cell, which is more easily proven and verified.

To modify an old proverb: "What is forgotten is doomed to be repeated without gain of new knowledge." Many current investigators do not cite or even know of crucial past efforts in the field of how cytokines, chemokines, and other growth-modulating factors influence hematopoietic stem cell (HSC) and hematopoietic progenitor cell (HPC) cell biology and of hematopoietic cell regulation. Thus they unfortunately lose out on knowledge that could help their research. For this reason, this review references the older, as well as more recent, literature on cytokines/interleukins (ILs)/chemokines, other growth factors, and their effects on cell regulation. Although this includes an extensive list of references, it is not an exhaustive list.

After many years of intensive research going back to at least the 1960s, it is recognized that cytokines are widely pleiotropic, with numerous cases of redundancy in action and with many overlapping functions. In fact, there are few cytokines that have been shown to have only one cell and mode of action, including production/release of other growth modulating factors, and it is likely even these few such cytokines will eventually be shown to be pleiotropic in action/activity. Individual cytokines can act as agents that induce either positive and/or negative effects or, when used in combination, additive to synergistically positive and/or negative effects. These different actions are sometimes dependent on the target cells(s) of action and depend on how they are combined for in vitro and in vivo assessments. It is highly likely that combined effects of cytokines occur with great frequency during in vivo regulatory events and in context of the microenvironment niche in which they are produced/released (e.g., in the bone marrow [BM]). Moreover, a cytokine can induce the production/release of other cytokines that can then produce effects that were originally ascribed to the cytokine that started this cascading set of regulatory events. Only by studying effects of cytokines at the single isolated target cell level, with and without serum/plasma (to eliminate effects of growth modulating factors present in serum/plasma) can one begin to truly understand the initial target cell(s) and the action(s) of the cytokine(s) of interest. Thus reductionist approaches of unraveling cytokine cell action(s) are more frequently used, as is single-cell ribonucleic acid (RNA) sequencing (RNA-seq), which is used to assess intracellular events. However, ultimately, to fully and clearly understand hematopoiesis and its regulation, it is necessary to put data together in a total context of interacting cytokines and target cells to get a more accurate picture of what is happening at a physiologic and pathologic level. This is necessary to better use our knowledge for eventual enhanced clinical efficacy for treatment of both malignant (e.g., leukemia, lymphoma) and nonmalignant (e.g., preleukemia, myelodysplastic syndromes [MDS], and myeloproliferative neoplasms [MPNs]) diseases.

Cytokines act through specific cell surface receptors to activate intracellular events, with some receptors acting in concert with other receptors to elicit their regulatory effects.[4,5] It is notable that as late as the mid-1970s not much was known about specific growth factors, other than that for erythropoietin (EPO), the red blood cell hormone, which was the first growth factor to be purified. Recombinant growth factors were not yet produced or known. Growth factors were identified by their purported actions present in crude preparations of cell or tissue extracts or in media conditioned by cells or tissues. The biologic activities attributed to these cell/tissue extracts or conditioned media could have been and, in fact, were in many cases due to combinations of proteins, and one had to rule out effects of confounding and contaminating agents such as bacterial lipopolysaccharide (LPS).[6] In fact, this contamination of even "purified" protein products is still a confounding problem because LPS can stimulate release of cytokines. An example of these growth regulatory factors were the terms for these activities: colony-stimulating activity (CSA), colony-inhibiting activity (CIA), or colony-stimulating factor (CSF), even before it was known what these proteins were. Presently, through biochemical purification procedures and then later through recombinant deoxyribonucleic acid (DNA) technology, the proteins from these crude preparations responsible for these biologic activities were found to include, in addition to EPO, the CSFs: granulocyte-macrophage (GM)-CSF (also known as CSF-2), granulocyte (G)-CSF (also known as CSF-3), interleukin-3 (IL-3) (previously termed multi-CSF), macrophage (M)-CSF (also known as CSF-1), and thrombopoietin (TPO). The CIA molecules were later identified as lactoferrin (LF), H-subunit (H)-ferritin, and some of the ILs, interferons (IFNs), tumor necrosis factors (TNFs), transforming growth factors (TGFs), and a large number of other, now well-defined and characterized, proteins. We now know that GM-CSF, G-CSF, IL-3, M-CSF, EPO, TPO, and two potent costimulating molecules, stem cell factor (SCF) and Flt3-ligand (FL), transmit their cascade of intracellular signaling molecules after binding to specific receptors (R). GM-CSFR is: GM-CSF-alpha (α), subunit cluster density (CD)116; G-CSFR is: CSF3R/CD114; IL-3R is: IL-3RA/CD123; M-CSFR is: tyrosine kinase CSF1R/c-FMS/CD115; EPO has two receptors, those on hematopoietic cells and those on other cells[7]; the TPOR is: myeloproliferative leukemia protein (MPL)/CD110; SCFR is: the tyrosine kinase c-kit/CD117 and is found as both a cell membrane and soluble protein; and FLR is: the tyrosine kinase, Flt3.

[†] Deceased

Class or type I cytokines (often referred to as hematopoietins) regulate development, differentiation, and activation of hematopoietic and immune cells. Their receptors are type I membrane proteins with an N-terminal extracellular and C-terminal intracellular orientation. Type I cytokine receptors include receptors for CSFs, ILs, EPO, TPO (Fig. 11.1),[5] and hormones such as growth hormone (GH) and leptin.

Class II cytokines consist of type I IFNs, which include 16 members that are produced by almost every nucleated cell, with approximately 20% to 60% sequence identity, including 12 subtypes of IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω. Type I IFNs initiate signaling by binding to the same receptor composed of two subunits called IFNAR1 and IFNAR2. Type II IFN consists of the single IFN-ϒ, which signals through a heterodimeric receptor composed of IFNGR1 and IFNGR2. Type III IFNs include IFN-λ1 (IL-29), IFN-λ2 (IL-28A), and IFN-λ3 (IL-28B). Some include the IL-10 family of cytokines (IL-10, IL-19, IL-20, IL-22, IL-24, IL-26) within the group of type III IFNs. The type III IFN receptor is composed of IL-10Rβ and IL-28R (Fig. 11.2).[5]

Structural similarities between the various type I cytokines were not immediately recognized. However, cloning of their receptors revealed significant homology in that the extracellular regions contain a common domain with four conserved cysteines (C4) in the N-terminal segment and a tryptophan-serine doublet near the C-terminal end.[3] Mutagenesis studies revealed a structural role for these amino acids

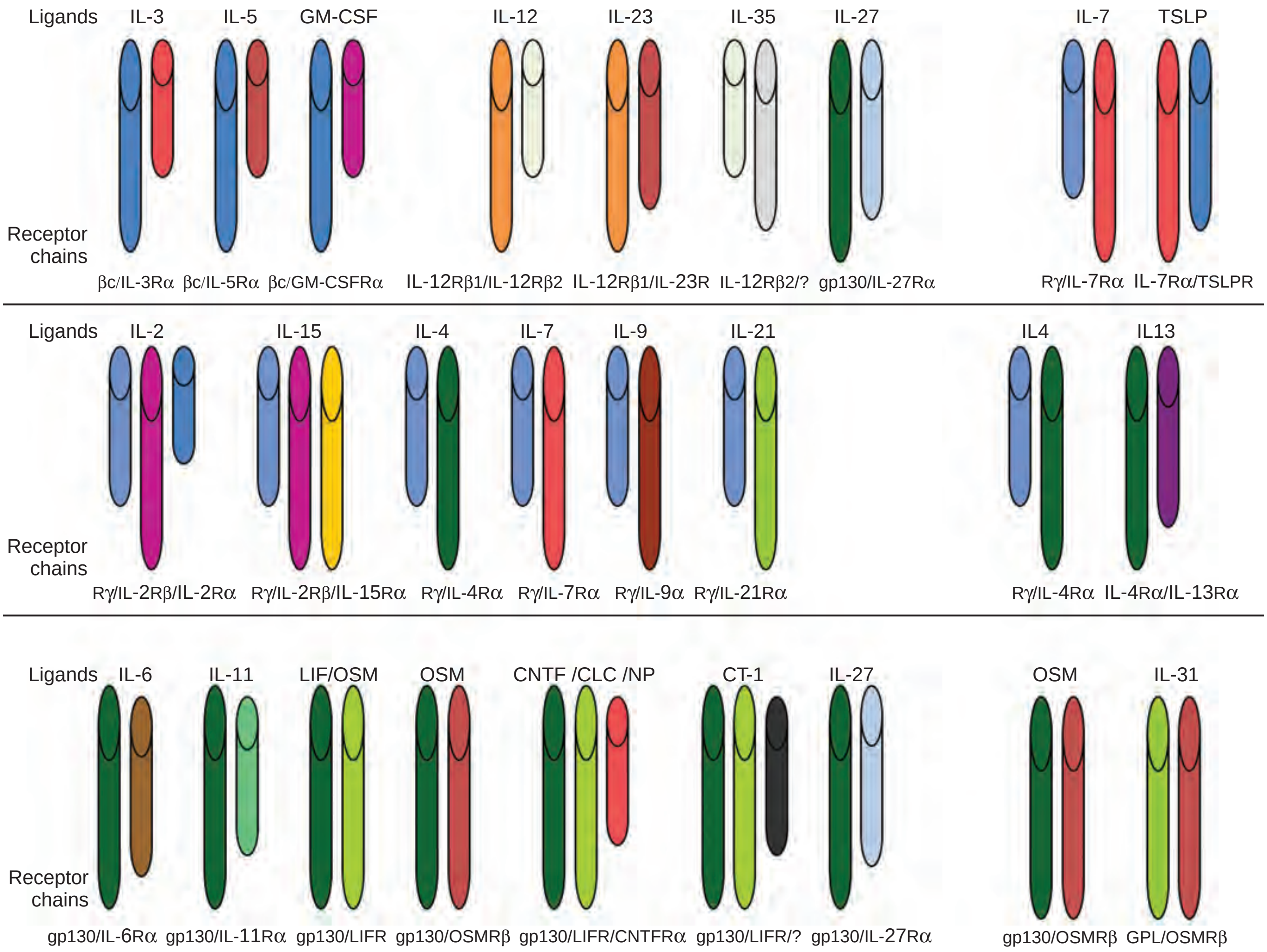

Ligands
IL-3
IL-5
GM-CSF
IL-12
IL-23
IL-35
IL-27
IL-7
TSLP
Receptor chains
βc/IL-3Rα
βc/IL-5Rα
βc/GM-CSFRα
IL-12Rβ1/IL-12Rβ2
IL-12Rβ1/IL-23R
IL-12Rβ2/?
gp130/IL-27Rα
Rγ/IL-7Rα
IL-7Rα/TSLPR
Ligands
IL-2
IL-15
IL-4
IL-7
IL-9
IL-21
IL4
IL13
Receptor chains
Rγ/IL-2Rβ/IL-2Rα
Rγ/IL-2Rβ/IL-15Rα
Rγ/IL-4Rα
Rγ/IL-7Rα
Rγ/IL-9α
Rγ/IL-21Rα
Rγ/IL-4Rα
IL-4Rα/IL-13Rα
Ligands
IL-6
IL-11
LIF/OSM
OSM
CNTF /CLC /NP
CT-1
IL-27
OSM
IL-31
Receptor chains
gp130/IL-6Rα
gp130/IL-11Rα
gp130/LIFR
gp130/OSMRβ
gp130/LIFR/CNTFRα
gp130/LIFR/?
gp130/IL-27Rα
gp130/OSMRβ
GPL/OSMRβ

Figure 11.1 CLASS OR TYPE I CYTOKINES (OFTEN REFERRED TO AS HEMATOPOIETINS) REGULATE DEVELOPMENT, DIFFERENTIATION, AND ACTIVATION OF HEMATOPOIETIC AND IMMUNE CELLS. Simple depiction of type I cytokine receptor subfamilies. Interleukin (IL)-3 receptor (R), IL-5R, and granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR) share a common βc chain that places them in one group. IL-12 family members include four cytokines. IL-12 and IL-23 share the IL12Rβ1 unit, and IL-12 and IL-35 share the IL-12Rβ2 unit. IL-27 shares gp130 with the IL-6 family. Multiple cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21) share the common Rγ chain, which is mutated in a subset of patients with severe combined immune deficiency. IL-4R shares a subunit with IL-13R. Both IL-4 and IL-13 drive T helper 2 (Th2) responses. IL-7R shares one subunit with the thymic stromal lymphopoietin (TSLP). This sharing of receptor subunit may explain why deletion of the gene encoding IL-7R affects the lymphoid system more severely than deleting the IL-7 gene. The IL-6 family includes multiple cytokines that all have the signal transducer gp130. Deleting gp130 results in embryonic lethality. Some IL-6 family members activate more than one receptor. Oncostatin M (OSM) can work through a heterodimer receptor consisting of gp130 with OSMRβ or gp130 with leukemia inhibitory factor receptor (LIF-R). Ciliary neurotrophic factor (CNTF), cardiotrophin-like cytokine (CLC), and neuropoietin (NP) engage the receptor composed of gp130, LIFR, and CNTFR. Cardiotrophin-1 (CT-1) uses a receptor composed of gp130, LIF-R, and another, yet to be identified subunit. IL-27 shares gp130 with its specific receptor unit. IL-31 receptor is composed of gp130-like receptor (GPL) and OSMRβ.

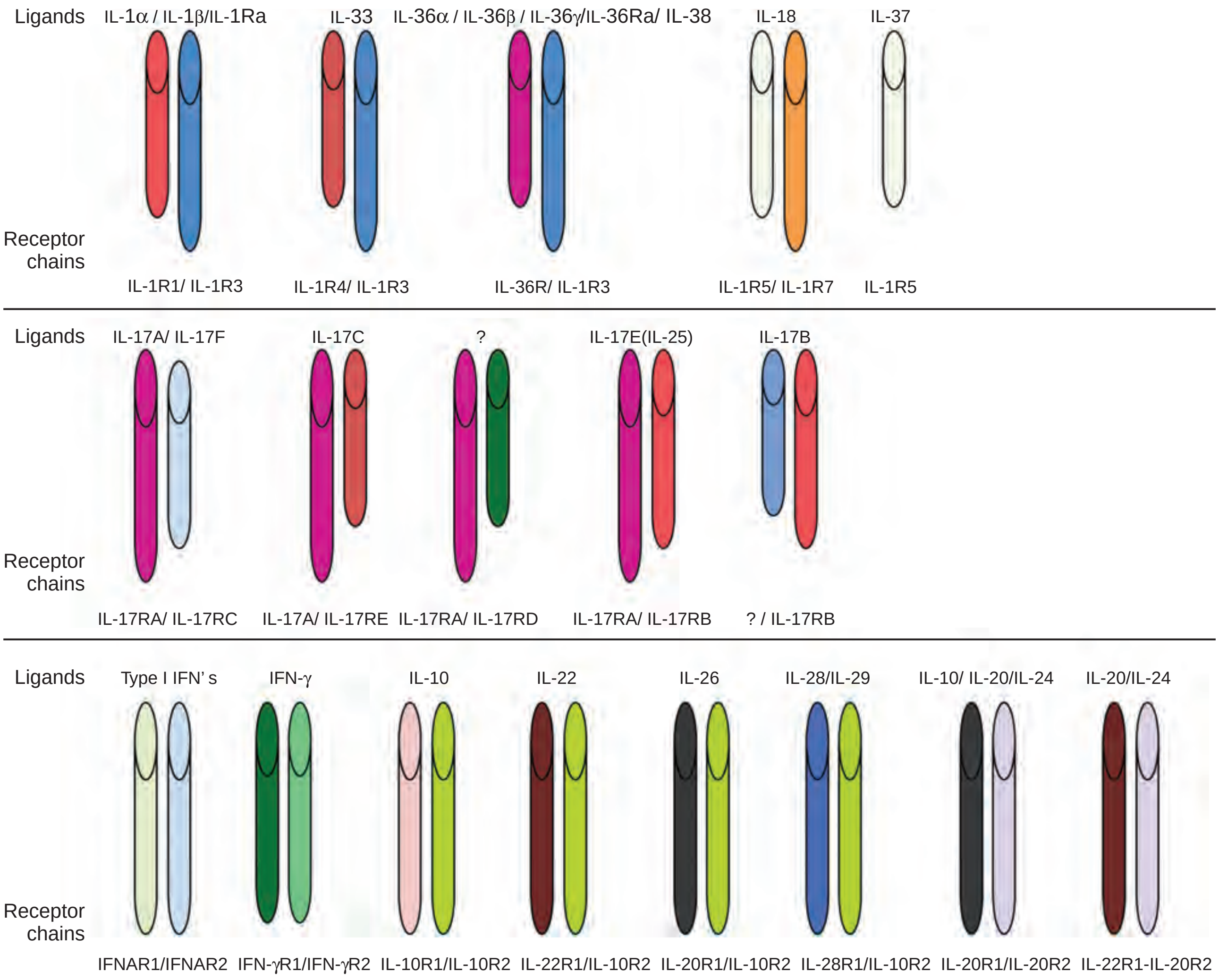

Ligands
IL-1α / IL-1β/IL-1Ra
IL-33
IL-36α / IL-36β / IL-36γ/IL-36Ra/ IL-38
IL-18
IL-37
Receptor chains
IL-1R1/ IL-1R3
IL-1R4/ IL-1R3
IL-36R/ IL-1R3
IL-1R5/ IL-1R7
IL-1R5
Ligands
IL-17A/ IL-17F
IL-17C
?
IL-17E(IL-25)
IL-17B
Receptor chains
IL-17RA/ IL-17RC
IL-17A/ IL-17RE
IL-17RA/ IL-17RD
IL-17RA/ IL-17RB
? / IL-17RB
Ligands
Type I IFN' s
IFN-γ
IL-10
IL-22
IL-26
IL-28/IL-29
IL-10/ IL-20/IL-24
IL-20/IL-24
Receptor chains
IFNAR1/IFNAR2
IFN-γR1/IFN-γR2
IL-10R1/IL-10R2
IL-22R1/IL-10R2
IL-20R1/IL-10R2
IL-28R1/IL-10R2
IL-20R1/IL-20R2
IL-22R1-IL-20R2

Figure 11.2 SCHEMATIC DIAGRAMS OF INTERLEUKIN (IL)-1, IL-17, AND TYPE II CYTOKINE FAMILY MEMBERS AND RECEPTORS. IL-1 family and receptors. The IL-1 family is associated with acute and chronic inflammation and plays an essential role in the host response to infection. IL-1α and IL-1β share the same receptor. IL-33 and the IL-36 subfamily share with IL-1 and the IL-1R3 receptors *(top panel)*; IL-17 family and receptors. IL-17 secretion defines the T helper 17 (Th17) cells that mediate host defensive mechanisms to various infections and that are involved in the pathogenesis of many autoimmune diseases. The five IL-17 receptors are not homologous to any known receptors and show considerable sequence divergence. They harbor extracellular domains composed of fibronectin type III domains and cytoplasmic SEFIR (a.k.a. SEF/IL-17R) domains that show loose homology to Toll–IL-1R domains *(middle panel)*; and type II cytokine receptor family. These include receptors for interferons (IFNs) and the IL-10 family *(lower panel)*.

in maintaining tertiary receptor structures without being involved in cytokine interactions. A 200-amino-acid region evolutionarily derived from a tandem of two ancestral fibronectin-like domains were named hematopoietin receptor domain or cytokine-binding homology region (CHR) because they mediate interactions with cytokines. α-Receptors of IL-2 and IL-15 of the γc family are atypical cytokine receptors; they do not contain a CHR, but rather they contain sushi domains. Two conserved Box 1/ Box 2 regions are located in the proximal intracytoplasmic segment (Fig. 11.3). By contrast, type II cytokine receptors contain two cysteine doublets (C2-C2) located in the C-terminal end of both fibronectin-derived domains. They retain Box 1/2 regions but lack a tryptophan-serine-x-serine-tryptophan motif. Both types of receptors bind ligands that display common spatial four α-helix bundle organizations; they use intracellular signaling mediators of Janus kinase (JAK) and signal transducer and activator of transcription (STAT) families.[5] Type I and II cytokine receptors represent homogeneous structural groups of proteins. Sequence homology is noted in limited numbers of cases, including for GH/prolactin (PRL) and for the IL-6 families. Evidence of the common derivation of cytokines is observed in a common four-helix bundle structures, in addition to similar intron-exon relationships and clustering observed for certain cytokine genes, such as genes of the IL-4 family.[8] Receptors can be composed of dimers of a single chain G-CSFR, EPO receptor (EPOR), or TPOR (c-MPL) or are heterodimeric with a common signaling subunit and a unique ligand-binding chain. Heterodimeric receptors are grouped into families based on sharing a common β-chain GM-CSFRα, IL-3Rα, IL-5Rα, or sharing a gp130 receptor (IL-6Rα, leukemia inhibitory factor [LIF] receptor β, ciliary neurotrophic factor receptor α [NTFRα], IL-11Rα IL-12R, IL-23R, oncostatin M receptor α) or sharing a common γ-chain (IL-2Rα, IL-2Rα, IL-4Rα, IL-7Rα, IL-9Rα, IL-13Rα, IL-15Rα, and IL-21Rα) (see Fig. 11.1).[5]

CYTOKINES AND THEIR MODIFYING INFLUENCES

Many CSFs have been implicated in the pathobiology of both hematopoietic and nonhematopoietic disorders or have been used

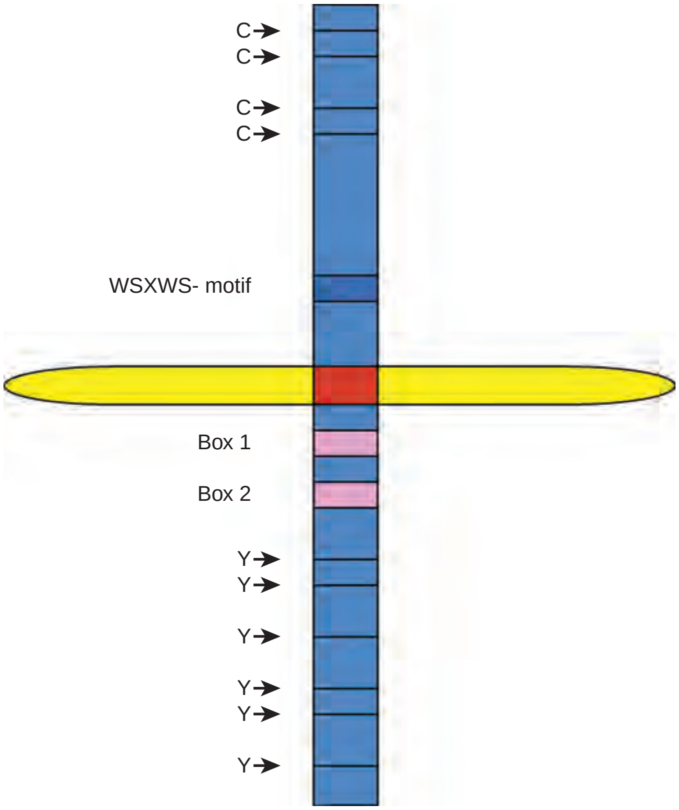


Figure 11.3 CYTOKINE RECEPTOR SUPERFAMILY. The general structure of cytokine receptor superfamily. In the extracellular cytokine receptor module, four conserved cysteine residues exist and are involved in disulfide bonds. A WSXWS (Tre, Ser, any, Tre, Ser) motif that is essential for receptor processing, ligand binding, and activation of the receptor is also located in the extracellular domain. In the intracellular portion, two short domains termed *Box 1* and *Box 2* are important for Janus kinase (JAK) binding. Tyrosine residues are present on the intracellular part to be phosphorylated upon receptor activation. Yellow oval represents the plasma membrane.

after production by recombinant DNA technology to treat patients. Cytokines are involved in regulation of hematopoiesis at the level of HSC, HPC, and more mature blood or other accessory cells. Accessory cells include stromal cells (which are themselves very heterogenous), endothelial cells, cells belonging to lymphoid lineages (T cells, B cells, natural killer [NK] cells, NKT cells), and innate immune cells (monocytes/macrophages, neutrophilic myeloid cells, dendritic cells [DCs]). Cell regulation is not necessarily easily dissectible, but strides have been made in elucidating these intricate cytokine-cell interactions. Cytokine receptor signaling has been reviewed.[3,5,8] Cytokines are involved in developmental processes during embryogenesis, through neonatal, and into adult life. They can fight infections and elicit immune responses. Redundancy in cytokine(s), their receptors, and actions are probably nature's way to protect the body against unwanted insults that may interfere with specific cytokines and their protective actions. ILs and chemokines are part of the cytokine superfamily of molecules. ILs, chemokines, and their receptors are covered in this chapter, as will other biologically active proteins that influence hematopoiesis. Cytokines are secreted by leukocytes, as well as other cells. They act on, but are not limited to, hematopoietic cells to regulate blood cell production; this includes effects on immune cells and during inflammation. Biologically active proteins influence in vitro and in vivo proliferation, differentiation, and survival/apoptosis (programmed cell death) at the level of HSC, HPC, and more mature cells of the hematopoietic system. It is clear that activities of these proteins can be modified by enzymatic cleavage, such as that mediated by dipeptidylpeptidase (DPP) 4 (a.k.a. CD26), and also in context of oxygen tensions that are vastly different in vivo (~1% to 5% O_2 in BM, and also low in blood and other tissues, from that of the ambient air conditions of ~21% O_2 in which most investigators have studied the in vitro and ex vivo activities of these growth regulators). These potent cytokines/chemokines/other growth regulators and their receptors are discussed throughout the chapter. It is not possible to truly understand the action(s) of cytokines and other growth-modulating factors in absence of these modulating/modifying influences on cytokine activities.

Importantly, cytokine-cell interactions can become dysregulated and induce or be involved in pathologic situations. Examples of some adverse pathologic effects of cytokines include the "cytokine storm" elicited following chimeric antigen receptor (CAR) T-cell therapy, during inflammation,[9] and after infection with viruses, such as SARS-CoV-2 (see Chapters 26, 67, 68, 79, 90, and 152).[10–13] Thus cytokine-cell interactions present a "double-edged sword" for both "good" (physiologic) and "bad" (pathologic) effects. Specific information on cytokines, chemokines, iron-binding proteins, other biologically active proteins, and growth regulation and their receptors follows.

SPECIFICS OF GROWTH-MODULATING FACTORS

There are many cytokines, including CSFs, ILs, and chemokines, which are enumerated as follows.

Colony-Stimulating Factors

Much of the original background on CSFs through the mid to late 1980s has been reviewed,[14–17] but it is important to understand that the purification and respective cloning of the ligands and receptors of GM-CSF,[18–23] G-CSF,[24,25] IL-3,[26–33] M-CSF,[34–45] EPO,[46–51] and TPO[52–55] have led to the preclinical analyses of their biologic activities in vitro and in vivo,[56–75] and the eventual evaluation of their effects in humans. For example, EPO has been used to enhance erythropoiesis and production of erythrocytes in humans under a variety of clinical scenarios,[76,77] GM-CSF, G-CSF, and TPO have been used to accelerate production of myeloid cells,[78–89] and G-CSF has also been used to mobilize HSC and HPC for eventual collection as autologous and allogeneic stem cell grafts.[90,91]

Interleukins

The IL family of cytokines now includes IL-1 through IL-38/40,[92–99] with the first IL, IL-1, purified to homogeneity in 1977.[94] Examples of some of the actions of the ILs and the cell(s) that produce them are summarized in Table 11.1. ILs are placed in different families based on sequence homology, receptor similarities, and their functional activities. Some of the IL receptors are shown in Fig. 11.4.[93] Many ILs, as do most members of the cytokine family, have redundant activities and have multiple effects on immune cells and hematopoiesis. Reviews on the IL-1,[94,100] IL-2,[101] IL-6,[95,96] IL-10,[97] IL-12,[98] and IL-17[99] families present in-depth information on many of the known IL molecules. Examples of the hematopoietic actions of some of the ILs from our lab and others, such as IL-1,[102–106] IL-3,[107–109] IL-4,[110,111] IL-5,[112] IL-6,[113–120] IL-7,[121,122] IL-8 (also known as the chemokine CXCL8),[123] IL-9,[124–126] IL-10,[127–129] IL-11,[130] IL-12,[131] IL-17,[132] IL-18,[133] IL-20,[134] IL-21,[135,136] IL-26,[137] IL-31,[138] IL-32,[139–142] and IL-33,[143–147] have been reported. The receptor for IL-33 is known as suppressor of tumorigenesis (ST2) which is primarily elevated during graft-versus-host disease (GVHD) and is a biomarker associated with a poor prognosis.[148–153] IL-33 signaling is also associated with hematologic malignancies, autoimmune disorders, allergic inflammation, and gastrointestinal cancers.[154–158] The ILs work in combination with other ILs, as well as other cytokines, to regulate hematopoiesis in an additive to synergistic fashion.

TABLE 11.1 Interleukins

Interleukin (IL)	Produced By	Actions
IL-1	Macrophages, B cells, endothelium, fibroblasts, astrocytes	Activates lymphocytes, stimulated macrophages; increases leukocyte/endothelial adhesion
IL-2	T cells	T and B cells proliferation and differentiation; activates NK cells antibody production
IL-3	T cells	Acts as a multi-CSF
IL-4	CD4$^+$ T cells (Th2)	Acts on T and B cells
IL-5	CD4$^+$ T cells (Th2)	Activates eosinophils and production of innate immune cells
IL-6	T and B cells, fibroblasts, macrophages	B-cell differentiation
IL-7	BM stromal cells	Acts on pre-B cells and T cells; stimulates B- and T-cell proliferation
IL-8	Monocytes and fibroblasts	Targets include mature myeloid cells
IL-9	Th9, Th2, Th17, Mast, NKT and TREG cells	Enhances survival of T cells and activates mast cells
IL-10	Th2 cells	Primary targets are Th1 cells; down regulates Th17 cells, and inhibits macrophage IL-12 production
IL-11	BM stromal cells and fibroblasts	Actions on hematopoietic progenitors and osteoclasts
IL-12	Monocytes	Induces Th1 cells, and T cells, and NK cell IFN-γ production
IL-13	Th2, NKT, and mast cells	Acts on monocytes, epithelial cells, and B cells
IL-14	T cells	Activates B cell proliferation and inhibits secretion of immunoglobulins
IL-15	Monocytes, epithelium	Acts on T cells and activated B cells
IL-16	CD8$^+$ T cells and eosinophils	CD4$^+$ T-cell chemoattractant
IL-17	Th17 cells	Acts on epithelial and endothelial cells; causes release of cytokines
IL-18	Macrophages	Cofactor in Th1 cell induction; induces IFN-γ and enhances NK cell activation
IL-19	Th2 cells, antiinflammatory molecules	Antiinflammatory molecule involvement
IL-20	Immune cells and activated epithelial cells	Plays a role in cellular communication
IL-21	NK and CD4$^+$ T cells	Acts on innate and adaptive immune cells, and erythroid progenitor cells
IL-22	T cells and other cells in the innate and acquired immunities	Inhibits IL-4 production
IL-23	Macrophages and dendritic cells	Maintenance of IL-17–producing T cells
IL-24	Monocytes and T and B cells	Involved in wound healing and protects against bacterial infections
IL-25	Dendritic cells	Th2 cells and their production of IL-4 and IL-13
IL-26	Th17 cells	Causes IL-10, IL-1β, IL-6, and IL-8 production
IL-27	T cells	Proinflammatory molecule
IL-28	TREGs	Acts on keratinocytes and melanocytes
IL-29	Virus-infected cells, dendritic cells, TREGs	Upregulates viral protective responses
IL-30	Monocytes in response to Toll-like receptor agonists such as bacterial lipopolysaccharides	Acts on many hematopoietic cells
IL-31	Th2 and dendritic cells	Acts as proinflammatory and chemotactic factor
IL-32	NK cells and monocytes	Induces cytokine production (IL-6, IL-1β) and inhibits IL-15 production
IL-33	Mast cells and Th2 cells	Mediates Th2 responses
IL-34	Phagocytes and epithelial cells	Enhances IL-6 production and is involved in differentiation of antigen-presenting cells
IL-35	Regulatory B cells	Immune suppressor
IL-36	Phagocytes	Acts on T and NK cells
IL-37	Phagocytes	Regulates innate immunity and causes immunosuppression
IL-38	Placenta, B cells, etc.	Acts on T cells, inhibits IL-17 and IL-22 production
IL-39	B cells	Acts on differentiation and expansion of neutrophils
IL-40	BM, fetal liver, activated B cells	Involved in development of humoral immune responses

BM, Bone marrow; *CSF*, colony-stimulating factor; *IFN*, interferon; *NK*, natural killer; *Th*, T helper; *TREG*, T regulatory.

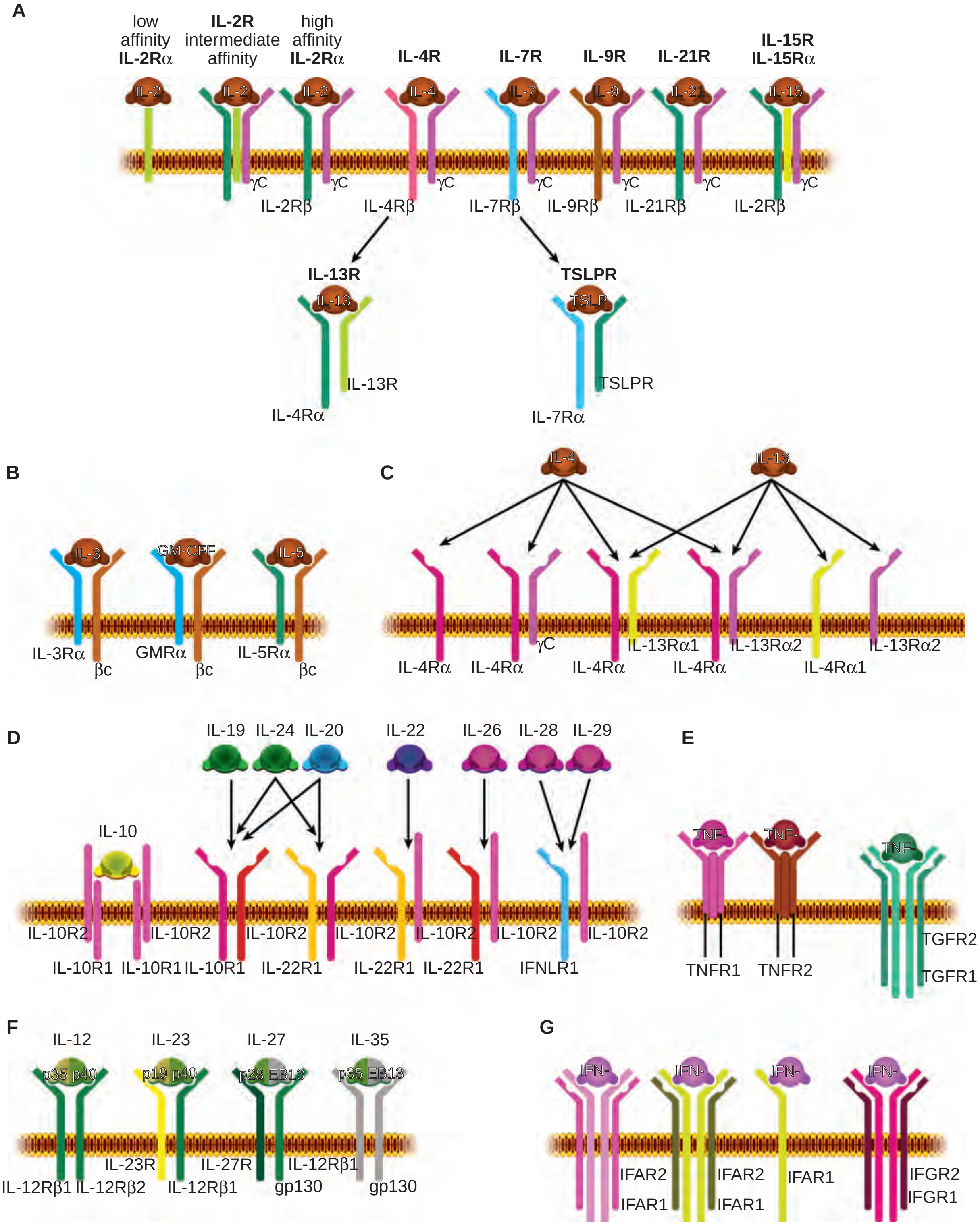

A
low affinity IL-2Rα
IL-2R intermediate affinity
high affinity IL-2Rα
IL-4R
IL-7R
IL-9R
IL-21R
IL-15R IL-15Rα
IL-2
IL-2
IL-2
IL-4
IL-7
IL-9
IL-21
IL-15
γC
γC
γC
γC
γC
γC
γC
IL-2Rβ
IL-4Rβ
IL-7Rβ
IL-9Rβ
IL-21Rβ
IL-2Rβ
IL-13R
TSLPR
IL-13
TSLP
IL-13R
IL-4Rα
TSLPR
IL-7Rα
B
IL-3
GM-CFF
IL-5
IL-3Rα
βc
GMRα
βc
IL-5Rα
βc
C
IL-4
IL-13
γC
IL-4Rα
IL-4Rα
IL-4Rα
IL-13Rα1
IL-4Rα
IL-13Rα2
IL-4Rα1
IL-13Rα2
D
IL-19
IL-24
IL-20
IL-22
IL-26
IL-28
IL-29
IL-10
IL-10R2
IL-10R2
IL-10R2
IL-10R2
IL-10R2
IL-10R2
IL-10R2
IL-10R1
IL-10R1
IL-10R1
IL-22R1
IL-22R1
IL-22R1
IFNLR1
E
TNF-
TNF-
TNF-
TGFR2
TGFR1
TNFR1
TNFR2
F
IL-12
IL-23
IL-27
IL-35
p35 p40
p19 p40
p28 EB13
p35 EB13
IL-23R
IL-27R
IL-12Rβ1
IL-12Rβ1
IL-12Rβ2
IL-12Rβ1
gp130
gp130
G
IFN-
IFN-
IFN-
IFN-
IFAR2
IFAR1
IFAR2
IFAR1
IFAR1
IFGR2
IFGR1

Figure 11.4 CYTOKINE RECEPTORS. (A) Receptors for the interleukin (IL)-2 family (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21); (B) receptors for IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF); (C) receptors for IL-4 and IL-13; (D) IL-10 receptor family members (IL-10, IL-19, IL-20, IL-24, IL-26, IL-28, IL-29); (E) tumor necrosis factor (TNF)-α (TNFR1, TNFR2) and transforming growth factor (TGF)-β (TGF-βR1, TGF-βR2) receptors; (F) IL-12 receptor (IL-12 β1 plus IL-12Rβ3), IL-23 receptor (IL-12Rβ plus IL-23R); and (G) interferon (IFN)-α and IFN-β bind IFNAR1 plus IFNAR2, and IFN-β also binds R1 plus IFN-γ R2 heterodimers. *(From Akdis M, Aab A, Altunbulakli C, et al. Interleukins (from IL-1 to IL-38), interferons, transforming growth factor β, and TNF-α: Receptors, functions, and roles in diseases. J Allergy Clin Immunol. 2016;138(4):984–1010, Fig. 1.)*

Chemokines and Chemokine Receptors

Chemokines, originally considered chemotactic cytokines, are a large family of cytokines that usually signal through G protein–coupled heptahelical receptors (Table 11.2 and Fig. 11.5).[159–161] There is redundancy within the chemokine family, with some chemokines binding multiple chemokine receptors and, in more rare occasions, nonchemokine receptors, and some chemokine receptors binding multiple chemokines. Fig. 11.6 diagrammatically denotes the many functions of chemokines and chemokine receptors.[159] Chemokines can act as monomers but can form homodimers,[162] heterodimers, and higher aggregates. Specific chemokines and their receptors have been implicated in various phases of HSC regulation.[163–166] They can act as positive and negative regulators of HSC and HPC and have been implicated in the survival, proliferation, and mobilization of these cells.[162,167–197] For example, IL-8/CXCL8, which acts through the chemokine receptors CXCR1 and CXCR2, has been implicated not only in the negative regulation of hematopoiesis,[176] but CXCL8 and CXCR2 have been implicated in leukemia/MDS.[195–197] Interestingly, DEK, a heterochromatin remodeling agent that has cytokine-like activity, can bind and signal through the chemokine receptor CXCR2 and can compete with IL-8/CXCL8, as well as macrophage inflammatory protein (MIP)-2, at the level of CXCR2.[196,197] Extracellular DEK potently enhances cytokine-induced ex vivo expansion of mouse BM and human cord blood (CB) HSCs,[196] plus has other effects.[198,199] Another well-recognized chemokine and its receptor is SDF-1/CXCL12 and CXCR4, respectively.[179–199] stromal cell-derived factor (SDF)-1/CXCL12 is involved in normal and leukemic HSC/HPC survival, proliferation, chemotaxis, migration, and mobilization. SDF-1/CXCL12 also binds to CXCR7,[192] but CXCR4 appears to be the main hematopoietic signaling receptor for SDF-1/CXCL12. Blocking of CXCR4 by the CXCR4 antagonist, AMD3100/plerixafor, has been used to induce the mobilization of peripheral blood HSC and HPC and to synergize with G-CSF in this mobilization.[90,187] Chemokines are affected by a number of posttranslational modifications,[159] including by DPP4, which is discussed later in this review. They are inducible by several different stimuli and are involved and are players in response to infection and inflammation.[159,160] There are also viral genes that encode chemokine receptors.[200] Chemokine receptors can also regulate infectivity to viruses. For example, individuals with specific mutations in CXCR5 are immune or less susceptible to infection with human immunodeficiency virus (HIV), but thus far only very few patients with HIV have been “cured” by allogeneic stem cell transplantation using donor cells from stem cell donors with this specific variant of mutated CXCR5.

The complex network of cytokines and chemokines and their overlapping and diverse effects on a variety of cellular processes is truly incredible and goes beyond regulation of normal cells.[201–207] Although redundant chemokine/chemokine receptor interactions are likely the body’s way to protect itself, this “protection” can elicit serious consequences. Many chemokines and their receptors are part of the triggered cytokine release syndrome (CRS), which represents a side effect following CAR-T-cell therapy and viral infections, such as that induced by SARS-Cov-2 during COVID-19 infections.[208–214] These events lead to excessive elaboration of chemokines and other cytokines and their receptors, representing the “double-edged sword” discussed earlier in terms of health benefits and disease-related problems. Greater knowledge of the production and actions of chemokines, and their intracellular signaling cascades that are induced through chemokine and cytokine receptors, is crucial for future benefits for normal cell regulation and their modification and to dampen their adverse effects during disease.

Role of Nuclear Factor-κB in Cytokine and Chemokine Gene Regulation, Inflammation, Immunity, and Cancer

NF-κB (nuclear factor kappa-light chain enhancer of activated B cells) is a protein complex controlling transcription of DNA, as well as cytokine production and cell survival.[215] NF-κB is involved in cellular responses to stress, cytokines, irradiation, and bacterial or viral antigens. Aberrant NF-κB regulation is associated with cancer, inflammation, autoimmune diseases, septic shock, viral infection, and disordered development of immune cells. In an inactivated state, NF-κB is located in the cytosol with inhibitory protein IκBα. Through a variety of extracellular signals including cytokine and chemokine, the enzyme IκB kinases is activated, which then phosphorylate IκBα, resulting in ubiquitination dissociation of IκBα from NF-κB and degradation of IκBα by the proteasome. Activated NF-κB then translocates to the nucleus and binds to specific sequences of DNA termed response elements. The DNA/NF-κB complex recruits other proteins. There are numerous NF-κB target genes that include cytokines, chemokines, and their modulators (see https://www.bu.edu/nf-kb/gene-resources/target-genes/). Upstream signal transduction of NF-κB activation has been described.[216] Reviews and articles are reported on a role for NF-κB in cytokine gene regulation,[217] chemokine gene transcription, and tumor growth,[218] inflammation, cancer, multiple myeloma, and thrombosis.[218–222] Many inhibitors of NF-κB signaling have been described.[223] Overall, the impact of NF-κB on cytokines and chemokines and resultant physiologic and pathologic effects cannot be underestimated. With the multitude of cytokines and other factors that can influence NF-κB signaling, it is not at all surprising that NF-κB can regulate hematopoiesis. NF-κB has been shown to play an important role in the differentiation of HSCs and HPCs down both the myeloid and lymphoid pathways. The Rel/NF-κB transcription factors are mainly composed of five members (c-Rel, p65/RelA, RelB, NFKB1/p50, NFKB2/p52). P65 and p50 are ubiquitous proteins expressed in all hematopoietic lineages. However, the distinct expression pattern of the other Rel/NF-κB factors in HPCs indicates their importance in HPC differentiation. Lymphocytes, monocytes, granulocytes, and erythrocytes express c-Rel. RelB is mostly expressed in DCs and lymphocytes. DCs, macrophages, and lymphocytes express p52. Using mouse knockout animal models, investigators have shown the importance of these factors in regulating hematopoietic differentiation.

Engineered Cytokines and Role of Cytokines and Other Reagents for Preclinical and Clinical Ex Vivo Expansion of Hematopoietic Stem Cells and Hematopoietic Progenitor Cells

Cytokines and/or chemokines when combined have additive to synergistic effects on proliferation of HPCs.[224–233] This is especially so for the potent costimulating cytokines SCF (which acts through the c-kit receptor)[234–249] and FL (which acts through the FL3 receptor).[250–259] Efforts have been made to specifically engineer cytokines and chemokines for greater efficacy.[260,261] This includes the GM-CSF/IL-3 fusion protein, PIXY32,[262,263] which has been used in a clinical trial to promote hematopoietic recovery following chemotherapy-induced multilineage myelosuppression in patients with sarcoma,[264,265] as well as IL-3/EPO,[266] and IL-8/PF4[174] fusion. These have had only limited clinical testing.[265] By contrast, molecularly engineered forms of small molecule agonists of the thrombopietin receptor (eltrombopag and romiplostim) are currently used to treat patients with immune thrombocytopenic purpura as well as aplastic anemia (see Chapters 31 and 128).

Enhancement of ex vivo expansion of HSCs/HPCs for both preclinical[267–280] and clinical use[272–285] is an ongoing area of research. Current clinical trials on ex vivo expansion have been described.[285] There are currently three sources of cells that have been used for clinical HCT. This includes BM, cytokine- and/or other reagent-induced mobilized peripheral blood, and umbilical cord blood. There have been a number of preclinical studies to expand numbers of CB HSCs and HPCs ex vivo. Some such as SR1, UM171, nicotinamide, prostaglandin $(PGE)_2$ analog, and other reagents are currently in clinical trials.[282–286] None of the ex vivo expansion procedures work without

TABLE 11.2 Chemokines

Name(s)	Human Receptor(s)	Mouse Receptor(s)
CCL Chemokines		
CCL1 (I-309)	CCR8, DARC	CCR8
CCL2 (MCP-1, MCAF)	CCR2, CCR4, CCR11, D6, DARC	CCR2, CCR4, D6, DARC (JE = mouse ligand along with MCP-1)
CCL3 (MIP-1α, LD78)	CCR1, CCR4, CCR5, D6	CCR1, CCR4, CCR5, D6
CCL3L1 (LD78β)	CCR1, CCR3, CCR5, D6	___
CCL3L3 (LD78β)	CCR1, CCR3, CCR5	___
CCL4 (MIP-1β)	CCR1, CCR5, CCR8, D6	CCR5, CCR1
CCL4L1	CCR1, CCR5	___
CCL5 (RANTES)	CCR1, CCR3, CCR5, CCR4, D6, DARC	CCR1, CCR3, CCR5, CCR4
CCL6	___	CCR1
CCL7 (MCP-3)	CCR1, CCR2, CCR3, D6, DARC	CCR1, CCR2, CCR3
CCL8 (MCP-2)	CCR1, CCR2, CCR5, CCR3, CCR11, D6, DARC	___
CCL9/10 (MIP-1γ)	___	CCR1, CCR3
CCL11 (Eotaxin)	CCR3, CCR5, D6, DARC	CCR3, DARC
CCL12 (MCP-5)	___	CCR2
CCL13 (MCP-4, CKβ)	CCR2, CCR3, CCR1, CCR5, CCR11, D6, DARC	___
CCL14 (HCC-1, MCIF, CKβ1)	CCR1, CCR3, CCR5, D6, DARC	___
CCL15 (MIP-1δ, MIP-1γ, LKN-1, MIP-5)	CCR1, CCR3	CCR1, CCR3
CCL16 (HCC-4, LEC, LMC)	CCR1, CCR2, CCR5, CCR8, H4, DARC	___
CCL17 (TARC)	CCR4, CCR8, D6, DARC	CCR4
CCL18 (PARC, CKβ7, MIP-4)	PITPNM3, CCR8, DARC	___
CCL19 (MIP-3β, ELC, Exodus-3)	CCR7, CCR11	CCR7, CCR11
CCL20 (MIP-3α, LARC, Exodus-1)	CCR6	CCR6
CCL21 (6CKine, Exodus-2)	CCR7, CCR11	CCR7, CCR11
CCL22 (MDC)	CCR4, D6	CCR4
CCL23 (MPIF, CKβ8, MIP3)	CCR1	CCR1
CCL24 (Eotaxin-2)	CCR3	CCR3
CCL25 (TECK, CKβ15)	CCR9, CCR11	CCR9, CCR11
CCL26 (Eotaxin-3, MIP-4α)	CCR3, CX$_3$CR1	___
CCL27 (CTACK, PESKY, Eskine)	CCR10	CCR10
CCL28 (MEK)	CCR3, CCR10	CCR10
CXC Chemokines		
CXCL1 (GRO-α, GRO1, NAP3 DCIP-1)	CXCR2, DARC	CXCR2, DARC
CXCL2 (GRO-β, MIP-2α, GRO2, MIP-2)	CXCR2, DARC	CXCR2, DARC
CXCL3 (GRO-γ, MIP-2β, KC)	CXCR3B, CXCR3, DARC	___
CXCL4L1 (PF4V1)	CXCR3B, CXCR3	CXCR3
CXCL5 (ENA-78)	CXCR2, DARC	___
CXCL6 (GCP-2)	CXCR1, CXCR2, DARC	CXCR1, CXCR2, DARC
CXCL7 (NAP2)	CXCR1, CXCR2	CXCR1, CXCR2
CXCL8 (IL-8, NAP-1, GCP-1)	CXCR1, CXCR2, DARC	___
CXCL9 (MIG)	CXCR3, CXCR3B, DARC	CXCR3
CXCL10 (IP-10)	CXCR3, CXCR3B, DARC	CXCR3
CXCL11 (I-TAC)	CXCR3, CXCR7, CXCR3B, DARC	CXCR3, CXCR7
CXCL12 (SDF-1)	CXCR4, CXCR7	CXCR4, CXCR7
CXCL13 (BCA-1, BLC)	CXCR3, CXCR5, DARC	CXCR5
CXCL14 (BRAK)	Unknown	Unknown
CXCL15 (Lungkine)	___	Unknown
CXCL16 (---)	CXCR6	CXCR6
CXCL17 (DMC)	Unknown	Unknown
LIX (---)	___	CXCR1, CXCR2, DARC

Continued

TABLE 11.2 **Chemokines, cont'd**

Name(s)	Human Receptor(s)	Mouse Receptor(s)
CX3C Chemokines		
CX3CL1 (Fractakine/Neurotactin)	CX3CR1	CX3CR1
C Chemokines		
XCL1 Lymphotactin, SCM-1α, ATAC)	XCR1	XCR1
XCL2 (SCM-1β)	XCR1	___

Adapted from R&D Systems Inc, Minneapolis MN. https://www.rndsystems.com/resources/technical-information/chemokine-nomenclature. Used with the permission of Bio-Techne.

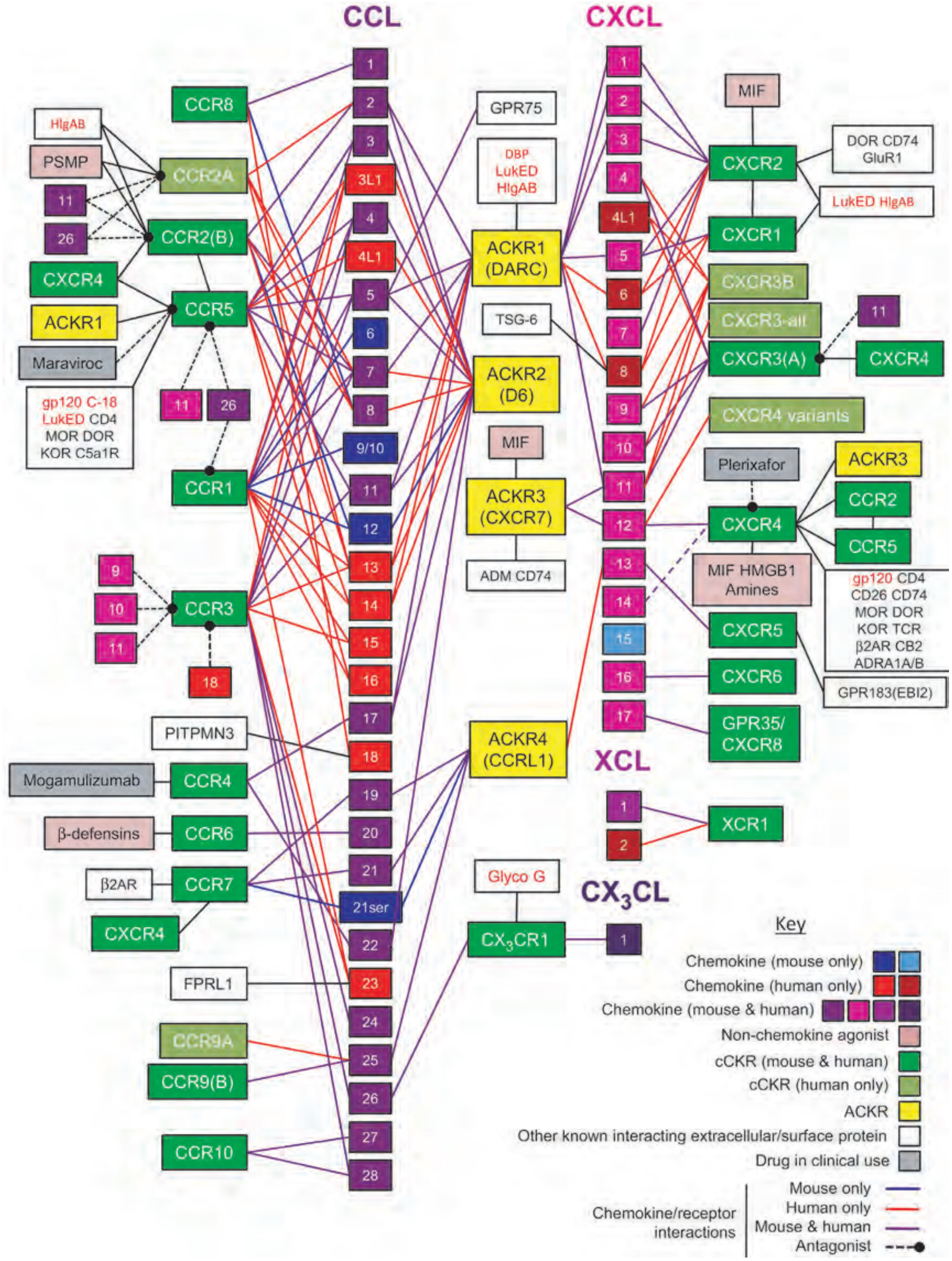

CCL
CXCL
1 2 3 3L1 4 4L1 5 6 7 8 9/10 11 12 13 14 15 16 17 18 19 20 21 21ser 22 23 24 25 26 27 28
1 2 3 4 4L1 5 6 7 8 9 10 11 12 13 14 15 16 17
CCR8
HlgAB
PSMP
CCR2A
11
26
CCR2(B)
CXCR4
ACKR1
CCR5
Maraviroc
gp120 C-18 LukED CD4 MOR DOR KOR C5a1R
11
26
CCR1
9
10
11
CCR3
18
PITPMN3
Mogamulizumab
CCR4
β-defensins
CCR6
β2AR
CCR7
CXCR4
FPRL1
CCR9A
CCR9(B)
CCR10
GPR75
DBP LukED HlgAB
ACKR1 (DARC)
TSG-6
ACKR2 (D6)
MIF
ACKR3 (CXCR7)
ADM CD74
ACKR4 (CCRL1)
Glyco G
CX3CR1
MIF
CXCR2
DOR CD74 GluR1
LukED HlgAB
CXCR1
CXCR3B
CXCR3-alt
11
CXCR3(A)
CXCR4
CXCR4 variants
Plerixafor
ACKR3
CCR2
CXCR4
CCR5
MIF HMGB1 Amines
gp120 CD4 CD26 CD74 MOR DOR KOR TCR β2AR CB2 ADRA1A/B
CXCR5
CXCR6
GPR183(EBI2)
GPR35/ CXCR8
XCL
1
2
XCR1
CX3CL
1
Key
Chemokine (mouse only) 🟦 🟦
Chemokine (human only) 🟥 🟥
Chemokine (mouse & human) 🟪 🟪 🟪 🟪
Non-chemokine agonist
cCKR (mouse & human) 🟩
cCKR (human only) 🟩
ACKR 🟨
Other known interacting extracellular/surface protein ☐
Drug in clinical use
Chemokine/receptor interactions
Mouse only
Human only
Mouse & human
Antagonist

Figure 11.5 MAMMALIAN CHEMOKINE RECEPTORS AND INTERACTIONS WITH CHEMOKINES AND KEY SECRETED CELL SURFACE AND PATHOGEN-ENCODED MOLECULES. *(From Hughes CE, Nibbs RJB. A guide to chemokines and their receptors. FEBS J. 2018;285[16]:2944–2971, Fig. 1.)*

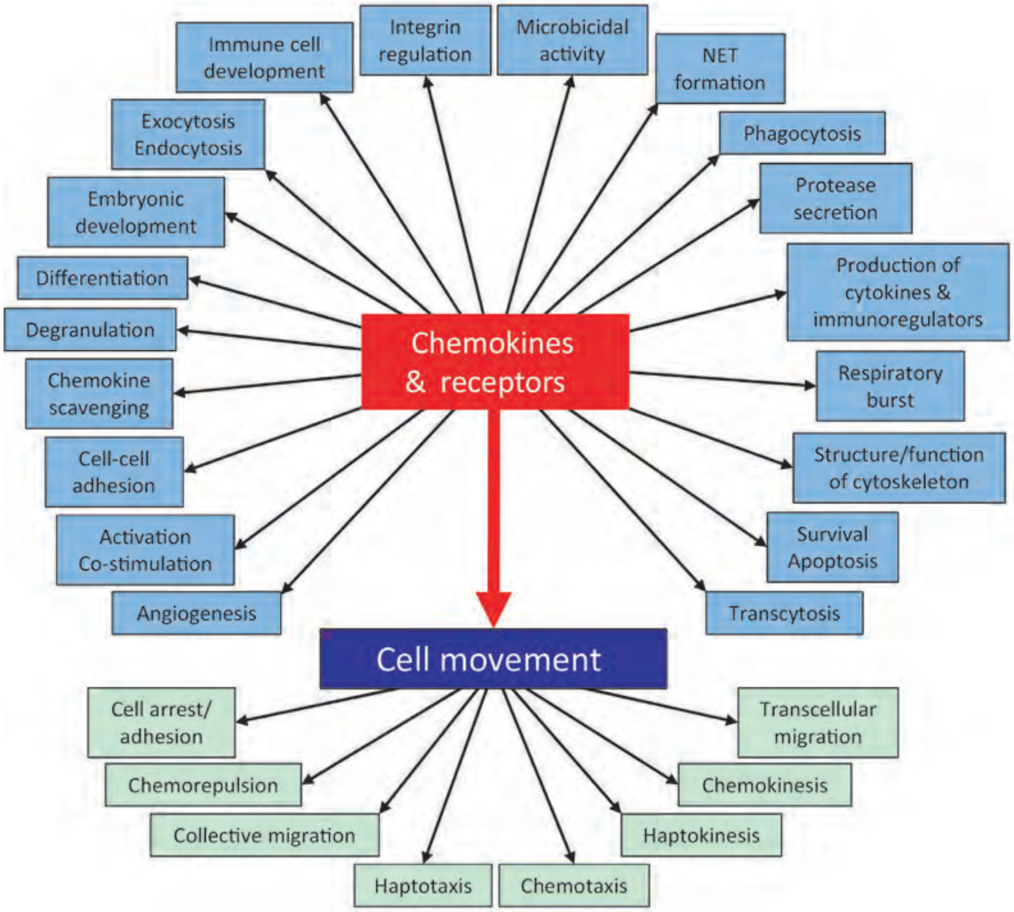

Immune cell development
Integrin regulation
Microbicidal activity
NET formation
Exocytosis Endocytosis
Phagocytosis
Embryonic development
Protease secretion
Differentiation
Production of cytokines & immunoregulators
Degranulation
Chemokines & receptors
Chemokine scavenging
Respiratory burst
Cell-cell adhesion
Structure/function of cytoskeleton
Activation Co-stimulation
Survival Apoptosis
Angiogenesis
Transcytosis
Cell movement
Cell arrest/ adhesion
Transcellular migration
Chemorepulsion
Chemokinesis
Collective migration
Haptokinesis
Haptotaxis
Chemotaxis

Figure 11.6 MULTIPLE CHEMOKINE/CHEMOKINE RECEPTOR FUNCTIONS. *(From Hughes CE, Nibbs RJB. A guide to chemokines and their receptors. FEBS J. 2018;285[16]:2944–2971, Fig. 2.)*

addition of combinations of cytokines such as SCF, TPO, and FL during the ex vivo culture period. Hence there is a need to add the reagent(s) of choice with SCF, TPO, and FL during the ex vivo culture period. Importantly, use of serum-free cultures and a short time of cell culture has benefits for potential use of the generated cells for clinical application. What is clear is that the most effective ex vivo expansion studies for clinical use is not yet known. Moreover, there are numerous other ex vivo expansion studies not yet tested clinically, and which of these may eventually be the procedure of choice for clinical use is as yet unknown.

OTHER GROWTH MODULATING PROTEINS/FACTORS

Iron-Binding Proteins

Although mainly known for their iron-binding capacity and influence on metabolism of iron, LF, transferrin, and H-ferritin also play a role in the regulation of hematopoiesis.[287–331] The iron-binding proteins LF,[295–312] transferrin,[313–315] and H-ferritin[316–331] have been implicated by in vitro and in vivo studies in the regulation of hematopoiesis. LF, originally named for being found in breast milk, has been shown to directly and/or indirectly suppress the production/release of CSFs from monocytes and macrophages and also to directly suppress hematopoiesis at the level of HPCs.[295–305] For LF suppressive activities to occur, the LF must be fully iron saturated.[295] Transferrin also suppresses release of growth factors but from subsets of lymphocytes.[314,315] H-ferritin has direct suppressive actions on HPCs mediated by its ferroxidase activity, and the iron content of H-ferritin does not appear to be required for its myelosuppressive activity.[331] The receptors for LF and H-ferritin have not been definitively defined to date.

Transforming Growth Factor-β, Associated-Inhibin Family Members, Bone Morphogenic Protein, Tumor Necrosis Factor-α, and Interferons

Transforming Growth Factor-β

TGF-β isoforms 1, 2, and 3 are part of a family of factors that influence growth and differentiation of cells with the capacity to both inhibit and/or stimulate growth.[332,333] TGF-β was originally isolated as a factor that induced cellular transformation and anchorage-independent fibroblast cell line growth.[334] Its many functional activities have been described.[332–345] Intracellular signaling of TGF-β through its receptors has been reported.[332] Although involved in normal cell regulation, abnormalities of the signaling of TGF-β have been associated with a number of diseases, including cancer.[333] Mammalian genomes encode 33 polypeptides related to known TGF-β family polypeptides. These include, among others, the activins (activin and inhibin which are discussed later), bone morphogenetic proteins (BMPs 2 to 10),[346–348] growth and differentiation factors (GDFs 1, 3, 5, 6, 7, 9, 10, 11, and 15), and nodal. Numerous studies have assessed roles for TGF-β in both positive and negative regulation of hematopoiesis and the immune system.[333–345] Modulation of the effects of TGF-β has been explored to treat HSC defects and BM failure states including Fanconi anemia and myelofibrosis.[349]

Inhibins (Activin and Inhibin)

Inhibins, belonging to the TGF-β family of polypeptides, are heterodimeric proteins that consist of an inhibin α and inhibin β chain, whereas activins are homodimers of inhibin/activin β chains. There are different inhibin chains (α and β) that have roles in a variety of developmental, physiologic, and disease states.[350,351] Activin has enhancement activity for erythroid progenitor cells (e.g., burst-forming unit–erythroid [BFU-E]) in vitro and in vivo, whereas inhibin has suppressive effects on BFU-E in vitro and in vivo.[352–355]

Bone Morphogenetic Protein

BMPs,[346–348] members of the TGF-β family, are secreted extracellular matrix (ECM) proteins that regulate a wide variety of development processes. They have been associated with a number of human disease states and in stem cell and organ formation,[356] but their role in hematopoiesis has yet to be rigorously elucidated.

Tumor Necrosis Factor

TNF was originally named for its capacity to cause the necrosis of selected tumors; it is derived from lymphocytes and macrophages and is distinguishable from lymphotoxin (LT).[357,358] TNF is also a powerful cachexia-inducing factor associated with the devastating cachexic wasting side effects of the progression of cancer,[359] and a pathogenetic role for TNF-α was proposed for the development of cachexia, arthritis, and autoimmunity due to tristetraprolin deficiency.[360] When the gene encoding tristrapolin is disrupted in mice, the animals develop a severe syndrome of arthritis, autoimmunity, cachexia, dermatitis, and myeloid hyperplasia. The severe inflammatory syndrome which accompanies loss of tristetraprolin function in mice is in part the result of TNF-α messenger RNA (mRNA) stabilization and hypersecretion; TNF family members play numerous negative and positive regulatory roles in hematopoiesis—some modulated by ligand/receptor intracellular events that cause the release of a number of other cytokines, each with their different effector functions.[361–367] There are large numbers of the TNF and TNF receptor superfamily members including, but not limited to, the receptors: CD40, FAS, KD30, 4-1BB, CD27, RANK, and the ligands (L): CD40L, FasL, OXr40L, Light, 4-1BBL, CD276, LT, Tweak, April, RANKL, and TRAIL/Apo2L.[356]

Interferons

IFNs form another large family of related molecules. IFN were originally described as antiviral agents, but have since shown to have a large variety of actions that can have positive or negative effects on the hematopoietic and immune systems, through direct and indirect actions that are well documented.[367–372] Indirect effects include induction of the release of other biologically active proteins.[372] The inhibitory effects of IFN-α have been exploited to treat patients with MPNs (see Chapter 70).

Additional Growth Modulating Factors

There are a number of other proteins and their families that also regulate cell growth. These include, but are not necessarily limited to, angiopoietins,[373] angiopoietin-like molecules,[374] adipocytokines,[375] epidermal growth factor (EGF),[376] fibroblast growth factor (FGF) and its receptors including FGF23 and FGFR3,[377–381] glial cell line–derived neurotrophic factor (GDNF), nerve growth factor (NGF),[382] early growth response (EGR)-1 proteins,[383] vascular endothelial cell growth factor (VEGF),[384,385] macrophage-stimulating factor (MSF),[385] DEK,[196–198] oncostatin M,[386] macrophage migration inhibitory factor,[387,388] and pleiotrophin. Pleiotrophin (PTN), also known as heparin-binding brain mitogen, heparin-binding growth factor 8, neurite growth-promoting factor 1, heparin affinity peptide, and heparin-binding growth factor–associated molecule, is an 18-kDa growth factor with an affinity for heparin. PTN is encoded by the PTN gene and is structurally related to midkine and retinoic acid–induced heparin-binding protein (see https://en.wikipedia.org/wiki/Pleiotrophin).[389] PTN has numerous effects on hematopoiesis, including retention and self-renewal of HSCs in the BM vascular niche,[390] HSC maintenance and regeneration,[391] and involvement in regulation of HSC/leukemia stem initiating cells from patients with chronic myeloid leukemia.[392] Leptin (Lep) and LepR have also been implicated in stem cell regulation at the level of the BM microenvironmental niche,[393] and LepR-positive BM HSCs have a high level of engrafting capability.[393,394] Although some have been implicated in regulation of hematopoiesis, it is likely that others will eventually be shown to modify hematopoietic cell regulation, either directly at the level of HSCs and HPCs, or indirectly at the level of accessory cells (e.g., monocytes/macrophages, lymphocytes, DCs, stromal cells) through their induced production/release of other cytokines and chemokines that affect hematopoiesis.

Prostaglandin E

Although not a protein, PGE is a potent effector molecule and modulator of hematopoiesis and immunity.[395–405] PGEs are eicosanoids (lipid hormone–like signaling molecules) that have been shown to modulate many biologic processes relating to inflammation, such as cellular proliferation, survival, and mobilization, angiogenesis, fever, tumorigenesis, and mature immune cell function. Much of PGEs' ability to augment HSC, HPC, and mature immune cell function is through direct and indirect modulation of cytokine/chemokine secretion and/or function (e.g., IL-1, TNF-α, IL-12, IL-10, IFN-γ, and CXCL2/GROβ) and by regulating the surface expression of cytokine/chemokine receptors (e.g., CXCR4 and IL-1R1). PGE (e.g., PGE_2) has been evaluated in preclinical[404] and clinical settings.[286]

Enzymes, Matrix Metalloproteinases, and Dipeptidylpeptidase 4, as Modifiers of Cytokine/Chemokine Biologic Activities

There is a much-neglected but important role for enzymes in biologically altering proteins that influence hematopoiesis. Matrix metalloproteinases (MMPs) are members of the metzincin group of proteases best known for their ability to collectively degrade a wide variety of components of the ECM and basement membrane.[406,407] The ECM and basement membranes of tissues (e.g., the BM) are often sites for the sequestration of cytokines, chemokines, growth factors, and other bioactive molecules. Thus the ability of MMPs to degrade components of these extracellular structures allows for the release and/or activation of these cytokines/chemokines/growth factors (e.g., TGF-β), increasing their bioavailability and thus allowing for their participation in physiologic functions such as regulating hematopoiesis, immunity, inflammation, angiogenesis, bone remodeling, and tumorigenesis, among others. One pivotal role of MPPs in vivo is to cleave membrane-bound growth factors, chemokines, cytokines, and their receptors from the cell surface, allowing for the shedding of these factors (e.g., several members of the TNF family and IL-6R). Important hematopoietic cytokines and chemokines processed by MMPs include SDF-1, IL-8/CXCL8, IL-1β, and TNF.

Another important modifier of cytokine, chemokine, and growth factor function is the serine protease DPP4. DPP4 truncates proteins at the penultimate amino acid from the N-terminus of the protein.[408–413] This usually occurs when that amino acid is an alanine or proline. However, it can cause this truncation when the penultimate amino acid is also a serine, glycine, or valine but with lower affinity than for alanine or proline. DPP4 exists as a type II membrane protein (CD26) present on many cell types, including HSCs and HPCs, but also exists as a soluble protein due to MMP-mediated cleavage

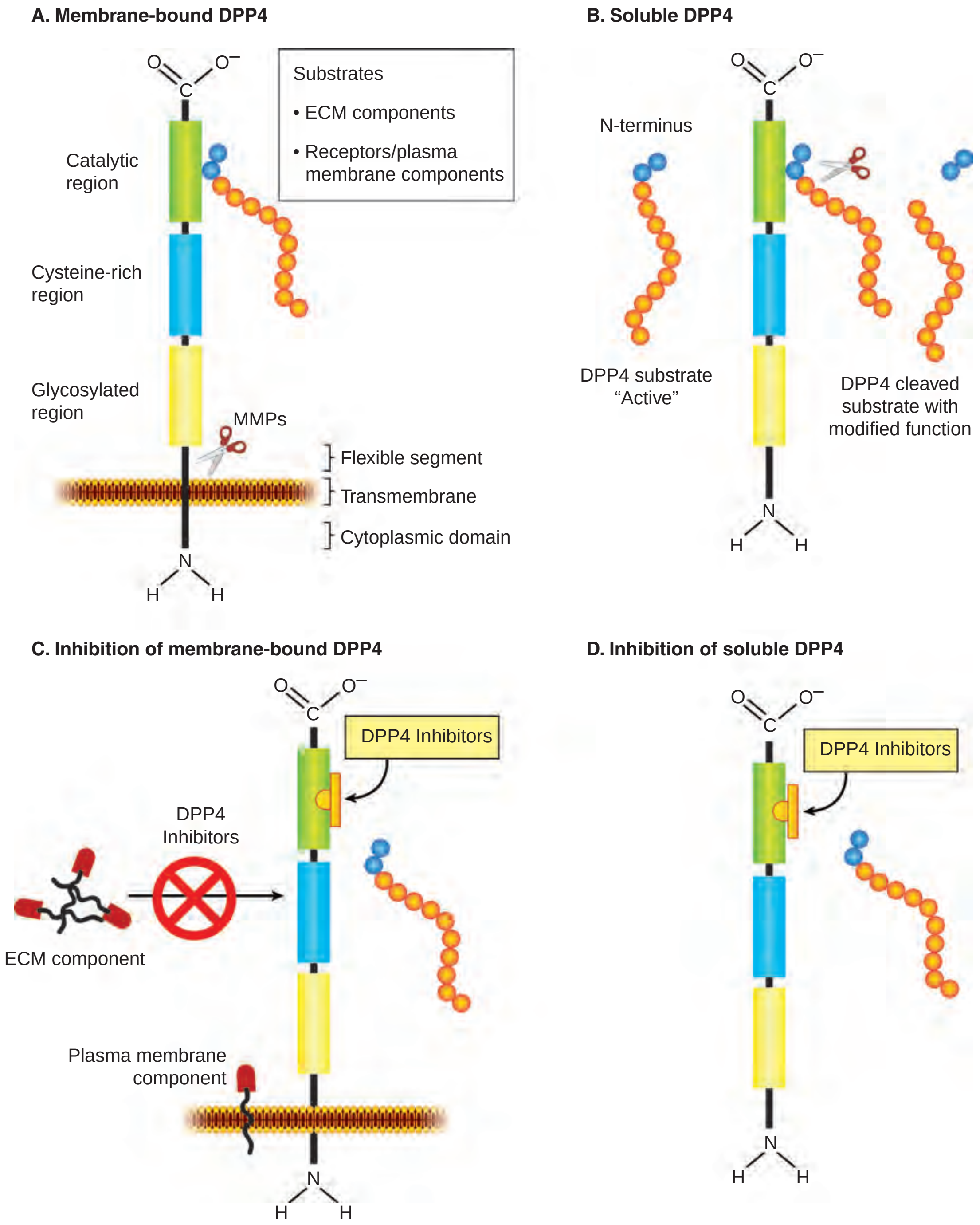

A. Membrane-bound DPP4
O
O−
C
Catalytic region
Cysteine-rich region
Glycosylated region
MMPs
Substrates
• ECM components
• Receptors/plasma membrane components
Flexible segment
Transmembrane
Cytoplasmic domain
N
H
H
B. Soluble DPP4
N-terminus
DPP4 substrate "Active"
DPP4 cleaved substrate with modified function
C. Inhibition of membrane-bound DPP4
DPP4 Inhibitors
DPP4 Inhibitors
ECM component
Plasma membrane component
D. Inhibition of soluble DPP4
DPP4 Inhibitors

Figure 11.7 SCHEMATIC REPRESENTATION OF DIPEPTIDYLPEPTIDASE (DPP)4. (A) Membrane-bound DPP4 consists of the C-terminal catalytic domain in which the enzymatic activity of DPP4 occurs, a cysteine-rich region domain in which the fibronectin and adenosine deaminase (ADA) binding sites reside, the glycosylated region domain, a flexible stalk in which matrix metalloproteinases (MMPs) can cleave DPP4, allowing for the shedding of the enzyme off the cell surface, a transmembrane region, and a cytoplasmic domain. DPP4 can transmit signals across cell membranes through its interaction with other membrane proteins. DPP4 can form tetramers with two soluble proteins or two membrane proteins through the ADA and/or fibronectin binding sites and possibly through binding of other protein/extracellular matrix (ECM) components other than fibronectin through specific interaction sites that have yet to be determined. Although DPP4 demonstrates a small amount of enzymatic activity in its monomeric form, experimental evidence suggests that for optimal DPP4 enzymatic activity, DPP4 must be in its more predominant form as a homodimer. (B) Circulating soluble DPP4 contains the same regions as the membrane-bound form of the protein; however, upon cleavage by MMPs at its flexible stalk, soluble DPP4 lacks its transmembrane and cytoplasmic domains. Soluble DPP4 can also initiate intracellular signaling pathways. This function appears to be independent of DPP4's catalytic domain and ADA-binding sites, and its mechanism of action remains poorly understood. (C) For membrane-bound DPP4, DPP4 inhibitors have been shown to inhibit both its ability to cleave substrates by binding to the catalytic site (as is the case with inhibitors such as sitagliptin, diprotin A, tripeptide ILE-PRO-ILE, etc.) and by blocking DPP4's interaction with ECM components and membrane-bound proteins. (D) For soluble DPP4, DPP4 inhibitors have been shown to block its ability to cleave DPP4 substrates, thus preventing the conversion of the substrates to their modified function.

of the cell surface (Fig. 11.7).[406,411] DPP4 shedding is enhanced under hypoxic conditions by specific cell types (i.e., smooth muscle cells), and this increase in DPP4 shedding is associated with increased expression of MMPs. The role of hypoxia in regulating soluble DPP4 concentrations in the BM microenvironment has not been examined.

Current strategies to enhance cord blood transplantation have been described.[414] Also in this strategy is the use of DPP4 inhibition such as sitagliptin.[415–418] Use of sitagliptin for CB transplantation not only resulted in enhanced time to neutrophil recovery but also to lowered GVHD than expected. Sitagliptin also decreased GVHD in patients receiving allogeneic G-CSF–mobilized peripheral blood.[419] A $CD26^+$ subset of polyfunctional $CD4^+$ T cells demonstrated potent antitumor activity.[420] It remains to be determined how these DPP4 inhibitory effects are being mediated, because DPP4 acts on numerous proteins.

There are a number of DPP4 inhibitors.[421] Inhibition of DPP4 on target donor cells[415] or in recipient mice[405] enhances engraftment of mouse BM cells in irradiated recipients. G-CSF and GM-CSF induce upregulation of DPP4, which downregulates SDF-1–induced chemotaxis and negatively regulates GM-CSF, G-CSF, IL-3, and EPO potency in stress conditions. Inhibitors of DPP4 include the orally active small molecule sitagliptin, which was first used to treat type 2 diabetes and subsequently used to enhance the time to engraftment of single unit CB grafts, thereby shortening the time to neutrophil recovery in patients with leukemia and lymphoma undergoing allogeneic stem cell transplantation.[416–418] Other DPP4 inhibitors that have shown efficacy in treating diabetes include saxagliptin, alogliptin, and linagliptin, among others.[410,412] There are many small molecule inhibitors of DPP4, including diprotin A and the tripeptide ILE-PRO-ILE. There are numerous cytokines/chemokines with DPP4-truncation sites or putative DPP4-truncation sites.[421] Examples of CSF proteins with DPP4 truncation sites whose biologic activities that are modified by DPP4 include GM-CSF, G-CSF, IL-3, TPO, and EPO. Of the CSF proteins,[421,422] M-CSF, however, does not have a DPP4 truncation site.[422] It has already been demonstrated that several of the CSF proteins with DPP4 truncation sites, such as GM-CSF, have decreased CSF activity when the protein is truncated by DPP4 as compared with the full-length CSF.[422] In addition, the DPP4-truncated CSF can downmodulate the activity of the full-length CSF.[422] These effects are at least in part due to the truncated CSF binding to these CSF receptors with higher affinity than the full-length CSF. This scenario also occurs with myelosuppressive chemokines, with the DPP4-truncated chemokine having less myelosuppressive activity than that of the full-length chemokine, thus blocking the myelosuppressive effects of the full-length chemokine.[423] Thus inhibition of DPP4 activity can enhance the activity of cytokines/chemokines, by both preventing truncation of these growth regulators and ablating the interfering effects of the truncated growth regulation from binding to and downmodulating the effects of full-length factors at their receptors.

Hence it is important to understand how DPP4 and other enzymes such as MMPs may influence the activity of biologically active proteins with regards to regulation of hematopoiesis. The totality of these interactions and effects have not yet been elucidated. Many studies, especially in clinical trials, assess the serum levels of cytokines/chemokines using enzyme-linked immunosorbent assay (ELISA), yet none of these immunoassays is able to distinguish full-length from truncated proteins. This may thus limit the value of these quantitative protein measurements. Until there are quantitative assays that distinguish full-length from DPP4 or other enzyme-truncated chemokines/cytokines, we cannot be sure how best to interpret such data based upon ELISAs for human cytokines. These limitations can be overcome by use of mass spectrometry to distinguish between a full-length fully functional cytokine and an inactive truncated form.[413]

Microenvironmental Niche and Role of Oxygen Tension in Hematopoiesis

The BM microenvironment is where HSCs and HPCs are mainly nurtured for their proliferation, survival, self-renewal, and differentiation.[424] The BM niche is a complex/dynamic structure encompassing a large number of cell types that act in context of cell-cell interactions and through the constitutive- and/or induced-release of cytokines and chemokines.[424–427] Dysregulated BM niches are associated with abnormalities during many diseases[428,429] such as MPNs, acute myeloid leukemia (AML), and multiple myeloma.[430] For example, microenvironmental cell signaling through notch is associated with development and disease.[431] Manipulating the BM niche accessory cells can also be used to treat/modulate disease severity. For example, it has been postulated that mesenchymal stem/stromal cell therapy may be used as a possible treatment for acute GVHD[427] as well as COVID-19,[432,433] although rigorous proof for use for patients with COVID-19 is currently being evaluated.

A crucial and sometimes ignored or underestimated aspect of the BM niche environment, and the study of cells collected from the BM, as well as the from cord blood and mobilized peripheral blood, for in vitro and ex vivo analysis is that of oxygen tension within the body.[434] The BM is quite hypoxic (1% to 5% oxygen), compared with that of ambient air (~21% oxygen), and even cord blood and adult blood can be relatively hypoxic (<10% oxygen).

Proliferation of HSCs and HPCs ex vivo is known to be more efficient when cells are cultured in an hypoxic environment of approximately 5% oxygen tension, than when these cells are cultured at ambient air O_2.[434–441] However, almost all studies have been based on numbers and activity of HSCs and HPCs first collected and processed in ambient air, regardless of whether the cells were subsequently cultured/studied at ambient air or hypoxic O_2 tension.[442] Hematopoietic cell populations from BM of young adult mice and human cord blood demonstrated a large loss of HSC numbers with an associated increase in HPC numbers within minutes of exposure of these cells to ambient air, via a phenomenon termed extraphysiologic oxygen shock/stress (EPHOSS).[442,443] Loss of HSCs from cells collected and processed in ambient air was due to EPHOSS-induced rapid differentiation of HSCs to HPCs, events not due to HSC death, but mediated through a mitochondrial permeability transition pore (MPTP)-p53-cyclophilin D axis associated with hypoxia inducing factor-1α and the hypoxamir, miR210. This involved rapid increased release of reactive oxygen species (ROS) due to ambient air exposure-induced opening of the MPTP.[442,443] Thus collecting/processing these cells in 3% O_2, such that they were never exposed to ambient air, resulted in increased numbers of phenotypic and functionally engrafting HSCs.[442] Additional studies have demonstrated enhancement in HSC numbers and functions when the cells have been collected and processed at 3% oxygen (= physioxia, in vivo hypoxia conditions).[444–446] Collecting/processing cells at 4°C mimics effects of low O_2, but whether the mechanisms are the same remains to be determined.[447] How cells respond to cytokines in vitro, and also likely in vivo, depends on the oxygen tension the cells are in, both in vitro[434–441] and in vivo.[442–446]

Exosomes/Extracellular Vesicles

Another way that cytokines, chemokines, and other growth-modulating factors can exert their paracrine activities is through their inclusion, along with other intracellular molecules, in extracellular vesicles (EVs). Ectosomes are vesicles that come off the surface of membranes through a process of binding. This includes microvesicles, microparticles, and vesicles which range from 50 nm to 1 µm in diameter. Alternatively, exosomes are EVs that average approximately 100 nm (approximately 40 to 160 nm in diameter; exosomes are endosomal in origin). Most, if not all, cytokines, chemokines, and other growth-modulating proteins/factors can be found encapsulated in EVs, especially in exosomes (see also https://en.wikipedia.org/wiki/Exosome_(vesicle).[448–454] Although the process of EVs/exosomes has been known for many decades, their roles in normal cell regulation and during leukemia, cancer, and other disorders have been especially appreciated in recent years. There are a number of recent reviews on this subject that highlighted their biology, function, biogenesis, and release; how they interact in the hematopoietic environmental niche[448–454]; and their role in leukemia.[454–464] For example, plasma exosomes from patients with AML and during relapse can inhibit colony formation by HPCs (CFU-GM, BFU-E, and CFU-GEMM) from normal donors in a dose-dependent fashion.[464] The exosomes from patients with AML in remission have greatly reduced inhibitory activity on the HPCs from normal donors. Although it is not clear how the combinations of intracellular components in EVs/exosomes manifest their effects, and which such components or combinations of components are actually involved, or exactly how they are involved, this is a rapidly advancing field of investigation. Exosomes from the plasma of patients with AML, as well as from established leukemia cell lines, contained DPP4, and the leukemia exosomes which suppressed HPC colony formation had much higher functional DPP4 activity than that of the exosomes from plasma of normal donors which manifested less or no inhibitory activity.[464] Of interest, the inhibition of DPP4 activity in the AML exosomes greatly or completely decreased the inhibitory activity of the AML exosomes on colony formation of HPC of donors.[464]

Aging in Context of Cell Regulation

Aging is associated with dysregulated hematopoiesis, including that regulated by cytokines, chemokines, and other growth-modulating molecules, and the BM niche.[446,465–468] There has been some evidence that aging-related abnormalities in cytokine/cytokine receptor signaling associated with dysregulated hematopoiesis is made worse upon removal of the aged HSCs and HPCs when processed and analyzed in ambient air, rather than their normal (more hypoxic) microenvironment.446 However, a better understanding of the aging process and hematopoietic cell (HSC, HPC) regulation is required.

Radiation Effects on Hematopoiesis

Radiation not only affects HSCs and HPCs but also accessory, bystander cells.[469–471] This has important implications for hematopoietic cell transplantation. Exactly how and what accessory cells and production of positive and negative growth modulatory factors are influenced remains to be determined.[471]

CONCLUDING THOUGHTS

Although we have learned a great deal about cytokines, ILs, chemokines, and other growth-modulating proteins/factors and their receptor-interactions and how they impact the regulation of HSCs, HPCs, and hematopoiesis, we will likely learn more about their activities and discover new hematopoietic effector molecules in the near future. Studies on effects of growth-modulatory molecules on hematopoiesis require rigorous assays to identify HSCs and HPCs both phenotypically by flow cytometry but more importantly by function. Such methodologies have been described.[472] These to-be-discovered effects will bode well for future preclinical and clinical efforts to treat and cure patients with hematologic and other disorders and malignancies, which in turn will enhance healthcare. One example of this knowledge is the recent report of the use of luspatercept in patients with lower-risk MDS[473] (see also https://en.wikipedia.org/wiki/Luspatercept). Luspatercept is a recombinant fusion protein from human activin R type IIb linked to a protein derived from immunoglobulin G and Actemra (tocilizumab) that blocks IL-6 and is used to treat rheumatoid arthritis (see https://www.verywellhealth.com/actemra-5072997). Hopefully, with increased interest in immune therapies, more applications of our growing knowledge of cytokines, chemokines, and other growth factors can lead to enhancements in clinical efficacy.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Akdis M, Aab A, Altunbulakli C, et al. Interleukins (from IL-1 to IL-38), interferons, transforming growth factor β, and TNF-α: receptors, functions, and roles in diseases. *J Allergy Clin Immunol*. 2016;138(4):984–1010.

Broxmeyer HE, Hoggatt J, O'Leary HA, et al. Dipeptidylpeptidase 4 negatively regulates colony stimulating factor activity and stress hematopoiesis. *Nature Med*. 2012;18:1786–1796.

Dinarello CA. Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol Rev*. 2018;281(1):8–27.

Kawasaki ES, Ladner MB, Wang AM, et al. Molecular cloning of a complementary DNA encoding human macrophage-specific colony-stimulating factor (CSF-1). *Science*. 1985;230(4723):291–296.

Liongue C, Sertori R, Ward AC. Evolution of cytokine receptor signaling. *J Immunol*. 2016;197(1):11–18.

Pelus LM, Broxmeyer HE. Peripheral blood stem cell mobilization: a look ahead. *Current Stem Cell Reports*. 2018;4(4):273–281.

Kang S, Narazaki M, Metwally H, et al. Historical overview of the interleukin-6 family cytokine. *J Exp Med*. 2020;217(5):e20190347.

Ouyang W, Rutz S, Crellin NK, et al. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu Rev Immunol*. 2011;29:71–109.

Tait Wojno ED, Hunter CA, Stumhofer JS. The immunobiology of the interleukin-12 family: room for discovery. *Immunity*. 2019;50(4):851–870.

Hughes CE, Nibbs RJB. A guide to chemokines and their receptors. *FEBS J*. 2018;285(16):2944–2971.

Mollica Poeta V, Massara M, Capucetti A, et al. Chemokines and chemokine receptors: new targets for cancer immunotherapy. *Front Immunol*. 2019;10:379.

Capitano ML, Mor-Vaknin N, Saha AK, et al. Secreted nuclear DEK regulates hematopoiesis through CXCR2 signaling. *J Clin Invest*. 2019;130:2555–2570.

Morikawa M, Derynck R, Miyazono K. TGF-β and the TGF-β family: context-dependent roles in cell and tissue physiology. *Cold Spring Harb Perspect Biol*. 2016;8(5):a021873.

Heldin C-H, Moustakas A. Signaling receptors for TGF-β family members. *Cold Spring Harb Perspect Biol*. 2016;8(8):a022053.

Namwanje M, Brown CW. Activins and inhibins: roles in development, physiology, and disease. *Cold Spring Harb Perspect Biol*. 2016;8(7):a021881.

Lyman SD, James L, Vanden Bos T, et al. Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. *Cell*. 1993;75(6):1157–1167.

Huang X, Guo B, Capitano M, et al. Past, present, and future efforts to enhance the efficacy of cord blood hematopoietic cell transplantation. *F1000Res*. 2019;8:F1000–F1833.

Mantel CR, O'Leary HA, Chitteti BR, et al. Enhancing hematopoietic stem cell transplantation efficacy by mitigating oxygen shock. *Cell*. 2015;161(7):1553–1565.

Shaheen M, Broxmeyer HE. Principles of cytokine signaling. In: Hoffman R, Benz EJJr, Silberstein LE, eds. *Hematology: Basic Principles and Practice*. 6th ed. Philadelphia, PA: Elsevier Saunders; 2013:136–146.

Sherr CJ, Rettenmier CW, Sacca R, et al. The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. *Cell*. 1985;41(3):665–676.

Souza LM, Boone TC, Gabrilove J, et al. Recombinant human granulocyte colony-stimulating factor: effects on normal and leukemic myeloid cells. *Science*. 1986;232(4746):61–65.

Spangler JB, Moraga I, Mendoza JL, et al. Insights into cytokine-receptor interactions from cytokine engineering. *Annu Rev Immunol*. 2015;33:139–167.

Wallach D. The TNF cytokine family: one track in a road paved by many. *Cytokine*. 2013;63(3):225–229.

Wendling F, Maraskovsky E, Debili N, et al. cMpl ligand is a humoral regulator of megakaryocytopoiesis. *Nature*. 1994;369(6481):571–574.

Williams DE, Eisenman J, Baird A, et al. Identification of a ligand for the c-kit proto-oncogene. *Cell*. 1990;63(1):167–174.

Wong GG, Witek JS, Temple PA, et al. Human GM-CSF: molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. *Science*. 1985;228(4701):810–815.

Yang YC, Ciarletta AB, Temple PA, et al. Human IL-3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. *Cell*. 1986;47(1):3–10.

Zhang L, Luo Q, Shu Y, et al. Transcriptomic landscape regulated by the 14 types of bone morphogenetic proteins (BMPs) in lineage commitment and differentiation of mesenchymal stem cells (MSCs). *Genes Dis*. 2019;6(3):258–275.

Zimran E, Papa L, Hoffman R. Ex vivo expansion of hematopoietic stem cells: finally transitioning from the lab to the clinic. *Blood Rev. 2021;50:100853.*

REFERENCES

1. Broxmeyer HE, Williams DE. The production of myeloid blood cells and their regulation during health and disease. *Crit Rev Oncol Hematol*. 1988;8(3):173–226.
2. Boulay J, O'Shea J, Paul W. Molecular phylogeny within type I cytokines and their cognate receptors. *Immunity*. 2003;19:159–163.
3. Bazan J. Structural design and molecular evolution of a cytokine receptor superfamily. *Proc Natl Acad Sci USA*. 1990;87:6934–6938.
4. Liongue C, Sertori R, Ward AC. Evolution of cytokine receptor signaling. *J Immunol*. 2016;197(1):11–18.
5. Shaheen M, Broxmeyer HE. Principles of cytokine signaling. In: Hoffman R, Benz EJ Jr, Silberstein LE, eds. *Hematology: Basic Principles and Practice*. 6th ed. Philadelphia, PA: Elsevier Saunders; 2013:136–146.
6. Broxmeyer HE, Van Zant G, Zucali JR, et al. Mechanisms of leukocyte production and release XII. A comparative assay of the leukocytosis-inducing factor (LIF) and the colony stimulating factor (CSF). *Proc Soc Expl Bio Med*. 1974;145:1262–1267.
7. Broxmeyer HE. Erythropoietin. Multi-targets, -actions, and -modifying influences for biological and clinical consideration. *J Exp Med*. 2013;210:205–208.
8. Boulay JL, Paul WE. The interleukin-4 family of lymphokines. *Curr Opin Immunol*. 4:294–298.
9. Hamilton JA. GM-CSF in inflammation. *J Exp Med*. 2020;217(1):e20190945.
10. Vardhana SA, Wolchok JD. The many faces of the anti-COVID immune response. *J Exp Med*. 2020;217(6):e20200678.
11. Brisse E, Wouters CH, Matthys P. Murine models of secondary cytokine storm syndromes. In: Cron R, Behrens E, eds. *Cytokine Storm Syndrome*. Cham: Springer; 2019:491–518.
12. Bonaventura A, Vecchié A, Wang TS, et al. Targeting GM-CSF in COVID-19 pneumonia: rationale and strategies. *Front Immunol*. 2020;11:1625.
13. Guihot A, Litvinova E, Autran B, et al. Cell-mediated immune responses to COVID-19 infection. *Front Immunol*. 2020;11:1662.
14. Metcalf D. The granulocyte-macrophage colony-stimulating factors. *Science*. 1985;229(4708):16–22.
15. Clark SC, Kamen R. The human hematopoietic colony-stimulating factors. *Science*. 1987;236(4806):1229–1237.
16. Sieff CA. Hematopoietic growth factors. *J Clin Invest*. 1987;79(6):1549–1557.
17. Walker F, Nicola NA, Metcalf D, et al. Hierarchical down-modulation of hemopoietic growth factor receptors. *Cell*. 1985;43(1):269–276.
18. Gough NM, Gough J, Metcalf D, et al. Molecular cloning of cDNA encoding a murine haematopoietic growth regulator, granulocyte-macrophage colony stimulating factor. *Nature*. 1984;309(5971):763–767.
19. Lee F, Yokota T, Otsuka T, et al. Isolation of cDNA for a human granulocyte-macrophage colony-stimulating factor by functional expression in mammalian cells. *Proc Natl Acad Sci USA*. 1985;82(13):4360–4364.
20. Wong GG, Witek JS, Temple PA, et al. Human GM-CSF: molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. *Science*. 1985;228(4701):810–815.
21. Cantrell MA, Anderson D, Cerretti DP, et al. Cloning, sequence, and expression of a human granulocyte/macrophage colony-stimulating factor. *Proc Natl Acad Sci USA*. 1985;82(18):6250–6254.
22. Park LS, Friend D, Price V, et al. Heterogeneity in human interleukin-3 receptors. A subclass that binds human granulocyte/macrophage colony stimulating factor. *J Biol Chem*. 1989;264(10):5420–5427.
23. Park LS, Friend D, Gillis S, et al. Characterization of the cell surface receptor for human granulocyte/macrophage colony-stimulating factor. *J Exp Med*. 1986;164(1):251–262.
24. Nicola NA, Metcalf D, Matsumoto M, et al. Purification of a factor inducing differentiation in murine myelomonocytic leukemia cells. Identification as granulocyte colony-stimulating factor. *J Biol Chem*. 1983;258(14):9017–9023.
25. Souza LM, Boone TC, Gabrilove J, et al. Recombinant human granulocyte colony-stimulating factor: effects on normal and leukemic myeloid cells. *Science*. 1986;232(4746):61–65.
26. Ihle JN, Keller J, Henderson L, et al. Procedures for the purification of interleukin 3 to homogeneity. *J Immunol*. 1982;129(6):2431–2436.
27. Clark-Lewis I, Kent SB, Schrader JW. Purification to apparent homogeneity of a factor stimulating the growth of multiple lineages of hemopoietic cells. *J Biol Chem*. 1984;259(12):7488–7494.
28. Cutler RL, Metcalf D, Nicola NA, et al. Purification of a multipotential colony-stimulating factor from pokeweed mitogen-stimulated mouse spleen cell conditioned medium. *J Biol Chem*. 1985;260(11):6579–6587.
29. Fung MC, Hapel AJ, Ymer S, et al. Molecular cloning of cDNA for murine interleukin-3. *Nature*. 1984;307(5948):233–237.
30. Campbell HD, Ymer S, Fung MC, et al. Cloning and nucleotide sequence of the murine interleukin-3 gene. *Eur J Biochem*. 1985;150(2):297–304.
31. Miyatake S, Yokota T, Lee F, et al. Structure of the chromosomal gene for murine interleukin 3. *Proc Natl Acad Sci USA*. 1985;82(2):316–320.
32. Yang YC, Ciarletta AB, Temple PA, et al. Human IL-3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. *Cell*. 1986;47(1):3–10.
33. Yang YC, Kovacic S, Kriz R, et al. The human genes for GM-CSF and IL 3 are closely linked in tandem on chromosome 5. *Blood*. 1988;71(4):958–961.
34. Stanley ER, Heard PM. Factors regulating macrophage production and growth. Purification and some properties of the colony stimulating factor from medium conditioned by mouse L cells. *J Biol Chem*. 1977;252(12):4305–4312.
35. Das SK, Stanley ER, Guilbert LJ, et al. Human colony-stimulating factor (CSF-1) radioimmunoassay: resolution of three subclasses of human colony-stimulating factors. *Blood*. 1981;58(3):630–641.
36. Das SK, Stanley ER. Structure-function studies of a colony stimulating factor (CSF-1). *J Biol Chem*. 1982;257(22):13679–13684.
37. Stanley ER. The macrophage colony-stimulating factor, CSF-1. *Methods Enzymol*. 1985;116:564–587.
38. Waheed A, Shadduck RK. Purification and properties of L cell-derived colony-stimulating factor. *J Lab Clin Med*. 1979;94(1):180–193.
39. Waheed A, Shadduck RK. Purification of colony-stimulating factor by affinity chromatography. *Blood*. 1982;60(1):238–244.
40. Kawasaki ES, Ladner MB, Wang AM, et al. Molecular cloning of a complementary DNA encoding human macrophage-specific colony-stimulating factor (CSF-1). *Science*. 1985;230(4723):291–296.
41. Wong GG, Temple PA, Leary AC, et al. Human CSF-1: molecular cloning and expression of 4-kb cDNA encoding the human urinary protein. *Science*. 1987;235(4795):1504–1508.
42. Ralph P, Ladner MB, Wang AM, et al. The molecular and biological properties of the human and murine members of the CSF-1 family. In: Webb DR, Pierce CA, Cohen S, eds. *Molecular Basis of Lymphokine Action*. Clifton, NJ: Humana Press; 1987:295–311.
43. Rajavashisth TB, Eng R, Shadduck RK, et al. Cloning and tissue-specific expression of mouse macrophage colony-stimulating factor mRNA. *Proc Natl Acad Sci USA*. 1987;84(5):1157–1161.
44. Ladner MB, Martin GA, Noble JA, et al. Human CSF-1: gene structure and alternative splicing of mRNA precursors. *EMBO J*. 1987;6(9):2693–2698.
45. Sherr CJ, Rettenmier CW, Sacca R, et al. The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. *Cell*. 1985;41(3):665–676.
46. Miyake T, Kung CK, Goldwasser E. Purification of human erythropoietin. *J Biol Chem*. 1977;252(15):5558–5564.
47. Wang FF, Kung CK, Goldwasser E. Some chemical properties of human erythropoietin. *Endocrinology*. 1985;116(6):2286–2292.
48. Goldwasser E. Erythropoietin: a somewhat personal history. *Perspect Biol Med*. 40(1):18–32.
49. Lin FK, Suggs S, Lin CH, et al. Cloning and expression of the human erythropoietin gene. *Proc Natl Acad Sci USA*. 1985;82(22):7580–7584.
50. Jacobs K, Shoemaker C, Rudersdorf R, et al. Isolation and characterization of genomic and cDNA clones of human erythropoietin. *Nature*. 1985;313(6005):806–810.
51. D'Andrea AD, Lodish HF, Wong GG. Expression cloning of the murine erythropoietin receptor. *Cell*. 1989;57(2):277–285.
52. Wendling F, Maraskovsky E, Debili N, et al. cMpl ligand is a humoral regulator of megakaryocytopoiesis. *Nature*. 1994;369(6481):571–574.
53. de Sauvage FJ, Hass PE, Spencer SD, et al. Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. *Nature*. 1994;369(6481):533–538.
54. Lok S, Kaushansky K, Holly RD, et al. Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. *Nature*. 1994;369(6481):565–568.
55. Bartley TD, Bogenberger J, Hunt P, et al. Identification and cloning of a megakaryocyte growth and development factor that is a ligand for the cytokine receptor Mpl. *Cell*. 1994;77(7):1117–1124.
56. Kaushansky K, Lok S, Holly RD, et al. Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. *Nature*. 1994;369(6481):568–571.
57. Donahue RE, Wang EA, Stone DK, et al. Stimulation of haematopoiesis in primates by continuous infusion of recombinant human GM-CSF. *Nature*. 1986;321(6073):872–875.
58. Metcalf D, Begley CG, Williamson DJ, et al. Hemopoietic responses in mice injected with purified recombinant murine GM-CSF. *Exp Hematol*. 1987;15(1):1–9.
59. Lord BI, Molineux G, Testa NG, et al. The kinetic response of haemopoietic precursor cells, in vivo, to highly purified, recombinant interleukin-3. *Lymphokine Res*. 1986;5(2):97–104.

60. Kindler V, Thorens B, de Kossodo S, et al. Stimulation of hematopoiesis in vivo by recombinant bacterial murine interleukin 3. *Proc Natl Acad Sci USA*. 1986;83(4):1001–1005.
61. Metcalf D, Begley CG, Johnson GR, et al. Effects of purified bacterially synthesized murine multi-CSF (IL-3) on hematopoiesis in normal adult mice. *Blood*. 1986;68(1):46–57.
62. Sieff CA, Niemeyer CM, Nathan DG, et al. Stimulation of human hematopoietic colony formation by recombinant gibbon multi-colony-stimulating factor or interleukin 3. *J Clin Invest*. 1987;80(3):818–823.
63. Kaushansky K, Broudy VC, Grossmann A, et al. Thrombopoietin expands erythroid progenitors, increases red cell production, and enhances erythroid recovery after myelosuppressive therapy. *J Clin Invest*. 1995;96(3):1683–1687.
64. Broudy VC, Lin NL, Kaushansky K. Thrombopoietin (c-mpl ligand) acts synergistically with erythropoietin, stem cell factor, and interleukin-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy in vitro. *Blood*. 1995;85(7):1719–1726.
65. Alexander WS, Roberts AW, Nicola NA, et al. Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. *Blood*. 1996;87(6):2162–2170.
66. Carver-Moore K, Broxmeyer HE, Luoh SM, et al. Low levels of erythroid and myeloid progenitors in thrombopoietin-and c-mpl-deficient mice. *Blood*. 1996;88(3):803–808.
67. Williams DE, Straneva JE, Shen RN, et al. Purification of murine bone marrow-derived granulocyte-macrophage colony-forming cells and partial characterization of their growth and regulation in vitro. *Exp Hematol*. 1987;15:243–250.
68. Broxmeyer HE, Williams DE, Cooper S. The influence in vivo of natural murine interleukin-3 on the proliferation of myeloid progenitor cells in mice recovering from sublethal dosages of cyclophosphamide. *Leukemia Res*. 1987;11:201–205.
69. Broxmeyer HE, Williams DE, Cooper S, et al. The comparative effects in vivo of recombinant murine interleukin-3, natural murine colony stimulating factor-1 and recombinant murine granulocyte-macrophage colony stimulating factor on myelopoiesis in mice. *J Clin Invest*. 1987;79:721–730.
70. Broxmeyer HE, Williams DE, Cooper S, et al. The influence in vivo of murine colony stimulating factor-1 on myeloid progenitor cells in mice recovering from sublethal dosages of cyclophosphamide. *Blood*. 1987;69:913–918.
71. Broxmeyer HE, Williams DE, Hangoc G, et al. The opposing action in vivo on murine myelopoiesis of purified preparations of lactoferrin and the colony stimulating factors. *Blood Cells*. 1987;13:31–48.
72. Williams DE, Straneva JE, Cooper S, et al. Interactions between purified murine colony stimulating factors (natural CSF-1, purified recombinant GM-CSF, and purified recombinant IL-3) on the in vitro proliferation of purified murine granulocyte-macrophage progenitor cells. *Exp Hematol*. 1987;15:1007–1012.
73. Chikkappa G, Broxmeyer HE, Cooper S, et al. Effects in vivo of multiple injections of low dosages of purified murine and recombinant human macrophage colony stimulating factor of mice. *Cancer Res*. 1989;;49:3558–3561.
74. Broxmeyer HE, Douglas GW, Hangoc G, et al. Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells. *Proc Natl Acad Sci USA*. 1989;86:3828–3832.
75. Broxmeyer HE, Hangoc G, Cooper S, et al. Growth characteristics and expansion of human umbilical cord blood and estimation of its potential for transplantation of adults. *Proc Natl Acad Sci USA*. 1992;89:4109–4113.
76. Erslev A. Erythropoietin coming of age. *N Engl J Med*. 1987;316(2):101–103.
77. Eschbach JW, Egrie JC, Downing MR, et al. Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. Results of a combined phase I and II clinical trial. *N Engl J Med*. 1987;316(2):73–78.
78. Broxmeyer HE, Cooper S, Williams DE, et al. Effects of purified recombinant human granulocyte-macrophage colony stimulating factor on growth characteristics of bone marrow hematopoietic progenitor cells in patients on a phase I clinical trial. *Exp Hematol*. 1988;16:594–602.
79. Vadhan-Raj S, Keating M, LeMaistre A, et al. Effects of recombinant human granulocyte-macrophage colony-stimulating factor in patients with myelodysplastic syndrome. *N Engl J Med*. 1987;317:1545–1552.
80. Vadhan-Raj S, Buescher S, LeMaistre A, et al. Stimulation of hematopoiesis in patients with bone marrow failure and in patients with malignancy by recombinant human granulocyte-macrophage colony-stimulating factor. *Blood*. 1988;72:134–141.
81. Vadhan-Raj S, Hittleman W, Broxmeyer HE, et al. In vivo biological activities of recombinant human granulocyte-macrophage colony-stimulating factor. *N Y Acad Sci*. 1989;554:231–240.
82. Vadhan-Raj S, Buescher S, Broxmeyer HE, et al. Stimulation of myelopoiesis in patients with aplastic anemia by recombinant human granulocyte-macrophage colony-stimulating factor. *N Engl J Med*. 1988;319:1628–1634.
83. Vadhan-Raj S, Broxmeyer HE, Spitzer G, et al. Stimulation of nonclonal hematopoiesis and suppression of neoplastic clone after treatment with recombinant human granulocyte-macrophage colony-stimulating factor in a patient with therapy-related myelodysplastic syndrome. *Blood*. 1989;74:1491–1498.
84. Broxmeyer HE, Cooper S, Vadhan-Raj S. Cell cycle status of erythroid (BFU-E) progenitor cells from the bone marrows of patients on a clinical trial with purified recombinant human granulocyte-macrophage colony-stimulating factor. *Exp Hematol*. 1989;17:455–459.
85. Vadhan-Raj S, Jeha SS, Buescher S, et al. Stimulation of myelopoiesis by recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) in a patient with congenital neutropenia. Biology and nature of response to GM-CSF. *Blood*. 1990;75:858–864.
86. Vadhan-Raj S, Hittelman WN, Lepe-Zuniga JL, et al. Regulation of endogenous erythropoietin levels in anemia associated with myelodysplastic syndromes. *Blood*. 1990;75:1749–1750.
87. Vadhan Raj S, Broxmeyer HE, Hittelman WN, et al. Abrogating chemotherapy-induced myelosuppression by recombinant granulocyte-macrophage colony-stimulating factor: Protection at the progenitor cell level. *J Clin Oncol*. 1992;10:1266–1277.
88. Vadhan-Raj S, Verschraegen CF, Bueso-Ramos C, et al. Recombinant human thrombopoietin attenuates carboplatin-induced severe thrombocytopenia and the need for platelet transfusions in patients with gynecologic cancer. *Ann Intern Med*. 2000;132:364–368.
89. Kaushansky K. Thrombopoietin. *N Engl J Med*. 1998;339(11):746–754.
90. Broxmeyer HE, Orschell CM, Clapp DW, et al. Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J Exp Med*. 2005;201:1307–1318.
91. Pelus LM, Broxmeyer HE. Peripheral blood stem cell mobilization: a look ahead. *Curr Stem Cell Rep*. 2018;4(4):273–281.
92. Justiz Vaillant AA, Qurie A. Interleukin. [Updated 2019 Jun 12]. In: StatPearls [Internet]. Treasure Island, FL: StatPearls Publishing; 2020.https://www.ncbi.nlm.nih.gov/books/NBK499840/.
93. Akdis M, Aab A, Altunbulakli C, et al. Interleukins (from IL-1 to IL-38), interferons, transforming growth factor β, and TNF-α: receptors, functions, and roles in diseases. *J Allergy Clin Immunol*. 2016;138(4):984–1010.
94. Dinarello CA. Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol Rev*. 2018;281(1):8–27.
95. Kang S, Narazaki M, Metwally H, et al. Historical overview of the interleukin-6 family cytokine. *J Exp Med*. 2020;217(5):e20190347.
96. Unver N, McAllister F. IL-6 family cytokines: key inflammatory mediators as biomarkers and potential therapeutic targets. *Cytokine Growth Factor Rev*. 2018;41:10–17.
97. Ouyang W, Rutz S, Crellin NK, et al. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu Rev Immunol*. 2011;29:71–109.
98. Tait Wojno ED, Hunter CA, Stumhofer JS. The immunobiology of the interleukin-12 family: room for discovery. *Immunity*. 2019;50(4):851–870.
99. Gu C, Wu L, Li X. IL-17 family: cytokines, receptors and signaling. *Cytokine*. 2013;64(2):477–485.
100. Chan AH, Schroder K. Inflammasome signaling and regulation of interleukin-1 family cytokines. *J Exp Med*. 2020;217(1):e20190314.
101. Pol JG, Caudana P, Paillet J, et al. Effects of interleukin-2 in immunostimulation and immunosuppression. *J Exp Med*. 2020;217(1):e20191247.
102. Zucali JR, Broxmeyer HE, Dinarello CA, et al. Regulation of early human hematopoietic (BFU-E and CFU-GEMM) progenitor cells in vitro by interleukin-1 induced fibroblast conditioned medium. *Blood*. 1987;69:33–37.
103. Williams DE, Broxmeyer HE. Interleukin-1-alpha enhances the in vitro survival of purified murine granulocyte-macrophage progenitor cells in the absence of colony-stimulating factors. *Blood*. 1988;72:1608–1615.
104. Hangoc G, Williams DE, Falkenburg JHF, et al. Influence of interleukin-1α on the survival of human bone marrow cells responding to hematopoietic colony stimulating factors. *J Immunol*. 1989;142:4329–4334.
105. Shen RN, Wu B, Lu L, et al. Recombinant human interleukin-1 alpha: a potent bio-immunomodifier in vivo in immunosuppressed mice induced by cyclophosphamide, retroviral infection and surgical stress. *In Vivo*. 1994;8:59–64.
106. de Mooij CEM, Netea MG, van der Velden WJFM, et al. Targeting the interleukin-1 pathway in patients with hematological disorders. *Blood*. 2017;129(24):3155–3164.
107. Hangoc G, Broxmeyer HE. Effects of purified recombinant human interleukin-3 on the growth of human hematopoietic progenitor cells in "long-term" bone marrow culture. *Biotechnol Therapeutics*. 1988;1(1):17–29.
108. Shibayama H, Anzai N, Ritchie A, et al. Interleukin-3 and Flt3-ligand induce adhesion of Baf3/Flt3 precursor B-lymphoid cells to fibronectin via activation of VLA-4 and VLA-5. *Cell Immunol*. 1998;187:27–33.
109. Shibayama H, Anzai N, Braun SE, et al. H-Ras is involved in the inside-out signaling pathway of IL-3 induced integrin activation. *Blood*. 1999;93:1540–1548.

110. Broxmeyer HE, Lu L, Cooper S, et al. Synergistic effects of purified recombinant human and murine B-cell growth factor-1/interleukin-4 on colony formation in vitro by hematopoietic progenitor cells: multiple actions. *J Immunol*. 1988;141:3852–3862.
111. Sadik A, Somarribas Patterson LF, Öztürk S, et al. IL4I1 is a metabolic immune checkpoint that activates the AHR and promotes tumor progression. *Cell*. 2020;182(5):1252–1270. e34.
112. Lu L, Lin ZH, Shen RN, et al. Influence of interleukins-3, -5 and -6 on the generation and growth of eosinophil progenitors in highly enriched human bone marrow in the absence of serum. *Exp Hematol*. 1990;18:1180–1186.
113. Gentile P, Broxmeyer HE. Interleukin-6 ablates the accessory cell mediated suppressive effects of lactoferrin on human hematopoietic progenitor cell proliferation in vitro. *Ann NY Acad Sci*. 1991;628:74–83.
114. Lu L, Shen RN, Broxmeyer HE. Effects in vivo of recombinant human interleukin-6, alone or in combination with local irradiation, on tumor growth in Lewis lung carcinoma-bearing mice. *Int J Cell Cloning*. 1991;9:511–520.
115. Jilka RL, Passeri G, Girasole G, et al. Estrogen loss causes an upregulation of several hematopoietic bone marrow progenitors in the mouse: a mediating role of interleukin-6. *Exp Hematol*. 1995;25:500–506.
116. Tie R, Li H, Cai S, et al. Interleukin-6 signaling regulates hematopoietic stem cell emergence. *Exp Mol Med*. 2019;51(10):1–12.
117. Valletta S, Thomas A, Meng Y, et al. Micro-environmental sensing by bone marrow stroma identifies IL-6 and TGFβ1 as regulators of hematopoietic ageing. *Nat Commun*. 2020;11(1):4075.
118. Delgobo M, Mendes DA, Kozlova E, et al. An evolutionary recent IFN/IL-6/CEBP axis is linked to monocyte expansion and tuberculosis severity in humans. *Elife*. 2019;8:e47013.
119. Bellido T, Jilka RL, Boyce BF, et al. Regulation of interleukin-6, osteoclastogenesis, and bone mass by androgens. The role of the androgen receptor. *J Clin Invest*. 1995;95(6):2886–2895.
120. Jilka RL, Hangoc G, Girasole G, et al. Increased osteoclast development after estrogen loss: mediation by interleukin-6. *Science*. 1992;257(5066): 88–91.
121. Lu L, Zhou Z, Wu B, et al. Influence of recombinant human interleukin (IL)-7 on disease progression in mice infected with Friend virus complex. *Int J Cancer*. 1992;52:261–265.
122. von Muenchow L, Alberti-Servera L, Klein F, et al. Permissive roles of cytokines interleukin-7 and Flt3 ligand in mouse B-cell lineage commitment. *Proc Natl Acad Sci USA*. 2016;113(50):E8122–E8130.
123. Broxmeyer HE, Cooper S, Cacalano G, et al. Interleukin-8 receptor is involved in negative regulation of myeloid progenitor cells *in vivo*: evidence from mice lacking the murine IL-8 receptor homolog. *J Exp Med*. 1996;184:1825–1832.
124. Williams DE, Morrissey PJ, Mochizuki DY, et al. The T cell growth factor p40 promotes the proliferation of myeloid cell lines and has erythroid potentiating activity by normal human bone marrow cells *in vitro. Blood*. 1990;76:906–911.
125. Miyazawa K, Hendrie PC, Yang YC, et al. Recombinant human IL-9 induces protein tyrosine phosphorylation and synergizes with steel factor to stimulate proliferation of the human factor dependent cell line, M07e. *Blood*. 1992;80:1685–1692.
126. Miyazawa K, Hendrie PC, Yang YC, et al. Recombinant human IL-9 induces protein tyrosine phosphorylation and synergizes with steel factor to stimulate proliferation of the human factor dependent cell line, M07e. *Blood*. 1992;80:1685–1692.
127. Saraiva M, Vieira P, O'Garra A. Biology and therapeutic potential of interleukin-10. *J Exp Med*. 2020;217(1):e20190418.
128. Li G, Hangoc G, Broxmeyer HE. Interleukin-10 in combination with M-CSF and IL-4 contributes to development of the rare population of CD14+CD16++ cells derived from human monocytes. *Biochem Biophys Res Comms*. 2004;322:637–643.
129. Saxton RA, Tsutsumi N, Su LL, et al. Structure-based decoupling of the pro- and anti-inflammatory functions of interleukin-10. *Science*. 2021;371(6535):eabc8433.
130. Hangoc G, Yin T, Cooper S, et al. *In vivo* effects of recombinant interleukin-11 on myelopoiesis in mice. *Blood*. 1993;81:965–972.
131. Xiao M, Li ZH, McMahel J, et al. Inhibitory effects of interleukin-12 on retroviral gene transduction into CD34+++ cord blood myeloid progenitors mediated by induction of tumor necrosis factor-α. *J Hematother*. 1996;5:171–177.
132. Broxmeyer HE, Starnes T, Ramsey H, et al. The IL-17 cytokine family members are inhibitors of hematopoietic progenitor proliferation. *Blood*. 2006;108:770.
133. Zhou T, Damsky W, Weizman OE, et al. IL-18BP is a secreted immune checkpoint and barrier to IL-18 immunotherapy. *Nature*. 2020;583(7817):609–614.
134. Liu L, Ding C, Zeng W, et al. Selective enhancement of multipotential hematopoietic progenitors in vitro and in vivo by IL-20. *Blood*. 2003;102:3206–3209.
135. Niu W, Xu Y, Zha X, et al. IL-21/IL-21R signaling aggravated respiratory inflammation induced by intracellular bacteria through regulation of CD4+ T cell subset responses. *J Immunol*. 2021;206(7):1586–1596.
136. Spolski R, Leonard WJ. Interleukin-21: a double-edged sword with therapeutic potential. *Nat Rev Drug Discov*. 2014;13(5):379–395.
137. Larochette V, Miot C, Poli C, et al. IL-26, a cytokine with roles in extracellular DNA-induced inflammation and microbial defense. *Front Immunol*. 2019;10:204.
138. Broxmeyer HE, Li J, Hangoc G, et al. Regulation of myeloid progenitor cell proliferation/survival by IL-31 receptor and IL-31. *Exp Hematol*. 2007;35:78–86.
139. Ribeiro-Dias F, Saar Gomes R, de Lima Silva LL, et al. Interleukin 32: a novel player in the control of infectious diseases. *J Leukoc Biol*. 2017;101(1):39–52.
140. Kim SH, Han SY, Azam T, et al. Interleukin-32: a cytokine and inducer of TNFalpha. *Immunity*. 2005;22(1):131–142.
141. Aass KR, Kastnes MH, Standal T. Molecular interactions and functions of IL-32. *J Leukoc Biol*. 2021;109(1):143–159.
142. Zahoor M, Westhrin M, Aass KR, et al. Hypoxia promotes IL-32 expression in myeloma cells, and high expression is associated with poor survival and bone loss. *Blood Adv*. 2017;1(27):2656–2666.
143. Capitano ML, Griesenauer B, Guo B, et al. The IL-33 receptor/ST2 acts as a positive regulator of functional mouse bone marrow hematopoietic stem and progenitor cells. *Blood Cells Mol Dis*. 2020;84:102435.
144. Swann JW, Koneva LA, Regan-Komito D, et al. IL-33 promotes anemia during chronic inflammation by inhibiting differentiation of erythroid progenitors. *J Exp Med*. 2020;217(9):e20200164.
145. Alt C, Yuan S, Wu F, et al. Long-acting IL-33 mobilizes high-quality hematopoietic stem and progenitor cells more efficiently than granulocyte colony-stimulating factor or AMD3100. *Biol Blood Marrow Transpl*. 2019;25(8):1475–1485.
146. Kim J, Kim W, Le HT, et al. IL-33-induced hematopoietic stem and progenitor cell mobilization depends upon CCR2. *J Immunol*. 2014;193(7):3792–3802.
147. Huang P, Li X, Meng Y, et al. Interleukin-33 regulates hematopoietic stem cell regeneration after radiation injury. *Stem Cell Res Ther*. 2019;10(1):123.
148. Paczesny S, Krijanovski OI, Braun TM, et al. A biomarker panel for acute graft-versus-host disease. *Blood*. 2009;113(2):273–278.
149. Vander Lugt MT, Braun TM, Hanash S, et al. ST2 as a marker for risk of therapy-resistant graft-versus-host disease and death. *N Engl J Med*. 2013;369(6):529–539.
150. Ponce DM, Hilden P, Mumaw C, et al. High day 28 ST2 levels predict for acute graft-versus-host disease and transplant-related mortality after cord blood transplantation. *Blood*. 2015;125(1):199–205.
151. Abu Zaid M, Wu J, Wu C, et al. Plasma biomarkers of risk for death in a multicenter phase 3 trial with uniform transplant characteristics post-allogeneic HCT. *Blood*. 2017;129(2):162–170.
152. Paczesny S. Biomarkers for posttransplantation outcomes. *Blood*. 2018;131 (20):2193–2204.
153. Srinagesh HK, Levine JE, Ferrara JLM. Biomarkers in acute graft-versus-host disease: new insights. *Ther Adv Hematol*. 2019;10: 2040620719891358.
154. Allegra A, Innao V, Tartarisco G, et al. The ST2/interleukin-33 axis in hematologic malignancies: the IL-33 paradox. *Int J Mol Sci*. 2019;20(20):5226.
155. Mager LF, Riether C, Schürch CM, et al. IL-33 signaling contributes to the pathogenesis of myeloproliferative neoplasms. *J Clin Invest*. 2015;125(7):2579–2591.
156. Liu X, Xiao Y, Pan Y, et al. The role of the IL-33/ST2 axis in autoimmune disorders: friend or foe? *Cytokine Growth Factor Rev*. 2019;50:60–74.
157. Chan BCL, Lam CWK, Tam LS, et al. IL33: Roles in allergic inflammation and therapeutic perspectives. *Front Immunol*. 2019;10:364.
158. Eissmann MF, Buchert M, Ernst M. IL33 and mast cells—the key regulators of immune responses in gastrointestinal cancers? *Front Immunol*. 2020;11:1389.
159. Hughes CE, Nibbs RJB. A guide to chemokines and their receptors. *FEBS J*. 2018;285(16):2944–2971.
160. Turner MD, Nedjai B, Hurst T, et al. Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. *Biochim Biophys Acta*. 2014;1843(11):2563–2582.
161. Mollica Poeta V, Massara M, Capucetti A, et al. Chemokines and chemokine receptors: new targets for cancer immunotherapy. *Front Immunol*. 2019;10:379.
162. Mantel C, Kim YJ, Cooper S, et al. Polymerization of murine macrophage inflammatory protein-1α inactivates its myelosuppressive effects *in vitro*. The active form is monomer. *Proc Natl Acad Sci USA*. 1993;90:2232–2236.

163. Broxmeyer HE, Kim CH. Regulation of hematopoiesis in a sea of chemokine family members with a plethora of redundant activities. *Exp Hematol*. 1999;27:1113–1123.
164. Youn BS, Mantel C, Broxmeyer HE. Chemokines, chemokine receptors and hematopoiesis. *Immunol Rev*. 2000;177:150–174.
165. Broxmeyer HE. Regulation of hematopoiesis by chemokine family members. *Int J Hematol*. 2001;74:9–17.
166. Broxmeyer HE. Chemokines in hematopoiesis. *Curr Opin Hematol*. 2008;15:49–58.
167. Graham GJ, Wright EG, Hewick R, et al. Identification and characterization of an inhibitor of haemopoietic stem cell proliferation. *Nature*. 1990;344(6265):442–444.
168. Dunlop DJ, Wright EG, Lorimore S, et al. Demonstration of stem cell inhibition and myeloprotective effects of SCI/rhMIP1 alpha in vivo. *Blood*. 1992;79(9):2221–2225.
169. Broxmeyer HE, Sherry B, Lu L, et al. Myelopoietic enhancing effects of murine macrophage inflammatory proteins 1 and 2 *in vitro* on colony formation by murine and human bone marrow granulocyte-macrophage progenitor cells. *J Exp Med*. 1989;170:1583–1594.
170. Broxmeyer HE, Sherry B, Lu L, et al. Enhancing and suppressing effects of recombinant murine macrophage inflammatory proteins on colony formation *in vitro* by bone marrow myeloid progenitor cells. *Blood*. 1990;76:1110–1116.
171. Broxmeyer HE, Sherry B, Cooper S, et al. Macrophage inflammatory protein (MIP)-1α abrogates the capacity of MIP-1α to suppress myeloid progenitor cell growth. *J Immunol*. 1991;147:2586–2594.
172. Broxmeyer HE, Sherry B, Cooper S, et al. Comparative analysis of the suppressive effects of the human macrophage inflammatory protein family of cytokines (chemokines) on proliferation of human myeloid progenitor cells. *J Immunol*. 1993;150:3448–3458.
173. Cooper S, Mantel C, Broxmeyer HE. Myelosuppressive effects *in vivo* with very low dosage of monomeric recombinant murine macrophage inflammatory protein-1α. *Exp Hematol*. 1994;22:186–193.
174. Daly TJ, LaRosa GJ, Dolich S, et al. High activity suppression of myeloid progenitor proliferation by chimeric mutants of interleukin 8 and platelet factor 4. *J Biol Chem*. 1995;270:23282–23292.
175. Broxmeyer HE, Cooper S, Hague N, et al. Human chemokines: enhancement of specific activity and effects *in vitro* on normal and leukemic progenitors and a factor dependent cell line and *in vivo* in mice. *Ann Hematol*. 1995;71:235–246.
176. Broxmeyer HE, Cooper S, Cacalano G, et al. Interleukin-8 receptor is involved in negative regulation of myeloid progenitor cells *in vivo*: evidence from mice lacking the murine IL-8 receptor homolog. *J Exp Med*. 1996;184:1825–1832.
177. Hromas R, Kim CH, Klemsz M, et al. Isolation and characterization of exodus-2, a novel c-c chemokine with a unique 37 amino acid carboxy terminal extension. *J Immunol*. 1997;159:2554–2558.
178. Han ZC, Maurer AM, Bellucci S, et al. Inhibitory effect of platelet factor 4 (PF4) on the growth of human erythroleukemia cells: proposed mechanism of action of PF4. *J Lab Clin Med*. 1992;120(4):645–660.
179. Kim CH, Broxmeyer HE. In vitro behavior of hematopoietic progenitor cells under the influence of chemoattractants: stromal cell-derived factor-1, steel factor and the bone marrow environment. *Blood*. 1998;91:100–110.
180. Broxmeyer HE, Cooper S, Kohli L, et al. Transgenic expression of stromal cell derived factor-1/CXCL12 enhances myeloid progenitor cell survival/anti-apoptosis in vitro in response to growth factor withdrawal and enhances myelopoiesis in vivo. *J Immunol*. 2003;170:421–429.
181. Broxmeyer HE, Kohli L, Kim CH, et al. Stromal cell-derived factor-1/CXCL12 directly enhances survival/antiapoptosis of myeloid progenitor cells through CXCR4 and G(alpha)i proteins and enhances engraftment of competitive, repopulating stem cells. *J Leuk Biol*. 2003;73(5):630–638.
182. Lee Y, Gotoh A, Kwon HJ, et al. Enhancement of intracellular signaling associated with hematopoietic progenitor cell survival in response to SDF-1/CXCL12 in synergy with other cytokines. *Blood*. 2002;99:4307–4317.
183. Okabe S, Fukuda S, Kim YJ, et al. Stromal cell derived factor-1-α/CXCL12 induced chemotaxis of T cells involves activation of the RasGAP-associated docking protein p62 Dok-1. *Blood*. 2005;105:474–480.
184. Basu S, Ray N, Atkinson S, et al. Protein phosphatase 2A plays an essential role in migration of CD34+ cells to SDF-1/CXCL12. *J Immunol*. 2007;179:3075–3085.
185. Tao W, Hangoc G, Cooper S, et al. SDF-1/CXCL12 enhances retroviral-mediated gene transfer into human and murine hematopoietic progenitor cells. *Gene Ther*. 2004;11:61–69.
186. Broxmeyer HE, Orschell CM, Clapp DW, et al. Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J Exp Med*. 2005;201:1307–1318.
187. Liles WC, Rodger E, Broxmeyer HE, et al. Augmented mobilization and collection of CD34+ hematopoietic cells from normal human volunteers stimulated with G-CSF by single-dose administration of AMD3100, a CXCR4 antagonist. *Transfusion*. 2005;45:295–300.
188. Broxmeyer HE, Cooper S, Hangoc G, et al. Stromal cell derived factor-1/CXCL12 selectively counteracts inhibitory effects of myelosuppressive chemokines on hematopoietic progenitor cell proliferation in vitro. *Stem Cell Dev*. 2005;14:199–203.
189. Guo Y, Hangoc G, Bian H, et al. SDF-1/CXCL12 enhances survival and chemotaxis of murine embryonic stem cells and production of primitive and definitive hematopoietic progenitor cells. *Stem Cells*. 2005;23:1324–1332.
190. Broxmeyer HE, Pelus LM, Kim CH, et al. Synergistic inhibition in vivo of bone marrow myeloid progenitors by myelosuppressive chemokines, and chemokine accelerated recovery of progenitors after treatment of mice with Ara-C. *Exp Hematol*. 2006;34:1069–1077.
191. Broxmeyer HE, Mejia JAH, Hangoc G, et al. SDF-1/CXCL12 enhances in vitro replating capacity of murine and human multipotential and macrophage progenitor cells. *Stem Cell Dev*. 2007;16:589–596.
192. Altenburg JD, Broxmeyer HE, Jin Q, et al. A naturally occurring splice variant of CXCL12/stromal cell-derived factor-1 is a potent HIV inhibitor with weak chemotaxis and cell survival activity. *J Virol*. 2007;81:8140–8148.
193. Broxmeyer HE. Myelosuppressive cytokines and peptides. In: Whetton T, Gordon T, eds. *Blood Cell Biochemistry, Vol. 7: Hemopoietic Growth Factors*. London, UK: Plenum; 1996:121–150.
194. Broxmeyer HE, Mantel CR, Aronica SM. Biology and mechanisms of action of synergistically stimulated myeloid progenitor cell proliferation and suppression by chemokines. *Stem Cells*. 1997;15(suppl 1):69–78.
195. Schinke C, Giricz O, Li W, et al. IL8-CXCR2 pathway inhibition as a therapeutic strategy against MDS and AML stem cells (published correction appears in *Blood*. 2015;126(3):425. Barreryo, Laura [corrected to Barreyro, Laura]). *Blood*. 2015;125(20):3144–3152.
196. Capitano ML, Mor-Vaknin N, Saha AK, et al. Secreted nuclear DEK regulates hematopoiesis through CXCR2 signaling. *J Clin Invest*. 2019;130:2555–2570.
197. Capitano ML, Broxmeyer HE. A role for intracellular and extracellular DEK in regulating hematopoiesis. *Curr Opin Hematol*. 2017;24(4):300–306.
198. Chen Z, Huo D, Li L, et al. Nuclear DEK preserves hematopoietic stem cells potential via NCoR1/HDAC3-Akt1/2-mTOR axis. *J Exp Med*. 2021;218(5):e20201974.
199. Capitano ML, Broxmeyer HE. CXCL12/SDF-1 and hematopoiesis. In: Bradshaw RA, Stahl PD, eds. *Encyclopedia of Cell Biology*. New York: Academic Press; 2016:624-631.
200. Krathwohl MD, Hromas R, Brown DR, et al. Functional characterization of the c-c chemokine-like molecules encoded by molluscum contagiosum virus types 1 and 2. *Proc Natl Acad Sci USA*. 1997;94:9875–9880.
201. Broxmeyer HE. Iron-binding proteins and the regulation of hematopoietic cell proliferation/differentiation. In: deSousa M, Brock JH, eds. *Iron, Immunity, Cancer, and Inflammation*. Chichester: John Wiley and Sons, Ltd; 1989:199–221.
202. Broxmeyer HE, Vadhan-Raj S. Preclinical and clinical studies with the hematopoietic colony stimulating factors and related interleukins. *Immunological Res*. 8:185–201.
203. Kany S, Vollrath JT, Relja B. Cytokines in inflammatory disease. *Int J Mol Sci*. 2019;20(23):6008.
204. Schuettpelz LG, Link DC. Regulation of hematopoietic stem cell activity by inflammation. *Front Immunol*. 2013;4:204.
205. Martin P, Goldstein JD, Mermoud L, et al. IL-1 family antagonists in mouse and human skin inflammation. *Front Immunol*. 2021;12:652846.
206. Pan W, Wang Q, Chen Q. The cytokine network involved in the host immune response to periodontitis. *Int J Oral Sci*. 2019;11(3):30.
207. Karagiannidis I, Salataj E, Said Abu Egal E, et al. G-CSF in tumors: aggressiveness, tumor microenvironment and immune cell regulation. *Cytokine*. 2021;142:155479.
208. Ropa J, Trinh T, Aljoufi A, et al. Consequences of coronavirus infections for primitive and mature hematopoietic cells: new insights and why it matters [published online ahead of print Mar 1]. *Curr Opin Hematol*. 2021. https://doi.org/10.1097/MOH.0000000000000645.
209. Ropa J, Cooper S, Capitano ML, et al. Human hematopoietic stem, progenitor, and immune cells respond ex vivo to SARS-CoV-2 spike protein. *Stem Cell Rev Rep*. 2021;17(1):253–265.
210. Ratajczak MZ, Bujko K, Ciechanowicz A, et al. SARS-CoV-2 entry receptor ACE2 is expressed on very small $CD45^-$ precursors of hematopoietic and endothelial cells and in response to virus spike protein activates the Nlrp3 inflammasome. *Stem Cell Rev Rep*. 2021;17(1):266–277.
211. Fajgenbaum DC, June CH. Cytokine storm. *N Engl J Med*. 2020;383(23):2255–2273.
212. Del Valle DM, Kim-Schulze S, Huang HH, et al. An inflammatory cytokine signature predicts COVID-19 severity and survival. *Nat Med*. 2020;26(10):1636–1643.

213. Huang W, Li M, Luo G, et al. The inflammatory factors associated with disease severity to predict COVID-19 progression. *J Immunol*. 2021;206(7):1597–1608.
214. Hojyo S, Uchida M, Tanaka K, et al. How COVID-19 induces cytokine storm with high mortality. *Inflamm Regen*. 2020;40:37.
215. Bottero V, Withoff S, Verma IM. NF-kappaB and the regulation of hematopoiesis. *Cell Death Differ*. 2006;13(5):785–797.
216. Chen F, Demers LM, Shi X. Upstream signal transduction of NF-kappaB activation. *Curr Drug Targets Inflamm Allergy*. 2002;1(2):137–149.
217. Blackwell TS, Christmas JW. The role of nuclear factor-κB in cytokine gene regulation. *Am J Respir Cell Mol Biol*. 1997;17:3–9.
218. Richmond A., NF-kappa B, chemokine gene transcription and tumour growth. *Nat Rev Immunol*. 2002;2(9):664–674.
219. Liu T, Zhang L, Joo D, et al. NF-κB signaling in inflammation. *Signal Transduct Target Ther*. 2017;2:17023.
220. Taniguchi K, Karin M. NF-κB, inflammation, immunity and cancer: coming of age. *Nat Rev Immunol*. 2018;18(5):309–324.
221. Mussbacher M, Salzmann M, Brostjan C, et al. Cell type-specific roles of NF-κB linking inflammation and thrombosis. *Front Immunol*. 2019;10:85.
222. Demchenko YN, Kuehl WM. A critical role for the NFkB pathway in multiple myeloma. *Oncotarget*. 2010;1(1):59–68.
223. Gilmore TD, Herscovitch M. Inhibitors of NF-kappaB signaling: 785 and counting. *Oncogene*. 2006;25(51):6887–6899.
224. Broxmeyer HE, Williams DE, Cooper S, et al. The comparative effects *in vivo* of recombinant murine interleukin-3, natural murine colony stimulating factor-1 and recombinant murine granulocyte-macrophage colony stimulating factor on myelopoiesis in mice. *J Clin Invest*. 1987;79:721–730.
225. Williams DE, Hangoc G, Cooper S, et al. The *in vivo* effects of purified recombinant IL-3 and purified natural CSF-1 on murine high proliferative potential colony forming cells: demonstration of *in vivo* synergism. *Blood*. 1987;70:401–403.
226. Broxmeyer HE, Williams DE, Hangoc G, et al. Synergistic myelopoietic action *in vivo* of combinations of purified natural murine colony stimulating factor-1, recombinant murine interleukin-3, and recombinant murine granulocyte-macrophage colony stimulating factor administered to mice. *Proc Natl Acad Sci USA*. 1987;84:3871–3875.
227. Williams DE, Straneva JE, Cooper S, et al. Interactions between purified murine colony stimulating factors (natural CSF-1, purified recombinant GM-CSF, and purified recombinant IL-3) on the *in vitro* proliferation of purified murine granulocyte-macrophage progenitor cells. *Exp Hematol*. 1987;15:1007–1012.
228. Broxmeyer HE, Cooper S, Lu L, et al. Effect of murine mast cell growth factor (c-kit proto-oncogene ligand) on colony formation by human marrow hematopoietic progenitor cells. *Blood*. 1991;77:2142–2149.
229. Broxmeyer HE, Hangoc G, Cooper S, et al. Influence of murine mast cell growth factor (c-kit ligand) on colony formation by mouse marrow hematopoietic progenitor cells. *Exp Hematol*. 1991;19:143–146.
230. Hendrie PC, Miyazawa K, Yang YC, et al. Mast cell growth factor (c-kit ligand) enhances cytokine stimulation of proliferation of human factor dependent cell line, M07e. *Exp Hematol*. 1991;19:1031–1037.
231. Carow C, Hangoc G, Cooper S, et al. Mast cell growth factor (c-kit ligand) supports the growth of human multipotential (CFU-GEMM) progenitor cells with a high replating potential. *Blood*. 1991;78:2216–2221.
232. Broxmeyer HE, Lu L, Cooper S, et al. Flt-3-ligand stimulates/co-stimulates the growth of myeloid stem/progenitor cells. *Exp Hematol*. 1995;23:1121–1129.
233. Balduini A, Braun SE, Cornetta K, et al. Comparative effects of retroviral-mediated gene transfer into primary human stromal cells of Flt3-ligand, IL-3, and GM-CSF on production of cord blood progenitor cells in long-term culture. *Stem Cells*. 1998;16:37–49.
234. Boswell HS, Mochizuki DY, Burgess GS, et al. A novel mast cell growth factor (MCGF-3) produced by marrow-adherent cells that synergizes with interleukin 3 and interleukin 4. *Exp Hematol*. 1990;18(7):794–800.
235. Williams DE, Eisenman J, Baird A, et al. Identification of a ligand for the c-kit proto-oncogene. *Cell*. 1990;63(1):167–174.
236. Anderson DM, Lyman SD, Baird A, et al. Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms [published correction appears in *Cell*. 1990;63(5):1112]. *Cell*. 1990;63(1):235–243.
237. Zsebo KM, Wypych J, McNiece IK, et al. Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver–conditioned medium. *Cell* 1990;63(1):195–201.
238. Martin FH, Suggs SV, Langley KE, et al. Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell*. 1990;63(1):203–211.
239. Zsebo KM, Williams DA, Geissler EN, et al. Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell*. 1990;63(1):213–224.
240. Nocka K, Buck J, Levi E, et al. Candidate ligand for the c-kit transmembrane kinase receptor: KL, a fibroblast derived growth factor stimulates mast cells and erythroid progenitors. *EMBO J*. 1990;9(10):3287–3294.
241. Huang E, Nocka K, Beier DR, et al. The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. *Cell*. 1990;63(1):225–233.
242. Broxmeyer HE, Hangoc G, Cooper S, et al. Influence of murine mast cell growth factor (c-kit ligand) on colony formation by mouse marrow hematopoietic progenitor cells. *Exp Hematol*. 1991;19(2):143–146.
243. Broxmeyer HE, Cooper S, Lu L, et al. Effect of murine mast cell growth factor (c-kit proto-oncogene ligand) on colony formation by human marrow hematopoietic progenitor cells. *Blood*. 1991;77(10):2142–2149.
244. Hendrie PC, Miyazawa K, Yang YC, et al. Mast cell growth factor (c-kit ligand) enhances cytokine stimulation of proliferation of human factor dependent cell line, M07e. *Exp Hematol*. 1991;19:1031–1037.
245. Maze R, Horie M, Hendrie P, et al. Differential responses of myeloid progenitor cells from patients with myeloid leukemia and myelodysplasia to the costimulating effects of steel factor *in vitro*. *Exp Hematol*. 1993;21:545–551.
246. Miyazawa K, Hendrie PC, Mantel C, et al. Comparative analysis of signaling pathways between mast cell growth factor (c-kit ligand) and granulocyte-macrophage colony stimulating factor in a human factor-dependent myeloid cell line involves phosphorylation of Raf-1, GTPase-activating protein and mitogen-activated protein kinase. *Exp Hematol*. 1991;19:1110–1123.
247. Miyazawa K, Williams DA, Gotoh A, et al. Membrane-bound steel factor induces more persistent tyrosine kinase activation and longer life span of c-kit encoded protein than its soluble form. *Blood*. 1995;85:641–649.
248. Lee YH, Broxmeyer HE. Synergistic activation of RSK correlates with c-fos induction in MO7e cells stimulated with GM-CSF plus steel factor. *Biochem Biophys Res Comm*. 2001;281:897–901.
249. Takahira H, Gotoh A, Ritchie A, et al. Steel factor enhances integrin-mediated tyrosine phosphorylation of focal adhesion kinase ($pp125^{FAK}$) and paxillin. *Blood*. 1997;89:1574–1584.
250. Lyman SD, James L, Vanden Bos T, et al. Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. *Cell*. 1993;75(6):1157–1167.
251. Rosnet O, Marchetto S, deLapeyriere O, et al. Murine Flt3, a gene encoding a novel tyrosine kinase receptor of the PDGFR/CSF1R family. *Oncogene*. 1991;6(9):1641–1650.
252. Lyman SD, James L, Zappone J, et al. Characterization of the protein encoded by the flt3 (flk2) receptor-like tyrosine kinase gene. *Oncogene*. 1993;8(4):815–822.
253. Hannum C, Culpepper J, Campbell D, et al. Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of haematopoietic stem cells and is encoded by variant RNAs. *Nature*. 1994;368(6472):643–648.
254. Lyman SD, James L, Johnson L, et al. Cloning of the human homologue of the murine flt3 ligand: a growth factor for early hematopoietic progenitor cells. *Blood*. 1994;83(10):2795–2801.
255. Broxmeyer HE, Lu L, Cooper S, et al. Flt-3-ligand stimulates/co-stimulates the growth of myeloid stem/progenitor cells. *Exp Hematol*. 1995;23:1121–1129.
256. Takahira H, Lyman SD, Broxmeyer HE. Flt3-ligand prolongs survival of CD34+++ human umbilical cord blood myeloid progenitors in serum-free culture medium. *Ann Hematol*. 1996;72:131–135.
257. Fukuda S, Broxmeyer HE, Pelus LM. Flt3-ligand and Flt3 regulate hematopoietic cell migration by modulating the SDF-1α (CXCL12)/CXCR4 axis. *Blood*. 2005;105:3117–3126.
258. Lisovsky M, Braun S, Ge Y, et al. Flt3-ligand production by human bone marrow stromal cells. *Leukemia*. 1996;10:1012–1018.
259. Braun SE, Aronica SM, Ge Y, et al. Retroviral-mediated gene transfer of Flt3 ligand enhances proliferation and MAP kinase activity of AML5 cells. *Exp Hematol*. 1997;25:51–56.
260. Spangler JB, Moraga I, Mendoza JL, et al. Insights into cytokine-receptor interactions from cytokine engineering. *Annu Rev Immunol*. 2015;33:139–167.
261. Li AW, Lim WA. Engineering cytokines and cytokine circuits. *Science*. 2020;370(6520):1034–1035.
262. Curtis BM, Williams DE, Broxmeyer HE, et al. Enhanced hematopoietic activity of a human GM-CSF/IL-3 fusion protein. *Proc Natl Acad Sci USA*. 1991;88:5809–5813.
263. Broxmeyer HE, Lu L, Hangoc G, et al. CD45 cell surface antigens are linked to stimulation of early human myeloid progenitor cells by IL-3, GM-CSF, a GM-CSF/IL-3 fusion protein and mast cell growth factor (a c-kit ligand). *J Exp Med*. 1991;174:447–458.
264. Broxmeyer HE, Benninger L, Cooper S, et al. Effects of *in vivo* treatment with PIXY321 (GM-CSF/IL-3 fusion protein) on proliferation kinetics of bone marrow and blood myeloid progenitor cells in patients with sarcoma. *Exp Hematol*. 1995;23:335–340.
265. Vadhan-Raj S, Papadopoulous NE, Burgess MA, et al. Effects of PIXY321 (granulocyte-macrophage colony stimulating factor/ interleukin-3 fusion protein) on chemotherapy-induced multilineage myelosuppression in patients with sarcoma. *J Clin Oncol*. 1994;12:715–724.

266. Lu L, Xiao M, Li ZH, et al. Influence *in vitro* of IL-3/Epo fusion proteins compared with the combination of IL-3 plus Epo in enhancing the proliferation of single isolated erythroid and multipotential progenitor cells from human umbilical cord blood and adult bone marrow. *Exp Hematol*. 1995;23:1130–1134.
267. Broxmeyer HE, Milano F. *Cord Blood Transplantation: State of the Science*. Cord Blood Association (CBA); 2020. http://www.cb-association.org/state-of-the-science.
268. Mayani H, Wagner JE, Broxmeyer HE. Cord blood research, banking, and transplantation: achievements, challenges, and perspectives. *Bone Marrow Transpl*. 2020;55(1):48–61.
269. Huang X, Guo B, Capitano M, et al. Past, present, and future efforts to enhance the efficacy of cord blood hematopoietic cell transplantation. *F1000Res*. 8:F1000–F1883.
270. Guo B, Huang X, Chen Y, Broxmeyer HE. Ex vivo expansion and homing of human cord blood hematopoietic stem cells. In: Zhao M, Qian P, eds. *Hematopoietic Stem Cells – Keystone of Tissue Development and Regenerative Medicine*. Springer Nature Publishers; 2020.
271. Delaney C, Heimfeld S, Brashem-Stein C, et al. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. *Nat Med*. 2010;16(2):232–236.
272. Islam P, Horwitz ME. Small-molecule nicotinamide for ex vivo expansion of umbilical cord blood. *Exp Hematol*. 2019;80:11–15.
273. Boitano AE, Wang J, Romeo R, et al. Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells [published correction appears in *Science*. 2011;332(6030):664]. *Science*. 2010;329(5997):1345–1348.
274. Fares I, Chagraoui J, Gareau Y, et al. Cord blood expansion. Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. *Science*. 2014;345(6203):1509–1512.
275. Guo B, Huang X, Lee MR, et al. Antagonism of PPAR-γ signaling expands human hematopoietic stem and progenitor cells by enhancing glycolysis. *Nat Med*. 2018;24(3):360–367.
276. Wang X, Broxmeyer HE. DUSP16 is a regulator of human hematopoietic stem and progenitor cells and promotes their expansion ex vivo [published online ahead of print Oct 19]. *Leukemia* 2020. https://doi.org/10.1038/s41375-020-01064-6.
277. Catelli LF, Saad STO. Ex vivo manufacture of megakaryocytes and platelets from stem cells: recent advances toward transfusion in humans. *Stem Cells Dev*. 2021;30(7):351–362.
278. Bari S, Zhong Q, Fan X, et al. Ex vivo expansion of CD34+ CD90+ CD49f+ hematopoietic stem and progenitor cells from non-enriched umbilical cord blood with azole compounds. *Stem Cells Transl Med*. 2018;7(5):376–393.
279. Li Z, Qian P, Shao W, et al. Suppression of m^{6}A reader Ythdf2 promotes hematopoietic stem cell expansion [published correction appears in *Cell Res*. 2018]. *Cell Res*. 2018;28(9):904–917.
280. Zhang J, Huang X, Guo B, et al. Effects of eupalinilide E and UM171, alone and in combination on cytokine stimulated ex-vivo expansion of human cord blood hematopoietic stem cells. *Blood Cells Mol Dis*. 2020;84:102457.
281. Stiff PJ, Montesinos P, Peled T, et al. Cohort-controlled comparison of umbilical cord blood transplantation using carlecortemcel-L, a single progenitor-enriched cord blood, to double cord blood unit transplantation. *Biol Blood Marrow Transpl*. 2018;24(7):1463–1470.
282. Wagner JE Jr, Brunstein CG, Boitano AE, et al. Phase I/II trial of StemRegenin-1 expanded umbilical cord blood hematopoietic stem cells supports testing as a stand-alone graft. *Cell Stem Cell*. 2016;18(1):144–155.
283. Horwitz ME, Chao NJ, Rizzieri DA, et al. Umbilical cord blood expansion with nicotinamide provides long-term multilineage engraftment. *J Clin Invest*. 2014;124(7):3121–3128.
284. Cohen S, Roy J, Lachance S, et al. Hematopoietic stem cell transplantation using single UM171-expanded cord blood: a single-arm, phase 1–2 safety and feasibility study. *Lancet Haematol*. 2020;7(2):e134–e145.
285. Zimran E, Papa L, Hoffman R. Ex vivo expansion of hematopoietic stem cells: finally transitioning from the lab to the clinic. *Blood Rev*. 2021;50:100853.
286. Cutler C, Multani P, Robbins D, et al. Prostaglandin-modulated umbilical cord blood hematopoietic stem cell transplantation. *Blood*. 2013;122(17):3074–3081.
287. Broxmeyer HE. Cytokine interactions regulating myelopoiesis/erythropoiesis. In: Freund M, Link H, Welte K, eds. *Cytokines in Hemopoiesis, Oncology and AIDS*. Berlin: Springer-Verlag; 1990:9–13.
288. Broxmeyer HE. Regulation of hematopoietic progenitor cell growth by stimulating and suppressing cytokines. In: Sachs L, Abraham NG, Weidemann C, et al., eds. *Molecular Biology of Hematopoiesis*. Andover, UK: Intercept; 1990:161–166.
289. Broxmeyer HE. The interacting effects of cytokines on hematopoietic stem and progenitor cells. In: Mertelsmann R, Herrmann F, eds. *Hematopoietic Growth Factors in Clinical Application*. New York: Marcel Dekker Inc; 1990:3–24.
290. Broxmeyer HE. Cytokine interactions on myeloid progenitor cells. In: Metcalf D, ed. *Therapeutic Strategies in Oncology. Cytokine Interactions; Implications for Cancer*. 8th ed. London: Mediscript; 1991:7–24.
291. Broxmeyer HE. H-ferritin: a regulatory cytokine that down modulates cell proliferation. *J Lab Clin Med*. 1992;120:367–370.
292. Broxmeyer HE. Interactions of colony stimulating factors, other modulating cytokines and hematopoietic progenitor cells. Laboratory and clinical studies. *Leukemia* 6:38-40.
293. Broxmeyer HE. New hematopoietic cytokines. In: Schattanuer L, Gadner FK, eds. *Haematology Trends*. Stuttgart, Germany: Schattauer Publishers; 1993:265–279.
294. Broxmeyer HE. Myelosuppressive cytokines and peptides. In: Whetton T, Gordon T, eds. *Blood Cell Biochemistry, Vol. 7: Hemopoietic Growth Factors*. London, UK: Plenum; 1996:121–150.
295. Broxmeyer HE, de Sousa M, Smithyman A, et al. Specificity and modulation of the action of the lactoferrin, a negative feedback regulator of myelopoiesis. *Blood*. 1980;55:324–333.
296. Broxmeyer HE. Lactoferrin acts on Ia-like antigen positive subpopulations of human monocytes to inhibit production of colony stimulatory activity *in vitro*. *J Clin Invest*. 1979;64:1717–1720.
297. Broxmeyer HE, Ralph P, Bognacki J, et al. A subpopulation of human polymorphonuclear neutrophils contains an active form of lactoferrin capable of inhibiting production of granulocyte-macrophage colony stimulating activities by human monocytes. *J Immunol*. 1980;125:903–909.
298. Pelus LM, Broxmeyer HE, Moore MAS. Regulation of human myelopoiesis by prostaglandin E and lactoferrin. *Cell Tissue Kinet*. 1981;14:515–526.
299. Pelus LM, Broxmeyer HE, de Sousa M, et al. Heterogeneity among resident murine peritoneal macrophages: separation and functional characterization of monocytoid cells producing granulocyte-macrophage colony stimulating factor (GM-CSF), and responding to regulation by lactoferrin. *J Immunol*. 1981;126:1016–1021.
300. Steinmann G, Broxmeyer HE, de Harven E, et al. Immuno-electron microscopic tracing of lactoferrin, a regulator of myelopoesis, into a subpopulation of human peripheral blood monocytes. *Br J Haematol*. 1982;50:75–84.
301. Bagby GC Jr, Rigas VD, Bennett RM, et al. Interaction of lactoferrin, monocytes, and T lymphocyte subsets in the regulation of steady-state granulopoiesis in vitro. *J Clin Invest*. 1981;68(1):56–63.
302. Bagby GC Jr, McCall E, Bergstrom KA, et al. A monokine regulates colony-stimulating activity production by vascular endothelial cells. *Blood*. 1983;62(3):663–668.
303. Broxmeyer HE, Platzer E. Lactoferrin acts on I-A and I-E/c antigen-positive subpopulations of mouse peritoneal macrophages in the absence of T-lymphocytes and other cell types to inhibit production of granulocyte-macrophage colony stimulatory factors *in vitro*. *J Immunol*. 1984;133:306–314.
304. Lu L, Broxmeyer HE, Moore MAS, et al. Abnormalities in myelopoietic regulatory interactions with acidic isoferritins and lactoferrin in mice infected with Friend virus complex: associations with altered expression of Ia-antigens on effector and responding cells. *Blood*. 1985;65:91–99.
305. Broxmeyer HE, Bicknell DC, Gillis S, et al. Lactoferrin: Affinity purification from human milk and polymorphonuclear neutrophils using monoclonal antibody (II2C) to human lactoferrin, development of an immunoradiometric assay using II2C, and myelopoietic regulation and receptor-binding characteristics. *Blood Cells*. 1986;11:429–446.
306. Broxmeyer HE, Williams DE, Hangoc G, et al. The opposing action *in vivo* on murine myelopoiesis of purified preparations of lactoferrin and the colony stimulating factors. *Blood Cells*. 1987;13:31–48.
307. Lu L, Hangoc G, Chen LT, et al. The protective influence of lactoferrin on mice infected with the polycythemia inducing strain of the Friend virus complex. *Cancer Res*. 1987;47:4184–4188.
308. Berman E, Rubin BY, Broxmeyer HE. Regulation of the proliferation of the established human monoblast cell line, U937, at the single cell level. *Cancer Res*. 1986;46:3309–3312.
309. Zucali JR, Broxmeyer HE, Levy D, et al. Lactoferrin decreases monocyte induced fibroblast production of myeloid colony stimulating activity by suppressing monocyte release of interleukin 1. *Blood*. 1989;74:1531–1536.
310. Miyazawa K, Mantel C, Lu L, et al. Lactoferrin-lipopolysaccharide interactions: effects on lactoferrin binding to monocyte/macrophage differentiated HL-60 cells. *J Immunol*. 1991;146:723–729.
311. Broxmeyer HE, Bicknell DC, Cooper S, et al. Qualitative functional deficiency of affinity purified lactoferrin from neutrophils and patients with chronic myelogenous leukemia, and lactoferrin/H-ferritin cell-interactions

in a patient with lactoferrin-deficiency with normal numbers of circulating leukocytes. *Pathobiol*. 1991;59:26–35.
312. Broxmeyer HE, Cooper S, Hangoc G, et al. Stromal cell derived factor-1/CXCL12 selectively counteracts inhibitory effects of myelosuppressive chemokines on hematopoietic progenitor cell proliferation in vitro. *Stem Cell Dev*. 2005;14:199–203.
313. Gentile P, Broxmeyer HE. Suppression of mouse myelopoiesis by administration of human lactoferrin *in vivo* and the comparative action of human transferrin. *Blood*. 1983;61:982–993.
314. Broxmeyer HE, Lu L, Bognacki J. Transferrin, derived from an OKT8-positive subpopulation of T-lymphocytes, suppresses the production of granulocyte-macrophage colony stimulatory factors from mitogen-activated T-lymphocytes. *Blood*. 1983;62:37–50.
315. Lu L, Bicknell DC, Piacibello W, et al. Purified human transferrin and "Transferrin" released from sorted T8+ lymphocytes suppress release of granulocyte-macrophage colony stimulating factors from sorted T4+ lymphocytes stimulated by phytohemagglutinin. *Exp Hematol*. 1986;14:955–962.
316. Broxmeyer HE, Bognacki J, Dorner MH, et al. The identification of leukemia-associated inhibitory activity (LIA) as acidic isoferritins: a regulatory role for acidic isoferritins in the production of granulocytes and macrophages. *J Exp Med*. 1981;153:1426–1444.
317. Broxmeyer HE. Relationship of cell-cycle expression of Ia-like antigen determinants on normal and leukemia human granulocyte-macrophage progenitor cells to regulation *in vitro* by acidic isoferritins. *J Clin Invest*. 1982;69:632–642.
318. Broxmeyer HE, Bognacki J, Ralph P, et al. Monocyte-macrophage derived acidic isoferritins: Normal feedback regulators of granulocyte-macrophage progenitor cells. *Blood*. 1982;60:595–607.
319. Broxmeyer HE. Detection of Ia-antigens for I-A and I-E/C subregions on mouse granulocyte-macrophage progenitor cells during DNA-synthesis: association with the action of acidic isoferritins *in vitro*. *J Immunol*. 1982;129:1002–1007.
320. Broxmeyer HE. Influence of acidic isoferritins and E-type prostaglandins on detection of the cycling status of granulocyte-macrophage progenitor cells. *Blood*. 1982;60:1042–1045.
321. Dorner MH, Broxmeyer HE, Silverstone A, et al. Biosynthesis of ferritin subunits from different cell lines of HL-60 human promyelocytic leukemia cells and the release of acidic isoferritin-inhibitory activity against normal granulocyte-macrophage progenitor cells. *Br J Haematol*. 1983;55:47–58.
322. Oblon DJ, Broxmeyer HE, Vellis K. Acidic isoferrin-inhibitory activity: a normal granulopoietic regulator within long term mouse bone marrow cultures. *Leukemia Res*. 1983;7:581–590.
323. Broxmeyer HE, Gentile P, Cooper S, et al. Functional activities of acidic isoferritins and lactoferrin *in vitro* and *in vivo*. *Blood Cells*. 1984;10:397–426.
324. Broxmeyer HE, Juliano L, Waheed A, et al. Release from mouse macrophages of acidic isoferritins that suppress hematopoietic progenitor cells is induced by purified L-cell colony stimulating factors and suppressed by human lactoferrin. *J Immunol*. 1985;135:3224–3231.
325. Broxmeyer HE, Lu L, Bicknell DC, et al. The influence of purified recombinant human acidic and basic isoferritins on colony formation *in vitro* by granulocyte-macrophage and erythroid progenitor cells. *Blood*. 1986;68:1257–1263.
326. Broxmeyer HE, Juliano L, Lu L, et al. HLA-DR human histocompatibility leukocyte antigens-restricted lymphocyte-monocyte interactions in the release from monocytes of acidic isoferritins that suppress hematopoietic progenitor cells. *J Clin Invest*. 1983;73:939–953.
327. Dezza L, Cazzola M, Piacibello W, et al. Effect of acidic and basic isoferritins on in vitro growth of human granulocyte-monocyte progenitors. *Blood*. 1986;67(3):789–795.
328. Moore RN, Joshi JG, Deana DG, et al. Characterization of a two-signal-dependent, Ia+ mononuclear phagocyte progenitor subpopulation that is sensitive to inhibition by ferritin. *J Immunol*. 1986;136(5):1605–1611.
329. Dezza L, Cazzola M, Bergamaschi G, et al. Effects of recombinant human H-subunit and L-subunit ferritins on in vitro growth of human granulocyte-monocyte progenitors. *Br J Haematol*. 1988;68(3):367–372.
330. Williams DE, Cooper S, Broxmeyer HE. The effects of hematopoietic suppressor molecules on the *in vitro* proliferation of purified murine granulocyte-macrophage progenitor cells (CFU-GM). *Cancer Res*. 1988;48:1548–1550.
331. Broxmeyer HE, Cooper S, Levi S, et al. Mutated recombinant human heavy-chain ferritins and myelosuppression *in vitro* and *in vivo*: a link between ferritin ferroxidase activity and biological function. *Proc Natl Acad Sci USA*. 1991;88:770–774.
332. Morikawa M, Derynck R, Miyazono K. TGF-β and the TGF-β family: context-dependent roles in cell and tissue physiology. *Cold Spring Harb Perspect Biol*. 2016;8(5):a021873.
333. Heldin C-H, Moustakas A. Signaling receptors for TGF-β family members. *Cold Spring Harb Perspect Biol*. 2016;8(8):a022053.
334. Roberts AB, Lamb LC, Newton DL, et al. Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction. *Proc Natl Acad Sci USA*. 1980;77(6):3494–3498.
335. Keller JR, Mantel C, Sing GK, et al. Transforming growth factor beta 1 selectively regulates early murine hematopoietic progenitors and inhibits the growth of IL-3-dependent myeloid leukemia cell lines. *J Exp Med*. 1988;168(2):737–750.
336. Keller JR, Sing GK, Ellingsworth LR, et al. Transforming growth factor beta: possible roles in the regulation of normal and leukemic hematopoietic cell growth. *J Cell Biochem*. 1989;39(2):175–184.
337. Ottmann OG, Pelus LM. Differential proliferative effects of transforming growth factor-beta on human hematopoietic progenitor cells. *J Immunol*. 1988;140(8):2661–2665.
338. Sing GK, Keller JR, Ellingsworth LR, et al. Transforming growth factor beta selectively inhibits normal and leukemic human bone marrow cell growth in vitro. *Blood*. 1988;72(5):1504–1511.
339. Aglietta M, Stacchini A, Severino A, et al. Interaction of transforming growth factor-beta 1 with hemopoietic growth factors in the regulation of human normal and leukemic myelopoiesis. *Exp Hematol*. 1989;17(3):296–299.
340. Dubois CM, Ruscetti FW, Palaszynski EW, et al. Transforming growth factor beta is a potent inhibitor of interleukin 1 (IL-1) receptor expression: proposed mechanism of inhibition of IL-1 action. *J Exp Med*. 1990;172(3):737–744.
341. Ruscetti FW, Jacobsen SE, Birchenall-Roberts M, et al. Role of transforming growth factor β1 in regulation of hematopoiesis. *Ann NY Acad Sci*. 1990;628:31–43.
342. Lu L, Heinrich MC, He J, et al. Retroviral mediated gene transduction of c-kit into single hematopoietic progenitor cells from cord blood enhances erythroid colony formation and decreases sensitivity to inhibition by TNF-α and TGF-β. *Blood*. 1999;94:2319–2332.
343. Lu L, Wang LS, Cooper RJ, et al. Suppressive effects of TNF-α, TGF-β1 and chemokines on megakaryocytic colony formation in CD34+ cells derived from umbilical cord blood compared with mobilized peripheral blood and bone marrow. *J Hematotherapy Stem Cell Res*. 2000;9:195–204.
344. Li G, Abediankenari S, Kim YJ, et al. TGF-β combined with M-CSF induces generation of immune inhibitory cord blood dendritic cells capable of enhancing cytokine-stimulated ex-vivo expansion of myeloid progenitors. *Blood*. 2007;110:2872–2879.
345. Basu S, Broxmeyer HE. Transforming growth factor-β1 modulates responses of $CD34^+$ cord blood cells to stromal cell derived factor-1/CXCL12. *Blood*. 2005;106:485–493.
346. Zhang L, Luo Q, Shu Y, et al. Transcriptomic landscape regulated by the 14 types of bone morphogenetic proteins (BMPs) in lineage commitment and differentiation of mesenchymal stem cells (MSCs). *Genes Dis*. 2019;6(3):258–275.
347. Mostafa S, Pakvasa M, Coalson E, et al. The wonders of BMP9: from mesenchymal stem cell differentiation, angiogenesis, neurogenesis, tumorigenesis, and metabolism to regenerative medicine. *Genes Dis*. 2019;6(3):201–223.
348. Yan S, Zhang R, Wu K, et al. Characterization of the essential role of bone morphogenetic protein 9 (BMP9) in osteogenic differentiation of mesenchymal stem cells (MSCs) through RNA interference. *Genes Dis*. 2018;5(2):172–184.
349. Zhang H, Kozono DE, O'Connor KW, et al. TGF-β inhibition rescues hematopoietic stem cell defects and bone marrow failure in Fanconi anemia. *Cell Stem Cell*. 2016;18(5):668–681.
350. Namwanje M, Brown CW. Activins and inhibins: roles in development, physiology, and disease. *Cold Spring Harb Perspect Biol*. 2016;8(7):a021881.
351. Aykul S, Corpina RA, Goebel EJ, et al. Activin A forms a non-signaling complex with ACVR1 and type II Activin/BMP receptors via its finger 2 tip loop [published online ahead of print Jun 9]. *Elife*. 2020;9:e54582.
352. Broxmeyer HE, Lu L, Cooper S, et al. Selective and indirect modulation of human hematopoietic progenitor cell proliferation by recombinant human activin and inhibin. *Proc Natl Acad Sci USA*. 1988;85:9052–9056.
353. Broxmeyer HE, Hangoc G, Zucali JR, et al. Effects *in vivo* of purified recombinant human activin and erythropoietin, alone and in combination, in mice. *Int J Hematol*. 1991;54:447–454.
354. Hangoc G, Carow CE, Schwall R, et al. Effects in vivo of recombinant human inhibin on myelopoiesis in mice. *Exp Hematol*. 1992;20:1243–1246.
355. Shav-Tal Y, Zipori D. The role of activin a in regulation of hemopoiesis. *Stem Cells*. 2002;20(6):493–500.

356. Brazil DP, Church RH, Surae S, et al. BMP signalling: agony and antagony in the family. *Trends Cell Biol*. 2015;25(5):249–264.
357. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell*. 2001;104(4):487–501.
358. Wallach D. The TNF cytokine family: one track in a road paved by many. *Cytokine*. 2013;63(3):225–229.
359. Beutler B, Cerami A. Cachectin and tumour necrosis factor as two sides of the same biological coin. *Nature*. 1986;320(6063):584–588.
360. Taylor GA, Carballo E, Lee DM, et al. A pathogenetic role for TNFα in the syndrome of cachexia, arthritis and autoimmunity resulting from tristetraprolin (TTP) deficiency. *Immunity*. 1996;4:445–454.
361. Degliantoni G, Murphy M, Kobayashi M, et al. Natural killer (NK) cell-derived hematopoietic colony-inhibiting activity and NK cytotoxic factor. Relationship with tumor necrosis factor and synergism with immune interferon. *J Exp Med*. 1985;162(5):1512–1530.
362. Broxmeyer HE, Williams DE, Lu L, et al. The suppressive influences of human tumor necrosis factor on bone marrow hematopoietic cells from normal donors and patients with leukemia: synergism of tumor necrosis factor and gamma interferon. *J Immunol*. 1986;136:4487–4495.
363. Lu L, Welte K, Gabrilove JL, et al. Effects of recombinant human tumor necrosis factor alpha, recombinant human interferon-gamma and prostaglandin E on colony formation of human hematopoietic progenitor cells stimulated by natural human pluripotent colony stimulating factor, pluripoietin-alpha and recombinant erythropoietin in serum-free cultures. *Cancer Res*. 1986;46:4357–4361.
364. Clapes T, Lefkopoulos S, Trompouki E. Stress and non-stress roles of inflammatory signals during HSC emergence and maintenance. *Front Immunol*. 2016;7:487.
365. Zucali JR, Broxmeyer HE, Gross MA, et al. Recombinant human tumor necrosis factors (TNF-alpha and TNF-beta) stimulate fibroblasts to produce hematopoietic growth factors *in vitro*. *J Immunol*. 1988;140:840–844.
366. Lu L, Walker D, Graham CD, et al. Enhancement of release from MHC-class II antigen-positive monocytes of hematopoietic colony stimulating factors by recombinant human tumor necrosis factor-alpha: synergism with recombinant human interferon gamma. *Blood*. 1988;72:34–41.
367. Lu L, Srour EF, Warren DJ, et al. Enhancement of release of granulocyte-colony stimulating factor from sorted subsets of PHA-stimulated human T-lymphocytes by recombinant human tumor necrosis factor-alpha: synergism with recombinant human interferon-gamma. *J Immunol*. 1988;141:201–207.
368. Broxmeyer HE, Lu L, Platzer E, et al. Comparative analysis of the influences of human gamma, alpha and beta interferons on human multipotential (CFU-GEMM), erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) progenitor cells. *J Immunol*. 1985;131:1300–1305.
369. Broxmeyer HE, Piacibello W, Juliano L, et al. Gamma interferon induces colony forming cells of the human monoblast cell line U937 to respond to inhibition by lactoferrin, transferrin and acidic isoferritins. *Exp Hematol*. 1986;14:35–43.
370. Piacibello W, Rubin BY, Broxmeyer HE. Prostaglandin E counteracts modulation of the gamma interferon induction of Ia antigens on U937 cells and induction of responsiveness of U937 colony forming cells to suppression by lactoferrin, transferrin, acidic isoferritins and prostaglandin E. *Exp Hematol*. 1986;14:44–50.
371. Broxmeyer HE, Rubin BY, Berman E, et al. Activities derived from established human myeloid cell lines reverse the suppression of cell line colony formation by lactoferrin and transferrin. *Exp Hematol*. 1986;14:51–59.
372. Piacibello W, Lu L, Aglietta M, et al. Human gamma interferon enhances release of granulocyte-macrophage colony stimulating factors from human T4+-lymphocytes stimulated with phytohemogglutin. *Blood*. 1986;68:1339–1347.
373. Fagiani E, Christofori G. Angiopoietins in angiogenesis. *Cancer Lett*. 2013;328(1):18–26.
374. Broxmeyer HE, Srour EF, Cooper S, et al. Angiopoietin-like-2 and -3 act through their coiled-coil domains to enhance survival and replating capacity of human cord blood hematopoietic progenitors. *Blood Cells Mol Dis*. 2012;48:25–29.
375. Cao H. Adipocytokines in obesity and metabolic disease. *J Endocrinol*. 2014;220(2):T47–T59.
376. Yarden Y. The EGFR family and its ligands in human cancer. signaling mechanisms and therapeutic opportunities. *Eur J Cancer*. 2001;37(suppl 4):S3–S8.
377. Beenken A, Mohammadi M. The FGF family: biology, pathophysiology and therapy. *Nat Rev Drug Discov*. 2009;8(3):235–253.
378. Noonan ML, Ni P, Agoro R, et al. HIF-PHI BAY85–3934 (Molidstat) improves anemia and is associated with reduced levels of circulating FGF23 in a CKD mouse model. *J Bone Miner Res*. 2021;36(6) 1117–1130.
379. Ishii S, Suzuki T, Wakahashi K, et al. FGF-23 from erythroblasts promotes hematopoietic progenitor mobilization. *Blood*. 2021;137(11):1457–1467.
380. Ran Q, Guo C, Sun C, et al. Loss of FGFR3 accelerates bone marrow suppression-induced hematopoietic stem and progenitor cell expansion by activating FGFR1-ELK1-cyclin D1 signaling. *Transplant Cell Ther*. 2021;27(1):45.e1–45.e10.
381. Fang T, Zhang Y, Chang VY, et al. Epidermal growth factor receptor-dependent DNA repair promotes murine and human hematopoietic regeneration. *Blood*. 2020;136(4):441–454.
382. Airaksinen MS, Saarma M. The GDNF family: signalling, biological functions and therapeutic value. *Nat Rev Neurosci*. 2002;3(5):383–394.
383. Li H, Lim HC, Zacharaki D, et al. Early growth response 1 regulates hematopoietic support and proliferation in human primary bone marrow stromal cells. *Haematologica*. 2020;105(5):1206–1215.
384. Broxmeyer HE, Cooper S, Li ZH, et al. Myeloid progenitor cell regulatory effects of vascular endothelial cell growth factor, a ligand for the tyrosine kinase receptor *Flk-1*. *Int J Hematol*. 1995;62:203–215.
385. Broxmeyer HE, Cooper S, Li ZH, et al. Macrophage-stimulating protein, a ligand for the *RON* receptor protein tyrosine kinase, suppresses myeloid progenitor cell proliferation and synergizes with vascular endothelial cell growth factor and members of the chemokine family. *Ann Hematol*. 1996;73:1–9.
386. Broxmeyer HE, Bruns H, Zhang S, et al. Th1 cells regulate hematopoietic progenitor cell homeostasis by production of oncostatin M. *Immunity*. 2002;16:815–825.
387. Martiney JA, Sherry B, Metz CN, et al. Macrophage migration inhibitory factor release by macrophages after ingestion of *Plasmodium chabaudi*-infected erythrocytes: Possible role in the pathogenesis of malarial anemia. *Infect Immun*. 2000;68:2259–2267.
388. Smith CA, Tyrell DJ, Kulkarni UA, et al. Macrophage migration inhibitory factor enhances influenza-associated mortality in mice. *JCI Insight*. 2019;4(13):e128034.
389. Deuel TF, Zhang N, Yeh HJ, et al. Pleiotrophin: a cytokine with diverse functions and a novel signaling pathway. *Arch Biochem Biophys*. 2002;397(2):162–171.
390. Himburg HA, Harris JR, Ito T, et al. Pleiotrophin regulates the retention and self-renewal of hematopoietic stem cells in the bone marrow vascular niche [published correction appears in *Cell Rep*. 2012;2(6):1774]. *Cell Rep*. 2012;2(4):964–975.
391. Himburg HA, Termini CM, Schlussel L, et al. Distinct bone marrow sources of pleiotrophin control hematopoietic stem cell maintenance and regeneration. *Cell Stem Cell*. 2018;23(3):370–381.
392. Himburg HA, Roos M, Fang T, et al. Chronic myeloid leukemia stem cells require cell-autonomous pleiotrophin signaling. *J Clin Invest*. 2020;130(1):315–328.
393. Trinh T, Broxmeyer HE. Role for leptin and leptin receptors in stem cells during health and diseases]. *Stem Cell Rev Rep*. 2021:1–12.
394. Trinh T, Ropa J, Aljoufi A, et al. Leptin receptor, a surface marker for a subset of highly engrafting long-term functional hematopoietic stem cells [published online ahead of print Nov 7]. *Leukemia*. 2021;35:2064–2075.
395. Kurland J, Bockman R, Broxmeyer HE, et al. Limitation of excessive myelopoiesis by the intrinsic modulation of macrophage-derived prostaglandin E. *Science*. 1978;199:552–555.
396. Kurland JI, Broxmeyer HE, Bockman RS, et al. Role for the monocyte-macrophage colony stimulating factor and prostaglandin E in the positive and negative feedback control of myeloid stem cell proliferation. *Blood*. 1978;52:388–407.
397. Pelus LM, Broxmeyer HE, Kurland JI, et al. Regulation of macrophage and granulocyte proliferation: specificities of prostaglandin E and lactoferrin. *J Exp Med*. 1979;150:277–292.
398. Pelus LM, Broxmeyer HE, Clarkson BD, et al. Abnormal responsiveness of colony forming cells (CFU-C) from patients with chronic myeloid leukemia to inhibition by prostaglandin E. *Cancer Res*. 1980;40:2512–2515.
399. Pelus LM, Broxmeyer HE, Moore MAS. Regulation of human myelopoiesis by prostaglandin E and lactoferrin. *Cell Tissue Kinet*. 1981;14:515–526.
400. Broxmeyer HE. Influence of acidic isoferritins and E-type prostaglandins on detection of the cycling status of granulocyte-macrophage progenitor cells. *Blood*. 1982;60:1042–1045.
401. Lu L, Pelus LM, Broxmeyer HE. Modulation of expression of Ia-antigens and the proliferation of human erythroid (BFU-E) and multipotential (CFU-GEMM) progenitor cells by prostaglandin E. *Exp Hematol*. 1984;12:741–748.
402. Piacibello W, Rubin BY, Broxmeyer HE. Prostaglandin E counteracts modulation of the gamma interferon induction of Ia antigens on U937 cells and induction of responsiveness of U937 colony forming cells to suppression by lactoferrin, transferrin, acidic isoferritins and prostaglandin E. *Exp Hematol*. 1986;14:44–50.
403. Lu L, Pelus LM, Piacibello W, et al. Prostaglandin E acts at two levels to enhance colony formation in vitro by erythroid (BFU-E) progenitor cells. *Exp Hematol*. 1987;15:765–771.
404. Martínez-Colón GJ, Moore BB. Prostaglandin E_2 as a regulator of immunity to pathogens. *Pharmacol Ther*. 2018;185:135–146.

405. Broxmeyer HE, Pelus LM. Inhibition of DPP4/CD26 and dmPGE$_2$ treatment enhances engraftment of mouse bone marrow hematopoietic stem cells. *Blood Cells Mol Dis*. 2014;53:34–38.
406. Hasan AA, Hocher B. Role of soluble and membrane-bound dipeptidyl peptidase-4 in diabetic nephropathy. *J Mol Endocrinol*. 2017;59(1):R1–R10.
407. Loffek S, Schilling O, Franzke CW. Biological role of matrix metalloproteinases: a critical balance. *Eur Respiratory J*. 2011;38(1):191–208.
408. Ropa J, Broxmeyer HE. An expanded role for dipeptidyl peptidase 4 in cell regulation. *Curr Opin Hematol*. 2020;27(4):215–224.
409. Deacon CF. A review of dipeptidyl peptidase-4 inhibitors. Hot topics from randomized controlled trials. *Diabetes Obes Metab*. 2018;20(suppl 1):34–46.
410. Metzemaekers M, Van Damme J, Mortier A, et al. Regulation of chemokine activity—a focus on the role of dipeptidyl peptidase IV/CD26. *Front Immunol*. 2016;7:483.
411. Rohrborn D, Eckel J, Sell H. Shedding of dipeptidyl peptidase 4 is mediated by metalloproteases and up-regulated by hypoxia in human adipocytes and smooth muscle cells. *FEBS Lett*. 2014;588:3870–3877.
412. Mulvihill EE, Drucker DJ. Pharmacology, physiology, and mechanisms of action of dipeptidyl peptidase-4 inhibitors. *Endocr Rev*. 2014;35(6):992–1019.
413. Wang X, Cho SY, Hu CS, et al. C-X-C motif chemokine 12 influences the development of extramedullary hematopoiesis in the spleens of myelofibrosis patients. *Exp Hematol*. 2015;43(2):100–109.
414. Gupta AO, Wagner JE. Umbilical cord blood transplants: current status and evolving therapies. *Front Pediatr*. 2020;8:570282.
415. Christopherson II KW, Hangoc G, Mantel C, et al. Modulation of hematopoietic stem cell homing and engraftment by CD26. *Science*. 2004;305:1000–1003.
416. Farag SS, Srivastava S, Messina-Graham S, et al. In vivo DPP-4 inhibition to enhance engraftment of single-unit cord blood transplants in adults with hematological malignancies. *Stem Cell Dev*. 2013;22:1007–1015.
417. Velez de Mendizabal N, Strother RM, Farag SS, et al. Modelling the sitagliptin effect on dipeptidyl peptidase-4 activity in adults with haematological malignancies after umbilical cord blood haematopoietic cell transplantation. *Clin Pharmacokinet*. 2014;53(3):247–259.
418. Farag SS, Nelson R, Cairo MS, et al. High-dose sitagliptin for systemic inhibition of dipeptidylpeptidase-4 to enhance engraftment of single cord umbilical cord blood transplantation. *Oncotarget*. 2017;8(66):110350–110357.
419. Farag SS, Abu Zaid M, Schwartz JE, et al. Dipeptidyl peptidase 4 inhibition for prophylaxis of acute graft-versus-host disease. *N Engl J Med*. 2021;384(1):11–19.
420. Nelson MH, Knochelmann HM, Bailey SR, et al. Identification of human CD4$^+$ T cell populations with distinct antitumor activity. *Sci Adv*. 2020;6(27):eaba7443.
421. Ou X, O'Leary HA, Broxmeyer HE. Implications of DPP4 modification of proteins that regulate stem/progenitor and more mature cell types. *Blood*. 2013;122:161–169.
422. Broxmeyer HE, Hoggatt J, O'Leary HA, et al. Dipeptidylpeptidase 4 negatively regulates colony stimulating factor activity and stress hematopoiesis. *Nat Med*. 2012;18:1786–1796.
423. Broxmeyer HE, Capitano M, Campbell T, et al. Modulation of hematopoietic chemokine effects in vitro and in vivo by DPP4/CD26. *Stem Cell Dev*. 2016;25:575–585.
424. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. *Nature*. 2014;505(7483):327–334.
425. Castro-Malaspina H, Gay R, Resnick G, et al. Characterization of human bone marrow fibroblast colony forming cells (CFU-F) and their progeny. *Blood*. 1980;56:289–301.
426. Hangoc G, Daub R, Maze RG, et al. Regulation of myelopoiesis by fibroblasts, adipocytes and heparan sulfate. *Exp Hematol*. 1993;21:502–508.
427. Le Blanc K, Frassoni F, Ball L, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet*. 2008;371(9624):1579–1586.
428. Yu WM, Liu X, Shen J, et al. Metabolic regulation by the mitochondrial phosphatase PTPMT1 is required for hematopoietic stem cell differentiation. *Cell Stem Cell*. 2013;12(1):62–74. 3.
429. Dong L, Yu WM, Zheng H, et al. Leukaemogenic effects of *Ptpn11* activating mutations in the stem cell microenvironment. *Nature*. 2016;539:304–308.
430. Curto-Garcia N, Harrison C, McLornan DP. Bone marrow niche dysregulation in myeloproliferative neoplasms. *Haematologica*. 2020;105(5):1189–1200.
431. Siebel C, Lendahl U. Notch signaling in development, tissue homeostasis, and disease. *Physiol Rev*. 2017;97(4):1235–1294.
432. Golchin A, Seyedjafari E, Ardeshirylajimi A. Mesenchymal stem cell therapy for COVID-19: present or future. *Stem Cell Rev Rep*. 2020;16(3):427–433.
433. Saldanha-Araujo F, Melgaço Garcez E, Silva-Carvalho AE, et al. Mesenchymal stem cells: a new piece in the puzzle of COVID-19 treatment. *Front Immunol*. 2020;11:1563.
434. Huang X, Trinh T, Aljoufi A, et al. Hypoxia signaling pathway in stem cell regulation: good and evil. *Curr Stem Cell Rep*. 2018;4(2):149–157.
435. Bradley TR, Hodgson GS, Rosendaal M. The effect of oxygen tension on haemopoietic and fibroblast cell proliferation in vitro. *J Cell Physiol*. 1978;97(3 Pt 2 suppl 1):517–522.
436. Rich IN, Kubanek B. The effect of reduced oxygen tension on colony formation of erythropoietic cells in vitro. *Br J Haematol*. 1982;52(4):579–588.
437. Lu L, Broxmeyer HE. Comparative influences of phytohemagglutinin-stimulated leukocyte conditioned medium, hemin, prostaglandin E, and low oxygen tension on colony formation by erythroid progenitor cells in normal human bone marrow. *Exp Hematol*. 1985;13(10):989–993.
438. Smith S, Broxmeyer HE. The influence of oxygen tension on the long-term growth in vitro of haematopoietic progenitor cells from human cord blood. *Br J Haematol*. 1986;63(1):29–34.
439. Broxmeyer HE, Cooper S, Gabig T. The effects of oxidizing species derived from molecular oxygen on the proliferation in vitro of human granulocyte-macrophage progenitor cells. *Ann N Y Acad Sci*. 1989;554:177–184.
440. Danet GH, Pan Y, Luongo JL, et al. Expansion of human SCID-repopulating cells under hypoxic conditions. *J Clin Invest*. 2003;112(1):126–135.
441. Shin DY, Huang X, Gil CH, et al. Physioxia enhances T-cell development ex vivo from human hematopoietic stem and progenitor cells. *Stem Cell*. 2020;38(11):1454–1466.
442. Mantel CR, O'Leary HA, Chitteti BR, et al. Enhancing hematopoietic stem cell transplantation efficacy by mitigating oxygen shock. *Cell*. 2015;161(7):1553–1565.
443. Broxmeyer HE, O'Leary HA, Huang X, et al. The importance of hypoxia and extra physiologic oxygen shock/stress for collection and processing of stem and progenitor cells to understand true physiology/pathology of these cells ex vivo. *Curr Opin Hematol*. 2015;22(4):273–278.
444. Aljoufi A, Cooper S, Broxmeyer HE. Collection and processing of mobilized mouse peripheral blood at lowered oxygen tension yields enhanced numbers of hematopoietic stem cells. *Stem Cell Rev Rep*. 2020;16(5):946–953.
445. Broxmeyer HE, Capitano ML, Cooper S, et al. Numbers of long-term hematopoietic stem cells from bone marrow of fanca and fancc knockout mice can be greatly enhanced by their collection and processing in physioxia conditions. *Blood Cell Mol Dis*. 2021;86:102492.
446. Capitano ML, Mohamad SF, Cooper S, et al. Mitigating oxygen stress enhances aged mouse hematopoietic stem cell numbers and function. *J Clin Invest*. 2021;131(1):e140177.
447. Broxmeyer HE, Cooper S, Capitano ML. Enhanced collection of phenotypic and engrafting human cord blood hematopoietic stem cells at 4°C. *Stem Cell*. 2020;38(10):1326–1331.
448. Durand C, Charbord P, Jaffredo T. The crosstalk between hematopoietic stem cells and their niches. *Curr Opin Hematol*. 2018;25(4):285–289.
449. Butler JT, Abdelhamed S, Kurre P. Extracellular vesicles in the hematopoietic microenvironment. *Haematologica*. 2018;103(3):382–394.
450. Kowal J, Tkach M, Théry C. Biogenesis and secretion of exosomes. *Curr Opin Cell Biol*. 2014;29:116–125.
451. Dreyer F, Baur A. Biogenesis and functions of exosomes and extracellular vesicles. *Methods Mol Biol*. 2016;1448:201–216.
452. Hessvik NP, Llorente A. Current knowledge on exosome biogenesis and release. *Cell Mol Life Sci*. 2018;75(2):193–208.
453. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. *Science*. 2020;367(6478):eaau6977.
454. Fitzgerald W, Freeman ML, Lederman MM, et al. A system of cytokines encapsulated in extracellular vesicles. *Sci Rep*. 2018;8(1):8973.
455. Laurenzana I, Lamorte D, Trino S, et al. Extracellular vesicles: a new prospective in crosstalk between microenvironment and stem cells in hematological malignancies. *Stem Cell Int*. 2018;2018:9863194.
456. Liu Y, Shi K, Chen Y, et al. Exosomes and their role in cancer progression. *Front Oncol*. 2021;11:792.
457. Boyiadzis M, Whiteside TL. Exosomes in acute myeloid leukemia inhibit hematopoiesis. *Curr Opin Hematol*. 2018;25(4):279–284.
458. Szczepanski MJ, Szajnik M, Welsh A, et al. Blast-derived microvesicles in sera from patients with acute myeloid leukemia suppress natural killer cell function via membrane-associated transforming growth factor-beta1. *Haematologica*. 2011;96(9):1302–1309.
459. Hong CS, Muller L, Whiteside TL, et al. Plasma exosomes as markers of therapeutic response in patients with acute myeloid leukemia. *Front Immunol*. 2014;5:160.

460. Boyiadzis M, Whiteside TL. The emerging roles of tumor-derived exosomes in hematological malignancies. *Leukemia*. 2017;31(6):1259–1268.
461. Shah R, Patel T, Freedman JE. Circulating extracellular vesicles in human disease. *N Engl J Med*. 2018;379(10):958–966.
462. Kalluri R. The biology and function of exosomes in cancer. *J Clin Invest*. 2016;126(4):1208–1215.
463. Hong CS, Sharma P, Yerneni SS, et al. Circulating exosomes carrying an immunosuppressive cargo interfere with cellular immunotherapy in acute myeloid leukemia. *Sci Rep*. 2017;7(1):14684.
464. Namburi S, Broxmeyer HE, Hong CS, et al. DPP4$^+$ exosomes in AML patients' plasma suppress proliferation of hematopoietic progenitor cells [published online ahead of print Nov 2] *Leukemia*. 2021;35(7):1925–1932.
465. Broxmeyer HE, Liu Y, Kapur R, et al. Fate of hematopoiesis during aging. What do we really know, and what are its implications? *Stem Cell Rev Rep*. 2020;16(6):1020–1048.
466. Ho YH, Méndez-Ferrer S. Microenvironmental contributions to hematopoietic stem cell aging. *Haematologica*. 2020;105(1):38–46.
467. He H, Xu P, Zhang X, et al. Aging-induced IL27Ra signaling impairs hematopoietic stem cells. *Blood*. 2020;136(2):183–198.
468. DeGregori J. Aging, inflammation, and HSC. *Blood*. 2020;136(2):153–154.
469. Hu L, Yin X, Zhang Y, et al. Radiation-induced bystander effects impair transplanted human hematopoietic stem cells via oxidative DNA damage. *Blood*. 2021;137(24):3339–3350.
470. Broxmeyer HE, Baker F, Galbraith PR. Relationship of colony stimulating activity to apparent kill of human colony forming cells by irradiation and hydroxyurea. *Blood*. 1976;47:403–412.
471. Broxmeyer HE. Associated guilt: radiation/bystanders. *Blood*. 2021;137(24):3314–3316.
472. Cooper SH, Broxmeyer HE, Capitano ML. Experimental mouse models of mouse and human hematopoietic stem cell transplantation. In: Pelus LM, Hoggatt J, eds. *Methods in Molecular Biology: Hematopoietic Stem Cells*. New York, NY: Springer Nature; 2020.
473. Fenaux P, Platzbecker U, Mufti GJ, et al. Luspatercept in patients with lower-risk myelodysplastic syndromes. *N Engl J Med*. 2020;382(2):140–151.

CHAPTER 12

ROLE OF CHEMOKINES IN LEUKOCYTE TRAFFICKING

Antal Rot, Elin Hub, Steffen Massberg, Alexander G. Khandoga,
and Ulrich H. von Andrian

The mammalian immune system has evolved to mount multifaceted molecular and cellular microbicidal responses tailored and custom-adapted to eliminate an endless variety of infectious agents and, at the same time, remain tolerant to self-antigens. Accomplishing these tasks requires continuous tightly controlled movement of billions of motile immune cells that roam throughout the body along distinct nonrandom traffic routes from one tissue to another using blood and lymphatic vessels as avenues for rapid access. Migratory pathways characteristic for distinct immune cell subsets are integral parts of their functional make-up determined in the process of cell differentiation and activation. During development in the bone marrow (BM) or thymus, or following stimulation by antigens or pathogen-associated molecules, immune cells acquire the expression of characteristic repertoires of cell surface molecules that enable and restrict their migration to defined tissues and microenvironments. For example, naive lymphocytes largely disregard inflammatory tissue sites, but migrate efficiently into secondary lymphoid organs (SLOs). Conversely, innate immune cells and antigen-experienced lymphocytes can respond to inflammation-induced traffic cues, although some subsets also enter noninflamed lymphoid and nonlymphoid target tissues.[1–4] Notably, not only mature leukocytes but also hematopoietic stem cells (HSCs) and progenitor cells (HPCs), and other rare BM-derived cell subsets recirculate throughout the body.[5–8] The characteristic trafficking routes of leukocyte subpopulations are determined by their expression of cell surface adhesion molecules (see Chapter 15) and chemoattractant receptors. Chemoattractants are produced in target tissues and organs in homeostasis as well as broadest scope of pathologies and signal through cognate receptors on leukocytes to induce their emigration from blood and directed locomotion within the tissues. Leukocyte chemoattractants can be of an exogenous origin; for example, bacterial N-formyl-methionine peptides,[9] tetrapeptides being the most potent and efficacious among them,[10] and many endogenous molecules produced by various host cells, including leukocytes. Endogenous chemoattractants include lipid mediators, e.g., arachidonic acid metabolites, leukotriene B4 in particular[11] or platelet-activating factor,[12] a phospholipid, anaphylatoxic complement fragments C3a, C5a, and C5a-desArg,[13] and most importantly, members of the chemokine family. This chapter discusses chemokines as master navigation signals for leukocyte trafficking and then focuses on specific trafficking pathways that direct leukocyte subsets to distinct target tissues.

CHEMOKINES

Chemokines (a clipping blend of *chemo*tactic cyto*kines*) are critical molecular messengers in the complex cellular communication network used by the immune system. Almost 50 human chemokine genes have been identified to date (Table 12.1).[14–18] The two major subclasses of chemokines are designated CC- or CXC-, reflecting the relative position of the two proximal to the N-terminus canonical cysteines, being either adjacent or separated by a single amino acid, respectively. XCL1 and XCL2, and CX3CL1 represent additional structural chemokine forms with one cysteine and three amino acids between the two canonical cysteines, respectively. CX3CL1 and another chemokine, CXCL16, are associated with a cell membrane via a long spacer sequence and anchored by a transmembrane domain; however, both these chemokines can be cleaved off their stalks and give rise to functional soluble molecules. All other chemokines are secreted proteins of 67 to 127 amino acids. Historically, chemokines have been grouped into functional subfamilies termed *inflammatory* and *homeostatic* chemokines. Former are induced by inflammatory signals and control the recruitment of effector leukocytes in infection, inflammation, tissue injury, and malignancies, whereas those belonging to the latter group navigate leukocytes during hematopoiesis in the BM and in the thymus, during initiation of adaptive immune responses in SLOs and in immune surveillance of healthy peripheral tissues. However, it is now clear that such functional distinction is largely blurred, as many "inflammatory" chemokines are produced under physiologic conditions and the expression of "homeostatic" chemokines is upregulated in inflammation.

Chemokine signals are transmitted through specific cell-surface G protein-coupled receptors (GPCRs) with seven transmembrane domains.[19–23] The human chemokine receptor repertoire identified at present consists of 20 different GPCRs (Table 12.2).[24] The tremendous specificity and plasticity of leukocyte homing and tissue localization is largely determined by the interactions of chemokines with their cognate receptors. Individual leukocyte subsets express highly characteristic fingerprints of chemokine receptors that determine their complex responses to chemokines and define their migratory paths in the body with different receptors playing either nonredundant, combinatorial or overlapping roles.[25] The interactions of individual chemokines and receptors are nonrandom and have been comprehensively characterized. According to new findings, still pending independent confirmation, the last remaining orphan chemokine, CXCL14, known to bind and allosterically modify chemokine receptors, including CXCR4, but not directly signal per se,[26,27] triggers its own GPCR and GPR85.[28] Each chemokine receptor binds either one chemokine or a defined set of them and any individual chemokine can ligate either one unique or several different receptors resulting in some overlap in specificities. However, there are multiple reasons why the apparent "promiscuity" of chemokine-receptor interactions is not a sign of chemokine redundancy and does not reflect a tautology of their signals. First, while the receptor use by different chemokines might overlap, the full spectra of receptors triggered by any particular chemokine are mostly distinct and characteristic for a chemokine, especially within the CC subfamily. Thus, individual chemokines can unmistakably be recognized, albeit in many cases not by one receptor, but by their entire system.[14] Second, messages encoded by individual chemokines that ligate the same receptor are largely unique, because different chemokines have highly disparate binding affinities, interact with distinct receptor moieties and consequently lead to biased signaling, i.e., they induce a dissimilar "texture" of intracellular secondary effectors, activating some and inhibiting others, ultimately resulting in unique spectra of downstream molecular and cellular responses.[29–34] For example, homologous chemokines CCL17 and CCL22 signaling through their cognate receptor CCR4 leads to differential molecular and functional outcomes.[35] CCL19 and CCL21 provide another example of differential, biased signaling through their common receptor CCR7,[36,37] whereby individually or in combination they can induce variations of directed migratory cell responses,

TABLE 12.1 Chemokines and Chemokine Receptors

Chemokine	Chemokine Receptor
CC Family	
CCL1 (I309)	CCR8[a]
CCL2 (MCP-1)	CCR2,[a] CCR5,[a] ACKR1,[b] ACKR2[b]
CCL3 (MIP-1α)	CCR1,[a] CCR5,[a] ACKR2[b]
CCL3L1 (MIP-1αP)	CCR1,[a] CCR3,[a] CCR5,[a] ACKR2[b]
CCL4 (MIP-1β)	CCR1,[c] CCR5,[a] ACKR2[b]
CCL4L1 (MIP-1β2)	CCR5,[a] ACKR2[b]
CCL5 (RANTES)	CCR1,[a] CCR3,[a] CCR5,[a] ACKR1,[b] ACKR2[b]
CCL7 (MCP-3)	CCR1,[a] CCR2,[a] CCR3,[a] CCR5,[c] CXCR3A,[c] CXCR3B,[c] ACKR1,[b] ACKR2[b]
CCL8 (MCP-2)	CCR1,[a] CCR3,[a] CCR5,[a] CCR8[a] (mouse), ACKR1,[b] ACKR2[b]
CCL11 (eotaxin)	CCR2,[c] CCR3,[a] CCR5,[a] CXCR3A,[c] CXCR3B,[c] ACKR1,[b] ACKR2[b]
CCL13 (MCP-4)	CCR1,[a] CCR2,[a] CCR3,[a] ACKR1,[b] ACKR2[b]
CCL14 (HCC1)	CCR1,[a] CCR5,[a] ACKR1,[b] ACKR2[b]
CCL15 (HCC2, MIP-1δ)	CCR1,[a] CCR3[a]
CCL16 (HCC4)	CCR1,[a] CCR2,[a] CCR5[a]
CCL17 (TARC)	CCR4,[a] ACKR1,[b] ACKR2[b]
CCL18 (PARC)	CCR1,[a] CCR3[c]
CCL19 (ELC)	CCR7,[a] ACKR4[b]
CCL20 (MIP-3β, LARC)	CCR6[a]
CCL21 (SLC)	CCR7,[a] ACKR4[b]
CCL22 (MDC)	CCR4,[a] ACKR2[b]
CCL23 (MPIF-1, SCYA23)	CCR1[a]
CCL24 (Eotaxin-2)	CCR3[a]
CCL25 (TECK)	CCR9,[a] ACKR4[b]
CCL26 (Eotaxin-3)	CCR3,[a] CCR2[c]
CCL27 (CTACK)	CCR10[a]
CCL28 (MEC)	CCR3,[a] CCR10[a]
CXC Family	
CXCL1 (GROα)	CXCR2,[a] ACKR1[b]
CXCL2 (GROβ)	CXCR2,[a] ACKR1[b]
CXCL3 (GROγ)	CXCR2,[a] ACKR1[b]
CXCL4 (PF4)	CXCR3B[a]
CXCL5 (ENA-78)	CXCR2,[a] ACKR1[b]
CXCL6 (GCP2)	CXCR1,[a] CXCR2,[a] ACKR1[b]
CXCL7 (NAP-2)	CXCR2,[a] ACKR1[b]
CXCL8 (interleukin-8)	CXCR1,[a] CXCR2,[a] ACKR1[b]
CXCL9 (MIG)	CXCR3A,[a] CXCR3B,[a] CCR3[c]
CXCL10 (IP-10)	CXCR3A,[a] CXCR3B,[a] CCR3[c]
CXCL11 (I-TAC)	CXCR3A,[a] CXCR3B,[a] CCR3,[c] ACKR1,[b] ACKR3[b]
CXCL12 (SDF-1)	CXCR4,[a] ACKR1,[b] ACKR3[b]
CXCL13 (BCA-1)	CXCR5,[a] ACKR4[b]
CXCL14 (BRAK)	GPR85[a] (?)
CXCL16 (SR-PSOX)	CXCR6[a]
CXCL17	CXCR8[a]
CX$_3$C Familiy	
CX3CL1 (fractalkine)	CX3CR1[a]
XC Family	
XCL1 (lymphotactin, SCM-1α)	XCR1[a]
XCL2 (SCM-1β)	XCR1[a]

[a] Agonistic.
[b] "Atypical" receptor.
[c] Antagonistic interaction.
[d] Nonagonist-nonantagonistic interaction.
ACKR, Atypical chemokine receptor; *BCA-1*, B cell chemoattractant 1; *CTACK*, cutaneous T cell-attracting chemokine; *ELC*, Epstein Barr virus-induced molecule 1 ligand chemokine; *ENA*, epithelial-cell-derived neutrophil-activating peptide; *GCP*, granulocyt e chemotactic protein; *GRO*, growth-regulated oncogene; *HCC*, hemofiltrate chemokine; *IP-10*, interferon-inducible protein 10; *I-TAC*, interferon-inducible T-cell alpha chemoattractant; *LARC*, liver and activation-regulated chemokine; *MCP*, monocyte chemoattractant protein; *MDC*, macrophage-derived chemokine; *MEC*, mammary-enriched chemokine; *MIG*, monokine induced by interferon-γ; *MIP*, macrophage inflammatory protein; *SDF-1*, stromal cell-derived factor-1; *SLC*, secondary lymphoid-tissue chemokine; *TARC*, thymus and activation-regulated chemokine; *TECK*, thymus-expressed chemokine.

including chemotaxis, haptotaxis or chemorepulsion.[38,39] Third, chemokines with an apparent overlap of their receptor specificity can behave fundamentally dissimilar due to their differential physicochemical properties, e.g., occurring primarily as soluble versus substrate-bound, as again exemplified by CCL19 and CCL21.[40] Fourth, individual chemokines that ligate the same receptor are produced in vivo in different tissues and/or derive from different cells and/or localize in alternate tissue microenvironments, thus do not act simultaneously but induce distinct, often sequential, steps of leukocyte migratory paths. This was recently shown for two CXCR2 ligands, CXCL1 and CXCL2, which are produced in the vessel wall and by migrating neutrophils, respectively, localize in distinct microanatomical foci thus induce sequential steps of neutrophil emigration.[41] In summary, there are no two chemokines alike and their endless possible combinations allow these structurally homologous signaling molecules to convey most diverse cellular messages acting as building blocks of a universal cell language.[14]

The total number of unique chemokines is further increased by single nucleotide polymorphisms of genes encoding them and their several known spliced variants.[42] Also, practically all chemokines undergo post-translational modifications of their main secreted forms with specific enzymes and protein-modifying agents inducing truncation, degradation, nitration or citrullination of chemokines.[43,44] Many of such modified chemokine isoforms have altered affinity and spectrum of agonistic activities on different receptors and, in some cases, are rendered completely inactive by their processing or gain receptor antagonistic profile.[43] Proteases prominently implicated in processing chemokines include dipeptidyl peptidase IV, also known as CD26, which cleaves two NH2-terminal amino acids of chemokines with alanine or proline at the N-terminal penultimate position, sometimes sequentially, elastase, the ADAM family, as well as matrix metalloproteases (MMPs), a family of more than 20 enzymes with important functions in matrix degradation and multifaceted roles in inflammatory leukocyte recruitment.[44–47] Conversely, some chemokines including CCL14, CCL15, CCL16, and CCL23 are secreted as precursors and their receptor interactive forms are generated upon proteolytic processing by serine proteases and MMPs abundant in the inflammatory tissue environments.[48–50] Also, chemokine processing by glutaminyl cyclase, the modifications to pyroglutamate of their N-terminal amino acids in CCL2 and CX3CL1, is required for protecting these chemokines from proteolysis in vivo and is required for their biological activity.[44,51,52] Further intricacy of chemokine signaling is achieved by the propensity of chemokines to dimerize and oligomerize with chemokine monomers and dimers differentially ligating

TABLE 12.2 Classical Chemokine Receptors

Receptor	Chemokine Ligands	Cell Types
CC Family		
CCR1	CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-1), CCL13 (MCP-4), CCL14 (HCC1), CCL15 (HCC2, MIP-1δ), CCL23 (MPIF)	T cells, monocytes, eosinophils, basophils
CCR2	CCL2 (MCP-1), CCL7 (MCP-3), CCL-11 (Eotaxin, partial agonist), CCL13 (MCP-4), CCL16 (HCC4)	Monocytes, dendritic cells (immature), memory T cells
CCR3	CCL11 (eotaxin), CCL13 (eotaxin-2), CCL7 (MCP-3), CCL5 (RANTES), CCL8 (MCP-2), CCL13 (MCP-4), CCL15 (MIP-1d), CCL24 (eotaxin-2), CCL26 (eotaxin-3), CCL28 (MEC)	Eosinophils, basophils, mast cells, Th2, platelets
CCR4	CCL17 (TARC), CCL22 (MDC)	T cells (Th2), dendritic cells (mature), basophils, macrophages, platelets
CCR5	CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL11 (eotaxin), CCL14 (HCC1), CCL16 (HCC4), CCL8 (MCP-2)	T cells, monocytes
CCR6	CCL20 (MIP-3 β, LARC)	T cells (T regulatory and memory), B cells, dendritic cells
CCR7	CCL19 (ELC), CCL21 (SLC)	T cells, dendritic cells (mature), antigen-experienced B cells
CCR8	CCL1 (I309), CCL8 (MCP-2, mouse)	T cells (Th2), dendritic cells
CCR9	CCL25 (TECK)	T cells, IgA^+ plasma cells
CCR10	CCL27 (CTACK), CCL28 (MEC)	T cells
CXC Family		
CXCR1	CXCL8 (interleukin-8), CXCL6 (GCP2)	Neutrophils, monocytes
CXCR2	CXCL8, CXCL1 (GROα), CXCL2 (GROβ), CXCL3 (GROγ), CXCL5 (ENA-78), CXCL6, CXCL7 (NAP-2)	Neutrophils, monocytes, microvascular endothelial cells
CXCR3-A	CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC)	Th1 helper cells, mast cells, mesangial cells
CXCR3-B	CXCL4 (PF4), CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC)	Microvascular endothelial cells, neoplastic cells
CXCR4	CXCL12 (SDF-1)	Widely expressed
CXCR5	CXCL13 (BCA-1)	B cells, follicular helper T cells (T_{FH})
CXCR6	CXCL16 (SR-PSOX)	$CD8^+$ T cells, natural killer cells, memory $CD4^+$ T cells
CXCR8	CXCL17	Monocytes, T cells, neutrophils, dendritic cells
CX_3C Familiy		
CX_3CR1	CX3CL1 (fractalkine)	Macrophages, NK-cells, endothelial cells, smooth-muscle cells
XC Family		
XCR1	XCL1 (lymphotactin), XCL2	T cells, natural killer cells

BCA-1, B cell chemoattractant 1; *CTACK*, cutaneous T cell-attracting chemokine; *ELC*, Epstein-Barr virus-induced molecule 1 ligand chemokine; *ENA*, epithelial-cell-derived neutrophil-activating peptide; *GCP*, granulocyte chemotactic protein; *GRO*, growth-regulated oncogene; *HCC*, hemofiltrate chemokine; *IP-10*, interferon-inducible protein 10; *I-TAC*, interferon-inducible T-cell alpha chemoattractant; *LARC*, liver and activation-regulated chemokine; *MCP*, monocyte chemoattractant protein; *MDC*, macrophage-derived chemokine; *MEC*, mammary-enriched chemokine; *MIG*, monokine induced by interferon-γ; *MIP*, macrophage inflammatory protein; *SDF-1*, stromal cell-derived factor-1; *SLC*, secondary lymphoid-tissue chemokine; *TARC*, thymus and activation-regulated chemokine; *TECK*, thymus-expressed chemokine.

receptors and causing biased signaling, as has been shown recently for CXCL12.[53] Moreover many, but not all, chemokines form heterodimers within and across their structural sub-family boundaries following a recently mapped pattern of nonrandom specificity.[54] The resultant chemokine heterodimers shift the use of their receptors as compared to the individual monomeric chemokines.[54]

SIGNALING BY CHEMOKINE RECEPTORS

Chemokine receptors, just like other GPCRs, function as allosteric molecular relays where chemokine binding to the extracellular portion modifies the receptor's tertiary structure. This allows the intracellular domain of the engaged receptor to bind to and activate heterotrimeric G proteins. In response, the activated G-proteins exchange GDP for GTP and in the process, dissociate into Gα and Gβγ subunits. Curiously, different chemokine receptors are able to associate with different subsets of Gα subtypes, though any particular functional relevance of such biased signaling is not yet clear.[55] The dissociated Gβγ subunits mediate large parts of chemokine-induced signals by activating different phosphatidylinositol 3-kinase (PI3K) isoforms, leading to the formation of phosphatidyl-3,4,5-triphosphate (PIP_3). PI3K and its product PIP_3 then translocate to the pseudopod at the leading edge of migrating leukocytes, where they colocalize with the small GTPase Rac.[56–59] PIP_3 activates Rac through specific guanine nucleotide exchange factors.[60,61] Rac in turn acts through the downstream effectors p21-activated kinase and the Wiskott-Aldrich protein homologue WAVE, which stimulate actin-related protein 2/3. Together, this process induces focal polymerization, required for the development and forward extension of the pseudopod, a critical step in leukocyte chemotaxis.[62] The importance of PI3K-dependent signaling for leukocyte chemotaxis is evidenced by the lack of migration of myeloid leukocytes to chemokines in mice lacking PI3Kγ[63–68] Notably, though, distinct signaling pathways or at least other PI3K isoforms appear to be involved in the trafficking of immune cells. For example, neutrophil and B-cell migration requires PI3Kδ,[69–72] whereas T-cell chemotaxis is not impaired in PI3K-deficient mice, but depends on the Rac guanine exchange factor DOCK2.[73–75]

Several pathways have been identified that can terminate chemokine signaling through their GPCRs. The Gα subunit possesses an intrinsic GTPase activity to hydrolyze GTP. In a negative-feedback loop, this GTPase activity allows the Gα subunits to reassociate with the Gβγ subunits, thereby restoring the heterotrimeric G protein to its inactive state. In addition, another class of molecules, known as regulators of G protein signaling (RGS), also modulates signaling through chemokine GPCRs. RGS are a large and diverse protein family initially identified as GTPase-activating proteins of heterotrimeric G- protein Gα-subunits.[76,77] At least some RGS can also influence Gα activity through either effector antagonism by competing with effector molecules for GTP-bound Gα-subunits or by acting as guanine nucleotide dissociation inhibitors.[76] To date, over three dozen genes have been identified within the human genome that encode proteins containing an RGS or RGS-like domain. Additionally, chemokine-induced receptor signaling is terminated by specific cytosolic enzymes known as GPCR kinases or GRKs.[78] GRKs are able to rapidly phosphorylate the intracellular domains of the receptors thus, on one hand, abolishing G-protein-mediated signaling and, on the other hand, permitting the association of β-arrestins with the receptors.[79] Such engagement of β-arrestins promotes the internalization of chemokine-triggered GPCRs from the cell membrane into intracellular vesicular compartments with the internalized chemokines targeted for lysosomal degradation and GPCRs either being also degraded or, alternatively, recycled back onto the cell membrane. Thus, pathways that involve GRKs and β-arrestins importantly mediate receptor "desensitization."[79] The activation of Src-family kinases downstream of the chemokine receptors via not yet fully mapped pathways involving G-proteins- and β-arrestins also contribute to the negative regulation of chemokine-induced migratory responses of myeloid cells.[80] In addition to its well-documented role in shutting down chemokine receptor signaling and contributing to receptor desensitization, β-arrestins also play important roles in mediating some of the cellular effects downstream of chemokine receptors by triggering the activation of multiple intracellular molecular signals, thus "redirecting" signaling from G-proteins to the G-protein-independent pathways.[32,81]

Remarkably, the molecular mechanisms involving RGS proteins and GRKs contributing to the "negative regulation" of chemokine signaling through their GPCRs might actually be required for optimal chemokine-induced in vitro and in vivo migratory leukocyte responses and their functions in host defense. A mutation (G184S) in Gαi2 protein abolishes its interactions with RGS proteins, thus allowing it to continuously activate the downstream effectors. Neutrophils of mice with such (G184S) mutation showed enhanced rate baseline locomotion but had reduced chemotactic responses in vitro and defective migratory responses in vivo.[82] Analogously, neutrophils deficient of GRK2, the enzyme dominantly involved in desensitization of their chemotactic migration in response to swarming-inducing autocrine chemoattractants, migrated faster, hence covered larger field areas but were functionally less effective, e.g., in locating and killing bacteria.[83]

ATYPICAL CHEMOKINE RECEPTORS

In addition to GPCRs, most chemokines also bind to one or multiple among the "atypical" chemokine receptors (ACKRs).[84,85] Currently four ACKRs have been included in the nomenclature (Table 12.3), which were shown to bind a broad range of chemokine ligands.[86] It is now clear that at least one additional ACKR exists, pending its inclusion in the systemic nomenclature.[87] Analogously to chemokine GPCRs, ACKRs are serpentine membrane receptors with seven transmembrane domains. However, unlike GPCRs, ACKRs lack or have an altered DRYLAIV consensus motive in the second intracellular loop, which is required for G-protein coupling and signaling through G-proteins. Therefore, upon chemokine binding ACKRs do not trigger signaling events characteristic of GPCRs and cannot mediate cell migration. However, ACKRs internalize chemokines and may target them either into lysosomes or alternatively into transcytotic cellular pathways.[88] ACKRs may also transmit intracellular signals, independently of G-proteins, for example, through triggering biochemical cascades downstream of β-arrestins or affect signaling through classical chemokine GPCRs expressed by the same cell, possibly through receptor heterodimerization or the consumption of secondary signaling molecules. Curiously, ACKRs can heterodimerize with the GPCRs which they share with chemokine ligands, e.g., CXCR4 and ACKR3[89] or do not share any, e.g., CXCR2 and CCRL2,[90] although, despite several reports, the latter might not be a bona fide ACKR as only its chemerin- but not chemokine-binding

TABLE 12.3 **Atypical Chemokine Receptors**

Receptor	Chemokine Ligands	Cell Types
ACKR1	CCL2 (MCP-1), CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CCL11 (eotaxin), CCL13 (MCP-4), CCL14 (HCC1), CCL17 (TARC) CXCL1 (GROα), CXCL2 (GROβ), CXCL3 (GROγ), CXCL5 (ENA-78), CXCL6 (GCP-2), CXCL7 (NAP-2), CXCL8 (IL-8), CXCL11 (I-TAC). CXCL12 (SDF1)	Erythrocytes, venular endothelial cells, cerebellar Purkinje cells and other neurons
ACKR2	CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CCL11 (eotaxin), CCL13 (MCP-4), CCL14 (HCC1), CCL17 (TARC), CCL22 (MDC),	Lymphatic endothelial cells, placenta syncytiotrophoblasts, innate-like B cells, marginal zone B cells, dendritic cells, monocytes, tissue-resident mast cells, macrophages
ACKR3	CXCL11 (I-TAC), CXCL12 (SDF-1)	Blood endothelial cells in brain, embryonic and neonatal tissues, marginal zone B cells, plasmablasts
ACKR4	CCL19 (ELC), CCL20 (MIP3 β), CCL21 (SLC), CCL2 (MDC), CCL25 (TECK), CXCL13 (BCA-1)	Lymphatic endothelial cells in collective vessels and lining the ceiling of the LN subcapsular sinus, skin keratinocytes, thymic epithelial cells, endothelial cells in "peri-marginal sinus" of the spleen red pulp
GPR182	CXCL13 (BCA-1), CCL19 (ELC), CCL21 (SLC), CCL25 (TECK),	Microvascular endothelial cells in lungs, bone marrow, LNs, Peyer patches, liver, and spleen

ACKR, Atypical chemokine receptor; *BCA-1*, B cell chemoattractant 1; *CTACK*, cutaneous T cell-attracting chemokine; *ELC*, Epstein-Barr virus-induced molecule 1 ligand chemokine; *ENA*, epithelial-cell-derived neutrophil-activating peptide; *GCP*, granulocyte chemotactic protein; *GRO*, growth-regulated oncogene; *HCC*, hemofiltrate chemokine; *IP-10*, interferon-inducible protein 10; *I-TAC*, interferon-inducible T-cell alpha chemoattractant; *LARC*, liver and activation-regulated chemokine; *LN*, lymph node; *MCP*, monocyte chemoattractant protein; *MDC*, macrophage-derived chemokine; *MEC*, mammary-enriched chemokine; *MIG*, monokine induced by interferon-γ; *MIP*, macrophage inflammatory protein; *SDF-1*, stromal cell-derived factor-1; *SLC*, secondary lymphoid-tissue chemokine; *TARC*, thymus and activation-regulated chemokine; *TECK*, thymus-expressed chemokine.

activity was independently confirmed.[91] Generally, the main role of ACKRs is to determine in which tissue microenvironments chemokines may or may not exert their activities, achieved either through chemokine scavenging or chemokine transport and presentation in salient tissue microenvironments.[84,85] Based on the chemokine-scavenging activity of some ACKRs, it has been postulated that the key role of this group of ACKRs is to counteract chemokine effects by reducing the chemokine availability in the tissues and in the organism as a whole. However, all ACKRs are expressed in discrete and often sparse cellular microenvironments, and they most effectively modify chemokine availability within the microenvironments of their cellular expression; although, of note, ACKRs expressed in tissues can affect the levels of chemokines in plasma,[85,92,93] with broadly variable impact on leukocyte responses.[14,94] Mechanistically, scavenging of chemokines by ACKRs also might buffer chemokine "noise" and keep chemokine concentrations within the range of optimal dissociation constants (K_d) for their cognate GPCRs.[95] Alternatively, by acting as chemokine sinks, ACKRs that are expressed in juxtaposition to the cellular sources of chemokines can facilitate the formation of functional chemokine gradients.[96] Both of such mechanistic outcomes have been suggested for ACKR4, and despite involving chemokine scavenging support the functions of cognate ligands in mediating cell homing into the lymph nodes (LNs) and spleen.[93,96,97]

CHEMOKINE INTERACTION WITH GLYCOSAMINOGLYCANS

In addition to their classical and atypical receptors, chemokines importantly bind to sulfated glycosaminoglycans (GAG) polysaccharides, heparan sulfate, chondroitin sulfate, and dermatan sulfate.[98] GAGs decorate cell membrane proteoglycans on multiple cells, most notably, endothelial cells where they importantly contribute to the function of the apical surface glycocalyx.[99] Extracellular matrices are also rich in GAGs and their immobilization of chemokines allows for the formation of haptotactic, i.e., substrate-immobilized chemokine gradients that facilitate extravascular leukocyte locomotion.[100,101] Such haptotactic gradients also spontaneously develop in vitro as chemokines avidly bind to plastics, and therefore Boyden-type and other in vitro chemotaxis assays measure haptotaxis and not chemotaxis, without recognizing this fact.[102] Chemokine binding to GAGs is characterized by a considerable selectivity on parts of both different GAGs molecules and different chemokines,[103–105] thus facilitating defined patterns of anatomical and microanatomical chemokine localization and availability. Critical involvement of GAGs supporting chemokine functions in vivo is illustrated by abrogated leukocyte homing to LNs[106] and disrupted chemokine-dependent growth and function of the thymus[107] that can be observed in the absence of local heparan sulfate expression. Mechanistically, GAG binding can augment chemokine activities in vitro[108] and in vivo,[109] by protecting them from proteolytic cleavage and promoting their oligomerization,[109–112] or alternatively, in a diametrically opposite scenario, depending on a cellular context, interfere with chemokine binding to GPCRs.[111–113] It has been suggested that trimolecular complexes of GAG-chemokine-GPCRs are not likely to exist, at least not for some chemokines, e.g., CXCL8.[114] If indeed, some chemokines are unable to bind to charged surfaces and GPCRs simultaneously, chemokine "presentation" would entail merely their retention on cells and in matrices and therefore should require chemokine release into solution to enable the subsequent binding to GPCRs.[115] However, this cannot be universally true as CCL21 induces in vitro haptotaxis when firmly substrate-attached, even printed on the surfaces as a stable photo-immobilized gradient.[116] In addition to GAGs, other charged molecules can also specifically bind chemokines. These include anionic phospholipid phosphatidylserine that decorates apoptotic cells, thus allowing for their rapid detection by phagocytes[113] and collagen IV, thus allowing chemokine immobilization around LN high endothelial venules (HEVs).[117]

Apart from inducing leukocyte migration, chemokines play multiple additional roles in health and disease. Several chemokines, including CXCL1, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14, CCL1, CCL19, CCL20, CCL21, CCL25, and CCL28, or their naturally occurring cleaved fragments have been ascribed direct microbicidal activities, albeit to a variable extent and at concentrations orders of magnitude higher than those required for inducing optimal leukocyte migration.[118] Some of these chemokines are generated in infections, and thus might contribute directly to the first line of anti-microbial host defenses. Others, however, are secreted constitutively, e.g., by epithelial cells of exocrine glands and secreted in sweat, saliva, tears, and colostrum and milk,[119–121] thus could play a prophylactic role in infections and in regulating skin and mucosal microbiota balance. Furthermore, chemokines are fundamentally involved in homeostatic brain functions,[122–124] pathophysiology of reproduction and birth,[125] embryogenesis, including the development of heart,[126] brain,[127] and lymphatic vasculature.[128] Furthermore, chemokines mediate angiogenic repair,[129] control hematopoiesis,[126] induce cell survival and apoptosis in leukocytes,[130] and other cells, including tumor cells, etc. Curiously "the mother of all chemokines," CXCL12, is involved in many of such effects outside leukocyte trafficking. As remarkable is the breadth of different chemokine activities, their main and best studied functions involve the regulation of leukocyte trafficking.

In general, leukocyte trafficking can be classified into three distinct patterns of migration: (1) **entry into tissues** from the circulation; (2) **migration within tissues**; and (3) **exit from tissues**. The following sections will discuss each of these steps in leukocyte trafficking.

LEUKOCYTE ENTRY INTO TISSUES

In order to leave the circulation and enter target tissues, leukocytes engage in several sequential steps of adhesion to the endothelial cells, which most often take place in the postcapillary and small venules.[56,131–136] Discrete adhesion steps are mediated by binding interactions of pairs of adhesion receptors and their counter-ligands expressed in trans-geometry by leukocytes and endothelial cells. The initial tethering of leukocytes to the endothelial cell is induced by adhesion molecules, which can rapidly bind their ligands with high tensile strength. Prime initiators of leukocyte tethering are selectins, expressed on leukocytes (L-selectin), endothelial cells (E- and P-selectin) and platelets (P-selectin). Selectins have constitutive optimal affinity for their counter-ligands, sialomucins that are decorated with oligosaccharides related to sialyl-Lewis^x, including P-selectin glycoprotein ligand (PSGL)-1 and the peripheral node addressin (PNAd). Selectin-mediated adhesion bonds that are formed in the bloodstream are transient and do not allow prolonged, firm leukocyte arrest. As tethered leukocytes are pushed along the vessel wall by the blood flow, selectin bonds continuously dissociate at the cells' upstream end and new ones form downstream, resulting in slow rolling motion characteristic for the leukocyte tethering. To undergo firm adhesion, the rolling leukocyte must engage additional adhesion receptors that belong to the integrin family, particularly CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1) and the α4 integrins, α4β1 (VLA-4), and α4β7. Without exception, individual integrins are expressed by the subsets of leukocytes and their counter-ligands by the endothelial cells.

To efficiently bind their endothelial cell counter-ligands, leukocyte integrins first need to be activated. Such activation is induced by chemoattractant signals that trigger a rapid reversible change in integrin conformation (leading to enhanced ligand binding affinity) and in integrin clustering (enhancing avidity), or both.[20] A subset of chemokines can associate with the luminal surface of microvascular endothelial cells,[137] allowing them to trigger integrin activation and efficiently induce leukocyte arrest, hence designated *arrest chemokines*.[138] The retention of chemokines on the vessel endothelium is mediated by GAGs in the luminal multifunctional glycocalyx,[139] heparan sulfate in particular.[110] Such apical GAG-immobilized chemokine gradients also drive intraluminal crawling of leukocytes,[140] allowing them to find transmigration hotspots, loci optimal for breaching the endothelial barrier, and migration into the extravascular space.[141] Many chemokines are produced by endothelial cells themselves and either secreted immediately or stored in specialized organelles, the Weibel-Palade bodies, and other storage vesicles and upon secondary stimulation released in the process of regulated exocytosis.[142–144] Chemokines that are produced extravascularly can be transported across the endothelial barrier to the luminal surface.[145] This is triggered by chemokine binding to the atypical chemokine receptor ACKR1[88] normally expressed in venular, but not capillary or arterial endothelial cells[146] but upregulated in endothelia of different vascular segments in inflammation. Such chemokine transcytosis by ACKR1 is especially important in microvascular beds characterized by tightest adhesions between EC, e.g., in the brain vasculature.[147] Similar transport mechanisms exist for other tissue-derived chemoattractant as C5a transcytosis across endothelium is accomplished by its atypical receptor C5aR2.[148] However, soluble tissue-derived chemokines, being small proteins, also can freely diffuse from the extravascular space through the endothelial cell junctions into the blood. As a consequence, the chemokine-encoded signals lose their attribution to the sites of their origin and become systemic stimuli in blood.[94] This process is counteracted by ACKR1, which salvages escaping into circulation chemokines by capturing them and presenting them in luminal and junctional membrane microdomains.[41,88] Importantly, chemokine binding to ACKR1, in contrast to other ACKRs, does not lead to chemokine targeting into lysosomes and their degradation.[88] ACKR1 has been initially described on the red blood cells as the Duffy blood group antigen/receptor for chemokines or DARC where it acts as a chemokine sink and reservoir as well as entry receptor of malarial parasite Plasmodium vivax.[149] Erythrocytes are devoid of transcription and translation, hence ACKR1 is expressed already by their precursors, nucleated erythroid cells of the BM, in which it mediates their cell contacts with HSCs and HPCs and thus contributes to the regulation of hematopoiesis.[150] ACKR1 is the most promiscuous of all chemokine receptors but binds its ligands within a very broad spectrum of affinities. Due to differential affinities of individual chemokines and their monomeric versus dimeric forms,[151] chemokines can compete for ACKR1 binding, shifting the equilibria of free and bound chemokines in the tissues and in plasma.[152]

Chemokines signaling via the Gα_i subfamily of large heterotrimeric G proteins can be inhibited by pertussis toxin (PTX). Consequently, intravital microscopy studies have shown that PTX-treated lymphocytes undergo normal tethering and rolling interactions in HEV in LNs and Peyer patches (PPs) but are unable to undergo integrin-dependent firm arrest. Chemokine receptor activation precipitates a cascade of intracellular signaling and adapter proteins, including Kindlin-3 and RAP-RAPL, that are involved in the so-called inside-out signaling that results in integrin activation. Modifications at the cytoplasmic tails of the integrin α and β chains are critical to regulating leukocyte adhesion to integrin ligands, such as the binding and spreading of neutrophils on intercellular cell adhesion molecule-1 (ICAM-1) and the complement C3 activation product, iC3b.[153] Surprisingly, some chemokines, e.g., CX3CL1 and CXCL12 can directly bind to the alternative bindings sites of leukocyte integrins αvβ3 and α4β1 and allosterically activate them without signaling through their respective GPCRs.[154,155] Once firmly adherent, leukocytes rapidly polarize and slowly migrate within the vessel in random directions.[156–158] The intralumenal crawling is thought to be essential to enable leukocytes to find exit points within the vessel through which they can leave the vasculature.[56] A subset of monocytes crawl within normal microvessels under steady-state conditions.[133,159,160] These patrolling monocytes are poised to provide immune surveillance of the endothelial cell surfaces and clear the intravascular debris[160] but may also enter the extravascular space in response to damage and infection. Once emigrated, some mononuclear cells may differentiate into macrophages or dendritic cells. In some tissues and organs, intestine, monocyte emigration contributes throughout life to the replenishing of the resident macrophage pool.[159,161,162] In other tissues, macrophage and dendritic cell precursors home only during the embryonic period from either the liver or yolk sack and the resident mature cells proliferate in situ to give rise to their progeny.[163,164]

The interactions between β2-integrins, LFA-1, and Mac-1, with endothelial ICAMs are required for intravascular adhesion and crawling. However, the specificity of these interactions differs between different leukocytes, e.g., neutrophils and monocytes.[156,159] Neutrophil luminal crawling is mainly mediated by Mac-1,[156] whereas monocytes and T cells use LFA-1,[1,159] Recent studies using blocking antibodies against Mac-1 and LFA-1 showed that crawling patterns of monocytes and neutrophils differ at steady-state versus inflammatory conditions; both LFA-1 and Mac-1 contribute to monocyte crawling; however, the LFA-1–dependent crawling in unstimulated venules becomes Mac-1 dependent upon inflammation. By contrast, Mac-1 alone is responsible for neutrophil crawling in both unstimulated and cytokine-stimulated venules.[165] This indicates that differences in monocyte and neutrophil crawling behavior result from involvement of different β2 integrins and consequently affect the next step of the leukocyte migration cascade, the transendothelial migration.

The transendothelial migration or diapedesis is a critical event allowing leukocytes to cross the vascular wall and enter their target tissue. Two main routes of leukocyte diapedesis have been observed: a paracellular route that dominates most extravasation processes and a transcellular route, reported for neutrophils and T cells.[166–172] Both routes involve the action of apical and junctional endothelial intercellular adhesion molecule-1 (ICAM-1) and, at least in some settings, also vascular cell adhesion molecule-1 (VCAM-1). Under inflammatory conditions, additional junctional endothelial ligands such as PECAM-1, VE-cadherin, ESAM, CD99, CD99L2, and junctional adhesion molecules (JAMs) can contribute to leukocyte diapedesis.[167,173–179] Different subsets of leukocytes use variations on these molecular mechanisms to emigrate into the tissues.[180]

After penetration of the endothelial barrier, leukocytes may move further within the interstitium towards their target destinations in the tissue. This locomotion is considered to reflect in vivo chemotaxis, increased rate directed cell locomotion driven by the putative gradients of chemoattractants.[181,182] Several signaling pathways have been proposed to be involved in this gradient-driven process, the most predominant being the PI3K pathway.[64] Thereby leukocytes use an "internal compass" for sensing the direction of chemotactic gradients, and undergo polarization characterized by the formation of lamellipodia at the leading edge of the cell and an uropod at the trailing edge.[183,184] Chemokines released by a broad range of tissue cells, epithelial cells, mast cells, smooth muscle cells, fibroblasts, myocytes, and tissue-resident immune cells may form gradients and mediate the leukocyte chemotaxis in the interstitium. In this context, the spatiotemporal formation of chemokine gradients in the interstitial tissue may be supported by GAGs, which immobilize chemokines and, thus, determine the position and temporal persistence of chemokine gradients.[185,186] It has been shown that immobilized, substrate-bound chemokines are effective in inducing directed leukocyte migration, haptotaxis.[102] It has also been suggested that efficient development of chemokine gradients requires, in addition to the free diffusion of chemokines from their cellular sources, also scavenging by their ACKRs expressed by cells in apposition to chemokine sources.[96] Additionally, responding leukocytes may also scavenge chemokines, thus participating in the formation of their functional gradients.[187] Leukocytes moving within the interstitial tissue receive signals from neighboring cells as well as from the extracellular matrix, activate intracellular processes, release inflammatory mediators, and upregulate adhesion molecules and release enzymes.[188] Neutrophils display enhanced expression of β1-integrins upon transmigration into the tissues, and integrins contribute but are not absolutely required for interstitial leukocyte migration. Importantly, the involvement of integrins is different in 2D versus 3D settings.[189] Integrins are dispensable for migration through the 3D tissue fiber networks that confine and mechanically anchor cells from all sides so that they intercalate alongside and perpendicular to tissue structures as shown for integrin-deficient DCs. However, migration on 2D surfaces was shown to be integrin-dependent.[189]

All consecutively occurring steps of (1) leukocyte tethering and rolling, (2) exposure to a chemotactic stimulus, (3) firm arrest, (4) post-adhesive strengthening and intralumenal crawling, (5) diapedesis, and (6) interstitial migration are essential for leukocytes to migrate to sites of inflammation. Accordingly, genetic defects in any of the molecules involved in either step lead to insufficient host defenses. Patients with leukocyte adhesion deficiency (LAD) syndrome, may have a genetic defect in β2 integrins (type 1) or in fucosylated selectin ligands (type 2) and neutrophils either cannot stop or cannot roll, respectively. LAD syndrome is characterized by marked leukocytosis and frequent and severe soft-tissue infections.[190–192] Another LAD variant referred to as LAD3 is caused by mutations affecting the FERMT3 gene that encodes focal adhesion protein kindlin-3, resulting either in the lack of this protein or its defective function in activating leukocyte and platelet integrins.[193]

Chemokine Control of Lymphocyte Homing to Secondary Lymphoid Organs

Migration of blood-borne lymphocytes to SLOs is the best-characterized example of leukocyte trafficking from the circulation into distinct target tissues.[2,4,194–199] Lymphocyte subsets constantly survey SLOs, which include the spleen, tonsils, appendix, PPs, and LNs. Such homing allows lymphocytes to encounter antigen that may pose a threat to the organism. Antigens are collected and presented to T cells by dendritic cells, in conjunction with signals by costimulatory molecules and cytokines. In contrast to the spleen, where the molecular mechanisms of leukocyte homing still are not entirely clear and differ from all other lymphoid organs,[93,200] the rules which govern homing of immune cells to LNs are well understood. Mature dendritic cells, that have acquired antigen in peripheral tissues, and some memory cells reach the LNs through afferent lymph vessels.[201–203] In contrast, circulating in blood T and B lymphocytes gain access to LNs through specialized postcapillary microvessels lined with cuboid endothelial cells that are known as *high endothelial venules* (HEVs).[204–207] HEV are also present in other SLOs and appear in tertiary lymphoid organs (TLOs) induced in multiple tissues by various pathologies, most notably autoimmunity[208] and cancer.[209] HEV in SLOs and TLOs express distinct patterns of trafficking molecules to serve as tethering platforms for defined subsets of lymphocytes. For example, HEV in LNs express PNAd, whereas HEV in PPs express mucosal addressin-cell adhesion molecule (MAdCAM-1). Other mucosa-associated lymphoid organs, such as mesenteric LNs express both MAdCAM-1 and PNAd. Although T and B lymphocytes are recruited by similar multistep cascades to home to SLOs, the roles of individual traffic molecules are not necessarily identical, even when both subsets interact with the same microvessel.[2]

The first step in the homing cascade in LNs is mediated by L-selectin/CD62L expressed on all lymphocytes, except effector/memory cells. PNAd, an O-linked sulfated core 1 carbohydrate moiety that is exclusively found in HEV, is the major endothelial L-selectin ligand.[210–214] Binding of L-selectin to PNAd initiates lymphocyte rolling in HEV and slows down and marginates the free-flowing lymphocytes.[2,136,213] While the L-selectin-PNAd interaction is required, it is not by itself sufficient to promote firm leukocyte adhesion. The subsequent firm arrest of rolling T and B lymphocytes is mediated by the α4β1 integrin, β2-integrin CD11a/CD18 (LFA-1), CD11b/CD18 (MAC-1) and β7-integrin, which bind intercellular adhesion molecules (ICAM), in particular ICAM-1 and ICAM-2, VCAM-1 or MAdCAM-1 on high endothelial cells.[213–218]

Chemokines that are presented on the apical side of HEV function as triggers of integrin activation.[219,220] On naive T cells, integrin activation is primarily mediated by CCL21 (previously known as SLC, TCA4, exodus 2 or 6-C-kine), which is constitutively expressed and secreted by HEV. The secreted chemokine is noncovalently bound to GAGs on the surface of HEV. Here, it activates rolling lymphocytes through binding to CCR7, which is expressed on naive B and T cells. Another CCR7 ligand, CCL19 (previously termed ELC or MIP3β), also supports T-cell homing to LNs.[220] CCL19 is not expressed by LN high endothelial cells themselves. However, CCL19 and other chemokines may be released by extravascular cells in LNs or in tissues that discharge lymph to a local LNs and readily trancytoses to the luminal aspect of HEV. Correspondingly, chemokines, including CCL2, CCL19, and CCL21, injected under the skin of mice accumulate on the luminal surface of the HEV in draining LNs, where they promote integrin activation on rolling leukocytes bearing the cognate receptors.[221–223] In this context, endothelial heparan sulfate has been shown to be essential for controlling chemokine presentation on the endothelium and thereby for recruitment of lymphocytes and dendritic cells to LNs.[106]

B cells use largely the same trafficking molecules as naive T cells to home to LNs. However, B cell-HEV interactions are only moderately affected by the absence of CCR7 or its ligands.[224] Correspondingly, LNs of mice lacking CCR7 contain few T cells, while the B cell compartment (and the memory T-cell compartment) is less affected.[225,226] Similar observations were made in *plt/plt* mice, which have a spontaneous genetic defect resulting in deletion of CCL19 and the HEV-expressed form of CCL21 (mice, unlike humans, have a second *ccl21* gene that is only expressed in lymph vessels of peripheral tissues), demonstrating that B cells are not absolutely dependent on CCR7 to adhere to HEV.[224] In fact, rolling B cells can be induced to arrest in HEVs by either CCR7 agonists or by CXCL12 (previously called stromal cell-derived factor [SDF-1]α), the ligand for CXCR4.[227] An additional chemokine pathway involving CXCL13 (also called BLC) and its receptor CXCR5 has also been implicated in B cell homing to secondary lymphoid tissues.[228] Of note, although B cells encounter several distinct integrin-activation signals in HEV, B cell homing to LNs is nonetheless less efficient than that of T cells. A likely reason is that the B cells express only approximately half the number of L-selectin molecules expressed on T cells, which greatly affects their ability to initiate the adhesion cascade in HEV.[207,229]

The requirement for a sequence of distinct molecular steps that each leukocyte must undergo to arrest within microvessels, explains why only certain leukocyte subsets gain readily access to lymphoid tissues, while others are largely excluded. Granulocytes, for example, express LFA-1 and L-selectin, but not CCR7. Consequently, although granulocytes can roll in HEV (via L-selectin), these leukocytes do not perceive an integrin-activating stimulus and, therefore, fail to massively accumulate in LNs or PPs. It has been clear for some time that alternative to CCL19/CCL21-CCR7 chemokine-receptor pathways can also be involved in leukocyte homing in HEVs,[223,230] thus some neutrophils can traverse HEVs, in low numbers under physiological conditions and in dramatically increased numbers in infection.[231] Mature dendritic cells also express CCR7 and CD11a/CD18, but not L-selectin. These cells when in circulation are incapable of rolling in HEV, and they fail to home to noninflamed LNs from the blood (although mature dendritic cells readily access LNs via afferent lymph). Hence, the GPCR-mediated integration activation step is critical for imparting specificity to the process of lymphocyte homing to LNs.

In HEVs of PPs, similar homing mechanisms are encountered as described above for LNs. However, the levels of L-selectin ligands (which are immunologically distinct from PNAd) expressed by HEV in PPs are considerably lower when compared with LNs.[232,233] As a result, L-selectin itself is not sufficient to initiate a successful homing cascade for most lymphocytes in PPs.[232] Indeed, HEV in PPs (and also in mucosa-associated LNs) express additionally MAdCAM-1, a ligand for the α4β7 integrin.[232–234] The α4β7 heterodimer, which comprises an α4 integrin chain (CD49d) linked to the β7 integrin chain, is expressed at low levels by naive T and B cells and is required for the successful homing of these cells in PP HEV.[232] Following formation of an initial L-selectin-dependent tether, the α4β7-MAdCAM-1 pathway stabilizes and slows the rolling lymphocytes without requiring chemokine activation. Once a chemokine signal has been transmitted, both α4β7 and LFA-1 become activated and jointly mediate firm cell arrest on the endothelium.[232]

Of note, α4β7 is strongly upregulated on gut-homing effector/memory lymphocytes, but completely absent on skin-homing memory T and B cells; these differential levels of α4β7 integrin expression allow certain antigen-experienced lymphocyte subsets to acquire tissue selectivity.[235] The mechanisms underlying this specificity and plasticity of lymphocyte homing will be addressed below. Like in LNs, chemokines are essentially involved in promoting integrin activation and allowing firm lymphocyte arrest in HEV of PPs. Thus, CXCR4, CXCR5, and CCR7 have been implicated in B cell homing, while CCR7 seems exclusively responsible for T-cell homing. Interestingly, while T and B cells are recruited across the same HEV in LNs, there is segmental segregation of T and B cell recruitment in PPs. HEV supporting B cell accumulation in PPs are concentrated in or near B follicles and present CXCL13, but not CCL21, whereas T cells preferentially accumulate in interfollicular HEV (i.e., within the T-cell area), which express high levels of CCL21, but not CXCL13.

Trafficking of Leukocytes From Blood Into Nonlymphoid Tissues

As outlined above, naive and subsets of memory lymphocytes migrate most efficiently to SLOs. Both innate immune cells and multiple subsets of lymphocytes can respond to inflammatory and/or activation signals by modulating the expression and/or activity of traffic molecules in a way that allows them to migrate to nonlymphoid tissues (4). For example, in response to an inflammatory stimulus, granulocytes, including neutrophils, eosinophils and basophils, are rapidly recruited to the affected site and provide the first line of defense. Thereafter, additional immune cells, including monocytes, dendritic cells, and effector as well as memory lymphocytes, may be recruited. Essentially, all these different recruitment events depend on distinct multistep adhesion cascades.

Many of the traffic molecules required for the access of inflammation-seeking leukocytes to nonlymphoid tissues are shared by their different subsets. The key receptors that initiate capture and mediate rolling of neutrophils, monocytes, natural killer cells, eosinophils, and effector T and B cells at peripheral sites of injury and inflammation are the three selectins, the leukocyte-expressed L-selectin and P- and E-selectin on stimulated endothelial cells.[236–240] In addition, interactions between leukocytes involving PSGL-1 and L-selectin can also support the accumulation of immune cells in inflamed tissues.[239,241–243] Subsequent to rolling firm arrest of leukocytes in nonlymphoid tissues involves integrins, including CD11a/CD18 (and its ligand ICAM-1 and possibly also ICAM-2), VLA-4 (and its ligand VCAM-1), as well as CD11b/CD18 (and its ligand ICAM-1).[1,56,244] As discussed above for lymphoid tissues, chemoattractants, including chemokines and other GPCR agonists, such as formyl peptides, complement fragment C5a and lipid mediators (e.g., PAF and LTB4), contribute essential integrin-activation signals for leukocytes at sites of inflammation. The molecular diversity of chemoattractants and their restricted temporal and spatial expression patterns combined with leukocyte-subset specific fingerprints of chemoattractant receptor expression provide a crucial mechanism for the fine-tuning of migratory cellular responses.[136,245,246]

In contrast, the migratory properties acquired by T and B lymphocytes in response to activation are diverse depending on the strength, the quality, and the context of the antigenic stimulus.[235] Specifically, antigen stimulation of naive lymphocytes results in generating effector and memory cells that express specific repertoires of trafficking molecules that guide them back to tissues containing the stimulatory antigen.[247–249] Thus, a cutaneous challenge generates preferentially a skin-tropic memory response, whereas orally ingested antigens induce preferentially gut-homing effector and memory cells. In addition to presenting antigen, dendritic cells in different lymphoid organs are endowed with information characteristic for the tissue in which the antigen was obtained. Dendritic cells in mucosa-associated lymphoid tissues, but not other lymphoid organs, possess the enzymatic machinery to synthesize retinoic acid (RA) from vitamin A.[250] Exposure of activated T cells to RA induces the expression of gut-homing receptors (i.e., α4β7 and CCR9) and suppresses skin-homing molecules.[248,251] In the absence of RA, T-cell stimulation induces few or no gut-homing molecules, but instead promotes the expression of P- and E-selectin ligands as well as CCR4, which are needed for homing to the skin. Additionally, when activated T cells are exposed to IL-12 and high levels of vitamin D3, which is physiologically induced by sunlight in the skin, they upregulate CCR10, the receptor for the epidermal chemokine CCL27.[252] This organ-specific information can reprogram and "imprint" the tissue-tropic memory cells as they differentiate from naive lymphocytes.[249]

Like T cells, B-cell subsets also express homing receptors that permit their selective trafficking to specific tissues. For example, distinct B cell subsets produce the immunoglobulin isotype IgA that is present in secreted body fluids, including tears, breast milk, and mucus. IgA$^+$ B cells are characterized by their expression of CCR10.[253,254] The ligand for CCR10, MEC/CCL28, is expressed predominantly in mucosal tissues that secrete IgA.[255] Hence, CCR10 may function as a homing receptor that allows IgA-secreting B cells to migrate to tissues where IgA is required. A large subset among the IgA secreting B cells are those in the small intestine, which in addition to CCR10 express the gut-homing receptors, α4β7 and CCR9. When naive B cells are activated in the presence of intestinal dendritic cells, they upregulate not only these two traffic receptors, but also undergo class-switching to IgA. This imprinting effect is dependent upon RA, which is sufficient to induce gut-homing receptors, but must be combined with dendritic cell-derived IL-5 or IL-6 to promote IgA class-switching.[251] More than 50 different subtypes of lymphocytes have been characterized in human blood and it is likely that multiple similar associations between homing receptors, immunological effector function, and tissue specificity will be revealed in the future.

Migration of Hematopoietic Stem Cells to the Bone Marrow and Mobilization in the Niche

The BM is the principal site of hematopoiesis in the adult body. Correspondingly, most HSCs are lodged in the BM cavity. Within the BM, maintenance of HSCs and regulation of their self-renewal and differentiation is thought to depend on the specific microenvironment, which has historically been termed "stem cell niche."[7,256–259] The central role of stem cell niches for HSC function has been recognized with the discovery that *Sl/Sl*^{d} (steel-Dickie) mice bearing a mutation in the gene encoding membrane-bound stem-cell factor (SCF, also known as KIT ligand) show failure of BM HSC maintenance.[260] However, the exact localization and the composition of BM HSC niches and the molecular crosstalk that controls the retention of HSC within the niches are the subject of intense ongoing research.[150,261–264]

Notably though, not all HSCs reside within the BM. In fact, it has been known for almost four decades that a small number of hematopoietic precursors are also present in peripheral blood.[265–268] Blood-borne HSCs continuously migrate back to the BM, presumably to fill any vacant stem cell niches.[268] Although the physiological relevance of blood-borne HSCs remains to be determined, and the magnitude might be limited, the intrinsic capacity of HSCs to home to the BM compartment is the prerequisite for successful clinical BM and stem cell transplantation. Homing of HSCs to BM is a rapid process, since IV-injected murine and human progenitors are quickly cleared from the recipient circulation.[268–270] Like mature lymphocytes, HSCs and HPCs interact through a multistep adhesion cascade with BM microvessels.[6,270–274] Initially, HPCs tether and roll along BM microvessels.[275,276] This process involves α4β7 integrin on HSC/HPC, which binds VCAM-1, as well as E- and P-selectin on BM sinusoidal endothelial cells, which bind α (1–3)-fucosylated ligands including CD44 and PSGL-1 on the surface of HPCs.[270] The subsequent firm arrest is mediated by activated α4β1 and VCAM-1, which is constitutively expressed in BM sinusoids. In addition to α4 β1 integrin, the integrins α4β7, α5β1, and α6β1, CD44 and JAM-B have recently been implicated in HSC homing to the BM.[274,277–280] Integrin-mediated adhesion is important for HSPC movement not only in adulthood, but also during embryogenesis. Thus, HSPCs in the yolk sac, AGM, and placenta express CD41 (GPIIb integrin encoded by the gene *Itga2b*). Expression gradually decreases during development, and adult HSPCs express little or no CD41.[6,281] In addition to CD41, β1 integrins tune the migration of fetal HSPCs. The use of chimeric mice generated with β1-integrin-deficient fetal HSPCs has revealed that fetal HSPCs lacking β1 integrins form and differentiate but they cannot colonize the FL, suggesting an essential role of β1 integrins in fetal HSPC trafficking. The role of the ß2-integrins LFA-1 (CD11a/CD18) and MAC-1 (CD11b/CD18) is controversially discussed. Although some studies have reported their involvement in HSPC retention,[282,283] others have indicated that the effect of β2 integrins becomes apparent only in synergy with α4β1.[284]

The chemokine CXCL12, the ligand for CXCR4 expressed by most hematopoietic cells including HSCs, is thought to play a pivotal role in BM homing of HSC. BM endothelial cells (in addition to immature osteoblasts and other stromal cells) constitutively express and secrete CXCL12. However, alternate pathways appear to exist since fetal liver-derived mouse HSCs home to the BM of adult recipients independent of CXCR4[285] and adult HSCs treated with a CXCR4 antagonist are still able to home sufficiently to the BM.[286] This indicates that HSCs may use different receptors and/or respond to distinct integrin-activation signals.

Of note, the CXCL12/CXCR4 axis is not only involved in the homing process of HSCs to the BM, but (among others) has also been linked to the retention of HSCs within stem cell niches and to the regulation of the maturation of more committed HPCs (in particular B cell progenitor cells) and PMNs.[287–292] Correspondingly, disruption of the CXCL12/CXCR4 pathway leads to release of HPCs and other BM cells into the peripheral blood.[293,294] HPCs lacking CXCR4 accumulate in the circulation and fail to undergo normal lymphopoiesis and myelopoiesis, most likely because the cells do not receive the required maturation signals. Interestingly, upregulation of metalloproteinases (see above), which cleave and inactivate CXCR4 and CXCL12, has recently been implicated in HSC mobilization.[295–298] Mechanisms that modulate the CXCR4/CXCL12 axis are also thought to play a role in the coordinated mobilization of HPCs in response to cytokines that are used for this purpose in clinical practice.[299] Rare autosomal dominant truncations of ten or more nucleotides that encode the C- terminal intracellular domain of human *CXCR4* result in a constitutively signaling CXCR4 and manifest in the WHIM syndrome (*W*arts, *H*ypogammaglobulinemia, *I*mmunodeficiency, and *M*yelokathexis).[300] Recently, described promising WHIM therapy involves chronic administration of the CXCR4 antagonist plerixafor, resulting in amelioration of the main symptoms, including disease-associated myelofibrosis, panleukopenia, anemia, and thrombocytopenia and the reduction of the papillomavirus-associated warts and carcinoma burden and frequency of other infections.[301]

In addition to the CXCL12, the egress from BM niches has been recently shown to be critically dependent on the signals provided by the nervous system. The UDP-galactose ceramide galactosyltransferase-deficient Cgt(-/-) mice exhibiting aberrant nerve conduction are characterized by absence of HSPC egress from BM following granulocyte colony-stimulating factor (G-CSF) or fucoidan administration.[302] Interestingly, norepinephrine (NE) signaling controlled bone CXCL12 downregulation and HSPC mobilization, whereas administration of a beta(2) adrenergic agonist enhanced HSPC mobilization. Therefore, the sympathetic nervous system regulates the attraction of stem cells to their niche via transduction of circadian information from the central pacemaker in the brain, the suprachiasmatic nucleus, to the BM microenvironment.[303] Recent data show that circadian regulation is regulated by beta-2 and beta-3 adrenergic receptors (beta-AR) expressed on HSCs, osteoblasts, and mesenchymal stem/progenitor cells. Moreover, beta(2)- and beta(3)-ARs have specific roles in stromal cells and cooperate during progenitor mobilization.[304] Whereas activation of beta(3)-AR downregulates Cxcl12, beta(2)-AR stimulation induces clock gene expression.[304] In addition, double deficiency in beta(2)- and beta(3)-ARs compromises enforced progenitor mobilization from the BM. For the clinical settings, the timing of stem cell harvest or infusion may influence the yield or engraftment, respectively, and may result in better therapeutic outcomes.[303]

Curiously, the paradigm of BM-derived hematopoietic cells entering blood to redistribute into the tissues is not absolute. Recent findings show that specialized shortcut channels between BM and dura mater of the meninges that surrounds the brain and spinal cord allow the resident in dura myeloid cells and B cells to be replenished directly from the scull- and vertebral BM without the cells ever entering systemic circulation.[305,306] Moreover, the inflammatory cell infiltrates into the brain and spinal cord in injury, experimental autoimmune encephalomyelitis, and the optic nerve crush model are also derived locally from the skull BM.[305] It is not clear if similar direct shortcuts from BM to surrounding tissues exist also in other anatomical locations.

LEUKOCYTE MIGRATION WITHIN TISSUES

Trafficking Patterns of Lymphocytes

After lymphocytes gain access to a tissue, they are able to migrate further to defined functional sites within. As discussed above, homing typically requires that the blood-borne leukocyte completes a complex tissue- and subset-specific multistep adhesion cascade with a possible exception for spleen where most blood-borne lymphocytes appear to leave the circulation in the absence of conventional traffic molecules. However, chemokines are essential in all lymphoid organs, including the spleen, to guide the newly arrived lymphocytes to their proper position within the organ.

Multiphoton intravital microscopy was used as a tool to decipher the mechanisms that control the extravascular traffic patterns of homed lymphocytes within lymphoid and nonlymphoid tissues.[307–315] For example, imaging experiments have shown that T cells that have entered a LN move incessantly within the paracortex (T-cell area). Here, they query the resident dendritic cells for the presence of antigens that activate their T-cell receptor (TCR). B cells that home to LNs migrate to the more superficial B-cell-rich follicles, where they may detect antigens presented by follicular dendritic cells (FDC). Activated B cells that encounter antigens then move to the margins of the B- and T-cell zones.[310] Here, they can receive help from antigen-specific CD4 T cells. Analogous specific microenvironments for T and B cells exist also in the other lymphoid tissues. In contrast to the step of trans-endothelial lymphocyte egress from blood, their crawling on the luminal endothelial cell surface preceding emigration,[316] as well as their chemokine-driven migration in the tissue, do not necessarily require integrins.[189,317] However, integrins contribute to the extravascular leukocyte motility, in either norm, or inflammation,[318] to T-cell migration in LNs, though acting not necessarily downstream of chemokine signaling but independently and in parallel to it. This suggests the involvement of differential molecular pathways downstream of chemokine receptors involved in leukocyte emigration versus their tissue migration.[319]

Migration of T Cells to T Zones Within Secondary Lymphoid Organs

After homing to SLOs, T cells migrate within the T zones. They engage in highly motile ameboid movement (average speed ~12 μm/min) and undergo multiple brief encounters with resident dendritic cells.[320–322] In this context, the fibroblastic reticular cell (FRC) network regulates naive T-cell access to the paracortex and also defines the limits of T-cell movement within this domain.[323,324] As a consequence of high velocity of T-cell motility, it has been estimated that every dendritic cell in a LN touches as many as 5000 naive T cells within one hour. When T cells encounter a specific antigen, they progressively decrease their motility, become activated and form long-lasting stable conjugates with DCs. Finally, antigen-experienced

T cells start to proliferate and resume their rapid migration while contacting DC only briefly.[322,325] The positioning and high motility of T cells in the T-cell area is dependent on CCR7 and its ligands CCL19 and CCL21.[195,225,226,307,326,327] Both ligands are abundantly expressed in T zones by the radiation-resistant stromal cells. Notably, ectopic expression of CCL21 induces the formation of LN-like structures in nonlymphoid tissues, e.g., the pancreas of mice.[328] The expression of CCL19 and CCL21, but also of CXCL13, a B-cell chemoattractant (see below), by stromal cells is strongly dependent on the cytokine lymphotoxin (LT)-α1β2 heterotrimers signaling via LT β receptor.[329–331] Correspondingly, mice deficient in LT have no morphologically detectable LNs or PPs.[332] Also, on one hand, CCL21 and CXCL13 have been shown to be transiently downregulated by a mechanism downstream of the cytokine interferon-γ altering lymphocyte and dendritic cell recruitment with possible consequences of mitigated T-cell responses to a subsequent second pathogen.[333,334] On the other hand, interferon-γ-inducible chemokines CXCL9 and CXCL10 produced in the LN by stromal cells and DCs, respectively, signal through CXCR3 on $CD4^+$ T helper 1 cells to optimize their LN positioning within interfollicular and medullary zones and promote their interaction with DCs and, consequently, effective differentiation.[335] Analogously, the same pair of chemokine ligands signaling through CXCR3 on CD8 T cells was shown to be responsible for their effector lineage differentiation and correlated with the segregation of CD8 memory and effectors at the LN periphery.[336] CD8 T cells that lacked CXCR3 remained in the LN center and differentiated into stem-like memory cell precursors.[337]

Positioning of B Cells Within Secondary Lymphoid Organs

Similar to T cells, B cells enter SLO from the blood to search for their specific antigens.[338] Also, the homing and entry of B cells into SLO such as the LNs and PPs depends on chemokine/receptor interactions that finally result in firm adhesion of integrins on the surface of HEV. This adherence is followed by movement into lymphoid tissue.[227,333,339,340] After entering SLO, the naive B cells travel to B-cell-rich areas, the B-cell follicles. This migration depends on the presence of CXCR5 on the surfaces of B cells and the localized expression of CXCL13 by follicular stromal cells.[194,331,340] Follicular B cells are also highly motile migrating on a network of FDCs, a process that is thought to be necessary to ensure optimal surveillance of the FDC for surface-displayed antigens. After a period of random migration within follicles, B cells that have not encountered a cognate antigen return to the circulation via the lymph or, in case of the spleen, via the blood. In contrast, B cells that become stimulated by antigen relocate to the B-T boundary area to solicit help from T cells, which is necessary for further differentiation. To achieve this repositioning, activated B cells rapidly upregulate CCR7. This permits their chemotaxis toward CCR7 ligands expressed in the T-cell-rich zones of the SLOs.[310] Real-time imaging has been performed to further characterize the timing of this relocalization process. Antigen-engaged follicular B cells initially reduce their migration velocity upon antigen exposure. About 6 hours later, the activated B cells move toward the follicle border with the T-cell-rich zone and undergo highly dynamic interactions with helper T cells during the following several days.[310]

In the spleen, a subpopulation of B cells is lodged in the marginal zone (MZ) immediately adjacent to the marginal sinus that surrounds the white pulp cords. The exact extent to which chemokine-induced attraction and adhesion affect the positioning of MZ B cells is still unclear. However, recent studies indicate that the lodgment of B cells in the MZ is dependent upon interactions of αLβ2 and α4β1 on MZ B cells with their ligands (ICAM-1 and VCAM-1, respectively).[341,342] As with follicular B cells in LN, antigen encounter of MZ B cells causes their rapid repositioning to the B-T boundary area. The retention of naive B cells in the MZ and their relocalization to the B-T boundary area upon antigen encounter is thought to involve signaling through the phospholipid sphingosine-1 phosphate (S1P) and its receptor $S1P_1$.[343]

LEUKOCYTE EXIT FROM TISSUES

While the coordinated role of adhesion molecules and chemokines governing lymphocyte entry into tissues has been examined in great detail, less is known about the exit of these cells from tissues. The final sections of this chapter will discuss examples of emerging research on the diverse mechanisms that regulate exit of distinct leukocyte subsets from tissues.

Reprogramming Dendritic Cells to Exit Tissues Toward Secondary Lymphoid Organs

Lymphocyte homing remains without consequence unless lymphocytes encounter dendritic cells that present their cognate antigen. Dendritic cells capture and present antigens to T cells more efficiently than any other antigen-presenting cell. In general, two routes of antigen delivery to LNs have been described to date. (1) Antigenic material becomes lymph borne and is taken up by dendritic cells that reside in the LN a priori; (2) Antigen is acquired by dendritic cells that reside in peripheral tissues and then transport the material to the draining LN. Dendritic cells constitutively patrol all tissues and engulf microorganisms, dead cells, and cellular debris. In the absence of inflammatory stimuli, the cells remain in an immature state that is only weakly immunogenic and often stimulates T-cell tolerance, rather than activation. However, multiple signals associated with infection or tissue damage can induce dendritic cell maturation. Immature dendritic cells express many chemokine receptors, including as CCR1, CCR5, and CCR6, which are believed to result in the constitutive homing of immature dendritic cells into tissues, particularly sites of inflammation where ligands for these receptors are abundant.[4,201,344–346] After exposure to a maturation stimulus, such as Toll-like receptor agonists (e.g., LPS, bacterial lipoproteins, peptidoglycans or CpG dinucleotides), which often originate from infectious pathogens,[347,348] dendritic cells lose CCR1, CCR5, and CCR6, while expression of GPCRs for lymphoid chemokines, in particular CCR7 and CXCR4 are upregulated.[349] Lymphatic endothelial cells in peripheral tissues express CCL21, the ligand for CCR7.[350] The loss of chemokine receptors that keep the dendritic cells within the tissue together with the increased expression of CCR7 results in the exit of the mature dendritic cells via the lymphatic drainage system.[225,351] Recently, using a microfluidic device that allows rapid establishment of stable gradients in three-dimensional matrices, it was shown that CCL21 is a more potent directional cue for DC migration than CCL19.[352] DC migration into the draining lymphatics requires also β2 integrin binding to ICAM-1 expressed by lymphatic endothelial cells. Moreover, junctional adhesion molecule A (JAM-A), which is expressed by DCs and the lymphatic endothelium—also affects DC migration to LNs, because the absence of JAM-A expression by DCs facilitates their migration to LNs.[203,353] The expression and activation of β1-integrins, which mediate the interaction of DCs with extracellular-matrix components, might favor retention of DCs in the periphery.[203]

While traveling to the draining LN, dendritic cells upregulate the expression of molecules for efficient antigen presentation and T-cell stimulation, and begin to generate chemokines and other cytokines, that allow them to attract and stimulate T cells.[351,354,355]

Egress of Lymphocytes From Secondary Lymphoid Organs

When naive lymphocytes do not encounter antigen on antigen-presenting dendritic cells after a period of random walk, they exit SLO through efferent lymph vessels or, in case of the spleen, by directly returning to the blood. Several adhesion receptors have been implicated in the egress of lymphocytes into lymphoid sinusoids, including PECAM-1 (CD31), the mannose receptor, which interacts with

L-selectin, and common lymphatic endothelial and vascular endothelial receptor 1 (CLEVER-1).[356]

We still have very limited information about the signals that determine the dwell time of lymphocytes in SLO (about 12 to 24 hours for T cells). However, the recent observation that the egress of both T and B cells from LN can be prevented by the immunosuppressant molecule FTY720, has revealed some of the principal mechanisms underlying lymphocyte egress. FTY720 is a synthetic derivative of myriocin, a metabolite of the fungus Isaria sinclairii, which has been used in Chinese traditional medicine. FTY720 induces lymphocyte sequestration in LN and causes profound lymphopenia. In animal models of transplantation and autoimmunity, FTY720 causes immunosuppression and it has recently been shown to exert significant therapeutic effects in a placebo-controlled clinical trial of relapsing multiple sclerosis.[357–359] While lymphocyte sequestration in LN and lymphopenia in response to FTY720 have been reported some time ago, the underlying molecular mechanisms were uncovered only recently.[199] Upon in vivo administration, FTY720 becomes rapidly phosphorylated and then binds to four of the five known S1P receptors (S1P$_1$and S1P$_{3–5}$).[194,360–362] Naive B and T cells express substantial levels of S1P$_1$, and it appears to be this receptor that plays a predominant role in lymphocytes egress from lymphoid tissues into the efferent lymph vessels. Studies using gene-targeted mice have shown that T lymphocytes deficient in S1P$_1$ cannot exit SLO (and in case of T cells also the thymus).[363,364] Reports using FTY720 and S1P$_1$-selective agonists also supported a role for S1P-S1P$_1$ signaling in the regulation of lymphocyte egress from SLOs. Notably, S1P$_1$ receptors not only regulate lymphocyte exit from tissues, but also modulate lymphocyte homing capacity.[206]

The sphingolipid S1P is abundant in blood and lymph while low levels of S1P are maintained within lymphoid tissues. This S1P gradient is established by the action of the S1P degrading enzyme S1P lyase.[365–367] Based on these findings, it has been proposed that S1P gradients between blood, lymphoid tissue, and lymph fluid together with cyclical ligand-induced modulation of S1P1 on recirculating lymphocytes regulates lymphocyte egress and determines the lymphoid organ transit time of lymphocytes.[256,368] Indeed, recent observations support that concept by showing that S1P1 on lymphocytes is downregulated in the blood, upregulated in lymphoid organs, and downregulated again in the lymph.[366] Notably, CD69, which is rapidly induced when T cells become activated negatively regulates S1P1 and thus promotes lymphocyte retention in lymphoid organs.[369,370]

Besides chemokines, other factors are also involved in the migration patterns of lymphocytes. By combining confocal, electron, and intravital microscopy, it has been found that the FRC network regulates naive T-cell access to the paracortex, whereas a distinct FDC network served as the substratum for movement of follicular B cells.[324] These results highlight the central role of stromal microanatomy in orchestrating cell migration within the LN.

Although the mechanism of HSPCs homing into the BM are well investigated, little is known about the signals regulating the exit of HSPCs out of tissues other than the BM. HSPCs arrive in peripheral organs via blood, but leave them predominantly via the draining lymphatics.[256,371] Interestingly, it has been shown that, like on the lymphocytes, S1P and S1P receptors mediate the egress of HSPCs from peripheral tissues.[6,7,256] The inhibition of S1P–S1P receptor signaling decreases the number of HSPCs in tissue-draining lymph due to impaired egress from the tissue. Stress signals that mimic an infection (induced by administration of a TLR4 agonist) reduce S1P1 expression on HSPCs, leading to the prolonged retention in peripheral organs, thus providing necessary time for HSPCs to differentiate into immune cells that are required to eliminate the danger. HSPC retention tuning in peripheral tissues could support innate immune responses by fostering a local and versatile supply of effector cells.

SUMMARY

In this chapter, we have outlined three distinct aspects of leukocyte trafficking: (1) **leukocyte entry into tissues** from the circulation; (2) **migration within tissues**; and (3) **exit from tissues**. Migration of leukocytes throughout the body is essential for the development of lymphocyte subsets and underpins their functions in immune surveillance and responses of effective adaptive immunity. The molecular reactions involved in leukocyte migration are tightly regulated and involve chemoattractants and adhesion molecules. Leukocyte migration is characterized by a considerable plasticity and specificity, since different leukocyte subsets express unique patterns of traffic molecules that enable their navigation into and within target tissues. Our expanding knowledge of the mechanisms that control leukocyte trafficking will likely influence the development of multiple therapeutic strategies, including the use of stem cells, cell immunotherapy of cancer, autoimmune, inflammatory, and infectious diseases.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Campbell DJ, Kim CH, Butcher EC. Chemokines in the systemic organization of immunity. *Immunol Rev*. 2003;195:58–71.

Carman CV, et al. Transcellular diapedesis is initiated by invasive podosomes. *Immunity*. 2007;26:784–797.

Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu Rev Immunol*. 2005;23:127–159.

Duchene J, et al. Atypical chemokine receptor 1 on nucleated erythroid cells regulates hematopoiesis. *Nat Immunol*. 2017;18:753–761.

Dyer DP, et al. Chemokine receptor redundancy and specificity are context dependent. *Immunity*. 2019;50:378–389. e375.

Girbl T, et al. Distinct compartmentalization of the chemokines CXCL1 and CXCL2 and the atypical receptor ACKR1 determine discrete stages of neutrophil diapedesis. *Immunity*. 2018;49:1062–1076.

Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. *Annu Rev Immunol*. 2014;32:659–702.

Laird DJ, von Andrian UH, Wagers AJ. Stem cell trafficking in tissue development, growth, and disease. *Cell*. 2008;132:612–630.

Lämmermann T, et al. Rapid leukocyte migration by integrin-independent flowing and squeezing. *Nature*. 2008;453:51–55.

Lapidot T, Petit I. Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. *Exp Hematol*. 2002;30:973–981.

Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*. 2007;7:678–689.

Matloubian M, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature*. 2004;427: 355–360.

McDermott DH, et al. Plerixafor for the treatment of WHIM syndrome. *N Engl J Med*. 2019;380:163–170.

Middleton J, et al. Transcytosis and surface presentation of IL-8 by venular endothelial cells. *Cell*. 1997;91:385–395.

Mora JR, von Andrian UH. T-cell homing specificity and plasticity: new concepts and future challenges. *Trends Immunol*. 2006;27:235–243.

Mueller SN, et al. Regulation of homeostatic chemokine expression and cell trafficking during immune responses. *Science*. 2007;317:670–674.

Muller WA. Mechanisms of leukocyte transendothelial migration. *Annu Rev Pathol*. 2011;6:323–344.

Nibbs RJB, Graham GJ. Immune regulation by atypical chemokine receptors. *Nat Rev Immunol*. 2013;13:815–829.

Nishikimi A, et al. Sequential regulation of DOCK2 dynamics by two phospholipids during neutrophil chemotaxis. *Science*. 2009;324:384–387.

Nomiyama H, Osada N, Yoshie O. A family tree of vertebrate chemokine receptors for a unified nomenclature. *Dev Comp Immunol*. 2011;35:705–715.

Pontejo SM, Murphy PM. Chemokines act as phosphatidylserine-bound "find-me" signals in apoptotic cell clearance. *PLoS Biol*. 2021;19:e3001259.

Proost P, et al. Chemokine isoforms and processing in inflammation and immunity. *J Autoimmun*. 2017;85:45–57.

Proudfoot AE, et al. Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc Natl Acad Sci: U S A*. 2003;100:1885–1890.

Pruenster M, et al. The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity. *Nat Immunol*. 2009;10:101–108.

Rot A, von Andrian UH. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. *Annu. Rev. Immunol*. 2004; 22:891–928.

Schumann K, et al. Immobilized chemokine fields and soluble chemokine gradients cooperatively shape migration patterns of dendritic cells. *Immunity*. 2010;32:703–713.

Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell*. 1994;76:301–314.

Ulvmar MH, et al. The atypical chemokine receptor CCRL1 shapes functional CCL21 gradients in lymph nodes. *Nat Immunol*. 2014;15:623–630.

Werth K, et al. Expression of ACKR4 demarcates the "peri-marginal sinus," a specialized vascular compartment of the splenic red pulp. *Cell Rep*. 2021; 36(2):109346.

Weber M, et al. Interstitial dendritic cell guidance by haptotactic chemokine gradients. *Science*. 2013;339:328–332.

REFERENCES

1. Steiner O, et al. Differential roles for endothelial ICAM-1, ICAM-2, and VCAM-1 in shear-resistant T cell arrest, polarization, and directed crawling on blood-brain barrier endothelium. *J Immunol*. 2010;185:4846–4855. doi:jimmunol.0903732 [pii] 10.4049/jimmunol.0903732.
2. von Andrian UH, Mackay CR. T-cell function and migration. *Two sides of the same coin. N Engl J Med*. 2000;343:1020–1034.
3. Tomura M, et al. Activated regulatory T cells are the major T cell type emigrating from the skin during a cutaneous immune response in mice. *J Clin Invest*. 2010;120:883–893. doi:40926 [pii] 10.1172/JCI40926.
4. Luster AD, Alon R, von Andrian UH. Immune cell migration in inflammation: present and future therapeutic targets. *Nat Immunol*. 2005;6:1182–1190.
5. Magnon C, Lucas D, Frenette PS. Trafficking of stem cells. *Methods Mol Biol*. 2011;750:3–24. https://doi.org/10.1007/978-1-61779-145-1_1.
6. Mazo IB, Massberg S, von Andrian UH. Hematopoietic stem and progenitor cell trafficking. *Trends Immunol*. 2011;32(10):493-503. doi:S1471-4906(11)00117-7 [pii] 10.1016/j.it.2011.06.011.
7. Massberg S, von Andrian UH. Novel trafficking routes for hematopoietic stem and progenitor cells. *Ann N Y Acad Sci*. 2009;1176:87–93. doi:NYAS4609 [pii] 10.1111/j.1749-6632.2009.04609.x.
8. Cuesta-Gomez N, Graham GJ, Campbell JDM. Chemokines and their receptors: predictors of the therapeutic potential of mesenchymal stromal cells. *J Transl Med*. 2021;19:156. https://doi.org/10.1186/s12967-021-02822-5.
9. Schiffmann E, Corcoran BA, Wahl SM. N-formylmethionyl peptides as chemoattractants for leucocytes. *Proc Natl Acad Sci U S A*. 1975;72:1059–1062. https://doi.org/10.1073/pnas.72.3.1059.
10. Rot A, Henderson LE, Copeland TD, Leonard EJ. A series of six ligands for the human formyl peptide receptor: tetrapeptides with high chemotactic potency and efficacy. *Proc Natl Acad Sci U S A*. 1987;84:7967–7971. https://doi.org/10.1073/pnas.84.22.7967.
11. Subramanian BC, Majumdar R, Parent CA. The role of the LTB_4-BLT1 axis in chemotactic gradient sensing and directed leukocyte migration. *Semin Immunol*. 2017;33:16–29. https://doi.org/10.1016/j.smim.2017.07.002.
12. Wardlaw AJ, Moqbel R, Cromwell O, Kay AB. Platelet-activating factor. A potent chemotactic and chemokinetic factor for human eosinophils. *J Clin Invest*. 1986;78:1701–1706. https://doi.org/10.1172/JCI112765.
13. Fernandez HN, Henson PM, Otani A, Hugli TE. Chemotactic response to human C3a and C5a anaphylatoxins. I. Evaluation of C3a and C5a leukotaxis in vitro and under stimulated in vivo conditions. *J Immunol*. 1978;120:109–115.
14. Rot A, von Andrian UH. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. *Annu Rev Immunol*. 2004;22:891–928.
15. Bromley SK, Mempel TR, Luster AD. Orchestrating the orchestrators: chemokines in control of T cell traffic. *Nat Immunol*. 2008;9:970–980. doi:ni.f.213 [pii] 10.1038/ni.f.213.
16. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med*. 2006;354:610–621.
17. Griffith JW, Sokol CL, Luster AD.DR,Chemokines and chemokine receptors: positioning cells for host defense and immunity.. *Annu Rev Immunol*. 2014;32:659–702.
18. Zlotnik A, Yoshie O. The chemokine superfamily revisited. *Immunity*. 2012;36:705–716. https://doi.org/10.1016/j.immuni.2012.05.008.
19. Wettschureck N, Offermanns S. Mammalian G proteins and their cell type specific functions. *Physiol Rev*. 2005;85:1159–1204.
20. Alon R, Shulman Z. Chemokine triggered integrin activation and actin remodeling events guiding lymphocyte migration across vascular barriers. *Exp Cell Res*. 2011;317:632–641. doi:S0014-4827(10)00565-3 [pii] 10.1016/j.yexcr.2010.12.007.
21. Nomiyama H, Osada N, Yoshie O. A family tree of vertebrate chemokine receptors for a unified nomenclature. *Dev Comp Immunol*. 2011;35:705–715. doi:S0145-305X(11)00020-6 [pii] 10.1016/j.dci.2011.01.019.
22. Sharma M. Chemokines and their receptors: orchestrating a fine balance between health and disease. *Crit Rev Biotechnol*. 2009;30:1–22. https://doi.org/10.1080/07388550903187418.
23. Horuk R, Proudfoot AEI. Drug discovery targeting the chemokine system–where are we? *Front Biosci (Elite Ed)*. 2009;1:209–219.
24. Murphy PM. International Union of Pharmacology. XXX. Update on chemokine receptor nomenclature. *Pharmacol Rev*. 2002;54:227–229.
25. Dyer DP, et al. Chemokine receptor redundancy and specificity are context dependent. *Immunity*. 2019;50:378–389. https://doi.org/10.1016/j.immuni.2019.01.009. e375.
26. Collins PJ, et al. Epithelial chemokine CXCL14 synergizes with CXCL12 via allosteric modulation of CXCR4. *FASEB J*. 2017;31:3084–3097. https://doi.org/10.1096/fj.201700013R.
27. Kouzeli A, et al. CXCL14 preferentially synergizes with homeostatic chemokine receptor systems. *Front Immunol*. 2020;11 https://doi.org/10.3389/fimmu.2020.561404.
28. Wang Y, et al. HIC1 deletion promotes breast cancer progression by activating tumor cell/fibroblast crosstalk. *J Clin Invest*. 2018;128:5235–5250. https://doi.org/10.1172/JCI99974.
29. Kenakin T, Onaran O. The ligand paradox between affinity and efficacy: can you be there and not make a difference? *Trends Pharmacol. Sci*. 2002;23:275–280.
30. Kenakin T. Ligand-selective receptor conformations revisited: the promise and the problem. *Trends Pharmacol. Sci*. 2003;24:346–354.
31. Steen A, Larsen O, Thiele S, Rosenkilde MM. Biased and g protein-independent signaling of chemokine receptors. *Front Immunol*. 2014;5:277. https://doi.org/10.3389/fimmu.2014.00277.
32. Smith JS, Lefkowitz RJ, Rajagopal S. Biased signalling: from simple switches to allosteric microprocessors. *Nat Rev Drug Discov*. 2018;17:243–260. https://doi.org/10.1038/nrd.2017.229.
33. Rajagopal S, et al. Biased agonism as a mechanism for differential signaling by chemokine receptors. *J Biol Chem*. 2013;288:35039–35048. https://doi.org/10.1074/jbc.M113.479113.
34. Eiger DS, Boldizsar N, Honeycutt CC, Gardner J, Rajagopal S. Biased agonism at chemokine receptors. *Cell Signal*. 2021;78:109862. https://doi.org/10.1016/j.cellsig.2020.109862.
35. Mariani M, Lang R, Binda E, Panina-Bordignon P, D'Ambrosio D. Dominance of CCL22 over CCL17 in induction of chemokine receptor CCR4 desensitization and internalization on human Th2 cells. *Eur J Immunol*. 2004;34:231–240. https://doi.org/10.1002/eji.200324429.
36. Kohout TA, et al. Differential desensitization, receptor phosphorylation, beta-arrestin recruitment, and ERK1/2 activation by the two endogenous ligands for the CC chemokine receptor 7. *J Biol Chem*. 2004;279:23214–23222. https://doi.org/10.1074/jbc.M402125200.
37. Hauser MA, Legler DF. Common and biased signaling pathways of the chemokine receptor CCR7 elicited by its ligands CCL19 and CCL21 in leukocytes. *J Leukoc Biol*. 2016;99:869–882. https://doi.org/10.1189/jlb.2MR0815-380R.
38. Schumann K, et al. Immobilized chemokine fields and soluble chemokine gradients cooperatively shape migration patterns of dendritic cells. *Immunity*. 2010;32:703–713. https://doi.org/10.1016/j.immuni.2010.04.017.
39. Nandagopal S, Wu D, Lin F. Combinatorial guidance by CCR7 ligands for T lymphocytes migration in co-existing chemokine fields. *PLoS One*. 2011;6:e18183. https://doi.org/10.1371/journal.pone.0018183.
40. Förster R, Davalos-Misslitz AC, Rot A. CCR7 and its ligands: balancing immunity and tolerance. *Nat Rev Immunol*. 2008;8:362–371. https://doi.org/10.1038/nri2297.
41. Girbl T, et al. Distinct compartmentalization of the chemokines CXCL1 and CXCL2 and the atypical receptor ACKR1 determine discrete stages of neutrophil diapedesis. *Immunity*. 2018;49:1062–1076. https://doi.org/10.1016/j.immuni.2018.09.018. e1066.
42. Colobran R, Pujol-Borrell R, Armengol MP, Juan M. The chemokine network. II. On how polymorphisms and alternative splicing increase the number of molecular species and configure intricate patterns of disease susceptibility. *Clin Exp Immunol*. 2007;150:1–12. https://doi.org/10.1111/j.1365-2249.2007.03489.x.
43. Proost P, et al. Chemokine isoforms and processing in inflammation and immunity. *J Autoimmun*. 2017;85:45–57. https://doi.org/10.1016/j.jaut.2017.06.009.
44. Vanheule V, Metzemaekers M, Janssens R, Struyf S, Proost P. How post-translational modifications influence the biological activity of chemokines. *Cytokine*. 2018;109:29–51. https://doi.org/10.1016/j.cyto.2018.02.026.
45. Liu Z, Christensson M, Forslöw A, De Meester I, Sundqvist KG. A CD26-controlled cell surface cascade for regulation of T cell motility and chemokine signals. *J Immunol*. 2009;183:3616–3624. doi:jimmunol.0804336 [pii] 10.4049/jimmunol.0804336.
46. Parks WC, Wilson CL, López-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat Rev Immunol*. 2004;4:617–629.
47. Lambeir AM, et al. Kinetic investigation of chemokine truncation by CD26/dipeptidyl peptidase IV reveals a striking selectivity within the chemokine family. *J Biol Chem*. 2001;276:29839–29845. https://doi.org/10.1074/jbc.M103106200.
48. Detheux M, et al. Natural proteolytic processing of hemofiltrate CC chemokine 1 generates a potent CC chemokine receptor (CCR)1 and

CCR5 agonist with anti-HIV properties. *J Exp Med*. 2000;192:1501–1508. https://doi.org/10.1084/jem.192.10.1501.

49. Starr AE, Dufour A, Maier J, Overall CM. Biochemical analysis of matrix metalloproteinase activation of chemokines CCL15 and CCL23 and increased glycosaminoglycan binding of CCL16. *J Biol Chem*. 2012;287: 5848–5860. https://doi.org/10.1074/jbc.M111.314609.
50. Berahovich RD, et al. Proteolytic activation of alternative CCR1 ligands in inflammation. *J Immunol*. 2005;174:7341. https://doi.org/10.4049/jimmunol.174.11.7341.
51. Cynis H, et al. The isoenzyme of glutaminyl cyclase is an important regulator of monocyte infiltration under inflammatory conditions. *EMBO Mol Med*. 2011;3:545–558. https://doi.org/10.1002/emmm.201100158.
52. Kehlen A, et al. N-terminal pyroglutamate formation in CX3CL1 is essential for its full biologic activity. *Biosci Rep*. 2017;37 https://doi.org/10.1042/bsr20170712.
53. Drury LJ, et al. Monomeric and dimeric CXCL12 inhibit metastasis through distinct CXCR4 interactions and signaling pathways. *Proc Natl Acad Sci U S A*. 2011;108:17655–17660. https://doi.org/10.1073/pnas.1101133108.
54. von Hundelshausen P, et al. Chemokine interactome mapping enables tailored intervention in acute and chronic inflammation. *Sci Transl Med*. 2017;9 https://doi.org/10.1126/scitranslmed.aah6650.
55. Corbisier J, Galés C, Huszagh A, Parmentier M, Springael J-Y. Biased signaling at chemokine receptors. *J Biol Chem*. 2015;290:9542–9554. https://doi.org/10.1074/jbc.M114.596098.
56. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*. 2007;7:678–689. doi:nri2156 [pii] 10.1038/nri2156.
57. Hawkins PT, Stephens LR, Suire S, Wilson M. PI3K signaling in neutrophils. *Curr Top Microbiol Immunol*. 2010;346:183–202. https://doi.org/10.1007/82_2010_40.
58. Huang YE, et al. Receptor-mediated regulation of PI3Ks confines PI(3,4,5)P3 to the leading edge of chemotaxing cells. *Mol Biol Cell*. 2003;14:1913–1922.
59. Gardiner EM, et al. Spatial and temporal analysis of Rac activation during live neutrophil chemotaxis. *Curr Biol*. 2002;12:2029–2034.
60. Welch HCE, et al. P-Rex1, a PtdIns(3,4,5)P3- and Gbetagamma-regulated guanine-nucleotide exchange factor for Rac. *Cell*. 2002;108:809–821.
61. Innocenti M, et al. Phosphoinositide 3-kinase activates Rac by entering in a complex with Eps8, Abi1, and Sos-1. *J Cell Biol*. 2003;160:17–23.
62. Costa C, et al. Negative feedback regulation of Rac in leukocytes from mice expressing a constitutively active phosphatidylinositol 3-kinase gamma. *Proc Natl Acad Sci U S A*. 2007;104:14354–14359. doi:0703175104 [pii] 10.1073/pnas.0703175104.
63. Liu L, Puri KD, Penninger JM, Kubes P. Leukocyte PI3Kgamma and PI3Kdelta have temporally distinct roles for leukocyte recruitment in vivo. *Blood*. 2007;110:1191–1198. doi:blood-2006-11-060103 [pii] 10.1182/blood-2006-11-060103.
64. Heit B, Liu L, Colarusso P, Puri KD, Kubes P. PI3K accelerates, but is not required for, neutrophil chemotaxis to fMLP. *J Cell Sci*. 2008;121:205–214. doi:121/2/205 [pii] 10.1242/jcs.020412.
65. Lokuta MA, et al. Type Igamma PIP kinase is a novel uropod component that regulates rear retraction during neutrophil chemotaxis. *Mol Biol Cell*. 2007;18:5069–5080. E07-05-0428 [pii] 10.1091/mbc.E07-05-0428.
66. Sarraj B, et al. Myeloid-specific deletion of tumor suppressor PTEN augments neutrophil transendothelial migration during inflammation. *J Immunol*. 2009;182:7190–7200. doi:182/11/7190 [pii] 10.4049/jimmunol.0802562.
67. Ghigo A, Damilano F, Braccini L, Hirsch E. PI3K inhibition in inflammation: Toward tailored therapies for specific diseases. *Bioessays*. 2010;32:185–196. https://doi.org/10.1002/bies.200900150.
68. Hirsch E, et al. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science*. 2000;287:1049–1053.
69. Ward SG. T lymphocytes on the move: chemokines, PI 3-kinase and beyond. *Trends Immunol*. 2006;27:80–87.
70. Sadhu C, Masinovsky B, Dick K, Sowell CG, Staunton DE. Essential role of phosphoinositide 3-kinase delta in neutrophil directional movement. *J Immunol*. 2003;170:2647–2654.
71. Hoellenriegel J, et al. The phosphoinositide 3'-kinase delta inhibitor, CAL-101, inhibits B-cell receptor signaling and chemokine networks in chronic lymphocytic leukemia. *Blood*. 2011 ;118(13):3603-3612. doi:blood-2011-05-352492 [pii] 10.1182/blood-2011-05-352492.
72. Fung-Leung WP. Phosphoinositide 3-kinase delta (PI3Kdelta) in leukocyte signaling and function. *Cell Signal*. 2010;23:603–608. doi:S0898-6568(10)00289-5 [pii] 10.1016/j.cellsig.2010.10.002.
73. Nishikimi A, et al. Sequential regulation of DOCK2 dynamics by two phospholipids during neutrophil chemotaxis. *Science*. 2009;324:384–387. doi:1170179 [pii] 10.1126/science.1170179.
74. Shulman Z, et al. DOCK2 regulates chemokine-triggered lateral lymphocyte motility but not transendothelial migration. *Blood*. 2006;108:2150–2158. doi:blood-2006-04-017608 [pii] 10.1182/blood-2006-04-017608.
75. Nombela-Arrieta C, et al. A central role for DOCK2 during interstitial lymphocyte motility and sphingosine-1-phosphate-mediated egress. *J Exp Med*. 2007;204:497–510.
76. Willars GB. Mammalian RGS proteins: multifunctional regulators of cellular signalling. *Semin Cell Dev Biol*. 2006;17:363–376.
77. Sethakorn N, Yau DM, Dulin NO. Non-canonical functions of RGS proteins. *Cell Signal*. 2010;22:1274–1281. doi:S0898-6568(10)00090-2 [pii] 10.1016/j.cellsig.2010.03.016.
78. Pitcher JA, Freedman NJ, Lefkowitz RJ. G protein-coupled receptor kinases. *Annu Rev Biochem*. 1998;67:653–692. https://doi.org/10.1146/annurev.biochem.67.1.653.
79. Moore CA, Milano SK, Benovic JL. Regulation of receptor trafficking by GRKs and arrestins. *Annu Rev Physiol*. 2007;69:451–482. https://doi.org/10.1146/annurev.physiol.69.022405.154712.
80. Zhang H, Meng F, Chu C-L, Takai T, Lowell CA. The Src family kinases Hck and Fgr negatively regulate neutrophil and dendritic cell chemokine signaling via PIR-B. *Immunity*. 2005;22:235–246. https://doi.org/10.1016/j.immuni.2005.01.004.
81. Brzostowski JA, Kimmel AR. Signaling at zero G: G-protein-independent functions for 7-TM receptors. *Trends Biochem Sci*. 2001;26:291–297.
82. Yan SL-S, et al. Unrestrained Gα_{i2} signaling disrupts neutrophil trafficking, aging, and clearance. *Front Immunol*. 2021;12 https://doi.org/10.3389/fimmu.2021.679856.
83. Kienle K, et al. Neutrophils self-limit swarming to contain bacterial growth in vivo. *Science*. 2021;372 https://doi.org/10.1126/science.abe7729.
84. Ulvmar MH, Hub E, Rot A. Atypical chemokine receptors. *Exp Cell Res*. 2011;317:556–568. https://doi.org/10.1016/j.yexcr.2011.01.012.
85. Nibbs RJB, Graham GJ. Immune regulation by atypical chemokine receptors. *Nat Rev Immunol*. 2013;13:815–829.
86. Bachelerie F, et al. International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. *Pharmacol Rev*. 2014;66:1–79. https://doi.org/10.1124/pr.113.007724.
87. Le Mercier A, et al. GPR182 is an endothelium-specific atypical chemokine receptor that maintains hematopoietic stem cell homeostasis. *Proc Natl Acad Sci U S A*. 2021;118 https://doi.org/10.1073/pnas.2021596118.
88. Pruenster M, et al. The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity. *Nat Immunol*. 2009;10:101–108. https://doi.org/10.1038/ni.1675.
89. Levoye A, Balabanian K, Baleux F, Bachelerie F, Lagane B. CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. *Blood*. 2009;113:6085–6093. https://doi.org/10.1182/blood-2008-12-196618.
90. Del Prete A, et al. The atypical receptor CCRL2 is required for CXCR2-dependent neutrophil recruitment and tissue damage. *Blood*. 2017;130: 1223–1234. https://doi.org/10.1182/blood-2017-04-777680.
91. Schioppa T, et al. Molecular basis for CCRL2 regulation of leukocyte migration. *Front Cell Dev Biol*. 2020;8 https://doi.org/10.3389/fcell.2020.615031.
92. Novitzky-Basso I, Rot A. Duffy antigen receptor for chemokines and its involvement in patterning and control of inflammatory chemokines. *Front Immunol*. 2012;3:266. https://doi.org/10.3389/fimmu.2012.00266.
93. Werth K, et al. Expression of ACKR4 demarcates the "peri-marginal sinus," a specialized vascular compartment of the splenic red pulp. *Cell Rep*. 2021;36 https://doi.org/10.1016/j.celrep.2021.109346.
94. Colditz IG, Schneider MA, Pruenster M, Rot A. Chemokines at large: in-vivo mechanisms of their transport, presentation and clearance. *Thromb Haemost*. 2007;97:688–693.
95. Lau S, et al. A negative-feedback loop maintains optimal chemokine concentrations for directional cell migration. *Nat Cell Biol*. 2020;22: 266–273. https://doi.org/10.1038/s41556-020-0465-4.
96. Ulvmar MH, et al. The atypical chemokine receptor CCRL1 shapes functional CCL21 gradients in lymph nodes. *Nat Immunol*. 2014;15: 623–630. https://doi.org/10.1038/ni.2889.
97. Bryce SA, et al. ACKR4 on stromal cells scavenges CCL19 to enable CCR7-dependent trafficking of APCs from inflamed skin to lymph nodes. *J Immunol*. 2016;196:3341–3353. https://doi.org/10.4049/jimmunol.1501542.
98. del Molino del Barrio I, Kirby J, Ali S. The role of chemokine and glycosaminoglycan interaction in chemokine-mediated migration in vitro and in vivo. *Methods Enzymol*. 2016;570:309–333. https://doi.org/10.1016/bs.mie.2015.09.002.

99. Reitsma S, Slaaf DW, Vink H, van Zandvoort MAMJ, oude Egbrink MGA. The endothelial glycocalyx: composition, functions, and visualization. *Pflugers Arch*. 2007;454:345–359. https://doi.org/10.1007/s00424-007-0212-8.
100. Weber M, et al. Interstitial dendritic cell guidance by haptotactic chemokine gradients. *Science*. 2013;339:328–332. https://doi.org/10.1126/science.1228456.
101. Sarris M, et al. Inflammatory chemokines direct and restrict leukocyte migration within live tissues as glycan-bound gradients. *Curr Biol*. 2012;22:2375–2382. https://doi.org/10.1016/j.cub.2012.11.018.
102. Rot A. Neutrophil attractant/activation protein-1 (interleukin-8) induces in vitro neutrophil migration by haptotactic mechanism. *Eur J Immunol*. 1993;23:303–306. https://doi.org/10.1002/eji.1830230150.
103. Dyer DP, Salanga CL, Volkman BF, Kawamura T, Handel TM. The dependence of chemokine-glycosaminoglycan interactions on chemokine oligomerization. *Glycobiology*. 2016;26:312–326. https://doi.org/10.1093/glycob/cwv100.
104. Sepuru KM, Rajarathnam K. Structural basis of chemokine interactions with heparan sulfate, chondroitin sulfate, and dermatan sulfate. *J Biol Chem*. 2019;294:15650–15661. https://doi.org/10.1074/jbc.RA119.009879.
105. Monneau Y, Arenzana-Seisdedos F, Lortat-Jacob H. The sweet spot: how GAGs help chemokines guide migrating cells. *J Leukoc Biol*. 2016;99:935–953. https://doi.org/10.1189/jlb.3MR0915-440R.
106. Bao X, et al. Endothelial heparan sulfate controls chemokine presentation in recruitment of lymphocytes and dendritic cells to lymph nodes. *Immunity*. 2010;33:817–829. doi:S1074-7613(10)00404-8 [pii] 10.1016/j.immuni.2010.10.018.
107. Hsu H-P, et al. Heparan sulfate is essential for thymus growth. *J Biol Chem*. 2021;296 https://doi.org/10.1016/j.jbc.2021.100419.
108. Webb LM, Ehrengruber MU, Clark-Lewis I, Baggiolini M, Rot A. Binding to heparan sulfate or heparin enhances neutrophil responses to interleukin 8. *Proc Natl Acad Sci U S A*. 1993;90:7158–7162. https://doi.org/10.1073/pnas.90.15.7158.
109. Proudfoot AE, et al. Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc Natl Acad Sci U S A*. 2003;100:1885–1890. https://doi.org/10.1073/pnas.0334864100.
110. Rot A. Chemokine patterning by glycosaminoglycans and interceptors. *Front Biosci (Landmark Ed)*. 2010;15:645–660. https://doi.org/10.2741/3638.
111. Metzemaekers M, et al. Glycosaminoglycans regulate CXCR3 ligands at distinct levels: protection against processing by dipeptidyl peptidase IV/CD26 and interference with receptor signaling. *Int J Mol Sci*. 2017;18 https://doi.org/10.3390/ijms18071513.
112. Kuschert GS, et al. Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses. *Biochemistry*. 1999;38:12959–12968. https://doi.org/10.1021/bi990711d.
113. Pontejo SM, Murphy PM. Chemokines act as phosphatidylserine-bound "find-me" signals in apoptotic cell clearance. *PLoS Biol*. 2021;19:e3001259. https://doi.org/10.1371/journal.pbio.3001259.
114. Joseph PRB, Sawant KV, Rajarathnam K. Heparin-bound chemokine CXCL8 monomer and dimer are impaired for CXCR1 and CXCR2 activation: implications for gradients and neutrophil trafficking. *Open Biol*. 2017;7 https://doi.org/10.1098/rsob.170168.
115. Graham GJ, Handel TM, Proudfoot AEI. Leukocyte adhesion: reconceptualizing chemokine presentation by glycosaminoglycans. *Trends Immunol*. 2019;40:472–481. https://doi.org/10.1016/j.it.2019.03.009.
116. Schwarz J, et al. Dendritic cells interpret haptotactic chemokine gradients in a manner governed by signal-to-noise ratio and dependent on GRK6. *Curr Biol*. 2017;27:1314–1325. https://doi.org/10.1016/j.cub.2017.04.004.
117. Yang B-G, et al. Binding of lymphoid chemokines to collagen IV that accumulates in the basal lamina of high endothelial venules: its implications in lymphocyte trafficking. *J Immunol*. 2007;179:4376–4382. https://doi.org/10.4049/jimmunol.179.7.4376.
118. Yung SC, Murphy PM. Antimicrobial chemokines. *Front Immunol*. 2012;3 https://doi.org/10.3389/fimmu.2012.00276. 276-276.
119. Rot A, Jones AP, Webb LM. Some aspects of NAP-1/IL-8 pathophysiology. II: chemokine secretion by exocrine glands. *Adv Exp Med Biol*. 1993;351:77–85. https://doi.org/10.1007/978-1-4615-2952-1_9.
120. Jones AP, Webb LM, Anderson AO, Leonard EJ, Rot A. Normal human sweat contains interleukin-8. *J Leukoc Biol*. 1995;57:434–437. https://doi.org/10.1002/jlb.57.3.434.
121. Michie CA, Tantscher E, Schall T, Rot A. Physiological secretion of chemokines in human breast milk. *Eur Cytokine Netw*. 1998;9:123–129.
122. Rostène W, Kitabgi P, Parsadaniantz SM. Chemokines: a new class of neuromodulator? *Nat Rev Neurosci*. 2007;8:895–903. https://doi.org/10.1038/nrn2255.
123. Milenkovic VM, Stanton EH, Nothdurfter C, Rupprecht R, Wetzel CH. The role of chemokines in the pathophysiology of major depressive disorder. *Int J Mol Sci*. 2019;20 https://doi.org/10.3390/ijms20092283.
124. Trettel F, Di Castro MA, Limatola C. Chemokines: key molecules that orchestrate communication among neurons, microglia and astrocytes to preserve brain function. *Neuroscience*. 2020;439:230–240. https://doi.org/10.1016/j.neuroscience.2019.07.035.
125. Kitaya K, Yamada H. Pathophysiological roles of chemokines in human reproduction: an overview. *Am J Reprod Immunol*. 2011;65:449–459. https://doi.org/10.1111/j.1600-0897.2010.00928.x.
126. Nagasawa T, et al. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature*. 1996;382:635–638. https://doi.org/10.1038/382635a0.
127. Tran PB, Miller RJ. Chemokine receptors: signposts to brain development and disease. *Nat Rev Neurosci*. 2003;4:444–455. https://doi.org/10.1038/nrn1116.
128. Cha YR, et al. Chemokine signaling directs trunk lymphatic network formation along the preexisting blood vasculature. *Dev Cell*. 2012;22:824–836. https://doi.org/10.1016/j.devcel.2012.01.011.
129. Bernardini G, et al. Analysis of the role of chemokines in angiogenesis. *J Immunol Methods*. 2003;273:83–101. https://doi.org/10.1016/s0022-1759(02)00420-9.
130. Vlahakis SR, et al. G protein-coupled chemokine receptors induce both survival and apoptotic signaling pathways. *J Immunol*. 2002;169:5546. https://doi.org/10.4049/jimmunol.169.10.5546.
131. Alon R, Ley K. Cells on the run: shear-regulated integrin activation in leukocyte rolling and arrest on endothelial cells. *Curr Opin Cell Biol*. 2008;20:525–532. doi:S0955-0674(08)00074-4 [pii] 10.1016/j.ceb.2008.04.003.
132. Vestweber D. Adhesion and signaling molecules controlling the transmigration of leukocytes through endothelium. *Immunol Rev*. 2007;218:178–196. doi:IMR533 [pii] 10.1111/j.1600-065X.2007.00533.x.
133. Hickey MJ, Kubes P. Intravascular immunity: the host-pathogen encounter in blood vessels. *Nat Rev Immunol*. 2009;9:364–375.
134. Muller WA. Mechanisms of leukocyte transendothelial migration. *Annu Rev Pathol*. 2011;6:323–344. https://doi.org/10.1146/annurev-pathol-011110-130224.
135. Nourshargh S, Hordijk PL, Sixt M. Breaching multiple barriers: leukocyte motility through venular walls and the interstitium. *Nat Rev Mol Cell Biol*. 2010;11:366–378. doi:nrm2889 [pii] 10.1038/nrm2889.
136. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell*. 1994;76:301–314.
137. Rot A. Endothelial cell binding of NAP-1/IL-8: role in neutrophil emigration. *Immunol Today*. 1992;13:291–294. https://doi.org/10.1016/0167-5699(92)90039-A.
138. Ley K. Arrest chemokines. *Front Immunol*. 2014;5 https://doi.org/10.3389/fimmu.2014.00150. 150-150.
139. Marki A, Esko JD, Pries AR, Ley K. Role of the endothelial surface layer in neutrophil recruitment. *J Leukoc Biol*. 2015;98:503–515. https://doi.org/10.1189/jlb.3MR0115-011R.
140. Massena S, et al. A chemotactic gradient sequestered on endothelial heparan sulfate induces directional intraluminal crawling of neutrophils. *Blood*. 2010;116:1924–1931. https://doi.org/10.1182/blood-2010-01-266072.
141. Grönloh MLB, Arts JJG, van Buul JD. Neutrophil transendothelial migration hotspots—mechanisms and implications. *J Cell Sci*. 2021;134 https://doi.org/10.1242/jcs.255653.
142. Rot A, et al. Some aspects of IL-8 pathophysiology. III: chemokine interaction with endothelial cells. *J Leukoc Biol*. 1996;59:39–44. https://doi.org/10.1002/jlb.59.1.39.
143. Wolff B, Burns AR, Middleton J, Rot A. Endothelial cell "memory" of inflammatory stimulation: human venular endothelial cells store interleukin 8 in Weibel-Palade bodies. *J Exp Med*. 1998;188:1757–1762. https://doi.org/10.1084/jem.188.9.1757.
144. Øynebråten I, Bakke O, Brandtzaeg P, Johansen F-E, Haraldsen G. Rapid chemokine secretion from endothelial cells originates from 2 distinct compartments. *Blood*. 2004;104:314–320. https://doi.org/10.1182/blood-2003-08-2891.
145. Middleton J, et al. Transcytosis and surface presentation of IL-8 by venular endothelial cells. *Cell*. 1997;91:385–395. https://doi.org/10.1016/s0092-8674(00)80422-5.
146. Thiriot A, et al. Differential DARC/ACKR1 expression distinguishes venular from non-venular endothelial cells in murine tissues. *BMC Biol*. 2017;15:45 https://doi.org/10.1186/s12915-017-0381-7.
147. Minten C, et al. DARC shuttles inflammatory chemokines across the blood-brain barrier during autoimmune central nervous system inflammation. *Brain*. 2014;137:1454–1469. https://doi.org/10.1093/brain/awu045.
148. Miyabe Y, Miyabe C, Mani V, Mempel TR, Luster AD. Atypical complement receptor C5aR2 transports C5a to initiate neutrophil adhesion and inflammation. *Sci Immunol*. 2019;4:eaav5951. https://doi.org/10.1126/sciimmunol.aav5951.
149. Rot A. Contribution of Duffy antigen to chemokine function. *Cytokine Growth Factor Rev*. 2005;16:687–694. https://doi.org/10.1016/j.cytogfr.2005.05.011.

150. Duchene J, et al. Atypical chemokine receptor 1 on nucleated erythroid cells regulates hematopoiesis. *Nat Immunol*. 2017;18:753–761. https://doi.org/10.1038/ni.3763.
151. Gutjahr JC, et al. The dimeric form of CXCL12 binds to atypical chemokine receptor 1. *Sci Signal*. 2021;14:eabc9012. https://doi.org/10.1126/scisignal.abc9012.
152. Mei J, et al. CXCL5 regulates chemokine scavenging and pulmonary host defense to bacterial infection. *Immunity*. 2010;33:106–117. https://doi.org/10.1016/j.immuni.2010.07.009.
153. Moser M, et al. Kindlin-3 is required for beta2 integrin-mediated leukocyte adhesion to endothelial cells. *Nat Med*. 2009;15:300–305. doi:nm.1921 [pii] 10.1038/nm.1921.
154. Fujita M, Takada YK, Takada Y. The chemokine fractalkine can activate integrins without CX3CR1 through direct binding to a ligand-binding site distinct from the classical RGD-binding site. *PLoS One*. 2014;9:e96372. https://doi.org/10.1371/journal.pone.0096372.
155. Fujita M, Davari P, Takada YK, Takada Y. Stromal cell-derived factor-1 (CXCL12) activates integrins by direct binding to an allosteric ligand-binding site (site 2) of integrins without CXCR4. *Biochem J*. 2018;475:723–732. https://doi.org/10.1042/BCJ20170867.
156. Phillipson M, et al. Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade. *J Exp Med*. 2006;203:2569–2575. doi:jem.20060925 [pii] 10.1084/jem.20060925.
157. Ryschich E, et al. Active leukocyte crawling in microvessels assessed by digital time-lapse intravital microscopy. *J Surg Res*. 2006;135:291–296. doi:S0022-4804(06)00067-9 [pii] 10.1016/j.jss.2006.02.020.
158. Wojciechowski JC, Sarelius IH. Preferential binding of leukocytes to the endothelial junction region in venules in situ. *Microcirculation*. 2005;12:349–359.
159. Auffray C, et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science*. 2007;317:666–670.
160. Carlin LM, et al. Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal. *Cell*. 2013;153:362–375. https://doi.org/10.1016/j.cell.2013.03.010.
161. Swirski FK, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science*. 2009;325:612–616. doi:325/5940/612 [pii] 10.1126/science.1175202.
162. Bain CC, et al. Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. *Nat Immunol*. 2014;15:929–937. https://doi.org/10.1038/ni.2967.
163. Hoeffel G, et al. Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. *J Exp Med*. 2012;209:1167–1181. https://doi.org/10.1084/jem.20120340.
164. Hashimoto D, et al. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity*. 2013;38:792–804. https://doi.org/10.1016/j.immuni.2013.04.004.
165. Sumagin R, Prizant H, Lomakina E, Waugh RE, Sarelius IH. LFA-1 and Mac-1 define characteristically different intralumenal crawling and emigration patterns for monocytes and neutrophils in situ. *J Immunol*. 2010;185:7057–7066. doi:jimmunol.1001638 [pii] 10.4049/jimmunol.1001638.
166. Feng D, Nagy JA, Pyne K, Dvorak HF, Dvorak AM. Neutrophils emigrate from venules by a transendothelial cell pathway in response to FMLP. *J Exp Med*. 1998;187:903–915.
167. Schulte D, et al. Stabilizing the VE-cadherin-catenin complex blocks leukocyte extravasation and vascular permeability. *EMBO J*. 2011 doi:emboj2011304 [pii] 10.1038/emboj.2011.304.
168. Cernuda-Morollón E, Gharbi S, Millán J. Discriminating between the paracellular and transcellular routes of diapedesis. *Methods Mol Biol*. 2010;616:69–82. https://doi.org/10.1007/978-1-60761-461-6_5.
169. Langer HF, Chavakis T. Leukocyte-endothelial interactions in inflammation. *J Cell Mol Med*. 2009;13:1211–1220. doi:JCMM811 [pii] 10.1111/j.1582-4934.2009.00811.x.
170. Carman CV, et al. Transcellular diapedesis is initiated by invasive podosomes. *Immunity*. 2007;26:784–797. doi:S1074-7613(07)00291-9 [pii] 10.1016/j.immuni.2007.04.015.
171. Carman CV, Springer TA. A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. *J Cell Biol*. 2004;167:377–388. doi:jcb.200404129 [pii] 10.1083/jcb.200404129.
172. Nieminen M, et al. Vimentin function in lymphocyte adhesion and transcellular migration. *Nat Cell Biol*. 2006;8:156–162. doi:ncb1355 [pii] 10.1038/ncb1355.
173. Johnson-Léger CA, Aurrand-Lions M, Beltraminelli N, Fasel N, Imhof BA. Junctional adhesion molecule-2 (JAM-2) promotes lymphocyte transendothelial migration. *Blood*. 2002;100:2479–2486.
174. Schenkel AR, Mamdouh Z, Chen X, Liebman RM, Muller WA. CD99 plays a major role in the migration of monocytes through endothelial junctions. *Nat Immunol*. 2002;3:143–150.
175. Bixel MG, et al. CD99 and CD99L2 act at the same site as, but independently of, PECAM-1 during leukocyte diapedesis. *Blood*. 2010;116:1172–1184. doi:blood-2009-12-256388 [pii] 10.1182/blood-2009-12-256388.
176. Wegmann F, et al. ESAM supports neutrophil extravasation, activation of Rho, and VEGF-induced vascular permeability. *J Exp Med*. 2006;203:1671–1677. doi:jem.20060565 [pii] 10.1084/jem.20060565.
177. Woodfin A, et al. JAM-A mediates neutrophil transmigration in a stimulus-specific manner in vivo: evidence for sequential roles for JAM-A and PECAM-1 in neutrophil transmigration. *Blood*. 2007;110:1848–1856. doi:blood-2006-09-047431 [pii] 10.1182/blood-2006-09-047431.
178. Nourshargh S, Krombach F, Dejana E. The role of JAM-A and PECAM-1 in modulating leukocyte infiltration in inflamed and ischemic tissues. *J Leukoc Biol*. 2006;80:714–718. doi:jlb.1105645 [pii] 10.1189/jlb.1105645.
179. Scheiermann C, et al. Junctional adhesion molecule-C mediates leukocyte infiltration in response to ischemia reperfusion injury. *Arterioscler Thromb Vasc Biol*. 2009;29:1509–1515. doi:ATVBAHA.109.187559 [pii] 10.1161/ATVBAHA.109.187559.
180. Schnoor M, Alcaide P, Voisin M-B, van Buul JD. Crossing the vascular wall: common and unique mechanisms exploited by different leukocyte subsets during extravasation. *Mediators Inflamm*. 2015;2015:946509. https://doi.org/10.1155/2015/946509.
181. Snyderman R, Goetzl EJ. Molecular and cellular mechanisms of leukocyte chemotaxis. *Science*. 1981;213:830–837.
182. Sixt M, Bauer M, Lämmermann T, Fässler R. Beta1 integrins: zip codes and signaling relay for blood cells. *Curr Opin Cell Biol*. 2006;18:482–490. doi:S0955-0674(06)00117-7 [pii] 10.1016/j.ceb.2006.08.007.
183. Franca-Koh J, Devreotes PN. Moving forward: mechanisms of chemoattractant gradient sensing. *Physiology (Bethesda)*. 2004;19:300–308. doi:10.1152/physiol.00017.2004 19/5/300 [pii].
184. Sánchez-Madrid F, Serrador JM. Bringing up the rear: defining the roles of the uropod. *Nat Rev Mol Cell Biol*. 2009;10:353–359. doi:nrm2680 [pii] 10.1038/nrm2680.
185. Tanino Y, et al. Kinetics of chemokine-glycosaminoglycan interactions control neutrophil migration into the airspaces of the lungs. *J Immunol*. 2010;184:2677–2685. doi:jimmunol.0903274 [pii] 10.4049/jimmunol.0903274.
186. Linhardt RJ, Toida T. Role of glycosaminoglycans in cellular communication. *Acc Chem Res*. 2004;37:431–438. https://doi.org/10.1021/ar030138x.
187. Tweedy L, Insall RH. Self-generated gradients yield exceptionally robust steering cues. *Front Cell Dev Biol*. 2020;8:133. https://doi.org/10.3389/fcell.2020.00133.
188. Schmidt S, Friedl P. Interstitial cell migration: integrin-dependent and alternative adhesion mechanisms. *Cell Tissue Res*. 2010;339:83–92. https://doi.org/10.1007/s00441-009-0892-9.
189. Lämmermann T, et al. Rapid leukocyte migration by integrin-independent flowing and squeezing. *Nature*. 2008;453:51–55. doi:nature06887 [pii] 10.1038/nature06887.
190. Gazit Y, et al. Leukocyte adhesion deficiency type II: long-term follow-up and review of the literature. *J Clin Immunol*. 2010;30:308–313. https://doi.org/10.1007/s10875-009-9354-0.
191. Meves A, Stremmel C, Gottschalk K, Fassler R. The Kindlin protein family: new members to the club of focal adhesion proteins. *Trends Cell Biol*. 2009;19:504–513. doi:S0962-8924(09)00152-4 [pii] 10.1016/j.tcb.2009.07.006.
192. Etzioni A. Genetic etiologies of leukocyte adhesion defects. *Curr Opin Immunol*. 2009;21:481–486. doi:S0952-7915(09)00140-X [pii] 10.1016/j.coi.2009.07.005.
193. Svensson L, et al. Leukocyte adhesion deficiency-III is caused by mutations in KINDLIN3 affecting integrin activation. *Nat Med*. 2009;15:306–312. https://doi.org/10.1038/nm.1931.
194. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu Rev Immunol*. 2005;23:127–159.
195. Worbs T, Förster R. T cell migration dynamics within lymph nodes during steady state: an overview of extracellular and intracellular factors influencing the basal intranodal T cell motility. *Curr Top Microbiol Immunol*. 2009;334:71–105. https://doi.org/10.1007/978-3-540-93864-4_4.
196. Marelli-Berg FM, Cannella L, Dazzi F, Mirenda V. The highway code of T cell trafficking. *J Pathol*. 2008;214:179–189. https://doi.org/10.1002/path.2269.
197. Henrickson SE, von Andrian UH. Single-cell dynamics of T-cell priming. *Curr Opin Immunol*. 2007;19:249–258. doi:S0952-7915(07)00065-9 [pii] 10.1016/j.coi.2007.04.013.
198. Mempel TR, Junt T, von Andrian UH. Rulers over randomness: stroma cells guide lymphocyte migration in lymph nodes. *Immunity*. 2006;25:867–869. doi:S1074-7613(06)00517-6 [pii] 10.1016/j.immuni.2006.11.002.

199. Massberg S, von Andrian UH. Fingolimod and sphingosine-1-phosphate—modifiers of lymphocyte migration. *N Engl J Med*. 2006;355:1088–1091.
200. Tadayon S, et al. Clever-1 contributes to lymphocyte entry into the spleen via the red pulp. *Sci Immunol*. 2019;4 https://doi.org/10.1126/sciimmunol.aat0297.
201. Bonasio R, von Andrian UH. Generation, migration and function of circulating dendritic cells. *Curr Opin Immunol*. 2006;18:503–511.
202. Villadangos JA, Young L. Antigen-presentation properties of plasmacytoid dendritic cells. *Immunity*. 2008;29:352–361. S1074-7613(08)00381-6 [pii] 10.1016/j.immuni.2008.09.002.
203. Randolph GJ, Angeli V, Swartz MA. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat Rev Immunol*. 2005;5:617–628. doi:nri1670 [pii] 10.1038/nri1670.
204. Umemoto E, et al. Novel regulators of lymphocyte trafficking across high endothelial venules. *Crit Rev Immunol*. 2011;31:147–169. 6fb1a82a406aab26,4446801b33e906ed [pii].
205. Galkina E, et al. T lymphocyte rolling and recruitment into peripheral lymph nodes is regulated by a saturable density of L-selectin (CD62L). *Eur J Immunol*. 2007;37:1243–1253. https://doi.org/10.1002/eji.200636481.
206. Halin C, et al. The S1P-analog FTY720 differentially modulates T-cell homing via HEV: T-cell-expressed S1P1 amplifies integrin activation in peripheral lymph nodes but not in Peyer patches. *Blood*. 2005;106:1314–1322.
207. Gauguet J-M, Rosen SD, Marth JD, von Andrian UH. Core 2 branching beta1,6-N-acetylglucosaminyltransferase and high endothelial cell N-acetylglucosamine-6-sulfotransferase exert differential control over B- and T-lymphocyte homing to peripheral lymph nodes. *Blood*. 2004;104:4104–4112.
208. Ruddle NH. High endothelial venules and lymphatic vessels in tertiary lymphoid organs: characteristics, functions, and regulation. *Front Immunol*. 2016;7 https://doi.org/10.3389/fimmu.2016.00491.
209. Milutinovic S, Abe J, Godkin A, Stein JV, Gallimore A. The dual role of high endothelial venules in cancer progression versus immunity. *Trends Cancer*. 2021;7:214–225. https://doi.org/10.1016/j.trecan.2020.10.001.
210. Kannagi R, et al. Sialylated and sulfated carbohydrate ligands for selectins and siglecs: involvement in traffic and homing of human memory T and B lymphocytes. *Adv Exp Med Biol*. 2011;705:549–569. https://doi.org/10.1007/978-1-4419-7877-6_29.
211. Streeter PR, Rouse BT, Butcher EC. Immunohistologic and functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes. *J. Cell Biol*. 1988;107:1853–1862.
212. Berg EL, Robinson MK, Warnock RA, Butcher EC. The human peripheral lymph node vascular addressin is a ligand for LECAM-1, the peripheral lymph node homing receptor. *J. Cell Biol*. 1991;114:343–349.
213. Xu B, et al. Lymphocyte homing to bronchus-associated lymphoid tissue (BALT) is mediated by L-selectin/PNAd, alpha4beta1 integrin/VCAM-1, and LFA-1 adhesion pathways. *J Exp Med*. 2003;197:1255–1267. https://doi.org/10.1084/jem.20010685jem.20010685.
214. Warnock RA, Askari S, Butcher EC, von Andrian UH. Molecular mechanisms of lymphocyte homing to peripheral lymph nodes. *J Exp Med*. 1998;187:205–216.
215. Sun H, et al. The CC' and DE loops in Ig domains 1 and 2 of MAdCAM-1 play different roles in MAdCAM-1 binding to low- and high-affinity integrin alpha4beta7. *J Biol Chem*. 2011;286:12086–12092. doi:M110.208900 [pii] 10.1074/jbc.M110.208900.
216. Park EJ, et al. Distinct roles for LFA-1 affinity regulation during T-cell adhesion, diapedesis, and interstitial migration in lymph nodes. *Blood*. 2010;115:1572–1581. doi:blood-2009-08-237917 [pii] 10.1182/blood-2009-08-237917.
217. Medoff BD, et al. CD11b+ myeloid cells are the key mediators of Th2 cell homing into the airway in allergic inflammation. *J Immunol*. 2009;182:623–635. doi:182/1/623 [pii].
218. Gorfu G, et al. Beta7 integrin deficiency suppresses B cell homing and attenuates chronic ileitis in SAMP1/YitFc mice. *J Immunol*. 2010;185:5561–5568. jimmunol.0903938 [pii] 10.4049/jimmunol.0903938.
219. Campbell DJ, Kim CH, Butcher EC. Chemokines in the systemic organization of immunity. *Immunol Rev*. 2003;195:58–71.
220. Stein JV, et al. CCR7-mediated physiological lymphocyte homing involves activation of a tyrosine kinase pathway. *Blood*. 2003;101:38–44. https://doi.org/10.1182/blood-2002-03-0841-2002-03-0841.
221. Stein JV, et al. The CC chemokine thymus-derived chemotactic agent 4 (TCA-4, secondary lymphoid tissue chemokine, 6Ckine, exodus-2) triggers lymphocyte function-associated antigen 1-mediated arrest of rolling T lymphocytes in peripheral lymph node high endothelial venules. *J Exp Med*. 2000;191:61–76.
222. Baekkevold ES, et al. The CCR7 ligand ELC (CCL19) is transcytosed in high endothelial venules and mediates T cell recruitment. *J Exp Med*. 2001;193:1105–1112.
223. Palframan RT, et al. Inflammatory chemokine transport and presentation in HEV: a remote control mechanism for monocyte recruitment to lymph nodes in inflamed tissues. *J Exp Med*. 2001;194:1361–1374.
224. Warnock RA, et al. The role of chemokines in the microenvironmental control of T versus B cell arrest in Peyer's patch high endothelial venules. *J Exp Med*. 2000;191:77–88.
225. Förster R, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell*. 1999;99:23–33.
226. Gunn MD, et al. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J Exp Med*. 1999;189:451–460.
227. Okada T, et al. Chemokine requirements for B cell entry to lymph nodes and Peyer's patches. *J Exp Med*. 2002;196:65–75.
228. Ebisuno Y, et al. Cutting edge: the B cell chemokine CXC chemokine ligand 13/B lymphocyte chemoattractant is expressed in the high endothelial venules of lymph nodes and Peyer's patches and affects B cell trafficking across high endothelial venules. *J Immunol*. 2003;171:1642–1646.
229. Tang ML, Steeber DA, Zhang XQ, Tedder TF. Intrinsic differences in L-selectin expression levels affect T and B lymphocyte subset-specific recirculation pathways. *J Immunol*. 1998;160:5113–5121.
230. Guarda G, et al. L-selectin-negative CCR7—effector and memory CD8+ T cells enter reactive lymph nodes and kill dendritic cells. *Nat Immunol*. 2007;8:743–752. https://doi.org/10.1038/ni1469.
231. Bogoslowski A, et al. Neutrophils recirculate through lymph nodes to survey tissues for pathogens. *J Immunol*. 2020;204:2552–2561. https://doi.org/10.4049/jimmunol.2000022.
232. Berlin C, et al. Alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell*. 1995;80:413–422.
233. Berg EL, McEvoy LM, Berlin C, Bargatze RF, Butcher EC. L-selectin-mediated lymphocyte rolling on MAdCAM-1. *Nature*. 1993;366:695–698.
234. Berlin C, et al. Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell*. 1993;74:185–195.
235. Mora JR, von Andrian UH. T-cell homing specificity and plasticity: new concepts and future challenges. *Trends Immunol*. 2006;27:235–243.
236. McEver RP. Selectins: lectins that initiate cell adhesion under flow. *Curr Opin Cell Biol*. 2002;14:581–586.
237. Zarbock A, Müller H, Kuwano Y, Ley K. PSGL-1-dependent myeloid leukocyte activation. *J Leukoc Biol*. 2009;86:1119–1124. doi:jlb.0209117 [pii] 10.1189/jlb.0209117.
238. Rosen SD. Ligands for L-selectin: homing, inflammation, and beyond. *Annu Rev Immunol*. 2004;22:129–156.
239. Sperandio M, et al. Alpha 2,3-sialyltransferase-IV is essential for L-selectin ligand function in inflammation. *Eur J Immunol*. 2006;36:3207–3215. https://doi.org/10.1002/eji.200636157.
240. Ley K, Kansas GS. Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation. *Nat Rev Immunol*. 2004;4:325–335. https://doi.org/10.1038/nri1351nri1351. [pii].
241. León B, Ardavín C. Monocyte migration to inflamed skin and lymph nodes is differentially controlled by L-selectin and PSGL-1. *Blood*. 2008;111:3126–3130. doi: blood-2007-07-100610 [pii] 10.1182/blood-2007-07-100610.
242. Sperandio M, et al. P-selectin glycoprotein ligand-1 mediates L-selectin-dependent leukocyte rolling in venules. *J Exp Med*. 2003;197:1355–1363.
243. Martinez M, et al. Regulation of PSGL-1 interactions with L-selectin, P-selectin, and E-selectin: role of human fucosyltransferase-IV and -VII. *J Biol Chem*. 2005;280:5378–5390. doi:M410899200 [pii] 10.1074/jbc.M410899200.
244. Porter JC, Hall A. Epithelial ICAM-1 and ICAM-2 regulate the egression of human T cells across the bronchial epithelium. *FASEB J*. 2009;23:492–502. doi:fj.08-115899 [pii] 10.1096/fj.08-115899.
245. Zabel BA, et al. Chemoattractants, extracellular proteases, and the integrated host defense response. *Exp Hematol*. 2006;34:1021–1032. doi: S0301-472X(06)00314-6 [pii] 10.1016/j.exphem.2006.05.003.
246. Luster AD. The role of chemokines in linking innate and adaptive immunity. *Curr Opin Immunol*. 2002;14:129–135. doi: S0952791501003089 [pii].
247. Campbell DJ, Butcher EC. Rapid acquisition of tissue-specific homing phenotypes by CD4$^{(+)}$ T cells activated in cutaneous or mucosal lymphoid tissues. *J Exp Med*. 2002;195:135–141.
248. Mora JR, et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature*. 2003;424:88–93.
249. Mora JR, von Andrian UH. Specificity and plasticity of memory lymphocyte migration. *Curr Top Microbiol Immunol*. 2006;308 83-11.
250. Iwata M, et al. Retinoic acid imprints gut-homing specificity on T cells. *Immunity*. 2004;21:527–538.
251. Mora JR, et al. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science*. 2006;314:1157–1160.
252. Sigmundsdottir H, et al. DCs metabolize sunlight-induced vitamin D3 to "program" T cell attraction to the epidermal chemokine CCL27. *Nat Immunol*. 2007;8:285–293.

253. Kunkel EJ, et al. CCR10 expression is a common feature of circulating and mucosal epithelial tissue IgA Ab-secreting cells. *J Clin Invest*. 2003; 111:1001–1010.
254. Lazarus NH, et al. A common mucosal chemokine (mucosae-associated epithelial chemokine/CCL28) selectively attracts IgA plasmablasts. *J Immunol*. 2003;170:3799–3805.
255. Pan J, et al. A novel chemokine ligand for CCR10 and CCR3 expressed by epithelial cells in mucosal tissues. *J Immunol*. 2000;165:2943–2949.
256. Massberg S, et al. Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. *Cell*. 2007;131: 994–1008. doi:S0092-8674(07)01356-6 [pii] 10.1016/j.cell.2007.09.047.
257. Oh IH, Kwon KR. Concise review: multiple niches for hematopoietic stem cell regulations. *Stem Cells*. 2010;28:1243–1249. https://doi.org/10.1002/stem.453.
258. Carlesso N, Cardoso AA. Stem cell regulatory niches and their role in normal and malignant hematopoiesis. *Curr Opin Hematol*. 2010;17:281–286. https://doi.org/10.1097/MOH.0b013e32833a25d8.
259. Schofield R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells*. 1978;4:7–25.
260. McCulloch EA, Siminovitch L, Till JE, Russell ES, Bernstein SE. The cellular basis of the genetically determined hemopoietic defect in anemic mice of genotype *Sl/Sl*^{d}. *Blood*. 1965;26:399–410.
261. Calvi LM, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature*. 2003;425:841–846.
262. Cheng T, et al. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science*. 2000;287:1804–1808.
263. Adams GB, et al. Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. *Nature*. 2006;439:599–603.
264. Scadden DT. The stem-cell niche as an entity of action. *Nature*. 2006;441: 1075–1079.
265. Dorie MJ, Maloney MA, Patt HM. Turnover of circulating hematopoietic stem cells. *Exp Hematol*. 1979;7:483–489.
266. Goodman JW, Hodgson GS. Evidence for stem cells in the peripheral blood of mice. *Blood*. 1962;19:702–714.
267. Fleming WH, Alpern EJ, Uchida N, Ikuta K, Weissman IL. Steel factor influences the distribution and activity of murine hematopoietic stem cells in vivo. *Proc Natl Acad Sci U S A*. 1993;90:3760–3764.
268. Wright DE, Wagers AJ, Gulati AP, Johnson FL, Weissman IL. Physiological migration of hematopoietic stem and progenitor cells. *Science*. 2001;294: 1933–1936.
269. Hidalgo A, Weiss LA, Frenette PS. Functional selectin ligands mediating human CD34$^{(+)}$ cell interactions with bone marrow endothelium are enhanced postnatally. *J Clin Invest*. 2002;110:559–569.
270. Frenette PS, Subbarao S, Mazo IB, von Andrian UH, Wagner DD. Endothelial selectins and vascular cell adhesion molecule-1 promote hematopoietic progenitor homing to bone marrow [see comments]. *Proc Natl Acad Sci U S A*. 1998;95:14423–14428.
271. Peled A, et al. The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34$^{(+)}$ cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. *Blood*. 2000; 95:3289–3296.
272. Grabovsky V, et al. Subsecond induction of alpha4 integrin clustering by immobilized chemokines stimulates leukocyte tethering and rolling on endothelial vascular cell adhesion molecule 1 under flow conditions. *J Exp Med*. 2000;192:495–506.
273. Peled A, et al. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. *Science*. 1999;283:845–848.
274. Katayama Y, Hidalgo A, Peired A, Frenette PS. Integrin alpha4beta7 and its counterreceptor MAdCAM-1 contribute to hematopoietic progenitor recruitment into bone marrow following transplantation. *Blood*. 2004;104: 2020–2026.
275. Narasipura SD, Wojciechowski JC, Charles N, Liesveld JL, King MR. P-Selectin coated microtube for enrichment of CD34^{+} hematopoietic stem and progenitor cells from human bone marrow. *Clin Chem*. 2008;54:77–85. doi:clinchem.2007.089896 [pii] 10.1373/clinchem.2007.089896.
276. Lapidot T, Dar A, Kollet O. How do stem cells find their way home? *Blood*. 2005;106:1901–1910. doi:2005-04-1417 [pii] 10.1182/blood-2005-04-1417.
277. Qian H, Tryggvason K, Jacobsen SE, Ekblom M. Contribution of alpha6 integrins to hematopoietic stem and progenitor cell homing to bone marrow and collaboration with alpha4 integrins. *Blood*. 2006;107:3503–3510.
278. Avigdor A, et al. CD44 and hyaluronic acid cooperate with SDF-1 in the trafficking of human CD34+ stem/progenitor cells to bone marrow. *Blood*. 2004;103:2981–2989.
279. Arcangeli ML, et al. JAM-B regulates maintenance of hematopoietic stem cells in the bone marrow. *Blood*. 2011;118:4609–4619. doi: blood-2010-12-323972 [pii] 10.1182/blood-2010-12-323972.
280. Méndez-Ferrer S, Frenette PS. Hematopoietic stem cell trafficking: regulated adhesion and attraction to bone marrow microenvironment. *Ann N Y Acad Sci*. 2007;1116:392–413. doi:1116/1/392 [pii] 10.1196/annals.1402.086.
281. Mikkola HK, Orkin SH. The journey of developing hematopoietic stem cells. *Development*. 2006;133:3733–3744. doi:133/19/3733 [pii] 10.1242/dev.02568.
282. Velders GA, et al. Enhancement of G-CSF-induced stem cell mobilization by antibodies against the beta 2 integrins LFA-1 and Mac-1. *Blood*. 2002;100:327–333.
283. Hidalgo A, Peired AJ, Weiss LA, Katayama Y, Frenette PS. The integrin alphaMbeta2 anchors hematopoietic progenitors in the bone marrow during enforced mobilization. *Blood*. 2004;104:993–1001. https://doi.org/10.1182/blood-2003-10-3702-2003-10-3702.
284. Papayannopoulou T, et al. Synergistic mobilization of hemopoietic progenitor cells using concurrent beta1 and beta2 integrin blockade or beta2-deficient mice. *Blood*. 2001;97:1282–1288.
285. Mazo IB, Quackenbush EJ, Lowe JB, von Andrian UH. Total body irradiation causes profound changes in endothelial traffic molecules for hematopoietic progenitor cell recruitment to bone marrow. *Blood*. 2002;99: 4182–4191.
286. Bonig H, Priestley GV, Papayannopoulou T. Hierarchy of molecular-pathway usage in bone marrow homing and its shift by cytokines. *Blood*. 2006;107:79–86.
287. Lapidot T, Petit I. Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. *Exp Hematol*. 2002;30:973–981.
288. Jujo K, et al. CXCR4 blockade augments bone marrow progenitor cell recruitment to the neovasculature and reduces mortality after myocardial infarction. *Proc Natl Acad Sci U S A*. 2010;107:11008–11013. doi:0914248107 [pii] 10.1073/pnas.0914248107.
289. Glodek AM, Honczarenko M, Le Y, Campbell JJ, Silberstein LE. Sustained activation of cell adhesion is a differentially regulated process in B lymphopoiesis. *J Exp Med*. 2003;197:461–473.
290. Honczarenko M, et al. SDF-1 responsiveness does not correlate with CXCR4 expression levels of developing human bone marrow B cells. *Blood*. 1999;94:2990–2998.
291. Moll NM, Ransohoff RM. CXCL12 and CXCR4 in bone marrow physiology. *Expert Rev Hematol*. 2010;3:315–322. https://doi.org/10.1586/ehm.10.16.
292. Honczarenko M, et al. CCR5-binding chemokines modulate CXCL12 (SDF-1)-induced responses of progenitor B cells in human bone marrow through heterologous desensitization of the CXCR4 chemokine receptor. *Blood*. 2002;100:2321–2329. https://doi.org/10.1182/blood-2002-01-0248.
293. Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature*. 1998;393:595–599.
294. Nagasawa T, et al. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature*. 1996;382:635–638.
295. Heissig B, et al. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell*. 2002;109:625–637.
296. Shirvaikar N, Marquez-Curtis LA, Shaw AR, Turner AR, Janowska-Wieczorek A. MT1-MMP association with membrane lipid rafts facilitates G-CSF—induced hematopoietic stem/progenitor cell mobilization. *Exp Hematol*. 2010;38:823–835. doi:S0301-472X(10)00189-X [pii] 10.1016/j.exphem.2010.05.002.
297. Vagima Y, et al. MT1-MMP and RECK are involved in human CD34+ progenitor cell retention, egress, and mobilization. *J Clin Invest*. 2009;119:492–503. doi:36541 [pii] 10.1172/JCI36541.
298. Iwakura A, et al. Estradiol enhances recovery after myocardial infarction by augmenting incorporation of bone marrow-derived endothelial progenitor cells into sites of ischemia-induced neovascularization via endothelial nitric oxide synthase-mediated activation of matrix metalloproteinase-9. *Circulation*. 2006;113:1605–1614. doi:CIRCULATIONAHA.105.553925 [pii] 10.1161/CIRCULATIONAHA.105.553925.
299. Petit I, et al. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat Immunol*. 2002;3:687–694.
300. Hernandez PA, et al. Mutations in the chemokine receptor gene CXCR4 are associated with WHIM syndrome, a combined immunodeficiency disease. *Nat Genet*. 2003;34:70–74. https://doi.org/10.1038/ng1149.
301. McDermott DH, et al. Plerixafor for the treatment of WHIM syndrome. *N Engl J Med*. 2019;380:163–170. https://doi.org/10.1056/NEJMoa1808575.
302. Katayama Y, et al. Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell*. 2006;124:407–421. doi:S0092-8674(05)01328-0 [pii] 10.1016/j.cell.2005.10.041.
303. Méndez-Ferrer S, Chow A, Merad M, Frenette PS. Circadian rhythms influence hematopoietic stem cells. *Curr Opin Hematol*. 2009;16:235–242. https://doi.org/10.1097/MOH.0b013e32832bd0f5.

304. Méndez-Ferrer S, Battista M, Frenette PS. Cooperation of beta(2)- and beta(3)-adrenergic receptors in hematopoietic progenitor cell mobilization. *Ann N Y Acad Sci*. 2010;1192:139–144. doi:NYAS5390 [pii] 10.1111/j.1749-6632.2010.05390.x.
305. Cugurra A, et al. Skull and vertebral bone marrow are myeloid cell reservoirs for the meninges and CNS parenchyma. *Science*. 2021;373:eabf7844. https://doi.org/10.1126/science.abf7844.
306. Brioschi S, et al. Heterogeneity of meningeal B cells reveals a lymphopoietic niche at the CNS borders. *Science*. 2021;373:eabf9277. https://doi.org/10.1126/science.abf9277.
307. Worbs T, Mempel TR, Bölter J, von Andrian UH, Forster R. CCR7 ligands stimulate the intranodal motility of T lymphocytes in vivo. *J Exp Med*. 2007;204:489–495.
308. Mempel TR, Scimone ML, Mora JR, von Andrian UH. In vivo imaging of leukocyte trafficking in blood vessels and tissues. *Curr Opin Immunol*. 2004;16:406–417.
309. Sumen C, Mempel TR, Mazo IB, Von Andrian UH. Intravital microscopy: visualizing immunity in context. *Immunity*. 2004;21:315–329.
310. Okada T, Cyster JG. B cell migration and interactions in the early phase of antibody responses. *Curr Opin Immunol*. 2006;18:278–285.
311. Cahalan MD, Parker I, Wei SH, Miller MJ. Two-photon tissue imaging: seeing the immune system in a fresh light. *Nat Rev Immunol*. 2002;2:872–880.
312. Cahalan MD, Parker I, Wei SH, Miller MJ. Real-time imaging of lymphocytes in vivo. *Curr Opin Immunol*. 2003;15:372–377.
313. Matheu MP, et al. Imaging of effector memory T cells during a delayed-type hypersensitivity reaction and suppression by Kv1.3 channel block. *Immunity*. 2008;29:602–614. doi:S1074-7613(08)00408-1 [pii] 10.1016/j.immuni.2008.07.015.
314. Huang JH, et al. Requirements for T lymphocyte migration in explanted lymph nodes. *J Immunol*. 2007;178:7747–7755.
315. Halin C, Mora JR, Sumen C, von Andrian UH. In vivo imaging of lymphocyte trafficking. *Annu Rev Cell Dev Biol*. 2005;21:581–603. https://doi.org/10.1146/annurev.cellbio.21.122303.133159.
316. Shulman Z, et al. Lymphocyte crawling and transendothelial migration require chemokine triggering of high-affinity LFA-1 integrin. *Immunity*. 2009;30:384–396. https://doi.org/10.1016/j.immuni.2008.12.020.
317. Woolf E, et al. Lymph node chemokines promote sustained T lymphocyte motility without triggering stable integrin adhesiveness in the absence of shear forces. *Nat Immunol*. 2007;8:1076–1085. https://doi.org/10.1038/ni1499.
318. Overstreet MG, et al. Inflammation-induced interstitial migration of effector CD4$^+$ T cells is dependent on integrin αV. *Nat Immunol*. 2013;14:949–958. https://doi.org/10.1038/ni.2682.
319. Hons M, et al. Chemokines and integrins independently tune actin flow and substrate friction during intranodal migration of T cells. *Nat Immunol*. 2018;19:606–616. https://doi.org/10.1038/s41590-018-0109-z.
320. Miller MJ, Wei SH, Parker I, Cahalan MD. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science*. 2002;296:1869–1873.
321. Bousso P, Robey E. Dynamics of CD8$^+$ T cell priming by dendritic cells in intact lymph nodes. *Nat Immunol*. 2003;4:579–585.
322. Mempel TR, Henrickson SE, Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature*. 2004;427:154–159.
323. Bajénoff M, Glaichenhaus N, Germain RN. Fibroblastic reticular cells guide T lymphocyte entry into and migration within the splenic T cell zone. *J Immunol*. 2008;181:3947–3954. doi:181/6/3947 [pii].
324. Bajénoff M, et al. Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. *Immunity*. 2006;25:989–1001.
325. Miller MJ, Safrina O, Parker I, Cahalan MD. Imaging the single cell dynamics of CD4+ T cell activation by dendritic cells in lymph nodes. *J Exp Med*. 2004;200:847–856.
326. Okada T, Cyster JG. CC chemokine receptor 7 contributes to Gi-dependent T cell motility in the lymph node. *J Immunol*. 2007;178:2973–2978.
327. Worbs T, Bernhardt G, Förster R. Factors governing the intranodal migration behavior of T lymphocytes. *Immunol Rev*. 2008;221:44–63. doi:IMR580 [pii] 10.1111/j.1600-065X.2008.00580.x.
328. Chen S-C, et al. Ectopic expression of the murine chemokines CCL21a and CCL21b induces the formation of lymph node-like structures in pancreas, but not skin, of transgenic mice. *J Immunol*. 2002;168:1001–1008.
329. Ansel KM, Cyster JG. Chemokines in lymphopoiesis and lymphoid organ development. *Curr Opin Immunol*. 2001;13:172–179.
330. Cyster JG. Lymphoid organ development and cell migration. *Immunol Rev*. 2003;195:5–14.
331. Yu P, et al. B cells control the migration of a subset of dendritic cells into B cell follicles via CXC chemokine ligand 13 in a lymphotoxin-dependent fashion. *J Immunol*. 2002;168:5117–5123.
332. De Togni P, et al. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science*. 1994;264:703–707.
333. Ortolano S, Hwang I-Y, Han S-B, Kehrl JH. Roles for phosphoinositide 3-kinases, Bruton's tyrosine kinase, and Jun kinases in B lymphocyte chemotaxis and homing. *Eur J Immunol*. 2006;36:1285–1295. https://doi.org/10.1002/eji.200535799.
334. Mueller SN, et al. Regulation of homeostatic chemokine expression and cell trafficking during immune responses. *Science*. 2007;317:670–674. doi:317/5838/670 [pii] 10.1126/science.1144830.
335. Groom JR, et al. CXCR3 chemokine receptor-ligand interactions in the lymph node optimize CD4+ T helper 1 cell differentiation. *Immunity*. 2012;37:1091–1103. https://doi.org/10.1016/j.immuni.2012.08.016.
336. Kastenmüller W, et al. Peripheral prepositioning and local CXCL9 chemokine-mediated guidance orchestrate rapid memory CD8$^+$ T cell responses in the lymph node. *Immunity*. 2013;38:502–513. https://doi.org/10.1016/j.immuni.2012.11.012.
337. Duckworth BC, et al. Effector and stem-like memory cell fates are imprinted in distinct lymph node niches directed by CXCR3 ligands. *Nat Immunol*. 2021;22:434–448. https://doi.org/10.1038/s41590-021-00878-5.
338. Cyster JG. B cell follicles and antigen encounters of the third kind. *Nat Immunol*. 2010;11:989–996. doi:ni.1946 [pii] 10.1038/ni.1946.
339. Fooksman DR, et al. Development and migration of plasma cells in the mouse lymph node. *Immunity*. 2010;33:118–127. doi: S1074-7613(10)00241-4 [pii] 10.1016/j.immuni.2010.06.015.
340. Pereira JP, Kelly LM, Cyster JG. Finding the right niche: B-cell migration in the early phases of T-dependent antibody responses. *Int Immunol*. 2010;22:413–419. doi:dxq047 [pii] 10.1093/intimm/dxq047.
341. Manevich-Mendelson E, et al. Talin1 is required for integrin-dependent B lymphocyte homing to lymph nodes and the bone marrow but not for follicular B-cell maturation in the spleen. *Blood*. 2010;116:5907–5918. doi: blood-2010-06-293506 [pii] 10.1182/blood-2010-06-293506.
342. Lu TT, Cyster JG. Integrin-mediated long-term B cell retention in the splenic marginal zone. *Science*. 2002;297:409–412.
343. Cinamon G, et al. Sphingosine 1-phosphate receptor 1 promotes B cell localization in the splenic marginal zone. *Nat Immunol*. 2004;5:713–720.
344. Alvarez D, Vollmann EH, von Andrian UH. Mechanisms and consequences of dendritic cell migration. *Immunity*. 2008;29:325–342. doi:S1074-7613(08)00377-4 [pii] 10.1016/j.immuni.2008.08.006.
345. Cravens PD, Lipsky PE. Dendritic cells, chemokine receptors and autoimmune inflammatory diseases. *Immunol Cell Biol*. 2002;80:497–505. doi:1118 [pii] 10.1046/j.1440-1711.2002.01118.x.
346. Sozzani S, Allavena P, Vecchi A, Mantovani A. Chemokines and dendritic cell traffic. *J Clin Immunol*. 2000;20:151–160.
347. Kawai T, Akira S. Innate immune recognition of viral infection. *Nat Immunol*. 2006;7:131–137.
348. Granucci F, Foti M, Ricciardi-Castagnoli P. Dendritic cell biology. *Adv Immunol*. 2005;88:193–233.
349. Macagno A, Napolitani G, Lanzavecchia A, Sallusto F. Duration, combination and timing: the signal integration model of dendritic cell activation. *Trends Immunol*. 2007;28:227–233. doi:S1471-4906(07)00079-8 [pii] 10.1016/j.it.2007.03.008.
350. Saeki H, Moore AM, Brown MJ, Hwang ST. Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *J Immunol*. 1999;162:2472–2475.
351. Johnson LA, Jackson DG. Inflammation-induced secretion of CCL21 in lymphatic endothelium is a key regulator of integrin-mediated dendritic cell transmigration. *Int Immunol*. 2010;22:839–849. doi:dxq435 [pii] 10.1093/intimm/dxq435.
352. Haessler U, Pisano M, Wu M, Swartz MA. Dendritic cell chemotaxis in 3D under defined chemokine gradients reveals differential response to ligands CCL21 and CCL19. *Proc Natl Acad Sci U S A*. 2011;108:5614–5619. doi:1014920108 [pii] 10.1073/pnas.1014920108.
353. Cera MR, et al. Increased DC trafficking to lymph nodes and contact hypersensitivity in junctional adhesion molecule-A-deficient mice. *J Clin Invest*. 2004;114:729–738. https://doi.org/10.1172/JCI21231.
354. Roediger B, Ng LG, Smith AL, Fazekas de St Groth B, Weninger W. Visualizing dendritic cell migration within the skin. *Histochem Cell Biol*. 2008;130:1131–1146. https://doi.org/10.1007/s00418-008-0531-7.
355. Milling S, Yrlid U, Cerovic V, MacPherson G. Subsets of migrating intestinal dendritic cells. *Immunol Rev*. 2010;234:259–267. doi:IMR866 [pii] 10.1111/j.0105-2896.2009.00866.x.
356. Salmi M, Koskinen K, Henttinen T, Elima K, Jalkanen S. CLEVER-1 mediates lymphocyte transmigration through vascular and lymphatic endothelium. *Blood*. 2004;104:3849–3857.

357. Zhi L, et al. FTY720 blocks egress of T cells in part by abrogation of their adhesion on the lymph node sinus. *J Immunol*. 2011;187:2244–2251. doi:jimmunol.1100670 [pii] 10.4049/jimmunol.1100670.
358. Brinkmann V, Lynch KR. FTY720: targeting G-protein-coupled receptors for sphingosine 1-phosphate in transplantation and autoimmunity. *Curr Opin Immunol*. 2002;14:569.
359. Kappos L, et al. Oral fingolimod (FTY720) for relapsing multiple sclerosis. *N Engl J Med*. 2006;355:1124–1140.
360. Brinkmann V, et al. Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nat Rev Drug Discov*. 2010:883–897. doi:nrd3248 [pii] 10.1038/nrd3248.
361. Rosen H, Goetzl EJ. Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. *Nat Rev Immunol*. 2005;5:560–570.
362. Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol*. 2003;4:397–407.
363. Ledgerwood LG, et al. The sphingosine 1-phosphate receptor 1 causes tissue retention by inhibiting the entry of peripheral tissue T lymphocytes into afferent lymphatics. *Nat Immunol*. 2008;9:42–53. doi:ni1534 [pii] 10.1038/ni1534.
364. Matloubian M, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature*. 2004;427:355–360.
365. Chi H. Sphingosine-1-phosphate and immune regulation: trafficking and beyond. *Trends Pharmacol Sci*. 2011;32:16–24. doi:S0165-6147(10)00202-6 [pii] 10.1016/j.tips.2010.11.002.
366. Schwab SR, et al. Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. *Science*. 2005;309:1735–1739.
367. Spiegel S, Milstien S. The outs and the ins of sphingosine-1-phosphate in immunity. *Nat Rev Immunol*. 2011;11:403–415. doi:nri2974 [pii] 10.1038/nri2974.
368. Obinata H, Hla T. Sphingosine 1-phosphate in coagulation and inflammation. *Semin Immunopathol*. 2012;34:73–91. https://doi.org/10.1007/s00281-011-0287-3.
369. Grigorova IL, Panteleev M, Cyster JG. Lymph node cortical sinus organization and relationship to lymphocyte egress dynamics and antigen exposure. *Proc Natl Acad Sci U S A*. 2010;107:20447–20452. doi:1009968107 [pii] 10.1073/pnas.1009968107.
370. Shiow LR, et al. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature*. 2006;440:540–544.
371. Laird DJ, von Andrian UH, Wagers AJ. Stem cell trafficking in tissue development, growth, and disease. *Cell*. 2008;132:612–630. doi:S0092-8674(08)00197-9 [pii] 10.1016/j.cell.2008.01.041.

CHAPTER 13

STEM CELL MODEL OF HEMATOLOGIC DISEASES

Omar Abdel-Wahab

CELL-OF-ORIGIN STUDIES IN HEMATOLOGIC MALIGNANCIES

One of the prevailing models of cancer development proposes that a cancer is initiated and maintained through the function of CSCs, which represent a rare population of cells within a cancer that have an indefinite proliferative potential and are ultimately responsible for the generation of the bulk of cancer cells. This so-called CSC hypothesis has been best studied in hematopoietic malignancies. The ability to purify hematopoietic cells more easily than cells from other tissues, combined with the well-defined cell-surface markers of hematopoietic cells, has allowed the prospective isolation of nearly every hematopoietic cell subset from humans as well as mice.

The CSC hypothesis proposes that cancers are organized into hierarchical populations like normal tissues. At the apex of hierarchy are largely quiescent long-lived CSCs with marked self-renewal capacity that sustain the disease and give rise to the majority of the bulk cancer cells that constitute the disease. While identification of a single normal hematopoietic cell subset as the target of the malignant transformation and the cellular reservoir for disease has been possible for a variety of myeloid leukemias and lymphomas, pinpointing a single cell as the target of malignant transformation has not yet proven possible for other hematopoietic malignancies. In this chapter, we discuss efforts to identify the malignant stem cell for each of the common forms of myeloid and lymphoid leukemias.

ACUTE MYELOID LEUKEMIA

The first evidence of a stem cell origin of malignancy came from studies in 1997 performed by Blair et al.,[1] as well as Bonnet and Dick[2] in acute myeloid leukemia (AML). These studies demonstrated that most leukemia cells were unable to proliferate extensively and that only a subset of cells was consistently clonogenic. In these studies, a small subset of human Thy1$^-$CD34$^+$CD38$^-$ AML cells (0.2% to 1.0%) was identified and shown to be the only cells capable of transferring human AML to immunodeficient mice. In humans, normal hematopoietic stem cells (HSCs) reside in the lineage-negative (Lin$^-$) CD34$^+$CD38$^-$CD90$^+$CD45RA$^-$ compartment and generate multipotent progenitors with lymphomyeloid potential (LMPPs) defined as Lin$^-$CD34$^+$CD38$^-$CD90$^-$CD45RA$^-$ cells, as well as more committed myeloid progenitors that are present in the CD34$^+$CD38$^+$ compartment (see Chapter 9).[3] Among the myeloid progenitors, the common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), and megakaryocyte-erythroid progenitors (MEPs) can be discriminated based on the differential expressions of CD123 (IL3RA), CD110 (MPL), and CD45RA.

The initial observation that AML leukemia-initiating cells (LICs) reside within the CD34$^+$CD38$^-$ compartment suggested that AML HSCs are rare cells that most closely resemble normal HSCs, sharing a common limited immunophenotype and being a rare population (Fig. 13.1). However, subsequent data have suggested that this conclusion is an oversimplification and that the cell of origin of any myeloid malignancy is likely dictated by a combination of the specific genetic and epigenetic alterations present in the individual patient as well as the cells in which these alterations occur.[4,5] For example, the aforementioned earliest studies of LICs in AML relied on their transplantation into immunodeficient nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (see box on Evolution of Immunodeficient Mouse Models) to assay the ability of a defined population of AML cells to give rise to AML in vivo. However, using more immunodeficient xenotransplant models, primary human cells from both CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ compartments have been shown to have LIC activity. In addition, work by Vyas and colleagues has revealed that two expanded populations, both with LIC activity, exist in CD34$^+$ AML (see Fig. 13.1).[9] One population shares the immunophenotype of normal LMPPs and the other mirrors the GMP population. The LMPP-like leukemic stem cell (LSC) population can give rise to the GMP-like LSC population, but either can give rise to AML in immunocompromised mice in vivo.

As described in the box on Functional Evaluation of Cell-of-Origin In Vivo, the leukemogenic effects of specific oncogenes directly depend on the specific oncogene as well as the target cell of expression. Based on these facts, consistent LICs may be most easily defined for specific genetically defined subsets of leukemias (such as specific chronic leukemias defined by specific translocations or point mutations) but are much more difficult to define for normal karyotype AML. For example, expression of the *AML1-ETO* fusion transcript, generated by the common t(8;21) translocation in AML, can

EVOLUTION OF IMMUNODEFICIENT MOUSE MODELS

Xenotransplantation of human hematopoietic cells into immunocompromised mice has been an important assay for studying normal and malignant human hematopoiesis for more than 50 years.[6] The first breakthrough in xenograft modeling of human hematopoiesis was the discovery of the severe combined immune-deficient (SCID) mouse. Transplantation of human bone marrow cells into the SCID mouse not only regenerated T and B cells, which the mice lacked genetically, but also continued to generate myeloid progenitors long term. SCID mice are limited by high levels of innate immune function that impede human engraftment, however. To circumvent this limitation, SCID mice were back-crossed onto nonobese diabetic (NOD) mice harboring innate immune defects. NOD-SCID mice support higher levels of human engraftment but are limited by the development of thymic lymphomas and the persistence of functioning natural killer (NK) cells. Both of these problems were addressed by deletion of the IL-2 receptor common gamma chain. Resultant NOD/SCID/gamma-null (NSG) mice have complete loss of B, T, and NK cells; and their defective cytokine signaling also prevents lymphomagenesis. NSG mice support higher levels of human donor cell engraftment and serve as assays that functionally assess the ability of potential cancer stem cells to initiate a malignancy. Newer types of humanized mice, such as the MISTRG mouse, have been developed that express human cytokines that are not cross-reactive with murine cells and are able to stimulate the growth of more human cell types in vivo.[7] Other humanization strategies include constitutive expression of HLA class I and II that strengthens T-cell function or constitutive expression of Sirpa, a transmembrane protein on macrophages that induces tolerance to human grafts. Loss-of-function mutations in KIT proto-oncogene receptor tyrosine kinase (KIT) has allowed for the creation of hematopoietic stem cell (HSC) xenografts without the need for irradiation of the recipient mouse.[8]

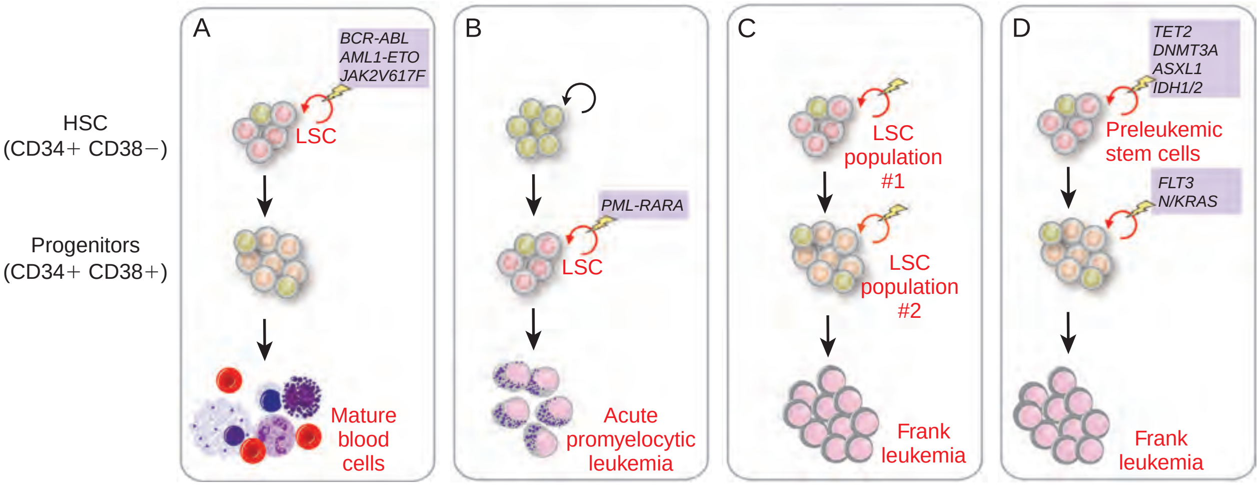


Figure 13.1 SCHEMATIC MODELS DEPICTING THE ORIGIN OF LEUKEMIC STEM CELLS (LSCS) IN VARIOUS MYELOID MALIGNANCIES. (A) Genetic evidence from some forms of acute and chronic myeloid leukemias reveals that the inciting genetic event occurs in a cell type in which normal hematopoietic stem cells (HSCs) are enriched (lineage-negative (Lin$^-$)CD34$^+$CD38$^-$). For example, the *AML1-ETO* and *BCR-ABL* translocations found in acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) are present in HSCs. In addition, the *JAK2*V617F mutation is present in HSCs. However, the presence of these mutations alone may not be sufficient to generate overt AML and may be associated with aberrant self-renewal (as is the case with *AML1-ETO*) or chronic myeloproliferation (as with *BCR-ABL* and *JAK2*V617F) alone. (B) In contrast, other forms of AML appear to be generated due to the acquisition of genetic alterations in a cell type more differentiated than HSCs. For example, *PML-RARA* translocations occur in Lin$^-$CD34$^+$CD38$^+$ cells in patients with acute promyelocytic leukemia (APL). (C) In addition, in a proportion of patients with de novo non-APL AML, LSC activity may be present in a cell with an immunophenotype distinct from HSCs. In some cases, multiple populations of LSCs may be present, each with a distinct immunophenotype as shown. (D) Finally, most recently, it has been proposed that genetic alterations may occur in a proportion of HSCs in patients with AML and that confer aberrant self-renewal properties to these cells. Further stepwise accumulation of genetic alterations then occurs in these preleukemic HSCs results in an overt malignant phenotype. As shown, mutations associated with the preleukemic HSCs include genes affecting DNA methylation and chromatin state, whereas mutations associated with frank leukemia include genetic alterations associated with increased cell proliferation.

be detected not only in leukemic cells but also in normal HSCs from patients in clinical remission from AML.[11] However, these *AML1-ETO*-expressing HSCs are not leukemic and can differentiate into myeloid and erythroid cells in vitro in a manner similar to HSCs without the *AML1-ETO* fusion transcripts (see Fig. 13.1). Similarly, analysis of mice expressing the *AML1-ETO* fusion from the endogenous *Aml1* locus in vivo has revealed that *AML1-ETO*-expressing HSCs have aberrant self-renewal capacity but do not develop overt leukemia unless additional genetic abnormalities are present. These data strongly suggest that the acquisition of additional genetic abnormalities in a subset of HSCs or their progeny is required to give rise to overt leukemia. In these studies, the HSCs bearing the *AML1-ETO* fusion reside within the Lin$^-$CD34$^+$CD38$^-$ subpopulation that is also the immunophenotype of normal human HSCs, suggesting that the initiating lesion must occur in a cell with an immunophenotype of normal HSCs. However, leukemic cells from 30% to 40% of patients with AML do not express CD34, and LICs from some patients with AML can actually be CD34$^-$. Interestingly, prior work evaluating the location of the *PML-RARA* transcript present in acute promyelocytic leukemia (APL) revealed that the PML-RARA translocation is actually present in CD34$^-$CD38$^+$ populations and not in CD34$^+$CD38$^-$ HSC-enriched populations (see Fig. 13.1).[12] These data clearly reveal that there is enormous heterogeneity in the cell-of-origin of AML.

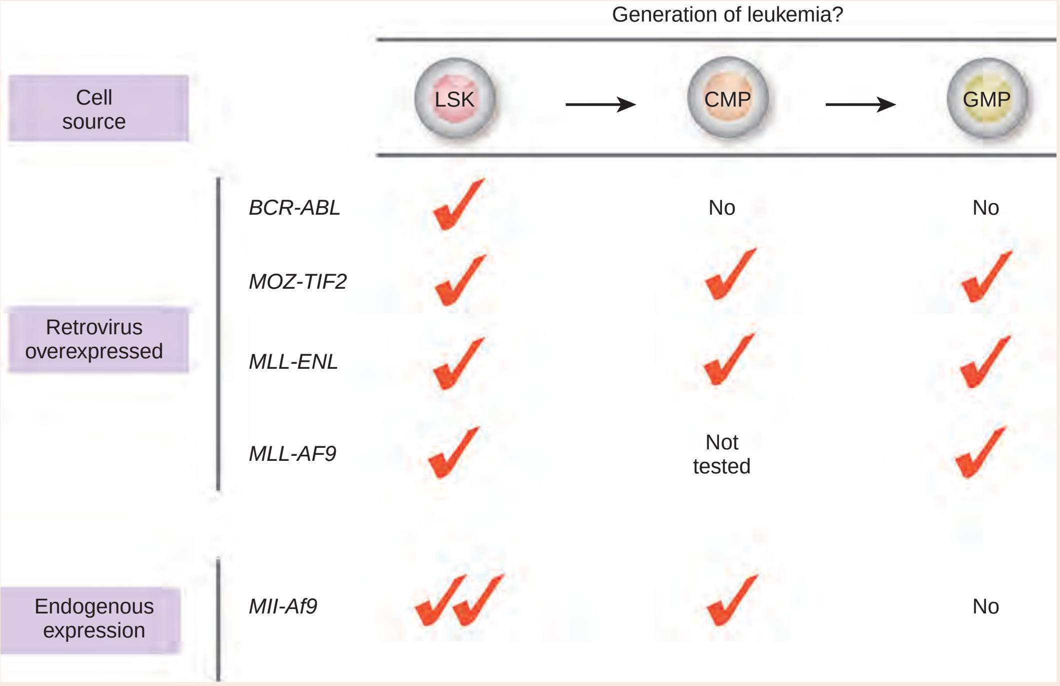
Advancement in techniques to map genetic alterations in cancer has allowed for much finer tracking of somatic mutations in AML and other hematopoietic malignancies with a normal karyotype. It is now believed that an average of five coding mutations is present in adults with de novo AML (see Chapter 59). Several groups have now studied the occurrence of somatic mutations in bulk AML cells and the remaining seemingly nonaffected HSCs. This work has clearly shown that the HSC compartment in patients with AML contains HSCs with none of the mutations found in the AML as well as HSCs with various combinations of genetic alterations similar to that present in the bulk malignant cells (see Fig. 13.1). These latter HSCs are now understood to be “preleukemic stem cells” that initiate AML and can also be identified in remission samples, indicating that they are able to survive induction chemotherapy (see box on Preleukemic Stem Cells).[19–21] Many of the mutations that occur in preleukemic HSCs confer growth properties that allow them to outcompete normal HSCs and presumably lead to relapse. Interestingly, mutations occurring in preleukemic HSCs are enriched in genes regulating DNA methylation, chromatin modifications, and the cohesin complex, while genetic alterations activating signaling are often present in more downstream overt malignant cells and absent from preleukemic HSCs. A series of recent studies performing single-cell mutational analysis of AML have further solidified these findings and identified that mutations in signaling genes (such as FLT3, NRAS, and KRAS) are often acquired in subclones of AML and can be acquired in multiple distinct subclones.[22–25]

MYELODYSPLASTIC SYNDROMES

Similar to AML, tracking major chromosomal abnormalities allowed earlier investigators to establish that clonal hematopoiesis in MDS originated in HSCs (see Chapter 61). Initially, deletion of the long arm of chromosome 5 (del(5q)) was identified in the HSCs of patients with del(5q)-MDS. Moreover, del(5q) CD34$^+$CD38$^-$ cells were shown to possess MDS-initiating potential using in vitro and in vivo stem cell assays. Systems subsequently improved phenotypic identification of HSCs that have allowed investigators to focus on Lin$^-$CD34$^+$CD38$^-$CD90$^+$CD45RA$^-$ cells as candidate CSCs in MDS. These studies showed that in patients with del(5q)-MDS, 99% of Lin$^-$CD34$^+$CD38$^-$CD90$^+$CD45RA$^-$ cells contained del(5q) and that they were molecularly and functionally distinct from clonally involved GMPs and MEPs.[26] By gene expression profiling and

FUNCTIONAL EVALUATION OF CELL-OF-ORIGIN IN VIVO

The earliest studies of cancer stem cells in acute myeloid leukemia (AML) suggested that AML is initiated by genetic alterations that take place in hematopoietic stem cells (HSCs). Alternatively, however, there is clear evidence that some leukemias may be initiated by mutations arising in more committed progenitors that provide these cells with aberrant self-renewal capacity that they otherwise lack. Numerous studies have attempted to directly address each of these possibilities through selective expression of specific oncogenes in isolated HSCs, common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), and even more mature myeloid cells (Fig. 13.2). Such experiments have been most thoroughly performed using the expression of mixed lineage leukemia (MLL) fusion oncoproteins. *MLL* encodes an epigenetic enzyme required for normal hematopoiesis due to its role in the maintenance of *HOX* gene expression (see Chapter 9). Translocation events fusing the N-terminus of MLL to over 50 different C-terminal partners are common in both AML and acute lymphoblastic leukemia (ALL). Overexpression of the *MLL-ENL* fusion oncoprotein in self-renewing HSCs as well as in myeloid progenitor populations with more restricted self-renewal potential including CMPs and GMPs result in the rapid onset of AML in vivo.[10] Similar results have been seen with retroviral overexpression of a different MLL fusion oncoprotein, *MLL-AF9*, as well as an unrelated AML-associated fusion, *MOZ-TIF2* (see Fig. 13.2). In contrast, overexpression of *BCR-ABL* is only able to transform HSCs but not committed downstream progenitors. These results unequivocally demonstrate (1) the ability of an MLL fusion oncoprotein to convert a myeloid progenitor cell population that is distinct from an HSC to acquire leukemogenic self-renewal activity and (2) that the cell-of-origin where a genetic alteration is expressed may regulate the resultant ability for a malignant disease to develop. It is also important to note that when the MLL-AF9 fusion is expressed under endogenous regulatory control, *Mll-Af9* transforms HSCs but not GMPs. These data clearly reveal that the dosage of oncogene expression may also regulate cellular transformation.



Cell source		Generation of leukemia? LSK	CMP	GMP
Retrovirus overexpressed	*BCR-ABL*	✓	No	No
	MOZ-TIF2	✓	✓	✓
	MLL-ENL	✓	✓	✓
	MLL-AF9	✓	Not tested	✓
Endogenous expression	*Mll-Af9*	✓✓	✓	No

Figure 13.2 LEUKEMIC CELL-OF-ORIGIN DEPENDS ON THE GENETIC ALTERATION, THE CELLULAR CONTEXT, AND GENE DOSAGE. Schematic of experiments testing the leukemogenic effects of retroviral overexpression of a number of leukemogenic fusions in hematopoietic stem and progenitor cell populations from mice followed by their transplantation into recipient mice. Mouse cell populations studied include the hematopoietic stem cell enriched LSK cell population as well as the common myeloid progenitor (CMP) and granulocyte-macrophage cell progenitor population. While retroviral overexpression of MLL-AF9 transforms LSK cells, CMP, and granulocyte-macrophage progenitor (GMP) populations, expression of MLL-AF9 from its endogenous locus efficiently transforms LSK cells but is only able to transform a proportion of CMP cells and is unable to transform GMPs.

principle component analysis of RNA sequencing data, the three cell populations were shown to be distinctively different, with the Lin$^-$CD34$^+$CD38$^-$CD90$^+$CD45RA$^-$ cells selectively expressing genes characteristic of normal HSCs. These candidate stem cells were exclusively able to sustain long-term generation of MDS myeloid progenitors in vitro that was not observed with GMPs, MEPs, or CMPs. Moreover, patients with del(5q)-MDS were found to have residual del(5q) clones in the CD34$^+$CD38$^-$CD90$^+$ stem cell compartment during clinical remission.[27] Over time, most of these patients experienced expansion of the clone, leading to cytogenetic and clinical progression.

The evidence for an MDS-initiating stem cell is not limited to isolated del(5q)-MDS, which may be a unique disease entity given its distinct clinical features. In fact, recent studies have shown that recurrent driver mutations occur at the HSC level in a broad panel of low to intermediate risk MDS and that the 5q deletion preceded any other identifiable recurrent driver mutations in isolated del(5q)-MDS. The same was observed with *SF3B1* mutation-positive MDS that is characterized by ringed sideroblasts, where *SF3B1* mutations are thought to be early and potentially initiating events. Targeted screening for mutations of a large number of genes known to be frequently mutated in MDS and other myeloid malignancies has been performed on bulk bone marrow from patients with MDS. After identifying the mutations specific to each patient, the different cell compartments (HSC, GMP, or MEP) were purified and the screen for mutations was repeated. It was hypothesized that if progenitor cells acquired self-renewal

PRELEUKEMIC STEM CELLS

Hematopoietic stem cells (HSCs) are rare (~1–2 per 10^8 bone marrow cells) and quiescent cells that rarely divide. However, all blood cells originate from progenitor cells that in turn originate from HSCs and thus HSCs must replicate/self-renew to continue the cycle. Mutations in HSCs can therefore occur during cell division and accumulate, sometimes speeding self-renewal divisions and leading to clonal hematopoiesis. Although able to confer a growth advantage to clones derived from mutated HSCs, these single mutations alone are not sufficient to transform cells, resulting in an overt malignant phenotype. Because growth-promoting gene mutations are common in cancer, it is thought that HSCs with mutations that cause clonal hematopoiesis might exist as preleukemic stem cells in patients with leukemia for months to years prior to diagnosis (see Chapter 59). Cooperative driver mutations are then acquired that cause cancer to develop. Acquired somatic mutations causing clonal hematopoiesis exist in healthy individuals and increase in frequency with age.[13–15] The risk of hematologic malignancy is higher in patients with clonal hematopoiesis compared with matched controls, providing evidence for leukemic predisposition (see Chapter 59). The most common mutations causing clonal hematopoiesis were found in the genes for *DNMT3A*, *TET2*, *SRSF2*, *SF3B1*, *U2AF1*, *IDH1/2*, and *ASXL1* which are also known to be recurrently mutated in myeloid malignancies. These genes encode proteins involved in epigenetic modifications of chromatin and DNA as well as in RNA splicing.[16] Functional experiments have shown that DNMT3A and TET2 actually increase the number of HSCs in the bone marrow due to impaired differentiation and increased self-renewal, respectively. Studies of a few patients with preleukemic stem cells who went on to develop leukemia have shown that at least in some cases, the leukemia developed only after acquisition of another driver mutation such as mutations in *FLT3*.[14] Additionally, different somatic mutations drive distinct patterns of clonal hematopoiesis, such as mutations in the splicing factors U2AF1, SF3B1, and SRSF2, which exclusively occur in patients over the age of 70 and may only confer a growth advantage under the selection pressures of an aging hematopoietic system.[17,18]

capacity, allowing them to persist long enough for the mutations to be responsible for the MDS phenotype, then the mutations would be identifiable in progenitors but not within HSCs. However, all of the mutations in MDS patients have been traced back to the HSC compartment ($Lin^-CD34^+CD38^-CD90^+CD45RA^-$ cells) and all of the mutations found in the $Lin^-CD34^+CD38^-CD90^+CD45RA^-$ MDS-initiating cells have also been identified in downstream GMPs and MEPs.[26] These findings suggest that MDS is propagated by a stem cell that develops and acquires mutations at an early stage in the disease.

Interestingly, Chen and colleagues provided evidence for a nonlinear, parallel manner by which MDS patient HSCs progress to AML.[28] They identified distinct progenitor populations that gave rise to MDS and MDS-related AML. They demonstrated that the dominant AML clone originated from a clone that was included in as pre MDS and/ or MDS HSCs but was undetectable in the MDS blast cells, indicating MDs stem cells are distinct from those that contribute to AML that follow MDS.

MYELOPROLIFERATIVE NEOPLASMS

The translocation between chromosomes 9 and 22, the so-called Philadelphia chromosome, is the hallmark of chronic myeloid leukemia (CML) (see Chapter 69) and gives rise to the *BCR-ABL* oncogene and its constitutively active protein tyrosine kinase product $p210^{BCR\text{-}ABL}$. In patients with CML, there is a clonal expansion of HSCs, and the BCR-ABL translocation can be detected in HSCs from patients with CML as well as all myeloid as well as even committed lymphoid cells generated by these HSCs (see Fig. 13.1). The data clearly identify chronic phase CML as a stem cell disorder in which the target cell of transformation is an HSC. This is a finding that has direct therapeutic implications as ABL kinase inhibitors, which are used clinically for CML, do not consistently eradicate the quiescent *BCR-ABL*–positive CML LICs.

In contrast to the chronic phase of CML where the normal HSC appears to be the target cell-of-origin, transformation of CML in the myeloid blast crisis appears to be associated with the expansion of a more committed myeloid progenitor population (which consists mostly of GMPs) rather than expansion of the HSC population. The GMPs from blast crisis patients with CML actually have the ability to serially replate in vitro, a proxy of aberrant self-renewal capacity not seen in normal GMPs. These data suggest that disease progression from chronic phase to myeloid blast crisis occurs due to aberrant acquisition of self-renewal potential within the committed GMP population, a possibility that has been directly studied in animal models (see Fig. 13.2 and box on Functional Evaluation of Cell-of-Origin In Vivo.)

The other chronic myeloproliferative neoplasm besides CML in which a specific genetic alteration is present in nearly 100% of patients is polycythemia vera (PV); a single mutation results in the substitution of phenylalanine for valine at codon 617 of the Janus kinase 2 (JAK2) tyrosine kinase in nearly every patient (see Chapter 70). Targeted sequencing of *JAK2* in patients with PV showed that the *JAK2*V617F mutation is present in cells with an HSC phenotype (see Fig. 13.1).[29] Moreover, HSCs from patients with PV are skewed toward the erythroid lineage at the HSC level already and there is also an expansion of the CMP pool, suggesting that expression of the *JAK2*V617F mutation affects HSC and progenitor cell populations. Interestingly, early efforts to study the effects of JAK inhibition in PV revealed that JAK inhibition exhibited inhibitory effects on the erythroid potential of PV HSCs but did not result in preferential eradication of *JAK2*V617F-mutant HSCs versus normal HSCs. Thus, this finding of an HSC origin for PV has direct therapeutic implications, as curative eradication of the disease would require therapy that eliminates rare quiescent bone marrow–based HSCs.

ACUTE LYMPHOBLASTIC LEUKEMIA

Unlike the myeloid malignancies, it has been less clear whether lymphoid malignancies also arise from an HSC or have a different cell-of-origin. In B-cell acute lymphoblastic leukemia (B-ALL) (see Chapter 66), considerable effort has gone into answering this question (Fig. 13.3). Early studies in specific subtypes of B-ALL supported a preleukemic stem cell such as identified in AML. *TEL/AML-1* rearranged ALL has been particularly well studied and is associated with a good prognosis. These studies showed that *TEL/AML-1* alone is insufficient for leukemogenesis. In fact, the frequency of *TEL/AML-1* in healthy infants far exceeds the incidence of ALL, suggesting that the development of leukemia requires a second hit. This is evident from studies of pairs of twins, both with evidence of a preleukemic *TEL/AML-1* $CD34^+CD38^-CD19^+$ population of cells, where one twin developed ALL and the other did not. The healthy twin retained the preleukemic population, which showed evidence of self-renewal and hierarchical differentiation. Later studies of high-risk ALL showed that leukemogenic potential may also be present in more differentiated cell populations, such as $CD34^-CD19^+$ cells (see Fig. 13.3). Recent extension of this work to B-ALL cases with a variety of chromosomal abnormalities has found that even mature blasts expressing CD20 were able to establish leukemia upon transplantation into immunodeficient mice. Also, as many as 1 in 40 B-ALL blasts retained leukemogenic potential. Overall, B-ALL blasts do not seem to be hierarchically arranged in a similar fashion as AML, and engraftment of further differentiated $CD34^-$ blasts can give rise to both $CD34^-$ and $CD34^+$ populations in vivo.[30] Together, these data suggest that B-ALL arises in a committed lymphoid progenitor rather than only a small population of cells accumulating mutations and retaining stem cell properties and that most

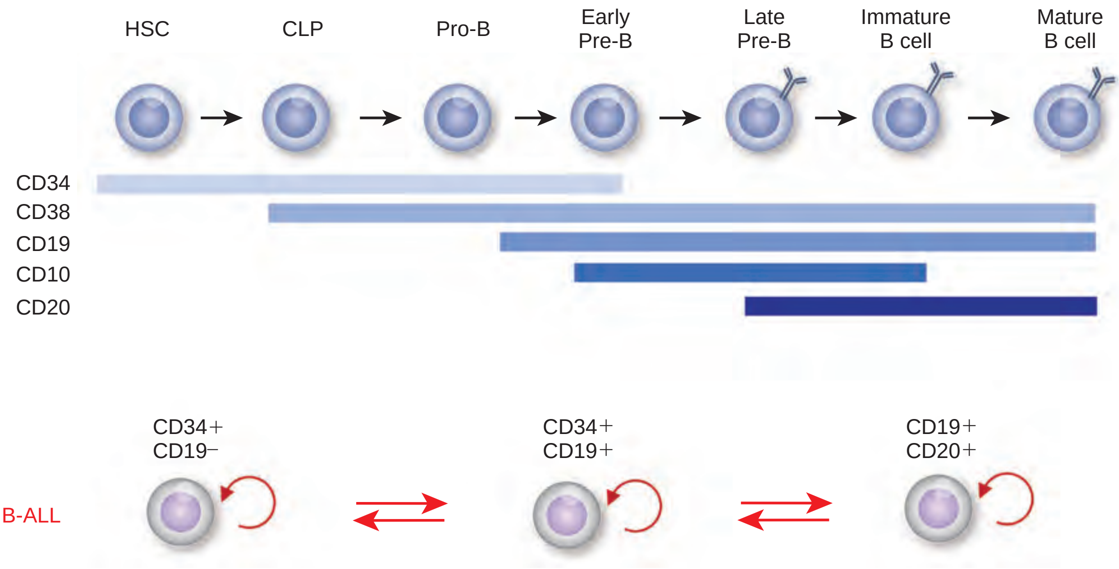


Figure 13.3 STAGES OF NORMAL EARLY B-CELL DIFFERENTIATION AND SCHEMATIC OF LEUKEMIC STEM CELLS IN B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (B-ALL). Based on several studies testing the ability of human B-ALL blasts to engraft in immune-deficient mice, it appears that multiple blast populations in B-ALL are able to establish the disease in immune-deficient mice. For example, CD34$^+$CD19$^+$ blasts as well as CD34$^-$CD19$^+$ blasts from individual patients have been shown to be able to transplant leukemia in vivo and to give rise to CD34$^+$ and CD34$^-$ leukemic cells. Thus, B-ALL does not appear to follow a hierarchical model in which blasts with a more mature immunophenotype lose their stem cell capacity, as has been seen in some cases of acute myeloid leukemia.

or even all of the leukemic blasts retain the ability to propagate the malignancy.

In contrast to *TEL/AML-1*, those patients with ALL carrying the BCR-ABL fusion protein have much worse clinical outcomes. However, like TEL/AML-1, BCR-ABL is also thought to be a primary mutation that is necessary but not sufficient for ALL transformation. Interestingly, the two BCR-ABL transcripts, P190 and P210, caused by different break points in the t(9;22) translocation, show distinct patterns of HSC and committed B-cell progenitor involvement. In most cases, the P190 BCR-ABL originates in a CD34$^+$CD38$^-$CD19$^+$ progenitor cell while P210 BCR-ABL originates in a multipotent HSC. When purified P210 BCR-ABL HSCs were transplanted into NOD/SCID mice, they exclusively reconstituted normal, BCR-ABL–negative multilineage hematopoiesis.[31] These data suggest that the purified HSC also contain normal HSCs that outcompete the P210 BCR-ABL HSCs, which are not the leukemia stem cells. In contrast, more committed CD19$^+$ P210 BCR-ABL–positive cells resulted in leukemic reconstitution upon transplantation into NOD/SCID mice that was enhanced following secondary transplant. These data complement recent findings suggesting that the primary BCR-ABL translocation in CML originates in HSCs, yet the leukemic transformation in blast crisis results in an LSC with a committed progenitor cell phenotype. This begs the question of whether P210 BCR-ABL–positive ALL represents de novo ALL or CML in lymphoid blast crisis.

MATURE B-CELL MALIGNANCIES

The classification of mature B-cell malignancies based on their histology and immunophenotypic resemblance to a particular stage of lymphoid differentiation has led to the theory that each lymphoma subtype originates within lymphocytes at distinct differentiation stages (Fig. 13.4). Another model predicts that cells acquire initial alterations at early stages, even at the HSC level, that may drive clonal expansion or resistance to apoptosis and that these cells then develop additional complementary alterations at a later stage that lead to overt malignancy. Recent studies have identified novel lymphoid associated mutations and alterations across large panels of lymphomas, creating the opportunity to define the clonal architecture and cell-of-origin in lymphoma subtypes.

CHRONIC LYMPHOCYTIC LEUKEMIA

Chronic lymphocytic leukemia (CLL) is a B-cell malignancy marked by the accumulation of clonal mature B cells in the lymph node, bone marrow, and/or blood. In nearly all cases, CLL is preceded by a preleukemic monoclonal B-cell lymphocytosis (MBL), though not all cases of MBL continue on to CLL (see Chapter 76). The precise cell within B-cell development that gives rise to CLL has been debated for decades, and two studies have implicated HSCs in the pathogenesis of CLL. Kikushige et al. investigated the cellular origin of CLL by isolating distinct subpopulations of cells and assaying their ability to initiate disease in immunodeficient mice. Only the CD34$^+$CD38$^-$CD90$^+$ fraction engrafted and this population, known to be highly enriched in HSCs, gave rise to both lymphoid and myeloid hematopoiesis. Interestingly, these engrafted mice showed an increased proportion of polyclonal B cells and a population of monoclonal B cells expressing CD5, akin to MBL.[32] Further examination determined that they had different variable, joining and diversity segments (V(D)J) rearrangement patterns compared to the prior CLL. The finding of aberrant HSCs in CLL suggests that they are primed to generate MBL cells, which are likely to accumulate additional mutations that eventually result in a clonal CLL disorder.

In a different approach, Damm et al. investigated whether molecular alterations present in CLL cells could be traced to cells in the earliest stages of hematopoietic development before committed B-cell development. In fact, acquired mutations were detected in HSCs in the majority of the patients with CLL studied.[33] These findings support a preleukemic phase and the clinical implications imply that treatments with true curative intent in CLL might require approaches other than those focused solely on cell-surface antigens restricted to B cells or B-cell receptor signaling pathways.

HAIRY CELL LEUKEMIA

Hairy cell leukemia (HCL) (see Chapter 77) is a chronic lymphoproliferative disorder characterized as a mature B-cell malignancy based on the expression of CD19, surface immunoglobulin, and the clonal rearrangement of immunoglobulin heavy and light chain genes. At the same time, HCL cells also express cell-surface markers not present on normal B cells, including CD103 and CD11c, which are typically expressed by dendritic cells and monocytes. In addition, patients with HCL have long been known to have clinical features disparate from most mature B-cell malignancies, including the absence of lymph node involvement and frequent splenomegaly due to extramedullary hematopoiesis (EMH). Recent identification of BRAFV600E mutations in nearly 100% of patients with classic HCL provided genetic insight into the pathogenesis of HCL and a clonal marker to track the origin and propagation of HCL. The BRAFV600E mutation was found in purified (Lin^-) $CD34^+$ CD38 $^-CD90^+CD45RA^-$ HSCs in primary samples from patients with HCL. These aberrant HSCs were also able to recapitulate BRAFV600E-mutant hematopoiesis after transplantation into NOD/SCID/gamma-null (NSG) mice. Additionally, conditional knock-in mice were generated, expressing BRAFV600E in either HSCs or $CD19^+$ cells. Those mice with BRAFV600E HSCs showed an HCL-like phenotype of anemia, thrombocytopenia, and EMH; however, no phenotypic hairy cells were seen. In contrast, mice with BRAFV600E expression limited to lineage-committed B cells did not have reduced survival or an overt phenotype. These data suggest that HCL is initiated within the HSC compartment but that the development of additional genetic alterations occurring along the course of hematopoiesis may be necessary to give rise to HCL cells. Indeed, in patients with genetic alterations in addition to BRAFV600E in HCL cells, these additional mutations were not found in the BRAF-mutated HSCs.[34]

DIFFUSE LARGE B-CELL LYMPHOMA

High-resolution genomic analyses of a large number of human diffuse large B-cell lymphoma (DLBCL) (see Chapters 81 and 85) samples identified copy number gain of chromosome 3q27.2 to be associated with the worst prognosis and the aggressive activated B-cell (ABC) disease subtype. The transcription factor BCL6 is likely the target oncogene affected by this copy number alteration at this locus given its propensity to be targeted by other alterations in DLBCL such as translocations and point mutations, which were mutually exclusive from the 3q27.2 gain. Other roles for BCL6 have been described in normal hematopoiesis and myeloid leukemia stem cells, prompting inquiries into whether BCL6 expression in HSCs has a role in the origins of DLBCL. This hypothesis was tested by the generation of a mouse model that expressed BCL6 only in HSCs under the control of a stem cell-specific promotor so that BCL6 was no longer expressed by the differentiated progeny. These mice developed a form of lymphoma that resembled DLBCL with an ABC phenotype that did not overexpress BCL6.[33] This led to a proposed model of “hit-and-run” oncogenesis, wherein BCL6 expression in hematopoietic precursors is sufficient to produce DLBCL without requiring sustained overexpression.[35]

FOLLICULAR LYMPHOMA

The most common subtype of lymphoma is follicular lymphoma (FL), which is so named because histologically the lymph node follicle B cell is affected (see Chapters 81 and 82). These aberrant B cells carry a hallmark translocation between chromosomes 14 and 18, resulting in the juxtaposition of the immunoglobulin heavy chain gene (IGH) and the antiapoptotic protein BCL-2, thus driving overexpression of BCL2 and resistance to programmed cell death. This malignancy usually follows an indolent course and is more common in elderly patients. It has been shown that up to 25% of healthy individuals harbor BCL-2/IGH rearranged cells yet do not have the disease, indicating that the hallmark lesion is not sufficient to cause lymphoma and that additional genetic mutations must be required for lymphomagenesis. As in other hematologic malignancies, newer sequencing technologies have allowed identification of novel recurrent mutations in FL, and some researchers have begun investigating the timing of those mutations. By taking the opportunity presented by an unfortunate but rare occurrence of donor-derived FL after HSC transplantation, investigators have shown that some of these genetic mutations occur in hematopoietic precursors prior to the cells in which the BCL-2/IGH rearrangement occurs.

HSC donors are screened for signs and symptoms of malignancy but are not currently subjected to genetic testing for premalignant conditions. Often, the donor is a brother or sister due to the 25% chance of being a complete HLA-match and the willingness of a sibling to donate. This inherently means that a sibling donor is at slightly higher risk of having a malignancy given their close relationship to someone with cancer (i.e., the recipient), yet it is still a rare occurrence that donor cells will transfer a malignancy to the recipient after stem cell transplantation. In the case mentioned previously,[36] the donor and the recipient both developed FL 7 years after the transplant. Both patients’ FL had exactly identical BCL-2/IGH rearrangement breakpoints and the same V(D)J recombination, indicating that they were derived from the same mutation. The recipient had received a donor-lymphocyte infusion (DLI) 7 years prior, which also harbored the same BCL-2/IGH and V(D)J rearrangements, thus confirming the donor-derived source. A sample of the original stem cell transplant was not available to test but the BCL-2/IGH rearrangement was not present in the cells other than $CD19^+$ mature B cells in the DLI sample. However, there were mutations known to occur in lymphoma present in the $CD34^+CD10^-CD19^-$ population that includes multipotent progenitors and HSCs.[37] These alterations presumably contributed to the development of FL prior to the acquisition of the hallmark translocation and occur in progenitors of the follicle B cell. These findings provide evidence of a possible role of stem cells in the development of mature B-cell malignancies.

CONCLUSION

The stem cell model of hematologic diseases has implications beyond a theoretical construct for the pathogenesis of disease. Not only does this model support stem cell transplant and gene therapy as curative treatments in some cases, but this model also has much broader application in the management of hematologic malignancies. Most of our current standard therapies are based on the principle of interrupting DNA replication in rapidly dividing cancer cells, yet stem cells are quiescent and spend little time in the active cell cycle. Many of the new and developing therapies are targeted at cell-surface markers or mutated genes whose expression may be restricted to the most mature malignant cells and may be lacking in the premalignant or malignant stem cells. It remains to be seen whether the same challenges will face immune response checkpoint modulators, but one could consider the possibility that neoepitopes recognized by activated T cells might have different expressions on different cell populations.

Remarkable progress has been made in the field of CSCs in hematologic malignancies due to the evolution of immune-deficient mouse models and the improvement of sequencing technologies that have allowed the mapping of clonal evolution and studies of mutational ontogeny. There are controversies from inside and outside of the field as to whether stem cells should be defined phenotypically, molecularly, or functionally; but as the evidence accumulates, there is broad agreement on the stem cell model of hematologic diseases.

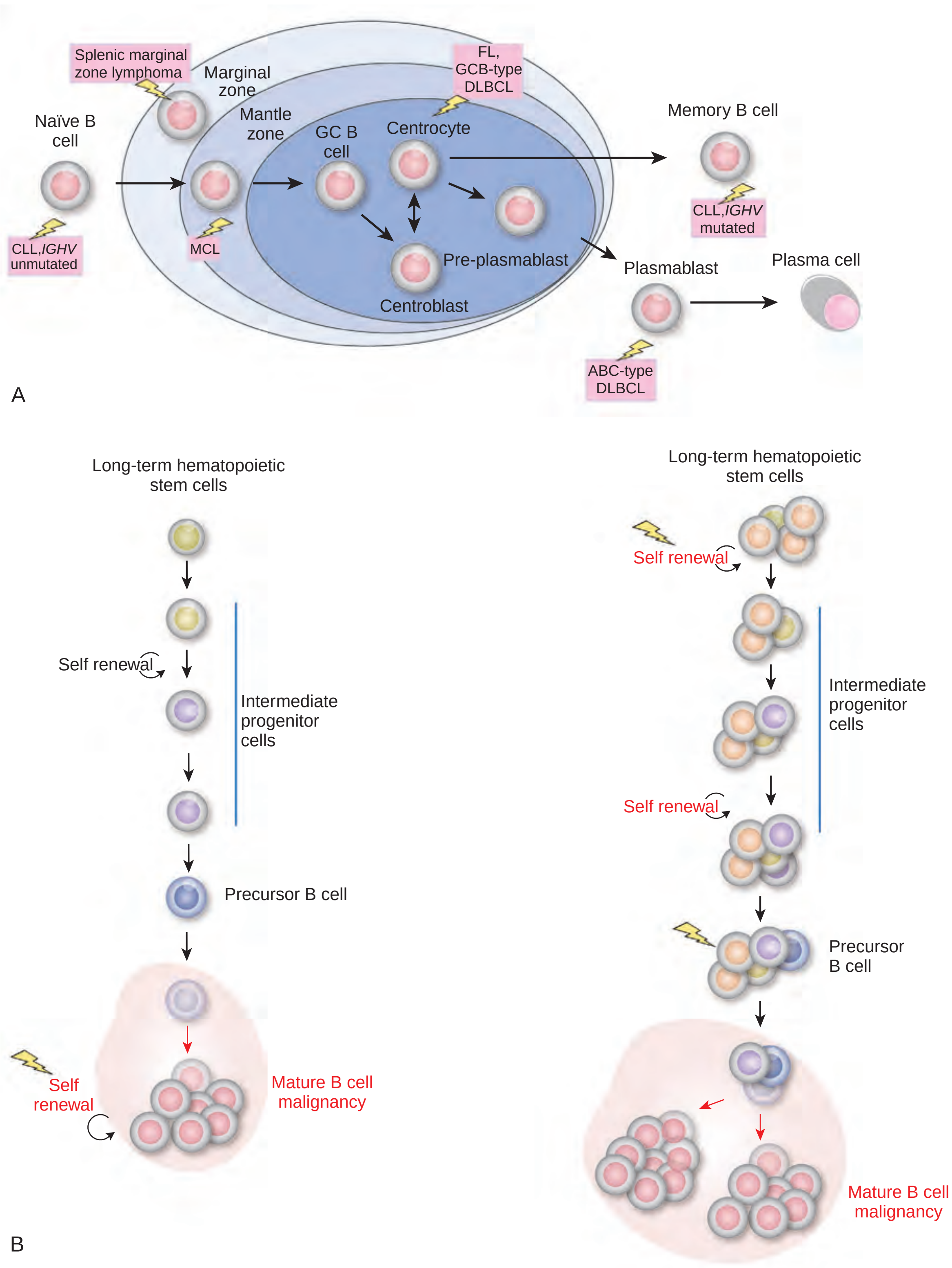

Splenic marginal zone lymphoma
Marginal zone
Naïve B cell
Mantle zone
GC B cell
Centrocyte
FL, GCB-type DLBCL
Memory B cell
CLL, IGHV unmutated
MCL
Pre-plasmablast
Centroblast
CLL, IGHV mutated
Plasmablast
Plasma cell
ABC-type DLBCL
A
Long-term hematopoietic stem cells
Self renewal
Intermediate progenitor cells
Precursor B cell
Self renewal
Mature B cell malignancy
Long-term hematopoietic stem cells
Self renewal
Intermediate progenitor cells
Self renewal
Precursor B cell
Mature B cell malignancy
B

Figure 13.4 MODELS OF LYMPHOMA ONTOGENY. (A) The conventional model of lymphoma classifies lymphomas based on the differentiation of mature peripheral B cells. This model is based on the presence of somatic hypermutation as well as immunophenotypic and gene expression analyses comparing the malignant cells to normal B-cell counterparts. (B) At the same time, mature B cells are derived from precursor B cells and earlier progenitor and hematopoietic stem cells (HSCs). In the conventional model *(left)*, it is assumed that cells more primitive than the peripheral B cell depicted in (A) are not involved with the lymphoma disease process. However, there is increasing evidence that HSCs and/or more intermediate progenitors may harbor aberrancies directly linked to mature B-cell malignancies. *ABC DLBCL*, Activated B-cell–like diffuse large B-cell lymphoma; *CLL*, chronic lymphocytic leukemia; *FL*, follicular lymphoma; *GCB*, germinal center B-cell–like DLBCL; *MCL*, mantle cell lymphoma.

REFERENCES

1. Blair A, Hogge DE, Ailles LE, et al. Lack of expression of Thy-1 (CD90) on acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo. *Blood*. 1997;89:3104–3112.
2. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med*. 1997;3:730–737.
3. Majeti R, Park CY, Weissman IL. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. *Cell Stem Cell*. 2007;1:635–645.
4. Velten L, Story BA, Hernández-Malmierca P, et al. Identification of leukemic and pre-leukemic stem cells by clonal tracking from single-cell transcriptomics. *Nat Commun*. 2021;12:1366–1379.
5. Stauber J, Greally JM, Steidl U. Preleukemic and leukemic evolution at the stem cell level. *Blood*. 2021;137:1013–1018.
6. Doulatov S, Notta F, Laurenti E, et al. Hematopoiesis: a human perspective. *Cell Stem Cell*. 2012;10:120–136.
7. Rongvaux A, Willinger T, Martinek J, et al. Development and function of human innate immune cells in a humanized mouse model. *Nat Biotechnol*. 2014;32:364–372.
8. Cosgun KN, Rahmig S, Mende N, et al. Kit regulates HSC engraftment across the human-mouse species barrier. *Cell Stem Cell*. 2014;15:227–238.
9. Goardon N, Marchi E, Atzberger A, et al. Coexistence of LMPP-like and GMP-like leukemia stem cells in acute myeloid leukemia. *Cancer Cell*. 2011;19:138–152.
10. Cozzio A, et al. Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors. *Genes Dev*. 2003;17:3029–3035.
11. Miyamoto T, Weissman IL, Akashi K. AML1/ETO-expressing nonleukemic stem cells in acute myelogenous leukemia with 8;21 chromosomal translocation. *Proc Natl Acad Sci USA*. 2000;97:7521–7526.
12. Turhan AG, et al. Highly purified primitive hematopoietic stem cells are PML-RARA negative and generate nonclonal progenitors in acute promyelocytic leukemia. *Blood*. 1995;85:2154–2161.
13. Busque L, Patel JP, Figueroa ME, et al. Recurrent somatic TET2 mutations in normal elderly individuals with clonal hematopoiesis. *Nat Genet*. 2012;44:1179–1181.
14. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med*. 2014;371:2488–2498.
15. Genovese G, Kähler AK, Handsaker RE, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med*. 2014;371:2477–2487.
16. Shih AH, Jiang Y, Meydan C, et al. Mutational cooperativity linked to combinatorial epigenetic gain of function in acute myeloid leukemia. *Cancer Cell*. 2015;27:502–515.
17. Xie M, Lu C, Wang J, et al. Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nat Med*. 2014;20:1472–1478.
18. McKerrell T, et al. Leukemia-associated somatic mutations drive distinct patterns of age-related clonal hemopoiesis. *Cell Rep*. 2015;10:1239–1245.
19. Corces-Zimmerman MR, Hong WJ, Weissman IL, et al. Preleukemic mutations in human acute myeloid leukemia affect epigenetic regulators and persist in remission. *Proc Natl Acad Sci USA*. 2014;111:2548–2553.
20. Jan M, et al. Clonal evolution of preleukemic hematopoietic stem cells precedes human acute myeloid leukemia. *Sci Transl Med*. 2012;4:149ra118.
21. Shlush LI, et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature*. 2014;506:328–333.
22. Ediriwickrema A, Aleshin A, Reiter JG, et al. Single-cell mutational profiling enhances the clinical evaluation of AML MRD. *Blood Adv*. 2020;4:943–952.
23. Taylor J, Mi X, North K, et al. Single-cell genomics reveals the genetic and molecular bases for escape from mutational epistasis in myeloid neoplasms. *Blood*. 2020;136 1477–148.
24. Morita K, et al. Clonal evolution of acute myeloid leukemia revealed by high-throughput single-cell genomics. *Nat Commun*. 2020;11:5327.
25. Miles LA, Bowman RL, Merlinsky TR, et al. Single-cell mutation analysis of clonal evolution in myeloid malignancies. *Nature*. 2020;587:477–482.
26. Woll PS, et al. Myelodysplastic syndromes are propagated by rare and distinct human cancer stem cells in vivo. *Cancer Cell*. 2014;25:794–808.
27. Tehranchi R, et al. Persistent malignant stem cells in del(5q) myelodysplasia in remission. *N Engl J Med*. 2010;363:1025–1037.
28. Chen J, et al. Myelodysplastic syndrome progression to acute myeloid leukemia at the stem cell level. *Nat Med*. 2019;25:103–110.
29. Jamieson CH, et al. The JAK2 V617F mutation occurs in hematopoietic stem cells in polycythemia vera and predisposes toward erythroid differentiation. *Proc Natl Acad Sci USA*. 2006;103:6224–6229.
30. McClellan JS, Majeti R. The cancer stem cell model: B cell acute lymphoblastic leukaemia breaks the mould. *EMBO Mol Med*. 2013;5:7–9.
31. Notta F, et al. Evolution of human BCR-ABL1 lymphoblastic leukaemia-initiating cells. *Nature*. 2011;469:362–367.
32. Kikushige Y, et al. Self-renewing hematopoietic stem cell is the primary target in pathogenesis of human chronic lymphocytic leukemia. *Cancer Cell*. 2011;20:246–259.
33. Damm F, et al. Acquired initiating mutations in early hematopoietic cells of CLL patients. *Cancer Discov*. 2014;4:1088–1101.
34. Chung SS, Kim E, Park JH, et al. Hematopoietic stem cell origin of BRAFV600E mutations in hairy cell leukemia. *Sci Transl Med*. 2014;6:238ra271.
35. Green MR, Vicente-Dueñas C, Romero-Camarero I, et al. Transient expression of Bcl6 is sufficient for oncogenic function and induction of mature B-cell lymphoma. *Nat Commun*. 2014;5:3904.
36. Weigert O, Kopp N, Lane AA, et al. Molecular ontogeny of donor-derived follicular lymphomas occurring after hematopoietic cell transplantation. *Cancer Discov*. 2012;2:47–55.
37. Weigert O, Weinstock DM. The evolving contribution of hematopoietic progenitor cells to lymphomagenesis. *Blood*. 2012;120:2553–2561.

CHAPTER 14

HEMATOPOIETIC MICROENVIRONMENT

David Scadden and Lev Silberstein

EVOLUTION OF THE NICHE CONCEPT

In 1868, Ernest Neumann first suggested that blood cells are being replenished throughout postnatal life, and this proposal led to the attempts to localize the place of hematopoiesis. His hypothesis that blood cell production takes place in the bone marrow (BM) was experimentally validated by selective lead shielding of limbs in irradiated animals almost a century later. Notably, these and other studies showed that differentiation pathways of immature blood cells are determined by their location and are different between the spleen and the BM. Based on this difference between BM and spleen, Schofield first proposed that there is a specialized place or niche where stem cells reside and are governed. He succinctly posed in 1978 that "stem cell is seen in association with other cells which determine its behavior."[1]

Trentin further clarified how different sites affected hematopoietic stem/progenitor cell (HSPC) differentiation. Although both spleen and marrow support multiple cell lineages (erythropoietic and granulocytopoietic, for example), the ratios of differentiating cells were distinct—spleen favored erythropoiesis, but BM predominantly supported granulopoiesis. This controlling influence of the surrounding cells was further illustrated by implanting BM stroma into the spleen and showing that hematopoietic cells abruptly changed from erythropoiesis to granulopoiesis at the spleen–BM demarcation. These observations suggest that immature differentiating progenitors require interactions with specific other cell types in a defined *micro*environment.

This chapter reviews the current knowledge of the hematopoietic microenvironment during development and in postnatal life in normal hematopoiesis and in myelodysplasia and leukemia. The opportunities for therapeutic manipulation of the niche in the treatment of these disorders are also discussed. For the related topics on stem cell mobilization, hematopoietic cytokines and the role of microenvironment in lymphoid malignancies, plasma cell disorders, and myeloproliferative conditions, readers are referred to other chapters of this book.

HEMATOPOIETIC MICROENVIRONMENT DURING DEVELOPMENT

In mammals, hematopoiesis during development takes place in distinct extra-embryonic and embryonic sites. Sequentially, it moves from the yolk sac to the aorta-gonad-mesonephros (AGM) region, fetal liver, placenta, and BM (for details, see Chapter 8).

The first definitive adult HSPCs emerge from the floor of the dorsal aorta, more precisely from the AGM region in the midgestation mouse embryo, and the HSPC clusters appear in close association with the aortic endothelium. Phenotypically defined HSPCs (Sca1+ c-kit + CD41+) arise directly from ventral aortic endothelial cells largely triggered by shear and pulsatile pressure forces.[2] Although direct cellular interactions during HSPCs emergence in the embryo remain to be dissected, bone morphogenetic protein 4 (BMP4), fibroblast growth factor (FGF), transforming growth factor (TGF), vascular endothelial growth factor (VEGF)-Flk1, YAP, and piezo signaling pathways are involved.

The placenta has also been identified as a hematopoietic organ during development.[3] Placenta is known to produce hormones that influence vascularization and therefore may affect blood cell production because hematopoiesis and vasculogenesis are tightly coupled. The hematopoiesis-promoting factors may be either produced by the placental trophoblast cells or enter via maternal circulation. Hematopoietic progenitors appear in the placenta at E9, but their number declines by E13. The cells and local factors providing placental hematopoietic support are currently unknown, but mesenchymal/stromal cells have been suggested as candidates. The placental microenvironment is thought to be geared toward supporting the expansion or maturation of HSPCs without their concomitant differentiation.

In the fetal liver, the HSPCs are first detected on day 9 of mouse embryonic development and approximately week 7 in the human. The liver remains the predominant site of blood production until the BM is invested with cells at approximately 11 weeks post conception and becomes the major blood production organ at week 20. Stromal cell lines obtained from the fetal liver are able to support primitive hematopoietic cells in ex vivo cultures. Some of these cells (termed *myelosupportive stroma*) are able to differentiate in vitro into mesenchymal components (osteoblasts, chondrocytes, and adipocytes). The fetal liver niche has been characterized as Nestin expressing periportal cells and hepatic stellate cells expressing stem cell factor (SCF).[4,5]

Despite the differences in the hematopoietic microenvironment between the sites of fetal and adult hematopoiesis, the key components of the molecular milieu are likely to be shared, as evidenced by successful (although limited) engraftment of HSPCs across developmental barriers. For example, AGM- or fetal liver–derived HSPCs are able to engraft in the adult BM. Notably, they have a competitive advantage over their BM-derived counterparts, with the long-term repopulating ability exceeding that of the BM by fivefold. Vice versa, BM HSPCs engraft in the fetal liver when transplanted in utero, although at low efficiency (less than 5% for the whole BM and 0.43% for highly enriched HSPCs), which may be partly attributable to the absence of pre-transplant conditioning.

Multilineage hematopoiesis during development occurs largely by the virtue of sequential HSPC migration from the AGM region to the fetal liver and the BM, as opposed to de novo HSPC generation. Failure of migration to the "next niche," as exemplified by the targeted disruption of the guanine-nucleotide–binding protein stimulatory α-subunit (GS-α), calcium-sensing receptor, or CXCL12/CXCR4 axis (discussed in detail in Chapter 11 on HSPC migration) leads to severe impairment in hematopoiesis. This suggests even in the absence of cell-intrinsic HSPC defects, proper progression of blood cell production throughout developmental critically depends on the ability of the HSPC to sequentially move to the appropriate microenvironmental compartments.

ADULT BONE MARROW MICROENVIRONMENT

Hematopoietic Stem Cell Niches

Location of the Hematopoietic Stem/Progenitor Cell Niche

In mammals, BM is a major site of hematopoiesis throughout life. The niche preserves and dynamically regulates the HSPC pool by providing signals required for maintenance, quiescence, and retention of HSPCs in the BM. However, the location of the HSPC niche within the marrow has been a subject of controversy.[6] The endosteal surface has long been considered the zone in which HSPCs are preferentially located.[7,8] In the setting of irradiation conditioning, this has been directly demonstrated by intravital imaging studies that allow dynamic assessment of the interaction between transplanted HSPC and the niche. Currently, in vivo imaging is limited to calvarial BM, an area in the mouse skull where the bone is very thin, thus permitting penetration of the laser beam into the BM cavity. Using this technique and simultaneous multicolor fluorescent labeling of osteolineage cells (OLCs), HSPCs, and the vasculature, studies showed that in irradiated recipients, transplanted HSPCs home closest to the endosteal surface and individual OLCs, as compared with more differentiated progenitors, and that they are "anchored" to their niches at least through 72 hours.[9] Preferential localization of primitive hematopoietic cells to the endosteal surface under the homeostatic conditions has been also demonstrated, although this analysis was performed using immunostaining of histological BM sections of either femoral bones or the sternum.

However, other studies performed under steady-state (not transplant) conditions indicate that most HSPCs are located in the central marrow in a perivascular position.[10] Vascular integrity is largely lost with transplant conditioning, so it is not surprising that there might be distinctive sites of hematopoiesis under homeostasis. The positioning of cells near vasculature is to some extent inevitable, as the marrow is richly endowed with sinusoidal vessels. Indeed, approximately 40% of the BM is vascular. Nonetheless, selective deletion of factors in primitive mesenchymal cells and endothelial cells provide evidence for them providing a regulatory and therefore, niche role.[11] Positioning of HSPC in BM by detailed 3-D analysis has indicated that the cells distribute equivalently to random dots when quiescent. Only upon activation do they have distinctive localization near CXCL12 cells.[12] Thus, the niche may be more macroarchitectural, with multiple cells contributing to the functional regulation of HSPC.

Functional roles for specific cells have been defined by the deletion of a key niche factor, such as SCF (kit-ligand), from either endothelial or perivascular cells. This was found to lead to decreases in HSPC providing an experimental basis for the notion of a perivascular niche. While the debate about the location of the HSPC niche—and consequently, cell types that serve as niche participants—continues, it is important to bear in mind that HSPCs themselves are molecularly and functionally heterogeneous and that several distinct niches may co-exist to support this heterogeneity, particularly under different conditions such as the stress of transplantation.

Cellular Components of the Hematopoietic Stem/Progenitor Cell Niche

Over recent years, animal studies revealed marked complexity in the cellular and molecular organization of the HSPC BM niche. Furthermore, single-cell analyses of stroma characterizing it by gene or protein expression has provided a complete inventory of the cell types present in the marrow and begun to define changes they undergo with stress.[13–15] Major cellular components of the HSPC niche and the factors that they produce (summarized in Table 14.1) are discussed in the following sections.

Osteolineage Cells

OLCs are a heterogeneous population of mesenchymal cells that line the endosteal surfaces of flat and trabeculated bones at the interface between the bone and the BM and become embedded within the bone matrix upon terminal differentiation. OLCs are thought to originate from mesenchymal stem cells (MSCs) and gradually progress from the early immature progenitors that express OLC-specific transcription factors Runx2 and osterix to mature osteoblasts expressing extracellular matrix protein osteocalcin and eventually to osteocytes.

Several lines of in vivo evidence support a functional role of the OLCs in the regulation of primitive hematopoietic cells. In the studies providing the first experimental evidence for a mammalian niche in vivo, mice with genetically modified OLCs had an increase in the number of activated OLCs and a corresponding increase in the number of HSPCs. This effect was associated with an increased trabecular bone area and an elevated number of trabecular osteoblasts that expressed the Notch ligand Jagged 1. When the OLCs were depleted, there was a reduction in the BM cellularity and migration of hematopoiesis to the extra-medullary sites. Thus, OLCs play a role in the regulation of HSPC pool size. In addition, OLCs participate in controlling HSPC quiescence through contributing to the production of CXCL12, Angiopoietin 1, thrombopoietin, pan-inhibitor of canonical Wnt signaling Dickkopf1 (Dkk1), noncanonical Wnt ligands, and ECM protein osteopontin. Finally, OLCs govern HSPC localization by controlling their egress into blood and return to the BM, a process that forms the basis for clinical peripheral blood stem cell collection for transplantation. When mature OLCs are deleted from bone, there is an increase in the number of circulating progenitors and a decrease in the mobilization of HSPC with G-CSF, indicating that OLC-derived signals retain primitive hematopoietic cells in the marrow. Following G-CSF-induced mobilization, the OLCs in the trabecular bone adapt a flattened morphology with short projections, which is associated with HSPC egress from the niche. Similar changes are seen after treatment with NSAIDs, which are known to enhance G-CSF-induced HSPC mobilization. When CXCL12, a major HSPC chemo-attractant and retention factor, is deleted from the osteoprogenitors using osterix-Cre promoter, increased HSPC mobilization is also observed. Thus, OLCs participate in the BM niche by regulating HSPC number, quiescence, and retention in the BM space.

Despite the evidence presented above, several studies have argued against the role of OLCs in the niche, citing the absence of HSPC changes either in genetic models associated with reduced OLC number or following OLC-specific deletion of HSPC regulators such as kit-ligand or CXCL12. Several experimental factors are likely to account for this discrepancy, including developmental adaptation when the genetic modification is present throughout the ontogeny or inability of genetic tools to target a specific subset within the OLC compartment that serves as a nodal point of the HSPC regulation: several studies suggest that immature OLCs are important for the niche function, while mature OLCs are dispensable. However, these latter cells appear to play a significant role in supporting lymphoid progenitors. They are critical for B-cell maturation, and osteocalcin-expressing cells regulate the production of T-competent progenitors (as shown in the following sections).

Endothelial Cells

Endothelial cells are known to secrete hematopoietic cytokines and express several adhesion molecules such as E-selectin, P-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1) that have been shown to participate in cellular interactions within the HSPC niche. The existence of vascular niche for the HSPCs has been supported by in vivo imaging studies showing early homing of transplanted BM progenitors to specific subdomains of the vascular tree as well as by histologic assessment of the BM using CD150 antibody when HSPCs were found to be in close proximity to the BM sinusoids.

Endothelium-derived factors have diverse effects on HSPCs in vivo. For example, E-selectin (which is expressed exclusively in the endothelial cells) negatively regulates HSPC quiescence; consequently, HSPCs from E-selectin KO mice are more quiescent and resistant to irradiation. On the other hand, endothelial-specific deletion of SCF or CXCL12 leads to the respective reduction of the HSPC pool and loss of repopulating capacity. Similar changes are seen upon endothelial-specific deletion of Notch ligand Jagged 1, which in contrast to E-selectin, promotes HSPC quiescence. The heparin-binding growth factor, pleiotrophin, that is produced by sinusoidal endothelial cells also plays a role in the retention and self-renewal of HSPC in BM. It is not established that among endothelial subpopulations it is the arteriolar cells that secrete the predominant regulator of HSPC, SCF.

TABLE 14.1 HSPC Niche Factors and Their Cellular Sources

Factor	Source	Effect and reference
Membrane-bound SCF	Lepr+ perivascular cells, Tie2$^+$ endothelial cells	Maintenance of HSC in the bone marrow[11]
CXCL12	Lepr$^+$ perivascular cells, Tie-2$^+$ endothelial cells	Retention of HSC in the bone marrow (Ding and Morrison, 2013)
	Prx-1$^+$ osteoprogenitors	Retention of HSC, myeloid and lymphoid progenitors in the bone marrow (Ding and Morrison, 2013; Greenbaum et al., 2013)
	Osx$^+$ osteoprogenitors and Col-2.3$^+$ osteoblastic cells	Retention of lymphoid progenitors in the bone marrow (Ding and Morrison, 2013; Greenbaum et al., 2013)
Notch signaling (Jagged1)	VE-cadherin$^+$ endothelial cells, osteoblastic cells (in PPR model)	Maintenance of HSC and regeneration posttransplantation (Poulos et al., 2013) Expansion of HSC and progenitors[7]
Wnt signaling (Canonical)	Col-2.3$^+$ osteoblastic cells (Wnt inhibitor DKK1)	Maintenance of HSC and quiescence (Fleming et al., 2008)
Wnt signaling (Noncanonical)	N-cadherin$^+$ osteoblastic cells	Maintenance of HSC and quiescence (Sugimura, 2012)
E-Selectin	CD-31$^+$ endothelial cells	Promotion of HSC cycling (Winkler et al., 2012)
Pleiotrophin	VEGFR3$^+$/VE-cadherin$^+$ endothelial cells and CXCL12 perivascular cells	Maintenance of HSC and retention in the bone marrow (Himburg et al., 2010, 2012)
Thrombopoietin	Alkaline phosphatase$^+$ osteoblastic cells	Maintenance of HSC and quiescence (Qian et al., 2007; Yoshihara et al., 2007)
Osteopontin	Osteoblastic and other microenvironmental cells	Negative regulation of HSC numbers and maintenance of quiescence (Nilsson et al., 2005; Stier et al., 2005)
TGF-β	Nonmyelinating Schwann cells, megakaryocytes	HSC quiescence (Yamazaki et al., 2011)
Angiopoietin-1	Osteocalcin$^+$ osteoblasts	Promotion of HSC quiescence (Arai et al., 2004)
Robo-4 ligand(s) (Slit2^a)	Bone marrow stromal cells (^{a}Slit2 identified at the mRNA level)	Promotion of HSC homing to the bone marrow (Smith-Berdan et al., 2011, 2012)
Junction adhesion molecule B (JamB)	Osteoblasts, MSCs, endothelial cells	HSC adhesion and quiescence (Archangeli, 2011)
CXCL4	Megakaryocytes	Promotion of HSC quiescence

Arai F. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell.* 2004;118(2):149–161.
Arcangeli ML, et al. JAM-B regulates maintenance of hematopoietic stem cells in the bone marrow. *Blood.* 2011;118(17):4609–4619.
Ding L, Morrison SJ. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature.* 2013;495:231–235.
Fleming HE, et al. Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. *Cell Stem Cell.* 2008;2(3):274–283.
Greenbaum A, et al. CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature.* 2013;495:227–230.
Himburg HA, et al. Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells. *Nat Med.* 2010;16(4):475–482.
Himburg HA, et al. Pleiotrophin regulates the retention and self-renewal of hematopoietic stem cells in the bone marrow vascular niche. *Cell Rep.* 2012;2(4):964–967.
Nilsson SK, et al. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood.* 2005;106(4):1232–1239.
Poulos MG, et al. Endothelial Jagged-1 is necessary for homeostatic and regenerative hematopoiesis. *Cell Rep.* 2013;4(5):1022–1034.
Qian H, et al. Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. *Cell Stem Cell.* 2007;1(6):671–684.
Smith-Berdan S, et al. Dynamic expression of the Robo ligand Slit2 in bone marrow cell populations. *Cell Cycle.* 2012;11(4):675–682.
Smith-Berdan S, et al. Robo4 cooperates with CXCR4 to specify hematopoietic stem cell localization to bone marrow niches. *Cell Stem Cell.* 2011;8(1):72–83.
Stier S, et al. Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. *J Exp Med.* 2005;201(11):1781–1791.
Sugimura R, et al. Noncanonical Wnt signaling maintains hematopoietic stem cells in the niche. *Cell.* 2012;150(2):351–365.
Winkler IG, et al. Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. *Nat Med.* 2012;18(11):1651–1657.
Yamazaki S, et al. Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. *Cell.* 2011;147(5):1146–1158.
Yoshihara H, et al. Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. *Cell Stem Cell.* 2007;1(6):685–697.

HSPC, Hematopoietic stem/progenitor cell; *MSC*, mesenchymal stem cell; *TGF*, transforming growth factor.
Updated and modified from Kfoury Y, Mercier F, Scadden DT. Snapshot: the hematopoietic stem cell niche. *Cell.* 2014;3;158(1).

Perivascular Cells

The observation that HSPCs co-localize with the marrow vasculature raised the possibility that perivascular cells may also play a role in the HSPC regulation by the niche. So far, three types of perivascular cells have been characterized—CXCL 12-abundant reticular cells (CAR cells), nestin-GFP^{dim}/leptinR+ MSCs, and nestin-GFP^{bright}/NG2+ pericytes.[11,16]

CAR cells were identified in a mouse model in which GFP was driven by the CXCL12 promoter. Similar to the other components of the niche, CXCL12-abundant reticular (CAR) cells are found in close proximity to HSPCs. It is likely that they are genetically and phenotypically related to nestin-GFP^{low} cells (see the following section) because their ablation also severely impaired adipogenic and osteogenic differentiation of nonhematopoietic BM cells in addition to reducing the number of HSPCs. Notably, the effect of deletion was not limited to HSPCs but also affected mature lineages, such as lymphoid cells, indicating a wider role for these cells in hematopoietic support.

MSCs are located in the peri-sinusoidal BM space and have been initially defined by low expression of GFP driven by gene regulatory elements of intermediate filament protein Nestin (nestin-GFP^{low}). Nestin-GFP^{low} cells express the genes associated with HSPC retention in the niche (Cxcl12, VCAM-1), which are down-regulated upon GCSF mobilization. Selective deletion of Nestin-positive cells in mice resulted in a 50% reduction in the number of long-term HSPCs and their relocation to the spleen, although it is not clear whether this effect was mediated directly by Nestin-positive cells or through their more differentiated downstream progeny. Subsequent studies revealed that a population that largely overlaps with nestin-GFP^{low} cells can be also defined by the expression of the leptin receptor. Specific deletion of known HSPC regulators (SCF, CXCL12) specifically from MSCs using Cre-recombinase driven by leptin receptor gene regulatory elements lead to a reduction in the HSPC number and long-term repopulating capacity, further delineating the functional significance of MSCs in the HSPC niche.

Leptin receptor-expressing cells (LepR+) are the single largest population of BM stromal cells. While the receptor itself does not appear to be critical, the cells are productive of high levels of SCF and CXCL12 and are shown to be important modifiers of HSPC number. With single-cell analyses, it has become clear that the LepR+ populations are very heterogeneous. It has also been shown that the cells differentiate into both osteoblasts and adipocytes. Since the studies defining the function of these cells have relied upon genetic models where any cell ever expressing LepR deletes the test gene, interpretation should be done with caution. However, these cells or their descendants are clearly important in regulating hematopoietic function and are therefore niche components.

Adipocytes

An observation that adipocyte-rich vertebrae in mice contained significantly fewer cycling HSPCs compared with adipocyte-poor thoracic vertebrae led to the discovery of their role in HSPC regulation. In a genetic mouse model of lipoatrophy (i.e., a condition with reduced adipocyte number), post-transplant hematopoietic recovery was accelerated, although a concomitant increase in trabecular bone could have contributed to this result. Subsequent studies have indicated that adipocytes are important for hematopoietic reconstitution after transplantation.[17]

Osteoclasts

Osteoclasts are BM-derived cells that are located in close proximity to stem cell-rich endosteum and play a critical role in bone remodeling. During stress and GCSF-induced mobilization, the activity of the osteoclasts increases and is accompanied by secretion of proteolytic enzymes and reduction in the endosteal niche components, as evidenced by downregulation of the osteopontin expression by the OLCs. Conversely, inhibition of the osteoclasts leads to reduced HSPC and progenitor egress.

Bone Marrow Macrophages

During experiments investigating the mechanisms of GCSF-induced HSPC mobilization, it was noted that in addition to previously reported reduction in the endosteal OLCs, BM macrophages were also decreased in number. In vivo depletion of this cell population by clodronate administration produced the same result. It therefore appears that macrophages play a critical role in supporting the OLC niche compartment. A similar role for the macrophages, but with regard to supporting nestin-GFP^{low} MSCs, has been suggested by another study that used clodronate-mediated macrophage ablation. Again, this resulted in HSPC egress from the BM, perhaps through the effect on nestin-GFP^{low}, that showed a marked downregulation of genes responsible for HSPC retention in the niche (see earlier discussion). Thus, macrophages appear to function at a level of regulation upstream of OLCs and nestin-GFP^{low} cells.

Megakaryocytes

Megakaryocytes are the most recent addition to a growing list of niche cell types and appear to be directly involved in the regulation of HSPC quiescence, as shown by cell deletion studies.[18] This effect is mediated by CXCL4, also known as platelet factor 4, and TGF-beta. Overall, only 20% of immunophenotypically defined HSPCs are associated with megakaryocytes and were spatially distinct from HSPCs that are located near the arterioles, suggesting the existence of a specific megakaryocytic niche. The presented findings also illustrate a feedback mechanism, by which mature hematopoietic cells may regulate HSPC number.

EXTRINSIC REGULATION OF THE HEMATOPOIETIC STEM CELL NICHE

Sympathetic Innervation

Sensory and autonomic innervation of the BM is critical for its ability to respond to hematopoietic stress. Increase in sympathetic tone promotes HSPC mobilization and down-regulates the components of the endosteal niche, mainly through the activation of β_2-adrenergic receptor.[19] Nestin-positive cells express both β_2 and β_3 receptors and act as another mediator between sympathetic signaling and HSPCs, again facilitating egress from the BM. In a mouse model of streptozocin-induced diabetes, diabetic autonomic dysfunction was shown to disrupt this regulatory circuit, alter the function of Nestin-positive cells, and lead to impaired GCSF-induced HSPC mobilization, providing a biologic explanation for a higher frequency of peripheral blood stem cell mobilization failure in diabetic patients. The sympathetic nervous system is also involved in the regulation of HSPC egress from the BM as governed by circadian rhythms, a remarkable discovery based on a chance observation that continuous exposure to light (because of a broken light switch in the animal house) significantly altered the number of HSPCs mobilized after GCSF administration. Thus, sympathetic niche innervation is essential for relaying and integrating extrinsic signals and acts as a responsive and finely tuned tool that regulates HSPC traffic between peripheral blood and the BM. It is also now apparent that the loss of adrenergic innervation contributes to the changing function of the niche with aging.

Not only sympathetic nerves, but the glial cells that surround them can regulate HSPC behavior in the marrow, as exemplified by the studies of non-myelinating Schwann cells. These cells ensheath sympathetic nerves, come into contact with HSPCs, and are responsible for activation of the latent form of TGF-beta. Denervation experiments lead to a significant reduction in the number of glial cells and the associated increase in HSPC cycling.

Hypoxia

The niche for quiescent HSPCs is thought to be hypoxic. These observations are based on studies that use an intracellular marker of reducing equivalents, pimonidazole, or direct measurement of oxygen tension in the BM using intravital microscopy. By direct measurement of oxygen, it is apparent that the absolute level of Po_2 in the BM is quite low (less than 32 mmHg) and reduces to ~10 mmHg deeper into the marrow space.[20] The vascular network in the marrow is extensive, and it appears that the low Po_2 levels reflect high consumption by the action of hematopoiesis.

Hypoxia is associated with upregulation of stromal cell-derived factor 1 (SDF-1) expression in the endosteal region and HSPC traffic to the BM; in contrast, hyperbaric oxygen (exposure to 100% oxygen under increased atmospheric pressure) mobilizes HSPC and progenitors away from the BM. Hypoxic responses in the HSPCs are mediated through a family of hypoxia-inducible factors (HIFs). The best-studied species of HIFs is HIF1-α, inducing SDF-1 expression and directing metabolic circuits within HSPCs toward anaerobic metabolism. HIF1-α also stimulates secretion of VEGF, thereby promoting bone formation and angiogenesis. HIF1a affects HSPC function, as HIF1a deletion from HSPCs leads to HSPC exhaustion.

What is a physiologic role of hypoxia? First, hypoxia is believed to protect HSPCs in the niche from oxidative stress. Indeed, HSPCs within the niche contain a lower level of reactive oxygen species. Moreover, it appears that hypoxic conditions are beneficial for the HSPC function because culturing human BM HSPCs under lower oxygen tension leads to an increase in their ability to engraft and repopulate nonobese diabetic/severe combined immune-deficient (NOD/SCI) mice. Finally, hypoxia may also protect the HSPC pool from exhaustion by promoting cell cycle quiescence. Of note, when radiation or cytotoxic chemotherapy are used, the Po_2 levels in marrow go dramatically higher raising the question of whether Po_2 levels participate in the hematopoietic regenerative response to injury.

LYMPHOID NICHES

BM is the site of *B-cell lymphopoiesis*. Several cell types involved in the HSPC niche also participate in the formation of the lymphoid niches.[11] Interestingly, B-cell niches correspond to maturation stages of the lymphoid cells—OLCs, osteoclasts, and CAR cells are necessary for the less mature stages of development, whereas interleukin-7 (IL-7) secreting cells and sinusoidal endothelial cells are important for more differentiated cells. These observations come from targeted deletion of each supporting cell population using genetic means and analyzing the effect on B-cell homeostasis. For example, deletion of the OLCs leads to a considerable decrease in pre-pro and pro-B cells. This process appears to be mediated by the heterotrimeric G protein α subunit because its deletion in the OLCs leads to a 60% decrease in the percentage of B-cell precursors in the BM. A similar phenotype is seen upon the deletion of CAR cells.

Naïve recirculating B and T cells are located in the perisinusoidal space and co-localize with dendritic cells, which are thought to deliver supportive signals, because their deletion leads to a significant decrease in B-cell number and reduction in IgM production after immunization.

Plasma cells are the product of the terminal differentiation of B cells after antigen exposure. In vitro and in vivo studies showed that plasma cells receive multiple extrinsic survival signals, including CXCL12, IL-6, BAFF (B-cell activating factor of the TNF family), and APRIL (a proliferation-inducing ligand), which may account for their longevity. Mice deficient in CXCR4 displayed impaired homing of plasmablasts, illustrating the involvement of CXCL12–CXCR4 axis in plasma cell trafficking. Eosinophil- and megakaryocyte-derived APRIL and BAFF appear to regulate the number of plasma cells, which is greatly reduced upon eosinophil or megakaryocyte deletion.

The majority of long-lived *memory T cells* reside in the BM and appear to require a close contact with IL-7 secreting stromal cells to ensure that they remain quiescent in the absence of antigen stimulation. The BM also contains a large proportion of *regulatory T cells*, which have recently been found to exclusively protect HSPCs and early progenitors from rejection after allogeneic transplantation, arguing that the endosteal surface act as an immune-privileged site. Surprisingly, BM harbors specific niche cells (osteocalcin+ OLCs) that provide instructive cues to T-competent progenitors that migrate from the BM to the thymus since deletion of the osteocalcin-expressing cells leads to decreased intrathymic T-cell precursors and impaired generation of mature T-cells in the presence of normal thymic function.[21] Specific OLCs therefore appear to be important in T-lymphopoiesis. Their damage in the settings of conditioning for transplantation or graft-versus-host disease may compromise the ability of BM to provide the early T-lineage cells for thymocyte and ultimately, T-cell production.

ERYTHROID NICHES

Erythroblastic islands were first described by a French hematologist Marcel Bessis more than 50 years ago and consist of developing erythroblasts surrounding a central macrophage. They are present in the BM, fetal liver, and the spleen and in in vitro long-term BM cultures. The number of erythroblasts per island ranges from 10 cells observed in sections of rat femur to 5 to 30 erythroblasts seen in human BM. Some islands are located adjacent to the BM sinusoids, and the others are scattered throughout the BM cavity. Within erythroid islands, the macrophage functions as a "nurse cell" providing iron to the developing erythroblasts and phagocytosing the extruded nuclei at the end of erythroid differentiation.

Adhesion between maturing erythroblasts and central macrophage is mediated by several molecules, including erythroblast macrophage protein (Emp via homophilic binding), α4β1 integrin (VCAM-1), and αv integrin (ICAM-4); antibody-mediated blockade of each of these molecule results in disruption of the islands. The most striking effect is seen with the blockade of Emp, which causes significant increase in proliferation, maturation, and apoptosis of maturing erythroblasts in vitro. Of note, Emp-null fetuses die in utero from severe anemia.

In addition to interaction within macrophages, maturing erythroblasts adhere to extracellular matrix proteins, fibronectin, and laminin for the maturation to proceed. Fibronectin protects erythroblasts from apoptosis, partly through anti-apoptotic bcl-xL, and laminin is thought to localize reticulocytes to sinusoids as the initial step before their release into circulation.

MEGAKARYOCYTIC NICHES

Megakaryocytes localize to BM endothelial cells in vivo and release platelets into the marrow intravascular–sinusoidal space or the lung capillaries. Although CXCL12 induces platelet production by megakaryocytes if preceded by migration through endothelial cells, this is not observed in the absence of endothelial cells, suggesting that megakaryocyte interaction with specific molecules present on the endothelial cells is necessary for thrombopoiesis. FGF4 and CXCL12 enhance the interaction of megakaryocytes with endothelial cells and restore thrombopoiesis in mice deficient in thrombopoietin or its receptor c-mpl. Thus, chemokine-mediated localization of megakaryocytes within a specific vascular microenvironment is necessary for their maturation and platelet production. Notably, the pulmonary vascular tree also appears to be a site where megakaryocyte production of platelets occurs.[22]

HUMAN BONE MARROW MICROENVIRONMENT

Because direct mechanistic studies of the human BM microenvironment cannot be undertaken, the bulk of our knowledge comes from experiments in xenotransplantation models. These initially involved fetal sheep and heavily irradiated or nude mice as recipients, but only a very low level of human hematopoietic engraftment was observed. The discovery of SCID mice led to the development of two powerful models. The first, known as SCID-hu mouse, was generated by engrafting the human thymus and fetal liver. This model was most informative for the study of human lymphoid development and used for testing novel HIV drugs. The second model, hu-SRC (for SCID-repopulating cell), through the pioneering work of John Dick and colleagues, enabled the investigation of human HSPC engraftment and differentiation. Further modifications of the SCID model led to the generation of a NOD/SCID–IL2 receptor γ chain knock-out (NSG) strain, which supports robust normal human multilineage (myeloid and lymphoid) hematopoietic engraftment, as well as engraftment of acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) cells from patients. The sensitivity of the transplant assay is further increased by direct intrafemoral injection into the BM cavity; remarkably, in NSG recipients, human hematopoietic engraftment can be detected after the intrafemoral transplantation of a single highly purified human HSPC. Further advances in the field of xenotransplantation is the development of mouse strains that express several human hematopoietic cytokines, thereby enabling support of innate immune cells (myeloid and NK cells). Several further iterations of the model are now available to permit the engraftment of human HSPC without conditioning.

Similar to mouse HSPCs, human HSPCs transplanted into mouse recipients preferentially traffic to the trabecular bone and home next to the endosteal surface. They are guided to their niches by the CXCL12–CXCR4 pathway and cell adhesion molecules such as very late antigen-4 (VLA-4), very late antigen-5 (VLA-5), and lymphocyte function-associated antigen-1 LFA-1; of note, CXCL12 is expressed by human OLCs, mesenchymal stromal, Nestin-positive, and endothelial cells. CD44 and hyaluronic acid cooperate with CXCL12 in human HSPC homing. Recent experiments identified α6 integrin CD49f as a novel marker for human HSPCs, alluding to the functional importance of HSPC anchorage within the BM microenvironment.

The cellular components of the human HSPC niche are yet to be identified, although single-cell cataloging of cell types has been conducted. One potential functional candidate is a population of mesenchymal subendothelial cells expressing CD146, which can be prospectively isolated from human BM. These perivascular cells were able to establish both bone and hematopoietic microenvironment upon subcutaneous transplantation; had a documented self-renewal capacity; and produced angiopoietin 1, a cytokine known to induce HSPC quiescence. CD271 has been suggested as another marker for the human hematopoiesis-supporting mesenchymal population; in addition to CD146+ perivascular cells, it labels CD146-endosteal population, which co-localizes with hematopoietic CD34+ cells in human BM.

The limitations of our knowledge of the human hematopoietic microenvironment restrict our ability to accomplish in vitro stem cell expansion. The potential benefits of doing so are especially evident in the context of cord blood transplantation when the number of donor cells is small and in the setting of gene-modified stem cells where gene transduction efficiencies are low. Several molecules have been tested. One of them is the sonic hedgehog protein, which has been shown to induce proliferation of primitive human hematopoietic cells when added to highly purified CD34+ CD38-lineage human cells; this effect translated into an increased level of progenitor expansion in NOD/SCID mice. The aryl hydrocarbon receptor antagonist, SR-1 also appears to increase HSPC and has improved hematopoietic reconstitution with SR-1 expanded cord blood transplantation. An engineered Notch ligand Delta 1 and pleiotrophin have both been shown to increase HSPC. Prostaglandin E_2 enhances murine HSPC localization in the BM after brief in vitro exposure. This resulted in a twofold to threefold increase in the number of HSPCs compared with control (vehicle-exposed) cells. PGE2-treated cord blood cells appeared to accelerate neutrophil recovery in a Phase I clinical trial. Other methods for expanding HSPC ex vivo continue to be developed, but the use of cord blood HSPC for transplant is declining, raising questions about the ultimate medical utility of expansion strategies.

HEMATOPOIETIC MICROENVIRONMENT IN ACUTE LEUKEMIA AND MYELODYSPLASIA

Given a critical role of the hematopoietic microenvironment in safeguarding cellular homeostasis in the BM, it is not surprising that alterations within it—either primary or induced by the presence of malignant cell population—have been proposed to contribute toward tumor initiation, maintenance, and resistance to treatment. Here, we summarize the data related to the role of the microenvironment in the pathogenesis of acute leukemia and myelodysplasia. For a review of this topic in other hematologic neoplasms, readers are referred to disease-specific chapters of this book.

Early in vitro studies alluded to the significant contribution of nonhematopoietic BM cells (collectively termed *stroma*) to the pathogenesis of acute leukemia. For example, fibroblastic stromal cells from patients with AML were unable to support normal granulocytic-macrophage colony formation in contrast to those obtained from normal individuals. However, when the stromal cells were tested from patients in remission, they maintained the growth of GM colonies similar to normal stroma. Strikingly, when the patients relapsed, this GM colony-supporting ability was lost. In another series of observations, when nonadherent cells from continuous marrow cultures or GM-CSF–dependent progenitor cell lines were co-cultured with mouse stromal cells that had been previously irradiated, they developed factor-independence and multiple distinct karyotypic abnormalities; upon subcutaneous injection, these newly transformed cell lines produced granulocytic monomyeloid tumors that spread to the spleen, lymph nodes, and BM. Although by no means definitive, these studies suggested that either the altered stromal cells may contribute to the emergence of leukemia, or leukemia itself may affect the nonhematopoietic compartment. Both of these hypotheses found confirmation in the later studies reviewed in the following sections.

Niche Contribution to Leukemia Development

The idea of "niche-induced oncogenesis," or contribution of the microenvironment to the emergence of malignant disease, stems from the clinical observation of donor-induced leukemia. In this condition, which has a reported incidence between 0.12% and 5%, the leukemic clone arises from an apparently normal donor hematopoietic cells after allogeneic BM transplantation. Although the etiology is clearly multifactorial, damage to the BM microenvironment, either because of previous chemotherapy or pre-transplant conditioning, may be an important contributing factor. In keeping with this notion, several experimental mouse models illustrate that microenvironmental alterations, either alone or in conjunction with corresponding molecular lesions in the hematopoietic compartment, can play a critical role in the initiation of malignant disease. For example, the mice with deficiency of phosphatase and tensin homolog (PTEN) both in the microenvironment and HSPC developed a myeloproliferative disorder, but PTEN deficiency in HSPCs alone did not result in the disease. Similarly, widespread deletion of retinoblastoma protein or retinoic acid receptor led to the development of myeloproliferative disorder, in the latter case purely because of gene deletion in the microenvironment. Using a cell type-specific approach, it was found that targeted deletion of the micro-RNA processing enzyme Dicer-1 in immature OLCs resulted in the development of myelodysplasia and acute leukemia associated with independent complex genetic changes.[23] The effect of Dicer-1 deletion was entirely attributable to the microenvironment because transplantation of Dicer 1-deleted BM into normal microenvironment resulted in reversal of the myelodysplastic phenotype. Remarkably, hematopoietic abnormalities were observed when Dicer-1 was deleted in very immature OLCs (osterix+) but not in those at a more mature differentiation stage (osteocalcin+). Deletion of the Schwachman-Diamond-Bodian syndrome gene in immature OLCs recapitulated the key features of the Dicer-1 deletion phenotype and implicated their role in its pathogenesis. In another striking example of niche involvement in leukemia generation, mice carrying an OLC-specific mutation of constitutively activated beta-catenin developed AML. The leukemic process was microenvironment-dependent and mediated by Notch signaling. Genetic or pharmacological inhibition of Notch signaling leads to a reversal of the leukemic phenotype. The findings of this study are clinically relevant, as 38% of patients with MDS and AML showed nuclear (activated) beta-catenin in osteoblasts.

Altogether, the presented studies illustrate that the perturbed BM microenvironment can act as a source of signals that promote malignant change in the hematopoietic compartment and highlight their potential role as therapeutic targets.

Niche Alterations by Leukemia

Just as the microenvironment can contribute to disordered hematopoiesis, it can be disrupted and modified by leukemic cells, leading to a competitive advantage over normal counterparts.[14,24,25]

Visualization of BM niches by intravital microscopy studies revealed that primary ALL and AML blasts are able to down regulate CXCL12 expression in the BM, causing the egress of normal CD34+ HSPCs from the BM and impairment of normal hematopoiesis. This process was mediated by the SCF secreted by leukemic blasts and reversed upon SCF neutralization. Similarly, in myeloproliferative disorders such as CML, it is thought that microenvironmental changes that are induced by malignant cells result in the formation of a self-reinforcing loop leading to preferential expansion of leukemic cells at the expense of their normal counterparts. Leukemia can also affect the niches indirectly, i.e., through perturbation of sympathetic innervation: both in CML and AML, "sympathetic neuropathy" promotes leukemogenesis through either reduction in quiescence-inducing nestin+ cells or altering their differentiation properties and frequency relative to nestin-GFP^{high}/NG2+ cells.[25] The "niche-modifying" ability of the leukemic cells in vivo is an area of ongoing investigation because it suggests that the leukemic niche can be molecularly distinct from the normal, thus creating an opportunity to alter the leukemic niche as a means of reducing the persistence of leukemia-initiating cells.

Therapeutic Targeting of Leukemic Niche

The CXCR4–CXCL12 pathway is critical for homing and subsequent adhesion of not only normal, but leukemic stem cells (LCSs) to the BM microenvironment. Of note, the level of CXCR4 is elevated in patients with AML and is associated with a poor outcome. The presence of Flt3 internal tandem duplication (a poor prognostic factor in AML) is in turn associated with increased CXCR4 expression. Experimentally, treatment of NOD/SCID mice transplanted with primary human AML cells using a neutralizing antibody against CXCR4 reduced the leukemic burden. Follow-up studies confirmed the antileukemic effect of blocking the CXCL12–CXCR4 axis using competitive antagonists of CXCR4 (AMD3100 and AMD3254) in mouse models of AML. These findings formed the basis for ongoing clinical trials of CXCR4 antagonist AMD3100 (Plerixafor) as a chemosensitizing agent in AML. So far, these studies have confirmed the safety and tolerability of this drug in combination with chemotherapy, but the effect on response rate and survival has not been demonstrated.

Another example of LSC niche dependence is a cell adhesion molecule CD44, which is present on the surface of leukemic cells and interacts with hyaluronan on the endosteal surface. Blocking the interaction between CD44 and hyaluronan using activating CD44 antibodies had a significant effect on LSC eradication and even cured some mice. The therapeutic effect of the antibody was more marked when it was administered soon after the injection of human leukemic cells compared with the animals with established disease, suggesting that it acts predominantly at the stage when LCSs engage their respective niches. It is also possible that some of the effect of the CD44 antibody was attributable to differentiation induction in the LSCs. Nevertheless, this study provided a proof-of-principle demonstration that LSCs interaction with the niche is required for their survival and leukemia progression and thereby raised the potential for targeting therapy.

Other molecular mediators of the LSC–microenvironment interaction have also been identified. B4 integrin (also known as very late antigen 4) mediates lodgment of leukemic cells in the BM and interacts with fibronectin to confer resistance to cytosine arabinoside-induced apoptosis. Integrin ligation triggers prosurvival pathways, and the blocking antibody leads to a reduction in the level of leukemic burden and a modest prolongation of the lifespan in human AML-transplanted animals. A similar protective role for AML blasts has been observed for β1 and β2 integrins. IL-3 receptor α chain (CD123) also contributes to LSC survival, at least partly through being involved in controlling LSC homing to the BM; the CD123 blocking antibody demonstrated considerable antileukemic activity, which was also attributable to promoting immune-mediating destruction of leukemic cells. This idea that has been explored further in the studies of blocking a macrophage-associated molecule CD47. Expression of CD47 on LSCs appears to protect the LSCs from phagocytosis. CD47 expression is associated with Flt3-ITD mutation and independently predicts worse prognosis. Mechanistically, CD47 acts as a "do not eat me" signal for the macrophages. Blocking CD47 antibody produces depletion of AML and MDS cells in human clinical trials in xenotransplantation models and specific eradication of LSCs. Phase 1 study of CD47 antibody in hematological malignancies and solid tumors is ongoing.

It has also been noted that leukemia induces a reorganization of the BM vasculature and that compromises normal HSPC support. Abrogating the vascular effects with deferoxamine was shown in mice to lead to improved normal hematopoiesis and responsiveness of AML to chemotherapy, resulting in improved survival.[26]

The innervation of the BM also contributes to leukemia. There is a loss of adrenergic stimulation that remodels the stroma to enhance AML growth; this appears to be mediated by the beta3 adrenergic receptor.[25] Further, metabolic changes in stromal cells contribute to sustaining AML cell survival, particularly during chemotherapy. A broad range of changes therefore accompany leukemia in the marrow; some may be primary, fostering leukemia induction, while others are secondary, fostering leukemic cell growth while compromising normal hematopoiesis.[27,28] Targeting these interactions may offer complementary therapies to those that are leukemia cell specific and certainly expand strategic considerations beyond that of leukemia cells themselves.

FUTURE DIRECTIONS

Although the concept of a specific microenvironment for different hematopoietic compartments was first proposed more than 100 years ago, it was not until recently that the existence of the "niches" has been experimentally proven and the molecular factors involved in cellular interactions have been discovered.

Our current knowledge of the hematopoietic microenvironment has been evolving in parallel and often leading that in other stem cell systems. It appears that fundamental components and molecular pathways are highly conserved among evolutionary diverse species, although their role in specific niches may vary. These include supporting stromal cells secreting soluble molecules regulating stem cell self-renewal (bone morphogenic protein and Wnt signaling), extracellular matrix proteins that serve as stem cell anchors (integrin ligands), blood vessels that are responsible for nutritional support and transit of stem cells in and away from the niche, and neural inputs for integrating signals from different systems. It is therefore likely that future studies in the spatial and molecular organization of other stem cell niches will inform the knowledge of hematopoietic niches and vice versa.

With a rapidly increasing number of cell types known to be involved in hematopoietic niches (and the number of different cytokines they produce, which will inevitably follow), it will be important to use a "network" approach—similar to the one used for the analysis of transcriptional networks—to understand how these multiple factors act in concert to control location, proliferation, and trafficking of HSPCs and more mature cells in the BM. It is possible that these factors work in a combinatorial manner, ultimately creating a "niche code" that is designed to suit a specific physiologic situation.

A particularly notable development over the recent years has been our improvement in understanding the role of the microenvironment in the initiation and maintenance of malignant disease, although many questions remain. We still know very little about the molecular mediators of "niche-induced oncogenesis" and those involved in microenvironment-induced chemoresistance. Recent advances in xenotransplantation assay using highly immunocompromised mouse strains for the study of normal and leukemic hematopoiesis, together with further molecular insights into the biology of LCSs, will provide an opportunity to address these issues.

Therapeutic manipulation of the hematopoietic microenvironment remains an ultimate goal of ongoing research. Clearly, the effort of the next several years will be focused on translating the wealth of data obtained from the animal models into human biology and the clinic. This work has already started with the clinical trials of ex vivo HSPC expansion before cord blood transplantation. A number of clinical trials are also underway to examine the efficacy of niche-directed therapies in hematologic malignancies. Although the animal data suggest that targeting the niche alone is often insufficient to achieve cure, especially in established disease, this approach has been successful in regaining leukemia chemosensitivity to commonly used agents and may become a valuable component of future treatment protocols, particularly in the setting of low tumor burden, such as minimal residual disease. Gaining a deeper insight into the molecular distinctions between normal and malignant niches will enable a better understanding of "niche competition" between normal and leukemic populations and lead to the development of novel approaches based on eradication of leukemic cells and fostering normal hematopoiesis through the manipulation of niche-derived signals.

REFERENCES

1. Schofield R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells*. 1978;4(1-2):7–25.
2. North TE, et al. Hematopoietic stem cell development is dependent on blood flow. *Cell*. 2009;137:736.
3. Gekas C, et al. The placenta is a niche for hematopoietic stem cells. *Dev Cell*. 2005;8:365.
4. Khan JA, et al. Fetal liver hematopoietic stem cell niches associate with portal vessels. *Science*. 2016;351:176080.
5. Lee Y, et al. Hepatic stellate and endothelial cells maintain hematopoietic stem cells in the developing liver. *J Exp Med*. 2021;218.
6. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. *Nature*. 2014;505:327.
7. Calvi LM, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature*. 2003;425:841.
8. Zhang J, et al. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature*. 2003;425:836.
9. Lo Celso C, et al. Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. *Nature*. 2009;457:92.
10. Nombela-Arrieta C, et al. Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. *Nat Cell Biol*. 2013;15:533.
11. Ding L, et al. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature*. 2012;481:457.
12. Kokkalkiaris KD, et al. Adult blood stem cell localization reflects the abundance of reported bone marrow niche cell types and their combinations. *Blood*. 2020;136:2296–2307.
13. Tikhonova AN, et al. The bone marrow microenvironment at single-cell resolution. *Nature*. 2019;569:222–228.
14. Baryawno N, et al. A cellular taxonomy of the bone marrow stroma in homeostasis and leukemia. *Cell*. 2019;177:1915–1932.
15. Severe N, et al. Stress-induced changes in bone marrow stromal cell populations revealed through single-cell protein expression mapping. *Cell Stem Cell*. 2019;25:570–583.
16. Mendez-Ferrer S, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature*. 2010;466:829.
17. Zhou BO, et al. Bone marrow adipocytes promote the regeneration of stem cell and hematopoiesis by secreting SCF. *Nat Cell Biol*. 2017;19:891–903.
18. Bruns I, et al. Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion. *Nat Med*. 2014;20:1315.
19. Katayama Y, et al. Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell*. 2006;124:407.
20. Spencer JA, et al. Direct measurement of local oxygen concentration in the bone marrow of live animals. *Nature*. 2014;508:269.
21. Yu VW, et al. Specific bone cells produce DLL4 to generate thymus-seeding progenitors from bone marrow. *J Exp Med*. 2015;212:759.
22. Lefrancais E, et al. The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature*. 2017;544:105–109.
23. Raaijmakers MH, et al. Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature*. 2010;464:852.
24. Colmone A, et al. Leukemic cells create bone marrow niches that disrupt the behavior of normal hematopoietic progenitor cells. *Science*. 2008;322:1861.
25. Hanoun M, et al. Acute myelogenous leukemia-induced sympathetic neuropathy promotes malignancy in an altered hematopoietic stem cell niche. *Cell Stem Cell*. 2014;15:365.
26. Duarte D, et al. Inhibition of endosteal vascular niche remodeling rescues hematopoietic stem cell loss in AML. *Cell Stem Cell*. 2018;22:64–77.
27. Forte D, et al. Bone marrow mesenchymal stem cells support acute myeloid leukemia bioienergetics and enhance antioxidant defense and escape from chemotherapy. *Cell Metab*. 2020;32:829–843.
28. van Gastel N, et al. Induction of a timed metabolic collapse to overcome cancer chemoresistance. *Cell Metab*. 2020;32:391–403.

CHAPTER 15

CELL ADHESION

Rodger P. McEver, Pilar Alcaide, and Francis W. Luscinskas

Cell adhesion is essential for the development and maintenance of multicellular organisms. Cell-to-cell and cell-to-matrix adhesion provide a mechanism for intercellular communication and to define the three-dimensional architecture of organs. The regulated nature of cell adhesion is particularly evident in the hematopoietic system, where blood cells routinely make transitions between nonadherent and adherent phenotypes during differentiation, and in response to stimuli in the circulation or extravascular space.

In the bone marrow (BM), hematopoietic stem cells reside in a specialized microenvironment called the *stem cell niche*, and their proliferation and differentiation are controlled not only by soluble growth factors but also by adhesion to stromal cells and matrix molecules (see Chapters 14 and 16 for more detailed discussion). Weakening of these adhesive interactions is required for mature blood cells to enter the circulation. Circulating erythrocytes normally remain nonadhesive until they become senescent and are finally cleared by the reticuloendothelial system (see Chapter 34 for more discussion). Other circulating blood cells often participate in regulated adhesive events during their lifespan. For example, prothymocytes adhere to thymic stromal cells where they undergo guided movement from the cortex to the medulla during maturation before reentering the circulation (see Chapter 24 for details of T cell development). T cells regularly adhere to the specialized high endothelial venules of lymph nodes in tissues, migrate into these organs for the sampling of processed antigens, and then exit via the lymphatics to recirculate in the blood (see Chapter 12 for more discussion). During inflammation, specific classes of leukocytes roll at very low velocity on acutely or chronically stimulated endothelium that line blood vessels, then adhere more tightly, and finally emigrate between endothelial cells into the tissues. There, neutrophils and monocytes phagocytose invading pathogens, and lymphocytes adhere to antigen-presenting cells, such as dendritic cells, B cells, and macrophages (see Chapter 123 for more discussion). During hemorrhage, platelets adhere to exposed subendothelial matrix components, spread, and recruit additional platelets into large aggregates that serve as an efficient surface for thrombin and fibrin generation. This is discussed in more detail later in Chapter 121. Leukocytes also adhere to activated platelets and to other leukocytes, and platelets also roll on the endothelium. When activated, endothelial cells increase the expression of molecules that affect the adhesiveness of platelets or leukocytes. Tight contacts between adjacent endothelial cells also regulate access of blood cells to the underlying tissues and passage of plasma proteins and fluid (edema).

ADHESION MOLECULES

Cells adhere through noncovalent bond formation between macromolecules on cell surfaces with macromolecules on other cell surfaces or in the extracellular matrix (ECM). These interactions involve either protein–protein or protein–carbohydrate recognition. Although some adhesion molecules are expressed only by blood or endothelial cells, most also are synthesized by other cells. Many adhesion molecules can be grouped into families according to related structural and functional features.

EXTRACELLULAR MATRIX PROTEINS

The ECM provides structural and mechanical support for many tissues and spatial cues that enable cell–cell communication and signaling. The principal constituents of the ECM are adhesive proteins and proteoglycans. The major proteins are collagens, von Willebrand factor (vWF), thrombospondins, elastin, fibronectin, laminin, and vitronectin. These proteins are large and often highly extended and consist of multiple domains with different binding functions. In some proteins such as fibronectin, alternative splicing can increase diversity by producing molecules with variable numbers of domains. In addition, stretching of fibronectin can expose cryptic binding sites. The many binding domains allow adhesive proteins to interact with each other as well as with cell-surface receptors, resulting in multipoint contacts that stabilize matrix structure. One adhesive protein, fibrinogen, is found predominantly in plasma but also may be deposited in the exposed subendothelial matrix after vascular injury. Fibronectin, vitronectin, thrombospondins, and vWF are located predominantly in the ECM but also are found in plasma in lower amounts. Several adhesive proteins also are stored in intracellular α-granules of platelets, where they are secreted after platelet activation at sites of vascular injury. Similarly, the endothelium stores adhesive proteins in cytoplasmic storage granules, called *Weibel–Palade bodies*, that are released upon injury or activation.

Proteoglycans contain protein cores to which are covalently attached many glycosaminoglycans—long linear polymers of repeating disaccharides. Most proteoglycans are in the ECM, but some are anchored on cell surfaces through a core protein that contains a membrane-spanning domain. Hyaluronan is a unique glycosaminoglycan that forms polymers with molecular masses up to several million daltons that are not covalently attached to a protein. Hyaluronan forms noncovalent interactions with globular domains on the protein core of proteoglycans and with a small molecule called *link protein*. The resultant hyaluronan–proteoglycan complexes can become very large, contributing to the structural stability of the matrix and function as space fillers during embryonic development. Hyaluronan can also bind to cell-surface receptors and is also abundantly produced during wound healing.

INTEGRINS

Integrins are a broadly distributed group of cell-surface adhesion receptors that consist of noncovalently associated α- and β-subunits (Fig. 15.1 and Table 15.1). There are 18 α-chains and eight β-chains that pair in many, but not all, of the possible combinations. All blood cells have multiple different integrins. The four β2 integrins, each paired with a unique α-subunit, are expressed only by leukocytes, and the αIIbβ3 integrin (glycoprotein IIb–IIIa [GPIIb–IIIa]) is expressed only by megakaryocytes and platelets. Multidomain adhesive proteins of the ECM are ligands for many integrins. Integrins are unusual adhesion molecules because they usually reside in an inactive state on the cell surface until they receive an activating signal. Some integrins bind to specific domains of several different proteins, and some adhesive proteins bind to several different integrins. These interactions generally mediate cell–matrix and cell–cell adhesion. A unique feature of integrins is the transmission of signals in both directions across the cell plasma membrane. Integrin binding to matrix informs the interior of the cell (outside-in), and intracellular signals or conditions inside cells transmit signals outward (inside-out) that regulate binding to matrix or to adhesion receptors on the surface of adjacent cells. Force can also

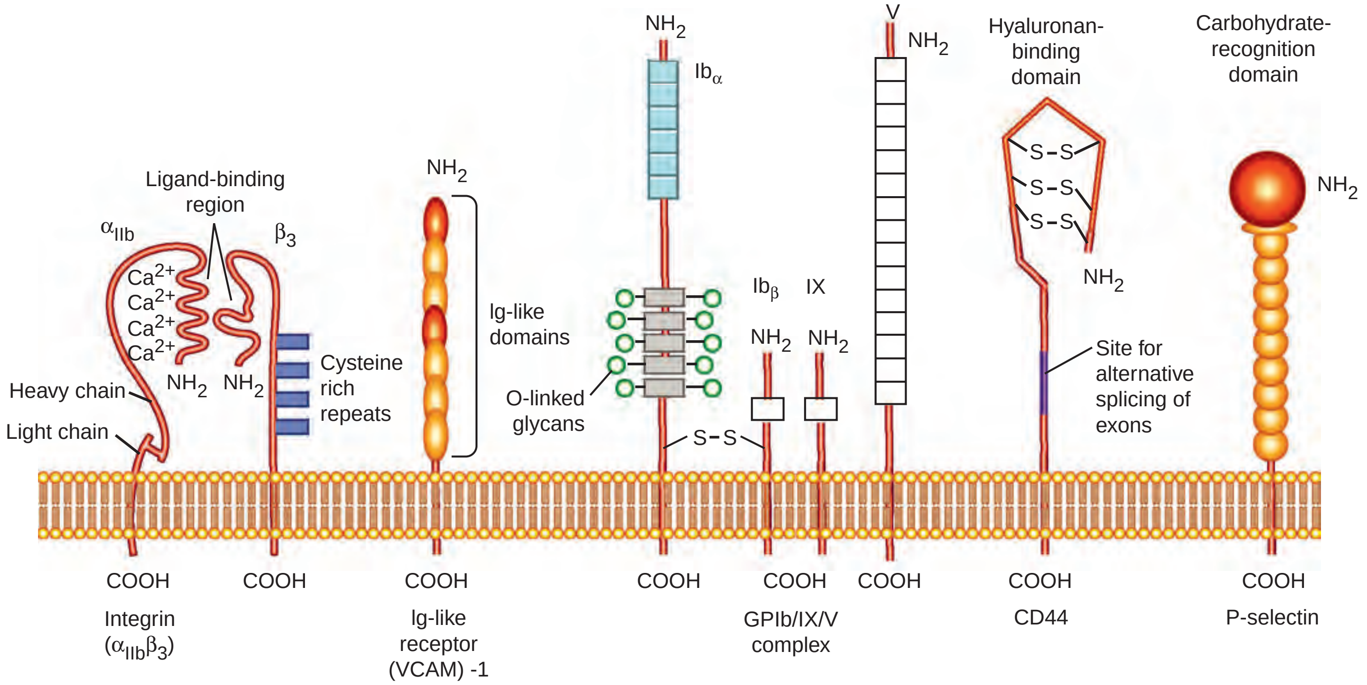

Ligand-binding region
α_{IIb}
β_3
Ca^{2+}
Ca^{2+}
Ca^{2+}
Ca^{2+}
Heavy chain
Light chain
NH_2
NH_2
Cysteine rich repeats
COOH
COOH
Integrin ($\alpha_{IIb}\beta_3$)
NH_2
Ig-like domains
COOH
Ig-like receptor (VCAM) -1
NH_2
Ib_α
O-linked glycans
S–S
Ib_β
IX
NH_2
NH_2
V
NH_2
COOH
COOH
COOH
GPIb/IX/V complex
Hyaluronan-binding domain
S–S
S–S
S–S
NH_2
Site for alternative splicing of exons
COOH
CD44
Carbohydrate-recognition domain
NH_2
COOH
P-selectin

Figure 15.1 SCHEMATIC DIAGRAMS OF SEVERAL TYPES OF CELL SURFACE ADHESION RECEPTORS. Integrins consist of noncovalently linked α- and β-subunits, both of which contribute to ligand binding. The platelet αIIbβ3 integrin is illustrated at *far left*. Ig-like receptors contain a variable number of Ig homology domains, of which some bind ligands and others extend the ligand-binding domains from the plasma membrane. Shown *second from left* is VCAM-1, which contains seven Ig domains; the two domains that bind to integrins are *shaded*. The platelet GPIb–IX–V complex, depicted in the *middle diagram*, consists of several leucine-rich protein subunits. CD44, illustrated next, contains an amino-terminal (*N*-terminal) domain that binds to hyaluronan. Each of the selectins contains an *N*-terminal carbohydrate-recognition domain that binds sialylated and fucosylated oligosaccharides on specific cell-surface GP ligands. Illustrated at the *far right* is P-selectin. *GP*, Glycoprotein; *Ig*, immunoglobulin; *VCAM-1*, vascular cell adhesion molecule-1.

TABLE 15.1 **Integrins on Blood Cells**

Integrin Designation	Other Name(s)	Expressed by	Ligand(s)	Function(s)
$\alpha_1\beta_1$	VLA-1	Leukocytes, other cells	Collagens, LM	Adhesion to ECM
$\alpha_2\beta_1$	VLA-2 GPIa/IIa	Leukocytes, platelets, other cells	Collagens, LM	Adhesion to ECM
$\alpha_3\beta_1$	VLA-3	Leukocytes, other cells	Collagens, LM, FN	Adhesion to ECM
$\alpha_4\beta_1$	VLA-4	Monocytes, lymphocytes, eosinophils	VCAM-1, FN	Adhesion to cells, ECM
$\alpha_5\beta_1$	VLA-5 GPIc/IIa	Leukocytes, platelets, other cells	FN	Adhesion to ECM
$\alpha_6\beta_1$	VLA-6 GPIc/IIa	Leukocytes, platelets, other cells	LM	Adhesion to ECM
$\alpha_9\beta_1$		Neutrophils	VCAM-1	Adhesion to ECs
$\alpha_L\beta_2$	LFA-1 CD11a/CD18	Leukocytes	ICAM-1, -2, -3	Leukocyte aggregation and adhesion
$\alpha_M\beta_2$	MAC-1 CR3 CD11b/CD18	Neutrophils, monocytes	ICAM-1, FIB, CR for iC3b	Neutrophil aggregation and adhesion to ECs
$\alpha_X\beta_2$	P150,95 CD11c/CD18	Neutrophils, monocytes	CR for iC3b	Adhesion to ECs
$\alpha_D\beta_2$	CD11d/CD18	Eosinophils, monocytes, lymphocytes	VCAM-1, ICAM-3	Adhesion to leukocytes and to ECs
$\alpha_{IIb}\beta_3$	GPIIb/IIIa	Platelets	FIB, FN, vWF, VN, TSP	Platelet adhesion and aggregation
$\alpha_V\beta_3$	VN receptor	Platelets, ECs	FIB, FN, vWF, VN, TSP, collagens	Platelet adhesion, angiogenesis
$\alpha_4\beta_7$	LPAM-1	Lymphocytes	VCAM-1, MAdCAM-1, FN	Lymphocyte adhesion to ECs and ECM

CR, Complement receptor; *EC*, endothelial cell; *ECM*, extracellular matrix; *FIB*, fibrinogen; *FN*, fibronectin; *GP*, glycoprotein; *LFA-1*, leukocyte function-associated antigen; *LM*, laminin; *LPAM-1*, lymphocyte Peyer patch adhesion molecule; *MAdCAM-1*, mucosal addressin cell adhesion molecule-1; *TSP*, thrombospondin; *VCAM-1*, vascular cell adhesion molecule-1; *VLA*, very late-appearing antigen; *VN*, vitronectin; *vWF*, von Willebrand factor.

regulate integrin adhesive function. The application of tension to integrins can increase ligand binding and a reduction in tension lessens integrin adhesiveness. Cell–cell interactions result from integrin recognition of cell-surface members of the immunoglobulin superfamily. Binding of fibrinogen to $\alpha\text{IIb}\beta3$ integrins on adjacent platelets creates a molecular bridge that promotes platelet aggregation (Fig. 15.2).

IMMUNOGLOBULIN-LIKE RECEPTORS

Immunoglobulin superfamily members contain a variable number of disulfide-stabilized motifs similar to those in antibodies, which are linked to transmembrane and cytoplasmic domains (Table 15.2; see also Fig. 15.1). The immunoglobulin-like motif provides a framework on which specific recognition structures for other proteins can be added. Some of these motifs also recognize glycoconjugates. The immunoglobulin-like molecules, intercellular adhesion molecule 1 and 2 (ICAM-1 and ICAM-2), and vascular cell adhesion molecule 1 (VCAM-1), expressed on endothelial cells, as well as ICAM-3, expressed on leukocytes, mediate cell–cell contact through recognition of specific integrins on leukocytes. ICAM-4, expressed on erythroid precursors, binds to integrins on stromal cells of BM, which may regulate erythropoiesis. ICAM-5 is restricted to neural tissues. The immunoglobulin-like GPVI on platelets promotes cell activation by binding to collagen in the subendothelial matrix exposed on damaged blood vessels. Interactions between immunoglobulin-like molecules help to mediate adhesion between T cells and antigen-presenting cells. Thus, whereas the immunoglobulin-like molecules CD8 and CD4 on T cells bind to the conserved membrane-proximal domains of class I and class II major histocompatibility complex (MHC) proteins, respectively, the α- and β-chains of the T-cell receptor (TCR) bind to the polymorphic antigen-presenting domain. In addition, the immunoglobulin-like proteins CD2 and CD28 on T cells bind to the immunoglobulin-like protein leukocyte function-associated antigen-3 (LFA-3) and B7-1 (CD80) and B7-2 (CD86), respectively, on antigen-presenting cells. The immunoglobulin-like receptor, platelet, and endothelial cell adhesion molecule-1 (PECAM-1; CD31) uses homotypical interactions to promote contacts between adjacent endothelial cells and to mediate transmigration of leukocytes across vascular endothelium (Fig. 15.3). The immunoglobulin-like junctional adhesion molecules (JAMs), expressed on endothelial and epithelial cells and leukocytes, regulate endothelial and epithelial cell junctions, paracellular permeability, and leukocyte trafficking between endothelial and epithelial cells by homotypical interactions or by heterotypical interactions with integrins. JAM-A, the founding member of this family, functions as a homodimer and transmits intracellular signals critical for its function in the regulation of endothelial and epithelial permeability.

OTHER ADHESION RECEPTORS THAT MEDIATE PROTEIN–PROTEIN INTERACTIONS

Cadherins are cytoskeletal-linked type 1 transmembrane proteins that mediate calcium-dependent, cell-to-cell contact in many organs through homotypical binding to cadherins on adjacent cells (Table 15.3). Cadherins are found on endothelial cells, where they form adherens junctions and regulate permeability and leukocyte migration across endothelial cell-to-cell borders, termed *diapedesis* or *transendothelial migration*. Cadherins are also expressed in the epithelium and help form cell-to-cell junctions.

The GPIb–IX–V complex on platelets consists of leucine-rich protein subunits (see Figs. 15.1 and 15.2). Under conditions of high shear stress such as those found in arterial circulation, this complex promotes the initial platelet adhesion to injured vessels by binding to vWF exposed in the subendothelial matrix. It also assists interactions with other platelets or with endothelial cells by binding to P-selectin, which normally binds to glycoconjugates, and it may assist platelet adhesion to leukocytes by binding to the integrin $\alpha_m\beta_2$.

CD36 is a multifunctional receptor with at least two membrane-spanning domains that is expressed on many cell types. On platelets, it has been implicated as a receptor for collagen and for thrombospondin and as a receptor to activate NADPH oxidase and

TABLE 15.2 **Immunoglobulin-Like Receptors**

Name	Other Name	Expressed by	Ligand	Function(s)
ICAM-1		Macrophages, EC, other cells	$\alpha_M\beta_2$, $\alpha_L\beta_2$, FIB	T-cell responses, leukocyte adhesion to EC
ICAM-2		EC	$\alpha_L\beta_2$	Leukocyte adhesion to EC
ICAM-3		Leukocytes	$\alpha_L\beta_2$	T-cell responses, leukocyte aggregation
ICAM-4		Erythroid precursors	$\alpha_4\beta_1$, $\alpha_V\beta_3$, $\alpha_{IIb}\beta_3$	Regulate erythropoiesis
ICAM-5	Telencephalin	Telencephalic neurons	ICAM-5, $\alpha L\beta 2$, $\alpha 5\beta 1$	Neuron-neuron and neuron-leukocyte
GPVI		Platelets	Collagen	Platelet adhesion and activation
PECAM-1	CD31	Leukocytes, platelets, EC	PECAM-1	EC junctions, leukocyte transmigration, cell signaling
VCAM-1		Activated EC, smooth muscle cells	$\alpha_4\beta_1$, $\alpha_4\beta_7$	Mononuclear cell adhesion to EC
MAdCAM-1		EC of Peyer patches	$\alpha_4\beta_7$	Lymphocyte homing
Siglecs		Leukocyte subsets	Sialylated glycans	Regulates B-cell activation, innate immunity?, hematopoiesis?
JAMs		EC	JAMs, $\alpha_L\beta_2$, $\alpha_4\beta_1$	EC junctions, leukocyte transmigration
CD2		T cells	LFA-3[a]	T-cell responses
CD4		T cells	Class II MHC[a]	T-cell responses
CD8		T cells	Class I MHC[a]	T-cell responses
CD3	T-cell receptor	T cells	Antigen on MHC[a]	T-cell responses
CD28	Costimulatory molecule	T cells	CD80, CD86	T-cell responses

[a] LFA-3 and classes I and II MHC molecules are also immunoglobulin-like receptors.

ICAM-1, -2, -3, -4, Intercellular adhesion molecules; *JAM,* junctional adhesion molecule; *MHC,* major histocompatibility complex; *PECAM-1,* platelet and endothelial cell adhesion molecules-1. For other abbreviations, see Table 15.1 footnotes.

TABLE 15.3 Other Adhesion Receptors

Name	Other Name	Expressed by	Ligand	Function(s)
Cadherins		EC, many other cells	Homotypic binding	Formation of EC junctions
GPIb/IX/V		Platelets	vWF	Platelet adhesion to ECM under shear
CD36	GPIV	Platelets, many other cells	Collagens, TSP	Platelet adhesion to ECM
CD44		Leukocytes, other cells	Hyaluronan, serglycin	Lymphopoiesis, lymphocyte activation
DC-SIGN		Dendritic cells	Mannosylated glycans, other glycans	Regulate T-cell–dendritic cell interactions, recognize pathogens
NK cell receptors		NK cells	MHC molecules	Recognition of virus-infected or other foreign cells

DC-SIGN, Dendritic cell-specific ICAM-3 grabbing nonintegrin; *MHC*, major histocompatibility complex; *NK*, natural killer. For other abbreviations, see Table 15.1 footnotes.

TABLE 15.4 Selectins

Name	Other Name (s)	Expressed by	Ligand	Ligands Expressed by	Function(s)
P-selectin	CD62P GMP-140 PADGEM	Thrombin-activated platelets and ECs, cytokine-activated ECs	PSGL-1, GPIbα	Leukocytes, platelets	Leukocyte adhesion to activated ECs and platelets
E-selectin	CD62E ELAM-1	Cytokine-activated ECs	PSGL-1, other sialylated and fucosylated GPs	Leukocytes	Leukocyte adhesion to activated ECs
L-selectin	CD62L LECAM-1 LAM-1	Leukocytes	PSGL-1, also GlyCAM-1, CD34, and other mucins on ECs of lymph nodes	Leukocytes, ECs or lymph nodes	Leukocyte adhesion to other leukocytes; lymphocyte homing to lymph nodes

The selectins bind to sialylated, fucosylated, and (in some cases) sulfated oligosaccharides on specific glycoproteins, of which only some have been identified. *EC*, Endothelial cell; *ELAM-1*, endothelial leukocyte adhesion molecule-1; *Gly-CAM-1*, glycosylation-dependent cell adhesion molecule-1; *GMP-140*, granule membrane protein-140; *LAM-1*, leukocyte adhesion molecule-1; *LECAM-1*, leukocyte endothelial cell adhesion molecule-1; *PADGEM*, platelet activation-dependent granule external membrane protein; *PSGL-1*, P-selectin glycoprotein ligand-1. For other abbreviations, see Table 15.1 footnotes.

generate reactive oxygen species (ROS); both processes could facilitate adhesion and activation at sites of hemorrhage.

LECTIN ADHESION RECEPTORS

CD44 is an unusual transmembrane GP expressed to variable degrees on many subsets of leukocytes (see Fig. 15.1). It has a membrane-distal domain that is structurally related to the link protein of the ECM, and similar to link protein, CD44 can bind to hyaluronan. The hyaluronan-binding function of CD44 may modulate a number of leukocyte responses. The most clearly demonstrated function is in lymphopoiesis, where maturation of lymphocyte precursors requires contact with BM stromal cells bearing surface hyaluronan. CD44–hyaluronate interactions also may promote lymphocyte entry to and transit through organized lymphoid tissues. The membrane-proximal regions of CD44 are structurally diverse because of the insertion of variable numbers of domains through alternative splicing. These insertions may regulate the ability of CD44 to bind hyaluronan and may mediate post-binding events that affect cell signaling, proliferation, differentiation, and motility.

The selectins are a group of three receptors that terminate in a membrane-distal carbohydrate-recognition domain related to those in Ca^{2+}-dependent (C-type) animal lectins such as the hepatic asialoglycoprotein receptor (see Fig. 15.1). L-selectin is expressed on leukocytes, E-selectin on cytokine-activated endothelium, and P-selectin on macrophages, platelets, and endothelial cells exposed to secretagogues such as thrombin or histamine (Table 15.4). The selectins mediate leukocyte adhesion to platelets, endothelium, or other leukocytes through Ca^{2+}-dependent interactions of the carbohydrate-recognition domains with cell-surface carbohydrates on opposing cells (see Fig. 15.3). High-affinity binding appears to require specific carbohydrate structures

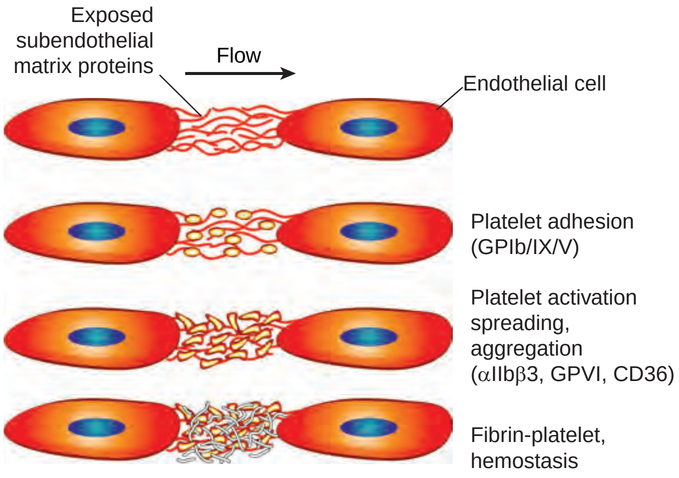

Exposed subendothelial matrix proteins
Flow
Endothelial cell
Platelet adhesion (GPIb/IX/V)
Platelet activation spreading, aggregation (αIIbβ3, GPVI, CD36)
Fibrin-platelet, hemostasis

Figure 15.2 PLATELET ADHESION, AGGREGATION, AND HEMOSTASIS. In response to arterial injury under high shear forces, platelets rapidly adhere to the subendothelial matrix of injured vessels. The initial contacts are made between GPIb–IX–V on platelets and von Willebrand factor (vWF) in the matrix. These molecular interactions help activate platelets, thereby increasing the affinity of several platelet integrins for other adhesive matrix proteins such as fibronectin, laminin, and collagens. GPVI further activates platelets by binding to collagen. CD36 also interacts with both collagen and thrombospondin. Fibrinogen cross-links activated platelets into aggregates by binding to $\alpha_{IIb}\beta_3$ integrins. The platelet plug then serves as an efficient surface for the generation of thrombin and fibrin. *GP*, Glycoprotein.

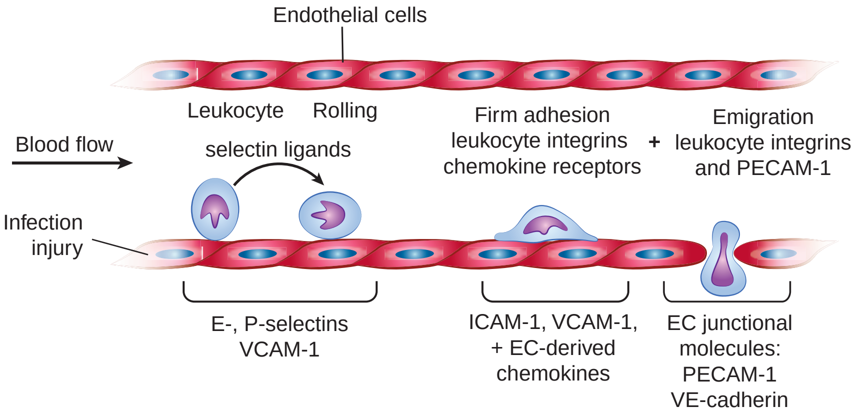


Figure 15.3 LEUKOCYTE ROLLING, SPREADING, AND EMIGRATION. At sites of tissue injury or infection, the vascular endothelium becomes hyperadhesive for leukocytes through activation by inflammatory cytokines tumor necrosis factor-α (TNF-α) or interleukin-1β (IL-1β), or by endotoxins (LPS). Leukocytes first roll on the activated endothelium in postcapillary venules. These transient adhesive interactions are mediated by TNF or LPS activation-induced transcription-dependent expression of E- or P-selectin and VCAM-1 on the endothelial cell surface. E- and P-selectin bind to glycosylated selectin ligands expressed on leukocytes and VCAM-1 binds VLA-4 integrins expressed by certain leukocytes. These molecular bonds can form under the shear forces in the venular circulation. The rolling leukocytes become activated by locally generated inflammatory mediators presented by endothelial cells that increase the affinity of β_2 integrins for intercellular adhesion molecule-1 (ICAM-1) on the endothelium. These bonds slow rolling and, in combination with endothelial-expressed chemokines, trigger firm adhesion to the endothelium. Leukocytes migrate to endothelial lateral borders and then through cell-cell borders by engaging adhesion molecules PECAM-1, CD99, CD47, ICAM-1, and others into tissues. *PECAM-1*, Platelet and endothelial cell adhesion molecule-1, CD31; *VCAM-1*, vascular cell adhesion molecule-1.

displayed on a limited number of membrane GPs. The best-characterized GP ligands for selectins are mucins, which have large numbers of clustered, sialylated *O*-linked oligosaccharides. Site-specific construction of *O*-glycans with specific sialylated, fucosylated, and (in some cases) sulfated moieties is required for these mucins to bind optimally to selectins. In the case of one mucin, P-selectin GP ligand-1 (PSGL-1), sulfation of tyrosine residues near a specific O-glycan is required for binding to P- and L-selectin.

Dendritic cells and related macrophages express a novel group of C-type lectins, of which the best characterized is dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN). DC-SIGN binds to particular oligosaccharides on ICAMs, thereby regulating T-cell and dendritic cell function during antigen presentation. It also binds to glycans on a variety of pathogens, which may have critical roles in innate immunity. Natural killer cells express a different group of proteins, with some containing membrane-distal C-type lectin-like domains (e.g., NKG2D). Although these receptors are important for interactions of natural killer cells with target cells, they may bind to proteins rather than to glycoconjugates.

Siglecs are a subgroup of membrane proteins of the immunoglobulin superfamily that bind to carbohydrates instead of to proteins (see Table 15.2). The first two amino-terminal (*N*-terminal) domains appear to be necessary and sufficient for carbohydrate recognition. The *N*-terminal domain is a V-type structure that includes an unusual disulfide bond that is not found in the more common C-type immunoglobulin domains. Siglecs bind well to sialylated glycans on some but not all GPs. Different siglecs preferentially recognize sialic acid that is linked α2,6-, α2,8-, or α2,3- to an underlying galactose residue. Most siglecs have immune receptor tyrosine-based inhibitory motifs and transmit inhibitory signals. Siglecs can form *cis* interactions with other GPs on the same cell or *trans* interactions with GPs on another cell. The best-characterized example is CD22, which negatively regulates B-cell activation when it engages sialylated GPs. Sialoadhesin, expressed on BM macrophages, may regulate hematopoietic cell differentiation.

LIGAND BINDING VERSUS CELL ADHESION

As with all noncovalent macromolecular interactions, adhesion molecules bind to each other with equilibrium affinities defined by association and dissociation rates. However, the efficiency of cell adhesion is not simply a function of the solution-phase equilibrium affinities of adhesion molecules for one another. Adhesion molecules in cell membranes and matrices are limited primarily to two dimensions, and even low-affinity molecular interactions stabilize adhesion if there is time for sufficient bonds to form, or to cluster together along the plane of cell contact. The efficiency of cell attachment and the ensuing strength of adhesion reflect multiple factors that dictate the probability of formation of bonds between adhesion molecules on cell or matrix surfaces. The kinetics of bond formation and dissociation are especially important for certain kinds of cell adhesion. Furthermore, interactions between cell adhesion molecules are subjected to force, affecting the lifetimes of adhesive bonds. This is particularly true in circulation, where platelets and leukocytes must rapidly adhere to the blood vessel wall and withstand forces applied by the wall shear stresses of flowing blood. Other factors that affect bond formation include the number of adhesion molecules on a cell or matrix surface, the distance the binding domain of an adhesion receptor protrudes from the cell membrane, the lateral mobility of receptors, receptor dimerization, and the clustering of receptors on microvilli or other membrane domains. Cell adhesion can be further stabilized by events that occur after the initial interactions of adhesion molecules. For example, the cytoplasmic domains of many adhesion molecules bind to cytoskeletal components, allowing clustering of receptors into surface patches that strengthen adhesion, thereby promoting cell spreading or migration.

REGULATION OF ADHESION RECEPTORS

To prevent inappropriate interactions of cells with each other or with the ECM, the expression and function of adhesion receptors must be tightly controlled. Three primary control mechanisms are used: (1) the rate of synthesis of the receptor, (2) the time during which the receptor is displayed on the cell surface, and (3) the binding affinity or avidity of the receptor for ligands (Table 15.5).

REGULATION OF SYNTHESIS

The synthesis of many adhesion receptors is regulated. Erythroid precursors synthesize integrins that mediate their interactions with

TABLE 15.5 **Regulation of Adhesion Receptors**

Mechanism	Example
Synthesis	Erythroid precursor synthesis of $\alpha_5\beta_1$
	Lymphocyte synthesis of CD44
	Cytokine-induced synthesis of E-selectin, P-selectin, ICAM-1, and VCAM-1 by endothelial cells
Surface expression	Proteolytic cleavage of L-selectin from leukocytes
	Redistribution of P-selectin from granule membranes to plasma membrane of platelets and endothelial cells
	Endocytosis of P- and E-selectin on endothelial cells
Ligand affinity	Activation-induced increased affinity of many integrins for their ligands
	Activation-induced increased affinity of CD44 for hyaluronan

For abbreviations, see Table 15.1 footnotes.

stromal cells and with ECM in the BM. As the precursors mature, synthesis ceases, resulting in loss of expression of cell-surface integrins by the time a mature erythrocyte enters the circulation. Lymphocyte precursors synthesize CD44 during differentiation in the BM, stop synthesis before release, and resume synthesis during maturation in the thymus. On exposure to antigens, immunologically naïve lymphocytes synthesize increased amounts of several adhesion receptors and chemokine receptors during their conversion to the effector phenotypes; this process presumably allows these cells to become more adhesive in response to a subsequent antigenic challenge. Endothelial cells in postcapillary venules of the peripheral vasculature express very low, if any, levels of adhesion molecules that bind leukocytes. When exposed to inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β(IL-1β) or bacterial endotoxins, endothelial cells transiently increase the synthesis of E- and P-selectin, ICAM-1, VCAM-1, and chemoattractants, resulting in a surface for leukocytes to adhere (see Fig. 15.3).

REGULATION OF SURFACE EXPRESSION

The surface expression of some adhesion receptors is tightly controlled. L-selectin is present on the plasma membrane of leukocytes, where it is available to bind to ligands on the endothelial cell surface. Stimulation of the leukocyte causes L-selectin to be shed into the plasma by proteolytic cleavage. P-selectin is constitutively synthesized by megakaryocytes (where it is incorporated into platelets) and by endothelial cells. Rather than being directly delivered to the plasma membrane, it is sorted into secretory storage granules: the α granules of platelets and the Weibel–Palade bodies of endothelial cells. On stimulation of these cells by agonists such as thrombin, P-selectin is rapidly transported to the cell surface during the fusion of granule membranes with the plasma membrane. When they are on the surface of the endothelium, both E-selectin and P-selectin are internalized and delivered to lysosomes for degradation. The net result of these events is to control the duration of exposure of E- and P-selectin on the endothelium, where they can mediate the adhesion of leukocytes. Activation of leukocytes also mobilizes a pool of $\alpha M\beta_2$ integrins from storage compartments to the plasma membrane, although some of these molecules are also constitutively expressed on the cell surface. Finally, platelet activation redistributes a portion of the GPIb–IX–V complexes from ligand-accessible positions on the plasma membrane to sequestered, invaginated membrane domains known as the *surface-connected canalicular system*. This process requires interactions of the cytoplasmic domain of GPIb–IX–V with the cytoskeleton and may serve to downregulate GPIb-mediated adhesion of platelets to immobilized vWF.

REGULATION OF BINDING AFFINITY

Regulation of binding affinity is an important control mechanism for other adhesion receptors. Many integrins are constitutively present on the cell surface but interact poorly with their ligands. Cell activation by a number of agonists induces conformational changes in integrins so that they effectively recognize their ligands. An example is the $\alpha_{IIb}\beta_3$ integrin, which requires platelet stimulation to bind fibrinogen; if this binding affinity were not regulated, circulating platelets would indiscriminately aggregate in the fibrinogen-rich plasma milieu. The cytoplasmic domains of integrins can exert both positive and negative influences on binding affinity. Binding of specific cytoplasmic proteins to these domains may propagate structural changes to the extracellular ligand-binding regions of the integrins. Three-dimensional structures of integrins suggest that the integrin "headpiece" that contains the ligand-binding site faces down toward the membrane in the inactive conformation and rapidly extends upward in a "switchblade"-like opening motion on activation. Low-affinity ligand binding may stabilize some active conformations of integrins, perhaps explaining why integrins on unactivated cells will sometimes bind to immobilized, multivalent adhesive proteins but not to the same proteins in solution. Cellular activation may also regulate the binding avidities of CD44, L-selectin, P-selectin, and some integrins through changes in membrane distribution engineered by interactions of their cytoplasmic domains with the cytoskeleton or with clathrin-coated pits.

CELL SIGNALING THROUGH ADHESION MOLECULES

In addition to their roles in cell–cell and cell–matrix contacts, adhesion molecules initiate cell signaling through indirect or direct mechanisms. Proteoglycans in the ECM can sequester growth factors that can be released to bind to surface receptors on nearby cells. Some chemoattractants, called chemokines, bind to proteoglycans on the surface of endothelial cells, where they can bind to G protein-coupled receptors (GPCRs) that activate rolling leukocytes (see Fig. 15.3). Binding of adhesive ligands to cell-surface integrins, GPIb–IX–V, CD44, cadherins, CD36, PECAM-1, selectins, ICAM-1, and VCAM-1, and perhaps other receptors can directly trigger intracellular events. The consequences of such signaling include changes in affinity or avidity of other adhesion receptors for their ligands, shape change, secretion, proliferation, synthesis of cytokines and other molecules, and migration. Most commonly, signaling requires cross-linking of several receptors through interactions with multivalent ligands in matrix or on opposing cells.

Many studies of adhesion receptor signaling have focused on integrins. Binding of the same ligand to different integrins can mediate different responses in the same cell. Furthermore, ligand binding to the same integrin expressed in different cells can result in different signals. These data suggest that very specific interactions occur between ligand-occupied integrins and intracellular components. The cytoplasmic domains of integrins are essential for initiating signaling. Tyrosine kinases have been localized at the interaction zones between integrins, the cytoskeleton and several adaptor and effector molecules, and tyrosine phosphorylation of a number of proteins accompanies integrin-mediated cell signaling. Tyrosine phosphorylation initiates a cascade of signaling events, including the activation of serine/threonine kinases, which cause a variety of cellular responses. Ligand binding to integrins also results in the generation of lipid second messengers, alkalization of the cytoplasm, and influxes of Ca^{2+}.

COOPERATIVE INTERACTIONS BETWEEN SIGNALING AND ADHESION MOLECULES

Signaling and adhesion molecules frequently function cooperatively in sequential cascades to enhance the specificity of cell adhesion. Three examples of how these cooperative interactions facilitate blood cell responses are described next.

Platelet Adhesion, Aggregation, and Hemostasis

At sites of blood vessel injury in the arterial circuit, platelets rapidly tether to and roll along the damaged vessel through reversible interactions of GPIb–IX–V receptors with immobilized vWF exposed in the subendothelial matrix of injured vessels (see Fig. 15.2). These interactions are facilitated by arterial flow and enabled by bond formation between GPIb and matrix vWF. An important feature of this initial reversible adhesive event is that prior activation of the platelets is not required. After adhesion, however, the interaction of immobilized vWF with GPIb receptors triggers intracellular signals that synergize with those produced by the engagement of collagen by its receptor GPVI. Platelet activation, in turn, further increases the affinity of platelet integrins for collagen and fibronectin in the exposed matrix to stabilize adhesion. Binding of these ligands transduces signals that propagate platelet spreading and secretion of granule contents that enhance the recruitment of additional platelets and leukocytes through cell–cell contact mediated by binding of fibrinogen to activated platelet $\alpha_{IIb}\beta_3$ and leukocyte αMβ2 integrins. This adhesion cascade allows unstimulated platelets and leukocytes to specifically recognize sites of vascular injury and then be activated by locally generated mediators.

Leukocyte Rolling, Spreading, and Migration Across Vascular Endothelium During Inflammation

Near sites of extravascular bacterial infections and injury, leukocytes first tether to and roll on the endothelial surface of postcapillary venules through the interactions of selectins with cell-surface carbohydrate ligands (see Fig. 15.3). Neutrophils are generally among the first leukocytes to exit the blood stream, are the most abundant in blood, and are the best studied in the different steps of the extravasation cascade. Little, if any, leukocyte adhesion occurs in nearby arterioles, except in specific disease conditions of the arterial vascular wall such as atherosclerosis. Leukocyte rolling on the endothelium occurs under shear forces, just as platelets adhere to subendothelial matrix under shear forces, although the shear flow in postcapillary venules is lower than that in arterioles. Rolling requires a balance between the formation of selectin–ligand bonds at the leading edge of the cell and the dissociation of bonds at the trailing edge of the cell. Whereas shear forces affect the lifetimes of selectin–ligand bonds, lower forces prolong lifetimes (catch bonds) and higher forces shorten lifetimes (slip bonds). Catch bonds help explain why a minimum shear force is required to support leukocyte rolling, particularly through L-selectin. Just as the initial adhesion to vWF does not require prior activation of platelets, selectin-mediated rolling does not require prior activation of leukocytes. Instead, locally generated inflammatory mediators induce expression of E- or P-selectin on the endothelial cell surface. The requirement for activation of endothelial cells rather than leukocytes allows the latter to adhere to vessels only at the site of vessel inflammation. Several infectious and sterile soluble mediators, such as endotoxins or pro-inflammatory cytokines, are involved in this critical step of endothelial cell activation that converts the endothelial anti-adhesive surface into a pro-adhesive surface, which expresses selectins and integrin ligands that interact with leukocyte selectin ligands and integrins. After being situated on the vessel wall through selectin-mediated contacts, however, the neutrophils become exposed to stimuli including a platelet-activating factor, a phospholipid signaling molecule, activated complement proteins, and chemokines like interleukin-8 (IL-8), which are presented on the surface of activated endothelial cells. These signals cooperate with others directed by engagement of selectin ligands and promote very slow rolling of neutrophils on the surface of activated endothelium. Neutrophil activation increases the affinity of β_2 integrins for immunoglobulin counterreceptors on the endothelial cell surface such as ICAM-1. Although flowing cells cannot form these bonds, leukocytes rolling on selectins can do so because of their slower velocities. The integrin–ICAM interactions further slow rolling and then arrest the cells near endothelial cell-to-cell junctions. The leukocytes then migrate, presumably because of disengagement of integrin–ICAM bonds and redistribution of integrins to the leading edge of the cell, where new bonds form. Interactions of leukocytes with endothelial cell junctional molecules such as PECAM-1, CD47, and other molecules facilitate transendothelial migration of the leukocytes into the underlying tissues. Adhesion of leukocytes to the endothelium disrupts cytoskeletal tethers to the endothelial cadherins; this disruption leads to dissociation of homotypical cadherin interactions that normally prevent the passage of leukocytes. Intracellular calcium in endothelial cells also contributes to leukocyte transendothelial migration during the integrin- and the PECAM-1–mediated adhesive events. These signals contribute to cytoskeletal redistributions in leukocytes and endothelial cells, which enhance leukocyte migr ation toward chemotactic molecules released in the vicinity of the infected or injured area. All of these steps are commonly shared by all leukocytes, although differences in responsiveness to certain chemokines and in expression/activation of adhesion molecules to mediate interactions with EC have been described. When leukocytes enter the tissues, integrin recognition of ECM protein ligands and inflammatory mediators, such as damage-associated molecular pattern molecules (DAMPs) and pathogen-associate pattern molecules (PAMPs), trigger secretion of proteolytic enzymes and production of ROS, cytokines, interferons, and chemokines that optimize bactericidal function.

Adhesion of T Cells to Antigen-Presenting Cells during Antigen Priming

The initial engagement of naïve T cells with antigen-presenting cells (APCs) requires that the TCR (αβ TCR) recognize antigen presented by the polymorphic domain of MHC molecules (Fig. 15.4). Subsequent interactions include the binding of CD8 or CD4 to MHC class I or II molecules, respectively. These molecular contacts are all of low affinity but are highly specific because they first require specific antigen presentation to the appropriate TCR. The combination of these binding events triggers inside-out signals that increase the affinity of LFA-1 ($\alpha_L\beta_2$), a β_2 integrin on T cells, for its ligand ICAM-1 on APCs, strengthening and prolonging the length of time cells stably adhere. During this time, the T cell is further activated by a second or costimulatory signal delivered by T-cell CD28 binding to CD80 and CD86 expressed by APCs. Together these interactions drive TCR-induced gene activation (interferon, chemokine, and cytokine production) and cell proliferation and differentiation into effector T cells that exit the secondary lymph node and migrate to immune reactions (see Chapter 24 for T cell-antigen priming).

The first principle of these three responses is that the initial adhesive event, although relatively limited, is highly specific. Thus, platelets bind to an exposed subendothelial matrix in injured vessels, neutrophils bind to activated endothelium at sites of infection, and T cells bind to antigen-loaded APC in secondary lymph nodes. The second principle is that subsequent activation events strengthen cell adhesion and lead to further responses such as secretion, fibrin formation, cellular migration, and release of mediators for cell activation and proliferation. Thus, adhesion and cell signaling are highly interrelated processes.

The process of reversing cell adhesion, although less well understood, is equally important for the control of cell behavior. Some molecules such as the selectins can be proteolytically cleaved or internalized. The activation-induced increases in the affinity of integrins

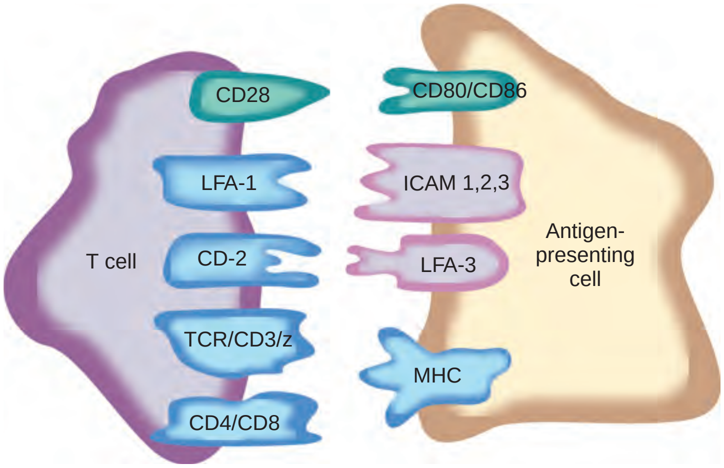


Figure 15.4 ADHESION BETWEEN T LYMPHOCYTES AND ANTIGEN-PRESENTING CELLS. The initial contact is mediated by the T-cell receptor (TCR) complex, composed of the TCR non-covalently linked to CD3 and ζ (z) proteins, which binds with low affinity but high specificity to a specific antigen peptide presented by an MHC molecule. Additional contacts, also of low affinity, are between CD4 (on helper cells) or CD8 (on cytotoxic cells) and MHC, between costimulatory molecules CD2 and LFA-3, and between CD28 with both CD80 (B7-1) and CD86 (B7-2). These interactions signal the T cell to transiently increase the affinity of its β_2 integrin LFA-1 for ICAM-1 on the antigen-presenting cell. These bonds strengthen adhesion, and the costimulatory molecules transduce sustained and additional signals to the T cell that causes increased gene transcription, proliferation, and cytokine secretion and produce effector and regulatory T cells. Not shown is the redistribution of these adhesion molecules into different regions of the contact zone as adhesion strengthens. *ICAM*, Intercellular adhesion molecule; *LFA-3*, leukocyte function-associated antigen-3; *MHC*, major histocompatibility complex.

TABLE 15.6 **Genetic Deficiencies in Adhesion Molecules**

Molecule	Disease	Laboratory Finding(s)	Clinical Finding(s)
$\alpha_{IIb}\beta_3$	Glanzmann thrombasthenia	Impaired platelet aggregation	Mucocutaneous bleeding
GPIb–IX–V	Bernard–Soulier syndrome	Impaired platelet adhesion to vWF	Mucocutaneous bleeding
β2 integrins	Leukocyte adhesion deficiency-1	Impaired adhesion of activated leukocytes to EC	Frequent infections
Selectin ligands	Leukocyte adhesion deficiency-2	Impaired fucose metabolism resulting in defective carbohydrate ligands for selectins, impaired rolling of leukocytes on venules	Frequent infections
β1, β2, β3 integrins	Leukocyte adhesion deficiency-3	Impaired platelet adhesion and aggregation and impaired adhesion of activated leukocytes to EC	Mucocutaneous bleeding and frequent infections

For abbreviations, see Table 15.1 footnotes.

and CD44 for their ligands are generally transient, but the mechanisms for return to the inactive conformation are obscure.

Genetic Deficiencies in Adhesion Molecules

Genetic deficiencies in platelet adhesion receptors such as the GPIb complex (as in Bernard–Soulier syndrome) and the $\alpha_{IIb}\beta_3$ integrin (as in Glanzmann thrombasthenia) result in hemorrhagic symptoms similar to those in patients with thrombocytopenia (Table 15.6). Genetic deficiencies in the leukocyte β_2 integrins (as in leukocyte adhesion deficiency-1, LAD-1) are associated with frequent severe bacterial infections and a failure of neutrophils to enter the infected tissues. Similar symptoms are seen in patients with a congenital defect in fucose metabolism that prevents the synthesis of the carbohydrate ligands for selectins (LAD-2). A recently identified set of patients has both hemorrhagic symptoms and life-threatening infections (LAD-3). The molecular mechanism is attributable to mutations in an intracellular protein kindlin-3, which binds to β1, β2, and β3 integrin cytoplasmic tails upon platelet and leukocyte activation. These patients have normal levels of integrin surface expression (see Table 15.6).

DYSREGULATED EXPRESSION OF ADHESION MOLECULES

Inappropriate expression of adhesion molecules has been implicated in thrombotic and inflammatory disorders and in tumor metastasis. For example, erythrocytes from patients with sickle cell anemia adhere to each other, to leukocytes, and to the endothelium, contributing to vasoocclusive crises. These adhesive events may reflect, in part, the expression of integrins and selectin ligands not normally found on mature erythrocytes. Inappropriate adhesion and activation of platelets on exposed atherosclerotic plaques may contribute to thrombosis and acute ischemic coronary artery syndromes. Dysregulated expression of selectins on the endothelium of ischemic blood vessels during myocardial infarction or shock may contribute to neutrophil-mediated tissue necrosis after reperfusion of the vessel. Mediators released while the neutrophils are adherent in the reperfused vessels may activate integrin function, strengthening adhesion and generating further signals that release destructive oxygen radicals and proteases within the vasculature. Finally, malignant cells appear to use molecules normally used for the adhesion of blood cells to promote metastatic spread through interactions with platelets, endothelial cells, and extravascular matrix.

These examples underscore the importance of proper regulation of adhesion molecule expression in the physiology of blood cells and their interactions with the vascular endothelium.

SUGGESTED READINGS

Alcaide P, Auerbach S, Luscinskas FW. Neutrophil recruitment under shear flow: it's all about endothelial cell rings and gaps. *Microcirculation*. 2009;16:43 Erratum in: *Microcirculation* 16:782, 2009.

Cambi A, Koopman M, Figdor CG. How C-type lectins detect pathogens. *Cell Microbiol*. 2005;7:481.

Cao H, Crocker PR. Evolution of CC33-related siglecs: regulating host immune functions and escaping pathogen exploitation? *Immunology*. 2010;132:18.

Coenen DM, Mastenbroek TG, Judith MEM, Cosemans JMEM. Platelet interaction with activated endothelium: mechanistic insights from microfluidics. *Blood*. 2017;130:2819.

Dalal PJ, William A, Muller WA, Sullivan DP. Endothelial cell calcium signaling during barrier function and inflammation. *Am J Pathol*. 2020;190:535.

Dinauer MC. Disorders of neutrophil function: an overview. *Methods Mol Biol*. 2014;1124:501–515.

Hogg N, Patzak I, Willenbrock F. The insider's guide to leukocyte integrin signalling and function. *Nat Rev Immunol*. 2011;11:416.

Hynes RO. Integrins: Bidirectional, allosteric signaling machines. *Cell*. 2002;110:673.

Kfoury Y, Scadden DT. Mesenchymal cell contributions to the stem cell niche. *Cell Stem Cell*. 2015;16:239.

Kumari S, Mak M, Poh YC, et al. Cytoskeletal tension actively sustains the migratory T-cell synaptic contact. *EMBO J*. 2020;39:e102783.

McEver RP, Zhu C. Rolling cell adhesion. *Annu Rev Cell Dev Biol*. 2010;26:363.

Mendelson A, Frenette PS. Hematopoietic stem cell niche maintenance during homeostasis and regeneration. *Nat Med*. 2014;20:833.

Ponta H, Sherman L, Herrlich PA. CD44: From adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol*. 2003;4:33.

Ruggeri ZM. Platelet adhesion under flow. *Microcirculation*. 2009;16:58.

Shattil SJ, Kim C, Ginsberg MH. The final steps of integrin activation: the end game. *Nat Rev Mol Cell Biol*. 2010;11:288.

Sperandio M, Gleissner CA, Ley K. Glycosylation in immune cell trafficking. *Immunol Rev*. 2009;230:97.

Springer TA, Wang JH. The three-dimensional structure of integrins and their ligands, and conformational regulation of cell adhesion. *Adv Protein Chem*. 2004;68:29.

Vestweber D. How leukocytes cross the vascular endothelium. *Nat Rev Immunol*. 2015;15:692–704.

Wang H, Lim D, Rudd CE. Immunopathologies linked to integrin signalling. *Semin Immunopathol*. 2010;32:173.

CHAPTER 16

CURRENT BIOLOGY OF STEM CELL HOMING AND MOBILIZATION: DYNAMIC INTERACTIONS BETWEEN HEMATOPOIETIC STEM AND PROGENITOR CELLS AND THEIR SURROUNDING BONE MARROW MICROENVIRONMENT

Orit Kollet, Montaser Haddad, Priyasmita Chakrabarti, Alejandra Ordonez-Moreno, and Tsvee Lapidot

INTRODUCTION

Hallmarks of hematopoietic stem and progenitor cell (HSPC) function include their dynamic metabolism, active bi-directional migration (bone marrow [BM] homing, egress, recruitment, and mobilization to the blood), durable multilineage BM and blood repopulation potential, self-renewal and chemotherapy resistance. Hematopoietic stem cells (HSCs) mainly reside in the BM, and their chemotherapy resistance requires their quiescence, adhesion, and metabolic interactions with bone-forming stromal cells. However, HSPCs are also actively released daily to the circulation at low levels as part of steady-state homeostasis and blood replenishment. This minute egress is profoundly accelerated via metabolic elevations of reactive oxygen species (ROS) levels leading to enhanced HSPC development, mobilization, and recruitment to the circulation during stress-induced hematopoiesis (e.g., blood loss and infections) as part of host defense and repair mechanisms. BM reconstitution requiring HSPC migration in the opposite direction, from the circulation across the blood-BM endothelial barrier into the BM and their lodgment in their stromal niches, also requires metabolically active directional navigation, a process termed homing. The ability of intravenously transplanted HSPCs to actively home to the BM is the first and essential step for successful engraftment and durable repopulation, following clinical BM transplantation. In recent years, several key players and mechanisms controlling HSPC function and dynamic regulation of the BM microenvironment, including the central role of the nervous, immune, coagulation, purinergic, and complement systems, have been revealed. In functional preclinical experimental mice models, during steady-state daily-light onset induces transient elevations of BM norepinephrine (NE) and tumor necrosis factor (TNF), which further lead to a transient rise in BM HSPC ROS levels. These dynamic changes metabolically program BM HSPC proliferation, differentiation, migration, and egress to replenish the blood with new mature cells, including the active daily release of immature HSPCs. Darkness onset induces lower transient elevations of BM NE and TNF, leading to a lower burst of BM HSPC ROS levels. These transient elevations at night activate local BM production and secretion of the darkness hormone melatonin, which together with other factors metabolically reprogram BM HSPC self-renewal and retention accompanied by increased short and long-term repopulation potential.

Stress signals caused by bleeding, injury, as well as by viral and bacterial infections induce massive proliferation and differentiation of BM HSPCs, accompanied by accelerated progenitor and mature cell recruitment to the circulation as part of host defense and repair. This process is mimicked by clinical mobilization protocols, which transiently expand the circulating HSPC pool, allowing the efficient harvest of stem and progenitor cells for clinical BM transplantation. Importantly, clinical mobilization of patients for autologous transplantation is often associated with poor yields of circulating HSPC due to the advanced disease state, heavy toxicity of clinical pretreatments, and old patient age. Both HSPC homing and mobilization involve metabolically active multi-step interactions with the BM microenvironment, including the adhesion machinery, proteolytic enzymes, cytokines, and chemokines. A dominant mechanism involves the interactions between the key BM stromal chemokine CXCL12 (also termed SDF-1) and its major receptor CXCR4, functionally expressed also by HSPCs. These interactions regulate HSPC motility (both homing and mobilization), proliferation, adhesion, BM retention, quiescence, and chemotherapy resistance. In addition to CXCL12, BM stromal and endothelial cells also functionally express CXCR4, and many mobilizing agents induce BM CXCL12 secretion to the blood, creating a gradient leading to HSPC mobilization. For clinical requirements, HSPC mobilization is induced by a variety of agents. Among them are repeated stimulation with the myeloid cytokine granulocyte colony-stimulating factor (G-CSF), the CXCR4 antagonist AMD3100 (also termed Plerixafor), and DNA damaging chemotherapy drugs such as cyclophosphamide and total-body radiation, which all induce BM CXCL12 secretion. HSPC mobilization involves in addition to the CXCL12/CXCR4 axis among others, also the stem cell factor (SCF)/c-Kit axis, the pro-inflammatory factors NE, TNF, sphingosine 1 phosphate (S1P), hepatocyte growth factor (HGF), and their receptors, complement, thrombin, and proteinase activated receptor one (PAR1).

This chapter discusses recent findings concerning the biology of HSPC homing and release during steady-state and their mobilization and recruitment during stress-induced conditions. This chapter emphasizes major signals controlling HSPC homing, BM retention, chemotherapy resistance, and clinical mobilization with a major focus on the CXCL12/CXCR4 axis.

LESSONS FROM STEADY-STATE HOMEOSTATIC REGULATION OF HEMATOPOIETIC STEM AND PROGENITOR CELLS

Daily Light- and Darkness-Onset Induced Signals Maintain Blood Replenishment Together With Self-Renewal of Long-Term Repopulating Hematopoietic Stem Cells

BM retained HSPCs daily give rise to the entire yet diverse repertoire of mature blood and immune cells while maintaining the BM reservoir of undifferentiated progenitor cell pool including long-term repopulating stem cells (LT-HSCs) throughout adult life. How these contrasting tasks, requiring induction of proliferation, differentiation, and migration versus quiescence, self-renewal, and BM retention are regulated and controlled is not fully understood. Recent studies revealed that daily light and darkness onset and circadian rhythms metabolically regulate mouse HSPC migration and development as well as their BM retention and self-renewal. One hour post light onset a transient increase in the level of ROS within BM retained HSPCs was observed. This increase is driven by and requires a transient increase in BM NE and TNF levels, induced by the onset of light. These modulations in the surrounding BM microenvironment including reduced levels of CXCL12 and higher surface expression of CXCR4 by HSPCs metabolically regulate HSPC differentiation and migration, accompanied by increased permeability of BM-blood vessels. Consequently, immature and maturing leukocytes are released from the BM to the blood, peaking five hours post light initiation, to replenish the blood. In contrast, one hour following the onset of darkness, similar triggering processes are observed, although weaker signals are generated: lower transient increases in ROS levels within BM retained HSPCs are following lower increases in BM NE and TNF. These darkness-onset mediated transient increases, metabolically inducing local synthesis and secretion of the dark hormone melatonin in the mouse BM. Melatonin together with other cytokines such as PGE2, directly and indirectly, promotes retention of HSPCs in the BM. Directly, ROS levels within HSPCs and primitive LT-HSCs are further reduced following melatonin and PGE2 stimulation. Indirectly, melatonin and PGE2 also reduce the permeability of mouse BM endothelial vessels via nocturnal S1P augmentation-mediated COX2 elevation. Acting together, retention of BM HSPCs is upregulated, and these primitive cells, when harvested for transplantation at darkness during the night, are endowed now with higher homing and LT-HSC repopulation potential. Taken together, these results reveal how light onset metabolically regulates HSPC differentiation and migration to replenish the blood with new mature blood and immune cells with a finite life span. Furthermore, the onset of darkness metabolically reprograms HSPCs, facilitating their BM retention, maintenance, and self-renewal via daily BM melatonin and PGE2 bursts.[1,2]

Mechanisms of Long-term Repopulating Stem Cell Chemotherapy Resistance via Anchorage to Their Supportive Bone Marrow Niches: The Dual Roles of the Coagulation-Related, Proteinase-Activated Receptor One Axis

The major coagulation receptor, PAR1, is well known in the context of coagulation and inflammation. PAR1 has two different signaling cascades leading to opposite outcomes. One cascade leading to pro-inflammatory and pro-coagulant activity is induced by thrombin-mediated PAR1 signaling. The other cascade, leading to anti-inflammatory and anti-coagulation, is initiated by interactions of activated protein C (aPC) with endothelial protein C receptor (EPCR) and PAR1. Recent studies reported that PAR1 is functionally expressed not only in the blood involving coagulation processes but also within the BM, a site with no coagulation. Hence, PAR1 is expressed by BM stromal, endothelial, and hematopoietic cells. EPCR as well is functionally expressed by BM endothelial cells and also by some other hematopoietic cells, including the most primitive human and mouse LT-HSCs that are endowed with the highest multilineage long-term repopulating potential in transplanted mice. Recent results revealed that PAR1 signaling in HSPCs determines their location, either targeting their retention in the BM (including LT-HSCs) or their egress and mobilization, independently of coagulation. In mice, thrombin-induced PAR1 mediated secretion of CXCL12 by BM stromal cells and increased the permeability of BM endothelial vessels. In parallel, thrombin/PAR1 signaling elevated generation of the signaling free radical nitric oxide (NO) in HSPCs, including in primitive EPCR$^+$ LT-HSCs. Other effects promoted by the NO elevation in HSPCs were: shedding of EPCR, increased expression of surface PAR1 and CXCR4, higher activity of the cell-cycle and motility regulator Cdc42, enhanced CXCL12/CXCR4-directed migration, which altogether led to rapid HSPC mobilization. Interestingly, despite the shedding of EPCR, primitive LT-HSCs still contain intracellular storage of EPCR that, upon their transplantation and homing to the BM, is re-expressed on the surface and is required for long-term BM repopulation. In contrast, aPC/EPCR/PAR1 signaling in primitive BM retained EPCR$^+$ LT-HSCs exert the opposite effects, namely, inhibition of NO generation, reduction of Cdc42 activity, and increase of VLA4-mediated adhesion, which all together boost BM retention. The dual activity of PAR1 is clinically relevant. Activation of this pathway in mouse BM retained EPCR$^+$ LT-HSCs is essential for acquiring chemotherapy resistance. Furthermore, the manipulation of NO levels can be applied to increase HSPC homing[3] or mobilization as well as for increasing their BM retention and chemotherapy resistance.[4,5]

Finally, expression levels of PAR1 by circulating human HSPCs and mature leukocytes is highly important for the outcome of clinical stem cell transplantation. PAR1 signaling is essential for CXCR4 mediated directional migration of both human and mouse HSPCs toward a gradient of CXCL12 in vitro. Importantly, in clinical settings, the levels of PAR1 expression by mature mononuclear cells in the blood of healthy donors during steady-state correlate with their CXCR4-dependent in vitro migration toward CXCL12. Moreover, upon G-CSF treatment in these healthy donors, the yield of their mobilized CD34$^+$ HSPCs also correlated with the levels of PAR1 expression by circulating HSPC and mature leukocytes before treatment. Furthermore, higher PAR1 basal expression correlated with faster kinetics of hematological recovery in allogeneic matched transplanted patients.[6]

Hematopoietic Stem and Progenitor Cell Regulation via Dynamic Interactions With the Blood-BM Endothelial Barrier and the Stromal Microenvironment

Mature red blood cells and platelets are present in the blood but are absent from the BM due to the selective blood-BM endothelial barrier. This selective endothelial barrier dynamically regulates the trafficking of HSPCs and mature leukocyte to and from the BM. Defining the architecture of the blood-BM barrier reveals different types of blood vessels with distinct roles, including the more permeable sinusoids, which are connected to the less-permeable capillaries. These, in turn, are continued by small arterioles. Trafficking of all leukocytes, including HSPCs, to and from the BM (homing and mobilization, respectively) occurs exclusively via the more permeable sinusoids. These vessels are associated with higher S1P and ROS levels, transferred from circulating red blood cells and platelets, conditions that induce HSPC migration and differentiation. In contrast, the less-permeable capillaries and arterioles provide low-ROS containing BM microenvironments also by physically shielding ROS entry from the circulation. As a result, low-ROS levels adjacent to the BM endosteal region provide niches for undifferentiated stem cells.[7] Importantly, total-body irradiation or chemotherapy that are applied as recipient preconditioning for BM transplantation transiently destroy the blood-BM endothelial barrier, allowing mature red blood cells and platelets to penetrate the BM. However, immature endothelial stem cells in the mouse BM vasculature can regenerate, and lead to the formation of new blood vessels and restoration of the barrier traits. The integrity of the BM-blood barrier is also deteriorating during aging. In old mice, the barrier becomes more permeable, allowing increased ROS levels to penetrate the BM, leading to conditions of chronic inflammation, which are associated with anemia, reduced host immunity, and osteoporosis. This inflammatory state can be attenuated and even reversed in old mice by activation of BM endothelial stem cells.[5]

The toxic effect of high ROS levels and the need to balance its levels in BM HSPCs is controlled by niche cells. An example is the BM non-myelinated Schwann cells, which are also ROS sensitive. These cells that are located in the low-ROS harboring BM endosteal region near less-permeable blood vessels provide a niche for

HSPCs via TGF secretion. Similarly, BM HSPCs are located adjacent to PGE2 secreting niche COX-2^+ monocyte/macrophages and megakaryocytes closed to sinusoids that both maintain their ROS^{low} state.[5,8] Another rescuing mechanism during chemotherapy-induced DNA damage is the ability of HSPCs to transfer their accumulating excess ROS levels to the attached BM niche stromal cells in order to prevent their apoptosis, which leads to lethal infection and hematology failure.[9] Preliminary results suggest that this excess ROS-transfer is a result of mitochondria transfer between LT-HSCs and their niche supporting BM stromal cells.[10] BM retaining HSPCs localize and anchor to their stromal niches via adhesion interactions that provide them with signals maintaining their quiescent, undifferentiated, non-motile, and low metabolic states. Different cell types, mainly stromal but also others, have been suggested as "HSC niche cells." Thus, mesenchymal stromal progenitor cells, including CXCL12- and SCF-expressing CAR, Nestin and leptin-receptor expressing stem cells, endothelial cells, non-myelinated Schwann cells, monocyte/macrophages, megakaryocytes, nerve fibers, and adipocytes have all been reported to regulate HSPCs and their function. The most primitive BM stromal cells of the niche have a common feature of high CXCL12 expression that is required for maintaining LT-HSC quiescence, BM retention, and chemotherapy resistance.[11–13] BM stromal niche-cells express CXCR4 as well. Functionally, CXCL12/CXCR4 signaling in these cells promotes niche regeneration after irradiation and recovery of hematopoiesis after transplantation.[14] Adult HSPC niches are also located within bone cavities and are essential for stem cell quiescence and chemotherapy resistance. Bone cavities are generated and regulated by signals originated by bone-forming osteoblasts and bone-resorbing osteoclasts. Studies in mice show that processes of bone formation are crucial for the development of BM hematopoiesis, while osteoclast activity facilitates both bone degradation and HSPC mobilization.[15–17] A recent study reported that active cycling and expanding primitive LT-HSCs are located in BM cavities with neighboring bone-remodeling activity,[18] emphasizing the major role of this region in HSC regulation. However, total-body irradiation disrupts the balance necessitating signals for restoration of new bone formation and controlled hematopoiesis. Interestingly, massive recovery of host osteoblasts following lethal body irradiation is mediated by host megakaryocytes that are recruited to the endosteal region in order to facilitate osteoblast proliferation and concurrently inhibit osteoclast activity.[19] These studies demonstrate the profound regulatory impact that bone-remodeling processes have on HSPC function and moreover suggest that a durable turnover of HSPC BM niches is required for the ongoing maintenance of the niche supportive function.

MEDICAL APPROACHES ORIGINATING FROM UNDERSTANDING MECHANISMS THAT REGULATE PHYSIOLOGICAL HSPC TRAFFICKING, FOR THE SAKE OF CURATIVE STEM CELL TRANSPLANTATION PROTOCOLS

Establishment of Functional Preclinical Immune-Deficient Mice Models to Study Human Hematopoietic Stem Cell Trafficking and the Major Role of the CXCL12/CXCR4 Axis

Mouse stem cell homing is mostly investigated in experimental BM transplantation assays. Recipient mice are preconditioned with total-body irradiation, followed by competitive transplantation of host BM cells with genetically matching donor HSPCs to prevent their rejection, but are genetically marked to track their donor cell engraftment. To identify and characterize human HSPCs and their repopulation potential in vivo, functional preclinical small animal models using immune-deficient NOD/SCID mice as recipients were developed based on conventional BM transplantation protocols. The NOD/SCID model, which is currently the gold standard for human HSC research allowed the identification of primitive human SCID-repopulating stem cells (SRC) and their hierarchy. This model was also utilized to understand the complex mechanisms regulating BM homing, engraftment, and mobilization of human HSPCs.[20] Human and murine CXCL12, the major stem cell chemokine, are cross-reactive and differ in only one amino acid, enabling homing and engraftment of human HSPCs, which functionally express its major receptor CXCR4, in transplanted immune-deficient mice. Homing and engraftment of immature human $CD34^+$ stem cells to the recipient BM requires host preconditioning with sublethal total-body irradiation or chemotherapy. These myeloablative regimens have been shown to disrupt the physical blood-BM endothelium barrier, induce a dramatic elevation of CXCL12 production and secretion by BM stromal cells, and create a chemotactic gradient toward the BM, which all facilitate the attraction of human transplanted $CD34^+CXCR4^+$ cells including the primitive $CD34^+CD38^+CXCR4^+$ stem cells to the BM. The CXCR4/CXCL12 axis is essential both for human stem cell homing and for their mobilization in transplanted NOD/SCID chimeric mice, as detailed in the homing and mobilization sections. Functional, preclinical immune-deficient murine models, which tolerate human HSPC xenografts, are important for the identification and characterization of human stem cells, for better understanding of the mechanisms involved and the molecules participating in the biological regulation of HSPC trafficking to and from the BM. The high homology of mechanisms regulating HSPC function shared between these preclinical functional BM transplantation models and transplanted human patients emphasize the clinical relevance and potential of these experimental models.

HEMATOPOIETIC STEM AND PROGENITOR CELL HOMING

Migration of HSPCs from the circulation and their extravasation across the physical barrier of the blood vessels into the BM require active steering, a process termed homing. Physiological homing occurs during development, when HSCs relocate from the fetal liver, the pre-natal hematopoiesis site, to the BM, the site of childhood, and adult hematopoiesis. Clinical stem cell transplantation mimics this process where HSPCs are infused into the blood of ablated recipients home to their BM as a critical and essential first step for successful engraftment of the BM, the predominant physiologic site of hematopoiesis. The BM endothelium is the first anchoring site for homing stem cells, exposing them to presented adhesion molecules (e.g., very late antigen 4 [VLA-4]/vascular cell adhesion molecule 1 [VCAM-1], and CD44/hyaluronic acid [HA] interactions) and stimulating chemokines (CXCL12/CXCR4 signaling). BM sinusoidal blood vessels, through which trans-endothelial migration takes place in mice[7] and likely in humans as well, are composed of specialized cell structures that regulate cell trafficking. The homing process is initiated by a CXCL12/CXCR4-mediated guiding signal, leading to firm adhesion and docking to the vascular sinusoidal wall, which induces cytoskeleton rearrangement and activation of integrins (e.g., VLA-4), and metalloproteinases (e.g., MMP2/9). These cellular alterations are followed by trans-migration across the physical BM-blood endothelial and extracellular matrix barriers.[20]

The Essential Role of the CXCL12/CXCR4 AXIS in the Regulation of Hematopoietic Stem and Progenitor Cell Homing

CXCL12/CXCR4 interactions and downstream signaling have been implicated in retention, migration, homing, and mobilization of HSPCs during steady-state homeostasis as well as during injury.[20] Both human and murine BM mesenchymal stromal cells, endothelial cells, and endosteal bone-lining osteoblasts, express and secrete high levels of CXCL12, observed in regions highly populated with HSPCs. Expression of CXCR4 is dynamically regulated by the levels of its ligand CXCL12, where a high concentration of soluble CXCL12 leads to CXCR4 internalization and reduced surface expression. Consequently, the expression of CXCR4 on transplanted circulating HSPCs is very dynamic and allows their direct chemo-attraction toward a high level of CXCL12 found in the BM, facilitating the homing process. A correlation observed between the migration potential of patients' CD34$^+$ HSPCs to a CXCL12 gradient in vitro, and hematopoietic recovery after autologous transplantation[21] demonstrates the clinical essential role of this axis in stem cell transplantation treatments. Importantly, in healthy donors (similar to PAR1 levels of expression[6]), the levels of surface CXCR4 expression by CD34$^+$ HSPCs correlated with their G-CSF-induced mobilization, CXCL12-induced migration in vitro, and hematopoietic recovery in matched transplanted patients.[21] Therefore, manipulations of this navigation machinery can facilitate or inhibit HSPC homing. For instance, CXCR4 expression by HSPCs can be upregulated ex-vivo, as a pretreatment aiming to accelerate their homing efficiency. Thus, genetic intervention by ex-vivo overexpression of CXCR4 enhanced the homing capacity of transplanted human HSPCs. CXCR4 expression can be also imposed by ex-vivo cytokine stimulation. A major cytokine in this context is stem cell factor (SCF, also termed c-Kit ligand). SCF is expressed by various BM stromal niche cells that co-express CXCL12 as part of the niche supporting traits. SCF regulates HSPC homing and plays an important role in hematopoiesis. Prestimulation of human or murine HSPCs with SCF increased their surface CXCR4 expression and in vivo homing through increased migration rates and adhesion via the VLA-4 and VLA-5 integrins. This superior homing capacity demonstrates the crucial role of adhesion interactions for proper rolling on the BM endothelium, which is essential for homing and retention of HSPCs in their stromal supportive niches. Similarly, the cytokine HGF as well, increased CXCR4 expression by human HSPCs and in line, their migration toward CXCL12. Raising the levels of cellular NO in HSPCs by exposure to NO donors achieves a similar effect, increasing CXCR4 expression and consequently, the homing and engraftment potential of human cord blood, BM or G-CSF-mobilized HSPCs as well as mouse HSPCs.[3,22] In contrast, the administration of neutralizing anti-CXCR4 antibodies impaired the homing of immature human CD34$^+$ HSPCs to the BM of transplanted immune-deficient mice.[20] Given the important clinical challenge of improving HSPC engraftment and BM repopulation, upregulation of CXCR4 expression prior to stem cell transplantation is a promising approach.

The dynamic expression of CXCL12 is also a key regulator of the HSPC function. The most prominent example is the rise of BM CXCL12 following total-body irradiation or chemotherapy treatment as demonstrated in mice, applied for host preconditioning. Once this rise is inhibited by injection of anti-CXCL12 neutralizing antibodies to the preconditioned immune-deficient mice, human HSPC homing and engraftment were abrogated. Administration of high doses of CXCL12 supports survival and quiescence of BM retaining HSPCs, while low doses promote the upregulation of CXCR4, proliferation, and migration. Accordingly, in vivo pretreatment with a high dose of CXCL12 locally increased the homing of human repopulating cells. Interestingly, physiologic mechanisms act to balance CXCL12 availability. Concentrations of this chemokine in the BM or plasma can reciprocally interchange and direct HSPC navigation. The noncleaved and active form of CXCL12 can transfer from the blood into the BM crossing the endothelial barrier in a CXCR4-dependent manner. This increase in concentrations of BM CXCL12 functionally enhances the homing capacity of human CD34$^+$ progenitors transplanted in NOD/SCID mice.[20] Inline, depletion of CXCL12-expressing stromal niche cells results in defective hematopoiesis and reduced homing of transplanted mouse HSPCs to the BM, demonstrating the crucial role for CXCL12 in homing of HSPCs.[12]

Taken together, enhancing CXCR4 expression on HSPCs and inhibiting CXCL12 degradation or raising its levels in the BM are promising approaches for creating a stable chemotactic response in order to direct HSPC homing into the BM.

HEMATOPOIETIC STEM AND PROGENITOR CELL MOBILIZATION: A DYNAMIC MULTI-FACET PROCESS

HSPCs, actively retained in the BM anchored to their stromal microenvironment, proliferate and differentiate to all mature leukocytes and erythrocytes to replenish blood and immune cells. In addition to mature and maturing leukocytes, low levels of HSPCs are also daily released to the blood as part of steady-state homeostasis. The timing of this daily HSPC egress is driven by light-induced signals derived from the nervous system.[1,2]

Hematologic stress situations such as bacterial-induced inflammation (mimicked by LPS or endotoxin), bleeding, and administration of cytotoxic agents such as radiation and chemotherapy all exert demands for accelerated hematopoiesis. Consequently, steady-state rates of HSPC egress are dramatically augmented leading to HSPC recruitment as part of host defense and repair mechanisms. This recruitment of human and mouse HSPCs from the BM to the blood is also achieved by clinical protocols, such as repeated G-CSF stimulations, which are used to expand and to mobilize the HSPC pool in order and to more easily harvest them from the blood for clinical BM transplantation. Stress-induced HSPC mobilization is a complex multi-step process involving activation of motility mechanisms; signals of various cytokines; and changes in chemotactic signals (e.g., CXCL12 and CXCR4), proteolytic enzymes, and other extrinsic factors that enable detachment of HSPCs from their BM niches. Some mobilizers such as repeated stimulations with the cytokine G-CSF first induce HSPC proliferation and differentiation while others rapidly acting are merely metabolically activating the mobilization machinery. Induction of mobilization settings modulates also the permeability of the BM-blood endothelial barrier and the anchoring-facilitating signals of BM HSPC niches.

The CXCL12/CXCR4 Axis as a Driving Force Leading to Hematopoietic Stem and Progenitor Cell Mobilization

The CXCL12/CXCR4 axis has important roles in HSPC homing to the BM, retention, quiescence, and chemotherapy resistance. Impairment of CXCL12 or CXCR4 expression levels in murine embryos results in multiple lethal defects, including lack of stem cell seeding of the BM. In order to circumvent lethality, conditional knock-out models were established. Induced-deletion of CXCR4 in the hematopoietic system of the adult mouse or ablation of CXCL12 in the BM stroma both led to severely reduced BM cellularity and HSC numbers as well as impaired competitive repopulation capacity. Conditional deletion of CXCR4 or CXCL12 also results in dramatically increased HSPC numbers in the peripheral blood and spleen since the cells were continuously cycling, losing their chemotherapy resistance[13] and revealing that the lack of CXCR4 hampers their retention in the BM. Selective chemical antagonists of CXCR4, such as AMD3100, were originally developed to block CXCR4 mediated HIV infection but failed to do so in vivo. Nevertheless, serendipitous research revealed that upon AMD3100 administration, mouse, primate, and human HSPCs undergo rapid mobilization within hours and the mobilized HSPCs retain a high in vivo repopulation potential. Mechanistically, AMD3100 enhanced secretion of CXCL12 from BM stromal cells and its release to the circulation, leading to the rapid CXCR4-dependent mobilization of HSPCs.[23] AMD3100 is the only chemokine receptor antagonist utilized clinically for inducing mobilization, either alone or in combination with G-CSF since it synergistically augments G-CSF-induced mobilization. The different kinetics and mode of action make AMD3100 an effective mobilizing agent in murine models, and human patients were found to poorly mobilize in response to G-CSF only.[23]

The essential role of CXCL12/CXCR4 signaling in HSPC retention can be inferred from the observations that following G-CSF administration, CXCL12 levels in the BM are transiently increased followed by its downregulation at both protein and mRNA levels, enabling transient and local CXCL12 gradients toward the blood. These modulations in the ligand levels following G-CSF treatment are associated with CXCR4 upregulation on immature murine BM HSPCs as well as on immature human $CD34^+$ HSPCs and the more primitive $CD34^+CD38^-$ stem cells resident in the BM of G-CSF-treated chimeric mice. Blocking either CXCR4 or CXCL12 blocks both G-CSF- and AMD3100-induced mobilization, demonstrating the key role of the CXCL12/CXCR4 axis in murine HSPC mobilization.[23]

Secreted CXCL12, as well as AMD3100, can activate the metalloprotease MMP-9, which in turn cleaves and degrades this chemokine, and induced the release of membrane-bound SCF, participating in the enhanced migration capacity and mobilization of HSPCs to the periphery.[23] Inline, repetitive daily administrations of CXCL12 for five consecutive days facilitated murine HSPC mobilization.[15] In summary, impeding CXCL12/CXCR4 signaling in the BM results in loss of retention since active membrane-bound CXCL12/CXCR4 signaling is required for stem cell adhesion, quiescence, and chemotherapy resistance in the BM.

Dynamics of the Hematopoietic Stem and Progenitor Cell Niches and the Bone Marrow Microenvironment During Mobilization

The two major roles of the BM microenvironment are to regulate hematopoiesis and mature blood cell production, together with providing quiescent, primitive BM retained LT-HSCs with chemotherapy resistance, in order to prevent lethal hematology failure and infections. Coupling of bone degradation by HSPC-derived and monocyte-derived osteoclasts and new bone formation carried out by mesenchymal stem cell (MSC)-derived osteoblasts, is part of a complex network implementing the process of bone remodeling. Noteworthy, osteoblasts and osteoclasts maintain bone equilibrium by acting also on the endosteum region in the vicinity of HSPCs. These regions are highly vascularized. Moreover, both osteoblasts and osteoclasts attached to the endosteal bone of mouse calvaria are observed close to BM sinusoidal capillaries.[24] Osteoclast activity has been shown to also mediate HSPC mobilization[15] but is also crucial for the normal function of BM HSPC niches,[25] demonstrating the major role these cells play in HSPC regulation. Thus, the endosteal neighboring HSPC niches are dynamically altered during bone remodeling, affecting HSPC function and maintenance during a steady state. Once mobilization is induced by clinical procedures the BM microenvironment experiences dynamic changes. G-CSF or cyclophosphamide injections, as part of clinically-oriented HSPC mobilization protocols, lead to the disappearance or altered morphology of mature bone-lining osteoblasts, resulting in their reduced function, which is also associated with reduced transcription of CXCL12. Of interest, upon G-CSF administration, osteoblasts rapidly expand, prior to their apoptosis, promoting HSPC proliferation, which in turn enables the loss of HSPC retention. The importance of BM stromal cells for the retention of HSPCs can be illustrated, for example, by the conditional deletion of the cell-cycle regulator Retinoblastoma protein (Rb). Not only the absence of Rb in the murine hematopoietic system but also the absence of Rb in the BM stroma alone is sufficient to increase the numbers of circulating HSPCs, in addition to a myeloproliferative-like disease and extramedullary hematopoiesis. Other BM cell types are also affected by the pro-inflammatory mobilization process, and in turn, exert signals influencing HSPCs. This list contains activated neutrophils and osteoclasts, which secrete proteolytic enzymes mediating the degradation of the extracellular matrix, CXCL12, and SCF.[15,23] Inline, depletion of osteal macrophages termed osteomacs lead to HSPC mobilization due to a reduction in BM CXCL12 and SCF.[26] BM endothelial vessels are also modulated during mobilization. Particularly, AMD3100 reduces expression of CXCL12 and SCF, particularly on BM sinusoids, and increases vascular permeability involving CXCR4 signaling.[5,7] Complement activity and purinergic signaling have also been shown to regulate HSPC egress and mobilization.[27] Taken together, HSPC mobilization is an active process that involves changes in metabolic activity, structural arrangement, and availability of BM HSC niche signaling components, all disrupting the adhesion and anchorage of HSPCs in their protecting niches.

CLINICAL RELEVANCE AND FUTURE DIRECTIONS

BM transplantation procedures, which are routinely used to treat patients with hematological malignancies, as well as inherited genetic disorders, require the collection of mobilized HSPCs from the donor's circulation. Therefore, the characterization of new agents for improved stem cell mobilization protocols has become a major focus in recent decades. The HSPC mobilization process is not credited only to key motility mechanisms, but also to microenvironmental dynamics. Clinical BM transplantation applying current mobilization protocols includes repeated G-CSF regimens and chemo-mobilization achieved by chemotherapy treatment combined with G-CSF. Despite the wide range of therapeutic strategies used, most of them have either failed to show a clear advantage to standard mobilization regimens or were associated with substantial adverse effects (e.g., chemo-mobilization). For the introduction of CXCR4 antagonists, e.g., AMD3100, there is a renewed optimization in the management of difficult to mobilize patients. AMD3100 mediates the rapid secretion of CXCL12 from BM stromal cells and its release to the circulation and increased BM endothelial cell permeability[7] result in stem and progenitor cell mobilization. Treatment with AMD3100 exhibits marked synergism with G-CSF, suggesting their distinct and complementary mechanisms of action to induce HSPC mobilization. Several studies have shown success in mobilization capacity of previously poor-mobilizing myeloma, non-Hodgkin lymphoma, and Hodgkin lymphoma patients.

As to HSPC homing, the paradox of preconditioning, having a “double-edged sword” effect, is the following: on one hand, killing dividing defective/malignant immune and blood progenitor cells and evacuating the BM niches for lodgment of donor transplanted BM HSPCs; on the other hand, generating stress conditions turn the HSPC migration machinery, the profile of niche signals, and BM barrier permeability into mobilization-favoring status, which sums up in lower homing efficiency. A solution for this complication is suggested by transplantation of a large amount of HSPCs without host preconditioning, leading to high durable donor stem cell engraftment levels.[28] The use of cord blood is limited at present to pediatric patients and adults of low body weight because of the low number of $CD34^+$ progenitors contained in each cord blood unit. For this reason, the major disadvantage of cord blood transplantation in adult patients is, delayed engraftment and the T cells in the cord blood are naïve and in low numbers. Attempts to overcome this problem consisted of double cord blood transplants or combined transplantation of mobilized or BM-derived $CD34^+$ cell infusion from a matched donor, achieving transient engraftment until the cord blood cells reconstitute the BM. A recent publication shares a protocol to expand mouse BM LT-HSCs in a 28-day-long in vitro culture derived from 50 sorted HSCs. These conditions afford between 236- and 899-fold expansions of functional HSCs that upon transplantation highly repopulated non-preconditioned host mice.[29] Finally, promising strategies are aiming at increasing CXCR4 expression prior to HSPC transplantation by in vitro exposure to NO donors, as shown for human and mouse HSPCs.[3,22,30] Interestingly, the HSC self-renewal agonist UM171 balances pro- and anti-inflammatory signals, with exclusive induction and expansion of human LT- HSCs. In vitro expansion of human CB $CD34^+$ HSCs with UM171 led to the appearance of a small population of $EPCR^+CD34^+$ HSCs. Notably, only $EPCR^+$ (but not $EPCR^-$) human CB HSCs were endowed with robust high-level multilineage repopulation and serial reconstitution capacity in transplanted immunodeficient NOD/SCID mice. Importantly, EPCR function protected human LT-HSCs from TNF and UM171 mediated hyper-inflammatory responses by preventing NO overproduction in primitive $EPCR^+$ LT-HSCs, potentially leading to cell-death and compromised HSC function.[30] These results are in agreement with chemotherapy resistance of mouse BM retained $EPCR^+$ LT-HSCs, which is induced via aPC/EPCR/PAR1-mediated NO inhibition, leading to reduced Cdc42-GTP activity and increased VLA-4 mediated stem cell adhesion and BM retention.[4]

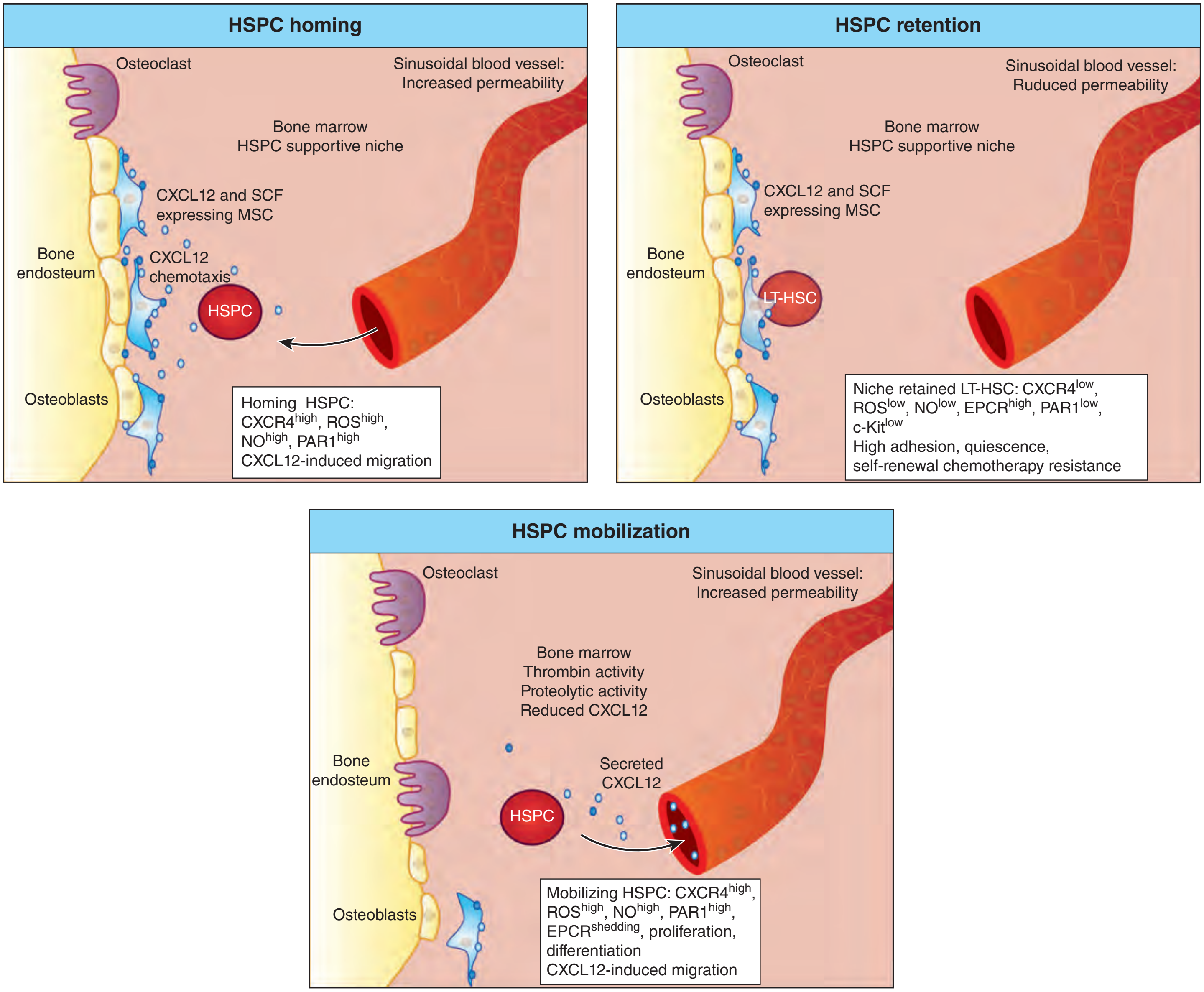

HSPC homing
Osteoclast
Sinusoidal blood vessel:
Increased permeability
Bone marrow
HSPC supportive niche
CXCL12 and SCF
expressing MSC
Bone
endosteum
CXCL12
chemotaxis
HSPC
Osteoblasts
Homing HSPC:
CXCR4^{high}, ROS^{high},
NO^{high}, PAR1^{high}
CXCL12-induced migration
HSPC retention
Osteoclast
Sinusoidal blood vessel:
Ruduced permeability
Bone marrow
HSPC supportive niche
CXCL12 and SCF
expressing MSC
Bone
endosteum
LT-HSC
Osteoblasts
Niche retained LT-HSC: CXCR4^{low},
ROS^{low}, NO^{low}, EPCR^{high}, PAR1^{low},
c-Kit^{low}
High adhesion, quiescence,
self-renewal chemotherapy resistance
HSPC mobilization
Osteoclast
Sinusoidal blood vessel:
Increased permeability
Bone marrow
Thrombin activity
Proteolytic activity
Reduced CXCL12
Bone
endosteum
Secreted
CXCL12
HSPC
Osteoblasts
Mobilizing HSPC: CXCR4^{high},
ROS^{high}, NO^{high}, PAR1^{high},
EPCR^{shedding}, proliferation,
differentiation
CXCL12-induced migration

Figure 16.1 MECHANISMS MEDIATING HOMING, RETENTION, AND MOBILIZATION OF BONE MARROW HEMATOPOIETIC STEM AND PROGENITOR CELLS. **Homing:** HSPC transplanted by intravenous infusion, home to the bone marrow crossing the sinusoidal barrier following CXCL12 gradients. Homing HSPCs express high levels of surface CXCR4 and PAR1 (required for CXCL12-induced migration), are motile, and are active metabolically, containing high levels of NO and ROS. **Retention:** BM retained LT-HSCs are attached via adhesion interactions to niche stromal cells expressing surface CXCL12 and SCF. These primitive and quiescent cells are endowed with high repopulation potential and are chemotherapy-resistant due to their adhesion and metabolic interactions with stromal niche cells. BM retained LT-HSCs express low levels of surface CXCR4 and c-Kit, together with high levels of EPCR, and demonstrate low metabolic activity manifested in low contents of NO and ROS. **Mobilization:** Stress-induced mobilization involves an increase in thrombin activity leading to EPCR-shedding, upregulation of CXCR4 and PAR1, high metabolic activity indicated by high NO and ROS content, and HSPC proliferation and differentiation and activation of the migration machinery, which together with the release of CXCL12 from BM stromal niche cells to the circulation lead to HSPC mobilization. *BM*, Bone marrow; *EPCR*, endothelial protein C receptor; *HSPC*, hematopoietic stem and progenitor cell; *LT-HSC*, long-term repopulating stem cell; *MSC*, mesenchymal stem cell; *NO*, nitric oxide; *PAR1*, proteinase activated receptor one; *ROS*, reactive oxygen species; *SCF*, stem cell factor

CONCLUDING REMARKS

Cell-intrinsic processes in BM HSPCs and BM microenvironmental extracellular signals join to control HSPC modulating programs either to maintain quiescence, self-renewal, long-term repopulation capacity, and chemotherapy resistance while retaining them in their BM niches or to cycle, proliferate, differentiate and activate their migration machinery in order to egress from the BM and replenish the blood. The steady-state dynamics, balancing these bi-directional processes, led by light/darkness circadian signals, are further accelerated upon stress conditions favoring HSPC expansion and mobilization. These processes require metabolic alterations in HSPCs involving their cytoskeletal adjustment to the state of navigation, diminishing or reducing niche-retaining signals, turning the chemotactic gradient toward the blood, and increasing permeability of the selective BM-blood endothelial barrier. In order to facilitate stem cell homing, as desired during BM transplantation, migration machinery in HSPCs needs to be better activated when they are infused to the blood. However, efficient homing and engraftment will also be achieved if the BM microenvironment is preconditioned to increase vascular permeability and turn-over of the chemotactic gradient toward the BM. Once homed, HSPCs require retention-facilitating signals in order to lodge in their niches and durably regenerate hematopoiesis.

Multiple studies have been engaged with defining the entity, features, and composite of the HSPC niches. The activity of the CXCL12/CXCR4 pathway has a prominent role in HSPC homing, mobilization, retention, self-renewal, quiescence, adhesion, and chemotherapy resistance. The high expression of CXCL12 by endosteal osteoblasts, endothelial cells, and stem cell-supporting MSCs, implies the pivotal role of the niche dynamic regulation in HSPC function. This chapter discusses previously discovered as well as recent updates of mechanisms and pathways involved in the regulation of HSPC homing, egress and mobilization, emphasizing the major roles of the CXCL12/CXCR4 axis in the regulation of these complex interactive processes (key players are illustrated in Fig. 16.1).

ACKNOWLEDGMENTS

We thank the following agencies for supporting our research: The Israel Science Foundation (ISF), a joint grant from the ISF and the National Natural Science Foundation of China (NSFC), Minerva Foundation and the Federal German Ministry for Education and Research, Steven B. Rubenstein Research Fund, Weizmann Stem Cell Institute, The WIS Moross Integrated Cancer Center, Asher Pertman and Wayne Pertman, and the Estate of David Levinson.

REFERENCES

1. Golan K, Kollet O, Markus RP, Lapidot T. Daily light and darkness onset and circadian rhythms metabolically synchronize hematopoietic stem cell differentiation and maintenance: The role of bone marrow norepinephrine, tumor necrosis factor, and melatonin cycles. *Exp Hematol*. 2019;78:1–10.
2. Mendez-Ferrer S, Chow A, Merad M, Frenette PS. Circadian rhythms influence hematopoietic stem cells. *Curr Opin Hematol*. 2009;16(4):235–242.
3. Xu D, Yang M, Capitano M, et al. Pharmacological activation of nitric oxide signaling promotes human hematopoietic stem cell homing and engraftment. *Leukemia*. 2021;35(1):229–234.
4. Gur-Cohen S, Kollet O, Graf C, et al. Regulation of long-term repopulating hematopoietic stem cells by EPCR/PAR1 signaling. *Ann N Y Acad Sci*. 2016;1370(1):65–81.
5. Ramasamy SK, Kusumbe AP, Itkin T, et al. Regulation of hematopoiesis and osteogenesis by blood vessel-derived signals. *Annu Rev Cell Dev Biol*. 2016;32:649–675.
6. Nevo N, Zuckerman T, Gur-Cohen S, et al. PAR1 Expression predicts clinical G-CSF CD34(+) HSPC mobilization and repopulation potential in transplanted patients. *Hemasphere*. 2019;3(5):e288.
7. Itkin T, Gur-Cohen S, Spencer JA, et al. Distinct bone marrow blood vessels differentially regulate haematopoiesis. *Nature*. 2016;532(7599):323–328.
8. Ludin A, Gur-Cohen S, Golan K, et al. Reactive oxygen species regulate hematopoietic stem cell self-renewal, migration and development, as well as their bone marrow microenvironment. *Antioxid Redox Signal*. 2014;21(11):1605–1619.
9. Singh AK, Cancelas JA. Gap junctions in the bone marrow lympho-hematopoietic stem cell niche, leukemia progression, and chemoresistance. *Int J Mol Sci*. 2020;21(3):796.
10. Golan K, Wellendorf A, Takihara Y, et al. Mitochondria transfer from hematopoietic stem and progenitor cells to Pdgfrα^{+}/Sca1^{-}/CD48^{dim} BM stromal cells via CX43 gap junctions and AMPK signaling inversely regulate ROS generation in both cell populations. *Blood*. 2016(128):5 ASH abst.
11. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. *Nature*. 2014;505(7483):327–334.
12. Pinho S, Frenette PS. Haematopoietic stem cell activity and interactions with the niche. *Nat Rev Mol Cell Biol*. 2019;20(5):303–320.
13. Sugiyama T, Omatsu Y, Nagasawa T. Niches for hematopoietic stem cells and immune cell progenitors. *Int Immunol*. 2019;31(1):5–11.
14. Singh P, Mohammad KS, Pelus LM. CXCR4 expression in the bone marrow microenvironment is required for hematopoietic stem and progenitor cell maintenance and early hematopoietic regeneration after myeloablation. *Stem Cells*. 2020;38(7):849–859.
15. Kollet O, Dar A, Lapidot T. The multiple roles of osteoclasts in host defense: bone remodeling and hematopoietic stem cell mobilization. *Annu Rev Immunol*. 2007;25:51–69.
16. Chan CK, Chen CC, Luppen CA, et al. Endochondral ossification is required for haematopoietic stem-cell niche formation. *Nature*. 2009;457(7228):490–494.
17. Miyamoto K, Yoshida S, Kawasumi M, et al. Osteoclasts are dispensable for hematopoietic stem cell maintenance and mobilization. *J Exp Med*. 2011;208(11):2175–2181.
18. Christodoulou C, Spencer JA, Yeh SA, et al. Live-animal imaging of native haematopoietic stem and progenitor cells. *Nature*. 2020;578(7794):278–283.
19. Dominici M, Rasini V, Bussolari R, et al. Restoration and reversible expansion of the osteoblastic hematopoietic stem cell niche after marrow radioablation. *Blood*. 2009;114(11):2333–2343.
20. Lapidot T, Dar A, Kollet O. How do stem cells find their way home? *Blood*. 2005;106(6):1901–1910.
21. Voermans C, Kooi ML, Rodenhuis S, et al. In vitro migratory capacity of CD34+ cells is related to hematopoietic recovery after autologous stem cell transplantation. *Blood*. 2001;97(3):799–804.
22. Zhang Y, Wittner M, Bouamar H, et al. Identification of CXCR4 as a new nitric oxide-regulated gene in human CD34+ cells. *Stem Cells*. 2007;25(1):211–219.
23. Lapid K, Glait-Santar C, Gur-Cohen S, et al. Egress and mobilization of hematopoietic stem and progenitor cells: a dynamic multi-facet process. In: StemBook [Internet]. Cambridge (MA): Harvard Stem Cell Institute; 2008–2012 Dec 10.
24. Kim J, Bixel MG. Intravital multiphoton imaging of the bone and bone marrow environment. *Cytometry A*. 2020;97(5):496–503.
25. Mansour A, Wakkach A, Blin-Wakkach C. Emerging roles of osteoclasts in the modulation of bone microenvironment and immune suppression in multiple myeloma. *Front Immunol*. 2017;8:954.
26. Winkler IG, Sims NA, Pettit AR, et al. Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. *Blood*. 2010;116(23):4815–4828.
27. Adamiak M, Ciechanowicz A, Skoda M, et al. Novel evidence that purinergic signaling—Nlrp3 inflammasome axis regulates circadian rhythm of hematopoietic stem/progenitor cells circulation in peripheral blood. *Stem Cell Rev Rep*. 2020;16(2):335–343.
28. Shimoto M, Sugiyama T, Nagasawa T. Numerous niches for hematopoietic stem cells remain empty during homeostasis. *Blood*. 2017;129(15):2124–2131.
29. Wilkinson AC, Ishida R, Kikuchi M, et al. Long-term ex vivo haematopoietic-stem-cell expansion allows nonconditioned transplantation. *Nature*. 2019;571(7763):117–121.
30. Chagraoui J, Lehnertz B, Girard S, et al. UM171 induces a homeostatic inflammatory-detoxification response supporting human HSC self-renewal. *PLoS One*. 2019;14(11):e0224900.

CHAPTER 17

CONTROL OF CELL DIVISION

Martin Fischer and James A. DeCaprio

THE CELL DIVISION CYCLE

The mammalian cell cycle is divided into four phases: mitosis (M), deoxyribonucleic acid (DNA) synthesis (S), and the gap phases G1 and G2 (Fig. 17.1). Mitosis is recognized when cells visibly undergo cell division and chromatin becomes condensed, sequentially progressing through prophase, metaphase, anaphase, and telophase. The G1 phase occurs immediately after mitosis has been completed and ends when DNA synthesis begins. During the S phase, cells duplicate their entire genome by DNA replication. G2 occurs after DNA synthesis has been completed and before chromosomal condensation in mitosis. Although the duration of the S, G2, and M phases is relatively constant for most mammalian cells, there can be a large degree of variability in the duration of G1. Among the earliest observations regarding the generation time for cells, it was shown that by varying the growth conditions, the length of a cell division cycle could change, with the length of G1 responsible for most of this variability. Although cells progress through S, G2, and M phases in relatively invariable time periods, the length of the G1 phase is highly variable, and this variability is dependent at least in part on the presence of growth factors.

Quiescence and Differentiation

Quiescence (G0) is a non-proliferative state in which viable cells have left the cell cycle and may remain for prolonged periods. Quiescent cells may be difficult to distinguish morphologically from cells in a prolonged G1 phase, but they can be distinguished by different markers. Terminally differentiated cells, such as neutrophilic granulocytes, muscle cells, and neurons, have irreversibly exited the cell cycle during the process of differentiation and are examples of cells that have irreversibly entered G0. Other cells, including stem cells, reversibly enter G0 and may be induced to re-enter the cell cycle with appropriate stimuli, such as growth factors. Differentiation provides the organism with a supply of cells to execute specific and specialized functions. In some cell types, such as muscle and nerve cells, differentiation and proliferation are mutually exclusive fates, and cells undergo "terminal differentiation." In other cell types, such as those of the hematopoietic lineage, proliferation may continue after cells acquire differentiated characteristics. For example, erythroblasts, myeloblasts, and megakaryoblasts are committed to particular differentiation pathways and possess lineage-specific markers yet continue to proliferate. T and B lymphocytes are fully differentiated and express antigen-specific receptors but can be induced to proliferate when appropriately stimulated.

G1 Phase

G1, which occupies the period or gap between M and S phases, is the interval between the completion of one round of cell division and initiation of the next. Its duration is the most variable, can be prolonged depending on the cell type, and is subject to regulation by environmental factors such as the availability of growth factors and nutrients. It is the period of cell growth, and as a first approximation, the amount of time a cell spends in G1 is inversely related to its rate of proliferation. A certain increase in mass usually is required before the cell initiates the next S phase. When conditions are unsuitable for proliferation (e.g., because of insufficient nutrients or absence of mitogens), cells arrest in G1, and those that are already in S, G2, or M phase usually complete the round to which they have been committed and arrest only when they reach G1 again. For example, when the 40 S ribosomal protein S6 is missing, cells stop proliferation. If cell size or mass were not regulated, S phase entry might cause cells to become progressively smaller or be at risk for DNA replication errors because of insufficient substrates. Cell size regulation is intimately linked to ribosome biogenesis and nutrient-sensing systems, central to which are the phosphoinositide 3-kinase (PI3K) and target of rapamycin (TOR) pathways. Notably, when the MYC transcription factor is missing, cells slow their growth and often do not enter the S phase. Myelocytomatosis (MYC) transcription factor regulates *cyclin* and *cyclin-dependent kinase (CDK)* genes as well as genes involved in ribosome biogenesis and translation.[1] In aggregate, studies of a variety of cell systems indicate that cell size regulation is linked to cell proliferation, except in specialized cells that undergo endoreplication or in embryos shortly after fertilization, when G1 is virtually undetectable, and there is no cell enlargement. In the latter, the original mass of egg cytoplasm is partitioned among thousands of cells within a few hours without a noticeable increase in size.

S Phase

S phase is the period of wholesale DNA synthesis during which the cell replicates its genetic content; a normal diploid somatic cell with a 2 N complement of DNA at the beginning of the S phase acquires a 4 N complement of DNA at its end. (Recall that $N = 1$ copy of each chromosome per cell [haploid]; $2N = 2$ copies [diploid].) The duration of the S phase may vary from only a few minutes in rapidly dividing, early embryo cells to a few hours in most somatic cells. Early embryo cells generally "live off" the accumulated stores of maternal ribonucleic acid (RNA) and proteins present in the egg and are transcriptionally silent, whereas cells in later development and mature organisms must actively transcribe subsets of their genes to survive and maintain specialized functions. The longer time required for the latter to complete the S phase probably allows these cells to coordinate DNA replication with transcription and to preserve higher-order gene and chromatin structural information that influences gene expression for transmission to progeny cells.

G2 Phase

G2 is the period or gap between S and M phases when cells have finished replicating their DNA, are preparing to divide, and have a 4 N DNA content. For most cells entering the S phase, passage through G2 is "automatic," and the duration of G2 is fixed, except under unusual circumstances. For example, G2 duration can be extremely short and is essentially undetectable in rapidly proliferating, early embryonic cells.

M Phase

Mitosis, or M phase, is the period of actual nuclear and cell division during which the duplicated chromosomes are divided equally

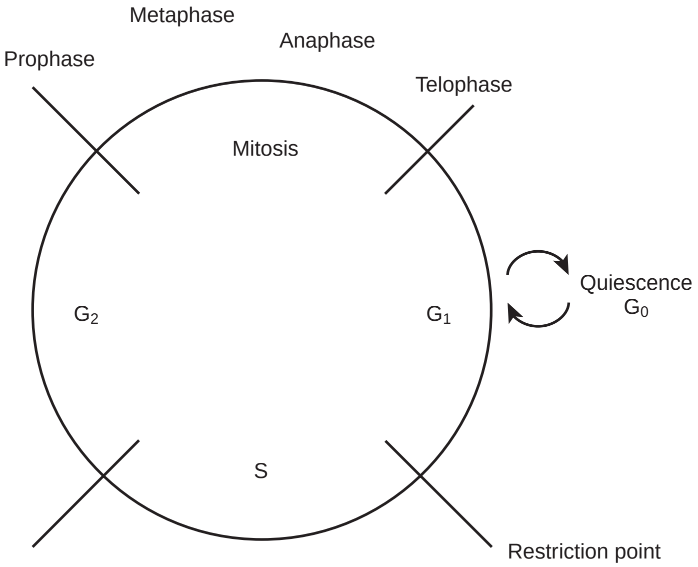


Figure 17.1 THE CELL DIVISION CYCLE. The mammalian cell cycle is divided into 4 phases that include mitosis (M), DNA synthesis (S), and the gap phases G1 and G2. Mitosis is subdivided into prophase, metaphase, anaphase, and telophase. Quiescence (G0) is a non-proliferative state in which viable cells have left the cell cycle and may remain for prolonged periods. A particularly important point in G1 is the restriction point, or R, which occurs near the G1–S boundary. After the cell passes the G1/S restriction point, it is committed to cell cycle progression.

between two progeny cells. It is obvious microscopically as the period of chromosome condensation and segregation, nuclear division (karyokinesis), and physical separation of the two daughter cells (cytokinesis). A cell entering the M phase has a 4 N DNA content and finishes as two cells, each with an identical 2 N complement of DNA. The complex sequence of changes that take place allows mitosis to be subdivided into prophase, prometaphase, metaphase, anaphase, and telophase. *Prophase* is the period of chromatin/chromosome condensation, centrosome separation/migration to opposite poles, and nuclear membrane breakdown. The centrosomes are microtubule organization centers that eventually give rise to the bipole mitotic spindle apparatus that will separate the sister chromatids of each duplicated chromosome. During *prometaphase*, chromosomes attach to microtubules of the mitotic spindle so that sister chromatids attach to opposite poles. In *metaphase*, the condensed chromosomes align at the equatorial plate. The cohesive "bond" between sister chromatids of duplicated chromosomes is dissolved, allowing *anaphase*, the period of sister chromatid separation, to proceed. On reaching their poles, nuclear membranes form to envelop each of the two separated sets of chromosomes, which also begin to decondense, marking *telophase* and karyokinesis. This is immediately followed by cytokinesis, during which an actin-myosin ring at the cell membrane contracts to move inwards until a midbody structure is formed. The abscission of the midbody marks the exit from mitosis. Following mitosis, cells reenter G1, and for approximately 3 hours, they are capable of leaving the cell cycle into quiescence when growth factors and nutrients are missing. Once past this point, cells are no longer sensitive to mitogen withdrawal and can commit to another round of cell division.

SIGNALING

To decide whether to proliferate, cells scan their environment with the help of cell surface receptors such as receptor tyrosine kinases (RTKs) that bind extracellular ligands and activate signaling pathways. RTKs become activated by ligand-stabilized dimerization that allows autophosphorylation.[2] The intracellular phosphotyrosine residues are recognized by SH2 (Src homology 2) or PTB (phosphotyrosine-binding) protein domains, leading to the recruitment of signaling effectors and formation of signaling complexes. In turn, these complexes permit activation of signaling pathways, such as rat sarcoma virus (RAS)–mitogen-activated protein kinase (MAPK), PI3K, and phospholipase Cγ (PLCγ). Together, these signaling pathways stimulate cell proliferation (Fig. 17.2).

T-Cell Receptor

Activation of T cells occurs upon ligation of the T-cell receptor (TCR) by major histocompatibility complex (MHC) molecules in antigen-presenting cells.[3] The TCR and its coreceptors CD4 and CD8 have no intrinsic enzymatic activity. Instead, the associated non–RTK LCK (lymphocyte-specific protein tyrosine kinase) binds to cytoplasmic domains of TCR coreceptors CD4 and CD8, and their activation leads to phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in CD3. Phosphorylated CD3 recruits the tyrosine kinase zeta-chain-associated protein kinase 70 (ZAP70), leading to a cascade of phosphorylation events that in turn activates LAT (linker for activation of T cells) complexes. The LAT signalosome triggers the release of intracellular Ca^{2+} and the production of diacylglycerol (DAG). The latter activates RAS-MAPK and protein kinase C (PKC)–nuclear factor κB (NF-κB) signaling pathways.

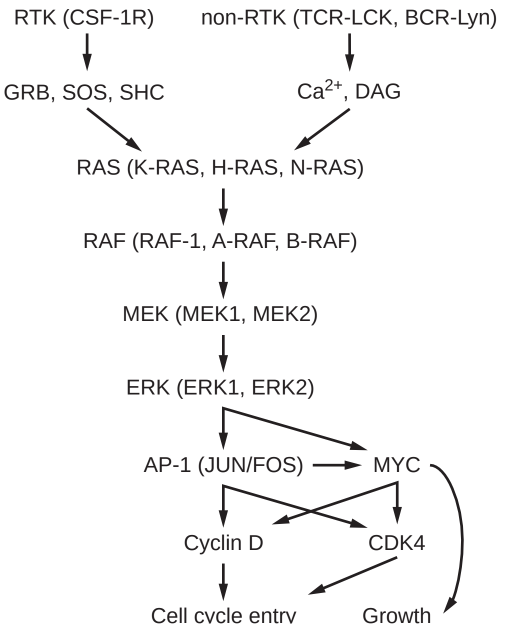


Figure 17.2 SIGNALING PATHWAYS, SUCH AS THE RAS-MAPK PATHWAY, CONTROL CELL CYCLE ENTRY. (See text for details and names of abbreviated items.) Cells scan their environment with the help of cell surface receptors such as receptor tyrosine kinases (RTKs). When RTKs become activated by ligand-stabilized dimerization, they can induce signaling pathways, in this case by activating GRB, SOS, SHC. Alternatively, a non-RTK receptor, like TCR-LCK or BCR-Lyn, can activate RAS by a pathway involving calcium influx and DAG. The RAS-MAPK signaling pathway plays a particularly important role in proliferation. RAS GTPases are activated by many receptors, such as TCR, BCR, and CSF-1R, and recruit MAPK complexes. These complexes are formed by scaffolds, such as KSR, binding to the three-tier MAPK module that comprises RAF, MEK, and ERK. When activated by RAS (H-RAS, K-RAS, N-RAS), the MAPKKK RAF (RAF-1, A-RAF, B-RAF) phosphorylates the MAPKK MEK (MEK1, MEK2), which in turn phosphorylates the MAPK ERK (ERK1, ERK2). Activated ERK kinases phosphorylate the transcription factors MYC and AP-1 (JUN/FOS), leading to transcription activation of Cyclin D1, CDK4, and CDK6. Monophosphorylation of retinoblastoma protein (RB) by Cyclin D-CDK4/6 is required for cells to leave quiescence and enter the cell cycle.

B-Cell Receptor

Every normal B cell has a unique B-cell receptor (BCR) consisting of pairs of immunoglobulin heavy (IgH) and light (IgL) chains. Each IgH and IgL has a unique variable region that allows the BCR to recognize and bind to diverse antigens, both soluble and on the surface of antigen-presenting cells. Antigen-induced aggregation of BCR leads to phosphorylation of ITAMs by the Src family tyrosine kinase LYN. This phosphorylation event initiates the assembly of intracellular signaling molecules, including SYK, PLCγ2, Bruton tyrosine kinase (BTK), VAV, and the adaptor B-cell linker (BLNK). The BCR coreceptor CD19 is also phosphorylated by LYN, leading to the recruitment of PI3K, BTK, and AKT.[4] Together, this signaling leads to the release of intracellular Ca^{2+} and production of DAG, and ultimately leads to activation of RAS-MAPK and PKC–NF-κB signaling pathways.

Macrophage Colony-Stimulating Factor Receptor

The known ligands of macrophage colony-stimulating factor 1 receptor (CSF-1R) are CSF-1 and interleukin (IL)-34.[5] CSF-1 was the first hemopoietic growth factor to be isolated, and it can promote the growth of pure colonies of macrophages from bone marrow progenitors. CSF-1R is an RTK that belongs to the platelet-derived growth factor family. The binding of CSF-1 or IL-34 to CSF-1R leads to dimerization of CSF-1R, which allows autophosphorylation. Numerous proteins, such as GRB2, SOS, SFK, CBL, and p85, are recruited to the intracellular domain phosphotyrosines, leading to the activation of the PI3K-AKT and RAS-MAPK pathways, among others.

RAS Pathway to Cyclin D

The RAS-MAPK pathway is evolutionarily conserved and controls many fundamental processes, including cell proliferation. RAS guanosine triphosphatases (GTPases) are activated by many receptors, such as TCR, BCR, and CSF-1R, and recruit MAPK complexes. These complexes are formed by scaffolds, such as KSR (kinase suppressor of RAS), binding to the three-tier MAPK module that comprises RAF, mitogen-activated protein kinase (MEK), and extracellular signal-regulated kinase (ERK). When activated by RAS (H-RAS, K-RAS, N-RAS), the MAPKKK RAF (RAF-1, A-RAF, B-RAF) phosphorylates the MAPKK MEK (MEK1, MEK2), which in turn phosphorylates the MAPK ERK (ERK1, ERK2). The principal function of RAS in cell cycle induction is to inactivate retinoblastoma protein (RB), relieving cells from its growth-inhibitory actions.[6] The RAS-MAPK signaling pathway is required to induce complexes of cyclin D1 and CDK4/CDK6. Activated ERK kinases phosphorylate the transcription factors MYC and activator protein 1 (AP-1), leading to transcription activation of cyclin D1, CDK4, and CDK6. Notably, PI3K-AKT and PKC–NF-κB pathways cooperate with RAS-MAPK in activating cyclin D1. Mono-phosphorylation of RB by cyclin D–CDK4/6 is required for cells to leave quiescence and enter the cell cycle. Given the important functions of RAS-MAPK signaling in cell proliferation, it is not surprising that cancer hijacks this pathway: K-RAS, N-RAS, and B-RAF are frequently mutated in cancer, leading to constant activation of the RAS-MAPK pathway.

CYCLINS AND CYCLIN-DEPENDENT KINASES

The 2001 Nobel Prize in Physiology or Medicine was awarded for discoveries concerning the control of the cell cycle: Leland Hartwell, Tim Hunt, and Paul Nurse discovered CDKs and cyclins that regulate the cell cycle. Identification and subsequent functional analysis of the factors and cofactors involved in mammalian cell cycle regulation have led to the current view that progression through the cell division cycle is driven by CDKs. These serine/threonine protein kinases play an essential role in promoting the cell division cycle.[7] There are many cyclins and CDKs, but only a limited number directly contribute to promoting cell cycle entry and progression (Fig. 17.3). Typically, the cyclin component binding to the CDK activates the kinase activity. Additionally, the cyclin component provides specificity to the substrate. During the G1 phase, cyclin D (cyclin D1, D2, or D3) binds to either CDK4 or CDK6. The principal targets of cyclin D–CDK4/6 are RB and the RB-related protein p130, which become monophosphorylated by cyclin D–CDK4/6.[8] Unphosphorylated and monophosphorylated RB can bind to the activating E2F (E2F1–E2F3) transcription factors and block E2F-dependent gene expression. In accordance with the important functions of cyclin D–CDK4/6 complexes in leaving quiescence and entering the cell cycle, they are frequently found overexpressed or mutated in some cancers. At the G1- to S phase transition, E-type cyclins (E1 and E2) bind to and activate CDK2, which promotes multiple phosphorylations (hyperphosphorylation) of RB and dissociation of RB from E2F. Consequently, E2F-dependent gene expression is enabled, leading to the expression of genes that encode for proteins required for DNA replication. Cyclin A binds to CDK2 at the end of the S phase and promotes entry into mitosis. CDK2 bound to cyclin E and cyclin A can phosphorylate multiple targets that contribute to DNA replication and cell cycle progression during S and G2 phases. Finally, cyclin B (B1 and B2) associates with CDK1 (CDC2) and contributes to the phosphorylation of many cellular proteins, driving cells through mitosis.

Control of cyclin-CDK activity occurs at many levels. First is the appearance and disappearance of different cyclins at specific phases of the cell cycle, which dictates the cyclin–CDK complexes that can form in each phase. Regulation at this level is a result of highly regulated synthesis and degradation of cyclin messenger RNA (mRNA) and protein at different points in the cell cycle. A second level of regulation is afforded by posttranslational modification of CDK kinases, which is often necessary to activate their function. Cyclin B–CDK1 complexes, for example, are initially inhibited by WEE1 kinase and are activated by CDC25C phosphatase when cells enter mitosis. A third level of regulation is provided by proteins that inhibit the activity of CDK kinases or cyclin-CDK complexes (see later).

Additional kinases and substrates that contribute to cell cycle progression include DDK (Dbf4-dependent kinase), PLKs (polo-like kinases), and Aurora kinases. The DDK CDC7, together with cyclin E–CDK2, coordinates the initiation of DNA replication. The polo-like kinase PLK1 activates, among others, CDC25C and deactivates WEE1, leading to active cyclin B–CDK1 complexes that drive mitosis, and PLK4 regulates centriole biogenesis during mitosis. Aurora kinases coordinate mitotic progression through phosphorylation of multiple proteins that function in chromosome segregation and cytokinesis.

CYCLIN-DEPENDENT KINASE INHIBITORS

Sharp control of CDK activity is achieved by two major classes of small polypeptide CDK inhibitors: the INK4 family (inhibitors of CDK4) comprising p16 (INK4A), p15 (INK4B), p18 (INK4C), and p19 (INK4D); and the CIP/KIP (CDK-interacting protein/kinase inhibitor protein) family that comprises p21 (CDKN1A), p27 (CDKN1B), and p57 (CDKN1C).[9] All members of the INK4 family specifically bind to CDK4 and CDK6, inhibiting their kinase activity by competing with their association with cyclin D. In contrast, CDK inhibitors of the CIP/KIP family bind to cyclin–CDK complexes and disturb their activities.

The first inhibitor to be identified and cloned in mammalian cells was p21 (CIP1, CDKN1A), which binds several different cyclin–CDK complexes. The proteins p27 (Kip1) and p57 (Kip2) were subsequently identified as CDK inhibitors with structural and functional similarities to p21. The regulation of p21 expression sheds light on its function:[10] Expression is transcriptionally induced by p53, the tumor suppressor protein activated by DNA damage, and induction of p21 expression provides a mechanism for halting cell proliferation

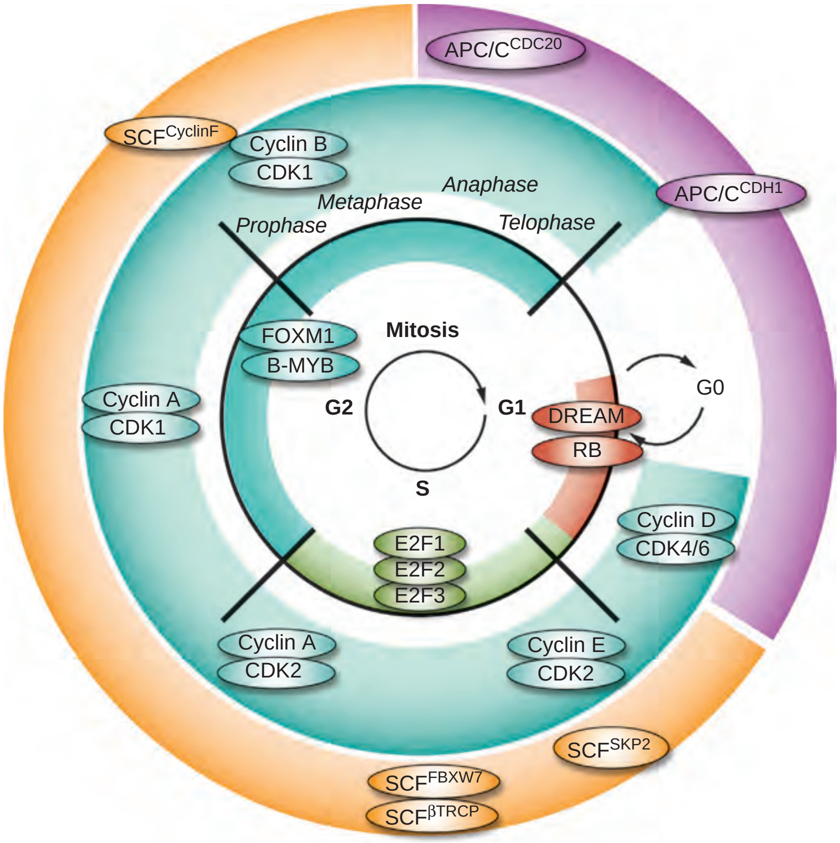


Figure 17.3 A MULTILAYERED NETWORK CONTROLS THE CELL CYCLE. Many proteins that carry out important functions during the cell cycle are encoded by genes that display a periodic expression pattern during the cell cycle. In G0 and early G1, DREAM and RB complexes repress the expression of cell cycle genes. In the late G1 and S phase, RB releases the activating E2F transcription factors that upregulate G1/S cell cycle genes encoding for important proteins in the process of DNA replication. When the S phase is completed, E2F7 and E2F8 will replace E2F1-3 and serve to repress the expression of the G1/S genes. In G2 and mitosis, B-MYB and FOXM1 transcription factors bind to the MuvB core and promote the expression of G2/M genes that encode important proteins for cell division. These transcription factors, as well as many other important cell cycle effectors, are controlled through phosphorylation by Cyclin-CDK complexes. Cyclin D-CDK4/6 complexes promote cell cycle entry and progression through the G1 phase. Cyclin E-CDK2 complexes stimulate S phase entry and progression, and Cyclin A-CDK2 complexes facilitate S phase completion. Cyclin A-CDK1 complexes promote G2 phase progression, and Cyclin B-CDK1 complexes regulate mitosis. These proteins are targets of an additional layer of cell cycle control that mediates their proteasomal degradation. APC/C$^{\text{CDH1}}$ becomes activated in anaphase and promotes exit from mitosis, and ensures proper G1 phase progression. SCF$^{\text{SKP2}}$ functions in the late G1 and early S phase, while SCF$^{\text{betaTRCP}}$ and SCF$^{\text{FBXW7}}$ are active throughout the S phase. SCF$^{\text{Cyclin F}}$ stimulates mitosis entry, and APC/C$^{\text{CDC20}}$ promotes progression through mitosis. Importantly, SCF, APC/C, and Cyclin-CDK complexes also regulate each other, and all these complexes contain effector proteins that are transcriptionally regulated by RB-E2F and MuvB complexes. This multilayered network ensures precise control of the cell cycle. (See text for names of abbreviated items.)

after DNA damage to allow time for damage assessment and repair. The p21 protein also can be expressed in cells lacking functional p53, indicating that p53-independent pathways of expression exist. These other pathways may account for increased p21 expression in other circumstances associated with cell cycle arrest, such as terminal differentiation. p21 inhibits cell cycle progression primarily through the inhibition of CDK2 activity: RB pocket proteins lose their hyperphosphorylated state, leading to the formation of RB-E2F and DREAM (dimerization partner, RB-like, E2F, and multivulva class B) complexes and downregulation of cell cycle genes. Interestingly, p21 promotes the formation of cyclin D–CDK4/6 complexes, and the resulting RB mono-phosphorylation is required for RB function in the DNA damage response program.[8] Inversely, cyclin D-CDK4/6 can sequester p21, preventing the inactivation of CDK2-containing complexes.

In marked contrast to the transcriptional regulation of p21, regulation of p27 (KIP1, CDKN1B) occurs post-transcriptionally, such that mRNA levels remain largely constant, whereas levels of the protein change. p27 protein levels are maximal during quiescence and early G1, and p27 protein binds and inhibits cyclin E–CDK2 complexes. The progressive decrease in p27 protein levels during G1 allows for activation of cyclin E–CDK2 complexes that are required for the transition into the S phase. Notably, to the end of the G1 phase, active cyclin E–CDK2 complexes phosphorylate p27, causing its ubiquitin-mediated degradation and leading to a dramatic decline in p27 protein levels. In accordance with the ability of p21 and p27 to inhibit cyclin-CDK activity and cell cycling, *p21* and *p27* are candidate tumor suppressor genes, but silencing or loss of these genes is very uncommon in cancers. Unlike its ubiquitous siblings, p57 has a tissue-specific expression pattern during embryogenesis and, in the adult, it is the only CDK inhibitor required for embryonic development.

Striking diversity in the pattern of expression of *INK4* genes suggests that this family of CDK inhibitors might have cell type–specific or tissue-specific functions. The two founding members, p16 and p15, were cloned as tumor suppressor genes, and the p18 and p19 proteins were subsequently cloned on the basis of homology to p16 and p15. INK4 CDK inhibitors block CDK4 and CDK6, and as RB family proteins are prime targets of cyclin D–CDK4/6 kinase, phosphorylation of these proteins is crucial for G1 progression; the INK4 inhibitor family induces cell cycle arrest in G1. Evidence that INK4 proteins inhibit cell proliferation by preventing phosphorylation of RB pocket proteins is provided by the observation that p16 overexpression inhibits proliferation only of cells containing functional RB pocket protein. p16 and p15 CDK inhibitors are inactivated by mutations in various cancers, providing evidence for their function as tumor suppressors. Importantly, p16 and p15 are neighboring genes on chromosome 9, and *CDKN2A*, the gene encoding for p16, also encodes for ARF using an alternative reading frame that produces a totally different protein. This overlap means that inactivating mutations affect p16 and ARF function. Because ARF is a positive regulator of p53 expression and a tumor suppressor protein in its own right, attribution of a tumor suppressor effect to each of these two genes is difficult. An interesting aspect of p16 expression is its upregulation in aging tissues and in response to oncogene activation. For example, in response to continuously high RAS or E2F activity, *CDKN2A* is transcriptionally induced, leading to cell cycle arrest and senescence.

TRANSCRIPTIONAL REGULATION: MYC, RB-E2F, AND DREAM/MUVB

Cell cycle entry and progression require specific genes to be expressed at certain times. When cell proliferation is triggered by growth conditions and cells leave quiescence, mitogen signaling leads to the activation of the MYC transcription factor.[1] MYC has the capability to drive cell proliferation because it activates cyclin D and CDK4/6, and it plays an important role in cell growth because it upregulates ribosomal RNA and proteins, leading to increased ribosome biogenesis and translation. Indeed, experiments showed that when MYC is missing, cell growth and proliferation slow down, and cells arrest primarily in the G1 phase. MYC activity ensures that cells reach a certain size before entering the S phase and progressing through the cell cycle.

The two main cell cycle events—DNA synthesis and mitosis—take place after the cell passes the G1/S restriction point and is committed to cell cycle progression. Entry into the S phase requires the expression of genes required for DNA replication. These genes are distinct from the ones required for entry into mitosis. In general, there are two major waves of gene expression, one occurring just before entry into the S phase and a second wave occurring just before entry into mitosis (see Figs. 17.3 and 17.4). The periodic expression of mRNA produces the specific protein factors required for DNA replication and cell division. Once the S phase is completed and cells have passed through mitosis, many of these protein factors are ubiquitinated and degraded by the proteasome, thereby ensuring one-way progression through the S and M phases of the cell cycle.

Proliferating cells require the expression of specialized genes for the synthesis of DNA during the S phase and for cell division during mitosis. These cell cycle–dependent genes are not typically required for the survival of quiescent cells. The expression of more than 1000 cell cycle–dependent genes is nearly absent during quiescence in G0 cells. For the most part, the expression of these cell cycle–dependent genes is silenced by the *trans*-repressor complexes DREAM and RB-E2F.[11] The DREAM complex is a multi-subunit protein complex that binds to promoters of cell cycle–regulated genes and contributes to their repression. The DREAM complex is comprised of the RB-like protein p130 (RBL2), E2F4 or E2F5, DP1 or DP2, and the 5-component MuvB complex containing LIN9, LIN37, LIN52, LIN54, and RBBP4.[12] Whereas the DREAM complex contributes to the expression of both S phase– and M phase–specific genes, the RB protein specifically represses S phase–specific genes.[13] RB binds to the activating E2F transcription factors (E2F1–E2F3) and blocks E2F-mediated activation of S phase genes. S and M phase genes possess distinct promoter elements that mediate the binding of RB-E2F and DREAM complexes. E2F promoter elements contain the canonical DNA sequence TTTCGCGC and specifically mediate binding of E2F transcription factors in S phase genes. In contrast, M phase genes possess cell cycle genes homology region (CHR) promoter elements that comprise the canonical DNA sequence TTTGAA and specifically mediate the binding of the MuvB complex.[14]

When a quiescent cell is stimulated to enter the cell cycle, it begins to express cyclin D that binds to CDK4 or CDK6 and is capable of

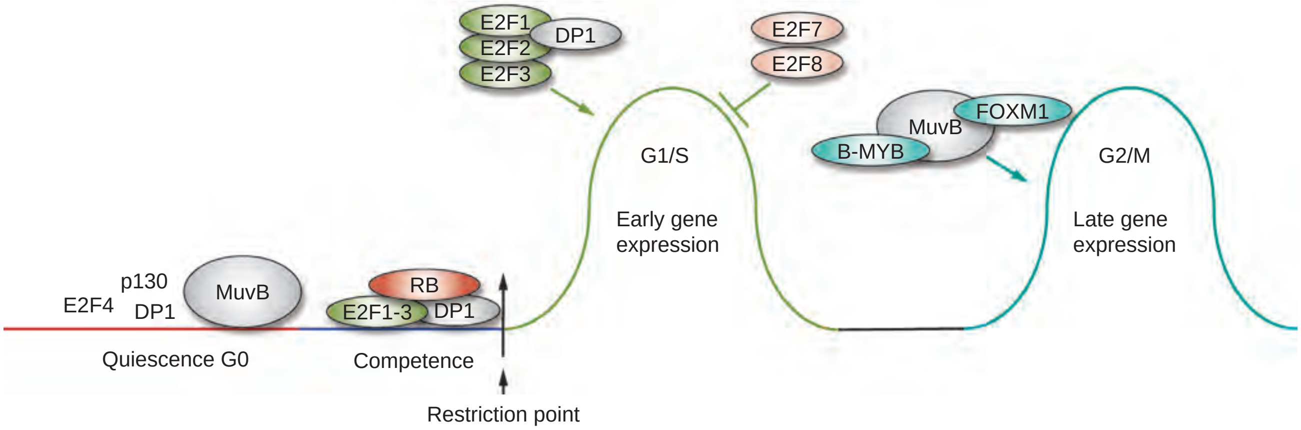


Figure 17.4 TRANSCRIPTION CONTROL OF G1/S AND G2/M CELL CYCLE GENES. There are two major waves of gene expression, with one occurring just prior to entry into the S phase and a second wave just prior to entry into mitosis. The DREAM complex represses both early and late cell cycle gene expression during quiescence *(red line)*. RB binds to the activating E2F transcription factors (E2F1–3) and blocks E2F-mediated activation of S phase genes. In the late G1 phase and S phase, the activating E2Fs dimerize with DP1 or DP2, bind to the promoters of genes required for DNA synthesis through E2F promoter elements, and promote their expression. During the late S phase, E2F7 and E2F8 will replace E2F1–3 and serve to repress the expression of the G1/S genes when DNA synthesis is completed. The B-MYB transcription factor binds to the MuvB core when p130, E2F4, and DP are released from the DREAM complex. The MMB complex recruits FOXM1 to promote the expression of genes in the late G2 phase and M phase.

monophosphorylating RB. In the late G1 phase, Cyclin E–CDK2 complexes multi-phosphorylate the monophosphorylated RB, leading to the release of the activating E2F transcription factors.[8] The activating E2Fs dimerize with DP1 or DP2, bind to the promoters of genes required for DNA synthesis through E2F promoter elements, and promote their expression during the late G1 phase and S phase. These G1/S genes encode many of the factors required for DNA replication. During the late S phase, E2F7 and E2F8, which are E2F targets themselves, will replace E2F1–E2F3 and serve to repress the expression of the G1/S phase genes when DNA synthesis is completed.

Cyclin D–CDK4/6 and cyclin E–CDK2 are also capable of phosphorylating p130, thereby enabling the release of p130 from the DREAM complex. The B-MYB (MYBL2) transcription factor is encoded by a G1/S gene and binds to the MuvB core when p130, E2F4, and DP are released from the DREAM complex. The newly formed MMB (B-MYB–MuvB) complex does not contain any E2F transcription factor and thus binds exclusively to genes that possess CHR promoter elements. These genes are highly expressed in late G2 phase and M phase and encode for proteins that carry out essential functions in mitosis. The MMB complex recruits FOXM1, a third transcription factor, to promote the expression of these G2/M genes. Notably, *FOXM1* is a G2/M gene itself, and together with the important role of FOXM1 in promoting the expression of G2/M genes, *FOXM1* has emerged as one of the most robust biomarkers for stratifying high-risk and low-risk cancers.[15] The B-MYB and FOXM1 factors are ubiquitinated and destroyed by the proteasome during late mitosis, resulting in a decrease in expression of the G2/M genes when a cell exits mitosis. Therefore the DREAM complex represses all cell cycle genes during quiescence, with the G1/S cell cycle genes being activated by E2F1–E2F3 and the G2/M late cell cycle genes being activated by B-MYB–MuvB–FOXM1.

Together, proliferation signals will enable MYC-dependent gene expression that contributes to the growth in the size of the cell, whereas the E2F and B-MYB/FOXM1-dependent gene expressions induce genes required for DNA replication during the S phase and cell division during mitosis. Given the strong link between the expression of cell cycle-regulated genes and cell proliferation, cell cycle genes have prognostic value for stratifying cancers because malignancy correlates with the number of proliferating cells in a tumor.[16]

UBIQUITINATION

Cells employ a variety of mechanisms to control the cell division cycle, ensuring one-way progression through S and M phases of the cell cycle. Cell cycle genes are periodically expressed at certain times, and cyclin-CDK complexes drive the cell cycle through the control of the cell cycle transcription machinery and regulation of critical events in S and M phases. The mRNA of many cyclins and CDKs is also cell cycle–dependently regulated, and in addition, cyclin-CDK activity is regulated by CDK inhibitors. Yet, cells employ an additional layer of cell cycle control through the timed destruction of essential cell cycle proteins, including cyclins, CDKs, and cell cycle transcription factors (see Fig. 17.3). Two ubiquitin ligases, the Skp, Cullin, F-box–containing complex (SCF) and the anaphase-promoting complex/cyclosome (APC/C), are responsible for the specific ubiquitination of many of these cell cycle proteins.[17] The SCF complex consists of the three invariable components RBX1, CUL1, and SKP1. RBX1 is a RING finger protein that functions as an E3 ubiquitin ligase, CUL1 is a scaffold protein, and SKP1 is an adaptor protein. In addition to the three invariable components, one variable coactivator, known as an F-box protein, binds through its F-box motif to SKP1 and is responsible for substrate recognition. Approximately 70 putative F-box proteins have been identified in humans, and at least four of them are thought to be involved in cell cycle control: SKP2, FBXW7, β-transducin repeats-containing proteins (β-TRCP), and cyclin F. The SCF complex mediates the degradation of cell cycle proteins during the progression from the late G1 phase through the S phase until the onset of mitosis. Important substrates include cyclin D and cyclin E; the transcriptional regulators MYC, E2F1, and p130; the CDK inhibitors p27 and p21; and WEE1 kinase. WEE1 kinase deactivates cyclin B–CDK1 complexes. Degradation of these important cell cycle regulators allows for proper S phase entry and the completion and onset of mitosis.

The APC/C complex consists of several invariable components, and the central ones are structurally similar to the SCF complex components. APC11 is a RING-finger protein related to RBX1, and APC2 is a scaffold protein related to CUL1. Similar to the SCF complex, APC/C contains a variable coactivator that confers substrate specificity. Two of such variable coactivators function in the cell cycle: CDC20 and CDH1. APC/C^{CDC20} mediates the degradation of substrates during mitosis, whereas APC/C^{CDH1} functions primarily in the G1 phase. Key substrates of APC/C^{CDC20} are cyclin A and cyclin B. With the onset of anaphase, activation of APC/C^{CDH1} leads to the degradation of CDC20, PLK1, and Aurora kinases.

Similar to the other cell cycle control systems, SCF and APC/C are controlled within the cell cycle, in part by their own substrates. Most important, SCF and APC/C can regulate each other. The cell cycle protein FBXO5 (EMI1) functions as an inhibitor of APC/ C^{CDC20} and is sent to proteasomal degradation by $SCF^{\beta\text{-TRCP}}$ when mitosis starts, and $SCF^{\text{cyclin F}}$ mediates degradation of CDH1. Similarly, ubiquitination of CDC20 and cyclin F by APC/C^{CDH1} leads to their destruction when cells exit mitosis. Further regulation occurs on the transcriptional level because substrate-specific components of SCF and APC/C are encoded by cell cycle–regulated genes, including *SKP2*, *FBXO5*, *Cyclin F*, and *CDC20*.

DEOXYRIBONUCLEIC ACID REPLICATION

The initiation of DNA replication represents a commitment to cell proliferation and is a central event in the growth and division of all organisms.[18] The assembly of replication machineries is coordinated by multiple proteins, which ensure that DNA synthesis begins at the correct chromosomal locus. Strict regulation of initiation is crucial to viability because inappropriate replication start is linked to genetic instability, including alterations in gene copy number and DNA damage. DNA replication starts with the assembly of prereplication complexes (pre-RCs) at multiple DNA replication origins during the G1 phase. At the transition from G1 to S phase, the replicative helicase is activated, leading to a change from pre-RCs to preinitiation complexes, which unwind DNA and initiate DNA synthesis. The recognition of pre-RC sites is known as replication origin licensing, and the activation of DNA synthesis is known as origin firing. The separation of these two steps is critical for preventing re-replication within the same cell cycle.

Replication origin licensing in the G1 phase involves the sequential assembly of different proteins. The origin recognition complex (ORC) consists of the six subunits ORC1–ORC6 and initially binds to replication origins. Then, CDC6 and CDT1 associate with the ORC, leading to the recruitment of the minichromosome maintenance (MCM) complex, which consists of six subunits: MCM2–MCM7. The MCM complex is a helicase that forms a double hexamer and encircles double-stranded DNA. Together, the inactive assembly of ORC, CDC6, CDT1, and MCM is known as the pre-RC. Notably, the Meier-Gorlin syndrome, a form of primordial dwarfism, is linked to mutation or depletion of pre-RC components, which impairs cell cycle progression.

During the transition from G1 to S phase, the MCM complex is phosphorylated by CDC7 and cyclin E–CDK2, leading to the recruitment of CDC45 and the four-protein DNA replication complex GINS. Together, CDC45, MCM, and GINS represent the CMG complex, and dissociation of the two MCM hexamers enables the formation of two preinitiation complexes at a replication origin. Cyclin E–CDK2 phosphorylates Treslin (TICRR) and RECQL4 that, with TOPBP1 and MCM10, facilitate the formation of the preinitiation complex. After replication origins are licensed, ORC1 phosphorylation by cyclin A–CDK1 inhibits rebinding of ORC1 to replication origins, and CDT1 is blocked by Geminin (GMNN) and sent to proteasomal degradation by SCF^{SKP2} to prevent relicensing during the S phase.

In the S phase, initiation of DNA replication by CDK2 promotes helicase activation, which leads to the unwinding of DNA and recruitment of DNA polymerases alpha (POLA), delta (POLD), and epsilon (POLE), together with PCNA (proliferating cell nuclear antigen) and RFC (replication factor C). The MCM complex translocates from replication origins to unwind double-stranded DNA, whereas POLA binds to single-stranded DNA and initiates DNA synthesis on both the leading and lagging strands by providing an RNA primer and synthesizing the first bases of DNA. POLE and POLD elongate these primers with a base substitution error rate of approximately 10^{-5}. When they occasionally incorporate a false nucleotide, it is usually removed by an exonuclease associated with these DNA polymerases. This so-called proofreading, together with DNA mismatch repair mechanisms, leads to mutation rates of as low as 10^{-9} per base and per cell division cycle.

An immediate consequence of DNA replication is the disruption of chromatin in front of the replication fork, which results in the release of parental histones. When the replication fork has passed, chromatin is rapidly reassembled onto old as well as newly replicated DNA, and in the reassembled chromatin, half of the histones are recycled from the parental chromatin, whereas the other half are newly synthesized.[19] Then, the multiprotein complex cohesin mediates cohesion between replicated sister chromatids in the S phase, which is essential for chromosome segregation in the M phase.

In addition to DNA replication, the centrosome also needs to be replicated to allow the formation of two daughter cells.[20] The centrosome is the major microtubule-organizing center and consists of two centrioles. Phosphorylation by cyclin E–CDK2 promotes the formation of one procentriole at each centriole. During S and G2 phases, procentrioles elongate until they reach the length of the older centrioles. In the early M phase, the two centrosomes separate to promote cytokinesis.

MITOSIS

The decision to enter mitosis is mediated by a network of proteins that regulate activation of the cyclin B–CDK1 complex.[21] In principle, a feedback system between CDK1, WEE1/CDC25C, and the CDK1-counteracting phosphatases generates a bistable switch, leading to robust directionality that prevents cells from going back and forth between G2 and M phases. Cyclin B and CDK1 levels increase in the late S phase and G2 phase, and the activity of newly made cyclin B–CDK1 complexes is blocked through phosphorylation by WEE1. Activation of the cyclin B–CDK1 complex is the key to cell entry into mitosis and occurs just before mitosis through the action of CDC25C, causing dephosphorylation of CDK1. The activities of WEE1 and CDC25C are themselves regulated with phosphorylation, inhibiting WEE1 function and enhancing CDC25C function. Once a small amount of cyclin B–CDK1 is activated, it can phosphorylate CDC25C and create a self-amplifying feedback loop that generates more active cyclin B–CDK1 from the large preexisting stock of inactive complex. What starts this sequence of events by initially phosphorylating and activating CDC25C is unclear, but PLK1 is a candidate kinase that can phosphorylate WEE1, CDC25C, and cyclin B, leading to nuclear localization of cyclin B and CDC25C. Cyclin B–CDK1 can phosphorylate serine/threonine residues in many cellular proteins that are essential for mitosis. Phosphorylation of lamins by cyclin B–CDK1 promotes nuclear lamina disassembly and envelope breakdown, which occurs during prophase. Phosphorylation of some kinesin motor proteins that function in spindle assembly, such as KIF11 and CENPE, by cyclin B–CDK1 increases their efficiency of microtubule binding and increases processive motility along microtubules. Other kinesins that function in midzone formation, such as KIF23, are blocked in their activity by cyclin B–CDK1–mediated phosphorylation.

After chromosome condensation, centrosome separation, and nuclear envelope breakdown during prophase, chromosomes attach to microtubules of the mitotic spindle apparatus in prometaphase. Kinetochores were originally called *centromeres* because they are located at the center of chromosomes. They are formed by centromere proteins during the G2 phase and prophase and link chromosomes to the mitotic spindle apparatus so that sister chromatids become attached to opposite poles. In metaphase, the chromosomes align at the equatorial plate, and cyclin B–CDK1 levels start to decline by APC/CCDC20-mediated degradation of cyclin B. The APC/CCDC20 further targets securin (PTTG1) and thereby activates separase (ESPL1), which cleaves the cohesin complexes linking sister chromatids. Once CDK1 activity is low, APC/CCDH1 becomes active and removes CDC20, PLK1, and Aurora kinases, as well as the mitotic cyclins and CDKs.

In anaphase, the spindle midzone forms between the separating chromosomes by coordinated actions of the microtubule cross-linker PRC1, the kinesin KIF4, and the multiprotein complexes centralspindlin and CPC (chromosomal passenger complex).[22] Centralspindlin consists of the kinesin KIF23 and the Rho GTPase RACGAP1, and the CPC consists of Aurora kinase B, the scaffold INCENP, survivin (BIRC5), and CDCA8. Phosphorylation by CPC promotes recruitment of centralspindlin to the spindle midzone through KIF23 and allows for multimerization and accumulation of centralspindlin, which in turn promotes localization of the RhoGEF ECT2 (epithelial cell transforming 2) to microtubules. ECT2 then fuels RhoA, which promotes the recruitment of effector contractile ring proteins. As the ring closes, the spindle midzone is remodeled to form the densely packed telophase midbody, which organizes the intracellular bridge. At this time in telophase, nuclear membranes form to envelop each of the two separated sets of chromosomes, which also begin to decondense. This is soon followed by the abscission event near the midbody, which completes mitosis.

CELL CYCLE CHECKPOINTS

Competence

Cells require the presence of nutrients and growth factors to switch from quiescence to a state of proliferation. When cells sense that conditions are suitable for proliferation, they leave quiescence into the G1 phase and become competent to enter the cell cycle. G1 has been subdivided into segments and regulatory points based largely on the study of the proliferative response of cells to sequential application of different growth factors, nutrients, and metabolic inhibitors. From the standpoint of cell cycle regulation, a particularly important point in G1 is the restriction point, or R, which occurs near the G1–S boundary. The period after mitosis, when cells can enter quiescence, is termed G1pm (postmitosis), and the period between quiescence and S phase is termed G1ps (pre-DNA synthesis) (Fig. 17.5). Notably, nearly all of the variability in the length of G1 can be accounted for by the G1ps interval. Experiments have shown that, to leave quiescence and to enter the cell cycle, cells require growth signals either continuous for several hours during G1 or, alternatively, as two discrete pulses of approximately 1 hour in duration and with a pause of several hours in between.[20] To become competent for cell cycle entry, initial activation of the MAPK pathway by mitogen signals is required that in turn activates essential metabolic programs. However, this initial increase in MAPK activity does not lead to induction of MYC and cyclin D; it leads only to the presence of growth signals several hours later that activate the PI3K pathway and MYC. Functions of MYC include transcriptional activation of *CDK4* and *Cyclin D*, as well as downregulation of CDK inhibitors. The following increase in cyclin D–CDK4 complex activity leads to phosphorylation of the principal target RB during the G1 phase.

Restriction Point

In 1974, Arthur Pardee published the first report on the restriction point and defined it as a point at which cells become committed to

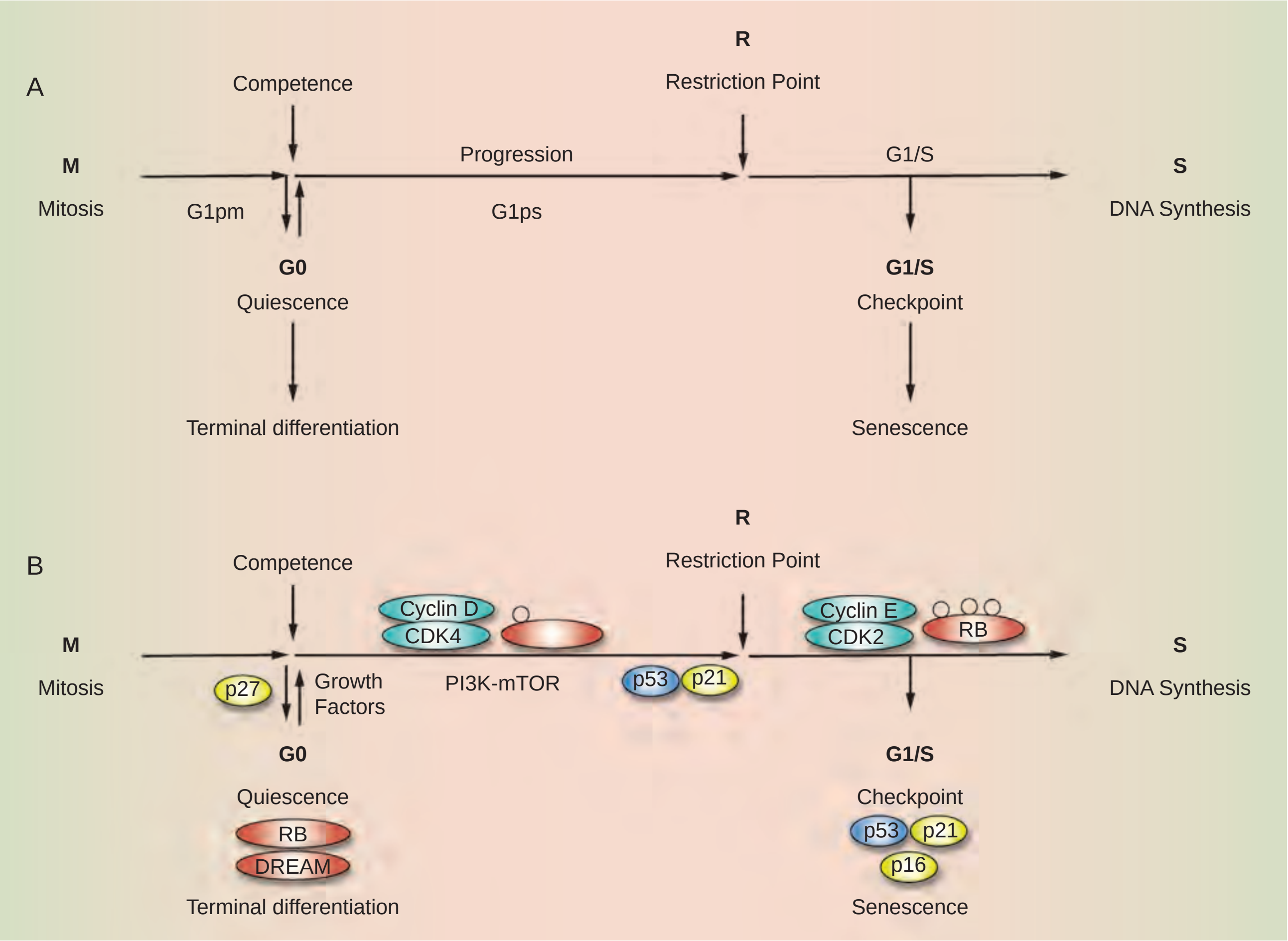


Figure 17.5 CHECKPOINTS OF CELL CYCLE ENTRY. (A) Quiescence is a non-proliferative state in which viable cells have left the cell cycle and may remain for prolonged periods. In contrast, terminally differentiated cells have irreversibly exited the cell cycle during the process of differentiation. When cells sense that conditions are suitable for proliferation, they leave quiescence into the G1 phase and become competent to enter the cell cycle. G1 has been subdivided into segments, and a particularly important point is the restriction point, or R, which occurs near the G1-S boundary. The period after mitosis, when cells can enter quiescence, is termed G1pm (post mitosis), and the period between quiescence and S phase is termed G1ps (pre-DNA synthesis). When DNA damage is recognized in G1, the G1/S checkpoint becomes activated, which blocks cells from S phase entry, although they may have passed the restriction point. If damage is not repaired timely, cells will enter senescence where they remain viable but not capable of re-entering the cell cycle. (B) During quiescence, the CDK inhibitor p27 prevents Cyclin-CDK activity, and DREAM and RB bind and repress cell cycle genes. When prompted by growth signals, cells enter the competent state. Activation of RAS-MAPK and PI3K signaling pathways is followed by activation of Cyclin D-CDK4/6, leading to mono-phosphorylation of RB. The progressive decrease in p27 protein levels during G1 allows for activation of Cyclin E-CDK2, which multi-phosphorylate the mono-phosphorylated RB, leading to the release of the activating E2F transcription factors. When DNA damage is recognized in G1, p53 becomes activated, and the p53 target gene *p21* promotes cell cycle arrest at the G1/S transition through inhibition of Cyclin E-CDK2 and activation of RB. Also, p16 can become activated by oncogenic stress, which leads to inhibition of Cyclin D-CDK4/6 and activation of the G1/S checkpoint. If stress signaling is not relieved, cells will enter a senescent state.

entering the S phase, regardless of subsequent availability of growth factors or essential nutrients.[23] He also correctly predicted that cancer cells undergo changes to become independent from growth factors and the restriction point. In the four decades that have passed since the initial description of the restriction point, many important insights have been gained that revealed the signaling events that contribute to proliferation and growth. In addition to the key contributions of signaling by MAPK, PI3K, and MYC to enable cell growth, it also became clear that there are restraining activities that can inhibit cell cycle entry and progression. As mentioned earlier, cyclin D levels increase during the progression phase of G1, and cyclin D–CDK4/6 complexes monophosphorylate RB, which restricts the activating E2F transcription factors.[8] Later in G1, cyclin E levels increase, and cyclin E binds specifically to CDK2. This leads to RB hyperphosphorylation and release of the activating E2Fs, enabling E2F-dependent gene expression. Once past this point, growth factors are no longer required for S phase entry. Therefore, expression and activation of cyclin E–CDK2 resulting in the hyperphosphorylation of RB enables a cell to pass the restriction point and become committed to cell cycle entry.

G1/S Checkpoint

The G1/S DNA damage checkpoint can be viewed as a point in the cell cycle when the cell has become fully committed to entering into the S phase and past the restriction point but is unable to enter the S phase because cyclin E–CDK2 and cyclin D–CDK4 are inactivated by p21 and p16, respectively, and RB remains capable of binding to and repressing the activating E2Fs, thereby decreasing levels of factors required for DNA synthesis. In G1, DNA damage is recognized by ataxia-telangiectasia mutated (ATM) kinase, which in turn phosphorylates histone variant H2AX to recruit repair factors and CHEK2 kinase.[24] CHEK2-mediated phosphorylation activates p53 and inactivates CDC25A, which is required for the activation of cyclin E–CDK2 complexes. The p53 target genes *p21* and *BTG2* further promote cell cycle arrest at the G1/S transition through inhibition of cyclin E–CDK2 and activation of RB. If the DNA damage or stress signals are repaired, cells can exit the G1/S checkpoint and re-enter the cell cycle.

S Phase Checkpoint

Under conditions that put DNA replication at risk, such as DNA damage or nucleotide depletion, the S phase checkpoint gets activated. Ataxia-telangiectasia and Rad3-related protein (ATR) is the main kinase that senses DNA damage during the S phase, and it phosphorylates CHEK1 kinase, which in turn activates p53. Similar to the G1/S checkpoint, inhibition of cyclin E–CDK2 is central to the S phase checkpoint. The inhibition of CDK2 activity blocks the loading of CDC45 onto replication origins and prevents the initiation of new origin firing. In addition to inhibiting cyclin E–CDK2, p21 directly interacts with PCNA to stop DNA replication. If the damage is repaired, cells continue DNA replication and cell cycle progression.

G2/M Checkpoint

The G2/M checkpoint prevents cells from initiating mitosis when DNA damage occurs during G2 or when cells progress into G2 with some unrepaired damage inflicted during previous S or G1 phases. The G2/M checkpoint also involves DNA damage recognition by ATM and ATR kinases and subsequent p53 activation through CHEK1 and CHEK2 kinases, and it ultimately requires activation of the p21 CDK inhibitor. If p21 is missing, both G1/S and G2/M checkpoints are abolished. In addition to p21, p53 induces GADD45A and 14-3-3 (stratifin (SFN)), which contribute to G2/M cell cycle arrest. 14-3-3 removes essential mitotic regulators from the nucleus and thereby promotes G2/M arrest. Moreover, inhibition of cyclin-CDK activity through p21 induces DREAM and RB-E2F complexes, which in turn repress the transcription of the cell cycle machinery.[11]

Senescence

If damage is not repaired timely, cells will enter a senescent state in which they remain viable but not capable of re-entering the cell cycle. Telomere shortening, which signals cell aging, also is recognized as a type of DNA damage and can trigger senescence.[25] RB is key in establishing the senescent state, which is activated downstream of p53 and the CDK inhibitors p16 and p21. During senescence, cells have committed to proliferation and presumably have passed the restriction point. In contrast to quiescence, senescent cells are unable to re-enter the cell cycle in response to external stimuli, such as growth signals.

Spindle Assembly Checkpoint

The spindle assembly checkpoint (SAC) involves the MAD (mitotic arrest deficient) proteins MAD1, MAD2, BUBR1 (MAD3), and BUB1.[26] To complete mitosis, the cell strictly requires the activity of cyclin B–CDK1. The main effector of the SAC is the mitotic checkpoint complex (MCC), which consists of MAD2, BUBR1, and BUB3 and binds to CDC20, the substrate-specific cofactor of the APC/C that mediates degradation of cyclin B and securin. Inhibition of CDC20 prolongs prometaphase until all chromosomes have become correctly orientated on the metaphase plate. Unattached kinetochores recruit MCC through BUB1, leading to active MCCs. Only when kinetochores are attached correctly to the mitotic spindle is the MCC deactivated, which in turn activates APC/CCDC20 and allows for mitosis progression.

SPECIAL HEMATOLOGY CELL CYCLE FEATURES

Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are characterized by their ability to execute multiple cell fate choices, including self-renewal, quiescence, and differentiation into the many different mature blood cell types. The stem cell niche forms the essential microenvironment for HSCs, and the primary HSC niche in adult organisms is located in the bone marrow. Stem cells are quiescent until prompted to proliferate by external stimuli. Stem cell quiescence is achieved largely through the CDK inhibitors p21, p27, and p57.[27] The pool of HSCs contains subsets that differ in self-renewal potential and cell division frequency. So-called short-term HSCs rapidly enter the cell cycle upon mitogen stimulation, whereas long-term HSCs exit quiescence later. This difference can result at least in part from varying CDK6 levels in HSC subsets. The absence of CDK6 in the long-term compared with short-term HSCs can result in a delay in quiescence exit, and the cumulative effect of this delay limits proliferation of these cells and ultimately preserves the long-term integrity of the HSC pool.

Endoreplication

A special type of cell cycle progression is featured in the differentiation of cells that have high metabolic profiles required for the synthesis of specific proteins, such as plasma proteins produced by hepatocytes, or for the production of platelets by megakaryocytes (MKs). Thrombopoietin (TPO) is the major regulator that directs the growth and development of MKs from HSCs. TPO binds to the MK-specific receptor MPL, leading to MPL dimerization and activation of JAK2 (Janus kinase 2). Initially, this signaling leads to endoreplication and accumulation of DNA content of up to well over 128 N before proceeding to final maturation and proplatelet formation.[28] Endoreplication requires impaired cyclin B–CDK1 kinase activity, which is achieved through upregulation of the CDK inhibitor p21 and induction of the APC/C subunit CDH1 after S phase completion. MK endoreplication also requires the prevention of active RhoA through downregulation of ECT2. During the S phase, cyclin E–CDK2 inactivates CDH1 to allow for proper DNA replication. As a result of the following CDK1 inhibition, mitosis is completely bypassed after DNA replication. Inhibition of CDK1 and RhoA and activation of ACP/CCDH1 promote bypass of mitosis and entry into the next G1 phase.

REFERENCES

1. Kress TR, Sabò A, Amati B. MYC: connecting selective transcriptional control to global RNA production. *Nat Rev Cancer*. 2015;15:593–607.
2. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell*. 2010;141:1117–1134.
3. Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. *Annu Rev Immunol*. 2009;27:591–619.
4. Treanor B. B-cell receptor: from resting state to activate. *Immunology*. 2012;136:21–27.
5. Stanley ER, Chitu V. CSF-1 receptor signaling in myeloid cells. *Cold Spring Harb Perspect Biol*. 2014:6.
6. Coleman ML, Marshall CJ, Olson MF. RAS and RHO GTPases in G1-phase cell-cycle regulation. *Nat Rev Mol Cell Biol*. 2004;5:355–366.
7. Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer*. 2009;9:153–166.
8. Narasimha AM, Kaulich M, Shapiro GS, et al. Cyclin D activates the Rb tumor suppressor by mono-phosphorylation. *Elife*. 2014;3:1068–1080.
9. Besson A, Dowdy SF, Roberts JM. CDK inhibitors: cell cycle regulators and beyond. *Dev Cell*. 2008;14:159–169.
10. Abbas T, Dutta A. P21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer*. 2009;9:400–414.
11. Fischer M, Grossmann P, Padi M, et al. Integration of TP53, DREAM, MMB-FOXM1 and RB-E2F target gene analyses identifies cell cycle gene regulatory networks. *Nucleic Acids Res*. 2016;44:6070–6086.

12. Sadasivam S, DeCaprio JA, et al. The DREAM complex: master coordinator of cell cycle-dependent gene expression. *Nat Rev Cancer*. 2013;13:585–595.
13. Schade AE, Oser MG, Nicholson HE, et al. Cyclin D-CDK4 relieves cooperative repression of proliferation and cell cycle gene expression by DREAM and RB. *Oncogene*. 2019;38:4962–4976.
14. Fischer M, Müller GA. Cell cycle transcription control: DREAM/MuvB and RB-E2F complexes. *Crit Rev Biochem Mol Biol*. 2017;52:638–662.
15. Gentles AJ, Newman AM, Liu CL, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med*. 2015;21:938–945.
16. Whitfield ML, George LK, Grant GD, et al. Common markers of proliferation. *Nat Rev Cancer*. 2006;6:99–106.
17. Teixeira LK, Reed SI. Ubiquitin ligases and cell cycle control. *Annu Rev Biochem*. 2013;82:387–414.
18. Fragkos M, Ganier O, Coulombe P, et al. DNA replication origin activation in space and time. *Nat Rev Mol Cell Biol*. 2015;16:360–374.
19. Stewart-Morgan KR, Petryk N, Groth A. Chromatin replication and epigenetic cell memory. *Nat Cell Biol*. 2020;22:361–371.
20. Nigg EA, Holland AJ. Once and only once: mechanisms of centriole duplication and their deregulation in diseases. *Nat Rev Mol Cell Biol*. 2018;19:297–312.
21. Mcintosh JR. Mitosis. *Cold Spring Harb Perspect Biol*. 2016;8:a023218.
22. Green RA, Paluch E, Oegema K. Cytokinesis in animal cells. *Annu Rev Cell Dev Biol*. 2012;28:29–58.
23. Pardee AB. A restriction point for control of normal animal cell proliferation. *Proc Natl Acad Sci USA*. 1974;71:1286–1290.
24. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. *Nature*. 2004;432:316–323.
25. Muñoz-Espín D, Serrano M. Cellular senescence: from physiology to pathology. *Nat Rev Mol Cell Biol*. 2014;15:482–496.
26. Musacchio A. The molecular biology of spindle assembly checkpoint signaling dynamics. *Curr Biol*. 2015;25:R1002–R1018.
27. van Velthoven CTJ, Rando TA. Stem cell quiescence: dynamism, restraint, and cellular idling. *Cell Stem Cell*. 2019;24:213–225.
28. Fox DT, Duronio RJ. Endoreplication and polyploidy: insights into development and disease. *Development*. 2013;140:3–12.

CHAPTER 18

CELL DEATH

Paolo Strati, Marina Konopleva, and William Wierda

CORE APOPTOSIS PATHWAYS

Apoptosis is a biochemically well-defined programmed cell death that is essential for normal development and cellular homeostasis; dysregulation of apoptosis is involved in several diseases. The classical morphological features of apoptosis include cell shrinkage, membrane blebbing, nuclear condensation, and deoxyribonucleic acid (DNA) fragmentation. The biochemical hallmarks of apoptosis include permeabilization of the outer mitochondrial membrane (MOMP), activation of caspases, and the externalization of phosphatidylserine, leading to phagocytosis of the apoptotic remnants without inducing inflammation.

In mammals, apoptosis is initiated by either internal or external stimuli and is governed by two molecular programs that terminate in caspase activation: the intrinsic (mitochondria-mediated) pathway and the extrinsic (death receptor-mediated) pathway. The intrinsic pathway is regulated by B-cell lymphoma (BCL-2) family proteins that determine whether MOMP occurs. Upon MOMP, cytochrome C is released into the cytosol, where it binds apoptotic protease activating factor 1 (APAF-1) and caspase-9 to form the "apoptosome," which in turn cleaves and activates the downstream effectors caspase-3 and caspase-7. Caspases (cysteinyl aspartate–specific protease) are a family of cysteine proteases that function as the central effectors of apoptosis. These executioner caspases cleave multiple cellular substrates, resulting in the nuclear condensation, DNA fragmentation, and membrane blebbing that are characteristic of apoptosis. Other apoptogenic factors released from mitochondria, including apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspase (SMAC)/Diablo, Omi/HtrA2, and endonuclease G, augment intrinsic apoptosis. The extrinsic pathway is initiated upon ligation of the death receptors of the tumor necrosis factor (TNF) receptor family, which recruit the adapter molecule FAS-associated death domain protein (FADD) and then caspases-8 and -10, leading to activation of other downstream caspases. Conventional, nonconventional, and other mechanisms of apoptosis and other modes of cell death are described in detail below (Table 18.1).

THE INTRINSIC APOPTOTIC PATHWAY

The intrinsic apoptotic pathway relies on the BCL-2 family of proteins, which include multiple proteins that share BCL-2-like homology domains 1-4 (BH1-BH4). The BCL-2 family proteins can be classified by their functions: suppressors (BCL2, BCL-XL, BCL-W, BCL2-A1, and myeloid cell leukemia [MCL-1]), activators (BCL2-interacting mediator of cell death [BIM] and p53 upregulated modulator of apoptosis [PUMA]), effectors (BCL2 associated X [BAX] and Bcl-2 homologous antagonist/killer [BAK]), and sensitizers (NOXA).[1,2] Activators and effectors oligomerize, creating pores in the mitochondrial outer membrane. Subsequently, cytochrome C is released through the membrane and caspase-9 activated, ultimately leading to proteolytic cell death. Suppressors bind to and counteract activators and effector, while sensitizers bind directly to suppressors, which causes their disassociation from effector and activator proteins (Fig. 18.1).

The proapoptotic proteins (including activators and effectors) share a homology only in their BH3 domain and have specific interaction with the five anti-apoptotic proteins (or suppressors) outlined above. By exposing mitochondria to known concentrations of BH3 peptides and measuring the resulting MOMP, it is possible to understand the specific antiapoptotic proteins on which a cell depends for its survival. This technique, called BH3 profiling, is considered the most accurate assessment of BCL-2 family member dependence.[3–5]

NONAPOPTOTIC ROLES OF BCL-2 FAMILY PROTEINS

Biochemical evidence indicates that select BCL-2 proteins interact with several non-BCL-2 protein partners that endow them with homeostatic roles beyond regulation of apoptosis. These functions include, but are not limited to, mitochondrial energy and nutrient metabolism, calcium signaling, cell cycle checkpoints, and DNA damage response.[6,7] In addition, several BCL-2 family proteins have been implicated in the regulation of mitochondrial electron transport chain activity in healthy cells that have not been stressed with any apoptotic signals. For example, BCL-XL interacts with the voltage-dependent anion channel protein that mediates ADP/ATP exchange across the mitochondrial membrane and facilitates mitochondrial respiration.[8,9] BCL2 was reported to be upregulated in quiescent leukemia stem cells (LSCs), where it maintains oxidative phosphorylation;[10] in another report, the combination of BCL2 inhibitor venetoclax and the hypomethylating agent azacitidine disrupted the tricarboxylic acid cycle in LSCs by targeting glutathionylation of mitochondrial complex II of the electron transport chain.[11] Proteolytic processing of MCL-1 produces an N-truncated MCL-1 isoform that is localized to the mitochondrial matrix, where it maintains normal inner mitochondrial membrane structure, regulates mitochondrial fusion, and supports mitochondrial bioenergetic functions.[12] This MCL-1 isoform does not have antiapoptotic activity. Furthermore, this inner mitochondrial membrane form of MCL-1 interacts with very-long-chain acyl-CoA dehydrogenase, a key enzyme in the mitochondrial fatty acid oxidation pathway.[13] Understanding the precise molecular mechanisms underlying these nonapoptotic functions is important for effective targeting of these proteins in disease settings.

THE DEATH RECEPTOR (EXTRINSIC) PATHWAY OF APOPTOSIS

The death receptor or extrinsic apoptotic pathway is initiated upon activation of the TNF death receptors (Fig. 18.2). TNF is the canonical member of the TNF superfamily; additional closely related family members include the TNF-related apoptosis-inducing ligand (TRAIL) and the CD95 ligand (CD95L, also known as FASLG and APO-1L). Upon ligation, signals from the death receptor CD95 (FAS) recruit the adapter FADD and, subsequently, the initiator caspase-8. This brings additional caspase-8 molecules that dimerize and are then activated and cleaved, inducing downstream cleavage of the executioners caspase-3 and caspase-7. This process can be inhibited by c-FLIP, which blocks the dimerization of caspase-8. The protein XIAP, an inhibitor of apoptosis, inhibits the executioner caspases. However, caspase-8 can also cleave the proapoptotic BH3-only protein BID which interferes with antiapoptotic BCL-2 family proteins and activates BAX and BAK, causing MOMP and release of Smac and Omi, which antagonize XIAP, facilitating execution of cell death.

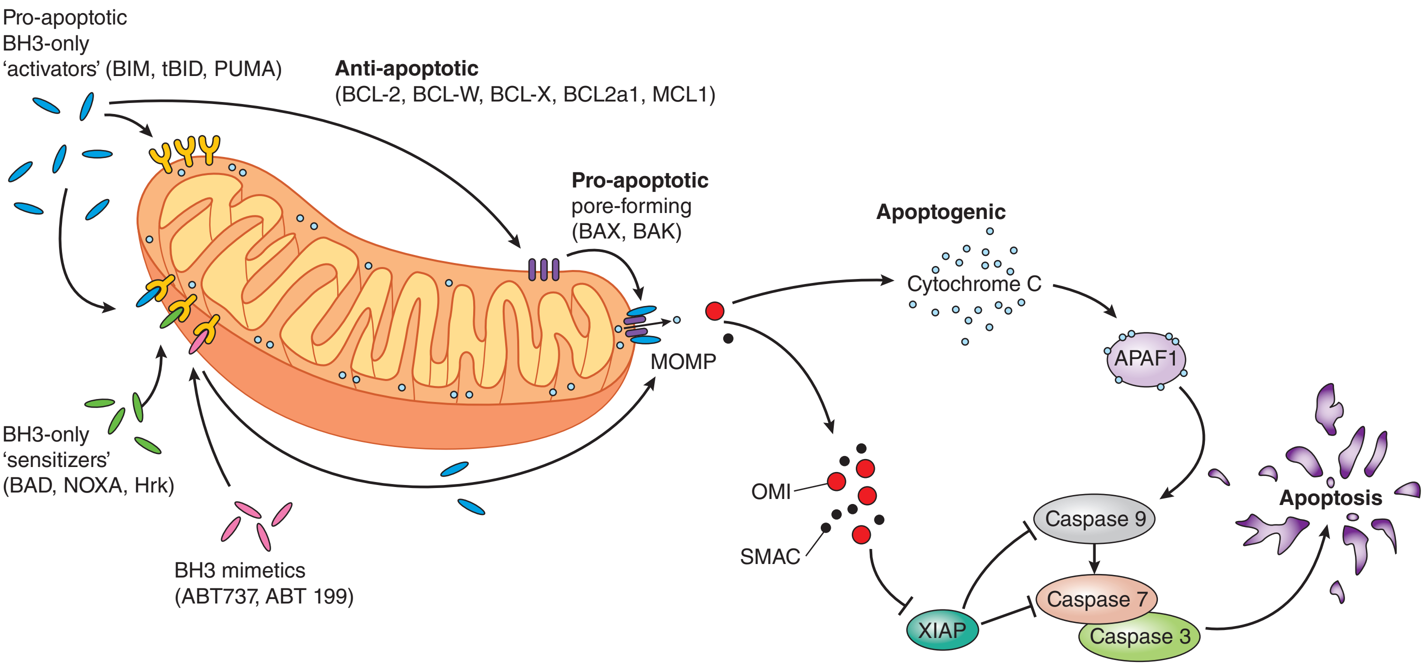

Pro-apoptotic
BH3-only
'activators' (BIM, tBID, PUMA)
Anti-apoptotic
(BCL-2, BCL-W, BCL-X, BCL2a1, MCL1)
Pro-apoptotic
pore-forming
(BAX, BAK)
Apoptogenic
Cytochrome C
APAF1
MOMP
BH3-only
'sensitizers'
(BAD, NOXA, Hrk)
BH3 mimetics
(ABT737, ABT 199)
OMI
SMAC
Caspase 9
Caspase 7
Caspase 3
XIAP
Apoptosis

Figure 18.1 MECHANISMS OF APOPTOSIS: INTRINSIC PATHWAY.

TABLE 18.1 Mechanisms of Apoptosis and Cell Death

Conventional Mechanisms
Mitochondrial (intrinsic) pathway
Death receptor (extrinsic) pathway
Nonconventional Mechanisms
Necroptosis
Ferroptosis
Pyroptosis
Other Mechanisms
Autophagy
Unfolded protein response

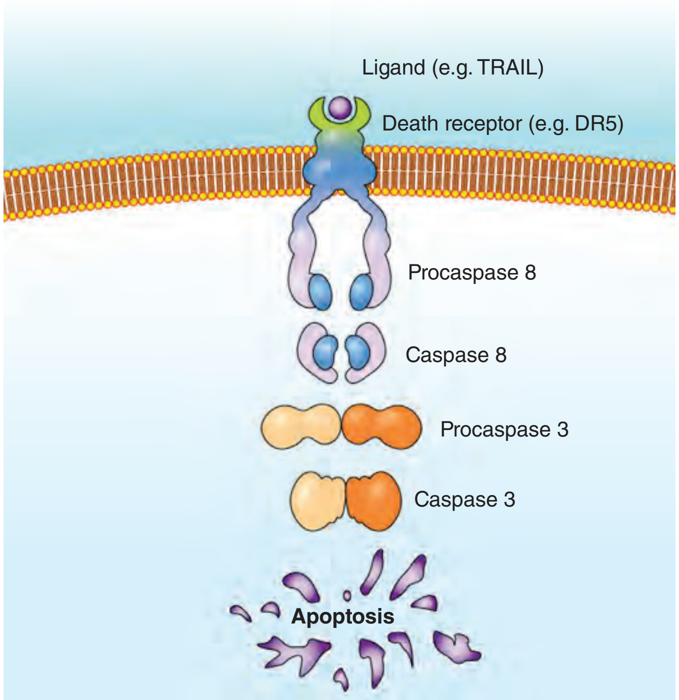

Ligand (e.g. TRAIL)
Death receptor (e.g. DR5)
Procaspase 8
Caspase 8
Procaspase 3
Caspase 3
Apoptosis

Figure 18.2 MECHANISMS OF APOPTOSIS: DEATH RECEPTOR (EXTRINSIC) PATHWAY.

NONAPOPTOTIC FORMS OF CELL DEATH

Necroptosis

Necroptosis (necrosis-like cell death) is a form of caspase-independent programmed cell death that is important in inflammation and viral infection. Necroptosis can be initiated by death receptors, such as FAS and TNFR1,[14–18] TLR3 and TLR4, which recognize pathogens (double-stranded RNA viruses or lipopolysaccharides, respectively), or by the intracellular nucleic acid sensor ZBP1.[19–21] Necroptosis is dependent on the activation of RIPK1 and RIPK3; the latter phosphorylates and activates the effector molecule mixed-lineage kinase domain-like protein (MLKL).[22,23] Phosphorylated MLKL oligomerizes and disrupts the plasma membrane through exposing phospholipids to trigger cell membrane permeabilization and cellular destruction.[24,25] The cell then dies by necroptosis, a process characterized by necrosis-like features, which include cell swelling, membrane disruption, and the release of the intracellular contents.[25,26] Death-receptor triggered necroptosis can only proceed when the caspase-8–c-FLIP complex is inhibited because caspase-8 can cleave RIPK1 and RIPK3, preventing necroptosis. Inhibition of caspases is observed during certain viral infections, in which case necroptosis proceeds as a dominant cell death pathway. Importantly, necroptotic cells release damage-associated molecular patterns (DAMPs) that can induce inflammatory responses.[27] They activate caspase-1, which in turn cleaves proinflammatory interleukin (IL)-1β and IL-18 into mature inflammatory forms. Release of IL-1β is dependent on RIPK3 through caspase-8 or an MLKL-dependent NLRP3 inflammasome. Among the three NLR inflammasomes, NLRP1 and NLRC4 inflammasomes recognize bacterial muramyl dipeptide and flagellins, respectively, while NLRP3 recognizes multiple stimuli, including saturated fatty acids, bacterial RNA, and urate crystals. An inflammatory cell death termed pyroptosis (see below) is initiated by inflammasome activation. Recent findings suggest that dysregulated necroptosis during hematopoiesis promotes bone marrow progenitor cell death that induces inflammation, impairs hematopoietic stem cells, and recapitulates the features of the bone marrow failure disorder myelodysplastic syndrome (MDS).[28] Genetic loss of Ripk1 in hematopoietic cells can be responsible for this bone marrow failure.[29,30] In cancer, necroptosis may facilitate metastasis through damaging endothelial cells.[31]

Ferroptosis

Ferroptosis is an iron-dependent form of regulated necrosis. In ferroptosis, lipid peroxidation occurs in the presence of free iron, which reacts with hydrogen peroxide produced by reactive oxygen species (ROS), a reaction that is further fueled by lipid oxidation in the presence of oxygen and the disruption of cellular membranes. The physiological role of ferroptosis in development and homeostasis remains to be elucidated. Ferroptotic cells exhibit a necrotic-like phenotype, including mitochondrial shrinkage, disrupted cristae, and a ruptured outer mitochondrial membrane, yet these features occur independently of caspase activation or necrosis.[32] Ferroptosis is inhibited by the lipid peroxidase GPX4, which in turn requires glutathione and NADPH for recycling. Accordingly, ferroptosis can be observed under conditions of cysteine/cystine deprivation or inhibition of the cystine/glutamate antiporter Xc^- (i.e., by sorafenib)[33] because these interfere with glutathione synthesis.[34] Reduction in NAPDH or inhibition of GPX4[35] (i.e., by the alkylator altretamine)[36] can also induce ferroptosis. Ferroptosis was first discovered in cancer cells treated with the VDAC2/3 inhibitor erastin,[37] which causes ferroptosis by promoting iron-dependent ROS accumulation. Ferroptosis can be blocked by depletion of free iron (i.e., iron chelation), inhibition of synthesis of polyunsaturated fatty acids, or ROS scavenging using lipophilic antioxidants such as ferrostatin-1. Identification of agents that can selectively induce ferroptosis is actively being pursued in cancer research.[38,39]

Pyroptosis

Pyroptosis is a cell-intrinsic inflammatory form of regulated cell death in response to bacterial, viral, fungal, and protozoan infections. It occurs upon cleavage and oligomerization of the effector molecule gasdermin D (GSDMD), which executes pyroptosis via forming large pores in the membranes. Pyroptosis requires activation of caspases distinct from those involved in apoptosis, including caspase-1 and caspases-4 and -5 in humans and their rodent homolog caspase-11.[40] Caspases-4, -5, and -11 are directly activated by cytosolic lipopolysaccharides from invading gram-negative bacteria and cleave GSDMD in monocytes and other cell types, triggering pyroptosis. In turn, activation of caspase-1 by pathogen-associated pattern recognition receptors like the NLRs and the cytosolic DNA sensor AIM2—or in some instances indirectly through an adapter protein, apoptosis-associated speck-like (ASC) protein—leads to formation of inflammasomes, caspase-1–mediated cleavage of GSDMD, and processing of the cytokines IL-1β and IL-18, which can be released through GSDMD pores.[41] Additional members of the gasdermin family were recently identified as mediators of pyroptosis, one of which, GSDME (also known as DFNA5), is cleaved by caspase-3 that has been activated by TNF or chemotherapy drugs and could be responsible for normal tissue toxicity from chemotherapy.[42] Further characterization of caspase-3 as an activator of GSDME/DFNA5 broadened the role of this type of cell death, which has been recently shown to augment mitochondrial apoptosis.[43]

CELL SURVIVAL AND CELL DEATH PATHWAYS AND ADAPTIVE RESPONSES TO STRESS

Autophagy

Autophagy is defined as "self-eating" and culminates in sequestration of the cytoplasmic material and organelles into a double-membrane structure, the autophagosome, which subsequently is delivered to the lysosome for degradation and recycling.[44] Autophagy permits the cell to maintain homeostasis when faced with metabolic or environmental stressors, including hypoxia, nutrient deprivation, and cytotoxic chemotherapy. In mammals, the core autophagy machinery includes the Atg1-Ulk1 protein complex, which initiates autophagy by recruiting the class III PI3K complex, which is required for recruitment of autophagic factors.[45] The core autophagy proteins, or ATGs, function to generate autophagosomes by encapsulating cytoplasmic cargo within a double-membrane vesicle. These autophagosomes subsequently fuse with acidic lysosomes, and the cytoplasmic material is degraded by pH-sensitive enzymes.[46]

The multistep process of macroautophagy includes phagophore initiation, vesicle nucleation, vesicle elongation, and autophagosome fusion with lysosomes (Fig. 18.3). Phagophores, or isolation membranes, are the precursors of autophagosomes. This pathway is initiated by the Unc-51–like kinase (ULK) complex, which phosphorylates a phosphatidylinositol 3-kinase, VPS34, which is part of the Beclin1 complex required for initiation of the phagophore.[45,47] The ULK complex can be regulated by nutrient availability via mTOR (mammalian target of rapamycin). Vesicle elongation depends on two ubiquitin (Ub)-like conjugation systems. ATG5 is conjugated to ATG12 by the E1-like enzyme ATG7 and the E2-like enzyme ATG10. The ATG5–ATG12 conjugate then binds to ATG16L1, and together they function as an E3-like enzyme (in coordination with ATG7 and ATG3) to facilitate the conjugation of the microtubule-associated LC3 family of proteins to phosphatidylethanolamine (PE). This second conjugation is also aided by ATG7 as well as the E1-like enzyme ATG3. Membrane-associated LC3-PE is incorporated into the autophagosome membrane. SNARE proteins, in conjunction with the small GTPases such as Rab7, facilitate fusion between fully formed autophagosomes and lysosomes. After fusion occurs, the cytoplasmic material within the autolysosome, as well as intravesicular LC3-II, is degraded by pH-sensitive enzymes found within the acidic compartments.

Regulation of autophagy under conditions of nutrient availability occurs primarily through the mammalian target of rapamycin complexes, mTORC1 and mTORC2,[48] as well as 5′ AMP-activated protein kinase (AMPK). Under conditions of amino acid deprivation, mTORC complexes are unable to phosphorylate and inhibit the ULK system, and reduced energy translates into decreased ATP:AMP ratios that induce AMPK, which in turn inhibits mTORC and directly activates the ULK complex.[49] Other cellular stressors, such as hypoxia, endoplasmic reticulum (ER) stress, and metabolic stress, generally regulate autophagy through the above-mentioned key nutrient-sensing pathways. Additionally, the basal autophagy state may be regulated transcriptionally through expression of the core ATGs; examples of transcription factors include TFEB,[50] the forkhead family of transcription factors (FOXO1 and FOXO3),[51] and the epigenetic regulator BRD4.[52]

Tumor cells commonly have high metabolic demands and experience hypoxia and nutrient deficiencies; thus, they use autophagy to promote their survival. For example, under hypoxic conditions, stabilization of hypoxia-inducible factor 1 alpha (HIF1-α) induces activation of proapoptotic proteins that induce autophagy without triggering cell death.[53] Similarly, under nutrient starvation, AMPK activates catabolic autophagy, providing nutrients that sustain tumor cell survival.[49] Certain oncogenes, such as *KRAS*, when mutated, activate autophagy to support primary tumor growth and facilitate metastasis.[54–56] This dependency on autophagy makes it an attractive therapeutic target, and several pharmacological autophagy inhibitors are in clinical development. Further, many cytotoxic agents and some targeted therapies, such as kinase inhibitors, have been shown to induce autophagy as a cytoprotective measure in cancer cells; thus, combinations of these agents with autophagy inhibitors may circumvent therapeutic resistance.[57–59]

Other forms of autophagy include microautophagy (mainly in yeast and plants) and chaperone-mediated autophagy (CMA), which facilitates direct delivery to the lysosome.[60] In CMA, substrates reach the lysosomes through LAMP2A translocation complexes at the lysosomal membrane; these are dismantled by lysosomal HSPA8, and the process does not require vesicles or membrane invagination.[61] CMA is involved in multiple physiological and pathophysiological processes, such as metabolic regulation, aging, neurodegeneration, and T-cell activation, and recently has been implicated in tumorigenesis.[62]

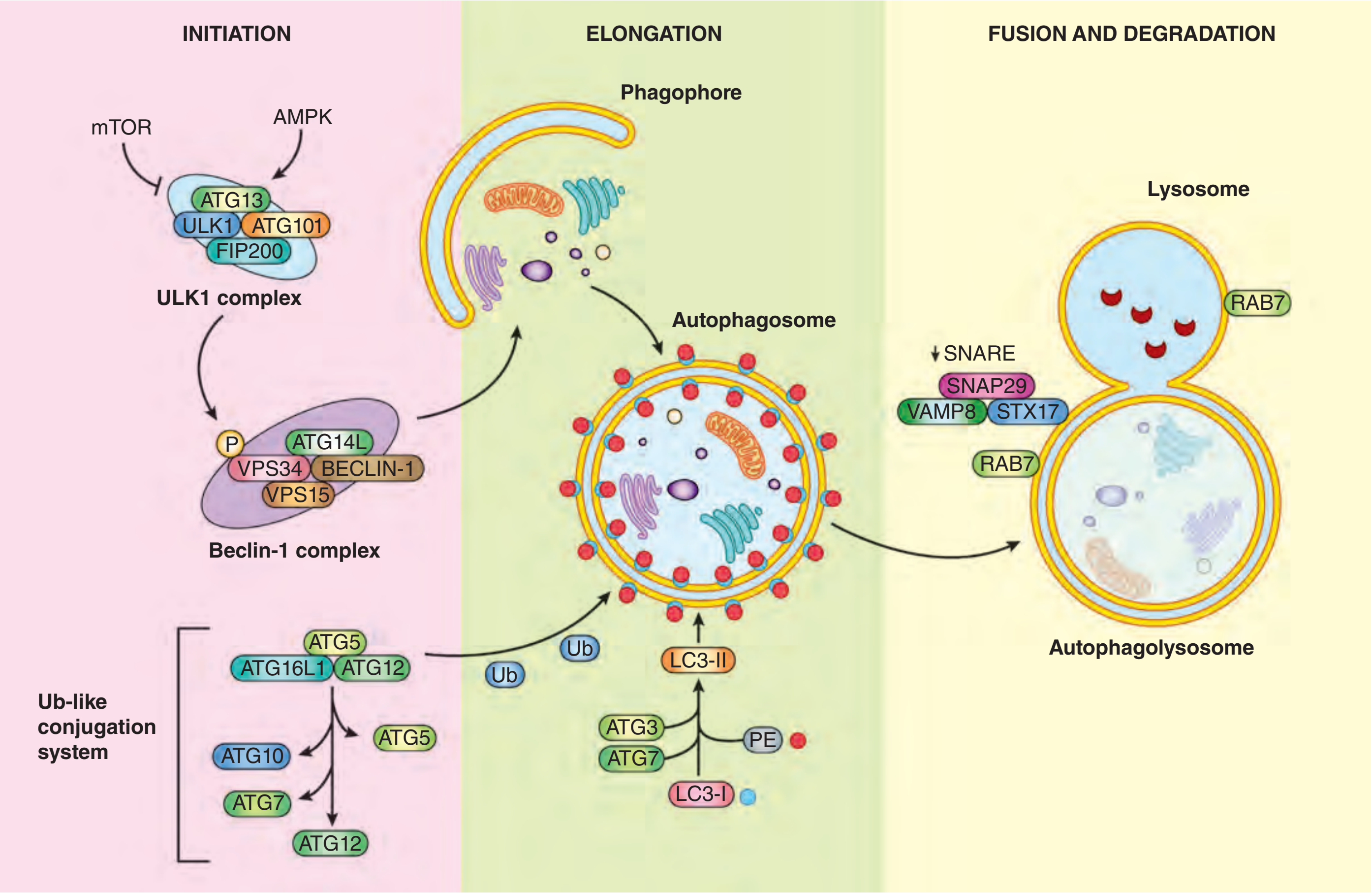


Figure 18.3 AUTOPHAGY SIGNALING.

While in a number of settings autophagy may facilitate cellular demise, this is generally viewed as a failed attempt to support survival. However, autophagic cell death (i.e., a cell death relying on autophagic machinery) has been reported in epithelial cells transformed with *RAS*[63] and in the setting of telomere dysfunction,[64] suggesting that in certain circumstances, autophagy might function to prevent oncogenic transformation.

Unfolded Protein Response

Protein stress responses that engage the unfolded protein response (UPR) during ER-induced stress may antagonize cell death or eventually lead to apoptosis. In the ER, over a third of all cellular proteins undergo processing, including protein folding, posttranslational manipulations, and structural maturation. Despite the robustness of the ER machinery, cells often function at the limit of their secretory capacity, and a wide variety of conditions can disrupt protein folding and result in the accumulation of misfolded proteins, known as ER stress. These triggers include nutrient deprivation, hypoxia, ROS accumulation, and loss of calcium homeostasis. The UPR engages three key signaling stressors located in the ER: PKR-like ER kinase (PERK), IRE1 (α and β), and activating transcription factor-6 (ATF6, α and β). These pathways regulate transcription of genes involved in resolving ER stress but also induce transcription of genes involved in apoptosis, causing the death of cells that are unable to manage the stress. Activation of PERK through autophosphorylation inhibits protein translation through the phosphorylation of eukaryotic translation initiator factor-2 (eIF2α) at serine 51, which also represents a convergence point for the so-called integrated stress response. eIF2α phosphorylation reduces the overall protein load but simultaneously induces mRNA translation of the transcription factor ATF4, which increases the antioxidant response and ER folding capacity and may also trigger macroautophagy. ATF4 also regulates the expression of genes involved in apoptosis, including the transcription factors TP53, C/EBP-homologous protein (CHOP), and growth arrest and DNA damage-inducible 34 (GADD34). CHOP inhibits expression of the antiapoptotic BCL2 and induces that of proapoptotic proteins such as BIM and NOXA. IRE1α is a transmembrane protein that, in response to unfolded proteins, catalyzes splicing of the XBP1 transcription factor, which in turn regulates transcription of genes involved in protein folding, secretion, and translocation. The second functional output of IRE1α is degradation of multiple mRNAs, microRNAs, and rRNAs in the cytosol and ER through a process called regulated IRE1-dependent decay, or RIDD, which is mediated by the RNase cytosolic domain of IRE1α. The RIDD function of IRE1α degrades specific microRNAs that affect the translation of caspase-2 to induce apoptosis via BID.[65] ATF6α is also an ER transmembrane protein that, under ER stress, undergoes cleavage and releases its cytosolic domain-containing transcription factor, ATF6f, which causes transcription of genes that regulate ER-associated degradation of unfolded proteins.

Emerging evidence from multiple experimental systems indicates that select protein modulators of UPR can promote either cell survival or cell death, depending on the extent of ER damage or the duration of UPR. Recent data indicate the important role of UPR in the regulation of the cancer-associated processes of metastasis, angiogenesis, and chemoresistance.[66] Although it remains to be determined whether agents targeting UPR regulators could be used as cancer therapeutics, recent studies have reported preclinical efficacy of small molecules that inhibit PERK or IRE1a in cancer models.[67]

CLINICAL APPLICATIONS

Given the central role of the BCL2 superfamily in the pathogenesis of cancer and in the mechanisms of chemoresistance, BCL2 targeting has in recent years been a highly sought-after therapeutic strategy in oncology, and its most successful application has taken place in the field of hematological malignancies.

PRECLINICAL ACTIVITY OF BCL2 ANTISENSE AND BH3-MIMETICS IN MYELOID MALIGNANCIES

Four BCL2-targeting agents have shown significant pre-clinical activity for the treatment of myeloid malignancies. These include a BCL2 antisense (oblimersen) and 4 BH3-mimetics (obatoclax, navitoclax, ABT-737, and venetoclax).

Preclinical Activity of Oblimersen in Acute Myeloid Leukemia

Oblimersen, formerly G3139, is a synthetic single-stranded phosphorothioate DNA oligonucleotide that downregulates BCL2 mRNA expression by selective hybridization. Formation of the RNA/antisense DNA duplex recruits RNase H, which degrades the RNA strand and prevents translation of the BCL2 protein.[68] In serum-free cultures of CD34+ normal marrow cells, BCL2+ myeloid cell lines, and acute myeloid leukemia (AML) cells, oblimersen treatment reduced BCL2 protein expression, decreased cell survival, and reduced the number of clonogenic cells. When oblimersen was used in combination with chemotherapeutic drugs, a synergistic killing effect was observed in AML cells.[69] While antisense mRNA compounds appear to be effective, subsequent preclinical efforts have been aimed at developing small-molecule BH3-mimetics that can occupy the BH3 binding grove of BCL2 and other antiapoptotic proteins and inhibit their activity.

Preclinical Activity of Obatoclax in Acute Myeloid Leukemia

Obatoclax, formerly called GX15-070, is a polypyrrole identified from a screen of natural compounds. It nonselectively inhibits BCL2 and multiple BCL2 alternative proteins, including BCL-XL, BCL-W, BCL2-A1, and MCL-1. It induces release not only of cytochrome C from mitochondria, but also of BAK from MCL-1 and BIM from both BCL2 and MCL-1, thereby inducing the formation of an active BAK:BAX complex, crucial effector proteins within the intrinsic apoptosis pathway. In preclinical studies, obatoclax inhibited growth of multiple AML cell lines and induced apoptosis in primary AML samples. In addition, it showed synergistic activity with chemotherapeutic agents and other BH3-mimetics such as ABT-737 (see below).[70]

Preclinical Activity of Navitoclax and ABT-737 in Acute Myeloid Leukemia

Navitoclax, formerly ABT-263, is a synthetic small-molecule BH3-mimetic that selectively inhibits BCL2, BCL-XL, and BCL-W, whose pre-clinical activity has been mostly observed in ALL models. It was derived by modification in three molecular positions of the first-generation, highly potent, synthetic small molecule BH3-mimetic ABT-737 to allow higher solubility, better oral bioavailability, and therapeutic use. In preclinical testing, ABT-737 induced the disruption of the BCL2:BAX complex and BAK-dependent but BIM-independent activation of the intrinsic apoptotic pathway, resulting in effective killing of AML blast, progenitor, and stem cells without affecting normal hematopoietic cells.[71] Unexpectedly, ABT-737 also induced activation of the extracellular receptor activated kinase (MAPK ERK) and of MCL-1 in AML cells, and its combination with MEK inhibitors resulted in potent synergistic killing of AML-derived cell lines, primary AML blasts, and CD34+CD38-CD123+ progenitor/stem cells.[72] A potent synergistic effect in inducing killing of primary AML and MDS cells was observed when ABT-737 was used in combination with hypomethylating agents (likely because of MCL-1 decrease) and FLT3 inhibitors.[73,74]

Preclinical Activity of Single-Agent Venetoclax in Acute Myeloid Leukemia

Venetoclax, formerly ABT-199, is a BH3-mimetic that is highly selective for BCL2.[75] Unlike previously investigated BH3-mimetics, venetoclax does not affect BCL-XL, on which platelet precursors are highly dependent.[70,71,76] This molecular feature has been critical for the subsequent clinical development of venetoclax for the treatment of AML, a disease typically associated with thrombocytopenia.[75]

In the first preclinical study of venetoclax in AML,[77] AML cell lines were similarly sensitive to venetoclax or the dual Bcl-2/XL inhibitor ABT-263. However, in BCL2-sensitive cell lines (such as MOLM-13), venetoclax more effectively induced cell killing, likely because of its five-fold higher affinity for BCL2.[75] These findings were confirmed in vivo in an aggressive mouse xenograft model of MOLM-13; venetoclax treatment significantly inhibited AML progression and extended mouse survival. Finally, ex vivo studies showed that both AML myeloblasts and AML stem/progenitor cells derived from patients' blood or bone marrow were highly sensitive to venetoclax, except for cases with complex karyotypes and *JAK2* mutations.

Combination Strategies to Boost Venetoclax Activity and Overcome Venetoclax Resistance

BH3 profiling has shown a consistent positive correlation between venetoclax sensitivity and BCL2 dependence and a negative correlation with MCL-1 dependence. Thus, studies have investigated combination strategies to overcome venetoclax resistance (Table 18.2).[77]

Combinations of daunorubicin or cytarabine with venetoclax have been investigated, both in AML cell lines and patient-derived samples.[78] These combinations reduced MCL-1 protein levels (which was instead increased by the use of single-agent venetoclax), resulting in synergistic apoptosis of AML cells. Synergistic activity was also reported for venetoclax and less-intensive chemotherapy agents, such as hypomethylating agents and histone deacetylase inhibitors. For instance, the use of venetoclax sensitized both AML cell lines and patient-derived AML samples to subsequent use of the hypomethylating agent azacitidine.[73,79] Interestingly, in vitro, agents such as navitoclax that can inhibit multiple BCL-2 family members exerted a stronger synergy than venetoclax in AML cell lines, but their potency was similar to that of venetoclax in ex vivo drug dose combination response assays in primary AML and MDS/chronic myelomonocytic leukemia samples.[73,80] This discrepancy was explained by the greater dependency of the AML-derived cell line panel on BCL-XL and/or BCL-W than of the primary samples analyzed ex vivo, as confirmed by BH3 profiling.[80] Importantly, it was recently observed that LSCs in de novo AML rely on amino acid metabolism for oxidative phosphorylation and survival.[11] Venetoclax plus azacitidine induced LSC toxicity in vitro by decreasing amino acid uptake, as confirmed by decreased α-ketoglutarate and increased succinate levels. These findings suggest that the combination of venetoclax and azacitidine inhibits its electron transport chain complex II, which was observed in LSCs derived from AML patients treated with this combination on a clinical trial.[81] The potential for this combination to eliminate LSCs is further supported by the fact that in a cohort of AML patients who were not eligible for intensive chemotherapy but were treated with venetoclax in combination with azacitidine or decitabine, 45% of those achieving complete remission also experienced eradication of minimal residual disease, with less than 10^{-3} leukemic cells observed in bone marrow samples at the time of response assessment.[82]

Moreover, in both AML cell lines and primary patient samples, the combination of venetoclax and the histone deacetylase inhibitor panobinostat synergistically induced apoptosis by upregulating BIM, and short hairpin RNA–mediated knockdown of BIM in AML cell

TABLE 18.2 Agents With Preclinical Evidence of Synergy With Venetoclax in Acute Myeloid Leukemia Models

Drug Name	Drug Class
Chemotherapy Agents	
Daunorubicin	Anthracycline
Cytarabine	Nucleoside analogue
Epigenetic Regulators	
Azacitidine	Hypomethylating agent
Panobinostat	Histone deacetylase inhibitor
Targeted Agents	
Alvocidib (flavopiridol)	CDK9 inhibitor
A-1592668	CDK9 inhibitor
MLN4924 (pevonedistat)	NED inhibitor
GDC-0980	PI3K/mTOR inhibitor
RG7388 (idasanutlin)	MDM2 antagonist
GDC-0973 (cobimetinib)	MEK inhibitor
A-1210477	MCL-1 inhibitor
VU661013	MCL-1 inhibitor
AMG 176	MCL-1 inhibitor
Quizartinib	FLT3 inhibitor
Enasidenib	IDH2 inhibitor

lines abrogated the benefit of the addition of panobinostat. These findings highlight the role of BIM as a potential target for overcoming MCL-1–mediated venetoclax resistance in AML.[83]

Multiple targeted agents that can directly or indirectly affect MCL-1 function have been investigated in combination with venetoclax in AML models. The cyclin-dependent kinases (CDKs) are a group of serine-threonine kinases; CDK9 regulates the transcription of prominent oncogenes, including *MYC* and *MCL1*. Alvocidib (also known as flavopiridol), a potent CDK9 inhibitor, has been shown to downregulate MCL-1 expression and increase BIM expression, thereby inducing synergistic apoptosis when combined with venetoclax in venetoclax-resistant AML cell lines (such as OCI-AML3), patient-derived samples, and a mouse xenograft model of OCI-AML3.[84] Similarly, A-1592668, an orally active CDK9 inhibitor, effectively downregulates RNA polymerase II phosphorylation at serine 2, resulting in the loss of MCL-1 protein expression; it synergistically induced cell death when combined with venetoclax in multiple myeloid cell lines and an AML mouse model.[85]

MLN4924 (also known as pevonedistat), an inhibitor of the Nedd8 activating enzyme NAE, has been demonstrated to inactivate E3 Cullin ring ligases (CRLs), causing accumulation of the CRL substrate c-Myc, which transactivates the *PMAIP1* gene (encoding NOXA), thus inducing upregulation of NOXA and subsequent MCL-1 inhibition. This synergistically led to apoptosis when MLN4924 was combined with venetoclax, both in cell lines and primary patient samples.[86] GDC-0980, a PI3K/mTOR inhibitor, has been shown to induce MCL-1 downregulation and BAX activation, resulting in marked apoptosis in venetoclax-resistant AML cell lines, patient-derived myeloblasts, and both cell line-derived and patient-derived xenograft mouse models of venetoclax-resistant AML.[87]

RG7388 (also known as idasanutlin), an MDM2 antagonist, activates p53, which negatively regulates the Ras/Raf/MEK/ERK pathway and activates GSK3 to modulate MCL-1 phosphorylation and promote its degradation.[88,89] In addition, p53 induces its downstream proapoptotic targets BAX and NOXA. When RG7388 was combined with venetoclax, it synergistically induced apoptosis in p53-intact AML cell lines and xenograft mouse models of venetoclax-resistant AML.[88 89]

The combination of GDC-0973 (also known as cobimetinib), a direct MEK inhibitor, with venetoclax was shown to specifically target leukemia progenitors that express high levels of BCL2 and to suppress the cytokine-induced pERK and pS6 signaling pathways. This combination also downregulated MCL-1 and disrupted both the BCL2:BIM and MCL1:BIM complexes, with the most marked effects in cell lines enriched with pathways including MYC, mTORC1, and p53. This produced significant growth-inhibitory activity both in AML cell lines (including those resistant to single agents) and patient-derived samples (including those with multiple genetic aberrations) and translated into significant reduction of the leukemia burden in xenograft OCI-AML3 and MOLM13 models.[90]

Finally, multiple direct MCL-1 inhibitors have been investigated in combination with venetoclax in preclinical models of AML, including A-1210477, VU661013, and AMG176. The activity of A-1210477 has been investigated both alone and in combination with venetoclax, with the combination showing synergistic induction of apoptosis both in AML cell lines and in patient-derived samples owing to decreased MCL-1–mediated sequestration of BIM.[91] VU661013 has been shown to destabilize the association between BIM and MCL-1, leading to apoptosis in venetoclax-resistant AML cells and patient-derived xenografts; the combination of VU661013 and venetoclax also showed significant activity in murine models of AML.[92] Similarly, AMG176 had synergistic effects when combined with venetoclax in AML tumor models and primary patient samples.[93]

Novel Venetoclax-Based Combinations

Multiple studies in recent years have investigated the efficacy of the combination of venetoclax with novel agents approved and clinically available for the treatment of patients with AML, such as FLT3 inhibitors and IDH1/2 inhibitors. The efficacy of quizartinib, an FLT3 inhibitor, in combination with venetoclax was investigated in a *FLT3*-mutated xenograft mouse model; the use of the combination resulted in more durable responses than did monotherapy.[94] Interestingly, the same study investigated the combination of venetoclax with BCL2, BCL-XL, and MCL-1 inhibitors in *FLT3*-ITD+ cells in vivo and found synergy between quizartinib and venetoclax but not between quizartinib and BCL-XL or MCL-1 inhibitors. It is important to note that earlier studies showed that *FLT3*-ITD or *PTPN11* mutations enhanced the expression of other members of the BCL-2 family, including BCL-XL and MCL-1, providing a potential explanation for venetoclax resistance in these contexts, which has been confirmed in a clinical setting.[95–97]

In a large-scale RNA interference screen aimed at identifying genes that were synthetically lethal with *IDH1/2* mutations in AML, *IDH1/2*-mutant primary human AML cells were more sensitive to venetoclax than were *IDH1/2* wild-type cells, both ex vivo and in xenograft mouse models.[98] Similar findings were reported for a chemical screen of about 300 drugs in a cohort of 38 primary human AML specimens.[99] By producing R-2-hydroxyglutarate, *IDH1/2*-mutant cells inhibit the activity of cytochrome c oxidase in the mitochondrial electron transport chain, lowering the mitochondrial threshold at which apoptosis is triggered upon engagement with venetoclax. Based on these findings, the combination of venetoclax with enasidenib, an oral IDH2 inhibitor, was investigated in three patient-derived xenograft models of human *IDH2*-mutant AML; the results showed that the combination reduced leukemia engraftment more effectively than did single-agent therapy.[100,101]

CLINICAL DEVELOPMENT OF BH3-MIMETICS FOR MYELOID MALIGNANCIES

Clinical Development of Oblimersen for Acute Myeloid Leukemia

The safety and efficacy of oblimersen was initially investigated in combination with FLAG (fludarabine, cytarabine, and granulocyte colony-stimulating factor) in a phase I study including 17 patients with refractory or relapsed AML.[102] Seven patients had a response, and no dose-limiting toxicity was observed, leading to a subsequent phase I trial of the combination of oblimersen, cytarabine, and daunorubicin in 29 patients with previously untreated AML.[103] Fourteen patients experienced remission, 7 of whom later had disease relapse, and no significant toxicity was observed. Of interest, after a 72-hour infusion of this combination, *BCL2* mRNA copies were significantly decreased from baseline in patients who went on to have remission. A phase II study further explored the efficacy of oblimersen in combination with gemtuzumab ozogamicin in older patients with AML at time of first relapse. This study showed a remission rate of 25% and durable responses.[104] Despite its clinical activity and limited toxicity, the clinical development of oblimersen has not moved forward, mainly because it requires weekly continuous infusions.

Clinical Development of Obatoclax for Acute Myeloid Leukemia

The safety and efficacy of single-agent obatoclax was initially investigated in a phase I study including 25 patients with relapsed AML and/or patients with previously untreated disease who were ineligible for induction chemotherapy.[105] A response was observed in only 1 patient, and most of the participants experienced transient neurological complications after the infusion. A subsequent phase II study investigated the activity of single-agent obatoclax in 24 patients with previously untreated MDS, but the study was stopped early because of limited efficacy.[106]

Clinical Development of Venetoclax for Acute Myeloid Leukemia

Single-agent venetoclax was initially investigated in a phase II study including 58 patients with relapsed or refractory AML. It showed limited efficacy, with an overall response rate of 19%, and a short median response duration of 48 days.[107] Notably, 4 of 12 patients with *IDH1/2* mutations had a complete response. This led to the further development of biologically rationally designed combination strategies. The combination of venetoclax and azacitidine or decitabine was investigated in a phase Ib study including 145 elderly patients with previously untreated AML. Thrombocytopenia, neutropenia, and febrile neutropenia were the most common grade 3 or higher adverse events; the complete response rate was 77% and median overall survival was 18 months.[108] The combination of venetoclax and low-dose cytarabine was investigated in a phase I/II study including older patients with previously untreated AML. This combination showed a similar toxicity profile to venetoclax and azacitidine, and among the 82% of patients who received the maximum tolerated dose of venetoclax, the complete response rate was 54% and median overall survival was 10 months.[109] Based on these data, in 2018 the FDA approved the combination of venetoclax and azacitidine or decitabine or low-dose cytarabine for the initial treatment of AML in adults 75 years of age or older or who have comorbidities that preclude the use of intensive induction chemotherapy. A confirmatory phase III randomized trial has been recently published, with results further supporting use of this combination.[110] In that study, 431 patients with previously untreated AML who were ineligible for standard chemotherapy were randomized to receive azacitidine alone or in combination with venetoclax. After a median follow-up of 21 months, patients treated with the combination had a significantly higher complete remission rate (37% vs 18%) and longer median overall survival (15 months vs 10 months), in spite of a higher rate of febrile neutropenia.

A list of published venetoclax-based combination studies is shown in Table 18.3. Multiple novel studies based on the above-outlined preclinical data are ongoing. These studies involve chemotherapy backbones, targeted agents, and immune oncology agents and may significantly improve the outcomes of patients affected by AML and other myeloid malignancies.

A deep understanding of apoptosis, a hallmark of cancer, has translated into the development of novel and effective biological agents for the treatment of hematological malignancies; a deeper knowledge of

TABLE 18.3 **Outcomes in Clinical Trials of Venetoclax-Based Combinations in Acute Myeloid Leukemia**

Regimen/Trial/Phase	Population Studied	FDA Approval	Indication	ORR (%)	CR (%)	CRi (%)	Median OS (months)	Median Duration of Response (months)
Venetoclax 400 mg/ azacitidine Dinardo et al.[108] Phase Ib	Newly diagnosed AML, age ≥ 65, ineligible for intensive chemotherapy	Yes	Age ≥ 75 or ineligible for induction chemotherapy due to comorbidities	76	44	27	16.9	21.2
Venetoclax 400 mg/ decitabine Dinardo et al.[108] Phase Ib	Newly diagnosed AML, age ≥ 65, ineligible for intensive chemotherapy	Yes	Age ≥ 75 or ineligible for induction chemotherapy due to comorbidities	71	55	19	16.2	15
Venetoclax 600 mg/ LDAC Wei et al.[109] Phase Ib/II	Newly diagnosed AML, age ≥ 65, ineligible for intensive chemotherapy	Yes	Age ≥ 75 or ineligible for induction chemotherapy due to comorbidities	54	26	28	10.1	8.1
Venetoclax 400 mg/ azacitidine Dinardo et al.[110] Phase III	Newly diagnosed AML, age ≥ 65, ineligible for intensive chemotherapy	Yes	Age ≥ 75 or ineligible for induction chemotherapy due to comorbidities	66	37	29	14.7	17.8

CR, complete remission; *Cri*, complete remission with incomplete hematological recovery; *LDAC*, low-dose cytarabine; *ORR*, overall response rate; *OS*, overall survival.

TABLE 18.4 Venetoclax-Based Treatment for Chronic Lymphocytic Leukemia

Study	Phase	Treatment	Population	N	ORR	CR/CRi	uMRD4 (%)	mPFS (months)	24-month PFS (%)	OS	NCT#
M12-175[114]	I/II	VEN-cont'	R/R	116	79%	20%	NP	NP	69% (15 mos)	84% (24 mos)	01328626
M13-982[116,135]	II	VEN-cont'	R/R Del(17p)	158	77%	20%	30% (PB)	27.2	54%	73% (24 mos)	01889186
M14-032[117,118]	II	VEN-cont'	R/R BTKi Ref/ Intol PI3Ki Ref/ Intol	91 36	65% 67%	9% 8%	26% (PB) 20% (PB)	24.7 NR	80% (12 mos) 79% (12 mos)	NR NR	02141282
M13-365[122]	Ib	VEN-cont' RIT-6 cycles	R/R	49	86%	51%	57% (BM)	NR	82%	NP	01682616
MURANO[122,123,136]	III	VEN-24 cycles RIT-6 cycles vs. BEN-6 cycles OBIN-6 cycle	R/R	194 195	92.3% 72.3%	8.2% 3.6%	62.4% (PB) 27.3% (BM) 13.3% (PB) 1.5% (BM)	53.6 17	84.9% 36.3%	82% (60 mos) 62% (60 mos)	02005471
GNE[120]	Ib	VEN-12 cycles OBIN-6 cycles	TN R/R	32 43	100% 95%	78% 37%	72/78% (PB/BM) 63/62% (PB/BM)	NR NR	90.6% 85.4%	NP NP	01685892
CLL14[121,124,137]	III	VEN-12 cycles OBIN-6 cycles vs. CHLOR-12 cycles OBIN-6 cycles	TN	216 216	84.7% 71.3%	49.5% 23.1%	76% (PB) 56.9% (BM) 35% (PB) 17.1% (BM)	NR 35.6	74% (48 mos) 35.4% (48 mos)	85.3% (48 mos) 83.1% (48 mos)	02242942
MDACC [127,138]	II	IBR-27+ cycles VEN-24 cycles	TN	80	100% (1-yr)	88% (1-yr)	56% (12 cycles combo) (BM) 66% (24 cycles combo) (BM)	NR	98% (12 mos)	99% (12 mos)	02756897
CLARITY[126]	II	VEN-12+ cycles IBR-14+ cycles	R/R	53	89% (1-yr combo)	51% (1-yr combo)	53% (PB) 36% (BM)	NR	NP	100%	NP
CAPTIVATE (MRD Cohort)[128]	II	IBR-15 cycles VEN-12 cycles	TN	164	NP	NP	75% (PB) 72% (BM)	NR	95.3% (30 mos)	NP	02910583
OSU[129]	Ib	IBR-13 cycles VEN-12 cycles OBIN-8 cycles	R/R	12	92%	42%	92% (PB) 58% (BM)	NR	92%	100%	02427451
DFCI-AVO[130,139]	II	ACA-15+ cycles VEN-12+ cycles OBIN-6 cycles	TN	44 36 for response	100%	43%	84% (PB) 78% (BM) (@ cycle 16)	NP	NP	NP	03580928
CLL2-BAG[140]	II	BEN-2 cycles OBIN-6 cycles, M VEN-cont'	TN R/R	34 29	100% 90%	41% 21%	91% (PB) 12%(BM) 83% (PB) 14% (BM)	NR NR	100% (15 mos) 83% (15 mos)	100% (15 mos) 90% (15 mos)	02401503

ACA, Acalabrutinib; *BEN*, bendamustine; *BM*, bone marrow; *cont'*, continuous; *CR/CRi*, complete remission/CR with incomplete count recovery; *GNE*, Genentech sponsored study; *IBR*, ibrutinib; *M*, maintenance; *MDACC*, MD Anderson Cancer Center; mos, months; *mPFS*, median PFS; *N,* number; *NP*, not provided; *NR*, not reached; *ORR*, overall response rate; *PB*, peripheral blood; *PFS*, progression-free survival; *R/R*, relapsed/refractory; *Ref/Intol*, refractory and/or intolerant; *RIT*, rituximab; *TN*, treatment naive; *uMRD*, undetectable minimal residual disease; *VEN*, venetoclax.

its interactions with other biological pathways is now contributing to the development of safer and more effective therapeutic strategies.

RATIONALE FOR USE OF BH-3 MIMETICS IN CHRONIC LYMPHOCYTIC LEUKEMIA

The majority of patients with chronic lymphocytic leukemia (CLL) harbor deletion of a portion of the long arm of chromosome 13 [del(13q)]. Two important microRNA (miR) genes, miR15 and miR16, were identified in the critical minimal deleted region of del(13q) and were noted to be deleted or down-regulated in more than two-thirds of all CLL cases.[111] MicroRNAs are non-translated small RNAs that function to regulate gene expression. Both miR15 and miR16 down-regulate *BCL-2* transcript levels. The absence of miR15 and miR16 in cases with del(13q) leads to increased BCL-2 expression[112] and resultant resistance to apoptosis.

In vitro treatment of CLL cells with navitoclax (ABT-263), the small molecule inhibitor of BCL-2, BCL-w, and BCL-xL, potently induced cell death. A phase I/II trial of orally administered navitoclax was conducted and generated promising results. The majority of patients treated in the study had greater than 50% reduction in leukemia counts and some patients experienced reduction in lymph node size.[113] The dose limiting toxicity was thrombocytopenia secondary to accelerated platelet senescence from inhibition of BCL-xL in platelets. Given this activity, venetoclax (ABT-199) was designed as a molecule with greater affinity for BCL-2 and reduced affinity for BCL-xL.[75]

This mechanism of action is independent of p53, making it particularly appealing for high-risk patients who lack *TP53*, such as those with del(17p) and patients who have mutated *TP53*.

CLINICAL DEVELOPMENT OF VENETOCLAX FOR CHRONIC LYMPHOCYTIC LEUKEMIA

Venetoclax is approved for treatment of patients with CLL, both as monotherapy and combined with CD20 antibody, developed through a series of phase I–III trials (Table 18.4). Venetoclax was initially studied in a phase I clinical trial that included patients with CLL as well as other lymphoid malignancies.[114,115] This trial identified tumor lysis syndrome (TLS) as a significant risk but did not identify a maximum tolerated dose or dose-limiting toxicity. The occurrence of clinical TLS was mitigated through a weekly ramp-up schedule for initiating venetoclax. The effective dose identified for CLL in this trial was 400 mg daily.

The pivotal trial that led to initial approval for venetoclax as continuous monotherapy was a phase II trial for relapsed or refractory del(17p) CLL(M13-982, see Table 18.4).[116] This trial demonstrated responses, including complete remissions, and undetectable MRD at 10^{-4} sensitivity (uMRD4) demonstrating the deepest remission, in a population of heavily previously treated patients with del(17p) CLL. Responses were durable; an estimated median progression-free survival (PFS) of 24 months was reported in this population.

Continuous monotherapy was also investigated in patients who were refractory or intolerant to B-cell receptor signaling pathway inhibitors, including ibrutinib and idelalisib (M14-032).[117,118] Responses were noted in both subgroups and were durable.

Venetoclax was initially combined with the CD20 antibody, rituximab, in a phase Ib trial exploring tolerability of this combination for relapsed and refractory patients with CLL (M13-365).[119] In addition, venetoclax was studied with obinutuzumab in another phase Ib trial that enrolled both first-line and relapsed CLL.[120] This trial evaluated schedules of treatment initiated first with obinutuzumab then venetoclax ramp-up and vice versa, and showed both schedules were safe. Similarly, the phase III CLL14 trial studied cycle 1 with obinutuzumab initiated alone; venetoclax ramp-up was initiated on day 22 of cycle 1.[121] Cycle 1 obinutuzumab single-agent was highly effective at reducing lymphocytosis prior to initiating venetoclax in these studies.

Larger randomized phase III clinical trials demonstrated improved PFS with venetoclax-based therapy over chemoimmunotherapy (see Table 18.4).[121–124] The MURANO trial evaluated venetoclax plus rituximab for patients with previously treated CLL.[122,123] The control arm received the standard 6 cycles bendamustine plus rituximab. The CLL14 trial evaluated venetoclax plus obinutuzumab in treatment naïve CLL and compared to treatment with chlorambucil plus obinutuzumab.[121] These trials showed remarkably improved outcomes and depth of remission associated with venetoclax-based therapy over chemoimmunotherapy.

In vitro studies demonstrated reduced MCL1 with BTKi treatment potentially making cells more vulnerable to BCL2i-induced apoptosis.[125] Combined targeted therapy with BTKi and BCL2i was evaluated in three early, phase I/II clinical trials.[126–128] Data were initially available for the MD Anderson[127] and CLARITY[126] studies, which clearly demonstrated high uMRD4 rate in both first-line and relapsed CLL. The CAPTIVATE study investigated 12 cycles of combined ibrutinib and venetoclax followed by randomization based on confirmed uMRD4 status.[128] Patients who were confirmed undetectable were randomized to continued ibrutinib single-agent versus placebo and those unconfirmed for uMRD4 were randomized to continued ibrutinib single-agent versus continued combined therapy. The post-randomized, 1-year disease-free survival rate was similar between those patients assigned to placebo versus continued ibrutinib. None of the three trials identified safety or tolerability concerns with this combination.

Venetoclax was also combined with BTKi (ibrutinib[129] or acalabrutinib,[130] a second-generation BTKi) and obinutuzumab (see Table 18.4). These trials showed similar tolerability and safety with these combinations.

MECHANISMS OF RESISTANCE TO VENETOCLAX IN CHRONIC LYMPHOCYTIC LEUKEMIA

Several mechanisms of acquired clinical resistance to venetoclax-based treatment have been described. These include increased expression of other anti-apoptotic BCL-2 family proteins, such as Mcl-1 and BCL-xL, which raise the threshold of apoptosis and decrease cell dependence on BCL-2.[131] One potential mechanism for increasing BCL-xL levels is through reduction in miR-377.[132] Furthermore, mutations that block venetoclax binding of BCL-2 have been described in clinical samples from patients who develop clinical resistance, including: G101V,[133] D103Y,[134] and F104I.[134] Finally, metabolic changes can also alter dependence and response to BH3-mimetics and regulate BCL-2 levels.

SUMMARY

Apoptosis, or programmed cell death, is an evolutionarily conserved, highly regulated mechanism for maintaining cell and tissue homeostasis in multicellular organisms. Physiological apoptotic process may proceed through an intrinsic, mitochondria-dependent pathway, or an extrinsic pathway that depends on the death signal and cell type involved. The BCL-2 family of proteins is situated upstream of irreversible cell damage in the apoptotic pathway, providing a pivotal checkpoint in the fate of a cell after a death stimulus. BH3-only BCL-2 members connect distinct upstream signal transduction pathways with the common, core apoptotic pathway. Cancer cells hijack this apoptotic machinery and utilize it to facilitate their survival. Understanding the molecular mechanisms of cell death and the critical dependencies of specific cancers has led to the discovery of several BH3-mimetics that are currently approved or are undergoing clinical trials for use in targeted cancer therapy.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Blombery P, et al. Acquisition of the Recurrent Gly101Val mutation in BCL2 confers resistance to venetoclax in patients with progressive chronic lymphocytic leukemia. *Cancer Discov*. 2019;9:342–353.

Campos L, Sabido O, Rouault JP, Guyotat D. Effects of BCL-2 antisense oligodeoxynucleotides on in vitro proliferation and survival of normal marrow progenitors and leukemic cells. *Blood*. 1994;84:595–600.

Certo M, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell*. 2006;9:351–365.

Cervantes-Gomez F, et al. Pharmacological and protein profiling suggests venetoclax (ABT-199) as optimal partner with ibrutinib in chronic lymphocytic leukemia. *Clin Cancer Res*. 2015;21:3705–3715.

Chan SM, et al. Isocitrate dehydrogenase 1 and 2 mutations induce BCL-2 dependence in acute myeloid leukemia. *Nat Med*. 2015;21:178–184.

DiNardo CD, et al. Azacitidine and venetoclax in previously untreated acute myeloid leukemia. *N Engl J Med*. 2020;383:617–629.

Fischer K, et al. Venetoclax and obinutuzumab in patients with CLL and coexisting conditions. *N Engl J Med*. 2019;380:2225–2236.

Jain N, et al. Ibrutinib and venetoclax for first-line treatment of CLL. *N Engl J Med*. 2019;380:2095–2103.

Kinsey CG, et al. Protective autophagy elicited by RAF→MEK→ERK inhibition suggests a treatment strategy for RAS-driven cancers. *Nat Med*. 2019;25:620–627.

Konopleva M, et al. Efficacy and biological correlates of response in a phase II study of venetoclax monotherapy in patients with acute myelogenous leukemia. *Cancer Discov*. 2016;6:1106–1117.

Lagadinou ED, et al. BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. *Cell Stem Cell*. 2013;12:329–341.

Letai A, et al. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell*. 2002;2:183–192.

Linkermann A, Green DR. Necroptosis. *N Engl J Med*. 2014;370:455–465.

Lock R, Kenific CM, Leidal AM, Salas E, Debnath J. Autophagy-dependent production of secreted factors facilitates oncogenic RAS-driven invasion. *Cancer Discov*. 2014;4:466–479.

Marcucci G, et al. Phase 1 and pharmacodynamic studies of G3139, a Bcl-2 antisense oligonucleotide, in combination with chemotherapy in refractory or relapsed acute leukemia. *Blood*. 2003;101:425–432.

Marcucci G, et al. Phase I study of oblimersen sodium, an antisense to Bcl-2, in untreated older patients with acute myeloid leukemia: pharmacokinetics, pharmacodynamics, and clinical activity. *J Clin Oncol*. 2005;23:3404–3411.

Nassour J, et al. Autophagic cell death restricts chromosomal instability during replicative crisis. *Nature*. 2019;565:659–663.

Pan R, et al. Selective BCL-2 inhibition by ABT-199 causes on-target cell death in acute myeloid leukemia. *Cancer Discov*. 2014;4:362–375.

Pan R, et al. Synthetic lethality of combined Bcl-2 inhibition and p53 activation in AML: mechanisms and superior antileukemic efficacy. *Cancer Cell*. 2017;32:748–760. e746.

Pollyea DA, et al. Venetoclax with azacitidine disrupts energy metabolism and targets leukemia stem cells in patients with acute myeloid leukemia. *Nat Med*. 2018;24:1859–1866.

Ramsey HE, et al. A novel MCL1 inhibitor combined with venetoclax rescues venetoclax-resistant acute myelogenous leukemia. *Cancer Discov*. 2018;8:1566–1581.

Rickard JA, et al. RIPK1 regulates RIPK3-MLKL-driven systemic inflammation and emergency hematopoiesis. *Cell*. 2014;157:1175–1188.

Rogers C, et al. Gasdermin pores permeabilize mitochondria to augment caspase-3 activation during apoptosis and inflammasome activation. *Nat Commun*. 2019;10:1689.

Seymour JF, et al. Venetoclax-rituximab in relapsed or refractory chronic lymphocytic leukemia. *N Engl J Med*. 2018;378:1107–1120.

Souers AJ, et al. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nat Med*. 2013;19:202–208.

Stilgenbauer S, et al. Venetoclax for patients with chronic lymphocytic leukemia with 17p deletion: results from the full population of a phase II pivotal trial. *J Clin Oncol*. 2018;36:1973–1980.

Strilic B, et al. Tumour-cell-induced endothelial cell necroptosis via death receptor 6 promotes metastasis. *Nature*. 2016;536:215–218.

Vercammen D, et al. Dual signaling of the Fas receptor: initiation of both apoptotic and necrotic cell death pathways. *J Exp Med*. 1998;188:919–930.

Wagner PN, et al. Increased Ripk1-mediated bone marrow necroptosis leads to myelodysplasia and bone marrow failure in mice. *Blood*. 2019;133:107–120.

Wang Y, et al. Chemotherapy drugs induce pyroptosis through caspase-3 cleavage of a gasdermin. *Nature*. 2017;547:99–103.

REFERENCES

1. Leverson JD, et al. Found in translation: how preclinical research is guiding the clinical development of the BCL2-selective inhibitor venetoclax. *Cancer Discov*. 2017;7:1376–1393.
2. Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat Rev Mol Cell Biol*. 2014;15:49–63.
3. Del Gaizo Moore V, Letai A. BH3 profiling—measuring integrated function of the mitochondrial apoptotic pathway to predict cell fate decisions. *Cancer Lett*. 2013;332:202–205.
4. Letai A, et al. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell*. 2002;2:183–192.
5. Certo M, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell*. 2006;9:351–365.
6. Giménez-Cassina A, Danial NN. Regulation of mitochondrial nutrient and energy metabolism by BCL-2 family proteins. *Trends Endocrinol Metab*. 2015;26:165–175.
7. Hardwick JM, Chen YB, Jonas EA. Multipolar functions of BCL-2 proteins link energetics to apoptosis. *Trends Cell Biol*. 2012;22:318–328.
8. Vander Heiden MG, Chandel NS, Schumacker PT, Thompson CB. Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. *Mol Cell*. 1999;3:159–167.
9. Harris MH, Thompson CB. The role of the Bcl-2 family in the regulation of outer mitochondrial membrane permeability. *Cell Death Differ*. 2000;7:1182–1191.
10. Lagadinou ED, et al. BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. *Cell Stem Cell*. 2013;12:329–341.
11. Jones CL, et al. Inhibition of amino acid metabolism selectively targets human leukemia stem cells. *Cancer Cell*. 2018;34:724–740. e724.
12. Perciavalle RM, et al. Anti-apoptotic MCL-1 localizes to the mitochondrial matrix and couples mitochondrial fusion to respiration. *Nat Cell Biol*. 2012;14:575–583.
13. Escudero S, et al. Dynamic regulation of long-chain fatty acid oxidation by a noncanonical interaction between the MCL-1 BH3 helix and VLCAD. *Mol Cell*. 2018;69:729–743. e727.
14. Degterev A, et al. Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat Chem Biol*. 2008;4:313–321.
15. Galluzzi L, Kepp O, Krautwald S, Kroemer G, Linkermann A. Molecular mechanisms of regulated necrosis. *Semin Cell Dev Biol*. 2014;35:24–32.
16. Degterev A, et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat Chem Biol*. 2005;1:112–119.
17. Vercammen D, et al. Dual signaling of the Fas receptor: initiation of both apoptotic and necrotic cell death pathways. *J Exp Med*. 1998;188:919–930.
18. Vercammen D, Vandenabeele P, Beyaert R, Declercq W, Fiers W. Tumour necrosis factor-induced necrosis versus anti-Fas-induced apoptosis in L929 cells. *Cytokine*. 1997;9:801–808.
19. Upton JW, Kaiser WJ, Mocarski ES. Virus inhibition of RIP3-dependent necrosis. *Cell Host Microbe*. 2010;7:302–313.
20. Upton JW, Kaiser WJ, Mocarski ES. DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. *Cell Host Microbe*. 2012;11:290–297.
21. Kaiser WJ, et al. Toll-like receptor 3-mediated necrosis via TRIF, RIP3, and MLKL. *J Biol Chem*. 2013;288:31268–31279.
22. Murphy JM, et al. The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. *Immunity*. 2013;39:443–453.
23. Linkermann A, Green DRNecroptosis. *N Engl J Med*. 2014;370:455–465.
24. Dondelinger Y, et al. MLKL compromises plasma membrane integrity by binding to phosphatidylinositol phosphates. *Cell Rep*. 2014;7:971–981.
25. Wang H, et al. Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3. *Mol Cell*. 2014;54:133–146.
26. Cai Z, et al. Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. *Nat Cell Biol*. 2014;16:55–65.
27. Kaczmarek A, Vandenabeele P, Krysko DV. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. *Immunity*. 2013;38:209–223.
28. Wagner PN, et al. Increased Ripk1-mediated bone marrow necroptosis leads to myelodysplasia and bone marrow failure in mice. *Blood*. 2019;133:107–120.
29. Roderick JE, et al. Hematopoietic RIPK1 deficiency results in bone marrow failure caused by apoptosis and RIPK3-mediated necroptosis. *Proc Natl Acad Sci USA*. 2014;111:14436–14441.
30. Rickard JA, et al. RIPK1 regulates RIPK3-MLKL-driven systemic inflammation and emergency hematopoiesis. *Cell*. 2014;157:1175–1188.
31. Strilic B, et al. Tumour-cell-induced endothelial cell necroptosis via death receptor 6 promotes metastasis. *Nature*. 2016;536:215–218.
32. Vanden Berghe T, Linkermann A, Jouan-Lanhouet S, Walczak H, Vandenabeele P. Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nat Rev Mol Cell Biol*. 2014;15:135–147.
33. Dixon SJ, et al. Pharmacological inhibition of cystine-glutamate exchange induces endoplasmic reticulum stress and ferroptosis. *Elife*. 2014;3:e02523.
34. Friedmann Angeli JP, et al. Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice. *Nat Cell Biol*. 2014;16:1180–1191.
35. Yang WS, et al. Regulation of ferroptotic cancer cell death by GPX4. *Cell*. 2014;156:317–331.
36. Woo JH, et al. Elucidating compound mechanism of action by network perturbation analysis. *Cell*. 2015;162:441–451.
37. Dixon SJ, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell*. 2012;149:1060–1072.
38. Ma S, Henson ES, Chen Y, Gibson SB. Ferroptosis is induced following siramesine and lapatinib treatment of breast cancer cells. *Cell Death Dis*. 2016;7:e2307.
39. Zhu S, et al. HSPA5 regulates ferroptotic cell death in cancer cells. *Cancer Res*. 2017;77:2064–2077.
40. Man SM, et al. Differential roles of caspase-1 and caspase-11 in infection and inflammation. *Scientific Reports*. 2017;7:45126.
41. Thi HTH, Hong S. Inflammasome as a therapeutic target for cancer prevention and treatment. *J Cancer Prev*. 2017;22:62–73.
42. Wang Y, et al. Chemotherapy drugs induce pyroptosis through caspase-3 cleavage of a gasdermin. *Nature*. 2017;547:99–103.
43. Rogers C, et al. Gasdermin pores permeabilize mitochondria to augment caspase-3 activation during apoptosis and inflammasome activation. *Nat Commun*. 2019;10:1689.
44. Rabinowitz JD, White E. Autophagy and metabolism. *Science*. 2010;330:1344–1348.
45. Russell RC, et al. ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. *Nat Cell Biol*. 2013;15:741–750.
46. Dikic I, Elazar Z. Mechanism and medical implications of mammalian autophagy. *Nat Rev Mol Cell Biol*. 2018;19:349–364.
47. He C, Levine B. The Beclin 1 interactome. *Curr Opin Cell Biol*. 2010;22:140–149.
48. Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. *Cell*. 2017;168:960–976.
49. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol*. 2011;13:132–141.
50. Settembre C, et al. TFEB links autophagy to lysosomal biogenesis. *Science*. 2011;332:1429–1433.
51. Audesse AJ, et al. FOXO3 directly regulates an autophagy network to functionally regulate proteostasis in adult neural stem cells. *PLoS Genet*. 2019;15:e1008097.
52. Sakamaki JI, et al. Bromodomain protein BRD4 is a transcriptional repressor of autophagy and lysosomal function. *Mol Cell*. 2017;66:517–532. e519.
53. Mazure NM, Pouysségur J. Atypical BH3-domains of BNIP3 and BNIP3L lead to autophagy in hypoxia. *Autophagy*. 2009;5:868–869.
54. Yang S, et al. Pancreatic cancers require autophagy for tumor growth. *Genes Dev*. 2011;25:717–729.
55. Lock R, Kenific CM, Leidal AM, Salas E, Debnath J. Autophagy-dependent production of secreted factors facilitates oncogenic RAS-driven invasion. *Cancer Discov*. 2014;4:466–479.
56. Guo JY, et al. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. *Genes Dev*. 2011;25:460–470.
57. Levy JM, et al. Autophagy inhibition improves chemosensitivity in BRAF(V600E) brain tumors. *Cancer Discov*. 2014;4:773–780.
58. Kinsey CG, et al. Protective autophagy elicited by RAF→MEK→ERK inhibition suggests a treatment strategy for RAS-driven cancers. *Nat Med*. 2019;25:620–627.
59. Bryant KL, et al. Combination of ERK and autophagy inhibition as a treatment approach for pancreatic cancer. *Nat Med*. 2019;25:628–640.
60. Kaushik S, Cuervo AM. The coming of age of chaperone-mediated autophagy. *Nat Rev Mol Cell Biol*. 2018;19:365–381.
61. Kaushik S, Cuervo AM. Chaperone-mediated autophagy: a unique way to enter the lysosome world. *Trends Cell Biol*. 2012;22:407–417.
62. Kon M, et al. Chaperone-mediated autophagy is required for tumor growth. *Sci Transl Med*. 2011;3:109ra117.
63. Elgendy M, Sheridan C, Brumatti G, Martin SJ. Oncogenic Ras-induced expression of Noxa and Beclin-1 promotes autophagic cell death and limits clonogenic survival. *Mol Cell*. 2011;42:23–35.

64. Nassour J, et al. Autophagic cell death restricts chromosomal instability during replicative crisis. *Nature*. 2019;565:659–663.
65. Upton JP, et al. IRE1α cleaves select microRNAs during ER stress to derepress translation of proapoptotic caspase-2. *Science*. 2012;338:818–822.
66. Urra H, Dufey E, Avril T, Chevet E, Hetz C. Endoplasmic reticulum stress and the hallmarks of cancer. *Trends Cancer*. 2016;2:252–262.
67. Hetz C, Chevet E, Harding HP. Targeting the unfolded protein response in disease. *Nat Rev Drug Discov*. 2013;12:703–719.
68. Klasa RJ, Gillum AM, Klem RE, Frankel SR. Oblimersen Bcl-2 antisense: facilitating apoptosis in anticancer treatment. *Antisense Nucleic Acid Drug Dev*. 2002;12:193–213.
69. Campos L, Sabido O, Rouault JP, Guyotat D. Effects of BCL-2 antisense oligodeoxynucleotides on in vitro proliferation and survival of normal marrow progenitors and leukemic cells. *Blood*. 1994;84:595–600.
70. Konopleva M, et al. Mechanisms of antileukemic activity of the novel Bcl-2 homology domain-3 mimetic GX15-070 (obatoclax). *Cancer Res*. 2008;68:3413–3420.
71. Konopleva M, et al. Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell*. 2006;10:375–388.
72. Konopleva M, et al. MEK inhibition enhances ABT-737-induced leukemia cell apoptosis via prevention of ERK-activated MCL-1 induction and modulation of MCL-1/BIM complex. *Leukemia*. 2012;26:778–787.
73. Bogenberger JM, et al. BCL-2 family proteins as 5-Azacytidine-sensitizing targets and determinants of response in myeloid malignancies. *Leukemia*. 2014;28:1657–1665.
74. Kohl TM, et al. BH3 mimetic ABT-737 neutralizes resistance to FLT3 inhibitor treatment mediated by FLT3-independent expression of BCL2 in primary AML blasts. *Leukemia*. 2007;21:1763–1772.
75. Souers AJ, et al. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nat Med*. 2013;19:202–208.
76. Tse C, et al. ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res*. 2008;68:3421–3428.
77. Pan R, et al. Selective BCL-2 inhibition by ABT-199 causes on-target cell death in acute myeloid leukemia. *Cancer Discov*. 2014;4:362–375.
78. Niu X, et al. Binding of released Bim to Mcl-1 is a mechanism of intrinsic resistance to ABT-199 which can be overcome by combination with daunorubicin or cytarabine in AML cells. *Clin Cancer Res*. 2016;22:4440–4451.
79. Tsao T, et al. Concomitant inhibition of DNA methyltransferase and BCL-2 protein function synergistically induce mitochondrial apoptosis in acute myelogenous leukemia cells. *Ann Hematol*. 2012;91:1861–1870.
80. Bogenberger JM, et al. Ex vivo activity of BCL-2 family inhibitors ABT-199 and ABT-737 combined with 5-azacytidine in myeloid malignancies. *Leuk Lymphoma*. 2015;56:226–229.
81. Pollyea DA, et al. Venetoclax with azacitidine disrupts energy metabolism and targets leukemia stem cells in patients with acute myeloid leukemia. *Nat Med*. 2018;24:1859–1866.
82. Pollyea DA, et al. Venetoclax in combination with hypomethylating agents induces rapid, deep, and durable responses in patients with AML ineligible for intensive therapy. *Blood*. 2018;132 285–285.
83. Schwartz J, et al. Synergistic anti-leukemic interactions between ABT-199 and panobinostat in acute myeloid leukemia ex vivo. *Am J Transl Res*. 2016;8:3893–3902.
84. Bogenberger J, et al. Combined venetoclax and alvocidib in acute myeloid leukemia. *Oncotarget*. 2017;8:107206–107222.
85. Phillips DC, et al. A novel CDK9 inhibitor increases the efficacy of venetoclax (ABT-199) in multiple models of hematologic malignancies. *Leukemia*. 2020;34:1646–1657.
86. Knorr KL, et al. MLN4924 induces Noxa upregulation in acute myelogenous leukemia and synergizes with Bcl-2 inhibitors. *Cell Death Differ*. 2015;22:2133–2142.
87. Rahmani M, et al. Cotargeting BCL-2 and PI3K induces BAX-dependent mitochondrial apoptosis in AML cells. *Cancer Res*. 2018;78:3075–3086.
88. Lehmann C, Friess T, Birzele F, Kiialainen A, Dangl M. Superior anti-tumor activity of the MDM2 antagonist idasanutlin and the Bcl-2 inhibitor venetoclax in p53 wild-type acute myeloid leukemia models. *J Hematol Oncol*. 2016;9:50.
89. Pan R, et al. Synthetic lethality of combined Bcl-2 inhibition and p53 activation in AML: mechanisms and superior antileukemic efficacy. *Cancer Cell*. 2017;32:748–760. e746.
90. Han L, et al. Concomitant targeting of BCL2 with venetoclax and MAPK signaling with cobimetinib in acute myeloid leukemia models. *Haematologica*. 2020;105:697–707.
91. Luedtke DA, et al. Inhibition of Mcl-1 enhances cell death induced by the Bcl-2-selective inhibitor ABT-199 in acute myeloid leukemia cells. *Signal Transduct Target Ther*. 2017;2:17012.
92. Ramsey HE, et al. A novel MCL1 inhibitor combined with venetoclax rescues venetoclax-resistant acute myelogenous leukemia. *Cancer Discov*. 2018;8:1566–1581.
93. Caenepeel S, et al. AMG 176, a selective MCL1 inhibitor, is effective in hematologic cancer models alone and in combination with established therapies. *Cancer Discov*. 2018;8:1582–1597.
94. Mali RS, et al. Venetoclax combines synergistically with FLT3 inhibition to effectively target leukemic cells in FLT3-ITD+ acute myeloid leukemia models. *Haematologica*. 2020
95. Chen L, et al. Mutated Ptpn11 alters leukemic stem cell frequency and reduces the sensitivity of acute myeloid leukemia cells to Mcl1 inhibition. *Leukemia*. 2015;29:1290–1300.
96. Kasper S, et al. Targeting MCL-1 sensitizes FLT3-ITD-positive leukemias to cytotoxic therapies. *Blood Cancer. J*. 2012;2:e60.
97. Chyla B, et al. Genetic biomarkers of sensitivity and resistance to venetoclax monotherapy in patients with relapsed acute myeloid leukemia. *Am J Hematol*. 2018
98. Chan SM, et al. Isocitrate dehydrogenase 1 and 2 mutations induce BCL-2 dependence in acute myeloid leukemia. *Nat Med*. 2015;21:178–184.
99. Bordeleau ME, et al. Genetic characterization of ABT-199 sensitivity in human AML. *Blood*. 2018;132 283–283.
100. Stein EM, et al. Enasidenib in mutant IDH2 relapsed or refractory acute myeloid leukemia. *Blood*. 2017;130:722–731.
101. Cathelin S, et al. Combination of enasidenib and venetoclax shows superior anti-leukemic activity against IDH2 mutated AML in patient-derived xenograft models. *Blood*. 2018;132 562–562.
102. Marcucci G, et al. Phase 1 and pharmacodynamic studies of G3139, a Bcl-2 antisense oligonucleotide, in combination with chemotherapy in refractory or relapsed acute leukemia. *Blood*. 2003;101:425–432.
103. Marcucci G, et al. Phase I study of oblimersen sodium, an antisense to Bcl-2, in untreated older patients with acute myeloid leukemia: pharmacokinetics, pharmacodynamics, and clinical activity. *J Clin Oncol*. 2005;23:3404–3411.
104. Moore J, et al. A Phase II study of Bcl-2 antisense (oblimersen sodium) combined with gemtuzumab ozogamicin in older patients with acute myeloid leukemia in first relapse. *Leuk Res*. 2006;30:777–783.
105. Schimmer AD, et al. A phase I trial of the small molecule Pan-Bcl-2 family inhibitor obatoclax mesylate (GX15-070) administered by continuous infusion for up to four days to patients with hematological malignancies. *Blood*. 2007;110 272a–272a.
106. Arellano ML, Borthakur G, Berger M, Luer J, Raza A. A phase II, multicenter, open-label study of obatoclax mesylate in patients with previously untreated myelodysplastic syndromes with anemia or thrombocytopenia. *Clin Lymphoma Myeloma Leuk*. 2014;14:534–539.
107. Konopleva M, et al. Efficacy and biological correlates of response in a phase II study of venetoclax monotherapy in patients with acute myelogenous leukemia. *Cancer Discov*. 2016;6:1106–1117.
108. DiNardo CD, et al. Venetoclax combined with decitabine or azacitidine in treatment-naive, elderly patients with acute myeloid leukemia. *Blood*. 2019;133:7–17.
109. Wei AH, et al. Venetoclax combined with low-dose cytarabine for previously untreated patients with acute myeloid leukemia: results from a phase Ib/II study. *J Clin Oncol*. 2019;37:1277–1284.
110. DiNardo CD, et al. Azacitidine and venetoclax in previously untreated acute myeloid leukemia. *N Engl J Med*. 2020;383:617–629.
111. Calin GA, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA*. 2002;99:15524–15529.
112. Cimmino A, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci USA*. 2005;102:13944–13949.
113. Roberts AW, et al. Substantial susceptibility of chronic lymphocytic leukemia to BCL2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease. *J Clin Oncol*. 2012;30:488–496.
114. Roberts AW, et al. Targeting BCL2 with venetoclax in relapsed chronic lymphocytic leukemia. *N Engl J Med*. 2016;374:311–322.
115. Davids MS, et al. Phase I first-in-human study of venetoclax in patients with relapsed or refractory non-Hodgkin lymphoma. *J Clin Oncol*. 2017;35:826–833.
116. Stilgenbauer S, et al. Venetoclax in relapsed or refractory chronic lymphocytic leukaemia with 17p deletion: a multicentre, open-label, phase 2 study. *Lancet Oncol*. 2016;17:768–778.
117. Jones JA, et al. Venetoclax for chronic lymphocytic leukaemia progressing after ibrutinib: an interim analysis of a multicentre, open-label, phase 2 trial. *Lancet Oncol*. 2018;19:65–75.
118. Coutre S, et al. Venetoclax for patients with chronic lymphocytic leukemia who progressed during or after idelalisib therapy. *Blood*. 2018;131:1704–1711.

119. Seymour JF, et al. Venetoclax plus rituximab in relapsed or refractory chronic lymphocytic leukaemia: a phase 1b study. *Lancet Oncol*. 2017;18:230–240.
120. Flinn IW, et al. Phase 1b study of venetoclax-obinutuzumab in previously untreated and relapsed/refractory chronic lymphocytic leukemia. *Blood*. 2019;133:2765–2775.
121. Fischer K, et al. Venetoclax and obinutuzumab in patients with CLL and coexisting conditions. *N Engl J Med*. 2019;380:2225–2236.
122. Seymour JF, et al. Venetoclax-rituximab in relapsed or refractory chronic lymphocytic leukemia. *N Engl J Med*. 2018;378:1107–1120.
123. Kater AP, et al. Fixed duration of venetoclax-rituximab in relapsed/refractory chronic lymphocytic leukemia eradicates minimal residual disease and prolongs survival: post-treatment follow-up of the MURANO phase III study. *J Clin Oncol*. 2019;37:269–277.
124. Al-Sawaf O, et al. Venetoclax plus obinutuzumab versus chlorambucil plus obinutuzumab for previously untreated chronic lymphocytic leukaemia (CLL14): follow-up results from a multicentre, open-label, randomised, phase 3 trial. *Lancet Oncol*. 2020;21:1188–1200.
125. Cervantes-Gomez F, et al. Pharmacological and protein profiling suggests venetoclax (ABT-199) as optimal partner with ibrutinib in chronic lymphocytic leukemia. *Clin Cancer Res*. 2015;21:3705–3715.
126. Hillmen P, et al. Ibrutinib plus venetoclax in relapsed/refractory chronic lymphocytic leukemia: the CLARITY study. *J Clin Oncol*. 2019;37:2722–2729.
127. Jain N, et al. Ibrutinib and venetoclax for first-line treatment of CLL. *N Engl J Med*. 2019;380:2095–2103.
128. Wierda WG, et al. Ibrutinib (Ibr) plus venetoclax (Ven) for first-line treatment of chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL): 1-year disease-free survival (DFS) results from the MRD cohort of the phase 2 CAPTIVATE study. *Blood*. 2020;136:16–17.
129. Rogers KA, et al. Phase 1b study of obinutuzumab, ibrutinib, and venetoclax in relapsed and refractory chronic lymphocytic leukemia. *Blood*. 2018;132:1568–1572.
130. Lampson BL, et al. Preliminary safety and efficacy results from a phase 2 study of acalabrutinib, venetoclax and obinutuzumab in patients with previously untreated chronic lymphocytic leukemia (CLL). *Blood*. 2019;134 32–32.
131. Haselager MV, et al. Changes in Bcl-2 members after ibrutinib or venetoclax uncover functional hierarchy in determining resistance to venetoclax in CLL. *Blood*. 2020;136:2918–2926.
132. Al-Harbi S, et al. miR-377-dependent BCL-xL regulation drives chemotherapeutic resistance in B-cell lymphoid malignancies. *Mol Cancer*. 2015;14:185.
133. Blombery P, et al. Acquisition of the recurrent Gly101Val mutation in BCL2 confers resistance to venetoclax in patients with progressive chronic lymphocytic leukemia. *Cancer Discov*. 2019;9:342–353.
134. Tausch E, et al. Venetoclax resistance and acquired BCL2 mutations in chronic lymphocytic leukemia. *Haematologica*. 2019;104:e434–e437.
135. Stilgenbauer S, et al. Venetoclax for patients with chronic lymphocytic leukemia with 17p deletion: results from the full population of a phase II pivotal trial. *J Clin Oncol*. 2018;36:1973–1980.
136. Kater AP, et al. Five-year analysis of murano study demonstrates enduring undetectable minimal residual disease (uMRD) in a subset of relapsed/refractory chronic lymphocytic leukemia (R/R CLL) patients (Pts) following fixed-duration venetoclax-rituximab (VenR) therapy (Tx). *Blood*. 2020;136:19–21.
137. Al-Sawaf O, et al. Clonal dynamics after venetoclax-obinutuzumab therapy: novel insights from the randomized, phase 3 CLL14 trial. *Blood*. 2020;136:22–23.
138. Jain N, et al. Combined ibrutinib and venetoclax for first-line treatment for patients with chronic lymphocytic leukemia (CLL): focus on MRD results. *Blood*. 2020;136:42–43.
139. Davids MS, et al. Updated safety and efficacy results from a phase 2 study of acalabrutinib, venetoclax and obinutuzumab (AVO) for frontline treatment of chronic lymphocytic leukemia (CLL). *Blood*. 2020;136:20–21.
140. Cramer P, et al. Bendamustine followed by obinutuzumab and venetoclax in chronic lymphocytic leukaemia (CLL2-BAG): primary endpoint analysis of a multicentre, open-label, phase 2 trial. *Lancet Oncol*. 2018;19:1215–1228.

CHAPTER

19

AGING AND HEMATOPOIESIS

Daozheng Yang, Arthur Flohr Svendsen, and Gerald de Haan

AGING

Increase in life expectancy has resulted in a major demographic global shift toward an elderly population. As a consequence, the prevalence of a multitude of age-associated diseases has increased gradually during the last decades, resulting in an increased number of hospitalizations of elderly individuals. A better understanding of the mechanisms that cause aging is crucial in order to mitigate age-associated clinical conditions.

Multiple "hallmarks" of cellular/molecular aging have been proposed.[1] Aging is a heterogeneous process, leading to impairment, malfunction, and failure of cells and tissues. One of the hallmarks of aging relates to stem cell exhaustion. Tissue homeostasis is maintained by stem cells, and their regenerative potential is severely reduced during normal aging. The hematopoietic system is affected by this process in different ways. In this chapter, we focus on the clinical hematological manifestations that are observed in elderly individuals and discuss the potential molecular causes of age-associated dysfunctions.

HEMATOPOIETIC STEM CELL POTENTIAL DECLINES WITH AGING

Hematopoietic stem cells (HSCs) are a rare population of bone marrow (BM) cells that harbor the potential to replenish every mature blood cell, thus maintaining hematopoietic homeostasis. HSCs are also able to self-renew—upon cell division, at least one of the daughter cells maintains stem cell properties—in order to maintain the stem cell pool throughout the lifespan. Yet, a wide variety of studies have documented numerous hematological changes in elderly individuals, suggesting that HSCs lose their full potential during the lifespan of an individual.

Quantitatively, there is an increase in the absolute number of phenotypical HSCs, while elderly individuals show a decreased number of more mature hematopoietic cells. The increased number of HSCs seems to be a compensatory mechanism in order to maintain blood production and also indicates that aged HSCs have a diminished functional capacity.[2] Indeed, allogenic BM transplantation studies demonstrated that HSCs derived from aged donors have diminished engraftment potential and reduced self-renewal capacity compared to HSCs from younger donors.[3]

Another characteristic of aged HSCs is their skewed differentiation potential, where differentiation toward myeloid progenitors is favored over differentiation to red blood cells (RBCs) and lymphocytes.[2,3] This myeloid skewing has profound implications for age-associated clinical manifestations, and it has been under intense investigation. We cover both aspects in the following sections. First, we describe the most common hematological age-associated clinical manifestations.

CLINICAL MANIFESTATIONS

Anemia

By far the most prevalent age-associated hematological condition is anemia. Studies demonstrated that 10% to 24% of elderly individuals develop some form of anemia, where idiopathic cytopenia of unknown significance with isolate anemia (ICUS-A), commonly known as "anemia at older age" is the most prevailing.[4] According to the World Health Organization (WHO), anemia in older individuals occurs when hemoglobin (Hb) levels are less than 13 g/dL in men and less than 12 g/dL in women.[4] Multiple epidemiological studies have associated low Hb levels as risk factors for cardiovascular diseases, cognitive impairment, and insomnia. Furthermore, anemia has been associated with diminished physical performance and more frequent and longer hospitalizations among elderly patients.[4]

The development of anemia at an older age is multifactorial. Malnutrition is the cause of the majority of cases, where deficiency of primarily iron but also folic acid and vitamin B12 hampers RBC production. Specifically in elderly patients, gastrointestinal (GI) tract problems, polypharmacy, and even social isolation have been reported to be significant contributors.[4] Chronic inflammation can also cause anemia, with as many as one-third of patients older than 65 years old displaying a chronic inflammatory state. Common age-related chronic diseases such as chronic kidney disease, rheumatoid arthritis, and different kinds of neoplasms affect systemic inflammatory cytokine levels. These inflammatory cytokines negatively affect erythroid precursors cells in the BM. In addition, erythropoietin (EPO)—the main regulator of erythropoiesis—is usually reduced, leading to decreased RBCs production.[4]

Immunosenescence

Elderly patients often display immunosenescence, which is defined by a reduced ability to combat new pathogens, the accumulation of memory T cells, and a chronic and systemic state of inflammation, termed "inflammaging" (see the discussion in the following sections).[5] One of the proposed causes of a hampered immune response in elderly individuals is the myeloid skewing of aged HSCs. Since myeloid differentiation is favored, the B and T cell population diversity is diminished and the adaptive immune response is impaired. The lack of an adequate immune response in conjunction with a chronic inflammatory state has profound implications and contributes to age-associated clinical manifestations such as susceptibility to infections, diverse types of cancer, autoimmune and neurodegenerative diseases, and atherosclerosis.[3]

Myelodysplastic Syndromes

Elderly individuals may display changes in hematopoiesis which are closely related to bone marrow failure (BMF) syndromes. The most prevalent of these, myelodysplastic syndrome (MDS), is composed of a heterogeneous group of clonal myeloid disorders. This syndrome is characterized by ineffective hematopoiesis, cytopenias—often severe anemia (<10 g/dL)—and dysmyelopoiesis. During normal aging, hematopoiesis displays similar changes as seen in MDS patients, and these pathological findings are associated with an increased risk for transformation into acute myeloid leukemia (AML).[3,6] Overall, phenotypes associated with normal aging and pathological BMF are generally caused by loss of HSC function, which in turn results in aberrant blood cell formation.[3]

Currently, there are a restricted set of approved drugs for MDS treatment, and none are curative. HSC transplantation is potentially the only curative intervention, but there are significant limitations in terms of donor/recipient compatibility and recipient age-associated comorbidities.[6]

The pathogenesis of MDS has been well studied and specific molecular mechanisms have been identified. Somatic mutations are consistently found in MDS patients and are associated with different prognoses. Mutations in genes such as *ASXL1*, *EZH2*, *RUNX1*, *SRSF2*, and *TP53* are associated with poor prognosis. Interestingly, some of these genes are also mutated in elderly patients with no clinical manifestations (see the clonal hematopoiesis section). Moreover, the acquisition of somatic mutations in non-hematopoietic cells in the BM microenvironment has been shown to also contribute to niche remodeling, favoring neoplastic hematopoietic progenitors that eventually can initiate disorders such as MDS and AML.[3,6]

Leukemia

There is a strongly increased incidence of many types of cancer across different tissues in elderly patients, including age-associated hematological malignancies. These malignancies are generally believed to be caused by time and the proliferation-dependent accumulation of somatic mutations.[3]

Specifically, in aged individuals, there is a high prevalence of and mortality from AML and chronic lymphoid leukemia (CLL) (Fig. 19.1). Although both types of malignancies are strongly age-dependent, they show distinct development, onset, and result from very distinct mutations.[7] Whereas CLL is considered to emerge from mature and differentiated lymphocytes, AML arises from undifferentiated hematopoietic stem and progenitor (HSPC) populations.

CLONAL HEMATOPOIESIS, CLONAL HEMATOPOIESIS OF INDETERMINATE POTENTIAL, AND AGE-RELATED CLONAL HEMATOPOIESIS

In the 1960s, it was established that X chromosomal inactivation (XCI) is a random process that takes place at the early female embryonic developmental stages. Since the same X chromosome remains inactive upon multiple cell divisions, XCI analysis allows for low-resolution clonal tracking. As hematological malignancies arise from single clones, clonal tracking is a powerful tool that allows for disease monitoring and understanding.[8]

Only recently, high-resolution clonality analyses of healthy individuals without malignant transformation demonstrated two remarkable features. First, mature blood cells from these individuals showed imbalanced clonality, suggesting that some HSC clones produced more mature blood cells than others, resulting in their progeny being overrepresented. This is referred to as (oligo-) clonal hematopoiesis. Second, when assessing the presence of clonal hematopoiesis in different age groups, 0.5% of individuals younger than 50 years old, 2% to 3% in elderly individuals, and a remarkable 10% to 20% of elderly individuals above 70 years old showed clonal hematopoiesis.[8–10] Thus, clonal hematopoiesis does not only occur during normal hematopoiesis but also is strongly correlated with age.

Insight in clonal hematopoiesis and its relationship with leukemia emerged when driver mutations were assessed in individuals with normal clonal hematopoiesis. *TET2* mutations were detected in 5% of older individuals with clonal hematopoiesis, but such mutations were not detected in individuals with non-clonal hematopoiesis. This was the first time that a mutation associated with myeloid leukemia was found to be present and compatible with normal hematopoiesis, corroborating the idea that leukemia arises from the acquisition of sequential mutations and that clonal hematopoiesis acts as a pre-leukemic state.[2,8–10]

With advancements in DNA exome sequencing, studies have revealed the mutation profile of large cohorts of individuals within different age categories and inclusion criteria (presence of hematological neoplasia, psychiatric and cardiovascular disorders). Individuals with clonal hematopoiesis showed a restricted pattern of mutations in genes involved in different cellular processes. However, the most striking mutation profile, found in more than two-thirds of these individuals, affected the coding sequence of a small number of epigenetic genes, particularly *DNMT3A*, *TET2*, and *ASXL1*.[2,8–10]

It may be relevant to distinguish between various types of clonal hematopoiesis. First, individuals with clonal hematopoiesis do not necessarily carry mutations in driver genes. Currently, it is a challenge to detect such clones due to low variant allele frequency (VAF). However, in individuals who do carry cancer-associated mutations, this process is referred to as clonal hematopoiesis of indeterminate potential (CHIP). Thus, in order to meet the definition of CHIP, one must carry driver mutations (*DNMT3A* and/or *TP53*, for example) at a VAF of 2% or higher.[9,10] CHIP by itself does not designate a malignant transformation or other clinical manifestations, but it does share common mutations that are recurrent in hematological disorders such as AML, MDS, myeloproliferative neoplasms (MPNs), and lymphomas.[10] Furthermore, for unknown reasons, only a fraction

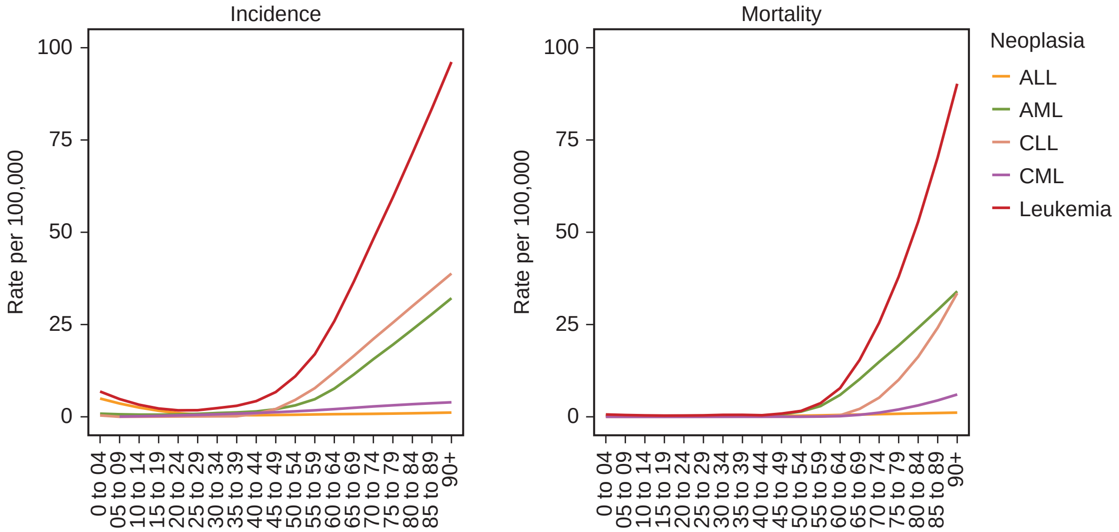


Figure 19.1 AGE-ASSOCIATION OF LEUKEMIA. Number of cases (incidence) and deaths (mortality) related to hematological malignancies. Rate represents the number of cases/mortality per 100, 000 habitants. *ALL*, Acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *CLL*, chronic lymphoid leukemia; *CML*, chronic myeloid leukemia. *(From Cancer Research UK, https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/leukaemia#heading-Zero. Accessed March 2020.)*

of individuals with CHIP will actually undergo malignant transformation.[8] However, individuals carrying CHIP have a significantly increased risk of mortality that is largely accounted for an overall increased risk for cardiovascular diseases. Large cohort studies showed that individuals with CHIP have double the risk of developing coronary heart diseases and a four times higher risk to develop early-onset myocardial infarction (MI). CHIP also increases the risk of venous thrombosis 12-fold and is correlated with worse survival outcomes of post-MI-related congestive heart failure. Overall, CHIP is a strong indicator of poor prognosis for several distinct disorders including the development of hematological malignancies and cardiovascular disorders.[10]

Although CHIP is significantly more prevalent in elderly individuals, a causal relation between clonal hematopoiesis and aging and possibly other factors remains to be determined. Indeed, many healthy elderly individuals display clonal hematopoiesis. Specifically, if the mutations that define clones are not leukemia-driver mutations—i.e., occur at a non-fully annotated genetic loci or in genes that have not yet been implicated in malignant transformation—this process is referred to as age-related clonal hematopoiesis (ARCH).[9] Similarly as individuals with CHIP, individuals who display ARCH also have a higher risk of mortality. As a full catalog of leukemia-drives genes is lacking, CHIP and ARCH could represent similar processes (Fig. 19.2).[9]

Although the molecular mechanism of how clonal hematopoiesis arises is far from understood, it is clear that perturbations that occur at the multipotent progenitor and/or HSCs level affect how such clones will behave. As an example in the context of clonal hematopoiesis, loss-of-function mutations in *DNMT3A* are among the most prevalent in individuals displaying CHIP. This event likely provides mutated clones with an aberrant DNA methylation profile that results in a selective advantage over time, leading to clonal outgrowth.[2]

Even in individuals who do not display detectable levels of clonal hematopoiesis, physiological aging is likely to render clone selection during a lifespan, where HSCs that become more adapted to age-associated changes eventually will replenish mature blood cells. Interestingly, studies in human and other animal models have demonstrated multiple age-associated changes (mechanisms) that play an important role in HSC aging, and those are discussed in the following sections.

MECHANISMS OF HEMATOPOIETIC STEM CELL AGING

Many studies have examined which factors, mechanistically, contribute to HSC aging. It is important to note that most of our understanding of HSC aging is based on murine models. In general, mechanisms that contribute to HSC aging can be divided into intrinsic factors (DNA damage, autophagy and reactive oxygen species [ROS], and epigenetic drift) or extrinsic factors (BM microenvironment, senescence, and inflammation) (Fig. 19.3).[11–13]

INTRINSIC FACTORS

DNA Damage

Genomic instability and DNA damage are hallmarks of aging in different tissues.[1] Acquired mutations at specific loci primarily caused by DNA damage in HSCs can lead to clonal hematopoiesis in elderly individuals, as discussed previously. This suggests that aged HSCs are more susceptible to DNA damage compared to young HSCs. Indeed, aged HSCs display an increased number of γH2A foci—a widely used marker of DNA damage—and have difficulty in removing these foci, indicating impaired DNA repair. However, to what extent the functional decline of aged HSCs is caused by DNA damage is not yet clear.[12,13] It has been demonstrated that both young and aged murine HSCs can repair DNA equally effectively following induced DNA damage.[13] Although HSCs from mice lacking distinct genomic maintenance pathways exhibit decreased reconstitution and self-renewal capacity, HSCs in these mice are reduced in numbers, in contrast to normal aging where HSCs expand.[12–14] Telomere shortening, another hallmark of aging, is a specific kind of DNA damage. Early studies demonstrated that telomere length inversely correlated with lifespan of a cell, where older cells display shorter telomeres and progressively shorten at each cell division. Interestingly, murine serial transplantation studies revealed no change in telomere length, and HSC repopulation capacity was not improved by overexpressing telomerase.[13] Collectively, more studies are needed to clarify to what extent normal HSC aging in fact is caused by DNA damage.

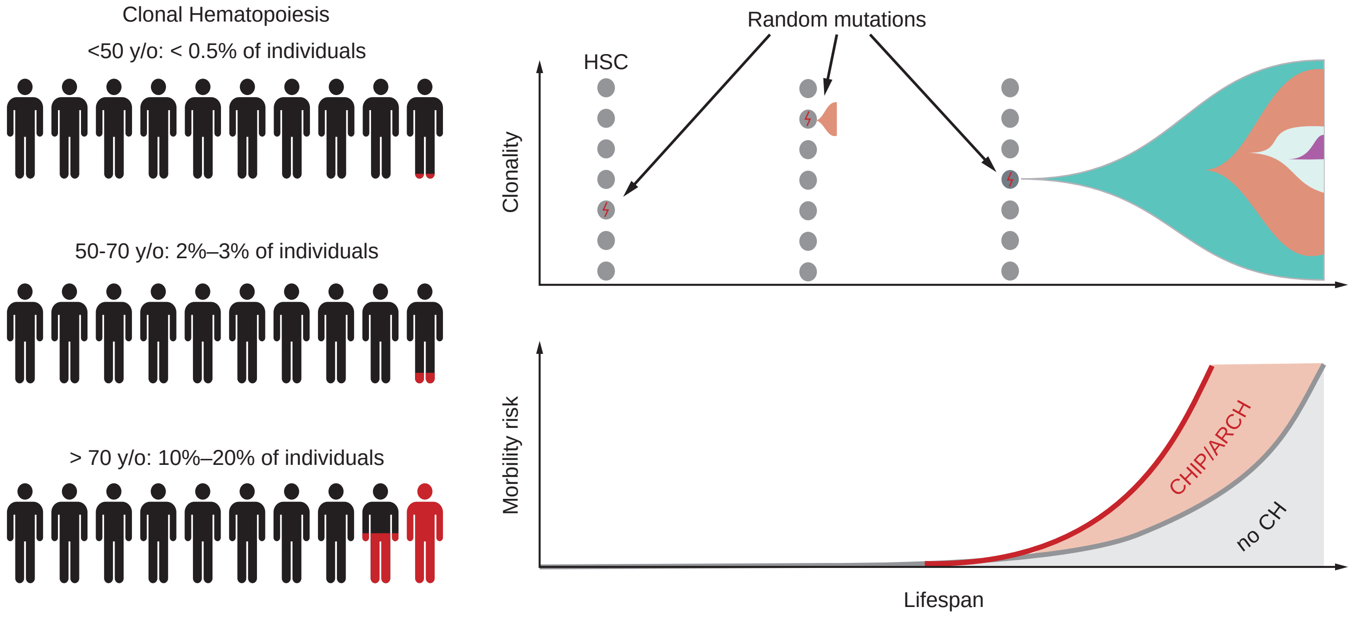

Clonal Hematopoiesis
<50 y/o: < 0.5% of individuals
50-70 y/o: 2%–3% of individuals
> 70 y/o: 10%–20% of individuals
Random mutations
HSC
Clonality
Morbility risk
CHIP/ARCH
no CH
Lifespan

Figure 19.2 CLONAL HEMATOPOIESIS. The incidence of clonal hematopoiesis increases with age *(left)*. Somatic mutations occur during normal aging and are typically inconsequential. Some clones may acquire a proliferative advantage, and eventually, blood production will be oligoclonal *(right, top)*. If dominant clones result from leukemia driving mutations (e.g., DNMT3A, TP53), individuals are diagnosed with clonal hematopoiesis of indeterminate potential *(CHIP)*. Age-related clonal hematopoiesis *(ARCH)* refers to the acquisition of non-driver mutations at an older age. Both processes significantly increase the odds of hematological malignancies and mortality *(right, bottom)* compared to individuals who do not carry clonal hematopoiesis *(CH)*. *HSC*, Hematopoietic stem cell.

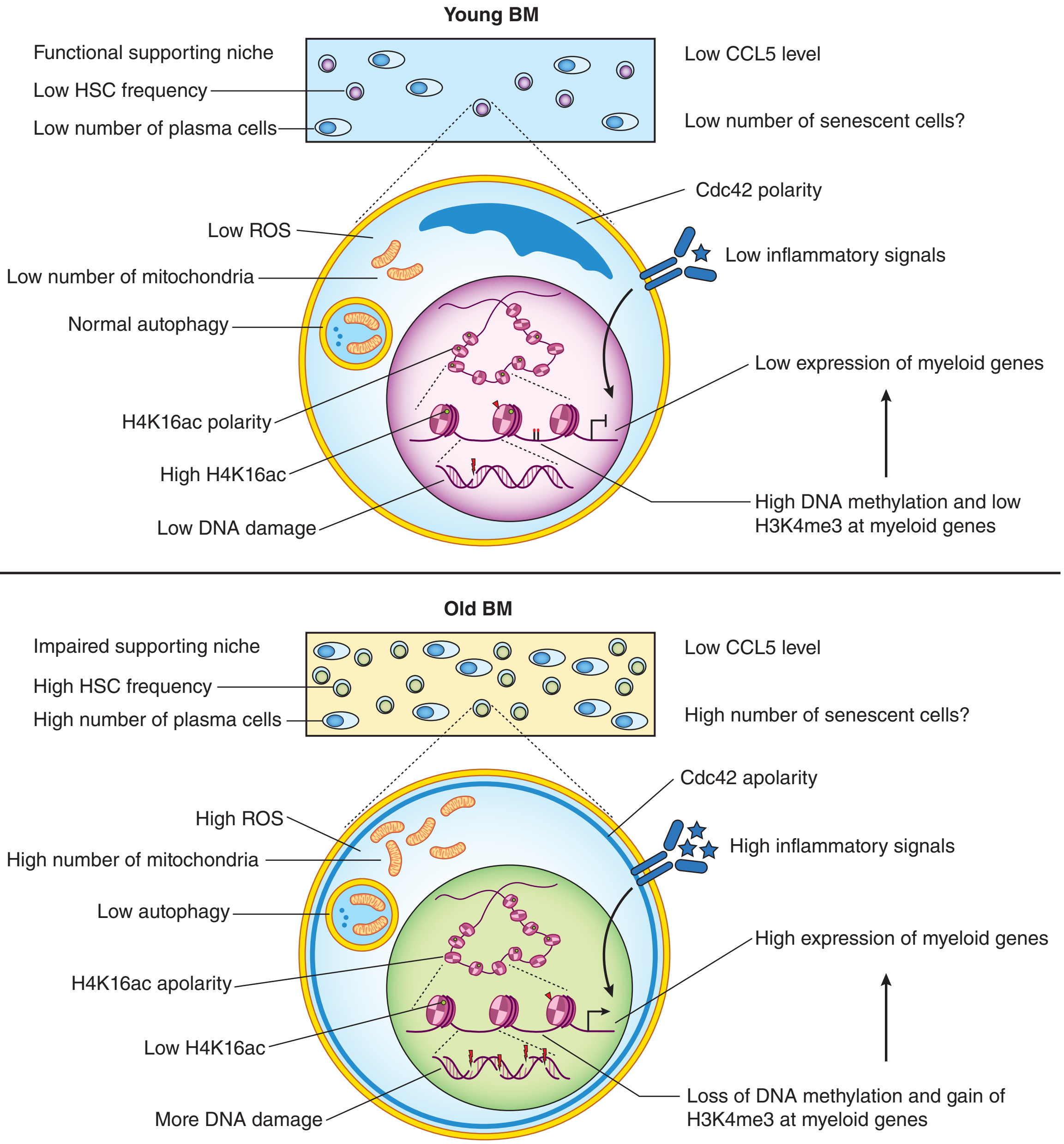

Young BM
Functional supporting niche
Low HSC frequency
Low number of plasma cells
Low CCL5 level
Low number of senescent cells?
Cdc42 polarity
Low ROS
Low number of mitochondria
Normal autophagy
Low inflammatory signals
Low expression of myeloid genes
H4K16ac polarity
High H4K16ac
Low DNA damage
High DNA methylation and low H3K4me3 at myeloid genes
Old BM
Impaired supporting niche
High HSC frequency
High number of plasma cells
Low CCL5 level
High number of senescent cells?
Cdc42 apolarity
High ROS
High number of mitochondria
Low autophagy
High inflammatory signals
High expression of myeloid genes
H4K16ac apolarity
Low H4K16ac
More DNA damage
Loss of DNA methylation and gain of H3K4me3 at myeloid genes

Figure 19.3 HALLMARKS OF HEMATOPOIETIC STEM CELL AGING. An overview of intrinsic factors and extrinsic factors that have been reported to contribute to HSC aging. *BM*, Bone marrow; *CCL5*, CC-chemokine ligand 5; *HSC*, hematopoietic stem cell; *ROS*, reactive oxygen species.

Autophagy and Reactive Oxygen Species

Autophagy is a self-catabolic process by which cellular components are sequestered into double-membrane vacuoles, generally associated with the recycling of organelles, and required for cellular hemostasis. Impaired autophagy can lead to the accumulation of toxic proteins and damaged mitochondria, which in turn induces metabolic stress.[12] Around one-third of aged murine HSCs exhibit high autophagy levels. This subpopulation shows a low metabolic state and a high regeneration potential, resembling young HSCs. The remaining population—with decreased autophagy levels—exhibits an activated metabolic state and impaired HSC self-renewal capacity.[13] Thus, loss of autophagy causes metabolic activation and similar myeloid bias as observed in aged HSCs.[13]

ROS—natural products of oxygen-based metabolic processes—are produced mainly by mitochondria. It has been shown that high levels of ROS generate oxidative cellular stress.[13,14] In general, HSCs are quiescent with low levels of metabolism and low ROS production. However, upon aging, HSCs start accumulating ROS, which then attenuates their fitness.[13,14] It has been shown that HSCs exposed to high ROS levels have age-associated phenotypes, for example, impaired long-term engraftment. The fact that the reduced colony-forming potential of ROS^{high} HSCs can be rescued by ROS inhibition further implies a role of ROS in HSC aging and presents potential pharmacological targets to counter age-associated HSC dysfunction.[13,14]

Epigenetic Modifications

Epigenetic modifications, including DNA methylation and histone modifications, are responsible for the regulation of gene expression without affecting the underlying DNA sequence—a change in phenotype without a change in genotype.[14] Epigenetic modifiers, generally characterized as writers, readers, or erasers, play essential roles in HSC self-renewal and differentiation.[15] As discussed, individuals displaying clonal hematopoiesis, specially CHIP, but also patients with MDS or full-blown AML, very frequently have acquired loss-of-function mutations in epigenetic genes, denoting the importance of this regulatory layer.[10,15] Moreover, in murine models, some epigenetic genes have been reported to be differentially expressed following HSC aging, suggesting that the epigenome is remodeled during aging.[13]

DNA methylation is often linked to transcriptional gene repression. Age-associated changes in the methylome occur, but their functional significance still remains controversial. However, regardless of whether overall hypo- or hypermethylation occurs in aged HSCs, similar patterns at specific loci have been found.[13] For instance, the Polycomb Repressive Complex-2 (PRC2), which deposits the repressive histone modification H3 lysine 27 tri-methylation (H3K27me3), targets genes that are DNA hypermethylated in aged HSCs,[13] suggesting a crosstalk between these two epigenetic marks.

Histone modifications, including active and repressive marks, impact gene expression by altering chromatin structure to render it more or less accessible to transcription factors and the transcriptional machinery or by recruiting "readers" of the histone code.[15] There are multiple histone modifications, but profiles of certain typical modifications occurring in young or aged HSCs have already provided insight into HSC aging.[13–16] For example, global levels of H4 lysine 16 acetylation (H4K16ac), an activating histone modification, have been shown to be elevated in aged HSCs. Interestingly, H4K16ac also affects HSC polarity changes that have been reported to be controlled by a small Rho GTPase, cell division control protein 42 (Cdc42).[13,14] The molecular function of Cdc42 is discussed in the following section.

Epigenetic regulation also provides a potential mechanism underlying myeloid skewing. It has been observed that some myeloid genes gain active histone modifications such as H3 lysine 4 tri-methylation (H3K4me3) and lose DNA methylation in aged HSCs, leading to transcriptional up-regulation of these genes.[16] This raises the possibility that targeting the epigenetic state can potentially restore the function of aged HSCs. Indeed, induced pluripotent stem (iPS) cells generated from reprogrammed young and aged hematopoietic progenitors showed similar repopulating capacity and appear to be rejuvenated.[13]

Chromatin Architecture and Polarity

Recently, overall chromatin architecture has been implicated as a pivotal player of gene expression, cell division, fate determination, growth and development, disease, and even genome evolution.[13] In HSC biology, disease-associated mutations of proteins involving 3D chromatin organization have occasionally been observed and linked to hematopoietic disorders in the elderly.[13] Studies of chromatin architecture during HSC cell division revealed that young HSCs display a more "asymmetrical" division pattern—where one of the daughter cells retains the stem cell properties—whereas aged HSCs have preferentially "symmetrical" division patterns due to loss of cell polarity, resulting in reduced regenerative capacity and lymphoid potential.[13] Cdc42 activity is elevated in aged HSCs and correlates with the loss of polarity of aged HSCs. Pharmacological reduction of Cdc42 activity restored the level and spatial distribution of H4K16ac to a similar status to that seen in young HSCs and eventually rejuvenated aged HSCs.[12–14]

EXTRINSIC FACTORS

Bone Marrow Microenvironment

The spatial location where HSCs reside also has a significant impact on their activity. This microenvironment, termed a BM "niche," is composed of a plethora of hematopoietic and non-hematopoietic cells such as mesenchymal stem cells, osteoblasts, endothelial cells, macrophages, among others.[17] These supporting populations are able to regulate quiescence, self-renewal, proliferation, and differentiation of HSCs.

Collectively, niche cells regulate HSCs through the production of diverse cytokines, extracellular matrix proteins, and adhesion molecules.[17] This raises the possibility that age-associated BM niche remodeling can influence the functioning of HSCs. Although recent studies have started to elucidate the mechanisms through which the niche regulates HSCs, limited data are available on the differences between young and aged HSC niches.[17]

Classic BM transplantation showed that a young BM niche failed to rescue aging stem cell phenotypes when aged HSCs were transplanted into irradiated young recipients, suggesting that features observed upon HSC aging are mainly caused by intrinsic changes.[12] However, young HSCs showed impaired homing and engraftment capability when transplanted into old recipients, while aged HSCs showed higher engraftment and less myeloid output when exposed to a young niche, suggesting a negative impact of the aged microenvironment on hematopoiesis.[17] Importantly, a recent study showed that BM plasma cells increased in number with age and that the depletion of these cells reduced myeloid cell production in aged mice.[18] In the future, greater effort is needed to investigate the roles of the BM niche in the context of HSC aging.

Senescence

Cellular senescence is another hallmark of aging, and the removal of senescent cells (SCs) can prevent or delay tissue dysfunction and extend the lifespan.[12,14] It has been reported that the senolytic drug navitoclax (ABT263), a specific inhibitor of anti-apoptotic proteins, selectively kills SCs in culture in a cell type- and species-independent manner by inducing apoptosis. ABT263 treatment also clears SCs in normally aged mice and improves the long-term engraftment potential of aged HSCs following serial transplantation.[14] It is likely SCs impair BM HSC function due to the senescence-associated secretory phenotype (SASP), where inflammatory cytokines, such as interleukin 6 (IL-6), are secreted. The impact of inflammatory cytokines will be further discussed in the following section.

Inflammation

Inflammation is defined as a protective immune response, which underlies a variety of pathophysiological processes that are in part caused by infection and tissue injury/damage. HSCs are needed for multi-lineage differentiation to replenish immune effector cells consumed during inflammation.[19] Actually, HSCs can also directly sense inflammatory signals since a broad spectrum of inflammatory cytokine/chemokine receptors are expressed by HSCs,[11,19] directly influencing HSC fate decisions. It has been shown that HSCs are activated and produce more myeloid cells but fewer lymphoid cells in response to inflammation.[11,19] Many studies have demonstrated that signaling via inflammatory cytokines impacts long-term HSC potential.[11,19]

In an aging context, the role of inflammation is more prominent. Chronic inflammation occurs frequently in the elderly and is associated with multiple age-related diseases—referred to as "inflammaging."[11,19] Age-related functional alterations (such as impaired repopulation potential and myeloid bias) of HSCs are very reminiscent of those seen under inflammatory stresses. Moreover, aged HSCs demonstrate increased myeloid output in response to inflammatory challenges as compared to young HSCs.[19] Elevated levels of the pro-inflammatory CC-chemokine ligand 5 (CCL5) have been reported in the old BM niche, which is likely responsible for myeloid skewing of aged HSCs.[14,17] Furthermore, it has been shown that tumor necrosis factor α (TNF-α) can drive myeloid regeneration, and the treatment of aged mice with interleukin 1 (IL-1) and TNF-α inhibitors reduces myelopoiesis.[18,20] Interestingly, an HSC-specific TNF-α signature is enriched in aged and malignant HSCs,[20] supporting the idea that inflammation plays an important role in HSC aging. To note, positive feedback between aging and inflammation may exist, as more myeloid cells, which secrete pro-inflammatory cytokines, are produced as HSCs age. As a consequence, those cytokines in turn promote myelopoiesis which then promotes HSC aging.[11]

FUTURE PERSPECTIVES

Hematopoietic aging is a very complex and multifactorial phenomenon, involving both intrinsic and extrinsic factors which directly impact HSCs, resulting in a plethora of age-associated clinical manifestations. In the context of clonal hematopoiesis, it remains unknown why ARCH/CHIP arises and what the dynamics of clonal growth within an individual are. For example, it is possible that clonal hematopoiesis is affected by inflammatory signals and that recurrent infections provoke the emergence of dominant clones. Further advancements and increased sensitivity of genomic sequencing will shed light on the actual frequency of mutations and should entail which—known or unknown—mutations are actually related to a poor prognosis. This layer of information, which is progressively being introduced into clinical diagnostics, provides two major advantages. First, a detailed characterization of clones should inform if patients carry premalignant traits and therefore will assist with early diagnosis. Second, the nature of such mutations can potentially directly impact the treatment regime for the patient, focusing on the most effective chemotherapeutic agent against a particular clone for the development of a chemopreventive clinical strategy. Possibly, age-associated premalignant clones may be targetable. Thus, the detection of premalignant stages may not only improve survival but also dictate the specificity of the treatment.

Yet, there are multiple aspects of HSC aging that have not been elucidated. This includes the identification of alternative, novel, molecular mechanisms that may impact the age-associated HSC fate. Most studies have focused on stem cell-intrinsic mechanisms, while our knowledge of extrinsic factors remains limited. The elusive stem cell niche plays an important role in HSC maintenance and its alterations with age very likely impact HSC aging. Many distinct cell types in the niche have been shown to be involved in HSC regulation, where the role of most cells in the BM microenvironment remains to be determined. Changes in intrinsic factors with age may well be the consequences of alterations in the niche. A series of studies have shown that targeting intrinsic aging drivers can delay or, to some extent, reverse the aging process. Intervention at the level of the niche provides novel targets for future therapies resulting in the delay of HSC aging and treating age-related hematopoietic disorders.

Moreover, recent efforts have been focused on understanding the mechanisms behind non-malignant and malignant HSC heterogeneity. These studies will provide fundamental aspects of clonal dynamics that will further refine our understanding and will allow the development of novel therapeutic approaches to mitigate the effect of aging on hematopoiesis.

REFERENCES

1. López-Otín C, Blasco MA, Partridge L, et al. The hallmarks of aging. *Cell*. 2013;153(6):1194. https://doi.org/10.1016/j.cell.2013.05.039.
2. Shlush LI, Zandi S, Itzkovitz S, Schuh AC. Aging, clonal hematopoiesis and preleukemia: not just bad luck? *Int J Hematol*. 2015;102(5):513–522. https://doi.org/10.1007/s12185-015-1870-5.
3. Chung SS, Park CY. Aging, hematopoiesis, and the myelodysplastic syndromes. *Hematology Am Soc Hematol Educ Program*. 2017;2017(1): 73–78.
4. Stauder R, Valent P, Theurl I. Anemia at older age: etiologies, clinical implications, and management. *Blood*. 2018;131(5):505–514. https://doi.org/10.1182/blood-2017-07-746446.
5. Aiello A, Farzaneh F, Candore G, et al. Immunosenescence and its hallmarks: how to oppose aging strategically? A review of potential options for therapeutic intervention. *Front Immunol*. 2019;10(Sep):2247. https://doi.org/10.3389/fimmu.2019.02247.
6. Montoro J, Yerlikaya A, Ali A, Raza A. Improving treatment for myelodysplastic syndromes patients. *Curr Treat Options Oncol*. 2018;19(12). https://doi.org/10.1007/s11864-018-0583-4.
7. Kikushige Y, Miyamoto T. Hematopoietic stem cell aging and chronic lymphocytic leukemia pathogenesis. *Int J Hematol*. 2014;100(4):335–340. https://doi.org/10.1007/s12185-014-1651-6.
8. Ayachi S, Buscarlet M, Busque L. 60 Years of clonal hematopoiesis research: from X-chromosome inactivation studies to the identification of driver mutations. *Exp Hematol*. 2020;83:2–11. https://doi.org/10.1016/j.exphem.2020.01.008.
9. Steensma DP. Clinical consequences of clonal hematopoiesis of indeterminate potential. *Blood Adv*. 2018;2(22):3404–3410. https://doi.org/10.1182/bloodadvances.2018020222.
10. Jaiswal S, Ebert BL. Clonal hematopoiesis in human aging and disease. *Science*. 2019;366(6465):eaan4673. https://doi.org/10.1126/science.aan4673.
11. Kovtonyuk LV, Fritsch K, Feng X, et al. Inflamm-aging of hematopoiesis, hematopoietic stem cells, and the bone marrow microenvironment. *Front Immunol*. 2016;7(Nov):1–13. https://doi.org/10.3389/fimmu.2016.00502.
12. De Haan G, Lazare SS. Aging of hematopoietic stem cells. *Blood*. 2018;131(5):479–487. https://doi.org/10.1182/blood-2017-06-746412.
13. Mejia-Ramirez E, Florian MC. Understanding intrinsic hematopoietic stem cell aging. *Haematologica*. 2020;105(1):22–37. https://doi.org/10.3324/haematol.2018.211342.
14. Akunuru S, Geiger H. Aging, clonality, and rejuvenation of hematopoietic stem cells. *Trends Mol Med*. 2016;22(8):701–712. https://doi.org/10.1016/j.molmed.2016.06.003.
15. Kramer A, Challen GA. The epigenetic basis of hematopoietic stem cell aging. *Semin Hematol*. 2017;54(1):19–24. https://doi.org/10.1053/j.seminhematol.2016.10.006.
16. Sun D, Luo M, Jeong M, et al. Epigenomic profiling of young and aged HSCs reveals concerted changes during aging that reinforce self-renewal. *Cell Stem Cell*. 2014;14(5):673–688. https://doi.org/10.1016/j.stem.2014.03.002.
17. Geiger H, De Haan G, Florian MC. The ageing haematopoietic stem cell compartment. *Nat Rev Immunol*. 2013;13(5):376–389. https://doi.org/10.1038/nri3433.
18. Pioli PD, Casero D, Montecino-Rodriguez E, et al. Plasma cells are obligate effectors of enhanced myelopoiesis in aging bone marrow. *Immunity*. 2019;51(2):351–366. https://doi.org/10.1016/j.immuni.2019.06.006. e6.
19. Chen Z, Ju Z. Inflamm-aging of hematopoietic stem cells. *Blood Sci*. 2019;1(2):141–143. https://doi.org/10.1097/BS9.0000000000000029.
20. Yamashita M, Passegué E. TNF-α coordinates hematopoietic stem cell survival and myeloid regeneration. *Cell Stem Cell*. 2019;25(3):357–372. https://doi.org/10.1016/j.stem.2019.05.019. e7.

PART III IMMUNOLOGIC BASIS OF HEMATOLOGY

CHAPTER 20

DENDRITIC CELL BIOLOGY

Cansu Cimen Bozkus and Nina Bhardwaj

Dendritic cells (DCs), originally discovered in 1973 by Ralph M. Steinman and Zanvil A. Cohn and named after their distinct stellate morphology, are important regulators of immune responses.[1] Specialized in antigen acquisition, processing, and presentation, the hallmark of DCs is their ability to induce naïve T cell activation and effector differentiation. DCs also interact with other immune cells, such as B cells, natural killer (NK) cells, innate lymphoid cells, and secrete immunomodulatory molecules, thereby mediating the induction and maintenance of immunity and tolerance.[2] This chapter describes the current knowledge of DC ontogeny and function as well as the utility of DCs as immunotherapeutic agents in clinical applications.

DENDRITIC CELL SUBSETS AND DEVELOPMENT

DCs are highly heterogeneous and classified into subsets based on their developmental origin, anatomical location, function, expression profile of transcription factors, and phenotypic markers. DC development involves four stages, namely, hematopoietic precursors, DC precursors (pre-DC), immature DCs (imDCs), and mature DCs (mDCs). All DCs originate in the bone marrow and are continuously produced in a steady rate from $CD34^+$ hematopoietic stem cells (HSCs) in a process primarily directed by Fms-like tyrosine kinase 3 ligand (Flt3L) and granulocyte-macrophage colony-stimulating factor (GM-CSF).[3] According to the classical model of hematopoiesis, HSCs give rise to common myeloid precursors (CMPs), excluding lymphoid lineage potential, and CMPs then develop into monocyte DC precursors (MDPs), excluding granulocyte potential. MDPs subsequently transition into common DC progenitors (CDPs). MDPs give rise to inflammatory DCs (iDCs), while CDPs give rise to pre-DCs that migrate out of bone marrow into lymphoid and nonlymphoid organs, where they differentiate into major DC subsets: conventional DCs (cDCs) and plasmacytoid DCs (pDCs).[4] Notably, pDCs are also produced by the lymphoid progenitors in the steady state (Fig. 20.1).[5–7] Therefore, the progressive lineage specification that limits alternative fate potential, described by the classical hematopoiesis model, should not imply a solid state of cell fates.

This section summarizes the transcriptional and phenotypical characteristics associated with distinct DC subsets while concentrating on human DCs with little reference to murine models. Readers are encouraged to seek additional information provided in other comprehensive reviews.[3,8]

Broadly, DCs are defined as mononuclear immune cells that lack key lineage markers for T cells (CD3), B cells (CD19 and CD20), and NK cells (CD56). Additionally, DCs express high levels of major histocompatibility complex (MHC) class II molecules, such as HLA-DR. Expression of distinct surface markers and transcription factors are utilized to classically categorize DCs into four major terminally differentiated subsets: cDCs, pDCs, iDCs, and Langerhans cells (LCs).[3]

Conventional Dendritic Cells

cDCs comprise two major subsets, cDC1s and cDC2s. Both subsets express CD11c and MHC class II molecules. In humans, cDC1s are characterized by the expression of CD141 (BDCA-3), CLEC9A, XCR1 and CADM1, whereas cDC2s express CD1c (BDCA-1), SIRPα (CD172), and CD11b. In mice, cDC1s also express XCR1, CLEC9A, CADM1, as well as CD8a/CD103 and cDC2s express SIRPα, CD4, and CD11b.[9] Although these markers define the cDC1 subset quite precisely, cDC2 markers are less specific and do not address the heterogeneity within the cDC2 subset. Recent studies by independent groups have identified that the classically defined cDC2 subset comprise multiple subpopulations,[10,11] which are detailed in the "New Developments in DC Classification" section.

The development of cDC subsets is regulated by distinct transcriptional programs. cDC1s express interferon regulatory factor (IRF) 8, basic leucine zipper transcriptional factor ATF-like 3 (Batf3), and DNA-binding protein inhibitor 2 (ID2). cDC2s express IRF4 and Kruppel-like factor 4 (Klf4). Notch signaling also plays an essential role in the development and differentiation of cDCs. Evidence for the importance of Notch signaling in DC programming comes from in vitro cultures both in murine and human systems, in which DCs are generated in the presence of OP9 cells, a stromal cell line supporting the differentiation of HSCs.[12] Specifically, in the context of human cells, expanding cord blood or peripheral blood $CD34^+$ precursor cells with key factors for DC growth and differentiation, namely Flt3-L, SCF, IL-7, and thrombopoietin (TPO) and subsequently inducing their differentiation in the presence of OP9 feeder cells, generated primarily pDCs with small frequencies of cDCs. In the same system, when Notch ligand DLL1 expressing OP9-DLL1 cells were used as feeders, pDC generation was suppressed and the proportion of cDCs, particularly cDC1s, were greatly increased, which transcriptionally, phenotypically, and functionally resembled their primary blood cDC1 counterparts.[13] Other studies in murine models have also demonstrated nonredundant roles for Notch signaling in cDC2 differentiation and function, particularly in T follicular helper (T_{FH}) cell differentiation and induction of B cell responses during infection.[14,15]

Both cDC subsets are known for their unique efficacy for priming naïve T cells and inducing their functional polarization. Additionally, cDC1s, expressing an array of immunoregulatory molecules, are key for tolerogenic regulation.[16] cDC1s are superior to cDC2s at antigen cross-presentation, a process through which exogenous antigens are acquired and presented on MHC class I molecules, thereby inducing $CD8^+$ T cell responses.[17] While cDC1s can also activate $CD4^+$ T cells and polarize them toward a Th1 phenotype, cDC2s are the main subset specialized in MHC class II-mediated antigen presentation. cDC2s efficiently activate $CD4^+$ T cells and induce their polarization toward Th1, Th2, and Th17 phenotypes in specific contexts. cDC2 is

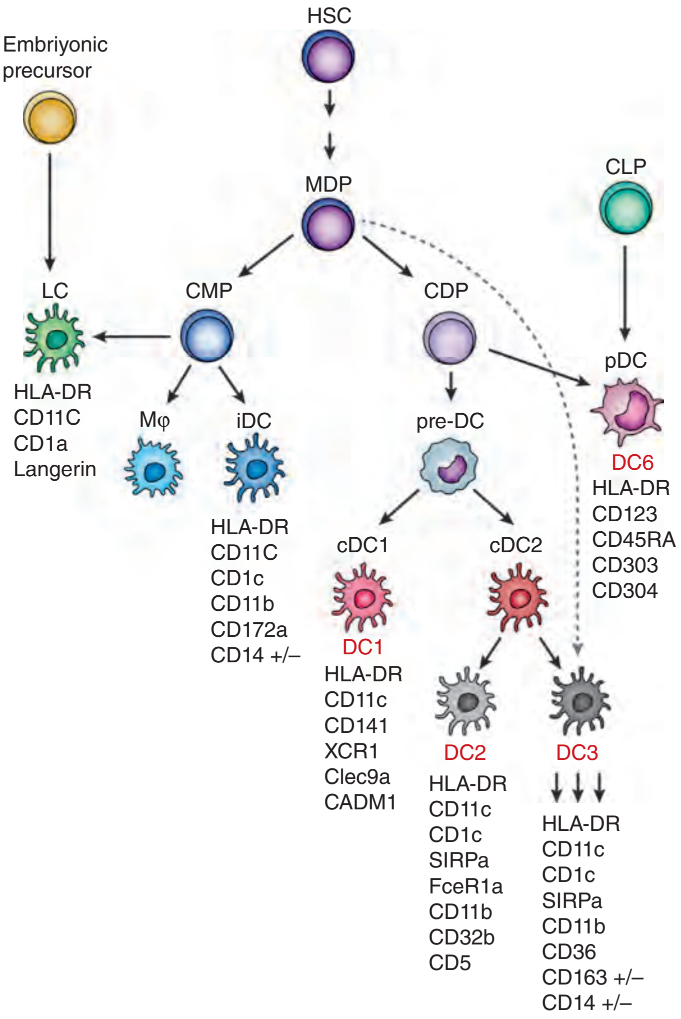


Figure 20.1 SIMPLIFIED MODEL OF DENDRITIC CELL HEMATOPOIESIS AND SUBSET CLASSIFICATION. The arrows indicate the lineage hierarchy. Classically defined DC subsets are also annotated in red based on their shared characteristics with more recently defined DC subset classifications, namely DC1-6, which are still undergoing modification. A simplified list of markers delineating different DC subsets is also described. *CDP*, Common dendritic cell progenitors; *CLP*, common lymphoid precursors; *CMP*, common myeloid progenitors; *HSC*, hematopoietic stem cells; *MDP*, macrophage dendritic cell progenitors; *Mφ*, macrophages.

the most frequent DC population and found in blood, lymphoid, and non-lymphoid tissues, while cDC1s are thought to primarily reside in the lymph nodes (LNs) and found in trace numbers in the blood. Another distinct feature between cDC1 and cDC2 subsets is the differential expression of pattern recognition receptors (PPRs), which recognize conserved molecules displayed by pathogens or damaged cells. cDC1s express Toll-like receptors (TLRs) 3 and 8. Due to TLR3 triggering, cDC1s have the unique ability to produce type III interferons, which contribute to antiviral and antitumor immunity.[16–19] cDC2s express a wide range of PRRs, TLRs 1–8, and produce IL-23, a critical cytokine for innate defense. Hence, cDC2s mediate antimicrobial responses against a greater variety of pathogens.[20]

Plasmacytoid Dendritic Cells

pDCs are distinguished by high levels of CD123, CD303 (BDCA-2), and CD304 (BDCA-4) and the lack of CD11c, AXL, Siglec-6 expression.[11] Transcription factor Tcf4 specifically regulates the development of pDCs.[21] pDCs can also be recognized by their distinct morphology resembling secretory plasma cells with a round shape and well-defined endoplasmic reticulum (ER).[22,23] Although small fractions of pDCs may be found in blood, they primarily reside in lymphoid tissues. The hallmark of pDCs is the ability to produce large quantities of type I interferons, IFN-α/β, although they have been shown to produce type III interferon, IFN-λ, as well.[24,25] pDCs produce interferons in response to recognition of viral RNA or DNA through TLR7 and TLR9, respectively, and exert potent antiviral effects. The activation of TLR7/9 may also lead to the production of tumor-necrosis factor-α (TNF-α) and IL-6 by pDCs, which contributes to pDC maturation, antibody production by B cells, and generation of tolerogenic T cells.[26–29]

pDCs lack the capacity for antigen presentation and T cell stimulation in an immature state, suggesting they are less important for the activation of naïve T cells. However, recent studies have shown that upon activation, a subset of pDCs differentiates into a more cDC-like state by adopting a dendritic morphology, upregulating HLA-DR, CD80, and CD11c and downregulating TCF4. This activated pDC subpopulation has antigen-presenting capacity and can prime T cells, highlighting the high degree of DC heterogeneity and plasticity.[23,30]

Inflammatory Dendritic Cells

iDCs appear under inflammatory conditions, accumulate at the inflammation site, and disappear upon resolution of inflammation. They are characterized by the expression of CD11c, CD11b, CD172a, CD14, and CD1c. iDCs also express the Zbtb46 transcription factor, which is specific to DC lineage. Functionally, iDCs can process and present antigens to T cells and induce T call activation and polarization. They can also produce inflammatory cytokines, including IL-6, IL-1β, and IL-23.[31] Based on their gene signature similarities, iDCs are proposed to be in vivo equivalents of monocyte-derived DCs (moDCs), which are differentiated in in vitro culture systems most commonly with the addition of GM-CSF and IL-4 (Fig. 20.2).[31] However, the role of GM-CSF for the generation of iDCs in vivo is not fully understood. In mice, while GM-CSF or its receptor were dispensable, M-CSF receptor was shown to be essential for iDC differentiation and function.[32]

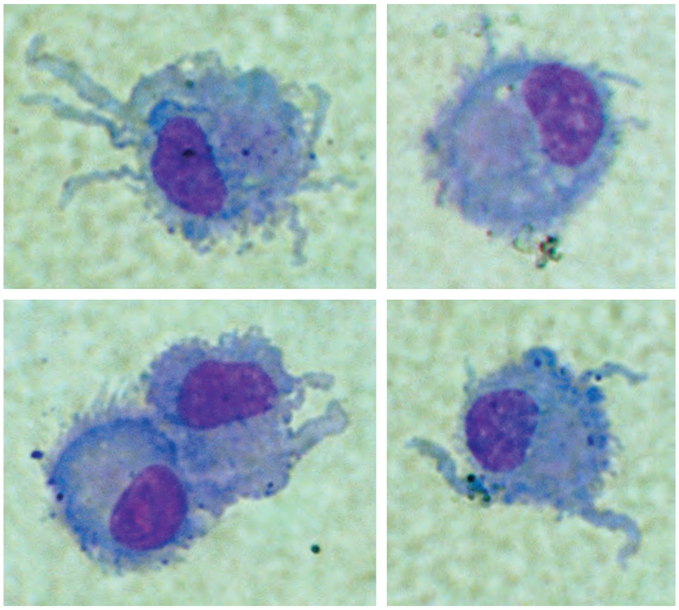


Figure 20.2 EXAMPLES OF MONOCYTE-DERIVED MATURE DENDRITIC CELLS. The monocytes were enriched by plastic adherence, cultured with interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF) for 6 days, and underwent maturation with IL-1, IL-6, tumor-necrosis factor-α (TNF-α), and prostaglandin E_2 (PGE_2) for 24 hours.

Langerhans Cells

LCs are tissue-resident cells found in the epidermal layer of the skin. LCs share common precursors with macrophages and are classified in the same lineage as macrophages. Moreover, like other tissue-resident macrophages, LCs can proliferate in differentiated states. Importantly, LCs also have DC-like capabilities, such as acquiring antigens, migrating to LNs, and activating T cells.[33–35] LCs express CD1a and Langerin (CD207). They also contain Birbeck granules in their cytoplasm, which distinguishes them from the other Langerin expressing dermal DCs.[36] Ontologically, LCs arise from yolk sac and fetal liver progenitors. These progenitors seed the skin during embryogenesis and differentiate into LCs in a transforming growth factor-β (TGF-β) and M-CSF receptor-dependent process.[35,37] Once established, LCs are maintained locally by self-renewal under steady state conditions.[38,39] However, LCs also have a hematopoietic origin as circulating hematopoietic precursor cells have been shown to replenish the LC population during skin inflammation.[33,38]

Serving as an immunological barrier to the external environment, LCs are efficient at recognizing foreign antigens since they express a variety of PRRs including Langerin and a large repertoire of TLRs.[40] Upon recognition of foreign antigens, LCs upregulate MHC class II and other co-stimulatory molecules and migrate to LNs, where they prime CD4$^+$ T cells and induce their polarization into Th1, Th2, Th17, and Th22 phenotypes.[41–43] LCs can also prime CD8$^+$ T cells under certain circumstances.[44] LCs also play a role in establishing immune tolerance, for example by presenting self-antigens to T cells in the LNs in homeostatic conditions,[45] as well as by inducing regulatory T cells in the skin that suppress allergic skin reactions.[46,47]

New Developments in Dendritic Cell Classification

The understanding of DC developmental pathways and subsets is continuously being improved due to advances in high-dimensional, single-cell technologies by more sensitively defining the identities of this highly heterogenous population. Utilizing blood cells isolated from healthy donors, which were enriched for HLA-DR expression and lack of lineage markers (CD3, CD56, CD19, CD14), Villani et al., performed single-cell RNA sequencing (scRNAseq) and unbiased clustering analysis to identify and compare DC populations. They identified six distinct DC subpopulations (DC1-6). Gene signatures of most of these clusters aligned well with traditionally defined subsets. DC1, DC6, and DC4 clusters mapped to cDC1, pDC, and CD16$^+$ monocytes, respectively. Two distinct clusters, DC2 and DC3, mapped to cDC2 subset, suggesting a new subdivision within the classical cDC2 population. Additionally, a previously unknown subset was identified. Despite expressing markers in common, cells in this cluster (DC5) differed from pDCs by their morphological resemblance to cDCs and their potent capacity to activate T cells. This novel subset was defined by the expression of AXL and SIGLEC6, hence named “AS DCs,” and corresponded to 2% to 3% of blood DCs.[11] Corroborating these results, Alcantara-Hernandez et al., unbiasedly evaluated the phenotypic heterogeneity of human blood and tissue DCs by utilizing mass spectrometry (CyTOF) and identified a cluster corresponding to AS DCs and subdivisions within the cDC2 population.[30] Such subdivisions within the cDC2s were further delineated by Dutertre et al., demonstrating that cDC2s comprise a CD5$^+$ and three CD5$^-$ subpopulations (CD163$^-$, CD163$^+$CD14$^-$, and CD163$^+$CD14$^+$ cells), with CD5$^+$ and CD5$^-$ cells resembling DC2 and DC3s, respectively, as described by Villani et al.[10] Another recent scRNA-seq study, by Bourdely et al., also identified the DC3 subset in blood and defined DC3s as a separate subset bearing monocyte markers (e.g., CD163, CD14) and features of cDC2s. Further supporting the distinction of the DC3 subset, they identified a DC3-restricted progenitor population.[48] Independently, Cytlak et al. found that the heterogeneity of DC2 and DC3 subsets was associated with distinct developmental pathways dependent on differential IRF8 requirements.[49]

DENDRITIC CELL MATURATION

Under steady state conditions DCs commonly exist in an immature form. Upon detecting tissue disturbances and danger signals through their PRRs, DCs undergo phenotypical and functional changes, enhancing their capacity to stimulate T cells. This process is called DC “maturation” or “activation.”

DC maturation can be induced by a variety of stimuli such as microbial products, danger signals released by damaged cells, cytokines, and cellular crosstalk (e.g., ligation of CD40).[50–52] To sense such diverse stimuli, DCs express a wide array of PRRs, localized both at the cell membrane and the cytoplasm. PRRs recognize pathogen-associated molecular patterns (PAMPs), derived from microorganisms, or danger-associated molecular patterns (DAMPs), secreted by damaged cells. Membrane-bound PRRs include TLRs and C-type lectin receptors (CLRs). Cytoplasmic PRRs include NOD-like receptors (NLRs) and retinoic acid (RA)-inducible gene (RIG)-I-like receptors (RLRs). Other receptors such as scavenger receptors (SRs) and integrins can also serve as PRRs.[53] Another major group of PRRs is secreted proteins, including complement receptors and collectins. PRRs are further classified into subgroups based on their ligand specificity and function. Table 20.1 summarizes the properties of PRRs.[54–58]

imDCs continuously sample their surroundings and capture antigens via endocytosis, a process by which cells internalize extracellular substances. DCs utilize multiple endocytic mechanisms to acquire external antigens: phagocytosis (ingestion of large particles, e.g., pathogens, apoptotic cells), micropinocytosis (engulfing extracellular liquid and dissolved molecules), and receptor-mediated endocytosis.[59] Upon engagement of PRRs by inflammatory stimuli and induction of maturation, DCs downregulate endocytosis, restricting antigen capture after the initial stimulation.[60] Concomitantly, DCs upregulate cell surface expression of MHC class II molecules and co-stimulatory molecules (e.g., CD80, CD86), which are facilitated by DC-LAMP and CD83, two key markers that distinguish mDCs.[61,62] Further augmenting the efficacy of antigen presentation to T cells, DC maturation increases the half-life of MHC molecules and enhances lysosomal acidification and antigen loading onto MHC-II. Mature DCs also upregulate CCR7, a homing receptor, and migrate to LNs in response to chemokines such as CCL-19 and CCL-21. In the LNs, mDCs present antigens to T cells, both non-self and self, and induce T cell polarization into cytotoxic or tolerogenic states, respectively.[63] The immunogenic or tolerogenic function of DCs also depends on the type of stimuli that induce their maturation. DCs may produce pro-inflammatory, for example, IL-12, IL-6, IL-1β, or anti-inflammatory, for example, IL-10, TGF-β, cytokines in response to different stimuli, which impacts T cell polarization.[64] Tolerogenic DCs may induce the formation of regulatory T cells (Tregs), suppressing immune responses.[65] Therefore, DCs are key mediators of both immunity and tolerance.

ANTIGEN PROCESSING AND PRESENTATION

DCs are the most efficient antigen-presenting cell (APC) population in vivo. Through specialized mechanisms, DCs capture both exogenous and endogenous antigens, process these antigens, and present them in the context of class I and class II MHC molecules to CD8$^+$ and CD4$^+$ T cells, respectively (Fig. 20.3). DCs also present lipid antigens to T cells by CD1 molecules.[66]

MHC Class I Antigen Presentation (Endogenous Route)

Like all nucleated cells, DCs express MHC-I molecules. Antigens bound to MHC-I molecules are typically generated endogenously within the cytosol. Such endogenous antigens can be derived from both self and non-self molecules, for example, mutated or microbial proteins, respectively. Therefore, the MHC-I antigen presentation

TABLE 20.1 **Pattern Recognition Receptors**[a]

PRRs		Localization	Natural Ligand	Source
TLRs	TLR2/ TLR1	Plasma membrane	Triacyl lipopeptides	Bacteria
	TLR2/ TLR6	Plasma membrane	Diacyl lipopeptides	Bacteria, viruses, fungi, parasites
	TLR3	Endolysosome	dsRNA	Viruses
	TLR4	Plasma membrane	LPS, HSPs	Bacteria, viruses, self
	TLR5	Plasma membrane	Flagellin	Bacteria
	TLR7	Endolysosome	ssRNA	Viruses
	TLR8	Endolysosome	ssRNA	Viruses
	TLR9	Endolysosome	CpG ODNs	Bacteria, viruses
	TLR10	Endolysosome	Unknown	Unknown
CLRs	MMR	Plasma membrane	Mannose	Bacteria, fungi, parasites
	DEC-205	Plasma membrane	CpG ODNs, Keratins	Bacteria, viruses, self
	Dectin-1	Plasma membrane	β-Glucan	Fungi
	DC-SIGN	Plasma membrane	Mannose, fucose	Bacteria, viruses
	CLEC9A	Plasma membrane	F-actin	Self
NLRs	NOD1	Cytoplasm	iE-DAP	Bacteria
	NOD2	Cytoplasm	MDP	Bacteria
	NLRPs	Cytoplasm	LT, particulate matters	Bacteria, viruses, self
ALRs	AIM2	Cytoplasm	dsDNA	Bacteria, viruses, self
	IFI16	Cytoplasm	dsDNA	Bacteria, viruses, self
RLRs	RIG-I	Cytoplasm	Short dsRNA	Viruses
	MDA5	Cytoplasm	Long dsRNA	Viruses
	LGP2	Cytoplasm	dsRNA	Viruses
	STING	ER	cyclic dinucleotides	Bacteria, viruses

[a] A list of some of the PRRs expressed by dendritic cells (DCs), their cellular localization at steady state, and their natural ligands.

ALR, Absent in melanoma (AIM) 2-like receptor; *CLR*, C-type lectin receptor; *CpG ODN*, deoxycytidyl-phosphate-deoxyguanosine oligodeoxynucleotides; *ds RNA*, double-stranded ribonucleic acid; *ER*, endoplasmic reticulum; *HSP*, heat shock protein; *iE-DAP*, g-D-glutamyl-meso-diaminopimelic acid; *LPS*, lipopolysaccharide; *LT*, lethal toxin; *MDA5*, melanoma differentiation-associated gene 5; *MDP*, muramyl dipeptide; *MMR*, macrophage mannose receptor; *NLR*, nucleotide-binding oligomerization domain (NOD)-like receptor; *PRRs*, pattern recognition receptors; *RLR*, retinoic acid-inducible gene (RIG)-I-like receptor; *ssRNA*, single-stranded ribonucleic acid; *STING*, stimulator of IFN genes; *TLR*, toll-like receptor.

pathway is critical for mediating immune detection of transformed or infected cells.

To bind to MHC-I molecules, antigens need to be broken down into certain lengths of peptides (commonly 8 to 11 amino acids). Antigenic peptides are initially generated by proteasome degradation and peptidase trimming in cytosol and then are translocated to the ER lumen by transporter associated with antigen processing (TAP). In the ER, a transient multi-subunit peptide loading complex (PLC) is formed, which comprises TAP, ERp57, and chaperon proteins tapasin, calreticulin, and calnexin. Interactions between these proteins and MHC-I heavy and light chains (β2-microglobulin) facilitate the assembly of MHC-I and retain empty MHC-I in a peptide receptive state within the PLC.[67] PLC mediates the loading of antigenic peptides onto newly synthesized MHC-I. Tapasin promotes the binding of high-affinity peptides to MHC-I by increasing the dissociation rate of suboptimal peptides.[68] Further enhancing the optimal peptide–MHC (pMHC) binding, antigenic peptides may be further trimmed by ER aminopeptidases (ERAP1/2) to increase conformational stability.[69] Once loaded with the optimal peptide, MHC-I molecules are stabilized and routed to the cell surface, where they can present the antigenic peptide to $CD8^+$ T cells, inducing their cytotoxic functions.

MHC Class II Antigen Presentation (Exogenous Route)

MHC-II molecules bind to peptides that are derived from extracellular proteins and from self-proteins that are degraded in the endosomal pathway. While all nucleated cells express MHC-I, only APCs express MHC-II. Although MHC-I and MHC-II molecules are structurally similar, the peptide-binding groove of MHC-II is more open and hence accommodates longer antigenic peptides (10 to 30 amino acids). Like MHC-I, the assembly of MHC-II molecules also occurs in the ER. MHC-II in the ER associates with the invariant chain (Ii) protein, which acts as a pseudopeptide to stabilize MHC-II and targets MHC-II molecules to late endosomal compartments called MHC class II compartment (MIIC) to encounter antigenic peptides. Antigenic peptides are generated in the MIIC by proteolytic degradation enabled by the acidic pH and resident proteases called cathepsins. The same proteases also degrade the Li protein bound to MHC-II, leaving a fragment called class II-associated invariant chain peptide (CLIP) intact as CLIP is bound to the MHC-II peptide-binding groove and hence protected. The chaperone protein HLA-DM catalyzes the replacement of CLIP by the antigenic peptides that bind to MHC-II with high affinity.[70,71] Antigen loading to MHC-II is further controlled by HLA-DO, another chaperone protein that pairs with HLA-DM and restricts HLA-DM activity only to acidic compartments.[72] Peptide-bound MHC-II are then transported to the cell membrane, where they can present the antigenic peptide to cognate $CD4^+$ T cells and induce helper T cell-mediated immunity.

Cross-Presentation

Cross-presentation is the process by which APCs present exogenous antigens on MHC class I molecules. This process is important for immune surveillance as it allows APCs, which are not transformed or infected themselves, to trigger cytotoxic immune responses or to induce cross-tolerance. DCs are considered the most efficient cross-presenting cells. The cross-presentation capacity of DCs varies among subsets and is influenced by the type of antigen, the inflammatory stimuli triggering PRRs and the endocytic receptor repertoire of DCs.[73,74]

DCs can acquire exogenous antigens from various sources, including apoptotic cells, necrotic cells, antibody-opsonized cells, immune complexes, heat shock proteins, exosomes, and exchange of antigen containing vesicles through dynamic synapses formed between DC subsets, and even nibbling of live cells.[75,76] Mechanistically, cross-presentation occurs via two major pathways, the cytosolic and the vacuolar pathways. In the cytosolic pathway, endocytosed antigens are transferred to cytosol, where they are degraded by the proteasome. These antigenic peptides then may translocate either to the ER or back to the endosomes to be loaded onto MHC-I. In the vacuolar pathway, antigens are degraded by lysosomal proteases and loaded onto recycling MHC-I in endocytic compartments. Signaling through TLRs has been reported to promote cross-presentation by inducing the accumulation of the MHC-I in the endosomes.[77] However, the exact

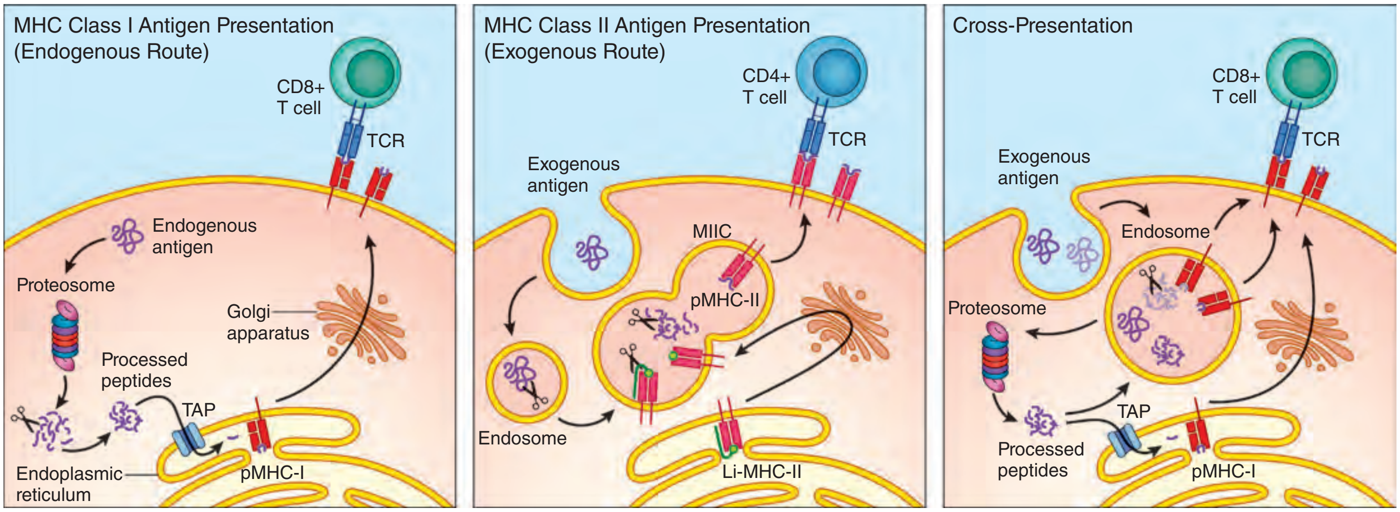


Figure 20.3 ANTIGEN PRESENTATION PATHWAYS IN DENDRITIC CELLS. The classical pathway for MHC class I antigen presentation *(left)* involves degradation of endogenous antigens into peptides by the proteasome followed by transport into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP). In the ER, peptides are loaded onto the newly synthesized MHC class I molecules, mediated by peptide loading complex, and further trimmed for conformational stability. Subsequently, peptide–MHC class I (pMHC-I) complexes are transported to the plasma membrane. For MHC class II antigen presentation *(middle)*, MHC-II molecules are assembled in the ER, where they associate with the invariant chain (Ii) protein. Li-MHC-II complex is then transported to endosomes called MHC class II compartment (MIIC). Proteases in the MIIC degrade Ii, leaving class II-associated invariant chain peptide (CLIP) intact in the MHC-II peptide-binding groove. HLA-DM catalyzes the exchange of CLIP by high-affinity antigenic peptides, which are endocytosed material (from both exogenous or endogenous sources) degraded by proteases in the MIIC. Peptide-bound MHC-II (pMHC-II) are then transported to the cell membrane. Two main pathways of cross-presentation *(right)* have been described that allow the presentation of exogenous antigens in association with MHC class I molecules. Antigens endocytosed or phagocytosed can be cleaved into peptides by proteases and loaded onto recycling MHC-I molecules within the same phagosome or on the cell surface (vacuolar pathway, shown in *light purple*). Alternatively, antigens may escape from the endosome and enter the cytosol (cytosolic pathway, shown in *dark purple*) to be processed via the classical MHC class I pathway. It has been suggested that elements of ER can be associated with phagosomes, allowing the transfer of antigens into the cytosol by the ER-associated degradation (ERAD) pathway and degradation by the phagosome-associated proteasome. The precise mechanisms and the locations of antigen processing in cross-presentation remain to be fully understood.

mechanisms and their relative contribution to cross-presentation are not yet fully understood.

An alternative mechanism for antigen presentation by DCs is cross-dressing. DCs can acquire fully functional pMHC complexes from other cells via trogocytosis (transfer of cell membrane patches), exosomes (vesicles that contain cellular constituents), or tunneling nanotubes. This process is called cross-dressing and allows rapid antigen presentation without the processing of antigens. DCs can acquire both MHC-I and MHC-II molecules by this method and elicit T cell responses.[78]

DENDRITIC CELL-MEDIATED IMMUNE RESPONSES

DCs are the sentinel cells of the immune system, constantly monitoring their external environment. Through their interactions with other immune cell populations, DCs initiate and orchestrate the appropriate immune responses against infectious diseases and cancer, as well as inducing immunological tolerance (Fig. 20.4).

T Cells

The hallmark function of DCs is to orchestrate T cell responses. By guiding T cell activation and differentiation in response to the inflammatory insults, DCs serve as a central bridge between innate and adaptive immunity. T cell priming by DCs takes place primarily at the LNs, which are specialized immune organs that are linked by lymphatic vessels. LNs are made up of the outer cortex and inner medulla surrounded by a fibrous capsule and mainly contain lymphocytes and B and T cells. T cells reside in the deeper part of the outer cortex, also called the paracortex or T cell zone. This is where T cells and DCs encounter one another. T cells (naïve and central memory) and DCs (primarily mDCs) both express the homing receptor CCR7, which permits trafficking in response to the CCR7 ligands, CCL19, and CCL21, expressed by cells in the LN paracortex.[79] T cell activation is initiated only when T cells encounter a DC presenting their cognate antigen. Increasing the likelihood of a successful encounter, both DCs and T cells are motile and continuously interact with each other in the absence of antigen recognition. DCs are estimated to contact approximately 500 different T cells per hour in the LN.[80] Upon successful encounters, T cell motility decreases, and T cells form stable, long-lasting interactions with DCs. This sustained engagement with DCs stimulates T cells and results in T cell expression of activation markers, effector cytokines, and subsequently clonal expansion. As activated T cells begin proliferating, they regain their motility and exit LNs and return to circulation.[81]

T cell responses can be categorized into three main stages: activation, differentiation, and memory formation. Fully efficient antigen-specific T cell activation requires three distinct signals. Signal 1 is the engagement of the T cell receptor (TCR) with the cognate peptide–MHC complex on APCs. Signal 2 is the co-stimulation and signal 3 is provided by the cytokine milieu. TCRs recognize antigenic peptides only in the context of MHC molecules. TCR binding to pMHC complex is the first trigger for T cell activation and initiates a cascade of downstream signaling, inducing a transcriptional program regulating cellular function. TCR triggering leads to membrane reorganization and formation of the immunological synapse between T cells and APCs. In addition to TCR clusters, co-stimulatory receptors are also recruited to the site. Co-stimulation acts to amplify or counteract the initial activating signals provided by TCR-pMHC interaction.[82] Co-stimulatory molecules are expressed on APC and include the B7.1 (CD80) and B7.2 (CD86), which ligate to CD28 on T cells. Notably, a new member of the B7 family expressed by DCs, B7-DC or programmed death ligand 2 (PD-L2), stimulates naïve T cells highly efficiently.[83] Co-stimulation may also lead to suppression of T cells responses, thereby preventing over-stimulation and autoimmune reactions. For example, binding of B7 ligands to cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antagonizes CD28 signaling and inhibits T cell activation. Similarly,

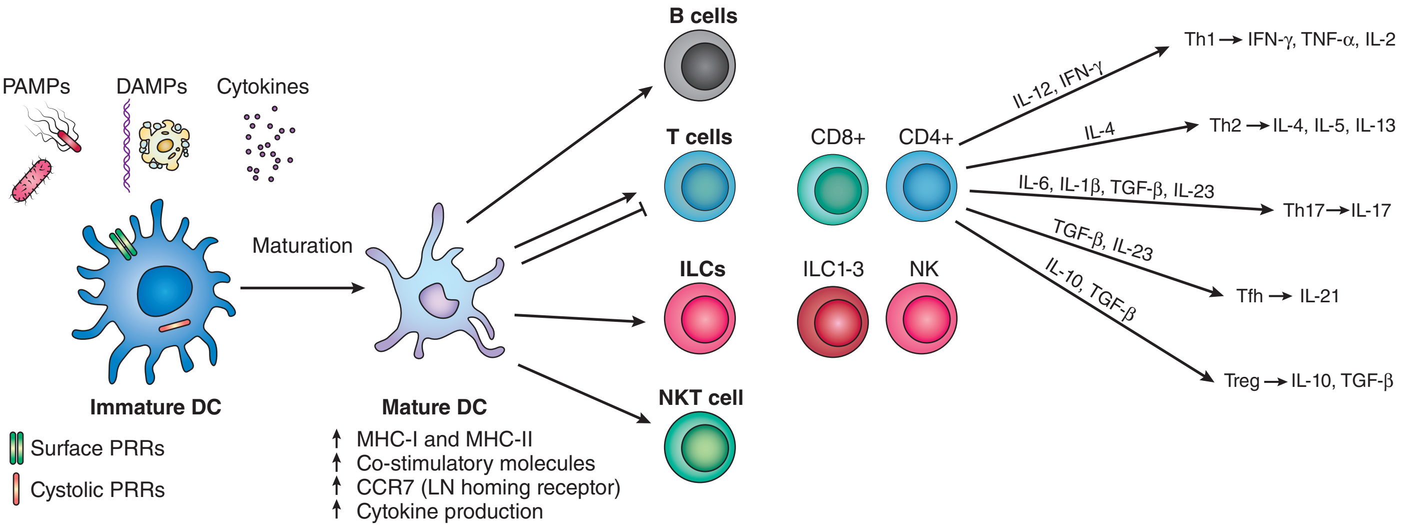


Figure 20.4 DENDRITIC CELLS LINK INNATE AND ADAPTIVE IMMUNITY. DCs sense tissue disturbances and danger signals through their pattern recognition receptors (PRRs) and undergo phenotypical and functional changes, called "maturation" or "activation." Upon maturation, DCs upregulate cell surface expression of MHC and co-stimulatory molecules, as well as cytokine production, pro- or anti-inflammatory. Collectively, these changes impact the DC-mediated differentiation, polarization, and immunomodulatory properties of various immune populations, including B and T cells, innate lymphoid cells (ILCs), and natural killer T cells (NKTs). *DAMP*, Danger-associated molecular pattern; *LN*, lymph node; *PAMP*, pathogen-associated molecular pattern.

engagement of PD-L1 and PD-L2 on DCs with PD-1 on T cells downregulates T cell responses.[84] Importantly, activation of T cells when co-stimulation is absent or suboptimal results in T cell anergy.[85] Co-stimulatory molecules also impact effector T cell differentiation.[84]

Signal 3, provided by cytokines in the microenvironment, determines the skewing of the T cell response such that naïve T cells may terminally differentiate toward different subsets with distinct functions and phenotypes. Classically, these phenotypes are grouped as T-helper cell types Th1 and Th2. IL-12 and IL-4, cytokines produced by DCs, are key determinants of initiation or amplification of Th responses. IL-12 and IFN-γ induce a Th1 phenotype, in which T cells produce IFN-γ, TNF-α, IL-2 and eradicate intracellular pathogens. IL-4 induces a Th2 phenotype with T cells producing IL-4, IL-5, and IL-13, which are important for controlling extracellular parasites.[86] The T cell skewing capacity of DCs depends on several variables. For example, DC expression of T-bet is required for the induction of Th1 responses.[87] DCs activated by human thymic stromal lymphopoietin (TSLP) skew T cells to a Th2 phenotype.[88] The duration of DC activation is also critical for T cell skewing. Prolonged activation causes IL-12 depletion and results in "exhausted DCs," which induce Th2 or nonpolarized T cells.[89] Antigen concentration also determines the direction of T cell skewing, where DCs skew T cells toward Th1 or Th2 phenotypes depending on whether they are presenting high or low amounts of antigen, respectively, which in turn depends on the maturation state of the DCs and consequences of environmental exposure.[90]

The differentiation of naïve T cells into different effector subsets is complex as the inflammatory milieu is comprised of a multitude of cytokines and distinct immune cell subsets. Therefore, a spectrum of T cell phenotypes beyond Th1 and Th2 exists, such as Th9, Th22, Th17, T_{FH} cells, and regulatory T (Treg) cells. DCs can polarize naïve T cells toward these phenotypes as well. For example, iDCs produce IL-6, IL-1β, TGF-β, and IL-23 and efficiently induce TH17 cell polarization.[91] IL-12, together with IL-23 and TGF-β, cytokines produced by activated DCs, induce the differentiation of naïve $CD4^+$ T cells into T_{FH} cells, which secrete IL-21 and regulates B cell responses.[91] Tolerogenic DCs secrete IL-10 and TGF-β and induce the generation of immunosuppressive Tregs.[92]

B Cells

Due to their ability to present antigens and mediate humoral responses, B cells are involved in both innate and adaptive immunity. DCs are important regulators of B cell responses. DCs influence B-cell proliferation, isotype switching, and plasma cell differentiation. DCs and B cells primarily interact in the outer cortex of LNs, as well as in the germinal centers of LNs or spleen. Unlike T cells, B cells recognize antigens in unprocessed native form. DCs possess specialized nondegradative pathways, in which endocytosed antigens are retained intact and recycled to the cell surface without antigen degradation. Therefore, DCs can present internalized antigens in their native state to B cells, engaging the B cell receptor (BCR).[93] Depending on the multivalency of the antigen triggering BCR and the engagement of co-stimulatory molecules, B cells may activate without needing further help. However, if the triggering antigens are of low valency, in order to activate, B cells require help from $CD4^+$ T cells, mainly for the engagement of CD40 by CD40L present on T cells. B cell activation can further be augmented by tumor-necrosis factor (TNF)-family ligands produced by DCs, such as B cell activating factor (BAFF) and APRIL.[94] Once activated, B cells begin to clonally expand and form germinal centers, where B cells undergo isotype switching. DCs may influence this process by mediating CD40-independent isotype switching through BAFF and APRIL.[95] Following these events, B cells differentiate into short-lived plasma cells or memory B cells. IFN-α and IL-6, primarily produced by pDCs, also play a role in inducing plasma cell differentiation.[28] Furthermore, follicular DCs, which are present in germinal centers, participate in the maintenance of B cell memory by providing survival signals.[96]

Innate Lymphoid Cells

Innate lymphoid cells (ILCs) are lymphocytes that lack uniquely rearranged antigen receptors such as those displayed by T and B cells. ILCs comprise three non-cytotoxic cell subsets, namely ILC1, ILC2, ILC3, and a cytotoxic NK cell subset. Due to their functional similarities, ILCs are considered as innate counterparts of T cells with ILC1s, ILC2s, and ILC3s resembling Th1, Th2, and Th17 cells, respectively, while NK cells resemble cytotoxic $CD8^+$ T cells.[97] The interactions between DCs and ILCs are complex and further underscore the role of DCs in linking innate and adaptive immunity.

DCs can promote the differentiation of ILC subsets. IL-12, IL-23, IL-1β, and RA produced by DCs regulate dynamic and reversible differentiation between ILC1 and ILC3 subsets.[98] DCs' ability to produce RA directly improves ILC3 activity by upregulating RORγt and IL-22 expression.[99] RA also controls tissue localization of ILCs by altering ILCs' expression of homing receptors, resulting in a migration of ILCs from lymphoid tissues to the gut.[100] ILCs can also regulate DCs. For example, RA production by DCs may be induced by ILC2-derived IL-13 and GM-CSF.[101] ILC2-derived IL-13 also controls the migration of activated lung DCs into draining LNs, inducing Th2 skewing of CD4 T cells.[102] Finally, ILC3s activate DCs through the expression of membrane-bound lymphotoxin, thereby promoting T cell responses, and in return DCs improve ILC functions, suggesting feedback loops between ILCs, T cells, and DCs.[103]

The crosstalk between DCs and NK cells impacts NK cell activation and cytolytic activity, as well as the maturation state of DCs. NK cells and DCs can form an immune synapse, potentially helping directional and confined secretion of cytokines as well as facilitating receptor–ligand interactions on one another. Activated NK cells contribute to DC maturation through TNF-α and IFN-γ secretion. In turn, activated DCs secrete IL-12, IL-18, IL-15, and IFN-α/β, which enhance the IFN-γ secretion, proliferation, and cytotoxicity of NK cells. In some conditions, NK cells can lyse imDCs through NKp30, although mDCs are protected from cytolysis. This might represent a form of "cellular editing" whereby immature and tolerogenic DCs are cleared by NK cells during an ongoing immune response.[104] The interaction between NK cells and DC is likely to take place early during an ongoing immune response. This allows DC to exploit the ability of NK cells to kill tumor or infected cells and to cross-present this material to T cells, suggesting DCs and NK cells play complementary roles in regulating T cell responses.[105]

Other Elements of the Immune System

DCs have proven to be quite versatile in their ability to interact with various constituents of the immune system. For example, they can activate NKT cells by the presentation of the synthetic ligand α-galactosyl ceramide on CD1, inducing the production of cytokines, such as IFN-γ, and resistance to tumors.[106] CD1-restricted γδ T cells, which respond to microbial antigens from *Mycobacterium tuberculosis* and other organisms, induce maturation of resting DC, and induce IL-12 production. This pathway and the IFN-γ secretion by activated γδ T cells provide the immune system with a source of activated APCs, which can polarize Th1 responses.[106]

In conclusion, the influence of DCs on other cell types of the immune system is broad and integral for the regulation of immunity. Future studies will further determine the roles of the interplay between DCs and the myriad of other immune cells and inform the design of novel therapies to better remedy immunopathology.

DENDRITIC CELLS IN DISEASE

Dendritic Cells and Autoimmunity

DCs play a pivotal role in the balance between immunity and tolerance. DCs are important in the induction of both central and peripheral tolerance. In the former, DCs play a role in the deletion of autoreactive T cells in the thymus. In the latter, DCs in their steady state induce T cell deletion, energy, or generation of regulatory T cells, which interfere with the IL-2 production and proliferation of effector T cells against self. Peripheral tolerance is critical as they limit harmful immune responses to antigens that may not have been available during thymic selection, as well as limiting autoreactive T cells that may have escaped central tolerance.[107] DCs induce peripheral tolerance via a variety of mechanisms, including the activation status of DCs such that resting DCs, in the absence of stimulatory signals necessary for their maturation, can induce robust T cell tolerance against immunodominant antigens.[108,109] These tolerogenic effects of DCs may be mediated through the engagement of inhibitory TCRs, such as PD-1 and CTLA-4, which act synergistically.[110] Additionally, tolerogenic DCs can produce immunosuppressive factors such as indoleamine 2, 3-dioxygenase (IDO), as well as IL-10, TGF-β, IL-35, which induce Tregs and inhibit effector T cell functions.[111] Dysregulation of mechanisms orchestrating tolerance induction by DCs leads to autoimmune diseases. Some of these autoimmune diseases are detailed as follows.

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease affecting multiple organ systems. SLE results from a dysregulated immune system, in which $CD4^+$ T cell and B cell tolerance is impaired, leading to high levels of circulating autoreactive antibodies, in particular against nuclear material.[112] Dysregulation of DCs, including alterations in subset frequency and localization, overactivation of cDCs and pDCs, and functional defects in DCs, is associated with the pathogenesis of SLE. DCs from SLE patients exhibit altered CD40, CD86, Fcγ receptor, and PD-L1 expression.[113–116] Activated cDCs may promote lupus pathogenesis by presenting RNA-associated proteins and chromatin to self-reactive T cells.[117] Abnormalities associated with pDC-derived type I IFN, due to for example persistent stimulation of TLR7 and TLR9 by endogenous nucleic acids, also contribute to the autoimmune responses in SLE.[118–121] Furthermore, DCs may also enhance B cell function and antinuclear autoantibody production.[121–124]

Another autoimmune disease that is associated with increased type I IFN activity is Aicardi–Goutières syndrome (AGS). AGS is an inflammatory encephalopathy that clinically mimics a congenital viral infection despite the absence of virus. AGS is caused by the mutations of several genes involved in nucleic acid metabolism.[125] These mutations may lead to the accumulation of endogenous nucleic acid products, which are then sensed by pDCs, upregulating type I IFN production that is associated with AGS pathogenesis.[126]

Rheumatoid arthritis (RA) is a common chronic autoimmune disease that causes joint pain and its pathogenesis is strongly associated with autoreactive $CD4^+$ T cell functions. Accumulating evidence supports DCs' involvement in RA pathogenesis. Both myeloid and pDCs are found at elevated numbers in the synovial fluid surrounding the joints of RA patients, where they produce pro-inflammatory molecules and contribute to joint inflammation. Although the exact mechanisms through which DCs contribute RA initiation and progression are not fully appreciated, DCs are shown to present MHC class II-restricted self-antigens and induce autoreactive $CD4^+$ T cells, which propagate RA.[127–129]

DCs are involved in the pathogenesis of other autoimmune diseases as well, including multiple sclerosis, type I diabetes, inflammatory bowel disease, and idiopathic inflammatory myopathies.[130] Overall, DCs' role in the development of autoimmune diseases is mediated either via direct activation of self-reactive T or B cells or indirectly by creating a pro-inflammatory environment that interrupts the maintenance of tolerance against self-antigens. When considering treatment approaches for these autoimmune diseases, there is a need to identify and define the balance between regulatory and pathogenic DC roles.

Dendritic Cells and Immunodeficiency

Immunodeficiency disorders can be congenital or acquired and involve an impaired immune system, including DC deficiency or dysfunction. Such diseases have been instrumental in demonstrating the nonredundant in vivo functions of DCs. Congenital immunodeficiencies, also called primary immunodeficiency disorders, are genetic disorders usually present at birth. Mutations in key regulators of DC development and differentiation pathways lead to DC deficiency syndromes, such as mutations in *GATA2*, *IRF8* and *IKZF1* genes. These mutations may result in an absolute deficiency of DCs or a reduction in their numbers. Some DC deficiency syndromes also cause DC dysfunction either through cell-intrinsic effects or by disrupting DC crosstalk with other cells. People with DC deficiencies suffer from increased susceptibility to bacterial, fungal, and viral infections, as well as cancer.[131]

Dendritic Cells and Pathogens

DC deficiency syndromes unequivocally demonstrated the nonredundant roles of DCs in mounting the appropriate antimicrobial immune response in infections. Therefore, pathogens have evolved different mechanisms to inhibit DC functions, allowing them to escape from immune recognition and persist in the host. The mechanisms employed by pathogens to inhibit DC functions can be broadly categorized as downregulation of antigen presentation machinery, impairment of microbial sensing, prevention of DC maturation, and induction of immunosuppressive cytokine production. Some examples of how pathogens modulate DC functions are detailed as follows.

Immunodeficiency syndromes can be acquired, which develop later in life through exposure to a causing agent. One example is the acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus (HIV) infecting $CD4^+$ T cells. As HIV infection occurs through mucosal areas that are rich in DCs, LCs, and imDCs at submucosa encounter and capture the virus early in the infection. HIV encounter may activate imDCs and lead to their migration to LNs, where they can induce antiviral immune responses. pDCs, especially, in response to HIV secrete high levels of type I IFN, which potently inhibits HIV replication.[132] However, HIV can downregulate the expression of co-stimulatory molecules and MHC molecules, which might diminish the efficacy of antiviral T cell induction.[133–135] DCs may be also susceptible to HIV infection, though much less effectively than $CD4^+$ T cells. The expression of multiple restriction factors in DCs that can block HIV replication renders the productive infection of DCs inefficient.[132] However, DCs may still contribute to HIV spread into $CD4^+$ T cells by hosting the virus and enabling its long-term transmission. Additionally, intracellular HIV might be protected from degradation within DCs and may be trafficked to DC-T synapse or released to extracellular milieu by exosomes, thereby mediating HIV transmission to T cells.[136,137] HIV may also be trapped on the cell surface of follicular DCs and transmitted to T cells through the formation of infectious synapses. DC-SIGN plays a key role in the formation of infectious synapses, in addition to its role as a receptor enabling HIV entry into DCs.[138] Like HIV, both Ebola and Dengue virus also use DC-SIGN to enter DCs.[139,140]

Human cytomegalovirus (HCMV), also called beta-herpes virus 5, infects the majority of the world population and establishes lifelong infection. Although commonly asymptomatic in healthy individuals, HCMV infection can be fatal in immunocompromised people. HCMV can non-productively infect DCs and modulate DC function. HCMV downregulates MHC class I and II expression in DCs by downregulating ERAP1, inhibiting MHC-II transcription, degrading MHC-II, and interfering with MHC-II and invariant chain interaction. MHC downregulation in DCs enables HCMV escape from T cell recognition. Notably, HCMV can also inhibit NK cell function and avoid NK cell-mediated lysis in absence of MHC-I molecules.[141] HCMV can mediate its inhibitory effects on DCs and T cells also by inducing immunosuppressive cytokine production, such as IL-10. HCMV can encode a functionally active viral IL-10 homolog, as well as induce DCs to produce IL-10.[142]

DENDRITIC CELLS AND CANCERS

The DC-mediated events leading to a successful antitumor immune response can be summarized as (1) DC access to tumor antigens in the tumor microenvironment (TME); (2) DC activation in response to DAMPs in the TME; (3) tumor antigen uptake by DCs; (4) DC migration to LNs; (5) cross-presentation of tumor antigens to T cells, activating tumor-specific $CD8^+$ cytotoxic T cells (CTL); (6) trafficking of CTL to TME; and (7) tumor cell killing by CTL (Fig. 20.5).[143] As they excel at cross-presentation, the cDC1 subset of DCs is particularly important for eliciting antitumor CTL responses.[17] Notably, $CD4^+$ T cells also play a crucial role in establishing antitumor immunity by enhancing the efficacy of CTL and directing sustained antitumor immune responses.[144,145] Moreover, tumor-specific $CD4^+$ T cells may also directly exert cytotoxic function.[146–148]

Increased susceptibility to cancer in people with DC deficiencies, as well as aggravated tumorigenesis observed in Batf3 knockout mice, which lack cross-presenting cDC1s, demonstrate the importance of DCs in mediating antitumor immunity.[131,149,150] The strong correlation between the abundance of tumor-infiltrating cDC1s and clinical outcome in cancer patients further corroborates these observations.[151] Therefore, inhibition of cDC1s is beneficial for tumor progression.

Tumors have evolved to escape immune responses through various mechanisms, including abrogating DC function. Tumor cells establish a highly immunosuppressive TME that perturbs DC recruitment, differentiation, survival, function, and exploits the tolerogenic capacity of DCs (see Fig. 20.5).[152] Tumor-derived factors can limit cDC1 recruitment to the tumor site by modulating DC-attractant chemokine production. For example, active β-catenin in tumors reduces CCL4 production.[153] Similarly, prostaglandin E_2 (PGE_2) impairs CCL5 and XCL1 production by NK cells.[154] Tumors can also inhibit cDC differentiation and survival through the downregulation of NK cell-derived Flt3L by secreting vascular endothelial growth factor (VEGF).[155,156] Likewise, PGE_2 can inhibit iDC differentiation, as well as cDC maturation and survival.[157] Moreover, IL-6 in the TME, produced by both tumor and immune cells, also inhibits cDC and iDC differentiation.[157] Tumors inhibit DC activation also by impairing sensing of DAMPs. For example, tumor-infiltrating cDCs upregulate TIM-3 expression, which interacts with an alarmin (HMGB1) and blocks nucleic acid recruitment into DC endosomes.[158] CD47 expression by tumors also impedes tumor DNA sensing by cDCs.[159] DC migration to LNs, a key step for the generation of antitumor T cell responses, also can be restrained by tumor-derived liver X receptor (LXRα) ligands.[160] Another mechanism employed by tumors to bypass DC-mediated immunity is to alter their antigen profile. Tumors can post-translationally modify their antigens, such as MUC1 and HER2 glycosylation, and render DCs unable to process these antigens by blocking antigen transport from early endosomes to downstream compartments.[161]

Tumors can also affect the cytokine secretion profile of DCs, polarizing them to a more tolerogenic state and perpetuating an immunosuppressive TME. For example, Versican, a tumor-derived TLR2 ligand, induces IL-10, IL-6, and their receptor expression in DCs.[162] IL-6 can downregulate MHC-II and CCR7.[163,164] IL-10 secretion, by DCs and macrophages within TME, can abolish IL-12 production by DCs.[165] IL-6 and IL-10, among other TME factors such as VEGF, also trigger signal transducer and activator of transcription 3 (STAT3) signaling, which is hyperactivated in cancer and contribute to tumor growth and immunosuppression in TME.[166] STAT3 signaling can upregulate the expression of immunoregulatory molecules in DCs, such as programmed cell death ligand 1 (PD-L1), which binds to the inhibitory receptor PD-1 on T cells.[167] Moreover, STAT3 activation can inhibit DC maturation, as well as DC expression of MHC-II, co-stimulatory molecules, and IL-12.[168] STAT3 signaling also plays a key role in the accumulation of an immature myeloid cell population with immunosuppressive functions, so called myeloid-derived suppressor cells (MDSCs), at the TME. MDSCs mediate their immunosuppressive effects through a myriad of mechanisms, including the activities of arginase-1 (ARG-1) and inducible nitric oxide synthase (NOS2), as well as the secretion of immunoregulatory cytokines, such as IL-10 and TGF-β.[169] In addition to directly inhibiting T cells, MDSCs can also impair the differentiation, maturation, and antigen presentation ability of DCs.[169] For example, TGF-β can induce epigenetic modifications that impair DC function and differentiation.[170]

Another immune escape mechanism utilized by tumors is skewing T cell differentiation. IL-10 and TGF-β induce Tregs, which inhibit effector $CD8^+$ and $CD4^+$ T cell functions.[171] Tregs may also modulate cDC2 function and migration, leading to inhibition of cDC2-mediated proinflammatory Th1 differentiation of antitumoral $CD4^+$ T cells.[172] Additionally, tumors overexpress matrix metalloproteinase-2 (MMP-2). MMP-2, through its binding to TLR2 on DCs, conditions DCs to produce low levels of IL-12 and to express OX40-L, thereby biasing antitumor $CD4^+$ T cells toward Th2 differentiation associated with anti-inflammatory responses.[173,174]

Finally, TME is highly hypoxic, deprived of adequate oxygen. One underlying mechanism for tumor-promoting effects of hypoxia is its contribution to immunosuppression, such as by inducing Tregs and MDSCs.[175] Hypoxia also mediates metabolic reprogramming

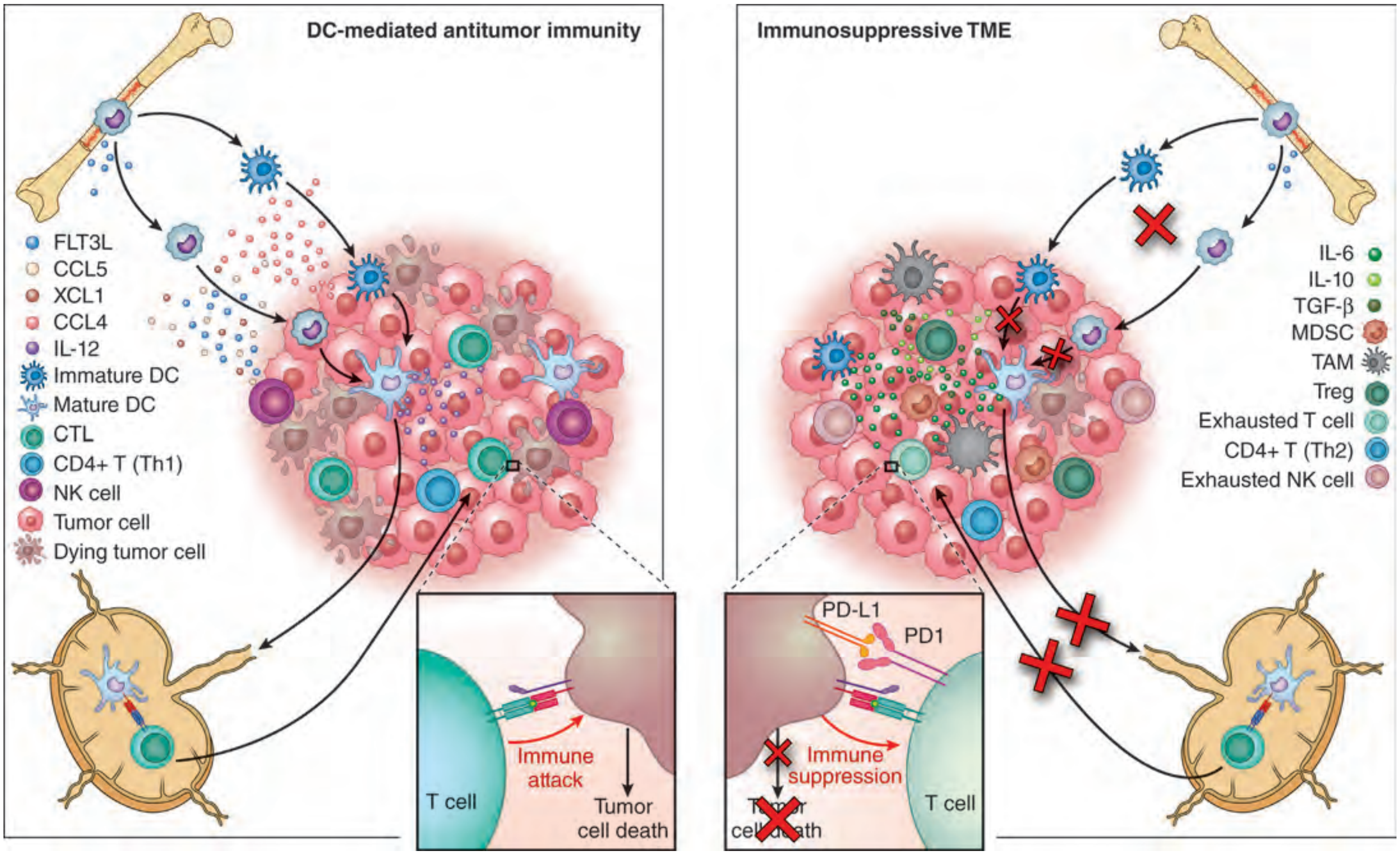


Figure 20.5 IMMUNE ESCAPE MECHANISMS EXPLOITED BY TUMORS. Summary of DC-mediated events leading to a successful antitumor immune response *(left)*: DCs are recruited to the tumor microenvironment (TME) in response to CCL5 and XCL1, produced by natural killer (NK) cells, and CCL4, produced by tumor cells. In the TME, DCs are activated in response to danger-associated molecular patterns (DAMPs) and uptake tumor antigen uptakes. Subsequently, DCs migrate to lymph nodes (LNs), where they cross-present tumor antigens to T cells, activating tumor-specific $CD8^+$ cytotoxic T cells (CTL). CTLs traffic back to the TME and kill tumor cells. However, tumors have evolved to escape immune responses through various mechanisms *(right)*, including inhibition of DC recruitment, differentiation, survival, function, and exploiting the tolerogenic capacity of DCs. Tumor-derived β-catenin and prostaglandin E_2 (PGE_2) block DC infiltration by inhibiting chemokine secretion by tumor and NK cells, respectively. Vascular endothelial growth factor (VEGF) secreted by tumors downregulates NK cell-derived Flt3L, thereby inhibiting DC differentiation and survival. Tumors also impair sensing of DAMPs by DCs and DC migration into LNs. Tumors can also affect the cytokine secretion profile of DCs, polarizing them to a more tolerogenic state, leading to the accumulation of immunoregulatory cells, such as myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and T regulatory cells (Tregs), in the TME, as well as the induction of checkpoint molecules and their ligand expressions, such as PD-1 on T cells and PD-L1 on tumor cells or myeloid cells.

in immune cells, including DCs. Hypoxia enhances the production of adenosine and IDO by DCs, whose activities can inhibit T cells and NK cells.[176,177] Hypoxia also causes ER stress, which can reduce DC maturation.[178] Moreover, ER stress leads to abnormal lipid accumulation in DCs, which inhibits the translocation of peptide-loaded MHC-I molecules to the cell surface, thereby blocking antigen cross-presentation.[179,180]

IMMUNOTHERAPEUTIC STRATEGIES AND CLINICAL TRIALS

The immune system can distinguish self from non-self and eliminate intruding or transformed material from the body. The advancement in the understanding of innate and adaptive immune responses and their regulation has enabled targeted strategies mobilizing the immune system to combat immunopathology. In recent years, immunotherapy has revolutionized the treatment of diseases, in particular cancer. An important type of immunotherapy is DC vaccination (Fig. 20.6). Numerous clinical trials conducted over the last two decades have established the safety and feasibility of DC vaccines aimed to generate immune responses against infectious pathogens, for example, HIV, and various tumor antigens.[181] Despite eliciting and strengthening antigen-specific immune responses, DC vaccination as monotherapy yields suboptimal clinical outcome. For example, the use of Sipuleucel-T, the first DC-based cancer vaccine approved by the US Food and Drug Administration (FDA), for the treatment of metastatic castration-resistant prostate cancer prolonged median survival time by 4.1 months compared to the placebo-treated patients.[182] In general, objective response rates with DC-based cancer vaccines rarely exceed 15%.[183] Similarly, according to a recent meta-analysis of 12 clinical trials, the overall success rate of DC vaccination for the treatment of HIV, defined as partial or at least transient viral load decrease, was estimated to be 38.2%.[184] The suboptimal clinical performance of DC vaccines highlights the need to understand the DC biology better and improve the design of therapeutic DC-based strategies.

DCs comprise a small fraction of blood cells. Therefore, the generation of sufficient DC numbers for vaccination strategies may be challenging. Various ex vivo protocols have been developed to generate patient-derived autologous DC subsets, for example, moDCs, cDCs, or pDCs, from distinct sources, such as peripheral blood monocytes or $CD34^+$ precursor cells mobilized from the bone marrow. Isolation techniques used to obtain the source population, as well as the culture method utilized to expand DCs, influence the phenotype, function, and potency of ex vivo generated DCs.[13,185,186] Due to the ease of ex vivo expansion protocols and their availability, moDCs have been

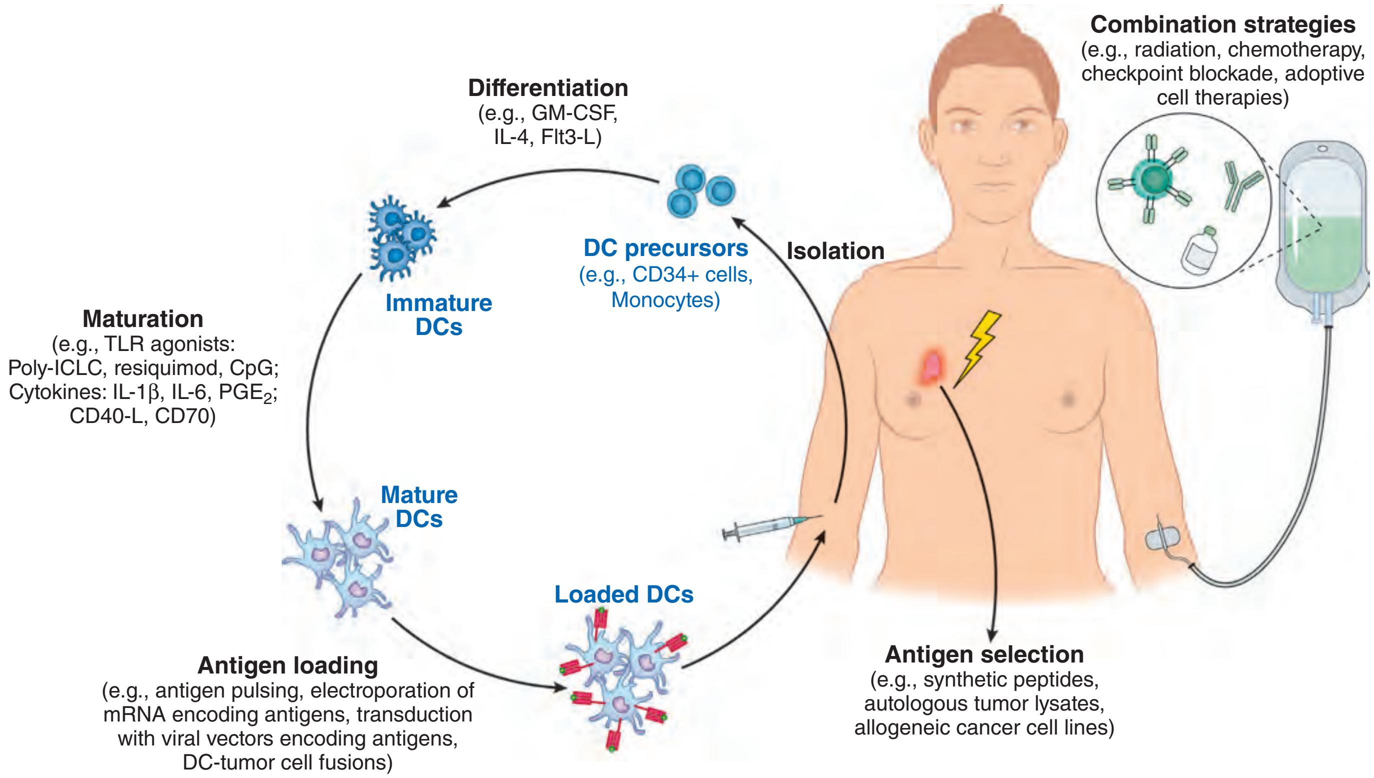

Differentiation
(e.g., GM-CSF,
IL-4, Flt3-L)
Combination strategies
(e.g., radiation, chemotherapy,
checkpoint blockade, adoptive
cell therapies)
DC precursors
(e.g., CD34+ cells,
Monocytes)
Isolation
Immature
DCs
Maturation
(e.g., TLR agonists:
Poly-ICLC, resiquimod, CpG;
Cytokines: IL-1β, IL-6, PGE2;
CD40-L, CD70)
Mature
DCs
Loaded DCs
Antigen loading
(e.g., antigen pulsing, electroporation of
mRNA encoding antigens, transduction
with viral vectors encoding antigens,
DC-tumor cell fusions)
Antigen selection
(e.g., synthetic peptides,
autologous tumor lysates,
allogeneic cancer cell lines)

Figure 20.6 DENDRITIC CELL-BASED CANCER VACCINES. Summary of some of the DC-based cancer vaccination strategies being used in clinical trials to induce antitumor adaptive immune responses. Tumor antigens can be loaded onto ex vivo generated DCs and administered to the patients alone or in conjunction with adjuvants. An alternative to ex vivo production of DC vaccines is in situ vaccination, in which the vaccine is generated in vivo. Combining DC-based cancer vaccines with different immunomodulatory treatments might provide therapeutic synergism. *Flt3L*, Fms-like tyrosine kinase receptor 3 ligand; *GM-CSF*, granulocyte-macrophage colony-stimulating factor; *mRNA*, messenger RNA; *PGE2*, prostaglandin E2; *Poly-ICLC*, polyinosinic-polycytidylic acid – poly-L-lysine carboxymethylcellulose; *TLR*, Toll-like receptor.

most frequently used for the preparation of DC vaccines.[187] However, as each DC subset is unique in their antigen presentation capacity and role in disease settings, the adaption of ex vivo culture systems that allow generation and expansion of different DC subsets (cDC1, cDC2, and pDC)[13] to clinical settings will be key to improve the efficacy of DC vaccines. Several studies evaluating the efficacy of pDC and cDC-based cancer vaccines are already underway.[188–191]

Antigen selection and antigen delivery are two key factors that contribute to the success of DC-based therapies. DC vaccines are intended to promote strong antigen-specific adaptive immunity. Therefore, the immunogenic potential of the targeted antigens impacts the magnitude of elicited immune responses. To date, most DC vaccines are prepared by loading autologous DCs with microbial or tumor antigens ex vivo prior to reinfusion into patients. Various antigen sources can be utilized to load DCs ex vivo: (1) synthetic peptides[192–194]; (2) whole tumor lysates[195,196] (e.g., NCT03803397 and NCT03990493 by PrimeVax); (3) DC-tumor cell fusions, hybrid cells that act as DCs and express complete tumor antigens[197–199]; (4) electroporation with mRNA encoding antigens[200]; and (5) transduction with viral vectors encoding antigens.[201] An alternative to ex vivo production of DC vaccines is in situ vaccination, in which the vaccine is generated in vivo.[202] This strategy eliminates the need for ex vivo cell manipulation, thereby simplifying the vaccine production pipelines. Agents utilized to form in situ DC vaccines include (1) antibodies binding to DC-specific cell surface molecules, such as Clec9A, Clec12A, Mannose receptor, DEC-205 and CD40 (e.g., NCT03358719, NCT02166905, NCT02376699, NCT02482168, NCT00648102, NCT00709462, and NCT01103635) that can mediate antigen delivery to DCs[203]; (2) viral vectors encoding antigens[204]; (3) oncolytic viruses, which induce tumor cell death, thereby releasing tumor antigens[205]; (4) nanoparticles[206,207]; (5) DC-activating agents[208]; (6) modified RNA or DNA vaccines (NCT02410733)[209]; and (7) GM-CSF-transduced tumor cells (GVAX platform).[210–213] Other platforms that combine ex vivo and in situ DC-based vaccination approaches have also been developed and tested in mouse models, such as the extracellular vesicle internalizing receptor (EVIR) platform, in which DCs are transduced with a chimeric receptor encoding lentivirus ex vivo that enables the selective uptake of tumor-derived EV in vivo.[214] Talimogene laherparepvec (T-VEC), a modified herpes simplex virus, is a first-in-class FDA-approved oncolytic in situ vaccine. T-VEC selectively replicates in tumor cells and lyses them, which release viral progeny and tumor antigens. Additionally, T-VEC expresses GM-CSF and promotes DC function.[215] FDA approved the use of T-VEC in patients with unresectable melanoma after a 16.3% durable response rate in patients receiving intralesional T-VEC versus 2.1% in patients receiving subcutaneous GM-CSF, as well as a 4.4-months prolonged median survival time in the T-VEC group.[216]

Another important factor impacting the potency of DC-based vaccinations is the method for DC activation. Various strategies mentioned previously for antigen loading to DCs can also be utilized for introducing DC-activating agents. DCs may be activated by TLR ligands, such as TLR3 agonist Poly-ICLC, TLR4 agonist LPS, TLR7 agonist imiquimod, TLR7 and TLR8 agonist resiquimod, and TLR9 agonist CpG DNA.[217] Pro-inflammatory cytokines, such as IFN-I, IL-1β, IL-6, TNF, and PGE_2, can also be used to activate DCs.[218] In addition, DCs can be engineered to express multiple immunostimulatory molecules. For example, the Trimix DCs express constitutively active TLR-4, as well as CD40-L and CD70.[219] Alternatively, expression of inhibitory molecules, such as PD-L1 and PD-L2, by DCs can be silenced, for example, by using siRNA (NCT02528682).[220] Intratumoral (IT) injection of DC-activating agents can lead to in situ activation of DCs. For example, IT administration of TLR9 agonist

CpG oligonucleotide PF-3512676, together with low-dose radiation, in patients with low-grade B-cell lymphoma induced tumor-specific $CD8^+$ T cells and led to complete or partial responses in a subset of patients.[221] Other immune modulators such as Poly-ICLC[222,223] and STING agonists[224] have also been tested as inducers of in situ DC activation. Moreover, stimulating local inflammation, e.g., by administering potent recall antigens[225] or Flt3L[208], is another strategy to enhance the efficacy of DCs. To enhance the elicited immunity, multiple agents can be incorporated into the design of the DC vaccine. For example, a recent phase II trial tested a regimen of Flt3L treatment together with Poly-ICLC and a vaccine comprising anti-DEC-205-NY-ESO-1 in high-risk melanoma patients, where Flt3L administration was randomized. Findings demonstrated that the addition of Flt3L enhanced the immunogenicity of the DC vaccine and induced potent and durable immune responses.[226]

The superior efficacy and clinical relevance of combination therapies over monotherapies, by therapeutic synergism while retaining safety profiles, have been demonstrated in many contexts.[227] Accordingly, to unleash the full power of DC-based therapies, combination strategies with different immunomodulatory treatments, for example, chemotherapy, radiotherapy, and checkpoint blockade, have been studied.[228] In addition to eliminating malignant calls and lowering tumor burden, chemotherapy, and radiotherapy-triggered tumor cell death releases tumor antigens and induces exposure of immunogenic molecules on tumor cells, which facilitate DC antigen uptake and activation.[228] Chemotherapy drugs can induce other immunogenic signals that further stimulate DCs. For example, taxane-induced release of cellular ATP potentiates inflammasome activation and IL-1β release, and cyclophosphamide-induced release of tumor nucleic acids can lead to type I IFN secretion.[229] Finally, chemotherapy can deplete immunosuppressive cells at the TME, rendering TME more sensitive to DC vaccine-induced antitumor T cell activity.[228] Checkpoint inhibition (ICI) can also improve the efficacy of DC-based therapies, both by reinvigorating DC-therapy-induced T cells (e.g., anti-PD-1, anti-CTLA-4) and blocking inhibitory molecules expressed by DCs (e.g., anti-PD-L1). In fact, several studies have indicated an enhanced efficacy of DC-therapy upon combination with ICI.[230]

Finally, other factors also influence the success of DC-based treatments, such as the route, dose, and schedule of vaccine administration, as well as the timing of vaccination. Although most DC vaccination trials to date have been conducted in patients with advanced disease, DC-therapy is likely to be most effective in patients with low tumor burden, as early neoadjuvant studies suggest (NCT01431196, NCT03387553).[231–233] Future controlled studies integrating these key components will improve the efficacy of DC-based therapies both in cancer and infectious disease settings.

FUTURE DIRECTIONS

As they bridge the innate and adaptive immunity, DCs are key targets for clinical applications in cancer, transplantation, and chronic infections. A deeper understanding of DC biology will improve the efficacy of current DC-based therapies and develop effective novel methodologies. Indeed, promising approaches enhancing DC recruitment and activity, which increase response rates, in various disease settings are already underway.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Alcantara-Hernandez M, et al. High-dimensional phenotypic mapping of human dendritic cells reveals interindividual variation and tissue specialization. *Immunity*. 2017;47(6):1037–1050.e6.

Amodio G, Gregori S. Dendritic cells a double-edge sword in autoimmune responses. *Front Immunol*. 2012;3:233.

Balan S, et al. Large-scale human dendritic cell differentiation revealing notch-dependent lineage bifurcation and heterogeneity. *Cell Rep*. 2018;24(7):1902–1915.e6.

Bergtold A, et al. Cell surface recycling of internalized antigen permits dendritic cell priming of B cells. *Immunity*. 2005;23(5):503–514.

Bhardwaj N, et al. Flt3 ligand augments immune responses to anti-DEC-205-NY-ESO-1 vaccine through expansion of dendritic cell subsets. *Nat Cancer*. 2020;1:1204–1217.

Bourdely P, et al. Transcriptional and functional analysis of CD1c(+) human dendritic cells identifies a CD163(+) subset priming CD8(+)CD103(+) T cells. *Immunity*. 2020;53(2):335–352.e8.

Brubaker SW, et al. Innate immune pattern recognition: a cell biological perspective. *Annu Rev Immunol*. 2015;33:257–290.

Chen DS, Mellman I. Elements of cancer immunity and the cancer-immune set point. *Nature*. 2017;541(7637):321–330.

Collin M, Bigley V. Human dendritic cell subsets: an update. *Immunology*. 2018;154(1):3–20.

Cruz FM, et al. The biology and underlying mechanisms of cross-presentation of exogenous antigens on MHC-I molecules. *Annu Rev Immunol*. 2017;35:149–176.

Dutertre CA, et al. Single-cell analysis of human mononuclear phagocytes reveals subset-defining markers and identifies circulating inflammatory dendritic cells. *Immunity*. 2019;51(3):573–589.e8.

Ferrer IR, et al. A wave of monocytes is recruited to replenish the long-term Langerhans cell network after immune injury. *Sci Immunol*. 2019;4(38).

Hammerich L, Binder A, Brody JD. In situ vaccination: Cancer immunotherapy both personalized and off-the-shelf. *Mol Oncol*. 2015;9(10):1966–1981.

Hasegawa H, Matsumoto T. Mechanisms of tolerance induction by dendritic cells in vivo. *Front Immunol*. 2018;9:350.

Huber A, et al. Current state of dendritic cell-based immunotherapy: opportunities for in vitro antigen loading of different DC subsets? *Front Immunol*. 2018;9:2804.

Iberg CA, Jones A, Hawiger D. Dendritic cells as inducers of peripheral tolerance. *Trends Immunol*. 2017;38(11):793–804.

Langenkamp A, et al. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. *Nat Immunol*. 2000;1(4):311–316.

Manches O, Frleta D, Bhardwaj N. Dendritic cells in progression and pathology of HIV infection. *Trends Immunol*. 2014;35(3):114–122.

Merad M, et al. Langerhans cells renew in the skin throughout life under steady-state conditions. *Nat Immunol*. 2002;3(12):1135–1141.

Mempel TR, Henrickson SE, Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature*. 2004;427(6970):154–159.

Pennock ND, et al. T cell responses: naive to memory and everything in between. *Adv Physiol Educ*. 2013;37(4):273–283.

Ridgway D. The first 1000 dendritic cell vaccinees. *Cancer Invest*. 2003;21(6):873–886.

Ruhland MK, et al. Visualizing synaptic transfer of tumor antigens among dendritic cells. *Cancer Cell*. 2020;37(6):786–799.e5.

Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med*. 1973;137(5):1142–1162.

Theisen D, Murphy K. The role of cDC1s in vivo: CD8 T cell priming through cross-presentation. *F1000Res*. 2017;6:98.

Thomas C, Tampe R. Proofreading of peptide-MHC complexes through dynamic multivalent interactions. *Front Immunol*. 2017;8:65.

van Gulijk M, et al. Combination strategies to optimize efficacy of dendritic cell-based immunotherapy. *Front Immunol*. 2018:92759.

Villani AC, et al. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science*. 2017;356(6335):eaah4573.

Vivier E, et al. Innate lymphoid cells: 10 years on. *Cell*. 2018;174(5):1054–1066.

Wculek SK, et al. Dendritic cells in cancer immunology and immunotherapy. *Nat Rev Immunol*. 2020;20(1):7–24.

REFERENCES

1. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med*. 1973;137(5):1142–1162.
2. Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. *Cell*. 2001;106(3):255–258.
3. Collin M, Bigley V. Human dendritic cell subsets: an update. *Immunology*. 2018;154(1):3–20.
4. Rieger MA, Schroeder T. Hematopoiesis. *Cold Spring Harb Perspect Biol*. 2012;4(12):a008250.
5. Kawamoto H, et al. A map for lineage restriction of progenitors during hematopoiesis: the essence of the myeloid-based model. *Immunol Rev*. 2010;238(1):23–36.
6. Naik SH, et al. Diverse and heritable lineage imprinting of early haematopoietic progenitors. *Nature*. 2013;496(7444):229–232.
7. Dress RJ, et al. Plasmacytoid dendritic cells develop from Ly6D(+) lymphoid progenitors distinct from the myeloid lineage. *Nat Immunol*. 2019;20(7):852–864.
8. Cabeza-Cabrerizo M, et al. Dendritic cells revisited. *Annu Rev Immunol*. 2021;39:131–166.
9. Vremec D, et al. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J Immunol*. 2000;164(6):2978–2986.
10. Dutertre CA, et al. Single-cell analysis of human mononuclear phagocytes reveals subset-defining markers and identifies circulating inflammatory dendritic cells. *Immunity*. 2019;51(3):573–589.e8.
11. Villani AC, et al. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science*. 2017;356(6335):eaah4573.
12. Kirkling ME, et al. Notch signaling facilitates in vitro generation of cross-presenting classical dendritic cells. *Cell Rep*. 2018;23(12):3658–3672.e6.
13. Balan S, et al. Large-scale human dendritic cell differentiation revealing notch-dependent lineage bifurcation and heterogeneity. *Cell Rep*. 2018;24(7):1902–1915.e6.
14. Lewis KL, et al. Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. *Immunity*. 2011;35(5):780–791.
15. Briseno CG, et al. Notch2-dependent DC2s mediate splenic germinal center responses. *Proc Natl Acad Sci U S A*. 2018;115(42):10726–10731.
16. Balan S, Radford KJ, Bhardwaj N. Unexplored horizons of cDC1 in immunity and tolerance. *Adv Immunol*. 2020;148:49–91.
17. Theisen D, Murphy K. The role of cDC1s in vivo: CD8 T cell priming through cross-presentation. *F1000Res*. 2017;6:98.
18. Lasfar A, et al. Interferon lambda: toward a dual role in cancer. *J Interferon Cytokine Res*. 2019;39(1):22–29.
19. Hubert M, et al. IFN-III is selectively produced by cDC1 and predicts good clinical outcome in breast cancer. *Sci Immunol*. 2020;5(46):eaav3942.
20. Satpathy AT, et al. Notch2-dependent classical dendritic cells orchestrate intestinal immunity to attaching-and-effacing bacterial pathogens. *Nat Immunol*. 2013;14(9):937–948.
21. Cisse B, et al. Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development. *Cell*. 2008;135(1):37–48.
22. Zhang H, et al. A distinct subset of plasmacytoid dendritic cells induces activation and differentiation of B and T lymphocytes. *Proc Natl Acad Sci U S A*. 2017;114(8):1988–1993.
23. Alculumbre SG, et al. Diversification of human plasmacytoid predendritic cells in response to a single stimulus. *Nat Immunol*. 2018;19(1):63–75.
24. Yin Z, et al. Type III IFNs are produced by and stimulate human plasmacytoid dendritic cells. *J Immunol*. 2012;189(6):2735–2745.
25. Coccia EM, et al. Viral infection and Toll-like receptor agonists induce a differential expression of type I and lambda interferons in human plasmacytoid and monocyte-derived dendritic cells. *Eur J Immunol*. 2004;34(3):796–805.
26. Vollmer J, et al. Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. *Eur J Immunol*. 2004;34(1):251–262.
27. Kadowaki N, et al. Natural interferon alpha/beta-producing cells link innate and adaptive immunity. *J Exp Med*. 2000;192(2):219–226.
28. Jego G, et al. Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. *Immunity*. 2003;19(2):225–234.
29. Ogata M, et al. Plasmacytoid dendritic cells have a cytokine-producing capacity to enhance ICOS ligand-mediated IL-10 production during T-cell priming. *Int Immunol*. 2013;25(3):171–182.
30. Alcantara-Hernandez M, et al. High-dimensional phenotypic mapping of human dendritic cells reveals interindividual variation and tissue specialization. *Immunity*. 2017;47(6):1037–1050.e6.
31. Segura E, Amigorena S. Identification of human inflammatory dendritic cells. *Oncoimmunology*. 2013;2(5):e23851.
32. Greter M, et al. GM-CSF controls nonlymphoid tissue dendritic cell homeostasis but is dispensable for the differentiation of inflammatory dendritic cells. *Immunity*. 2012;36(6):1031–1046.
33. Ferrer IR, et al. A wave of monocytes is recruited to replenish the long-term Langerhans cell network after immune injury. *Sci Immunol*. 2019;4(38).
34. Doebel T, Voisin B, Nagao K. Langerhans cells—the macrophage in dendritic cell clothing. *Trends Immunol*. 2017;38(11):817–828.
35. Otsuka M, Egawa G, Kabashima K. Uncovering the mysteries of Langerhans cells, inflammatory dendritic epidermal cells, and monocyte-derived Langerhans cell-like cells in the epidermis. *Front Immunol*. 2018;9:1768.
36. Valladeau J, Dezutter-Dambuyant C, Saeland S. Langerin/CD207 sheds light on formation of birbeck granules and their possible function in Langerhans cells. *Immunol Res*. 2003;28(2):93–107.
37. Chorro L, et al. Langerhans cell (LC) proliferation mediates neonatal development, homeostasis, and inflammation-associated expansion of the epidermal LC network. *J Exp Med*. 2009;206(13):3089–3100.
38. Merad M, et al. Langerhans cells renew in the skin throughout life under steady-state conditions. *Nat Immunol*. 2002;3(12):1135–1141.
39. Hemmerling J, et al. Human epidermal Langerhans cells replenish skin xenografts and are depleted by alloreactive T cells in vivo. *J Immunol*. 2011;187(3):1142–1149.
40. Flacher V, et al. Human Langerhans cells express a specific TLR profile and differentially respond to viruses and Gram-positive bacteria. *J Immunol*. 2006;177(11):7959–7967.
41. Fujita H, et al. Human Langerhans cells induce distinct IL-22-producing CD4+ T cells lacking IL-17 production. *Proc Natl Acad Sci U S A*. 2009;106(51):21795–21800.
42. Furio L, et al. Human Langerhans cells are more efficient than CD14(−) CD1c(+) dermal dendritic cells at priming naive CD4(+) T cells. *J Invest Dermatol*. 2010;130(5):1345–1354.
43. Mathers AR, et al. Differential capability of human cutaneous dendritic cell subsets to initiate Th17 responses. *J Immunol*. 2009;182(2):921–933.
44. Elnekave M, et al. Second-generation Langerhans cells originating from epidermal precursors are essential for CD8+ T cell priming. *J Immunol*. 2014;192(4):1395–1403.
45. Ghigo C, et al. Multicolor fate mapping of Langerhans cell homeostasis. *J Exp Med*. 2013;210(9):1657–1664.
46. West HC, Bennett CL. Redefining the role of Langerhans cells as immune regulators within the skin. *Front Immunol*. 2017;8:1941.
47. Deckers J, Hammad H, Hoste E. Langerhans cells: sensing the environment in health and disease. *Front Immunol*. 2018;9:93.
48. Bourdely P, et al. Transcriptional and functional analysis of CD1c(+) human dendritic cells identifies a CD163(+) subset priming CD8(+) CD103(+) T cells. *Immunity*. 2020;53(2):335–352.e8.
49. Cytlak U, et al. Differential IRF8 transcription factor requirement defines two pathways of dendritic cell development in humans. *Immunity*. 2020;53(2):353–370.e8.
50. Matzinger P. The danger model: a renewed sense of self. *Science*. 2002;296(5566):301–305.
51. Garg AD, Dudek AM, Agostinis P. Cancer immunogenicity, danger signals, and DAMPs: what, when, and how? *Biofactors*. 2013;39(4):355–367.
52. Ma DY, Clark EA. The role of CD40 and CD154/CD40L in dendritic cells. *Semin Immunol*. 2009;21(5):265–272.
53. Gianni T, et al. αvβ3-integrin is a major sensor and activator of innate immunity to herpes simplex virus-1. *Proc Natl Acad Sci U S A*. 2012;109(48):19792–19797.
54. Brubaker SW, et al. Innate immune pattern recognition: a cell biological perspective. *Annu Rev Immunol*. 2015;33:257–290.
55. Yin Q, et al. Structural biology of innate immunity. *Annu Rev Immunol*. 2015;33:393–416.
56. Baizabal-Aguirre VM, et al. Control and resolution mechanisms of the inflammatory response. *Mediators Inflamm*. 2014;2014:387567.
57. Figdor CG, van Kooyk Y, Adema GJ. C-type lectin receptors on dendritic cells and Langerhans cells. *Nat Rev Immunol*. 2002;2(2):77–84.
58. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell*. 2010;140(6):805–820.
59. Kumari S, Mg S, Mayor S. Endocytosis unplugged: multiple ways to enter the cell. *Cell Res*. 2010;20(3):256–275.
60. Sallusto F, et al. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med*. 1995;182(2):389–400.
61. Li Z, et al. CD83: Activation marker for antigen presenting cells and its therapeutic potential. *Front Immunol*. 2019;10:1312.
62. de Saint-Vis B, et al. A novel lysosome-associated membrane glycoprotein, DC-LAMP, induced upon DC maturation, is transiently expressed in MHC class II compartment. *Immunity*. 1998;9(3):325–336.

63. Kim MK, Kim J. Properties of immature and mature dendritic cells: phenotype, morphology, phagocytosis, and migration. *RSC Advances*. 2019;9(20):11230–11238.
64. Lutz MB, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol*. 2002;23(9):445–449.
65. Kushwah R, Hu J. Role of dendritic cells in the induction of regulatory T cells. *Cell Biosci*. 2011;1(1):20.
66. Schiefner A, Wilson IA. Presentation of lipid antigens by CD1 glycoproteins. *Curr Pharm Des*. 2009;15(28):3311–3317.
67. Raghavan M, et al. MHC class I assembly: out and about. *Trends Immunol*. 2008;29(9):436–443.
68. Thomas C, Tampe R. Proofreading of peptide-MHC complexes through dynamic multivalent interactions. *Front Immunol*. 2017;8:65.
69. Chen H, et al. ERAP1-ERAP2 dimers trim MHC I-bound precursor peptides; implications for understanding peptide editing. *Sci Rep*. 2016;6:28902.
70. Neefjes J. CIIV, MIIC and other compartments for MHC class II loading. *Eur J Immunol*. 1999;29(5):1421–1425.
71. Garstka MA, Neefjes J. How to target MHC class II into the MIIC compartment. *Mol Immunol*. 2013;55(2):162–165.
72. Denzin LK, et al. Right place, right time, right peptide: DO keeps DM focused. *Immunol Rev*. 2005;207:279–292.
73. Nierkens S, et al. Antigen cross-presentation by dendritic cell subsets: one general or all sergeants? *Trends Immunol*. 2013;34(8):361–370.
74. Gutierrez-Martinez E, et al. Cross-presentation of cell-associated antigens by MHC class I in dendritic cell subsets. *Front Immunol*. 2015;6:363.
75. Cruz FM, et al. The biology and underlying mechanisms of cross-presentation of exogenous antigens on MHC-I molecules. *Annu Rev Immunol*. 2017;35:149–176.
76. Ruhland MK, et al. Visualizing synaptic transfer of tumor antigens among dendritic cells. *Cancer Cell*. 2020;37(6):786–799.e5.
77. Nair-Gupta P, et al. TLR signals induce phagosomal MHC-I delivery from the endosomal recycling compartment to allow cross-presentation. *Cell*. 2014;158(3):506–521.
78. Campana S, et al. Cross-dressing: an alternative mechanism for antigen presentation. *Immunol Lett*. 2015;168(2):349–354.
79. Groom JR. Moving to the suburbs: T-cell positioning within lymph nodes during activation and memory. *Immunol Cell Biol*. 2015;93(4):330–336.
80. Bousso P, Robey E. Dynamics of CD8+ T cell priming by dendritic cells in intact lymph nodes. *Nat Immunol*. 2003;4(6):579–585.
81. Mempel TR, Henrickson SE, Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature*. 2004;427(6970):154–159.
82. Pennock ND, et al. T cell responses: naive to memory and everything in between. *Adv Physiol Educ*. 2013;37(4):273–283.
83. Tseng SY, et al. B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J Exp Med*. 2001;193(7):839–846.
84. Magee CN, Boenisch O, Najafian N. The role of costimulatory molecules in directing the functional differentiation of alloreactive T helper cells. *Am J Transplant*. 2012;12(10):2588–2600.
85. Schwartz RH. T cell anergy. *Annu Rev Immunol*. 2003;21:305–334.
86. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol*. 2010;28:445–489.
87. Lugo-Villarino G, et al. T-bet is required for optimal production of IFN-gamma and antigen-specific T cell activation by dendritic cells. *Proc Natl Acad Sci U S A*. 2003;100(13):7749–7754.
88. Soumelis V, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol*. 2002;3(7):673–680.
89. Langenkamp A, et al. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. *Nat Immunol*. 2000;1(4):311–316.
90. Kalinski P, et al. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunol Today*. 1999;20(12):561–567.
91. Agalioti T, et al. TH17 cell plasticity: the role of dendritic cells and molecular mechanisms. *J Autoimmun*. 2018;87:50–60.
92. Hasegawa H, Matsumoto T. Mechanisms of tolerance induction by dendritic cells in vivo. *Front Immunol*. 2018;9:350.
93. Bergtold A, et al. Cell surface recycling of internalized antigen permits dendritic cell priming of B cells. *Immunity*. 2005;23(5):503–514.
94. MacLennan I, Vinuesa C. Dendritic cells, BAFF, and APRIL: innate players in adaptive antibody responses. *Immunity*. 2002;17(3):235–238.
95. Litinskiy MB, et al. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. *Nat Immunol*. 2002;3(9): 822–829.
96. Park CS, Choi YS. How do follicular dendritic cells interact intimately with B cells in the germinal centre? *Immunology*. 2005;114(1):2–10.
97. Vivier E, et al. Innate lymphoid cells: 10 years on. *Cell*. 2018;174(5):1054–1066.
98. Bernink JH, et al. Interleukin-12 and -23 control plasticity of CD127(+) group 1 and group 3 innate lymphoid cells in the intestinal lamina propria. *Immunity*. 2015;43(1):146–160.
99. Mielke LA, et al. Retinoic acid expression associates with enhanced IL-22 production by gammadelta T cells and innate lymphoid cells and attenuation of intestinal inflammation. *J Exp Med*. 2013;210(6):1117–1124.
100. Kim MH, Taparowsky EJ, Kim CH. Retinoic acid differentially regulates the migration of innate lymphoid cell subsets to the gut. *Immunity*. 2015;43(1):107–119.
101. Dalmas E, et al. Interleukin-33-activated islet-resident innate lymphoid cells promote insulin secretion through myeloid cell retinoic acid production. *Immunity*. 2017;47(5):928–942.e7.
102. Halim TY, et al. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity*. 2014;40(3):425–435.
103. Kruglov AA, et al. Nonredundant function of soluble LTalpha3 produced by innate lymphoid cells in intestinal homeostasis. *Science*. 2013;342(6163):1243–1246.
104. Moretta A. The dialogue between human natural killer cells and dendritic cells. *Curr Opin Immunol*. 2005;17(3):306–311.
105. Iyoda T, et al. The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. *J Exp Med*. 2002;195(10):1289–1302.
106. Fujii S, et al. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol*. 2002;3(9):867–874.
107. Iberg CA, Jones A, Hawiger D. Dendritic cells as inducers of peripheral tolerance. *Trends Immunol*. 2017;38(11):793–804.
108. Probst HC, et al. Inducible transgenic mice reveal resting dendritic cells as potent inducers of CD8+ T cell tolerance. *Immunity*. 2003;18(5):713–720.
109. Hawiger D, et al. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med*. 2001;194(6):769–779.
110. Probst HC, et al. Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. *Nat Immunol*. 2005;6(3):280–286.
111. Devi KS, Anandasabapathy N. The origin of DCs and capacity for immunologic tolerance in central and peripheral tissues. *Semin Immunopathol*. 2017;39(2):137–152.
112. Pisetsky DS, Lipsky PE. New insights into the role of antinuclear antibodies in systemic lupus erythematosus. *Nat Rev Rheumatol*. 2020;16(10):565–579.
113. Scheinecker C, et al. Alterations of dendritic cells in systemic lupus erythematosus: phenotypic and functional deficiencies. *Arthritis Rheum*. 2001;44(4):856–865.
114. Gerl V, et al. Blood dendritic cells in systemic lupus erythematosus exhibit altered activation state and chemokine receptor function. *Ann Rheum Dis*. 2010;69(7):1370–1377.
115. Carreno LJ, et al. Disease activity in systemic lupus erythematosus is associated with an altered expression of low-affinity Fc gamma receptors and costimulatory molecules on dendritic cells. *Immunology*. 2009;128(3):334–341.
116. Mozaffarian N, Wiedeman AE, Stevens AM. Active systemic lupus erythematosus is associated with failure of antigen-presenting cells to express programmed death ligand-1. *Rheumatology (Oxford)*. 2008;47(9):1335–1341.
117. Ganguly D, et al. The role of dendritic cells in autoimmunity. *Nat Rev Immunol*. 2013;13(8):566–577.
118. Klarquist J, et al. Dendritic cells in systemic lupus erythematosus: from pathogenic players to therapeutic tools. *Mediators Inflamm*. 2016;2016:5045248.
119. Pascual V, Farkas L, Banchereau J. Systemic lupus erythematosus: all roads lead to type I interferons. *Curr Opin Immunol*. 2006;18(6):676–682.
120. Farkas L, et al. Plasmacytoid dendritic cells (natural interferon- alpha/beta-producing cells) accumulate in cutaneous lupus erythematosus lesions. *Am J Pathol*. 2001;159(1):237–243.
121. Gilliet M, Cao W, Liu YJ. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nat Rev Immunol*. 2008;8(8):594–606.
122. Wan S, et al. Direct B cell stimulation by dendritic cells in a mouse model of lupus. *Arthritis Rheum*. 2008;58(6):1741–1750.
123. Sang A, et al. Dysregulated cytokine production by dendritic cells modulates B cell responses in the NZM2410 mouse model of lupus. *PLoS One*. 2014;9(8):e102151.
124. Zumaquero E, et al. IFNgamma induces epigenetic programming of human T-bet(hi) B cells and promotes TLR7/8 and IL-21 induced differentiation. *Elife*. 2019;8:e41641.

125. Lee-Kirsch MA, Wolf C, Gunther C. Aicardi-Goutieres syndrome: a model disease for systemic autoimmunity. *Clin Exp Immunol*. 2014;175(1):17–24.
126. Crow YJ, Manel N. Aicardi-Goutières syndrome and the type I interferonopathies. *Nat Rev Immunol*. 2015;15(7):429–440.
127. Wehr P, et al. Dendritic cells, T cells and their interaction in rheumatoid arthritis. *Clin Exp Immunol*. 2019;196(1):12–27.
128. Yu MB, Langridge WHR. The function of myeloid dendritic cells in rheumatoid arthritis. *Rheumatol Int*. 2017;37(7):1043–1051.
129. Leung BP, et al. A novel dendritic cell-induced model of erosive inflammatory arthritis: distinct roles for dendritic cells in T cell activation and induction of local inflammation. *J Immunol*. 2002;169(12):7071–7077.
130. Amodio G, Gregori S. Dendritic cells a double-edge sword in autoimmune responses. *Front Immunol*. 2012;3:233.
131. Bigley V, Cytlak U, Collin M. Human dendritic cell immunodeficiencies. *Semin Cell Dev Biol*. 2019;86:50–61.
132. Manches O, Frleta D, Bhardwaj N. Dendritic cells in progression and pathology of HIV infection. *Trends Immunol*. 2014;35(3):114–122.
133. Lore K, et al. Accumulation of DC-SIGN+CD40+ dendritic cells with reduced CD80 and CD86 expression in lymphoid tissue during acute HIV-1 infection. *AIDS*. 2002;16(5):683–692.
134. Schwartz O, et al. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. *Nat Med*. 1996;2(3):338–342.
135. Stumptner-Cuvelette P, et al. HIV-1 Nef impairs MHC class II antigen presentation and surface expression. *Proc Natl Acad Sci U S A*. 2001;98(21):12144–12149.
136. Wu L, KewalRamani VN. Dendritic-cell interactions with HIV: infection and viral dissemination. *Nat Rev Immunol*. 2006;6(11):859–868.
137. Ahmed Z, et al. The role of human dendritic cells in HIV-1 infection. *J Invest Dermatol*. 2015;135(5):1225–1233.
138. Arrighi JF, et al. DC-SIGN-mediated infectious synapse formation enhances X4 HIV-1 transmission from dendritic cells to T cells. *J Exp Med*. 2004;200(10):1279–1288.
139. Alvarez CP, et al. C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in trans. *J Virol*. 2002;76(13):6841–6844.
140. Tassaneetrithep B, et al. DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J Exp Med*. 2003;197(7):823–829.
141. Gredmark-Russ S, Soderberg-Naucler C. Dendritic cell biology in human cytomegalovirus infection and the clinical consequences for host immunity and pathology. *Virulence*. 2012;3(7):621–634.
142. Chang WL, et al. Human cytomegalovirus-encoded interleukin-10 homolog inhibits maturation of dendritic cells and alters their functionality. *J Virol*. 2004;78(16):8720–8731.
143. Chen DS, Mellman I. Elements of cancer immunity and the cancer-immune set point. *Nature*. 2017;541(7637):321–330.
144. Zander R, et al. CD4(+) T cell help is required for the formation of a cytolytic CD8(+) T cell subset that protects against chronic infection and cancer. *Immunity*. 2019;51(6):1028–1042.e4.
145. Alspach E, et al. MHC-II neoantigens shape tumour immunity and response to immunotherapy. *Nature*. 2019;574(7780):696–701.
146. Quezada SA, et al. Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. *J Exp Med*. 2010;207(3):637–650.
147. Xie Y, et al. Naive tumor-specific CD4(+) T cells differentiated in vivo eradicate established melanoma. *J Exp Med*. 2010;207(3):651–667.
148. Sledzinska A, et al. Regulatory T cells restrain interleukin-2- and Blimp-1-dependent acquisition of cytotoxic function by CD4(+) T cells. *Immunity*. 2020;52(1):151–166.e6.
149. Hildner K, et al. Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science*. 2008;322(5904):1097–1100.
150. Theisen DJ, et al. WDFY4 is required for cross-presentation in response to viral and tumor antigens. *Science*. 2018;362(6415):694–699.
151. Broz ML, et al. Dissecting the tumor myeloid compartment reveals rare activating antigen-presenting cells critical for T cell immunity. *Cancer Cell*. 2014;26(5):638–652.
152. Wculek SK, et al. Dendritic cells in cancer immunology and immunotherapy. *Nat Rev Immunol*. 2020;20(1):7–24.
153. Spranger S, Bao R, Gajewski TF. Melanoma-intrinsic beta-catenin signalling prevents anti-tumour immunity. *Nature*. 2015;523(7559):231–235.
154. Bottcher JP, et al. NK cells stimulate recruitment of cDC1 into the tumor microenvironment promoting cancer immune control. *Cell*. 2018;172(5):1022–1037.e14.
155. Barry KC, et al. A natural killer-dendritic cell axis defines checkpoint therapy-responsive tumor microenvironments. *Nat Med*. 2018;24(8):1178–1191.
156. Ohm JE, et al. Effect of vascular endothelial growth factor and FLT3 ligand on dendritic cell generation in vivo. *J Immunol*. 1999;163(6):3260–3268.
157. Zong J, et al. Tumor-derived factors modulating dendritic cell function. *Cancer Immunol Immunother*. 2016;65(7):821–833.
158. Chiba S, et al. Tumor-infiltrating DCs suppress nucleic acid-mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1. *Nat Immunol*. 2012;13(9):832–842.
159. Xu MM, et al. Dendritic cells but not macrophages sense tumor mitochondrial DNA for cross-priming through signal regulatory protein alpha signaling. *Immunity*. 2017;47(2):363–373.e5.
160. Villablanca EJ, et al. Tumor-mediated liver X receptor-alpha activation inhibits CC chemokine receptor-7 expression on dendritic cells and dampens antitumor responses. *Nat Med*. 2010;16(1):98–105.
161. Hiltbold EM, et al. The mechanism of unresponsiveness to circulating tumor antigen MUC1 is a block in intracellular sorting and processing by dendritic cells. *J Immunol*. 2000;165(7):3730–3741.
162. Tang M, et al. Toll-like receptor 2 activation promotes tumor dendritic cell dysfunction by regulating IL-6 and IL-10 receptor signaling. *Cell Rep*. 2015;13(12):2851–2864.
163. Kitamura H, et al. IL-6-STAT3 controls intracellular MHC class II alphabeta dimer level through cathepsin S activity in dendritic cells. *Immunity*. 2005;23(5):491–502.
164. Hegde S, Pahne SJ, Smola-Hess S. Novel immunosuppressive properties of interleukin-6 in dendritic cells: inhibition of NF-kappaB binding activity and CCR7 expression. *FASEB J*. 2004;18(12):1439–1441.
165. Ruffell B, et al. Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells. *Cancer Cell*. 2014;26(5):623–637.
166. Johnson DE, O'Keefe RA, Grandis JR. Targeting the IL-6/JAK/STAT3 signalling axis in cancer. *Nat Rev Clin Oncol*. 2018;15(4):234–248.
167. Wolfle SJ, et al. PD-L1 expression on tolerogenic APCs is controlled by STAT-3. *Eur J Immunol*. 2011;41(2):413–424.
168. Su YL, et al. STAT3 in Tumor-associated myeloid cells: multitasking to disrupt immunity. *Int J Mol Sci*. 2018;19(6).
169. Yang Y, et al. Myeloid-derived suppressor cells in tumors: from mechanisms to antigen specificity and microenvironmental regulation. *Front Immunol*. 2020;11:1371.
170. Huang Y, et al. Global mapping of H3K4me3 and H3K27me3 reveals chromatin state-based regulation of human monocyte-derived dendritic cells in different environments. *Genes Immun*. 2012;13(4):311–320.
171. Hsu P, et al. IL-10 potentiates differentiation of human induced regulatory T cells via STAT3 and Foxo1. *J Immunol*. 2015;195(8):3665–3674.
172. Binnewies M, et al. Unleashing type-2 dendritic cells to drive protective antitumor CD4(+) T cell immunity. *Cell*. 2019;177(3):556–571.e16.
173. Godefroy E, et al. Matrix metalloproteinase-2 conditions human dendritic cells to prime inflammatory T(H)2 cells via an IL-12- and OX40L-dependent pathway. *Cancer Cell*. 2011;19(3):333–346.
174. Godefroy E, et al. Activation of toll-like receptor-2 by endogenous matrix metalloproteinase-2 modulates dendritic-cell-mediated inflammatory responses. *Cell Rep*. 2014;9(5):1856–1870.
175. Vito A, El-Sayes N, Mossman K. Hypoxia-driven immune escape in the tumor microenvironment. *Cells*. 2020;9(4).
176. Vigano S, et al. Targeting adenosine in cancer immunotherapy to enhance T-cell function. *Front Immunol*. 2019;10:925.
177. Liu M, et al. Targeting the IDO1 pathway in cancer: from bench to bedside. *J Hematol Oncol*. 2018;11(1):100.
178. Luis A, et al. Oxidative stress-dependent activation of the eIF2alpha-ATF4 unfolded protein response branch by skin sensitizer 1-fluoro-2, 4-dinitrobenzene modulates dendritic-like cell maturation and inflammatory status in a biphasic manner [corrected]. *Free Radic Biol Med*. 2014;77:217–229.
179. Veglia F, et al. Lipid bodies containing oxidatively truncated lipids block antigen cross-presentation by dendritic cells in cancer. *Nat Commun*. 2017;8(1):2122.
180. Cubillos-Ruiz JR, et al. ER stress sensor XBP1 controls anti-tumor immunity by disrupting dendritic cell homeostasis. *Cell*. 2015;161(7):1527–1538.
181. Ridgway D. The first 1000 dendritic cell vaccines. *Cancer Invest*. 2003;21(6):873–886.
182. Kantoff PW, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med*. 2010;363(5):411–422.
183. Anguille S, et al. Clinical use of dendritic cells for cancer therapy. *Lancet Oncol*. 2014;15(7):e257–e267.
184. Coelho AV, et al. Dendritic cell-based immunotherapies to fight HIV: how far from a success story? A systematic review and meta-analysis. *Int J Mol Sci*. 2016;17(12).
185. Elkord E, et al. Human monocyte isolation methods influence cytokine production from in vitro generated dendritic cells. *Immunology*. 2005;114(2):204–212.

186. Vopenkova K, et al. Complex evaluation of human monocyte-derived dendritic cells for cancer immunotherapy. *J Cell Mol Med*. 2012;16(11):2827–2837.
187. Huber A, et al. Current state of dendritic cell-based immunotherapy: opportunities for in vitro antigen loading of different DC subsets? *Front Immunol*. 2018;9:2804.
188. Charles J, et al. An innovative plasmacytoid dendritic cell line-based cancer vaccine primes and expands antitumor T-cells in melanoma patients in a first-in-human trial. *Oncoimmunology*. 2020;9(1):1738812.
189. Fu C, et al. Plasmacytoid dendritic cells cross-prime naive CD8 T cells by transferring antigen to conventional dendritic cells through exosomes. *Proc Natl Acad Sci U S A*. 2020;117(38):23730–23741.
190. Westdorp H, et al. Blood-derived dendritic cell vaccinations induce immune responses that correlate with clinical outcome in patients with chemo-naive castration-resistant prostate cancer. *J Immunother Cancer*. 2019;7(1):302.
191. Tel J, et al. Natural human plasmacytoid dendritic cells induce antigen-specific T-cell responses in melanoma patients. *Cancer Res*. 2013;73(3):1063–1075.
192. Mukherji B, et al. Induction of antigen-specific cytolytic T cells in situ in human melanoma by immunization with synthetic peptide-pulsed autologous antigen presenting cells. *Proc Natl Acad Sci U S A*. 1995;92(17):8078–8082.
193. Salem ML. The use of dendritic cells for peptide-based vaccination in cancer immunotherapy. *Methods Mol Biol*. 2014;1139:479–503.
194. Carreno BM, et al. Cancer immunotherapy. A dendritic cell vaccine increases the breadth and diversity of melanoma neoantigen-specific T cells. *Science*. 2015;348(6236):803–808.
195. Chiang CL, et al. A dendritic cell vaccine pulsed with autologous hypochlorous acid-oxidized ovarian cancer lysate primes effective broad antitumor immunity: from bench to bedside. *Clin Cancer Res*. 2013;19(17):4801–4815.
196. Liau LM, et al. First results on survival from a large Phase 3 clinical trial of an autologous dendritic cell vaccine in newly diagnosed glioblastoma. *J Transl Med*. 2018;16(1):142.
197. Akasaki Y, et al. Phase I/II trial of combination of temozolomide chemotherapy and immunotherapy with fusions of dendritic and glioma cells in patients with glioblastoma. *Cancer Immunol Immunother*. 2016;65(12):1499–1509.
198. Rosenblatt J, et al. Individualized vaccination of AML patients in remission is associated with induction of antileukemia immunity and prolonged remissions. *Sci Transl Med*. 2016;8(368):368ra171.
199. Rosenblatt J, et al. Vaccination with dendritic cell/tumor fusions following autologous stem cell transplant induces immunologic and clinical responses in multiple myeloma patients. *Clin Cancer Res*. 2013;19(13):3640–3648.
200. Aarntzen EH, et al. Vaccination with mRNA-electroporated dendritic cells induces robust tumor antigen-specific CD4+ and CD8+ T cells responses in stage III and IV melanoma patients. *Clin Cancer Res*. 2012;18(19):5460–5470.
201. Sundarasetty BS, et al. Lentivirus-induced 'Smart' dendritic cells: pharmacodynamics and GMP-compliant production for immunotherapy against TRP2-positive melanoma. *Gene Ther*. 2015;22(9):707–720.
202. Hammerich L, Binder A, Brody JD. In situ vaccination: cancer immunotherapy both personalized and off-the-shelf. *Mol Oncol*. 2015;9(10):1966–1981.
203. Dhodapkar MV, et al. Induction of antigen-specific immunity with a vaccine targeting NY-ESO-1 to the dendritic cell receptor DEC-205. *Sci Transl Med*. 2014;6(232):232ra51.
204. Goyvaerts C, Breckpot K. The journey of in vivo virus engineered dendritic cells from bench to bedside: a bumpy road. *Front Immunol*. 2018;9:2052.
205. Cawood R, et al. Recombinant viral vaccines for cancer. *Trends Mol Med*. 2012;18(9):564–574.
206. Yang W, et al. In situ dendritic cell vaccine for effective cancer immunotherapy. *ACS Nano*. 2019;13(3):3083–3094.
207. Ni J, et al. Dendritic cell vaccine for the effective immunotherapy of breast cancer. *Biomed Pharmacother*. 2020;126:110046.
208. Hammerich L, et al. Systemic clinical tumor regressions and potentiation of PD1 blockade with in situ vaccination. *Nat Med*. 2019;25(5):814–824.
209. Jabulowsky RA, et al. A first-in-human phase I/II clinical trial assessing novel mRNA-lipoplex nanoparticles encoding shared tumor antigens for immunotherapy of malignant melanoma. *Abstract Immunother Cancer*. 2018;29(Suppl 8):viii439.
210. Nemunaitis J. Vaccines in cancer: GVAX, a GM-CSF gene vaccine. *Expert Rev Vaccines*. 2005;4(3):259–274.
211. Ho VT, et al. Biologic activity of irradiated, autologous, GM-CSF-secreting leukemia cell vaccines early after allogeneic stem cell transplantation. *Proc Natl Acad Sci U S A*. 2009;106(37):15825–15830.
212. Burkhardt UE, et al. Autologous CLL cell vaccination early after transplant induces leukemia-specific T cells. *J Clin Invest*. 2013;123(9):3756–3765.
213. Borrello IM, et al. Granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting cellular immunotherapy in combination with autologous stem cell transplantation (ASCT) as postremission therapy for acute myeloid leukemia (AML). *Blood*. 2009;114(9):1736–1745.
214. Squadrito ML, et al. EVIR: chimeric receptors that enhance dendritic cell cross-dressing with tumor antigens. *Nat Methods*. 2018;15(3):183–186.
215. Harrington KJ, et al. Clinical development of talimogene laherparepvec (T-VEC): a modified herpes simplex virus type-1-derived oncolytic immunotherapy. *Expert Rev Anticancer Ther*. 2015;15(12):1389–1403.
216. Andtbacka RH, et al. Talimogene laherparepvec improves durable response rate in patients with advanced melanoma. *J Clin Oncol*. 2015;33(25):2780–2788.
217. Santos-Sierra S. Developments in anticancer vaccination: budding new adjuvants. *Biol Chem*. 2020;401(4):435–446.
218. Cintolo JA, et al. Dendritic cell-based vaccines: barriers and opportunities. *Future Oncol*. 2012;8(10):1273–1299.
219. Bonehill A, et al. Single-step antigen loading and activation of dendritic cells by mRNA electroporation for the purpose of therapeutic vaccination in melanoma patients. *Clin Cancer Res*. 2009;15(10):3366–3375.
220. Hobo W, et al. siRNA silencing of PD-L1 and PD-L2 on dendritic cells augments expansion and function of minor histocompatibility antigen-specific CD8+ T cells. *Blood*. 2010;116(22):4501–4511.
221. Brody JD, et al. In situ vaccination with a TLR9 agonist induces systemic lymphoma regression: a phase I/II study. *J Clin Oncol*. 2010;28(28):4324–4332.
222. Saxena M, et al. Poly-ICLC, a TLR3 agonist, induces transient innate immune responses in patients with treated HIV-infection: a randomized double-blinded placebo controlled trial. *Front Immunol*. 2019;10:725.
223. Salazar AM, et al. Therapeutic in situ autovaccination against solid cancers with intratumoral poly-ICLC: case report, hypothesis, and clinical trial. *Cancer Immunol Res*. 2014;2(8):720–724.
224. Su T, et al. STING activation in cancer immunotherapy. *Theranostics*. 2019;9(25):7759–7771.
225. Mitchell DA, et al. Tetanus toxoid and CCL3 improve dendritic cell vaccines in mice and glioblastoma patients. *Nature*. 2015;519(7543):366–369.
226. Bhardwaj N, et al. Flt3 ligand augments immune responses to anti-DEC-205-NY-ESO-1 vaccine through expansion of dendritic cell subsets. *Nat Cancer*. 2020;1:1204–1217.
227. Bayat Mokhtari R, et al. Combination therapy in combating cancer. *Oncotarget*. 2017;8(23):38022–38043.
228. van Gulijk M, et al. Combination strategies to optimize efficacy of dendritic cell-based immunotherapy. *Front Immunol*. 2018;9:2759.
229. Kroemer G, et al. Natural and therapy-induced immunosurveillance in breast cancer. *Nat Med*. 2015;21(10):1128–1138.
230. Zhao J, et al. Safety and efficacy of therapeutic cancer vaccines alone or in combination with immune checkpoint inhibitors in cancer treatment. *Front Pharmacol*. 2019;10:1184.
231. Garg AD, et al. Integrating next-generation dendritic cell vaccines into the current cancer immunotherapy landscape. *Trends Immunol*. 2017;38(8):577–593.
232. Urrizola A, Solans B, Sanchez Bayona R, et al. Addition of dendritic cell vaccines to neoadjuvant chemotherapy in HER2 negative breast cancer patients. *Ann Oncol*. 2020;31(4):S710.
233. Hatem S, Hogue D, Han H, et al. A pilot study utilizing a HER2 directed dendritic cell vaccine during neoadjuvant therapy of HER2+ breast cancer [abstract]. *American Association for Cancer Research*. 2020;80(4).

CHAPTER 21

NATURAL KILLER CELL IMMUNITY AND THERAPY

William E. Carson III

Natural killer (NK) cells are large, granular lymphocytes that comprise about 10% to 15% of the circulating peripheral blood lymphoctyes.[1–3] These unique cells were first characterized by their ability to lyse targets independently of activating or initiating stimuli.[4] NK cells are a critical cellular component of the innate immune system that have the ability to target and lyse malignant and virally infected cells. In addition, NK cells secrete cytokines that help to organize and shape the innate and adaptive immune response to infectious insults and malignant transformation. There is increased interest in NK cells as therapeutic agents as discoveries in the laboratory and the clinic have led to a better understanding of the important role played by NK cells in molding the early immune response.[5] NK cells play a key role in maintaining host defense, as illustrated by human NK cell deficiency syndromes which carry increased susceptibility to overwhelming viral, intracellular, and atypical mycobacterial infections.[6] Further, murine models of NK cell deficiency are characterized by a distinct susceptibility to development of cancer, and reduced NK cell activity in humans is associated with a higher risk for developing malignancy.[7–9] This chapter provides a review of the current understanding of NK cell biology, the role of NK cells in human disease, and the recent clinical applications of NK cells to the therapy of cancer.

NATURAL KILLER CELL FUNDAMENTAL BIOLOGY

NK cells are critical cellular components of the innate immune response and are particularly important in the early control of viral infections and the elimination of malignant cells. NK cells express a number of receptors, both activating and inhibitory, that enable them to identify cellular targets that are not part of the host's normal tissues. The overall balance between activating signals and the absence of inhibitory signals will lead to NK cell activation and the initiation of effector cell programs. NK cells also constitutively express a number of cytokine receptors including receptors for interleukin (IL)-2, IL-10, IL-12, IL-15, IL-18, and IL-21. The ability of NK cells to sense very low levels of these cytokines allows them to be intimately attuned to the current status of the immune microenvironment. And unlike T cells, NK cells do not require antigenic stimulation in order for cytokine receptor expression to occur. For example, resting NK cells express high levels of a functional IL-2 receptor and are able to rapidly respond to this cytokine. NK cells will embark upon the immediate cytolytic destruction of cells that do not display the appropriate "self molecules"; however, they also produce large amounts of multiple cytokines with potent effects on other immune compartments such as interferon-gamma (IFN-γ), tumor necrosis factor (TNF)-α, and granulocyte–macrophage colony-stimulating factor (GM-CSF). Notably, IFN-γ is described as the prototypical macrophage activating factor, and NK cells are known to interact with and facilitate the function of this cellular compartment as well as dendritic cells and T cells. Under the appropriate conditions, NK cells are also potent producers of chemokines such as IL-8, macrophage inflammatory protein-1-alpha (MIP-1α), and RANTES (regulated upon activation, normal T Cell expressed and presumably secreted) that have the ability to attract T cells and macrophages to sites of immunologic activity. NK cell mobilization is aided by the expression of chemokine receptors that direct their trafficking during development and differentiation and promote their recruitment to sites where an immune response is developing. NK cell adherence to the vascular endothelium is the first step in their deployment to sites of infection or malignancy and this process is facilitated via their expression of abundant levels of multiple adhesion molecules. NK cell expression of cellular adhesion molecules also promotes the formation of conjugates between NK cells and susceptible target cells. These characteristics of the NK cell compartment are indicative of a complex immune cell type with potent effector functions.[1–3]

Natural Killer Cell Subsets

NK cells are phenotypically recognized by surface expression of CD56 (also called *neural cell adhesion molecule*) and the absence of the T-cell receptor as evidenced by lack of CD3 expression.[10,11] Two functional subsets of NK cells may be discriminated from one another based on the intensity of CD56 surface expression, the so-called CD56^{bright} and CD56^{dim} subsets. CD56^{dim} NK cells comprise 85% to 90% of the NK cells in the peripheral circulation and are generally considered to be potent mediators of cytotoxicity. About 10% to 15% of NK cells in the blood are CD56^{bright}, and upon activation, this subset is capable of robust cytokine and chemokine production.[12] Fig. 21.1 graphically depicts these NK subsets, and Table 21.1 summarizes major surface antigens associated with each NK cell subset. While the use of CD56 expression is useful in understanding NK cell biology and function, it is also apparent that the differential cytolytic function and cytokine output of the bright and dim subsets is not absolute and can be altered (and even reversed) by specific environmental cues.

CD56^{dim} Natural Killer Cells

CD56^{dim} NK cells are strongly cytolytic and are able to kill infected as well as tumor cell targets without prior sensitization.[13] They constitutively express the interleukin-2/15 (IL-2/IL-15) receptor (R) β- and common γ-receptor chains, which together form a receptor complex through which cells may respond to stimulation following exposure to either IL-2 or IL-15.[14,15] CD56^{dim} NK cells can lyse tumor cell targets through at least three distinct mechanisms. First, they can execute cytotoxicity through the exocytosis of lytic granules containing the pore-forming protein perforin and proteases called granzymes (e.g., granzyme B or GrzB) which initiate an apoptotic program via cleavage of specific protein substrates. Second, cytotoxicity can be mediated through NK cell expression of Fas ligand (CD95L) and TNF-related apoptosis-inducing ligand (TRAIL) which interact with their respective receptors CD95/Fas and TRAIL-R1 and -R2 on the target cell surface.[16] This binding event leads to recruitment of the Fas-associated protein with death domain (FADD), recruitment and activation of procaspase 8 and 10, and the initiation of a caspase cascade that results in target cell apoptosis. This process is associated with NK cell production of cytokines, IFN-γ, TNF-α, and GM-CSF.[17] Third, CD56^{dim} NK cells can mediate antibody-dependent cytotoxicity (ADCC) via the high-level surface expression of CD16 (FcγRIIIA), a low-affinity receptor for IgG which mediates a lytic program against antibody-coated target cells.[18] Compared with CD56^{bright} NK cells, freshly isolated, unstimulated CD56^{dim} NK cells have intrinsically greater cytotoxicity in vitro against NK-sensitive tumor targets such as the well-studied K562 chronic myelogenous

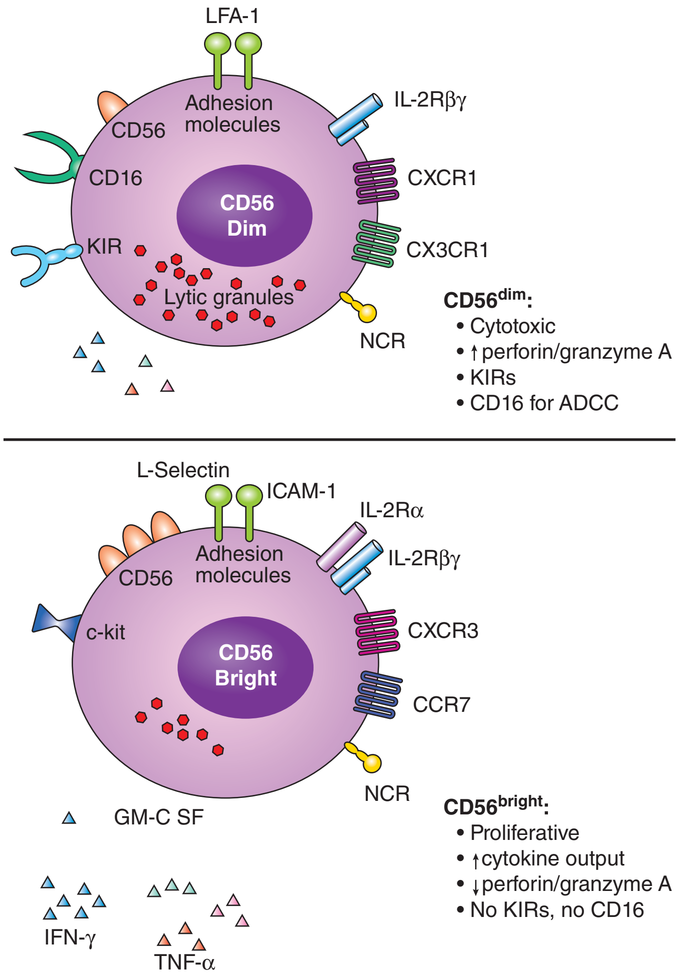


Figure 21.1 SIMPLIFIED REPRESENTATION OF NATURAL KILLER CELL SUBSETS. CD56^{bright} cells have immunoregulatory function, whereas CD56^{dim} cells have cytolytic function. *ADCC*, Antibody-dependent cellular cytotoxicity; *CCR7*, C-C chemokine receptor type 7; *CXCR1*, C-X-C motif chemokine receptor 1; *CXCR3*, C-X-C motif chemokine receptor 3; *CX3CR1*, C-X3-C motif chemokine receptor 1; *GM-CSF*, granulocyte macrophage-colony stimulating factor; *ICAM-1*, intercellular adhesion molecule 1; *IFN*, interferon; *IL*, interleukin; *KIR*, killer immunoglobulin-like receptor; *LFA-1*, lymphocyte function-associated antigen 1; *NCR*, natural cytotoxicity receptor; *R*, receptor; *TNF*, tumor necrosis factor.

leukemia cell line,[19] and they exhibit relatively high surface density expression of killer-cell immunoglobulin-like receptors (KIRs), a family of polymorphic activating and inhibitory receptors that are critically important in regulating NK cell surveillance of host tissues for cells with "altered self" (see below).[20]

CD56^{bright} Natural Killer Cells

CD56^{bright} NK cells appear to play an immunoregulatory role in the innate immune response. CD56^{bright} NK produce multiple cytokines and chemokines, have a relatively high proliferative capacity, reside primarily in the parafollicular T cell–rich region of secondary lymphoid tissue (SLT), and have modest levels of cytolytic granules, low expression of KIR and FcγRIII (see Table 21.1).[13] CD56^{bright} NK cells are unique among cytotoxic effector cells in their constitutive expression of the heterotrimeric high-affinity IL-2 Rαβγ complex, making them responsive to picomolar concentrations of IL-2 released by activated T cells in the SLT parafollicular region.[21] As noted, CD56^{bright} NK cells comprise only about 10% of the circulating NK population but predominate almost to the exclusion of the CD56^{dim} NK subset in SLT.[22] This distribution likely results from their selective expression of a number of receptors that assist in homing cells to SLT and retaining them there (e.g., CCR7 and CD62L).[13,23] NK cell precursor expression of the C-type lectin-like surface activating receptor NKp80 correlates with NK cell functional maturity in SLTs. NKp80 may play an important regulatory role during NK maturation in SLTs related to the acquisition of cytotoxic functions and/or cytokine-production.[24]

Chemokine receptor repertoires (and thus migration patterns) are differentially aligned among NK cell sub-populations. Compared to CD56^{dim} NK cells, CD56^{bright} NK cells express higher levels of chemokine (C-X-C motif) receptor 4 (CXCR4) which is the receptor for CXCL12 (also knowns as SDF-1α/β). CXCL12/SDF-1 stimulates NK cell migration and its production by trophoblasts is responsible for maintaining a population of CD56^{bright} NK cells within the decidua of the uterus during pregnancy. The CD 56^{bright} subset is directed to lymph nodes via their expression of CCR7, and they preferentially express CXCR3 which binds the interferon-inducible chemokines CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC). This pattern of chemokine receptor expression, along with the high levels of the adhesion molecule L-Selectin (CD62L), helps to explain the prevalence of CD56^{bright} NK cells in secondary lymphoid tissues. In contrast, CD56^{dim} NK cells uniquely express high levels of receptors specific for inflammatory chemokines such as CXCR1, CXCR2, and CX3CR1.[25] CXCR1 binds IL-8 (CXCL8), the levels of which are increased in the setting of acute viral infections and correlate with disease severity.[26] Fractalkine (CX3CL1), the ligand for CX3CR1, is induced at sites of inflammation and acts as an adhesion protein for cells with CX3CR1 expression, resulting in retention of NK cells on vessel walls. As a member of the seven transmembrane domain G protein-coupled receptor family, CX3CR1 activation leads to NK cell stimulation via calcium mobilization and the initiation of mitogen-activated protein kinase (MAPK) and PI3 kinase signaling.[27] Thus, expression of these chemokine receptors allows NK cells to traffic to local areas of inflammatory response to augment and modify the host immune response. Altered expression of chemokine ligand/receptor pairs can also promote malignant disease. In murine models of multiple myeloma, there is increased bone marrow expression of CXCL9 and CXCL10 which leads to downregulation of CXCR3 on NK cells and reduced migration and retention of NK cells to this site.[28]

Not unexpectedly, NK cell expression of adhesion molecules also differs between the two CD56 subsets. An analysis of CD56^{dim} NK cells reveals that they express CD11a at a high level as compared to the bright subset. CD11a is the α chain of the $\alpha\gamma_L\beta 2$ integrin (also known as leukocyte function associated antigen 1 [LFA-1]. Integrins bind to a wide variety of ligands in the extracellular matrix, on the surface of other cells and also soluble proteins. In contrast, CD56^{bright} NK cells exhibit high-level expression of CD11c. They also express high levels of CD2, CD44, CD49e, CD54, and CD62L as compared to the dim subset. CD44 binds to hyaluronic acid in the extracellular matrix.[12] CD49e belongs to the integrin alpha chain family and associates with CD29 (integrin beta1) to form the VLA-5 receptor which binds to fibronectin and fibrinogen. CD54 is upregulated during activation and strengthens the NK-target during the lytic process. L-selectin (CD62L) is a type-I transmembrane glycoprotein that has been identified as a tethering/rolling receptor. As a result of these differing repertoires of adhesion molecules and chemokine receptors, the migratory properties of NK cells diverge quite significantly. The CD56^{bright} subset migrates preferentially to secondary lymphoid organs whereas CD56^{dim} NK cells are attracted to sites of acute inflammation.[12]

The ability of CD56^{bright} NK cells to produce an abundant variety of cytokines and chemokines compared with the CD56^{dim} subset likely relates more to the differential expression of both negative and positive regulators of cytokine/chemokine production and less to constitutive expression of cytokine-activating receptors. For example, CD56^{bright} NK cells have little or no expression of two negative regulators of cytokine/chemokine production, namely SHIP-1 (Src homology 2 domain-containing inositol 5-phosphatase 1) and HLX (H2.0-like homeobox 1).[29,30] CD56^{dim} NK cells lack constitutive expression of a positive regulator of cytokines called *SET*.[31] Single-cell transcriptomic analyses generally support the use of CD56 as a means of identifying NK cell subsets with mature populations expressing high levels of CXCR1, TIM-3 (inhibitory checkpoint molecule), and ZEB2.[32]

TABLE 21.1 Differential Properties of CD56$^{\text{bright}}$ and CD56$^{\text{dim}}$ Natural Killer Cells

Factor/Molecule[a] (Other Name)	Binding Partner/Function	CD56$^{\text{bright}}$	CD56$^{\text{dim}}$
CD56 (NCAM-1)	NK cell marker	++	+
CD16 (FcγRIIIa)	Binds Fc region of IgG	−(± in some individuals)	++
c-kit (CD117)	Binds stem cell factor (SCF)	+	−
CD57 (Leu-7)	Marker of differentiated NK cells with high cytotoxic/low proliferative potential	−	+
KIR-inhibitory (e.g., KIR2DL, 3DL)	Binds HLA C2 (inhibitory signal)	−	+
KIR-stimulatory (e.g., KIR2DS, 3DS)	Binds HLA C1 (stimulatory signal)	−	+
NKG2A (forms heterodimer w/ C-type lectin CD94)	Binds HLA-E (inhibitory signal)	+	±
NKG2C (heterodimer w/ CD94)	Binds HLA-E (activating signal)	±	±
NKG2D	Activating Binds MICA, MICB, and 6 members of ULBP family	+	+
NKp30	Natural cytotoxicity receptor (NCR) Recognizes N-terminal IgV domain of B7-H6/hemagglutinin (HA) of vaccinia virus	++	+
NKp44	NCR, expressed after NK activation Recognizes HA of influenza and Sendai viruses	±	±
NKp46	NCR Recognizes HA of influenza, vaccinia viruses Upregulated upon cytokine stimulation	++	+
DNAM-1 DNAX accessory molecule-1 (CD226)	Promotes NK cell cytotoxicity and IFN-γ release upon binding to ligands poliovirus receptor (PVR, nectin-like molecule 5, CD155) or herpes entry mediator (B—HVEB, Nectin-2, CD112)	+	++
IL-2R alpha (CD25)	High affinity IL-2 receptor	++	−
IL-2R beta (CD122)	Intermediate affinity IL-2 receptor	++	+
CCR5	Receptor for CCL3, 4, 5 (MIP-1α, MIP-1, RANTES)β	+	+
CCR7	Receptor for CCL19, 21 (MIP-3β, 6Ckine)	++	−
CXCR1	Receptor for CXCL1 (IL-8)	−	++
CXCR2 (IL-8R beta)	Receptor for CXCL8 (IL-8)	+	−
CXCR3	Receptor for CXCL9, 10, 11 (MIG, IP-10, ITAC)	++	±
CXCR4	Receptor for CXCL12 (SDF-1α/β)	±	±
CX3CR1	Receptor for CX3CL1 (fractalkine)	−	+
CD44	Binds hyaluronic acid Enhances NK cell functions via increased Ras, MAPK, and PI3K signals	++	+
CD2	Adhesion molecule Binds to other adhesion molecules (e.g., LFA-3)	+ +	+
L-selectin (CD62L)	Adhesion molecule Binds to CD34, GlyCAM1, MadCAM1	++	±
ICAM-1 (CD54)	Binds to integrins CD11a/CD18, or CD11b/CD18	++	±

Continued

TABLE 21.1 **Differential Properties of CD56$^{\text{bright}}$ and CD56$^{\text{dim}}$ Natural Killer Cells—cont'd**

Factor/Molecule[a] (Other Name)	Binding Partner/Function	CD56$^{\text{bright}}$	CD56$^{\text{dim}}$
LFA-1 (CD11a/CD18, αLβ2 integrin)	Binds ICAMs Initiates immune synapse formation and provides activating signals	+	++
Perforin	Pore-forming cytolytic protein found in granules	–	++
Granzyme A	Serine protease induces apoptosis by cleaving/inactivating diverse substrates (e.g., mitochondrial e- transport protein)	+	++
Granzyme B	Serine protease that induces apoptosis by activation of caspases	–	++
Granzyme K	Granzyme K (GrK) and GrA have trypsin-like activity and cleave after basic residues arginine and lysine; GrB cleaves after aspartic acid residues	+	–

[a] Expression levels of the listed molecules may vary across the CD56$^{\text{bright}}$ and CD56$^{\text{dim}}$ NK cell subsets due to donor type and variability, NK cell isolation protocols, flow cytometry gating strategies, antibody reagents being employed, and NK cell activation status.

NATURAL KILLER CELL DEVELOPMENT

NK cells are prototypic, founding members of a population of cells referred to as innate lymphoid cells (ILC).[33] Three populations of ILC have been described based on differential expression of specific transcription factors and cytokine production. NK cells are believed to arise from group 1 ILCs, which are characterized by T-bet and EOMES expression.[34] T-bet and EOMES are T-box transcription factors that serve as master regulators of lymphoid cell differentiation. Acquisition of the IL-15 receptor is likely a first step toward NK cell differentiation from CD34$^+$ hematopoietic stem cells and a subsequent common lymphoid precursor cell. Moreover, IL-15 is required for NK cell development in mice and humans,[35–37] and the NK cell maturation process appears to occur outside the bone marrow.[3,38] In humans, IL-15 has been found to regulate an NK cell signaling pathway that employs the serine-threonine kinase AKT and the transcription factor XBP1s which controls genes belonging to the unfolded protein response family.[39] IL-15 induced accumulation of XBP1s in the NK cell nucleus enables increased T-BET-mediated transcription of the granzyme B gene. XBP1s promotes NK cell cytotoxicity and is critical for IL-15-mediated NK cell survival.

Freud et al. identified a CD34$^{\text{dim}}$CD45RA$^+\alpha_4\beta_7^{\text{bright}}$ cell to be the only CD34$^+$ subset in SLT.[22] Found within the parafollicular T cell–rich region of SLT in the same region as the CD56$^{\text{bright}}$ NK cell, the CD34$^{\text{dim}}$CD45RA$^+\alpha_4\beta_7^{\text{bright}}$ cell can differentiate into a CD56$^{\text{bright}}$ NK cell in the presence of IL-15. Five novel, discrete stages of NK cell development were characterized in situ within the same parafollicular region of SLT, each with differential expression of CD34, CD117, and CD94.[33,40,41] As development proceeds along this continuum, the acquisition of phenotypic markers occurs in a progressive, orderly manner and cells acquire the ability to secrete cytokines (e.g., IFN-γ) and display natural cytotoxicity. This orderly development in SLT from a CD34$^+$ subset to CD56$^{\text{bright}}$ NK cells suggests that CD56$^{\text{dim}}$ NK cells represent a terminally differentiated NK stage that follows CD56$^{\text{bright}}$ NK development.[42–44] The abundance of CD56$^{\text{dim}}$ NK cells in blood versus SLT and their loss of both CD117 (c-kit) expression and proliferative capacity, along with their acquisition of KIR, FcγRIII (CD16), and cytolytic granules, are all consistent with this notion.[41] CD57 has been identified as a surface marker of terminally differentiated NK cells.[45]

NATURAL KILLER CELL RECEPTORS

In place of antigen-specific receptors, NK cells express a complex repertoire of surface molecules that allow them to efficiently recognize malignant and virally infected cells as non-self and rapidly initiate an appropriate immune response.[46,47] NK cell receptors may be activating or inhibitory. The binding of the NK cell receptor to its cognate ligand expressed on the surface of a target cell may either activate or suppress a functional NK cell response. Such receptors fall into three general categories: (1) those that are members of the KIR superfamily, (2) receptors that belong to the C-type lectin receptor (CTLR) superfamily, and (3) natural cytotoxicity receptors.[48] Currently, investigators are working from a model in which NK cell reactivity is determined in large part by the recognition of target cell major histocompatibility complex (MHC) class I alleles, with the presence of other target cell antigens providing additional signals to the NK cell (Fig. 21.2). MHC alleles are expressed on most human cells and may be classified as human leukocyte antigen A [HLA-A],[49] HLA-B,[50] and HLA-C[51] and their role in suppressing NK cytotoxicity is well-established.[52]

Killer-Cell Immunoglobulin-Like Receptors

KIRs provide one method by which NK cells recognize self from non-self in order to mediate an appropriate cytotoxic response. In total, 16 KIR genes have been identified on chromosome 19q13.4. Structurally, the KIR protein contains two or three extracellular immunoglobulin-like domains that have the ability to recognize MHC class I proteins, namely HLA-A, HLA-B, and HLA-C.[53] KIRs may be either inhibitory or activating, a functional feature that derives from the structure and enzymatic activity of the intracellular tyrosine-based motif of the KIR protein. An examination of KIR nomenclature provides information on the number of extracellular domains, the function of the intracellular motif, and the existence of polymorphic versions. The designation 2D or 3D indicates the number of immunoglobulin-like extracellular domains, while the length of the intracytoplasmic tail may be either long (L) and inhibitory, or short (S) and activating. A suffix numeral appearing at the very end of the KIR nomenclature indicates the existence of a polymorphic form of that particular receptor. For example, the terms KIR2DS2 and KIR2DS3 indicate the polymorphic forms of an activating KIR that displays two extracellular domains. The KIRs have been designated as members of the CD158 series.[53]

The KIR molecules provide NK cells with the ability to sense the absence of self-HLA molecules on potential target cells—a process which was originally referred to by K. Karre and early investigators as the detection of "missing self."[54–56] The 16 KIR genes are encoded by a family of homologous genes 10-16 kb in size that have been mapped to chromosome 19q13.4 where they are tightly arranged in a head-to-tail fashion. There are 14 KIR genes and 2 KIR pseudogenes. There are eight inhibitory KIR (KIR2DL1, 2DL2, 2DL3, 2DL5, 3DL1, 3DL2, and 3DL3) and six activating KIR (KIR2DS1, 2DS2, 2DS3, 2DS4, 2DS5, and 3DS1). The pseudogenes are designated KIR2DP1 and KIR3DP1). The long tails of the inhibitory KIRs contain 1 or 2 copies of immunoreceptor tyrosine-based inhibition motifs (ITIMs) that undergo phosphorylation following interaction with specific

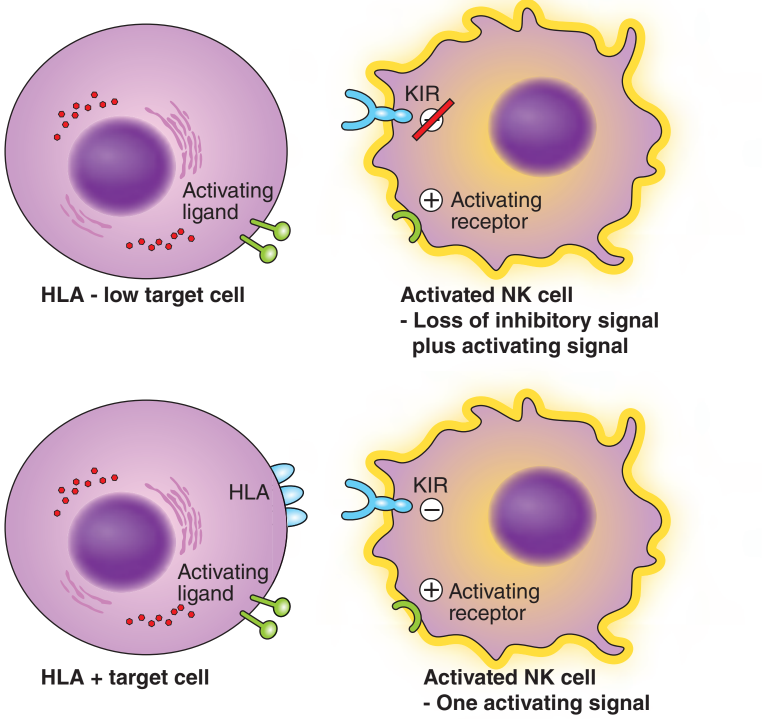


Figure 21.2 SIMPLIFIED REPRESENTATION OF NATURAL KILLER CELL CYTOTOXICITY MEDIATED THROUGH THE BALANCE OF ACTIVATING AND INHIBITORY SIGNALING IN RESPONSE TO LIGANDS ON POTENTIAL TARGETS. The NK cell in the top panel is stimulated to lyse the target cell due to the presence of activating ligands and absence of cognate HLA molecules, which leads to loss of inhibition through KIR. The NK cell in the bottom panel receives inhibitory signals by way of HLA-KIR interactions but is stimulated to lyse the target cell due to its expression of activating ligands. *HLA*, Human leukocyte antigen; *KIR*, killer immunoglobulin-like receptor; *NK*, natural killer.

HLA class I ligand and recruit Src homology region 2 domain containing phosphatase 1 and 2 (SHP-1, -2) that switch off the NK cell response. The short-tails of the activating KIRs lack ITIMs and instead are characterized by a positively charged amino acid residue in the transmembrane region that promotes the interaction with the 12 kDa DNAX activation protein (DAP-12). DAP12 contains immunoreceptor tyrosine-based activation motifs which initiate activating signals in response to ligand binding.[53]

Since they are organized in the form of a tightly linked cluster, the KIR genes are inherited as haplotypes with an individual receiving maternal and paternal contributions. KIR3DL3, 3DP1, 2DL4, and 3DL2 are present in every haplotype and are referred to as "framework" genes. KIR3DL3 is located at the 5′ centromeric end of the haplotype and KIR3DL2 is located at the 3′ telomeric end with KIR3DP1 and KIR2DL4 being located in the middle of the KIR gene complex separated by a 14 kb sequence that is enriched with L1 repeats. Thus, the centromeric half of the KIR haplotype is delimited by KIR3DL3 and 3DP1, and the telomeric half is delimited by KIR2DL4 and 3DL2. The KIR genes contained within these centromeric and telomeric regions enable the KIR haplotypes to be broadly classified into two categories, namely group A or group B. Group A haplotypes exhibit a fixed gene content that consists of KIR3DL3…2DL3…2DP1…2DL1…3DP1…2DL4…3DL1…2DS4…3DL2 in that order, with the framework genes having been underlined. In contrast, group B haplotypes are variable both in the number and combinations of KIR genes and contain several KIR genes (2DL2, 2DL5, 2DS1, 2DS2, 2DS3, 2DS5, 3DS1) that are not part of the A haplotype. Group A haplotypes contain only one activating gene (KIR2DS4), but group B haplotypes may contain up to five different activating KIRs, namely KIR2DS1, 2DS2, 2DS3, 2DS5, and 3DS1.

KIRs recognize specific peptide motifs of HLA class I molecules which are the products of MHC genes located on chromosome 6. The human MHC encodes for six functional HLA class I genes. HLA-A, HLA-B, and HLA-C represent the so-called classical class I genes and are highly polymorphic. The genes for HLA-E, HLA-F, and HLA-G encode non-classical class I molecules that are conserved. All of these class I molecules interact with NK cell receptors with the receptors for HLA-F having only recently been identified (Fig. 21.3). The alleles of the HLA-C locus can be placed into two groups of ligands (C1 and C2) based on the amino acid present at position 80 of the protein. HLA-C group 1 with asparagine at position 80 provides the ligand for KIR2DL2 and KIR2DL3, whereas HLA-C group 2 with lysine at position 80 provides the ligand for KIR2DL1. The HLA-Bw4 epitope at residues 77–83 of the HLA-B α1 domain is recognized by KIR3DL1. HLA-A3/11 serves as the ligand for KIR3DL2. While the ligands for inhibitory KIR have become well-defined, the ligand specificity of the activating KIR remains a subject of investigation. Recent studies indicate that KIR2DL4 is able to mediate both inhibitory and activating functions. Also, in addition to recognition of HLA-C1, KIR2DL2/L3 can weakly recognize some HLA-C2 alleles and the HLA-B*4601 and HLA-B*7301 alleles that bear the HLA-C1 epitope.[53,56]

Genotyping efforts reveal that all human populations possess group A and group B haplotypes, but their frequencies vary considerably. People of European descent and African Americans commonly carry both A and B haplotypes, whereas the homozygous group-A KIR haplotype (AA genotype) is common in Northeast Asians (i.e., Japanese, Koreans, and Chinese). The AB or BB genotypes are common in the Indigenous populations of India, Australia, and America and the NK cells of these individuals may contain up to 6 activating

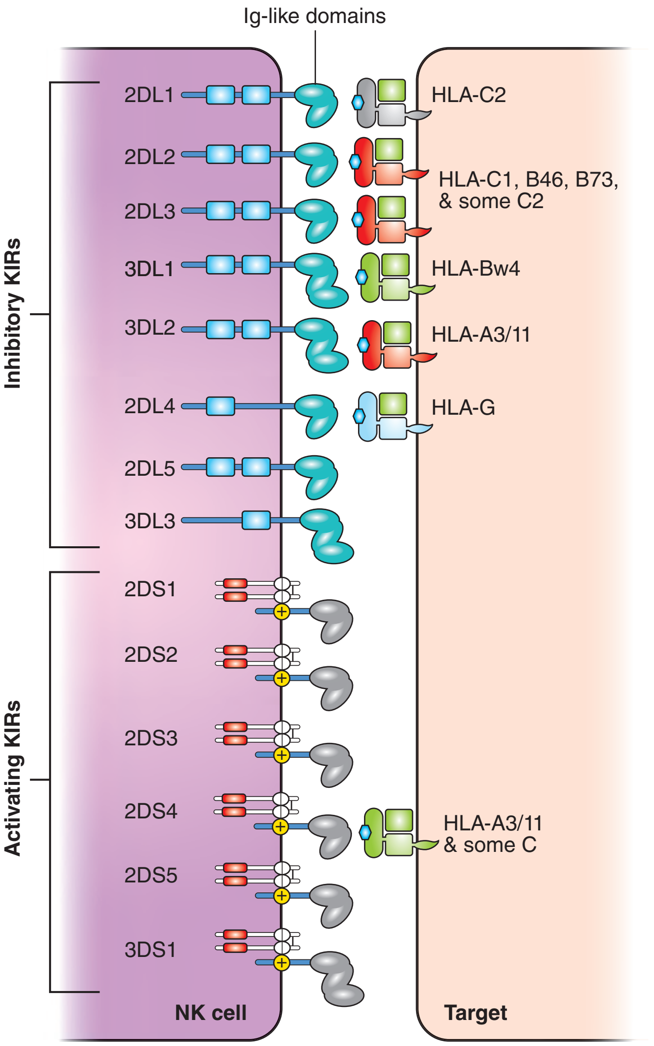


Figure 21.3 SIMPLIFIED REPRESENTATION OF HUMAN KIR MOLECULES AND THEIR HLA LIGANDS. There are 16 human KIR genes, 8 inhibitory, 6 activating, and 2 pseudogenes (not shown). The known HLA ligands are presented as binding partners. The ligands for several of the stimulatory KIRs have not yet been confirmed. *HLA*, Human leukocyte antigen; *Ig*, immunoglobulin; *KIR*, killer immunoglobulin-like receptor. *(From Rajalingam R. Diversity of killer cell immunoglobulin-like receptors and disease. Clin Lab Med. 2018;38(4):637–653.)*

KIRs. The prehistoric migrations of these populations may have provided environmental selective pressure in favor of haplotypes enriched in activating KIR.[57]

Individual KIR genes exhibit a high level of allelic polymorphism that can affect protein expression, affinity for HLA molecules, strength of the inhibitory/activating signal, and downstream cytotoxicity and cytokine release. The inhibitory KIR exhibit greater sequence polymorphism than the activating KIRs. Generally, inhibitory receptors bind with greater avidity or attraction for a corresponding HLA antigen than activating receptors.[58] Thus, if an NK cell expresses both activating and inhibitory KIR for an identical ligand, the NK cell will generally be inhibited from killing. Combinations of HLA variant and KIR genes exert an influence of resistance to viral infections, susceptibility to some autoimmune diseases, several pregnancy syndromes as well as the outcome of bone marrow transplantation.[59] Specific KIR/HLA allele pairings have been associated with improved outcomes in HIV[60,61] and hepatitis C infection.[62]

KIR2DL4 is present in all KIR haplotypes, and the presence of a positively charged arginine in its transmembrane region and a single ITIM moiety enables it to generate both a weak inhibitory signal as well as an activation program that leads to the secretion of proinflammatory and proangiogenic cytokines. HLA-G is expressed almost exclusively in fetal trophoblast cells at the maternal-fetal interface and its soluble form is the only known ligand for KIR2DL4 which is localized to NK cell endosomes. Uptake of soluble HLA-G by NK cell endosomal KIR2DL4 leads to endosomal signaling and the secretion of a collection of factors that favors embryo implantation, placental development, and successful pregnancy.[63,64]

C-Type Lectin Receptors

The CTLRs, located on human chromosome 12p12.3, share a common subunit (CD94) covalently bonded to one of four closely related gene products of the NKG2 family.[2,65] CTLRs promote a second type of NK cell receptor–mediated killing and include NKG2A (and splice variant B), NKG2C, NKG2E (and splice variant H), and NKG2F.[65] NKG2D, which does not bind CD94 and shares little sequence homology to other NKG2 proteins, is discussed below. The CTLRs expressed on NK cells and cytotoxic T lymphocytes are activating except for CD94/NKG2A which is inhibitory.[66] CD94/NKG2A specifically recognizes the non-classical HLA-E class I molecule.[67] HLA-E*0101 and HLA-E*0103 are the only two alleles present worldwide. Tumor cells may exhibit upregulation of HLA-E expression, presumably to avoid NK cell cytotoxicity. High-level expression of HLA-E is associated with poor prognosis in several cancers, and blockade of this ligand-receptor interaction can enhance NK cell anti-tumor activity.[68] HLA-E is able to present leader peptides from other HLA molecules which provides an additional mechanism for NK cells to sense the expression of class I MHC molecules on the cell surface. As is seen with KIR interactions, binding of CD94/NKG2A to HLA-E is more avid than binding of activating CTLRs to other epitopes.[69]

NKG2D is a CTLR; however, it does not associate with CD94 and has limited sequence homology with other members of the NKG2 family. NKG2D exists as a homodimer and does not have intrinsic signaling capacity, but instead interacts with the DAP10 adapter molecule which is able to provide signals that recruit the p85 subunit of phosphatidylinositol 3-kinase and a complex of GRB2 and VAV1.[70] This signal transduction arrangement effectively isolates the NKG2D signal from inhibitory signals that can suppress NK cell activation by other CTLRs. NKG2D is constitutively expressed at high levels on all NK cells, as well as on γδ T cells and $CD8^+$ T cells.[71] NKG2D binds to two different classes of MHC-I-like ligands whose expression is markedly induced by cellular stress, such as viral infection or malignant transformation. This binding event then leads to enhanced degranulation/cytotoxicity and production of IFN-γ. The ligands for NKG2D include the highly polymorphic MHC class I chain–related proteins MICA and MICB and 6 members of the UL16 binding proteins (ULBPs).[72–74] MICA and MICB expression is under the control of promoter elements similar to those that regulate the heat shock proteins which are upregulated in malignant cells and also those infected with cytomegalovirus (CMV).[75–78] UL16 is a virus-encoded type I transmembrane protein expressed in the setting of CMV infection that binds to ULBP-1 and ULBP-2 and allows the virus-infected cell to escape NK cell surveillance.[74,79] Similarly some human tumors downregulate expression of NKG2D ligands or release soluble forms of MICA as a mechanism of immune escape from NK cells.[80–83] Conversely, MICA expression by normal monocytes could provide a stimulus to NK cell activity against antibody-coated tumor cells.[84]

Natural Cytotoxicity Receptors

A third family of NK receptors that participate in the process of target cell recognition and elimination mediate cell killing are referred to as natural cytotoxicity receptors (NCRs). The three major NCRs that have been found to impact NK cell activation are NKp46, NKp30, and NKp44. NKp46 and NKp30 are uniquely and constitutively expressed on NK cells, and NKp44 is expressed on NK cells after cytokine stimulation (e.g., IL-2) and also on some γδ T cells.[85–87] Studies with neutralizing mAbs demonstrated that these receptors were recognizing ligands present on the surface of NK-susceptible targets, such as tumor cells and virally infected cells. The NCRs are type I transmembrane proteins that possess one or two immunoglobulin-like extracellular domains and a positively charged lysine or arginine in their hydrophobic transmembrane domain that allows them to interact with the ITAM-containing proteins and transduce an activating signal upon ligand binding. The CD56^{bright} and CD56^{dim} populations both express NKp46 and activation of this receptor promotes its association with CD3zeta or FcRgamma and drives the production of IFN-γ and TNF-α. NKp30 is expressed constitutively on all resting and activated NK cells. Its transmembrane region possesses a charged arginine residue that promotes a linkage with CD3zeta or FcRγ much like NKp46. Alternative transcript splicing gives rise to six different isoforms of NKp30, one of which (NKp30c) promotes NK cell secretion of the immunosuppressive cytokine IL-10 and is associated with reduced overall survival in patients with GIST tumors and neuroblastoma. NKp44 possesses a single extracellular V-type Ig domain that is connected to a lysine-containing transmembrane segment by a long stalk. The charged lysine residue enables the association of NKp44 with the DAP12 ITAM adapter which generates a stimulatory signal upon ligand binding. Notably, the intracellular region of the NKp44 protein contains an ITIM and thus has the ability to transmit both inhibitory and stimulatory signals. NK cell expression of NKp44 is markedly upregulated in the presence of IL-2, IL-15, or IL-1β.[88]

A large number of NCR activating ligands have been identified which permit NK cell recognition and elimination of malignant and pathogen-infected cells.[88] The costimulatory molecule B7-H6 is expressed at high levels on tumor cells and has been identified as an activating ligand for NKp30 that induces NKp30-dependent cytotoxicity. Some tumors shed a soluble form of B7-H6 that is associated with NK cell dysfunction and impaired tumor surveillance.[89,90] Heparin sulfate (HS) glycosaminoglycans are long chains of branched anionic polysaccharides that promote tumorigenesis by altering the extracellular milieu and providing a docking site for the basic portions of secreted growth factors. Each NCR recognizes a distinct HS sequence and mediates an activating signal upon binding.[91] NKp44 is also able to recognize nuclear proteins that undergo cell surface expression in the setting of malignant transformation or viral infection such as proliferating cell nuclear antigen (PCNA) that is normally expressed in the nucleus but can become complexed with HLA on the surface of lymphoma cells to form an inhibitory ligand for NKp44.[92]

NKp44 and NKp46 recognize hemagglutinin-neuraminidases (HN) expressed on the surface of cells infected with parainfluenza, Newcastle and Sendai virus, and also hemagglutinins (HA) from influenza infected cells. HA from the vaccinia poxvirus also bind to NKp30 and NKp46. Virus-mediated cleavage of sialic acid residues from NKp46 disrupts its binding to HA and inhibits NK cell elimination of infected cells.[93,94] NCR have the ability to recognize a broad array of pathogen-derived molecules. There is evidence that NKp44 and NKp46 play a role in the control of *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*. NKp30 and NKp46 are able to recognize proteins on the surface of erythrocytes infected with *Plasmodium falciparum* (cause of malaria) as well as fungal antigens from *Candida glabrata*, *C. neoformans*, and *C. albicans*. These beneficial effects of NCR-driven NK cell activity are balanced by studies that implicate these receptors in allergic disease and autoimmune conditions.[88]

Other Activating Receptors

DNAX accessory molecule-1 (DNAM-1) is a member of the immunoglobulin-like superfamily that is constitutively expressed on the surface of NK cells as well as T cells and macrophages. The ligands for DNAM-1 are the poliovirus receptor (PVR, or CD155) and Nectin-2 (herpes entry mediator B—HVEB or CD112). PVR and Nectin-2 become upregulated in response to cellular stress such as occurs with viral infection or malignant transformation. Engagement of DNAM-1 on the NK cell by PVR or Nectin-2 on the target cell initiates a signaling cascade that results in activation of ERK and AKT followed by calcium mobilization and the onset of NK cell effector functions. DNAM-1-mediated NK cell activation is counterbalanced by CD96 (also known as TACTILE) and TIGIT, two inhibitory NK cell receptors that belong to the same immunoglobulin-like superfamily as DNAM-1. CD96 is able to bind to PVR while TIGIT recognizes both Nectin-2 and PVR. These nectins may thus deliver either activating or inhibitory signals to the NK cell.[95] Also, the ability of a soluble, tumor-derived PVR molecule to inhibit NK cell degranulation and promote the pulmonary spread of tumor in a murine model of melanoma suggests a role for DNAM-1 in NK cell anti-cancer activity.[96]

CD244 (2B4) is an NK cell immune regulatory transmembrane receptor belonging to the signaling lymphocyte activation (SLAM) family. Its two extracellular Ig-like domains permit binding of CD48 (receptor on hematopoietic cells) and its cytoplasmic domain contains four immunoreceptor tyrosine—based switch motifs (ITSMs) that generate inhibitory or activating signals based upon the binding of distinct adaptor molecules with positive (SAP-Signaling lymphocyte activation molecule-associated protein) or negative (SHP-1, SHP-2, SHIP) effects. The expression levels, availability, and relative binding affinity of these adaptor molecules determine the output of CD244 in terms of its ultimate inhibitory or activating effects.[97,98] CMV viruses produce a soluble CD48 decoy protein that masks the 2B4 protein and inhibits the NK cell anti-virus responses.[99]

ADAPTIVE IMMUNE PROPERTIES OF NATURAL KILLER CELLS

NK cells have traditionally been thought of reacting to target cells in a largely stochastic fashion based on the presence or absence of specific receptors and their corresponding ligands. However, emerging data suggest that NK cells demonstrate sophisticated adaptive capabilities that improve the response to pathogens while limiting autoreactivity. Cues in the tissue microenvironment originating from interactions with normal cells and target cells can have a long-lasting impact on the NK cell compartment.[100]

Natural Killer Cell Education

The location of the KIR gene locus on chromosome 19 and the existence of the HLA locus on chromosome 6 has significant impact on the optimal functioning of the NK cell compartment. The potential for NK cell autoreactivity exists because NK cells in any one individual may lack inhibitory receptors for a particular self molecule, or the NK cell population may actually express activating receptors for self ligands. This situation can occur because the receptor array expressed on NK cells is determined in a random fashion through inheritance, and the ligands corresponding to these receptors are inherited independently. We now know that there exists a system of "education" whereby potentially autoreactive NK cells are not clonally deleted but rather rendered hyporesponsive in order to generate a state of self-tolerance. Conversely, NK cells that have engaged self-MHC via the appropriate inhibitory receptors may be thought of as being "licensed" to respond to subsequent stimulatory signals provided by target cells that lack self-MHC molecules.[101] Two different models of NK cell licensing have been proposed to explain the differential responsiveness of NK cell populations. The "arming" model proposes that NK cells lacking inhibitory receptors self-MHC simply fail to achieve full reactivity, whereas a "disarming" model predicts that NK cells that lack the inhibitory receptors for self MHC class I molecules are rendered hyporesponsive due to chronic low-level stimulation by activating receptors.[102] Evidence for the arming models comes from experimental murine models in which the functional maturation of NK cell precursors is enhanced following interactions with normal cells that express cognate MHC class I molecules during the course of development.[103–105] Support for the disarming model derives from experiments in which NK cell hyporesponsiveness is achieved when normal cells provide excessive stimulatory signals in the absence of inhibitory signals.[106–108] Although there is evidence to support each model, it is also possible that these two systems of education are both active and working together simultaneously. In fact, a so-called rheostat model has been proposed that describes a continuous process in which the availability of receptor ligands within the normal environment leads to differential and ever-changing engagement of the NK cell receptor repertoire such that the cellular response becomes "tuned" to an optimal set point.[102]

Natural Killer Cell Memory

In contrast to T and B cells, NK cells do not express the recombinase genes necessary for generating receptor variability and have a limited repertoire of germline encoded receptors for the identification of target cells that should be eliminated. Immunologic memory is a hallmark of the adaptive immune system wherein lymphocytes recognize a previously encountered antigen and rapidly respond with a highly specific immune response. However, recent data suggest that NK cells possess a form of memory as well. This idea was first demonstrated in a recombinase-activating gene 1 (RAG-1)-deficient mouse lacking T and B cells that were treated with haptens—that is small molecules that interact with host proteins to form immunogenic adducts. Despite the absence of adaptive immunity, mice rechallenged with the hapten developed a measurable hypersensitivity type recall response that was dependent on the existence of "memory" NK cells residing in the liver and expressing Thy1 and CXCR6.[100] The concept that murine NK cells can exhibit an adaptive immune response to haptens has also been demonstrated in the setting of viral infection with vesicular stomatitis virus, HIV-1, influenza, vaccinia, varicella-zoster virus, and hepatitis B.[109–113]

A second example of adaptive NK cell responses has been identified in the setting of human and murine CMV (MCMV) infection. Ly49H$^+$ NK cells recognize the MCMV m157 glycoprotein, resulting in NK cell–mediated control of the disease. These Ly49H$^+$ NK cells preferentially expand in the setting of infection and contract under the direction of the proapoptotic factor Bim once the infection is controlled.[114,115] However, "memory" NK cells can be detected months after infection, and, upon restimulation, these NK cells exhibited augmented cytotoxicity and cytokine production against MCMV.[81] These NK cells stably express KLRG1, a cadherin-recognizing inhibitory receptor, and could be detected two months after infection control. CMV infection in humans is associated with expansion of NK cells expressing NKG2C, the human homologue of murine Ly49H that respond to CMV reactivation with enhanced anti-viral effector functions.[116,117]

A third type of functional NK cell memory has been reported by Cooper et al. in murine NK cells that were briefly pre-activated with IL-12, IL-15, and IL-18 and exhibited enhanced IFN-γ output in response to restimulation for up to 3 weeks.[118] This training effect could be passed on to subsequent generations of NK cells and was associated with increased expression of CD94, NKG2A, NKp30, NKp44, NKp46, CD62L, and CD25 expression and no change in levels of CD57 and KIR molecules.[119] Many of the features associated these cytokine-induced memory-like (CIML) NK cells are also seen in human NK cells and their response to tumor targets. This observation has provided a rationale for infusion of CIML NK cells in the clinical setting.[120]

THE ROLE OF NATURAL KILLER CELLS IN HUMAN DISEASE

Studies of individuals with impaired or dysfunctional NK cell activity have shed new light on the genes and mechanisms responsible for proper NK development and function. Several syndromes have been linked to increased susceptibility to infection, while others may predispose to autoimmune disease and possibly the development of malignancy.

Natural Killer Deficiency Syndromes Linked to Increased Infectious Risks

Pure NK cell deficiency syndromes may be the result of a profound decrease in the number of circulating NK cells, or may reflect a serious defect in NK cell function but with normal numbers of NK cells in the peripheral blood.[121] Patients with classical NK cell deficiency present with a history of severe, recurrent infections caused by members of the Herpesviridae and Papillomaviridae families that usually have a benign course in normal individuals. CD56+ NK cells are either absent or represent less than 1% of circulating lymphocytes. Biron and colleagues reported in 1989 on the case of a young patient who presented with life-threatening varicella infection who exhibited an absolute absence of CD56$^+$CD16$^+$ NK cells. This individual subsequently developed CMV pneumonia and cutaneous HSV (herpes simplex virus) infection.[6] A second patient with a similar lack of peripheral blood NK cells presented with disseminated *Mycobacterium avium* infection and went on to die as a result of systemic varicella infection.[122] Other patients have been described with an isolated deficiency of CD56$^+$/CD3$^-$ lymphocytes but with normal or even increased populations of CD56$^+$/CD3$^+$ cells. One such patient presented with severe, recurrent condylomatous disease caused by the human papilloma virus.[123] An example of a gene mutation causing a functional NK deficiency in NK cell cytotoxicity is the substitution of "T → A" at gene position 230 in *FCGR3*A (codes for FcγRIIIa) resulting in the replacement of a histidine residue with lysine. Affected patients experience increased susceptibility to severe and disseminated HSV infections as well as Epstein-Barr virus and varicella infections.[124,125]

The Role of Natural Killer Cells in Autoimmunity

NK cells have been implicated in both the regulation and pathogenesis of autoimmune disorders. NK cells have been implicated in preventing the progression of paralysis in murine experimental models of MS.[126] Recently, investigators have identified CD56$^{\text{bright}}$ NK cells as a possible biomarker of response in MS following treatment with interferon-beta.[127] NK cells have also been shown to control inflammation in an experimental model of autoimmune colitis.[128,129] Experimental evidence supports the idea that NK cells may promote development of type 1 diabetes mellitus through targeted elimination of pancreatic islet β cells after viral infection.[130] This pathobiology may be mediated via an as yet unidentified NKp46 ligand located in the insulin granules of beta cells.[131,132] Other studies suggest that NK cells can promote humorally mediated autoimmune diseases such as myasthenia gravis through potentiation of autoreactive B cells.[133] Synoviocytes of patients with rheumatoid arthritis (RA) have been shown to express abnormally high levels of MICA, a ligand for NKG2D.[134] NK cells present in acute RA joint effusions may perpetuate this autoimmune inflammatory response,[135] and an analysis of synovial fluid NK cells in patients with severe RA revealed a marked expansion of a CD56$^{\text{bright}}$ population with high-level expression of CD69 and NKp46.[136] There is also evidence for NK cell participation in the process of RA bone destruction.[137]

Polymorphisms of the FcγRIIIA gene have been implicated in the pathogenesis and progression of autoimmune disease such as systemic lupus erythematosus (SLE) and RA.[138–141] There is evidence for an association between NK cell KIR expression and the onset of several autoimmune diseases. The frequency of KIR2DS2 has been identified as being significantly elevated in individuals with SLE.[142] The KIR2DS gene has also been associated with the onset of psoriatic arthritis.[143] Behçet disease is a rare autoimmune disease affecting blood vessels. A comprehensive analysis of HLA and KIR genotypes in over 300 patients suggested that the NK cell KIR repertoire is very likely involved in Behçet disease pathogenesis.[144] Particular KIR genes have also been linked to the development of RA, MS, type 1 diabetes, and ankylosing spondylitis, among others.[145]

THE THERAPEUTIC POTENTIAL OF NATURAL KILLER CELLS

In order to generate an antitumor immune response and eliminate malignant cells, T lymphocytes require the presence of a tumor-specific antigen. These targets have been difficult to identify for the majority of non-viral neoplasms. In contrast, it has long been recognized that NK cells are capable of mediating an antitumor effect in the absence of identifiable tumor antigens. As our understanding of NK cell biology has progressed, investigators have developed a number of innovative approaches to cancer therapy based on the use of NK cells (Table 21.2).

Natural Killer Cells and Hematopoietic Cell Transplantation

In greater than 95% of cases of patients undergoing hematopoietic cell transplantation (HCT) for leukemia, haploidentical individuals are available to act as donors of bone marrow or peripheral blood stem cells. Mismatches between donor KIR and recipient HLA create the potential for donor NK cell reactivity against leukemic cells as well as normal tissues within the recipient. Donor NK cells do not mediate significant graft-versus-host disease (GVHD) in the setting of HCT, likely due to the absence of significant activating signals being delivered by normal cells. In contrast, an effective NK cell-mediated graft-versus-leukemia (GVL) effect is possible when KIR on donor NK cells fail to encounter cognate HLA ligands on residual leukemic cells within the recipient. In 2002, Ruggeri et al. were the first to establish that the risk of relapse in acute myeloid leukemia (AML) following haploidentical HCT was markedly lowered when NK cell-based alloreactivity was present based on KIR-HLA mismatch in the GVL direction and the existence of alloreactive NK cell clones with the ability to lyse recipient leukemic cells.[146] These results were confirmed in a larger study of 112 AML patients where transplantation in the setting of complete remission with a NK cell alloreactive donor was associated with a significantly reduced relapse rate of 3% compared to 47%. Similar results were obtained for patients transplanted in relapse (6% vs. 34%).[147] Variable results have been obtained in subsequent studies that reported the outcomes of KIR mismatched HCT that utilized various donor sources, such as umbilical cord

TABLE 21.2 Anti-Cancer Therapies That Employ or Activate Natural Killer Cells

Therapy	Example	Advantages	Disadvantages
Exogenous cytokines	IL-2 IL-12 Interferon-alpha	Availability/low cost Can be combined with other agents	Toxic at high doses Off-target effects
Monoclonal antibodies ±NK cell stimulatory agents	Trastuzumab (anti-HER2 mAb)	Relatively inexpensive Known toxicity profiles Repeat dosing possible	Limited number of targets mAb therapy usually given w/ chemotherapy which can deplete NK cells
KIR blockade	Lirilumab (blocks KIR2DL1/2/3)	Blocks key NK inhibitory pathway	Ineffective as single agent
Checkpoint blockade	anti-TIM-3 mAb anti-TIGIT mAb	Ease of mAb administration	Checkpoints not universally expressed on all NK cell subsets
Engage activating receptor	Agonistic Abs to NKG2D, NKp46, CD137	Multiple targets on NK cells	Inhibitory pathways still active
Multivalent molecules	Bispecific killer engager (BiKE) to engage CD16 and tumor antigens	Molecule directs and activates NK cells	Tumor antigen target is required
Expansion of NK cells in vivo via cytokine administration	Low-dose IL-2 infusions IL-15 infusion	Well-tolerated Effectively expands NK cell populations	IL-2 can induce Treg cells Low anti-tumor activity as a single agent
Autologous NK cell Infusions	Lymphokine-activated killer (LAK) cells±IL-2	Relatively low side-effects	Need for expansion of the graft ex vivo Limited persistence of graft in recipient
Allogeneic NK cell Infusions	IL-2-activated NK cells from related haplo-identical donors	Availability of graft	Requirement for lymphodepleting chemotherapy Short-term persistence of graft
Hematopoietic cell transplantation (HCT) for leukemia	HCT for AML with haploidentical donor	GVL effect Less chance for GVHD	Requirement for lymphodepleting chemotherapy
CAR-NK cells	Multiple approaches	Highly modifiable Multiple cell sources	Need for genetic modification of NK cells Need for sophisticated manufacturing processes Limited lifespan Given after lymphodepleting chemotherapy

For abbreviations, see text.

blood, and sibling or unrelated donors.[148] The precise mechanisms involved in the generation of an NK vs. leukemia effect are complex given the independent inheritance of KIR and HLA genes, and the large variety of KIR interactions that can occur post-transplant. Also, the strength of this effect can be influenced by the type of preparative chemotherapeutic regimen, graft dose, T-cell depletion strategy, and post-transplant conditioning regimens.[149] Since a KIR mismatch requires the absence of a host KIR ligand that is present in the donor, NK alloreactivity is taking place in the setting of a major HLA-class I mismatch, and thus the extent of graft T-cell depletion becomes important. Indeed, the presence of T cells in the graft can adversely impact NK cell reconstitution.[150] Given the diversity of KIR expression and the existence of multiple alleles with variable binding capacity and levels of expression, it is recommended that KIR genotyping for HCT be accompanied by phenotyping via cytometric methods or quantitative PCR with an evaluation of the ability of donor NK cells to kill allogeneic recipient targets, such as PHA lymphoblasts and/or leukemia cells.[151] In adults, the beneficial effects of NK cell GVL activity are most prominent for AML rather than ALL; however the event-free survival post-HCT of pediatric ALL patients was significantly better for KIR B haplotype donors than for A haplotypes.[152] The association of the KIR B haplotype with improved survival is also seen in the setting of unrelated donor HCT for AML and non-Hodgkin lymphoma.[153,154] Activating KIR can also play a role in HCT outcomes, as evidenced by a significant reduction in infectious complications and non-relapse mortality when there was a GVL effect driven by the activating KIR2DS1 and KIR3DS1.[155] Finally, in a study of over 600 patients receiving reduced intensity HCT, CMV reactivation was associated with improved disease-free survival and the expansion of a CMV-responsive population of NK cells with reduced expression of CD56 and elevated surface levels of CD57 and NKG2C.[156]

Infusion of Autologous Natural Killer Cells

In studies conducted in the 1980s, peripheral blood mononuclear cells (PBMC) from patients with metastatic melanoma were expanded in vitro with IL-2 and the resulting lymphokine-activated killer cell (LAK) preparations (which likely had CD56+ NK cells as their precursors) were re-infused, usually with the addition of intravenous high-dose IL-2.[157] Complete responses were seen in about 10% of patients, but IL-2 administration was associated with a vascular leak syndrome and liver damage in some individuals. Given the evidence for NK cell anti-tumor activity in the setting of HCT, direct infusion of pure NK cell preparations has been performed as primary therapy in patients with leukemia and solid tumors.[158,159] However, the infusion of unmodified autologous NK cells has not shown significant anti-tumor activity. No clinical responses were observed when autologous NK cells were isolated from T-cell-depleted peripheral blood, cultured with IL-2, OKT3 (stimulatory anti-CD3 mAb) and irradiated autologous peripheral blood cells for 10 days and then infused into lymphodepleted patients with metastatic melanoma or renal cell carcinoma.[160] Similarly, patients with advanced breast cancer or lymphoma did not respond to the infusion of an autologous lymphapheresis product that had been cultured overnight with IL-2.[161]

Infusion of Allogeneic Natural Killer Cells

Outside the setting of HCT, the persistence of infused allogeneic NK cells is restricted by the recipient's immune system which is able to reject the graft. However, rejection of the donor NK cells can be delayed by pre-treating the recipient with lymphodepleting chemotherapy. Miller et al. employed this principle in a study that administered IL-2-activated NK cells (1×10^5–2×10^7 cells/kg) from related haploidentical donors to patients with metastatic solid tumors and also to individuals with poor-prognosis AML. Low-dose IL-2 (10 MU thrice weekly) was administered post-infusion. A lower intensity chemotherapy regimen was associated with NK cell persistence but did not lead to NK cell expansion. In contrast, AML patients receiving a more intensive inpatient regimen of high-dose cyclophosphamide and fludarabine exhibited expansion of the NK cell graft, endogenous production of IL-15, and complete remission of their leukemia in 5 of 19 cases.[162] A follow-up phase II study of a similar regimen in patients with recurrent breast or ovarian cancer did not result in significant expansion of the infused NK cells.[163]

The procedures being used for allogeneic NK cell infusions have undergone substantial advances in terms of the source of NK cells and methods used for cell expansion.[164] Peripheral blood NK cells have been used in the majority of clinical studies to date. PBMC obtained by density gradient centrifugation of a leukapheresis product from a normal donor can be further purified via T-cell depletion. Increased NK cell purity can be achieved via magnetic bead selection for CD56+ cells.[165] Peripheral blood NK cells from healthy persons are in a resting state. Therefore, IL-2 has been used to activate and expand the NK cell graft with a four-fold expansion being observed after one week of culture.[166] The use of IL-15 in a clinically applicable culture protocol led to increased expression of genes supporting cell metabolism and respiration.[167] Combinations of cytokines have also been used (e.g., IL-12, IL-15, and IL-18) in an effort to generate an NK cell product with greater cytotoxic activity and longevity.[118,168] Inclusion of irradiated feeder cells such as the K562 cell line will stimulate NK cell expansion and genetically engineered K562 cell lines have been used to present combinations of stimulatory signals such as membrane bound IL-15 (mbIL-15) and 4-1BBL, the ligand for the OX40 activating receptor.[169] Umbilical cord blood can serve as a source of CD34+ hematopoietic stem cells that can be expanded and then differentiated into NK cells via culture in a cocktail of cytokines that includes stem cell factor, IL-7, and IL-15.[170] Induced pluripotent stem cells (iPSCs) are derived from somatic cells such as fibroblasts that have been transduced with reprogramming genes (classically, Oct4, Sox2, cMyc, and Kl4) and may therefore serve as a starting source of cells. iPSC are first cultured in conditions that induce a hematopoietic differentiation program followed by cytokine conditions that promote the differentiation of NK cells which are then propagated with a stimulatory cell line expressing a membrane bound cytokine (e.g., IL-15 or IL-21).[171,172] Prior to the infusion of allogeneic NK cells, chemotherapeutic agents such as cyclophosphamide and fludarabine are used to induce a lymphodepleted state which transiently depletes the immune cell compartment and permits the temporary engraftment of the infused NK cells.[173] IL-2 or IL-15 may be administered to the patient post-transplantation in order to support NK cell engraftment.[174] A study of s.c. rhIL-15 administered after lymphodepleting chemotherapy and infusion of haploidentical NK cells led to NK-cell expansion by day 14 in 27% of patients with remission being achieved in 40% of cases, but this regimen was associated with previously unreported cytokine release syndrome.[175] While most clinical studies have focused on the treatment of patients with hematologic malignancies (AML most commonly), studies have been conducted with some success in patients with neuroblastoma, melanoma, renal cell carcinoma, ovarian cancer, and breast cancer.[163] Manufacture of the NK cell product is resource intensive, and therefore some investigators have made use of the NK-92 cell line in place of primary NK cells. This $CD56^+CD3$ cell line has cytotoxic capabilities similar to NK cells, but the cells must be irradiated prior to infusion in order to prevent tumor transfer to the recipient, and this prevents further cell proliferation.[176] Strategies to enhance the efficacy of infused haploidentical NK cells involve the use of novel cell sources, improved expansion techniques, elimination of immune suppressor cells (e.g., regulatory T cells and myeloid-derived suppressor cells), and the blockade of inhibitory checkpoint molecules.

Expansion of Natural Killer Cells In Vivo via Cytokine Administration

NK cells have been successfully expanded in vivo in patients with cancer through the exogenous administration of recombinant human cytokines. IL-2 was the first cytokine to be employed as a means of driving NK cell proliferation in humans. Caligiuri and colleagues discovered that the CD56^{bright} subset of NK cells expressed a high-affinity IL-2R that was comprised of the IL-2R beta and gamma chains in combination with the alpha chain (IL-2Rα). This heterotrimeric receptor is capable of binding picomolar doses of IL-2 and mediates a very strong proliferative signal. Prolonged infusion of rIL-2 at doses of 1.5×10^5 and 4.5×10^5 U/m^2/day was well-tolerated in cancer patients and resulted in a 6- to 19-fold expansion of NK cells. Similar effects were seen in post-remission AML patients and individuals with HIV-associated malignancies.[177,178] Subsequent analyses indicated that IL-2 administration could induce the unwanted expansion of regulatory T cells.[179] The first in-human trial of intravenous rhIL-15 was associated with a greater than 10-fold expansion of NK cells with virtually no change in the regulatory T-cell population.[180] A novel superagonist form of IL-15 (ALT-803) is now in clinical development that consists of an IL-15/IL-15Rα complex linked to IgG1 Fc, in which IL-15 is additionally mutated (asn72asp) to further increase biological activity.[181] Phase I studies have demonstrated that this agent is well tolerated and induced the expansion of NK cells. Combination studies with other immune-activating agents (e.g., anti-PD-1 mAb) are now being conducted.[182,183] NK cell expression of receptors for stimulatory cytokines such as IL-12, IL-18, and IL-21 provide a rationale for clinical studies that administer these factors to patients with advanced malignancies, yet consistent single-agent anti-tumor activity has not been achieved. IL-12 (originally known as NK cell stimulatory factor) significantly enhances NK cell anti-tumor activity in pre-clinical models but was associated with IFN-γ-induced toxicity that has limited its clinical utility. Several innovative alternate routes of IL-12 administration are under investigation.[184]

Killer-Cell Immunoglobulin-Like Receptors Blockade

The interaction between inhibitory NK receptors and MHC class I receptors can be interrupted by means of monoclonal antibodies. Murine models lend support to the notion that tumor MHC class I molecules can engage inhibitory NK cell receptors to induce NK tolerance.[23] When antibody fragments were introduced to disrupt this ligand–receptor interaction, increased NK cytotoxicity and decreased tumor growth were observed. Thus blockade of inhibitory NK cell receptors represents a novel form of cancer immunotherapy.[185,186] Phase I clinical trials of an anti-KIR antibody that blocks KIR2DL1/2/3 (lirilumab) have shown that greater than 90% KIR occupancy could be achieved.[187,188] The combination of lirilumab with lenalidomide in patients with multiple myeloma was well-tolerated as was the combination of lirilumab with the anti-PD-1 mAb nivolumab; however, the observed clinical activity did not support further investigation in these settings.[189,190] It has been hypothesized that KIR blockade might prevent full maturation of the NK cell anti-tumor response and thus additional combinatorial strategies are being explored.

Natural Killer Cell Checkpoint Blockade

NK cells either constitutively or transiently express several other inhibitory molecules that represent negative regulators of immune activity or so-called immune checkpoints. These checkpoint molecules include PD-1, CTLA-4, TIM-3, LAG-3, IL-1R8, and TIGIT.[191] There is evidence that inhibition of these pathways with neutralizing antibodies could augment NK cell anti-tumor activity. A subset of CD56^{dim} NK cells expresses PD-1 and this correlates with reduced anti-tumor function and poor outcome in some cancer patients.[192–194] TIM-3 is a type I transmembrane protein belonging to the Ig superfamily that recognizes multiple ligands including phosphatidylserine and galectin-9. High-level TIM 3 expression correlates with NK cell exhaustion, which can be reversed by TIM-3 blockade.[195,196] LAG-3 is an NK-expressed inhibitory receptor that belongs to the Ig superfamily and recognizes several tumor-expressed proteins.[197] IL-1R8 is a member of the interleukin-1 receptor (IL1R) family and acts as a negative regulator of activation signals mediated by IL-1Rs and toll-like receptors (TLRs). IL-1R8 is able to down-regulate NK cell maturation and anti-tumor/anti-viral function.[198] TIGIT competes with DNAM for binding to ligands of the nectin family and when ligated mediates an inhibitory signal that leads to reduced NK cell cytotoxicity and IFN-γ production. Blockade of TIGIT in combination with IL-15 in a murine model of melanoma led to NK cell-mediated destruction of MHC class I-deficient tumors that were refractory to CD8+ T cell-mediated immunity.[199] These studies highlight the ability of inhibitory pathways to modulate NK cell activity.

Natural Killer Cell Antibody-Dependent Cytotoxicity

The ability of NK cells to recognize antibody-coated target cells and conduct ADCC and produce immune activating cytokines represents an anti-tumor mechanism that can be manipulated for therapeutic benefit. While antigen-specific mAbs are able to exert a direct antitumor effect, there is evidence that FcR-bearing immune effector cells (e.g., FcγRIIIa+ CD56^{dim} NK cells) may also be involved in the elimination of tumor cells.[200] Clynes et al. reported that the anti-tumor effects of trastuzumab in a murine model of breast cancer required the expression of functional FcγR by host immune effector cells.[201] And patients with single nucleotide polymorphisms that confer enhanced FcR binding to IgG may have improved outcomes to some mAb therapies.[202,203] Although granulocytes and monocytes co-express both activating and inhibitory FcγR, NK cells primarily express only the activating FcγRIIIa receptor.

Multiple strategies have been employed to enhance the NK cell response to antibody-coated tumor targets. The N-terminal glycosylation pattern of human IgG at Asn297 in the Cγ2 domain markedly impacts antibody affinity for the Fc receptor. Removal of the fucose group from the pentasaccharide core creates a non-fucosylated antibody with greater affinity for FcγRIIIa and an enhanced ability to mediate ADCC. Obinutuzumab is a non-fucosylated antibody with superior ADCC induction capabilities that recognizes CD20 and has clinical efficacy.[204] Cytokine stimulation of NK cells has been found to enhance NK cell ADCC and cytokine production.[205] Notably, in a murine model of HER2-expresssing cancer, co-administration of IL-12 with an anti-HER2 mAb generated synergistic anti-tumor activity that was dependent on NK cell production of IFN-γ.[206] In a phase I study of IL-12 in combination with the anti-HER2 mAb trastuzumab, sustained NK cell production of IFN-γ was observed only in patients who experienced clinical benefit.[207] In a follow-up study of IL-12 in combination with trastuzumab and paclitaxel, 50% of patients experienced tumor shrinkage or prolonged disease stabilization along with high levels of IFN-γ. NK cells were shown to be the source of cytokine production, and co-localization of the FcR and IL-12R was found to be responsible for synergistic NK cell activation.[208,209] IL-2 has also been used in combination with both trastuzumab and rituximab in order to expand NK cell numbers; however, Treg induction inhibited NK cell functioning.[210,211] Pre-clinical models support the use of other NK cell activating agents in combination with mAbs such as IL-15, IL-21, and TLR agonists.[212–214] Dual stimulation of NK cells via the FcγRIIIa and a cytokine receptor (e.g., IL-12R) results in the synergistic release of IFN-γ, TNF-γ, and GM-CSF, and T-cell chemokines including IL-8, MIP-1γ, MIP-1β, MDC, MCP-1, and RANTES[208]

Protein engineering techniques have permitted the development of novel reagents with multiple binding moieties that promote the engagement of NK cells with tumor targets and the generation of an immune synapse that stimulates an NK cell cytotoxic response. One group has created a novel bivalent protein that recognizes NKG2D on NK cells and CD138, a protein overexpressed by malignancies. This construct strongly activated NK cells and inhibited tumor growth in mice.[215] NK cell cytotoxicity can be triggered using bispecific molecules that simultaneously bind to CD16 on NK cells and a tumor antigen. Bispecific killer engagers (so-called BiKEs) are comprised of two single-chain variable fragments (scFv) which are fusion proteins of the variable regions of the heavy and light chains of an antibody (VH and VL) connected with a short linker peptide. In a BiKE, one scFv is specific for CD16 and the other for the tumor antigen of interest, such as CD19.[216] Tri-specific molecules that incorporate an activating cytokine are also being tested.[217]

Genetic Engineering of Natural Killer Cells

NK cells can be genetically modified to express new proteins that augment their ability to eliminate target cells and then infused for therapeutic purposes. The use of HLA-mismatched NK cells provides for an expanded menu of cell types that can serve as starting material. Strategies that have been employed include the expression of (1) modified activating receptors, (2) machinery to downregulate inhibitory receptors, (3) soluble or membrane-bound cytokine, and (4) chimeric activating receptors (CARs). Genetically modified NK cells may then be infused into cancer patients following lymphodepleting chemotherapy.

Based on the clinical success of CAR-T-cell therapy for CD19-expressing lymphomas[218] there has been considerable interest in generating analogous NK cell products or so-called CAR-NK. This approach has several theoretical advantages over CAR-T-cell therapies. While CAR-T-cell therapy requires genetic manipulation of autologous or HLA-matched T cells, CAR-NK may be generated from multiple different cell sources, and as is the case with HCT, HLA mismatches can be beneficial to the NK cell therapeutic effect. Reduced expression of MHC class I molecules or target antigen severely limits the cytotoxic capacity of infused CAR-T cells and represents an important mechanism of resistance to this form of cell therapy. In contrast, NK cells possess an innate ability to detect and eliminate such target cells due to the ability of NK cells to detect activating/stress ligands and missing self. Although NK cells have the ability to conduct serial killing, infused NK cells have a limited lifespan and have not been known to cause significant side effects or GVHD. On the other hand, CAR-T-cell therapy can result in serious and sometimes fatal toxicities including cytokine release syndrome and neurotoxicity in the form of immune effector cell-associated neurologic syndrome (ICANS). It is not always possible to obtain sufficient numbers of high-quality T cells from chemotherapy-refractory patients. Also, the manufacturing process can take 2 to 4 weeks and patients must have therapy held for at least 2 weeks prior to apheresis. Thus, the timely administration of CAR-T cells can be problematic in patients who have proven refractory to standard therapies. CAR-T-cell therapy requires post-treatment hospitalization for several weeks. In contrast, it is possible to envision "off-the-shelf" CAR-NK cell products being administered in the outpatient setting as a bridging therapy to patients experiencing recurrent disease.[218,219]

Advances made in the field of CAR-T-cell therapy have been applied to the development of CAR-NK cells.[220] The CAR structure usually consists of an extracellular scFv derived from an antibody against a tumor antigen, a hinge region connecting this antigen-recognition sequence to a transmembrane domain (TM), and one or more intracellular signaling domains. The scFv consists of a heavy and a light variable fragment (VH, VL) joined by a flexible linker. The binding affinities and order of the variable fragments as well as the length of the linker can impact CAR affinity for an antigen. The hinge sequence is typically based on known proteins with similar structure (CD8alpha, CD28, CH2/3 domains), and its length can be adjusted to optimize antigen binding. The TM domain is a hydrophobic region that crosses the cell membrane and serves to anchor the CAR. The choice of TM is important since some sequences can promote activation-induced cell death or alter CAR cell surface distribution. First-generation CAR-T constructs contained only a CD3zeta activating domain. However, subsequent generations included one or more additional co-stimulatory domains derived from either the TNF receptor family (4-1BB, CD27, OX40) or the CD28 family. CAR-NK constructs may contain NK-specific activating domains (e.g., DAP10 or DAP12) in place of or alongside CD3zeta. Co-stimulatory domains derived from 2B4 and CD137 have also been evaluated.[220]

As with NK cell infusions for leukemia, there are multiple cell types that can be used for the generation of CAR-NK cells.[163] Peripheral blood CD56+ NK cells can be obtained through apheresis, depleted of T and B cells, and expanded in a stimulatory environment. These NK cells possess a mature phenotype and effectively eliminate tumor cells but exhibit low proliferation. Lymphocytes derived from umbilical cord blood have a high proportion of NK cells of an immature phenotype that exhibit reduced expression of CD16, perforin, and granzyme B. However, their proliferative capacity is much greater than that of NK cells derived from peripheral blood.[221] iPST-derived CAR-NK cells exhibit a high proliferative capacity but have low levels of CD16 and KIR and high levels of inhibitory NKG2A. These deficits can be rectified via insertion of missing cytotoxic components. Also, the NK-92 cell line can be genetically modified to express a CAR molecule and then infused following irradiation. Low expression of KIR and CD16 by this cell line is a limiting factor.

NK cells are notoriously resistant to viral transduction with retrovirus- or lentivirus-based constructs. The use of modified viral vectors, cationic transfection reagents, and cytokine activation can help improve the transduction efficiency. Electroporation can result in a high rate of gene uptake; however, stable integration of the construct is not possible and consequently there is transient expression of the CAR construct. The use of transposon technology and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 systems in conjunction with electroporation or viral approaches can improve the efficiency of gene uptake and dictate the site of integration.[220]

Expansion of the NK cell product pre-infusion can be achieved with various combinations of cytokines, and these can be chosen with the goal of directing the NK cell population toward a particular phenotype, such as the use of IL-12, IL-15, and IL-18 to induce a memory NK cell.[222,223] However, if high concentrations of these factors are used there is the potential for cytokine addiction and reduced in vivo efficacy. Genetically engineered feeder cells represent an alternate method for driving the expansion of NK cells. NK cells have a limited ability to proliferate, and unlike T cells, infused NK cells cannot be detected in a patient's circulation past 1 to 2 weeks post-transfusion. For this reason, the systemic administration of IL-2 or IL-15 has been considered as a means of lengthening the time frame of CAR-NK cell activity. The delivery of CAR-NK constructs in combination with anti-tumor mAbs, checkpoint inhibitors, and targeted drugs to eliminate immune suppressor cells is being investigated.

FUTURE DIRECTIONS

Rapid secretion of powerful immunomodulatory cytokines and chemokines supports the role of NK cells as "first responders" to immune insults and foreign invaders, facilitating mobilization and tailoring of the innate and adaptive immune responses. Potent natural cytotoxicity, unrestricted by the need for classical antigen presentation and costimulation required for adaptive immune cells suggest that NK cells have an important, complementary role to that of cytotoxic T lymphocytes. Further understanding of NK cell biology will certainly shed more light on the therapeutic potential for NK cells in the treatment of human disease.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Colonna M, Samaridis J. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science*. 1995;268:405–408.

Freud AH, Becknell B, Roychowdhury S, et al. A human CD34+ subset resides in lymph nodes and differentiates into CD56bright NK cells. *Immunity*. 2005;22:295–304.

Gang M, Marin ND, Wong P, et al. CAR-modified memory-like NK cells exhibit potent responses to NK-resistant lymphomas. *Blood*. 2020;136:2308–2318.

Liu E, Marin D, Banerjee P, Macapinlac HA, et al. Use of CAR-transduced natural killer cells in CD19-positive lymphoid tumors. *N Engl J Med*. 2020;382:545–553.

Miller JS, Soignier Y, Panoskaltsis-Mortari A, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood*. 2005;105:3051.

Parihar R, Dierksheide J, Hu Y, et al. IL-12 enhances the natural killer cell cytokine response to Ab-coated tumor cells. *J Clin Invest*. 2002;110:983–992.

Rajalingam R. Diversity of killer cell immunoglobulin-like receptors and disease. *Clin Lab Med*. 2018;38:637–653.

Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. 2002;295:2097–2100.

Shimasaki N, Jain A, Campana D. NK cells for cancer immunotherapy. *Nat Rev Drug Discov*. 2020;19:200–218.

Sun JC, Beilke JN, Lanier LL. Adaptive immune features of natural killer cells. *Nature*. 2009;457:557–561.

Yu J, Freud AG, Caligiuri MA. Location and cellular stages of natural killer cell development. *Trends Immunol*. 2013;34:573–582.

REFERENCES

1. Caligiuri MA. Human natural killer cells. *Blood*. 2008;112:461–469.
2. Moretta L, Montaldo E, Vacca P, et al. Human natural killer cells: origin, receptors, function, and clinical applications. *Int Arch Allergy Immunol*. 2014;164:253–264.
3. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer cell subsets. *Trends Immunol*. 2001;22:633–640.
4. Herberman RB, Holden HT. Natural cell-mediated immunity. *Adv Cancer Res*. 1978;27:305–377.
5. Abel AM, Yang C, Thakar MS, et al. Natural killer cells: development, maturation, and clinical utilization. *Front Immunol*. 2018;9:1869.
6. Biron CA, Byron KS, Sullivan JL. Severe herpesvirus infection in an adolescent without natural killer cells. *N Engl J Med*. 1989;320:1731–1735.
7. Kim S, IIzuka K, Aguila HL, et al. In vivo natural killer cell activities revealed by natural killer cell deficient mice. *Proc Natl Acad Sci USA*. 2000;97:2731–2736.
8. Smyth MJ, Thia KY, Street SE, et al. Differential tumor cell surveillance by natural killer (NK) and NKT cells. *J Exp Med*. 2000;191:661–668.
9. Imai K, Matsuyama S, Miyake S, et al. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet*. 2000;356:1795–1979.
10. Lanier LL, Le AM, Civin CI, et al. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J Immunol*. 1986;136:4480–4486.
11. Lanier LL, Testi R, Bindl J, et al. Identity of Leu-19 (CD56) leukocyte differentiation and neural cell adhesion molecule. *J Exp Med*. 1989;169:2233–2238.
12. Poli A, Michel T, Thérésine M, et al. CD56bright natural killer (NK) cells: an important NK cell subset. *Imunology*. 2009;126:458–465.
13. Cooper MA, Fehniger TA, Turner SC, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood*. 2001;97:3146–3151.
14. Siegel JP, Sharon M, Smith PL, et al. The IL-2 receptor beta chain (p70): role in mediating signals for LAK, NK, and proliferative activities. *Science*. 1987;238:75–78.
15. Carson WE, Giri JG, Lindemann MJ, et al. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J Exp Med*. 1994;180:1395–1403.
16. Prager I, Liesche C. van Ooijen Het al. NK cells switch from granzyme B to death receptor-mediated cytotoxicity during serial killing. *J Exp Med*. 2019;216:2113–2127.
17. Belizário JE, Neyra JM, Setúbal Destro Rodrigues MF. When and how NK cell-induced programmed cell death benefits immunological protection against intracellular pathogen infection. *Innate Immun*. 2018;24:452–465.
18. Grier JT, Forbes LR, Monaco-Shawver L, et al. Human immunodeficiency-causing mutation defines CD16 in spontaneous NK cell cytotoxicity. *J Clin Invest*. 2012;122:3769–3780.
19. Nagler A, Lanier LL, Cwirla S, et al. Comparative studies of human FcRIII-positive and negative natural killer cells. *J Immunol*. 1989;143:3183–3191.
20. Wendt K, Wilk E, Buyny S, et al. Gene and protein characteristics reflect functional diversity of CD56dim and CD56bright NK cells. *J Leukoc Biol*. 2006;80:1529–1541.
21. Fehniger TA, Cooper MA, Nuovo GJ, et al. CD56bright natural killer cells are present in human lymph nodes and are activated by T cell derived IL-2: a potential new link between adaptive and innate immunity. *Blood*. 2003;101:3052–3057.
22. Freud AH, Becknell B, Roychowdhury S, et al. A human CD34+ subset resides in lymph nodes and differentiates into CD56bright NK cells. *Immunity*. 2005;22:295–304.
23. Vitale M, Della Chiesa M, Carlomagno S, et al. The small subset of CD56brightCD16- natural killer cells is selectively responsible for both cell proliferation and interferon-gamma production upon interaction with dendritic cells. *Eur J Immunol*. 2004;34:1715–1722.
24. Freud AG, Keller KA, Scoville SD, et al. NKp80 Defines a critical step during human natural killer cell development. *Cell Rep*. 2016;16:379–391.
25. Bernardini G, Antonangeli F, Bonanni V, et al. Dysregulation of chemokine/chemokine receptor axes and NK cell tissue localization during diseases. *Front Immunol*. 2016;7:402.
26. Betakova T, Kostrabova A, Lachova V, et al. Cytokines induced during influenza virus infection. *Curr Pharm Des*. 2017;23:2616–2622.
27. Korbecki J, Simińska D, Kojder K, et al. Fractalkine/CX3CL1 in neoplastic processes. *Int J Mol Sci*. 2020;21:3723.
28. Ponzetta A, Benigni G, Antonangeli F, et al. Multiple myeloma impairs bone marrow localization of effector natural killer cells by altering the chemokine microenvironment. *Cancer Res*. 2015;75:4766–4777.
29. Trotta R, Parihar R, Yu J, et al. Differential expression of SHIP1 in CD56bright and CD56dim natural killer cells provides a molecular basis for distinct functional responses to monokine stimulation. *Blood*. 2005;105:3011–3018.
30. Becknell B, Hughes TL, Freud AG, et al. The Hlx homeobox transcription factor negatively regulates interferon-γ production in monokine-activated natural killer cells. *Blood*. 2007;109:2481–2487.
31. Trotta R, Ciarlariello D, Dal Col J, et al. The PP2A inhibitor SET regulates natural killer cell IFN-gamma production. *J Exp Med*. 2007;204:2397–2405.
32. Yang C, Siebert JR, Burns R, et al. Heterogeneity of human bone marrow and blood natural killer cells defined by single-cell transcriptome. *Nat Commun*. 10:3931.
33. Yu J, Freud AG, Caligiuri MA. Location and cellular stages of natural killer cell development. *Trends Immunol*. 2013;34:573–582.
34. Spits H, Artis D, Colonna M, et al. Innate lymphoid cells—a proposal for uniform nomenclature. *Nat Rev Immunol*. 2013;13:145–149.
35. Carson WE, Fehniger TA, Haldar S, et al. A potential role for interleukin-15 in the regulation of human natural killer cell survival. *J Clin Invest*. 1997;99:937–943.
36. Vosshenrich CA, Ranson T, Samson SI, et al. Roles for common cytokine receptor γ-chain-dependent cytokines in the generation, differentiation and maturation of human NK cell precursors and peripheral NK cells in vivo. *J Immunol*. 2005;174:1213–1221.
37. Kawamura T, Koka R, Ma A, et al. Differential roles for IL-15Ra-chain in NK cell development and Ly-49 induction. *J Immunol*. 2003;171:5085–5090.
38. Bonanni V, Sciumè G, Santoni A, et al. Bone marrow NK cells: origin, distinctive features, and requirements for tissue localization. *Front Immunol*. 2019;10:1569.
39. Wang Y, Zhang Y, Yi P, et al. The IL-15-AKT-XBP1s signaling pathway contributes to effector functions and survival in human NK cells. *Nat Immunol*. 2019;20:10–17.
40. Freud AG, Yokohama A, Becknell B, et al. Evidence for discrete stages of human natural killer cell differentiation in vivo. *J Exp Med*. 2006;203:1033–1043.
41. Freud AG, Yu J, Caligiuri MA. Human natural killer cell development in secondary lymphoid tissues. *Semin Immunol*. 2014;26:132–137.
42. Yu J, Mao HC, Wei M, et al. CD94 surface density identifies a functional intermediary between the CD56bright and CD56dim human NK-cell subsets. *Blood*. 2010;15:274.
43. Chen L, Youssef Y, Robinson C, et al. CD56 expression marks human group 2 innate lymphoid cell divergence from a shared NK cell and group 3 innate lymphoid cell developmental pathway. *Immunity*. 2018;49:464–476.
44. Freud AG, Mundy-Bosse BL, Yu J, et al. The broad spectrum of human natural killer cell diversity. *Immunity*. 2017;47:820–833.
45. Lopez-Verges S, Milush J, Pandey S, et al. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. *Blood*. 2010;116:3865–3874.
46. Karre K, Ljunggren HG, Piontek G, et al. Selective rejection of H-2 deficient lymphoma variants suggests alternative immune defense strategy. *Nature*. 1986;319:675–678.
47. Market M, Angka L, Martel AB, et al. Flattening the COVID-19 curve with natural killer cell based immunotherapies. *Front Immunol*. 2020;11:1512.
48. Sivori S, Vacca P, Del Zotto G, et al. Human NK cells: surface receptors, inhibitory checkpoints, and translational applications. *Cell Mol Immunol*. 2019;16:430–441.
49. Dohring C, Scheidegger D, Samaridis J, et al. A human killer inhibitor receptor specific for HLA-A1, 2. *J Immunol*. 1996;156:3098–3101.
50. Litwin V, Gumperz J, Parham P, et al. NKB1: A natural killer cell receptor involved in the recognition of polymorphic HLA-B molecules. *J Exp Med*. 1994;180:537–543.
51. Wagtmann N, Rajagopalan S, Winter CC, et al. Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. *Immunity*. 1995;3:801–809.
52. Colonna M, Samaridis J. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science*. 1995;268:405–408.
53. Rajalingam R. Diversity of killer cell immunoglobulin-like receptors and disease. *Clin Lab Med*. 2018;38:637–653.
54. Kärre K, Ljunggren HG, Piontek G, et al. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature*. 1986;319:675–678.
55. Borrego F. The first molecular basis of the "missing self" hypothesis. *J Immunol*. 2006;177:5759–5760.

56. Moesta AK, Norman PJ, Yawata M, et al. Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. *J Immunol*. 2008;180:3969–3979.
57. Manser AR, Weinhold S, Uhrberg M. KIR repertoires: shaped by genetic diversity and evolution. *Immunol Rev*. 2015;267:178–196.
58. Vales-Gomez M, Reyburn HT, et al. Kinetics of interaction of HLA-C ligands with natural killer cell inhibitor receptors. *Immunity*. 1998;9:337–344.
59. Parham P, Guethlein LA. Genetics of natural killer cells in human health, disease, and survival. *Ann Rev Immunol*. 2018;36:519–548.
60. Flores-Villanueva PO, Yunis EJ, Delgado JC, et al. Control of HIV-1 viremia and protection from AIDS are associated with HLA-Bw4 homozygosity. *Proc Natl Acad Sci USA*. 2001;98:5140–5145.
61. Martin MP, Gao X, Lee JH, et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet*. 2002;31:429–434.
62. Khakoo SI, Thio CL, Martin MP, et al. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science*. 2004;305:872–874.
63. Ashkar AA, DiSanto JP, Croy BA. Interferon γ contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. *J Exp Med*. 2000;192:259–270.
64. Díaz-Peña R, de Los Santos MJ, Lucia A, et al. Understanding the role of killer cell immunoglobulin-like receptors in pregnancy complications. *J Assist Reprod Genet*. 2019;36:827–835.
65. Borrego F, Masilamani M, Marusina AT. The CD94/NKG2 family of receptors: from molecules and cells to clinical relevance. *Immunol Res*. 2006;35:263–278.
66. Moser JM, Gibbs J, Jensen PE, et al. CD94-NKG2A receptors regulate antiviral CD8+ T cell responses. *Nat Immunol*. 2002;3:189–195.
67. Borrego F, Ulbrecht M, Weiss EH, et al. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell mediated lysis. *J Exp Med*. 1998;187:813–818.
68. Kamiya T, Seow SV, Wong D, et al. Blocking expression of inhibitory receptor NKG2A overcomes tumor resistance to NK cells. *J Clin Invest*. 2019;129:2094–2106.
69. Llano M, Lee N, Navarro F, et al. HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/NKG2 receptors: preferential response to an HLA-G-derived nonamer. *Eur J Immunol*. 1998;28:2854–2863.
70. Lanier LL. NKG2D receptor and its ligands in host defense. *Cancer Immunol Res*. 2015;3:575–582.
71. Raulet DH, Gasser S, Gowen BG, et al. Regulation of ligands for the NKG2D activating receptor. *Annu Rev Immunol*. 2013;31:413–441.
72. Bauer S, Groh V, Wu J, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress inducible MICA. *Science*. 1999;285:727–729.
73. Groh V, Rhinehart R, Secrist H, et al. Broad tumor-associated expression and recognition by tumor-derived γδ T cells of MICA and MICB. *Proc Natl Acad Sci USA*. 1999;96:6879–6884.
74. Kaye J, Browne H, Stoffel M, et al. The UL16 gene of human cytomegalovirus encodes a glycoprotein that is dispensable for growth in vitro. *J Virol*. 1992;66:6609–6615.
75. Groh V, Bahram S, Bauer S, et al. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. *Proc Natl Acad Sci USA*. 1996;93 12445–12450.
76. Pignoloni B, Fionda C, Dell'Oste V. Distinct roles for human cytomegalovirus immediate early proteins IE1 and IE2 in the transcriptional regulation of MICA and PVR/CD155 expression. *J Immunol*. 2016;197:4066–4078.
77. Zhao Y, Chen N, Yu Y, et al. Prognostic value of MICA/B in cancers: a systematic review and meta-analysis. *Oncotarget*. 2017;8:96384–96395.
78. Mastaglio S, Wong E, Perera T, et al. Natural killer receptor ligand expression on acute myeloid leukemia impacts survival and relapse after chemotherapy. *Blood Adv*. 2018;2:335–346.
79. Dunn C, Chalupny NJ, Sutherland CL, et al. Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity. *J Exp Med*. 2003;197:1427–1439.
80. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature*. 2002;419:734–738.
81. Waldhauer I, Steinle A. Proteolytic release of soluble UL16-binding protein 2 from tumor cells. *Cancer Res*. 2006;66:2520–2526.
82. Cosman D, Mullberg J, Sutherland CL, et al. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL-16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity*. 2001;14: 123–133.
83. Sutherland CL, Rabinovich B, Chalupny NJ, et al. ULBPs, human ligands of NKG2D receptor, stimulate tumor immunity with enhancement by IL-15. *Blood*. 2006;108:1313–1319.
84. Campbell AR, Duggan MC, Suarez-Kelly LP, et al. MICA-expressing monocytes enhance natural killer cell Fc receptor-mediated antitumor functions. *Cancer Immunol Res*. 2017;5:778–789.
85. Pende D, Parolini S, Pessino A, et al. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human killer cells. *J Exp Med*. 1999;190:1505–1516.
86. Vitale M, Bottino C, Sivori S, et al. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex restricted tumor cell lysis. *J Exp Med*. 1998;187:2065–2072.
87. Sivori S, Vitale M, Morelli L, et al. p46, a novel natural killer cell-specific surface molecule that mediates cell activation. *J Exp Med*. 1997;186:1129.
88. Barrow AD, Martin CJ, Colonna M. The natural cytotoxicity receptors in health and disease. *Front Immunol*. 2019;10:909.
89. Brandt CS, Baratin M, Yu EC, et al. The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. *J Exp Med*. 2009;206:1495–1503.
90. Schlecker E, Fiegler N, Arnold A, et al. Metalloprotease-mediated tumor cell shedding of B7-H6, the ligand of the natural killer cell-activating receptor NKp30. *Cancer Res*. 2014;74:3429–3440.
91. Hecht ML, Rosental B, Horlacher T, et al. Natural cytotoxicity receptors NKp30, NKp44 and NKp46 bind to different heparan sulfate/heparin sequences. *J Proteome Res*. 2009;8:712–720.
92. Horton NC, Mathew SO, Mathew PA. Novel interaction between proliferating cell nuclear antigen and HLA I on the surface of tumor cells inhibits NK cell function through NKp44. *PLoS One*. 2013;8:e59552.
93. Mandelboim O, Lieberman N, Lev M, et al. Recognition of hemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature*. 2001;409:1055–1060.
94. Bar-On Y, Seidel E, Tsukerman P, et al. Influenza virus uses its neuraminidase protein to evade the recognition of two activating NK cell receptors. *J Infect Dis*. 2014;210:410–418.
95. Cifaldi L, Doria M, Cotugno N, et al. DNAM-1 activating receptor and its ligands: how do viruses affect the NK cell-mediated immune surveillance during the various phases of infection? *Int J Mol Sci*. 2019;20:3715.
96. Okumura G, Iguchi-Manaka A, Murata R, et al. Tumor-derived soluble CD155 inhibits DNAM-1-mediated antitumor activity of natural killer cells. *J Exp Med*. 2020;217:1.
97. Lee KM, Forman JP, McNerney ME, et al. Requirement of homotypic NK-cell interactions through 2B4(CD244)/CD48 in the generation of NK effector functions. *Blood* 107:3181–3188.
98. Agresta L, Hoebe KHN, Janssen EM. The emerging role of CD244 signaling in immune cells of the tumor microenvironment. *Front Immunol*. 2018;9:2809.
99. Martínez-Vicente P, Farré D, Sánchez C, et al. Subversion of natural killer cell responses by a cytomegalovirus-encoded soluble CD48 decoy receptor. *PLoS Pathog*. 2019;15:e1007658.
100. Stary V, Stary G. NK cell-mediated recall responses: memory-like, adaptive, or antigen-specific? *Front Cell Infect Microbiol*. 2020;10:208.
101. Jonsson AH, Yokoyama WM. Natural killer cell tolerance licensing and other mechanisms. *Adv Immunol*. 2009;101:27–79.
102. Boudreau JE, Hsu KC. Natural killer cell education and the response to infection and cancer therapy: stay tuned. *Trends Immunol*. 2018;39:222–239.
103. Kim S, Poursine-Laurent J, Truscott SM, et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature*. 2005;436:709–713.
104. Elliott JM, Wahle JA, Yokoyama WM. MHC class I deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I-sufficient environment. *J Exp Med*. 2010;207:2073–2079.
105. Joncker NT, Shifrin N, Delebecque F, et al. Mature natural killer cells reset their responsiveness when exposed to an altered MHC environment. *J Exp Med*. 2010;207:2065–2072.
106. Coudert JD, Scarpellino L, Gros F, et al. Sustained NKG2D engagement induces cross-tolerance of multiple distinct NK cell activation pathways. *Blood*. 2008;11:3571–3578.
107. Pittari G, Liu XR, Selvakumar A, et al. NK cell tolerance of self-specific activating receptor KIR2DS1 in individuals with cognate HLA-C2 ligand. *Immunol*. 2013;190:4650–4660.
108. Sun JC, Lanier LL. Tolerance of NK cells encountering their viral ligand during development. *J Exp Med*. 2008;205:1819–1828.
109. Paust S, Gill HS, Wang BZ, et al. Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. *Nat Immunol*. 2010;11:1127–1135.

110. Gillard GO, Bivas-Benita M, Hovav AH, et al. Thy1+ NK [corrected] cells from vaccinia virus-primed mice confer protection against vaccinia virus challenge in the absence of adaptive lymphocytes. *PLoS Pathog*. 2011;7:e1002141.
111. Nikzad R, Angelo LS, Aviles-Padilla K, et al. Human natural killer cells mediate adaptive immunity to viral antigens. *Sci Immunol*. 2019;4:eaat8116.
112. Wijaya RS, Read SA, Truong NR, et al. HBV vaccination and HBV infection induces HBV-specific natural killer cell memory. *Gut*. 2020;30 gutjnl-2019-319252.
113. Reeves RK, Li H, Jost S, et al. Antigen-specific NK cell memory in rhesus macaques. *Nat Immunol*. 2015;16:927–932.
114. Sun JC, Beilke JN, Lanier LL. Adaptive immune features of natural killer cells. *Nature*. 2009;457:557–561.
115. Min-Oo G, Bezman NA, Madera S, et al. Proapoptotic Bim regulates antigen-specific NK cell contraction and the generation of the memory NK cell pool after cytomegalovirus infection. *J Exp Med*. 2014;211:1289–1296.
116. Gumá M, Budt M, Sáez A, et al. Expansion of CD94/NKG2C+ NK cells in response to human cytomegalovirus-infected fibroblasts. *Blood*. 2006;107:3624–3631.
117. Foley B, Cooley S, Verneris MR, et al. Human cytomegalovirus (CMV)-induced memory-like NKG2C(+) NK cells are transplantable and expand in vivo in response to recipient CMV antigen. *J Immunol*. 2012;189:5082–5088.
118. Cooper MA, Elliott JM, Keyel PA, et al. Cytokine-induced memory-like natural killer cells. *Proc Natl Acad Sci USA*. 2009;106:1915–1919.
119. Romee R, Schneider SE, Leong JW, et al. Cytokine activation induces human memory-like NK cells. *Blood*. 2012;120:4751–4760.
120. Gang M, Marin ND, Wong P, et al. CAR-modified memory-like NK cells exhibit potent responses to NK-resistant lymphomas. *Blood*. 2020;136:2308–2318.
121. Eidenschenk C, Dunne J, Jouanguy E, et al. A novel primary immunodeficiency with specific natural killer cell deficiency maps to the centromeric region of chromosome 8. *Am J Hum Genet*. 2006;78:721–727.
122. Wendlend T, Herren S, Yawalkar A, et al. Strong αβ and γδ TCR response in a patient with disseminated *Mycobacterium avium* infection and lack of NK cells and monocytopenia. *Immunol Lett*. 2000;72:75–82.
123. Ballas Z, Turner JM, Turner DA, et al. A patient with simultaneous absence of "classical" natural killer cells (CD3−, CD16+ and NKH1+) and expansion of CD3+, CD4−, CD8−, NKH1+ subset. *J Allergy Clin Immunol*. 1990;85:453–459.
124. Jawahar S, Moody C, Chan M, et al. Natural killer (NK) cell deficiency associated with an epitope-deficient Fc receptor type IIIa (CD16-II). *Clin Exp Immunol*. 1996;103:408–413.
125. De Vries E, Koene HR, Vossen JM, et al. Identification of an unusual Fc gamma receptor IIIa (CD16) on natural killer cells in a patient with recurrent infections. *Blood*. 1996;88:3022–3027.
126. Zhang B, Yamamura T, Kondo T, et al. Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells. *J Exp Med*. 1997;186:1677–1687.
127. Laroni A, Ucelli A. CD56bright natural killer cells: a possible biomarker of different treatments in multiple sclerosis. *J Clin Med*. 2020;9:1450.
128. Fort MM, Leach MW, Rennick DM. A role for NK cells as regulators of CD4+ T cells in a transfer model of colitis. *J Immunol*. 1998;161:3256–3261.
129. Kakimoto K, Inoue T, Nishikawa T, et al. Primary CD56+ NK/T-cell lymphoma of the rectum accompanied with refractory ulcerative colitis. *Gastroenterol*. 2008;43:576–580.
130. Flodstrom M, Maday A, Balakrishna D, et al. Target cell defense prevents the development of diabetes after viral infection. *Nat Immunol*. 2002;3:373–382.
131. Gur C, Enk J, Weitman E, et al. The expression of the β-cell derived autoimmune ligand for the killer receptor NKp46 is attenuated in type 2 diabetes. *PLoS One*. 2013;8:e74033.
132. Gur C, Porgador A, Elboim M, et al. The activating receptor NKp46 is essential for the development of type 1 diabetes. *Nat Immunol*. 2010;11:121–128.
133. Shi FD, Wang HB, Li H, et al. Natural killer cells determine the outcome of B cell-mediated autoimmunity. *Nat Immunol*. 2000;1:245–251.
134. Groh V, Bruhl A, El-Gabalawy H, et al. Stimulation of T cell autoreactivity by anomalous expression of NKG2D and its MIC ligands in rheumatoid arthritis. *Proc Natl Acad Sci USA*. 2003;100:9452–9457.
135. Pridegon C, Lennon GP, Pazmany L, et al. Natural killer cells in the synovial fluid of rheumatoid arthritis patients exhibit a CD56bright, CD94bright, CD158negative phenotype. *Rheumatology (Oxford)*. 2003;42: 870–878.
136. Yamin R, Berhani O, Peleg H, et al. High percentages and activity of synovial fluid NK cells present in patients with advanced stage active rheumatoid arthritis. *Sci Rep*. 2019;9:1351.
137. Dalbeth N, Callan MF. A subset of natural killer cells is greatly expanded within inflamed joints. *Arthritis Rheum*. 2002;46:1763–1772.
138. De Haas M, Koene HR, Kleijer E, et al. A triallelic Fcγ receptor type IIIA polymorphism influences the binding of human IgG by NK cell FcγRIIIA. *J Immunol*. 1996;156 2948–2945.
139. Wu J, Edberg JC, Redecha PB, et al. A novel polymorphism of FcgammaRIIIa (CD16) alters receptor function and predisposes to autoimmune disease. *J Clin Invest*. 1997;100:1059–1070.
140. Edberg JC, Langefeld CD, Wu J, et al. Genetic linkage and association of Fcγ receptor IIIA (CD16A) on chromosome 1q23 with human systemic lupus erythematosus. *Arthritis Rheum*. 2002;46:2132–2140.
141. Morgan AW, Keyte VH, Babbage SJ, et al. FcγRIIIA-158V and rheumatoid arthritis: A confirmation study. *Rheumatology (Oxford)*. 2003;42:528–533.
142. Gambino CM, Di Bona D, Aiello A, et al. HLA-C1 ligands are associated with increased susceptibility to systemic lupus erythematosus. *Hum Immunol*. 2018;79:172–177.
143. Chandran V, Bull SB, Pellett FJ, et al. Killer-cell immunoglobulin-like receptor gene polymorphisms and susceptibility to psoriatic arthritis. *Rheumatology (Oxford)*. 2014;53:233–239.
144. Mohammad-Ebrahim H, Kamali-Sarvestani E, Mahmoudi M, et al. Association of killer cell immunoglobulin-like receptor (KIR) genes and their HLA ligands with susceptibility to Behçet's disease. *Scand J Rheumatol*. 2018;47:155–163.
145. Agrawal S, Prakash S. Significance of KIR like natural killer cell receptors in autoimmune disorders. *Clin Immunol*. 2020;216:108449.
146. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. 2002;295:2097–2100.
147. Ruggeri L, Mancusi A, Capanni M, et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. *Blood*. 2007;110:433–440.
148. Gill S, Olson JA, Negrin RS. Natural killer cells in allogeneic transplantation: effect on engraftment, graft-versus-tumor, and graft-versus-host responses. *Biol Blood Marrow Transplant*. 2009;15:765–776.
149. Baumeister SHC, Rambaldi B, Shapiro RM, et al. Key aspects of the immunobiology of haploidentical hematopoietic cell transplantation. *Front Immunol*. 2020;11:191.
150. Cooley S, McCullar V, Wangen R, et al. KIR reconstitution is altered by T cells in the graft and correlates with clinical outcomes after unrelated donor transplantation. *Blood*. 2005;106:4370–4376.
151. Ruggeri L, Mancusi A, Urbani E, et al. Identifying NK alloreactive donors for haploidentical hematopoietic stem cell transplantation. *Methods Mol Biol*. 2016;1393:141–145.
152. Oevermann L, Michaelis SU, Mezger M, et al. KIR B haplotype donors confer a reduced risk for relapse after haploidentical transplantation in children with ALL. *Blood*. 2014;124:2744–2747.
153. Bachanova V, Weisdorf DJ, Wang T, et al. Donor KIR B genotype improves progression-free survival of non-Hodgkin lymphoma patients receiving unrelated donor transplantation. *Biol Blood Marrow Transplant*. 2016;22:1602–1607.
154. Cooley S, Trachtenberg E, Bergemann TL, et al. Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia. *Blood*. 2009;113:726–732.
155. Mancusi A, Ruggeri L, Urbani E, et al. Haploidentical hematopoietic transplantation from KIR ligand-mismatched donors with activating KIRs reduces nonrelapse mortality. *Blood*. 2015;125:3173–3182.
156. Cichocki F, Cooley S, Davis Z, et al. CD56dimCD57+NKG2C+ NK cell expansion is associated with reduced leukemia relapse after reduced intensity HCT. *Leukemia*. 2016;30:456–546.
157. Rosenberg SA, Lotze M, Muul L, et al. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N Engl J Med*. 1987;316:889–897.
158. Vacca P, Pietra G, Tumino N, et al. Exploiting human NK cells in tumor therapy. *Front Immunol*. 2020;10:3013.
159. Kim DH, Sohn SK, Lee NY, et al. Transplantation with higher dose of natural killer cells associated with better outcomes in terms of non-relapse mortality and infectious events after allogeneic peripheral blood stem cell transplantation from HLA-matched sibling donors. *Eur J Haematol*. 2005;75:299–308.
160. Parkhurst MR, Riley JP, Dudley ME, et al. Adoptive transfer of autologous natural killer cells leads to high levels of circulating natural killer cells but does not mediate tumor regression. *Clin. Cancer Res*. 2011;17:6287–6297.

161. Burns LJ, Weisdorf DJ, DeFor TE, et al. IL-2-based immunotherapy after autologous transplantation for lymphoma and breast cancer induces immune activation and cytokine release: a phase I/II trial. *Bone Marrow Transplant*. 2003;32(2):177–186.
162. Miller JS, Soignier Y, Panoskaltsis-Mortari A, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood*. 2005;105:3051.
163. Geller M, Cooley S, Judson PL, et al. A phase II study of allogeneic natural killer cell therapy to treat patients with recurrent ovarian and breast cancer. *Cytotherapy*. 2011;13:98–107.
164. Shimasaki N, Jain A, Campana D. NK cells for cancer immunotherapy. *Nat Rev Drug Discov*. 2020;19:200–218.
165. Williams SM, Sumstad D, Kadidlo D, et al. Clinical-scale production of cGMP compliant CD3/CD19 cell-depleted NK cells in the evolution of NK cell immunotherapy at a single institution. *Transfusion*. 2018;58:1458–1467.
166. Huenecke S, Zimmermann SY, Kloess S, et al. IL-2-driven regulation of NK cell receptors with regard to the distribution of CD16+ and CD16- subpopulations and in vivo influence after haploidentical NK cell infusion. *J Immunother*. 2010;33:200–210.
167. Mao Y, van Hoef V, Zhang X, et al. IL-15 activates mTOR and primes stress-activated gene expression leading to prolonged antitumor capacity of NK cells. *Blood*. 2016;128:1475–1489.
168. Leong JW, Chase JM, Romee R, et al. Preactivation with IL-12, IL-15, and IL-18 induces CD25 and a functional high-affinity IL-2 receptor on human cytokine-induced memory-like natural killer cells. *Biol Blood Marrow Transplant*. 2014;20:463–473.
169. Fujisaki H, Kakuda H, Shimasaki N, et al. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. *Cancer Res*. 2009;69:4010–4017.
170. Dolstra H, Roeven MWH, Spanholtz J, et al. Successful transfer of umbilical cord blood CD34(+) hematopoietic stem and progenitor-derived NK cells in older acute myeloid leukemia patients. *Clin Cancer Res*. 2017;23:4107–4118.
171. Knorr DA, Ni Z, Hermanson D, et al. Clinical-scale derivation of natural killer cells from human pluripotent stem cells for cancer therapy. *Stem Cells Transl Med*. 2013;2:274–283.
172. Zhu H, Blum RH, Bjordahl R, et al. Pluripotent stem cell-derived NK cells with high-affinity noncleavable CD16a mediate improved antitumor activity. *Blood*. 2020;135:399–410.
173. Fehniger TA, Miller JS, Stuart RK, et al. A phase 1 trial of CNDO-109-activated natural killer cells in patients with high-risk acute myeloid leukemia. *Biol Blood Marrow Transplant*. 2018;24:1581–1589.
174. Shaffer BC, Le Luduec JB, Forlenza C, et al. Phase II study of haploidentical natural killer cell infusion for treatment of relapsed or persistent myeloid malignancies following allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2016;22:705–709.
175. Cooley S, He F, Bachanova V, et al. First-in-human trial of rhIL-15 and haploidentical natural killer cell therapy for advanced acute myeloid leukemia. *Blood Adv*. 2019;3:1970–1980.
176. Tam YK, Martinson JA, Doligosa K, et al. Ex vivo expansion of the highly cytotoxic human natural killer-92 cell-line under current good manufacturing practice conditions for clinical adoptive cellular immunotherapy. *Cytotherapy*. 2003;5:259–272.
177. Caligiuri MA, Murray C, Soiffer RJ, et al. Extended continuous infusion low-dose recombinant interleukin-2 in advanced cancer: prolonged immunomodulation without significant toxicity. *J Clin Oncol*. 1991;9(12):2110–2119.
178. Caligiuri MA, Murray C, Robertson MJ, et al. Selective modulation of human natural killer cells in vivo after prolonged infusion of low dose recombinant interleukin 2. *J Clin Invest*. 1993;91:123–132.
179. Ye C, Brand D, Zheng SG. Targeting IL-2: an unexpected effect in treating immunological diseases. *Signal Transduct Target Ther*. 2018;3:2.
180. Conlon KC, Lugli E, Welles HC, et al. Redistribution, hyperproliferation activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. *J Clin Oncol*. 2015;33:74–82.
181. Xu W, Jones M, Liu B, et al. Efficacy and mechanism-of-action of a novel superagonist interleukin-15: interleukin-15 receptor αSu/Fc fusion complex in syngeneic murine models of multiple myeloma. *Cancer Res*. 2013;73:3075–3086.
182. Romee R, Cooley S, Berrien-Elliott MM, et al. First-in-human phase 1 clinical study of the IL-15 superagonist complex ALT-803 to treat relapse after transplantation. *Blood*. 2018;131:2515–2527.
183. Wrangle JM, Velcheti V, Patel MR, et al. ALT-803, an IL-15 superagonist, in combination with nivolumab in patients with metastatic non-small cell lung cancer: a nonrandomised, open-label, phase 1b trial. *Lancet Oncol*. 2018;19:694–704.
184. Berraondo P, Etxeberria I, Ponz-Sarvise M, et al. Revisiting interleukin-12 as a cancer immunotherapy agent. *Clin Cancer Res*. 2018;24:2716–2718.
185. Koh CY, Blazar BR, George T, et al. Augmentation of antitumor effects by NK cell inhibitory receptor blockade in vitro and in vivo. *Blood*. 2001;97:3132–3137.
186. Romagne F, Andre P, Spee P, et al. Preclinical characterization of 1-7F9, a novel human anti-KIR receptor therapeutic antibody that augments natural-killer mediated killing of tumor cells. *Blood*. 2009;114:2667–2677.
187. Benson DM, Hofmeister CC, Padmanabhan S, et al. A phase 1 trial of the anti-KIR antibody IPH2101 in patients with relapsed/refractory myeloma. *Blood*. 2012;120:4324–4333.
188. Vey N, Bourhis JH, Boissel N, et al. A phase 1 trial of the anti-inhibitory KIR mAb IPH2101 for AML in complete remission. *Blood*. 2012;120:4317–4323.
189. Benson Jr DM, Cohen AD, Jagannath S, et al. A phase I trial of the anti-KIR antibody IPH2101 and lenalidomide in patients with relapsed/refractory multiple myeloma. *Clin Cancer Res*. 2015;21:4055–4061.
190. Armand P, Lesokhin A, Borrello I, et al. A phase 1b study of dual PD-1 and CTLA-4 or KIR blockade in patients with relapsed/refractory lymphoid malignancies. *Leukemia*. 2020
191. Zhang C, Liu Y. Targeting NK cell checkpoint receptors or molecules for cancer immunotherapy. *Front Immunol*. 2020;11:1295.
192. Pesce S, Greppi M, Tabellini G, et al. Identification of a subset of human natural killer cells expressing high levels of programmed death 1: A phenotypic and functional characterization. *J Allergy Clin Immunol*. 2017;139:335–346. e3.
193. Liu Y, Cheng Y, Xu Y, et al. Increased expression of programmed cell death protein 1 on NK cells inhibits NK-cell-mediated anti-tumor function and indicates poor prognosis in digestive cancers. *Oncogene*. 2017;36:6143–6153.
194. Concha-Benavente F, Kansy B, Moskovitz J, et al. PD-L1 mediates dysfunction in activated PD-1 NK cells in head and neck cancer patients. *Cancer Immunol Res*. 2018;6:1548–1560.
195. Gleason MK, Lenvik TR, McCullar V, et al. Tim-3 is an inducible human natural killer cell receptor that enhances interferon gamma production in response to galectin-9. *Blood*. 2012;119:3064–3072.
196. Da Silva IP, Gallois A, Jimenez-Baranda S, et al. Reversal of NK-cell exhaustion in advanced melanoma by Tim-3 blockade. *Cancer Immunol Res*. 2014;2:410–422.
197. Merino A, Zhang B, Dougherty P, et al. Chronic stimulation drives human NK cell dysfunction and epigenetic reprograming. *J Clin Invest*. 2019;129:3770–3785.
198. Molgora M, Bonavita E, Ponzetta A, et al. IL-1R8 is a checkpoint in NK cells regulating anti-tumour and anti-viral activity. *Nature*. 2017;551:110–114.
199. Chauvin JM, Ka M, Pagliano O, et al. IL-15 stimulation with TIGIT blockade reverses CD155-mediated NK cell dysfunction in melanoma. *Clin Cancer Res*. 2020;26:5520–5533.
200. Sanseviero E. NK cell-Fc receptors advance tumor immunotherapy. *J Clin Med*. 2019;8:1667.
201. Clynes RA, Towers TL, Presta LG, et al. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat Med*. 2000;6:443–446.
202. Liu D, Tian Y, Sun D, et al. The FCGR3A polymorphism predicts the response to rituximab-based therapy in patients with non-Hodgkin lymphoma: a meta-analysis. *Ann Hematol*. 2016;95:1483–1490.
203. Gavin PG, Song N, Kim SR, et al. Association of polymorphisms in FCGR2A and FCGR3A with degree of trastuzumab benefit in the adjuvant treatment of ERBB2/HER2–positive breast cancer: analysis of the NSABP B-31 trial. *JAMA Oncol*. 2017;3:335–341.
204. Goede V, Fischer K, Busch R, et al. Obinutuzumab plus chlorambucil in patients with CLL and coexisting conditions. *N Engl J Med*. 2014;370:1101–1110.
205. Parihar R, Dierksheide J, Hu Y, et al. IL-12 enhances the natural killer cell cytokine response to Ab-coated tumor cells. *J Clin Invest*. 2002;110:983–992.
206. Jaime-Ramirez AC, Mundy-Bosse BL, et al. IL-12 enhances the antitumor actions of trastuzumab via NK cell IFN-γ production. *J Immunol*. 2011;186:3401–3409.
207. Parihar R, Nadella P, Lewis A, et al. A phase I study of interleukin 12 with trastuzumab in patients with human epidermal growth factor receptor-2-overexpressing malignancies: analysis of sustained interferon gamma production in a subset of patients. *Clin Cancer Res*. 2004;10:5027–5037.
208. Bekaii-Saab TS, Roda JM, Guenterberg KD, et al. A phase I trial of paclitaxel and trastuzumab in combination with interleukin-12 in patients with HER2/neu-expressing malignancies. *Mol Cancer Ther*. 2009;8:2983–2991.
209. Kondadasula SV, Roda JM, Parihar R, et al. Colocalization of the IL-12 receptor and FcgammaRIIIa to natural killer cell lipid rafts leads to

activation of ERK and enhanced production of interferon-gamma. *Blood*. 2008;111:4173–4183.

210. Khan KD, Emmanouilides C, Benson Jr DM, et al. A phase 2 study of rituximab in combination with recombinant interleukin-2 for rituximab-refractory indolent non-Hodgkin's lymphoma. *Clin Cancer Res*. 2006;12:7046–7053.
211. Fleming GF, Meropol NJ, Rosner GL, et al. A phase I trial of escalating doses of trastuzumab combined with daily subcutaneous interleukin 2: report of cancer and leukemia group B 9661. *Clin Cancer Res*. 2002;8:3718–3727.
212. Pinette A, McMichael E, Courtney NB. An IL-15-based superagonist ALT-803 enhances the NK cell response to cetuximab-treated squamous cell carcinoma of the head and neck. *Cancer Immunol Immunother*. 2019;68:1379–1389.
213. McMichael EL, Jaime-Ramirez AC, Guenterberg KD, et al. IL-21 enhances natural killer cell response to cetuximab-coated pancreatic tumor cells. *Clin Cancer Res*. 2017;23:489–502.
214. Roda JM, Parihar R, Carson WE. CpG-containing oligodeoxynucleotides act through TLR9 to enhance the NK cell cytokine response to antibody-coated tumor cells. *J Immunol*. 2005;175:1619–1627.
215. Von Strandmann EP, Hansen HP, Reiners KS, et al. A novel bispecific protein (ULBP2-BB4) targeting the NKG2D receptor on natural killer (NK) cells and CD138 activates NK cells and has potent antitumor activity against human multiple myeloma in vitro and in vivo. *Blood*. 2006;107:195562.
216. Gleason MK, Ross JA, Warlick ED, et al. CD16xCD33 bispecific killer cell engager (BiKE) activates NK cells against primary MDS and MDSC CD33+ targets. *Blood*. 2014;123:3016–3026.
217. Vallera DA, Felices M, McElmurry R, et al. IL15 trispecific killer engagers (TriKE) make natural killer cells specific to CD33+ targets while also inducing persistence, in vivo expansion, and enhanced function. *Clin Cancer Res*. 2016;22:3440–3450.
218. Pfefferle A, Huntington ND. You have got a fast CAR: chimeric antigen receptor NK cells in cancer therapy *Cancers (Basel)*. 122020706.
219. Guedan S, Ruella M, June CH. Emerging cellular therapies for cancer. *Annu Rev Immunol*. 2019;37:145–171.
220. Zhao Y, Zhou X. Engineering chimeric antigen receptor-natural killer cells for cancer immunotherapy. *Immunotherapy*. 2020;12:653–664.
221. Liu E, Marin D, Banerjee P, et al. Use of CAR-transduced natural killer cells in CD19-positive lymphoid tumors. *N Engl J Med*. 2020;382: 545–553.
222. Gang M, Marin ND, Wong P, et al. CAR-modified memory-like NK cells exhibit potent responses to NK-resistant lymphomas. *Blood*. 2020;136:2308–2318.
223. Ljunggren HG, Kärre K. In search of the "missing self": MHC molecules and NK cell recognition. *Immunol Today*. 1990;11:237–244.

CHAPTER 22

B-CELL DEVELOPMENT

Kenneth Dorshkind, Dinesh S. Rao, and David J. Rawlings

B lymphocytes are the subset of white blood cells specialized to synthesize and secrete immunoglobulin (Ig). Their name derives from the finding that the avian *b*ursa of Fabricius is a site of B-cell production. However, post-natal B-cell production in mammals takes place in the bone marrow. Following their production, newly generated B lymphocytes migrate into secondary lymphoid organs such as the spleen where they undergo final maturation. Mature B cells may remain there or relocate via the circulation to additional tissues such as lymph nodes where they are poised to respond to antigenic challenge.

This chapter focuses on adult B-cell development and the regulation of that process, although we briefly discuss fetal B lymphopoiesis. We then outline B-cell maturation in secondary lymphoid tissues. The information presented provides a basis for understanding abnormalities of B-cell development such as leukemia, lymphoma, and immunodeficiency states. Although we emphasize the human literature as much as possible, frequent reference to findings in mice are made as studies in that species have contributed much to what is known about B-cell development.

STAGES OF B-CELL DEVELOPMENT

As a result of advances in the development of monoclonal antibodies to leukocyte cell surface antigens and flow cytometry, it is possible to resolve various stages of murine and human B-cell development from the hematopoietic stem cell (HSC) to newly produced, surface IgM expressing B cells.

Murine B-Cell Development

As the progeny of murine HSCs differentiate, they generate multipotent progenitors (MPPs). MPPs include four phenotypically separable subsets; MPP1s are short-term HSCs, MPP2s and MPP3s are myeloerythroid biased, and MPP4s primarily generate lymphoid progeny. MPP4s are presumably the precursors of most lineage-negative (Lin^-) $\mathrm{CD117(c\text{-}kit)}^{\mathrm{low}}$ $\mathrm{Sca\text{-}1}^{\mathrm{low}}$ $\mathrm{CD127}^+$ (interleukin-7 receptor α) common lymphoid progenitors (CLPs). Lin^- indicates that the cells lack expression of determinants present on mature myeloid, erythroid, and lymphoid lineage cells. CLPs then mature through pre-pro-B, pro-B, pre-B-, and B-cell stages of development that can be phenotypically resolved based on their expression of various cell surface and cytoplasmic determinants (Fig. 22.1). The stages of development depicted in Fig. 22.1 are based on the scheme originally defined by Hardy.[1]

Human B-Cell Development

The human MPP compartment is not as well defined as in the mouse, but various downstream stages of development which parallel those in the mouse can be phenotypically identified.[2] For example, CLPs sequentially generate pro-B, pre-B, and B cells each of which expresses distinct combinations of cell surface determinants.[2–4] Fig. 22.1 shows that human pre-B and newly produced B cells express CD20. It is relevant to note this determinant because antibodies that recognize it (rituximab) are in widespread clinical use for the treatment of lymphoma and, increasingly, autoimmune diseases. Developing and mature B-lineage cells express additional cell surface determinants that include CD21, CD22, CD24, CD38, and CD40, several of which are linked to critical intracellular signaling pathways.

B LINEAGE SPECIFICATION AND COMMITMENT

As differentiation of HSCs and immature precursors into the B lineage occurs, genetic programs that promote B lymphopoiesis are activated while those used by non-B lineage cells are silenced. This results in *specification* of progenitors toward the B-cell lineage. For example, cells at the CLP stage of development are destined to generate B cells, but they retain limited T and myeloid potential. Because of this, CLPs can be thought of as B lineage *specified*. However, pro-B cells only generate B lymphocytes and are B lineage *committed*. Specification and commitment are dependent on the interdependent expression of a cascade of transcription factors and other regulatory molecules in developing B lineage cells.[5]

Transcriptional Regulation of B-Cell Development

The generation of lymphoid cells from HSCs is dependent on expression of the Ets family member PU.1. Mice in which *Spi1*, the gene encoding PU.1, is not expressed produce erythroid and megakaryocytic but not monocytic, granulocytic, and lymphoid cells and die during the fetal or neonatal period. PU.1 regulates several events that promote B lineage specification, including expression of CD135 (Flt3), which is expressed on immature progenitors such as the CLP, CD45R(B220), and CD127 (IL-7Rα). However, deletion of *Spi1* at the CLP stage of development has no negative effect on B-cell development, indicating that PU.1 expression is not required once B-cell specification has initiated.

Further B lineage specification is dependent on expression of additional transcription factors including early B-cell factor (EBF) and the E2A-encoded splice variants E12 and E47. Each of these DNA-binding proteins regulates the expression of a variety of B-lineage target genes. For example, Ebf1 regulates the expression of Igα, VpreB, λ5, and Pax5 and represses genes associated with alternative lineage fates. Mice in which EBF and E2A are not expressed exhibit an almost complete block in B-cell development at the pro-B-cell stage.

Ebf1 and E2A-expressing progenitors can still exhibit some non-B lineage potential, indicating that the expression of these DNA binding proteins does not result in absolute B lineage commitment. Instead, this is dependent on expression of the Pax5 transcription factor.[6] Phenotypically identifiable B-cell precursors are present in *Pax5* knock-out mice, and these cells can differentiate into myeloid, T, and natural killer (NK) cells. However, if the gene encoding Pax5 is introduced into *Pax5*-deficient precursors, this developmental promiscuity ceases. Thus, a critical function of Pax5 is to suppress non-B lineage potential. For example, Pax5 may repress expression of myeloid growth factor receptors and inhibit T-cell potential by antagonizing expression of Notch1, a cell-surface receptor required for commitment to the T-cell lineage. Continued Pax5 expression is necessary to maintain lineage fidelity even in relatively mature B cells.

Additional transcription factors, such as Ikaros, Satb1, Foxo1, IRF4, IRF8, c-Myb, Gfi1, Miz-1, Bcl6, and Bach2, function at various times during B-cell development. For example, IRF4 is involved

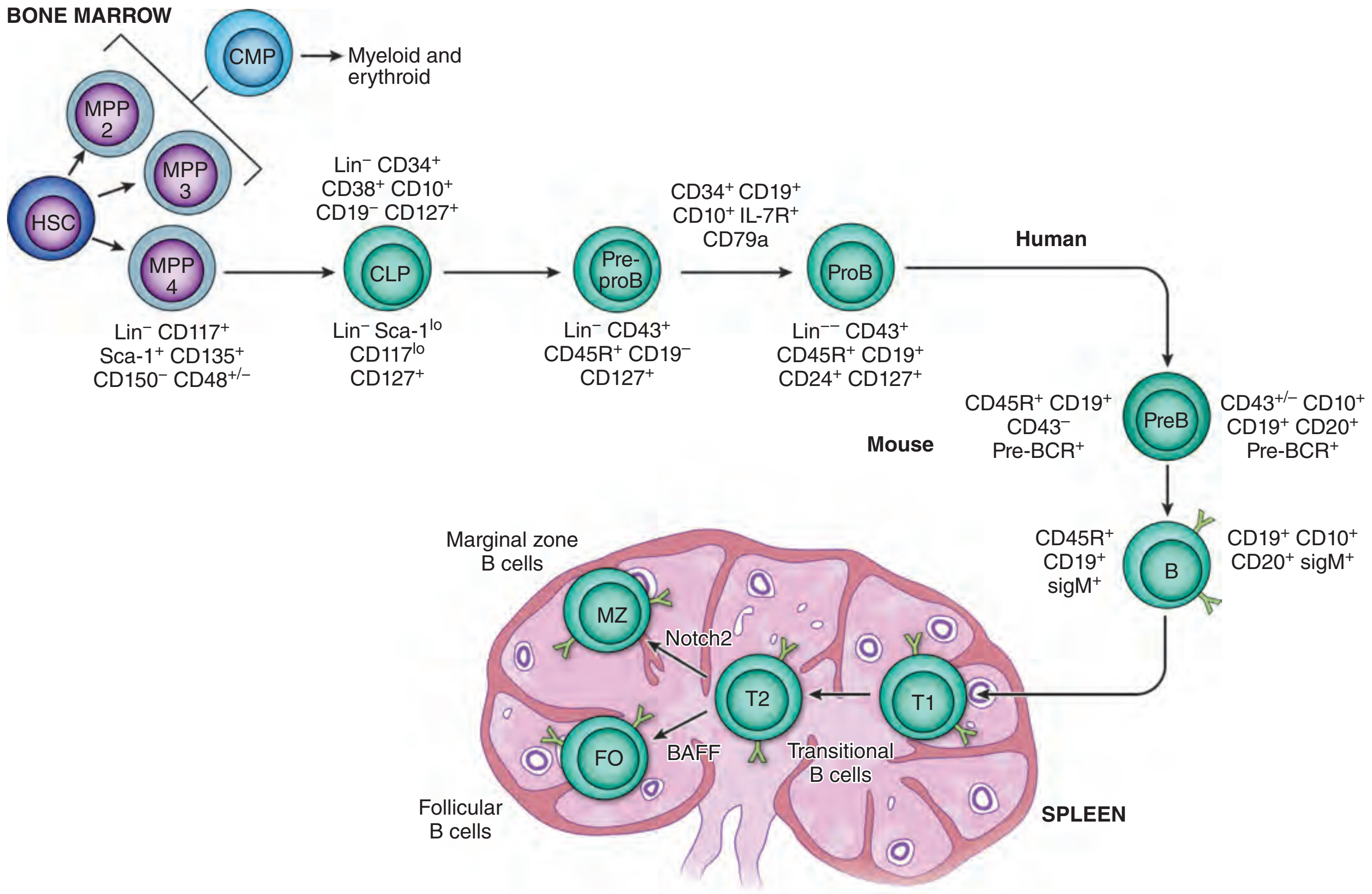

BONE MARROW
CMP
Myeloid and erythroid
MPP 2
MPP 3
HSC
MPP 4
Lin$^-$ CD117$^+$ Sca-1$^+$ CD135$^+$ CD150$^-$ CD48$^{+/-}$
Lin$^-$ CD34$^+$ CD38$^+$ CD10$^+$ CD19$^-$ CD127$^+$
CLP
Lin$^-$ Sca-1^{lo} CD117^{lo} CD127$^+$
CD34$^+$ CD19$^+$ CD10$^+$ IL-7R$^+$ CD79a
Pre-proB
Lin$^-$ CD43$^+$ CD45R$^+$ CD19$^-$ CD127$^+$
ProB
Lin^{--} CD43$^+$ CD45R$^+$ CD19$^+$ CD24$^+$ CD127$^+$
Human
Mouse
CD45R$^+$ CD19$^+$ CD43$^-$ Pre-BCR$^+$
PreB
CD43$^{+/-}$ CD10$^+$ CD19$^+$ CD20$^+$ Pre-BCR$^+$
CD45R$^+$ CD19$^+$ sigM$^+$
B
CD19$^+$ CD10$^+$ CD20$^+$ sigM$^+$
Marginal zone B cells
MZ
Notch2
T2
T1
FO
BAFF
Transitional B cells
Follicular B cells
SPLEEN

Figure 22.1 HEMATOPOIESIS WITH AN EMPHASIS ON B-CELL DEVELOPMENT. Human and mouse B-cell differentiation and selected cell surface and cytoplasmic determinants used to distinguish various stages of development are shown. Newly produced B cells migrate to the spleen and mature through transitional cell stages into marginal zone or follicular B cells. *CLP*, Common lymphoid progenitor; *CMP*, common myeloid progenitor; *FO*, follicular B cells; *HSC*, hematopoietic stem cell; *MPP*, multipotential progenitor; *MZ*, marginal zone B cells; *T1*, transitional 1 B cells; *T2*, transitional 2 B cells.

with Ig recombination and the attenuation of IL-7 signaling, thus promoting the transition from the pre-B to B cell stages of maturation. IRF8, along with PU.1, regulates EBF expression. Ikaros plays a role in regulating expression of key B lineage genes such as IL-7Rα and EBF and promoting B lineage commitment. c-Myb has been shown to synergize with PU.1 to activate IL-7 receptor (IL-7R) gene transcription. Focused reviews should be consulted for a full discussion of these and additional transcriptional regulators of B lymphopoiesis.

MicroRNAs

MicroRNAs (miRNAs) are 19 to 23 nucleotide-long RNA molecules that are processed from longer RNA precursors. miRNAs act post-transcriptionally, by promoting degradation of mRNA targets or by blocking their translation. However, like transcriptional regulators, a single miRNA can potentially regulate many targets to provide coordinated and simultaneous regulation of a network of genes.

Mice with conditional deletion of Dicer, an enzyme necessary for miRNA synthesis do not develop B lymphocytes, indicating the importance of miRNA regulatory mechanisms during B-cell differentiation. Work is now ongoing to identify the role of specific miRNAs at distinct stages of B-cell development. For example, deletion of miR-17∼92 cluster blocks pro-B-cell maturation.[7]

IMMUNOGLOBULIN GENE REARRANGEMENT

The defining feature of a B lymphocyte is the expression of cell surface Ig, which is formed by two heavy chains and two light chains, each of which is encoded by multiple gene segments. The process of Ig gene rearrangement occurs in a step-wise manner as murine and human B cells mature through the cellular stages of development just described.[8,9]

Heavy Chain Gene Rearrangement

The initial Ig rearrangement event occurs at the heavy chain locus located on human chromosome 14 (Fig. 22.2). The Ig heavy chain locus includes multiple variable (V), diversity (D), joining (J), and constant (C) region gene segments that are separated from one another by introns. The V region genes are located at the $5'$ end of the Ig heavy chain locus, and each consists of approximately 300 base pairs. These genes, which are separated by short intron sequences, are organized into families based on sequence homology. There are ∼25 human D region genes located $3'$ to the V region that are also grouped into families based on sequence homology. Downstream of the D region are six human J region genes. Finally, 10 C region genes representing alternative Ig isotypes are arranged in tandem.

The transcription of the unrearranged heavy chain locus occurs prior to actual Ig gene recombination. This results in production

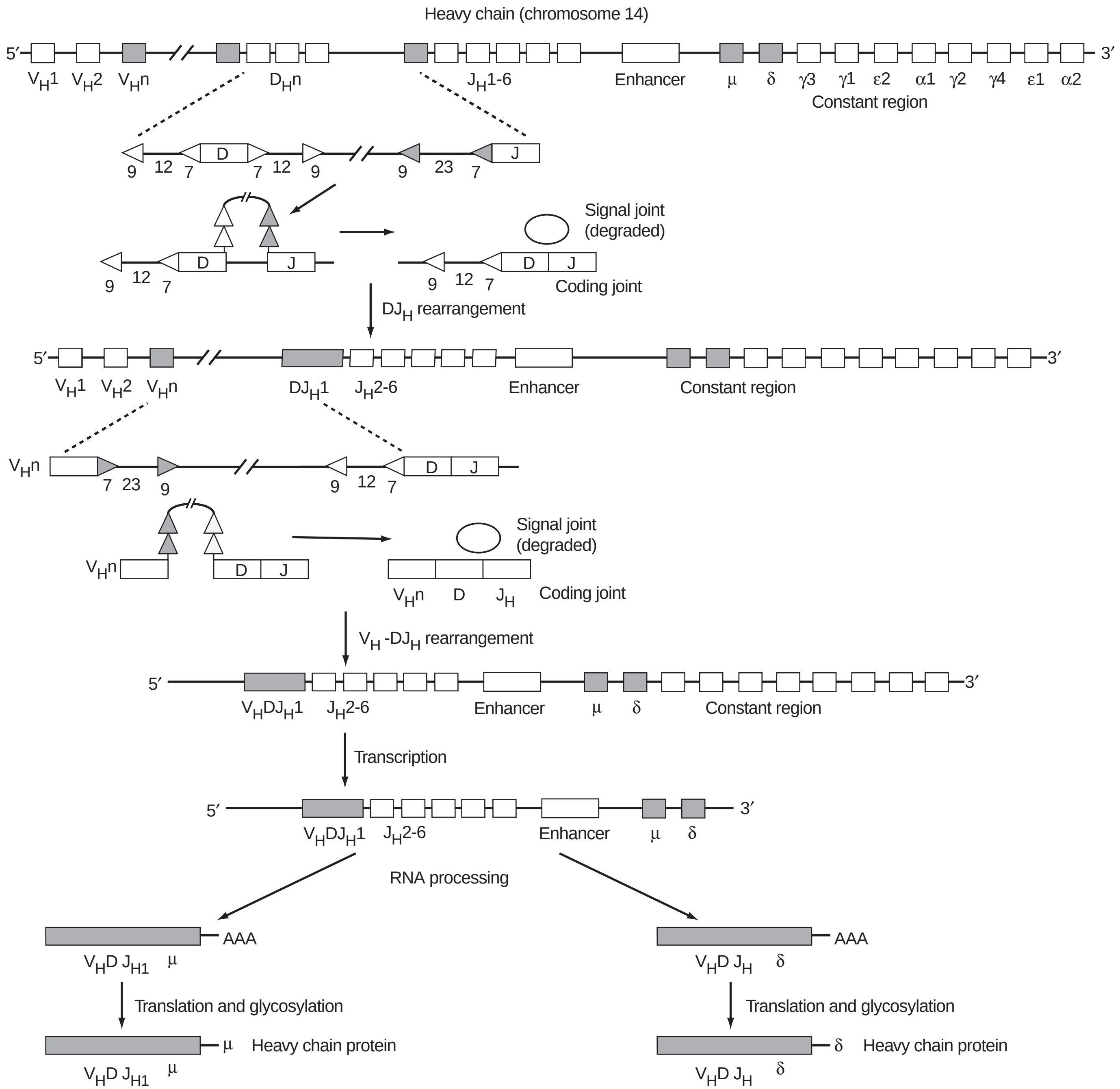

Heavy chain (chromosome 14)
5′
3′
V_H1
V_H2
V_Hn
D_Hn
J_H1-6
Enhancer
μ
δ
γ3
γ1
ε2
α1
γ2
γ4
ε1
α2
Constant region
D
J
9
12
7
7
12
9
9
23
7
Signal joint (degraded)
Coding joint
DJ_H rearrangement
DJ_H1
J_H2-6
V_Hn
7
23
9
9
12
7
V_Hn
D
J_H
V_H-DJ_H rearrangement
V_HDJ_H1
Transcription
RNA processing
AAA
V_HD J_{H1}
V_HD J_H
Translation and glycosylation
Heavy chain protein

Figure 22.2 REARRANGEMENT AND EXPRESSION OF THE HUMAN IMMUNOGLOBULIN HEAVY CHAIN GENE. The figure shows the Ig heavy chain gene and the signal sequences 3′ of each V region locus, 5′ and 3′ of each D region locus, and 5′ of each J region locus. These consist of heptamer and nonamer sequences separated by either 12 or 23 base pairs. During immunoglobulin (Ig) recombination, a signal sequence of 12 base pairs can only join to another of 23 base pairs (the so-called 12–23 rule). The initial heavy chain gene rearrangements form coding joints between D and J regions as well as signal joints that are ultimately degraded. Subsequently, the joining of the V region gene to the DJ complex occurs. After a successful rearrangement, the VDJ complex, the μ intron, and portions of the constant regions are transcribed. RNA processing and differential splicing results in formation of an mRNA molecule that is then translated. In the example shown, the rearranged VDJ complex and the constant region, with the μ and δ C region genes, is transcribed. After RNA processing and translation, a particular B cell could then express μ protein, δ protein, or both.

of developmentally regulated transcripts of unrearranged Ig genes, referred to as germline or sterile transcripts. Multiple species of sterile transcripts have been described, and some could conceivably encode proteins. A mechanistic link between transcription and Ig gene rearrangement has been hypothesized. For example, transcription could make unrearranged Ig genes accessible to both RNA polymerase and V(D)J recombinase, the germline transcripts could function in the rearrangement reaction, or transcription could alter structural characteristics of DNA, making the recombination signal sequences described below better targets for recombination.

The initial event during heavy chain gene rearrangement occurs by the CLP stage and juxtaposes a D region segment to a J region segment. Although in theory any D region gene can join with equal frequency to any J region gene, there may be preferential utilization of selected D and J region genes at various times during fetal and adult B-cell development. The next recombination event involves the rearrangement of a V region gene to the D–J complex, and this occurs at the pro-B-cell stage of development. The biased usage of J proximal V genes may occur in the newly generated repertoire of neonatal mice and humans. The heavy chain C region remains separated from the rearranged VDJ complex by an intron, and this entire sequence is transcribed. RNA processing subsequently leads to deletion of the intron between the VDJ complex and the most proximal C region genes. After translation, μ heavy chain protein is expressed in the cytoplasm of pre-B cells (see Fig. 22.2).

The E2A encoded transcription factors are particularly important for Ig gene recombination and mediate their effects via binding to specific promoter sequences located 5′ of each heavy chain V region and enhancer regions located 3′ of the J region genes and downstream from the CH region genes (see Fig. 22.2). Before Ig gene rearrangement, E12 and E47 proteins may be in an inactive state owing to their heterodimeric association with another protein known as Id. Successful transition from the pro-B to pre-B-cell stage is dependent on cessation of Id expression, a conclusion consistent with the fact that mice expressing an Id transgene have a complete block in B-cell differentiation.

Each pro-B cell has two Ig heavy chain genes, but only one of these encodes heavy chain protein in any given cell. This phenomenon is known as allelic exclusion. One theory for how this occurs is that functional Ig rearrangements are rare, so the chance that two functional rearrangements will occur in an individual cell is extremely low. Alternatively, the expression of heavy chain protein from a successfully rearranged allele may inhibit rearrangements at the other heavy chain allele.

Light Chain Gene Rearrangement

Ig light chain protein can be encoded by the kappa (κ) or lambda (λ) genes. Greater than 90% of murine B cells express κ protein. However, the proportions of human κ and λ proteins are more equivalent, with approximately 60% of human B cells expressing κ light chain protein.

The human κ gene is located on chromosome 2 and includes around 40 Vκ region genes, five functional Jκ region genes, and one C κ region gene. The human λ locus is located on human chromosome 22. Approximately 30 human Vλ genes exist. There are seven human Cλ genes, four of which are functional and three of which are pseudogenes. Each Cλ gene is located 3′ of a respective J λ gene. Light chain genes do not include D region loci.

The initial event in light chain gene recombination involves the joining of a V region to a J region. The VJ complex remains separated from the light chain C region by an intron, the entire complex is transcribed, and further splicing of the intron results in formation of a mature V–J–C transcript. Light chain rearrangements in mice occur initially at the kappa locus. If rearrangements at the first κ allele are unsuccessful, attempts are made to rearrange the second κ gene, and the λ locus is used if this fails.

The Immunoglobulin Recombinatorial Machinery

Ig heavy and light chain gene rearrangements are dependent on enzymes that delete intronic sequences and join coding segments of DNA. The enzymes that mediate these functions act by recognition of recombination signal sequences located 3′ of each heavy chain V region exon, 5′ of each heavy chain J segment, and 5′ and 3′ of each heavy chain D region gene (see Fig. 22.2). Each recombination signal sequence consists of conserved heptamer and nonamer sequences separated by nonconserved DNA segments of 12 or 23 base pairs. During Ig gene recombination, these recognition sequences form loops of DNA, which in turn bring the coding exons in apposition to one another. These noncoding loops are subsequently deleted and degraded.

The expression of two highly conserved proteins, referred to as recombinase-activating genes-1 (RAG-1) and RAG-2, is required for heavy and light chain gene recombination,[8,9] and failure to express *Rag* results in a complete block in B- as well as T-cell development. Results from cell-free systems that measure V(D)J recombination indicate that RAG proteins are involved in cleavage of DNA at recombination signal sequences and the subsequent joining of coding sequences to one another. In addition to the RAG proteins, general DNA repair enzymes, such as those encoded by the Ku complex of genes, also play a critical role in Ig gene recombination.

Generation and Selection of the Primary B-Cell Repertoire

Each newly generated B cell expresses Ig with a specific antigen binding capability. Thus, humoral immunity is dependent on the production of a large number of B cells to ensure that a B lymphocyte capable of responding to a particular antigenic challenge is present. Several mechanisms have evolved to ensure that B cells with a diverse Ig repertoire are generated.

First, heavy and light chain proteins are encoded by multiple germline V, J, and, in the case of the heavy chain, D region genes, and the combinatorial diversity among them is enormous. Second, nucleotides not encoded in the germline can be added to D-J and VDJ junctions by a nuclear enzyme known as terminal deoxynucleotidyl transferase (TdT). Third, DNA joints that form during recombination are often imprecise and can occur at any of several nucleotides in the germline. Although out-of-frame joints that cannot be transcribed may result, this junctional diversity has the potential to generate different amino acid sequences, resulting in added diversity of the Ig repertoire. Finally, somatic mutation of V region genes can occur, usually in secondary lymphoid tissues. This latter process, which results in an increased affinity of the antibody for antigen, is discussed in more detail in the section on secondary B-cell development.

Because the process of Ig gene recombination is random, self-reactive B cells may be produced, and several mechanisms have been proposed to account for their fate. In some cases, the presence of self-antigen may not activate self-reactive B cells. This scenario may result from weak B-cell affinity for the antigen or the autoantigen may be present at an extremely low concentration. In other instances, interaction of antigen with the autoreactive B cell may result in anergy. The level of membrane Ig on anergic B cells may be reduced up to 20-fold, the cell's ability to proliferate may be impaired, and differentiation into Ig-secreting cells may be blocked. Finally, self-reactive B cells may be clonally deleted. Clonal deletion may result from cytolysis by other cells, such as BM macrophages, or autoreactive B cells may undergo a physiologic change resulting in cell death after receptor engagement.[10]

The recognition of self-antigen by a B cell may not necessarily result in anergy or deletion but instead may lead to receptor editing. In this process, rearranged κ light chain alleles can be replaced by secondary rearrangements of upstream Vκ genes to downstream, unrearranged Jκ segments. These secondary rearrangements, which may delete the primary VκJκ complex or separate it from Cκ by inversion, are possible because of the continual presence of unrearranged Vκ regions upstream of the joined VκJκ coding segments.

THE PRO-B- AND PRE-B-CELL CHECKPOINTS

B-cell progenitors progress through two critical checkpoints as they mature into B lymphocytes. The first occurs at the pro-B- to pre-B-cell transition. The second transition occurs as pre-B cells mature into B cells.

The Pro-B- to Pre-B-Cell Transition

The key event during the pro-B- to pre-B-cell transition is the rearrangement and expression of Ig heavy chain genes. Ig heavy chain gene rearrangements are productive in only around one-third of pro-B cells, so the majority of pro-B cells do not navigate this transition. Those cells with nonproductive Ig gene rearrangements undergo apoptosis and are eliminated from the BM by resident macrophages and stromal cells.

However, if Ig heavy chain recombination is productive and μ heavy chain protein is expressed, it appears on the surface of the pre-B cells in association with two additional molecules referred to as the surrogate light chains. The surrogate light chain proteins, Vpre-B and λ5, are encoded by genes located on chromosome 16 in mice and on chromosome 22 in humans and are non-covalently linked to one another. λ5 in turn is covalently linked to the CH1 domain of the μ heavy chain via a carboxyl-terminal (C-terminal) cysteine. One role of the surrogate light chains is to select heavy chains that will ultimately be capable of pairing with conventional light chains.[11] If this does not occur, then these cells will likely be deleted.

The μ heavy chain-surrogate light chain complex is additionally associated with two transmembrane proteins, Igα (CD79a) and Igβ (CD79b), and the entire complex is referred to as the pre-B-cell receptor (*pre-BCR*). The intracellular tails of both Igα and Igβ contain immunoreceptor tyrosine activation motifs (ITAMs) critical to the signaling function of the pre-BCR (Fig. 22.3, *upper panel*). Lipid rafts that contain mediators of intracellular signaling such as Lyn are constitutively associated with the pre-BCR in human pre-B cells. Cross-linking of the pre-BCR leads to an increase in Lyn kinase activity, phosphorylation of the Igβ chain, and recruitment and activation within the pre-BCR complex of additional signaling intermediates, including spleen tyrosine kinase (Syk), B-cell linker protein (BLNK), phosphoinositide-3 kinase (PI3K), Bruton's tyrosine kinase (Btk), VAV, and phospholipase C-γ (PLCγ2). These events lead to calcium flux and activation of signaling cascades within the pre-B cell (see Fig. 22.3, *lower panel*).

These signaling pathways are crucial in developing pre-B cells. One of the best examples of this requirement is the prototypical humoral immunodeficiency, X-linked agammaglobulinemia (XLA). XLA results from mutations within the gene segments that encode the nonreceptor tyrosine kinase, Btk. In males who express a defective Btk protein, pre-B-cell clonal expansion is markedly depressed, and there is an almost complete loss of immature B cells in the BM and in secondary lymphoid organs. As a result, affected males develop recurrent bacterial infections early in life because of a profound decrease in circulating Ig. A nearly identical clinical phenotype has been observed in persons with mutations in additional components of the pre-BCR signaling complex, including the μ–heavy chain, λ5, Igα, Igβ, the key B-cell adaptor protein BLNK, and the lipid kinase PLCγ2.

How signaling through the pre-BCR is initiated is unclear. It has been suggested that this occurs by binding of the extracellular portion of the pre-BCR to an environmental ligand. The identification of such ligands has been difficult, although galectin-1 has been suggested to function in this capacity. It has also been proposed that constitutive signaling occurs after pre-BCR surface expression. Structural studies which suggest that the pre-BCR constitutively assembles as an oligomer provide a potential mechanism for this behavior.[12]

The Pre-B to B-Cell Transition

Pre-BCR expressing cells undergo several rounds of proliferation in response to the stromal cell derived cytokine IL-7, and this expands the size of the clone that expresses a particular μ heavy chain. However, IL-7R signaling inhibits *Rag* gene expression. Thus, in order to reactivate the recombinatorial machinery and initiate light chain gene recombination, downregulation of the IL-7 response must occur.[13] There is general agreement that this is accomplished by movement of pre-BCR expressing cells from IL-7 high to IL-7 areas of the bone marrow.

Different mechanisms for how this is achieved have been proposed, but one current model is that pre-B cells are more motile compared to pro-B cells and thus spend a reduced amount of time in contact with IL-7 producing stromal cells (Fig. 22.4).[14] This occurs because, in contrast to pro-B cells that tightly adhere to stromal cells via expression of adhesion molecules that include focal adhesion kinase (FAK) and very late antigen 4 (VLA-4), these receptors are expressed at lower levels of pre-B cells which thus allows them to move away from the IL-7 secreting cells. As a result, IL-7R signaling is attenuated and pre-BCR signaling predominates. This represses proliferation, likely through activation of the RAS–extracellular-signal-regulated kinase (ERK) pathway as well as via limiting PI3K activity through BLNK mediated signals leading to expression of transcription factors, including E2A, IRF4, and PAX5, that induce cell cycle exit, RAG expression and light chain gene recombination (see Fig. 22.4).[13] As with heavy chain recombination, functional light chain gene rearrangements do not occur in all pre-B cells. However, when they are successful, the heavy and light chains assemble and are expressed on the surface of a newly produced B lymphocyte as the mature B-cell receptor (BCR).

Events that disrupt the pre-B-cell transition such as activating lesions in IL7 receptor subunits or downstream mediators significantly enhance the risk for development of pre-B acute lymphoblastic leukemia. An improved understanding of these changes will likely provide new therapeutic targets to inhibit leukemia cell growth.[15]

The B-Cell Receptor

Once light chain expression occurs, a complete Ig molecule is expressed on the surface of newly produced B cells. These cells express IgM, but others may co-express cell surface IgD. This occurs because the primary heavy chain transcript includes the rearranged VDJ heavy chain complex, the μ and δ C regions, and the intron separating these exons. If RNA processing results in association of the Cμ region with the VDJ complex, the B cell expresses IgM. Alternatively, if the Cμ exon is deleted along with the heavy chain intron, the VDJ complex and the Cδ exon become contiguous and the B cell expresses IgD.

The complex of cell surface Ig, along with Igα and Igβ, is referred to as the *BCR* (see Fig. 22.3). The cytoplasmic tail of the Ig heavy chain is relatively short, and, as noted above, both Igα and Igβ contain an ITAM in their intracellular tails that is required for signal transduction following antigen binding to the BCR. Antigen engagement initiates assembly of a lipid raft, BCR-associated "signalosome," composed of multiple signaling molecules that include tyrosine kinases, serine/threonine kinases, lipid kinases, lipases, phosphatases, and linkers and adaptors. This signalosome mediates a cascade of intracellular signals that includes the initiation of calcium influx. Additional calcium-dependent and -independent downstream signals that include the mitogen-activated protein (MAP) kinase cascade (c-jun N-terminal kinase [JNK], p38, ERK) and activation of key transcription factors that include JUN, c-fos, nuclear factor of activated T cells (NFAT), and nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) in turn mediate transcriptional events leading to cell proliferation, survival, and differentiation. The level and duration of receptor activation and hence transcriptional output are further modified by a series of cell surface co-receptors or "response modifiers" that bind to complement or to receptors on the surface of stromal cells, activated T cells, or other populations present in secondary lymphoid organs.

THE HEMATOPOIETIC MICROENVIRONMENT

Hematopoiesis takes place in the intersinusoidal spaces of the medullary cavity in association with several types of hematopoietic and non-hematopoietic cells that together form the hematopoietic microenvironment (see Fig. 22.4). These populations include mesenchymal stromal cells, adipocytes, osteoblasts, sympathetic nervous system fibers, and endothelial cells.[16]

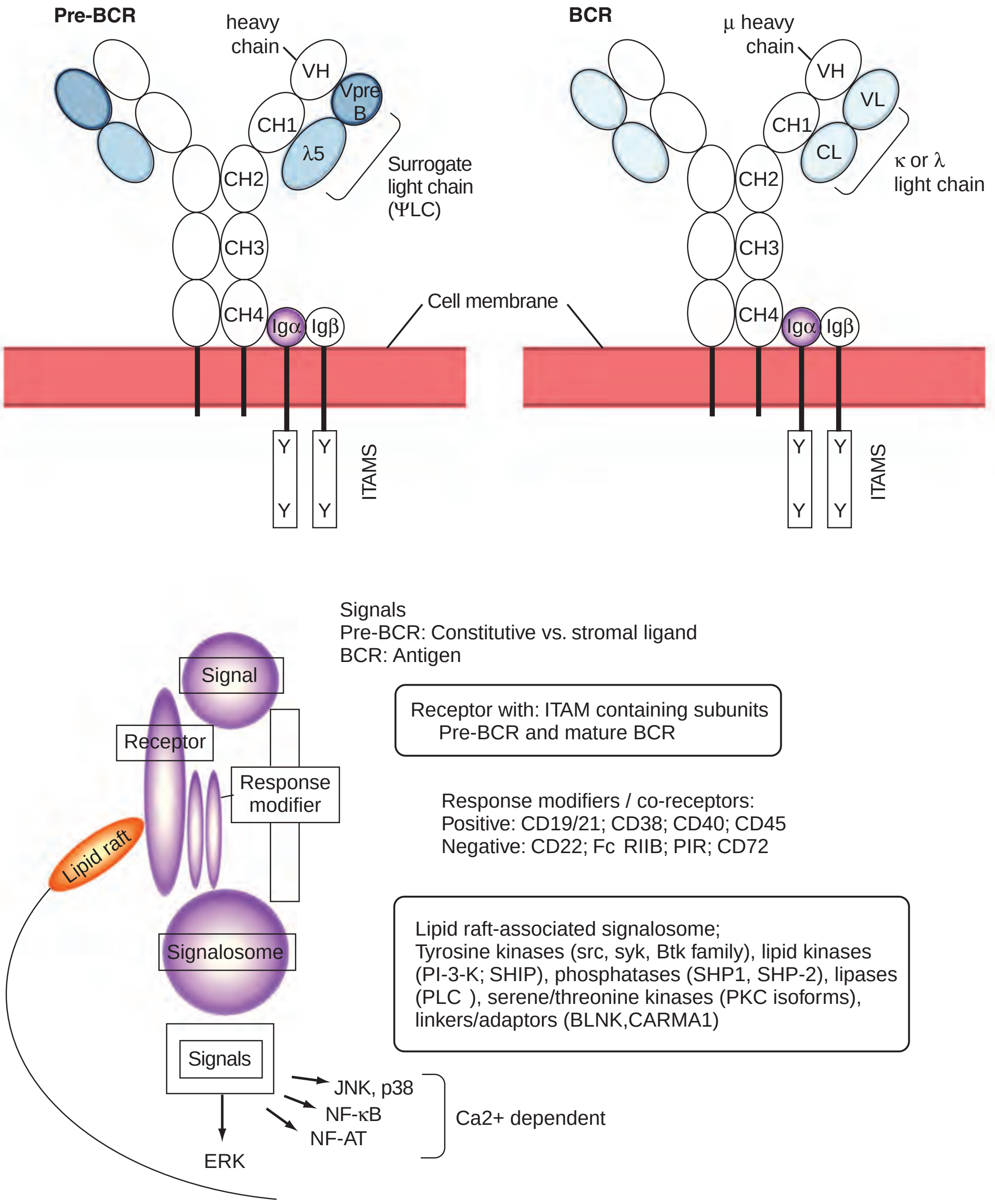

Pre-BCR
heavy chain
VH
Vpre B
CH1
λ5
CH2
Surrogate light chain (ΨLC)
CH3
CH4
Igα
Igβ
Cell membrane
Y
Y
Y
Y
ITAMS
BCR
μ heavy chain
VH
VL
CH1
CL
κ or λ light chain
CH2
CH3
CH4
Igα
Igβ
Y
Y
Y
Y
ITAMS
Signals
Pre-BCR: Constitutive vs. stromal ligand
BCR: Antigen
Signal
Receptor
Response modifier
Lipid raft
Receptor with: ITAM containing subunits
Pre-BCR and mature BCR
Response modifiers / co-receptors:
Positive: CD19/21; CD38; CD40; CD45
Negative: CD22; Fc RIIB; PIR; CD72
Signalosome
Lipid raft-associated signalosome;
Tyrosine kinases (src, syk, Btk family), lipid kinases (PI-3-K; SHIP), phosphatases (SHP1, SHP-2), lipases (PLC), serene/threonine kinases (PKC isoforms), linkers/adaptors (BLNK,CARMA1)
Signals
JNK, p38
NF-κB
NF-AT
Ca2+ dependent
ERK

Figure 22.3 THE PRE-B-CELL RECEPTOR AND B-CELL RECEPTOR AND ASSOCIATED SIGNALING INTERMEDIATES. *Top*, μ heavy chain protein in pre-B cells is associated with the surrogate light chains v-pre-B and λ5 *(left)* to form the pre-B-cell receptor (pre-BCR). In newly produced B lymphocytes, μ heavy chain is associated with conventional light chains *(right)* to form the BCR. Associated with heavy chain in both pre-B and B cells are two additional transmembrane proteins, Igα and Igβ, that contain immunoreceptor tyrosine activation motifs (ITAMs) critical to the signaling function. *Bottom*, Expression of the pre-BCR (or possibly its binding to a stromal ligand) or binding of antigen to the mature BCR, respectively, initiates the assembly of a lipid raft, BCR-associated "signalosome" composed of multiple signaling molecules, ultimately leading to transcriptional events that promote cell proliferation, survival, and differentiation. *ERK*, Extracellular-signal-regulated kinase; *Ig*, immunoglobulin; *JNK*, Janus kinase; *NF-κB*, nuclear factor kappa-light-chain enhancer of activated B cells; *NFAT*, nuclear factor of activated T cells; *SHIP*, src homology 2-containing inositol phosphatase; *SHP*, src homology 2-containing protein tyrosine phosphatase.

Several cell surface determinants, some of which have been introduced above, mediate adhesion between developing B lineage cells and stromal cells. Both murine and human pre-B cells express the VLA-4 integrin that interacts with a stromal cell ligand identified as vascular cell adhesion molecule-1 (VCAM-1). Pro- and pre-B cells also express varying amounts of FAK involved in stromal cell binding via mediation of integrin signaling. FAK is an intracellular kinase typically linked to surface integrins. The hematopoietic microenvironment is also a source of cytokines that regulate B lymphopoiesis and include CXCL12 (SDF1), Flt3 ligand, Stem Cell Factor, IL-7, Wnt family members, TGF-β family members, and thymic stromal lymphopoietin (TSLP).

A full discussion of the cytokines that regulate B-cell development is beyond the scope of this chapter, but the focus can be narrowed when only those with obligate effects on B-cell development are considered. The critical B lymphopoietic cytokine in mice is IL-7, which

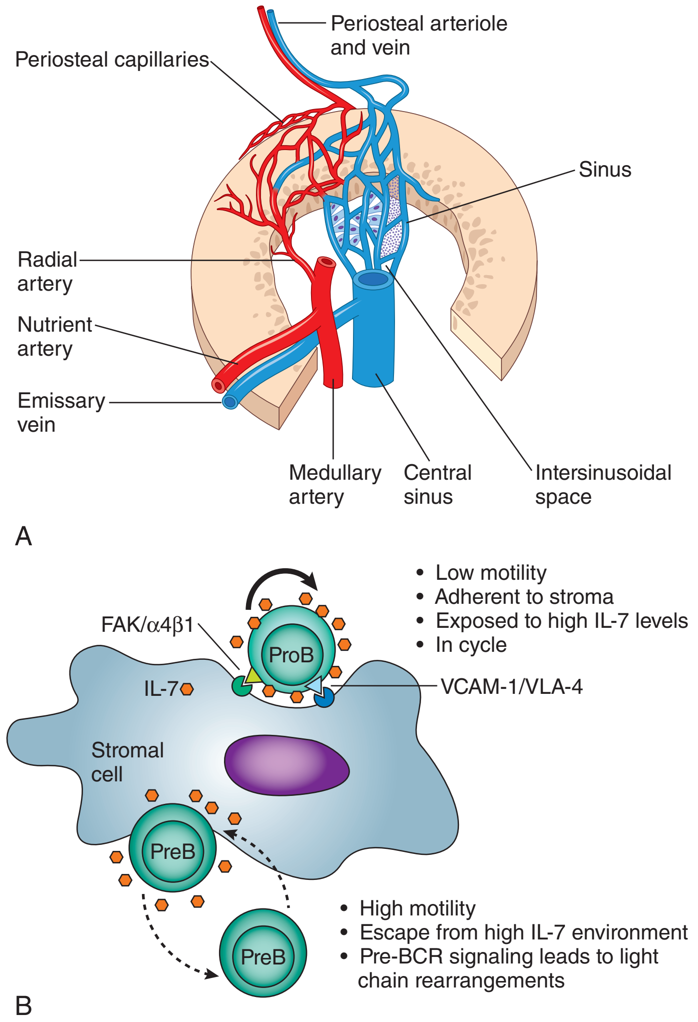

Periosteal arteriole and vein
Periosteal capillaries
Sinus
Radial artery
Nutrient artery
Emissary vein
Medullary artery
Central sinus
Intersinusoidal space
A
FAK/α4β1
ProB
IL-7
VCAM-1/VLA-4
Stromal cell
PreB
PreB
• Low motility
• Adherent to stroma
• Exposed to high IL-7 levels
• In cycle
• High motility
• Escape from high IL-7 environment
• Pre-BCR signaling leads to light chain rearrangements
B

Figure 22.4 THE HEMATOPOIETIC MICROENVIRONMENT. (A) Cross-section of bone showing elements of the medullary circulation and the marrow sinusoids. Stromal cells that support developing blood cells are located in the intersinusoidal spaces. (B) Pro-B cells are tightly adherent to IL-7 producing stromal cells via their expression of adhesion molecules such as focal adhesion kinase and vascular cell adhesion molecule-1. Following the successful expression of Ig μ heavy chain and assembly of the pre-B-cell receptor (pre-BCR), pre-B cells must ultimately attenuate IL-7 receptor signaling in order to recombine Ig light chain genes and mature into BCR expressing B lymphocytes. A current model proposes that they are less adherent to stromal cells compared to pro-B cells and as a result migrate away from IL-7 rich areas. This results in attenuated IL-7 receptor signaling while pre-BCR signaling in these cells induces cell cycle exit, RAG expression, and light chain gene recombination. *(A, From Dorshkind K. Regulation of hematopoiesis by bone marrow stromal cells and their products. Annu Rev Immunol. 1990;8:111. Reprinted by permission from the Annual Review of Immunology. B, Based on concepts reviewed and depicted in reference 14.)*

binds to the IL-7R.[17] IL-7 signaling potentiates the recombination of a VH region gene segment to an already rearranged DJH complex and, as discussed above, is critical for pro-B-cell proliferation which expands the size of clones expressing a particular Ig heavy chain.

The IL-7R is formed by the IL-7R α chain and the common cytokine γ chain. The Janus kinases (JAK) JAK3 and JAK1 are associated with the γc and IL-7 Rα, respectively and are critical for IL-7R mediated signaling. When IL-7 binds to the IL-7R, these JAK kinases are phosphorylated, and this in turn recruits signal transducer and activator of transcription 5 A (STAT5A) and STAT5B. The STAT molecules can then translocate to the nucleus where they act as transcriptional activators. The requirement for IL-7 in murine B-cell development is demonstrated by studies showing a block in B-cell development at approximately the pro-B-cell stage in IL-7 and IL-7R knock-out mice.

The precise role of IL-7 during human B-cell development is unclear. Human CD34$^+$CD19$^+$ pro-B cells proliferate in response to IL-7, but human B-lymphopoiesis may not be dependent on IL-7, because B cells are present in patients whose B-lineage cells express a mutated IL-7Rα chain. In addition, B cells are present in patients with X-linked severe combined immunodeficiency; these individuals have mutations in the gene encoding the cytokine common γ chain, which is part of the receptor for IL-7.[18]

FETAL B-CELL DEVELOPMENT

Most knowledge of fetal hematopoiesis is based on studies in mice. It has been long recognized that some blood cells in that species are generated prior to the appearance of HSCs in an early wave of hematopoiesis that occurs in tissues such as the yolk sac. It has been thought that this pre-HSC wave of development is restricted to the production of erythroid and selected myeloid cells, but there is strong evidence that innate-like B-1 and γδ T cells are also generated.[19] The contribution of B cells generated in this pre-HSC phase to the adult immune system remains to be determined. HSCs are generated at embryonic day 10.5 of murine gestation, and their B lineage progeny can be detected several days thereafter in fetal liver, bone marrow, and spleen.

Hematopoiesis initiates in the human yolk sac at 3 weeks of gestation, pre-B cells are present in human fetal liver by week 8 of gestation, and surface IgM$^+$ cells are detectable at week 9. IgM expressing cells have also been observed in additional human fetal tissues that include the omentum, the peritoneal cavity, and the spleen. Studies of human fetal B-cell development are limited and this is an area in need of further investigation.

B-1 B Cells

The B cells produced in adult BM, which constitute the majority of B cells in spleen and lymph nodes, are referred to as B-2 B cells. This nomenclature serves to contrast them with another functionally distinct population of mature B cells termed B-1 B cells. B-1 B cells are best characterized in mice where they constitute around 5% of total B lymphocytes. Murine B-1 B cells preferentially localize in serous cavities and in those sites can be distinguished by their unusual phenotype that includes high levels of sIgM, low levels of sIgD, and expression of CD11b, a determinant typically expressed on myeloid cells. B-1 B cells can be further subdivided into B-1a B cells that are CD5$^+$ and B-1b B cells that do not express this determinant. Antibodies from B-1 B cells, at least in mice, are required for protection against pathogens such as *Streptococcus pneumoniae*.

Mouse B-1 B cells are generated most efficiently from precursors that arise in the fetus.[20] Thus, while HSCs from fetal liver can efficiently generate both B-1 and B-2 B cells, those from the adult primarily produce B-2 B lymphocytes. These observations support the long-held view that B-1 cells are a distinct B-cell lineage preferentially derived from fetal precursors. The description of a phenotypically identifiable B-1 B-cell–specified progenitor that is preferentially generated in the fetus provided strong support for this view.

The regulation of B-1 and B-2 development is distinct, providing further support for the conclusion that these are distinct B-cell populations. For example, while B-2 B-cell development is blocked in IL-7 deficient mice, B-1 development can occur. The preferential generation of B-1 B cells from fetal progenitors has been linked to the expression of the Lin28b miRNA binding factor by fetal HSCs and B lineage progenitors. Adult HSCs are Lin28b$^-$, but ectopic Lin28b expression in adult hematopoietic stem/progenitor cells results in their ability to generate B-1 B cells, and B-1a B cells in particular.[21]

B-1a B cells in mice are CD5$^+$, and many studies have claimed to have identified human B-1 cells based on their simultaneous expression of CD5 and sIgM. These reports must be viewed with caution because CD5 is not a B-1–restricted determinant. The identification of B-1 B cells in humans has proven elusive, but a CD20$^+$ CD27$^+$ CD43$^+$ CD70$^-$ population of cord blood B cells that has properties consistent with their being classified as human B-1 cells has been described.[22] The further characterization of human B-1 B cells and studies to define their origin remain areas of active investigation.

SECONDARY LYMPHOID TISSUES

The bone marrow and the thymus are the sites of primary, antigen-independent lymphocyte development and are distinguished from secondary lymphoid organs that include the spleen, lymph nodes, and epithelial and mucosa-associated lymphoid tissues such as Peyer patches in the small intestine. Secondary lymphoid tissues are the sites of B-cell maturation and the initiation of humoral immune responses.

Anatomic Organization of Secondary Lymphoid Tissues

The spleen is divided into white pulp and red pulp (Fig. 22.5).[23] Red pulp functions as a site of extramedullary hematopoiesis early in fetal life and is a storage site for iron, erythrocytes, and platelets throughout life. White pulp is composed mainly of lymphoid cells and includes three regions referred to as the periarteriolar lymphoid sheath (PALS), the marginal zone (MZ), and primary and secondary follicles (see Fig. 22.5). In mice, the PALS contains abundant T cells and is found surrounding the central artery, but few T cells are present in this region in humans. The MZ is located at the outer portion of the white pulp.

The spleen lacks afferent lymphatics and samples foreign antigens in blood rather than lymphatic fluid. Unlike other organs that have a closed vascular circulation in which blood flows from arteries to veins through capillary beds, branches of the splenic artery penetrate the white pulp, forming an open sinusoidal network termed the *marginal sinuses*. This architecture ensures the interposing of blood, and blood-borne antigens, with the lymphoid areas of the white pulp. From the marginal sinuses, blood filters through the white pulp regions of the spleen and encounters resident B and T cells. Blood is drained via branches of the splenic vein, but an efferent lymphatic circulation also collects and drains the spleen. Beyond the white pulp, the splenic artery sends additional branches into the red pulp for further filtration and subsequent blood antigen surveillance that is accomplished by macrophages (see Fig. 22.5).

In contrast to the spleen which samples blood-borne antigens, fluid and cells gain entry to the convex surface of lymph nodes via afferent lymphatic vessels that drain into the subcapsular sinus. Lymphatic fluid in the subcapsular sinus then courses into the trabecular sinus network that runs perpendicular to the capsule through an area called the *cortex*. The cortex is composed of follicles and interfollicular zones. Follicles consist mainly of B cells, some T cells, and antigen presenting cells such as macrophages and follicular dendritic cells while interfollicular zones consist mainly of T cells and additional antigen presenting cells. These zones are separate but contiguous compartments where B cells and T cells initially encounter antigen. The net effect of antigenic exposure is the proliferation of antigen-specific lymphocytes (see Fig. 22.5).

In addition to lymph nodes and spleen, mucosa-associated lymphatic tissue (MALT) is a critical part of the secondary lymphoid system. As the name implies, the MALT is in physical proximity with the mucosa (i.e., the epithelium and associated connective tissue that line the surfaces of the body) and is found at sites where antigens most commonly breach these epithelial barriers in the gastrointestinal, respiratory, and genitourinary tracts. In some tissues, MALT forms relatively large structures that can be clearly distinguished histologically, such as the Peyer patches in the ileum and in the lymphoid tissue under the epithelium of the appendix. In these sites, perhaps because of the constant stimulation by microbial pathogens in the intestine, the MALT resembles lymphatic tissue in the spleen and lymph nodes, with well-demarcated primary and secondary follicles that contain primarily B cells and intervening T-cell-rich zones.

Transitional B Cells

Even though they express a BCR, newly produced B cells are functionally immature, and they complete their maturation in secondary lymphoid tissues soon after their arrival from the bone marrow. These events are well defined in the mouse spleen where newly generated B cells mature through transitional stages of development before generating MZ or follicular (FO) B cells (see Fig. 22.1).

The most immature transitional cells, referred to as transitional 1 (T1) B cells, localize at the outer edge of the PALS. T1 B cells give rise to a more mature population of B cells, referred to as transitional 2 (T2) cells (see Fig. 22.1). The T1 and T2 population can be phenotypically distinguished, respond differentially to developmental stimuli, and undergo a considerable degree of selection during the T1 to T2 transition. For example, T1 cells with BCR specificities for

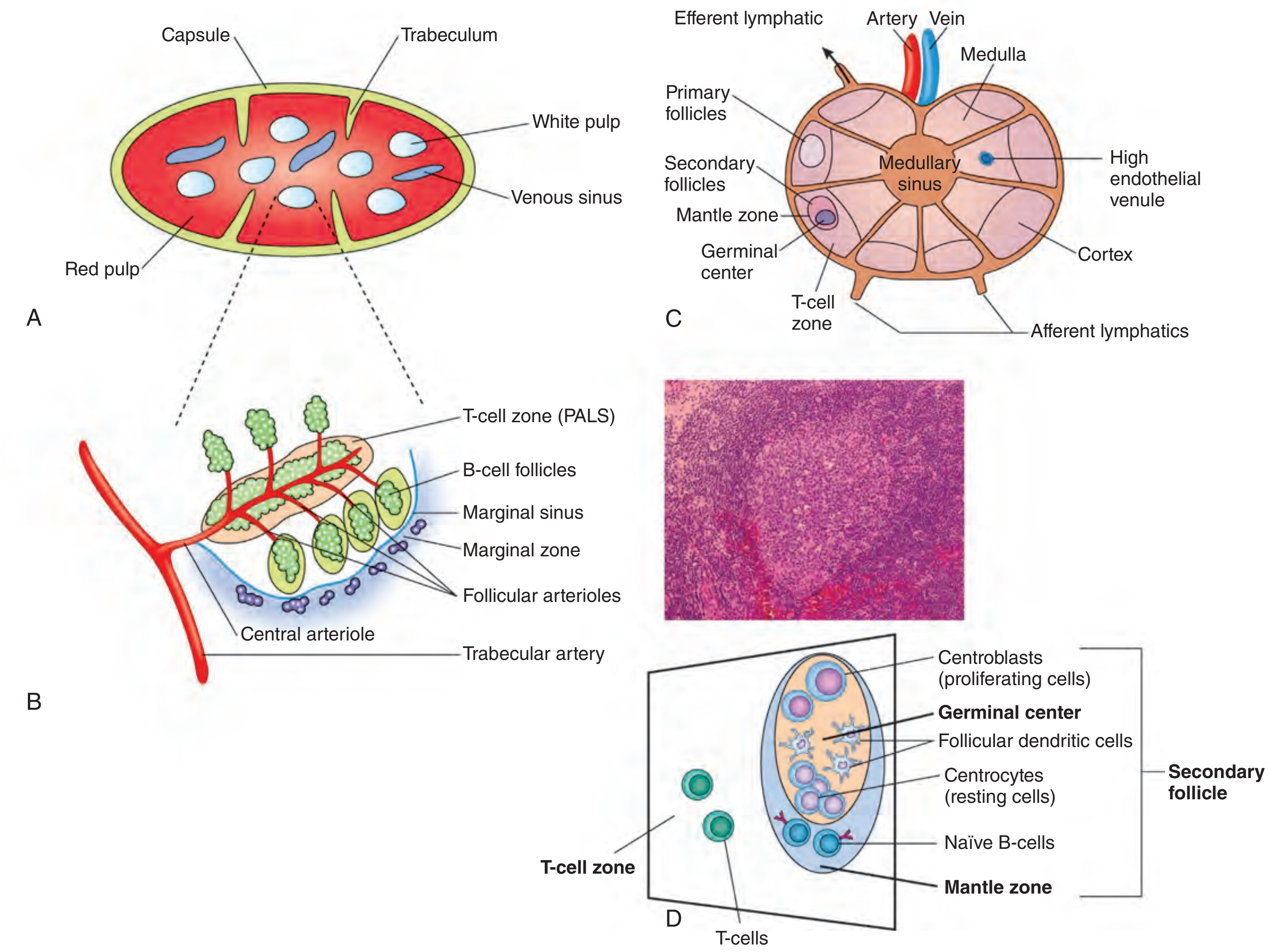

Capsule
Trabeculum
White pulp
Venous sinus
Red pulp
A
T-cell zone (PALS)
B-cell follicles
Marginal sinus
Marginal zone
Follicular arterioles
Central arteriole
Trabecular artery
B
Efferent lymphatic
Artery
Vein
Medulla
Primary follicles
Secondary follicles
Medullary sinus
High endothelial venule
Mantle zone
Germinal center
Cortex
T-cell zone
C
Afferent lymphatics
Centroblasts (proliferating cells)
Germinal center
Follicular dendritic cells
Centrocytes (resting cells)
Secondary follicle
Naïve B-cells
Mantle zone
T-cell zone
D
T-cells

Figure 22.5 ORGANIZATION OF B CELLS IN SECONDARY LYMPHOID ORGANS. (A) The spleen consists of the red pulp and white pulp. The red pulp is the site of myeloid cells which ingest and remove opsonized antigens and damaged red blood cells from the circulation. (B) The white pulp consists of lymphoid cells, with a periarteriolar T-cell sheath (PALS), a marginal zone, and mixed B- and T-cell follicles. (C) Lymph nodes are surrounded by a capsule. Afferent lymphatics draining tissues enter the node on the convex side into the capsule. Fluid and cells drain through the node and collect in the medullary sinus, where the fluid leaves the node through efferent lymphatics to rejoin the lymphatic circulation. The outer rim of the node is called the cortex and contains primary follicles composed of naïve, nonproliferating B cells that have not encountered antigens and secondary follicles with proliferating B cells in the germinal center. (D) A section of lymph node showing a high magnification of a follicle. The schematic shows the regions of the follicle.

blood-borne self-antigens are deleted by negative selection. Positive selection via BCR signaling must occur, and if it does not, the T2 cells will die by neglect. The survival of T2 cells, but not T1 cells, is also dependent on the B-cell growth factor BAFF (BLyS, TALL-1, THANK, zTNF4), which is produced by the splenic microenvironment. A fraction of T2 cells are no longer in Go phase of the cell cycle, suggesting they are in a more activated state than is the case for T1 cells. An additional population, transitional 3 (T3) B cells, has also been described and proposed to be either an anergic population or a stage of development between T2 and naïve B cells.

Whether transitional cells mature into MZ or FO B cells is influenced by the extrinsic signals they receive.[24] Weak signaling through the BCR along with engagement of the Notch2 receptor promotes entry into the MZ B-cell compartment. There is a marked depletion of MZ B cells when the Notch2 pathway is blocked. Self-reactive B cells are enriched within the MZ population, suggesting that weak self-antigens may play an important role in their generation. This feature may permit them to respond rapidly to cross-reactive epitopes on pathogens. BCR signals, along with activation of the alternative NF-κB pathway, are required for T2 cells to mature into an FO B cell. It is estimated based on murine studies that only 1% to 3% of splenic transitional B cells develop into mature, naïve B cells.

In addition to MZ and FO B cells, an additional population of B regulatory (Breg) cells exists. Bregs have been identified by their production of IL-10 but they also function via IL-10 independent mechanisms. The developmental origin(s) of these cells and their role in the regulation of immune responses is an area of active investigation.[25]

Immune Responses in Secondary Lymphoid Tissues

A feature of the spleen that distinguishes it from other secondary lymphoid tissues is the presence of an MZ, and this is the site where MZ B cells localize along with macrophages and dendritic cells. MZ

B cells play a role in T-independent responses elicited by polymeric antigens, such as polysaccharides, that are composed of repetitive antigenic epitopes. On antigen binding, MZ B cells undergo rapid proliferation and maturation into plasma cells that secrete low-affinity IgM and IgG3 antibodies that provide a first line of defense. The rapid response of MZ B cells to antigen has led to the idea that this effector population, similar to B-1 B cells, constitutes a key element of the innate immune response to bacterial and other selected pathogens. Human MZ B cells are heterogeneous and include a large proportion of $CD27^+$ IgM^+ unswitched memory B cells with somatically mutated Ig heavy chains. The origin of this cell population is unclear but is presumed to be antigen driven yet may not require T-cell help. Although MZ B cells in rodents appear to be a static, nonrecirculating population, cells with a $CD27^+$ IgM^+ MZ phenotype are clearly present in human peripheral blood as well as other lymphatic tissues. However, it remains unclear if these circulating memory cells can reenter the splenic MZ. The poor response of infants to some types of T-independent antigens correlates with the fact that the MZ is not fully formed until the age of 1 to 2 years. In addition, splenectomized individuals are more susceptible to infection with some bacteria, owing to the deficient antibody response to capsular polysaccharides.

FO B cells circulate and take up residence in follicles found in lymphoid tissues that include lymph nodes, intestinal Peyer patches, and the spleen following their generation. The B cells in *primary* follicles are mature FO cells that have not yet encountered antigen and initiated an immune response. Although some B cells in the MZ can respond to T-dependent antigens, most B cells that do so are FO B cells. After their binding of a T-dependent antigen (as soluble antigen, indirectly via presentation by a local antigen presenting cell, or as an immune complex) mature, naïve B cells in primary follicles undergo a blastogenic response. Some of these cells will immediately mature into plasma cells that secrete low-affinity IgM to provide a rapid initial response to infection. In response to T-cell help, however, other B cells undergo further proliferation and differentiation forming *secondary* follicles. The histologic appearance of the follicle changes as these events evolve. The nonresponsive B cells form an outer mantle zone surrounding the proliferating, antigen-responsive B cells in a central *germinal center*. Germinal center B cells are shielded from soluble antigens and are exposed only to a unique set of antigens presented by follicular dendritic cells.

Germinal Centers

Germinal centers are classically divided into two compartments, denoted as the dark and light zones based on their appearance under light microscopy (see Fig. 22.5). Dark zones are located adjacent to the T-cell areas and contain a high density of proliferating B cells termed *centroblasts*, which are large cells with a high nuclear:cytoplasmic ratio that do not express surface BCR. The light zone has a lower cellular density due to the presence of an extensive loose network of follicular dendritic cells, and this imparts the "light" appearance. B cells in the light zone are termed *centrocytes*, which in contrast to the centroblasts, are small B cells expressing surface BCR.

Within the germinal center, the series of sequential shuttling and reentry of B cells into the dark and light zones is termed the germinal center reaction or the B-cell selection process. The cyclic reentry model proposes that centroblasts in the dark zone undergo cell division, class switching, and somatic hypermutation, discussed in the next section. Next, they exit the cell cycle and migrate into the light zone to interact with antigen-presenting follicular dendritic cells and T follicular helper cells (Tfhs). In light zones, B cells with increased affinity for antigen are preferentially selected for survival by receiving vital signals from Tfh cells; in contrast, B cells with impaired or absent antigen binding undergo apoptosis and clearance by resident macrophages, known as tingible body macrophages.

Selected centrocytes in the light zone are thought to return to the dark zone to undergo further rounds of proliferation, affinity maturation, and selection to improve the affinity of B-cell repertoire. Positively selected germinal center B cells eventually leave the germinal center, differentiating into memory B cells or plasma cells possessing somatically mutated Ig genes that encode for a high-affinity BCR. The modified capability of antigen-selected memory B cells to generate a fast, highly specific humoral immune response upon a second encounter with the same pathogen forms the mechanistic basis of humoral memory. Plasma cells are long-lived cells that take up residence in the BM and spleen, and are responsible for maintaining high levels of Ig seen in the serum.

Immunoglobulin Class Switching and Affinity Maturation

Germinal center B cells undergo three distinct modifications to increase the affinity of the Ig for antigen. Ig class switching involves deletions of germline DNA resulting in religation of the VDJ complex to downstream heavy chain C region genes, such as γ3, γ1 γ2b, γ2a, ε, and α. These DNA deletions are believed to occur at or near nucleotide sequences called switch regions that are located in the intron 5′ to each CH exon. These class-switching events are highly regulated, secondary-differentiation events potentiated by helper T cells and their secreted products. Note that Ig class switching is distinct from the previously discussed differential splicing events that allows the newly produced B cell to express the same VDJ complex associated with either the μ or δ heavy chain C regions.[26]

B cells may also undergo receptor editing. Receptor editing usually involves modifications of the existing light chain in which an upstream V region segment joins to a downstream J region gene. As a result, the genetic region encoding the originally expressed light chain is deleted. RAG-1 and RAG-2 expression are required for this process to occur. It has been proposed that B cells in germinal centers might reactivate *Rag* gene expression to mediate events such as receptor editing. However, that this occurs has been questioned. Instead, receptor editing in splenic B cells may be limited to a small subset of recent immature BM immigrants that enter germinal centers before their *Rag* expression has been extinguished.

Somatic hypermutation provides the primary means to increase antibody affinity. During this process, single-nucleotide exchanges, deletions, and mutations are introduced into the genes encoding the antibody-binding regions of the Ig receptor. A B-cell–specific gene that encodes activation-induced cytidine deaminase (AID), which is expressed in germinal center B cells, has been identified. AID is a putative RNA-editing enzyme that acts as a cytidine deaminase and has been shown to be indispensable for somatic hypermutation and class switch recombination.[27]

As discussed in the previous section, these events are dependent on signals delivered to the antigen-responsive B cells by antigen-specific Tfh lymphocytes that migrate into the germinal center from the PALS or the T-cell zone within lymph nodes. Tfh cells mediate their effects on B cells through the secretion of cytokines as well as through direct intercellular contacts, and these stimuli result in B-cell growth, differentiation, and Ig class switching. For example, CD40 is a T-cell–surface glycoprotein encoded by a member of the tumor necrosis gene family, and its ligand is expressed on B cells. CD40 ligand knockout mice do not form germinal centers, and humans who do not express CD40 ligand have X-linked hyper IgM immunodeficiency. Additional key T-cell costimulatory signals include cytokines such as IL-4, IL-10, IL-21, and interferon γ secreted by T cells in response to their activation via the "inducible costimulator" ICOS. Humans lacking expression of ICOS on T cells have adult-onset common variable immune deficiency, leading to a severe deficit in generation of class-switched and memory B cells.

There are two unintended consequences of affinity maturation. One is that autoreactive clones may be inadvertently generated. The other is the development of B-cell lymphoma. Lymphomagenesis results in part from the fact that vigorous B-cell proliferation, combined with the changes at the DNA level that lead to molecular alterations, may promote malignant transformation.[28] Numerous studies have assigned B-cell lymphomas to each of the normal B-cell counterparts shown in Fig. 22.1. Events that limit differentiation of immature or activated mature B cells can also promote malignant transformation. Importantly, many genetic lesions leading to B-cell lymphoma directly impact the BCR signaling cascade, and an improved understanding of these signaling events may lead to new therapies. These include application of Btk-specific small molecule inhibitors in a broad range of human lymphomas.[29]

EFFECTS OF AGING ON B-CELL DEVELOPMENT AND FUNCTION

Studies of both rodents and humans have demonstrated that the quality of the immune response is diminished with age. Such declines are not incompatible with life, but they may become a factor when the individual is required to mount an immune response to a novel pathogen or respond to vaccination.[30]

The production of B cells from HSCs is severely attenuated with age, and the frequency and number of CLPs, pro-B cells, and pre-B cells is significantly reduced in the BM of old mice. Similar age-related reductions in B lymphopoiesis also occur in humans. B-cell progenitors from young and old mice have been compared, and this has revealed that expression of the *Ink4a* and *Arf* genes increases with age and is a factor that contributes to the diminished proliferation and increased apoptosis of aging B-cell progenitors.

In addition to declines in primary B lymphopoiesis, which reduces the number of newly generated naïve B cells that are produced, aging also affects mature B cells resident in peripheral lymphoid tissues. For example, in addition to an accumulation of memory B cells in the spleen of old mice, the Igs they produce are less protective because of low titer and affinity. Some of these defects may be intrinsic to the B cells but others may be secondary to age-related defects in T cells.

While the molecular changes that underlie the aging of HSCs and B lineage cells are being delineated, the events that trigger the aging process are incompletely defined. One possibility is that HSCs and lymphoid progenitors are genetically programmed to age. An alternative hypothesis is that these age-related events occur secondary to changes in the local and systemic environments.

REFERENCES

1. Hardy RR, Kincade PW, Dorshkind K. The protean nature of cells in the B lymphocyte lineage. *Immunity*. 2007;26(6):703–714.
2. Levesque MC, Clair St. EW. B cell-directed therapies for autoimmune disease and correlates of disease response and relapse. *J Allergy Clin Immunol*. 2008;121:13–21.
3. Blom B, Spits H. Development of human lymphoid cells. *Annu Rev Immunol*. 2006;24:287–320.
4. Ichii M, Oritani K, Kanakura Y. Early B lymphocyte development: similarities and differences in human and mouse. *World J Stem Cells*. 2014;66(4):421–431.
5. Mandel EM, Grosschedl R. Transcription control of early B cell differentiation. *Curr Opin Immunol*. 2010;22(2):161–167.
6. Cobaleda C, Schebesta A, Delogu A, Busslinger M. Pax5: the guardian of B cell identity and function. *Nat Immunol*. 2007;8(5):463–470.
7. Zheng B, Xi Z, Liu R, et al. The function of microRNAs in B-cell development, lymphoma, and their potential in clinical practice. *Front Immunol*. 2018;9:936.
8. Lin SG, Ba Z, Alt FW, Zhang Y. RAG chromatin scanning during V(D)J recombination and chromatin loop extrusion are related processes. *Adv Immunol*. 2018;139:93–135.
9. Schatz D, Swanson P. V(D)J recombination: mechanisms of initiation. *Annu Rev Genet*. 2011;45:167–202.
10. Yarkoni Y, Getahun A, Cambier J. Molecular underpinning of B-cell anergy. *Immunol Rev*. 2010;237(1):249–263.
11. Mårtensson I, Keenan R, Licence S. The pre-B-cell receptor. *Curr Opin Immunol*. 2007;19(2):137–142.
12. Reth M, Neilsen P. Signaling circuits in early B-cell development. *Adv Immunol*. 2014;122:129–175.
13. Clark M, Manda lM, Ochiai K, Singh H. Orchestrating B cell lymphopoiesis through interplay of IL-7 receptor and pre-B cell receptor signalling. *Nat Rev Immunol*. 2014;14(2):69–80.
14. Zehentmeier S, Pereira JP. Cell circuits and niches controlling B cell development. *Immunol. Rev*. 2019;289:142–157.
15. Buchner M, Swaminathan S, Chen Z, Müschen M. Mechanisms of pre-B-cell receptor checkpoint control and its oncogenic subversion in acute lymphoblastic leukemia. *Immunol Rev*. 2015;263(1): 192–209.
16. Pinjo S, Frenette PS. Haematopoietic stem cell activity and interactions with the niche. *Nat Rev Mol Cell Biol*. 2019;20:303–320.
17. Barata JT, Durum SK, Seddon B. Flip the coin: IL-7 and IL-7R in health and disease. *Nat Immunol*. 2019;20:1584–1593.
18. Giliani S, Mori L, de Saint Basile G, et al. Interleukin-7 receptor alpha (IL-7Ralpha) deficiency: cellular and molecular bases. Analysis of clinical, immunological, and molecular features in 16 novel patients. *Immunol Rev*. 2005;203:110–126.
19. Hadland B, Yoshimoto M. Many layers of embryonic hematopoiesis: new insights into B-cell ontogeny and the origin of hematopoietic stem cells. *Exp Hematol*. 2018;60:1–9.
20. Montecino-Rodriguez E, Dorshkind K. B-1 B cell development in the fetus and adult. *Immunity*. 2012;36:13–19.
21. Yuan J, Nguyen C, Liu X, Kanellopoulou C, Muljo S. Lin28b reprograms adult bone marrow hematopoietic progenitors to mediate fetal-like lymphopoiesis. *Science*. 2012;335(6073):1195–1200.
22. Rothstein TL, Quach TD. The human counterpart of mouse B-1 cells. *Ann N Y Acad Sci*. 2015;1362:143–152.
23. Chadburn A. The spleen: anatomy and anatomical function. *Semin Hematol*. 2000;37:13–21.
24. Pillai S, Cariappa A. The follicular versus marginal zone B lymphocyte cell fate decision. *Nat Rev Immunol*. 2009;9(11):767–777.
25. Alhabbab RY, Nova-Lamperti E, Aravena O, et al. Regulatory B cells: development, phenotypes, functions, and role in transplantation. *Immunol Rev*. 2019;292:164–179.
26. Matthews A, Zheng S, DiMenna L, Chaudhuri J. Regulation of immunoglobulin class-switch recombination: choreography of noncoding transcription, targeted DNA deamination, and long-range DNA repair. *Adv Immunol*. 2014;122:1–57.
27. Pavri R, Nussenzweig M. AID targeting in antibody diversity. *Adv Immunol*. 2011;110:1–26.
28. Mlynarczyk C, Fontan L, Melnick A. Germinal center-derived lymphomas: the darkest side of humoral immunity. *Immunol Rev*. 2019;288:214–239.
29. Young RM, Phelan JD, Wilson WH, Staudt LM. Pathogenic B-cell receptor signaling in lymphoid malignancies: new insights to improve treatment. *Immunol Rev*. 2019;291:190–213.
30. Montecino-Rodriguez E, Berent-Maoz B, Dorshkind K. Causes, consequences and reversal of immune system aging. *J Clin Invest*. 2013;123(3):958–965.

CHAPTER 23

COMPLEMENT AND IMMUNOGLOBULIN BIOLOGY LEADING TO CLINICAL TRANSLATION

David J. Araten, David E. Isenman, and Michael C. Carroll

Complement and immunoglobulins represent the two major arms of humoral immunity and are discussed in detail in respective major subsections of this chapter. The complement section begins with a comprehensive overview of pathway activation, regulation, and effector mechanisms, followed by discussion of the pathological consequences of various complement deficiencies and polymorphisms. Next discussed is complement's integral role in B-cell and T-cell immunity. Additional topics include the surprisingly detrimental role of complement in cancer control by the immune system, and links between the complement and coagulation systems. The immunoglobulin section similarly begins with an overview of fundamentals and proceeds to discuss how human- and animal-derived immunoglobulins are employed as pharmacologic agents. This is followed by discussion of monoclonal antibody technologies, which have led to a vast array of new drug approvals. To bring this chapter "full circle," we discuss how monoclonal antibodies that target complement have revolutionized the treatment of paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS). Finally, a promising newly approved anti-complement peptide is described along with a glimpse at some other exciting new complement-targeted drugs on the horizon.

THE COMPLEMENT SYSTEM: AN OVERVIEW

Complement refers to a group of greater than 40 distinct proteins that play a pivotal role in host defense against infection. In the 1880s, the serum factors involved in host response to pathogens were placed into two categories based on sensitivity to heat. Whereas the heat-stable component, antibody, was recognized as being specific for the invading pathogen and arose after immunization, the heat-labile (>56°C) fraction displayed nonspecific killing activity. The heat-labile fraction acted to complement the antibody-mediated lytic killing of targeted organisms.[1–3]

In addition to its lytic role in the effector arm of the antibody response, the complement system serves several other functions. First, components of the complement system are involved in clearance of targeted microorganisms by the process of opsonization. *Opsonization* is the coating of a particle with proteins that facilitate phagocytosis of the particle by tissue macrophages and activated follicular dendritic cells (FDCs) as well as binding by receptors on peripheral blood cells.[4,5] Second, complement promotes inflammation by releasing small peptide fragments from complement proteins. These peptides cause mast cell degranulation, smooth muscle contraction, and directed migration (chemotaxis) of motile cells to sites of inflammation.[6,7]

Complement can be activated via three distinct pathways: classical, lectin, and alternative. Although all depend on different molecules for their initiation, eventually they converge to generate the same set of effector molecules. Each of these pathways is described here (Fig. 23.1).

Classical Pathway

The classical pathway (CP), so called because it was the earliest studied arm of the complement system, directly links the innate and acquired immune systems. There are nine proteins in the CP. As a matter of terminology, each CP component protein is designated with an uppercase C followed by a number. Fragments of these proteins generated by cleavage during the complement cascade are designated with a lower-case letter suffix (e.g., C3a, C3b). In general, the smaller product arising from a proteolytic activation step is given the fragment designation "a," and the larger product is designated "b." The sole exception to this rule is for the naming of the C2 proteolytic activation fragments, where, for historical reasons, the larger fragment is C2a and the smaller one is C2b.

C1, the first component of the CP, binds and is activated by the Fc portion of the antibody molecule. C1q is a macromolecule complex composed of three individual protein subunits: C1q, C1r, and C1s.[8–12] The largest of these subunits, C1q, is an 18-chain molecule with six copies each of three chains: A, B, and C. Structurally, C1q consists of a central core with six radiating arms. Each arm possesses a triple helical structure similar to collagen that is capped at the end with a head region consisting of a quaternary assembly of the globular domains from each of chains A, B, and C. C1q, largely through ionic interactions, links the C1 complex to the antibody molecule. In addition to its capacity for binding to antibody molecules, C1q possesses the capability to bind directly to the surfaces of some microorganisms and apoptotic cells, not unlike mannan-binding lectin (MBL; see Lectin Pathway discussion, later).

Associated with C1q are two molecules each of C1r and C1s. In unactivated C1, C1r and C1s are proenzyme serine esterases. Upon binding of C1q with an array of target-associated immunoglobulin G (IgG) Fc regions, which themselves have a propensity to form hexamers on the target surface,[13] or directly to surface molecules of the pathogen, a conformational distortion of the C1q arms occurs that leads to reciprocal autoactivation of the associated C1r molecules. The active form of C1r then cleaves its associated C1s to generate an active serine protease.

Activated C1s is responsible for cleaving C4 and C2, the next two proteins in the complement pathway. Cleavage of C4 yields two fragments: C4a and C4b. C4b possesses a highly reactive thioester group within its C4d/TED region (structurally analogous to the C3d/TED region of C3 discussed further below) that allows it to bind covalently to molecules in the immediate vicinity of its active site. Only a small proportion of the C4b produced binds to proteins or carbohydrates on the targeted surface; the rest is inactivated by reaction with water in the surrounding milieu. This helps to prevent inadvertent C4b binding to surrounding host cells.

C2, the next substrate in the CP cascade, is susceptible to cleavage by C1s. Upon association with C4b, C2 is cleaved by activated C1s into two fragments: C2a and C2b. C2a, which is now an active serine protease, remains bound to C4b, thereby confining it to the targeted surface. C4b2a is termed the *C3 convertase*, an enzymatic complex that is responsible for binding and cleaving C3, the next component in the cascade. The function of the C3 convertase is to cleave large numbers of C3 molecules to produce C3b and C3a. Nascently activated C3b, similar to C4b, also possesses a highly reactive thioester bond, allowing a portion of the nascent C3b to covalently bind to the targeted surface (opsonization of the target) and thereby mark it for phagocytosis. The activated thioester in the bulk of the nascent C3b becomes water hydrolyzed and can no longer bind to a target. By

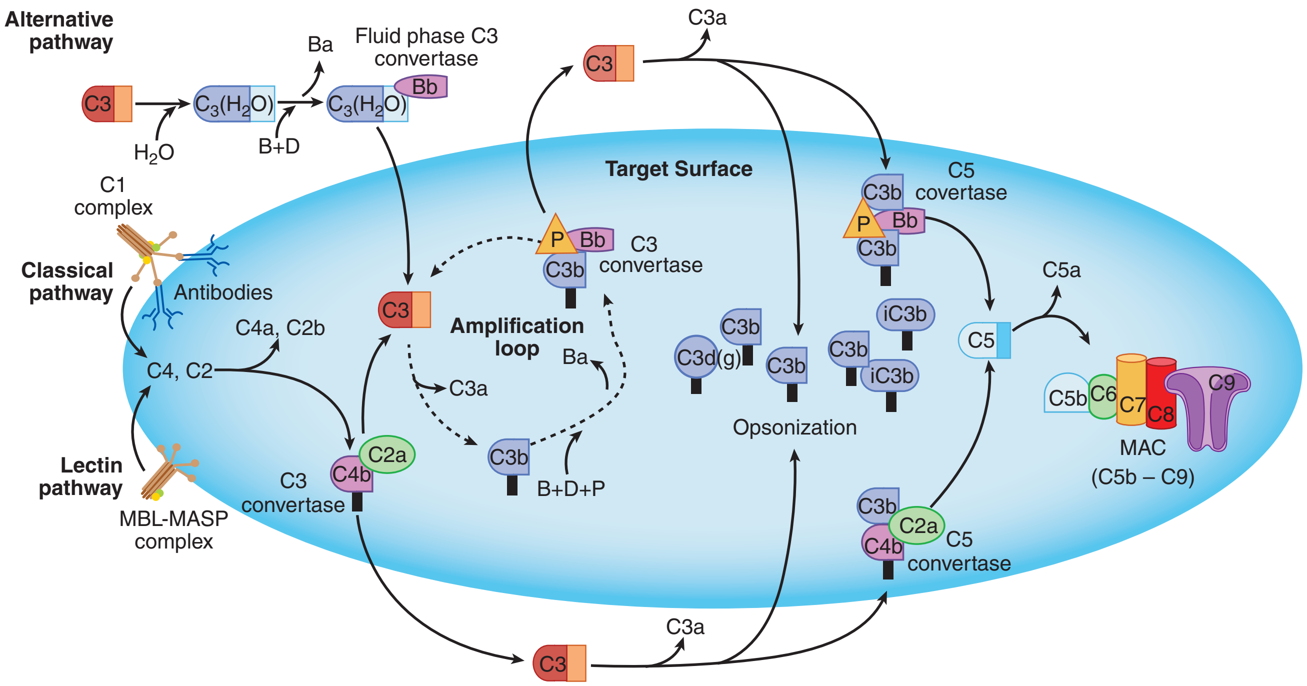


Figure 23.1 SCHEMATIC OVERVIEW OF THE COMPLEMENT CASCADE. The initiation events of the classical, lectin, and alternative pathways are at the left. The C1 complex of the CP recognizes an array of antibody Fc regions via its C1q pattern recognition entity. The induced conformational distortion of the C1q arms leads to the autoactivation of the associated $C1r_2C1s_2$ enzymatic complex. MBL of the lectin pathway's MBL-MASP complex is similarly a pattern recognition molecule for pathogen-specific carbohydrate arrays, whose binding leads to the activation of the associated MASP1 or MASP2 enzymatic complexes, albeit via a distinct mechanism from C1 (see text). Both activated C1s and activated MASP-2 can cleave C4, with some of the resulting nascent C4b covalently binding to the target surface (covalent bond denoted by thick black vertical stroke). The binding of C2 to the target-bound C4b, followed by C2's cleavage by either activated forms of C1s, MASP-2, or MASP-1 yields the CP C3 convertase, C4b2a. Some nascently activated C3b molecules deposit on the target as opsonins, where they are ligands for immune cell-associated complement receptors either as C3b, or as its subsequent degradation products iC3b, C3dg, and C3d. If nascent C3b binds to the CP C3 convertase, the latter becomes capable of cleaving C5. The AP initiates spontaneously in the fluid phase when factor B is recruited to the thioester-hydrolyzed C3b-like entity, $C3(H_2O)$. Cleavage by factor D yields a fluid-phase initial AP C3 convertase $C3(H_2O)Bb$. Some of the C3b generated by this entity may covalently bind to the target surface, whereupon a much more efficient surface-bound, and properdin-stabilized, AP C3 convertase, C3bBbP, assembles. A surface-bound AP C3 convertase can also assemble using C3b deposited via the CP as a nidus. Via the depicted amplification loop, the AP C3 convertase deposits many opsonic C3b molecules on the target surface. As for the CP, the association of a second C3b molecule with the AP C3 convertase enables the latter to cleave C5. The generation of C5b by either the CP or AP C5 convertase initiates the self-assembly of the $C5b678(9)_n$ membrane attack complex (MAC). Of the liberated fluid-phase complement split products, namely C4a, Ba, C2b, C3a, and C5a, the latter two have well established roles as inflammatory mediators. *(Adapted from Fig. 1 of Chen, JY, Cortes, C, and Ferreira, V. Properdin: a multifaceted molecule involved in inflammation and disease. Mol Immunol. 2018;102:588–572.Copyright © 2018 Elsevier Ltd. All rights reserved.)*

contrast, all of the C3a fragment remains in solution, where it initiates a local inflammatory response.

An Ig-independent mechanism for activation of C1q has been identified.[14] The lectin protein Sign R1, which is expressed on a subset of macrophages within the outer marginal zone sinus of the spleen, is capable of capturing to the surface of the marginal zone macrophage both C-polysaccharide–containing bacteria and C1q. The recruited C1 becomes activated and propagates the CP to deposit C3b on the captured bacterium. Sign R1 was also identified on the surface of resident dendritic cells (DC) in draining lymph nodes (LN) and shown to be important in capture and transport of inactivated influenza virus.[15] This novel pathway provides an alternative innate recognition of pathogens leading to activation of the CP of complement.

Lectin Pathway

Before continuing with the discussion of the complement cascade at the point of C3 cleavage by convertase, we turn our attention to the other two complement-activating pathways: the lectin pathway (LP) and the alternative pathway (AP). What will become evident is that all of these pathways converge at C3.

The LP is a relatively recently described pathway for complement activation.[16,17] MBL, similar to C1q, is a triple helical structure with collagen-like arms coupled to C-type lectin globular domains, which form carbohydrate recognition domains (CRDs) that bind repeating polysaccharides present on the surfaces of many microorganisms. Most commonly MBL circulates as an oligomer of three or four collagenous stem-associated subunits, where each subunit consists of a homotrimer of MBL chains. The MBL CRDs attach to the termini of polymeric carbohydrate chains in the following order: mannose > GlcNAc > fucose > glucose. The greatest avidity appears to be for repeating mannose-based structural patterns typical of microbial surfaces. On vertebrate cells, these sugars are not as dense as on microbial surfaces, thus decreasing the avidity of the MBL-binding interaction, and furthermore, they often are covered by sialic acid residues, thus limiting recognition by MBL. The MBL-associated serine proteases MASP-1 and MASP-2, whose domain architecture is similar to C1r and C1s, predominantly bind as homodimeric zymogens to separate MBL oligomers. Upon MBL binding to polysaccharides on a pathogen surface, MASP-1 and MASP-2 become activated. The mechanism of activation has recently been clarified and is distinctly different from the conformational distortion-based intracomplex mechanism described above for the C1 activation. Although it had originally been thought that MASP-2 was capable of autoactivating itself when MBL bound to its target, and that MASP-1 played a non-essential and ill-defined augmentary role,[14] under physiological conditions autoactivated MASP-1 has now been firmly established to be the obligatory activator of MASP2.[18,19] Although the relationship between MASP-1 and MASP-2 parallels that of C1r to C1s in the C1 activation mechanism, what is decidedly different is that MASP-1 autoactivation is intercomplex, as is MASP-2 activation by autoactivated MASP-1. Specifically, it is the juxtaposition through clustering of the MBL–MASP-1 and MBL–MASP-2 complexes brought about by MBL binding to the target surface that leads to the intercomplex autoactivation of MASP-1, and the subsequent cleavage of MASP-2 by autoactivated MASP-1 that is on a neighboring complex.[20] Activated MASP-2 acts similar to C1s, cleaving C4 and C2 and thereby forming a C3 convertase, C4b2a, as found in the CP.[21] Besides its role in cleaving zymogen MASP-2, activated MASP-1 also cleaves C2, but not C4. Nevertheless, given the approximately 24-fold higher serum concentration of MASP-1 relative to MASP-2, this would ensure the efficiency of C2 activation on C4b deposited near an MBL-MASP2 complex.

While MBL was the microbial pattern recognition molecule initially identified in the LP, collectin-10 (also known as collectin-liver 1, CL-L1), collectin-11 (also known as collectin-kidney 1, CL-K1), and the ficolins M, L, and H (also known as ficolins-1, -2, -3) are collagen triple helix-containing paralogues of MBL in serum, which also associate with MASP-1 and MASP-2[22] and which undergo activation in a similar manner to the MBL-MASP complexes.[20] CL-L1 and CL-K1, like its collectin family member MBL, have C-type lectin CRDs, and have a carbohydrate binding spectrum that largely overlaps that of MBL. However, unlike the homotrimeric subunit composition of MBL, CL-K1 and CL-L1 mostly circulate as heteromeric subunit complexes, referred to as CL-LK, with each arm consisting of CL-K1 and CL-L1 in a 2:1 ratio.[23] In contrast to the collectins, the globular regions of the respective ficolin chains bear a fibrinogen-like domain fold and they recognize acetyl groups, be they on carbohydrate (e.g., *N*-acetylglucosamine) or non-carbohydrate entities (e.g., *N*-acetylglycine or acetylcholine).[22]

MBL serum concentration can differ by up to 1000-fold among individuals, with those having low circulating MBL apparently more vulnerable to infections. MBL insufficiency appears to be a particular risk factor for infections in infants and individuals undergoing chemotherapy or immunosuppression treatment.[24]

Gene-targeted knock-out mouse models deficient in MBL components have been described. In general, in pathogenic microbe infection models, such as *Candida albicans* or *Staphylococcus aureus*, MBL knock-out mice showed increased susceptibility to systemic infection and relatively much higher mortality compared to wild type.[25,26]

Alternative Pathway

The AP may represent one of the earliest forms of innate immunity. Unlike the CP or LP pathway, the AP can be fully activated in the absence of specific pathogen binding by a “recognition” equivalent to C1q or MBL.[27] In fact, the AP is always “on” at a low level. In addition, the AP forms and uses the distinct C3 convertase C3bBb.[28]

Complement C3 is a disulfide-linked two-chain protein, denoted as α and β, and having a combined apparent molecular weight of approximately 200 kDa. The crystal structure of native C3, shown as a domain-colored ribbon model in Fig. 23.2A, identified 13 distinct domains, including the thioester domain (TED), which contained the covalent binding site.[30] In the native molecule, the intramolecular thioester bond, formed between the side chains of cysteine and glutamine residues within the sequence CGEQ, is buried within a hydrophobic interface formed between the TED and MG8 domains, which is nevertheless close to the protein’s surface. The subsequent determination of the atomic structure of the activated form of C3 (i.e., C3b) demonstrated a dramatic shift in the location of the TED.[31–33] Proteolytic cleavage releases the C3a anaphylatoxin peptide, and the TED becomes fully exposed to engage potential targets (see structure-based depiction of C3b in Fig. 23.2B). Thus the dramatic shift in structure also exposes potential binding sites for factor B of the AP and competing sites for regulators of C3b, such as factor H (FH), membrane cofactor protein (MCP/CD46), complement receptor type 1 (CR1/CD35), and decay accelerating factor (DAF/CD55; all described later in this section). At a low so-called “tick-over” level, the thioester bond undergoes spontaneous hydrolysis, forming $C3(H_2O)$. This conformationally altered C3b-like form of C3 (see Fig. 23.2B) allows for binding to factor B, a plasma protein. Factor B is a serine protease that is approximately 30% identical to C2. The binding of factor B by $C3(H_2O)$ allows factor D, another protease, to cleave factor B to form Ba and Bb. Bb remains associated with $C3(H_2O)$ to form the $C3(H_2O)Bb$ complex. Factor D appears to function as a serine protease in its native state but can cleave factor B only when bound to C3. Recently, there has been an interesting connection found between factor D and a component of the LP. MASP-1 and MASP-3 are alternative splice products of the same gene with the difference being in the exons encoding the serine protease domain. It was found that a *MASP-1/MASP-3* knockout mouse completely lacked AP functionality. Upon further investigation, it was determined that the secreted factor D in this mouse possessed a five-residue propeptide at its amino terminus, suggesting that either MASP-1 or MASP-3 mediated its removal.[34] Subsequently, it was determined that under physiologic conditions, it was solely MASP-3 that was responsible for removing the propeptide from zymogen FD and further, that unlike MASP-1 or MASP-2, MASP-3 circulated predominantly in a proteolytically active form.[35]

$C3(H_2O)Bb$ is an enzymatic complex capable of cleaving native C3. This complex is a fluid-phase C3 convertase. Although it is formed only in small amounts, it can cleave many molecules of C3. Much of the C3b produced in this process is inactivated by hydrolysis, but some attaches covalently to the surface of host cells or pathogens. C3b bound in this way is able to bind factor B, allowing its cleavage by factor D to yield Ba and Bb. The result is the formation of C3bBb, a C3 convertase akin to C4b2a found in the classical and MBL pathways, with the capability of initiating an amplification cascade (see Fig. 23.1).

In light of the nonspecific nature of C3b binding in the AP, it is not surprising that a number of complement regulators exist both in the plasma and on host cell membranes to prevent complement activation on self-tissues. Some of these regulatory components are mentioned now for the sake of clarity; more detailed attention is provided later in this chapter (Table 23.1). CR1 (CD35) and DAF (CD55) compete with factor B for binding to C3b on the cell surface and can displace Bb from a convertase that has already formed.[36] Factor I (FI), a serum protease, in concert with CR1 or MCP (CD46) can prevent convertase formation by converting C3b into its inactive derivative, iC3b.[37] CR1 is unique among the FI cofactors in facilitating an additional proteolytic cleavage of iC3b to yield C3c and C3dg (see Fig. 23.2B). Trimming of the latter by noncomplement proteases yields the proteolytic limit fragment C3d, which structurally corresponds to the TED domain (see Fig. 23.2B). Another complement regulatory protein found in the plasma is FH. FH binds C3b and is able to compete with factor B and displace Bb from the convertase. FH also acts as a cofactor for FI to convert C3b to iC3b. In addition to interaction sites for C3b, FH possesses two distinct binding sites for polyanionic molecules, particularly various sulfated glycosaminoglycans (e.g., heparan sulfate) or arrays of sialic acid (e.g., from membrane surface glycoproteins) found on host surfaces in contact with blood plasma. Although these polyanion binding sites are not required for FH to regulate fluid phase AP C3 convertase, they are required for its activity on surface-bound C3bBb. In fact, this is the basis for FH being able to discriminate between AP C3 convertase adventitiously deposited on host tissue versus that deposited on a microbial surface because the latter do not possess either the sulfated glycosaminoglycans or the sialic acid arrays.[38,39]

Pathogen surfaces are normally not afforded the protection offered by these regulators. Persistence of the C3bBb convertase on microbial surfaces may additionally be favored by the positive regulator properdin (P). The positive modulation of the AP by properdin has traditionally been thought to be attributable to its ability to prolong the lifetime of the AP C3 convertase by forming a C3bBbP complex in which properdin contacts segments in both C3b and Bb[40] (see Fig. 23.1). This mechanism may indeed be the dominant one exhibited by properdin,[41]

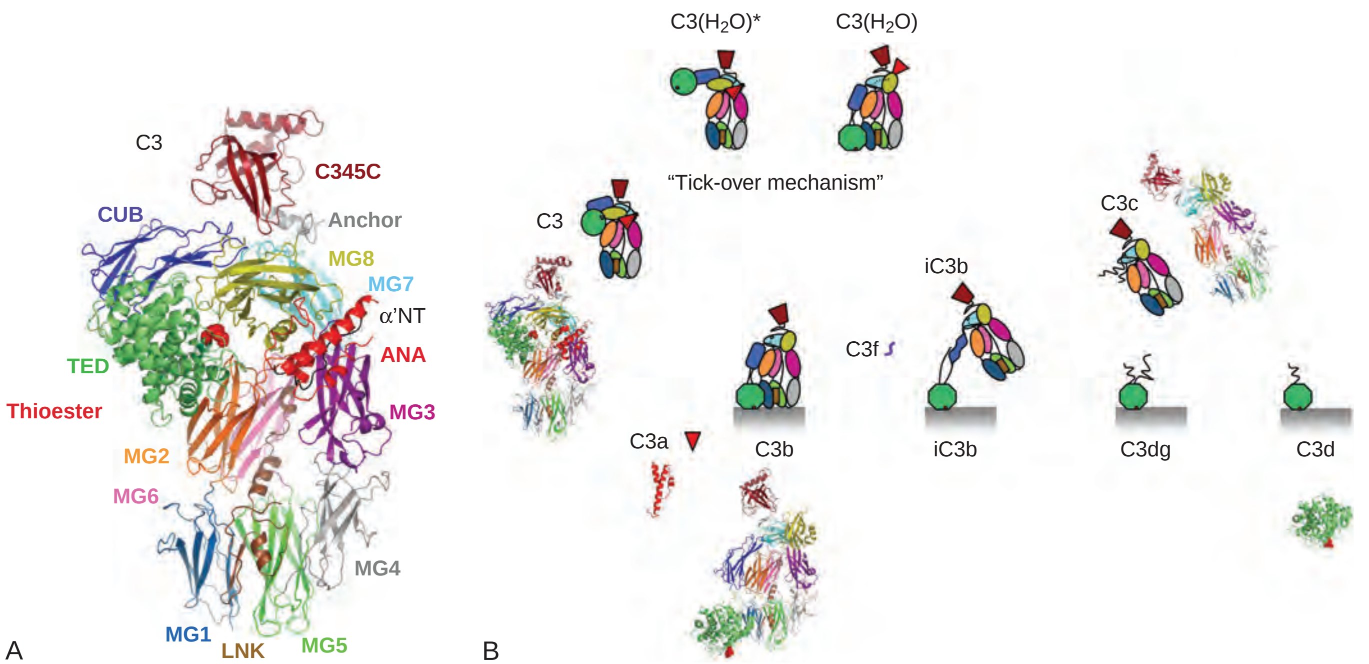


Figure 23.2 THE STRUCTURE OF NATIVE C3, ITS CONFORMATIONAL INTERMEDIATES, AND ITS CLEAVAGE FRAGMENTS. (A) Ribbon diagram representation of the X-ray crystal structure of native C3 indicating the 13 domains (bold lettering, color-coded the same as the domain) of which it is composed. (B) Structure-based cartoon representation of the conformational states of intact C3, as well as its cleavage fragments. Where these cartoons are derived from X-ray structures, those structures are depicted as ribbon diagrams adjacent to the cartoon. The remaining cartoons are based on electron micrograph images,[29] as well as established biochemical data. In all cases, the domain colors in the cartoons correspond to those in the ribbon diagrams. Proteolytic activation of C3 to C3b results in an approximate 90-Å downward movement of the thioester domain (TED), a significant repositioning of the CUB (complement C1r/C1s, urchin EGF, bone morphogenic protein 1) domain, and a flipping of the positions macroglobulin 7 (MG7) and MG8 domains. The reorientation of these domains creates binding sites for ligands of C3b that were not present in the native molecule. The reactive thioester produced during this conformational transition is capable of binding a portion of the C3b molecules covalently to a target surface *(gray-shaded boxes)*. Subsequent cleavage of C3b by factor I releases a small C3f fragment and results in a reorientation of the C3c portion of the molecule relative to C3d/TED within iC3b, a molecule that remains bound to the target. This reorientation relative to C3b relieves the steric blockage by MG1 of a portion of the binding site for CR2/CD21, as iC3b is an equivalent ligand to C3dg and C3d with respect to CR2 binding. C3dg and C3c are the products of an additional cleavage by factor I within the CUB domain. A noncomplement protease removes an N-terminal segment from C3dg, yielding the still target-associated C3d fragment. The remaining "squiggle" on C3d represents 16 residues at its C-terminus that are sufficiently flexible that they were not visible in the x-ray crystal structure of C3d. Although the thioester in native C3 is protected from the solvent, native C3 is in conformational equilibrium with a stable conformational intermediate, $C3(H_2O)^*$, in which the thioester become susceptible to hydrolysis. Although the equilibrium strongly favors the native state, if hydrolysis of the thioester in $C3(H_2O)^*$ occurs, it cannot reform, and the molecule undergoes a unidirectional conformational change to the $C3(H_2O)$ stage, which adopts both a C3b-like conformation and functional profile. This conformational transition of intact C3 is the basis of the "tick-over mechanism" for alternative pathway initiation. *(Modified from P. Gros, Utrecht University; contains elements previously published in Gros P, Milder FJ, Janssen BJ. Complement driven by conformational changes. Nat Rev Immunol. 2008;8:48.)*

TABLE 23.1 **Control Proteins of the Classical and Alternative Pathways**

Name	Role in the Regulation of Complement Activation
C1 inhibitor (C1INH)	Binds to activated C1r, C1s, removing it from C1q
C4-binding protein (C4BP)	Binds C4b, displacing C2a; cofactor for C4b cleavage by factor I
Complement receptor 1 (CR1/CD35)	Binds C4b, displacing C2a, or C3b displacing Bb; cofactor for FI
Factor H (FH)	Binds C3b, displacing Bb; cofactor for factor I
Factor I (FI)	Serine protease that cleaves C3b and C4b: aided by factor H, MCP, C4BP, or CR1
Decay-accelerating factor/CD55	Membrane protein that displaces Bb from C3b and C2a from C4b
Membrane cofactor protein/CD46	Membrane protein that promotes C3b and C4b inactivation by factor I
CD59	Membrane protein that prevents formation of membrane attack complex on autologous cells

however, there is also evidence for properdin displaying pattern recognition functionality for some, but not all, AP targets. For example, native properdin, which circulates predominantly as a trimer, binds to zymosan (yeast cell walls), *Chlamydia pneumoniae*, and necrotic, or late apoptotic, mammalian cells, but not to *Neisseria meningitidis* or *Neisseria gonorrhoeae*.[42] Because of properdin's trimeric nature, even if it uses two of its subunits to bind to the target surface, one is still left that can recruit C3b, or $C3(H_2O)$, from the fluid phase to the target surface. The properdin-bound $C3b/C3(H_2O)$ may then act as a platform for recruiting factors B and D, thereby forming a surface-bound AP C3 convertase.[43] Although this mechanism can be demonstrated to function in vitro, its physiologic relevance has yet to be established.

After forming, the C3bBb convertase rapidly cleaves more C3 to C3b, which can participate in the formation of more molecules of C3bBb convertase. The AP thereby activates an amplification loop that can proceed on the surface of a pathogen but not on a host cell (see Fig. 23.1). An additional point regarding amplification by the AP is that C3b deposited on a target as a result of activation of either the CP or the LP can act as a nidus for the formation of an AP C3 convertase. It has been argued that this AP augmentation mechanism is responsible for upwards of 80% of the downstream complement effector mechanisms initiated via the classical or LPs.[44]

Although specific antibody is not required for AP activation, many classes of immunoglobulin can facilitate AP activation.[45] The mechanism by which this occurs remains elusive, although some evidence indicates that C3b covalently bound to IgG displays a reduced rate of inactivation to iC3b by factors H and I.[46] However, in contrast to CP activation, which requires Fc, AP activation can occur with $F(ab)'_2$ fragments.

An instructive demonstration for the role of antibody in continuing the AP cascade, with possible ramifications for human disease, comes from a murine model of rheumatoid arthritis.[47] Mice do not spontaneously develop rheumatoid arthritis. However, a murine model has been developed in which expression of antibodies specific for the ubiquitously expressed cytoplasmic protein glucose-6-phosphate can cause joint destruction reminiscent of human rheumatoid arthritis. Interestingly, the disease state, through complement-mediated joint destruction, can occur even if the specific antibodies are of isotypes incapable of fixing complement through the CP. The response may be localized to the joints because of the absence of complement cascade regulators on cartilage.

C3, C5, and the Membrane Attack Complex

The formation of the C3 convertase, C4b2a (CP and LP) and C3bBb (AP), is the point at which the three pathways converge (see Fig. 23.1). The function of these complexes is to convert C3 to C3a and C3b. C3 is the most abundant complement protein in plasma, occurring at a concentration of 1.2 mg/mL, and up to 1000 molecules of C3b can bind in the vicinity of a single C3 convertase.[48]

The covalent attachment of C3b to either C4b2a or C3bBb converts this enzyme into a trimeric complex (C5 convertase) capable of binding and cleaving C5 into C5a and C5b. Mechanistically, the "adduct" C3b molecule increases the binding affinity of the C5 convertase for its substrate C5 such that its Michaelis constant (KM) is now well below the physiologic concentration of C5 in plasma.[49] In essence, the adduct C3b molecule "primes" C5 for cleavage by the respective C3 convertase enzymatic entities. C5b generation is the initiating event of terminal component assembly into the membrane attack complex (MAC). The MAC is a multiprotein complex whose components are C5b, C6, C7, C8, and multiple C9s.[50,51]

In addition to the above discussed canonical mechanisms of C5 activation leading to MAC assembly, two non-canonical routes have recently come to light that bear mentioning here as they are relevant to a subsequently discussed clinical observation of breakthrough terminal pathway activity even in cases of ordinarily complete pharmacologic inhibition of either C3 or C5, but under conditions where there is strong complement activation occurring in the individual. Mannes et al.[52] have shown that even in the absence of C3b deposition on a target, if there is robust CP activation leading to a dense array of C4b molecules on the target, these C4b molecules have sufficient affinity for C5 to prime it for cleavage by the CP C3 convertase, C4b2a. These authors also present data suggesting that when C5 cleavage is precluded by a pharmacologic agent, in the presence of a dense array of C3b molecules on a target surface, the binding of C5 to this priming array induces, in at least a portion of the C5 molecules, a conformational change to a C5b-like state (i.e., with C5a still present) which can bind C6 and initiate MAC assembly. As the domain architectures, and indeed respective overall structures of the C3/C4/C5 family of molecules are highly similar[53] in essence the proposed C5b-like molecule is structurally analogous to the thioester-cleaved, but peptide chain-intact, $C3(H_2O)$ molecule discussed above in the context of AP initiation.

The MAC, when viewed by electron microscopy, resembles a cylinder that possesses a hydrophobic outer face and a hydrophilic central core. If assembled near a lipid bilayer, such as a cell or the bacterial membrane of a gram-negative strain, the MAC can associate with and insert into the lipid bilayer. Such insertion can be thought as "punching holes" into the membrane, allowing for passage of water and small ions into the cell. Osmotic equilibrium is thereby lost, leading to eventual lysis of the targeted cell or bacterium. C5b678 are sufficient to form small pores in the target membrane. The role of C9 appears to be to enlarge the channel through multiple C9 polymerization, thereby causing more rapid loss of membrane function and lysis. Deficiencies in complement components C5 to C9 have only been associated with increased susceptibility to *Neisseria* species–based infections, such as gonorrhea and bacterial meningitis. Also, the extended cell wall peptidoglycan layer of gram-positive strains of bacteria make them resistant to the lytic arm of complement. It can be concluded from these observations that the requirement for MAC is limited in host protection.

Complement Receptors and Their Role in Immune Complex Clearance and Activation

As described in the previous section, complement can act by the direct lysis of targeted cells. Another important function of complement in host protection is facilitating the uptake and destruction of pathogens by phagocytic cells. This occurs by the specific recognition of C3b/C4b–coated (opsonized) particles by complement receptors.[54–56]

The best characterized complement receptor for the uptake of C4-coated immune complexes is CR1 (CD35). CR1 binds C4b/C3b–bearing immune complexes. CR1, similar to most proteins that bind activation products of C4 and C3 molecules, shares a structural motif known as the short consensus repeat (SCR). Each SCR consists of approximately 60 amino acids. CR1 in humans is composed of 30 linked SCRs and possesses three binding sites for C4b and two for C3b.

CR1 is expressed on a wide variety of cell types in humans, including erythrocytes, macrophages, polymorphonuclear leukocytes, B cells, monocytes, and FDCs. The role of CR1 expression on B cells and FDCs in activating and maintaining the adaptive immune response is detailed subsequently. For now, the focus is on the other cell types that express CR1.

Because CR1 is not directly associated on its cytoplasmic side with any intracellular signaling molecules, binding of C3b by CR1 expressed on phagocytic cells is not in itself capable of inducing endocytosis of the C3b-opsonized target. A secondary signal is required to induce phagocytosis. This second signal can be provided by IgG binding to the phagocyte's Fc receptor, by carbohydrates commonly found on bacterial surfaces, or by exposure of the phagocytic cell to the appropriate cytokines. In addition, some phagocytic cells, such as macrophages, are activated by binding of C5a through C5a receptor (C5aR1, [CD88]) (see Biologic Activity of C3a and C5a, later). What these secondary ligands have in common is that they all bind to receptor domains that are the ligand recognition units of a cell signaling molecule or complex.

The largest pool of CR1-expressing cells is erythrocytes.[57] Erythrocytes bearing opsonized material are removed from the circulation presumably to prevent deposition in tissue sites such as the renal glomerulus. Erythrocytes bearing opsonized material traverse the sinusoids of the liver and spleen, where they come into close contact with fixed phagocytic cells. These phagocytic cells affect the transfer of opsonized material from the erythrocyte onto their own membranes. The transfer of complexes is enhanced by cleavage of C3b to iC3b by FI, as iC3b is a poor ligand for CR1, but is a good ligand for CRIg, a complement receptor of the Ig superfamily present on tissue-resident phagocytic cells (see later for further discussion of CRIg).

Given its central position in the complement cascade, the presence of C3b is tightly regulated. This regulation is brought about by cleaving C3b into inactive derivatives that cannot participate in forming an active convertase. One of the conformationally altered inactive derivatives of C3b, iC3b (see Fig. 23.2B), can act as an opsonin in its own right for complement receptors CR2 (CD21), CR3 (CD11b/CD18), and CR4 (CD11c/CD18). CR3 binds iC3b and plays a major role in inducing phagocytosis but probably not activation in the absence of a second signal (e.g., Fc receptor or pattern recognition receptor). CR4 also binds iC3b-opsonized particles, resulting in direct endocytosis. Although its role as a phagocytic receptor is not well characterized, CD11c is the major marker for DCs. It is important to understand the functional importance of this complement receptor on DC and how it participates in uptake of antigen for presentation to T cells.

CR2 expressed on B cells augments cognate antibody receptor signaling (see later section). This receptor recognizes targets that are coated with iC3b, as well as the subsequent degradation products C3dg and C3d, all of which remain covalently bound to the target (see Fig. 23.2B). CR2 is the only well-defined complement receptor that recognizes C3d/TED on its own as its functionally-relevant ligand. However, the CR2 binding site on TED only becomes accessible after degradation of C3 to at least the iC3b stage. Activation of complement plays a contributing role in producing a strong antibody response. An interesting aside is that CR2 is the cell surface receptor on human B cells that is recognized by the Epstein-Barr virus.[58]

CRIg is a more recently described complement receptor that plays an important role in the clearance of C3b opsonized complexes by phagocytic cells of the liver.[59] It is also expressed on subsets of macrophages, but less is known about this role.

Biologic Activity of C3a and C5a

The role of the complement fragments C3a and C5a in the immune response is to produce localized inflammation.[60] C3a and C5a are anaphylatoxins and are structurally similar to chemokines and they bind to their respective receptors, C3aR and C5aR1 (CD88), which in turn are canonical G protein–coupled receptor (GPCR) signaling molecules.[56] When produced in large amounts or injected systemically, C3a and C5a induce a generalized circulatory collapse and shock-like syndrome similar to that seen in a systemic allergic reaction involving IgE antibodies.[61]

Of the two fragments, C5a is the most stable and possesses the best characterized and possibly highest specific biologic activity. Both C3a and C5a induce smooth muscle contraction and increased vascular permeability. C5a and C3a also act on endothelial cells lining blood vessels to induce adhesion molecule expression.[62,63] In addition, C3a and C5a can activate the mast cells that populate submucosal tissues and line vessels throughout the body to release histamine, tumor necrosis factor α (TNF-α), and protease.[6] The changes induced by C3a and C5a recruit antibody, complement, and phagocytic cells to the site of infection, thereby hastening immune clearance. C5a also induces the upregulation of CR1 and CR3 on the surfaces of these cells. C5a is the only complement chemotactic agent for neutrophils, macrophages, and basophils. By contrast, both C3a and C5a possess chemotactic activity for mast cells.[64] Although a similar fragment, C4a, is produced in the course of C4 activation, its physiologic relevance as a "classical" anaphylatoxin has long been questioned.[65] Specifically, human C4a binds to neither C3aR nor C5aR1 and a specific C4a-binding entity has not been identified.

There is a second high-affinity receptor for C5a, namely C5aR2 (previously known as C5L2), whose function has not been fully delineated. Although highly related in protein sequence to C5aR1, C5aR2 nevertheless contains several critical sequence differences that preclude it from binding G proteins, and thus it does not act as a canonical GPCR signaling entity. It seems that at least one of its functions is to be a downregulator of C5aR1-mediated inflammatory function, both by sequestering excessive C5a as a "decoy" receptor, and through heterodimerization with C5aR1, which facilitates internalization from the cell surface of both cargo-loaded receptors.[66]

Regulation of Complement Activation

Activation of the complement system must be tightly regulated to prevent autologous tissue damage (see Table 23.1).[67] Some of the proteins involved in regulating complement action have been described (see Alternative Pathway, earlier). In addition to these regulators, a number of other checkpoints limit the scope and target of complement activation.

As a result of binding to antibody or pathogen, conformational changes to C1q induce the enzymatic activity of C1r and C1s. Both of these enzymes are regulated by the C1 inhibitor (C1-INH). C1-INH is a member of a family of *ser*ine *p*rotease *in*hibitors termed *serpins*.[68] Serpins provide a bait sequence that mimics the active site of the substrate. When C1r or C1s proteolytically attacks this sequence, the net result is that their respective active site serine hydroxyls become permanently covalently bound to the C1-INH bait site, thereby destroying their proteolytic activity. C1-INH works in a similar fashion in regulating the activated MASP proteases of the LP. Finally, C1-INH is also responsible for preventing spontaneous fluid-phase activation of C1 in plasma, but this activity can be overridden by immune complexes.

Although C1 is capable of cleaving multiple C4 molecules, only approximately 10% of the produced C4b clusters about the targeted antigen, the rest being released into the fluid phase.[69] C4b in the fluid phase is rapidly bound by C4 binding protein (C4bp), which is a cofactor for FI. Factor I cleaves C4b into two fragments, C4c and C4d, which are quickly cleared from the circulation.

In addition to their FI cofactor activities, the soluble regulators C4bp and FH, respectively, promote the dissociation of the CP (C4b2a) and AP (C3bBb) C3 convertases into their constituent components. This decay-dissociation is unidirectional because neither C2a nor Bb can reassociate on its own with their respective C3 convertase subunits. The membrane-bound regulators CR1 and DAF similarly possess decay-accelerating functionality toward both the CP and AP C3 convertases. The importance of CR1 or CR1-like molecules in curbing the complement response can be witnessed in a rather unexpected condition. Complement receptor 1–related gene (*Crry*) is a murine homologue of the human *CR1* gene, although its near-ubiquitous tissue distribution more closely resembles that of MCP (a somewhat more distant homologue).[70,71] Mice lacking Crry are unable to properly regulate C3. Crry-deficient mice spontaneously abort because of C3-dependent injury to the fetus. This presumably is the result of uncontrolled C3 deposition on the placenta. This observation in mice sheds light on the possibility that defective MCP (or perhaps CR1) plays a role in recurrent fetal loss manifest in patients with antiphospholipid syndrome.

Biologic Consequences of Complement Cascade Deficiencies

The important role of the complement system in preventing disease is witnessed in cases in which components of the system are absent either because of random mutation in the human population or by design in gene-targeted "knock-out" mice. Some complement cascade deficiencies have been described. This section focuses on deficiencies in complement cascade activation that have profound biologic consequences followed by a discussion on deficiencies in complement regulatory proteins.

Homozygous deficiencies in C1q, the most common form of C1 deficiency in humans, is a powerful susceptibility factor for the development of systemic lupus erythematosus (SLE).[72,73] Patients lacking C1q nearly always present with SLE. They have increased susceptibility to viral and bacterial infections, but it is not nearly as pronounced as in C3 deficiency (see later discussion). C1q knock-out mice show increased mortality, with up to 25% of mice having histologic evidence of glomerulonephritis.

C4 in humans is encoded by two separate loci giving rise to two distinct, albeit highly similar (>99% sequence identity), protein products, C4A and C4B.[74,75] Complete C4 deficiency correlates with a 75% prevalence of SLE in humans. However, at least in certain human populations, the absence, or even haploinsufficiency, of C4A, but not C4B, is associated with elevated risk for development of autoimmune diseases such as SLE and other lupus-like autoimmune disease.[76] The protective role of C4A with respect to SLE has recently been confirmed through genetic analyses of very large cohorts of European ancestry (6748 cases, 11,516 controls) and African American ancestry

(1458 cases, 5908 controls) individuals and it was also confirmed to be independent of any linkage disequilibrium to neighboring HLA alleles.[77] This study found that the protective effect of the expression of a *C4A* allele copy was equivalent to the expression of 2.3 *C4B* allele copies. The relative protective effect of *C4A* gene expression over *C4B* expression was found to be identical (i.e., 2.3-fold) in a European ancestry cohort of Sjögren syndrome individuals (673 cases, 1153 controls), this being a systemic autoimmune disease targeting mainly exocrine glands. The reason for the protective effect of C4A is not settled, but it is worth noting that the one indisputable functional difference between C4A and C4B is in the nature of the covalent bond formed upon target deposition. Whereas C4A transacylates onto amino group nucleophiles, forming amide bonds, C4B shows a strong preference for forming ester linkages to hydroxyl group nucleophiles. The nature of the covalent bond is in turn determined by the C-terminal-most residue of the so-called isotypic region sequence, a sequence located ~110 residues C-terminal to the thioester-forming residues of C4's TED domain and is respectively ^{1101}**PC**PVL**D**^{1106} in C4A and ^{1101}**LS**PV**IH**^{1106} in C4B.[78] The presence of the His1106 imidazole side chain in C4B isotype allows for a catalytic transacylation mechanism to hydroxyl group nucleophiles involving an initial formation of an intramolecular acylimidazole intermediate with the thioester carbonyl of nascently-activated C4b fragment. By contrast, the corresponding Asp1106 carboxylate side chain of C4A isotype cannot form such a covalent intermediate and thus only amino groups are nucleophilic enough to directly attack the exposed thioester carbonyl of nascently-activated C4A isotype.[79] It therefore follows that the approximately threefold greater propensity of C4A, relative to C4B, to bind to amino group–rich C1-bearing IgG aggregates,[78] as would be present in immune complexes in need of complement-dependent clearance, is one possible reason for the association of C4A null states with SLE.

As with C1q, mice deficient in C4 are also predisposed to SLE-like disease. Mice only express one complement pathway-functional isoform of C4, with the corresponding isotypic region sequence being a hybrid of the human isotypic sequences, namely **PC**PV**IH**. Nevertheless, the presence of a C-terminal His residue dictates that mouse C4 displays a human C4B-like covalent binding preference.[79] Novel strains of mC4A and mC4B mice were constructed using CRISPR-cas 9 to replace the mouse isotypic region with the corresponding human *C4A* or *C4B* nucleotides, generating mC4A and mC4B "like" strains, respectively. Breeding the mice with a lupus mouse (564 Igi) showed that autoreactive B cells reactive to nuclear antigens in the mC4A mice were more efficiently eliminated than in the mC4B strain. Thus, the C4A isotypic form of mouse C4 was more protective in lupus mice. These results suggest one explanation for the finding that C4A is more protective than C4B in lupus patients may be due to its more efficient elimination of autoreactive B cells specific for nuclear antigens[80] (see also "Autoimmunity and Complement Deficiencies" section below regarding potential mechanisms).

C2 deficiency appears to be relatively benign.[81] Humans lacking C2 appear to have a normally functioning immune system, although autoimmune disorders and, less commonly, infections are observed with increased frequency.

In light of the central role of C3 in the complement cascade, it is not surprising that C3 deficiency has dire consequences for the host organism. Of all known cases of C3 deficiency among humans, no patients have been reported as disease free. Infectious complications, predominantly pyogenic in nature, occur frequently and recurrently. *Streptococcus pneumoniae* and *N. meningitidis* are the major pathogens reported. In addition, SLE, vasculitic syndromes, and glomerulonephritis have been documented in up to 21% of C3-deficient patients. Mice deficient in C3 show, similar to humans, greatly increased susceptibility to streptococcal infection and death.[82] The 50% lethal dose (LD_{50}) is 50-fold less for C3-deficient mice than for C3-sufficient control subjects. This may be attributable in large part to the inability of mice deficient in C3 to effectively opsonize the bacteria. Moreover, the deficient mice have an impaired humoral response (see later section).

Biologic Consequences of Complement Regulatory Protein Deficiencies and Function-Affecting Mutations

Deficiencies in C1-INH have been observed in the human population.[83] C1-INH deficiency can be inherited as an autosomal dominant trait or can result from autoantibodies that recognize C1-INH, blocking its function.[68] The inherited form of this deficiency is the cause of hereditary angioedema (HAE). Patients with HAE experience chronic spontaneous complement activation leading to the production of excess cleaved fragments of C4 and C2. Although the increased vascular permeability and edema symptoms that are the hallmarks of HAE were initially thought to be mediated by a C2a secondary degradation peptide referred to as C2 kinin, it is now recognized that the primary mediator of these effects is in fact not complement-derived, but rather is bradykinin, a product of the kallikrein-kinin contact system. Bradykinin is produced in an uncontrolled fashion in this disease as a result of the lack of inhibition of another plasma protease, kallikrein, which is activated by tissue damage and is also regulated by C1-INH. Although C1 is unregulated in patients with HAE, large-scale cleavage of C3 is prevented by complement regulatory mechanisms that proteolytically degrade C4b and that dissociate any CP C3 convertase that has formed in solution or on host cells. An increased risk of infection is therefore not associated with C1-INH deficiency. HAE flare-ups can be fully corrected by infusion of purified human serum-derived, or recombinant, C1-INH. A bradykinin-B2-receptor antagonist (icatibant),[84] and inhibitors of kallikrein (ecallantide, lanadelumab, and berotralstat) represent additional treatment options. Plasmin is important in the generation of bradykinin, and this may explain the effect of tranexamic acid in preventing attacks.[85,86] Attacks can be precipitated by female hormones and especially pregnancy, which can be managed by plasma derived C1-INH. Conversely, danazol is effective but limited to specific groups due to side effects, and its use may be obviated by newer approaches.

Acquired C1-INH deficiency may be associated with lymphoproliferative disorders and in most cases represents development of an autoantibody that binds to and neutralizes C1-INH. In two examined cases, autoantibodies abrogate C1-INH activity by preventing formation of the C1s–C1-INH complex. However, after the complex formed, the autoreactive antibodies had no effect on C1-INH function. Icatibant is an effective therapy for these patients, as is C1-INH concentrate, and treatment of the underlying lymphoma.[87,88]

The role of FI in complement cascade regulation can be witnessed in patients with FI deficiency.[89] In the presence of a cofactor protein, FI cleaves C3b, producing iC3b, the inactive form of C3b. iC3b is incapable of reacting with factor B to form the AP C3 convertase, thereby preventing uncontrolled AP activation. In the absence of FI, unrestrained C3 consumption occurs secondary to accelerated spontaneous AP turnover. Patients with FI deficiency have recurrent infections caused by pyogenic organisms, including meningococcal meningitis.

Likewise, mice deficient in the central protein FH exhibit unrestrained C3 activation via the AP, leading to near total depletion of serum C3. An important outcome of the failure to regulate C3 activation is glomerulonephritis. Strikingly, mice deficient in FH develop a disease resembling the human disorder membrane glomerulonephritis. The phenotype of the mice confirms the general notion that the AP is always "on" and that failure to regulate activated C3 results in consumption of circulating C3 and tissue injury.

Another example of the importance of FH regulation is the reports of genetic association between variant alleles of FH and the human diseases age-related macular degeneration (AMD) and aHUS. Whereas AMD is a fairly common condition—indeed, it is the leading cause of blindness in the Western world—it has been the elucidation of the etiology of the much rarer aHUS condition (two cases per million) that has led to a fuller appreciation of the diverse ways through which dysregulation of the AP of complement can give rise to severe pathology. Classically, HUS is a clinical triad of microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. The disease is characterized by a precipitating injury of endothelial cells. In contrast to the fairly common classical form of HUS, which is diarrhea-associated and is usually caused by a Shiga toxin–secreting pathogen, the atypical form of HUS is nondiarrheal and, although it can be idiopathic, or result from autoantibodies to FH (see Chapter 132), it is more frequently caused by genetic predisposition. Even haploinsufficiency of variants of FH, MCP, and FI resulting from either loss of expression—or more commonly, loss of regulatory function—results in disease pathology. In addition, gain-of-function variants of factor B have been described that either form the AP C3 convertase more efficiently than wild-type factor B or are more resistant to decay-dissociation by FH or DAF. Finally, several C3 variants have been described in aHUS patients that are gain of function in the sense that as C3b there is decreased binding affinity for MCP and FH and thus AP C3 convertases formed with this C3b as subunit would have a prolonged lifetime relative to wild-type C3b.[90,91]

Because FH mutations account for at least 30% of reported aHUS cases and approximately 70% of these are caused by missense mutations in SCR domains 19 and 20, the molecular basis for this disease association has been intensively investigated, and the findings of these studies are best understood in the context of a structure-based domain model of FH bound to C3b on a nonactivator (i.e., host) surface (Fig. 23.3).[92] FH consists of 20 SCR domains, where some domains in the middle of the molecule appear to play mainly a structural role, likely allowing the molecule to bend back on itself, but domain clusters near the ends mediate specific functions. SCRs 1 to 4 bind to C3b and mediate both decay-accelerating and FI-cofactor functionalities. Indeed, FH (SCR1 to 4) on its own is able to regulate a fluid-phase AP C3 convertase, but it cannot do so for surface-bound AP C3 convertases. For regulation of the latter, there are three additional binding interactions that become relevant. Two of these are located within SCRs 19 to 20, specifically, a site localized mainly to SCR19 binds to the C3d/TED domain of the surface-bound C3b molecule, and a site within SCR20 binds to surface-associated polyanions such as sulfated glycosaminoglycans or sialic acid arrays. The aHUS-associated missense mutations found within SCRs 19 to 20 affect one or other of these two binding functions and lead to dysregulation of the AP C3 convertase at the surface of host tissue. In particular, complement-mediated damage to the kidney basement membrane is often a hallmark of aHUS. As a tissue devoid of the membrane-associated complement regulators MCP, DAF, or CR1, but rich in sulfated glycosaminoglycans, the functionality of the soluble AP regulator FH becomes even more crucial for host protection and likely explains the high incidence of missense mutations within SCRs 19 to 20 in aHUS patients. Interestingly, missense mutations in FH SCRs 19 to 20 do not result in systemic C3 consumption, as would be the case for complete deficiencies of FH. This is because SCRs 1 to 4 of the mutant molecule are still capable of regulating spontaneously formed AP C3 convertases in the fluid phase.

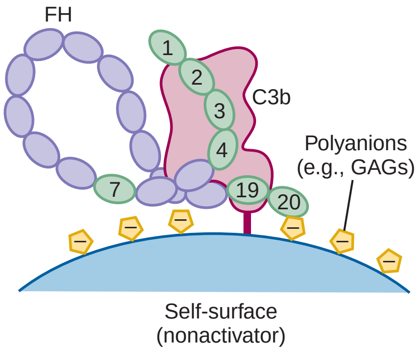


Figure 23.3 A STRUCTURE-BASED MODEL OF THE FACTOR H (FH)–MEDIATED REGULATION OF THE ALTERNATIVE PATHWAY ON HOST CELLS BEARING ADVENTITIOUSLY DEPOSITED C3B. Whereas the depicted interaction of FH domains SCR(1–4) with C3b is sufficient to prevent C3b in solution from becoming a subunit of an AP C3 convertase, for surface-bound C3b, at least two additional interactions are necessary. The first is the interaction indicated between FH SCR19 and the thioester domain (TED)/C3d domain of the C3b molecule. The second is between FH SCR20 and cell surface–associated sulfated glycosaminoglycans (GAGs) or arrays of sialic acid containing glycans, in both cases denoted by pentagons with an internal minus sign. Mutations affecting either the C3d binding site or the polyanion binding site within FH SCR(19–20) lead to alternative pathway dysregulation and the disease atypical hemolytic uremic syndrome (aHUS). There is an additional polyanion binding site in SCR7, which appears to be important for regulating the alternative pathway on some host surfaces, most particularly Bruch's membrane in the eye because the SCR7 Y402H polymorphism is a risk factor for AMD. *(Adapted from Kajander T, Lehtinen MJ, Hyvärinen S, et al. Dual interaction of factor H with C3d and glycosaminoglycans in host-nonhost discrimination by complement. Proc Nat Acad Sci U S A 2011;108:2897; Reproduced with permission of the National Academy of Science.)*

In addition to the polyanion binding site in FH SCR 20, there is also one in SCR 7. This SCR is the site of an amino acid polymorphism in FH (tyrosine to histidine at residue 402, Y402H) that is a significant risk factor for AMD, but interestingly does not correlate with disease susceptibility for aHUS. Heterozygotes and homozygotes for H402 are respectively 2.7-fold and 7.4-fold more at risk for AMD than homozygous Y402 individuals, and this single polymorphism can account for up to 50% of the risk of AMD.[93,94] Two significant functional differences have been observed for the Y402 and H402 variants of FH. First, the affinity and specificity for a spectrum of sulfated glycosaminoglycans is different for the two variants of FH. Secondly, the affinity of the H402 variant of FH for C-reactive protein (CRP), an acute-phase protein that binds to damaged tissue, is substantially lower than that of the Y402 variant. It is notable that Bruch's membrane of the macula, similar to the kidney basement membrane, is devoid of membrane-associated complement regulators and so is expected to be highly dependent on FH for local AP regulation. Indeed, the spectrum of sulfated glycosaminoglycans found on the Bruch membrane appear to be more dependent on the polyanionic binding site in SCR 7 for the interaction than that in SCRs 19 to 20 because even with non-AMD eye tissue, there is preferential binding of the Y402 variant to the Bruch membrane.[95]

SCR 7 is also present in a splice variant of the FH gene, FHL-1 (factor H-like 1), which encodes a truncated protein consisting of SCRs 1 to 7, followed by a unique tetrapeptide C-terminus. The question was then asked whether it is FHL-1, or the parent FH, which was responsible for the association of the Y402H polymorphism with AMD. It was found that not only is FHL-1 more highly expressed by retinal pigment epithelium cells than is FH, but owing to its smaller size, only FHL-1, and not intact FH, can diffuse into and across the Bruch membrane of the human macula. Also, the 402H variant of FHL-1 was confirmed to show lower binding to two different tissue sources of heparan sulfate than did the 402Y variant. Finally, the binding of FHL-1 to Bruch's membrane was shown to be heparan sulfate-dependent. It was therefore suggested that the Y402H polymorphism association with AMD is predominantly driven by the locally-produced FHL-1 splice variant, and not by full-length FH.[96] The lower binding affinity of the H402 FHL-1 variant, coupled with age-related changes in the biosynthesized spectrum of sulfated glycosaminoglycans on Bruch's membrane, could account for the dysregulation of the AP in the macula with the ensuing inflammation of the macula seen in AMD patients.[97] There may also be a contribution from the differential binding of the FH/FHL-1 Y402H variants to CRP present on the particulate debris (drusen) residing in between the retinal pigment epithelium and the Bruch membrane.

Before leaving the topic of AP dysregulation in the context of complement-mediated pathologies, it is necessary to discuss the emerging role of the factor H-related proteins, FHR-1, -2, -3, -4A, -4B, and -5. These proteins, which generally consist of 4 to 5 SCR domains each (FHR-4B is the exception having 9 SCRs), are encoded by the *CFHR1-5* genes, which themselves originated from the *CFH* parent gene by tandem gene duplication events and are arrayed just downstream of the *CFH* gene. The sequence similarities of the SCR domains within the FHRs are highest to the C-terminal domains 19 to 20 of FH and to domains 6/7, these being precisely the SCR domains of FH which possess its surface recognition sites. Indeed reflecting these sequence similarities, the FHRs all bind to C3b/iC3b/C3d and most bind to the model sulfated glycosaminoglycan heparin. Importantly, none of the FHRs possess domains corresponding to FH SCRs 1 to 4, which mediate the decay acceleration and FI cofactor activities of FH. Thus the emerging picture (as reviewed in Jozsi et al., 2015)[98] is that the FHRs do not act as supplementary regulatory molecules of the AP, but rather they can act as modulators of FH-mediated regulation by competing with it for its binding sites on cell surface structures and for the C3d binding site located in FH SCRs 19 to 20. When C3b is adventitiously deposited on normal host tissue, the competition of the FHRs with FH is thought to be quite limited for a number of reasons, including their much lower circulating concentrations relative to FH, the total absence of the largest contact site for C3b, i.e., that mediated by SCRs 1 to 4, and a much lower affinity than FH for normal host surface structures, such as sialic acid arrays. However, the nature of the tandem array of the *CFHR* genes in proximity to the *CFH* gene sometimes results in the formation of hybrid molecules through homologous recombination events resulting in either loss of function in FH or gain of function in the CFRs, both of which become risk factors for complement-mediated pathologies. For example, a hybrid molecule in which SCR 20 of FH is replaced by its homologue in FHR-1 (namely SCR5) results in the loss of complement regulation on human host cell surfaces and is a risk factor for aHUS.[99] Conversely, the substitution of FHR-1 SCR 5 by FH SCR 20 confers upon this hybrid the host surface binding properties of FH, allowing it to compete with FH and thus being a risk factor for aHUS.[100] Another type of gene rearrangement involves the complete deletion of the adjacent genes encoding FHR-1 1nd FHR-3. This is actually a fairly common polymorphism and has been found to be protective with respect to the development of AMD,[101] presumably because these FHRs will not compete with FH and FHL-1 to regulate the AP at the Bruch membrane. In contrast to the AMD situation, the deletion of the *CFHR1* and *CFHR3* genes is a risk factor for SLE,[102] possibly indicating that overregulation of complement leads to a decrease in the opsonophagocytic removal of autoantigens present in the blebs of apoptotic cells. A final example of a disease-associated gain of function mutation in an FHR protein involves the formation of higher order oligomers of FHR-1, FHR-2, and FHR-5, which would increase their avidity for ligand and thus be able to compete with FH. These three FHR proteins share near identical SCR 1 to 2 domains and these normally act as head-to-tail dimerization domains, forming both homo- and heterodimers. The gain of function mutation involves the duplication of the exons encoding SCRs 1 to 2 in FHR-1, -2, or -3, which in turn will mediate the formation of higher homo- and hetero-oligomers. These higher oligomers are in turn risk factors for C3 glomerulonephritis (C3G), which involves massive C3 deposition on the glomerular basement membrane.[103] The MAC is one mechanism used by the host to rid itself of certain microorganisms. Host cells are protected from MAC-mediated lysis by CD59 (protectin), a membrane-bound protein. CD59 performs its function by inhibiting the binding of C9 to the C5b–C6–C7–C8–C9 complex. CD59 and DAF (CD55) are linked to the cell surface by a glycosylphosphatidylinositol glycolipid (GPI) anchor. PIG-A, the enzyme responsible for the first step in the synthesis of GPI, is encoded on the X chromosome. An acquired somatic mutation of this gene in hematopoietic stem cells leads to a failure to synthesize the GPI anchor and with it an inability to express CD59 or CD55 on the derived blood cell surfaces.[104–106] This results in the complement mediated hemolysis and thrombosis seen in PNH, as discussed below and in Chapter 32. Rare individuals with biallelic mutations of CD59 can display a similar phenotype, but can also develop neuropathy.[105,107] In contrast, biallelic inactivation of CD55 results in the CHAPLE syndrome (hyperactivation of complement, angiopathic thrombosis, and protein-losing enteropathy)[108–110] rather than hemolysis, as well as loss of the Cromer blood group antigens. In contrast to PNH, where the disorder is limited to blood cells, germline mutations of PIG-A, or other genes involved in the biosynthesis of GPI, results in a severe neurologic phenotype.[109,110]

Autoimmunity and Complement Deficiencies

There exists a strong correlative relationship between the lack of certain components of the complement system (i.e., C1 and C4) and autoimmune disease, particularly SLE. Two general non-mutually exclusive hypotheses have been put forward to explain the increased incidence of SLE among complement deficient individuals: the clearance hypothesis and the tolerance hypothesis.[73,111,112] The clearance hypothesis is based on the known role of the CP of complement in binding to foreign antigens and transporting them to the liver and spleen for degradation and removal from the circulation. Thus defects in clearance of apoptotic cells or debris would lead to inappropriate accumulation of self-antigen and over-stimulation of self-reactive lymphocytes.

The tolerance model proposes that innate immunity protects against SLE by delivering lupus autoantigens to sites where immature B lymphocytes are tolerized, thereby facilitating their negative selection. SLE is characterized by high-affinity antibodies specific for autoantigens such as double-stranded DNA (dsDNA), ribonuclear proteins, and histones. Validation of the model comes in part from studies with human B cells demonstrating that self-reactive B cells are eliminated or anergized at two major checkpoints, bone marrow (BM) and spleen. Thus counterselection of potentially pathogenic B cells is an active process and most likely involves components of innate immunity.

Recent studies in a lupus mouse model (strain 564 Igi) in which the B cells express an Ig receptor specific for the lupus antigens such as Ro-60 and SSB/LA suggests a third possible explanation for why C4 is critical for protection against SLE. Accordingly, this hypothesis suggests that C4-dependent defects in clearance of immune complexes leads to a loss of tolerance of certain autoreactive B cells. Thus failure to clear immune complexes composed of lupus antigens or apoptotic cells that bear DNA or ribonucleoprotein (RNP) ligands that trigger Toll-like receptors (TLRs) TLR 7 and TLR 9 may induce myeloid cells to release excess type I interferon (IFN-α). In a feed-forward loop, IFN-α release induces increased sensitivity of TLR 7 and 9 receptors, in particular on B cells, such that the combined effects of engagement of DNA or RNP self-antigen by the B-cell receptor (BCR) and increased TLR 7 and 9 signaling leads to escape of B-cell tolerance.[113,114]

The preceding parts of the complement section familiarized the reader with the general aspects of the complement system. Much of the remainder of this section focuses on the role of the complement system in the initiation and propagation of the adaptive immune response, beginning with a description of natural antibody. Two final complement biology sections of this chapter deal with this system's surprisingly detrimental role in immune-mediated control of cancer and with the interplay between the complement and coagulation systems of blood.

Natural Antibody

Natural antibody, in contrast to antibody secreted in response to active immunization, is continuously released, mostly by the B1 subpopulation of lymphocytes. Predominantly IgM but also IgA and IgG3 (in mice) natural antibodies tend to be polyreactive, with low-affinity binding for antigens such as nuclear proteins, DNA, and phosphatidylcholine, which are common structures among both pathogens and host tissue. These antibodies rarely show evidence of somatic mutation. It has been speculated that the variable region genes that predominate among natural antibodies have been selected evolutionarily for their ability to recognize pathogens and act as a rapid response to infection, thereby acting as a stop gap to provide sufficient time for the adaptive immune response to form. Natural antibody mediates its protective effects via the CP of complement.

IgM natural antibody is important in initiating the CP, leading to enhanced humoral immunity. In addition to its role in protecting against pathogens, natural antibody protects against lupus-like disease based on studies in mice. Thus, similar to C1q and C4, deficiency in IgM predisposes to an SLE-like phenotype.

Complement Links Innate and Adaptive Immune Responses

One of the critical functions of CP complement is providing a bridge between innate and acquired immune systems.[115] The process is achieved through attachment of complement products to the antigen or pathogen, either directly to the surface or via antibody (see earlier section). This complement "tag" consists of breakdown products of C3 (i.e., C3b, C3dg, and C3d) that facilitate recognition of pathogens by the immune system. The recognition phase is mediated principally through complement receptors CD21 (CR2) and CD35 (CR1). This section details complement-dependent mechanisms of immune detection and humoral responses to thymus (T)-dependent antigens.

Soluble Complement Mediators of Antibody Responses

The first clue that complement is important in regulating B-lymphocyte responses came from the observation that B lymphocytes bind activated C3 fragments.[116] Soon thereafter, it was noted that mice depleted of serum C3 by treatment with cobra venom factor had diminished responses to T-dependent antigens.[117] The discovery of naturally occurring genetic deficiencies in C3, C4, and C2 in species as diverse as guinea pigs,[118,119] dogs,[120] and humans[121,122] allowed description of impaired antibody responses as well. Because the impaired responsiveness is comparable among animals deficient in CP activators (C4, C2) and C3-deficient or C3-depleted animals, a model emerged suggesting that the effect is mediated through the CP of the complement system. That the impaired responsiveness is comparable among diverse animal species indicated the importance of CP complement in regulating antibody responses to T-dependent antigens.

The advance of gene-targeting technology in the murine system led to development of engineered strains devoid of various components of CP complement. C1q-, C4-, and C3-deficient mouse strains generate reduced antibody responses to T-dependent antigens.[123–126] Furthermore, these strains fail to switch Ig isotypes normally, suggesting that germinal center responses are impaired.[125] Germinal centers are microanatomic structures whose purpose is to provide for increasing affinity of serum antibody for antigens (affinity maturation) and development and differentiation of memory B lymphocytes and plasma cells.[127] Consistent with this theory, immunized complement-deficient mice produce fewer and smaller germinal centers compared with immunized wild-type mice.[125] Importantly, humoral responses in each of the C1q-, C4-, and C3-deficient strains can be rescued by transplantation of wild-type BM.[72,128,129] Therefore BM-derived cells can produce sufficient complement to reconstitute antibody responses to T-dependent antigens administered intravenously.

It is suggested that the CP potentiates antibody responses through involvement of immune complex formation. The implication is that natural antibodies or specific IgM released early in the response by B cells responding to antigen recognize and bind pathogens, thereby activating the CP. In support of this model, genetically engineered mice producing only membrane IgM (i.e., with gene-targeted deletion of secretory signals) produce significantly reduced antibody responses to T-dependent antigens.[130] A second mechanism for initial CP activation on the antigen, which may be relevant to a subset of antigens bearing a repeating epitope, involves binding of the antigen by B cells through the surface IgM of two or more BCRs. The cross-linking and distortion that is imparted to the Fc_μ regions of adjacent BCRs is sufficient to activate the CP at the B-cell surface. Indeed, this mechanism does not work if the BCR μ-chain contains a mutation that abolishes C1q binding.[131] Finally, a third permutation of these mechanisms may apply to monovalent soluble T-dependent antigens. The antigen is first captured by the BCR, creating an antigen array on the B-cell surface, to which low-affinity natural repertoire IgM can bind by virtue of avidity effects and initiate the CP.[132] As illustrated by these examples, immune complex formation is only important for initiating the CP, leading to the deposition of C3 activation products on the antigen or immune complex. Indeed, antigens directly conjugated to C3b or C3d fragments are more potent immunogens compared to unconjugated antigen.[133,134] Furthermore, the magnitude of the immune response is directly influenced by the number of C3d fragments conjugated to the antigen.[133] Therefore activated products of complement component C3 act as a natural adjuvant in driving efficient antibody responses.

Complement Receptors and Antibody Responses

B-Lymphocyte Coreceptors

The effects of complement-coated antigens on antibody responses are mediated primarily through complement receptors CD21 (CR2) and CD35 (CR1). CD21 and CD35 are expressed predominantly on B lymphocytes and FDCs.[135,136] CD35 is also found on polymorphonuclear cells, macrophages, mast cells, and DCs.[135] CD21 and CD35 are encoded for by separate yet closely linked genes in humans.[137] In mice, CD21 and CD35 originate from the same locus (*Cr2*) and are generated by alternative splicing events at the RNA level.[138,139]

Two novel sets of experiments demonstrated that CD21 and CD35 are important in regulating B-lymphocyte responses to T-dependent antigens. In the first set of experiments, antibodies specific for both CD21 and CD35 or CD35 alone were administered to immunized mice.[140–143] In the second set of experiments, a soluble form of CD21 was administered to immunized mice, thereby competing for C3d-coupled antigen interactions.[144] In both sets of experiments, treatment impaired antibody responses. In the first approach, the antibody that specifically blocked the interaction of C3d with CD21 was much more effective at blocking antibody responses compared with anti-CD35 antibody treatment, which blocked only the binding of C3b to CD35. This suggested that although both receptors contribute, CD21 is more important in regulating antibody responses.[141]

Because CD21 and CD35 are found on B lymphocytes and FDCs, two important cell types for humoral responses, two non-mutually exclusive models are proposed for their function. In the first model, CD21 augments antibody responses through activity as a coreceptor on B lymphocytes (Fig. 23.4A).[145] The second model proposes that CD21/CD35 on FDCs trap and focus antigen such that B lymphocytes can efficiently cross-link their antigen receptor to become activated (see Fig. 23.4B).[146]

As is apparent from the schematics in Fig. 23.4A and B, and as will be elaborated upon further in the ensuing discussion, the key ligand receptor–receptor interaction mediating the linkage between complement and the adaptive humoral immune system is that between the C3d fragment that is covalently coupled to antigen and CD21 (CR2) present on B cells and FDC. The extracellular region of CD21 is composed of 15 or 16 SCR domains (because of the usage of alternative splice sites for exon 11), but the C3d binding site is confined to the two N-terminal–most SCR domains.[147] In what is an instructive lesson on the need to have concordance between x-ray crystallographic structures and biochemical data, the nature of this important interface had been hotly debated for a decade because of discrepancies between a structure of the CR2 (SCR1-2):C3d complex published in 2001[148] with both preexisting and subsequent biochemical data in the literature. A 2011 de novo structure of this complex,[149] depicted in Fig. 23.4C, appears to have resolved the issue because the interactions seen in the new structure are fully supported by the biochemical data in the literature. For example, the biochemical data suggesting that there should be multiple ionic bonds mediating the binding is fully rationalized in terms of the five such bonds seen in the structure between a very negatively charged interface on a concave face of C3d that is remote from the covalent attachment site and positively charged lysine and arginine side chains from CR2 sticking down and interacting with oppositely charged residues on the C3d interface, as can be appreciated in Fig. 23.4C.

As a coreceptor, engagement of CD21 by complement-coupled antigen on the surface of a B lymphocyte, in combination with membrane Ig (BCR) cross-linking, would lower the threshold of signal through the BCR required to activate the cell.[145] Accordingly, naïve B

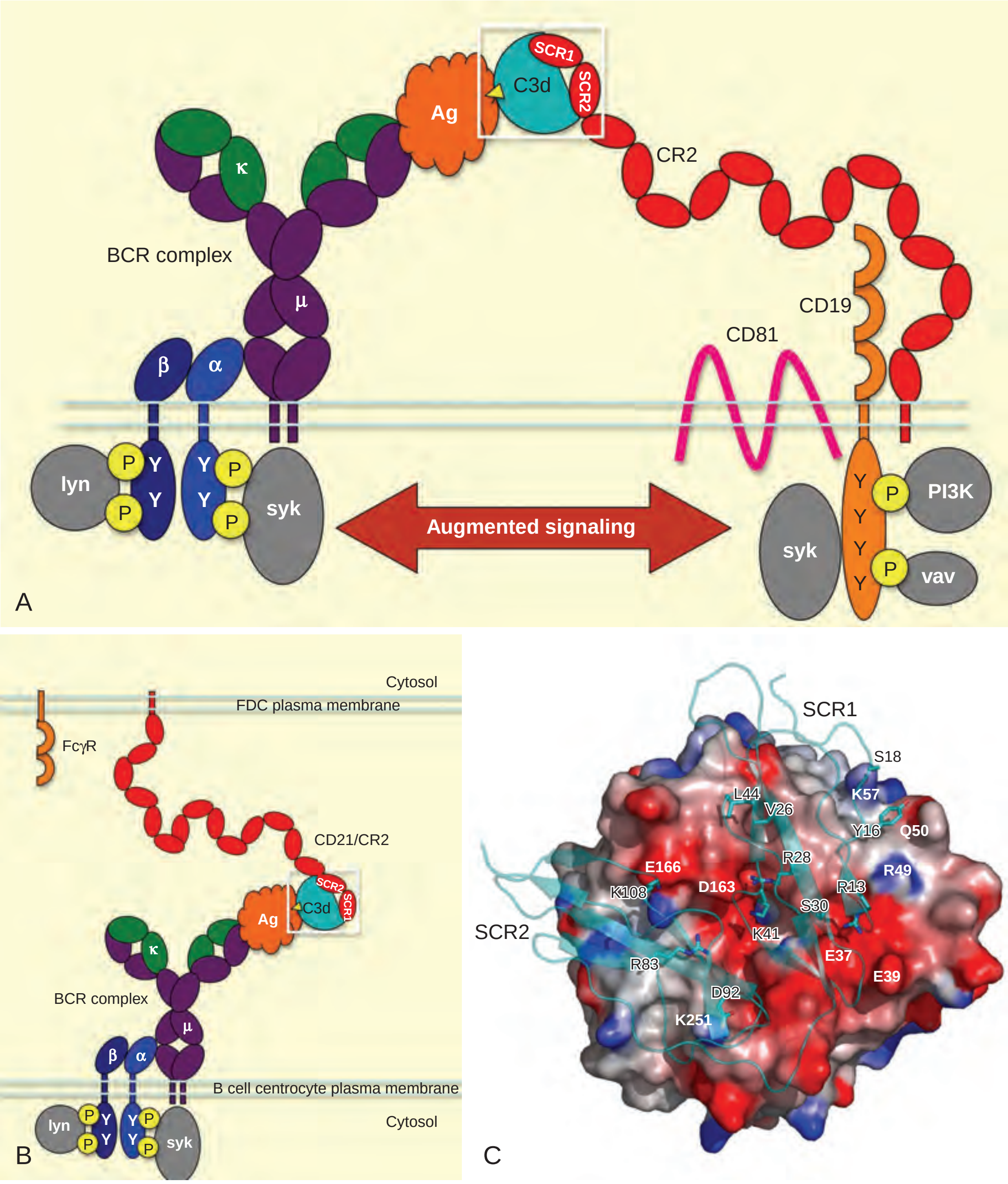


Figure 23.4 COUPLING OF C3D TO ANTIGEN ALTERS ITS FATE IN B-CELL RESPONSE. (A) Coligation of the B-cell receptor (BCR) with the CD19/CD21/CD81 complex by antigen coated with C3d regulates essential functions for naïve B-cell activation. The *boxed area* indicates the key binding interaction between CD21/CR2(SCR1-2), and the C3d fragment that is covalently bound *(yellow triangle)* to the antigen recognized by this B cell's BCR. (B) C3d-coated antigens are also captured on the surface of the follicular dendritic cells (FDCs) by CD21, allowing for efficient stimulation of previously antigen-engaged B-cell centrocytes in the germinal centers during the process of affinity maturation and the generation of memory B cells. (C) The structure of the CR2(SCR1–2):C3d complex as a surface representation of C3d colored for electrostatic potential (*red*, negative; *blue*, positive) and an overlayed, semitransparent, ribbon diagram of CR2(SCR1–2) showing stick models of the side chains of some of the interacting residues. Note the charge complementarity for many of the interacting amino acids. *(C, Reproduced with permission from van den Elsen JM, Isenman DE. A crystal structure of the complex between human complement receptor 2 and its ligand C3d. Science. 2011;332:608.)*

lymphocytes bear low-affinity receptors for antigen; therefore, especially under conditions of limiting antigen, as would be the case during initial encounter with a microbial pathogen, additional signaling by the CD21 coreceptor is required for efficient activation. This was demonstrated in vitro by culturing B lymphocytes with cognate antigen, either uncoupled or coupled to C3d. By measuring intracellular Ca^{2+} levels as a measure of cell activation, it was estimated that 100- to 1000-fold less C3d-conjugated antigen was required to activate B lymphocytes compared with unconjugated antigen.[133]

The opportunity to test the importance of CD21 and CD35 as B-lymphocyte coreceptors in vivo came from studies using mice with targeted disruption in the *Cr2* locus. Importantly, *Cr2*-deficient mice have impaired humoral responses similar to C1q-, C4-, and C3-deficient mice (Fig. 23.5).[150–152] Using embryonic stem cells with a disrupted *Cr2* locus, Croix et al.[153] used blastocyst complementation of $Rag2^{-/-}$ mice, such that chimeric mice expressed CD21/CD35 on FDCs but not on B lymphocytes. These chimeric mice displayed impaired antibody responses to the T-dependent antigen NP-KLH compared with control subjects. Therefore CD21/CD35 on B lymphocytes is important for normal antibody responses. Although CD21/CD35 on FDC is on its own insufficient for normal antibody responses, as discussed in the next section, CD21/CD35 on FDC does have a specific role in the memory response of B-cell–mediated immunity.

The covalent attachment of complement to antigen engages CD21 as a complex with CD19/CD81 and BCR on the cell surface (see Fig. 23.4A).[145,154,155] Dual binding of CD21/CD19/CD81 with BCR generates a stronger signal compared with BCR engagement

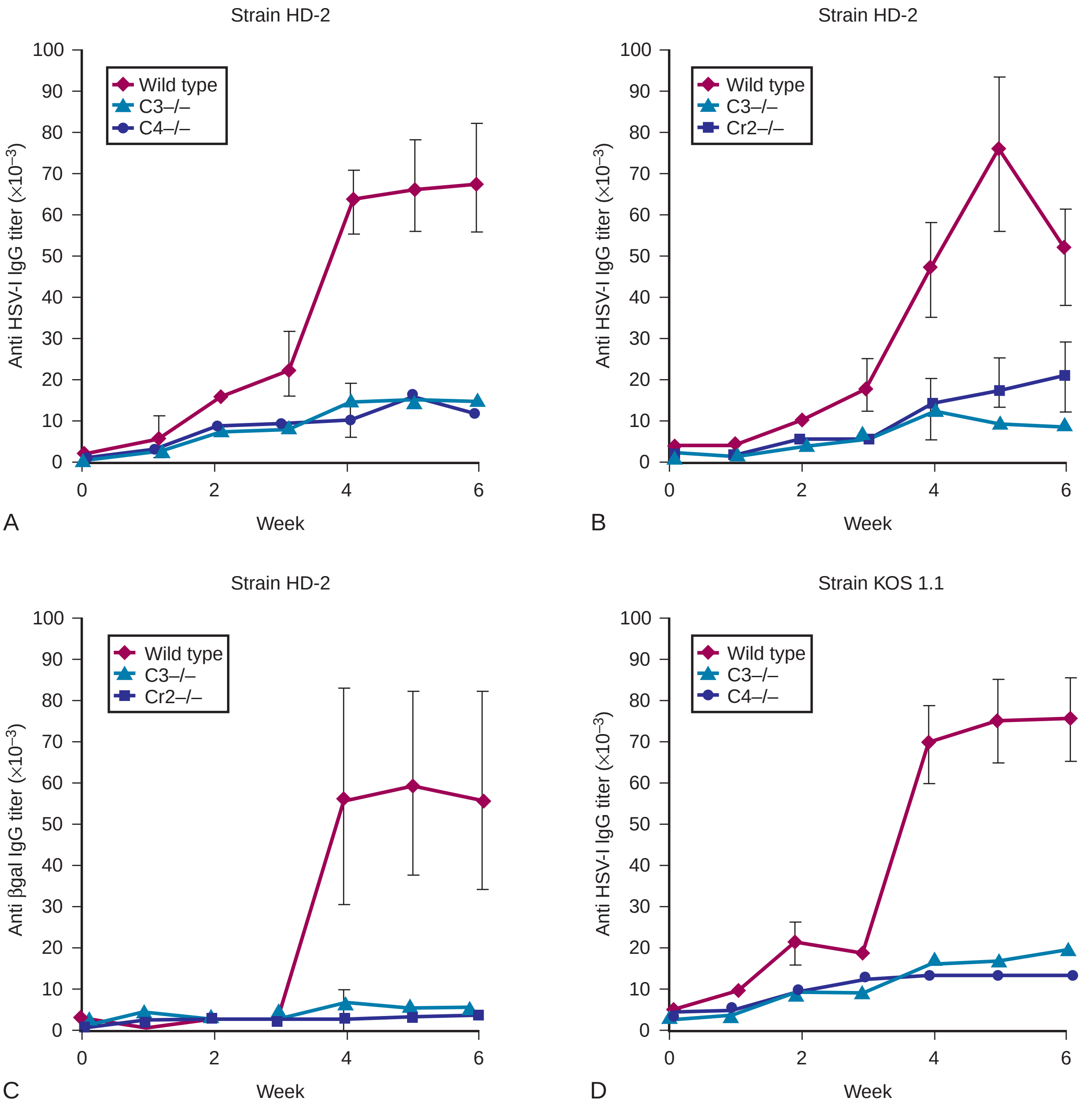


Figure 23.5 Classical pathway complement and complement receptors CD21/CD35 are required for the humoral response to replication-defective HD-2 virus or replication-sufficient KOS1.1 wild-type (WT) virus. Mice were injected at days 0 and 21 with 2×10^6 plaque-forming units of replication-defective (A–C) or replication-sufficient (D) virus, HD-2, and KOS1.1, respectively. Antibody titers were determined by enzyme-linked immunosorbent assay. Mean titer ± SD represents at least five mice analyzed in two separate experiments. (A) Deficiency in either C3 or C4 results in an impaired secondary humoral response to infectious herpes simplex virus (HSV). (B) $Cr2^{-/-}$ mice have an impaired secondary response similar to mice deficient in C3. (C) Humoral response to recombinant virus-expressed heterologous protein (β-galactosidase) is also impaired in mice deficient in C3 or CD21/CD35. (D) Secondary humoral response to replication-sufficient HSV-1 (strain KOS1.1) depends on complement C3 and C4. *(From Da Costa XJ, Brockman MA, Alicot E, et al. Humoral response to herpes simplex virus is complement-dependent. Proc Nat Acad Sci U S A 1999;96:12708. Reproduced with permission of the National Academy of Science.)*

alone.[145] If the combined signal is sufficient, the B lymphocyte is activated. If insufficient, then the B lymphocyte is likely eliminated by apoptosis.[128,156–162] The major ligand-binding receptor within the CD21/CD19/CD81 complex is CD21. The major role of CD19 is in initiating a signaling cascade within the cell.[163] CD81 is a tetraspanning molecule that stabilizes the complex within the membrane. After coligation of the BCR with the CD21/CD19/CD81 complex, CD81 gets *S*-palmitoylated on a cysteine side chain, and this in turn mobilizes the coligated complexes to a special compartment of the plasma membrane known as a lipid raft. Localization to this compartment facilitates prolonged intracellular signaling because the compartment is rich in signal-propagating phosphokinases but is relatively devoid of the regulatory phosphatases.[164] Absence of any of the CD21/CD19/CD81 components adversely affects antibody responses to T-dependent antigens, although the degree of impairment varies.[150,165–167]

Focusing Antigen on Follicular Dendritic Cells

The second role of complement receptors CD21 and CD35 in regulating humoral responses is that they permit FDCs to trap antigen (Fig. 23.6).[146,168] FDCs concentrate in regions of ongoing immune responses, such as germinal centers, and they appear necessary for

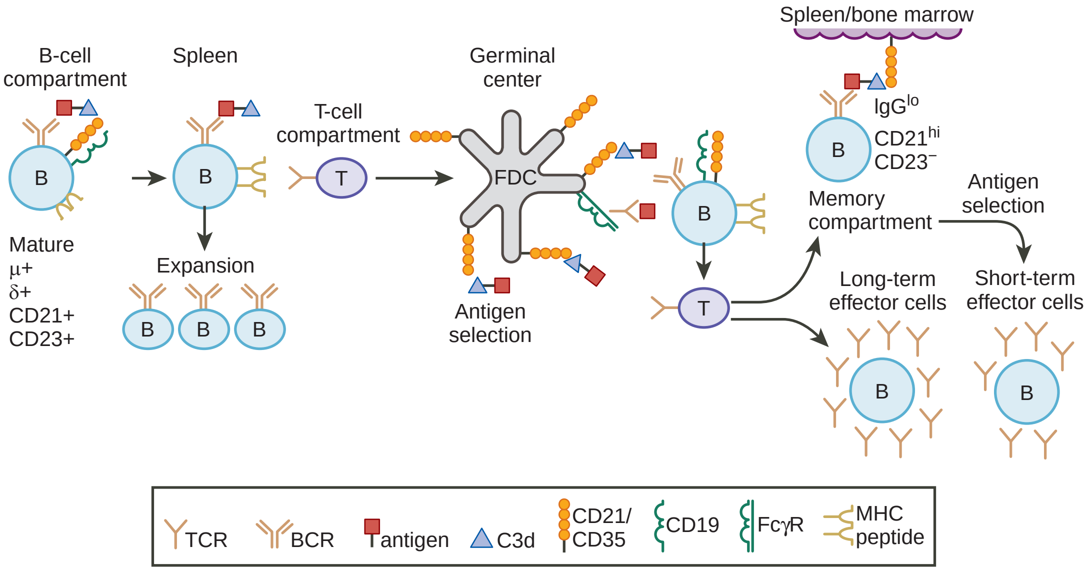


Figure 23.6 ROLE OF COMPLEMENT-TAGGED ANTIGEN IN DIRECTING B-LYMPHOCYTE ACTIVATION AND FORMATION OF MEMORY B LYMPHOCYTES. Mature B lymphocytes survey secondary lymphoid tissues in search of antigen. Survival of mature B lymphocytes after antigen contact and T-cell help within splenic follicles depends on coreceptor signals through CD21/CD35. Lymphocytes receiving requisite signals expand and continue to differentiate within germinal centers, where CD21/CD35 is again important. B lymphocytes not receiving complement–ligand interactions in germinal centers die. In addition, complement-mediated deposition may localize antigen to follicular dendritic cells (FDCs), thereby providing the substrate for B-lymphocyte selection. Selection and differentiation in germinal centers lead to production of long-lived memory B lymphocytes and effector cells. The lifespan of memory B lymphocytes may also depend on continued interaction of antigen deposited on FDCs with CD21/CD35 in the spleen and in bone marrow. *IgG*, Immunoglobulin G.

antibody responses. Germinal centers (see earlier section) promote somatic hypermutation within Ig heavy- and light-chain genes along with production of memory B lymphocytes and plasma cells. They can be divided into two regions, dark zone and light zone. To gain entry into the dark zone, B lymphocytes are activated by receiving above threshold signals from the CD21/CD19/CD81 co-receptor complex and BCR in combination with co-stimulation from helper T lymphocytes.[169–171] Within the dark zone, activated B lymphocytes divide and mutate their Ig receptor genes.[170–173] After several rounds of proliferation in the dark zone, B lymphocytes enter the light zone, where they are subjected to selection on antigen deposited on FDCs (i.e., clonal selection).[174,175] The selection of high-affinity B lymphocyte clones into memory B-lymphocyte and plasma cell pools ensures future protection against repeat antigen exposure.

How antigen is retained on FDCs, both for primary B-lymphocyte responses and for long-term memory responses, is subject to intense research. However, supporting evidence indicates that complement receptors on FDCs are important in both short- and long-term B-lymphocyte responses. Papamichail et al.[146] demonstrated that retention of antigen–IgG immune complexes on FDCs was reduced upon depletion of C3 using cobra venom factor. Therefore it appears that immune complex deposition on FDCs is complement dependent. In addition, antibody production in vitro using FDCs demonstrates that antibody production is dependent on CD21/CD35.[176] More recent studies on trafficking of complement-tagged immune complexes into skin draining LN using intravital imaging reported a two-step pathway. Initial binding of the C3 tagged complexes by CR3 expressed on sinus lining macrophages resulted in their transfer into the underlying B-cell compartment where they were "handed off" to naïve B cells in a CD21-dependent mechanism. Subsequently, the C3d-tagged complexes were stripped from the B-cell surface and internalized into the early endosomal compartment as they came in contact with CD21 receptors expressed on FDC.[157] Thus, CD21 has a dual role in the B-cell delivery of C3d-tagged immune complexes from sinus macrophages to the stromal FDC.

Availability of *Cr2*-deficient mice has shed light on the importance of FDC-derived CD21/CD35 on humoral responses. Because FDCs are radioresistant, it was possible to generate chimeric mice that restricted CD21/CD35 expression to B lymphocytes by BM transplantation. Ahearn et al.[150] made chimeric mice with *Cr2*-deficient FDCs by transplanting wild-type BM (B-lymphocyte $Cr2^{+/+}$) into lethally irradiated *Cr2*-deficient recipient mice (FDC-$Cr2^{-/-}$). After secondary challenge with antigen, the chimeric mice failed to sustain high-level antibody production, suggesting that CD21/CD35 on FDCs is important for recall or memory responses. Fang et al.[177] came to a similar conclusion regarding the importance of CD21/35 expression on FDC for a strong immune response.

CD21/CD35 do appear important for persistence of antibody titers, normal frequencies of memory B lymphocytes and plasma cells, and affinity maturation. Adoptively transferring memory B lymphocytes into recipient mice lacking FDC-derived CD21/CD35 demonstrated that complement receptors on recipient mice stroma were required for each of these elements of memory.[156] Importantly, chimeric mice lacking CD21/CD35-bearing FDCs had severely impaired recall responses several months after transfer of memory B lymphocytes compared with wild-type recipients.[156] Subsequent studies identified a mechanism explaining long-term retention of complement-opsonized immune complexes. Heesters et al.[157,158] found that FDC internalize C3d-opsonized complexes via the CD21 receptor into a non-degradative cycling endosomal compartment that periodically exposes intact immune complexes on the cell surface for recognition by cognate B cells as discussed above. These studies suggest that CD21/CD35 on FDCs have an important role in long-term storage of antigen, thereby facilitating B-lymphocyte memory.

Complement and T-Cell Immunity

The complement system is important not only in humoral immunity; it also enhances responses by both CD4^{+} and CD8^{+} T cells.[178] Studies with influenza in C3-deficient mice first identified an important role for C3 in both the CD8^{+} and CD4^{+} response to infectious virus.[179] Although this was initially thought to be dependent on serum-derived complement components, similar to the observations referred to in a previous section that the humoral immune response in C1q$^{-/-}$, C4$^{-/-}$, and C3$^{-/-}$ mice could be reconstituted by BM-derived immune cells locally producing complement components, so too it was found that antigen-induced Th1 cell activation in CD4$^+$ T cells was serum complement-independent, but was instead dependent on C3, C5, FB, and FD being expressed and secreted by both the antigen-presenting cell (APC) and the T cell.[180–182] C3a and C5a produced by extracellular C3 and C5 convertases bind to their respective C3aR and C5aR1 receptors on the APC and to C3aR on the T cell in an autocrine/paracrine manner, leading to maturation of both partners as evidenced by their cytokine production.[178,183] In humans, autocrine engagement of the complement regulatory protein CD46 on the T-cell surface by T-cell-produced C3b is also required for the induction of a Th1 phenotype of CD4$^+$ T cells. It should be noted that CD46 is a signaling receptor, as both of its differentially spliced cytoplasmic tails (Cyt-1 and Cyt-2) are substrates for kinases. Notably, mice do not express CD46 on hematopoietic cells, but a case has been made that one or more members of the Notch receptor family are the functional homologs to CD46 in mice.[178]

Before outlining the main features of the current working model for complement's role in the human CD4$^+$ Th1 response, at present the best understood area of complement's involvement in T-cell immunity, there is one additional site of complement activation involving T cells to consider, specifically the intracellular complement system, sometimes referred to as the complosome. Although sharing with the extracellular complement system the presence of the anaphylatoxin receptors C3aR and C5aR1, the engagement of these receptors by their respective ligands intracellularly results in distinct signaling pathways and cellular responses compared to those that occur when engagement is at the cell surface. Intracellular complement activation is essential for both the maintenance of resting CD4$^+$ T cells, as well as their activation to a Th1 phenotype. In the resting cells, an intracellular (endosomal and lysosomal) source of C3 is continuously cleaved into C3a and C3b by the lysosomal endoprotease cathepsin L (CTSL). Engagement of lysosomal membrane-resident C3aR by intracellularly-generated C3a provides a survival signal for such resting T cells via the activation of mTOR (mammalian target of rapamycin). Indeed, inhibition of CTSL proteolytic activity results in the resting T cells undergoing apoptotic death.[184] Upon activation of CD4$^+$ cells via their T-cell receptor (TCR), the intracellularly-generated C3a and C3b translocate to the cell surface where they engage their respective receptors C3aR and CD46. Although the protease is as yet unknown, intracellular C5 also undergoes cleavage to produce C5a as a ligand for intracellular C5aR1 (organellar location at present unknown), whose ligation results in an increased production of mitochondrial-derived reactive oxygen species (ROS). The ROS triggers the assembly of the NLRP3 inflammasome, which in turn initiates caspase-1-dependent IL-1β secretion.[185] Collectively these autocrine events are crucial for the induction of an IFN-γ-producing Th1 phenotype. Since C3 and C5 are normally exported from the cell via the secretory pathway, their intracellular presence requires either importation from the extracellular milieu, or some form of retrotranslocation from the organelles of the secretory pathway. In the case of C3, it has been suggested that C3(H_2O), the thioester-hydrolyzed form of the protein, is imported from the extracellular milieu in the course of a receptor-specific recycling process, whereupon the non-re-exported portion of C3(H_2O) is cleaved into C3a and C3b by CTSL.[186]

As an indication of the importance of CD46 in T-cell functionality, patients deficient in CD46, like those deficient in C3, suffer recurrent infections and when their CD4$^+$ T cells are stimulated in vitro with anti-TCR and anti-CD28, they fail to secrete IFN-γ, which is typical of Th1 response, but do secrete normal levels of Th2 response cytokines IL-4 and IL-5.[187]

After successful Th1 expansion leading to pathogen clearance, there is "tune down" of the Th1 response that is required for maintenance of immune homeostasis. Such contraction of the Th1 response involves ligation of CD46 in concert with the binding of the cytokine IL-2 to its receptor, where the latter cytokine would have accumulated to fairly high concentrations during the successful expansion of the Th1 response. This leads to switch from the effector Th1 phenotype to a Treg-like phenotype, including upregulation and secretion of the regulatory cytokine IL-10.[188]

In contrast to the CD4$^+$ T-cell situation, much less is mechanistically known about the role of complement in CD8$^+$ T-cell responses. A recent report found that, although CD8$^+$ T cells possess much of the same autocrine complement, complosome, and inflammasome activities described for CD4$^+$ T cells, whereas CD46-mediated signals are obligatory for Th1 differentiation by CD4$^+$ T cells, they are not obligatory for *basal* level effector functions in CD8$^+$ T cells, but are critical for *optimal* cytotoxic activity by these cells.[189]

Complement and Cancer

Novel entities expressed on the surfaces of tumor cells have long been known to activate all three pathways of the complement system. Nevertheless, complement activation, even with interventional tumor-specific complement activating antibodies, does not generally lead to tumor elimination, or even control of its progression. Conventionally, this has been ascribed to the tumor cell's anti-complement protective mechanisms,[190] the most prominent of which is the increased expression of the membrane complement regulators CD46, CD55, and CD59 on the tumor cells relative to the surrounding normal tissue. Indeed, in human tumor xenograft mouse model systems, combinations of anti-tumor antibody with blocking membrane complement regulatory protein antibodies were in some cases shown to be effective in promoting the survival of the animal, although to be translatable to humans, approaches such as the use of bispecific antibodies, with one Fab directed against the tumor-specific antigen and the other directed against the membrane complement regulator, would likely be necessary to not cause complement dysregulation on normal tissue (reviewed in Gancz and Fishelson[190]). However, in the absence of such complement regulatory protein-targeted interventions, far from complement activation just being ineffective in eliminating tumor progression, over the past decade a compelling picture has emerged that tumor-associated complement activation leads to an immunosuppressive state in the tumor's microenvironment and results in an attenuations of the tumor-specific cytotoxic T-cell responses. As reviewed by Pio et al.[191] and Kolev and Markiewski,[183] the mechanisms underlying this observation are both varied and complex. Although a full discussion of these would be beyond the scope of this chapter, we would like to briefly highlight what is probably the most prevalent and best characterized of the mechanisms, as it is also a potential therapeutic target. Specifically, it involves the C5a:C5aR1 axis in recruiting C5aR1-bearing myeloid-derived suppressor cells (MDSC) to the site of the tumor through the chemotactic activity of C5a. Beyond just acting as a chemotaxis receptor, binding of C5a to the MDSC results in signal-mediated metabolic changes leading to the synthesis and secretion of both reactive oxygen and reactive nitrogen species, both of which inhibit antigen-specific responses in CD8$^+$ T cells. Other effects of C5a-activated MDSC in the tumor microenvironment include the production of immunosuppressive cytokines (e.g., TGF-β1 and IL-10) leading to the accumulation of Tregs and changes in CD4$^+$ T-cell polarization from the anti-tumor Th1 phenotype to the tumor-promoting Th2 phenotype. C5aR1 signaling also affects macrophages in the tumor environment by causing a change from the pro-inflammatory M1 phenotype to the immunosuppressive, and therefore tumor-promoting, M2 phenotype.

Despite the emergence of T-cell checkpoint inhibitor-based immunotherapy, i.e., anti-PD-1 on the T cell, or anti-PD-L1 on the tumor cell, as a potent tool in the treatment of several cancer types, including melanoma, non-small cell lung cancer, kidney cancer, colon cancer, and Hodgkin lymphoma, many patients either are or become resistant to this monotherapy and some cancer types do not respond at all. Given the above described importance of the C5a:C5aR1 axis in promoting a T-cell immunosuppressive state in the tumor microenvironment, a combination therapy targeting both the PD-1:PD-L1 and C5a:C5aR1 axes has been evaluated in preclinical models of lung cancer, melanoma, and colon cancer.[192,193] Relative to each monotherapy, the combined immunotherapy showed synergistic effects in the reduction of both tumor growth and metastatic progression that correlated with an increase in CD8$^+$ T cells and a decrease in the number, or inhibitory activity, of MDSC at the site of the tumor. Based on these preclinical findings, there is an ongoing human phase I/II clinical trial (STELLAR-001, Innate Pharma, and MedImmune) in which the anti-PD-L1 therapeutic antibody durvalumab is being evaluated in combination with an anti-C5aR1 monoclonal (IPH5401) in patients having advanced solid tumors, including non-small cell lung cancer and hepatocellular carcinoma.

Complement and Coagulation

Coagulation and complement are both proteolytic cascades with amplification and regulatory components, each with APs, and there are indeed links between the two cascades.[194] For example, thrombin can activate C5 even in the absence of C3,[195] and factor IXa, Xa, XIa, thrombin and plasmin can all activate C3 and C5.[196] Conversely, complement can result in platelet activation and generation of microparticles and thrombin and thus may initiate coagulation.[197–202] This may proceed in an exaggerated manner on platelets deficient in the complement inhibitors CD59 and CD55, as occurs in PNH, perhaps explaining the marked prothrombotic state of the disorder.[202–206] Other conditions in which the connections between complement and coagulation may be important include aHUS, sepsis, organ transplantation, and infection with SARS-CoV-2.[194,207] Interestingly, antithrombin III may have a downregulatory effect on complement, which may be potentiated by heparin.[208]

Conclusion

Over the past 30 years, a new appreciation for the complement system has come to light. Not only is the complement system required for host protection and innate immunity, but it also plays a critical role in "directing" the humoral response to thymus-dependent and thymus-independent antigens. Covalent attachment of split products of C3 (i.e., C3d) alters the fate of antigen through multiple steps of adaptive immunity such as lowering the threshold for B-cell activation, transport of immune complexes, and loading of antigens on FDC for enhancement of B-cell response. Other studies are uncovering additional roles for complement in the regulation of self-reactive B cells. Strikingly, allelic differences between human C4A and human C4B can be important in protection in human disease. For example, human C4A is more protective in lupus than human C4B probably due to the former's increased efficiency in binding and clearing of dying cells. The next decade will likely witness a similar revolution on our understanding of how complement participates in early development in the central nervous system (CNS) and protection against neuro-psychiatric diseases such as schizophrenia and neuropsychiatric lupus. Remarkably, copy number of human C4A expression is a risk factor for schizophrenia.[209,210]

IMMUNOGLOBULINS

Properties and Structure

The mammalian immune system responds to the almost unlimited array of antigens by producing antibodies that react specifically with the molecules that induced their production. During the immune response, the structure of the inducing antigen is imprinted on the immune system, and subsequent challenges with the same or structurally related molecule(s) causes a more rapid rise in antibody levels to much greater concentrations than were achieved after the primary antigenic challenge. Thus the hallmarks of the humoral immune system include induction, specific protein interaction, and memory.

Antibodies belong to the family of proteins called the *immunoglobulins*. The basic structure of all immunoglobulins consists of a monomer that contains four polypeptide chains: two identical heavy (H) chains and two identical light (L) chains covalently linked by disulfide bonds (Fig. 23.7).[211] The X-ray crystallographic structure of a monomeric immunoglobulin, specifically a mouse IgG2a monoclonal antibody (mAb), is shown depicted in both ribbon and space-filling models in Fig. 23.8.[212] Depending on the angle between the constituent Fab (fragment antigen-binding) monomers, an immunoglobulin monomer consists of a Y- or T-like structure. The size of the Fab arms is 80 × 50 × 40 Å, and the size of base, called the Fc (fragment crystallizable) region, is approximately 70 × 45 × 40 Å according to the X-ray structure models. The Ig molecule exhibits considerable flexibility. In electron microscopic, low-angle X-ray scattering, transient electric birefringence, and resonance energy transfer studies, the angle between the Fab domains has been observed to vary from 0 to 180 degrees. All antibodies have two identical combining sites for antigen located at the ends of the Fab domains.

Fab and Fc represent functional domains in immunoglobulins. They were discovered by performing limited proteolytic digestion of the molecule. Both the H and L chains contribute amino acids that constitute the antigen-binding site in Fab. The monovalent Fab fragment will bind to, but will not precipitate, multivalent antigens, in contrast to native IgG. A fragment can be prepared, called $F(ab')_2$, that is devoid of Fc but still precipitates antigen. This form of immunoglobulin consists of two Fabs disulfide bonded at a part of the molecule called the *hinge region*. The hinge region is the part of the Ig molecule that is responsible for the molecular flexibility exhibited by all immunoglobulins. The other major function of immunoglobulins, binding to specific receptors on cells and certain effector proteins such as C1q, is associated with binding sites also found in Fc. The Fc region of IgG, one of the classes of immunoglobulin, also interacts with protein A, an immune evasion molecule on the cell walls of

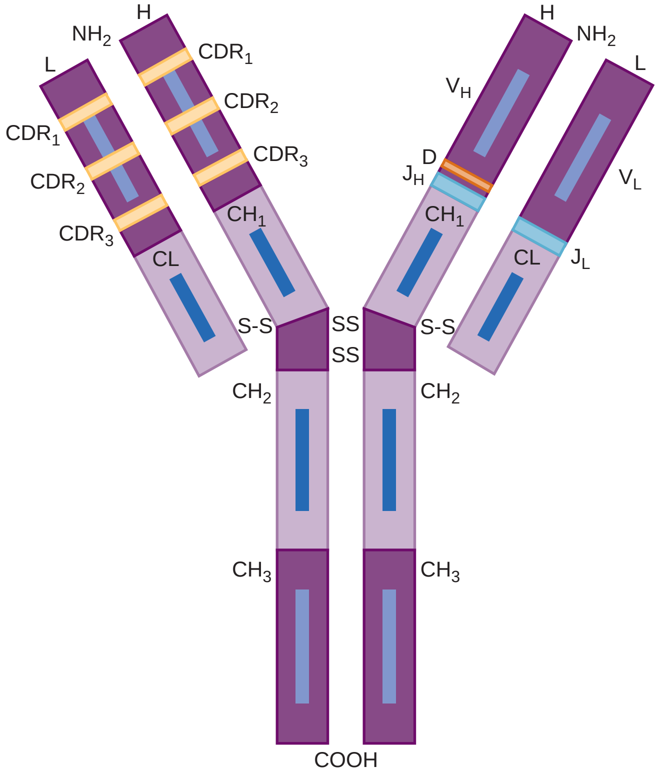


Figure 23.7 DIAGRAMMATIC REPRESENTATION OF THE STRUCTURAL FEATURES OF AN IMMUNOGLOBULIN G (IGG) MOLECULE. NH_2 indicates the N-terminus and COOH the C-terminus. VH, CH$_1$, VL, and CL homology domains are shown as *boxes*. Only the disulfide linkages that join H and L chains are shown. *Left*, Approximate boundaries of the complementarity-determining region (CDR) regions in the VL and VH regions. *Right*, Sequences encoded by VH, D, JH, VL, and JL segments in the VH and VL regions.

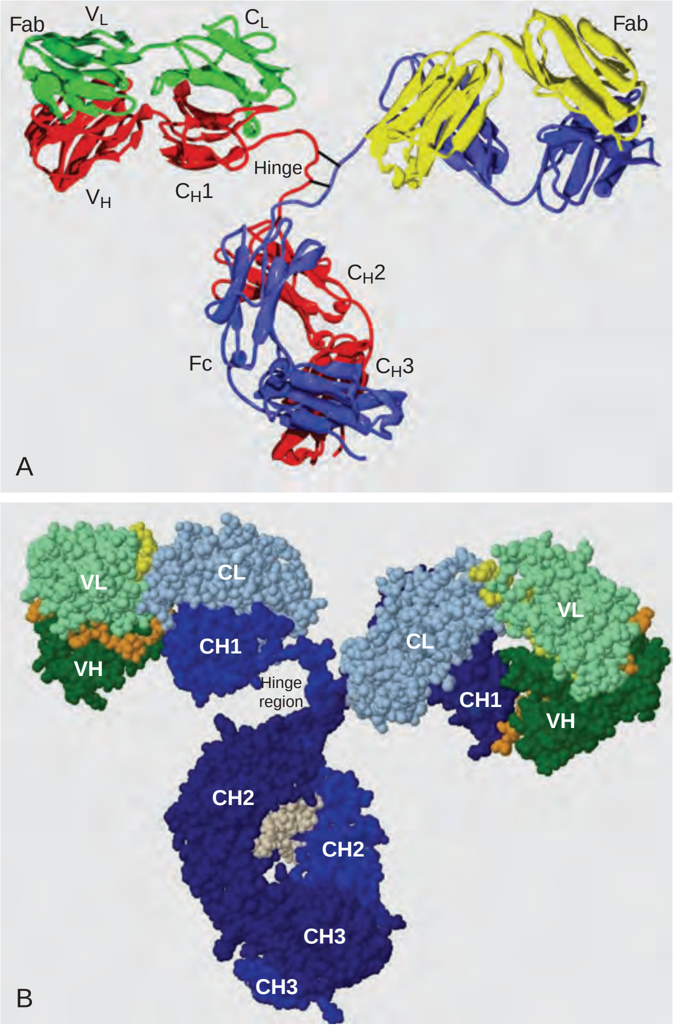


Figure 23.8 X-RAY CRYSTALLOGRAPHIC STRUCTURE OF AN INTACT IGG MOLECULE SHOWN AS A RIBBON DIAGRAM (A), OR A SPACE-FILLING MODEL (B). The structure is that of a mouse immunoglobulin G2a (IgG2a) monoclonal antibody (protein data base [PDB] file 1IGT) and it was the first intact IgG to have its structure determined. (A) The two-layer β-sandwich characteristic of the "immunoglobulin fold" is clearly visible within each of the constituent domains of the γ-heavy chains *(blue and red)* and κ-light chains *(green and yellow)*, respectively. *Black lines* indicate the positions of inter-heavy chain disulfide bonds in the hinge region. (B) The constant domains of the heavy chains and light chains are in various shades of *blue*, and the glycan chain lining a region between apposing C_H2 domains is in *white*. The variable regions are colored according to the genetic segment encoding them. *Dark green* denotes the polypeptide region encoded by the V segment of V_H and *orange* the DJ segment of V_H. *Light green* denotes the polypeptide encoded by the V segment of V_L and *yellow* that encoded by the J segment of V_L. *(A, Modified from* http://proteopedia.org/wiki/index.php/Image:Opening_1igt.png*; B, From* http://www.imgt.org/IMGTeducation/Tutorials/IGandBcells/_UK/3Dstructure/Figure2.html.*)*

S. aureus. When bound to protein A, the binding of IgG to host effector molecules such as C1q is sterically interfered with.

The chain structure of immunoglobulins explains neither antibody structural diversity nor antibody binding to antigen. The discovery of variable and constant regions of amino acid sequence formed the basis for understanding both phenomena. Thus in the L chain, the 100 or so amino acids in the amino-terminal half of the protein (variable region [V_L]) vary among antibody molecules, but in the second half (constant region [C_L]), there is virtual complete correspondence in amino acids, position for position, to the carboxy-terminus. The H chains exhibit a similar pattern and can be divided likewise into V_H and C_H1, C_H2, and C_H3. Comparison of the amino acid sequence of many V_Ls has revealed that whereas certain parts of the variable region exhibit excess variability, others are less variable. The former regions are called *hypervariable* or *complementarity-determining regions* (CDRs). The latter framework regions function as a structural scaffold to support the CDRs. Antigen binding is mediated by six CDRs, three in each of the V_H and V_L domains. The combining site for antigen is a trough, cavity, or even flat surface composed of parts of the hypervariable regions of both the H and L chains. It is a small region, representing only 25% of the antibody V region. The region that interacts directly with the epitope on the antigen is even smaller and is formed by the association of the CDR regions, each of which consists of approximately 20 amino acids. Thus the variation in a few amino acids accounts for the specificity and diversity of antibodies with respect to antigen binding.[213]

Immunoglobulins exhibit additional physical heterogeneity, which imparts to each immunoglobulin a special effector function that is reflected in unique biologic properties independent of antigen-binding activity. In the pregenome era of immunochemical research, heterologous and autologous antisera raised against immunoglobulins were used to classify three types of physical heterogeneity. The first kind is based on the antigenic heterogeneity exhibited by immunoglobulin when it is used as an immunogen in other species. This is called *class* or *isotypic variation*. In humans, five isotypes can be distinguished based on unique antigenic (isotypic) determinants found on the H chain. These are designated by capital Roman letters as IgG, IgM, IgA, IgD, and IgE. The H chain of each class is designated by the lower-case Greek letter corresponding to the Roman letter of the class. Thus the H chain for IgG is γ, for IgM is μ, for IgA is α, for IgD is δ, and for IgE is ε. Some of the immunoglobulin classes are composed of polymers of the basic monomer. In humans, the two antigenic varieties of the L chain are kappa (κ) and lambda (λ). Each Ig has two identical L chains; the κand λ are shared by all classes. The monomeric form of any immunoglobulin is described by its chain structure. The molecular mass of the immunoglobulins can vary from 150 to 1000 kDa. This variation is attributable to polymerization of the basic monomer form. None of the immunoglobulins are polymeric forms of another class. IgG is the most prevalent, constituting 75% of the total Ig in blood. It is present in normal adults at concentrations of 600 to 1500 mg/dL. IgG is designated γ2κ2 or γ2λ2. It is the only class of Ig that crosses the placenta (Table 23.2).[214]

The isotype IgM is predominantly a pentamer consisting of five monomeric units disulfide linked at the C-terminus of the H chain. Each monomer of IgM is 180 kDa because of the presence of an additional C_H domain, specifically the Cμ2 domain, which replaces the hinge segment. The complete protein has a sedimentation coefficient of 19 S, which corresponds to a molecular mass of 850 kDa. IgM is designated $(\mu 2\kappa 2)_5$ or $(\mu 2\lambda 2)_5$. IgM also contains a 15-kDa protein called the *J chain*. In the current structural model of IgM, the J chain forms a disulfide-bonded clasp at the C-terminus of two H chains (Fig. 23.9).[211]

The structure of the other isotypes of immunoglobulins are summarized as follows. The isotype IgA has a variable number of monomeric units and is designated $(\alpha 2\kappa 2)_n$ or $(\alpha 2\lambda 2)_n$, where *n* = 1–5. Serum IgA constitutes 20% of the total serum immunoglobulin, and 80% of this is monomeric. The remainder exists as polymers, where *n* = 2–5. The other form of IgA is found in external secretions such as saliva, tracheobronchial secretions, colostrum, milk, and genitourinary secretions. Secretory IgA consists of four components: a dimer of two monomeric molecules, a 70-kDa secretory component that binds noncovalently to the IgA dimer, and the 15-kDa J chain that is believed to form a disulfide-bonded clasp at the C-terminus of the H chains (see Fig. 23.9). The isotype IgD has a molecular mass of 180 kDa. Its serum concentration is very low, approximately 3 mg/dL. IgD apparently functions as a membrane molecule, being associated on mature but unstimulated B cells in association with IgM. IgE is the homocytotropic or reaginic Ig and mediates immediate hypersensitivity. It has a molecular mass of 180 kDa and, similar to IgM, has four C domains. The Fc portion of IgE binds strongly to a receptor on mast cells, FcεR, and this is how this immunoglobulin exerts its

TABLE 23.2 **Human Immunoglobulins: Properties and Functions**

	IgG1	IgG2	IgG3	IgG4	IgM	IgA1	IgA2	IgD	IgE
H chain	γ1	γ2	γ3	γ4	μ	α1	α2	δ	ε
Molecular weight (kDa)	146	146	170	146	970	160	160	194	199
Molecular weight of H chain (kDa)	51	51	60	51	65	56	52	70	73
Number of H-chain domains	4	4	4	4	5	4	4	4	5
Carbohydrate (%)	2–3	2–3	2–3	2–3	12	7–11	7–11	9–14	12
Hinge inter-heavy chain disulfides	2	5	11	2	NA	2	1	1	NA
Serum concentration (mg/dL)	900	300	100	50	150	300	50	3	0.005
Classical pathway complement fixation	++	+	+++	–	+++	–	–	–	
Alternative pathway complement activity			–			+	+		–
Placental transfer	+	+	+	+	+				–
Binding to mononuclear cells	+	–	+					–	–
Binding to mast cells and to basophils	–	–	–	–	–			–	+++
Reaction with protein A from *Staphylococcus aureus*	+	+	–	+	–			–	–
Half-life (days)	21	20	7	21	10	6	6	3	2
Distribution (% intravascular)	45	45	45	45	80	42	42	75	50
Fractional catabolic rate (% Intravascular pool catabolized/day)	7	7	17	7	9	25	25	37	71
Synthetic rate (mg/kg/day)	33	33	33	33	33	24	24	0.4	0.002

Data from Golub ES. *Immunology: A Synthesis.* Sunderland, MA: Sinaur; 1987.

particular activity. The overall properties of the immunoglobulins are summarized in Table 23.2.

Subclasses of isotypes IgG, IgA, and IgM have been identified. The structural basis for this antigenic heterogeneity is variation in amino acid sequence in the Fc portion of the H chain of a given class. The subclasses of human IgG, called IgG1, IgG2, IgG3, and IgG4, are the best characterized. Each has a slightly different structure, with the most notable differences being in the length of the hinge and in the number of interchain disulfide bonds (see Fig. 23.9 and Table 23.2). IgG1 constitutes 70% of the total IgG and IgG2 20%. IgG3 and IgG4 constitute 8% and 2%, respectively, of the total IgG. The subclasses of IgG exhibit different catabolic rates and bind differentially to cell-associated Fc receptors (FcγR) and to C1q. Specifically, IgG2 does not bind to the FcγRs and IgG4 binds about 10-fold less well than do IgG1 and IgG3. For C1q binding, the rank order of affinities is IgG3 > IgG1 > IgG2 ≫ IgG4. Despite the most obvious sequence differences among the human IgG isotypes being in their hinge regions, studies using engineered domain-swapped chimeric molecules have demonstrated that it is the more subtle amino acid sequence differences within the respective Cγ2 domains that account for the differences in binding to C1q and to the FcγRs. Transport across the placenta is mediated by the Fc-neonatal receptor (FcRn) and for this functional activity IgG2 crosses the placenta slightly more slowly than the other three subclasses. The other known subclasses of Ig isotypes are associated with IgM (IgM1 and IgM2) and IgA (IgA1 and IgA2). The properties and function of these subclasses are less well known.

The second type of variation is called *allotypic variation*. It is attributable to genetically controlled antigenic determinants found on both the H and L chains. Although each human has all immunoglobulin isotypes, an individual has only one form of each allotype on his or her immunoglobulin molecules. Allotypes are codominantly expressed, but an individual B lymphocyte secretes only one of the parental forms. This phenomenon is called *allelic exclusion*.

The third type of variation is attributable to antigenic determinants that are unique to each particular antibody molecule produced by an individual. These markers are called *idiotypic determinants*, and they are associated with a single species of antibody. The antiidiotypic antibodies that recognize a particular idiotype will not react with any other immunoglobulins in the donor other than the purified antibody that was used to raise the antiidiotype antibody. In most cases, the immune response to an antigen results in a mixture of several antibodies, each of which has identical binding specificity but distinct idiotypic determinants. Thus there can be many idiotypes for a given antigenic specificity, which has been interpreted as being a reflection of physical heterogeneity in or near the antibody combining site, for example, in the variable region domains. In some species (notably certain strains of mice), the response to antigen results in a predominant idiotype on all antibodies of a given specificity. Because this quality is inherited, the idiotypes are called major, cross-reactive, or public. Some public idiotypes have been found in certain species (again, most notably mice) to be genetically linked to allotypes. Three kinds of antiidiotype antibodies have been described: those that function as an internal image of the original antigen by mimicking the antigen structure, those that recognize antibody combining site-associated idiotypes, and those that are specific for framework-associated determinants. The internal image antiidiotypic antibodies are of clinical interest.

Every immunoglobulin is a glycoprotein, and the critical glycan is attached to the H chain in the Fc domain at the conserved asparagine at position 297 (Asn297). This single, N-linked glycan is essential for maintaining an open conformation of the two H chains as it lines the opposing faces of the pair of C_H2 subdomains of Fc (see Fig. 23.8B). The core structure of the N-linked glycan is a biantennary heptapolysaccharide containing *N*-acetylglucosamine plus additional sugars (fucose, galactose), with bisecting *N*-acetylglucosamine and sialic acid variably present. Effector functions depend on the Asn297-linked glycan and are influenced by its structure.[215] Deglycosylated IgG does not interact effectively with Fcγ receptors (FcγRs) and cannot support in vivo effector responses, including antibody-dependent cell-mediated cytotoxicity or complement-dependent cytotoxicity.[216] Individual glycoforms contribute to modulating inflammatory responses and have disease association. For example, glycosylation differs in patients with rheumatoid arthritis[217] or vasculitis[218] compared with the normal population. Addition of sialic acid to the N-linked glycan reduces binding of IgG to FcγRs and reduces in vivo cytotoxicity. Regulation of sialylation of IgG contributes to the antiinflammatory homeostasis of serum IgG. Upon antigen challenge, reduced sialic acid–IgG

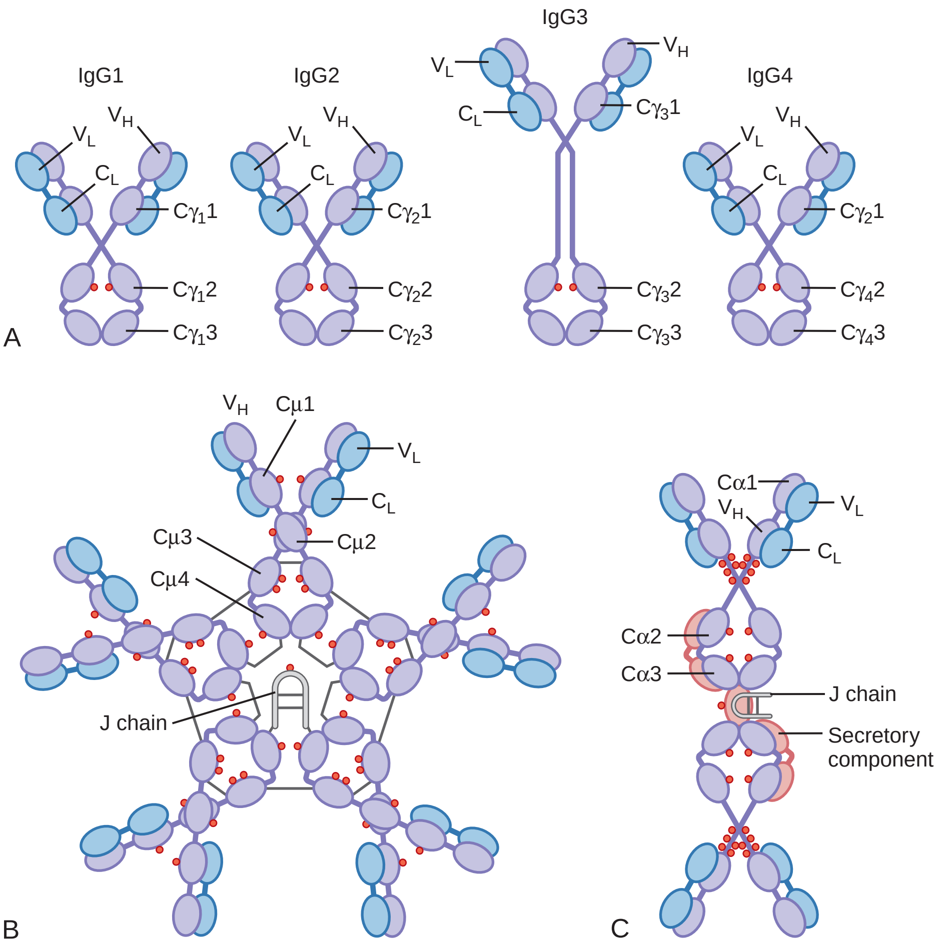


Figure 23.9 (A) Structure of the four subclasses of human immunoglobulin G (IgG). Constant region domains are indicated by C_nN, where n is the subclass and N is the domain. (B) Structure of human IgM. The J chain is shown in the model as disulfide linked to two μ-chains. Other models have been proposed. *Filled circles* indicate carbohydrate. (C) Structure of human secretory IgA. This model shows the possible arrangement of the two IgA monomers in relation to the secretory component and J chain. As the IgA molecule passes through the epithelial cells, the secretory components are synthesized and attached covalently to the Fc domain of the α-chains that have previously been joined to the J chain with disulfide links. Light chains are shown in *blue*, heavy chains in *purple*, disulfide bonds as *gray lines*, and carbohydrates as *red circles*. *(From Turner M. Molecules which recognize antigens. In: Roitt DK, ed. Immunology. London: Gower; 1989:51.)*

can mediate immune clearance and protective immunity through interaction with subclass-specific FcγRs. Kaneko et al.[219] have proposed that the protective effect of intravenous immunoglobulin (IVIg) therapy is attributable to the minor fraction of sialylated IgG species in the total IVIg preparation and that the high doses required (1 to 3 g/kg body weight) for antiinflammatory activity could be significantly reduced by increasing the percentage of sialylated IgG.

THERAPEUTIC USE OF IMMUNOGLOBULIN

Intravenous Immunoglobulin

IgG was one of the first plasma proteins prepared in a purified state as a therapeutic drug for treatment of clinical disorders. It remains, along with albumin and α-proteinase inhibitor, the most widely used therapeutic plasma derivative and is currently the major plasma product on the global market. Polyvalent human immunoglobulin preparations have been used to reconstitute humoral immunity in agammaglobulinemic patients for more than three decades. Until 30 years ago, intramuscular treatment was the mode of administration. Intramuscular preparations caused severe adverse reactions when injected intravenously.[220–222] The most serious were anaphylactoid reactions and were probably complement mediated. Efforts to reduce anticomplementary activity and the prekallikrein activator activity were initiated in the early 1980s and safer IVIg preparations became available.

IVIg is prepared from pooled human plasma pools of 3000 to 50,000 L. The World Health Organization requires more than 1000 donors per lot. The majority of IVIg is produced by cold ethanol fractionation procedures,[223,224] with filtration and polishing chromatography steps added to increase yield and decrease pathogen transmission.[225,226] Gamunex (Talecris Biotherapeutics) is produced from cold ethanol fractionation followed by caprylate precipitation and chromatograpy.[225,227] This is the first significant change in commercial IVIg production in 20 years. IVIg contains concentrated IgG with normal plasma ratios of IgG1 and IgG2, lower percentages of IgG3 and IgG4, and only trace amounts of IgA and IgM. It retains the antibody repertoire, reflecting the combined immunologic experience of the donors.[228,229] Hyperimmune IVIg is purified from donor plasma selected for high titer toward a specific pathogen. Prophylaxis for cytomegalovirus and respiratory syncytial virus are two approved clinical applications.[229,230]

The availability of safe IVIg preparations and the fortuitous observation that IgG treatment of a patient with thrombocytopenia and IgG deficiency increased the patient's platelet count began an intense period of clinical use of IVIg for indications other than primary immune deficiency. In 1990, the National Institutes of Health sponsored a Consensus Development Conference, which produced the first consensus statement on IVIg clinical indications.[231] As a result, six disease indications—primary immunodeficiency, Kawasaki syndrome, chronic lymphocytic leukemia, human immunodeficiency virus (HIV) infections during childhood to prevent infections, BM transplantation to prevent graft-versus-host disease or bacterial infections in adults, and idiopathic purpura—were approved by the US Food and Drug Administration (FDA) for labeling and marketing. The licensed indications remain unchanged, but off-label uses include more than 100 conditions (for further discussion, see Chapter 116).[232,233] Recently, hyaluronidase has been used to facilitate subcutaneous administration of immune globulin preparation, and this has now become an option for the treatment of patients with immune deficiency. These preparations can be self-administered and may result in fewer reactions. However, they need to be given more frequently, there are subcutaneous reactions, they are not licensed for the treatment of hematologic conditions, and it is recommended they not be used for ITP.[234]

The experience with IVIg clinical development has been largely empiric and anecdotal. The mechanisms for patient benefit or harm are poorly understood, especially for high-dose immune modulation therapy. Various known and some yet undiscovered functions of immunoglobulins in immune homeostasis may contribute, including modulation of the function and expression of Fc receptors, interaction with complement and cytokine systems, antiidiotypic antibodies, and regulation of T-cell and B-cell function.[235–237]

Many effects of IVIg are explained by mechanisms beyond antigenic recognition of pathogens.[238] IVIg preparations contain up to 30% dimers composed of idiotype–antiidiotype antibody pairs. These dimers appear to be very effective as a sink for activated complement and can inhibit complement activation.[239] Benefit for treatment of immune thrombocytopenia purpura seems to be mediated by Fc-receptor blockade of the reticuloendothelial cell salvage receptor, also known as FcRn. Therefore, if the FcRn is saturated with infused immunoglobulin, this may decrease the half-life of any autoantibodies that are being produced. This, together with an antiidiotypic neutralization of antiplatelet antibody will eliminate antiplatelet antibodies from the plasma.[240] Similarly, these mechanisms may explain the rapid therapeutic effect of IVIg in patients with acquired inhibitors to factor VIII.[241] Other indications of antibody neutralization can be seen in IVIg treatment of myasthenia gravis. The dramatic success of IVIg in treating Kawasaki syndrome may be attributable to several mechanisms, including antiidiotypic neutralization of antiendothelial antibodies, inhibition of cytokine production and function, and elimination of causative superantigens.[242,243] IVIg inhibits B-cell activation and autoantibody production by enhancing $CD8^+$ suppressor T-cell function. Cell-mediated immunity is also affected.[235] As mentioned earlier, Kaneko et al.[219] ascribe much of the effect of IVIg to a small fraction of it that is sialylated. A 2011 report from this group suggests a mechanism for the way that sialylated IgG in IVIg downmodulates the inflammatory response of the immune system.[244] They suggest that the sialylated IgG Fc region binds to DC-SIGN, a molecule on the surface of "regulatory" myeloid cells, including DCs. In response to DC-SIGN ligation by sialylated IgG, these cells secrete the cytokine IL-33, which in turn stimulates IL-4 production by basophils. IL-4 upregulates the synthesis of the "inhibitory" class of FcγR on effector macrophages, namely FcγRIIB. Because ligation of this class of FcγR by immune complexes actually results in the recruitment of regulatory phosphatases, which shut down intracellular signaling cascades, the net effect is to increase the activation threshold required to initiate inflammation by these effector cells.

Adverse Events Related to Intravenous Immunoglobulin Infusion

Adverse events associated with IVIg can be characterized as (1) early systemic events, (2) infectious disease transfer, and (3) high-dose treatment-related adverse effects.[221]

Early Systemic Events

Common transfusion-related early events are listed in Table 23.3. Most early events are self-limiting and infusion rate dependent. Premedication with steroids, aspirin, or acetaminophen and antihistamines (such as diphenhydramine and the H2 blocker famotidine) often decreases symptoms. Prophylaxis with propranolol can be effective for induced migraine. Aseptic meningitis is a rare early event, is observed 1 to 2 days postinfusion, is unrelated to infusion rate, and can be treated with intravenous steroids and analgesics.[221,245]

The frequency of reported adverse events varies considerably, ranging from 10% to 85%.[221,231,246–248] There are many reasons for this high variability in reporting, including (1) differences in product,[226,246,249] (2) infusion rate, (3) dose and frequency of dosing, (4) patient population, and (5) relative experience of patient and physician. Both patients and physicians become steeled to the adverse events, and because incidents are not life threatening and often respond to prophylaxis medication, they are ignored as "normal." Nonetheless, these events are common and affect health and quality of life of patients.[232,246]

Infectious Disease Transfer

A few early preparations of IVIg transmitted hepatitis C virus. Manufacturers have added viral inactivation and partitioning steps, and current licensed products are safe with respect to HIV, hepatitis C virus, hepatitis B virus, and other blood-borne pathogens (see Chapter 117).[250] The industry has responded to the threat of prions with process validation,[247,251] donor screening, donor testing, inventory management (look back), and plasma pool testing.

High-Dose Treatment–Related Adverse Events

IVIg treatment for immune modulation of neurologic diseases requires doses of 1 to 2 g/kg body weight or two to five times the dose recommended for replacement therapy. Adverse events with high-dose administration include those listed in Table 23.3 and occasionally thromboembolic events, renal complications, and anemia.[221,248,252–255] Thromboembolic events include deep venous thrombosis, pulmonary embolism, myocardial infarction, and stroke. Thromboembolic events and renal failure seem to be independent of infusion rate. The cause of thromboembolic events is not known. Dalakas[256] has suggested that increased serum viscosity plays a role. Factor XIa has also been identified in IVIg preparations.[249] Factor XIa could directly lead to shortening of coagulation time and risk of thrombosis. Renal complications are rare but result in high morbidity and mortality.

TABLE 23.3 Early Systemic Adverse Events Associated With Intravenous Immunoglobulin Infusion

Fever	Rash or urticaria
Chills	Chest tightness
Sore throat	Dyspnea
Face flush	Wheezing
Tachycardia	Low or high blood pressure
Palpitations	Shock
Lumbar pain	Anxiety
Abdominal pain	Nervousness
Nausea	Headache
Vomiting	Migraine
Shaking	Anaphylaxis
Fatigue	Malaise
Myalgia	Leukopenia

Whether IgG, contaminants, or excipients are responsible is not clear. Of the 88 renal adverse events reported to the FDA, 90% were associated with products stabilized with sucrose.[221,245] Whether the adverse events observed with IVIg treatment of neurologic diseases are related to a preexisting medical condition or the high doses required for treatment is not clear. Individuals deficient in IgA, such as those with common variable immune deficiency (CVID), can develop an anaphylactic reaction to IVIg,[257] and products with low levels of IgA are recommended for such patients. Hemolysis can result in recipients who are not of blood type O, due to passive transfer of antibodies against A or B antigens.[258] IVIg administration can also produce false-positive serologic tests for prior infections.[259]

Therapeutic Passive Immunization and Monoclonal Antibody Therapy

Passive immunization in the broadest sense represents the transfer of antibodies to a human recipient who is unable to produce the antibody due to the acuity of an infection, immunodeficiency, or immune tolerance to the target antigen. The use of plasma from patients who have recovered from Ebola or SARS-CoV-2 viruses to treat those acutely ill with these infections[260,261] is an example of the simplest form of antibody transfer, where neither the antigenic epitope nor the sequence of the antibodies are known, and there is no purification step. Indeed, the pathogen need not be identified; it is only necessary that antibodies can clear the infection.[262] It is critical, however, that the antibodies not paradoxically increase viral infectivity, as thought to occur in dengue infections.[262]

With the use of IVIg (described earlier) to normalize IgG levels in patients with inherited immunodeficiencies (e.g., CVID or X-linked hypogammaglobulinemia) or chronic lymphoproliferative disorders, the goal is to provide a wide range of IgG immunoglobulins specific for antigens encountered by the general population, using a product purified from plasma donated by a large pool of healthy donors. Specialized IVIg products have also been developed that are enriched for antibodies that recognize specific pathogens by prescreening donors. Examples include VariZIG (for varicella), CytoGam (CMV), HBIG (hepatitis B) as well as products for rabies, botulinum toxin, and tetanus.

Immunized animals once served as the major source of therapeutic immunoglobulins; indeed, the modern medical era can be traced to the late 1800s, when serum from horses immunized with diphtheria toxin was first used therapeutically. The antigen used to immunize the animal need not be infectious: antivenom products can be used to treat bites from coral snakes, pit vipers, black widow spiders, and scorpions. Some of these products are treated with proteolytic enzymes to produce Fab fragments, such as for the anti-venom product crotalidae polyvalent immune FAB (CroFab). Digibind is another such Fab product, derived from sheep immunized with digoxin bound to human albumin, and is used to treat digoxin overdoses.

When the intended antigen is of human origin, there is the challenge that human plasma donors are likely to be tolerant to the antigen. A notable exception is the Rh-D antigen, which, being genetically polymorphic, is immunogenic to Rh-negative individuals, and human-derived anti-Rh preparations can be used to prevent sensitization during pregnancy and also as a treatment for immune thrombocytopenic purpura.[263] However, apart from this special case, targeting human antigens traditionally required a sensitized animal, a prominent example being antithymocyte globulin (ATG). Here, purified serum products from animals sensitized with human thymocytes can induce lymphopenia as a treatment for aplastic anemia or as part of an immunosuppressive regimen in organ transplantation.

Polyclonal antibodies can be advantageous in some cases (especially for ATG and antivenin), but the epitopes recognized and the biologic activity may be variable between different lots of the product. In retrospect, then, it is not surprising how Kohler and Milstein's technique for the generation of monoclonal antibodies[264] quickly revolutionized medical diagnostics and almost all of biomedical research. Monoclonal antibodies as therapies came more slowly, and there were initial concerns about the generation of "HAMA" (human antimouse antibodies). However, now there are modifications to partially or fully replace mouse sequences with human sequences (Fig. 23.10A). Furthermore, fully human antibodies can be generated using mice that are lacking mouse immunoglobulin genes and are transgenic for the human sequences.[271] Using mice with a germline knockout for the gene encoding the antigen of interest can enable the generation of antibodies that have been previously difficult to obtain.[272] Four fully human monoclonal antibody products (ranibizumab, adalimumab, belimumab, and ramucirumab) now on the market were developed by an alternative method, phage display (see Fig. 23.10B).[266–270] Human antibodies can also be obtained from isolated human B cells that are immortalized by EBV or fusion with an appropriate cell line, and from which immunoglobulin sequences can be obtained by RT-PCR.[273–276]

Monoclonal antibodies can affect target cells by activation-induced cell death, blockage of ligand-receptor interactions, activation of complement, antibody-dependent cell-mediated cytotoxicity, and uptake of antibody-coated cells in the reticuloendothelial system. To increase cytotoxicity, immunoglobulins can be conjugated to toxins (e.g., brentuximab vedotin, targeting CD30; trastuzumab emtansine, targeting human epidermal growth factor receptor 2 [HER2]; and gemtuzumab ozogamicin, targeting CD33), belantamab mafodotin, targeting BCMA, and loncastuximab tesirine, targeting CD19), or radionuclides (e.g., ibritumomab tiuxetan or I131-tositumomab). In some of these cases the toxicities are largely the result of the toxin conjugate.

A chimeric molecule consisting of bispecific single chain variable antibody sequences (scFvs) has been developed (blinatumomab), to engage cytotoxic CD3+ T cells with CD19 expressing acute lymphoblastic leukemia (ALL) cells.[277] Similarly, bispecific molecules can approximate two clotting proteins in patients with hemophilia.[278] Faricimab and amivantamab are bispecifics that are designed to inhibit 2 targets simultaneously with one drug. Tebentafusp is a bispecific that engages T cells with uveal melanoma cells, where one moiety is an scFV that binds to CD3, and the other moiety is a fragment of a TCR that recognizes gp100 in the context of HLA-A*02:01 on the target cells of individuals who express that HLA-allele. A particularly interesting development is the use of single chain antibodies derived from camel "heavy-chain only" immunoglobulin sequences: the smaller molecular size was considered to be advantageous in the development of a drug for TTP targeting a very large molecule, vWF.[279] Obinutuzumab kills CD20-positive cells through a mechanism somewhat different from rituximab due to glycoengineering such that its Fc moiety has increased affinity for Fc receptors.[280] As for some IVIg preparations, the combination of monoclonal antibodies with hyaluronidase has allowed for subcutaneous injections for increased convenience (e.g., for anti CD20 therapy) and has been accompanied by fewer reactions in the case of anti-CD38 therapy. Antibodies can be engineered for longer half-life as described below in the case of C5 complement inhibition, for increased patient convenience and for more stable pharmacodynamics.

Regarding their antibody-secreting hybridomas, Kohler and Milstein wrote in 1975, "such cultures could be valuable for medical and industrial use," which is probably one the greatest understatements in the history of medicine. Indeed, there are currently almost 100 FDA approved therapeutic monoclonal antibodies (Table 23.4, not counting some generics and biosimilars which are now already in use) that are based upon their work for which they shared in the 1984 Nobel Prize. About half of these products have been approved in the last 5 years. The 2018 Nobel Prize was awarded to Alison and Honjo for the development of immune checkpoint inhibitors as a treatment for cancer—to date, all of these drugs are monoclonal antibodies. This strategy has opened up new options for patients with Hodgkin disease in addition to solid tumors. mAbs targeting malignant or autoantibody producing blood cells have impacted practically every hematologic, malignant, and autoimmune condition and allogeneic transplantation.[281] In addition to surface molecules, mAbs can target plasma components—in theory, any protein—and this may increase their clearance from the circulation and inhibit protein-protein interactions or ligand-receptor binding. Antibodies binding to plasma

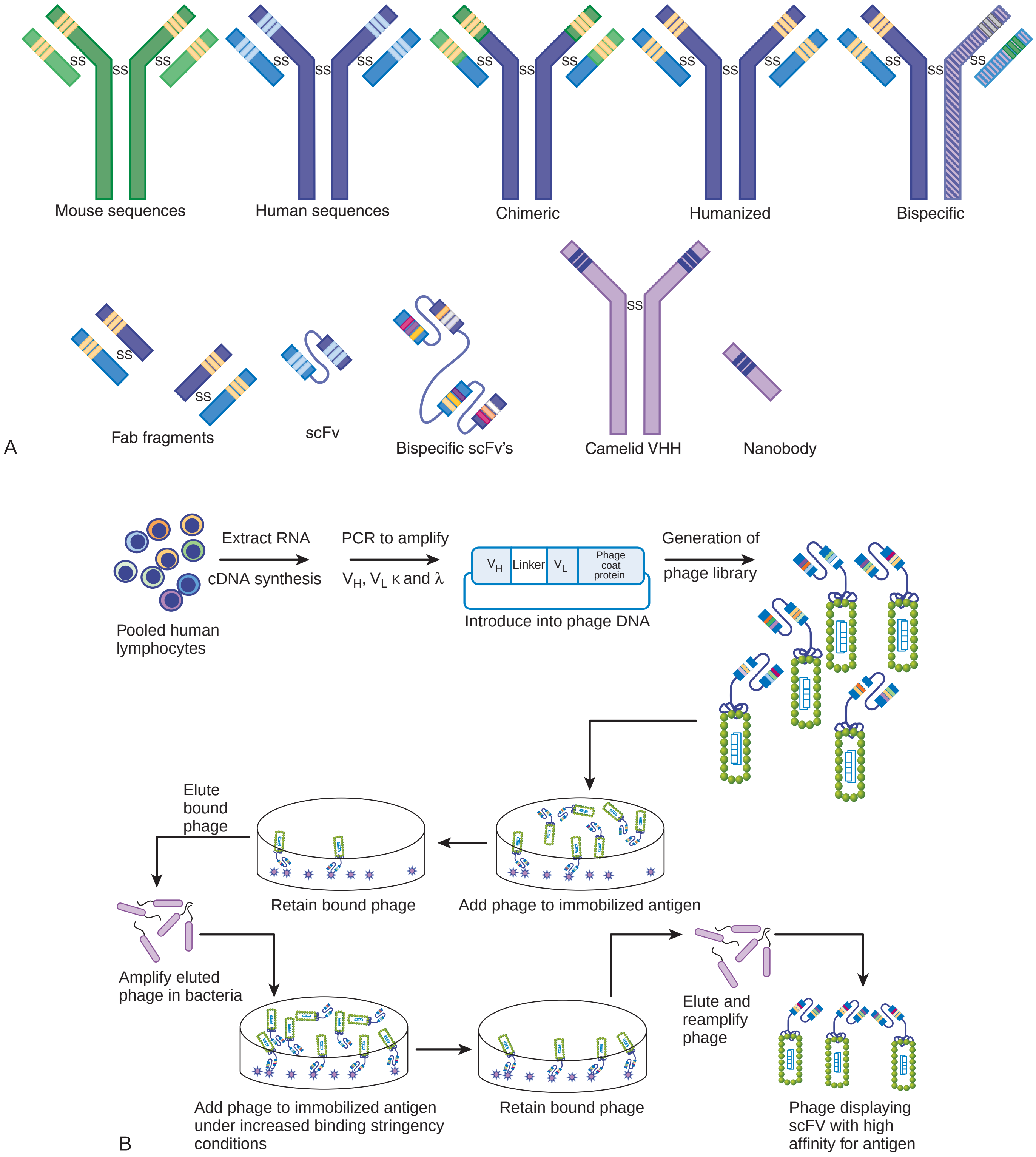

SS
Mouse sequences
Human sequences
Chimeric
Humanized
Bispecific
Fab fragments
scFv
Bispecific scFv's
Camelid VHH
Nanobody
A
Pooled human lymphocytes
Extract RNA
cDNA synthesis
PCR to amplify
V_H, V_L κ and λ
V_H
Linker
V_L
Phage coat protein
Introduce into phage DNA
Generation of phage library
Add phage to immobilized antigen
Retain bound phage
Elute bound phage
Amplify eluted phage in bacteria
Add phage to immobilized antigen under increased binding stringency conditions
Retain bound phage
Elute and reamplify phage
Phage displaying scFV with high affinity for antigen
B

Figure 23.10 GENERATION, STRUCTURE, AND MODIFICATION OF MONOCLONAL ANTIBODIES. To generate monoclonal antibodies, based on the technique of Kohler and Milstein,[264,265] first, a mouse is immunized repeatedly with the antigen, using an adjuvant. After verification of an antibody response, the spleen is removed. Polyethylene glycol is then used to fuse isolated splenic lymphocytes with a mouse nonsecretory myeloma cell line carrying a mutation in the X-linked *Hgprt* gene, which is essential for the purine salvage pathway. The mutation ensures that the myeloma cell line will not grow in HAT (hypoxanthine, aminopterin, thymidine) media. Normal cells can survive despite the presence of aminopterin, which disrupts the *de novo* purine synthesis pathway, because they have an intact purine salvage pathway and can use hypoxanthine. (Because aminopterin interferes with folate metabolism, which also affects pyrimidine synthesis, provision of thymidine is required.) Fusions (hybridomas) between the lymphocytes and the myeloma cell line can grow in HAT only if they have incorporated an X chromosome with the normal *Hgprt* gene from the mouse lymphocyte, restoring the purine salvage pathway. Unfused lymphocytes, on the other hand, have no stimulus to grow. Hybridomas growing in HAT are then cloned by limiting dilution (e.g., in 96-well plates); some of these hybridomas will have retained the chromosomes containing the rearranged immunoglobulin light and heavy chain of the original lymphocyte. Because these genes are now present in a plasma cell with the cellular apparatus for immunoglobulin secretion, immunoglobulin expressed by the original mouse lymphocyte will now be secreted into the media. The supernatant of each hybridoma clone must be screened for the presence of the desired antibody.

proteins are approved to treat hemophilia, sickle cell disease, TTP, hypereosinophilia, and complement disorders (described below) and to reverse dabigatran anticoagulation. Omalizumab is a special case of a humanized IgG immunoglobulin molecule that targets a whole class of immunoglobulin: IgE.[282]

The side effects of any monoclonal or polyclonal antibody therapy depend on the source of the antibody, the target, and the dose. The administration of human-derived IVIg, which involves a very large dose of immunoglobulin, is typically preceded by acetaminophen and diphenhydramine, and sometimes famotidine,[283] to prevent infusional reactions, as described above. For products derived from animal serum, anaphylactoid reactions are commonly seen, requiring premedication regimens that include high-dose steroids, for example, for equine ATG administration. When the patient later makes an antibody response against horse proteins, this can result in immune complex deposition, leading to serum sickness, characterized by fever, rash, and arthritis. Purified chimeric, humanized, or human monoclonal antibodies will typically not result in these reactions. Rather, side effects are dependent, typically, on "on target" effects. Notable examples of this are the febrile infusion reactions that occur with the initial use of rituximab caused by the lysis of CD20-expressing (malignant and nonmalignant) B cells, the immunosuppressive effects of drugs targeting TNF-α, C5, and integrins, and the bleeding risk associated with targeting von Willebrand factor in TTP. Indeed, one of the worst disasters in the history of drug development was due to the excessive "on target" effect of a monoclonal anti-CD28 antibody.[284] However, a true "off target" side effect could, theoretically, occur if a mAb were to cross-react with unintended epitopes on plasma proteins or extracellular surface proteins. Monoclonal antibodies can sometimes be detected on an immunofixation, which can confound the analysis of minimal residual disease in multiple myeloma.[285] Because red cells express CD38, administration of daratumumab and isatuximab are detected as panagglutinins which can mask the development of true allo-immunization.[286]

The overall half-life of IgG is approximately 20 days[287]; when targeting an abundant surface protein or rapidly produced plasma protein, the half-life may be shorter. Most monoclonal antibodies are administered less often than weekly; based on their affinity constants, doses required are generally high enough that, with some exceptions (e.g., the antibody-hyaluronidase combinations), they must be given by intravenous rather than subcutaneous injection. The naming of monoclonal antibodies follows a convention[288] such that the first syllable is coined by the company developing the drug, the second syllable indicates the use (e.g., "ci," for "cardiovascular," "li" for "immune system," "tu" or "ta" for "tumor," and "so" for bone) and the penultimate syllable indicates whether the antibody is derived from mouse sequences ("–omab"), chimeric mouse-human sequences ("–ximab"), more fully humanized molecules ("–zumab"), or fully human sequences ("–umab"). For example, ce*tu***xi**mab is a **chimeric** antibody used to treat *tumors*, whereas ecu*li***zu**mab is a **humanized** antibody that targets the *immune* system.

DRUGS TARGETING THE COMPLEMENT SYSTEM

Eculizumab

The humanized anti-C5 mAb, eculizumab (Soliris), represents an interesting link between the previous sections of this chapter. Eculizumab has already dramatically affected the management of patients with two hematologic disorders: PNH (see Chapter 32) and aHUS (see Chapter 132). C5 was originally thought to be the ideal target because it is essential for all three pathways of complement activation and it is present in a considerably lower circulating concentration than C3 (by a factor of about 16.[289]) Furthermore, congenital absence of C5 and other downstream members of the MAC results in a predisposition mainly to *N. meningitidis* infections[290,291] but not to other infections. In contrast, inherited mutations in C3 result in a broader spectrum of infections[292] as well as autoimmunity. However, in spite of these earlier concerns, C3 inhibition in PNH has now also been shown to be effective—and it seems to be safe, with a short follow-up so far.[293]

Eculizumab is derived from the sequence of an immunoglobulin from a mouse hybridoma, generated by immunizing mice with purified human C5, followed by screening of thousands of clones. The antibody secreted by the selected clone, m5G1.1mAb, inhibited complement activation at a 0.5:1 ratio (as expected given the bivalency of IgG) in a standard hemolytic assay.[294–296] The CDRs were cloned and grafted onto the respective human light and heavy chain sequences. An IgG2/IgG4 hybrid was chosen for the constant regions, because IgG2 binds Fc receptors minimally, and IgG4 activates complement minimally, given that these were two functions not desired for this particular drug.[297] The humanized antibody retains the affinity of the mouse antibody, with a dissociation constant (K_d) of 120 pM. It has a half-life in humans of approximately 11 days, and it has been suggested that a minimum trough level of 35 μg/mL is required for sustained inhibition of terminal complement in humans.[298] Although there is currently no way to measure drug levels, testing for CH50, which is inhibited by eculizumab, is widely available and predicts clinical responses.[299] This is particularly of importance in pregnancy where the metabolism of the drug may increase[300] and the dose or treatment interval may need to be adjusted. Similarly, CH50 testing in patients who have had life-threatening thromboses can be used to verify continual complement blockade throughout the treatment cycle.

Early development of eculizumab focused on testing the drug (and in some cases a related single chain variant) in patients with rheumatoid arthritis, lupus, coronary bypass, myocardial infarction, and membranous nephritis, and in these studies definitive evidence of efficacy was not established.[301–304] However, the strategy of therapeutically inhibiting terminal complement in PNH was validated by a pilot study, a randomized study, and an open label study of eculizumab, where dramatic reductions in the serum hemolytic activity, lactate dehydrogenase (LDH), visible hemoglobinuria, and transfusion requirements—as well as improvements in male erectile dysfunction and esophageal spasms—were shown after a series of loading

---

(A) Modifications of immunoglobulins derived from monoclonal antibody technology. Chimeric mAbs contain the mouse variable region and retain the human constant regions. Humanized antibodies retain only the mouse sequences from the CDRs, which recognize antigens. Bispecific antibodies can be used to engage two different antigens, be they on two different cells, the same cell, two soluble proteins, or combinations of cell-associated and soluble proteins. Fabs are also shown, which lack the constant region of the heavy chain. scFVs (single chain variable fragments) contain only the N-terminal sequences of the heavy and the light chain required for antigen recognition. Because they no longer have any disulfide bond to connect the heavy and the light chain, a linker peptide must be introduced. In the example shown, the carboxy terminus of the light chain is linked to the amino terminus of the heavy chain. The structure of a bispecific scFv is also shown, which can engage two separate ligands simultaneously, bringing together two separate cell types (e.g., immune effector cells with malignant target cells). The structure of a camel–derived single domain antibody (VHH) as well as a smaller "nanobody" is shown. (B) Isolation of human scFvs by phage display.[266–270] This is a newer technique that does not require mouse immunization. First, a library of human variable chain sequences is generated by amplification of cDNA from pooled human lymphocytes. These sequences are then cloned into the DNA of a filamentous phage, such that one of the phage coat proteins is linked to the scFv. Each phage displays a different scFv, and a critical step is to ensure the diversity of the scFvs contained in the library (typically 10^9 to 10^{10} unique clones are desirable). The phage expressing an scFv can be captured by an immobilized antigen (on a plate, or on the surface of a cell) and the retained phage can be recovered and amplified in bacteria. The phage obtained from this step can be further purified in one or more additional capture steps, and at the end, a phage expressing an scFv highly specific for antigen can be recovered, allowing the human immunoglobulin sequences that recognize the antigen to be determined.

TABLE 23.4 Monoclonal Antibodies Approved by the FDA as Therapies

Generic Drug Name	Year FDA Approved	Target	Indication
Muromonab-CD3	1986	CD3	Transplant rejection
Rituximab	1994	CD20	Lymphoma, CLL, microscopic polyarteritis nodosa, Wegener, rheumatoid arthritis
Abciximab	1994	GPIIb/IIIa	Angioplasty
Daclizumab	1997	CD25	Transplant rejection (no longer available)
Trastuzumab	1998	HER-2	Breast cancer, gastric and GE junction tumors
Palivizumab	1998	RSV	RSV prophylaxis
Basiliximab	1998	CD25	Transplant rejection
Infliximab	1998	TNF-alpha	Crohn disease, ankylosing spondylitis, plaque psoriasis, psoriatic arthritis, rheumatoid arthritis, ulcerative colitis
Gemtuzumab ozogamicin	2000	CD33	AML
Alemtuzumab	2001	CD52	CLL, T-cell lymphoma, multiple sclerosis
Adalimumab	2002	TNF-alpha	Rheumatoid arthritis, psoriatic arthritis, Crohn disease
Ibritumomab tiuxetan	2002	CD20	Lymphoma
Omalizumab	2003	IgE	Severe (allergic) asthma, idiopathic urticaria
Tositumomab-I131	2003	Cd20	Lymphoma (no longer available)
Cetuximab	2004	EGFR	Colon cancer, head and neck cancer,
Natalizumab	2004	α4β1	Multiple sclerosis, Crohn disease
Bevacizumab	2004	VEGF	Colon renal cervical, ovarian, and lung cancer
Ranibizumab	2006	VEGF	Wet AMD, diabetic macular edema, macular edema following retinal vein occlusion
Panitumumab	2006	EGFR	Colorectal cancer
Eculizumab	2007	Complement Factor 5	PNH, aHUS, myasthenia, neuromyelitis optica spectrum disorder
Certolizumab pegol	2008	TNF alpha	Crohn disease, psoriatic arthritis
Canakinumab	2009	IL-1b	Cryopyrin-associated periodic syndromes, familial cold urticaria, systemic onset juvenile chronic arthritis, Muckle-Wells syndrome
Golimumab	2009	TNF	Rheumatoid and psoriatic arthritis, ankylosing spondylitis
Ustekinumab	2009	IL-12/23	Psoriatic arthritis, plaque psoriasis
Ofatumumab	2009	CD20	CLL
Denosumab	2010	RANK-L	Bone metastases, osteoporosis
Tocilizumab	2010	IL-6 receptor	Rheumatoid arthritis, systemic juvenile idiopathic arthritis
Brentuximab vedotin	2011	CD30	Hodgkin, anaplastic large cell lymphoma
Belimumab	2011	BlyS	Systemic lupus
Ipilimumab	2011	CTLA-4	Melanoma
Raxibacumab	2012	anthrax protective antigen	Inhalational anthrax
Pertuzumab	2012	HER2	Breast cancer
Obinutuzumab	2013	CD20	CLL
Ado trastuzumab emtansine	2013	HER2	Breast cancer
Nivolumab	2014	PD-1	Melanoma, lung cancer
Blinatumomab	2014	CD19 and CD3 bispecific	ALL
Pembrolizumab	2014	PD-1	Melanoma
Ramucirumab	2014	VEGFR2	Gastric, GE junction, and lung cancer
Vedolizumab	2014	α4β7 integrin	Crohn disease, ulcerative colitis
Siltuximab	2014	IL-6	Multicentric Castleman disease
Dinutuximab	2015	gangliosideGD2	Neuroblastoma
Evolocumab	2015	PCSK9	Hypercholesterolemia
Idarucizumab	2015	dabigatran	Reversal of anticoagulation effect of dabigatran
Alirocumab	2015	PCSK9	Hypercholesterolemia
Necitumumab	2015	EGFR	Squamous non-small cell lung cancer
Mepolizumab	2015	IL-5	Asthma
Daratumumab	2015	CD38	Myeloma

Continued

TABLE 23.4 **Monoclonal Antibodies Approved by the FDA as Therapies—cont'd**

Generic Drug Name	Year FDA Approved	Target	Indication
Secukinumab	2015	IL-17a	Plaque psoriasis, psoriatic arthritis, ankylosing spondylitis
Elotuzumab	2015	SLAMF7	Myeloma
Ixekizumab	2016	IL-17	Psoriasis
Reslizumab	2016	IL-5	Asthma with eosinophilic phenotype
Obiltoxaximab	2016	anthrax protective antigen	Inhalational anthrax
Atezolizumab	2016	PD-L1	Small and non-small cell lung, liver, urothelial, melanoma, and triple-negative breast cancer
Bezlotoxumab	2016	*C. diff* toxin B	*Clostridioides difficle* infection
Daclizumab	2016	CD25	Multiple sclerosis
Avelumab	2017	PD-L1	Merkel, urothelial and renal cell cancer
Benralizumab	2017	IL5R	Eosinophilic asthma
Brodalumab	2017	IL17R	Plaque psoriasis
Dupilumab	2017	IL4Ra	Eosinophilic asthma, eosinophilic asthma, sinusitis with nasal polyps
Durvalumab	2017	PD-L1	Small cell and non-small cell lung cancer, urothelial cancer
Emicizumab	2017	Coagulation factors IX and X bispecific	Hemophilia A
Guselkumab	2017	IL-23	Plaque psoriasis and psoriatic arthritis
Inotuzumab ozogamicin	2017	CD22	B-cell ALL
Ocrelizumab	2017	CD20	Multiple sclerosis
Sarilumab	2017	IL6R	Rheumatoid arthritis
Burosumab	2018	FGF23	X-linked hypophosphatemia, tumor-induced osteomalacia
Cemiplimab	2018	PD-1	Squamous skin cancer
Emapalumab	2018	Interferon gamma	Familial HLH
Erenumab	2018	CGRP Receptor	Migraine
Fremanezumab	2018	CGRP	Migraine
Galcanezumab	2018	CGRP	Migraine, episodic cluster headache
Ibalizumab	2018	CD4	HIV
Lanadelumab	2018	Kallikrein	Hereditary angioedema
Mogamulizumab	2018	CCR4	Mycosis fungoides, Sézary syndrome
Moxetumomab pasudotox	2018	CD22	Hairy cell leukemia
Ravulizumab	2018	Complement Factor 5	PNH, aHUS
Tildrakizumab	2018	IL23	Plaque psoriasis
Brolucizumab	2019	VEGF-A	Exudative age-related macular degeneration
Caplacizumab	2019	vWF	TTP
Crizanlizumab	2019	P-selectin	Sickle cell disease
Polatuzumab-vedotin	2019	CD79b	Diffuse large B-cell Lymphoma
Risankizumab	2019	IL23a	Plaque psoriasis
Romosozumab	2019	Sclerostin	Osteoporosis
Enfortumab vedotin	2019	nectin-4	Bladder cancer
Atoltivimab, maftivimab, and odesivimab	2020	Zaire ebolavirus glycoprotein	Zaire ebolavirus
Belantamab mafodotin	2020	BCMA	Multiple myeloma
Eptinezumab	2020	CGRP	Migraines
Inebilizumab	2020	CD19	Neuromyelitis optica spectrum disorder
Isatuximab	2020	CD38	Multiple myeloma
Sacituzumab govitecan	2020	Trop-2	Triple-negative breast cancer
Satralizumab	2020	IL-6R	Neuromyelitis optica spectrum disorder
Tafasitamab	2020	CD19	Diffuse large B-cell lymphoma
Teprotumumab	2020	IGF1 receptor	Thyroid eye disease
Bamlanivimab	2020	SARS-CoV-2 spike protein	COVID-19

Continued

TABLE 23.4 Monoclonal Antibodies Approved by the FDA as Therapies—cont'd

Generic Drug Name	Year FDA Approved	Target	Indication
Naxitamab	2020	GD2	High-risk neuroblastoma
Margetuximab	2020	HER-2	HER-2 positive breast cancer
Ansuvimab	2020	Ebola glycoprotein	Ebola infection
Casirivimab and imdevimab	2020	SARS-CoV-2 spike protein	COVID-19
Atoltivimab, maftivimab, and odesivimab	2020	Ebola glycoprotein	Ebola infection
Loncastuximab tesirine	2021	CD19	Large B-cell lymphoma
Evinacumab	2021	Angiopoietin-like 3	Homozygous familial hypercholesterolemia
Dostarlimab	2021	PD-1	Endometrial cancer
Aducanumab	2021	Amyloid beta	Early Alzheimer disease
Tralokinumab	2021	IL-13	Atopic dermatitis
Anifrolumab	2021	Type 1 Interferon receptor	Systemic lupus
Amivantamab	2021	EGFR and MET bispecific	EGFR exon 20-mutated non-small cell lung cancer
Tisotumab vedotin	2021	Tissue factor	Cervical cancer
Sotrovimab	2021	SARS-CoV-2 spike protein	Mild/moderate COVID, at risk populations
Tixagevimab, cilgavimab	2021	SARS-CoV-2 spike protein	Pre-exposure prophylaxis, at risk populations
Tezepelumab	2021	TSLP	Severe asthma
Faricimab	2022	VEGF-A and Ang-2 bispecific	Age-related macular degeneration, diabetic macular edema
Tebentafusp	2022	gp100 A*02:01, CD3 TCR arm-scFv bispecific	Unresectable/metastatic uveal melanoma if HLA-A*02:01+

aHUS, Atypical hemolytic uremic syndrome; *AMD*, age-related macular degeneration; *AML*, acute myeloid leukemia; *ALL*, acute lymphoblastic leukemia; *CLL*, chronic lymphocytic leukemia; *EGFR*, epidermal growth factor receptor; *GE*, gastroesophageal; *HLH*, hemophagocytic lymphohistiocytosis; *IL*, interleukin; *PNH*, paroxysmal nocturnal hemoglobinuria; *RSV*, respiratory syncytial virus; *TNF*, tumor necrosis factor; *TTP*, thrombotic thrombocytopenic purpura.

doses of 600 mg weekly for 4 weeks followed then by 900 mg, repeated every 2 weeks.[305–308] While these studies were dramatic enough for drug approval in 2007, the question could have arisen as to whether correction of anemia and amelioration of transfusion requirements was an outcome important enough to justify the risk of meningococcal infection, a potentially fatal outcome, whose incidence was not known at the time. However, that same year, it was reported that the use of eculizumab dramatically decreased the rate of thromboses,[308] which are often recurrent,[309] and which represent the most important predictor of mortality.[310] The effect of eculizumab on thrombosis points to a role for complement (e.g., activation of CD59-deficient platelets derived from the PNH clone) among the proposed mechanisms to explain hypercoagulability in PNH.[311] It is probably due to anticomplement therapy that survival curves in treated patients are now similar to age-matched controls.[312] Whereas pregnancies had been fraught with thrombotic complications, there is now evidence that not only is eculizumab safe for the fetus, but it also may be instrumental in reducing maternal mortality and morbidity.[313] As expected, *Neisseria* infections, as seen in patients with inherited deficiencies of terminal complement, have indeed been seen in patients treated with eculizumab, sometimes despite immunization.[314] In Japan, there have been 2 confirmed deaths reported among 3559 patient years of exposure to eculizumab, and 15 additional deaths that were suspected to be due to the infection; the rate of meningococcemia is therefore elevated above that of the general population by at least 3 to 4 orders of magnitude.[315] All patients on eculizumab can now be vaccinated with a quadrivalent MenB (meningococcal B) vaccine (i.e., Trumenba or Bexsero) in addition to one of the quadrivalent MenACWY vaccines (e.g., Menactra or Menveo). The CDC recommends for immunocompromised patients to receive a booster of the MenACWY vaccine at 8 weeks and then every 5 years, and a booster of MenB at 1 year after the initial series and then every 2 to 3 years.[309] In individual cases, antibody titer levels for the meningococcal A,C,W, and Y strains might inform decisions about booster vaccinations. All patients must be instructed to report to an emergency room for blood cultures and immediate empiric treatment with antibiotics (e.g., ceftriaxone) in the event of any fever over 100.0°F or 37.7°C. Patients should also carry with them a letter from their physician explaining their condition (or wear an alert bracelet) and should carry on their persons at all times an antibiotic that covers meningococcal infection, such as azithromycin or ciprofloxacin, as was the protocol for the randomized trial of eculizumab, in case it is impossible for them to obtain emergency care.[306] For patients who otherwise could obtain urgent medical care, the prior self-administration of antibiotics will make it much harder to detect the infection through blood cultures, creating uncertainty as to the duration of therapy for the patient, and making it impossible to know whether exposed family members would need to receive prophylactic antibiotics, as is the case for sporadic cases of meningococcemia. Interestingly, meningococcal infections in hypocomplementemic patients seem different than in normal hosts, and generally do not result in CNS infections but rather septicemia, such that lumbar punctures are rarely required for patients on eculizumab. Therefore, imaging and LP procedures should never delay empiric treatment with antibiotics in a febrile patient on complement inhibition. As for adults who have been splenectomized, in the United Kingdom, all patients on eculizumab are treated prophylactically with penicillin, if they are not allergic—a practice that is now being adopted in the United States as well. While there have been penicillin-resistant strains of *N. meningitides* in patients on eculizumab who are taking penicillin, there is reason to believe that at least some cases can be prevented by long-term penicillin use.[316] While it has been hard to document an increased risk of other infections in patients on eculizumab, there have been case series of disseminated and fatal gonococcal infections[317] as well as a case of leptospirosis.[318]

Patients with PNH may fail to respond well to eculizumab because of (1) underlying aplastic anemia; (2) rapid drug clearance, as manifested by a nonsuppressed CH50 level indicating a low eculizumab level on day 14 of the treatment cycle; (3) the Arg885His polymorphism in the C5 gene, as seen in about 3% of the Japanese population, such that the drug does not recognize the C5 protein at all,[319] as well as other substitutions in the same codon in other populations having the same effect[320]; (4) extravascular hemolysis of C3d/iC3b/C3b-opsonized red cells in the reticuloendothelial system; or (5) formation of the MAC in spite of saturating levels of eculizumab ("breakthrough hemolysis"), which can occur in the setting of very high levels of C3b or C4b formation, as might occur in the course of an infection—as per the recently proposed non-canonical C5 activation mechanism described above.[52,321]

Extravascular hemolysis as described by Risitano et al.[322,323] is most interesting, reflecting a mechanism that has been "unmasked" by eculizumab. Despite blockade at the C5 level, the PNH red cell is still lacking CD55, which normally functions to inhibit the classical and alternative C3 and C5 convertases.[324] The PNH red cell is also lacking CD59, which may inhibit not only the MAC formation, but also the deposition of C3d on the red cell in the setting of loss of CD55.[325] Indeed, in some patients on eculizumab, C3d deposition on the red cell can be demonstrated by the direct antiglobulin test or by flow cytometry; such cells would have been lysed by the MAC in untreated patients. Opsonization of red cells by C3 split products can account for persistently elevated reticulocyte counts, the requirement for occasional transfusions, and progressive iron overload in some patients on eculizumab. Polymorphisms in the *CR1* gene, which affect levels of expression of this complement-regulating protein, may predict which patients have a partial versus complete response to eculizumab.[326]

aHUS (see Chapter 132), a different complement-mediated disorder, is the other FDA-approved hematologic indication for eculizumab. aHUS is characterized by thrombocytopenia, hemolysis, an elevated LDH, microangiopathy, and dysfunction of many organs, particularly the kidneys. Other entities with similar presentations that need to be distinguished include thrombotic thrombocytopenia purpura (TTP), malignant hypertension, Shiga toxin–producing *Escherichia coli* (STEC), calcineurin inhibitors (and other medications), lupus, and the antiphospholipid antibody syndrome, the latter two being potential triggers for aHUS itself. The disease can be recognized by identifying schistocytes on the peripheral smear, an elevated LDH, and a disintegrin and metalloproteinase with thrombospondin motifs 13 (ADAMTS 13) level (drawn before empiric pheresis) that is not significantly decreased, and a variable and often inadequate response to plasmapheresis. C5b-9 staining on renal biopsies or unaffected skin can point to diagnosis of aHUS[327] and may help justify the empiric use of eculizumab in cases where there could be alternative diagnoses, such as cell mediated renal graft rejection.

Compared with TTP, the degree of thrombocytopenia is not as severe, there are fewer schistocytes, but the degree of renal insufficiency is often much more severe. aHUS is genetically complex, in that it can result from inherited mutations in the genes encoding the inhibitory complement factors H, I, membrane cofactor protein (CD46), or thrombomodulin—or gain of function mutations in the effector complement factors B or C3. Autoantibodies against factor H can produce a similar syndrome, and in a substantial proportion, no mutation is ever found. The inheritance is typically dominant with incomplete penetrance.[328]

The FDA granted approval to eculizumab for the treatment of aHUS based on two single arm studies reporting rapid improvement in thrombocytopenia, typically by day 7, and a more gradual improvement in the renal function, in many cases, allowing for discontinuation of dialysis.[329,330] As in PNH, where eculizumab only masks an underlying defect, relapses have been seen upon drug discontinuation.[331] Since recurrences after renal transplantation are common in aHUS, initiation and maintenance of eculizumab before and after transplant may be critical for maintaining graft function. Since undertreatment would be more serious here than in PNH, the recommended dose in aHUS is higher.

Patients with aHUS are often first treated with plasma exchange until the diagnosis of TTP can be excluded. Since this will replace any of the plasma proteins that might be defective in aHUS, patients with defects in complement regulation can respond to this.[332] However, once eculizumab is initiated, one must be aware that it would be removed by pheresis, and so it is generally given immediately after the last planned pheresis session. This is in contrast to the situation when patients with microangiopathies are treated with rituximab, which is expected to be bound to CD20 on B cells and still have an effect as long as it is given at a sufficient interval before pheresis.

Eculizumab is now also FDA approved for Devic disease (neuromyelitis optica)[333] and myasthenia gravis.[334] There is a growing literature on successful "off label" use of eculizumab, for example, for catastrophic antiphospholipid antibody syndrome,[335] cold agglutinin disease,[336] HUS due to Shiga toxin–associated *E. coli*,[337] antibody-mediated renal graft rejection and dense deposit disease,[338] calcineurin inhibitor-induced thrombotic microangiopathy,[339] lupus nephritis,[340] hyperhemolysis in sickle cell disease,[341] and COVID pnemonia[342]—about 35 off-label indications in all.[343]

Ravulizumab

Ravulizumab is a derivative of eculizumab that was approved in 2018 for PNH[318,344] and subsequently for aHUS. The molecule incorporates four amino acid substitutions into the original heavy chain sequences[345]: Y27H, S57H, M428L, and N434S. The latter two substitutions, in the Fc region, were previously known to increase the affinity of therapeutic immunoglobulins for FcRn by approximately 11-fold in the acidic environment of the endosome.[346] The two histidine substitutions, respectively within the CDR1 and CDR2 segments of the heavy chain, act as "pH switches" to preferentially decrease the affinity of the drug for C5 at the acidic pH of the endosome, while having a minimal effect on the extent of C5-drug complex formation under plasma conditions. Whereas in the case of eculizumab the persistence of the antibody-C5 complex in the endosome led to the degradation of both members of the complex, the efficient release of C5 from ravulizumab in the endosome allowed the antibody to be more fully protected from degradation through its interaction with FcRn, an interaction also made stronger by the engineered Fc region mutations. The drug is then efficiently recycled back to the cell surface where, upon encountering the neutral pH of the extracellular fluid, it is released from FcRn back into circulation.[347] This results in a drug with a half-life that is about four times as long as the original mAb[348] which allows for dosing every 8 weeks rather than every 2 weeks. The main advantage, then, is convenience for the patient—and more leeway in the case of a delay in administration due to a hospitalization or an interruption in the drug supply. Ravulizumab met a non-inferiority endpoint based on hemolysis when compared to eculizumab that was based on hemolysis in two clinical trials,[318,344] and presumably the drug will have the same effect as eculizumab in preventing thrombosis. Suppression of free C5 levels seems to be superior with ravulizumab.[349] Whereas with eculizumab, low CH50 levels are a good indicator of therapeutic drug levels[350] this is not uniformly the case for ravulizumab.[351] (For ravulizumab, the pH at which the test happens to be performed may be a determinant of the in vitro effect.) While the drug is priced such that it is somewhat less expensive to treat a patient for a year, because a single dose is considerably more expensive than eculizumab, hospital pharmacies are expected to be less willing to stock the newer drug for inpatient use in places where inpatient and outpatient medications are reimbursed differently. Eculizumab has been shown to be safe in pregnancy for the fetus and seems to have minimal effect on the newborn's complement system[352]—and in fact has tremendously reduced the risks of pregnancy for women with PNH as mentioned above.[353] However, the same has not yet been demonstrated for ravulizumab, which is expected to cross the placenta to a greater degree due to its increased affinity for the FcRn. Therefore, there will continue to be a clinical need for eculizumab for hospitalized patients and women of childbearing years. Because ravulizumab binds to the same epitope as eculizumab, it will not be effective in patients with the Arg885His polymorphism in the C5 gene. On the other hand, when switching from eculizumab to ravulizumab, drug-target-drug

TABLE 23.5 FDA-Approved Complement Inhibitors and Some New Drugs Under Development

Company	Drug	Target	Structure/Derivation	Route	Stage of Development
Alexion	Eculizumab	C5	Humanized monoclonal antibody	Intravenous	FDA approved for PNH, aHUS, myasthenia gravis, and anti-AQ4+ NMOSD
ViroPharma, CSC Behring	C1 esterase inhibitor: plasma derived	C1 esterase	Human protein	Intravenous	FDA approved for hereditary angioedema
Pharming-Salix	C1 esterase inhibitor: recombinant	C1 esterase	Protein analogue, produced in rabbits	Intravenous	FDA approved for hereditary angioedema
Alexion	Ravulizumab	C5	Monoclonal antibody FcRN optimized, derived from eculizumab	Intravenous	Approved for PNH and aHUS
Apellis	ALP-2 (pegcetacoplan)	C3	Pegylated compstatin (cyclic peptide) analogue	Subcutaneous	Successful phase II study in C3 glomerulopathy; successful phase III study compared with eculizumab in PNH, FDA approved for PNH
Amgen	ABP959	C5	Monoclonal antibody--eculizumab biosimilar	Intravenous	Phase III studies ongoing
Sanofi	Sutimlimab (BIVV009, TNT 009)	C1s	Humanized monoclonal antibody	Intravenous	FDA approval for cold agglutinin disease
AO Generium	Elizaria	C5	Monoclonal antibody-eculizumab biosimilar	intravenous	Successful randomized study in PNH
Bioced	BCD-148	C5	Monoclonal antibody-eculizumab biosimilar	Intravenous	Phase III study in progress
Chemocentryx	CCX168/Avacopan	C5aR	Small molecule	Oral	Phase III studies in vasculitis
Novartis	LNP023	Factor B	Small molecule	Oral	Successful phase II study when added to a regimen of eculizumab in PNH
Achillion/Alexion	ACH-014471/ Danicopan	Factor D	Small molecule	Oral	Successful phase II study when added to a regimen of eculizumab in PNH
Regeneron	REGN3918/ pozelimab	C5	Monoclonal antibody	Intravenous/ subcutaneous	Successful phase II study in PNH
AKARI	Coversin/rVA576/ nomacopan	C5	17kDa peptide/tick saliva	Subcutaneous	Successful phase II study in PNH/studies in aHUS
Ra Pharmaceuticals	Zilucoplan/ RA101495	C5	15 amino acid macrocyclic peptide	Subcutaneous	Successful phase II studies in myasthenia gravis and PNH
BioCryst	BCX9930	Factor D	Small molecule	Oral	Successful phase II study in PNH
Samsung Bioepis	SB12	C5	Monoclonal antibody-eculizumab biosimilar	Intravenous	Phase III study planned
Roche	SKY59/RO7112689/ Crovalimab	C5	Monoclonal antibody, recycling technology	Intravenous and subcutaneous	Successful phase II study
Omeros	OMS721/ Narsoplimab	MASP-2	Human monoclonal antibody	Intravenous	Phase II studies in thrombotic microangiopathies and other renal disorders
Annexon Biosciences	ANX005	C1q	Humanized monoclonal antibody	Intravenous	Guillain-Barré syndrome, phase II/III clinical trial
Amyndas	AMY-101	C3	Cyclic peptide compstatin analog Cp40	Subcutaneous or intravenous	Successful phase II studies completed in gingivitis
Alexion(Taligen)	TT30	C3 convertase	Factor H-CR2 fusion	Intravenous/ subcutaneous	Human studies
Novartis	LFG 316/ Tesidolumab	C5	mAb	Intravenous/ Intravitreal	Human studies
Achillion/Alexion	ACH-0145228/ ALXN2050	Factor D	Small molecule	Oral	Successful phase I study
Innate Pharma	Avdoralimab (IPH5401)	C5aR	Monoclonal antibody		Human studies for solid tumors in combination with durvalumab
Amyndas	Mini Factor H	C3 convertase	Derived from factor H		Preclinical studies
Alnylam	ALN-CC5/ Cemdisiran	C5 RNA	RNAi conjugate	Subcutaneous	Preclinical studies
Lindofer et al.	3E7/H17	C3b	mAb		Preclinical studies
Elusys	H17	C3b, iC3b	Monoclonal		Preclinical studies
Mubodina	Adienne	C5	Mini-antibody		Preclinical studies

AQ4+ NMOSD, aquaporin-4 antibody-positive neuromyelitis optica spectrum disorder; *aHUS*, atypical hemolytic uremic syndrome; *PNH*, paroxysmal nocturnal hemoglobinuria.

immune complexes do not form—which can occur when two antibodies simultaneously bind to distinct epitopes on the same plasma protein.[354] As expected, meningococcal infections have occurred.[348]

New Anticomplement Drugs: Pegcetacoplan and Beyond

In the realm of anti-complement drugs, it will soon be an embarrassment of riches (Table 23.5). Beyond eculizumab and ravulizumab, there are biosimilars under development, as well as antibodies and peptides that will also target C5, regardless of the polymorphisms in residue 885 in the C5 gene that confer eculizumab resistance. Examples of the latter include zilucoplan[355,356] and the tick-derived coversin analogue nomacopan.[320,357,358] Sutimlimab, targeting C1s in the CP, is showing promise in cold agglutinin disease.[359] There are several drugs under investigation to specifically target proteins in the AP, including peptide inhibitors of C3, and small molecule inhibitors of factors B and D, which may prevent intravascular and extravascular hemolysis in PNH.[360]

Of the new inhibitors of the AP, pegcetacoplan is the first to receive FDA approval. This is an analogue of compstatin, which targets C3 and was originally discovered by phage display.[361] Pegcetacoplan is highly specific for primate C3, it binds with a subnanomolar affinity, and prevents C3 activation by the convertases.[362] This compound is a pentadecapeptide that combines the cyclic tridecapeptide compstatin derivative and a 2-amino acid linker, with one moiety coupled to each end of a linear 40 kDa PEG chain, providing two targeting peptides per molecule of the drug.[293] It is given by a self-administered subcutaneous infusion twice weekly, and it takes several weeks to achieve steady state levels. In a randomized study of 80 patients with PNH who failed to achieve a hemoglobin greater than 10.5 g/dL on eculizumab, transitioning to pegcetacoplan was superior to remaining on eculizumab for its effect on hemoglobin (a mean increase of 3.8 g/dL), and for achieving red cell transfusion independence (85% versus 15%). Not surprisingly, fatigue scores improved. Unlike in patients on eculizumab, reticulocyte counts normalized in most patients on pegcetacoplan. The most common side effects are injection site reactions and diarrhea. Although the follow-up was short, no patient on pegcetacoplan developed meningococcemia or thrombosis, though there was one episode of sepsis in a patient during the period of transition from eculizumab to pegcetacoplan.

The clinical need for these new anti-complement drugs stems from the incomplete responses seen in patients with PNH mentioned above, and slow and incomplete recovery of renal function in many patients with aHUS, which leaves some patients still requiring dialysis.[330] It is unclear whether the best strategy for drug development and patient care will be to add additional anti-complement agents to the approved C5 inhibitors for patients who are responding poorly or slowly, or to switch to another agent completely. For PNH, which is by nature paroxysmal, it is unclear whether additional or alternative agents need to be maintained, or used to abort "breakthrough" attacks. It is not clear whether the easily measured outcomes (such as normalization of LDH) will correlate with the important long-term outcomes—recovery of renal function for aHUS and prevention of thrombosis for PNH. As above, to date, safety in pregnancy has been demonstrated only for eculizumab. Because inherited deficiency of C3 carries a risk of a much broader spectrum of infections than inherited deficiency of the terminal complement proteins,[363] interference with the upstream complement proteins might carry a higher risk of infection. Indeed, the randomized study of pegcetacoplan mandated vaccination not only against *N. meningitidis* types A, C, W, Y, and B, but also against *S. pneumoniae*, and *Haemophilus influenzae* type B. It is now known that eculizumab does not completely block the lysis of red cells under conditions that strongly activate complement, whereas a combination of eculizumab and an additional C5 targeting drug, coversin is much more completely protective of the red cells in vitro.[321] It may be that this accounts for both the relative safety of eculizumab, as well as the occurrence of breakthrough hemolysis, especially during times of infection. Likewise, C3 inhibition with compstatin alone does not completely block lysis of red cells under strong complement activation,[52] which may be why pegcetacoplan, fortunately, does not seem to confer the same infectious risk seen in inherited C3 deficiency. However, infections with encapsulated organisms might occur if different complement inhibitors are combined, or during a period of transition from one to the other. The challenge for drug development and for clinicians will be to identify the subset of patients and conditions where dysregulated complement is clearly injurious, whose response to established complement inhibition is so clearly inadequate, that a more complete complement blockade is warranted, despite what will probably be a greater risk of infection.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians

Ahearn JM, Fischer MB, Croix D, et al. Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen. *Immunity*. 1996;4:251–262.

Carroll MC, Isenman DE. Regulation of humoral immunity by complement. *Immunity*. 2012;37:199–207.

Carter RH, Fearon DT. CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes. *Science*. 1992;256:105–107.

Clark SJ, Schmidt CQ, White AM, et al. Identification of factor H-like protein 1 as the predominant complement regulator in Bruch's membrane: implications for age-related macular degeneration. *J Immunol*. 2014;193: 4962–4970.

Degn SE, Kjaer TR, Kidmose RT, et al. Complement activation by ligand-driven juxtaposition of discrete pattern recognition complexes. *Proc Natl Acad Sci USA*. 2014;111:13445–13450.

Dempsey PW, Allison ME, Akkaraju S, et al. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science*. 1996;271: 348–350.

Ferreira VP, Pangburn MK, Cortes C. Complement control protein factor H: the good, the bad, and the inadequate. *Mol Immunol*. 2010;47:2187–2197.

Gros P, Milder FJ, Janssen BJC. Complement driven by conformational changes. *Nat Rev Immunol*. 2008;8:48–58.

Hanlon A, Metjian A. Caplacizumab in adult patients with acquired thrombotic thrombocytopenic purpura. *Ther Adv Hematol*. 2020;11 20406207720902904.

Hayashi M, Machida T, Ishida Y, et al. Cutting edge: role of MASP-3 in the physiological activation of factor D of the alternative complement pathway. *J Immunol*. 2019;203:1411–1416.

Heesters BA, van der Poel CE, Das A, et al. Antigen presentation to B cells. *Trends Immunol*. 2016;37:844–854.

Hillmen P, Muus P, Dührsen U, et al. Effect of the complement inhibitor eculizumab on thromboembolism in patients with paroxysmal nocturnal hemoglobinuria. *Blood*. 2007;110:4123–4128.

Hillmen P, Szer J, Weitz I, et al. Pegcetacoplan versus eculizumab in paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 2021;384:1028–1037.

Józsi M, Tortajada A, Uzonyi B, et al. Factor H-related proteins determine complement-activating surfaces. *Trends Immunol*. 2015;36:374–384.

Kavanaugh D, Richards A, Atkinson J. Complement regulatory genes and hemolytic uremic syndromes. *Annu Rev Med*. 2008;59:293–309.

Kelly R, Arnold L, Richards S, et al. The management of pregnancy in paroxysmal nocturnal haemoglobinuria on long term eculizumab. *Brit J Haematol*. 2010;149:446–450.

Kitazawa T, Shima M. Emicizumab, a humanized bispecific antibody to coagulation factors IXa and X with a factor VIIIa-cofactor activity. *Int J Hematol*. 2020;111:20–30.

Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*. 1975;256:495–497.

Legendre CM, Licht C, Muus P, et al. Terminal complement inhibitor eculizumab in atypical hemolytic-uremic syndrome. *N Engl J Med*. 2013;368:2169–2181.

Matsumura Y. Risk analysis of eculizumab-related meningococcal disease in Japan using the Japanese adverse drug event report database. *Drug Healthc Patient Saf*. 2020;12:207–215.

Pansri P, Jaruseranee N, Rangnoi K, et al. A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. *BMC Biotechnol*. 2009;9:1–16.

Pickering MC, Botto M, Taylor PR, et al. Systemic lupus erythematosus, complement deficiency, and apoptosis. *Adv Immunol*. 2001;76: 227–324.

Pio R, Ajona D, Ortiz-Espinosa S, et al. Complementing the cancer-immunity cycle. *Front Immunol*. 2019;10:774.

Risitano AM, Notaro R, Marando L, et al. Complement fraction 3 binding on erythrocytes as additional mechanism of disease in paroxysmal nocturnal hemoglobinuria patients treated by eculizumab. *Blood*. 2009;113:4094–4100.

Roth A, Barcellini W, D'Sa S, et al. Sutimlimab in cold agglutinin disease. *N Engl J Med*. 2021;384:1323–1334.

Schwab I, Nimmerjahn F. Intravenous immunoglobulin therapy: how does IgG modulate the immune system? *Nat Rev Immunol*. 2013;13:176–189.

Sheridan D, Yu ZX, Zhang Y, et al. Design and preclinical characterization of ALXN1210: A novel anti-C5 antibody with extended duration of action. *PloS One*. 2018;13:e0195909.

van den Elsen JM, Isenman DE. A crystal structure of the complex between human complement receptor 2 and its ligand C3d. *Science*. 2011;332: 608–611.

Verschoor A, Kemper C, Kohl J: Complement Receptors. eLS Wiley Online Library https://doi.org/10.1002/9780470015902.a0000512.pub3, 2017.

West EE, Kolev M, Kemper C. Complement and the regulation of T cell responses. *Annu Rev Immunol*. 2018;36:309–338.

REFERENCES

1. Silverstein AM. *A History of Immunology*. San Diego, CA: Academic Press; 1989:422.
2. Frank MM. Introduction and historical notes. In: Volanakis JE, Frank MM, eds. *The Human Complement System in Health and Disease*. New York: Marcel Dekker; 1998:1–8.
3. Weiler JM. Ch. 1, Introduction. In: Whaley K, Weiler JM, Loos M, eds. *Complement in Health and Disease*. London: Kluwer Academic Publishers; 1993:1–37.
4. Zhang P, Summer WR, Bagby GJ, et al. Innate immunity and pulmonary host defense. *Immunol Rev*. 2000;173:39–51.
5. Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol*. 1999;17:593–623.
6. Hartmann K, Henz BM, Krüger-Krasagakes S, et al. C3a and C5a stimulate chemotaxis of human mast cells. *Blood*. 1997;89:2863–2870.
7. Höpken UE, Lu B, Gerard NP, et al. The C5a chemoattractant receptor mediates mucosal defence to infection. *Nature*. 1996;383:86–89.
8. Cooper NR. The classical complement pathway: activation and regulation of the first complement component. *Adv Immunol*. 1985;37:151–216.
9. Loos MPF, Colomb M. Complement component C1 and the collectins: preface. *Immunobiology*. 1998;199:1–2.
10. Rosse WF. Fixation of the first component of complement (C'la) by human antibodies. *J Clin Invest*. 1969;47:2430–2445.
11. Sim RB, Reid KB. C1: molecular interactions with activating systems. *Immunol Today*. 1991;12:307–311.
12. Arlaud GJ, Thielens NM, Illy C. Assembly of the C1 complex. *Behring Inst Mitt*. 1993;93:189–195.
13. Diebolder CA, Beurskens FJ, de Jong RN, et al. Complement is activated by IgG hexamers assembled at the cell surface. *Science*. 2014;343:1260–1263.
14. Kang YS, Do Y, Lee HK, et al. A dominant complement fixation pathway for pneumococcal polysaccharides initiated by SIGN-R1 interacting with C1q. *Cell*. 2006;125:47–58.
15. Gonzalez SF, Lukacs-Kornek V, Kuligowski MP, et al. Capture of influenza by medullary dendritic cells via SIGN-R1 is essential for humoral immunity in draining lymph nodes. *Nat Immunol*. 2010;11:427–434.
16. Takahashi M, Iwaki D, Kanno K, et al. Mannose-binding lectin (MBL)-associated serine protease (MASP)-1 contributes to activation of the lectin complement pathway. *J Immunol*. 2008;180:6132–6138.
17. Fraser IP, Koziel H, Ezekowitz RA. The serum mannose-binding protein and the macrophage mannose receptor are pattern recognition molecules that link innate and adaptive immunity. *Semin Immunol*. 1998;10:363–372.
18. Héja D, Kocsis A, Dobó J, et al. Revised mechanism of complement lectin-pathway activation revealing the role of serine protease MASP-1 as the exclusive activator of MASP-2. *Proc Natl Acad Sci USA*. 2012;109:10498–10503.
19. Degn SE, Jensen L, Hansen AG, et al. Mannan-binding lectin-associated serine protease (MASP)-1 is crucial for lectin pathway activation in human serum, whereas neither MASP-1 nor MASP-3 is required for alternative pathway function. *J Immunol*. 2012;189:3957–3969.
20. Degn SE, Kjaer TR, Kidmose RT, et al. Complement activation by ligand-driven juxtaposition of discrete pattern recognition complexes. *Proc Natl Acad Sci USA*. 2014;111:13445–13450.
21. Thiel S, Vorup-Jensen T, Stover CM, et al. A second serine protease associated with mannan-binding lectin that activates complement. *Nature*. 1997;386:506–510.
22. Matsushita M. Ficolins in complement activation. *Mol Immunol*. 2013; 55:22–26.
23. Hansen SW, Ohtani K, Roy N, et al. The collectins CL-L1, CL-K1 and CL-P1, and their roles in complement and innate immunity. *Immunobiology*. 2016;21:058–067.
24. Thompson C. Protein proves to be a key link in innate immunity. *Science*. 1995;269:301–302.
25. Held K, Thiel S, Loos M, et al. Increased susceptibility of complement factor B/C2 double knockout mice and mannan-binding lectin knockout mice to systemic infection with Candida albicans. *Mol Immunol*. 2008; 45:3934–3941.
26. Shi L, Takahashi K, Dundee J, et al. Mannose-binding lectin-deficient mice are susceptible to infection with *Staphylococcus aureus*. *J Exp Med*. 2004; 199:1379–1390.
27. Müller-Eberhard HJ, Schreiber RD. Molecular biology and chemistry of the alternative pathway of complement. *Adv Immunol*. 1980;29:1–53.
28. Kolb WP, Morrow PR, Tamerius JD. Ba and Bb fragments of factor B activation: fragment production, biological activities, neoepitope expression and quantitation in clinical samples. *Complement Inflamm*. 1989;6:175–204.
29. Nishida N, Walz T, Springer TA. Structural transitions of complement component C3 and its activation products. *Proc Natl Acad Sci USA*. 2006;103:19737–19742.
30. Janssen BJ, Huizinga EG, Raaijmakers HC, et al. Structures of complement component C3 provide insights into the function and evolution of immunity. *Nature*. 2005;437:505–511.
31. Janssen BJ, Christodoulidou A, McCarthy A, et al. Structure of C3b reveals conformational changes that underlie complement activity. *Nature*. 2006;444:213–216.
32. Wiesmann C, Katschke KJ, Yin J, et al. Structure of C3b in complex with CRIg gives insights into regulation of complement activation. *Nature*. 2006;444:217–220.
33. Gros P, Milder FJ, Janssen BJC. Complement driven by conformational changes. *Nat Rev Immunol*. 2008;8:48–58.
34. Takahashi M, Ishida Y, Iwaki D, et al. Essential role of mannose-binding lectin-associated serine protease-1 in activation of the complement factor D. *J Exp Med*. 2010;207:29–37.
35. Hayashi M, Machida T, Ishida Y, et al. Cutting edge: role of MASP-3 in the physiological activation of factor D of the alternative complement pathway. *J Immunol*. 2019;203:1411–1416.
36. Brodbeck WG, Mold C, Atkinson JP, et al. Cooperation between decay-accelerating factor and membrane cofactor protein in protecting cells from autologous complement attack. *J Immunol*. 2000;165:3999–4006.
37. Morgan BP, Harris CL. *Complement Regulatory Proteins*. San Diego, CA: Academic Press; 1999:382.
38. Ferreira VP, Pangburn MK, Cortés C. Complement control protein factor H: the good, the bad, and the inadequate. *Mol Immunol*. 2010;47:2187–2197.
39. Pangburn MK. Cutting edge: localization of the host recognition functions of complement factor H at the carboxyl-terminal: implications for hemolytic uremic syndrome. *J Immunol*. 2002;169:4702–4706.
40. Pedersen DV, Gadeberg TAF, Thomas C, et al. Structural basis for properdin oligomerization and convertase stimulation in the human complement system. *Front Immunol*. 2019;10:2007. https://doi.org/10.3389/fimmu.2019.02007.
41. Zhou HF, Yan HM, Stover CM, et al. Antibody directs properdin-dependent activation of the complement alternative pathway in a mouse model of abdominal aortic aneurysm. *Proc Natl Acad Sci USA*. 2012;109:E415–E422.
42. Chen JY, Cortes C, Ferreira VP. Properdin: a multifaceted molecule involved in inflammation and diseases. *Mol Immunol*. 2018;102:58–72.
43. Spitzer D, Mitchell LM, Atkinson JP, et al. Properdin can initiate complement activation by binding specific target surfaces and providing a platform for de novo convertase assembly. *J Immunol*. 2007;179:2600–2608.
44. Harboe M, Ulvund G, Vien L, et al. The quantitative role of alternative pathway amplification in classical pathway induced terminal complement activation. *Clin Exp Immunol*. 2004;138:439–446.
45. Ratnoff WD, Fearon DT, Austen KF. The role of antibody in the activation of the alternative complement pathway. *Springer Semin Immunopathol*. 1983;6:361–371.
46. Fries LF, Gaither TA, Hammer CH, et al. C3b covalently bound to IgG demonstrates a reduced rate of inactivation by factors H and I. *J Exp Med*. 1984;160:1640–1655.
47. Matsumoto I, Maccioni M, Lee DM, et al. How antibodies to a ubiquitous cytoplasmic enzyme may provoke joint-specific autoimmune disease. *Nat Immunol*. 2002;3:360–365.
48. Volanakis JE. Participation of C3 and its ligands in complement activation. *Curr Top Microbiol Immunol*. 1990;153:1–21.
49. Rawal N, Pangburn MK. Formation of high affinity C5 convertase of the classical pathway of complement. *J Biol Chem*. 2003;278:38476–38483.
50. Bhakdi S, Tranum-Jensen J. Complement lysis: a hole is a hole. *Immunol Today*. 1991;21 12:318-20; discussion.
51. Esser AF, Big MAC. attack: complement proteins cause leaky patches. *Immunol Today*. 1991;21 12:316-8; discussion.
52. Mannes M, Dopler A, Zolk O, et al. Complement inhibition at the level of C3 or C5: mechanistic reasons for ongoing terminal pathway activity. *Blood*. 2021;137:443–455.
53. Schatz-Jakobsen J, Pedersen D, Andersen G. Structural insight into proteolytic activation and regulation of the complement system. *Immunol Rev*. 2016;274:59–73.
54. Ahearn JM, Fearon DT. Structure and function of the complement receptors, CR1 (CD35) and CR2 (CD21). *Adv Immunol*. 1989;46:183–219.
55. Fischer E, Appay MD, Cook J, et al. Characterization of the human glomerular C3 receptor as the C3b/C4b complement type one (CR1) receptor. *J Immunol*. 1986;136:1373–1377.
56. Verschoor A, Kemper C, Kohl J: Complement Receptors. eLS Wiley Online Library https://doi.org/10.1002/9780470015902.a0000512.pub3, 2017.

57. Walport MJ, Lachmann PJ. Erythrocyte complement receptor type 1, immune complexes, and the rheumatic diseases. *Arthritis Rheum*. 1988;31:153–158.
58. Fearon DT, Ahearn JM. Complement receptor type 1 (C3b/C4b receptor; CD35) and complement receptor type 2 (C3d/Epstein-Barr virus receptor; CD21). *Curr Top Microbiol Immunol*. 1990;153:83–98.
59. Helmy KY, Katschke Jr. KJ, Gorgani NN, et al. CRIg: a macrophage complement receptor required for phagocytosis of circulating pathogens. *Cell*. 2006;124:915–927.
60. Ember JA, Jagels MA, Hugli TE. Characterization of complement anaphylatoxins and their biological responses. In: Volanakis JE, Frank MM, eds. *The Human Complement System in Health and Disease*. New York: Marcel Dekker; 1998:241–284.
61. Finkelman FD, Khodoun MV, Strait R. Human IgE-independent systemic anaphylaxis. *J Allergy Clin Immun*. 2016;137:1674–1680.
62. DiScipio RG, Daffern PJ, Jagels MA, et al. A comparison of C3a and C5a-mediated stable adhesion of rolling eosinophils in postcapillary venules and transendothelial migration in vitro and in vivo. *J Immunol*. 1999;162:1127–1136.
63. Foreman KE, Glovsky MM, Warner RL, et al. Comparative effect of C3a and C5a on adhesion molecule expression on neutrophils and endothelial cells. *Inflammation*. 1996;20:1–9.
64. Kohl J. Anaphylatoxins and infectious and non-infectious inflammatory diseases. *Mol Immunol*. 2001;38:175–187.
65. Barnum SR. C4a: An anaphylatoxin in name only. *J Innate Immun*. 2015;7:333–339.
66. Li XX, Lee JD, Kemper C, et al. The complement receptor C5aR2: a powerful modulator of innate and adaptive immunity. *J Immunol*. 2019;202:3339–3348.
67. Liszewski MK, Farries TC, Lublin DM, et al. Control of the complement system. *Adv Immunol*. 1996;61:201–283.
68. Davis AE 3rd. C1 inhibitor and hereditary angioneurotic edema. *Annu Rev Immunol*. 1988;6:595–628.
69. Ollert MW, Kadlec JV, David K, et al. Antibody-mediated complement activation on nucleated cells. A quantitative analysis of the individual reaction steps. *J Immunol*. 1994;153:2213–2221.
70. Holers VM, Kinoshita T, Molina H. The evolution of mouse and human complement C3-binding proteins: divergence of form but conservation of function. *Immunol Today*. 1992;13:231–236.
71. Xu C, Mao D, Holers VM, et al. A critical role for murine complement regulator crry in fetomaternal tolerance. *Science*. 2000;287:498–501.
72. Petry F. Molecular basis of hereditary C1q deficiency. *Immunobiology*. 1998;199:286–294.
73. Walport MJ, Davies KA, Botto M. C1q and systemic lupus erythematosus. *Immunobiology*. 1998;199:265–285.
74. Carroll MC, Campbell RD, Bentley DR, et al. A molecular map of the human major histocompatibility complex class III region linking complement genes C4, C2 and factor B. *Nature*. 1984;307:237–241.
75. Belt KT, Yu CY, Carroll MC, et al. Polymorphism of human complement component C4. *Immunogenetics*. 1985;21:173–180.
76. Pickering MC, Botto M, Taylor PR, et al. Systemic lupus erythematosus, complement deficiency, and apoptosis. *Adv Immunol*. 2001;76:227–324.
77. Kamitaki N, Sekar A, Handsaker RE, et al. Complement genes contribute sex-biased vulnerability in diverse disorders. *Nature*. 2020;582:577–581.
78. Carroll M, Fathallah D, Bergamaschini L, et al. Substitution of a single amino acid (aspartic acid for histidine) converts the functional activity of human complement C4B to C4A. *Proc Natl Acad Sci USA*. 1990;87:6868–6872.
79. Law S, Dodds A. The internal thioester and the covalent binding properties of the complement proteins C3 and C4. *Protein Sci*. 1997;6:263–274.
80. Simoni L, Presumey J, van der Poel CE, et al. Complement C4A regulates autoreactive B cells in murine lupus. *Cell Rep*. 2020;33:108330.
81. Bala Subramanian V, Liszewski MK, Atkinson JP. The complement system and autoimmunity. In: Lahita R, Chiorazzi N, Reeves W, eds. *Textbook of Autoimmune Diseases*. Philadelphia, PA: Lippincott-Raven; 2000.
82. Carroll MC. The role of complement in B cell activation and tolerance. *Adv Immunol*. 2000;74:61–88.
83. Cicardi M, Bergamaschini L, Cugno M, et al. Pathogenetic and clinical aspects of C1 inhibitor deficiency. *Immunobiology*. 1998;199:366–376.
84. Bork K. Acquired and hereditary forms of recurrent angioedema: update of treatment. *Allergol Select*. 2018;2:121–131.
85. Caccia S, Suffritti C, Cicardi M. Pathophysiology of hereditary angioedema. *Pediatr Allergy Immunol Pulmonol*. 2014;27:159–163.
86. Horiuchi T, Hide M, Yamashita K, et al. The use of tranexamic acid for on-demand and prophylactic treatment of hereditary angioedema—a systematic review. *J Cutan Immunol Allergy*. 2018;1:126–138.
87. Bekos C, Perkmann T, Krauth M, et al. Acquired C1 esterase inhibitor deficiency in lymphomas: prevalence, symptoms, and response to treatment. *Leuk Lymphoma*. 2016;57:2033–2036.
88. Bork K, Staubach-Renz P, Hardt J. Angioedema due to acquired C1-inhibitor deficiency: spectrum and treatment with C1-inhibitor concentrate. *Orphanet J Rare Dis*. 2019;14:65.
89. Densen P. Complement deficiencies and infection. In: Volanakis JE, Frank MM, eds. *The Human Complement System in Health and Disease*. New York: Marcel Dekker; 1998:409–421.
90. Kavanaugh D, Richards A, Atkinson J. Complement regulatory genes and hemolytic uremic syndromes. *Annu Rev Med*. 2008;59:293–309.
91. Fremeaux-Bacchi V, Miller EC, Liszewski MK, et al. Mutations in complement C3 predispose to development of atypical hemolytic uremic syndrome. *Blood*. 2008;112:4948–4952.
92. Kajander T, Lehtinen MJ, Hyvarinen S, et al. Dual interaction of factor H with C3d and glycosaminoglycans in host-nonhost discrimination by complement. *Proc Natl Acad Sci USA*. 2011;108:2897–2902.
93. Edwards AO, Ritter R 3rd, Abel KJ, et al. Complement factor H polymorphism and age-related macular degeneration. *Science*. 2005;308:421–424.
94. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science*. 2005;308:385–389.
95. Clark SJ, Bishop PN, Day AJ. Complement factor H and age-related macular degeneration: the role of glycosaminoglycan recognition in disease pathology. *Biochem Soc Trans*. 2010;38:1342–1348.
96. Clark SJ, Schmidt CQ, White AM, et al. Identification of factor H-like protein 1 as the predominant complement regulator in Bruch's membrane: implications for age-related macular degeneration. *J Immunol*. 2014;193:4962–4970.
97. Keenan TDL, Pickford CE, Holley RJ, et al. Age-dependent changes in heparan sulfate in human Bruch's membrane: implications for age-related macular degeneration. *Invest Ophth Vis Sci*. 2014;55:5370–5379.
98. Józsi M, Tortajada A, Uzonyi B, et al. Factor H-related proteins determine complement-activating surfaces. *Trends Immunol*. 2015;36:374–384.
99. Venables JP, Strain L, Routledge D, et al. Atypical haemolytic uraemic syndrome associated with a hybrid complement gene. *PLoS Med*. 2006;3:e431.
100. Valoti E, Alberti M, Tortajada A, et al. A novel atypical hemolytic uremic syndrome-associated hybrid CFHR1/CFH gene encoding a fusion protein that antagonizes factor H-dependent complement regulation. *J Am Soc Nephrol*. 2015;26:209–219.
101. Hughes AE, Orr N, Esfandiary H, et al. A common CFH haplotype, with deletion of CFHR1 and CFHR3, is associated with lower risk of age-related macular degeneration. *Nat Genet*. 2006;38:1173–1177.
102. Zhao J, Wu H, Khosravi M, et al. Association of genetic variants in complement factor H and factor H-related genes with systemic lupus erythematosus susceptibility. *PLoS Genet*. 2011;7:e1002079.
103. Tortajada A, Yebenes H, Abarrategui-Garrido C, et al. C3 glomerulopathy-associated CFHR1 mutation alters FHR oligomerization and complement regulation. *J Clin Invest*. 2013;123:2434–2446.
104. Atkinson JP, Bessler M. Paroxysmal nocturnal hemoglobinuria. In: Stamatoyannopoulos JA, Perlmutter JS, Majerus PW, eds. *The Molecular Basis of Blood Disease*. Philadelphia: WB Saunders; 2000:564–577.
105. Motoyama N, Okada N, Yamashina M, et al. Paroxysmal nocturnal hemoglobinuria due to hereditary nucleotide deletion in the HRF20 (CD59) gene. *Eur J Immunol*. 1992;22:2669–2673.
106. Takeda J, Miyata T, Kawagoe K, et al. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. *Cell*. 1993;73:703–711.
107. Hochsmann B, Dohna-Schwake C, Kyrieleis HA, et al. Targeted therapy with eculizumab for inherited CD59 deficiency. *N Engl J Med*. 2014;370:90–92.
108. Ozen A, Comrie WA, Ardy RC, et al. CD55 deficiency, early-onset protein-losing enteropathy, and thrombosis. *N Engl J Med*. 2017;377:52–61.
109. Almeida AM, Murakami Y, Layton DM, et al. Hypomorphic promoter mutation in PIGM causes inherited glycosylphosphatidylinositol deficiency. *Nat Med*. 2006;12:846–851.
110. Johnston JJ, Gropman AL, Sapp JC, et al. The phenotype of a germline mutation in PIGA: the gene somatically mutated in paroxysmal nocturnal hemoglobinuria. *Am J Hum Genet*. 2012;90:295–300.
111. Carroll MC. The lupus paradox. *Nat Genet*. 1998;19:3–4.
112. Paul E, Carroll MC. SAP-less chromatin triggers systemic lupus erythematosus. *Nat Med*. 1999;5:607–608.
113. Chatterjee P, Agyemang AF, Alimzhanov MB, et al. Complement C4 maintains peripheral B-cell tolerance in a myeloid cell dependent manner. *Eur J Immunol*. 2013;43:2441–2450.
114. Das A, Heesters BA, Bialas A, et al. Follicular dendritic cell activation by TLR ligands promotes autoreactive B cell responses. *Immunity*. 2017;46:106–119.
115. Carroll MC, Isenman DE. Regulation of humoral immunity by complement. *Immunity*. 2012;37:199–207.

116. Nussenzweig V, Bianco C, Duker P, et al. Receptors for C3 of B lymphocytes: possible role in the immune response. In: Amos B, ed. *Progress in Immunology*. New York: Academic Press; 1971.
117. Pepys MB. Role of complement in induction of antibody production in vivo. Effect of cobra factor and other C3-reactive agents on thymus-dependent and thymus-independent antibody responses. *J Exp Med*. 1974;140:126–145.
118. Bitter-Suermann D, Burger R. C3 deficiencies. *Curr Top Microbiol Immunol*. 1990;153:223–233.
119. Bottger EC, Metzger S, Bitter-Suermann D, et al. Impaired humoral immune response in complement C3-deficient guinea pigs: absence of secondary antibody response. *Eur J Immunol*. 1986;16:1231–1235.
120. O'Neil KM, Ochs HD, Heller SR, et al. Role of C3 in humoral immunity. Defective antibody production in C3-deficient dogs. *J Immunol*. 1988; 140:1939–1945.
121. Jackson CG, Ochs HD, Wedgwood RJ. Immune response of a patient with deficiency of the fourth component of complement and systemic lupus erythematosus. *N Engl J Med*. 1979;300:1124–1129.
122. Finco O, Li S, Cuccia M, et al. Structural differences between the two human complement C4 isotypes affect the humoral immune response. *J Exp Med*. 1992;175:537–543.
123. Cutler AJ, Botto M, van Essen D, et al. T cell-dependent immune response in C1q-deficient mice: defective interferon gamma production by antigen-specific T cells. *J Exp Med*. 1998;187:1789–1797.
124. Da Costa XJ, Brockman MA, Alicot E, et al. Humoral response to herpes simplex virus is complement-dependent. *Proc Natl Acad Sci USA*. 1999; 96:12708–12712.
125. Fischer MB, Ma M, Goerg S, et al. Regulation of the B cell response to T-dependent antigens by classical pathway complement. *J Immunol*. 1996;157:549–556.
126. Ochsenbein AF, Pinschewer DD, Odermatt B, et al. Protective T cell-independent antiviral antibody responses are dependent on complement. *J Exp Med*. 1999;190:1165–1174.
127. Kelsoe G. B cell diversification and differentiation in the periphery. *J Exp Med*. 1994;180:5–6.
128. Fischer MB, Goerg S, Shen L, et al. Dependence of germinal center B cells on expression of CD21/CD35 for survival. *Science*. 1998;280:582–585.
129. Gadjeva M, Verschoor A, Brockman MA, et al. Macrophage-derived complement component C4 can restore humoral immunity in C4-deficient mice. *J Immunol*. 2002;169:5489–5495.
130. Boes M, Esau C, Fischer MB, et al. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J Immunol*. 1998;160:4776–4787.
131. Rossbacher J, Shlomchik MJ. The B cell receptor itself can activate complement to provide the complement receptor 1/2 ligand required to enhance B cell immune responses in vivo. *J Exp Med*. 2003;198: 591–602.
132. Manderson AP, Quah B, Botto M, et al. A novel mechanism for complement activation at the surface of B cells following antigen binding. *J Immunol*. 2006;177:5155–5162.
133. Dempsey PW, Allison ME, Akkaraju S, et al. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science*. 1996;271:348–350.
134. Ross TM, Xu Y, Bright RA, et al. C3d enhancement of antibodies to hemagglutinin accelerates protection against influenza virus challenge. *Nat Immunol*. 2000;1:127–131.
135. Kinoshita T, Takeda J, Hong K, et al. Monoclonal antibodies to mouse complement receptor type 1 (CR1). Their use in a distribution study showing that mouse erythrocytes and platelets are CR1-negative. *J Immunol*. 1988;140:3066–3072.
136. Kinoshita T, Fujita T, Tsunoda R. Expression of complement receptors CR1 and CR2 on murine follicular dendritic cells and B lymphocytes. In: Imai Y, Tew JG, Hoefsmit ECM, eds. *Dendritic Cells in Lymphoid Tissues*. Amsterdam: Elsevier; 1991:127.
137. Carroll MC, Alicot EM, Katzman PJ, et al. Organization of the genes encoding complement receptors type 1 and 2, decay-accelerating factor, and C4-binding protein in the RCA locus on human chromosome 1. *J Exp Med*. 1988;167:1271–1280.
138. Kurtz CB, O'Toole E, Christensen SM, et al. The murine complement receptor gene family. IV. Alternative splicing of Cr2 gene transcripts predicts two distinct gene products that share homologous domains with both human CR2 and CR1. *J Immunol*. 1990;144:3581–3591.
139. Molina H, Kinoshita T, Inoue K, et al. A molecular and immunochemical characterization of mouse CR2. Evidence for a single gene model of mouse complement receptors 1 and 2. *J Immunol*. 1990;145:2974–2983.
140. Gustavsson S, Kinoshita T, Heyman B. Antibodies to murine complement receptor 1 and 2 can inhibit the antibody response in vivo without inhibiting T helper cell induction. *J Immunol*. 1995;154:6524–6528.
141. Heyman B, Wiersma EJ, Kinoshita T. In vivo inhibition of the antibody response by a complement receptor-specific monoclonal antibody. *J Exp Med*. 1990;172:665–668.
142. Thyphronitis G, Kinoshita T, Inoue K, et al. Modulation of mouse complement receptors 1 and 2 suppresses antibody responses in vivo. *J Immunol*. 1991;147:224–230.
143. Wiersma EJ, Kinoshita T, Heyman B. Inhibition of immunological memory and T-independent humoral responses by monoclonal antibodies specific for murine complement receptors. *Eur J Immunol*. 1991;21:2501–2506.
144. Hebell T, Ahearn JM, Fearon DT. Suppression of the immune response by a soluble complement receptor of B lymphocytes. *Science*. 1991;254: 102–105.
145. Carter RH, Fearon DT. CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes. *Science*. 1992;256:105–107.
146. Papamichail M, Gutierrez C, Embling P, et al. Complement dependence of localisation of aggregated IgG in germinal centres. *Scand J Immunol*. 1975; 4:343–347.
147. Kalli KR, Ahearn JM, Fearon DT. Interaction of iC3b with recombinant isotypic and chimeric forms of CR2. *J Immunol*. 1991;147:590–594.
148. Szakonyi G, Guthridge JM, Li D, et al. Structure of complement receptor 2 in complex with its C3d ligand. *Science*. 2001;292:1725–1728.
149. van den Elsen JM, Isenman DE. A crystal structure of the complex between human complement receptor 2 and its ligand C3d. *Science*. 2011;332: 608–611.
150. Ahearn JM, Fischer MB, Croix D, et al. Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen. *Immunity*. 1996;4:251–262.
151. Haas KM, Hasegawa M, Steeber DA, et al. Complement receptors CD21/35 link innate and protective immunity during *Streptococcus pneumoniae* infection by regulating IgG3 antibody responses. *Immunity*. 2002;17:713–723.
152. Molina H, Holers VM, Li B, et al. Markedly impaired humoral immune response in mice deficient in complement receptors 1 and 2. *Proc Natl Acad Sci USA*. 1996;93:3357–3361.
153. Croix DA, Ahearn JM, Rosengard AM, et al. Antibody response to a T-dependent antigen requires B cell expression of complement receptors. *J Exp Med*. 1996;183:1857–1864.
154. Bradbury LE, Kansas GS, Levy S, et al. The CD19/CD21 signal transducing complex of human B lymphocytes includes the target of antiproliferative antibody-1 and Leu-13 molecules. *J Immunol*. 1992;149:2841–2850.
155. Cherukuri A, Cheng PC, Sohn HW, et al. The CD19/CD21 complex functions to prolong B cell antigen receptor signaling from lipid rafts. *Immunity*. 2001;14:169–179.
156. Barrington RA, Pozdnyakova O, Zafari MR, et al. B lymphocyte memory: role of stromal cell complement and FcgammaRIIB receptors. *J Exp Med*. 2002;196:1189–1199.
157. Heesters BA, Chatterjee P, Kim YA, et al. Endocytosis and recycling of immune complexes by follicular dendritic cells enhances B cell antigen binding and activation. *Immunity*. 2013;38:1164–1175.
158. Heesters BA, van der Poel CE, Das A, et al. Antigen presentation to B cells. *Trends Immunol*. 2016;37:844–854.
159. Bonnefoy JY, Henchoz S, Hardie D, et al. A subset of anti-CD21 antibodies promote the rescue of germinal center B cells from apoptosis. *Eur J Immunol*. 1993;23:969–972.
160. Fischer MB, Ma M, Hsu NC, et al. Local synthesis of C3 within the splenic lymphoid compartment can reconstitute the impaired immune response in C3-deficient mice. *J Immunol*. 1998;160:2619–2625.
161. Roberts T, Snow EC. Cutting edge: recruitment of the CD19/CD21 coreceptor to B cell antigen receptor is required for antigen-mediated expression of Bcl-2 by resting and cycling hen egg lysozyme transgenic B cells. *J Immunol*. 1999;162:4377–4380.
162. Barrington R, Zhang M, Fischer M, et al. The role of complement in inflammation and adaptive immunity. Immunol Rev. 2001;180:5–15.
163. Matsumoto AK, Martin DR, Carter RH, et al. Functional dissection of the CD21/CD19/TAPA-1/Leu-13 complex of B lymphocytes. *J Exp Med*. 1993;178:1407–1417.
164. Cherukuri A, Carter RH, Brooks S, et al. B cell signaling is regulated by induced palmitoylation of CD81. *J Biol Chem*. 2004;279: 31973–31982.
165. Engel P, Zhou LJ, Ord DC, et al. Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. *Immunity*. 1995;3:39–50.
166. Maecker HT, Do MS, Levy S. CD81 on B cells promotes interleukin 4 secretion and antibody production during T helper type 2 immune responses. *Proc Natl Acad Sci USA*. 1998;95:2458–2462.
167. Rickert RC, Rajewsky K, Roes J. Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. *Nature*. 1995;376:352–355.

168. Klaus GG, Humphrey JH, Kunkl A, et al. The follicular dendritic cell: its role in antigen presentation in the generation of immunological memory. *Immunol Rev*. 1980;53:3–28.
169. Kelsoe G. The germinal center reaction. *Immunol Today*. 1995;16:324–326.
170. Hardie DL, Johnson GD, Khan M, et al. Quantitative analysis of molecules which distinguish functional compartments within germinal centers. *Eur J Immunol*. 1993;23:997–1004.
171. Berek C. Somatic mutation and memory. *Curr Opin Immunol*. 1993;5: 218–222.
172. Jacob J, Kelsoe G, Rajewsky K, et al. Intraclonal generation of antibody mutants in germinal centres. *Nature*. 1991;354:389–392.
173. Jacob J, Przylepa J, Miller C, et al. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. III. The kinetics of V region mutation and selection in germinal center B cells. *J Exp Med*. 1993; 178:1293–1307.
174. Kelsoe G. Life and death in germinal centers (redux). *Immunity*. 1996;4: 107–111.
175. Liu YJ, Joshua DE, Williams GT, et al. Mechanism of antigen-driven selection in germinal centres. *Nature*. 1989;342:929–931.
176. Qin D, Wu J, Carroll MC, et al. Evidence for an important interaction between a complement-derived CD21 ligand on follicular dendritic cells and CD21 on B cells in the initiation of IgG responses. *J Immunol*. 1998;161:4549–4554.
177. Fang Y, Xu C, Fu YX, et al. Expression of complement receptors 1 and 2 on follicular dendritic cells is necessary for the generation of a strong antigen-specific IgG response. *J Immunol*. 1998;160:5273–5279.
178. West EE, Kolev M, Kemper C. Complement and the regulation of T cell responses. *Annu Rev Immunol*. 2018;36:309–338.
179. Kopf M, Abel B, Gallimore A, et al. Complement component C3 promotes T-cell priming and lung migration to control acute influenza virus infection. *Nat Med*. 2002;8:373–378.
180. Liu J, Miwa T, Hilliard B, et al. The complement inhibitory protein DAF (CD55) suppresses T cell immunity in vivo. *J Exp Med*. 2005;201: 567–577.
181. Heeger PS, Lalli PN, Lin F, et al. Decay-accelerating factor modulates induction of T cell immunity. *J Exp Med*. 2005;2005:1523–1530.
182. Strainic MG, Liu J, Huang D, et al. Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. *Immunity*. 2008;28:425–435.
183. Kolev M, Markiewski MM. Targeting complement-mediated immunoregulation for cancer immunotherapy. *Semin Immunol*. 2018;37: 85–97.
184. Liszewski MK, Kolev M, Le Friec G, et al. Intracellular complement activation sustains T cell homeostasis and mediates effector differentiation. *Immunity*. 2013;39:1143–1157.
185. Arbore G, Kemper C. A novel "complement-metabolism-inflammasome axis" as a key regulator of immune cell effector function. *Eur J Immunol*. 2016;46:1563–1573.
186. Elvington M, Liszewski MK, Bertram P, et al. C3(H20) recycling pathway is a component of the intracellular complement system. *J Clin Invest*. 2017;127:970–981.
187. Le Friec G, Sheppard D, Whiteman P, et al. The CD46-Jagged1 interaction is critical for human TH1 immunity. *Nat Immunol*. 2012;13: 1213–1221.
188. Cardone J, Le Friec G, Vantourout P, et al. Complement regulator CD46 temporally regulates cytokine production by conventional and unconventional T cells. *Nat Immunol*. 2010;11:862–871.
189. Arbore G, West EE, Rahman J, et al. Complement receptor CD46 co-stimulates optimal human CD8(+) T cell effector function via fatty acid metabolism. *Nat Commun*. 2018;9:4186.
190. Gancz D, Fishelson Z. Cancer resistance to complement-dependent cytotoxicity (CDC): Problem-oriented research and development. *Mol Immunol*. 2009;46:2794–2800.
191. Pio R, Ajona D, Ortiz-Espinosa S, et al. Complementing the cancer-immunity cycle. *Front Immunol*. 2019;10:774.
192. Ajona D, Ortiz-Espinosa S, Moreno H, et al. A combined PD-1/C5a blockade synergistically protects against lung cancer growth and metastasis. *Cancer Discov*. 2017;7:694–703.
193. Zha H, Han X, Zhu Y, et al. Blocking C5aR signaling promotes the anti-tumor efficacy of PD-1/PD-L1 blockade. *Oncoimmunology*. 2017; 6:e1349587.
194. Oikonomopoulou K, Ricklin D, Ward PA, et al. Interactions between coagulation and complement—their role in inflammation. *Semin Immunopathol*. 2012;34:151–165.
195. Huber-Lang M, Sarma JV, Zetoune FS, et al. Generation of C5a in the absence of C3: a new complement activation pathway. *Nat Med*. 2006;12: 682–687.
196. Amara U, Flierl MA, Rittirsch D, et al. Molecular intercommunication between the complement and coagulation systems. *J Immunol*. 2010;185:5628–5636.
197. Polley MJ, Nachman R. The human complement system in thrombin-mediated platelet function. *J Exp Med*. 1978;147:1713–1726.
198. Sims PJ, Faioni EM, Wiedmer T, et al. Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. *J Biol Chem*. 1988;263:18205–18212.
199. Sims PJ, Wiedmer T. The response of human platelets to activated components of the complement system. *Immunol Today*. 1991;12:338–342.
200. Wiedmer T, Sims PJ. Effect of complement proteins C5b-9 on blood platelets. Evidence for reversible depolarization of membrane potential. *J Biol Chem*. 1985;260:8014–8019.
201. Wiedmer T, Sims PJ. Participation of protein kinases in complement C5b-9-induced shedding of platelet plasma membrane vesicles. *Blood*. 1991; 78:2880–2886.
202. Zimmerman TS, Kolb WP. Human platelet-initiated formation and uptake of the C5-9 complex of human complement. *J Clin Invest*. 1976;57:203–211.
203. Devine DV, Siegel RS, Rosse WF. Interactions of the platelets in paroxysmal nocturnal hemoglobinuria with complement. Relationship to defects in the regulation of complement and to platelet survival in vivo. *J Clin Invest*. 1987;79:131–137.
204. Dixon RH, Rosse WF. Mechanism of complement-mediated activation of human blood platelets in vitro: comparison of normal and paroxysmal nocturnal hemoglobinuria platelets. *J Clin Invest*. 1977;59:360–368.
205. Parker CJ, Wiedmer T, Sims PJ, et al. Characterization of the complement sensitivity of paroxysmal nocturnal hemoglobinuria erythrocytes. *J Clin Invest*. 1985;75:2074–2084.
206. Wiedmer T, Hall SE, Ortel TL, et al. Complement-induced vesiculation and exposure of membrane prothrombinase sites in platelets of paroxysmal nocturnal hemoglobinuria. *Blood*. 1993;82:1192–1196.
207. Magro C, Mulvey JJ, Berlin D, et al. Complement associated microvascular injury and thrombosis in the pathogenesis of severe COVID-19 infection: a report of five cases. *Transl Res*. 2020;220:1–13.
208. Weiler JM, Linhardt RJ. Antithrombin III regulates complement activity in vitro. *J Immunol*. 1991;146:3889–3894.
209. Sekar A, Bialas AR, de Rivera H, et al. Schizophrenia risk from complex variation of complement component 4. *Nature*. 2016;530:177–183.
210. Yilmaz M, Yalcin E, Presumey J, et al. Overexpression of schizophrenia susceptibility factor human complement C4A promotes excessive synaptic loss and behavioral changes in mice. *Nat Neurosci*. 2021;24:214–224.
211. Turner M. Molecules which recognize antigens. In: Roitt IM, Brostoff J, Male DK, eds. *Immunology*. 2nd ed. London: Gower; 1989:51.
212. Harris LJ, Larson SB, Hasel KW, et al. The three-dimensional structure of an intact monoclonal antibody for canine lymphoma. *Nature*. 1992; 360:369–372.
213. Alt FW, Blackwell TK, Yancopoulos GD. Development of the primary antibody repertoire. *Science*. 1987;238:1079–1087.
214. Golub ES. *Immunology: A Synthesis*. Sunderland, MA: Sinaur; 1987:60.
215. Krapp S, Mimura Y, Jefferis R, et al. Structural analysis of human IgG-Fc glycoforms reveals a correlation between glycosylation and structural integrity. *J Mol Biol*. 2003;325:979–989.
216. Nimmerjahn F, Ravetch JV. Fcgamma receptors: old friends and new family members. *Immunity*. 2006;24:19–28.
217. Parekh RB, Dwek RA, Sutton BJ, et al. Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature*. 1985;316:452–457.
218. Holland M, Yagi H, Takahashi N, et al. Differential glycosylation of polyclonal IgG, IgG-Fc and IgG-Fab isolated from the sera of patients with ANCA-associated systemic vasculitis. *Biochim Biophys Acta*. 2006;1760:669–677.
219. Kaneko Y, Nimmerjahn F, Ravetch JV. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science*. 2006;313:670–673.
220. Boshkov LK, Kelton JG. Use of intravenous gammaglobulin as an immune replacement and an immune suppressant. *Transfus Med Rev*. 1989;3:82–120.
221. Eibl MM. Intravenous immunoglobulins in neurological disorders: safety issues. *Neurol Sci*. 2003;24(Suppl 4):S222–S226.
222. Eibl MM, Wedgwood RJ. Intravenous immunoglobulin: a review. *Immunodefic Rev*. 1989;1(Suppl):1–42.
223. Kistler P, Nitschmann H. Large scale production of human plasma fractions. Eight years experience with the alcohol fractionation procedure of Nitschmann, Kistler and Lergier. *Vox Sang*. 1962;7:414–424.
224. Oncley JL, Melin M, Richert DA, et al. The separation of the antibodies, isoagglutinins, prothrombin, plasminogen and beta1-lipoprotein into subfractions of human plasma. *J Am Chem Soc*. 1949;71:541–550.
225. Buchacher A, Iberer G. Purification of intravenous immunoglobulin G from human plasma—aspects of yield and virus safety. *Biotechnol J*. 2006; 1:148–163.

226. Gelfand EW. Differences between IGIV products: impact on clinical outcome. *Int Immunopharmacol*. 2006;6:592–599.
227. Martin TD. IGIV: contents, properties, and methods of industrial production—evolving closer to a more physiologic product. *Int Immunopharmacol*. 2006;6:517–522.
228. McIver J, Grady GF. Immunoglobulin preparations. In: Churchill W, Kuntz SR, eds. *Transfusion Medicine. Oxford*: Blackwell Scientific; 1990:189–209.
229. Snydman DR, Werner BG, Heinze-Lacey B, et al. Use of cytomegalovirus immune globulin to prevent cytomegalovirus disease in renal-transplant recipients. *N Engl J Med*. 1987;317:1049–1054.
230. Casadevall A, Scharff MD. Return to the past: the case for antibody-based therapies in infectious diseases. *Clin Infect Dis*. 1995;21:150–161.
231. Intravenous immunoglobulin: prevention and treatment of disease. Summary of the NIH Consensus Development Conference. *Transfus Med Rev*. 5:171-2, 1991.
232. Darabi K, Abdel-Wahab O, Dzik WH. Current usage of intravenous immune globulin and the rationale behind it: the Massachusetts General Hospital data and a review of the literature. *Transfusion*. 2006;46:741–753.
233. Kumar A, Teuber SS, Gershwin ME. Intravenous immunoglobulin: striving for appropriate use. *Int Arch Allergy Immunol*. 2006;140:185–198.
234. Ness S. Differentiating characteristics and evaluating intravenous and subcutaneous immunoglobulin. *Am J Manag Care*. 2019;25:S98–S104.
235. Bayary J, Dasgupta S, Misra N, et al. Intravenous immunoglobulin in autoimmune disorders: an insight into the immunoregulatory mechanisms. *Int Immunopharmacol*. 2006;6:528–534.
236. Dalakas MC. Mechanisms of action of IVIg and therapeutic considerations in the treatment of acute and chronic demyelinating neuropathies. *Neurology*. 2002;59:S13–S21.
237. Larroche C, Chanseaud Y, Garcia de la Pena-Lefebvre P, et al. Mechanisms of intravenous immunoglobulin action in the treatment of autoimmune disorders. *BioDrugs*. 2002;16:47–55.
238. Schwab I, Nimmerjahn F. Intravenous immunoglobulin therapy: how does IgG modulate the immune system? *Nat Rev Immunol*. 2013;13:176–189.
239. Asghar SS. Pharmacological manipulation of the complement system in human diseases. *Front Biosci*. 1996;1:e15–e25.
240. Jin F, Balthasar JP. Mechanisms of intravenous immunoglobulin action in immune thrombocytopenic purpura. *Hum Immunol*. 2005;66:403–410.
241. Yamamoto K, Takamatsu J, Saito H. Intravenous immunoglobulin therapy for acquired coagulation inhibitors: a critical review. *Int J Hematol*. 2007;85:287–293.
242. Meissner HC, Leung DY. Immunoglobulin therapy in Kawasaki syndrome and RSV prophylaxis. *Front Biosci*. 1996;1:e55–e64.
243. Rosen FS. Putative mechanisms of the effect of intravenous gamma-globulin. *Clin Immunol Immunopathol*. 1993;67:S41–S43.
244. Anthony RM, Kobayashi T, Wermeling F, et al. Intravenous gammaglobulin suppresses inflammation through a novel T(H)2 pathway. *Nature*. 2011;475:110–113.
245. Hamrock DJ. Adverse events associated with intravenous immunoglobulin therapy. *Int Immunopharmacol*. 2006;6:535–542.
246. Immune Deficiency Foundation. *Treatment Experiences and Preferences of Patients with Primary Immune Deficiency Disease: First National Survey*. Towson: Immune Deficiency Foundation; 2003.
247. Boschetti N, Stucki M, Spath PJ, et al. Virus safety of intravenous immunoglobulin: future challenges. *Clin Rev Allergy Immunol*. 2005;29:333–344.
248. Siegel J. Safety considerations in IGIV utilization. *Int Immunopharmacol*. 2006;6:523–527.
249. Shelton BK, Griffin JM, Goldman FD. Immune globulin IV therapy: optimizing care of patients in the oncology setting. *Oncol Nurs Forum*. 2006;33:911–921.
250. Miller JL, Petteway Jr. SR, Lee DC. Ensuring the pathogen safety of intravenous immunoglobulin and other human plasma-derived therapeutic proteins. *J Allergy Clin Immunol*. 2001;108:S91–S94.
251. Van Holten RW, Autenrieth S, Boose JA, et al. Removal of prion challenge from an immune globulin preparation by use of a size-exclusion filter. *Transfusion*. 2002;42:999–1004.
252. CDC (Centers for Disease Control and Prevention) Renal insufficiency and failure associated with immune globulin intravenous therapy—United States, 1985–1998. *MMWR Morb Mortal Wkly Rep*. 1999;48:518–521.
253. Brannagan 3rd TH, Nagle KJ, Lange DJ, et al. Complications of intravenous immune globulin treatment in neurologic disease. *Neurology*. 1996;47:674–677.
254. Caress JB, Cartwright MS, Donofrio PD, et al. The clinical features of 16 cases of stroke associated with administration of IVIg. *Neurology*. 2003;60:1822–1824.
255. Stangel M, Muller M, Marx P. Adverse events during treatment with high-dose intravenous immunoglobulins for neurological disorders. *Eur Neurol*. 1998;40:173–174.
256. Dalakas MC. High-dose intravenous immunoglobulin and serum viscosity: risk of precipitating thromboembolic events. *Neurology*. 1994;44:223–226.
257. Williams SJ, Gupta S. Anaphylaxis to IVIG. *Arch Immunol Ther Exp (Warsz)*. 2017;65:11–19.
258. Kahwaji J, Barker E, Pepkowitz S, et al. Acute hemolysis after high-dose intravenous immunoglobulin therapy in highly HLA sensitized patients. *Clin J Am Soc Nephrol*. 2009;4:1993–1997.
259. Hanson K, Gabriel N, Mchardy I, et al. False-positive serologic results attributable to IVIG therapy. *Open Forum Infect Dis*. 2018;5(Suppl 1):S591–S5922018.
260. WHO. *Use of Convalescent Whole Blood or Plasma Collected from Patients Recovered from Ebola Virus Disease for Transfusion, as an Empirical Treatment during Outbreak*. Geneva: World Health Organization; 2014.
261. Libster R, Perez Marc G, Wappner D, et al. Early high-titer plasma therapy to prevent severe Covid-19 in older adults. *N Engl J Med*. 2021;384:610–618.
262. Kulkarni R. Antibody-dependent enhancement of viral infections. In: Bramhachari P, ed. *Dynamics of Immune Activation in Viral Diseases*. Singapore: Springer Nature; 2020:9–41.
263. Scaradavou A, Woo B, Woloski BM, et al. Intravenous anti-D treatment of immune thrombocytopenic purpura: experience in 272 patients. *Blood*. 1997;89:2689–2700.
264. Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*. 1975;256:495–497.
265. Greenfield EA. Generating monoclonal antibodies. In: *Antibodies: A Laboratory Manual*. 2nd ed. Long Island, NY : Cold Spring Harbor Laboratory Press; 2014.
266. Ahmad Z, Yeap S, Ali A, et al. scFv antibody principles and clinical application. *Clin Dev Immunol*. 2012;2012:1–15. ID980250.
267. Dantas-Barbosa C, Brigido MdM, Maranhao AQ. Antibody phage display libraries: contributions to oncology. *Int J Mol Sci*. 2012;13:5420–5440.
268. Hammers C, Stanley J. Antibody phage display: technique and applications. *J Invest Dermatol*. 2014;134:1–5.
269. Nixon A, Sexton D, Lander R. Drugs derived from phage display: from candidate identification to clinical practice. *MAbs*. 2014;6:73–85.
270. Pansri P, Jaruseranee N, Rangnoi K, et al. A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. *BMC Biotechnol*. 2009;9:1–16.
271. Lonberg N, Huszar D. Human antibodies from transgenic mice. *Int Rev Immunol*. 1995;13:65–93.
272. Hrabovska A, Bernard V, Krejci E. A novel system for the efficient generation of antibodies following immunization of unique knockout mouse strains. *PLoS One*. 2010;5:e12892.
273. Corti D, Misasi J, Mulangu S, et al. Protective monotherapy against lethal Ebola virus infection by a potently neutralizing antibody. *Science*. 2016;351:1339–1342.
274. Devilder MC, Moyon M, Saulquin X, et al. Generation of discriminative human monoclonal antibodies from rare antigen-specific B cells circulating in blood. *J Vis Exp*. 2018;Feb 6;(132):56508. https://doi.org/10.3792/56508.
275. Guthmiller J, Dugan H, Neu K, et al.: An efficient method to generate monoclonal antibodies from human B cells. In: Steinitz M, ed. *Human Monoclonal Antibodies: Methods and Protocols*. 2nd ed. Methods in Molecular Biology. Totowa, NJ: Humana Press; 2019, 109–45.
276. Soni P, Yasuhara A, Takenaga T, et al. Evaluation of the fusion partner cell line SPYMEG for obtaining human monoclonal antibodies against influenza B virus. *J Vet Med Sci*. 2018;80:1020–1024.
277. Zhao J, Song Y, Liu D. Recent advances on blinatumomab for acute lymphoblastic leukemia. Exp Hematol Oncol. 2019;8:28.
278. Kitazawa T, Shima M. Emicizumab, a humanized bispecific antibody to coagulation factors IXa and X with a factor VIIIa-cofactor activity. *Int J Hematol*. 2020;111:20–30.
279. Hanlon A, Metjian A. Caplacizumab in adult patients with acquired thrombotic thrombocytopenic purpura. *Ther Adv Hematol*. 2020;11 2040620720902904.
280. Tobinai K, Klein C, Oya N, et al. A review of obinutuzumab (GA101), a novel type II anti-CD20 monoclonal antibody, for the treatment of patients with B-cell malignancies. *Adv Ther*. 2017;34:324–356.
281. Morris EC, Rebello P, Thomson KJ, et al. Pharmacokinetics of alemtuzumab used for in vivo and in vitro T-cell depletion in allogeneic transplantations: relevance for early adoptive immunotherapy and infectious complications. *Blood*. 2003;102:404–406.
282. MacGlashan Jr. DW, Bochner BS, Adelman DC, et al. Down-regulation of Fc(epsilon)RI expression on human basophils during in vivo treatment of atopic patients with anti-IgE antibody. *J Immunol*. 1997;158:1438–1445.
283. Lin R. Rituximab infusion reaction prophylaxis using histamine receptor blockade, prednisone and colchicine. *Internet J Asthma Allergy Immunol*. 2003;3:1–3.

284. Attarwala H. TGN1412: From discovery to disaster. *J Young Pharm*. 2010;2:332–336.
285. Tang F, Malek E, Math S, et al. Interference of therapeutic monoclonal antibodies with routine serum protein electrophoresis and immunofixation in patients with myeloma: frequency and duration of detection of daratumumab and elotuzumab. *Am J Clin Pathol*. 2018;150:121–129.
286. Lancman G, Arinsburg S, Jhang J, et al. Blood transfusion management for patients treated with anti-CD38 monoclonal antibodies. *Front Immunol*. 2018;9:2616.
287. Mankarious S, Lee M, Fischer S, et al. The half-lives of IgG subclasses and specific antibodies in patients with primary immunodeficiency who are receiving intravenously administered immunoglobulin. *J Lab Clin Med*. 1988;112:634–640.
288. WHO. 09.251 General policies for monoclonal antibodies. Geneva: World Health Organization International Nonproprietary Names (INN) Working Document; 2009:1–3.
289. Holers VM. Complement and its receptors: new insights into human disease. *Ann Rev Immunol*. 2014;Vol 32(32):433–459.
290. Pfarr N, Prawitt D, Kirschfink M, et al. Linking C5 deficiency to an exonic splicing enhancer mutation. *J Immunol*. 2005;174:4175–4177.
291. Wang X. Inherited human complement C5 deficiency nonsense mutations in exons 1 (Gln' to Stop) and 36 (Arg1458t o Stop) and compound heterozygosity in three African-American families. *J Immunol*. 1995;154:5464–5471.
292. Ross SC, Densen P. Complement deficiency states and infection: epidemiology, pathogenesis and consequences of neisserial and other infections in an immune deficiency. *Medicine (Baltimore)*. 1984;63:243–273.
293. Hillmen P, Szer J, Weitz I, et al. Pegcetacoplan versus eculizumab in paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 2021;384:1028–1037.
294. Evans M, Rollins S, Wolff D, et al. In vitro and in vivo inhibition of complement activity by a single chain Fv fragment recognizing human C5. *Mol Immunol*. 1995;32:1183–1195.
295. Matis L, Rollins S. Complement specific antibodies: designing novel anti-inflammatories. *Nat Med*. 1995;8:839–842.
296. Thomas T, Rollins S, Rother R, et al. Inhibition of complement activity by humanized anti C5 antibody and single chain Fv. *Mol Immunol*. 1996; 33:1389–1401.
297. Mueller J, Giannoni M, Hartman S, et al. Humanized porcine VCAM-specific monoclonal antibodies with chimeric IgG2/G4 constant regions block human leukocyte binding to porcine endothelial cells. *Mol Immunol*. 1997;34:441–452.
298. Hill A, Hillmen P, Richards SJ, et al. Sustained response and long-term safety of eculizumab in paroxysmal nocturnal hemoglobinuria. *Blood*. 2005;106:2559–2565.
299. de Latour RP, Fremeaux-Bacchi V, Porcher R, et al. Assessing complement blockade in patients with paroxysmal nocturnal hemoglobinuria receiving eculizumab. *Blood*. 2015;125:775–783.
300. Patriquin C, Leber B. Increased eculizumab requirements during pregnancy in a patient with paroxysmal nocturnal hemoglobinuria: case report and review of the literature. *Clin Case Rep*. 2015;3:88–91.
301. Rother RP, Mojcik CF, McCroskery EW. Inhibition of terminal complement: a novel therapeutic approach for the treatment of systemic lupus erythematosus. *Lupus*. 2004;13:328–334.
302. Verrier ED, Shernan SK, Taylor KM, et al. Terminal complement blockade with pexelizumab during coronary artery bypass graft surgery requiring cardiopulmonary bypass: a randomized trial. *JAMA*. 2004;291: 2319–2327.
303. Armstrong PW, Granger CB, Adams PX, et al. Pexelizumab for acute ST-elevation myocardial infarction in patients undergoing primary percutaneous coronary intervention: a randomized controlled trial. *JAMA*. 2007;297:43–51.
304. Appel GB, Waldman M, Radhakrishnan J. New approaches to the treatment of glomerular diseases. *Kidney Int*. 2006;70:S45–S50.
305. Hillmen P, Hall C, Marsh J, et al. Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 2004;350:552–559.
306. Hillmen P, Young NS, Schubert J, et al. The complement inhibitor eculizumab in paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 2006;355:1233–1243.
307. Brodsky RA, Young NS, Antonioli E, et al. Multicenter phase 3 study of the complement inhibitor eculizumab for the treatment of patients with paroxysmal nocturnal hemoglobinuria. *Blood*. 2008;111:1840–1847.
308. Hillmen P, Muus P, Dührsen U, et al. Effect of the complement inhibitor eculizumab on thromboembolism in patients with paroxysmal nocturnal hemoglobinuria. *Blood*. 2007;110:4123–4128.
309. Araten DJ, Thaler HT, Luzzatto L. High incidence of thrombosis in African-American and Latin-American patients with paroxysmal nocturnal haemoglobinuria. *Thromb Haemost*. 2005;93:88–91.
310. de Latour RP, Mary JY, Salanoubat C, et al. Paroxysmal nocturnal hemoglobinuria: natural history of disease subcategories. *Blood*. 2008;112:3099–3106.
311. Araten DJ, Boxer D, Zamechek L, et al. Analysis of platelets by flow cytometry in patients with paroxysmal nocturnal hemoglobinuria (PNH). *Blood Cells Mol Dis*. 2020;80:102372.
312. Kelly R, Hill A, Arnold LM, et al. Long-term treatment with eculizumab in paroxysmal nocturnal hemoglobinuria: sustained efficacy and improved survival. *Blood*. 2011;117:6786–6792.
313. Kelly R, Arnold L, Richards S, et al. The management of pregnancy in paroxysmal nocturnal haemoglobinuria on long term eculizumab. *Br J Haematol*. 2010;149:446–450.
314. Dmytrijuk A, Robie-Suh K, Cohen MH, et al. FDA report: Eculizumab (Soliris (R)) for the treatment of patients with paroxysmal nocturnal hemoglobinuria. *Oncologist*. 2008;13:993–1000.
315. Matsumura Y. Risk analysis of eculizumab-related meningococcal disease in Japan using the Japanese adverse drug event report database. *Drug Healthc Patient Saf*. 2020;12:207–215.
316. Crew PE, McNamara L, Waldron PE, et al. Antibiotic prophylaxis in vaccinated eculizumab recipients who developed meningococcal disease. *J Infect*. 2020;80:350–371.
317. Crew PE, Abara WE, McCulley L, et al. Disseminated gonococcal infections in patients receiving eculizumab: a case series. *Clin Infect Dis*. 2019;69:596–600.
318. Lee JW, Sicre de Fontbrune F, Wong Lee Lee L, et al. Ravulizumab (ALXN1210) vs eculizumab in adult patients with PNH naive to complement inhibitors: the 301 study. *Blood*. 2019;133:530–539.
319. Nishimura J, Yamamoto M, Hayashi S, et al. Genetic variants in C5 and poor response to eculizumab. *N Engl J Med*. 2014;370:632–639.
320. Schols S, Nunn MA, Mackie I, et al. Successful treatment of a PNH patient non-responsive to eculizumab with the novel complement C5 inhibitor coversin (nomacopan). *Br J Haematol*. 2020;188:334–337.
321. Harder MJ, Kuhn N, Schrezenmeier H, et al. Incomplete inhibition by eculizumab: mechanistic evidence for residual C5 activity during strong complement activation. *Blood*. 2017;129:970–980.
322. Risitano AM. Anti-complement treatment in paroxysmal nocturnal hemoglobinuria: where we stand and where we are going. *Transl Med UniSa*. 2014;8:43–52.
323. Risitano A, Notaro R, Marando L, et al. Complement fraction 3 binding on erythrocytes as additional mechanism of disease in paroxysmal nocturnal hemoglobinuria patients treated by eculizumab. *Blood*. 2009;113:4094–4100.
324. Brodbeck WG, Kuttner-Kondo L, Mold C, et al. Structure/function studies of human decay-accelerating factor. *Immunology*. 2000;101:104–111.
325. Holguin MH, Martin CB, Bernshaw NJ, et al. Analysis of the effects of activation of the alternative pathway of complement on erythrocytes with an isolated deficiency of decay accelerating factor. *J Immunol*. 1992;148: 498–502.
326. Rondelli T, Risitano AM, Peffault de Latour R, et al. Polymorphism of the complement receptor 1 gene correlates with the hematologic response to eculizumab in patients with paroxysmal nocturnal hemoglobinuria. *Haematologica*. 2014;99:262–266.
327. Magro CM, Momtahen S, Mulvey JJ, et al. Role of the skin biopsy in the diagnosis of atypical hemolytic uremic syndrome. *Am J Dermatopathol*. 2015;37:349–356. quiz 57-9.
328. Zuber J, Fakhouri F, Roumenina LT, et al. Use of eculizumab for atypical haemolytic uraemic syndrome and C3 glomerulopathies. *Nat Rev Nephrol*. 2012;8:643–657.
329. El-Husseini A, Hannan S, Awad A, et al. Thrombotic microangiopathy in systemic lupus erythematosus: efficacy of eculizumab. *Am J Kidney Dis*. 2015;65:127–130.
330. Legendre CM, Licht C, Muus P, et al. Terminal complement inhibitor eculizumab in atypical hemolytic-uremic syndrome. *N Engl J Med*. 2013;368:2169–2181.
331. Ardissino G, Testa S, Possenti I, et al. Discontinuation of eculizumab maintenance treatment for atypical hemolytic uremic syndrome: a report of 10 cases. *Am J Kidney Dis*. 2014;64:633–637.
332. Hans R, Sharma RR, Marwaha N, et al. Efficacy and safety of therapeutic plasma exchange by using apheresis devices in pediatric atypical hemolytic uremic syndrome patients. *J Clin Apher*. 2016;31:381–387.
333. Pittock SJ, Lennon VA, McKeon A, et al. Eculizumab in AQP4-IgG-positive relapsing neuromyelitis optica spectrum disorders: an open-label pilot study. *Lancet Neurol*. 2013;12:554–562.
334. Howard Jr. JF, Barohn RJ, Cutter GR, et al. A randomized, double-blind, placebo-controlled phase II study of eculizumab in patients with refractory generalized myasthenia gravis. *Muscle Nerve*. 2013;48:76–84.
335. Shapira I, Andrade D, Allen SL, et al. Brief report: induction of sustained remission in recurrent catastrophic antiphospholipid syndrome via

inhibition of terminal complement with eculizumab. *Arthritis Rheum*. 2012;64:2719–2723.

336. Roth A, Huttmann A, Rother RP, et al. Long-term efficacy of the complement inhibitor eculizumab in cold agglutinin disease. *Blood*. 2009;113:3885–3886.
337. Lapeyraque AL, Malina M, Fremeaux-Bacchi V, et al. Eculizumab in severe Shiga-toxin-associated HUS. *N Engl J Med*. 2011;364:2561–2563.
338. Barnett AN, Asgari E, Chowdhury P, et al. The use of eculizumab in renal transplantation. *Clin Transplant*. 2013;27:E216–E229.
339. Broome C, McCloskey J, Girlanda R. Successful management of calcineurin induced thrombotic microangiopathy (TMA) with eculizumab after non-renal solid organ transplantation. *ASH Annual Meeting*. 2013; 122:122.
340. Coppo R, Peruzzi L, Amore A, et al. Dramatic effects of eculizumab in a child with diffuse proliferative lupus nephritis resistant to conventional therapy. *Pediatr Nephrol*. 2015;30:167–172.
341. Kirui L, Scully M, Mcqueen N, et al. Use of eculizumab for the treatment of hyperhaemolysis in pregnancy in sickle cell disease: a case report. *Blood*. 2018;132(Supplement 1). 49222018.
342. Annane D, Heming N, Grimaldi-Bensouda L, et al. Eculizumab as an emergency treatment for adult patients with severe COVID-19 in the intensive care unit: a proof-of-concept study. *EClinicalMedicine*. 2020;28:100590.
343. Kim MS, Prasad V. The clinical trials portfolio for on-label and off-label studies of eculizumab. *JAMA Intern Med*. 2020;180:315–317.
344. Kulasekararaj AG, Hill A, Rottinghaus ST, et al. Ravulizumab (ALXN1210) vs eculizumab in C5-inhibitor-experienced adult patients with PNH: the 302 study. *Blood*. 2019;133:540–549.
345. Sheridan D, Yu ZX, Zhang Y, et al. Design and preclinical characterization of ALXN1210: a novel anti-C5 antibody with extended duration of action. *PLoS One*. 2018;13:e0195909.
346. Zalevsky J, Chamberlain AK, Horton HM, et al. Enhanced antibody half-life improves in vivo activity. *Nat Biotechnol*. 2010;28:157–159.
347. Kuo TT, Aveson VG. Neonatal Fc receptor and IgG-based therapeutics. *MAbs*. 2011;3:422–430.
348. Roth A, Rottinghaus ST, Hill A, et al. Ravulizumab (ALXN1210) in patients with paroxysmal nocturnal hemoglobinuria: results of 2 phase 1b/2 studies. *Blood Adv*. 2018;2:2176–2185.
349. Brodsky RA, Peffault de Latour R, Rottinghaus ST, et al. Characterization of breakthrough hemolysis events observed in the phase 3 randomized studies of ravulizumab versus eculizumab in adults with paroxysmal nocturnal hemoglobinuria. *Haematologica*. 2021;106:230–237.
350. Peffault de Latour R, Fremeaux-Bacchi V, Porcher R, et al. Assessing complement blockade in patients with paroxysmal nocturnal hemoglobinuria receiving eculizumab. *Blood*. 2015;125:775–783.
351. Cataland S, Ariceta G, Chen P, et al. Discordance between Free C5 and CH50 complement assays in measuring complement C5 inhibition in patients with aHUS treated with ravulizumab. *Blood*. 2019;134 (Supplement 1):1099.
352. Hallstensen RF, Bergseth G, Foss S, et al. Eculizumab treatment during pregnancy does not affect the complement system activity of the newborn. *Immunobiology*. 2015;220:452–459.
353. Kelly RJ, Hochsmann B, Szer J, et al. Eculizumab in pregnant patients with paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 2015;373:1032–1039.
354. Roth A, Nishimura JI, Nagy Z, et al. The complement C5 inhibitor crovalimab in paroxysmal nocturnal hemoglobinuria. *Blood*. 2020;135:912–920.
355. Gorman DM, Lee J, Payne CD, et al. Chemical synthesis and characterisation of the complement C5 inhibitory peptide zilucoplan. *Amino Acids*. 2021;53:143–147.
356. Howard Jr. JF, Nowak RJ, Wolfe GI, et al. Clinical effects of the self-administered subcutaneous complement inhibitor zilucoplan in patients with moderate to severe generalized myasthenia gravis: results of a phase 2 randomized, double-blind, placebo-controlled, multicenter clinical trial. *JAMA Neurol*. 2020;77:582–592.
357. Fredslund F, Laursen NS, Roversi P, et al. Structure of and influence of a tick complement inhibitor on human complement component 5. *Nat Immunol*. 2008;9:753–760.
358. Weston-Davies W, Nunn M, Pinto F, et al. Clinical and immunological characterisation of coversin, a novel small protein inhibitor of complement C5 with potential as a therapeutic agent in PNH and other complement mediated disorders. ASH Annual Meeting. 2014 Abstract 4280.
359. Roth A, Barcellini W, D'Sa S, et al. Sutimlimab in cold agglutinin disease. *N Engl J Med*. 2021;384:1323–1334.
360. Risitano AM, Kulasekararaj AG, Lee JW, et al. Danicopan: an oral complement factor D inhibitor for paroxysmal nocturnal hemoglobinuria. *Haematologica*. 2021;106(12). https://doi.org/10.3324/haematol.2020.261826.
361. Mastellos DC, Yancopoulou D, Kokkinos P, et al. Compstatin: a C3-targeted complement inhibitor reaching its prime for bedside intervention. *Eur J Clin Invest*. 2015;45:423–440.
362. Qu H, Ricklin D, Bai H, et al. New analogs of the clinical complement inhibitor compstatin with subnanomolar affinity and enhanced pharmacokinetic properties. *Immunobiology*. 2013;218:496–505.
363. Reis E, Falcao D, Isaac L. Clinical aspects and molecular basis of primary deficiencies of complement component C3 and its regulatory proteins factor I and factor H. *Scand J Immunol*. 2006;63:155–168.

CHAPTER 24

T-CELL IMMUNITY

Shannon A. Carty, Matthew J. Riese[†], and Gary A. Koretzky

Thymus-derived lymphocytes (T cells) play an essential role in the immune response to pathogens and against host cells that have undergone malignant transformation. T cells are critical regulators of the immune system that act through production of soluble mediators and direct interactions between ligands on the T-cell surface and receptors on other immune cells. This chapter reviews T-cell activation after engagement with specific antigens and describes how signals delivered by the antigen receptors shape the repertoire of mature T cells in secondary lymphoid organs. The mechanism by which different populations of mature T cells exert their effector functions is then discussed. Because homeostasis of the immune system requires not only that T cells become activated under appropriate conditions but also that their activity be curtailed once the pathogenic challenge has been met, the processes by which T-cell activation is terminated are also described. Finally, we review how the molecular understanding of T-cell activation has resulted in important advances in the treatment of human diseases.

T-CELL ACTIVATION

T-cell activation begins when T cells encounter a specific antigen that engages and then initiates signal transduction through the T-cell antigen receptor (TCR). Unlike B cells that respond to soluble antigens, T cells are stimulated by small peptides presented on the surface of other cells. These peptides are incorporated into the binding groove of proteins of the major histocompatibility complex (MHC, known in humans as human leukocyte antigen [HLA] complexes) through a process called *antigen presentation*. Thus, the ligand for the TCR is a peptide surface generated from both amino acids in the antigenic peptide and residues found in the MHC molecules themselves. Engagement of peptide–MHC complexes by the TCR induces a series of intracellular biochemical events that culminate in T-cell activation. Although T cells make use of many of the same biochemical pathways used by other cells for activation, there are a number of molecules unique to immune cells that are critical for T-cell activation. This section discusses TCR signal transduction, focusing on immune cell-specific molecular events.

Antigen Presentation: Creating the Ligand for the T-Cell Receptor

Invading pathogenic bacteria and viruses use different strategies to survive within infected hosts. Many bacteria, such as the pathogens *Staphylococcus, Streptococcus*, and various enteric Gram-negative bacilli, survive in the extracellular milieu, whereas viruses and other bacteria, such as *Listeria*, survive inside host cells. Successful elimination of pathogens in each of these locations requires distinct responses from the host immune system. T cells play a central role in the control of extracellular and intracellular pathogens; however, the responding subset of T cells differs for each type of pathogen, with subsets of T cells expressing the cell surface marker CD4 most important for protection against extracellular pathogens and those expressing the CD8 marker essential for control of intracellular organisms. Stimulated CD4$^+$ T cells act on other cells of the immune system by producing cytokines, soluble mediators that elicit a variety of cellular responses important for clearance of extracellular pathogens, whereas CD8$^+$ T cells function largely by directly lysing host cells that have become infected with an intracellular organism. It is therefore critical for antigens derived from extracellular sources to stimulate CD4$^+$ T cells and for intracellular antigens to stimulate CD8$^+$ T cells. Whether a particular antigenic peptide activates a CD4$^+$ versus a CD8$^+$ T cell is determined by which MHC proteins present the peptide to the TCR.

Class II MHC proteins are found on cells of the innate immune system known as "professional" antigen-presenting cells (APCs) as well as B cells and the thymic epithelium. Professional APCs include dendritic cells (DCs) and various tissue macrophages, which engulf extracellular organisms (often after these are coated with host antibodies), host cells that have undergone apoptosis (programmed cell death), and cellular debris through an endocytic pathway that brings the ingested material into contact with degradative enzymes. The peptides that are formed in these reactions are bound to the MHC class II proteins for presentation to T cells. The MHC class II complex is a dimer consisting of a single α chain and a single β chain. Both α and β chains contribute to peptide binding and interaction with the TCR. During synthesis within the cell, MHC class II complexes bind invariant chain (Ii), a protein that directs the newly formed MHC proteins into an acidic vesicle. During this trafficking event, a portion of the Ii occupies the peptide-binding site. Once the MHC class II protein reaches the acidic vesicle, Ii is proteolyzed by cathepsin S, leaving behind a small fragment that remains lodged within the peptide-binding cleft of the MHC class II complex. This fragment is termed *the class II–associated invariant chain peptide (CLIP)*. The MHC class II–containing vesicles then fuse with other vesicles containing the peptide fragments from the endocytosed particles. There, CLIP is replaced with a peptide, thus stabilizing the MHC class II complex and allowing it to be transported to the cell surface, where it interacts with the TCR on CD4$^+$ T cells (Fig. 24.1).

All cells of the body are at risk of being infected with intracellular pathogens or becoming transformed. Because protection against such challenges requires a CD8$^+$ T-cell response, all nucleated cells in the body express class I MHC, the protein complex that presents antigen to CD8$^+$ T cells. Like class II MHC, class I MHC is a protein dimer. However, in contrast to class II, only the α chain of class I MHC is variable. This α chain is associated with β_2 microglobulin, which stabilizes the complex but plays no direct role in antigen presentation. During its assembly in the endoplasmic reticulum (ER), the MHC class I complex comes into contact with peptides derived from proteins undergoing translation within the cell. During protein synthesis, small amounts of protein are modified by ubiquitinylation. This serves as a targeting sequence, directing the modified protein to the proteasome, where it is degraded into small peptide fragments. These fragments are transported back into the ER by the transporters associated with antigen processing (TAP-1 and TAP-2), where they become available for binding to the newly synthesized MHC class I complexes. Peptide association completes the folding and assembly of MHC class I, which is then transported to the cell surface, where it can be recognized by CD8$^+$ T cells (Fig. 24.1).

T cells can respond to antigenic peptides only if these peptides fit into the binding pocket of either MHC class I or MHC class II. Although a large number of peptides are able to bind to a specific MHC complex, the diversity of antigen presentation is enhanced through expression of three different MHC class I alleles (in humans, HLA A, B, and C) and class II alleles (in humans, HLA DR, DP,

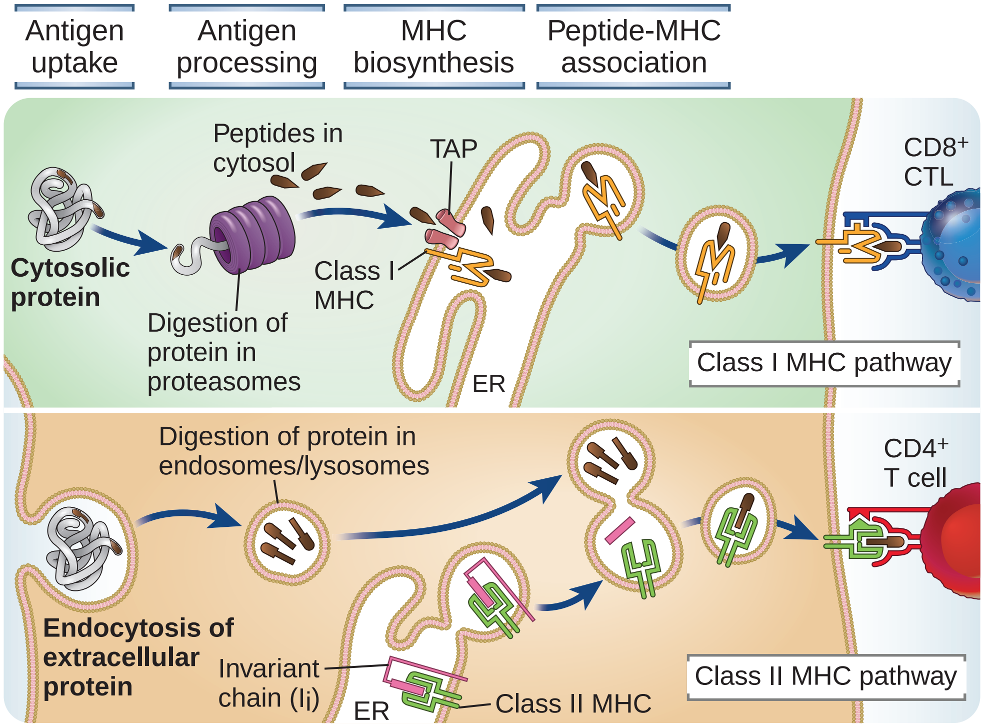


Figure 24.1 ANTIGEN PROCESSING AND PRESENTATION. Presentation of peptides by major histocompatibility complex (MHC) class I and class II molecules occurs by different mechanisms. *Top*, In the class I MHC pathway, protein antigens in the cytosol are processed by proteasomes, and peptides are transported into the endoplasmic reticulum (ER), where they bind to class I MHC molecules. *Bottom*, In the class II MHC pathway, protein antigens are endocytosed from the extracellular milieu and degraded in lysosomes. These peptides subsequently bind to class II MHC molecules, displacing the class II-associated invariant chain peptide (CLIP) in the antigen presentation cleft of the class II molecule. *ER*, Endoplasmic reticulum; *TAP*, transporter associated with antigen processing. *(From Abbas AK, Lichtman AH, Pillai S, Baker DL, Baker A. Cellular and Molecular Immunology. 9th ed. Philadelphia: Elsevier; 2017.)*

and DQ). To further increase the spectrum of peptides any particular cell may present, MHC alleles are always codominantly expressed. Thus, any individual expresses a large number of different class II dimers on its APCs and class I dimers on all nucleated cells, providing broad protection against potential pathogenic organisms. It is possible, however, that even with this degree of potential for antigen presentation, pathogens may evolve that do not possess unique proteins with sequences that can be efficiently presented by MHC. To circumvent this problem, the MHC locus has evolved to be highly polymorphic, thus providing enormous diversity within the population for antigen presentation, ensuring that some individuals will express MHC dimers that can present antigens from virtually any pathogen. Interestingly, predominant MHC alleles exist in different parts of the world, suggesting that there is local pressure, perhaps based upon prevailing microorganisms, that shapes selection of MHC expression.

Neither MHC class I nor MHC class II distinguishes foreign from host peptides as they fill their peptide binding grooves. Because MHC class II are capable of presenting any ingested antigen and MHC class I present peptides produced within the cell, the majority of the MHC complexes are filled with self-peptides. The T cell must distinguish self from non-self to ensure that a response is directed only against peptides generated from foreign peptides. Control over which antigens elicit a T-cell response is accomplished through selection of a population of T cells expressing appropriate TCRs (see T-Cell Development section later).

The T-Cell Receptor Complex

The TCR is a multimolecular complex with separate components able to bind ligand or to transduce an activating signal to the cell. The peptide–MHC binding regions of the TCR consist of an α/β heterodimer in the majority of T cells, and the related γ/δ heterodimer in a smaller subset of T cells. α and β as well as γ and δ consist of variable and constant regions. Similar to antibodies (see Chapter 22), the variable regions of the TCR antigen-binding proteins arise from rearranging gene segments that are imprecisely joined during T-cell development. This process allows for an extraordinarily diverse repertoire of potential antigen reactivity, although there are in total only several hundred genes that make up the α, β, γ, and δ loci. The germline configurations of the α and β loci are different, such that the α-chain locus comprises about 70 variable (V) segments, 60 joining (J) segments, and 1 constant (C) segment, whereas the β-chain locus comprises 50 V regions, 2 diversity (D) segments, 13 J segments, and 2 C regions. Greater diversity is generated by the addition of nucleotides between the V and J gene segments on α chains and the V, D, and J segments in β chains during the formation of the mature TCR. In total, it has been calculated that approximately 10^{18} different TCRs can be created from these segments, although the functional population is much smaller because of the requirements for selection during maturation in the thymus (see T-Cell Development section later). Thus, once it has completed its developmental program, an individual T cell expresses a unique TCR encoded by a combination of gene segments that have been altered and rearranged (Fig. 24.2). The T cells circulating through the lymphatics, lymph nodes (LNs), and spleen possess sufficient diversity for nearly all pathogens encountered to express an antigenic sequence recognized by a circulating T cell, which will then expand in number to combat that pathogen.

Soon after identification of the genes encoding TCR α and β, it became apparent that although they were sufficient to bind peptide–MHC,[1] the α/β heterodimer was not capable of transmitting an intracellular signal once ligand was bound. A series of studies demonstrated that the signal transduction function of the TCR complex resides in a protein complex that noncovalently associates with the α/β dimer. This complex, CD3, is required both for stable expression of the ligand-binding components of the TCR and for signal transduction.[2] CD3 is composed of three subunits, δ, ε, and γ, expressed as heterodimers (γ/ε and δ/ε) along with the ζ subunit, which is present

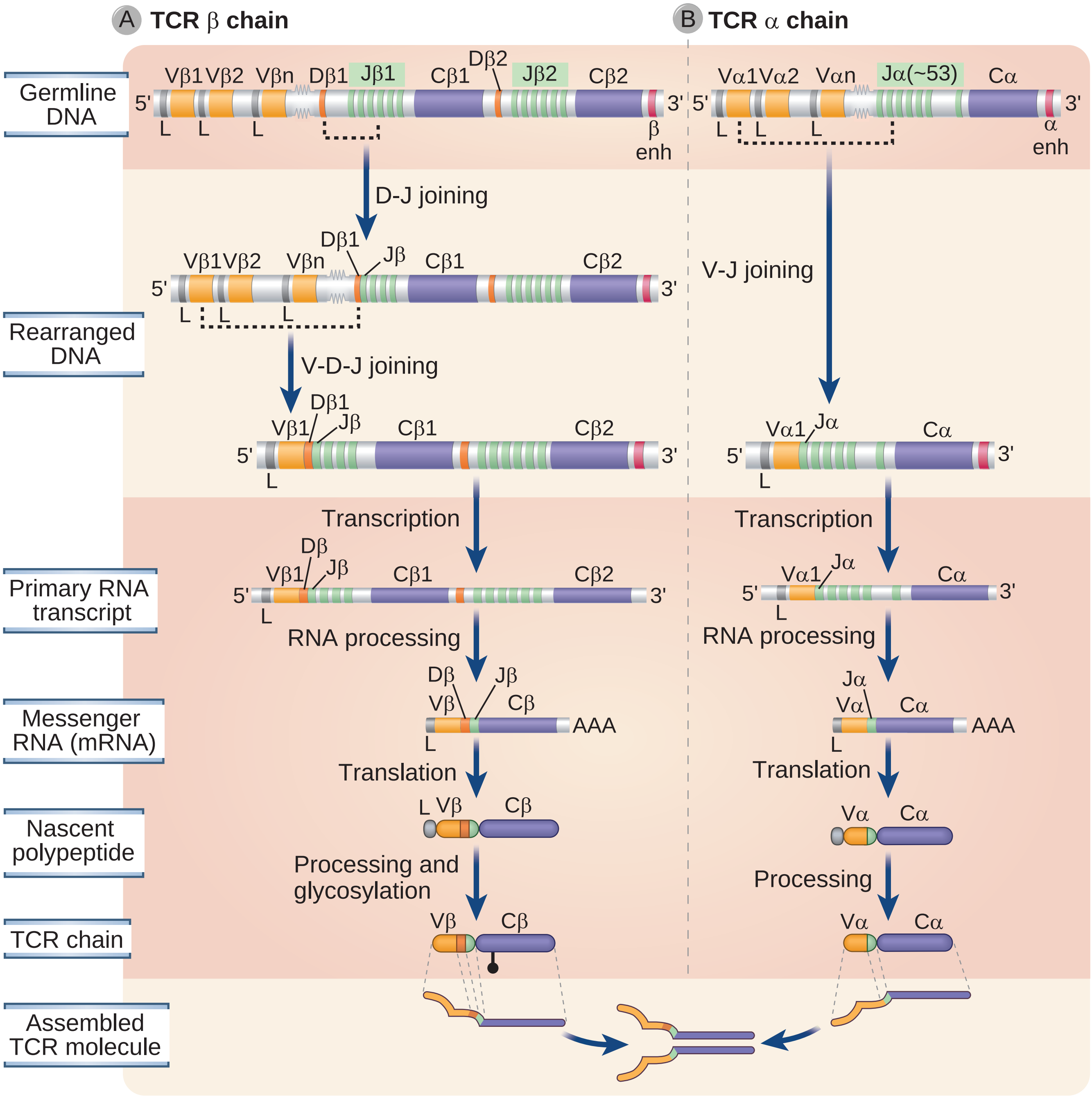

A TCR β chain
B TCR α chain
Germline DNA
5'
Vβ1 Vβ2 Vβn Dβ1 Jβ1 Cβ1 Dβ2 Jβ2 Cβ2
3'
L L L
β enh
5'
Vα1 Vα2 Vαn Jα(~53) Cα
3'
L L L
α enh
D-J joining
Rearranged DNA
Vβ1 Vβ2 Vβn Dβ1 Jβ Cβ1 Cβ2
5' 3'
L L L
V-J joining
V-D-J joining
Dβ1
Vβ1 Jβ Cβ1 Cβ2
5' 3'
L
Vα1 Jα Cα
5' 3'
L
Transcription
Primary RNA transcript
Dβ
Vβ1 Jβ Cβ1 Cβ2
5' 3'
L
Transcription
Vα1 Jα Cα
5' 3'
L
RNA processing
Messenger RNA (mRNA)
Dβ Jβ
Vβ Cβ
AAA
L
RNA processing
Jα
Vα Cα
AAA
L
Translation
Nascent polypeptide
L Vβ Cβ
Translation
Vα Cα
Processing and glycosylation
TCR chain
Vβ Cβ
Processing
Vα Cα
Assembled TCR molecule

Figure 24.2 GENERATION OF DIVERSITY OF THE T-CELL ANTIGEN RECEPTOR. To generate the diverse repertoire of antigen receptors needed for protective T-cell immunity, the genetic loci encoding the two proteins of the T-cell antigen receptor (TCR) undergo multiple rearrangements to form the mature α and β chains. The sequence of recombination and gene expression events is shown for the TCR β chain (A) and the TCR α chain (B). In the example shown in A, the variable (V) region of the rearranged TCR β chain includes the Vβ1 and Dβ1 gene segments and the third J segment in the Jβ1 cluster. The constant (C) region in this example is encoded by the exons of the Cβ1 gene, depicted for convenience as a single exon. Note that at the TCR β chain locus, rearrangement begins with D-to-J joining followed by V-to-DJ joining. In humans, 14 Jβ segments have been identified, and not all are shown in the figure. In the example shown in B, the V region of the TCR α chain includes the Vα1 gene and the second J segment in the Jα cluster. (This cluster is made up of at least 61 Jα segments in humans; not all are shown here.) *(From Abbas AK, Lichtman AH, Pillai S, Baker DL, Baker A. Cellular and Molecular Immunology. 9th ed. Philadelphia: Elsevier; 2017.)*

as a homodimer. Each subunit contains immunoreceptor tyrosine-based activation motifs (ITAMs), a stretch of amino acids with discretely placed tyrosine residues: one ITAM in δ, ε, and γ and three ITAMS in ζ. The ITAM tyrosines are inducibly phosphorylated upon engagement of the α/β TCR chains by peptide–MHC and become docking sites for other proteins that initiate the signaling cascade for T-cell activation. Notably, the CD4 or CD8 protein also plays a role in mediating signal transduction. These coreceptors bind both the appropriate MHC complex (MHC I for CD8, MHC II for CD4) and, via their cytoplasmic tails, the signaling molecule Lck, one of the kinases capable of phosphorylating the ITAMs (Fig. 24.3).

T-Cell Receptor Signal Transduction

Once the genes were cloned for each TCR complex component, it became clear that, unlike many other cell surface receptors that transduce activating signals, neither the ligand-binding domains nor

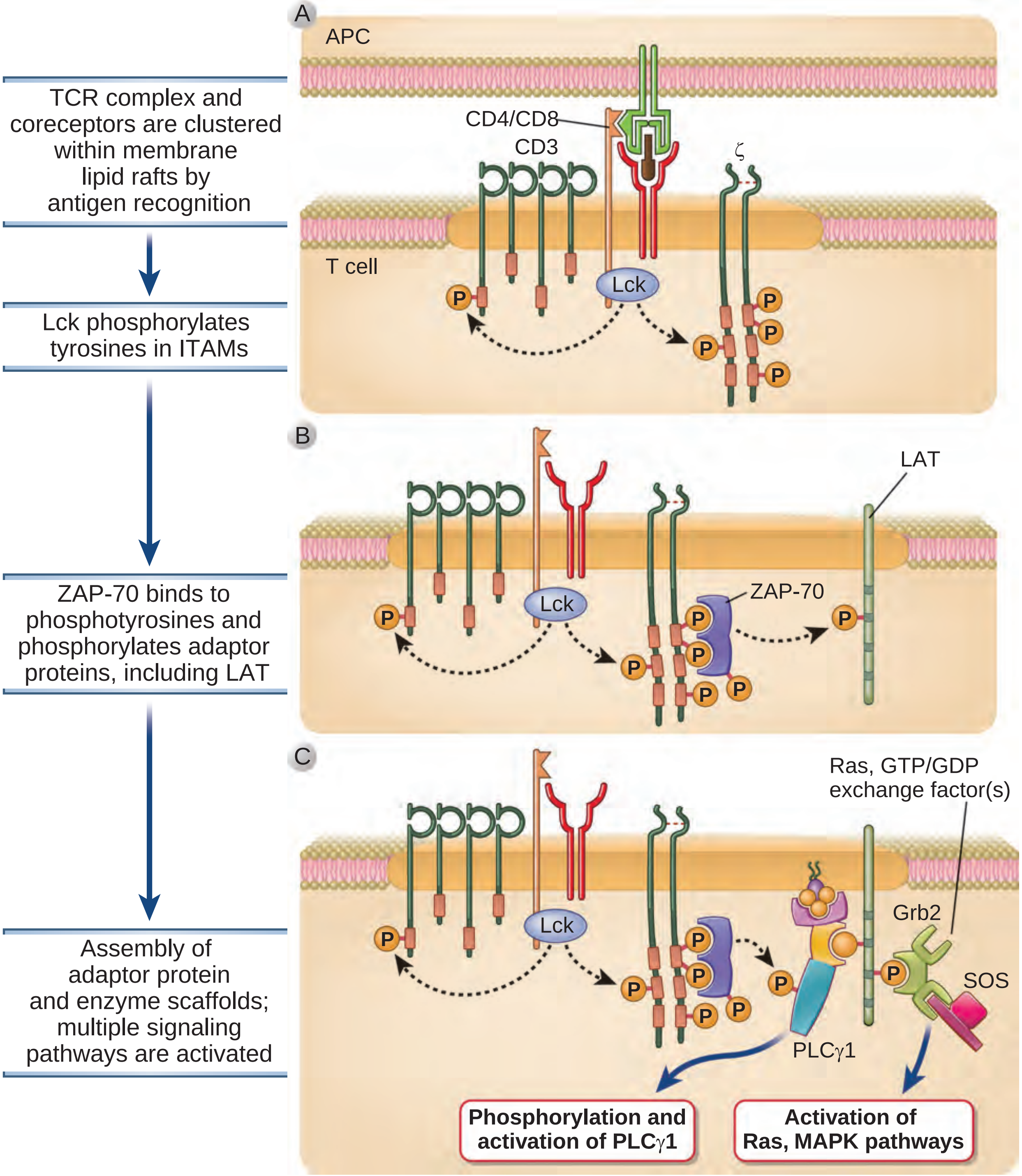

TCR complex and coreceptors are clustered within membrane lipid rafts by antigen recognition
Lck phosphorylates tyrosines in ITAMs
ZAP-70 binds to phosphotyrosines and phosphorylates adaptor proteins, including LAT
Assembly of adaptor protein and enzyme scaffolds; multiple signaling pathways are activated
A
APC
CD4/CD8
CD3
ζ
T cell
Lck
P
B
LAT
ZAP-70
C
Ras, GTP/GDP exchange factor(s)
Grb2
SOS
PLCγ1
Phosphorylation and activation of PLCγ1
Activation of Ras, MAPK pathways

Figure 24.3 PROXIMAL T-CELL RECEPTOR SIGNAL TRANSDUCTION. Upon antigen recognition, there is clustering of T-cell antigen receptor (TCR) complexes with coreceptors (CD4, in this case). CD4-associated Lck becomes active and phosphorylates tyrosines in the immunoreceptor tyrosine-based activation motifs (ITAMs) of CD3 and ζ chains (A). ζ-associated protein of 70 kDa (ZAP-70) binds to the phosphotyrosines of the ζ chains and is itself phosphorylated and activated. (The illustration shows one ZAP-70 molecule binding to two phosphotyrosines of one ITAM in the ζ chain, but it is likely that initiation of a T-cell response requires the assembly of multiple ZAP-70 molecules on each ζ chain.) Active ZAP-70 then phosphorylates tyrosines on various adaptor molecules, such as linker of activated T cells (LAT) (B). The adaptors become docking sites for cellular enzymes such as PLCγ1 and GDP-GTP exchange factors that activate Ras and other small G proteins upstream of MAP kinases (C), and these enzymes activate various cellular responses. *MAPK*, Mitogen activated protein kinase; *PLC γ1*, phospholipase C γ1. *(From Abbas AK, Lichtman AH, Pillai S, Baker DL, Baker A. Cellular and Molecular Immunology. 9th ed. Philadelphia: Elsevier; 2017.)*

the CD3 proteins of the complex have intrinsic enzymatic function. Engagement of the TCR by the peptide–MHC was found to result in the rapid activation of protein tyrosine kinases (PTKs) within the T cells. Exactly how TCR engagement initiates PTK activation remains unclear; however, clustering of TCRs on the cell surface with resultant conformational changes in the CD3 proteins appears critical in the process. Src family (Lck and Fyn) PTKs are activated first following TCR stimulation, and the tyrosines within the CD3 and ζ ITAMs are substrates of these kinases. Phosphorylation of the ITAM tyrosines makes these residues able to bind to Src homology 2 (SH2) domains of other proteins. The most important SH2 domain-containing protein that is recruited to the ITAMs is ζ-associated protein of 70 kDa (ZAP-70), a PTK itself and a member of the Syk family of proteins.[3] Thus binding of the TCR by ligand converts an enzymatically inactive receptor complex into an active PTK through recruitment and activation of cytosolic proteins.

Activation of ZAP-70 leads to tyrosine phosphorylation of a number of substrates, including enzymes that catalyze reactions generating second messengers important for T-cell activation. Phospholipase Cγ1 (PLCγ1) is activated by its tyrosine phosphorylation to cleave phosphatidylinositol-(4,5)-bisphosphate (PIP$_2$) into the second messengers diacylglycerol (DAG) and inositol-(1,4,5)-triphosphate (IP$_3$). DAG is a membrane-bound lipid second messenger that binds to and activates downstream signaling components, including protein kinase C θ (PKCθ) and the Ras guanine exchange factor RasGRP. PKCθ, a serine/threonine kinase, regulates numerous effectors of gene transcription and T-cell effector function development, including the transcription factors nuclear factor κB (NFκB) and activator protein 1 (AP-1). RasGRP is responsible for activating the small molecular weight guanosine triphosphate (GTP)-binding protein Ras by enhancing Ras release of GDP, allowing it to assume its activated GTP-bound form. Active Ras collaborates with PKC family members to stimulate transcription of new genes by activating mitogen-activated protein kinase (MAPK) family members. IP$_3$ mobilizes calcium stores from the ER. This increase in calcium is important for enzyme function, most notably the phosphatase calcineurin that dephosphorylates nuclear factor of activated T cells (NFAT), allowing it to translocate to the nucleus and transactivate genes necessary for T-cell proliferation, such as the gene encoding interleukin 2 (IL-2).

Although early TCR signal transduction studies demonstrated the importance of TCR-initiated PTK activity for T-cell activation, the mechanistic basis by which PTK activation drove the many critical second-messenger cascades required more precise study. These findings were elucidated with the identification and characterization of adapter proteins, which possess modular domains important for intermolecular interactions. Two central adapters in the TCR signaling pathway are linker of activated T cells (LAT) and SH2 domain-containing leukocyte protein of 76 kDa (SLP-76).[4] LAT is a transmembrane protein with seven cytoplasmic tyrosines that are phosphorylated by the PTKs activated by the TCR. SLP-76 is a cytosolic adapter protein that is also phosphorylated by these PTKs. Because these tyrosine phosphorylation events create docking sites for other proteins with SH2 domains, once the TCR is engaged, SLP-76 and LAT nucleate a large complex of signaling molecules at the membrane, in the vicinity of the activated TCR. This cluster of molecules initiates the signaling cascades that are integrated to result in T-cell activation. Key proteins in this complex are Vav1, a guanine nucleotide exchange factor important for cytoskeletal reorganization; inducible T-cell kinase (ITK), a member of the Tec family of PTKs (a third family of PTKs essential for T-cell activation); adhesion and degranulation-promoting adapter protein (ADAP), an adapter that is a key regulator of integrins to promote T-cell interactions with other cells; PLCγ, the enzyme described earlier that initiates both the calcium and Ras/MAPK pathways in T cells; and growth factor receptor-bound protein 2 (Grb2) and Son of Sevenless (SOS), two proteins, like RasGRP, capable of activating Ras (Fig. 24.4).

Co-Stimulation

For T-cell immunity to be effective, T cells must possess TCRs that are exquisitely sensitive to specific antigen. Because the TCR is generated through random reassortment and alteration of gene segments, it is impossible to prevent generation of TCRs that have the potential to respond to self-antigens. Although the developmental program of T cells in the thymus provides a mechanism to eliminate

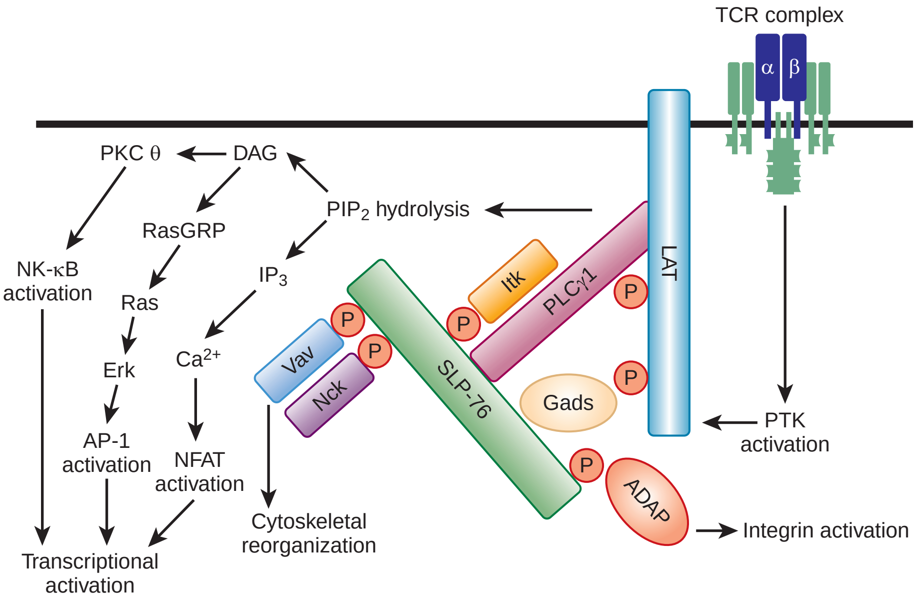


Figure 24.4 INTEGRATION OF T-CELL RECEPTOR SIGNALS BY ADAPTER PROTEINS. Following engagement of the T-cell antigen receptor (TCR) and activation of protein tyrosine kinases, several hematopoietic-specific adapter proteins are phosphorylated, enabling the formation of a multimolecular signaling complex. The transmembrane adapter protein linker of activated T cells (LAT) recruits SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) through the growth factor receptor-bound protein 2 (Grb2) family member Grb2-related adaptor downstream of Shc (Gads). This SLP-76 nucleated complex associates with phospholipase Cγ1 (PLCγ1), inducible T-cell kinase (Itk), Vav1, and adhesion and degranulation-promoting adapter protein (ADAP). After phosphorylation by Itk, PLCγ1 catalyzes the cleavage of phosphatidylinositol-(4,5)-bisphosphate (PIP$_2$) into inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ induces calcium flux from the endoplasmic reticulum, leading to activation of the transcription factor nuclear factor of activated T cells (NFAT). DAG binds and activates proteins important in signaling such as protein kinase Cθ (PKCθ), a kinase whose substrates initiate the activation of the transcription factor nuclear factor κB (NFκB), and Ras guanine exchange factor (RasGRP), a Ras-activating protein that induces activation of extracellular signal-regulated kinase (Erk) and formation of the transcription factor activator protein 1 (AP-1). Apart from transcriptional changes, T cells also undergo cytoskeletal changes after TCR stimulation mediated in part by Vav1, an activating protein for the actin-modulating protein Rac1, and activation of cell surface integrins, mediated in part by the adapter protein ADAP. *PTK*, Protein tyrosine kinase.

most potentially self-reactive T cells (see T-Cell Development section later), this process is not 100% effective. Hence mechanisms exist to prevent mature T cells from responding against normal host tissues. One such mechanism is the requirement for T cells to receive two signals to become activated, one mediated by the TCR and the second through a costimulatory receptor. Although several different T-cell molecules can provide this costimulatory function, the best studied is the surface protein CD28.[5] This additional requirement for T-cell activation helps to prevent autoimmunity because the ligands for CD28 (CD80 and CD86) are upregulated on APCs only in the presence of pathogen-associated molecular signals generated largely by bacterial and viral components or in the setting of cellular stress. (The mechanism of how bacterial and viral components signal through Toll-like receptors to activate APCs is described in Chapter 20.)

For CD28 engagement to provide the second signal for T-cell activation, it must also initiate signal transduction pathways (Fig. 24.5). CD28 signaling not only augments those signals stimulated by the TCR (described earlier) but also delivers independent signals. The interaction between CD28 and its ligands triggers the activation of phosphatidylinositol 3-kinase (PI3K), a protein that phosphorylates PIP_2 to form phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3). Although the formation of PIP_3 induces broad changes within cells, the PIP_3 effector pathways that have been studied most intensively include two serine/threonine kinases: Akt, a PIP_3-binding protein responsible for regulating the T-cell metabolism to favor cell division, and PKCθ, a protein required for cytokine production in T cells that is dependent upon PIP_3 generation for full activation. The importance of CD28 costimulation of T cells goes beyond its requirement for T-cell activation because engagement of the TCR in the absence of CD28 signaling in naive (i.e., antigen-inexperienced) T cells induces an impaired functional state within T cells termed *anergy* (see Anergy section later in this chapter).

Although CD28 is the prototypical and best studied costimulatory receptor, a multitude of other costimulatory molecules are expressed on T cells and regulated in a spatiotemporal manner in response to environmental cues, including CD27, inducible costimulator (ICOS), and 4-1BB. These costimulatory molecules bind an array of ligands on other cells (primarily APCs). Stimulation of individual costimulatory molecules can influence T-cell activation, effector function, and survival. One of the mechanisms by which distinct costimulatory molecules play unique roles in T-cell responses is likely due to the differential activation of discrete signaling pathways through their intracellular signaling domains. For instance, ICOS contains a binding motif that recruits the more active subunit of PI3K, leading to enhanced AKT signaling relative to CD28 activation. These differences have been exploited in building chimeric antigen receptor constructs to enhance T-cell-based adoptive cellular therapy (see Therapeutic Manipulation of T-Cell-Mediated Immunity section below and Chapter 26).

Spatial Coordination of T-Cell Receptor Signal Transduction: The Immunologic Synapse

As the biochemical signaling events that occur following TCR engagement by peptide–MHC became known, investigators sought to define the topography of the activation events. Sophisticated imaging technologies were applied to visualize the contact site between the APC and the T cell, and this interaction was modeled by visualizing the contact between key receptors on T cells and ligands fixed to a solid support. These studies revealed a stepwise reorganization of the T-cell membrane at the contact site called the *immunologic synapse* (IS).[6] The first step in IS formation is an interaction between integrins on the surface of the T cell and their ligands on the APC that brings the T cell and APC into close proximity. If a productive interaction occurs between the TCR and peptide–MHC, the next event is clustering of TCRs in the central portion of the developing IS (the so-called central supramolecular activation complex [cSMAC]) with the activated integrins forming the peripheral supramolecular activation complex (pSMAC), a ring around the clustered TCRs. Although ligands on the APC initially direct the formation of the IS, changes within the T cell, including reorganization of the actin cytoskeleton, are also critical for stabilization of this structure. As sophistication of imaging in real time has advanced and with the advent of tools to visualize smaller and smaller numbers of molecules, it has become clear that the IS is a dynamic structure that includes not only the TCR and integrins but also many of the signaling molecules essential for T-cell activation. Functionally, the IS has been demonstrated to regulate TCR signaling and polarized secretion of effector molecules, including cytokines and lytic granules.

T-Cell Proliferation

The number of naïve T cells potentially responsive to any particular peptide antigen (the precursor frequency of the responding population) is quite small, yet a large number of antigen-specific T cells are required to combat pathogens. Accordingly, a consequence of TCR plus costimulatory receptor engagement is the clonal expansion of an activated T cell. One outcome of the second-messenger cascades stimulated by the TCR and CD28 is the production of IL-2, an essential cytokine for T-cell proliferation. Another outcome of TCR signaling is upregulation of the high-affinity receptor for IL-2, thus making the activated T cell able to respond to local concentrations of this cytokine. Signaling through the IL-2 receptor is necessary for the proliferative response. Similar to the TCR, the IL-2 receptor makes use of cytoplasmic PTKs (in this case members of the Janus kinase [JAK] family) to initiate a cascade of second messengers that lead ultimately to T-cell proliferation. Additionally, other cytokines such as IL-7 and IL-15 also regulate T-cell proliferation in antigen-experienced (i.e., memory) T cells. The details of IL-2 and other cytokine receptor signaling are provided in Chapter 11.

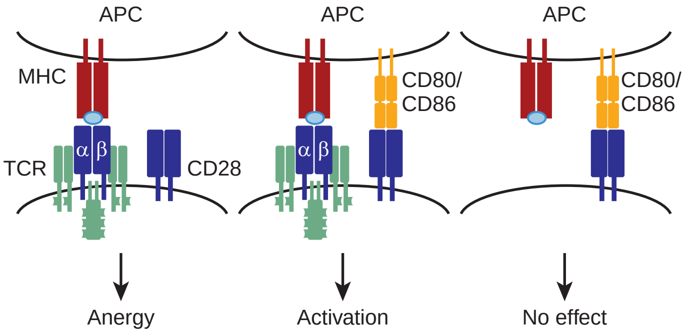


Figure 24.5 TWO SIGNALS ARE REQUIRED TO ACTIVATE T CELLS. T-cell activation requires two signals, one through the T-cell antigen receptor (TCR) and one mediated by a costimulatory molecule, such as CD28. An antigen-presenting cell (APC) will activate a T cell if it presents appropriate peptide–major histocompatibility complex (MHC) to the T cell and also expresses a ligand to engage CD28. If CD28 is engaged without a concomitant TCR signal, the T cell is neither activated nor inactivated. However, if a T cell is stimulated through the TCR in the absence of costimulation through CD28, then it becomes anergic, unresponsive to the initial as well as subsequent stimulations.

T-CELL DEVELOPMENT

Protective T-cell immunity requires populating the secondary lymphoid organs with a large number of mature T cells. Unlike most hematopoietic cells that complete the transition from progenitors to mature cells in the bone marrow, T cells develop primarily in the thymus.[7] This population collectively must possess a diverse TCR repertoire capable of recognizing the enormous number of foreign antigens that will be encountered over the lifetime of an individual. Because the TCR binds antigenic peptide plus amino acid residues of self-MHC molecules, it is essential that only cells with a TCR able to recognize self-MHC, albeit with limited affinity, be exported from the thymus to the periphery. It is also critical, however, that the population of peripheral T cells be restricted to those that respond to foreign antigens, and cells possessing TCRs recognizing self-peptides plus MHC must not be allowed to complete their developmental program. Ensuring that only those cells with an appropriate TCR mature in the thymus relies heavily on many of the same TCR signal transduction events described earlier.

Of the T-cell lineages, those expressing the α/β TCR cells are the best studied and most numerous. However, γ/δ T cells, another population that possesses an antigen receptor generated through combinatorial rearrangement of gene segments, as well as natural killer (NK) T cells, a subtype of lymphocytes that has characteristics of both T and NK cells (see Chapter 21), are also generated in the thymus. It has become clear recently that additional small populations of T cells possessing unique characteristics are also produced in the thymus. This chapter focuses primarily on α/β T cells and touches briefly on γ/δ T-cell development.

Early T-Cell Development

Identifying T-cell progenitors is an area of intense investigation because developing tools to manipulate these cells has great therapeutic potential for increasing the speed at which T-cell repopulation may occur following hematopoietic stem cell transplant. A population of bone marrow-derived thymic settling progenitors (TSPs) that can give rise to mature T-cell populations has been identified. Understanding the steps that arise following settling of these cells in the thymus will be key to advancing approaches to quickly reconstitute T-cell function after bone marrow ablation.

The thymus is composed of developing T lymphocytes, DCs, epithelial cells, and mesenchymal components. Histologically, it is divided into two principal zones, the cortex and the medulla. TSPs enter the thymus at the corticomedullary junction, transition into early thymic progenitors (ETPs), which then develop into double-negative (DN) T cells, characterized by lack of expression of the CD4 or CD8 coreceptors (Fig. 24.6). As these early T cells progress though the DN stage, they are further subdivided into DN1, DN2, DN3, and DN4 stages on the basis of the cell surface receptors they express. During DN1, developing thymocytes lose the ability to differentiate into non-T lineages and begin to proliferate in the deep cortex of the thymus. As these early thymocytes progress to the DN2 phase,

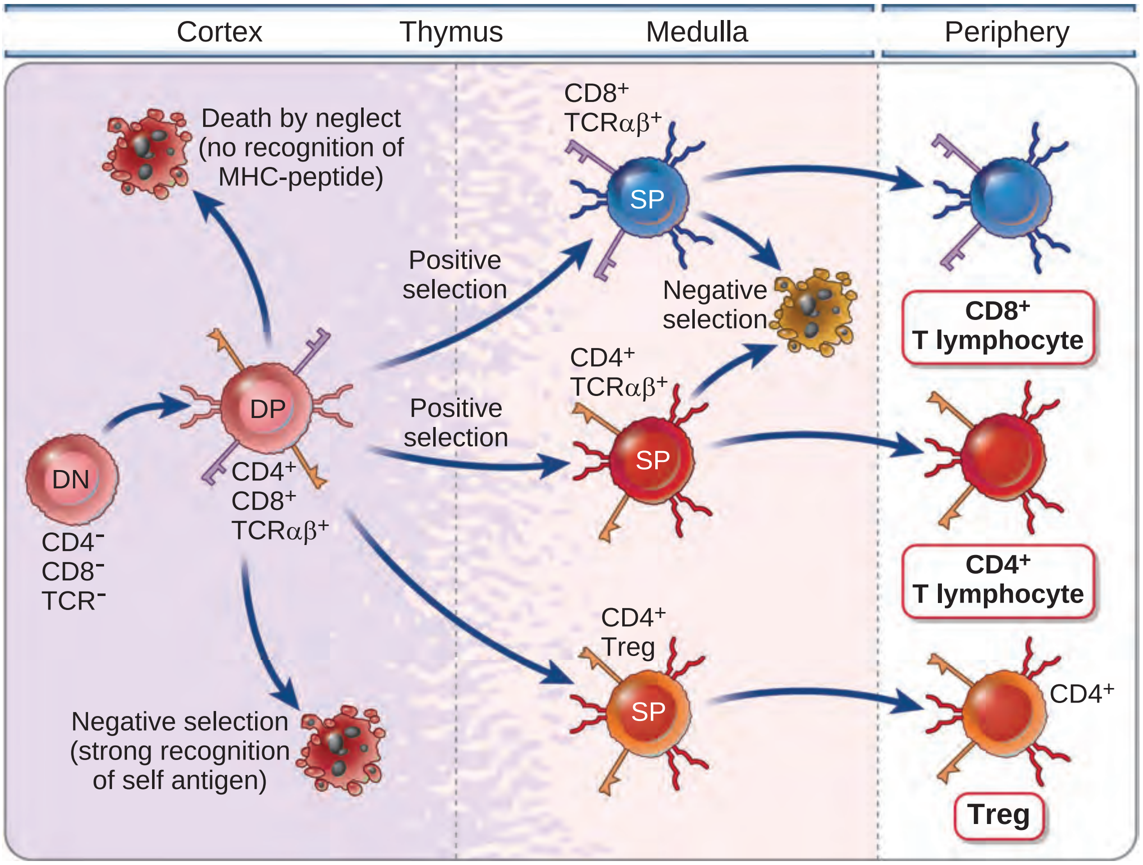


Figure 24.6 T-CELL DEVELOPMENT IN THE THYMUS. Thymic settling precursors (TSPs) travel from the bone marrow through the blood to the thymus. The progenitors of αβ cells are double-negative (DN) T cells. In the thymic cortex, these cells begin to express TCRs and CD4 and CD8 coreceptors. Double-positive (DP) thymocytes undergo positive selection if their TCR is able to recognize self-major histocompatibility complex (MHC) molecules; otherwise, they undergo "death by neglect." Negative selection occurs in the thymic medulla, when cells bearing TCRs that bind with strong avidity to self-MHC with self-peptide undergo apoptosis, thereby promoting central tolerance. These selection processes promote survival of thymocytes whose TCRs bind self MHC molecules with low affinity. Functional and phenotypic differentiation into CD4 or CD8 SP T cells occurs in the medulla, and mature T cells migrate into the circulation. Some double-positive cells differentiate into CD4 regulatory T cells (Treg). The development of γδ T cells is not depicted. *(From Abbas AK, Lichtman AH, Pillai S, Baker DL, Baker A. Cellular and Molecular Immunology. 9th ed. Philadelphia: Elsevier; 2017.)*

they begin to express T-cell–specific markers, such as Thy-1 (CD90), CD24, and CD25, and initiate TCR gene rearrangement at the TCR-γ, TCR-δ, and TCR-β loci. Throughout the DN1 to DN3 stages, as the cells migrate from the cortex to the subcapsular zone, interactions between Notch receptors on the developing T cells and specific Notch ligands collaborate with signaling through the IL-7 cytokine receptor to regulate lineage commitment and developmental progression.

During the DN3 stage, rearrangement of TCR-γ, TCR-δ, and TCR-β loci occurs with maximal efficiency, and initial expression of the TCR proteins these genes encode occurs. From this time onward in T-cell development, the proliferation and survival of the developing thymocytes depend on TCR signals. Two key checkpoints must be passed for full T-cell development to occur. First, upon productive rearrangement of the TCR-β locus, the TCR-β protein forms a "pre-TCR" complex with an invariant cytosolic protein designated *pre-Tα*. This complex engages the TCR signaling machinery, including the PTKs Lck, Fyn, and Syk (a ZAP-70-related PTK) and the adapters SLP-76 and LAT, to initiate the TCR signaling cascade. The resultant biochemical second messengers suppress rearrangement of the other β allele resulting in "allelic exclusion," or silencing of the non-rearranged allele, to ensure each T cell expresses only one TCR specificity. These signals also induce continued T-cell development by promoting rearrangements at the α locus, maintaining cellular survival, initiating a proliferative burst, and inducing expression of CD4 and CD8. For effective signaling to occur, the rearranged β locus must encode a protein that folds correctly and pairs with pre-Tα. Because the rearrangement of the genes that eventually make up the β chain is a random process, it is often the case that the rearranged allele encodes a dysfunctional protein. In this circumstance, signaling does not occur, and the cell initiates rearrangement at the other β chain allele. If this again does not result in a functional protein, no signaling occurs, and the cell undergoes apoptosis. In other cells, a similar rearrangement process occurs in the γ and δ loci. Productive rearrangements of these gene families create a functional, mature γ/δ TCR that also associates with the TCR signaling complex to propagate signals to trigger further cellular development.

Although the determining factors that result in either γ/δ or α/β T-cell development have not been fully elucidated, several molecular events are thought to contribute. The expression of a TCR gene rearrangement product likely plays a role in lineage determination because there is evidence suggesting that developing thymocytes with a functional γ/δ TCR are often excluded from the α/β cell fate. However, TCR expression is not the only factor in determining lineage fate; cytokine signals and TCR signal strength may also play a role. Experiments have shown that DN2 thymocytes distinguished according to IL-7 receptor expression differentiate into α/β or γ/δ T cells, with DN2 cells expressing high IL-7 receptor levels preferentially developing into γ/δ T lymphocytes and those with lower expression more likely to differentiate into the α/β lineage. Other studies have suggested that the strong signals propagated by the γ/δ TCR in comparison to those of the pre-TCR complex may promote γ/δ lineage commitment.

Positive Selection

Developing α/β T cells that have passed the first checkpoint demonstrating functional β-chain rearrangement transition into the double-positive (DP; $CD4^+CD8^+$) stage and complete TCR-α rearrangement to produce a mature α/β TCR heterodimer. The stochastic nature of TCR gene rearrangements guarantees that a significant proportion of cells expressing TCR-α/β complexes will not be able to interact with self-MHC proteins and hence would not be stimulated by peptide–MHC complexes in the periphery. DP thymocytes therefore undergo a series of tests, collectively known as *positive* and *negative selection*, to determine TCR fitness. If the TCR is not stimulated via peptide–MHC complexes presented by thymic APCs, the developing cell undergoes "death by neglect" through apoptosis. Approximately 90% of developing α/β DP thymocytes express a TCR that cannot weakly recognize self-peptide–MHC and thus die by neglect. In contrast, those DP thymocytes that interact with self-peptide–MHC complexes on thymic cortical epithelial cells with sufficient strength pass this "positive selection" test and are protected from apoptosis.

CD4 and CD8 Lineage Commitment

The MHC specificity of the TCR on a positively selected DP thymocyte influences lineage fate. Cells signaled through a MHC class I–restricted TCR develop into CD8 single-positive (SP; $CD4^-CD8^+$) cells, and those that receive signals via MHC class II–restricted TCRs develop into CD4 SP T cells. The underlying molecular mechanisms governing CD4/CD8 lineage choice is much debated. Most recently, a kinetic signaling model has emerged. It proposes that CD4 or CD8 lineage fate is determined by TCR signal duration.

Among the many proteins that are involved in CD4 or CD8 lineage choice are key transcription factors. T-helper-inducing POZ/Krüppel-like factor (Th-POK), a zinc finger protein that is expressed exclusively in $CD4^+$ T cells and not in $CD8^+$ T cells, and RUNX3, a member of the Runx transcription factor family, are required for CD4 and CD8 lineage commitment, respectively. Additional studies have identified a network of key transcription factors and signaling proteins important for lineage choice in the thymus, underscoring the complexity of this stage of T-cell development.

Negative Selection

Although positive selection ensures that the random combinatorial rearrangement of gene segments results in a TCR that recognizes antigen presented by self-MHC proteins, until this point in T-cell development, there is no guard against the emergence of T cells that possess TCRs with high reactivity against self-peptides in the MHC binding pockets. Thus, to prevent autoimmunity, there must also be a mechanism to eliminate developing T cells with TCRs expressing these potentially autoreactive specificities. This process is called *negative selection*. Negative selection occurs primarily in the thymic medulla, where thymocytes serially interact with medullary thymic epithelial cells (mTECs) and other thymic APCs including DCs. At this stage, if thymocytes with TCRs engage peptide–MHC complexes with high affinity, the strong TCR signal initiates apoptosis. Whereas it is easy to see how this model allows for deletion of developing thymocytes with reactivity against self-antigens generated within the thymus itself, it was difficult to imagine how cells with reactivity against antigens known to be expressed outside the thymus would also be deleted. An explanation for how this occurs came from the discovery of the autoimmune regulator (AIRE) protein. Initially identified as the gene product mutated in a rare human autoimmune disorder, autoimmune polyendocrinopathy-candidiasis-ectoderm dystrophy (APECED), AIRE was later found to be essential for the expression of peripheral tissue-specific antigens by mTECs.[8] Although AIRE does not regulate thymic expression of all peripheral antigens, its contribution to the elimination of autoreactive cells is highlighted by the widespread, multiorgan autoimmunity seen in patients with APECED. Identifying additional mechanisms responsible for thymic expression of tissue-specific genes is an area of active investigation.

Negative selection is one mechanism for development of "tolerance" or immune unresponsiveness to self-antigens; however, the process is not perfect in eliminating all self-reactive T cells. Hence, other means exist to promote self-tolerance after T cells leave the thymus. One such mechanism relies on development of regulatory T cells (Tregs), which actively interfere with effector T-cell function. Like conventional αβ T cells, a subset of Tregs (previously known as *natural* or *nTregs* and more recently designated *thymic* or *tTregs*) also develops in the thymus. Tregs are characterized by the surface expression of CD4 and CD25 (the high affinity α chain of the IL-2 receptor) and depend on the transcription factor *forkhead box protein 3* (*FoxP3*) for their lineage commitment.[9] The gene encoding FoxP3 was originally identified as the causal mutation in a rare, and frequently fatal, human autoimmune disease called *immunodysregulation, polyendocrinopathy, and enteropathy, X-linked (IPEX) syndrome*. A mutation in the mouse gene for *FoxP3* causes a similar disease (*scurfy* mice). These naturally occurring loss-of-function mutations demonstrate the necessity for Tregs in maintaining self-tolerance. In the thymus, development into a tTreg is enhanced in cells that have high-affinity TCR-peptide-MHC interactions, suggesting that these cells develop specifically to counter autoreactive responses. The exact mechanism that drives these cells to adopt a Treg fate and avoid negative selection during development is being investigated.

The path of developing γ/δ thymocytes contrasts with that of α/β T-cell development, which is likely related to the function of mature γ/δ T cells. In the periphery, γ/δ T cells reside in secondary lymphoid organs with conventional α/β T cells but also are enriched in epithelial tissues of various organs, such as the skin, intestinal epithelium, reproductive tract, and lung. In these distinct settings, the TCR diversity of the γ/δ T cells is more restricted, suggesting that these subsets may preferentially recognize ligands expressed at these anatomic locations during times of infection or tissue damage.

T-CELL FUNCTION

As T cells leave the thymus, they circulate in the bloodstream through secondary lymphoid tissues. Secondary lymphoid organs include the spleen, LNs, and mucosa-associated lymphoid tissues (MALT). Before interaction with their cognate antigen, these cells are designated naive T cells. Naive T cells leave the blood to enter LNs through specialized vessels called *postcapillary venules* and travel through the T cell–rich interfollicular zones. Naive T cells exit from these postcapillary venules, and upon antigenic recognition, remain in the node to proliferate and differentiate. If the naive T cells do not encounter cognate antigens, the cells exit LNs via lymphatic fluid back to the bloodstream and recirculate.

As naive T cells migrate through peripheral lymphoid organs, they sample various peptide–MHC complexes on APCs. These APCs include cells residing in the secondary lymphoid organs as well as those in tissues that sample their local environment and then migrate to the secondary lymphoid organs, hence concentrating antigen in these locations. If a naive T cell does not encounter its specific antigen, it leaves the lymphoid tissue via the lymphatic system to reenter the bloodstream and repeat this process.

When a naive T cell recognizes its cognate antigen on an APC, a program of proliferation and differentiation transforms the naïve T cell into an effector T cell, now primed to respond rapidly upon encountering its corresponding antigen in the tissues. One important difference between naive and activated T cells is the cell surface expression of chemokine receptors and integrins. These receptors direct the cell to the appropriate tissue where the effector T cell is needed. Thus, as a part of the T-cell activation process, those receptors that direct the naive T cell in its pathway recirculating between the lymphatic organs and blood vessels are altered for those that direct the activated cell to the tissues, so that the effector T cell reaches the site of pathogen challenge.

$CD4^+$ and $CD8^+$ T cells undergo analogous differentiation processes to acquire functional maturity but play distinct roles in the adaptive immune response to infection. Naive cells of both lineages are activated through peptide-MHC interaction with their TCRs, and their differentiation is influenced by a combination of signals, including TCR signal strength, co-stimulation by ligands that interact with other T-cell surface receptors, and the local cytokine environment during antigen encounter. Integration of these signals promotes expression of signature transcription factors and key effector molecules, which allow the mature cell to perform its individualized function. Activated $CD8^+$ T cells possess the machinery to induce death in host cells that express the appropriate peptide within the binding groove of MHC class I (see later), whereas $CD4^+$ T cells exert their functions through the production of cytokines or by interacting with other immune cells through direct cell–cell contact following restimulation of their TCR by peptide presented by class II MHC. These so-called helper functions marshal and activate other cells of the immune system (Fig. 24.7). Until they encounter peptide–MHC, naive $CD4^+$ T cells have the potential to develop into one of several effector subsets, including Th1, Th2, Th17, and T follicular helper (Tfh) cells. Additional subsets have been defined recently, but these remain less well characterized and are not discussed here. Additionally, there is

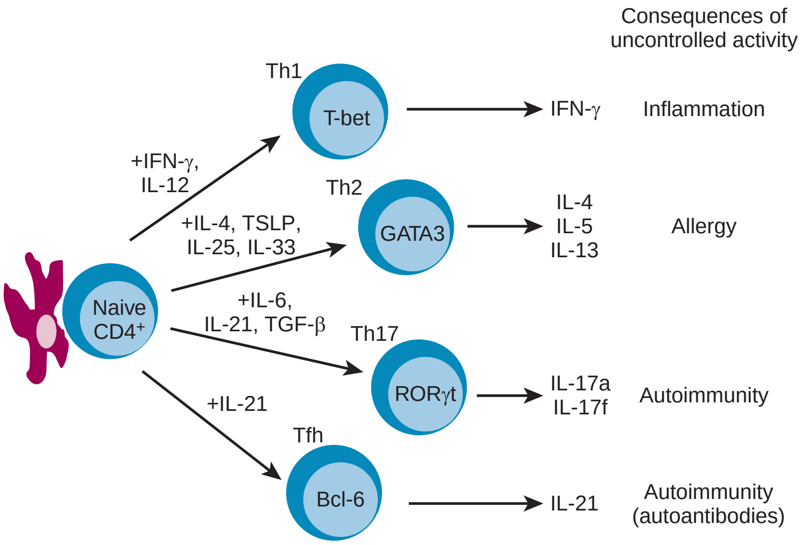


Figure 24.7 DIFFERENTIATION OF $CD4^+$ T-HELPER SUBSETS. When activated, $CD4^+$ T cells differentiate into distinct, functionally mature effector subsets. Various factors, including the cytokine milieu, promote the expression of signature transcription factors and effector molecules. $CD4^+$ helper subsets are defined largely by their cytokine production driven by these key transcription factors. Th1 cells are induced by interferon γ (IFN-) and interleukin 12 (IL-12), express the transcription factor T-bet, and produce IFN-γ. IL-4 is the primary cytokine that promotes Th2 differentiation. Th2 cells are characterized by expression of GATA3 and production of IL-4, IL-5, and IL-13. Naive $CD4^+$ cells that are activated in the presence of IL-6 and IL-21 differentiate into Th17 cells, typified by the expression of retinoic acid receptor–related orphan receptor γt (ROR-γt) and production of the IL-17 family of cytokines. T follicular helper (Tfh) cell differentiation is mediated by IL-21. These cells are characterized by the transcription factor B-cell lymphoma 6 protein (Bcl-6) and production of IL-21. If $CD4^+$ helper differentiation and activity are not adequately controlled, imbalanced responses can lead to pathologic conditions.

evidence that some CD4+ T cells have direct cytotoxic function, similar to their CD8+ counterparts (see below), but a full discussion of these cells is beyond the scope of this chapter.

Th1 Cells

Th1 cells activate macrophages, NK cells, and CD8+ T cells to combat intracellular pathogens. Th1 cells also stimulate immunoglobulin class switching in B cells for the production of immunoglobulin G2a (IgG2a) antibodies that optimize clearance of viruses and extracellular bacteria (see Chapter 22). During priming of naive CD4+ T cells, several factors combine to promote differentiation along the Th1 pathway, including characteristics of the antigen, costimulatory signals from the presenting APC, and the cytokine microenvironment. Several cytokines are implicated in Th1 differentiation, but the two most critical are interferon γ (IFN-γ) and IL-12. IFN-γ produced by innate immune cells promotes Th1 differentiation by activating signal transducer and activator of transcription 1 (STAT1), a key signaling molecule that regulates T-bet, one of the signature transcription factors associated with Th1 cells. IL-12, produced by activated APCs and other innate immune cells, acts through a separate STAT4-dependent pathway to promote IFN-γ production. IL-12 also signals to upregulate its own receptor and the IL-18 receptor, thereby allowing IL-18 to act in concert with IL-12 to promote IFN-γ production, thus creating a "feedforward" cycle to amplify the Th1 response.

T-bet, a T-box family member, is the key transcription factor associated with Th1 differentiation and function. T-bet-deficient T cells are defective in their ability to differentiate into Th1 cells either in vitro or in vivo, and T-bet-deficient mice are unable to control *Leishmania major* infection, a well-characterized intracellular pathogen model that depends on the characteristic Th1 cytokines for clearance. Whereas T-bet is considered the "essential" factor that directs Th1 lineage determination, other transcription factors can play roles in optimal Th1 function.

Once differentiated, Th1 effector cells are characterized by production of proinflammatory cytokines such as IFN-γ and tumor necrosis factor-α (TNF-α) that stimulate macrophages, NK cells, and CD8+ T cells to promote pathogen clearance. It is clear, however, that Th1 function must be balanced. Evidence from both animal models and human patients indicates that overexuberant Th1 responses drive inflammatory conditions and may lead to tissue destruction.

Th2 Cells

Th2 cells are critical for the immune response against extracellular parasites, such as helminths, through production of IL-4, IL-5, and IL-13. At initial sites of parasitic infection, epithelial cells of the target organs, including the skin, lungs, and intestines, and resident cells of the innate immune system sense parasite-derived products and produce Th2-inducing cytokines, including thymic stromal lymphopoietin (TSLP), IL-4, IL-25, and IL-33. These cytokines then act on innate immune cells, including basophils and DCs, as well as directly on naive CD4+ cells to promote Th2 differentiation.

IL-4 signaling is particularly critical to promote Th2 differentiation. Through interaction with its receptor, IL-4 activates STAT6. STAT6 plays a vital role in Th2 differentiation, as evidenced by the profound reduction in development of this lineage in *Stat6*-deficient mice. STAT6 activation leads to its nuclear translocation and subsequent induction of the transcription factor GATA binding factor 3 (GATA3), which, like T-bet for Th1 cells, is considered the master regulator of Th2 differentiation. GATA3 regulates Th2 cytokine production by binding and activating the "Th2 locus," which includes the genes encoding IL-4, IL-5, and IL-13. When GATA3 function is abrogated, Th2 differentiation is virtually absent both in vitro and in vivo. Repression of Th1 differentiation occurs at least partially through GATA3-dependent inhibition of STAT4, thus interfering with *Ifng* gene transcription.

IL-4 produced by mature Th2 cells acts in a positive feedback loop to promote further Th2 cell differentiation in naive T cells as they encounter antigen. Th2-derived IL-4 also mediates IgE class switching in B cells. Soluble IgE binds to and crosslinks its high-affinity receptor FcεRI on basophils and mast cells, promoting production of histamine and serotonin as well as several cytokines, including IL-4, IL-13, and TNF-α. IL-5 produced from Th2 cells recruits eosinophils, whereas Th2-derived IL-13 promotes both the expulsion of helminths during parasitic infection and also the induction of airway hypersensitivity.

Th2 responses are critical for immunity against extracellular parasites, but excessive Th2 responses are associated with the pathologic conditions of allergy and airway hypersensitivity. The increase in asthma in the developed world has been linked to an imbalance of Th subsets with skewing toward "Th2-ness" in the population. Additional work is necessary to more firmly establish a molecular immunologic link to the epidemiology of these diseases. These studies will be enhanced by information gleaned from clinical trials of modulators of Th2 cytokines in asthma and other hypersensitivity disorders.

Th17 Cells

The original description of Th1 and Th2 cells, indicating that not all mature CD4+ T cells were alike, led to the search for other CD4+ subsets. Extensive analyses of IL-17 and the cells that produce this cytokine demonstrate that Th17 cells are important for the control of extracellular bacterial and fungal infections. With excessive activity, however, these cells also appear to play an important role in autoimmune diseases through the production of proinflammatory cytokines, including IL-17A, IL-17F, IL-21, and IL-22.

Although IL-23 is a key regulator of Th17 cells, the IL-23 receptor is not expressed on naive CD4+ cells and hence could not explain the differentiation of cells into the Th17 subset. Subsequent studies demonstrated that the combination of transforming growth factor-β (TGF-β) with either IL-16 or IL-21 induces Th17 differentiation. The cytokines that are key mediators of Th17 differentiation and survival, including IL-6, IL-21, and IL-23, all activate STAT3. The critical role of this STAT family member was demonstrated in murine studies, when its deletion abrogated the ability of T cells to undergo Th17 differentiation. In humans, the importance of STAT3 was highlighted when it was identified as the genetic mutation present in many patients with hyper-IgE syndrome (HIES, or Job syndrome). HIES is a rare immunodeficiency syndrome characterized by recurrent staphylococcal skin abscesses, elevated serum IgE, and pneumatocele-forming pneumonias. Patients with HIES with STAT3 mutations have an impaired ability to form Th17 cells, which may explain part of their immunodeficiency. STAT3 regulates expression of many cytokine and cytokine receptor genes involved in Th17 generation or function, including IL-17A, IL-17F, IL-21, IL-21R, and IL-23R.

STAT3 is also important for induction of the signature Th17 transcription factor ROR-γt, which is a member of the retinoic acid–related orphan receptor (ROR) family. In naive CD4+ cells, ROR-γt induces IL-17 gene transcription and promotes expression of the IL-23 receptor. ROR-γt deficiency only partially affects Th17 cells in vivo because of expression of the related transcription factor ROR-α, which is also expressed in T cells and is induced by IL-6/TGF-β in a STAT3-dependent manner. Cells deficient in both ROR-γt and ROR-α lose the ability to undergo Th17 differentiation, both in vitro and in vivo.

Th17 cells are induced during the response to extracellular bacteria and fungi, including *Klebsiella pneumoniae*, *Bacteroides* species, and *Candida albicans*. Indeed, some patients with chronic mucocutaneous candidiasis have been shown to have mutations in IL-17F and the IL-17 receptor genes. Excessive Th17 cell function also plays a role in autoimmune diseases, such as rheumatoid arthritis, psoriasis, and Crohn disease, and therapies targeting the Th17 axis have been approved or are under active clinical investigation for treating these disorders.

Tfh Cells

In addition to Th1, Th2, and Th17 subsets, naive CD4$^+$ cells develop other functions dependent on the cytokines produced. Examples include recently described Th9, Th22, and Tfh cells. This latter subset enhances the humoral immune response by providing help to B cells during germinal center reactions. Tfh cells express high levels of CXCR5, the receptor for the chemokine CXCL13. The expression of CXCR5 permits differentiating Tfh cells to migrate from the T-cell zone to the CXCL13-rich B-cell follicle in the LN, thereby allowing Tfh cells to interact with B cells and exert their function. In addition to CXCR5 expression, other signals, such as TCR signal strength and costimulatory molecules, are important for Tfh differentiation. A study using adoptive transfer of naive CD4$^+$ cells expressing high- and low-affinity transgenic TCRs demonstrated that high-affinity TCR interactions preferentially developed into the Tfh subset. Tfh cells have higher expression of multiple costimulatory molecules, including CD40L, ICOS, and OX40, than other T helper subsets. Because costimulatory molecules enhance B-cell differentiation, the higher expression of these molecules on Tfh cells is hypothesized to positively correlate with the enhanced ability to facilitate B-cell antibody production. It appears that the expression of costimulatory molecules on Tfh cells is important not only for their function but also for their development and/or maintenance, because both mice and humans deficient in ICOS have fewer Tfh cells with reduced germinal center formation.

Similar to other CD4$^+$ helper subsets, Tfh programming depends on a signature transcription factor, in this case B-cell lymphoma 6 protein (Bcl-6). In Tfh cells, Bcl-6 acts as a transcriptional repressor. Studies employing complementary methods of T cell–specific Bcl-6 deficiency and overexpression demonstrated that Bcl-6 expression in T cells is both necessary and sufficient for Tfh differentiation in vivo.

CD4$^+$ Th Plasticity

Although CD4$^+$ T-helper differentiation was classically thought to be a model of lineage specification and differentiation, it is clear that there is more plasticity in the CD4$^+$ Th subsets than was originally appreciated. Traditionally, Th subsets are associated with a signature cytokine(s) and transcription factor. However, recent data demonstrate CD4$^+$ Th cells can express more than one cytokine, particularly in vivo, and even the transcriptional "master regulators" can be coexpressed in the same cell. The mechanisms that underlie this plasticity and its functional relevance are areas of active investigation.

CD8$^+$ Cytotoxic T Cells

The principal function of CD8$^+$ cytotoxic T cells (CTLs) is to kill host cells that have been infected with pathogens or that have undergone deleterious changes, such as malignant transformation. Like CD4$^+$ cells, naive CD8$^+$ cells initially encounter peptide antigen and MHC on the surface of APCs in the secondary lymphoid organs. However, unlike CD4$^+$ cells that are stimulated by class II MHC alleles on the APCs, CD8$^+$ cells are engaged by class I MHC plus peptide. For many years it remained unclear how APCs, which acquire peptide antigens largely by engulfing materials generated outside the cell, are able to present MHC class I–restricted peptides, which typically are generated within the cell (see earlier). This conundrum was solved with the identification of "cross-presentation," a mechanism by which APCs present engulfed antigens on both class I and class II alleles. Thus, tissue-resident phagocytic cells ingest virally-infected or malignantly transformed host cells, degrade the ingested material, and present the peptide antigens in the binding grooves of both class I and class II MHC alleles. These activated phagocytic cells then migrate to the LNs, where they encounter recirculating naive CD8$^+$ cells. TCR engagement of foreign peptide–MHC class I complexes triggers activation of the CD8$^+$ T cells and initiates CTL differentiation. As part of its activation program, the CTL changes its expression of integrins and chemokine receptors so that it can leave the circulation and enter the tissues, looking for host cells displaying the same antigen that induced CTL activation by the APC in the LN.

Once an appropriate target cell is identified in the tissues, the CTL is again stimulated through its TCR, this time by the peptide–MHC class I combination on the target cells. A structure similar to the IS forms between the CTL and the target cell. The CTL contains specialized granules that are transported to the contact site between the CTL and target. These granules are modified lysosomes that contain effector proteins, including perforin, granzymes, and granulysin. Perforin facilitates the entry of the granzymes into the cytosol of the target cell. The granzyme family, consisting of granzyme A, granzyme B, granzyme H, granzyme K, and granzyme M, are proteases that degrade host cell proteins. Granzyme B is the best-studied family member and is known to cleave caspase 3, activating a proteolytic cascade leading to DNA degradation and apoptosis of the target cell (Fig. 24.8). Granzyme B also promotes cell death in a caspase-independent manner through cleavage of the proapoptotic protein Bid, promoting its migration to and disruption of the outer mitochondrial membrane, resulting in the release of cytochrome c. CTLs also produce cytokines, including IFN-γ, TNF-α, and IL-2. IFN-γ acts to inhibit viral replication in the affected tissues and also induces increased class I MHC expression, thus improving the ability of cells to stimulate the TCR on CTLs. IFN-γ synergizes with TNF-α for macrophage activation.

The transcription factors important for CD8$^+$ T-cell effector differentiation include two members of the T-box family, T-bet and Eomesodermin (Eomes). Initially identified as the master Th1-determining transcription factor in CD4$^+$ cells, T-bet also plays an essential role in CD8$^+$ effector cell differentiation. Recent work has shown that T-bet expression is highest in short-lived effector cells and lower in CD8$^+$ T cells destined to become memory cells (see later), suggesting that a gradient of T-bet expression controls the balance between different CD8$^+$ effector fates. Eomes cooperates with T-bet in CTL function, and cells deficient in both factors are unable to generate CTLs in response to viral infection.

MATURATION OF T-CELL-MEDIATED IMMUNITY

T-Cell Memory

The activation of naive T cells does not complete their maturation process; instead, it is the starting point for the changes that result in T-cell-mediated immunity. At the initiation of an infection, individual antigen-specific T cells become activated and expand robustly to combat the pathogen. As the pathogen is eradicated, the large population of activated T cells must contract dramatically to ensure homeostasis of the immune system. However, a discrete but small population of antigen-specific T cells persists. These long-lived T cells have properties distinct from naive or activated T cells, including self-renewal through homeostatic proliferation and the ability to rapidly proliferate and regain effector function upon re-exposure to antigen. These are the cardinal features of cell-mediated immunologic memory.

Immunologic memory refers to the observation that after an initial exposure and mounting of an effective immune response to a pathogen, subsequent interactions with that pathogen elicit rapid and robust T-cell activation, with more efficient clearance of the pathogen. Memory is the foundation of vaccination because immunization with pathogen-specific antigens induces a memory response so that first exposure of the host to the intact pathogen results in a rapid, effective response, thus abrogating signs and symptoms of the infection.

Within days of infection, subsets of activated effector CD8$^+$ T cells can be identified with different cell fates: those that are terminally differentiated and those that have the potential to develop into memory cells. Single-cell adoptive transfer experiments demonstrate that individual naive CD8$^+$ T cells have the ability to differentiate into a heterogeneous pool of short-lived effector and long-lived memory cells, likely in response to differences in antigen specificity and

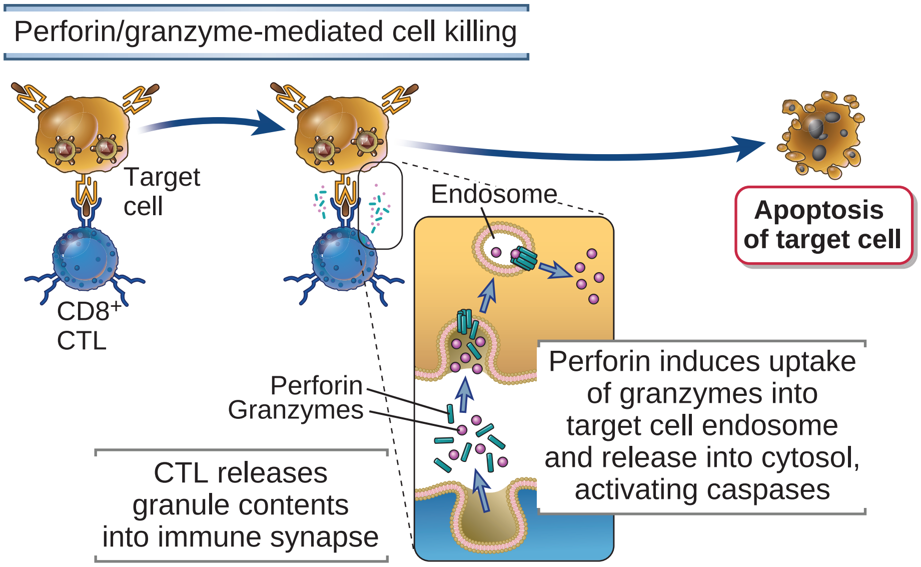

Perforin/granzyme-mediated cell killing
Target cell
CD8+ CTL
Endosome
Apoptosis of target cell
Perforin
Granzymes
CTL releases granule contents into immune synapse
Perforin induces uptake of granzymes into target cell endosome and release into cytosol, activating caspases

Figure 24.8 CD8+ T-CELL CYTOLYTIC FUNCTION . Cytotoxic CD8+ T lymphocytes (CTLs) function primarily to kill host cells that have been infected by intracellular pathogens or that have undergone malignant transformation. After naive CD8+ cells encounter peptide–major histocompatibility complex (MHC) class I plus costimulation in secondary lymphoid organs, these activated CTLs leave the circulation and enter the tissues. There, upon interaction with a target expressing that same peptide–MHC class I, a CTL forms a lytic synapse, similar to the immunologic synapse, with the target. Complexes of perforin and granzymes are released from the CTL by granule exocytosis and enter target cells. The granzymes are delivered into the cytoplasm of the target cells by a perforin-dependent mechanism, and they induce apoptosis. *(From Abbas AK, Lichtman AH, Pillai S, Baker DL, Baker A. Cellular and Molecular Immunology. 9th ed. Philadelphia: Elsevier; 2017.)*

duration of stimulation, precursor frequency, and the inflammatory environment.

Different subsets of memory cells are observed after resolution of infection. The two main classes are effector memory and central memory T cells. Effector memory T cells, characterized by lack of expression of LN homing molecules CD62L and CCR7, rapidly produce cytokines in response to restimulation with previously encountered antigen, thereby allowing for rapid responses to invading pathogens. These cells preferentially reside in the circulation. In contrast, central memory cells express high levels of CD62L and CCR7, are more prevalent in lymphoid tissues, and mount a robust proliferative response after reencountering antigen and are longer-lived. More recently, tissue-resident memory cells have been identified. These tissue-resident memory cells have been identified in the lungs, skin, liver, and the female reproductive tract. They are characterized by expression of the integrin CD103, which aids in tissue entry, and CD69, which promotes tissue retention.

As with differentiation of naive T cells into efficient effectors, cytokines play an important role in memory T-cell development and maintenance. IL-2 is essential for initial memory cell differentiation, whereas IL-7 and IL-15 are crucial for memory cell persistence. Other signals, such as the strength of antigenic and inflammatory signals during T-cell activation, also influence memory cell development and maintenance. An important consideration for memory development is cell–cell interactions because $CD4^+$ T cells are required during initial priming of $CD8^+$ cells for development of fully functional $CD8^+$ memory cells. A number of infectious disease models have demonstrated that in the absence of $CD4^+$ T-cell help, fewer $CD8^+$ memory T cells are maintained, and those that do persist are of the central memory phenotype.

Although great progress has been made in elucidating the molecular underpinnings of immunologic memory, much remains to be learned. Recent data have emerged on the importance of the cellular metabolic state in the control of memory T-cell differentiation. As $CD8^+$ T cells are activated, they transition from using primarily oxidative phosphorylation to generate basal energy in the quiescent state to using glycolysis during the effector phase and then back to using oxidative phosphorylation as memory cells. In experimental models, manipulations of the cell's metabolic profile can influence effector function and memory differentiation. As additional discoveries are made, it is anticipated that new approaches will develop to improve T-cell responses to vaccines against infectious agents, to promote T-cell recall responses to pathogens that today result in chronic infections, and to harness host T-cell responses to combat tumors.

T-Cell Exhaustion: Impaired Response after Chronic Antigen Exposure

Under most circumstances, acute infection results in the expansion of T lymphocytes specific for the inciting pathogen, clearance of the pathogen, and the development of memory T cells able to eliminate that pathogen more effectively upon re-exposure of the host. However, some pathogens cannot be efficiently cleared from infected hosts and persist throughout the lifetime of the organism, despite the formation of pathogen-specific T cells. Examples of such pathogens include human immunodeficiency virus and hepatitis viruses B and C. These persistent infections result in chronic antigen exposure, which, instead of continuing to induce maximal productive T-cell responses, leads to the generation of "exhausted" T cells that have reduced ability to kill and produce cytokines in response to infection. The development of T-cell memory and the exhaustion response are initiated in similar ways, with the formation of cells that are capable of responding to antigen challenge through proliferation and the secretion of cytokines. However, during exhaustion, the persistence of pathogen causes T cells to become increasingly less responsive to stimulation. At early time points in this process, exhausted $CD8^+$ T cells lose the ability to secrete IL-2 or TNF-α and cannot induce cytolysis of infected host cells. At later time points, $CD8^+$ T cells become completely unresponsive and ultimately undergo apoptosis. The induction of exhaustion is thought to represent a functional adaption that permits some degree of control of chronic infection while limiting immune-induced tissue damage.

Concurrent with the loss of functional responses, exhausted cells upregulate inhibitory cell surface receptors. The best studied of these inhibitory receptors is programmed death 1 (PD-1), which binds its ligands, PD-L1 and PD-L2, expressed on activated macrophages and other APCs. Engagement of PD-1 dampens the T-cell response, likely by recruiting phosphatases that oppose the PTKs necessary for T-cell activation. PD-1 is normally expressed on T cells after initial activation, presumably as a means to prevent excessive responses, and is then downregulated as T cells acquire a memory phenotype after the pathogen clearance. Exhausted T cells, however, continue to express this inhibitory receptor.[10] Early during exhaustion, PD-1 blockade is capable of reversing T-cell exhaustion in experimental models to some degree; however, epigenetic changes in T cells that take place during exhaustion prevent the T cells from ever regaining full function. Moreover, as chronic antigen exposure continues, other inhibitory receptors such as Lag-3 and Tim3 become expressed, further diminishing the functional potential of the exhausted T cell. Blockade of PD-1 with these other receptors has been shown to improve T-cell responsiveness, even at later stages of exhaustion. Therapeutic targeting of the PD-1 axis and other key inhibitory receptors is an exciting new avenue for immunotherapy against malignancies (see later) and chronic infections.

INHIBITION OF T CELL-MEDIATED IMMUNITY

Efficient signaling through the TCR and other cell surface molecules is required for initial T-cell activation. Similarly, appropriate maturation of the T-cell response to generate effector and memory cells is critical for adequate responses to pathogens. However, because of the potential for activated T cells to damage host tissues, an integral aspect of the immune system is also to negatively regulate T-cell activities. The mechanisms for inhibiting T-cell responses are critical for the prevention of inappropriate activation of naive T cells at the initiation of an immune response, for limiting the robustness of an appropriate T-cell response as effector cell functions are developed, and for terminating the T-cell response once an antigenic challenge has been met. This section discusses examples of how T-cell activation is modulated at each of these three critical steps of T-cell immunity.

Prevention of Inappropriate Initiation of T-Cell Responses

Given the enormous power of immune effector cells to damage tissues, it is essential that the immune system be nonreactive (tolerant) to self. As described earlier, T-cell tolerance is achieved centrally through the requirement to pass selection checkpoints during thymic development. However, negative selection in the thymus is not sufficient to eliminate all cells with potential autoreactivity, and some T cells bearing TCRs that may respond to self-antigens are exported from the thymus to the periphery. Mechanisms are in place to prevent these cells from becoming active effectors as they encounter antigen. Two such mechanisms are anergy, a process by which T cells limit their own responsiveness based upon engagement of particular cell surface receptors (a cell-intrinsic path to inactivation), and the action of Treg cells, which instruct potential effectors to remain quiescent.

Anergy

One means of limiting T-cell responses against host tissues is a process of self-inactivation termed *anergy*. As noted earlier, T cells require signaling through both the TCR and costimulatory receptors such as CD28 to become activated (see Fig. 24.5). Stimulation of the TCR alone in the absence of adequate co-stimulation produces T cells that fail to secrete IL-2 or upregulate high-affinity receptors for this cytokine and hence fail to clonally expand. Cells that have been rendered anergic fail to respond to subsequent stimulation, even if ligands for both the TCR and CD28 or other costimulatory receptors are available. This two-signal requirement ensures that only APCs activated by pathogens or other "danger signals" can initiate an immune response, because ligands for costimulatory receptors, such as CD80 and CD86, for CD28 are upregulated only in activated APCs. Thus, under circumstances of pathogen invasion, APCs present peptide antigens to T cells in addition to CD28 ligands. In the absence of an immune challenge, APCs express only low levels of CD80 or CD86. If a T cell encounters an APC that presents a stimulatory peptide–MHC complex but lacks sufficient expression of CD28 ligands, the T cell does not become activated. In this situation, the absence of ligands for CD28 implies that there is no "danger" and that the antigen being recognized is derived from a self-protein. The result of such an encounter leaves the T cell in an anergic state, refractory to activation even in the face of subsequent TCR stimulation by an activated APC.

The role of anergy in human immunology remains unclear, as investigators have largely used in vitro model systems and/or animal models. However, several lines of evidence indicate that self-antigen–reactive T cells that remain quiescent can be identified in normal human hosts. The biochemical basis of anergy also remains incompletely understood, but intriguing models suggest that an imbalance between the strength of Ras versus calcium signaling may be crucial. In this paradigm, it is the activation of calcium-dependent transcription factors, such as NFAT, in the absence of transcription factors activated by Ras signaling, such as AP-1, that confers an anergic state. Although anergy is classically thought to persist indefinitely, under some circumstances there is apparent plasticity, as exposure of T cells to high concentrations of IL-2 can improve functional responses in previously anergic cells. Thus, the physiologic importance of anergy in limiting endogenous T-cell activation and preventing autoimmunity and whether there are times when anergy must be reversed for appropriate immune responses are areas of active investigation.

Regulatory T Cells

Tregs are a subset of $CD4^+$ T cells that suppress the proliferation and cytokine production of activated T cells whose TCRs have been engaged by peptide–MHC, even in the presence of co-stimulation. Hence, as opposed to anergy, which operates in a cell-intrinsic fashion, Tregs block responsiveness in *trans*, by modulating responses of other cells. Tregs arise in two ways: "thymic" Tregs (tTregs, also termed natural Tregs, nTregs) that acquire function during development in the thymus (described earlier) and "inducible" Tregs (iTregs) that are generated through the differentiation of naive $CD4^+$ T cells in the periphery. Both tTregs and iTregs are characterized by expression of the key transcription factor FoxP3 and by surface expression of CD25, a subunit of the IL-2 receptor.

As noted, there are multiple steps and checkpoints that occur during development of T cells in the thymus. After reaching the DP stage, T cells test their TCR for reactivity against peptide–MHC complexes presented by thymic APCs and epithelial cells. Cells bearing TCRs with no reactivity undergo apoptosis (failed positive selection) as do cells with very strong TCR reactivity (through negative selection). Only cells whose TCRs have moderate affinity for peptide–MHC continue to mature. Within this continuum of permitted reactivity, those cells with TCRs exhibiting the highest affinity for peptide–MHC are induced to express FoxP3 and develop into Tregs. In the periphery, these cells respond to TCR stimulation by diminishing the response of "conventional" T effector cells, thus downregulating immune responses.

iTregs act similarly to tTregs, but these cells do not leave the thymus poised to have suppressive function. Instead, these cells arise from naive T cells that encounter antigen in the secondary lymphoid structures. Similar to other $CD4^+$ subsets, iTregs are induced on the basis of prevailing cytokine conditions and which receptor-ligand interactions predominate during this initial antigen encounter. Regardless of whether they arise in the thymus or are induced in the periphery, Tregs exert their immunosuppressive functions on a variety of immune cells, including $CD4^+$ and $CD8^+$ T cells, DCs, B cells, macrophages, and NK cells, within their microenvironment. Tregs mediate these immunosuppressive effects through the secretion of suppressor cytokines such as IL-10, IL-35, and TGF-β, the consumption of local concentrations of IL-2, and the induction of apoptosis or cell cycle arrest through direct cell-to-cell contact, likely through direct presentation of TGF- β by cell surface receptors on Tregs (Fig. 24.9).

Limiting T-Cell Responses After Stimulation by Foreign Antigen

Even when stimulated appropriately to combat an invading pathogen, it is essential to limit T-cell activation. Unchecked T-cell effector functions present a danger to the host through production of proinflammatory cytokines that recruit other cells of the immune system and through direct damage of self-tissues. T-cell effector functions are limited by modulating the T-cell activation pathways through activation of signaling molecules that counter the second messengers stimulated by TCR engagement, through inducible expression of cell surface receptors that compete with activating receptors on the T cell, or by targeting key activating proteins for destruction, thus limiting their ability to promote T-cell effector function. Additionally, the local environment in which the T cell exists may change, with cell extrinsic factors (e.g., inhibitory cytokines) becoming available to dampen T-cell responses (Fig. 24.10).

Limitation of T-Cell Activity from Cell-Intrinsic Components

Protein Tyrosine Phosphatases

As noted earlier, the most proximal known biochemical event to occur following engagement of the TCR by peptide–MHC results is activation of PTKs, including Lck and Zap-70, enzymes central to the T-cell activation program. Thus, one means by which to limit TCR signaling is to oppose the activating PTKs with deactivating protein tyrosine phosphatases, reversing the phosphorylation events that drive T-cell activation. Several such phosphatases have now been identified, including SH2 domain-containing phosphatase 1 (SHP-1) and protein tyrosine phosphatase, nonreceptor type 1 (PTPN1). Although the direct targets of these phosphatases have yet to be demonstrated conclusively, there is increasing evidence in murine systems that they are important for control of T-cell activation as well as for regulating the function of other cells of the immune system. Experiments show that, compared with wild-type cells, SHP-1-deficient T cells demonstrate enhanced proliferation and cytokine production after stimulation. These cells also show prolonged phosphorylation of TCR signaling molecules, consistent with a role for SHP-1 in reversing these events. Overexpression of SHP-1 within T-cell lines inhibits TCR-mediated signaling events. Furthermore, SHP-1 is recruited into the IS after engagement of the TCR, thus providing an appropriate physical localization for SHP-1 to directly engage targets of the TCR-stimulated PTKs. SHP-1 inhibitory activity appears to be crucial in vivo because mice that lack functional SHP-1 develop fatal autoimmunity, likely secondary to alterations of function of both innate and adaptive immune cells. There is accumulating evidence that other phosphatases are also critical for interfering with T-cell activation, both in animal models and more recently in studies of patients. Polymorphisms in the genes encoding several protein tyrosine phosphatases, including CD45

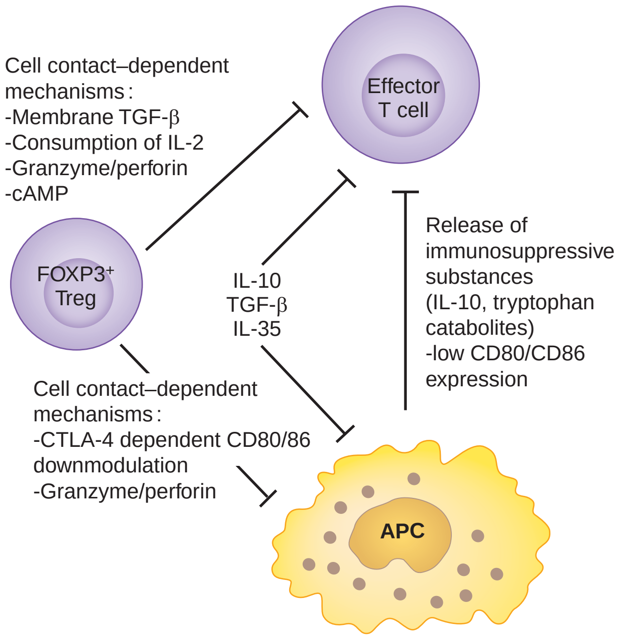


Figure 24.9 REGULATORY T-CELL ACTIONS. T-regulatory cells (Tregs) act to suppress other T cells through a multitude of mechanisms. Tregs can act in a cell contact–dependent manner by competing directly for stimulatory ligands on the antigen-presenting cell (APC), by absorbing essential growth factors, such as interleukin-2 (IL-2), or by directly transmitting an as-yet uncharacterized negative signal to either the T cell or the APC. Alternatively, Tregs can use long-range suppressive mechanisms by means of the cytokines IL-10, IL-35, and transforming growth factor-β (TGF-β). *(From Rich RR, et al. Clinical Immunology. 5th ed. Elsevier; 2019.)*

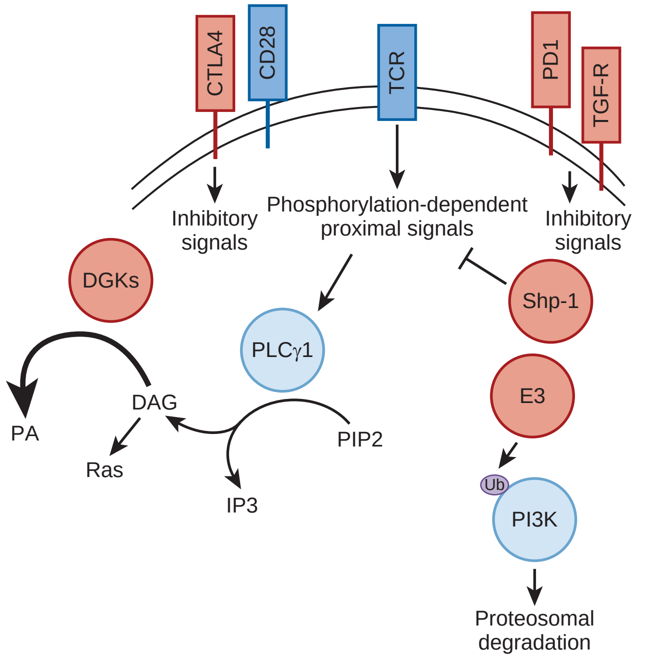


Figure 24.10 INHIBITORY PATHWAYS IN T CELLS Negative influences on T cells and T-cell antigen receptor (TCR) signaling take place at multiple levels within T cells and are crucial for the prevention of autoimmunity. Examples (indicated in *red*) include the protein tyrosine phosphatase SH2 domain-containing phosphatase-1 (SHP-1) that opposes early phosphorylation events mediated by kinases after TCR activation, E3 ubiquitin ligases such as Casitas b-lineage lymphoma-b (Cbl-b) that ubiquitinate key signaling mediators, such as phosphatidylinositol 3-kinase (PI3K), resulting in proteosome-mediated degradation and diacylglycerol kinases (DGKs), which terminate TCR signaling by metabolizing signaling intermediates such as diacylglycerol (DAG). Cytotoxic T-lymphocyte antigen-4 (CTLA-4), a T-cell surface receptor upregulated after activation, also induces T-cell inhibition, both by sequestering CD80/CD86 away from the activating costimulatory molecule CD28 and by transducing its own inhibitory signals after CD80/CD86 binding. Other well-established inhibitory T-cell surface receptors are programmed death 1 (PD-1), which is expressed under prolonged antigenic stimulation or "exhaustion," and the transforming growth factor β receptor (TGF-β-R), a receptor for one cytokine key for regulatory T-cell-mediated suppression. *IP_3*, Inositol 1,4,5-trisphosphate; *PA*, phosphatidic acid; *PIP_2*, phosphatidylinositol-(4,5)-bisphosphate; *PLCγ1*, phospholipase Cγ1.

and *PTPN22*, align with susceptibility to human immune-mediated disorders. These intriguing findings are being pursued actively by researchers in a number of laboratories to uncover the molecular basis of how these phosphatases exert their control on immune cell function.

Cytotoxic T Lymphocyte Antigen-4

A second strategy to limit T-cell activity is through the induced expression and activation of inhibitory cell surface receptors, such as cytotoxic T lymphocyte antigen-4 (CTLA-4). As discussed earlier, activation of T cells requires two independent signals, one through the TCR and a second through a costimulatory receptor such as CD28. Two to five days after initial T-cell activation, however, another member of the CD28 superfamily, CTLA-4, becomes upregulated on T cells. CTLA-4 differs from CD28 in that, instead of serving as an essential costimulatory receptor, engaged CTLA-4 actively interferes with T-cell activation. Moreover, CTLA-4 binds CD80 and CD86 with much higher affinity than CD28, thus sequestering these key ligands away from CD28. CTLA-4 likely functions to limit T-cell co-stimulation in the presence of minute amounts of CD28 ligand unlikely to have resulted from bacterial or viral-mediated activation of innate immune responses. The importance of CTLA-4 in controlling immune reactions was highlighted in the study of CTLA-4-deficient mice, which were found to die as a result of autoimmune disease at 3 to 4 weeks of age.[11] Targeting CTLA-4 with blocking antibodies to augment T-cell responses is a new therapeutic strategy for human cancer treatment (see below). Conversely, providing soluble CTLA-4 to patients with autoimmunity has been shown to be effective at blocking T-cell activation, presumably by acting as a competitive antagonist and interfering with the ability of CD28 to bind to its ligands, resulting in an anergizing signal to T cells.

E3 Ubiquitin Ligases

TCR signaling is also limited through the targeted destruction of proteins required for TCR signal transduction. E3 ubiquitin ligases are a class of proteins that target intracellular proteins for degradation by the proteasome, the large multisubunit cytosolic complex essential for protein turnover. In T cells, several E3 ubiquitin ligases target components of TCR signal transduction for degradation after TCR activation. These include Casitas b-lineage lymphoma-b (Cbl-b), c-Cbl, and Itch, among many others.[12] As with other negative modulators of TCR signaling, genetic deletion of E3 ubiquitin ligases, either alone or in combination, results in dysregulation of immune function or the development of frank autoimmune disease in mice. The targeted degradation of crucial signaling modulators after T-cell activation thus serves as an additional physiologic mechanism to limit T-cell responses.

Diacylglycerol Kinases

Intrinsic cellular components limit T-cell activity through degradation of second messengers of T-cell signal transduction, such as metabolism of DAG by diacylglycerol kinases (DGKs). As described above, engagement of the TCR results in the activation and recruitment of phospholipase C gamma 1 (PLCγ1) that cleaves PIP_2 into the second messengers DAG and IP_3. DAG levels are regulated in T cells through the activity of DGKs that metabolize DAG to terminate its ability to transduce signals. Two DGK isoforms, DGK-α and DGK-ζ, are important for limiting TCR signaling, as deletion of either in mice results in enhanced proliferation and cytokine production after TCR stimulation. Moreover, deletion of DGK-α leads to impaired induction of T-cell anergy, presumably by augmenting function of Ras-dependent second messengers. Mice deficient in either isoform of DGK do not develop overt autoimmune disease, likely because of some biochemical redundancy between the isoforms, and, in the case of DGK-ζ, enhanced numbers of tTregs. However, enhanced functional responses to viral infection and tumors have been reported in DGK-deficient T cells, defining an important role for DGKs in limiting immune responses.

Limitation of T-Cell Activity From Cell-Extrinsic Components

Extrinsic factors also help limit the function and activation state of T cells. The predominant influences of T cells in this respect are inhibitory cytokines that bind cell surface receptors and influence transcriptional changes that favor decreased activation. Two cytokines that serve as a paradigm for understanding cytokine-mediated inhibition of T cells are IL-10 and TGF-β.

IL-10 is a major negative regulator of immune effector function. Its central role is underscored by the fact that pathogenic viruses, such as cytomegalovirus and Epstein-Barr virus, use homologs of IL-10 to subvert immunologic activity and create environments more favorable for viral spread and replication. IL-10 is produced by both innate and adaptive immune cells in response to activation. As with other cytokines, binding of IL-10 to the IL-10 receptor induces signaling through JAKs, resulting in the nuclear translocation of STAT proteins and the implementation of a transcriptional program that results in decreased expression of inflammatory cytokines and in antagonism of crucial signaling molecules.

IL-10 exerts broad changes within the immune system. In monocytes, IL-10 decreases the production of inflammatory mediators and antigen presentation. In T cells, the effects of IL-10 are generally inhibitory, resulting in decreased capacity for proliferation and a decreased capacity to secrete cytokines. These effects vary by T-cell subtype, however, as IL-17 secretion by Th17 cells is not impaired in the presence of IL-10. As in other proteins important in the negative regulation of T cells, *IL10* germline deletion often results in fatal autoimmunity, in this case a gastrointestinal disease resulting from the inability to control inflammation caused by commensal bacteria.

TGF-β is a pleiotropic cytokine with functions in many organ systems that acts as a potent inhibitor of immune responses. As noted, TGF-β is important both in its capacity to upregulate the transcription factor FoxP3, required for the generation of Tregs, and in inducing more global changes that favor immunosuppression. TGF-β binds its cell surface receptor complex and subsequently induces the phosphorylation, activation, and nuclear transport of intracellular effector Smad proteins (Smad 2/3) proteins. Effector Smad proteins exert their effects by directly coordinating transcriptional programs that inhibit immune responsiveness. Like IL-10, TGF-β acts on numerous cell types. It has been shown to inhibit the differentiation of effector Th cells; induce the conversion of naive T cells into Tregs; suppress the proliferation and production of IL-2 by T cells; and inhibit the activity of macrophages, DCs, and APCs. Mice lacking TGF-β1 develop autoimmune-mediated multiorgan failure and die shortly after birth, underscoring the important role that this molecule plays in attenuating immune reactions.

Terminating Immune Responses After Pathogen Clearance

The simplest way in which T-cell responses end following clearance of a pathogenic challenge is by the removal of antigen, which limits the perpetuation of T-cell activation and abrogates the recruitment of new effector cells. Effector functions of those T cells that were stimulated to respond to the pathogen challenge also diminish as the inhibitory mechanisms described above exert their effects. However, immune homeostasis also requires that the majority of those T cells that emerged from the clonal expansion of antigen-stimulated cells (at its peak representing several percent of the hosts' T-cell pool) be eliminated, retaining only a small population of memory T cells responsive to the inciting antigens. Elimination of the expanded population occurs through activation-induced cell death (AICD).

AICD is initiated when CD95 (also called Fas), a T-cell surface receptor present on the activated effector cells, is engaged by its ligand

(CD95 ligand), expressed on multiple immune cells, including the activated cells themselves. CD95 is a member of the TNF family of receptors and, when stimulated, recruits the adapter molecule Fas-activating via death domain (FADD). FADD creates a multimolecular complex that triggers the activation of several intracellular caspases that induce DNA damage and apoptosis of the effector T cell. During T-cell activation, both CD95 and CD95 ligand are upregulated on the surface of the cell, and all of the machinery is present to initiate AICD. Hence the default pathway for activated T cells is apoptosis, an event that is blocked when T cells are appropriately stimulated to respond to antigen. Once antigen is cleared and the stimulatory events cease, AICD takes over, reducing the expanded population of cells.

Experiments of nature have taught us much about the biology and importance of both CD95 and CD95 ligand. Loss of these proteins as well as components of their signaling machinery results in the human disease autoimmune lymphoproliferative syndrome (ALPS).[13] ALPS is characterized by massive enlargement of lymphoid organs, autoimmune cytopenias, and an increased risk of hematologic malignancy.

THERAPEUTIC MANIPULATION OF T-CELL-MEDIATED IMMUNITY

A comprehensive description of the myriad ways in which the manipulation of T cells has led to important clinical advances is beyond the scope of this chapter. Thus, only a subset of the ways in which an enhanced understanding of the molecular basis for T-cell-mediated immunity has resulted in changes in clinical practice is described here. Many human diseases are related to T-cell dysfunction, both in cases of overexuberant immune responses, as in autoimmune diseases and rejection of transplanted organs, and in insufficient immune responses, as in the case of some chronic infections and in uncontrolled malignancy. Here, we briefly address T-cell responses in graft rejection and in malignancy as paradigms for how T-cell immunity can be modulated therapeutically.

Modulating T Cells to Permit Allograft Transplantation

The success of solid organ transplant depends greatly on the ability to control the immune response of the recipient against the donor organ. Donor tissues express foreign MHC alleles and other proteins to which endogenous T cells have not been exposed (and therefore tolerized against) during thymic development, and thus these tissues serve as potential targets for T-cell-mediated destruction. Initially, the only medications capable of permitting graft survival were high-dose steroids, medications with potent effects in essentially all organ systems and with severe side effects not limited to the immune system. Subsequently, however, several classes of medications were identified that act more specifically on T cells, first cyclosporine and then tacrolimus and sirolimus. These agents target the IL-2 axis: cyclosporine and tacrolimus inhibit IL-2 transcription, and sirolimus inhibits mammalian target of rapamycin (mTOR), which is critical for facilitating IL-2 signal transduction. Because T cells, depending on the treatment modality, are unable to either produce IL-2 or respond to IL-2, they fail to proliferate, despite conditions favorable for stimulation, leading to impaired T-cell-mediated immunity and improved survival of transplanted organs.

Cyclosporine and rapamycin were originally identified in screens of compounds that interfered with immune cell function, and their mechanism of action was discerned only after much of the basic biology of T-cell activation was understood. Other agents currently in use in the clinic were designed precisely because of insights that emerged from studies probing the molecular basis of immune cell function. For example, antibodies directed against CD3 are potent T-cell inhibitors and are now used in the setting of acute solid organ transplant rejection. Similarly, blocking the IL-2 receptor with monoclonal antibodies prevents IL-2 receptor signaling and thus abrogates division of stimulated T cells, thereby quelling T cell–mediated immune destruction.

Given the importance of co-stimulation for T-cell activation and the success in interfering with CD28 signaling in various autoimmune disorders, recent studies have demonstrated efficacy in transplantation with blockade of the CD28/CD80-CD86 interaction using soluble CTLA-4 as a competitive inhibitor of the interaction between CD28 and CD80/CD86. A soluble CTLA-4 fusion protein has been approved for use in the setting of transplant rejection. Additional studies are in progress to examine ways in which modulation of other costimulatory receptors, alone or in combination with soluble CTLA-4, may be used to preserve allografts. As our understanding of how different T-cell subsets are induced is becoming more precise, new therapeutics are on the horizon that are being designed to redirect immune responses by changing the balance of the various effector subsets that emerge as the recipient responses to the transplanted organ. Additional agents directed against receptors and signaling molecules discovered to be key for T-cell activation are currently being tested for clinical efficacy and safety and likely will soon be available to block T-cell responses in the setting of solid organ transplant.

Manipulating T Cells to Improve Activity Against Malignancy

In contrast to the need to impede immune responses following organ transplant, in the setting of malignancy, the goal is to enhance T-cell activity. T cells face several hurdles in their response to spontaneous malignancy. First, they must recognize peptides and proteins that are unique to tumor tissue. These include oncogenic mutant proteins, fusion proteins that may have formed during the course of tumor development or aberrantly expressed embryonic proteins that result from altered transcription often found in malignant tissue (see Chapter 25). Apart from generation of aberrant peptides, these peptides also require efficient presentation by MHC on the cell surface to permit recognition by T cells. Second, T cells must overcome the limited co-stimulation provided by tumor cells. Because tumor cells originate from normal host tissue, they fail to generate the bacterial or viral products crucial for activating APCs, although pathogen-independent inflammation plays a significant role in the immunogenicity of some cancers, such as melanoma and cancer of the bladder. Third, T cells must overcome the generally immunosuppressive microenvironment within tumor tissue, which may include an abundance of TGF-β, Tregs, immunosuppressive macrophages, and/or the induction of an anergy-like state.

The first successful approach to enhance T-cell-mediated responses to tumors also makes use of the biology of CTLA-4 (Fig. 24.11). In this case, however, instead of using soluble CTLA-4 as an agent to inhibit T-cell responses by interfering with co-stimulation, antibodies against CTLA-4 are used to block the ability of CTLA-4 expressed on activated T cells to inhibit T-cell function. Phase III studies have shown that CTLA-4 blocking antibodies may prolong T-cell activation in response to malignancy, and their use has resulted in long-term disease remission in approximately 15% of patients with metastatic melanoma, a previously uniformly fatal disease. Some antibodies against CTLA-4 may also function by depleting Tregs from immune organs and the tumor microenvironment. Whether CTLA-4 antibodies mediate their effect by inducing the expansion of newly activated tumor-specific cells or by eliminating Tregs from the immunosuppressive microenvironment continues to be studied; however, one major concern of using antibodies against CTLA-4 has been the generation of severe autoimmune colitis in a significant fraction of patients, and poor tolerance in older patients, which constitutes a significant fraction of cancer patients.

The initial success achieved by blocking CTLA-4 on the surface of T cells led to the search for other molecules that might similarly be targeted with blocking antibodies. One such candidate molecule identified was PD-1, the inhibitory receptor present on activated and exhausted T cells. Because many, if not all, patients with

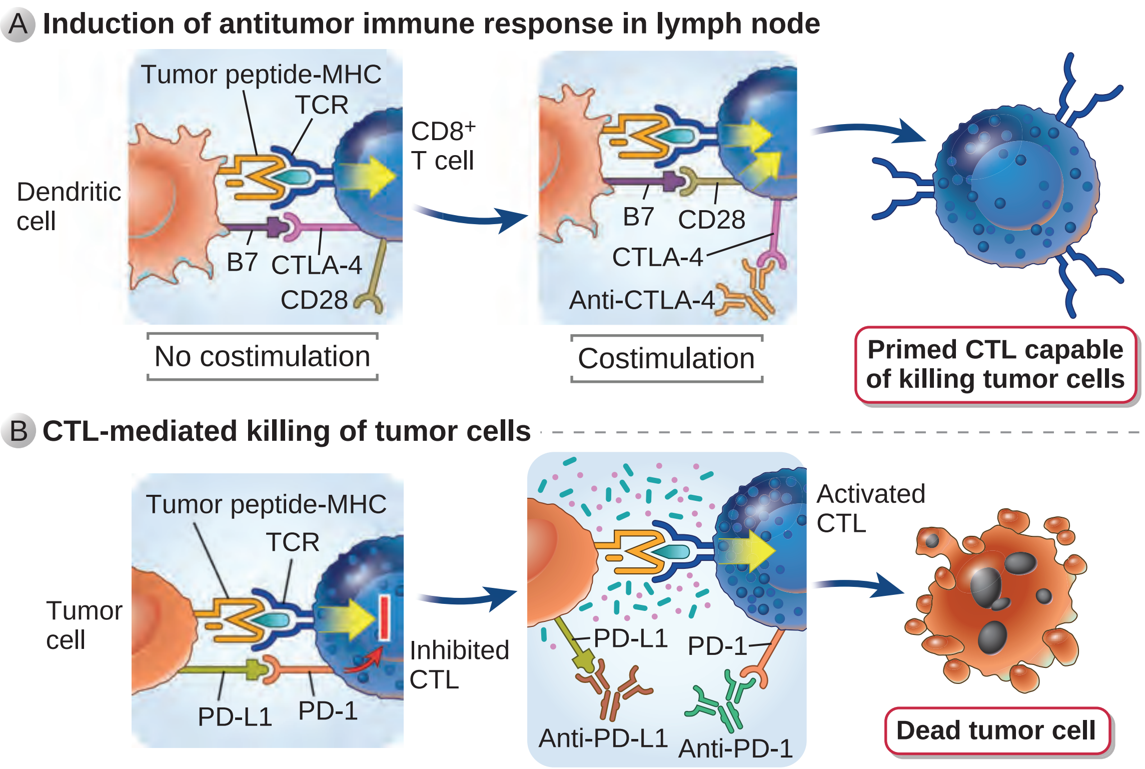


Figure 24.11 IMMUNE CHECKPOINT BLOCKADE. Cancer patients often mount ineffective T-cell responses to their tumors, thus recent advances in immunotherapies manipulate T-cell activity to promote antitumor immune responses. Upregulation of inhibitory receptors such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death 1 (PD-1) on the tumor-specific T cells, and expression of the ligand PD-L1 on the tumor cells, blunts tumor-specific T-cell responses. Blocking anti-CTLA4 antibodies (*top*) or anti-PD-1 or anti-PD-L1 antibodies (*bottom*) are highly effective in treating several types of advanced tumors, by releasing the inhibition of tumor-specific T cells by these molecules. Anti-CTLA-4 may work by blocking CTLA-4 on effector T cells (shown) or on Tregs. *(From Abbas AK, Lichtman AH, Pillai S, Baker DL, Baker A. Cellular and Molecular Immunology. 9th ed. Philadelphia: Elsevier; 2017.)*

cancer have circulating T cells capable of binding tumor antigen, albeit with limited responsiveness, it was speculated that relieving exhaustion of tumor-specific T cells with antibodies that block PD-1 would permit improved T-cell responses against malignancy. In fact, therapies targeting PD-1 and its ligand have demonstrated profound activity in patients with malignancy, with overall survival rates as high as 35% in patients with advanced melanoma, and activity in many other cancers, such as highly mutated colorectal cancer, classical Hodgkin lymphoma, lung and bladder cancers.[14] Surprisingly, in studies described to date, autoimmune disease occurs much less frequently in patients treated with antibodies targeting PD-1 versus those targeting CTLA-4. The biologic basis of this finding is unclear, but it suggests that PD-1 represents a superior target to boost T cell–mediated activity against tumors. Emerging research has identified factors that correlate with higher response rates to anti-PD-1 therapies. These include high levels of expression of PD-L1 on tumor cells (as in Hodgkin lymphoma), high mutational burden (as in highly mutated colorectal cancer), and the presence of significant immune infiltrate (as in bladder cancer). Further research using next-generation sequencing of immune cell infiltrates should identify additional biocorrelates that can better refine choices of immunotherapies. Apart from CTLA-4 and PD-1, other potential targets include exhaustion receptors LAG-3 and Tim3, and certain isoforms of the epacadostat receptor on T cells. Ideal efficacy will likely result by combining immunotherapies. For instance, simultaneous targeting of CTLA-4 and PD-1 results in an approximate 70% response rate and 50% five-year survival in melanoma, and 42% response rate in renal cell carcinoma. The combination therapy is approved as first-line therapy in both diseases.

In addition to targeting inhibitory receptors on T cells to augment antitumor responses, another major approach for using T cells as anti-cancer therapy has been through engineering T cells to directly target tumor antigens. Knowledge gained through fundamental studies of proximal signaling events important for T-cell activation has led investigators to engineer chimeric antigen receptors (CARs), which permit direct activation of T cells by tumor cells.[15] These "designer" molecules have a modular structure: an extracellular binding domain for antigens on tumor cells, transmembrane domains from CD8a or other cell surface proteins, cytoplasmic signaling components of the ζ chain of the TCR complex, and a costimulatory domain(s) containing other key activating co-receptors (Fig. 24.12). T cells are removed from patients, genetically engineered to express CARs, and reintroduced into patients with the anticipation that these T cells will engage the tumor through the CAR, resulting in T-cell activation. These activated T cells, when effective, generate robust antitumor responses, bolstering antitumor immunity sufficiently to eliminate the cancer.

One of the most well-studied tumor antigens selected for CAR-T-cell therapy is CD19, a cell surface costimulatory receptor found exclusively on B cells. In patients with refractory B-cell leukemias and lymphomas, treatment with CD19-directed CAR-T cells have demonstrated striking success, with a significant proportion of patients achieving complete and durable remission. As predicted from selection of CD19 as the cellular target, these patients also develop B-cell aplasia and hypogammaglobulinemia resulting from the elimination of healthy B cells by CAR-T cells; however, this long-term side effect can be effectively managed by antibody infusions or coupling CAR-T-cell therapy with subsequent bone marrow transplantation. Identification of appropriate target antigens for CAR-T cells is a key challenge because many tumor antigens may also be expressed on normal tissues and "on-target, off-tumor" effects of CAR-T cells could potentially lead to unacceptable toxicity. These concerns will require careful evaluation of each CAR targeting domain for both efficacy against the tumor and potential deleterious effects on normal tissues. Currently,

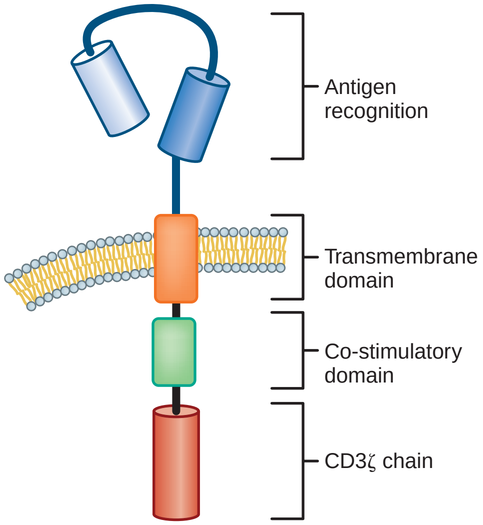


Figure 24.12 CHIMERIC ANTIGEN RECEPTOR T CELLS. The modular design of successful chimeric antigen receptors (CARs) uses the knowledge gained through the study of fundamental properties of antigen recognition and signaling pathways in immune cells. The extracellular antigen recognition domain is typically derived from a single-chain variable fragment of an antibody specific for a surface antigen expressed by the tumor cells. This domain is coupled to a transmembrane domain, which has been derived from either CD8 or CD28 molecules. The CAR transmits an activation signal through the costimulatory domains and the CD3ζ chain to intracellular T-cell signaling pathways. The costimulatory domain contains one (or more) signaling domains derived from costimulatory molecules, including CD28, CD27, 4-1BB, and inducible costimulator (ICOS). This costimulatory domain significantly augments signaling from the CD3ζ chain and has been shown to improve CAR-T-cell function, proliferation, and persistence.

trials are underway testing multiple targets in hematologic malignancies (see Chapter 26) and solid tumors. Additionally, it has been observed that certain CD19+ tumors can develop resistance to CAR-T cells by downregulation of CD19. Thus, newer CAR-T cell therapies in B cells engineer T cells to recognize two or more B-cell antigens.

Apart from identifying the best targets for CARs, much effort has also been placed on optimal construction of the CAR signaling domains. First-generation CARs, which contain only the ζ chain of the TCR complex, led to suboptimal antitumor responses. The incorporation of an additional intracellular signaling domain derived from costimulatory molecules, such as CD28 or 4-1BB, into second-generation CARs augmented CAR-T-cell activation and antitumor efficacy, and third-generation CARs include signaling domains from two costimulatory receptors. Currently, most CARs adhere to the second-generation model. Which intracellular costimulatory domains will work best in CARs and how many costimulatory domains are required for optimal T-cell activation and antitumor efficacy are under active investigation. Other outstanding questions in the biochemistry of CARs concern the optimal number of functional ITAMs present in ζ chain domains and the length and composition of the transmembrane hinge domain and interdomain junctions. Defining the biochemistry and signal transduction of CARs should permit broadening its use to more common malignancies.

A major side effect of CAR-T-cell therapies has been the development of cytokine release syndrome (CRS). Rapid lysis of target cells can result in overexuberant inflammation that results in systemic pathology including vasodilation and hypotension, multi-organ failure, and neurologic changes. Early identification of CRS and appropriate intervention with steroids and antibodies that target IL-6 and TNFα are capable of blunting many sequelae of CRS (see Chapter 96 for more details).

A third approach for directing T-cell responses against malignancies is with Bispecific T-cell Engagers (BiTEs). BiTEs are fusion proteins containing two antibody recognition domains – one that recognizes the CD3 complex on T cells, and a second that recognizes a receptor present on tumor cells. BiTE binding to T cells in the absence of tethering to a tumor surface protein is insufficient to generate T-cell activation. However, dual binding of BiTEs to a tumor antigen and T cells in close proximity result in activation of cytotoxic T-cell and tumor cell lysis, independent of the TCR specificity of the T cell. In some B-cell malignancies, BiTE therapies have demonstrated efficacy similar to CAR-T cells, and do not require T-cell engineering. Similar to CAR-T-cell therapies, CRS remains a significant hurdle.

The examples presented here are only a small subset of novel approaches in use or being tested to modulate immune cell function based upon our understanding of the molecular basis of T-cell activation. It is anticipated that as more is learned about the molecules and pathways critical for control of T-cell-mediated immunity, additional new agents with greater efficacy and improved safety profiles will become available for clinical use. The advent of these new therapeutics and their potential to improve treatments for serious human diseases underscore the importance of continued efforts to understand the mechanisms of T-cell development and function.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Anderson MS, Venanzi ES, Klein L, et al. Projection of an immunological self shadow within the thymus by the aire protein. *Science*. 2002;298(5597):1395–1401.

Chan AC, Iwashima M, Turck CW, Weiss A. ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell*. 1992;71(4):649–662.

Clements JL, Yang B, Ross-Barta SE, et al. Requirement for the leukocyte-specific adapter protein SLP-76 for normal T cell development. *Science*. 1998;281(5375):416–419.

Day CL, Kaufmann DE, Kiepiela P, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature*. 2006;443(7109):350–354.

Dembic Z, Haas W, Weiss S, et al. Transfer of specificity by murine alpha and beta T-cell receptor genes. *Nature*. 1986;320(6059):232–238.

Dustin ML. The immunological synapse. *Cancer Immunol Res*. 2014;2(11):1023–1033.

Esensten JH, Helou YA, Chopra G, et al. CD28 Costimulation: from mechanism to therapy. *Immunity*. 2016;44(5):973–988.

Irving BA, Weiss A. The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell*. 1991;64(5):891–901.

June CH, O'Connor RS, Kawalekar OU, et al. CAR T cell immunotherapy for human cancer. *Science*. 2018;359(6382):1361–1365.

Li X, Gong L, Gu H. Regulation of immune system development and function by Cbl-mediated ubiquitination. *Immunol Rev*. 2019;291(1):123–133.

Ohkura N, Kitagawa Y, Sakaguchi S. Development and maintenance of regulatory T cells. *Immunity*. 2013;38(3):414–423.

Rieux-Laucat F, Magerus-Chatinet A, Neven B. The Autoimmune Lymphoproliferative Syndrome with Defective FAS or FAS-Ligand Functions. *J Clin Immunol*. 2018;38(5):558–568.

Shah DK, Zuniga-Pflucker JC. An overview of the intrathymic intricacies of T cell development. *J Immunol*. 2014;192(9):4017–4023.

Waterhouse P, Penninger JM, Timms E, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science*. 1995;270(5238):985–988.

Wei SC, Duffy CR, Allison JP. Fundamental mechanisms of immune checkpoint blockade therapy. *Cancer Discov*. 2018;8(9):1069–1086.

REFERENCES

1. Dembic Z, Haas W, Weiss S, et al. Transfer of specificity by murine alpha and beta T-cell receptor genes. *Nature*. 1986;320(6059):232–238. https://doi.org/10.1038/320232a0.
2. Irving BA, Weiss A. The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell*. 1991;64(5):891–901. https://doi.org/10.1016/0092-8674(91)90314-o.
3. Chan AC, Iwashima M, Turck CW, Weiss A. ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell*. 1992;71(4):649–662. https://doi.org/10.1016/0092-8674(92)90598-7.
4. Clements JL, Yang B, Ross-Barta SE, et al. Requirement for the leukocyte-specific adapter protein SLP-76 for normal T cell development. *Science*. 1998;281(5375):416–419. https://doi.org/10.1126/science.281.5375.416.
5. Esensten JH, Helou YA, Chopra G, et al. CD28 Costimulation: from mechanism to therapy. *Immunity*. 2016;44(5):973–988. https://doi.org/10.1016/j.immuni.2016.04.020.
6. Dustin ML. The immunological synapse. *Cancer Immunol Res*. 2014;2(11):1023–1033. https://doi.org/10.1158/2326-6066.CIR-14-0161.
7. Shah DK, Zuniga-Pflucker JC. An overview of the intrathymic intricacies of T cell development. *J Immunol*. 2014;192(9):4017–4023. https://doi.org/10.4049/jimmunol.1302259.
8. Anderson MS, Venanzi ES, Klein L, et al. Projection of an immunological self shadow within the thymus by the aire protein. *Science*. 2002;298(5597):1395–1401. https://doi.org/10.1126/science.1075958.
9. Ohkura N, Kitagawa Y, Sakaguchi S. Development and maintenance of regulatory T cells. *Immunity*. 2013;38(3):414–423. https://doi.org/10.1016/j.immuni.2013.03.002.
10. Day CL, Kaufmann DE, Kiepiela P, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature*. 2006;443(7109):350–354. https://doi.org/10.1038/nature05115.
11. Waterhouse P, Penninger JM, Timms E, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science*. 1995;270(5238):985–988. https://doi.org/10.1126/science.270.5238.985.
12. Li X, Gong L, Gu H. Regulation of immune system development and function by Cbl-mediated ubiquitination. *Immunol Rev*. 2019;291(1):123–133. https://doi.org/10.1111/imr.12789.
13. Rieux-Laucat F, Magerus-Chatinet A, Neven B. The Autoimmune Lymphoproliferative Syndrome with Defective FAS or FAS-Ligand Functions. *J Clin Immunol*. 2018;38(5):558–568. https://doi.org/10.1007/s10875-018-0523-x.
14. Wei SC, Duffy CR, Allison JP. Fundamental mechanisms of immune checkpoint blockade therapy. *Cancer Discov*. 2018;8(9):1069–1086. https://doi.org/10.1158/2159-8290.CD-18-0367.
15. June CH, O'Connor RS, Kawalekar OU, et al. CAR T cell immunotherapy for human cancer. *Science*. 2018;359(6382):1361–1365. https://doi.org/10.1126/science.aar6711.

CHAPTER 25

UNMODIFIED EX VIVO EXPANDED T CELLS

Ifigeneia Tzannou, Wingchi Leung, and Premal Lulla

T lymphocytes have the natural ability to destroy viral-infected or tumor target cells by a range of mechanisms that are initiated upon recognition of target peptides presented by histocompatibility antigens. Coupled with their ability to traffic through multiple tissues and self-renew, these properties make T lymphocytes an appealing cell-type for adoptive immunotherapy of cancer. Allogeneic hematopoietic stem cell transplantation (HSCT) is enduring "proof-of-principle" for adoptive immunotherapy with unmodified T cells. The introduction into an allogeneic recipient of an unselected donor T-cell repertoire by HSCT or through donor lymphocyte infusions (DLIs) induces a potent "graft-versus-tumor (GvT)" immune effect that can cure patients of their primary malignancy. However, this effect is non-specific and is frequently accompanied by life threatening "graft-versus-host disease (GVHD)." Additionally, as our understanding of the molecular basis of immunotherapy has increased, we have uncovered many pathways of tumor-mediated immune evasion that must be overcome for T cell-based immunotherapies to be curative.

In this chapter, we review ongoing efforts to refine the antigen-specificity of adoptively transferred unmodified T cells (Fig. 25.1), as well as strategies to overcome tumor-mediated inhibition of immunotherapy with these T cells (Fig. 25.2).

ADOPTIVE IMMUNOTHERAPY OF VIRAL INFECTIONS WITH VIRUS-SPECIFIC T CELLS

Viral infections with latent (e.g., cytomegalovirus [CMV], Epstein–Barr virus [EBV], BK virus [BKV]) and community-acquired respiratory viruses cause substantial morbidity and mortality in the HSCT setting. Since antiviral drugs are not always effective, investigators found that adoptive transfer of virus-specific T cells (VSTs) derived from the original stem cell donor, or more recently from "third-party" allogeneic donors, can control these infections (Table 25.1).

Methods to Generate Virus-Specific T Cells

Rapid Selection

Two rapid selection approaches to generate VSTs have been clinically tested to date. In the first approach, multimers are used to capture epitope-specific memory T cells from donors, while in the second donor T cells that secrete interferon gamma (IFNγ) or express activation markers like 4-1BB after exposure to viral antigens are captured using magnetic beads. The former strategy has been successful in controlling CMV, EBV, and adenoviral (ADV) infections without inducing toxicities, but it selects a monoclonal CD8+ population and is limited to specific human leukocyte antigen (HLA)-haplotypes and epitopes. By contrast, the second approach selects both CD4+ and CD8+ presumably multi-epitope VSTs and also demonstrated efficacy in controlling CMV, EBV, BKV and ADV reactivations post-HSCT. The CliniMACS Prodigy Cytokine Capture System, a closed system for IFNγ selection, was used to generate third-party VSTs that were infused in nine pediatric patients with CMV, EBV, and ADV infections. Seven of these patients achieved a complete response and only one experienced manageable toxicity in the form of mild cytokine release syndrome (CRS).[8] A key limitation of both rapid-selection strategies is the requirement of large volumes of blood (usually a leukapheresis product) from which a relatively small number of VSTs can be isolated, sufficient for only one dose.

Ex Vivo Expansion

To overcome the small cell numbers generated by rapid selection, investigators have explored expanding VSTs ex vivo by stimulating them with engineered antigen-presenting cells (APCs) that express viral peptides or EBV-transformed lymphoblastoid cell lines (LCLs). Pioneering work from Fred Hutchinson Cancer Research Center and St. Jude Children's Research Hospital demonstrated the feasibility of this method. Investigators prepared CMV- and EBV-reactive T cells, respectively, for clinical use and found them effective as prophylaxis and treatment of active infections. However, the manufacturing process was laborious, requiring prolonged cultures over multiple weeks.[8,18] Efforts have since focused on simplifying manufacture, leading to the development of a 10-day process where clinical grade VSTs are generated by peptide library stimulation and peripheral blood mononuclear cells (PBMCs) serve as APCs. These rapidly expanded VSTs simultaneously targeting EBV, CMV, ADV, BKV, and human herpes virus-6 (HHV6) produced a 94% response rate without any increase in toxicity in a phase I study at Baylor College of Medicine.[13]

Off-the-Shelf T-Cell Banks

Banks of VSTs that are prospectively manufactured from seropositive transplant eligible donors and available for urgent administration as an "off the shelf" product represents a major step toward broader implementation of this therapy. Haque et al. first used partially HLA-matched VSTs to treat EBV-lymphoproliferative disease (LPD) in solid organ and HSC transplant recipients. Researchers found that, despite the HLA disparity, third-party VSTs could effectively treat infections without increasing the risk of GVHD.[12] Other groups in larger trials confirmed these proof-of-concept observations using banked VSTs with multiple specificities, including CMV, EBV, ADV, BKV, John Cunningham (JC), and HHV6, with response rates ranging from 74% to 93% (see Table 25.1).[19] To be broadly applicable, a key feature of this approach is to develop a bank that can encompass diverse HLA-types. To this end, a recent study demonstrated that a bank of only eight carefully selected donors with diverse HLA-types could supply over 95% of all patients with a third-party VST product, while a scale-up expansion protocol could provide more than 2000 cell doses from just one donor.[20] Taken together, VSTs have demonstrated the capacity to effectively control viral infections/reactivations, even with allogeneic and partially HLA-matched VST products.

ADOPTIVE IMMUNOTHERAPY OF CANCER

Antigen-specific T cells have been used as cancer therapies by targeting (i) viral peptides in virus-associated malignancies, (ii) germline antigens that have limited expression on normal tissues (e.g., cancer-testis antigens [CTAs]), and (iii) cancer mutation derived neoantigens. We summarize the clinical outcomes of selected clinical trials using unmodified antigen-specific T-cell immunotherapy in Table 25.2.

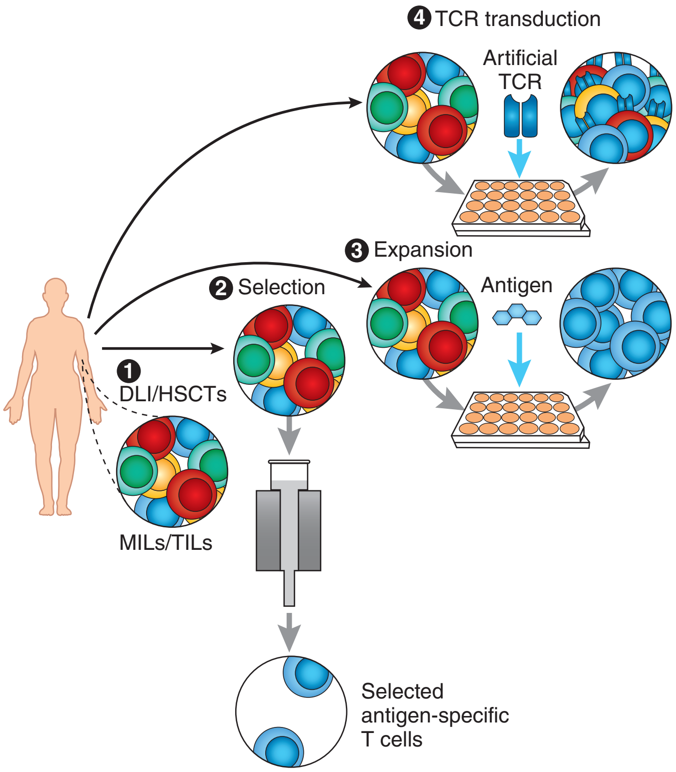


Figure 25.1 TYPES OF NON-CAR-BASED ADOPTIVE T-CELL THERAPY. (1) Antigen agnostic approaches. Donor lymphocyte infusions (*DLI*), hematopoietic stem cell transplantation (*HSCT*), tumor infiltrating lymphocytes (*TIL*) or marrow infiltrating lymphocytes (*MIL*) . (2) Antigen-driven selection of reactive T cells. (3) Antigen-driven ex vivo expansion of reactive T cells. (4) Transduction of an artificial, antigen-reactive T cell receptor (*TCR*).

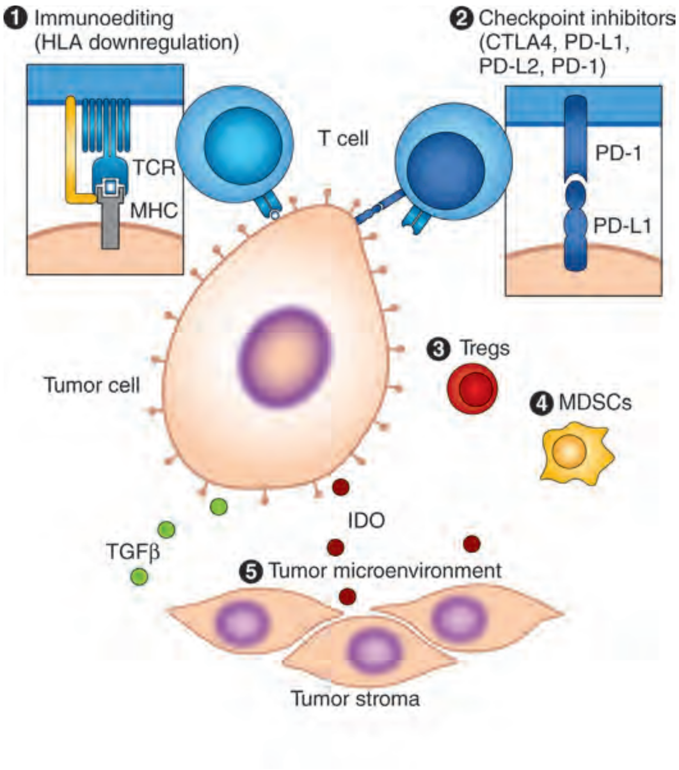


Figure 25.2 EMERGING CHALLENGES TO ADOPTIVE T-CELL IMMUNOTHERAPY. (1) Immunoediting of target-antigen presentation. (2) Overexpression of immune checkpoints. (3) Proliferation of regulatory T cells (*Tregs*). (4) Proliferation of myeloid derived suppressor cells (*MDSCs*). (5) Immunosuppressive factors within the tumor microenvironment (e.g., stromal cells, transforming growth factor-β [*TGFβ*], indoleamine 2,3-dioxygenase [*IDO*], etc.). *MHC*, Major histocompatibility complex; *TCR*, T-cell receptors.

Adoptive Immunotherapy of Virus-Associated Malignancies

Viruses cause approximately 12% of cancers. These cancers express viral oncoproteins that can serve as targets for adoptive cell therapy. Early successes with adoptive immunotherapy with EBV–VSTs as prophylaxis and treatment of post-transplant LPD, which arises in immunocompromised patients, led to the extension of this strategy to other EBV+ tumors (lymphoma and nasopharyngeal cancer) that develop in immunocompetent patients. Unlike EBV-LPD, which expresses a diverse array of viral latency antigens, including the highly immunogenic EBNA3 and EBNA2, other EBV+ tumors are less immunogenic and express few poorly processed (EBNA1) or weakly stimulatory (latent membrane protein: LMP1 and LMP2) EBV-derived antigens. Nevertheless, infusion of polyclonal LMP2-specific $CD4^+$ and $CD8^+$ T cells[21] or $CD8^+$ LMP2-specific T cells (restricted to HLA-A2 and A24 only) increased EBV peptide-specific T-cell responses and led to sustained complete tumor regression in patients with a diverse array of relapsed/resistant EBV+ lymphoma. A multicenter cooperative group phase III clinical trial is ongoing to assess the efficacy of autologous EBV-specific T cells for the treatment of refractory EBV+ lymphomas.

In order to increase the antitumor activity of EBV-directed T cells, a separate trial infused EBV-specific T cells genetically engineered to overcome transforming growth factor-β (TGFβ)-mediated T-cell inhibition to seven patients with EBV+ lymphomas. Of these, four had a sustained response with persisting LMP-specific T cells for more than 4 years following infusion.[35] Thus, genetic modifications of these cells appear safe and may be necessary to overcome the suppressive tumor microenvironment and increase T-cell activity in immunocompetent patients.

Adoptive Immunotherapy of Virus-Independent Malignancies

For malignancies not associated with viruses, T cells must target alternative antigens to achieve clinical responses. A wide variety of germline "tumor-associated antigens (TAAs)" or mutated "neoantigens" are suitable T-cell targets due to qualities such as tumor-exclusive expression or overexpression, T-cell immunogenicity, and the association between endogenous responses to these antigens and clinical outcomes. Compared to VSTs, manufacturing TAA-specific T cells is limited by physiologic and pathologic barriers. Physiologically, the vast majority of T cells with high affinity for self-antigens are deleted during T-cell development, while those that persist are frequently anergic because of the highly immunosuppressive tumor environment. Thus, ex vivo expansion strategies using single peptide/antigen expressing stimulator cells in culture result in considerable variability in the anti-tumor function of manufactured products. To overcome this limitation, investigators have taken the approach to (i) harvest tumor infiltrating lymphocytes (TILs) that have been shown to be enriched for TAA-reactive T cells and expand them non-specifically; (ii) stimulate and expand T cells with specificity for not just single but multiple TAAs, thereby improving the chances of manufacturing a product with antitumor function coupled with broader targeting to address tumor heterogeneity; or (iii) transduce T cells with peptide-specific T-cell receptors (TCRs).

In the clinic, adoptive T-cell approaches have centered on targeting neoantigens or two major sources of non-mutated, non-viral antigens expressed by tumor cells: (1) Mismatched histocompatibility antigens and (2) "cancer-testis" or equivalent antigens, where target peptides are sufficiently foreign to elicit robust T-cell responses.

TABLE 25.1 Selected Published Studies Using Allogeneic Unmodified Virus-Specific T Cells for Viral Infections

Publication	Target	N	Donor	Safety	Response
Selection					
Cobbolt (2005)-Tetramer[1]	CMV	9	Donor derived	0	8 CR
Uhlin (2012)-Pentamer[2]	EBV	1	Donor derived,[2]	0	EBV: 1 CR
	ADV	1	Third party[3]		ADV: 1 NR
	CMV	6			CMV: 4 CR
Neuenhahn (2017)-Streptamer[4]	CMV	16	Donor derived,[5] Third party[5]	1 aGVHD 1 cGVHD	9 CR
Feuchtinger (2006)-IFNγ capture[6]	ADV	9	Donor derived	1 GVHD	4 CR
Moosman (2010)- IFNγ capture[7]	EBV	6	Donor derived	0	3 CR
Peggs (2011)-IFNγ capture[3]	CMV	18	Donor derived	8 GVHD	Prophy: 7/7 CCR Treat: 11 CR
Kallay (2017)-IFNγ capture[8]	CMV	3	Third party	1 CRS	CMV: 2 CR
	EBV	2			EBV: 2 CR
	ADV	1			ADV: 1 CR
	CMV+ADV	2			CMV+ADV: 2
	CMV+EBV	1			CR CMV+EBV: 1 CR
Ex Vivo Expansion					
Walter et al. (1995)[5]	CMV	14	Donor derived	7 GVHD	Prophylaxis
Heslop (2010)[9]	EBV	114	Donor derived	8 aGVHD 13 cGVHD	Prophy: 101/101 CCR Treat: 11 CR, 2 deaths
Leen (2006)[10]	EBV	3	Donor derived	0	EBV: 3 CR
	ADV	5			ADV: 5 CR
	CMV	3			CMV: 3 CR
Doubrovina (2012)[11]	EBV	19	Donor derived,[12] Third party[7]	0	13 CR
Papadopoulou (2014)[13]	EBV	5	Donor derived	0	EBV: 5 CR
	ADV	1			ADV: 1 CR
	CMV	3			CMV: 2 CR
	BKV	7			BKV: 5 CR HHV6: 2 CR
	HHV6	2			Prophy: 3/3 CCR
Haque (2007)[12]	EBV	2	Third party	0	2 CR
Vickers (2014)[14]	EBV	11	Third party	1 aGVHD	8 CR
Leen (2013)[15]	EBV	9	Third party	8 aGVHD	EBV: 2 CR
	ADV	23			ADV: 9 CR
	CMV	18			CMV: 7 CR
Tzannou (2017)[16]	EBV	2	Third party	3 de novo GVHD	EBV: 2 CR
	ADV	9		3 GVHD reactivations	ADV: 5 CR
	CMV	19			CMV: 9 CR
	BKV	20			BKV: 6 CR
	HHV6	4			HHV6: 3 PR
Prockop (2020)[17]	EBV	33	Third party	1 aGVHD	19 CR

ADV, Adenovirus; *BKV*, BK virus; *CCR*, continue response; *CMV*, cytomegalovirus; *CR*, complete response; *CRS*, cytokine release syndrome; *EBV*, Ebstein–Barr virus; *GVHD*, graft-versus-host disease (a = acute, c = chronic); *HHV6*, human herpes virus-6; *N*, sample size; *NR*, no response; *PR*, partial response; prophy, prophylaxis; Treat, cells administrated for treatment.

Neoantigens

Neoantigens are novel protein sequences arising from non-synonymous mutations in malignant cells that can be presented by HLA to induce an anti-tumor immune response. Neoantigens arise from translocations (e.g., BCR-ABL1 t(9;22)), point mutations (e.g., KRAS G12D), or insertions/deletions and lead to an alternate reading frame and consequently a novel peptide, rendering them attractive immunotherapeutic targets due to their restricted expression.[36] Furthermore, as neoantigens are considered “foreign,” cognate T cells

TABLE 25.2 Selected Published Studies Using Tumor-Specific T Cells (Through TCR) for Cancer

Publication	Target	Modification	Prior chemo	Donor	Patients	Outcomes	Grade ≥ III AEs
VSTs for Cancer							
Bollard et al. (2013)[21]	LMP1 and 2	Unmodified	None	Autologous	29 lymphomas of varying subtypes in CR and 21 in relapse	29 of 29 sustained CR and 11 of 21 with active lymphoma entered CR	2 with self-limited inflammatory reactions
Eom et al. (2016)[22]	LMP2A-CD8+T cells	Unmodified	None	Autologous	2 with NK/T cell lymphoma,1 DLBCL, 1 Hodgkin	1 CR,1 PR	None
Minor Histocompatibility Antigen (miHA)–and Tumor Associated Antigen (TAA)–Specific T Cells							
Warren et al. (2010)[23]	mHA Ags	Unmodified	None	HSC-donor	8 various leukemias		1 severe pulmonary toxicity
Meij et al. (2012)[24]	HA-1	Unmodified	None	HSC-donor	3 AML	0 of 3 responses	None
Chapuis et al. (2013)[25]	WT1 (HLA-A*02 peptide)	Unmodified	None	HSC-donor	6 AML in CR, 2 in relapse	1 of 2 CRs in relapse group, 3 of 6 eventually relapse in CR group	None
Lulla et al. (2020)[26]	NYESO1, MAGEA4, SSX2, PRAME, Survivin	Unmodified	None	Autologous	12 with MM residual disease and 9 MM in CR	3 of 12 had objective response and 7 of 9 had long-term (>2 year) CR	None
Tawara et al. (2017)[27]	WT1 (HLA-A*24 peptide)	Yes, retro-v transduction with si-RNA for native TCR	None	Autologous	8 AML in relapse	Transient responses only	None
Chapuis et al. (2019)[28]	WT1 (HLA-A*02 peptide)	Yes, lenti-v transduced TCR	Cy/Flu and post-IL-2	HSC-donor	12 AML/MDS in CR	12 of 12 remain in CR (44 months), no durable responses in relapse arm	None
Stadtmauer et al. (2020)[29]	NYESO1	Yes, lenti-v transduced TCR and CRISPR edited (PD-1 knockout)	Cy/Flu	Autologous	2 refractory MM and 1 sarcoma	2 of 3 had SD with reduction in NYESO1 expression	Cytopenias in all treated patients, thought to be chemo related
Neo-STs for Cancer[a]							
Tran et al. (2014)[30]	Mutated ERBB2IP	Unmodified TIL	Post-IL-2	Autologous	1 metastatic cholangiocarcinoma	Tumor regression with prolonged SD. Subsequent PD treated with second dose induced tumor regression	None
Tran et al. (2016)[31]	KRAS G12D	Unmodified TIL	Cy/Flu and post-IL-2	Autologous	1 patient with metastatic colorectal cancer	Initial regression of all 7 pulmonary lesions, later PD due to loss of HLA gene	None
Ott et al. (2017)[32]	Personalized neoantigens	Peptide vaccination with TLR3 agonist	None	N/A	6 patients with surgically resected metastatic melanoma	4/6—no recurrence at 25 months post-vaccination; 2/6—PD but complete regression with pembrolizumab	None
Comoli et al. (2017)[33]	BCR-ABL (p190)	Unmodified	None	Auto and HSC-donor	3 with Ph+ALL in relapse	3 of 3 achieved CR with concurrent ABL kinase inhibitors	

Continued

TABLE 25.2 Selected Published Studies Using Tumor-Specific T Cells (Through TCR) for Cancer—cont'd

Publication	Target	Modification	Prior chemo	Donor	Patients	Outcomes	Grade ≥ III AEs
Zacharakis et al. (2018)[34]	Mutated SLC3A2, KIAA0368, CADPS2 and CTSB)	Unmodified (TIL)	Cy/Flu, pembrolizumab and post-IL2	Autologous	1 patient with refractory metastatic breast cancer	CR > 22 months	Cytopenia during Cy/Flu, hypophosphatemia, febrile neutropenia

[a]Published reports mainly on solid cancers. but hematologic malignancy studies underway.
AE, adverse events; *AML*, acute myeloid leukemia; *BCR-ABL*, fusion of t(9;22); *CR*, complete remission; *Cy/Flu*, cytoreductive chemotherapy with cyclophosphamide and fludarabine; *DLCBL*, diffuse large B-cell lymphoma; *ERBB2IP*, Erbb2 interacting protein; *Flu*, fludarabine; *HA-1*, histocompatibility antigen 1; *HSC*, hematopoeitic stem cell; lenti-v, lentiviral; *LFTs*, liver function tests; *LMP*, latent membrane protein; *MAGEA4*, melanoma-associated antigen 4; *mHA Ags*, minor histocompatibility antigens; *MM*, multiple myeloma; *Neo-STs*, neoantigen-specific T cells; *NYESO1*, New York esophageal squamous cell carcinoma 1; *PD*, progressive disease; *Ph+ALL*, Philadelphia chromosome–positive acute lymphoblastic leukemia; *Post-IL-2*, interleukin-2 injections post cellular therapy; *PR*, partial remission; *PRAME*, preferentially expressed antigen in melanoma; *retro-v*, retroviral; *SD*, stable disease; *SSX2*, synovial sarcoma X2; *si-RNA*, small interfering *RNA*; *TCR*, T-cell receptor; *TIL*, tumor-infiltrating lymphocytes; *TLR3*, Toll-like receptor 3; *VSTs*, virus-specific T cells; *WT1*, Wilms' tumor antigen 1.

clones are likely spared from negative thymic selection, thereby allowing persistence of high affinity TCRs that have enhanced activation and cytotoxicity.

Determining immunogenic antigens and their major histocompatibility complex (MHC) restriction remains challenging but is crucial to developing efficacious neoantigen-directed immunotherapies. Pioneering work by Rosenberg et al. demonstrated the presence of neoantigen-specific T cells that mediate complete responses in patients with a variety of solid tumors (e.g., colon cancer, cholangiocarcinoma, melanoma, breast cancer, etc.) in expanded TILs.[30,31,34] The discovery that relapses following TIL therapy can be attributable to loss of neopeptide presentation is further proof of their immune effect on tumors. Vaccination offers an alternative strategy to target neoantigens. While therapy with TILs is a form of passive immunity which involves the adoptive transfer of ex vivo expanded tumor-directed T cells, vaccinations stimulate activity immunity in endogenous T cells for anti-tumor activity and has been tested in phase I clinical trials for melanoma and glioblastoma.[33] Recent advances in high-throughput genome sequencing uncovered an increasing list of tumor mutations that may encode for neoantigens presented by MHC molecules. While *in silico* algorithms can predict clinically relevant, neoepitope:MHC complexes based on the mutated sequences, mass spectrometry approaches that immunoprecipitate MHC complexes with their peptides are currently being used to design personalized neoantigen targeted immunotherapies, as these techniques can directly identify candidate neoantigens, including those that are modified during translation/transcription of the mutated sequences.[37] In Table 25.2, we describe clinical activity of published reports of neoantigen-specific T cells used as cancer immunotherapy.

Mismatched Histocompatibility Antigens

In the allogeneic setting, alloreactivity toward mismatched major or minor histocompatibility antigens (miHA) on recipient tissues are a major source of non-mutated target antigens driving both GVT and GVHD. In a pivotal report, Warren et al. administered donor T lymphocytes that were reactive to a range of recipient expressed miHAs and observed both on-target GVT as well as on-target but off-tumor pulmonary toxicity.[23] Since then, the focus of targeting miHAs has shifted to those that have natural target-tissue specific expression (e.g., histocompatibility antigen 1 [HA-1] that is expressed only on hematopoietic tissues and hematologic malignancies) and are thus less likely to induce reactions in normal non-target tissues.[24]

Non-Histocompatibility, Germline Antigens

Outside of mismatched histocompatibility antigens, "cancer-testis" antigens represent the major source of germline-derived target antigens for T-cell immunotherapy. Most CTAs (e.g., NYESO1, MAGE) were discovered by studying the target antigen profile of TILs. These antigens are selectively overexpressed by tumor cells with normal expression restricted to immune-privileged gonadal tissues. Thus, T lymphocytes with specificities for CTAs may not be deleted by the mature thymus during central tolerance. Though not true of CTAs, a number of antigens have been discovered that have limited normal tissue expression but are overexpressed by cancer cells by virtue of altered epigenetics (e.g., WT1) or rapid cell cycling (e.g., Cyclin A1, Survivin).

As an extension of TIL therapy in solid tumors, Noonan et al. treated myeloma patients with analogous "marrow-infiltrating lymphocytes" or MILs.[38]

Since TILs or MILs are not always available and have considerable variability in quality and quantity among patients, another approach is to expand TAA-reactive T cells from the periphery by stimulating with TAAs ex vivo. In this regard, our group and others have demonstrated the clinical safety of administering expanded T cells with natural TAA reactivity to patients with a variety of refractory cancers. These mostly small cohort studies target TAAs that are predicted or known to be expressed by tumor cells, and the products manufactured target single or multiple TAAs simultaneously (see Table 25.2). While encouraging efficacy has been demonstrated in these reports, larger, multi-institutional, efficacy-based trials are ongoing.

Other groups have transduced T cells with artificial TCRs with defined specificity for single peptides derived from a TAA. Notably, in their proof-of-principle clinical trial, Rappaport et al. demonstrated the safety of targeting a peptide derived from NYESO1/LAGE1 in patients with myeloma and sarcoma. In this trial, these T cells substantially improved progression-free survival in patients (15 of 20 remained in remission for over 1 year). The authors also observed loss of the target antigen in relapsing tumors as a mechanism of immune escape.[39] Similarly, two groups demonstrated leukemia-directed efficacy without GVHD of engineering donor-T cells to express WT1-peptide specific TCRs in patients with AML.[27,28]

Targeting germline antigens with artificial, affinity enhanced TCRs carries the risk for self-reactivity. Indeed, in two patients who received T cells transduced with a TCR specific for a peptide derived from the TAA MAGE-A3 had cardiac toxicity that led to death. An elegant investigation demonstrated that the transduced TCR was "codon-optimized," resulting in high level avidity and affinity for the MAGE-A3 peptide, but also a peptide from titin, which is an unrelated germline antigen, highly expressed by normal cardiac myocytes.[40]

FUTURE DIRECTIONS

Unmodified ex vivo expanded tumor-specific T cells are subject to evasion tactics mediated by tumor cells or its microenvironment (see Fig. 25.2). After adoptive transfer of tumor-specific T cells, some of these escape mechanisms occur at the level of the tumor cell, such as downregulation/loss of target antigen, overexpression of T-cell checkpoint ligands, loss of HLA molecules required for antigen presentation, or the disruption of antigen processing and presentation machinery. In the tumor microenvironment, secretion of immunosuppressive cytokines (e.g., TGFβ, etc.), infiltration with regulatory T cells or myeloid derived suppressor cells, as well as upregulation of T-cell checkpoints, block T-cell activity. Ongoing clinical trials of unmodified VST and tumor-specific T cells are exploring combinatorial approaches to overcome these barriers, for instance, by infusing T cells in combination with exogenously administered type 1 helper (Th1) polarizing cytokines, checkpoint inhibitors, or drugs that alter the epigenome. Similarly, infused T cells are being engineered to resist tumor evasion tactics with decoy receptors for immunosuppressive molecules, Th1 polarizing cytokine receptors, or gene edited to delete T-cell checkpoint genes such as programmed cell death protein 1 (PD-1). In the future, we expect clinical investigation of immunotherapeutic T cells to involve synergistic approaches that combine the antigen specificity of the transferred cells with costimulatory and/or cytokine signals to overcome tumor-mediated immune-suppression.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Biernacki MA, Bleakley M. Neoantigens in hematologic malignancies. *Front Immunol*. 2020;11:121.

Bollard CM, Gottschalk S, Torrano V, et al. Sustained complete responses in patients with lymphoma receiving autologous cytotoxic T lymphocytes targeting Epstein–Barr virus latent membrane proteins. *J Clin Oncol*. 2014;32(8):798–808.

Bollard CM, Tripic T, Cruz CR, et al. Tumor-specific T-cells engineered to overcome tumor immune evasion induce clinical responses in patients with relapsed Hodgkin lymphoma. *J Clin Oncol*. 2018;36(11):1128–1139.

Braun DA, Wu CJ. Antigen discovery and therapeutic targeting in hematologic malignancies. *Cancer J*. 2017;23(2):115–124.

Chapuis AG, Egan DN, Bar M, et al. T cell receptor gene therapy targeting WT1 prevents acute myeloid leukemia relapse post-transplant. *Nat Med*. 2019;25(7):1064–1072.

Chapuis AG, Ragnarsson GB, Nguyen HN, et al. Transferred WT1-reactive CD8+ T cells can mediate antileukemic activity and persist in post-transplant patients. *Sci Transl Med*. 2013;5(174):174ra27.

Comoli P, Basso S, Riva G, et al. BCR-ABL-specific T-cell therapy in Ph+ ALL patients on tyrosine-kinase inhibitors. *Blood*. 2017;129(5):582–586.

Doubrovina E, Oflaz-Sozmen B, Prockop SE, et al. Adoptive immunotherapy with unselected or EBV-specific T cells for biopsy-proven EBV lymphomas after allogeneic hematopoietic cell transplantation. *Blood*. 2012;119:2644–2656.

Feuchtinger T, Matthes-Martin S, Richard C, et al. Safe adoptive transfer of virus specific T-cell immunity for the treatment of systemic adenovirus infection after allogeneic stem cell transplantation. *Br J Haematol*. 2006;134:64–76.

Haque T, Wilkie GM, Jones MM, et al. Allogeneic cytotoxic T-cell therapy for EBV-positive posttransplantation lymphoproliferative disease: results of a phase 2 multicenter clinical trial. *Blood*. 2007;110:1123–1131.

Heslop HE, Slobod KS, Pule MA, et al. Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood*. 2010;115:925–935.

Leen AM, Bollard CM, Mendizabal AM, et al. Multicenter study of banked third-party virus-specific T cells to treat severe viral infections after hematopoietic stem cell transplantation. *Blood*. 2013;121:5113–5123.

Leen AM, Myers GD, Sili U, et al. Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. *Nat Med*. 2006;12:1160–1166.

Linette GP, Stadtmauer EA, Maus MV, et al. Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. *Blood*. 2013;122(6):863–871.

Lulla PD, Tzannou IT, Vasileiou S, et al. The safety and clinical effects of administering a multiantigen-targeted T cell therapy to patients with multiple myeloma. *Sci Transl Med*. 2020;12(554):eaaz3339.

Meij P, Jedema I, van der Hoorn MA, et al. Generation and administration of HA-1-specific T-cell lines for the treatment of patients with relapsed leukemia after allogeneic stem cell transplantation: a pilot study. *Haematologica*. 2012;97(8):1205–1208.

Ott PA, Hu Z, Keskin DB, et al. An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature*. 2017;547(7662):217–221.

Papadopoulou A, Gerdemann U, Katari UL, et al. Activity of broad-spectrum T cells as treatment for AdV, EBV, CMV, BKV, and HHV6 infections after HSCT. *Sci Transl Med*. 2014;6:242ra83.

Rapoport AP, Stadtmauer EA, Binder-Scholl GK, et al. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *Nat Med*. 2015;21(8):914–921.

Rooney CM, Smith CA, Ng CY, et al. Use of gene-modified virus-specific T lymphocytes to control Epstein–Barr-virus-related lymphoproliferation. *Lancet*. 1995;345:9–13.

Tawara I, Kageyama S, Miyahara Y, et al. Safety and persistence of WT1-specific T-cell receptor gene-transduced lymphocytes in patients with AML and MDS. *Blood*. 2017;130(18):1985–1994.

Tran E, Robbins PF, Lu YC, et al. T-cell transfer therapy targeting mutant KRAS in cancer. *N Engl J Med*. 2016;375(23):2255–2262.

Tran E, Turcotte S, Gros A, et al. Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. *Science*. 2014;344(6184):641–645.

Tzannou I, Papadopoulou A, Naik S, et al. Off-the-shelf virus-specific T cells to treat BK virus, human herpesvirus 6, cytomegalovirus, Epstein–Barr virus, and adenovirus infections after allogeneic hematopoietic stem-cell transplantation. *J Clin Oncol*. 2017;35:3547–3557.

Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N Engl J Med*. 1995;333:1038–1044.

Warren EH, Fujii N, Akatsuka Y, et al. Therapy of relapsed leukemia after allogeneic hematopoietic cell transplantation with T cells specific for minor histocompatibility antigens. *Blood*. 2010;115(19):3869–3878.

Zacharakis N, Chinnasamy H, Black M, et al. Immune recognition of somatic mutations leading to complete durable regression in metastatic breast cancer. *Nat Med*. 2018;24(6):724–730.

REFERENCES

1. Cobbold M, Khan N, Pourgheysari B, et al. Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. *J Exp Med*. 2005;202:379–386.
2. Uhlin M, Gertow J, Uzunel M, et al. Rapid salvage treatment with virus-specific T cells for therapy-resistant disease. *Clin Infect Dis*. 2012;55:1064–1073.
3. Peggs KS, Thomson K, Samuel E, et al. Directly selected cytomegalovirus-reactive donor T cells confer rapid and safe systemic reconstitution of virus- specific immunity following stem cell transplantation. *Clin Infect Dis*. 2011;52:49–57.
4. Neuenhahn M, Albrecht J, Odendahl M, et al. Transfer of minimally manipulated CMV-specific T cells from stem cell or third-party donors to treat CMV infection after allo-HSCT. *Leukemia*. 2017;31:2161–2171.
5. Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N Engl J Med*. 1995;333:1038–1044.
6. Feuchtinger T, Matthes-Martin S, Richard C, et al. Safe adoptive transfer of virus specific T-cell immunity for the treatment of systemic adenovirus infection after allogeneic stem cell transplantation. *Br J Haematol*. 2006;134:64–76.
7. Moosmann A, Bigalke I, Tischer J, et al. Effective and long-term control of EBV PTLD after transfer of peptide-selected T cells. *Blood*. 2010;115:2960–2970.
8. Kallay K, Kassa C, Reti M, et al. Early experience with CliniMACS Prodigy CCS (IFN-gamma) system in selection of virus-specific T cells from third-party donors for pediatric patients with severe viral infections after hematopoietic stem cell transplantation. *J Immunother*. 2018;41:158–163.
9. Heslop HE, Slobod KS, Pule MA, et al. Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood*. 2010;115:925–935.
10. Leen AM, Myers GD, Sili U, et al. Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. *Nat Med*. 2006;12:1160–1166.
11. Doubrovina E, Oflaz-Sozmen B, Prockop SE, et al. Adoptive immunotherapy with unselected or EBV-specific T cells for biopsy-proven EBV lymphomas after allogeneic hematopoietic cell transplantation. *Blood*. 2012;119:2644–2656.
12. Haque T, Wilkie GM, Jones MM, et al. Allogeneic cytotoxic T-cell therapy for EBV-positive posttransplantation lymphoproliferative disease: results of a phase 2 multicenter clinical trial. *Blood*. 2007;110:1123–1131.
13. Papadopoulou A, Gerdemann U, Katari UL, et al. Activity of broad-spectrum T cells as treatment for AdV, EBV, CMV, BKV, and HHV6 infections after HSCT. *Sci Transl Med*. 2014;6:242ra83.
14. Vickers MA, Wilkie GM, Robinson N, et al. Establishment and operation of a good manufacturing practice-compliant allogeneic Epstein–Barr virus (EBV)-specific cytotoxic cell bank for the treatment of EBV-associated lymphoproliferative disease. *Br J Haematol*. 2014;167:402–410.
15. Leen AM, Bollard CM, Mendizabal AM, et al. Multicenter study of banked third-party virus-specific T cells to treat severe viral infections after hematopoietic stem cell transplantation. *Blood*. 2013;121:5113–5123.
16. Tzannou I, Papadopoulou A, Naik S, et al. Off-the-shelf virus-specific T cells to treat BK virus, human herpesvirus 6, cytomegalovirus, Epstein–Barr virus, and adenovirus infections after allogeneic hematopoietic stem-cell transplantation. *J Clin Oncol*. 2017;35:3547–3557.
17. Prockop S, Doubrovina E, Suser S, et al. Off-the-shelf EBV-specific T cell immunotherapy for rituximab-refractory EBV-associated lymphoma following transplantation. *J Clin Invest*. 2020;130:733–747.
18. Rooney CM, Smith CA, Ng CY, et al. Use of gene-modified virus-specific T lymphocytes to control Epstein–Barr-virus-related lymphoproliferation. *Lancet*. 1995;345:9–13.
19. Balduzzi A, Lucchini G, Hirsch HH, et al. Polyomavirus JC-targeted T-cell therapy for progressive multiple leukoencephalopathy in a hematopoietic cell transplantation recipient. *Bone Marrow Transplant*. 2011;46:987–992.
20. Tzannou I, Watanabe A, Naik S, et al. “Mini” bank of only 8 donors supplies CMV-directed T cells to diverse recipients. *Blood Adv*. 2019;3:2571–2580.
21. Bollard CM, Gottschalk S, Torrano V, et al. Sustained complete responses in patients with lymphoma receiving autologous cytotoxic T lymphocytes targeting Epstein–Barr virus latent membrane proteins. *J Clin Oncol*. 2014;32(8):798–808.
22. Eom H-S, Choi BK, Youngjoo L, et al. Phase 1 clinical trial of 4-1BB-based adoptive T-cell therapy for Epstein–Barr Virus (EBV)-positive tumors. *J Immunother*. 2016;39(3):140–148.
23. Warren EH, Fujii N, Akatsuka Y, et al. Therapy of relapsed leukemia after allogeneic hematopoietic cell transplantation with T cells specific for minor histocompatibility antigens. *Blood*. 2010;115(19):3869–3878.
24. Meij P, Jedema I, van der Hoorn MA, et al. Generation and administration of HA-1-specific T-cell lines for the treatment of patients with relapsed leukemia after allogeneic stem cell transplantation: a pilot study. *Haematologica*. 2012;97(8):1205–1208.
25. Chapuis AG, Ragnarsson GB, Nguyen HN, et al. Transferred WT1-reactive CD8+ T cells can mediate antileukemic activity and persist in post-transplant patients. *Sci Transl Med*. 2013;5(174):174ra27.
26. Lulla PD, Tzannou IT, Vasileiou S, et al. The safety and clinical effects of administering a multi-antigen targeted T cell therapy to patients with multiple myeloma. *Sci Transl Med*. 2020;12(554):eaaz3339.
27. Tawara I, Kageyama S, Miyahara Y, et al. Safety and persistence of WT1-specific T-cell receptor gene-transduced lymphocytes in patients with AML and MDS. *Blood*. 2017;130(18):1985–1994.
28. Chapuis AG, Egan DN, Bar M, et al. T cell receptor gene therapy targeting WT1 prevents acute myeloid leukemia relapse post-transplant. *Nat Med*. 2019;25(7):1064–1072.
29. Stadtmauer EA, Fraietta JA, Davis MM, et al. CRISPR-engineered T cells in patients with refractory cancer. *Science*. 2020;367(6481):eaba7365.
30. Tran E, Turcotte S, Gros A, et al. Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. *Science*. 2014;344(6184):641–645.
31. Tran E, Robbins PF, Lu YC, et al. T-cell transfer therapy targeting mutant KRAS in cancer. *N Engl J Med*. 2016;375(23):2255–2262.
32. Ott PA, Hu Z, Keskin DB, et al. An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature*. 2017;547(7662):217–221.
33. Comoli P, Basso S, Riva G, et al. BCR-ABL-specific T-cell therapy in Ph+ ALL patients on tyrosine-kinase inhibitors. *Blood*. 2017;129(5):582–586.
34. Zacharakis N, Chinnasamy H, Black M, et al. Immune recognition of somatic mutations leading to complete durable regression in metastatic breast cancer. *Nat Med*. 2018;24(6):724–730.
35. Bollard CM, Tripic T, Cruz CR, et al. Tumor-specific T-cells engineered to overcome tumor immune evasion induce clinical responses in patients with relapsed Hodgkin lymphoma. *J Clin Oncol*. 2018;36(11):1128–1139.
36. Biernacki MA, Bleakley M. Neoantigens in hematologic malignancies. *Front Immunol*. 2020;11:121.
37. Braun DA, Wu CJ. Antigen discovery and therapeutic targeting in hematologic malignancies. *Cancer J*. 2017;23(2):115–124.
38. Noonan KA, Huff CA, Davis J, et al. Adoptive transfer of activated marrow-infiltrating lymphocytes induces measurable antitumor immunity in the bone marrow in multiple myeloma. *Sci Transl Med*. 2015;7(288):288ra78.
39. Rapoport AP, Stadtmauer EA, Binder-Scholl GK, et al. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *Nat Med*. 2015;21(8):914–921.
40. Linette GP, Stadtmauer EA, Maus MV, et al. Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. *Blood*. 2013;122(6):863–871.

CHAPTER 26

TREATMENT OF HEMATOLOGIC MALIGNANCIES WITH GENETICALLY MODIFIED T CELLS

Eben I. Lichtman, Malcolm K. Brenner, and Gianpietro Dotti

Conventional modalities for treating cancer remain unsatisfactory. Despite the introduction of small molecules that target specific molecular lesions or pathways within the cancer cells, cure rates for many common tumors remain low, while adverse events are still distressingly high. Cancer immunotherapy represents a promising extension of highly targeted cancer therapy with potentially favorable toxicity and pharmacoeconomic profiles. Until recently, most attention has been on the development of conventional monoclonal antibodies that target specific tumor-expressed antigens. Over the past few years, the focus of monoclonal antibody therapy has shifted to agents that recruit innate or adaptive immune responses against the tumor, either by blocking immune regulation by tumors or by simultaneously engaging tumor cells and effector lymphocytes (bispecific antibodies).[1,2] More recently, however, strikingly beneficial results with direct (adoptive) transfer of immune system cells are now being reported.[3] Although to date these have primarily been obtained in patients with leukemia, lymphoma, multiple myeloma, melanoma, or neuroblastoma, methodologies are being developed to allow us to extend the tumor range.

Many human tumors express tumor-specific antigens (TSAs) or tumor-associated antigens (TAAs) that can be recognized by the host immune system and induce antitumor cell-mediated and humoral immune responses. Although these responses may be transient and are not always associated with clinical responses, they provide evidence for the existence of tumor-directed immunity in humans that may also have antitumor activity. Several barriers block the development of more effective antitumor immunity in people with cancer. First, many human tumors express few major histocompatibility complex (MHC) molecules or have poor processing of their potential tumor antigens. Even when TAA/TSA are processed and presented, most tumors lack the costimulatory molecules necessary to implement long-lived and effective immune responses. In addition to these passive defenses against immunity, many tumors can “edit” the immune system to their advantage, secreting cytokines such as transforming growth factor beta (TGFβ) or by expressing molecules such as programmed death-ligand 1 (PD-L1) that act as inhibitory or check point signals to cytotoxic effector T-cell growth, function, and survival, or that favor expansion of Th2/regulatory T cells rather than effector T cells. Finally, intensive chemotherapy and radiotherapy can themselves severely reduce immune function by destroying antigen presenting cells and dividing T lymphocytes.

As our understanding of the molecular basis of tumor immune escape has increased, it has been possible to derive countermeasures that may allow us to induce more potent antitumor immune responses, and that will soon allow us to extend effective therapies to a broad range of common tumors.

TYPES OF CELLULAR IMMUNOTHERAPY

Cellular immunotherapy may use cell-based vaccines derived from tumor cells or antigen-presenting cells expressing TAA/TSA from proteins/peptides, or may depend on the direct adoptive transfer of viable immune cells. The former approach relies on the intact afferent and efferent immune system of the host responding to the stimulus with an effective antitumor response, while the latter is the cellular equivalent of antibody serotherapy, in which the transferred immune cells are expected to attack the tumor cells directly, albeit with a phase of in vivo expansion, and to subsequently establish a pool of memory cells to provide long-term protection against resurgent disease. Several cell subsets are currently being studied in adoptive transfer protocols, including activated T lymphocytes (ATL), tumor infiltrating T lymphocytes, antigen-specific cytotoxic T lymphocytes (CTL), natural killer (NK) cells, γβ T cells, and natural killer T (NKT) cells. In this chapter, we discuss adoptive transfer of genetically modified ATL and CTL.

Adoptive Cell Therapy With T Lymphocytes

In principle, lymphocytes have the ability to traffic through multiple tissue planes and to be self-renewing. These assets, coupled with their ability to destroy tumor or viral infected target cells through a range of mechanisms makes them an appealing resource for adoptive transfer, and a multiplicity of clinical studies using this approach have now been described. Adoptive lymphocyte therapies may use allogeneic or autologous cells, which may be of tightly defined specificity (e.g., T-cell clones) or broad phenotype and activity (e.g., tumor infiltrating lymphocytes). As we have learned more about the molecular basis of immune recognition and immune regulation, it has become possible to genetically modify the infused lymphocytes to alter their specificity or behavior. In this section, we describe examples of each type of T-cell adoptive transfer and discuss the relative merits and limitations of each.

Allogeneic Donor Lymphocyte Infusion

It has long been apparent that the curative effects of allogeneic hematopoietic stem cell transplants (HSCTs) for many hematologic malignancies can be attributed to a graft-versus-leukemia (GVL) effect largely mediated by the incoming T cells within the donor graft. Thus, patients with chronic graft-versus-host disease (GVHD) were well recognized as having a lower probability of relapse than individuals without this unpleasant complication. Similarly, recipients of syngeneic grafts have the lowest rate of GVHD and the highest risk of relapse. In 1990, Kolb and colleagues took advantage of this observation and deliberately infused donor lymphocytes in an attempt to eliminate recurrent disease in patients with chronic myeloid leukemia (CML). Their positive results have been confirmed in multiple studies worldwide, and remission can be induced in more than 50% of CML patients who relapse after transplantation by stopping immunosuppressive treatment or infusing donor lymphocytes. Unfortunately, donor lymphocyte infusion (DLI) is much less effective at treating other types of relapsed leukemias after transplantation, with a 29% remission rate for acute myeloid leukemia (AML) and only 5% for acute lymphoblastic leukemia (ALL). It is not clear why these differences occur, since all these leukemias present the minor histocompatibility antigens (mHags) that are likely the targets of this GVL effect, although many mHags have yet to be defined. DLI therapy may also produce severe adverse effects, since the frequency of broadly alloreactive effector cells is usually much higher than the frequency of lymphocytes targeted exclusively to the relapsed malignancy. As a consequence, patients receiving DLI often develop GVHD. This complication of DLI usually increases in frequency and severity if the donor and recipient are either unrelated or human leukocyte antigen (HLA) haploidentical. Strategies aimed at retaining the benefits of GVL while preventing GVHD have included the depletion of alloreactive T cells in the donor lymphocyte product and the incorporation of suicide genes into the infused donor T cells so that they may be killed if the GVHD activity exceeds the benefits from GVL.[4] Manipulation of the stem cell graft to deplete only the αβ T-cell receptor (TCR)+ T lymphocytes while retaining the γδTCR+ T-cell compartment may reduce GVHD without compromising stem cell engraftment and may retain some protection against opportunistic infections.[5] Ultimately, investigators may wish to identify tumor-restricted target antigens on the malignant cells and infuse antigen-specific T cells directed to them. As discussed in Chapter 25 the potential efficacy of this approach has been demonstrated in preliminary studies, although scalability remains a challenge. Furthermore, genetic evolution and neoantigen depletion on tumor cells has been demonstrated as a mechanism of resistance to the GVL effect following allogeneic hematopoietic stem cell transplantation[6] and could similarly limit the efficacy of infusing autologous T cells that target tumor-restricted antigens.

Infusion of Activated T Lymphocytes

When T cells are polyclonally stimulated, for example by simultaneously cross-linking their CD3 and CD28 receptors by CD3/CD28 monoclonal antibodies on beads, they proliferate, secrete tumoricidal cytokines such as tumor necrosis factor alpha (TNFα), and can mediate MHC-unrestricted cytotoxicity toward a range of tumor target cells. Efforts have been made to harness these effects by producing large numbers of CD3/CD28-activated T cells for cancer patients and infusing them. Although infusion of CD3/CD28-ATL after autologous stem cell transplant may improve patients' T-cell reconstitution, there is not yet evidence to suggest improved antitumor activity.[7] ATL that are additionally primed with interferon gamma (IFN-γ) and interleukin (IL)-2 (so called cytokine-induced killer [CIK] cells) may have superior clinical potential for hematologic malignancies and early phase clinical trials have shown clinical benefits.[8]

GENETIC MODIFICATION OF T CELLS

Early clinical studies using genetic modification only attempted to "mark" the T-cell infused to follow their fate in the peripheral blood or other tissues. More recently, efforts have been devoted to "redirecting" the antigen-specificity of T lymphocytes and thus providing them with robust antitumor activity. To overcome the low affinity of tumor-specific CTLs detected in vivo, investigators have cloned T-cell receptor α and β chains (αβTCR) of high affinity.[9] Alternatively, tumor-specificity has been generated by the construction of chimeric antigen receptors (CARs),[10] which are most commonly composed of the binding domains of a monoclonal antibody and the ξ signaling domain of the CD3αβ TCR as well as components of costimulatory molecules to ensure signaling and T-cell activation once the CAR has been engaged (Fig. 26.1). Finally, interest in genetic modification of T cells has also arisen as a means of incorporating countermeasures to the multiplicity of immune evasion strategies used by potentially immunogenic tumor cells or to enhance the "survival' of T cells in vivo (see Fig. 26.1). Because T lymphocytes can be long-lived cells and may proliferate extensively in vivo, most gene transfer studies have used integrating vectors such as gamma-retroviral vectors, lentiviral vectors, or transposon/transposase integrating plasmids to ensure long-term expression of the therapeutic transgene.[10]

Artificial αβT-Cell Receptors

The large-scale culture of T lymphocytes to enrich the scanty precursors specific for weak TAAs is often unsuccessful and always tedious. This process can be bypassed by introducing additional TCR genes with predetermined specificity and high affinity for the weak tumor antigen into a polyclonal population of T cells. Technical improvements in retroviral transduction mean that greater than 30% of polyclonal T lymphocytes can now be induced to express a transgenic TCR with high affinity for TAAs including melanoma-associated antigen recognized by T cells (MART)-1, melanoma antigen (MAGE)-3, mouse double minute 2 homolog (MDM2), Wilms' tumor 1 (WT1), New York esophageal squamous cell carcinoma 1 (NY-ESO-1), survivin, and for mHags such as HA1 and infectious

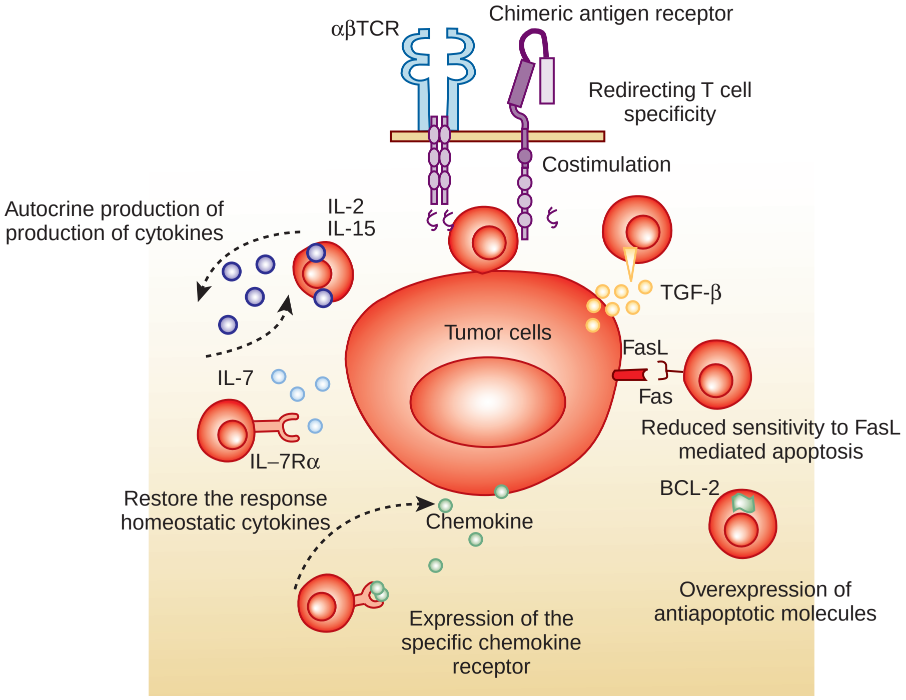

αβTCR
Chimeric antigen receptor
Redirecting T cell specificity
Costimulation
ζ ζ
ζ
Autocrine production of production of cytokines
IL-2
IL-15
TGF-β
Tumor cells
FasL
Fas
Reduced sensitivity to FasL mediated apoptosis
IL-7
IL–7Rα
BCL-2
Restore the response homeostatic cytokines
Chemokine
Overexpression of antiapoptotic molecules
Expression of the specific chemokine receptor

Figure 26.1 GENETIC MODIFICATIONS OF T LYMPHOCYTES FOR ADOPTIVE T-CELL THERAPY.

agents such as human immunodeficiency virus (HIV)-1 and Epstein-Barr virus (EBV).[11]

Early studies, primarily among solid tumor patients, have been hampered by "on target, off tumor" toxicities in normal tissues that physiologically express the target antigen at low level. Melanoma patients treated with polyclonal T cells expressing transgenic MART-1 specific αβTCRs developed toxicity in normal tissues containing melanocytes (e.g., skin and uvea).[9] Patients with metastatic colon carcinoma developed colitis after treatment with T cells expressing a TCR directed to the carcinoembryonic antigen that is also expressed at low level in normal gut epithelia cells.[12]

Nonetheless, promising results have been reported among patients with AML and multiple myeloma. In a trial including 12 AML patients who were required to be HLA-A*0201 (HLA-A2)-positive, EBV-specific CD8$^+$ T cells (to minimize GVHD and enhance T-cell survival) were engineered to express an HLA-A2-restricted WT1-targeted TCR and were infused following allogeneic HSCT. No on-target, off-tissue toxicities were identified and, at a median follow up of 44 months, no patient had relapsed.[13] Another study among MM patients evaluated NY-ESO-1–specific TCR-engineered T cells, which did not appear to cause any significant toxicities and were associated with a 19-month progression-free survival when infused following autologous HSCT.[14]

The major problem of TCR gene transfer in polyclonal T lymphocytes harboring their own native αβTCR was hypothesized to be the "cross-pairing" between transgenic α or β receptor chains and the reciprocal endogenous TCR α- and β-chains, that could create loss of function or—and potentially worse—gain-of-function receptors that may produce autoimmune disease, an adverse effect clearly demonstrable in mouse models.[15] Although these events have not been reported in clinical trials so far, the issue of potential "cross-pairing" has been addressed in one recent study via clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9-mediated knockout of the endogenous TCR.[16] In prior studies, other unexpected toxicities have unfortunately been observed. For example, patients with myeloma or melanoma were given T cells modified to express a TCR specific for MAGE-A3 that had been synthetically affinity-enhanced. Two of the recipients rapidly developed lethal cardiotoxicity caused by an unanticipated cross-reactivity of the transgenic TCR against peptide epitopes derived from Titin, which were expressed only by cardiac myocytes.[17] These results strongly indicate that T cells engineered with high-affinity TCR can be effective, but can also reveal unexpected and lethal cross-reactivity with other peptide epitopes that would not be recognized by TCR with more physiologic binding affinity.[11,17] While such unanticipated toxicities may be avoided by ever more extensive preclinical evaluation, reliance on a "superaffinity" TCR may be intrinsically hazardous.

Chimeric Antigen Receptors

The cytotoxic activity of T cells through their native or transgenic TCR is MHC-restricted so that multiple distinct transgenic αβTCRs would be required to recognize tumor antigens associated with the multiple MHC polymorphisms in a human population, precluding a "universal" receptor. In addition, tumor cells can downregulate MHC molecules and avoid immune recognition by conventional TCRs. In an attempt to overcome this limitation, MHC unrestricted CARs have been generated.[10] CARs are typically prepared by joining the light and heavy chain variable regions of a monoclonal antibody expressed as a single-chain Fv (scFv) molecule to the cytoplasmic signaling domains derived from CD3ζ cytoplasmic and intracytoplasmic endodomains derived from costimulatory molecules such as CD28, 4-1BB, and OX40.[10] Thus when CARs are expressed by polyclonal T lymphocytes or virus-specific T cells (VSTs), they can combine the antigen specificity of an antibody with the cytotoxic properties of T cells, together with the costimulatory signals provided by professional antigen presenting cells that allow survival and proliferation of ATLs. Since CARs bind to target antigens in an HLA-unrestricted manner, they are resistant to many of the tumor immune evasion mechanisms, such as downregulation of HLA class I molecules or failure to process or present proteins, used by tumor cells to escape immune attack. Adoptive transfer of polyclonal activated T cells expressing a second-generation CD19-specific CAR have now led to impressive clinical responses in multiple studies among patients with ALL and non-Hodgkin lymphomas, with greater than 75% complete response rates in relapsed/refractory ALL patients in phase II clinical trials and 52% to 85% response rates in phase II trials among patients with relapsed/refractory lymphomas (Table 26.1). These results have led to the first Food and Drug Administration (FDA) approvals for CAR-T-cell therapy in the United States.

Clinical Results of Chimeric Antigen Receptor T Cells in Hematologic Malignancies

CD19

Tisagenlecleucel (tisa-cel), the first FDA-approved CAR-T-cell therapy, is a CD19-specific CAR containing the 4-1BB costimulatory domain and was initially approved for relapsed/refractory B-ALL based on the phase II ELIANA trial, which evaluated tisa-cel among 75 pediatric and young adult patients with B-ALL and demonstrated a complete remission rate of 81% and overall survival of 70% at 18 months based on longer-term follow-up data.[18] Cytokine release syndrome (CRS) and neurotoxicity (both discussed in more detail below), were seen in a substantial percentage of patients (see Table 26.1). Because the CD19 target molecule is also expressed by normal B cells, the eradication of B-cell leukemia is also associated with B-cell aplasia, which is typically sustained during the period of robust CAR-T cell engraftment. Among responders to tisa-cel who ultimately relapsed, 74% were found to have loss of CD19 expression on leukemic cells despite ongoing B-cell aplasia.[18]

The phase II JULIET study evaluated tisa-cel in 93 patients with relapsed/refractory large B-cell lymphoma, and demonstrated overall and complete response rates of 52% and 40%, respectively, leading to FDA-approval for this indication as well. Axicabtagene ciloleucel (axi-cel) is another CD19-directed CAR which contains the CD28 costimulatory domain and was approved for the treatment of relapsed/refractory large B-cell lymphoma based on the phase II ZUMA-1 trial, which demonstrated overall and complete response rates of 82% and 54%, respectively.[18] Finally, lisocabtagene maraleucel (liso-cel), a CD19-directed CAR containing the 4-1BB costimulatory domain with a modified hinge and transmembrane domain, has also been approved for relapsed/refractory large cell lymphoma based on the TRANSCEND NHL 001 trial, which demonstrated complete and overall response rates of 73% and 53%, respectively.[18]

The efficacy of CD19-directed CAR-T-cell therapy has also been demonstrated in mantle cell lymphoma (MCL). The phase II ZUMA-2 trial evaluated brexucabtagene autoleucel, a CD19-directed CAR containing the CD28 costimulatory domain, in 74 patients with relapsed/refractory MCL, demonstrating overall and complete response rates of 85% and 59%, respectively.[18]

B-Cell Maturation Antigen

CAR-T-cell therapies for multiple myeloma have also achieved promising results. B-cell maturation antigen (BCMA) has emerged as an important target antigen in multiple myeloma, and several BCMA-directed CAR-T-cell therapies are under development, with phase II studies demonstrating overall response rates of greater than 70% with impressive associated progression free survival benefits among patients with relapsed/refractory disease (see Table 26.1). Idecabtagene vicleucel (ide-cel), a BCMA-targeted CAR containing the 4-1BB costimulatory domain, was the first CAR-T-cell therapy to receive FDA approval for multiple myeloma. The phase II KarMMa study evaluated ide-cel among 140 patients with relapsed/refractory myeloma and demonstrated overall and complete response rates of 73% and 33%, respectively with a median progression-free survival of 8.8 months (see Table 26.1).[19]

TABLE 26.1 Selected Clinical Trials Using Chimeric Antigen Receptors–Modified and T-Cell Receptor–Modified T Cells

Reference (Trial Name)	Disease	Target	Product	Phase	*n*	Lympho-depletion	Outcome	Gr ≥3 CRS; Gr ≥3 Neurotox.
CAR-T Cells: B-Cell Malignancies								
[a]Maude[18] (ELIANA)	B-ALL	CD19	[c]Tisagenlecleucel (formerly CTL019; Kymriah)	II	75	Flu, Cy (95%)	81% OR; 81% CR/CRi	46%; 13%
[a]Schuster[18] (JULIET)	DLBCL			II	93	Flu, Cy or Benda	52% OR; 40% CR	22%; 12%
[a]Neelapu[18] (ZUMA-1)	DLBCL, PMBCL, tFL	CD19	[c]Axicabtagene ciloleucel (axi-cel; formerly KTE-C19; Yescarta)	II	111	Flu, Cy	82% OR; 54% CR	13%; 28%
[a]Wang[18] (ZUMA-2)	MCL	CD19	[c]Brexucabtagene autoleucel (formerly KTE-X19; Tecartus)	II	74	Flu, Cy	85% OR; 59% CR	15%; 31%
[a]Abramson[18] (TRANSCEND NHL 001)	DLBCL	CD19	[c]Lisocabtagene maraleucel (liso-cel; formerly JCAR017; Breyanzi)	I	256	Flu, Cy	73% OR; 53% CR	2%; 10%
[a]Ramos[20] (UNC: LCCC 1532-ATL; BCM: RELY-30)	HL/NHL	CD30	ATLCAR.CD30	I/II	42	Flu, Cy or Flu, Benda	72% ORR; 59% CR; 36% 12 m PFS	–; –
CAR-T Cells: Multiple Myeloma								
[a]Raje[20] (CRB-401)	MM	BCMA	[c]Idecabtagene vicleucel (ide-cel; formerly bb2121; Abecma)	I	33	Flu, Cy	85% OR; 45% CR; mPFS 11.8 m	6%; 3%
[a,b]Munshi[19,20] (KarMMa)				II	140	Flu, Cy	73% OR; 33% CR/sCR; mPFS 8.8 m	5% (1 death); 3%
[a]Zhao[20] (LEGEND-2)	MM	BCMA	Ciltacabtagene autoleucel (cilta-cel; formerly LCAR-B38M and JNJ-4528)	I	57	Cy	88% OR; 68% CR; mPFS 15 m	7%; –
[a,b]Madduri[20] (CARTITUDE-1)				I/II	97	Flu, Cy	95% OR; 56% CR/sCR; mPFS NR (87% 6 m PFS)	4% (one death); 10% (one death)
[a,b]Mailankody[20] (EVOLVE)	MM	BCMA	Orvacabtagene autoleucel (orva-cel; formerly JCARH125)	I/II	51	Flu, Cy	91% OR; 39% CR/sCR; mPFS NR	2%; 4%
TCR-Engineered T Cells								
Rapoport[14]	MM	NY-ESO-1	NY-ESO_c259 TCR–engineered T cells	I/II	20	Melphalan (2 days post-autoHCT)	90% OR; 80% ≥VGPR; 19.1 m mPFS	–; –
Chapuis[13]	AML	WT1	T_TCR-C4 TCR-engineered T cells	I/II	12	None (median 97 days post-allo HSCT)	NE (NED at baseline); 100% PFS at median 44 m follow up	2%; –
Stadtmauer[16]	AML, sarcoma	NY-ESO-1	NY-ESO-1 TCR-engineered T cells with CRISPR-Cas9 knockout of PD-1 endogenous TCR	I	3	Flu, Cy	SD in 2/3 patients	–; –

[a] References 18 and 20 refer to review articles that summarize these clinical trials.

[b] Results reported in abstract form only.

[c] Currently FDA-approved.

–, None; *allo HSCT*, allogeneic hematopoietic stem cell transplantation; *AML*, acute myeloid leukemia; *B-ALL*, B-cell acute lymphoblastic leukemia; *BCMA*, B-cell maturation antigen; *Benda*, bendamustine; *CR*, complete response; *Cy*, cyclophosphamide; *DLBCL*, diffuse large B-cell lymphoma; *FDA*, Food and Drug Administration; *Flu*, fludarabine; *HL*, Hodgkin lymphoma; *m*, months; *MCL*, mantle cell lymphoma; *MM*, multiple myeloma; *mPFS*, median progression free survival; *NE*, not evaluable; *NED*, no evidence of disease; neurotox, neurotoxicity; *NHL*, non-Hodgkin lymphoma; *NR*, not reached; *OR*, overall response; *PFS*, progression-free survival; *PMBCL*, primary mediastinal B-cell lymphoma; *SD*, stable disease; *TCR*, T-cell receptor; *tFL*, transformed follicular lymphoma; *VGPR*, very good partial response; *WT1*, Wilms tumor 1.

TABLE 26.2 **Target Antigens Under Evaluation for Chimeric Antigen Receptors T-cell Therapy**

Disease	Target Antigen
B-ALL, NHL	CD19, CD20, CD22, CD37, CD79b
HL	CD30, CD123
T-cell leukemia/ lymphoma	CD5, CD7, CD30, TRBC1, TRBC2
MM	BCMA, CD138, CD38, SLAMF7, GPRC5D, CD44, TACI, kappa, lambda
AML	CD123, CD33, CD13

ALL, Acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *BCMA*, B-cell maturation antigen *CD*, cluster of differentiation; *GPRC5D*, G protein–coupled receptor class C group 5 member D; *HL,* Hodgkin lymphoma; *MM*, multiple myeloma; *NHL*, non-Hodgkin lymphoma; *SLAMF7*, signalling lymphocytic activation molecule family member 7; *TACI*, transmembrane activator and calcium-modulator and cyclophilin ligand interactor.
Modified from Frigault MJ, Maus MV. State of the art in CAR T cell therapy for CD19+ B cell malignancies. *J Clin Invest*. 2020;130:1586–1594; and Grover NS, Tschernia N, Dotti G, et al. Extending the promise of chimeric antigen receptor T-cell therapy beyond targeting CD19(+) tumors. *J Clin Oncol*. 2021;39:499–513.

CD30

CAR-T-cell therapies for other hematologic malignancies are earlier in development, although several promising therapies are under investigation. For Hodgkin lymphoma and CD30$^+$ non-Hodgkin lymphoma, CD30-directed CAR-T cells have also shown significant potential with a phase II study demonstrating an overall response rate of 72% (see Table 26.1).[20]

Acute Myeloid Leukemia Targets

The identification of suitable target antigens for AML has remained a challenge largely because most AML-associated surface antigens are also expressed on normal hematopoietic stem cells, creating the potential for unacceptable on-target, off-tumor toxicities.[21] Nonetheless, several ongoing trials are evaluating CD33- and CD123-specific CAR-T cells and other targets are also being evaluated (Table 26.2). Although few results have been reported to date, in a preliminary report of a phase I trial of CD123-specific CAR-T cells including 6 patients with AML, there were no significant myeloablative toxicities observed, and clinically meaningful responses were seen in two patients, one of whom achieved a CR and proceeded to a second allogeneic HSCT.[20] One potential strategy to overcome limitations surrounding on-target, off-tumor hematopoietic toxicity in the treatment of AML is to use gene-editing techniques to remove the target antigen from donor hematopoietic stem cells prior to allogeneic HSCT, coupled with infusion of donor-derived allogeneic CAR-T cells. This was recently demonstrated in pre-clinical models using CRISPR/Cas9 editing to knock out CD33 from normal HSCs, followed by the infusion of CD33-specific CAR-T cells; however, the clinical feasibility of this approach is not yet clear.[22] It remains to be seen whether toxicities, or efficacy concerns in the setting of possible antigen-negative relapse, will limit the feasibility of targeting a single antigen in myeloid malignancies and there is thus significant pre-clinical interest in combinatorial approaches, allowing the targeting of multiple antigens.[21]

T-Cell Antigens

Targeting of T-cell malignancies such as T-cell ALL (T-ALL) and T-cell lymphomas (TCL) with CAR-T cells has also been hampered by several added challenges. Because T-cell linage-associated antigens are typically shared by both normal and malignant T cells, CAR–T-cell fratricide can interfere with the manufacturing process. CD5 is one potential target antigen that may be associated with less fratricide due to rapid downregulation of CD5 surface-expression on CAR-T cells. An initial clinical trial of CD5-targeted CAR-T cells among patients with T-cell malignancies has demonstrated promising preliminary results, with objective responses seen in 4 of 9 evaluable patients.[20] CRISPR/Cas9 gene editing to remove T-cell associated antigens from CAR-T cells is another technique that is being evaluated to prevent fratricide, and this strategy is currently being evaluated in several ongoing trials of both autologous and allogeneic CAR-T cells targeting CD7, which is highly expressed on both T and NK cells.[20] CD30 is another potential target for certain TCLs, including anaplastic large cell lymphoma (ALCL). CD30 is expressed on only a small subset of activated T and B cells, so the risk of both fratricide and on-target, off-tumor toxicity is low. CD30-targeted CAR-T cells are being evaluated in several ongoing clinical trials and responses have been reported in a small number of patients with ALCL.[20]

Factors Affecting Response

Clinical trials, including those mentioned above, have demonstrated improved outcomes when lymphopenia is induced prior to CAR-T cell infusion, typically with the use of cyclophosphamide and fludarabine. Much remains unknown regarding how the composition of the CAR affects the outcome and patients can achieve remissions regardless of the type of CAR costimulation (CD28 or 4-1BB), although 4-1BB costimulation appears to be associated with longer CAR-T-cell persistence and a lower incidence of certain toxicities.

Toxicities of Chimeric Antigen Receptor T Cells

Despite their remarkable success, CAR-T cell administration is associated with potentially lethal acute toxicity caused by a profound perturbation of the immune system, termed the CRS, which may manifest as hyperpyrexia, hypotension, and/or respiratory distress and is associated with high levels of circulating proinflammatory cytokines. In addition, CAR-T administration can trigger significant neurologic dysfunction, referred to as the immune effector cell-associated neurotoxicity syndrome (ICANS), which may cause headaches, speech/language alterations, hallucinations, encephalopathy, seizures, and potentially lethal cerebral edema. ICANS often follows the development of CRS, although it can also occur independently. Although the pathophysiology is poorly understood, the incidence of ICANS appears to be higher in trials of CAR-T cell products containing the CD28 costimulatory domain.

Severe CRS can often be controlled with rapid administration of glucocorticoids and the IL-6 receptor antagonist, tocilizumab. The management of severe ICANS typically involves treatment with glucocorticoids, antiepileptic medications, and supportive care. Despite multiple FDA-approved CAR-T-cell therapies, management of these complications remains a challenge and their occurrence continues to present a barrier to the larger-scale implementation of these therapies. As discussed below, additional strategies are under investigation, including the use of so-called safety switches.

Allogeneic Banked Cells

Financial barriers, technical challenges (e.g., inadequate leukapheresis or rapid disease progression), and disease-related obstacles (e.g., T-cell dysfunction in certain malignancies) often limit the applicability of CAR-T-cell therapies, and there has thus been significant interest in the development of allogeneic, or so-called “universal,” CAR-T products. To accomplish this, several groups have reported the use of gene-editing techniques such as CRISPR-Cas9, transcription activator-like effector nucleases, and zinc-finger nucleases to knock out the endogenous αβ-TCR in donor T cells, thereby mitigating the risk of GVHD associated with the infusion of donor-derived CAR-T cells. Several universal CAR-T products are currently being evaluated in clinical trials, although it remains to be seen how such approaches will affect CAR-T-cell function, persistence, and associated toxicities.[20]

Additional strategies for the development of allogenic, "off-the-shelf," cellular therapies include the use of alternative immune effectors that do not mediate significant alloreactivity, such as gamma delta (γδ) T cells, NK cells, NKT cells, and VSTs. NK cells are effector cells of the innate immune system and are known to exhibit significant anti-tumor activity via multiple cytotoxic mechanisms despite lacking an antigen-specific receptor analogous to the TCR. CAR-NK cells have several potential advantages over CAR-T cells; the possibility of both CAR-dependent and CAR-independent mechanisms for activation; a decreased risk of on-target, off-tumor toxicity; a decreased risk of GVHD; and a lower risk of cytokine-mediated toxicities. Several early-phase clinical trials are currently underway, including trials evaluating CAR-NK cells targeting CD19, BCMA, CD33, and CD7.[23]

NKT cells express NK lineage markers in addition to a highly invariant αβTCR (in contrast to the highly diverse, antigen-specific TCRs of T cells). The invariant αβTCRs of NKT cells recognize lipid antigens presented by the MHC class-1-like molecule, CD1d. CAR-NKT cells can exhibit antitumor activity via CAR-mediated activation, but also via cytokine secretion leading to activation of T and NK cells. Similar to NK cells, allogeneic donor-derived NKT cells have a lower risk of alloreactivity compared with T cells. The feasibility of CAR-NKT has been demonstrated in several pre-clinical studies, and initial clinical trials are currently underway.[23]

Gamma-delta T cells represent another potentially useful T-cell subset for use in both autologous and allogeneic CAR-T-cell therapy. γδ T cells represent a small peripheral blood T-cell subset with features of both the innate and adaptive immune system, expressing NK cell receptors that target stress-inducible surface molecules, while also expressing clonally rearranged γδ TCRs that recognize antigens in an MHC/HLA-independent fashion. The HLA-independence of γδ TCRs likely explains the lower potential for alloreactivity. Gene-modified γδ T cells have been evaluated in multiple pre-clinical studies, though this approach is still early in development.[23]

Adoptive cellular therapy with VSTs has been used for many years to target CMV, EBV, and other viruses common after allogeneic HSCT. VSTs were initially derived from allogeneic stem cell donors, but patients have also been successfully treated with banked VSTs derived from partially HLA-matched healthy donors. Because the latter approach is associated with a low incidence of GVHD, allogeneic CAR-VSTs have been identified as another potentially useful "off-the-shelf" cellular therapy strategy with a low risk of complications related to alloreactivity.[23]

Optimizing T-Cell Trafficking and Overcoming Tumor Immune Evasion

The expression of transgenic TCRs or CARs in T lymphocytes confers potent cytotoxic activity and potential long-term persistence to these cells. However, other functional T-cell properties may need to be addressed to maximize their antitumor effects. Table 26.3 and Fig. 26.1 summarize some of the T-cell modifications that may optimize the antitumor activity of T lymphocytes. For example, CD19-specific CAR-T cells may be more effective for the treatment of ALL than of lymphoma in part because they more efficiently eliminate tumor cells from the circulation and bone marrow than the lymph nodes. Many lymphomas are characterized by a particular chemokine milieu to which engineered T cells can be adapted. For example, Hodgkin lymphomas may produce high levels of TARC, and T cells coexpressing a CAR specific for the Hodgkin disease–associated CD30 antigen and a transgenic chemokine receptor CCR4 have significantly enhanced traffic king to the tumor and consequently better antitumor activity in animal models.[10] Even when tumor-specific T cells efficiently reach the tumor environment, other tumor-associated factors may hamper T-cell survival and function. For example, many tumors, including hematologic malignancies, and their tumor-associated stroma produce TGF-β, which favors the development of immune tolerance and T-cell anergy, inducing T effector cell growth arrest with induction of regulatory T cells. Transfection of a dominant negative form of TGF-β RII (dnTGF-β RII) confers resistance to the antiproliferative effects of TGF-β and improves the persistence of T cells and antitumor effects in preclinical models.[10] Such an approach has been studied in patients with refractory EBV-associated Hodgkin lymphoma, with the infusion of TGF-β-resistant tumor-specific T cells leading to clinical responses in four out of seven evaluable patients, two of which achieved durable complete responses lasting over 4 years.[24]

Achievement of sustained clinical responses upon T-cell transfer is strongly dependent on in vivo T-cell expansion and persistence, which in turn requires the infused T cells to contain a population with a stemness/memory signature and the availability of cytokines that sustain T-cell replication and survival. We do not yet know the optimal means by which stem/memory T cells can be preserved before adoptive transfer.[25,26] Nonetheless it is clear that infusion of tumor-specific T cells in a lymphodepleted host benefits T-cell expansion, likely because the infused T cells can exploit the favorable homeostatic cytokine milieu (including production of IL-7 and IL-15) and the transient depletion of regulatory T cells. Exogenous cytokines such as recombinant IL-2 can also be infused but may cause significant toxicity and concomitant expansion of regulatory T cells. Recombinant IL-15 infusions were anticipated to be more effective and better tolerated than IL-2, but toxicity remains problematic.[27] Thus investigators have developed T-cell engineering strategies that make tumor-directed T cells which produce their own cytokines or express receptors for specific cytokines.[10] While these approaches are effective in preclinical models, and multiple clinical trials are ongoing, we do not yet know if they can replace or augment the use of lymphodepleting agents before adoptive transfer.

The molecular pathways responsible for the regulation and contraction of the T-cell immune response (immune check-points) have become a major focus of effective immunotherapies, and monoclonal antibodies that interrupt pathways such as the CD28/CTLA-4 and the PD-1/PD-L1 axes have emerged as potent new agents for the treatment of cancer, inducing sustained clinical responses in tumors likely mediated by the functional release of suppressed tumor-specific T cells recognizing neoantigens.[1] Many investigators therefore believe

TABLE 26.3 Causes of Immunosuppression in Cancer Patients

Immunosuppression Induced by Tumors
Release of chemokine by tumor cells that attract immunosuppressive T lymphocytes
Antigen-specific CD4+/CD8+ T-cell tolerance
Defective proximal TCR signaling (decreased expressions of CD3δ chain, p56^{lck}, p59^{fyn} tyrosine kinases)
Impairment of antigen-processing machinery (TAP, LMP2, LMP7) or downregulation of MHC molecules and costimulatory molecules
Activation of negative costimulatory signals (CTLA-4, PD-1, B7-H4, BTLA)
Tumor-derived immunosuppressive cytokines (TGF-β, IL-10, VEGF, PGE_2)
Expression of immunomodulatory or proapoptotic molecules by tumor (tryptophan-depleting enzyme IDO, galectin-1, FasL, TRAIL)
Recruitment and expansion of immunosuppressive cell populations (regulatory T cells, myeloid/plasmacytoid dendritic cells)
Immunosuppression Induced by Therapy
Neutropenia, depletion and functional impairment of monocytes
Hypogammaglobulinemia (decreased levels of IgA and IgM)
Defective T-ell-mediated immune response

BTLA, B- and T-lymphocyte attenuator; *CTLA*, cytotoxic T lymphocyte antigen; *FasL*, Fas-Fas ligand; *Ig*, immunoglobulin; *IL*, interleukin; *LMP7*, latent membrane protein 7; *MHC*, major histocompatibility complex; *PD-1*, programmed death-1; *PGE2*, prostaglandin E_2; *TAP*, transporter associated with antigen processing; *TCR*, T-cell receptor; *TGF-β*, transforming growth factor-β; *TRAIL*, tumor necrosis factor–related apoptosis-inducing ligand; *VEGF*, vascular endothelial growth factor.

that the adoptive transfer of tumor-specific T cells and potentially CAR-T cells generated ex vivo will synergize with infusion of checkpoint antibodies, and this is an active area of clinical research.

Despite the remarkable successes of the CAR-T cell therapies outlined above and in Table 26.1, most patients will ultimately relapse. In addition to the factors discussed above, loss of expression of the target antigen is another important mechanism by which tumors may develop resistance to targeting by transgenic TCR- or CAR-expressing T cells. Such therapies exert significant selection pressure, and patients with B-ALL who have relapsed following CD19-directed CAR–T-cell therapy have been found to harbor a variety of potential genetic alterations, including frameshift mutations leading to a loss of the CD19 transmembrane domain, expression of splice variants with a loss of the exon encoding the epitope targeted by the CAR scFv, or splice variants preventing expression of the anchoring transmembrane domain.[18] In AML and MM especially, there is also concern for so-called leukemic and myeloma "stem cell" populations, respectively. These "stem cell" populations are hypothesized to have increased resistance to traditional chemotherapy agents, but may also lack expression of cell surface proteins targeted by existing CAR-T therapies. To overcome these potential limitations, there has thus been significant interest in bispecific CAR-T cells and multiple technical variations of this concept are under investigation. There are currently multiple clinical trials underway evaluating bispecific CAR-T cells, primarily for B-cell malignancies (e.g., CD19/CD20 and CD19/CD22).[18]

T Lymphocytes, Transfer of Safety Genes, and Strategies to Improve Tumor Cell Specificity

A major problem of any successful cell therapy is that adverse events produced by the infused cells may persist and worsen if the cells survive and proliferate. A classic example is the GVHD that occurs when allogeneic donor T cells are transferred with the hematopoietic graft. It is also clear, however, that even nonalloreactive T cells may cause serious and even lethal toxicities, particularly if they are genetically modified to target highly expressed self-antigens present both on tumors and normal tissues. Examples include CRS and ICANS as discussed above with CAR-T-cell therapy, as well as potential on-tumor, off-target toxicities. In addition, efforts to enhance the survival and expansion of T cells could lead to uncontrolled expansion of the manipulated T cells, an event that might even occur as a result of retroviral genotoxicity alone, although this has been observed primarily in clinical studies of hematopoietic stem cells transduced by murine oncoretroviral vectors and has not emerged as a significant limitation of CAR-T-cell therapy. For all these reasons, there has been increasing interest in the incorporation of safety switches or suicide genes in any T cell that is adoptively transferred to humans.

Safety or suicide genes have been best studied in the recipients of DLI in patients with hematologic malignancies relapsed after allogeneic HSCT to prevent the occurrence of GVHD. Adequate doses of donor T cells can only be safely given if there is some means by which unwanted alloreactivity can be abrogated in vivo. Efforts have been made to achieve this aim by genetically modifying T cells through the introduction of suicide genes.

Herpes simplex thymidine kinase (HSVtk) was the first such transgene to be studied in the context of adoptive cellular therapy.[28] HSVtk phosphorylates specific nucleoside analogues, including ganciclovir, to nucleoside monophosphates. These compounds block effective DNA synthesis and kill dividing cells. In several clinical trials, this gene has been transferred to donor T lymphocytes, which have then been given to the allogeneic stem cell transplant recipient to prevent or treat relapse. While HSVtk gene-modified T cells have been shown to persist in the circulation in most patients, and to be removed after the administration of ganciclovir, often with an improvement in GVHD,[28] several problems have limited the use of this approach. HSVtk is a viral protein and in some patients, a cell-mediated immune response against HSVtk is detected, causing undesired premature elimination of transgenic cells. Other drawbacks of HSVtk include the unintended elimination of gene-modified cells when ganciclovir is used for treatment of CMV reactivation, ganciclovir resistance that may occur from truncated HSVtk generated from cryptic splice donor and acceptor sites, and slow elimination of transgenic cells as HSVtk requires DNA synthesis to be active; such delayed activity may be undesirable if T cells are acutely toxic. Alternative strategies have included the genetic modification of T cells to express CD20 or truncated epidermal growth factor receptor (EGFRt), allowing the use of rituximab or cetuximab, respectively, to eliminate the modified T cells via complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC). While these approaches are being incorporated into multiple ongoing clinical trials, there are significant potential limitations, including the biodistribution of the infused monoclonal antibody and on-target toxicity from the antibody.

Investigators attempted to overcome some of these limitations by developing new suicide genes based on human molecules that are potentially less immunogenic and which overcome the limitations related to antibody bioavailability. In particular, suicide genes have been generated based on chimeric molecules derived from human proteins involved in the apoptotic pathway, which have been modified to be activated by a small molecule (inducible Fas and inducible Caspase9 [iC9]). Two clinical studies have used the *iC9* gene, which consists of the human FK506-binding protein sequence with an F36V mutation, connected to human caspase-9 deleted for its endogenous activation and caspase-activating recruitment domain. FKBP12-F36V binds with high affinity an otherwise bioinert small molecule dimerizing agent (AP1903). In the presence of the drug, the iCasp9 promolecule dimerizes and activates the intrinsic apoptotic pathway leading to cell death. The study infused donor-derived iCaspase9-T cells after haploidentical stem cell transplant and if patients developed GVHD, administered a single dose of the dimerizing drug. There was rapid destruction of greater than 95% of the cells with prompt resolution of GVHD and faster immune reconstitution that allowed robust protection against opportunistic infection.[4] This system has been incorporated into CAR-T-cell constructs being evaluated in multiple ongoing clinical trials, including an ongoing trial of CD19-specific CAR-T cells for relapsed/refractory ALL.

The safety genes discussed above will likely become increasingly important tools to minimize potentially severe toxicities such as CRS and ICANS. However, these strategies can only be utilized once such toxicities have already developed. A preventative strategy will depend on an improved understanding of the mechanisms underlying these syndromes.

Although less of a concern for B-cell malignancies given the relative tolerability of the off-tumor, on-target toxicity directed at normal B cells that accompanies CD19-directed cellular therapies, potential on-target toxicities remain a significant barrier toward the wider applicability of TCR- and CAR-engineered T-cell therapies. For most other malignancies, the applicability of CAR-T-cell therapies has been limited by the difficulty in identifying a single target antigen that can be safely targeted without the risk of unacceptable on-target toxicities. There has thus been significant pre-clinical work toward the development of strategies that could improve the specificity of CAR-T cells for tumor cells. Dual antigen recognition strategies have been hypothesized that would allow better discrimination between tumor cells and normal tissue. One compelling pre-clinical example involves a combinatorially activated T-cell circuit, referred to as a "two antigen AND-gate circuit," in which activation of a synthetic Notch receptor for one antigen induces expression of a CAR targeted toward a second antigen.[29] At this point, however, the clinical feasibility of such strategies has yet to be demonstrated.

FUTURE APPLICATIONS AND IMPLEMENTATION OF CELL THERAPIES FOR CANCER

Beginning with the approval of the first cell therapy for cancer in 2010 (sipuleucel-T), followed by the approval of the first CAR-T-cell therapy in 2017 (tisagenlecleucel), and now in the setting of multiple subsequent FDA approvals and hundreds of ongoing clinical trials involving cellular immunotherapies, many reporting unprecedented clinical responses, it has become increasingly clear that this methodology is finally taking its place as an important component of cancer therapeutics. T-cell therapies with engineered T cells for B-cell lymphoid and plasma-cell malignancies are currently a clinical reality and T-cell therapies targeting many other malignancies are being evaluated in clinical trials. Despite this trend, much remains to be done to ensure the effectiveness and safety of these therapies. Equally important, and as illustrated by the fate of sipuleucel-T, much remains to be learned about development of a sustainable economic model for their affordable and broad clinical application. Nonetheless the integration of cellular therapies with other biologic agents and small molecules continues to offer the prospect of truly transformative therapies for the treatment of hematologic and other malignancies.

ACKNOWLEDGMENT

Dr Brenner's disclosures can be found at https://www.bcm.edu/academic-centers/cell-and-gene-therapy/research/disclosure-of-outside-interests.

REFERENCES

1. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med*. 2012;366:2443–2454.
2. Bargou R, Leo E, Zugmaier G, et al. Tumor regression in cancer patients by very low doses of a T cell-engaging antibody. *Science*. 2008;321:974–977.
3. Maude SL, Frey N, Shaw PA, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med*. 2014;371:1507–1517.
4. Di Stasi A, Tey SK, Dotti G, et al. Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med*. 2011;365:1673–1683.
5. Bertaina A, Merli P, Rutella S, et al. HLA-haploidentical stem cell transplantation after removal of αβ+ T and B cells in children with nonmalignant disorders. *Blood*. 2014;124:822–826.
6. Bachireddy P, Ennis C, Nguyen VN, et al. Distinct evolutionary paths in chronic lymphocytic leukemia during resistance to the graft-versus-leukemia effect. *Sci Transl Med*. 2020 12:eabb7661.
7. Rapoport AP, Aqui NA, Stadtmauer EA, et al. Combination immunotherapy using adoptive T-cell transfer and tumor antigen vaccination on the basis of hTERT and survivin after ASCT for myeloma. *Blood*. 2011;117:788–797.
8. Zhang Y, Schmidt-Wolf IGH. Ten-year update of the international registry on cytokine-induced killer cells in cancer immunotherapy. *J Cell Physiol*. 2020;235:9291–9303.
9. Morgan RA, Dudley ME, Wunderlich JR, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science*. 2006;314:126–129.
10. Dotti G, Gottschalk S, Savoldo B, et al. Design and development of therapies using chimeric antigen receptor-expressing T cells. *Immunol Rev*. 2014;257:107–126.
11. Arber C, Feng X, Abhyankar H, et al. Survivin-specific T cell receptor targets tumor but not T cells. *J Clin Invest*. 2015;125:157–168.
12. Parkhurst MR, Yang JC, Langan RC, et al. T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. *Mol Ther*. 2011;19:620–626.
13. Chapuis AG, Egan DN, Bar M, et al. T cell receptor gene therapy targeting WT1 prevents acute myeloid leukemia relapse post-transplant. *Nat Med*. 2019;25:1064–1072.
14. Rapoport AP, Stadtmauer EA, Binder-Scholl GK, et al. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *Nat Med*. 2015;21:914–921.
15. Bendle GM, Linnemann C, Hooijkaas AI, et al. Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. *Nat Med*. 2010;16:565–570. 1p following 70.
16. Stadtmauer EA, Fraietta JA, Davis MM, et al. CRISPR-engineered T cells in patients with refractory cancer. *Science*. 2020;367 eaba7365.
17. Linette GP, Stadtmauer EA, Maus MV, et al. Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. *Blood*. 2013;122:863–871.
18. Frigault MJ, Maus MV. State of the art in CAR T cell therapy for CD19+ B cell malignancies. *J Clin Invest*. 2020;130:1586–1594.
19. Munshi NC, Anderson Jr LD, Shah N, et al. Idecabtagene vicleucel in relapsed and refractory multiple myeloma. *N Engl J Med*. 2021;384:705–716.
20. Grover NS, Tschernia N, Dotti G, et al. Extending the promise of chimeric antigen receptor T-cell therapy beyond targeting CD19(+) tumors. *J Clin Oncol*. 2021;39:499–513.
21. Perna F, Berman SH, Soni RK, et al. Integrating proteomics and transcriptomics for systematic combinatorial chimeric antigen receptor therapy of AML. *Cancer Cell*. 2017;32:506–519. e5.
22. Kim MY, Yu KR, Kenderian SS, et al. Genetic inactivation of CD33 in hematopoietic stem cells to enable CAR T cell immunotherapy for acute myeloid leukemia. *Cell*. 2018;173:1439–1453. e19.
23. Perez C, Gruber I, Arber C. Off-the-shelf allogeneic T cell therapies for cancer: opportunities and challenges using naturally occurring "universal" donor T cells. *Front Immunol*. 2020;11:583716.
24. Bollard CM, Tripic T, Cruz CR, et al. Tumor-specific T-cells engineered to overcome tumor immune evasion induce clinical responses in patients with relapsed Hodgkin lymphoma. *J Clin Oncol*. 2018;36:1128–1139.
25. Gattinoni L, Lugli E, Ji Y, et al. A human memory T cell subset with stem cell-like properties. *Nat Med*. 2011;17:1290–1297.
26. Xu Y, Zhang M, Ramos CA, et al. Closely related T-memory stem cells correlate with in vivo expansion of CAR.CD19-T cells and are preserved by IL-7 and IL-15. *Blood*. 2014;123:3750–3759.
27. Conlon KC, Lugli E, Welles HC, et al. Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. *J Clin Oncol*. 2015;33:74–82.
28. Ciceri F, Bonini C, Stanghellini MT, et al. Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I-II study. *Lancet Oncol*. 2009;10:489–500.
29. Roybal KT, Rupp LJ, Morsut L, et al. Precision tumor recognition by T cells with combinatorial antigen-sensing circuits. *Cell*. 2016;164:770–779.

PART IV DISORDERS OF HEMATOPOIETIC CELL DEVELOPMENT

CHAPTER 27

BIOLOGY OF ERYTHROPOIESIS, ERYTHROID DIFFERENTIATION, AND MATURATION

Thalia Papayannopoulou and Anna Rita Migliaccio

The production of erythroid cells is a dynamic and exquisitely regulated process. The mature red cell is the final phase of a complex but orderly series of genetic events that is initiated when a multipotent stem cell commits to the erythroid program. Expression of the erythroid program occurs several divisions later in a greatly amplified population of erythroid cells, which have a characteristic form and structure, maturation sequence, and function. These maturing cells are termed *erythroid precursor cells* and *reticulocytes*. Terminally differentiated cells have a finite life span, and they are constantly replenished by influx from earlier compartments of progenitor cells that are irreversibly committed to express the erythroid phenotype. During ontogeny, successive waves of erythropoiesis occur in distinct anatomic sites. Erythroid cells developing in these sites have distinguishable phenotypes and intrinsic programs that are dependent on gestational time and their microenvironment. At each site, erythroid cells are in intimate contact with other cells (e.g., stromal cells, hematopoietic accessory cells, and extracellular matrix) comprising their microenvironment. Within this microenvironment, erythroid development is influenced by cytokines, which are either elaborated by microenvironmental cells or produced elsewhere and then entrapped in the extracellular matrix.

Knowledge of the properties of erythroid progenitor and precursor cells and their complex interactions with the microenvironment is essential for understanding the pathophysiology of erythropoiesis. Aberrations in the generation and/or amplification of fully mature and functional erythroid cells or in the regulatory influences of microenvironmental cells or their cytokines/chemokines form the basis for various clinical disorders, including aplasias, dysplasias, and neoplasias of the erythroid tissue.

ERYTHROID CELL COMPARTMENTS

Erythropoiesis involves progressive changes in the epigenome, transcriptome, proteasome, and metabolome culminating in changes in the architecture of the cells which acquire the morphology of biconcave red cells unlike that of any other cell type (Fig. 27.1). Changes at the epigenomic and transcriptomic levels occur mostly at the levels of progenitor cells in a process defined commitment. Changes at the proteosomic, metabolic, and structural levels occur instead at the precursor cell level and involve a process defined terminal erythroid maturation. Both commitment and terminal erythroid maturation are carefully regulated by unique intrinsic (transcription factors) and extrinsic (growth factors and microenvironmental) cues. This detailed regulation confers great levels of plasticity that, on one hand, allow the system to promptly respond to various environmental challenges, but, on the other, expose the system to injuries.

Phenotypic and Functional Properties of Erythroid Progenitor Cells

These cells are functionally situated between the multipotent stem cell and the morphologically distinguishable erythroid precursor cells (Chapter 9). This compartment contains a spectrum of cells with a parent-to-progeny relationship, all committed to erythroid differentiation. A complete understanding of how erythroid commitment is achieved at the biochemical or molecular level is finally starting to emerge in all its complexity (see intrinsic control of erythropoiesis) (most of the references published before 2016 are available in Papayannopoulou and Migliaccio[1]). Although all erythroid progenitor cells share the irreversible commitment to express the erythroid phenotype, the properties of these cells progressively diverge as the cells become separated by several divisions. Over time, increases in the sophistication of the technologies used to determine these properties have also increased the precision with which we define these cells.

Erythroid progenitor cells are sparse and difficult to isolate in sufficient purity and numbers for study. For these reasons, the existence and characteristics of these cells were first inferred by functional assays based on their ability to generate hemoglobinized progeny in vitro in clonal erythroid cultures. Two classes of progenitors have been identified using this approach. The first, more primitive class consists of the burst-forming unit-erythroid (BFU-E), named for the ability of BFU-E to give rise to multiclustered colonies (erythroid bursts) of hemoglobin-containing cells. BFU-E represent the earliest progenitors committed exclusively to erythroid differentiation and a quiescent reserve, with only 10% to 20% in cycle at any given time. However, once stimulated to proliferate in the presence of appropriate cytokines, BFU-Es demonstrate a significant proliferative capacity in vitro, giving rise to colonies of 30,000 to 40,000 cells, which become fully hemoglobinized after 2 to 4 weeks, with a peak incidence at 14 to 16 days. They have a limited self-renewal capacity; at least a subset of BFU-E is capable of generating secondary colonies upon replating. In contrast to this class of progenitor cells, a second, more differentiated class of progenitors consists of the colony-forming unit–erythroid (CFU-E). Most (60% to 80%) of these progenitors already are in cycle and thus proliferate immediately after initiation of culture, forming erythroid colonies within 7 days. Because CFU-E are more differentiated than BFU-E, they require fewer divisions to generate colonies of hemoglobinized cells, and the colonies are small (8 to 64 cells per colony).

Although BFU-E and CFU-E appear distinct from each other, in reality progenitor cells constitute a continuum, with graded changes in their properties. Only progenitor cells at both ends of the differentiation spectrum have distinct properties. Perhaps the earliest cell with the potential to generate hemoglobinized progeny is an oligopotent progenitor, which is capable of giving rise to mature cells of at least one

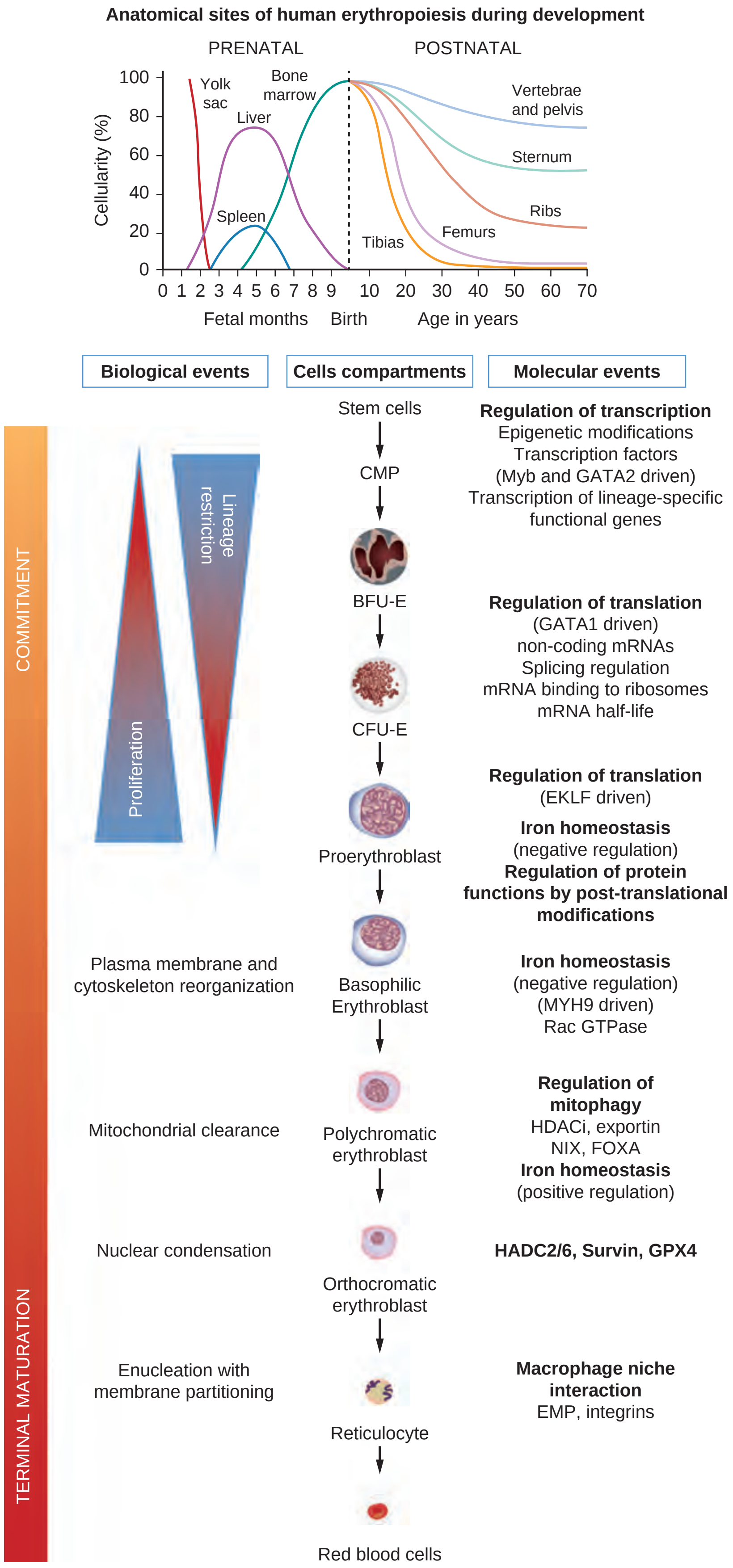

Anatomical sites of human erythropoiesis during development
PRENATAL
POSTNATAL
Cellularity (%)
100
80
60
40
20
0
Yolk sac
Bone marrow
Liver
Spleen
Vertebrae and pelvis
Sternum
Ribs
Femurs
Tibias
0 1 2 3 4 5 6 7 8 9
10 20 30 40 50 60 70
Fetal months
Birth
Age in years
Biological events
Cells compartments
Molecular events
COMMITMENT
TERMINAL MATURATION
Lineage restriction
Proliferation
Stem cells
CMP
BFU-E
CFU-E
Proerythroblast
Basophilic Erythroblast
Polychromatic erythroblast
Orthocromatic erythroblast
Reticulocyte
Red blood cells
Regulation of transcription
Epigenetic modifications
Transcription factors
(Myb and GATA2 driven)
Transcription of lineage-specific functional genes
Regulation of translation
(GATA1 driven)
non-coding mRNAs
Splicing regulation
mRNA binding to ribosomes
mRNA half-life
Regulation of translation
(EKLF driven)
Iron homeostasis
(negative regulation)
Regulation of protein functions by post-translational modifications
Plasma membrane and cytoskeleton reorganization
Iron homeostasis
(negative regulation)
(MYH9 driven)
Rac GTPase
Mitochondrial clearance
Regulation of mitophagy
HDACi, exportin
NIX, FOXA
Iron homeostasis
(positive regulation)
Nuclear condensation
HADC2/6, Survin, GPX4
Enucleation with membrane partitioning
Macrophage niche interaction
EMP, integrins

Figure 27.1 SNAPSHOT OF HUMAN ERYTHROPOIESIS. *Top panel:* Summary of the anatomic sites sequentially recruited for erythropoiesis during human ontogeny (see Ontogeny of Erythropoiesis). *Bottom panel:* Sequence of the distinctive phases involved in the generation of a red blood cell starting from the hematopoietic stem cell. The biological events are summarized on the left, the cellular compartments are depicted in the middle, and the major molecular events are summarized on the right. See Erythroid Cell Compartments and Intrinsic Control of Erythropoiesis for further detail.

other lineage (granulocytic, macrophage, or megakaryocytic) in addition to the erythroid. This progenitor, a multilineage colony-forming unit (CFU) called a *colony-forming unit–granulocyte, erythrocyte, macrophage, megakaryocyte* (CFU-GEMM) or *common myeloid progenitor*, and the most primitive BFU-E have physical and functional properties that are shared by both pluripotent stem cells and progenitor cells committed to non-erythroid lineages. These properties include high proliferative potential, low cycling rate, response to a combination of cytokines, and presence of specific surface antigens or surface receptors. In contrast, the most differentiated CFU-E have many similarities with erythroid precursor cells and have little in common with primitive BFU-E. Their proliferative potential is limited, they cannot self-renew, they lack the cell surface antigens common to all early progenitors, and they are exquisitely sensitive to erythropoietin (EPO).

Although clonal erythroid cultures are indispensable for the study of erythroid progenitors, they do not faithfully reproduce the in vivo kinetics of red cell differentiation/maturation, and many maturing cells have a megaloblastic appearance and lyse before they reach the end stage of red cell development. In vivo, erythropoiesis probably occurs faster than predicted from culture data. For example, studies in dogs with cyclic hematopoiesis, a genetic stem cell defect leading to pulses of hematopoiesis (Chapter 30), provide evidence that BFU-E mature to CFU-E over 2 to 3 days in vivo, although this process may require 5 to 6 days in canine marrow cultures.

BFU-E and their immediate progeny (but not CFU-E) are motile cells found in significant numbers in peripheral blood. As with BFU-E, the ability of stem cells and progenitor cells to circulate is physiologically important for the redistribution of marrow cells in cases of local damage to the microenvironment and for reconstitution of hematopoiesis after transplantation. The spectrum of BFU-E in circulation probably is narrower (consisting mostly of early, quiescent BFU-E) than that of BFU-E in the bone marrow; otherwise, their properties are similar to those of marrow BFU-E. The number of circulating BFU-E (along with other progenitors and stem cells) can increase to significant levels after cytokine/chemokine treatments and after chemotherapy, a finding that has been exploited for transplantation purposes. At present, mononuclear cells contained in the blood from subjects mobilized with granulocyte colony-stimulating factor (G-CSF) are routinely used as a source of stem/progenitor cells for autologous and allergenic transplantation, alone or in combination with AMD3100, an inhibitor of CXCR4, the receptors expressed on the progenitor cells that by binding with SDF1 (also known as CXCL12) produced by stromal cells, retain the cells in the bone marrow (Chapter 16). Apart from SDF1 another important pathway for retaining stem/progenitor cells in BM is represented by VLA-4 integrins ($\alpha 4\beta 1$).[2] These pathways are independently regulated, but they work in concert.

In addition to forming colonies in semisolid medium, hematopoietic progenitors from different sources can generate erythroid cells in liquid culture. Liquid cultures do not allow progenitor cell enumeration but may generate more differentiated cells per progenitor cell than occurs in semisolid cultures. This culture system is often used for modeling erythroid disorders and in theory may generate numbers of erythroid cells equivalent to 1 unit of blood from discarded stem cell sources (cord blood and leukoreduced products of blood donations),[3,4] which has led to the belief that red blood cells (RBCs) generated ex vivo may one day be used for transfusion therapies.

The hematopoietic compartments can also be defined on the basis of their antigenic profiling based on the expression on the plasma membrane of specific proteins recognized by monoclonal antibodies. These studies have first provided a robust definition of the progenitor cells present in bone marrow of normal mice where the prospectively isolated cells may be functionally tested not only in vitro but also in vivo.

The best representative antigen expressed by human BFU-E is the CD34 antigen, which has been successfully exploited for isolation of BFU-E and other progenitors. CD34 is a highly *O*-glycosylated cell surface glycoprotein expressed by all hematopoietic progenitors and vascular endothelial cells that may serve as a bumper that prevents close contacts among these cells. Additional clinically important antigens expressed by human erythroid progenitors are the histocompatibility antigens. Like other hematopoietic progenitors, BFU-E display human leukocyte antigen (HLA) class I (A, B, C) and class II (DP, DQ, DR) antigens on their surface. Class II antigens (especially the products of the DR locus), in contrast to class I, are variably expressed by BFU-E. Furthermore, use of antibodies or conjugated ligands determined that, as most of the other immature hematopoietic progenitor cells, BFU-E display receptors for KIT ligand (KL, also known as stem cell factor, SCF), EPO, granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin (IL)-3 (the alpha and common beta subunit for both), gp130, the signaling subunit of the IL-6 receptor, and IL-11. They share with late colony-forming unit–megakaryocyte (CFU-Mk) progenitors the expression of the thrombopoietin (TPO) receptor (c-Mpl or TPO-R) and glycoprotein Iib/IIIa (CD41), an antigen previously thought to be restricted to megakaryocytes, which marks the divergence between definitive hematopoiesis and endothelial cells during development. However, the majority of BFU-E, in contrast to myeloid progenitors, do not express the restricted hematopoietic phosphatase CD45R and aldehyde dehydrogenase activity, an enzyme lost in humans during the transition from CMP to MEP.

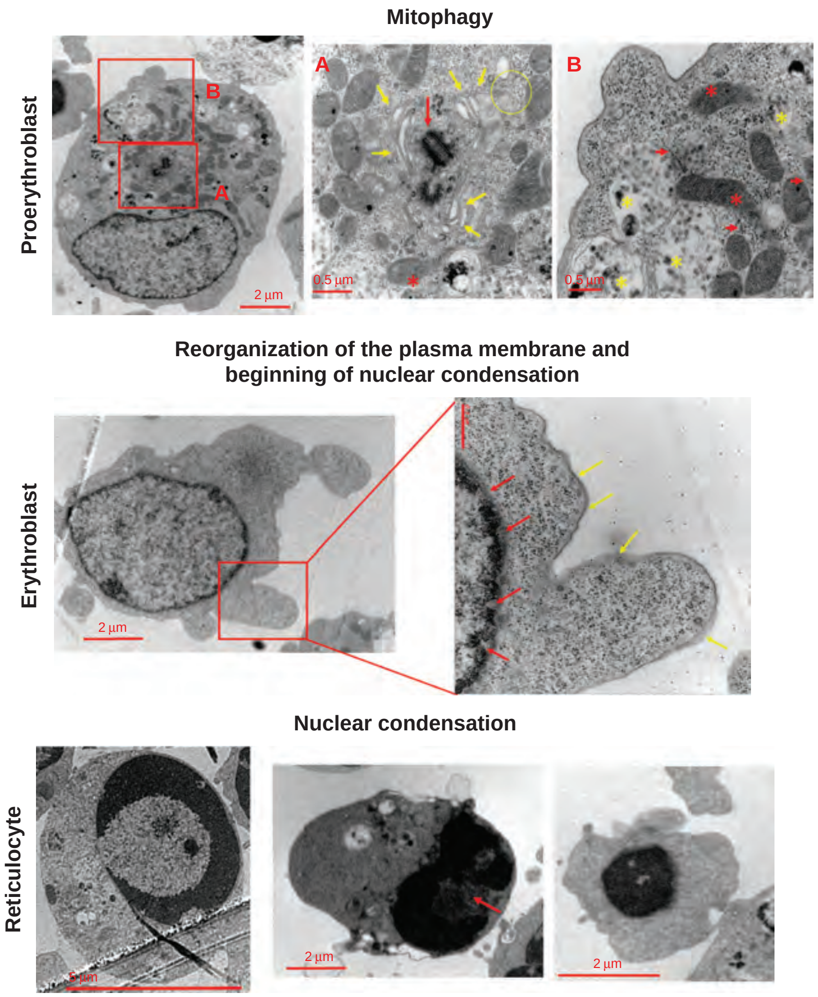
As BFU-E mature to the CFU-E stage, they begin to express surface proteins characteristic of erythroblasts, the morphologically recognizable erythroid cells. For example, CFU-E expresses Rh antigens and the erythroid-specific sialoglycoprotein glycophorin A. Blood group antigens of the ABH Ii type are detectable in a subset of CFU-E. In contrast, CD34 molecules, class II antigens, and certain growth factor receptors (i.e., IL-3R, KIT) are greatly diminished or virtually absent at the CFU-E stage. Conversely, the EPO-receptor is greatly expressed at the CFU-E stage but barely detectable on BFU-E. Thus, CFU-E, in contrast to BFU-E, cannot survive in vitro even for a few hours in the absence of EPO.

In conclusion, the Weissman laboratory has defined the phenotype of human erythroid progenitors as lineage$^-$, CD34$^+$, CD38$^+$, IL-3 receptor α^-, and CD34RA$^-$ for MEP and Lineage$^-$, CD34$^+$, CD38$^+$, IL-3 receptor α^-, CD34RA$^-$, CD71$^{\text{intermediate}}$, and CD105$^+$ for erythroid restricted progenitor cells[5] while other investigators have defined the phenotype for human BFU-E as CD45$^+$, GPA$^-$, IL-3 receptor$^-$, CD34$^+$, CD36$^-$, and CD71$^{\text{low}}$ and that for CFU-E as CD45$^+$,GPA$^-$, IL-3R$^-$, CD36$^+$, and CD71$^{\text{high}}$.[6]

More recent analyses of single cell transcriptome profiling has identified significant levels of variability even in cells purified at homogeneity on the basis of rigorous antigenic criteria.[7–10] This analysis may lead to the identification of alternative differentiation routes that may play important roles under pathological or stress conditions.[11] These studies are still too preliminary and further information should shed more light on these issues and may require the development of specific cell culture conditions.[12]

THE MORPHOLOGICALLY RECOGNIZABLE ERYTHROID PRECURSOR CELL COMPARTMENT

The erythroid precursor cell compartment, also termed the *erythron*, includes cells that, in contrast to the erythroid progenitor cells, are defined by morphologic criteria. The earliest recognizable erythroid cell is the *proerythroblast*, which after four to five mitotic divisions and serial morphologic changes gives rise to mature erythroid cells. Its progeny includes basophilic erythroblasts, which are the earliest daughter cells, followed by polychromatophilic and orthochromatic erythroblasts. Their morphologic characteristics reflect the accumulation of erythroid-specific proteins (i.e., hemoglobin) and the decline in nuclear activity and are acquired through a process defined terminal erythroid maturation that involves elimination of unwanted organelles (mitochondria, ribosomes, and other intracytoplasmic organelles) by autophagy, intense membrane trafficking, cytoskeleton reorganization, and nuclear condensation (Fig. 27.2). The last mitotic division is an asymmetric partitioning of the remaining cell components between two morphologically distinct daughter



Mitophagy

Reorganization of the plasma membrane and beginning of nuclear condensation

Nuclear condensation

Figure 27.2 ULTRASTRUCTURAL DETAIL OF TERMINAL ERYTHROID MATURATION. *Top panels:* Transmission electron microscopy snapshots of one proerythroblast showing the overall cellular architecture of mitophagy. The red squares in the left panel (magnification at 2000X) are shown at greater magnification in the central (A) and right (B) panels (magnification 20,000X). The panel on the left shows the localization of the mitochondria at one pole of the cell surrounded by the cisternae of the rough endoplasmic reticulum and of autophagosomes. The greater magnification in A shows the centrioles *(red arrow)* surrounded by the cisternae of the Golgi apparatus *(yellow arrows)* and contoured by mitochondria, some of which are already included in autophagic vesicles *(red asterisk)*, and mature phagosomes *(yellow asterisk)*. The *yellow circle* indicates an immature phagosome. Panel B shows additional mitochondria *(red arrowheads)* surrounded by autophagic vesicles *(yellow asterisks)* containing portions of the rough endoplasmic reticulum. *Middle panels:* Transmission electron microscopy snapshots of one erythroblast showing morphological indications for reorganization of the plasma (*yellow arrows* on the right) and of the nuclear (*red arrows* on the right) membranes. The panel on the right shows the presence of a split plasma membrane and of large pores in the nuclear membrane localized in proximity of condensed chromatin areas. (Magnification 4400× on the left and 20,000× on the right, respectively). *Bottom panels:* Transmission electron microscopy of three reticulocytes at different stages of nuclear condensation. The nucleus of the reticulocyte on the left panel is localized at one pole of the cell and displays a large nucleolus in the middle of the euchromatin areas. The nucleus of the reticulocyte in the middle panel is almost completely condensed and surrounds a large autophagic vesicle *(red arrow)* while the nucleus of the reticulocyte in the right panel is completely condensed and is localized in the middle of the cytoplasm. Magnification, ×5800 in all the panels. *(Courtesy of Dr. Maria Zingariello).*

cells: one nucleated, the pyrenocyte, and one enucleated, the reticulocyte. During this last mitosis, the inactive dense nucleus of the orthochromatic erythroblast moves to one side of the cell and is extruded, encased by a thin cytoplasmic layer, the pyrenocyte, that is ingested by marrow macrophages through recognition antigens present in the pyrenocytes. Since all mammals have enucleated cells in their circulation, it is possible that enucleation provides an evolutionary advantage by allowing for greater red cell deformability when traveling through the small vasculature, and/or to minimize cardiac workload.

Maturation from proerythroblast to reticulocyte likely does not always adhere to a rigid sequence in which each division is associated with the production of two more differentiated and morphologically distinct daughter cells (i.e., basophilic erythroblast gives rise to two polychromatophilic ones). Rather, significant flexibility, both in the number and rate of divisions and in the rate of enucleation, may be allowed. Such deviations from the normal orderly maturation sequence may be dictated by the level of EPO or "stress" conditions. Thus, in cases of acute demand for red cell production (because of blood loss or hemolysis), the kinetics of formation of new reticulocytes is significantly more rapid. Resulting red cells may be larger (i.e., with increased mean corpuscular volume). This has led to the concept of "skipped" divisions. The orderly unilineage differentiation pathway shown in Fig. 27.1 is likely restricted to conditions of steady-state hematopoiesis. Similar to occurrences in the lymphoid system, alternative routes are taken under conditions of "stress."

The morphologic alterations that occur as erythroid precursor cells mature (see Fig. 27.2) are determined by complex biochemical changes, which accommodate the accumulation of erythroid-specific proteins and the progressive decline in proliferation. Compared with erythroid progenitor cells, erythroid precursor cells have been more accessible to study, and considerable information is available about their maturation-related biochemical changes.[13] Quantification by mass spectrometry of the content of 6130 erythroid-specific proteins during erythroid commitment and maturation has confirmed that protein modifications follow those observed at the transcriptome level with a breakpoint at the basophilic levels with as many as 1300 of them differentially partitioned between the reticulocyte and the pyrenocyte during enucleation.[14,15]

Plasma Membrane and Cytoskeleton Reorganization

The shape and deformability of red cells are determined by the appropriate assembly of their membrane proteins with the cytoplasmic cytoskeleton (Chapter 48). Red cells survive shear forces in the microvasculature because of two transmembrane complexes, ankyrin and 4.1, embedded in the lipid bilayer and attached to the cytoskeleton, ensuring its flexibility. The extracellular part of these complexes contains the clinically relevant blood group antigens determined by genetic polymorphisms in proteins of these complexes. The intracellular parts contain the docking sites for actin filaments, interconnected with $(\alpha1\beta1)_2$ tetramers forming a supporting network below the lipid bilayer of the plasma membrane.[16,17] Formation of this network is facilitated by the actin-motor protein non-muscle myosin IIA encoded by the *MYH9* gene.[18,19] A recently recognized player required for actin assembly in red cells, Rac GTPase, has been identified.

Most membrane cytoskeletal proteins (spectrin, glycophorin, band 3, band 4.1, and ankyrin) accumulate after the CFU-E stage (i.e., within the precursor cell compartment). Specifically, expression of membrane glycoproteins such as band 3 and band 4.1 is greatly enhanced at the later stages of erythroid maturation. Likewise, the quantity of polylactosaminoglycan, a specific carbohydrate chain that carries blood group ABH and Ii antigenic determinants, is much higher in mature erythrocytes than in erythroblasts (Chapter 111). Whereas a linear, virtually unbranched polylactosamine structure is present in fetal and newborn erythroid cells (reflected by i antigenic reactivity), a branched polylactosaminyl structure is present in adult erythroblasts (reflected by I antigenic reactivity), and branching increases further as maturation progresses.

Glycophorins, especially glycophorin A, are expressed fully at the CFU-E or proerythroblast level just before expression of globin, and few changes occur during maturation. In contrast, the membrane glycoproteins p105 and p95 decline during the later stages of maturation, and yet other membrane glycoproteins, such as vimentin (an intermediate filament protein), are totally lost. Loss of vimentin expression at the late erythroblastic stages most likely facilitates enucleation.

In addition to quantitative changes that occur during maturation, gradual switches in subunit composition of some cytoskeletal proteins occur. For example, exclusively erythroid subunits of α- and β-spectrin are displayed only in end-stage cells. Likewise, multiple transcripts of ankyrin or protein 4.1 have been identified, and the ratios of these transcripts change during maturation. Initial expression of many of these membrane components likely begins at the progenitor cell level. However, in these cells, final assembly may be discouraged because of the higher turnover of these proteins, which minimizes mutual interactions, or because of asynchrony in protein synthesis. Prevention of cytoskeletal assembly at these early stages may secure more membrane fluidity and cell motility needed during this proliferative phase of differentiation. Because molecular probes for many of the red cell cytoskeletal components have been developed, detailed information about the transcription and processing of most of these proteins is beginning to emerge. For example, band 3, the major anion transport protein of human erythrocytes, is a key component of a multicomplex that also contains protein 4.2. Appropriate display of this protein complex on the cell membrane is dependent on critical interactions established between newly synthesized band 3 and protein 4.2 already at the proerythroblast stage.

Expression of the majority of genes encoding cytoskeletal components is not restricted to red cells. Dissecting hematopoietic from non-hematopoietic consequences of abnormalities in these genes has been difficult, but the development of mouse models that mimic defects found in human diseases has been helpful in this respect. It has been presently identified that the erythroid specificity of the expression of these proteins is achieved through alternative splicing of the corresponding messenger RNA (mRNA) mediated by spliceosomes the expression of which increases with the progression of maturation.

Mitophagy and Ribosome and Mitochondria Clearance

The mature red cells do not contain mitochondria, ribosomes, and other cellular elements such as the spindle motors. These structures are eliminated by a specialized process of autophagy-defined mitophagy that begins at the pro-erythroblast levels (see Fig. 27.2) and involves clustering of the mitochondria pool at one pole of the cells where they start to display crests with morphological features predictive of degeneration. This region of the cell also contains the rough endoplasmic reticulum. These alterations are probably determined by increased reactive oxygen species (ROS) levels due to iron accumulation (Chapter 36). Mitochondria, and the surrounding rough endoplasmic reticulum, are then embedded into autophagic vesicle where they are posed for degradation. This process requires the proteins NIX and FOXA and is completed at the reticulocyte level (Figs. 27.1 and 27.2). As a consequence of mitophagy, the energy metabolism of erythroid cells becomes exquisitely dependent on the anaerobic pathway. Mitochondria are retained by RBCs in the blood of patients with sickle cell anemia and are thought to be responsible for increased oxygen consumption rates and ROS generation that mediate the high level of hemolysis in these patients.[20]

Nuclear Condensation and Enucleation

The process of erythroblast enucleation involves the remodeling of the nuclear membrane, which loses its connection with the plasma membrane and displays larger nuclear pores, chromatin condensation to form pyknotic nuclei, and formation of spindle-independent motors driving the separation of the reticulocyte from the pyrenocyte (see Fig. 27.2). Partitioning of erythroblast plasma membrane components to reticulocytes is regulated by the degree of skeletal linkage and by the Coimbra domain of band 3. The absence of band 3 in human erythrocytes has only been reported in a single homozygous band 3 Coimbra patient. Band 3 Coimbra is a V488M mutation in the fourth transmembrane helix of band 3 which in the heterozygous state results in a typical mild form of hereditary spherocytosis and a partial reduction in band 3 expression. In fact, red cells from patients with homozygous band 3 Coimbra express reduced levels of multiple cell surface antigens which are all rescued in vitro by forced expression of normal band 3. Chromatin condensation may require DNA demethylation, since with maturation the total methylation state of the DNA greatly decreases down to barely detectable levels and is mediated by the histone deacetylases (HDAC) 2 and 6,[21] suggesting that impairment of HDAC activity contributes to the anemia observed in epigenomic-based cancer treatments. Chromatin condensation begins in the region surrounding the nuclear membrane and also requires shedding from the DNA of key transcription factors that are exported to the cytoplasm, where they are posed for degradation, through dedicated pores (see Figs. 27.1 and 27.2).[22]

Recent evidence indicates that erythroblast enucleation is also dependent on appropriate vesicle trafficking. During enucleation vesicles and vacuoles move toward and accumulate close to the extruding nucleus and enucleation rates in vitro are reduced or increased by treating human reticulocytes with vesicle-trafficking inhibitors or with vacuolin-1, a cell-permeable lysosomal exocytosis inhibitor that induces vacuole fusion, respectively. In addition, survivin, a member of the inhibitor of apoptosis (IAP) family, is highly expressed in erythroid cells and is required for proper erythropoiesis.[23,24] Survivin forms a multi-protein complex with two proteins involved in endocytic vesicle trafficking, the epidermal growth factor receptor substrate15 (EPS15) and clathrin. This complex directly promotes enucleation since survivin deficiency causes defective enucleation which is rescued by vacuolin-1.[25]

Additional proteins that promote enucleation are GPX4,[26] PI3K and its downstream signaling p38α,[27,28] and HDAC6.[21] PI3K/p38α promote enucleation by downregulating the pro-apoptotic signal p21 while HDAC6 deacetylates and activates mDia2, which in turns promotes the association between actin and non-muscle myosin that, in the absence of the spindle degradation by mitophagy, provides the motor driving the separation between the reticulocyte and the pyrenocyte.

The enucleation process is caspase independent but erythroblast macrophage protein (EMP) dependent. EMP, also known as macrophage-erythroblast attacher (MAEA), is expressed by both erythroblasts and macrophages but recent evidence suggests that presence of EMP on erythroblasts (for homotypic interaction with macrophages) is dispensable and EMP+-macrophages interact through an as yet unidentified ligand present on erythroblasts.[29]

Reticulocytes are released in the blood, where they reduce the number of ribosomes to become mature red cells. This process is mediated by autophagic machinery, whereas engulfment of pyrenocytes, and subsequent degradation by macrophages, occurs only after pyrenocytes are totally disconnected from reticulocytes. Phosphatidylserine, the “eat me” flag for apoptotic cells, is also used for engulfment of pyrenocytes expelled from erythroblasts, whereas expression of CD47, the “eat me not” signal, by interacting with SIRP1α expressed by the macrophages, prevents engulfment and destruction of erythroblasts and reticulocytes.[29]

Presence of molecules enhancing adherence of erythroblasts to central macrophages are thought to be important mediators of the interaction of erythroblasts with macrophages. Several of these molecules have been described and summarized in recent reviews. Among these are the VLA4 interacting with vascular cellular adhesion molecule-1 (VCAM-1), the intercellular adhesion molecule-4 (ICAM-4) on erythroblasts interacting with αv and α4 on macrophages, the CD163 on macrophages serving as the Hb-Hp binding receptor, or the Palladin, on macrophages with an unclear counter receptor on erythroblasts.

Gene activity during erythroid maturation is dominated by globin expression. Globin represents less than 0.1% of protein at the proerythroblast level but constitutes 95% of all protein at the reticulocyte level. Globin expression has been extensively studied, and its gene regulation is well understood in molecular terms. Major steps in globin transcription and processing are known in considerable detail and are summarized elsewhere in this text (Chapter 34). The globin type synthesized by adult precursors is hemoglobin A (HbA; $\alpha_2\beta_2$). In addition, two other minor globin components, HbA$_2$ ($\alpha_2\delta_2$) and HbF ($\alpha_2\gamma_2$), are present. Of significant biologic interest are the low amounts of HbF that continue to be synthesized throughout life.

The small amount of HbF, which is present in all normal individuals, has the following characteristics: (1) It is confined to a small fraction of red cells, called *F cells*, which are detected by sensitive immunofluorescence assays or acid elution techniques and usually constitute 2% to 5% of all red cells. Within each F cell, HbF or γ-globin constitutes 14% to 25% of total globin. (2) The number of F cells is genetically determined, and the gene(s) linked or nonlinked to the β-locus is responsible for F-cell formation. (3) F cells do not display other features of “fetalness” because their membrane components and enzymes are characteristically adult. (4) Synthesis of HbF peaks earlier than that of HbA, so the proportion of HbF is higher in immature cells compared with mature, fully hemoglobinized cells. (5) F cells and cells that contain only HbA are not derived from distinct stem cell populations but from a common adult stem cell. Whether the latter will form F or non-F (i.e., A) cells is determined at the BFU-E level and throughout the CFU-E level. In vitro the great majority of BFU-E have the potential to express HbF, whereas in vivo only a very small proportion of red cells contain HbF. This potential appears to be lost during normal cell differentiation and maturation in vivo. This concept links the potential for HbF expression to the pathway of erythroid differentiation and thus may have implications for interpreting the reactivation of HbF that occurs in adults under diverse circumstances (e.g., after chemotherapy or with acute bleeding). Many of these circumstances seem to influence HbF levels by directly or indirectly modifying the kinetics of the normal differentiation/maturation process, or the result of changes in stress signaling molecules.

The patterns of transferrin receptor (TfR, CD71) and glycophorin A (CD235A) expression during erythroid maturation have been exploited to create flow cytometric criteria that distinguish the different populations of erythroid precursors in mice and men. By coupling size and forward scatter (both progressively reduced) with CD71 and TER-119, murine erythroid precursors were divided into the classes TER-119$^{\text{med}}$CD71$^{\text{high}}$, TER-119$^{\text{high}}$CD71$^{\text{high}}$, TER-119$^{\text{high}}$CD71$^{\text{med}}$, and TER-119$^{\text{high}}$CD71$^{\text{low}}$, which correspond to proerythroblasts and basophilic, chromatophilic, and orthochromatophilic erythroblasts, respectively. TER-119 recognizes a protein associated with the murine equivalent of glycophorin A. However, such distinction is not conserved in all mouse strains. For example, in C57Bl/6 mice, CD71 expression levels remain constant during maturation. CD44/glycophorin A expression provides a better flow cytometric definition of the maturation stage of murine erythroblasts. Although reduced levels of CD44 do not correlate with the maturation of human erythroblasts, it marks loss of proliferation potential of these cells. Double CD71/glycophorin A staining is therefore still used as criteria to define human erythroblast precursors by flow cytometry. However, the pattern of CD71 expression during erythroid maturation presents a high level of donor variability. Given that downmodulation of CD36 (the thrombospondin receptor) expression during erythroid maturation is relatively independent of genetic variability, an alternative flow cytometric definition of erythroblast subclasses is proposed by the phenotype CD36$^{\text{high}}$/glycophorin A$^{\text{medium}}$, CD36$^{\text{high}}$/glycophorin A$^{\text{high}}$, and CD36$^{\text{low}}$/glycophorin A$^{\text{high}}$, corresponding to basophilic, polychromatic, and orthochromatic erythroblasts, respectively. The most current phenotypes for human erythroid cells, defined as CD45$^{\text{neg}}$GPA$^{\text{pos}}$, progressing along the maturation pathway are: αIV integrin$^{\text{high}}$ and band3$^{\text{neg}}$ (pro-erythroblasts), αIV integrin$^{\text{high}}$ and band3$^{\text{low}}$ (early basophilic), αIV integrin$^{\text{high}}$ and band3$^{\text{med}}$ (late basophilic), αIV integrin$^{\text{med}}$ and band3$^{\text{med}}$ (polychromatophilic), and αIV integrin$^{\text{low}}$ and band3$^{\text{high}}$ (orthochromatic).[30] It should be noted that since expression of some of these markers is determined by the functional content of some erythroid transcription factors (such as EKLF), this definition may be valid for normal cells but may be impaired in cells from patients, especially those in which the intrinsic control is disturbed.[31] Flow cytometric criteria for reticulocytes and red cells are instead provided by size (reticulocytes and red cells are distinctively smaller than erythroblasts) and by lack of reactivity for DNA (both reticulocytes and red cells) and RNA (reticulocyte only) staining.

INTRINSIC CONTROL OF ERYTHROPOIESIS

The development of advanced and sensitive molecular biology techniques and flow cytometric criteria to prospectively isolate different erythroid cell compartments has allowed the identification of the sequence of events involved in the execution of the genetic program encoding the making of an erythroid cell. Evidence from the epigenetic profiling of prospectively isolated cells suggests that commitment to a specific hematopoietic lineage is accomplished not by acquisition of new genetic information but rather by restriction to specific programs from a wider repertoire available to pluripotent progenitor cells.

The restriction to the erythroid program occurs in multiple steps. It requires closing the configuration of not-relevant genetic loci while opening that of lineage-specific enhancers and establishing contact between these enhancers and the promoter region of the genes to allow recruitment of the mRNA polymerase. Gene transcription is then initiated by removing factors bound downstream to the promoter region that pause the mRNA polymerase, and finally, when the mRNA is transcribed, its translation is finely tuned by the efficiency of the ribosome machinery (Chapter 2).

Genome-wide analyses have recently detailed the timing when these epigenetic events occur during the process of erythroid maturation. Erythroid-specific enhancers and promoters are already in an active configuration at the progenitor levels. During erythroid maturation, the expression of these genes is mainly regulated posttranscriptionally (mRNA stability and splicing, and binding to the ribosome translational machinery). Erythroid-specific micro- and long-noncoding RNAs may play important roles in the posttranscriptional regulation of erythroid genes at late stages of maturation (see Fig. 27.1).

The first epigenetic step involved in preparing an erythroid-permissive chromatin configuration is represented by changes in the methylation state of specific DNA and histone proteins. Actively expressed genes are localized in areas on the chromosome in an open configuration. The DNA switch from a closed to an open configuration is determined by the tightness of its binding to the histones by which it is surrounded. A series of enzymes regulates the chromosome configuration state by modifying either the DNA (cytosine methylation mediated by specific methylases) or the histones (e.g., histone acetyltransferase [HAT] and deacetylase [HDAC], polycomb repressive complexes). HAT exerts a positive effect (promoting the formation of an open configuration state), whereas methylases and HDAC exert a negative effect (inducing a closed chromatin configuration state) on gene expression. A global methylation status of erythroid cells as they mature has been documented. The DNA methylation status increases as cells progress along the commitment process, probably to favor nuclear condensation, and late stages of terminal maturation.

The link between epigenetic and transcriptional control of gene expression in erythroid cells is emerging. The conformation of the chromatin of erythroid cells is shaped into positive and negative compartments, contact domains, and chromosomal loops[32] and is determined by negative and positive protein complexes, some of which, like the transcriptional repressor CTCF,[33] cohesion,[34,35] and YY1,[36,37] are common to all lineages, while others, glued together by the adaptor protein Ldb1, contain the erythroid transcription factors that, by binding to erythroid-specific consensus sequences, provide lineage specificity.[38]

The majority of erythroid-specific transcription factors has been identified from cloning of breakpoints or translocations associated with human leukemias or from expression libraries obtained from erythroid cell lines. The precise role exerted by each of these factors in erythropoiesis was later clarified by painstaking experiments with somatic cell fusions and in transgenic mice. Most recent data using single cell analyses and lineage tracing technologies have confirmed that cell fate decisions are determined by quantitative changes in lineage-specific transcription factor expression.[39,40]

Genome wide association studies identified polymorphism at the c-*MYB* locus as one of the traits significantly associated with the variability in erythroid traits observed in the human population. Since MYB is the only transcription factor known to regulate the expression of KIT, the receptor for KL (see extrinsic control of erythropoiesis), MYB is the very first transcription factor involved in establishing an erythroid-specific transcriptional landscape.[41,42]

Two genes of the GATA family of transcription factors, *GATA1* and *GATA2*, play important roles in programming erythroid differentiation. *GATA2* is expressed at high levels in multipotential progenitors and affects expansion of all hematopoietic lineages. With erythroid commitment, the expression ratios between *GATA2* and *GATA1* change and *GATA1* is the factor most abundantly expressed in CFU-E and more mature cells. The switch from *GATA2* to *GATA1* expression is known as the GATA switch and is thought to represent the event which initiates the translation of erythroid specific genes.

Once the commitment process is initiated by the transcription factor GATA1, the founder of the GATA family is of paramount importance. The GATA1 protein controls erythroid differentiation at several levels by controlling (in cooperation with GATA2) the proliferative capacity of erythroid progenitor/precursor cells, the apoptotic rate of erythroblasts, and the expression of lineage-specific genes. These effects are mediated through activation of expression of target genes by binding to specific sequences (WGATAR) present in the regulatory domains of virtually any erythroid gene. However, WGATAR binding sites are also present in genes specific for megakaryocytic, eosinophilic, mast cell, and dendritic lineages, as well as in genes expressed in testicular Sertoli cells. Insights into the specificity of GATA1 in erythroid differentiation have been provided by studies on the organization of WGATAR sites in erythroid-specific regulatory sequences. A minimal erythroid transcription-activation sequence that consists of a core-binding motif flanked by two canonical GATA1 binding sites has been identified. The core-binding motif is composed of one SCL binding site and one GATA binding site separated by 10 bp. Different domains of the GATA1 protein are responsible for binding to the core and the flanking sequences. At least three functional domains in the GATA1 protein have been identified: two zinc finger domains (amino-terminal [N-terminal] finger [NF] and C-terminal finger [CF]) and an active N-terminal domain.

The NF domain is required for association with Friend of GATA1 (FOG-1), a protein encoded by a gene identified using GATA1 as bait in the two-hybrid yeast assay. FOG-1 contains 10 zinc finger domains, only the first of which is required for GATA1 binding. The function of its other nine zinc finger domains is not clear because they appear to be dispensable in structure-function studies, but they are well conserved in evolution. The GATA1-FOG-1 heterodimeric complex binds to the two flanking sites of the minimal erythroid transcription activation domain. Experimentally induced genetic mutations, such as GATA1^{V205M}, impairing GATA1-FOG-1 interaction in mice lead to impaired megakaryocytopoiesis and absence of definitive erythropoiesis, whereas primitive erythropoiesis is normal. Rescue experiments indicate that GATA1^{V205M} newborns are severely anemic with anisocytosis and spherocytosis with striking reduction mainly in the expression of genes encoding membrane proteins, whereas expression of other erythroid-specific genes, such as *Alas2*, are not affected. These results indicate that DNA binding of the GATA1-FOG-1 complex is necessary for activation of a subset of GATA1 target genes in definitive erythroid cells but is dispensable for their activation in primitive erythroblasts.

The CF domain, on the other hand, recognizes and binds to the GATA site localized in the core of the minimal erythroid transcription sequence 10 bp downstream to the SCL binding site. SCL and GATA1 bind simultaneously to their respective sites of the core as multimeric complexes formed by SCL/E47/LMO2 on the one hand and by GATA1/LMO2 on the other. Binding of the two complexes to the core is stabilized by Lbd1, which forms a physical bridge between them. The paramount importance of the CF finger for GATA1 function is proved by the fact that *GATA1* genes lacking the region encoding this domain are unable to rescue erythroid differentiation in *GATA1*^{null} embryonic stem cells (ESCs), whereas minigenes containing only the CF of either GATA1 or GATA2 are sufficient to induce megakaryocytic differentiation of myeloid cell lines. In addition to forming heterodimers with LMO2, CF can form complexes with Sp1 and PU.1, two factors essential for myelomonocytic cell differentiation. The GATA1-PU.1 complex is unable to bind DNA, so its function might be to establish either an erythroid- or a myeloid-permissive cellular environment depending on which factor is expressed at the highest concentration. The presence of relatively higher concentrations of GATA1 would favor the formation of GATA1-LMO2 complexes leading to activation of erythroid-specific genes, whereas the presence of relatively higher concentrations of PU.1 would lead mainly to the formation of the transcriptionally inactive GATA1-PU.1 complexes.[22] This hypothesis has been confirmed by recent single cell profiling of prospectively isolated murine progenitor cells that has identified that GATA1 and PU.1 are both expressed at the level of common myeloid progenitor cells and that their expression diverge in cells committed to the erythroid and myeloid lineage, respectively.[43,44]

Knock-in experiments in mice have indicated that the N-terminal domain of GATA1, although dispensable for primitive erythropoiesis, is required for appropriate production of definitive red cells.[45] A truncated *GATA1* gene lacking the N-terminal domain is 10 times less efficient than the full-length gene in rescuing erythroid differentiation in *GATA1*$^{\text{null}}$ mice.[45] This experiment suggests that interaction of the N-terminal domain of *GATA1* with a suitable partner(s) is required for optimal definitive erythropoiesis. Structure function studies have identified that the N-terminal domain of GATA1 interacting with the product of the retinoblastoma (RB) gene is essential for proper terminal erythroid maturation, providing a unifying mechanism for the similar phenotype of several *GATA1* and *RB* mouse mutants and of human diseases associated with mutations in these two genes. The *GATA1* gene is translated from two alternative promoters, one containing the sequences encoding the N-terminal domain of GATA1, GATA1 full length, and another one encoding a protein lacking this domain (GATA1 short).[46] Mutations leading to the transcription only of the short GATA1 mRNA are associated with the megakaryocyte leukemia of Down syndrome (Chapters 29 and 30) and Diamond-Blackfan anemia (DBA) (Chapter 30). Since with erythroid maturation, the mRNA encoding the full length GATA1 prevails and the CAP site of the mRNA encoding GATA1short is less efficient than that of the full-length mRNA to bind the small ribosomal subunit, these mutations impair erythroid maturation by reducing the translation on GATA1 mRNA and, ultimately, the GATA1 content of the cells.

In addition to all the evidence pointing to *GATA1* as exerting a predominant but ontogenetic-specific role in the control of erythroid differentiation, other evidence indicates that this gene exerts exquisite control in the differentiation of other hematopoietic lineages, such as megakaryocytes, mast cells, eosinophils, and dendritic cells. The mechanism used by one single factor in guiding differentiation along different lineages does not rely on specific domains in the GATA1 protein itself. In fact, the structure of all the GATA proteins is so well conserved among different family members and in evolution that *GATA1*$^{\text{null}}$ ESCs are rescued not only by reintroduction of the *GATA1* gene itself but also by introducing any other member of the GATA family, such as *GATA3*. The lineage-specific action of *GATA1* in regulating gene expression is achieved through the presence of lineage-specific regulatory sequences in the promoter regions of its target genes.[47] Therefore, the relative concentration of GATA1, as opposed to the levels of a few key regulatory partners, may establish a lineage-permissive microenvironment. Furthermore, the existence of lineage-specific regulatory sequences in the *GATA1* gene itself ensures that such concentrations are achieved only in the right cell. Although GATA1 is expressed in erythroid, megakaryocytic, mast, dendritic, and eosinophilic cells, its level of expression differs greatly among the various cell types, with erythroid cells expressing the most. Three DNase hypersensitive sites (HS) have been recognized within the 8 Kb upstream and the first intron of the murine *GATA1* gene, defined as HSI, HSII, and HSIII. Targeted deletion mutants in the mouse have shown that each of these sites functions as an enhancer in different cell types: HSI in megakaryocytes and mast cells, and also for upregulation of GATA1 expression during the process of antigen presentation in dendritic cells and during the progression of erythroid maturation; HSIII sustains low levels of GATA1 expression in erythroid and dendritic cells and HSII is dispensable for erythroid and megakaryocyte expression but absolutely required for gene expression in eosinophils.

GATA1 mRNA has an unusually long half-life (>9 hours). Two GATA1 bands, corresponding to the native and processed (acetylated and phosphorylated) forms of the protein, have been detected by Western blot analysis. The processed form binds DNA with higher affinity than the native form. Furthermore, although the half-life of the native form is short (approximately 0.5 hour) and stabilized by EPO, the processed form is extremely stable (half-life >6 hours) and EPO-independent. Because the cell cycle of hematopoietic cells in vivo is as short as 6 hours, erythroid cells accumulate GATA1 mRNA and protein as they proliferate. Because maturation is dependent on the levels of GATA1 expressed by cells, the cellular GATA1 content might represent the biologic clock that, by controlling the number of precursors, determines the cellular output of the differentiation process. This hypothesis suggests that EPO-induced GATA1 processing through the ubiquitin–proteasome pathway is an important element in the regulation of erythroid differentiation. Furthermore, in preparation of DNA synthesis, in the G1 phase of the cell cycle the DNA of erythroid cells lose most of the binding to regulatory proteins with the exception of GATA1 that, once the DNA is duplicated, works as a bookmark that recruits back the regulatory complexes in G2 allowing that epigenetic information is inherited by the daughter cells.[48] It has been suggested that the cell cycle exerts a primary role in determining the fate specification of the bipotent megakaryocyte-erythroid progenitor cells.[49]

The GATA1 content is negatively regulated by caspase 3, the end element of the TRAIL-Bruton kinase death pathway. Caspase 3 is unable to cleave GATA1 if the protein is complexed in the nucleus with the chaperone protein heat shock protein 70 (Hsp70). EPO-receptor signaling counteracts the apoptotic pathway by favoring Hsp70-GATA1 colocalization in the nucleus.

The equilibrium between TRAIL and EPO-dependent control on Hsp70 localization may be perturbed under pathologic conditions. As an example, defective nuclear localization of Hsp70 and increased GATA1 cleavage is associated with dyserythropoiesis in myelodysplastic disorders (Chapter 61). Similarly, by sequestering Hsp70, free α-globin promotes GATA1 degradation and induces ineffective erythropoiesis in β-thalassemia. The biochemical studies detailing the link between EPO and TRAIL from one side and GATA1 from the other are consistent with additional data indicating that EPO signaling also induces GATA1 phosphorylation at Ser310 and that this phosphorylation plays an important role in regulating GATA1 function in erythroid cell lines. Although GATA1 mutants expressing only the native form of GATA1 do not have a detectable erythroid phenotype under steady-state conditions, more studies on the response of these mice to erythroid stress will clarify the role of GATA1 processing in stress erythropoiesis.

The relationship between chromatin architecture and transcription factor occupancy in the loci encoding key erythrocyte membrane proteins has also been established. In addition to binding GATA1, FOG-1 is also capable of binding NuRD, a complex that contains HDAC1. The multicomplex GATA1/FOG-1/NuRD is responsible for appropriate activation/repression of several erythroid specific genes, including the GATA2-GATA1 switch described above.

In addition to the participation of GATA1 in complexes which activate the expression of erythroid genes described above, several studies indicate that GATA1 form distinct repressive complexes with FOG-1 and MeCP1 (the latter consisting of the methyl-DNA binding protein MBD2, p66/p68 and the multi-subunit Mi-2/NuRD complex) and with Gfi-1b and the co-repressor complex LSD1/CoREST.[50] In addition, the PRC2 complex (containing EED, Ezh1/2, and Suz12) is involved in GATA1-gene repression during erythroid differentiation.[51]

Another factor with special importance in the erythroid lineage is the CACCC binding protein designated EKLF (also known as KLF1), which is expressed at all stages of erythropoiesis and binds preferentially to CACCC sites in the β-globin promoter. EKLF is a zinc finger protein that binds not only DNA, but also, after appropriate posttranslational modifications, is a key regulatory protein that modulates chromatin structure of the β-globin locus. Mice lacking EKLF (*EKLF*$^{\text{Null}}$) die of a thalassemic-like defect because of severe deficiency of β-globin expression.[52] Microarray analysis of *EKLF*$^{\text{null}}$ erythroid cells and promoter-specific expression of reported genes in *EKLF*$^{\text{null}}$ cells have identified that the first GATA1-dependent molecular control of erythroid differentiation is followed by a second EKLF-dependent phase. In erythroid cells *EKLF* expression is first activated by GATA2 and, once the GATA2 to GATA1 switch is completed, it is regulated by GATA1 which increase its transcription by several folds. In addition, the EKLF content in erythroid cells is posttranscriptionally regulated by the G-protein pathway suppressor 2 (GPS2), which stabilizes the protein.[53] Primarily GATA1-dependent genes include, in addition to *EPOR* and those involved in the control of apoptosis, α- and δ-globin while EKLF-dependent genes, in addition to β-globin and α-hemoglobin-stabilizing protein (AHSP), are represented by those required for appropriate membrane assembly, such as β-spectrin, ankyrin, and band 3 (but not α-spectrin). These results are consistent with the notion that, in erythroid differentiation, activation of α-globin gene expression precedes that of β-globin and that loss of GATA1 binding sites in the promoter of the gene is found in α-thalassemia, in the Greek nondeletion hereditary persistence of fetal hemoglobin (HPFH) (guanine to adenine at nucleotide position −117 of γ-globin), and in δ-thalassemia (point mutation leading to G→A substitution at position +69 of the δ-globin gene), whereas loss of EKLF binding site is present in other forms of HPFH. In addition to regulating globin gene expression directly, EKLF inhibits γ-globin expression indirectly by activating *BCL11A* expression. The notion that EKLF controls mainly late stages of erythroid maturation is further confirmed by the fact that individuals harboring genetic EKLF variants have reduced or absent (Lutheran phenotype) expression of RBC antigens and that the loss of function EKLF-E235K mutation, which alters the DNA binding site of the protein, induces congenital dyserythropoietic anemia (Chapter 30), a phenotype associated with the presence in the circulation of binucleated red cells, a sign of inefficient enucleation.[54,55]

More recently, it has been identified that EKLF also promotes terminal erythroid maturation in a noncell-autonomous fashion by regulating expression of DNase IIα, in the central macrophage of fetal liver erythroblastic islands thus facilitating digestion of the DNA of engulfed pyrenocytes, in the central macrophage of the erythroblastic island present in mouse fetal liver. These observations have been recently refined by a single cell transcriptomic profile of F4/80 macrophages from mouse fetal liver and adult bone marrow which indicates that the three subpopulations that are capable to engage in the formation of erythroblastic islands are all EKLF positive.

Additional transcription factors exerting an intrinsic control on erythroid differentiation are IKAROS and proteins that, until expressed, prevent terminal cell maturation. The most studied of these last group of protein is encoded by the inhibitor of differentiation 1 gene *(ID1)*, which inhibits differentiation along almost all mesenchymal cell lineages, including the erythroid one. ID1 appears to act between GATA1 and EKLF by preventing EKLF from executing its program.

Taken together, these studies have significantly expanded our understanding of the molecular basis of hematopoietic cell development in general and of erythropoiesis in particular. The emerging picture is that certain genes, such as *SCL*, are absolutely required for hematopoietic development, whereas other genes, such as *GATA2*, *c-Myb, CBF, TEL*, and some downstream signal transducing molecules such as gp30 and SHP2, are responsible for expansion and maintenance of a normal pool of fetal liver and adult hematopoietic progenitors. The participation of many of these molecules in multicomponent molecular complexes with protein/protein and protein/DNA interactions (i.e., LM02/Lbd1/SCL/E2A/GATA) during the early proliferative stages of hematopoiesis[56,57] may underlie their role in the proliferation and maintenance of immature progenitor/precursor pools in erythropoiesis. Other genes such as *GATA1*, its partner *FOG*-1, and *EKLF* are necessary to direct high levels of function of erythroid-specific genes in cells already committed to terminal differentiation (see Fig. 27.1).[58]

Extrinsic Control of the Erythropoiesis

Erythropoiesis is finely regulated by a combination of hormones and microenvironmental cues that have been carefully identified using a combination of cell culture techniques and loss-of-function studies in mice. In addition to serum containing media, erythroid progenitor cells can be cultured in serum-depleted media, a condition that limits the confounding effects due to the multiple, often unknown, factors present in serum. Conditions that imitate lower oxygen pressures, found in bone marrow in vivo, favorably influence erythroid development in culture and may be advantageous.

Erythropoietin

The physiologically obligatory growth factor for erythroid development is EPO, a 35-kDa glycoprotein produced mainly by the peritubular cells of the kidney in response to the oxygen need sensed by a heme-containing protein. Through the interaction of EPO with receptor-bearing cells within the bone marrow, physiologic oxygen demands are translated into increased red cell production. Thus, EPO is a true hormone, manufactured at one anatomic site and transported through the bloodstream to the site of activity.

According to the prevailing model of hematopoiesis, progenitor cells committed to erythroid differentiation are generated in a stochastic fashion from pluripotent stem cells.[59] According to this model, neither EPO nor other lineage-restricted regulators play any role in determining lineage commitment. This view is supported by the observation that BFU-E and CFU-E can be generated in vitro and in vivo, in the absence of EPO or EPOR (in EPO or EPOR^{null} mice). At the progenitor level, EPO influences erythroid differentiation by rescuing (from apoptosis) cells that express its receptor, EPOR, and amplifying them further. In addition to the permissive role of EPO ascribed by the stochastic theory, experiments in vivo, in anemic states, or after pharmacologic doses of EPO suggest that high levels of EPO hasten the transition from BFU-E to hemoglobin-synthesizing cells by decreasing either the number of divisions required for this transition or the resting periods between cell divisions.

Autoradiographic studies of purified BFU-E populations indicate that EPORs are detectable only in a subset of BFU-E and that their number increases as BFU-E mature to CFU-E, with the highest level observed at the CFU-E/proerythroblast boundary. That the transition from BFU-E to CFU-E occurs under the influence of EPO suggests ligand (EPO)-induced receptor upregulation. Whether the magnitude of such upregulation is dependent on EPO dose and whether it can modulate the rate of entry of these cells into the maturing compartment is unclear.

For CFU-E, EPO seems to affect their survival and not their cycling status since CFU-E are irrevocably lost after one cycle of DNA synthesis if EPO is not present. It also stimulates all the biochemical processes required to initiate the terminal maturation process (i.e., heme synthesis, globin synthesis, and synthesis of cytoskeletal proteins).

EPORs decrease progressively (from approximately 1000 to <300 receptors per cell) as proerythroblasts mature, and they are undetectable at the reticulocyte level. Through these receptors, EPO exerts its proliferative influence on proerythroblasts and basophilic erythroblasts, but maturation beyond these stages can proceed in the absence of EPO and is instead regulated by thyroid hormone.[60] The reduced number of EPO receptors observed in the most mature erythroid cells is probably instrumental to facilitate enucleation, a process that would be inhibited by the oxidative stress induced by EPO in these cells.[61]

The exquisite role of EPO in determining red cell numbers in the circulation has been clearly established by direct correlations between hematocrit and EPO plasma concentrations in individuals exposed to hypoxia and in patients with compromised kidney functions (Chapters 70 and 150). However, the variabilities around the mean of hematocrit and EPO plasma levels found in normal individuals under steady-state conditions are not correlated, indicating that other factors (sex and age) cooperate with EPO in determining the fluctuations in red cell mass under steady-state hematopoiesis.

The EPOR polypeptide is a 66-kDa membrane protein that is a member of the cytokine receptor superfamily which includes the receptors for IL-3, GM-CSF, and IL-5. Like other members of this superfamily, EPOR contains four conserved cysteine residues and a WSXWS motif in the extracellular region. The extracytoplasmic region of EPOR contains the EPO binding activity of the receptor. The cytoplasmic region of EPOR does not contain a tyrosine kinase catalytic domain; instead, it interacts with cytoplasmic tyrosine kinases. Cross-linking of radiolabeled EPO to cell surface EPOR results in formation of at least two major cross-linked protein complexes of 140 and 120 kDa. The molecular composition of these complexes remains unsolved but suggests that EPOR contains additional subunits or accessory proteins, one of which is represented by second gene for TfR (TfR2),[62] a potential therapeutic target for β-thalassemia.[63]

The dimeric structure of the activated receptor has been confirmed by crystallization of the extracytoplasmic region of EPOR.[64–66] These crystallization studies have also allowed the identification of small synthetic peptides capable of inducing EPOR dimerization, suggesting a profitable avenue to design EPO-mimetic and EPO-antagonist drugs possibly more effective than the native protein, especially with regard to the activity of the growth factor in nonhematopoietic tissues. In addition to EPO mimetics, erythroid stimulating agents under development are represented by modulators of HIF-1α expression, IgA2, an immunoglobin that selectively increases under conditions of anemic stress and TGF-β superfamily ligand traps.[67,68]

EPOR mRNA, originally isolated from murine and human erythroid cell lines, has been found in nonerythroid cells as well. EPO promotes the differentiation of megakaryocytes and chemotaxis of endothelial cells, at physiologic concentrations of hormone, suggesting the presence of functional cell surface EPORs in these cells. In addition, cell surface EPOR, detected by radiolabeled EPO cross-linking, has been found in rat and mouse placenta and in neural and smooth muscle cells. The presence of EPOR in the placenta may be related to the fact that this organ is an erythropoietic site in first-trimester fetuses. Adverse effects in cancer patients treated with EPO have been attributed to the effects of EPO on tumor cells.[69]

The existence of naturally occurring splice variants of the EPOR gene encoding EPOR polypeptides of variable length and activity has been shown. The soluble secreted form of EPOR binds EPO and thereby competes with the cell surface receptor isoform.[70] The biologic function of these alternative forms, including a truncated form of EPOR found in early progenitors, remains unknown but may be related either to differential EPO signaling and responses (survival, proliferation, differentiation, and epigenomic modeling[71]) at different stages in erythroid development or to the establishment of erythroid-specific versus myeloid-specific niches in the marrow microenvironment.

Considerable progress has been made in our understanding of EPOR-mediated signal transduction. As mentioned above, EPO induces homodimerization of EPOR and phosphorylation of its first signaling element, Janus-activated kinase 2 (JAK2), a tyrosine kinase constitutively docked at the membrane proximal domain of the receptor required for its appropriate Golgi processing and cell surface expression. The essential role played by JAK2 in delivering EPO signaling has been demonstrated by the observation that *JAK2*^{null} mice die at an early embryonic stage. Phosphorylation activates the catalytic domain of JAK2 that next phosphorylates several tyrosine residues of the cytoplasmic tail of EPOR downstream to its docking site. Once phosphorylated, these tyrosine residues serve as docking sites for other cytoplasmic effector proteins containing Src homology 2 (SH2) domains, such as the p85 subunit of phosphatidylinositol 3-kinase,[72] the adaptor protein Shc, and the signal transducer and activator of transcription 5 (STAT5). Once docked on EPOR, these proteins become tyrosine phosphorylated and engage other downstream signaling events. In addition, JAK2 directly activates the Ras/Raf/MAPK (mitogen-activated protein kinase) pathway, further contributing to the EPO-induced mitogenic signal. The most carboxyterminal tyrosine is a negative regulatory domain that recruits the phosphatase SHP1. Once activated, SHP1 rapidly dephosphorylate JAK2, downregulating its activity, and EPOR, setting the system to rest. Failure to recruit the SHP1 phosphatase results in increased EPOR signaling and polycythemia (Chapter 70).

Activation of the JAK2/STAT5 signaling has been studied in considerable detail. Upon EPOR tyrosine phosphorylation, STAT5 binds to a specific phosphorylated tyrosine residue of EPOR. Following EPOR binding, STAT5 itself becomes phosphorylated at amino acid Y694. Activated STAT5 then disengages from EPOR, undergoes homodimerization, and translocates to the cell nucleus, where it activates transcription of EPO-inducible genes. Some EPO-inducible genes, such as *MYC* and *FOS*, are common to other hematopoietic growth factor signaling pathways. Other EPO-inducible genes are specifically expressed in erythroid cells and are not shared by other growth factor responses.

Other signal transduction pathways downstream from cytokine receptors have been identified. For instance, EPO (and IL-3) activates the signaling protein CBL and the subsequent binding and activation of CrkL. Inositide-specific phospholipases C (PLCs) and the protein kinase C (PKC) pathways also are involved in EPO signaling. PLCs catalyze hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol and inositol 3,4,5-bisphosphate, a well-known intracellular messenger for PKC activation and intracellular Ca^{2+} mobilization. In fact, the involvement of PLCs in erythroid differentiation was suggested for the first time by studies demonstrating that stimulation of EPOR in primary erythroid cells results in increased calcium ion flux. PLCs are classified into four families (α, β, γ, and δ), each one with multiple isoforms.[73] More recent studies demonstrated that primary erythroblasts express only some (i.e., PLC β_1, β_2, β_3, δ_1, γ_1, and γ_2) PLC isoforms. Among these, PLCβ_1 most likely is involved in EPO signaling since its expression is induced within 6 hours of stimulation with the growth factor.[74]

PKC represents a family of nine different serine-threonine kinases genes, encoding a total of 12 different isoforms, involved in the regulation of many cellular functions. These enzymes exert their biologic functions as a cytoplasmic-nuclear shuttle of the transduction machinery and become phosphorylated, and hence activated, in response to a variety of stimuli. Human CD34$^+$ cells express all of the PKC isoforms. Commitment of these cells along the erythroid lineage is associated with suppression of some isoforms. Of those, PKCε exerts positive control on erythropoiesis, because its inhibitors specifically impair the ability of erythroid cells to respond to EPO. It is also possible that different PKC isoforms are active at different ontogenic stages, because differentiation of neonatal or adult erythroblasts is associated with activation of PKCα or PKCδ, respectively.

EPO signaling also activates Lyn, a tyrosine kinase of the Src family physically associated with EPOR that acts upstream to both STAT5 and PLCγ2/PI3K.[75] Failure to activate Lyn prevents erythroid differentiation of the J2E cell line and Lyn^{null} mice have a phenotype remarkably similar to that of mice hypomorphic for GATA1 (normal hematocrit thanks to extramedullary spleen hematopoiesis). Lyn also activates Liar, a Lyn-binding nuclear/cytoplasmic shuttling protein specifically responsible for downregulating KIT expression in response to EPO. In humans, Lyn is responsible for the phosphorylation of several membrane proteins, and failure to activate Lyn results in the formation of acanthocytic red cells, a diagnostic marker of chorea-acanthocytosis, a rare autosomal recessive neurodegenerative disorder.

The development of robust methods for unbiased phosphoproteomic investigations has recently permitted the identification of as many as 22 novel transducers of EPOR signaling in human erythroid cells,[76] including thioredoxin-interacting protein (TXNIP) and protein tyrosine phosphatase non-receptor type 18 (PTPN18).[76]

A critical question in the field of EPOR signaling is the mechanism of erythroid specificity since most, if not all, of the signaling pathways activated by EPOR are also activated by other hematopoietic cytokine receptors. Several models are possible. First, EPOR may activate unique but unknown signaling pathways specific to EPOR and distinct from other cytokine receptors. Alternatively, EPOR may activate pathways in common with other cytokine receptors but the specificity of the signal is provided not by EPOR itself but from interactions with other developmentally programmed events, such as expression of erythroid-specific transcription factors. Experiments exist in support of the latter hypothesis. Activation of EPOR in the murine IL-3–dependent cell line Ba/F3 induce both mitogenesis and globin accumulation while activation of other cytokine receptors, such as IL-3R and IL-2R, drive proliferation but not β-globin synthesis.[77] By contrast, the murine IL-2–dependent cell line CTLL-2 engineered to express EPOR grows in EPO but does not differentiate into globin-bearing cells. Therefore, EPOR may be necessary for erythroid differentiation but not sufficient alone. Other erythroid-specific markers, such as the transcription factors GATA1 or EKLF, are likely required for cells to differentiate down the erythroid pathway. Taken together, these results suggest that EPOR generates a differentiation-specific signaling within the context of a proper transcriptional environment.

The tyrosine residues involved in EPOR signaling were identified by structure-function studies in cell lines expressing mutant forms of the receptor. Expression of the full-length, wild-type EPOR polypeptide in these cells resulted in EPO-dependent growth and partial EPO-induced erythroid differentiation. By contrast, expression of its truncated forms resulted in variable growth responses. For instance, truncation of the membrane proximal region of EPOR demonstrated a critical positive regulatory domain of EPOR required for mitogenesis. Furthermore, truncation of the carboxy-terminal (C-terminal) 40 amino acids of EPOR resulted in increased EPO-dependent growth, providing indication that the C-terminal region contain the negative regulatory domain required for down-modulating EPOR signals.

The detailed analysis of the EPOR receptor domain and chromosomal mapping of EPOR (to chromosome 9 in mice and chromosome 19p in men) led to the identification of EPOR mutation in the rare congenital disease familial erythrocytosis (Chapter 70). Familial erythrocytosis is a heterogeneous group of hereditary conditions characterized by erythrocytosis in the setting of low serum EPO levels. A few families that demonstrate autosomal dominant inheritance have been identified. The linkage between the EPOR gene and familial erythrocytosis was first established by the observation that a mutant EPOR allele segregates with the disease in one familial erythrocytosis kindred. This allele contains a nonsense mutation in the coding region of the gene that results in synthesis of a truncated EPOR that lacks the negative regulatory domain of its C-terminal region.[78] Since that report, several other frameshift and deletion EPOR gene mutations, all encoding C-terminal–truncated forms of the protein providing EPO hypersensitivity and resulting in familial erythrocytosis, have been reported.

Congenital polycythemia may arise not only from gene mutations leading to abnormal EPOR signaling but also from gene mutations altering EPO production. The T598T mutation in one of the genes controlling oxygen sensing (von Hippel-Lindau *[VHL]* gene), which leads to increased EPO production by the kidney, is associated with congenital erythrocytosis, the Chuvash polycythemia (Chapter 70).[79,80] The *C598T VHL* mutation is endemic in Chuvashia, Russian Federation, and in the small island of Ischia, in southern Italy.

Polycythemia may also arise from somatic mutations leading to constitutive EPOR signaling. Several investigators have reported a mutation in the *JAK2* gene resulting in a valine→phenylalanine substitution at position 617 of the protein (*JAK2*^{V617F} mutation) in patients with Ph-negative myeloproliferative disorders (Chapter 70).[81–84]

Studies have suggested that EPO functions synergistically with other multilineage growth factors, such as KL and IL-3, produced by the microenvironment (see later). EPO and KL function together, resulting in increased erythroid colony cell growth in methylcellulose culture. Studies with the EPOR polypeptide suggest a molecular mechanism for such synergy. In fact, in erythroid cells EPOR and KIT are physically associated and activation of KIT by KL results in transphosphorylation of EPOR at the cell surface. Physical interaction between EPOR and the β common chain of the IL-3 receptor has been demonstrated[85] and may be involved in the neuroprotective action exerted by EPO. In fact, a carbamylated derivative of EPO prevents motoneuron degeneration in vitro and in vivo and promotes recovery in several in vivo models of brain and heart injuries, such as chronic autoimmune encephalomyelitis in mice, radiosurgery- or ischemia-induced brain injury, and myocardium ischemia-reperfusion injury in rats. (For a review of the nonhematopoietic activity of EPO, see reference Papayannopoulou and Migliaccio[1]). Taken together, these results suggest that receptor cross-talk at the cell surface may account, at least in part, for the physiologic interaction of some cytokines in controlling hematopoietic versus non-hematopoietic effects of EPO.

Proliferation and/or survival of early erythroid progenitors in vitro are dependent on the presence of numerous cytokines, elaborated by stromal and accessory cells within the microenvironment. Among the cytokines, KL, which is produced by stromal cells, and IL-3, which is produced by a subset of T cells, alone and in synergy, promote proliferation of BFU-E and its progeny. Other cytokines, such as GM-CSF, IL-11, and TPO, stimulate a subset of BFU-E. In addition to proliferation, cytokines also control BFU-E survival since most of them die when cultured for more than a few days in the absence of cytokines. Cytokines exert their effects through interaction with specific receptors present on the BFU-E surface. These receptors are also expressed by the leukemic counterparts of normal BFU-E and by leukemic cell lines. Additional important positive effects on erythropoiesis are exerted by vitamins (such as vitamin A and D)[86] and hormones, such as insulin growth factor 1 (IGF1),[87,88] which facilitate the cell entry into the S phase of the cycle. A polymorphism at the IGF1 locus is associated with anemia in men.[89] In addition, targeted ablation and naturally existing mutations have disclosed the importance, even if indirect, played by the vascular endothelial growth factor (VEGF)/Flk-1 receptor in the control of erythropoiesis, since Flk-1 deletion affects both endothelial and hematopoietic development through its presumed presence in the hemangioblast, the common endothelial/hematopoietic stem cell.

Hematopoietic Microenvironment

In invertebrates such as worms and sessile marine creatures, erythropoiesis occurs adjacent to peritoneal and endothelial cells (Chapter 14). In premammalian species, the spleen is the primary site of erythropoiesis (Chapter 156). With evolutionary advancement, the function gradually shifts to the liver and the sinusoidal cavities of bones. These observations suggest that sufficient oxygen, a stagnated flow of blood to avoid dispersion of factors produced locally, and extensive and redundant surfaces for cell–cell interactions are essential to supporting red cell production. Similar sites support erythropoiesis during human development (see Fig. 27.1 and "Ontogeny of Erythropoiesis"). During both phylogeny and ontogeny, the liver and spleen are primarily erythropoietic organs; granulocytic cells dominate in the bone marrow. Within the bone marrow, hematopoiesis is restricted to the extravascular space, where compact collections of cells are interspersed among venous sinuses. These sinuses originate adjacent to the endosteal bone surface and empty into a central longitudinal vein. Studies in mice demonstrate that BFU-E follows a bimodal distribution with peaks adjacent to the periosteum and mid cavity, whereas CFU-E and later erythroid cells have a broad distribution with highest incidence toward the axis of the femur, adjacent to the central vein, thus suggesting that the local anatomy (specialized niches?) influences the maturation of erythroid cells.

The bone marrow microenvironment consists of three broad components: stromal cells (e.g., fibroblasts, endothelial cells, mesenchymal stem cells and their diverse descendant progeny), accessory cells (monocytes, macrophages, megakaryocytes, T cells), and extracellular matrix (a protein-carbohydrate scaffold) (Chapter 14). In the bone marrow, although early studies described two distinct hematopoietic niches for hematopoietic stem cells/progenitor cells (i.e., the "endosteal" niche and the "endothelial or vascular niche" within the medulla), more recent studies suggest that this distinction may be artificial, because both cellular structures can be intimately associated in trabecular bone. Thus mesenchymal stem cell–derived osteoprogenitor cells and stromal reticular cells (nestin$^+$ or leptinR$^+$) are intimately associated with sinusoidal endothelium and seem to be pivotal organizers of the bone marrow niche.[91] Reciprocal communication of hematopoietic stem cells with cells/matrix in their bone marrow niche ensures both their quiescent state and self-renewal dynamics.

EPO, in addition to promoting erythropoiesis directly, enhances erythropoiesis indirectly by decreasing the interaction of hematopoietic stem cells with their niches, reducing the amount of trabecular bone and downregulating CXCR4 expression, the receptor for CXCL12/SDF1 expressed by stromal cells, on hematopoietic stem cells.

Accessory cells are progeny of hematopoietic stem cells; hence after marrow transplantation these cells are of donor origin, whereas stromal cells remain mostly host derived. Extracellular matrix molecules are synthesized and secreted by microenvironmental cells and include collagens (types I, III, IV, and V), glycoproteins (fibronectin, laminins, thrombospondins, hemonectin, and tenascin), and glycosaminoglycans (hyaluronic acid, chondroitin, dermatan, and heparan sulfate). The production of extracellular matrix proteoglycans by mesenchymal stromal cells may be regulated by the Wnt pathway.

Besides providing structure to the marrow space and a surface for cell adhesion, the microenvironment is important for hematopoietic cell homing, engraftment, migration, and the response to physiologic stress and homeostasis. Although the functional consequences of the microenvironment ultimately must be defined by in vivo studies in mice, dissection of the cellular components of the microenvironment, definition of the cytokines that are produced by individual cells, and the nature of cell–cell interactions have been aided by in vitro models. Long-term bone marrow cultures provide an experimental approach for such studies. Under these in vitro conditions, murine hematopoiesis can be maintained for 8 to 10 months and human hematopoiesis for 2 to 3 months. An adherent layer consisting of fibroblasts, adipocytes, and macrophages is a crucial component of the culture system. Progenitor cells adherent to stroma are generally quiescent (dormant), whereas those in the non-adherent cell compartment are in active cell cycle.[91]

In vitro studies have demonstrated that stromal cells, including endothelial cells and fibroblasts, elaborate cytokines such as GM-CSF, G-CSF, IL-1, IL-3, IL-6, IL-11, KL, Flt-3 ligand, activin A, and basic fibroblast growth factor, which influence, alone or in combination, the growth of adjacent marrow progenitors.[91] In addition to positive regulators of replication and differentiation, stromal cells elaborate factors such as TGF-β, TPO, CXCL12, IFN-γ, TNF-α, and TRAIL, which exert a negative influence on proliferation and may help maintain a dormant (noncycling) state.[91–94] These negative regulators are responsible, at least in part, for the anemia associated with chronic inflammatory states. The effects of TNF-α and TRAIL are mediated through induction of apoptosis at specific stages of erythroid maturation. In the case of TRAIL, a complex system of signaling and decoy receptor isoforms determines the precise cell window susceptible to TRAIL-induced apoptosis.[95] TRAIL probably induces apoptosis by competing with EPO for activation of Bruton tyrosine kinase. Its effects are counteracted by KL and PKCε signaling. TRAIL is also involved in the pathobiology of the anemia associated with multiple myeloma (TRAIL is overproduced by the malignant plasma cells of these patients) and myelodysplastic syndrome (MDS) (myelodysplastic erythroid progenitors overexpress the adaptor Fas-associated death domain of the TRAIL receptor). On the other hand, the negative effects of TGF-β are mainly achieved by accelerating cell differentiation, whereas data from mouse models indicate that chronic exposure of IFN-γ reduces the erythrocyte life span and inhibits erythropoiesis by promoting the expression of PU.1, a transcription factor that antagonizes GATA1, a master transcriptional regulator of erythropoiesis. Because some regulators inhibit differentiation along certain lineages but not others, there is an intriguing possibility that lineage-specific regulation within the microenvironment can be achieved through negative, rather than positive, factors.

Among the factors elaborated by stromal cells, KL has the most profound effect on erythropoiesis as emphasized by genome-wide association studies (GWAS) that have identified several single-nucleotide polymorphisms (SNPs) lying close to a prominent DNase hypersensitive region ~115 kb upstream of the gene encoding its receptor, *KIT*, associated with variability in RBC counts, mean RBC volume, and mean RBC hemoglobin content observed in the normal population.[41,96] Stromal cells produce a membrane bound form of KL that may be cleaved in a soluble form and released in the circulation. Mice with KIT mutations (W mutations), leading to absence of or compromised kinase activity of the intracellular domain of the receptor, and steel mice, with mutations of KL, have disproportionate and severe reduction of the numbers of late erythroid progenitors, CFU-E, and differentiated erythroid precursors resulting in anemia.[97] Steel (Sl) mice unable to synthesize KL die in utero because of severe anemia while steel-Dickie (Sl^d) mice that are unable to make the membrane-restricted form of KL are viable but severely anemic, whereas other lineages in these animals are marginally affected or not affected by this defect. The fact that erythropoiesis is abnormal, despite high levels of circulating EPO and the presence of soluble KL, suggests that normal erythroid differentiation and maturation require both a functional membrane-restricted KL/KIT and an EPO signaling pathway. Several studies have investigated the relationship between KIT signaling and erythroid cell fate that may involve cross-phosphorylation of EPOR following activation of KIT/KL signaling. Furthermore, data suggest that tyrosine cross-phosphorylation of EPOR is sustained longer when cells are cultured on steel stromal cells engineered to express the membrane-restricted form of KL than cells expressing the soluble form.

KIT is a member of the immunoglobulin-domain containing receptor family and contains 6 major autophosphorylation tyrosines (Y568, Y703, Y721, Y730, and Y900) on its intracellular domain.[98] Upon KL/KIT interaction, these tyrosines are sequentially phosphorylated and dock proteins that activate specific signaling pathway. Y568 is the first Y to be phosphorylated and is the docking site for EPOR responsible for the KL-mediated EPOR transactivation discussed above.[99] By interaction with EPOR, Y568 may indirectly activate STAT5. In fact, whether KL activates the STAT5 pathway in erythroid cells has been controversial. KL was found to be unable to activate STAT5 in prospectively isolated human erythroid progenitor cells but more recent single-cell analyses indicate that KL activates STAT5 in bipotent erythroid/megakaryocytic but not in myelomonocytic progenitor cells. This site also docks Src activating the MEK signaling.[100] The MEK signaling is also activated directly by binding of MEK to Y703. In human and murine erythroid progenitors, KL induces rapid (within 15 minutes) activation of the MEK downstream effector ERK1/2, which lasts only 1 hour. In human erythroleukemic K562 and myeloid MO7e cells, the rapid KL-dependent ERK activation is associated with proliferation, whereas the late sustained ERK activation is responsible for differentiation. Y721 docks and activates PI3K, which in turn activates the mTOR downstream protein mTORC1 (promoting ribosomal protein biosynthesis) and mTORC2/AKT (promoting survival).[101] Activation of the PI3K/AKT pathway has been reported in murine erythroid progenitors and in human MO7e cells. Y721 also docks CD63, which prime KIT for endocytosis.[102] By tuning the speed of endocytosis, CD63 regulates the strength of the overall response of erythroid cells to KL. Once in the cytoplasm, KIT is phosphorylated at Y900 which recruits CRKI/CRKII and c-Cbl, posing the receptor for lysosomal degradation.[103] Degradation of KIT triggers de novo *KIT* gene expression, maturation, and export to the cell surface for new KL interaction. Y730 is an interesting docking site because it engages PLCγ increasing interaction with EPOR and sustaining hyperproliferation of erythroid progenitor cells.[104] Y730 is phosphorylated upon KIT binding to stromal but not to soluble KL. These complex signaling pathways indicate that regulation of KIT metabolism is at least as important as that of its signaling in determining the extent of the response of the erythron to KL stimulation (see also reference Federici et al.[105]).

The soluble form of KL is produced by proteolytic cleavage of membrane KL and is released in the circulation. Soluble KL effectively supports erythroid maturation in vitro[106] but is dispensable for steady-state erythropoiesis, because targeted mutant mice expressing exclusively the more stable membrane isoform of KL, KL2, lacking the major proteolytic cleavage site, have normal hematocrit values. However, these mice recover poorly from radiation-induced anemia. In wild-type mice, sublethal radiation induced a transient fourfold increase in KL in the serum (from <0.5 up to >2 ng/mL), reaching a peak after 7 days. In contrast, the proteolytic-cleavage mutant $KL^{KL2/KL2}$ mice did not release soluble KL into the serum after sublethal radiation, and survival was significantly diminished because of anemia. This phenotype is remarkably similar to that of mice lacking the dimerization domain of glucocorticoid receptor (GR). The observation that soluble KL specifically stabilizes through its ERK1/2 signaling GRα in human erythroid cells suggests that soluble KL initiates the response to stress by priming erythroid cells to respond to glucocorticoids.

Besides cell–cytokine interactions, (paracrine) cell–cell adhesion and adhesion of cells to the extracellular matrix are important functions of the microenvironment. Perhaps most studied are the β_1 integrins VLA4 and VLA5, which mediate the adherence of hematopoietic cells to stromal cells, fibronectin, or other components of the extracellular matrix. In β_1 integrin/ko mice, hematopoietic stem cells fail to colonize the fetal liver during embryonic development. Conditional deletion of β_1 integrin in adults leads to mild anemia with increased ROS formation and decreased RBC survival at steady state and in impairment in erythroid stress response (Fig. 27.3).[107] Milder responses to stress are seen with conditional deletion of α4 integrin.[108] In addition to intrinsic effects on erythropoiesis, antibodies to VLA4 or to the VCAM-1 (a VLA4 ligand on endothelial cells) influence the retention of stem/progenitor cells (and many erythroblasts in case of anti-α4) in bone marrow and thereby impair their homing and lead to their mobilization in adult mice, in primates, and in MS patients.[109] In in vitro studies, hematopoietic progenitors bind to specific domains of fibronectin in a differentiation-dependent manner (long-term culture-initiating cell and day 12 CFU-spleen in mice adhere mainly through the heparin-binding domain and CS-1). BFU-E and other progenitor cells adhere to both the cell-binding (Arg-Gly-Asp-Ser [RGDS]) and heparin-binding domains, whereas CFU-E preferentially bind the RGDS sequence, and reticulocytes fail to adhere to fibronectin. This differential binding could influence the proliferation and especially the maturation and survival of developing erythroid cells, particularly under stress as well as the migration of progenitor cells in and out of the bone marrow cavity. Hematopoietic cytokines/chemokines present in the microenvironment can also modulate the affinity of β1 integrins for ligand, adding complexity to the regulation of erythropoiesis within the marrow microenvironment.

In histologic sections of normal marrow, islands of maturing erythroblasts (erythroblastic islands) often surround a central macrophage, termed a *nurse cell* (Chapter 36). It has been suggested that the central macrophage supports terminal erythroid maturation by producing specific growth factors, assuring proper stage-specific iron supply, by establishing nanotubules which restrain mitochondria numbers before the process of mitophagy is in place, by providing a hub that promotes enucleation, and finally by engulfing and degrading the pyrenocytes. For this reason, the proteins that mediate the interaction between the two cell types have been the subject of intense investigation. Adhesion may be mediated through the binding of VLA4 (on erythroid cells) to VCAM-1 (on central macrophages), or through several other molecules, such as EMP discussed earlier.[29] These molecular interactions have been identified as being critical for erythroblastic island integrity.[110] In addition, the erythroblast ICAM-4 links erythroblasts to macrophages by interacting with αv integrin expressed in macrophages. Mice with targeted deletion of EMP are severely anemic and die at an embryonic stage,[111] and ICAM-4^{null} mice have markedly reduced erythroblastic islands. Of interest, RB-deficient macrophages do not bind RB^{null} erythroblasts, and failure of this interaction may mediate the defect in fetal liver erythropoiesis observed in RB^{null} mice. RB normally stimulates macrophage differentiation by counteracting inhibition of Id2 (a helix-loop-helix protein) on PU.1, a transcription factor crucial in macrophage differentiation. In addition to the aforementioned pathways, macrophage CD163 can serve as an erythroblast adhesion receptor in erythroblastic islands, promoting erythroid proliferation and/or survival. Whether a specific number or type of macrophages is required for optimal responses is not clear.[107] This hypothesis is supported by recent data on single cell expression profiling of macrophages that have identified that at least three different macrophage populations are capable of interacting with erythroid cells and may promote their maturation. However, these studies are still at their infancy and have yet to generate definite conclusions regarding the existence on specialized "erythroid niches" and on their complex function. Of note, tissue macrophages express RNA for EPO and may also influence erythropoiesis through this mechanism. The picture of the role of macrophages in erythropoiesis is further complicated by the fact that during stress erythropoiesis, activation of GR induces stress-specific erythroid progenitor cells to generate specific types of macrophages that are capable of promoting their proliferation even in the presence of maximal concentrations of growth factors.[112]

Erythropoiesis and Iron Regulation

Iron plays an essential role in numerous cellular functions, either as free ion or chelated with porphyrin to form heme, by facilitating the activities of numerous enzymes (Chapter 36). In erythroid cells, in addition to cytochromes, heme is a functional element of hemoglobin, the most abundant red cell protein. It is therefore not surprising that iron is at the same time an important regulator and effector of erythropoiesis and that any step of its metabolism in these cells is carefully regulated (uptake, storage, and efflux). Iron affects erythropoiesis directly and indirectly through events that restrict iron availability in the body. Erythroid cells, in turn, release feedback messages to modulate iron metabolism.

Iron circulates in the blood complexed with a specific transporter, Tf, and is taken up by the cells through the TfR. TfRs belong to a large group of receptors that internalize their ligand through receptor-mediated endocytosis. This cycle allows for reuse both of the ligand, which is released in the blood for re-saturation with iron, and of the receptor, for entering another round of endocytosis.

Iron is so important for erythropoiesis that erythroid progenitors are distinguished from other marrow progenitors by the presence of high levels of TfRs. TfRs are found in characteristic abundance in erythroid cells (300,000 to 800,000 TfRs per cell) and reach peak levels on CFU-E and erythroid precursors and decrease with maturation with the lowest level observed on reticulocytes. After the reticulocyte stage, receptors appear to be shed as small lipid vesicles.

An inverse relationship exists between receptor density and iron availability. Deprivation of iron results in receptor induction, and excess iron results in receptor suppression. However, the mechanisms that regulate the number of TfRs throughout the maturation of precursors (even within progenitors) are largely unknown.

Erythroid precursor cells differ from nonerythroid cells not only by requiring a higher number and higher occupancy of TfRs, but also by displaying immunologically distinct receptor isoforms. In addition to TfR1, a TfR2 has been identified. TfR1 and TfR2 are members of a family of genes encoding at least seven different homologous proteins in primates. TfR1 is a type II membrane glycoprotein that, as a cell surface homodimer, binds iron-loaded transferrin as part of the process of iron transfer and uptake. TfR2 is expressed in a membrane (TfR2-α)- and non-membrane (TfR2-β)-bound form, both of which bind transferrin with low affinity. The specific role of TfR2 in hematopoietic cells is unclear. In cells from 67 patients with de novo acute myeloid leukemia (AML), high levels of TfR2-α expression were correlated with an improved prognosis, and higher levels of both TfR2-α and TfR2-β were associated with longer survival, suggesting that TfR-independent iron uptake plays a role in in vivo proliferation of AML cells. Recent data in TfR2-deficient mouse models suggest that this receptor may also modulate the erythroblast sensitivity to EPO.[62]

In addition to its direct effect, TfR2 may also play an indirect role in erythropoiesis as the key regulator of iron metabolism in the liver. In fact, maintenance of stable extracellular iron concentrations requires the coordinated regulation of iron transport into plasma from dietary sources in the duodenum, recycled senescent red cells in macrophages, and storage in hepatocytes. The presence of diferric transferrin in the liver is regulated by a complex machinery involving, in addition to TfR2, the product of the hereditary hemochromatosis (HFE) gene (a protein of the major histocompatibility complex class I) and the product of the hemojuvelin (HJV) gene (also known as HFE2). Given that the levels of TfR2 expression are exclusively regulated by holotransferrin, TfR2 expressed by hepatocytes is likely the first element of the iron sensory pathway in the liver. In turn,

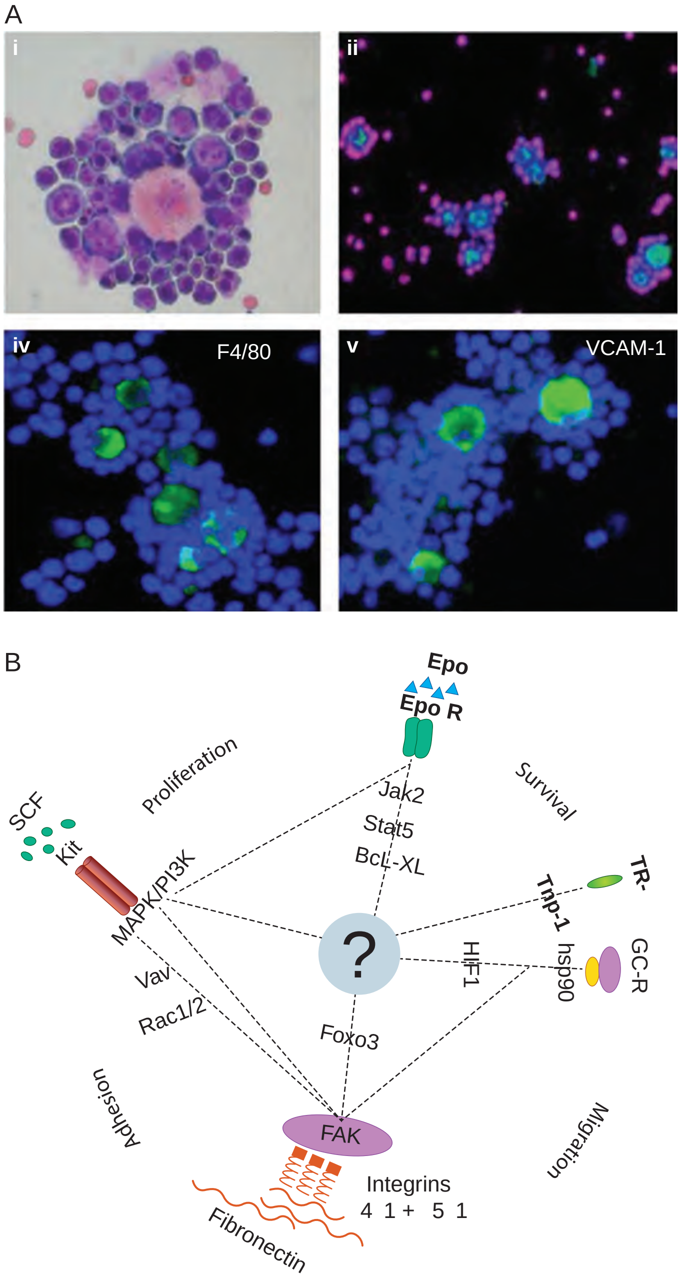


Figure 27.3 STRESS ERYTHROPOIESIS AND THE MACROPHAGE NICHE. (A) Macrophage subsets in WT mice at steady state and during response to stress. Morphology and expression of F4/80, CD11b, and vascular cellular adhesion molecule-1 (VCAM-1) was analyzed in preparations of erythroid islands isolated from spleens of PHZ-treated mice on day 4. (i): Hematoxylin staining of an erythroid island: note the different maturation stages of the erythroblasts that adhere to themselves and to the central macrophage engorged in damaged red cells after PHZ treatment; light green stain for F4/80 and magenta nuclear stain (assigned color) for surrounding erythroblasts (ii); light green F4/80 cells and DAPI-stained erythroblasts (iv); and green VCAM-1 positivity on central macrophages (v). (B) Important participating pathways leading to proliferative expansion of erythroid cells (mainly in the spleen) during stress in mice. Following genetic impairment of several pathways (i.e., Kit, GC-R, TR-α, and β1 integrins in a cell intrinsic manner), proliferative erythroid expansion in the spleen is severely compromised even in the presence of adequate levels of erythropoietin (EPO). It is possible that β1 integrins (specifically α5β1) intercept at least some of these known pathways, but the molecular details and cooperating/interacting molecules are missing (illustrated by *dashed lines*). Interpretations of the influence of these pathways and their signaling intermediates are complicated by the fact that these pathways have not been selectively eliminated in erythroid cells. Consequently, it is not clear at what stage of erythroid differentiation the FAK- or Src-dependent signaling is activated, early or late or both, and future studies should clarify these issues. *(Part A from Ulyanova T, Phelps SR, Papayannopoulou T. The macrophage contribution to stress erythropoiesis: when less is enough. Blood. 2016;128:1756–1765. Part B modified from Ulyanova T, et al. Erythroid cells generated in the absence of specific β1-integrin heterodimers accumulate reactive oxygen species at homeostasis and are unable to mount effective antioxidant defenses. Haematologica. 2013;98[11]:1769–1777, Suppl Fig 3B.)*

hepatocytes respond to iron sensing by modulating hepcidin expression and secretion. Hepcidin, a 25-amino acid disulfide-rich peptide, acts as a systemic iron regulatory hormone that regulates both dietary iron absorption by the enterocytes and iron recycling by the macrophages. Because ferroportin shuttles iron from the enterocytes to the macrophages and hepcidin is required for ferroportin internalization and degradation, decreased hepcidin expression blocks iron export in the two cell types. Each gene involved in iron metabolism has a role in regulating the expression of the other genes. In particular, reduced expression of HEF, TfR2, and HJV reduces expression of hepcidin. It is not surprising then that mutations altering the function of all of these genes, including hepcidin, have been found to be associated with hereditary hemochromatosis (see Chapter 37). In addition to its role in determining the pathobiology of hereditary hemochromatosis, hepcidin plays an important role in determining the anemia of chronic diseases and inflammation (see Chapters 36 and 38). In inflammation, the increased hepcidin synthesis traps iron in macrophages, decreases plasma iron concentrations, and causes iron-restricted erythropoiesis characteristic of the anemia of inflammatory states. Hepcidin might inhibit proliferation of erythroid progenitors at low EPO concentrations. On the basis of these data, a hepcidin assay has been proposed as a useful tool for diagnosing iron disorders and monitoring their treatment and hepcidin agonists and antagonists have been suggested as useful therapeutics for treatment of iron disorders and polycythemia vera (PV) (Chapters 37, 41, and 70).

The stringent need for iron during erythroid maturation led Dr. Clement Finch to first hypothesize the existence of signals released by mature erythroid cells that controls iron metabolism. This hypothesis has been recently confirmed by the identification of erythroferrone (ERFE), a protein released by erythroid cells that suppresses hepcidin expression in mice under conditions of stress.[113] ERFE-deficient mice fail to suppress hepcidin after hemorrhage and exhibit a delayed recovery after blood loss. Data from additional mouse models suggest that ERFE also contributes to recovery from anemia after inflammation.

Synthesis of heme appears to be coordinated with synthesis of globins throughout erythroid maturation so that functional hemoglobin tetramers are formed rapidly and spontaneously after release of newly synthesized globins from polysomes. Information about the accumulation of heme and its synthetic intermediaries has been provided by crude biochemical approaches. However, now that the genes for several enzymes in the heme synthetic pathway (e.g., δ-5aminolevulinic acid synthase, porphobilinogen deaminase, ferrochelatase) have been cloned, further information about their regulation is available (Chapter 39).

At the early stages of differentiation when globin chain synthesis is scanty, upregulation of heme biosynthesis is restrained by specific iron effluxers, such as the export proteins first identified as the receptor for the feline leukemia virus, which restrict iron availability in the cytoplasm.[114] As maturation progresses, an important role in coordinating heme and globin chain assembly during hemoglobin production is exerted by AHSP. AHSP is a protein abundantly expressed in erythroid cells whose function is to bind free α-chains, stabilizing their structure and limiting their ability to participate in chemical reactions that generate ROS. In addition, AHSP binding increases the affinity of α-chains for β-chains, accelerating the formation of Hb tetramers. The essential role exerted by this gene in erythroid development has been demonstrated by the fact that its deletion in normal mice impairs red cell production. The red cells from AHSP^{null} mice have a decreased half-life, contain Hb precipitates, and exhibit signs of oxidative damage.[115] The observation that double AHSP−/− b-thalassemic mutant mice have an exacerbated phenotype suggests that AHSP is a gene modifier that, like the HPFH mutations, may ameliorate the phenotype of thalassemic patients. However, gene mapping, direct genomic sequencing, and extended haplotype analysis have not revealed any mutations or specific association between haplotypes of AHSP in 120 β-thalassemic patients. On the other hand, a polymorphism in the putative AHSP promoter leading to a threefold higher expression of the gene in reticulocytes has been observed in the normal population, but the clinical consequences of this observation are unknown.

CELLULAR DYNAMICS IN ERYTHROPOIESIS: STEADY STATE AND STRESS ERYTHROPOIESIS

The primary function of the mature red cell, which is the end-product of erythropoiesis, is to transport oxygen efficiently through the circulation to the tissues. To achieve this goal, the adult marrow must release approximately 3×10^9 new red cells or reticulocytes per kilogram per day. This number of reticulocytes represents (1%) of the total red cell mass and is derived from an estimated 5×10^9 erythroid precursors per kilogram. In addition to maintaining homeostasis (i.e., a stable hematocrit), the erythron must be able to respond quickly and appropriately to increased oxygen demands, either acute (e.g., following red cell loss) or chronic (e.g., with hypoxia from pulmonary disease or a right-to-left cardiac shunt). It is well established that EPO is responsible both for maintaining normal erythropoiesis and for increasing red cell production in response to oxygen needs. However, the overall marrow response is complex and requires not only the participation of erythroid cells responsive to EPO but also a structurally intact microenvironment and an optimal iron supply within the marrow.

EPO stimulation elicits two types of measurable responses: changes in proliferative activity (including improved survival) and changes in maturation rates. The first detectable response to increased serum EPO is amplification of CFU-E and erythroid precursors, cells that are extremely sensitive to EPO. Because virtually all these cells are already in cycle, increases in their numbers cannot be achieved by increasing their fraction in cycle. Either additional divisions are involved, or new cells must be recruited to the CFU-E pool (from a pre–CFU-E pool). Additional divisions of CFU-E or precursor cells would increase their transit time within the marrow and potentially delay the delivery of new red cells to the periphery. Because a shortened maturation time has been observed instead and the proliferative potentials of CFU-E and proerythroblasts are finite, high levels of amplification cannot be achieved through this mechanism. Therefore, such needs are met by influx into the CFU-E and precursor pools of newly differentiating cells from earlier progenitor compartments.

Such a surge of newly produced cells has been observed in prior experiments. A rapid influx of fresh cells was particularly notable in polycythemic mice that were experimentally depleted of CFU-E and erythroid precursors at the time the stimulus was applied. Because of the rapidity of response (i.e., within 24 hours in the polycythemic animals), it appeared that the orderly progression from BFU-E to CFU-E to proerythroblast had been compressed. Such acceleration of differentiation is possible through shortened intermitotic intervals, fewer mitotic divisions, or differentiation without divisions. This short-circuiting in differentiation requires high serum levels of EPO and adequate numbers of BFU-E (i.e., these conditions are met in a previously hypertransfused, polycythemic animal stimulated by EPO or in marrow suddenly recovering from acquired pure erythroid aplasia). Once CFU-E and precursors are expanded through this mechanism, most persisting erythropoietic demands can be met through this pool without excess input from pre–CFU-E pools. Thus, acute demand for erythropoiesis is met by influx from pre–CFU-E pools through an accelerated differentiation and maturation sequence. Demonstration of such an event was seen in mice with conditional deletions of integrins using the EPOR-Cre model deleting cells at the post–CFU-E level. In contrast, chronic demands (i.e., demands because of a chronic hemolytic anemia) are mainly satisfied through a greatly amplified late erythroid pool and with a minimum distortion in the differentiation sequence.[117] The fact that the kinetics of erythroid differentiation/maturation are different in acute versus chronic marrow regeneration is supported by differing qualitative changes in the newly formed red cells. An increase in i antigen and HbF expression as well as an increase in cells with higher mean corpuscular volumes is seen with an acute response, whereas these alterations are minimal or less pronounced with chronic responses.[117] When severe anemia persists from birth onward, erythroid production can increase up to 10-fold above baseline. This is possible not only because of maximally expanded erythropoietic pools but also because the sites of active erythropoiesis may extend to include those that support red cell differentiation during fetal life. Thus although the marrow space in axial bones (vertebrae, pelvis, ribs, sternum, clavicles) is sufficient for normal erythropoiesis or for a response to moderate anemia, the femur, humerus, spleen and/or liver, and (rarely) thymus may support red cell production in children with congenital hemolytic anemia (e.g., thalassemia major). Expanded erythropoiesis may lead to skeletal deformities, hepatosplenomegaly, or erythropoiesis in the soft tissues adjacent to bone.

Quantitative assessments of changes in erythroid progenitor cell pools in response to EPO stimulation can be made through cultures of bone marrow cells. Despite sampling errors,[117] erythroid cultures can provide rough estimates of relative progenitor abundance within an aspirated marrow specimen and have shown consistent increases in the frequency of CFU-E in proportion to the level of EPO stimulation. Conversely, with increases in the hematocrit or in polycythemic animals, a decrease in CFU-E frequency has been observed. In contrast to CFU-E, the incidence of BFU-E was found to fluctuate less with either acute or chronic expansion of erythropoiesis, probably because a few BFU-E can generate several thousand cells. Furthermore, BFU-E can increase their fraction in cycle and thus increase the number of differentiated progeny without a significant change in their total numbers. Most BFU-E detectable in marrow or blood erythroid cultures probably represent a reservoir of progenitors not normally participating in day-to-day erythropoiesis. The parameters needed to maintain a healthy or appropriate BFU-E pool in hematopoiesis are not defined. That hematopoietic expansion is curtailed in mice with steel mutations and anemia develops in mice treated with anti-KIT antibody suggests that adequate levels of normal KL may be crucial for early erythropoietic expansion.

The rate of red cell production can also be accurately evaluated by ferrokinetic studies (i.e., study of iron incorporation into developing red cells). In addition, a marrow scan, typically with technetium Tc 99 m, can document the extent of active erythropoiesis. However, these approaches are seldom necessary in clinical practice because estimates of erythropoiesis can be obtained from the reticulocyte index. First, the observed percentage of reticulocytes is normalized to the hematocrit to calculate the total marrow output of reticulocytes. Alternatively, the absolute number of reticulocytes per microliter can be counted directly using fluorescent RNA labeling. However, because younger reticulocytes are prematurely released into the circulation under conditions of acute need, the total number of reticulocytes overestimates the true level of red cell production as measured by iron kinetics. Therefore a second correction is made to account for the maturation of early circulating reticulocytes, or "shift" cells (polychromatophilic red cells), when present in the blood smear. The resulting reticulocyte index provides an excellent estimate of effective red cell production.

Although the presence, density, or both of EPORs on developing erythroid cells determines the responses to EPO, other properties (e.g., surface antigens on BFU-E versus CFU-E versus end-stage red cells) may provide the basis for selective suppression of CFU-E versus BFU-E or selective immune destruction of red cells versus erythroblasts. For example, suppression of CFU-E or erythroblasts can occur in acquired pure red cell aplasia or B19 parvovirus infection (Chapter 33), respectively, whereas the BFU-E compartment in both these conditions remains largely unperturbed. Thus, the boundary from BFU-E to CFU-E and erythroblast may be biologically important for the pathophysiology of these disease states. Furthermore, in acquired hemolytic anemia, selective destruction at a given stage of maturation (of red cells only or of both erythroblasts and red cells) can be observed depending on the type of antibody produced and the density of its antigen present on maturing erythroid cells.

Detailed knowledge of the structural and functional properties of erythroid cells throughout their differentiation may provide significant insights into the pathogenesis of hematopoietic disorders affecting the red cell lineage. These studies have identified qualitative aberrations in the response of erythroid progenitors to cytokines or EPO that may underlie the abnormalities of congenital erythroid hypoplasia (Diamond-Blackfan syndrome) (Chapter 30).[118] Analogous qualitative or functional defects can be observed in neoplastic erythropoiesis, because erythroid progenitors from patients with PV and other myeloproliferative neoplasms have altered sensitivities to EPO (Chapter 70).

The correlation between the antigenic profiling and the function of erythroid cells described in the section "The Erythroid Compartment" is not maintained under conditions of perturbed or stressed erythropoiesis. Stress activates bone morphologic protein 4 (BMP4)/Hedgehog signaling, which induces the generation of erythroid progenitor cells with a unique phenotype, KIT^{pos}, CD71^{pos}, and TER-119^{pos} (TER-119 recognizes the murine equivalent of glycophorin A). The expression on these cells of "true" markers of terminal erythroid maturation suggests that stress-specific erythroid progenitors may be related to the proerythroblasts with extensive proliferative potential generated in mice after EPO treatment or anemia challenge, thereby indicating that stress may uncouple proliferation and differentiation programs during terminal erythroid maturation. Interestingly, human erythroblasts generated in the presence of dexamethasone also express high levels of KIT and acquire self-renewal potential. These observations challenge the notion that erythroblasts are capable of a limited (at most two to four) number of divisions.

Murine models have been developed to address phenotype-function cell relationships during recovery from acute and chronic erythroid stress (see Fig. 27.3).[108] A model for acute stress is represented by the hemolytic anemia induced by phenylhydrazine treatment. Recovery from this acute anemia involves recruitment of the spleen as an additional erythropoietic site and is dependent on EPO. The amount of ^{3}H-thymidine incorporated by splenic erythroblasts produced in response to this stress initially represented the biologic assay for EPO. Genetic evidence indicates that recovery from this hemolytic anemia is controlled by a receptor complex formed between the EPOR and a truncated version of the Stk receptor encoded by Fv2^{s}, a locus that also determines strain susceptibility to Friend virus infection. An additional control on the response to acute erythroid stress in mice is exerted by the GR, because mice in which this receptor is targeted recover poorly from phenylhydrazine treatment. On the other hand, experimentally induced mutations in genes involved in the regulation of erythroid differentiation, such as STAT5^{null}[119] and GATA1^{LOW}, or an inability to respond to ROS challenge (i.e., Foxo3 deficiency) increase the rate of erythroblast apoptosis.

Although clinical observations indicating that the GR ligand such as dexamethasone stimulates erythropoiesis have been available since 1961, the precise role of GR in human erythropoiesis is still unclear. GR activation increases the erythroid output by inducing BFU-E-self replication (this effect is mediated by ZFP36L2 in cooperation with PARP)[120] and by blocking the terminal maturation of proerythroblasts which proliferate for longer period of time.[121] This later effect requires the chaperone protein, calreticulin (CALR), which retains GR in the cytoplasm restoring EPOR signaling.[114]

Great individual variability in the response to glucocorticoids exists in the human population. In fact, the murine GR is not polymorphic, whereas the human GR (GR/NR3C1 located in the 5q31-32 region of chromosome 5 and deleted in 5q-syndrome) contains several SNPs. Because of this genetic diversity, human cells may express more than 260 isoforms with slightly or greatly different biologic activities. The most studied isoform is GRα, an isoform similar to the murine GR. Alternative splicing between exon 3 and 4 generates GRγ, an isoform containing an additional arginine in the DNA-binding domain that reduces the transactivation potential by half. An alternative splicing of exon 9 generates mRNA encoding the dominant-negative GRβ isoform. It is generally accepted that responses to GR ligands depend on the signal transduction potential of the GR isoforms expressed by different cells and tissues. Studies in human non-erythroid cell types have identified that GR isoform expression predicts the variegation of cellular response to dexamethasone in vitro. Recently, clinicians have established important correlations between GR haplotype and variability in patients' responses to glucocorticoids and in the development of glucocorticoid resistance in several disorders. GR polymorphism and/or epigenetic changes are emerging as the leading cause for dexamethasone unresponsiveness or for development of dexamethasone resistance in patients with inflammatory and autoimmune diseases (i.e., Crohn disease, systemic lupus) and in chronic depression. The frequency of the rs6198 SNP is also greater than normal in patients with the Philadelphia-negative myeloproliferative neoplasm PV (55%, $P = .0028$) and primary myelofibrosis and with DBA (43%, $P = .03$), suggesting that genetic conditions favoring GRβ expression may represent host genetic modifiers in diseases with altered terminal erythroid differentiation.

Additional functions of the red cells are starting to emerge: RBCs are thought to initiate clotting by binding to the endothelial cells and trapping platelets to the binding site. Since they express high levels of lipoprotein receptors, RBCs have been proposed to function as lipid scavengers that regulate the lipid content in blood and may affect the onset of atherosclerosis and stroke. In addition, the similarity between the amino acid sequence of the blood group antigens and that of proteins present on the surface of bacteria and the increased frequency of certain blood group antigens in regions with a high incidence of malaria suggest that the blood group antigens present on red cells may facilitate development of innate immunoreactivity toward opportunistic infections.[122]

ONTOGENY OF ERYTHROPOIESIS

During human development, distinct anatomic areas for production of erythroid cells are recruited sequentially, in a temporal succession that allows overlap (see Fig. 27.1). In addition, parallel changes occur in the morphologic and functional properties of the erythroid cells themselves (Chapter 9).

During the phase of embryonic erythropoiesis in the blood islands of yolk sac, aggregates of immature erythroid cells undergo maturation synchronously as a single cohort. Before their maturation is completed, they begin to circulate, and by gestational week 5 they are found in the vascular spaces of the rudimentary liver. At about the same time, foci of immature erythroid cells emerge within the fetal liver as the fetal (or hepatic) phase of erythropoiesis commences. From week 7 onward, the liver is progressively filled with erythroid precursors and becomes the dominant site of erythroid cell production until approximately gestational week 30. Although some red cell production can be found in the thymus, the spleen, or occasionally in the lymph nodes, these other sites are never dominant. However, recently placenta has been recognized as an important local erythropoietic site. From month 6 onward, the cavities of long bones are invaded by vascular sprouts and become competent to support red cell development. However, by contrast with the liver where erythropoiesis prevails, myelopoiesis is the dominant lineage that differentiates in the fetal bone marrow. Shortly after birth, all bone cavities become actively engaged in erythroid production, and the hepatic (fetal) phase of erythropoiesis comes to an end, as the final (adult) phase of erythropoiesis unfolds exclusively within the bone marrow. This neonatal switch of erythroid site is thought to be responsible for the transient anemia of the newborn and is controlled by microenvironmental cues.

The anatomic shifts in the sites of erythropoiesis are associated with shifts in the phenotypic characteristics of the erythroid cells. Embryonic (primitive) erythroid cells (derived from the yolk sac) are large (~200 μm), circulate as nucleated cells, and have a megaloblastic appearance. These embryonic erythrocytes eventually undergo enucleation in the fetal liver at late stages of development, once significant numbers of macrophages start to mature in this organ and after acquisition of α4-integrin expression.[123] As BFU-E present in yolk sac, fetal liver, and fetal bone marrow have a definitive-like progeny and these progenitors were not present after ablation of core binding factor (CBF)-β,[124] despite the presence of normal embryonic erythropoiesis, the derivation of embryonic erythroblasts from a distinct and unique progenitor, not present in subsequent life, remains a viable hypothesis.

Fetal liver erythroid cells are smaller than embryonic cells (~125 μm) but have a macrocytic appearance compared with adult normocytic red cells (~80 μm). Like adult cells and in contrast to the great majority of primitive cells, fetal liver erythroid cells eject their nuclei during maturation before being released in the blood.

Apart from variations in size and morphology, embryonic and fetal erythroid cells differ from each other and from their adult counterparts in several other properties, including growth factor requirements, proliferative status, transplantation potential, and surface antigenic expression. For example, whereas fetal erythropoiesis is under the control of EPO, the extent of EPO's influence on embryonic erythropoiesis is disputed. Most convincing are the results of EPO/EPOR knockout mice[125] that showed only partial effects on embryonic erythropoiesis, in contrast to fetal erythropoiesis. Single-lineage transcriptomic analyses indicate that TGF-β may represent a primary regulator of embryonic erythropoiesis. EPO levels increase between weeks 9 and 32 of gestation, and fetuses respond to hypoxia or anemia with increased EPO as early as 24 weeks. Fetal erythroid progenitors when studied in vitro appear more sensitive to EPO and KL than adult progenitors. In contrast, their in vitro response to IL-3 or GM-CSF is minimal compared to that of adult erythroid progenitors. In addition, fetal erythroid progenitors are characterized by a higher proliferative potential and shorter doubling times than adult cells when cultured in vitro. The dependency of stem/progenitor cells on KL changes during ontogeny. Although generation of repopulating stem cells is minimally affected during fetal life in mice that cannot produce KL (steel-Dickie (Sl/Sl^d) mutant mice) adult mice which produce only soluble KL display greatly impaired erythropoiesis and hematopoiesis, suggesting that the KL/KIT pathway plays a role in the recruitment and self-renewal behavior of adult but less so of fetal stem cells in vivo.

The homing properties of fetal stem cells transplanted into adult irradiated recipients were found to be inferior to those of their adult counterparts. Whether this finding is related to their increased cycling or other reasons is unclear. However, fetal liver stem cells, despite their reduced homing potential, have higher engraftment levels, likely because of their proliferative prowess compared to adult stem cells.

The surface antigenic profiles of erythroid progenitors and precursors are distinct at each ontogenetic stage. For example, HLA class I and class II antigens are not detected in embryonic erythroid progenitor cells but reach adult levels at approximately gestational week 9. $CD34^+$ hematopoietic progenitors present in yolk sac express Mac-1 but are negative for stem cell antigen 1 (Sca-1), which is expressed by fetal and adult $CD34^+$ murine progenitor cells. On the other hand, adult $CD34^+$ progenitors lack Mac-1 and AA4.1, which are expressed by fetal $CD34^+$ progenitor cells. Fetal BFU-E and CFU-E express similar levels of HLA class II antigens, whereas adult CFU-E are largely devoid of these antigens. β_1 integrins, especially $\alpha_4\beta_1$ and α_5, are expressed widely by all hematopoietic cells, including nucleated erythroid cells. However, in the latter, they display a differentiation-dependent, developmentally segregated pattern of expression, because they are absent in embryonic murine erythroblasts, and among adult cells, stem/progenitor cells express them in a constitutively active form in contrast to more mature cells. Fetal red cells display a straight, unbranched polylactosaminyl chain (i antigen) on their surface, whereas in adult cells, this structure, which bears ABH blood group determinants, is highly branched (I antigen).[126] The enzymatic activity of several enzymes in the glycolytic pathway is greater in fetal than in adult red cells. In contrast, carbonic anhydrase levels are very low during intrauterine and early neonatal life. Distinct isozyme patterns for several enzymes (i.e., phosphoglycerate kinase, acetylcholinesterase) also distinguish fetal from adult red cells.

The most widely studied changes during red cell ontogeny are the shifts or "switches" in globin types. Embryonic erythroblasts are characterized by their avid accumulation of iron, which is stored as ferritin (0.3% to 1% of total protein) and by the synthesis of the unique hemoglobins Gower I ($\zeta_2\varepsilon_2$), Gower II ($\alpha_2\varepsilon_2$), and Portland ($\zeta_2\gamma_2$). The ζ- and ε-globin chains are embryonic α-like and β-like chains, respectively. These three embryonic types of hemoglobin are most likely synthesized in succession, because the concentration of Gower I is highest in smaller embryos. Thus a switch from ζ- to α- and ε- to γ-globin gene production begins during the embryonic phase of erythropoiesis but is not complete until fetal erythropoiesis is well established. During the transition from yolk sac to fetal liver erythropoiesis (6 to 9 weeks), erythroid precursors within the fetal liver co-express embryonic (ζ- or ε-) and fetal (α- or γ-) globin. The predominant type of hemoglobin synthesized during fetal liver erythropoiesis is HbF ($\alpha_2\gamma_2$), with a high proportion of γ^G: γ^A (7:3). Adult HbA ($\alpha_2\beta_2$), which is detectable at the earliest stages of fetal liver erythropoiesis, is synthesized as a minor component throughout this period and HbA_2 ($\alpha_2\delta_2$), which is a minor hemoglobin in the adult, is undetectable in these early stages. From about gestational week 30 onward, β-globin synthesis steadily increases so that, by term, 50% to 55% of hemoglobin synthesized is HbA. By 4 to 5 weeks of postnatal age, 75% of the hemoglobin is HbA. This percentage increases to 95% by 4 months as the fetal-to-adult hemoglobin switch is completed. HbF levels in circulating red cells are at a plateau for the first 2 to 3 weeks (as a result of the decline in total erythropoiesis that follows birth), but HbF levels gradually decline so that normal levels (<1%) are achieved by 200 days after birth.

Several in vitro and in vivo approaches have been used to study the basis of globin switching through development. Beyond its biologic interest, rigorous research in this area was propelled by the possibility of manipulating globin switching to increase HbF production in adults and ameliorate the clinical symptoms of disorders of the β-globin locus (e.g., sickle cell anemia, thalassemia). The most important determinant of fetal-to-adult hemoglobin switching seems to be post-conceptual age, with the sharpest period for transition between 30 and 52 weeks. The fetal-to-adult switch appears to be unaffected by the time at which birth occurs or by changes in the kinetics of erythropoiesis induced by perinatal hemolysis. A delay in switching usually is observed in cases of general developmental retardation, in patients with certain chromosomal abnormalities (e.g., trisomy 13), and in diabetic infants because of increased circulating levels of α-aminobutyric acid, which directly affects HbF synthesis, inspiring the use of butyrate for increasing HbF in vivo.

Transplantation experiments and ablative endocrine maneuvers in the sheep model and transplantation of human fetal liver cells to adult recipients have failed to provide convincing support for the effects of environmental cues on the switching process. In fact, integration of data from experiments in transgenic mice and in hybrid cells (produced by fusion of human with mouse cells),[127] as well as by isolation of stage-specific transcription factors in other erythroid systems (e.g., avian) indicates that developmental control of globin switching is mostly intrinsic to erythroid cells.

Genetic linkage and GWAS in individuals with increased levels of HbF or subjects with HPFH syndromes have provided important insights concerning the control of fetal to adult globin switching. These studies have identified elements that control the switching both in *cis* (deletions in β-globin cluster or mutations in the γ-globin gene promoters, i.e., in DNA regions which provide hubs for transcription factors) and in *trans* (genes on other chromosomes that encode the transcription factors that bind the hubs that repress the fetal globin genes) of the β-globin locus. Transcriptional factors like BCL11A, ZBTB7A (an oncogene also known as LRF), and HBS1L-Myb are examples of HbF repressors. The full-length BCL11A is expressed in adult but not fetal erythroid cells. In addition to transcriptional control, the ontogenic-specific regulation of BCL11A expression is exerted at the transcriptional level by LIN28B that suppresses the translation of BCL11A mRNA by the ribosome machinery.[128] Furthermore, a group of adult individuals with high hemoglobin F harbor loss-of-function BCL11A mutations that reduce its expression in adult cells. In addition, suppression of BCL11A expression reactivates HbF expression in adult erythroid cells in vitro, and deletion of BCL11A interferes with fetal hemoglobin silencing during development and rescues the phenotype of a mouse model of sickle cell disease. These observations suggest that genome editing of BCL11A in autologous stem cells followed by transplantation may represent a useful strategy to ameliorate the clinical picture of hemoglobinopathies such as sickle cell disease. Genome editing approaches based on zinc fingers, TALENS, and Crisper/cas technology are rapidly being developed and allow precise targeting either of BCL11A or its erythroid-specific enhancer-binding sites in g-promoter. ZBTB7A is a C2H2 zinc finger that binds at the site −200 bp of the γ-globin promoter and has been shown to effectively and independently represses the human γ-globin gene.

Myb-HBS1L is downregulated in individuals with elevated HbF levels and overexpression of Myb inhibits γ-globin in human erythroleukemia cells. The levels of Myb were found to be controlled by micro-RNA 15a and 16-1 in patients with human trisomy 13 and high HbF levels. In addition, the observation that EKLF mutations are relatively more common in a thalassemia-endemic region where they are associated with milder β-thalassemia phenotypes support a role for EKLF, the major erythroid transcriptional regulator, as a suppressor of fetal globin gene. Valuable insights of the effect of EKLF on the regulation of globin genes were obtained from families with haploinsufficiency of EKLF (missense mutations affecting DNA binding) and increased fetal hemoglobin levels. However, this was not always the case, and a compound heterozygosity was required in other families for high HbF expression. It appears that EKLF directly targets genes, such as *BCL11A*, *EPB4*.9, and *CD44*, which are very sensitive to its activity, whereas its effects on other genes, like γ-globin, or *BCAM* (carrying the Lutheran blood group antigens) are variable. Collectively, these data indicate that EKLF affects the globin gene switching both directly, by activating β-globin, and indirectly, by suppressing γ-globin through its control of *BCL11A*. In addition to EKLF another zinc finger containing protein, pogo transposable element with zinc (POGZ-finger domain), positively regulates *BCL11A* expression in mouse and human cells by binding to *BCL11A* promoter and enhancer elements.[129] Beyond that, POGZ may be a direct EKLF target.[130]

Beyond the above transcriptional factors influencing *BCL11A* expression there is a multilayer control through chromatin remodeling complexes governing histone acetylation/methylation and DNA methylation which dictates differences in fetal globin expression between fetal and adult cells. Any member of the epigenetic subunits of the NuRD silencing complex may be sufficient to repress HbF and any of these (i.e., CHD4, MBD2, DNMT1, DRED complex, PRMTs, etc.) can serve as a potentially druggable target for increasing fetal globin.[131–136] Furthermore redirecting the LCR to the silenced gamma-promoter by targeted tethering of the Ldb1SA domain will increase fetal globin with a reciprocal decrease in beta globin.[137] New highly innovative base-editing approaches to either correct the sickle mutation or possibly creating novel binding sites in fetal promoters may obviate potential genotoxicity issues associated with double-strand DNA brakes, although they may not be entirely free of any off-target effects of their own.[138]

Whereas all the above-cited approaches represent potential strategies to increase fetal globin for therapeutic purposes in severe β-thalassemia and sickle cell disease, the easy administration of a small molecule addressing any of the aforementioned targets may have a greater worldwide impact.

ERYTHROID CELLS FROM IN VITRO GENERATED SOURCES

The need of severely anemic patients for regular blood transfusions leads not infrequently to the development over time of alloantibodies. These events have led investigators to generate RBCs in vitro from dedicated sources for use as an alternative blood source for transfusion therapy. These studies were prompted by advances made in the 2000s to generate in vitro large numbers of human erythroid cells. Initially attractive sources for creating cultured RBC products were either CD34+ cells from discarded leukoreduced blood products from individuals with rare blood types or low volume cord blood collections.[139] However, these promising efforts were met with challenges in obtaining suitable HSC sources and producing clinical-grade RBCs in numbers and quality necessary for transfusion.

Alternative sources for erythroid cells have included erythroid progenitor cell lines derived from mouse ESCs. These lines were shown to produce functional RBCs that when transplanted into mice subjected to hemolysis significantly ameliorated the degree of acute anemia and increased survival. This proof of principle was followed by additional studies which identified conditions to generate erythroid cells in vitro from human ESC lines. The discovery of methods to generate $CD34^+$ HSC from induced pluripotent stem cells (iPSCs) has further advanced our ability to model in vitro erythroid diseases. In these studies, $CD34^+$ HSC can be obtained from either iPSCs generated from the patient's somatic cells or from iPSCs from normal donors modified by gene editing to carry the mutations that lead to these red cell disorders in man.[140] However, the use of ESC- or iPSC-derived erythroid cells for transfusion purposes has proven cumbersome, expensive, and associated with limitations for generating sufficient enucleated RBCs needed for a clinically accepted transfusion product.

Recently, the ability to generate human erythroblast cell lines capable of extensive proliferation that may be induced to mature on demand have presented new opportunities for unlimited sources for generating RBCs.[141,142] Although the availability of these cell lines, with inherent potential for gene editing,[143] has provided incredible opportunities to generate RBCs with patient-specific blood group antigens, to generate surrogate models to study inherited and acquired erythroid disorders, and to serve as a source of RBCs for transfusion therapy, this approach has encountered the same difficulties of delivering RBCs in the absence of any nucleated erythroid cells. Furthermore, direct lineage conversion or enforced differentiation of somatic cells to erythroid lineage cells has also provided new avenues to produce blood products from patient-specific cells.[144] These approaches suffer from the same shortcomings described for earlier approaches and, until improvements in providing safe and functional transfusable RBCs at a large scale are found, one may suggest that for the time being establishment of banks of iPSCs or direct reprogramming of cells from alloimmunized patients and those with rare blood groups as a reasonable direction for future clinical development.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Braccioli L, de Wit E. CTCF: a Swiss-army knife for genome organization and transcription regulation. *Essays Biochem*. 2019;63:157–165.

Bresnick EH, Jung MM, Katsumura KR. Human GATA2 mutations and hematologic disease: how many paths to pathogenesis? *Blood Adv*. 2020;4:4584–4592.

Conboy JG. RNA splicing during terminal erythropoiesis. *Curr Opin Hematol*. 2017;24:215–221.

Crispino JD, Horwitz MS. GATA factor mutations in hematologic disease. *Blood*. 2017;129:2103–2110.

Dijon M, Chabannon C, Tonnelle C. Ikaros and erythropoiesis. *Med Sci (Paris)*. 2008;24:1065–1070.

Dorshkind K. Regulation of hemopoiesis by bone marrow stromal cells and their products. *Annu Rev Immunol*. 1990;8:111–137.

Fraser NS, Knauth CM, Moussa A, et al. Genetic variants within the erythroid transcription factor, KLF1, and reduction of the expression of Lutheran and other blood group antigens: review of the In(Lu) phenotype. *Transfus Med Rev*. 2019;33:111–117.

Gnanapragasam MN, Bieker JJ. Orchestration of late events in erythropoiesis by KLF1/EKLF. *Curr Opin Hematol*. 2017;24:183–190.

Huang J, Liu X, Li D, et al. Dynamic control of enhancer repertoires drives lineage and stage-specific transcription during hematopoiesis. *Dev Cell*. 2016;36:9–23.

Jelkmann W. Activin receptor ligand traps in chronic kidney disease. *Curr Opin Nephrol Hypertens*. 2018;27:351–357.

Katsumura KR, Bresnick EH. The GATA factor revolution in hematology. *Blood*. 2017;129:2092–2102.

Kereny MA, Orkin SH. Networking erythropoiesis. *J Exp Med*. 2012;207:2537–2541.

Lennartsson J, Rönnstrand L. Stem cell factor receptor/c-Kit: from basic science to clinical implications. *Physiol Rev*. 2012;92:1619–1649.

Liggett LA, Sankaran VG. Unraveling hematopoiesis through the lens of genomics. *Cell*. 2020;182:1384–1400.

Menon V, Ghaffari S. Erythroid enucleation: a gateway into a "bloody" world. *Exp Hematol*. 2021;95:13–22.

Nigra AD, Casale CH, Santander VS. Human erythrocytes: cytoskeleton and its origin. *Cell Mol Life Sci*. 2020;77:1681–1694.

Papayannopoulou T. Control of fetal globin expression in man: new opportunities to challenge past discoveries. *Exp Hematol*. 2020;92:43–50.

Paulson RF, Hariharan S, Little JA. Stress erythropoiesis: definitions and models for its study. *Exp Hematol*. 2020;89:43–54. e2.

Perkins A, Xu X, Higgs DR, et al. Krüppeling erythropoiesis: an unexpected broad spectrum of human red blood cell disorders due to KLF1 variants. *Blood*. 2016;127:1856–1862.

Pinho S, Frenette PS. Haematopoietic stem cell activity and interactions with the niche. *Nat Rev Mol Cell Biol*. 2019;20:303–320.

Psaila B, Mead AJ. Single-cell approaches reveal novel cellular pathways for megakaryocyte and erythroid differentiation. *Blood*. 2019;133:1427–1435.

Shimizu R, Engel JD, Yamamoto M. GATA1-related leukaemias. *Nat Rev Cancer*. 2008;8:279–287.

Smith EM, Tangpricha V. Vitamin D and anemia: insights into an emerging association. *Curr Opin Endocrinol Diabetes Obes*. 2015;22:432–438.

Stamatoyannopoulos JA. The genomics of gene expression. *Genomics*. 2004;84:449–457.

Stohlman F, Ebbe S, Morse B, et al. Regulation of erythropoiesis. XX Kinetics of red cell production. *Ann N Y Acad Sci*. 1968;149(1):156–172.

Varricchio L, Migliaccio AR. The role of glucocorticoid receptor (GR) polymorphisms in human erythropoiesis. *Am J Blood Res*. 2014;4:53–72.

Vermunt MW, Zhang D, Blobel GA. The interdependence of gene-regulatory elements and the 3D genome. *J Cell Biol*. 2019;218:12–26.

Xavier-Ferrucio J, Krause DS. Concise review: bipotent megakaryocytic-erythroid progenitors: concepts and controversies. *Stem Cells*. 2018;36:1138–1145.

Yien YY, Bieker JJ. EKLF/KLF1, a tissue-restricted integrator of transcriptional control, chromatin remodeling, and lineage determination. *Mol Cell Biol*. 2013;33:4–13.

Yuan G-C, Cai L, Elowitz M, et al. Challenges and emerging directions in single-cell analysis. *Genome Biol*. 2017;18:84.

REFERENCES

1. Papayannopoulou T, Migliaccio AR. Biology of erythropoiesis, erythroid differentiation, and maturation. In: Hoffman R, Benz E, Silberstein LE, Heslop EE, Weitz JI, Anastasi J, Salama ME, Abutalib SA, eds. *Hematology: Basic Principles and Practice*. Seventh Edition Philadelphia, PA: Churchill Livingstone Elsevier; 2018:297–320.
2. Rettig MP, Ansstas G, Dipersio JF. Mobilization of hematopoietic stem and progenitor cells using inhibitors of CXCR4 and VLA-4. *Leukemia*. 2012;26:34–53.
3. Anstee DJ, Gampel A, Toye AM. Ex-vivo generation of human red cells for transfusion. *Curr Opin Hematol*. 2012;19:163–169.
4. Bouhassira EE. Concise review: production of cultured red blood cells from stem cells. *Stem Cells Transl Med*. 2012;1:927–933.
5. Mori Y, Chen JY, Pluvinage JV, et al. Prospective isolation of human erythroid lineage-committed progenitors. *Proc Natl Acad Sci U S A*. 2015;112:9638–9643.
6. An X, Chen L. Flow cytometry (FCM) analysis and fluorescence-activated cell sorting (FACS) of erythroid cells *Methods in Molecular Biology*. vol. 1698. Totowa, NJ: Humana Press Inc; 2018:153–174.
7. Doulatov S, et al. Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. *Nat Immunol*. 2010;11:585–593.
8. Pellin D, et al. A comprehensive single cell transcriptional landscape of human hematopoietic progenitors. *Nat Commun*. 2019;10:1–15.
9. Huang P, et al. Putative regulators for the continuum of erythroid differentiation revealed by single-cell transcriptome of human BM and UCB cells. *Proc Natl Acad Sci U S A*. 2020;117:12868–12876.
10. Hay SB, et al. The Human Cell Atlas bone marrow single-cell interactive web portal. *Exp Hematol*. 2018;68:51–61.
11. Loughran SJ, et al. Lineage commitment of hematopoietic stem cells and progenitors: insights from recent single cell and lineage tracing technologies. *Exp Hematol*. 2020;88:1–6.
12. Mettananda S, et al. Phenotypic and molecular characterization of a serum-free miniature erythroid differentiation system suitable for high-throughput screening and single-cell assays. *Exp Hematol*. 2018;60:10–20.
13. Nguyen AT, et al. UBE2O remodels the proteome during terminal erythroid differentiation. *Science*. 2017;357 eaan0218.
14. Gautier EF, et al. Comprehensive proteomic analysis of human erythropoiesis. *Cell Rep*. 2016;16:1470–1484.
15. Trakarnsanga K, et al. Qualitative and quantitative comparison of the proteome of erythroid cells differentiated from human iPSCs and adult erythroid cells by multiplex TMT labelling and nanoLC-MS/MS. *PLoS One*. 2014;9:e100874.
16. Fowler VM. The human erythrocyte plasma membrane: a Rosetta Stone for decoding membrane-cytoskeleton structure. *Curr Top Membr*. 2013;72:39–88.
17. Gokhin DS, Fowler VM. Feisty filaments: actin dynamics in the red blood cell membrane skeleton. *Curr Opin Hematol*. 2016;23:206–214.
18. Alimohamadi H, Smith AS, Nowak RB, et al. Non-uniform distribution of myosin-mediated forces governs red blood cell membrane curvature through tension modulation. *PLoS Comput Biol*. 2020;16:1–26.
19. Smith AS, et al. MYH9-related disease mutations cause abnormal red blood cell morphology through increased myosin-actin binding at the membrane. *Am J Hematol*. 2019;94:667–677.
20. Jagadeeswaran R, et al. Pharmacological inhibition of LSD1 and mTOR reduces mitochondrial retention and associated ROS levels in the red blood cells of sickle cell disease. *Exp Hematol*. 2017;50:46–52.
21. Li X, et al. Histone deacetylase 6 regulates cytokinesis and erythrocyte enucleation through deacetylation of formin protein mDia2. *Haematologica*. 2017;102:984–994.
22. Pop R, et al. A key commitment step in erythropoiesis is synchronized with the cell cycle clock through mutual inhibition between PU.1 and S-phase progression. *PLoS Biol*. 2010;8:e1000484.
23. Gurbuxani S, Xu Y, Keerthivasan G, Wickrema A, Crispino JD. Differential requirements for survivin in hematopoietic cell development. *Proc Natl Acad Sci U S A*. 2005;102:11480–11485.
24. Leung CG, et al. Requirements for survivin in terminal differentiation of erythroid cells and maintenance of hematopoietic stem and progenitor cells. *J Exp Med*. 2007;204:1603–1611.
25. Nunomura W, Sawada K. Survivin: a new player during erythroblast maturation. *Haematologica*. 2012;vol. 97:1456–1458.
26. Ouled-Haddou H, et al. A new role of glutathione peroxidase 4 during human erythroblast enucleation. *Blood Adv*. 2020;4:5666–5680.
27. Wang J, et al. Mammalian erythroblast enucleation requires PI3K-dependent cell polarization. *J Cell Sci*. 2012;125:340–349.
28. Schultze SM, et al. p38α controls erythroblast enucleation and Rb signaling in stress erythropoiesis. *Cell Res*. 2012;22:539–550.
29. Wei Q, et al. Maea expressed by macrophages, but not erythroblasts, maintains postnatal murine bone marrow erythroblastic islands. *Blood*. 2019;133:1222–1232.
30. Hu J, et al. Isolation and functional characterization of human erythroblasts at distinct stages: implications for understanding of normal and disordered erythropoiesis in vivo. *Blood*. 2013;121:3246–3253.
31. O'Brien KA, et al. Molecular convergence in ex vivo models of Diamond-Blackfan anemia. *Blood*. 2017;129:3111–3120.
32. Beagan JA, Phillips-Cremins JE. On the existence and functionality of topologically associating domains. *Nat Genet*. 2020;52:8–16.
33. Behera V, et al. Exploiting genetic variation to uncover rules of transcription factor binding and chromatin accessibility. *Nat Commun*. 2018;9:782.
34. Dixon JR, et al. Topological domains in mammalian genomes identified by analysis of chromatin interactions. *Nature*. 2012;485:376–380.
35. Zhang H, et al. Chromatin structure dynamics during the mitosis-to-G1 phase transition. *Nature*. 2019;576:158–162.
36. Atchison ML. Function of YY1 in long-distance DNA interactions. *Front Immunol*. 2014;5:45.
37. Pan X, et al. YY1 controls Igκ repertoire and B-cell development, and localizes with condensin on the Igκ locus. *EMBO J*. 2013;32:1168–1182.
38. Deng W, et al. Controlling long-range genomic interactions at a native locus by targeted tethering of a looping factor. *Cell*. 2012;149:1233–1244.
39. Palii CG, et al. Single-cell proteomics reveal that quantitative changes in co-expressed lineage-specific transcription factors determine cell fate. *Cell Stem Cell*. 2019;24:812–820. e5.
40. Brand M, Morrissey E. Single-cell fate decisions of bipotential hematopoietic progenitors. *Current Opinion in Hematology*. 2020;vol. 27:232–240.
41. Kamatani Y, et al. Genome-wide association study of hematological and biochemical traits in a Japanese population. *Nat Genet*. 2010;42:210–215.
42. Nandakumar SK, et al. Gene-centric functional dissection of human genetic variation uncovers regulators of hematopoiesis. *Elife*. 2019;8:1–29.
43. Drissen R, et al. Distinct myeloid progenitor-differentiation pathways identified through single-cell RNA sequencing. *Nat Immunol*. 2016;17:666–676.
44. Wu Z, et al. Sequencing of RNA in single cells reveals a distinct transcriptome signature of hematopoiesis in GATA2 deficiency. *Blood Adv*. 2020;4:2656–2670.
45. Shimizu R, et al. In vivo requirements for GATA-1 functional domains during primitive and definitive erythropoiesis. *EMBO J*. 2001;20:5250–5260.
46. Byrska-Bishop M, et al. Pluripotent stem cells reveal erythroid-specific activities of the GATA1 N-terminus. *J Clin Invest*. 2015;125:993–1005.
47. Ferreira R, et al. Dynamic regulation of Gata factor levels is more important than their identity. *Blood*. 2007;109:5481–5490.
48. Kadauke S, et al. Tissue-specific mitotic bookmarking by hematopoietic transcription factor GATA1. *Cell*. 2012;150:725–737.
49. Lu YC, et al. The molecular signature of megakaryocyte-erythroid progenitors reveals a role for the cell cycle in fate specification. *Cell Rep*. 2018;25:2083–2093. e4.
50. Rodriguez P, et al. GATA-1 forms distinct activating and repressive complexes in erythroid cells. *EMBO J*. 2005;24:2354–2366.
51. Yu M, et al. Insights into GATA-1-mediated gene activation versus repression via genome-wide chromatin occupancy analysis. *Mol Cell*. 2009;36:682–695.
52. Perkins AC, Sharpe AH, Orkin SH. Lethal beta-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF. *Nature*. 1995;375:318–322.
53. Ma W-B, et al. GPS2 promotes erythroid differentiation by control of the stability of EKLF protein. *Blood*. 2020;135:2302–2315.
54. Varricchio L, et al. Genetic disarray follows mutant KLF1-E325K expression in a congenital dyserythropoietic anemia patient. *Haematologica*. 2019;104:2372–2381.
55. Kulczynska-Figurny K, Bieker JJ, Siatecka M. Severe anemia caused by dominant mutations in Krüppel-like factor 1 (KLF1). *Mutat Res*. 2020;786:108336.
56. Tripic T, et al. SCL and associated proteins distinguish active from repressive GATA transcription factor complexes. *Blood*. 2009;113:2191–2201.
57. Cai W, et al. Enhancer dependence of cell-type-specific gene expression increases with developmental age. *Proc Natl Acad Sci U S A*. 2020;117:21450–21458.
58. Cheng Y, et al. Erythroid GATA1 function revealed by genome-wide analysis of transcription factor occupancy, histone modifications, and mRNA expression. *Genome Res*. 2009;19:2172–2184.
59. Ogawa M, Porter PN, Nakahata T. Renewal and commitment to differentiation of hemopoietic stem cells (An interpretive review). *Blood*. 1983;61:823–829.

60. Gao X, et al. Thyroid hormone receptor beta and NCOA4 regulate terminal erythrocyte differentiation. *Proc Natl Acad Sci U S A*. 2017;114: 10107–10112.
61. Zhao B, Mei Y, Yang J, Ji P. Erythropoietin-regulated oxidative stress negatively affects enucleation during terminal erythropoiesis. *Exp Hematol*. 2016;44:975–981.
62. Forejtnikovà H, et al. Transferrin receptor 2 is a component of the erythropoietin receptor complex and is required for efficient erythropoiesis. *Blood*. 2010;116:5357–5367.
63. Artuso I, et al. Transferrin receptor 2 is a potential novel therapeutic target for β-thalassemia: evidence from a murine model. *Blood*. 2018;132: 2286–2297.
64. Jolliffe LK, et al. Erythropoietin receptor: application in drug development. *Nephrol Dial Transplant*. 1995;10(Suppl 2):28–34.
65. Middleton SA, et al. Identification of a critical ligand binding determinant of the human erythropoietin receptor: evidence for common ligand binding motifs in the cytokine receptor family. *J Biol Chem*. 1996;271: 14045–14054.
66. Barbone FP, et al. Mutagenesis studies of the human erythropoietin receptor: establishment of structure-function relationships. *J Biol Chem*. 1997;272:4985–4992.
67. Verma A, et al. Biological basis for efficacy of activin receptor ligand traps in myelodysplastic syndromes. *J Clin Invest*. 2020;130:582–589.
68. Feld J, Navada SC, Silverman LR. Myelo-deception: Luspatercept and TGF-Beta ligand traps in myeloid diseases and anemia. *Leuk Res*. 2020;97: 106430.
69. Vaupel P, et al. Erythropoietin to treat head and neck cancer patients with anaemia undergoing radiotherapy: randomised, double-blind, placebo-controlled trial. *Lancet*. 2004;363:992–994.
70. Yoshida S, et al. Lack of relationship between soluble erythropoietin receptor levels and erythroid parameters in anemic patients. *Blood*. 1996;88:3246–3247.
71. Perreault AA, et al. Epo reprograms the epigenome of erythroid cells. *Exp Hematol*. 2017;51:47–62.
72. Damen JE, et al. Phosphatidylinositol 3-kinase associates, via its Src homology 2 domains, with the activated erythropoietin receptor. *Blood*. 1993;81:3204–3210.
73. Rhee SG. Regulation of phosphoinositide-specific phospholipase C. *Annu Rev Biochem*. 2001;70:281–312.
74. Di Giacomo V, et al. Expression of signal transduction proteins during the differentiation of primary human erythroblasts. *J Cell Physiol*. 2005;202: 831–838.
75. Boudot C, et al. Involvement of the Src kinase Lyn in phospholipase C-gamma 2 phosphorylation and phosphatidylinositol 3-kinase activation in Epo signalling. *Biochem Biophys Res Commun*. 2003;300:437–442.
76. Held MA, et al. Phospho-PTM proteomic discovery of novel EPO-modulated kinases and phosphatases, including PTPN18 as a positive regulator of EPOR/JAK2 signaling. *Cell Signal*. 2020;69:109554.
77. Pless M, et al. Receptors that induce erythroid differentiation of Ba/F3 cells: structural requirements and effect on STAT5 binding. *Blood*. 1997;89:3175–3185.
78. Arcasov MO, Degar BA, Harris KW, Forget BG. Familial erythrocytosis associated with a short deletion in the erythropoietin receptor gene. *Blood*. 1997;89:4628–4635.
79. Ang SO, et al. Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. *Nat Genet*. 2002;32:614–621.
80. Pastore YD, et al. Mutations in the VHL gene in sporadic apparently congenital polycythemia. *Blood*. 2003;101:1591–1595.
81. Baxter EJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet*. 2005;365:1054–1061.
82. Ugo V, James C, Vainchenker W. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Med Sci*. 2005;21: 669–670.
83. Kralovics R, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med*. 2005;352:1779–1790.
84. Levine RL, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell*. 2005;7:387–397.
85. Jubinsky PT, et al. The beta chain of the interleukin-3 receptor functionally associates with the erythropoietin receptor. *Blood*. 1997;90:1867–1873.
86. Dewamitta SR, Joseph C, Purton LE, Walkley CR. Erythroid-extrinsic regulation of normal erythropoiesis by retinoic acid receptors. *Br J Haematol*. 2014;164:280–285.
87. Ratajczak J, et al. The role of insulin (INS) and insulin-like growth factor-I (IGF-I) in regulating human erythropoiesis. Studies in vitro under serum-free conditions—comparison to other cytokines and growth factors. *Leukemia*. 1998;12:371–381.
88. Kadri Z, et al. Erythropoietin and IGF-1 signaling synchronize cell proliferation and maturation during erythropoiesis. *Genes Dev*. 2015;29:2603–2616.
89. Marini MA, et al. A polymorphism at IGF1 locus is associated with anemia. *Oncotarget*. 2017;8:32398–32406.
90. Tikhonova AN, et al. The bone marrow microenvironment at single-cell resolution. *Nature*. 2019;569:222–228.
91. Verfaillie CM, Catanzaro P. Direct contact with stroma inhibits proliferation of human long-term culture initiating cells. *Leukemia*. 1996;10:498–504.
92. Qian H, et al. Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. *Cell Stem Cell*. 2007;1:671–684.
93. Yoshihara H, et al. Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. *Cell Stem Cell*. 2007;1:685–697.
94. Nie Y, Han YC, Zou YR. CXCR4 is required for the quiescence of primitive hematopoietic cells. *J Exp Med*. 2008;205:777–783.
95. Zamai L, et al. TNF-related apoptosis-inducing ligand (TRAIL) as a negative regulator of normal human erythropoiesis. *Blood*. 2000;95: 3716–3724.
96. van der Harst P, et al. Seventy-five genetic loci influencing the human red blood cell. *Nature*. 2012;492:369–375.
97. Russell ES. Hereditary anemias of the mouse: a review for geneticists. *Adv Genet*. 1979;20:357–459.
98. Waters MJ, Brooks AJ. JAK2 activation by growth hormone and other cytokines. *Biochem J*. 2015;466:1–11.
99. Hong L, et al. KIT associated intracellular tyrosines play an essential role in EpoR co-signaling. *Cell Signal*. 2008;20:1513–1520.
100. Broudy VC, et al. Signaling via Src family kinases is required for normal internalization of the receptor c-Kit. *Blood*. 1999;94:1979–1986.
101. Kim M-S, Kuehn HS, Metcalfe DD, Gilfillan AM. Activation and function of the mTORC1 pathway in mast cells. *J Immunol*. 2008;180:4586–4595.
102. Anzai N, et al. C-kit associated with the transmembrane 4 superfamily proteins constitutes a functionally distinct subunit in human hematopoietic progenitors. *Blood*. 2002;99:4413–4421.
103. Shimizu Y, Ashman LK, Du Z, Schwartz LB. Internalization of Kit together with stem cell factor on human fetal liver-derived mast cells: new protein and RNA synthesis are required for reappearance of Kit. *J Immunol*. 1996;156:3443–3449.
104. Maddens S, et al. Kit signaling inhibits the sphingomyelin-ceramide pathway through PLC gamma 1: implication in stem cell factor radioprotective effect. *Blood*. 2002;100:1294–1301.
105. Federici G, et al. Phosphoproteomic landscaping identifies non-canonical cKIT signaling in polycythemia vera erythroid progenitors. *Front Oncol*. 2019;9:1245 2019.
106. Durand B, et al. Long-term generation of human mast cells in serum-free cultures of CD34+ cord blood cells stimulated with stem cell factor and interleukin-3. *Blood*. 1994;84:3667–3674.
107. Ulyanova T, Phelps SR, Papayannopoulou T. The macrophage contribution to stress erythropoiesis: when less is enough. *Blood*. 2016;128:1756–1765.
108. Ulyanova T, Georgolopoulos G, Papayannopoulou T. Reappraising the role of α5 integrin and the microenvironmental support in stress erythropoiesis. *Exp Hematol*. 2020;81:16–31. e4.
109. Hamamura K, et al. A critical role of VLA-4 in erythropoiesis in vivo. *Blood*. 1996;87:2513–2517.
110. Chow A, et al. $CD169^+$ macrophages provide a niche promoting erythropoiesis under homeostasis and stress. *Nat Med*. 2013;19:429–436.
111. Soni S, et al. Absence of erythroblast macrophage protein (Emp) leads to failure of erythroblast nuclear extrusion. *J Biol Chem*. 2006;281:20181–20189.
112. Falchi M, et al. The Calreticulin control of human stress erythropoiesis is impaired by JAK2V617F in polycythemia vera. *Exp Hematol*. 2017;50: 53–76.
113. Kautz L, et al. Identification of erythroferrone as an erythroid regulator of iron metabolism. *Nat Genet*. 2014;46:678–684.
114. Keel SB, et al. A heme export protein is required for red blood cell differentiation and iron homeostasis. *Science*. 2008;319:825–828.
115. Kihm AJ, et al. An abundant erythroid protein that stabilizes free α-haemoglobin. *Nature*. 2002;417:758–763.
116. Stamatoyannopoulos G, Nienhuis AW. Hemoglobin switching. In: Stamatoyannopoulos G, Nienhuis AW, Leder P, Majerus PW, eds. *Molecular Basis of Blood Diseases*. Philadelphia:WB Saunders; 1987.
117. Blau CA, et al. Fetal hemoglobin in acute and chronic states of erythroid expansion. *Blood*. 1993;81:227–233.
118. Freedman MH. Erythropoiesis in Diamond-Blackfan anemia and the role of interleukin 3 and steel factor. *Stem Cells*. 1993;11(Suppl 2):98–104.
119. Socolovsky M, et al. Ineffective erythropoiesis in Stat5a(−/−)5b(−/−) mice due to decreased survival of early erythroblasts. *Blood*. 2001;98: 3261–3273.

120. Lee HY, et al. PPAR-α and glucocorticoid receptor synergize to promote erythroid progenitor self-renewal. *Nature*. 2015;522:474–477.
121. Migliaccio G, et al. Under HEMA conditions, self-replication of human erythroblasts is limited by autophagic death. *Blood Cells, Mol Dis*. 2011;47:182–197.
122. Cartron JP, Colin Y. Structural and functional diversity of blood group antigens. *Transfus Clin Biol*. 2001;8:163–199.
123. McGrath KE, et al. Enucleation of primitive erythroid cells generates a transient population of "pyrenocytes" in the mammalian fetus. *Blood*. 2008;111:2409–2417.
124. Sasaki K, et al. Absence of fetal liver hematopoiesis in mice deficient in transcriptional coactivator core binding factor beta. *Proc Natl Acad Sci U S A*. 1996;93:12359–12363.
125. Kieran MW, Perkins AC, Orkin SH, Zon LI. Thrombopoietin rescues in vitro erythroid colony formation from mouse embryos lacking the erythropoietin receptor. *Proc Natl Acad Sci U S A*. 1996;93:9126–9131.
126. Hakomori S. Blood group ABH and Ii antigens of human erythrocytes: chemistry, polymorphism, and their developmental change. *Semin Hematol*. 1981;18:39–62.
127. Papayannopoulou T, Enver T, Takegawa S, Anagnou NP, Stamatoyannopoulos G. Activation of developmentally mutated human globin genes by cell fusion. *Science*. 1988;242:1056–1058.
128. Basak A, et al. Control of human hemoglobin switching by LIN28B-mediated regulation of BCL11A translation. *Nat Genet*. 2020;52:138–145.
129. Gudmundsdottir B, et al. POGZ is required for silencing mouse embryonic β-like hemoglobin and human fetal hemoglobin expression. *Cell Rep*. 2018;23:3236–3248.
130. Su MY, et al. Identification of biologically relevant enhancers in human erythroid cells. *J Biol Chem*. 2013;288:8433–8444.
131. Gnanapragasam MN, et al. p66Alpha-MBD2 coiled-coil interaction and recruitment of Mi-2 are critical for globin gene silencing by the MBD2-NuRD complex. *Proc Natl Acad Sci U S A*. 2011;108:7487–7492.
132. Liu N, et al. Direct promoter repression by BCL11A controls the fetal to adult hemoglobin switch. *Cell*. 2018;173:430–442. e17.
133. Masuda T, et al. Transcription factors LRF and BCL11A independently repress expression of fetal hemoglobin. *Science*. 2016;351:285–289.
134. Rivers A, et al. The LSD1 inhibitor RN-1 recapitulates the fetal pattern of hemoglobin synthesis in baboons (P. anubis). *Haematologica*. 2016;101:688–697.
135. Sher F, et al. Rational targeting of a NuRD subcomplex guided by comprehensive in situ mutagenesis. *Nat Genet*. 2019;51:1149–1159.
136. Verheul TCJ, Trinh VT, Vázquez O, Philipsen S. Targeted protein degradation as a promising tool for epigenetic upregulation of fetal hemoglobin. *ChemMedChem*. 2020;15:2436–2443.
137. Deng W, et al. Reactivation of developmentally silenced globin genes by forced chromatin looping. *Cell*. 2014;158:849–860.
138. Koblan LW, et al. In vivo base editing rescues Hutchinson-Gilford progeria syndrome in mice. *Nature*. 2021;589:608–614.
139. Giarratana M-C, et al. Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. *Nat Biotechnol*. 2005;23:69–74.
140. Lapillonne H, et al. Red blood cell generation from human induced pluripotent stem cells: perspectives for transfusion medicine. *Haematologica*. 2010;95:1651–1659.
141. Hirose S-I, et al. Immortalization of erythroblasts by c-MYC and BCL-XL enables large-scale erythrocyte production from human pluripotent stem cells. *Stem Cell Reports*. 2013;1:499–508.
142. Vinjamur DS, Bauer DE. Growing and genetically manipulating human umbilical cord blood-derived erythroid progenitor (HUDEP) cell lines. *Methods Mol Biol*. 2018;1698:275–284.
143. Hawksworth J, et al. Enhancement of red blood cell transfusion compatibility using CRISPR-mediated erythroblast gene editing. *EMBO Mol Med*. 2018;10:e8454.
144. Capellera-Garcia S, et al. Defining the minimal factors required for erythropoiesis through direct lineage conversion. *Cell Rep*. 2016;15:2550–2562.

CHAPTER 28

GRANULOCYTOPOIESIS AND MONOCYTOPOIESIS

Frederick D. Tsai, Arati Khanna-Gupta, and Nancy Berliner

GRANULOCYTOPOIESIS

Granulocytes (neutrophils, eosinophils, and basophils) are short-lived cells that are critical to both antimicrobial and inflammatory responses. The bone marrow (BM) produces granulocytes, particularly neutrophils, at a prodigious rate to supply the baseline need for circulating cells that survive in the peripheral blood for only 3 to 24 hours. It also has the capacity to sharply increase granulocyte production in response to a wide range of stresses. Granulocyte production is controlled by a variety of cytokines that induce the myeloid differentiation program through carefully orchestrated interactions of multiple general and myeloid-specific transcription factors. Understanding this intricate maturation sequence provides important insights into normal neutrophil responses to infectious, inflammatory, and allergic stresses, as well as into the dysregulation of differentiation that leads to myelodysplasia and leukemia.

Granulocyte Ontogeny

Stages of Neutrophil Differentiation

Granulocytes differentiate from early progenitors in the BM in a process that takes 7 to 10 days. The cells progress through several identifiable maturation stages, during which they acquire the morphologic appearance and granule contents that characterize the mature granulocyte. The earliest identifiable granulocyte precursor is the myeloblast, a minimally granulated cell with scant cytoplasm and a prominent nucleolus (Fig. 28.1). Transition to the promyelocyte stage is associated with the acquisition of abundant primary granules. Primary granules are found in both granulocytes and monocytes and contain proteins necessary for intracellular killing of microbes. The transition to the myelocyte stage is associated with the acquisition of secondary or "specific" granules, which give the characteristic staining pattern that differentiates neutrophils from eosinophils and basophils.

Neutrophil precursors account for approximately half the cells in the BM of normal individuals, with a majority of these at the metamyelocyte stage and beyond. Promyelocytes and myelocytes represent the primary proliferative pool of granulocyte precursors in the BM. Beyond the myelocyte stage, cells mature as nondividing cells. Bands and segmented neutrophils constitute greater than 50% of the total granulocyte mass, primarily as a storage pool of mature cells in the BM. Only 5% of total neutrophils circulate in the periphery, of which 60% are marginated in the spleen and on vessel walls. Mature neutrophils circulate in the peripheral blood for 3 to 24 hours and then migrate to the tissues, where they survive for 2 to 3 days. Therefore the peripheral neutrophil count reflects approximately 2% of the total neutrophil cell mass during approximately 1% of the neutrophil lifespan.

Biochemical events that accompany these physical changes include the sequential acquisition of primary granules and their content proteins (including myeloperoxidase [MPO], lysozyme, neutrophil elastase [NE; encoded by the *ELANE* gene], defensins, myeloblastin), secondary granules and their content proteins (lactoferrin [LF], neutrophil collagenase [matrix metalloproteinase (MMP) 9], neutrophil gelatinase [matrix metalloproteinase (MMP) 8], neutrophil gelatinase-associated lipocalin [NGAL], transcobalamin 1), and tertiary granules containing neutrophil gelatinase (Table 28.1). The progressive gain of these phenotypic changes and granule acquisition is accompanied by a loss of proliferative potential. This carefully coordinated process is disrupted in acute myeloid leukemia (AML), in which a block in the myeloid maturation pathway usually results in the circulation of immature blasts in the peripheral blood.

Markers of Granulocytic Maturation

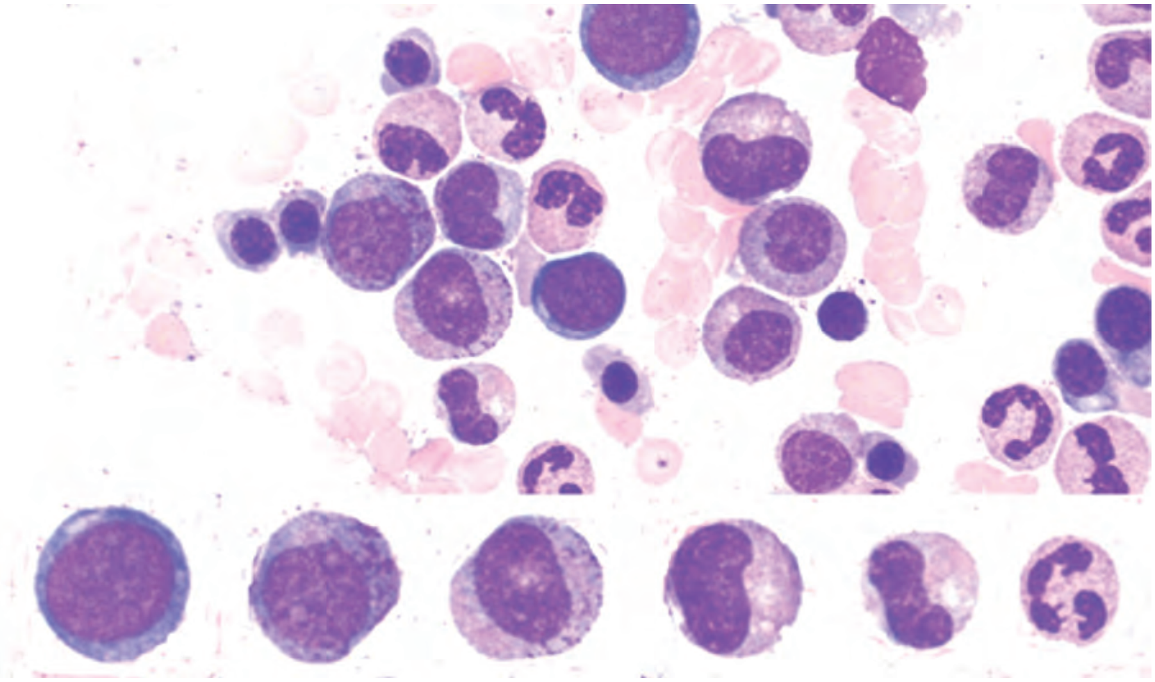
Stem cells have been characterized primarily by their marrow repopulating potential, as outlined in Chapter 9. Early granulocytic progenitors form hematopoietic colonies in vitro and their more differentiated progeny express specific cell surface proteins that are critically important to myeloid differentiation and function. They mediate both the adhesion of precursors within the BM and the vascular adhesion of mature neutrophils needed for normal neutrophil activation. Other proteins serve as receptors that recognize pathogens or as stimulatory peptides that facilitate activation of phagocytosis and killing of organisms. Appropriate expression of these surface proteins plays an important role in normal neutrophil function, and abnormalities of their expression are implicated in a wide range of diseases affecting the neutrophil compartment. For example, congenital abnormalities in the surface expression of integrin proteins are responsible for failure of neutrophil adhesion in leukocyte adhesion deficiency, whereas acquired abnormalities of expression of the same proteins are thought to underlie the abnormal circulation of immature precursors in myeloproliferative neoplasms.[1] These markers also serve to help distinguish among the stages of myeloid commitment and maturation.

The phenotype of the early hematopoietic stem cells is $CD34^+CD38^-CD33^-$, with absence of lineage-specific markers. The common myeloid progenitor, colony-forming unit–granulocyte–erythrocyte–macrophage–megakaryocyte (CFU-GEMM), is characterized by the coexpression of CD33. CD33 is expressed at high levels on committed myeloid progenitors and on early precursors of both the granulocytic and monocytic lineages. Expression of CD33 wanes with granulocytic maturation and is absent or nearly absent beyond the myelocyte stage. CD33 is a member of the sialic acid–binding immunoglobulin (Ig)-like lectins (siglecs), which mediate cell-cell interactions and cell signaling pathways involved in immune responses and inflammation. This class of receptors recognizes endogenous sialoglycans as "self-associated molecular patterns" (SAMPs), dampening immune responses via cytosolic Src homology-2 (SH2) domain–containing protein tyrosine phosphatase 1 (SHP1) and SHP2 tyrosine phosphatases and the suppressor of cytokine signaling 3 (SOCS3).[2]

Characteristic granulocyte markers acquired as the early myeloid progenitor cells become committed to the neutrophil lineage include CD45RA, MPO, and CD38, all of which are expressed by myeloblasts. Further differentiation beyond the myelocyte stage is associated with expression of CD16, CD11b/CD18 (Mac-1), and leukocyte alkaline phosphatase (LAP), all of which are expressed at high levels in mature neutrophils.

Neutrophil Granules and Their Content Proteins

The acquisition of granules and their content proteins is a critical part of the developmental program of the granulocyte.[3] Arising at specific stages of neutrophil maturation, these intracellular and secretory



	Myeloblast	Promyelocyte	Myelocyte	Metamyelocyte	Band	Segmented neutrophil
Proliferation	++	+++	+++	+/–	––	––
Granule production						
1°		+++	+			
2°			+++	+		
3°				+++	+	

Figure 28.1 NEUTROPHIL MATURATION STAGES WITH ASSOCIATED ACQUISITION OF STAGE-SPECIFIC GRANULES.

organelles contain many of the requisite enzymes that mediate the oxidative and nonoxidative killing functions of the neutrophil (see Table 28.1).

Primary (azurophilic) granules are acquired at the promyelocyte stage and contain a wide array of proteins, including MPO, defensins, cathepsins, and NE. *Secondary* granules are secretory granules acquired at the transition to the myelocyte stage. Neutrophil secondary granules contain LF, the vitamin B_{12}–binding protein transcobalamin I, the metalloproteinases (neutrophil collagenase and gelatinase), and NGAL. With the exception of gelatinase, which is also expressed by monocytes, expression of the secondary granule proteins is restricted within the hematopoietic lineage to neutrophils. Secondary granules and the synthesis of their contents therefore constitute a definitive marker of commitment to terminal neutrophil maturation. As discussed later, characteristic secondary granules are acquired at the same stage by eosinophils and basophils. *Tertiary* granules, containing primarily gelatinase, are formed during later stages of neutrophil maturation. *Secretory vesicles* are formed by endocytosis and contain plasma proteins.[4]

On stimulation, the neutrophil first mobilizes secretory vesicles, which contribute their membrane proteins, including abundant integrin receptors, to the plasma membrane. This increases cellular adhesion by upregulating surface integrin expression in response to selectin stimulation or inflammatory mediators. Primary granules fuse with the phagosome and contribute to bacterial killing. Secondary and tertiary granules have multiple functions. Like primary granules, they fuse intracellularly with the phagosome to help promote bactericidal activity. They also contribute membrane proteins to the plasma membrane and are the source of the surface integrin receptor Mac-1 (CD11b/CD18) that is prominently upregulated during neutrophil activation. Secondary and tertiary granules also release the matrix-modifying metalloproteinase collagenase (MMP8) and gelatinase (MMP9) into the extracellular milieu, enhancing neutrophil penetration into sites of inflammation. LF and transcobalamin I are thought to contribute to the antimicrobial response by sequestering iron and cobalamin, respectively, away from infecting organisms. LF has also been implicated in modulating neutrophil extracellular traps (NETs) by inhibiting their release.

The fusion of azurophilic and peroxidase-negative granules allows for cross-exposure of their contents within the phagosome. Under resting conditions, their constituent proteins are carefully sequestered in separate organelles, preventing premature activation and damage to the resting neutrophil. When granule fusion occurs, their protein contents cooperate to generate the antimicrobial response. Hydrogen peroxide, a by-product of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in secondary granules, combines with MPO from the primary granules to produce hypochlorous acid, a highly toxic microbicidal agent. The secondary granule proteins neutrophil gelatinase and neutrophil collagenase are produced as zymogens and are converted to their active forms by the action of NE released from the primary granules.

Current evidence largely supports the hypothesis that the content of the neutrophil granules is dictated primarily by the timing of synthesis of their respective content proteins. Multiple studies have demonstrated that each distinct granule population is generated not by differences in protein sorting but rather by a highly regulated

TABLE 28.1 Neutrophil Granules: Major Classes and Contents

Primary (Azurophilic)	Secondary (Specific)	Tertiary
Microbial Agents		
Lysozyme	Lysozyme	
Myeloperoxidase		
Defensins		
Cationic proteins		
Bactericidal permeability–increasing agent (BPI)		
Proteases		
Elastase	Collagenase	Gelatinase
Cathepsin G		
Other proteases		
Acid Hydrolases		
N-acetylglucuronidase		
Cathepsins B and D		
β-Glucuronidase		
β-Glycerophosphatase		
α-Mannosidase		
Other		
Kinin-generating enzyme		
C5a-inactivating factor	Lactoferrin	
Vitamin B_{12}–binding protein		
Plasminogen activator		
Cytochrome *b*[a]		
CD11/1B complex[a]		
Formyl peptide receptor[a]		
Histaminase[a]		
Neutrophil gelatinase-associated lipocalin		

[a] These granule constituents are conventionally assigned to the secondary granule, but their exact compartment remains controversial. Some may be located in the tertiary granule or possibly in one of the other, heterogeneous small-granule populations.

Modified from Boxer LA, Smolen JE. Neutrophil granule constituents and their release in health and disease. *Hematol Oncol Clin North Am.* 1988;2:101.

transcriptional process that results in sequential gene expression. Expression of MPO occurs between the promyelocyte and the myelocyte stages of neutrophil development, and as a result it is packaged into the primary granules. Secondary granule proteins such as LF are expressed between the myelocyte and the metamyelocyte stages and hence are packaged into the secondary granules. Overexpression of the secondary granule protein NGAL in HL60 cells, a leukemic cell line that is arrested at the myeloblast stage of differentiation, results in its incorporation into primary granules, supporting the notion that gene expression and protein sorting into granules are coordinated events. However, this model may be an oversimplification because there is some overlap of expression between certain primary and secondary granule protein genes, and primary granule protein gene expression is much less synchronous than that of secondary granule proteins. The defensins are expressed later than other primary granule proteins, and defensin transcription appears to be regulated by the same transcriptional regulatory pathway as for the secondary granule proteins gelatinase and LF.[5] The defensins are a family of small, variably cationic proteins that are highly abundant in the granules of mammalian phagocytes. Three defensins, HNP-1, -2, and -3, comprise 30% to 50% of total protein in the azurophilic granules of human neutrophils. Some defensins are broadly antimicrobial, antiviral, and cytotoxic, whereas others are chemotactic or opsonic or may modulate hormonal responses. The defensins are the only primary granule proteins that are absent in patients with neutrophil specific granule deficiency (SGD), which would predict targeting to the secondary granule.[6] How defensins are packaged exclusively in the primary granule remains unclear.

CONTROL OF GRANULOPOIESIS

Granulocytes arise from pluripotent hematopoietic stem cells by a process of commitment, proliferation, and differentiation. Stem cells are long-lived cells capable of both self-renewal and differentiation to lineage-specific–committed progenitors (see Chapter 9). The process governing the cell fate decision that takes a stem cell down the path to lineage commitment and the subsequent factors that regulate lineage-specific differentiation have been the subjects of intense study for many years. Three models have been proposed for the mechanism underlying lineage commitment and differentiation of the pluripotent stem cell. The first, or *inductive*, model suggests that lineage commitment and differentiation are the results of external stimuli (e.g., growth factors, stroma). The second model, the *stochastic* model, emphasizes intrinsic cellular factors as being critical to hematopoiesis, and the third model combines the attributes of the first two. It appears that the transition from a stem cell to a committed progenitor is largely stochastic, although subsequent maturation from progenitor to precursor to mature neutrophil requires cytokine activity. Controversy remains as to whether cytokines and the BM microenvironment play instructive or permissive roles in influencing stem cell commitment and in inducing the proliferation and maturation of committed progenitors.

Recent observations in mouse models of hematopoietic stem cell transplantation suggest that neutrophils themselves play active roles in modulating the BM microenvironment by driving sinusoidal regeneration following transplantation. Aging neutrophils reenter the BM as well as extramedullary tissues from the peripheral circulation, and their elimination by resident macrophages activates feedback loops that regulate granulopoiesis through cytokine signaling (reviewed by Cossio et al.[7]). The interplay between neutrophil life cycle, BM niche, and cross-talk with extramedullary organ systems currently remains an active area of investigation.

Cytokine Regulation of Myeloid Proliferation and Differentiation

Early progenitor cells express receptors for multiple cytokines, but their expression becomes more restricted as cells become committed to a specific lineage.[8] As a consequence of the broad range of cytokine receptor expression, early progenitors respond to combined growth factors, many of which show synergistic activity. The early-acting cytokines include the interleukins IL-1 and IL-6, stem cell factor (SCF), FLT3 ligand, and several others, including granulocyte colony-stimulating factor (G-CSF) and thrombopoietin (TPO). IL-3 is important in directing the pluripotent stem cell toward the myelomonocytic lineage, giving rise to the mixed myeloid progenitor (CFU-GEMM). Subsequent stages leading to commitment- and lineage-restricted differentiation are governed by late-acting cytokines (Fig. 28.2).

The major cytokines that mediate neutrophil maturation are G-CSF and granulocyte–macrophage colony-stimulating factor (GM-CSF). G-CSF not only supports the survival and proliferation of developing myeloid cells at all stages of differentiation but also increases the functional activity of mature neutrophils. G-CSF binding to its receptor, G-CSFR, leads to homodimerization and signal activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) and the Ras/mitogen-activated protein kinase (MAPK) pathways.[9] The role of G-CSF as both an early- and late-acting cytokine is underscored by the successful use of G-CSF

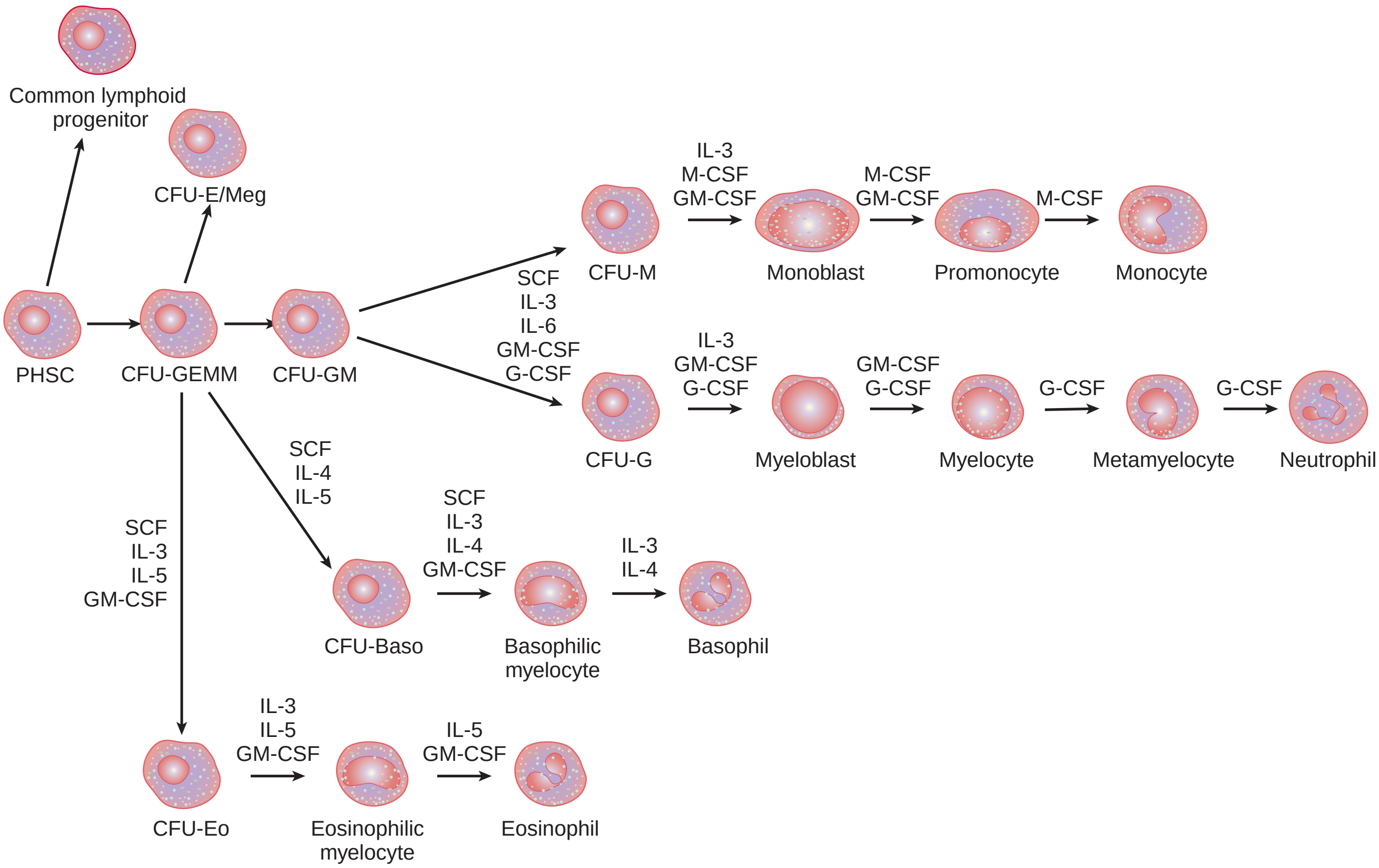


Figure 28.2 CYTOKINE REGULATION OF GRANULOCYTIC PROGENITORS. *CFU-Baso*, Colony-forming unit–basophil; *CFU-E/Meg*, colony-forming unit–erythrocyte/megakaryocyte; *CFU-Eo*, colony-forming unit–eosinophil; *CFU-G*, colony-forming unit–granulocyte; *CFU-GEMM*, colony-forming unit–granulocyte/erythrocyte/macrophage/megakaryocyte; *CFU-GM*, colony-forming unit–granulocyte/macrophage; *CFU-M*, colony-forming unit–macrophage; *G-CSF*, granulocyte colony-stimulating factor; *GM-CSF*, granulocyte–macrophage colony-stimulating factor; *IL*, interleukin; *M-CSF*, monocyte colony-stimulating factor; *PHSC*, pluripotent hematopoietic stem cell; *SCF*, stem cell factor.

to mobilize early progenitors into the peripheral blood for stem cell collection and to speed neutrophil recovery following chemotherapy. Although the major role of G-CSF is thought to be induction of neutrophil proliferation and differentiation, the G-CSFR is also expressed on a wide range of cell types. In addition to myeloid progenitors and precursors at all stages of neutrophil differentiation, the G-CSFR is expressed on platelets, monocytes, and lymphocytes, as well as some nonhematopoietic tissues, including endothelium and placenta.

The importance of G-CSF in myeloid proliferation and differentiation has been studied in G-CSF–null and G-CSFR–null mice. Mice lacking G-CSF or G-CSFR have markedly decreased myeloid progenitors and impaired neutrophil production, with low circulating neutrophil counts. In addition, G-CSF–null mice have impaired neutrophil mobilization, and mature neutrophils from G-CSFR–null mice have increased susceptibility to apoptosis, supporting the role of the G-CSF pathway in sustaining the mobilization, survival, and function of mature neutrophils. Despite these abnormalities, G-CSF/G-CSFR–knockout mice are still capable of some neutrophil production, suggesting that there are alternative cytokine pathways that allow for granulocyte development.

GM-CSF also induces proliferation and differentiation of myeloid precursors. The GM-CSF receptor (GM-CSFR) is a heterodimeric protein composed of an α- and a β-subunit. The α-subunit binds GM-CSF. The β-subunit is shared by GM-CSFR and the receptors for IL-3, IL-5, and IL-6. The β-subunit does not bind ligand but is necessary for high-affinity ligand binding to the αβ-heterodimer of each receptor. Signaling through GM-CSFR activates both the JAK/STAT pathway and the Ras/MAPK pathway. Of note, GM-CSF–null mice have no defect in hematopoiesis, whereas mice with null mutations in both G-CSF and GM-CSF have more profound neutropenia in the perinatal period but the same levels of neutrophils in adulthood as those of mice lacking G-CSF alone.

Transcriptional Regulation of Myeloid Differentiation

Lineage-specific maturation of committed hematopoietic progenitors is driven by transcription factors controlling cellular proliferation, differentiation, and survival of the totipotent stem cell, and these roles have been well established with the aid of mammalian animal models.[10] Studies of the regulation of individual genes that show tissue- and stage-specific myeloid expression have implicated a small number of transcription factors that are responsible for directing both phenotypic myeloid maturation and the expression of functionally important myeloid genes. Furthermore, pathognomonic chromosomal translocations resulting in the dysregulation of transcription factor expression and activity, leading to defects in myeloid-specific gene expression, have been well described in AML.

On the basis of experimental observations using genetic knockout techniques, overexpression studies, and multicolor flow cytometry, critical transcription factors have been identified and are classified into two major categories. The first category includes stem cell leukemia transcription factor (SCL), GATA-2, and AML transcription factor-1 (AML-1; now known as Runx1), all of which influence

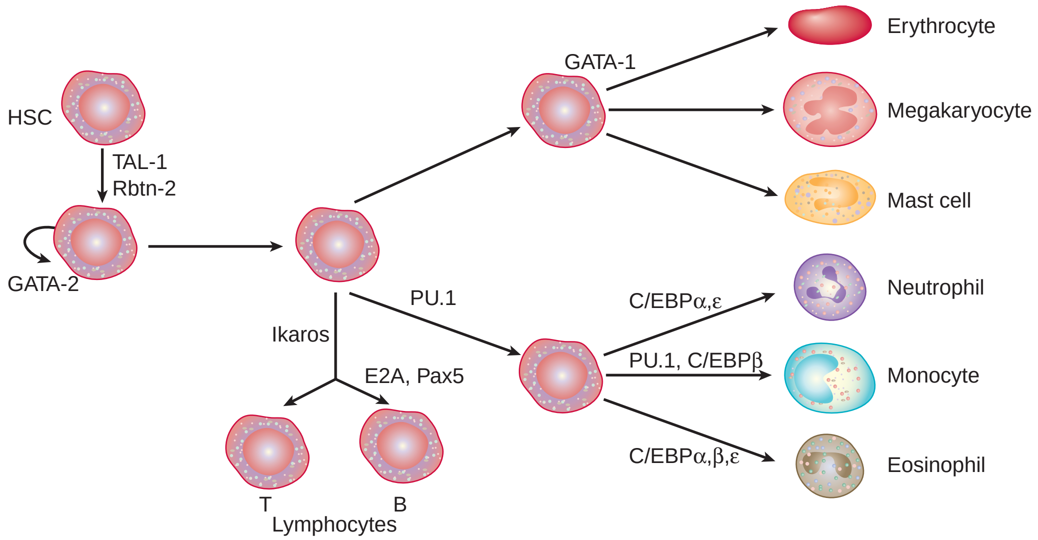


Figure 28.3 SIMPLIFIED SCHEMA OF TRANSCRIPTIONAL REGULATION OF HEMATOPOIESIS. *C/EBP*, CCAAT enhancer-binding protein; *HSC*, hematopoietic stem cell; *SCL*, stem cell leukemia transcription factor.

differentiation of all hematopoietic lineages. The second category comprises the master regulators of lineage development, including GATA-1, PU.1, and CCAAT enhancer-binding protein-α (C/EBPα). These factors not only promote lineage-specific gene expression but also suppress alternative lineage pathways. Fig. 28.3 summarizes the postulated role of several key transcription factors during hematopoietic development. Myeloid progenitors exhibit multilineage patterns of transcription factor gene expression. Laslo et al.[11] elegantly demonstrated that cell fate determination is dependent upon subtle changes in expression levels of transcription factors that regulate differential lineage maturation. For example, levels of PU.1 expression are increased by Egr-1/Nab-2 in developing macrophages; at the same time, Egr-1 inhibits the expression of the neutrophil-specific transcription factor growth factor independence-1 (Gfi-1), thereby simultaneously repressing the neutrophil development program (Fig. 28.4).

Transcription Factors Regulating Myeloid Differentiation and Myeloid-Specific Gene Expression

Runx1

Runx1, previously known as AML-1, belongs to a family of highly conserved transcription factors that harbor a 128-amino-acid motif referred to as the Runt domain. The Runt domain functions in DNA binding, protein-protein interaction, adenosine triphosphate (ATP) binding, and nuclear localization.[12] This family of transcription factors, known as the core binding factor (CBF) family, has been implicated in specification of cell fate and has roles in myeloid differentiation and lineage-specific granulocytic function.

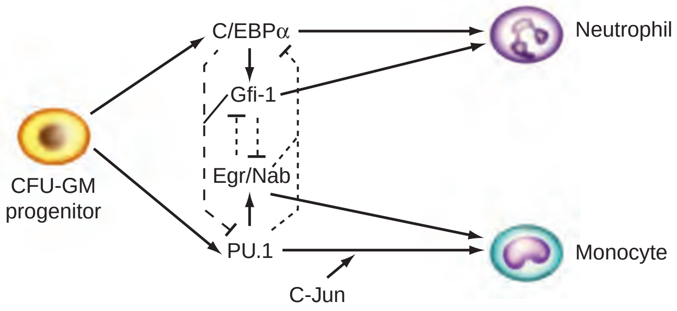


Figure 28.4 TRANSCRIPTION FACTOR CROSS-TALK AFFECTING NEUTROPHIL AND MONOCYTE DEVELOPMENT. *C/EBP*, CCAAT enhancer-binding protein; *CFU-GM*, colony-forming unit–granulocyte/macrophage; *Gfi-1*, growth factor independence-1.

Runx1 is the DNA-binding α-subunit of the CBF complex. Together with CBFβ, a widely expressed protein that enhances the DNA-binding affinity of the α-subunit, Runx1 binds the consensus DNA motif 5′ Pu ACCPuCA 3′ as a dimer. Disruption of the *Runx1* gene in mice results in embryonic lethality due to failure of definitive hematopoiesis in the fetal liver. Although high levels of Runx1 expression have been reported in the early stages of myeloid differentiation, its expression decreases beyond the promyelocytic stage. Runx1 has been implicated in regulating a number of genes expressed early in the myeloid development pathway, including GM-CSF, macrophage colony-stimulating factor (M-CSF) receptor, MPO, NE, and IL-3. In addition to activating lineage-specific myeloid markers, Runx1 has been shown to stimulate the G_1 to S transition in myeloid and lymphoid cell lines.

A significant percentage (10% to 20%) of human leukemia has been found to be associated with mutations in the *RUNX1* gene (see Chapters 59 and 60). The most common is the t(8;21) translocation, which generates the fusion protein RUNX1/RUNX1T1 (previously known as AML1-ETO), where the Runt domain of Runx1 is fused in frame with the RUNX1T1 (ETO) transcriptional corepressor. This fusion protein is thought to function predominantly as a repressor that inhibits expression of genes normally activated by Runx1. For example, the tumor suppressor $p14^{ARF}$, a critical Runx1 target encoded from the *CDKN2A* gene and which is necessary for p53 activation, is normally activated by Runx1 but is repressed by RUNX1-RUNX1T1. The mechanisms underlying Runx1 function through its target genes are not yet fully understood. However, studies in sea urchins have suggested that Runx1 regulates genes that contribute to chromatin architecture during cell proliferation. Runx1 functions within a narrow window during development by assisting in the opening of chromatin associated with genes that are vital to hematopoietic development, and for the formation of transcription factor complexes on these genes.[13]

Studies involving mouse knockin models of Runx1-RUNX1T1 expression have demonstrated that the fusion protein alone is not sufficient to cause leukemia. However, these animals are more susceptible to mutagen-induced AML, suggesting that Runx1-RUNX1T1 is part of a multistep process that contributes to leukemogenesis.

CCAAT Enhancer-Binding Protein Family of Transcription Factors.

C/EBPs are a family of basic region-leucine zipper (b-ZIP) transcription factors that recognize the consensus DNA-binding sequence 5′TKN NGYAAK3′ (Y = C or T; K = T or G) within the regulatory regions of target genes. C/EBP family proteins bind DNA as either homodimers or heterodimers. This family of transcription factors, which plays a crucial role in hematopoiesis, includes C/EBPα, -β, -γ, -δ, -ε, and -ζ (CHOP-GADD 153). These proteins share highly homologous C-terminal leucine zipper dimerization domains and positively charged basic region DNA-binding motifs but differ in their N-terminal transactivation domains.[9] CHOP-GADD 153 lacks this domain altogether and instead dimerizes with and inhibits transactivation by C/EBPα, -β, and -ε.

With the exception of C/EBPε, which is expressed exclusively in the late stages of granulopoiesis and in T lymphocytes, the C/EBP family members are expressed in a wide variety of tissues, including liver, adipose tissue, lung, intestine, adrenal gland, peripheral blood mononuclear cells, and placenta. Both C/EBPβ and C/EBPδ are expressed at high levels in late-stage granulocytes. The C/EBP family members are known to exert pleiotropic effects in the tissues in which they are expressed. This may be because of their tissue- and stage-specific expression, their ability to dimerize with members of their own family and of the Fos/Jun and ATF/CREB families of transcription factors, and their ability to interact with other transcription factors such as nuclear factor-κB (NF-κB) and specificity protein-1 (Sp-1).

The C/EBP factors have been implicated in regulating differentiation in a variety of tissues. C/EBPα plays a role in adipocyte differentiation: inhibition of C/EBPα blocks adipocyte differentiation, and overexpression of C/EBPα induces adipocyte differentiation. Regulation of constitutive hepatic genes as well as acute phase response genes in the liver involves several C/EBP family members, in particular C/EBPα. Modulation of myelomonocytic differentiation is also attributed to the activity of C/EBP family members. The importance of this family of transcription factors in myeloid differentiation has been further demonstrated by the study of hematopoietic abnormalities observed in mice with targeted disruption of C/EBPα, -β, and -ε.

C/EBPα

C/EBPα is thought to be a master regulator of the granulopoietic developmental program. It is expressed at high levels throughout myeloid differentiation and has been shown to bind to the promoters of multiple myeloid-specific gene promoters regulating gene expression at many different stages of myeloid maturation. Although *Cebpa*$^{-/-}$ mice die perinatally because of defects in gluconeogenesis that result in fatal hypoglycemia, they also have a selective early block in the differentiation of granulocytes without affecting either monocyte/macrophage maturation or the differentiation of other hematopoietic lineages. Myeloid cells of *Cebpa*$^{-/-}$ mice lack the G-CSFR, and it is thought that lack of mature neutrophils in these mice occurs as a result. However, the myeloid defect in *Cebpa*$^{-/-}$ mice is more severe than that seen in G-CSFR knockout mice, suggesting that C/EBPα has additional functions vital to granulocytic maturation.

CEBPA is a single exon gene, but it is expressed as two isoforms that arise from alternate translation start sites giving rise to a full-length C/EBPα p42 and a truncated dominant negative C/EBPα p30 isoform.[14] Translational control of C/EBPα isoform expression is orchestrated by a conserved upstream open reading frame (uORF) in the 5′ untranslated region (UTR). This region is thought to be responsive to the activities of the translation initiation factors eIF4E and eIF2 (reviewed by Khanna-Gupta[15]) such that an increase in the activity of eIF4E or eIF2 results in an increase in expression of the shorter p30 isoform (reviewed by Calkhoven et al.[14]).

Several groups have reported loss of function mutations in the *CEBPA* gene in a subset of patients (~10%) with normal-karyotype AML (see Chapters 59 and 60) (reviewed by Muller and Pabst[16]). These mutations can be broadly classified into two categories. The first includes in-frame mutations clustered in the highly conserved C-terminus of the C/EBPα protein. The second category involves frameshift mutations at the N-terminus of C/EBPα resulting in the premature termination of the full-length C/EBPα p42 isoform while keeping the truncated C/EBPα p30 protein intact.[17] The remaining C/EBPα p42 is thought to be rendered inactive by the dominant negative activity of the p30 isoform, although the mechanism is unknown. Mice that express a vector inducing overexpression of C/EBPα p30 from the *Cebpa* locus develop AML with complete penetrance.[18] Thus changes in the expression ratio of the two C/EBPα isoforms play a role in cell fate and in leukemogenesis (reviewed by Muller and Pabst[16] and Kirstetter et al.[18]).

The expression of C/EBPα is associated with growth arrest and differentiation of granulocyte precursor cells. This block in proliferation is thought to occur via the interaction of C/EBPα with, and inhibition of, the cyclin-dependent protein kinases cdk2 and cdk4. In addition, C/EBPα inhibits E2F-dependent transcription, which in turn leads to inhibition of cell proliferation and induction of differentiation associated with C/EBPα-induced granulopoiesis.

C/EBPβ

Expression of C/EBPβ increases during myeloid maturation and is important for monocyte/macrophage gene expression and development. Mice lacking the *Cebpb* gene demonstrate reduced B-cell numbers and defects in macrophage activation and function and increased susceptibility to microbial infections. Knockout studies reveal that C/EBPβ is not essential for myeloid development, although knockin of *Cebpb* into the *Cebpa* locus of *Cebpa*$^{-/-}$ mice rescues granulopoiesis. Several monocyte/macrophage-specific genes are activated by C/EBPβ, including G-CSFR, lysozyme, CD11c, monocyte chemoattractant protein-1 (MCP-1), IL-6, IL-8, and nitric oxide synthase. As with C/EBPα, multiple isoforms of C/EBPβ are generated from a single transcript through three alternate translation initiation sites and a leaky ribosome scanning mechanism. The shortest of these isoforms, initiated at the most distal start codon, results in the formation of liver-enriched inhibitory protein (LIP), which lacks the N-terminal activation domain present in full-length C/EBPβ and has been implicated as a negative regulator of C/EBPβ function. It has been suggested that the ratio of C/EBPβ to LIP may affect cellular proliferation and differentiation. The activity of C/EBPβ is regulated through protein-protein interactions and posttranslational modifications. For example, in early myeloid progenitor cells, C/EBPβ is found in an unphosphorylated state in the cytoplasm, whereas on differentiation, C/EBPβ becomes phosphorylated and translocates to the nucleus.[19]

C/EBPβ in Emergency Granulopoiesis

As short-lived cells, neutrophils must be continuously produced in the BM under normal steady-state conditions. The large storage pool of neutrophils is sufficient to provide an immediate increase in circulating neutrophils in response to acute infection. However, if there is a severe or persistent demand for neutrophils due to a prolonged or particularly severe infection, a switch from steady-state to emergency granulopoiesis occurs to meet this increased demand. This marked de novo increase in neutrophil production is defined as “emergency granulopoiesis” and is critical for survival of the host. As a rule, the emergency granulopoiesis pathway follows three distinct steps: pathogen sensing and alerting the innate immune system of infection, triggering of the molecular events that lead to increased neutrophil production in the BM, and finally, restoration of steady-state conditions following clearance of the pathogen from the system (reviewed by Dao et al.[20]).

Transcriptional Networks in Emergency Granulopoiesis

The role of transcription factors in emergency granulopoiesis has only recently begun to be elucidated. In an elegant study, expression levels of C/EBP family members were measured in early granulocytic precursor cells following induction of emergency granulopoiesis with cytokines or *Candida albicans* infection. Although expression of C/EBPα, C/EBPδ, and C/EBPε was downregulated in the granulocytic precursor cells, C/EBPβ levels remained elevated, suggesting that C/EBPβ may play an important role in emergency granulopoiesis.[4] C/EBPα-null hematopoietic precursor cells are capable of generating neutrophils in substantial numbers upon cytokine stimulation[4–6] or upon infection with *C. albicans*,[4] but C/EBPβ-null mice were unable to support emergency granulopoiesis in response to cytokine stimulation,[4,7] even though steady-state granulopoiesis remained unaffected. Based on these observations, C/EBPα and C/EBPβ appear to function specifically and antagonistically toward one another during steady-state and emergency granulopoiesis.[4] C/EBPα functions as a master regulator of steady-state granulopoiesis by limiting proliferation via inhibition of expression of the cyclin-dependent kinases, cdk2 and cdk4,[8] and of c-Myc,[9] and by promoting granulocytic differentiation. C/EBPβ may promote emergency granulopoiesis largely because it does not block the expression of cdk2, cdk4, or c-Myc and thereby allows for proliferation of granulocytic progenitors and increasing neutrophil numbers during emergency granulopoiesis.

According to current models, the switch from steady-state to emergency granulopoiesis is thought to be regulated by increased levels of cytokines such as G-CSF and, to less extent, GM-CSF and IL-6. These cytokines are known to be upregulated in response to pathogen invasion. G-CSF signaling activates the JAK/STAT pathway, in particular, STAT3, a known regulator of granulopoiesis[10] that together with C/EBPβ binds to the *MYC* promoter and activates gene expression. This also prevents binding of C/EBPα to the *MYC* promoter, resulting in C/EBPβ-mediated gene expression prevailing over that of C/EBPα during emergency granulopoiesis.[11]

C/EBPγ

C/EBPγ is a ubiquitously expressed C/EBP family member that was first identified by its affinity for *cis*-regulatory sites in the Ig heavy chain promoter and enhancer. C/EBPγ contains a C/EBP-like b-Zip domain but lacks an N-terminal transactivation domain and can inhibit transcriptional activation of other C/EBP members in some cell types. Impairment of natural killer (NK) cytotoxic activity and of interferon-γ production has been reported in *Cebpg*$^{-/-}$ mice.

C/EBPδ

C/EBPδ is expressed at low or undetectable levels in several tissues of adult mice and humans. Expression has been shown to dramatically increase upon induction with bacterial lipopolysaccharide (LPS) and inflammatory cytokines, suggesting a role for C/EBPδ in the acute phase and inflammatory response. Experiments with double knockout of C/EBPβ and C/EBPδ suggest a synergistic role for these two C/EBP family members in controlling terminal adipocyte differentiation. Both C/EBPβ and C/EBPδ are also expressed during late neutrophil development, suggesting a parallel role in late neutrophil gene expression.

C/EBPε

The human *CEBPE* gene resides on chromosome 14 and is transcribed under the control of two alternative promoters, Pα (thought to function in mature neutrophils) and Pβ (thought to function in BM). A combination of differential splicing and alternate promoter use results in four messenger RNA (mRNA) isoforms 2.6 kb and 1.3 to 1.5 kb in size, from which three proteins of size 32.2, 27.8, and 14.3 kDa have been identified. *Cebpe*$^{-/-}$ mice produce hyposegmented granulocytes that are functionally defective, and these mice develop myelodysplasia later in life. Absence of C/EBPε is thought to block the later steps in terminal differentiation of mature segmented granulocytes. Mutant mice usually survive 2 to 5 months and eventually succumb to low-pathogenicity bacterial infections. C/EBPε thus plays a crucial role in terminal granulocytic differentiation.

Cebpe$^{-/-}$ mice have wild-type levels of the G-CSFR, and the defects manifested in these mice are confined to late-stage gene expression associated with the function of the mature neutrophil. It has been shown that the ability of G-CSF to regulate myeloid differentiation is dependent on the induction of C/EBPε. Verbeek and colleagues demonstrated that the mRNAs of several genes, including p47 phox (a component of the neutrophil–NADPH oxidase complex), as well as the secondary granule protein genes, are either absent or abnormal in the BM of *Cebpe*$^{-/-}$ mice. These observations suggest that C/EBPε also plays a critical role in the regulation of host antimicrobial defense (see Chapter 51).

Neutrophils from *Cebpe*$^{-/-}$ mice have morphologic and biochemical features similar to those observed in patients with neutrophil SGD. SGD is an extremely rare congenital disorder that is characterized by frequent and severe bacterial infections. Patients with SGD have defects in neutrophil function, including atypical nuclear morphology, impaired bactericidal activity, and abnormalities in neutrophil migration; they also lack both neutrophil and eosinophil secondary granule proteins. Sequence analysis of genomic DNA from two patients with SGD revealed mutations within the *CEBPE* gene, resulting in the expression of a mutant protein lacking the dimerization and DNA-binding domains and with impaired transcriptional activity. Loss of C/EBPε activity is thought to underlie the observed pathology in these patients.

C/EBPζ

C/EBPζ-C/EBP homologous protein (CHOP) is a C/EBP family member that was originally identified as a gene product induced in response to DNA-damaging agents. It has subsequently been shown to also be induced by various extracellular or intracellular (endoplasmic reticulum [ER]) stresses. The basic region of CHOP is less well conserved than that of the other C/EBP family members, and CHOP does not seem to bind to canonical C/EBP *cis* elements. CHOP has been shown to interfere with the transcriptional activity of C/EBPβ in a manner dependent on its leucine zipper.

PU.1

PU.1 (encoded on the *SPI1* gene) is a member of the Ets family of transcription factors and is expressed abundantly in B cells and macrophages. Expression of PU.1 has also been reported in granulocytes and eosinophils as well as in CD34^{+} hematopoietic progenitor cells. High levels of PU.1 expression in fetal mouse liver preferentially direct macrophage development, whereas low levels of PU.1 result in B-cell development. c-Jun, another member of the b-Zip family of transcription factors, serves as a coactivator of PU.1, and overexpression of c-Jun in myeloid progenitor cells results in macrophage development. Downregulation of c-Jun by C/EBPα is necessary for granulocytic maturation and appears to be a primary mechanism through which C/EBPα blocks macrophage development. C/EBPα not only binds to the promoter of the *JUN* gene and decreases its expression but also binds PU.1, thereby inhibiting its activity.

PU.1-binding sites have been reported in almost all myeloid-specific promoters reported to date, including those for M-CSF, GM-CSF, and G-CSF receptors, all of which play critical roles in myeloid cell development. PU.1 activity is modulated both by protein-protein interactions and posttranslational modifications; phosphorylation of PU.1 by casein kinase II or by JNK kinase leads to increased transcriptional activity.

Abrogation of PU.1 expression in PU.1-null mice results in perinatal lethality accompanied by the absence of mature monocytes/macrophages and B cells and delayed and reduced granulopoiesis. Following in vitro differentiation, embryonic stem (ES) cells derived from PU.1-null blastocysts fail to express mature myeloid cell markers, suggesting that PU.1 is not essential for the initial events associated with myeloid lineage commitment but is necessary for the later stages of development.

Growth Factor Independence-1

The *GFI1* gene was first identified as a target of proviral insertion following infection with Moloney murine leukemia virus (MoMuLV) resulting in IL-2 factor independence in a rat lymphoma cell line (reviewed by van der Meer et al.[21]). *GFI1* is a highly conserved gene that encodes a 55-kDa nuclear proto-oncogene that harbors six C_2H_2-type zinc finger domains at the C-terminus and a 20-amino-acid stretch at the N-terminus known as the SNAG domain. The SNAG domain appears to be conserved in the Snail/Slug family of proteins and confers transcriptional repressor activity on Gfi-1. The human *GFI1* gene is located on chromosome 1p22, and its closely related paralog *GFI1B* maps to chromosome 9q34. Gfi-1 is expressed at high levels in the thymus and BM, whereas Gfi-1b expression is confined to the BM and spleen. Homozygous knockout of Gfi-1b results in embryonic lethality at day E15, despite the fact that normal myelopoiesis occurs. Death in these mice has been attributed instead to a failure of erythropoiesis and megakaryopoiesis.

The essential role of Gfi-1 in neutrophil differentiation became apparent following two reports of gene disruption in mice. Gfi-1–null mice are severely neutropenic and eventually succumb to bacterial infections. In addition, these mice lack mature neutrophils and their granulocyte precursors are unable to differentiate into mature neutrophils upon induction with G-CSF. These cells also lack secondary granule protein expression, as is seen in *Cebpe*$^{-/-}$ granulocytes. *Gfi1*$^{-/-}$ BM contains an atypical Gr1$^+$Mac1$^+$ myeloid precursor cell that shares characteristics of both granulocyte and macrophage precursors. Ectopic expression of Gfi-1 in ex vivo–sorted *Gfi1*$^{-/-}$ progenitor cells restores G-CSF–mediated neutrophil maturation to these cells. These observations provide evidence for the critical role of Gfi-1 in the neutrophil maturation program. Other studies have further demonstrated that Gfi-1 synergizes with C/EBPε to transactivate the promoters of late myeloid genes. This synergy is lost in patients with SGD who have a heterozygous substitution mutation in the *CEBPE* gene and decreased levels of Gfi-1 in the BM.

Heterozygous dominant negative mutations in the *GFI1* gene have been described in two patients with severe congenital neutropenia (SCN), underscoring the role of Gfi-1 in the neutrophil maturation pathway. It has been suggested that mutant Gfi-1 in these patients alters the expression of NE, mutations in which are commonly associated with SCN (see later). This observation confirms the vital role that Gfi-1 plays in human granulopoiesis.

CCAAT Displacement Protein

CCAAT displacement protein (CDP)/cut is a ubiquitously expressed, highly conserved, homeodomain (HD) protein with extensive homology to the *Drosophila* cut protein. CDP acts as a repressor of developmentally regulated genes, including the phagocyte-specific cytochrome heavy chain gene (gp91 phox, also known as Nox2 or cytochrome b-245 heavy chain), which is expressed exclusively in differentiating granulocytes (reviewed by Nepveu[22]). Overexpression of CDP in 32Dcl3 myeloid cells blocks G-CSF–induced expression of *SGP* genes without blocking phenotypical maturation. CDP therefore acts as a negative regulator of stage-specific expression of both early and late neutrophil-specific genes.

The CDP homeobox protein contains three highly conserved DNA-binding repeats referred to as *cut repeats* (CR1, CR2, CR3) and an HD, each of which is capable of recognizing and binding specific DNA motifs in target genes. This may explain why the CDP molecule as a whole does not have a well-defined consensus DNA-binding sequence; cut repeats cannot bind DNA as monomers but in combination exhibit high DNA-binding affinity. It has further been suggested that CDP-binding activity is restricted to proliferating cells, in which CDP target genes are repressed. These targets are upregulated as cells undergo cell cycle arrest and terminal differentiation, in association with a decrease in CDP binding. Target genes of CDP are numerous and include *MYC*, *MOS*, thymidine kinase *(TK)*, cdk inhibitor p21 *(WAF1/CIP1)*, cystic fibrosis transmembrane conductance regulator *(CFTR)*, transforming growth factor-β (TGF-β) receptor 2 *(TGFBR2)*, gp91 phox *(NOX2/CYBB)*, major histocompatibility complex (MHC) class I locus, and neutrophil *SGP* genes.

During myeloid differentiation, CDP binding has been shown to regulate genes that are expressed at widely disparate stages of differentiation. For example, CDP represses the *CYBB* gene, which is expressed at a much earlier time in myelopoiesis than is the case for the *LF* gene. The mechanisms by which CDP mediates repression and modulates stage-specific gene expression at different stages of differentiation within a single lineage are not fully understood. CDP is reported to have repressive activity associated with its ability to be displaced by a positive *trans*-acting factor involving the CR1 and CR2 cut repeats. However, other modes of repressive activity involving the two active repression domains within the C-terminus of CDP have also been reported. CDP has been shown to function as a repressor of transcription via chromatin modification by recruitment of histone deacetylases (HDACs), consistent with the hypothesis that transcriptional silencing is associated with hypoacetylated histone markers. Both acetylation and phosphorylation of CDP are posttranscriptional modifications that have been postulated to regulate CDP function. Thus differential modification, by phosphorylation or acetylation, of CDP-DNA complexes bound to the promoters of target genes could result in the observed differential repression exerted by CDP during neutrophil development.

ROLE OF DEVELOPMENTALLY IMPORTANT NEUTROPHIL-SPECIFIC GENES IN DISEASE

Our understanding of the role of neutrophil-specific genes has been enhanced by the study of mice in which targeted disruption of a gene results in phenotypically important defects in neutrophil differentiation and function. Similarly, their importance has been underscored by the analysis of naturally occurring genetic events within these genes that result in human disease. The links between some genes and the diseases induced by their dysfunction may be anticipated by their important roles in neutrophil differentiation and function, whereas the pathophysiologic links of other genes to disease remain elusive (Table 28.2).

Disruption of neutrophil transcriptional regulation is a recurring theme in the pathogenesis of leukemia. Nearly half of patients with AML have pathognomonic translocations resulting in the fusion of a transcription factor with a tissue-specific gene. These translocations have been shown to interfere with appropriate myeloid differentiation and emphasize the role of transcription factors in that process.

As discussed previously, the same transcription factors that are implicated in the induction of neutrophil differentiation also direct the expression of genes encoding neutrophil-specific functional proteins. The link between morphologic differentiation and synthesis

TABLE 28.2 **Differentiation-Specific Genes Implicated in Neutrophil Disorders**

Transcription Factors
CEBPA, *SPI1* (PU.1), *RARA*, *RUNX1*, and others in AML
CEBPE and *GFI1* in specific granule deficiency
GFI1 in neutropenia
Granule and Functional Proteins
ELANE (neutrophil elastase) in severe congenital neutropenia and cyclic neutropenia
CYBB (*NOX2*, gp91 phox) in chronic granulomatous disease
Adhesion Molecules, Receptors
Common β-chain of integrin receptors in leukocyte adhesion deficiency
G-CSF receptor mutations in AML arising in patients with Kostmann syndrome

AML, Acute myeloid leukemia; *G-CSF*, granulocyte colony-stimulating factor.

of neutrophil functional proteins is illustrated by the observation that disruption of C/EBPε signaling results in SGD, associated with both morphologic abnormalities and increased infections attributable to neutrophil functional defects. C/EBP$\varepsilon^{-/-}$ mice share these abnormalities while also demonstrating a predilection for the development of myelodysplasia. Although the development of myelodysplastic syndrome (MDS) or AML has not been reported in patients with SGD, long-term follow-up is limited because SGD is a rare disease described in only fewer than a dozen patients.

Other diseases have been linked to defects in functionally important neutrophil proteins. Abnormalities in integrin expression, notably loss of the common β-chain of the integrin receptors, result in leukocyte adhesion deficiency, whereas absence of any one of the components of the reduced NADPH oxidase complex leads to chronic granulomatous disease (see Chapter 51).

Abnormalities in granule protein gene expression again underscore the complexity of the granulocyte functional program. Congenital absence of many individual granule proteins, including MPO, LF, and transcobalamin, has been described. In the absence of the more global defects seen in SGD, which presumably reflect more complex abnormalities than simple protein deficiency, these defects tend to be observed as incidental laboratory findings with minimal or no associated pathology.

One prominent exception is the association between point mutations in the *ELANE* gene (previously known as *ELA2*, which encodes NE) and SCN. The pathogenesis of SCN originally was attributed to defects in G-CSFR, supported by the observation of a truncation mutation in G-CSFR in select patients with Kostmann syndrome (reviewed by Berliner[23]). It was later found that these were instead acquired mutations that may predispose the patient to secondary AML but by themselves did not constitute the pathologic basis for SCN.

Severe Congenital Neutropenia and the Unfolded Protein Response

SCN is a rare BM failure syndrome characterized by maturation arrest at the promyelocyte/myelocyte stage during neutrophil maturation in the BM (reviewed by Coffman[12] and Lichtinger et al.[13]). Because of profound neutropenia, the disease was almost uniformly fatal as a result of bacterial infections prior to the advent of G-CSF therapy (reviewed by Calkhoven et al.[14]). In addition to having an increased risk of infection, patients with SCN treated with G-CSF also have an underlying predisposition for the later development of MDS/AML (reviewed by Lichtinger et al.[13]). Although originally described as an autosomal recessive disorder, SCN is genetically heterogeneous, with multiple modes of inheritance including autosomal recessive (30% of patients), autosomal dominant (60% of patients), X-linked, and sporadic. Pathogenic mutations associated with SCN have been identified in a number of genes, including *ELANE*, *HAX1*, *GFI1*, *WAS*, *CSF3R*, and *G6PC3* (reviewed by Coffman[12]). Regardless of the mode of inheritance or gene mutation, newborns with SCN present with severe neutropenia. In contrast, patients with cyclic neutropenia (CN) have regular and consistent oscillations of their circulating neutrophils, with a mean cycle of 21 days. Patients with CN tend to have milder symptoms that often manifest later in life, respond well to lower doses of G-CSF, and do not progress to developing MDS/AML.[15]

Mutations in *ELANE*, which encodes the primary granule protein NE, are the most common causes of both SCN (35% to 60%) and CN (80% to 100%).[12] More than 200 distinct pathologic mutations in *ELANE* have been identified, spanning the entire coding sequence, and which occur in both patients with SCN and with CN with autosomal dominant inheritance. These mutations cause structural impairments in NE, leading to protein misfolding and intracellular mislocalization and accumulation.[12,14] As a result, ER stress occurs, activating the unfolded protein response (UPR) and causing apoptosis within the granulocyte compartment.[13] Different *ELANE* mutations appear to impact NE processing and trigger UPR activation to varying degrees, accounting for differences in disease severity and, in particular, the milder phenotype associated with CN (see Chapter 49).

Role of MicroRNAs in Controlling Gene Expression in Granulopoiesis

MicroRNAs (miRNAs) are 18- to 24-nucleotide-long noncoding RNAs that regulate eukaryotic gene expression by binding to specific sites in the 3′ UTR of target genes and altering expression by destabilizing mRNA or blocking mRNA translation. miRNAs are encoded in the genome and are transcribed by RNA polymerase II as long primary transcripts referred to as primary miRNAs (pri-miRNAs). These transcripts are recognized and processed by a ribonuclease, Drosha, into 60- to 80-nucleotide intermediates called precursor miRNAs (pre-miRNAs), which are then exported to the cytoplasm, where a second ribonuclease, Dicer, cleaves pre-miRNAs to generate double-stranded miRNAs. The miRNAs are then incorporated into the RNA-induced silencing complex (RISC), a large protein complex that also contains the Argonaute or mRNA-cleaving proteins. The miRNA guides the RISC complex to target complementary regions in the 3′ UTRs of mRNAs, leading to repression of translation or mRNA destabilization by deadenylation (reviewed by Manikandan et al.[24]).

An increasing body of evidence implicates miRNA activity in mediating both normal and abnormal myelopoiesis (reviewed by Pelosi et al.[25]). miRNAs have been shown to activate or be activated by myeloid-specific transcription factors such as C/EBPα and Gfi-1. For example, miR-223 is thought to be a direct target of C/EBPα, and its expression increases during granulopoiesis. Ablating miR-223 in mice results in the expansion of granulocyte precursor cells resulting from a cell-autonomous increase in the number of granulocytic progenitors.[26] In addition, overexpression of miR-223 in acute promyelocytic leukemia (APL) cells results in an enhanced capacity for granulocytic differentiation.[27] miR-223 is thus thought to be a positive regulator of granulopoietic differentiation. In addition, miR-223 targets E2F1, a master cell cycle regulator, by inhibiting translation of its mRNA. Thus granulopoiesis appears to be regulated by a C/EBPα–miR-223–E2F1 axis, wherein miR-223 functions as a key regulator of myeloid cell proliferation associated with E2F1 in a mutual negative feedback loop.[28]

Eiring et al. have demonstrated another role for miRNAs in granulopoiesis by demonstrating that miR-328 is downregulated in CML patients in blast crisis. miR-328 expression restores differentiation by interaction with both the C/EBPα translational inhibitor hnRNP-E2 and the mRNA for *PIM1*, a survival factor. The interaction with hnRNP-E2 leads to the release of *CEBPA* mRNA from hnRNA-E2–mediated translational inhibition through an interaction that is independent of its seed sequence. miR-328 appears to control cell fate by its ability to base pair with the 3′ UTR of target mRNAs (*PIM1*) as well as by acting as a decoy for hnRNP binding, thus interfering with cell fate by releasing C/EBPα from translational inhibition.[29]

A role for miR-27 in granulopoiesis has also been documented. miR-27 targets the myeloid transcription factor *RUNX1*, leading to decreased expression during granulocytic differentiation. Anti–miR-27 treatment of immature myeloid progenitors resulted in an increase in the expression of *RUNX1* and impaired granulocytic differentiation.[30] In a separate study, the transcription factor Gfi-1 was shown to bind to the promoter of miR-196b and repress its expression, thereby promoting granulocytic maturation while repressing monocytic lineage development. Overexpression of miR-196b blocked granulopoiesis in granulocyte monocyte precursors (GMPs).[31]

EOSINOPHIL PRODUCTION

Eosinophil precursors constitute approximately 3% of BM progenitors, of which approximately two-thirds are myelocyte precursors and the remainder maturing eosinophils. Eosinophilic myelocytes are large cells with a bilobed nucleus (Fig. 28.5). The characteristic specific granules of eosinophils contain major basic protein, eosinophil cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin. Eosinophils also contain primary granules that contain Charcot-Leyden crystal protein. Mature eosinophils are released into the peripheral blood, where they circulate up to 18 hours before migrating to the tissues.

Eosinophils proliferate and mature under the influence of IL-3, IL-5, and GM-CSF. Evidence suggests that these cytokines are secreted by T cells as the stimulus to eosinophil production in many disorders associated with eosinophilia. Recent studies of idiopathic hypereosinophilic syndrome have described both activating mutations in and fusion partners of the platelet-derived growth factor receptor-α (PDGFR-α) that result in constitutive tyrosine kinase activation and eosinophil proliferation (see Chapter 74).

Transcriptional regulation of eosinophilic differentiation is mediated through PU.1, C/EBPα, and C/EBPβ. Because these same factors also induce myeloid differentiation, how the choice between myeloid and eosinophilic lineage is modulated is not fully understood. It has been proposed that levels of GATA-1 may determine whether C/EBP expression induces the eosinophilic or the myeloid maturation program. The transcription factor interferon regulatory factor-8 (IRF8) has also been implicated in associating with PU.1 and inhibiting C/EBPα to preferentially direct monocytic and eosinophilic differentiation over neutrophilic production.

BASOPHIL AND MAST CELL PRODUCTION

Basophils and mast cells mediate allergic responses and are centrally involved in IgE-induced immune responses to parasites and other allergens. Both cell types are derived from BM precursors, although they have a different ontogeny.

Basophils have a single-lobed nucleus and characteristic intensely staining purple granules that may obscure the nucleus when seen on microscopy of peripheral blood and BM (see Fig. 28.5). These granules contain glycosaminoglycans, predominantly heparin. Basophils arise from BM progenitors and are released from the BM as mature cells where they circulate with a brief lifespan similar to that of neutrophils. Maturation occurs in response to IL-3, which serves both to induce basophilic differentiation and to mediate activation of mature basophils. Although IL-3 is the primary mediator of basophil development, studies of IL-3–null mice have demonstrated that it is not required for baseline production of basophils. However, IL-3 is required for the induction of basophilia in response to parasitic infection. Other cytokines that influence basophil proliferation include GM-CSF, IL-5, and SCF.

Mast cells arise from BM precursors but are released into the circulation as immature cells. They circulate only briefly in the peripheral blood before migrating to the tissues, where they complete their maturation. There remains some question about whether mast cells and basophils arise from a common precursor. In vitro studies showed that adding SCF and IL-3 to cultured CD34$^+$ cells results in an increased proliferation and maturation of both basophils and mast cells but did not establish a common progenitor cell for the two lineages. It is clear that SCF is especially effective in inducing mast cell proliferation: activating mutations in c-Kit, the SCF receptor, is the underlying molecular defect in most cases of systemic mastocytosis (see Chapter 75).

MONOCYTOPOIESIS

Monocyte Ontogeny

Stages of Monocyte Differentiation

Monocytes originate in the BM from promonocytes, which constitute approximately 3% of the total cells in the normal BM (Fig. 28.6). Promonocytes have round nuclei and basophilic cytoplasm. Differentiation occurs rapidly, with a maturation time of 50 to 60 hours, associated with two rounds of replication and morphologic maturation marked by progressive lobulation of the nucleus. Stress-induced release of monocytes occurs primarily through their

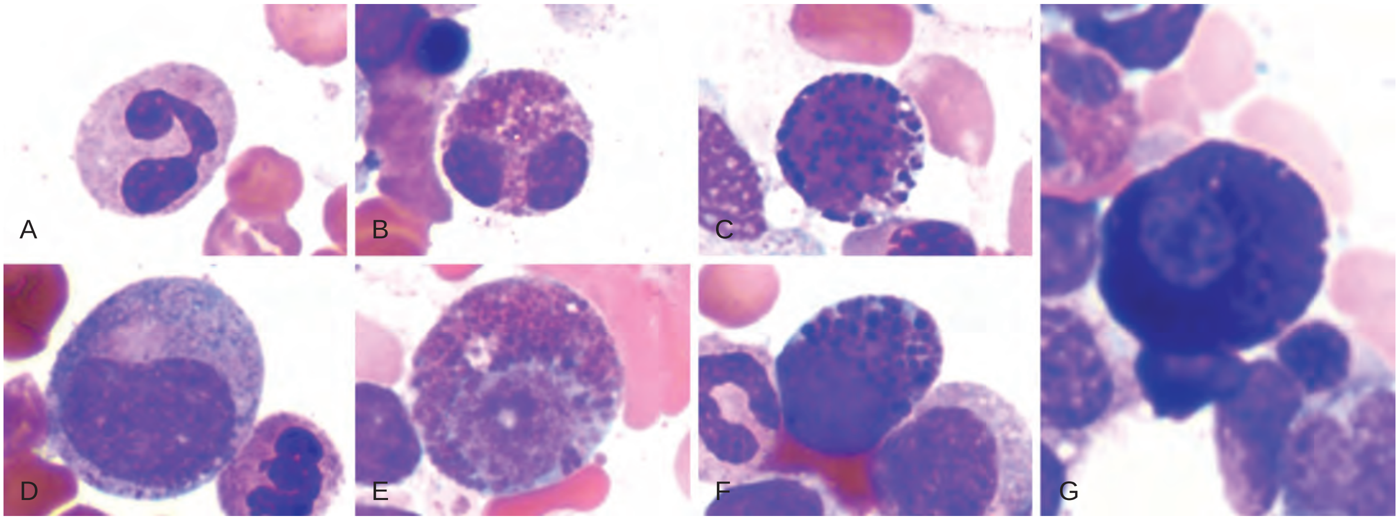


Figure 28.5 NEUTROPHILIC, EOSINOPHILIC, AND BASOPHILIC GRANULOCYTES; THEIR MYELOCYTE PRECURSORS; AND MAST CELLS. Eosinophilic and basophilic granulocytes are always best viewed compared with neutrophilic granulocytes because staining can vary from laboratory to laboratory and even from case to case. When compared with a neutrophil (A), the eosinophil (B) has larger and more red/orange-colored and almost refractile granules than the pink and barely perceivable granules in the neutrophil. Mature eosinophils are also frequently bilobed, whereas neutrophils tend to have three or four lobes. The basophil (C) has dense, dark blue granules that frequently overlie the condensed and lobulated nucleus. These granules are much larger than the primary granules of promyelocytes. The myelocyte stage of maturation is when the secondary granules first appear (D–F). In the neutrophilic myelocyte (D), the secondary granules first appear in the Golgi region and have been described as resembling the sunrise over the horizon, the "dawn of neutrophilia." The eosinophilic and basophilic myelocytes (E, F) are relatively infrequent in the bone marrow and are usually enumerated together with the mature forms. Mast cells (G) must be distinguished from basophils and basophilic myelocytes. Mast cells are larger and have numerous granules mostly in the cytoplasm. Mast cells have round nuclei, which are frequently obscured by the granules. A toluidine blue cytochemical reaction will be positive (metachromatic) in the granules of both basophils and mast cells. However, a mast cell tryptase immunostain used on a tissue section will be positive only in mast cells.

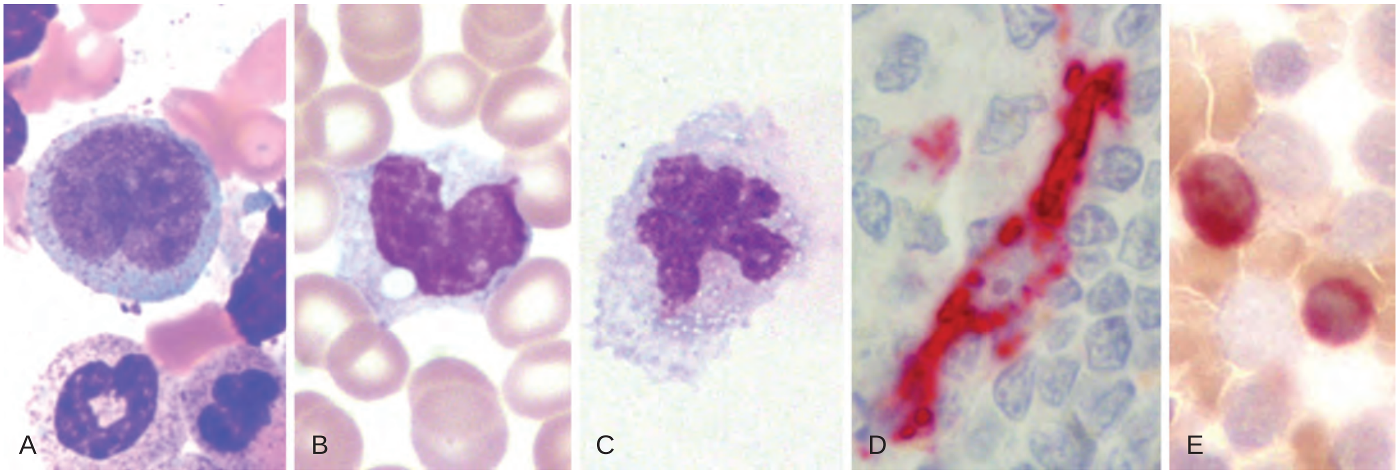


Figure 28.6 A PROMONOCYTE, MONOCYTE, MACROPHAGE, AND HISTIOCYTE. Promonocytes are rare in the bone marrow (BM) (A) and are more frequently seen during recovery or during marrow regeneration after chemotherapy or other insult. They are typically slightly folded and have nucleoli. The cytoplasm is moderately abundant and blue/gray with very faint granules. Monocytes seen in the blood (B) typically have more folded or horseshoe-shaped nuclei and more abundant gray cytoplasm frequently with vacuoles and rare granules. The macrophage (C) is seen in body fluid specimens such as peritoneal fluid or cerebrospinal fluid and resembles the blood monocyte. Tissue histiocytes are fixed in the tissues and are seen on tissue sections. The histiocyte shown (D) is stained with CD68 and is from a lymph node. It likely represents a dendritic cell in a germinal center. Monocytes in the BM can be more easily enumerated with a nonspecific esterase cytochemical reaction such as alpha-naphthyl acetate esterase, which gives an orange/brown reaction product (E).

premature release from the proliferating pool. Survival in the blood is short, approximately 8 to 72 hours. Monocytes then enter the tissues, where they develop into macrophages that may survive for 2 to 3 months. Tissue-resident macrophages are found in the lung (alveolar macrophages), liver (Kupffer cells), spleen, and central nervous system (glial cells).

Monocytes can also serve as precursors to a subset of dendritic cells. Dendritic cells are professional antigen-presenting cells that arise from both myeloid and lymphoid precursor cells. The myeloid subset of dendritic cells arises from a precursor that can alternatively differentiate into macrophages. Similar cells have been generated for immunotherapy by exposing peripheral blood monocytes to GM-CSF and IL-4 in vitro (see Chapter 20).

Markers of Monocyte Maturation

Unique surface markers of monocyte maturation have been difficult to identify. In the mouse, the marker F4/80 was identified as a nearly universal marker of monocytes and macrophages; this antigen has been shown to be homologous to the human EGF module containing mucin-like hormone receptor 1. The function of this receptor is unknown because the knockout mouse has no phenotype. Monocyte precursor cells express the M-CSF receptor, lysozyme, the Fcγ receptor (II/III), and the scavenger receptor. Mature monocytes, like neutrophils, show high-level expression of CD11b/CD18. Following differentiation to macrophages, the cells acquire expression of macrosialin (CD68), a glycoprotein of unknown function that may play a role in lipoprotein metabolism. Macrophages also express sialoadhesin, a member of the sialic acid–binding receptor family. Although its precise function has not been proven, sialoadhesin mediates binding to sialic acid moieties on cell surfaces and probably plays a role in macrophage cell-cell and cell–extracellular matrix interactions.

CD14 is a major functional surface protein of the monocyte/macrophage lineage. CD14 is the receptor for LPS, leading to monocyte/macrophage activation. More recent studies have suggested that CD14 may also have a role in apoptosis.

Monocytes contain both primary (peroxidase-positive) and secondary (peroxidase-negative) granules. The primary granules of monocytes, like those of neutrophils, contain MPO. Secondary granule fusion with the membrane upon monocyte stimulation results in upregulation of Mac1 and p150, which are thought to play roles in adhesion and diapedesis of activated monocytes.

Control of Monocytopoiesis

Cytokine Regulation of Monocyte Proliferation and Differentiation

The primary regulator of mononuclear phagocyte production is colony-stimulating factor 1 (CSF-1; also known as M-CSF). Its effects are mediated by the high-affinity receptor tyrosine kinase CSF-1 receptor (CSF-1R), which was first identified as the gene product of the c-*fms* proto-oncogene. Alternative splicing of *CSF1* results in five distinct mRNA transcripts and three isoforms of the CSF-1 protein: a secreted proteoglycan, a secreted glycoprotein, and a membrane-spanning cell surface proteoglycan.

The phenotypes of Csf1-null mice and of mice harboring an inactivating mutation in the coding region of *Csf1* (*Csf1*$^{op/op}$, osteopetrotic mice) are virtually identical; features include toothlessness, low body weight, low growth rate, defects in fertility, and deficient tissue macrophages. Compared with their wild-type littermates, splenic erythroid burst-forming unit and high-proliferative-potential colony-forming cell counts in both *Csf1*$^{op/op}$ and *Csf1*$^{-/-}$ mice were significantly elevated, supporting a negative regulatory role for CSF-1 in erythropoiesis and in the maintenance and proliferation of primitive hematopoietic progenitors. Plasma CSF-1 levels in CSF receptor–null (*Csf1R*$^{-/-}$) mice were elevated 20-fold, consistent with previous reports demonstrating clearance of circulating CSF-1 by CSF-1R–mediated endocytosis. Despite their overall similarity, several phenotypic characteristics of the *Csf1R*$^{-/-}$ mice are more severe than those of the *Csf1*$^{op/op}$ mice. These observations suggest that CSF-1 signals exclusively through CSF-1R, but CSF-1R may mediate other effects from CSF-1–independent activation.

Signaling through CSF-1R appears to be critical for monocyte/macrophage development. Although little is known about the events that lead to stimulation of a monocyte/macrophage–specific array of genes, it is clear that several transcription factors, probably stimulated by CSF-1–related signaling, play vital roles in the development of this lineage. It should be noted that the ability of phorbol esters to induce monocytic differentiation of myeloid cell lines through activation of the protein kinases Cα and Cδ suggests a role for the PKC pathway in monopoiesis.

IL-3, G-CSF, and tumor necrosis factor (TNF) have all been shown to synergize with CSF-1 in the proliferation of macrophages. G-CSF induces increased mobilization of monocytes as an indirect effect dependent on the presence of CSF-1. Monocytes have also been shown to have functional G-CSFRs, although G-CSF appears to function mainly to decrease monokine secretion rather than to increase monocyte proliferation.

Transcriptional Regulation of Monocyte Differentiation

Of the several transcription factors known to regulate the development of the monocyte/macrophage lineage, PU.1 appears to be the most significant because abrogation of PU.1 expression in PU.1-null mice results in perinatal lethality accompanied by the absence of mature monocytes/macrophages and B cells, and delayed and reduced granulopoiesis. A number of factors, most notably c-Jun, cooperate with PU.1 to regulate monocyte-specific genes.

c-Jun

The *JUN* proto-oncogene encodes the transcriptional activator protein AP-1. As an early response gene, *JUN* is rapidly and transiently activated in response to external proliferative signals. Expression of c-Jun as well as related family members JunB and JunD is upregulated during monocytic differentiation. In addition, overexpression of c-Jun in M1, U937, or WEHI-B D^+ myeloid cell lines, as well as in myeloid progenitor cells, results in partial monocytic differentiation. However, $Jun^{-/-}$ fetal liver cells are capable of reconstituting hematopoiesis in syngeneic recipients, suggesting that c-Jun is not required for myeloid development. This finding may reflect a compensatory role played by other Jun family proteins.

During macrophage development, c-Jun serves as a coactivator of PU.1. Recent studies have revealed that downregulation of *JUN* by C/EBPα is necessary for granulocytic maturation and appears to be the mechanism through which C/EBPα blocks macrophage development (see Fig. 28.4). C/EBPα not only binds to the promoter of the *JUN* gene and decreases its expression but also binds to PU.1, inhibiting its activity. Such transcription factor cross-talk, resulting in subtle changes in the levels of transcription factors within a given lineage, appears to define a paradigm by which master regulators of lineage specification, such as C/EBPα and PU.1, direct lineage-specific development by directly upregulating lineage-specific genes and by blocking the progression of alternate lineages.

Other Transcription Factors Modulating Monocyte Development

Egr-1. Egr-1 belongs to a family of zinc finger transcription factors and is expressed in a number of tissues and at various points in development, including the terminal stages of macrophage and neutrophil differentiation. Egr-1 is necessary for monocytic differentiation of myeloid cell lines U937 and M1 and prevents factor-induced granulocytic differentiation of HL60 and 32Dcl3 cells. In addition, ectopic expression of Egr-1 in myeloid BM progenitors results in an increase in the number of CFU-M at the expense of CFU-G progenitors. However, mice lacking Egr-1 develop normal numbers of macrophages, a phenomenon attributed to the possible compensatory effects of other Egr family members.

C/EBPβ. As discussed earlier, expression of C/EBPβ increases during myeloid maturation and has been shown to be important for monocyte/macrophage gene expression and development.

MafB and c-Maf. The transcription factors MafB and c-Maf belong to a family of b-Zip factors that bind DNA as dimers. The Maf proteins can dimerize with members of other b-Zip family proteins including c-Jun, fos, and NF-E2 in erythroid cells. Ectopic expression of MafB in myeloblasts resulted in macrophage differentiation, whereas overexpression of c-Maf in HL60 and U937 myeloid cells resulted in monocytic differentiation.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Bjerregaard MD, Jurlander J, Klausen P, et al. The in vivo profile of transcription factors during neutrophil differentiation in human bone marrow. *Blood*. 2003;101:4322.

Cowland JB, Borregaard N. Granulopoiesis and granules of human neutrophils. *Immunol Rev*. 2016;273:11.

Crocker PR, Paulson JC, Varki A. Siglecs and their roles in the immune system. *Nat Rev Immunol*. 2007;7:255.

Dao C, Metcalf D, Zittoun R, et al. Normal human bone marrow cultures in vitro: cellular composition and maturation of the granulocytic colonies. *Br J Haematol*. 1977;37:127.

D'Alo' F, Johansen LM, Nelson EA, et al. The amino terminal and E2F interaction domains are critical for C/EBPalpha-mediated induction of granulopoietic development of hematopoietic cells. *Blood*. 2003;109:3163.

Dinauer MC. Inflammatory consequences of inherited disorders affecting neutrophil function. *Blood*. 2019;133:2130.

Hattori T, Ohoka N, Hayashi H, et al. C/EBP homologous protein (CHOP) up-regulates IL-6 transcription by trapping negative regulating NF-IL6 isoform. *FEBS Lett*. 2003;541:33.

Hock H, Hamblen MJ, Rooke HM, et al. Intrinsic requirement for zinc finger transcription factor Gfi-1 in neutrophil differentiation. *Immunity*. 2003;18:1092003.

Jafar-Nejad H, Bellen HJ. Gfi/Pag-3/senseless zinc finger proteins: a unifying theme? *Mol Cell Biol*. 2004;24:8803.

Jongstra-Bilen J, Harrison R, Grinstein S. Fcgamma-receptors induce Mac-1 (CD11b/CD18) mobilization and accumulation in the phagocytic cup for optimal phagocytosis. *J Biol Chem*. 2003;278:45720.

Keeshan K, Santilli G, Corradini F, et al. Transcription activation function of C/EBPalpha is required for induction of granulocytic differentiation. *Blood*. 2003;102:12672003.

Khan I, Eklund EE, Gartel AL. Therapeutic vulnerabilities of transcription factors in AML. *Mol Cancer Ther*. 2021;20:229.

Khanna-Gupta A, Hong S, Zibello T, et al. Growth factor independence-1 (Gfi-1) plays a role in mediating specific granule deficiency (SGD) in a patient lacking a gene-inactivating mutation in the C/EBPepsilon gene. *Blood*. 2007;109:4181.

Khanna-Gupta A, Zibello T, Sun H, et al. Chromatin immunoprecipitation (ChIP) studies indicate a role for CCAAT enhancer binding proteins alpha and epsilon (C/EBP alpha and C/EBP epsilon) and CDP/cut in myeloid maturation-induced lactoferrin gene expression. *Blood*. 2003;101:3460.

Laslo P, Spooner CJ, Warmflash A, et al. Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. *Cell*. 2006;126:755.

Lawrence SM, Corriden R, Nizet V. The ontogeny of a neutrophil: mechanisms of granulopoiesis and homeostasis. *Microbiol Mol Biol Rev*. 2018;82 e00057-17.

Manz M, Boettcher S. Emergency granulopoiesis. *Nat Rev Immunol*. 2014;14:302.

Mevel R, Draper JE, Lie-a-Ling M, et al. RUNX transcription factors: orchestrators of development. *Development*. 2019;146:dev148296.

Person RE, Li FQ, Duan Z, et al. Mutations in proto-oncogene GFI1 cause human neutropenia and target ELA2. *Nat Genet*. 2003;34:308.

Rangatia J, Vangala RK, Singh SM, et al. Elevated c-Jun expression in acute myeloid leukemias inhibits C/EBPalpha DNA binding via leucine zipper domain interaction. *Oncogene*. 2003;22:4760.

Skokowa J, Dale DC, Touw IP, et al. Severe congenital neutropenias. *Nat Rev Dis Primers*. 2017;3:17032.

Subramanian P, Mitroulis I, Hajishengallis G, et al. Regulation of tissue infiltration by neutrophils: role of integrin α3β1 and other factors. *Curr Opin Hematol*. 2016;23:36.

Yuan-Charvet L, Ng LG. Granulopoiesis and neutrophil homeostasis: a metabolic, daily balancing act. *Trends Immunol*. 2019;40:598.

REFERENCES

1. Verfaillie C. Adhesion receptors as regulators of the hematopoietic process. *Blood*. 1998;92:2609.
2. Ulyanova T, Blasioli J, Woodford-Thomas TA, et al. The sialoadhesin CD33 is a myeloid-specific inhibitory receptor. *Eur J Immunol*. 1999;29:3440.
3. Borregaard N, Theilgaard-Mönch K, Sørensen OE, et al. Regulation of human neutrophil granule protein expression. *Curr Opin Hematol*. 2001;8:23.
4. Borregaard N, Kjeldsen L, Rygaard K, et al. Stimulus-dependent secretion of plasma proteins from human neutrophils. *J Clin Invest*. 1992;90:86.
5. Khanna-Gupta A, Zibello T, Sun H, et al. Chromatin immunoprecipitation (ChIP) studies indicate a role for CCAAT enhancer binding proteins alpha and epsilon (C/EBP alpha and C/EBP epsilon) and CDP/cut in myeloid maturation-induced lactoferrin gene expression. *Blood*. 2003;101:3460.
6. Johnston JJ, Boxer LA, Berliner N. Correlation of messenger RNA levels with protein defects in specific granule deficiency. *Blood*. 1992;80:2088.
7. Cossio I, Lucas D, Hidalgo A. Neutrophils as regulators of the hematopoietic niche. *Blood*. 2019;133:2140.
8. Metcalf D. Hematopoietic regulators: redundancy or subtlety? *Blood*. 1993;82:3515.
9. Avalos BR. Molecular analysis of the granulocyte colony-stimulating factor receptor. *Blood*. 1996;88:761.
10. Tenen DG. Disruption of differentiation in human cancer: AML shows the way. *Nat Rev Cancer*. 2003;3:89.
11. Laslo P, Spooner CJ, Warmflash A, et al. Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. *Cell*. 2006;126:755.
12. Coffman JA. Runx transcription factors and the developmental balance between cell proliferation and differentiation. *Cell Biol Int*. 2003;27:315.
13. Lichtinger M, Hoogenkamp M, Krysinska H, et al. Chromatin regulation by RUNX1. *Blood Cells Mol Dis*. 2010;44:287.
14. Calkhoven CF, Müller C, Leutz A. Translational control of C/EBPalpha and C/EBPbeta isoform expression. *Genes Dev*. 2000;14:1920.
15. Khanna-Gupta A. Regulation and deregulation of mRNA translation during myeloid maturation. *Exp Hematol*. 2011;39:133.
16. Muller BU, Pabst T. C/EBPalpha and the pathophysiology of acute myeloid leukemia. *Curr Opin Hematol*. 2006;13:7.
17. Pabst T, Muller BU, Zhang P. Dominant-negative mutations of CEBPA encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. *Nat Genet*. 2001;27:263.
18. Kirstetter P, Schuster MB, Bereshchenko O, et al. Modeling of C/EBPalpha mutant acute myeloid leukemia reveals a common expression signature of committed myeloid leukemia-initiating cells. *Cancer Cell*. 2008;13:299.
19. Ford AM, Bennett CA, Healy LE, et al. Regulation of the myeloperoxidase enhancer binding proteins PU1, C-EBP alpha, -beta, and -delta during granulocytic-lineage specification. *Proc Natl Acad Sci USA*. 1996;93:108381996.
20. Dao C, Metcalf D, Zittoun R, et al. Normal human bone marrow cultures in vitro: cellular composition and maturation of the granulocytic colonies. *Br J Haematol*. 1977;37:127.
21. van der Meer LT, Jansen JH, van der Reijden BA. Gfi1 and Gfi1b: key regulators of hematopoiesis. *Leukemia*. 2010;24:1834.
22. Nepveu A. Role of the multifunctional CDP/cut/cux homeodomain transcription factor in regulating differentiation, cell growth and development. *Gene*. 2001;270:1.
23. Berliner N. Lessons from congenital neutropenia: 50 years of progress in understanding myelopoiesis. *Blood*. 2008;111:5427.
24. Manikandan J, Aarthi JJ, Kumar SD, et al. Oncomirs: the potential role of non-coding microRNAs in understanding cancer. *Bioinformation*. 2008;8:3302008.
25. Pelosi E, Labbaye C, Testa U. MicroRNAs in normal and malignant myelopoiesis. *Leuk Res*. 2009;33:1584.
26. Johnnidis JB, Harris MH, Wheeler RT, et al. Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. *Nature*. 2008;451:1125.
27. Fazi F, Rosa A, Fatica A, et al. A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell*. 2005;123:819.
28. Pulikkan JA, Dengler V, Peramangalam PS, et al. Cell-cycle regulator E2F1 and microRNA-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia. *Blood*. 2010;115:1768.
29. Eiring AM, Harb JG, Neviani P, et al. miR-328 functions as an RNA decoy to modulate hnRNP E2 regulation of mRNA translation in leukemic blasts. *Cell*. 2010;140:652.
30. Feng J, Iwama A, Satake M, et al. MicroRNA-27 enhances differentiation of myeloblasts into granulocytes by post-transcriptionally downregulating Runx1. *Br J Haematol*. 2009;145:412.
31. Velu CS, Baktula AM, Grimes HL. Gfi1 regulates miR-21 and miR-196b to control myelopoiesis. *Blood*. 2009;113:4720.

CHAPTER 29

THROMBOCYTOPOIESIS

Camelia Iancu-Rubin and Alan B. Cantor

Platelets, once regarded simply as "blood dust," are now recognized to play essential roles in hemostasis. Not only do they form a hemostatic plug and initiate thrombus formation in the event of vascular injury, but they also repair minute vascular damage that occurs on a daily basis. Platelets also participate in wound healing and angiogenesis via the delivery of key growth factors to sites of vascular injury and interact with the innate immune system. Disorders associated with platelet production carry significant morbidity and mortality in humans because of hemorrhage, thrombosis, bone marrow (BM) fibrosis, BM failure, and/or hematologic malignancy. Thrombocytopoiesis is a complex process in which platelets are generated from their precursor cells, megakaryocytes, via a complex process. For a long time, the extreme rarity of megakaryocytes significantly hampered studies aimed at understanding the molecular mechanisms underlying platelet biogenesis. However, the purification and cloning (in 1994) of thrombopoietin (TPO), the major megakaryocyte cytokine, has enabled considerable progress to be made. Recent application of whole exome and genome DNA sequencing has further stimulated the discovery of new disease-associated genes involved in human thrombocytopoiesis. These new insights provide an important foundation for improved diagnosis and treatment of disorders of thrombocytopoiesis. This chapter reviews the current understanding of megakaryocyte biology and platelet production, highlighting connections with human disease.

MEGAKARYOCYTE BIOLOGY

Megakaryocytopoiesis

Although platelets were described as early as the 1840s, it was not until 1906, in a seminal study by James Homer Wright, that their origin from megakaryocytes was first recognized.[1] Megakaryocytes are large polyploid cells that reside predominantly within the BM during postnatal life. They are rare cells, constituting only about 0.05% to 0.1% of nucleated BM cells under normal steady-state conditions. They develop from hematopoietic stem cells (HSCs) through a process known as megakaryocytopoiesis. According to the classical model of megakaryocytopoiesis, HSCs give rise to multipotent progenitor cells (MPPs) which sequentially transition to common myeloid progenitor (CMP) cells, bipotential megakaryocyte-erythroid progenitor (MEP) cells, and Mk progenitor (MkP) cells which will ultimately generate platelet-forming mature megakaryocytes (Fig. 29.1). In the past decade, single-cell transplantation and lineage tracing studies have demonstrated the existence of lineage-biased HSCs and underscored the heterogeneity of traditionally defined HSC and MPP cell compartments. Thus, the canonical model of hematopoietic hierarchy has been challenged by the emerging concept that hematopoiesis is a dynamic continuous process in which lineage priming and multilineage potential coexist.[2] Notably, megakaryocytopoiesis has been at the center of this paradigm shift. The first functional evidence that megakaryocytes derive from HSCs bypassing the MPP stage was suggested by Adolfsson and colleagues in 2005. Subsequently, several groups have shown that megakaryocyte-biased HSCs exist in both murine and human hematopoiesis supporting the concept that megakaryocytic lineage branches out earlier than previously appreciated. In vitro culture and transplantation assays have established that a common MEP capable of Mk-erythroid differentiation represents an intermediate step between MMP and MkP. Recent evidence employing lineage tracing in situ has challenged the existence of MEPs during unperturbed hematopoiesis thus underscoring the potential limitations of stress hematopoiesis such as cell culture and transplantation models. Furthermore, the concept that megakaryocytic lineage undergoes a "fast-tracked" differentiation process has been also documented in response to acute inflammation in murine models and in patients with myelofibrosis (MF) which is a condition characterized by Mk lineage hyperplasia (Chapter 72). According to the revised model of megakaryocytopoiesis (see Fig. 29.1), megakaryocyte differentiation is initiated earlier during hematopoiesis from a pool of lineage primed HSCs and/or MMPs the fate of which is differentially modulated in conditions such as steady-state, stress hematopoiesis or inflammation. In recognizing the complexity and plasticity unveiled by this emerging research, it is conceivable that the new and the canonical pathways of megakaryocytic differentiation exist in the context of different physiological or experimental conditions.

Cellular Hierarchy of Megakaryocyte Development

Like other hematopoietic progenitor cells, once committed to the megakaryocytic lineage, MkPs undergo a series of dramatic maturational steps ultimately tailored to their final task of platelet production. These include changes in proliferative capacity, cell size, nuclear content, organelle biogenesis, membrane development, and cytoskeletal rearrangement.

This process can be conceptually divided into three broad stages: proliferating MkPs, which contain normal DNA content (2 N/4 N), nonproliferating immature megakaryocytes (4 N to 8 N DNA content), and nonproliferating mature megakaryocytes (DNA content 8 N to 128 N). In vitro culture using semisolid media and mitogenic cytokine cocktails revealed that within the MkP compartment reside cells with high-proliferative-potential colony-forming cell (Mk-HPP-CFC), burst-forming unit-megakaryocyte (BFU-Mk) cells and more mature MkPs with very limited proliferative potential, the colony-forming cell-megakaryocyte (CFU-Mk). The proliferative capacity of the cells within the MkPs compartment can be demonstrated in vitro by the size of the colonies and the number of Mk-HPP-CFC, BFU-Mk, and CFU-Mk.

The immature megakaryocytes have an intermediate DNA content and are transitional cells intermediate between proliferating progenitor cells and postmitotic, mature megakaryocytes. They cease to proliferate and switch to *endomitosis*, also known as abortive mitosis, a process in which cells replicate their DNA but fail to undergo cytokinesis. This is accompanied by an increase in cell size and DNA content (up to 64 to 128 N), organelle biogenesis, membrane development, and cytoskeletal rearrangement. This culminates with the generation of mature, polyploid megakaryocytes that reach diameters of 50 to 100 μm and DNA content as high as 64 to 128 N. On BM specimens, morphologically recognizable megakaryocytes can be defined as megakaryoblasts (stage I), promegakaryocytes (stage II) and granular or "platelet shedding" megakaryocytes (stages II and IV) (Fig. 29.2).

Mature megakaryocytes contain a large multilobulated polyploid nucleus and have abundant cytoplasm, which contains platelet-specific

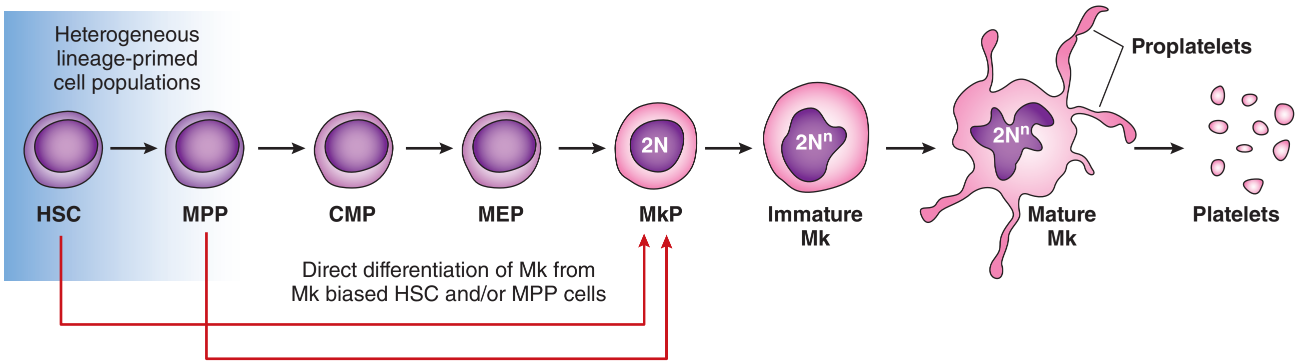

Heterogeneous lineage-primed cell populations
HSC
MPP
CMP
MEP
2N
MkP
2N^n
Immature Mk
2N^n
Proplatelets
Mature Mk
Platelets
Direct differentiation of Mk from Mk biased HSC and/or MPP cells

Figure 29.1 MEGAKARYOCYTOPOIESIS. In the classical model of megakaryocytopoiesis, hematopoietic stem cells (HSCs) give rise to multipotent progenitor cells (MPP) which sequentially transition to common myeloid progenitor (CMP) cells, common bipotential megakaryocyte-erythroid progenitor (MEP) cells and megakaryocyte progenitor (MkP) cells which undergo proliferation and maturation events to generate platelet-forming megakaryocytes (Mk). These latter events include an increase in cell size, acquisition of a polyploid DNA content (>2 N), proplatelet formation and, the release of platelets into the circulation. This model has been challenged by recent evidence indicating that the HSC and MPP cell compartments are highly heterogeneous and exist as a ”continuum” comprising cells primed towards a megakaryocyte lineage capable of generating MkP directly.

secretory granules, alpha (α-) granules, and dense granules (see Fig. 29.2).[3] The biogenesis of α-granules and dense granules begins in immature megakaryocytes, and both granule types develop concomitantly. α-Granules are 200 to 500 nm in diameter and have a dense center and fine granular matrix. Megakaryocytes synthesize many of the constituents of α-granules and target them to the granules. These include vWF, fibronectin, P-selectin, fibrinogen receptors, PF4, coagulation factor V, and plasminogen activator inhibitor-1, among others. In addition, some constituents, such as fibrinogen, are taken up by megakaryocytes via endocytosis and/or pinocytosis and stored in α-granules. It was once thought that α-granules were a homogeneous population of vesicles. However, it has become clear that there are distinct populations of α-granules containing different constituents, and that these can be differentially released during platelet activation.[4] Dense granules are 200 to 300 nm in diameter and consist of a halo encircling an electron opaque core. They contain many soluble hemostatic factors such as serotonin, catecholamines, adenosine, adenosine 5′-diphosphate, adenosine 5′-triphosphate, and calcium. Their limiting membranes contain glycoproteins such as αIIbβ3, glycoprotein Ib (GPIb), and P-selectin, which are also present in α-granules, as well as unique membrane proteins such as granulophysin. Multivesicular bodies serve as intermediates in the biogenesis of both α-granules and dense granules. It has been proposed that they constitute a sorting compartment between α-granule and dense granule components.

Mutations in the *NBEAL2* gene have recently been linked to gray platelet syndrome (GPS) (OMIM 139090), a disorder of impaired platelet α-granule synthesis. This gene encodes a large BEACH domain containing protein that shares homology with the *LYST* gene product. LYST is involved in vesicular trafficking and is mutated in Chediak–Higashi syndrome (OMIM 214500), a disorder that includes impaired platelet dense granule biogenesis (Chapter 123).

The megakaryocyte cytoplasm contains at least two complex membranous systems: the demarcation membrane system (DMS) and the dense tubular network (DTS) (see Fig. 29.2). The DMS consists of an extensive network of tubular and flattened membranous structures that interconnect with one another and communicate with the extracellular space. Whole-cell patch-clamp studies in living rat megakaryocytes show that they are electrophysiologically contiguous with the plasma membrane. The open canalicular system of platelets share many features of the megakaryocyte DMS and may represent a remnant of this structure. The DMS serves as a vast membrane reservoir for proplatelet and platelet formation. The DTS of megakaryocytes is distinct from the DMS. Unlike the DMS, it fails to stain with surface membrane tracer dyes, indicating a lack of communication with the plasma membrane. The DTS is thought to be a site of platelet prostaglandin synthesis.

Relationship Between Megakaryocytes and Hematopoietic Stem Cells

The recent work uncovering that megakaryocytes are hierarchically closer to HSCs than previously appreciated underscores the similarities between the two cell entities.[5] HSCs and megakaryocytes have common signaling pathways (TPO signaling), surface and cytoplasmic molecules expression (CD41, CD150, CXCR4, TPO receptor, von Willebrand factor), and transcription factors (RUNX1, GATA2, TAL1, ETV6, FLI1, and MEIS1). TPO signaling is not only important for megakaryocyte proliferation and development but also plays a role in HSCs survival, self-renewal, and expansion.[6] Lastly, HSCs and megakaryocytes occupy the same niche at the BM vascular sinusoids, where they physically contact one another. One recent study also suggests that megakaryocytes are necessary to maintain HSC quiescence. The teleologic explanation for such a close relationship between HSCs and megakaryocytes remains to be further explored.

UNIQUE FEATURES OF MEGAKARYOCYTES

Endomitosis and Polyploidization

The Endomitotic Cell Cycle

Megakaryocytes derive their name from their large and complex nuclei. This arises from an atypical cell cycle, termed the *endomitotic* cell cycle (Fig. 29.3) (see comprehensive review by Mazzi et al.[7]). Like normal diploid cells, the cycle begins with a G1 phase, followed by S phase (DNA replication), and a G2 phase. The cells then enter the M phase, but unlike normal diploid mitotic cells, fail to complete late cytokinesis and karyokinesis. The defective abscission leads to the accumulation of the replicated DNA in the same cell. The process is repeated in subsequent rounds of DNA replication leading to a polyploid cell with a multilobulated nucleus encapsulated by a single nuclear membrane. Mature human megakaryocytes have been observed to reach ploidy levels as high as 128 N. The term *endoreduplication* has at times been used erroneously to describe megakaryocyte polyploidization. *Endoreduplication* correctly refers to a cell cycle that involves DNA replication but no entry into the M phase enabling polyploidization in *Drosophila*, plants and trophoblasts.

Role of Endomitosis in Thrombocytopoiesis

The reason that megakaryocytes undergo endomitosis is not known. It has been speculated that it provides a means for generating the

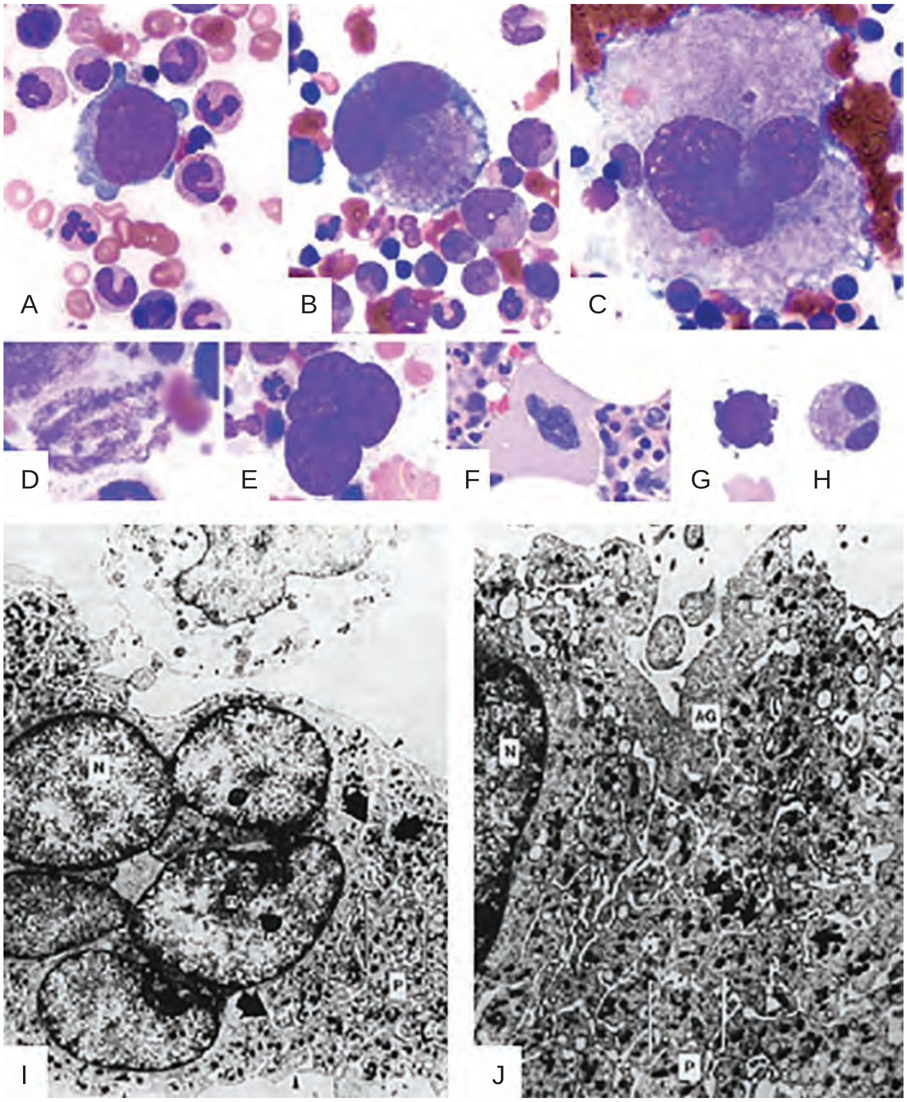


Figure 29.2 MEGAKARYOCYTOPOIESIS AND MEGAKARYOCYTES. (A) Megakaryoblast (stage I) with intermediate ploidy level. The cytoplasm is scant. Note prominent cytoplasmic pseudopods. (B) Promegakaryocyte with early platelet production (stage II). (C) Mature, high-ploidy megakaryocyte (stage III or IV) with abundant cytoplasm. Note cells traveling through the cytoplasm. This is referred to as *emperipolesis* and is not uncommonly seen in large megakaryocytes. (D) A portion of megakaryocyte cytoplasm in a long strand. Fragments of these can sometimes be seen in the blood and are referred to as *proplatelets*. (E) Megakaryocyte nucleus denuded of its platelets and cytoplasm. (F) Mature megakaryocyte seen in a tissue section of bone marrow biopsy. (G) Megakaryoblast from a patient with acute megakaryoblastic leukemia. Note cytoplasmic pseudopods. (H) Micromegakaryocyte from a patient with myelodysplasia. Note small, low-ploidy (2–4 N) nucleus, but mature cytoplasm. (I and J) Transmission electron micrographs of two stage III and IV human megakaryocytes. Openings of the demarcation membrane system (*arrowheads*). *AG*, α-Granules; *n*, nucleolus; *N*, nucleus; *P*, a platelet field within the megakaryocyte cytoplasm. ***(Courtesy** Dr. Maryann Weller.)*

abundant membrane, protein, biosynthetic cargo, and energy required for the dramatic final stages of proplatelet elaboration and platelet release. Several circumstantial pieces of evidence support this model. First, it is known that megakaryocyte DNA content correlates with megakaryocyte cell size, mRNA content, protein production, and eventual numbers of platelets released. Second, an increased DNA content of megakaryocytes precedes increases in platelet count during recovery from acute thrombocytopenia. Third, increases in cytoplasmic volume and maturation occur predominantly, if not completely, in stage II and III megakaryocytes, which do not synthesize DNA. Fourth, in polyploid megakaryocytes (4 N to 32 N), all alleles of the genes studied (i.e., ITGA2B [GPIIb], VWF, ACTB [β-actin], HSPA1 [HSP70], MPL, FLI1, and ZFPM1 [FOG-1]) have been found to be transcriptionally active.

Mechanisms of Endomitosis in Megakaryocytes

The molecular mechanisms mediating endomitosis in megakaryocytes are incompletely understood. Studies investigating endomitosis have been hampered by the rarity of megakaryocytes, difficulty separating direct effects from general perturbations of cell maturation, complications associated with synchronizing the cell cycle, use of transformed cell lines, and potential differences between rodent and human megakaryocytes.

Cyclins and Cyclin-Dependent Kinases

Two classes of proteins control the cell cycle in mammalian cells. These are the cyclins, so named for their cyclical synthesis and degradation during the cell cycle, and cell division kinases (Cdks, also

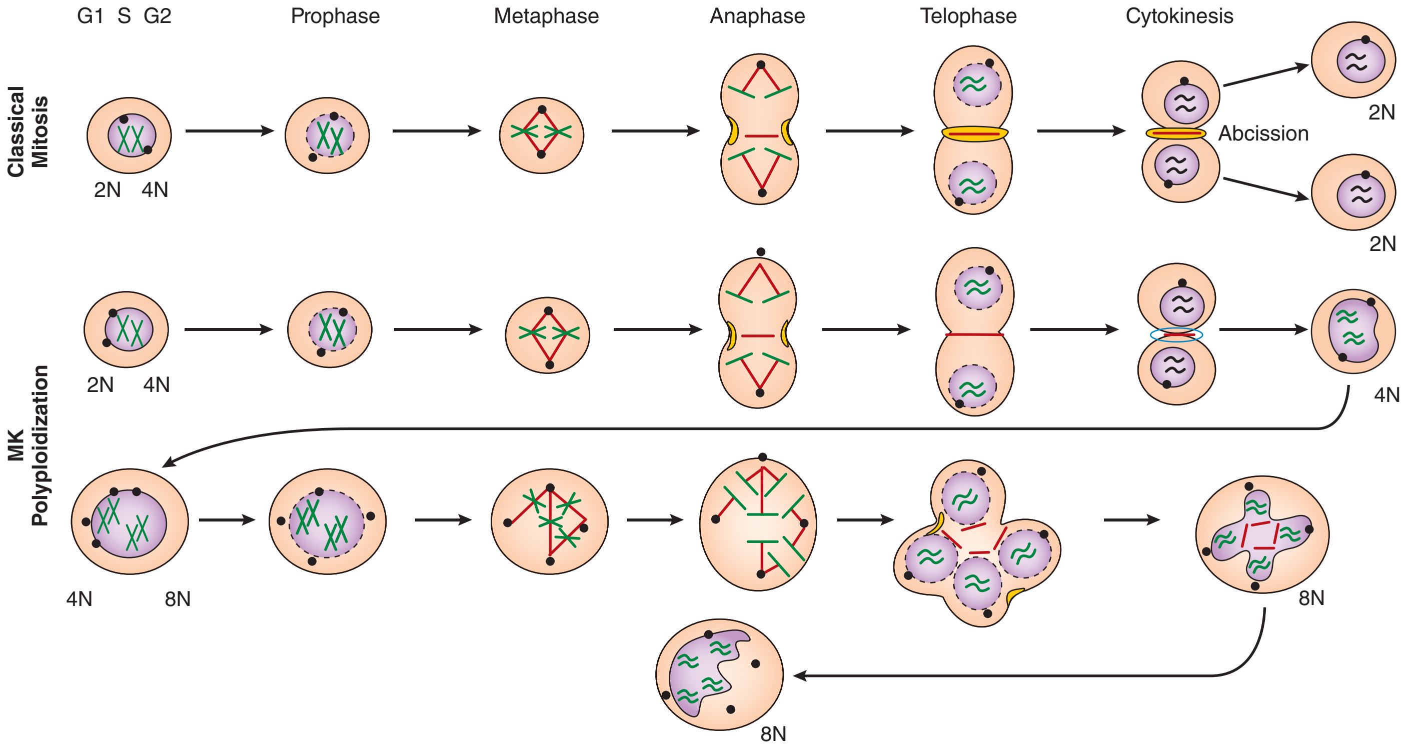


Figure 29.3 THE ENDOMITOTIC CELL CYCLE IN MEGAKARYOCYTES. Schematic diagram depicting stages of the classical mitotic cell cycle (*top*) and endomitotic cell cycle leading to megakaryocytes polyploidization (*bottom*). Like normal mitosis, megakaryocytes enter mitosis and proceed normally through metaphase, anaphase, telophase but fail to complete cytokinesis. This "aborted" cytokinesis is due to abnormal accumulation of actomyosin at the cleavage furrow which prevents abscission or the separation of the two daughter cells. A single nuclear membrane is thus formed enclosing the replicated DNA after each round of endomitosis. Chromosomes are depicted in *green*, mitotic spindles are shown as *red lines* and centrosomes are shown as *black dots*. *(Adapted with permission from Mazzi S, Lordier L, Debili N, et al. Megakaryocyte and polyploidization. Exp Hematol. 2018;57:1–13.)*

known as *cyclin-dependent kinases*). Together, these two families of proteins form a protein-kinase complex in which the regulatory unit is the cyclin and the catalytic unit is the Cdk. The role of these kinase complexes in cell cycle control is complex. At least seven members of the cyclin gene family and seven distinct *Cdk* genes have been identified.

Given the importance of cyclins and Cdks in controlling the cell cycle, they have been the focus of considerable attention in investigations of the mechanisms underlying megakaryocyte endomitosis. The most compelling evidence probably exists for the role of the D-type cyclins in megakaryocyte endomitosis. The D-type cyclins are unique in that their activity can be modulated by extracellular mitogens. Megakaryocytes express cyclin D3 and, to a lesser extent, cyclin D1. Levels of both of these factors increase after treatment with TPO. Overexpression of cyclin D3 results in increased megakaryocyte ploidy in transgenic mouse models. Complexes of cyclin D3 and its major kinase subunit, Cdk2, show high kinase activity in polyploid cells. Antisense knockdown of cyclin D3 levels suppresses endomitosis and abrogates normal development of primary mouse megakaryocytes.

Cyclin D1 is a direct target gene of GATA1, a transcription factor required for megakaryocyte polyploidization and maturation. Overexpression of cyclin D1 in transgenic mice increases megakaryocyte modal ploidy compared with nontransgenic littermates, and the combination of cyclin D1 and Cdk4 kinase activity restores polyploidization of GATA1-deficient murine megakaryocytes. Conversely, enforced expression of $p16^{ink4a}$, a cell cycle inhibitor of Cdk4/6, blocks polyploidization in murine megakaryocytes. $p16^{ink4a}$ is also potently repressed by GATA1.

Cyclin $E^{-/-}$ mice have impaired megakaryocytopoiesis with reduced modal ploidy. These mice also have defective trophoblast development, another tissue characterized by atypical mitosis. Cyclin B1/CDC2 is a mitotic cyclin complex. Yeast strains deficient in cyclin B1 or CDC2 undergo an additional round of DNA replication without cytokinesis. Several studies have shown that low levels of cyclin B1/CDC2 are required for the progression of endomitosis in megakaryocytic cell lines. However, studies of primary megakaryocytes have shown normal cyclin B1 and CDC2 levels and functional mitotic activity during endomitosis.

Other Mitotic Kinases

Aurora-B kinase (also called *AIM-1 kinase*) is involved in late anaphase and cytokinesis, and mRNA transcript levels of Aurora-B kinase have been reported to decrease during polyploidization of primary megakaryocytes and megakaryocytic cell lines. This suggests that Aurora-B kinase may play a mechanistic role in megakaryocyte endomitosis. However, functional activity of Aurora-B kinase appears normal in late anaphase of endomitotic primary megakaryocytes, indicating that a simple deficiency of Aurora-B kinase activation is an unlikely mechanism to explain endomitosis. A polo-like kinase (PLK-1) is a serine-threonine kinase required for assembly of the mitotic spindle, separation of chromosomes during anaphase, and exit from mitosis. PLK-1 mRNA and protein levels decrease during polyploidization of murine megakaryocytes, and enforced expression of PLK-1 in primary murine megakaryocytes impairs endomitosis. However, the effects of overexpression are modest, preferentially affect lower-ploidy megakaryocytes, and are complicated by alterations in cell cycle kinetics.

Microtubules and the Mitotic Spindle

Microtubules play key roles in mitosis by enabling the formation of the mitotic spindle. Therefore, factors that regulate their assembly have also been investigated as candidates involved in megakaryocyte endomitosis. The protein regulator of cytokinesis 1 (PRC-1) is involved in mitotic spindle elongation and cytokinesis. However, no differences in PRC-1 levels were detected in primary murine megakaryocytes undergoing polyploidization compared with nonendomitotic precursors. Stathmin is a microtubule-depolymerizing factor that plays an important role in the regulation of the mitotic spindle. Levels of stathmin are inversely related to the level of ploidy of megakaryocytic cell lines and primary megakaryocytes. Inhibition of stathmin in K562 cells increases their propensity to undergo endomitosis when induced to differentiate into megakaryocytes, and overexpression of stathmin prevents the transition from mitotic to endomitotic cell cycles. Conversely, lentiviral-mediated overexpression of stathmin in primary megakaryocytes delayed cytoplasmic maturation and impaired their ability to achieve high levels of ploidy. Together, these findings support a possible role of stathmin in modulating endomitosis.

The Spindle Checkpoint. During mitosis of normal diploid cells, a spindle assembly checkpoint prevents the progression of anaphase until all of the chromosomes are aligned with the mitotic spindle and each sister chromatid is properly attached to spindle microtubules originating from the opposing spindle pole. This ensures that each daughter cell receives the proper complement of chromosomes. The anaphase-promoting complex (APC) is a multisubunit protein complex with ubiquitin ligase activity that regulates chromosome segregation and anaphase progression by targeting key factors for degradation. Since some chromosomal missegregation occurs during megakaryocyte endomitosis, several groups have examined the expression levels and/or activity of certain APC components and associated factors. These studies have shown no significant difference in protein levels of the core APC protein CDC27 or the kinetochore-associated signaling protein hsMAD2 in primary murine megakaryocytes undergoing polyploidization compared with nonendomitotic precursors. Haploinsufficiency of BUBR1, a key component of the spindle checkpoint, perturbs megakaryocyte development and polyploidization in mice but does not cause alterations in circulating platelet counts.

Contractile Ring Activity

Cytokinesis requires the assembly and activity of an actin-based contractile ring which enables cell separation. This process is regulated by RhoA involving actin polymerization, myosin II accumulation, and activation which provide the contractile forces for abscission. The failure to complete cytokinesis during endomitotic cell cycles in megakaryocytes involves functional and quantitative defects in the RhoA, Rock, F actin, and myosin II. Silencing of nonmuscle myosin heavy chain IIB (Myh10) by the transcription factor RUNX1 is also required for efficient megakaryocyte polyploidization. Further studies will be required to fully dissect the molecular pathways involved in megakaryocyte endomitosis.

Platelet Biogenesis

It has been estimated that each megakaryocyte produces between a few hundred to several thousand platelets. The exact mechanism by which this occurs has been controversial, with several competing models proposed in the past. It was initially suggested that the DMS established platelet fields, which defined territories of prepackaged platelet contents. These fields would generate platelets directly upon the breakdown of the megakaryocyte cytoplasm. However, prevailing evidence supports an alternate model in which platelets are released from dynamic megakaryocyte pseudopod extensions called *proplatelets*. This model was first proposed by Becker and De Bruyn[8] in 1976 and supported by ultrastructural studies later in the 1980s. Italiano et al.,[9] extended these earlier studies on proplatelet formation and platelet biogenesis using the videomicroscopy of cultured murine megakaryocytes (see Chapter 123). Briefly, platelet biogenesis begins with a reorganization of unique cortical microtubules within the megakaryocyte to produce large pseudopodia structures from one pole of the megakaryocyte. This spreads across the megakaryocyte, generating extensions that elongate into complex branching tubular proplatelet processes. During this time, organelles travel along microtubules within the shafts of the proplatelets and are loaded into the proplatelet tips where they are captured.[10] This phenomenon likely accounts for the ability of each megakaryocyte to generate such a large number of platelets. The DMS serves as an extensive membrane reservoir for these processes. Proplatelet formation is regulated by a pathway involving Rho GTPase proteins, Rho-associated kinase (ROCK), the *MYH9* gene product myosin IIA, and myosin light-chain kinase.

Megakaryocytopoiesis in Ontogeny

Hematopoiesis develops in distinct waves during embryonic development. In mammals, the first hematopoietic progenitors are found inside blood islands of the yolk sac. These give rise to a distinct population of large erythrocytes, termed *primitive erythrocytes*, which express unique globin genes and retain their nucleus longer than adult-type or ”definitive” erythrocytes. ”Definitive” hematopoiesis arises later during embryogenesis from HSCs that develop de novo from the ventral aspect of the dorsal aorta in the aorto-gonad-mesonephros (AGM) region. These then seed the fetal liver, which serves as a major site of hematopoiesis during gestation. Eventually, hematopoiesis shifts to the BM (and spleen in mice), where it is sustained postnatally.

MkPs have been detected in the yolk sac as early as embryonic day 7.5 (e7.5) of mouse development. They are capable of generating proplatelets and platelets after in vitro culture. Circulating platelets have been detected in the mouse embryo as early as e10.5. Megakaryocytes cultured from early yolk sac have features somewhat distinct from those cultured from adult BM, such as lower modal ploidy, smaller size, different cytokine requirements, and faster kinetics of platelet generation. These unique progenitors disappear by e13.5. In addition, mixed erythroid-megakaryocyte colonies derived from the early yolk sac give rise to primitive erythrocytes. It has therefore been suggested that a separate wave of ”primitive megakaryocytes,” akin to ”primitive erythrocytes,” exists during the early yolk sac stages of hematopoiesis. These rapidly maturing megakaryocytes may prevent hemorrhages from the developing vasculature until definitive hematopoiesis is available to provide a steady supply of platelets.

Several pieces of evidence suggest that fetal megakaryocytes also have unique features compared with adult BM-derived megakaryocytes.[11] They are smaller, have a greater proliferative capacity in vitro, have lower ploidy and form fewer platelets. This could be caused by either cell-intrinsic differences or possibly their interactions with a distinct microenvironment. The developmental differences between fetal and adult megakaryocytes are reflected by disease conditions that affect neonates.[12] In response to increased platelet demand, adult megakaryocytes increase their number, size, and ploidy which replenish the platelet counts. By contrast, neonatal megakaryocytes are not “equipped” to respond to increased platelet demand in the same manner which is believed to contribute, at least in part, to neonatal thrombocytopenia. Two congenital disorders of megakaryocytopoiesis in humans, Down syndrome-associated transient myeloproliferative disorder (DS-TMD) and thrombocytopenia with absent radii (TAR) resolve spontaneously after the newborn period, suggesting specific effects on fetal megakaryocytopoiesis. Congenital amegakaryocytic thrombocytopenia (CAMT) is a disorder in which megakaryocytes are absent or present in very low numbers due to mutations in the gene encoding for c-MPL which is the receptor for TPO, the master regulator of megakaryocytopoiesis. In adults, this defect is consistently associated with severe thrombocytopenia, while in infants it coexists in some cases with relatively normal platelet counts or in other cases with the appropriate number of megakaryocytes in the BM. These observations and recent findings in murine models indicate that developmental differences exist in the response to abnormal c-MPL/TPO signaling in neonatal and adult megakaryocytes.

The features of fetal megakaryocytes have additional clinical implications including neonatal thrombocytopenia and the delayed platelet engraftment often observed when umbilical cord blood is used as a graft source for human stem cell transplantation.

Conversely, due to their proliferative potential, neonatal megakaryocytes represent a valuable cell source for expansion and manipulation towards ex vivo manufacturing of megakaryocyte or platelet-based cellular therapies.

Tissue Distribution and Spatial Cues During Megakaryocytopoiesis

There is mounting evidence that the proliferation and terminal maturation of MkPs occur in distinct spatial compartments within the BM (see Chapter 14). In a simplified model, the BM space can be conceptually divided into distinct regions, a space adjacent to the cortical bone (an "osteoblastic niche"), an intermediate zone, and a "vascular niche" containing sinusoidal vessels lined with specialized BM endothelial cells. HSCs are thought to reside in a quiescent state adjacent to the bone. Under appropriate conditions, they are recruited to generate hematopoietic multipotent progenitor cells which are then subject to expansion and lineage commitment under the influence of various cytokines and likely other signaling molecules. This is where TPO is postulated to affect MkP proliferation and survival. Rafii and colleagues have shown that the chemokines stromal-derived factor-1 (SDF-1; also called CXCL12) and fibroblast growth factor-4 (FGF-4) promote migration and attachment of murine MkP cells (which express the receptor for SDF-1, CXCR4) to the vascular endothelium, where they physically attach, mature, and produce intercalating pseudopod structures.[13] In fact, exogenous SDF-1 and FGF-4 restores thrombocytopoiesis in TPO$^{-/-}$ or TPO receptor (c-Mpl)$^{-/-}$ mice to near wild-type (WT) levels. This occurs in the absence of enhanced MkP proliferation and requires direct physical interaction with BMECs. Based on these findings, Avecilla et al., have proposed a model in which MkPs proliferate in an immature developmental state (in response to TPO) in a nonvascular niche (Fig. 29.4). However, once the progenitors reach and adhere to the sinusoidal vessels in the vascular niche in response to chemokines, proliferation ceases, and terminal maturation and platelet release ensue. Work from other investigators supports this model. Multiple electron microscopic studies have captured megakaryocytes extending proplatelet processes through vascular endothelium and into BM sinusoids, and in vivo imaging studies have documented this process in living mice.[14]

In addition to the BM, the lung and the spleen represent important sites of residence for megakaryocytes. Historically, the presence of platelet-forming megakaryocytes in the lung have been documented in humans as early as 1893, described in detail by Howell and Donahue in 1936[15] and later confirmed by several reports. The significance of such observations has been controversial but recent evidence confirms that the lung is not only a tissue of residence for megakaryocytes but a site conducive to platelet biogenesis. It has been postulated that two types of megakaryocytes exist in the lung; those residing in the pulmonary interstitium and those originating in the BM which are present in the lung vasculature where they release platelets. It remains debatable whether lung-based thrombocytopoiesis is a physiological process, driven by increased platelet demand or both. Furthermore, it is not clear to what degree lung-generated platelets contribute to the total pool of circulating platelets.[16]

Megakaryocytes are normally present in the spleen in rodents where the spleen remains a site of hematopoiesis throughout adult life. In humans, the presence of megakaryocytes in the spleen is a consequence of pathological conditions such as extramedullary hematopoiesis (EMH). Prototypical EMH occurs in primary MF which is also characterized by abnormal and hyperplasic megakaryocytes (Chapter 72).

Therapeutic Applications of Ex Vivo Megakaryocytopoiesis

The increased understanding of the biological events underlying the generation of platelets from megakaryocytes led to the development of novel strategies that might eventually supplement or replace donor-derived platelet transfusions. Ex vivo manufacturing of platelets from primary HSCs or induced pluripotent stem cells (iPSCs) has been pursued and near-clinical doses of platelets have been achieved by utilizing highly optimized ex vivo cultures and/or state-of-the-art bioreactors. In addition, megakaryocyte-biased cellular products that have the ability to naturally release platelets in the patient's own body after infusion has been proposed to mitigate repeated platelet transfusions.[17] Clinical implementation of these approaches has the potential to overcome the limitations associated with donor-dependent platelet transfusions and mitigate the increasing demand for donor platelet transfusions which is compounded by dependency on volunteer donors, short storage time, and lifespan after infusion as well as shortages in supply due to inclement weather and holidays.

Furthermore, gene therapy of megakaryocytes derived ex vivo from CD34$^+$ HSCs or iPSCs has also been explored with the goal of correcting inherited bleeding disorders such as Glanzmann thrombasthenia, hemophilia or Bernard-Soulier syndrome.

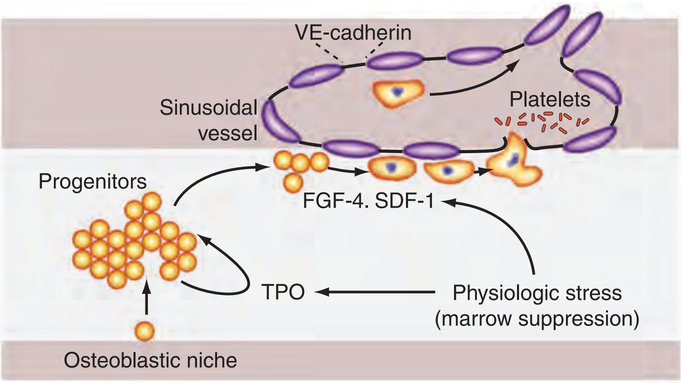


Figure 29.4 MODEL OF TERMINAL MATURATION OF MEGAKARYOCYTES AT THE BONE MARROW VASCULAR SINUSOID. Schematic diagram showing hematopoietic stem cells located predominantly adjacent to the cortical bone ("osteoblast niche"), megakaryocyte progenitors proliferating in the bone marrow space under the influence of thrombopoietin (TPO), and migration of progenitor cells to the vascular sinusoid ("vascular niche") under the influence of chemokines such as stromal-derived factor 1 (SDF-1) and fibroblast growth factor 4 (FGF-4). Once attached to the sinusoidal vascular endothelium, progenitor cells cease proliferating, undergo terminal maturation and proplatelet formation, and shed platelets into the vascular sinusoidal space. *(Reproduced with permission from Avecilla ST, Hattori K, Heissig B, et al. Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. Nat Med. 2004;10:64.)*

CYTOKINE REGULATION OF THROMBOCYTOPOIESIS

Thrombopoietin Signaling

Thrombopoietin

It has been estimated that an adult human produces nearly 2×10^{11} platelets per day, and this number can increase fourfold to eightfold during times of increased demand. The regulation of this process has been the subject of intense investigation. Kelemen first used the term *thrombopoietin* in 1958 to describe a humoral substance responsible for enhancing platelet production following the onset of thrombocytopenia.[18] However, it was not until 1994 that five independent groups succeeded in purifying and cloning the responsible cytokine, now known as TPO (previously referred to as *cMpl ligand, megakaryocyte growth and development factor [MGDF]*, and *megapoietin*).[19] The gene for TPO is located on chromosome 3q27. It encodes a 30 kDa glycoprotein of 353 amino acids that can be divided into two structural domains: an amino-terminal region with homology to human erythropoietin (EPO), and a carboxyl-terminal region that contains multiple N- and O-linked oligosaccharides. The amino terminal 155 residues of human TPO share 21% sequence identity and 46% overall sequence similarity to human EPO. This region mediates binding to the TPO receptor (c-Mpl). The carboxyl region does not share sequence homology with any known protein. TPO is reported to enhance multiple stages of megakaryocyte maturation, including cell size, cell ploidy, and platelet production. The predominant sites of TPO production are the liver and kidney, which secrete it in a generally constitutive fashion. Expression of TPO has been detected by more sensitive methods in BM stroma and spleen in the setting of thrombocytopenia, although this likely accounts for only a minor fraction of total TPO production. A low-level expression has also been reported in the amygdala and hippocampus of the brain.

Thrombopoietin Receptor (c-Mpl)

The receptor for TPO (TPO receptor; c-Mpl) is the normal homologue of the oncogene *v-Mpl*, the transforming gene of murine myeloproliferative leukemia virus. It is a 635 amino acid protein that contains a number of distinct functional domains: a 25-amino acid signal peptide, a 465 amino acid extracellular domain, a 22-residue transmembrane domain, and an intracellular domain that contains two conserved motifs, termed Box 1 and Box 2 (Fig. 29.5). The extracellular domain contains a distal region that negatively influences TPO signaling. It is a member of the type I cytokine receptor superfamily. Like the EPO receptor, it is thought to function as a homodimer. The TPO receptor is expressed on MkPs, as well as earlier multipotential progenitors, including MEPs, CMPs, and HSCs. TPO receptors are present on the surface of platelets at an estimated density of 20 to 200 receptors per platelet and bind TPO with an affinity of 200 to 560 pM. The binding of TPO to platelets plays an important role in the regulation of total body platelet mass by the TPO–TPO receptor system. Both TPO receptor$^{-/-}$ (c-Mpl$^{-/-}$) and TPO (TPO$^{-/-}$) knock-out mice contain ≈85% to 90% lower platelet and megakaryocyte numbers as compared with WT mice. The structure of the megakaryocytes and platelets in these animals is normal, reinforcing the notion that TPO signaling plays an important role in the expansion and development of MkPs, but not in terminal maturation and proplatelet release. In addition, the residual platelet production in these mice suggests alternate cytokine, or possibly cytokine-independent, pathways for thrombocytopoiesis. Interbreeding experiments of TPO receptor$^{-/-}$ mice with knock-out mice for IL-3, IL-6, IL-11, or leukemia inhibitory factor (LIF) or their receptors, show that these other cytokines are not responsible for the residual platelet production.

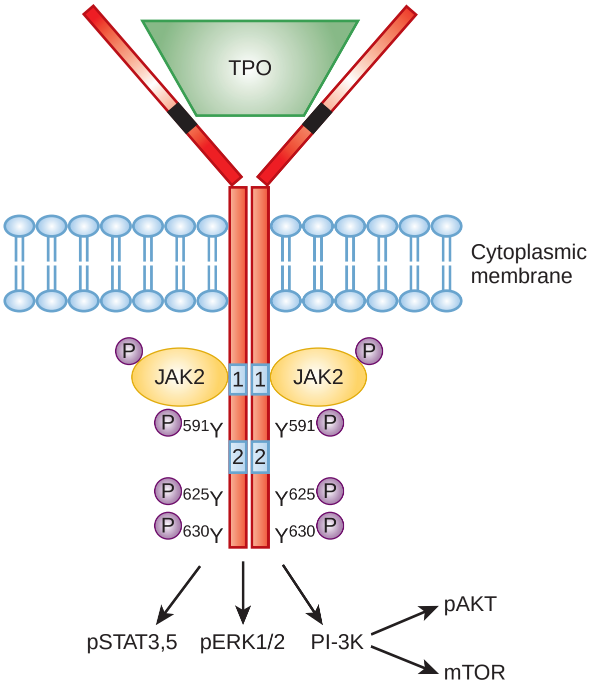


Figure 29.5 THE THROMBOPOIETIN RECEPTOR. Schematic diagram of the thrombopoietin (TPO) receptor depicted as a homodimer with TPO bound. The binding of Janus-kinase 2 (JAK2) at Box 1 of the cytoplasmic tail is shown. Conformational changes in the TPO receptor upon TPO binding result in a juxtaposition of the two cytoplasmic tails, as well as JAK2 autophosphorylation and JAK2-mediated phosphorylation of the c-Mpl cytoplasmic tail (Tyr^{591}, Tyr^{625}, and Tyr^{630}). Activation of signal transducers and activators of transcription, ERK, phosphoinositol-3 kinase (PI-3K)-Akt, and PI3K-mTOR signaling pathways then occurs. *mTOR*, mammalian target of rapamycin; *STAT*, signal transducers and activators of transcription. *(Reproduced with permission from Geddis AE. Megakaryopoiesis. Semin Hematol. 2010;47:212.)*

Thrombopoietin Receptor Downstream Signaling Pathways

The TPO receptor lacks intrinsic tyrosine kinase activity. Instead, ligand binding is thought to induce a conformational change in the homodimeric receptor and stimulates the cytoplasmic tyrosine kinase Janus-kinase 2 (JAK2), which binds to Box 1 of the cytoplasmic tail. This results in tyrosine phosphorylation of multiple targets, including signal transducers and activators of transcription (STATs), Shc adaptor protein, and the TPO receptor itself (Tyr^{591}, Tyr^{625}, and Tyr^{630}). Additional signaling pathways activated upon TPO receptor engagement include the mitogen-activated protein kinase (MAPK) p38, p42/p44 extracellular signal-regulated kinase 1 (ERK1/ERK2), phosphoinositol-3-kinase-AKT (PI3K-AKT), and PI3K-Mammalian target of rapamycin (mTOR) signaling pathways.

Several of these downstream signaling pathways have been shown to be functionally important in TPO-mediated effects on megakaryocytopoiesis. Double STAT5a/STAT5b–deficient mice have impaired platelet production as well as defects in early multipotent progenitor cells. Moreover, megakaryocyte-selective overexpression of a dominant-negative mutant STAT3 in transgenic mice reduces platelet recovery following 5-fluorouracil–induced myelosuppression. These findings suggest a functional role for STAT family members in thrombocytopoiesis.

Studies in primary megakaryocytes show a requirement for PI3-AKT signaling in TPO-induced cell cycling. This involves the silencing of the Forkhead O family of transcription factors. Activation of the p42/p44-MAPK plays an important role in TPO-induced maturation and endomitosis. The mTOR signaling pathway is involved in TPO-mediated megakaryocytic progenitor proliferation and possibly terminal megakaryocyte size determination, ploidy, and cellular maturation.

Negative Regulation of Thrombopoietin Signaling

As with other receptor-mediated signaling processes, feedback mechanisms exist to limit or turn off the signal once initiated to avoid uncontrolled growth. Lnk, an adaptor protein implicated in immunoreceptor and cytokine receptor signaling negatively modulates TPO signaling in megakaryocytes. Overexpression of Lnk decreases TPO-dependent megakaryocyte growth and polyploidization in BM-derived cultures. Conversely, loss of Lnk expression by gene targeting results in increased numbers of megakaryocytes, accentuated megakaryocyte polyploidization, and a myeloproliferative disorder in mice. This correlates with enhanced and prolonged TPO-mediated induction of STAT3, STAT5, AKT, and MAPK signaling pathways.

Following TPO binding, the TPO receptor is internalized and subsequently degraded. This process depends on dileucine repeats, and Tyr^{591} and Tyr^{625} within the TPO receptor cytoplasmic tail, and involves ubiquitinylation via the E3 ubiquitin ligase c-Cbl.

Regulation of Platelet Mass by Thrombopoietin

Platelet counts are typically held at a relatively fixed level in humans, ranging from 150,000 to 400,000/mm^3. The maintenance of platelet numbers by the TPO-TPO receptor system involves an unusual homeostatic mechanism among hematopoietic cytokine-mediated regulation. This is sometimes referred to as the "sponge" model (Fig. 29.6). Unlike other cytokines, TPO is secreted predominantly in a constitutive manner, mostly from the liver and kidney. High-affinity TPO receptors present on the platelet surface bind free TPO and internalize it, where it is degraded. Therefore, when platelet counts are low, less TPO is removed, and more are available to stimulate megakaryocytopoiesis in the BM. Conversely, when platelet counts rise above a given set point, they act as a "sink" for TPO, binding and destroying it before it can stimulate megakaryocytopoiesis in the BM. Thus total platelet mass is preserved, rather than absolute platelet numbers. This may explain the mild to moderate thrombocytopenia seen in certain disorders associated with large platelets, such as Bernard–Soulier syndrome.

Several pieces of evidence support this model. First, it has been known for over 40 years that the peripheral blood platelet count varies inversely with plasma TPO activity. Second, TPO receptor-deficient mice (c-Mpl$^{-/-}$) have elevated levels of circulating TPO, and this is reduced when the mice are transfused with washed platelets from normal mice. Third, in contrast to platelets from TPO receptor-deficient mice, platelets from normal mice bind purified radiolabeled TPO and degrade it. Fourth, TPO levels are low to intermediate in normal individuals and in those with idiopathic thrombocytopenic purpura (where the bound TPO is destroyed along with the platelets). However, following chemotherapy, or in individuals with aplastic anemia, levels are markedly elevated.

Although the model described above likely explains the predominant basal regulation of platelet number by the TPO–TPO receptor signaling system, overlying inducible mechanisms also probably exist. It has been shown that the *TPO* gene is transcriptionally activated in BM stroma and spleen during times of thrombocytopenia, although the degree to which this may contribute to total TPO levels is uncertain. In addition, IL-6 mediates upregulation of hepatic TPO mRNA transcripts in inflammation-related thrombocytosis. Recent work shows that binding of desialylated platelets to the hepatic Ashwell-Morell receptor triggers *TPO* gene transcription and protein production via a JAK 2/STAT3 pathway, linking platelet turnover directly to TPO production.[20]

Thrombopoietin Signaling in Disease Conditions

Congenital Amegakaryocytic Thrombocytopenia (Chapter 30)

Biallelic mutations in the TPO receptor gene cause CAMT (OMIM 604498). In this disorder, megakaryocytes are absent or greatly diminished in number in the BM. Patients typically present shortly

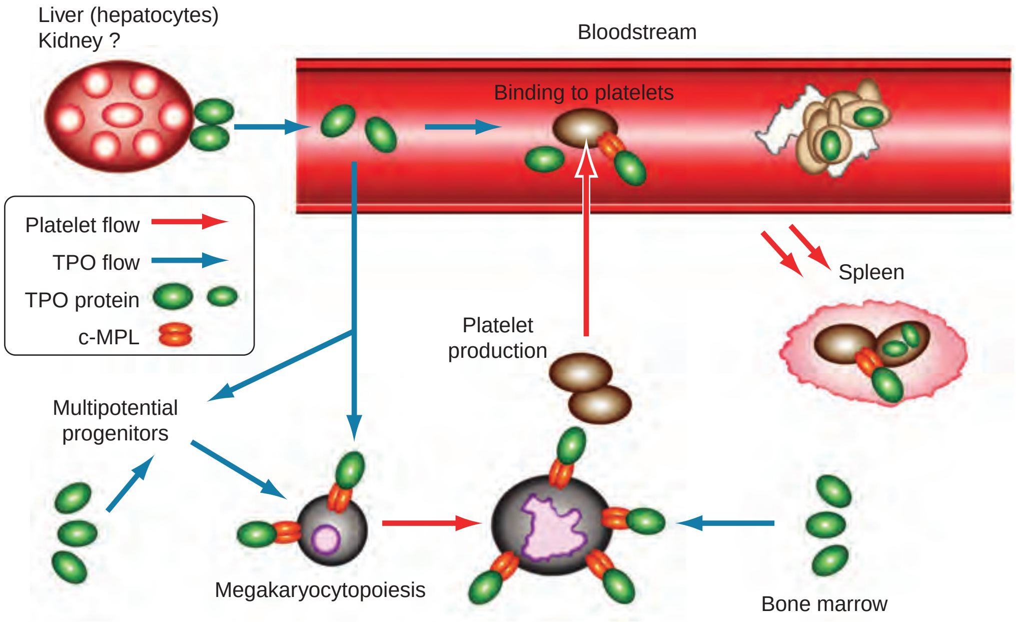


Figure 29.6 REGULATION OF PLATELET COUNT BY THROMBOPOIETIN (TPO): THE "SPONGE" MODEL. TPO is secreted at a constitutive rate primarily in the liver, and perhaps other sources such as the kidney, into the circulation. There it binds with high affinity to TPO receptors (c-Mpl) present on the surface of platelets. The TPO is then internalized by the platelets and degraded. Free TPO (i.e., TPO not bound to platelets) enters the bone marrow and stimulates megakaryocytopoiesis. Thus in the presence of high platelet counts, little free TPO is available to stimulate megakaryocytopoiesis. Conversely, low platelet numbers lead to increased free TPO and active megakaryocytopoiesis. The net result is the preservation of total platelet mass.

after birth with petechiae, bruising, or bleeding. Patients with severe CAMT are at high risk for developing progressive BM failure, typically within the first few years of life. Recently, biallelic loss-of-function mutations in the TPO gene (*THPO*) have also been linked to BM failure. These observations are consistent with the role of TPO signaling in maintaining HSCs and/or multipotential progenitor cells. It should also be noted that in contrast to humans, TPO receptor$^{-/-}$ (as well as TPO$^{-/-}$) mice do not develop BM failure states. The reason for this discrepancy is not known, but it highlights important differences between human and mouse hematopoiesis.

Essential Thrombocythemia (Chapter 71)

Essential thrombocythemia (ET) is a chronic myeloproliferative neoplasm (MPN) associated with sustained excessive megakaryocyte hyperproliferation, thrombocytosis, and abnormal platelet function leading to either hemorrhage or thrombosis (see Chapter 71). Mutation in c-MPL, Janus activated kinase (JAK2V617F) and calreticulin (CALR) are major drivers of transformation in ET and its related MPNs, polycythemia vera (PV) and MF. While c-MPL mutations are relatively rare, V617F JAK2 mutations are found in ~50% of patients with ET, ~95% of patients with PV, and ~50% of patients with MF. Mutations involving exon 9 of *CALR* gene were identified in 67% to 88% of cases of MPN which do not carry V617F JAK2 or MPL mutations, particularly ET and MF. Recently, several lines of evidence have established that all three driver mutations activate TPO-c-mpl signaling axis and its downstream pathways (Chapters 71 and 72).[21]

Mutations leading to constitutive activation of the TPO receptor or enhanced translation efficiency of the *TPO* gene have also been reported in rare cases of familial thrombocytosis (Chapter 71). These two classes of disorders can be distinguished by measuring circulating TPO levels, which are elevated with mutations enhancing TPO mRNA translation efficiency, and decreased with mutations leading to constitutive TPO receptor activation.

Additional Cytokines Involved in Megakaryocytopoiesis

Although TPO is the major cytokine regulating megakaryocytopoiesis, other cytokines have been shown to be active in vitro and in vivo at various stages of megakaryocyte development (see comprehensive review by Behrens and Alexander[22]). These include SCF, IL-3, IL-6, Il-9, IL-11, FL, LIF, GM-CSF, VEGF, and IGF-1 which were shown to play a role in steady-state megakaryocytopoiesis. Other soluble factors such as SDF-1 and FGF play a role in megakaryocyte migration and interaction with the BM niche. A number of cytokines, including IL-1a and Angptl4, have been implicated in stress megakaryocytopoiesis. None of these factors is megakaryocyte-specific but act as synergistic coregulators with TPO. Only SCF and TPO have been shown to affect megakaryocyte development and platelet production in vivo using genetic ablation experiments in mice.

Therapeutic Cytokine Stimulation of Megakaryocytopoiesis

Since the identification of TPO as a major activator of megakaryocyte growth and maturation, there has been considerable interest in developing recombinant forms of TPO for clinical use in the treatment of chemotherapy-related thrombocytopenia and immune-mediated thrombocytopenia. Small pilot studies using a polyethyleneglycol (PEG)-related, truncated form of human TPO (PEG-MGDF) showed activity in stimulating megakaryocyte growth and maturation, resulting in elevated platelet counts. However, some recipients subsequently developed thrombocytopenia as a result of the generation of a neutralizing anti-TPO antibody that cross-reacted with endogenous TPO. The agent was, therefore, withdrawn from further testing. Since then, several nonimmunogenic thrombopoietic peptides and small, nonpeptide molecules have been developed. Romiplostim (formerly called *AMG 531*), a synthetic molecule consisting of an immunoglobulin Fc domain fragment linked to two identical peptide chains that activate the TPO receptor, stimulates platelet production and has been approved by the US. Food and Drug Administration (FDA) for the treatment of adults with chronic immune thrombocytopenia purpura (ITP). It is given intravenously or subcutaneously. Eltrombopag, an orally administered small molecule that binds to a portion of the TPO receptor distinct from the normal TPO binding site, also stimulates thrombocytopoiesis and is FDA approved for the treatment of chronic ITP. It has also been shown to improve trilineage hematopoiesis in refractory aplastic anemia, likely through its effect on HSC TPO receptor signaling pathways (Chapters 31, 128, and 129). Second generation TPO agonists such as Avatrombopag and Lusutrombopag have been recently approved by the FDA for the treatment of thrombocytopenia in adults with chronic liver disease.

IL-11 has multiple effects on in vivo and in vitro megakaryocytopoiesis. It affects IL-3–dependent megakaryocyte colony formation and has a potent effect on megakaryocyte maturation. Administration of recombinant IL-11 to mice results in increased numbers of MkPs, increased megakaryocyte polyploidization, and increased peripheral platelet counts. Recombinant IL-11 has also been approved for use in humans for the treatment of chemotherapy-induced thrombocytopenia.

TRANSCRIPTIONAL CONTROL OF MEGAKARYOCYTOPOIESIS

Since platelets do not contain nuclei, all transcriptional regulation of platelet-specific genes must occur at the level of the megakaryocyte. Significant strides have been made in identifying key transcription factors involved in megakaryocyte development and platelet-specific gene expression. Importantly, mutations in a large number of these factors are linked to various human thrombocytopoiesis disorders providing significant new insights into the pathogenesis of these diseases (see box on Inherited Causes of Thrombocytopenia).

INHERITED CAUSES OF THROMBOCYTOPENIA

Although the most common cause of thrombocytopenia is immune thrombocytopenia purpura (ITP), it is important to maintain a high index of clinical suspicion for inherited disorders of thrombocytopoiesis. This is a particular problem because ITP is essentially a diagnosis of exclusion, and many inherited disorders mimic the macrothrombocytopenia seen in ITP. Making the correct diagnosis early is paramount, since it may spare patients unnecessary treatment with corticosteroids, other immunosuppressants, and/or splenectomy. In addition, it may be important in guiding decisions about surveillance for myelodysplasia or leukemia, screening for additional associated clinical problems, and/or possible family planning. Obtaining a careful family history, and sometimes obtaining blood counts of first-degree relatives, is important in fully evaluating patients with chronic thrombocytopenia. Associated abnormalities may provide important clues to the presence of nonimmune familial thrombocytopenia. For instance, associated erythroid abnormalities and/or an X-linked inheritance pattern (GATA1, FLNA, WASP mutations) (obligate female carriers may have dimorphic populations of platelets); leukocyte Döhle bodies, +/− nephritis, sensorineural hearing loss, and early-onset cataracts (Myh9 mutations); family history of myelodysplasia or myeloid leukemia (RUNX1, ANKRD26, and ETV6 mutations); developmental delay, congenital cardiac anomalies, hand/face dysmorphogenesis (Paris–Trousseau/Jacobsen syndrome; Fli-1 [ETS-1] mutations); bleeding diathesis out of proportion to degree of thrombocytopenia (Bernard-Soulier syndrome). A superb review of inherited thrombocytopenias and an excellent diagnostic algorithm has been provided by Balduini et al., Table 29.1 summarizes genes involved in normal thrombocytopoiesis that are known to be mutated in human platelet disorders.

TABLE 29.1 Genetic Causes of Human Thrombocytopoiesis Disorders

Disease	Inheritance	Mutated Gene	Theme	Comments	Diagnosis
Large-Sized Platelets					
Bernard–Soulier syndrome	AR-AD	*GPIBA, GPIBB, GP9*	Glycoprotein receptor for VWF	Giant platelets, bleeding diathesis in biallelic forms	Platelet aggregations; flow
Platelet-type VWD	AD	*GPIBA*	Increased binding to VWF	Platelet count decreases with stress	Ristocetin titration; VWF multimer analysis; VWF binding studies
Filamin-related TP	XL	*FLNA*	Cytoskeletal defect	Rare; platelets small to large size; females affected; PNH; splenic dysfunction; Platelet function abnormal.	DNA sequencing
MYH9-related disease	AD	*MYH9*	Cytoskeletal defect	Can include nephritis, sensorineural hearing loss, cataracts, Dohle bodies. Mild bleeding tendency.	Immunofluorescence DNA sequencing
Tubulin-related TP	AD	*TUBB1*	Cytoskeletal defect	Rare; giant platelets	DNA sequencing
ACTN1-related TP	AD	*ACTN1*	Cytoskeletal defect	Mild bleeding	DNA sequencing
TP associated with sitosterolemia	AR	*ABCG5, ABCG8*	Channelopathy	Rare; stomatocytes, possible anemia, tendon xanthomas, atherosclerosis	DNA sequencing
Gray platelet syndrome	AD-AR XL	*NBEAL2, GATA1, GFI1b*	Absence of alpha granules; transcription factor	Giant "pale" platelets. Variable bleeding disorder. Mild RBC anisopoikilocytosis in some	Platelet EM; DNA sequencing
Paris–Trousseau; Jacobsen syndrome	AD	Large deletions at 11q23; FLI1	Transcription factor	Cardiac and facial anomalies ± developmental delay. Mks/platelets with giant fused alpha granules	FISH
FLI1-related TP	AD/AR	FLI1 point mutations	Transcription factor	Mild TP; platelet-dense granule secretion defects; impaired collagen-induced platelet aggregation	DNA sequencing
GATA1-related disease	XL	*GATA1*	Transcription factor	Can also have anemia, β-thalassemia and/or dyserythropoiesis	DNA sequencing
CDC42-related TP	De novo, het	*CDC42*	GTPase	Rare: developmental delay; ± inflammatory syndrome; ± myelofibrosis	DNA sequencing
PRKACG-related TP	AR	*PRKACG*	cAMP-dependent kinase	Rare; platelet dysfunction; bleeding phenotype	DNA sequencing
GNE myopathy with congenital TP	AR	*GNE*	Sialic acid synthesis pathway enzyme	Progressive weakness; renal disease	DNA sequencing
Normal-Sized Platelets					
TP with absent radii (TAR)	AR	*RBM8A* (1q21.1 del)	RNA binding protein	Bilateral radial aplasia ± other malformations; TP often resolves by 12–24 months	X-ray; FISH (for microdeletion)
Congenital amegakaryocytic TP (CAMT)	AR	*MPL, THPO*	Cytokine receptor, cytokine	Reduced Mks; can evolve into full BM failure	DNA sequencing; TPO levels
ANKRD26-related TP (THC2)	AD	*ANKRD26*	Cytoskeletal defect?	Associated platelet dysfunction; risk of leukemia	DNA sequencing
Familial platelet disorder with propensity to develop AML (FPD/AML)	AD	*RUNX1*	Transcription factor	Associated platelet dysfunction; ~35%–40% risk of developing MDS/leukemia; platelets of variable size; can have giant fused alpha granules	DNA sequencing

Continued

TABLE 29.1 Genetic Causes of Human Thrombocytopoiesis Disorders—cont'd

Disease	Inheritance	Mutated Gene	Theme	Comments	Diagnosis
ETV6-related TP (THC5)	AD	*ETV6*	Transcription factor	Increased risk of leukemia/MDS and other cancers; GI symptoms in some patients; RBC macrocytosis in some patients	DNA sequencing
Congenital TP with radio-ulnar synostosis (RUSAT I)	AD	*HOXA11*	Transcription factor	Reduced Mks; cannot pronate distal arms; risk for BM failure/leukemia?	X-ray; DNA sequencing
Radio-Ulnar Synostosis with Amegakaryocytic Thrombocytopenia II (RUSAT II)	De novo	*MECOM (EVI1)*	Transcription factor	Reduced Mks, congenital anemia; cannot pronate distal arms; finger abnormalities (5th digit); risk for BMF/leukemia? Pulmonary HTN?	X-ray; DNA sequencing
CYCS-related TP	AD	*CYCS*	Cytochrome C	Rare	DNA sequencing
IKZF5 (Pegasus)-related TP	AD	*IKZF5*	Transcription factor	Reduced alpha granules	DNA sequencing; platelet EM
Small-Sized Platelets					
Wiskott–Aldrich syndrome (WAS)	XL	*WAS*	Cytoskeletal defect	Severe immunodeficiency; eczema; platelet dysfunction	Western blot; flow cytometry; DNA sequencing
X-linked TP	XL	*WAS*	Cytoskeletal defect	No or mild immunodeficiency; platelet dysfunction	Western blot; flow cytometry; DNA sequencing
PTPRJ-related TP	AR	*PTPRJ*	Tyrosine phosphatase	Non-syndromic TP; mild to moderate bleeding tendencies	DNA sequencing
FYB-related TP (THC3)	AR	*FYB1*	Cell signaling adaptor protein	Reduction of mature Mks; bleeding tendencies	DNA sequencing

AD, Autosomal dominant; *AML*, acute myeloid leukemia; *AR*, autosomal recessive; *BM*, bone marrow; *CAMT*, congenital amegakaryocytic thrombocytopenia; *EM*, electron microscopy; *FISH*, fluorescence in situ hybridization; *GI*, gastrointestinal; *GPIb*, glycoprotein Ib; *MDS*, myelodysplastic syndrome; *Mks*, megakaryocytes; *PNH*, periventricular nodular heterotopia; *RBC*, red blood cells; *RUSAT*, radio-ulnar synostosis with amegakaryocytic thrombocytopenia; *TP*, thrombocytopenia; *TPO*, thrombopoietin; *vWD*, von Willebrand disease; *vWF*, von Willebrand factor; *XL*, X-linked.

GATA Family Transcription Factors

GATA1

GATA transcription factors comprise a family of zinc finger proteins that bind the consensus DNA sequence (T/A)GATA(A/G). There are six known members of the GATA family in vertebrates. GATA1, GATA2, and GATA3 play roles predominantly, although not exclusively, within the hematopoietic system. GATA4, GATA5, and GATA6 are expressed in nonhematopoietic tissues and play diverse developmental roles within the cardiac, gastrointestinal, endocrine, and gonadal systems. Functionally important binding sites for GATA factors have been identified in cis-acting regulatory elements of essentially every megakaryocytic and erythroid gene that has been studied. GATA1, the founding member of this family, is highly expressed in erythroid and megakaryocytic cells and, to a lesser extent, in eosinophils and mast cells. GATA1 plays an essential role in erythroid development, with loss of function resulting in blocked erythroid maturation and apoptosis of erythroid progenitor cells. GATA1 is also required for megakaryocyte maturation and growth control. Lineage-selective loss of GATA1 in megakaryocytes results in marked thrombocytopenia in mice with platelet counts of only ≈15% of WT littermates. Megakaryocytes are present in the mutant animals but have a disorganized DMS, paucity of platelet-specific granules, reduced expression of multiple megakaryocyte-specific genes (including *GPIbα*, *GPIbβ*, *PF4*, c-*Mpl*, and *p45 NF-E2*), and marked hyperproliferation as compared with WT mice. Gene expression studies of GATA1-deficient versus WT murine megakaryocytes have revealed a large number of potential GATA1 target genes, and many of these have been found to be bound by GATA1 in genome-wide chromatin occupancy studies. Mice containing a reduced megakaryocyte-specific expression of GATA1 (GATA1^{low}) develop MF as they age, a frequent finding with disorders of MkP hyperproliferation (Chapter 72). A GATA binding site mutation in the *GPIbβ* promoter has been described in a patient with Bernard-Soulier syndrome, which is characterized by a deficiency of the GPIb/IX/V complex and a bleeding diathesis. Taken together, these findings suggest that GATA1 acts as a master regulator of megakaryocyte maturation and proliferative control.

Friend of GATA (ZFPM1)

All vertebrate GATA factors contain two zinc fingers. The carboxyl zinc finger mediates high-affinity DNA binding, whereas the amino zinc finger stabilizes the DNA interaction at certain double GATA sites. The amino zinc finger also interacts with a friend of GATA (FOG) proteins, a family of large multitype zinc finger transcriptional cofactors. This interaction occurs on the surface of the zinc finger opposite to its DNA binding surface. FOG-1 (also called zfpm1), the founding member, is expressed predominantly within erythroid and megakaryocytic cells. Knockout of FOG-1 in mice results in embryonic lethality caused by severe anemia from a block in erythroid maturation similar to that observed in GATA1^{-} mice. In addition, FOG-1$^{-/-}$ mice have a complete failure of megakaryocytopoiesis, establishing FOG-1 as the first identified transcription-associated factor selectively required to generate the entire megakaryocyte lineage. FOG-1's role in megakaryocyte and erythroid development requires direct physical interaction with GATA factors. The discrepancy between the relatively late block in megakaryocyte development seen in GATA1-deficient animals and the complete loss of megakaryocytopoiesis in FOG-1$^{-/-}$ mice is explained by overlapping FOG-dependent roles of GATA1 and GATA2 during the early stages of megakaryocytopoiesis.

X-Linked Dyserythropoietic Anemia and Thrombocytopenia Caused by GATA1 Mutations

Germline GATA1 mutations that impair binding to FOG-1 and/or DNA have been identified in several families with X-linked macrothrombocytopenia and/or anemia (GATA1 is located on the X chromosome in both humans and mice) (Chapter 30). The first case, reported by Nichols et al., involved a woman with mild chronic thrombocytopenia who had two pregnancies with male offspring that were both complicated by severe fetal anemia and thrombocytopenia requiring in utero transfusions.[23] BM examination after birth revealed marked dyserythropoiesis and an overabundance of immature-appearing, dysplastic megakaryocytes that share many of the features of GATA1^{low} murine megakaryocytes. Remarkably, sequencing of the *GATA1* gene from affected family members identified a substitution of valine by methionine at codon 205 within the amino zinc finger. This mutation (GATA1^{V205M}) significantly impairs FOG-1 binding but retains normal DNA affinity based on electromobility shift assays using synthetic oligonucleotides. This is consistent with the location of this residue on the surface of the zinc finger opposite the DNA binding face.

Several other GATA1 mutations have been linked to cases of familial X-linked macrothrombocytopenia with or without anemia. All of these substitutions impair FOG-1 binding, although to different degrees. Substitution of glycine by serine at codon 208 (GATA1^{G208S}) results in moderate to severe thrombocytopenia and mild dyserythropoiesis, but no anemia. Substitution of the same residue by arginine (GATA1^{G208R}) results in thrombocytopenia with anemia and severe dyserythropoiesis. Similarly, the substitution of aspartic acid by glycine at codon 218 (GATA1^{D218G}) leads only to thrombocytopenia, whereas substitution of this same codon by tyrosine (GATA1^{D218Y}) leads to severe thrombocytopenia, moderate anemia, and marked dyserythropoiesis. The severity of the phenotype appears to correlate with the degree of FOG-1 binding impairment, suggesting that megakaryocytic development is more sensitive to affinity changes in GATA1–FOG-1 interactions than is erythroid development.

X-Linked Thrombocytopenia and Thalassemia Caused by GATA1 Mutations

Mutations mapping to the DNA binding surface of the amino zinc finger of GATA1 have also been described (GATA1^{R216Q}). As expected, this reduces DNA affinity to double (palindromic) GATA sites but not to single GATA sites. FOG-1 binding is not substantially altered. Affected family members exhibit an X-linked β-thalassemia syndrome characterized by an imbalance of alpha- and beta-globin chain synthesis, reticulocytosis, and hemolysis. They also have mild to moderate thrombocytopenia. In vitro platelet aggregation studies are normal, but there is a prolonged bleeding time. Substitution of the same residue by tryptophan (R216W) produces thrombocytopenia, β-thalassemia intermedia, and congenital erythropoietic porphyria (CEP). The CEP is likely caused by dysregulation of the *GATA1* target gene uroporphyrinogen III synthase.

X-Linked Gray Platelet–Like Syndrome

GPS refers to a disorder of large platelets with absent or markedly reduced α-granules and/or α-granule proteins. Platelets from individuals with GATA1^{R261Q} share some features with classical GPS. Ultrastructural studies of platelets from a different family with GATA1-related X-linked macrothrombocytopenia (GATA1^{G208S}) also demonstrate hypogranular platelets that contain small vacuoles, likely representing membranes of empty α-granules. However, the GATA1 mutant platelets also possess unique features such as masses of dense tubular system channels, dense double membranes, and platelets within platelets, not seen in classical GPS, suggesting a more general disorder of platelet biogenesis. GPS is characterized by the release of proteins normally contained in α granules into the marrow, causing MF. GPS is primarily inherited in an autosomal recessive manner, and the mutated gene has been mapped to chromosome 3p and identified as NBEAL2.

GATA1 Mutations in Down Syndrome Transient Myeloproliferative Disorders and Acute Megakaryoblastic Leukemia

About 10% of children with DS (trisomy 21) are born with a transient myeloproliferative disorder (also termed "Down Syndrome—Transient Abnormal Myelopoiesis [DS-TAM]"), which is characterized by an abundance of circulating erythromegakaryocytic precursor cells, pancytopenia, and in some cases, severe liver fibrosis. Remarkably, this myeloproliferation resolves spontaneously over the first few months of life. In about 20% to 30% of cases, DS-associated acute megakaryocytic leukemia (DS-AMKL, also termed "Myeloid Leukemia of Down Syndrome [ML-DS]") develops within a few years, sometimes preceded by a myelodysplastic phase. In 2002, Wechsler et al. reported that DS-AMKL cells harbor acquired mutations in their *GATA1* gene.[24] Since then, many groups have identified similar mutations in DS-TMD cells. Although a wide spectrum of mutations has been found, including missense, deletion, insertion, and splice-site mutations, they all involve exon 2 (or rarely exon 3) and result in the same outcome: generation of an amino-terminal truncated protein (loss of amino acids 1 to 83) because of translation initiation from a downstream ATG codon. This removes a region that functions as a transcriptional activation domain in transient transfection reporter assays. The mutations are detectable in BM from DS-AMKL patients but disappear when patients enter remission, indicating a strong correlation between the mutated clone and the leukemic phenotype. Mutations involving exon 2 of *GATA1* are highly specific for DS-AMKL and DS-TMD, or AMKL with acquired trisomy 21. There is only one reported case of such a mutation in AMKL without trisomy 21, and mutations have not been detected in DS-acute lymphoblastic leukemia or a large number of healthy individuals.

Analysis of stored neonatal blood spots shows the coexistence of several different GATA1 mutations (all resulting in the generation of GATA1s) in patients who subsequently developed DS-AMKL, suggesting an oligoclonal expansion. In a few cases in which material was available, identical GATA1 mutations have been found in both the DS-TMD and DS-AMKL cells from the same patient. DS-AMKL cells often harbor additional genetic abnormalities, such as trisomy 8 or tetrasomy 21, not observed in DS-TMD cells. Acquisition of secondary loss-of-function mutations in members of the cohesion complex, and other epigenetic factors, is also common. Taken together, these findings support a clonal evolution model of DS-AMKL, with GATA1 mutations associated with an early initiating event.

Generation of knock-in mice that recapitulate these truncating GATA1 mutations show unexpected stage-specific effects on megakaryocytopoiesis. During fetal liver hematopoiesis, the mutant megakaryocytes markedly hyperproliferate, similar to what is observed for GATA1-deficient megakaryocytes. However, during adult-stage BM hematopoiesis, megakaryocytopoiesis and thrombocytopoiesis appear normal. This suggests that the fetal liver and BM cellular contexts interact differentially with the GATA1 truncated molecule. This may also explain the restriction of TMD to the neonatal period. A family has been described with members containing a germline *GATA1* gene splice site mutation (G332C) that results in the exclusive production of the GATA1s protein product. Affected individuals exhibit a unique phenotype characterized by trilineage BM dysplasia, macrocytic anemia, and neutropenia. None of the family members has developed leukemia, suggesting that trisomy 21 plays a role in DS-TMD progression to DS-AMKL.

Of note, Calligaris et al., previously reported that GATA1s is produced naturally at low levels in erythroid cells.[25] They proposed that this might serve a regulatory role during normal hematopoiesis by acting as a dominant-negative molecule at specific times/environmental stimuli. Endogenous GATA1s have also been detected in normal mouse fetal liver megakaryocytes and adult human BM megakaryocytes. Thus, it has been proposed that the ratio of GATA1 to GATA1s plays a role in developmental aspects of megakaryocytopoiesis and that acquired GATA1 mutations observed in DS-TMD and DS-AMKL, or germline mutations in the family described earlier, perturb hematopoiesis by altering this ratio.

E26 Transformation Specific Family Transcription Factors

A common feature of megakaryocyte-specific genes is the presence of tandem binding sites for GATA and E26 transformation specific (ETS) family transcription factors in their promoters and enhancers. The ETS transcription factor family is composed of a diverse group of proteins that share a common ETS DNA-binding domain, which recognizes a GGAA core sequence. Over 30 different ETS factors have been identified, at least 10 of which (ELF1, ELF2, Fli-1, PU.1, TEL, GABPα, ETS1, ETS2, ELK4, ERG) are expressed in megakaryocytes. Functional studies have implicated several of these, including Fli-1, ETS1, ETV6 (TEL), ERG, and GABPα, in megakaryocytopoiesis.

Fli-1

The role of Fli-1 megakaryocytopoiesis is the best characterized of the ETS factors in terms of its functional role in megakaryocyte development. Fli-1$^{-/-}$ mice die during embryogenesis from hemorrhage, likely caused by both vascular defects and dysmegakaryocytopoiesis. Colony assays show an increased number of MkPs in Fli-1$^{-/-}$ embryos as compared with WT mice. However, the megakaryocytes from these colonies are small, contain a high nuclear/cytoplasmic ratio, and have hypolobulated nuclei, disorganized platelet demarcation membranes, and reduced numbers of α-granules. Expression of the late megakaryocyte marker gene *GPIX* is markedly reduced, whereas expression of the early genes, TPO receptor, and αIIb are normal or mildly reduced, consistent with a role of Fli-1 in late megakaryocyte maturation. Fli-1 is involved in the synergistic transcriptional activation of several megakaryocyte-specific genes by GATA1, FOG-1, and RUNX1. Different ETS factors act in a stage-specific manner during megakaryocytopoiesis, with GABPα predominantly regulating genes active during early stages of megakaryocytopoiesis and Fli-1 during later stages. Fli-1 has been also implicated in the lineage commitment of bipotent erythroid-MkP cells to the megakaryocyte pathway.

Paris-Trousseau syndrome (OMIM 188925) and Jacobsen syndrome (OMIM 147791) (Chapter 30) are overlapping contiguous gene-deletion disorders in humans involving the long arm of chromosome 11 (11q23). The constellation of findings in these syndromes includes severe congenital cardiac abnormalities, trigonocephaly, developmental delay, dysmorphogenesis of the hands and face, and macrothrombocytopenia. The etiology of the thrombocytopenia in these patients appears to be related to impaired platelet production since platelet survival time is normal. Examination of BM reveals significant dysmegakaryocytopoiesis with an abundance of micromegakaryocytes and the death of large numbers of megakaryocytes during terminal stages of maturation. Peripheral blood platelets contain giant α-granules, which are thought to arise from aberrant α-granule fusion during prolonged residence in the BM. The minimal chromosome regions deleted in Paris-Trousseau and Jacobsen syndromes associated with thrombocytopenia include the genes for the ETS factors *Fli-1* and *ETS-1*. Lentiviral expression of Fli-1 in CD34$^+$ cells from patients with Paris-Trousseau thrombocytopenia rescues megakaryocyte differentiation in vitro, providing evidence that it is the deficiency of Fli-1 that is the cause of impaired thrombocytopoiesis in these patients. Using iPS cells derived from patients with Paris-Trousseau syndrome, Poncz and colleagues also demonstrated that FLI1 overexpression improved MK yield and functionality.[26] Of interest, Raslova et al., have shown that in normal individuals, expression of Fli-1 is mostly monoallelic in early megakaryocytic progenitors (CD41$^+$/CD42$^-$ cells) but predominantly biallelic in later stages. They propose that the different populations of megakaryocytes seen in patients with Paris-Trousseau disorder arise from the expression of the normal allele in the normally differentiating megakaryocytes, and the deleted allele (leading to complete loss of Fli-1 expression) in the dying population of megakaryocytes.

Germline heterozygous missense *Fli-1* gene mutations have recently been reported in patients with a familial platelet defect. Most of these mutations involve the DNA binding domain and abolish transcriptional activity. The small number of patients reported have been presented with mild macrothrombocytopenia and a storage pool-type platelet defect.

ETS-Related Gene

ETS-related gene (ERG) is closely related to Fli-1. Mouse studies show that ERG and Fli-1 play compensatory roles in murine thrombopoiesis. The *ERG* gene is located on chromosome 21 in humans and has been suggested to play a role in DS-TMD and AMKL.

ETV6 (TEL)

Generation of a fusion protein between ETV6 and RUNX1 is the most frequent chromosomal translocation in childhood pre-B cell acute lymphoblastic leukemia (Chapter 66). Although ETV6 is required for the ontogeny of all definitive hematopoiesis, a conditional knock-out study of the *ETV6* gene in mice demonstrates its specific requirement for adult-stage megakaryocytopoiesis. Heterozygous germline ETV6 mutations have recently been described as a cause of autosomal dominant thrombocytopenia. The mutations described to date involve either the DNA binding domain (and disrupt DNA interaction) or a common site in a linker region located between the DNA binding domain and the Pointed protein-protein interaction domain. Megakaryocytes from these patients are generally small and contain hypolobulated nuclei and underdeveloped cytoplasm. There is also variable red blood cell macrocytosis. Importantly, affected individuals have an increased risk for the development of hematologic malignancies.

Other Transcription Factors

RUNX1

In 1999, Song et al. used positional cloning to identify the genetic cause of a rare dominant disorder characterized by thrombocytopenia, an aspirin-like functional platelet defect, and increased risk for developing acute myeloid leukemia (FPD/AML; OMIM 601399).[27] They identified nonsense mutations, intragenic deletions, or missense mutations on one allele of the gene for RUNX1 (formerly called *AML-1* and *CBFA2*) that co-segregated with the disease in six separate pedigrees. These mutations all resulted in a loss of function, indicating that haploinsufficiency of RUNX1 plays a causal role in this disorder. BM or peripheral blood from these patients were characterized by reduced megakaryocyte colony formation, indicating that RUNX1 dosage affects megakaryocytopoiesis.

RUNX1 is a member of an evolutionarily conserved family of transcription factors that share a conserved 128 amino acid domain in their amino half with homology to *Drosophila runt* gene. This region mediates binding to DNA (consensus [C/T]G[C/T]GGT), as well as to its heterodimeric binding partner CBF-β via protein-protein interactions. RUNX1 is the most frequently mutated transcription factor in human leukemia. In addition, acquired mutations in RUNX1 have been identified in a significant number of patients with myelodysplastic syndrome, particularly those that progress to AML. The homozygous knockout of either RUNX1 or CBF-β in mice is embryonic-lethal because of a complete failure of definitive hematopoiesis. This is thought to arise from a defect in the ontogeny of HSCs in the AGM region. Conditional knockout of RUNX1 in adult hematopoiesis demonstrate a specific role of RUNX1 in megakaryocytopoiesis. BM deletion of RUNX1 in adult mice results in up to ≈80% reduction in peripheral blood platelet numbers, although no bleeding diathesis. BM megakaryocytes are small, lack lobulated nuclei, have poorly developed demarcation membranes, and reduced polyploidization. These findings are reminiscent of the abnormal "micromegakaryocytes" seen in humans with myelodysplastic syndromes and MF. Paradoxically, there is an increase in in vitro megakaryocyte colony plating efficiency, suggesting an expansion of early MkPs. These effects are cell-autonomous. No defects are seen in the erythroid lineage. Reduced dosage of RUNX1's essential cofactor CBF-β also perturbs megakaryocytopoiesis in vivo. Taken together, these findings indicate a specific role of RUNX1/CBF-β in megakaryocyte terminal maturation. In a recent study, Li et al. identified NOTCH4 as a RUNX1 target gene and demonstrated that genetic or pharmacologic inhibition of NOTCH4 stimulates MK production by human iPSC or cord blood-derived $CD34^+$ cells.

NF-E2 p45

NF-E2 is a heterodimeric transcription factor composed of two basic region-leucine zipper (bZip) subunits: a hematopoietic-specific 45 kDa protein (p45) and a widely expressed 18 kDa subunit (p18). NF-E2 p45 is expressed in erythroid, megakaryocytic, and mast cell lineages. In vitro studies implicated NF-E2 p45 as a critical factor for β-globin expression. Unexpectedly, NF-E2 $p45^{-/-}$ mice were found to have only mild perturbations of the erythroid lineage. However, these mice fail to produce platelets secondary to a maturational arrest in the megakaryocyte lineage and succumb to hemorrhage in the neonatal period. Since the initial studies, NF-E2 p45 has been recognized as being a major regulator of terminal megakaryocyte maturation and platelet release. Notably, although NF-E2 $p45^{-/-}$ mice are severely thrombocytopenic, they have normal serum levels of TPO. In addition, megakaryocytes from these animals proliferate in vivo in response to TPO administration. These findings suggest that NF-E2 p45 regulates target genes independent of the action of TPO. Pahl and co-workers have reported the overexpression of NF-E2 in MPN patients and showed that elevated NF-E2 levels in vivo cause an MPN phenotype which predisposes to leukemic transformation in transgenic mice. Acquired insertion and deletion mutations in the NF-E2 gene have been reported in MPN patients that result in truncated NF-E2 proteins which enhance WT NF-E2 function and cause erythrocytosis and thrombocytosis in a murine model. NF-E2 mutant cells acquire a proliferative advantage, witnessed by clonal dominance over WT NF-E2 cells in MPN patients.

Several important target genes of NF-E2 p45 have been identified, including β-*1 tubulin*, 3β-hydroxysteroid dehydrogenase *(3β-HSD)*, thromboxane synthase, caspase 12, and Rab27b. β-1 tubulin is a megakaryocyte-restricted isoform of β-tubulin that plays a key role in the marginal band structure of platelets and is essential for their discoid shape. Deficiency of β-1 tubulin leads to spherocytic platelets. Heterozygosity for a polymorphism (Q43P, because of the double nucleotide substitution AG>CC) in the human *β1-tubulin* gene is present in about 11% of individuals in a White Northern European population and correlates with a reduced risk for cardiovascular disease in humans. This may be caused by alterations in platelet structure and function. 3β-HSD catalyzes autocrine biosynthesis of estradiol within megakaryocytes and plays an important role in proplatelet formation.

SCL (TAL1)

SCL (also known as *TAL1*) is a member of the basic helix-loop-helix member of transcription factors and is expressed predominantly in megakaryocytic and erythroid cells. Dysregulated expression of SCL because of chromosomal translocation is associated with certain cases of T-cell acute lymphoblastic leukemia. SCL forms obligate heterodimers with ubiquitously expressed E proteins (such as E12 and E47), which bind to E-box motifs (sequence CANNTG). It participates in multiprotein complexes that include E2A, GATA1, LMO2, LDB1, and the repressor ETO-2. $SCL^{-/-}$ mice die during embryogenesis as a result of a failure of all hematopoiesis and defective vasculogenesis. However, conditional SCL knock-out models show a specific role for SCL in the late stages of megakaryocytopoiesis and stress thrombocytopoiesis during adult hematopoiesis. SCL-null megakaryocytes have disorganized DMSs and a reduced number of platelet granules. SCL modulates thrombocytopoiesis, in part, by direct transcriptional activation of NF-E2 p45.

Gfi-1b

Gfi-1b (*Gfi* standing for "growth factor independent") is a member of a family of hematopoietic expressed zinc finger transcription factors that contain a unique amino-terminal transcriptional repressor Snail/Gfi-1 (SNAG) domain. It represses target genes by recruitment of histone lysine methyltransferases. The knockout of Gfi-1b in mice results in embryonic lethality because of severe anemia. The fetal liver of mutant mice contains erythroid and megakaryocytic progenitors that are blocked in their maturation. The culture of these cells in the presence of TPO, in contrast to those from WT animals, generates only small colonies and the cells are acetylcholinesterase negative (a marker of maturing megakaryocytes in mice). They contain markedly reduced mRNA transcript levels of vWF, NF-E2 p45, c-Mpl, and GPIIb, compared with WT, suggesting a requirement for Gfi-1b in at least relatively early stages of megakaryocytopoiesis. Germline dominant-negative *Gfi1b* gene mutations have recently been reported as a cause of familial GPS. The patients have macrothrombocytopenia, platelet dysfunction, and red blood cell anisopoikilocytosis. Megakaryocytes from the patients are dysplastic-appearing with some having hypolobulated nuclei and others with multiple separated nuclei. All of the mutations described to date involve the carboxyl-terminal zinc finger domain and result in truncated protein products.

HOX-Related Genes

Homeobox containing transcription factors (HOX factors) play a central role in embryonic patterning and development. Several of these factors have specific functions in megakaryocytopoiesis. The best evidence is for MEIS1, a homeodomain protein belonging to the Transcription activator-like effector (TALE) subfamily. MEIS1 knock-out mice fail to produce megakaryocytes. Genome-wide chromatin occupancy studies show high enrichment for binding near megakaryocyte and platelet function-specific genes. Heterodimers of MEIS1 and the homeobox protein PBX1 have been shown to functionally regulate the *PF4* gene in megakaryocytopoiesis.

Other HOX genes have also been implicated in megakaryocytopoiesis. Mutations in the *HOXA11* gene cause a rare syndrome of congenital thrombocytopenia with radio-ulnar synostosis (CTRUS; OMIM #605432). The described mutations reduce DNA binding and lead to impaired in vitro megakaryocyte differentiation. There are also limited data suggesting a role for HOXA10 in murine megakaryocytopoiesis.

MECOM (MDS1-EVI1)

The zinc finger transcription factor MECOM is produced from the splicing of exons from the *MDS1* and *EVI1* gene loci located on human chromosome 11q26. Germline mutations in *MECOM* cause a syndrome of congenital hypomegakaryocytic thrombocytopenia with various additional features such as radio-ulnar synostosis, clinodactyly, cardiac and renal malformation, B-cell deficiency, and hearing loss (RUSAT2; OMIM #616738).[28] This disorder frequently manifests as neonatal thrombocytopenia and can progress to full BM failure.

c-Myb

Carpinelli et al. performed an N-ethyl-N-Nitrosourea (ENU) mutagenesis screen in TPO receptor$^{-/-}$ mice to identify factors that might influence thrombocytopoiesis.[29] They identified two independent loss-of-function alleles of the transcription factor c-Myb (substitution of valine for aspartic acid at residue 152 within the DNA binding domain and residue 384 within the leucine zipper domain). Both TPO receptor$^{-/-}$ and WT mice containing these mutations have supraphysiologic production of platelets as a result of excessive megakaryocytopoiesis, at the expense of erythroid and lymphocyte development. Megakaryocytes from these animals have a 200-fold increased sensitivity to GM-CSF, suggesting dysregulation of signaling pathways. Similar megakaryocytic hyperplasia and thrombocytosis occur in mice containing germline c-Myb mutations that disrupt binding the transcriptional coactivator p300. Thus c-Myb may play an important negative regulatory function in megakaryocytopoiesis and thrombocytopoiesis.

Hypoxia-Inducible Factor 1-Alpha

Hypoxia-inducible factor 1-alpha (HIF-1α) is an oxygen-sensing transcription factor known for its role in promoting engraftment and differentiation of HSCs. HIF-1α was recently found at low levels in BM supernatants from patients with immune thrombocytopenia (ITP). In a subgroup of ITP patients with low megakaryocyte and platelet numbers the levels of HIF-1α were further reduced. In vitro studies showed that pharmacological activation of HIF-1α stimulated CD41 expression and polyploidization of murine megakaryocytes suggesting that low HIF-1α levels negatively impact thrombocytopoiesis. Other groups have reported that TPO modulates HIF-1α expression in murine embryonic and HSCs. Taken together, these observations warrant additional functional studies to define the role of HIF-1α in megakaryocyte development and its potential as a therapeutic target in ITP or other disorders associated with defective megakaryocyte differentiation.

MICRO RNAS IN MEGAKARYOCYTOPOIESIS

MicroRNAs (miRNAs) are a class of small (typically 19 to 25 nucleotide) non-coding RNAs that interact in a sequence-specific manner with mRNAs (typically in their 3′ untranslated region in mammals) and modulate gene expression through either enhanced mRNA decay or inhibiting translation. They play roles in development and differentiation by fine-tuning tissue-specific transcription factor expression. Each miRNA can have multiple target genes, and conversely, each mRNA can be subject to regulation by multiple miRNA species. In addition, the transcription of miRNAs themselves are mediated by RNA polymerase II and are subject to control by transcription factors. Therefore complex regulatory networks can exist between miRNAs and transcription factors. A number of miRNAs have been shown to influence megakaryocytopoiesis during ontogeny and at various stages of differentiation.[30] miR-150 enhances megakaryocytopoiesis at the expense of erythropoiesis, suggesting a critical role in the cell fate decision of bipotent MEP cells. This is mediated, at least in part, via targeting the 3′-UTR of c-MYB mRNA transcripts. TPO signaling increases miR-150 levels. miR-155 inhibits megakaryocytopoiesis by targeting ETS1 and MEIS1 transcription factors. Other miRNAs have been implicated in controlling thrombopoiesis, but the evidence supporting a functional role is not as strong as for miR-150 and miR-155. For instance, miR-22 has been suggested to promote megakaryocyte differentiation through repression of GFI1 and miR-142 stimulates polyploidization, maturation and proplatelet release by targeting actin cytoskeleton regulators. miRNAs have also been detected in platelets and have been suggested as potential biomarkers for platelet activation. Further studies are needed to examine their roles in platelet activation and function.

FUTURE DIRECTIONS

Although the molecular details regarding the regulation and generation of platelets remain to be fully elucidated, considerable progress has been made over the past few decades. This has been significantly facilitated by the isolation of TPO and its receptor. Important models of thrombocytopoiesis have now been tested rigorously in vivo, yielding new insights into the final stages of platelet formation and shedding. These studies highlight the efficient mechanisms that have been developed to satisfy the demands for dynamic and high-output platelet production. Several important transcription factors have been identified that regulate different stages of megakaryocytopoiesis, and mutations in these, and other genes, have been linked to human disorders of thrombocytopoiesis. The role of miRNAs in controlling thrombocytopoiesis is beginning to be appreciated. Although mouse models have played important roles in the analysis of these genes, it is becoming clear that they do not always faithfully recapitulate human disease. In addition, several studies have documented important differences between rodent and human platelets, including differences in size, circulating numbers, and DMS ultrastructural features. Thus some caution must be exercised when extrapolating results of mouse studies to human thrombocytopoiesis. The advent of megakaryocyte in vitro differentiation systems using human CD34^{+} cells, embryonic stem cells, and iPSCs coupled with new single-cell technologies are providing important tools for additional studies geared toward understanding and treating human disorders of megakaryocytopoiesis and thrombocytopoiesis.

SUGGESTED REFERENCES

A list including supplementary References is available at Elsevier eBooks for Practicing Clinicians.

1. Wright J. The origin and nature of blood platelets. *Boston Med Surg J.* 1906:23.
2. Zhang Y, Gao S, Xia J, Liu F. Hematopoietic hierarchy—an updated roadmap. *Trends Cell Biol.* 2018;28(12):976–986.
3. King SM, Reed GL. Development of platelet secretory granules. *Semin Cell Dev Biol.* 2002;13(4):293–302.
4. Battinelli EM, Thon JN, Okazaki R, et al. Megakaryocytes package contents into separate α-granules that are differentially distributed in platelets. *Blood Adv.* 2019;3(20):3092–3098.
5. Huang H, Cantor AB. Common features of megakaryocytes and hematopoietic stem cells: what's the connection? *J Cell Biochem.* 2009;107(5):857–864.
6. Kimura S, Roberts AW, Metcalf D, Alexander WS. Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. *Proc Natl Acad Sci USA.* 1998;95(3):1195–1200.
7. Mazzi S, Lordier L, Debili N, et al. Megakaryocyte and polyploidization. *Exp Hematol.* 2018;57:1–13.
8. Becker RP, De Bruyn PP. The transmural passage of blood cells into myeloid sinusoids and the entry of platelets into the sinusoidal circulation; a scanning electron microscopic investigation. *Am J Anat.* 1976;145(2):183–205.

9. Italiano Jr. JE, Lecine P, Shivdasani RA, Hartwig JH. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J Cell Biol*. 1999;147(6):1299–1312.
10. Richardson JL, Shivdasani RA, Boers C, et al. Mechanisms of organelle transport and capture along proplatelets during platelet production. *Blood*. 2005;106(13):4066–4075.
11. Elagib KE, Brock AT, Goldfarb AN. Megakaryocyte ontogeny: clinical and molecular significance. *Exp Hematol*. 2018;61:1–9.
12. Davenport P, Liu Z-J, Sola-Visner M. Changes in megakaryopoiesis over ontogeny and their implications in health and disease. *Platelets*. 2020;31(6):692–699.
13. Avecilla ST, Hattori K, Heissig B, et al. Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nat Med*. 2004;10(1):64–71.
14. Junt T, Schulze H, Chen Z, et al. Dynamic visualization of thrombopoiesis within bone marrow. *Science*. 2007;317(5845):1767–1770.
15. Howell WH, Donahue DD. The production of blood platelets in the lungs. *J Exp Med*. 1937;65(2):177–203.
16. Lefrançais E, Looney MR. Platelet biogenesis in the lung circulation. *Physiology (Bethesda)*. 2019;34(6):392–401.
17. Sim X, Poncz M, Gadue P, French DL. Understanding platelet generation from megakaryocytes: implications for in vitro-derived platelets. *Blood*. 2016;127(10):1227–1233.
18. Cserhati I, Tanos B, Kelemen E. Acute prolonged thrombocytosis in mice induced by the serum of patients having thrombocythemia; postulated human thrombopoietin. *Orv Hetil*. 1958;99(16):540–541.
19. Kaushansky K. The molecular mechanisms that control thrombopoiesis. *J Clin Invest*. 2005;115(12):3339–3347.
20. Grozovsky R, Begonja AJ, Liu K, et al. The Ashwell-Morell receptor regulates hepatic thrombopoietin production via JAK2-STAT3 signaling. *Nat Med*. 2015;21(1):47–54.
21. Vainchenker W, Plo I, Marty C, et al. The role of the thrombopoietin receptor MPL in myeloproliferative neoplasms: recent findings and potential therapeutic applications. *Expert Rev Hematol*. 2019;12(6):437–448.
22. Behrens K, Alexander WS. Cytokine control of megakaryopoiesis. *Growth Factors*. 2018;36(3-4):89–103.
23. Nichols KE, Crispino JD, Poncz M, et al. Familial dyserythropoietic anaemia and thrombocytopenia due to an inherited mutation in GATA1. *Nat Genet*. 2000;24(3):266–270.
24. Wechsler J, Greene M, McDevitt MA, et al. Acquired mutations in GATA1 in the megakaryoblastic leukemia of Down syndrome. *Nat Genet*. 2002;32(1):148–152.
25. Calligaris R, Bottardi S, Cogoi S, et al. Alternative translation initiation site usage results in two functionally distinct forms of the GATA-1 transcription factor. *Proc Natl Acad Sci USA*. 1995;92(25):11598–11602.
26. Vo KK, Jarocha DJ, Lyde RB, et al. FLI1 level during megakaryopoiesis affects thrombopoiesis and platelet biology. *Blood*. 2017;129(26):3486–3494.
27. Song WJ, Sullivan MG, Legare RD, et al. Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. *Nat Genet*. 1999;23(2):166–175.
28. Germeshausen M, Ancliff P, Estrada J, et al. MECOM-associated syndrome: a heterogeneous inherited bone marrow failure syndrome with amegakaryocytic thrombocytopenia. *Blood Adv*. 2018;2(6):586–596.
29. Carpinelli MR, Hilton DJ, Metcalf D, et al. Suppressor screen in Mpl-/- mice: c-Myb mutation causes supraphysiological production of platelets in the absence of thrombopoietin signaling. *Proc Natl Acad Sci USA*. 2004;101(17):6553–6558.
30. Raghuwanshi S, Dahariya S, Musvi SS, et al. MicroRNA function in megakaryocytes. *Platelets*. 2019;30(7):809–816.

REFERENCES

1. Adolfsson J, Månsson R, Buza-Vidas N, et al. Identification of Flt3$^+$ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell*. 2005;121(2):295–306.
2. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*. 2000;404(6774):193–197.
3. Avecilla ST, Hattori K, Heissig B, et al. Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nat Med*. 2004;10(1):64–71.
4. Balduini CL, Pecci A, Noris P. Diagnosis and management of inherited thrombocytopenias. *Semin Thromb Hemost*. 2013;39(2):161–171.
5. Battinelli EM, Thon JN, Okazaki R, et al. Megakaryocytes package contents into separate α-granules that are differentially distributed in platelets. *Blood Adv*. 2019;3(20):3092–3098.
6. Becker RP, De Bruyn PP. The transmural passage of blood cells into myeloid sinusoids and the entry of platelets into the sinusoidal circulation; a scanning electron microscopic investigation. *Am J Anat*. 1976;145(2):183–205.
7. Behrens K, Alexander WS. Cytokine control of megakaryopoiesis. *Growth Factors*. 2018;36(3-4):89–103.
8. Bersenev A, Wu C, Balcerek J, et al. Lnk constrains myeloproliferative diseases in mice. *J Clin Invest*. 2010;120(6):2058–2069.
9. Bruns I, Lucas D, Pinho S, et al. Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion. *Nat Med*. 2014;20(11):1315–1320.
10. Bunting S, Widmer R, Lipari T, et al. Normal platelets and megakaryocytes are produced in vivo in the absence of thrombopoietin. *Blood*. 1997;90(9):3423–3429.
11. Calligaris R, Bottardi S, Cogoi S, Apezteguia I, Santoro C. Alternative translation initiation site usage results in two functionally distinct forms of the GATA-1 transcription factor. *Proc Natl Acad Sci U S A*. 1995;92(25):11598–11602.
12. Carpinelli MR, Hilton DJ, Metcalf D, et al. Suppressor screen in Mpl-/- mice: c-Myb mutation causes supraphysiological production of platelets in the absence of thrombopoietin signaling. *Proc Natl Acad Sci U S A*. 2004;101(17):6553–6558.
13. Carrelha J, Meng Y, Kettyle LM, et al. Hierarchically related lineage-restricted fates of multipotent haematopoietic stem cells. *Nature*. 2018;554(7690):106–111.
14. Chang Y, Auradé F, Larbret F, et al. Proplatelet formation is regulated by the Rho/ROCK pathway. *Blood*. 2007;109(10):4229–4236.
15. Chapnik E, Rivkin N, Mildner A, et al. miR-142 orchestrates a network of actin cytoskeleton regulators during megakaryopoiesis. *Elife*. 2014;3:e01964.
16. Cserhati I, Tanos B, Kelemen E. Acute prolonged thrombocytosis in mice induced by the serum of patients having thrombocythemia; postulated human thrombopoietin. *Orv Hetil*. 1958;99(16):540–541.
17. Davenport P, Liu Z-J, Sola-Visner M. Changes in megakaryopoiesis over ontogeny and their implications in health and disease. *Platelets*. 2020;31(6):692–699.
18. Debili N, Coulombel L, Croisille L, et al. Characterization of a bipotent erythro-megakaryocytic progenitor in human bone marrow. *Blood*. 1996;88(4):1284–1296.
19. Elagib KE, Brock AT, Goldfarb AN. Megakaryocyte ontogeny: clinical and molecular significance. *Exp Hematol*. 2018;61:1–9.
20. Fuentes R, Wang Y, Hirsch J, et al. Infusion of mature megakaryocytes into mice yields functional platelets. *J Clin Invest*. 2010;120(11):3917–3922.
21. Gao Y, Smith E, Ker E, et al. Role of RhoA-specific guanine exchange factors in regulation of endomitosis in megakaryocytes. *Dev Cell*. 2012;22(3):573–584.
22. Germeshausen M, Ancliff P, Estrada J, et al. MECOM-associated syndrome: a heterogeneous inherited bone marrow failure syndrome with amegakaryocytic thrombocytopenia. *Blood Adv*. 2018;2(6):586–596.
23. Grozovsky R, Begonja AJ, Liu K, et al. The Ashwell-Morell receptor regulates hepatic thrombopoietin production via JAK2-STAT3 signaling. *Nature Med*. 2015;21(1):47–54.
24. Gurney AL, Carver-Moore K, de Sauvage FJ, Moore MW. Thrombocytopenia in c-mpl-deficient mice. *Science*. 1994;265(5177):1445–1447.
25. Haas S, Hansson J, Klimmeck D, et al. Inflammation-induced emergency megakaryopoiesis driven by hematopoietic stem cell-like megakaryocyte progenitors. *Cell Stem Cell*. 2015;17(4):422–434.
26. Harker LA, Finch CA. Thrombokinetics in man. *J Clin Invest*. 1969;48(6):963–974.
27. Howell WH, Donahue DD. The production of blood platelets in the lungs. *J Exp Med*. 1937;65(2):177–203.
28. Iancu-Rubin C, Gajzer D, Tripodi J, et al. Down-regulation of stathmin expression is required for megakaryocyte maturation and platelet production. *Blood*. 2011;117(17):4580–4589.
29. Italiano Jr. JE, Lecine P, Shivdasani RA, Hartwig JH. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J Cell Biol*. 1999;147(6):1299–1312.
30. Ito Y, Nakamura S, Sugimoto N, et al. Turbulence activates platelet biogenesis to enable clinical scale ex vivo production. *Cell*. 2018;174(3):636–648. e618.
31. Junt T, Schulze H, Chen Z, et al. Dynamic visualization of thrombopoiesis within bone marrow. *Science*. 2007;317(5845):1767–1770.
32. Kaufman RM, Airo R, Pollack S, Crosby WH. Circulating megakaryocytes and platelet release in the lung. *Blood*. 1965;26(6):720–731.
33. Kaushansky K. The molecular mechanisms that control thrombopoiesis. *J Clin Invest*. 2005;115(12):3339–3347.
34. Kimura S, Roberts AW, Metcalf D, Alexander WS. Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. *Proc Natl Acad Sci U S A*. 1998;95(3):1195–1200.
35. King SM, Reed GL. Development of platelet secretory granules. *Semin Cell Dev Biol*. 2002;13(4):293–302.
36. Lefrançais E, Looney MR. Platelet biogenesis in the lung circulation. *Physiology (Bethesda)*. 2019;34(6):392–401.
37. Lefrançais E, Ortiz-Muñoz G, Caudrillier A, et al. The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature*. 2017;544(7648):105–109.
38. Levine RF, Eldor A, Shoff PK, Kirwin S, Tenza D, Cramer EM. Circulating megakaryocytes: delivery of large numbers of intact, mature megakaryocytes to the lungs. *Eur J Haematol*. 1993;51(4):233–246.
39. Li Y, Jin C, Bai H, et al. Human NOTCH4 is a key target of RUNX1 in megakaryocytic differentiation. *Blood*. 2018;131(2):191–201.
40. Lordier L, Bluteau D, Jalil A, et al. RUNX1-induced silencing of non-muscle myosin heavy chain IIB contributes to megakaryocyte polyploidization. *Nat Commun*. 2012;3:717.
41. Lorenz V, Ramsey H, Liu Z-J, et al. Developmental stage-specific manifestations of absent TPO/c-MPL signalling in newborn mice. *Thromb Haemost*. 2017;117(12):2322–2333.
42. Mazzi S, Lordier L, Debili N, Raslova H, Vainchenker W. Megakaryocyte and polyploidization. *Exp Hematol*. 2018;57:1–13.
43. Monteferrario D, Bolar NA, Marneth AE, et al. A dominant-negative GFI1B mutation in the gray platelet syndrome. *N Engl J Med*. 2014;370(3):245–253.
44. Nichols KE, Crispino JD, Poncz M, et al. Familial dyserythropoietic anaemia and thrombocytopenia due to an inherited mutation in GATA1. *Nat Genet*. 2000;24(3):266–270.
45. Notta F, Zandi S, Takayama N, et al. Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. *Science*. 2016;351(6269):aab2116.
46. Olnes MJ, Scheinberg P, Calvo KR, et al. Eltrombopag and improved hematopoiesis in refractory aplastic anemia. *N Engl J Med*. 2012;367(1):11–19.
47. Pleines I, Nieswandt B. RhoA/ROCK guides NMII on the way to MK polyploidy. *Blood*. 2016;128(26):3025–3026.
48. Pramono A, Zahabi A, Morishima T, Lan D, Welte K, Skokowa J. Thrombopoietin induces hematopoiesis from mouse ES cells via HIF-1 α-dependent activation of a BMP4 autoregulatory loop. *Ann N Y Acad Sci*. 2016;1375(1):38–51.
49. Prins D, Arias CG, Klampfl T, Grinfeld J, Green AR. Mutant calreticulin in the myeloproliferative neoplasms. *Hemasphere*. 2020;4(1):e333.
50. Psaila B, Wang G, Rodriguez-Meira A, et al. Single-cell analyses reveal megakaryocyte-biased hematopoiesis in myelofibrosis and identify mutant clone-specific targets. *Mol Cell*. 2020;78(3):477–492. e478.
51. Qi J, You T, Pan T, Wang Q, Zhu L, Han Y. Downregulation of hypoxia-inducible factor-1alpha contributes to impaired megakaryopoiesis in immune thrombocytopenia. *Thromb Haemost*. 2017;117(10):1875–1886.
52. Raghuwanshi S, Dahariya S, Musvi SS, et al. MicroRNA function in megakaryocytes. *Platelets*. 2019;30(7):809–816.
53. Raslova H, Komura E, Le Couédic JP, et al. FLI1 monoallelic expression combined with its hemizygous loss underlies Paris-Trousseau/Jacobsen thrombopenia. *J Clin Invest*. 2004;114(1):77–84.
54. Richardson JL, Shivdasani RA, Boers C, Hartwig JH, Italiano Jr. JE. Mechanisms of organelle transport and capture along proplatelets during platelet production. *Blood*. 2005;106(13):4066–4075.
55. Rodriguez-Fraticelli AE, Wolock SL, Weinreb CS, et al. Clonal analysis of lineage fate in native haematopoiesis. *Nature*. 2018;553(7687):212–216.
56. Sanjuan-Pla A, Macaulay IC, Jensen CT, et al. Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. *Nature*. 2013;502(7470):232–236.

57. Scheinin TM, Koivuniemi AP. Megakaryocytes in the pulmonary circulation. *Blood*. 1963;22:82–87.
58. Schulze H, Korpal M, Hurov J, et al. Characterization of the megakaryocyte demarcation membrane system and its role in thrombopoiesis. *Blood*. 2006;107(10):3868–3875.
59. Shin JY, Hu W, Naramura M, Park CY. High c-Kit expression identifies hematopoietic stem cells with impaired self-renewal and megakaryocytic bias. *J Exp Med*. 2014;211(2):217–231.
60. Sim X, Poncz M, Gadue P, French DL. Understanding platelet generation from megakaryocytes: implications for in vitro-derived platelets. *Blood*. 2016;127(10):1227–1233.
61. Song WJ, Sullivan MG, Legare RD, et al. Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. *Nat Genet*. 1999;23(2):166–175.
62. Sparger KA, Ramsey H, Lorenz V, et al. Developmental differences between newborn and adult mice in response to romiplostim. *Platelets*. 2018;29(4):365–372.
63. Sunderland N, Skroblin P, Barwari T, et al. MicroRNA biomarkers and platelet reactivity: the clot thickens. *Circ Res*. 2017;120(2):418–435.
64. Thon JN, Dykstra BJ, Beaulieu LM. Platelet bioreactor: accelerated evolution of design and manufacture. *Platelets*. 2017;28(5):472–477.
65. Tijssen MR, Cvejic A, Joshi A, et al. Genome-wide analysis of simultaneous GATA1/2, RUNX1, FLI1, and SCL binding in megakaryocytes identifies hematopoietic regulators. *Dev Cell*. 2011;20(5):597–609.
66. Vainchenker W, Plo I, Marty C, Varghese LN, Constantinescu SN. The role of the thrombopoietin receptor MPL in myeloproliferative neoplasms: recent findings and potential therapeutic applications. *Expert Rev Hematol*. 2019;12(6):437–448.
67. Vo KK, Jarocha DJ, Lyde RB, et al. FLI1 level during megakaryopoiesis affects thrombopoiesis and platelet biology. *Blood*. 2017;129(26):3486–3494.
68. Wang Y, Hayes V, Jarocha D, et al. Comparative analysis of human ex vivo-generated platelets vs megakaryocyte-generated platelets in mice: a cautionary tale. *Blood*. 2015;125(23):3627–3636.
69. Wechsler J, Greene M, McDevitt MA, et al. Acquired mutations in GATA1 in the megakaryoblastic leukemia of Down syndrome. *Nat Genet*. 2002;32(1):148–152.
70. Weiss CN, Ito K. microRNA-22 promotes megakaryocyte differentiation through repression of its target, GFI1. *Blood Adv*. 2019;3(1):33–46.
71. Wilcox DA. Megakaryocyte- and megakaryocyte precursor-related gene therapies. *Blood*. 2016;127(10):1260–1268.
72. Wright J. The origin and nature of blood platelets. *Boston Med Surg J*. 1906:23.
73. Xavier-Ferrucio J, Krause DS. Concise review: bipotent megakaryocytic-erythroid progenitors: concepts and controversies. *Stem Cells*. 2018;36(8):1138–1145.
74. Zhang MY, Churpek JE, Keel SB, et al. Germline ETV6 mutations in familial thrombocytopenia and hematologic malignancy. *Nat Genet*. 2015;47(2):180–185.
75. Zhang Y, Gao S, Xia J, Liu F. Hematopoietic hierarchy—an updated roadmap. *Trends Cell Biol*. 2018;28(12):976–986.

CHAPTER 30

INHERITED BONE MARROW FAILURE SYNDROMES

Yigal Dror

INTRODUCTION

Inherited bone marrow (BM) failure is defined herein as decreased production of one or more of the major hematopoietic lineages; i.e., red blood cells (RBCs), neutrophils, and platelets, due to germline mutations that were derived from the parents or occurred de novo (Table 30.1). Although outdated, the term "constitutional" has been used interchangeably with "inherited" and similarly implies that a genetic abnormality causes the BM dysfunction. The designation "congenital" has a looser connotation and refers to conditions that manifest early in life, often at birth, but does not imply a particular causation. Therefore, "congenital BM failure" is not necessarily inherited and may be caused by acquired factors such as viruses, drugs, or environmental toxins. Nevertheless, some inherited BM failure syndromes (IBMFSs) have been historically termed congenital (e.g., severe congenital neutropenia [SCN]) and remain so.

Hematopoiesis is an orderly but complex interplay of stem and progenitor cells, growth factors, BM stromal elements, and positive and negative cellular and humoral regulators. Thus, BM failure can potentially occur at several critical points in the hematopoietic lineage pathways. With regard to IBMFSs, germline mutations interfere with orderly hematopoiesis and cause the BM failure. The discovery of specific, high-penetrance mutant alleles associated with discrete IBMFSs provides evidence for this. Many of these alleles are of genes that directly affect physiologic cell survival, differentiation, and function in pathways that are essential for normal hematopoiesis (e.g., DNA repair, telomere maintenance, ribosome biogenesis, microtubule stabilization, chemotaxis, signaling from hematopoietic growth factors, signal transduction related to hematopoietic cell differentiation, and granulocytic enzymes). Modifying genes, epigenetic processes, acquired factors, and chance effects may also be operative and interact with the mutant genes to produce overt disease with varying clinical expression. Hence, the disorders listed in Table 30.1 are transmitted in a Mendelian pattern determined primarily by mutant genes with inheritance patterns of autosomal dominant, autosomal recessive, or X-linked types. Newly discovered IBMFSs may follow similar inheritance patterns or be multifactorial in origin caused by an interaction of multiple genes and a variety of exogenous or environmental determinants.

Data from the Canadian Inherited Marrow Failure Registry (CIMFR) suggest an incidence of about 65 cases diagnosed per million live births per year. As of December 2020, the most prevalent IBMFSs in this registry according to decreasing order are Diamond-Blackfan anemia (DBA) (22.2%), Fanconi anemia (FA) (14.2%), Shwachman-Diamond syndrome (SDS) (13.9%), dyskeratosis congenita (DC) (11.0%), Kostmann/SCN (8.0%), thrombocytopenia absent radii (TAR) syndrome (1.9%), and congenital amegakaryocytic thrombocytopenia (CAMT) (1.3%). It is noteworthy that 30% of the patients in this registry are unclassified and cannot be assigned a syndromic or genetic diagnosis. In the Israeli registry the frequency of IBMFS in decreasing order were reported as FA (52%), SCN (17%), DBA (14%), CAMT (6%), DC (5%), SDS (2%), and TAR syndrome (2%). Importantly, none of these syndromes is restricted to the pediatric age group. Patients with IBMFSs may be detected for the first time in adulthood. Reported cases include patients with FA, SDS, DC, DBA, and SCN among others, who first became evident when reaching adulthood. The genetics and pathophysiology of IBMFSs are closely linked to several common pathological and physiological process such as cancer and aging.

Historically, the IBMFSs were classified as "benign" disorders in contrast sharply with hematologic cancers. Patients with IBMFSs often die early in life from complications of cytopenias. However, in the current era of advanced supportive care and availability of recombinant cytokines and other effective therapeutics, patients with these conditions usually survive the early years of life and beyond. With the extended lifespan of patients, the natural history of these disorders has dramatically changed. One of the most sobering observations is that the many IBMFSs confer an inordinately high predisposition to developing myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). These include conditions such as SCN, SDS, FA, DC, CAMT, DBA, and TAR syndrome, among others. Thus, the distinction between "benign" and "malignant" hematology in the context of the IBMFSs has become blurred, and a new clinical and hematologic continuum is evident. Clearly, these disorders are leukemia-predisposition syndromes and several of them (e.g., FA, DC, and DBA) are broader cancer-predisposition syndromes. There is reason to believe that the first genetic "hit" or leukemia-initiating step may be the syndrome-specific inherited genetic abnormality itself, which initially manifests as the single- or multiple-lineage marrow failure state. The "predisposed" progenitor, already initiated, could conceptually develop decreased responsiveness to the signals that regulate homeostatic growth, terminal cell differentiation, or programmed cell death. Leukemic promotion and progression with clonal expansion leading to MDS or AML could then ensue readily.

INHERITED BONE MARROW FAILURE SYNDROME WITH MULTI-LINEAGE CYTOPENIAS

Fanconi Anemia

Background

FA is inherited in an autosomal recessive manner in 98% of cases. In rare cases, it is transmitted in an X-linked recessive or autosomal dominant mode.

Although the original report of FA in 1927 by Dr. Guido Fanconi described pancytopenia combined with physical anomalies in three brothers, many publications thereafter have underscored the clinical variability of the condition. FA is a *genomic instability disorder* characterized by chromosomal fragility and breakage, a defect in DNA repair, progressive BM cell underproduction, peripheral blood cytopenias, developmental anomalies, and a strong propensity for hematologic and solid tumor cancers.

FA patients may present with either physical anomalies but normal hematology, or normal physical features but abnormal hematology, normal physical features *and* normal hematology, or physical anomalies and abnormal hematology (Fig. 30.1). There can also be sibling heterogeneity in presentation with discordance in clinical and hematologic findings, even in affected monozygotic twins. Using published information, the median age at diagnosis of FA is about 6.5 years with a reported range from birth to 49 years. However, advances in genetic diagnostic tools may result in early diagnosis of young siblings or adults who do not have apparent clinical manifestations.

TABLE 30.1 **Inherited Bone Marrow Failure Syndromes and Familial Myelodysplastic Syndrome/Acute Myeloid Leukemia: Inheritance and Mutated Genes**

Disorder	Inheritance	Gene
IBMFSs With Multilineage Cytopenia and Familial MDS/AML		
Fanconi anemia	AR	*FANCA, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG/XRCC9, FANCI, FANCJ/BRIP1, FANCL/PHF9, FANCM, FANCN/PALB2, FANCO/RAD51C, FANCP/SLX4, FANCQ/ERCC4, FANCR/RAD51, FANCS/BRCA1, FANCT/UBE2T, FANCU/XRCC2, FANCV/REV7, FANCW/RFWD3*
	XLR	*FANCB*
Mixed Fanconi anemia/xeroderma pigmentosa/Cockayne syndrome)	AR	*ERCC1/XPF*
Shwachman-Diamond syndrome	AR	*SBDS, DNAJC21, EFL1*
Dyskeratosis congenita	XLR	*DKC1*
	AD	*TINF2, TERC, TERT, RTEL1, PARN, ACD(TPP1)*
	AR	*TERT, RTEL1, PARN, ACD(TPP1), NOP10, NHP2, WRAP53(TCAB1), CTC1, POT1, RPA1*
Congenital amegakaryocytic thrombocytopenia	AR	*MPL*
SRP72-associated hereditary aplastic anemia/MDS	AD	*SRP72*
ERCC6L2-associated hereditary aplastic anemia/MDS	AR	*ERCC6L2*
THPO-associated hereditary aplastic anemia/MDS	AR/AD	*THPO*
Reticular dysgenesis	AR	*AK2*
Cartilage-Hair hypoplasia	AR	*RMRP, POP1, NEPRO*
Pearson syndrome	Maternal	*mDNA*
Familial thrombocytopenia with predisposition to AML	AD	*RUNX1/CBFA2*
	AD	*ETV6*
GATA2-associated disorders (MonoMac syndrome, Emberger syndrome, familial MDS syndrome)	AD	*GATA2*
Bone marrow failure and diabetes		*DUT*
Familial MDS/AML (Others)		*CEBPA*
Familial MDS/AML (Others)		*DDX41*
Seckel syndrome	AR	CEP152, CENPJ, CEP63, NIN, PLK4, CDK5RAP2, ATR, RBBP8, ATRIP, DNA2
Schimke immunoosseous dysplasia	AR	*SMARCAL1*
Dubowitz syndrome	AR	*NSUN2, LIG4,*
	AD	-14q32, -17q24, -19q13
Rothmund-Thomson syndrome	AR	*RECQL4*
Nijmegen breakage syndrome	AR	*NBN*
IBMFSs With Predominantly Anemia		
Diamond-Blackfan anemia	AD	*RPS7, RPS10, RPS15, RPS15a, RPS17, RPS19, RPS24, RPS26, RPS27, RPS27a, RPS28, RPS29, RPL5, RPL9, RPL11, RPL15, RPL18, RPL26, RPL27, RPL31, RPL35, RPL35a*
	XL	*TSR2*
		GATA1
	AR	*EPO*
	AR	*ADA2/CECR1*
Inherited sideroblastic anemia	XL	*ALAS2*
	XL	*ABCB7*
	AR	*SLC19A2, GLRX5, PUS1, SLC25A38, YARS2, TRNT1*
	Maternal	*MT-ATP6*
Congenital dyserythropoietic anemia type I	AR	*CDAN1, CDIN1*
Congenital dyserythropoietic anemia type II	AR	*SEC23B*
Congenital dyserythropoietic anemia type III	AD	*KIF23*
Congenital dyserythropoietic anemia - unclassified	AR	*KLF1*

Continued

TABLE 30.1 **Inherited Bone Marrow Failure Syndromes and Familial Myelodysplastic Syndrome/Acute Myeloid Leukemia: Inheritance and Mutated Genes—cont'd**

Disorder	Inheritance	Gene
IBMFSs With Predominantly Neutropenia		
Kostmann/Severe congenital neutropenia	AD	*ELA2, GFI1, TCIRG1*
	AR	*HAX1, CSF3R, G6PC3, VPS45, JAGN1*
	XL	*WAS*
Cyclic neutropenia	AD	*ELA2*
WHIM syndrome	AD	*CXCR4*
Glycogen storage diseases Ib	AR	*G6PT1/SLC37A4*
Barth syndrome	XL	*TAZ*
Poikiloderma with neutropenia	AR	*USB1/C16orf57*
Neutropenia, immune deficiency, skeletal dysplasia and glycosylation defect	AR	*PGM3*
Cohen syndrome	AR	*COH1/VPS13B*
Dominant intermediate Charcot-Marie-Tooth	AD	*DNM2*
Hermansky-Pudlak syndrome, type 2 with neutropenia	AR	*AP3B1*
Hyper-IgM syndrome	XL	*CD40LG*
Autosomal dominant severe congenital neutropenia	AD	*SEC61A1*
Mitochondrial DNA depletion syndrome 13	AR	*FBXL4*
IBMFSs With Predominantly Thrombocytopenia		
Thrombocytopenia absent radii syndrome	AR	*RBM8A*
Thrombocytopenia with radio-ulnar synostosis	AD	*HOXA11*
Familial autosomal dominant non-syndromic thrombocytopenia	AD	*MASTL, ANKRD26, ACBD5, CYCS*
Familial platelet disorder with AML	AD	*CDC25C*
X-linked thrombocytopenia	XL	*WASP*
Mediterranean platelet disorder	AD	*GP1BA*
Familial thrombocytopenia	AD	*GFI1B*
Familial thrombocytopenia	AR	*FYB, SBF2*
Gray platelet syndrome	AR	*NBEAL2*
Epstein/Fechtner/Sebastian/May-Hegglin/Alport syndrome	AD	*MYH9*
Familial macro-thrombocytopenia	AR	FLNA, ABCG5, ABCG8, ACTN1, MYSM1, *PRKACG*
Familial macro-thrombocytopenia	AD	TUBB1, ITGA2, ITGA2B, ITGB3
Stormorken syndrome (thrombocytopenia with anemia)	AD	*STIM1*
Autosomal dominant non-syndromic sensorineural deafness type DFNA and thrombocytopenia	AD	*CDCL1 (MCM2)*

Modified from Dror Y. Inherited bone marrow failure syndromes: genetic complexity of monogenic disorders. In: *Genetic Disorders*. InTech Open Access Publisher. Available at http://www.intechweb.org.
AD, Autosomal dominant; *AML*, acute myeloid leukemia; *AR*, autosomal recessive; *IBMFSs, inherited* bone marrow failure syndromes; *MDS*, myelodysplastic syndrome; *UK*, unknown; *WHIM*, warts, hypogammaglobulinemia, infections, and myelokathexis; *XL*, X-linked recessive.

Epidemiology

The overall prevalence of FA is 1 to 5 cases per million with a carrier frequency of 1 in 200 to 300 in most populations. Data from the CIMFR showed a prevalence of 11.4 cases per million live births per year. It occurs in all racial and ethnic groups. Spanish Gypsies have the world's highest prevalence of FA with a carrier frequency of 1 in 64 to 1 in 70 for a common founder mutation. A founder effect has also been demonstrated in Afrikaners in South Africa in whom one specific mutation is common (frequency, 1 in 83), as well as in Ashkenazi Jews (1 in 89), Moroccan Jews, Tunisians, sub-Saharan African Blacks, Indian, Israeli Arabs, Brazilians, and Japanese.

Genetics

FA genes are all involved in DNA repair. The first clue for this defect was the abnormal chromosome fragility that is readily seen in metaphase preparations of peripheral blood lymphocytes or skin fibroblasts cultured with phytohemagglutinin (PHA) and is enhanced by adding a DNA interstrand cross-linking agent, such as mitomycin C (MMC) or diepoxybutane (DEB) (see Abnormal Chromosome Fragility section later). This feature was utilized to discover the first FA genes by complementation analysis. Complementation is considered when fusion of FA patient cells with cells from an individual who does not have mutations in the same gene (i.e., cell hybridization with cells from a healthy subject or a FA patient with mutations in another FA gene) result in correction of MMC or DEB hypersensitivity in growth inhibition or chromosomal fragility assays. Complementation has also been determined by transducing known FA gene cDNA into ungenotyped patient cells. The mutated gene was determined by a failure of fused cells with known mutation to correct (complement) the abnormal chromosome breakage in the patient's T cells in culture on exposure to DEB/MMC. This is why FA genes are called FANC

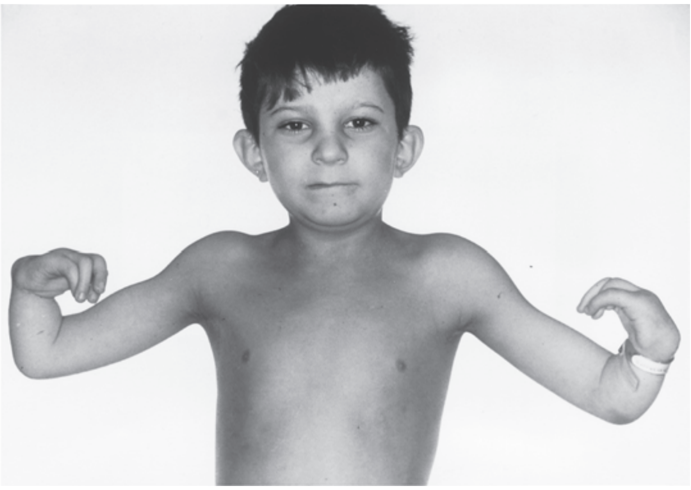


Figure 30.1 CLASSIC PHENOTYPE OF FANCONI ANEMIA. The patient has pigmentary changes around the neck, shoulders, and trunk; short stature; absent radii and absent thumbs bilaterally; microcephaly; and low-set ears.

genes (FA complementation). Recently, next generation gene panel assays replaced complementation testing for identifying the mutant FA gene, and whole exome/genome sequencing replaced the complementation testing for FA gene discovery. So far, 22 genetic groups (termed types A, B, C, D1, D2, E, F, G, I, J, L, M, N, O, P, Q, R, S, T, U, V, W) have been proposed (see Table 30.1). The first FA gene, *FANCC*, was discovered in 1992 in Toronto, and then the other genes, corresponding to each of the other complementation groups, were subsequently cloned: *FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ/BACH1/BRIP1, FANCL, FANCM, FANCN/PALB2, FANCO/RAD51C, FANCP/SLX4, FANCQ/ECCR4, FANCR/EXCC2*, and *FANCS/BRCA1, FANCT/UBE2T, FANCU/XRCC2, FANCV/REV7, FANCW*.

The most commonly mutated genes based on data from the International Fanconi Anemia Registry (IFAR) are FANCA (~61%), FANCC (~16%), and FANCG (~10). As of 2020, the most commonly used tool for genetic investigation of FA patients is by either next generation sequencing gene panel assays or single gene analysis. Partial or complete gene deletions are common in FA; hence, analysis of deletions should always be considered when nucleotide sequencing does not reveal pathogenic variants. Deletion analysis can be done by either subjecting next generation sequencing gene panel data to special copy number variation analysis software programs, or by utilizing multiplex ligation-dependent probe amplification) or microarray copy number variation (CNV).

Pathophysiology

Fanconi Anemia Genes and DNA Damage Repair

Cells and cell lines from FA patients are phenotypically similar regardless of the complementation group that they represent. A hypothesis was therefore formulated and subsequently substantiated that the various wild-type FA proteins function in a common response pathway to repair DNA damage incurred during DNA replication. A major function of FA pathway genes is to repair interstrand DNA crosslinks. Many exogenous agents (e.g. cisplatin, nitrogen mustards, and MMC) and endogenous agents (e.g. aldehydes and free oxygen radicals) can induce formation of interstrand DNA crosslinks.

There are three general steps in the FA DNA damage response pathway: (1) *Core complex*. Nine wild-type FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM, and FANCT) and additional proteins (e.g., FAAP20, FAAP24, and FAAP100) form a single large nuclear protein "core complex" as the first step. The core complex functions as a ubiquitin ligase of which FANCT, an E2 ubiquitin-conjugating enzyme and FANCL, an E3 ubiquitin ligase, are the catalytic subunits. The FA core complex is recruited to fork-like DNA structures via FAAP24 and FANCM. (2) *ID2 complex*. The activated *core complex* converts a downstream heterodimer composed of two proteins, FANCI and FANCD2 (called the *"ID2 complex"*), from unubiquitinated isoforms to monoubiquitinated isoforms. Monoubiquitination does not occur if the *core complex* upstream of the *ID2 complex* is not intact, and therefore FA cells from patients with upstream mutations do not show the monoubiquitinated FANCI/FANCD2. Recently, it has been shown that monoubiquitination occurs after the ID2 complex binds to an arrested replication fork at an ICL. (3) *Downstream effector complexes*. In normal cells after monoubiquitination of the ID2 complex, it forms a binding interface for single and double stranded DNA and downstream effector complexes with additional FA and other proteins. The scaffold protein FANCP/SLX4 binds first, followed by the endonucleases FANCQ(ERCC4), MUS81, SLX1, and ERCC1 that cleave DNA interstrand crosslinks, resulting in DNA adducts and dsDNA breaks. DNA adducts are resolved by the exonucleases FANCV/REV7, REV1, REV3 after forming a translesion synthesis complex. dsDNA breaks are repaired by homologous recombination. The exonucleases CTIP, MRN, XO1 cut the dsDNA breaks and generate ssDNA with 3′ overhang. Replication protein A (RPA) is recruited to the ssDNA break. FANCR/RAD51 recombinase localizes and loaded to the ssDNA/RPA complex by FANCD1/BRACA2 and FANCS/BRACA1. FANCR/RAD51 removes RPA and prevents it from self-binding. A recombination filament is generated by FANCR/RAD51 and the additional proteins that bind to the site in parallel or subsequently: FANCD1/BRCA2, FANCS/BRCA1, FANCO/RAD51C, FANCJ/BRIP1, FANCN/PALB2, and FANCU/XRCC1. Consequently, the recombination filament searches for homologous bases to repair DNA crosslinks.

There are three main DNA repair processes that the FA genes cooperate with: (1) *nucleotide excision repair* that excises one DNA strand flanking the interstrand crosslink (via interaction between FANCP/SLX4 and MUS81, SLX1, and others) followed by ligation. This process is utilized in quiescent cells; (2) *translesion synthesis* that involves one strand incisions around the interstrand crosslink (ICL), unhooking of the ICL, and extension of the uncut strand (via recruitment of translesion polymerase); (3) homologous recombination is initiated after nucleolytic incisions by endonucleases (FANCQ/ERCC4), MUS81, SLX1, ERCC1) and generation of dsDNA break. The dsDNA break is cut by exonucleases to generate a ssDNA break, to which FANCR/RAD51, FANCD1, FANCS, FANCN, FANCJ, FANCO, FANCU, and other proteins are recruited to form a recombination filament that searches for homologous bases for further repair. Other DNA-repair proteins such as MRE11-RAD50-NBS1, PCNA, and BLM are also involved in the later stages of the DNA repair response.

The exact link between the impaired ability to repair interstrand crosslinks and FA phenotype is still to be defined, but may be related to accumulation of DNA adducts, a failure to arrest DNA synthesis in response to DNA damage, impaired homologous recombination, defective nonhomologous end joining, abnormal induction of p53, induction of P53/CHK1 dependent G2/M cell cycle arrest, and increased apoptosis. In addition, homologous recombination and several FA proteins play a role in replication of telomeric G4 structures and possibly prevention of replication induced telomere damage. Loss of these functions may thus lead to short telomeres.

Fanconi Anemia Genes, Cell Survival, and Balancing Oxidative Stress

There are important protein-protein interactions between FA proteins and non-FA "binding partners" for cell survival. FANCC and FANCD2 form complexes with members of the signal transducer and activator of transcription (STAT) family of transcription factors in cytokine-mediated biologic responses. Secondly, heat shock proteins provide several cell survival functions, and FANCC protein specifically facilitates the anti-apoptotic role of Hsp70. FANCC also interacts with cdc2, PKR, and p53, suggesting that FANCC has other roles that are independent of DNA damage recognition and repair. GSTP1 is an enzyme that detoxifies byproducts of redox stress and xenobiotics and FANCC protein enhances GSTP1 activity in cells exposed to apoptosis inducers.

Several studies suggested a role of oxidative stress in the evolution of BM failure and leukemia in FA. Reactive oxygen species (ROS) were shown to be elevated in FA cells and high oxidative stress causes increased DNA damage, increased hematopoietic stem cell (HSC) senescence and a decreased HSC pool, thereby leading to BM failure. Further, in vivo and in vitro studies have demonstrated the ability of the antioxidant N-acetylcysteine to reduce DNA damage, reduce HSC senescence, and improve HSC reconstitution ability. Therefore, it is possible that patients with FA are particularly sensitive to ROS-induced DNA damage due to impaired DNA repair mechanisms. This increased sensitivity may be caused, at least in part, by impaired detoxification of ROS and naturally produced aldehydes. A deficiency in superoxide dismutase and poor cell growth at ambient oxygen have also been demonstrated in FA cells.

In FA patients' skin fibroblasts, N-acetylcysteine was able to reduce ROS levels and apoptosis as measured by activation of caspase-3 and poly(ADP-ribose)polymerase (PARP) cleavage. In fancc$^{-/-}$ mice, N-acetylcysteine rescues hematopoietic colony formation that is impaired by spontaneous secretion of TNFα. It also reduces TNFα-mediated hematopoietic colony formation and HSC senescence and HSC reconstitution potential. Using a fancd2$^{-/-}$ mouse model, treatment with the antioxidant drug resveratrol has also been shown to preserve HSC quiescence, partially correct the abnormal cell cycle status, and significantly improve the spleen colony-forming capacity of BM cells. Importantly, treatment of FA mice with N-acetylcysteine has been shown to reduce the accumulation of cytogenetic abnormalities (that are commonly seen in FA patients who transform to MDS/AML). In one study, the antioxidant tempol delayed cancer in tumor-prone fancd2$^{-/-}$/Trp53$^{+/-}$ mice. However, in another study neither N-acetylcysteine nor the antioxidant resveratrol had this property in this mouse model.

Cell lines from FA patients have also been shown to feature increased autophagy and mitophagy that was attributed to elevated levels of mitochondrial fission caused by high oxidative stress. In another study, interestingly, cells from FA patients showed impairment of mitochondrial functions as evidenced by a high frequency of mtDNA genetic variants, downregulation of mtDNA complex-I and complex-III encoding genes of OXPHOS, and reduced expression of certain mitophagy-related genes (ATG, Beclin-1, and MAP1-LC3) that may lead to reduced ability to clear damaged mitochondria.

The level at which oxidative stress is linked to FA phenotype independently of DNA damage is still to be defined. The high oxidative stress and oxygen sensitivity phenotype of FA cells shorten cell survival. A cardinal phenotype of FA cells is an abnormality in cell cycle distribution with an increased number of cells with 4 N DNA content arising from a delay in the G_2/M or late S phase of the cell cycle. The strongest evidence supporting an oxygen metabolism deficiency in FA is a *reduction* of FA cells with 4 N DNA content when grown at *low* oxygen levels and the unexpected appearance of 4 N DNA content when normal cells are grown at *high* oxygen levels. Of note, some wild-type FA proteins play a role in redox-related functions. FANCC associates with NADPH (nicotinamide adenine dinucleotide phosphate), cytochrome P-450 reductase, and glutathione S-transferase, proteins with redox functions. FANCA and FANCG are redox-sensitive proteins that multimerize after H_2O_2 treatment, prompting the notion that the FA pathway may function in oxidative stress management.

Pathogenesis of Bone Marrow Failure

Hematologic abnormalities in FA are evident at the hematopoietic stem and progenitor cell (HSPC) level with marked reduction in both multipotent (HSC, and multipotent progenitors, MPP) and oligopotent cells (common myeloid progenitors, CMP, megakaryocyte-erythroid progenitors, MEP), but a modest reduction in granulocyte-monocyte progenitors (GMP). Cure of FA BM failure by HSC transplantation (HSCT) supports the hypothesis that the hematopoietic defect starts at the HSC level. Clonogenic assays show reduced frequencies of CFU-E (colony-forming unit-erythroid), BFU-E (burst-forming unit-erythroid), and CFU-GM (colony-forming unit-granulocyte macrophage) colony-forming cells in almost all patients after aplastic anemia ensues as well as in a few patients before the onset of aplastic anemia. Besides low progenitor numbers, decreased colony numbers in these studies can also be interpreted as faulty proliferative properties which lead to an inability to form colonies in vitro. Indeed, there is a defective proliferative response of CFU-GEMM (colony-forming unit granulocyte, erythrocyte, macrophage, megakaryocyte), BFU-E, and CFU-GM progenitors to GM-CSF plus stem cell factor (SCF) (c-kit ligand) or to IL-3 plus SCF.

Additional factors are operative in FA BM failure. Telomeres, the non-encoding DNA at each end of chromosomes, shorten with each round of cell division in normal human somatic cells. Their length is a reflection of the mitotic history of the cell. Telomerase, a ribonucleoprotein reverse transcriptase that can restore telomere length, is variably present in hematopoietic progenitors. Leukocyte telomere length is significantly shortened in FA patients despite increased telomerase activity. In parallel, increased BM cell apoptosis has been demonstrated in FA patients and in knock-out mouse models and is mediated by Fas, a membrane glycoprotein receptor containing an integral death domain. FA cells exposed to tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), MIP-1α, Fas ligand, and double-stranded RNA undergo exaggerated apoptotic responses.

Studies of cytokines in FA patients have shown varied abnormalities. FA fibroblasts showed no deficiencies in SCF or M-CSF (macrophage colony-stimulating factor) production. Importantly, IL-6 production was found to be reduced in FA patients and TNF-α generation was markedly increased.

Initial attempts to generate induced pluripotent stem cells (iPSCs) from FA patients have been difficult since reprogramming causes increased DNA double-strand breaks and the FA pathway needs to be activated. This barrier could be bypassed by either correcting the genetic defect before reprogramming or performing the reprogramming under hypoxic conditions. Successful reprogramming resulted in cells that recapitulate the hematopoietic defect and identify the early pathogenetic defect at the stage of hemoangiogenic progenitors.

Interestingly, transforming growth factor-β (TGF-β) signaling was found to suppress FA cells. Blocking this pathway improved the survival and proliferation of HSPCs derived from FA mice and from FA patients. Further, inhibition of TGF-β signaling in FA HSPCs resulted in elevated homologous recombination repair with a decrease in non-homologous end-joining.

Mouse Models

Multiple FA mouse models have been generated in which targeted disruption of genes like *Fanca*, *Fancc*, *Fancd1*, *Fancd2*, *Fancg, Fancn,* among others has been achieved. Knock-out mouse models largely do not recapitulate the marrow hypocellularity and cytopenias that characterizes FA, with a few exceptions (e.g., $Slx4^{-/-}$ or combined $Fancc^{-/-}/Fancg^{-/-}$). Consistent findings in some or all of the mice include impaired proliferation of BM hematopoietic progenitors, hypogonadism, impaired fertility, growth retardation, microphthalmia, development of cancers, hypersensitivity of BM progenitor cells to MMC, as well as to INF-γ or TNF-α in vitro and in vivo. The phenotype of these mutant mice shows abnormal G_2/M progression of the cell cycle similar to FA patients. Interestingly, double knockout of several FA genes together with genes that play a role in balancing oxidative stress and other genotoxic agents (e.g., $\text{Fancd2}^{-/-}/\text{Foxo3a}$, $\text{Fancc}^{-/-}/\text{Sod1}^{-/-}$, $\text{Fancd2}^{-/-}/\text{Aldh2}^{-/-}$) leads to a phenotype that more closely resembles human FA. For example, $Fancc^{-/-}/Sod1^{-/-}$ mice develop a hypocellular BM; $Fancd2^{-/-}/Foxo3a^{-/-}$ mice feature an initial expansion followed by a progressive decline of BM stem and progenitor cells, and $Fancd2^{-/-}/Aldh2^{-/-}$ have reduced progenitor cell numbers and develop leukemia. $FancD2^{-/-}p53^{+/-}$ mice have a significantly increased incidence of tumors relative to either single mutant strain.

Clinical Features

The diagnosis of FA can readily be made based on signs and symptoms related to aplastic anemia and the presence of characteristic congenital physical anomalies. However, about 30% of the patients have no physical anomalies, and about 25% may be diagnosed with FA based on physical anomalies without yet developing cytopenias. Interestingly, family screening may detect affected family members who have neither physical malformations nor cytopenias at the time of diagnosis (about 7% of the patients).

Table 30.2 lists the characteristic physical abnormalities and their approximate frequency based on more than 2000 published case reports. The two most common anomalies are *skin hyperpigmentation* and *short stature*, each with a frequency of 40% of cases. Characteristically, the hyperpigmentation is a generalized brown melanin-like splattering that is most prominent on the trunk, neck, and intertriginous areas that becomes more obvious with age. Café-au-lait spots are also common. Hypopigmentation and vitiligo may also be seen. In the minority of cases with short stature, growth failure is associated with endocrine abnormalities. In one report, spontaneous overnight growth hormone secretion was abnormal in all patients tested, and 44% had a subnormal response to growth hormone stimulation. Approximately 40% of patients also have overt or compensated hypothyroidism, sometimes in combination with growth hormone deficiency.

TABLE 30.2 **Characteristic Physical Anomalies in More Than 2000 Published Case Reports of Patients With Fanconi Anemia**

Anomalies	Approximate Frequency (%)
Skin pigment changes or café-au-lait spots	40
Short stature	40
Upper limb anomalies (thumbs, hands, radii, ulnae)	35
Hypogonadal and genitalia changes (mostly male)	27
Other skeletal findings (head or face, neck, spine)	25
Eye, eyelid, or epicanthal fold anomalies	20
Renal malformations	20
Gastrointestinal or cardiopulmonary malformations	11
Ear anomalies (external and internal), deafness	10
Hips, legs, feet, toe abnormalities	5
Central nervous system imaging anomalies	3

From Shimamura A, Alter BP. Pathophysiology and management of inherited bone marrow failure syndromes. *Blood Rev.* 2010;24:101.

Malformations involving the upper limbs are common, especially hypoplastic, supernumerary, bifid, or absent thumbs. Hypoplastic or absent radii are always associated with hypoplastic or absent thumbs in contrast to TAR syndrome in which thumbs are always present. Less often, anomalies of the feet are seen, including toe syndactyly, short toes, a supernumerary toe, clubfoot, and flat feet. Congenital hip dislocation and leg abnormalities are occasionally seen. Male patients often have gonadal and genital abnormalities, including undescended, atrophic, or absent testes, hypospadias, an underdeveloped penis or micropenis, phimosis, and an abnormal urethra. Female patients occasionally have malformations of the vagina, uterus, or ovary. Renal anomalies such as ectopic, pelvic, or horseshoe kidneys are detected often, as are duplicated, hypoplastic, dysplastic, or absent organs. Occasionally, hydronephrosis or a hydroureter is present.

Many patients have a "*Fanconi facies,*" and unrelated patients can resemble each other almost as closely as siblings. The head and facial changes vary but commonly consist of microcephaly; small eyes; epicanthal folds; and abnormal shape, size, or positioning of the ears (see Fig. 30.1). Anomalies in the tympanic membrane and middle ear ossicles are seen in almost 70% of patients, resulting in hearing loss in most affected patients. Approximately 10% of FA patients have cognitive deficiencies.

Laboratory Findings

Peripheral Blood and Bone Marrow Findings

A cardinal feature is the gradual onset of BM failure with declining values in one or more hematopoietic lineages in the first decade of life, usually between 4 and 8 years of age. Fewer than 5% of patients develop hematological changes during the first year of life. Of 754 FA patients followed prospectively by the IFAR, 80% had hematologic abnormalities other than acute leukemia or MDS. The cumulative incidence of BM failure by 40 years of age was 90%. Thrombocytopenia with RBC macrocytosis usually develops initially, with subsequent onset of granulocytopenia and then anemia. Severe BM aplasia eventually ensues in most cases, but the degree of pancytopenia is variable and evolves over a period of months to years. The development of aplastic anemia can be accelerated by intercurrent infections or by certain drugs. Within families, there is a tendency for the hematologic changes to occur at approximately the same age in affected siblings, but this is not consistent.

The RBCs are macrocytic with mean corpuscular volumes (MCVs) often above 100 fL even before the onset of significant anemia. Erythropoiesis is characterized by increased fetal hemoglobin (HbF) levels. The increased HbF production has a heterogeneous distribution in contrast to most cases of hereditary persistent fetal hemoglobin. Elevation of RBC MCV and HbF is substantially higher than that seen in acquired aplastic anemia. Ferrokinetic studies indicate that most patients have an element of ineffective erythropoiesis. The RBC lifespan may be slightly shortened, but this is a minor contributory factor to the anemia.

In the early stages of the disease, the BM may not be hypocellular and can even show erythroid hyperplasia, sometimes with dyserythropoiesis, dysplastic changes, and even megaloblastic-appearing cells. Dysplastic changes may be very prominent with nuclear–cytoplasmic dyssynchrony, hypolobulated megakaryocytes, and binucleated erythroid cells; the findings are difficult to distinguish from MDS. As the disease progresses, the BM becomes hypocellular and fatty, sometimes in a patchy manner, and shows a relative increase in lymphocytes, plasma cells, reticulum cells, and mast cells. When full-blown BM failure occurs, the morphology of the BM biopsy is identical to severe acquired aplastic anemia.

Abnormal Chromosome Fragility. Abnormal chromosome fragility is the hallmark of FA and the recommended diagnostic test. A major finding in FA is abnormal chromosome breakage seen in metaphase preparations of peripheral blood lymphocytes cultured with PHA. The karyotype is characterized by chromatid breaks, rearrangements, gaps, endoreduplications, and chromatid exchanges. Cultured skin fibroblasts also show the abnormal karyotype, underscoring the systemic nature of the disorder. The abnormal lymphocyte chromosome patterns and the number of breaks per cell have no direct correlation with the hematologic or clinical course of individual patients.

Although the breakage is increased in these baseline lymphocyte cultures, it is strikingly enhanced by adding a DNA interstrand cross-linking agent. Many oncogenic and mutagenic inducers such as ionizing radiation, SV40 viral transformation, alkylating agents (e.g., cyclophosphamide and nitrogen mustard), and platinum compounds. Nevertheless, DEB and MMC have supplanted all the above for diagnostic testing.

For a definitive diagnosis of FA, the IFAR has determined increased numbers of chromosome breaks per cell occurring after exposure to DEB with a range of 1.06 to 23.9 compared with the normal control range of 0.00 to 0.10. Further supportive features are unusual chromosome abnormalities such as tri-radial and quadriradial figures. This pattern of abnormal chromosome breakage can also be used to make a prenatal diagnosis of FA (see below). Results of DEB testing of heterozygote carriers overlap with the results from healthy individuals.

A small proportion of patients with clinical FA do not show increased number of cells with chromosome breakage when treated with DEB or MMC. These patients usually have hematopoietic cell somatic mosaicism as a result of a genetic correction in a hematopoietic stem or progenitor cell, resulting in one normal allele. The mechanisms for this phenomenon include gene conversion events, back mutations, or compensatory deletions or insertions. The end result is mixed populations of somatic cells, some with two abnormal alleles and some with one. However, combined analysis of a number of cells with breaks, number of breaks per cell, and number of breaks per aberrant cell may help overcome most of these challenges. If FA is strongly suspected and the chromosome fragility test on peripheral blood cells is negative, a skin biopsy is performed to assess chromosomal breakage in cultured fibroblasts with DEB or MMC rather than in lymphocyte cultures.

Immunoblotting for FANCD2. Immunoblotting of the FANCD2 protein has been proposed as a diagnostic test for most cases of FA and/or as a tool to direct specific gene testing. In this assay, FANCD2 protein is analyzed in primary lymphocytes or fibroblasts after exposure to MMC or radiation by immunoblotting. The blot can distinguish the unubiquitinated and monoubiquitinated FANCD2. The results may suggest one of three situations: (1) in FA patients with a positive chromosomal breakage assay, no detection of FANCD2 suggests biallelic null mutations in this gene; (2) detection of FANCD2 without its monoubiquitinated form suggests mutations in one of the upstream core complex genes are predicted; and (3) detection of monoubiquitinated FANCD2 suggests mutation in one of the downstream FA genes such as FANCD1/BRCA2, FANCJ/BACH1/BRIP1, FANCN/PALB2, FANCO/RAD51C, FANCP/SLX4 etc. Monoubiquitination of FANCD2 is normal in other chromosomal breakage disorders. With the development of relatively rapid and affordable molecular diagnostic tests such as next generation sequencing this test is no longer used for diagnostic purposes.

FA cells (e.g., lymphocytes and skin fibroblasts) exposed to DNA interstrand crosslinking agents arrest in the G_2/M phase of the cell cycle, leading to a resultant 4 N DNA cellular content. This alteration can be detected by flow cytometry and has been used to diagnose FA. Transfection of a wild-type FA gene reduces G_2/M arrested cells as determined by cell cycle kinetics using flow cytometry, thereby pinpointing the mutant gene. Due to concerns about specificity and reproducibility and instrumentation cost, this method is not used clinically for the diagnosis of FA.

The majority of FA patients have stable, elevated levels of serum α-fetoprotein expressed constitutively that are independent of liver disease or androgen therapy. Levels are also unchanged after HSCT. The clinical utility of these findings is limited.

Imaging Studies. Ultrasonographic examination of the abdomen may reveal intraabdominal anomalies in various organs such as kidneys, urogenital system, and the gastrointestinal tract. Echocardiography may reveal cardiac anomalies. Radiography and computed tomography (CT) can be informative in revealing bone, intestinal, or other anomalies; however, imaging using radiation should be minimized as much as possible because of the carcinogenic risk, and should be replaced by magnetic resonance imaging (MRI).

Predisposition to Malignancy

A major feature of the FA phenotype is the propensity to develop cancer. The chromosome fragility, defects in DNA repair, genomic instability, oxidative stress, and the cellular damage that occur in FA patients translate into a significant predisposition to develop a malignancy. Because many genes are associated with FA and because alterations in the FA pathway are relevant to the pathogenesis of common types of cancers, the disorder is a critical human model of the genetic determinants of hematologic cancers and solid tumors.

FA can be considered as a member of two families of cancer predisposition syndromes. The first is composed of genetic disorders of DNA repair that include ataxia telangiectasia, xeroderma pigmentosum, and Bloom syndrome. The close relationship between FA and these syndromes is underscored by data showing convergence of signaling pathways in these conditions and the identification of *ERCC4* mutations in patients who manifest a complex phenotype of FA, xeroderma pigmentosa and Cockayne syndrome. The second family of predisposition syndromes consists of other inherited BM failure disorders described herein, including SDS and DC that have a propensity for malignant myeloid transformation or development of solid tumors.

The magnitude of the risk of developing malignancy in FA has been evaluated in several studies including literature reviews and analyses of registry data. In the National Cancer Institute study the observed to expected ratio of any malignancy was 19 in FA patients who have not received HSCT and was 55 in patients who received HSCT. The median patient age for the development of all cancers in a literature review was 16 years of age, which is strikingly different from the median age of 68 years for the same types of cancer in the general population. The risk of developing solid tumors in FA increases with advancing age.

In a study of the Canadian cohort, the cumulative risk of clonal and malignant myeloid transformation (of MDS and AML) after censoring patients transplanted for severe BM failure was 75% by the age of 18 years. The IFAR data indicate that the risk of acquiring clonal cytogenetic abnormalities was 67% by 30 years of age; the actuarial risk of MDS or AML was 52% by 40 years of age; and the cumulative incidence of leukemia was 33% by 40 years of age. This steady tempo of leukemic evolution implies a stepwise acquisition over time of additional, critical genetic “hits” before overt MDS/AML develops. In a literature review of 320 published FA cases, 14 patients developed leukemia. The risk of MDS was estimated as 5% to 10% and of leukemia as 5% to 10%. Studies from the IFAR showed that the risk of developing MDS and AML was higher for patients in whom a prior clonal BM cytogenetic abnormality had been detected. Monosomy 7, rearrangement or partial loss of 7q, rearrangements of 1p36 and 1q24-34, and rearrangements of 11q22-25 are frequent recurring cytogenetic abnormalities. Additional data indicate a strong correlation in FA BM cells of chromosome 3q26q29 partial trisomies and tetrasomies and rapid progression to MDS or AML. When interpreting the significance of clonal cytogenetic abnormalities in FA patients, note that clonal variation is frequent, including appearances of new clones, inability to detect established clones on repeat examination, and clonal evolution. In a study from France the investigators used SNP arrays to analyze whole marrow cells of FA patients with MDS/leukemia. They identified a relatively high frequency of somatic *RUNX1* gene disruption compared to that typically seen in patients with de novo MDS/AML. In a study of FA patients without apparent MDS/AML the GMP-cell population was reduced, but to a lesser degree than other multipotent and oligopotent progenitors. These cells harbored higher mutation rates than control subjects with a nucleotide variant signature that was similar to that seen in AML; increased G>A/C>T variants, decreased A>G/T>C variants, increased trinucleotide mutations at Xp(C>T)pT, and lower mutation rates at Xp(C>T)pG sites compared to other Xp(C>T)pX sites. The same pattern was seen in blast cells from a patient with FA and AML. These data suggest that the CFU-GM represent a cellular reservoir for clonal evolution.

The IFAR data indicates that by the age of 40 years, the cumulative incidence of nonhematologic cancers is 28%. In a literature review of 320 published FA cases, 25 non-transplanted cases had one to three separate types of solid tumors. Among the NCI cohort 21 of 163 FA patients developed solid tumors with an observed/expected ratio of 19. The most frequent solid tumor reported was squamous cell carcinoma involving the head and neck (mostly tongue) and upper and lower esophagus followed by the vulva or anus, cervix, brain, and skin. There were additional cases of tongue and oral squamous cell carcinoma as well as thyroid cancer and lymphoproliferative diseases that occurred after HSCT. Liver tumors, benign and malignant, were the second most frequent. Most of the patients with hepatomas and adenomas had received prior androgen therapy for aplastic anemia. Androgen administration has therefore been implicated in liver tumor pathogenesis. In descending order of frequency, cancers were also reported in the brain, kidney, breast, and adrenal gland.

Heterozygote Phenotype

Heterozygote carriers of FA gene mutations do not develop peripheral blood cytopenias or aplastic anemia, and cell lines from heterozygote carriers do not show excessive chromosome fragility in culture when exposed to DEB or MMC. The mean chromosomal breakage level of lymphocytes from FA carriers tested in cultures with a clastogenic agent may be higher than controls, but individual carrier testing may show overlap with normal values and severely limits its diagnostic utility. Literature from the early 1980s describes congenital anomalies of the hand and the genitourinary system in relatives of patients with FA, and parents of children with FA may have short stature. FA carriers may have increased levels of HbF, decreased natural killer (NK) cell counts, and diminished reactivity to mitogen stimulation. However, these results need to be reexamined using modern genetic diagnostic testing to exclude homozygosity and coexisting other conditions.

Monoallelic carriers for several FA genes are at increased risk of developing cancer, including for example, *FANCD1, FANCS*, *FANCN, FANCJ, FANCM*, *FANCP*, and *FANCO*. Female carriers of *FANCD1/BRCA2* and *FANCS/BRCA1* have an increased risk of breast cancer ranging from 40% at age 80 to a lifetime risk of about 80% and of ovarian cancer with a risk of up to 20% at age 70. Male carriers have a 7% risk of breast cancer and a 20% risk of prostate cancer before age 80. Other FA genes that are more prevalent in patients with breast cancer than in the general population, but to a much lower degree than FANCD1 and FANCS, are FANCN, FANCJ, FANCO, FANCP, FANCU, FANCD2.

Heterozygous mutations in FANCN are associated with pancreatic cancer, medulloblastoma, and high-grade glioma. Heterozygous mutations in *FANCJ*, *FANCP*, and *FANCO* are also associated with ovarian cancer.

Genotype–Phenotype Correlations

The clinical severity of FA is partly determined by the type of mutation and by the specific *FA* gene involved. Correlation between genotype and phenotype have been described but they were not consistently observed in different cohorts, which may be due to phenotype modifying variants that differ between cohorts and are possibly related to different ethnic backgrounds.

Several studies have found that patients with null mutations (e.g., deletions or nonsense point mutations in *FANCA*, *FANCB*, and *FANCG*) or missense mutations that lead to little residual protein function tend to have more severe physical malformations and an earlier onset of BM failure than patients with milder mutations. An earlier onset of leukemia was also found to be associated with null mutations in *FANCG*.

With regard to the impact of specific genes, several studies have shown association between specific genetic groups and certain phenotypes.

Severe physical anomaly phenotype is associated with *FANCB*, *FANCC* IVS4+4 A>T in Ashkenazi Jews, *FANCD1/BRCA2*, *FANCD2*, *FANCI, FANCN(PALB2)*

Early-onset BM failure is associated with *FANCC* (IVS4+4 A>T in Ashkenazi Jews and FANCI.

A significant predisposition to develop solid tumors, AML is associated with *FANCD1(BRCA2), FANCN(PALB2), and FANCM. FANCM* mutations were found in patients with cancer, but without physical malformations or BM failure. Patients with FANCA mutations developed cancer at a significantly older age as compared to patients with mutations in other Fanconi genes.

VACTERL-H association (combination of vertebral, anal, tracheoesophageal, renal, limb, and cardiac abnormalities) is often seen in patients with mutations in *FANCD1/BRCA2*, *FANCC* (IVS4+4 A>T in Ashkenazi Jews), and *FANCB*.

Ethnic backgrounds may affect phenotype severity in patients with the same mutations. For example, non-hematological phenotype that may include VACTERL-H association is often seen in Ashkenazi Jewish patients and mutations in *FANCC* (IVS4+4 A>T in), but not in Japanese patients with the same mutation.

Differential Diagnosis

About 30% of FA patients do not have physical anomalies, and such individuals may not be recognized until they present with aplastic anemia, MDS, AML, unilineage cytopenias, or macrocytic RBCs. Thus, FA should be part of the differential diagnosis in children and adults with unexplained cytopenias; characteristic birth defects; a diagnosis of aplastic anemia, MDS, or AML in patients mainly up to the age of 40 years, but sometimes also higher; unusual sensitivity to chemo- or radiotherapy; cancers typical of FA but at an atypical age such as cancer of the cervix when younger than 30 years or squamous cell carcinoma of the head and neck when younger than 50 years of age. Any of these should prompt consideration of FA as the underlying problem. All patients with idiopathic aplastic anemia who are younger than 40 years should have chromosomal fragility testing. However, if the test was not performed at diagnosis, patients with "idiopathic" aplastic anemia who fail to respond to immunosuppressive therapy with ATG and cyclosporine should be tested.

Although neutropenia is a consistent feature of ***SDS***, anemia or thrombocytopenia (or both) is seen in more than 50% of the patients and can be confused with FA. Because growth failure is also a manifestation of SDS, differentiating between the two disorders can initially be difficult. The major difference between them is that SDS is a disorder of exocrine pancreatic dysfunction that may or may not produce gut malabsorption. This can be confirmed by fecal fat analysis, by showing reduced levels of serum trypsinogen, serum isoamylase, or fecal elastase, and by reduced levels of fat-soluble vitamins such as A, D, and E. Nowadays pancreatic stimulation studies using intravenous secretin or cholecystokinin and measuring enzyme secretion is rarely done. Ultrasonography, MRI of the pancreas may also demonstrate fatty changes within the pancreas. Other skeletal features found in some patients with SDS include short flared ribs, thoracic dystrophy at birth, delayed bone maturation, and metaphyseal dysostosis of the long bones. Chromosomal analyses do not show spontaneous breaks in SDS, and increased breakage after clastogenic stress testing using DEB or MMC does not occur in SDS. Genetic testing for mutations in one of the SDS genes can help distinguish SDS from FA.

Dyskeratosis congenita (DC) shares some features with FA, including development of pancytopenia, a predisposition to develop solid tumors and leukemia, and skin pigmentary changes. However, the pigmentation pattern is somewhat different in DC and manifests with a lacy reticulated pattern affecting the face, neck, chest, and arms, often with a telangiectatic component. At some point, usually in the first decade of life, DC patients also develop dystrophic nails of the hands and feet and, somewhat later, leukoplakia involving the oral mucosa, especially the tongue. Other findings seen only in DC and not in FA are teeth abnormalities with dental decay and early tooth loss, hair loss, and hyperhidrosis of the palms and soles. Chromosomal fragility with DEB testing is typically normal in DC patients, which contrasts sharply with FA patients. Molecular analysis of DC genes is positive in about three-quarters of the patients (see Table 30.1).

Congenital amegakaryocytic thrombocytopenia (CAMT) and ***TAR syndrome*** both present in the neonatal period with thrombocytopenia. Patients with CAMT develop impairment in other blood cell lineages soon after presentation. A neonatal hematologic presentation is atypical for FA; fewer than 5% of patients are diagnosed during the first year of life. Neither CAMT nor the various thrombocytopenia syndromes above show chromosome fragility, which separates them from FA. Genetic testing is available for many of these disorders (see Table 30.1). In the TAR syndrome, thumbs are always preserved and intact despite the absence of radii, but in FA, the thumbs are hypoplastic or absent when the radii are absent.

Seckel syndrome, or "bird-headed dwarfism" manifests with short stature; microcephaly; cognitive delay; sinopulmonary infections; and a predisposition to developing lymphomas, pancytopenia, and AML. Some patients may show increased chromosomal breakage in lymphocyte cultures with DEB or MMC and mimic FA. There are several genes that have been linked to Seckel syndrome: mutant *ATR* has been associated with Seckel Type 1, *RBBP8* with Type 2, *CENPJ* with Type 4, *CEP152* with Type 5, *CEP63* with Type 6, and *ATRIP* with Type 8. Genotyping will distinguish FA from Seckel syndrome.

Nijmegen breakage syndrome (NBS) is an autosomal recessive disorder caused by mutations in the *NBS1* gene and is characterized by stunted growth, microcephaly, a distinctive facies, café-au-lait spots, immunodeficiency, and a predisposition to develop lymphoid malignancies. Some patients resemble those with FA, have BM failure, and may show increased chromosome breakage in lymphocyte cultures with MMC. The genetic defect is a mutant *NBS1* gene whose wild-type protein product is involved in DNA repair. Because NBS can mimic and be confused with FA, genotyping is essential and diagnostic.

Cells from patients with ***Bloom syndrome*** show abnormal spontaneous breakage, but unlike FA cells, the breakage does not increase in vitro in response to DEB. ***Ataxia telangiectasia*** is characterized by sister chromatid exchange without hypersensitivity to DEB or BM failure.

Natural History and Prognosis

The most serious early consequence in most FA patients is BM failure. The exceptions are patients with biallelic *FANCD1/BRCA2* mutations who have a cumulative probability of 97% of developing a malignancy by age 6 years, including AML, Wilms tumor, and medulloblastoma. By age of 40 years, patients with mutations in genes other than *FANCD1* have a cumulative risk of marrow failure, AML, and solid tumors of about 60%, 10%, and 15%, respectively. The risk of marrow failure plateaus at 70% at about 60 years of age, but the risk of solid tumors continues to rise. Treatment for cancer imposes additional problems and probably increases the risk for additional cancers secondary to therapy. Thus, the major causes of death in FA patients are sepsis and bleeding from BM failure, complications of HSCT, and progressive cancer or consequences of its treatment.

Despite these serious issues, the prognosis for FA patients is improving. Based on a literature review of more than 2000 FA case reports, the median survival from 1927 to 1999 was 21 years. In contrast, the median survival age from 2000 to 2009 was 29 years and 39 years in 2018. More than 80% of patients reach age 18 years or more. Earlier diagnosis, especially of mild cases, diagnosis of FA in young adults with AML or a solid tumor, comprehensive clinical and laboratory surveillance programs, timely therapeutic interventions, and HSCT are attributed to the improved outlook.

Therapy

Because of their clinical and psychosocial complexity, patients with FA should be supervised by a hematologist at a tertiary care center using a comprehensive and multidisciplinary approach. On the initial visit, the practitioner should take a detailed personal and family history, a careful physical examination with emphasis on physical anomalies, complete blood counts and chemistries, a BM biopsy for cellularity and morphology, and an aspirate for additional morphology, cytogenetics, and an iron stain looking for ringed sideroblasts. Chromosome fragility testing with and without DEB and/or MMC on peripheral blood lymphocytes on patients should be arranged. If FA is confirmed by DEB or MMC testing, genetic diagnosis should be offered to the family. Subsequently, imaging studies should be requested to search for internal anomalies. Because of the carcinogenic risk, imaging using radiation should be limited to those that may change management and may provide information that cannot be obtained by other methods or in a timely fashion. When all the results from the workup have been compiled, a discussion should be held with the patient and guardians about the diagnosis, management options, and prognosis. A referral to a genetic counselor should ensue. Blood counts, hemoglobin variant analysis, and chromosome fragility testing of siblings should be offered. High-resolution human leukocyte antigen (HLA) typing of the patient and immediate family members is recommended shortly after the diagnosis is established to determine potential matched-related donors in case HSCT becomes necessary.

If the patient is stable, has only minimal to moderate hematologic changes, and does not have transfusion requirements, a period of observation is indicated. During this time, subspecialty consultations (e.g., with orthopedic surgeons, urologists, gynecologists, and otolaryngologists) can be arranged. Blood counts should be frequently monitored to determine their stability. In a stable patient with mild cytopenias, blood counts can be monitored every 3 months, and BM evaluation should be performed annually. Falling counts, a clonal BM cytogenetic abnormality, or prominent multilineage dysplasia require more frequent clinic visits and blood and BM sampling to monitor for progression to severe aplastic anemia, MDS, or AML. Spectral karyotyping (SKY), fluorescent in situ hybridization (FISH), and comparative genomic hybridization of BM cells can enhance the diagnostic capability (Chapter 57). Analysis of mutations in specific cancer-related genes may be introduced into clinical practice in the future, but more research is necessary to determine their ability to predict progression to MDS/AML.

A surveillance program for solid cancers should be initiated at least annually. After the age of 10 years or one year after HSCT, the oral cavity should be examined every 6 months for signs of malignant changes because the risk in untransplanted FA patients is 700-fold that of the general population. Dentists, oral surgeons, or head and neck surgeons should be periodically recruited after the age of 10 years or after HSCT to screen for head and neck squamous cell carcinomas by rhinopharyngoscopy using a flexible endoscope. Beginning at age 13 years, all women with FA should be offered an annual gynecologic screening because the relative risk of vulvar squamous cell carcinoma is 4000-fold higher and cervical cancer is 200-fold higher than that of the general population. Human papilloma virus (HPV) DNA can be detected in 84% of FA squamous cell carcinoma specimens from various anatomic sites. Although the role of HPV in FA carcinogenesis is controversial, quadrivalent HPV vaccine is still recommended for boys and girls with FA at 9 years of age as a possible preventive approach.

Growth should be serially documented, and if growth velocity or stature falls below expectations, endocrine evaluation is needed to identify growth hormone deficiency. Diabetes mellitus occurs more commonly in FA, and random glucose levels should be evaluated annually or biannually. Based on the degree of hyperglycemia found on initial testing, fasting glucose levels and glucose tolerance tests should be performed. Screening for hypothyroidism should also be performed annually.

Androgens

Androgen therapy has been used to treat FA for decades. The overall response rate in the literature is about 50% heralded by reticulocytosis and a rise in hemoglobin within 1 to 2 months. If the other lineages respond to androgens, white blood cells increase next and then platelets, but it may take many months to achieve the maximum response. Accepted indications for treating with androgens are one or more of the following: hemoglobin level less than 8 g/dL or symptoms from anemia, platelet count less than 30,000/mm^3, and neutrophil count less than 500/mm^3. Oxymetholone, an oral 17-α alkylated androgen, is used most frequently at 1 to 5 mg/kg once a day. Depending on disease severity, oxymetholone can initially be administered either at 5 mg/kg/day and be decreased to the lowest effective dose or be started at a low dose of about 1 mg/kg/day and be increased monthly if there are no major side effects and an insufficient response. Although unproven, some clinicians add corticosteroids to offset androgen-induced growth acceleration and to prevent thrombocytopenic bleeding by promoting vascular stability. For this purpose, 5 to 10 mg of prednisone is given orally every second day. There are increasing data on the efficacy of the attenuated androgen, danazol, in FA; however, there are no comparative data with oxymetholone. Claims of reduced masculinizing side effects in female FA patients treated with danazol compared with those treated with oxymetholone have not yet been validated in clinical trials. A maintenance danazol dose of 2 to 5 mg/kg/day is probably sufficient to maintain good blood counts in those who respond. Another androgen, oxandrolone, has been evaluated for FA at Cincinnati Children's Hospital. Nine subjects completed this study and were followed for a median of 99 weeks (46 to 136 weeks). A third of the subjects developed mild sub-clinical virilization and continued treatment with a dose reduction. None of the patients had adverse behavioral changes. Two patients developed elevated liver function tests at 42 and 105 weeks. Seven patients had a hematologic response. Based upon this limited study, these investigators concluded that oxandrolone was well-tolerated, had a favorable toxicity profile in patients with FA, and may serve as an alternative androgen for the treatment of BM failure in FA patients.

If an injectable androgen is preferred to decrease the risk of liver toxicity and growth of hepatic tumors, nandrolone decanoate, 1 to 2 mg/kg/week, is given intramuscularly followed by the application of local pressure and ice packs to prevent the development of hematomas. When the response is deemed maximal or sufficient, the androgens should be slowly tapered but not stopped entirely.

Almost all patients relapse when androgens are stopped. The few who successfully discontinue treatment are often undergoing puberty when temporary "spontaneous hematologic remissions" have been observed. Most patients on long-term androgens eventually become refractory to therapy as BM failure progresses. Potential side effects include masculinization, which is especially troublesome in female patients, and elevated hepatic enzymes, cholestasis, *peliosis hepatis*, and liver tumors. Five complications of androgen therapy require consideration.

1. *Peliosis hepatis* is a cystic dilation of hepatic sinusoids that fill with blood and can be life threatening if they rupture. They may be clinically silent or produce right upper quadrant pain. Liver function test results are normal. Ultrasonographic examination is a safe way to diagnose the abnormality. The lesions may regress after stopping the androgens.
2. *Androgens also damage hepatocytes* nonspecifically. This may be manifest as cholestatic jaundice or elevated liver enzymes. Stopping androgen therapy usually leads to complete resolution. Hepatic cirrhosis may develop in patients on continued androgen therapy. If resolution of enzyme elevation does not occur after androgen withdrawal, a liver biopsy is indicated.
3. *Hepatocellular adenomas* are associated with androgen therapy. These are benign, noninvasive tumors. They can, however, rupture, leading to life-threatening bleeding. FA patients may develop these tumors rapidly, but they can be readily detected by imaging. The tumor may regress after stopping the androgens. If persistent, surgical resection or radiofrequency ablation may be necessary.
4. *Hepatocellular carcinoma* (HCC; hepatoma) occurs with androgen use, and some studies have suggested that FA patients on treatment may be at increased risk for HCC. The HCC associated with androgens is characteristically not associated with serum, α-fetoprotein elevations distinguishing it from de novo HCC. Patients developing HCC should discontinue androgen therapy.
5. Androgen therapy for FA patients has been found to be an adverse prognostic factor for those receiving a HSCT in one European study, but not in others. Unfortunately, comparative studies between androgen administration and HSCT from related or unrelated donors are not available and are probably not feasible due to the rarity of the disease.

Those receiving androgens should be evaluated serially with liver enzyme profiles every 2 to 3 months and ultrasonography of the liver every 6 to 12 months. If liver enzymes increase to above normal or if abnormalities appear on imaging, the androgen dose should be decreased or stopped.

Hematopoietic Growth Factors

Both granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) can induce a neutrophil response in neutropenic FA patients. G-CSF is indicated for a patient with recurrent or serious bacterial infection, especially if the neutrophil counts are less than 500/mm^3. In a published clinical trial of G-CSF in 12 FA patients, all 12 had an increase in absolute neutrophil numbers, 5 had a significant increment in hemoglobin levels, and 4 had an increase in platelet counts. Concurrent with the impressive improvements in blood counts, 8 of 10 patients who finished 40 weeks of G-CSF treatment showed elevations in the percentage of BM and peripheral blood CD34$^+$ cells. The starting dose for subcutaneous G-CSF is 5 μg/kg/day, and after a neutrophil response occurs, the dose can be decreased to every second day or two to three times a week. Long-acting pegylated G-CSF has not been studied in FA.

In another published clinical trial, combination cytokine therapy consisting of subcutaneous G-CSF 5 μg/kg once daily with erythropoietin 50 units/kg administered subcutaneously or intravenously three times a week was given to FA patients. Androgen therapy was added if the response was inadequate. Of 20 patients treated, 19 had improved neutrophil numbers, 6 had an increase in hemoglobin levels, and 4 achieved a sustained rise in platelets.

Because genomic instability and a marked predisposition to leukemia and cancer are features of FA, the wisdom of using granulopoietic growth-promoting cytokines on a long-term basis for FA is an issue. There may be a heightened risk of inducing or promoting expansion of a leukemic clone, especially one with monosomy 7. Therefore, before starting cytokine therapy, a baseline BM aspirate and biopsy is recommended, which then should be repeated every 6 months to document changes in morphology and cytogenetics.

Hematopoietic Stem Cell Transplantation

HSCT is the only clinically available curative therapy for the hematologic abnormalities of FA: aplastic anemia, MDS, and AML, though this may change if ongoing clinical trials of gene therapy are proven feasible, effective, and safe. The best donor source is an HLA-matched sibling in whom thorough history, physical examination, blood counts, HbF, chromosome breakage testing, and ideally genetic testing have excluded a diagnosis of FA.

Absolute indications for a matched sibling donor HSCT are (1) severe underproductive cytopenias (platelet $<20 \times 10^9$ L, ANC $<0.5 \times 10^9$ L, hemoglobin <70 g/L) and/or transfusion dependency; (2) high-risk MDS with chromosomal clonal abnormalities like monosomy 7, partial trisomies and tetrasomies of 3q26q29 and deteriorating counts, or a BM blast count of greater than 5%; or (3) overt AML. Decision to transplant for milder, relative indications should be made on a case-by-case basis.

Three caveats about transfusional supportive care before HSCT are (1) more than 20 exposures to blood products is a risk factor that adversely affects engraftment and survival posttransplant; (2) use of directed donations from family members may cause alloimmunization to an antigen that can increase the risk of graft rejection after a matched sibling donor or haploidentical related donor; and (3) single-donor apheresis platelets should be requested when required and available and the product should be leukodepleted and irradiated.

Initial efforts to transplant FA patients using standard myeloablative preparative regimens and graft-versus-host disease (GVHD) prophylaxis were plagued by two serious and often lethal problems, severe cytotoxicity from chemotherapy and irradiation and exaggerated GVHD. In vivo studies confirmed hypersensitivity of FA patients to standard doses of radiation. These observation resulted in developing reduced-intensity HSCT protocols that remain myeloablative for FA patients for both matched related donors (cyclophosphamide 20 mg/kg, single dose of TAI/TBI 400 to 500 cGy and ATG, resulting in 75% to 100% overall survival) and for alternative donors (cyclophosphamide 40 mg/kg, TAI/TBI 400 to 500 once, ATG, resulting in ~35% overall survival).

Over time, conditioning protocols utilizing irradiation have been almost entirely abandoned due to higher toxicity, lower long-term overall survival, and a potential increase in post HSCT solid tumors compared to regimens without radiation. Fludarabine, a purine antimetabolite with potent immunosuppressive and myeloablative properties with minimal toxicity to other tissues, has gained favor as an effective adjunct to preparative regimens. In the late 1990s/early 2000s fludarabine was introduced almost universally to conditioning regimens for patients with FA.

Modern protocols for HSCT incorporating fludarabine (e.g., cyclophosphamide 20–40 mg, fludarabine 140–180 mg/m^2 and horse or rabbit ATG, T-cell depleted BM or umbilical cord blood, and GVHD prophylaxis (based on cyclosporine or sirolimus + MMF or methylprednisolone) have resulted in 5-year overall survival of greater than 90%.

Using HLA-mismatched related donors, matched or one-antigen mismatched unrelated donors (MUDs) or unrelated cord blood carry a higher risk of complications and a lower disease-free survival than matched sibling donor HSCT (Chapters 105 and 106). Provided that no family members are suitable, BM or peripheral blood stem cell donors or cord blood from unrelated donors are sought and identified by searching donor and umbilical cord blood registries. Ideally, an unrelated donor is fully matched by high-resolution typing for all HLA-A, B, C, and DRB1 antigens, a so-called *8/8 match*. A second choice is a one-antigen mismatch unrelated donor. Matched or one-antigen mismatched banked unrelated umbilical cord blood is equally suitable to a one-antigen mismatched BM donor in FA and is an option. The last choice of an unrelated donor is a two-antigen mismatched cord blood. Using cord blood cells from unrelated donors, engraftment and survival are comparable to related or unrelated BM, although engraftment is slower with cord blood. The incidence of acute and chronic GVHD is reduced with cord blood grafts even in 1 or 2 HLA antigen mismatched transplants.

Recently, radiation-free HSCT in FA with T-cell depletion has been shown to be highly promising in FA patients transplanted with a MUD. In a multicenter prospective study conducted between 2009 and 2014, 45 patients age 4.3 to 44 years with BM failure (76%) or MDS (11% MDS) or other indications (13%) were transplanted. The donors were matched unrelated (56%), misMUD (31%), or misMRD (13%). The graft source was peripheral blood $CD34^+$ cells (1.1 to 63 × 10^6 cells/kg, Aim >2.5), CD3 cell depleted (0 to 5 × 10^4 cells/kg, Aim <5). The conditioning was intravenous busulfan (2.4 to 4 mg/kg with adjustment to <350 ng/mL), cyclophosphamide (40 mg/kg), fludarabine (140 mg/m^2), and rabbit ATG. One-year probabilities of overall and disease-free survival for the entire cohort were 80% and 78%, respectively

The best outcome predictors are recipients younger than 10 years old, recipient seronegativity for cytomegalovirus (CMV), history of fewer than 20 exposures to blood products, and use of fludarabine in the cytoreductive regimen. FA phenotype with three or more congenital malformations and prior administration of androgen therapy have been shown to adversely affect HSCT outcomes in some studies but not in others. Indications for an alternate donor HSCT are identical to those for a matched sibling donor HSCT.

HSCT from a haploidentical related donor have gained interest due to the almost universal availability of such donors (parents). Initially $CD34^+$ selected cells with or without ex vivo T-cell-depletion have been used in few FA patients. Recently a study from India and a study from a group of investigators from the USA and Brazil published results of haploidentical HSCT using in vivo T-cell depletion by post HSCT cyclophosphamide in 19 and 30 patients with FA, respectively. The group from India used fludarabine (180 mg/m^2), cyclophosphamide (10 mg/kg), TBI 200cG, post-HSCT cyclophosphamide 25 m/kg day +3+4, N-acetylcysteine during cyclophosphamide, and peripheral blood (81%) or BM (19%) (median CD34 dose: 5 × 10^6/kg). The group from USA/Brazil used fludarabine 150 mg/m^2, TBI 200 to 300 cGy, ± cyclophosphamide 10 mg/kg, ± rabbit ATG, post-HSCT cyclophosphamide 25 m/kg day +3+4, and unmanipulated BM graft. The 5-year overall survival was 68% in the study in India and 67% in the USA/Brazilian study.

Treatment of patients with FA with MDS/AML is challenging, at least in part due to the limited ability to high-intensity induction therapy. A collaborative French/Brazilian clinical trial used tandem FLAG (fludarabine/ARA-C/G-CSF) followed by HSCT regardless of response. Eighteen FA patients with advanced MDS or AML were enrolled. The trial showed relatively promising results. The transplant conditioning included cyclophosphamide 40 mg/kg, fludarabine 120 mg/m^2, TBI 2 Gy, ATG. In case of haploidentical transplants the patients also received post HSCT cyclophosphamide 30 mg/kg on day +4 and +5. The results were relatively promising; at 7 years, overall survival was 53% and GVHD-free/relapse free was 48%. In an EBMT retrospective study patients with FA and MDS/AML were transplanted with busulfan-based or TBI-based HSCT regimens with or without pre-HSCT chemotherapy. Overall survival and event-free survival were 42% and 39%, respectively. However, in this study there was no statistical difference in outcome if the patients received pre-HSCT chemotherapy or not.

Molecular technology has led to ***preimplantation genetic diagnosis*** (PGD) coupled with in vitro fertilization and selection of HLA-matched embryos for implantation. This is an option for parents who have a child with FA and a defined FA mutation but without a matched sibling donor. The mother's fertile eggs are harvested and fertilized with the father's sperm in vitro, resulting in a number of blastomeres. Using single-cell polymerase chain reaction (PCR) technology, isolated cells from several blastomeres can be tested for an HLA match and for absence of biallelic FA mutation. The selected HLA-compatible normal blastomeres can then be transferred and implanted in utero, resulting in a successful pregnancy and subsequent birth of a matched unaffected sibling. Cord blood from the PGD-selected healthy infant siblings can be banked for use as a graft for a HSCT for the affected sibling. The notion of "designer babies" is still debated in ethical circles.

Despite the successes of HSCT in correcting the BM failure of FA patients, there is a subset of survivors who develop ***cancers***, particularly squamous cell carcinoma of the head and neck (see also the section Predisposition to Malignancy above). These malignancies reflect the ongoing genetic susceptibility of FA nonhematopoietic tissue to cancer despite successful transplantation for aplastic anemia, MDS, or AML. Published data comparing cancer risks in transplanted and nontransplanted FA patients show a 4.4-fold increase in age-specific hazard rate of squamous cell carcinoma in the transplanted cohort. The causes for the increased cancer risk above the baseline seen in FA patients who have not been transplanted are not proven, but GVHD, especially chronic, and the preparative regimens of chemotherapy and irradiation are highly suspect. T-cell depletion has been introduced in some protocols to reduce GVHD, and irradiation has been reduced or eliminated in others to address this issue.

Gene therapy. Spontaneous mutation correction is common in FA patient cells and such cells may outnumber biallelically mutated cells. This is evident by mosaicism in chromosome breakage assays. Therefore, corrected cells through gene therapy are expected to have a growth advantage and expand in the BM. However, thus far gene therapy trials have not shown long-term preservation of substantial number of corrected FA cells in the transplanted patients or hematological improvement.

There are several challenges to gene therapy in FA. Mobilization, collection, and cryopreservation of enough peripheral blood CD34$^+$ cells from FA patients is feasible, particularly with usage G-CSF or plerixafor. However, this must efficiently be done before the onset of severe pancytopenia, since at this stage the HSC pool has been exhausted.

Several gene therapy studies have been conducted in FA and several others are currently being conducted in Europe and USA. Potential steps that may heighten the success with gene therapy are improving the vectors and/or correction methods, transduction of true HSCs, infusion of sufficient numbers of corrected HSCs, better preparation of patients and possibly giving conditioning before cell infusion, confusion of mesenchymal stem cells to improve stromal function, ensuring safety and eliminating off-target effects.

Genetic Counseling

Genetic counseling should be offered to all patients and families with FA. The discussion should include the implications of the identified (or not identified) underlying genetic lesion, important genotype phenotype correlations (e.g., high risk of early cancer in patients with *FANCD1/BRAC2* mutations), mode of transmission, risk of having the disease in family members, risk of recurrence in future pregnancies, available diagnostic tests during pregnancy, and PGD/IVF and selection of HLA-matched embryos who do not have FA as potential donors for HSCT. Screening of all first-degree relatives should be offered according to the methods available: medical history, physical examination, complete blood counts, hemoglobin F level, chromosome fragility, and mutation analysis.

During pregnancy, gene testing and/or the abnormal chromosome breakage patterns characteristic of FA can be used to make a prenatal diagnosis of FA. Diagnostic testing can be performed on fetal amniotic fluid cells obtained at week 16 of gestation or on chorionic villus biopsy specimens at 9 to 12 weeks of gestation.

A manual for the management of FA patients is available by the Fanconi Anemia Research Fund (see www.fanconi.org for *Fanconi Anemia Guidelines for Diagnosis and Management*, 4th edition, 2014).

Future Directions

The premise for gene therapy in FA is based on the assumption that corrected hematopoietic cells would have a growth advantage. Strengthening this supposition are FA patients with hematopoietic somatic mosaicism who show spontaneous disappearance of cells with the FA phenotype. These *mosaic* patients may show spontaneous hematologic improvement, suggesting that hematopoiesis was derived from stem cells with a normal phenotype.

Despite encouraging preclinical studies since the early 2000s using retroviral vectors showing that wild-type *FANCC* and *FANCA* can be integrated into normal and FA CD34$^+$ cells, the ensuing clinical trials in *FANCC* and *FANCA* patients using retroviral vectors were disappointing. A central problem was suboptimal wild-type gene integration into FA cells in culture. Because of the apoptotic phenotype and the sensitivity to oxidative stress, FA cells die rapidly in vitro before efficient gene transfer is accomplished. Changing the tissue culture conditions (e.g., usage of low oxygen condition) and introducing lentiviral vectors that can infect noncycling human cells were deemed the solutions. A few clinical trials for FA-A were opened in the late 2010s. Rio and colleagues reported promising results demonstrating successful engraftment of CD34$^+$ hematopoietic stem and progenitor cells carrying lentivector-mediated corrected FANCA gene in FA patients without conditioning. The procedure was well tolerated, hematopoiesis from CD34$^+$ cells was improved, but clinical efficiency could not be assessed due to the small number of patients. One caveat: a successful FA gene therapy protocol may correct BM failure and possibly the propensity for MDS and AML, but the predisposition for cancer in other tissues will continue unchecked.

Shwachman-Diamond Syndrome

SDS is a multisystem disorder characterized by varying degrees of BM failure, a high risk of leukemia, and exocrine pancreatic insufficiency. Additional features may include short stature and skeletal abnormalities. SDS is considered a ribosomopathy, as almost all patients have mutations in genes involved in the last stages of pre-60S ribosome subunit maturation.

Epidemiology

SDS has been reported among all ethnic groups. Older studies suggested a higher incidence in males. However, recent data suggest an equal distribution between genders as expected from a mainly autosomal recessive disorder. Based on data from the CIMFR, SDS is the third most common IBMFS with an incidence of 1 in 118,000 to 1 in 175,000 live births.

Genetic Basis

SDS is considered a ribosome-related disorder (ribosomopathy). The vast majority of patients with an SDS phenotype have mutations in genes involved in the last stages of pre-60S ribosome subunit maturation (*SBDS*, *DNAJC21*, *EFL1*). Some patients have a mutation in a gene related to the cotranslation protein-targeting pathway (*SRP54*), which also cause severe congenital neutropenia phenotype.

The first identified SDS gene is the *Shwachman-Boyden-Diamond-syndrome* (*SBDS*) gene that is mutated in 85% to 90% of SDS patients. The described mutations in this gene include null and splicing alterations, but never biallelic null, suggesting that a complete absence of SBDS protein is lethal. The common *SBDS* mutations are composed of sequences that are homologous to its pseudogene, *SBDSP*, and are believed to result from genetic recombination events in which *SBDSP1* acts as the donor. These recombinational events result in three common gene conversion mutations in exon 2 that account for 75% of *SDS* alleles: (1) a splice-site mutation, c.258+2T>C, which may either cause premature truncation of the **SBDS protein** by frameshift (p.C84fs3) or use an alternative splice site; (2) a nonsense mutation, c.183_184TA>CT that introduces an in-frame stop codon (p.K62X); and (3) an extended conversion mutation, c.183_184TA>CT and c.258+2T>C, that encompasses both mutations. In the Toronto database of 210 SDS families, 89% of unrelated SDS individuals carry a gene conversion mutation on one allele, and 60% carry conversion mutations on both alleles. The majority of patients are compound heterozygotes with respect to p.K62X and p.C84fsx3. Additional rare mutations in the *SBDS* gene have been identified in SDS patients. These include dozens of insertion, deletion, and missense mutations that have not arisen from gene conversion events. Most *SBDS* mutations alter the N-terminal domain of the protein and lead to markedly reduced protein levels.

DNAJC21 is the second gene identified to be associated with SDS. Biallelic mutation in *DNAJC21* can be identified in about 10% of SDS patients. *DNAJC21*-associated SDS is autosomal recessive. Both biallelic missense mutations and biallelic null mutations have been reported. Additional groups identified rarer SDS genetic groups with biallelic mutations in *EFL1* and monoallelic mutations in *SRP54*. All reported patients with biallelic *EFL1* mutations had at least one missense mutations.

Pathobiology

SDS genes encode highly conserved proteins that are ubiquitously expressed. SBDS protein has three main domains (N-terminal, middle, and C-terminal) with predicted protein-protein, protein-DNA, and protein-RNA binding motifs. SBDS protein is essential for life because patients with homozygous null mutations have not been reported, and small levels of residual protein can usually be detected in SDS patients. Furthermore, a complete loss of the protein in mice causes developmental arrest before embryonic day 6.5 and early lethality. In contrast to *SBDS*, biallelic null mutations in *DNAJC21* have been identified in SDS patients, indicating the complete loss of this protein is still compatible with life.

SBDS, DNAJC21, and EFL1 play a direct role in ribosome biogenesis. SBDS binds EFL1 on the 60 S large ribosome subunit, which through GTP hydrolysis causes release of the dissociation factor eIF6 and binding of 60 S to a mRNA-loaded 40 S subunit. Patient-related mutations in SBDS and in EFL1 have been shown to disturb this function. DNAJC21 is required for the release of the dissociation factor PA2G4 from the pre-60S ribosome subunit, which is critical for 60 S subunit maturation and association with 40 S. This function of DNAJC21 homolog in yeast (Jjjj1) is also required for the release of eIF6 homolog (Tif6) by SBDS homolog (Sod1).

Yeast strains deleted in *SBDS* and *DNAJC21* homologs accumulate their targets in the cytosol (eIF6 and Arx1, respectively), grow poorly at low temperatures, accumulate halfmer ribosomes and have reduced levels of mature ribosomes, all hallmarks of dysfunctional 60 S ribosomal subunit biogenesis. Loss of SBDS or DNAJC21 in human cells results in markedly reduced global translation.

As the first identified disease-related gene, *SBDS* functions have been studied more extensively than other SDS genes. It has been shown that SBDS is critical for several cellular pathways, including cell survival, telomere maintenance, mitotic spindle stabilization, chemotaxis, and marrow stromal function, besides ribosome biogenesis.

The SBDS protein can be detected in human cell nuclei and cytoplasm. It concentrates in the nucleolus during G1 and G2. SBDS interacts with multiple proteins with diverse molecular functions; many of them are involved in ribosome biogenesis, such as RPL4, and DNA metabolism, such as RPA70.

SBDS is critical for cell survival. When *SBDS* is lost in SDS BM cells or in *SBDS*-knockdown K562 and HeLa cells, the cells undergo accelerated apoptosis. The accelerated apoptosis in BM cells and *SBDS*-knockdown cells seems to be through the Fas pathway and not through the Bax/Bcl-2/Bcl-XL pathway. SBDS deficiency in primary SDS cells and in *SBDS*-knockdown cells results in abnormal accumulation of functional Fas (transcript 1) at the plasma membrane level.

Interestingly, knocking down *SBDS* in CD34$^+$ cells and in cell lines increased the levels of ROS, and antioxidants reduced Fas-mediated cell death and improved hematopoiesis from primary SDS cells. This suggests that *SBDS* promotes balanced levels of ROS, thereby protecting hematopoietic cells from cell death.

Patients with SDS have a defect in leukocyte chemotaxis. Consistent with this observation, the *SBDS* homologue in amoeba was found to localize to the pseudopods during chemotaxis. These observations suggest that the *SBDS* protein deficiency in SDS causes a chemotaxis defect in patients.

SBDS has been shown to colocalize to the mitotic spindle and bind microtubules and stabilize them. Its deficiency results in centrosomal amplification and multipolar spindles.

Shwachman-Diamond Syndrome Genes and Bone Marrow Failure

The pathophysiologic link between mutations in SDS genes and BM failure is still unclear. Colony forming assays have indicated a defect in CFU-GM, BFU-E, and CFU-GEMM colony formation in most patients, compatible with a defective stem cell origin of the BM failure. The BM phenotype is summarized in Table 30.3. SDS BM is characterized by decreased numbers of CD34$^+$ cells as well as an impaired ability for CD34$^+$ cells to form multilineage hematopoietic colonies in vitro, confirming that they are intrinsically defective. A recent study showed decreased levels of multipotent HSCs, MPPs, CMPs, MEPs, and, GMPs, though GMPs were the relatively more preserved than other HSC/HPCs.

To a certain extent, the hematopoietic failure is likely related directly to reduced global protein synthesis. Indeed human myeloid cell lines feature reduced translation, which is accentuated during differentiation, at least toward erythroid cells.

TABLE 30.3 Hematopoietic Abnormalities in Shwachman-Diamond Syndrome Patients

- Decreased bone marrow cellularity
- Decreased BM total CD34$^+$ cells, hematopoietic stem cells, multipotent cells, common myeloid progenitors, megakaryocyte-erythroid progenitors, granulocyte-monocyte progenitors
- Decreased colonies formation from CD34$^+$ cells
- Increased apoptosis of BM cells
- Apoptosis is mediated by Fas pathway
- Increased levels of reactive oxygen species
- Increased BM microvessel density
- BM cell upregulation of specific oncogenes
- Accentuation of the ribosome biogenesis defects with reduced ribosome subunits, ribosomes, and polysomes
- Accentuation of the protein translation defect during differentiation (toward erythroid cells)
- Abnormal telomere shortening of leukocytes
- Abnormal lymphoid immune function
- Impaired BM stromal cell function

BM, Bone marrow.

Several studies pinpointed apoptosis through the FAS pathway as a central pathogenetic mechanism that lead to reduced numbers of hematopoietic cells. As mentioned above, patients' BM cells overexpress FAS, and show increased patterns of apoptosis after preincubation with activating anti-FAS antibody. Induction of differentiation (at least toward the erythroid lineage) results in markedly accelerated apoptosis in SBDS-deficient cells, with only a minimal effect on proliferation.

Importantly, oxidative stress is also increased during differentiation of SBDS-deficient erythroid cells, and antioxidants enhance the expansion capability of both differentiating *SBDS*-knockdown K562 cells and colony production of SDS HSCs and progenitors. These studies indicate that when *SBDS* protein is deficient, several biologic pathways may be dysfunctional during hematopoietic cell development; this may be the cause of the high predilection for BM failure in patients with SDS.

A group from Boston used iPSCs from SDS patients and demonstrated an alternative mechanism for cell death. During differentiation of iPSCs to promyelocytes protease levels were increased and apoptosis was enhanced. Supplementing the culture media with protease inhibitors provided a rescue. Interestingly, TGF-β signaling pathway was also shown to be activated in SDS models (iPSCs, zebrafish, mouse) and in patient CD34$^+$ cells, and inhibition of the pathway improved hematopoiesis.

When the averages of telomere lengths adjusted to age were compared with those of control participants, a tendency toward shortening of telomeres was found in patient leukocytes, which may reflect premature cellular aging. This may represent either an inherent defect in telomere maintenance or compensatory stem cell hyperproliferation.

In addition to an inherent hematopoietic defect, it has also been shown that the BM stroma is markedly defective in terms of its ability to support and maintain normal hematopoiesis, which may contribute to the hematopoietic defect and the high frequency of engraftment failure during HSC transplantation in patients with SDS.

Clinical Features

The many clinical manifestations that occur in varying combinations have been reported in several publications and are shown in Table 30.4. Most patients present in infancy with evidence of growth failure, feeding difficulties, diarrhea, and infections. Steatorrhea and abdominal discomfort are frequent. Approximately 50% of patients exhibit a modest improvement in pancreatic function and do not require further pancreatic enzyme replacement therapy. Hepatomegaly is a common physical finding in young children but typically resolves with age and does not have clinical significance.

Patients with SDS are particularly susceptible to bacterial and fungal infections, including otitis media, bronchopneumonia, osteomyelitis, septicemia, and recurrent furuncles. Overwhelming sepsis is a well-recognized fatal complication of this disorder, particularly early in life.

Short stature is a common feature of the syndrome; the 50th percentile of SDS charts for height is positioned on the 3rd percentile of regular charts, both for boys and girls. When treated with pancreatic enzyme replacement, most patients show a normal growth velocity yet remain consistently below the third percentile for height and weight, indicating an intrinsic growth defect. Although metaphyseal dysplasia is a common radiologic abnormality (44% to 77% of patients), particularly in the femoral head and the proximal tibia, in most patients it fails to produce any symptoms. Occasional patients have clinical joint deformities, resulting in pain, functional impairment, or cosmetic problems, necessitating surgery. Some patients present at birth with respiratory distress caused by thoracic dystrophy. Others may have asymptomatic short and flared ribs.

The majority of the patients have deficits in cognitive abilities at varying levels of severity. These include delayed language development, low intellectual ability, impaired visual-motor integration, and failure to achieve higher order language functioning and problem solving. About one-fifth of the children have behavioral challenges such as attention deficit hyperactivity disorder, pervasive developmental disorder, or oppositional defiant disorder.

Some additional clinical features are seen very infrequently in SDS. Endocrine abnormalities include insulin-dependent diabetes, growth hormone deficiency, hypogonadotropic hypogonadism, hypothyroidism, and delayed puberty. Cardiomyopathies have been noted in some cases. Urinary tract anomalies, renal tubular acidosis, and cleft palate also occur. Retinopathy has been reported in SDS patients with *DNAJC21* mutations.

It is important to note that SDS phenotype might be mild and a diagnosis may be made only as part of a comprehensive genetic screening of adults with MDS. Nevertheless, to see if a subset of previously undiagnosed SDS patients presented for the first time with AML, 48 BM samples at remission were studied for mutations in SBDS, but none were found.

TABLE 30.4 Clinical and Hematologic Features of Shwachman–Diamond Syndrome Patients

Feature	Patients (%)
Pancreatic insufficiency (decreased digestive enzymes)	86–100
Neutropenia	81–100
Thrombocytopenia	24–70
Anemia	16–66
Pancytopenia	10–44
MDS/AML	≈30
Delayed bone maturation	100
Low-turnover osteoporosis	~100
Short stature	50 (38-
Metaphyseal dysplasia	44–77
Rib cage anomalies	32–52
Hepatomegaly or elevated enzymes	<50
Poor oral health (caries, ulcers, tooth loss)	>50
Learning and behavioral problems	>50

AML, Acute myeloid leukemia; *MDS*, myelodysplastic syndrome.

Laboratory Findings

Peripheral Blood and Bone Marrow Findings. The spectrum of hematologic findings has been reported in several publications and is summarized in Table 30.4. Neutropenia is present in almost all patients on at least one occasion. The neutropenia can be chronic or intermittent. Neutropenia has been identified in some SDS patients in the neonatal period during an episode of sepsis. Anemia is recorded in about half of the patients, but may fluctuate. RBC MCV and fetal hemoglobin are elevated in 60% and 75% of the patients, respectively, after the age of 1 year, which represents stress hematopoiesis. The combination of isolated neutropenia and high MCV or high HbF after the first year of life is seen in up to 28% of SDS patients and rarely in other IBMFSs. Reticulocyte responses are inappropriately low for the levels of hemoglobin in 75% of patients. Thrombocytopenia can be seen in about 40% of the patients and similar to anemia and neutropenia can also appear intermittently.

More than one lineage can be affected, and multi-lineage cytopenias have been observed in up to 65% of cases. Multi-lineage cytopenias can be due to profound severe aplastic anemia (Fig. 30.2). However, BM biopsies and aspirates vary widely with respect to cellularity; varying degrees of BM hypoplasia and fat infiltration are the usual findings. BM with normal or even increased cellularity has also been observed, typically in young children. The severity of neutropenia does not always correlate with BM cellularity, nor is the severity of the pancreatic insufficiency concordant with the hematologic abnormalities.

SDS neutrophils may have defects in mobility, migration, and chemotaxis. There appears to be a diminished ability of SDS neutrophils to orient toward a gradient of *N*-formyl-methionyl-leucyl-phenylalanine. An unusual surface distribution of concanavalin A has also been reported that reflects a cytoskeletal defect in SDS neutrophils. Whatever the magnitude of the chemotaxis abnormality is in vitro in SDS, neutrophil recruitment into abscesses or empyemas ensues robustly in vivo.

Immune Dysfunction. Impaired immune function can be significant in SDS and contribute to recurrent infections even if adequate numbers of neutrophils are present. Patients have various B-cell abnormalities, including one or more of the following: low immunoglobulin G (IgG) or IgG subclasses, low percentage of circulating B lymphocytes, decreased in vitro B-cell proliferation, and lack of

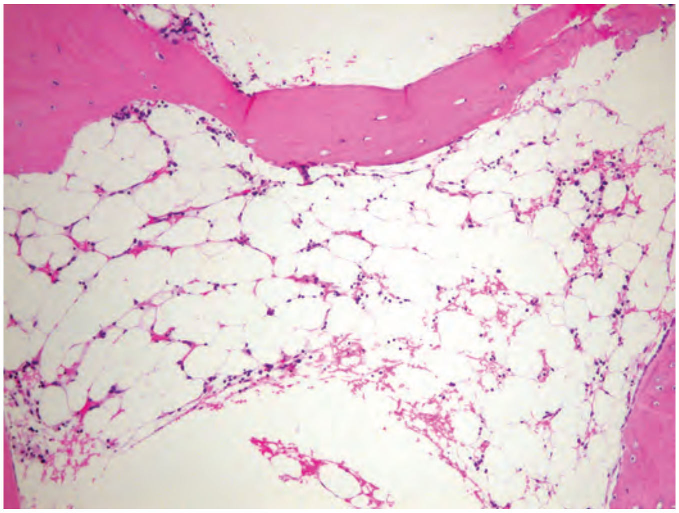


Figure 30.2 BONE MARROW BIOPSY IN SEVERE SHWACHMAN-DIAMOND SYNDROME SHOWING STRIKING HYPOCELLULARITY, FATTY CHANGES, AND TRILINEAGE APLASIA. *(Courtesy Dr. Mohamed Abdelhaleem, Toronto.)*

specific antibody production. Patients may also have T-cell abnormalities, including a low percentage of circulating T lymphocytes or subsets or NK cells, and decreased in vitro T-cell proliferation. Inverted CD4:CD8 ratios have also been described.

Exocrine Pancreatic Tests. The exocrine pancreatic pathology is caused by failure of pancreatic acinar development (Fig. 30.3). Pathologic studies reveal normal ductular architecture but extensive fatty replacement of pancreatic acinar tissue, which can be visualized by ultrasonography, CT, or MRI.

During the first 3 years of life, serum trypsinogen is typically reduced and can be used for diagnostic purposes. After the age of 3 years serum trypsinogen levels in SDS patients may normalize, which reduces its diagnostic usefulness at this stage. Serum isoamylase levels are reduced in SDS patients of all ages. However, normal children younger than 3 years have low isoamylase levels, so its measurement is not diagnostically useful at this age. Fecal elastase is another pancreatic enzyme that is reduced in SDS. Approximately 50% of patients exhibit a modest improvement in enzyme secretion with advancing age and normal fat absorption when assessed by 72-hour fecal fat balance studies. These patients do not require further pancreatic enzyme replacement therapy.

Pancreatic function studies using intravenous secretin or cholecystokinin can confirm the presence of markedly impaired enzyme secretion averaging 10% to 14% of normal but with preserved ductal function. Because of its invasive nature, this test has been replaced by measuring the levels of pancreatic enzymes in the serum.

Imaging Studies. Radiographs of the bone are useful as a screening diagnostic test for SDS. Osteopenia is seen in most patients but rarely results in clinical osteoporosis. Metaphyseal dysplasia has been reported in about 50% of the patients, particularly of the femoral heads, knees, humeral heads, wrists, ankles, and vertebrae. Rib-cage abnormalities can be found in 30% to 50% of patients. These include a narrow rib cage, short ribs, flared anterior rib ends, and costochondral thickening. Digital abnormalities such as clinodactyly, syndactyly, and supernumerary thumbs have been reported but are rare. Spinal deformities, including kyphosis and scoliosis, have been reported.

Patients with SDS do not have macroscopic brain malformations by MRI testing. However, they may have a decreased global brain volume (both gray matter and white matter) and a smaller posterior fossa, cerebellar vermis, corpus callosum, brainstem, and occipitofrontal head circumference compared with control participants. These anomalies might be the basis for the neurocognitive and neurobehavioral difficulties.

The French registry found cardiac anomalies in 11% of SDS patients. These include dilated and non-dilated cardiomyopathy, and structural malformations such as atrial septal defect, ventricular septal defect, coarctation of the aorta, and tetralogy of Fallot. Circumferential strain as measured by echocardiography was found to be decreased by the USA SDS registry, suggesting systolic dysfunction. Endocardial fibrosis and reduced left ventricular strain were also reported.

Cancer Predisposition. SDS is characterized by a high propensity to develop MDS and leukemia, particularly AML. The published crude rate for MDS/AML in patients with SDS ranges from 8% to 33%. In data from the CIMFR and the French Severe Chronic Neutropenia Registry, the cumulative risk of MDS/AML by the age of 18 and 20 years was 20% and 19%, respectively. The risk of leukemia in the French registry was 36% by the age of 30 years.

There is an increased frequency of BM clonal cytogenetic abnormalities as the sole evidence for a clonal disease in an otherwise hypocellular BM without excess blast counts or prominent multilineage cellular dysplasia. The incidence is roughly estimated to be 7% to 41% based on pooled published data; however, not all clones progress and some clones may not be detected on subsequent testing. Isochromosome 7q [i(7q)], an extremely uncommon finding rarely described in primary MDS or AML, was seen in up to 44% of SDS patients. This high occurrence suggests that it is a fairly specific marker for SDS and might be related to the mutant gene on 7q(11). Other chromosome 7 abnormalities are seen in 33% of SDS patients and include monosomy 7, i(7q) combined with monosomy 7 and deletions or translocations involving part of 7q. The prognostic significance of the cytogenetic changes requires prospective monitoring for clarification. Of the patients with i(7q), progression to advanced MDS with excess blasts or to AML has rarely been reported, but development of severe cytopenias and additional clones was described in three of four patients after long-term follow-up in the Canadian registry. Among a group of six patients with i(7q) from several hospitals in the United Kingdom, none progressed to advanced MDS/AML. In contrast, approximately 40% of patients with the other chromosomal 7 abnormalities progressed to either advanced MDS or to AML. Similarly, only rare cases with SDS patients and del(20q) evolve into advanced MDS/AML.

The pathophysiologic link between *SBDS* mutations and propensity to MDS and AML is unknown. Patients with SDS cells develop more frequent mutations than healthy subjects, possibly because of genomic instability due to mitotic spindle dysregulation or telomere shortening. It is also possible that impaired ribosome biogenesis and accelerated apoptosis cause a growth disadvantage for SDS BM cells, allowing for a growth advantage and expansion of malignant clones. Although molecular and cellular parameters do not distinguish SDS patients with transformation from SDS patients without

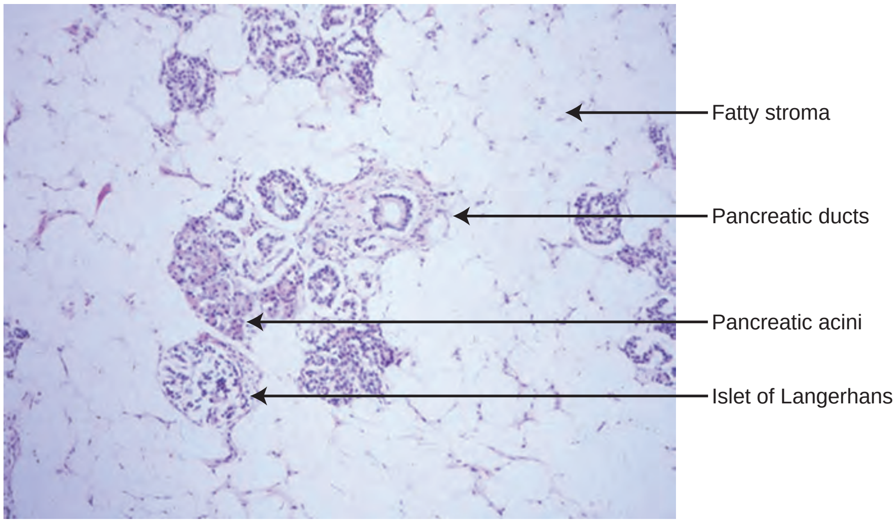


Figure 30.3 PANCREATIC TISSUE PATHOLOGY IN SEVERE SHWACHMAN-DIAMOND SYNDROME. The two classic features, deficiency of acinar tissue and fatty replacement, are shown. Islets of Langerhans are intact. *(Provided by Dr. Peter Durie, Toronto.)*

transformation, it is remarkable that SDS BM demonstrates many features characteristic of MDS. These include impaired BM stromal support of normal hematopoiesis, increased BM cell apoptosis mediated by the Fas pathway, telomere shortening of leukocytes, increased BM neovascularization, high frequency of clonal cytogenetic abnormalities, and abnormal leukemia-related gene expression in BM progenitor cells, e.g. overexpression of the oncogenes *TAL1* and *LARG*.

Similar to clonal evolution in FA, deep immunotyping analysis of early progenitors in SDS BM samples demonstrated relative selective survival of cells with GMP markers. Whole-exome and targeted sequencing of GMP-like cells in leukemia-free patients revealed a higher mutational load than in healthy controls and molecular changes that are characteristic of AML including increased G>A/C>T variants, decreased A>G/T>C variants, increased trinucleotide mutations at Xp(C>T)pT (X indicates any nucleotide), and decreased mutation rates at Xp(C>T)pG sites compared with other Xp(C>T)pX sites. Serial analysis of GMPs from an SDS patient who progressed to leukemia revealed a gradual increase in the mutational burden, enrichment of G>A/C>T signature, and emergence of new clones. Importantly, the molecular signature of marrow cells from a SDS patient with leukemia was similar to that of SDS patients without transformation. The predicted founding clones in SDS-derived AML harbored mutations in several genes, including TP53. The results suggest that GMP-like cells might represent a cellular reservoir for clonal evolution.

Monoallelic deletion of 20q10-11 and point mutations in EIF6 are commonly seen in SDS, and alleviate the SDS ribosome joining defect and translation by inactivating eIF6. In contrast, somatic monoallelic TP53 mutations, which are also frequently seen in SDS, decrease checkpoint activation and may progress to leukemia after developing a second mutation on the other allele, as demonstrated in one SDS patient and AML.

The vast majority of published cases of SDS-associated MDS/AML developed without previous G-CSF therapy. None of the six patients with SDS-associated MDS/AML from our institution were treated with G-CSF before transformation. However, it is still unclear whether G-CSF increases the risk of developing leukemia or promotes the expansion of existing malignant clones. Because G-CSF might increase neutrophil counts and prevent infections in SDS, a fraction of the reported patients with SDS-associated MDS/AML had been previously treated with G-CSF.

SDS patients with MDS/AML have common *SBDS* mutations, and no specific mutations have been shown to be associated with a higher risk of MDS/AML.

Several cases of solid tumors have been described in SDS. These include two cases of pancreatic ductal adenocarcinoma, one brain frontal lobe B-cell lymphoma, one dermatofibrosarcoma protuberans, and one breast cancer. However, more data are needed to determine whether the risk of solid tumors is higher than in the general population.

Genotype Phenotype Correlation

Despite the very similar function of *SBDS* and *DNAJC21* in ribosome biogenesis, some important phenotypic differences between these two SDS genetic groups have been observed. (1) Many *SBDS*-associated SDS have neutropenia without prominent anemia and thrombocytopenia. By contrast, hematopoietic failure in *DNAJC21*-associated SDS is often more global and affects more lineages and can be more severe. (2) Retinal disease is associated with *DNAJC21* mutations, but not with *SBDS* mutations. ELF1 mutations are associated with multi-lineage cytopenia, pancreatic insufficiency, and skeletal dysplasia. Patients with a *SRP45* mutation have a variable phenotype ranging from severe congenital neutropenia resembling ELANE-related disease to classical SDS.

Differential Diagnosis

The introduction of single gene analysis and later of comprehensive genomic testing has improved the ability to diagnose the disorder and particularly has helped identify cases with an atypical presentation. The diagnostic criteria have been summarized in an international consensus document and include having at least two of the following: (1) chronic BM failure, (2) exocrine pancreatic insufficiency, (3) positive genetic testing results or a first degree-relative with SDS. Several syndromes with overlapping features have to be excluded.

The syndrome of refractory sideroblastic anemia with vacuolization of BM precursors, or **Pearson syndrome**, is clinically similar to SDS but characterized by very different BM morphology. Severe anemia requiring transfusions rather than neutropenia is often present at birth and by 1 year of age in all cases. In contrast to SDS, the major BM morphologic findings are ringed sideroblasts with decreased erythroblasts and prominent vacuolation of erythroid and myeloid precursors. The disorder shares clinical similarities with SDS because of exocrine pancreatic dysfunction. Malabsorption and severe failure to thrive occur in approximately half of cases within the first 12 months of life. Qualitative pancreatic function tests show depressed acinar function and reduced fluid and electrolyte secretion. Approximately 50% of reported patients die early in life from sepsis, acidosis, and liver failure; the others appear to improve spontaneously with reduced transfusion requirements. At autopsy, the pancreas shows acinar cell atrophy and fibrosis, but fatty infiltration as seen in SDS is not a prominent feature. Patients may need pancreatic enzyme replacement. These patients have a diagnostic deletion of mitochondrial deoxyribonucleic acid (mtDNA). mtDNA encodes enzymes in the mitochondrial respiratory chain that are relevant to oxidative phosphorylation, including the reduced form of nicotinamide adenine dinucleotide dehydrogenase (NADH), cytochrome oxidase, adenosine triphosphatase (ATPase), mitochondrial transfer ribonucleic acids (tRNAs), and mitochondrial ribosomal RNAs. The degree of heteroplasmy affects the disease expression.

SDS shares some manifestations with **FA** such as BM dysfunction and growth failure, but patients with SDS can usually be distinguished because of malabsorption syndrome, fatty changes within the pancreatic body that can be visualized by imaging, and characteristic skeletal abnormalities not seen in patients with FA. In difficult cases with incomplete disease expression, the distinction relies on normal clastogenic stress-induced chromosome fragility testing and genetic testing of SDS and FA genes.

Atypical SDS cases with only little evidence of pancreatic changes can be difficult to distinguish from early-onset **dyskeratosis congenita** with no mucocutaneous manifestations. Telomere length testing showing very short telomeres (below 1% for age) is far more commonly seen in DC; nevertheless, when the diagnosis is unclear, genetic testing can help define the diagnosis.

Prognosis

Because of the carried clinical phenotype in SDS, the number of undiagnosed patients with mild or asymptomatic disease is unknown. Hence, the overall prognosis may be better than previously thought.

From a literature review, the projected median survival of SDS patients was calculated as 35 years. During infancy, morbidity and mortality are mostly related to infections, thoracic dystrophy, and malabsorption. Later in life, the major problems are hematologic or complications related to their treatment. Cytopenias tend to fluctuate in severity but do not fully resolve spontaneously. The most common cause of death in late childhood or adulthood is related to MDS/AML.

The impact of different SDS genetic groups on long-term outcome may vary, but needs to be further studied.

Therapy

Patient management is ideally shared by a multidisciplinary team consisting of a hematologist and a gastroenterologist as core members and other subspecialists such as an orthopedic surgeon, a dentist, and a psychologist as required. All patients with DNAJC21 mutations should also have an eye examination by an ophthalmologist.

The malabsorption component of SDS responds to treatment with oral pancreatic enzyme replacement with meals and snacks using guidelines similar to those for cystic fibrosis. Supplemental fat-soluble vitamins are also usually required. When monitored over time, approximately 50% of patients convert from pancreatic insufficiency to sufficiency because of spontaneous improvement in pancreatic enzyme secretion. This improvement is particularly evident after 4 years of age.

A long-term plan should be initiated for early detection of severe cytopenias that require corrective action or malignant myeloid transformation. There are currently no data about the cost effectiveness of a specific leukemia surveillance program in SDS. However, in a consensus document on SDS management it is generally recommended that surveillance should include periodic blood counts with differentials and blood smears every 3 to 4 months, a clinical evaluation by a hematologist every 6 months, and BM testing every 1 to 3 years. The latter includes aspirates for smears and cytogenetics analyses. Concomitant BM biopsies are recommended when the patient's clinical status changes. Recently it has been shown that an SDS BM testing may reveal evidence of MDS before changes are seen in the peripheral blood counts.

G-CSF

G-CSF given for profound neutropenia has been very effective in inducing a clinically beneficial neutrophil response. A report from the Severe Chronic Neutropenia International Registry (SCNIR) and unpublished data from the CIMFR demonstrated that treatment with G-CSF results in a brisk neutrophil response in about 90% of the patients. The response can be sustained in some cases for over 10 years

Androgens and Other Medications

A smaller number of patients have received androgens with or without steroids similar to their use in treating FA, and improved BM function has been observed in some. In the CIMFR database three patients were treated with androgens. Two had *DNAJC21* mutations, one of them responded. One had *SBDS* mutation and responded. One patient on the CIMFR was treated with eltrombopag without improvement [unpublished data]. Anecdotal cases treated with cyclosporine or erythropoietin do not allow broad therapeutic conclusions. A few patients who were treated with corticosteroids with some hematologic improvement have been reported in the 1980s.

Blood Products and Other Supportive Care

Anemia and thrombocytopenia are managed with transfusions of RBCs or platelets when symptoms appear or prophylactically for profound cytopenias. Antifibrinolytic therapy with tranexamic acid can also be given for mild mucosal bleeding. Broad-spectrum antibiotics are indicated for febrile episodes and severe neutropenia.

Hematopoietic Stem Cell Transplantation

At present, the only curative option for severe BM failure and MDS/AML in SDS is allogeneic HSCT. The indications for HSCT include bone marrow failure (BMF) with severe or symptomatic cytopenias, MDS with excess blasts (5% to 29%), or AML. Published data are limited and derived from relatively small case series with a mix of sibling and MUD.

The European Group for Blood and Bone Marrow Transplantation (EBMT) Registry reported 74 transplanted SDS patients. The indications included BMF (n = 61), MDS (n = 7), and AML (n = 6). Fifty-four percent were transplanted with myeloablative conditioning regimens that included busulfan or total-body irradiation and 46% with reduced intensity. The majority (68%) of the donors were unrelated, 24% were matched siblings, and 8% were parents. Overall survival at 3.5 years of those with BMF was 71% compared to 29% among patients with MDS/AML. Overall survival of patients with BMF remained 71% at 6 years post HSCT. Complications include graft failure (15%), acute GVHD grade I–IV (55%), chronic GVHD (20%), as well as relapse, infections, and organ toxicity. In this study the difference in overall survival between myeloablative (69%) and reduced intensity (57%) conditioning regimens was not significant (P = .7). The overall survival of patients who received TBI was 38% and of those who did not receive TBI was 79%, but the difference did not reach statistical significance (P = .1). The results of this study showed slight improvement from a previous publication from the same group in 2006.

In another retrospective multicenter study from the USA, 39 patients were transplanted either with reduced intensity (n = 26) or myeloablative (n = 13) conditioning from 2000 to 2017. Median age at transplant was 7 years (range from 0.4 to 51 years). Five-year overall survival of patients transplanted for BMF was 72% compared to 15% of those who were transplanted for MDS/AML (<0.001). Main causes of death among the patients with BMF were GVHD and graft failure. In contrast most common causes of death among the patients with MDS/AML were relapse and toxicity.

A note of caution is sounded regarding HSCT for SDS. Left ventricular fibrosis and necrosis without coronary arterial lesions have been reported in 50% of SDS patients at autopsy, suggesting that there may be an increased risk of cardiotoxicity as well as other problems with the intensive preparatory chemotherapy used in HSCT. Indeed, published data emphasized that complications are more common in SDS patients who receive chemotherapy or undergo HSCT than in patients with acquired aplastic anemia. Complications include toxicity (cardiac, neurologic renal, pulmonary, and venoocclusive disease), graft failure, and severe GVHD. The heightened HSCT-related toxicity can be explained by increased sensitivity to chemotherapy and radiation, resulting in massive apoptosis in various organs. The hypersensitivity might be related to shorter telomeres than in individuals in the general population, mitotic spindle destabilization, and to genomic instability. Increased risk of graft failure might be related to BM stromal defects that are not corrected by the allograft and might be aggravated by the conditioning regimen.

Reduced-intensity HSCT regimens have been proposed for patients with SDS, particularly if they have BMF without MDS/AML. Data from small studies utilizing Campath-1H, fludarabine, and melphalan or fludarabine, treosulfan, melphalan plus Campath-1H, or rabbit ATG demonstrated promising results. However, the larger and recent retrospective studies described above did not show superiority of this approach. The high risk of graft failure with both reduced intensity and myeloablative conditioning emphasize the need for innovative approaches to transplant SDS patients.

Future Directions

Several other clinical and basic research questions in SDS must be addressed. First, the various biochemical functions of SDS genes require further studies. How the protein products maintain normal hematopoiesis and protect from apoptosis as well as cancer is unclear. The natural history and risk factors for the development of complications need to be clarified. There is also a need to understand the mechanism for the heightened sensitivity of SDS patients to chemotherapy and irradiation and to develop effective and less toxic regimens for HSCT. Determining risk factors and molecular events during malignant myeloid transformation might prompt strategies for prevention and screening for complications. Further research is needed to develop new drugs that improve growth potential of HSCs and relieve the severity of the cytopenias. Clinical trials to explore efficacy and safety of agents that target pathways that were shown to be abnormal in SDS (e.g., translation, oxidative stress, and TGF-beta signaling) may help identify such drugs. Recently Ataluren, which promotes mRNA reading by the ribosome through premature stop codons, has shown to increase SBDS protein levels, improve CFU-GEMM and CFU-GM colony formation, and neutrophil maturation from SDS BM mononuclear cells as well as neutrophil survival. Ataluren has been approved for the treatment of patients with Duchenne muscular dystrophy and nonsense mutations, and might also be effective in SDS which will require evaluation in a clinical trial.

Dyskeratosis Congenita

Background

DC is an inherited multisystem disorder of the mucocutaneous and hematopoietic systems in association with a wide variety of other somatic abnormalities. Originally, it was considered a dermatologic disease, and was termed *Zinsser-Cole-Engman syndrome* after the first investigators who described it. The term "dyskeratosis congenita with pigmentation" was first proposed by Cole and colleagues, Rauschkolb, and Toomey in 1930. The traditional diagnostic ectodermal triad consists of reticulate skin pigmentation of the upper body, mucosal leukoplakia, and nail dystrophy. The skin and nail findings usually become apparent during the first 10 years of life, but the oral leukoplakia is commonly observed later. These manifestations tend to progress as patients get older. Age of presentation varies according to the genetic group.

Hematologic manifestations were reported in the 1950s, and in the 1960s/1970s BMF was recognized to be a major component of the syndrome and responsible for substantial morbidity and mortality. Indeed, the full diagnostic dermatologic triad is present in only about 46% of the patients, but BM failure of varying severity is reported in up to 90% of cases. With the recent advances in understanding the molecular basis of the disease, patients with hematologic abnormalities but without dermatologic findings have been identified that dramatically changed the historical definition of the disease. DC patients also have a predisposition to develop solid tumors and MDS/AML.

Epidemiology

The estimated incidence of DC in childhood is about 4 cases per million per year. In older literature, most DC patients were reported as males. However, with better understanding and broadening of the genetic and clinical spectrum of the disease and with more autosomal cases being identified, the proportion of males is much lower.

Genetics

DC is a telomere-related disorder (telomeropathy), and all the identified DC genes play a role in telomere homeostasis (see Table 30.1). DC genes encode components of the telomerase complex (*TERT*, *DKC1*, *TERC*, *NOP10*, and *NHP2*), T-loop assembly protein (*RTEL1*), telomere capping (*CTC1*), the telomere shelterin complex (*TINF2*), and the telomerase trafficking protein (*TCAB1*); all of which are critical for telomere maintenance.

The X-linked recessive disease is a common form of DC. It was originally estimated to comprise as many as 75% of DC cases, but with the identification of more DC genes and more patients with autosomal dominant inheritance, the true incidence is approximately 30%. The X-linked disease is caused by mutations in *DKC1* on chromosome Xq28. The encoded protein, dyskerin, is part of a complex (with *TERC*) that stimulates the telomerase (TERT). It is also involved in rRNA biogenesis.

The **autosomal recessive** forms of DC are caused by biallelic mutations in *RTEL1*, *PARN*, *NOP10*, *NHP2*, *TCAB1*, *CTC1*, *ACD*(*TPP1*), *or TERT*. Interestingly, several DC gene (*RTEL1*, *PARN* or *TERT, ACD*(*TPP1*)) mutations can be associated with a severe disease (Hoyeraal-Hreidarsson syndrome) when both alleles are mutated, or a milder disease when one allele is affected.

Several genes are mutated in families with autosomal dominant inheritance. *TINF2* is probably the most commonly mutated gene in this group and accounts for approximately 11% to 25% of the DC families. The vast majority of *TINF2* mutations are de novo, probably due to the severity of disease. Heterozygous mutations in *TERT, TERC, PARN, ACD*(TPP1), or *RTEL1* also result in autosomal dominant disease. In autosomal dominant DC where family members from several generations are affected, younger generations tend to have longer telomeres and more severe disease than their ancestors.

DC is a complex disorder and several clinicogenetic sub-types have been described. **Classical DC** that includes BMF and typical extra hematological manifestations can be caused by mutations in any of the DC genes (Table 30.3). DC with either hemizygous *DKC1* mutations or a heterozygous *TINF2* mutation or biallelic mutations in *TERT*, *PARN, RTEL1, WRAP53*(TCAB1), or *ACD*(TPP1) can result in a severe form of DC called **Hoyeraal-Hreidarsson syndrome**. It is characterized by hematologic and dermatologic manifestations of DC in addition to cerebellar hypoplasia. Immune deficiency is common when this syndrome is caused by *DKC1* mutations. **Revesz syndrome** is a combination of classical manifestations of DC and exudative retinopathy. It is caused by mutations in *TINF2* and is an autosomal dominant form of the disease. Some cases of **moderate/severe aplastic anemia** without physical anomalies are caused by mutations in *TINF2, TERT*, and *TERC*. In contrast to cases with biallelic *TERT* mutations, heterozygosity for mutations in *TERT* is associated with a milder phenotype, late presentation, severe aplastic anemia without physical malformations, isolated pulmonary fibrosis, isolated hepatic fibrosis, or a combination of these clinical sequelae. Heterozygosity for mutations in *TERC* is associated with a milder phenotype, late presentation and severe aplastic anemia, or MDS without physical malformations**. Coats plus syndrome** is caused by mutations in the *CTC1* gene. It is characterized by retinal telangiectasia and exudates, intracranial calcification, leukodystrophy, brain cysts, osteopenia, gastrointestinal bleeding, and portal hypertension caused by the development of vascular ectasias in the stomach, small intestine, and liver. Some patients with this disease have the additional manifestations of DC, which include sparse and gray hair, dystrophic nails, and anemia. Telomeres are short.

It is noteworthy that mutations in certain telomere-related genes can cause partial DC phenotype without BMF; for example, monoallelic mutations in *NAF1* or *ZCCHC8* causing pulmonary fibrosis and biallelic mutations in *STN1* causing Coats plus.

Pathobiology

The hallmark of DC is short telomeres. Most children have severely short telomeres (less than the first percentile for age). DC genes play a role in telomere length maintenance in several ways and can be divided into those who belong to telomerase complex (*TERT, TERC)*, other telomere replication-related factors (*RTEL1*, *CTC1*, *RPA1*), telomerase trafficking (*WRAP53*/TCAB1), TERC-associated factors that stimulate telomerase activity (*DKC1, NOP10, NHP2*), TERC-maturation factors (*PARN*), shelterin complex (*TINF2, POT1, TPP1*)

Telomerase complex (*TERT, TERC) TERT* encodes for the enzyme component of telomerase. Telomerase is a ribonucleoprotein polymerase that maintains telomere ends by synthesis and addition of G-rich telomere repeat TTAGGG at the 3′-hydroxy DNA terminus of telomeres using the *TERC* RNA as a template. *TERC* encodes for the RNA component of telomerase and has a 3′ H/ACA small nucleolar RNA -like domain. The H/ACA box at the 3′-end of TERC is essential for its stability and assembly into the telomerase pre-ribonucleoprotein complex.

Other telomere replication-related factors (*RTEL1*, *CTC1*, *RPA1*). RTEL1 is a helicase that resolves DNA secondary structures. It is recruited to telomers by TRF2 and unwinds the telomeric t-loop, thereby allowing telomere replication. CTC1 is a member of the CTC1-STN1-TEN1 (CST) trimeric complex that helps synthesize telomeric C-strand by binding to the telomeric 3′ overhang and stimulating DNA polymerase a-primase. RPA1 binds to telomeric 3′ overhangs during late S-phase to unfold G-quadruplexes and facilitate telomerase activity. A gain of function mutation was identified in a patient with classic DC.

WRAP53(TCAB1) is involved in **telomerase trafficking** to telomeres. *TCAB1* interacts with the CAB box on TERC, promoting localized telomerase in Cajal bodies and trafficking it to telomeres.

Mutations in the TCAB1 gene (*WRAP53*) impair this trafficking activity and lead to misdirection of telomerase RNA to nucleoli, thereby preventing elongation of telomeres by telomerase.

Several DC genes encode TERC-associated factors that stimulate telomerase activity: *DKC1, NOP10, NHP2. GAR1, TEP1,* and *NAF1* are also associated with TERC, but have not been shown to be mutated in DC as of December 2020. Dyskerin (encoded by *DKC1*) binds H/ACA classes of RNA. It binds to the 3′ H/ACA small nucleolar RNA-like domain of the *TERC*. This stimulates telomerase to synthesize telomeric repeats during DNA replication. Dyskerin is also involved in maturation of nascent rRNA. It binds to small nucleolar RNA through the 3′ H/ACA domain and catalyzes the isomerization of uridine to pseudouridine through its pseudouridine synthase homology domain. This might be the mechanism for impaired translation from internal ribosome entry sites seen in mice and human DC cells.

In the telomerase complex, the H/ACA domain of nascent human *TERC* forms a pre-ribonucleoprotein with dyskerin, NOP10, NHP2, and NAF1. Initially, the core trimer dyskerin-NOP10-NHP2 forms to enable incorporation of NAF1, and efficient reverse transcription of telomere repeats. NOP10 and NHP2 also play an essential role in the assembly and activity of the H/ACA class of small nucleolar ribonucleoproteins that catalyze the isomerization of uridine to pseudouridine in rRNAs.

Poly(A)-specific ribonuclease (PARN) is a TERC-maturation factor. It is one of the major mammalian deadenylases that trims single-stranded poly(A) tails of mRNAs, but also of oligoadenylated tails of H/ACA box snoRNAs and microRNAs. As such it trims excessive adenylate residues that are added to TERC by PARD5. In patient cells oligoadenylated TERC forms are more abundant in PARN-deficient patient cells than those of controls, although its total levels were slightly reduced.

The shelterin complex is composed of *TINF2, POT1*, and *TPP1*, which are mutated in DC, as well as *TRF1, TRF2*, and *RAP1* that as of December 2020 have not been shown to harbor mutations that cause DC. TINF2 protein binds to and protects telomeres by allowing cells to distinguish between telomeres and regions of DNA damage. In the complex, TINF2 binds to TRF1, TRF2, and TPP1.

In several acquired and IBMFSs besides DC, telomeres are shorter than in age-matched control participants. However, because the telomere maintenance pathway is profoundly impaired in DC, the telomeres in this disease are much shorter than in other disorders. Most children with DC have telomeres that are very short (lower than the first percentile of the normal range). Average telomere length in adults with DC is shorter than that of healthy subjects, but it is commonly at the low range of normal for age. Shortening of telomeres results in cellular senescence, apoptosis (“cellular crisis”), or chromosome instability. However, some cells may survive the crisis by harboring compensatory genetic mutations that confer proliferative advantage and neoplastic potential.

DC is a chromosome “instability” disorder of a different type than FA. Results of clastogenic stress studies of DC cells are typically normal. There is no significant difference in chromosomal breakage between patient and normal lymphocytes with or without exposure to bleomycin, DEB, MMC, or γ-radiation. This contrasts sharply with FA cells and distinguishes one disorder from the other. However, metaphases of cultured patient peripheral blood cells, BM cells, and fibroblasts show numerous spontaneous unbalanced chromosome rearrangements such as dicentrics, tricentrics, and translocations. These are probably caused by short telomeres.

Most studies of the pathogenesis of the aplastic anemia in DC have shown a marked reduction or absence of CFU-GEMM, BFU-E, CFU-E, and CFU-GM. Long-term DC BM cultures have shown that hematopoiesis is severely defective in all patients with a low frequency of colony-forming cells. The function of DC BM stromal cells is normal in their ability to support growth of hematopoietic progenitors from normal BM, but generation of progenitors from DC BM cells seeded over normal stroma is reduced, suggesting that the defect in DC is of stem cell origin. Telomerase is activated in HSCs and might be necessary for HSC self-renewal capacity and prevention of senescence. The BM failure in this disorder may be a result of a progressive attrition and depletion of HSCs. This is supported by studies showing reduced numbers of CD34$^+$/CD38$^-$ cells in patient BMs. Alternatively, the BM dysfunction may represent a failure of replication, maturation, or both.

iPSCs from DC patients have been shown to have defects in telomere elongation during programming in a manner that is concordant with the mutated gene in the patients. In iPSCs from patients with heterozygous mutations in *TERT*, telomerase activity is directly affected. iPSCs from a patient with *TCAB1* mutations featured mislocalization of telomerase from Cajal bodies to nucleoli. iPSCs from patients with mutant *DKC1* display reduced telomerase activity because of impaired telomerase assembly. Extended culture of *DKC1*-mutant iPSCs leads to progressive telomere shortening and eventual loss of self-renewal. In contrast, another group studied telomerase reactivation and *TERC* regulation during reprogramming and showed that reprogramming restores telomere elongation in DC cells despite genetic lesions affecting telomerase. This group showed that *TERC* upregulation is a feature of the pluripotent state and that several telomerase components are targeted by pluripotency-associated transcription factors.

Clinical Features

Clinical manifestations in DC often appear during childhood. The skin pigmentation and nail changes typically appear first; mucosal leukoplakia and excessive ocular tearing appear later; and by the mid-teens, the serious complications of BM failure and malignancy begin to develop. In a portion of the patients, BM abnormalities appear before or without the skin manifestations.

The DC Registry data from England have detailed the prevalence of somatic abnormalities in families with classic DC. Cutaneous findings are a typical feature of the syndrome. Lacy reticulated skin pigmentation affecting the face, neck, chest, and arms is a common finding (89%). The degree of pigmentation increases with age and can involve the entire skin surface. There may also be a telangiectatic erythematous component. Nail dystrophy of the hands and feet is the next most common finding (88%) (Fig. 30.4). It usually starts with longitudinal ridging, splitting, or pterygium formation and may progress to complete nail loss. Leukoplakia usually involves the oral mucosa (78%), especially the tongue (Fig. 30.5), but may also be seen in the conjunctiva, anal, urethral, or genital mucosa. Hyperhidrosis of the palms and soles is common, and hair loss is sometimes seen. Eye abnormalities are observed in approximately 50% of cases. Excessive tearing (epiphora) secondary to nasolacrimal duct obstruction is

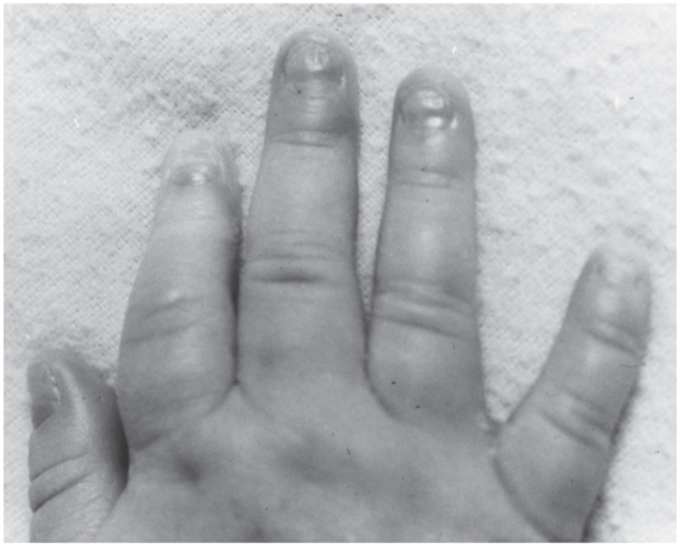


Figure 30.4 DYSTROPHIC NAILS IN DYSKERATOSIS CONGENITA.

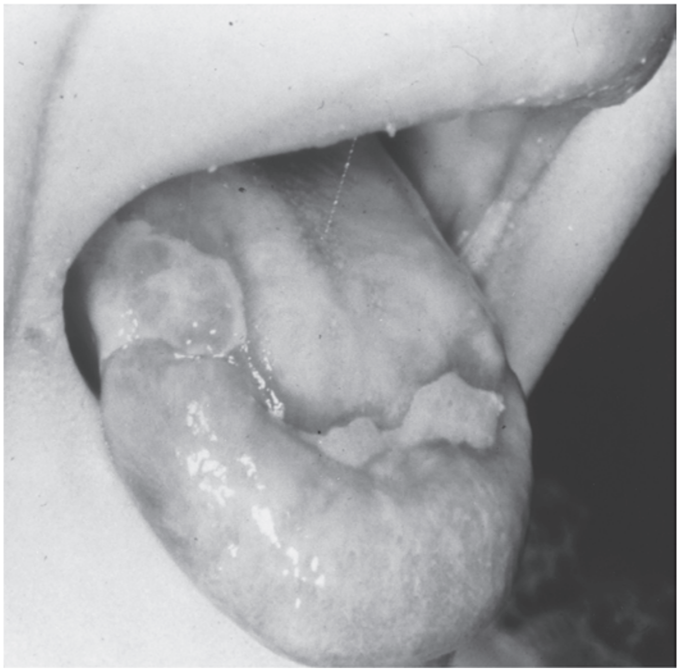


Figure 30.5 LEUKOPLAKIA OF THE TONGUE IN DYSKERATOSIS CONGENITA.

common. Other ophthalmologic manifestations include conjunctivitis, blepharitis, loss of eyelashes, strabismus, and cataracts and optic atrophy. Abnormalities of the teeth, particularly an increased rate of dental decay and early loss of teeth, are common. Skeletal abnormalities such as osteoporosis with recurrent long bone fractures, avascular necrosis, abnormal bone trabeculation, scoliosis, and mandibular hypoplasia are seen in approximately 20% of cases. Genitourinary abnormalities include hypoplastic testes, hypospadias, phimosis, and urethral stenosis and horseshoe kidneys. Gastrointestinal findings, such as esophageal strictures, hepatomegaly, or cirrhosis, are seen in 10% of cases. A subset of patients develops pulmonary fibrosis with a reduced diffusion capacity or a restrictive defect. In fatal cases, lung tissue shows pulmonary fibrosis and abnormalities of the pulmonary vasculature. Hepatic fibrosis may also occur. Vasculopathy of the gut, kidneys, liver, chest, or other organs is seen in severe cases and may cause massive bleeding.

Laboratory Findings

Peripheral Blood, Bone Marrow, and Immunologic Findings. The incidence of cytopenias caused by BM failure has been reported in up to 90% of the patients. Severe aplastic anemia occurs in about 50% of the patients. When BM failure is evident, most patients already have physical manifestations of DC, but this is variable. The initial hematologic change is usually thrombocytopenia, anemia, or both followed by full-blown pancytopenia caused by aplastic anemia. The RBCs are often macrocytic, and the fetal hemoglobin can be elevated. It is noteworthy that early BM specimens and biopsies may be normocellular or hypercellular; however, with time, the cellular elements decline with a symmetrical decrease in all hematopoietic lineages. Ferrokinetic studies at this point are consistent with aplastic anemia. Some patients with DC, particularly those with *DKC1* mutations, have immunologic abnormalities, including reduced immunoglobulin levels, reduced B- or T-lymphocyte numbers, and reduced or absent proliferative responses to PHA. Severe immunodeficiency necessitating HSCT has also been described.

Imaging studies can reveal a small-sized cerebellum which may provide a clue to the diagnosis in patients with atypical presentations. Imaging of the skeleton usually shows nonspecific osteopenia.

Telomere length is a useful screening test for DC. In the vast majority of patients, the telomeres are very short (i.e., lower than the first percentile adjusted to age).

Cancer Predisposition

Similar to FA, DC patients can develop solid tumors as well as MDS/AML, though the published risks of both solid tumors and MDS/AML in DC are lower than those in FA. At the age of 65 to 70 years, the cumulative incidence of solid cancers and leukemia were estimated as 20% and 5% to 10%, respectively. The cumulative incidence of MDS was estimated as 20% by the age of 50 years. Most of the cancers are squamous cell carcinomas or adenocarcinomas, and the oropharynx and gastrointestinal tract are involved most frequently. Some patients have multiple separate primaries in different sites involving the tongue and nasopharynx. Thus, the sites of most of the cancers involve areas known to be abnormal in DC, such as mucous membranes and the gastrointestinal tract.

Differential Diagnosis

Several physical findings can be used to distinguish **FA** from DC. The following abnormalities are seen only in DC and not FA: nail dystrophy, leukoplakia, abnormalities of the teeth, hyperhidrosis of the palms and soles, and hair loss. Specific presentations of DC may have prominent overlap with other syndromes. The Hoyeraal-Hreidarsson syndrome variant of DC and the Revesz syndrome variant of DC are two examples. **The ataxia–pancytopenia syndrome** at least in some families is a variant of DC with mutations in *TINF2*. The spectrum of manifestations in **Coats plus syndrome** may lead to an investigative workup directed toward inherited eye disorders and degenerative brain disease, but screening for DC by telomere length should be considered.

Natural History and Prognosis

Different genetic groups vary in natural history and prognosis. Patients with severe DC (e.g., Hoyeraal-Hreidarsson syndrome due to mutations in *DKC1*, *TINF2*, or others) have a multiorgan disorder and often an early onset of cytopenias. Such patients are at risk of early death from hematological and extrahematological complications. In moderate (historically considered “classical DC”), nail dystrophy and skin pigmentation occur first, often in the first 10 years of life. BM failure usually follows in the teenage years and 20s. The primary causes of death are hemorrhage secondary to thrombocytopenia or intestinal vascular anomalies, pulmonary fibrosis, sepsis from severe neutropenia, and complications after HSCT. In the patients who develop cancer or MDS/AML, these complications or its treatment can prove fatal. Pulmonary fibrosis can develop in 20% of cases and is typically progressive and culminates in death caused by respiratory failure. Considerable clinical heterogeneity exists even within the same family, and some patients live into their forties with only moderate nail changes and mild cytopenias. The median survival in the cases reported in the past decade was estimated at 49 years.

Therapy

Androgens

The management of aplastic anemia is similar to treatment for FA. Androgens improve BM function in about 70% to 80% of patients. If a response is achieved and deemed to be sufficient or maximal, the minimal androgen dose to maintain this response should be considered. Low doses of androgens (e.g., 0.5 to 2 mg/kg/day of danazol) are frequently sufficient to elicit response. As in FA, patients typically become refractory to androgens as the aplastic anemia progresses. Immunosuppressive therapy is not effective for this disorder, and a portion of the patients with DC are only diagnosed after failure to respond to immunosuppressive therapy for severe aplastic anemia. Since androgens have been shown to activate telomerase activity, the question whether they can alleviate symptoms related to non-hematological complications of DC needs to be answered.

G-CSF

A small number of patients have been reported who responded to G-CSF therapy with significant increases in absolute neutrophil counts (ANCs). Similarly, other patients received GM-CSF therapy that resulted in improved neutrophil numbers. G-CSF with erythropoietin has resulted in a trilineage hematologic response. G-CSF plus androgens was associated with splenic peliosis and rupture in DC in some cases and HSCT should be considered as an alternative long-term solution if a donor is available. Although the reports are scanty, cytokine therapy appears to offer potential benefit, at least for the short term, especially for improving granulopoiesis.

Hematopoietic Stem Cell Transplantation

Publications focusing on HSCT in DC are mostly isolated case reports or a small series that limit one's ability to make meaningful correlations with the types of regimens, donors, and indications with outcome. The older literature consists of patients who received myeloablative regimens resulting in a median survival of approximately 3 to 4 years after HSCT. Causes of death include either transplant-related toxicity (infections, graft failure, GVHD) or DC-related complications that are not prevented by HSCT such as vascular lesions of the gut, kidneys, liver, and lung (≈50% of the patients) and fibrosis involving the lung and liver (≤40% of the patients). A review of the Center for International Blood and Marrow Transplant Research database identified 34 DC patients who had been transplanted between 1981 and 2009. The overall 10-year survival among this group was 30%. Early post-transplant deaths were due to graft failure and other transplant-related complications. Late mortality was related to pulmonary failure. These striking complications after HSCT probably reflect the natural history of the disease. However, it is not known whether HSCT can accelerate their course. These unusual complications, uniquely seen in DC, have not been reported in other IBMFSs such as FA.

DC is a disorder with chromosomal instability caused by flawed telomere maintenance. Short telomeres may not be sufficiently replicated after damage by the conditioning regimen, which may increase genomic instability and cell death. This might explain the hypersensitivity to irradiation and chemotherapy. The increased hypersensitivity of DC patients to transplant conditioning can be related to the telomere shortening from DC itself combined with the accelerated telomere shortening that occurs after HSCT. Further, because of the high degree of mucocutaneous involvement, DC patients may be more susceptible to endothelial damage, which occurs after HSCT as a result of various factors, including the conditioning regimen, cyclosporine A, infectious diseases, GVHD, and a cytokine storm. Lastly, DC is characterized by a high tendency to generate oxidative stress that can cause additional DNA damage during HSCT e.g., in the lungs.

The increased predisposition to posttransplant complications and the tendency to develop tumors highlight the need to avoid certain conditioning agents such as busulfan and irradiation and possibly reduce the intensity of the transplant preparative regimens.

The strategy of using low-intensity fludarabine-based protocols for HSCT in DC was first reported in 2003 and has produced encouraging results. A retrospective study of 94 patients transplanted with non-myeloablative conditioning was published by a collaborative European/USA group. The indications were mostly BMF, but 8 had MDS. 81% of the patients were less than 20 years old. About three quarters had a MUD or mismatched donors. Three-year overall survival and event free survival were 66% and 62%, respectively. An interim analysis of an ongoing multicenter study led by a group from Boston using an alkylator free/radiation free conditioning regimen for patients with DC was presented in the American Society for Transplantation and Cellular Therapy Meeting 2019 and showed 85% rate of sustained engraftment and 90% 2-year overall survival.

Non-myeloablative regimens appear to be well tolerated and can induce prompt engraftment without significant complications. However, the benefit in reducing the risk of non-hematological disease-related complications, such as bleeding from vascular lesions and respiratory failure caused by pulmonary fibrosis, is not clear. Post-transplant deaths among published cases in which reduced intensity transplant regimens were incorporated include such complications. Indeed, a literature review published in 2019 showed that reduced intensity is associated with better survival at 4 years post HSCT, but unfortunately it declines and loses statistical significance after 5 years. Last, the role of these conditioning regimens in increasing the additive risk of cancer caused by HSCT is still to be determined.

Future Directions

So far, about 75% to 80% of the patients with DC can be genotyped. Clearly, there is a need to discover additional DC genes. Furthermore, the mechanism by which impaired activity, transport, and stability of telomerase and other ribonucleoprotein complexes influence HSC function requires clarification. The complete spectra of disorders and phenotypes caused by mutations in these genes are still to be defined. Effective therapies with reduced toxicity are necessary to prevent devastating complications such as BM failure, pulmonary fibrosis, and vascular anomalies. Further studies are necessary to optimize and explore the efficacy of reduced intensity HSCT in preventing devastating DC-related complications. Last, there is a need for studies focusing on translating this genetic knowledge into a gene therapy approach.

Congenital Amegakaryocytic Thrombocytopenia

Background

CAMT is an autosomal recessive syndrome that typically presents in infancy with isolated thrombocytopenia caused by reduced or absent BM megakaryocytes with preservation initially of granulocytic and erythroid lineages. Aplastic anemia subsequently ensues in the vast majority of the patients, usually in the first few months or years of life. Most patients do not have physical malformations; therefore, the diagnosis depends on the exclusion of other acquired and inherited causes of thrombocytopenia in early life. Biallelic mutations of the thrombopoietin receptor gene, *MPL*, have been identified as the main cause of this disease. *CAMT* is a distinct genetic entity, but mutations in other genes associated with inherited thrombocytopenias and inherited aplastic anemias must be considered in the differential diagnosis (Table 30.5).

Epidemiology

CAMT is rare. The incidence of diagnosed cases is estimated at one case per million births per year. In the CIMFR, CAMT is 10-fold less common than FA. Since some patients have late onset disease, it is important to test patients with thrombocytopenia and patients with aplastic anemia who do not respond to immunosuppressive therapy for mutations in *MPL*.

Genetics and Pathobiology

The defect in CAMT is directly related to mutations in *MPL*, the gene for the thrombopoietin receptor that maps to 1p34 in 94% of the patients with a CAMT clinical phenotype. Rare patients with a similar phenotype to that of *MPL*-associated CAMT were found to have mutations in the gene for thrombopoietin (*THPO)* and **the**

TABLE 30.3 Miscellaneous Inherited Thrombocytopenia Disorders and Their Major Hematologic Features

Disorder	Genetics	Mutant Gene	Platelet Size[a]	Features
Amegakaryocytic thrombocytopenia	AR	*MPL*	Normal	Progression to aplastic anemia, MDS, AML, rarely physical anomalies (~10%)
Thrombocytopenia absent radii	AR	*RBM8A*	Normal	Physical anomalies
Familial platelet disorder with predisposition to AML	AD	*RUNX1, ETV6, ANKRD26*	Normal	MDS, AML
X-linked macrothrombocytopenia	XL	*GATA1*	Large	Anemia, dyserythropoiesis, thalassemia
Thrombocytopenia and radio-ulnar synostosis	AD	*HOXA11*	Normal	Fused radius, limited range of motion
Familial dominant thrombocytopenia	AD	*MASTL*	Normal	No associated features
MYH9-related thrombocytopenia: May-Hegglin anomaly	AD	*MYH9*	Large	Neutrophil inclusions
Fechtner syndrome	AD	*MYH9*	Large	Neutrophil inclusions, hearing loss, nephritis
Epstein syndrome	AD	*MYH9*	Large	No inclusions, hearing loss, nephritis
Sebastian syndrome	AD	*MYH9*	Large	Neutrophil inclusions
Wiskott-Aldrich syndrome	XL	*WAS*	Small	Immune deficiency, eczema
X-linked thrombocytopenia	XL	*WAS*	Small	No associated features
Paris-Trousseau thrombocytopenia	AD	*FLI1* (hemizygous deletion)	Large	Dysmegakaryocytopoiesis, Jacobsen syndrome
Bernard-Soulier syndrome	AR	*GP1BA*	Large	No associated features
Bernard-Soulier carrier/Mediterranean macrothrombocytopenia	AD	*GP1BA*	Large	No associated features

AD, Autosomal dominant; *AML*, acute myeloid leukemia; *AR*, autosomal recessive; *MDS*, myelodysplastic syndrome; *MPV*, mean platelet volume; *XL*, X-linked recessive.
[a] Platelet size: small, MPV <7 fL; normal, MPV 7 to 11 fL; large or giant, MPV >11 fL.

MDS1 and EVI1 (MECOM) gene which is also known as the **ecotropic virus integration site 1 protein homolog** (EVI-1) or **positive regulatory domain zinc finger protein 3** (PRDM3), a protein that in humans is encoded by the *MECOM* gene, though these genes are more frequently mutated in other IBMFSs. Heterozygote carriers of the mutant MPL gene have normal blood cell counts. Affected individuals have mutations in both alleles in either homozygous or compound heterozygous state. Mutations have been found throughout the *MPL* gene, including nonsense, missense, frameshift, and splicing mutations. The mutations cause either reduced cell surface receptor expression or defective thrombopoietin binding and receptor activation. A genotype–phenotype correlation has been identified in CAMT patients and two prognostic groups were established, types I and II.

Type 1

Frameshift, nonsense, and splicing mutations result in a complete loss of function of and signaling from the thrombopoietin receptor in type I by deletion of all or most of the intracellular domain. This causes persistently low platelet counts and a rapid progression to pancytopenia. Thrombopoietin plays a critical role in the proliferation, survival, and differentiation of early and late megakaryocytes. This clearly explains the thrombocytopenia. However, *MPL* is also highly expressed by HSCs and promotes their quiescence and survival. Thus, MPL protein insufficiency may account for depletion of HSCs and pancytopenia. Evolution into severe aplastic anemia is particularly common in type I.

Type II

CAMT with missense and certain splicing mutations cause reduced expression of the protein, reduced localization to the plasma membrane (e.g., R102P in the extracellular domain), or an inability to bind thrombopoietin (e.g., F104S). Patients with these mutations have a milder course; a transient increase in platelet counts during the first years of life; and delayed onset, if any, of pancytopenia, indicating residual receptor function.

Serial studies of CAMT hematopoiesis using clonogenic assays have been informative. Initially, when the only hematologic abnormality is isolated thrombocytopenia, the numbers of hematopoietic progenitors are comparable to those of control participants, including the number of CFU-MK. As the disease evolves into aplastic anemia, the peripheral blood counts decline, and assayable colony numbers from progenitors belonging to each myeloid lineage also decline in parallel. Stromal cells established in short- and long-term cultures of patient BM show normal proliferative activity and yield a “fertile” BM microenvironment for patient and control BM colony growth. The findings are consistent with current knowledge about *MPL* mutations, namely, that the central problem in CAMT is an intrinsic HSC defect rather than an abnormality of the BM milieu.

Other data demonstrate measurable numbers of CFU-MK progenitors in vitro from patients with CAMT when studied early in the disease in response to IL-3, GM-CSF, or a combination of both but defective CFU-MK colony formation in response to recombinant human thrombopoietin that fits with *MPL* mutations. Plasma thrombopoietin levels in patients with CAMT are always elevated and are among the highest seen in any patient population.

The pathogenesis of the associated neurologic abnormalities is less understood; however, MPL is expressed in the neuronal cells and might be important for their development.

Clinical Features

Almost all patients present with a petechial rash, bruising, or bleeding during the first year of life. Three-quarters of the patients have thrombocytopenia at birth or within the first 2 months. Most cases evolve into multilineage cytopenias and aplastic anemia. Overall, about three quarters of the patients develop pancytopenia before the age of 4 year.

The majority of patients with *MPL* mutations have normal physical and imaging features, but isolated cases with anomalies have been identified. The commonest anomalies are neurologic, including varying degrees of cerebellar hypoplasia or agenesis, cerebral atrophy, cortical dysplasia and lissencephaly, hypoplasia of the corpus callosum and brainstem, Dandy Walker malformation, and developmental delay. Patients may also have microcephaly and abnormal facies.

Congenital heart disease with a variety of malformations have been reported, including atrial septal defects, ventricular septal defects, patent ductus arteriosus, tetralogy of Fallot, and coarctation of the aorta. Some of these occur in combination. Other anomalies include abnormal hips or feet, kidney malformations, eye anomalies, and cleft or high-arched palate. Some affected sibships manifested both normal and abnormal physical findings in the same family.

Laboratory Findings

Thrombocytopenia is the major laboratory finding with normal hemoglobin levels and white blood cell counts initially. Although there are usually measurable but reduced platelet numbers, peripheral blood platelets may be totally absent. Those that can be identified are of normal size and appearance. Similar to several other IBMFSs, RBCs may be macrocytic. HbF is increased in most but not all patients. BM aspirates and biopsies initially show normal cellularity with markedly reduced or absent megakaryocytes (Fig. 30.6). In patients who develop aplastic anemia, BM cellularity is decreased with fatty replacement, and the erythroid and granulocytic lineages are symmetrically reduced.

Predisposition to Leukemia

Cases with CAMT have been reported with secondary clonal BM cytogenetic abnormalities such as monosomy 7 and trisomy 8, MDS, or AML. Several published cases clearly demonstrate a typical progression of thrombocytopenia, aplastic anemia, and clonal or malignant myeloid transformation. One boy with a normal physical appearance had amegakaryocytic thrombocytopenia from day 1 of life, developed aplastic anemia at 5 years of age, responded poorly to androgens and steroids, and then developed AML at age 16 with death at age 17. A girl had thrombocytopenia at 2 months of age, pancytopenia at 5 months, and thereafter developed a preleukemic picture with clonal abnormalities involving chromosome 19. Another patient had thrombocytopenia at 6 months of age, developed progressive aplastic anemia over the next 2 years, acquired monosomy 7 in BM cells at age 5, and then developed MDS with an acquired activating RAS oncogene mutation. These cases support the notion that CAMT is a pre-leukemic IBMFS. The risk or incidence of malignant conversion is difficult to determine because of the rarity of the disease and the paucity of published data, and because patients frequently require early HSCT, which eliminate the risk of clonal hematopoiesis.

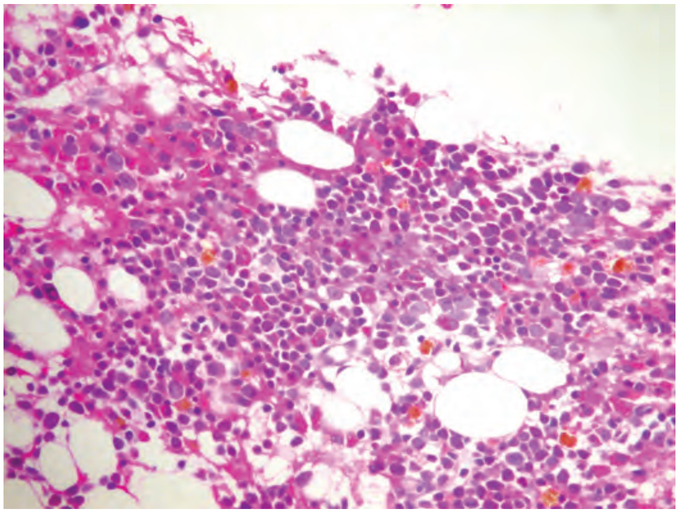


Figure 30.6 LOW-POWER VIEW OF A BONE MARROW ASPIRATE FROM A NEWLY DIAGNOSED PATIENT WITH CONGENITAL AMEGAKARYOCYTIC THROMBOCYTOPENIA. The three findings are normal cellularity, normal granulopoiesis and erythropoiesis, and absent megakaryocytes. *(Photomicrograph prepared by Dr. Mohamed Abdelhaleem, Toronto.)*

Differential Diagnosis

If CAMT presents at birth or shortly after, it must be distinguished from other causes of severe neonatal thrombocytopenia, which most commonly are caused by severe systemic infections, e.g., by bacteria or viruses. Usually, these infectious etiologies are characterized by increased peripheral destruction of platelets or a combination of peripheral destruction and BM suppression. Congenital infections collectively designated as the **TORCH** (***To****xoplasma gondii*, **r**ubella, **c**ytomegalovirus, and **h**erpes simplex virus) **syndrome** should be considered.

Passive transplacental passage of IgG antiplatelet antibodies into fetal circulation can cause rapid destruction of fetal platelets. This occurs in two circumstances: a (1) **maternal autoimmune disease** such as idiopathic thrombocytopenic purpura or systemic lupus erythematosus and (2) in **neonatal alloimmune thrombocytopenia** by alloimmunization of the pregnant mother to fetal antigens inherited from father but absent in the mother. In the former situation, the mother has thrombocytopenia or a history of such; in the latter situation, the mother has a normal platelet count and serum antibodies to human platelet alloantigens. Increased platelet destruction also occurs in newborns with giant benign hemangiomas of skin, liver, or spleen, the so-called **Kasabach-Merritt syndrome**.

Thrombocytopenia with absent radii syndrome can be distinguished from CAMT since in TAR the patient's radii are absent. Peripheral blood chromosomal analysis is not associated with increased breakage with DEB or MMC clastogenic stress testing, which allows CAMT to be distinguished from **FA**. Also, FA very rarely leads to cytopenias in infancy.

Other inherited forms of thrombocytopenia should be considered (see Table 30.5). These can generally be classified according to inheritance pattern (autosomal dominant, autosomal recessive, or X-linked recessive), size of the platelets (small, normal, or large or giant), and presence or absence of associated clinical features. Identification of the specific mutant gene for each disorder confirms the diagnosis.

If CAMT presents beyond the neonatal age period, it must be distinguished from causes of peripheral platelet destruction such as in chronic immune thrombocytopenic purpura, acquired amegakaryocytic thrombocytopenia or aplastic anemia, other IBMFSs, MDS, and acute leukemias. The medical history of the patient and family, physical examination, and initial laboratory test results may help to exclude other disorders. However, a BM aspirate and biopsy will point to the diagnosis, and a *MPL* mutational analysis will confirm the diagnosis.

Therapy and Prognosis

Supportive treatment has been largely unsatisfactory to date, and the mortality rate from thrombocytopenic bleeding, complications of aplastic anemia, or malignant myeloid transformation has been very close to 100%. For that reason, HLA typing of family members should be performed as soon as the diagnosis is confirmed to determine if a matched related donor for HSCT exists. If not, a search for a suitable alternative donor (unrelated donor, cord blood, or haploidentical family member) should ensue as soon as platelet counts show deterioration toward severely low levels with or without similar involvement of other blood lineages. The need for transfusional support is a cogent indication.

Platelet transfusions should be used discretely to minimize the risk of developing antibodies and becoming refractory to transfusions. Single-donor filtered platelets are preferred to multiple unfiltered random donor platelets to minimize sensitization. Maintaining platelet counts above 10×10^9/L is a reasonable approach. Since HSCT is the mainstay of treatment for CAMT patients, all blood products should be free of cytomegalovirus and irradiated.

Efficacy data about androgens in CAMT is limited, but a sufficient and sustained response is unlikely. In the CIMFR three patients received androgens and none responded. Androgens can be considered when HSCT is contraindicated or as a temporizing measure until an HSCT donor is available. Corticosteroids have been used for thrombocytopenia with no apparent efficacy.

Thrombopoietin has not been tried for the treatment of *MPL*-associated CAMT patients because endogenous thrombopoietin levels are markedly increased and its receptor is mutated. Nevertheless, thrombopoietin agonists that either bypass the patient mutations at the extracytoplasmic (e.g., romiplostim) or binds to the transmembrane domain (e.g., eltrombopag and LGD-4665) may prove efficacious in CAMT. For example, the in vitro effect of LGD-4665 on megakaryopoiesis from cells carrying the F104S *MPL* mutation has been demonstrated. Also, two patients with CAMT and p.R99W or p.R157 mutations in *THPO* have responded to romiplostim. Ideally, further use of thrombopoietin agonists should optimally be assessed as part of a clinical trial because of a potential risk of promoting the development of a hematologic malignancies.

Based on the in vitro augmentation of megakaryocyte colony growth in response to IL-3, a small phase I/II clinical trial was conducted for CAMT patients. IL-3 resulted in improved platelet counts in two of five patients and decreased bleeding and transfusion requirements in the other three. Prolonged IL-3 administration in two additional patients also resulted in platelet increments. However, IL-3 is no longer commercially available. GM-CSF has a positive in vitro effect but not in vivo.

BM failure in CAMT can be cured by HSCT, and most often is used in patients between 1 and 5 years of age. Most of the recent published cases have had successful outcomes. Matched sibling donor sources are ideal even if the donor is a carrier with one mutant allele. Reduced-intensity conditioning regimens for CAMT have been successfully used in both related and unrelated donor setting. Such an approach was successful even in a case with monosomy 7 reported by the CIMFR who received BM from an unrelated donor. HSCT using T cell–depleted BM with relatively high $CD34^+$ cell numbers and enhanced T cell–specific immunosuppression in the transplant cytoreductive regimens that have also been successful.

Future Directions

Novel therapy that can activate the mutated thrombopoietin receptor may be suitable for some patients with CAMT. Alternatively, drugs that stimulate downstream targets of the receptor might be effective. The cellular consequences of specific gene mutations need to be further studied because this might help to develop novel strategies in patients who cannot be transplanted. CAMT is also a candidate disease for gene therapy because restoration of wild-type *MPL* would provide in vivo selection of corrected HSCs.

Other Inherited Syndromes With Associated Pancytopenia

Several additional IBMFSs with pancytopenia are briefly described in this section and/or listed in Table 30.1.

GATA2-related disorders can present in several different ways: cytopenias (single or multilineage), MDS/AML, and lymphedema (Emberger syndrome); monocytopenia, B and NK cell lymphopenia, infections (nontuberculous mycobacteria, mainly mycobacterium avium complex [MAC], viral, fungal, and bacterial opportunistic infections), cytopenias progressing to MDS/AML (monoMAC syndrome). The MDS is typically associated with monosomy 7. The underlying genetic cause is monoallelic mutations in *GATA2*.

SAMD9/9L-related disorder can present as MDS, infection, restriction of growth, adrenal hypoplasia, genital phenotypes, and enteropathy (MIRAGE syndrome). Patients may also have developmental delay, chronic lung disease, central nervous system abnormalities and other physical malformations. The disease can also present as ataxia and pancytopenia, as single lineage cytopenia, or isolated MDS/AML (typically with monosomy 7). These phenotypes are caused by a gain-of-function mutation in the sterile alpha motif domain containing 9 protein (SAMD9) or sterile alpha motif domain containing 9-like protein (SAMD9L) due to paralogous genes on the long arm of chromosome 7 (7q21.2, which function in endosome fusion, innate immune responses and cell growth). They mediate down regulation of growth factor signaling via internalization of growth factor receptors.

SRP72-related disorder is inherited in an autosomal dominant fashion. It is a syndrome characterized by familial aplastic anemia and MDS. Some patients in the described families also had deafness. *SRP72* encodes for a signal recognition particle 72 protein. The protein is part of a ribonucleoprotein complex that mediates the targeting of secretory proteins to the endoplasmic reticulum.

ERCC6L2-related disorder is an autosomal recessive disorder with the following clinical features: pancytopenia, MDS, and physical malformations. Neurological manifestations are common and include developmental delay, microcephaly, ataxia, dysmetria, generalized brain volume loss, retinal dystrophy, and low-set ears. ERCC6L2 is involved in regulating RNA Pol II-mediated transcription via its interaction with DNA-PK to resolve R loops and minimize transcription-associated genome instability. The inherited BMF syndrome caused by biallelic variants in *ERCC6L2* can be considered as a primary transcriptional disorder rather than a DNA repair defect.

Dubowitz Syndrome

This is an autosomal recessive disorder characterized by a peculiar facies, infantile eczema, small stature, and mild microcephaly. The face is small with a shallow supraorbital ridge, a nasal bridge at the same level as the forehead, short palpebral fissures, variable ptosis, and micrognathia. This is a rare disorder, and incidence rates for complications are difficult to establish; however, there appears to be a predilection to develop cancer as well as hematopoietic disorders. Patients have developed acute leukemia, neuroblastoma, and lymphoma. Approximately 10% of patients also develop hematologic abnormalities varying from hypoplastic anemia, moderate pancytopenia, and full-blown aplastic anemia. A genetic cause can be identified in about one third of the patients and include homozygous mutations in *NSUN2* or LIG4 and one copy deletion in several chromosome sites including 14q32, 17q24, and -14q32, -17q24, -19q13. The *NSUN2* gene encodes a conserved RNA methyltransferase, the expression of which is lacking in patient cells.

Seckel Syndrome

Sometimes called *bird-headed dwarfism*, patients with this autosomal recessive developmental disorder have marked intrauterine and postnatal growth failure, mental deficiency, severe microcephaly, a hypoplastic face with a receding forehead and chin, a prominent curved nose, and low-set or malformed ears. Some patients may show increased chromosomal breakage in lymphocyte cultures with DEB or MMC and mimic FA, thus genotyping may distinguish between the syndromes. About 25% of patients develop aplastic anemia or malignancies. There are several genes that have been linked to Seckel syndrome. The genes play a role in centriole biogenesis and centrosome related functions (CEP152, CENPJ, CEP63, NIN, PLK4, and CDK5RAP2), and in maintenance of genomic stability (ATR, RBBP8, ATRIP, and DNA2). Deficiency of the encoded proteins disrupts cell proliferation, which may account for the reduced global body mass as seen in the syndrome.

Reticular Dysgenesis

Reticular dysgenesis is a combined immunodeficiency and BM failure disorder. It is characterized by severe lymphopenia and agranulocytosis. Anemia, thrombocytopenia, and aplastic anemia may be evident. Immunologically, the disorder is a variant of severe combined immune deficiency in which cellular and humoral immunity are absent. A striking feature is absent lymph nodes and tonsils and an absent thymic shadow on radiographs. Because of profoundly compromised immunity, the syndrome presents early with severe infections at birth or shortly thereafter. BM specimens are hypocellular with markedly reduced myeloid and lymphoid elements. Assayable hematopoietic progenitors are consistently reduced to absent, indicating that the disorder has its origins at the HSC level. The mode of inheritance is autosomal recessive caused by biallelic mutations in mitochondrial *AK2*. The only curative therapy is HSCT.

Schimke Immunoosseous Dysplasia

Schimke immunoosseous dysplasia is an autosomal recessive disorder caused by mutations in the chromatin remodeling gene *SMARCAL1* in 50% to 60% of patients. Patients manifest spondyloepiphyseal dysplasia with exaggerated lumbar lordosis and a protruding abdomen. They have pigmentary skin changes with abnormally discolored and configured teeth. Renal dysfunction can be problematic with proteinuria and nephrotic syndrome. Approximately 50% of patients have hypothyroidism and 50% have cerebral ischemia; 50% have anemia, 50% have neutropenia, 30% have thrombocytopenia, and 10% have aplastic anemia. Lymphopenia and altered cellular immunity are present in 80% of patients. HSCT has been applied in five cases with severe BM failure/immunodeficiency, one of which survived.

Cartilage-Hair Hypoplasia

Cartilage-hair hypoplasia (CHH) is an autosomal recessive syndrome characterized by metaphyseal dysostosis; short-limbed dwarfism; and fine, sparse hair. Additional skeletal findings include scoliosis, lordosis, chest deformity, and varus lower limbs. Aganglionic megacolon and other gastrointestinal abnormalities have been reported. Most patients reported in the literature are Finnish or Amish. Mutations in the noncoding RNA gene *RMRP* (RNA Component of Mitochondrial RNA Processing Endoribonuclease) are seen in more than 80% of cases. Mutations in *POP1* (Processing Of Precursors 1) and in *NEPRO* (Nucleolus and Neural Progenitor Protein), whose encoded proteins are part of or interact with the RNAse-MRP endoribonuclease complex cause similar phenotype of CHH, but without immunodeficiency. The complex plays a role in rRNA processing and mitochondrial RNA processing by cleavage at a priming site during replication. Macrocytic anemia of varying severity is seen in the majority of patients. Most patients have mild and self-limited anemia, but some are severe and persistent resembling Diamond-Blackfan anemia and require RBC transfusions. Severe immunodeficiency can occur, often with the severe anemia. HSCT has been used successfully to reconstitute the immune system. Neutropenia has been reported in 25% of CHH cases and lymphopenia in 65%. Lymphomas and basal cell carcinoma also occur at an increased frequency.

Pearson Syndrome

Pearson syndrome is an IBMFS which in 30% of cases is characterized by impaired exocrine pancreatic function caused by acinar cell atrophy and fibrosis. Patients with Pearson syndrome have a maternally inherited diagnostic deletion of mtDNA that encodes enzymes that are critical to oxidative phosphorylation. The genetic deletion results in a syndrome of refractory anemia with ringed sideroblasts and prominent vacuolization of BM erythroid and myeloid precursors. Physical malformations are rarely observed. Severe anemia requiring transfusions is present within the first year of life, sometimes at birth. Pancytopenia may occur alone or in association with hepatic failure and a renal tubulopathy leading to lactic acidosis. The projected median survival time is 4 years. Anemia is managed with RBC and platelet transfusional support. Erythropoietin has been used for the anemia of renal failure. G-CSF is indicated for severe neutropenia. HSCT has been utilized in two cases with Pearson syndrome; both patients engrafted and achieved normal blood counts. One of the children survived at 3 years follow-up post HSCT. The second patient developed AML 12 months post HSCT and died. Interestingly, HSCT was associated not only with improved hematopoiesis, but also with resolution of lactacidemia and acidosis. Although HSCT was traditionally not recommended for the hematopoietic complications of Pearson syndrome due to their tendency to improve spontaneously in most cases, further studies are necessary to decipher the role of HSCT in this disease. The need for pancreatic enzyme replacement is unclear.

Unclassified Inherited Forms of Bone Marrow Failure

BMF can cluster in families, but many of these cases cannot be readily classified into discrete diagnostic entities such as FA, SDS, or DC. In other cases, the patients present with chronic BMF shortly after birth or have physical malformations and most likely also have IBMFSs. The phenotype of these unclassifiable cases can be complex with varying combinations of cytopenias, macrocytosis, elevated levels of HbF, hypocellular BM, immunologic deficiency, physical malformations, and predisposition to develop leukemia. The BMF might be the result of mutations in novel IBMFS genes or atypical presentations of classified syndromes. The complex interplay of mutations in multiple genes, modifying genes, epigenetic processes, acquired factors, and chance effects that may be specific to each affected case cannot be excluded.

Using the Canada-wide database of the CIMFR, a unique study was launched on IBMFS cases that were deemed unclassifiable at study entry. Of 162 enrolled patients at the time of the study, 39 were registered as having an unclassifiable disorder. Although the hematologic phenotypes were similar to the classified syndromes in the registry (single- or multilineage cytopenia, severe aplastic anemia, MDS, AML, and cancer), the patients presented at an older age (median, 9 months vs. median 1 month for classified), and the variation in clinical presentation was substantial. Grouping patients according to physical abnormalities and hematologic phenotype was not always sufficient to characterize or diagnose a condition because affected members from several families fit into different phenotypic groupings. It was difficult to formulate a sensible and cost-effective diagnostic workup based on the family histories and hematologic and physical findings. Compared with workups of classifiable syndromes, clastogenic chromosomal fragility testing and extensive genotyping efforts of the unclassifiable cases required use of several-fold higher specific diagnostic tests at a cost that was 4.5 times higher per evaluated patient. Despite these efforts and the huge, recent explosion of gene discovery, only 20% of unclassified patients were eventually diagnosed with a specific syndrome, underscoring ongoing diagnostic limitations for these disorders.

Treatment of Unclassified Familial Forms of Bone Marrow Failure

Because these disorders are rare, broad conclusions about management are difficult to formulate. For full-blown aplastic anemia with a hypocellular, fatty BM or for MDS/AML, curative therapy with HSCT remains the treatment of choice if a suitable donor is identified. In the familial cases, potential related stem cell donors must be thoroughly assessed clinically, hematologically, and with diagnostic laboratory testing to ensure that latent or masked BM dysfunction is not present. If a matched sibling donor is not available, a search for an alternative donor (unrelated, cord blood, or haploidentical) should be initiated. In the interim, principles of medical management with androgens and supportive care similar to other IBMFSs can be used.

INHERITED BONE MARROW FAILURE SYNDROMES PREDOMINANTLY WITH ANEMIA

Diamond-Blackfan Anemia

Background

Diamond-Blackfan anemia, previously called *congenital hypoplastic anemia*, is an inherited form of pure RBC aplasia. The syndrome is heterogeneous with respect to genetic causes, clinical and laboratory findings, in vitro behavior, and therapeutic outcomes. DBA is the first disease to be identified as a *ribosomopathy*. The vast majority of DBA genes are components of the small or large ribosome subunits. The predominant inheritance pattern is autosomal dominant with variable disease penetrance and expression.

Epidemiology

Based on data from a European registry of DBA patients, the estimated incidence of the disorder as assessed in France over a 13-year period was 7.3 cases per million live births. Data from the CIMFR show an incidence of 10.4 cases per million live births as assessed over a 9-year period. DBA is the most common IBMFS in the CIMFR. Although the majority of published patients are White, DBA has been recognized in multiple ethnic groups, including African Blacks, Arabs, East Indians, and Japanese. In terms of gender distribution, both sexes are equally affected. About 80% of DBA cases are sporadic.

Genetics

The discovery of DBA genes (see Table 30.1) demonstrates predominantly heterozygosity of mutations in ribosomal protein genes consistent with an **autosomal dominant pattern of** inheritance. In most DBA cases the peripheral blood karyotype is normal. Discovery of a balanced reciprocal translocation t(x;19) in a sporadic female case of DBA and the identification of microdeletions of chromosome 19 in other DBA patients led to the identification of the first DBA gene, *RPS19*. Subsequent studies revealed mutations in one allele of the gene in 25% to 36% of patients, and it is currently the most common known mutant DBA mutation. RPS19 protein is a component of the ribosomal 40 S subunit. Multiple other genes encoding either the 40 S small ribosome subunit or 60 S ribosome subunit have been subsequently identified in DBA patients. The second most commonly mutated gene in DBA is *RPL5*. It is mutated in 12% to 21% of patients. Other mutated genes are *RPL11* (7% to 16% of the patients) and *RPS26* (~10%). Other genes are less commonly involved.

Mutations in two DBA genes are inherited in an **X-linked** manner: *TSR2*, that plays a role in rRNA processing and the erythroid transcription factor gene, *GATA1*. Biallelic *EPO* mutations cause **autosomal recessive** disease. Rare cases with pure red cell aplasia and biallelic mutations in *CECR1/ADA2* have been published; however, mutations in this gene usually cause an autoimmune disease with systemic manifestations and autoantibodies, rather than classical BMF.

About 75% to 80% of the patients with DBA can now be genotyped. It is important to note that about 20% of the patients with DBA have a large deletion in one of the DBA genes rather than nucleotide-level mutations. Therefore, metaphase cytogenetics and molecular karyotyping to detect microscopic and submicroscopic deletions, respectively, should be included in the genetic testing process if an abnormal sequence of a known DBA gene is not detected.

Pathophysiology

RP genes encode structural components of either the small or large ribosome subunits, but many of them have various other functions. RPS19 associates with the ribosomal subunit 40 S. It is critical for normal maturation of rRNA because its deficiency causes defective cleavage of the pre-rRNA at the internal transcribed spacer 1 (ITS1) sequence and abnormal maturation of the 40 S subunit. This leads to accumulation of faulty pre-40S ribosome subunits.

Other ribosomal proteins that are mutated in DBA are also critical for ribosome biogenesis. For example, it has been shown that the yeast RPL11 is positioned at the intersubunit cleft of the large ribosome subunit central protuberance, thereby forming an intersubunit bridge with the small subunit protein S18. Mutations in this region such as F96 and A66 lead to halfmer formation. Mutations in *RPS24* also impair pre-rRNA processing of the 18 S rRNA and decrease the production of the 40 S ribosomal subunit. On the other hand, depletion of RPL35A reduces the amount of the 60 S subunit and of the mature 80 S ribosomes.

The mutations in RP genes likely cause protein haploinsufficiency. In support of this hypothesis, two classes of *RPS19* mutations have been described: quantitative defects resulting in undetectable protein from the affected allele and hotspot mutations leading to loss of function.

Links between *RP* gene mutations and erythropoiesis have been clearly established. For example, defective erythropoiesis occurs when *RPS19* is knocked down in cellular models. In addition, transfer of wild-type forms of the gene corrects the defective erythropoiesis in *RPS19*-deficient DBA CD34^{+} cells resulting in a threefold increase in erythroid colony formation. In yeast, introduction of *RPS19* mutations resulted in a defect in the processing of pre-rRNA similar to that observed in DBA cells with decreased expression of *RPS19*.

A large body of evidence indicates that the erythroid progenitor compartment is intrinsically defective in DBA. The frequency and clonogenicity of DBA early erythroid progenitors (defined by LinCD34^{+}CD38^{+} CD45RA^{-}CD123^{-}CD71^{+}CD41a^{-}CD105^{-}CD36^{-}) and late erythroid progenitors (as defined by Lin-CD34$^{+/-}$CD38^{+}CD45RACD123^{-}-D71^{+}CD41a-CD105^{+}CD36^{+}) are significantly decreased. A study from Toronto determined that the earliest defect of erythropoiesis in DBA patients occurred at the erythroid producing CMP-F2/F3 and MEP-F2/F3 compartments. Standard clonogenic assays for CFU-E and BFU-E progenitors consistently have shown reduced or absent erythroid colonies in most DBA patients and intermediate, normal, or occasionally increased numbers in the rest. Functional analysis of HSC/HPC sub-populations in single-cell colony assays showed that in contrast to controls, F2/F3 CMP/MEP of DBA patients had limited erythroid colony-forming potential, but maintained the ability to develop myeloid colonies.

The DBA erythroid progenitors are relatively insensitive to erythropoietin in vitro and to burst-promoting activity, but the hyporesponsiveness to erythropoietin can be corrected in some cases by the addition of glucocorticoids in vitro or by administering prednisone to DBA patients.

The data underscore the fact that the intrinsic defect of DBA erythroid progenitors is an inability to respond normally to inducers of erythroid proliferation, differentiation, or both, while maintaining normal differentiation along megakaryocytic and granulocytic pathways.

The combination of recombinant IL-3 and SCF increases the in vitro clonogenicity of DBA BM progenitors. The size and number of DBA BFU-E colonies are dramatically increased. The combination of IL-9 to SCF, IL-3, and erythropoietin also potentiates DBA BFU-E colony formation. The data on the effect of IL3, SCF, or GM-CSF as single agents are less conclusive. FLT-3 ligand has no effect on DBA BM colony growth.

There are significant age-related changes in erythroid and granulopoietic progenitors in DBA patients. Despite profound anemia, 7 of 10 patients studied within 1 year of diagnosis had normal numbers of CFU-E and BFU-E that showed a normal response to cytokines. In contrast, 12 of 14 patients followed for more than 3 years had decreased numbers of assayable CFU-E and BFU-E and, in seven cases, decreased CFU-GM. The data are consistent with the idea that the DBA defect involves other hematopoietic lineages and worsens with time.

Strong support for this conclusion comes from a detailed study that examined the interaction between DBA CD34$^+$ cells and the hematopoietic microenvironment using long-term BM cultures. Stromal adherent layers from DBA patients did not show evidence of any morphologic, phenotypic, or functional abnormality, and the stroma sustained the proliferation of normal CD34$^+$ cells. A major finding was an impaired capacity of DBA CD34$^+$ cells in the presence of normal stromal cells to proliferate and differentiate along not only the erythroid pathway but also along the granulocytic–macrophage pathway. These results indicate an intrinsic defect of a hematopoietic progenitor with at least bilineage potential that places it earlier along the hematopoietic hierarchy than previously suspected and that was only unmasked by the evaluation using long-term marrow cultures. This observation, however, is in keeping with the clinical observation that in addition to anemia, patients may have neutropenia and thrombocytopenia. These findings were extended with evidence in long-term culture initiating cell assays for a trilineage defect in DBA refractory to treatment. The data broaden the definition of DBA and explain generalized BM dysfunction and hypoplasia in some cases of DBA that have puzzled investigators for years.

Although a negative impact of RP gene mutations on erythropoiesis has been established, the molecular mechanism that links ribosome protein haploinsufficiency to the erythroid defect is unclear. *One hypothesis* is that it is related to global translation insufficiency. It is well known that during early stages of erythropoiesis, translation is increased. It is possible that the need for protein synthesis is not met during this critical developmental stage. Interestingly, it has recently been shown that not only global translation is affected in DBA but also translation of the specific erythroid related gene, *GATA1*.

Another hypothesis is that *RP* gene mutations lead to accumulation of abnormal rRNA precursors as well as dysregulation of multiple ribosomal protein genes and protein expression as shown with *RPS19*. This leads to defective ribosome biogenesis, unassembled ribosome proteins, and *cellular stress*. Indeed, loss of ribosome proteins have been shown to increase the levels of S6 kinase phosphorylation due to increased ROS, which in turn results in stimulating erythroid cell autophagy.

It has also been hypothesized that defective ribosome biogenesis leads to *activation of p53*, thereby causing apoptosis and cell cycle arrest. A role for p53 is supported by a mouse model with mutations in *RPS19* that is characterized by RBC underproduction and small mouse size and by zebrafish models of *RPS19* inhibition that are characterized by impaired erythropoiesis and malformations. Activation of p53 may involve interactions of MDM2 with specific ribosomal proteins such as RPL5, RPL11, and RPL23. These interactions may lead to dissociation of p53 from MDM2, impairment of p53 targeting to the proteosome, and prevention of p53 degradation. However, these models do not explain how haploinsufficiency of RPL5 and RPL11 leads to p53 activation. Further, co-inhibition of TP53 activity in five different zebrafish models of ribosome protein knockdown rescued the morphological malformations associated with the ribosome protein knockdown, but did not alleviate the erythroid aplasia. This suggests that ribosomal protein deficiency causes erythroid failure at least in part in a TP53-independent manner.

Several studies have also linked *toxic accumulation of heme* to DBA pathogenesis. The heme exporter FLVCR1 is critical for CFU-E development. Knocking out FLVCR1 in mice causes impaired CFU-E development. A partial block in human FLVCR1 in CD34$^+$ HSCs recapitulates the hematologic features of DBA, including CD36$^+$/CD135a$^+$ erythroid progenitor cell development but not myeloid cell development. Importantly, 55% to 95% of the *FLVCR1* transcript is alternatively spliced in DBA cells compared with 4% to 24% in normal immature erythroid cells. The spliced variants in DBA encode *FLVCR1* proteins that are defective in their cellular and surface expression and in their function. It is possible that expression of *FLVCR1* splicing variants leads to impaired export of intracellular heme, accumulation of heme in erythroid progenitors/precursors, and consequently apoptosis.

Last, a potential role of the *PIM-1* oncoprotein has also been proposed. RPS19 has been shown to interact with PIM-1. PIM-1 is a ubiquitous serine-threonine kinase. Its expression in erythropoietic cells can be induced by several growth factors, including erythropoietin.

Clinical Features

DBA registries which provide longitudinal data and a summary of published cases supply comprehensive information about the clinical aspects of the disorder. Aside from findings associated with anemia, about half of infants at presentation appear healthy and are normal physically. Unless the baby develops cardiac failure as a result of anemia, hepatosplenomegaly and edema are absent.

Pregnancy, birth history, or both are often abnormal. In a survey from the French and German DBA registries of 64 pregnancies in 26 women with DBA, complications were seen in 42 pregnancies (66%) and included abortion, preeclampsia, in utero fetal death, in utero growth retardation, retroplacental hematoma, and preterm delivery. Thirteen of 34 children born alive had DBA. Fetal DBA with hydrops fetalis has been reported. More than 90% of cases present in the first 12 months of life, at a median age of 2 months. Due to the increasing availability of genetic testing for adult patients and family members, more patients with a mild to moderate phenotype are being diagnosed later on in life. The median age at diagnosis in a study published in 2018 was 18.5 months (range: 1 to 479 months). After diagnosis, family screening may identify the parents or older siblings as being affected.

About 30% to 47% of patients present with one or more congenital anomalies. Most of these phenotypic abnormalities belong to the following categories: (1) craniofacial dysmorphism, including hypertelorism, microcephaly, microphthalmos, congenital cataracts or glaucoma, strabismus, microretrognathism, and a high-arched palate or cleft palate; (2) prenatal or postnatal growth failure independent of steroid therapy; (3) neck anomalies, which may consist of a pterygium coli or the fusion of cervical vertebrae with flaring of the trapezius muscle (Klippel-Feil syndrome), giving a Turner syndrome-like appearance or there may also be the Sprengel deformity (congenital elevation of the scapula) as an isolated anomaly or a combination of the two anomalies; and (4) thumb malformations, such as bifid thumb (Fig. 30.7), duplication, subluxation, hypoplasia, or absence of the thumb. There is a characteristic association of triphalangeal thumbs with DBA (Fig. 30.8) commonly referred to as "Aase syndrome II" or "Aase-Smith syndrome." In addition, some patients have a flat, hypoplastic thenar eminence, weak or absent radial pulses, or both, which probably represent variations in the thumb malformations.

Some patients have a characteristic facial appearance. The facies of individuals with DBA is said to consist of tow-colored hair, a snub nose, wide-set eyes, a thick upper lip, and an intelligent expression. Another facies observed in two unrelated girls of markedly different ancestries consists of small heads, almond-shaped eyes with a slight antimongoloid slant, a "fish-like" smile, and a pointed chin. These patients resemble each other more than they resemble their own family members (Figs. 30.9A and B). Some patients with DBA have a phenotype indistinguishable from Treacher-Collins syndrome, a disorder of ribosome biogenesis caused by *TCOF1* mutations.

Various other anomalies are occasionally reported in association with DBA. There may be urogenital malformations, such as dysplastic or horseshoe kidneys, duplication of ureters, or renal tubular acidosis. There may also be congenital heart disease, mainly ventricular and atrial septal defects, or hypogonadism, ear malformations, mental retardation, congenital hip dislocation, or tracheoesophageal fistula.

Laboratory Findings

Peripheral Blood and Bone Marrow. The main hematologic findings in DBA are summarized in Table 30.6. The anemia is usually

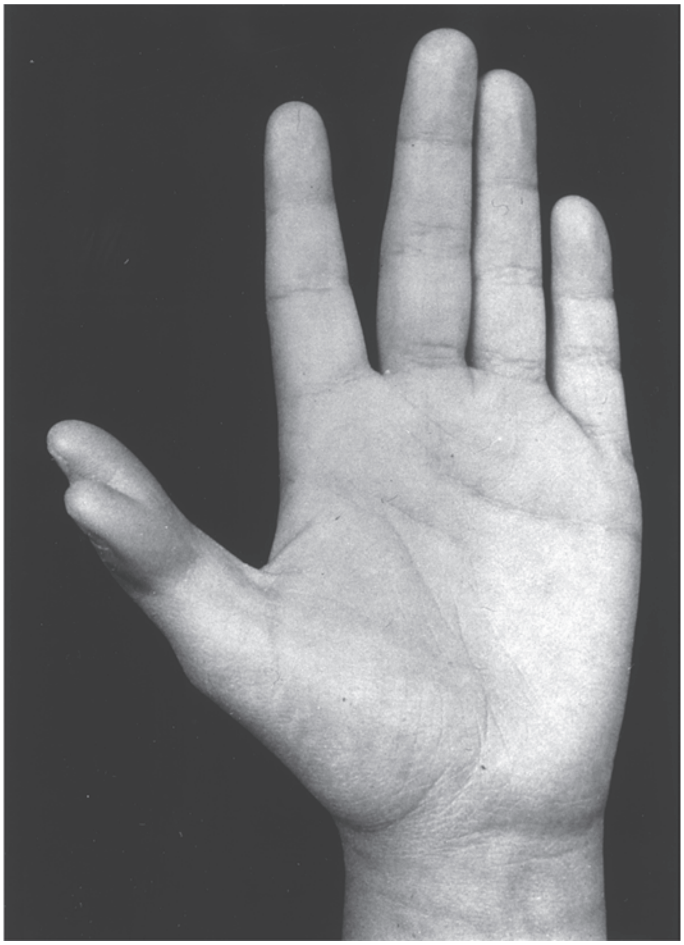


Figure 30.7 BIFID THUMB IN DIAMOND-BLACKFAN ANEMIA.

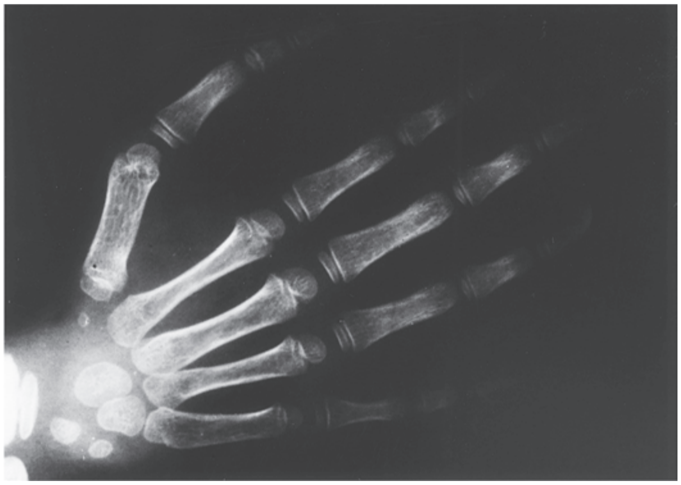


Figure 30.8 RADIOGRAPH OF A TRIPHALANGEAL THUMB IN DIAMOND-BLACKFAN ANEMIA.

TABLE 30.6 **Hematologic Features in Diamond-Blackfan Anemia Patients at Diagnosis Based on Data on 21 Toronto Cases and on 41 Cases From the Canadian Inherited Marrow Failure Registry**

Hematologic Parameters	Laboratory Findings
Mean hemoglobin value (range)	
Newborns younger than 2 months of age	6.5 g/dL (1.7–9.1 g/dL)
Children 2 months of age or older	4.0 g/dL (1.8–7.4 g/dL)
High MCV for age after the age of 1 year	87%
Low reticulocyte for the degree of anemia	100% (usually markedly decreased to <1%)
Increased HbF for age after 1 year of age	100%
RBC adenosine deaminase activity	77%
RBC i antigen	Expression increased beyond first year of life
RBC enzymes	Fetal pattern
Neutropenia	31%
Thrombocytopenia	11%
BM cellularity	Normal or increased in 90%; mildly reduced in 10%
BM erythropoiesis	Markedly reduced/absent erythroid precursors in >90% of cases
BM myeloid and megakaryocytic lineages	Normal in 100% of the cases

BM, Bone marrow; *HbF*, fetal hemoglobin; *MCV*, mean corpuscular volume; *RBC*, red blood cell.

profound at the time of diagnosis. Hemoglobin levels average 6.5 g/dL in patients diagnosed during the first 2 months of life (range, 1.7 to 9.1 g/dL) and 4.0 g/dL (range, 1.8 to 7.4 g/dL) in those diagnosed later. In the vast majority of patients, the MCV is above the expected value for age. The peripheral blood smear may show, in addition to macrocytes, a mild degree of nonspecific anisocytosis and poikilocytosis. The aregenerative component of the anemia is reflected by the absence of both polychromasia and nucleated RBCs on the blood film. Decreased RBC production is confirmed by the absence of a reticulocyte response and by characteristic findings on BM examination.

In more than 90% of patients, the BM aspirate is normocellular, but erythroblasts are markedly decreased or absent. Proerythroblasts, if present, account for less than 3% of all nucleated elements, with a myeloid-to-erythroid ratio of 10 to 1 (Fig. 30.10). In 5% to 10% of cases, at presentation erythroid precursors may be present in normal numbers, with or without a maturation arrest at the proerythroblast stage. Repeat BM testing after several weeks or months may reveal typical picture of pure red cell aplasia. White blood cell counts and platelet counts are usually normal at diagnosis, but platelets may be increased with normal function. Among DBA patients enrolled in the CIMFR mild to moderate neutropenia and thrombocytopenia occurred in 34% and 21% of the patients, respectively, either at presentation or at follow-up. Progression of the single-lineage erythroid deficiency of DBA into pancytopenia and severe aplastic anemia is rare but occurs. Of 36 deaths reported to the DBA Registry in the USA, one died from severe aplastic anemia.

Erythrocyte Findings. Erythrocytes in DBA express a number of fetal characteristics. The level of HbF is increased persistently even during remission. It remains at a level of about 10% after the age of 6 months and has a heterogeneous distribution in RBCs. The HbF has a specifically fetal amino-acid profile with a high glycine-to-alanine ratio (G-γ:A-γ). Similarly, the i antigen, which normally disappears from the erythrocyte surface by 1 year of age, is expressed at near fetal levels in older patients with DBA.

The precise cause of this fetal-like erythropoiesis is unclear. It is clearly distinct from the fetal erythropoiesis implicated in various types of leukemia, notably in juvenile myelomonocytic leukemia in which the fetal RBCs presumably arise from the leukemic clone. The situation in DBA may be analogous to that in other forms of

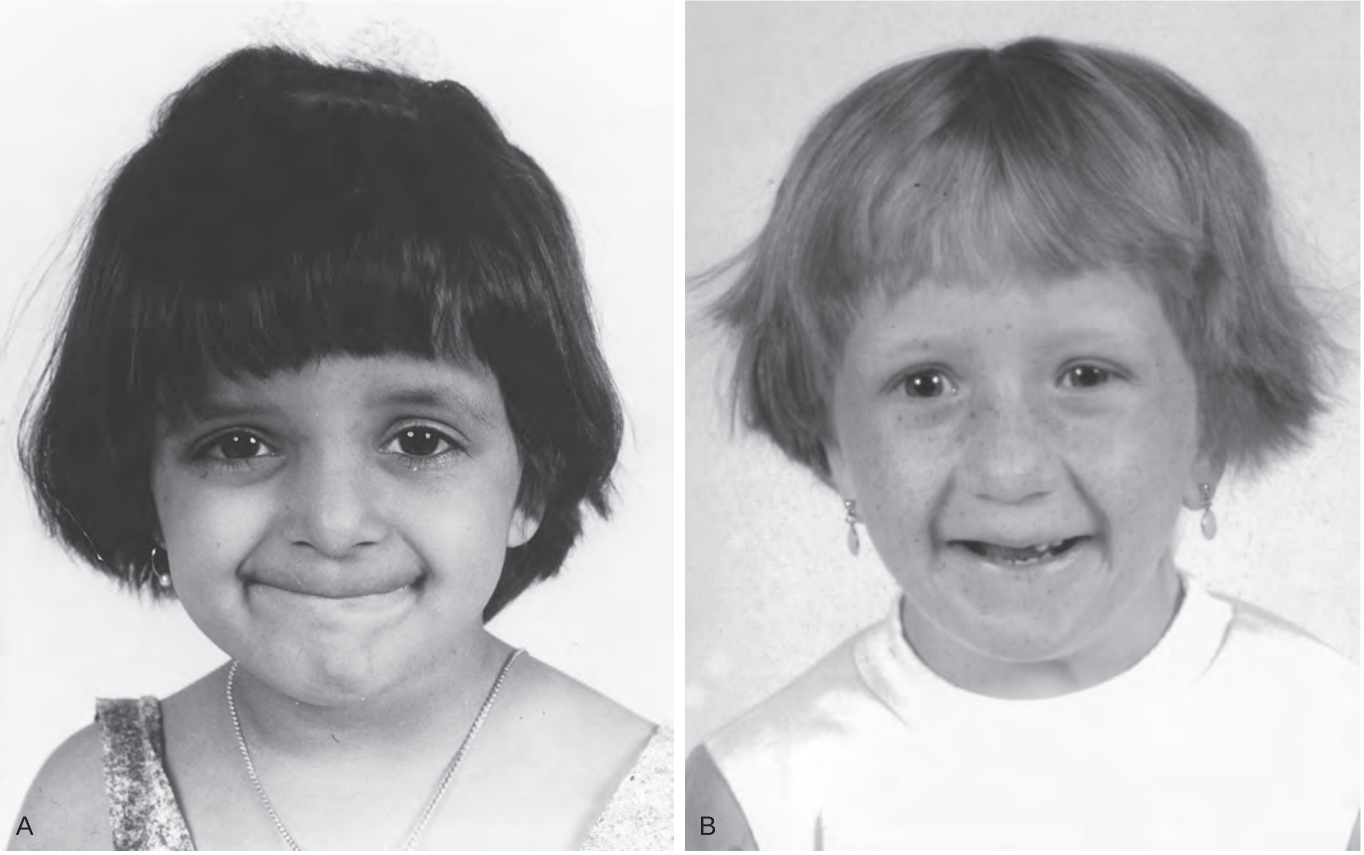


Figure 30.9 SIMILAR DIAMOND-BLACKFAN FACIES IN TWO UNRELATED GIRLS OF DIFFERENT ANCESTRIES (A AND B) CONSISTING OF A SMALL HEAD, ALMOND-SHAPED EYES WITH A SLIGHT ANTIMONGOLOID SLANT, A "FISH-LIKE" SMILE, AND A POINTED CHIN.

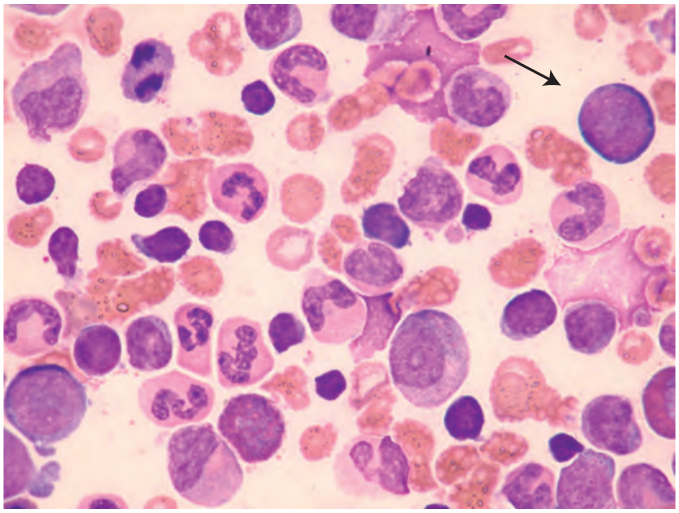


Figure 30.10 HIGH-POWER VIEW OF A BONE MARROW ASPIRATE FROM A NEWLY DIAGNOSED INFANT WITH DIAMOND-BLACKFAN ANEMIA. The findings are active granulopoiesis; normal lymphoid activity for age; and an isolated pronormoblast *(arrow)* with total absence of early-, intermediate-, and late-stage nucleated red blood cells. *(Photomicrograph prepared by Dr. Mohamed Abdelhaleem, Toronto.)*

BM failure, hemoglobinopathies and in the hematologic recovery phase after SCT. In all of these conditions, the fetal (or "stress") erythropoiesis may represent an accelerated recapitulation of RBC ontogeny in the face of an increased demand for new RBCs in peripheral blood by alternative expressing alleles in Xmn1-HBG2.

RBC enzymes often display an abnormal pattern of activity that reflects a fetal expression pattern of RBC glycolytic and hexose monophosphate shunt enzyme activities. Enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, phosphofructokinase, and glutathione peroxidase, have increased activity in patients with DBA compared with normal children and adults and in patients with transient erythroblastopenia of childhood (TEC). For some enzymes, this increased activity is comparable to cord blood RBCs. In apparent contradiction, carbonic anhydrase isoenzyme B, which is not normally present in fetal RBCs, was detected in hemolysates from three patients with DBA. Also, the RBCs of two of the three patients had an adult hexokinase isoenzyme distribution by isoelectric focusing.

Abnormalities in purine and pyrimidine metabolism are reflected by increased activity of RBC adenosine deaminase (ADA) in 60% to 90% of the patients with DBA. Also, increased orotidine decarboxylase (ODC) activity is seen in some patients. ADA activity is raised in DBA erythrocytes but not in cord blood RBCs from normal newborns or from patients with any of several hematologic conditions associated with "stress" erythropoiesis. Thus, this enzymatic abnormality cannot be simply attributable to a "reversion" to fetal erythropoiesis. Raised ADA activity may also be detected in some hemolytic anemias and acute leukemias, which limits the utility of this assay as a specific diagnostic marker for DBA. However, increased ADA activity does appear to be useful in differentiating DBA from acquired pure red cell anemias such as TEC and for epidemiologic testing of DBA pedigrees to identify family members with a mild phenotype.

Miscellaneous Findings. Serum levels of various factors involved in RBC production, such as erythropoietin, iron, vitamin B_{12}, and folate, are appropriately elevated in DBA. These findings are compatible with any form of chronic hypoplastic anemia. Riboflavin levels are normal in the serum but not in the erythrocytes. This observation initially aroused interest because experimental riboflavin deficiency may be corrected by corticosteroids similar to DBA. However, administration of large doses of riboflavin to several DBA patients did not result in a hematopoietic response. RBC serology is usually unremarkable at the time of diagnosis, but alloantibodies can be detected in chronically transfused patients.

Imaging Studies. Imaging studies are frequently informative and assist in establishing a diagnosis. Skeletal radiography may define abnormalities suspected from physical examination, such as hypoplastic, absent, or extra phalanges. Ultrasound of the abdomen may reveal malformations such as of the urogenital system. Echocardiography may reveal undiagnosed cardiac defects.

Predisposition to Malignancy

DBA is associated with hematologic cancer and with solid tumors, albeit to a much lesser degree than FA, DC, SDS, and Kostmann/SCN. The link between DBA and blood cancers is more understandable from the data described herein that implicate an early pluripotent BM progenitor in the pathobiology of DBA. Analysis of cancer incidence among 702 patients enrolled in the DBA registry in the USA identified 28 patients with tumors whose incidence can be compared to that in the general population. By age 46 years, the cumulative incidence of solid cancers and leukemia was 22% among the whole group and 13.7% among those who did not have HSCT. The median age at first malignancy was 35 years (range 11 to 70 years). In this study the identified cancers from highest to lowest frequency were MDS/AML, colon cancer, osteogenic sarcoma, breast cancer and isolated cases of gastroesophageal cancer, esophageal cancer, soft tissue sarcoma, testicular cancer, uterine cancer, cervical cancer, vaginal squamous cell carcinoma, melanoma, oral squamous cell carcinoma, lung cancer, chordoid meningioma of the lung. In the literature the following other cancers have been reported: Hodgkin lymphoma, non-Hodgkin lymphoma, acute lymphoblastic leukemia, HCC, breast carcinoma, gastric carcinoma, vaginal melanoma, and malignant fibrous histiocytoma.

Among pediatric patients enrolled in the CIMFR one of 72 patients developed MDS at the age of 17 years. Therefore, the role and schema for surveillance for detection of early malignancy in children with DBA are still to be defined.

The published cases implicate a genetic predisposition to develop cancer, though to a lower degree than FA, DC, and SDS. It is noteworthy that inhibition of ribosome protein genes in zebrafish is associated with the development of cancer.

Genotype-Phenotype Correlation

Patients with mutations in *RPL5* and *RPL11* are more likely to have multiple physical malformations. For example, thumb anomalies are seen in 56% and 39% of the patients with *RPL5* and *RPL11* mutations, respectively, compared with 7% in patients who have *RPS19* gene mutations. Interestingly, cleft lip or palate was reported in 42% of the patients with *RPL5* mutations compared with 6% and 0% of the patients with *RPL11* and *RPS19* gene mutations, respectively.

A study from the CIMFR analyzed genotype-phenotype correlation among patients with mutations in *RPS19*, *RPL11*, *RPL5*, and *RPS26*. In this study, most patients with *RPL11* mutations (71%) were not reliant on chronic treatment for severe anemia, while most of the other patients did. For example, 88% of the patients with *RPS19* mutations were reliant on chronic transfusions. Patients with *RPS19* mutations were more likely to continue to respond and be maintained on corticosteroid-therapy than other groups. Regarding extrahematological manifestations, most patients with either *RPL5* or *RPL11* or *RPS26* mutations had at least one organ malformation, whereas only 31% of patients with *RPS19* mutations had at least one organ malformation. Patients with *RPS19* mutations had significantly fewer organ malformations than patients with *RPL5* mutations. Patients with *RPL5* mutations had the greatest proportion of skeletal malformations (particularly hand malformations, cleft lips or palate). Genitourinary malformations were found only in patients with mutations in *RPL5, RPL11*, and *RPS26* and not in those with *RPS19* mutations.

Differential Diagnosis

The diagnosis of DBA is made if the patients have at least two of the following criteria (1) pure RBC aplasia as documented by normochromic-macrocytic anemia, relative reticulocytopenia, and normocellular BM with a selective deficiency of RBC precursors; (2) classical constellation of physical malformations; (3) a first-degree relative with DBA or a mutation in a DBA gene.

In clinical practice, after excluding a viral etiology, particularly parvovirus B_{19}, **TEC** is usually the main diagnosis that is confused with DBA (Table 30.7). Both entities share the same morphologic findings in the BM. However, TEC is a self-limited disorder with an excellent prognosis and needs no specific therapy except for RBC transfusions in the most profoundly anemic patients. The definition of TEC includes the following features: (1) gradual onset of symptoms (pallor and/or weakness) in previously healthy children usually 1 to 4 years of age (85% of cases); (2) normochromic-normocytic anemia with varying reticulocytopenia unless recovery has already ensued; (3) BM erythroid hypoplasia (60% of cases) or aplasia (10% of cases) or a recovery picture (30% of cases); and (4) spontaneous recovery usually within 4 to 8 weeks without recurrence, with rare exceptions.

There are some additional important features of TEC. It can occur in siblings simultaneously and in seasonal "clusters" from June to October and from November to March. Of concern are the transient

TABLE 30.7 **Distinguishing Features Between Patients with Diamond–Blackfan Anemia and Transient Erythroblastopenia of Childhood**

	DBA	TEC
Etiology	Genetic	Acquired
Immune mediated	None	Common
Family history	≈10%	Occasional siblings with concurrent TEC
Antecedent history	None	Viral infection
Age at diagnosis	90% by 1 year	6 months to 4 years
Physical anomalies	≈50%	None
Neurologic findings	None	Occasional
Transfusion dependence	Yes, if steroid refractory	None
Course	Chronic	Full recovery
Risk of cancer	Increased	Not increased
Risk of MDS or leukemia	Increased	Not increased
Laboratory findings at diagnosis:		
RBC size	Macrocytic	Normocytic
HbF	Increased	Normal[a]
i Antigen	Increased	Normal[a]
RBC enzyme activities	Fetal levels	Adult levels
RBC adenosine deaminase	Increased in 40%–90%	Normal

DBA, Diamond–Blackfan Anemia; *HbF*, Fetal hemoglobin; *MDS*, myelodysplastic syndrome; *RBC*, red blood cell; *TEC*, transient erythroblastopenia of childhood.

[a] During spontaneous recovery, values may be increased.

neurologic changes that can accompany TEC. Affected children may have one or more of the following: hemiparesis, papilledema, abnormal extraocular movements, seizures, and unsteadiness of gait. The affected patients in the published reports recovered without sequelae, and the precise relationship of these neurologic changes to the pathogenesis of TEC has not been determined.

It was claimed initially that only the erythroid lineage was affected in TEC and all other hematopoietic lineages were normal. Nevertheless, significant neutropenia also occurs in many patients with TEC, being associated in some with hypocellular BM or with a granulopoietic maturational arrest. The neutropenia may be caused by a common pathogenetic mechanism that produces anemia. An unusual presentation of TEC with active erythropoiesis has been recorded, possibly reflecting a recovery stage.

Although two cases of TEC were reported to be associated with detection of the parvovirus B19 genome, no data firmly incriminate other infectious agents in the etiology of TEC. Nevertheless, a history of a preceding viral-like illness can be obtained in more than half of the patients. The most plausible explanation proposed to date is that TEC is caused by transient immunosuppression of erythropoiesis and possibly of granulopoiesis in those with neutropenia. Increased numbers of $CD10^+$ lymphoid cells in BM of TEC patients might be an indication for such a mechanism. Most supportive evidence for this hypothesis comes from in vitro studies. Two reports described an inhibitory effect of TEC serum and fractionated IgG on erythroid colony formation that disappeared as TEC improved. An IgG inhibitor of erythropoiesis was discovered in one case and an IgM inhibitor in a second patient. A summary of other published studies suggests that more than 60% of TEC patients have autologous or allogeneic serum inhibitors of erythroid colony formation. Autologous or allogeneic cell-mediated immune suppression of erythropoiesis has also been identified in about 25% of cases. All of the in vitro studies have generated varying patterns of erythroid colony growth in TEC. Colony numbers can be normal, but reduced numbers of BFU-E (burst forming unit - erythroid) and CFU-E (colony forming unit - erythroid) progenitors have been recorded in 30% and 50% of cases, respectively.

TEC cases are not caused by DBA mutations. However, anemia in DBA may be mild, not present, or transiently triggered or exacerbated by viral infections. Therefore, cases with transient anemia and reduced BM erythropoiesis should be carefully evaluated for the possibility of an underlying undiagnosed DBA and a period of follow-up with serial blood counts is recommended.

In summary, TEC has an autoimmune pathogenesis. The transient nature of TEC is similar to other autoimmune hematologic disorders of childhood such as autoimmune neutropenia of infancy, immune thrombocytopenia purpura, and some cases of autoimmune hemolytic anemia. The decreased activities of virtually all RBC enzymes in TEC compared with control participants probably relate to the aged population of peripheral blood erythrocytes being tested.

Regarding **viral causes** of non-DBA RBC aplasia, Epstein-Barr virus, hepatitis virus, human T-cell leukemia virus-1, and human immunodeficiency virus-1 have all been implicated and should be excluded if the etiology of the anemia remains unclear. Parvovirus B_{19} stands out as a major causal agent of RBC aplasia in the context of an underlying chronic hemolytic anemia in infants and children with chronic congenital and acquired forms of immunosuppression. Fetuses are uniquely susceptible to parvovirus infection, and in utero transmission is a well-documented cause of nonimmune hydrops fetalis. Parvovirus infection should be ruled out in every case of childhood RBC aplasia by serial measurements of serum IgM and IgG and by BM examination for the characteristic giant pronormoblasts. Parvovirus may also be detected in BM by gene amplification using PCR or direct in situ hybridization.

An important differential diagnosis is a milder form of **mitochondrial DNA deletion syndrome** or **Pearson syndrome**. This disorder can also present with macrocytic anemia and may mimic DBA. In a recent study, about 3% of the patients with a clinical diagnosis of DBA were eventually diagnosed with Pearson syndrome. In some of these cases erythroid hypoplasia was demonstrated with variable vacuolated progenitors and without ringed sideroblasts.

Occasionally, the initial hematologic manifestation of several other IBMFSs such as **FA, DC**, and **congenital hair hypoplasia** is isolated macrocytic anemia. Specific screening testing for these conditions might be necessary.

Natural History and Prognosis

Historically, the only treatment for DBA was blood transfusions. Without this, patients die of anemia. When corticosteroids were introduced as an effective therapy for DBA, all patients were assigned to one of the two therapeutic interventions, and the "natural history" of the disorder took on a different dimension.

A notable phenomenon is spontaneous remission that occurs in about 20% of cases that allows patients to discontinue whatever treatment they are receiving, either chronic transfusion therapy or corticosteroids. Data from the DBA Registry in USA indicate that 75% of these patients remit before their 10th birthday, and in most cases, the remission is sustained. It appears that an equal number of patients remit from either corticosteroids or transfusions. A relapse of DBA requires reintroduction of treatment.

The overall actuarial survival for DBA patients greater than 40 years of age is 75% ± 4.8%. For those in sustained spontaneous remission, it is 100%; for corticosteroid responders, it is 57% ± 8.9%; and for transfusion-dependent patients, it is 8.9%. Causes of death mostly relate directly to the development of cancer or its treatment, HSCT-related complications, and transfusional hemosiderosis. Rarely, immunosuppression during the initial stage of prednisone therapy causes fatal infections.

Therapy

In younger children and infants, it is important to determine whether the RBC aplasia is DBA or TEC (see Table 30.7). Until a firm diagnosis is established, the initial treatment in children is almost always transfusions. This allows the flexibility to complete the workup for acquired and inherited causes and to await a spontaneous remission if the anemia is thought to be caused by TEC or another self-limited condition. Demonstration of a mutant DBA gene would clinch the diagnosis, but negative molecular analysis does not rule out this diagnosis.

If transfusions are used, it is recommended to aim for a moderate but not full correction of anemia so that erythropoiesis is not suppressed and recovery from TEC not delayed. Generally, the nadir should not be less than 6 g/dL and should prevent the development of significant symptoms. Most patients with TEC usually recover within a few weeks after receiving only one transfusion. Occasionally, recovery from TEC is slow and may mimic DBA in chronicity. If there is confusion about the proper diagnosis, it is appropriate to withhold corticosteroids in favor of additional transfusions to allow more observation time.

Transfusions for Patients With Diamond-Blackfan Anemia

Before the first transfusion, it is recommended that a full RBC phenotype be performed on the patient. This information is valuable for prevention and management of alloantibody formation caused by sensitization. For patients in whom corticosteroids are either ineffective or excessively toxic, a regular program of RBC transfusions is usually required. During the course of this program, a small number of steroid-resistant patients may show responsiveness to corticosteroids when retreated or even proceed to a spontaneous transient or prolonged remission. If not, leukocyte-depleted packed RBCs are given monthly to keep the hemoglobin concentration at a level compatible with normal activity, usually above 9 g/L. CMV-negative packed cells should be used if HSCT is contemplated. Several complications may arise from transfusions such as bloodborne infections and sensitization, but the major long-term threat is iron overload, which causes delayed puberty, growth retardation, diabetes mellitus, hypoparathyroidism, and eventually liver cirrhosis and cardiac failure. These complications can be delayed and possibly prevented by the early administration of an iron chelator (Chapter 37).

Two iron chelators are available in North America. The first, deferoxamine (Desferal), is administered by a battery-powered pump as a daily 8- to 24-hour subcutaneous infusion at a rate of 20 to 40 mg/kg/day). It has been the main chelator used since 1968. Deferasirox (Exjade) is an effective oral iron chelator in patients with iron overload and is approved for children older than 2 years of age. In some countries it is only approved for children age 2 to 5 if they cannot be adequately treated with deferoxamine. The initial dose is 20 to 30 mg/kg by mouth once daily. In a randomized trial of Desferal versus Exjade in patients with transfusional iron overload, the two chelators showed similar efficacy. In an international multicenter study in which the efficacy of Exjade was assessed in patients with anemia of various etiologies, 30 patients with DBA were included. Successful chelation was observed in 54% of the DBA patients as defined by reduction of the liver iron concentration to less than 7 mg/g dry weight within 1 year. Given its oral route of administration, Exjade has been replacing Desferal as the iron chelator of choice if the former is not contraindicated Jadenau is a new formulation of deferasirox that can be given with or without food at doses between 14 to 28 mg/kg per day.

There are uncertainties about the optimal age at which to start patients younger than 2 years old with transfusion-dependent anemia with Desferal therapy. There have been reports of abnormal linear growth and metaphyseal dysplasia in patients with thalassemia major treated with deferoxamine before the age of 3 years. This adverse event has prompted recommendations for starting deferoxamine later. However, a progressively rising serum ferritin level or, more accurately, excessive hepatic iron concentration obtained by FerriScan (using R2-MRI imaging technology) after a period of regular transfusions would be an appropriate indication to commence chelation therapy. Initial and periodic liver biopsies to assess liver iron levels were used in the past but have been almost completely replaced by FerriScan. The daily starting subcutaneous dose of deferoxamine should not exceed 50 mg/kg. Ascorbate supplementation should be considered if there is sustained loss of efficacy of deferoxamine, especially if tissue ascorbate concentrations are reduced.

Corticosteroids

Upon administration of prednisone at a dose of 2 mg/kg/day in two to three divided doses, varying degrees of response are observed in 50% to 75% of DBA patients. Reticulocytosis is usually seen within 1 to 4 weeks and is followed by a rise in hemoglobin concentration. When the hemoglobin level reaches 9.0 to 10.0 g/dL, prednisone can be slowly tapered by reducing the number of daily doses. If a single daily dose of prednisone maintains the desired hemoglobin level, the dose can be doubled and given on alternate days, but this may not prevent significant steroid toxicity.

The dose of prednisone is reduced by small decrements (10% to 15%) initially on a weekly basis and gradually spaced to every 2 weeks to 3 months until the minimal effective dose is determined. This dose is extremely variable. A few patients can be maintained on minute, nonpharmacologic doses, but other patients need large doses that preclude long-term therapy because of serious side effects such as Cushingoid features, pathologic fractures, cataracts, growth failure, diabetes, and avascular necrosis of the femoral or humeral heads. There is no known predictor of steroid responsiveness or any way to anticipate the type of individual response. In general, a corticosteroid dose equivalent of prednisone, 0.5 mg/kg/day, is suggested as a maximum "maintenance" dose after the initial dose of 2 mg/kg/day. About one-third of patients can maintain a response at a low prednisone dose. The rest are usually managed with chronic RBC transfusions.

There are several patterns of response to corticosteroid therapy, some of which may occur at different times in the same patient. Most children who respond to steroids cannot be completely weaned off the medication and become steroid dependent. About one-third of these patients, however, enter steroid-free remission after a prolonged period of treatment. Between 1% and 5% of responders immediately enter a durable steroid-independent remission. However, late relapses, sometimes precipitated by an infectious illness or by hormonal changes such as in pregnancy or with the use of birth control pills, are common. In other cases, a progressive resistance to steroids occurs, requiring escalating doses of prednisone or alternative therapy. After a relapse, some patients are responsive to steroids again, but others are refractory to subsequent trials. Initial refractoriness to steroids is observed in 36% of cases. In more than 60% of patients, long-term steroid therapy is hampered by the development of resistance or by side effects of the treatment. A study from the CIMFR showed that 70% of DBA patients respond to corticosteroids, and the probability of remaining corticosteroid-responsive among the whole DBA cohort was 70% and 55% at 5 years and 10 years post treatment initiation, respectively. In adolescent responders on long-term steroids, an option is to stop prednisone temporarily to allow for a normal growth spurt to occur. Infants with DBA on high doses of steroids are at risk for pneumocystis jirovecii pneumonia and should be given prophylactic antibiotics.

Megadose steroid therapy for DBA patients who were refractory to conventional-dose prednisone has resulted in partial or complete responses in some patients, but all of these patients relapsed with a taper and became transfusion dependent. Thus, this treatment is not recommended.

Cytokine Therapy

Serum erythropoietin levels are elevated in DBA, and attempts at treatment with high-dose erythropoietin have been ineffective, except for patients with biallelic mutations in EPO. Because of the "corrective" effect on erythropoiesis by IL-3 in vitro, clinical trials were introduced for steroid-refractory and steroid-dependent DBA patients and for those in whom HSCT was considered too risky. IL-3 was effective in only 10% to 20% of such cases. Currently, IL-3 is not available for clinical use.

Hematopoietic Stem Cell Transplantation

HSCT is a therapeutic option for DBA, but the risks must be weighed against the benefits on a case-by-case basis. The fundamental issue centers on the defined mortality rate with HSCT when used for a nonlethal medical disorder, at least a disorder that is nonlethal in the short term. In steroid-responsive patients on low-dose maintenance therapy HSCT is widely considered to not be indicated. In transfusion-dependent and adequately chelated patients who do not have severe thrombocytopenia, severe neutropenia, severe aplastic anemia, or MDS/AML, HSCT is ultimately a choice made by the parents and patients. Advances have been made in both, the management of patients who need chronic transfusions (e.g., developing of oral chelators and MRI-based imaging of iron overload) and HSCT approaches.

Experience has broadened since the first HSCT was performed for DBA in 1976. Preparative regimens, supportive measures, and GVHD management have progressively become more refined, thereby reducing the overall risks of the procedure. For consideration in the decision-making process, though, there are still higher risks when transplanting patients with DBA who have certain congenital malformations involving for example the kidneys and heart. Iron overload may also increase the risk. Otherwise, there is no known specific hypersensitivity of DBA patients to HSCT as seen in FA, DC, and SDS patients. Until 2014, most data showed more favorable results of HSCT when a MRD was used compared to a MUD. A report from the German and French registries demonstrated a major outcome improvement in pediatric DBA patients who were transplanted using MRD and MUD after the year of 2000, with overall survival of 100% and 87%, respectively ($P = .06$). The conditioning regimens included either busulfan/cyclophosphamide or busulfan/other drugs or treosulfan/thiotepa/fludarabine. Most patients also received antithymocyte globulin or antilymphocyte globulin. Eleven percent suffered from chronic GVHD. A recent study from Brazil showed overall survival of 80% and 73% when rMRD or MUDs were used, respectively; however, the results with mismatched donors were significantly poorer (29%). A small study from Japan showed no outcome differences between the use of myeloablative or reduced intensity conditioning regimens.

Data from the DBA Registry in the USA indicated that the survival rate for patients younger than the age of 10 years receiving matched related HSCT was greater than 90%. Umbilical cord blood as a stem cell source has been used for DBA HSCT with favorable results.

Given the generally favorable results with related donors, this procedure can now be offered to patients approximately after the age of 5 years when spontaneous remission is uncommon. HSCT from an unrelated donor should still be a part of a clinical trial unless the patient has severe thrombocytopenia, severe neutropenia, severe aplastic anemia, MDS, or leukemia.

This success with related donors has sparked interest in preimplantation genetic diagnosis with in vitro fertilization to "create" an HLA-matched sibling donor without a mutated DBA gene, and a number of patients worldwide have been successfully transplanted using umbilical cord stem cells from donors produced in this way. The religious, ethical, and economic questions generated by preimplantation genetic diagnosis to find a healthy matched donor for DBA and other inherited BM failure transplantations is ongoing.

Other Therapeutic Options

Based on the role of the DBA genes in ribosome biogenesis and global protein translation and in vitro studies aimed to stimulate translation in BM cells, a clinical trial was performed in which the branched amino acid leucine was administered to patients who were transfusion-dependent. However, the results were disappointing as only 5% of the patients became transfusion independent. Leucine stimulates translation by enhancing the activation of translation initiation factors that regulate mRNA binding to the ribosomal complex and by activation of the ribosomal protein S6 kinase and the mTOR (mammalian target of rapamycin) pathway. Thus far, the efficacy of leucine has been documented in cellular models of DBA, in several ribosomal protein deficient zebrafish models, and in a *RPS19*-knockdown mouse model where doxycycline-regulatable specific shRNA was used.

Several novel therapeutic targets are currently being investigated in clinical trials. The *TGFβ* pathway is activated in *RPS19* and in *RPL5* DBA, and TGF-β inhibits proliferation and self-renewal of BFU-E. Sotatercept and luspatercept are drugs that bind *TGF-β family* ligands. The drugs were shown to increase hemoglobin levels in patients with thalassemia and MDS. Consequently, a clinical trial of Sotatercept in adult patients with DBA is ongoing.

Calmodulin is an intracellular target of the secondary messenger Ca^{2+}. It is activated by Ca^{2+} and regulates downstream kinases and phosphatases. The antipsychotic drug trifluoperazine is also a calmodulin inhibitor and has been shown to rescue the hematopoietic phenotype in several DBA models. Trifluoperazine has been shown to rescue the hematopoietic phenotype in zebrafish and mouse DBA models and of DBA patient $CD34^+$ in vitro. Trifluoperazine is currently being investigated in a clinical trial in adult patients with DBA.

Nemo-like kinase (NLK) is chronically hyperactivated in committed erythroid progenitors and precursors in human and murine models of DBA. Inhibition of NLK expression or activity improves human erythroid expansion ex vivo and in vivo murine models of DBA. Metformin is a NLK inhibitor that improved erythropoiesis in human and zebrafish models of DBA through induction of miR-26a and suppression of NLK expression. miR-26a recognizes a binding site within the NLK 3′-UTR to facilitate transcript degradation. However, clinical trials to assess the effectiveness of repurposing this drug to DBA patients is necessary.

A number of uncontrolled therapeutic trials have been performed in steroid-refractory patients using various medications and treatments with varying anecdotal successes in a few patients. The medications include cyclosporine, metoclopramide, lenalidomide, androgens, riboflavin, vitamin B_{12}, folate, iron and other "hematinic" agents, 6-mercaptopurine, cyclophosphamide with antilymphocyte globulin, and antithymocyte globulin alone. There is a case report claiming efficacy of valproic acid and another report of an 8-month transfusion-free remission after rituximab therapy. Plasmapheresis has also been tried. Splenectomy, used in the past, shows no effect on erythropoiesis.

Future Directions

Registries and DBA patient databases will continue to broaden our understanding of the genetic origins and epidemiology of DBA. Specimen collection and distribution to qualified research laboratories globally will identify the remaining DBA genes. Genetically based DBA diagnosis and pedigree analysis will underscore the broad dimensions of the DBA phenotype, from clinically silent to life-threatening severe. Genotype–phenotype correlations will facilitate HSCT donor selection, allow counseling for reproductive options, and be predictive of cancer risk. Deciphering the pathogenesis of BM failure and other disease manifestations using animal models and iPSCs may allow the development of effective erythropoietic stimulators for use in this disease

Ex vivo experiments using gene reintroduction in patient cells to rescue the phenotype have been conducted. Correction of anemia in a mouse model using a lentiviral vector has also been achieved. However, thus far, clinical trials of gene therapy have not been initiated in DBA patients.

Congenital Dyserythropoietic Anemias

The designation *congenital dyserythropoietic anemia* (CDA) refers to a family of inherited refractory anemias characterized by BM erythroid multinuclearity, ineffective erythropoiesis, and secondary hemosiderosis. The ineffective erythropoiesis is reflected by BM erythroid hyperplasia, inappropriately low reticulocyte counts for the degree of anemia, and intramedullary RBC destruction. Splenomegaly and chronic or intermittent jaundice are additional features. Granulopoiesis and thrombopoiesis are normal. These disorders are genetically transmitted and result in anemia with a blunted erythropoietic response. Some patients, especially with CDA type I, have congenital anomalies.

Several classical forms of CDA have been described as well as a number of variants. The classification is based on the inheritance pattern, peripheral blood and BM morphology, and serologic findings. Based on reported CDA cases, type II is the most common CDA, type I is next, and CDA III is the rarest of the three major forms.

Etiology, Genetics, Pathophysiology, and Clinical Features

CDA Type I

CDA type I has been clinically defined as a disorder with macrocytosis, megaloblastic erythroid precursor cells, 2% to 5% binucleated erythroid precursor cells, internuclear chromatin bridges involving polychromatic erythroblasts, and a negative acidified serum lysis test (Ham test).

The onset of anemia, jaundice, and other symptoms may be noted at any age, especially in neonates. Eighty percent of infants in one large series required blood transfusions during the first month of life. Cases with anemia in utero requiring intrauterine exchange transfusions during the third trimester have been reported. Affected patients often have some degree of icterus and splenomegaly.

CDA type I can be associated with a variety of congenital anomalies in about 65% of patients: patches of brown skin pigmentation, syndactyly in the feet, absence of phalanges and nails in the fingers and toes, an additional phalanx, duplication or hypoplasia of metatarsals, short stature, pigeon chest deformity, varus deformity of hips, flattened vertebral bodies, a hypoplastic rib, congenital ptosis, Madelung deformity of the wrist, and deafness. The pigmentation, syndactyly, and absence of phalanges and nails are not common in CDA I patients but appear to be quite specific for this subtype. Neonatal pulmonary hypertension and sensorineural deafness, and a lack of motile sperm cells were reports in a few cases.

The degree of anemia is usually mild to moderate (hemoglobin in the range of 6.6 to 11.6 g/dL), and RBCs appear macrocytic. Peripheral blood RBC morphology is characterized by anisocytosis and poikilocytosis, and occasionally Cabot rings are seen. Cabot rings appear to be unique to CDA I and are not seen in types II and III. Cabot rings are thin, red-violet staining, threadlike strands in the shape of a loop or figure of 8 present in RBCs. They are believed to be microtubules that are remnants from the mitotic spindle. White blood cells and platelets are normal. Examination of the BM reveals erythroid hyperplasia with some megaloblastic erythropoiesis and a small number of erythroblasts with dyserythropoietic features. The unique morphologic abnormality seen in CDA I is the presence of chromatin bridges between nuclei of two separate erythroblasts, a reflection of impaired cellular division (Fig. 30.11). This internuclear bridging of erythroblasts seen with light microscopy is also a common feature in MDS. Electron microscopy reveals additional abnormalities that include widening of the nuclear membrane pore space with cytoplasmic invagination into the nucleus, separation of nuclear chromatin, and chromatin condensation, all of which give the general appearance of a spongy nucleus (Fig. 30.12). Dyserythropoiesis seems limited mostly to more mature RBC precursors. In contrast to CDA II, there are no unique serologic features.

The disorder is caused by biallelic mutations in *CDAN1* or in *CDIN1* (CDAN1 interacting nuclease 1, *C15ORF41*). *CDAN1* was first identified in highly inbred population of Israeli Bedouins. The gene product, codanin-1, may be involved in nuclear envelope integrity. Codanin-1 binds to the nuclease, CDIN1, and stabilizes it. Codanin-1 also shifts CDIN1 from the nucleus to the cytoplasm. The defect in CDA I is at the stem cell level. The numbers of CFU-E and BFU-E colonies are normal but contain a mixture of normal and abnormal cells when examined by electron microscopy. This suggests that the abnormality is expressed variably in the mature progeny of each stem cell. Erythroid precursors also are characterized by S phase arrest and morphologic features of apoptosis. In some CDA I patients, hemoglobin A_2 levels are increased. Also, some cases show unbalanced globin chain synthesis. Patients do not have thalassemia, and the cause of these findings is not known.

CDA Type II (HEMPAS)

CDA Type II is an autosomal recessive disorder with normocytic RBCs, normoblastic erythroid precursor cells, 10% to 40% binucleated late normoblasts, and a positive acidified serum lysis test (Ham test). CDA Type II is also known as HEMPAS, an acronym for hereditary erythroblastic multinuclearity with a positive acidified serum test.

Anemia is often more severe than in CDA type I. Patients with CDA II may develop progressive, lifelong iron overload even in the absence of transfusions, and approximately 20% develop cirrhosis. Splenomegaly occurs in the majority of patients with CDA II. A number of other clinical associations with CDA II have been reported such as mental retardation, Sweet syndrome, von Willebrand disease, and Dubin-Johnson syndrome, among others. Rather than true associations, it is likely that the majority represent coincidental occurrences. Mild hyperbilirubinemia is common, and about half of the patients develop gallstones.

The disorder is caused by biallelic mutant *SEC23B*. The wild-type gene encodes the SEC23B component of the coat protein (COP) II complex. COP II vesicles transport secretory proteins from the endoplasmic reticulum to the Golgi complex. Mutations result in misglycosylation and an impaired clearance of the endoplasmic reticulum cisternae past a given point during erythroid differentiation. The mutations in *SEC23B* are localized along the entire coding sequence of the gene, in splicing sites, and in regulatory regions. No case with biallelic nonsense mutations has been described, suggesting an essential role of the gene.

A large study from Italy and Turkey identified a relationship between three genetic groups and clinical phenotypes: patients carrying two missense alleles (Group 1), patients carrying a missense allele in compound heterozygosity with a nonsense/hypomorphic allele (Group 2), and patients carrying either two hypomorphic alleles or a nonsense allele in compound heterozygosity with a hypomorphic allele (Group 3). The degree of anemia, hyperferritinemia, and transfusion dependency was most severe in patients who belonged to Group 2, followed by the patients in Group 1. The patients in group 3 had the mildest phenotype.

In vitro cultures of CDA II erythroid progenitors produced CFU-E and BFU-E colonies with erythroblast multinuclearity. Initially, studies of peripheral blood CDA II RBCs identified a number of chemical

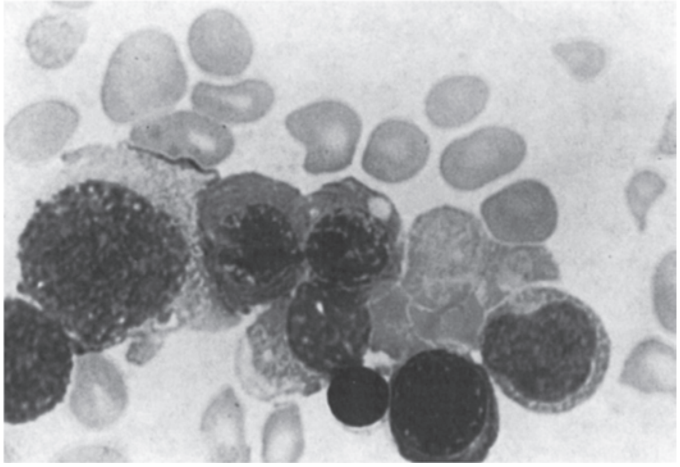


Figure 30.11 BONE MARROW FROM PATIENT WITH CONGENITAL DYSERYTHROPOIETIC ANEMIA TYPE I. Erythroblasts are connected by internuclear bridges between two cells. *(Provided by Dr. Jean Shafer, Rochester, NY.)*

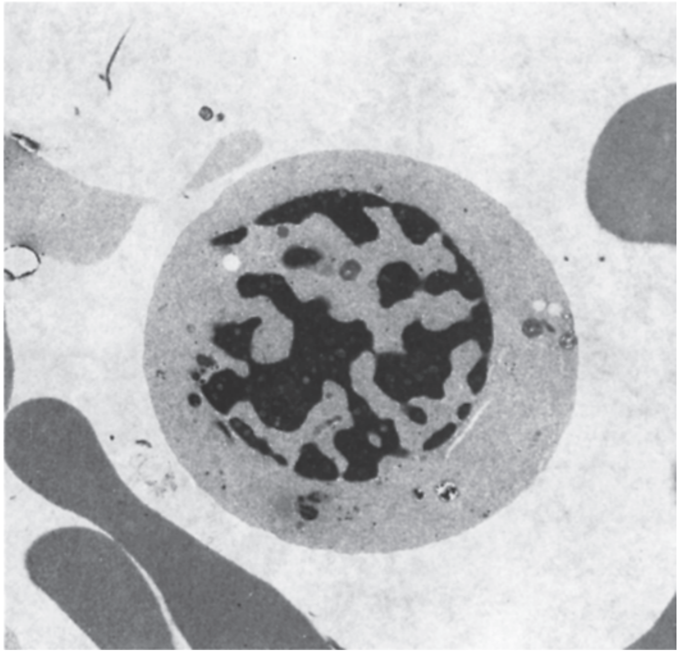


Figure 30.12 ELECTRON MICROGRAPH OF BONE MARROW FROM PATIENT WITH CONGENITAL DYSERYTHROPOIETIC ANEMIA TYPE I. Note the “spongy” appearance of the nucleus resulting from uneven chromatin with cytoplasmic invagination into the nucleus. *(Provided by Dr. Raoul Fresco, Maywood, IL.)*

abnormalities, including unbalanced globin chain synthesis, increased membrane glycolipids, and altered RBC membrane protein patterns demonstrated by two-dimensional electrophoresis. Furthermore, glycoproteins on CDA II RBCs were found to have an abnormal carbohydrate structure, leading to aberrant reactivity with anti-i sera. Additional data suggested that the IgM antibody responsible for hemolysis in the acidified-serum lysis test recognized an abnormal glycolipid structure sharing homology with i and I antigens. Thus, a variety of data predicted and confirmed that abnormalities in the glycosylation pathway were involved in the etiology of CDA II.

The two major defects in the CDA II glycosylation enzymatic pathway are a deficiency of α-mannosidase II and of *N*-acetylglucosaminyl transferase II. A third defect in a CDA II variant is deficient levels of the membrane-bound form of galactosyl transferase. All three of these enzymatic deficiencies lead to abnormal oligosaccharides on major erythrocyte proteins such as the anion transporter band 3 that could cause disruption of the structural network of erythrocytes and their precursors, thereby leading to their premature demise. Defective glycosylation of the RBC surface may also affect the regulation of complement on the surface of erythrocytes. Enhanced functional activity of the alternative pathway C3 convertase and of the membrane attack complex may result from the improper glycosylation of glycophorin A, which has been proposed to serve as a complement regulatory protein. These abnormalities are not a consequence of quantitative or functional deficiencies of the complement regulatory proteins CD55 or CD59.

There is overlap between the clinical and laboratory manifestations of CDA I and CDA II, but there are three major differences. The first is that the magnitude of anemia is usually more severe, and patients, especially children, often require RBC transfusions. Peripheral blood RBCs are usually normocytic but show anisocytosis and poikilocytosis (Fig. 30.13). The second difference is that the BM in CDA II reveals greater numbers of abnormal erythroblasts with binuclearity in up to 35% late erythroblasts, as well as multinuclearity and abnormal lobulation (Fig. 30.14). These nuclear abnormalities are seen only in late erythroblasts, not in basophilic erythroblasts. Karyorrhexis is commonly observed, and pseudo-Gaucher cells may be present, representing the ingestion of debris by histiocytic cells from ineffective erythropoiesis. Electron microscopy of late erythroblasts also reveals an excess of endoplasmic reticulum parallel to the cell membrane, giving the appearance of a double cell membrane (Fig. 30.15). A third difference, which is also a pathognomonic finding, is that CDA II RBCs are lysed by acidified (pH, 6.8) sera obtained from approximately 30% to 60% of fresh ABO-compatible sera from normal persons (i.e., a positive Ham test result), but there is no lysis when RBCs are incubated with the patient's own acidified serum. This lysis is a result of a naturally occurring IgM antibody that recognizes an antigen on CDA II cells and binds complement; this antibody can be removed by preincubating normal sera with HEMPAS erythrocytes. However, the specific HEMPAS antigen recognized by this antibody is not known. In contrast to HEMPAS, the erythrocytes of patients with paroxysmal nocturnal hemoglobinuria (PNH) undergo lysis when the acidified serum is from the PNH patient or from normal donors. Another difference is that PNH erythrocytes undergo lysis in isotonic sucrose (sugar water test), but HEMPAS RBCs do not lyse in isotonic sucrose.

The erythrocytes from patients with CDA II also exhibit an increased agglutinability and lysis to anti-i and anti-I sera and manifest increased expression of both antigens. These surface antigens are complex carbohydrate structures found predominantly on fetal

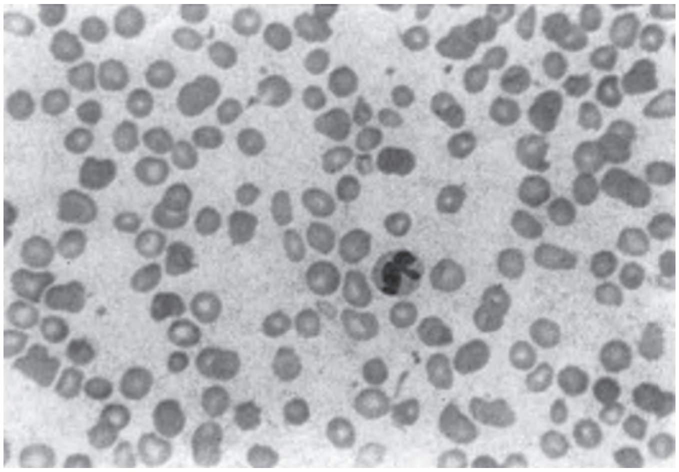


Figure 30.13 PERIPHERAL BLOOD SMEAR FROM A PATIENT WITH CONGENITAL DYSERYTHROPOIETIC ANEMIA TYPE II. Note the marked variation in red blood cell size and shape.

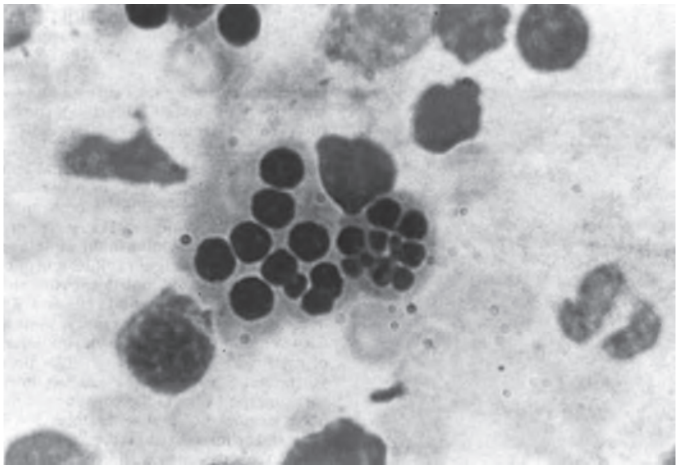


Figure 30.14 BONE MARROW ASPIRATE FROM A PATIENT WITH HEMPAS (CONGENITAL DYSERYTHROPOIETIC ANEMIA TYPE II) SHOWING ERYTHROID HYPERPLASIA AND MULTINUCLEATED ERYTHROBLASTS. *(Provided by Dr. Jean Shafer, Rochester, NY.)*

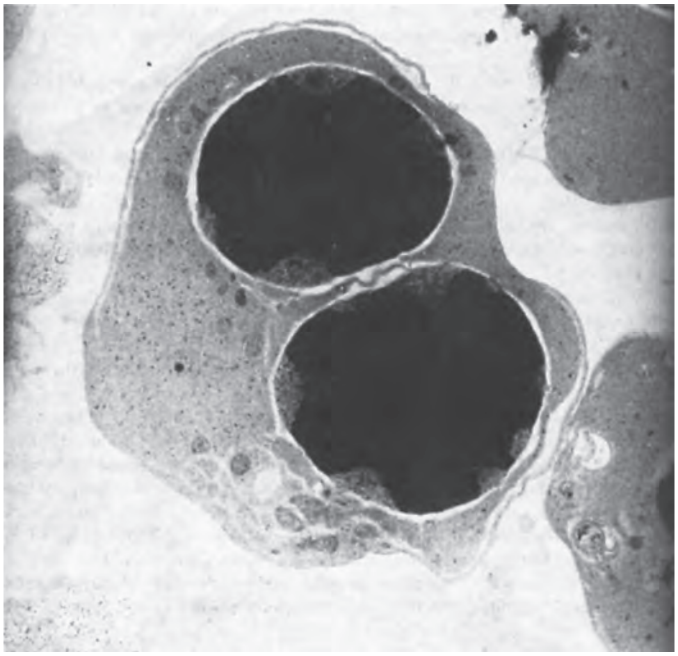


Figure 30.15 ELECTRON MICROGRAPH OF A BONE MARROW ERYTHROBLAST FROM A PATIENT WITH HEMPAS (CONGENITAL DYSERYTHROPOIETIC ANEMIA TYPE II). Note the appearance of a double cell membrane, reflecting an excess of endoplasmic reticulum. *(Provided by Dr. Raoul Fresco, Maywood, IL.)*

and adult RBCs, respectively. Increased expression of i antigen can be demonstrated on all RBCs in CDA II using fluorescent labels. Relatives of patients with CDA II who have normal BM but increased agglutinability to anti-i appear to be heterozygote carriers of this disorder. HEMPAS erythrocytes bind a normal amount of complement (C1), but more antibody and less C4 than normal. This causes binding of an excess of C3 and hemolysis.

As in CDA I, the defect in CDA II is at the level of erythroid progenitor cells and is expressed variably in more mature erythroblasts.

CDA Type III

CDA Type III was clinically defined as an autosomal dominant disorder with macrocytosis, megaloblastic erythroid maturation, giant multinucleated erythroid precursors with up to 12 nuclei per cell, and a negative acidified serum lysis test (Ham test).

In contrast to the other two forms, CDA III is inherited as an autosomal dominant disorder. The responsible gene is *KIF23*, which encodes mitotic kinesin-like protein 1 (MKLP1) that localizes to the midbody ring. In vitro studies showed that the mutations in the genes result in cytokinesis failure. It has also been shown that ubiquitination of MKLP1 is required for midbody ring degradation by autophagy; thus, abrogation of this process may cause cytokinesis failure and multinuclearity.

In a Swedish family with 31 cases inherited in an autosomal dominant mode, an excess number of cases with a monoclonal gammopathy and myeloma have occurred. Also, an adult patient with CDA III was described with T-cell non-Hodgkin lymphoma. These cases, plus a case of Hodgkin disease occurring in an additional patient, may indicate an increased incidence of lymphoproliferative diseases in CDA III.

In CDA III, splenomegaly is usually minimal or absent. The anemia is usually mild to moderate, but transfusion-dependent patients have been observed. The circulating RBCs can be normal or mildly macrocytic. BM examination shows erythroid hyperplasia. Giant erythroblasts with up to 12 nuclei are the most distinctive feature of CDA III observed on light microscopic examination of the BM. These may appear similar to some of the large multinucleated cells seen in CDA II (see Fig. 30.14). Abnormally large lobulated nuclei and discordance in nuclear maturation are also found. Although they are hallmarks of CDA III, these findings are not pathognomonic and may be seen in erythroleukemia. Electron microscopy demonstrates nuclear clefts and blebs, autolytic areas within the cytoplasm, and iron-filled mitochondria. In some cases of CDA III with presumed autosomal dominant inheritance and in some sporadic cases, electron microscopy reveals that an occasional erythroblast section contains stellate or branching electron-dense intracytoplasmic inclusions. These are morphologically indistinguishable from those in HbH disease and consist of precipitated β-globin chains.

The acidified-serum lysis test result is negative in CDA III. Agglutination and lysis of erythrocytes to anti-i antibody has only been examined in a few cases of CDA III with conflicting findings. Serum thymidine kinase was measured in 20 CDA III patients and 10 healthy siblings. Elevated thymidine kinase was found in all 20 cases but was normal in the siblings. It is suggested that measuring thymidine kinase levels can allow clinicians to discriminate between affected individuals and healthy siblings without performing a BM aspirate.

CDA Group IV. This group has severe anemia, transfusion dependence from birth, and sometimes presentation as hydrops fetalis. Patients have increased expression of embryonic and fetal hemoglobins. BMs are characterized by marked erythroid hyperplasia, normoblastic or mild to moderate megaloblastic changes, basophilic stippling of polychromatic erythroblasts and erythrocytes, marked abnormalities of nuclear shape, up to 8% BM erythroblasts with markedly irregular or karyorrhectic nuclei, and an absence of precipitated protein within erythroblasts by electron microscopy. The spleen is enlarged. It is caused by mutations in the ***KLF1*** gene that encodes an erythroid transcription factor.

Other CDAs. ***CDA type V*** refers to patients with normal or near-normal hemoglobin levels, a normal or slightly elevated MCV, and an increased serum unconjugated bilirubin. The BM shows marked erythroid hyperplasia and normoblastic or mild to moderate megaloblastic changes. The spleen may be palpable. The condition has been previously described as "primary shunt hyperbilirubinemia." Inheritance is variable and appears to be autosomal recessive in some cases but possibly autosomal dominant or X-linked in others. In CDA group V, the BM macrophages engulf morphologically normal but functionally abnormal erythroblasts. ***CDA Group VI*** is characterized by marked macrocytosis (MCV 119 to 125 fL) with little or no anemia and grossly megaloblastic erythropoiesis. There may be mild jaundice and an increased serum bilirubin. The differential diagnosis includes orotic aciduria and thiamine-responsive anemia. Vitamin B_{12} and RBC folate levels are normal.

Patients with CDA Group VII have severe transfusion-dependent anemia from birth, marked erythroid hyperplasia, normoblastic or nonspecific dysplastic changes, markedly abnormal nuclear shapes in many erythroblasts, and intraerythroblastic inclusions resembling precipitated α globin chains. The inclusions, however, do not react with monoclonal antibodies against α and β globin chains. The diagnosis requires the exclusion of β-thalassemia trait in the parents. ***Unclassifiable cases of CDA*** have been reported with lifelong anemia that was probably inherited. These cases were characterized by marked aniso- and poikilocytosis and occasional teardrop and fragmented erythrocytes in the peripheral blood. Hyperplastic BM showed megaloblastoid features without multinuclearity or ringed sideroblasts, but a case with prominent ringed sideroblasts was also described. Unlike classical CDA, neutropenia or thrombocytopenia has been observed in some of these patients. Cytogenetic studies of the BM did not reveal clonal chromosomal abnormalities. Reticulocyte responses to anemia are absent or inappropriately low. Most studies of parents failed to reveal abnormalities, suggesting an autosomal recessive mode of transmission. Dyserythropoietic anemia has been described in cases with mutations in genes that are typically associated with other phenotypes such as ***GATA1*** (with Val205Met mutation), the erythroid anion exchanger **band 3**, ***MVK*** (mevalonate kinase), and ***COX412***.

Differential Diagnosis

Marrow erythroblast multinuclearity is seen with other hematologic disorders, but these can be readily distinguished from CDA. The **megaloblastic anemias** have a different clinical presentation and are identified by the presence of hypersegmented neutrophils, decreased RBC folate values, or reduced serum levels of vitamin B_{12}. The **MDS** may manifest as an isolated refractory anemia, but they often present with bi- or trilineage cytopenias that contrast sharply with CDA. The MDS subsets may also show BM granulocytic and megakaryocytic morphologic abnormalities, the presence of myeloblasts, ringed sideroblasts, and clonal cytogenetic changes. **Erythroleukemia** (AML, M6) is another cause of marked dyserythropoiesis, but typically there is pancytopenia, the erythroblasts are avidly positive for the periodic acid-Schiff stain, and the BM cells may show a clonal cytogenetic marker. The **β-thalassemia syndromes** differ from CDA by the presence of marked microcytosis with elevated levels of hemoglobin A_2, fetal hemoglobin, or both.

Therapy and Prognosis

In general, the CDAs are associated with a favorable long-term prognosis. For example, CDA I can be diagnosed late in life. One reported case was associated with a relatively benign course over a 30-year follow-up. Clinical manifestations of the CDAs may include intermittent jaundice and dark urine caused by increased hemoglobin catabolism or signs and symptoms of anemia. Rarely, hyperbilirubinemia without anemia may be the initial presentation of CDA patients. Cholelithiasis may be present as a consequence of chronic hyperbilirubinemia.

Anemia is typically mild in most cases of CDA and requires no intervention, but in more severe presentations, especially those requiring transfusional support, splenectomy may be beneficial. Most of the experience showing an improvement in hemoglobin levels after splenectomy has been in CDA II; the benefit of splenectomy in CDA I is less clear. Splenectomy may cause a persistent thrombocytosis in CDA types I and II and be associated with the development of Budd-Chiari syndrome and portal vein thrombosis.

Treatment of all forms of CDA with androgens, corticosteroids, vitamin E, vitamin B_{12}, folic acid, pyridoxine, or iron is ineffective. Iron therapy is also contraindicated because of the underlying propensity for hemosiderosis secondary to ineffective erythropoiesis, transfusional iron overload, increased gut iron absorption, and downregulation of the primary regulator of iron absorption, hepcidin. Even if regular RBC transfusions are not initiated, patients should be routinely monitored for evidence of iron overload. Iron chelation has probably been underused in CDA even though deaths have been recorded from hemochromatosis.

Phlebotomy has been carried out to remove iron in selected CDA patients, but this could result in a worsening of the anemia and theoretically enhance gut iron absorption. An additional concern is that CDA patients seem predisposed to develop cirrhosis irrespective of body iron burden. The cause of this association is unclear.

Interferon-α-2a or INF-α-2b therapy given two or three times a week or peg-INF-α-2a once a week increases hemoglobin levels and decreases the degree of iron overload in most CDA I patients who require repeated transfusions for moderately severe anemia. Erythrokinetic studies demonstrate a striking reduction of ineffective erythropoiesis in patients receiving INF therapy, and electron microscopy shows a reduction in nuclear structural abnormalities. INF therapy should also be considered in moderately anemic CDA I patients before treatment of iron overload with phlebotomy. Patients with CDA II do not respond to INF-α.

HSCT has been curative in a few severe cases of CDA, including transfusion-dependent CDA type I, CDA type II, Ham test–negative "CDA type II," and an unclassifiable CDA.

Asymptomatic extramedullary hematopoiesis may mimic tumors of the mediastinum, abdomen, and vertebral column. Because of the increased RBC production that occurs, the development of sites of extramedullary hematopoiesis may result in an amelioration of the degree of anemia. Technetium-99m sulfur colloid scintigraphy is useful in delineating the extent of these regions.

Future Directions

Identification of the various mutant genes for the CDA types will facilitate a more precise classification than the phenotype-based system used currently. The wild-type protein product of the CDA genes requires further research to clarify their function in health and their role in producing CDA when mutated. Strategies are required for managing iron overload similar to those used for Diamond-Blackfan anemia and thalassemia major.

INHERITED BONE MARROW FAILURE SYNDROMES WITH PREDOMINANTLY NEUTROPENIA

Hereditary neutropenias are genetically and phenotypically heterogenous groups of disorders (Table 30.8). They can be classified according to either their inheritance pattern or phenotype, or gene function. Conditions with **autosomal dominant** inheritance include patients with mutations in neutrophil elastase (*ELANE*) that can cause classic SCN or classic cyclic neutropenia. *GFI1* is mutated in patients with neutropenia and lymphopenia. The rest of the hereditary neutropenias with autosomal dominant inheritance are typically associated with a broad phenotype that include extra hematological manifestations: *TCIRG1* (hemangiomas), *CXCR4* (wart, hypoglobulinemia, infection, and myelokathexis [WHIM] syndrome), *DNM2* (intermediate Charcot-Marie-Tooth syndrome), and *SEC61A1* (common variable immunodeficiency and glomerulocystic kidney disease). The phenotype associated with *SRP54* mutations ranges from classical SCN to classical SDS.

All hereditary neutropenias that are transmitted in an **autosomal recessive** mode are associated with extra hematological manifestations either in a minority or majority of the patients. These include SCNs that are caused by mutations in *HAX1*, *GCPC3 VPS45, JAGN1, USB1, PGM3, G6PT1/SLC37A4, COH1/VPS13B, AP3B1*, and *FBXL4*.

The cases of X-linked hereditary neutropenias comprise several syndromes that are associated with the following genes: *WAS*, *TAZ*, *CD40LG*. The mutations in the *WAS* genes are in the C-terminus of the encoded protein. They cause gain of function and are different from the inactivating mutations underlying Wiskott-Aldrich syndrome.

In this section we will first describe hereditary neutropenias caused by mutations in *ELANE, HAX1, GFI1*, and *CSFR3* that result in classical SCN. This will be followed by a description of several other hereditary neutropenias. The complete list of hereditary neutropenias described thus far is in Tables 30.1 and 30.8.

Severe Congenital Neutropenia

Background

Classical SCN refers to inherited types of neutropenia with onset in early childhood of profound neutropenia (ANC <200/μL), recurrent life-threatening infections, and a maturation arrest of myeloid precursors at the promyelocyte-myelocyte stage of differentiation.

The initial description of the syndrome made by Dr. Kostmann in 1956 included several neutropenic patients in a large intermarried Swedish kinship, and the condition was named Kostmann syndrome. An autosomal recessive mode of inheritance in 24 cases was deduced by inference because of hematologically normal parents with two or more neutropenic children in several families. In 2007 homozygous germline *HAX1* mutations was identified in a portion of patients with SCN, including some from the original pedigree described by Dr. Kostmann, confirming an autosomal recessive inheritance in these families. Nevertheless, it is now clear that the SCN group is genetically heterogeneous despite a shared hematologic phenotype (see Table 30.1). The first identified SCN gene was the neutrophil elastase 2 gene *(ELA2* or *ELANE)*, which was found to be heterozygously mutated in patients with SCN, indicating an autosomal dominant inheritance in many cases and constitutes the largest genetic groups. Mutations in *ELANE* were found in patients of various ethnic extracts; however, mutations in *HAX1* are largely restricted to those of Swedish or Turkish origin.

Epidemiology

SCNs are rare. The prevalence was estimated as 3 to 8.5 cases per million individuals. The estimated incidence based on data from CIMFR from 2001 to 2010 was 4.7 cases per million live births per year (1). There is an equal distribution of the disease between genders. There might be different frequency of specific genetic groups in different countries. For example, *ELANE* is the most commonly mutated gene in SCN, but the prevalence of ELANE mutations is higher in North America than in Europe. *HAX1* mutations are seen in 20% to 25% of the patients in Europe, but only in rare patients who emigrated from certain European countries in North America.

Genetics

The discovery of heterozygous mutations in the ***ELANE*** encoding neutrophil elastase in 22 of 25 sporadic and dominantly inherited patients with SCN was the entry point for understanding the molecular basis of the disorder in many patients. Among patients with hereditary neutropenias from various cohorts the most commonly mutated gene is *ELANE* (40% to 60%). Mutant *ELANE* also occurs in all cases of classical cyclic neutropenia, but the mutations cluster in exon 4 or 5 of

TABLE 30.8 Inherited Neutropenia Disorders Ordered According to Mode of Inheritance

Diagnosis	Inheritance	Mutant Gene	Features Besides Neutropenia That Appear in Some or Most Patients
SCN and cyclic neutropenia	AD	*ELA2*	Osteopenia in patients with SCN
SCN	AR	*HAX1*	Seizures, developmental delay
SCN	AD	*TCIRG1*	Hemangiomas
SCN	AR	*JAGN1*	Short stature, amelogenesis imperfecta, bone defects, osteoporosis
SCN	AR	*CSF3R*	None
SCN	AD	*GFI1*	Lymphopenia
SRP54-related disorder	AD	*SRP54*	Some patients have partial or complete SDS phenotype
WHIM syndrome	AD	*CXCR4*	Warts, ↓IgG, infections, myelokathexis
WAS-related neutropenia	XL	*WAS*	Lymphopenia, monocytopenia, ↓natural killer cells, reduced phagocyte activity
Barth syndrome	XL	*TAZ*	Cardiomyopathy, myopathy, short stature, 3-methylglutaconic aciduria, quantitative and qualitative cardiolipin abnormalities
Glycogen storage disease Ib	AR	*G6PT1/SLC37A4*	Glycogen storage disease type I phenotype, colitis, pancreatitis, osteoporosis
G6PC3-related SCN	AR	*G6PC3*	Thrombocytopenia, ↓T-cells, cardiac defects, prominent superficial veins, skin hyperelasticity, urogenital malformations, endocrine abnormalities, colitis
Myelofibrosis with neutropenia	AR	*VPS45*	BM fibrosis, anemia, thrombocytopenia Splenomegaly, osteosclerosis, cortical blindness, hearing loss, thin corpus callosum, developmental delay, nephromegaly
Poikiloderma with neutropenia	AR	*USB1/C16ORF57*	Poikiloderma, hyperkeratosis of palms and soles, dystrophic nails, short stature, pulmonary infections
Cohen syndrome	AR	*VPS13B/COH1*	Retinopathy/myopia, skeletal dysplasia, psychomotor retardation, hypotonia, microencephaly, truncal obesity,
STK4 deficiency	AR	*STK4*	Monocytopenia, ↓T-cells, ↓B-cells, warts, atrial septal defects, lymphomas
Charcot-Marie-Tooth syndrome, type 2	AD	*DMN2*	Axonal demyelinating neuropathy
Common variable immunodeficiency and glomerulocystic kidney disease	AD	*SEC61A1*	Anemia, thrombocytopenia, common variable immunodeficiency, small kidneys, glomerulocystic kidney disease.
P14 deficiency	AR	*LAMTOR2*	Immunodeficiency, oculocutaneous albinism, stunted growth
Neutropenia, immune deficiency, skeletal dysplasia and glycosylation defect	AR	*PGM3*	Immune deficiency, skeletal dysplasia and glycosylation defect
Fumarase deficiency[a]	AR	*FH*	Fumaric aciduria, neurological abnormalities
Mitochondrial DNA depletion syndrome 13	AR	*FBXL4*	Lymphopenia, encephalomyopathy, lactic acidosis, growth failure, neurological abnormalities, heart abnormalities, cataract, optic atrophy, sensorineural hearing loss
Hermansky-Pudlak syndrome, type 2	AR	*AP3B1*	↓IgG, partial albinism, platelet dysfunction
Griscelli syndrome, type 1	AR	*MYO5A*	Neurologic dysfunction, partial albinism
Griscelli syndrome, type 2	AR	*RAB27A*	Same as type 1 plus hemophagocytosis
Chediak-Higashi syndrome	AR	*LYST (CHSI)*	Immunodeficiency, partial albinism
Hyper IgM syndrome, type 1	XL	*CD40LG*	↓IgG, IgA, IgE, autoimmune cytopenias

Data compiled from Pubmed and Online Mendelian Inheritance in Man (http://ncbi.nlm.nih.gov/omim).
AD, Autosomal dominant; *AR*, autosomal recessive; *Ig*, immunoglobulin; *SCN*, severe congenital neutropenia; *XL*, X-linked recessive.
[a] In addition to the above patients with the following autosomal recessive metabolic diseases may have intermittent neutropenia: propionic aciduria, maple syrup urine disease, methylmalonic aciduria, methylglucagonic acid type VII. The related genes are *PCCA*, *PCCB*, *DBT*, BCKDHA, *BCKDHB*, *MMUT*, *CLPB*.

the gene or at the junction of exon 4 with intron 4. Patients with congenital neutropenia have mutations more widely distributed over exons 2, 3, 4, and 5. Various types of mutations in *ELANE* have been reported: missense frameshift, termination, intronic, and in-frame deletion/insertion. In a thorough analysis of the mutations all frame-shift mutations were associated with the SCN, and specific mutations (i.e., G214R or C151Y) had a high risk for evolution to AML.

In 2007 ***HAX1*** was found to be mutated in patients with SCN. In Europe *HAX1* is mutated in 11% to 25% of patients. HAX-1 has two isoforms. Mutations affecting both isoforms (e.g., p.R86X and p.Q190X) cause SCN with neurologic abnormalities such as seizures and developmental delay. Mutations that affect only one isoform (e.g., p.W44X) lead to SCN without neurologic involvement.

Biallelic mutations in the extracytoplasmic domain of G-CSF receptor, CSF3R, cause SCN. The mutations abrogate the binding of G-CSF ligand to its receptor. Heterozygous mutations in the repressor transcription factor *GFI1* causes SCN with lymphopenia. *Mutations in SRP54* are associated most frequently with a SCN phenotype; however, some patients have a partial complete SDS phenotype.

Although typical SCN patients present early in life with severe neutropenia and life-threatening infections, rare cases with *ELANE* mutations in phenotypically healthy family members were reported. For example, two siblings with congenital neutropenia inherited the same heterozygous *ELANE* mutation from their hematologically normal father. In another family, a healthy father of a congenital neutropenia patient was mosaic for his daughter's Cys42Arg mutation. The mutation was found in about 50% of his T-lymphocytes but only 10% of his neutrophils. This is congruent with a phenotype associated with a lack of recurrent infections in the father. The mutation was evident in myeloid precursors but was selectively lost during myelopoiesis.

Pathophysiology

ELANE encodes neutrophil elastase, a glycoprotein synthesized in promyelocytes/myelocytes and packaged in the azurophilic cytoplasmic granules. It is released in response to infection and inflammation. There are several proposed mechanisms for how mutations in *ELANE* cause neutropenia. The wild-type neutrophil elastase diffusely localizes throughout the cytoplasm. It has been shown that the mutated protein is not reduced, but mistrafficked and is abnormally concentrated in the nucleus and plasma membrane. Interestingly, mutations that disrupt the ATG translation initiation codon or the immediately adjacent Kozak sequence cause translation from downstream in-frame initiation codons, yielding a protein that lacks the amino-terminally domain sequences that are important for ER-localizing. A second theory suggests that the mutant protein leads to accumulation of nonfunctional protein in the endoplasmic reticulum, activation of unfolded protein response, and apoptosis of SCN neutrophils. Related to this theory, decreased expression of Bcl-2 was observed in SCN myeloid progenitor cells along with constitutive mitochondrial release of cytochrome C and excessive cellular apoptosis. Of note, administration of G-CSF restored Bcl-2 expression and improved survival of myeloid progenitor cells. Evidence supporting a detrimental effect of the mutant elastase is demonstrated by the correction of the cellular phenotype by deleting the mutant allele in SCN iPSCs. Another proposed mechanism is downregulation of lymphoid enhancer-binding factor 1 (*LEF-1*) in SCN. This leads to reduced transcription of *LEF1*-target genes such as *C/EBP*-α and impaired granulocytic differentiation. In contrast, the expression of monopoiesis-activating transcription factor PU.1 (PU.1) is unaffected or slightly elevated, explaining the monocytopenia seen in patients with SCN.

HAX1 localizes to the mitochondria. It contains two domains reminiscent of a BH1 and BH2 of the BCL-2 family. It promotes normal potential of the inner mitochondrial membrane and protects myeloid cells from apoptosis. The direct function of HAX1 in promoting survival may explain the accelerated apoptosis reported in SCN neutrophils. *HAX1-mutant* SCN cells are also characterized by reduced LEF1 levels as in *ELANE-mutant* SCN cells. HAX-1 partners with hematopoietic lineage cell-specific protein (HCLS1), which is required for G-CSF receptor signaling. Thus, it is possible that HAX1 mutations reduce HCLS1-dependent G-CSF receptor signaling, which leads to reduced granulopoiesis and neutropenia.

A constitutively activating mutation in the Wiskott-Aldrich syndrome protein encoded by the *WAS* gene was first discovered in five males from a three-generation family. The phenotype was composed of severe neutropenia from birth, bacterial infections, monocytopenia, and shifts of lymphocyte subsets. The BM was characterized by a selective maturation arrest at the promyelocyte/myelocyte stage similar to SCN. Mutant *WAS* leads to constitutive activation of the WASP protein because of disruption of an autoinhibitory domain in the wild-type protein. This increased WASP protein activity produces marked abnormalities of cytoskeletal structure and dynamics, disruption of mitosis, genomic instability, and apoptosis of neutrophils.

Mutations in the proto-oncogene *GFI1* also cause SCN with severe neutropenia and a maturation arrest at the promyelocyte–myelocyte stage. GFI1-deficient mice exhibit severe neutropenia with accumulation of abnormal arrested progenitors and increased HSC/HPC proliferation. GFI1 is a transcriptional repressor of several transcriptional programs by recruiting histone-modifying enzymes to promoters and enhancers of target genes. Patient-related mutations disrupt the C-terminal zinc finger domains of GFI1. One of the transcriptional programs modulated by GFI1 is active at the progenitor stage. During this stage, GFI1 downregulates the HoxA9-Pbx1-Meis1 transcription factor complex. Because HOXA9 drives progenitor proliferation, GFI1 deficiency leads to uncontrolled HOXA2 activation and accumulation of arrested-differentiation myeloid progenitors. Another transcription program is activated during terminal granulopoietic differentiation. During this stage, GFI1 represses genes that promote differentiation of nongranulocytic cells such as CSF1. Patient-related mutations also disable the GFI1 repressor activity on *ELANE* expression, which leads to accumulation of neutrophil elastase in all subcellular compartments and might underlie the mechanism for premature apoptosis. An additional potential mechanism for neutropenia in GFI1 deficient patients might be related to loss of GFI1-mediated regulation of expression of the micoRNAs miR-21 and miR-196b, which regulate myeloid maturation.

A constitutive point mutation was first discovered in the extracellular domain of the G-CSF receptor (GCSFR or *CSF3R*) in a patient with SCN who also had a mutant *ELANE* gene. The receptor mutation affected ligand–receptor complex formation with severe consequences for intracellular signal transduction; the patient was totally unresponsive to G-CSF therapy. As demonstrated in vitro and then clinically, corticosteroids combined with G-CSF produced a corrective action through synergistic activation of STAT5, and the patient responded to G-CSF therapy. Importantly, four SCN patients from two different families who did not respond to G-CSF treatment were found to have biallelic mutations in *GCSFR*. Some of these patients respond to GM-CSF.

Regarding the cellular pathology of SCN, many cell culture studies performed in the 1970s and 1980s provided clonogenic data that pointed to intrinsically defective granulocytic progenitors. Further reports confirmed this and excluded other possible pathogenetic factors. To summarize: (1) SCN BM myeloid colony growth is defective, (2) SCN serum contains normal or increased levels of G-CSF, (3) endogenous SCN G-CSF has normal biologic activity, (4) G-CSF receptors are expressed in slightly increased numbers on myeloid cells from SCN patients, and (5) the binding constant for G-CSF to its receptor in SCN is normal.

Clinical Features

Approximately half of the patients with SCN develop clinically significant infections within the first month of life and almost all others develop them by 6 months. Skin abscesses are common, but deep-seated tissue infections and septicemias also occur. Data from various SCN registries illustrate examples of every conceivable form of bacterial and fungal infection in the precytokine era. Especially troublesome in survivors were recurrent episodes of otitis media and pneumonia; advanced gingival stomatitis, sometimes with tooth loss; and in the extreme, gut bacterial flora overgrowth, leading to malabsorption requiring total parenteral nutritional therapy.

In contrast to some of the other IBMFSs, physical malformations are uncommon in most patients with SCN. These observations derived from the fact that *ELANE* is the most commonly mutated genes. Birth weights are generally unremarkable, and physical examination findings are usually normal. Data from the SCN International Registry indicate that some patients with SCN, including those with *ELANE* mutations, develop bone demineralization that may be an intrinsic component of the disorder; it has been observed before and during G-CSF therapy. The underlying pathogenesis is unclear, but patients can develop bone pain and unusual fractures. Osteopenia is a common theme with the IBMFSs.

The clinical features associated with the various hereditary neutropenias are summarized in Table 30.8, and the major ones are described after the section on SCN.

Laboratory Findings

Neutropenia is profound and persistent in SCN, usually less than 200/µL. A small number of patients have intermittent cycling patterns with regular periodicity that range from a low to severely low neutrophil count. A compensatory two- to fourfold increase in monocytes is seen, sometimes accompanied by eosinophilia. At diagnosis, platelet numbers are normal or increased, and hemoglobin values are usually normal. In survivors in the precytokine era, anemia of chronic disease associated with recurrent infections and inflammation was common. Aside from neutropenia, humoral and cellular immunity is completely normal in most types of SCN.

BM specimens are usually normocellular. The striking classical finding is a maturation arrest at the promyelocyte or myelocyte stage with a paucity of more mature elements. Promyelocytes are abundant and may have atypical nuclei and the cytoplasm may be vacuolated. Neutrophils and bands are usually absent. BM eosinophilia and monocytosis is common. The other hematopoietic lineages are normal, and maturation is undisturbed.

Predisposition to Leukemia and Myelodysplastic Syndrome

Clearly, SCN is associated a high risk of developing leukemia. There were three case reports of patients who developed AML before the use of hematopoietic growth factors and one more recent patient diagnosed with acute leukemia before starting G-CSF. As a rough estimate, in the literature, there were 128 cases of SCN reported and 3 cases of AML up to 1989 (the first year that G-CSF was available for general use), leading to a crude estimated risk of leukemia of 2%. Nevertheless, because most patients with SCN died at a young age from bacterial sepsis or pneumonia in the precytokine era, the true risk of patients with SCN developing MDS/AML was not defined. Specific mutations (i.e., G214R or C151Y) were associated in one study with a high risk for evolution to AML.

G-CSF therapy completely changed clinical outcomes of SCN. Before G-CSF, the median duration of survival was about 3 years; the current median age is more than 40 years. The number of documented cases of MDS/AML has dramatically increased since 1989, which likely reflects the natural history of the disease that is now allowed to manifest by prolonging life. However, whether G-CSF increases this risk or hastens the appearance of leukemia is still debatable.

The risk of MDS/AML is higher in patients requiring higher doses of G-CSF. According to SCN International Registry data, less responsive patients, defined as those having ANCs of less than 2.1×10^9/L on G-CSF doses greater than 8 µg/kg/day, had a cumulative incidence of MDS/AML of 34% after 15 years; more responsive patients, defined as having ANCs of greater than 2.1×10^9/L on G-CSF doses lower than 8 µg/kg/day, had a cumulative inci dence of 15%. The data were interpreted as indicating that a poor response to G-CSF defines an "at-risk" population and predicts an adverse outcome. The data do not necessarily support a cause-and-effect relationship between development of MDS/AML and G-CSF therapy. The results may only mean that patients requiring higher G-CSF therapy have a more severe clinical and hematologic phenotype. A report from the French Severe Chronic Neutropenia Study Group confirmed that increased exposure to G-CSF with respect to dose and duration in congenital neutropenia patients was associated with a heightened risk of MDS/AML.

Conversion to MDS/AML in SCN patients is associated with cellular and genetic abnormalities that provide insight into the pathobiology of the transformation and may be useful in identifying patients who are at high risk. Several cellular and genetic changes have been found in the BM of patients with SCN who received G-CSF. Whether these changes are related to G-CSF therapy is unknown. Remarkably, the abnormalities have similar characteristics in most patients and underscore a fairly specific multistep pathogenesis in the evolution into MDS/AML. At varying time points after starting G-CSF therapy, about half of the congenital neutropenia patients who transform acquire the same activating RAS oncogene mutation, namely a GGT (glycine) to GAT (aspartic acid) substitution at codon 12. More than 90% of patients who transform also have an acquired cytogenetic clonal alteration in BM cells, usually −7 or 7q- but also +21 or +8. Complex karyotypes (e.g., −7 and +21) have also been identified. More than 80% of patients develop one or more *GCSFR* point mutations. These *GCSFR* mutations are nonsense mutations that result in the truncation of the C-terminal cytoplasmic region, a subdomain that is crucial for G-CSF–induced granulocytic maturation, while the upstream intracellular domain that promotes proliferation is intact. The acquired mutation is directly operative in the conversion to MDS/AML. In murine models, the mutation results in impaired ligand internalization, defective receptor downmodulation, and enhanced growth signaling that produces an exaggerated hyperproliferative effect in response to G-CSF. This also confers resistance to apoptosis and enhances cell survival that favors clonal expansion in vivo. The detection of *GCSFR* mutations places patients at high risk for malignant conversion, but the time course from detection of mutations to overt MDS/AML varies considerably and may take years.

Although patients requiring higher doses of G-CSF to attain safe neutrophil levels are at a greater risk of developing MDS/AML, there is no definitive evidence that G-CSF directly causes malignant transformation. G-CSF may simply be an "innocent bystander" that corrects the neutropenia, prolongs patient survival, and allows time for the malignant predisposition to declare itself. Alternatively, G-CSF may accelerate the propensity for MDS/AML in the genetically altered stem and progenitor cells. G-CSF may rescue malignant clones that would otherwise be destined for apoptosis. The clinical interplay between G-CSF and the receptor mutation was underscored in the report of a patient with congenital neutropenia on G-CSF who developed a receptor mutation and AML. When G-CSF was stopped, the blast count fell to undetectable levels on two occasions without giving chemotherapy, although the mutant receptor was persistently detectable during the remissions. A similar patient has been observed in Toronto (Y. Dror, unpublished data).

An axiom of oncogenesis is that rapidly dividing cells are more susceptible to mutational events. Because t G-CSF provides a powerful proliferative signal for BM cells, it is a reasonable hypothesis that congenital neutropenia BM progenitors acquire new mutations. From the evidence cited herein, acquisition of a G-CSF receptor mutation in the face of therapeutic G-CSF in SCN can provide the hyperresponsive replicative scenario that can relentlessly evolve into MDS/AML. Is recombinant human G-CSF a carcinogen? This would seem highly unlikely. As a physiologic regulator of hematopoiesis, it would be unexpected for G-CSF to break molecular bonds and cause DNA damage even when used in therapeutic dosages.

In one patient with SCN who progressed to MDS/AML, clonal evolution was assessed by exome sequencing of whole marrow cells. A pro-proliferative, differentiation-defective *GCSFR* mutation was found, which persisted and acquired secondary mutations. Subsequently, *RUNX1* mutations developed and their frequency are high (65%) among SCN patients who develop MDS/AML. Mutations in *RUNX1* and *GCSFR* cooperate to promote clonal expansion. RAS mutations have also been reported in SCN-associated MDS/AML and are considered a late event.

Genotype-Phenotype Correlations

ELANE mutations are associated with severe and early-onset neutropenia with differentiation arrest at the stage of promyelocyte-myelocyte. Typically, the patients do not have physical malformations. The patients have a high risk of MDS/AML but no known risk of solid tumors. It is noteworthy that patients with the same *ELANE* mutations may develop either SCN or cyclic neutropenia, including patients who belong to the same family.

Patients with mutations in *HAX1* typically have severe and early-onset neutropenia with differentiation arrest at the stage of promyelocyte–myelocyte. They also have a high risk of MDS/AML but no known risk for solid tumors. About 30% of the patients with *HAX1* mutations have neurologic abnormalities such as seizures, learning disabilities, and developmental delay. This is attributable to nonsense mutations (e.g., p.Gln155ProfsX14) that affect the *HAX1* transcript that is expressed in the central nervous system in addition to that expressed in hematopoietic cells.

Mutations in *WAS* are associated with moderate to severe neutropenia, reduced phagocyte activity, monocytopenia, lymphopenia, reduced NK cells, reduced lymphocyte proliferation, and recurrent infections (but usually not as frequent as in classical SCN). MDS/monosomy 7 has also been reported.

GFI1 mutations are associated with severe to moderate neutropenia, and monocytosis, reduced B and T cells with normal lymphocytic function. There are no clear data about the BM findings in this type of neutropenia, and the risk of MDS/AML is unknown.

Differential Diagnosis

The commonest cause of isolated neutropenia in very young children is **viral-induced BM suppression**. An antecedent history of good health, the occurrence of a viral illness, and the transient nature of the neutropenia distinguish this disorder from SCN.

Autoimmune neutropenia of infancy is recognized as a fairly specific syndrome of early childhood. Low neutrophil numbers are often discovered during the course of routine investigation for a benign febrile illness. The illness abates, but the neutropenia persists, sometimes for months and occasionally for years. A BM biopsy is normocellular, and an aspirate shows active granulopoiesis up to the band stage; mature neutrophils may be normally represented, reduced, or absent. One should bear in mind that the BM in some patients with congenital neutropenia do not show maturation arrest at the myelocyte/metamyelocytes stage. The neutropenia in autoimmune neutropenia of infancy is caused by increased peripheral destruction and the diagnosis can be supported by demonstrating specific antigranulocyte antibodies on neutrophils, though the specificity and particularly the sensitivity of most available tests are low to moderate. The prognosis is good, the neutropenia is self-limited albeit protracted, and patients seldom develop serious bacterial infections. Most patients recover at the age of 3 to 5 years. Persistent neutropenia beyond this age should trigger evaluation of a hereditary neutropenia. It is noteworthy that some patients with hereditary neutropenia (e.g., SDS) may have intermittent neutropenia and periods of normal counts. Therefore, resolution of neutropenia may not necessarily be an indication of a recovery from an acquired disorder.

Other infrequent acquired causes of severe, isolated neutropenia in this age group include BM suppression from a **drug or toxin** and neutrophil sequestration as part of a **hypersplenism** syndrome.

Of the IBMFSs, **SDS** can also manifest as isolated neutropenia but can be identified because of growth failure, the malabsorption component caused by pancreatic insufficiency, fatty changes in the pancreas seen on CT scanning or ultrasonography, and characteristic skeletal abnormalities. **Glycogen storage disease type 1b (GSD-1b)** and **Barth syndrome** are also in the differential diagnosis. Neutropenia can also be a prominent part of **antibody deficiency syndromes** and **cellular immunodeficiency disorders** (see Table 30.8); investigation of chronic neutropenia of childhood should include an immunoglobulin electrophoresis, T-cell proliferative studies, and quantitation of T-cell subsets and NK cell activity. **Cyclic neutropenia** is distinguished by predictable symptomatology, especially mouth sores about every 3 weeks (19 to 23 days), often associated with chronic gingivitis. A complete blood count two or three times a week for 4 to 8 weeks demonstrates the diagnostic oscillation pattern with a cyclic nadir. Other **unclassified inherited neutropenia syndromes** with vertical transmission or in siblings have been described. The neutropenia in such cases are typically mild to moderate. When severe **neutropenia is diagnosed in the newborn period**, the cause may be passive transfer transplacentally of IgG antineutrophil antibodies from the mother. This can occur if the mother has an autoimmune disorder with neutropenia or by alloimmunization caused by fetomaternal incompatibility for a neutrophil-specific antigen.

Therapy and Prognosis

Before the introduction of G-CSF as a specific therapy of SCN, there was limited treatment. Antibiotics were the mainstay of management for active infections and for prophylaxis. Attempts to mobilize neutrophils with lithium had limited success.

Cytokine Therapy

G-CSF has supplanted all other forms of management because more than 90% of SCN patients respond. G-CSF treatment markedly increased the levels of NAMPT and SIRT1 in patients' myeloid cells and plasma, thereby activating the NAMPT- and SIRT1-induced C/EBPβ-dependent granulopoiesis. It is recommended to begin G-CSF therapy as front-line treatment when the diagnosis is established. The specific target of ANC when G-CSF is given to SCN patients has not been carefully studied. Levels of 1.0 to 5.0 × 10^9/L are considered as a target by some investigators. However, an ANC range of 0.75 to 2.5 × 10^9/L that is not associated with infections might also be acceptable. If the ANC remains below the target after initiation of G-CSF at 5 µg/kg/dose once a day subcutaneously, the dose may be escalated to 10 µg/kg/dose and then by increments of 10 µg/kg/dose at 7- to 14-day intervals until a response is seen. The occurrence of bacterial infections is reduced dramatically with an ANC of 1 × 10^9/L or above. Patients who are proven to be infection free with ANC of 0.75 to 1.0 may continue on the same G-CSF doses. The G-CSF dose can be reduced if the ANC increases above the target range to find the lowest dose necessary for maintaining a neutrophil count at the desired range. Nonresponders are defined as patients who do not respond to G-CSF levels exceeding 120 µg/kg/day. Partial responders show an increase of their ANC to 0.5 to 1.0 × 10^9/L with the highest dose, but they still experience bacterial infections. In these patients, the dose of G-CSF cannot be increased because of the large volume and frequency of injections required. The only currently available treatment for patients who do not demonstrate sufficient response to G-CSF treatment is HSCT (see comment below about GM-CSF treatment). In one published case of a G-CSF nonresponder, the addition of a small dose of prednisone (5 mg/day) to a standard G-CSF dose of about 5 µg/kg resulted in a long-term complete response. HSCT should be considered for patients who require high doses of G-CSF, because of the association with high risk of developing MDS/AML.

All patients on G-CSF therapy should be seen by a physician every 3 to 6 months. Patients requiring more than 8 µg/kg/day are at higher risk for MDS/AML and should be evaluated more often. Blood counts (white blood cells, hemoglobin, platelets, and differential blood counts) should be obtained and a physical examination performed at least every 3 months, including assessment for weight and height in pediatric patients and documentation of intercurrent infections. BM examination for morphology and karyotype is recommended once a year to search for acquired cytogenetic abnormalities such as monosomy 7 or trisomy 21 and other early signs of MDS.

From SCN International Registry data, a sustained hematologic response in patients treated with G-CSF for more than 15 years has been confirmed. With therapy, neutrophil counts rise in more than 90% of SCN patients and are maintained at a plateau for protracted periods, resulting in vast clinical benefits. In no instance has there been BM or hematopoietic lineage "exhaustion" or depletion with G-CSF therapy.

GM-CSF in crossover trials with G-CSF for SCN is not as effective and does not induce a neutrophil response consistently but might be tried when the patient does not respond to G-CSF. In particular, patients with *CSF3R* typically do not respond to G-CSF but may respond to GM-CSF.

Recently, addition of nicotinamide (vitamin B3) has been shown to improve responses to G-CSF in patients with SCN, including those with minimal response on high doses of G-CSF.

Hematopoietic Stem Cell Transplantation

Several cohorts of patients with SCN undergoing HSCT have been reported. The indications were severe neutropenia with inadequate response to G-CSF or development of MDS/AML. Patients transplanted for severe neutropenia using related or unrelated matched donors and myeloablative (e.g., busulfan/cyclophosphamide-based) or non-myeloablative conditioning had a favorable overall survival of 81% to 89% and resolution of neutropenia.

In a European and Middle Eastern study, the results of 136 patients with SCN transplanted between 1990 and 2012 were reported. Thirty-nine had mutations in *ELANE*, 5 in HAX1, 2 in G6PC3, and 90 cases had unknown mutations. Most patients (63) were transplanted for neutropenia, 14 for MDS/AML, 59 for unknown indications. Overall survival was 82%. Patients transplanted after the age of 10 years and patients transplanted from a mismatched donor had a worse outcome.

Data from the SCNIR showed that all patients who received standard AML chemotherapy w/o HSCT died. Early transplantation of patients with MDS/AML with matched sibling or unrelated donors resulted in poor outcomes and overall survival below 20%. However, in a follow-up study patients with MDS/AML received less intense chemotherapy comprised of low-dose ara-C and thioguanine to lower the leukemic blast load and organomegaly, followed by HSCT with standard conditioning and the 5-year survival was approximately 80%. The morbidity was related to aGVHD, cGVHD, infections, and secondary malignancies. The authors attributed the improvement in outcomes to the change in pre-transplant treatment strategy and to surveillance and treatment at the MDS stage before progression to AML. In a small series of six transplanted patients who developed MDS or AML, two with MDS who underwent the procedure without being given induction chemotherapy survived, but four with AML given induction chemotherapy had significantly more morbidity and died posttransplant, raising questions about conditioning strategies for these patients. Two other cases with AML from Japan were published in 2013. In both cases HSCT without prior AML-type chemotherapy was successfully applied.

A multicenter retrospective analysis of umbilical cord blood transplants for IBMFs in Europe included 16 patients with SCN. The conditioning regimens were myeloablative in nature. One patient received a related cord blood graft, and the other 15 received unrelated grafts. Three patients were transplanted for AML or MDS using unrelated cord blood grafts; two died. The other 13 patients were transplanted because of a lack of response to G-CSF. At a median follow-up of 41 months, 11 of the 13 were alive; one received a related graft, and 10 received unrelated grafts.

The discovery of an isolated BM clonal cytogenetic abnormality without other evidence of MDS or AML in patients with congenital neutropenia raises management issues. One option is to perform HSCT if there is a matched donor as soon as feasible. This has generally been recommended for patients with −7, 7q-, or +21. The a priori argument is that the chance for cure is higher when the patient is well and has a low burden of malignant cells. The problem with this decision centers on not knowing the tempo of progression from cytogenetic evolution to clear-cut MDS or AML. Thus, there may not be a need to rush to transplant in all patients. Instead, one recommendation is to lower the G-CSF dose to the lowest dose that maintains neutrophil counts greater than 0.5×10^9/L and to monitor the patient regularly with blood counts and by serial BM testing.

Opinions also vary about the best way to manage patients with somatic mutations involving *CSF3R* or *RAS* genes without clonal BM cytogenetic abnormalities or morphologic evidence of transformation. Implementing close monitoring with regular blood counts and BM testing is an acceptable option.

Therapeutic options for newly diagnosed patients with SCN must be constantly reevaluated. It is largely accepted that G-CSF induces robust neutrophilic responses and eliminates infections almost completely in more than 90% of SCN patients. Therefore, it should be the first treatment choice. HSCT has been regarded as “salvage” therapy for patients who either acquire evidence of malignant myeloid transformation or fail to respond to G-CSF altogether or require high doses of G-CSF (e.g., over 20 to 30 μg/kg/day) to maintain neutrophils above 0.5×10^9/L. HSCT from a fully matched donor before transforming to MDS/AML and in stable health provides a high chance for cure. In such cases, not only is the neutropenia fully corrected by HSCT, but the risk of MDS/AML is also eliminated.

Bisphosphonates for Osteoporosis

In 50% of SCN patients on G-CSF, bone density measurements show varying degrees of osteopenia and osteoporosis. It is unclear whether the osteopenia in SCN is due to G-CSF or the underlying disease or a combination of both. Most of the cases with osteopenia are subclinical and asymptomatic, but some patients complain of bone pain and have fractures. Evidence shows that bisphosphonates are effective treatment for the majority of these cases.

Future Directions

The list of SCN genes is by no means complete, and further studies are necessary to discover novel genes. It is also not clear why some patients develop to MDS/AML and others do not, but studies are ongoing to try to answer this clinical question. At present, diagnosis of cyclic and congenital neutropenia still depends primarily on observations of serial blood cell counts, but it is expected that additional biomarkers beyond the *ELANE* mutation will become a routine part of making the diagnosis and the establishment of a prognosis for these patients in the future. Several pathways including apoptosis have been implicated in SCN hematopoiesis; however, further studies are necessary to determine the mechanism of *maturation arrest*. It appears that cellular models involving transfection of the mutant genes into human myeloid cell lines provide evidence of how neutropenia occurs. Potentially, these models can also be used to examine new approaches to preventing apoptosis and serve to provide clues to new and more effective therapies. Considerable data now confirm the effectiveness of G-CSF in the treatment of various forms of SCN. However, the onerous burden of daily subcutaneous injections, financial expenses, potential long-term effects, and lack of response in 10% of the patients require the development of alternative therapies. Gene therapy is still to be developed.

Cyclic Neutropenia

Cyclic neutropenia is an autosomal dominant disorder characterized by a regular, repetitive reduction in peripheral blood neutrophils for 3 to 4 days every 19 to 23 days. Between nadirs, the patients have normal or nearly normal neutrophil counts. Patients usually present in infancy or childhood and have a less severe infectious course compared with those with SCN. However, life-threatening infections have been reported. A proportion of the patients need treatment. Daily administration of G-CSF typically improves symptoms in most patients.

Cyclic neutropenia is caused by heterozygous mutations in the *ELANE* gene that encodes neutrophil elastase. The mutations usually occur at the active site of neutrophil elastase without disrupting the enzymatic substrate cleavage. The mutations seem to disturb a predicted transmembrane domain, leading to excessive granular accumulation of elastase and defective membrane localization of the enzyme. The myeloid precursors are characterized by cyclic increases in apoptosis. However, the precise molecular mechanism for the cycling has not been defined. It is also not clear why the same mutations in *ELANE* are associated with both cyclic and SCN phenotypes. Prognosis is favorable and life expectancy is probably close to normal. Few cases of leukemia have been reported in patients with cyclic neutropenia; however, the incidence is very low, and a surveillance program should not be as strict as that recommended for patients with SCN.

Myelokathexis and WHIM Syndrome

Myelokathexis (from the Greek, meaning "retained in the bone marrow") is a rare autosomal dominant disorder with recurrent bacterial infections caused by a reduced number and function of neutrophils. Neutropenia is typically moderate to severe. Degenerative changes in the granulocytes are characteristic and include pyknotic nuclear lobes, fine chromatin filaments, and hypersegmentation. The BM is usually hypercellular with granulocytic hyperplasia. The pathophysiology of myelokathexis has been attributed to a defective release of BM cells into the peripheral blood. Neutrophil precursors are characterized by decreased expression of BCL-XL and accelerated apoptosis. The mainstay of treatment for patients with WHIM syndrome is G-CSF and sometimes also IVIG. G-CSF ameliorates the neutropenia and leads to clinical improvement during episodes of bacterial infection.

WHIM syndrome refers to the association of myelokathexis with other features (**w**arts, **h**ypogammaglobulinemia, **i**nfections, and **m**yelokathexis). Most cases are caused by mutations in the chemokine receptor gene *CXCR4* but dysfunction of GRK3 (without documented mutations), a negative regulator of CXCR4, has also been implicated. *CXCR4* mutations result in enhanced chemotactic response of neutrophils in response to the CXCR4 ligand CXCL12 (stroma-derived factor 1) and pathologic retention of neutrophils in the BM. Patients with wild type *CXCR4* might have other genetic defects that lead to an enhanced interaction between CXCR4 and CXCL12 and an enhanced chemotactic response, such as reduced inhibition of CXCL12-promoted internalization, and desensitization of CXCR4 by G protein–coupled receptor (GPCR) kinase-3 caused by decreased transcription of the GPCR kinase-3. G-CSF induces a prompt increase in neutrophil numbers and gamma globulin levels may also increase. Twice daily injections of plerixafor (CXCR4 antagonist) are associated with an increase in neutrophil counts, fewer infections, and improvement of warts in combination with imiquimod. Immunoglobulin levels and specific antibody response were not fully corrected.

G6PC3-Associated SCN (Dursun Syndrome)

This syndrome is an autosomal recessive form of SCN caused by biallelic mutant *G6PC3*. High prevalence (25%) and a unique pattern of mutations in G6PC3 was found in Israel. Wild-type *G6PC3* encodes glucose-6-phosphatase catalytic subunit 3, which, when mutated, confers an increased susceptibility of neutrophils to apoptosis. BM morphology varies among the reported patients, but includes mild to moderate decreases in mature neutrophils, normal appearing BM, or promyelocyte-myelocyte maturation arrest. Neutropenia is usually severe, rendering patients susceptible to severe bacterial infections. Lymphopenia and thrombocytopenia may occur. Structural heart defects, urogenital abnormalities, venous angiectasias on the trunk and extremities, intellectual disability, inflammatory bowel disease, and a broad range of other physical abnormalities are additional features. MDS and AML have been reported in these patients.

Barth Syndrome

Barth syndrome is a rare multisystem metabolic disorder inherited in an X-linked recessive mode. The primary causative factor is an alteration of cardiolipin remodeling. Cardiolipin is a component of the inner mitochondrial membrane necessary for proper functioning of the electron transport chain.

The findings in typical cases are mild to severe neutropenia, dilated or hypertrophic cardiomyopathy, underdeveloped skeletal musculature and muscle weakness, exercise intolerance, growth delay, cardiolipin abnormalities, and 3-methylglutaconic aciduria. ANCs are variable, but total agranulocytosis has been reported. BM examination has shown normocellular specimens in most cases. A maturation arrest at the myelocyte stage is commonly seen. On electron microscopy, mitochondria show concentric, tightly packed cristae and occasional inclusion bodies in various tissues, including granulocyte precursors. Clinically, the cardiomyopathy dominates the clinical picture, but gingivitis, oral problems, and bacterial sepsis from neutropenia can be problematic. Most patients do not need treatment with G-CSF. When needed, G-CSF is highly effective in correcting the neutropenia and preventing infections. The myriad of clinical problems requires a multidisciplinary approach. Female carriers are healthy and hematologically normal, likely because of extreme skewing of X-inactivation. The initial impression that Barth syndrome was a lethal infantile disease has been modified; age distribution in 54 living patients ranges from 0 to 49 years and peaks around puberty.

The Barth syndrome gene was mapped to Xq28, which led to the cloning of the *TAZ* gene and the various mutations that account for the phenotype. *TAZ* produces several different mRNAs with resultant proteins called tafazzins. Mutations in *TAZ* result in a decrease in tetralinoleoyl species of cardiolipin and an accumulation of monolysocardiolipin within cells. A murine model of Barth syndrome has been developed. Knocked-down cellular models and patient BM cells are characterized by accelerated cell death. However, a recently proposed mechanism for the neutropenia involves exposure of cell surface phosphatidylserine because of ROS, and consequently increased clearance of neutrophils by tissue macrophages.

Glycogen Storage Disease Type 1b

Glycogen storage disease type 1b is caused by a deficiency in glucose-6-phosphate translocase (transporter) because of mutant *G6PT1* (*SLC37A4*). GSD-1b patients experience disturbed glucose homeostasis and quantitative or qualitative neutrophil abnormalities. The translocase transports glucose-6-phosphate into the lumen of the endoplasmic reticulum, where it is hydrolyzed into glucose and inorganic phosphate. Absence of translocase results in an inability to liberate glucose from glucose-6-phosphate. Consequently, patients with GSD-1b are susceptible to fasting hypoglycemia, lactic acidosis, hepatomegaly, poor linear growth, delayed pubertal development, and other systemic complications.

Most patients have mild to severe neutropenia. A strict correlation between genotype and the degree of neutropenia has not been established. Some studies reported hypocellular BM; however, BM testing on nine patients from the City of Hope National Medical Center in 2001 before G-CSF therapy revealed normal hypercellularity in all patients. Maturation arrest at the myelocyte stage or later is a feature. Neutrophil apoptosis appears to be a central mechanism leading to granulopoietic failure. Neutrophil dysfunction with defective chemotaxis and an impaired respiratory burst is an additional feature. Patients are consequently susceptible to recurrent infections and to inflammatory bowel disease. Infections most commonly involve the skin, perirectal area, ears, and urinary tract, but septicemia, pneumonia, and meningitis may also occur. The most frequently isolated organisms include *Staphylococcus aureus*, group A streptococci, *Streptococcus pneumoniae, Escherichia coli*, and *Pseudomonas.*

G-CSF therapy is extremely effective in almost all GSD-1b patients. BM cellularity increases, the ANCs exuberantly, and impaired oxygen radical formation is corrected. AML while on G-CSF is a rare event. Prospective data on GSD-1b patients receiving G-CSF therapy from the SCNIR identified 40% with splenomegaly before starting G-CSF, 81% with splenomegaly by the first year of treatment, and 100% with splenomegaly by 3 years. Hypersplenism and associated pain can occur in GSD-1b, and may limit the ability to provide adequate doses of G-CSF. Nevertheless, this is reversal and can be overcome by reducing the G-CSF dosage or by splenectomy. Histology of the surgically excised spleens shows extramedullary hematopoiesis. For unknown reasons, G-CSF also improves the symptoms of enterocolitis of GSD-1b, though to a limited degree.

Other Inherited Neutropenias

Other inherited neutropenia disorders are associated with specific mutant genes, but they do not necessarily have the SCN BM phenotype, nor is their pathophysiology necessarily similar (see Table 30.8). Immune deficiency appears to be an important component in some of these syndromes. These miscellaneous forms tend to have distinguishing physical abnormalities such as partial albinism and are not predisposed to MDS/AML.

INHERITED BONE MARROW FAILURE SYNDROMES WITH PREDOMINANTLY THROMBOCYTOPENIA

Thrombocytopenia With Absent Radii Syndrome

Background

TAR syndrome has two essential features, hypomegakaryocytic thrombocytopenia and bilateral radial aplasia with thumbs present. It is one of a group of IBMFSs that includes FA, DBA, and radioulnar synostosis with radial ray anomalies. The manifestations of the phenotype vary widely, and patients can present with abnormalities involving skeletal, skin, gastrointestinal, brain, renal, and cardiac systems. The identification of biallelic mutations in *RBM8A* in patients with TAR syndrome determined the autosomal recessive inheritance of this disorder. Almost always, parents of patients with TAR are phenotypically normal. Women with TAR syndrome can conceive and give birth to hematologically and phenotypically normal offspring. Cases of AML and acute lymphoblastic leukemia in TAR syndrome patients have been reported with a rate that is higher than that expected in the general population.

Genetics

TAR patients have submicroscopic deletions at 1q21.1 or less often a truncating mutation in one allele of *RBMA8* in 100% of cases. The other allele carries one of several low-frequency SNPs in the regulatory 5′-translated or 3′-untranslated regions of *RBM8A*. *RBM8A* encodes the Y14 subunit of exon-junction complex, which processes mRNA. The mutations cause reduced expression of the protein in platelets from affected individuals.

Pathophysiology

Y14 is a 174 amino-acid protein with an RNA recognition motif. It is a component of the exon junction complex together with the MAGOH, EIF4AIII, and CASC3 proteins. The complex is involved in mRNA splicing and maturation, mRNA nuclear export, and activation of nonsense mediated RNA decay of proteins with a premature stop codons.

Thrombocytopenia in TAR syndrome is the result of a defect in megakaryopoiesis and thrombocytopoiesis. Thrombopoietin levels in plasma or serum are consistently elevated in TAR syndrome, thereby excluding a cytokine production defect as a cause for thrombocytopenia. BM CFU-Meg (CFU-MK) progenitors are either absent or are present in low to normal frequencies but produce small colonies in vitro with an abnormal morphology. CFU-GM and BFU-E colony growth is often increased.

In a detailed study of CD34$^+$ cells, the thrombocytopenia of TAR syndrome was associated with a dysmegakaryocytopoiesis characterized by cells remaining at an early stage of differentiation. Cells expressing CD41 without CD42 accumulated, while cells expressing CD41/CD42 underwent apoptosis and had reduced c-mpl transcript expression and mpl protein. The response of platelets to adenosine diphosphate or to the thrombin receptor agonist peptide SFLLRN (TRAP) is normal in TAR patients. However, in contrast to control participants, platelets from TAR patients do not undergo activation *in vitro* in response to recombinant thrombopoietin as measured by testing thrombopoietin synergism to adenosine diphosphate and TRAP. Thrombopoietin-induced tyrosine phosphorylation of platelet proteins in this setting is completely absent or markedly decreased. The results indicate that there is a lack of response to thrombopoietin downstream of the c-mpl signal transduction pathway.

No recurrent chromosomal changes are seen in TAR syndrome. Some karyotypic abnormalities have been found in a few patients and are of unclear significance. Clastogenic induced chromosomal breakage analysis in TAR syndrome is normal.

Clinical Features

The diagnosis is typically made during the newborn period because of the absent radii, and thrombocytopenia. Complete blood count testing is done either because of the congenital anomalies or because of bleeding symptoms such as petechial rash and bloody diarrhea. Patients have bilateral radial aplasia (Fig. 30.16) with preservation of the thumbs and fingers on both sides. Additional upper extremity deformities include radial club hands; hypoplastic carpals and phalanges; and hypoplastic ulnae, humeri, and shoulder girdles. Syndactyly and clinodactyly of the toes and fingers are also seen. Characteristic findings include a selective hypoplasia of the middle phalanx of the fifth finger and altered palmar contours. Upper extremity involvement ranges from isolated absent radii to true, often asymmetric, phocomelia. The lower extremities are involved in about half of cases. Malformations include hip dislocation, coxa valga, femoral torsion, tibial torsion, abnormal tibiofibular joints, small feet, and valgus and varus foot deformities. Abnormal toe placement is commonly seen, especially the fifth toe overlapping the fourth. Similar to upper limb involvement, lower extremity deformities range from minimal involvement to complete phocomelia. An asymmetric first rib, a cervical rib, cervical spina bifida, and a fused cervical spine can occur, but trunk involvement is usually minimal. Micrognathia has been associated with the TAR syndrome in up to 65% of cases.

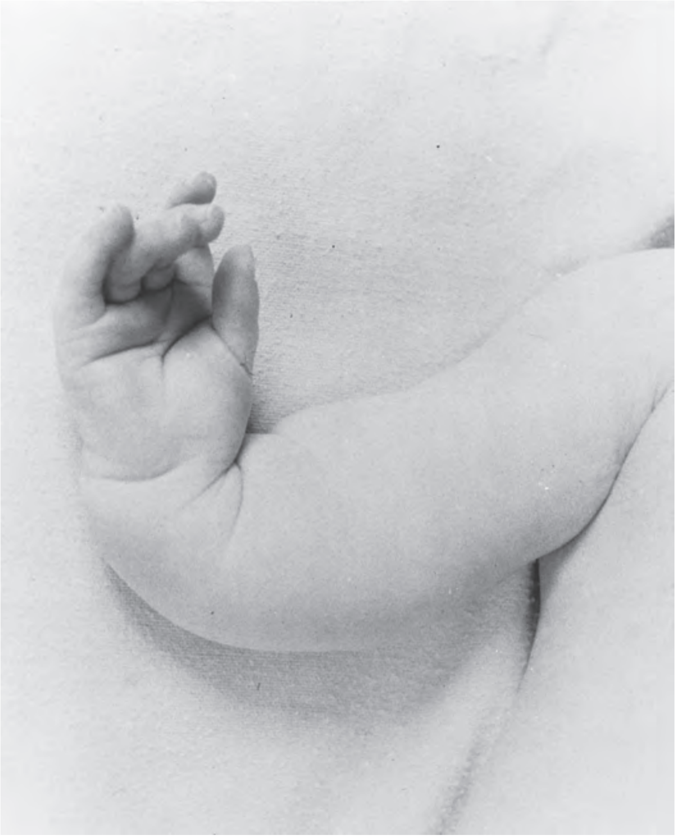


Figure 30.16 RADIAL APLASIA WITH PRESERVATION OF THE THUMB IN A NEWBORN WITH THROMBOCYTOPENIA WITH ABSENT RADII SYNDROME.

Cardiac abnormalities occur in 15% of the patients, including atrial septal defects, tetralogy of Fallot, and ventricular septal defects. Capillary hemangiomas are common (24%) as well as redundant nuchal folds. Genitourinary tract malformations are detected in 23% of cases. About 95% of patients have short stature, 76% have macrocephaly, and 53% show facial dysmorphism. Structural brain abnormalities may be present. Additional findings are dorsal pedal edema, hyperhidrosis, and gastrointestinal disturbances such as diarrhea and feeding intolerance; almost 50% of patients are intolerant of cow's milk.

Prenatal diagnosis can be made by genetic testing, by ultrasound imaging of absent radii with thumbs present, and by measuring platelet numbers obtained by fetoscopy or cordocentesis. A published case describes a prenatal diagnosis of TAR followed by an in utero platelet transfusion to facilitate safe delivery.

Laboratory Findings

Thrombocytopenia as a result of BM underproduction is a consistent finding. BM specimens show normal to increased cellularity with decreased to absent megakaryocytes. The erythroid and myeloid lineages are normally represented. When a few megakaryocytes can be identified in biopsies, they are small, contain few nuclear segments, and show immature nongranular cytoplasm. If platelet counts increase spontaneously in patients after the first year of life, megakaryocytes increase in parallel and appear more mature. At diagnosis, leukocytosis is seen in the majority of patients and is sometimes greater than 100,000/μL with a "left shift" to immature myeloid forms. The cause of this leukemoid reaction is unclear, but it is usually transient and subsides spontaneously. If anemia is present, it is likely attributable to blood loss caused by thrombocytopenia. When platelet numbers are adequate for study, their size is generally normal, and routine testing of function is unremarkable, although some patients may show abnormal platelet aggregation and storage pool defects. Compared with other IBMFSs, RBC size and fetal hemoglobin levels are normal.

Cancer Predisposition

Four cases of AML and two case of acute lymphoblastic leukemia in TAR syndrome patients have been reported, which is about a 1% to 2% crude rate among published cases. This suggests a predisposition to the development of hematologic malignancies.

Differential Diagnosis

Important clinical differences distinguish TAR syndrome from **FA**. In FA, when radii are absent, the thumbs are hypoplastic or absent. FA patients do not have skin hemangiomas like some patients with TAR, and TAR patients rarely have prominent skin pigmentation defects like 40% of FA patients. Importantly, in FA thrombocytopenia is very rarely evident in the first year of life. Confirmation of FA is made by the clastogenic chromosome stress test showing increased fragility and by mutational analysis. TAR patients do not have increased chromosomal breakage.

Some infants with **trisomy 18** (+18) have absence or hypoplasia of radii and thrombocytopenia. However, in +18, thumbs are absent if radii are absent, and the disorder can also be distinguished from TAR syndrome cytogenetically. There are several syndromes with radial abnormalities but with *normal* platelet counts that can be diagnosed by mutational gene analysis. These include **Roberts syndrome** (mutant *ESC02*); **Holt-Oram syndrome** (mutant *TBX5*); and the clinical spectrum of three disorders caused by mutant *RECQL4:* **Rothmund-Thomson syndrome, Baller-Gerold syndrome**, and **RAPADILINO syndrome**.

Therapy and Prognosis

The risk of hemorrhage is greatest in the first year of life. Deaths are usually caused by intracranial or gastrointestinal bleeding. If patients survive the first year, platelet counts spontaneously increase inexplicably to levels that are hemostatically safe and do not require platelet transfusional support. A minority of patients have sustained, profound thrombocytopenia that does not improve spontaneously. An actuarial survival curve plateau of 80% by age 1 to 2 years has been reported. Many reports of TAR patients antedated the modern use of platelet transfusions, so the survival is likely much better currently. TAR patients do not develop pancytopenia, but 1% to 2% may develop AML.

Platelet Transfusions

As in other IBMFSs associated with thrombocytopenia, platelet transfusions should be used judiciously. Bleeding and prophylaxis for orthopedic surgical procedures are appropriate indications. Persistent platelet counts below 10,000/μL may require preventive platelet transfusions on a regular basis, especially during the first year of life when the expectation is that a spontaneous improvement in platelet number will ensue with time in most infants. Single-donor platelets are preferred to multiple random donor platelets to minimize the risk of alloimmunization. HLA-partially matched or fully matched donors for platelets may be required if patients become refractory to transfusions.

Other Therapies

Supportive management is the mainstay, but in exceptional situations, profound, persistent life-threatening thrombocytopenia can be successfully treated with HSCT. There has been one report of a patient with TAR syndrome who had an increment in platelet numbers after treatment with romiplostim (a fusion protein analog of thrombopoietin) or IL-11. This is intriguing as the elevated serum thrombopoietin and IL-11 levels at baseline in TAR patients may predict a poor response to these products. Androgens, corticosteroids, and splenectomy are ineffective therapies for TAR syndrome.

SUGGESTED READINGS

General

Alter BP, Giri N, Savage SA, Rosenberg PS. Cancer in the National Cancer Institute inherited bone marrow failure syndrome cohort after fifteen years of follow-up. *Haematologica*. 2018;103(1):30–39.

Collins J, Dokal I. Inherited bone marrow failure syndromes. *Hematology*. 2015;20(7):433–434.

Ghemlas I, Li H, Zlateska B, et al. Improving diagnostic precision, care and syndrome definitions using comprehensive next-generation sequencing for the inherited bone marrow failure syndromes. *J Med Genet*. 2015;52(9):575–584.

Wegman-Ostrosky T, Savage SA. The genomics of inherited bone marrow failure: from mechanism to the clinic. *Br J Haematol*. 2017;177(4):526–542.

Fanconi Anemia

Auerbach AD. Fanconi anemia and its diagnosis. *Mutat Res*. 2009;668:4.

Bhattacharjee S, Nandi S. DNA damage response and cancer therapeutics through the lens of the Fanconi anemia DNA repair pathway. *Cell Commun Signal*. 2017;15(1):41.

Debureaux PE, Sicre de Fontbrune F, Bonfim C, et al. FLAG-sequential regimen followed by bone marrow transplantation for myelodysplastic syndrome or acute leukemia in patients with Fanconi anemia: a Franco-Brazilian study. *Bone Marrow Transplant*. 2021;56(1):285–288.

Ebens CL, DeFor TE, Tryon R, et al. Comparable outcomes after HLA-matched sibling and alternative donor hematopoietic cell transplantation for children with Fanconi anemia and severe aplastic anemia. *Biol Blood Marrow Transplant*. 2018;24(4):765–771.

Fiesco-Roa MO, Giri N, McReynolds LJ, et al. Genotype-phenotype associations in Fanconi anemia: a literature review. *Blood Rev*. 2019;37:100589.

Giardino S, de Latour RP, Aljurf M, et al. Outcome of patients with Fanconi anemia developing myelodysplasia and acute leukemia who received allogeneic hematopoietic stem cell transplantation: a retrospective analysis on behalf of EBMT group. *Am J Hematol*. 2020;95(7): 809–816.

Heidemann S, Bursic B, Zandi S, et al. Cellular and molecular architecture of hematopoietic stem cells and progenitors in genetic models of bone marrow failure. *JCI Insight*. 2020;5(4).

Longerich S, Li J, Xiong Y, et al. Stress and DNA repair biology of the Fanconi anemia pathway. *Blood*. 2014;124(18):2812–2819.

Quentin S, Cuccuini W, Ceccaldi R, et al. Myelodysplasia and leukemia of Fanconi anemia are associated with a specific pattern of genomic abnormalities that includes cryptic RUNX1/AML1 lesions. *Blood*. 2011;117:e161–e170.

Rio P, Navarro S, Wang W, et al. Successful engraftment of gene-corrected hematopoietic stem cells in non-conditioned patients with Fanconi anemia. *Nat Med*. 2019;25(9):1396–1401.

Steinberg-Shemer O, Goldberg TA, Yacobovich J, et al. Characterization and genotype-phenotype correlation of patients with Fanconi anemia in a multi-ethnic population. *Haematologica*. 2020;105(7):1825–1834.

Uppuluri R, Swaminathan VV, Ramanan KM, et al. Haploidentical stem cell transplantation with post-transplant cyclophosphamide in Fanconi anemia: improving outcomes with improved supportive care in India. *Biol Blood Marrow Transplant*. 2020;26(12):2292–2298.

Wang AT, Smogorzewska A. SnapShot: Fanconi anemia and associated proteins. *Cell*. 2015;160(1-2). 354-e1.

Shwachman-Diamond Syndrome

Boocock GR, Morrison JA, Popovic M, et al. Mutations in SBDS are associated with Shwachman-Diamond syndrome. *Nat Genet*. 2003;33(1):97–101.

Cesaro S, Pegoraro A, Sainati L, et al. A prospective study of hematologic complications and long-term survival of Italian patients affected by Shwachman-Diamond syndrome. *J Pediatr*. 2020;219:196–201.

D'Amours G, Lopes F, Gauthier J, et al. Refining the phenotype associated with biallelic DNAJC21 mutations. *Clin Genet*. 2018;94(2):252–258.

Dhanraj S, Matveev A, Li H, et al. Biallelic mutations in DNAJC21 cause Shwachman-Diamond syndrome. *Blood*. 2017;129(11):1557–1562.

Donadieu J, Fenneteau O, Beaupain B, et al. Classification of and risk factors for hematologic complications in a French national cohort of 102 patients with Shwachman-Diamond syndrome. *Haematologica*. 2012;97(9):1312–1319.

Dror Y, Freedman MH. Shwachman-Diamond syndrome: an inherited preleukemic bone marrow failure disorder with aberrant hematopoietic progenitors and faulty marrow microenvironment. *Blood*. 1999;94(9):3048–3054.

Finch AJ, Hilcenko C, Basse N, et al. Uncoupling of GTP hydrolysis from eIF6 release on the ribosome causes Shwachman-Diamond syndrome. *Genes Dev*. 2011;25:917.

Hashmi SK, Allen C, Klaassen R, et al. Comparative analysis of Shwachman-Diamond syndrome to other inherited marrow failure syndromes and genotype-phenotype correlation. *Clin Genet*. 2011;79:448.

Heidemann S, Bursic B, Zandi S, et al. Cellular and molecular architecture of hematopoietic stem cells and progenitors in genetic models of bone marrow failure. *JCI Insight*. 2020;5(4).

Kennedy AL, Myers KC, Bowman J, et al. Distinct genetic pathways define pre-malignant versus compensatory clonal hematopoiesis in Shwachman-Diamond syndrome. *Nat Commun*. 2021;12(1):1334.

Myers K, Hebert K, Antin J, et al. Hematopoietic stem cell transplantation for Shwachman-Diamond syndrome. *Biol Blood Marrow Transplant*. 2020;26(8):1446–1451.

Ruiz-Gutierrez M, Bolukbasi OV, Alexe G, et al. Therapeutic discovery for marrow failure with MDS predisposition using pluripotent stem cells. *JCI Insight*. 2019:5.

Sen S, Wang H, Nghiem CL, et al. The ribosome-related protein, SBDS, is critical for normal erythropoiesis. *Blood*. 2011;118:6407.

Watanabe K, Ambekar C, Wang H, et al. SBDS-deficiency results in specific hypersensitivity to Fas stimulation and accumulation of Fas at the plasma membrane. *Apoptosis*. 2009;14(1):77–89.

Dyskeratosis Congenita

Alter BP, Giri N, Savage SA, et al. Cancer in dyskeratosis congenita. *Blood*. 2009;25 113:6549.

Fioredda F, Iacobelli S, Korthof ET, et al. Outcome of haematopoietic stem cell transplantation in dyskeratosis congenita. *Br J Haematol*. 2018;183(1): 110–118.

Grill S, Nandakumar J. Molecular mechanisms of telomere biology disorders. *J Biol Chem*. 2020;296:100064.

Niewisch MR, Savage SA. An update on the biology and management of dyskeratosis congenita and related telomere biology disorders. *Expert Rev Hematol*. 2019;12(12):1037–1052.

Townsley DM, Dumitriu B, Liu D, et al. Danazol treatment for telomere diseases. *N Engl J Med*. 2016;374(20):1922–1931.

Congenital Amegakaryocytic Thrombocytopenia

Germeshausen M, Ballmaier M. CAMT-MPL: congenital amegakaryocytic thrombocytopenia caused by MPL mutations—heterogeneity of a monogenic disorder—a comprehensive analysis of 56 patients. *Haematologica*. 2020

Diamond-Blackfan Anemia

Arbiv OA, Cuvelier G, Klaassen RJ, et al. Molecular analysis and genotype-phenotype correlation of Diamond-Blackfan anemia. *Clin Genet*. 2018;93(2):320–328.

Da Costa L, Leblanc T, Mohandas N. Diamond-Blackfan anemia. *Blood*. 2020;136(11):1262–1273.

Ellis SR, Gleizes PE. Diamond Blackfan anemia: ribosomal proteins going rogue. *Semin Hematol*. 2011;48:89.

Iskander D, Psaila B, Gerrard G, et al. Elucidation of the EP defect in Diamond-Blackfan anemia by characterization and prospective isolation of human EPs. *Blood*. 2015;125(16):2553–2557.

Liu Y, Dahl M, Debnath S, et al. Successful gene therapy of Diamond-Blackfan anemia in a mouse model and human CD34$^+$ cord blood hematopoietic stem cells using a clinically applicable lentiviral vector. *Haematologica*. 2022;107(2):446-456.

Strahm B, Loewecke F, Niemeyer CM, et al. Favorable outcomes of hematopoietic stem cell transplantation in children and adolescents with Diamond-Blackfan anemia. *Blood Adv*. 2020;4(8):1760–1769.

Vlachos A, Rosenberg PS, Atsidaftos E, et al. Increased risk of colon cancer and osteogenic sarcoma in Diamond-Blackfan anemia. *Blood*. 2018;132(20):2205–2208.

Congenital Dyserythropoietic Anemias

Iolascon A, Andolfo I, Russo R. Congenital dyserythropoietic anemias. *Blood*. 2020;136(11):1274–1283.

Kostmann/Severe Congenital Neutropenia

Nasri M, Ritter M, Mir P, et al. CRISPR/Cas9-mediated ELANE knockout enables neutrophilic maturation of primary hematopoietic stem and progenitor cells and induced pluripotent stem cells of severe congenital neutropenia patients. *Haematologica*. 2020 Mar;105(3):598–609.

Rosenberg PS, Zeidler C, Bolyard AA, et al. Stable long-term risk of leukaemia in patients with severe congenital neutropenia maintained on G-CSF therapy. *Br J Haematol*. 2010;150:196.

Skokowa J, Dale DC, Touw IP, et al. Severe congenital neutropenias. *Nat Rev Dis Primers*. 2017;3:17032.

Skokowa J, Steinemann D, Katsman-Kuipers JE, et al. Cooperativity of RUNX1 and CSF3R mutations in severe congenital neutropenia: a unique pathway in myeloid leukemogenesis. *Blood*. 2014;123:2229–2237.

Thrombocytopenia Absent Radii Syndrome

Boussion S, Escande F, Jourdain AS, et al. TAR syndrome: Clinical and molecular characterization of a cohort of 26 patients and description of novel noncoding variants of RBM8A. *Hum Mutat*. 2020;41(7):1220–1225.

Letestu R, Vitrat N, Masse A, et al. Existence of a differentiation blockage at the stage of a megakaryocyte precursor in the thrombocytopenia and absent radii (TAR) syndrome. *Blood*. 2000;95(5):1633–1641.

CHAPTER 31

APLASTIC ANEMIA

Neal S. Young and Jaroslaw P. Maciejewski

Aplastic anemia (AA), the paradigm of the bone marrow (BM) failure syndromes, is most simply defined as peripheral blood pancytopenia and a hypocellular BM (Fig. 31.1). AA occurring as a primary hematologic disorder is historically denoted idiopathic, but it is now understood as immune-mediated. Constitutional marrow failure syndromes share a similar pathology of an apparently “empty” marrow (see Chapter 30) and can be distinguished from immune AA by personal and family history, age of onset, involvement of other organs, and genomic testing. Physical and chemical damage (most frequently as radiation and chemotherapy) also can lead to an aplastic marrow. However, the diagnosis of AA requires excluding other causes of pancytopenia, especially hypocellular myelodysplasia (see Chapter 61). Even typical AA can vary in its clinical course, from a fulminant illness marked by hemorrhage and infections to an indolent process manageable by transfusions. Immune AA is treated by hematopoietic stem cell transplantation and also immunosuppressive therapies; both result in excellent outcomes. The reader is referred to previous editions of this textbook for references, as well as to the authors’ recent reviews.[1]

HISTORY

The study of BM failure dates to 1888, when Paul Ehrlich described a young woman who died after an explosive short illness, marked by severe anemia, bleeding, and high fever. As a pathologist, Ehrlich was struck by the absence of nucleated RBCs and the fatty quality of the femoral BM. Vaquez and Aubertin, in a 1904 case report of “pernicious anemia with yellow BM,” named the disease and emphasized a pathophysiology of *anhematopoiesis*. The etymologic root of the term *aplastique* is the Greek verb πλάϑω, to create and give shape to (άπλαζτκή, the adjective, unformed).

CLASSIFICATION

Historically, AA has been classified by clinical associations (Table 31.1). A hypocellular marrow is typical following irradiation and exposure to cytotoxic chemotherapy. AA has been associated with the use of chemicals and drugs, viral infections, and with other diseases (Table 31.2). The same pathology occurs in constitutional syndromes, often as a component of multiorgan involvement (see Chapter 30). Historical associations of environmental exposures and causation are interesting but should be considered with some skepticism because of biases of observation and reporting, and lack of direct evidence. Most patients with sporadic disease have been considered “idiopathic” but their AA can now be considered immune-mediated. A simple classification of AA divides the cases into three categories: (1) marrow damage from toxicity, which is usually dose-dependent and reversible; (2) constitutional, as part of a recognizable gene defect and due to an identifiable genetic aberration; and (3) immune AA.

EPIDEMIOLOGY

Incidence, Geographic and Age Distribution

The International AA and Agranulocytosis Study (IAAAS) was conducted in Europe and Israel from 1980 to 1984.[2] This study was performed prospectively and applied strict case definition to pathologically confirmed cases. Using stringent criteria, the overall annual incidence of AA was 2 cases per 1 million people. In Asia, similar methodology was applied by Thai investigators to determine a higher annual incidence, 4.0 cases per 1 million people in Bangkok, and 5.6 cases per 1 million people in the northeastern Thai province of Khonkaen.[3] In general, from published, hospital-based series, personal communications, and first-hand observations, AA appears more prevalent in less developed regions of the world. There are no major sex or racial differences in the occurrence of AA. AA is a disease of the young (Fig. 31.2). Most patients present between 15 and 25 years of age or older than 60 years of age.

Epidemiologic Clues to Causality

Population-based studies have investigated possible causal associations. Drugs are implicated in approximately 25% of the cases of AA in the West; while in Thailand, AA was attributed to drug exposure in only approximately 15% of the cases.[3] There are associations with chemical exposures, exposures to viruses, hepatitis, and occupation. There is evidence that geographic variation in the incidence of AA might result from environmental causes and also genetic predispositions.

Genetic Aspects

In children and young adults, acquired AA should be distinguished from the main inherited forms of BM failure, Fanconi anemia (FA) and telomere biology disease (TBD) (see Chapter 30). Identification of constitutional AA has important therapeutic implications. Patients with FA and TBD can lack typical physical anomalies, and the pancytopenia can develop long after childhood, mimicking acquired disease. Genomic approaches to the study of AA are likely to uncover other genetic contributions to susceptibility to BM failure.[4,5]

A few histocompatibility types have also been associated with AA, most consistently human leukocyte antigen (HLA)-DR2. HLA-DR subtypes predicted response to immunosuppressive therapy in a large cohort of US AA patients, in which HLA-DR15 was associated with the presence of a paroxysmal nocturnal hemoglobinuria (PNH) clone (see Chapter 32) and responsiveness to immunosuppression. Genetic predisposition may be responsible for some idiosyncratic reactions to drugs and chemicals leading to the development of AA. Polymorphisms in cytokine genes, associated with an increased immune response, are also more prevalent in AA. Genome-wide

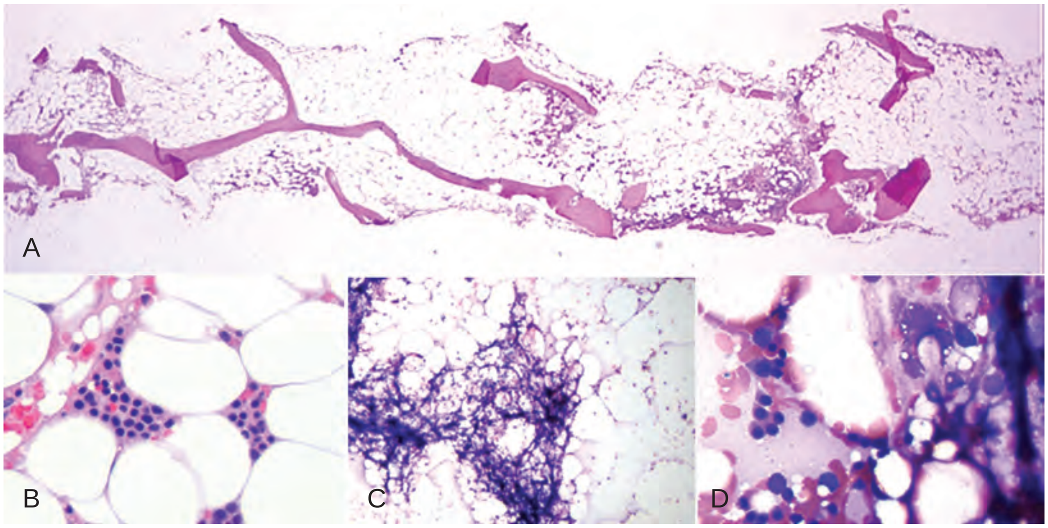


Figure 31.1 BONE MARROW MORPHOLOGY IN SEVERE APLASTIC ANEMIA. Bone marrow biopsy specimen, of sufficient length (A) shows severe hypocellularity. The corresponding aspirate (B, C, D) shows empty marrow spicules and residual stoma including lymphoid cells, plasma cells, histiocytes and mast cells.

transcriptional analysis of T and natural killer cells from AA patients has implicated pathologic expression of components of innate immunity, including Toll-like receptors.

ETIOLOGY AND PATHOGENESIS

Hematopoiesis in Bone Marrow Failure

Stem Cells

A consistent laboratory finding for patients with AA is a very low number of hematopoietic progenitor cells. Deficient in vitro colony formation by BM cells from AA patients is observed, even in the presence of high levels of hematopoietic growth factors. The total number of progenitors in a BM sample is reduced, and the number of progenitor cells assayed from a purified CD34$^+$ cell population is low. The numbers of long-term culture-initiating cells (LTC-ICs), and in vitro surrogate stem-cell assays (see Chapter 9) are also profoundly deficient in patients with severe AA. At clinical presentation, the number of LTC-ICs is usually less than 10% of normal; combined with a reduction in total BM cellularity to 10% or less, the stem-cell number is estimated to be reduced to 1% or less than normal in patients with AA (Fig. 31.3).[6]

Telomeres and Bone Marrow Failure

One peculiar feature of white blood cells in some cases of AA is short telomeres. The discovery by linkage analysis in large pedigrees that the X-linked form of dyskeratosis congenita (DKC) was caused by mutations in *DKC1* and subsequently purposeful identification of mutations in *TERC* in some autosomal dominant patients with this constitutional BM failure syndrome provided a genetic basis for DKC (see Chapter 30). Central to the repair machinery is an RNA template, encoded by *TERC*, on which telomerase, a reverse transcriptase encoded by *TERT*, elongates the nucleotide repeat structure; other proteins, including the *DKC1* gene product dyskerin, are associated with the telomere repair complex. Systematic surveys of DNA disclosed first telomerase RNA component (*TERC*) and later telomerase reverse transcriptase (*TERT*) mutations in some patients with apparently acquired AA, including older adults.[4] Family members who share the mutation, despite normal or near-normal blood counts, have hypocellular marrows, reduced CD34$^+$ cell counts and poor hematopoietic colony formation, increased hematopoietic growth factor levels, and of course short telomeres. However, clinical presentation is much later than in typical DKC, and physical anomalies are often absent. Chromosomes are also protected by shelterin proteins that bind directly to telomeres. Mutations in the shelterin gene lead to very severe DKC. Some inherited sequence variants/polymorphism in genes that repair or protect telomeres may be genetic risk factors in acquired AA, probably because they confer a quantitatively reduced hematopoietic stem cell compartment that may also be qualitatively inadequate to sustain immune-mediated damage. Accelerated telomere attrition in AA not currently explained by mutations may be caused by subtle or obscure genetic lesions, or follow BM stress and excessive stem cell turnover.

Stromal and Hematopoietic Growth Factors

Stromal cell function is usually not defective in cases of AA. Adherent cells from patients support hematopoiesis by normal CD34$^+$ cells, whereas no hematopoietic colonies develop when AA patient CD34$^+$ cells are cultured in the presence of normal stroma (Fig. 31.4). Stromal cells cultured from patient's BM generally produce normal quantities of hematopoietic growth factors. Serum levels of erythropoietin, thrombopoietin, granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) are almost always normal or elevated. Adequate stromal function is implicit in the success of BM transplantation in AA because important stromal elements remain of host origin.

PATHOPHYSIOLOGIC PATHWAYS LEADING TO APLASTIC ANEMIA

Direct Hematopoietic Injury

The most common cause of BM hypoplasia is iatrogenic; transient BM failure routinely follows treatment with cytotoxic chemotherapeutic drugs or irradiation. Certain chemical or physical agents directly injure proliferating and quiescent hematopoietic cells. However, patients with community-acquired AA rarely have a history of exposure to such physicochemical agents. Even benzene, which can act as a particularly inefficient cytotoxic chemical, is an infrequent cause of AA in developed countries. Drugs are associated with acquired AA, and in some instances, they can directly cause BM damage. Compared with chemotherapeutic agents, which are delivered in high doses, relatively low total quantities of ingested drug apparently cause idiosyncratic hematologic reactions. In addition to their direct toxic effects, chemicals and viruses may induce complex and not well-understood immune reactions leading to BM failure.

TABLE 31.1 **Differential Diagnosis of Pancytopenia**

Pancytopenia With Hypocellular Bone Marrow
Acquired aplastic anemia
Inherited aplastic anemia (Fanconi anemia and others)
Some myelodysplasia syndromes
Rare aleukemic leukemia (acute myelogenous leukemia)
Some acute lymphoblastic leukemias
Some lymphomas of bone marrow
Pancytopenia With Cellular Bone Marrow
Primary bone marrow diseases
Myelodysplasia syndromes
Paroxysmal nocturnal hemoglobinuria
Myelofibrosis
Some aleukemic leukemias
Myelophthisis
Bone marrow lymphoma
Hairy cell leukemia
Secondary to systemic diseases
Systemic lupus erythematosus, Sjögren syndrome
Hypersplenism
Vitamin B_{12}, folate deficiency (familial defect)
Overwhelming infection
Alcohol
Brucellosis
Ehrlichiosis
Sarcoidosis
Tuberculosis and atypical mycobacteria
Hypocellular Bone Marrow ± Cytopenia
Q fever
Legionnaires disease
Mycobacteria
Tuberculosis[a]
Anorexia nervosa, starvation
Hypothyroidism

[a] Pancytopenia in tuberculosis only rarely is associated with a hypocellular bone marrow at biopsy or autopsy. Marrow failure in the setting of tuberculosis is almost always fatal; exceptional patients probably had underlying myelodysplasia or acute leukemia.

TABLE 31.2 **A Classification of Aplastic Anemia**

Acquired Aplastic Anemia
Secondary aplastic anemia
Irradiation
Drugs and chemicals
Regular effects
Cytotoxic agents
Benzene
Idiosyncratic reactions
Chloramphenicol
Nonsteroidal antiinflammatory drugs
Antiepileptics
Gold
Other drugs and chemicals
Viruses
Epstein-Barr virus (infectious mononucleosis)
Hepatitis virus (non-A, non-B, non-C, non-G hepatitis)
Parvovirus (transient aplastic crisis, some pure red cell aplasia)
Human immunodeficiency virus (acquired immunodeficiency syndrome)
Immune diseases
Eosinophilic fasciitis
Hyperimmunoglobulinemia
Thymoma and thymic carcinoma
Graft-versus-host disease in immunodeficiency
Paroxysmal nocturnal hemoglobinuria
Pregnancy
Immune aplastic anemia
Constitutional Aplastic Anemia
Fanconi anemia
Dyskeratosis congenita
Shwachman-Diamond syndrome
Reticular dysgenesis
Amegakaryocytic thrombocytopenia
Familial aplastic anemias
Preleukemia (e.g., monosomy 7)
Nonhematologic syndromes (e.g., Down, Dubowitz, Seckel)

Immune-Mediated Bone Marrow Failure

In the 1970s, Mathé and colleagues observed an unexpected improvement of pancytopenia due to AA after failed BM transplantation. They speculated that the immunosuppressive conditioning regimen, intended to allow engraftment of the donor BM, might instead have promoted the recovery of host BM function. The effectiveness of diverse treatments that reduce lymphocyte number or block T-cell function, and the superior results obtained when agents are combined strongly suggest that such therapeutic success is caused by the immunosuppressive effects of the drugs used. AA shares clinical and pathophysiologic features with other autoimmune or immune-mediated human diseases that are also characterized by T-cell–mediated, tissue-specific organ destruction (inflammatory bowel disease, type 1 diabetes, multiple sclerosis, uveitis, and others).

Immune system destruction of BM occurs in animal models of graft-versus-host disease (GVHD) and in humans with transfusion-associated GVHD, in which AA is the cause of death. Very small numbers of effector cells, which have been conveyed by residual lymphocytes contained within the transfusion product or with solid organ transplants, are sufficient to mediate GVHD under these conditions. AA is associated with rheumatologic syndromes, such as eosinophilic fasciitis, and with systemic lupus erythematosus. AA occasionally occurs in individuals with hypogammaglobulinemia or congenital immunodeficiency syndrome, thymoma, thymic hyperplasia, and thymic carcinoma. AA is a rare complication of immunotherapy of cancers, both checkpoint inhibitors and chimeric antigens receptor T cells (CAR-T cells).

Laboratory support for the immune hypothesis first came from coculture experiments in which mononuclear cells from AA patient's blood or BM were shown to suppress in vitro colony formation by hematopoietic progenitor cells. T-cell depletion sometimes improved colony formation in vitro. Patient's blood and BM cells were shown to produce a soluble factor that inhibited hematopoiesis, ultimately identified as interferon (IFN)-γ. Patient's cells overproduce IFN-γ and tumor necrosis factor (TNF), two cytokines that inhibit hematopoietic proliferation. The T-box transcription factor, Tbet, which is critical to Th1 polarization, is constitutionally expressed in a majority of AA patients. AA blood and BM also contain elevated numbers of

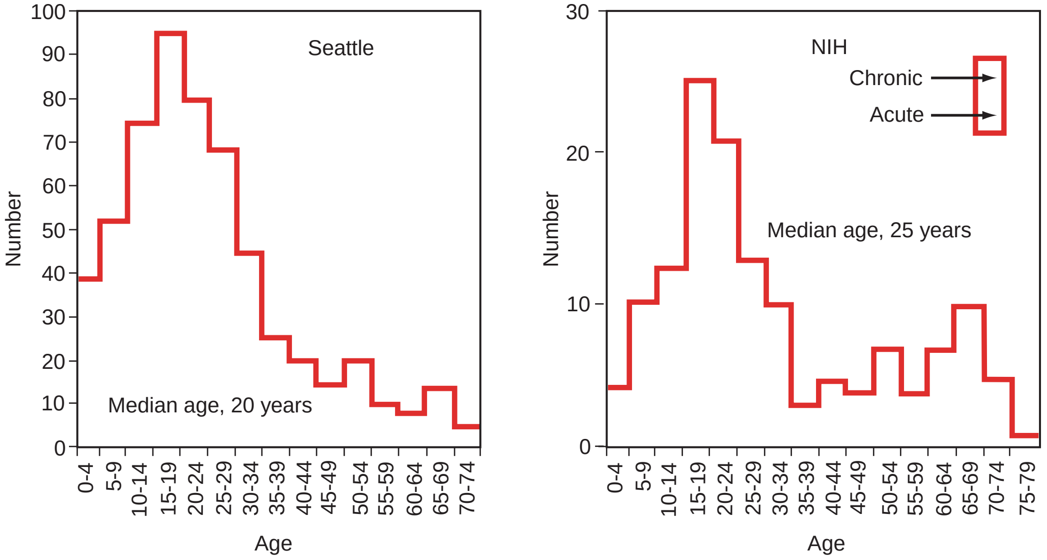


Figure 31.2 DISTRIBUTION OF APLASTIC ANEMIA BY AGE. For patients at the University of Washington (Seattle), a major transplantation center, the age given is at the time of first treatment. For the patients at the National Institutes of Health (NIH), where immunosuppressive therapy is offered, the age given is at the time of diagnostic bone marrow biopsy. Acute disease is defined as less than 3 months between diagnosis and presentation at NIH, and chronic disease is defined as more than 3 months. *(Seattle statistics are courtesy of Rainer Storb, University of Washington.)*

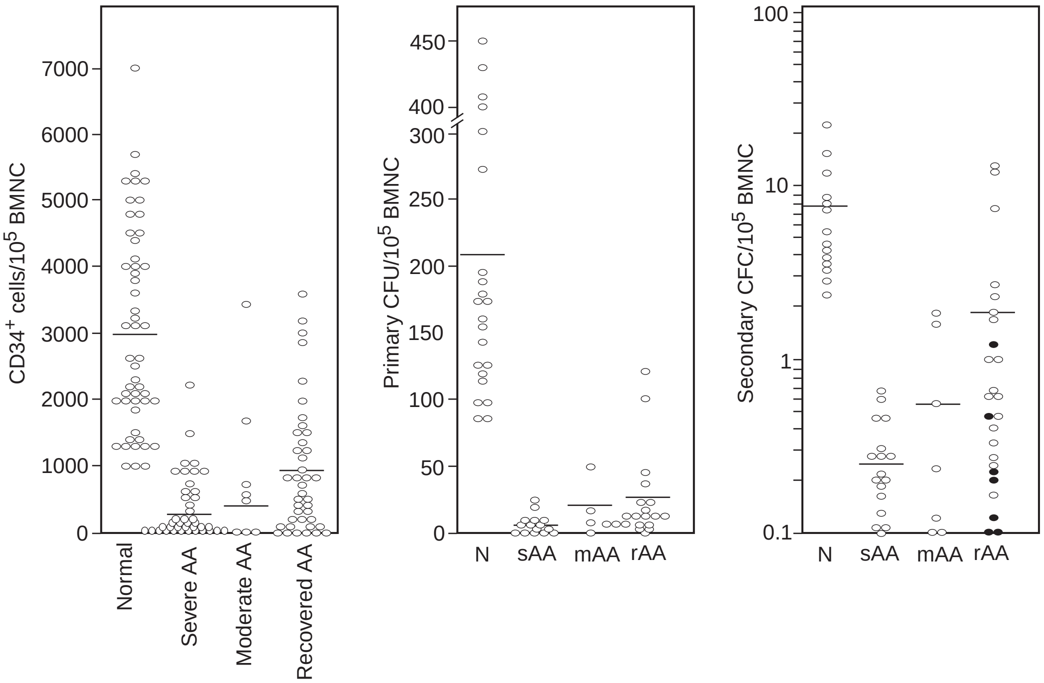


Figure 31.3 STEM AND PROGENITOR CELL DEFICIT IN AA. The numbers of CD34 cells, primary CFC, and LTC-ICs were measured in the bone marrow of AA patients. Each dot represents an individual patient's sample studied. sAA includes patients at presentation, cases refractory to immunosuppressive therapy, and patients who relapsed after a period of recovery. Primary CFC were measured in short-term methylcellulose cultures. Secondary CFC after long-term bone marrow cultures reflect LTC-IC numbers. *AA*, Aplastic anemia; *BMNC*, blood mononuclear cell; *CFC*, colony-forming cell; *CFU*, colony-forming unit; *LTC-IC*, long-term culture-initiating cell; *mAA*, moderate aplastic anemia; *N*, normal; *rAA*, recovered from aplastic anemia; *sAA*, severe aplastic anemia.

activated cytotoxic lymphocytes, and activity and levels of these cytotoxic cells are decreased with antithymocyte globulin (ATG) therapy. T regulatory cells, as in other human immune-mediated diseases, are decreased in AA. IFN-γ and TNF negative effects on the proliferation of early and late hematopoietic progenitor, and stem cells are far more potent when these cytokines are secreted into the BM microenvironment than when they were simply added to in vitro cultures. IFN-γ and TNF can suppress hematopoiesis by inhibiting cell proliferation, inducing Fas-mediated apoptosis, and blocking hematopoietic growth factor intracellular signals. The early immune system events that must precede the global destruction of hematopoietic cells are not clear. Involvement of CD4 lymphocytes has been suggested based on the overrepresentation of HLA-DR15 among patients with immune-mediated AA. Clones of HLA-DR–restricted T cells derived from a few patients have been shown to proliferate in response to BM cells.

Many features of human AA can be reproduced in mouse models of GVHD, in which the donor inoculum lacks stem cells. Major and minor histocompatibility mismatches demonstrate the potency and specificity of small numbers of T cells, the role of cytokines, efficacy

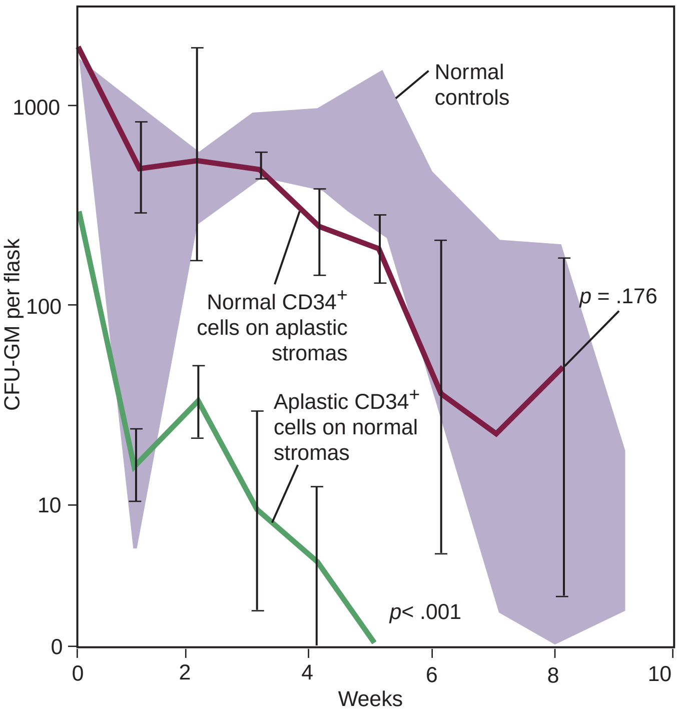


Figure 31.4 NORMAL STROMAL CELL FUNCTION IN LONG-TERM CULTURE OF APLASTIC ANEMIA BONE MARROW. *CFU-GM*, Colony-forming unit granulocyte-macrophage. *(Courtesy Dr. Judith Marsh, St. George's Hospital Medical School, London.)*

of immunosuppressive therapies, an "innocent bystander effect," and roles for specific lymphocyte regulatory and effector T-cell subsets.[7]

Radiation

BM aplasia is a major acute toxic effect of radiation (Fig. 31.5); a dose-related pancytopenia occurs 2 to 4 weeks after exposure to radiation. Mortality from hematologic toxicity is a function of the ability of BM to tolerate damage to stem cells. The capacity for hematopoietic recovery after even massive single irradiation exposures is considerable, reflecting the resistance of the quiescent stem cell damage and enormous BM repopulating potential. At intermediate radiation doses around the median lethal dose (LD_{50}), at which BM toxicity limits survival, supportive efforts can dramatically alter outcomes. Autopsies of atomic bomb victims in Japan showed acellular BMs in the first weeks of the explosion, but later regenerating BM was frequently present. The histologic picture of radiation-mediated aplasia includes necrosis, nuclear pyknosis and karyorrhexis, nuclear lysis, and ultimately cytolysis; the associated phagocytosis, marked congestion, and hemorrhage are rapidly followed by fatty replacement. BM hypoplasia occurs with radiation doses higher than 1.5 to 2 Gy to the whole body. Precise LD_{50} figures for humans do not exist, and estimates are based on limited direct human data and extrapolation from animal experiments. The LD_{50} is highly dependent on the quality of medical care, and improved support may double the tolerated radiation dose. From assessment of the outcome of radiation accidents and high-dose therapeutic irradiation, the LD_{50} has been estimated at approximately 4.5 Gy (see Fig. 31.5).

Although the immediate management of pancytopenia after a single large dose of irradiation is similar to that for treating AA, some unique points should be made concerning immediate evaluation and long-term prognosis. The type and intensity of the source of radiation, and the distance and shielding of the subject are the major determinants of radiation injury. However, these factors are often

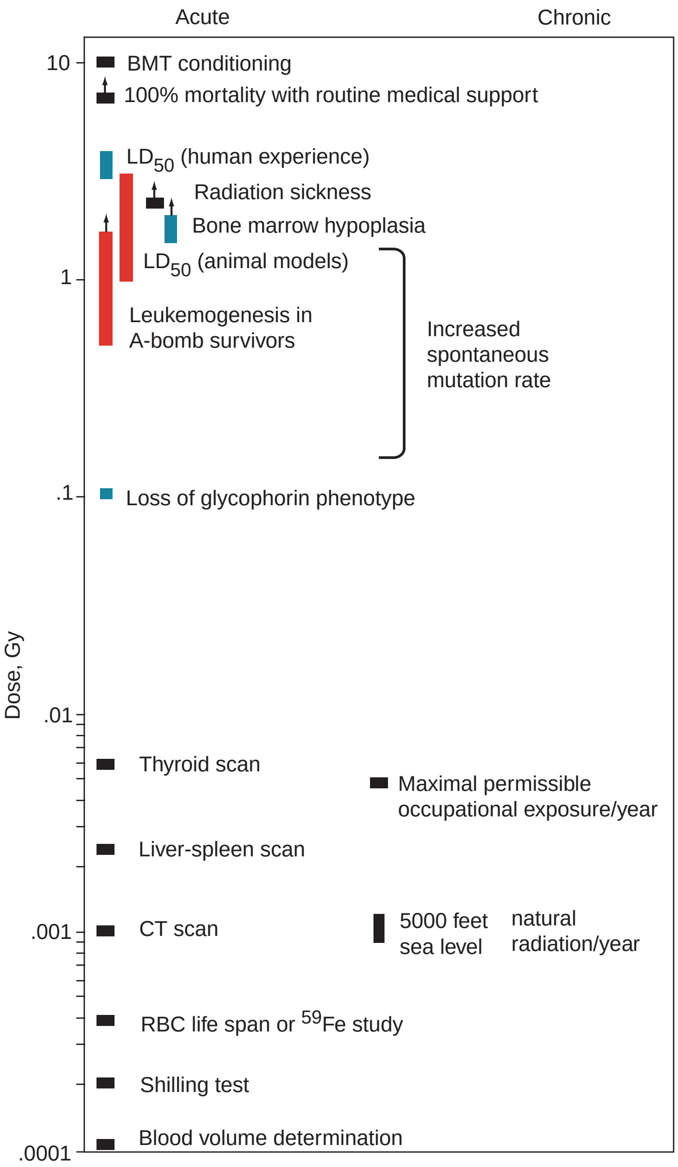


Figure 31.5 SCALE OF WHOLE-BODY RADIATION DOSES. A Gray (Gy) is a measure of absorbed dose equivalent to 1 J/kg unit mass, and 1 Gy equals 100 rads. Radiation represents radiant energy. When absorbed by biologic tissue, radiant energy causes release of electrons and molecular ionization, which result in further energy release. Radiant energy can directly break chemical bonds and indirectly damage macromolecules through generation of high-energy free radical forms. The relationship between increased mutation rate and radiation dose is very approximate *(hatched bars)*. Measurement of the phenotype of an autosomal recessive gene such as for glycophorin would be expected to be a very sensitive indicator. Because malignant transformation is almost certainly a two-step process, increased leukemogenesis is probably an underestimation of the effect of radiation on a single gene. Even the extensive data on the atomic bomb survivors of Hiroshima are subject to statistical errors because of the small number of cases; a linear or exponential curve fit gives various results, and very high doses of radiation may not be associated with as high a risk of leukemia because of stem cell death. Other data that can bear on mutation frequency lie outside the range shown. In a patient with ankylosing spondylitis who underwent irradiation of the spine, leukemogenesis was observed at relatively low doses (doubling of the leukemia rate can be extrapolated to approximately 7 Gy), but such individuals can be predisposed to leukemia. An increased risk of thyroid cancer after irradiation of the mediastinum in childhood occurred at approximately 4 Gy. *BMT*, Bone marrow transplantation; *CT*, computed tomography; *LD_{50}*, median lethal dose; *RBC*, red blood cell.

difficult to assess. Early recognition of the nature of the accident provides the best opportunity for dosimetry by accident reconstruction and use of blocking, displacement, or chelation agents. Exposure correlates well with the degree of pancytopenia. Because lymphocytes are particularly sensitive to radiation, their rate of decline can be used to estimate the dose of total body exposure to a level of approximately 3 Gy. At higher doses, the fall in the numbers of granulocytes and the severity of thrombocytopenia and reticulocytopenia can be used as gauges. The survival of some patients who received doses higher than 9 Gy suggests that autologous BM reconstitution may occur in most persons who survive the immediate consequences of accidental radiation exposure.

AA is not well documented as a delayed event after radiation exposure. A variety of hematologic abnormalities are associated with chronic low-level radiation exposure, most commonly lymphocytosis, neutropenia, dysmorphic leukocytes, and giant platelets (see Fig. 31.5). Cytogenetic abnormalities accumulate with time after chronic exposure, but they may not be reliably related to dose. AA does not appear to be more frequent among nuclear power plant or thorium processing factory workers. The excessive risk of death from AA previously reported after therapeutic irradiation of the spine for ankylosing spondylitis may have been overestimated. Cancer patients who had received therapeutic irradiation or higher exposure to natural background radiation were not found to have an increased risk of AA.

Drugs and Chemicals

AA is frequently associated with medical drug use (Table 31.3). At the end of the 19th century, chemicals were linked to BM function through observations of benzene effects on workers. Establishment of a relationship between amidopyrine analgesics and agranulocytosis in the early 20th century and an apparent epidemic of AA after the introduction of chloramphenicol in the 1960s also supported this concept. Initially suggested by the accumulation of case reports, drug associations have been established in formal case-control population-based epidemiologic studies. In the IAAAS, relative risks were estimated for individual drugs and large classes of pharmaceutical agents, including nonsteroidal antiinflammatory drugs (NSAIDs), drugs affecting thyroid function, certain cardiovascular agents, some psychotropics, and sulfa-based antibiotics (Table 31.4).[2] Approximately 25% of the cases of AA identified in the IAAAS could be attributed to drug use. Drug use as a risk factor was also assessed by similar methods in Thailand, where the incidence of AA is higher than in the West. Surprisingly, chloramphenicol was not shown to be a risk factor; the etiologic fraction for drugs was only 15%.

Associations between drug exposure and AA can be divided into two classes. Drugs used in cancer chemotherapy are selected for their cytotoxicity, and their regular, dose-dependent induction of BM aplasia is an expected effect. Most AA associated with medical drug use in the community is described as idiosyncratic, meaning that its occurrences are unexpected and rare. Many of the drugs implicated in AA also appear to cause other, milder forms of BM suppression such as neutropenia. Although difficult to prove, some dose relationship probably does exist even for idiosyncratic reactions. In most case reports, patients received normal or high doses of the agent, usually for a period of weeks to months. Drug-induced aplasia cannot be distinguished by history from idiopathic forms of the disease; the clinical course, including the favorable response to immunosuppressive therapy, is the same as in idiopathic disease.

The low probability of developing AA after a course of drugs may be a reflection of the gene variant frequency for metabolic enzymes (for direct chemical effects) or immune response genes. The rarity of idiosyncratic drug reactions could then arise from the infrequent combination of unusual circumstances: exposure, genetic variations in drug metabolism, the physical properties of the agent, enzymatic pathways that chemically alter the drug, and the susceptibility of the host to the action of a toxic compound. Examples of detoxifying enzyme systems directly applicable to BM failure that also demonstrate genetic variability include arylhydrocarbon hydroxylase (e.g., benzene toxicity), epoxide hydrolases (e.g., phenytoin toxicity), *S*-methylation (e.g., 6-mercaptopurine, 6-thioguanine, azathioprine) and *N*-acetylation (e.g., sulfa drugs). Genomic approaches have revealed the complex role of genetic variation in metabolic pathways that process arylhydrocarbons and even links to immune function.

TABLE 31.3 **Classification of Drugs and Chemicals Associated With Aplastic Anemia**

I. Agents That Regularly Produce Marrow Depression as a Major Toxic Effect When Used in Commonly Used Doses or Normal Exposures
Cytotoxic drugs used in cancer chemotherapy
Alkylating agents (busulfan, melphalan, cyclophosphamide)
Antimetabolites (antifolic compounds, nucleotide analogs) antimitotics (vincristine, vinblastine, colchicine)
Some antibiotics (daunorubicin, doxorubicin [Adriamycin])
Benzene (and less often benzene-containing chemicals: kerosene, carbon tetrachloride, Stoddard solvent, chlorophenols)
II. Agents Probably Associated With Aplastic Anemia but With a Relatively Low Probability Relative to Their Use
Chloramphenicol
Insecticides
Antiprotozoals (quinacrine and chloroquine)
Nonsteroidal antiinflammatory drugs (including phenylbutazone, indomethacin, ibuprofen, sulindac, diclofenac, naproxen, piroxicam, fenoprofen, fenbufen, aspirin)
Anticonvulsants (hydantoins, carbamazepine, phenacemide, ethosuximide)
Gold, arsenic, and other heavy metals such as bismuth and mercury
Sulfonamides as a class
Antithyroid medications (methimazole, methylthiouracil, propylthiouracil)
Antidiabetes drugs (tolbutamide, carbutamide, chlorpropamide)
Carbonic anhydrase inhibitors (acetazolamide, methazolamide, mesalazine)
D-Penicillamine
2-Chlorodeoxyadenosine
III. Agents More Rarely Associated With Aplastic Anemia
Antibiotics (streptomycin, tetracycline, methicillin, ampicillin, mebendazole and albendazole, sulfonamides, flucytosine, mefloquine, dapsone)
Antihistamines (cimetidine, ranitidine, chlorpheniramine)
Sedatives and tranquilizers (chlorpromazine, prochlorperazine, piperacetazine, chlordiazepoxide, meprobamate, methyprylon, remoxipride)
Antiarrhythmics (tocainide, amiodarone)
Allopurinol (can potentiate marrow suppression by cytotoxic drugs)
Ticlopidine
Methyldopa
Quinidine
Lithium
Guanidine
Canthaxanthin
Thiocyanate
Carbimazole
Cyanamide
Deferoxamine
Amphetamines

TABLE 31.4 Drugs Associated With Aplastic Anemia in the International Aplastic Anemia and Agranulocytosis Study[a]

Drug	Stratified Risk Estimate (95% CI)	Multivariate Relative Risk Estimate (95% CI)
Nonsteroidal Analgesics		
Butazones	3.7 (1.9–7.2)	5.1 (2.1–12)
Indomethacin	7.1 (3.4–15)	8.2 (3.3–20)
Piroxicam	9.8 (3.3–29)	7.4 (2.1–26)
Diclofenac	4.6 (2.0–11)	4.2 (1.6–11)
Antibiotics		
Sulfonamides[b]	2.8 (1.1–7.3)	2.2 (0.6–7.4)
Antithyroid drugs	16 (4.8–54)	11 (2.0–56)
Cardiovascular Drugs		
Furosemide	3.3 (1.6–7.0)	3.1 (1.2–8.0)
Psychotropic Drugs		
Phenothiazines	3.0 (1.1–8.2)	1.6
Corticosteroids	5.0 (2.8–8.9)	3.5 (1.6–7.7)
Allopurinol	7.3 (3.0–17)	5.9 (1.8–19)
Gold	29 (9.7–89)	

[a] The multivariate model included the following factors: age, gender, geographic area, date of interview, reliability of the patient, person interviewed, transfer from another hospital, history of blood disorder or tuberculosis, exposure to benzene and related chemicals, and use of other suspected drugs.
[b] Other than trimethoprim-sulfonamide combination.
CI, Confidence interval.
From Kaufman DW, Kelly JP, Levy M, Shapiro S. *The Drug Etiology of Agranulocytosis and Aplastic Anemia.* New York: Oxford University Press; 1991.

Benzene

Benzene exposure is linked to AA. Benzene myelotoxicity can be placed between the predictable effects of chemotherapeutic agents and idiosyncratic drug reactions. Industrial emissions add greatly to the biologic sources of ambient benzene. Significant benzene exposure can also occur outside of industry. Although the concentrations of benzene to which consumers are exposed are orders of magnitude lower than those observed in industrial workers, the effect of low-dose chronic exposure is uncertain, but genetic variations in metabolizing enzymes may influence susceptibility to BM suppression at these levels. Benzene metabolites are also generated from the diet. Water-soluble products of benzene metabolism such as phenols, hydroquinones, and catechols mediate toxicity to the BM. Benzene and its intermediate metabolites covalently and irreversibly bind to BM DNA, inhibit DNA synthesis, and introduce DNA strand breaks. Benzene acts as a "mitotic poison" and as a mutagen. Acutely, the more mature, actively cycling BM precursor cells are preferentially damaged over more primitive progenitors. Intermittent exposure may be more damaging to the stem cell compartment than is continuous exposure. BM stroma can also be damaged by benzene.

The range of hematologic disease attributable to benzene is broad, from relatively frequent mild alterations in blood counts to AA or leukemia. Studies of exposed North American workers earlier in the 20th century suggested that the risk of AA was 3% to 4% in men exposed to concentrations higher than 300 ppm and that 50% of individuals exposed to 100 ppm developed some blood cell count depression. Leukopenia, anemia, thrombocytopenia, and lymphocytopenia are common consequences of benzene; other manifestations include macrocytosis, an acquired Pelger-Huet anomaly, eosinophilia, basophilia, and less often, polycythemia, leukocytosis, thrombocytosis, or splenomegaly. The BM is usually normocellular but can show hypocellularity or hypercellularity; a hypercellular phase can precede complete aplasia.

Aromatic Hydrocarbons

The common perception that other molecules resembling benzene or containing a benzene ring can also cause AA is not well supported by evidence. Neither the closely related alkylbenzenes nor pure toluene or xylene are established BM toxins. Often, an aromatic hydrocarbon has been implicated as causative by a clinician only for lack of another apparent etiology. For some substances, toxicity might result from the presence of benzene as a contaminant of the synthesis of the molecule or in the petroleum distillates used to dissolve the compound. However, the total number of AA cases reported with aromatic hydrocarbon exposures is small when the large populations exposed to this heterogeneous group of chemicals are considered. For example, the significance of a handful of case reports associated with insecticide exposure in the context of the vast use of these compounds is questionable. However, the very high prevalence of aromatic hydrocarbons in daily life would greatly amplify even a small individual risk. Pesticides and insecticides have been associated with AA for decades, with almost 300 medical case reports appearing in the medical literature. The most frequently cited insecticides are chlordane, lindane, and dichlorodiphenyltrichloroethane.

Chloramphenicol

The structural similarity of chloramphenicol to amidopyrine, a drug known to cause agranulocytosis, led to early prediction of possible hematotoxicity associated with the administration of this antibiotic. During the period of its unrestrained use, chloramphenicol was considered the most common cause of AA in the United States, accounting for 20% to 30% of total cases and 50% of drug-associated cases. Estimates of the risk of AA after a course of chloramphenicol ranged from 1 case per 20,000 to 1 case per 800,000 people. Based on these figures, a course of chloramphenicol was estimated to increase the risk of AA 13-fold. Although the introduction of chloramphenicol into the US market was perceived as having increased the total number of cases of AA, this assumption was only weakly supported by epidemiologic data, and the mortality from AA remained essentially constant during the period of chloramphenicol's introduction, extensive use, and after the withdrawal of chloramphenicol from the market. Chloramphenicol has not been associated with AA in Thailand, despite its high rate of use there. In Hong Kong, where the use of chloramphenicol is almost 100 times higher than in the West, drug-associated AA occurs infrequently.

Nonsteroidal Antiinflammatory Drugs

Compared with chloramphenicol, it took far longer to associate phenylbutazone with AA. Mortality estimates have ranged from 1 case per 100,000 to 1 case per 1 million treatment courses. The use of other NSAIDs is associated with case reports of AA. A large case-control led investigation in Europe confirmed the risk of AA with phenylbutazone use and identified even higher probabilities with other NSAIDs. There was a suggestion of increased risk with drugs taken regularly for a prolonged period at very high doses, and in some cases, hematologic reactions were reproduced on repeat exposure.

Neuroleptics and Psychotropic Drugs

A variety of drugs used to treat disorders of the central nervous system have been associated with AA: the hydantoins and carbamazepine, antidepressants, tranquilizers, and the anticonvulsant felbamate. The marketing of felbamate was severely affected by the occurrence of AA in more than 30 patients. Monitoring of drug blood levels and peripheral blood counts in patients receiving carbamazepine was recommended despite fewer than two dozen AA cases reported by 1982. There was doubt about the validity of the many cases reported, as well as several large series of patients who did not develop hematologic toxicity and an estimated AA case rate of approximately 1 in 200,000 treated patients, having led to questions concerning the relationship between carbamazepine and AA.

TYPICAL AND ATYPICAL PRESENTATIONS

Most patients with AA seek medical attention for symptoms that occur as a result of low blood counts (Table 31.5). Some patients are diagnosed incidentally and often show few symptoms despite severely depressed blood counts. All of the blood elements can be depressed or a single lineage cytopenia can dominate. The differential diagnosis of pancytopenia includes a variety of diseases (see Table 31.1). Most patients do not have systemic symptoms: weight loss, persistent fever, pain, and loss of appetite point to an alternative diagnosis.

Bleeding is the most alarming manifestation of pancytopenia and frequently provokes the patient to see a doctor. Thrombocytopenia usually does not cause massive bleeding. Instead, the patient reports easy bruisability and the appearance of red spots, especially over dependent surfaces; gum bleeding with tooth brushing and episodic nose bleeds are common. Heavy menstrual flow or irregular vaginal bleeding can occur in younger women. In AA associated with PNH (see Chapter 32), red or dark urine is due to hemoglobinuria, but generally visible bleeding from the genitourinary and gastrointestinal tracts is rare. Extensive hemorrhage from any organ can occur but usually late in the course of the disease and almost always associated with infections, drug therapy, or invasive procedures.

The ability to adapt to a gradual reduction in hemoglobin concentration is remarkable. The anemic patient might mention fatigue, lassitude, shortness of breath, or ringing in the ears, but some individuals can tolerate a very low hemoglobin levels without complaint. Even abrupt cessation of erythropoiesis leads to only a slow decline in hemoglobin.

Infection is an uncommon initial symptom in patients with AA. The sore throat associated with agranulocytosis is not often observed, presumably because other alarming symptoms appear earlier.

Retrospective studies of AA associated with drugs and viruses and the observation of the occasional patient with serially monitored blood counts suggest a latent period of 6 to 8 weeks between the inciting event and the onset of pancytopenia. The interval can be more prolonged when pancytopenia is well tolerated or moderate.

With identification of genetic etiologies, the family history has assumed great importance in the evaluation of pancytopenia. Leukemia and myelodysplastic syndrome (MDS) (see Chapter 61) can occur in FA and TBD pedigrees (see Chapter 30). In the telomeropathies secondary to *TERT* and *TERC* mutations, hematologic findings in other family members may be mild, such as modest thrombocytopenia or macrocytosis with or without anemia. These mutations also cause cirrhosis and pulmonary fibrosis, which may be present as diagnoses in the pedigree. Early graying of the hair is sometimes prominent in TBD pedigrees. Inherited mutations of other genes predispose to BM failure and myeloid neoplasms (see Chapter 30). For instance, germline mutations in runt-related transcription factor 1 (*RUNX1*), ETS variant transcription factor 6 (*ETV-6*), CCAAT enhancer binding protein alpha (*CEBPa)*, and myeloproliferative leukemia protein (*MPL*) cause childhood AA or leukemia. Germline mutations in sterile alpha motif domain containing 9 (SAMD9) and sterile alpha motif domain containing 9 like (SAMD9L), or GATA binding protein 2 (GATA2) cause BM failure and later evolution to MDS associated with monosomy-7 or deletion 7q. Genetic testing for genes that are mutated in constitutional BM failure is commercially available as genomic screening.

Findings on physical examination usually reflect the severity of the pancytopenia (Table 31.6). However, patients with severe disease can look well. The patient can present with subtle variations from normal or with a dramatic, even toxic appearance. Petechiae are often present over the pretibial surface of the lower legs, the dorsal aspects of the forearms and wrists, in the oropharynx, and on the palate. Ecchymoses typically appear in areas exposed to minor trauma. With severe thrombocytopenia, retinal hemorrhages can be observed on funduscopic examination, there can be gingival oozing or blood in the nares, and hemorrhage can be apparent at the uterine cervical os. The stool can contain traces of heme. Pallor is common and is best appreciated on the mucous membranes and palmar surfaces. The newly diagnosed patient can be febrile, but specific or localizing signs of infection are uncommon on presentation. Cachexia, splenomegaly, and lymphadenopathy are not associated with AA, and these findings should strongly suggest another diagnosis. The examiner should look carefully for café-au-lait spots and other physical anomalies of FA, typical nail changes, leucoplakia, and hypopigmentation of the skin and gray hair of telomere disease in children and adults (see Chapter 30).

Several atypical presentations of AA should be noted. A physician might encounter an elderly patient with pancytopenia in whom subsequent BM examination reveals dysplastic features (see box on Diagnostic Algorithm in Aplastic Anemia and Table 31.7). Although the history of the illness in a newly diagnosed patient is typically short—in the range of months—some patients may recall a longer history of bruising, anemia, and low blood-cell counts reported to them by previous physicians during routine examinations. These patients can have a moderately severe, chronic disease, and pancytopenia from childhood should suggest a constitutional AA.

CLINICAL ASSOCIATIONS

A number of clinical syndromes, usually revealed through a careful history and physical examination, are associated with AA (see box on Diagnostic Algorithm in Aplastic Anemia and Tables 31.2 and 31.3).

TABLE 31.5 Presenting Symptoms of Aplastic Anemia

Symptoms	Number of Patients
Bleeding	41
Anemia	27
Bleeding and anemia	14
Bleeding and infection	6
Infection	5
Routine examination	8
Total	101

Adapted from Williams DM, Lynch RE, Cartwright GE. Drug induced aplastic anemia. *Semin Hematol.* 1973;10(3):195–223.

TABLE 31.6 Severity of Aplastic Anemia as Defined by Laboratory Studies

Severe Aplastic Anemia

Bone marrow cellularity <30%

Two of three peripheral blood criteria:

Absolute neutrophil count <500 cells/mm^3

Platelet count <20,000 cells/mm^3

Reticulocyte count <40,000 cells/mm^3

No other hematologic disease

Moderate Aplastic Anemia

Patients with pancytopenia who do not fulfill the criteria of severe disease

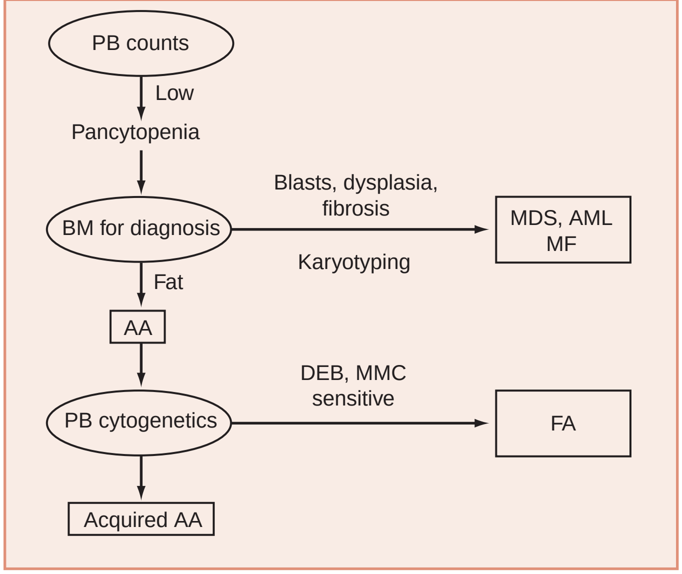
TABLE 31.7 **Bone Marrow Morphologic and Molecular Findings That Discriminate Myelodysplasia From Aplastic Anemia**

Characteristic[a]	Myelodysplasia	Aplastic Anemia
Cellularity	Usually normal to increased	Decreased
Cytogenetics	50% abnormal, if presence usually consistent with MDS	Normal, transient or small number of metaphases affected
Somatic mutations	Typical myeloid driver mutations with high clonal burden and complex clonal architecture (multiple mutations)	Usually single somatic hits, low clonal burden, typical BCOR/BCORL, DNMT3A, other can be also present RNUX1, CBL, SETBP1, ASXL1 as single hits but warrant follow-up
Erythropoiesis		
Megaloblastic	Very common	Common
Dyserythropoietic	Very common	Unusual
Maturation defects	Common	Not found
Ringed sideroblasts	Common	Not found
Myelopoiesis		
Monocyte prominence	Very common	Unusual
Midmyeloid predominance	Very common	Unusual
Increased blasts	Yes	Not found
Megakaryocytes		
Atypical morphology	Very common	Not found

[a] Ringed sideroblasts and myeloblasts are observed by definition, in some of the myelodysplastic syndromes. Dyserythropoietic red blood cell precursors show bizarre forms with multiple or irregular nuclei. Megakaryocytes can show defective nuclear polyploidization and increased internuclear spaces, or they can be small with only a few nuclei and peculiar granulation.

Adapted from Bagby GC. The preleukemic syndrome (hematopoietic dysplasia). In: Shahidi NT, ed. *Aplastic Anemia and Other Bone Marrow Failure Syndromes*. New York: Springer-Verlag; 1990:199 (provides percentages for myelodysplasia).

DIAGNOSTIC ALGORITHM IN APLASTIC ANEMIA



AA, Aplastic anemia; *AML*, acute myeloid leukemia; *BM*, bone marrow; *DEB*, diepoxybutane; *FA*, Fanconi anemia; *MDS*, myelodysplastic syndrome; *MF*, myelofibrosis; *MMC*, mitomycin C; *PB*, peripheral blood.

Posttransfusion and Post Solid Organ Transplant Associated Graft-Versus-Host Disease

Almost uniformly fatal AA is a constant feature of transfusion-associated GVHD, produced by the transfusion of competent lymphocytes into immunodeficient hosts, including children with congenital syndromes, solid organ transplant patients, cancer patients receiving high-dose chemotherapy, and patients with adoptive cellular immunotherapy for leukemia.[8] In rare circumstances, competent donor lymphocytes can be also transferred with the transplanted organ. Rarely, posttransfusion GVHD occurs in an apparently immunocompetent recipient in the special circumstance in which the donor is homozygous for an HLA haplotype also shared by the recipient, as can occur among first-degree family members. Small numbers of lymphocytes are sufficient to produce the syndrome, which is resistant to immunosuppressive therapy.

Pregnancy

Pregnancy is common in the age groups most susceptible to BM failure, and in many cases, its association is probably only coincidental. The true frequency of AA in pregnancy is unknown, but from the number of cases reported, it appears rare, although BM hypoplasia may be relatively common during pregnancy. A causal relationship is suggested by the temporal relationship between the onset of pancytopenia and that of pregnancy and by resolution after delivery and spontaneous or induced abortion; some patients who have developed AA are remitted after each delivery. Survival rates for AA in pregnancy have been relatively high for the mother and baby, with most pregnancies successful. Hemorrhage is the most common cause of death from AA during pregnancy. The published data are insufficient to guide management of a pregnant woman with AA, especially because it is clear that AA, in some cases, is serendipitously diagnosed and can persist beyond parturition. A woman who desires a child can be maintained with transfusions, with the understanding that any clinical deterioration is a criterion for interruption or termination of the pregnancy. The hazard of pregnancy to the woman who has recovered from immune AA is unknown, but most hematologists, recognizing the risk of relapse, advise patients not to become pregnant, especially if thrombocytopenia and a PNH clone are present. The risk associated with PNH has been now greatly reduced with the use of complement inhibitors.

Hepatitis

Hepatitis-associated AA has several distinctive features.[9] Typically, an uneventful episode of apparent viral hepatitis in a young man is followed in 1 to 2 months, during convalescence from the liver inflammation, by very severe pancytopenia. Depression of blood cell counts during the course of hepatitis is common; leukopenia, atypical lymphocytosis, macrocytosis, and thrombocytopenia mimic in milder forms the hematologic changes of AA. However, posthepatitis AA has a very poor prognosis, with early estimates of mortality of 90% at one year, and a history of AA with hepatitis has been considered an indication for early stem cell transplantation. Patients with posthepatitis AA can successfully undergo stem cell transplantation without an increased risk of venoocclusive disease. Patients with hepatitis-associated aplasia have markers of immune system activation and respond to intensive immunosuppressive therapy. Almost all cases have been non-A, non-B, non-C hepatitis. Posthepatitis AA is linked to fulminant hepatitis of childhood and acute seronegative hepatitis. Acute viral hepatitis that is seronegative differs clinically from hepatitis C disease; parenteral exposure is not a risk factor, liver functions abnormalities are more severe during the acute phase, and late complications are more common. Even next generation sequencing has failed to find evidence of infection in acute seronegative hepatitis, making the likely mechanism immune.

Postmononucleosis Aplastic Anemia

Acute infection with Epstein-Barr virus (EBV) causes infectious mononucleosis that is commonly associated with neutropenia and other hematologic abnormalities, but, like acute hepatitis, is only rarely complicated by AA (see Chapter 55). Pancytopenia can be first observed during the acute mononucleosis syndrome or shortly thereafter. Some patients have recovered spontaneously, and others after therapy with corticosteroids or antithymocyte globulin (ATG). EBV can occasionally be demonstrated in the BM cells of patients with AA, in association with serologic evidence of a primary or reactivated viral infection.

Hemophagocytic Syndrome

The BM is hypocellular in approximately one-third of the cases with hemophagocytic syndrome. In this disorder, there can be progression from BM hypercellularity to aplasia; myelofibrosis is also common. Pancytopenia occurs in most cases; anemia is a universal finding; thrombocytopenia and neutropenia are also common. In contrast to typical AA, these patients are systemically ill and have a fulminant course, with fever and constitutional symptoms, and peripheral blood count depression is often associated with abnormalities of other organ systems: hepatosplenomegaly, elevation of transaminases lymphadenopathy, cutaneous eruptions, and pulmonary infiltrates. The syndrome is associated with a wide variety of diseases. In the infectious category, viral infections are most common and include EBV, cytomegalovirus (CMV), herpes simplex, herpes zoster, B19 parvovirus, and HIV-1; bacterial and parasitic infections have also been associated with hemophagocytosis. Hemophagocytosis can be observed on supravital or Wright-Giemsa staining of the BM of patients with immune AA, and it is also a morphologic feature of graft rejection after BM transplantation. In virus-associated hemophagocytosis, there is evidence of immune system activation. The sera of patients contain high levels of IFN-γ, TNF-α, interleukin (IL)-6, soluble CD8, and soluble IL-2 receptor, and T cells overproduce IFN-γ in vitro. The clinical response to cyclosporine or anti-IFN-γ is consistent with a T cell–mediated hematopoietic failure state. In younger patients, hereditary forms of the syndrome (familial hemophagocytic lymphohistiocytosis [HLH])) are most common and should be investigated by appropriate testing for germline alterations, chiefly *PRF1* or *UNC13D* genes. Secondary hemophagocytic syndrome in adults can be a presentation of lymphomas.

Paroxysmal Nocturnal Hemoglobinuria and Aplastic Anemia

There is a strong association between AA and PNH (see Chapter 32). These diseases are frequently diagnosed concurrently or sequentially in the same individual, and they share similar clinical and pathologic features. The presence of an expanded PNH clone is associated with HLA-DR15, and has been reported as a good prognostic marker for responsiveness to immunosuppressive therapy. Clinical BM failure can be present at the onset of PNH or can develop after diagnosis. By flow cytometry of granulocytes for glycosylphosphoinositol-anchored proteins, there is expansion of a PNH clone in 50% or more of AA cases at presentation. Longitudinal studies of patients with de novo PNH or PNH developing from AA indicate a low probability of spontaneous remission; in most patients, the contribution of the PNH clones remains stable for years, buthemolytic PNH with frank hemolysis can develop in up to 15% of patients.

Rheumatologic Diseases

AA is a component of the collagen vascular syndrome called eosinophilic fasciitis. This severe, scleroderma-like disease is characterized by fibrosis of subcutaneous and fascial tissue, localized skin induration, eosinophilia, hypergammaglobulinemia, and an elevated erythrocyte sedimentation rate. The rheumatologic symptoms of fasciitis respond to corticosteroids, but the associated AA has a very poor prognosis. A few patients have survived after BM transplantation or immunosuppressive therapy. More rarely, AA has complicated systemic lupus erythematosus and rheumatoid arthritis, but in many cases, the role of concomitant drug therapy is confounding. Rarely, AA can accompany Sjögren syndrome, multiple sclerosis, and immune thyroid disease. AA occasionally occurs in individuals with hypogammaglobulinemia or congenital immunodeficiency syndrome, thymoma, or thymic hyperplasia.

LABORATORY EVALUATION

Peripheral Blood

In typical cases of AA, all the blood cell counts are depressed. The blood smear usually shows a paucity of platelets and leukocytes but normal RBC morphology; toxic granulation can be present in neutrophils. Automated cell counting shows erythrocyte macrocytosis and a normal RBC distribution of width. Platelet size is normal and not increased as in immune peripheral destruction, but the low number can cause greater heterogeneity of size. Prior transfusions alter platelet numbers, relative reticulocyte counts, and hemoglobin values. Although relative lymphocytosis is common, most patients also have decreased absolute numbers of monocytes and lymphocytes. The severity of AA can be graded based on the peripheral blood cell counts (see Table 31.6 and box on Diagnostic Algorithm in Aplastic Anemia).

DIAGNOSIS OF APLASTIC ANEMIA

Although the ultimate diagnosis of AA rests on the interpretation of an adequate BM biopsy specimen, important clues to the cause of pancytopenia can be obtained from the history, physical examination, and laboratory data. Pancytopenia that is not primarily hematologic in origin but secondary to other disease processes is usually an obvious diagnosis. Patients with severe liver disease and splenomegaly, systemic lupus erythematosus, or overwhelming sepsis can have low blood cell counts, but the clinical presentation is not subtle. Similarly, BM aplasia following cytotoxic drug therapy for cancers and a variety of nonmalignant diseases is anticipated. In the challenging case, obvious medical causes of pancytopenia have usually already been excluded. Pancytopenia almost never results from peripheral blood cell destruction alone. In AA, the blood smear does not show reticulocytosis, band forms, or the large platelets typical of increased compensatory BM efforts.

Immune AA is a disease of younger people, as is constitutional aplasia. Patients with FA often, but not always, have physical abnormalities. In the absence of a suggestive family history or the presence of physical anomalies, the distinction between acquired and constitutional disease depends on the results of a clastogenic-stress culture of peripheral lymphocytes (for FA), and the telomere length of leukocytes (for the telomeropathies), and in recent years genomic panels that incorporate dozens of genes etiologic in constitutional syndromes.

In older patients the major differential diagnosis is between AA and myelodysplasia (see Chapter 61). There is a gray area between hypocellular myelodysplasia and moderate AA, and even competent hematologists might not agree on the final diagnosis. BM cytogenetics, genomic screens of peripheral blood leukocytes, can help in establishing the proper diagnosis.

Myelofibrosis can also lead to pancytopenia, but the BM is not aspirable, the spleen is often enlarged, and the peripheral blood smear shows characteristic abnormalities (Chapter 72). Acute leukemia in children and the elderly can manifest as BM hypocellularity, requiring a careful search for lymphoblasts or myeloblasts, including phenotypic analysis by flow cytometry. Blood flow cytometry for glycophosphoinositol-anchored proteins should be performed to diagnose PNH (see Chapter 32).

The patient's history can provide clues, such as benzene exposure for myelodysplasia and acute leukemia, or a suspicious drug history for AA. Discontinuation of exposure to the incriminated drugs or chemicals is mandatory, and in some instances, patients may then recover. However, given the difficulty of assigning blame with absolute certainty to environmental agents, we treat all patients similarly and do not advocate protracted observation for possible spontaneous recovery. For patients with severe disease (see Table 31.6), early preparation for stem cell transplant should be undertaken or immunosuppression begun, whereas for those with moderate disease, the clinical status should be evaluated, and serial blood cell counts are required to assess progression of the disease.

BONE MARROW

The BM must be assessed quantitatively and qualitatively for cellularity and the morphology of residual cells (Fig. 31.6 and see Fig. 31.1). BM aspiration and biopsy should always be performed, and the core specimen should be at least 1 cm long. There should no hesitation in performing a second procedure if required.

BM cellularity is estimated from the core biopsy. Point counting under microscopic cross hairs in many parts of a histologic section is the most accurate method of determining cellularity, but hematologists commonly rely on visual estimation only. A crude "eyeball" approximation is almost always adequate in severe aplasia, because the hematopoietic content of the BM specimen is usually close to zero. Estimates of BM cellularity based on examination of the aspirate smear and biopsy specimen are correlated, but dilution of the aspirate by sinusoidal blood often occurs, and the aspirate can be hypocellular when the biopsy specimen is hypercellular or shows focal areas of active hematopoiesis. BM cellularity decreases considerably with age, a variation that is of some importance in assessing the older patient for aplasia or myelodysplasia. In autopsy samples from normal, young children, approximately 80% of the BM space of the iliac crest is cellular. BM cellularity gradually decreases from age 20 to 70 years and more precipitously in the very elderly, to approximately 30% in the eighth decade of life (see Chapter 157). For practical purposes, the lower limit of normal BM cellularity in adults is accepted at 30%, but the differences at the extremes of life should be recalled when evaluating infants and the elderly. In most patients with AA, total BM cellularity is extremely low, but there can be significant residual lymphocytosis. The increase in BM fat in aplasia is caused by increases in the size and number of individual fat cells. "Hot pockets" of hematopoiesis can be present. The BM tends to contract centripetally with age, and a similar process can be observed in pathologic states, so the sternal BM can be more cellular than iliac crest samples.

Examination of the BM (Fig. 31.7, and see also Figs. 31.1 and 31.6) is critical for establishing the diagnosis of most primary hematologic causes of pancytopenia (see Table 31.1 and box on Diagnostic Algorithm in Aplastic Anemia). A fatty, even watery specimen can usually be aspirated without difficulty from an aplastic patient, whereas a truly dry tap is more typical of a packed or fibrotic BM. The morphology of individual cells is best seen in the Wright-Giemsa–stained aspirate smear, and the architecture of the BM is appreciated in a biopsy section. In acellular specimens, the only cells visible are usually lymphocytes, plasma cells, and stromal elements (fibroblastoid and histiocytic cells). Some degree of dyserythropoiesis is common, usually the megaloblastoid features of macrocytosis and some nuclear-cytoplasmic maturation asynchrony, but sometimes more complex degenerative changes in nuclei and cytoplasm can be observed (see Fig. 31.6). These features are common to AA and myelodysplasia (see Table 31.7), which can be very difficult to distinguish. Hemophagocytosis of RBCs can also occur in AA. Examination of cells close to the spicules of a sparse aspirate smear can disclose a distinctive population of leukemic blasts; increased numbers of myeloblasts are not seen in AA and are evidence of aleukemic leukemia or leukemia in evolution. Histochemistry for

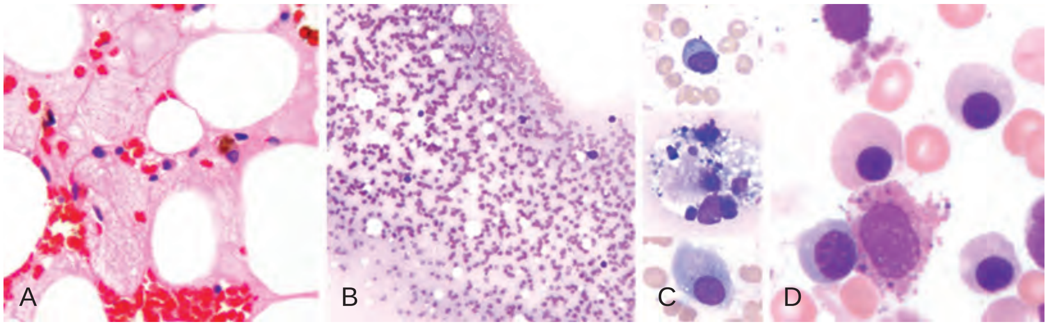


Figure 31.6 SOME MORPHOLOGIC FEATURES OCCASIONALLY OBSERVED IN PATIENTS WITH APLASTIC ANEMIA. Empty marrow with eosinophilic ground substance consistent with serous atrophy or stromal injury (A), possibly indicative of marrow damage. Scanty marrow aspirate in severe disease (B) showing only rare nucleated elements many of which are from blood. Presence of plasma cells, histiocytes and osteoblasts (C) confirms marrow nature of aspirate. Note: sometimes the histiocytes can show hemophagocytosis. Megaloblastoid erythropoiesis (D) is sometimes seen in aplastic anemia and in recovery.

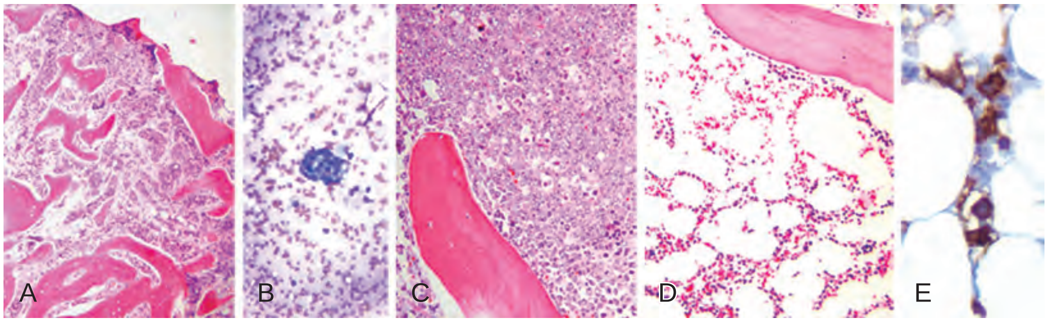


Figure 31.7 MORPHOLOGY OF OTHER DISEASES THAT MAY MANIFEST WITH PANCYTOPENIA. Bone marrow biopsy from patient with pancytopenia showing myelofibrosis and osteosclerosis associated with metastatic prostate cancer (A). The aspirate was hypocellular but did show occasional tumor clusters (B). Another case where the patient presented with pancytopenia and was found to have a bone marrow packed with lymphoma cells (C). Hairy cell leukemia can present with pancytopenia and with a hypocellular bone marrow (D) difficult to distinguish from aplastic anemia. The diagnosis rests on identifying a B-cell infiltrative process with immunohistochemical stains (E, CD20).

$CD34^+$ cells should show staining only of vascular elements in AA, and increased $CD34^+$ cell numbers is typical of MDS and acute myeloid leukemia (AML).

Karyotyping of BM cells is diagnostically important. Unfortunately, the yield of cells from a hypocellular BM can be inadequate to perform cytogenetic analysis. Chromosome analysis is usually normal in AA but frequently reveals a clonal abnormality in myelodysplasia. Cytogenetic studies, including interphase fluorescent in situ hybridization (FISH) and single nucleotide polymorphisms array–based karyotyping may produce informative results, including detection of cryptic chromosomal abnormalities (see Chapter 57).[10]

DIFFERENTIAL DIAGNOSIS OF PANCYTOPENIA

AA is not the most common cause of pancytopenia (see Table 31.1). A rational diagnostic algorithm can be very helpful in establishing a correct diagnosis (see box on Diagnostic Algorithm in Aplastic Anemia). Pancytopenia is unlikely to be the presenting feature of hypersplenism in cirrhosis or of Evans syndrome (autoimmune hemolytic anemia and thrombocytopenia) in systemic lupus erythematosus. Findings on physical examination can point strongly toward another diagnosis. For example, the patient with myelofibrosis usually has splenomegaly, which is very unusual in AA. Although vitamin B_{12} and folate deficiencies have been reported to be associated with erythroid hypoplasia, this must be an exceedingly rare event. For the practicing hematologist, the most important and difficult task is distinguishing the primary BM disorder in patients with pancytopenia.

In moderate AA, the modest depression of BM cellularity can muddle the single most reliable diagnostic criterion. BM cellularity is imprecisely quantitated at best, and further uncertainty is introduced by sampling errors. “Hot spots” of hematopoietic activity in an otherwise acellular specimen may reflect biologic heterogeneity in the pattern of cell loss. In patients with a syndrome of transient pancytopenia, spontaneous recovery occurs within a few months; although the blood cell counts can be severely depressed, the BM is much more commonly normo/hypercellular than hypoplastic. In patients with chronic BM failure, serial BM specimens may not be identical because of sampling error or because the original disease was misdiagnosed or has changed its character. Some patients with AA are not pancytopenic; they do not have uniform depressions of RBCs, neutrophils, and platelets, despite an empty BM, and their clinical course is dominated by failure in two cell lines or a single hematopoietic lineage. Related conditions such as pure red cell aplasia, amegakaryocytic thrombocytopenia, and agranulocytosis, although usually distinctive in their clinical presentation, can evolve into more generalized BM failure (see Chapter 33). A hypocellular BM often precludes the proper morphologic diagnosis, especially in hypoplastic BM (see Table 31.7).

BM cytogenetics, if positive for chromosome abnormalities, usually leads to a diagnosis of MDS or leukemia (see Chapters 57, 59 and 61). However, some random chromosomal abnormalities may be transient, and some experts believe that typical AA is not incompatible with an abnormal karyotype. Often, an acellular specimen precludes successful culture and generation of metaphase smears. In such cases, single nucleotide polymorphisms arrays–based karyotyping can disclose chromosome abnormalities. Screening for monosomy 7 and trisomy 8 can be also performed using interphase FISH.

Molecular diagnostics has entered the BM failure clinic. To establish the diagnosis of constitutional AA, commercial panels are now available to screen germline DNA for genes responsible for FA, telomeropathy, and Schwachman-Bodian-Diamond syndrome, and many other genes implicated in inherited BM failure syndromes.

The presence of multiple somatic mutations typical for myeloid neoplasia at high clonal burden, especially in spliceosome genes, supports the diagnosis of MDS. Acquired mutations of recurrently mutated genes in MDS/AML can be detected in circulating white blood cells in about one-third of otherwise typical AA patients. Some mutated clones occur transiently and disappear upon recovery (see Table 31.7). Clones may reflect coexisting clonal hematopoiesis of indeterminate potential, be a result of oligoclonality or correspond to potential founder hits for clonal evolution. In contrast to MDS and AML, somatic mutations in AA occur in a limited subset of genes, and the clone size, as estimated by variant allele frequency, is also small. Somatic hits in *RUNX1*, *DNMT3A*, *ASXL*, *CBL* and *SETBP1* correlate with patient age but independently predict for a poorer long-term outcome. Some of these mutations can be also found to expand during progression of MDS. Mutations in *PIGA* and *BCOR*/*BCORL* correlate with responsiveness to immunosuppressive therapy (see Table 31.7). Detection of unfavorable mutations in a patient who has failed treatment may influence the decision to undertake hematopoietic stem cell transplantation.[11]

TREATMENT

AA should be considered a medical emergency. Lives are lost, mainly because the grave consequences of severe pancytopenia go unrecognized. The ultimate benefits of definitive therapies such as transplantation or immunosuppression will be unrealized if the patient succumbs to an early clinical catastrophe. A haphazard transfusion policy increases the risk of graft rejection after BM transplantation, but an overly conservative approach to transfusion can jeopardize the patient’s life and increase morbidity. Supportive management requires meticulous attention to the daily problems that occur as a consequence of pancytopenia and appreciation of their impact on the ultimate possibilities for cure or amelioration of AA. AA can be cured by replacement of stem cells, by BM transplantation, and by immunosuppressive therapy. Androgens and hematopoietic growth factors have secondary roles (see box on Treatment Algorithm in Aplastic Anemia).

Supportive Management

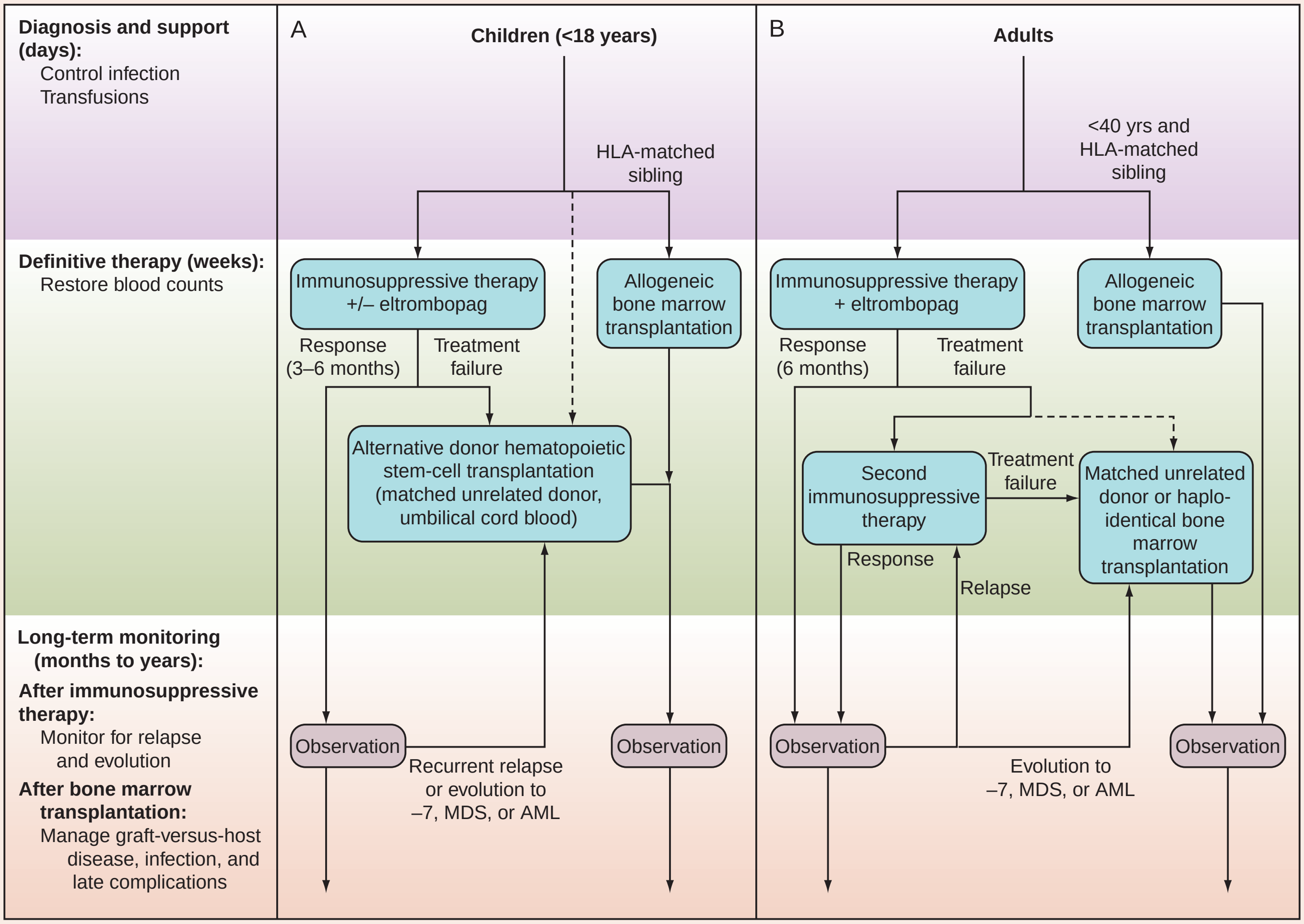
Bleeding

Bleeding was historically a common symptom in AA, and death from hemorrhaging occurred frequently in the premodern era. Platelet transfusions have substantially improved survival in patients with this disease. Measurable correction of the platelet count by transfusion therapy almost always alleviates the minor mucocutaneous bleeding common in thrombocytopenic patients. Major bleeding is usually not caused by thrombocytopenia alone, and other explanations for massive hemorrhage should be sought. The bleeding time improves after erythrocyte transfusion in patients with anemia, and there is a strong inverse correlation between the hematocrit and bleeding. The treatment of serious hemorrhage should include correction of severe anemia with RBC transfusions.

Modern transfusion practice has made platelets readily available and safe to administer (see Chapters 112 and 113). Other than cost and convenience, the major problem related to platelet transfusions is the development of alloimmunization in the recipient. The life span of the transfused platelet in the circulation is dramatically shortened by host antibodies, almost always directed to HLA-A and HLA-B antigens. Alloimmunization is suggested by a limited increase of the 1-hour posttransfusion platelet count and confirmed by finding specific HLA antibodies in serum. Refractoriness can often be overcome by selection of HLA-matched donors. Alloimmunization can be prevented or delayed by the use of single-donor platelets rather than pooled platelets, and by leukocyte depletion, by filtration or ultraviolet treatment of blood products. Avoidance of platelet transfusions except when there is active bleeding is another alternative to prevent alloimmunization, but the dose relationship between exposure to different donors' platelets and the probability of developing refractoriness is not clearly established.

The primary indication for platelet prophylaxis is to prevent intracranial hemorrhage, but the risk of this complication in the chronically thrombocytopenic patient, although real, is low. Prophylactic platelet transfusions have not been shown to alter patient survival. Nevertheless, the beneficial effects of avoiding bleeding complications and improving the quality of life justify their use. Although the 20,000 platelets/μL value has long been used to trigger transfusion,

TREATMENT ALGORITHM IN APLASTIC ANEMIA



Diagnosis and support (days):
Control infection
Transfusions
Definitive therapy (weeks):
Restore blood counts
Long-term monitoring (months to years):
After immunosuppressive therapy:
Monitor for relapse and evolution
After bone marrow transplantation:
Manage graft-versus-host disease, infection, and late complications
A
Children (<18 years)
HLA-matched sibling
Immunosuppressive therapy +/– eltrombopag
Allogeneic bone marrow transplantation
Response (3–6 months)
Treatment failure
Alternative donor hematopoietic stem-cell transplantation (matched unrelated donor, umbilical cord blood)
Observation
Recurrent relapse or evolution to –7, MDS, or AML
Observation
B
Adults
<40 yrs and HLA-matched sibling
Immunosuppressive therapy + eltrombopag
Allogeneic bone marrow transplantation
Response (6 months)
Treatment failure
Second immunosuppressive therapy
Treatment failure
Matched unrelated donor or haplo-identical bone marrow transplantation
Response
Relapse
Observation
Evolution to –7, MDS, or AML
Observation

Algorithm-based selection of treatment for patients with aplastic anemia. *AML*, Acute myeloid leukemia; *HLA*, human leukocyte antigen; *MDS*, myelodysplastic syndrome.

many reports have suggested little difference in the risk of bleeding over a wide range of platelet counts between 5000 and 100,000 platelets/μL. In a randomized trial of patients with AML, the risk of major bleeding was no different when 10,000 or 20,000 platelets/μL were chosen as the threshold, but the lower value led to a 20% reduction in platelet use. Any prophylaxis program must be modified to address the individual patient, but a goal of maintaining platelet counts greater than 10,000 platelets/μL is reasonable. In patients who have experienced serious hemorrhage, as for examples intracranial and intraocular, thresholds often are higher, greater than 50,000/μL, to prevent recurrence. Major surgery can be accomplished in the setting of thrombocytopenia. In one study blood loss and morbidity rates were low even at platelet counts of less than 30,000 platelets/μL.

Anemia

The goal of the usual transfusion regimen is to prevent symptoms of anemia. After equilibrium is achieved, a constant amount of blood will be required to maintain a given hemoglobin concentration. Physically fit individuals are usually not symptomatic at hemoglobin concentrations higher than 7 g/dL; patients with underlying cardiovascular disease should be maintained at a higher level (≥9 g/dL). Iron chelation should be introduced for unresponsive chronic anemia when there is a reasonable expectation of survival. Chelation may be avoided in the patient who proceeds to transplant soon after diagnosis or who responds in a few months to immunosuppressive therapy. In patients with chronic anemia, institution of a regimen of oral deferasirox (Exjade) or subcutaneous deferoxamine (Desferal) at adequate doses may be initiated as transfusion numbers accumulate and serum ferritin levels rise (see Chapter 37).

Alloimmunization due to blood product administration increases the probability of graft rejection and mortality after BM transplantation. Blood products from potential BM donors such as a sibling or a parent (who share histocompatibility antigens) should be avoided. Small numbers of transfusions do not have a major deleterious effect on survival. The 5% risk of graft rejection after transplantation in entirely untransfused patients was increased to 15% with receipt of 1 to 40 units and to higher than 25% in more heavily transfused patients. Graft rejection would be anticipated to be lower with the implementation of leucocyte-depletion methods for platelet transfusion preparations. Speed in arranging tissue typing and transfer to an appropriate center has a greater impact on the survival of the patient than the judicious transfusion of a few units of RBCs to a severely anemic patient or platelets to a bleeding patient. Transfusions should not be withheld from an older patient for whom immunosuppressive therapy will be the first-line therapy.

Infection

There are very few specific reports of infections and their therapy in patients with AA. The duration of neutropenia is the major difference between the neutropenia of BM failure and that induced by cytotoxic chemotherapy. With longer periods of neutropenia, the probability of serious bacterial or fungal infection increases. A second major difference is that neutropenia is part of a complex of problems associated with malignant disease and its therapy. In AA, the immune system is activated, and with the exception of intravenous catheter placement, the integument is preserved. Studies of cancer patients have usually identified a low-risk category of neutropenia, determined by the relatively brief period of neutropenia; by this criterion, almost all unresponsive patients with AA are at high risk.

In classic studies of leukemic children, neutropenia was shown to increase susceptibility to bacterial infections, and the number of infectious episodes correlated with the degree and duration of neutropenia. Susceptibility to infection is extremely high with an absolute neutrophil count of less than 200/μL, and this value has been used to define a category of very severe AA. As profound granulocytopenia becomes prolonged, infection is inevitable. Recommendations for initiation of empirical antibiotic therapy are similar for AA patients and other patients with neutropenia. The cardinal rule is, if the absolute neutrophil count is less than 500 cells/μL and infection is suspected, broad-spectrum parenteral antibiotic therapy should begin immediately. Any regimen may require modification based on the results of cultures, new symptoms or signs, or a deteriorating clinical course. Bacteremia is present in only 20% of febrile neutropenic episodes, and in only approximately 40% of those can a microbiologic cause or localizing physical findings be identified. Early discontinuation of antibiotics when cultures are unrevealing in persistently neutropenic patients is dangerous. Some experienced practitioners use prophylactic antibiotics in neutropenic patients, a practice that results in reduction of febrile episodes, emergency visits and hospital admissions.

Patients often remain febrile despite antibiotic therapy, or fever reappears. In the absence of additional microbiologic data or clinical clues from the patient's complaints or physical examination, antifungal therapy should be instituted in patients who have remained febrile despite adequate antibacterial therapy. Current infectious disease guidelines recommend introduction of antifungal therapy after 5 days, but earlier addition can be advisable in the AA patient, especially if there are findings on chest tomography and a positive test for galactomannan protein. Fungemia during an initial febrile episode is rare, but fungal infection becomes more likely with repeated courses of antibiotics and ultimately is the major cause of death in AA patients in whom definitive therapy fails. *Candida* and *Aspergillus* species account for almost all fungal diseases in AA. Early aggressive treatment can reverse fungal disease. Large, randomized trials have shown that newer antifungal agents such as voriconazole and caspofungin are less toxic, and as effective or superior compared with amphotericin and liposomal amphotericin for treatment of both persistent neutropenic fever and established *Candida* and *Aspergillus* infection. Improved survival in severe AA is in some part a benefit of both more and better tolerated antifungal drugs.[12]

Polymorphonuclear cells have a relatively brief life span in the circulation and their major activity is in infected tissue. There are no simple measures of the therapeutic efficacy of white blood cell transfusions. The absolute neutrophil count is not measurably increased by standard transfusions. Several controlled trials performed in the 1970s reported improved survival in patients who received granulocyte transfusions; negative studies may have used relatively low numbers of granulocytes transfused ($<10^{10}/m^2$/day). Granulocyte transfusions are expensive and associated with serious toxicity: severe febrile reactions, pulmonary capillary leak syndrome, an increased risk of infection, and inevitable alloimmunization. Administration of G-CSF to normal donors greatly increases the yield of granulocyte leukapheresis products without adverse effects on the volunteers. Excellent results can be obtained from community donors, and the use of allocompatible granulocytes (as determined by a lymphocytotoxicity screening assay) can further improve clinical results of granulocyte transfusions. Large numbers of neutrophils can be obtained, allowing dramatic increases in the absolute granulocyte levels in neutropenic recipients, which can improve clinical outcomes; case reports and phase I and II studies have suggested that G-CSF–mobilized granulocyte transfusions can be helpful in the treatment of life-threatening fungal infections in severely neutropenic patients.[13]

Definitive Therapy

Hematopoietic Stem Cell Transplantation

Allogeneic stem cell transplantation is curative therapy for AA patients. Transplant options have expanded dramatically with the availability of unrelated donor registries with large numbers of possible volunteers and effective strategies to reduce the formidable barriers to haploidentical donor transplant.

Stem cell transplantation in AA is the subject of a large literature of reports and reviews.[1,14,15] For AA, multiple studies favor BM over mobilized peripheral blood stem cell grafts because of the lower risk of chronic GVHD.[16] The first studies of allogeneic stem cell transplants conclusively demonstrated their value compared with conventional supportive therapy. Results with stem cell transplantation have improved over time due to a combination of factors: modification of conditioning regimens and lower transplant-related early mortality, improved transfusion support and antibiotic regimens, and the introduction of cyclosporine as prophylactic therapy for GVHD. Most referral centers have very high rates of survival. Between 1990 and 1994, the European Group for Bone Marrow Transplantation (EGBMT) reported a 72% survival rate at 3.5 years, and 80% at 5 years.[17] Favorable risk factors were identified and allowed stratification into prognostic categories.[18] When young patients, age less than 20 years old, are transplanted within 180 days of diagnosis with BM rather than blood as the stem cell source, receive ATG in the conditioning regimen, and their CMV status were combined, low-risk patients had a 90% long-term survival (high-risk patients survival was 67%). Age remains a major risk factor, and results with conventional allogeneic stem cell transplant have been static at about 50% survival for patients above 40 years old.

Graft rejection and GVHD are the major complications of stem cell transplantation in AA. Graft rejection is a strong predictor of posttransplantation survival. The rate of graft rejection decreased with intensification of the immunosuppressive conditioning regimen. Graft rejection may be related to the pathophysiology of AA, a finding supported by the unexpectedly high proportion of failures in unprepared patients receiving syngeneic transplants, in adequately preconditioned patients receiving matched sibling donor transplants, and as a late consequence in some cases. In a group of untransfused patients who received allogeneic stem cells, the incidence of graft rejection was 10%, indicating that AA patients may be particularly sensitive to alloimmunization. Nevertheless, the influence of the number of transfusions on graft rejection is relative, and modest numbers of blood donations (40 units in the International Bone Marrow Transplant Registry experience and less than 10 units of erythrocytes or 40 units of platelets in Seattle) did not greatly increase the risk of graft rejection.[19]

Matched Sibling Donor Hematopoietic Stem Cell Transplantation

Intensification of immunosuppressive conditioning regimens with the use of total-body or lymphoid irradiation, cyclosporine, or ATG reduces the risk of graft rejection. Such measures, however, have not been shown to influence long-term survival.[20] The effect of the conditioning program on graft rejection probably is achieved through elimination of the recipient's lymphocytes and of subsequent mixed hematologic chimerism, which is associated with rejection. More rapid regeneration of stem cell grafts has been observed when cyclosporine is used, and second transplantations have been successful when ATG has been added to the conditioning regimen. In a prospective trial, ATG in the conditioning regimen did not significantly improve outcomes, but ATG has appeared advantageous in retrospective analysis of large numbers of patents. Rabbit ATG is more lymphocyte-depleting than is horse ATG, and more efficacious in clinical practice. Matched sibling transplants should be accomplished without irradiation, and successful nonradiation conditioning regimens have included substitution with ATG, alemtuzumab, or fludarabine, which achieve engraftment and avoid many of the long-term complications of irradiation especially late cancers. With added ATG, sustained engraftment can achieved by more than 90%, and patients who reject the graft can still be successfully retransplanted. The combination of cyclophosphamide plus fludarabine, with or without ATG, or regimens including alemtuzumab, have also achieved high rates of graft acceptance and survival even in heavily transfused patients who were transplanted with mobilized peripheral blood stem cells, months after proving refractory to immunosuppressive drugs. Overall, reported rejection rates range between 10% and 15%.[21]

Rates of chronic GVHD vary and are related to patient selection and treatment regimens. Age remains the dominant risk factor for the development of chronic GVHD. Chronic GVHD is more frequent and more severe in older patients, and children, including adolescents, have a much lower probability of suffering and dying from chronic GVHD. In a EGBMT analysis, a significant survival difference was observed between those younger than 20 years of age (65%) and those older than 20 years of age (56%), but there was no survival difference between patients 21 to 30 years old and those 31 to 55 years old. Young adults have fared better in other series, although morbidity from severe GVHD disease was more prevalent in the young adults than in children (43% vs. 10%). Overall, the acute GVHD rate between 1991 and 1997 was approximately 20% for patients younger than 20 years of age and 40% for those over 40 years of age. In general, similar numbers have been cited for chronic GVHD. In more recent reports, the Seattle team's regimen of cyclophosphamide, ATG, cyclosporine, and methotrexate in children produced 100% 5-year survival, with low rates of graft rejection, 7%; high-grade acute GVHD, 3%, and chronic GVHD, 10%; the European collective experience is similar for first-line transplant in very young children (91% long-term survival; 2% rejection, 8% acute GVHD, and 6% chronic GVHD). In contrast, patients over the age of 40 years (who are often transplanted after failing immunosuppressive treatment), have three- to fourfold higher probabilities of developing acute and chronic GVHD.

In summary, excellent survival rates and low morbidity in younger patients make allogeneic stem cell transplantation the treatment of choice for children and adolescents. Older adults have a higher risk of transplant-related morbidity and mortality. Younger adults have an excellent opportunity for cure with transplantation but face more complications than do children. In addition to age, a prolonged interval between diagnosis and transplantation, multiple transfusions, and serious infections before transplantation are poor risk factors.

Transplant From Alternative Donors

Haplotype sharing between parents occasionally has allowed identification and successful transplantation between phenotypically matched relatives (see Chapter 105). Long-term survival after even one-locus-mismatched family donation is inferior to genotypically matched transplants, mainly because of graft rejection and GVHD. In the large European experience, for phenotypically identical family matches, the actuarial survival rate was 45%; for patients with a single-locus mismatch, it was 25%; and for those with two to three loci mismatched, the survival rate was 11%. In a report from Seattle although all patients who received fully HLA-matched transplants survived, those with mismatches at one or more loci had a much poorer outcome, and even with total-body irradiation added to the conditioning regimen, the survival rate was only 50%.

Until recently, most large studies of matched unrelated donor (MUD) transplants have shown inferior long-term survival and higher rates of complications as compared with matched sibling transplants. Even more than in standard sibling transplants, age is a crucial risk factor in unrelated transplants. The degree of match clearly impacts the outcome of the unrelated BM transplantation, and selection of donors based on high resolution typing is responsible for improving outcomes (see Chapter 106). Different conditioning regimens have been used at various centers, and results are often difficult to compare between institutions because of the large number of variables. Nevertheless, in a summary of the large European experience of transplants performed between 2005 and 2009, overall survival was equivalent to matched sibling procedures (76% vs. 83%), but with more high-grade GVHD (10% vs. 5%) and chronic extensive GVHD (11% vs. 6%). Factors predictive of a favorable MUD outcome were transplant using BM as a source of stem cells, transplant within 180 days of diagnosis, patient age, use of ATG in the conditioning regimen, and CMV status.[18] MUDs are sufficiently effective in children that they have been tested as first-line therapy in pilot trials, with good outcomes.

Retrospective analysis of 71 AA patients treated with umbilical cord transplantation showed the estimated probability of 3-year overall survival of 38% (median follow-up of 35 months); the cell dose appeared to be the most important factor impacting survival.[22]

Haploidentical transplant is increasingly popular (see Chapter 107). A donor is almost always readily available, avoiding delays typical in arranging unrelated donor transplants, and effective strategies have reduced typical complications of GVHD. In China, pretransplant lymphocyte depletion is utilized, with very high survival rates for children as well as adults. Johns Hopkins pioneered the addition of cyclophosphamide posttransplant, to eradicate immune cells responsible for GVHD; small numbers of patients have been reported, with very favorable survival and little chronic GVHD. Patients ordinarily rejected as too old for transplant care do remarkably well, especially when younger donors within a family are selected; haploidentical donors have been preferred often to older sibling donors for this reason. Ongoing controlled trials will produce comparisons of haploidentical transplant to immunosuppressive therapy in treatment-naïve AA. Haploidentical transplant was initially introduced for refractory marrow failure but more recently has been extended to first-line. However, because of graft rejection in some cases, the Hopkins regimen now includes low dose irradiation. Retrospective case series from Brazil and Europe report lower survival (about 70%) than the striking outcomes from Baltimore. Extension of haploidentical transplant, broader criteria for selection of recipients, and refinement of the preparative and post-transplant regimens should result in data that in practice clarify the validity of this approach over the next few years.

Late Complications of Stem Cell Transplantation

Late complications after transplantation include effects on gonadal function, growth and development, avascular necrosis, as well as compromised function of endocrine, neurologic, or other organ systems. About 10% of long-term survivors have significant late effects after matched sibling transplants, and a higher proportion after MUDs. A high rate of secondary malignancies has been recorded after transplantation. In a National Cancer Institute retrospective analysis of almost 20,000 transplantations, the risk of late-onset cancer was eightfold higher at 10 years than in the general population and even higher for young patients, for whom the risk of malignancy was increased approximately 40-fold. For AA, multivariate analyses repeatedly implicate radiation as the major risk factor for the development of malignancies, usually solid cancers. For AA, among 320 patients who received transplants in Seattle, four developed cancer, leading to a calculated risk seven times higher than for normal controls. In a recent update, 12% of patients who survived more than 2 years after transplantation developed solid tumors. In a French survey, 4 of 147 AA patients developed solid tumors, an 8-year cumulative incidence rate of 22%. In an analysis of 700 transplantation patients with AA and FA, the risk of developing a secondary malignancy was 14% at 20 years. The hazard of lymphoid malignancies decreased with time after transplantation, whereas the risk of solid tumors progressively increased. In general, the rates of secondary malignancies after stem cell transplantation for AA and other diseases are similar. Immune events such as acute GVHD, treatment with ATG or monoclonal antibodies, in addition to total-body irradiation have been linked to the development of secondary malignancies. Patients with these secondary cancers, except for carcinoma of the skin, have a poor prognosis. The risk of cancer after stem cell transplantation must be evaluated in the context of other therapeutic options, especially immunosuppression, because a significant risk of a late onset malignancy exists in AA patients independent of transplantation therapy. The risk of malignancy in the large registry of the EGBMT was equivalent for patients who received immunosuppression and for those who underwent transplantation. Overall, the rate of malignancy after stem cell transplantation has been calculated to be 3.8-fold higher than in the age-matched population.

Immunosuppression

Antithymocyte Globulins

Immunosuppressive therapy is an effective alternative treatment for patients who are not candidates for BM transplantation (see box on Treatment Algorithm in Aplastic Anemia).[1]

Immunoglobulin preparations made from the sera of horses immunized against human thymocytes are the mainstays of current regimens. Horse ATG is licensed for use in the United States as anti-thymocyte globulin (ATGAM) and severe AA is an approved indication. Thymoglobulin, a rabbit ATG, is more widely available worldwide.

The efficacy of antilymphocyte globulin (ALG) in BM failure was discovered serendipitously in the late 1960s, when Mathé observed recovery of autologous hematopoietic function in patients who received ALG as conditioning for BM transplantation. Observations and formal studies in Europe showed that 40% to 70% of patients responded with hematologic improvement and better survival. Similar results were also obtained in US randomized and multicenter protocols, in which about one-half of patients treated with ATG or ALG showed hematologic improvement, broadly defined as an end to transfusions dependence and a neutrophil level protective against infection.

The putative cause of AA is not a factor that predicts response. Virus- and drug-induced aplasia and posthepatitis AA respond similarly compared with idiopathic AA. Cytogenetic abnormalities do not preclude a response because AA with chromosomal abnormalities and some cases of frank myelodysplasia improve after ATG. The response rate is not increased by the addition of androgens or very high doses of corticosteroids.

A hematologic response to ATG is usually apparent within a few months of therapy; in some cases, all blood counts rise dramatically, and in others, increases in platelets or RBCs can be delayed (see box on Treatment Algorithm in Aplastic Anemia). Continued improvement without further therapy commonly occurs after 3 months; nevertheless, clinical status by 3 months is strongly correlated with long-term survival. Blood counts above the critical values for severity and platelets and reticulocyte counts of more than 50,000 cells/μL are highly prognostic. Reticulocyte count, perhaps reflecting the stem cell reserve, has been the best predictor of response and survival. With new and better tolerated antifungal drugs, survival even of nonresponders has improved and a very low neutrophil count may no longer have prognostic value.

ATG has three major toxic effects: immediate allergic phenomena, serum sickness, and transient blood cell count depression. Fever, rigors, and an urticarial cutaneous eruption are common on the first or second day of ATG therapy, and these symptoms respond to antihistamines and meperidine. Anaphylaxis is rare but can be fatal. Corticosteroids are administered in moderate doses (1 mg/kg of prednisone or methylprednisolone) during the first 2 weeks to ameliorate the symptoms of serum sickness. While rabbit ATG is more potent by weight than horse ATG, a randomized trial showed it markedly inferior to horse ATG in achieving hematologic response, and survival was poorer as well.[23]

ALGs are immunosuppressive. ATGs contain a heterogeneous mix of antibody specificities for lymphocytes. Horse sera fix human complement efficiently, and all preparations are T-cell cytotoxic in vitro, with little difference among ATGs or among lots for lymphocyte killing in vitro. In vitro ALGs efficiently inhibit T-cell proliferation and block IL-2 and IFN-γ production and IL-2 receptor expression. ATG induces Fas-mediated apoptosis of T cells, especially after activation. In patients the administration of ATG results in rapid reduction in the number of circulating lymphocytes, usually to less than 10% of starting values, and lymphopenia persists for several days after discontinuing therapy. Although lymphocyte numbers return to pretreatment values by 3 months, reductions in activated lymphocyte numbers in recovered patients persist. It seems likely that these inhibitory effects on T cells are responsible for the efficacy of ALGs in AA. Nevertheless, the ability of ATG to stimulate lymphocyte function by acting as a mitogen may also play a role in their therapeutic efficacy. Notably, rabbit ATG can stimulate T regulatory cell development in vitro and in patients, but this effect in AA is overwhelmed by rabbit ATG's more potent CD4 cell depletion in comparison to horse ATG.

Cyclosporine

Historical studies suggested efficacy of cyclosporine A (CSA) in patients refractory to ALG or ATG alone, with salvage rates of approximately 50%, but in current practice cyclosporine is combined with ATG as first-line therapy, or used to treat moderate AA, and after relapse. The optimal doses and regimen have not been determined. In the United States, CSA has usually been used in high doses (12 mg/kg/day for adults and 15 mg/kg/day for children), with adjustment according to plasma drug concentrations and serum creatinine levels. In Europe lower doses (3 to 7 mg/kg/day) have been reported as equally efficacious. Some experts advocate prolonged low dose therapy to prevent relapse after initial immunosuppression, to avoid a remitting-relapsing course and reintroduction of high dose therapy.

CSA has considerable toxicity. Hypertension and azotemia are the most common serious side effects; hirsutism and gingival hypertrophy are also frequent complaints. Increasing serum creatinine levels are an indication for dose reduction. Chronic CSA nephropathy characterized by interstitial fibrosis and tubular atrophy can be irreversible. The risk of nephropathy is increased by the use of high CSA doses and longer duration of therapy, and occurs more commonly in older than in younger patients.

Combined or Intensive Immunosuppressive Therapy

The combination for the treatment of AA of an agent that lyses lymphocytes (ATG) with a drug that blocks lymphocyte function is rational. The strategy has resulted in a striking increase in the response rate to immunosuppressive therapy in randomized and multicenter trials, to 60% to 80% at 1-year compared to about 40% with ATG alone. There are also more complete responses and better 5-year survival rates for responding patients at 80% to 90%. Children do especially well with combined therapy, elderly patients less so, partly because of greater toxicity and poor tolerance of pancytopenia-related complications in the presence of comorbidities.

In disease refractory, to initial therapy with ATG and CSA, a repeat course of rabbit ATG and CSA or alemtuzumab can be administered as a salvage regimen and can rescue about a third or more of patients.

Cyclophosphamide

Cyclophosphamide in high doses (45 to 50 mg/kg/day for 4 days) and "moderate" dose (50 mg/kg/day for 2 days) without stem cell rescue can induce hematologic recovery in severe AA, with an overall response rate claimed similar to that achieved with ATG therapy, but a higher proportion of complete responses and less relapse and clonal evolution. However, cyclophosphamide severely depresses the neutrophil count, resulting in prolonged hospitalization for suspected or actual infections and a higher risk of invasive fungal infections and death in comparison to ATG and CSA.

Corticosteroids

Methylprednisolone in modest doses (1 mg/kg/day) is administered with ATG to ameliorate the symptoms of serum sickness. Very high-dose corticosteroids regimens can be effective, especially in recently diagnosed patients. High-dose methylprednisolone has also been added to ATG therapy, with inconsistent results. However, ATG is associated with better response rates and many fewer associated toxic effects than is high-dose steroid therapy and is generally preferable as initial therapy. Corticosteroids do not have roles in the treatment of AA except in combination with ATG.

Late Complications of Immunosuppressive Therapy

Relapse after immunosuppressive therapy is common. About one-third or more of responding patients may be expected to require reinstitution of immunosuppressive drugs or another course of ATG. Relapse can manifest as a gradual decline in one blood count, need for transfusion after a period of transfusion-independence, or abrupt recurrence of severe pancytopenia. Most patients who relapse respond to retreatment, and there is no clear relationship with inferior survival.

A much more serious complication is the development of late-onset clonal hematologic disorders, especially myelodysplasia and AML. Some of these events likely represent part of the natural history of AA. Before recent improvements in treatment, leukemia was considered an unusual complication, but late-onset clonal disorders do not appear to be the result of the introduction of immunosuppressive therapy. In European and US reports, the overall rate of clonal evolution is 12% to 15% at about a decade. Children appear to be at similar risk for the development of clonal complications as adults, and evolution to MDS can occur in responders and in refractory patients. MDS that develops after AA can transform to AML but also can be surprisingly indolent. Cytogenetic analyses are almost always abnormal during clonal evolution and thus if cytogenetic analysis is not successful SNP array-based karyotyping or FISH study should be performed. Monosomy 7 is the most frequent finding and has a poor prognosis. Trisomy 8 and 6 and 13q− behave benignly in most cases. A PNH clone can be detected in up to 50% of patients at the time of presentation. Usually the clone size remains stable and it may decrease; over time, only a minority of patients will develop a large clone and the hemolytic or thrombotic form of PNH: the evolution rate to manifest hemolytic PNH is about 15% at 10 years. Preexisting PNH greater than 5% clone of granulocytes has been identified as risk factor of a frank PNH after AA. The presence of somatically mutated clones, and rapid telomere attrition have been associated with the development of MDS and AML after treatment of AA.

Immunosuppression Versus Bone Marrow Transplantation

Immunosuppression and transplantation are both effective therapies for AA (Fig. 31.8). Stem cell transplantation offers a permanent cure. Its disadvantages are cost, procedure-related morbidity and mortality (especially GVHD in older patients), and an increased incidence of solid organ malignancies. Immunosuppressive therapy is easier and initially cheaper. However, many patients do not achieve normal blood cell counts and remain at high risk for relapse, and the more serious complications of late-onset clonal hematologic disease, especially MDS.

Retrospective analyses of large number of European patients reported to the EGBMT show consistently improved results with both therapies, but a survival advantage for transplantation over immunosuppression has not been demonstrated. Certain categories of patients, defined by neutrophil number and age, probably benefit from one therapy or the other. In general, stem cell transplantation yields superior results in children, immunosuppression in older adults

Remarkable improvements in transplant results using unrelated donors have made this approach available to many patients who lack a sibling donor. Increasingly, children who have failed a single course of immunosuppression and adults refractory to multiple courses of ATG are offered this procedure. In adults, similar survival is achieved with unrelated as with related donors, with some increase in transplant-complications. Haploidentical transplant is

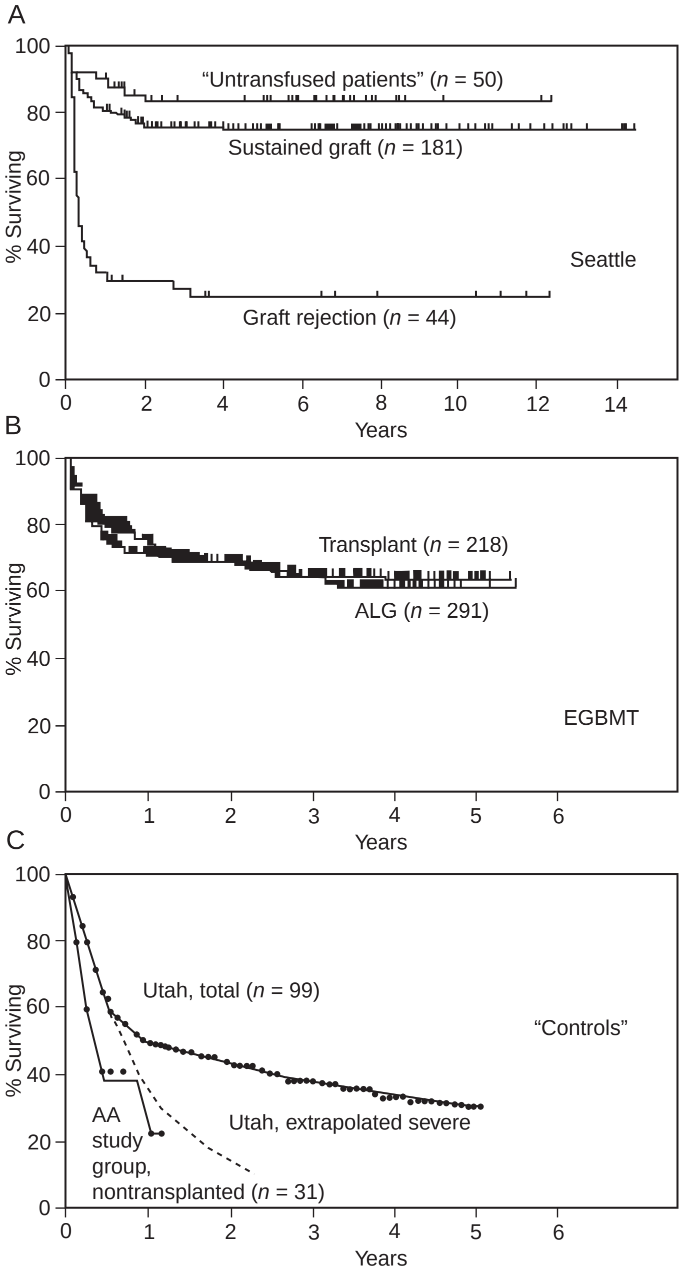


Figure 31.8 ACTUARIAL SURVIVAL RATES FOR PATIENTS WITH APLASTIC ANEMIA. (A) Data on bone marrow transplantation from the University of Washington. (B) Data from the EGBMT on bone marrow transplantation versus immunosuppression with ALG. (C) Natural history as indicated by survival with supportive and other treatments. Two groups are illustrated. Extrapolated survival curves for patients with severe disease are derived from retrospective reviews from the University of Utah of 101 records collected from the late 1940s to early 1970s. The patients received blood transfusions and, later in this period, also received platelets. Almost all were treated with corticosteroids, and one-half were also treated with androgens. Data for patients who did not receive transplants come from a multicenter study of the efficacy of marrow transplantation performed in the early 1970s; this control group was treated with androgens. *AA*, Aplastic anemia; *ALG*, antilymphocyte globulin; *EGBMT*, European Group for Bone Marrow Transplantation.

a preferred option at many Chinese centers and ongoing studies will define its role in Europe and the United States. For adults, a single round of immunosuppression is advised, with early alternative donor transplant if the patient deteriorates or fails to improve blood counts at three months.

Hematopoietic Growth Factors

Hematopoietic growth factor production is normal or increased in AA. Nonetheless patients are treated with pharmacologic high doses of cytokines, often with uncertain justification. G-CSF and GM-CSF in some cases can increase neutrophil numbers in AA. In general, neutrophil responses to growth factors are transient, dependent on their continuous administration, and usually restricted to patients with less severe AA. Only occasional bilineage and trilineage responses have been observed.

A concern has been the possibility that prolonged administration of G-CSF might increase the probability of late clonal disease, especially monosomy 7. In retrospective analyses of Japanese children and adults with severe AA, this syndrome appeared to occur most frequently among patients who had received growth factor; a modest increase in the risk of myelodysplasia and leukemia and monosomy 7 was also seen in European cases.

Anemia and pancytopenia in rare patients have responded to prolonged administration of high doses of erythropoietin alone, GM-CSF and erythropoietin, and with IL-3 and G-CSF. In one randomized protocol, the combination of G-CSF and high doses of erythropoietin improved hemoglobin values, mainly in patients with moderate disease.

Growth factors have also been combined with definitive medical therapy to improve neutrophil counts during the early phase of immunosuppression. Neither small pilot trials nor large randomized studies have shown substantial benefit for the routine use of GM-CSF or G-CSF in preventing infection, improving the response rate to immunosuppression, or in survival. A brief therapeutic trial of G-CSF or GM-CSF is often used in severely neutropenic patients who are persistently or seriously infected in the hope of clinical benefit. Growth factors, alone or in combination, are rarely effective in refractory patients.

Eltrombopag

Thrombopoietin mimetics are an exception to the generally unfavorable profile of hematopoietic growth factors in AA. In an NIH protocol, the oral c-mpl agonist eltrombopag increased not only platelet counts but led to robust erythrocyte and granulocyte improvement in almost half of the patients with chronic refractory severe AA, allowing them to discontinue transfusion support. Responses were durable and did not require chronic drug administration. However, about 15% of patients progressed to 7- or complex cytogenetics, temporally related to eltrombopag, necessitating stem cell transplant. A prospective single arm trial at NIH combined standard immunosuppression with eltrombopag: the overall response rate was about 80%, and about half of responses were associated with normal or near normal blood counts, superior to historic controls (65% and 15%, respectively). Both NIH protocols resulted in Food and Drug Administration (FDA) approval, of eltrombopag monotherapy in refractory AA and the combination for treatment-naïve AA. To date, the rate of clonal evolution to poor prognosis cytogenetic abnormalities and MDS/AML does not appear to be elevated by the addition of eltrombopag.

The benefit of eltrombopag in combination with immunosuppression has been confirmed, using complete response as the primary endpoint, in a randomized European trial. Eltrombopag and the similar romiplostim mimetic have shown favorable results in studies in Asia as well. Benefits of monotherapy and combined therapy in children is uncertain. Eltrombopag treatment improved blood counts in a prospective trial in moderate AA, without any patients showing evidence of evolution.

The mpl receptor is expressed by hematopoietic stem cells, and laboratory and clinical data indicate physiologic stimulation of stem cells by thrombopoietin. Mimetics are efficient iron chelators, and they also may beneficially increase regulatory T cells.

Androgens

Testosterone and synthetic anabolic steroids were introduced into hematology in the 1960s. The high response rates in some early series

THERAPY FOR APLASTIC ANEMIA

After the diagnosis of aplastic anemia (AA) has been established, treatment options must be identified, considered carefully, and chosen with alacrity (see box on Treatment Algorithm in Aplastic Anemia). For patients with moderate disease, an expectant approach can be chosen based on a stable course and adequate blood counts, or for patients dependent on transfusion support, horse antithymocyte globulin (ATG; 40 mg/kg/day for 4 days) can be given. After assessment of the response, patients who improve should be monitored for hematologic signs of relapse, and nonresponders can be offered alternative therapy, such as androgens or cyclosporine. In severe disease for which the prognosis with blood transfusion and antibiotic support alone is poor, HSCT from a histocompatible sibling or immunosuppression are accepted and effective therapies. Although large, retrospective analyses have shown that long-term survival rates from transplantation or immune therapy are equivalent, each has its own advantages and disadvantages. For children, stem cell transplantation remains the treatment of choice if an appropriate family donor is available, because these patients have a low rate of graft-versus-host disease (GVHD), and the BM disease is cured by stem cell replacement. The risk of therapy-related cancers can be increased, especially in children, after BM transplantation, and is ameliorated by avoiding radiation in the conditioning regimen. Adults with AA also successfully receive transplants, although the risk and severity of chronic GVHD and other treatment-related complications increase with age. Stem cell transplantation has been performed in patients older than 50 years of age, and it is a reasonable approach in a younger adult, less than 40 years old, especially with more severe degrees of neutropenia. Most studies show that transplant early in the course of disease, within 6 months of diagnosis, is desirable.

When transplantation is not performed immunosuppression is the treatment of choice. Patients with severe disease should receive a combination of ATG and cyclosporine. We recommend a regimen consisting of 40 mg/kg/dayof horse ATG on days 1–4, followed by cyclosporine for 6 months at a dose of 12 mg/kg and elthrombopag (now approved in the first line setting, at 150 mg/day po). Corticosteroids are added in moderate doses (1 mg/kg of prednisone or methylprednisolone) during the first 2 weeks to ameliorate serum sickness. Improvement should be expected within 6 months. This regimen has produced hematologic responses in approximately 80% of treated patients, who then have an excellent 5-year survival rate. Of note is that addition of eltrombopag in primary setting in pediatric patients did not result in improved response rate.

Although immunosuppressive therapy is generally well tolerated, patients frequently relapse and require further treatment; however, relapse is not associated with a poor prognosis. More serious is the development of late-onset hematologic clonal diseases, paroxysmal nocturnal hemoglobinuria (which may not be clinically significant), myelodysplastic syndrome, and acute myeloid leukemia.

For patients in whom immunosuppressive therapy fails, there are a number of options. For children, alternative donor stem cell transplantation should be considered early; at the best transplant centers, survival rates now are almost as good as with sibling donors. In children and adults, well-matched unrelated donor stem cells provide similar long-term survival as do matched sibling donors, but the rate of chronic and severe chronic GVHD is higher. Patients with AA and telomere disease can respond to androgen therapy. Repeated immunosuppression in a patient in whom a first course of ATG and cyclosporine has failed is successful in about 30% of cases. Eltrombopag is approved for use in patients with refractory AA but approval for front-line therapy may limit availability of this option as most of the patients will have received eltrombopag upfront.

Patients with severe AA should not be subjected to useless early trials of corticosteroids or hematopoietic growth factors as the primary treatment. For the occasional patient who must decide between stem cell transplantation and immunosuppression, the advice of experts familiar with this disease and careful counseling of the patient are advisable. Age and severity of neutropenia are decisive factors. A limited number of transfusions may be necessary to optimize the patient's condition before definitive therapy and are acceptable. Single-donor platelets should be given and can be obtained from HLA-compatible donors. In severely neutropenic patients, granulocyte colony-stimulating factor therapy can decrease the risk of life-threatening infections. In the absence of overt bleeding, platelet counts as low as 5000 cells/μL appear to be safe.

may be retrospectively attributed to the inclusion of patients with moderate acquired and constitutional AA. For severe AA, controlled trials in general have not demonstrated efficacy, as measured by survival rates or hematologic improvement. When added to immunosuppressive therapies, androgens failed to result in any increase in response rates.

Androgens may be helpful in occasional patients when used as a second-line therapy. Most hematologists have observed patients who appeared to respond or even to develop hormone dependence. Androgen therapy remains popular in developing countries because it is inexpensive, well tolerated, and seemingly effective. Various preparations of androgens at different doses have resulted in similar response rates of 35% to 60% after 6 months of therapy. Sex hormones increase telomerase gene transcription, and danazol has been effective in marrow failure due to telomeropathy.[24]

Useful androgens include nandrolone decanoate, oxymetholone, and danazol. The hemoglobin response is frequently more impressive than improvements in granulocyte or platelet levels. An adequate trial is considered to be a full dose given for at least 3 months. Complications occur infrequently, although some are serious and can limit effective therapy, especially in the elderly. The associated liver cholestasis is usually reversible. Hepatotoxicity (e.g., bile duct proliferation, peliosis, atypical hepatocyte hyperplasia, tumors) are less common with parenteral formulations. Children appear to tolerate high doses of androgens without lasting effects on growth or maturation.

PROGNOSIS

The initial blood cell counts of a patient with AA are important indicators of prognosis. The popular "Camitta" criteria used to define severe disease are the presence of two of the following three: neutrophil count of less than 500 cells/μL, platelet count of less than 20,000 cells/μL, and corrected reticulocyte level of less than 1% (<40,000 cells/μL). In the more modern era absolute reticulocytes (>25,000/μL) and absolute lymphocytosis predicted a good response to immunosuppression and survival. The robustness of the platelet and reticulocyte response after immunosuppression also correlates with long-term survival. Clonal evolution, especially −7del(7q), is a poor prognostic factor, and age-adjusted telomere length of leukocytes at diagnosis may predict this serious complication.[25]

The rate of spontaneous recovery is difficult to estimate, but most observers believe it to be low. Untreated severe disease is almost invariably fatal. In contrast, moderate AA has a good prognosis, and some patients with minimal blood cell count depression recover normal blood cell counts with limited or no therapy.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Adkins DR, Goodnough LT, Shenoy S, et al. Effect of leukocyte compatibility on neutrophil increment after transfusion of granulocyte colony-stimulating factor-mobilized prophylactic granulocyte transfusions and on clinical outcomes after stem cell transplantation. *Blood*. 2000;95:3605.

Bacigalupo A. How I treat acquired aplastic anemia. *Blood*. 2017;129:1428–1436.

Bacigalupo A. Alternative donor transplants for severe aplastic anemia. *Hematology Am Soc Hematol Educ Program*. 2018(1):467–473.

Bacigalupo A, Socie G, Hamladji RM, et al. Current outcome of HLA identical sibling vs. unrelated donor transplants in severe aplastic anemia: an EBMT analysis. *H aematologica*. 2015;100(5):696–702.

Bono E, McLornan D, Travaglino E, et al. Clinical, histopathological and molecular characterization of hypoplastic myelodysplastic syndrome. *Leukemia*. 2019;33(10):2495–2505.

Champlin RE, Perez WS, Passweg JR, et al. Bone marrow transplantation for severe aplastic anemia: a randomized controlled study of conditioning regimens. *Blood*. 2007;109(10):4582–4585.

Chen J, Lipovsky K, Ellison FM, et al. Bystander destruction of hematopoietic progenitor and stem cells in a mouse model of infusion-induced bone marrow failure. *Blood*. 2004;104:1671.

Clay J, Kulasekararaj AG, Potter V, et al. Nonmyeloablative peripheral blood haploidentical stem cell transplantation for refractory severe aplastic anemia. *Biol Blood Marrow Transplant*. 2014;20(11):1711–1716.

Cooper J, Young NS. Clonality in context: hematopoietic clones in their marrow environment. *Blood*. 2017;130(22):2363–2372.

Deeg HJ, Leisenring W, Storb R, et al. Long-term outcome after marrow transplantation for severe aplastic anemia. *Blood*. 1998;91:3637.

Desmond R, Townsley DM, Dumitriu B, et al. Eltrombopag restores trilineage hematopoiesis in refractory severe aplastic anemia that can be sustained on discontinuation of drug. *Blood*. 2014;123:1818–1825.

DeZern AE, Zahurak M, Symons H, et al. Alternative donor transplantation with high-dose post-transplantation cyclophosphamide for refractory severe aplastic anemia. *Biol Blood Marrow Transplant*. 2017;23(3):498–504.

DeZern AE, Zahurak ML, Symons HJ, et al. Haploidentical BMT for severe aplastic anemia with intensive GVHD prophylaxis including posttransplant cyclophosphamide. *Blood Adv*. 2020;4(8):1770–1779.

Dufour C, Pillon M, Socié G, et al. Outcome of aplastic anaemia in children. A study by the severe aplastic anaemia and paediatric disease working parties of the European group blood and bone marrow transplant. *Br J Haematol*. 2015;169(4):565–573.

Dufour C, Veys P, Carraro E, et al. Similar outcome of upfront-unrelated and matched sibling stem cell transplantation in idiopathic paediatric aplastic anaemia. A study on behalf of the UK Paediatric BMT Working Party, Paediatric Diseases Working Party and Severe Aplastic Anaemia Working Party of EBMT. *Br J Haematol*. 2015;171(4):585–594.

Dumitriu B, Feng X, Townsley DM, et al. Telomere attrition and candidate gene mutations preceding monosomy 7 in aplastic anemia. *Blood*. 2015;125:706–709.

Esteves I, Bonfim C, Pasquini R, et al. Haploidentical BMT and post-transplant Cy for severe aplastic anemia: a multicenter retrospective study. *Bone Marrow Transplant*. 2015;50(5):685–689.

Giudice V, Cardamone C, Triggiani M, Selleri C. Bone marrow failure syndromes, overlapping diseases with a common cytokine signature. *Int J Mol Sci*. 2021;22(2):705.

Heckman KD, Weiner GJ, Davis CS, et al. Randomized study of prophylactic platelet transfusion threshold during induction therapy for adult acute leukemia: 10,000/microL versus 20,000/microL. *J Clin Oncol*. 1997;15:1143.

Herbrecht R, Denning DW, Patterson TF, et al. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. *N Engl J Med*. 2002;347:408.

Hughes WT, Armstrong D, Bodey GP, et al. Guidelines for the use of antimicrobial agents in neutropenic patients with cancer. *Clin Infect Dis*. 2002;34(730):2002.

Iftikhar R, Chaudhry QUN, Anwer F, et al. Allogeneic hematopoietic stem cell transplantation in aplastic anemia: current indications and transplant strategies. *Blood Rev*. 2020;100772

International Agranulocytosis and Aplastic Anemia Study. Risks of agranulocytosis and aplastic anemia: a first report of their relation to drug use with special reference to analgesics. *JAMA*. 1986;256:1749.

Kallen ME, Dulau-Florea A, Wang W, Calvo KR. Acquired and germline predisposition to bone marrow failure: diagnostic features and clinical implications. *Semin Hematol*. 2019;56(1):69–82.

Koh KN, Im HJ, Kim BE, et al. Haploidentical haematopoietic stem cell transplantation using CD3 or CD3/CD19 depletion and conditioning with fludarabine, cyclophosphamide and antithymocyte globulin for acquired severe aplastic anaemia. *Br J Haematol*. 2012;157(1):139–142.

Kojima S, Matsuyama T, Kato S, et al. Outcome of 154 patients with severe aplastic anemia who received transplants from unrelated donors: the Japan Marrow Donor Program. *Blood*. 2002;100:799.

Lu Y, Sun RJ, Zhao YL, et al. Unmanipulated haploidentical hematopoietic stem cell transplantation achieved outcomes comparable with matched unrelated donor transplantation in young acquired severe aplastic anemia. *Biol Blood Marrow Transplant*. 2018;24(9):1881–1887.

Marsh J, Pearce R, Koh M, et al. Retrospective study of alemtuzumab vs ATG-based conditioning without irradiation for unrelated and matched sibling donor transplants in acquired severe aplastic anemia: a study from the British Society for Blood and Marrow Transplantation. *Bone Marrow Transplant*. 2014;49(1):42–48.

Marsh J, Schrezenmeier H, Marin P, et al. Prospective randomized multicenter study comparing cyclosporin alone versus the combination of antithymocyte globulin and cyclosporin for treatment of patients with nonsevere aplastic anemia: a report from the European Blood and Marrow Transplant (EBMT) Severe Aplastic Anaemia Working Party. *Blood*. 1999;93:2191.

Morgan GJ, Alveres CL. Benzene and the hemopoietic stem cell. *Chem Biol Interact*. 2005;30:153.

Mustjoki S, Young NS. Somatic mutations in "benign" disease. *N Engl J Med*. 2021;384(21):2039–2052.

Nazha A, Seastone D, Radivoyevitch T, et al. Genomic patterns associated with hypoplastic compared to hyperplastic myelodysplastic syndromes. *Haematologica*. 2015;100(11):e434–e437. 1.

Rosenfeld SJ, Follman D, Nunez O, et al. Antithymocyte globulin and cyclosporine for severe aplastic anemia. Association between hematologic response and long-term outcome. *JAMA*. 2003;289:1130.

Shimamura A, Alter BP. Pathophysiology and management of inherited bone marrow failure syndromes. *Blood Rev*. 2010;24(3):101–122.

Socie G, Stone JV, Wingard JR, et al. Long-term survival and late deaths after allogeneic bone marrow transplantation. Late effects working committee of the international bone marrow transplant registry. *N Engl J Med*. 1999;341:14.

Stanley N, Olson TS, Babushok DV. Recent advances in understanding clonal haematopoiesis in aplastic anaemia. *Br J Haematol*. 2017;177(4):509–525.

Tichelli A, Socie G, Marsh J, et al. Outcome of pregnancy and disease course among women with aplastic anemia treated with immunosuppression. *Ann Intern Med*. 2002;137:164.

Tisdale JF, Dunn DE, Geller N, et al. High-dose cyclophosphamide in severe aplastic anaemia: a randomised trial. *Lancet*. 2000;356:1554.

Townsley D, Dumitriu B, Liu D, et al. Danazol treatment for telomere diseases. *N. Engl. J. Med*. 2016;374(20):1922–1931.

Townsley DM, Sheinberg P, Winkler T, et al. Eltrombopag added to standard immunosuppression for aplastic anemia. *N Engl J Med*. 2017;376(16):1540–1550.

Wegman-Ostrosky T, Savage SA. The genomics of inherited bone marrow failure: from mechanism to the clinic. *Br J Haematol*. 2017;177(4):526–542.

Winkler T, Fan X, Cooper J, et al. Treatment optimization and genomic outcomes in refractory severe aplastic anemia treated with eltrombopag. *Blood*. 2019;133(24):2575–2585.

Xu LP, Jin S, Wang SQ, et al. Upfront haploidentical transplant for acquired severe aplastic anemia: registry-based comparison with matched related transplant. *J Hematol Oncol*. 2017;10(1).

Xu LP, Wang SQ, Wu DP, et al. Haplo-identical transplantation for acquired severe aplastic anaemia in a multicentre prospective study. *Br J Haematol*. 2016;175(2):265–274.

Xu LP, Zhang XH, Wang FR, et al. Haploidentical transplantation for pediatric patients with acquired severe aplastic anemia. *Bone Marrow Transplant*. 2017;52(3):381–387.

Xu ZL, Huang XJ. Haploidentical stem cell transplantation for aplastic anemia: the current advances and future challenges. *Blood Marrow Transplant*. 2021;56(4):779–785.

Ye L, Zhang F, Kojima S. Current insights into the treatments of severe aplastic anemia in China. *Int J Haematol*. 2020;112(3):292–299.

Yoshizato T, Dumitriu B, Hosokawa K, et al. Somatic mutations and clonal hematopoiesis in aplastic anemia. *N Engl J Med*. 2015;373:35–47.

Young NS. Aplastic Anemia. *N Engl J Med*. 2018;379(17):1643–1656.

Young NS, Calado R, Scheinberg P. Current concepts in the pathophysiology and treatment of aplastic anemia. *Blood*. 2006;108:2511.

REFERENCES

1. Young NS. Aplastic anemia. *N Engl J Med*. 2018;379(17):1643–1656.
2. Kaufman DW, Kelly JP, Levy M, et al. *The drug etiology of agranulocytosis and aplastic anemia*. New York: Oxford; 1991.
3. Issaragrisil S, Kaufman D, Anderson T, et al. The epidemiology of aplastic anemia in Thailand. *Blood*. 2006;107:1299–1307.
4. Townsley DM, Dumitriu B, Young NS. Bone marrow failure and the telomeropathies. *Blood*. 2014;124:2775–2783.
5. Dokal I, Vulliamy T. Inherited aplastic anaemias/bone marrow failure syndromes. *Blood Rev*. 2008;40:719–722.
6. Maciejewski JP, Selleri C, Tadatsugu S, et al. A severe and consistent deficit in marrow and circulating primitive hematopoietic cells (long-term culture-initiating cells) in acquired aplastic anemia. *Blood*. 1996;88:1983–1991.
7. Scheinberg P, Chen J. Aplastic anemia: what have we learned from animal models and from the clinic. *Semin Hematol*. 2013;50:156–164.
8. Kopolovic I, Ostro J, Tsubota H, et al. A systematic review of transfusion-associated graft-versus-host disease. *Blood*. 2015;126:406–414.
9. Brown KE, Tisdale J, Dunbar CE, et al. Hepatitis-associated aplastic anemia. *N Engl J Med*. 1997;336:1059–1064.
10. Afable MG, Wlodarski M, Makishima H, et al. SNP array-based karyotyping: differences and similarities between aplastic anemia and hypocellular myelodysplastic syndromes. *Blood*. 2011;117:6876–6884.
11. Yoshizato T, Dumitriu B, Hosokawa K, et al. Somatic mutations and clonal hematopoiesis in aplastic anemia. *N Engl J Med*. 2014;373:35–47.
12. Valdez JM, Scheinberg P, Nunez O, et al. Decreased infection-related mortality and improved survival in severe aplastic anemia in the past two decades. *Clin Infect Dis*. 2011;52:726–735.
13. Leitman SF, Oblitas JM, Emmons R, et al. Clinical efficacy of daily G-CSF-recruited granulocyte transfusions in patients with severe neutropenia and life-threatening infections [abstract]. *Blood*. 1996;88(Suppl 1):331a.
14. Miano M, Dufour C. The diagnosis and treatment of aplastic anemia: a review. *Int J Hematol*. 2015;101:527–535.
15. Bacigalupo A. Bone marrow transplantation for acquired severe aplastic anemia. *Hematol Oncol Clin North Am*. 2014;28:1145–1155.
16. Schrezenmeier H, Passweg JR, Marsh JCW, et al. Worse outcome and more chronic GVHD with peripheral blood progenitor cells than bone marrow in HLA-matched sibling donor transplants for young patients with severe aplastic anemia. *Blood*. 2007;110:1397–1400.
17. Locasciulli A, Oneto R, Bacigalupo A, et al. Outcome of patients with acquired aplastic anemia given first line bone marrow transplantation or immunosuppressive treatment in the last decade: a report from the European Group for Blood and Marrow Transplantation. *Haematologica*. 2007;92:11–18.
18. Bacigalupo A, Socie G, Hamladji RM, et al. Current outcome of HLA identical sibling vs. unrelated donor transplants in severe aplastic anemia: an EBMT analysis. *Haematologica*. 2015;100:696–702.
19. Horowitz MM. Current status of allogeneic bone marrow transplantation in acquired aplastic anemia. *Semin Hematol*. 2000;37:30–42.
20. Passweg JR, Marsh JC. Aplastic anemia: first-line treatment by immunosuppression and sibling marrow transplantation. *Hematol Am Soc Hematol Educ Program*. 2010;2010:36–42.
21. Aderka D, Praff G, Santo M, et al. Bleeding due to thrombocytopenia in acute leukemias and reevaluation of the prophylactic platelet transfusion policy. *Am J Med Sci*. 1986;291:147–151.
22. de Peffault LR, Purtill D, Ruggeri A, et al. Influence of nucleated cell dose on overall survival of unrelated cord blood transplantation for patients with severe acquired aplastic anemia: a study by eurocord and the aplastic anemia working party of the European group for blood and marrow transplantation. *Biol Blood Marrow Transpl*. 2011;17:78–85.
23. Scheinberg P, Nunez O, Weinstein B, et al. Horse versus rabbit antithymocyte globulin in acquired aplastic anemia. *N Engl J Med*. 2011;365:430–438.
24. Townsley DM, Bogdan D, Delong L, et al. Danazol treatment for telomere diseases. *N Engl J Med*. 2016;374:1922–1931.
25. Scheinberg P, Cooper JN, Sloand EM, et al. Association of telomere length of peripheral blood leukocytes predicts hematopoietic relapse, malignant transformation, and survival in severe aplastic anemia. *JAMA*. 2010;304:1358–1364.

CHAPTER 32

PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

David J. Araten and Robert A. Brodsky

INTRODUCTION AND HISTORY

Paroxysmal nocturnal hemoglobinuria (PNH) is an opportunity to expound upon three major topics: hemolytic anemia, autoimmune-mediated bone marrow failure, and thrombosis. For the physician explaining a new diagnosis to the patient, PNH is now a cause for cautious optimism, as we have seen a tremendous improvement in the outcome over the past decades. Indeed, we can now alleviate symptoms and prevent complications in almost all patients, and life expectancy of a newly diagnosed patient is now close to that of age-matched controls. The complement-mediated hemolysis in PNH has inspired the development of a new class of drugs that target complement proteins (see Chapter 23), and C5 blockade has been shown to not only ameliorate hemolysis but also reduce the risk of thrombosis in PNH. A novel C3 inhibitor was approved recently for PNH and may be particularly useful for those with persistent hemolytic anemia despite C5 inhibition. Anti-complement therapy has also revolutionized the treatment of another hematologic disorder (atypical hemolytic anemic syndrome, aHUS) (see Chapter 132), as well as two neurologic disorders, with more drugs and additional indications likely to come.

In considering the earliest descriptions of patients who might have had PNH,[1] we must keep in mind that most patients throughout history with dark urine, for sure, have not had this rare disorder. However, William Gull reported on a patient in 1866 with an intermittent bloody-appearing urine that did not contain red cells, with the color of the morning samples more pronounced than later in the day[2]—possibly the first reported case. Paul Strübing in 1882 described a young man with urine that appeared black or dark red (Fig. 32.1A), with associated fatigue and abdominal pain.[3] He made the critical observation that the attacks were not precipitated by exposure to cold, differentiating this from paroxysmal cold hemoglobinuria (PCH), which, due to its association with untreated syphilis, was then much more common. Strübing deduced that the hemolysis was intravascular, as the patient's plasma turned red after severe attacks (see Fig. 32.1B). Van den Bergh[4] later characterized the disorder in a patient, having ruled out PCH by the Donath Landsteiner test, and demonstrated that red cells under conditions of acidification with CO_2 were lysed by autologous and ABO blood type-matched donor serum. Unlike antibody-mediated hemolysis, hemolysis in vitro could be abrogated by heat treatment of the serum, which was known to inactivate complement. Soon after, it was determined that urine hemosiderin staining could be seen between attacks, and we now know that in spite of the paroxysms that give the disease its name, hemolysis is, in fact, an ongoing process. The disorder was briefly named for the authors Marchiafava and Micheli who recognized this point[5,6] and was eventually termed paroxysmal nocturnal hemoglobinuria by Enneking in 1928.[7]

A major breakthrough occurred in the late 1930s with the development of the Ham test, which provides an estimate of the percentage of red cells that are susceptible to lysis by activation of the alternative pathway, after the acidification of serum in conditions of optimized magnesium concentration.[8–11] Of note, there are always some red cells that will not lyse in the Ham test even upon repeat exposure to acidified serum, and the severity of the condition roughly correlated with the percentage of cells that were susceptible. Rosse and Dacie in the 1960s delineated these two populations and showed that the abnormal cells were exquisitely sensitive to low levels of human complement[12,13] in an assay that activated the classical pathway. In 1970, Oni, Osunkoya, and Luzzatto made the critical observation, using G6PD allozymes to infer X-inactivation patterns, that the abnormal red cells in PNH belong to a clone derived from a single cell.[14]

By that time, it had already been recognized that there was a cellular defect in acetylcholinesterase in PNH erythrocytes and alkaline phosphatase in neutrophils,[15–17] but the lack of these particular enzymes on a clonal population did not yet explain complement sensitivity. That came with the discovery of a regulator of early complement proteins, CD55[18,19] (decay accelerating factor)—and the observation by Anne Nicholson-Weller and her colleagues that this protein is missing on PNH red cells and leucocytes.[20–23] It was soon appreciated that there is a second regulator, membrane inhibitor of reactive lysis (MIRL, CD59), which inhibits the late complement proteins that can form a membrane attack complex, and it was found that like CD55, CD59 is also missing on blood cells in PNH (Fig. 32.2).[24–29]

The missing membrane proteins (Table 32.1) have in common the glycosylphosphatidylinositol (GPI) anchor, which is covalently bound to the carboxy terminus of certain proteins and tethers them to the membrane.[30,31] Taroh Kinoshita's group and colleagues in Osaka first showed in the early 1990s that PNH cells are defective in the first step in the biosynthesis of GPI[32] and then went on to isolate the gene responsible for this step by complementation cloning in cell lines. They named this gene *PIGA* (phosphatidylinositol glycan A), and demonstrated that there is an acquired somatic mutation in this gene in all patients with PNH they studied.[33] While there are over 20 genes necessary for the production and trafficking of GPI (Table 32.2), it was understood that *PIGA* was the only gene found to be mutated in PNH because it maps to the X-chromosome, whereas all the other genes in the pathway are autosomal. Thus, the clonal GPI-negative population would be much more likely to be produced by a single event on the X-chromosome, rather than two separate events involving any of the other genes in the pathway. Identifying the membrane defect in PNH led to a simple flow cytometry test performed on peripheral blood (see Fig. 32.2), which by the 1990s was replacing the Ham test.[34] The identification of abnormal leucocyte populations with the same surface defect as red cells and harboring the *PIGA* mutation confirmed that PNH is indeed not just a red cell disease, but actually is a stem cell disorder.

The link between the mutation, GPI-anchor synthesis defect, and loss of complement inhibition now closed the circle on the most prominent clinical phenotype—hemolytic anemia. But this failed to explain the paradox of why this stem cell clone—whose mature red cell progeny, at least, seem to be weakened—would have a growth advantage. To explain this, in 1989 Rotoli and Luzzatto[35] considered the critical earlier observation of Dacie and Lewis[36] that PNH, a rare disease, is highly associated with another rare and seemingly very different hematologic disorder—immune-mediated aplastic anemia (see Chapter 31) (Fig. 32.3). This had been commented upon by Dameshek in 1967 and then revisited by Young in 1992.[37,38] The working hypothesis for the past 32 years has thus been the "immune escape model," whereby the PNH stem cell has a selective growth advantage in an aplastic marrow environment, because it is less sensitive to immune attack than normal stem cells. This model has not been proven, but it explains many key features of PNH and there is experimental evidence supporting it.

The other major piece of the puzzle has been to understand the pro-thrombotic tendency in PNH, noted 70 years ago by Crosby to be a frequent cause of death, often involving unusual sites such as the

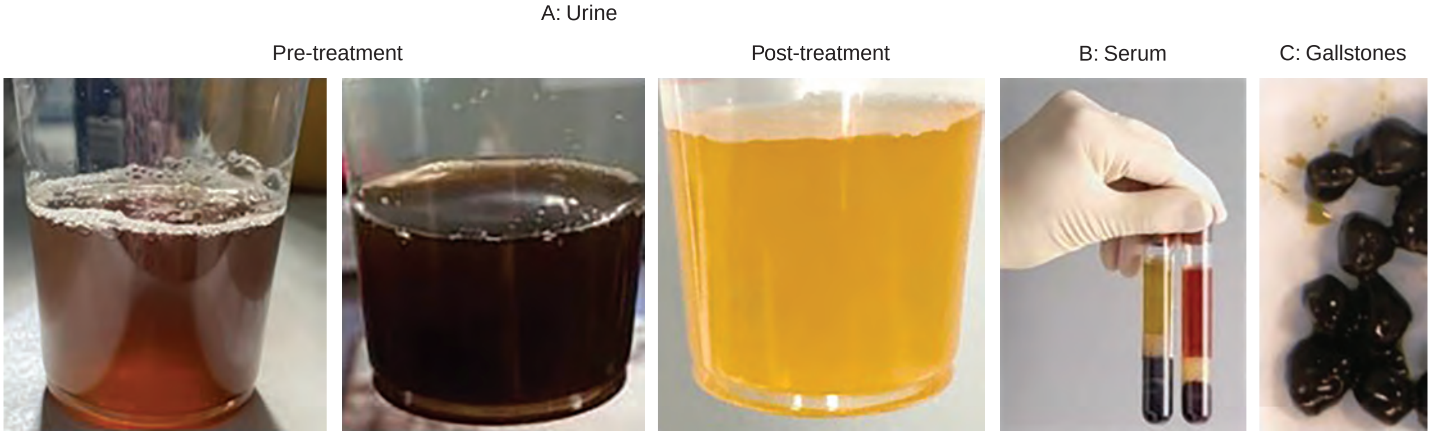


Figure 32.1 (A) Two urine samples from a patient with PNH with a large PNH clone, taken several days apart, prior to treatment. The urine color then normalized soon after starting ravulizumab. Note that since the darkening of the urine is "paroxysmal," the normalization of the urine color is not sufficient to demonstrate an effect of any therapy– but rather the response is most rapidly demonstrated by the decrease in the lactate dehydrogenase (LDH). (B) Blood samples in a serum separator tube from a patient with PNH compared with the normal donor. Note the red color of the serum above the separator plug and the decreased size of the clotted red cells below the plug, consistent with anemia and intravascular hemolysis. (C) Symptomatic bilirubin gallstones from a cholecystectomy specimen in a patient with PNH on long-term treatment with a C5 inhibitor. Stone formation is a consequence of chronic extravascular hemolysis of various etiologies and is seen in some patients on eculizumab or ravulizumab. This complication was not seen historically in untreated patients with PNH in whom the hemolysis is largely intravascular. The reason for this is that in intravascular hemolysis, free hemoglobin is largely excreted in the urine and is not broken down to bilirubin, whereas for extravascular hemolysis, hemoglobin is broken down in the cells of the reticuloendothelial system, resulting in hyperbilirubinemia. Likewise, patients with PNH who are treated with C5 inhibition can develop iron overload, as seen in other forms of extravascular hemolysis, but this is not typically seen in intravascular hemolysis where iron is lost in the urine.

TABLE 32.1 **Glycosylphosphatidylinositol-Anchored Surface Proteins Absent on Paroxysmal Nocturnal Hemoglobinuria Blood Cells**

Antigen	Hematopoietic Lineage	Classification
CD55: decay accelerating factor	All blood cells	Complement regulator
CD59: membrane inhibitor of reactive lysis	All blood cells	Complement regulator
CD58: lymphocyte function associated antigen-3	All blood cells	Adhesion molecule
Acetylcholinesterase	Red blood cells	Enzyme
CD14: monocyte differentiation antigen	Granulocytes, monocytes, macrophages	Endotoxin-binding receptor
CD16: Fcγ receptor III	Granulocytes, NK cells	Receptor
CD66b	Granulocytes	Adhesion
Neutrophil alkaline phosphatase	Granulocytes	Enzyme
CD87: urokinase (plasminogen activator) receptor	Monocytes, granulocytes	Receptor
Leukocyte alkaline phosphatase	Granulocytes	Enzyme
CD52: Campath-1 antigen	Lymphocytes, monocytes	Small peptide of unknown function, target of Campath
CD24	B lymphocytes, granulocytes	B-cell differentiation
CD48	All leukocytes	Adhesion molecule
CD73: ecto-5′-nucleotidase	Some B and T lymphocytes	Enzyme
Dombrock-Holley/Gregory-bearing protein	Red blood cells	Blood group antigen
Folate receptor	Myeloid and erythroid cells	Receptor
CD109	Activated platelets and T cells	Unknown
CD157	Mature monocytes	Adhesion and transmigration of monocytes

cerebral dural venous sinuses and intra-abdominal veins (Fig. 32.4).[39] While there are a few proposed mechanisms for this, the group in the United Kingdom made two major breakthroughs. First, using historical controls, they demonstrated that thrombotic complications in PNH could be largely prevented by primary prophylaxis with warfarin.[40] Then, as reported by Hillmen et al.,[41] they pioneered in 11 patients the use of eculizumab, a monoclonal antibody targeting the C5 complement protein (see review by Rother et al.[42] and Chapter 23). This led to the first randomized trial in PNH (the TRIUMPH Study), and the drug's approval in 2007[43] as a treatment for hemolysis in PNH, for which it can be highly effective. Most importantly, it was subsequently shown that eculizumab reduces thrombotic risk by more than an order of magnitude[44]—from 14 per 100 observation-years in untreated patients to approximately 1 per 100 observation-years in treated patients. Probably as a result of this effect, the UK group has now demonstrated a near normal life-expectancy in treated patients.[45]

Eculizumab has now been engineered in a clever manner with a set of substitutions that increase its half-life about fourfold. However, unfortunately, some patients respond only partially to C5 inhibition. In 2009, Risitano and colleagues made the critical observation that despite

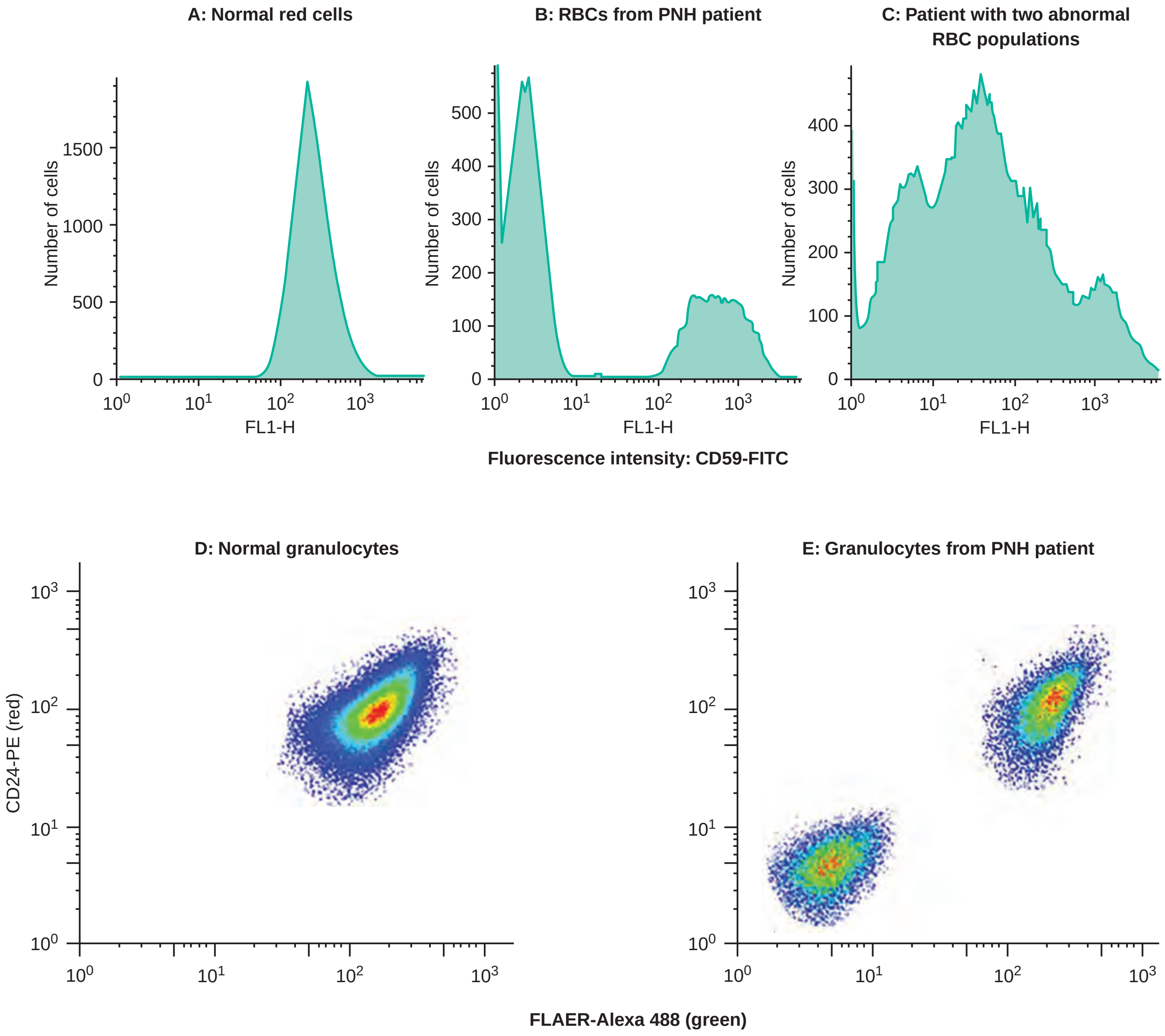


Figure 32.2 FLOW CYTOMETRY ANALYSES. After staining of red cells with a mouse anti-human antibody specific for CD59 conjugated to fluorescein isothiocyanate *(FITC)*, cells from the normal donor (A) pick up high levels of the antibody and fluoresce brightly in a unimodal distribution, as seen on this histogram plot. In contrast, in a patient with PNH (B), abnormal red cells that do not express CD59 co-exist with a normal cell population, producing a bimodal distribution. Some patients with PNH will have a trimodal population (C) with a population of red cells with intermediate levels of expression (the middle peak), known as PNH II cell populations. The PNH abnormality can be readily demonstrated in granulocytes as well. Here, in this illustration, cells are stained with a phycoerythrin *(PE)* conjugated mouse antibody specific for human CD24 (a GPI-linked antigen) giving red fluorescence and are simultaneously stained with FLAER (fluorescent aerolysin), which binds to cells in a GPI-dependent manner, giving green fluorescence. Normal granulocytes (D) fluoresce brightly in the red and green channels simultaneously and are illustrated as a single population on this pseudocolor density plot. Patients with PNH (E) have a normal population of granulocytes as well as a population that binds to neither FLAER nor to the anti-CD24 antibody, registering in the lower left quadrant. *RBC*, red blood cell. *(Illustration after Borowitz MJ, Craig FE, Digiuseppe JA, et al. Guidelines for the diagnosis and monitoring of paroxysmal nocturnal hemoglobinuria and related disorders by flow cytometry. Cytometry B Clin Cytom. 2010;78:211–230.)*

C5 blockade, C3 degradation products can still opsonize PNH red cells (due to their lack of CD55), resulting in their uptake in the reticuloendothelial system—a milder form of *extravascular* hemolysis that had previously been masked by the severe *intravascular* hemolysis seen in untreated patients (see Fig. 32.1).[46] These partial responders represented an unmet clinical need, which has led to the development of the first-ever inhibitor of the C3 complement protein—pegcetacoplan. This drug was designed to prevent both forms of hemolysis and has recently been approved by the Food and Drug Administration (FDA) for the treatment of PNH,[47] after it compared favorably with eculizumab in a randomized trial with an endpoint of hemolytic anemia.

EPIDEMIOLOGY AND CLASSIFICATION

The incidence of PNH in the United Kingdom is reported to be 1.3 per million per year, with a prevalence of approximately 16 per million.[48] In some Asian countries such as Thailand,[49] where the incidence of aplastic anemia is higher, PNH is probably also more common, though still only a few cases per million.[50] Approximately 30% of patients with PNH will have a prior history of acquired aplastic anemia,[51] and this is by far the greatest risk factor for PNH. In contrast, there is a single case report of PNH arising out of an inherited

TABLE 32.2 Enzymatic Steps in the Synthesis of Glycosylphosphatidylinositol

Step	Genes	Enzymatic Function	Human Neurologic Phenotype	Human Hematologic Phenotype
1	*PIGA*	UDP-GlcNAc:PI α1–6 N-acetylglucosaminyltransferase (GlcNAc-T)	Yes (MCAHS2)	Acquired mutation→PNH Inherited hypomorphic mutations→no hemolysis reported
1	*PIGH*	GlcNAc-T	Yes	No
1	*PIGC*	GlcNAc-T	Yes	No
1	*PIGQ*	GlcNAc-T	Yes	No
1	*PIGP*	GlcNAc-T	Yes	No
1	*DPM2*	GlcNAc-T	Yes	No
1	*PIGY*	GlcNAc-T	Yes (HPMRSG)	No
2	*PIGL*	GlcNAc-PI deacetylase	Yes (CHIME syndrome)	No
3	*PIGW*	Inositol acyltransferase	Yes, hyperphosphatasia	No
4	*PIGM*	Mannose transferase	Yes	Inherited promoter mutation→Budd Chiari syndrome.
4	*PIGX*	Mannose transferase	No human cases reported	No
5	*PIGV*	Mannose transferase	Yes	No
6	*PIGN*	Ethanolamine phosphate transferase	Yes	No
7	*PIGB*	Mannose transferase	Germline bi-allelic mutations resulting in severe neurologic phenotype	Inherited mutation and CN-LOS→PNH
8	*PIGO*	Ethanolamine phosphate transferase	Yes, hyperphosphatasia	No
8	*PIGF*	Ethanolamine phosphate transferase	No human cases reported	No
9	*PIGG*	Ethanolamine phosphate transferase	Yes	No
9	*PIGF*	Ethanolamine phosphate transferase	Yes	No
10	*GPAA1*	GPI transamidase (transfers peptide to the anchor)	Yes	No
10	*PIGK*	GPI transamidase (transfers peptide to the anchor)	Yes	No
10	*PIGT*	GPI transamidase (transfers peptide to the anchor)	Germline bi-allelic inactivation: multiple congenital abnormalities with hypotonic seizures (MCAHS)	Inherited mutation combined with an acquired mutation: hemolysis responding to eculizumab, aseptic meningitis, urticaria, bowel inflammatory condition (PIGT-PNH)
10	*PIGS*	GPI transamidase (transfers peptide to the anchor)	Yes	No
10	*PIGU*	GPI transamidase (transfers peptide to the anchor)	Yes	No

CHIME, Congenital heart disease ichthyosiform dermatosis, developmental delay, ear abnormalities; *HPMRS6*, hyperphosphatasia with developmental delay; *MCAHS*, multiple congenital anomalies, hypotonic seizures.

Table after Ferguson M, Hart G, Kinoshita T. Glycosylphosphatidylinositol anchors. In: Varki A, Esko J, eds. *Essentials of Glycobiology*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 2015–2017; and Kinoshita T. Biosynthesis and biology of mammalian GPI-anchored proteins. *Open Biol*. 2020;10:190290.

bone marrow failure syndrome (see Chapter 30), and the emergence of PNH is essentially unheard of after chemotherapy or radiation-induced marrow hypoplasia.[52] Approximately 7.5% of patients with Budd Chiari syndrome will have a concurrent diagnosis of PNH as an underlying cause,[53] and PNH flow cytometry should be considered part of the hypercoagulability workup for unusual site thromboses, or thrombosis with an abnormal CBC or LDH. The median age of patients at diagnosis is about 40,[54] children can be affected,[55] and males and females are at similar risk.

Patients with a flow cytometry test indicating a PNH clone can be classified as "Classic PNH," "PNH with another hematologic disorder," and "laboratory/subclinical PNH." Patients with classic PNH typically present with intravascular hemolysis or thrombosis. Patients with "PNH and another hematologic disorder" typically have aplastic anemia (AA/PNH) and their PNH clone is often detected based on routine screening or an elevated LDH. PNH/MDS and PNH/MPN overlap syndromes are rare. Patients with "laboratory/subclinical PNH" will have a PNH clone detected as part of a workup for mild cytopenias that defy explanation, or a mildly elevated LDH, MCV, or reticulocyte count, and they by definition do not require treatment. Nevertheless, the identification of a small subclinical clone helps to classify the patient as having an immune-mediated marrow injury. Some patients with PNH will have a population of cells that have only a partial deficiency in expression of CD59 (termed PNH II cells) often in co-existence with cells with a total lack of CD59 (PNH III cells) as well as normal (PNH I) cells (see Fig. 32.2C). Those patients whose abnormal red cell populations are mainly of the PNH II type may have less severe hemolysis but can have serious thrombotic complications.

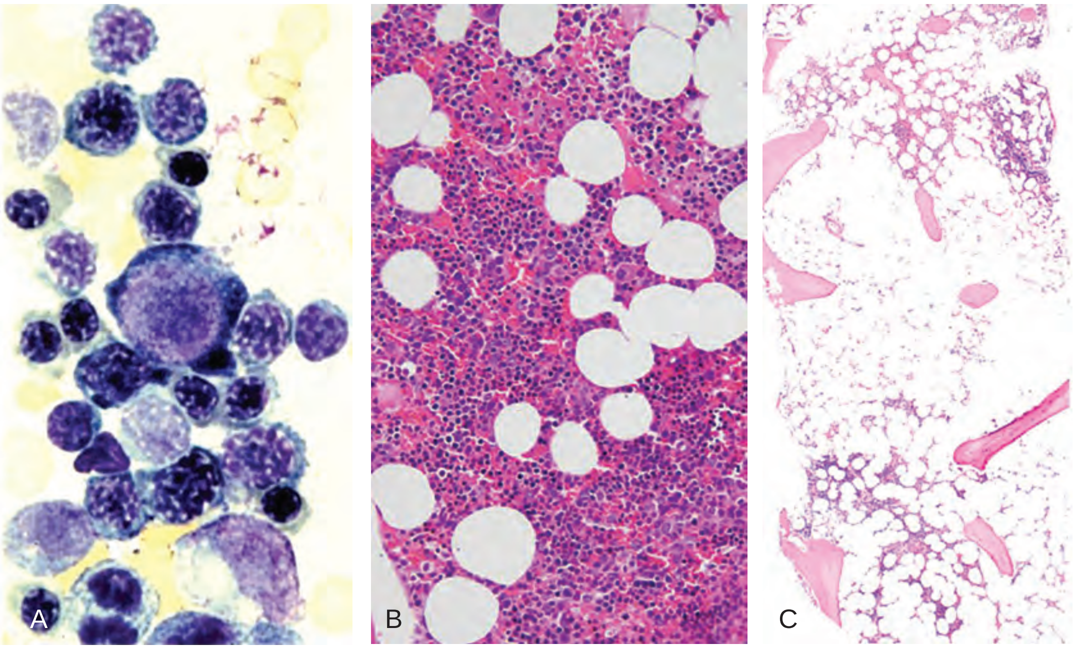


Figure 32.3 THE MARROW CAN HAVE A WIDELY VARIABLE APPEARANCE IN PAROXYSMAL NOCTURNAL HEMOGLOBINURIA. (A) Hypercellularity with erythroid hyperplasia (Wright stain, aspirate), as might be seen in any hemolytic anemia; (B) H&E-stained section showing overall normocellularity; (C) marked hypocellularity, as would be seen in aplastic anemia. In this case there is a patchy distribution to the marrow with some cellular areas with increased erythropoiesis interspersed with acellular areas. The immune escape hypothesis proposes that it is the aplastic marrow environment that favors the *PIGA* mutant clones.

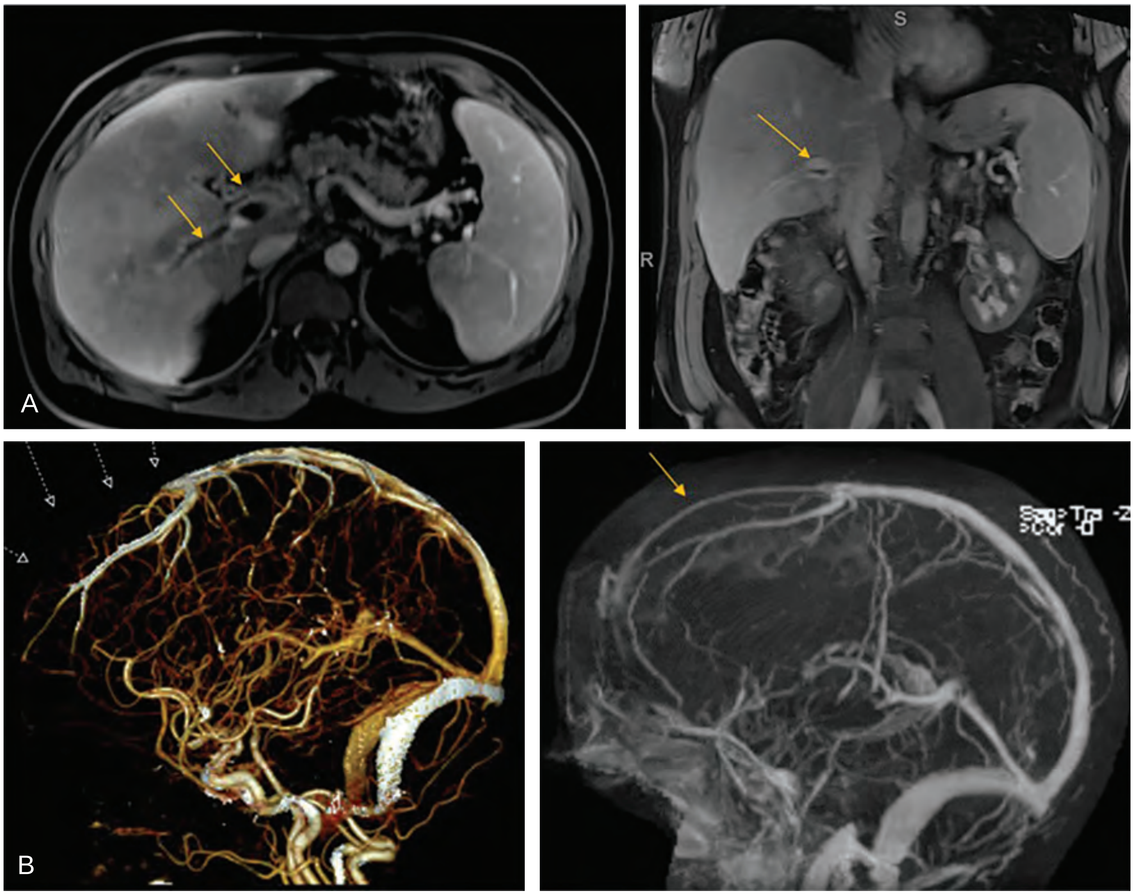


Figure 32.4 (A) Hepatic MRI images indicative of portal vein thrombosis in a patient with paroxysmal nocturnal hemoglobinuria. (B) Diminutive appearance of the anterior sagittal sinus seen on CT angiogram *(left)* and MR venogram *(right)*.

CLINICAL MANIFESTATIONS

Patients with classic PNH will present with dark urine or symptoms of anemia and features of *intravascular* hemolysis: an elevated LDH and reticulocyte count, anemia, a positive urine hemosiderin stain, with low or absent haptoglobin. The chronic loss of hemoglobin in the urine can result in iron deficiency. The patient will often report that the urine is darkest in the morning; it is unclear if this is due to increased hemolysis at night, as once thought, or increased concentration of the urine due to nocturnal fasting. To clarify what patients mean by "dark urine" it is helpful to ask them to compare the color to some familiar liquid. Patients will often describe their darkest urine as appearing as cola, black ink, soy sauce—or appearing red. This may depend upon the oxidation status of the iron in the free hemoglobin. Bouts of darkened urine are interspersed with periods of time with apparently normal appearing urine, as per the term "paroxysmal," sometimes with upward swings of a chronically elevated LDH and an exacerbation of anemia.

Patients will sometimes have had extensive urologic evaluations prior to referral to a hematologist: while a dipstick that is positive for "blood" in the absence of red cells on a microscopic examination will be a clue that the problem is not hematuria, some patients with PNH will actually have microscopic hematuria as well. In contrast, jaundice, hyperbilirubinemia, dark gallstones, and iron overload are features of *extravascular* hemolysis not typically seen on presentation—but are seen commonly in patients on C5 inhibitors. In untreated patients, free hemoglobin in the plasma can scavenge nitrous oxide and result in symptoms of smooth muscle dysfunction: abdominal pains, esophageal spasms, and male erectile dysfunction. Pulmonary arterial hypertension and renal dysfunction can occur, but this is usually subclinical. Sometimes a darkening of the kidneys is picked up on magnetic resonance images (MRI) as an incidental finding, a result of hemosiderin deposition. Attacks of hemolysis can be triggered by infection and fever, and due to abdominal discomfort, it may be difficult for patients to follow the typical instructions to keep well-hydrated. This can require a visit to the emergency room (ER) or infusion center for intravenous fluids, even if transfusions are not required at the time.

Patients with classic PNH can also present with thrombosis (with or without symptomatic hemolysis), typically involving unusual sites, such as the cerebral dural venous sinuses, the portal or hepatic veins, or other intra-abdominal veins, such as the inferior vena cava, the renal veins, splenic veins, or inferior or superior mesenteric veins. Thromboses in most of these sites can be detected by a sonogram with Doppler or a contrast computed tomography (CT), but mesenteric vein thrombosis can be detected only on an MR venogram, which is also sensitive enough to identify thromboses in smaller branches of the hepatic veins as well. Splenic vein and mesenteric vein thromboses are particularly dangerous because they can lead to splenic rupture and bowel infarction respectively: both are surgical emergencies. Patients with thrombosis and secondary gastrointestinal hemorrhage (or central nervous system hemorrhage due to extensive cerebral vein thrombosis) also represent a particular challenge. Hypersplenism or marrow failure can result in thrombocytopenia, and PNH is therefore one of the conditions where the difficult combination of thrombocytopenia and thrombosis can occur. While the aspartate aminotransferase (AST) can be elevated simply due to hemolysis, an elevated alanine transaminase (ALT) in patients with PNH must be taken as a sign of Budd-Chiari syndrome until proven otherwise. Dermal venous thrombosis has been reported[56] and can be complicated by necrosis. More familiar sites of thrombosis such as extremity deep venous thrombosis, pulmonary emboli, arterial cerebral vascular accidents (CVAs), and myocardial infarction can also occur in patients with PNH.

Patients presenting with AA/PNH fall along a spectrum, where they can resemble patients with aplastic anemia, or classic PNH, or both. As mentioned above, patients with AA/PNH can have thrombosis and thrombocytopenia at the same time due to marrow failure, resulting in a situation where anticoagulation would be dangerous. In AA/PNH, the reticulocyte count and neutrophil count can be decreased as well. Aplastic anemia can precede the diagnosis of PNH, or can occur later. A fall in blood counts in a patient with PNH who has been previously stable should prompt a bone marrow biopsy and aspirate to rule out progression to MDS or AML.

The peripheral smear findings will often show various red cell abnormalities, but none is specific to PNH. However, the smear will be useful to rule out findings suggestive of competing causes of intravascular hemolysis and can indicate concurrent iron deficiency. In those with an adequate erythropoietic response and an elevated reticulocyte count, the most prominent feature on the smear can be polychromasia. The mean corpuscular volume (MCV) is usually elevated, even in the absence of reticulocytosis, as occurs in aplastic anemia.

The bone marrow in patients with classic PNH is typically hypercellular with an erythroid hyperplasia, as can be seen in any patient with reticulocytosis (see Fig. 32.3). However, the marrow can range from hypocellular to hypercellular, and there is often a patchy distribution of the marrow. A normocellular or even hypercellular marrow does not rule out AA/PNH, and cytopenias may still respond to immune suppression. An increase in mast cells is sometimes noted. Almost a quarter of patients with PNH will have a cytogenetic abnormality, which is sometimes transient and by itself does not make a diagnosis of myelodysplasia (MDS) (see Chapter 61).[57] Likewise, morphological abnormalities can often be seen in PNH that overlap with those seen in MDS. However, excess blasts and complex cytogenetic abnormalities are diagnostic of MDS and are an indication for referral for stem cell transplantation—as may be the case for abnormalities of chromosome 7, which is associated with a poor prognosis when seen in aplastic anemia.[58] Elevated platelets, white blood cell counts, or fibrosis are rare findings but can be seen in the PNH/MPN overlap syndrome (see Chapters 69 and 72)[59–62] and should prompt testing for mutations in *JAK2* as well as *BCR-ABL* fusions.

PATHOPHYSIOLOGY

The Glycosylphosphatidylinositol Anchor

Understanding the pathophysiology of PNH requires a consideration of the GPI anchor. Although there are variations between species as well as modifications, the core GPI structure consists of protein-ethanoloamine-*P*-6Manα1–2Manα1–6Manα1 (P-ethanolamine)-4Glucoseamine- phosphatidylinositol (Fig. 32.5A).[63] Unlike in yeast, GPI is not essential for the survival of mammalian cells but is essential for embryogenesis. The anchor is synthesized in the endoplasmic reticulum (ER) membrane and involves 10 reactions and almost 30 different genes (Table 32.2). The first two steps take place on the cytoplasmic side and the remaining steps in the lumen of the ER. The first step is the transfer of N-acetylglucosamine (GlcNAc) from uridine diphosphate (UDP)-GlcNAc to phosphatidylinositol (PI) to yield GlcNAc-PI: this is the step that is deficient in PNH. This reaction is catalyzed by GlcNAc-PI α1–6 GlcNAc transferase, an enzyme whose subunits are encoded by seven different genes: *PIGA*, *PIGC*, *PIGH*, *PIGQ*, *PIGY*, *PIGP*, and *DPM2* (see Fig. 32.5B).[64–66] The *PIGA* gene encodes a membrane protein in the endoplasmic reticulum that has the active site of this enzyme complex; it has a nonfunctional intron-less pseudogene on chromosome 12.[67]

Because of embryonic lethality, the mutations seen in PNH are almost always acquired somatic mutations occurring in a hematopoietic stem cell. Because *PIGA* is X-linked (Fig. 32.6), a mutational "single hit" will generate a GPI-negative (PNH) phenotype. Of note, although *PIGA* is near the pseudo-autosomal region, it is completely silenced on the inactive X-chromosome in female cells.[68] Therefore, on the cellular level, any female with a mutation on the active X chromosome that disrupts *PIGA* will have an equally severe phenotype as the same mutation in a hemizygous male. Thus females are as severely affected as males.

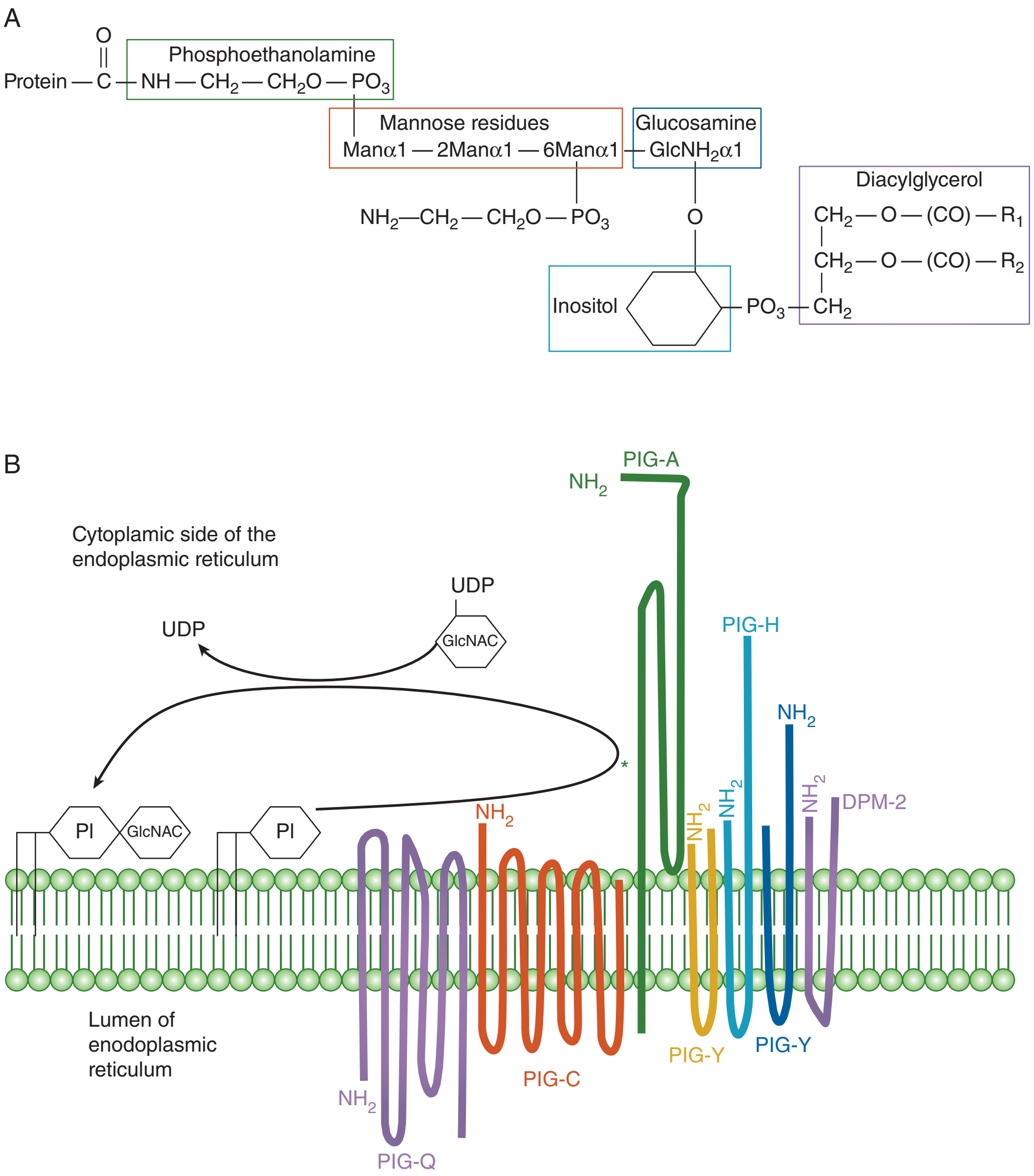

A
Protein — C(=O) — NH — CH2 — CH2O — PO3
Phosphoethanolamine
Mannose residues
Manα1 — 2Manα1 — 6Manα1
Glucosamine
GlcNH2α1
NH2—CH2—CH2O—PO3
O
Diacylglycerol
CH2—O—(CO)—R1
CH2—O—(CO)—R2
CH2
Inositol
PO3
B
Cytoplamic side of the endoplasmic reticulum
UDP
UDP
GlcNAC
PI
GlcNAC
PI
*
PIG-A
NH2
PIG-H
NH2
NH2
NH2
NH2
DPM-2
NH2
Lumen of enodoplasmic reticulum
NH2
PIG-Q
PIG-C
PIG-Y
PIG-Y

Figure 32.5 (A) The core structure of GPI. A protein is covalently linked to the ethanolamine moiety of this structure by a peptide bond. The diacylglycerol moiety is inserted in cell phospholipid membrane bilayer, which keeps the structure localized to the surface of the cell. This provides lateral mobility in the membrane for the attached protein. (B) It requires 7 proteins to catalyze the first step in the biosynthesis of GPI in the endoplasmic reticulum. The reaction occurs on the cytoplasmic side but later steps occur on the luminal side. *PIGA* has the active site of the enzyme complex, indicated with the asterisk. This is the step of GPI-anchor synthesis that is deficient in PNH. *(Based on Ferguson MAJ, et al. In Varki A, et al, eds. Essentials of Glycobiology. Cold Spring Harbor, 2015–2017.)*

PIGA Gene Mutations

The *PIGA* gene was identified in the early 1990s by expression cloning and was found to correct the defect in GPI-anchor synthesis when expressed in cell lines from patients with PNH. Since then, *PIGA* has been found to be mutated in almost all patients.[32,33,69] The *PIGA* gene encodes a protein of 484 amino acids, with a transmembrane domain inserted in the endoplasmic reticulum. Most of the protein is on the cytoplasmic side (see Fig. 32.5B), with the GlcNAc transferase homologous region containing the active site near the transmembrane domain. There are 6 exons, the longest is exon 2, which contains the translation initiation codon (see Fig. 32.6); 600 bp of upstream sequence is required for full gene expression,[70] which has features of a housekeeping gene promoter, and *PIGA* is listed as a housekeeping gene—as is the case for almost all of the genes required for GPI-anchor assembly.[71]

The somatic inactivating *PIGA* mutations in PNH are quite diverse and include missense mutations, in frame deletions, nonsense and frameshift mutations, splice site mutations, deletions of hundreds of base pairs[72]—and much larger deletions that also involve contiguous genes on the X-chromosome (see Fig. 32.6).[73] Missense mutations are often seen in patients where there is a partial loss of CD59 (PNH II cells), suggesting that there is some residual enzymatic activity, whereas truncating mutations are generally associated with complete loss of CD59.[74] It is not uncommon to observe 3 distinct populations of cells demonstrated by flow: normal cells, PNH II cells with a partial deficiency, and PNH III cells with a complete deficiency of CD59, corresponding to differing degrees of complement senstitivity (see Fig. 32.2).[12,13] In keeping with this, multiple *PIGA* mutations have been identified in some patients.[75] The *oligo*clonality sometimes seen in PNH must be taken into account in any model of how *PIGA* mutant clones expand.

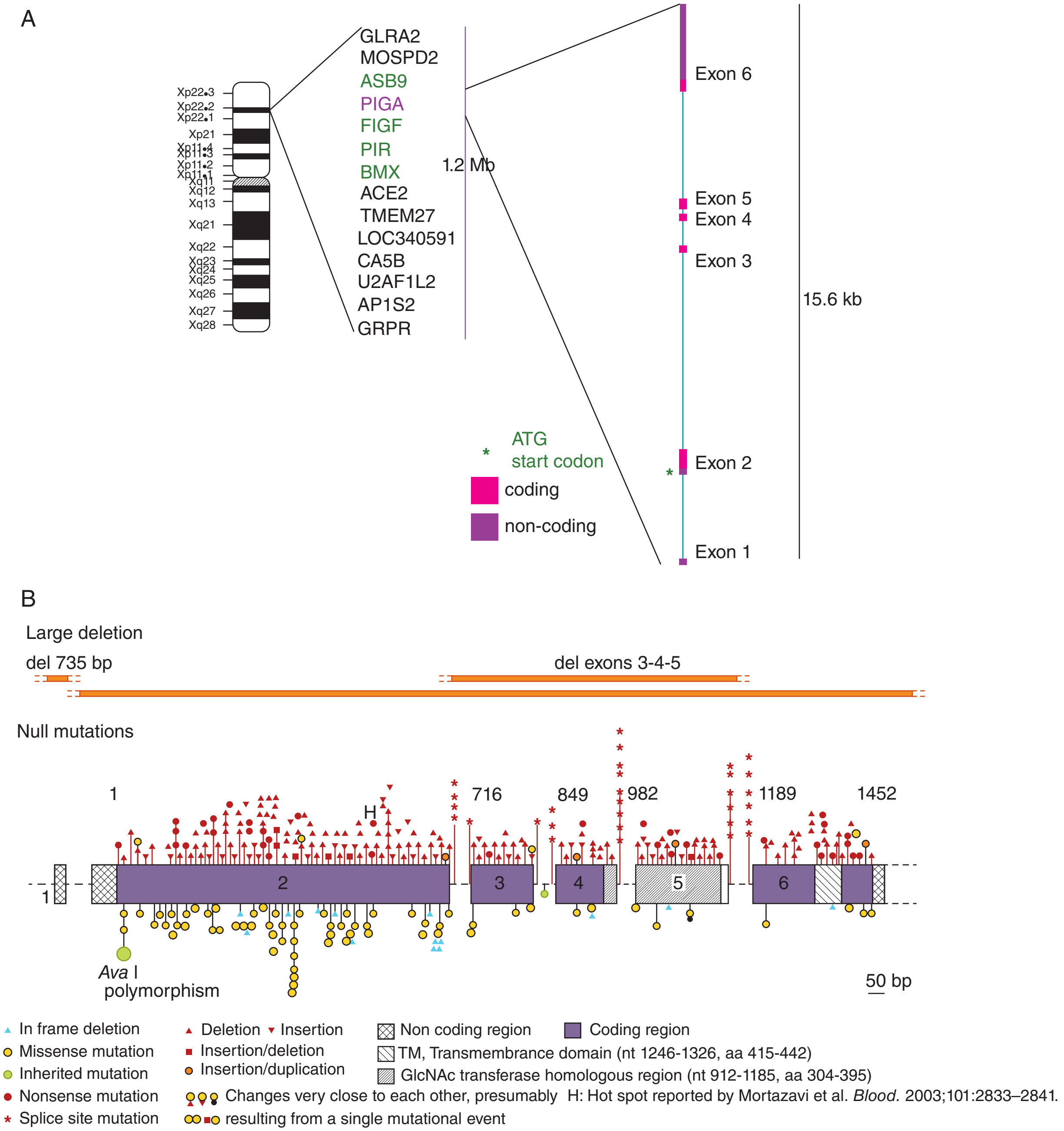

A
Xp22.3
Xp22.2
Xp22.1
Xp21
Xp11.4
Xp11.3
Xp11.2
Xp11.1
Xq11
Xq12
Xq13
Xq21
Xq22
Xq23
Xq24
Xq25
Xq26
Xq27
Xq28
GLRA2
MOSPD2
ASB9
PIGA
FIGF
PIR
BMX
ACE2
TMEM27
LOC340591
CA5B
U2AF1L2
AP1S2
GRPR
1.2 Mb
Exon 6
Exon 5
Exon 4
Exon 3
Exon 2
Exon 1
15.6 kb
* ATG start codon
coding
non-coding
B
Large deletion
del 735 bp
del exons 3-4-5
Null mutations
1
716
849
982
1189
1452
H
1
2
3
4
5
6
Ava I polymorphism
50 bp
In frame deletion
Missense mutation
Inherited mutation
Nonsense mutation
Splice site mutation
Deletion
Insertion
Insertion/deletion
Insertion/duplication
Changes very close to each other, presumably resulting from a single mutational event
Non coding region
Coding region
TM, Transmembrance domain (nt 1246-1326, aa 415-442)
GlcNAc transferase homologous region (nt 912-1185, aa 304-395)
H: Hot spot reported by Mortazavi et al. Blood. 2003;101:2833–2841.

Figure 32.6 (A) The location of the *PIGA* gene on the X-chromosome. Large deletions that also involve neighboring genes (shown in *green*) have been reported occasionally in patients with PNH. (B): A broad spectrum of mutations in the *PIG-A* gene have been identified in patients with PNH. *(Illustration after Luzzatto L, Nafa K. Genetics of PNH. In: Young N, Moss J, eds. PNH and GPI-Linked Proteins. Academic Press; 2000;21–47.)*

The *PIGA* gene has also proven useful to other fields. Particularly, because flow cytometry can identify rare mutants among a much larger population of normal cells, *PIGA* represents an excellent reporter gene for measuring the frequency and rate of spontaneous and induced mutations in human cells[76,77] and in animals.[78,79] This provides an important tool in the preclinical evaluation of the genotoxic effects of medications. Furthermore, because of the ease of cell sorting for GPI-positive and GPI-negative cells, and because mutants can be selected by aerolysin[80] (which selectively kills normal cells in a GPI-dependent manner) *PIGA* represents a selectable and counter-selectable marker for genome editing and genome writing projects.[81,82]

Loss of Glycosylphosphatidylinositol in Blood Cells Due to Mutations in Genes Other Than *PIGA*

There is a recently discovered alternative pathway to produce the PNH phenotype: germline loss of one of the autosomal genes involved in GPI-anchor synthesis and a second acquired event that eliminates the remaining allele in hematopoietic stem cells. This was first reported for the *PIGT* gene on chromosome 20q, and the resulting syndrome has been termed "PIGT-PNH."[83–85] Here the first event is an inherited chain-terminating mutation on the maternal allele and the second mutation is a deletion of chromosome 20q ranging from 8 to 18 Mb on the paternal allele. In addition to features of PNH, these patients also develop recurrent aseptic meningitis, urticaria, arthralgia, and bowel inflammation. Of note, the *PIGT* mutation affects the 10th step in the pathway, where the protein is transferred to the anchor, and accumulation of the completed but un-conjugated anchor may result in the inflammatory phenotype. Recently, a family has been reported where something similar occurs in the *PIGB* gene on chromosome 15q.[86] Here there is a germline missense mutation in *PIGB* as well as a 70 kb deletion that is telomeric to *PIGB* on the same chromosome that may greatly increase the rate of copy neutral loss of heterozygosity events (CN-LOH). A woman with a history of aplastic anemia was found to have the inherited *PIGB* mutation, the 70 kb deletion, and CN-LOH resulting in the PNH phenotype in almost 100% of blood cells. Her asymptomatic mother, who inherited the same mutations, was found to have approximately 4% PNH leukocytes. Another individual in the pedigree with the missense mutation but without the deletion did not have PNH blood cells.

A partial loss of GPI is not embryonic lethal, and inherited germline hypomorphic alleles have been reported for many of the genes involved, including for *PIGA*.[87–91] This produces a severe neurologic phenotype (see Table 32.2), but there is generally sufficient residual GPI anchor production so that these patients do not have thrombosis or hemolysis. An exception to this has been the patient reported by Almeida et al. with a *PIGM* promoter mutation resulting in Budd Chiari syndrome as well as seizures—which responded to epigenetic modification therapy.[92,93]

Glycosylphosphatidylinositol Anchored Proteins and Complement-Mediated Hemolysis

The proteins that require the GPI-anchor have in common a lack of a hydrophobic transmembrane domain, the presence of a cleaved N-terminal signal that localizes the protein to the endoplasmic reticulum, and a c-terminal sequence that is cleaved in the process of transfer of the peptide to the anchor (see Table 32.1).[64] Alkaline phosphatase was the first GPI-linked protein discovered, by virtue of its release from the cell surface by bacterial phospholipase C. GPI–linked proteins are important in the biology of malaria, trypanosomes, and prion diseases. In addition to complement regulators, GPI-anchored proteins in humans can serve as enzymes, blood group antigens, receptors, and adhesion molecules (see Table 32.1). Certain proteins (e.g., CD58 [LFA3] and CD16 [FcγRIII]) may exist in both GPI-linked and transmembrane forms. Membrane inhibitor of reactive lysis (CD59) and decay accelerating factor (CD55)—both complement regulatory proteins—are the most widely expressed GPI-anchored proteins and can be found on cells belonging to all hematopoietic lineages including $CD34^{+}CD38^{-}$ stem cells. Their function is discussed further in the chapter on complement biology (see Chapter 23).

The loss of CD59 is most consequential in untreated patients with PNH, as it works to inhibit the assembly of the C5b-9 membrane attack complex; pharmacologic C5 inhibition compensates for this defect. CD55, in contrast, works upstream, by inhibiting the alternative and classic C3 convertases. In patients who are treated with C5 inhibitors, the lack of CD55 becomes more consequential, in that unregulated C3 conversion results in C3 degradation products that opsonize the red cell and result in uptake in the reticuloendothelial system, thus leading to extravascular hemolysis. In untreated patients, C3 activation on these red cells would have instead led to the full progression of the complement cascade, leading to lysis by the membrane attack complex. Flow cytometry demonstrates that this process occurs only on the GPI-negative cell population,[46] and in some patients receiving C5 inhibitors, the DAT (direct Coombs test) is positive. The severity of hemolysis in patients with PNH is likely affected by inter-individual differences in complement activation, for example, as a result of polymorphisms in the *CR1* gene.[94] An extreme case of this is inherited deficiency of C9, which can partially mask the hemolytic phenotype.[95]

In patients with partial deficiency in GPI (PNH II populations) the degree of hemolysis is generally less severe. Of note, when there is a limiting amount of GPI, CD55 may take precedence over CD59 for acquiring the anchor.[96] Therefore the PNH II populations are best delineated with antibodies specific for CD59. Interestingly, bi-allelic mutations in CD59 recapitulate PNH and can be treated with C5 inhibition,[97,98] whereas bi-allelic inactivation of CD55 results in the CHAPLE syndrome (complement hyperactivity, angiopathic thrombosis, and protein-losing enteropathy)[99] rather than hemolysis.

Clonal Expansion of Glycosylphosphatidylinositol-Negative Populations in Paroxysmal Nocturnal Hemoglobinuria

Since 1989, the leading hypothesis on the expansion of GPI-negative populations has been the immune escape model, which posits that the *PIGA* mutation is the first "hit" and immune-mediated marrow failure is the "second hit."[35] In this model, the PNH stem cells are relatively spared and are able to repopulate the marrow after an immune-mediated injury.[35,38,100] According to this hypothesis, in some patients there is a prior history of aplastic anemia, and in others the marrow failure is subclinical. This hypothesis explains several key features of PNH: (1) the fact that platelet and neutrophil counts are often decreased in PNH even without a history of aplastic anemia; (2) the fact that PNH is sometimes oligoclonal; (3) the associations between HLA-DR2 alleles and PNH[101]; and (4) the observation that patients with PNH clones often respond well to immunosuppression when they develop cytopenias.[101,102]

This model holds that *PIGA* mutations by themselves do not provide a growth advantage. As predicted, normal individuals harbor tiny PNH cell populations on the order of $1{:}10^5$ to $1{:}10^6$, as a result of spontaneous mutations of the *PIGA* gene—and yet these clones do not go on to expand in normal individuals.[77] Likewise, in a tissue-specific model involving female mice heterozygous for a floxed Pig-A allele, PNH clones arise as a result of the expression of Cre over time, in cells with the deleted Piga on the active X-chromosome. However, the PNH cells that arise do not outcompete GPI-positive cells that have the deleted allele on the inactive X-chromosome.[103,104] Likewise, in the majority of patients followed over time, PNH clones either remain stable in size or sometimes regress.[105] Of note, *PIGA* mutations are not turning up in genome wide sequencing analysis of malignancies, and it has long been observed that it is easy to eradicate PNH clones by allogeneic stem cell transplantation.[106–108]

This model posits subclinical aplasia in patients without a history of PNH, and stem cell numbers are indeed reduced in PNH,[109] with a specific growth defect in the GPI-positive population.[110] As further evidence of the immune escape model, oligoclonal T-cell expansions have been demonstrated.[111,112] Since the autoantigens in aplastic anemia are still unknown, the escape mechanism has not been completely worked out. An obvious theory is that there is an autoantigen that is GPI-linked. One possibility involves the UL-16 binding proteins (ULBPs), which are NKGD2 ligands.[113] Alternatively, the GPI-anchor itself can fit into the groove of CD1d and can be presented to T cells[114–117]; indeed GPI-reactive T cells have been demonstrated in some patients with PNH. This model of immune selection may therefore be analogous to the loss of HLA allele expression due to chromosome 6 loss of heterozygosity in some patients with aplastic anemia.[118]

The immune escape model does not quite explain the increased relative risk of leukemia and MDS that is seen in PNH, though this can also occur in immune-mediated bone marrow failure without PNH. The hypermutability model, which attempts to explain this feature and account for oligoclonality at the same time posits that PNH patients are prone to mutations. However, an increase in mutant frequency or mutation rate has not been observed in cells from PNH patients,[119,120] who are not prone to solid tumors or lymphoid neoplasms. On the other hand, some patients with PNH do have a second *genetic* hit: mutations in *TET2*,[121] *JAK2*,[62] *HMGA*,[122] *SUZ12*, *U2AF1*,[123] *ASXL1*, *BCOR*, *MECOM*, and *RIT1*,[124] the Philadelphia chromosome,[125] as well as other cytogenetic abnormalities (which are sometimes transient)[57] have all been reported. Cytogenetic abnormalities and mutations in some of these genes can be seen in aplastic anemia, and second genetic hits in PNH are not mutually exclusive with the immune escape model. In some cases, the mutation or cytogenetic abnormality occurs within the PNH clone, and in some cases it is within the GPI-positive population.[57,124] In some cases, the mutant allele burden is sufficiently high and the nature of the mutation is such that these second genetic events are probably contributing to the expansion of the PNH clone.[126] An alternative explanation posits that the PNH clone expands as a result of stochastic effects,[127] which is also not mutually exclusive with immune escape.

Thrombosis

As for hemolysis, the size of the PNH clone is strongly associated with the risk of thrombosis,[128] which is almost never seen in patients who have aplastic anemia and small PNH clones.[129] Historically, there has been a marked geographic disparity, where the rates of thrombosis are higher in Western countries than in Asia. It was observed in the era before routine anticoagulation and before the availability of anti-complement therapy that African-American and Latin American patients had a higher risk of thrombosis; it is not clear if that is still the case.[129] Because vessel wall injury and stasis can occur due to previous occlusions, it can be said here, as in other disorders, that “clot begets clot.” Indeed, those with a prior history of thrombosis have a high risk of second events despite therapeutic anticoagulation.[130] The combination of PNH with another hypercoagulable state such as the *JAK2*-mutated myeloproliferative syndromes (PNH/MPN)[62] or Factor V Leiden can produce a severe thrombotic phenotype, but these are rarely seen.[131]

Chronic hemolysis can be associated with thrombosis in some other conditions (e.g., sickle cell anemia and autoimmune hemolytic anemia), but not others (e.g., heart valve dysfunction) and so hemolysis may not be the sole explanation. Other possible mechanisms include: (1) decreased fibrinolysis due to abnormal expression of the GPI-linked uPAR receptor[132]; (2) increased thrombin generation on platelet-derived microparticles[133–135]; (3) lack of a GPI-linked tissue factor pathway inhibitor[136,137]; and (4) increased complement-mediated activation of CD59-negative platelets.[22,138–142] While using flow cytometry to demonstrate GPI-negative platelets is technically difficult, it can be done, and the results correlate with the percentage of PNH granulocytes.[143] The presence of PNH platelets may explain why unusual patients who have large PNH granulocyte populations but small PNH red cell populations are at a particularly high risk of thrombosis.[143,144] As reviewed in Chapter 23, while complement can activate platelets, coagulation factors can activate complement, and there may be a vicious cycle. Of note, while the explanations above are not mutually exclusive, the success of eculizumab in reducing thrombosis supports a central role for complement.

DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS

The differential diagnosis of the patient with dark urine includes other causes of intravascular hemolysis such as mechanical damage, antibody mediated hemolysis (such as in PCH, mycoplasma infection, and cold agglutinin disease), unstable hemoglobin variants (such as hemoglobin Zurich), and G6PD deficiency. Myoglobinuria, porphyria, and some medications can also darken the urine. PNH can mimic MDS, as well as Budd Chiari syndrome produced by a myeloproliferative neoplasm and other hypercoagulable states. The bouts of abdominal pain in PNH can be confused with porphyria, pancreatitis, and familial Mediterranean fever. The combination of thrombocytopenia and thrombosis can be seen in a few other disorders, such as DIC (see Chapter 137), the antiphospholipid antibody syndrome (see Chapter 139), aHUS, and TTP (see Chapter 132). Patients with PNH will often have a prior (mis)diagnosis of hematuria or immune thrombocytopenic purpura (ITP). “Laboratory PNH”/“Subclinical PNH” produces mild cytopenias and macrocytosis and can be confused with B12 or folate deficiency or alcoholism.

Fortunately, the diagnosis of PNH by flow cytometry is now straightforward if done following standard recommendations.[34] For red cells, loss of CD59 is highly sensitive and specific (see Fig. 32.2A–C), though the proportion of abnormal red cells is typically underestimated due to hemolysis and transfusions. For the analysis of granulocytes and monocytes, a combination of an anti-CD24 antibody conjugated to PE together with the FLAER reagent (Alexa-488 conjugated to a non-pore forming variant of proaerolysin that binds to cells in a GPI-dependent manner) is particularly robust (see Fig. 32.2D and E). The flow cytometry analysis should be performed only on anticoagulated peripheral blood and never on marrow. Red cells should be identified by forward and side scatter on a log-log scale. Unlike most flow cytometry protocols, here the abnormality is identified as a population that *lacks* expression of a marker, so it is important to ensure that the entire cell population comes in contact with the staining reagents. An antibody specific for non-GPI-linked proteins can be used to identify specific lineages, and this renders the method extremely sensitive to small populations, provided that a large number of events are collected.[77] This can identify patients who have “laboratory PNH” or “subclinical PNH” at a low level, and this can have implications for prognosis and treatment in patients who might otherwise have a diagnosis of MDS,[145] for example.

Congenital loss of CD59 can present similarly to PNH, from which it can be distinguished if an appropriate panel of reagents is used in the flow cytometry protocol. Of historical interest, type II congenital dyserythropoietic anemia[146] can produce a false-positive Ham test, a condition known as HEMPAS (hereditary erythroblastic multinuclearity with positive acidified serum lysis test), but will produce a negative flow cytometry test (see Chapter 30).

EVALUATION OF NEWLY DIAGNOSED PATIENTS

The LDH is helpful in estimating the intensity of hemolysis in newly diagnosed patients. The normal range differs between laboratories and should be interpreted as the ratio of the level compared to the upper limit of normal. The reticulocyte count will also give an estimate of whether the marrow response is appropriate to the degree of anemia. In patients who are anemic, whose absolute reticulocyte count is below 200,000/μL, it is reasonable to check the erythropoietin level, especially in the elderly. However, erythropoietin levels are almost always elevated in PNH, and exogenous supplementation is rarely needed. The B12, folate, and iron, TIBC, and ferritin are important parameters and deficiencies should be corrected, and these levels monitored periodically. To identify additional risk factors for thrombosis, a panel that includes protein C, protein S, antithrombin III, either the APC resistance assay or a genetic test for the factor V Leiden allele, and the lupus anticoagulant, beta 2 glycoprotein 1, and cardiolipin antibodies is indicated. Baseline MR venograms of the brain and abdomen to identify occult thrombotic events is also useful. Venous Doppler studies and a CT angiogram are likewise sometimes indicated. Soon after the diagnosis of PNH, a bone marrow biopsy and aspirate should be performed to rule out features of the rare PNH/MDS overlap syndrome—excess blasts, complex cytogenetic abnormalities, and abnormalities that affect chromosome 7.

TREATMENT

Supportive Care

As for any hemolytic anemia, patients with PNH should be given folic acid pre-emptively, to avoid megaloblastic crisis, and those with inadequate intake should be given B12 as well. Iron deficiency may need to be corrected with oral supplements or dietary modification, but there is no information on the safety of intravenous iron preparations, which may activate complement[147] and would thus be best avoided. Conversely, patients on C5 inhibitors or who have AA/PNH need to be monitored for iron overload and should generally avoid iron supplements. Patients should be instructed to maintain oral hydration and should be offered intravenous fluids if they are having gastrointestinal symptoms that might make it difficult to maintain fluid balance. Because of the often-subtle effects of PNH on renal function, patients should be advised to avoid nonsteroidal anti-inflammatory drugs.

Free hemoglobin scavenges nitrous oxide and affects smooth muscle tone, which can result in erectile dysfunction, abdominal pain, and esophageal spasms. Those with erectile dysfunction may benefit from sildenafil, which inhibits phosphodiesterase 5 (PDE5). Esophageal spasms are sometimes treated with nitroglycerin, which must not ever be given together with sildenafil or any other drugs in that class. Nowadays, such patients would almost always be offered anti-complement therapy instead.

Transfusion of red cells was once the mainstay of treatment and may still be necessary even in patients on complement inhibitors. As for any patient with bone marrow failure, irradiated filtered blood products should be provided. Patients should only receive type-specific red cells (i.e., a type AB recipient should receive only type AB red cells). This is because the small amount of plasma in a type O red cell unit could contain sufficient anti-A or anti-B isohemagglutinins to initiate the complement cascade. Washing of the red cells should not be necessary and may decrease the efficacy of transfusions,[148] but would be required if type-specific blood cannot be provided.

Prevention and Treatment of Thrombosis

One of the challenges in caring for the patient with PNH is to recognize that the greatest threat to the patient's well-being is not the darkening of the urine, anemia, or smooth muscle dysfunction, but rather the risk of thrombosis—and to explain this effectively to the patient. It has been suggested that a PNH clone size representing 50% of the granulocytes would be an appropriate cut-off value above which to initiate a strategy to prevent thrombosis,[149] and this is supported by analysis of receiver operator curves.[143] Warfarin is the only anticoagulant that has been studied in large numbers of patients, and probably should be overlapped with an injectable anticoagulant to prevent the transient acute thrombotic tendencies seen with warfarin-induced protein C deficiency.[40] Full therapeutic doses of low-molecular-weight heparin and fondaparinux are probably equivalent to warfarin and have the obvious advantage that they are less likely to be overdosed or underdosed (see Chapter 143). Less is known about the novel/direct acting anticoagulants, but these are being increasingly used for most other indications[150]; of these, apixaban is probably preferred if there is a possibility of renal dysfunction developing. It is important to obtain imaging to be sure that the patient has not had any previous occult thrombosis, in which case *both* anticoagulation and anticomplement therapy are indicated, since patients can develop thrombosis despite being on either agent alone.[44,130]

Patients who are initiated on primary anticoagulation prophylaxis should be continued on this until they initiate anti-complement therapy or until their PNH granulocyte clone regresses in size to below 50%. Those with PNH cell populations below this cutoff can be offered aspirin therapy, but it is unclear whether this is effective. As for any patient on anticoagulation, it is critical to counsel the patient regarding the risks associated with CNS trauma and the signs and management of GI bleeding. Since many patients with PNH will have some degree of thrombocytopenia, which together with anticoagulation can increase the risk of bleeding, platelet counts will need to be monitored closely. Some of these patients can be given anticomplement therapy instead—as is the case for any patient who is at risk of trauma. Women with PNH should not be treated with certain oral or implantable hormonal contraceptives due to the thrombogenic complications seen with these drugs. Hormone-eluting intrauterine devices and barrier methods are likely to be safer options.

For patients who have developed acute thrombotic complications, full anticoagulation as well as urgent initiation of anticomplement therapy is indicated. Those who develop refractory life-threatening thrombotic complications can be treated in an intensive care unit with tissue plasminogen activator, which is given by a slow intravenous infusion[130] (see Chapter 143). This needs to be done, however, before fibrosis develops in the affected vessel. For those with established thromboses resulting in hypersplenism, selective splenic artery embolization can be effective.[151] Bowel infarction as a result of mesenteric vein thrombosis is a surgical emergency.

Treatment of Marrow Failure

Patients with the AA/PNH syndrome now have an excellent survival,[152] probably due to the widespread recognition that these patients respond well to immunosuppressive therapy. A full discussion of the treatment of aplastic anemia is beyond the scope of these pages and can be found in Chapter 31, but there are a few points to be made here. (1) Those with large PNH red cell populations can develop an acute hemolytic event triggered by the infusion reactions that commonly occur with equine antithymocyte globulin (ATG). This can be avoided by giving transfusions of red cells over a course of a few weeks before a planned admission for ATG, with the goal of bringing the percentage of PNH red cells down to below 10%, as verified by flow cytometry. (If the patient is on anti-complement therapy, this might not be necessary.) (2) The nephrotoxicity of cyclosporin can compound the mild renal insufficiency seen in severe hemolytic PNH, and particular attention to maintaining hydration and avoiding concurrent nephrotoxic drugs is critical. (Here too, this might be less of an issue in patients on anticomplement therapy.) (3) It should be remembered that patients with substantial PNH cell populations were excluded from the initial studies of eltrombopag in aplastic anemia (see Chapter 31).[153] The concern here is that as in ITP, thrombopoietin-mimetic drugs can be pro-thrombotic and they might also be in this clinical scenario. More recently, it has been reported that the presence of a PNH cell population is a positive predictor of survival in patients treated with eltrombopag,[154] though the sample size and clone sizes were small. (4) The decision to offer allogeneic stem cell transplantation versus immunosuppression for severe aplastic anemia is usually based on the age and neutrophil count on presentation. The presence of the PNH clone, however, clearly predicts a good outcome and survival with immunosuppression[155] and can be factored into decision-making. (5) Patients with PNH with cytopenias can respond well to immune suppression even if their marrow is normocellular. (6) Some patients with PNH with cytopenias not meeting Camitta's criteria for severe aplastic anemia can respond to cyclosporin alone without ATG.[156] (7) Hypersplenism due to thrombosis can mimic the AA/PNH overlap syndrome and an abdominal sonogram with Doppler should be done to rule this out before treating for aplastic anemia.

Anticomplement Therapy

The biology, history, and clinical use of complement inhibitors is covered extensively in Chapter 23. Here we will focus on the indications for anticomplement therapy, the strategies for reducing risk of infectious complications, the appropriate follow-up of treated patients, and the evaluation of non-responding or partially responding patients.

The C5 inhibitor eculizumab was approved by the FDA in 2007 for the treatment of hemolysis in PNH, based on reduction in transfusion requirement. Typically the LDH starts to normalize, the urine color clears, and the hemoglobin starts to increase within one week. Because the sudden reduction in intravascular hemolysis ameliorates nitric oxide scavenging, vasodilatation can result in a transient headache soon after the first dose. For the treatment of PNH, eculizumab is given weekly for 4 weeks at a dose of 600 mg, on week 5 the dose is increased to 900 mg, and it is subsequently given again every 2 weeks. During treatment, the percentage of PNH red cells will typically increase due to their improved survival. Discontinuation of the drug can thus trigger an acute attack of severe hemolysis and, in theory, thrombotic events as well. This can be preempted by transfusions and anticoagulation.

Ravulizumab is a second-generation C5 inhibitor. It differs from eculizumab by 4 amino acids, which increases the half-life by about 4 fold[157] (Fig. 32.7). Ravulizumab is given as an initial weight-based dose, then as a maintenance dose 2 weeks later, and then given approximately every 8 weeks afterward. The drug met a non-inferiority endpoint based on hemolysis when compared to eculizumab in 2 clinical trials[158,159] and was approved by the FDA in 2018 for the treatment of adults with PNH. Ravulizumab has not been definitively shown to be as effective as eculizumab in preventing thrombosis,[41,43,44,160] but it may be better at suppressing free C5 levels under certain circumstances, and it is therefore probably at least equivalent.[161] Eculizumab produces a low CH50 test, which can be monitored to verify complement blockade. For technical reasons, this test may not always be sufficiently sensitive to the presence of ravulizumab.[162,163]

Eculizumab or ravulizumab should be offered to any patient with symptomatic anemia regardless of transfusion requirement, renal failure, male erectile dysfunction, episodes of fatigue, abdominal pains, or esophageal spasms. But most significantly, this treatment should be very strongly suggested to any patient with a history of thrombosis if they still have a significant PNH clone, even if they are not symptomatic. Similarly, eculizumab should be used to prevent pregnancy complications in any patient with a significant PNH clone (see Chapter 149).[164] While the definition of “significant” in these two cases is arbitrary, it probably should be lower than the proposed cutoff value of 50% of the granulocytes for most other patients and a value closer to 20% might be reasonable. Because ravulizumab has increased affinity for the neonatal Fc-receptor (FcRn), eculizumab rather than ravulizumab should be given in pregnancy or for any woman who thinks this is a possibility. An additional indication for eculizumab or ravulizumab would be a patient with over 50% PNH granulocytes who cannot be safely anticoagulated, due to risk of falls, their occupation, or athletic pursuits—or a low platelet count.

Two weeks prior to treatment with eculizumab or ravulizumab, patients must be vaccinated with the first dose of a tetravalent meningococcal vaccine that covers strain A,C,W, and Y, such as Menveo or Menactra. Boosters should be given at 2 months and then probably again every 3 years, and it is possible to measure levels of antibodies against these strains. If patients cannot wait the full 2 weeks (the time period needed for a primary immune response) they should start an antibiotic that is effective against *Neisseria* (e.g., ciprofloxacin) with their first dose of the C5 inhibitor, to continue until 2 weeks after the vaccine. Patients should also be vaccinated against meningococcal strain B (with the series of either Bexsero or Trumenba), and then with a booster one year later. However, vaccination alone is clearly not sufficient[165] and the rate of infection remains between 0.5% and 1% per year. Therefore, additional strategies are mandated, for example: (1) penicillin VK 500 mg twice daily, for patients who are not allergic; (2) explicit instructions for patients to carry with them a thermometer and to report to an ER with any temperature over 100°F (37.8°C); (3) a letter addressed to the ER staff outlining the protocol for such patients (blood cultures and prompt administration of iv antibiotics that cover *Neisseria* (such as ceftriaxone); (4) medical alert bracelets. Furthermore, patients on the TRIUMPH study carried with them on their person a filled prescription for ciprofloxacin, which seems a reasonable step to allow for self-administration for the situation of a patient with a fever who is unable to come to the ER, or if the patient might not be seen and given iv antibiotics in a timely manner after arriving in an ER.[43] Of note, patients with complement deficiency who develop meningococcemia generally develop septicemia but not meningitis and almost never require a lumbar puncture. Patients should be made aware that meningococcus can be spread by saliva and reasonable precautions should be taken. Cases of disseminated *Neisseria gonorrhoeae* have been reported and appropriate precautions should be taken to prevent this infection as well.

Patients may have a poor response to eculizumab or ravulizumab therapy for several reasons: (1) the PNH clone is relatively small and their reason for anemia is actually AA/PNH; (2) the patient carries the Arg885His polymorphism in the *C5* gene, as seen in about 3% of the Japanese population, which affects the drug’s binding site on its target[166]; (3) rapid metabolism of the drug, which can be seen in pregnancy and would be evidenced by a rising LDH and a CH50 level that is not completely suppressed at the end of the treatment cycle; (4) breakthrough hemolysis, such that infections can overwhelm the complement cascade even in situations where there are saturating levels of an anti-C5 drug (e.g., via a non-canonical complement activation mechanism[167]); (5) extravascular hemolysis, as evidenced by a high indirect bilirubin and reticulocyte count, and, possibly, a positive DAT. Of note, patients on C5 blockade with extravascular hemolysis can develop iron overload, as evidenced by hyperferritinemia,[168] and therefore iron supplementation should generally be discontinued when initiating anti-complement therapy.[169] Those who develop a serum ferritin over 1000 ng/mL can often be treated with therapeutic phlebotomy; chelation is rarely necessary. Similarly, some patients will require cholecystectomy for bilirubin gallstones (see Fig. 32.1C).[170]

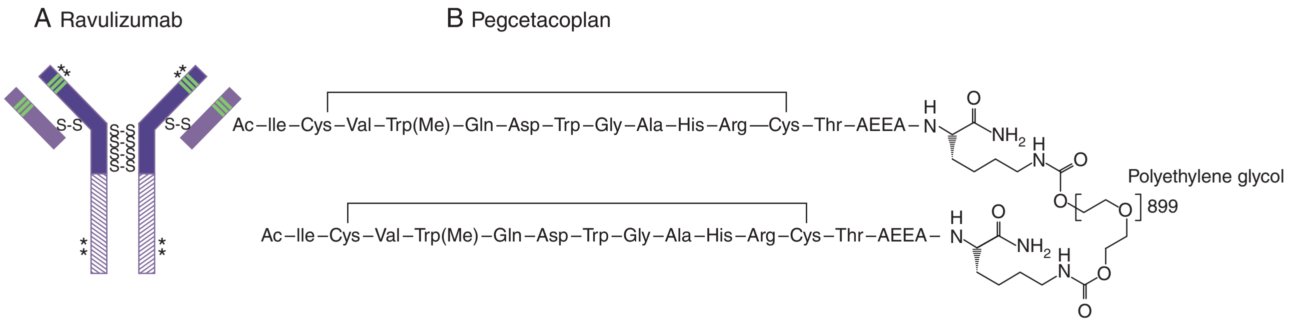

A Ravulizumab
B Pegcetacoplan
S-S
S-S
S-S
S-S
*
*
*
*
Ac–Ile–Cys–Val–Trp(Me)–Gln–Asp–Trp–Gly–Ala–His–Arg—Cys–Thr–AEEA–
Ac–Ile–Cys–Val–Trp(Me)–Gln–Asp–Trp–Gly–Ala–His–Arg–Cys–Thr–AEEA–
H
N
O
NH2
H
N
O
Polyethylene glycol
O
O
899

Figure 32.7 (A) Structure of ravulizumab and eculizumab. The green bars represent mouse CDR sequences from the original mouse hybridoma incorporated into the structure of human immunoglobulin kappa and heavy chains. The heavy chain constant region is derived from a fusion of IgG2 *(solid)* and IgG4 *(hashed)* sequences. Compared with eculizumab, ravulizumab has 4 substitutions *(*)* in the heavy chain: Y27H, S57H in the antigen recognition region and M429L and N435S in the Fc portion of the IgG2/IgG4 chimera. The latter two substitutions increase the affinity for the neonatal Fc receptor, and this increases recycling of the drug from the endosome back to the surface of cells that have taken it up, from which it is released back into the extracellular space and the circulation. The substitutions in the antigen recognition region decrease the affinity of the drug for C5 in the acidic environment of the endosome, which further facilitates this recycling process. This results in a drug that has a half-life that is 4 times as long as eculizumab, allowing for every-8-week dosing. (B) Structure of pegcetacoplan. Disulfide bonds between cysteine residues are shown for the peptides. This sequence is derived from compstatin, which was originally identified by its ability to bind to C3 by phage display. The modified amino acids acetylisoleucine and methyltryptophan are incorporated into this structure. Two C3-binding cyclic peptides are linked to a polyethylene glycol polymer, with a molecular weight of approximately 43.5 kd. AEEA aminoethoxyethoxyacetic acid. *(Illustration after pegcetacoplan package insert.)*

The presence of C3 on the red cell in patients receiving C5 inhibitors predicts persistent anemia and a need for red cell transfusions, which will be required by approximately 1/3 of patients despite C5 blockade.[171] Such patients have inspired the search for complement inhibitors that work upstream of C5 (see Chapter 23). This would prevent the formation of the complement protein fragments that lead to opsonization, such as C3dg, as well as prevent the formation of the membrane attack complex, and thus prevent both intravascular and extravascular hemolysis. Recently, a novel C3 inhibitor, pegcetacoplan, has been approved for the treatment of PNH (see Fig. 32.7). This is a compstatin peptide derivative, coupled to polyethylene glycol,[47] and it is given by a self-administered subcutaneous infusion twice weekly at a dose of 1080 mg, through an infusion pump. Because inherited deficiency of C3 results in susceptibility to a broader range of infections than deficiency in terminal complement proteins, it is required that patients must be vaccinated against not only the meningococcal ACWY and B strains, but also against *Streptococcus pneumonia* and *Haemophilus influenzae* type B. Because it takes several weeks to achieve steady state drug levels, patients who are already on a C5 inhibitor need an overlap period. In a randomized study of 80 patients with PNH who failed to achieve a hemoglobin greater than 10.5 g/dL on eculizumab, transitioning to pegcetacoplan was superior to remaining on eculizumab for its effect on hemoglobin (a mean increase of 3.8 g/dL), and for achieving red cell transfusion independence (85% vs. 15%). Not surprisingly, fatigue scores improved. Unlike patients on eculizumab, reticulocyte counts normalized in most patients on pegcetacoplan. The most common side effects are injection site reactions and diarrhea. Although the follow-up was short, patients on pegcetacoplan did not develop meningococcemia or thrombosis, though there was one episode of sepsis in a patient during the period of transition from eculizumab to pegcetacoplan. Based on this data, pegcetacoplan was approved in May 2021 for the treatment of PNH, regardless of prior treatment with a C5 inhibitor.

It should be said that the rate of thrombosis in patients treated with pegcetacoplan is not yet known. Presumably, the effects of the drug on platelets will be similar to the effects on red cells, and it may be essentially equivalent to eculizumab, or perhaps even superior. However, coagulation proteins can in theory activate complement downstream of C3 (e.g., thrombin can activate C5 even in the absence of C3,[172] and factor IXa, Xa, XIa, thrombin, and plasmin can all activate C5 as well as C3[173]), and one can posit situations where a vicious cycle occurs involving platelet activation and C5b-9 deposition, which could bypass an upstream inhibitor of complement. It will therefore be critical to re-evaluate data on thrombosis after a sufficient number of patient-exposure years are observed, to allow historical comparisons with untreated patients and those on eculizumab.[44] Similarly, post marketing data on infections will be critical in determining the ultimate place of pegcetacoplan in the treatment of patients with PNH.

Until then, in addition to ascertaining patients' preferences for the route and frequency of administration, the following points must be considered: (1) any patient who is on C5 inhibition who is symptomatic with extravascular hemolysis, resulting in anemia, iron overload, or gallstones, is likely to benefit from switching to pegcetacoplan[47]; (2) those who have had thromboses need to be advised that there is insufficient data to know that C3 inhibition will prevent thrombosis. Some patients and physicians will conclude that it is safer to wait for additional information before switching away from a C5 inhibitor; (3) similarly, those who have had a splenectomy or are on cyclosporin should be cautioned that the infectious risks of pegcetacoplan compared to C5 inhibition are not known—and there is theoretical reasons why it might be more immunosuppressive; (4) for those who have not had a thrombosis and would be a good candidate to switch to pegcetacoplan, they also should be cautioned about the points regarding thrombosis and infection, and any patient who is thought to be at high risk (e.g., a carrier of the factor V Leyden allele) should probably be offered concurrent anticoagulation at the time of switching to pegcetacoplan. (Indeed, this option should probably be discussed with all patients until more information is available.) For previously untreated patients, these points must also be considered, but they of course have an additional option: trying a C5 inhibitor first and switching to pegcetacoplan if they have a sub-optimal response.

Transplant

Allogenic bone marrow transplantation (allo-BMT) is the only curative therapy for PNH, but is associated with significant morbidity and mortality.[174] Antin et al. reported on successful outcomes in four patients without any recurrence of PNH, which was notable since three of them received a regimen (procarbazine, cyclophosphamide, and antithymocyte serum) that would not be considered myeloablative.[106] The International Bone Marrow Transplant Registry (IBMTR) reported a 2-year survival probability of 56% in 48 recipients of HLA-identical sibling transplants between 1978 and 1995. The European Blood and Marrow Transplant group reported a 5-year survival rate of 70% following allo-BMT for PNH; however, only 54% met criteria for classic PNH. Graft failure occurred in 6% of patients and acute and chronic graft-versus-host disease occurred in 15% and 20% of patients, respectively.

More recently, the Gruppo Italiano Trapianto Midollo Osseo (GITMO) published a retrospective study of 26 patients (median age, 32 years) who received an allo-BMT for PNH (4 AA/PNH) in Italy between 1988 and 2006. HLA-matched sibling donors were used as a stem cell source for 22 patients; there was one patient who received stem cells from a matched unrelated donor and 3 who received stem cells from mismatched donors (2 related and one unrelated). A myeloablative conditioning regimen (busulfan + cyclophosphamide) was used for 15 of the patients. The remaining 11 received a variety of different reduced intensity conditioning regimens, most being cyclophosphamide or fludarabine based. The 10-year probability of survival was 57% for all patients, with a median follow-up of 131 months. There was one primary graft failure in a patient receiving a myeloablative conditioning regimen and one secondary graft failure in a patient who received a non-myeloablative allo-BMT; both patients eventually died from complications of a second transplant. Acute graft-versus-host-disease (aGVHD) greater than stage 2 occurred in 3 of the 26 patients; chronic GVHD (cGVHD) occurred in 10 of 20 evaluable patients with four (16%) experiencing extensive cGVHD. The transplant-related mortality at one year was 26% in patients receiving a myeloablative conditioning regimen and 63% in the group that received a non-myeloablative regimen; however, this was likely due to the fact that all three patients in the non-myeloablative group received a graft from a non-identical donor.

Peffault de Latour et al. have reported on matched pair analysis and concluded that patients with PNH with a history of thromboembolism who are transplanted fare worse than those who are not transplanted,[174] which was not seen for the group transplanted for aplasia. Recent developments such as post-transplant high-dose cyclophosphamide may mitigate the risk of GVHD.[175] Particularly, post-transplant cyclophosphamide with a non-myeloablative regimen of cyclophosphamide, fludarabine, ATG, and TBI provided excellent results for relapsed/refractory as well as treatment-naïve patients receiving a haploidentical graft for aplastic anemia, with a cumulative incidence of grade II-IV acute GVHD at day 100 of 11% and a cumulative index of chronic GVHD at 2 years of 8%.[176] This promising approach is likely to greatly increase the proportion of patients who would have a suitable donor and may be applicable to patients with PNH. Of note, eculizumab can serve as a "bridge" to transplant, and this may decrease the reactions associated with the conditioning regimen.[177]

In summary, allo-BMT should not be offered as initial therapy for most patients with classic PNH given the morbidity and mortality. Exceptions are PNH patients in countries where eculizumab is not available or patients who do not have a good response to anticomplement therapies. Patients with severe AA/PNH and life-threatening cytopenias continue to be reasonable candidates for allo-BMT, if they meet criteria based on their age and neutrophil count. As mentioned above, however, the presence of the PNH clone would predict a good response to the alternative—immunosuppression. A myeloablative conditioning regimen is not required to eradicate the PNH clone. Whether or not there is an advantage to one approach over the other will require further study; however, non-myeloablative regimens may be preferable in young patients seeking to maintain fertility or patients with moderate organ dysfunction who may not tolerate a myeloablative regimen. Lastly, since allo-BMT is the only curative therapy available for PNH, continued use and investigation of this approach in selected patients is reasonable. As noted below, transformation to MDS or AML would require stem cell transplantation to achieve long-term survival.

Transformation to MDS/AML

The development of AML in PNH patients is well reported in the literature[178]; the risk is probably of the order of 2% and is lower than the rate of leukemia occurring in patients with aplastic anemia.[124] However, this is much higher than the rates in the general population and so PNH must be considered, like aplastic anemia, to be a preleukemic condition. Interestingly, the leukemic blasts can be of either a GPI-positive or a GPI-negative phenotype. The simplest model is a corollary of the immune escape model: an abnormal marrow environment that selects for a variety of clones that are able to withstand the immune attack on the marrow through a variety of mechanisms. In many cases there will be an intermediary stage of myelodysplasia with excess blasts, and the challenge is to identify this subset of patients in a timely manner. Therefore, any patient whose peripheral blood counts decline after a period of stability, or demonstrate an increase in basophils, immature myeloid cells, or blasts in the peripheral blood should have a bone marrow sampling procedure performed.

Pregnancy

Pregnancy in patients with PNH was once fraught with complications for the mother and fetus. There are probably 3 reasons: (1) hormonal effects on the production of clotting factors and inhibitors of coagulation in the liver; (2) the gravid uterus compressing intra-abdominal veins; (3) the known effects of pregnancy on lowering the blood counts and inducing relapses of aplastic anemia. Pregnancy now is much safer in women treated with anticomplement therapy together with an anticoagulant. For both classes of drugs, we are limited to those that will not cross the placenta: eculizumab and either low-molecular-weight heparin or fondaparinux are the options. The half-life of eculizumab and low-molecular-weight heparin can decrease during the pregnancy—this should be monitored with trough CH50 assays and peak anti Xa assays respectively, and the doses (or, for eculizumab, the dosing interval) should be adjusted accordingly. Labor should be induced before these pregnancies go past their due dates. Issues related to maintenance of hemoglobin levels to ensure adequate transplacental oxygen delivery, as well as the effect of thrombocytopenia and anticoagulation on bleeding risks during delivery and C-sections, and how these factors impact decisions regarding the timing and safety of epidural anesthesia are absolutely critical and are beyond the scope of this chapter. Since thrombosis risk continues for weeks after pregnancy,[179] anticoagulation, if interrupted for delivery, should be resumed as soon as possible and should be given, together with anticomplement therapy, probably for at least 2 months postpartum. Since eculizumab can often be given only as an outpatient, timing is critical to avoid missing doses.

Pregnancy can induce relapses and worsening cytopenias in those with a history of aplastic anemia.[180] This is particularly a problem in women whose platelet counts fall below the point at which it is safe to continue anticoagulation. While cyclosporin is probably safe in pregnancy, there is inadequate information about ATG and eltrombopag. Prior to any planned pregnancy, in anticipation of falling blood counts, it is best to perform a marrow procedure to rule out progression to MDS/AML. The decision to conceive must be made in consultation with a high-risk obstetrician, and delivery should planned at a hospital with a neonatal intensive care unit (ICU). Women with PNH who are considering conception should be counselled that there is a possibility that they will be advised to undergo premature induction of labor or to terminate the pregnancy in the event of life-threatening aplasia or thrombotic complications. In the era of surrogacy, all reproductive options should be discussed since the risks of an oocyte collection procedure are likely to be less than for a pregnancy.

MONITORING/FOLLOW-UP OF PATIENTS

Given time, there are three possible outcomes: (1) stability in the size of the PNH clone; (2) further expansion of the PNH clone; (3) spontaneous regression of the PNH clone.[105] A patient who does not initially require either anticomplement therapy or anticoagulation prophylaxis needs to be monitored regularly, with flow cytometry performed perhaps yearly and any time there is an increase in the LDH (measured as an easy surrogate for the PNH red cell clone). Conversely, those who are on anticoagulation or anticomplement therapy should be monitored to see if the clone is regressing to the point where such therapy is no longer needed. Thrombosis should be detected and treated as soon as possible by having a low threshold for imaging of any symptomatic complaint suggestive of an occlusive event, and education of the patient about warning symptoms is important. Likewise, there should be a low threshold for repeating a bone marrow sampling to promptly identify the rare patients who go on to develop MDS/AML.

SUGGESTED READINGS

Araten DJ, Boxer D, Zamechek L, et al. Analysis of platelets by flow cytometry in patients with paroxysmal nocturnal hemoglobinuria (PNH). *Blood Cells Mol Dis*. 2020;80:102372.

Badri P, Jiang X, Borodovsky A, et al. Pharmacokinetic and pharmacodynamic properties of cemdisiran, an RNAi therapeutic targeting complement component 5, in healthy subjects and patients with paroxysmal nocturnal hemoglobinuria. *Clin Pharmacokinet*. 2021;60:365–378.

Brodsky RA. How I treat paroxysmal nocturnal hemoglobinuria. *Blood*. 2021;137:1304–1309.

Crew PE, Abara WE, McCulley L, et al. Disseminated gonococcal infections in patients receiving eculizumab: a case series. *Clin Infect Dis*. 2019;69:596–600.

Crew PE, McNamara L, Waldron PE, et al. Antibiotic prophylaxis in vaccinated eculizumab recipients who developed meningococcal disease. *J Infect*. 2020;80:350–371.

DeZern AE, Jones RJ, Brodsky RA. Eculizumab bridging before bone marrow transplant for marrow failure disorders is safe and does not limit engraftment. *Biol Blood Marrow Transplant*. 2018;24:e26–e30.

Dragoni F, Chiarotti F, Iori AP, et al. Antithrombotic therapy with rivaroxaban in five patients with paroxysmal nocturnal haemoglobinuria and thrombotic events. *Thromb J*. 2018;16:26.

Fattizzo B, Ireland R, Dunlop A, et al. Clinical and prognostic significance of small paroxysmal nocturnal hemoglobinuria clones in myelodysplastic syndrome and aplastic anemia. *Leukemia*. 2021

Gorman DM, Lee J, Payne CD, et al. Chemical synthesis and characterisation of the complement C5 inhibitory peptide zilucoplan. *Amino Acids*. 2021;53:143–147.

Griffin M, Hillmen P, Munir T, et al. Significant hemolysis is not required for thrombosis in paroxysmal nocturnal hemoglobinuria. *Haematologica*. 2019;104:e94–e96.

Harder MJ, Kuhn N, Schrezenmeier H, et al. Incomplete inhibition by eculizumab: mechanistic evidence for residual C5 activity during strong complement activation. *Blood*. 2017;129:970–980.

Hillmen P, Szer J, Weitz I, et al. Pegcetacoplan versus eculizumab in paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 2021;384:1028–1037.

Kawamoto M, Murakami Y, Kinoshita T, et al. Recurrent aseptic meningitis with PIGT mutations: a novel pathogenesis of recurrent meningitis successfully treated by eculizumab. *BMJ Case Rep*. 2018:2018.

Kelly RJ, Hochsmann B, Szer J, et al. Eculizumab in pregnant patients with paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 2015;373:1032–1039.

Kim MS, Prasad V. The clinical trials portfolio for on-label and off-label studies of eculizumab. *JAMA Intern Med*. 2020;180:315–317.

Kulasekararaj AG, Hill A, Rottinghaus ST, et al. Ravulizumab (ALXN1210) vs eculizumab in C5-inhibitor-experienced adult patients with PNH: the 302 study. *Blood*. 2019;133:540–549.

Kulesekararaj A, Risitano AM, Maciejewski JP, et al. Phase 2 study of danicopan in paroxysmal nocturnal hemoglobinuria patients with an inadequate response to eculizumab. *Blood*. 2021

Langemeijer S, Schaap C, Preijers F, et al. Paroxysmal nocturnal hemoglobinuria caused by CN-LOH of constitutional PIGB mutation and 70-kbp microdeletion on 15q. *Blood Adv*. 2020;4:5755–5761.

Lee JW, Sicre de Fontbrune F, Wong LLL, et al. Ravulizumab (ALXN1210) vs eculizumab in adult patients with PNH naive to complement inhibitors: the 301 study. *Blood*. 2019;133:530–539.

Lobry C, Bains A, Zamechek LB, et al. Analysis of TET2 mutations in paroxysmal nocturnal hemoglobinuria (PNH). *Exp Hematol Oncol*. 2019;8:17.

Luzzatto L, Karadimitris A. Paroxysmal nocturnal haemoglobinuria (PNH): novel therapies for an ancient disease. *Br J Haematol*. 2020;191:579–586.

Mannes M, Dopler A, Zolk O, et al. Complement inhibition at the level of C3 or C5: mechanistic reasons for ongoing terminal pathway activity. *Blood*. 2021;137:443–455.

Matsumura Y. Risk analysis of eculizumab-related meningococcal disease in Japan using the Japanese adverse drug event report database. *Drug Healthc Patient Saf*. 2020;12:207–215.

Risitano AM, Kulasekararaj AG, Lee JW, et al. Danicopan: an oral complement factor D inhibitor for paroxysmal nocturnal hemoglobinuria. *Haematologica*. 2020 Online ahead of print.

Roth A, Nishimura JI, Nagy Z, et al. The complement C5 inhibitor crovalimab in paroxysmal nocturnal hemoglobinuria. *Blood*. 2020;135:912–920.

Schols S, Nunn MA, Mackie I, et al. Successful treatment of a PNH patient non-responsive to eculizumab with the novel complement C5 inhibitor coversin (nomacopan). *Br J Haematol*. 2020;188:334–337.

Shin TH, Baek EJ, Corat MAF, et al. CRISPR/Cas9 PIG—a gene editing in nonhuman primate model demonstrates no intrinsic clonal expansion of PNH HSPCs. *Blood*. 2019;133:2542–2545.

Sun L, Babushok DV. Secondary myelodysplastic syndrome and leukemia in acquired aplastic anemia and paroxysmal nocturnal hemoglobinuria. *Blood*. 2020;136:36–49.

REFERENCES

1. Rosse W. A brief history of PNH. In: Young N, Moss J, eds. *PNH and GPI-Linked Proteins*. Academic Press;; 2000:1–20.
2. Gull W. A case of intermittent haematinuria, with remarks. *Guy's Hospital Rep*. 18666;12:381–392.
3. Strubing P. Paroxysmale hamoglobinurie. *Dtche Med Wochenschr*. 1882;8:1–8.
4. vandenBergh H. Ictere hemolytique avec crises hemoglobinuriques. Fragilite globuliare. *Rev Med*. 1911;31:63–69.
5. Marchiafava E. Anemia emolitica con emosiderinuria perpetua. *Policlinico [Med]*. 1928;35:105–117.
6. Micheli F. Uno caso di anemia emolitica con emosiderinuria perpetua. *G Accad Med Torino*. 1931;13:148.
7. Enneking J. Eine neue form intemittierender haemoglobinurie (hemoglobinuria paroxysmalis noctura). *Klin Wschr*. 1928;7:2045–2047.
8. Dacie J, Israels M, Wilkinson J. Paroxysmal nocturnal hemoglobinuria of the marchiafava type. *Lancet*. 1939;1:479–482.
9. Ham T, Dingle J. Studies on destruction of red blood cells. II. Chronic hemolytic anemia with paroxysmal nocturnal hemoglobinuria: certain immunological aspects of the hemolytic mechanism with special reference to serum complement. *J Clin Invest*. 1938;18:657–672.
10. Jordan F. Etudes sur l'hemoglobinurie. *Acta Med Scand*. 1938;95:319–340.
11. May J, Rosse W, Frank M. Paroxysmal nocturnal hemoglobinuria. alternative complement-pathway-mediated lysis induced by magnesium. *N Engl J Med*. 1973;289:705–709.
12. Rosse WF, Dacie JV. Immune lysis of normal human and paroxysmal nocturnal hemoglobinuria (PNH) red blood cells. I. The sensitivity of PNH red cells to lysis by complement and specific antibody. *J Clin Invest*. 1966;45:736–748.
13. Rosse WF, Dacie JV. Immune lysis of normal human and paroxysmal nocturnal hemoglobinuria (PNH) red blood cells. II. The role of complement components in the increased sensitivity of PNH red cells to immune lysis. *J Clin Invest*. 1966;45:749–757.
14. Oni SB, Osunkoya BO, Luzzatto L. Paroxysmal nocturnal hemoglobinuria: evidence for monoclonal origin of abnormal red cells. *Blood*. 1970;36:145–152.
15. Auditore J, Hartmann R, Flexner R, et al. The erythrocyte acetylcholinesterase enzyme in paroxysmal nocturnal hemoglobinuria. *Arch Pathol*. 1960;69:534–540.
16. Beck W, Valentine W. Biochemical studies on leucocytes II phosphatase activity in chronic lymphatic leukemia, acute leukemia, and miscellaneous hematologic conditions. *J Lab Clin Med*. 1951;38:245–253.
17. Lewis S, Dacie J. Neutrophil (leucocyte) alkaline phosphatase in paroxysmal nocturnal hemoglobinuria. *Br J Haematol*. 1965;11:549–557.
18. Hoffman EM. Inhibition of complement by a substance isolated from human erythrocytes. I. Extraction from human erythrocyte stromata. *Immunochemistry*. 1969;6:391–403.
19. Hoffmann EM. Inhibition of complement by a substance isolated from human erythrocytes. II. Studies on the site and mechanism of action. *Immunochemistry*. 1969;6:405–419.
20. Nicholson-Weller A, Burge J, Fearon DT, et al. Isolation of a human erythrocyte membrane glycoprotein with decay-accelerating activity for C3 convertases of the complement system. *J Immunol*. 1982;129:184–189.
21. Nicholson-Weller A, March JP, Rosenfeld SI, et al. Affected erythrocytes of patients with paroxysmal nocturnal hemoglobinuria are deficient in the complement regulatory protein, decay accelerating factor. *Proc Natl Acad Sci USA*. 1983;80:5066–5070.
22. Nicholson-Weller A, Spicer DB, Austen KF. Deficiency of the complement regulatory protein, "decay-accelerating factor," on membranes of granulocytes, monocytes, and platelets in paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 1985;312:1091–1097.
23. Nicholson-Weller A, Wang CE. Structure and function of decay accelerating factor CD55. *J Lab Clin Med*. 1994;123:485–491.
24. Holguin MH, Fredrick LR, Bernshaw NJ, et al. Isolation and characterization of a membrane protein from normal human erythrocytes that inhibits reactive lysis of the erythrocytes of paroxysmal nocturnal hemoglobinuria. *J Clin Invest*. 1989;84:7–17.
25. Holguin MH, Wilcox LA, Bernshaw NJ, et al. Relationship between the membrane inhibitor of reactive lysis and the erythrocyte phenotypes of paroxysmal nocturnal hemoglobinuria. *J Clin Invest*. 1989;84:1387–1394.
26. Holguin MH, Wilcox LA, Bernshaw NJ, et al. Erythrocyte membrane inhibitor of reactive lysis: effects of phosphatidylinositol-specific phospholipase C on the isolated and cell-associated protein. *Blood*. 1990;75:284–289.
27. Meri S, Morgan BP, Davies A, et al. Human protectin (CD59), an 18,000–20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers. *Immunology*. 1990;71:1–9.
28. Nose M, Katoh M, Okada N, et al. Tissue distribution of HRF20, a novel factor preventing the membrane attack of homologous complement, and its predominant expression on endothelial cells in vivo. *Immunology*. 1990;70:145–149.
29. Sugita Y, Nakano Y, Oda E, et al. Determination of carboxyl-terminal residue and disulfide bonds of MACIF (CD59), a glycosyl-phosphatidylinositol-anchored membrane protein. *J Biochem*. 1993;114:473–477.
30. Davitz MA, Low MG, Nussenzweig V. Release of decay-accelerating factor (DAF) from the cell membrane by phosphatidylinositol-specific phospholipase C (PIPLC). Selective modification of a complement regulatory protein. *J Exp Med*. 1986;163:1150–1161.
31. Okazaki I, Moss J. The function of GPI-anchored proteins. In: Young N, Moss J, eds. *PNH and the GPI-Linked Proteins*. Academic Press; 2000.
32. Takahashi M, Takeda J, Hirose S, et al. Deficient biosynthesis of N-acetylglucosaminyl-phosphatidylinositol, the first intermediate of glycosyl phosphatidylinositol anchor biosynthesis, in cell lines established from patients with paroxysmal nocturnal hemoglobinuria. *J Exp Med*. 1993;177:517–521.
33. Miyata T, Yamada N, Iida Y, et al. Abnormalities of PIG-A transcripts in granulocytes from patients with paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 1994;330:249–255.
34. Borowitz MJ, Craig FE, Digiuseppe JA, et al. Guidelines for the diagnosis and monitoring of paroxysmal nocturnal hemoglobinuria and related disorders by flow cytometry. *Cytometry B Clin Cytom*. 2010;78:211–230.
35. Rotoli B, Luzzatto L. Paroxysmal nocturnal haemoglobinuria. *Baillieres Clin Haematol*. 1989;2:113–138.
36. Dacie JV, Lewis SM. Paroxysmal nocturnal haemoglobinuria: clinical manifestations, haematology, and nature of the disease. *Ser Haematol*. 1972;5:3–23.
37. Dameshek W. Riddle: what do aplastic anemia, paroxysmal nocturnal hemoglobinuria (PNH) and "hypoplastic": leukemia have in common? *Blood*. 1967;30:241–254.
38. Young NS. The problem of clonality in aplastic anemia: Dr Dameshek's riddle, restated. *Blood*. 1992;79:1385–1392.
39. Crosby W. Paroxysmal nocturnal hemoglobinuria. *Blood*. 1953;8:769–812.
40. Hall C, Richards S, Hillmen P. Primary prophylaxis with warfarin prevents thrombosis in paroxysmal nocturnal hemoglobinuria (PNH). *Blood*. 2003;102:3587–35891.
41. Hillmen P, Hall C, Marsh JC, et al. Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 2004;350:552–559.
42. Rother RP, Rollins SA, Mojcik CF, et al. Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria. *Nat Biotechnol*. 2007;25:1256–1264.
43. Hillmen P, Young NS, Schubert J, et al. The complement inhibitor eculizumab in paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 2006;355:1233–1243.
44. Hillmen P, Muus P, Duhrsen U, et al. Effect of the complement inhibitor eculizumab on thromboembolism in patients with paroxysmal nocturnal hemoglobinuria. *Blood*. 2007;110:4123–4128.
45. Kelly RJ, Hill A, Arnold LM, et al. Long-term treatment with eculizumab in paroxysmal nocturnal hemoglobinuria: sustained efficacy and improved survival. *Blood*. 2011;117:6786–6792.
46. Risitano AM, Notaro R, Marando L, et al. Complement fraction 3 binding on erythrocytes as additional mechanism of disease in paroxysmal nocturnal hemoglobinuria patients treated by eculizumab. *Blood*. 2009;113:4094–4100.
47. Hillmen P, Szer J, Weitz I, et al. Pegcetacoplan versus eculizumab in paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 2021;384:1028–1037.
48. Hill A, Platts P, Smith A, et al. The incidence and prevalence of paroxysmal nocturnal hemoglobinuria (PNH) and survival of patients in Yorkshire. *Blood*. 2006;108:985.
49. Kruatrachue M, Wasi P, Na-Nakorn S. Paroxysmal nocturnal haemoglobinuria in Thailand with special reference to as association with aplastic anaemia. *Br J Haematol*. 1978;39:267–276.
50. Issaragrisil S, Kaufman DW, Anderson T, et al. The epidemiology of aplastic anemia in Thailand. *Blood*. 2006;107:1299–1307.
51. Hillmen P, Lewis SM, Bessler M, et al. Natural history of paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 1995;333:1253–1258.
52. Wainwright L, Brodsky RA, Erasmus LK, et al. Paroxysmal nocturnal hemoglobinuria arising from Fanconi anemia. *J Pediatr Hematol Oncol*. 2003;25:167–168.
53. Hoekstra J, Leebeek FW, Plessier A, et al. Paroxysmal nocturnal hemoglobinuria in Budd-Chiari syndrome: findings from a cohort study. *J Hepatol*. 2009;51:696–706.

54. Schrezenmeier H, Roth A, Araten DJ, et al. Baseline clinical characteristics and disease burden in patients with paroxysmal nocturnal hemoglobinuria (PNH): updated analysis from the International PNH Registry. *Ann Hematol*. 2020;99:1505–1514.
55. Curran KJ, Kernan NA, Prockop SE, et al. Paroxysmal nocturnal hemoglobinuria in pediatric patients. *Pediatr Blood Cancer*. 2012;59: 525–529.
56. Hsieh F, Chen T, Lee J. Severe cutaneous thrombosis with hemorrhagic necrosis in a patient with paroxysmal nocturnal hemoglobinuria: a case report and review of literature. *Dermatologica Sinica*. 2017;35:138–141.
57. Araten DJ, Swirsky D, Karadimitris A, et al. Cytogenetic and morphological abnormalities in paroxysmal nocturnal haemoglobinuria. *Br J Haematol*. 2001;115:360–368.
58. Maciejewski JP, Risitano A, Sloand EM, et al. Distinct clinical outcomes for cytogenetic abnormalities evolving from aplastic anemia. *Blood*. 2002;99:3129–3135.
59. Dameshek W, Fudenberg H. Paroxysmal nocturnal hemoglobinuria. *AMA Arch Intern Med*. 1957;99:202–208.
60. Luzzatto L, Familusi JB, Williams CK, et al. The PNH abnormality in myeloproliferative disorders: association of PNH and acute erythremic myelosis in two children. *Haematologica*. 1979;64:13–30.
61. Nakahata J, Takahashi M, Fuse I, et al. Paroxysmal nocturnal hemoglobinuria with myelofibrosis: progression to acute myeloblastic leukemia. *Leuk Lymphoma*. 1993;12:137–142.
62. Sugimori C, Padron E, Caceres G, et al. Paroxysmal nocturnal hemoglobinuria and concurrent JAK2 (V617F) mutation. *Blood Cancer J*. 2012;2:e63.
63. Ferguson M, Hart G, Kinoshita T. Glycosylphosphatidylinositol anchors. In: Varki A, Esko J, eds. *Essentials of Glycobiology*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 2015–2017.
64. Kinoshita T. Biosynthesis and biology of mammalian GPI-anchored proteins. *Open Biol*. 2020;10:190290.
65. Murakami Y, Siripanyaphinyo U, Hong Y, et al. The initial enzyme for glycosylphosphatidylinositol biosynthesis requires PIG-Y, a seventh component. *Mol Biol Cell*. 2005;16:5236–5246.
66. Watanabe R, Murakami Y, Marmor MD, et al. Initial enzyme for glycosylphosphatidylinositol biosynthesis requires PIG-P and is regulated by DPM2. *EMBO J*. 2000;19:4402–4411.
67. Nagarajan S, Brown CJ, Medof ME. Identification of a PIG-A related processed gene on chromosome 12. *Hum Genet*. 1995;95:691–697.
68. Carrel L, Willard HF. X-inactivation profile reveals extensive variability in X-linked gene expression in females. *Nature*. 2005;434:400–404.
69. Takeda J, Miyata T, Kawagoe K, et al. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. *Cell*. 1993;73:703–711.
70. Iida Y, Takeda J, Miyata T, et al. Characterization of genomic PIG-A gene: a gene for glycosylphosphatidylinositol-anchor biosynthesis and paroxysmal nocturnal hemoglobinuria. *Blood*. 1994;83:3126–3131.
71. Eisenberg E, Levanon EY. Human housekeeping genes, revisited. *Trends Genet*. 2013;29:569–574.
72. Luzzatto L, Nafa K. Genetics of PNH. In: Young N, Moss J, eds. *PNH and GPI-Linked Proteins*. Academic Press; 2000:21–47.
73. O'Keefe CL, Sugimori C, Afable M, et al. Deletions of Xp22.2 including PIG-A locus lead to paroxysmal nocturnal hemoglobinuria. *Leukemia*. 2011;25:379–832.
74. Nafa K, Bessler M, Castro-Malaspina H, et al. The spectrum of somatic mutations in the PIG-A gene in paroxysmal nocturnal hemoglobinuria includes large deletions and small duplications. *Blood Cells Mol Dis*. 1998;24:370–384.
75. Nafa K, Bessler M, Deeg HJ, et al. New somatic mutation in the PIG-A gene emerges at relapse of paroxysmal nocturnal hemoglobinuria. *Blood*. 1998;92:3422–3427.
76. Araten DJ, Golde DW, Zhang RH, et al. A quantitative measurement of the human somatic mutation rate. *Cancer Res*. 2005;65:8111–8117.
77. Araten DJ, Nafa K, Pakdeesuwan K, et al. Clonal populations of hematopoietic cells with paroxysmal nocturnal hemoglobinuria genotype and phenotype are present in normal individuals. *Proc Natl Acad Sci USA*. 1999;96:5209–5214.
78. Dertinger SD, Bhalli JA, Roberts DJ, et al. Recommendations for conducting the rodent erythrocyte Pig-a assay: a report from the HESI GTTC Pig-a Workgroup. *Environ Mol Mutagen*. 2021;62:227–237.
79. Peruzzi B, Araten DJ, Notaro R, et al. The use of PIG-A as a sentinel gene for the study of the somatic mutation rate and of mutagenic agents in vivo. *Mutat Res*. 2010;705:3–10.
80. Brodsky RA, Mukhina GL, Nelson KL, et al. Resistance of paroxysmal nocturnal hemoglobinuria cells to the glycosylphosphatidylinositol-binding toxin aerolysin. *Blood*. 1999;93:1749–1756.
81. Brosh R, Laurent JM, Ordonez R, et al. A versatile platform for locus-scale genome rewriting and verification. *Proc Natl Acad Sci USA*. 2021:118.
82. Li D, Sun X, Yu F, et al. Application of counter-selectable marker PIGA in engineering designer deletion cell lines and characterization of CRISPR deletion efficiency. *Nucleic Acids Res*. 2021;49:2642–2654.
83. Hochsmann B, Murakami Y, Osato M, et al. Complement and inflammasome overactivation mediates paroxysmal nocturnal hemoglobinuria with autoinflammation. *J Clin Invest*. 2019;129: 5123–5136.
84. Kawamoto M, Murakami Y, Kinoshita T, et al. Recurrent aseptic meningitis with PIGT mutations: a novel pathogenesis of recurrent meningitis successfully treated by eculizumab. *BMJ Case Rep*. 2018;2018
85. Krawitz PM, Hochsmann B, Murakami Y, et al. A case of paroxysmal nocturnal hemoglobinuria caused by a germline mutation and a somatic mutation in PIGT. *Blood*. 2013;122:1312–1315.
86. Langemeijer S, Schaap C, Preijers F, et al. Paroxysmal nocturnal hemoglobinuria caused by CN-LOH of constitutional PIGB mutation and 70-kbp microdeletion on 15q. *Blood Adv*. 2020;4:5755–5761.
87. Belet S, Fieremans N, Yuan X, et al. Early frameshift mutation in PIGA identified in a large XLID family without neonatal lethality. *Human Mutation*. 2014;35:350–355.
88. Johnston JJ, Gropman AL, Sapp JC, et al. The phenotype of a germline mutation in PIGA: the gene somatically mutated in paroxysmal nocturnal hemoglobinuria. *Am J Human Genet*. 2012;90:295–300.
89. Kato M, Saitsu H, Murakami Y, et al. PIGA mutations cause early-onset epileptic encephalopathies and distinctive features. *Neurology*. 2014;82:1587–1596.
90. Swoboda KJ, Margraf RL, Carey JC, et al. A novel germline PIGA mutation in ferro-cerebro-cutaneous syndrome: a neurodegenerative X-linked epileptic encephalopathy with systemic iron-overload. *Am J Med Genet A*. 2014;164A:17–28.
91. van der Crabben SN, Harakalova M, Brilstra EH, et al. Expanding the spectrum of phenotypes associated with germline PIGA mutations: a child with developmental delay, accelerated linear growth, facial dysmorphisms, elevated alkaline phosphatase, and progressive CNS abnormalities. *Am J Med Genet A*. 2014;164A:29–35.
92. Almeida AM, Murakami Y, Baker A, et al. Targeted therapy for inherited GPI deficiency. *N Engl J Med*. 2007;356:1641–1647.
93. Almeida AM, Murakami Y, Layton DM, et al. Hypomorphic promoter mutation in PIGM causes inherited glycosylphosphatidylinositol deficiency. *Nat Med*. 2006;12:846–851.
94. Rondelli T, Risitano AM, Peffault de Latour R, et al. Polymorphism of the complement receptor 1 gene correlates with the hematologic response to eculizumab in patients with paroxysmal nocturnal hemoglobinuria. *Haematologica*. 2014;99:262–266.
95. Yonemura Y, Kawakita M, Koito A, et al. Paroxysmal nocturnal haemoglobinuria with coexisting deficiency of the ninth component of complement: lack of massive haemolytic attack. *Br J Haematol*. 1990;74:108–113.
96. Chen R, Knez JJ, Merrick WC, et al. Comparative efficiencies of C-terminal signals of native glycophosphatidylinositol (GPI)-anchored proproteins in conferring GPI-anchoring. *J Cell Biochem*. 2001;84:68–83.
97. Hochsmann B, Dohna-Schwake C, Kyrieleis HA, et al. Targeted therapy with eculizumab for inherited CD59 deficiency. *N Engl J Med*. 2014;370:90–92.
98. Motoyama N, Okada N, Yamashina M, et al. Paroxysmal nocturnal hemoglobinuria due to hereditary nucleotide deletion in the HRF20 (CD59) gene. *Eur J Immunol*. 1992;22:2669–2673.
99. Ozen A, Comrie WA, Ardy RC, et al. CD55 deficiency, early-onset protein-losing enteropathy, and thrombosis. *N Engl J Med*. 2017;377:52–61.
100. Luzzatto L, Bessler M, Rotoli B. Somatic mutations in paroxysmal nocturnal hemoglobinuria: a blessing in disguise? *Cell*. 1997;88:1–4.
101. Maciejewski JP, Follmann D, Nakamura R, et al. Increased frequency of HLA-DR2 in patients with paroxysmal nocturnal hemoglobinuria and the PNH/aplastic anemia syndrome. *Blood*. 2001;98:3513–3519.
102. Dunn DE, Tanawattanacharoen P, Boccuni P, et al. Paroxysmal nocturnal hemoglobinuria cells in patients with bone marrow failure syndromes. *Ann Intern Med*. 1999;131:401–408.
103. Keller P, Payne JL, Tremml G, et al. FES-Cre targets phosphatidylinositol glycan class A (PIGA) inactivation to hematopoietic stem cells in the bone marrow. *J Exp Med*. 2001;194:581–589.
104. Keller P, Tremml G, Rosti V, et al. X inactivation and somatic cell selection rescue female mice carrying a Piga-null mutation. *Proc Natl Acad Sci USA*. 1999;96:7479–7483.
105. Araten DJ, Bessler M, McKenzie S, et al. Dynamics of hematopoiesis in paroxysmal nocturnal hemoglobinuria (PNH): no evidence for intrinsic growth advantage of PNH clones. *Leukemia*. 2002;16:2243–2248.

106. Antin JH, Ginsburg D, Smith BR, et al. Bone marrow transplantation for paroxysmal nocturnal hemoglobinuria: eradication of the PNH clone and documentation of complete lymphohematopoietic engraftment. *Blood*. 1985;66:1247–1250.
107. Araten DJ, Luzzatto L. Allogeneic bone marrow transplantation for paroxysmal nocturnal hemoglobinuria. *Haematologica*. 2000;85:1–2.
108. Kawahara K, Witherspoon RP, Storb R. Marrow transplantation for paroxysmal nocturnal hemoglobinuria. *Am J Hematol*. 1992;39:283–288.
109. Elebute M, Rizzo S, Tooze J, et al. Evaluation of the haemopoietic reservoir in de-novo haemolytic paroxysmal nocturnal haemoglobinuria. *Br J Haematol*. 2003;123:552–560.
110. Chen R, Nagarajan S, Prince GM, et al. Impaired growth and elevated fas receptor expression in PIGA(+) stem cells in primary paroxysmal nocturnal hemoglobinuria. *J Clin Invest*. 2000;106:689–696.
111. Karadimitris A, Li K, Notaro R, et al. Association of clonal T-cell large granular lymphocyte disease and paroxysmal nocturnal haemoglobinuria (PNH): further evidence for a pathogenetic link between T cells, aplastic anaemia and PNH. *Br J Haematol*. 2001;115:1010–1014.
112. Karadimitris A, Manavalan JS, Thaler HT, et al. Abnormal T-cell repertoire is consistent with immune process underlying the pathogenesis of paroxysmal nocturnal hemoglobinuria. *Blood*. 2000;96:2613–2620.
113. Hanaoka N, Nakakuma H, Horikawa K, et al. NKG2D-mediated immunity underlying paroxysmal nocturnal haemoglobinuria and related bone marrow failure syndromes. *Br J Haematol*. 2009;146:538–545.
114. Gargiulo L, Lastraioli S, Cerruti G, et al. Highly homologous T-cell receptor beta sequences support a common target for autoreactive T cells in most patients with paroxysmal nocturnal hemoglobinuria. *Blood*. 2007;109:5036–5042.
115. Gargiulo L, Papaioannou M, Sica M, et al. Glycosylphosphatidylinositol-specific, CD1d-restricted T cells in paroxysmal nocturnal hemoglobinuria. *Blood*. 2013;121:2753–2761.
116. Joyce S, Woods AS, Yewdell JW, et al. Natural ligand of mouse CD1d1: cellular glycosylphosphatidylinositol. *Science*. 1998;279:1541–1544.
117. Karadimitris A, Gadola S, Altamirano M, et al. Human CD1d-glycolipid tetramers generated by in vitro oxidative refolding chromatography. *Proc Natl Acad Sci USA*. 2001;98:3294–3298.
118. Imi T, Katagiri T, Hosomichi K, et al. Sustained clonal hematopoiesis by HLA-lacking hematopoietic stem cells without driver mutations in aplastic anemia. *Blood Adv*. 2018;2:1000–1012.
119. Araten DJ, Luzzatto L. The mutation rate in PIG-A is normal in patients with paroxysmal nocturnal hemoglobinuria (PNH). *Blood*. 2006;108:734–736.
120. Araten DJ, Zamechek L, Halverson G. No evidence of hypermutability in red cells from patients with paroxysmal nocturnal hemoglobinuria using the XK gene. *Haematologica*. 2014;99:e142–e144.
121. Lobry C, Bains A, Zamechek LB, et al. Analysis of TET2 mutations in paroxysmal nocturnal hemoglobinuria (PNH). *Exp Hematol Oncol*. 2019;8:17.
122. Inoue N, Izui-Sarumaru T, Murakami Y, et al. Molecular basis of clonal expansion of hematopoiesis in 2 patients with paroxysmal nocturnal hemoglobinuria (PNH). *Blood*. 2006;108:4232–4236.
123. Shen W, Clemente MJ, Hosono N, et al. Deep sequencing reveals stepwise mutation acquisition in paroxysmal nocturnal hemoglobinuria. *J Clin Invest*. 2014;124:4529–4538.
124. Sun L, Babushok DV. Secondary myelodysplastic syndrome and leukemia in acquired aplastic anemia and paroxysmal nocturnal hemoglobinuria. *Blood*. 2020;136:36–49.
125. Chen Y, Tao S, Deng Y, et al. Chronic myeloid leukemia transformation in a patient with paroxysmal nocturnal hemoglobinuria: a rare case report with literature review. *Int J Clin Exp Med*. 2015;8:8226–8229.
126. Ikeda K, Mason PJ, Bessler M. 3'UTR-truncated Hmga2 cDNA causes MPN-like hematopoiesis by conferring a clonal growth advantage at the level of HSC in mice. *Blood*. 2011;117:5860–5869.
127. Dingli D, Luzzatto L, Pacheco JM. Neutral evolution in paroxysmal nocturnal hemoglobinuria. *Proc Natl Acad Sci USA*. 2008;105:18496–18500.
128. Moyo VM, Mukhina GL, Garrett ES, et al. Natural history of paroxysmal nocturnal haemoglobinuria using modern diagnostic assays. *Br J Haematol*. 2004;126:133–138.
129. Araten DJ, Thaler HT, Luzzatto L. High incidence of thrombosis in African-American and Latin-American patients with paroxysmal nocturnal haemoglobinuria. *Thromb Haemost*. 2005;93:88–91.
130. Araten DJ, Notaro R, Thaler HT, et al. Thrombolytic therapy is effective in paroxysmal nocturnal hemoglobinuria: a series of nine patients and a review of the literature. *Haematologica*. 2012;97:344–352.
131. Nafa K, Bessler M, Mason P, et al. Factor V Leiden mutation investigated by amplification created restriction enzyme site (ACRES) in PNH patients with and without thrombosis. *Haematologica*. 1996;81:540–542.
132. Ronne E, Pappot H, Grondahl-Hansen J, et al. The receptor for urokinase plasminogen activator is present in plasma from healthy donors and elevated in patients with paroxysmal nocturnal haemoglobinuria. *Br J Haematol*. 1995;89:576–581.
133. Hugel B, Socie G, Vu T, et al. Elevated levels of circulating procoagulant microparticles in patients with paroxysmal nocturnal hemoglobinuria and aplastic anemia. *Blood*. 1999;93:3451–3456.
134. Sims PJ, Faioni EM, Wiedmer T, et al. Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. *J Biol Chem*. 1988;263:18205–18212.
135. Wiedmer T, Hall SE, Ortel TL, et al. Complement-induced vesiculation and exposure of membrane prothrombinase sites in platelets of paroxysmal nocturnal hemoglobinuria. *Blood*. 1993;82:1192–1196.
136. Girard TJ, Tuley E, Broze Jr GJ. TFPIbeta is the GPI-anchored TFPI isoform on human endothelial cells and placental microsomes. *Blood*. 2012;119:1256–1262.
137. Ott I, Miyagi Y, Miyazaki K, et al. Reversible regulation of tissue factor-induced coagulation by glycosyl phosphatidylinositol-anchored tissue factor pathway inhibitor. *Arterioscler Thromb Vasc Biol*. 2000;20:874–882.
138. Devine DV, Siegel RS, Rosse WF. Interactions of the platelets in paroxysmal nocturnal hemoglobinuria with complement. Relationship to defects in the regulation of complement and to platelet survival in vivo. *J Clin Invest*. 1987;79:131–137.
139. Dixon RH, Rosse WF. Mechanism of complement-mediated activation of human blood platelets in vitro: comparison of normal and paroxysmal nocturnal hemoglobinuria platelets. *J Clin Invest*. 1977;59:360–368.
140. Jin JY, Tooze JA, Marsh JC, et al. Glycosylphosphatidyl-inositol (GPI)-linked protein deficiency on the platelets of patients with aplastic anaemia and paroxysmal nocturnal haemoglobinuria: two distinct patterns correlating with expression on neutrophils. *Br J Haematol*. 1997;96:493–496.
141. Polley MJ, Nachman RL. Human complement in thrombin-mediated platelet function: uptake of the C5b–9 complex. *J Exp Med*. 1979;150:633–645.
142. Zimmerman TS, Kolb WP. Human platelet-initiated formation and uptake of the C5–9 complex of human complement. *J Clin Invest*. 1976;57:203–211.
143. Araten DJ, Boxer D, Zamechek L, et al. Analysis of platelets by flow cytometry in patients with paroxysmal nocturnal hemoglobinuria (PNH). *Blood Cells Mol Dis*. 2020;80:102372.
144. Griffin M, Hillmen P, Munir T, et al. Significant hemolysis is not required for thrombosis in paroxysmal nocturnal hemoglobinuria. *Haematologica*. 2019;104:e94–e96.
145. Wang H, Chuhjo T, Yasue S, et al. Clinical significance of a minor population of paroxysmal nocturnal hemoglobinuria-type cells in bone marrow failure syndrome. *Blood*. 2002;100:3897–3902.
146. Schwarz K, Iolascon A, Verissimo F, et al. Mutations affecting the secretory COPII coat component SEC23B cause congenital dyserythropoietic anemia type II. *Nat Genet*. 2009;41:936–940.
147. Faria B, Gaya da Costa M, Poppelaars F, et al. Administration of intravenous iron formulations induces complement activation in-vivo. *Front Immunol*. 2019;10:1885.
148. Sloand EM, Maciejewski JP, Dunn D, et al. Correction of the PNH defect by GPI-anchored protein transfer. *Blood*. 1998;92:4439–4445.
149. Parker C, Omine M, Richards S, et al. Diagnosis and management of paroxysmal nocturnal hemoglobinuria. *Blood*. 2005;106:3699–3709.
150. Dragoni F, Chiarotti F, Iori AP, et al. Antithrombotic therapy with rivaroxaban in five patients with paroxysmal nocturnal haemoglobinuria and thrombotic events. *Thromb J*. 2018;16:26.
151. Araten DJ, Iori AP, Brown K, et al. Selective splenic artery embolization for the treatment of thrombocytopenia and hypersplenism in paroxysmal nocturnal hemoglobinuria. *J Hematol Oncol*. 2014;7:27.
152. de Latour RP, Mary JY, Salanoubat C, et al. Paroxysmal nocturnal hemoglobinuria: natural history of disease subcategories. *Blood*. 2008;112:3099–3106.
153. Townsley DM, Scheinberg P, Winkler T, et al. Eltrombopag added to standard immunosuppression for aplastic anemia. *N Engl J Med*. 2017;376:1540–1550.
154. Fattizzo B, Kulasekararaj AG, Hill A, et al. Clinical and morphological predictors of outcome in older aplastic anemia patients treated with eltrombopag. *Haematologica*. 2019;104:e494–e496.
155. Yoshizato T, Dumitriu B, Hosokawa K, et al. Somatic mutations and clonal hematopoiesis in aplastic anemia. *N Engl J Med*. 2015;373:35–47.
156. Marsh J, Schrezenmeier H, Marin P, et al. Prospective randomized multicenter study comparing cyclosporin alone versus the combination of antithymocyte globulin and cyclosporin for treatment of patients with

nonsevere aplastic anemia: a report from the European blood and marrow transplant (EBMT) Severe Aplastic Anaemia Working Party. *Blood*. 1999;93:2191–2195.

157. Kuo TT, Aveson VG. Neonatal Fc receptor and IgG-based therapeutics. *MAbs*. 2011;3:422–430.
158. Lee JW, Sicre de Fontbrune F, Wong LLL, et al. Ravulizumab (ALXN1210) vs eculizumab in adult patients with PNH naive to complement inhibitors: the 301 study. *Blood*. 2019;133:530–539.
159. Kulasekararaj AG, Hill A, Rottinghaus ST, et al. Ravulizumab (ALXN1210) vs eculizumab in C5-inhibitor-experienced adult patients with PNH: the 302 study. *Blood*. 2019;133:540–549.
160. Brodsky RA, Young NS, Antonioli E, et al. Multicenter phase 3 study of the complement inhibitor eculizumab for the treatment of patients with paroxysmal nocturnal hemoglobinuria. *Blood*. 2008;111:1840–1847.
161. Lee JW, Kulasekararaj AG. Ravulizumab for the treatment of paroxysmal nocturnal hemoglobinuria. *Expert Opin Biol Ther*. 2000;20:227–237.
162. Cataland S, Ariceta G, Chen P, et al. Discordance between free C5 and CH50 complement assays in measuring complement C5 inhibition in patients with aHUS treated with ravulizumab. *Blood*. 2019;134(suppl 1):1099.
163. Peffault de Latour R, Fremeaux-Bacchi V, Porcher R, et al. Assessing complement blockade in patients with paroxysmal nocturnal hemoglobinuria receiving eculizumab. *Blood*. 2015;125:775–783.
164. Kelly RJ, Hochsmann B, Szer J, et al. Eculizumab in pregnant patients with paroxysmal nocturnal hemoglobinuria. *N Engl J Med*. 2015;373:1032–1039.
165. Reher D, Fuhrmann V, Kluge S, et al. A rare case of septic shock due to *Neisseria meningitidis* serogroup B infection despite prior vaccination in a young adult with paroxysmal nocturnal haemoglobinuria receiving eculizumab. *Vaccine*. 2018;36:2507–2509.
166. Nishimura J, Yamamoto M, Hayashi S, et al. Genetic variants in C5 and poor response to eculizumab. *N Engl J Med*. 2014;370:632–639.
167. Mannes M, Dopler A, Zolk O, et al. Complement inhibition at the level of C3 or C5: mechanistic reasons for ongoing terminal pathway activity. *Blood*. 2021;137:443–455.
168. Roth A, Hock C, Konik A, et al. Chronic treatment of paroxysmal nocturnal hemoglobinuria patients with eculizumab: safety, efficacy, and unexpected laboratory phenomena. *Int J Hematol*. 2011;93:704–714.
169. Risitano AM, Imbriaco M, Marando L, et al. From perpetual haemosiderinuria to possible iron overload: iron redistribution in paroxysmal nocturnal haemoglobinuria patients on eculizumab by magnetic resonance imaging. *Br J Haematol*. 2012;158:415–418.
170. Araten D, Pachter HL, Dring R, et al. Symptomatic bilirubin gallstones in patients with PNH treated with eculizumab. *Blood*. 2019;134(suppl 1).
171. Hill A, Rother RP, Arnold L, et al. Eculizumab prevents intravascular hemolysis in patients with paroxysmal nocturnal hemoglobinuria and unmasks low-level extravascular hemolysis occurring through C3 opsonization. *Haematologica*. 2010;95:567–573.
172. Huber-Lang M, Sarma JV, Zetoune FS, et al. Generation of C5a in the absence of C3: a new complement activation pathway. *Nat Med*. 2006;12:682–687.
173. Amara U, Flierl MA, Rittirsch D, et al. Molecular intercommunication between the complement and coagulation systems. *J Immunol*. 2010;185:5628–5636.
174. Peffault de Latour R, Schrezenmeier H, Bacigalupo A, et al. Allogeneic stem cell transplantation in paroxysmal nocturnal hemoglobinuria. *Haematologica*. 2012;97:1666–1673.
175. Brodsky RA, Luznik L, Bolanos-Meade J, et al. Reduced intensity HLA-haploidentical BMT with post transplantation cyclophosphamide in nonmalignant hematologic diseases. *Bone Marrow Transplant*. 2008;42:523–527.
176. DeZern AE, Zahurak ML, Symons HJ, et al. Haploidentical BMT for severe aplastic anemia with intensive GVHD prophylaxis including posttransplant cyclophosphamide. *Blood Adv*. 2020;4:1770–1779.
177. DeZern AE, Jones RJ, Brodsky RA. Eculizumab bridging before bone marrow transplant for marrow failure disorders is safe and does not limit engraftment. *Biol Blood Marrow Transplant*. 2018;24:e26–e30.
178. Harris JW, Koscick R, Lazarus HM, et al. Leukemia arising out of paroxysmal nocturnal hemoglobinuria. *Leuk Lymphoma*. 1999;32:401–426.
179. de Guibert S, Peffault de Latour R, Varoqueaux N, et al. Paroxysmal nocturnal hemoglobinuria and pregnancy before the eculizumab era: the French experience. *Haematologica*. 2011;96:1276–1283.
180. Tichelli A, Socie G, Marsh J, et al. Outcome of pregnancy and disease course among women with aplastic anemia treated with immunosuppression. *Ann Intern Med*. 2002;137:164–172.

CHAPTER 33

ACQUIRED DISORDERS OF RED CELL, WHITE CELL, AND PLATELET PRODUCTION

Francis R. LeBlanc, Jaroslaw P. Maciejewski, and Thomas P. Loughran, Jr.

Acquired pure red cell aplasia (PRCA) is characterized by the presence of an acquired severe normochromic, most frequently normocytic anemia associated with a complete disappearance of reticulocytes and erythroid precursors in the marrow and normal production of myeloid cells and platelets. Consequently, it is presumed that the defect lies within erythroid precursors and not within stem cells as seen in aplastic anemia. Initially described as *progressive anemia with exclusive absence of erythroid series in the bone marrow*, PRCA is a rare bone marrow failure disorder without geographic or racial predilection. All ages can be affected, but if present in children, it is called transient erythroblastopenia of childhood (TEC) and may be difficult to distinguish from congenital causes of anemia, mainly Diamond-Blackfan anemia (DBA) (see Chapter 30). Former nosology included various terms such as *erythrophthisis, chronic hypoplastic anemia,* and *pure red cell agenesis.*

ETIOLOGY AND CLASSIFICATION

Acquired forms of PRCA must be distinguished from congenital forms of PRCA, which usually manifest themselves early in life (see Chapter 30). Acquired PRCA occurring in childhood may be difficult to distinguish from DBA. As an acquired disease, PRCA may be a primary disorder or secondary to a variety of systemic diseases, including a number of hematologic malignancies (Table 33.1).

PATHOGENESIS

The inciting events in the development of PRCA are not known. However, as with idiopathic aplastic anemia, viruses or exposure to chemicals can serve as potential triggers (Fig. 33.1). Theoretically, a viral infection could lead to depletion of erythroid precursors. Studies of B19 parvovirus (discussed later) suggest such an etiology. Because hematopoietic stem cells are not affected by B19 parvovirus, myeloid cells and platelets are normally produced, and upon clearance of the virus, normal erythroid production can resume. Similarly, in immune-mediated PRCA, the mechanism of erythroid inhibition may vary and may include (1) antibodies to proteins specific to erythroblasts, (2) direct cytotoxic T lymphocyte (CTL)–mediated killing of erythroid precursors, and (3) production of soluble products by CTLs such as inhibitory or proapoptotic cytokines that directly affect the erythroid series.

Historically, initial studies concentrated on examining the effects of soluble serum inhibitors of erythropoiesis.[39,40] These investigations revealed a decline in erythroid colony formation in the presence of patient serum or failure to induce erythroid colony formation in the presence of erythropoietin. Such serum inhibitors can be detected in 40% of patients with PRCA.[39–41] In 60% of patients, erythroid colony formation can be induced in vitro with hematopoietic growth factors.[39–41] The inhibitory activity is localized to the immunoglobulin (Ig)G fraction and disappears upon remission. The antigenic targets for autoantibodies have not been well characterized, but various stages of erythroid differentiation can be affected (also called PRCA type A), as seen in the reduction of burst-forming unit–erythroid or colony-forming unit–erythroid.[42,43] In certain cases of antibody-mediated PRCA, the involvement of the complement system is a prerequisite to disease causation.[44] Perhaps the exception and a model for antibody-induced red cell aplasia is the identification of PRCA associated with anti-erythropoietin antibodies (also called PRCA type B) in rare cases.[1,2] Consistent with the specificity of the antibodies, myeloid colony formation is not impaired, making it unlikely that a more ubiquitous inhibitory cytokine mediates the specific erythroid inhibition.

Experimental and clinical observations have suggested that PRCA may also be mediated by CTLs, which specifically recognize and kill erythroid precursors similar to CTL-mediated killing of cells in aplastic anemia.[7,8,45] Although such a T cell–mediated erythroid response is likely to be polyclonal, rare instances of T-cell large granular lymphocyte (T-LGL) leukemia associated with PRCA or erythroid inhibition may represent an extreme form of the clonal continuum of CTL responses (see T-Cell Large Granular Lymphocyte–Associated PRCA). In addition to CTLs expressing α/β T-cell receptors (TCRs), T cells with a γ/δ TCR can mediate PRCA.[46,47] The antigens/antigenic peptides triggering such a response have not been well described.[48,49] However, it has been shown that T-LGLs from PRCA patients are directly capable of suppressing erythropoiesis of CFU-E in vitro.[50] Similarly, natural killer (NK) cells have also been implicated in mediating cytotoxicity directed against erythroid precursors.[9,51] NK cells, like γ/γ T lymphocytes and unlike α/β CTLs, do not rely on major histocompatibility complex (MHC)–restricted cytotoxicity, but may use killer-cell immunoglobulin-like receptors (KIRs).[52,53] KIRs inhibit cytolysis when they encounter a cell bearing human leukocyte antigen (HLA) class I molecules. A lack of appropriate KIRs may predispose cells to increased attack by NK cells or γ/δ CTLs. Physiologic downregulation of KIRs has been implicated in the pathogenesis of PRCA in a patient with concomitant γ/δ T-LGL clonal proliferation.[46,54] An alternative NK-cell cytotoxic

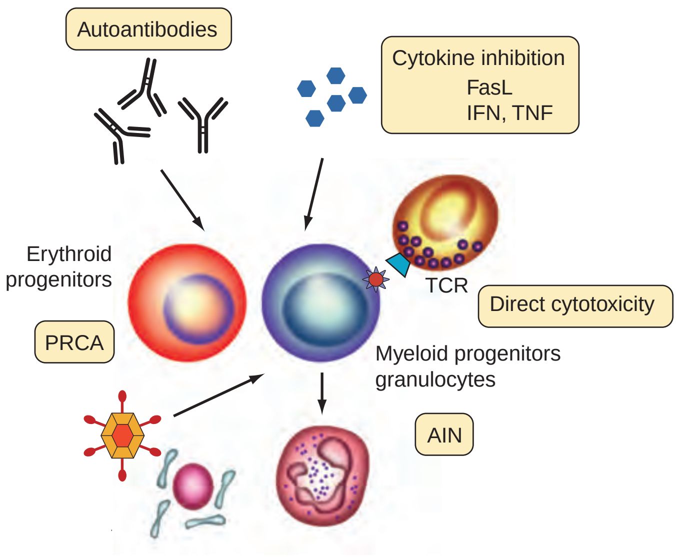


Figure 33.1 PATHOGENESIS OF PURE RED CELL APLASIA. *AIN*, Autoimmune neutropenia; *FasL*, Fas ligand; *IFN*, interferon; *PRCA*, pure red cell aplasia; *TCR*, T-cell receptor; *TNF*, tumor necrosis factor.

TABLE 33.1 Classification of Pure Red Cell Aplasia

Congenital (DBA)		
Primary	Autoimmune Idiopathic	
Secondary	Thymoma[1–4] Hematologic malignancies	CLL[5,6]
		T-LGL/chronic NK-LGL leukemia[7–13]
		Myeloma[14]
		NHL[15,16]
		MDS[17]
		ALL[18–20]
	Solid tumors	Renal cell carcinoma[21]
		Thyroid cancer[22]
		Various adenocarcinomas[23,24]
	Infections	Parvovirus B19[25]
		EBV,[26] mumps
		HIV,[27] HTLV-1[10]
		CMV
		Viral hepatitis (hepatitis A,[28] hepatitis B[29])
		Leishmaniasis[30]
		Gram-positive systemic infections (e.g., staphylococcemia)
		Meningococcemia
	Autoimmune conditions	SLE[31]
		RA[32]
		Sjögren syndrome[33]
		Mixed connective tissue disease[34]
		Autoimmune hepatitis[35]
		Anti-EPO antibodies[36]
		ABO-incompatible BMT
		Minor incompatibility[37,38]
	Drugs and chemicals	
	Pregnancy	
	Severe nutritional deficiencies	
	Renal failure	

ALL, Acute lymphoblastic leukemia; *BMT*, bone marrow transplantation; *CLL*, chronic lymphocytic leukemia; *CMV*, cytomegalovirus; *DBA*, Diamond-Blackfan anemia; *EBV*, Epstein-Barr virus; *EPO*, erythropoietin; *HIV*, human immunodeficiency virus; *HTLV-1*, human T-lymphotropic virus-1; *MDS*, myelodysplastic syndrome; *NHL*, non-Hodgkin lymphoma; *NK-LGL*, natural killer large granular lymphocyte; *RA*, refractory anemia; *SLE*, systemic lupus erythematosus; *T-LGL*, T-cell large granular lymphocyte.

mechanism independent of KIR has been reported in healthy individuals.[55]

Peripheral Th lymphocyte polarization has been implicated in the pathogenesis of PRCA.[49,56] Polarization toward the Th2 functional subtype during disease relapse and normalization of Th1/Th2 ratio after effective treatment has been associated with both monoclonal gammopathy- and thymoma-associated PRCA.

PRIMARY PURE RED CELL APLASIA

Primary PRCA occurs in the absence of any underlying disorder. It may be acute and self-limited or may be a chronic and refractory condition. Acute forms are uncommon. Most cases are protracted and chronic, unlike TEC, which is an acute and self-limited disorder. Most of the cases of classic primary PRCA are autoimmune in origin, but a significant proportion will remain idiopathic in origin in spite of an exhaustive workup.

SECONDARY FORMS OF PURE RED CELL APLASIA

Clinical Associations

B-Cell Chronic Lymphocytic Leukemia–Associated Pure Red Cell Aplasia

In B-cell chronic lymphocytic leukemia (CLL), PRCA can be observed in up to 6% of cases.[5,6] A recent study found 0.5% of their 1750 CLL patients has PRCA.[57] This diagnosis should be considered in CLL patients who demonstrate anemia and reticulocytopenia. Definite diagnosis requires bone marrow biopsy which will show absence of erythroid precursors and red cell aplasia. The underlying pathogenetic mechanisms are not clear, and the inhibition of the erythroid series does not appear to be mediated by a soluble factor.[58,59] The distinction between whether the PRCA is a result of the primary B-cell CLL disease or its therapy becomes difficult in circumstances when PRCA presents as a late event. In most cases, PRCA cannot be attributed simply to infiltration of the marrow by lymphoma cells.

T-Cell Large Granular Lymphocyte–Associated Pure Red Cell Aplasia

Although neutropenia is a typical finding in T-LGL leukemia, PRCA with varying degrees of erythroblastopenia can also be observed in 10% to 68% of patients with T-LGL leukemia, with much higher rates in Asian populations.[10–13,45,51,60,61] It is found to be commonly associated with signal transducer and activator of transcription (STAT)3 mutant T-LGL leukemia.[62] In such a setting, PRCA is often accompanied by red cells with an increased mean corpuscular volume. It is possible that PRCA associated with T-LGL leukemia represents an extreme form of the T-cell–mediated disease that, if polyclonal, might be classified as idiopathic PRCA.

Thymoma-Associated Pure Red Cell Aplasia

Thymoma is associated with PRCA; thus a chest X-ray examination or computed tomographic (CT) scan should be included in the workup for PRCA. Antibodies with direct inhibitory effects against erythroid precursors may be present.[1–4,63] T-cell–mediated inhibition of erythropoiesis has also been implicated in the pathogenesis of PRCA associated with either benign or malignant thymomas.[64–67] A late onset immunodeficiency condition, Good syndrome, which is characterized by hypogammaglobulinemia and thymoma is associated with PRCA in 33% of the patients.[68] Thymectomy is the usual initial treatment approach[63]; however, incomplete responders, nonresponders, and patients who relapse are common,[67] necessitating additional therapies in the form of azathioprine,[26] intravenous immunoglobulins (IVIg),[69] and cyclosporine A (CsA).[70]

Pregnancy-Associated Pure Red Cell Aplasia

Pregnancy-associated PRCA is a self-limited syndrome that may occur at any age of gestation.[71] It has a high risk for relapse during subsequent pregnancies and can be safely managed with either blood transfusions or corticosteroids.[72,73] Patients with other forms of PRCA may also be more prone to relapse during pregnancy.[74]

Parvovirus B19 and Other Viral-Induced Pure Red Cell Aplasias

Parvovirus B19 is a single-stranded deoxyribonucleic acid (DNA) virus, which in normal individuals causes fifth disease (erythema infectiosum) in children and arthropathy in adults.[75–77] It is of particular concern in patients with sickle cell disease.[75] The cellular receptor for the virus is the P antigen, a blood group antigen also responsible for the agglutination reaction that occurs in the presence of the virus. Detection of parvovirus B19–specific IgM without antiparvovirus B19 IgG supports the diagnosis of acute infection, whereas the parvovirus B19–specific IgG suggests immunity.[25] Addition of parvovirus B19 in vitro to cultures of erythroid progenitor cells completely abolishes erythroid colony formation.[76,77] Primary infection causes lifelong immunity; however, it is possible that a latent virus may persist in a healthy individual for years.

A transient aplastic crisis is a typical complication of a primary parvovirus B19 infection in patients with increased red cell turnover (usually chronic hemolysis, e.g., hemoglobinopathies, and hereditary red blood cell [RBC] membrane disorders, e.g., hereditary spherocytosis).[78–80] In typical cases, acute reticulocytopenia results in a sudden drop in hemoglobin (Hb)/hematocrit levels as RBC destruction is not supported by a suppressed marrow. Occasionally, characteristic giant pronormoblasts may be seen in marrow aspirates (Fig. 33.2). Aplastic crisis is often self-limiting with the evolution of a protective IgG response. Viral titers in the serum of affected patients may be high.

A more chronic form, parvovirus B19–related PRCA, may develop in immunocompromised patients as, for example, in acquired immunodeficiency syndrome (AIDS) (see Chapter 152). In such cases, IVIg can produce remarkable responses. High doses of IVIg are required (>2 g/kg) because an insufficient dose may not produce the desired effect. DNA dot blot hybridization is the best diagnostic test for the detection of viremia. Parvovirus B19 can also be detected by polymerase chain reaction (PCR), a routinely available test, but this method may provide a high rate of false-positive results. However, if negative, it excludes B19 parvovirus–mediated disease.[25] Improved tests have been developed that allow for the detection of neutralizing antibodies and infectivity of parvovirus B19.[81]

Several other viral infections, including viral hepatitis (A[28,82] and C[29,83]), Epstein-Barr virus (EBV),[84] cytomegalovirus (CMV),[84] human T-Lymphotropic virus (HTLV)-1,[10] and human immunodeficiency virus (HIV), have been implicated as causative agents of PRCA. Little is known about the exact mechanisms underlying these disorders, but they likely involve T-cell–mediated suppression as observed during HTLV-1 infection and EBV or antibody-mediated destruction of RBC precursors, as in hepatitis C–induced PRCA.[1,29] Frequently the presence of multiple comorbidities and medications makes it difficult to isolate the causative agent of PRCA.[85,86]

Connective Tissue Disease–Associated Pure Red Cell Aplasia

The majority of connective tissue diseases associated with PRCA are autoimmune in nature. Several rheumatologic diseases have been associated with PRCA, including adult-onset Still disease,[87,88] dermatomyositis,[89] mixed connective tissue disease,[34] polymyositis,[90] rheumatoid arthritis,[32,91] Sjögren syndrome,[33,92,93] and systemic lupus erythematosus.[31,94,95] The pathogenesis of the PRCA in this setting may vary and includes autoantibody-mediated erythroid inhibition,[96] autoantibody directed against erythropoietin,[97] and CTL-mediated killing of erythroid precursors.[94]

Drug-Induced Pure Red Cell Aplasia

Various chemical agents and drugs have been associated with PRCA. The mechanisms responsible for erythroid inhibition may be diverse depending on the offending agent (Table 33.2) but may include induction of antibodies targeting the drugs or drugs bound to cellular and plasma proteins. Another possible mechanism involves drug-mediated triggering of T-cell responses, or direct toxicity to the erythroid series as seen with diphenylhydantoin.[98]

Erythropoietin Antibody–Associated Pure Red Cell Aplasia

Recombinant erythropoietin is used in the treatment of anemia of various origins, including anemia of chronic disease, renal disease, and a variety of bone marrow failure syndromes, particularly myelodysplastic syndromes (MDS). Cases of PRCA have developed as a consequence of antibody formation against endogenous erythropoietin[99] or while receiving treatment with recombinant erythropoietin.[100–102] The latter condition has been referred to as *epoetin-induced PRCA* or *EPO-PRCA* with initial cases related to exposure to epoetin alpha (Eprex; 92%) and epoetin beta (NeoRecormon; 8%).[100] There are reports of human erythropoietin (HuEpo) neutralizing antibody PRCA related to the use of biosimilar recombinant HuEpo in Thailand. There are several risk factors associated with the development of EPO-PRCA, including subcutaneous (SC) route of administration, use of epoetin alpha stabilized in a human serum albumin (HSA)–free formulation, use of silicone oil as lubricant in prefilled Eprex syringes, and use in patients with chronic renal disease.[103]

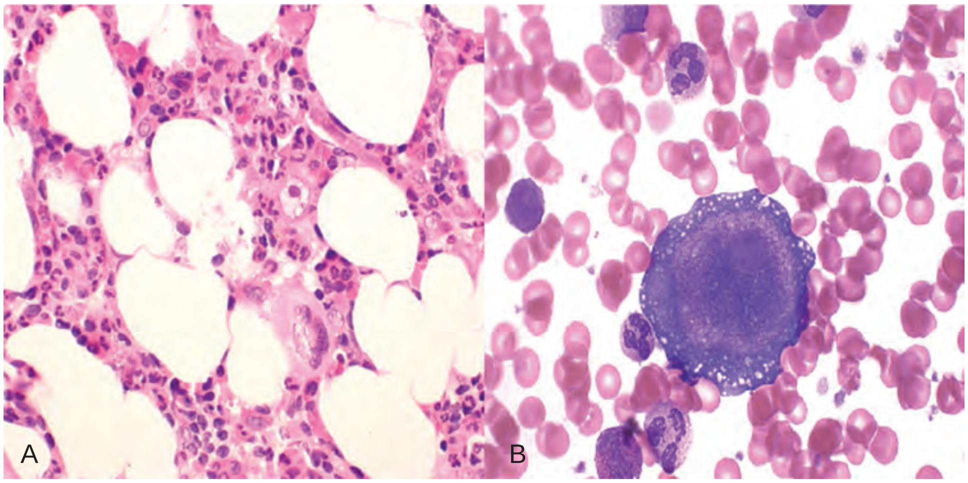


Figure 33.2 PARVOVIRUS B19–MEDIATED PURE RED CELL APLASIA. (A) Bone marrow biopsy and (B) bone marrow aspirate demonstrated giant pronormoblasts.

This observation has led to modification in the storage, handling, and administration of Eprex favoring IV administration, especially with Eprex stabilized with HSA-free formulation, and avoidance of the SC non-HSA–stabilized Eprex. Diagnostic criteria have also been proposed incorporating major features (treatment with epoetin for at least 3 weeks, decrease in Hb by 0.1 g/dL/day without transfusions or transfusion requirement of about 1 unit/week to keep Hb levels stable, reticulocyte count less than 10×10^9/L, no major drop in other blood lineages), minor features (skin and systemic allergic reactions), and accessory investigations to exclude other causes. The most commonly used tests to detect antibodies are enzyme-linked immunosorbent assay, radioimmunoprecipitation assay, and surface plasmon resonance. Once antibodies are detected, their neutralizing ability is tested using an in vitro bioassay. Following establishment of diagnosis, management should include discontinuation of exogenous erythropoietin, administration of immunosuppressive agents, or, in cases of anemia secondary to renal insufficiency, renal transplantation.[104]

Pure Red Cell Aplasia Following Allogeneic Stem Cell Transplantation

In contrast to HLA matching, ABO blood group incompatibility plays a minor role in the success of allogeneic hematopoietic stem cell transplantation (HSCT). However, PRCA may be associated with major ABO incompatibility between the donor and recipient, leading to inhibition of donor erythroid precursors by residual host isoagglutinins. This complication is more commonly observed following the use of nonmyeloablative conditioning regimens. PRCA may also be resistant to the withdrawal or decrease of immunosuppression, or donor lymphocyte infusions. Responses to rituximab,[105,106] erythropoietin,[107] plasma exchange,[108] and azathioprine[107] have been reported. A case responsive to a purified $CD34^+$ cell infusion has also been reported.[37] Multiple cases of successful treatment with daratumumab (IgG1k monoclonal antibody directed against CD38) have also been reported.[109–111] Resolution of PRCA is generally associated with a decrease and subsequent disappearance of host isoagglutinins.[37,38,112–115]

Pure Red Cell Aplasia Associated With Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-Linked Syndrome

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is a rare X-linked recessive condition typically seen during infancy. Clinical features may include type 1 diabetes mellitus, eczema, and autoimmune hepatitis. Anemia can be severe at diagnosis because the fall in Hb occurs over a protracted period of time and patients often exhibit a good degree of adaptation. Arrest of erythropoiesis is obvious with a profound reticulocytopenia.[116]

LABORATORY EVALUATION

A complete blood cell count with differential, peripheral smear review, reticulocyte count, and a bone marrow examination remain the cornerstone in the diagnosis of PRCA. The classic hematologic picture of PRCA includes a normocytic, normochromic anemia (anemia associated with T-LGL leukemia is often macrocytic) with a normal white blood cell and platelet count. The reticulocyte count is reduced to less than 1% when corrected for the degree of anemia (a reticulocyte level greater than 2% is not compatible with the diagnosis of PRCA) (Fig. 33.3).

The bone marrow examination generally shows absence of cells belonging to the erythroid lineage and the normal appearance of granulocytic and monocytic precursors and megakaryocytes. Erythroid precursors, if present, are usually less than 1%, and only a few residual proerythroblasts or basophilic erythroblasts may be seen. Blast cell numbers and cellularity are within normal limits. There are no dysplastic changes, ringed sideroblasts, or reticulin fibrosis. Cytogenetic evaluation is normal. In some cases, neutropenia,[117] mild thrombocytopenia, eosinophilia,[49] thrombocytosis,[49] leukocytosis, or relative lymphocytosis may be seen. Cytogenetic abnormalities, if present, may indicate concomitant MDS and is a poor prognostic marker for both response to treatment and propensity to leukemic transformation.[118,119] During the course of PRCA patients, ineffective erythropoiesis characterized by a maturation arrest at the proerythroblast or basophilic erythroblast stage may be observed and signifies either partial response to treatment or initial recovery from treatment or a prelude to the development of full-blown PRCA.

TABLE 33.2 **Drugs and Chemicals Implicated in Pure Red Cell Aplasia**

Alemtuzumab
Allopurinol
α-Methyldopa
Aminopyrine
Azathioprine
Benzene
Carbamazepine
Cephalothin
Chloramphenicol
Chlorpropamide
Cladribine
Clopidogrel
Cotrimoxazole
D-Penicillamine
Erythropoietin
Estrogens
Fludarabine
FK506
Gold
Halothane
Interferon-α
Isoniazid
Lamivudine
Linezolid
Maloprim
Mycophenolate mofetil
Penicillin
Phenylbutazone
Phenytoin
Procainamide
Ribavirin
Rifampicin
Sulfasalazine
Sulfathiazole
Sulindac
Tacrolimus
Thiamphenicol
Ticlopidine
Valproic acid
Zidovudine

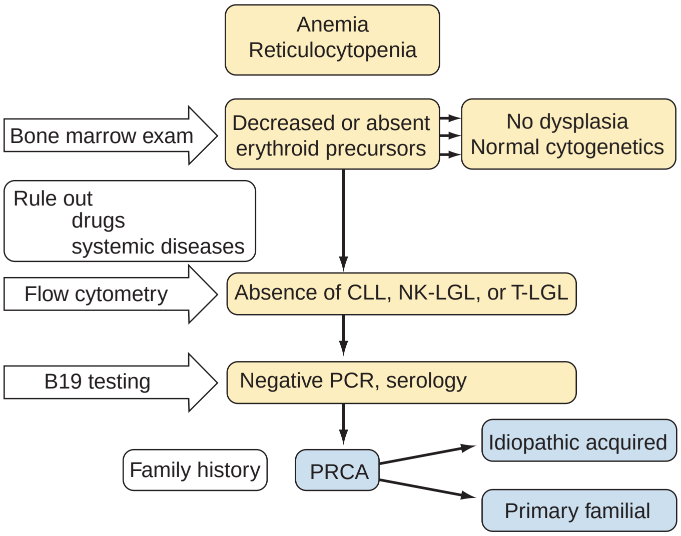


Figure 33.3 DIAGNOSTIC ALGORITHM IN PURE RED CELL APLASIA. *CLL*, Chronic lymphocytic leukemia; *NK-LGL*, natural killer large granular lymphocyte; *PCR*, polymerase chain reaction; *PRCA*, pure red cell aplasia; *T-LGL*, T-cell large granular lymphocyte.

It is important to exclude vitamin B_{12} and folate deficiencies, and depending on the etiology and associated disease, other blood and bone marrow findings may be seen. The presence of giant and vacuolated pronormoblasts in the bone marrow examination should raise the suspicion for parvovirus B19 infection.[25] The presence of large granular lymphocytosis, neutropenia, and/or thrombocytopenia, expansion of $CD3^+CD8^+CD57^+$ T cells, clonal cytotoxic T cells as documented by TCR gene rearrangement, expansion of specific TCR Vβ region family gene segment, and splenomegaly may point toward concomitant T-LGL leukemia.[120,121] B-cell lymphocytosis, especially of the $CD5^+/CD19^+/CD20^+/CD23^+/\text{cyclin } D^-/SmIg^{-dim}$ phenotype with concomitant lymphadenopathy, hepatosplenomegaly, hypogammaglobulinemia, and thrombocytopenia may be very suggestive of a B-cell CLL. Another laboratory finding that may help point to a secondary cause of PRCA includes the presence of monoclonal gammopathy.[49] Parvovirus B19 DNA titers (DNA hybridization and amplification techniques) may show high levels of the virus at 10 genome copies per milliliter, but it is important to note that serologic (IgM and IgG) titers are usually absent.[25] Erythropoietin antibodies,[100] antinuclear antibodies,[122,123] and/or complement consumption may point toward a specific disease mechanism.[124] A radiographic workup may also be useful in the clinical workup because chest X-ray examination or CT scan may show evidence of a thymoma.

Differential Diagnosis

PRCA can be easily differentiated from aplastic anemia and other types of bone marrow failure syndromes. A distinction between MDS with erythroid hypoplasia and idiopathic PRCA may be more difficult. In childhood, TEC has to be distinguished from DBA, but a history of normal blood counts, late onset of manifestations, and a transient disease course are characteristic of TEC.

Therapy

The distinction between primary and secondary forms of PRCA is essential because many secondary types have specific and very effective therapies (Table 33.3). All potentially offending drugs should be discontinued, and drug-associated PRCA should remit within 3 to 4 weeks. Nutritional deficiencies (B_{12} and folic acid) should be excluded and treated if present. The therapy of primary and secondary forms of PRCA refractory to the treatment of an underlying disease may be challenging and should include a sequential trial of various immunosuppressive agents until a response is achieved. Spontaneous remissions have been reported.

Surgery or Radiation

In cases associated with thymoma, thymectomy is the usual initial treatment of choice before immunosuppression and may induce remission with return of erythropoiesis in 4 to 8 weeks in about 30% to 40% of patients.[132–134] Patients who fail to respond to surgery should be treated as patients with idiopathic PRCA. The removal of a thymoma may improve responsiveness to immunosuppressive therapy.[133] Thymectomy in the absence of a thymoma in other forms of PRCA is not recommended. In circumstances where surgical resection of thymoma is contraindicated, radiation therapy with or without chemotherapy may be administered.[135]

MEDICAL

Supportive

Supportive care includes blood transfusions and iron chelation with deferasirox (Exjade) which is an oral iron chelator developed for treating transfusion-related iron overload syndromes.[136]

Immunosuppression

Prednisone therapy is associated with significant response rates (approximately 40%) and should constitute the initial therapeutic approach.[119] Initial responses are generally observed after 4 to 6 weeks. A slow taper of prednisone is suggested over a period of 3 to 4 months.[118,128] The disease may relapse, and the minimal maintenance dose of corticosteroids may need to be established to maintain the desired Hb levels.[137] Trials of prednisone therapy without clinical response longer than 8 weeks are not warranted.[118,119,128]

Alternative therapies may include cyclosporine, oral cyclophosphamide, azathioprine, antithymocyte globulin (ATG), rituximab, and alemtuzumab (see Table 33.3). Erythropoietin and darbepoeitin[138,139] are usually not effective as a sole agent but may hasten recovery following an adequate trial of cyclophosphamide. No randomized trials exist to favor a particular treatment based on efficacy. The choice of therapy may be influenced by clinical clues. For example, the presence of LGLs may suggest the use of cyclophosphamide or CsA, hypogammaglobulinemia may be corrected with IVIg, whereas detection of hypergammaglobulinemia or monoclonal protein may suggest a choice of rituximab. Most refractory cases may require administration of ATG. The age of the patient may influence the choice of the cytotoxic agent, which may pose a significant risk for the development of secondary leukemias, especially with a prolonged administration.

TABLE 33.3 Therapy for Pure Red Cell Aplasia and Its Results

	Study								
Agent	**Chikkappa[125]**	**Means[126]**	**Au[127]**	**Dessypris[128]**	**Zecca[129]**	**Lacy[119]**	**Charles[118]**	**Sloand[130]**	**Abkowitz[131]**
Steroids				18/41		9/29	9/36	—	
Cytotoxic agents				24/54		14/29	8/27	—	
Antithymocyte globulin				2/6		0/1	8/12		6/6
Cyclosporine A	6/7	6/9		3/4		4/5	2/3		
Splenectomy				4/23		0/1	0/1		
Daclizumab (Zenapax)								6/15	
Rituximab					1/1				
Alemtuzumab (Campath)			2/2						
Methotrexate							2/37		

Danazol is a synthetic attenuated androgen that has been used for many years for the treatment of a variety of hematologic disorders, mainly myelofibrosis. Danazol is given at 200 mg orally (PO) twice per day up to 600 mg daily. It has been used in combination with steroids or other agents and has shown efficacy in PRCA secondary to multiple etiologies.[140–143]

IVIg is also effective in several types of PRCA.[144–147] Higher doses of usually 2 g/kg of IVIg for 5 days are necessary for the treatment of parvovirus B19 virus–induced PRCA. In AIDS patients with parvovirus B19 virus–induced PRCA, a regimen consists of induction therapy with 1 g/kg daily for 1 to 2 days followed by 1 g/kg for 2 days.[148]

Azathioprine is an imidazolyl derivative of mercaptopurine that inhibits DNA synthesis by inhibition of purine metabolism. In PRCA, it may be given at a dose of 2 to 3 mg/kg/day IV and has been found to be effective in patients nonresponsive to cyclophosphamide.[26,149]

Oral cyclophosphamide may be started at a dose of 50 mg orally (PO) daily, with a maximal dose of no more than 150 mg daily. Blood counts should be monitored, and the dose may be escalated accordingly. Trials of therapy longer than 3 months without signs of response are not warranted. Monitoring of the reticulocyte count may allow for the early assessment of response. Often, a delayed response may be seen when cyclophosphamide is withdrawn, reflective of balance between immunosuppression and cytotoxicity.[150] It has been shown to be efficacious in combination with CsA in T-LGL-associated PRCA.[151]

Rituximab given by IV infusion weekly for 4 weeks has been found to be efficacious in PRCA. PRCA in a variety of settings, including B-cell CLL, EBV-associated posttransplant lymphoproliferative disease, ABO-incompatible allogeneic HSCT for acute myeloid leukemia, and hairy cell leukemia variant, has been successfully treated with rituximab.[105,152–156] In cases refractory to immunosuppressive agents affecting T-cell function, rituximab or low-dose alemtuzumab constitute a reasonable option.[152] Rituximab is effective in patients with PRCA owing to ABO incompatibility following HSCT.[105,106]

CsA can be administered at a dose of 5 to 10 mg/kg PO daily in divided doses. CsA can be combined with prednisone at doses of 20 to 30 mg PO. The trough levels of CsA should be monitored. An adequate trial of therapy is considered 3 months of therapy. The response rates may be as high as 60% to 80%. After a response is achieved, the therapy should be continued for 6 months followed by a slow taper.[67,157–159]

Horse ATG may be given to refractory cases at a dose of 40 mg/kg IV daily for 4 days with prednisone at 1 mg/kg. Concomitant prednisone should be administered and then tapered over 2 to 3 weeks. A therapeutic response should occur within 3 months post-therapy, although responses at or beyond 6 months may be observed.[131,160,161]

Methotrexate (MTX), an antimetabolite, at low doses (7.5 to 15 mg/week PO) is useful in treating PRCA, especially in patients with concomitant LGL leukemia. The responses are generally sustained, and therapy is well tolerated. MTX may be given in conjunction with other therapies like cyclophosphamide or CsA.[45,118]

Alemtuzumab (Campath, anti-CD52 monoclonal antibody) is a recombinant DNA–derived humanized monoclonal antibody directed against the cell surface glycoprotein CD52, which is expressed on the surface of normal and malignant B and T lymphocytes. It is currently approved for multiple sclerosis and available on compassionate use for other indications. Former IV dosing has been replaced by SC administration (initial dose of 3 mg, and subsequent doses at 10 mg once or twice weekly). The usual cumulative dose before response is around 50 to 100 mg. Alemtuzumab has been tested in a variety of lymphoproliferative disorders, including B-cell lymphomas (BCLs) and T-LGL leukemia. Similarly, PRCA occurring in the context of these conditions previously unresponsive to other therapies has been shown to be responsive to this agent.[127,162–164]

Daratumumab (Darzalex, anti-CD38 monoclonal antibody) is an IgG1k human monoclonal antibody directed against the cell surface glycoprotein CD38 expressed on many immune cells, including plasma cells. It is currently approved for treatment of refractory multiple myeloma (see Chapter 91). It has been shown to have efficacy in multiple case reports in refractory PRCA secondary to ABO mismatched allo-HSCT.[109–111]

HEMATOPOIETIC STEM CELL TRANSPLANTATION

Despite advances in immunosuppressive regimens, subsets of patients with PRCA remain refractory and are very difficult to treat. As with other bone marrow failure disorders with autoimmune pathogenesis like aplastic anemia, HSCT is an important treatment option especially for refractory and relapsed PRCA cases. Matched sibling donor allogeneic HSCT results in restoration of normal hematopoiesis in patients with refractory PRCA. In another case, a patient with relapsed PRCA underwent matched sibling donor allogeneic HSCT combined with donor lymphocyte infusions resulting in full donor engraftment and subsequent return of normal hematopoiesis, suggesting a graft-versus-autoimmunity effect as the likely mechanism for response.

Prognosis

The prognosis of secondary PRCA depends upon the underlying disease. Idiopathic PRCA may be very refractory to treatment. Ultimately, remission may be achieved in a significant proportion (approximately 68%) of patients, especially when sequential regimens are used. Spontaneous remissions are observed in 5% to 10% of cases. Relapses are common, especially during the first year post-remission but are usually responsive to the same regimen that induced remission.[118,128] Chronic, low-dose immunosuppressive therapy may be needed in certain cases that have relapsed. Median survival has been reported to be 12 to 14 years.[60] Unlike in amegakaryocytic thrombocytopenic purpura, evolution to aplastic anemia is rare and very few patients (3% to 5%) evolve into acute leukemia.[118,128]

Acquired White Blood Cell Production Disorders

Neutropenia is a common condition (see Chapter 49). The majority of cases are secondary to a variety of causes, including systemic or hematologic diseases. We will describe a primary, isolated form of neutropenia in which other hematopoietic lineages are not affected. In such a setting, neutropenia may be the result of peripheral destruction or perhaps less frequently the result of the absence of myeloid progenitors in the marrow. The cutoff value for the diagnosis of neutropenia is an absolute neutrophil count (ANC) of less than 1500 cells/μL. This value is generally accepted as a definition for neutropenia for all ages and ethnic backgrounds except for newborn infants.[165] Clinically, the most concerning consequence of neutropenia is the propensity to develop infections. However, the correlation between ANC and the propensity for infection is variable in different circumstances and determined by marrow neutrophil reserves, duration of neutropenia, and clinical context.[166–169] This is best illustrated in patients with chronic benign neutropenia of childhood and infancy, where patients may have an ANC as low as or less than 250 cells/μL and yet they may be devoid of infections or only have mild infections.[170]

Classification of Acquired Neutropenias

Neutropenia as a primary disease should be distinguished from inherited forms of neutropenia, which commonly present during early childhood (see Chapter 30), and secondary forms of neutropenia associated with systemic disorders. In addition, idiopathic neutropenia is distinct from the constitutional or familial benign neutropenia frequently seen in African Americans,[171,172] Yemenites, and Falasha (northern Ethiopian) Jews or Black Bedouins.[173] The degree of neutropenia is often mild, and there is no propensity to develop infections. Most cases of neutropenia are secondary to a variety of disorders. Primary autoimmune neutropenia (AIN) and idiopathic neutropenia are less common. We will limit our description to isolated forms of neutropenia (Table 33.4).

TABLE 33.4 **Classification of Neutropenia**

Congenital		
Primary	Autoimmune neutropenia	
	Pure white cell aplasia	
	Idiopathic	
	Thymoma	
	Hematologic malignancies (e.g., T-LGL leukemia)	
	Infections/postinfectious	
	Viral	
		Measles, mumps, roseola, rubella, RSV, influenza
		Hepatitis A, B, and C
		CMV, EBV, HIV
		Parvovirus
	Bacterial	
		Tuberculosis
		Brucellosis
		Tularemia
		Typhoid fever
	Rickettsial	
		Rocky Mountain spotted fever
		Ehrlichiosis.
	Fungal	
		Histoplasmosis
	Parasitic	
		Malaria, leishmaniasis
	Autoimmune conditions, (e.g., SLE, RA)	
	Drugs and chemicals	
	Neutropenia associated with immunodeficiency	
	Severe nutritional deficiencies	
	Neutropenia caused by increased margination	
	Iatrogenic (e.g., hemodialysis)	

CMV, Cytomegalovirus; *EBV*, Epstein-Barr virus; *HIV*, human immunodeficiency virus; *RA*, refractory anemia; *RSV*, respiratory syncytial virus; *SLE*, systemic lupus erythematosus; *T-LGL*, T-cell large granular lymphocyte.

Primary Neutropenia

Most cases of neutropenia are secondary to various hematologic and systemic diseases. However, in a small proportion of cases, an inciting cause cannot be identified despite intensive testing. Such idiopathic cases are most likely immune mediated.

Chronic Idiopathic Neutropenia in Adults

Chronic idiopathic neutropenia in adults compared with those in infancy and early childhood have less tendency toward spontaneous remission, although it does generally remain clinically benign. There may be concomitant anemia or thrombocytopenia that may portend a higher incidence of splenomegaly, infectious complications, and antineutrophil antibodies (ANAs) that are complement fixing.[174] A bone marrow biopsy may show evidence of an arrest in myeloid maturation and mild hypercellularity. ANAs may be seen in 36% of patients, suggesting an immunologic pathogenesis.[174] An altered TCR Vβ repertoire representing immunodominant CTL clones,[175] telomere shortening,[176] and activation of Toll-like receptor 4 have been reported to play a role in pathogenesis of chronic idiopathic neutropenia.

Idiopathic neutropenias may be chronic and benign in nature or may be associated with significant morbidity. In certain instances, idiopathic neutropenia may be associated with a hemolytic anemia or with immune thrombocytopenia, but these forms likely represent a distinct nosologic entity. Idiopathic neutropenia can occur at any age with a median age at diagnosis being 28.3 years and affects predominantly females (85%). Autoimmune idiopathic neutropenia (AIN) may be associated with moderate and severe depression of neutrophil counts. Monocytosis is frequently present.[167,168] Some cases present with splenomegaly, which is to be distinguished from cytopenias associated with hypersplenism in Felty syndrome.[177] The frequency of infectious complications rarely correlates with the severity of neutropenia, and patients with severe neutropenia may remain asymptomatic for long periods.

Conceptually, AIN may be caused by peripheral autoimmune destruction of neutrophils caused by lineage-specific inhibition of myeloid precursors, with its extreme form being pure white cell aplasia (PWCA) (see Fig. 33.3). Consequently, increased peripheral destruction is associated with marrow hypercellularity and an increased number of myeloid precursors or, if myeloid progenitors are the targets, decreased myeloid precursors and a myeloid maturation arrest. Autoimmune processes have been implicated in the pathogenesis of AIN and could be mediated by both peripheral destruction of neutrophils and inhibition of myelopoiesis.[178,179] The antigens involved in these processes include neutrophil antigens NA1, NA2, ND1, ND2, and NB1 (Table 33.5). Both IgG and IgM antibodies have been implicated. Neutrophil-specific antibodies can be detected using many assays, including specific enzyme-linked immunosorbent, opsonization,[180,181] leukoagglutination,[182] and direct antibody binding assays.[183] There are several proposed effector mechanisms as to how ANAs can result in neutropenia or affect neutrophil integrity. For example, ANAs may act as opsonins and directly enhance neutrophil destruction.[181] Alternatively, ANAs can indirectly activate, complement, and facilitate opsonization.[184] Immune complexes may also bind to the neutrophil Fc portion, leading to increased neutrophil clearance by the reticuloendothelial system,[185] and finally, ANAs may recognize and damage myeloid precursors.[186] The currently available diagnostic assays used to detect ANAs are generally based on immunofluorescence and agglutination assays. The former allows for the detection of IgM and IgG antibodies from a suspected patient that are attached to normal donor neutrophils detected by flow cytometry performed with the patient's serum and antihuman IgG. The second technique, called an agglutination assay, uses serum that leads to agglutination of normal neutrophils into either small or large clumps. These tests suggest an underlying immunologic process but cannot establish the definite cause. If there is a high index of suspicion that ANAs are the causative factor, some authors suggest that a minimum of two methods be used to detect ANAs.[187]

In addition to antibodies, T-cell responses may be associated with neutropenia. The most extreme example of such responses is T-LGL associated with neutropenia and polarized proliferation of CTLs (see later). In AIN, CTL responses are polyclonal (but demonstrate an

TABLE 33.5 **Human Neutrophil Alloantigens and Autoantigens**

		Nomenclature
Integrin α M chain	CD11b	HNA-4a (MART)
Integrin α L chain	CD11a	HNA-4a (OND)
Gp50-64	CD177	HNA-2a (NB1) PRV-1
Gp70-95		HNA-3a/5b
Integrin β2 chain	CD18	
FcγIII	CD16	
• FCGR3B-01		HNA-1a (NA1)
• FCGR3B-02		HNA-1b (NA2)
• FCGR3B-03		HNA-1c (SH/NA3)

immunodominant clone) and are often accompanied by the simultaneous presence of antibodies. It is likely that specialized CTL clones are capable of recognizing and killing myeloid precursors, interrupting granulocyte production.[175] Consequently, some cases of AIN may be amenable to therapy with immunosuppressive agents directed against T cells.

Chronic Benign Neutropenia of Infancy and Childhood

Chronic benign neutropenia of infancy and childhood is considered the most common cause of chronic neutropenia in the pediatric age group. The majority of cases are autoimmune in nature and show clinical overlap with childhood idiopathic thrombocytopenic purpura and autoimmune hemolytic anemia. It is therefore considered a type of AIN.[188–193] This form of neutropenia typically presents in children less than 3 years of age, with a median age of 8 to 11 months and with a predominance of females.[188,190,191] The ANC is usually less than 250 cells/μL with normal morphologic characteristics and normal Hb and platelet count. Occasionally monocytosis, eosinophilia, and mild thrombocytopenia may be present. In this condition, neutropenia is caused by chronic depletion of mature granulocytes and is accompanied by a compensatory myeloid left shift in the marrow.[170] Most frequent clinical signs and symptoms are oral infections, including bothersome ulcers, but these are often associated with additional functional defects of neutrophils and cellulitis of the labia majora. Of importance is the normal neutrophil count at birth and absence of a history of familial forms of neutropenia. The etiology of this condition is unknown, but the pathogenesis involves ANAs, detectable in the majority of patients.[188,190] The antibodies are generally directed against similar antigens as seen in adult AIN, especially those involving *NA*, *NB*, and *ND* loci. In about 25% of cases, the antibody is against an allele of neutrophil FcγRIII opsonin receptor called NA1.[188,190,191] Immunosuppressive therapy leads frequently to responses supporting the immune pathogenesis of this disease. Although the neutrophil count can be severely depressed, serious infectious complications are uncommon, and therefore treatment to raise the ANC is generally not indicated except if recurrent infections occur. Antibiotics are used to treat infections, and granulocyte colony-stimulating factor (G-CSF) has been shown to be effective in elevating neutrophil counts.[194]

SECONDARY FORMS OF NEUTROPENIAS

Clinical Associations

Drug-Induced Neutropenia/Agranulocytosis

Drug-induced neutropenia is common.[195] The association was first identified when agranulocytosis was observed in patients taking aminopyrine. The incidence in various studies is 1 to 3.4 cases per million per year.[196–200] Drugs may induce granulocytopenia by (1) direct toxicity leading to inhibition of myelopoiesis frequently observed in drugs like valproic acid,[201] carbamazepine,[202] and β-lactam antibiotics[203]; (2) immune mediated (either antibody- or complement-mediated) as seen with penicillin[204] or immune complex–mediated (quinidine)[205] destruction of myeloid progenitors and mature neutrophils; and (3) induction of CTL responses.[206] Polymorphisms in various genes coding for cytokines and cytokine receptors can lead to genetic predisposition for drug-induced neutropenia/agranulocytosis. This has been demonstrated for clozapine with polymoprhisms in tumor necrosis factor and HLA genes.[207] Of interest are drugs that directly antagonize important vitamin cofactors necessary in normal bone marrow development. Neutropenia observed in patients treated with trimethoprim-sulfamethoxazole is caused by the inhibitory effects on granulopoiesis by trimethoprim, owing to its antifolate action, which is reversed by folinic acid.[208] A similar finding can be seen with MTX owing to its antifolate action.

Most patients present with either asymptomatic neutropenia discovered on routine examination or symptomatic neutropenia with infectious complications, including fever, angular stomatitis, or pneumonia. The reported mortality rates vary from 1% to 25%. Most patients recover with withdrawal of the offending drug without further complications. It has been estimated that drugs account for 72% of cases of agranulocytosis. The usual time to development of overt neutropenia is around 1 to 2 weeks, and neutropenia resolves upon discontinuation of the offending drug within a 2-week period, although time to recovery may vary.[209] The recovery in neutrophil counts is usually preceded by increases in peripheral blood monocytes and immature granulocytes.[210] The International Agranulocytosis and Aplastic Anemia Study has identified the most commonly associated agents and the relative odds ratios for developing agranulocytosis (Table 33.6).[200] In some instances, the severity of neutropenia is related to the dose and duration of the therapy. The incidence of drug-induced neutropenia/agranulocytosis increases with age and is nearly twice as frequent in women. The therapy includes discontinuation of the potentially offending agents. In some instances, associated with infections or prolonged recovery, G-CSF may be administered.

Neutropenia as a Manifestation of Systemic Diseases

Postinfectious Neutropenia

Neutropenia is commonly associated with viral infections, particularly in children. Various mechanisms have been implicated in neutropenia associated with systemic viral infections, including inhibition of hematopoiesis, granulocyte sequestration, margination, and peripheral destruction. Neutropenia generally improves when the viremia resolves. Neutropenia has been associated with hepatitis A,[211] B,[211,212] and C[83,211] viruses, EBV,[213–215] influenza,[216] measles,[217] roseola,[218] CMV,[219–222] and parvovirus B19 infections.[223–225] Neutropenia is also frequently encountered in patients with HIV/AIDS, with approximately 70% of patients being neutropenic during their illness.[226] The mechanisms vary and may include antibody formation against neutrophils,[227–229] direct viral inhibition of hematopoietic progenitor cells, abnormal expression of growth factors and other cytokines, and inhibitory effects exerted by HIV-infected accessory cells.[230] The HIV virus not only suppresses hematopoiesis but also increases the risk for acquiring other infections. There has been a correlation between CD4$^+$ counts and persistent neutropenia that is associated with a lower nadir.[231] Furthermore, therapy with antiretroviral agents may dramatically decrease neutrophil counts (see Table 33.4).

Systemic bacterial, fungal, and parasitic infections can be accompanied by neutropenia, including typhoid fever,[232] shigella,[233] tularemia,[234] brucellosis,[235] mycobacterial infections,[236] histoplasmosis,[237] malaria,[238] leishmaniasis,[30] and ehrlichiosis.[239] The pathogenesis includes margination and sequestration of leukocytes as observed in malaria, in which there is reduction in the circulating neutrophil pool and enlargement of the marginating granulocytes, primarily in the spleen and lung. Neutropenia can be present during sepsis, especially in newborns or debilitated individuals.[240,241] In such situations,

TABLE 33.6 Drugs Associated With Agranulocytosis

	Etiologic Fraction	
	Agranulocytosis (%)	Aplastic Anemia (%)
Overall	64	62
IAAAS	12	27
United States	72	17
Thailand	70	2

IAAAS, International Agranulocytosis and Aplastic Anemia Study.

neutropenia may be caused by inhibition of hematopoiesis by inflammatory cytokines, exhaustion of marrow reserves, or redistribution.[242]

Neutropenia Association With Nutritional Deficiency and Nutritional Excess

Protein-calorie malnutrition, dietary restrictions, malabsorptive states, and concomitant intake of inhibitory drugs are just a few common causes of nutritional deficiencies that may lead to neutropenia. Vitamin B_{12} and folate deficiency, frequently associated with megaloblastic anemia, can also be associated with neutropenia. Lack of these essential vitamin cofactors results in the impairment of normal DNA synthesis, leading to abnormal granulopoiesis. A frequently observed morphologic feature is the presence of hypersegmented neutrophils.

Copper deficiency may also be associated with neutropenia.[243,244] Possible mechanisms may include arrest in maturation of neutrophil development as shown in studies in mice[245] and increased antineutrophil antibody formation.[246] Most cases have been found in malnourished infants or in patients with zinc intoxication[247,248] and malabsorption states.[249] Copper deficiency is often accompanied by a normocytic anemia, whereas platelet counts are invariably normal.[250] Other clinical and laboratory manifestations associated with copper deficiency include the presence of ringed sideroblasts in the bone marrow,[251,252] macrocytic anemia,[253] low ceruloplasmin levels,[251] myeloneuropathy,[254] and skeletal abnormalities.[255] Zinc intoxication in the absence of concomitant copper deficiency has also been associated with neutropenia generally in conjunction with severe anemia.[256]

Neutropenia Associated With Metabolic Disorders

Various acquired or inherited metabolic conditions may be associated with neutropenia. For example, neutropenia has been observed in patients with ketoacidosis and hyperglycemia, orotic aciduria, or methylmalonic aciduria.[257–260] Similarly, glycogen storage disease type IB is commonly associated with neutropenia responsive to myeloid growth factors.[261,262]

Acquired Neonatal Neutropenias

Neutropenia has been described in infants of hypertensive mothers. In this syndrome, the ANC can be severely depressed for up to 1 month postpartum.[263–265] This type of neutropenia is associated with an increased risk for early-onset sepsis in neonates, a prolonged duration of neutropenia, and an increased risk for neonatal nosocomial infections. Granulocyte kinetic investigations suggested that the neutropenia is the result of diminished neutrophil production. An inhibitor released by the placenta and present in cord blood serum has been shown to play a role in this syndrome.[263,266]

Moderate to severe neutropenia has also been observed secondary to IgG antibodies transferred from mother to infant. This is a condition called *isoimmune neonatal neutropenia* or *neonatal alloimmune neutropenia* and is a result of an incompatibility between maternal and fetal immune systems due to the mother developing alloantibodies against neutrophil antigens the fetus inherits from the father.[267] In most cases, antibodies are directed against antigens on neutrophil FcγRIIIb (anti-NA1, anti-NA2, and anti-SH)[268] and NB1,[269] but in rare circumstances maternal neutrophil-specific isoantibodies are also produced when there is deficiency of the *FcγRIIIb* gene.[270] The incidence of this condition can be as high as 2 : 1000 live births. In both neutropenia occurring in hypertensive mothers and isoimmune neonatal neutropenia, differentiation from congenital forms of neutropenia may be difficult. Clinically, this can present as pneumonia, meningitis, sepsis, and most often omphalitis. Treatment with G-CSF is usually effective in neutropenia of infants of hypertensive mothers, but higher doses may be needed because preeclampsia-associated inhibitor of rhG-CSF may be present.[271] IVIg[272] and G-CSF[271] are both effective for treatment of isoimmune neonatal neutropenia.

Neutropenia and Hypersplenism

Hypersplenism may be associated with neutropenia, but in most instances other cytopenias will also be present. However, hypersplenism may be a sign of diseases that can result in neutropenia, such as in T-LGL leukemia[120,121] and Felty syndrome.[273,274] Other than direct sequestration, another potential mechanism leading to neutropenia may be increased neutrophil apoptosis that normalizes after splenectomy.[275] A high incidence of *Helicobacter pylori* infection has also been noted among individuals with neutropenia and splenomegaly. Splenectomy either laparoscopically[276] or through laparotomy[277] may be effective in most cases, although in situations precluding splenectomy, intraoperative splenic artery embolization is also effective.[278]

Pure White Cell Aplasia

PWCA is a rare condition with pathophysiologic overlap with some forms of AIN associated with myeloid suppression. It has also shown to occasionally be associated with LGL leukemia. Similar to PRCA, the pathogenesis may vary and includes antibody-mediated suppression of granulopoiesis,[204,279] T-cell–mediated suppression of granulopoiesis, direct myelotoxicity as seen with certain drugs,[280] opsonization of neutrophil precursors in the bone marrow leading to its destruction by macrophages within the bone marrow[280] and formation of an antibody-drug complex that may damage myeloid progenitors.[281] A bone marrow examination reveals either a total absence of myeloid precursors or arrest at the promyelocyte stage, with megakaryocytes and the erythroid series remaining quantitatively and qualitatively normal. In many cases, a thymoma is present.[282,283] PWCA, if associated with thymoma, has a variable clinical outcome. The complete absence of granulocytic precursors portends a poor response to both immunosuppression and thymectomy and is often fatal, whereas the presence of a maturation arrest at the promyelocyte stage may respond to immunosuppressive therapy.[279,284,285] PWCA has been described in connection with imipenem-cilastatin,[286] ibuprofen,[287] mesalamine,[288] and chlorpropramide.[186] The discontinuation of the offending drug leads to rapid improvement.

Therapeutic options may include azathioprine,[279] G-CSF combined with plasmapheresis especially if the disease process is antibody-mediated,[289] methylprednisolone,[286] IV cyclophosphamide combined with plasmapheresis and G-CSF,[290] and thymectomy.[282] Severe depression of counts may require ATG therapy (see later).

Neutropenia Associated With Immunologic Abnormalities

Acquired and inherited defects of the cellular and humoral immune system may be accompanied by secondary neutropenias (see Chapter 50). In the inherited immunodeficiency syndromes, the initial presentation is neutropenic in children and may be associated with failure to thrive. X-linked agammaglobulinemia is a primary immunodeficiency disorder caused by mutations in the gene for Bruton tyrosine kinase (Btk) expressed in both myeloid and B cells that result in the absence of development of B lymphocytes and hypogammaglobulinemia. Neutropenia is seen in 15% to 26% of patients with X-linked agammaglobulinemia, and most suffer from upper respiratory tract infections.[291–293] The exact pathogenetic mechanism is not clear but is believed to be related to the crucial role of Btk in myeloid survival under stress. Neutropenia is also seen in 40% to 50% of patients with X-linked hyper-IgM syndrome and has been associated with defects of myelopoiesis.[294] The most common form of hyper IgM syndrome is caused by mutations in the *CD40* gene. This defect also leads to a decrease in IgG and IgA. In addition to chronic anemia, children suffer from various infectious complications. They typically lack ANAs and show an arrest at the promyelocyte-myelocyte stage of neutrophil development.[295] Allogeneic HSCT has been curative in some instances.[296] Common variable immunodeficiency can be associated with neutropenias that can be either chronic or episodic.[297] In addition, hypergammaglobulinemia and hypogammaglobulinemia and T- and NK-cell abnormalities of various causes (both inherited and acquired) can also be associated with neutropenia.

LABORATORY EVALUATION

Laboratory studies aim at the identification of the primary causes of neutropenia, as outlined in Fig. 33.4. Idiopathic and AIN remain, in most instances, diagnoses of exclusion. A careful history, including family history and initial age of first abnormal counts, help to distinguish familial neutropenias (see Chapter 30). Should primary causes such as drugs or systemic diseases be identified, the diagnostic evaluation will concentrate on disease-specific tests. In addition to a complete blood cell count and differential, which will help establish the severity of neutropenia, and tests to diagnose a specific disorder such as T-LGL leukemia, a bone marrow examination is required. Lack of morphologic or cytogenetic signs of primary hematologic diseases (e.g., MDS or aplastic anemia) and peripheral destruction will be supported by the observation of a left shifted myeloid predominance. Inhibition of myeloid production is exemplified by an increased erythroid:myeloid ratio.

In some instances, auxiliary tests may help identify the cause of the neutropenia, including vitamin B_{12}, zinc, folate, or copper levels. Determination of Ig levels may be helpful to establish the presence of immunodeficiency. Detection of ANAs has a limited significance because most of the currently available tests are not very sensitive and may not be specific. The presence of antibodies, however, may be helpful and supports the diagnosis of autoimmune forms of neutropenia.

Differential Diagnosis

Differential diagnostic consideration aims to distinguish primary from secondary forms of neutropenia and to exclude familial hematologic diseases. Suspicion of neutropenia secondary to a primary hematologic disorder requires a bone marrow examination that may be consistent with an early form of aplastic anemia or MDS.

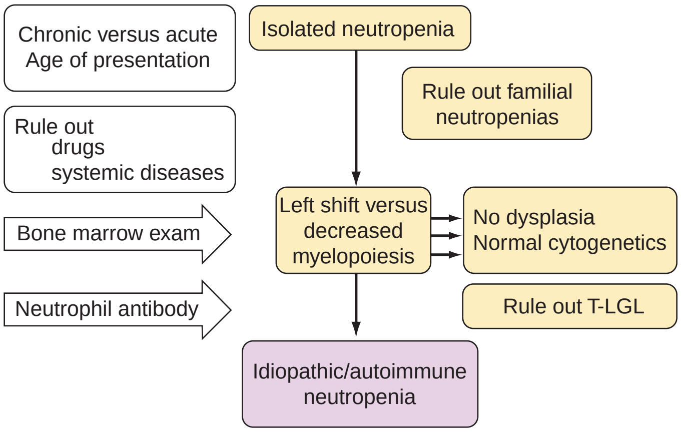


Figure 33.4 EVALUATION OF PRIMARY AND SECONDARY NEUTROPENIA. *T-LGL*, T-cell large granular lymphocyte.

Therapy

For secondary neutropenias, the therapy is aimed at the primary disease. If potentially offending drugs are present, they should be discontinued. G-CSF may be used for severe cases of both primary and secondary neutropenias. It may help to speed up recovery, but if the primary cause persists, it will have only a temporary effect. Primary or idiopathic neutropenias most often have an autoimmune cause. Prednisone may be used as first-line therapy,[298] but other B cell–targeted agents may be needed, including rituximab.[299] IVIg is effective in certain cases of PWCA. Finally, similar to the treatments applied for T-LGL leukemia, T cell–targeted approaches, such as CsA, can be used. A trial of at least 6 weeks is recommended.[300,301] Similarly use of ATG has also been effective for cases of PWCA[302] and other idiopathic forms of neutropenia.[303] Weekly oral MTX is effective in treating neutropenia related to Felty syndrome[304] and T-LGL leukemia.[120,305] Of note is that in many instances, isolated neutropenias may be asymptomatic, and therapy may not be needed. Alemtuzumab has been used with success in treating thymoma-associated PWCA and other cases of neutropenias.[306]

Large Granular Lymphocyte Leukemia

LGL leukemia is a chronic clonal lymphoproliferation of cytotoxic T cells (T-LGL) or NK cells (NK-LGL), often associated with cytopenias, including neutropenia, red cell aplasia, and thrombocytopenia. Pancytopenia is less frequently encountered and may be related to splenomegaly. T-LGL leukemia may be an indolent disorder and present with leukopenia or with lymphocytosis. LGLs observed on a peripheral smear are characteristic of T-LGL leukemia, but their frequency can vary.

T-LGL leukemia results from a proliferation of CTLs and often resembles reactive CTL expansion. It is associated with rheumatoid arthritis (11% to 36%) and B-cell malignancies (5% to 7%)[307] and to a lesser extent with other autoimmune diseases such as Sjögren syndrome,[308] celiac disease,[309] pulmonary hypertension,[310] hematologic malignancies like MDS,[311,312] and HSCT. Most reactive processes are polyclonal, but immunodominant CTL clones may be present, making a distinction between true T-LGL and a reactive process difficult (Fig. 33.5). A reduction in the variability of the CTL repertoire can occur in older adults, and clonal or oligoclonal expansion of CTL populations may be more frequent in older individuals. If

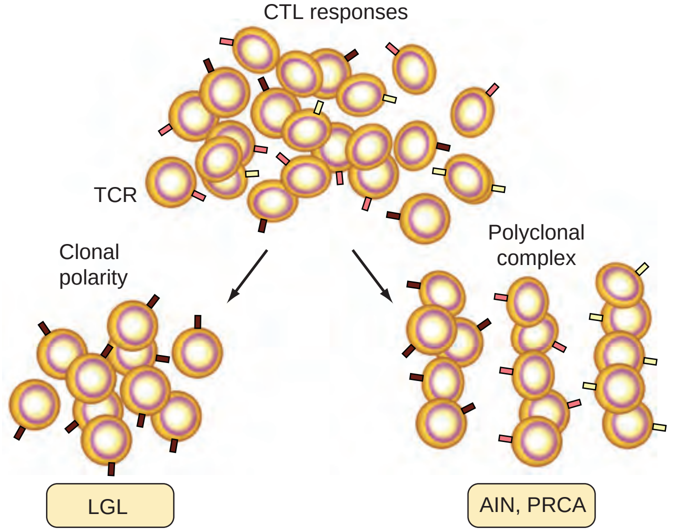


Figure 33.5 PATHOPHYSIOLOGY OF CYTOTOXIC T-LYMPHOCYTE RESPONSES. *AIN*, Autoimmune neutropenia; *CTL*, cytotoxic T lymphocyte; *LGL*, large granular lymphocyte; *PRCA*, pure red cell aplasia; *TCR*, T-cell receptor.

asymptomatic, this disorder has been termed *monoclonal clonopathy of unclear significance*.

Pathogenesis

Inciting Events

T-LGL leukemia frequently arises in the context of a reactive polyclonal CTL expansion undergoing transformation in a manner similar to that proposed for CLL.[313] It is possible also that in T-LGL leukemia one of the effector CTL clones may be initially driven by an inciting antigen, may transform, and consequently the cells fail to undergo apoptosis. The initial or initiating polyclonal response may be a component of the pathophysiologic process associated with infectious agents (such as retroviruses[314]), rheumatoid arthritis, or other autoimmune disorders.

An initial T cell–mediated process may be responsible for cytopenias in the absence of clonal predominance. In concurrence with this hypothesis, the clinical spectrum of T-LGL leukemias is determined by the specificity of the TCR: for example, if myeloid precursors are targets of clonal CTL, neutropenia will be a clinical manifestation. Conversely, if erythroid progenitors are affected, patients will present with anemia (see Figs. 33.1 and 33.6). However, unlike the cytopenias that resolve following immunosuppression, the CTL clone may persist at a certain level, suggesting that other disease mechanisms involving soluble factors play a role in the development of the cytopenias. Various soluble agents, including (FasL) and perforin, have been implicated in the pathophysiology of the cytopenias in T-LGL leukemia.[315–317]

Clonal Transformation

Clinically, T-LGL leukemia does not behave like a typical leukemia: excessive accumulation of malignant cells is often absent, and progression to a more malignant phenotype is rare.[120,318] Instead, the expanded clone in T-LGL leukemia resembles a normal antigen-activated $CD8^+CD57^+$ terminally differentiated effector memory T cell; both normal and malignant LGL constitutively express perforin and FasL and can suppress neutrophil development in vitro.[316,319,320] Typical clonal LGL cells seem to be terminally differentiated and cannot be effectively expanded in vitro by polyclonal mitogens.

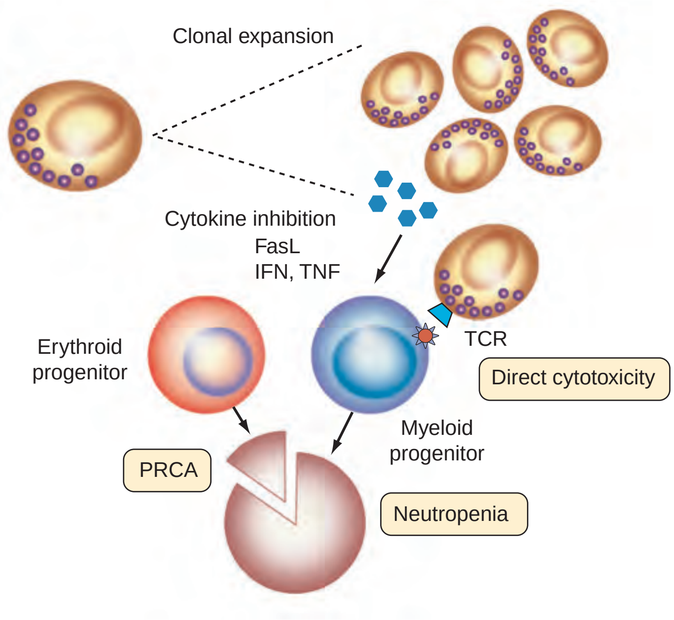


Figure 33.6 PATHOPHYSIOLOGY OF CYTOPENIAS IN T-CELL LARGE GRANULAR LYMPHOCYTE LEUKEMIA. *FasL*, Fas ligand; *IFN*, interferon; *PRCA*, pure red cell aplasia; *TCR*, T-cell receptor; *TNF*, tumor necrosis factor.

It is likely that a polyclonal CTL response predates the outgrowth of the immunodominant T-LGL leukemia clone (see Fig. 33.3). The putative transforming event most likely involves a memory cell that feeds into the mature effector CTL compartment.[321] Under normal physiologic circumstances, activated effector T cells are deleted after antigen-driven expansion by Fas-mediated apoptosis. The failure of an activated memory and/or effector clone to undergo apoptosis may result in its persistent expansion.[322–324] LGL leukemia cells express high levels of Fas/FasL, yet themselves are resistant to Fas-mediated apoptosis.[325] It is conceivable that persistent LGL leukemia cell expansion may result from this resistance to homeostatic apoptosis. In addition to the high surface expression of Fas/FasL, soluble FasL has been detected in sera from T-LGL leukemia patients and may contribute to the induction of apoptosis of neutrophil precursors in the bone marrow.[305]

An LGL clone persists mostly in the G_0/G_1 phase of cell cycle, and clonal transformation may also be because of a constitutive overexpression of prosurvival and antiapoptotic transcription factors.[326] Dysregulation of the JAK/STAT pathway is a core aspect of LGL leukemia pathobiology.[327,328] *STAT3* has been shown to be involved in cellular transformation along with an active Src family kinase[329] and appears to be constitutively activated in T-LGL leukemia cells.[327,328,330] In addition, a constitutive activation of an Src family kinase in T-LGL leukemia (likely Lck or Fyn) has been reported that may be related to this increased STAT phosphorylation. It has been proposed that STAT3 activation in T-LGL may inhibit apoptosis downstream of Fas receptor signaling by induction of myeloid cell leukemia-1 (MCL1), a member of the BCL 2 family of antiapoptotic proteins.[330,331] This finding is further supported by data showing that blockade of STAT signaling in T-LGL cells leads to the reversal of Fas resistance. Similarly, constitutive activation of the extracellular signal-related kinase mitogen-activated protein kinase pathway seems to play a role in survival of NK- and T-LGL leukemia cells.[332]

Extreme Clonal Expansion and the Nonrandom Nature of the T-Cell Large Granular Lymphocyte

Molecular analysis of the TCR repertoire in T-LGL leukemia has revealed a spectrum of expansion of the T-cell clone in individual patients. In some cases, up to 98% of the $CD8^+$ repertoire consists of only one clone, a surprising finding given the absence of immunodeficiency among T-LGL leukemia patients. More recent studies using deep sequencing of the TCR have verified this with significantly decreased diversity of the T-cell repertoire in T-LGL leukemia.[333] In healthy controls, even the most predominant clones, most likely reactive to ubiquitous antigens, represent around 1% of the entire TCR repertoire.[334] It is possible that structurally similar clonotypes present in some patients with T-LGL arise in the context of initial polyclonal CTL response and the initial transformation step is not random (see Fig. 33.3). Once a pathogenic immunodominant clonotype is identified and characterized, its sequence may be used for molecular tracking.

Genetic Alterations in Large Granular Lymphocyte

No recurrent chromosomal aberrations or mutations are associated with LGL leukemia. Massively parallel second-generation sequencing technology has been used successfully to uncover the genetic background of LGL leukemia. Whole exome sequencing has aided in the discovery of somatic *STAT3* mutations, an oncogene located in chromosome 17 in 40% of the LGL cases.[327,328] The *STAT3* missense mutations (D661V, D661Y, D661H, Y640F, N647I, and K658N), as well as the insertion mutation (Y657_K658insY), are located in the SH2 domain on the dimerization interface that mediates *STAT3* activation. *STAT3* mutations are detected in one-third of cases of

NK–LGL unifying T- and NK-cell lymphoproliferative pathogenesis.[327] Several studies have identified *STAT3* and *STAT5b* mutations specific to LGL at various frequencies. Of interest, patients with *STAT3* mutations were more likely to have autoimmune conditions and neutropenia and tend to respond to treatment.[335] Sequencing of *STAT3* wildtype cases revealed activating mutations in *STAT5b* in 2% of the cases.[336] *STAT3* and *STAT5b* mutations can be used as molecular markers for LGL leukemia diagnostics, and they present novel therapeutic targets for STAT3 and STAT5b inhibitors.[337,338] Interestingly, LGL leukemia patients with *STAT5b* mutations (specifically N642H) were associated with highly aggressive and fatal disease, which is different from the relatively indolent course of typical LGL leukemia.[336] Furthermore, not only were *STAT* mutations identified in aplastic anemia and MDS cases with concomitant LGL, but they were found in AA (7%) and MDS (2.5%) patients without LGL.[312]

Clinical Presentation and Physical Features

Patients with T-LGL leukemia present at a median age of about 55 years, with an equal male/female distribution. The clinical course may be indolent and chronic. Patients are asymptomatic in one-third of cases. The most common clinical presentation is neutropenia (observed in approximately 85% of patients) often accompanied by infections such as recurrent aphthous ulcerations or neutropenic fever due to bacterial infections.[307,318,339–341] However, in contrast to neutropenia associated with other hematologic disorders, LGL leukemia patients may remain surprisingly free of infectious complications for extended periods of time regardless of the depressed ANC. Despite the extreme clonality within the T-cell population (suggesting a decreased antigen recognition spectrum), opportunistic infections are rare. Other single-lineage cytopenias, including PRCA and immune-mediated thrombocytopenia, accompany T-LGL leukemia less frequently than neutropenia.[342,343] Pancytopenia may be related to splenomegaly reported in 20% to 50% of patients. Anemia is also seen with 10% to 30% of anemic patients becoming transfusion-dependent.[307] Hepatomegaly is present in a minority of patients (10% to 20%). Lymphadenopathy and B symptoms may also occur; however, this is uncommon.[318] It has been reported that pregnancy can improve neutropenia in women with LGL leukemia. Clinical transformation to a more malignant form is rare. The clinical presentation of NK-cell LGL lymphocytosis is very similar to that seen in T-LGL leukemia with regard to lymphocyte counts, associated conditions, treatment responses, and survival.[307,344]

Clinical Overlap and Associations

In some clinical circumstances, natural or pathologic immune responses can resemble T-LGL expansions. For example, responses to viruses such as CMV or EBV, although of an oligoclonal or polyclonal nature, may display a strong clonal dominance mimicking at times a true clonal process.[345] Consequently, polarized CTL responses in the context of infections have to be distinguished from true LGL leukemia.

T-LGL leukemia can occur concomitantly with several autoimmune diseases. Rheumatoid arthritis is likely the most common association, occurring in one-third of patients with T-LGL leukemia, but additional diseases including ulcerative colitis, Sjögren syndrome, Behçet disease, systemic lupus erythematosus, multiple sclerosis, and a number of other (auto)immune conditions have been described.[346–353] Felty syndrome is characterized by neutropenia with rheumatoid arthritis and splenomegaly; 80% of cases express the HLA-DR4 allele, a finding also observed in T-LGL leukemia. This common immunogenetic link and similar patterns of cytotoxic clonal expansion with T-cell infiltration suggest that Felty syndrome and T-LGL leukemia represent components of the same disease process.[352,354,355] In addition, PRCA and immune-mediated thrombocytopenia may also be associated with LGL lymphoproliferation.[356] Clonal expansions that characterize T-LGL leukemia can appear similar to oligoclonal CTL responses elicited by strong immunodominant antigens, including certain viruses—thus the distinction between T-LGL leukemia and a reactive lymphoproliferative process. LGL leukemia has also been described after HSCT and solid organ transplantation, perhaps initiated by an alloantigen or an infectious agent such as EBV.[357]

LGL-like cell expansions may also be present in other hematologic disorders, including MDS, aplastic anemia, and paroxysmal nocturnal hemoglobinuria (PNH), and may coincide with a number of lymphoproliferative disorders as well.[358–365] In MDS the prognosis is usually determined by the presence of MDS, but the presence of LGL may provide a rational target for immunosuppressive therapy. In MDS, T-LGL leukemia has been reported to negatively affect the outcome of therapy directed against the CTL clone.[361] LGL leukemia has also been described in conjunction with hemolytic anemia following bone marrow transplantation, perhaps a process initially driven by an alloantigen or infectious agent such as EBV.[13] As with infections, distinction between an LGL leukemia and reactive lymphoproliferation may be blurred. For example, neutropenia may be associated with various degrees of clonality, with an LGL leukemia representing the most extreme form of this process.

Laboratory Diagnosis

Diagnostic criteria remain a subject of considerable discussion (Table 33.7). Traditionally, LGL lymphocytosis (identified by morphologic characteristics and flow cytometry) is a significant diagnostic criterion with either an increased number of LGL cells (>2000/μL) or an LGL count between 400 and 2000/μL when associated with clinical or hematological features compatible with the diagnosis.[307,366] T-LGL cells exhibit a mature post-thymic phenotype. Most investigators consider the presence of an expanded homogeneous CD3^{+}, TCR-αβ^{+}, CD8^{+}, CD16^{+}, CD45RA^{+}, CD57^{+}, and CD4^{-}, CD5$^{\mathrm{dim}}$, CD27^{-}, CD28^{-}, CD45RO^{-} cell population as diagnostic of T-LGL leukemia. Additionally, for NK-LGL leukemia, NK-LGL cells display CD2^{+}, sCD3^{-}, CD3ε^{+}, TCR-αβ^{-}, CD4^{-}, CD8^{+}, CD16^{+}, CD56^{+}, CD57$^{(\mathrm{variable})}$ for NK-LGL leukemia.[367–369] In almost all patients the expanded clone is CD8^{+} (only very rarely CD4^{+}), and in the majority of cases this population also expresses CD57, but LGL leukemia cases without this marker have been observed.[369] Clinical correlations based on immunophenotypic characteristics have been defined; CD8$^{+(\mathrm{dim})}$/CD57^{+} LGLs are associated with clonal T-LGL leukemia and neutropenia, CD16 expression with complete or partial loss of CD5 is

TABLE 33.7 Immunophenotype and Laboratory Features of T-Cell Large Granular Lymphocyte Leukemia

Laboratory features	
Relative/absolute lymphocytosis	
LGL on peripheral blood smear	
CD4/CD8 ratio reversed	
Vβ family skewing (flow cytometry)	
Immunophenotype	CD2^{+}, CD5^{+}, CD3^{+}
	Majority CD8^{+}, few CD4^{+}/CD8^{+} or CD4^{+}
	CD27^{+}CD28^{-}
	CD57^{+}CD16^{+} perforin/granzyme^{+}
	CD56^{+} associated with more aggressive forms
TCR rearrangement	TCR-γ PCR
	Southern blot

LGL, Large granular lymphocyte; *PCR*, polymerase chain reaction; *TCR*, T-cell receptor.

associated with T-LGL leukemia but not cytopenias, and CD8$^{+(\text{dim})}$/CD57^{+} with loss of CD5 expression is associated with T-LGL leukemia with severe neutropenia.[370] In addition, clinically aggressive T-LGL leukemia is characterized by expression of CD56 and has been associated with mutations of *STAT5b* gene.[336] Additionally, there has been a distinct subtype of LGL leukemia identified with a CD3^{+}, CD4^{+}, CD8^{+}, CD56^{+} that may also be associated with *STAT5b* mutations but instead has an indolent clinical course.[336] In most cases of LGL leukemia, CD94 is expressed at increased levels, and other receptors for class I MHC molecules are abnormally expressed. Some investigators have suggested that a pool of CD8^{+} memory cells exists that lack CD57 expression but feed into the mature CD57 effector compartment.[321,367]

The size of the abnormal clone defining T-LGL leukemia remains controversial. It is likely that the size of the leukemic T-cell population influences the detection of the clonal TCR γ-chain (G) rearrangement by PCR; thus, this is considered mandatory for diagnosis.[371,372] TCR γ can be used for both α/β and γ/δ T cell lineages. These methods may detect a clonal population that represents 15% of the cell population, but it is also likely that smaller CTL numbers may be consistent with latent T-LGL detected only if more precise methods are used. T-LGL can express CD4, but both CD4^{+} as well as double-positive CD4^{+}/CD8^{+} cases are rare. Cytogenetic analyses are not useful, although cases with chromosomal aberrations have been described.[373,374]

Flow cytometric analysis of Vβ utilization pattern with antibodies directed against most of the Vβ-chain types may be helpful in making the diagnosis. The Vβ family expansion by flow cytometry does not prove clonality, but it may help assess the contribution of the T-LGL clone to the CD8^{+} or CD4^{+} population (Fig. 33.7).

Identifying clonality in NK-LGL leukemia is a more difficult task due to the absence of TCR recombination or expression. The use of immunophenotyping assays that measure the expression patterns of KIRs may be useful in diagnosis.

Rare immunophenotypic variants of T-LGL exist that co-express CD4 and CD8, lack both of these markers, or use γ/δ TCR instead of α/β TCR chains. Vβ flow cytometry has been used to assess the size of the LGL clone and its Vβ use; Vβ use can be identified in 80% of patients. The current Vβ antibody panel does not cover 25% to 35% of the Vβ spectrum. A monotypic expression pattern of KIR can be found with monoclonal antibodies to CD157b, CD158a, and CD158e (corresponding to the most prevalent *KIR* genes) in about 50% of patients.[375]

Additional supportive tests include a reticulocyte count, which is low in cases presenting with red cell aplasia. The mean corpuscular volume is usually high. Serologic studies or a DNA titer to detect evidence of an EBV infection is usually not needed but may be helpful to distinguish reactive immunologic responses. The rheumatoid factor is frequently positive. Antinuclear antibodies and ANAs may also at times be positive (see box on Approach to the Diagnosis and Treatment of Acquired PRCA, AIN, and T-LGL).[376,377]

Differential Diagnosis

Differential diagnostic considerations include reactive processes such as viral infections. Occasionally MDS may be present simultaneously or serve as an alternative diagnosis; T-cell oligoclonality may accompany MDS.

Therapy

A significant proportion of patients will be asymptomatic, and in such cases therapy may be delayed. Lymphocytosis may be significant, but absolute lymphocyte counts more than 40,000/μL is unusual. Symptomatic splenomegaly may be an indication for splenectomy.

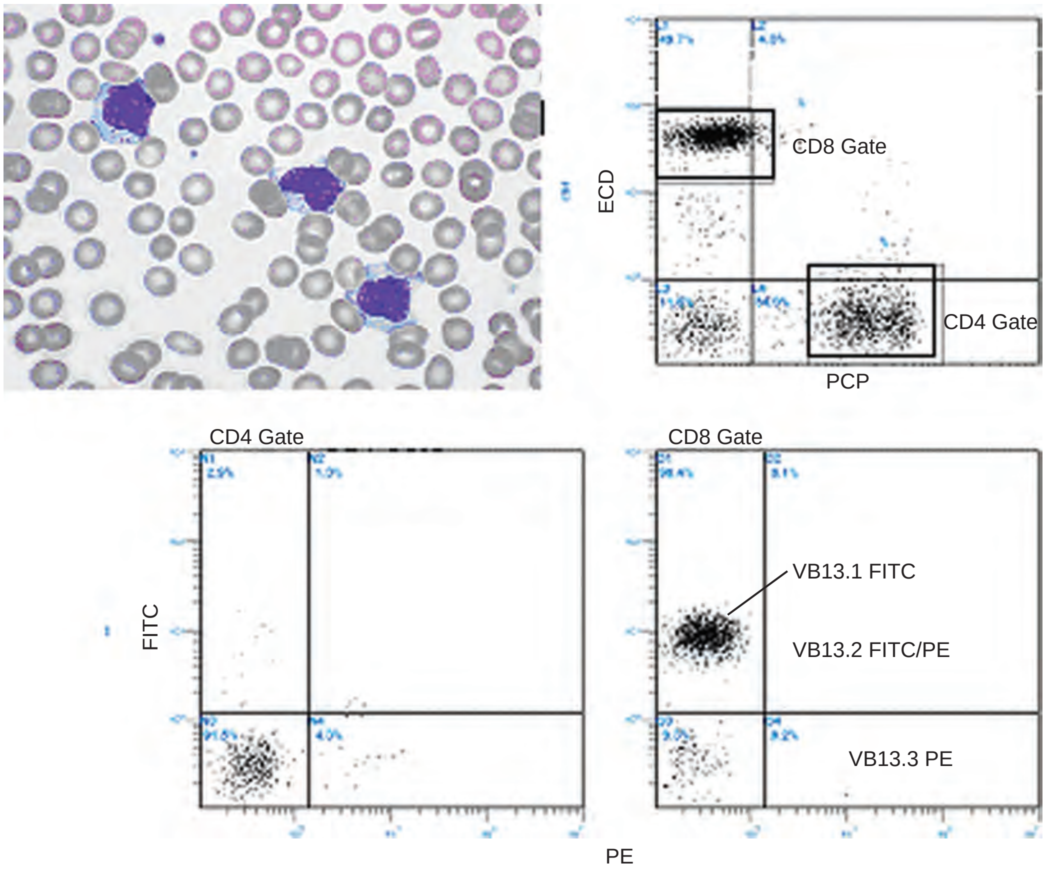


Figure 33.7 MORPHOLOGIC FEATURES AND FLOW CYTOMETRY OF T-CELL LARGE GRANULAR LYMPHOCYTE. *ECD*, Ethyl cysteinate dimer; *FITC*, fluorescein isothiocyanate; *PCP*, phencyclidine hydrochloride; *PE*, phycoerythrin.

APPROACH TO THE DIAGNOSIS AND TREATMENT OF ACQUIRED PURE RED CELL APLASIA, AUTOIMMUNE NEUTROPENIA, AND T-LARGE GRANULAR LYMPHOCYTE

Acquired PRCA is characterized by reticulocytopenia, but the diagnosis is based on the morphologic absence of erythroid precursors in the bone marrow. Congenital forms of PRCA, including Diamond-Blackfan anemia, need to be distinguished when presenting in young children. In adults, primary idiopathic disease has to be differentiated from secondary forms of red cell aplasia associated with hematologic diseases such as B-cell chronic lymphocytic leukemia, myeloma, T-LGL leukemia, and parvovirus B19–associated chronic reticulocytopenia or acute transient aplastic crisis. The diagnosis of parvovirus B19 infection can be made on the basis of the presence of parvovirus B19–specific IgM and by DNA hybridization techniques. Parvovirus B19–specific polymerase chain reaction can help rule out an ongoing infection. This diagnosis is important because therapy with IVIg can be curative. The therapy of secondary red cell aplasia includes treatment of the underlying condition. It is also important to distinguish red cell aplasia from myelodysplastic syndromes, which can be associated with erythroid hypoplasia but carry a significantly worse prognosis.

In cases with thymoma, thymectomy is the usual initial treatment approach; however, incomplete responders, nonresponders, and patients who relapse are common, necessitating the addition of immunosuppressive therapy. In patients with idiopathic PRCA, therapy includes immunosuppressive agents such as prednisone, CsA, or oral cyclophosphamide. Second-line therapies include ATG or rituximab.

Neutropenia may be associated with severe infections, but the risk associated with neutropenia depends on its clinical context, severity, and duration. The management of neutropenia must account for its clinical presentation and the risk for possible life-threatening complications and includes supportive care, clinical monitoring, and implementation of prophylactic antibiotics and/or hematopoietic growth factors. Neutropenia in childhood may be caused by congenital diseases, be associated with viral infections, or be immune mediated. These three causes are usually self-limiting. In adults, most neutropenias are secondary to other conditions, including underlying hematologic or systemic diseases. In general, drug reactions are a very common cause of neutropenia. Idiopathic or AIN as a primary disease is a diagnosis of exclusion. The pathogenesis involves T-lymphocyte/NK–mediated inhibition of myelopoiesis or ANAs. Immunosuppressive therapy may be used and includes prednisone, methotrexate, CsA, IVIg, cyclophosphamide or rituximab or in conjunction with myeloid growth factors.

T-LGL leukemia is a chronic, often indolent clonal lymphoproliferation of cytotoxic T cells associated with immune-mediated cytopenias. It may be a part of a continuum of reactive cytotoxic T-cell responses ranging from polyclonal, oligoclonal, to monoclonal expansions as seen in T-LGL leukemia. The pathophysiology of cytopenias includes cytokine effects and direct antigen-specific cytotoxicity. Most patients present with neutropenia. PRCA and pancytopenia are less common. Hemolytic anemia and pancytopenia may be the result of splenomegaly present in a significant minority of patients. B symptoms and lymphadenopathy are uncommon, and many patients remain asymptomatic. The diagnosis is established according to the presence of characteristic LGL lymphocytosis, but in some patients the LGL count may not be very high. The immunophenotype is $CD3^+$, $CD8^+$, $CD57^+$, $CD16^+$, $CD56^-$, and $CD28^-$. CD56 antigen–expressing LGL may be characterized by a more aggressive course. Some cases may coexpress CD4 and CD8. The diagnosis includes detection of a T-cell receptor rearrangement. In most instances, expansion of the involved Vβ family may be detected using Vβ flow cytometric clonotyping. T-LGL is often associated with autoimmune diseases, including rheumatoid arthritis and Felty syndrome. T-LGL can accompany MDS and, in rare instances, aplastic anemia or PNH. Reactive, often viral infection–associated, CTL proliferation may be difficult to document. Asymptomatic cases are monitored, and development of systemic symptoms or symptomatic cytopenias may prompt therapy. Current treatments include immunosuppressive agents such as prednisone, CsA, oral methotrexate, or cyclophosphamide. Chronic long-term therapy may be more effective than high-dose combination chemotherapy applied in B-cell lymphomas. Second-line treatments may involve alemtuzumab or ATG. The prognosis is generally good, and transformation to a more aggressive lymphoproliferative disorder is rare.

Unless additional hematologic diseases, such as MDS, are suspected, bone marrow examination may not be required. Bone marrow biopsy and aspirate should be obtained in cases of pancytopenia or involvement of several lineages. Morphologic hallmarks of MDS and cytogenetic analysis may help establish a diagnosis of MDS.

AIN, Autoimmune neutropenia; *ATG*, anti-thymocyte globulin; *CsA*, cyclosporine A; *CTL*, cytotoxic lymphocytes; *IVIg*, intravenous immunoglobulin; *MDS*, myelodysplastic syndrome; *PRCA*, pure red cell aplasia; *T-LGL*, T-cell large granular lymphocyte.

Pancytopenia may be a result of splenomegaly, and the procedure aids in the treatment of a hemolytic anemia that can be present in some patients.

The therapeutic approach to LGL leukemia has historically relied on immunosuppressive therapy with MTX, cyclophosphamide, and cyclosporine. These remain the standard treatment options but there have been other more recent options proposed.[307]

MTX is used as a first-line therapy based on prospective studies performed with or without prednisone as an adjunct therapy.[378,379] The use of MTX in the treatment of RA makes its use especially beneficial in LGLL patients with RA. MTX is administered orally at $10\,mg/m^2$ per week. The monitoring of MTX requires assessment of both hepatic and lung function for toxicity. Importantly, the largest prospective trial was a phase II multicenter clinical trial analyzing treatment of 59 patients that found a response rate of 38% (complete and partial responders) to frontline MTX therapy (Table 33.8).[335] This response is lower than what has been observed for cyclophosphamide as a first-line treatment (see below). To address this, a randomized prospective trial is ongoing that directly compares MTX and cyclophosphamide efficacy (NCT01976182). Patients may tolerate significant degrees of neutropenia for many years. Indications for treatment include neutropenic complications or transfusion dependence (Fig. 33.8). G-CSF therapy will increase counts in some patients, but a significant proportion of patients will be refractory. Of interest is the observation that high-dose therapy with typical lymphoma regimens may be ineffective and therefore should not be used. Cases refractory to HSCT have been described.[380] Monotherapy with prednisone may relieve some of the symptoms and improve neutropenia, but remissions are usually not durable. CsA represents a reasonable first-line therapy; however, a course of sufficient duration has to be given, with a response expected after 8 to 10 weeks of therapy.[379] Patients with

TABLE 33.8 Meta-Analyses of Studies for Response to Therapy in Large Granular Lymphocyte Leukemia

Study	*N*	MTX (N)	OR (%)	CsA (N)	OR (%)	CPM (N)	OR (%)
French Registry[a]	229	62	34 (55)	24	5 (21)	32	21 (66)
Battiwalla et al.[b]	25			25	14 (56)		
Moignet et al.[a]	45					45	32 (71)
Osuji et al.[a]	29	8	6 (85)	23	18 (78)	4	1 (25)
Dhodapkar[b]	68	2	2 (100)			16	11 (69)
Loughran[c]	59	55	21 (38)			14	9 (64)

[a] Retrospective analysis from multiple centers.
[b] Retrospective analysis in single center.
[c] Multicenter phase II trial.
CPM, Cyclophosphamide; *CsA*, cyclosporine A; *MTX*, methotrexate; *OR*, overall response.

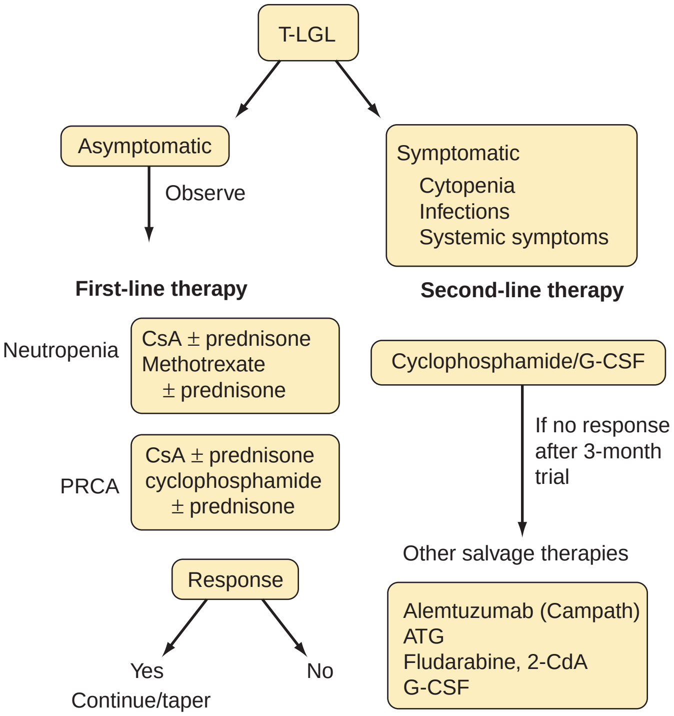


Figure 33.8 THERAPY OF T-CELL LARGE GRANULAR LYMPHOCYTE LEUKEMIA. *ATG*, Antithymocyte globulin; *2-CdA*, 2-chlorodeoxyadenosine; *CsA*, cyclosporine A; *G-CSF*, granulocyte colony-stimulating factor; *PRCA*, pure red cell aplasia; *T-LGL*, T-cell large granular lymphocyte.

LGL leukemia–associated PRCA may be better treated with oral cyclophosphamide instead of MTX with a dose between 50 and 100 mg/day. In LGL leukemia with associated PRCA, responses may be delayed, and the PRCA may recur after discontinuation of cyclophosphamide therapy, or if insufficient treatment (<6 to 8 weeks) was administered.[62,307] In the above-mentioned trial, overall response rate to cyclophosphamide was 64% following initial MTX therapy.[335] Therapy with cyclophosphamide in neutropenic patients may be difficult because of myelosuppression. In most refractory cases, ATG has also been used with success.[379] Successful therapy with alemtuzumab has been reported. CD52 expression determined by flow cytometry is an important predictive factor for response to alemtuzumab.[381,382] High response rates with the Janus-activated kinase inhibitor tofacitinib citrate have been observed (66%) when used as a salvage therapy.[383] Currently such a therapy is approved in cases of LGL with rheumatoid arthritis. Purine analogues like fludarabine given in combination with mitoxantrone and dexamethasone or with dexamethasone alone have been shown to produce impressive responses in 79% of cases, although these were confined to a small group of patients.[384] Rituximab has been used for RA-associated T-LGL with a 100% ORR in a small case series.[385] Allogeneic HSCT has also been used successfully in some cases.[386,387] Relapses are frequent but are usually responsive to the previously effective therapy. Certain patients may require low-dose maintenance therapy with CsA. In some cases, remarkable improvement of cytopenia can be achieved with splenectomy.[388,389]

Prognosis

LGL leukemia is a chronic condition and may be indolent. In general, mortality is low. Transformation to more aggressive forms of lymphomas or leukemias is uncommon. In cases of T-LGL leukemia associated with a primary hematologic disease such as MDS, the prognosis is dependent on the therapy of the underlying problem.

Acquired Platelet Production Disorder

Megakaryocytes, like all formed elements of the peripheral blood, are ultimately derived from undifferentiated hematopoietic stem cells that exist in a developmental continuum (see Chapters 9 and 29).[390] Through a series of still incompletely understood events, stem cells undergo an asynchronous division that gives rise to two daughter cells. One daughter cell remains a stem cell, fulfilling the requirement for self-renewal of the stem cell compartment, and the other commits to developing within a given lineage, likely through the induction of specific transcription factors such as GATA1, FOG-1, and Fli-1, in the case of megakaryocytes,[391,392] and perhaps by downmodulation of other transcription factors, such as c-Myb.[393,394] Lineage-committed progenitor cells are characterized by a loss of "plasticity" and a remarkable capacity for proliferation. The latter is required because approximately 15 × 10^6 megakaryocytes/kg body weight must be available to produce the roughly 100 × 10^9 new platelets that are needed daily to maintain a normal platelet count of 150 to 400 × 10^9/L.[395] As progenitor cell divisional activity proceeds, maturation, as defined by the acquisition of lineage-specific proteins, ensues, largely under the control of the hematopoietic cytokine thrombopoietin.[396–398] After a variable number of mitoses, proliferative activity eventually declines, giving rise to many daughter cells, which are known as *precursors*. Precursor cells are essentially postmitotic and are capable of one or two additional cell divisions at most. They are often morphologically identifiable as belonging to a given lineage and are primarily engaged in the terminal maturation steps that allow them to function as competent members of their lineage. In the case of megakaryocytes, precursor cells undergo nuclear endoreduplication to increase their ploidy (to a mean of approximately 16 N), a characteristic unique to cells of the megakaryocyte lineage.[391,398–404] Nuclear endoreduplication is accompanied by an increase in megakaryocyte cytoplasm and thereby the number of platelets that an individual megakaryocyte can produce.

As discussed in Chapter 29, the process of platelet formation, or thrombopoiesis, occurs during megakaryocyte terminal maturation.[405,406] It is initiated by the development of the demarcation membrane system in the megakaryocyte's cytoplasm.[407] Among the functions of the demarcation membrane system is delineation of platelet fields. These fields are filled with the granules and proteins that ultimately make up the contents of mature platelets. The latter are shed from pseudopods that mature megakaryocytes extend through endothelial cell junctions into the lumen of marrow capillaries. The pseudopods fracture, because of shear stress in the lumen of these capillaries, and release shards of megakaryocytic cytoplasm, or proplatelets, that are the immediate antecedents of circulating platelets. A fully mature megakaryocyte is estimated to produce approximately 1 to 1.5 × 10^3 platelets. The molecular regulation of this process is beginning to be better understood. It has been shown, for example, that the apoptosis-stimulating gene *Bax* promotes platelet production.[408] Interestingly, the life span of circulating platelets is also regulated by the apoptosis proteins. Using the strategy of ethylnitrosourea-inducted mutations, Mason et al.[222] have recently demonstrated that mutations in the *Bcl-x$_L$* gene lead to synthesis of a form of the protein that no longer inhibits *Bax*, and that this in turn leads to accelerated platelet death and a heritable form of thrombocytopenia (Fig. 33.9).

Failure in the process of either megakaryocytopoiesis or thrombopoiesis will result in thrombocytopenia. Under either circumstance, platelet production is characterized as "ineffective," either because there is an absolute decrease in available megakaryocyte cytoplasm (failure of megakaryocytopoiesis) or because cytoplasmic development is defective (failure of thrombopoiesis). Selective impairment of megakaryocytopoiesis may also result from damage to the progenitor cell compartment (the burst-forming units–megakaryocyte or colony-forming units–megakaryocyte [CFU-Mk]; see Chapter 29) or rarely from a compromised ability to synthesize thrombopoietin, the chief cytokine regulator of this compartment (Fig. 33.10).[409] Inherent or acquired defects in megakaryocyte precursor cells may lead to ineffective thrombopoiesis (see Chapter 30).[409,410] The relative

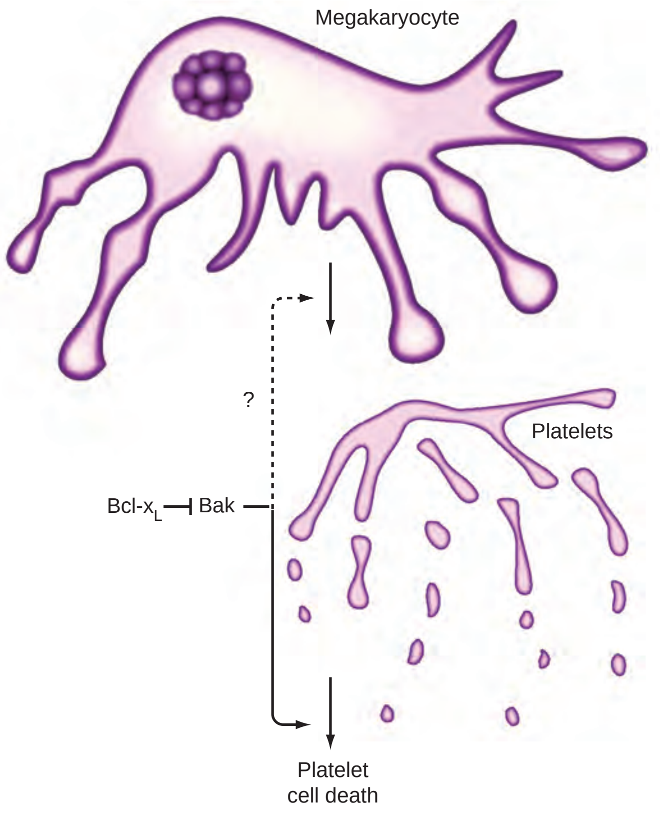


Figure 33.9 B-CELL LYMPHOMA (Bcl)-xL AND PLATELET LIFE SPAN. Mason et al.[222] subjected mice to ethylnitrosourea mutagenesis and screened their first-generation offspring for platelet deficiency. They identified two mutations in the gene encoding the antiapoptotic factor Bcl-x$_L$ that give rise to a dominantly inherited reduction in platelet count. Bcl-x$_L$ appears to promote platelet survival through inhibition of the proapoptotic activity of Bak. Bax promotes production of platelets,[223] and overexpression of antiapoptotic Bcl-x$_L$ impairs the fragmentation of megakaryocytes.[224] *(Modified from Qi B, Hardwick JM. A Bcl-xL timer sets platelet life span. Cell. 2007;128:1035.)*

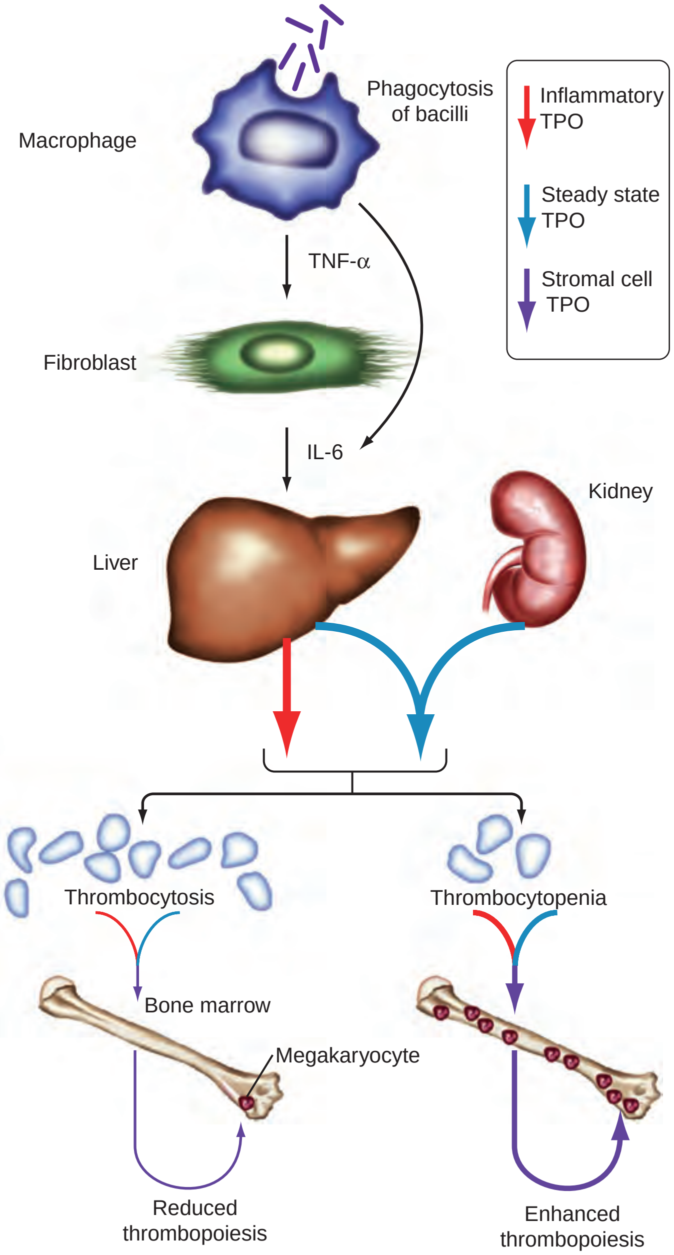


Figure 33.10 THE REGULATION OF THROMBOPOIETIN LEVELS. A steady-state amount of hepatic thrombopoietin (TPO) is regulated by platelet c-Mpl receptor–mediated uptake and destruction of the hormone. Hepatic production of the hormone is depicted. Upon binding to platelet c-Mpl receptors, the hormone is removed from the circulation and destroyed, which reduces blood levels. In the presence of inflammation, interleukin-6 (IL-6) is released from macrophages and, through tumor necrosis factor-α (TNF-α) stimulation, from fibroblasts and circulates to the liver to enhance TPO production. Thrombocytopenia also leads to enhanced marrow stromal cell production of TPO, although the molecular mediator(s) of this effect is not yet completely understood. *(Modified from Kaushansky K. The molecular mechanisms that control thrombopoiesis. J Clin Invest. 2005;115:3339.)*

effectiveness of platelet production can be calculated by measuring platelet mass turnover, which is defined as the product of the mean megakaryocyte cytoplasmic volume multiplied by the total number of marrow megakaryocytes.[411–413] A disparity between cytoplasmic mass and platelet delivery to blood (platelet count divided by platelet survival, corrected for splenic pooling) is the hallmark of ineffective platelet production (Fig. 33.11). An examination of the peripheral blood smear is the first step in the initial assessment of patients who present with thrombocytopenia. The presence of platelet clumps, indicative of pseudothrombocytopenia, or abnormally large, or small, platelets can be very useful in generating a differential diagnosis, as can the presence of inclusion bodies in neutrophils. Nevertheless, the current gold standard for diagnosing thrombocytopenia caused by ineffective platelet production is a bone marrow aspirate and biopsy. At the moment, direct visualization of the marrow and its cellular contents is the only way to judge the quantity and quality of the megakaryocyte population (Fig. 33.12). However, noninvasive methods for making a diagnosis of ineffective platelet production are being developed. For example, the concentration of serum glycocalicin, the soluble fragment of glycoprotein Ib, has been shown to be significantly diminished in patients with platelet production abnormalities when compared with normal control patients.[414] Reticulated platelets, like RBC reticulocytes, contain ribonucleic acid (RNA). It has been suggested that as is true for RBC reticulocytes, the presence of residual RNA in platelets indicates that they have been newly formed.[414,415] Thus, they may be useful for assessing the dynamics of platelet production under baseline conditions and after marrow insults such as chemotherapy or irradiation.[416–419] Platelet RNA can be detected by staining with dyes such as thiazole orange,[415] and it has been suggested that assessing the mean thiazole orange staining can be used to construct a reticulated platelet maturation index.[416] Another approach to assessing platelet production is the measurement of serum thrombopoietin levels.[415,420,421] Thrombopoietin is synthesized constitutively in the liver and then binds to its receptor c-Mpl on megakaryocytes and platelets (see Chapter 29). Accordingly, in patients with disorders in which megakaryocytes are reduced in the

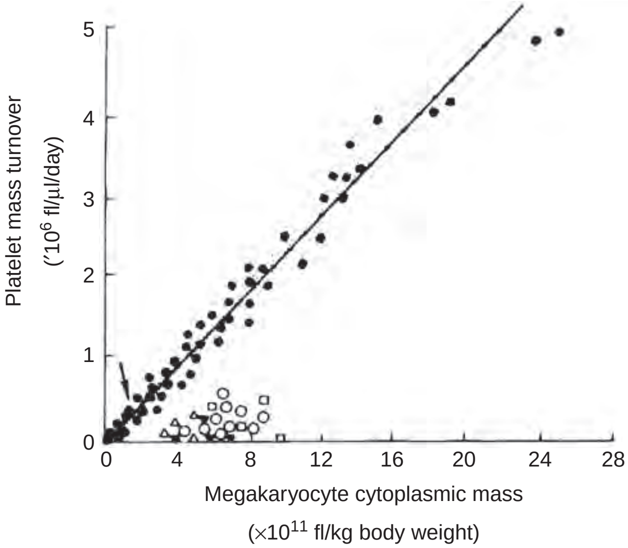


Figure 33.11 INEFFECTIVE PLATELET PRODUCTION. In thrombocytopenia, the relationship between marrow megakaryocyte cytoplasmic mass and the turnover of platelet mass in the peripheral blood is usually direct. Platelet mass turnover represents the product of the mean megakaryocyte cytoplasmic volume multiplied by the total number of marrow megakaryocytes. The results in normal patients are indicated by the arrow, and the stippled area represents 95% of confidence limits in thrombocytopenic patients with effective production. Ineffective thrombocytopoiesis is identified as a disparity between available marrow substrate (megakaryocyte cytoplasmic mass) and delivery of platelet mass to the peripheral blood (platelet mass turnover). Results in patients with autosomal dominant thrombocytopenia (open circles), Wiskott-Aldrich syndrome (open triangles), megaloblastic anemia (open squares), and preleukemia (closed triangles) are characterized by ineffective platelet production. *(Data from Thompson A, Harker L. Quantitative platelet disorders. In: Manual of Hemostasis and Thrombosis. Philadelphia, PA: FA Davis; 1983: p 65.)*

marrow, thrombopoietin levels rise. However, the wide variation in "normal" thrombopoietin concentrations in serum make this determination somewhat problematic as well. Some of this variability may be attributed to the fact that thrombopoietin synthesis is inducible in marrow stromal cells, perhaps by platelet α-granule proteins.[422,423] Reports attesting to the increased reliability and precision of measuring several of these parameters at once have appeared,[423] but it remains unclear whether the expense and time involved will prove cost-effective when compared with the relative simplicity of a bone marrow examination.

As is true for the congenital thrombocytopenias, acquired thrombocytopenia can be caused by a failure of either megakaryocytopoiesis or thrombopoiesis. Of these two possibilities, ineffective thrombopoiesis is the more likely cause, because pure megakaryocyte aplasia or hypoplasia is quite rare. Indeed, thrombocytopenia secondary to decreased marrow megakaryocytes is much more likely to be a prodrome of aplastic anemia, or an early form of MDS. Clues to these conditions can be found in the marrow, where often subtle abnormalities of other hematopoietic lineages, such as macrocytosis or dyserythropoiesis, can be observed.[424]

Selective Megakaryocyte Aplasia

Acquired selective amegakaryocytic thrombocytopenia is quite rare. It is almost always because of an autoimmune mechanism, either antibody- or cell-mediated. Autoantibodies reacting with megakaryocytes or their progenitor cells, presumably leading to their destruction, have been described.[425] There have been case reports of association with LGL leukemia.[426,427] In one particular case of acquired amegakaryocytic thrombocytopenia, autoantibodies against c-Mpl were described.[428] Antibodies directed to cytokines that regulate megakaryocyte development, in particular thrombopoietin, might also play a role in the biogenesis of such disorders. Cases of cell-mediated suppression of megakaryocytopoiesis leading to a complete selective megakaryocyte aplasia have also been described.[429] In these cases, suppression was shown in one case to be caused by autoreactive T

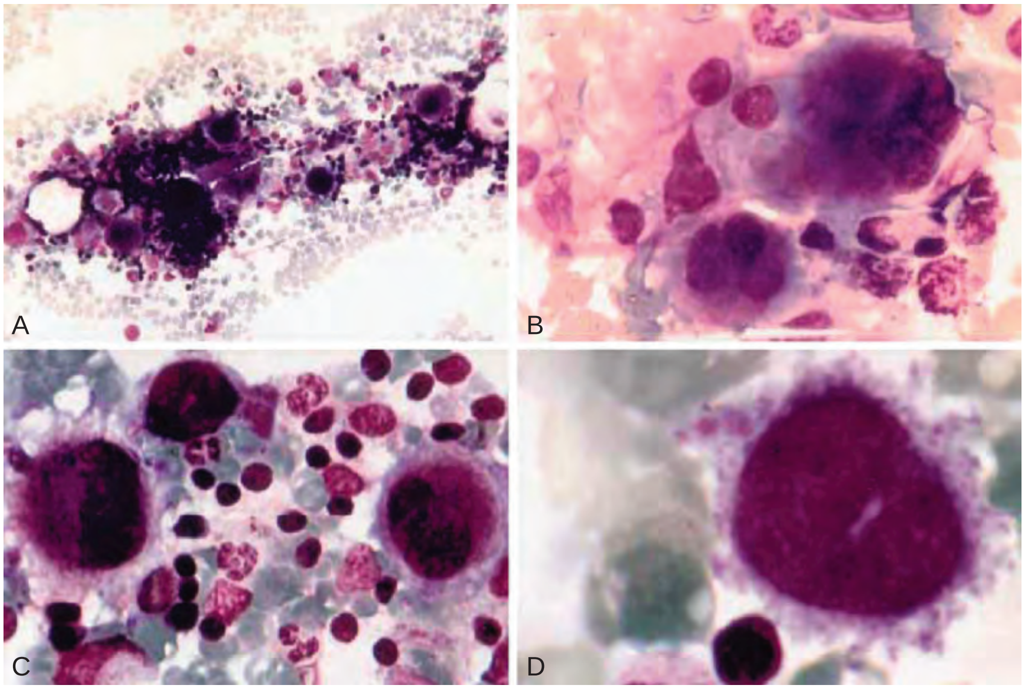


Figure 33.12 MARROW ASPIRATE OBTAINED FROM A CHILD WITH THROMBOCYTOPENIA AND DYSMEGAKARYOCYTOPOIESIS. The megakaryocytes are small and hypolobular, with diminished cytoplasm. Cells are viewed at magnifications of 250× (A), 1000× (B), 200× (C), and 1600× (D). *(From van den Oudenrijn S, Bruin M, Folman CC, et al. Three parameters: plasma thrombopoietin levels, plasma glycocalicin levels, and megakaryocyte culture, distinguish between different causes of congenital thrombocytopenia. Br J Haematol. 2002;117:390.)*

lymphocytes, whereas a macrophage-derived “factor” was implicated in the other.

Patients in whom an autoimmune mechanism is operative may respond to treatment with cyclosporine and ATG, achieving durable remissions.[430] Cytotoxic antibodies directed toward the CFU-Mk may be treated with corticosteroids, plasmapheresis, IVIg, danazol, cyclosporine, or cyclophosphamide.[431] Patients with T cell–mediated inhibition of megakaryocytopoiesis may respond to ATG, cyclosporine, or hematopoietic growth factors. If a particular drug or toxin exposure is believed to be responsible, for example ethanol or a thiazide diuretic, then withdrawal of the offending agent is obviously indicated. If the cause is viral, IVIg or anti-HIV therapies are indicated.[432] Despite the various causes of ineffective thrombopoiesis, immunosuppressive therapy was found to be effective in 8 out of 30 patients.[433]

Infection

Many infectious diseases are associated with thrombocytopenia, and it is likely that infection is the greatest non-iatrogenic cause of ineffective platelet production.[434] Infectious agents associated with decreased platelet counts include mycoplasma,[433] mycobacteria,[435] ehrlichiosis,[436] and malaria.[437] In these disorders, the cause of the thrombocytopenia is believed to be diminished platelet production although immune-mediated thrombocytopenia has also been described in some patients.[433]

Viral infections are by far the most common infectious agents associated with thrombocytopenia caused by ineffective megakaryocyte or platelet production. Thrombocytopenia has been reported in cases of mumps, rubella, measles, varicella, CMV, infectious mononucleosis, chickenpox, dengue and other hemorrhagic fevers, hepatitis, and parvovirus infections.[438–442] Live measles virus vaccination can also induce thrombocytopenia arising from decreased production. The mechanism responsible for viral suppression of platelet counts is not completely clear. It is known that megakaryocytes are capable of being infected by a variety of viruses. Infected cells may appear dysplastic, with inclusion bodies, vacuoles, or degenerating nuclei. Naked megakaryocyte nuclei may be seen in particular after HIV infection.[442] That such cytopathic cells might have trouble producing platelets is not difficult to imagine. Recently it has been demonstrated that dengue directly binds to dendritic cell-specific intracellular adhesion molecule-3 on the surface of platelets, and could replicate its viral RNA by usurping the translational machinery of platelets and raising the possibility that other (+)ssRNA viruses may be similarly propagated by these anucleate cytoplasts.[443]

Perhaps the best studied virally induced thrombocytopenia is that associated with HIV infection (see Chapter 152).[444] Mild to moderate reduction in platelet counts is quite common in patients with this disease. In a large study of HIV-positive patients with hemophilia, the cumulative frequency of thrombocytopenia 6 years after seroconversion was 16% for children and 18% for adults. At 10 years, the frequency increased to 27% in children and 43% in adults.[442,445] In another study, the frequency of thrombocytopenia was 16% among 103 homosexual men and 37% among 182 IV drug users with a new diagnosis of HIV infection. Except for patients who acquire HIV in the background of hemophilia, bleeding secondary to thrombocytopenia is unusual, because the counts are rarely less than 50,000/μL.[446] The principal cause of thrombocytopenia appears to vary with the stage of disease.

Examination of a bone marrow aspirate and biopsy specimens may be required to assess whether infiltration by a granulomatous infection or a malignancy is contributing to, or causing, the thrombocytopenia in an HIV patient. Assuming no other obvious cause of the thrombocytopenia, and the presence of typical megakaryocytic morphologic abnormalities, antiretroviral therapy is the principal treatment. For patients with severe and/or symptomatic thrombocytopenia, immune thrombocytopenia purpura (ITP) regimens, including splenectomy, may well be effective.[447]

Chemotherapy and Irradiation

Chemotherapy and irradiation reliably damage the bone marrow in a dose-dependent fashion. Megakaryocytes and their progenitors seem to be particularly sensitive to the effects of these agents. As a result, thrombocytopenia is one of the most frequent adverse effects of total body irradiation[448] and chemotherapy. Allogeneic or autologous HSCT is often complicated by prolonged thrombocytopenia, which may persist long after restoration of neutrophil and RBC counts. Various strategies to ameliorate this problem have been tried, including the use of peripheral blood “stem cells,” which may lead to a faster rate of platelet recovery when compared with marrow transplantation. More recently, attempts have been made to expand megakaryocyte progenitor cells, with cMpl agonists including romiplostim (Nplate) and eltrombopag (Promacta).

Alkylating agents in general produce more prolonged thrombocytopenia than antimetabolites. It has been claimed that some alkylating agents spare megakaryocytes (e.g., cyclophosphamide), but this is a relative phenomenon. Agents such as busulfan, the nitrosoureas, or platinum may cause cumulative damage of the more primitive progenitor cells. Other chemotherapeutic agents, such as the vinca alkaloids, may not decrease the platelet count significantly.

Various potential mechanisms for the relative sparing of platelet production by certain chemotherapeutic regimens have been investigated.[449] For patients who suffer from severe or prolonged thrombocytopenia, reducing the intensity of the chemotherapy is the most appropriate approach to management. It had been anticipated that the use of recombinant thrombopoietin or Promacta might significantly ameliorate this problem.[450] However, to date there is no certain conclusions that thrombopoietin receptor-agonists prevent chemotherapy-induced thrombocytopenia or prevent the recurrence of chemotherapy-induced thrombocytopenia in patients with solid tumors.

At the present time, in addition to cMpl-agonists Nplate and Promacta, supportive therapy with platelet transfusions and drugs such as ε-aminocaproic acid for patients who have become refractory to platelet transfusions remain the mainstays of therapy.[450,451]

NUTRITIONAL DEFICIENCIES

Thrombocytopenia of various degrees can be observed in patients with either folate or vitamin B_{12} deficiency. The mechanism of thrombocytopenia is ineffective platelet production.[452,453] Megakaryocyte numbers are normal or increased in the marrow, and platelet survival is normal or slightly shortened. Vitamin B_{12} deficiency was reported to cause a case of amegakaryocytic thrombocytopenia. Folate deficiency is frequently associated with ethanol abuse, and the etiology of the thrombocytopenia in patients who abuse ethanol is often complex.

Patients with iron deficiency typically exhibit thrombocytosis, but rare patients may become thrombocytopenic.[454] The extremely rapid increase in platelet counts after initiation of iron therapy suggested an essential role for iron in a late stage of thrombopoiesis.[455]

Marrow Infiltration

It is not rare for marrow infiltrative diseases of any type to cause ineffective hematopoiesis. Blood cell production disorders are commonly observed when the marrow is involved with metastatic cancer, lymphoma, or leukemia (see Chapter 72). Table 33.3 categorizes the infiltrative processes associated with thrombocytopenia. Physical replacement of marrow is the cause of the thrombocytopenia in many cases; it is also possible that inhibitory factors produced by the infiltrating cells are toxic to the cells of the megakaryocytic lineage or interfere with normal regulatory mechanisms. The diagnosis of infiltrative disease is made by marrow examination, although diagnostic clues are usually provided by history, physical examination, and a leukoerythroblastic blood smear. The marrow shows decreased megakaryocytes, which may be larger than normal because of a compensatory physiologic response to the thrombocytopenia.

Ethanol-Related Disorders

Ethanol abuse is very commonly associated with thrombocytopenia, which may result from several different mechanisms.[456–458] These include, most commonly, increased splenic pooling as a result of portal hypertension and ineffective production related to folate deficiency (which may lead to severe thrombocytopenia). Ethanol itself can be directly toxic to the marrow.[459] In vitro studies have shown that alcohol concentrations achievable in vivo inhibit megakaryocyte maturation but do not inhibit CFU-Mk.[459] Megakaryocyte numbers usually are normal, but markedly decreased megakaryocytes have been observed.

Other Drug-Related Disorders

A variety of drugs and toxins have been implicated in the etiology of isolated platelet production defects. Estrogen, for example, has been reported to decrease platelet counts through an unknown mechanism. Thrombocytopenia arising from thiazide diuretics has been reported frequently.[460] Although the cause of the thrombocytopenia in most cases is probably increased clearance, decreased marrow megakaryocyte numbers have been noted. Interferon and IL-2 may induce thrombocytopenia.[461] The most likely explanation is inhibition of CFU-Mk. Anagrelide is a very useful drug for lowering platelet counts in patients with myeloproliferative neoplasms and appears to work by reducing megakaryocyte size and ploidy and by disrupting maturation.[462]

Paroxysmal Nocturnal Hemoglobinuria

PNH is a clonal disorder resulting from mutations in the X-linked gene *PIGA* that encodes for an enzyme required in the initial step of biosynthesis of glycosylphosphatidylinositol anchors (PNH is discussed in Chapter 32).[463] Approximately 25% of patients with PNH have significant marrow aplasia.[464] Thrombocytopenia at diagnosis is a poor prognostic indicator. Because platelet survival is usually normal in PNH,[465] thrombocytopenia is caused by decreased or ineffective platelet production. Megakaryocyte progenitors have a decreased proliferative activity and exhibit increased sensitivity to complement.[466] Treatment with ATG or G-CSF and cyclosporine has ameliorated the thrombocytopenia whereas complement blockade with eculizumab has little impact on platelet count.[467]

Refractory Thrombocytopenia Caused by Myelodysplasia

MDSs may present as isolated thrombocytopenias rarely (see Chapter 61).[468] The diagnosis of MDS should be considered when there are clonal chromosome abnormalities. The clinical course of patients with this type of disorder is progressive. Additional cytopenias invariably develop. A significant number of cases will evolve into an acute myeloid leukemia.[468,469] Some patients with a full-blown MDS associated with marked thrombocytopenia and less than 10% blasts have been reported to experience increases in platelet counts after androgen therapy. Amifostine may be beneficial for some patients. It has been reported that some of these patients have been misdiagnosed as having ITP. Thrombopoietin agonists like romiplostim have also been used in patients with MDS. In an initial study involving 44 patients, a durable platelet response was achieved in 46% of patients with less bleeding events and transfusions seen in patients who achieved a durable response A meta-analysis concluded that treatment with thrombopoietin mimetics resulted in a lower number of MDS patients suffering from bleeding events. There was no evidence for a difference between study groups regarding transfusion requirements. Other treatment strategies such as treatment with cytokines and immunomodulating drugs have shown limited activity (box on Diagnosing Thrombocytopenia Caused by Impaired Thrombopoiesis).

DIAGNOSING THROMBOCYTOPENIA CAUSED BY IMPAIRED THROMBOPOIESIS

Compiling a thorough patient history is the first step in a complete workup of a thrombocytopenic patient. Many potential causes will be revealed by a good history, including obtaining a family history of thrombocytopenia, recent infection, medication or substance ingestion, radiation, or chemotherapy.

A careful physical examination could also contribute to making a diagnosis. For example, physical findings suggesting any of the inherited disorders described earlier might be discerned, as might findings suggestive of malignancy such as enlarged lymph nodes. Splenomegaly itself is not indicative of a platelet production abnormality but is often found in patients with lymphoma or other processes associated with marrow infiltration and damage that might cause impaired thrombopoiesis.

The peripheral blood smear is next examined, and this is needed to rule out pseudothrombocytopenia. Moreover, the blood smear provides additional clues to both the pathophysiologic mechanism of the thrombocytopenia and the diagnosis. For example, giant platelets suggest a hereditary or myelodysplastic syndrome; oval macrocytosis and hypersegmented neutrophils suggest a folate or vitamin B_{12} deficiency; and a leukoerythroblastic smear points to an infiltrative process.

Examination of the bone marrow is also required to evaluate megakaryocyte number and morphologic features. A biopsy specimen is more reliable than an aspirate to determine whether megakaryocytes are decreased in number. However, an aspirate showing abundant megakaryocytes in the presence of thrombocytopenia is sufficient to suggest platelet destruction or ineffective production. Megakaryocytes are not evenly distributed throughout the marrow, so examination of many fields is required to determine if adequate numbers of cells are present. Megakaryocyte morphologic characteristics are also useful to observe. The normal compensatory response to thrombocytopenia is enlargement of the cells with increased ploidy. Small, microlobulated or hypolobulated megakaryocytes may be seen in myelodysplastic syndromes. Dysmorphic megakaryocytes may also be observed in viral infections, including human immunodeficiency virus. In the future, flow cytometry may provide a more objective analysis of megakaryocytes.

Ultimately a diagnosis of ineffective thrombopoiesis is made by exclusion. The marrow examination reveals quantitatively normal megakaryocytes, and the apparent absence of peripheral platelet destruction together with the appropriate clinical circumstances (e.g., folate or vitamin B_{12} deficiency) often point to this mechanism. Platelet function tests may be helpful in distinguishing ineffective production from platelet destruction. In destructive processes such as immune thrombocytopenia, function is normal, whereas in ineffective platelet production impaired function is not uncommon, as noted earlier. In complex cases, platelet survival studies may be necessary to show that consumption or splenic pooling are not significant contributors to the thrombocytopenia; however, survival studies are rarely required for clinical purposes. In the future, flow cytometric estimation of platelet production rate and measurement of thrombopoietin levels may permit a more facile approach to the differential diagnosis of thrombocytopenia.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Azhar W, Zaidi F, Hannan A. Isoniazid induced pure red blood cell aplasia. *Cureus*. 2020;12(2):e7112.

Balasubramanian SK, Sadaps M, Thota S, et al. Rational management approach to pure red cell aplasia. *Haematologica*. 2018;103(2):221–230.

Broun ER, Greist A, Tricot G, Hoffman R. Excessive zinc ingestion. A reversible cause of sideroblastic anemia and bone marrow depression. *JAMA*. 1990;264:1441–1443.

Casadevall N, et al. Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N Engl J Med*. 2002;346:469–475.

Charles RJ, Sabo KM, Kidd PG, Abkowitz JL. The pathophysiology of pure red cell aplasia: implications for therapy. *Blood*. 1996;87:4831–4838.

Cheon H, Dziewulska KH, Moosic KB, et al. Advances in the diagnosis and treatment of large granular lymphocytic leukemia. *Curr Hematol Malig Rep*. 2020;15:103–112.

Gmür JP, et al. Pure red cell aplasia of long duration complicating major ABO-incompatible bone marrow transplantation. *Blood*. 1990;75:290–295.

Gurnari C, Maciejewski JP. How I manage acquired pure red cell aplasia in adults. *Blood*. 2021;137(15):2001–2009.

Hoffman R, Briddell RA, van Besien K, et al. Acquired cyclic amegakaryocytic thrombocytopenia associated with an immunoglobulin blocking the action of granulocyte-macrophage colony-stimulating factor. *N Engl J Med*. 1989;321:97–102.

Hoffman R, Kopel S, Hsu SD, et al. T cell chronic lymphocytic leukemia: presence in bone marrow and peripheral blood of cells that suppress erythropoiesis in vitro. *Blood*. 1978;52:255–260.

Hoffman R, Zaknoen S, Yan HH, et al. An antibody cytotoxic to megakaryocyte progenitor cells in a patient with immune thrombocytopenic purpura. *N Engl J Med*. 1985;312:1170–1174.

Krantz SB, Kao V. Studies on red cell aplasia. I. Demonstration of a plasma inhibitor to heme synthesis and an antibody to erythroblast nuclei. *Proc Natl Acad Sci U S A*. 1967;58:493–500.

Lai DW, Loughran Jr TP, Maciejewski JP, et al. Acquired amegakaryocytic thrombocytopenia and pure red cell aplasia associated with an occult large granular lymphocyte leukemia. *Leuk Res*. 2008;32(5):823–827.

Lamy T, Loughran TP. Large granular lymphocytic and other rare lymphoid leukemias. *Best Pract Res Clin Haematol*. 2019;32:193–195.

Lamy T, Moignet A, Loughran Jr. TP. LGL leukemia: from pathogenesis to treatment. *Blood*. 2017;129:1082–1094.

Loughran Jr. TP. Clonal diseases of large granular lymphocytes. *Blood*. 1993;82:1–14.

Loughran Jr. TP, Kidd PG, Starkebaum G. Treatment of large granular lymphocyte leukemia with oral low-dose methotrexate. *Blood*. 1994;84:2164–2170.

Marco-Ayala J, Gómez-Seguí I, Sanz G, et al. Pure red cell aplasia after major or bidirectional ABO incompatible hematopoietic stem cell transplantation: to treat or not to treat, that is the question. *Bone Marrow Transplant*. 2021;56:769–778.

Means Jr. RT. Pure red cell aplasia. *Blood*. 2016;128(21):2504–2509.

Thompson DF, Gales MA. Drug-induced pure red cell aplasia. *Pharmacotherapy*. 1996;16:1002–1008.

Perzova R, Loughran Jr. TP. Constitutive expression of Fas ligand in large granular lymphocyte leukaemia. *Br J Haematol*. 1997;97:123–126.

Vlachonikola E, Stamatopoulos K, Chatzidimitriou A. T cells in chronic lymphocytic leukemia: a two-edged sword. *Front Immunol*. 2021;11:612244.

Young NS, Mortimer PP, Moore JG, Humphries RK. Characterization of a virus that causes transient aplastic crisis. *J Clin Invest*. 1984;73:224–230.

REFERENCES

1. Marmont A, Peschle C, Sanguineti M, Condorelli M. Pure red cell aplasia (PRCA): Response of three patients of cyclophosphamide and/or antilymphocyte globulin (ALG) and demonstration of two types of serum IgG inhibitors to erythropoiesis. *Blood*. 1975;45:247–261.
2. Jepson JH, Lowenstein L. Inhibition of erythropoiesis by a factor present in the plasma of patients with erythroblastopenia. *Blood*. 1966;27:425–434.
3. Field EO, Caughi MN, Blackett NM, Smithers DW. Marrow-suppressing factors in the blood in pure red-cell aplasia, thymoma and Hodgkin's disease. *Br J Haematol*. 1968;15:101–110. https://doi.org/10.1111/j.1365-2141.1968.tb01516.x.
4. Jepson JH, Vas M. Decreased in vivo and in vitro erythropoiesis induced by plasma of ten patients with thymoma, lymphosarcoma, or idiopathic erythroblastopenia. *Cancer Res*. 1974;34:1325–1334.
5. Chikkappa G, Zarrabi MH, Tsan MF. Pure red-cell aplasia in patients with chronic lymphocytic leukemia. *Medicine (Baltimore)*. 1986;65:339–351. https://doi.org/10.1097/00005792-198609000-00006.
6. Mangan KF, Chikkappa G, Farley PC. T gamma (T gamma) cells suppress growth of erythroid colony-forming units in vitro in the pure red cell aplasia of B-cell chronic lymphocytic leukemia. *J Clin Invest*. 1982;70:1148–1156. https://doi.org/10.1172/jci110713.
7. Hoffman R, Kopel S, Hsu SD, Dainiak N, Zanjani ED. T cell chronic lymphocytic leukemia: presence in bone marrow and peripheral blood of cells that suppress erythropoiesis in vitro. *Blood*. 1978;52:255–260.
8. Lipton JM, et al. Evidence for genetic restriction in the suppression of erythropoiesis by a unique subset of T lymphocytes in man. *J Clin Invest*. 1983;72:694–706. https://doi.org/10.1172/JCI111019.
9. Tefferi A, Windebank KP, Veeder MH, Kiely JM. Steroid-responsive pure red cell aplasia associated with natural killer cell lymphocytosis. *Am J Hematol*. 1989;31:211–212. https://doi.org/10.1002/ajh.2830310313.
10. Levitt LJ, et al. Human T cell leukemia virus-I-associated T-suppressor cell inhibition of erythropoiesis in a patient with pure red cell aplasia and chronic T gamma-lymphoproliferative disease. *J Clin Invest*. 1988;81:538–548. https://doi.org/10.1172/JCI113352.
11. Abkowitz JL, Kadin ME, Powell JS, Adamson JW. Pure red cell aplasia: lymphocyte inhibition of erythropoiesis. *Br J Haematol*. 1986;63:59–67. https://doi.org/10.1111/j.1365-2141.1986.tb07495.x.
12. Hara T, et al. Human gamma delta T-cell receptor-positive cell-mediated inhibition of erythropoiesis in vitro in a patient with type I autoimmune polyglandular syndrome and pure red blood cell aplasia. *Blood*. 1990;75:941–950.
13. Tanaka Y, et al. T-gamma delta large granular lymphocyte leukemia preceded by pure red cell aplasia and complicated with hemophagocytic syndrome caused by Epstein-Barr virus infection. *Intern Med*. 2006;45:631–635. https://doi.org/10.2169/internalmedicine.45.1594.
14. Orchard J, Myint H, Hamblin TJ. A patient with myeloma who still has pure red cell aplasia despite the most intensive immune modulation. *Leuk Res*. 1997;21:353–354. https://doi.org/10.1016/s0145-2126(97)00028-3.
15. Singh VP, et al. Non-Hodgkin's lymphoma with pure red cell aplasia. *J Assoc Physicians India*. 2001;49:1123–1124.
16. Suzuki A, et al. Pure red cell aplasia associated with non-Hodgkin's lymphoma and hemolytic anemia. *Jpn J Clin Oncol*. 1991;21:384–387.
17. Grigg AP, O'Flaherty E. Cyclosporin A for the treatment of pure red cell aplasia associated with myelodysplasia. *Leuk Lymphoma*. 2001;42:1339–1342. https://doi.org/10.3109/10428190109097761.
18. de Alarcon PA, Miller ML, Stuart MJ. Erythroid hypoplasia. An unusual presentation of childhood acute lymphocytic leukemia. *Am J Dis Child*. 1978;132:763–764.
19. Imamura N, Kuramoto A, Morimoto T, Ihara A. Pure red cell aplasia associated with acute lymphoblastic leukaemia of pre-T-cell origin. *Med J Aust*. 1986;144:724.
20. Sallan SE, Buchanan GR. Selective erythroid aplasia during therapy for acute lymphoblastic leukemia. *Pediatrics*. 1977;59:895–898.
21. Maekawa K, Shibata J, Nakagawa S, Imamura N. Pure red cell aplasia associated with renal cell carcinoma. *Nihon Naika Gakkai Zasshi*. 1989;78:1002–1003. https://doi.org/10.2169/naika.78.1002.
22. Iannucci A, Perini A, Pizzolo G. Acquired pure red cell aplasia associated with thyroid carcinoma: case report. *Acta Haematol*. 1983;69:62–63. https://doi.org/10.1159/000206843.
23. Kobayashi M, Nukui A, Morita T. Pure red cell aplasia in a prostate cancer patient treated with leuprolide acetate and chlormadinone acetate. *Int J Urol*. 2005;12:1010–1011. https://doi.org/10.1111/j.1442-2042.2005.01195.x.
24. Gajwani B, Zinner EN. Pure red-cell aplasia. Associated with adenocarcinoma of stomach. *N Y State J Med*. 1976;76:2177–2179.
25. Young NS, Brown KE. Parvovirus B19. *N Engl J Med*. 2004;350:586–597. https://doi.org/10.1056/NEJMra030840.
26. Eridani S, Whitehead S, Sawyer B, Dalton R. Pure red cell aplasia and thymoma: demonstration of persisting inhibition of erythropoiesis after thymectomy and resolution after immune suppressive treatment. *Clin Lab Haematol*. 1986;8:181–185. https://doi.org/10.1111/j.1365-2257.1986.tb00094.x.
27. Stricker RB, Goldberg B. AIDS and pure red cell aplasia. *Am J Hematol*. 1997;54:264.
28. Chehal A, Sharara AI, Haidar HA, Haidar J, Bazarbachi A. Acute viral hepatitis A and parvovirus B19 infections complicated by pure red cell aplasia and autoimmune hemolytic anemia. *J Hepatol*. 2002;37:163–165. https://doi.org/10.1016/s0168-8278(02)00090-9.
29. al-Awami Y, et al. Pure red cell aplasia associated with hepatitis C infection. *Am J Med Sci*. 1997;314:113–117. https://doi.org/10.1097/00000441-199708000-00013.
30. Badaro R, et al. Recombinant human granulocyte-macrophage colony-stimulating factor reverses neutropenia and reduces secondary infections in visceral leishmaniasis. *J Infect Dis*. 1994;170:413–418. https://doi.org/10.1093/infdis/170.2.413.
31. Cavalcant J, Shadduck RK, Winkelstein A, Zeigler Z, Mendelow H. Red-cell hypoplasia and increased bone marrow reticulin in systemic lupus erythematosus: reversal with corticosteroid therapy. *Am J Hematol*. 1978;5:253–263. https://doi.org/10.1002/ajh.2830050310.
32. Dessypris EN, Baer MR, Sergent JS, Krantz SB. Rheumatoid arthritis and pure red cell aplasia. *Ann Intern Med*. 1984;100:202–206. https://doi.org/10.7326/0003-4819-100-2-202.
33. Giordano N, Senesi M, Battisti E, DeRegis FM, Gennari C. Sjogren's syndrome and pure red cell aplasia. *Clin Exp Rheumatol*. 1996;14:344–345.
34. Julkunen H, Jantti J, Pettersson T. Pure red cell aplasia in mixed connective tissue disease. *J Rheumatol*. 1989;16:1385–1386.
35. Trinchet JC, et al. Association of autoimmune chronic active hepatitis and acquired erythroblastopenia cured by cyclophosphamide. *Gastroenterol Clin Biol*. 1986;10:841–844.
36. Jacob A, et al. Antibody-mediated pure red cell aplasia in a dialysis patient receiving darbepoetin alfa as the sole erythropoietic agent. *Nephrol Dial Transplant*. 2006;21:2963–2965. https://doi.org/10.1093/ndt/gfl331.
37. Bolan CD, et al. Delayed donor red cell chimerism and pure red cell aplasia following major ABO-incompatible nonmyeloablative hematopoietic stem cell transplantation. *Blood*. 2001;98:1687–1694. https://doi.org/10.1182/blood.v98.6.1687.
38. Lee JH, et al. Anti-A isoagglutinin as a risk factor for the development of pure red cell aplasia after major ABO-incompatible allogeneic bone marrow transplantation. *Bone Marrow Transplant*. 2000;25:179–184. https://doi.org/10.1038/sj.bmt.1702121.
39. Zalusky R, Zanjani ED, Gidari AS, Ross J. Site of action of a serum inhibitor of erythropoiesis. *J Lab Clin Med*. 1973;81:867–875.
40. Krantz SB, Kao V. Studies on red cell aplasia. I. Demonstration of a plasma inhibitor to heme synthesis and an antibody to erythroblast nuclei. *Proc Natl Acad Sci U S A*. 1967;58:493–500. https://doi.org/10.1073/pnas.58.2.493.
41. Krantz SB, Kao V. Studies on red cell aplasia. II. Report of a second patient with an antibody to erythroblast nuclei and a remission after immunosuppressive therapy. *Blood*. 1969;34:1–13.
42. Katz LJ, Hoffman R, Ritchey AK, Dainiak N. The proliferative capacity of pure red cell aplasia bone marrow cells. *Yale J Biol Med*. 1981;54:89–94.
43. Lacombe C, Casadevall N, Muller O, Varet B. Erythroid progenitors in adult chronic pure red cell aplasia: relationship of in vitro erythroid colonies to therapeutic response. *Blood*. 1984;64:71–77.
44. Takahashi M, et al. Serum erythropoietic inhibitors in patients with pure red cell aplasia. *Ann Hematol*. 1991;63:9–14. https://doi.org/10.1007/BF01714954.
45. Go RS, Li CY, Tefferi A, Phyliky RL. Acquired pure red cell aplasia associated with lymphoproliferative disease of granular T lymphocytes. *Blood*. 2001;98:483–485. https://doi.org/10.1182/blood.v98.2.483.
46. Handgretinger R, et al. Pure red-cell aplasia associated with clonal expansion of granular lymphocytes expressing killer-cell inhibitory receptors. *N Engl J Med*. 1999;340:278–284. https://doi.org/10.1056/NEJM199901283400405.
47. Sandberg Y, et al. TCRgammadelta$^+$ large granular lymphocyte leukemias reflect the spectrum of normal antigen-selected TCRgammadelta+ T-cells. *Leukemia*. 2006;20:505–513. https://doi.org/10.1038/sj.leu.2404112.
48. Mori KL, Furukawa H, Hayashi K, Sugimoto KJ, Oshimi K. Pure red cell aplasia associated with expansion of CD3$^+$ CD8$^+$ granular lymphocytes expressing cytotoxicity against HLA-E+ cells. *Br J Haematol*. 2003;123:147–153. https://doi.org/10.1046/j.1365-2141.2003.04553.x.

49. Matsuhashi Y, et al. Increased expression of c-maf in pure red cell aplasia secondary to plasma cell dyscrasia. *Leuk Lymphoma*. 2003;44:523–524. https://doi.org/10.1080/1042819021000032890.
50. Hansen RM, et al. T-cell chronic lymphocytic leukemia with pure red cell aplasia: laboratory demonstration of persistent leukemia in spite of apparent complete clinical remission. *Am J Hematol*. 1986;22:79–86. https://doi.org/10.1002/ajh.2830220112.
51. Partanen S, Ruutu T, Vuopio P, Andersson LC. Acquired pure red-cell aplasia: a consequence of increased natural killer cell activity? *Leuk Res*. 1984;8:117–122. https://doi.org/10.1016/0145-2126(84)90039-0.
52. Fisch P, et al. Gamma/delta T cell clones and natural killer cell clones mediate distinct patterns of non-major histocompatibility complex-restricted cytolysis. *J Exp Med*. 1990;171:1567–1579. https://doi.org/10.1084/jem.171.5.1567.
53. Fisch P, et al. Control of B cell lymphoma recognition via natural killer inhibitory receptors implies a role for human Vgamma9/Vdelta2 T cells in tumor immunity. *Eur J Immunol*. 1997;27:3368–3379. https://doi.org/10.1002/eji.1830271236.
54. Casado LF, et al. High expression of the ILT2 (LIR-1) inhibitory receptor for major histocompatibility complex class I molecules on clonal expansions of T large granular lymphocytes in asymptomatic patients. *Haematologica*. 2001;86:457–463.
55. Grau R, et al. Cytotoxic activity of natural killer cells lacking killer-inhibitory receptors for self-HLA class I molecules against autologous hematopoietic stem cells in healthy individuals. *Exp Mol Pathol*. 2004;76:90–98. https://doi.org/10.1016/j.yexmp.2003.12.002.
56. Fujisao S, Tsuda H. Th1/Th2 balance alteration in the clinical course of a patient with pure red cell aplasia and thymoma. *Br J Haematol*. 1998;103:308–310. https://doi.org/10.1046/j.1365-2141.1998.01011.x.
57. Zent CS, et al. The prognostic significance of cytopenia in chronic lymphocytic leukaemia/small lymphocytic lymphoma. *Br J Haematol*. 2008;141:615–621. https://doi.org/10.1111/j.1365-2141.2008.07086.x.
58. Peschle C, et al. Pure red cell aplasia: studies on an IgG serum inhibitor neutralizing erythropoietin. *Br J Haematol*. 1975;30:411–417. https://doi.org/10.1111/j.1365-2141.1975.tb01855.x.
59. Mangan KF, Chikkappa G, Scharfman WB, Desforges JF. Evidence for reduced erythroid burst (BFUE) promoting function of T lymphocytes in the pure red cell aplasia of chronic lymphocytic leukemia. *Exp Hematol*. 1981;9:489–498.
60. Hirokawa M, et al. Long-term outcome of patients with acquired chronic pure red cell aplasia (PRCA) following immunosuppressive therapy: a final report of the nationwide cohort study in 2004/2006 by the Japan PRCA collaborative study group. *Br J Haematol*. 2015;169:879–886. https://doi.org/10.1111/bjh.13376.
61. Kwong YL, Au WY, Leung AY, Tse EW. T-cell large granular lymphocyte leukemia: an Asian perspective. *Ann Hematol*. 2010;89:331–339. https://doi.org/10.1007/s00277-009-0895-3.
62. Ishida F, et al. STAT3 gene mutations and their association with pure red cell aplasia in large granular lymphocyte leukemia. *Cancer Sci*. 2014;105:342–346. https://doi.org/10.1111/cas.12341.
63. al-Mondhiry H, Zanjani ED, Spivack M, Zalusky R, Gordon AS. Pure red cell aplasia and thymoma: loss of serum inhibitor of erythropoiesis following thymectomy. *Blood*. 1971;38:576–582.
64. Mangan KF, Volkin R, Winkelstein A. Autoreactive erythroid progenitor-T suppressor cells in the pure red cell aplasia associated with thymoma and panhypogammaglobulinemia. *Am J Hematol*. 1986;23:167–173. https://doi.org/10.1002/ajh.2830230211.
65. Masuda M, Arai Y, Okamura T, Wada M, Mizoguchi H. Pure red cell aplasia (PRCA) with thymoma: a possible distinct clinical entity distinct from large granular lymphocyte (LGL) leukemia. *Am J Hematol*. 2000;63:102. doi:10.1002/(sici)1096-8652(200002)63:2<102::aid-ajh9>3.0.co;2-p.
66. Soler J, Estivill X, Ayats R, Brunet S, Pujol-Moix N. Chronic T-cell lymphocytosis associated with pure red call aplasia, thymoma and hypogammaglobulinemia. *Br J Haematol*. 1985;61:582–584. https://doi.org/10.1111/j.1365-2141.1985.tb02865.x.
67. Thompson CA, Steensma DP. Pure red cell aplasia associated with thymoma: clinical insights from a 50-year single-institution experience. *Br J Haematol*. 2006;135:405–407. https://doi.org/10.1111/j.1365-2141.2006.06295.x.
68. Malphettes M, et al. Good syndrome: an adult-onset immunodeficiency remarkable for its high incidence of invasive infections and autoimmune complications. *Clin Infect Dis*. 2015;61:e13–19. https://doi.org/10.1093/cid/civ269.
69. Larroche C, et al. Successful treatment of thymoma-associated pure red cell aplasia with intravenous immunoglobulins. *Eur J Haematol*. 2000;65:74–76. https://doi.org/10.1034/j.1600-0609.2000.9c212.x.
70. Fukushima K, et al. Pure red cell aplasia developing after treatment of pleural recurrence of thymoma, successfully treated with cyclosporin A but not with tacrolimus. *Intern Med*. 2006;45:485–486. https://doi.org/10.2169/internalmedicine.45.1587.
71. Lehman G, Alcoff J. Reversible pure red cell hypoplasia in pregnancy. *JAMA*. 1982;247:1170–1171.
72. Choudry MA, Moffett BK, Laber DA. Pure red-cell aplasia secondary to pregnancy, characterization of a syndrome. *Ann Hematol*. 2007;86:233–237. https://doi.org/10.1007/s00277-006-0211-4.
73. Bambery P, et al. Prolonged, pregnancy-related pure red cell aplasia; a case report. *Eur J Obstet Gynecol Reprod Biol*. 1991;42:233–235. https://doi.org/10.1016/0028-2243(91)90227-c.
74. Banavali SD, et al. Corticosteroid-responsive pure red cell aplasia in rheumatoid arthritis and its association with pregnancy: a case report. *Am J Hematol*. 1989;31:58–61. https://doi.org/10.1002/ajh.2830310112.
75. Anderson MJ, et al. Occurrence of infection with a parvovirus-like agent in children with sickle cell anaemia during a two-year period. *J Clin Pathol*. 1982;35:744–749. https://doi.org/10.1136/jcp.35.7.744.
76. Young N, Mortimer P. Viruses and bone marrow failure. *Blood*. 1984;63:729–737.
77. Young NS, Mortimer PP, Moore JG, Humphries RK. Characterization of a virus that causes transient aplastic crisis. *J Clin Invest*. 1984;73:224–230. https://doi.org/10.1172/JCI111195.
78. Pattison JR, et al. Parvovirus infections and hypoplastic crisis in sickle-cell anaemia. *Lancet*. 1981;1:664–665. https://doi.org/10.1016/s0140-6736(81)91579-8.
79. Serjeant GR, et al. Outbreak of aplastic crises in sickle cell anaemia associated with parvovirus-like agent. *Lancet*. 1981;2:595–597. https://doi.org/10.1016/s0140-6736(81)92739-2.
80. Kelleher JF, Luban NL, Mortimer PP, Kamimura T. Human serum "parvovirus": a specific cause of aplastic crisis in children with hereditary spherocytosis. *J Pediatr*. 1983;102:720–722. https://doi.org/10.1016/s0022-3476(83)80243-1.
81. Wong S, Brown KE. Development of an improved method of detection of infectious parvovirus B19. *J Clin Virol*. 2006;35:407–413. https://doi.org/10.1016/j.jcv.2005.12.008.
82. Tomida S, et al. Severe acute hepatitis A associated with acute pure red cell aplasia. *J Gastroenterol*. 1996;31:612–617. https://doi.org/10.1007/BF02355068.
83. Ramos-Casals M, et al. Severe autoimmune cytopenias in treatment-naive hepatitis C virus infection: clinical description of 35 cases. *Medicine (Baltimore)*. 2003;82:87–96. https://doi.org/10.1097/00005792-200303000-00003.
84. Xu LH, et al. Pure red cell aplasia associated with cytomegalovirus and Epstein-Barr virus infection in seven cases of Chinese children. *Hematology*. 2013;18:56–59. https://doi.org/10.1179/1607845412Y.0000000044.
85. Majluf-Cruz A, Luna-Castanos G, Trevino-Perez S, Santoscoy M, Nieto-Cisneros L. Lamivudine-induced pure red cell aplasia. *Am J Hematol*. 2000;65:189–191. doi:10.1002/1096-8652(200011)65:3<189::aid-ajh2>3.0.co;2-6.
86. Manickchund N, et al. Emtricitabine-induced pure red cell aplasia. *South Afr J HIV Med*. 2019;20:983. https://doi.org/10.4102/sajhivmed.v20i1.983.
87. Chung JW, et al. Pure red cell aplasia and adult-onset Still's disease. *Clin Rheumatol*. 2004;23:368–370. https://doi.org/10.1007/s10067-004-0899-2.
88. Janssen HL, et al. Severe hepatitis and pure red cell aplasia in adult Still's disease: good response to immunosuppressive therapy. *Dig Dis Sci*. 1999;44:1639–1642. https://doi.org/10.1023/a:1026631514623.
89. Fong PH, Wee A, Chan HL, Tan YO. Primary thymic carcinoma and its association with dermatomyositis and pure red cell aplasia. *Int J Dermatol*. 1992;31:426–428. https://doi.org/10.1111/j.1365-4362.1992.tb02675.x.
90. Katabami S, et al. Polymyositis associated with thymoma and the subsequent development of pure red cell aplasia. *Intern Med*. 1995;34:569–573. https://doi.org/10.2169/internalmedicine.34.569.
91. Rodrigues JF, Harth M, Barr RM. Pure red cell aplasia in rheumatoid arthritis. *J Rheumatol*. 1988;15:1159–1161.
92. Fujiu K, et al. Triad of thymoma, myasthenia gravis and pure red cell aplasia combined with Sjogren's syndrome. *Jpn J Thorac Cardiovasc Surg*. 2004;52:345–348. https://doi.org/10.1007/s11748-004-0068-1.
93. Ramakrishna R, Chaudhuri K, Sturgess A, Manoharan A. Haematological manifestations of primary Sjogren's syndrome: a clinicopathological study. *Q J Med*. 1992;83:547–554.
94. Arcasoy MO, Chao NJ. T-cell-mediated pure red-cell aplasia in systemic lupus erythematosus: response to cyclosporin A and mycophenolate mofetil. *Am J Hematol*. 2005;78:161–163. https://doi.org/10.1002/ajh.20237.
95. Meyer RJ, Hoffman R, Zanjani ED. Autoimmune hemolytic anemia and periodic pure red cell aplasia in systemic lupus erythematosus. *Am J Med*. 1978;65:342–345. https://doi.org/10.1016/0002-9343(78)90829-x.
96. Bailey FA, Lilly M, Bertoli LF, Ball GV. An antibody that inhibits in vitro bone marrow proliferation in a patient with systemic lupus erythematosus and aplastic anemia. *Arthritis Rheum*. 1989;32:901–905.

97. Linardaki GD, Boki KA, Fertakis A, Tzioufas AG. Pure red cell aplasia as presentation of systemic lupus erythematosus: antibodies to erythropoietin. *Scand J Rheumatol*. 1999;28:189–191. https://doi.org/10.1080/03009749950154293.
98. Dessypris EN, Redline S, Harris JW, Krantz SB. Diphenylhydantoin-induced pure red cell aplasia. *Blood*. 1985;65:789–794.
99. Casadevall N, et al. Autoantibodies against erythropoietin in a patient with pure red-cell aplasia. *N Engl J Med*. 1996;334:630–633. https://doi.org/10.1056/NEJM199603073341004.
100. Casadevall N, et al. Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N Engl J Med*. 2002;346:469–475. https://doi.org/10.1056/NEJMoa011931.
101. Eckardt KU, Casadevall N. Pure red-cell aplasia due to anti-erythropoietin antibodies. *Nephrol Dial Transplant*. 2003;18:865–869. https://doi.org/10.1093/ndt/gfg182.
102. Prabhakar SS, Muhlfelder T. Antibodies to recombinant human erythropoietin causing pure red cell aplasia. *Clin Nephrol*. 1997;47:331–335.
103. Locatelli F, et al. Erythropoiesis-stimulating agents and antibody-mediated pure red-cell aplasia: here are we now and where do we go from here? *Nephrol Dial Transplant*. 2004;19:288–293. https://doi.org/10.1093/ndt/gfg489.
104. Bennett CL, et al. Long-term outcome of individuals with pure red cell aplasia and antierythropoietin antibodies in patients treated with recombinant epoetin: a follow-up report from the Research on Adverse Drug Events and Reports (RADAR) Project. *Blood*. 2005;106:3343–3347. https://doi.org/10.1182/blood-2005-02-0508.
105. Helbig G, et al. Successful treatment of pure red cell aplasia with repeated, low doses of rituximab in two patients after ABO-incompatible allogeneic haematopoietic stem cell transplantation for acute myeloid leukaemia. *Haematologica*. 2005;90(Suppl):ECR33.
106. Zhu KE, Li JP, Zhang T, Zhong J, Chen J. Clinical features and risk factors of pure red cell aplasia following major ABO-incompatible allogeneic hematopoietic stem cell transplantation. *Hematology*. 2007;12:117–121. https://doi.org/10.1080/10245330601111540.
107. Helbig G, et al. Pure red-cell aplasia following major and bi-directional ABO-incompatible allogeneic stem-cell transplantation: recovery of donor-derived erythropoiesis after long-term treatment using different therapeutic strategies. *Ann Hematol*. 2007;86:677–683. https://doi.org/10.1007/s00277-007-0304-8.
108. Dellacasa CM, et al. Efficacy of plasmapheresis for the treatment of pure red blood cell aplasia after allogeneic stem cell transplantation. *Transfusion*. 2015;55:2979–2982. https://doi.org/10.1111/trf.13269.
109. Bathini S, et al. Refractory postallogeneic stem cell transplant pure red cell aplasia in remission after treatment with daratumumab. *Am J Hematol*. 2019;94:E216–E219. https://doi.org/10.1002/ajh.25515.
110. Chapuy CI, Kaufman RM, Alyea EP, Connors JM. Daratumumab for delayed red-cell engraftment after allogeneic transplantation. *N Engl J Med*. 2018;379:1846–1850. https://doi.org/10.1056/NEJMoa1807438.
111. Salas MQ, Alahmari A, Lipton JH. Successful treatment of refractory red cell aplasia after allogeneic hematopoietic cell transplantation with daratumumab. *Eur J Haematol*. 2020;104:145–147. https://doi.org/10.1111/ejh.13343.
112. Rabitsch W, et al. Prolonged red cell aplasia after major ABO-incompatible allogeneic hematopoietic stem cell transplantation: removal of persisting isohemagglutinins with Ig-Therasorb immunoadsorption. *Bone Marrow Transplant*. 2003;32:1015–1019. https://doi.org/10.1038/sj.bmt.1704264.
113. Maschan AA, et al. Successful treatment of pure red cell aplasia with a single dose of rituximab in a child after major ABO incompatible peripheral blood allogeneic stem cell transplantation for acquired aplastic anemia. *Bone Marrow Transplant*. 2002;30:405–407. https://doi.org/10.1038/sj.bmt.1703668.
114. Gmur JP, et al. Pure red cell aplasia of long duration complicating major ABO-incompatible bone marrow transplantation. *Blood*. 1990;75:290–295.
115. Grigg AP, Juneja SK. Pure red cell aplasia with the onset of graft versus host disease. *Bone Marrow Transplant*. 2003;32:1099–1101. https://doi.org/10.1038/sj.bmt.1704271.
116. Bae KW, et al. A novel mutation and unusual clinical features in a patient with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. *Eur J Pediatr*. 2011;170:1611–1615. https://doi.org/10.1007/s00431-011-1588-1.
117. Ergas D, Resnitzky P, Berrebi A. Pure red blood cell aplasia associated with parvovirus B19 infection in large granular lymphocyte leukemia. *Blood*. 1996;87:3523–3524.
118. Charles RJ, Sabo KM, Kidd PG, Abkowitz JL. The pathophysiology of pure red cell aplasia: implications for therapy. *Blood*. 1996;87:4831–4838.
119. Lacy MQ, Kurtin PJ, Tefferi A. Pure red cell aplasia: association with large granular lymphocyte leukemia and the prognostic value of cytogenetic abnormalities. *Blood*. 1996;87:3000–3006.
120. Lamy T, Loughran Jr. TP. Clinical features of large granular lymphocyte leukemia. *Semin Hematol*. 2003;40:185–195. https://doi.org/10.1016/s0037-1963(03)00133-1.
121. Rose MG, Berliner N. T-cell large granular lymphocyte leukemia and related disorders. *Oncologist*. 2004;9:247–258. https://doi.org/10.1634/theoncologist.9-3-247.
122. La Montagna G, Baruffo A, Abbadessa A, Felaco T. Pure red cell aplasia in Felty's syndrome: a case report of successful reversal after cyclosporin A treatment. *Clin Rheumatol*. 1999;18:244–247. https://doi.org/10.1007/s100670050093.
123. Ustun C, Karavelioglu D, Ilhan O, Aydintug O, Beksac M. A case report of a patient who has pure red cell aplasia and rheumatoid arthritis. *Int J Hematol*. 1997;66:505–512.
124. Higuchi T, Mori H, Niikura H, Omine M. Hypocomplementemia and hematological abnormalities in immunoblastic lymphadenopathy and immunoblastic lymphadenopathy-like T cell lymphoma. *Acta Haematol*. 1996;96:68–72. https://doi.org/10.1159/000203718.
125. Chikkappa G, Pasquale D, Phillips PG, Mangan KF, Tsan MF. Cyclosporin-A for the treatment of pure red cell aplasia in a patient with chronic lymphocytic leukemia. *Am J Hematol*. 1987;26:179–189. https://doi.org/10.1002/ajh.2830260209.
126. Means Jr. RT, Dessypris EN, Krantz SB. Treatment of refractory pure red cell aplasia with cyclosporine A: disappearance of IgG inhibitor associated with clinical response. *Br J Haematol*. 1991;78:114–119. https://doi.org/10.1111/j.1365-2141.1991.tb04392.x.
127. Au WY, Lam CC, Chim CS, Pang AW, Kwong YL. Alemtuzumab induced complete remission of therapy-resistant pure red cell aplasia. *Leuk Res*. 2005;29:1213–1215. https://doi.org/10.1016/j.leukres.2005.02.018.
128. Clark DA, Dessypris EN, Krantz SB. Studies on pure red cell aplasia. XI. Results of immunosuppressive treatment of 37 patients. *Blood*. 1984;63:277–286.
129. Zecca M, De Stefano P, Nobili B, Locatelli F. Anti-CD20 monoclonal antibody for the treatment of severe, immune-mediated, pure red cell aplasia and hemolytic anemia. *Blood*. 2001;97:3995–3997. https://doi.org/10.1182/blood.v97.12.3995.
130. Sloand EM, Scheinberg P, Maciejewski J, Young NS. Brief communication: Successful treatment of pure red-cell aplasia with an anti-interleukin-2 receptor antibody (daclizumab). *Ann Intern Med*. 2006;144:181–185. https://doi.org/10.7326/0003-4819-144-3-200602070-00006.
131. Abkowitz JL, Powell JS, Nakamura JM, Kadin ME, Adamson JW. Pure red cell aplasia: response to therapy with anti-thymocyte globulin. *Am J Hematol*. 1986;23:363–371. https://doi.org/10.1002/ajh.2830230408.
132. Jacobs EM, Hutter RV, Pool JL, Ley AB. Benign thymoma and selective erythroid aplasia of the bone marrow. *Cancer*. 1959;12:47–57. doi:10.1002/1097-0142(195901/02)12:1<47::aid-cncr2820120110>3.0.co;2-q.
133. Hirst E, Robertson TI. The syndrome of thymoma and erythroblastopenic anemia. A review of 56 cases including 3 case reports. *Medicine (Baltimore)*. 1967;46:225–264. https://doi.org/10.1097/00005792-196705000-00001.
134. Masaoka A, et al. Thymomas associated with pure red cell aplasia. Histologic and follow-up studies. *Cancer*. 1989;64:1872–1878. doi:10.1002/1097-0142(19891101)64:9<1872::aid-cncr2820640920>3.0.co;2-0.
135. Weide LG, Ulbright TM, Loehrer Sr. PJ, Williams SD. Thymic carcinoma. A distinct clinical entity responsive to chemotherapy. *Cancer*. 1993;71:1219–1223. doi:10.1002/1097-0142(19930215)71:4<1219::aid-cncr2820710408>3.0.co;2-b.
136. Vichinsky E, et al. A randomised comparison of deferasirox versus deferoxamine for the treatment of transfusional iron overload in sickle cell disease. *Br J Haematol*. 2007;136:501–508. https://doi.org/10.1111/j.1365-2141.2006.06455.x.
137. Finkel HE, Kimber RJ, Dameshek W. Corticosteroid-responsive acquired pure red cell aplasia in adults. *Am J Med*. 1967;43:771–776. https://doi.org/10.1016/0002-9343(67)90119-2.
138. Fujisawa S, et al. Pure red cell aplasia after major ABO-incompatible bone marrow transplantation: two case reports of treatment with recombinant human erythropoietin. *Transpl Int*. 1996;9:506–508. https://doi.org/10.1007/BF00336830.
139. Santamaria A, et al. Successful treatment of pure red cell aplasia after major ABO-incompatible T cell-depleted bone marrow transplantation with erythropoietin. *Bone Marrow Transplant*. 1997;20:1105–1107. https://doi.org/10.1038/sj.bmt.1701012.
140. Cervantes F, Alvarez-Larran A, Domingo A, Arellano-Rodrigo E, Montserrat E. Efficacy and tolerability of danazol as a treatment for the anaemia of myelofibrosis with myeloid metaplasia: long-term results in 30 patients. *Br J Haematol*. 2005;129:771–775. https://doi.org/10.1111/j.1365-2141.2005.05524.x.

141. Lippman SM, Durie BG, Garewal HS, Giordano G, Greenberg BR. Efficacy of danazol in pure red cell aplasia. *Am J Hematol*. 1986;23:373–379. https://doi.org/10.1002/ajh.2830230409.
142. Tsai CY, et al. Pure red cell aplasia in a man with RA. *Scand J Rheumatol*. 1997;26:329–331. https://doi.org/10.3109/03009749709105325.
143. Chan AY, Li EK, Tam LS, Cheng G, Choi PC. Successful treatment of pure red cell aplasia associated with systemic lupus erythematosus with oral danazol and steroid. *Rheumatol Int*. 2005;25:388–390. https://doi.org/10.1007/s00296-004-0521-6.
144. Castelli R, Vismara A, Pavia G, Dagani R, Porro T. Relapsing pure red cell aplasia associated with B-cell chronic lymphocytic leukemia successfully treated by intravenous immunoglobulin concentrate. *Ann Ital Med Int*. 2002;17:47–50.
145. Mouthon L, Guillevin L, Tellier Z. Intravenous immunoglobulins in autoimmune- or parvovirus B19-mediated pure red-cell aplasia. *Autoimmun Rev*. 2005;4:264–269. https://doi.org/10.1016/j.autrev.2004.10.004.
146. Baldus M, Moller M, Walter H, Brass H, Queisser W. A case of pure red cell aplasia: follow-up on different immunosuppressive regimens. *Clin Investig*. 1994;72:1051–1055. https://doi.org/10.1007/BF00577754.
147. Sherer Y, Levy Y, Fabbrizzi F, Shoenfeld Y. Treatment of hematologic disorders other than immune thrombocytopenic purpura with intravenous immunoglobulin (IVIg) - report of seven cases and review of the literature. *Eur J Intern Med*. 2000;11:85–88. https://doi.org/10.1016/s0953-6205(00)00067-4.
148. Koduri PR, Kumapley R, Valladares J, Teter C. Chronic pure red cell aplasia caused by parvovirus B19 in AIDS: use of intravenous immunoglobulin—a report of eight patients. *Am J Hematol*. 1999;61:16–20. doi:10.1002/(sici)1096-8652(199905)61:1<16::aid-ajh4>3.0.co;2-y.
149. Firkin FC, Maher D. Cytotoxic immunosuppressive drug treatment strategy in pure red cell aplasia. *Eur J Haematol*. 1988;41:212–217. https://doi.org/10.1111/j.1600-0609.1988.tb01183.x.
150. Bottiger LE, Rausing A. Pure red cell anemia: immunosuppressive treatment. *Ann Intern Med*. 1972;76:593–597. https://doi.org/10.7326/0003-4819-76-4-593.
151. Peng G, et al. Moderate-dose cyclophosphamide in the treatment of relapsed/refractory T-cell large granular lymphocytic leukemia-associated pure red cell aplasia. *Hematology*. 2016;21:138–143. https://doi.org/10.1080/10245333.2015.1101977.
152. Dungarwalla M, et al. Lack of clinical efficacy of rituximab in the treatment of autoimmune neutropenia and pure red cell aplasia: implications for their pathophysiology. *Ann Hematol*. 2007;86:191–197. https://doi.org/10.1007/s00277-006-0202-5.
153. Ghazal H. Successful treatment of pure red cell aplasia with rituximab in patients with chronic lymphocytic leukemia. *Blood*. 2002;99:1092–1094. https://doi.org/10.1182/blood.v99.3.1092.
154. Narra K, Borghaei H, Al-Saleem T, Hoglund M, Smith MR. Pure red cell aplasia in B-cell lymphoproliferative disorder treated with rituximab: report of two cases and review of the literature. *Leuk Res*. 2006;30:109–114. https://doi.org/10.1016/j.leukres.2005.05.017.
155. Quach H, Januszewicz H, Westerman D. Complete remission of hairy cell leukemia variant (HCL-v) complicated by red cell aplasia post treatment with rituximab. *Haematologica*. 2005;90(Suppl):ECR26.
156. Zecca M, et al. Rituximab for the treatment of refractory autoimmune hemolytic anemia in children. *Blood*. 2003;101:3857–3861. https://doi.org/10.1182/blood-2002-11-3547.
157. Totterman TH, Nisell J, Killander A, Gahrton G, Lonnqvist B. Successful treatment of pure red-cell aplasia with cyclosporin. *Lancet*. 1984;2:693. https://doi.org/10.1016/s0140-6736(84)91245-5.
158. Debusscher L, Paridaens R, Stryckmans P, Delwiche F. Cyclosporine for pure red cell aplasia. *Blood*. 1985;65:249.
159. Williams DL, Mageed AS, Findley H, Ragab AH. Cyclosporine in the treatment of red cell aplasia. *Am J Pediatr Hematol Oncol*. 1987;9:314–316. https://doi.org/10.1097/00043426-198724000-00007.
160. Hocking W, Champlin R, Mitsuyasu R. Transient response of pure red cell aplasia to anti-thymocyte globulin in a patient with T-cell chronic lymphocytic leukemia. *Am J Hematol*. 1987;24:285–291. https://doi.org/10.1002/ajh.2830240308.
161. Krantz SB. Studies on red cell aplasia. 3. Treatment with horse antihuman thymocte gamma globulin. *Blood*. 1972;39:347–360.
162. Ru X, Liebman HA. Successful treatment of refractory pure red cell aplasia associated with lymphoproliferative disorders with the anti-CD52 monoclonal antibody alemtuzumab (Campath-1H). *Br J Haematol*. 2003;123:278–281. https://doi.org/10.1046/j.1365-2141.2003.04609.x.
163. Thachil J, Salim R. Campath-1H induced pure red cell aplasia in a patient with chronic lymphatic leukaemia. *Leuk Res*. 2007;31:1025–1026. https://doi.org/10.1016/j.leukres.2006.08.012.
164. Thota S, et al. Therapeutic outcomes using subcutaneous low dose alemtuzumab for acquired bone marrow failure conditions. *Br J Haematol*. 2018;183:133–136. https://doi.org/10.1111/bjh.14907.
165. Manroe BL, Weinberg AG, Rosenfeld CR, Browne R. The neonatal blood count in health and disease. I. Reference values for neutrophilic cells. *J Pediatr*. 1979;95:89–98. https://doi.org/10.1016/s0022-3476(79)80096-7.
166. Wright DG, Meierovics AI, Foxley JM. Assessing the delivery of neutrophils to tissues in neutropenia. *Blood*. 1986;67:1023–1030.
167. Kyle RA. Natural history of chronic idiopathic neutropenia. *N Engl J Med*. 1980;302:908–909. https://doi.org/10.1056/NEJM198004173021608.
168. Dale DC, Guerry 4th D, Wewerka JR, Bull JM, Chusid MJ. Chronic neutropenia. *Medicine (Baltimore)*. 1979;58:128–144. https://doi.org/10.1097/00005792-197903000-00002.
169. Boxer L, Dale DC. Neutropenia: causes and consequences. *Semin Hematol*. 2002;39:75–81. https://doi.org/10.1053/shem.2002.31911.
170. Stahlie TD. Chronic benign neutropenia in infancy and early childhood; report of a case with a review of the literature. *J Pediatr*. 1956;48:710–721. https://doi.org/10.1016/s0022-3476(56)80201-1.
171. Phillips D, Rezvani K, Bain BJ. Exercise induced mobilisation of the marginated granulocyte pool in the investigation of ethnic neutropenia. *J Clin Pathol*. 2000;53:481–483. https://doi.org/10.1136/jcp.53.6.481.
172. Mason BA, Lessin L, Schechter GP. Marrow granulocyte reserves in black Americans. Hydrocortisone-induced granulocytosis in the "benign" neutropenia of the black. *Am J Med*. 1979;67:201–205. https://doi.org/10.1016/0002-9343(79)90391-7.
173. Shoenfeld Y, Alkan ML, Asaly A, Carmeli Y, Katz M. Benign familial leukopenia and neutropenia in different ethnic groups. *Eur J Haematol*. 1988;41:273–277. https://doi.org/10.1111/j.1600-0609.1988.tb01192.x.
174. Logue GL, et al. Idiopathic neutropenia: antineutrophil antibodies and clinical correlations. *Am J Med*. 1991;90:211–216.
175. Wlodarski MW, Nearman Z, Jiang Y, Lichtin A, Maciejewski JP. Clonal predominance of CD8(+) T cells in patients with unexplained neutropenia. *Exp Hematol*. 2008;36:293–300. https://doi.org/10.1016/j.exphem.2007.11.011.
176. Pavlaki KI, et al. Abnormal telomere shortening of peripheral blood mononuclear cells and granulocytes in patients with chronic idiopathic neutropenia. *Haematologica*. 2012;97:743–750. https://doi.org/10.3324/haematol.2011.053983.
177. Balint GP, Balint PV. Felty's syndrome. *Best Pract Res Clin Rheumatol*. 2004;18:631–645. https://doi.org/10.1016/j.berh.2004.05.002.
178. Palmblad J, Papadaki HA, Eliopoulos G. Acute and chronic neutropenias. What is new? *J Intern Med*. 2001;250:476–491. https://doi.org/10.1046/j.1365-2796.2001.00915.x.
179. Starkebaum G. Chronic neutropenia associated with autoimmune disease. *Semin Hematol*. 2002;39:121–127. https://doi.org/10.1053/shem.2002.31918.
180. Boxer LA, Stossel TP. Effects of anti-human neutrophil antibodies in vitro. Quantitative studies. *J Clin Invest*. 1974;53:1534–1545. https://doi.org/10.1172/JCI107704.
181. Hadley AG, Holburn AM, Bunch C, Chapel H. Anti-granulocyte opsonic activity and autoimmune neutropenia. *Br J Haematol*. 1986;63:581–589. https://doi.org/10.1111/j.1365-2141.1986.tb07536.x.
182. Ducos R, Madyastha PR, Warrier RP, Glassman AB, Shirley LR. Neutrophil agglutinins in idiopathic chronic neutropenia of early childhood. *Am J Dis Child*. 1986;140:65–68. https://doi.org/10.1001/archpedi.1986.02140150067039.
183. Cines DB, et al. Granulocyte-associated IgG in neutropenic disorders. *Blood*. 1982;59:124–132.
184. Rustagi PK, Currie MS, Logue GL. Activation of human complement by immunoglobulin G antigranulocyte antibody. *J Clin Invest*. 1982;70:1137–1147. https://doi.org/10.1172/jci110712.
185. Caligaris-Cappio F, Camussi G, Gavosto F. Idiopathic neutropenia with normocellular bone marrow: an immune-complex disease. *Br J Haematol*. 1979;43:595–605. https://doi.org/10.1111/j.1365-2141.1979.tb03792.x.
186. Levitt LJ. Chlorpropamide-induced pure white cell aplasia. *Blood*. 1987;69:394–400.
187. Bux J, Chapman J. Report on the second international granulocyte serology workshop. *Transfusion*. 1997;37:977–983. https://doi.org/10.1046/j.1537-2995.1997.37997454028.x.
188. Bux J, Mueller-Eckhardt C. Autoimmune neutropenia. *Semin Hematol*. 1992;29:45–53.
189. Jonsson OG, Buchanan GR. Chronic neutropenia during childhood. A 13-year experience in a single institution. *Am J Dis Child*. 1991;145:232–235. https://doi.org/10.1001/archpedi.1991.02160020126032.
190. Lalezari P, Khorshidi M, Petrosova M. Autoimmune neutropenia of infancy. *J Pediatr*. 1986;109:764–769. https://doi.org/10.1016/s0022-3476(86)80690-4.

191. Neglia JP, et al. Autoimmune neutropenia of infancy and early childhood. *Pediatr Hematol Oncol*. 1993;10:369–376. https://doi.org/10.3109/08880019309029518.
192. Lyall EG, Lucas GF, Eden OB. Autoimmune neutropenia of infancy. *J Clin Pathol*. 1992;45:431–434. https://doi.org/10.1136/jcp.45.5.431.
193. Conway LT, et al. Natural history of primary autoimmune neutropenia in infancy. *Pediatrics*. 1987;79:728–733.
194. Komiyama A, et al. Increases in neutrophil counts by purified human urinary colony-stimulating factor in chronic neutropenia of childhood. *Blood*. 1988;71:41–45.
195. van der Klauw MM, Wilson JH, Stricker BH. Drug-associated agranulocytosis: 20 years of reporting in The Netherlands (1974–1994). *Am J Hematol*. 1998;57:206–211. doi:10.1002/(sici)1096-8652(199803)57:3<206::aid-ajh4>3.0.co;2-z.
196. Kaufman DW, et al. Drugs in the aetiology of agranulocytosis and aplastic anaemia. *Eur J Haematol Suppl*. 1996;60:23–30. https://doi.org/10.1111/j.1600-0609.1996.tb01641.x.
197. Andrès E, et al. Nonchemotherapy drug-induced agranulocytosis in elderly patients: the effects of granulocyte colony-stimulating factor. *Am J Med*. 2002;112:460–464. https://doi.org/10.1016/s0002-9343(02)01064-1.
198. van der Klauw MM, et al. A population-based case-cohort study of drug-associated agranulocytosis. *Arch Intern Med*. 1999;159:369–374. https://doi.org/10.1001/archinte.159.4.369.
199. Böttiger LE, Furhoff AK, Holmberg L. Drug-induced blood dyscrasias. A ten-year material from the Swedish Adverse Drug Reaction Committee. *Acta Med Scand*. 1979;205:457–461.
200. Risks of agranulocytosis and aplastic anemia. A first report of their relation to drug use with special reference to analgesics. The International Agranulocytosis and Aplastic Anemia Study. *JAMA*. 1986; 256:1749–1757.
201. Watts RG, Emanuel PD, Zuckerman KS, Howard TH. Valproic acid-induced cytopenias: evidence for a dose-related suppression of hematopoiesis. *J Pediatr*. 1990;117:495–499. https://doi.org/10.1016/s0022-3476(05)81105-9.
202. Irvine AE, French A, Daly A, Ranaghan L, Morris TC. Drug-induced neutropenia due to direct effects on CFU-C—ten years of culture experience. *Eur J Haematol*. 1994;52:21–27. https://doi.org/10.1111/j.1600-0609.1994.tb01280.x.
203. Neftel KA, Hauser SP, Müller MR. Inhibition of granulopoiesis in vivo and in vitro by beta-lactam antibiotics. *J Infect Dis*. 1985;152:90–98. https://doi.org/10.1093/infdis/152.1.90.
204. Levitt LJ, Ries CA, Greenberg PL. Pure white-cell aplasia. Antibody-mediated autoimmune inhibition of granulopoiesis. *N Engl J Med*. 1983;308:1141–1146. https://doi.org/10.1056/NEJM198305123081908.
205. Eisner EV, Carr RM, MacKinney AR. Quinidine-induced agranulocytosis. *JAMA*. 1977;238:884–886.
206. Papadaki T, et al. Evidence for T-large granular lymphocyte-mediated neutropenia in rituximab-treated lymphoma patients: report of two cases. *Leuk Res*. 2002;26:597–600. https://doi.org/10.1016/s0145-2126(01)00183-7.
207. Turbay D, et al. Tumor necrosis factor constellation polymorphism and clozapine-induced agranulocytosis in two different ethnic groups. *Blood*. 1997;89:4167–4174.
208. Bjornson BH, McIntyre AP, Harvey JM, Tauber AI. Studies of the effects of trimethoprim and sulfamethoxazole on human granulopoiesis. *Am J Hematol*. 1986;23:1–7. https://doi.org/10.1002/ajh.2830230102.
209. Sievers EL, Dale DC. Non-malignant neutropenia. *Blood Rev*. 1996;10:95–100. https://doi.org/10.1016/s0268-960x(96)90038-7.
210. Heimpel H. Drug-induced agranulocytosis. *Med Toxicol Adverse Drug Exp*. 1988;3:449–462. https://doi.org/10.1007/BF03259896.
211. Amarapurkar DN, Amarapurkar AD. Extrahepatic manifestations of viral hepatitis. *Ann Hepatol*. 2002;1:192–195.
212. Iwamoto J, et al. A case of agranulocytosis associated with severe acute hepatitis B. *Hepatol Res*. 2001;21:181–185. https://doi.org/10.1016/s1386-6346(01)00083-3.
213. Savard M, Gosselin J. Epstein-Barr virus immunosuppression of innate immunity mediated by phagocytes. *Virus Res*. 2006;119:134–145. https://doi.org/10.1016/j.virusres.2006.02.008.
214. Imashuku S, Tabata Y, Teramura T, Hibi S. Treatment strategies for Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis (EBV-HLH). *Leuk Lymphoma*. 2000;39:37–49. https://doi.org/10.3109/10428190009053537.
215. Corcione A, et al. Transforming growth factor beta-1 (TGF-beta 1) released by an Epstein-Barr virus (EBV) positive spontaneous lymphoblastoid cell line from a patient with Kostmann's congenital neutropenia inhibits the growth of normal committed haemopoietic progenitors in vitro. *Br J Haematol*. 1993;85:684–691. https://doi.org/10.1111/j.1365-2141.1993.tb03209.x.
216. Lewis DE, Gilbert BE, Knight V. Influenza virus infection induces functional alterations in peripheral blood lymphocytes. *J Immunol*. 1986;137:3777–3781.
217. Arakawa Y, Matsui A, Sasaki N, Nakayama T. Agranulocytosis and thrombocytopenic purpura following measles infection in a living-related orthotopic liver transplantation recipient. *Acta Paediatr Jpn*. 1997;39:226–229. https://doi.org/10.1111/j.1442-200x.1997.tb03587.x.
218. Hashimoto H, et al. Hematologic findings associated with thrombocytopenia during the acute phase of exanthem subitum confirmed by primary human herpesvirus-6 infection. *J Pediatr Hematol Oncol*. 2002;24:211–214. https://doi.org/10.1097/00043426-200203000-00010.
219. Takikawa T, Hayashibara H, Harada Y, Shiraki K. Liver dysfunction, anaemia, and granulocytopenia after exanthema subitum. *Lancet*. 1992;340:1288–1289. https://doi.org/10.1016/0140-6736(92)92989-s.
220. Ivarsson SA, Ljung R. Neutropenia and congenital cytomegalovirus infection. *Pediatr Infect Dis J*. 1988;7:436–437. https://doi.org/10.1097/00006454-198806000-00019.
221. Avanzini A, Colombo T, Santucci S. Neutropenia in a cytomegalovirus (CMV) infected VVLBW infant. *Acta Paediatr Scand*. 1991;80:738–739. https://doi.org/10.1111/j.1651-2227.1991.tb11940.x.
222. Almeida-Porada GD, Ascensão JL. Cytomegalovirus as a cause of pancytopenia. *Leuk Lymphoma*. 1996;21:217–223. https://doi.org/10.3109/10428199209067603.
223. McClain K, Estrov Z, Chen H, Mahoney Jr. DH. Chronic neutropenia of childhood: frequent association with parvovirus infection and correlations with bone marrow culture studies. *Br J Haematol*. 1993;85:57–62. https://doi.org/10.1111/j.1365-2141.1993.tb08645.x.
224. Koch WC, Massey G, Russell CE, Adler SP. Manifestations and treatment of human parvovirus B19 infection in immunocompromised patients. *J Pediatr*. 1990;116:355–359. https://doi.org/10.1016/s0022-3476(05)82820-3.
225. Doran HM, Teall AJ. Neutropenia accompanying erythroid aplasia in human parvovirus infection. *Br J Haematol*. 1988;69:287–288. https://doi.org/10.1111/j.1365-2141.1988.tb07636.x.
226. Zon LI, Groopman JE. Hematologic manifestations of the human immune deficiency virus (HIV). *Semin Hematol*. 1988;25:208–218.
227. Murphy MF, et al. Incidence and mechanism of neutropenia and thrombocytopenia in patients with human immunodeficiency virus infection. *Br J Haematol*. 1987;66:337–340. https://doi.org/10.1111/j.1365-2141.1987.tb06920.x.
228. Ribera E, et al. Autoimmune neutropenia and thrombocytopenia associated with development of antibodies to human immunodeficiency virus. *J Infect*. 1989;18:167–170. https://doi.org/10.1016/s0163-4453(89)91206-1.
229. McCance-Katz EF, Hoecker JL, Vitale NB. Severe neutropenia associated with anti-neutrophil antibody in a patient with acquired immunodeficiency syndrome-related complex. *Pediatr Infect Dis J*. 1987;6:417–418. https://doi.org/10.1097/00006454-198704000-00015.
230. Scadden DT, Zon LI, Groopman JE. Pathophysiology and management of HIV-associated hematologic disorders. *Blood*. 1989;74:1455–1463.
231. Moore DA, Sullivan A, Hilstead P, Gazzard BG. A retrospective study of neutropenia in HIV disease. *Int J STD AIDS*. 2000;11:8–14. https://doi.org/10.1258/0956462001914832.
232. Abdool Gaffar MS, Seedat YK, Coovadia YM, Khan Q. The white cell count in typhoid fever. *Trop Geogr Med*. 1992;44:23–27.
233. Fried D, Maytal J, Hanukoglu A. The differential leukocyte count in shigellosis. *Infection*. 1982;10:13–14. https://doi.org/10.1007/BF01640829.
234. Syrjälä H. Peripheral blood leukocyte counts, erythrocyte sedimentation rate and C-reactive protein in tularemia caused by the type B strain of *Francisella tularensis*. *Infection*. 1986;14:51–54. https://doi.org/10.1007/BF01644441.
235. al-Eissa Y, al-Nasser M. Haematological manifestations of childhood brucellosis. *Infection*. 1993;21:23–26. https://doi.org/10.1007/BF01739305.
236. Maartens G, Willcox PA, Benatar SR. Miliary tuberculosis: rapid diagnosis, hematologic abnormalities, and outcome in 109 treated adults. *Am J Med*. 1990;89:291–296. https://doi.org/10.1016/0002-9343(90)90340-j.
237. Davis KA, Finger DR, Shparago NI. Disseminated histoplasmosis mimicking Felty's syndrome. *J Clin Rheumatol*. 2002;8:38–43. https://doi.org/10.1097/00124743-200202000-00009.
238. Dale DC, Wolff SM. Studies of the neutropenia of acute malaria. *Blood*. 1973;41:197–206.
239. Aguero-Rosenfeld ME, et al. Human granulocytic ehrlichiosis: a case series from a medical center in New York State. *Ann Intern Med*. 1996;125:904–908. https://doi.org/10.7326/0003-4819-125-11-199612010-00006.
240. Christensen RD, Bradley PP, Rothstein G. The leukocyte left shift in clinical and experimental neonatal sepsis. *J Pediatr*. 1981;98:101–105. https://doi.org/10.1016/s0022-3476(81)80553-7.

241. Manroe BL, Rosenfeld CR, Weinberg AG, Browne R. The differential leukocyte count in the assessment and outcome of early-onset neonatal group B streptococcal disease. *J Pediatr*. 1977;91:632–637. https://doi.org/10.1016/s0022-3476(77)80522-2.

242. Craddock PR, Hammerschmidt DE, Moldow CF, Yamada O, Jacob HS. Granulocyte aggregation as a manifestation of membrane interactions with complement: possible role in leukocyte margination, microvascular occlusion, and endothelial damage. *Semin Hematol*. 1979;16:140–147.

243. Becton DL, Schultz WH, Kinney TR. Severe neutropenia caused by copper deficiency in a child receiving continuous ambulatory peritoneal dialysis. *J Pediatr*. 1986;108:735–737. https://doi.org/10.1016/s0022-3476(86)81056-3.

244. Zidar BL, Shadduck RK, Zeigler Z, Winkelstein A. Observations on the anemia and neutropenia of human copper deficiency. *Am J Hematol*. 1977;3:177–185. https://doi.org/10.1002/ajh.2830030209.

245. Karimbakas J, Langkamp-Henken B, Percival SS. Arrested maturation of granulocytes in copper deficient mice. *J Nutr*. 1998;128:1855–1860. https://doi.org/10.1093/jn/128.11.1855.

246. Higuchi S, Higashi A, Nakamura T, Yanabe Y, Matsuda I. Anti-neutrophil antibodies in patients with nutritional copper deficiency. *Eur J Pediatr*. 1991;150:327–330. https://doi.org/10.1007/BF01955933.

247. Willis MS, et al. Zinc-induced copper deficiency: a report of three cases initially recognized on bone marrow examination. *Am J Clin Pathol*. 2005;123:125–131. https://doi.org/10.1309/v6gvyw2qtyd5c5pj.

248. Igic PG, Lee E, Harper W, Roach KW. Toxic effects associated with consumption of zinc. *Mayo Clin Proc*. 2002;77:713–716. https://doi.org/10.4065/77.7.713.

249. Hayton BA, Broome HE, Lilenbaum RC. Copper deficiency-induced anemia and neutropenia secondary to intestinal malabsorption. *Am J Hematol*. 1995;48:45–47. https://doi.org/10.1002/ajh.2830480109.

250. Harless W, Crowell E, Abraham J. Anemia and neutropenia associated with copper deficiency of unclear etiology. *Am J Hematol*. 2006;81:546–549. https://doi.org/10.1002/ajh.20647.

251. Hirase N, et al. Anemia and neutropenia in a case of copper deficiency: role of copper in normal hematopoiesis. *Acta Haematol*. 1992;87:195–197. https://doi.org/10.1159/000204758.

252. Broun ER, Greist A, Tricot G, Hoffman R. Excessive zinc ingestion. A reversible cause of sideroblastic anemia and bone marrow depression. *JAMA*. 1990;264:1441–1443. https://doi.org/10.1001/jama.264.11.1441.

253. Oppenheimer SM, Hoffbrand BI, Dormandy TL, Parker N, Wickens DG. Macrocytic anaemia due to copper deficiency in a patient with late onset hypogammaglobulinaemia. *Postgrad Med J*. 1987;63:205–207. https://doi.org/10.1136/pgmj.63.737.205.

254. Kumar N. Copper deficiency myelopathy (human swayback). *Mayo Clin Proc*. 2006;81:1371–1384. https://doi.org/10.4065/81.10.1371.

255. Allen TM, Manoli 2nd A, LaMont RL. Skeletal changes associated with copper deficiency. *Clin Orthop Relat Res*. 1982:206–210.

256. Fessatou S, et al. Severe anemia and neutropenia associated with hyperzincemia and hypercalprotectinemia. *J Pediatr Hematol Oncol*. 2005;27:477–480. https://doi.org/10.1097/01.mph.0000179958.19524.9c.

257. Childs B, Nyhan WL, Borden M, Bard L, Cooke RE. Idiopathic hyperglycinemia and hyperglycinuria: a new disorder of amino acid metabolism. I. *Pediatrics*. 1961;27:522–538.

258. Soriano JR, Taitz LS, Finberg L, Edelmann Jr. CM. Hyperglycinemia with ketoacidosis and leukopenia. Metabolic studies on the nature of the defect. *Pediatrics*. 1967;39:818–828.

259. Huguley Jr. CM, Bain JA, Rivers SL, Scoggins RB. Refractory megaloblastic anemia associated with excretion of orotic acid. *Blood*. 1959;14:615–634.

260. Rosenberg LE, Lilljeqvist AC, Hsia YE. Methylmalonic aciduria. An inborn error leading to metabolic acidosis, long-chain ketonuria and intermittent hyperglycinemia. *N Engl J Med*. 1968;278:1319–1322. https://doi.org/10.1056/NEJM196806132782404.

261. D'Eufemia P, et al. Absence of severe recurrent infections in glycogen storage disease type Ib with neutropenia and neutrophil dysfunction. *J Inherit Metab Dis*. 2007;30:105. https://doi.org/10.1007/s10545-006-0511-9.

262. Garty BZ, Douglas SD, Danon YL. Immune deficiency in glycogen storage disease type 1B. *Isr J Med Sci*. 1996;32:1276–1281.

263. Koenig JM, Christensen RD. Incidence, neutrophil kinetics, and natural history of neonatal neutropenia associated with maternal hypertension. *N Engl J Med*. 1989;321:557–562. https://doi.org/10.1056/NEJM198908313210901.

264. Greco P, et al. Neutropenia in neonates delivered of women with pre-eclampsia. *Acta Biomed Ateneo Parmense*. 1997;68(Suppl 1):91–94.

265. Zuppa AA, et al. Influence of maternal preeclampsia on recombinant human granulocyte colony-stimulating factor effect in neutropenic neonates with suspected sepsis. *Eur J Obstet Gynecol Reprod Biol*. 2002;102:131–136. https://doi.org/10.1016/s0301-2115(01)00601-7.

266. Koenig JM, Christensen RD. The mechanism responsible for diminished neutrophil production in neonates delivered of women with pregnancy-induced hypertension. *Am J Obstet Gynecol*. 1991;165:467–473. https://doi.org/10.1016/0002-9378(91)90118-b.

267. Arneth B. Neonatal immune incompatibilities between newborn and mother. *J Clin Med*. 2020;9 https://doi.org/10.3390/jcm9051470.

268. Curtis BR, Reno C, Aster RH. Neonatal alloimmune neutropenia attributed to maternal immunoglobulin G antibodies against the neutrophil alloantigen HNA-1c (SH): a report of five cases. *Transfusion*. 2005;45:1308–1313. https://doi.org/10.1111/j.1537-2995.2005.00199.x.

269. Lalezari P, Murphy GB, Allen Jr. FH. NB1, a new neutrophil-specific antigen involved in the pathogenesis of neonatal neutropenia. *J Clin Invest*. 1971;50:1108–1115. https://doi.org/10.1172/JCI106582.

270. Maślanka K, Guz K, Uhrynowska M, Zupanska B. Isoimmune neonatal neutropenia due to anti-Fc(gamma) RIIIb antibody in a mother with an Fc(gamma) RIIIb deficiency. *Transfus Med*. 2001;11:111–113. https://doi.org/10.1046/j.1365-3148.2001.00294.x.

271. Gilmore MM, Stroncek DF, Korones DN. Treatment of alloimmune neonatal neutropenia with granulocyte colony-stimulating factor. *J Pediatr*. 1994;125:948–951. https://doi.org/10.1016/s0022-3476(05)82014-1.

272. Hanada T, Shin R, Hosoi M, Saito K, Takita H. Intravenous gammaglobulin in treatment of isoimmune neonatal neutropenia. *Eur J Pediatr*. 1988;148:218–219. https://doi.org/10.1007/BF00441407.

273. Fishman D, Isenberg DA. Splenic involvement in rheumatic diseases. *Semin Arthritis Rheum*. 1997;27:141–155. https://doi.org/10.1016/s0049-0172(97)80013-3.

274. Campion G, et al. The Felty syndrome: a case-matched study of clinical manifestations and outcome, serologic features, and immunogenetic associations. *Medicine (Baltimore)*. 1990;69:69–80.

275. Aref SE, Mahmoud LA, El Refie MF, Abdel Wahab M, Abou Samara N. Assessment of neutrophil apoptosis ex vivo in hepatosplenic patients with neutropenia pre and post splenectomy. *Hematology*. 2003;8:265–272. https://doi.org/10.1080/1024533031000135676.

276. Rosen M, et al. Outcome of laparoscopic splenectomy based on hematologic indication. *Surg Endosc*. 2002;16:272–279. https://doi.org/10.1007/s00464-001-8150-6.

277. Trueblood HW, Gustafson JR, Williams DK. Splenectomy for hypersplenism. *Am Surg*. 1978;44:517–522.

278. Naoum JJ, et al. Concomitant intraoperative splenic artery embolization and laparoscopic splenectomy versus laparoscopic splenectomy: comparison of treatment outcome. *Am J Surg*. 2007;193:713–718. https://doi.org/10.1016/j.amjsurg.2006.09.043.

279. Mathieson PW, et al. Antibody-mediated pure neutrophil aplasia, recurrent myasthenia gravis and previous thymoma: case report and literature review. *Q J Med*. 1990;74:57–61.

280. Weitzman SA, Stossel TP. Drug-induced immunological neutropenia. *Lancet*. 1978;1:1068–1072. https://doi.org/10.1016/s0140-6736(78)90915-7.

281. Murphy MF, Minchinton RM, Metcalfe P, Grint P, Waters AH. Neutropenia due to beta lactamine antibodies. *Br Med J (Clin Res Ed)*. 1984;288:795. https://doi.org/10.1136/bmj.288.6419.795.

282. Fumeaux Z, et al. Complete remission of pure white cell aplasia associated with thymoma, autoimmune thyroiditis and type 1 diabetes. *Eur J Haematol*. 2003;70:186–189. https://doi.org/10.1034/j.1600-0609.2003.00023.x.

283. Ackland SP, Bur ME, Adler SS, Robertson M, Baron JM. White blood cell aplasia associated with thymoma. *Am J Clin Pathol*. 1988;89:260–263. https://doi.org/10.1093/ajcp/89.2.260.

284. Yip D, Rasko JE, Lee C, Kronenberg H, O'Neill B. Thymoma and agranulocytosis: two case reports and literature review. *Br J Haematol*. 1996;95:52–56. https://doi.org/10.1046/j.1365-2141.1996.d01-1880.x.

285. Weir 3rd AB, Dow LW. Response of agranulocytosis to thymectomy in a patient with thymoma and chronic lymphocytic leukemia. *Med Pediatr Oncol*. 1989;17:58–61. https://doi.org/10.1002/mpo.2950170112.

286. Kalambokis G, Vassou A, Bourantas K, Tsianos EV. Imipenem-cilastatin induced pure white cell aplasia. *Scand J Infect Dis*. 2005;37:619–620. https://doi.org/10.1080/00365540510040205.

287. Mamus SW, et al. Ibuprofen-associated pure white-cell aplasia. *N Engl J Med*. 1986;314:624–625. https://doi.org/10.1056/NEJM198603063141006.

288. Frattini F, Crestani S, Vescovi PP, Franchini M. Pure white cell aplasia induced by mesalazine in a patient with ulcerative colitis. *Hematology*. 2013;18:181–182. https://doi.org/10.1179/1607845412Y.0000000043.

289. Iida S, et al. [Pure white cell aplasia (PWCA) with an inhibitor against colony-forming unit of granulocyte-macrophage (CFU-GM)]. *Rinsho Ketsueki*. 1990;31:1726–1730.

290. Postiglione K, Ferris R, Jaffe JP, Stroncek D. Immune mediated agranulocytosis and anemia associated with thymoma. *Am J Hematol*. 1995;49:336–340. https://doi.org/10.1002/ajh.2830490412.
291. Winkelstein JA, et al. X-linked agammaglobulinemia: report on a United States registry of 201 patients. *Medicine (Baltimore)*. 2006;85:193–202. https://doi.org/10.1097/01.md.0000229482.27398.ad.
292. Kanegane H, Taneichi H, Nomura K, Futatani T, Miyawaki T. Severe neutropenia in Japanese patients with x-linked agammaglobulinemia. *J Clin Immunol*. 2005;25:491–495. https://doi.org/10.1007/s10875-005-5370-x.
293. Farrar JE, Rohrer J, Conley ME. Neutropenia in X-linked agammaglobulinemia. *Clin Immunol Immunopathol*. 1996;81:271–276. https://doi.org/10.1006/clin.1996.0188.
294. Levy J, et al. Clinical spectrum of X-linked hyper-IgM syndrome. *J Pediatr*. 1997;131:47–54. https://doi.org/10.1016/s0022-3476(97)70123-9.
295. Notarangelo LD, Duse M, Ugazio AG. Immunodeficiency with hyper-IgM (HIM). *Immunodefic Rev*. 1992;3:101–121.
296. Scholl PR, O'Gorman MR, Pachman LM, Haut P, Kletzel M. Correction of neutropenia and hypogammaglobulinemia in X-linked hyper-IgM syndrome by allogeneic bone marrow transplantation. *Bone Marrow Transplant*. 1998;22:1215–1218. https://doi.org/10.1038/sj.bmt.1701512.
297. Cham B, Bonilla MA, Winkelstein J. Neutropenia associated with primary immunodeficiency syndromes. *Semin Hematol*. 2002;39:107–112. https://doi.org/10.1053/shem.2002.31916.
298. Tamura H, et al. Pure white cell aplasia: report of the first case associated with primary biliary cirrhosis. *Int J Hematol*. 2007;85:97–100. https://doi.org/10.1532/IJH97.06018.
299. Kim JJ, Thrasher AJ, Jones AM, Davies EG, Cale CM. Rituximab for the treatment of autoimmune cytopenias in children with immune deficiency. *Br J Haematol*. 2007;138:94–96. https://doi.org/10.1111/j.1365-2141.2007.06616.x.
300. Burks EJ, Loughran Jr. TP. Pathogenesis of neutropenia in large granular lymphocyte leukemia and Felty syndrome. *Blood Rev*. 2006;20:245–266. https://doi.org/10.1016/j.blre.2006.01.003.
301. Sood R, et al. Neutropenia associated with T-cell large granular lymphocyte leukemia: long-term response to cyclosporine therapy despite persistence of abnormal cells. *Blood*. 1998;91:3372–3378.
302. Firkin FC, Prewett EJ, Nicholls K, Moran J. Antithymocyte globulin therapy for pure white cell aplasia. *Am J Hematol*. 1987;25:101–105. https://doi.org/10.1002/ajh.2830250111.
303. Chudwin DS, Cowan MJ, Greenberg PL, Wara DW, Ammann AJ. Response of agranulocytosis to prolonged antithymocyte globulin therapy. *J Pediatr*. 1983;103:223–227. https://doi.org/10.1016/s0022-3476(83)80349-7.
304. Rashba EJ, Rowe JM, Packman CH. Treatment of the neutropenia of Felty syndrome. *Blood Rev*. 1996;10:177–184. https://doi.org/10.1016/s0268-960x(96)90024-7.
305. Liu JH, et al. Chronic neutropenia mediated by fas ligand. *Blood*. 2000;95:3219–3222.
306. Alvares CL, Svasti-Salee D, Rowley M, Gordon-Smith EC, Marsh JCW. Remission induced by Campath-1H for thymoma-associated agranulocytosis. *Ann Hematol*. 2004;83:398–400. https://doi.org/10.1007/s00277-003-0834-7.
307. Lamy T, Loughran Jr. TP. How I treat LGL leukemia. *Blood*. 2011;117:2764–2774. https://doi.org/10.1182/blood-2010-07-296962.
308. Franco G, Palazzolo R, Liardo E, Tripodo C, Mancuso S. T cell large granular lymphocytic leukemia in association with Sjogren's syndrome. *Acta Haematol*. 2010;124:5–8. https://doi.org/10.1159/000314900.
309. Lopez P, Morris DG, Galbraith PR, Lillicrap DP, Pross HF. Lymphoproliferative disease of "LAK cell" precursor large granular lymphocytes in association with celiac disease. *Am J Hematol*. 1993;43:116–122. https://doi.org/10.1002/ajh.2830430209.
310. Cherel B, et al. Large granular lymphocyte leukemia and precapillary pulmonary hypertension. *Chest*. 2020;158(6):2602–2609. https://doi.org/10.1016/j.chest.2020.07.094.
311. Komrokji RS, et al. Characterization of myelodysplastic syndromes (MDS) with T-cell large granular lymphocyte proliferations (LGL). *Leukemia*. 2020;34(11):3097–3099. https://doi.org/10.1038/s41375-020-0928-4.
312. Jerez A, et al. STAT3 mutations indicate the presence of subclinical T-cell clones in a subset of aplastic anemia and myelodysplastic syndrome patients. *Blood*. 2013;122:2453–2459. https://doi.org/10.1182/blood-2013-04-494930.
313. Herve M, et al. Unmutated and mutated chronic lymphocytic leukemias derive from self-reactive B cell precursors despite expressing different antibody reactivity. *J Clin Invest*. 2005;115:1636–1643. https://doi.org/10.1172/JCI24387.
314. Nyland SB, et al. Retroviral sero-reactivity in LGL leukaemia patients and family members. *Br J Haematol*. 2020;188:522–527. https://doi.org/10.1111/bjh.16223.
315. Perzova R, Loughran Jr. TP. Constitutive expression of Fas ligand in large granular lymphocyte leukaemia. *Br J Haematol*. 1997;97:123–126. https://doi.org/10.1046/j.1365-2141.1997.d01-2113.x.
316. Oshimi K, Shinkai Y, Okumura K, Oshimi Y, Mizoguchi H. Perforin gene expression in granular lymphocyte proliferative disorders. *Blood*. 1990;75:704–708.
317. Kothapalli R, et al. Constitutive expression of cytotoxic proteases and down-regulation of protease inhibitors in LGL leukemia. *Int J Oncol*. 2003;22:33–39.
318. Greer JP, Kinney MC, Loughran Jr. TP. T cell and NK cell lymphoproliferative disorders. *Hematology Am Soc Hematol Educ Program*. 2001:259–281. https://doi.org/10.1182/asheducation-2001.1.259.
319. Mollet L, et al. Leukemic CD3+ LGL share functional properties with their CD8+ CD57+ cell counterpart expanded after BMT. *Leukemia*. 1999;13:230–240. https://doi.org/10.1038/sj.leu.2401266.
320. Coakley G, Iqbal M, Brooks D, Panayi GS, Lanchbury JS. CD8$^+$, CD57$^+$ T cells from healthy elderly subjects suppress neutrophil development in vitro: implications for the neutropenia of Felty's and large granular lymphocyte syndromes. *Arthritis Rheum*. 2000;43:834–843. doi:10.1002/1529-0131(200004)43:4<834:AID-ANR14>3.0.CO;2-H.
321. Melenhorst JJ, Sorbara L, Kirby M, Hensel NF, Barrett AJ. Large granular lymphocyte leukaemia is characterized by a clonal T-cell receptor rearrangement in both memory and effector CD8(+) lymphocyte populations. *Br J Haematol*. 2001;112:189–194. https://doi.org/10.1046/j.1365-2141.2001.02509.x.
322. Suda T, Nagata S. Purification and characterization of the Fas-ligand that induces apoptosis. *J Exp Med*. 1994;179:873–879. https://doi.org/10.1084/jem.179.3.873.
323. Suda T, et al. Expression of the Fas ligand in cells of T cell lineage. *J Immunol*. 1995;154:3806–3813.
324. Suda T, Hashimoto H, Tanaka M, Ochi T, Nagata S. Membrane Fas ligand kills human peripheral blood T lymphocytes, and soluble Fas ligand blocks the killing. *J Exp Med*. 1997;186:2045–2050. https://doi.org/10.1084/jem.186.12.2045.
325. Lamy T, Liu JH, Landowski TH, Dalton WS, Loughran Jr. TP. Dysregulation of CD95/CD95 ligand-apoptotic pathway in CD3(+) large granular lymphocyte leukemia. *Blood*. 1998;92:4771–4777.
326. Aprile JA, Russo M, Pepe MS, Loughran Jr. TP. Activation signals leading to proliferation of normal and leukemic CD3$^+$ large granular lymphocytes. *Blood*. 1991;78:1282–1285.
327. Jerez A, et al. STAT3 mutations unify the pathogenesis of chronic lymphoproliferative disorders of NK cells and T-cell large granular lymphocyte leukemia. *Blood*. 2012;120:3048–3057. https://doi.org/10.1182/blood-2012-06-435297.
328. Koskela HL, et al. Somatic STAT3 mutations in large granular lymphocytic leukemia. *N Engl J Med*. 2012;366:1905–1913. https://doi.org/10.1056/NEJMoa1114885.
329. Yu CL, et al. Enhanced DNA-binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein. *Science*. 1995;269:81–83. https://doi.org/10.1126/science.7541555.
330. Epling-Burnette PK, et al. Inhibition of STAT3 signaling leads to apoptosis of leukemic large granular lymphocytes and decreased Mcl-1 expression. *J Clin Invest*. 2001;107:351–362. https://doi.org/10.1172/JCI9940.
331. Schade AE, Wlodarski MW, Maciejewski JP. Pathophysiology defined by altered signal transduction pathways: the role of JAK-STAT and PI3K signaling in leukemic large granular lymphocytes. *Cell Cycle*. 2006;5:2571–2574. https://doi.org/10.4161/cc.5.22.3449.
332. Epling-Burnette PK, et al. ERK couples chronic survival of NK cells to constitutively activated Ras in lymphoproliferative disease of granular lymphocytes (LDGL). *Oncogene*. 2004;23:9220–9229. https://doi.org/10.1038/sj.onc.1208122.
333. Clemente MJ, et al. Deep sequencing of the T-cell receptor repertoire in CD8$^+$ T-large granular lymphocyte leukemia identifies signature landscapes. *Blood*. 2013;122:4077–4085. https://doi.org/10.1182/blood-2013-05-506386.
334. Wlodarski MW, et al. Pathologic clonal cytotoxic T-cell responses: nonrandom nature of the T-cell-receptor restriction in large granular lymphocyte leukemia. *Blood*. 2005;106:2769–2780. https://doi.org/10.1182/blood-2004-10-4045.
335. Loughran Jr. TP, et al. Immunosuppressive therapy of LGL leukemia: prospective multicenter phase II study by the Eastern Cooperative Oncology Group (E5998). *Leukemia*. 2015;29:886–894. https://doi.org/10.1038/leu.2014.298.
336. Rajala HL, et al. Discovery of somatic STAT5b mutations in large granular lymphocytic leukemia. *Blood*. 2013;121:4541–4550. https://doi.org/10.1182/blood-2012-12-474577.
337. Rajala HLM, Mustjoki S. STAT5b in LGL leukemia—a novel therapeutic target? *Oncotarget*. 2013;4:808–809. https://doi.org/10.18632/oncotarget.1035.

338. Kuusanmaki H, et al. Drug sensitivity profiling identifies potential therapies for lymphoproliferative disorders with overactive JAK/STAT3 signaling. *Oncotarget*. 2017;8:97516–97527. https://doi.org/10.18632/oncotarget.22178.
339. Loughran Jr. TP, Starkebaum G. Large granular lymphocyte leukemia. Report of 38 cases and review of the literature. *Medicine (Baltimore)*. 1987;66:397–405.
340. Loughran Jr. TP. Clonal diseases of large granular lymphocytes. *Blood*. 1993;82:1–14.
341. Dhodapkar MV, Li CY, Lust JA, Tefferi A, Phyliky RL. Clinical spectrum of clonal proliferations of T-large granular lymphocytes: a T-cell clonopathy of undetermined significance? *Blood*. 1994;84:1620–1627.
342. Kwong YL, Wong KF. Association of pure red cell aplasia with T large granular lymphocyte leukaemia. *J Clin Pathol*. 1998;51:672–675. https://doi.org/10.1136/jcp.51.9.672.
343. Loughran Jr. TP, et al. Leukemia of large granular lymphocytes: association with clonal chromosomal abnormalities and autoimmune neutropenia, thrombocytopenia, and hemolytic anemia. *Ann Intern Med*. 1985;102:169–175. https://doi.org/10.7326/0003-4819-102-2-169.
344. Tefferi A, et al. Chronic natural killer cell lymphocytosis: a descriptive clinical study. *Blood*. 1994;84:2721–2725.
345. Molad Y, Okon E, Stark P, Prokocimer M. Sjogren's syndrome associated T cell large granular lymphocyte leukemia: a possible common etiopathogenesis. *J Rheumatol*. 2001;28:2551–2552.
346. Marlton P, Taylor K, Elliott S, McCormack J. Monoclonal large granular lymphocyte proliferation in SLE with HTLV-I seroreactivity. *Aust N Z J Med*. 1992;22:54–55. https://doi.org/10.1111/j.1445-5994.1992.tb01711.x.
347. Lima M, et al. Immunophenotypic analysis of the TCR-Vbeta repertoire in 98 persistent expansions of CD3(+)/TCR-alphabeta(+) large granular lymphocytes: utility in assessing clonality and insights into the pathogenesis of the disease. *Am J Pathol*. 2001;159:1861–1868. https://doi.org/10.1016/s0002-9440(10)63032-5.
348. Charlanne H, et al. Large granular lymphocyte leukaemia associated with systemic sclerosis. *Rheumatology (Oxford)*. 2004;43:1197–1198. https://doi.org/10.1093/rheumatology/keh293.
349. Valmori D, Ayyoub M, Hesdorffer CS, Keilholz U, Scheibenbogen C. A monoclonal melanoma-specific T-cell population phenotypically indistinguishable from CD3+ LGL-leukemia. *Blood*. 2003;101:4643–4644. https://doi.org/10.1182/blood-2003-03-0828.
350. Kondo H, Watanabe J, Iwasaki H. T-large granular lymphocyte leukemia accompanied by an increase of natural killer cells (CD3-) and associated with ulcerative colitis and autoimmune hepatitis. *Leuk Lymphoma*. 2001;41:207–212. https://doi.org/10.3109/10428190109057973.
351. Kaplanski G, et al. "NK-like" T cytotoxicity against B lymphocytes in a hypogammaglobulinemic patient. *Immunol Invest*. 1992;21:601–612. https://doi.org/10.3109/08820139209069396.
352. Starkebaum G, Loughran Jr. TP, Gaur LK, Davis P, Nepom BS. Immunogenetic similarities between patients with Felty's syndrome and those with clonal expansions of large granular lymphocytes in rheumatoid arthritis. *Arthritis Rheum*. 1997;40:624–626. https://doi.org/10.1002/art.1780400406.
353. Berliner N, Horwitz M, Loughran Jr. TP. Congenital and acquired neutropenia. *Hematology Am Soc Hematol Educ Program*. 2004:63–79. https://doi.org/10.1182/asheducation-2004.1.63.
354. Bowman SJ, et al. Large granular lymphocyte expansions in patients with Felty's syndrome: analysis using anti-T cell receptor V beta-specific monoclonal antibodies. *Clin Exp Immunol*. 1995;101:18–24. https://doi.org/10.1111/j.1365-2249.1995.tb02271.x.
355. Bowman SJ, Hall MA, Panayi GS, Lanchbury JS. T cell receptor alpha-chain and beta-chain junctional region homology in clonal $CD3^+$, $CD8^+$ T lymphocyte expansions in Felty's syndrome. *Arthritis Rheum*. 1997;40:615–623. https://doi.org/10.1002/art.1780400405.
356. Fogarty PF, Stetler-Stevenson M, Pereira A, Dunbar CE. Large granular lymphocytic proliferation-associated cyclic thrombocytopenia. *Am J Hematol*. 2005;79:334–336. https://doi.org/10.1002/ajh.20375.
357. Au WY, Lam CC, Lie AK, Pang A, Kwong YL. T-cell large granular lymphocyte leukemia of donor origin after allogeneic bone marrow transplantation. *Am J Clin Pathol*. 2003;120:626–630. https://doi.org/10.1309/VA75-5A03-PVRV-9XDT.
358. Risitano AM, et al. Large granular lymphocyte (LGL)-like clonal expansions in paroxysmal nocturnal hemoglobinuria (PNH) patients. *Leukemia*. 2005;19:217–222. https://doi.org/10.1038/sj.leu.2403617.
359. Karadimitris A, et al. Association of clonal T-cell large granular lymphocyte disease and paroxysmal nocturnal haemoglobinuria (PNH): further evidence for a pathogenetic link between T cells, aplastic anaemia and PNH. *Br J Haematol*. 2001;115:1010–1014. https://doi.org/10.1046/j.1365-2141.2001.03172.x.
360. Go RS, Tefferi A, Li CY, Lust JA, Phyliky RL. Lymphoproliferative disease of granular T lymphocytes presenting as aplastic anemia. *Blood*. 2000;96:3644–3646.
361. Saunthararajah Y, et al. Coincident myelodysplastic syndrome and T-cell large granular lymphocytic disease: clinical and pathophysiological features. *Br J Haematol*. 2001;112:195–200. https://doi.org/10.1046/j.1365-2141.2001.02561.x.
362. Kochenderfer JN, Kobayashi S, Wieder ED, Su C, Molldrem JJ. Loss of T-lymphocyte clonal dominance in patients with myelodysplastic syndrome responsive to immunosuppression. *Blood*. 2002;100:3639–3645. https://doi.org/10.1182/blood-2002-01-0155.
363. Papadaki T, et al. Clonal T-large granular lymphocyte proliferations associated with clonal B cell lymphoproliferative disorders: report of eight cases. *Leukemia*. 2002;16:2167–2169. https://doi.org/10.1038/sj.leu.2402643.
364. Lesesve JF, et al. Association of B-chronic lymphocytic leukaemia and T-large granular lymphocyte leukaemia. *Clin Lab Haematol*. 2000;22:121–122. https://doi.org/10.1046/j.1365-2257.2000.00294.x.
365. Kingreen D, et al. Lymphocytosis of large granular lymphocytes in patients with Hodgkin's disease. *Am J Hematol*. 1995;50:234–236. https://doi.org/10.1002/ajh.2830500403.
366. Lamy T, Loughran Jr. TP. Current concepts: large granular lymphocyte leukemia. *Blood Rev*. 1999;13:230–240. https://doi.org/10.1054/blre.1999.0118.
367. Melenhorst JJ, et al. T-cell large granular lymphocyte leukemia is characterized by massive TCRBV-restricted clonal CD8 expansion and a generalized overexpression of the effector cell marker CD57. *Hematol J*. 2003;4:18–25. https://doi.org/10.1038/sj.thj.6200212.
368. Herling M, et al. A systematic approach to diagnosis of mature T-cell leukemias reveals heterogeneity among WHO categories. *Blood*. 2004;104:328–335. https://doi.org/10.1182/blood-2004-01-0002.
369. Morice WG, Kurtin PJ, Leibson PJ, Tefferi A, Hanson CA. Demonstration of aberrant T-cell and natural killer-cell antigen expression in all cases of granular lymphocytic leukaemia. *Br J Haematol*. 2003;120:1026–1036. https://doi.org/10.1046/j.1365-2141.2003.04201.x.
370. Ohgami RS, et al. Refining the diagnosis of T-cell large granular lymphocytic leukemia by combining distinct patterns of antigen expression with T-cell clonality studies. *Leukemia*. 2011;25:1439–1443. https://doi.org/10.1038/leu.2011.107.
371. Ryan DK, Alexander HD, Morris TC. Routine diagnosis of large granular lymphocytic leukaemia by Southern blot and polymerase chain reaction analysis of clonal T cell receptor gene rearrangement. *Mol Pathol*. 1997;50:77–81. https://doi.org/10.1136/mp.50.2.77.
372. Berliner N, et al. T cell receptor gene rearrangements define a monoclonal T cell proliferation in patients with T cell lymphocytosis and cytopenia. *Blood*. 1986;67:914–918.
373. Man C, Au WY, Pang A, Kwong YL. Deletion 6q as a recurrent chromosomal aberration in T-cell large granular lymphocyte leukemia. *Cancer Genet Cytogenet*. 2002;139:71–74. https://doi.org/10.1016/s0165-4608(02)00605-2.
374. Wong KF, Chan JCW, Liu HSY, Man C, Kwong YL. Chromosomal abnormalities in T-cell large granular lymphocyte leukaemia: report of two cases and review of the literature. *Br J Haematol*. 2002;116:598–600. https://doi.org/10.1046/j.0007-1048.2001.03315.x.
375. Fischer L, Hummel M, Burmeister T, Schwartz S, Thiel E. Skewed expression of natural-killer (NK)-associated antigens on lymphoproliferations of large granular lymphocytes (LGL). *Hematol Oncol*. 2006;24:78–85. https://doi.org/10.1002/hon.777.
376. Gentile TC, Wener MH, Starkebaum G, Loughran Jr. TP. Humoral immune abnormalities in T-cell large granular lymphocyte leukemia. *Leuk Lymphoma*. 1996;23:365–370. https://doi.org/10.3109/10428199609054840.
377. Viny AD, Lichtin A, Pohlman B, Loughran T, Maciejewski J. Chronic B-cell dyscrasias are an important clinical feature of T-LGL leukemia. *Leuk Lymphoma*. 2008;49:932–938. https://doi.org/10.1080/10428190801932635.
378. Loughran Jr. TP, Kidd PG, Starkebaum G. Treatment of large granular lymphocyte leukemia with oral low-dose methotrexate. *Blood*. 1994;84:2164–2170.
379. Bargetzi MJ, et al. Severe neutropenia in T-large granular lymphocyte leukemia corrected by intensive immunosuppression. *Ann Hematol*. 1996;73:149–151. https://doi.org/10.1007/s002770050218.
380. Osuji N, Matutes E, Wotherspoon A, Catovsky D. Lessons from a case of T-cell large granular lymphocytic leukaemia suggesting that immunomodulatory therapy is more effective than intensive treatment. *Leuk Res*. 2005;29:225–228. https://doi.org/10.1016/j.leukres.2004.05.023.
381. Osuji N, et al. CD52 expression in T-cell large granular lymphocyte leukemia—implications for treatment with alemtuzumab. *Leuk Lymphoma*. 2005;46:723–727. https://doi.org/10.1080/10428190500052156.

382. Mohan SR, et al. Therapeutic implications of variable expression of CD52 on clonal cytotoxic T cells in $CD8^+$ large granular lymphocyte leukemia. *Haematologica*. 2009;94:1407–1414. https://doi.org/10.3324/haematol.2009.009191.
383. Bilori B, et al. Tofacitinib as a novel salvage therapy for refractory T-cell large granular lymphocytic leukemia. *Leukemia*. 2015;29:2427–2429. https://doi.org/10.1038/leu.2015.280.
384. Ma SY, et al. Fludarabine, mitoxantrone and dexamethasone in the treatment of indolent B- and T-cell lymphoid malignancies in Chinese patients. *Br J Haematol*. 2004;124:754–761. https://doi.org/10.1111/j.1365-2141.2004.04852.x.
385. Lobbes H, et al. Rituximab for rheumatoid arthritis-associated large granular lymphocytic leukemia, a retrospective case series. *Semin Arthritis Rheum*. 2020;50:1109–1113. https://doi.org/10.1016/j.semarthrit.2020.05.020.
386. La Nasa G, et al. Allogeneic hematopoietic stem cell transplantation in a patient affected by large granular lymphocyte leukemia and multiple sclerosis. *Ann Hematol*. 2004;83:403–405. https://doi.org/10.1007/s00277-003-0801-3.
387. Ebihara Y, et al. Successful treatment of natural killer (NK) cell leukemia following a long-standing chronic active Epstein-Barr virus (CAEBV) infection with allogeneic bone marrow transplantation. *Bone Marrow Transplant*. 2003;31:1169–1171. https://doi.org/10.1038/sj.bmt.1704078.
388. Gentile TC, Loughran Jr. TP. Resolution of autoimmune hemolytic anemia following splenectomy in $CD3^+$ large granular lymphocyte leukemia. *Leuk Lymphoma*. 1996;23:405–408. https://doi.org/10.3109/10428199609054846.
389. Subbiah V, et al. Outcomes of splenectomy in T-cell large granular lymphocyte leukemia with splenomegaly and cytopenia. *Exp Hematol*. 2008;36:1078–1083. https://doi.org/10.1016/j.exphem.2008.04.005.
390. Lemischka IR. The haematopoietic stem cell and its clonal progeny: mechanisms regulating the hierarchy of primitive haematopoietic cells. *Cancer Surv*. 1992;15:3–18.
391. Raslova H, et al. Megakaryocyte polyploidization is associated with a functional gene amplification. *Blood*. 2003;101:541–544. https://doi.org/10.1182/blood-2002-05-1553.
392. Shivdasani RA. Molecular and transcriptional regulation of megakaryocyte differentiation. *Stem Cells*. 2001;19:397–407. https://doi.org/10.1634/stemcells.19-5-397.
393. Metcalf D, et al. Anomalous megakaryocytopoiesis in mice with mutations in the c-Myb gene. *Blood*. 2005;105:3480–3487. https://doi.org/10.1182/blood-2004-12-4806.
394. Sandberg ML, et al. c-Myb and p300 regulate hematopoietic stem cell proliferation and differentiation. *Dev Cell*. 2005;8:153–166. https://doi.org/10.1016/j.devcel.2004.12.015.
395. Kaushansky K. The molecular mechanisms that control thrombopoiesis. *J Clin Invest*. 2005;115:3339–3347. https://doi.org/10.1172/JCI26674.
396. Kaushansky K. Thrombopoietin: accumulating evidence for an important biological effect on the hematopoietic stem cell. *Ann N Y Acad Sci*. 2003;996:39–43. https://doi.org/10.1111/j.1749-6633.2003.tb03230.x.
397. Deutsch VR, Tomer A. Megakaryocyte development and platelet production. *Br J Haematol*. 2006;134:453–466. https://doi.org/10.1111/j.1365-2141.2006.06215.x.
398. Gewirtz AM. Megakaryocytopoiesis: the state of the art. *Thromb Haemost*. 1995;74:204–209.
399. Jackson CW. Megakaryocyte endomitosis: a review. *Int J Cell Cloning*. 1990;8:224–226. https://doi.org/10.1002/stem.5530080405.
400. Kikuchi J, et al. Polyploidization and functional maturation are two distinct processes during megakaryocytic differentiation: involvement of cyclin-dependent kinase inhibitor p21 in polyploidization. *Blood*. 1997;89:3980–3990.
401. Levine RF, Hazzard KC, Lamberg JD. The significance of megakaryocyte size. *Blood*. 1982;60:1122–1131.
402. Mazur EM, Arriaga M, South K, Cohen JL. Polyploidization of human megakaryocytes in culture. *Prog Clin Biol Res*. 1986;215:193–199.
403. Odell Jr. TT, Jackson CW. Polyploidy and maturation of rat megakaryocytes. *Blood*. 1968;32:102–110.
404. Paulus JM. Cytophotometric measurements of DNA in thrombopoietic megakaryocytes. *Exp Cell Res*. 1968;53:310–313. https://doi.org/10.1016/0014-4827(68)90383-2.
405. Hunt P, Hokom MM, Wiemann B, Leven RM, Arakawa T. Megakaryocyte proplatelet-like process formation in vitro is inhibited by serum prothrombin, a process which is blocked by matrix-bound glycosaminoglycans. *Exp Hematol*. 1993;21:372–381.
406. Vitrat N, et al. Compared effects of Mpl ligand and other cytokines on human MK differentiation. *Stem Cells*. 1998;16(Suppl 2):37–51. https://doi.org/10.1002/stem.5530160707.
407. Schulze H, et al. Characterization of the megakaryocyte demarcation membrane system and its role in thrombopoiesis. *Blood*. 2006;107:3868–3875. https://doi.org/10.1182/blood-2005-07-2755.
408. Dale GL, Friese P. Bax activators potentiate coated-platelet formation. *J Thromb Haemost*. 2006;4:2664–2669. https://doi.org/10.1111/j.1538-7836.2006.02211.x.
409. Kaushansky K. Thrombopoietin: a tool for understanding thrombopoiesis. *J Thromb Haemost*. 2003;1:1587–1592. https://doi.org/10.1046/j.1538-7836.2003.00273.x.
410. Sheehan RG. Thrombopoiesis and thrombokinetics—an approach to the evaluation of thrombocytopenia. *Am J Med Sci*. 1985;289:168–176. https://doi.org/10.1097/00000441-198504000-00008.
411. Harker LA. Platelet production. *N Engl J Med*. 1970;282:492–494. https://doi.org/10.1056/NEJM197002262820906.
412. Harker LA. The kinetics of platelet production and destruction in man. *Clin Haematol*. 1977;6:671–693.
413. Harker LA, Finch CA. Thrombokinetics in man. *J Clin Invest*. 1969;48:963–974. https://doi.org/10.1172/JCI106077.
414. Beer JH, Buchi L, Steiner B. Glycocalicin: a new assay—the normal plasma levels and its potential usefulness in selected diseases. *Blood*. 1994;83:691–702.
415. Ault KA, Knowles C. In vivo biotinylation demonstrates that reticulated platelets are the youngest platelets in circulation. *Exp Hematol*. 1995;23:996–1001.
416. Wang C, Smith BR, Ault KA, Rinder HM. Reticulated platelets predict platelet count recovery following chemotherapy. *Transfusion*. 2002;42:368–374. https://doi.org/10.1046/j.1537-2995.2002.00040.x.
417. Watanabe K, et al. Automated measurement of reticulated platelets in estimating thrombopoiesis. *Eur J Haematol*. 1995;54:163–171. https://doi.org/10.1111/j.1600-0609.1995.tb00209.x.
418. Salvagno GL, et al. Evaluation of platelet turnover by flow cytometry. *Platelets*. 2006;17:170–177. https://doi.org/10.1080/09537100500437851.
419. Thomas-Kaskel A-K, Mattern D, Kohler G, Finke J, Behringer D. Reticulated platelet counts correlate with treatment response in patients with idiopathic thrombocytopenic purpura and help identify the complex causes of thrombocytopenia in patients after allogeneic hematopoietic stem cell transplantation. *Cytometry B Clin Cytom*. 2007;72:241–248. https://doi.org/10.1002/cyto.b.20163.
420. Robinson MS, et al. Flow cytometric analysis of reticulated platelets: evidence for a large proportion of non-specific labelling of dense granules by fluorescent dyes. *Br J Haematol*. 1998;100:351–357. https://doi.org/10.1046/j.1365-2141.1998.00563.x.
421. Emmons RV, et al. Human thrombopoietin levels are high when thrombocytopenia is due to megakaryocyte deficiency and low when due to increased platelet destruction. *Blood*. 1996;87:4068–4071.
422. Sungaran R, et al. The role of platelet alpha-granular proteins in the regulation of thrombopoietin messenger RNA expression in human bone marrow stromal cells. *Blood*. 2000;95:3094–3101.
423. Sungaran R, Markovic B, Chong BH. Localization and regulation of thrombopoietin mRNa expression in human kidney, liver, bone marrow, and spleen using in situ hybridization. *Blood*. 1997;89:101–107.
424. Vila L, et al. Correlations between cytogenetics and morphology in myelodysplastic syndromes. *Blut*. 1990;60:223–227. https://doi.org/10.1007/BF01728788.
425. Hoffman R, et al. An antibody cytotoxic to megakaryocyte progenitor cells in a patient with immune thrombocytopenic purpura. *N Engl J Med*. 1985;312:1170–1174. https://doi.org/10.1056/NEJM198505023121807.
426. Kouides PA, Rowe JM. Large granular lymphocyte leukemia presenting with both amegakaryocytic thrombocytopenic purpura and pure red cell aplasia: clinical course and response to immunosuppressive therapy. *Am J Hematol*. 1995;49:232–236. https://doi.org/10.1002/ajh.2830490309.
427. Lai DW, et al. Acquired amegakaryocytic thrombocytopenia and pure red cell aplasia associated with an occult large granular lymphocyte leukemia. *Leuk Res*. 2008;32:823–827. https://doi.org/10.1016/j.leukres.2007.08.012.
428. Son B, et al. A case of acquired amegakaryocytic thrombocytopenia with anti-c-mpl autoantibody: comparison with idiopathic thrombocytopenic purpura. *Acta Haematol*. 2019;142:239–243. https://doi.org/10.1159/000499523.
429. Gewirtz AM, Sacchetti MK, Bien R, Barry WE. Cell-mediated suppression of megakaryocytopoiesis in acquired amegakaryocytic thrombocytopenic purpura. *Blood*. 1986;68:619–626.
430. Oh H, Nakamura H, Yokota A, Asai T, Saito Y. Serum thrombopoietin levels in cyclic thrombocytopenia. *Blood*. 1996;87:4918.
431. Hoffman R, et al. Acquired cyclic amegakaryocytic thrombocytopenia associated with an immunoglobulin blocking the action of granulocyte-macrophage colony-stimulating factor. *N Engl J Med*. 1989;321:97–102. https://doi.org/10.1056/NEJM198907133210207.

432. Nicolle M, et al. Normal platelet numbers correlate with plasma viral load and $CD4^+$ cell counts in HIV-1 infection. *Eur J Haematol*. 1998;61:216–217. https://doi.org/10.1111/j.1600-0609.1998.tb01088.x.
433. Manoharan A, Williams NT, Sparrow R. Acquired amegakaryocytic thrombocytopenia: report of a case and review of literature. *Q J Med*. 1989;70:243–252.
434. Aird WC. The hematologic system as a marker of organ dysfunction in sepsis. *Mayo Clin Proc*. 2003;78:869–881. https://doi.org/10.4065/78.7.869.
435. Levin M. Acute hypersplenism and thrombocytopenia: a new presentation of disseminated mycobacterial infection in patients with acquired immunodeficiency syndrome. *Acta Haematol*. 1994;91:28–31. https://doi.org/10.1159/000204240.
436. Standaert SM, et al. Ehrlichiosis in a golf-oriented retirement community. *N Engl J Med*. 1995;333:420–425. https://doi.org/10.1056/NEJM199508173330704.
437. Yamaguchi S, et al. Severe thrombocytopenia suggesting immunological mechanisms in two cases of vivax malaria. *Am J Hematol*. 1997;56:183–186. doi:10.1002/(sici)1096-8652(199711)56:3<183::aid-ajh9>3.0.co;2-u.
438. Lefrere JJ, Got D. Peripheral thrombocytopenia in human parvovirus infection. *J Clin Pathol*. 1987;40:469. https://doi.org/10.1136/jcp.40.4.469-b.
439. Chesney PJ, Taher A, Gilbert EM, Shahidi NT. Intranuclear inclusions in megakaryocytes in congenital cytomegalovirus infection. *J Pediatr*. 1978;92:957–958. https://doi.org/10.1016/s0022-3476(78)80375-8.
440. Ninomiya N, Maeda T, Matsuda I. Thrombocytopenic purpura occurring during the early phase of a mumps infection. *Helv Paediatr Acta*. 1977;32:87–89.
441. Srivastava A, et al. Parvovirus B19-induced perturbation of human megakaryocytopoiesis in vitro. *Blood*. 1990;76:1997–2004.
442. Carbonara S, et al. Response of severe HIV-associated thrombocytopenia to highly active antiretroviral therapy including protease inhibitors. *J Infect*. 2001;42:251–256. https://doi.org/10.1053/jinf.2001.0833.
443. Lozach P-Y, et al. Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN)-mediated enhancement of dengue virus infection is independent of DC-SIGN internalization signals. *J Biol Chem*. 2005;280:23698–23708. https://doi.org/10.1074/jbc.M504337200.
444. Ramratnam B, et al. Short course dexamethasone for thrombocytopenia in AIDS. *Am J Med*. 1996;100:117–118. https://doi.org/10.1016/s0002-9343(96)90020-0.
445. Eyster ME, et al. Human immunodeficiency virus-related conditions in children and adults with hemophilia: rates, relationship to CD4 counts, and predictive value. *Blood*. 1993;81:828–834.
446. Glatt AE, Anand A. Thrombocytopenia in patients infected with human immunodeficiency virus: treatment update. *Clin Infect Dis*. 1995;21:415–423. https://doi.org/10.1093/clinids/21.2.415.
447. Lord RV, Coleman MJ, Milliken ST. Splenectomy for HIV-related immune thrombocytopenia: comparison with results of splenectomy for non-HIV immune thrombocytopenic purpura. *Arch Surg*. 1998;133:205–210. https://doi.org/10.1001/archsurg.133.2.205.
448. Mendenhall NP, Noyes WD, Million RR. Total body irradiation for stage II-IV non-Hodgkin's lymphoma: ten-year follow-up. *J Clin Oncol*. 1989;7:67–74. https://doi.org/10.1200/JCO.1989.7.1.67.
449. Pertusini E, et al. Investigating the platelet-sparing mechanism of paclitaxel/carboplatin combination chemotherapy. *Blood*. 2001;97:638–644. https://doi.org/10.1182/blood.v97.3.638.
450. Haznedaroglu IC, Goker H, Turgut M, Buyukasik Y, Benekli M. Thrombopoietin as a drug: biologic expectations, clinical realities, and future directions. *Clin Appl Thromb Hemost*. 2002;8:193–212. https://doi.org/10.1177/107602960200800301.
451. Antony AC, et al. Megaloblastic hematopoiesis in vitro. Interaction of anti-folate receptor antibodies with hematopoietic progenitor cells leads to a proliferative response independent of megaloblastic changes. *J Clin Invest*. 1991;87:313–325. https://doi.org/10.1172/JCI114989.
452. Cowan DH, Hines JD. Thrombokinetics in dietary-induced folate deficiency in human subjects. *J Lab Clin Med*. 1973;81:577–586.
453. Carrascosa M, Pérez-Castrillón JL, Pascual F, Olalla JI, Valle R. Pernicious anemia presenting with acute generalized thrombocytopenic purpura. *Am J Hematol*. 1995;48:125. https://doi.org/10.1002/ajh.2830480211.
454. Berger M, Brass LF. Severe thrombocytopenia in iron deficiency anemia. *Am J Hematol*. 1987;24:425–428. https://doi.org/10.1002/ajh.2830240412.
455. Go RS, Porrata LF, Call TG. Thrombocytopenia after iron dextran administration in a patient with severe iron deficiency anemia. *Ann Intern Med*. 2000;132:925. https://doi.org/10.7326/0003-4819-132-11-200006060-00025.
456. Cowan DH, Hines JD. Thrombocytopenia of severe alcoholism. *Ann Intern Med*. 1971;74:37–43. https://doi.org/10.7326/0003-4819-74-1-37.
457. Gardner FH, Bessman JD. Thrombocytopenia due to defective platelet production. *Clin Haematol*. 1983;12:23–38.
458. Gewirtz AM, Hoffman R. Transitory hypomegakaryocytic thrombocytopenia: aetiological association with ethanol abuse and implications regarding regulation of human megakaryocytopoiesis. *Br J Haematol*. 1986;62:333–344. https://doi.org/10.1111/j.1365-2141.1986.tb02937.x.
459. Nakao S, Harada M, Kondo K, Mizushima N, Matsuda T. Reversible bone marrow hypoplasia induced by alcohol. *Am J Hematol*. 1991;37:120–123. https://doi.org/10.1002/ajh.2830370210.
460. Lundh B, Hasselgren KH. Hematological side effects from antihypertensive drugs. *Acta Med Scand Suppl*. 1979;628:73–75. https://doi.org/10.1111/j.0954-6820.1979.tb00782.x.
461. Sacchi S, et al. Alfa-interferon in the treatment of essential thrombocythemia: clinical results and evaluation of its biological effects on the hematopoietic neoplastic clone. Italian Cooperative Group on ET. *Leukemia*. 1998;12:289–294. https://doi.org/10.1038/sj.leu.2400931.
462. Mazur EM, Rosmarin AG, Sohl PA, Newton JL, Narendran A. Analysis of the mechanism of anagrelide-induced thrombocytopenia in humans. *Blood*. 1992;79:1931–1937.
463. Wang H, Chuhjo T, Yasue S, Omine M, Nakao S. Clinical significance of a minor population of paroxysmal nocturnal hemoglobinuria-type cells in bone marrow failure syndrome. *Blood*. 2002;100:3897–3902. https://doi.org/10.1182/blood-2002-03-0799.
464. Louwes H, Vellenga E, de Wolf JT. Abnormal platelet adhesion on abdominal vessels in asymptomatic patients with paroxysmal nocturnal hemoglobinuria. *Ann Hematol*. 2001;80:573–576. https://doi.org/10.1007/s002770100350.
465. Basara N, Antunovic P, Sefer D, Radosevic N, Gotic M. Megakaryocyte progenitors in paroxysmal nocturnal haemoglobinuria are sensitive to complement. *Eur J Haematol*. 1996;57:227–229. https://doi.org/10.1111/j.1600-0609.1996.tb01368.x.
466. Dessypris EN, Gleaton JH, Clark DA. Increased sensitivity to complement of megakaryocyte progenitors in paroxysmal nocturnal haemoglobinuria. *Br J Haematol*. 1988;69:305–309. https://doi.org/10.1111/j.1365-2141.1988.tb02367.x.
467. Schubert J, Scholz C, Geissler RG, Ganser A, Schmidt RE. G-CSF and cyclosporin induce an increase of normal cells in hypoplastic paroxysmal nocturnal hemoglobinuria. *Ann Hematol*. 1997;74:225–230. https://doi.org/10.1007/s002770050289.
468. Ridell B, Kutti J, Swolin B, Wadenvik H. Dysplastic megakaryopoiesis with thrombocytopenia and chromosomal aberration. *Am J Clin Pathol*. 1992;98:227–230. https://doi.org/10.1093/ajcp/98.2.227.
469. Tricot G, Criel A, Verwilghen RL. Thrombocytopenia as presenting symptom of preleukaemia in 3 patients. *Scand J Haematol*. 1982;28:243–250. https://doi.org/10.1111/j.1600-0609.1982.tb00522.x.

PART V RED BLOOD CELLS

CHAPTER 34

PATHOBIOLOGY OF THE HUMAN ERYTHROCYTE AND ITS HEMOGLOBINS

Martin H. Steinberg, Edward J. Benz, Jr., and Benjamin L. Ebert

Anemia, polycythemia, and functional derangements of the human erythrocyte together represent a common group of human disorders with a significant impact on public health. Sickle cell disease, hemoglobin E (HbE)–associated disorders, and the thalassemias are humankind's most common single-gene diseases, but the relevance of red blood cell (RBC) disorders to general medicine extends even beyond their individual clinical severities or the number of patients affected. A critical added dimension of erythrocyte disorders is the extraordinarily detailed knowledge available about the basic biochemistry, physiology, and molecular biology of the human RBC and its membrane, metabolism, and major component, hemoglobin (Hb). RBCs are especially abundant, relatively simple, and readily accessible for repeated testing in individual patients. These features have facilitated the rapid application of the techniques of cellular and molecular biology to studies of the RBC, its component molecules and structures, and syndromes resulting from abnormalities of these entities. Taken as a group, erythrocyte disorders are better understood at the molecular and cellular levels than disorders of any other cell or tissue. It is for this reason that these conditions merit particularly careful scrutiny by students of hematology.

This chapter reviews the concepts about normal RBC homeostasis that form the essential knowledge base for understanding anemias, polycythemias, and functional erythrocyte disorders. The primary focus and the object for detailed discussion within this chapter is Hb, the major component, both quantitatively and qualitatively, of the erythrocyte. Hb molecules dominate the pathophysiology of many RBC disorders and modulate most of the others, in part because of their sheer quantitative predominance in the RBC cytoplasm. The other major relevant aspects of human RBCs—the membrane, the enzymes used for intermediary metabolism, differentiation and development, and the process of destruction—are discussed in detail in the introductory portions of other chapters. This chapter surveys these areas only briefly. Detailed descriptions of the RBC membrane can be found in Chapter 46. RBC enzymes and enzymopathies are described in Chapter 45; differentiation and development are described in Chapters 9 and 27; regulation of the RBC mass by erythropoietin is discussed in Chapter 27; and the necessary aspects of RBC destruction are considered in Chapters 44, 47, and 48.

ESSENTIAL FEATURES OF RED BLOOD CELL HOMEOSTASIS

As discussed in Chapter 27, the mature RBC is the product of a complex and orderly set of differentiation and maturation steps beginning with the pluripotent stem cell. By complex partially understood mechanisms involving hierarchic networks of cytokines, a portion of these cells becomes committed to differentiate along the erythroid pathway. Commitment to erythropoiesis provokes a progressively increasing sensitivity to the stimulatory actions of the hormone erythropoietin (see Chapters 9 and 27). As differentiation proceeds, there is preprogramming of certain genes whose expression at high levels will be required during the maturation phase of erythropoiesis. Genes coding for molecules defining the RBC phenotype (e.g., globin) are poised for activation at later maturation steps.

Intermediate progenitor cells arising during differentiation have been characterized experimentally, including the burst-forming unit-erythroid (BFU-E) and the colony-forming unit-erythroid (CFU-E) stages. BFU-Es are progenitor cells that in culture produce bursts or clusters of erythroid colonies, are relatively less sensitive to erythropoietin, and are more plastic with respect to important gene expression parameters, such as the synthesis of adult or fetal Hb (HbF) by their descendants. CFU-Es produce single colonies, exhibit considerably higher sensitivity to erythropoietin, and appear to be more fixed in their potential to express a particular subset of globin genes. CFU-Es give rise to the first morphologically recognizable erythroid cells, the proerythroblasts. At this "primitive" morphologic stage, the program of erythroid cell expression has already been essentially predetermined. The cell is predestined to undergo only a limited additional number of cell divisions, culminating in the formation of the enucleate reticulocyte. The terminal maturation stages are morphologically recognizable as erythroblasts exhibiting progressive hemoglobinization of the cytoplasm, condensation, and eventual ejection of the nucleus and remodeling of the plasma membrane. The actual expression of the preprogrammed genes occurs during the 5- to 7-day period of erythroblast maturation.

As discussed in Chapters 9 and 27, the actual reconfiguration of chromatin for activation of the genes and activation itself appears to require the concerted and complex interaction of a diverse but limited group of transcription factors and associated epigenetic regulators. These regulatory proteins recognize a specific array of promoter and enhancer sequences that are embedded as recurrent motifs in and around the appropriate target genes. Even though an enormous amount of information has been gathered about sequences such as the GATA enhancers and their cognate transcription factors (e.g., GATA, FOG, ETS), the precise means by which these sequences and factors cause erythroid differentiation remains under investigation. (The current state of understanding is outlined in Chapter 27.) At this time, this information is of limited clinical relevance to anemias or polycythemias. Major changes in the balance of post transcriptional regulatory molecules such as pre-mRNA splicing factors, mRNA stabilization factors, and translation factors also occur during maturation. Some of these, notably certain splicing factors, are proving to be relevant to myelodysplastic and myeloproliferative disorders (see Chapters 61 and 70). The orderly 14- to 21-day sequence of differentiation and maturation becomes progressively influenced by the levels of erythropoietin available to the progenitor cells, possibly because of increasing density and affinity of erythropoietin receptors on their cell surfaces. Within 24 hours after enucleation, the reticulocyte traverses the bone marrow–blood barrier membrane and enters the circulation as an immature erythrocyte. These cells retain remnants of nucleated precursors in the form of a relatively small number of polyribosomes actively translating messenger ribonucleic acid (RNA) (greater than 90% of which is globin messenger RNA), a cell membrane that retains some molecules and structures reminiscent of its earlier stages of differentiation, and the complement of enzymes, phospholipids, and cytoskeletal proteins that the cell will possess throughout its remaining life span.

During its first 24 hours in the circulation, the reticulocyte spends considerable amounts of time in the spleen, during which its membrane is "polished." This is a poorly understood remodeling process by which some lipids and proteins, including adhesive molecules such as fibronectin, are removed. The content of polyribosomes and other nucleic acids progressively declines so that stainability with methylene blue is lost by the end of the first day. At this time, the RBC is regarded as a mature erythrocyte, and it circulates relatively unchanged for the remainder of its 120-day life span.

Perhaps the most remarkable feature of the human RBC is its durability, given that it is an enucleated cell devoid of organelles that are critical for the survival and function of most other cell types. The RBC has no mitochondria available for efficient oxidative metabolism; no ribosomes for regeneration of lost or damaged proteins; a very limited metabolic repertoire that largely precludes de novo synthesis of lipids; and no nucleus to direct regenerative processes, adaptation to circulatory stresses, or cell division to replenish itself. Given these handicaps, the 120-day survival of these cells is even more striking considering the multiple and often exceedingly hostile environments they must traverse. Mechanical stresses of the circulation include high hydrostatic pressure and turbulence and the shear stresses inherent in a microcirculation networked with many capillaries having diameters only one-third to one-half that of the normal RBC. Biochemical stresses include osmotic and redox fluxes associated with travel through the collecting system of the kidney; the sluggish vascular beds of the spleen, muscle, and bone; and the rapid changes in ambient oxygen pressures occurring in the lungs. All conspire to damage RBCs. Their 4-month survival is truly remarkable.

The ability of the RBC to persist in the circulation depends on its simple but exquisitely adaptive membrane structures; its pathways of intermediary energy metabolism and redox regulation; and its ability to maintain its largest cytoplasmic component, Hb, in a soluble and non-oxidized state. The membrane and enzymes of the RBC appear to be exquisitely crafted to protect the cell from the external ravages of the circulation and the potential internal assaults of the massive amount of iron-rich and potentially oxidizing protein represented by its complement of Hb molecules. For these reasons, a few basic features of these membrane and enzyme systems merit comment before considering the Hb molecule itself.

MAJOR FEATURES OF THE RED BLOOD CELL MEMBRANE

Chapter 46 describes the RBC membrane in considerable detail. Only a few major aspects of that discussion bear repeating for the purposes of this chapter. The RBC membrane and its underlying cytoskeleton have evolved to provide mechanical strength and the necessary pliability and resilience to withstand the mechanical, osmotic, and chemical stresses of the circulation. Because a lipid bilayer membrane essentially has the physical properties of a soap bubble, it would rapidly be emulsified in the circulation. Strength and order are provided to the lipid bilayer by the hexagonal arrays of the highly helical protein spectrin, which forms a latticework underlying the membrane.

The spectrin meshwork is held together by adaptor molecules, such as protein 4.1, adducin, p55, and ankyrin, arrayed at defined points along the highly coiled, rod-like structure of the spectrin oligomers (see figures in Chapter 46). These protein–protein interactions appear to be critical for holding the latticework together in what has been described as the "horizontal" dimension that permits resistance to shear stress. The involvement of intermediate-length actin fibers and the variability of binding affinities governed by phosphorylation and other biochemical alterations appear to provide some flexibility and pliability at these points of interaction. Strength in the "vertical" dimension is provided by additional molecules or additional binding functions of the same molecule, whereby the latticework is attached to the lipid bilayer. For the most part, the physiologically important attachments appear to be indirect. Linkage is mediated through the interaction of the adaptor proteins, such as ankyrin and protein 4.1, with both the cytoplasmic domains of abundant transmembrane proteins and specific domains within the spectrin molecule. These proteins traverse and are embedded in the lipid bilayer, providing a firm anchor. The two most critical of these molecules appear to be band 3 (i.e., the anion transport channel) and a glycophorin, probably glycophorin C/D. A possible additional stabilizing role for the Rh protein complex has been suggested. The construction of these attachments by multiple "hinge" or coupling molecules appears to provide for the flexibility and distensibility of the RBC membrane, a property essential to its ability to flow through small capillaries.

As described in Chapter 46, the complex structure of the membrane is exquisitely sensitive to perturbations impinging on any of its components. In particular, the membrane cytoskeleton and phospholipid structures are each highly susceptible to oxidation, particularly by partially proteolyzed molecules of Hb, which denature to form highly toxic compounds called *hemopyrroles*. This interaction of denatured Hb with the RBC membrane is clinically important, as illustrated by its impact on the pathophysiology of sickle cell anemia (see Chapters 42 and 43) or of oxidized and precipitated globin inclusion bodies in thalassemia (see Chapter 41) and unstable Hb disorders (see Chapter 44). In this chapter, it is sufficient to note that alterations of the proteins of the RBC membrane/cytoskeleton complex can contribute to shortening the life span of the RBC. Damage can result from intrinsic defects in the cytoskeletal proteins themselves or from the susceptibility of these proteins to direct oxidation or attack by oxidized or denatured Hb molecules. Readers are referred to Chapters 44–48 for detailed descriptions of the relevant phenomena.

ENZYMES OF RED BLOOD CELL INTERMEDIARY METABOLISM

Mammalian erythrocytes possess a highly specialized but remarkably simplified set of metabolic pathways. As discussed in Chapter 45, there are essentially three relevant sets of pathways. The first two are interconnected by the enzyme glucose-6-phosphate dehydrogenase (G6PD). Glucose entering the RBC is metabolized by an anaerobic pathway, the Embden–Meyerhof pathway, which terminates with the enzyme lactic dehydrogenase, forming lactate. Despite its inefficiency (a net of only two adenosine triphosphate [ATP]/glucose molecules generated per glucose molecule consumed), this pathway is the sole source of usable ATP in the cell. Moreover, the pathway generates reduced nicotinamide adenine dinucleotide (NADH), a molecule necessary for driving the reduction of methemoglobin to Hb (see Chapters 45 and 48). A shunt within this pathway, the Rapoport–Luebering shunt, generates the compound 2,3-bisphosphoglycerate (bis[phosphoglyceric acid]) (2,3-BPG), an important cofactor that, when bound to Hb, reduces the affinity of Hb for oxygen (see Hemoglobin Function). The ATP generated is necessary for kinase reactions controlling phosphorylation of membrane and signaling components, for fueling ion pumps and channels, and for maintaining phospholipid levels.

The anaerobic metabolic pathway generates, as one of its intermediates, glucose-6 phosphate, which is the substrate for G6PD. G6PD appears to be the rate-limiting enzyme for a linked pathway called the *oxidative hexose monophosphate shunt*. This pathway involves a cascade of reactions culminating in the reduction of oxidized glutathione to reduced glutathione. Reduced glutathione is used to reverse the oxidation of critical structures, including Hb, cytoskeletal proteins, and membrane lipids. Anaerobic glycolysis generates NADH for methemoglobin reduction, 2,3-BPG for modulation of Hb–oxygen affinity, and ATP for metabolic energy requirements. Its end product is lactate. The oxidative hexose monophosphate shunt generates NADH phosphate (NADPH) and reduced glutathione for use as the major erythrocyte antioxidant.

During the past decade, most of the enzymes (or at least the erythroid isoforms of these enzymes) involved in RBC intermediary metabolism have been characterized at the molecular level by cloning of their cDNAs, genomic loci, or both. Some of the more relevant information arising from this progress is discussed in Chapter 45. The erythrocyte possesses membrane-based signaling receptors and cytoplasmic signal transduction elements similar, although perhaps less elaborate, than those of nucleated cells. The relevance of these systems to the pathophysiology of RBC disorders is just becoming apparent.

RED BLOOD CELL SENESCENCE AND DESTRUCTION

Erythrocytes, despite their impressive adaptations to circulatory stresses, eventually wear out and are destroyed. RBC survival in humans appears to be remarkably uniform under normal circumstances, spanning approximately 120 days from release of the reticulocyte into circulation to sequestration of the senescent RBC in the reticuloendothelial cells of the liver and spleen. The precise signal, or signals, marking RBCs for destruction remain incompletely understood, as does the underlying pathophysiology within the RBC or on its surface. However, several interrelated theories have emerged; these are discussed only briefly because they are mentioned in other chapters.

RBCs accumulate surface blemishes during their lives in circulation. These appear to result in part from the accumulation of small amounts of oxygen damage to membrane structures. The altered regions are sensed by the reticuloendothelial cells during passage of the erythrocytes through the liver and spleen. Removal or pitting of these damaged regions from RBC membranes can be documented microscopically; small amounts of the normal membrane are also lost during the process.

The biconcave disk shape of the RBC, so important to its distensibility, depends on a high ratio of surface area to volume. This requires a redundant membrane surface area. The membrane surface area of the normal biconcave disk is approximately 140 μm^2. To enclose a sphere containing a normal RBC volume (≈90 fL), only approximately 95 μm^2 would be needed. Progressive loss of membrane surface by means of the pitting phenomenon ultimately causes the aging erythrocyte to assume a more rigid spherical shape. A sphere is inevitably far less distensible and far less capable of "squeezing" through small apertures than a disk, especially in the sluggish and tortuous circulation of the spleen. The entrapped RBCs become engulfed by macrophages and catabolized. This geometric mechanism contributes to the eventual destruction of RBCs.

RBCs progressively lose some of the critical enzymes needed for intermediary metabolism and antioxidant capacity. G6PD levels, for example, progressively decline during the circulating life span, as do levels of several other enzymes. The decline of certain enzymes can be used as a crude means of estimating the relative age of different RBC populations. The biochemical or oxidative mechanism of destruction postulates that aged RBCs are eventually depleted of critical enzymes needed for the maintenance of redox status. Oxidation of critical membrane proteins, lipids, and Hb would then ensue, causing distortion and rigidity of the RBC membrane, with accelerated loss as previously described. The end product would be spherocytes incapable of traversing the splenic vascular bed and escaping engulfment by the reticuloendothelial cell.

It has been proposed that an immune mechanism can contribute to normal and pathologic RBC senescence. This hypothesis is based on the observation that oxidative damage, regardless of cause, promotes a clustering, or *capping*, of oligomers of band 3 on the RBC surface. Under normal circumstances, band 3 molecules form monomers, dimers, or tetramers. Higher-order aggregates appear to be recognized by an endogenous isoantibody possessed by most people. Any RBC accumulating oxidative damage from wear and tear in the circulation, from the depletion of enzymes, or from internal pathologic processes such as denaturation of Hb in certain hemoglobinopathies can accumulate these aggregates. The aggregates would then be bound by antibodies and be removed by the reticuloendothelial cells as antigen–antibody complexes using the same means used by reticuloendothelial cells to recognize any immune complex. This mechanism could also provide for the pitting or polishing of damaged RBC membranes. All three of the proposed mechanisms are interrelated by their inception with oxidative damage.

Other membrane-related changes might influence RBC destruction. Bcl-X_L, a suppressor of apoptosis, is present in erythrocyte membranes, and its antagonization may promote cell death. This may be mediated by calcium accumulation and phosphatidylserine exposure. Cholesterol and fatty acids accumulate on the aging RBC membrane and might be targets for oxidation induced by reactive oxygen species.

RBCs are removed from the circulation by splenic macrophages, probably by several mechanisms. SHPS-1, a surface glycoprotein and a member of the immunoglobulin superfamily that interacts with RBC membrane CD47, is abundant in macrophages. Studies using mice expressing a mutant SHPS-1 suggested that this molecule might negatively regulate phagocytosis, influencing cell life span. Increasing phosphatidylserine exposure and reduced aminophospholipid translocase activity during aging might induce oxidative damage to the cell. It is probable that these mechanisms leading to cell destruction are not mutually exclusive, that no single effect predominates, and that these events occur at different times at different sites of RBC damage.

Regardless of the mechanism(s) fostering eventual senescence and destruction of RBCs, the process itself involves components clinically useful for the assessment of anemias associated with accelerated destruction. Chief among these is the generation of indirect or unconjugated bilirubin, the byproduct of heme catabolism occurring within the reticuloendothelial cells. In markedly accelerated states of RBC destruction, hypertrophy of the liver and spleen can also occur, providing a useful physical indicator of hemolytic anemia. These indirect clinical features, coupled with the reticulocyte count remain more useful for detecting clinical hemolysis than complicated studies of RBC kinetics.

HEMOGLOBIN SYNTHESIS, STRUCTURE, AND FUNCTION

Basic Features

Hbs are the major oxygen-carrying pigments of the body. They are packaged into RBCs in quantities sufficient to carry enough oxygen from the lungs to the tissues to meet the needs of those cells for oxidative metabolism. These quantities are enormous—almost 2 pounds of Hb are present in the body of a reasonably sized human at any given time. Because free Hb in the bloodstream is catabolized and excreted renally in a matter of minutes, packaging in erythrocytes is essential to preserve the newly synthesized molecules for the entire 4-month life span of the RBC. Otherwise, the caloric and biosynthetic resources needed to replace the daily losses of Hb would be prohibitive. The RBC's major function is to encase Hb and protect it so it can function as an oxygen transporter for a prolonged period. An additional function of Hb is to modulate vascular tone by its transport of nitric oxide (NO) and possibly nitrous oxide.

The cellular content of blood influences its viscosity; in particular, the hemodynamics are adversely compromised by the presence of too many circulating erythrocytes because blood viscosity correlates especially with hematocrit. To provide for adequate oxygen transport (i.e., enough Hb molecules) in a number of RBCs compatible with tolerable viscosity, each cell must enclose a high concentration of Hb (32 to 35 g/100 mL of cytoplasm). This concentration is close to the solubility limit of Hb in physiologic solutions. It follows that even minor perturbations within these molecules (e.g., oxidation) or in the milieu (e.g., changes in pH or ionic strength) can have potentially devastating effects on the solubility of Hb. Because polymerized or precipitated Hbs derange intracellular viscosity, trigger proteolytic reactions that lead to oxidative damage of erythrocytes, and compromise oxygen transport, it is not surprising that the fate of the RBC is inextricably interwoven with the state of its enormous complement of Hb molecules.

Hemoglobin Structure

The Hb tetramer consists of two pairs of unlike globin polypeptide chains, each associated with a heme group. Normal Hb has two α-globin and two non-α-globin chains; the interaction of these chains is responsible for the quaternary structure of the Hb molecule and normal oxygen transport. Functionally, the second exon of each globin gene encodes the major component of the heme-binding pocket, and the α and non-α contacts are regulated by the third exon.

The behavior of Hb is determined by its primary structure, the covalent linking of amino acids to form the polypeptide globin. The higher-order structures of Hb depend on the sequence of amino acid residues that make up the globin chain. The α-globin chains contain 141 residues, and the β-globin–like chains are 146 amino acids long (Fig. 34.1). There is considerable homology among these globins, especially among the non-α-globin chains. Whereas the α-globin genes *(HBA2, HBA1)* result from a very ancient gene duplication, the non-α-globin genes *(HBE, HBG2, HBG1, HBD, HBB)* are the result of more recent gene duplications and are more akin to each other than they are to the α-like globin genes. Gene conversion events also ensure the similarity of duplicated genes.

Elements of the secondary structure of globin are shown in Figs. 34.1 and 34.2. Approximately 75% of the globin polypeptide chain forms an α-helix. There are eight helical segments, A through H, separated by short stretches from which the α-helix is absent. These nonhelical segments permit folding of the polypeptide on itself and are often dictated by the presence of prolyl residues, which are generally unable to participate in the formation of α-helices. Although the helical segments of the α-globin and non-α-globin chains do not exactly correspond, it is possible to align amino acid residues in all globin peptides by their helical and nonhelical residue numbers, as indicated in Fig. 34.3. This permits greater appreciation of the homology among globins. Some of the amino acids of globin are invariant, or conserved, in the sense that they are preserved during phylogeny. These residues occur at portions of the molecule that are critical for its stability and function, such as heme-binding residues, hydrophobic amino acids of the interior of the molecule, and certain subunit contacts at the α_1–β_2 interface. The introduction of prolyl residues into α-helical segments by mutation leads to interruption of the α-helix and instability of the resulting Hb molecule.

The poorly understood laws that govern the folding of proteins are responsible for the tertiary structure of globin, shown in Fig. 34.3. This folding pattern places polar residues exteriorly and provides a hydrophobic niche for the heme ring between the E and F helices. Numerous noncovalent bonds are formed between the heme and surrounding amino acid residues of globin. An iron atom in the center of the porphyrin ring of heme forms an important bond with the F8 or proximal histidine and through the linked oxygen with the E7 or distal histidine residue. Oxygenation and deoxygenation of Hb occur at the heme iron. Folding of globin and association of chains into dimers and tetramers was once thought to occur spontaneously. However, it is now clear that these processes are assisted by chaperone proteins, which are described in Chapter 7.

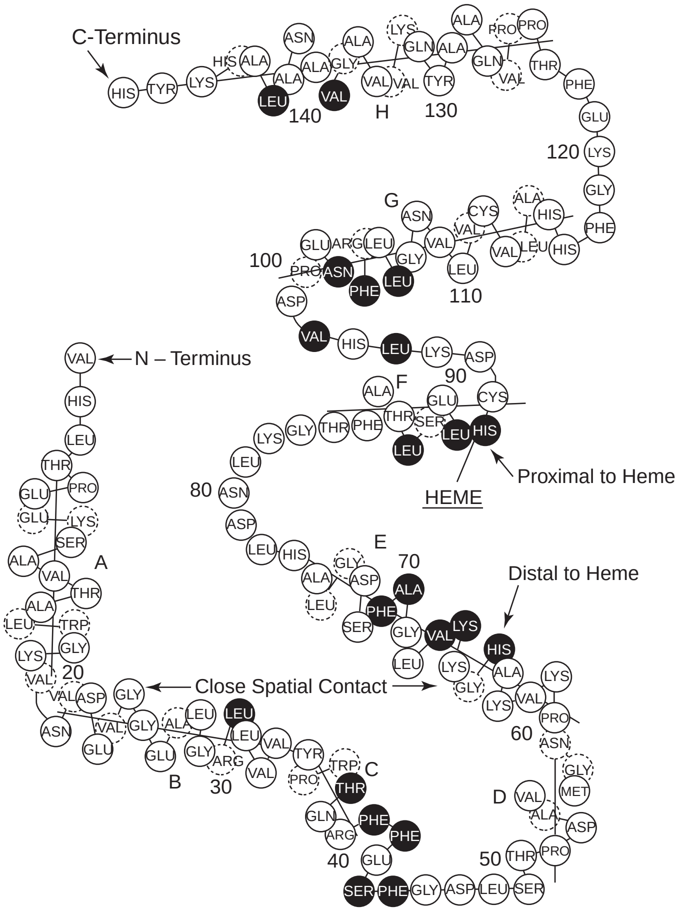


Figure 34.1 THE B-GLOBIN CHAIN SHOWING HELICAL AND NONHELICAL SEGMENTS. The helical segments are labeled A through H, and the nonhelical segments are designated NA for residues between the N terminus and the A helix, CD for residues between the C and D helices, and so forth. *(Reproduced with permission from Huisman THJ, Schroeder WA. New Aspects of the Structure, Function, and Synthesis of Hemoglobin. Boca Raton, FL: CRC Press; 1971.)*

Two α-globin chains and two non-α-globin chains fit together specifically to form a Hb tetramer with a molecular mass of approximately 64,000 Da and with the quaternary structure shown in Fig. 34.4. The motion of individual globin chains, as well as the movement of globin chains relative to each other during oxygenation and deoxygenation, gives Hb its unique usefulness as a respiratory protein.

Hemoglobin Function

Evolution has honed the Hb tetramer in different species into a molecular variant ideally suited for its tasks. Human Hb, adapted for life largely near sea level, must behave differently than that of high-altitude dwelling species or species inhabiting hypoxic locales. Many different variants of the same basic molecular design have thus evolved. Because of the exigencies of molecular evolution, we find in the genome of all animals, including humans, attempts by nature to propagate a variety of different globin genes. The crystallographic studies of Perutz et al. defined the oxygenated and deoxygenated structures of Hb at Ångström-unit resolution and provided an exquisitely detailed picture of how the globin chains and individual amino acid residues respond to the loading and unloading of oxygen. All of these, however, share the properties of highly reversible oxygen binding and high solubility in cytoplasm. We know more about the function of Hb than about virtually any other protein, and the knowledge

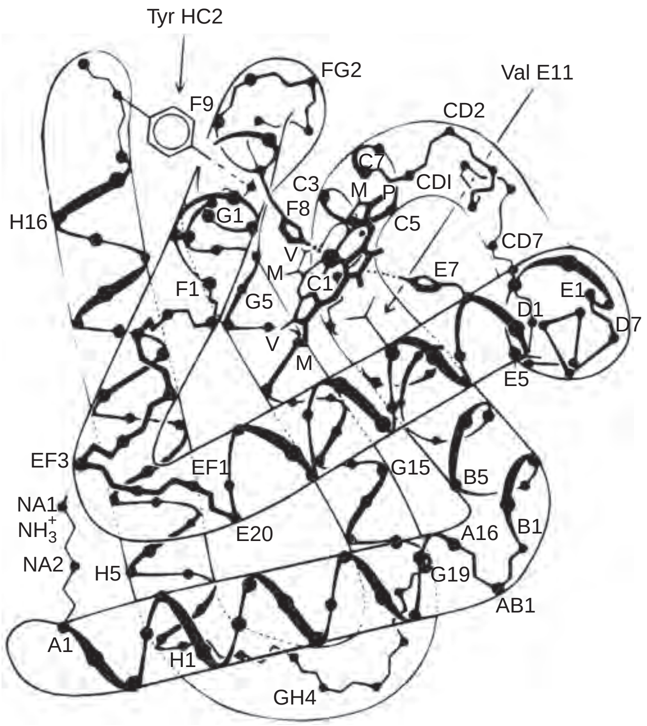


Figure 34.2 TERTIARY STRUCTURE OF A GLOBIN CHAIN. Globin folds into a tertiary structure such that polar or charged amino acids are located on the exterior of the molecule and the heme ring resides in a hydrophobic niche between the E and F helices. Linked to the heme are the proximal (F8) histidine and the distal (E7) histidine. *(Reproduced with permission from Perutz MF. Molecular anatomy, physiology, and pathology of hemoglobin. In: Stamatoyannopoulos G, Neinhuis AW, Leder P, et al., eds. The Molecular Basis of Blood Diseases. Philadelphia, PA: Saunders; 1987:127.)*

of this mechanism provides a beautiful and intellectually satisfying culmination to decades of study by many investigators.

The oxygen dissociation curve of Hb, shown in Fig. 34.3, describes the percent saturation of Hb with oxygen at different oxygen tensions. The sigmoidal shape of this curve is a result of interaction among the subunits of Hb. Communication within the tetramer is called heme–heme interaction or cooperativity. This implies that the four heme groups do not undergo simultaneous oxygenation or deoxygenation but rather that the state of each heme unit with regard to the presence or absence of bound oxygen influences the binding of oxygen to other heme groups. Myoglobin, a heme-containing protein with virtually the same tertiary structure as globin, exists in muscle as a monomer. The oxygen equilibrium curve of myoglobin is a rectangular hyperbola; in physiologic terms, it rapidly becomes fully saturated at low oxygen tensions and remains saturated as the oxygen tension plateaus. The difference in the oxygen equilibrium curves of myoglobin and Hb lies in the tetrameric nature of the Hb molecule and the cooperativity permitted by the association of similar but unlike subunits. Compared with Hb, myoglobin has a very low P_{50} (i.e., oxygen partial pressure at which the molecule is one-half saturated). It therefore has an extremely high oxygen affinity and would not be useful for delivering oxygen to tissues. The oxygen in myoglobin is passed on to the mitochondria, where oxidative metabolism occurs. The sigmoidal shape of the oxygen dissociation curve of Hb indicates that the totally deoxygenated Hb tetramer is slow to become oxygenated, but as oxygenation proceeds, the reaction of heme with oxygen accelerates. Perutz has drawn an analogy in which the “appetite” of heme for oxygen grows with the “eating,” and conversely, loss of oxygen by heme lowers the oxygen affinity of the remaining heme groups. The Hill coefficient, *n*, that can be calculated from plots of oxygen equilibrium curves is a description of heme–heme interaction or cooperativity that explains in part the oxygen-binding properties of Hb and myoglobin. The Hill coefficient for myoglobin is 1, indicating no cooperativity; *n* is approximately 3 for the normal human HbA molecule.

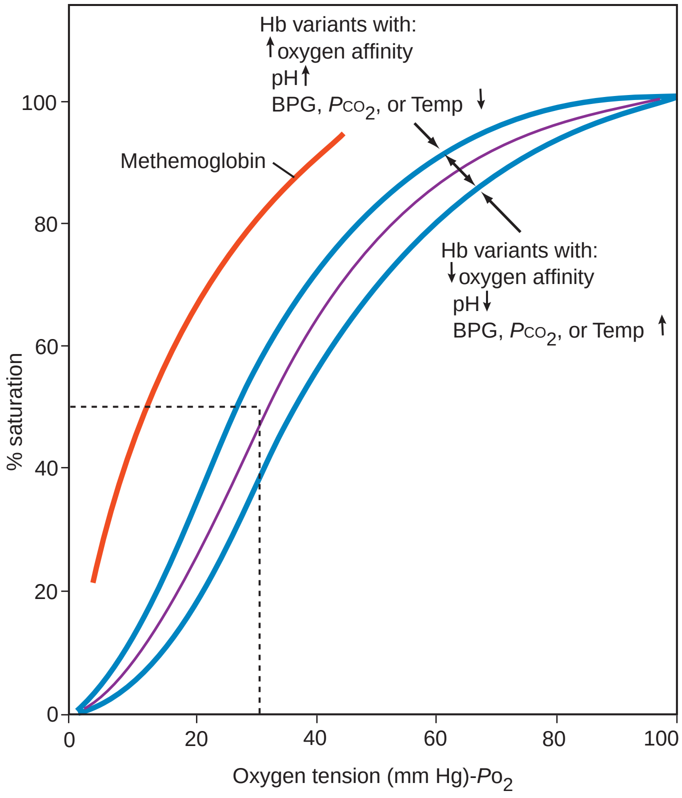


Figure 34.3 OXYGEN DISSOCIATION CURVE OF HEMOGLOBIN. The percent saturation of hemoglobin (Hb) with oxygen at different oxygen tensions is depicted by the *red sigmoidal curve*. The P_{50} (i.e., oxygen tension at which the Hb molecule is one-half saturated) is approximately 27 mmHg in normal erythrocytes *(dotted lines)*. Heterotopic modifiers of Hb function can shift the curve leftward by increasing or rightward by decreasing its oxygen affinity. *BPG*, Bisphosphoglycerate $P\text{CO}_2$, partial pressure of carbon dioxide; $P\text{O}_2$, partial pressure of oxygen. *(Reproduced with permission from Benz EJ, Jr. Synthesis, structure, and function of hemoglobin. In: Kelly WN, DeVita VT, eds. Textbook of Internal Medicine. vol 1. Philadelphia, PA: JB Lippincott; 1989:236.)*

The oxygen affinity of Hb within the erythrocyte does not depend solely on the intrinsic properties of the tetramer. The position of the Hb oxygen dissociation curve, and therefore the P_{50}, can be influenced by a number of heterotropic modifiers, including temperature, pH, and small organic phosphate molecules in the cell. The effects of these modifiers on P_{50} are shown in Fig. 34.3.

Hb is the prototype of an allosteric protein; its structure and function are influenced by other molecules. The major intracellular modulator of Hb–oxygen affinity in human erythrocytes is 2,3-BPG, an intermediate product of glycolysis that is present within the erythrocyte at concentrations equimolar to Hb. The synthesis of 2,3-BPG is enzymatically regulated, and its levels can change depending on the conditions extant. 2,3-BPG is able to bind stereospecifically within the central cavity of the Hb tetramer. Hb prepared in the absence of 2,3-BPG has a very high oxygen affinity, but as 2,3-BPG is added to a Hb solution, the oxygen affinity progressively decreases. 2,3-BPG is a polyanion that binds strongly to the deoxygenated form of Hb but poorly to its oxygenated or other liganded forms. Specific amino acids are involved in the binding of 2,3-BPG; these β-chain residues include the N-terminal valines, H21 histidine (position 143), and EF6 lysine (position 82). In oxyhemoglobin, the H helices of the β-chains are insufficiently spread to permit firm binding of 2,3-BPG; this, along with other conformational changes, favors the binding of

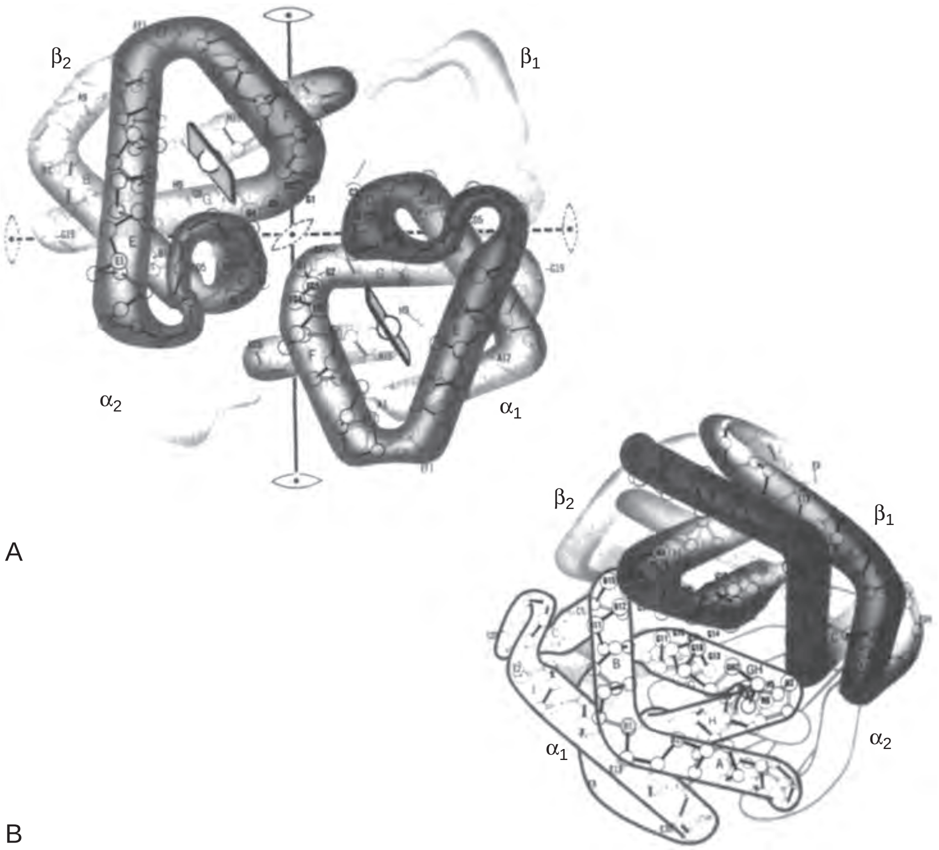


Figure 34.4 QUATERNARY STRUCTURE OF HEMOGLOBIN. The contacts between subunits are shown as circled amino acids. In the front view (A), α_1–β_2 contacts are shown, and in the side view (B), α_1-β–β_1 contacts are depicted. *(Reproduced with permission from Dickerson RE, Geis I. Hemoglobin: Structure, Function, and Evolution Pathology. Menlo Park, CA: Benjamin-Cummings; 1983.)*

this anion to the deoxygenated rather than the oxygenated form of Hb. The binding of 2,3-BPG stabilizes the tense (T) structure of the deoxygenated form at the expense of the relaxed (R) structure of the oxyhemoglobin tetramer.

The transition from the deoxy (T) to the oxy (R) form of Hb is accompanied by rotation of the αβ dimers along the α_1–β_2 contact region (Fig. 34.5). The T structure is stabilized by salt bridges, which are broken as the molecule switches into the R structure. Some abnormal Hbs with an intrinsically high oxygen affinity, or low P_{50}, occur as a result of an amino acid substitution that leads to the loss of bonds that stabilize the tetramer in the T conformation. Hydrogen ions, chloride ions, and carbon dioxide all decrease the affinity of Hb for oxygen by strengthening the salt bridges that lock the molecule into its T conformation. The corollary of the lowering of Hb–oxygen affinity by protons is the combination of Hb with protons on deoxygenation. This is known as the Bohr effect and is responsible for carbon dioxide transport in blood, another critical function of the Hb molecule. Deoxyhemoglobin binds the hydrogen ion liberated by the reaction of carbon dioxide with water, increasing the concentration of bicarbonate. Within the lungs, hydrogen ions are lost as Hb binds oxygen; therefore, carbon dioxide leaves the solution and is excreted from the body through the lungs. Deoxyhemoglobin can also directly bind carbon dioxide; however, this process involves the minority of carbon dioxide exchanged by the RBCs.

RBCs containing high levels of Hb F have high oxygen affinity because it binds to 2,3-BPG poorly. Physiologically, this predicts that the Hb of fetuses should be oxygenated at the expense of the maternal HbA. The high oxygen affinity of HbF is accounted for by a single change in its primary structure, the presence of a serine residue at helical position H21 in place of the histidine found in the β-globin chain. This weakens the binding of 2,3-BPG and leads to the stabilization of the molecule in its R state.

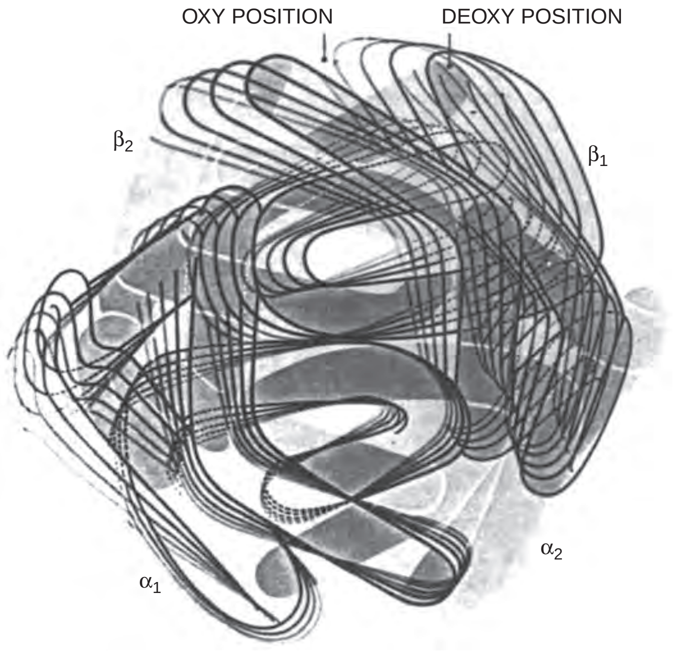


Figure 34.5 SUBUNIT MOTION IN THE HEMOGLOBIN TETRAMER. The relative motion of hemoglobin subunits on oxygenation and deoxygenation is shown. The $\alpha_1\beta_1$ dimer *(black)* is moving relative to the $\alpha_2\beta_2$ dimer *(shaded)*. The oxyhemoglobin tetramer (R state) is more compact than the deoxyhemoglobin configuration (T state). *(Reproduced with permission from Dickerson RE, Geis I. Hemoglobin: Structure, Function, and Evolution Pathology. Menlo Park, CA: Benjamin-Cummings; 1983.)*

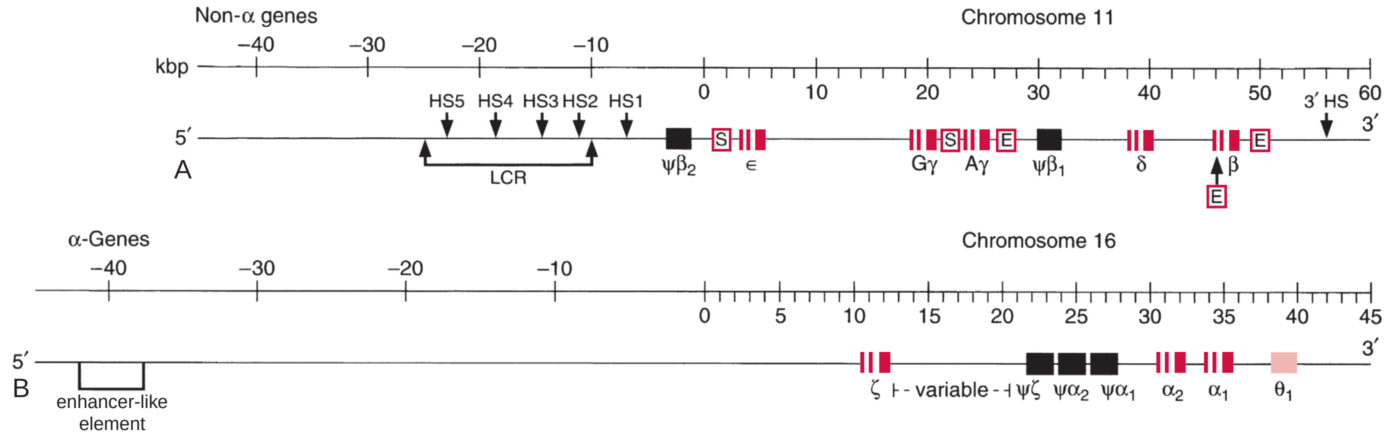


Figure 34.6 MAPS OF THE B-LIKE AND A-LIKE GLOBIN GENE CLUSTERS LOCATED ON CHROMOSOME 11 (A) AND CHROMOSOME 16 (B). Within each gene cluster are pseudogenes, which are remnants of previously expressed globin genes that have become inactivated as a result of mutation. Active genes are shown in *red boxes* filled with clear introns; inactive or pseudogenes genes are shown in *black boxes*, and the θ-globin gene is shown as a *pink box*. Although this gene is transcribed, it is not clear whether it is represented in a cellular protein. The distance between the functional ζ-globin and pseudo-ζ-globin gene is variable because of the presence of repeated elements. *E*, Enhancer; *HS*, DNase hypersensitive site; *S*, silencer.

Interactions of Hb with NO have been a recent focus of investigation. NO, generated from L-arginine by NO synthases, activates soluble guanylate cyclase to produce the second messenger cyclic guanosine monophosphate. As a potent vasodilator, NO is an important regulator of vascular tone. The reaction of free NO with erythrocytes is diffusion limited. Normally, the primary NO–Hb adduct is nitrosyl (heme) Hb (HbFe[II]NO). Within the erythrocyte, β93 cysteine is reduced and seems incapable of NO storage and delivery by *S*-nitrosohemoglobin, as originally proposed. NO was thought to form *S*-nitrosylhemoglobin in the lungs, where Hb is in its R or oxygenated state, and liberate NO in the microcirculation, where the transition of the R to T conformation induced by deoxygenation released NO from Hb. However, studies suggest that NO binding to heme groups is physiologically a rapidly reversible process. This view supports a model of Hb delivery of NO distinct from its dissociation from the β93 cysteine residues. Small nitrosothiol molecules could also be involved in NO transfer. The thiol groups of Hb can exchange NO with small nitrosothiols derived from free cysteine and glutathione. Accordingly, the thiol groups of Hb could bind and transfer NO or exchange NO with small shuttle molecules, increasing the perfusion of hypoxic tissues. It has been suggested that cytoskeletal and other erythrocyte proteins slow NO influx into the cell and, coupled with NO heme binding, preserve NO bioactivity. NO–Hb interactions, whether through *S*-nitrosohemoglobin formation at the β93 cysteine or the formation of nitroso intermediates, are likely to be physiologically important. Hb liberated from intravascularly hemolyzed RBCs rapidly inactivates NO. As the RBC lyses, arginase is also released and destroys the substrate for NO synthases, L-arginine. Together, this leads to a reduction in biologically active NO. With hemolysis, as in sickle cell disease or thalassemia, reduced NO bioavailability is associated with disease complications such as pulmonary hypertension, leg ulcers, priapism, and perhaps increased risk of stroke. Lactic dehydrogenase also released from the RBC in hemolytic anemia is an excellent marker of these complications.

In summary, the primary amino acid structure of α-globin and non-α-globin chains dictates the inevitable quaternary structure, within which resides the ability of Hb to serve as a respiratory protein. Cooperativity ensures rapid binding of oxygen in the lungs and unloading in tissues. Similarly, carbon dioxide is transported from tissues to lungs. The function of Hb may be influenced by mutation and by heterotropic effectors such as protons and 2,3-BPG. The molecule itself changes shape as it provides oxygen for metabolism; it is a lung in miniature, breathing as it allows the body to respire.

Globin Gene Clusters

The amounts and types of human Hb produced at any given age are determined by the selective expression of the individual genes encoding each globin chain. The globin genes of humans are located in two clusters (Fig. 34.6): α-like genes in approximately 30 kb of DNA on the short arm of chromosome 16 between band p13.2 and the telomere and β-like genes in approximately 70 kb of DNA on the terminal portion of the short arm of chromosome 11 (p15). Each gene shares certain basic organizational features. Each contains three exons separated by two introns. Both introns of the α-gene are small (100 bp to 300 bp); non-α-genes have one small and one large (1000 bp to 1200 bp) intron. The second exon of each globin gene encodes the major components of the heme-binding pocket, and the third encodes the α and non-α contact points.

Flanking each gene at the 5′ and 3′ ends are groups of conserved nucleotides. In conjunction with protein factors, these influence the promotion of gene transcription, ensure the fidelity of the transcript and its translatability, specify sites for the initiation and termination of translation, and improve the stability of the newly synthesized mRNA (Fig. 34.7). Also encoded within the genes are signals that permit the enzymatic machinery within the nucleus to excise precisely the introns from the mRNA precursor and splice together the exons to form a contiguous "mature" mRNA. The spliced mRNA is transported to the cytoplasm and translated into protein. These conserved signals lie at the junction of the exon and intron and within the introns themselves. They are recognized by small nuclear ribonucleoprotein particles, which participate in the formation of a spliceosome, or splicing complex. Their preservation is critical for the splicing process to occur. When mutations occur within splice signal sites, globin synthesis is often impaired. The 5′ end of the mRNA contains a cap structure, and the 3′ end contains a poly(A) tail, as described in Chapter 1. Chapter 4 provides a detailed explanation of the processes of pre-mRNA splicing, capping, polyadenylation, exporting to the cytoplasm and stabilization, and translation.

Conserved nucleotide clusters located 5′ to the coding portion of each globin gene in the aggregate act as promoters (see Fig. 34.7). Globin promoters are modular. Some modules are located relatively close to the initiation site of mRNA translation, and some are more distally placed. Promoters ultimately form the binding sites for the RNA polymerase complexes that catalyze gene transcription. Mutations within the promoter can affect the level of gene transcription and the amount of globin made. Surrounding and within each gene are other sequence elements that play important roles in its transcriptional regulation (see Fig. 34.6). These clusters, called *enhancers*

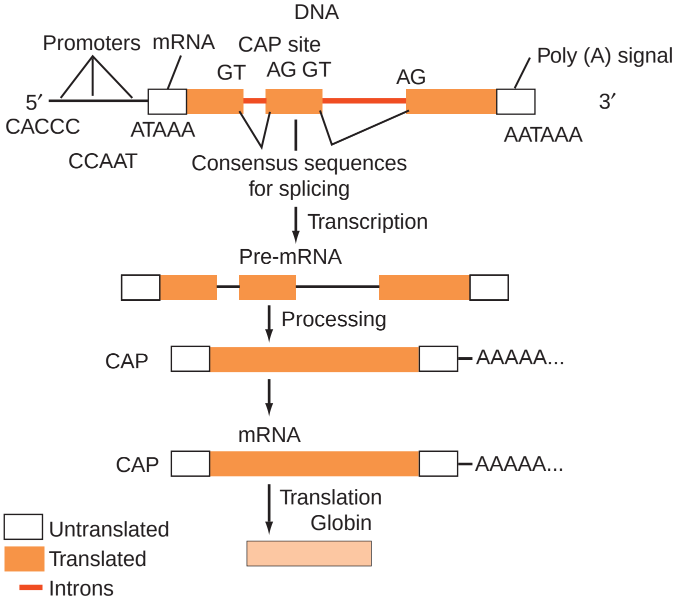


Figure 34.7 PATHWAY OF GLOBIN BIOSYNTHESIS. Transcription of the globin gene results in a large pre-mRNA molecule containing intervening sequences. During intranuclear processing of this molecule, the intervening sequences are excised and the coding sequences ligated to form a contiguous stretch of RNA, which codes for the globin protein. The message is further processed by the addition of a CAP and a poly(A) tail. The mature message is transported from the nucleus to cytoplasm, where it is translated on polyribosomes by the addition of activated amino acids to a growing polypeptide chain. Globin acquires heme and α: non-α dimers are formed and a hemoglobin tetramer is assembled. *(Reproduced with permission from Steinberg, MH. Hemoglobinopathies and thalassemias. In: Stein JH, ed. Internal Medicine. 4th ed. St. Louis: Mosby-Year Book; 1994:852.)*

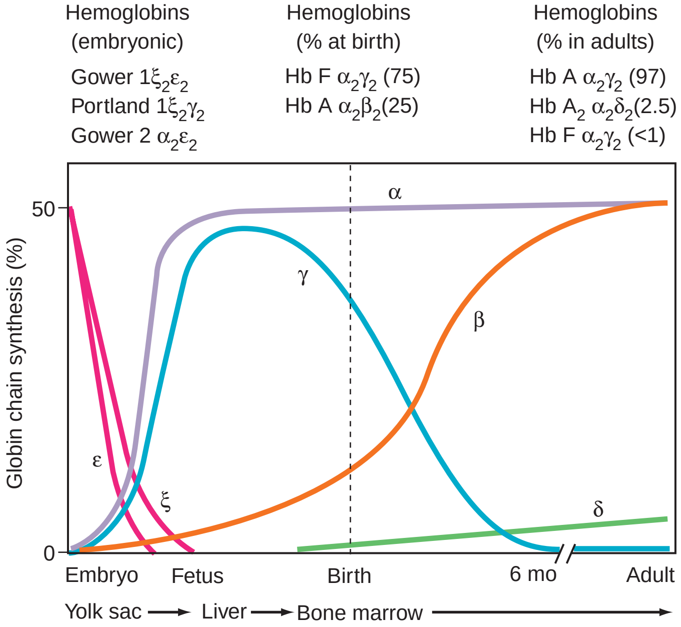


Figure 34.8 HEMOGLOBIN (HB) SWITCHING DURING EMBRYONIC, FETAL, AND ADULT DEVELOPMENT. The ζ and ε genes are transcribed during embryonic development and are soon replaced by the fetal γ-globin and adult α-globin gene. At birth, fetal Hb (HbF) forms approximately 75%, and HbA forms 25% of the total. Transcription of the γ gene begins to decrease before birth, and by 6 months of age, this gene is expressed only at very low levels. Expression of the δ-globin gene begins near birth. In adults, HbA makes up approximately 97%, HbA_2 approximately 2.5%, and HbF less than 1% of the total. *(Reproduced with permission from Steinberg MH. Hemoglobinopathies and thalassemias. In: Stein JH, ed. Internal Medicine. 4th ed. St. Louis: Mosby-Year Book; 1994:852.)*

and *silencers* (see Chapter 1), may lie within introns or 5′ and 3′ to the coding sequences; in some instances, they are quite remote from the gene. The higher-order structure of DNA in chromatin may permit close approximation of these remote enhancers to the gene during transcription by forming loops that bring otherwise distant sequences together spatially. Enhancers play important roles in the tissue-specific regulation of globin gene expression. Representative regulatory sequences near the globin genes are shown in Fig. 34.6 (enhancer-like element). DNA elements controlling globin genes are described in more detail later.

The α-like and β-like globin genes are ordered in the 5′ to 3′ direction in the same sequence expressed during embryonic, fetal, and adult development (Fig. 34.8). The functional significance of this arrangement is unclear. However, evidence suggests that the ordering of the ε, γ, δ, and β genes could be an important factor influencing the ability of each locus to interact with distant control elements at different developmental stages.

The α-like and β-like gene clusters probably are the result of an ancient duplication of a primordial globin gene that existed early in the history of vertebrates, approximately 500 million years ago. Each gene cluster probably developed from the duplication of ancestral genes and subsequent divergence through eons of evolution. Within the α-like gene cluster, the ζ-globin gene (*HBZ*) is expressed only very early in embryogenesis and participates in the formation of embryonic Hbs. A μ, α-like globin gene *(HBM)*, originally considered a pseudogene (*ψα2*), codes for a 141 amino acid α-globin–like chain, is expressed in erythroid cells in a highly regulated fashion; however, an associated protein has not been found.

The α-globin genes (*HBA2, HBA1*) are duplicated, and their encoded amino acid sequences are identical; therefore, only a single α-globin polypeptide results. Minor differences within the second intervening sequence and the 3′ flanking regions of the α-globin gene permit identification of transcripts from each gene. The 5′ or α_2-gene is expressed more efficiently than the 3′ or α_1-gene, so abnormalities of this gene are more likely to be clinically apparent. Both clusters contain genes that are actively transcribed, as well as pseudogenes whose defective structures prohibit expression at any time.

The gene 3′ to the α_1-gene is the Θ-gene (*HBQ1*), a somewhat mysterious element of the α-gene cluster. Although Θ-gene transcripts are found in fetal tissue and adult erythroid marrow, it is unclear whether this gene's translation product is able to participate in the formation of a functional tetramer. The Θ-globin protein has been found in vivo, but deletion of the Θ-globin gene does not appear to have any implications for developing fetuses. In vitro, Θ-globin mRNA is correctly spliced, and Θ-globin cDNA can direct the synthesis of a translatable mRNA and a Θ-globin protein.

The β-like–globin gene cluster consists of the embryonic ε-gene (*HBE*), transcribed only during the first 6 to 11 weeks of life; the duplicated γ-globin genes (*HBG2, HBG1*) that code for the dominant non-α-globin of fetal life; and the δ- (*HBD*) and β-globin (*HBB*) genes that code for the Hbs of adults. The coding sequences of the two γ-globin genes are identical, except at codon 136, where the 5′ or Gγ-gene codes for glutamic acid; the 3′ or Aγ-gene encodes an alanine residue. These genes are unequally expressed during fetal development. A switch in their relative rates of expression leads to a similar disparity between the amounts of Gγ and Aγ chains in adults. Although the Gγ/Aγ switch is interesting from the standpoint of the control of gene expression, it is of little clinical importance. HbF in fetuses and adults contains a mixture of Gγ and Aγ chains; the functional qualities of these Hbs are identical.

The δ- and β-globin genes are probably the result of a duplication event that occurred more than 40 million years ago. The β-globin gene has become the predominant gene, coding for most non-α-globin chains of adults. The δ-globin gene promoter has undergone mutation in several critical areas, and its expression is greatly curtailed. Its product, a minor fraction of adult Hb (HbA_2), has become functionally insignificant by virtue of its very low level in the erythrocyte. It is likely that the δ-globin gene is a "pseudogene in evolution." HbA_2 is clinically useful, however, for characterizing hemoglobinopathies such as β-thalassemia.

The pseudogenes dispersed within both globin gene clusters provide interesting glimpses into the evolutionary history of globin genes. Pseudogenes are inactive remnants of previously expressed genes. As a result of relaxed selection, their mutation rates are higher than those of surrounding active genes. Because of this, the expression of the δ-globin gene might be totally abolished as it acquires an inactivating mutation.

The expression of the human globin genes is highly regulated. Until recently, globin was thought to be synthesized in only one tissue—erythroid cells—and only during a narrowly defined stage of erythroid progenitor cell differentiation—the 5 to 7 days that commence with the proerythroblast stage and end when the enucleated reticulocyte loses the last traces of its RNA. Recently, however, evidence has been found for the expression of small amounts of α-globin in the myoendothelial junctions of the endothelium. Its presence there may be involved in NO regulation of vascular tone. Within the confines of these tissue-specific and differentiation stage-specific boundaries, the globin genes are extraordinarily active in erythroblasts. By the late normoblast and reticulocyte stages, 90% to 95% of all protein synthesis in these cells is globin synthesis.

Individual globin genes are expressed at different levels in developing erythroblasts of human embryos, fetuses, and "adults" (i.e., 37 to 38 weeks of gestation and beyond). Different subsets of α-genes and non-α-genes are expressed and silenced at each developmental stage. Moreover, the overall balance of non-α-globin, α-globin, and heme production is maintained throughout each of these complex switching events. The complex mechanisms ensuring the proper tissue-specific, differentiation stage-specific, and ontologic stage-specific expression are incompletely defined. However, much information about relevant DNA control elements and transcription factors is emerging. These topics are discussed after a review of the ontogeny of Hb.

Ontogeny of Hemoglobin

The Hb composition of the erythrocyte depends on when in gestation or postnatal development it is measured. This is a result of sequential activation and inactivation (i.e., switching) among genes within the α-globin and non-α-globin gene clusters (see Fig. 34.8). What controls these switches in globin gene transcription is not understood. The two early embryonic Hbs consist of ζ- and ε-globin chains (Hb Gower-1) and α- and ε-globin chains (Hb Gower-2). The ζ-globin gene is akin to the α-globin genes but is expressed only during early embryogenesis. The ε-embryonic globin chain is a β-like element. The combination of ζ- and γ-globin chains forms Hb Portland. These early Hbs are made primarily in yolk-sac erythroblasts and are detectable only during the very earliest stages of embryogenesis except in certain pathologic states, in which they may persist until gestation is complete. The major Hb of intrauterine life is HbF, which consists of two α- and two γ-globin chains. Expression of the γ-globin gene begins early in embryogenesis, peaks during mid-gestation, and begins a rapid decline just before birth. By 6 months of age in normal infants, only a remnant of prior γ-globin gene expression remains. The level of HbF in the blood declines rapidly thereafter to less than 1% of the total. Expression of the α-globin gene starts early in the first trimester, peaks quickly, and is sustained for life. Expression of the β-globin gene also commences early in gestation and reaches its zenith within a few months after birth. The combination of α-globin with β-globin chains forms HbA, the predominant Hb of postnatal life. Adult cells also contain HbA_2. The δ-globin gene, which directs the synthesis of the non-α-globin chain of HbA_2, is very inefficiently expressed. Only low levels of HbA_2 are present; defects in the δ-globin gene are of no clinical consequence. In adult blood, HbF is not evenly distributed among erythrocytes and is present in only a very small number of RBCs, called F cells. HbA_2 is present in all RBCs, albeit at levels less than 3.5% of the total Hb in adult life.

Hemoglobin Biosynthesis and Its Regulation

Throughout development, genes coding for α-globin, non-α-globin, and heme exhibit coordinated expression. Almost equal amounts of each of the moieties that ultimately constitute the Hb tetramer are made. Excess unpaired globin chains and mutant globins are removed from the cell by ATP-dependent proteases, ensuring a balance between accumulation of α-globin and non-α-globin chains. Balanced chain synthesis and coordination of globin chain production with the synthesis of heme are important because Hb tetramers are highly soluble, but the components of Hb (i.e., unpaired chains, protoporphyrin, and iron) are not. Precipitation of any of these is deleterious to cell survival. Erythroblast proteases are not efficient enough to eliminate the substantial excesses of unpaired chains that accumulate when an α-gene or non-α-gene is selectively impaired by severe thalassemia mutations. The mechanisms regulating heme production and some of the interactions between heme and globin synthesis are discussed in Chapter 39.

The proper production of the individual globin chains within erythroid tissues at the appropriate states of differentiation and development is predominantly ensured by regulation at the level of transcription. The onset of phenotypic maturation at the proerythroblast stage is marked by the onset of globin mRNA biosynthesis in dramatically increasing quantities. Expression of α-globin and non-α-globin genes begins at essentially the same time, although some studies suggest a slightly earlier onset for α-globin gene expression. Transcription persists at a high level throughout most of the remainder of erythropoiesis, declines as the nucleus condenses, and is eventually lost in late erythroblasts. Even as the absolute rates of globin gene transcription begin to decrease, however, the relative percentage of total transcriptional activity devoted to globin gene expression continues to increase; this reflects the silencing of transcription of almost every other gene in the erythroblast.

The transcriptional activation of the globin genes is the major event that must be understood to define and manipulate therapeutically the regulation of Hb biosynthesis and Hb switching. However, posttranscriptional mechanisms contribute to the final distribution of globin and nonglobin mRNAs and to the balance of α-globins and non-α-globins within the erythroblasts. When compared with many other mRNAs, such as cytokine mRNAs, globin mRNAs are extraordinarily stable. Their half-lives have been estimated at 30 to 50 hours. Most other mRNAs have turnover rates, or half-lives, measured within the range of a few minutes to 5 or 6 hours. The increase in the percentage of total mRNA that is globin mRNA is greatly accentuated because the newly transcribed globin mRNAs accumulate and remain quite stable in the cell, while non-globin mRNAs, which are no longer being produced, are also disappearing at a faster rate. Consequently, the mRNA content of the reticulocytes consists of 90% to 95% globin mRNA.

The transcription rates of the α-globin and non-α-globin genes are not precisely equal. (This phenomenon has been studied in detail only in adult erythroid cells expressing the α- and β-globin genes.) A slight, but reproducibly detectable, excess of α-globin mRNA is present in erythroblasts. However, β-globin mRNA is translated somewhat more efficiently than α-globin mRNA. These counterbalancing forces result in almost equal syntheses of α- and β-globin polypeptide chains. There is a very slight excess of α-globin production, resulting in a small pool of free α-globin chains.

Alpha Hb-stabilizing protein (AHSP), a small protein present at high concentrations in RBCs, binds specifically to the α-globin polypeptide, protecting the unstable free α-globin chain by inhibiting heme loss and oxidant-mediated chain precipitation. It remains unclear whether mutations of this protein can modify the phenotype of β-thalassemia by increasing the imbalance in globin chain synthesis. Some α-globin chain variants, because the mutations alter AHSP binding, are associated with mild thalassemia-like features.

Newly synthesized β-globin chains are rapidly and completely incorporated into αβ dimers that spontaneously associate as tetramers. Hb tetramers are remarkably stable throughout the life span of the circulating RBC by virtue of their long half-lives. Only small amounts sustain oxidative or proteolytic damage.

Hb molecules are exposed for prolonged periods to chemically active compounds in the milieu of the bloodstream. They often become nonenzymatically modified by such processes as glycosylation, acetylation, and sulfation. Glycosylation occurs more extensively during periods of hyperglycemia and leads to elevated levels of the glycosylated form of HbA, HbA_{1c}. This phenomenon is the basis of a useful test for control of the blood sugar in diabetes. Other posttranslational modifications are of little clinical importance except as already noted for 2,3-BPG, carbon dioxide, and NO.

Transcriptional Regulation of Globin Gene Expression

Precise regulation of the globin gene clusters involves a complex interplay between *trans*-acting proteins, such as transcription factors, and *cis*-acting sequences that act as promoters, enhancers, and silencers of gene activity. DNA-binding proteins interact with sequences in regulatory regions of the globin gene cluster and with other proteins through specific protein–protein interactions, forming DNA–protein complexes that regulate gene transcription. *Trans*-acting factors mediate the remodeling of chromatin structure, influencing gene expression for the entire globin gene clusters. Mutations in the *cis*-acting sequences or *trans*-acting proteins cause dysregulated expression of globin genes, resulting in thalassemia-like syndromes, although most thalassemia-causing mutations are in the cognate globin gene clusters. Elucidating the full extent of sequences required for appropriate expression of globin genes will inform the development of constructs for gene therapy.

The nuclei of erythroid cells contain numerous proteins that have been identified as transcription factors, including GATA1, NFE2, LRF, and erythroid Kruppel-like factor (EKLF). GATA1 is named on the basis of the DNA sequence motif (T/A) GATA (A/G), the GATA motif that it recognizes and binds. It is a zinc finger class DNA-binding protein (see Chapters 22 and 26). The activity of GATA1 requires binding to a zinc finger protein cofactor called FOG1 (named for Friend of GATA1). NFE2 recognizes the DNA sequence motif (T/C) GCT GA (C/G) TCA (T/C). It is a member of the B-zip class of transcriptional activators. GATA1 and NFE2 were originally identified and cloned on the basis of their interactions with their cognate sequences in the globin genes. EKLF, also called KLF1, may be the most specific of the erythroid transcription factors yet discovered. EKLF interacts specifically with the β-globin gene promoter and may influence the γ–β switch. Mice homozygous for disruption of the *EKLf* gene have lethal β-thalassemia. Alone, GATA1, NFE2, EKLF, and FOG1 cannot be the sole determination of tissue specificity of the globin genes. Together, they form a robust transcriptional network that regulates erythroid genes, including the globin genes. Mutations in GATA1 or FOG1 can cause β-thalassemia and thrombocytopenia in patients.

The regions of the globin gene clusters with essential regulatory sequences and erythroid-specific chromatin remodeling extend far beyond the coding sequences of the globin genes. The key regulatory elements for the α-globin gene (HS–48, 40, and 33) lies in erythroid-specific DNase I hypersensitive sites about 40 kb upstream from the α-globin gene. The locus control region (LCR) is critical for high-level expression of the β-globin gene cluster consisting of five sites that are hypersensitive to DNase I (HS 1–5) (see Chapters 1 and 4). Patients with deletion of the HS–40 sites exhibit α-thalassemia, and patients with deletions of the β-globin LCR develop β-thalassemia; however, the thalassemia can be a result of changes in chromatin caused by the large deletion. Similarly, transgenic mice bearing deletions of these critical regulatory regions have severely restricted expressions of the respective globin genes.

The LCRs contain binding sites for the major erythroid transcription factors, including GATA1, EKLF, and NFE2, as well as sites for transcription factors found more widely distributed in many cell types. The LCRs loop to interact directly with the promoters of individual globin genes, resulting in a complex termed the *active chromatin hub*. The resulting structure enables high-level expression of globin genes in erythroid cells at the appropriate developmental stages. Additional elements act as insulators, protecting expressed genes from gene silencing through the regulation of chromatin structure.

BCL11A is a transcriptional repressor that decreases HbF expression in adult tissues. Polymorphisms in the *BCL11A* gene are powerfully associated with HbF levels, including in patients with sickle cell disease. The inhibition of *Bcl11a* increases HbF levels and attenuates the phenotype of sickle cell disease in a murine model. The erythroid-specific enhancers of BCL11A have been identified and are an attractive target for genome editing as a therapeutic strategy for the treatment of sickle cell disease and β-thalassemia. The transcription factor LRF or ZBTB7A is also a powerful silencer of HbF gene expression. When this gene and *BCL11A* are knocked out in human erythroid cells, the HbF concentration is more than 90% of the total Hb.

Transcription factors recruit enzymes that remodel chromatin structure. "Open" chromatin, or euchromatin, generally appears cytogenetically uncondensed and is associated with hyperacetylated histones, unmethylated CpG dinucleotides, and active transcription. ATRX is a protein that has been implicated in the modulation of chromatin structure at the α-globin locus. Mutations in the *ATRX* gene, located on the X chromosome, cause a syndrome of α-thalassemia, severe mental retardation, facial dysmorphism, and urogenital abnormalities. ATRX, a member of the SNF2 family of helicase/ATPases, localizes to pericentromeric heterochromatin during interphase and mitosis and contains a plant homeodomain-like motif that is found in chromatin-associated proteins. Cells with a mutated *ATRX* gene have altered patterns of DNA methylation. The ATRX protein therefore exemplifies the connections among DNA methylation, chromatin remodeling, and expression of the α-globin genes.

Chromatin structure can be manipulated pharmacologically through the influence of drugs on methylation and histone acetylation. Cytidine analogs such as 5-azacytidine and its less toxic derivative, decitabine, inactivate DNA methyltransferases, inducing γ-globin gene expression and increasing HbF levels in patients with sickle cell anemia. Histone deacetylase inhibitors are being studied as agents to increase γ-globin expression.

Posttranscriptional, Translational, and Posttranslational Mechanisms

Processed globin mRNA is exported from the nucleus to the cytoplasm by complex mechanisms described in Chapter 4. mRNA translation occurs in the cytoplasm (see Fig. 34.7). The triplet codons or mRNA are recognized by the anticodons of specific tRNAs that bring activated amino acid residues to the nascent polypeptide chains. The process of translation, in which an mRNA template directs the synthesis of protein, is typically divided into three phases: initiation, elongation, and termination (see Chapters 1 and 4). Each phase is regulated by a variety of protein factors.

The globin mRNA molecule becomes associated with four to six ribosomes, forming the polyribosome. At least 11 eukaryotic translation initiation factors interact with the polyribosome. They mediate stabilization of a preinitiation complex, binding of the initiator methionine tRNA to ribosomal subunits, binding of mRNA to the preinitiation complex, stabilization of mRNA binding, recognition of the cap site at the 5′ end of mRNA, and release of initiation factors from the preinitiation complex. Several elongation and termination factors have also been defined. Mutations of some of these have been associated with malignancies. The initiation or an early step in the elongation process is the rate-limiting factor.

The first posttranslational step in tetramer formation is the combination of α-globin and non-α-globin chains to form dimers, an event that appears to depend on the relative charge of each globin subunit. The dimers then form tetrameric Hb. Because of charge differences among non-α-globin chains, there is a hierarchy or affinity of these chains for α-globin chains. The combination of α- and β-globin chains is most favored followed by a combination of α-, γ-, and δ-globin chains. Certain mutant Hbs that have gained or lost a charge may alter this hierarchic arrangement. This may influence the proportion of variant Hb present, especially when the patient also inherits an α-thalassemia syndrome, in which the synthesis of α-globin chains is reduced. The supply of available α-globin chains is then limited, and non-α-globin chains compete with one another to form tetramers with the limiting α-globin chain pool.

Globin chain biosynthesis and heme synthesis are mutually regulated. Heme plays a role in the regulation of the initiation complex. A deficiency of heme (e.g., in iron deficiency) is associated with the accumulation of a repressor of translation initiation factors. Translation of β-globin mRNA appears to be initiated more efficiently than α-globin mRNA, conferring on the associated anemia some of the features of mild α-thalassemia. This phenomenon occurs because heme deficiency depresses the availability of initiating factors for which the less efficient α-mRNA must compete with the more efficient β-mRNA.

The identification of genetic mutations that cause congenital and acquired forms of anemia provide further insight into the pathways required for the coordinated production of globin and heme. More than half of patients with Diamond-Blackfan anemia, a disorder characterized by a severe macrocytic anemia and a paucity of erythroid progenitor cells, have heterozygous germline mutations in the *RPS19* gene or other genes encoding ribosomal proteins. Similarly, the macrocytic anemia in patients with myelodysplastic syndrome and a deletion of chromosome 5q is caused by heterozygous deletion of another ribosomal protein gene, *RPS14*. Haploinsufficiency for these ribosomal protein genes activates the p53 pathway, leading to cell cycle arrest and apoptosis selectively in the erythroid progenitor cells.

Refractory anemia with ring sideroblasts (RARS) is a subtype of myelodysplastic syndrome characterized by iron-loaded mitochondria evident on Prussian blue staining. In the majority of RARS cases, somatic mutations are present in the *SF3B1* gene, encoding a core member of the RNA splicing machinery. The precise targets of SF3B1 have not been identified.

NOSOLOGY OF HEMOGLOBINOPATHIES

Inherited abnormalities of the Hb molecules that cause morbidity are called hemoglobinopathies and thalassemias. Many of these conditions produce diseases (e.g., sickle cell anemia, thalassemia, unstable Hbs, Hbs with altered oxygen affinity, M Hbs) that are especially important to hematologists. A few acquired conditions lead to modifications of Hb (e.g., carbon monoxide exposure, producing carboxyhemoglobinemia, nitrite exposure causing methemoglobinemia) that produce clinical abnormalities. These situations are summarized by the term acquired hemoglobinopathies or dyshemoglobinemias.

Most of the more than 1200 mutations of the globin gene that have been described produce no disease or only trivial clinical effects. The remainder can be classified according to the hematologic and clinical phenotypes that cause reduced solubility with hemolytic anemia (unstable Hbs and polymerizing Hbs, such as sickle Hb); Hbs with altered oxygen affinity; Hbs predisposing to methemoglobin formation; and the thalassemias involving abnormal synthesis of one or more globin chains with anemia, hemolysis, and alterations of erythropoiesis. Some mutations, such as that responsible for HbE, can alter the structure and synthesis of the molecule. A classification of hemoglobinopathies and thalassemias is provided in Table 34.1. Individual conditions are discussed in the chapters already cross-referenced in earlier sections of this chapter.

TABLE 34.1 Classification of Hemoglobinopathies and Thalassemias

Structural hemoglobinopathies—mutations altering the amino acid sequence of a globin chain and altering physical or chemical properties of the hemoglobin tetramer in such a way that function is deranged

Abnormal Hemoglobin Polymerization—Sickle Cell Hemoglobin (HbS); Hemolysis, Vasoocclusion

- Abnormal Hb crystallization (e.g., HbC)
- High oxygen affinity—polycythemia (Hb Zurich)
- Low oxygen affinity—cyanosis (Hb Kansas)
- Hbs that oxidize or precipitate too readily—unstable Hbs (Hb Köln)
- M Hbs—methemoglobinemia, cyanosis (e.g., Hb Milwaukee)

Thalassemia—Defective Production of Globin Chains With Hypochromia, Anemia, Hemolysis, Altered Erythropoiesis

- α-Thalassemia
- β-Thalassemia
- δβ-Thalassemias, γδβ-thalassemias, αβ-thalassemias

"Thalassemic" Hemoglobinopathies and Dominantly Inherited Thalassemias—Mutations Altering the Synthesis and Structure or Function of the Hemoglobin Gene Products (e.g., HbE, Hb Terre Haute, Hb Lepore, Hb Constant Spring)

- Hereditary persistence of fetal hemoglobin (HbF)—persistence of high levels of HbF into adult life
- Pancellular—high HbF levels in all RBCs
- Nondeletion forms
- Deletion forms
- Hb Kenya
- Heterocellular—inherited increases in the percentage of F cells
- Acquired hemoglobinopathies

Methemoglobinemia Caused by Toxic Exposures

- Sulfhemoglobinemia caused by toxic exposures
- Carboxyhemoglobinemia caused by toxic exposures
- HbH in erythroleukemias
- Acquired elevations in F cells and HbF
- Erythroid stress (e.g., recovery from BM suppression)
- BM dysplasias
- Exposure to agents altering stem cells or gene expression (e.g., hydroxyurea, butyric acid)

BM, Bone marrow; *Hb*, hemoglobin; *HbE*, hemoglobin E; *RBC*, red blood cell.

SUGGESTED READINGS

Bank A. Regulation of human fetal hemoglobin: new players, new complexities. *Blood*. 2006;107:435.

Boas FE, Forman L, Beutler E. Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. *Proc Natl Acad Sci USA*. 1998;95:3077.

Burgess-Beusse B, Farrell C, Gaszner M, et al. The insulation of genes from external enhancers and silencing chromatin. *Proc Natl Acad Sci USA*. 2002;99:16433.

Cantor AB, Orkin SH. Transcriptional regulation of erythropoiesis: an affair involving multiple partners. *Oncogene*. 2002;21:3368.

Chakalova L, Carter D, Debrand E, et al. Developmental regulation of the beta-globin gene locus. *Prog Mol Subcell Biol*. 2005;38:183.

Chiu CH, Schneider H, Slightom JL, et al. Dynamics of regulatory evolution in primate β-globin gene clusters: *cis*-mediated acquisition of simian gamma fetal expression patterns. *Gene*. 1997;205:47.

Dzierzak E. The emergence of definitive hematopoietic stem cells in the mammal. *Curr Opin Hematol*. 2005;12:197.

Feng L, Zhou S, Gu L, et al. Structure of oxidized alpha-haemoglobin bound to AHSP reveals a protective mechanism for haem. *Nature*. 2005;435:697.

Gibbons RJ, Picketts DJ, Villard L, et al. Mutations in a putative global transcriptional regulator cause X-linked mental retardation with α-thalassemia (ATR-X syndrome). *Cell*. 1995;80:837.

Gladwin MT, Wang X, Reiter CD, et al. *S*-nitrosohemoglobin is unstable in the reductive erythrocyte environment and lacks O_2/NO-linked allosteric function. *J Biol Chem*. 2002;277:27818.

Gow AJ, Stamler JS. Reactions between nitric oxide and haemoglobin under physiological conditions. *Nature*. 1998;391:169.

Hardison R, Riemer C, Chui DH, et al. Electronic access to sequence alignments, experimental results, and human mutations as an aid to studying globin gene regulation. *Genomics*. 1998;47:429.

Higgs DR, Garrick D, Anguita E, et al. Understanding alpha-globin gene regulation: Aiming to improve the management of thalassemia. *Ann N Y Acad Sci*. 2005;1054:92.

Jenuwein T, Allis CD. Translating the histone code. *Science*. 2001;293:1074.

Kato GJ, McGowan V, Machado RF, et al. Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension, and death in patients with sickle cell disease. *Blood*. 2006;107:2279.

Kihm AJ, Kong Y, Hong W, et al. An abundant erythroid protein that stabilizes free alpha-haemoglobin. *Nature*. 2002;417:758.

Li Q, Peterson KR, Fang X, et al. Locus control regions. *Blood*. 2002;100:3077.

Miller IJ, Bieker JJ. A novel erythroid cell-specific murine transcription factor that binds to the CACCC element and is related to the Kruppel family of nuclear proteins. *Mol Cell Biol*. 1993;13:2776.

Rother RP, Bell L, Hillmen P, et al. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease. *JAMA*. 2005;293:1653.

Stamatoyannopoulos G. Control of globin gene expression during development and erythroid differentiation. *Exp Hematol*. 2005;33:259.

Stamler JS, Jia L, Eu JP, et al. Blood flow regulation by *S*-nitrosohemoglobin in the physiological oxygen gradient. *Science*. 1997;276:2034.

Steinberg MH, Forget BG, Higgs DR, et al. *Disorders of Hemoglobin: Genetics, Pathophysiology, And Clinical Management*. 2nd ed. Cambridge: Cambridge University Press; 2009.

Viprakasit V, Tanphaichitr VS, Chinchang W, et al. Evaluation of alpha hemoglobin stabilizing protein (AHSP) as a genetic modifier in patients with beta thalassemia. *Blood*. 2004;103:3296.

Walsh M, Lutz RJ, Cotter TG, et al. Erythrocyte survival is promoted by plasma and suppressed by a Bak-derived BH3 peptide that interacts with membrane-associated Bcl-X(L). *Blood*. 2002;99:3439.

Weatherall DJ, Clegg JB. *The Thalassaemia Syndromes*. 4th ed. Oxford: Blackwell Science Limited; 2001:818.

Weiss MJ, Zhou S, Feng L, et al. Role of alpha-hemoglobin-stabilizing protein in normal erythropoiesis and beta-thalassemia. *Ann N Y Acad Sci*. 2005;1054:103.

CHAPTER 35

APPROACH TO ANEMIA IN THE ADULT AND CHILD

Judith C. Lin and Edward J. Benz, Jr.

Anemia is the clinical state of red cell mass inappropriately low for the individual's physiologic state. Anemias are among the most commonly encountered laboratory findings and clinical disorders in hematology. They encompass a broad range of clinical disorders and diseases with a spectrum of subtle to severe clinical impact on health. The approach to anemias can be direct if the cause is a common, singular or monogenic, easily tested and identified one; but in challenging cases, the approach may require a careful, systematic investigation and deduction due to the diverse span of possible pathologies and pathogenesis to the essential components determinative of the red cell mass: circulating red cells, erythropoiesis, and apoptosis. Examination of the patient's blood cell morphology by peripheral smear supports the analysis of anemia and is an integral skill for anemia diagnosis. Epidemiologic studies on the global burden of anemias show that in recent decades iron deficiency accounts for the majority of anemias, with increasing predominance in females and the very young (under 5 years of age). Anemias due to hemoglobinopathies, immune destruction of erythroid cells, nutritional deficiencies, parasitic infections, chronic inflammation, and chronic kidney disease are among the more common causes significantly impacting quality of life.[1]

When analyzing anemia in individuals the approach should be systematic and is often initially categorical (e.g., by relative rates of red cell production and destruction or by characteristic indices and morphologies of red blood cells [RBCs]). Additionally, the evaluation of anemia in the adult and child differ in the changing normal ranges for red cells and hemoglobin during childhood as well as the prevalence and onset of congenital hematopoietic diseases and the risks and causes of acquired anemias at different ages in the adult. As population demographics continuously change over time, the evaluation of anemia may also require consideration of the frequent causes of anemia that are endemic in the region of origin of the patient.[2]

The evaluation of anemia includes the initial systematic review of the history, physical examination, and laboratory data obtained from the complete blood count (CBC), reticulocyte count, and peripheral blood smear and consideration of how they reflect alterations in the process of RBC production, erythropoiesis.

OVERVIEW OF ERYTHROPOIESIS

Erythropoiesis is the regulated process leading to the production of mature RBCs or erythrocytes. Bone marrow (BM) stem cells stimulated by the hormone erythropoietin and other factors, proliferate and differentiate along a pathway of recognizable erythroid precursors that ultimately leads to extrusion of the nucleus to facilitate efficient RBC rheology after the production and accumulation of a high concentration of hemoglobin and RBC enzymes (Fig. 35.1).[1] Reticulocytes, the early maturing RBCs, are released with residual RNAs and ribosomes, which they lose by degradation within a day, but no other organelles. Special staining for RNA identifies *reticulocytes*. The reticulocyte count is useful for both qualitative and quantitative measure of the relative rate of peripheral blood erythropoiesis. Mature RBCs survive in blood circulation 100 to 120 days before being removed from the circulation by macrophages in the spleen and other cells of the reticuloendothelial system.[2] At steady state under physiologic conditions, the production and destruction of erythrocytes are equivalent. Control of this homeostatic system process is driven in large part by the hormone erythropoietin, which is produced in a regulated fashion by periglomerular cells in the kidney (~90%) and constitutively by the liver (~10%).[3] Because preservation of oxygen delivery to tissues is so important, the oxygen-sensing regulatory proteins located in the kidney respond to decreased oxygen tension from any cause such as blood loss, high altitude, or cardiac shunts with the production of erythropoietin (Fig. 35.2). This hormone then travels through the bloodstream and stimulates RBC production in the bone marrow. Provided that there are adequate nutrients, including folate, vitamin B_{12}, and iron, the precursors in the BM proliferate and mature and are released into the circulation, ultimately expanding the pool of erythrocytes.[4] The increase in oxygen delivery to the kidney then reduces the stimulus for erythropoietin production.

DEFINITION, PHYSIOLOGIC CONSEQUENCES AND SYMPTOMS OF ANEMIA

Anemia is typically defined as a reduction in the RBC mass, but a clinically useful definition requires a more nuanced expansion of that statement. While there are established "normal" values based on age and gender (Table 35.1, discussed in more detail below), these values are based on results obtained by testing large populations of healthy individuals at rest under normal conditions of oxygenation, altitude, etc. In terms of its clinical implications, "anemia" has to be defined in somewhat relative terms, relative to the person's physiological circumstances, environment, and chronic baseline values. For example, a hematocrit in the "normal" range would be abnormally low in someone living in the high altitude, low oxygen environment of the high Andes plains, or a child with right to left shunting caused by congenital heart defects such as tetralogy of Fallot. Similarly, some whose hematocrit has stably dropped by four points from the high-normal to the normal range is actually anemic relative to his or her baseline and requires evaluation. With regard to this latter consideration, the clinical importance of anemia does not always correlate with its severity. A modest drop in red cell mass in a 50-year-old individual could reflect chronic low grade blood loss due to an otherwise clinically silent colon cancer, while a severe and symptomatic anemia due to iron or deficiencies are readily corrected with iron or vitamin supplementation, and, depending on the underlying cause of the deficiency (e.g., gastritis due to overuse of aspirin or a non-steroidal anti-inflammatory drug [NSAID]), may have an excellent prognosis. Similarly, the degree to which an individual is physiologically compromised or symptomatic does not always correlate with the severity of the anemia. Some children with profound iron deficiency anemia (so called milk-fed babies) may be only modestly symptomatic while some elderly patients with compromised cardiopulmonary status can be significantly compromised and at risk for ischemic events with only a modest reduction in red cell mass. Any evaluation of anemia must therefore be approached in the context of the patient's comprehensive medical history, including family and social history, living environment, physical examination, and comorbidities. This is essential not just for establishing the basis of the anemia but, importantly, for determining the kinds and urgency of therapeutic interventions.

Finally, it is important to recognize that the laboratory values evaluating red cell mass are themselves relative values. The hemoglobin, hematocrit, and red cell count are measured per unit volume of whole

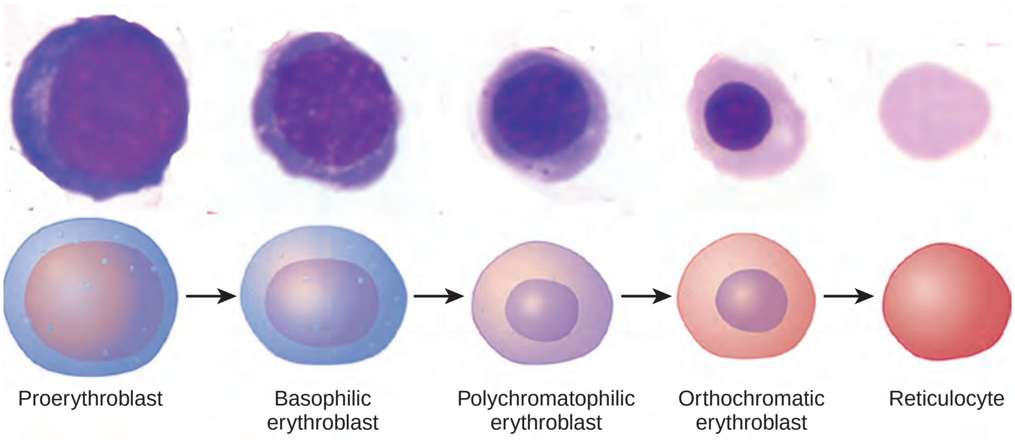


Figure 35.1 OVERVIEW OF ERYTHROPOIESIS.

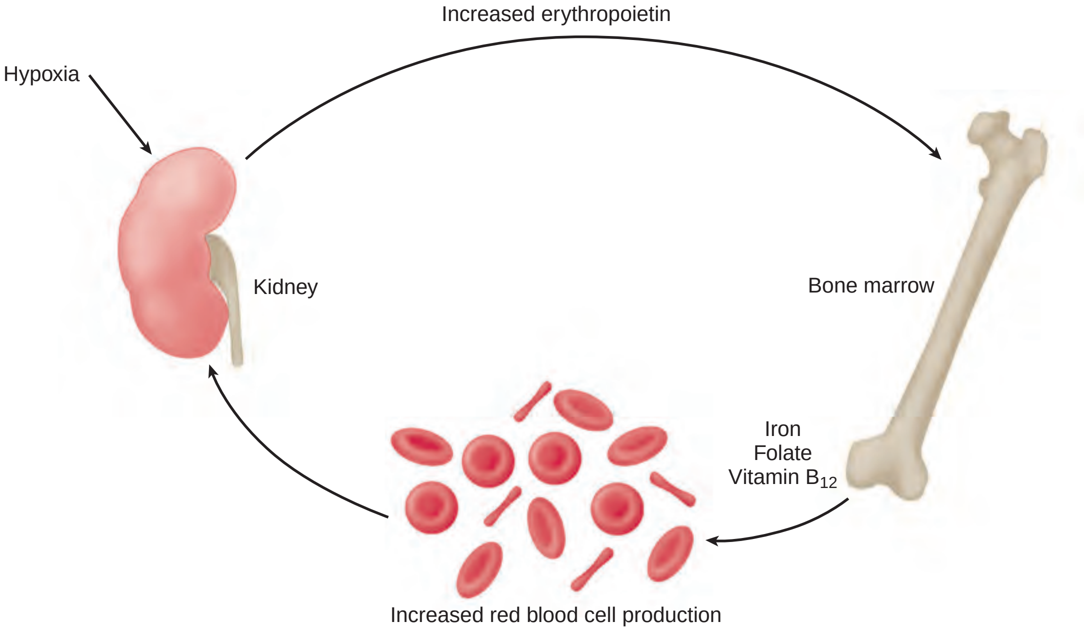


Figure 35.2 REGULATION OF ERYTHROPOIESIS.

blood. Dehydration can artifactually elevate counts, by reducing the relative volume of plasma, while overhydration artifactually lowers counts by increasing the relative volume of plasma.

The RBC mass normally changes during the lifespan of an individual and may be different in males and females.[5] Understanding the changes that occur is critical to appropriately identifying what constitutes anemia (see Table 35.1). The relatively elevated levels of hemoglobin present at birth reflect the lower arterial oxygen saturation extant *in utero* and the predominance of fetal hemoglobin, which exhibits a higher oxygen affinity, and, therefore, is less efficient at delivering oxygen to tissues. The hemoglobin level declines over the first few months of life, as fetal hemoglobin is replaced by adult hemoglobin, to levels that are lower than those seen in adulthood. In later childhood, the hemoglobin values are similar and increase modestly over time. Around puberty, girls have reached adult levels of hemoglobin, but androgenic steroids lead to a continued increase in hemoglobin in boys through about age 18 years. This approximately 1.5 g/dL difference between males and females persists through much of adult life until about age 70 years, when the hemoglobin value in men begins to decline. Over the next two decades, the hemoglobin value declines by about 1 g/dL in men while decreasing by only approximately 0.2 g/dL in women.[6] Thus, at age 90 years, there is only a modest difference between the mean hemoglobin values observed in men and women (14.1 vs. 13.8 g/dL).

The physiologic impact of anemia, defined in context as just discussed, arises from the primary function of red cells and their hemoglobin, which is to deliver oxygen to tissues. The primary symptoms of virtually all anemias are thus those due to inadequate tissue oxygenation: reduced exercise tolerance, fatigue, malaise, palpitations, dyspnea on exertion, anginal or claudication symptoms to cardiac or muscle hypoxia, and changes in mental state. Pallor results from the effect of reduced hemoglobin pigment content of blood perfusing the skin. As discussed later, anemias arising at least in part from accelerated RBC destruction may generate findings reflecting the accelerated catabolism of the RBCs and hemoglobin: jaundice or premature biliary tract disease due to the accelerated turnover of heme; hepatosplenomegaly, due to the destruction of red cells in liver and spleen; elevated red cell lactate dehydrogenase (LDH) levels in serum, etc. A large array of other symptoms and findings (e.g., pagophagia in iron deficiency anemia, long tract neurological signs in pernicious anemias, vaso-occlusive crises in sickle cell anemia) depend on the etiology of the anemia and are discussed extensively in the chapters in this section that focus on these conditions.

TABLE 35.1 Normal Red Blood Cell Values

	Hemoglobin (g/dL)		Hematocrit (%)		Red Blood Cell Count (10^{12}/L)		MCV (fL)		MCH (pg)		MCHC (g/dL)	
Age	**Mean**	**–2 SD**	**Mean**	**–2 SD**	**Mean**	**–2 SD**	**Mean**	**–2 SD**	**Mean**	**–2 SD**	**Mean**	**–2 SD**
Birth (cord blood)	16.5	13.5	51	42	4.7	3.9	108	98	34	31	33	30
1–3 days (capillary)	18.5	14.5	56	45	5.2	4.0	108	95	34	31	33	29
1 week	17.5	13.5	54	42	3.1	3.9	107	88	34	28	33	28
2 weeks	16.5	12.5	51	39	4.9	3.6	105	86	34	28	33	28
1 month	14.0	10.0	43	31	4.2	3.0	104	85	34	28	33	29
2 months	11.5	9.0	35	28	3.8	2.7	96	77	30	26	33	29
3–6 months	11.5	9.5	35	29	3.8	3.1	91	74	30	25	33	30
0.5–2 years	12.0	11.0	36	33	4.5	3.7	78	70	27	23	33	30
2–6 years	12.5	11.5	37	34	4.6	3.9	81	75	27	24	34	31
6–12 years	13.5	11.5	40	35	4.6	4.0	86	77	29	25	34	31
12–18 years												
Female	14.0	12.0	41	36	4.6	4.1	90	78	30	25	34	31
Male	14.5	13.0	43	37	4.9	4.5	88	78	30	25	34	31
18–49 years												
Female	14.0	12.0	41	36	4.6	4.0	90	80	30	26	34	31
Male	15.5	13.5	47	41	5.2	4.5	90	80	30	26	34	31

MCH, Mean corpuscular hemoglobin; *MCHC*, mean corpuscular hemoglobin concentration; *MCV*, mean corpuscular volume.
From Oski FA. Pallor. In: Kaye R, Oski FA, Barness LA, eds. *Core Textbook of Pediatrics*. 3rd ed. Philadelphia: Lippincott; 1989:62.

As already mentioned, individual patients vary widely in their symptomatology relative to the degree of the anemia. This is because anemia triggers an array of compensatory responses that can mitigate the impact of reduced red cell mass on tissue oxygenation. One response is increased cardiac output combined with reduced afterload, likely due to systemic vasodilatation. This has the effect of delivering more blood per minute to counterbalance the presence of less oxygen content per unit volume of blood due to the anemia. For complex reasons, anemia also triggers a reduction on red cell 2, 3-DPG, a cofactor that, when bound to hemoglobin, increases it O_2 affinity. Reduced 2, 3-DPG leads to a lower affinity, meaning that the hemoglobin gives up more oxygen at the oxygen tension of capillary blood (see Chapters 34 and 44). These and other adaptations, such as increased minute ventilation, point to the importance of the cardiopulmonary and vascular status of the anemic patient in modulating symptoms and functional tolerance of the anemia.

MECHANISMS OF ANEMIA

Although a complete review of all of the mechanisms leading to anemia is beyond the scope of this chapter, an appreciation of some of the mechanisms is useful before approaching the diagnosis of anemia in adults and children. Three broad categories of anemia are blood loss anemia, hypoproliferative anemia, and hemolytic anemia.

Blood Loss Anemia

Blood loss may occur acutely or chronically. When blood is lost acutely through hemorrhage, it may take several hours before a decline in hemoglobin concentration is observed because of the time required for restoration of the plasma volume and equilibration. Several days may elapse before an appropriate reticulocytosis is noted. Chronic blood loss ultimately leads to hypoproliferative anemia because of iron deficiency. In most of the latter patients, the underlying cause of the chronic blood loss is usually more determinative of prognosis than the anemia itself.

Hypoproliferative Anemia

When used broadly, the term *hypoproliferative anemias* refers to entities that manifest as an inability to produce an adequate number of erythrocytes in response to appropriate signals. Although there are many different causes, the hallmark of hypoproliferative anemia is a low reticulocyte count (Table 35.2). The etiology underlying this class of disorders may relate to the hypoproliferation of precursors within the BM, such as may be seen in aplastic anemia, when there is BM replacement (myelophthisis), or where there is ineffective erythropoiesis, due to abnormal maturation of precursors in the BM, as is encountered in thalassemia, megaloblastic anemia (folate deficiency, vitamin B_{12} deficiency, myelodysplastic syndromes [MDSs]), and others. In thalassemia and megaloblastic anemia, the BM often is packed with early erythroid progenitors. However, intramedullary demise of precursors prevents the formation and release of mature RBCs.

By far the most common cause of hypoproliferative anemia globally is iron deficiency.[7] It is estimated that about 2% of infants and children may become iron deficient purely because of inadequate dietary intake. About 4% of women ages 20 to 49 years of age in the United States have iron-deficiency anemia primarily because of inadequate dietary intake in the setting of menstruation and childbirth. Iron deficiency is also commonly encountered in older individuals as well (∼2% of individuals older than age 50 years), and it should provoke a thorough search for its etiology, which in both men and nonmenstruating women frequently is gastrointestinal blood loss. After iron deficiency, acute or chronic inflammation and renal disease are common etiologies of anemia.[8,9] BM failure states and BM replacement caused by hematologic malignancies or solid tumors are less common causes of anemia and are often accompanied by other hematologic manifestations, such as leukopenia and thrombocytopenia.

Hemolytic Anemia

The causes of hemolytic anemia are quite varied and may be congenital or acquired.[10] The hallmark of hemolytic anemia is an elevated reticulocyte count (see Table 35.2). However, if hemolysis occurs in a setting where erythropoiesis is also suppressed or ineffective, the reticulocyte count may not be as high as expected for the degree of anemia. Other features commonly associated with hemolytic anemia include an elevated LDH level, increased unconjugated (indirect) bilirubin level, and decreased haptoglobin level. Hemolytic anemia also may manifest with distinctive changes on the peripheral blood smear (e.g., spherocytes, sickle cells, or schistocytes). Congenital causes include the hemoglobinopathies, enzymopathies (predominantly glucose-6-phosphate dehydrogenase [G6PD] deficiency), and membrane disorders.[11] Acquired conditions include autoimmune hemolytic anemia, microangiopathic hemolytic anemia, hemolysis related to infections, and acquired membrane disorders such as those caused by liver disease (spur cell of anemia) and paroxysmal nocturnal hemoglobinuria.[12]

TABLE 35.2 **Usefulness of the Reticulocyte Count in the Diagnosis of Anemia[a]**

Diagnosis	Value
Hypoproliferative anemias	Absolute reticulocyte count <75,000/µL
Anemia of chronic disease	
Anemia of renal disease	
Congenital dyserythropoietic anemias	
Effects of drugs or toxins	
Endocrine anemias	
Iron deficiency	
Bone marrow replacement	
Maturation abnormalities	Absolute reticulocyte count <75,000/µL
Vitamin B_{12} deficiency	
Folate deficiency	
Sideroblastic anemia	
Appropriate response to blood loss or nutritional supplementation	Absolute reticulocyte count ≥100,000/µL
Hemolytic anemias	Absolute reticulocyte count ≥100,000/µL
Hemoglobinopathies	
Immune hemolytic anemias	
Infectious causes of hemolysis	
Membrane abnormalities	
Metabolic abnormalities	
Mechanical hemolysis	

[a] Note that reticulocyte counts in the range of 75,000–100,000/µL can sometimes be associated with appropriate response to blood loss or hemolytic anemia.

COMPARISON OF ETIOLOGIES OF ANEMIA IN ADULTS AND CHILDREN

As already noted, the designation that anemia is present relies on comparison of the patient's hemoglobin or hematocrit with an age- and sex-appropriate normal range (see Table 35.1). Although many types of anemia may occur across the age spectrum, certain types tend to be identified more commonly in either adults or children, and some are primarily identified in neonates. In children, the most common causes of anemia are related to nutritional deficiency or to a primary hematologic process, either hereditary or acquired. In contrast, the most common causes of anemia in adults are iron deficiency caused by blood loss or anemia caused by systemic illness, such as renal failure, inflammatory conditions, or malignancy (Table 35.3).

Anemia in Children

Hypoproliferative anemia in children may be associated with either acquired or congenital etiologies. Acquired cases are most commonly caused by nutritional deficiency but also include those caused by acquired aplastic anemia, transient erythroblastopenia of childhood (TEC), the anemia of acute inflammation, and marrow replacement caused by malignancy.[13] Congenital causes include Diamond-Blackfan anemia and other rare syndromes, including refractory sideroblastic anemia and the congenital dyserythropoietic anemias.[14] Iron deficiency may occur in children because of a diet that is rich in cow's milk to the exclusion of other iron-containing foods. This is particularly common during the first 2 years of life. The anemia may be quite severe and may

TABLE 35.3 **Comparison of the More Common Causes of Anemia in Children and Adult**

Type of Anemia	Children	Adults
Hypoproliferative	• Nutritional deficiency (most commonly iron deficiency) • Acute inflammation • Transient erythroblastopenia of childhood • Acquired aplastic anemia • Marrow replacement caused by malignancy	• Iron deficiency • Anemia of inflammation (anemia of chronic disease) • Anemia of renal disease • Folate or vitamin B_{12} deficiency • Drugs or toxins • Pure RBC aplasia (viral or idiopathic) • MDS
Hemolytic	• Inherited hemoglobinopathies • Inherited membrane disorders • Autoimmune hemolytic anemia • Microangiopathic hemolytic anemia	• Inherited hemoglobinopathies with milder manifestations • Inherited membrane disorders with milder manifestations • G6PD deficiency • Autoimmune hemolytic anemia • Microangiopathic hemolytic anemia (DIC, TTP, HUS, aHUS)

aHUS, Atypical hemolytic uremic syndrome; *DIC*, disseminated intravascular coagulation; *G6PD*, glucose-6-phosphate dehydrogenase; *HUS*, hemolytic uremic syndrome; *MDS*, myelodysplastic syndrome; *RBC*, red blood cell; *TTP*, thrombotic thrombocytopenic purpura.

be associated with a mean corpuscular volume (MCV) of 50 to 65 fL. Acquired aplastic anemia, as opposed to pure RBC aplasia, is associated with bicytopenia or pancytopenia. TEC is an acquired disorder that generally occurs during the first 3 years of life in otherwise healthy children, although it can be seen in children from 6 months to 10 years old. It is thought to have a viral or immunologic cause and resolves without specific intervention. The anemia of acute inflammation may be encountered in children who are hospitalized and is generally transient, resolving when the underlying condition has improved. Leukemia may result in BM replacement and is usually associated with abnormalities in other cell lineages in addition to RBCs.

Hemolytic anemia in children is most commonly associated with inherited disorders of hemoglobin or the RBC membrane.[15] However, acquired causes such as autoimmune hemolytic anemia and microangiopathic hemolytic anemia, particularly *Shiga* toxin–associated hemolytic uremic syndrome (HUS), also occur.[16] In older children, many etiologies of hemolytic anemia overlap with those considered in adults, and a similar diagnostic algorithm may be appropriate. However, in newborns, jaundice as an indicator of the inherited causes of hemolytic anemia must be distinguished from more pronounced cases of the physiologic hyperbilirubinemia that occurs during the perinatal period. After true hemolysis has been identified in an infant, the differential diagnosis is relatively limited (Fig. 35.3). Immune-mediated hemolysis may result from ABO, Rh, or minor blood group incompatibility.[17] Other causes include metabolic disorders and disorders of the RBC membrane, and some forms of α-thalassemia. Of note, however, is the fact that hemoglobinopathies, such as sickle cell disease and β-thalassemia, are silent during the newborn period and only become manifest at 4 to 6 months of age when the fetal-to-adult hemoglobin transition has been completed. Newborn screening programs in the United States may provide salient information in this regard on the presence or absence of a hemoglobinopathy. Alternatively, ethnic background and family history may be helpful in suspecting the appropriate diagnosis.

Anemia in Adults

Hypoproliferative anemia in adults is relatively common. If acute blood loss is excluded, hypoproliferative causes are the most common entities associated with anemia in adults. These are iron deficiency, inflammation (anemia of chronic disease), and renal disease (Fig. 35.4). The megaloblastic anemias that represent maturation abnormalities, including folate and vitamin B_{12} deficiency, are often categorized along with the hypoproliferative anemias because they present with a low reticulocyte count as well. Myelosuppressive drugs and toxins such as ethanol can also be associated with hypoproliferative anemia. Pure RBC aplasia may be associated with other diseases (thymoma) or viral infection (parvovirus B19) or be idiopathic.[18] Finally, MDS may present with hypoproliferative anemia, as may an infiltrative process such as metastatic malignancies, myelofibrosis, or acute leukemia. The distinction between the various causes of anemia is facilitated by historical factors, physical findings, and concomitant laboratory abnormalities in conjunction with review of the MCV and RBC distribution width (RDW) along with the peripheral blood smear. In the setting of a low reticulocyte count, MCV values below 70 fL are most commonly associated with iron-deficiency anemia, and those above 120 fL are most commonly associated with folate or vitamin B_{12} deficiency. The differential diagnosis broadens for MCV values that fall just outside of the normal range. For example, in the setting of a low reticulocyte count, MCV values in the range from 75 to 80 fL may be associated with iron-deficiency anemia, the anemia of inflammation, and endocrine causes of anemia. MCV values between 100 and 110 fL may be associated with folate or vitamin B_{12}

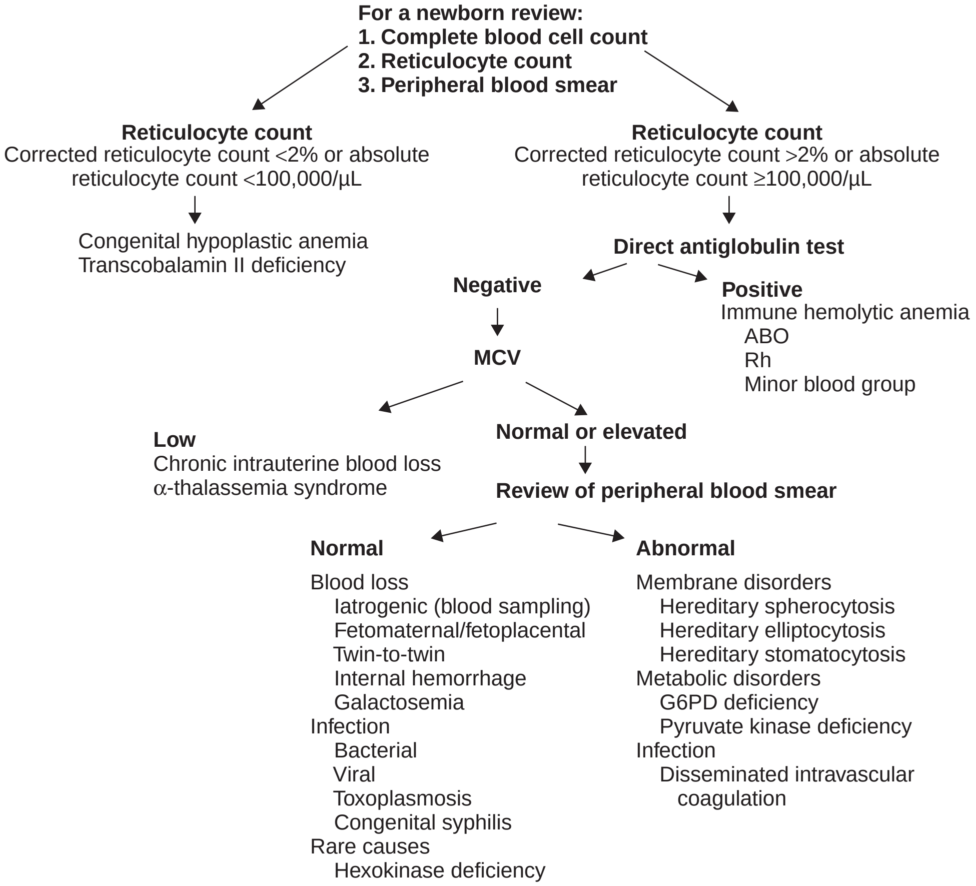

For a newborn review:
1. Complete blood cell count
2. Reticulocyte count
3. Peripheral blood smear
Reticulocyte count
Corrected reticulocyte count <2% or absolute reticulocyte count <100,000/µL
Congenital hypoplastic anemia
Transcobalamin II deficiency
Reticulocyte count
Corrected reticulocyte count >2% or absolute reticulocyte count ≥100,000/µL
Direct antiglobulin test
Negative
Positive
Immune hemolytic anemia
ABO
Rh
Minor blood group
MCV
Low
Chronic intrauterine blood loss
α-thalassemia syndrome
Normal or elevated
Review of peripheral blood smear
Normal
Blood loss
Iatrogenic (blood sampling)
Fetomaternal/fetoplacental
Twin-to-twin
Internal hemorrhage
Galactosemia
Infection
Bacterial
Viral
Toxoplasmosis
Congenital syphilis
Rare causes
Hexokinase deficiency
Abnormal
Membrane disorders
Hereditary spherocytosis
Hereditary elliptocytosis
Hereditary stomatocytosis
Metabolic disorders
G6PD deficiency
Pyruvate kinase deficiency
Infection
Disseminated intravascular coagulation

Figure 35.3 APPROACH TO THE DIFFERENTIAL DIAGNOSIS OF ANEMIA IN A NEWBORN. *G6PD*, Glucose-6-phosphate dehydrogenase; *MCV*, mean corpuscular volume.

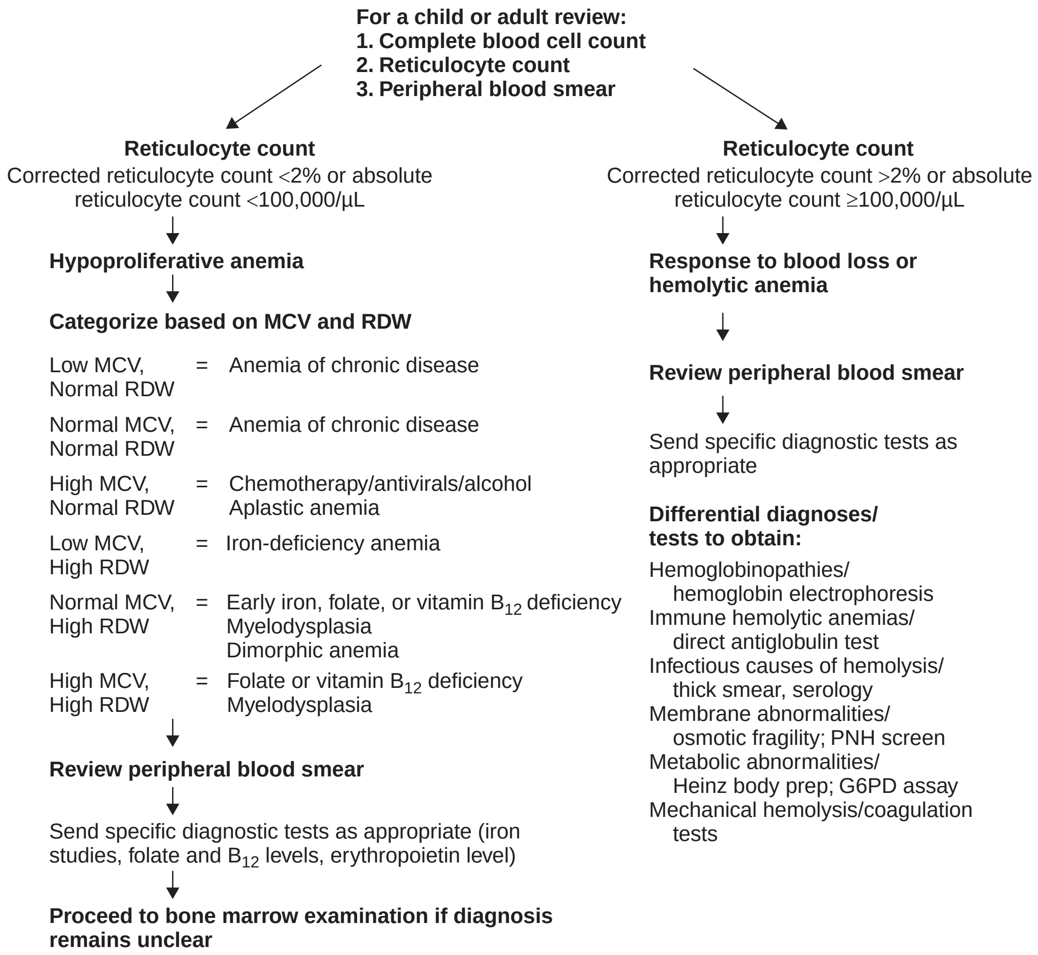

For a child or adult review:
1. Complete blood cell count
2. Reticulocyte count
3. Peripheral blood smear
Reticulocyte count
Corrected reticulocyte count <2% or absolute reticulocyte count <100,000/μL
Hypoproliferative anemia
Categorize based on MCV and RDW
Low MCV, Normal RDW = Anemia of chronic disease
Normal MCV, Normal RDW = Anemia of chronic disease
High MCV, Normal RDW = Chemotherapy/antivirals/alcohol Aplastic anemia
Low MCV, High RDW = Iron-deficiency anemia
Normal MCV, High RDW = Early iron, folate, or vitamin B12 deficiency Myelodysplasia Dimorphic anemia
High MCV, High RDW = Folate or vitamin B12 deficiency Myelodysplasia
Review peripheral blood smear
Send specific diagnostic tests as appropriate (iron studies, folate and B12 levels, erythropoietin level)
Proceed to bone marrow examination if diagnosis remains unclear
Reticulocyte count
Corrected reticulocyte count >2% or absolute reticulocyte count ≥100,000/μL
Response to blood loss or hemolytic anemia
Review peripheral blood smear
Send specific diagnostic tests as appropriate
Differential diagnoses/ tests to obtain:
Hemoglobinopathies/ hemoglobin electrophoresis
Immune hemolytic anemias/ direct antiglobulin test
Infectious causes of hemolysis/ thick smear, serology
Membrane abnormalities/ osmotic fragility; PNH screen
Metabolic abnormalities/ Heinz body prep; G6PD assay
Mechanical hemolysis/coagulation tests

Figure 35.4 APPROACH TO THE DIFFERENTIAL DIAGNOSES OF ANEMIA IN ADULTS AND CHILDREN. *G6PD*, Glucose-6-phosphate dehydrogenase; *MCV*, mean corpuscular volume; *PNH*, paroxysmal nocturnal hemoglobinuria; *RDW*, red blood cell distribution width.

deficiency, aplastic anemia, MDS, liver disease, and immune hemolytic anemias.

Hemolytic anemia in adults is less common than hypoproliferative anemia, and the differential diagnosis is broad. Congenital causes associated with mild to moderate hemolysis may be clinically silent until detected later in life.[19] This is particularly the case for milder cases of β-thalassemia intermedia, sickle cell (SC) disease and sickle-β^+-thalassemia, and hereditary spherocytosis. Additionally, the most common RBC enzymopathy (which is also one of the most common human enzyme defect deficiencies), G6PD deficiency, does not present until individuals encounter oxidant stress either because of infection or drugs such as sulfonamides and antimalarials.[20] Acquired hemolytic anemias include autoimmune hemolytic anemia, which is often associated with hematologic malignancies or rheumatologic disorders, and the microangiopathic hemolytic anemias, including disseminated intravascular coagulation (DIC), thrombotic thrombocytopenic purpura (TTP), and HUS.[21] Distinction of the various causes of hemolytic anemia is also facilitated by the associated historical features, physical findings, and laboratory abnormalities of the clinical presentation. For these disorders, review of the peripheral blood smear may be particularly revealing as to the etiology.

SYSTEMIC APPROACH TO ANEMIA

The correct diagnosis of anemia can often be determined by combining a thorough history and physical examination with review of the CBC, concentrating particularly on the MCV and RDW, along with review of the reticulocyte count and the peripheral blood smear.

History and Physical Examination

Anemia can be a primary disorder of the erythron or secondary to other systemic processes, thus a careful history and physical examination provide valuable insight into the potential cause. Fatigue often accompanies anemia, but it is very nonspecific and may be related to systemic illness. Nonetheless, determining the concomitant presence of a systemic inflammatory disorder, infection, or malignancy that may be associated with fatigue can be critical in determining the underlying causes of anemia in both adults and children. The medical history may also be quite informative. For example, a history of diabetes mellitus can be associated with significantly impaired renal production of erythropoietin, even in the setting of only a mildly elevated creatinine level. Because certain medications may be associated with BM depression or, alternatively, the development of autoimmune hemolytic anemia, all pharmacologic agents, including those prescribed and over the counter, including alternative medicines, should be reviewed. Occupational history is occasionally relevant, as in the case of individuals, such as welders, who might have been exposed to lead or other potentially BM-toxic agents. Social history can be important. A history of intravenous drug use might suggest the possibility of virally transmitted diseases, such as human immunodeficiency virus (HIV), which may be associated with anemia. Dietary history is also very important, particularly in young and elderly individuals with anemia. The finding of pica in adults (most commonly ice chips or cornstarch) is well known to be associated with iron-deficiency anemia.[22] Ingestion of paint chips may suggest the need to investigate the possibility of toxic lead ingestion. A family history of anemia is highly relevant in the evaluation of children with anemia. However, it is also relevant in adults because certain congenital anemias, such as milder forms of sickle β^+-thalassemia and

TABLE 35.4 Usefulness of the Mean Corpuscular Value and Red Blood Cell Distribution Width in the Diagnosis of Anemia

	Low MCV (<80 fL)	Normal MCV (80–99 fL)	High MCV (≥100 fL)
Normal RDW	Anemia of chronic disease	Acute blood loss	Aplastic anemia
	α- or β-thalassemia trait	Anemia of chronic disease	Chronic liver disease
	Hemoglobin E trait	Anemia of renal disease	Chemotherapy, antivirals, or alcohol
Elevated RDW	Iron deficiency	Early iron, folate, or vitamin B_{12} deficiency	Folate or vitamin B_{12} deficiency
	Sickle cell-β–thalassemia	Dimorphic anemia (for example, iron + folate deficiency)	Immune hemolytic anemia
		Sickle cell anemia	Cytotoxic chemotherapy
		Sickle cell disease	Chronic liver disease
		Chronic liver disease	Myelodysplasia
		Myelodysplasia	Hereditary spherocytosis, hereditary elliptocytosis, congenital hemoglobinopathies and RBC enzymopathies

MCW, Mean corpuscular value; *RDW*, red blood cell distribution width.

hereditary spherocytosis, occasionally first become clinically apparent in adulthood.

The significance of pallor on physical examination is in many ways similar to the historic feature of fatigue: it is a common but nonspecific finding. More specific findings may be found in certain types of anemia. For example, angular cheilitis (cracking at the edges of the lips) and koilonychia (spooning of the nails) may accompany iron-deficiency anemia. Splenomegaly may be present in patients with anemia arising from a wide variety of different causes. When present early in life, it is suggestive of a congenital hemolytic anemia, such as thalassemia, sickle cell disease, or hereditary spherocytosis. When found for the first time later in life, splenomegaly may indicate an acquired disorder, such as autoimmune hemolytic anemia, lymphoproliferative disease, or a myeloproliferative disease such as myelofibrosis. Other physical findings can also sometimes provide insight relevant to the investigation of anemia when combined with historical features and laboratory data. Although anemia itself may lead to the presence of systolic cardiac murmurs, the finding of an increased cardiac murmur in an anemic patient with a prosthetic aortic valve and new microangiopathic change on peripheral smear may indicate that investigation into the possibility of perivalvular leak or prosthetic dysfunction is in order.[23] Finally, because neurologic manifestations can accompany or even predate the anemia associated with vitamin B_{12} deficiency, findings such as loss of vibration or position sense in the extremities may be relevant.[24]

Reticulocyte Count

As a marker of RBC production, the reticulocyte count provides essential information in directing the initial investigation of anemia. Modern flow cytometers accurately determine the reticulocyte count using fluorescent probes that bind to the residual ribonucleic acid present in newly released RBCs.[25] These measurements are useful, accurate, and reflect the state of erythropoiesis. However, when significant numbers of nucleated RBCs or nuclear debris are present in the peripheral blood, this diagnostic accuracy declines, and manual counting methods are generally preferable.

When the reticulocyte count is reported as a percentage, it needs to be adjusted for the total number of RBCs present. This correction can be made by multiplying the reticulocyte count by the patient's hematocrit divided by an age- and sex-appropriate normal hematocrit. No such correction is necessary when the reticulocyte count is reported as an absolute number or when it is converted to an absolute number by multiplying the percentage by the RBC number (in RBC/μL).

In the absence of anemia, the normal absolute reticulocyte count is between 25,000 and 75,000/μL. In the presence of anemia, an absolute reticulocyte count of less than 75,000/μL is indicative of a hypoproliferative process, and an absolute reticulocyte count of greater than 100,000/μL is indicative of hemolysis or an appropriate erythropoietic response to blood loss (see Table 35.2). Reticulocyte counts between 75,000 and 100,000/μL require interpretation in the context of other available clinical data, including the severity of anemia present.

Mean Corpuscular Volume and Red Blood Cell Distribution Width From the Complete Blood Count

Automated cell counters provide a wealth of information regarding the size, shape, and hemoglobin content of RBCs. The two parameters most useful in classifying anemia are the MCV and the RDW. MCV is reported in femtoliters (fL) and reflects average cell size. RDW is often reported in percent and represents the standard deviation of RBC volume divided by the mean volume. It reflects the variation in cell size in the population of RBCs.[26] These two parameters are useful because relatively reproducible changes in the MCV and RDW are associated with certain types of anemia (Table 35.4). The MCV and RDW can significantly narrow the differential diagnosis, particularly when combined with the reticulocyte count (Table 35.5).

TABLE 35.5 Combining the Reticulocyte Count and Red Blood Cell Parameters for Diagnosis

MCV, RDW	Reticulocyte Count <100,000/μL	Reticulocyte Count ≥100,000/μL
Low, normal	Anemia of chronic disease	
Normal, normal	Anemia of chronic disease	
High, normal	Chemotherapy, antivirals, or alcohol Aplastic anemia	Chronic liver disease
Low, high	Iron-deficiency anemia	Sickle cell-β–thalassemia
Normal, high	Early iron, folate, vitamin B_{12} deficiency Myelodysplasia	Sickle cell anemia, sickle cell disease
High, high	Folate or vitamin B_{12} deficiency Myelodysplasia	Immune hemolytic anemia Chronic liver disease

MCV, Mean corpuscular volume; *RDW*, red blood cell distribution width.

SYSTEMATIC APPROACH TO THE DIAGNOSIS OF ANEMIA

Integration of historic features and physical findings with thoughtful review of the results of the automated complete blood cell count and peripheral smear often significantly narrows down the differential diagnosis of anemia. For example, a patient who has had a gastric bypass eating a normal diet who presents with gradual onset of fatigue accompanied by the more recent onset of distal paresthesias and a finding of decreased vibration sense in the setting of anemia with significantly elevated mean corpuscular volume and red blood cell (RBC) distribution width values and numerous six-lobed polymorphonuclear leukocytes on peripheral blood smear almost certainly has vitamin B_{12} deficiency. This is suggested even before the return of specific laboratory testing because of the relatively narrow differential diagnosis for megaloblastic anemia and the fact that neurologic abnormalities are not associated with folate deficiency. For the purposes of diagnostic efficiency, the rewards of correlation of historic features and physical findings with a careful review of the peripheral blood smear cannot be overstated.

Special stains of the peripheral blood smear can be helpful in elucidating the cause of anemia. If there is significant nuclear debris present, the reticulocyte count obtained by automated methods can be inaccurate. In such cases, manual counting after staining with new methylene blue, which stains residual ribonucleic acid (RNA) in reticulocytes, permits accurate enumeration. If bite cells are detected on peripheral smear, supravital staining with methyl crystal violet can reveal Heinz bodies. These are aggregates of denatured hemoglobin reflecting an oxidative insult, most commonly caused by glucose-6-phosphate dehydrogenase deficiency or, less frequently, by the presence of an unstable hemoglobin (see Fig. 35.5H).

Several commonly encountered findings can be seen in RBCs on the peripheral blood smear (Table 35.6 and Fig. 35.5). Whereas microcytic, hypochromic RBCs are suggestive of iron-deficiency anemia or thalassemia (see Fig. 35.5F) macrocytic RBCs with ovalocytes (oval RBCs) are suggestive of megaloblastic anemias (see Fig. 35.5G). Some findings reflect organ dysfunction, such as echinocytes (burr cells) in uremia (see Fig. 35.5R) or acanthocytes (spur cells) in severe liver disease (see Fig. 35.5S), although acanthocytes may also be seen in rare conditions such as abetalipoproteinemia. Target cells may be seen in cases of liver disease but may also be present in hemoglobinopathies, including sickle cell disease and thalassemia (see Fig. 35.5W). The presence of schistocytes or RBC fragmentation often reflects systemic disease, such as disseminated intravascular coagulation (DIC), thrombotic thrombocytopenic purpura (TTP), or hemolytic uremic syndrome (HUS) (see Fig. 35.5P). Finding spherocytes on a smear is suggestive of autoimmune hemolytic anemia or hereditary spherocytosis (see Fig. 35.5H). Occasionally, the clue to the correct diagnosis of a systemic illness comes in the form of the observation of intraerythrocytic inclusions, such as malarial (see Fig. 35.5O) or babesial forms, and examination of a thick blood smear may be useful for the diagnosis of these disorders when a low parasite burden is suspected.

TABLE 35.6 Features of the Peripheral Blood Smear

Red Blood Cell Morphology	Definition	Interpretation
Polychromasia	Large, bluish RBCs lacking normal central pallor on peripheral blood smear; bluish stain is the result of residual ribonucleic acid	Rapid production and release of RBCs from BM; elevated reticulocyte count; most commonly seen in any hemolytic anemia and states of increased RBC turnover
Basophilic stippling	Many small bluish dots in portion of erythrocytes; comes from staining of clustered polyribosomes in young circulating RBCs	Seen in a variety of erythropoietic disorders, including acquired (e.g., myelodysplasia) and congenital hemolytic anemias and occasionally in lead poisoning
Pappenheimer bodies	Several grayish, irregularly shaped inclusions in a portion of erythrocytes visible on peripheral smear; composed of aggregates of ribosomes, ferritin, and mitochondria	Erythropoietic malfunction in congenital anemias such as hemoglobinopathies, particularly with splenic hypofunction or acquired anemias such as megaloblastic anemia
Heinz bodies	Several grayish, round inclusions visible after supravital staining with methyl crystal violet of the peripheral blood smear, often in the context of bite cells; represent aggregates of denatured hemoglobin	Indicative of oxidative injury to the erythrocyte, such as occurs in G6PD deficiency and other RBC enzymopathies or unstable hemoglobins
Howell-Jolly bodies	Usually, one or, at most, a few purplish inclusion(s) in the erythrocyte visible on the routine peripheral blood smear; represent residual fragments of nuclei containing chromatin	Associated with states of splenic hypofunction, splenic atrophy, splenic thrombosis or after splenectomy
Schistocytes	RBCs that are fragmented into a variety of shapes and sizes, including helmet-shaped cells; indicative of shearing of the erythrocyte within the circulation	Associated with microangiopathic hemolytic anemias, including DIC, TTP, HUS or aHUS, as well as other mechanical causes of hemolysis, such as prosthetic heart valves or severe cardiac valvular stenosis
Spherocytes	RBCs that have lost their central pallor and appear spherical; indicative of loss of cytoskeletal integrity from internal or external causes	Associated with hereditary spherocytosis, autoimmune hemolytic anemia; may also be observed in addition to schistocytes in the presence of microangiopathic hemolytic anemia
Teardrop cells	Pear-shaped erythrocytes visible on peripheral blood smear; indicative of mechanical stress on the RBC during release from the BM or passage through the spleen	Seen in a variety of conditions along with other poikilocytes including severe iron deficiency anemia, congenital anemias such as thalassemias, hemoglobinopathies, and acquired disorders such as megaloblastic anemia. As isolated poikilocyte, teardrop RBCs may be initial changes of myelophthisis (BM replacement or infiltration), e.g., myelodysplastic syndrome or myelofibrosis.
Burr cells (echinocytes)	RBCs that have smooth undulations present on the surface circumferentially; pathogenesis unknown	Indicative of uremia when present on a properly made peripheral blood smear
Spur cells (acanthocytes)	RBCs that have spiny points present on the surface circumferentially; reflective of abnormal lipid composition of RBC membrane	Most commonly indicative of hemolytic anemia of advanced liver disease when present in significant numbers; also seen in abetalipoproteinemia and in RBCs lacking the Kell blood group antigen

aHUS, Atypical hemolytic uremic syndrome; *BM*, bone marrow; *DIC*, disseminated intravascular coagulation; *G6PD*, glucose-6-phosphate dehydrogenase; *HUS*, hemolytic uremic syndrome; *RBC*, red blood cell; *TTP*, thrombotic thrombocytopenic purpura.

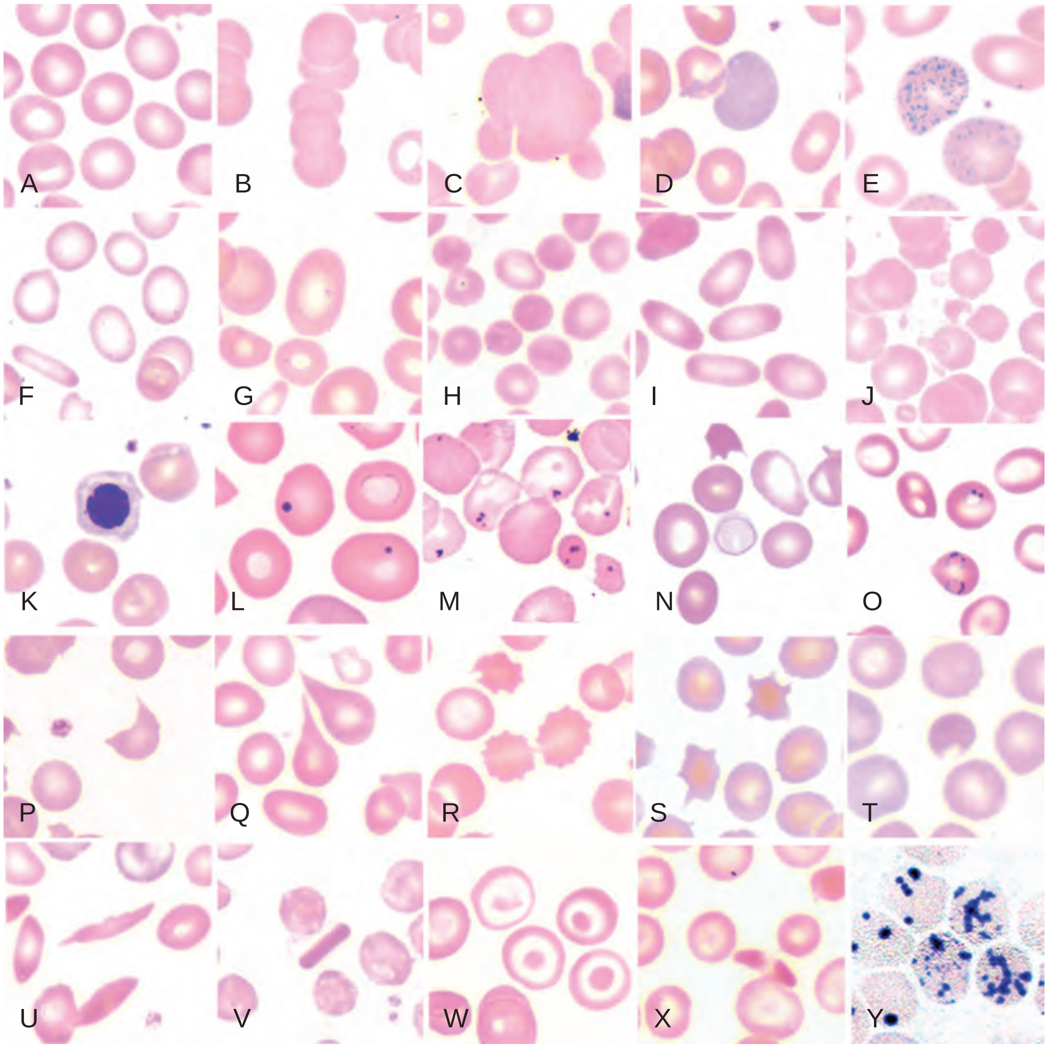


Figure 35.5 USEFUL PERIPHERAL BLOOD AND RED BLOOD CELL FEATURES IN THE EVALUATION OF ANEMIA. (A) Normal red blood cells (RBCs). Note the central pallor is one-third the diameter of the entire cell. (B) Rouleaux formation is indicative of increased plasma protein. (C) Agglutination indicates an antibody-mediated process such as cold agglutinin disease. (D) Polychromatophilic cell. The gray-blue color is attributable to RNA and the cell is equivalent to a reticulocyte, which must be identified with a reticulocyte stain. (E) Basophilic stippling. This also is attributable to increased RNA caused either by a left shift in erythroid cells or lead toxicity. (F) Hypochromic microcytic cells typical of iron-deficiency anemia. Note the widened central pallor and the "pencil" cell in the *lower left*. (G) Macroovalocyte as can be seen in either megaloblastic anemia or myelodysplastic syndrome. (H) Microspherocytes typical of hereditary spherocytosis. (I) Elliptocytes (ovalocytes) from a patient with hereditary elliptocytosis. (J) RBC fragments from thermal injury (burn patient). (K) Nucleated RBC. (L) Howell-Jolly bodies indicative of splenic dysfunction or absence. (M) Pappenheimer bodies from a patient with sideroblastic anemia. (N) Cabot ring, as can be seen in megaloblastic anemia or MDS. (O) Malarial parasites *(Plasmodium falciparum)*. (P) Schistocyte typical of a microangiopathic hemolytic anemia. (Q) Tear-drop form indicates marrow fibrosis and extramedullary hematopoiesis. (R) Echinocyte (Burr cell) with rounded edges. (S) Acanthocyte (spur cell) with more irregular pointed ends. This was from a patient with neuroacanthocytosis. They can also be seen in patients with liver disease and lipid abnormalities. (T) "Bite" cell from a patient with glucose-6-phosphate dehydrogenase (G6PD) deficiency. (U) Sickle cell, from a patient with homozygous sickle cell disease. (V) Hemoglobin C crystal. (W) Target cells. (X) Hemoglobin C disease. Note that the RBC in center has condensed hemoglobin at each pole. (Y) Heinz body preparation (supravital stain) from a patient with G6PD deficiency. Note that the cells to the right have increased precipitated hemoglobin.

Examination of the Peripheral Blood Smear

Despite the development and availability of more sophisticated diagnostic testing, review of a well-made peripheral blood smear remains one of the most informative and rewarding diagnostic procedures.[27] It offers the chance to confirm the findings of the automated CBC count, which can be inaccurate in the presence of nucleated RBCs or rouleaux formation. Review of the blood smear also allows for evaluation of other cell lineages, which might suggest a primary BM or infiltrative disease. For example, the finding of hypersegmented neutrophils suggests a megaloblastic process, and this morphologic abnormality can be seen in the blood smear before there are significant changes in the hemoglobin or MCV (see box on Systematic Approach to the Diagnosis of Anemia). Also, only the blood smear reveals the unique morphologic changes occurring with several of the various hemolytic disorders.

Bone Marrow Examination

Bone marrow aspiration and biopsy permit evaluation of cellular morphology and BM architecture, respectively. Special stains, flow cytometry, cytogenetic analysis, fluorescence in situ hybridization (FISH), and molecular testing performed on the BM can provide a wealth of diagnostic information.[28] Because of the discomfort involved in the procedure, however, careful consideration should be given to determining the array of tests required, so that repeated BM aspirates or biopsies need not be performed. If there is any consideration of the possibility of myelodysplasia, leukemia, or lymphoma, an aliquot of anticoagulated aspirate should be set aside at the time of the initial procedure that can be sent, if necessary, for flow cytometry or cytogenetics after review of the aspirate smear. It should be noted that even when properly performed, difficulty obtaining a BM aspirate is commonly observed in certain situations, including myelofibrosis, erythroblastic leukemia (M6), and hairy cell leukemia. In these cases, touch preps of the BM biopsy may help expedite diagnosis.

Diagnostic uncertainty in the setting of hypoproliferative anemia is an indication for BM biopsy. Hematologic disorders such as myelodysplasia, leukemia, lymphoma, or myeloma may be identified. Myelodysplasia in the marrow classically includes megaloblastic change and nuclear budding in maturing erythroblasts, as well as morphologic abnormalities in other lineages, such as hypolobated megakaryocytes and hypogranulation of the myeloid lineage.[29] A variety of infiltrative (myelophthisic) processes may be observed.[30] These include malignancies such as small-cell lung, breast, and prostate cancers, which frequently can appear in advanced stages with BM involvement. Alternatively, granulomas may be present, suggesting the possible presence of mycobacterial disease. In children, disseminated neuroblastoma and rhabdomyosarcoma occasionally can appear as a myelophthisic anemia.

FUTURE DIRECTIONS

Anemia may represent a primary hematologic disorder or may represent the manifestation of a systemic process. In children, the former tends to be somewhat more common than the latter, and in adults, the converse is true. However, in both children and adults, a systematic approach to the evaluation of anemia that includes careful review of historic features, the CBC count, and peripheral smear facilitates an efficient diagnosis and minimizes unnecessary testing.

REFERENCES

1. Kassebaum NJ, Jasrasaria R, Naghavi M, et al. A systematic analysis of global anemia burden from 1990 to 2010. *Blood*. 2014;123:615.
2. Vichinsky EP. Changing patterns of thalassemia worldwide. *Ann N Y Acad Sci*. 2015;1054:18.
3. Cantor AB, Orkin SH. Transcriptional regulation of erythropoiesis: an affair involving multiple partners. *Oncogene*. 2002;13:3368.
4. Rosse W. The spleen as a filter. *N Engl J Med*. 1987;317:704.
5. Semenza GL. Involvement of oxygen-sensing pathways in physiologic and pathophysiologic erythropoiesis. *Blood*. 2009;114 2015.
6. Hoffbrand AV, Herbert V. Nutritional anemias. *Semin Hematol*. 1999; 36:13.
7. Kelly A, Munan L. Haematologic profile of natural populations: red cell parameters. *Br J Haematol*. 1977;35:153.
8. Nilsson-Ehle H, Jagenburg R, Landahl S, et al. Blood haemoglobin values in the elderly: Implications for reverence intervals from age 70 to 88. *Eur J Haematol*. 2000;65:297.
9. Centers for Disease Control and Prevention Iron deficiency—United States, 1999–2000. *MMWR Morb Mortal Wkly Rep*. 2002;51:897.
10. Fishbane S. Anemia treatment in chronic renal insufficiency. *Semin Nephrol*. 2002;22:474.
11. Roy CN, Weinstein DA, Andrews NC. E. Mead Johnnson Award for Research in Pediatrics Lecture: The molecular biology of the anemia of chronic disease: a hypothesis. *Pediatr Res*. 2002;53:507 2003.
12. Tabbara IA. Hemolytic anemias: diagnosis and management. *Med Clin North Am*. 1992;76:649.
13. Beutler E. Glucose-6-phosphate dehydrogenase deficiency: a historical perspective. *Blood*. 2008;111:16.
14. Gehrs BC, Freidberg RC. Autoimmune hemolytic anemia. *Am J Hematol*. 2002;69:258.
15. Freedman MH, Saunders EF. Transient erythroblastopenia of childhood: varied pathogenesis. *Am J Hematol*. 1983;14:247.
16. Lipton JM, Ellis SR. Diamond-Blackfan anemia: diagnosis, treatment, and molecular pathogenesis. *Hematol Oncol Clin North Am*. 2009;23:261.
17. Atweh GF, DeSimone J, Saunthararajah Y, et al. Hemoglobinopathies. *Hematology Am Soc Hematol Educ Program*. 2003:14.
18. Boyce TG, Swerdlow DL, Griffin PM. *Escherichia coli* O157:H7 and the hemolytic-uremic syndrome. *N Engl J Med*. 1995;333:364.
19. Lee AI, Kaufman RM. Transfusion medicine and the pregnant patient. *Hematol Oncol Clin North Am*. 2011;25:393.
20. Sawada K, Fujishima N, Hirokawa M. Acquired pure red cell aplasia: updated review of treatment. *Br J Haematol*. 2008;142:505.
21. Tse WT, Lux SE. Red blood cell membrane disorders. *Br J Haematol*. 1999;104:2.
22. Ammus S, Yunis AA. Drug-induced red cell dyscrasias. *Blood Rev*. 1989;3:71.
23. George JN. Evaluation and management of patients with thrombotic thrombocytopenic purpura. *J Intensive Care Med*. 2007;22:82.
24. Moore Jr DF, Sears DA. Pica, iron deficiency, and the medical history. *Am J Med*. 1994;97:390.
25. Lam BK, Cosgrove DM, Bhudia SK, et al. Hemolysis after mitral valve repair: mechanisms and treatment. *Ann Thorac Surg*. 2004;77:191.
26. Lindenbaum J, Healton EB, Savage DG. Neuropsychiatric disorders caused by cobalamin deficiency in the absence of anemia or macrocytosis. *N Engl J Med*. 1720;1988:318.
27. Corberand JX. Reticulocyte analysis using flow cytometry. *Hematol Cell Ther*. 1996;38:487.
28. Lombarts AJ, Koevoet AL, Leijnse B. Basic principles and problems of haemocytometry. *Ann Clin Biochem*. 1986;23:390.
29. Bain B. Diagnosis from the blood smear. *N Engl J Med*. 2005;353:498.
30. Hyun BH, Stevenson AJ, Hanau CA. Fundamentals of bone marrow examination. *Hematol Oncol Clin North Am*. 1994;8:651.

CHAPTER 36

IRON HOMEOSTASIS AND ITS DISORDERS

Tomas Ganz

Iron is an essential trace element required for energy production, oxygen transport and utilization, and cellular proliferation. Iron is a catalyst, acting as an electron donor and an electron acceptor by readily interconverting between ferric (Fe^{3+}) and ferrous (Fe^{2+}) forms. Iron functions mainly in association with proteins, either within prosthetic groups (heme, iron-sulfur clusters) or bound directly by amino acid side chains. Important heme-containing proteins include the oxygen carrier hemoglobin, oxygen storage protein myoglobin, and redox enzymes called cytochromes. Iron-sulfur cluster proteins are prominently involved in mitochondrial energy metabolism. Proteins that directly coordinate iron include a family of prolyl hydroxylases that stabilize collagen and mediate oxygen sensing. The ease with which iron can gain and lose electrons also allows the pathological formation of highly reactive oxygen species that can damage lipids, proteins, and DNA and injure subcellular organelles, resulting in cellular dysfunction, apoptosis, and necrosis. Animals have evolved mechanisms that tightly control total body iron and the amount of iron within each cell to ensure adequate iron availability and avoid its toxicity.

Humans have no regulated means for iron excretion, and obligatory losses are normally minuscule, 1 to 2 mg/day or less than 0.05% of the total body iron each day. The amount of body iron is therefore determined by controlling iron absorption. Human iron homeostasis is dependent on the highly efficient recycling of iron (Fig. 36.1).

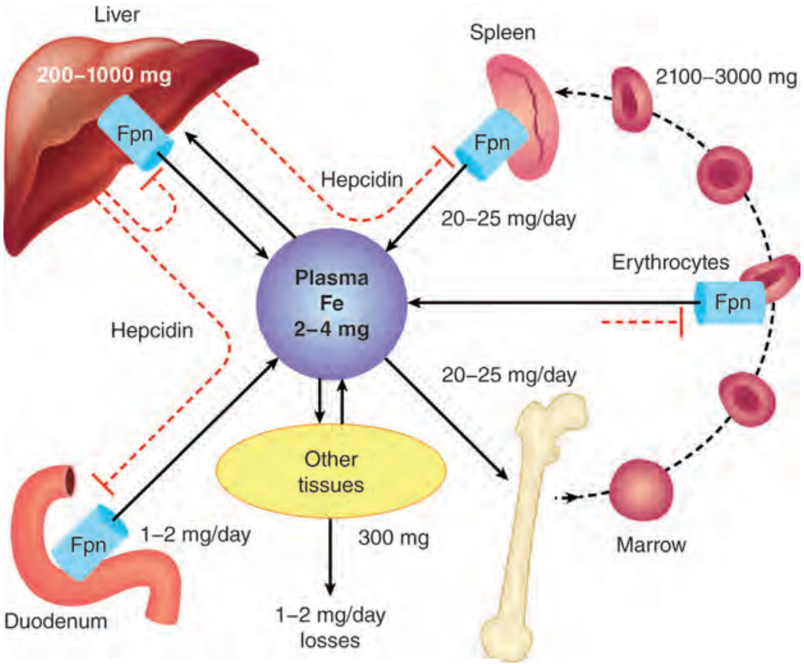
Although all cells require iron, most of the iron in the body is found in the hemoglobin of erythroid cells, and most of the daily movement of iron (approximately 80%) cycles through the erythroid compartment. Iron contained in nonerythroid cells is also recycled. The normal losses of iron of about 1 to 2 mg/day are attributable to iron contained within desquamated epithelial cells from the skin and the intestinal tract. These losses are physiologically compensated by the absorption of similar amounts of dietary iron in the duodenum. In women who menstruate, the losses of iron can be higher, and compensation for them requires more iron absorption.

Iron bound to the plasma iron carrier protein *transferrin* is carried into the erythroid marrow and incorporated into hemoglobin, thereby entering the circulation within red blood cells (RBCs). At the end of their lifespan, normally about 120 days, RBCs are phagocytized by a select population of macrophages in the spleen, liver, and marrow. Macrophages degrade the RBCs, releasing their iron to plasma transferrin. Hepatocytes and some macrophages in the liver, spleen, and marrow function as a dynamic storage compartment for iron. After examining the intricate interrelationship between intracellular and systemic iron homeostasis, this chapter considers in turn each portion of the pathway of iron transport, use, storage, and absorption (see Fig. 36.1). Altogether, iron homeostasis is maintained by effective use of iron for erythropoiesis, efficient recycling of iron from senescent erythrocytes, hepcidin-controlled storage and release of iron by macrophages and hepatocytes, and fine regulation of intestinal iron absorption.[1–3]

REGULATION OF CELLULAR AND SYSTEMIC IRON HOMEOSTASIS

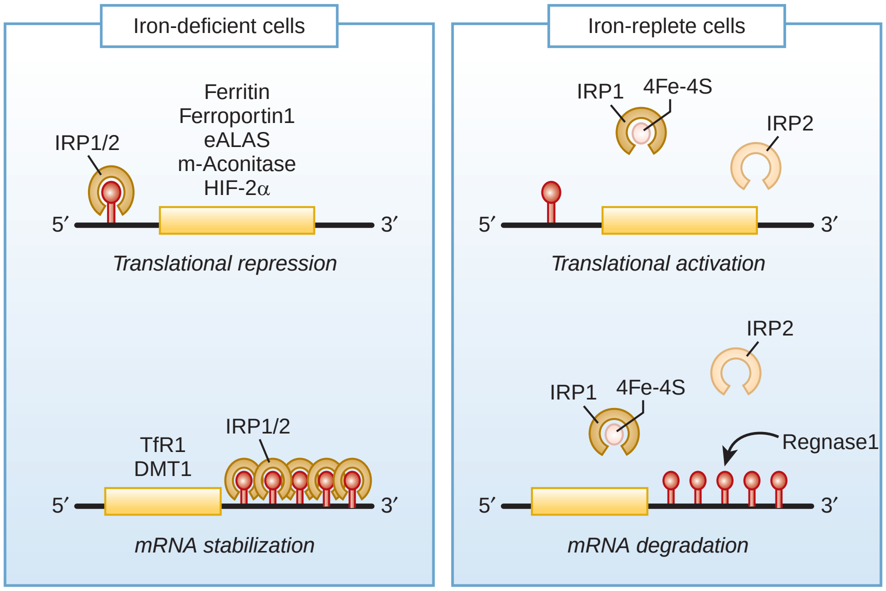
Each cell in the body regulates its iron uptake, content, and utilization. The transport protein *transferrin* functions as the physiologic carrier of iron in the plasma and extracellular fluid. Each cell captures its share of circulating transferrin-bound iron using *transferrin receptor 1*, a glycoprotein on cell membranes that binds the transferrin-iron complex. The transferrin receptor with its cargo is internalized in an endocytic vesicle, where iron is released, and then the complex of the receptor and iron-free (apo)transferrin returns to the cell membrane, liberating apotransferrin into the plasma.[4–6] Within the cell, the iron released from the endosome is either used for the synthesis of iron-containing biomolecules or stored in cytosolic *ferritin*, a protein that holds iron in a nontoxic form ready for mobilization when needed.[7] The main determinant of iron uptake by each cell is the number of transferrin receptors on the cell surface. Within each cell, the intracellular availability of iron homeostatically regulates its cellular uptake and storage through the iron-regulatory proteins 1 and 2 (IRP1 and IRP2) whose levels are negatively regulated by available intracellular iron (Fig. 36.2). When iron is available in the cytoplasm, IRP1 acquires an iron-sulfur cluster to become a cytoplasmic enzyme, aconitase, and loses its iron-regulatory function, while IRP2 is degraded by an iron-dependent process. When available iron is low, the IRP concentrations rise and IRPs bind to RNA stem–loop structures called iron-responsive elements (IREs).[8] IREs within the 3′-untranslated region of a messenger (m) RNA (e.g., transferrin receptor 1 mRNA) bind IRPs that protect the bound mRNA from degradation, making more mRNA available for translation, thus *increasing* protein production. Under the same conditions, IRP binding to IREs located in the 5′-untranslated region of an mRNA (e.g., cytosolic ferritin mRNA) *impedes* protein translation. Accordingly, a *decrease* in intracellular iron availability *enhances* transferrin receptor 1 protein synthesis, increasing iron import, and *reduces* cytosolic ferritin and iron storage. Conversely, an *increase* in intracellular iron availability reduces transferrin receptor 1 protein synthesis, inhibiting iron import, and augments cytosolic ferritin protein production and iron storage. In iron-replete cells with sufficient oxygen, F box and leucine-rich repeat protein 5 (FBXL5), a subunit of a ubiquitin ligase complex, monitors cytosolic iron and initiates iron-dependent degradation of iron-regulatory protein 2.[9] Another system that contributes to cellular iron homeostasis is the regulated release of ferritin iron stores via a process called ferritinophagy. Here, a cargo carrier protein NCOA4 (nuclear receptor coactivator 4) delivers ferritin to the lysosomal compartment for the breakdown and release of its iron content. When cellular iron levels are high, NCOA4 itself is targeted for iron-dependent degradation via the ubiquitin proteasome system, so that NCOA4 concentrations in the cytoplasm are low, decreasing ferritinophagy and increasing ferritin iron storage. Conversely, under low cellular iron conditions, NCOA4 is increased, enhancing ferritinophagy and releasing more iron from ferritin to restore cellular iron levels.[10]

Altogether, *regulation of intracellular iron homeostasis* is mediated principally through iron-regulatory proteins 1 and 2 by their reciprocal control of the synthesis of transferrin receptor and ferritin and, in specialized cells, by controlling the synthesis of other essential proteins involved in iron homeostasis, including erythroid δ-aminolevulinic acid synthase 2 (eALAS), mitochondrial aconitase, hypoxia-inducible factor 2α (HIF-2α), intestinal divalent metal transporter 1 (DMT1) isoform I, and ferroportin.[11] The NCOA4 system and other iron-dependent regulatory processes also contribute to cellular iron homeostasis.[10]



Liver
200–1000 mg
Fpn
Spleen
2100–3000 mg
Fpn
Hepcidin
20–25 mg/day
Erythrocytes
Fpn
Plasma Fe 2–4 mg
Hepcidin
20–25 mg/day
Other tissues
300 mg
Fpn
1–2 mg/day
Duodenum
1–2 mg/day losses
Marrow

Figure 36.1 BODY IRON SUPPLY AND STORAGE. A schematic representation of the iron economy of the body and the routes of iron movement *(black arrows)* in an adult. Iron flows into plasma include iron efflux from splenic and hepatic macrophages involved in recycling of erythrocyte iron, from hepatocytes that store iron and duodenal enterocytes that absorb iron from the diet, and from erythroblasts and erythrocytes that release excess iron. By far the largest movement of iron is generated by recycling senescent erythrocytes in the splenic and hepatic macrophages, exporting the iron to plasma transferrin and returning the iron to the marrow to generate new erythrocytes. Smaller amounts of iron are recycled in other tissues. The liver (and to a lesser extent the spleen) functions as a storage organ for iron. Duodenal iron absorption normally balances out small losses, mainly from epithelial shedding. Hepcidin controls the iron outflows into plasma through ferroportin (Fpn), as indicated by *dashed red lines*.



Iron-deficient cells
Ferritin
Ferroportin1
eALAS
m-Aconitase
HIF-2α
IRP1/2
5′
3′
Translational repression
TfR1
DMT1
IRP1/2
5′
3′
mRNA stabilization
Iron-replete cells
IRP1
4Fe-4S
IRP2
5′
3′
Translational activation
IRP2
IRP1
4Fe-4S
Regnase1
5′
3′
mRNA degradation

Figure 36.2 REGULATION OF CELLULAR IRON HOMEOSTASIS BY THE IRON-REGULATORY PROTEINS (IRP1 AND IRP2). The iron-regulatory proteins bind to RNA stem–loop structures called iron-responsive elements (IREs) when iron is absent and dissociate when iron is present. Binding IREs within the 3′-untranslated region of mRNA (e.g., transferrin receptor 1 [TfR1]) and some intestinal divalent metal transporter 1 (DMT1) isoforms increases mRNA stability, increasing protein synthesis. In contrast, binding IREs in the 5′-untranslated region of mRNA (e.g., cytosolic ferritin, ferroportin 1, erythroid aminolevulinic acid synthase [eALAS], mitochondrial aconitase [m-Aconitase], and hypoxia-inducible factor 2α [HIF-2α]) inhibits protein expression when iron is absent. In iron-replete cells, IRP1 assembles a cubane Fe/S cluster, acquiring aconitase activity while losing the ability to bind to IREs. In iron-replete cells, IRP2 interacts with F box and leucine-rich repeat protein 5 (FBXL5), a subunit of a ubiquitin ligase complex, leading to its ubiquitination and degradation by the proteasome. See text for details. *(Reproduced with permission from Wallander ML, Leibold EA, Eisenstein RS. Molecular control of vertebrate iron homeostasis by iron-regulatory proteins. Biochim Biophys Acta.) 2006;1763:668.)*

Regulation of organismal iron content and tissue distribution is accomplished by the control of the entry of iron into plasma for transport by transferrin.[1–3] Circulating iron is delivered to transferrin by specialized cells that can export iron, primarily splenic and hepatic macrophages that recycle iron from senescent RBCs ("iron-recycling macrophages"), hepatocytes that can mobilize iron from stores, and duodenal enterocytes that provide iron absorbed from the diet. To enter plasma, iron in these cells must pass through *ferroportin* (SLC40A1, a member of the solute carrier family of transporters), a 12-transmembrane-segment protein that is the sole known cellular iron exporter.[12] *Hepcidin*, a small 25-amino acid peptide hormone secreted principally by hepatocytes, posttranslationally controls ferroportin iron transport and ferroportin membrane concentration. Hepcidin acts by binding to and occluding ferroportin and inducing its internalization, ubiquitination, and degradation, thereby inhibiting iron export from duodenal enterocytes, iron-recycling macrophages, and iron-storing hepatocytes to plasma (Fig. 36.3).[13] Hepatic hepcidin synthesis is stimulated by increases in body iron stores, plasma iron concentration, infection, and inflammation and is inhibited by hypoxemia and increased erythropoietic demand.[14] The amount of iron in plasma in adults at any given time is only about 2 to 4 mg, while the daily amount of iron required for erythropoiesis is about 20 to 25 mg, so the iron in plasma turns over approximately every 2 to 4 hours. Increments in plasma hepcidin reduce the export capacity of ferroportin and the amount of ferroportin in cell membranes, thereby decreasing iron delivery to plasma. Continuing iron consumption for erythropoiesis and other processes then causes a prompt fall in plasma iron concentration as the small amounts of iron in plasma are rapidly depleted. Conversely, decrements in plasma hepcidin concentration increase the amount of ferroportin, producing a rise in plasma iron concentration.

Less well understood is the role of microRNAs, short (approximately 22 nucleotides), noncoding RNAs that act as antisense regulators of target RNAs, providing a further degree of control of both cellular and systemic iron homeostasis.[15]

USE OF IRON FOR ERYTHROPOIESIS

The major traffic of iron in the body is to the erythroid marrow (Fig. 36.4). Each day, almost 200 billion RBCs are produced in a normal adult to replace a similar number reaching the end of their lifespan, amounting to about 24 mL of packed RBC/day containing about 24 mg of iron. Erythroid precursors and most other iron-utilizing cells require iron complexed by transferrin.[4] Apotransferrin, a form of transferrin not carrying iron, is a single-chain glycoprotein with two structurally similar lobes. The binding of a ferric (Fe^{3+}) ion to one of these lobes yields monoferric transferrin; binding of ions to both yields diferric transferrin. The transferrin saturation is the proportion of the available iron-binding sites on transferrin that are occupied by iron atoms, expressed as a percentage. In humans, almost all circulating plasma apotransferrin is synthesized by hepatocytes. Apotransferrin is not lost in delivering iron but is reused repeatedly as the half-life of the protein is about 8 days. After delivering iron to cells, apotransferrin is promptly returned to the plasma to function again as an iron transporter, completing 100 to 200 cycles of iron delivery during its lifetime in the circulation.

Transferrin receptors on the cell surface bind monoferric or diferric transferrin. Two different forms of the transferrin receptor exist, encoded by two separate genes. Transferrin receptor 1 is ubiquitously expressed and functions as the physiologic transferrin iron importer on all iron-requiring cells. Transferrin receptor 2 is expressed in hepatocytes, functioning in the control of iron supply by regulating hepcidin expression (see later), and in erythroid precursors,[4] coordinating erythropoiesis with iron availability (see later). Transferrin receptor 1 is a transmembrane glycoprotein dimer composed of two identical subunits linked by a disulfide bond. Each transferrin receptor 1 can bind two molecules of transferrin; if each transferrin is diferric, the dimeric receptor can carry a total of four atoms of transferrin-bound iron. The affinity of transferrin receptor 1 for transferrin depends both on the iron content of transferrin and on the pH. Transferrin

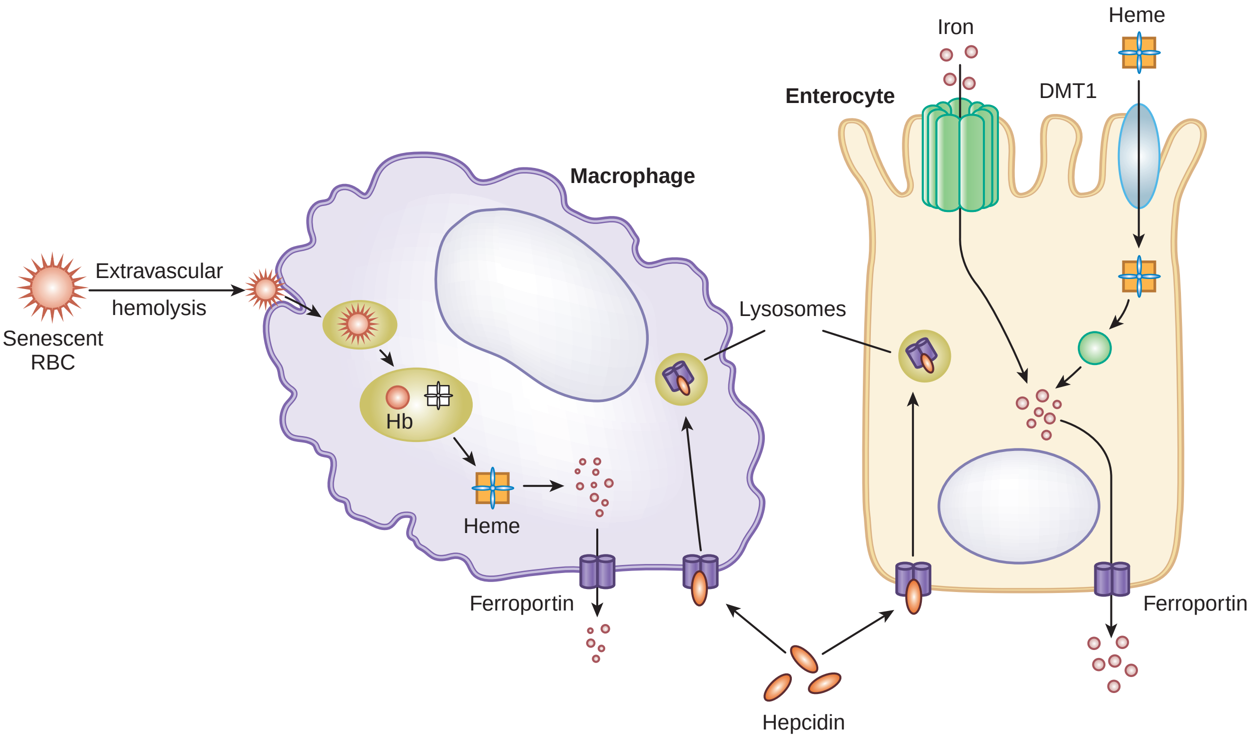


Figure 36.3 CONTROL OF IRON ENTRY INTO PLASMA BY FERROPORTIN AND HEPCIDIN IN THE REGULATION OF SYSTEMIC IRON HOMEOSTASIS. Ferroportin, a multitransmembrane-spanning protein that is the only known iron exporter in humans, is expressed at high concentrations on the basolateral membrane of duodenal enterocytes, reticuloendothelial macrophages, and hepatocytes (not shown). Plasma hepcidin binds to a site in the cavity of the "open out" conformation of ferroportin, occluding iron transport and inducing a conformational change that exposes a cytoplasmic loop for ubiquitination, leading to endocytosis and subsequent degradation of ferroportin in the lysosome. See text for details. *DMT1*, Divalent metal transporter 1; *Hb*, hemoglobin; *RBC*, red blood cells.

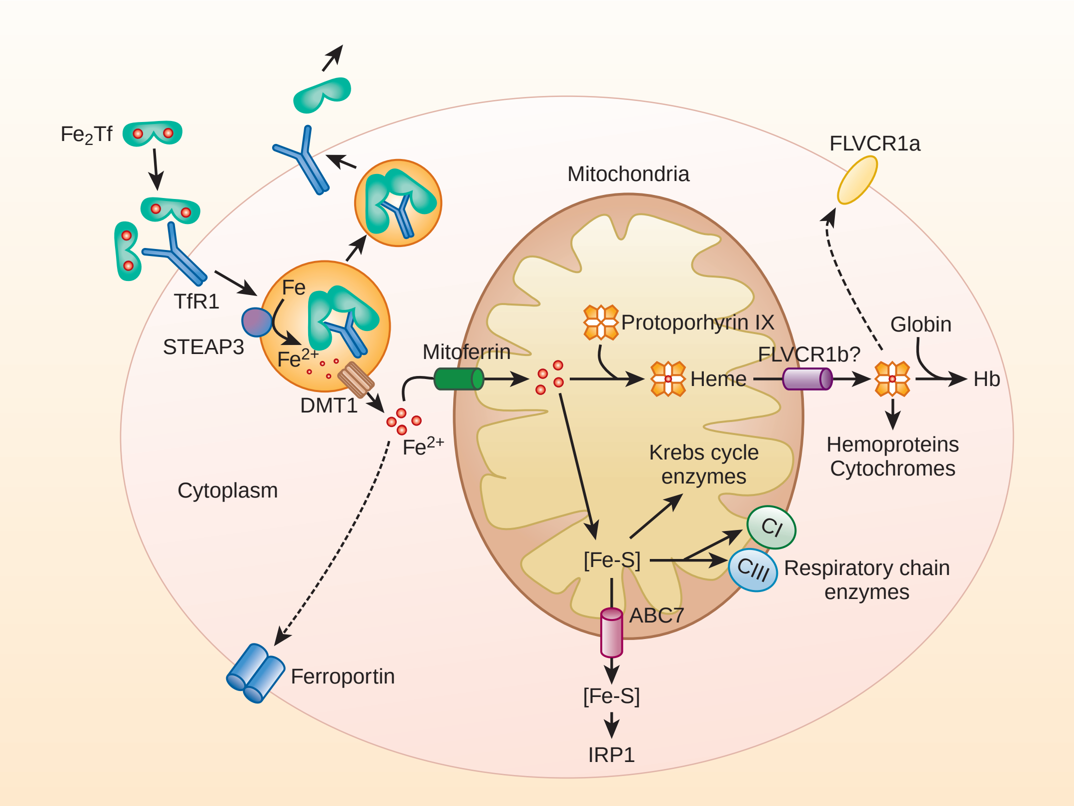


Figure 36.4 ACQUISITION AND USE OF IRON BY ERYTHROID PRECURSORS. Iron is imported in the transferrin (Tf) cycle and principally used for the synthesis of heme. See text for details. *ABC7*, Adenosine triphosphate–binding cassette, subfamily B *(MDR/TAP)*, member 7; *DMT1*, divalent metal transporter 1; *Fe_2Tf*, diferric transferrin; *FLVCR*, feline leukemia virus subgroup C cellular receptor; *Hb*, hemoglobin; *IRP1*, iron-regulatory protein 1; *STEAP3*, six-transmembrane epithelial antigen of the prostate 3; *TfR1*, transferrin receptor 1. *(Modified with permission from Beaumont C, Delaby C. Recycling iron in normal and pathological states. Semin Hematol. 2009;46:328.)*

receptor 1 has a low affinity for apotransferrin; an intermediate affinity for monoferric transferrin; and the highest affinity for diferric transferrin, estimated at 2×10^{-9} to 7×10^{-9} M. Under physiologic conditions, the affinity of transferrin receptor 1 for diferric transferrin is more than fourfold greater than that for monoferric transferrin. At a pH of about 5 in the endosome, the affinity of transferrin receptor 1 for apotransferrin increases to that of diferric transferrin.

Iron delivery to an erythroid cell (see Fig. 36.4) begins with the binding of one or two molecules of monoferric or diferric transferrin to transferrin receptor 1.[4] The efficiency of iron delivery to the cell depends on the amounts of monoferric and diferric plasma transferrin available, with the iron distributed between the two forms according to the principles of chemical equilibrium, which also govern the interaction of the two forms with transferrin receptor 1. During normal erythropoiesis and a normal transferrin saturation of about 33%, most of the iron supply to cells is derived from diferric transferrin, providing four atoms of iron with each cycle. At a transferrin saturation of about 19%, equal amounts of iron are provided by monoferric and diferric transferrin; at lower saturations, most of the iron is derived from the monoferric form. Whether monoferric or diferric, the fate of transferrin bound to the transferrin receptor is the same. When bound, the iron-bearing transferrin–receptor complex rapidly clusters with other transferrin–receptor complexes in a clathrin-coated pit that is promptly internalized. Within the cytoplasm, the coated vesicle is rapidly stripped of clathrin, and the uncoated vesicles fuse to become multivesicular endosomes. A proton pump then acidifies the endosome internal pH to less than 6. In the acidic environment of the endosome, both transferrin and transferrin receptor 1 undergo conformational changes that cause iron release. The released ferric iron is reduced by the ferrireductase six-transmembrane **e**pithelial antigen of the prostate 3 (STEAP3) to the ferrous form and then transported across the endosomal membrane through DMT1 (SLC11A2). Low pH in the endosome increases the affinity of the now iron-free apotransferrin for the transferrin receptor so that the complex remains intact as it is transported back to the cell surface within the endosome. On exposure to the neutral pH of the plasma, the apotransferrin loses its affinity for the transferrin receptor and is released from the membrane, making both the apotransferrin and the transferrin receptor 1 available for reuse (see Fig. 36.4).

Most of the iron transported across the endosomal membrane through DMT1 is then directed to the mitochondria for use in the synthesis of heme and iron-sulfur clusters (see Fig. 36.4) with the rest destined for cytoplasmic proteins that coordinate iron through their amino acid side chains and for cytoplasmic ferritin that stores an inorganic iron complex in the cavity of a shell-like structure.

Iron can be imported from the cytosol across the mitochondrial membrane by the transmembrane protein mitoferrin 1 (MFRN1; SLC25A37). Transport of iron from endosomes into mitochondria for heme synthesis by direct contact between the organelles ("kiss and run"), avoiding the cytosol, also has been proposed. Heme (ferrous protoporphyrin IX), a planar molecule consisting of an atom of ferrous iron in the center of a tetrapyrrole ring, is then synthesized in eight biochemical reactions, with the first and final three reactions catalyzed by mitochondrial enzymes and the four intermediate reactions taking place in the cytoplasm (see Chapter 39). Most heme is then bound to α- or β-globin subunits that combine to form α–β dimers that in turn join to form the functional α_2-β_2-tetramer of hemoglobin (see Fig. 36.4). Small amounts of heme are incorporated into heme enzymes and cytochromes. Iron is also used for another class of prosthetic groups, iron-sulfur clusters, assembled within mitochondria and in the cytosol (see Fig. 36.4).[16] The cytosolic iron chaperones poly(rC)-binding proteins 1 and 2 (PCBP1, PCBP2) may ferry surplus iron to multiple intracellular destinations: cytosolic ferritin for storage, to some cytosolic nonheme enzymes, and in specialized cell types for export into plasma to meet the iron needs of the organism.[17] Transferrin receptor 2 (TfR2), which binds iron-loaded transferrin with an affinity some 25-fold less than that of transferrin receptor 1, functions as a sensor of iron bound to transferrin and is not involved in cellular iron uptake. In erythroid precursors, transferrin receptor 2 coordinates erythropoiesis with iron availability, a vital mechanism for adaptation to iron deficiency.[18] Transferrin receptor 2, a component of the erythropoietin receptor complex, stabilizes the receptor on the cell surface and modulates the sensitivity of the developing erythroid cells to erythropoietin. By simultaneously sensing the concentration of iron-loaded transferrin in developing erythroid cells and in hepatocytes (see later), transferrin receptor 2 permits erythropoiesis to adapt to the level of the iron supply while modulating iron absorption and the release of iron from stores to meet the erythropoietic (and organismal) requirement for iron.[18] Erythroid precursors have multiple other mechanisms to coordinate their iron usage with systemic iron availability, to conserve iron, and to share excess iron with the rest of the organism. These mechanisms likely evolved to avoid the adverse consequences of the erythropoietic system using more than its share of iron at the expense of the rest of the organism.

- Reduction of responsiveness to erythropoietin: Iron regulation of erythroid differentiation helps match the rate of erythropoiesis to iron supply. With iron deficiency, an TfR2-dependent pathway also reduces the responsiveness of erythroid progenitors to erythropoietin.[19] During iron deficiency, decreased erythroid use for RBC production helps preserve the supply of iron for vital functions in other tissues.
- Inhibition of erythroid heme synthesis: Heme synthesis is coordinated with iron availability through an iron-regulatory element in the 5′-untranslated region of the mRNA for eALAS, the erythroid-specific initial enzyme in the heme synthetic pathway. If intracellular iron availability is low, binding of an iron-regulatory protein will inhibit heme synthesis by preventing translation of the mRNA.[11]
- Inhibition of globin and other protein translation: If the lack of iron leads to heme deficiency, the heme-regulated translational inhibitor (HRI) is activated and, acting through the α-subunit of eukaryotic initiation factor 2, slows most protein synthesis to coordinate the translation of globin mRNAs with the intracellular heme concentration. This action of the HRI is responsible for the physiologic adaptation that produces hypochromic, microcytic erythrocytes in iron deficiency. More recent data indicate that mTorc1 (mechanistic target of rapamycin complex 1), the master regulator of cell growth in proportion to available nutrients, coordinates with HRI to regulate the synthesis of cellular macromolecules and thereby determines the size of erythrocytes.[20]
- Export of iron through ferroportin: Developing erythroblasts synthesize ferroportin to export iron. Their expression of ferroportin is regulated principally by hepcidin, providing another means to coordinate erythroid iron use with systemic iron availability. In erythroid precursors (and in duodenal enterocytes; see later), two ferroportin transcripts are present: the ubiquitously expressed ferroportin (FPN1A), with an iron-responsive element in its 5′ untranslated region, and FPN1B, which lacks the iron-responsive element. During erythroid cell differentiation, FPN1B is therefore not subject to translational repression by the iron-regulatory protein system, thereby permitting the export of iron from erythroid precursor cells during the critical period when cells commit to proliferation and differentiation, express high levels of transferrin receptor 1, and rapidly accumulate iron. As a consequence, erythropoiesis may be partially suppressed when nonerythropoietic tissues are at risk for iron deficiency. Iron export from erythroblasts via FPN1B may account for the development of iron deficiency anemia as an initial, early manifestation of systemic iron deficiency. Nonetheless, when the cells begin to produce hemoglobin, FPN1B expression diminishes and FPN1A predominates, allowing erythroid cells to limit iron export through the iron-responsive element iron-regulatory protein system and to efficiently manufacture heme.
- Export of excess heme: Erythroblasts have the capacity to export excess heme through the feline leukemia virus subgroup C cellular receptor and avoid heme toxicity.[21]
- Storage of excess iron: Cytosolic ferritin can be synthesized and utilized to deposit surplus iron in a safe form from which iron can be recovered when needed. Also, a mitochondrial ferritin, consisting of homopolymers of a nuclear gene-encoded H-type ferritin (see later), can be expressed to protect against mitochondrial iron accumulation in sideroblastic anemia and some other disorders.
- Recovery of the nonfunctional iron content during terminal differentiation: Orthochromatic erythroblasts, with nuclei that are unable to synthesize DNA, gradually lose most mitochondria and halt RNA synthesis but continue to produce hemoglobin. The pyknotic nucleus is finally extruded through the erythroblast membrane with the loss of about 5% to 10% of the hemoglobin that had been synthesized previously, with the extruded material recycled by erythroblastic island macrophages.[22] The resultant reticulocyte continues to synthesize hemoglobin for another 2 to 3 days until the cellular supply of mRNA is exhausted, producing as much as 30% of the total hemoglobin complement of the RBC. Eventually, the reticulocyte is released from the marrow, remodeled, and pitted of siderotic granules and debris within the spleen, and these excess materials are recycled by splenic macrophages.

RECYCLING OF ERYTHROCYTE IRON BY MACROPHAGES

The normal lifespan of erythrocytes is approximately 120 days. During their time in the bloodstream, RBCs undergo progressive modifications (oxidant damage, metabolic depletion, increasing intracellular calcium concentrations, dehydration, decrease in cell volume, phosphatidylserine exposure, formation of "senescent" antigens, and others) that lead to their selective removal by specialized recycling macrophages in the liver, and spleen (Fig. 36.5).[23] Macrophages in the bone marrow also cull defective immature erythroid cells to prevent their release into the circulation and remove some deposits of erythrocyte ferritin from developing RBCs.

Quality control of RBCs is also performed by the spleen as evidenced after splenectomy by the appearance of abnormal erythrocytes in circulation. On average, each of these iron-recycling macrophages can phagocytize one erythrocyte per day. After ingesting the erythrocyte in a phagosomal vacuole known as an *erythrophagolysosome*, the erythrocyte membrane is lysed. The hemoglobin within then undergoes oxidative precipitation. proteolysis and liberation of heme (see Fig. 36.5). Heme is then transported from the erythrophagolysosome into the cytosol via the heme transporter HRG1 (SLC48A1), a heme-transporting permease.[24]

A small proportion of aged or damaged erythrocytes undergo intravascular hemolysis. With normal erythropoiesis, this portion of the total iron flux is minor but can increase substantially in disorders with increased ineffective erythropoiesis or intravascular hemolysis. The hemoglobin released into plasma is rapidly bound by haptoglobin, a glycoprotein synthesized in the liver.[25] The hemoglobin–haptoglobin complex (M_r 150,000) is too large to be filtered by the kidneys, a feature that helps restrict the renal loss of iron with hemoglobinemia. Macrophages (and hepatocytes; see later) remove the haptoglobin–hemoglobin complex from plasma by binding through the cluster of differentiation 163 (CD163) receptor and after endocytosis digest the complex in lysosomes, liberating heme. In an analogous fashion, any heme released into plasma by intravascular hemolysis complexes with hemopexin and is removed by macrophages (and hepatocytes) expressing the low-density lipoprotein receptor–related protein 1 (LRP1).[25]

In macrophages, heme from all these sources is degraded by an enzymatic complex containing nicotinamide adenine dinucleotide phosphate–cytochrome c reductase, the microsomal enzyme heme oxygenase 1, and biliverdin reductase, yielding carbon monoxide (this

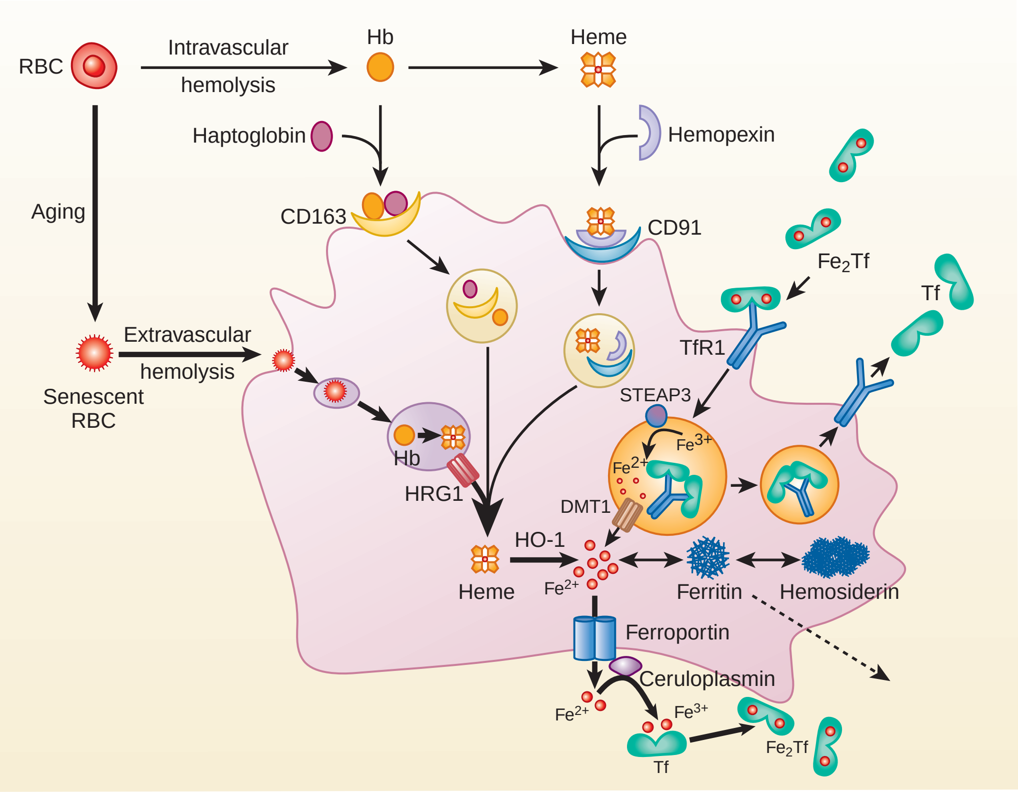


Figure 36.5 RECYCLING OF ERYTHROCYTE IRON BY MACROPHAGES. Most erythrocyte iron is acquired by erythrophagocytosis of senescent red blood cells, but smaller amounts are derived from hemoglobin–haptoglobin and heme–hemopexin complexes. Iron derived from plasma transferrin is a minor portion of the total iron flux. Heme is catabolized, and the iron is exported through ferroportin and oxidized by ceruloplasmin. In the absence of iron deficiency, a portion of the iron is retained as ferritin and hemosiderin. See text for details. *DMT1*, Divalent metal transporter 1; *Fe*$_2$*Tf*, diferric transferrin; *FLVCR*, feline leukemia virus subgroup C cellular receptor; *Hb*, hemoglobin; *HO-1*, heme oxygenase-1; *RBC*, red blood cells; *Tf*, transferrin; *TfR1*, transferrin receptor 1; *HRG1*: heme importer; *STEAP3*, six-transmembrane epithelial antigen of the prostate 3. *(Modified with permission from Beaumont C, Delaby C. Recycling iron in normal and pathological states. Semin Hematol. 2009;46:328.)*

is its sole physiologic source in the body), bilirubin, and iron (see Fig. 36.5). Both DMT1 and natural resistance–associated macrophage protein 1 (NRAMP1; SLC11A1), a DMT expressed within the late endosomal and phagolysosomal membranes of iron-recycling macrophages, seem to be involved in the efficient recycling of this iron. The export of iron from the erythrophagolysosome may occur in parallel with that of heme, considering the evidence that heme export from the macrophage erythrophagolysosome is essential for iron recycling.[24]

Ferroportin is the sole conduit for the export of elemental iron from macrophages in the bone marrow, liver, and spleen to plasma apotransferrin, normally the largest single flux of iron from cells in the body, greatly exceeding the flow of iron from dietary iron absorption in the duodenum. Ferroportin transcription increases in response to both iron and heme. FPN1A levels are also regulated posttranscriptionally through an iron-responsive element in the 5′-untranslated region, with increases in cytosolic iron resulting in increased ferroportin translation. Iron export through ferroportin is facilitated by ferroxidase activity, provided by the multicopper oxidase ceruloplasmin in macrophages and by hephaestin in duodenal enterocytes (see later). Ceruloplasmin oxidation may generate a concentration gradient that helps move the ferric iron out of the macrophage. The ferric iron can then be bound by transferrin and transported back to erythroid and other iron-requiring tissues.

Plasma hepcidin regulates iron efflux from macrophages by decreasing the number of functioning ferroportin molecules available for iron export.[13] The multimeric composition of ferroportin has not been determined definitively, but there is immunohistochemical evidence for a dimeric structure, which is also the simplest explanation for the autosomal dominant inheritance of loss of function ferroportin mutations (see Chapter 37). For the most part, ferroportin mutations either interfere with iron export by decreasing the amount of functional ferroportin on the cell surface, resulting in retention and accumulation of macrophage iron, or produce ferroportin resistance to internalization and degradation by hepcidin, resulting in loss of control of macrophage iron export that leads to parenchymal iron loading. Following hepcidin binding, conformational change and ubiquitination, ferroportin is degraded after entering the multivesicular body that fuses with lysosomes. Hepcidin can also block the transport function of ferroportin directly by occluding the cavity involved in iron transport.[13] This mechanism may be important in cell types that are not actively endocytic, such as mature erythrocytes.

Under normal circumstances, the macrophages in the liver, spleen, and bone marrow that are specialized in reprocessing hemoglobin iron from senescent erythrocytes partition the recovered iron between storage in ferritin and release through ferroportin. Synthesis of cytosolic ferritin is induced in response to erythrophagocytosis, and in the absence of iron deficiency, some of the iron derived from the ingested erythrocyte is retained within the macrophage inside cytosolic ferritin. With increasing amounts of storage iron within the macrophage, an increasing proportion of iron is stored within amorphous, insoluble masses as hemosiderin.

Iron preparations for intravenous use are carbohydrate-coated iron nanoparticles that are effectively targeted to macrophages. These preparations are relatively nontoxic because the release of iron from these nanoparticles to transferrin is subject to the same regulation as the release of iron from erythrophagocytosis. The use of intravenous iron at high doses would be expected to cause macrophage iron loading which accelerates the synthesis of ferroportin, thereby counteracting the depletion of ferroportin by hepcidin and promoting the export of iron.

LIVER REGULATION OF SYSTEMIC IRON HOMEOSTASIS AND IRON STORAGE

The liver (Fig. 36.6) is the central controller of systemic iron homeostasis, the source of plasma hepcidin, and the major iron storage organ.[2] The dual blood supply of the liver from the portal and systemic circulation allows monitoring of both plasma iron in the systemic circulation and newly absorbed iron in the portal circulation. Hepatocytes can acquire iron from plasma transferrin via the transferrin cycle; from hemoglobin–haptoglobin and heme–hemopexin complexes via endocytosis after binding to CD163 and LRP1 receptors, respectively; from lactoferrin, apparently by receptor-mediated endocytosis; and from plasma nontransferrin-bound iron (see Fig. 36.6). Plasma non-transferrin-bound iron forms when the rate of iron influx into plasma exceeds the rate of iron acquisition by transferrin. Plasma non-transferrin-bound plasma iron enters specific cells independently of the transferrin mechanism, particularly hepatocytes, pancreatic acinar cells, cardiomyocytes, and anterior pituitary cells, producing toxic accumulations in some forms of iron overload (see Chapter 37). In mice, the ZRT/IRT-like protein 14 (ZIP14; SLC39A14), whose nonredundant role is as a manganese transporter required for regulating the total amount of manganese in the body, is also the major route for cellular uptake of plasma non-transferrin-bound iron by hepatocytes and pancreatic acinar cells.[26] In the heart, ZIP14 is not required for uptake of plasma non-transferrin-bound iron and iron loading; L- or T-type Ca^{2+} channels are possible alternative transporters.

The liver functions as the central controller of systemic iron homeostasis by being by far the predominant organ secreting hepcidin into blood circulation. The biologically active 25-amino acid peptide is produced by proteolytic processing of an 84-amino acid prepropeptide by furin. After secretion, hepcidin circulates in plasma weakly bound to α_2-macroglobulin and is rapidly cleared by the kidneys or degraded after binding to ferroportin. As detailed earlier, hepcidin controls the entry of iron into plasma by decreasing the number of ferroportin molecules available for iron export from macrophages, hepatocytes, and duodenal enterocytes.

Plasma hepcidin concentrations increase in response to increased iron concentrations in plasma and in response to the amount of iron stored in hepatocytes. A separate regulatory mechanism increases the production of hepcidin during infection or inflammation. Plasma hepcidin concentrations decrease with iron deficiency, hypoxia, and increased erythropoietic requirements for iron, increasing plasma iron. The hepatocyte coordinates the influences of iron, inflammation, and erythropoietic demand to determine hepcidin secretion and thereby the systemic supply of iron. Because plasma iron in the steady state is replaced every 2 to 3 hours, changes in plasma hepcidin that substantially affect iron influx into plasma are followed within a few hours by changes in plasma iron.

Regulation of hepcidin seems to be entirely transcriptional, integrating signals for induction and inhibition of synthesis both from within and outside the hepatocyte.[2] Intensive investigation has revealed a complex and still incompletely characterized signaling network for transcriptional regulation of hepcidin (summarized graphically in Fig. 36.7). The available evidence indicates that major regulators of hepatic hepcidin synthesis include iron (hepatic iron

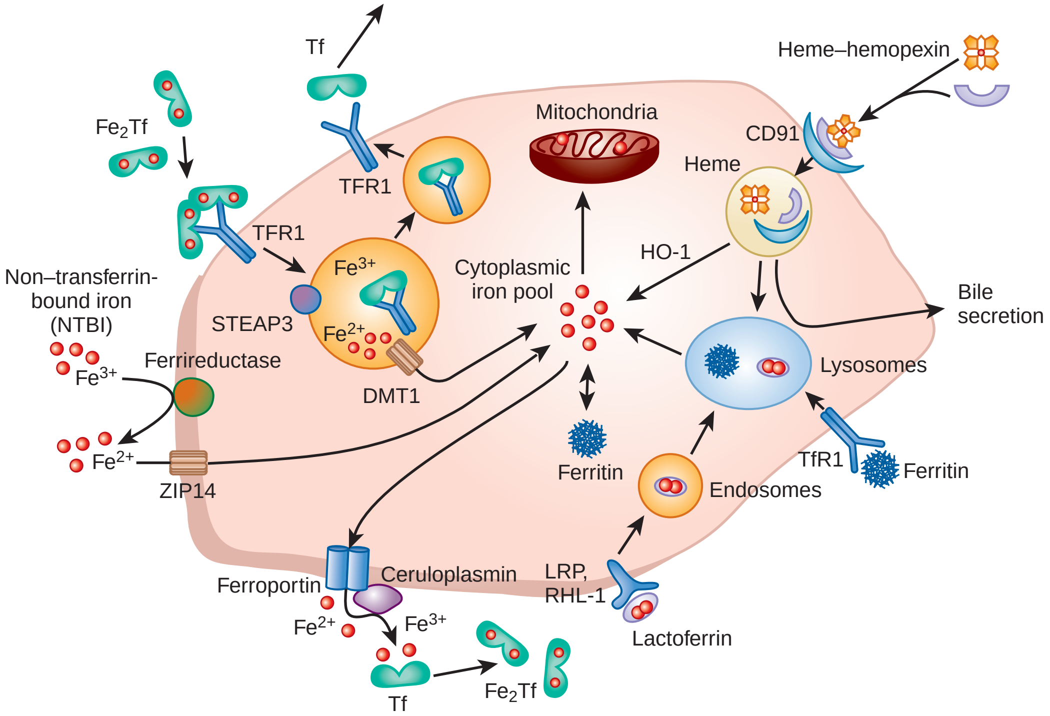


Figure 36.6 ACQUISITION, USE, STORAGE, AND EXPORT OF IRON BY HEPATOCYTES. Hepatocytes can acquire iron from plasma transferrin (Tf) via the Tf cycle; from heme–hemopexin complexes via endocytosis after binding to CD91 receptors; from lactoferrin, apparently by receptor-mediated endocytosis; from iron-rich ferritin by TfR1-mediated endocytosis and from plasma non-Tf-bound iron (NTBI) using ZIP14. Iron is used for the synthesis of heme and nonheme enzymes, with any excess stored in ferritin and hemosiderin. Iron is exported through ferroportin and oxidized by ceruloplasmin before being taken up by plasma Tf. See text for details. *DMT1*, Divalent metal transporter 1; *Fe_2Tf*, diferric transferrin; *FLVCR*, feline leukemia virus subgroup C cellular receptor; *HO-1*, heme oxygenase-1; *LRP*, low-density lipoprotein receptor-related protein; *RHL-1*, rat hepatic lectin-1 subunit of the asialoglycoprotein receptor; *STEAP3*, six-transmembrane epithelial antigen of the prostate 3; *TfR1*, transferrin receptor 1; *ZIP14*, Zrt- and Irt-like protein 14 (SLC39A14, solute carrier family 39, member 14). *(Reproduced with permission from Graham RM, Chua ACG, Herbison CE, et al. Liver iron transport. World J Gastroenterol. 2007;13:4725.)*

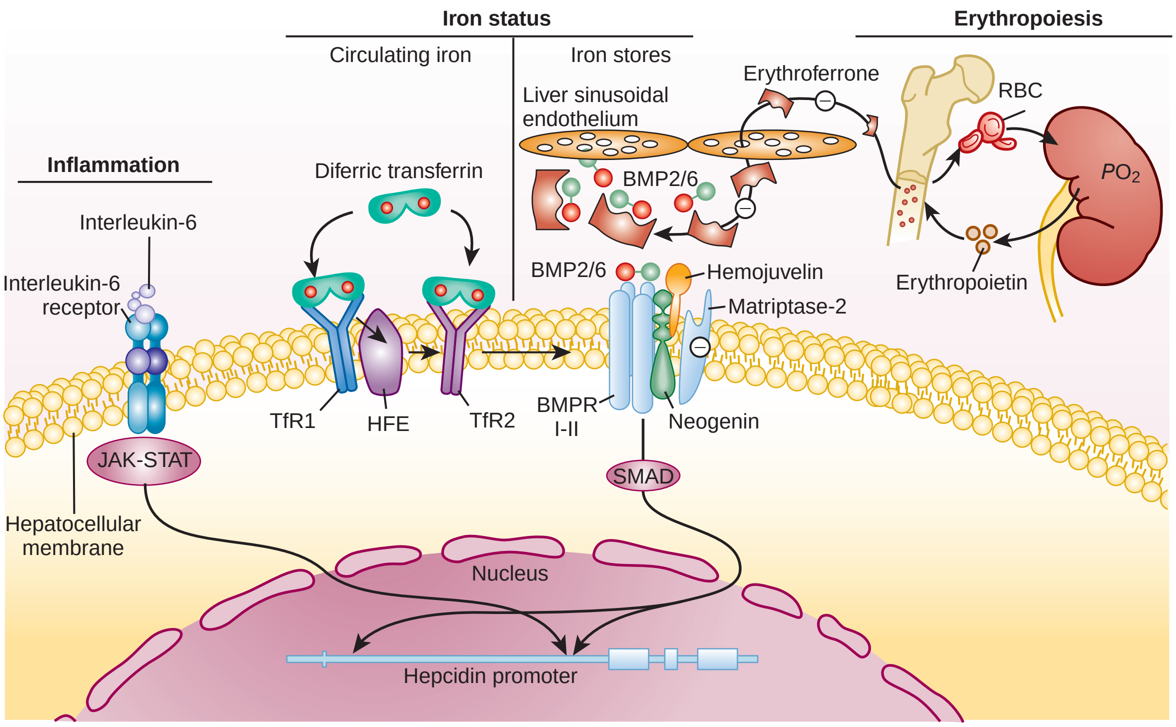


Figure 36.7 TRANSCRIPTIONAL REGULATION OF HEPCIDIN EXPRESSION IN HEPATOCYTES. Hepatic hepcidin synthesis is regulated by iron, erythropoietic iron requirements, inflammation, and endoplasmic reticulum (ER) stress. Bone morphogenetic proteins 2 and 6 (BMP2/6) are the key endogenous regulators of hepcidin synthesis, possibly acting as the BMP2/6 heterodimer. BMP2/6 initiates a signaling cascade by binding to the BMP coreceptor hemojuvelin and to two type I and two type II BMP receptors (BMPR I-II) on the surface of hepatocytes. Neogenin may act to stabilize hemojuvelin. BMP2/6 binding is followed by phosphorylation of sons of mothers against decapentaplegic (SMAD)1/5/8 and formation of the SMAD1/5/8–SMAD4 complex, which translocates to the nucleus and activates the promoter of the hepcidin gene (HAMP). TMPRSS6 (transmembrane protease, serine 6; matriptase-2) inhibits BMP2/6 induction of hepcidin synthesis by cleaving hemojuvelin from the cell membrane. HFE (the hemochromatosis protein) interacts with TfR1 and perhaps also with TfR2 to modulate hepcidin synthesis through the BMP6-HJV-SMAD pathway. The erythroid-derived hormone erythroferrone has been identified as a mediator of hepcidin suppression by stress erythropoiesis and acts by binding to BMP2/6 and preventing its access to the BMP receptor complex. The inflammatory cytokine interleukin-6 induces hepcidin expression through a Janus kinase–signal transducer and activator of transcription (JAK-STAT) signaling pathway. See text for details. *RBC*, red blood cells. *(Modified from Kroot JJ, Tjalsma H, Fleming RE, et al. Hepcidin in human iron disorders: diagnostic implications. Clin Chem. 2011;57:1650.)*

stores, absorbed dietary iron, plasma iron in the systemic circulation), hypoxia, erythropoietic iron requirements, and inflammation and endoplasmic reticulum (ER) stress. Recent studies have found that the control of hepcidin synthesis is further modulated by a variety of other signal transduction pathways, including nutrient-sensitive mammalian target of rapamycin (mTOR) and proliferative rat sarcoma/rapidly accelerated fibrosarcoma mitogen-activated protein kinase (Ras/RAF MAPK), signaling[1] that link hepcidin regulation to nutrient metabolism, cytokines, growth factors, and cellular proliferation.

Iron Regulation of Hepcidin Expression

Bone morphogenetic proteins 2 and 6 (BMP2 and 6), members of the transforming growth factor-β (TGF-β) superfamily, are the key endogenous regulators of hepcidin production (see Fig. 36.7).[2] BMP6 is produced primarily by hepatic sinusoidal endothelial cells in response to hepatocyte iron stores. BMP2 is produced by the same cells. It is required for hepcidin regulation but appears to be less responsive to hepatic iron load. BMP2 and 6, perhaps acting as a heterodimer, initiate a signaling cascade by binding to hemojuvelin (HJV), a membrane glycophosphatidylinositol-linked BMP coreceptor essential for effective induction of hepcidin, and to BMP receptors on the surface of hepatocytes. BMP2/6 binding is followed by phosphorylation of sons of mothers against decapentaplegic (SMAD)1/5/8 and formation of the SMAD1/5/8–SMAD4 complex, which translocates to the nucleus and activates the promoter of the hepcidin gene *(HAMP)*.[2] HJV is a critical potentiator of the BMP2/6-SMAD regulatory pathway. Some mutations in *HJV*, the gene for HJV, can profoundly decrease the synthesis of hepcidin and other mutations in *HAMP* may even completely abolish the synthesis of hepcidin, resulting in juvenile forms of hemochromatosis (types 2A and 2B, respectively; see Chapter 37) with severe iron loading.

Neogenin, a Deleted in Colorectal Cancer (DCC) family member, seems to stabilize HJV, thereby enhancing BMP6 signaling and hepcidin expression. Furin, a proprotein convertase, cleaves membrane-bound HJV to produce a soluble form of HJV that acts as a competitive antagonist of membrane-bound HJV, inhibiting hepcidin activation. TMPRSS6, a transmembrane serine protease, inhibits BMP6 induction of hepcidin synthesis by cleaving HJV and possibly other proteins of the BMPR complex from the cell membrane.[1] A variety of inactivating mutations in the *TMPRSS6* gene produce high levels of hepcidin that are responsible for iron-refractory iron-deficiency anemia (see Chapter 37).

Plasma iron, in the form of diferric transferrin, is believed to modulate hepcidin synthesis through a distinct pathway that involves HFE, an atypical major histocompatibility complex class I protein that forms a complex with β_2-microglobulin, transferrin receptor 1, and transferrin receptor 2 (see Fig. 36.7).[2,27] Mutations in the genes encoding these proteins, *HFE* (hemochromatosis gene) and *TFR2* (transferrin receptor 2 gene), respectively, are responsible for adult forms of hemochromatosis (types 1 and 3, respectively; see Chapter 37). In these adult forms of hemochromatosis, hepcidin is produced but does not get appropriately upregulated as iron stores increase; iron loading is generally less severe than in the juvenile forms. The regulatory mechanism may involve the binding of circulating diferric transferrin to transferrin receptor 1 (TfR1), displacing HFE to form a complex with the BMP receptor. TfR2 also interacts with BMP signaling. The two transferrin receptors and HFE may effectively act as a sensor of extracellular concentration of diferric transferrin that modulates BMP signaling.[2,27]

Erythropoietic Regulation of Hepcidin Expression

Increased erythropoietic demand for iron reduces hepatic hepcidin synthesis and can sometimes override competing influences that induce hepcidin expression, such as iron overload and inflammation (see later). Hemolysis, hemorrhage, and administration of erythropoietin lower circulating hepcidin concentrations. Patients with marked ineffective erythropoiesis, such as those with β-thalassemia intermedia, have very low or absent plasma hepcidin, increased iron absorption, and high plasma iron despite severe iron overload (see Chapters 37 and 41). Neither hypoxia nor erythropoietin decreases hepcidin transcription directly. Studies of earlier candidate mediators, growth differentiation factor 15 (GDF15) and twisted gastrulation protein (TWSG1), have found that neither is involved in the downregulation of hepcidin synthesis after acute blood loss. Erythroferrone, a newly identified hormone produced by erythroblasts in response to erythropoietin, is the physiologic regulator of hepcidin expression that suppresses secretion during stress erythropoiesis.[14] Erythroferrone likely suppresses hepcidin transcription by inhibiting signaling through BMP-SMAD pathway. A specific erythroferrone receptor has not been identified, but there is evidence for a non-receptor mechanism involving direct binding of BMP2/6 by erythroferrone and erythroferrone-mediated interference with BMP2/6 access to the BMP receptor.[28] Still other signal transduction pathways seem likely to be involved in regulating hepatic hepcidin production in response to increased erythropoietic requirements. Erythropoietin-induced increase in transferrin receptor 1 expression on erythroid precursors may selectively deplete its preferred ligand, diferric transferrin, from plasma. As diferric transferrin also functions as an extracellular iron signal for hepcidin regulation, its removal will at least transiently suppress hepcidin.

Inflammatory and Endoplasmic Reticulum Stress-Related Regulation of Hepcidin Expression

Inflammation increases plasma hepcidin, resulting in the retention of iron within macrophages, reduced iron absorption, and hypoferremia.[29] The inflammatory cytokine interleukin-6 (IL-6) induces hepcidin expression (see Fig. 36.7). IL-6 activates the Janus kinase–signal transducer and activator of transcription (JAK-STAT) signaling pathway, stimulating hepcidin production through STAT3 interactions with a STAT3-binding element in the hepcidin promoter. Other cytokines and the BMP6-HJV-SMAD pathway may also be involved. In addition, the acute inflammatory response is linked to ER stress (see Fig. 36.7), resulting from the accumulation of unfolded or misfolded proteins with disruption of ER homeostasis.

Within hepatocytes and other cells, cytosolic iron is present physiologically in low-molecular-weight forms destined for incorporation into functional compounds or, if present in amounts exceeding cellular requirements, for storage. Recent evidence suggests that protein chaperones and other specialized carriers, membrane transporters, and glutathione provide for the distribution of iron within cells. The cytosolic iron chaperone PCBP1 can deliver excess iron to ferritin, whose structure maintains large amounts of iron in solution in a compact yet bioavailable form, diffusely distributed within the cytosol.[17] A closely related molecule PCBP2 may help transport iron that is destined for cellular export by ferroportin. Cytosolic ferritin is a heteropolymer consisting of 24 subunits of heavy (H) and light (L) peptides that form a hollow sphere into which as many as 4500 atoms of iron may be deposited in an iron core composed of the hydrous ferric oxide mineral ferrihydrite ($5Fe_2O_3{\cdot}9H_2O$). Exit of iron from ferritin may occur through gated pores but mainly by autophagy and lysosomal degradation of ferritin. The ferritin cargo receptor NCOA4 delivers ferritin to the autophagic system where its iron content is liberated.[10] A soluble, relatively iron-poor form of ferritin is found in blood plasma. This form is a 24-subunit polymer containing mostly L-ferritin and is derived primarily from macrophages and hepatocytes. Recent studies indicate that iron-loaded ferritin may be released from cells by a non-canonical secretion pathway, with subsequent TfR1-mediated selective reuptake of iron-loaded ferritin by neighboring cells, presumably as a means of distributing iron within tissues to cells that are relatively iron-deficient. This process selectively depletes the iron-loaded form of ferritin from extracellular fluid, leaving relatively iron-poor ferritin in circulation.[7] Serum concentrations of ferritin correlate with iron stores, with exceptions reflecting pathological situations in which the macrophages are much less or much more iron-loaded than parenchymal tissue, or situations where ferritin synthesis is primarily driven by inflammation.

At very high iron concentrations, ferritin protein subunits may aggregate, resulting in the formation of amorphous, insoluble masses of hemosiderin that may protect against iron toxicity by sequestering the excess iron away from the cytosol, enclosed within siderosome membranes. Depending on the cellular type and iron supply and use, the half-life of cellular ferritin may range from less than 20 to 96 hours. Hemosiderin characteristically has a much slower cellular turnover than ferritin. Altogether, for short-term storage of iron, cytosolic iron is in rapid equilibrium with soluble, dispersed ferritin, but for long-term sequestration, the aggregates of iron within hemosiderin undergo slow and limited exchange. Nonetheless, with phlebotomy or iron-chelating therapy, all of the iron within hemosiderin deposits eventually can be mobilized.

INTESTINAL IRON ABSORPTION

Because humans are unable to excrete excess iron, iron balance is physiologically maintained by the control of iron absorption in the proximal portion of the duodenum. Iron overload develops if regulation of iron balance is bypassed by parenteral injections of iron or transfusion. Normally, only about 1 to 1.5 mg of iron of the 10 to 20 mg in the adult diet is absorbed to balance obligatory losses. Both nonheme iron and heme iron enter through the microvillous brush border at the apical (luminal) surface of the intestinal enterocytes (Fig. 36.8). Nonheme dietary iron is predominantly ferric (Fe^{3+}) and, before absorption, is converted to ferrous (Fe^{2+}) iron either by the reducing action of ascorbate or by the action of brush border ferrireductases, such as membrane-associated duodenal cytochrome B (DCYTB) and likely others. The ferrous iron is then absorbed through DMT1, the same ferrous iron transporter that provides an exit for iron from the endosome (see earlier). To transport ferrous iron, DMT1 requires an inward proton gradient that is generated by the mucosa-acidifying sodium/proton exchangers NHE2 and NHE3.

The exact means by which heme iron is absorbed are still uncertain, but when inside the enterocyte, inducible heme oxygenase 1 releases the iron from protoporphyrin, apparently into a common pathway with absorbed dietary nonheme iron. In the enterocyte cytosol, the iron can be (1) retained for cellular requirements or stored in cytosolic ferritin and then lost when the enterocyte is exfoliated or (2) exported through ferroportin on the enterocyte basolateral membrane. Iron export through ferroportin requires oxidation by membrane-bound hephaestin or circulating ceruloplasmin to the ferric form for binding by plasma transferrin. Control of duodenal iron uptake is intricate,[30] depending on both systemic factors (hepcidin control of ferroportin) and local modulation of iron absorption through transcriptional (HIF-2α) and posttranscriptional (by the iron-regulatory protein/iron-responsive element system) mechanisms. Expression of FPN1B, which lacks the iron-responsive element in its 5′ untranslated region, allows enterocytes to bypass iron-regulatory protein repression of ferroportin iron export even when cells throughout the body are iron-deficient.

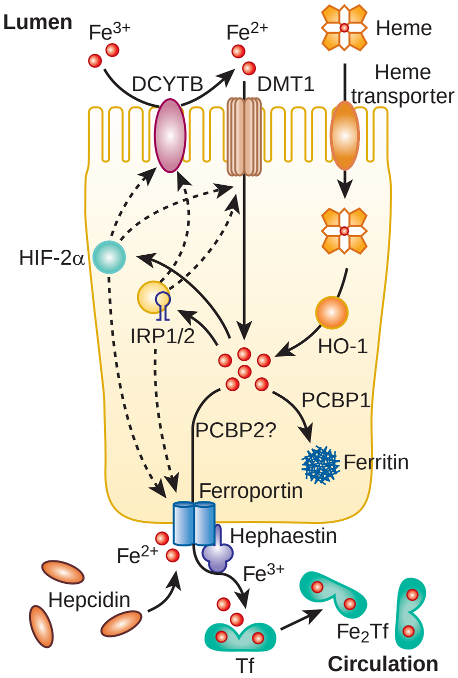


Figure 36.8 ABSORPTION OF DIETARY IRON BY THE DUODENAL ENTEROCYTE. In the gastrointestinal lumen, dietary iron is presented to the enterocyte as heme or nonheme iron. Heme iron uptake is not well characterized, and the specific membrane transporter remains uncertain. After absorption, heme oxygenase 1 (HO-1) releases iron from heme into a common cytosolic pool. Nonheme dietary iron is predominantly ferric (Fe^{3+}) and, before absorption, is converted to ferrous (Fe^{2+}) iron either by the reducing action of other dietary constituents or by the action of brush border ferrireductases, such as membrane-associated duodenal cytochrome B (DCYTB) and likely others. Ferrous iron is then transported across the apical membrane by the divalent metal transporter 1 (DMT1) into the common cytosolic iron pool. Iron is delivered to ferroportin by a chaperone (likely poly(rC)-binding protein 2 [PCB2]), exported through ferroportin, regulated by hepcidin, with hephaestin or circulating ceruloplasmin acting as ferrioxidases that convert Fe^{2+} to Fe^{3+} before loading it onto transferrin (Tf). Cytosolic iron in excess of systemic needs may be carried to ferritin by the cytosolic iron chaperone poly(rC)-binding protein 1 (PCBP1), retained, and then lost when the enterocyte is shed. In addition to regulation by hepcidin, enterocyte iron absorption is modulated by hypoxia-inducible factor 2α *(HIF-2α)* and the iron-regulatory proteins (IRP1 and IRP2). See text for details. *Fe2Tf*, Diferric transferrin. *(Modified with permission from Anderson GJ, Frazer DM, McLaren GD. Iron absorption and metabolism. Curr Opin Gastroenterol. 2009;25:129.)*

FUTURE DIRECTIONS

Remarkable progress has been made in unraveling the molecular mechanisms underlying systemic iron homeostasis, but much remains to be done. Some important unknowns include the mechanism of dietary heme absorption, the role of ferritin secretion by macrophages and hepatocytes, the cellular and molecular mechanisms of iron sensing in the liver, mechanisms and regulation of placental iron transport, and the role of autocrine and paracrine iron regulation in individual organs and tissues. We need to learn more about the genetic basis for individual susceptibilities both to iron deficiency and to iron overload. We need to know much more about iron homeostasis in the three areas in the body that are outside systemic control: the central nervous system, the testis, and the retina. Nonetheless, a pivotal point has been reached when the advances already made will begin to yield therapeutic benefits from new approaches to biologic therapy using agonists and antagonists to the components of the iron-regulatory pathways summarized in this chapter.

REFERENCES

1. Camaschella C, Nai A, Silvestri L. Iron metabolism and iron disorders revisited in the hepcidin era. *Haematologica*. 2020;105(2):260–272.
2. Wang CY, Babitt JL. Liver iron sensing and body iron homeostasis. *Blood*. 2019;133(1):18–29.
3. Coffey R, Ganz T. Iron homeostasis: an anthropocentric perspective. *J Biol Chem*. 2017;292(31):12727–12734.
4. Kawabata H. Transferrin and transferrin receptors update. *Free Radic Biol Med*. 2019;133:46–54.
5. Bartnikas TB. Known and potential roles of transferrin in iron biology. *Biometals*. 2012;25(4):677–686.
6. Luck AN, Mason AB. Transferrin-mediated cellular iron delivery. *Curr Top Membr*. 2012;69:3–35.
7. Truman-Rosentsvit M, Berenbaum D, Spektor L, et al. Ferritin is secreted via 2 distinct nonclassical vesicular pathways. *Blood*. 2018;131(3):342–352.
8. Wilkinson N, Pantopoulos K. The IRP/IRE system in vivo: insights from mouse models. *Front Pharmacol*. 2014;5:176.
9. Ruiz JC, Walker SD, Anderson SA, et al. F-box and leucine-rich repeat protein 5 (FBXL5) is required for maintenance of cellular and systemic iron homeostasis. *J Biol Chem*. 2013;288(1):552–560.
10. Mancias JD, Pontano Vaites L, Nissim S, et al. Ferritinophagy via NCOA4 is required for erythropoiesis and is regulated by iron dependent HERC2-mediated proteolysis. *Elife*. 2015;4:e10308.
11. Kuhn LC. Iron regulatory proteins and their role in controlling iron metabolism. *Metallomics*. 2015;7(2):232–243.
12. Deshpande CN, Ruwe TA, Shawki A, et al. Calcium is an essential cofactor for metal efflux by the ferroportin transporter family. *Nat Commun*. 2018;9(1):3075.
13. Aschemeyer S, Qiao B, Stefanova D, et al. Structure-function analysis of ferroportin defines the binding site and an alternative mechanism of action of hepcidin. *Blood*. 2018;131(8):899–910.
14. Ganz T. Erythropoietic regulators of iron metabolism. *Free Radic Biol Med*. 2019;133:69–74.
15. Zumbrennen-Bullough KB, Wu Q, Core AB, et al. MicroRNA-130a is up-regulated in mouse liver by iron deficiency and targets the bone morphogenetic protein (BMP) receptor ALK2 to attenuate BMP signaling and hepcidin transcription. *J Biol Chem*. 2014;289(34):23796–23808.
16. Lill R, Freibert SA. Mechanisms of mitochondrial iron-sulfur protein biogenesis. *Annu Rev Biochem*. 2020;89:471–499.
17. Ryu MS, Zhang D, Protchenko O, et al. PCBP1 and NCOA4 regulate erythroid iron storage and heme biosynthesis. *J Clin Invest*. 2017;127(5):1786–1797.
18. Silvestri L, Nai A, Pagani A, Camaschella C. The extrahepatic role of TFR2 in iron homeostasis. *Front Pharmacol*. 2014;5:93.
19. Khalil S, Delehanty L, Grado S, et al. Iron modulation of erythropoiesis is associated with Scribble-mediated control of the erythropoietin receptor. *J Exp Med*. 2018;215(2):661–679.
20. Zhang S, Macias-Garcia A, Ulirsch JC, et al. HRI coordinates translation necessary for protein homeostasis and mitochondrial function in erythropoiesis. *Elife*. 2019;8:e46976.
21. Korolnek T, Hamza I. Like iron in the blood of the people: the requirement for heme trafficking in iron metabolism. *Front Pharmacol*. 2014; 5:126.
22. Hom J, Dulmovits BM, Mohandas N, Blanc L. The erythroblastic island as an emerging paradigm in the anemia of inflammation. *Immunol Res*. 2015;63(1):75–89.
23. Theurl I, Hilgendorf I, Nairz M, et al. On-demand erythrocyte disposal and iron recycling requires transient macrophages in the liver. *Nat Med*. 2016;22(8):945–951.
24. Pek RH, Yuan X, Rietzschel N, et al. Hemozoin produced by mammals confers heme tolerance. *Elife*. 2019;8:e49503.
25. Schaer DJ, Vinchi F, Ingoglia G, et al. Haptoglobin, hemopexin and related defense pathways-basic science, clinical perspectives and drug development. *Front Physiol*. 2014;5:415.
26. Knutson MD. Non-transferrin-bound iron transporters. *Free Radic Biol Med*. 2019;133:101–111.
27. Silvestri L, Nai A, Dulja A, Pagani A. Hepcidin and the BMP-SMAD pathway: an unexpected liaison. *Vitam Horm*. 2019;110:71–99.
28. Wang CY, Xu Y, Traeger L, et al. Erythroferrone lowers hepcidin by sequestering BMP2/6 heterodimer from binding to the BMP type I receptor ALK3. *Blood*. 2020;135(6):453–456.
29. Ganz T. Anemia of inflammation. *N Engl J Med*. 2019;381(12): 1148–1157.
30. Schwartz AJ, Das NK, Ramakrishnan SK, et al. Hepatic hepcidin/intestinal HIF-2alpha axis maintains iron absorption during iron deficiency and overload. *J Clin Invest*. 2019;129(1):336–438.

CHAPTER 37

DISORDERS OF IRON HOMEOSTASIS: IRON DEFICIENCY AND OVERLOAD

Clara Camaschella

Iron is an essential nutrient for every cell of the body. Both decreased and increased total body iron, as well its inappropriate tissue distribution may be clinically important. In iron deficiency, limitation of the synthesis of physiologically active iron-containing compounds can have harmful consequences. In iron overload when iron exceeds the body's capacity for safe transport and storage, iron toxicity may produce widespread cell (and organ) damage. Altered distribution may lead to selected organ deficiency. The body has no effective means to excrete excess iron and relies upon control of iron absorption to maintain homeostasis. This chapter focuses on clinical disorders arising from derangement of the molecular mechanisms that preserve iron balance.

Systemic iron homeostasis is maintained by the hepcidin-ferroportin axis. Hepcidin is the iron regulatory hormone that controls iron absorption, use, and storage by binding to, blocking, and inducing the degradation of the cellular iron export channel ferroportin, limiting iron entry into circulation from stores and absorption sites (see Chapter 36). Hepcidin expression is suppressed by iron deficiency, hypoxia, and increased erythropoiesis, while it is stimulated by iron, inflammation, and infection. Genetic and acquired disorders leading to hepcidin deficiency or ferroportin resistance to the hepcidin effect result in iron overload. Hepcidin excess due to genetic causes leads to iron-deficiency anemia, while acquired forms, such as those associated with inflammatory disorders, result in iron sequestration in stores, iron-restricted erythropoiesis, and anemia.

In normal adults total body iron content approximates 3 to 4 g, most present as heme iron in hemoglobin of red blood cells and erythroid precursors (>2.0 g). Myoglobin in skeletal muscles and cardiomyocytes accounts for an other 300 mg. Functional iron represents a minimal amount in all cells, but those minute amounts are essential for mitochondrial metabolism and enzyme-based processes, such as DNA synthesis and repair. Circulating iron bound to transferrin accounts for only 3 to 4 mg, and turns over several times to supply about 25 mg iron daily to tissues, about 80% of which is used to sustain erythropoiesis. More than 20 mg iron are provided daily by macrophages that recycle the metal derived from the breakdown of senescent and damaged erythrocytes, while the contribution of iron absorption is small (1 to 2 mg/day). Release of iron to tissues occurs through the interaction of diferric transferrin (transferrin bound to two iron molecules) with the ubiquitous cell iron importer transferrin receptor (see Chapter 36 for details). Iron is stored in hepatocytes and macrophages by ferritin that with time aggregates into hemosiderin, a complex of molecules of denatured ferritin that can be visualized with Perls' Prussian blue stain on histological preparations. The amount of stored iron is variable from 750 to 1000 mg in adult males to much lower amounts (200 to 300 mg) in females. Daily iron losses balance the amount of absorbed iron (1 to 2 mg) and occur mainly through cell desquamation from skin, genitourinary system, gastrointestinal tract, and menses in females. The distribution of body iron is schematically illustrated in Fig. 37.1.

Since there is no active mechanism to excrete excess iron, iron absorption and recycling are tightly regulated. Disruption of this regulation leads to disorders of iron homeostasis, broadly classified as iron deficiency and overload that can be either genetic or acquired. Other processes such as inflammation that modify iron homeostasis are described in Chapters 36 and 38. Diseases causing local alterations of iron distribution are briefly mentioned, but not discussed in detail in this chapter.

IRON DEFICIENCY

Introduction

Absolute or true iron deficiency is the reduction of total body iron, with depletion of iron stores, a condition that can be present with or without anemia, respectively indicated as iron deficiency anemia and isolated iron deficiency. Functional iron deficiency, that is, the reduced iron availability for tissues, especially erythropoiesis in the presence of replete stores, frequently occurs in acute and chronic inflammation (see box on Definitions and Acronyms Used in Iron Deficiency) (see also Chapter 38). Treatment with erythropoiesis stimulating agents (ESA) may increase erythroid demand beyond iron supply causing functional iron deficiency that is also referred to as *iron restricted erythropoiesis*.

DEFINITIONS AND ACRONYMS USED IN IRON DEFICIENCY

Iron deficiency: decrease of total body iron, including iron stores in the absence of anemia.
Iron deficiency anemia: decrease of total body iron including iron stores, in the presence of anemia.
Iron-refractory iron deficiency anemia (IRIDA): a rare genetic disease caused by *TMPRSS6* mutations resulting in high hepcidin levels and iron deficiency anemia unresponsive to oral iron.
Functional iron deficiency: insufficient iron supply in face of increased iron needs (usually referred to erythropoiesis: it could also apply to other organs).
Iron-restricted erythropoiesis: reduced iron supply to erythropoiesis irrespective of stored iron.
Anemia of inflammation or anemia of chronic disease: anemia associated with up regulation of hepcidin by inflammatory cytokines, leading to iron sequestration in stores and functional iron deficiency.

Most transferrin iron is utilized for heme synthesis and hemoglobinization of erythroid precursors; for this reason, anemia is the more evident and easily recognizable manifestation of iron deficiency and the terms iron deficiency anemia and iron deficiency are sometimes used as synonyms. However, defective iron supply may affect every tissue that needs a minimal but essential amount of iron for the function of iron-containing proteins and enzymes. Thus the two conditions should be kept distinct, since in iron deficiency tissues other than erythropoiesis may be affected, even in the absence of anemia[1].

Epidemiology

Iron deficiency anemia is the most frequent anemia worldwide, estimated to affect over 1.2 billion individuals, and is among the five top causes of years lived with disability burden, according to the Global Burden of Disease Study[2]. About 40% of pre-school children and

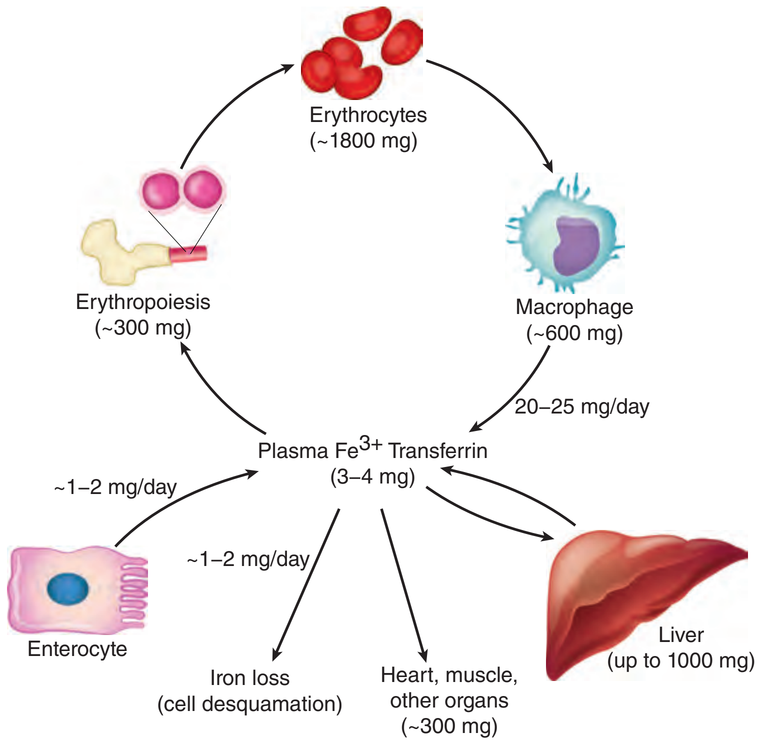


Figure 37.1 DISTRIBUTION OF BODY IRON. Iron (Fe) circulates bound to transferrin that interacts with transferrin receptor that all tissues/organs use to take up iron. Only 1 to 2 mg of dietary iron is taken up daily by duodenal enterocytes and the same amount is lost through cell desquamation and blood losses; 20 to 25 mg of iron are recycled by macrophages from destruction of senescent red blood cell (RBC). Most iron is supplied to the bone marrow for RBC production. Iron that is not utilized is stored in liver and macrophages in variable amount. Arrows indicate direction, numbers (in mg) indicate a mean estimated value. *(Adapted from Camaschella C, Nai A, Silvestri L. Iron metabolism and iron disorders revisited in the hepcidin era. Haematologica. 2020;105:260–272.)*

pregnant women and 32% of young women are anemic worldwide, although variations among countries are huge.[3] More than 50% of the populations in low-income African and Asian countries are estimated to be iron deficient, with the highest prevalence among individuals whose diet contains low bioavailable iron, have helminthic infection resulting in gastrointestinal blood loss, or both. A significantly lower prevalence (around 10% to 15%) is recorded where the diet is adequate as in Western Europe[3] and even lower in the United States because of food fortification and the use of iron supplements. The prevalence of isolated iron deficiency is estimated to be higher than that of iron deficiency anemia, but epidemiological data are lacking. There is increasing awareness that absolute iron deficiency may coexist with anemia of inflammation in several common chronic disorders, causing a mixed type of iron deficiency-inflammation anemia (see Chapter 38). This further increases the relevance of iron deficiency that, irrespective of its prevalence, often remains undiagnosed and/or undertreated.

Etiology

Everywhere in the world, even in the high-income countries, iron deficiency affects mainly children and young women because of their increased iron requirements.[2,3] Physiologic variations in iron demand during life explain differences of age- and gender-related prevalence. Preterm and low-birth-weight newborns have low iron stores and because of rapid postnatal rate of growth—the body weight normally triples in the first year of life—are at high risk of iron deficiency. Infants fed cow's milk need iron supplementation because of the small bioavailable iron content of cow's milk. Preschool children are at risk because of rapid growth. Iron requirements decline into childhood but rise again (around 1.5 mg/day) with the adolescent growth spurt and with menarche in females. Iron need in men and postmenopausal women is about 1 mg/day while in women of childbearing age is approximately double, because of menses. Pregnancy enhances iron demands up to 3 and 5 mg daily in the second and third trimester respectively for the expansion of maternal and fetal erythroid mass. The global iron need for pregnancy, including the amount to replace partum blood loss, ranges from 600 to 800 mg. In all these cases the high iron need may be unmet by limited dietary supply, especially in low-income countries, where hypoferremia may also be sustained by concomitant infections.

Bioavailability of non-heme iron is influenced by other diet components that inhibit iron absorption, such as tannins of tea and coffee and phytates of cereals. Reduced intake and dietary habits, such as vegetarians and vegans, who refuse heme-containing food, cause iron deficiency. The other causes are pathological and related to disorders of iron absorption or chronic blood loss as detailed in Table 37.1. Search for the cause/s is a fundamental step in the diagnostic workup of iron deficiency both with and without anemia.[1] It is especially important in individuals who do not belong to a risk category, because it may lead to diagnose a relevant gastrointestinal pathological process such as peptic ulcer or even colon cancer.

Reduction of chloride production in atrophic gastritis or after gastric surgery impairs the concentration of ferrous iron available for absorption. Bariatric surgery to correct obesity causes iron deficiency and refractoriness to oral iron therapy by reducing gastric size or by passing the duodenum, the key site of iron absorption. Obesity, because of a mild chronic inflammation and increased hepcidin, is itself a cause of iron deficiency (see Table 37.1). The widespread *Helicobacter pylori* infection may decrease iron absorption by competing for dietary iron or causing bleeding from gastric microerosions. Celiac disease is a common gluten mediated enteropathy that should be searched for in iron-deficient children; in adults, who are affected by milder forms, the disease is often undiagnosed and revealed by iron deficiency. Gluten-free diet promotes mucosal recovery and restored iron absorption. In inflammatory bowel diseases anemia may be

TABLE 37.1 Etiology of Iron Deficiency

“Physiological” High Iron Requests
Rapid growth (infancy, adolescence)
Pregnancy (2nd and 3rd trimester)
Blood donations
Insufficient Intake of Bioavailable Iron
Malnutrition
Vegetarians, vegans
Disorders of Iron Absorption
Gastrectomy, duodenal bypass (bariatric surgery)
Helicobacter pylori infection
Atrophic gastritis
Celiac disease
Drugs: Proton pump inhibitors, histamine receptor blockers
Genetic iron-refractory iron deficiency anemia
Chronic Blood Loss
GI tract
Esophagitis
Erosive gastritis
Peptic ulcer
Gastric cancer
Diverticulitis
Benign tumors
Intestinal cancer
Inflammatory bowel disease (Crohn disease, ulcerative colitis)[a]
Angiodysplasia
Hemorrhoids
Obscure source
Hookworm infestation[a]
Genitourinary system
Heavy menses
Menorrhagia
Hemoglobinuria (paroxysmal nocturnal hemoglobinuria, mechanical hemolytic anemia)
Schistosomiasis
Systemic bleeding
Hemostatic disorders: hereditary hemorrhagic telangiectasia, von Willebrand disease
Drugs: Anticoagulants, corticosteroids, salicylates, NSAID
Functional iron deficiency
Anemia of inflammation (anemia of chronic disease)
Chronic kidney disease[a]
Inflammatory bowel disease[a]
Chronic systolic heart failure[a]
Obesity[a]
Erythropoiesis stimulating agents (ESA)

NSAID, Non-steroidal anti-inflammatory drugs.
[a] Frequently mixed anemia (IDA and AI).

multifactorial, resulting from combination of blood losses, iron and vitamin deficiency, inflammatory cytokines, and sometimes treatment secondary effects. Anemia of inflammation prevails in flare up, while true iron deficiency becomes manifest in remission.

Young females are frequently iron deficient because of voluntary dietary restriction, heavy menstrual bleeding, or bleeding from fibroid. Common defects of hemostasis such as mild von Willebrand disease, inherited platelet disorders, and other rare bleeding disorders may cause heavy menstrual bleeding and postpartum hemorrhages and may present as iron deficiency anemia in young women. Bleeding lesions of the gastrointestinal tract may occur at any age but are the main cause of iron deficiency anemia in males and postmenopausal females. Bleeding may be overt or occult and the responsible lesion may be evident as in the case of hemorrhoids or a symptomatic peptic ulcer or silent as a colonic polyp or a malignant tumor. Gastrointestinal bleeding is of special concern in the elderly because of frequent mucosal lesions such as angiodysplasia, or even colon cancer.[4] The elderly do not have increased iron needs but are at risk of deficiency because of reduced iron intake, multiple comorbidities, and the chronic use of drugs that interfere with iron absorption, such as proton pump inhibitors, or contribute to blood loss, such as anticoagulants (see Table 37.1). Chronic recurrent epistaxis, hemoptysis, and hematuria are uncommon causes of iron deficiency. In low-income countries hookworm and other helminthic (*Schistosoma*) infections may cause chronic blood losses, adding this effect to the widespread low iron diet.

Among healthy individuals frequent blood donors are at risk of iron deficiency and female donors often require iron supplementation.[5] Since 1 mL of blood contains about 0.4 mg iron, each donation removes about 200 mg. Athletes may be at risk of iron deficiency because of blood loss or intravascular hemolysis and consequent urinary iron loss during strenuous exercise and often have elevated levels of hepcidin because of training-induced inflammation.

Absolute iron deficiency may coexist with anemia of chronic disorders characterized by either overt or silent inflammation, as in rheumatoid arthritis, inflammatory bowel disease, colon cancer, chronic kidney disease, obesity, and diabetes.[1,6] In chronic infections such as malaria iron deficiency increases the severity of anemia caused by the parasite. Multiple causes often concur to decrease body iron. An example is chronic heart failure, where iron deficiency anemia is reported in 30% to 50% of patients due to decreased iron absorption, decreased renal function, blood loss, and inflammation; importantly, both anemia and iron deficiency are independently associated with increased mortality in heart failure.[7]

Iron deficiency is usually acquired. However, research on hepcidin regulation led to identification of a genetic form called “iron-refractory iron deficiency anemia IRIDA)” (see box on Definitions and Acronyms Used in Iron Deficiency). It is a rare condition caused by recessive mutations in *TMPRSS6*, a gene encoding a liver transmembrane protease that acts as the main hepatic inhibitor of hepcidin expression (see Chapter 36). In subjects with iron deficiency TMPRSS6 suppresses hepcidin production to facilitate iron entry to the circulation. Patients homozygous or compound heterozygous for *TMPRSS6* mutations have constitutionally high hepcidin levels that, blocking iron absorption, lead to iron deficiency anemia refractory to oral iron treatment since childhood.[8] According to several genome-wide association studies *TMPRSS6* genetic variants associate to variant erythroid and iron traits. By modulating hepcidin production, they might influence the mucosal dietary and pharmacological iron absorption in normal subjects, contributing to a variable susceptibility to iron deficiency.

Pathobiology

Cellular and systemic mechanisms are active in responses to iron deficiency to compensate for the lack of iron. When compensatory mechanisms are overwhelmed depletion of total body iron and iron deficiency anemia occur. The cell iron sensor and regulatory proteins (IRPs) increase the synthesis of the iron importer transferrin receptor to take up more iron, while the translation of the iron store ferritin and iron exporter ferroportin is suppressed (see Chapter 36 for detailed coverage of the mechanisms described herein). “Ferritinophagy,” a process of degradation of ferritin in lysosomes, mediated by the cargo protein

Nuclear Receptor Coactivator 4 (NCOA4), is activated to recover iron stored in ferritin. Suppression of hepcidin production to increased iron supply is a key mechanism to compensate for iron deficiency. With increased ferroportin activity macrophages recycle more iron to transferrin and iron absorption is enhanced. Inhibition of hepcidin expression is more marked in iron deficiency with than without anemia because the tissue hypoxia accompanying anemia further contributes to hepcidin suppression.

In the duodenal mucosa the effect of hepcidin on ferroportin is coupled to the function of hypoxia inducible factor-2alpha (HIF-2α).[9] In the presence of anemia tissue hypoxia stabilizes HIF-2α, which controls the expression of iron transporters divalent metal transporter 1 (DMT1) and duodenal cytochrome B (DCYTB) on luminal and ferroportin on the basolateral membrane, increasing the flux of iron into the circulation. Microorganisms compete with the human host for iron, leading to the hypothesis that gut microbiota might contribute to systemic iron homeostasis. Studies in mice suggest that some *Lactobacillae* species increased in iron deficiency produce metabolites that inhibit HIF-2α function in duodenal mucosa to favor bacterial iron acquisition when availability is limited.[10] Whether a similar competition occurs also in humans has to be verified.

The synthesis of erythropoietin (EPO) is modulated not only by hypoxia but also by iron. In iron deficiency without anemia EPO synthesis is attenuated because high IRPs partially suppressed translation of HIF-2α; this limits erythropoiesis, saving iron for other vital functions. In the presence of anemia, the need for oxygen delivery by erythroid cells prevails, HIF-2α is fully stabilized, and EPO is strongly increased. The sensitivity of erythroid cells to EPO may be iron-modulated also *via* transferrin receptor 2, which contributes to hepcidin activation in the liver and is a partner of the EPO receptor in erythroid cells. In absolute iron deficiency, erythroblasts and erythrocytes release excess iron to plasma through ferroportin, a mechanism of protection from oxidative stress that likely becomes a compensatory mechanism in iron deficiency.

Deficiency of iron for mitochondrial function is likely the most important event that affects skeletal muscle, cardiomyocytes, and brain. The hematological effects of iron deficiency are easily recognizable. Reduction of transferrin bound iron impairs the iron uptake by maturing erythroblasts and anemia develops, paralleling the severity of iron deficiency. Initially red cell count is preserved, although erythrocytes become smaller with reduced Hb content (Fig. 37.2).

Signs of iron deficiency may be evident in epithelial cells that require iron for continuous renewal; the recognition of the consequences of iron deficiency in other organs is more difficult in the lack of specific laboratory tests.

In inflammation the enhanced expression of hepcidin induced by pro-inflammatory cytokines like IL-6 withholds iron in macrophages and decreases plasma iron. Hypoferremia coupled with inappropriate EPO production impairs erythroid activity[11] (see Chapter 36). When true iron deficiency develops in the context of inflammation, adaptation to iron deficiency prevails and hepcidin decreases, provided the inflammatory trigger is not too powerful.

Clinical Manifestations

Patients with iron deficiency anemia or isolated iron deficiency are often asymptomatic or show non-specific symptoms and come to medical attention because of abnormal blood tests. In other cases symptoms are directly related to anemia or to the underlying disorder responsible for iron deficiency. In young individuals iron deficiency anemia may go unnoticed until severe, because slow development allows cardiovascular adaptation mechanisms, promoting increased cardiac output and thus maintaining tissue oxygenation. On the contrary, anemia in the elderly may trigger dyspnea, palpitations, or angina due to ischemic cardiomyopathy, heart failure, and/or arrhythmias.

Complaints such as pallor, fatigue, weakness, headache, and dizziness are common but nonspecific. Epithelial signs of iron deficiency such as frail and broken nails, koilonychia, alopecia, atrophic glossitis, angular cheilitis, and rarely dysphagia, due to Plummer-Vinson esophageal webs, are more specific but occur almost exclusively in severe longstanding deficiency. Pica is a compulsive behavior to eat non-nutritive substances such as clay, paper, and ice (pagophagia); this rare, suggestive symptom may worsen iron deficiency. Restless leg syndrome, a distressing condition of uncontrollable leg movements, likely reflects a neurological iron deficiency. Elderly patients may present with tachycardia, increased cold sensitivity due to peripheral vasoconstriction, high systolic with low diastolic blood pressure in the context of a high-output syndrome when anemia is severe. Cardiac ejection murmurs may be present at heart auscultation.

Decreased attention and concentration ability is common in school-age children and adolescents, while in the elderly iron deficiency may accelerate cognitive decline. Decreased physical performance, exercise tolerance, and work productivity ascribed to iron deficiency in adults have important socioeconomic impact. Iron deficiency in pregnancy may have deleterious effects on both mother and fetus, with adverse outcomes including increased risk of preterm labor and low neonatal weight.

A correct diagnosis of iron deficiency and iron deficiency anemia should always include the investigation of the cause. The cause can be "physiologic"

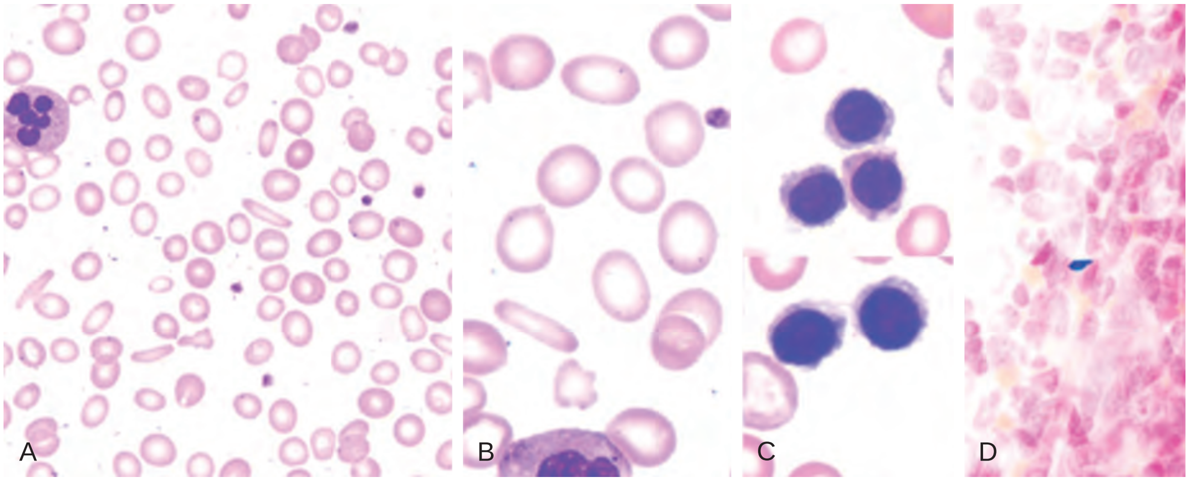


Figure 37.2 IRON DEFICIENCY ANEMIA. Peripheral blood smear (A, B), bone marrow (BM) aspirate (C), and Prussian blue stain of BM aspirate (D) from a 16-year-old girl with hemoglobin 6.7 g/dL, hematocrit 22.6%, and mean corpuscular volume 59.2 fL. Peripheral smear shows hypochromic microcytic red blood cells (A), with widening of the central pallor and "pencil" cells (B). Polychromatophilic erythroid precursors in the aspirated specimen have scanty cytoplasm that is irregular and vacuolated (C). The Prussian blue-stained aspirate shows no iron stores in multiple spicules (D).

as in case of high, unmet iron needs of children and young menstruating or pregnant women. However, even in these cases a careful history and physical examination should be taken to exclude unlikely pathological causes. In males, postmenopausal females, and the elderly it is mandatory to exclude gastrointestinal blood losses; in young females a search for a gastrointestinal cause is recommended in symptomatic, iron unresponsive or relapsing cases. In the presence of comorbidities and especially in old individuals, discontinuation of drugs that interfere with iron absorption or favor blood loss has to be considered whenever possible.

The detection of iron deficiency superimposed on the anemia of inflammation may be challenging because symptoms of the underlying disorder may prevail, mask iron deficiency anemia, or cause fatigue, the same cardinal symptom of anemia. Usually the diagnosis is based on laboratory tests.[6,11]

Laboratory Manifestations

Anemia, defined by WHO criteria as Hb < 13 g/dL in males, < 12 g/dL in females, and < 11 g/dL in pregnant women, is absent in isolated iron deficiency; when present it may be mild, moderate, or severe. The absolute reticulocyte count is low, consistent with decreased erythrocyte production.

Circulating ferritin is an indirect estimate of body iron stores; it is an important tool to evaluate iron disorders in general, and especially iron deficiency. In the absence of inflammation serum ferritin <30 μg/L appears to be the most reliable and cost-effective test for the diagnosis of iron deficiency. The cut-off of 30 μg/L has 92% sensitivity and 83% specificity, as established comparing ferritin levels with bone marrow Perls' staining (Fig. 37.3), the recognized *gold standard* for iron deficiency, although these results were obtained in a small study. Isolated iron deficiency without anemia is defined by serum ferritin <30 μg/L and normal Hb levels. Serum iron is reduced, while total iron binding capacity (TIBC), which measures the functional capacity of transferrin as iron carrier, or transferrin levels are increased, resulting in decreased transferrin saturation (the ratio of iron on TIBC). However, as discussed below transferrin saturation may be low also in anemia of inflammation. Thus for a precise evaluation of an individual case and for the differential diagnosis with other conditions characterized by altered iron parameters it is important to assess both serum ferritin and transferrin saturation.[1]

When anemia develops serum iron and transferrin saturation are even lower than in isolated iron deficiency. Usually transferrin saturation is <16 % and serum ferritin <12 μg/L. However, the use of ferritin <30 μg/L as the lower limit of normal captures most cases with the exception of some situations in the elderly. Thresholds of serum ferritin to diagnose iron deficiency in the elderly should be higher, considering concomitant morbidities and inflammation.[4]

Erythrocyte morphology on peripheral blood smear inspection is abnormal, with small, hypochromic erythrocytes showing the pale central area larger than usual (see Fig. 37.2). Automated counters better assess microcytosis and hypochromia by providing mean MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), and MCHC (mean corpuscular hemoglobin concentration) values. Erythrocyte indexes are not altered in all cases. Reduction of MCV (<80 fl), MCH (<27 pg), MCHC (<32 g/L) and increased hypochromic red cells (HYPO, with MCH < 28 pg) are relatively late changes compared to iron parameters, due to the long erythrocyte lifespan. The same applies to the increased red cell distribution width (RDW), an index of anisocytosis, indicating the presence of erythrocytes of different size (Table 37.2). Iron deficiency anemia of recent onset is usually normocytic and normochromic and only Hb content of reticulocytes (CHr) may be reduced (<27 pg). This early indicator of iron deficiency may signal the need of iron supplementation after treatment with ESA, while an early increase in CHr (day 4) indicates a positive response to iron treatment. The percentage of circulating hypochromic red cells (>6%) is a sensitive marker of iron deficiency in patients with chronic kidney disease; experience in other conditions is limited. Erythrocyte indexes can be deceitfully normal in case of combined iron and vitamin B_{12} or folic acid deficiency.

In all cases of iron deficiency, zinc is preferentially chelated into protoporphyrin-IX (PPF). Elevated ZnPPF levels (>80 μg/dL) persist throughout the lifespan of erythrocytes, providing a screening test for iron deficiency. However, ZnPPF levels may be elevated in other conditions of low iron utilization, such as anemia of inflammation, hemolytic and sideroblastic anemia, and in chronic lead and other heavy metal poisoning.

Iron deficiency increases transferrin receptor (TFR) synthesis in all cells. However, when TFR is not stabilized by its ligand because diferric transferrin is low/absent, its extracellular domain is shed from the membrane and released into the circulation as a soluble component (sTFR). Erythroid precursors that express the largest number of receptors are the major contributors to sTFR concentration. Although its function remains unknown, serum sTFR levels measured by ELISA provide a sensitive and quantitative measure of iron deficiency (see Table 37.2) in the absence of erythroid

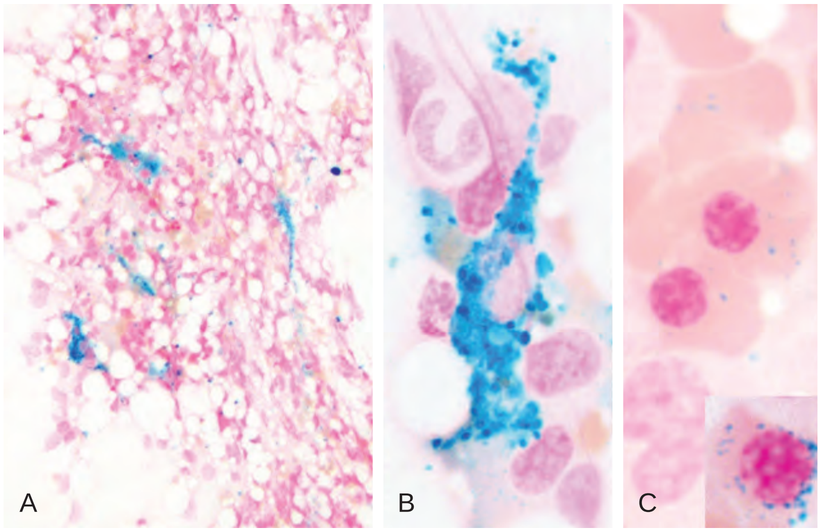


Figure 37.3 ASSESSMENT OF IRON STORES ON A BONE MARROW ASPIRATE. Iron stores are better assessed on the aspirate as opposed to the biopsy because the decalcification procedure required for processing the biopsy leaches out the iron and can lead to a false conclusion of absent stores. On the aspirate, a Prussian blue stain evaluates iron. This can demonstrate iron stores (blue reaction product), particularly in the cytoplasm of macrophages and histiocytes (A, B). Iron can also be seen in the cytoplasm of some erythroblasts (tiny blue cytoplasmic specks), which would allow these cells to be designated *sideroblasts* (C). These are in contrast to red blood cell precursors with abnormal mitochondria iron accumulation around the nucleus, or "ring sideroblasts" (C, *inset*).

TABLE 37.2 **Laboratory Tests for Differential Diagnosis of Iron Deficiency Anemia**

	IDA	IRDA	AI	IDA+AI	Normal Values
Serum iron (μMol/L)	Low	Low	Low	Low	10–30
Tsat (%)	<16	<10	N/<20	<20	16–45
Serum ferritin (μg/L)	<10	N/low	>100**	<100**	30–200(F) 40–300(M)
Hb (g/dL)	Low	Low	Low	Low	>12 (F) > 13 (M)
MCV (fL)	<80	<75	N/<80	<80	90 ± 10
MCH (pg)	<27	Very low	N/low	Low	27–34
CHr (pg)	Low	Low	Low	Low	31.2 ± 1.6
ZnPP (μmol/mol heme)	>80	>80	60–80	>80	<60
sTFR	High	High	N/low	N/high	[a]
sTFR/log ferritin	>2	-	<1	>2	1
Serum hepcidin	Very low	N/high	High	N	[a]
Iron in BM Perls staining	Absent	+	+++	+	+/++

AI, Anemia of inflammation; *BM*, bone marrow; *CHr*, reticulocyte hemoglobin content; *F*, females; *Hb*, hemoglobin; *IDA*, iron deficiency anemia; *IRIDA*, iron-refractory iron deficiency anemia; *M*, males; *MCV*, mean corpuscular volume; *MCH*, mean corpuscular hemoglobin; *N*, normal; *sTFR*, soluble transferrin receptor; *sTFRC/log ferritin*, ratio of soluble transferrin receptor concentration on logarithm transformed ferritin; *Tsat*, transferrin saturation; *ZnPP*, zinc protoporphyrin of red blood cells.

[a] Normal values are according to the method used.

hyperplasia. Unfortunately the use of this test is limited by the scarce availability and the absence of standardization and harmonization in different laboratories.

The diagnosis of iron deficiency in the setting of chronic inflammatory disorders is challenging since ferritin may increase as an acute phase protein. No specific test assesses tissue iron deficiency when ferritin levels are unreliable. It is generally agreed that iron deficiency can be ruled out only when ferritin levels are >100 μg/L; a higher cut-off to diagnose iron deficiency (<300 μg/L) is accepted in special conditions, such as chronic heart failure, provided that transferrin saturation is low (<20%).[6] Inflammation is characterized by hypoferremia, hyperferritinemia, and normal/decreased levels of sTFR (see Table 37.2). The shedding of the receptor persists at an elevated level when inflammation is associated with iron deficiency. The discrepancy between levels of sTFR (high in iron deficiency and normal/low in inflammation) and serum ferritin (low in iron deficiency and normal/high in inflammation) can be utilized to calculate the ratio between sTFR and log-transformed serum ferritin. This ratio is 1 when iron is normal, <1 in anemia of inflammation, and >2 in iron deficiency and in anemia of mixed type due to both iron deficiency and inflammation (see Table 37.2), representing a valuable diagnostic tool. Unfortunately, the reported limitations with regard to standardization present an obstacle to routine use.

Prussian blue (Perls') staining directly assesses iron stores revealing the presence/absence of iron in bone marrow macrophages (see Fig. 37.3) and offering a semi-quantitative grading of iron. Absence of bone marrow iron at Perls' staining is still considered the reference test for iron deficiency. However, the test is rarely used nowadays, unless other disorders that require bone marrow aspiration are suspected. Hepcidin levels are low/undetectable in absolute iron deficiency, except in patients with genetic IRIDA. The latter have low transferrin saturation, strikingly decreased MCV and MCH, and normal/high hepcidin and ferritin levels. Serum hepcidin levels are high in inflammation as are serum ferritin and C-reactive protein (CRP). Whether the hepcidin concentration may help in assessing mixed anemia (due to both iron deficiency and inflammation) is still under investigation.[12] Table 37.2 reports the results of tests currently used to differentiate iron deficiency from other conditions characterized by altered iron parameters. In individual patients it is important to evaluate the global clinical and laboratory picture rather than relying on single tests.[1] The efficiency of iron absorption might be tested by an oral iron challenge (see the online chapter for additional text on Iron Challenge to Test Iron Absorption). Finally the suspected diagnosis of iron deficiency may be retrospectively confirmed by the positive outcome of a therapeutic trial of iron supplementation.

Differential Diagnosis

The differential diagnosis of iron deficiency anemia should take into account other microcytic hypochromic anemias and/or other conditions of altered iron tests.

Apart from lack of iron supply to erythropoiesis, microcytic erythrocytes may result from defective globin or heme production in thalassemia syndromes and congenital sideroblastic anemias respectively. Red blood cell abnormalities in iron deficiency are less severe than in thalassemia where typical target cells are present on the peripheral blood smear. MCV and MCH are less decreased and RCDW wider in iron deficiency than in thalassemia. Heterozygous carriers of β-thalassemia usually have mild or no anemia but severe MCV and MCH reduction and compensatory Hb A_2 increase at hemoglobin electrophoresis. Carriers of deletion or non-deletion α-thalassemia may be silent or have microcytic red cells depending on the severity of the molecular defect. Only tests based on α-globin gene sequencing can establish the correct diagnosis. Diagnosis of thalassemia syndromes is discussed in Chapter 41.

Congenital sideroblastic anemia may be microcytic and hypochromic especially in males with X-linked sideroblastic anemia due to delta-aminolevulinate synthase 2 (ALAS2) mutations and in recessive forms due to mutations in the glycine mitochondrial importer *SLC25A38* gene (see Chapter 39). However, serum iron and ferritin are high, ringed sideroblasts are present at bone marrow Perls' staining, and siderocytes may be observed in the peripheral blood smears (see Fig. 37.2). Precise molecular diagnosis of sideroblastic anemia requires genetic testing. Rare atypical microcytic anemias such as atransferrinemia, mutations of DMT1, and aceruloplasminemia are associated with increased body iron (see iron overload).

Anemia of inflammation is usually normocytic unless the underlying disorder is severe, longstanding, or complicated by absolute iron deficiency. As discussed above, transferrin saturation may be normal or decreased (<20%), total iron-binding capacity low-to-normal, while ferritin is normal/high (>100 μg/L) (see Table 37.2). The differential diagnosis relies on the discrepancy between low transferrin saturation and high ferritin. Usually the diagnosis is suggested by both the underlying disorder and elevated CRP or erythrocyte sedimentation rate (see Chapter 38).

Prognosis

The prognosis of iron deficiency/iron deficiency anemia treated with iron compounds is excellent: most cases are responsive to iron treatment and both anemia and abnormalities of iron tests revert to normal. The ultimate outcome depends on the underlying cause and can be of concern when iron deficiency is the first symptom of a gastrointestinal cancer.

Therapy

The patient's diet has to be integrated with heme-rich food whenever possible. However, due to the low percentage of dietary iron absorbed (5% to 15% in normal subjects and about 25% to 30% in iron deficiency), the diet alone is insufficient to overcome iron deficiency and pharmacological iron has to be supplemented. Iron preparations are available for either oral or intravenous therapy.

Oral Iron Therapy

Oral iron is the first-line therapy in stable patients with isolated iron deficiency or mild-moderate anemia. A plethora of compounds are available: the most effective and inexpensive are iron salts, such as ferrous gluconate, sulfate, and fumarate (containing from 36 to 100 mg elemental iron/tablet). Traditionally, the recommended therapy was 100 to 200 mg elemental iron daily supplemented as iron salts in divided doses at fast and between meals.[1] However, a modified schedule may increase the efficacy and reduce the side effects of oral iron treatment. Several requirements have to be met for oral iron to be efficacious. Patients must be compliant to the treatment schedule, gastric chloride production should be adequate, duodenal mucosa should be intact, and hepcidin levels should be suppressed to allow iron export by ferroportin into plasma. Unfortunately compliance to oral iron treatment is low, because of gastrointestinal side effects and because of the treatment duration. To correct anemia and replete the exhausted stores—that means reaching a target ferritin of 50 to 100 µg/L—may require 3 to 6 months of treatment and few patients comply with such a long schedule. Nausea, epigastric discomfort, vomiting, constipation, dark stools, and metallic taste occur in 30% to 70% of the cases according to different studies, often leading to premature therapy discontinuation.[13] Side effects are ascribed to the toxicity of non-absorbed iron on intestinal mucosa through generation of reactive oxygen species (ROS). Dose reduction or iron administration at meals may induce a better tolerance, although prolonging treatment. A further problem of non-absorbed iron concerns the modification of the intestinal microbiota, with reduction of *Lactobacillae* and *Bifidobacteria* and enhanced growth of pathogens such as enterobacteria, causing dysbiosis.

Slow-release iron formulations are better tolerated than iron salts, but less effective since beyond the duodenum iron absorption is negligible. Ferric iron is insoluble and compounds such as ferric saccharide complexes are less efficacious than iron salts. Ferric citrate, an intestinal phosphate binder, is useful in chronic kidney disease patients to correct both anemia and hyperphosphatemia. Preparations of iron plus multivitamins are useless, the exception being ascorbic acid that increases iron salts absorption.

Recent studies suggest a modified schedule of iron salts administration to increase the therapeutic efficacy and decrease adverse effects. Short-term trials with stable iron isotopes administered as iron sulfate at variable doses (from 60 to 240 mg) to iron-deficient non-anemic women showed that each dose acutely increases serum hepcidin levels, thus decreasing the fractional iron absorption of the consecutive doses up to 48 hours. The conclusion is that in iron deficiency without anemia a low iron dose (60 mg) administered as a single dose on alternate days is more effective and tolerated than higher refracted doses.[14] Studies in iron deficiency with anemia are limited and less clear-cut; more intensive dosing (up to 100 mg/day or 200 mg on alternate days) is needed to reach adequate absorption. Thus in the presence of anemia the schedule choice may consider the rapidity of the desired response, the prolonged course in case of alternate day administration, the common adverse effects when high doses are prescribed, and in some cases also the patient's preference.

Response to oral iron therapy is indicated by raised reticulocyte count in 1 week, enhanced reticulocyte Hb content even earlier, and increased Hb by the second week. In non-responding patients possible causes of iron refractoriness must be investigated (see box on Refractoriness to Oral Iron).

REFRACTORINESS TO ORAL IRON

Refractoriness to oral iron therapy is classically defined as Hb increment < 1 g/dL after 4 weeks of therapy.[15] For practical purposes lack of Hb increase in the second week (or lack of reticulocyte Hb content increase after one week) indicates inadequate response. In non-responding patients the schedule of drug administration should be reviewed to distinguish between inappropriate treatments (compound type, insufficient iron dose, modalities of administration, premature discontinuation), real intolerance for important side effects, and real inefficacy due to reduced intestinal absorption, high hepcidin levels, and/or concomitant inflammation. In some cases it is important to reassess the diagnosis in order to exclude disorders of iron absorption, excessive bleeding, or other causes of microcytic anemia. *Helicobacter pylori* infection should be eradicated in iron-resistant cases to improve iron absorption. Iron refractoriness in celiac disease may revert after gluten-free diet and villi normalization. Refractoriness to oral iron in IRIDA, due to excessive hepcidin levels, is a model of lack of oral iron response in inflammation; both require parenteral iron treatment.

In severe anemia or when oral iron treatment is inconvenient for whatever reason, the alternative option is parenteral, intravenous, iron administration.

Intravenous Iron Therapy

Indications to intravenous iron therapy have been limited in the past by the risk of severe hypersensitivity reactions with old iron compounds, especially high-molecular-weight iron dextran that is no longer available. True refractoriness, poor intestinal iron absorption due to medical or surgical pathological conditions, excessive needs not met by oral therapy (e.g., chronic bleeding not compensated by oral iron therapy), severe anemia, and need of quick recovery are established indications. The increased safety of currently available formulations has broadened the indications to parenteral iron therapy (Table 37.3). Intravenous iron is more effective than oral iron in conditions of mixed anemia (iron deficiency and inflammation), such as in inflammatory bowel disease, chronic heart failure, and chronic kidney disease (CKD). In acute flares of inflammatory bowel disease iron deficiency should be exclusively treated by intravenous iron, considering the high hepcidin levels, and to avoid further mucosal damage and microbiota alteration. Patients in remission phase may respond to oral iron. Intravenous iron improves symptoms and physical performance, such as the 6-minute walk distance test, and quality of life in patients with iron deficiency and chronic heart failure with reduced ejection fraction. This occurs even independently from correction of anemia suggesting a direct effect of iron on cardiomyocyte function. Parenteral iron is more effective than oral iron in combination with erythropoiesis stimulating agents in CKD and has blood transfusions and ESA dose sparing properties[16] that are relevant considering the negative ESA-induced cardiovascular side effects.

The use of intravenous preparation is increasing because of the increased safety profile of available preparations. The contemporary generations of compound have a structure (Table 37.4) based on a carbohydrate shell surrounding the iron core, which confers increased stability,

TABLE 37.3 Indications to Parenteral Iron Therapy

Established Indications
Failure of oral therapy
Iron intolerance
Allergy
Severe side effects
Iron refractoriness
Post gastrectomy or duodenal bypass
Helicobacter pylori infection
Atrophic gastritis
Celiac disease
Genetic IRIDA
Need for a quick Hb recovery
Severe anemia in the 2nd, 3rd trimester of pregnancy
Bleeding uncontrolled by oral iron therapy
Hereditary hemorrhagic telangiectasia, congenital coagulation disorders
Heavy uterine bleeding
Substitute for blood transfusions unaccepted for religious tenet
ESA treatment in CKD
Extended Indications
Inflammatory bowel disease: acute flares
ID in heart failure even in the absence of anemia
Anemia of CKD (irrespective of ESA)
Cancer anemia (selected cases under chemotherapy ± ESA)
Perioperative anemia: Transfusion sparing strategy in patients undergoing elective surgery[a]
Inability to adhere to an oral schedule for cognitive impairment.

CKD, Chronic kidney disease; *ESA*, erythropoiesis stimulating agents; *ID*, iron deficiency; *IDA*, iron deficiency anemia; *IRIDA*, Iron-refractory iron deficiency anemia.

[a] Patient blood management programs.

TABLE 37.4 Iron Preparations for Parenteral Intravenous Use

Compound	Single Dose (mg)	Total Replacement in One/Two Infusions	Maximum Dose (mg)	Infusion Time (min)
Iron gluconate	125	No	125	60
Iron sucrose	200	No	200	15–60
LMW dextran iron	100	Yes	1000	60
Ferumoxytol	510	Yes	1020[a]	15
Ferric carboxymaltose	750–1000	Yes	750 –1000[b]	15
Ferric derisomaltose[c]	1000	Yes	1000	15

A black box warning for anaphylaxis reaction applies to iron dextran and ferumoxytol.

LMW, Low molecular weight.

[a] Alternatively two infusions of 510 mg one week apart.

[b] Up to two 750 mg or 1000 mg doses, according to body weight at least one week apart.

[c] Previously iron isomaltoside.

preventing premature iron release. On one hand this reduces the acute adverse effects ascribed to labile free iron released in the circulation and on the other it allows high-dose infusion in a single injection.

The currently available preparations have equivalent efficacy. The most important difference is between low-dose compounds that require repeated infusions and high-dose compounds. The latter have gained popularity because the total iron amount needed is replaced in one/two infusion(s) with a good safety profile (see Table 37.4). The dose of iron to be replaced with multiple infusions is calculated based on body weight, the Hb deficit plus the amount (calculated in 500 mg) needed to restore iron stores. For the high-dose compounds a simplified formula is used based on Hb levels and body weight (see the online chapter for additional text on Intravenous Iron Treatment: How to Calculate the Correct Dose). Since Hb increase and stores reconstitution occur more promptly with high-dose preparations these compounds are convenient when a rapid recovery is necessary or in patients with limited hospital access; the main disadvantage is the high cost, which is nevertheless balanced by reduced hospital accesses. In practice the choice of the drug, besides what is approved/available at different centers, often depends on the clinical status such as age, sex, longstanding vs recent onset or recurrent anemia, the reversibility of the underlying cause, cost evaluation, and ultimately also patient's preference.

In general, intravenous iron supplementation is safe. Minor/moderate infusion reactions (nausea, pruritus, urticaria, flushing, back or thoracic pain) often self-limited with infusion interruption are reported in 1:200 infusions with different preparations. Delayed reactions (headache, myalgia, arthralgia) may occur up to 48 hours after the infusion. Severe reactions (hypotension, dyspnea) are infrequent, estimated around 1:200,000. Guidelines to minimize the infusion risk are available and training of the personnel is important (see the online chapter for additional text on How to Manage Hypersensitivity Reactions to Intravenous Iron).[17]

Contraindications to intravenous iron are limited. Immediate discontinuation is required in acute infections since microorganisms may use iron as a growth factor. Intravenous iron should not be used in the first trimester of pregnancy because of the lack of safety data and in patients with previous severe hypersensitivity reactions. Of some concern is the incidence of hypophosphatemia in patients treated with ferric carboxymaltose, an incidence significantly higher than with other preparations. Hypophosphatemia alteration is usually mild, transient, and asymptomatic. Occasionally, after repeated doses, it persists and causes osteomalacia with bone pain and risk of fractures. Phosphate control is recommended in the rare patients receiving multiple injections of high-dose ferricarboxymaltose.

Concerns exist about overcorrecting iron depletion with intravenous iron in subjects with heart failure,[7] CKD, or inflammatory conditions. Possible late complications of excess iron in terms of increased risk of oxidative stress and atherosclerosis are not assessed in available trials. Increased susceptibility to infections in CKD patients who receive chronic iron therapy is controversial; a recent large trial of prolonged iron treatment in hemodialysis patients showed that the infection rate was similar irrespective of the high or low iron dose used.[16,17] In the choice of oral vs intravenous treatment, risks and benefits should be evaluated on an individual basis.

Red blood cell transfusions should not be used to treat iron deficiency anemia unless anemia is life threatening or patients are at risk of cardiac or cerebral ischemia.

Prevention Strategies

Prevention strategies should target populations at risk, especially children and pregnant women. Diet changes implementing heme-iron rich food and avoiding non-bioavailable iron-containing food are ideal, but unrealistic in some contexts. Food (flour, rice, or others) iron fortification proved effective in preventing iron deficiency in children in different world areas. Supplementation with 30 to 60 mg elemental iron for 3 months/year as prevention of iron deficiency in young women and lower adapted doses in children have been recommended by WHO in areas with high prevalence of anemia. These protocols are not recommended in regions endemic for infection, such as malaria, unless supplementation is associated with antimalarial treatment, since iron may potentiate *Plasmodium* replication.

A type of prevention of iron deficiency anemia involves the screening of anemia before elective surgery and/or the use of intravenous iron to increase Hb and iron stores before major surgical procedures (e.g., orthopedic or abdominal) predicted to induce heavy blood loss.[18] Several centers worldwide are implementing perioperative intravenous iron use under general programs of "Patient blood management." The aim is to limit both blood losses during surgery and the consequent need for blood transfusions to treat postoperative anemia, because these are associated with infections and negative outcome in high-risk interventions.[19]

Future Directions

Novel iron preparations with high efficacy and low side effects are needed to render prolonged oral treatment more feasible. The efficacy of the alternate day protocol should be explored for extended periods of time and more extensively evaluated in iron deficiency anemia. Extended follow-up of patients treated with high-dose intravenous iron should clarify possible long-term side effects. In patients treated with ESA or in the setting of inflammatory conditions, new compounds able to mobilize endogenous iron sequestered in macrophages could eventually replace intravenous iron. The availability of multiple options for iron therapy will offer the best-personalized treatment to individual iron-deficient patients.

IRON OVERLOAD

Introduction

Iron overload is an increase of total body iron resulting from iron supply in excess of iron requirements. Mild body iron increase is clinically silent; clinical complications due to iron toxicity are usually evident when iron exceeds about 4 to 5 g total body content. This may occur in hereditary hemochromatosis, in iron-loading anemias, and in all patients receiving blood transfusions for chronic anemia such as thalassemia major and other congenital or acquired refractory anemias.

In hemochromatosis and iron-loading anemias excessive and deregulated iron absorption causes an increased saturation of transferrin that leads to the appearance of non-transferrin-bound iron (NTBI) in the circulation. This toxic iron species is avidly taken up by parenchymal cells of liver and other organs, while macrophages remain iron-free. In chronically transfused patients excess iron is initially safely stored in macrophages; when the storing capacity is overcome, transferrin becomes fully saturated and the increased NTBI, as in hemochromatosis, damages liver and other organs. The main causes of iron overload are summarized in Table 37.5.

Epidemiology

The most common form of iron overload in populations of northern European descent is hereditary hemochromatosis due to a recessive mutation (C282Y) in the HFE gene. The estimated prevalence of the C282Y homozygous genotype in the largest study available is 0.44% in non-Hispanic whites, 0.014% in black individuals, and 0.000039% in Asians.[20] A small proportion of patients have mutations in other genes leading to disorders collectively known as "non-HFE hemochromatosis" (see Table 37.5). No epidemiological data are available for the latter disorders; they are rarely reported in different countries, often as a consequence of consanguineous marriages that increase the rate of recessive diseases in offspring.

Iron-loading anemias include several disorders characterized by ineffective erythropoiesis and development of iron overload in the absence of blood transfusions. The most studied disease is beta-thalassemia; however, the same pathophysiology of iron loading is shared by other congenital anemias characterized by ineffective erythropoiesis (see Table 37.5). Thalassemia major and other forms of iron-loading anemia are important public health problems in the Mediterranean area, Southwest and Southeast Asia, and the Indian subcontinent. However, due to population migrations, patients with these disorders are now present everywhere. Other inherited types of systemic iron overload such as atransferrinemia, DMT1 mutations, and aceruloplasminemia are even more rare. Rare as well are conditions leading to focal sequestration of iron, such as idiopathic pulmonary hemosiderosis or disorders leading to specific patterns of brain iron deposition and associated neurodegeneration.

Genetic Aspects

Most hemochromatosis patients carry a homozygous cysteine to tyrosine substitution at position 282 (C282Y) of the HFE protein. C282Y is an ancient mutation whose origin dates back about 6000 years ago. The highest C282Y frequency occurs in Northern Europe especially in Brittany, Scotland, and Ireland, resulting from a founder effect followed by positive selection. The heterozygous carriers of the mutation were likely protected from iron deficiency in times of restricted dietary iron availability,[21] an advantage especially relevant for the outcome of pregnancies. A second change, a histidine to aspartic acid substitution at amino acid 63 (H63D), is a polymorphic change found at high frequency (around 15%) worldwide. Less than 5% of patients, usually mildly affected, are C282Y/H63D compound heterozygotes. Other *HFE* point mutations/deletions associated with iron overload are private mutations in individual families.[22]

Homozygous or compound heterozygous mutations in genes encoding hemojuvelin (*HJV*), transferrin receptor 2 (*TFR2*), and hepcidin (*HAMP*) cause a more severe phenotype that is defined "non-HFE hemochromatosis" (see Table 37.5). More specifically *HJV* and *HAMP* mutations cause juvenile hemochromatosis characterized by severe and early onset iron overload; *TFR2*-hemochromatosis may affect both young and adult patients. A dominant form of the disease is caused by mutations in *SLC40A1*, the gene that encodes the hepcidin receptor ferroportin. *SLC40A1* mutations are functionally heterogeneous and may lead to two opposite phenotypes with iron accumulation respectively in macrophages and hepatocytes (see box on Macrophage vs. Hepatocyte Iron Overload in Ferroportin Mutations).

MACROPHAGE *VS.* HEPATOCYTE IRON OVERLOAD IN FERROPORTIN MUTATIONS

Ferroportin is the sole iron exporter of mammalian cells. Mutations of ferroportin gene *SLC40A1* occur at the heterozygous state; full inactivation of ferroportin gene is likely lethal. *SLC40A1 loss of function* mutations impair iron export and lead to iron accumulation in macrophages without iron toxicity and clinical complications. Iron recycling is in part compromised and transferrin saturation is normal/low, never increased. Anemia is absent or marginal but may become evident if phlebotomy is performed. This phenotype is considered the true "ferroportin disease."[23] *Gain of function* mutations, which are extremely rare, affect residues of the hepcidin binding site, cause resistance of ferroportin to the hepcidin effect and unrestrained iron export, recapitulating the pathobiology and clinical complications of hemochromatosis (dominant hemochromatosis) (see Table 37.5). Ferroportin mutations strengthen the notion that iron sequestration in macrophages is safer than in hepatocytes.

Most iron-loading anemias are genetically determined and include thalassemia syndromes (see Chapter 41), congenital dyserythropoietic and sideroblastic anemias (see Chapter 39), and some chronic hemolytic anemias. A subtype of myelodysplastic syndromes with ringed sideroblasts (MDS-RS), once called RARS (refractory anemia with ringed sideroblasts) due to somatic mutations of the hematopoietic stem cell, is associated with iron overload (see Chapter 61). All these conditions are characterized by different degrees of ineffective

TABLE 37.5 Classification of Iron Overload

	Inheritance	Gene	Phenotype
Genetic Iron Overload			
HFE-HH (type 1)	AR	*HFE*	Inappropriate low hepcidin Adult-onset iron overload
Non-HFE HH (type 2)	AR	*HJV* *HAMP*	Very low/undetectable hepcidin Juvenile iron overload
Non-HFE HH (type 3)	AR	*TFR2*	Low hepcidin. Early or adult-onset iron overload
Non-HFE HH (type 4)	AD	Gain of function *SCL40A1* mutations	Hepcidin resistance Severe iron overload
Ferroportin disease	AD	Loss of function *SCL40A1* mutations	Macrophage iron overload
Genetic Iron Loading Anemias			
Thalassemia syndromes			Microcytic anemia. Transfusion-independent iron overload
α-thalassemia	AR	*HBA*	
β-thalassemia	AR	*HBB*	
Congenital sideroblastic anemia (non-syndromic)	X-linked AR	*ALAS2* *SLC25A38* *GLRX5*	Microcytic anemia Ringed sideroblasts Iron overload
Congenital dyserythropoietic anemia			Anemia, jaundice, splenomegaly, erythroblasts multinuclearity, iron overload
Type 1	AR	*CDAN1*	
Type 2, HEMPAS	AR	*SEC23B*	
Type 3	AR	*KIF23*	
Atransferrinemia[a]	AR	*TF*	Microcytic anemia Iron overload
DMT1 deficiency	AR	*SLC11A2*	Microcytic anemia Iron overload
Aceruloplasminemia	AR	*CP*	Iron accumulation in basal ganglia, liver, and spleen; diabetes, neuro-, retinal- degeneration, anemia
Acquired Iron Overload			
Chronic blood transfusions (congenital and acquired anemias)			Iron overload requiring chelation therapy (e.g., thalassemia major)
Intravenous iron administered in excess of needs			Iron accumulation in macrophages
Acquired Iron Loading anemias			
MDS RS	Clonal disorder with somatic mutations	*SF3B1*	Macrocytic anemia. Ringed sideroblasts. Iron overload
Regional Iron Accumulation			
Pulmonary hemosiderosis	Idiopathic		Repeated alveolar hemorrhages Iron loading of pulmonary macrophages
Associated with neurologic abnormalities (examples)			
Ferritinopathy	AD	*FTL*	Brain iron accumulation and neurodegeneration + cardiac iron overload
Friedreich's ataxia	AR	*FRDA*	

AD, Autosomal dominant; *AR*, autosomal recessive; *DMT1*, divalent metal transporter 1; *FT*, ferritin; *FTL*, ferritin light chain; *HH*, hereditary hemochromatosis; *HEMPAS*, Hereditary Erythroblastic Multinuclearity with Positive Acidified Serum lysis test; *MDS RS*, myelodysplastic syndrome with ringed sideroblasts, both single lineage and multilineage dysplasia; *SLC40A1*, the gene encoding ferroportin.

[a] The term atransferrinemia is incorrect. The disease should indeed be an hypotransferrinemia since mutations in all cases do not fully suppress the protein synthesis.

erythropoiesis that drive increased iron absorption and iron overload even independently from blood transfusions.

Many disorders requiring chronic red blood cell transfusions are hereditary, including thalassemia major, sickle cell disease, and other chronic refractory anemias.

Pathobiology of Hemochromatosis

The disruption of systemic iron homeostasis in hemochromatosis leads to iron overload of liver and other organs. All proteins mutated in recessive hemochromatosis act in the liver as components of the bone morphogenetic protein (BMP) pathway that activates hepcidin (see Chapter 36). The HFE gene, an atypical MHC class I-like gene on the short arm of chromosome 6, encodes a ubiquitously expressed protein that on cell surface is associated to beta$_2$-microglobulin and has a dual function in iron homeostasis: it binds transferrin receptor 1 (TFR1) and enters the cell through the same endosomal pathway. Competing with diferric transferrin for binding the same sequence of TFR, HFE protein is displaced from the receptor when serum iron is abundant, thereby behaving as a sensor of the iron taken up by cells. In the liver HFE participates to the complex that activates hepcidin in response to iron increase (see Chapter 36), as clearly demonstrated in mouse models. Both *Hfe* knock out mice and *Hfe*^{C282Y} knock in mice recapitulate the iron overload of hemochromatosis. However, only conditional inactivation of *Hfe* in hepatocytes leads to iron overload, while iron homeostasis is normal when *Hfe* is selectively knocked down in enterocytes or macrophages. In the presence of diferric transferrin, HFE, free from TFR1 binding, upregulates hepcidin synthesis in the liver, likely contrasting the degradation of the essential BMP receptor ALK3. Both the ubiquitous and hepatic HFE functions are lost in hemochromatosis, where mutant HFE, unable to bind beta$_2$-microglobulin, is not exposed on cell surface. The elucidation of the hemochromatosis genetic heterogeneity clearly demonstrates that the severity of iron overload depends on the function of the altered protein in the hepcidin pathway. The deficiency of hepcidin and the entity of iron overload are variable but greater in non-HFE than in HFE-hemochromatosis. HJV is essential for BMP signaling and its inactivation leads to the same severe phenotype as the inactivation of the *HAMP* gene. TFR2, a sensor of diferric transferrin, participates in hepcidin activation, although the molecular mechanisms remain unclear. In *HFE* C282Y patients' serum hepcidin is low/normal, but inappropriate to iron overload and the hepcidin response to a low dose of oral iron is blunted. In *TFR2* and *HJV* disease hepcidin deficiency is more severe accounting for both the early onset and the magnitude of iron overload. The inability to regulate iron homeostasis explains the hallmarks of all recessive hemochromatosis: increased iron absorption, increased serum iron and transferrin saturation, hepatocyte iron loading with periportal distribution, and relatively iron-poor macrophages. Dominant mutations of ferroportin cause different phenotypes according to the mutation (*loss* vs. *gain* of function) and consequently the cell type (hepatocyte vs. macrophage) that accumulates iron.[23]

In atransferrinemia the strong reduction of the physiological carrier of iron leads to NTBI increase in the circulation and iron overload, while the limited iron supply to erythropoiesis causes severe microcytic anemia. In *DMT1* mutations microcytic anemia and iron overload are due to defective iron utilization by the erythroblasts, as DMT1 is essential to release iron from endosome to mitochondria. In aceruloplasminemia defective iron release from macrophages, pancreatic islets, and glial cells results in iron overload and late-onset diabetes mellitus, retinal damage, and neurodegeneration.[24]

Neonatal hemochromatosis is a type of liver insufficiency started in fetal life that, among other causes, may be the result of gestational alloimmune liver disease susceptible to treatment by intravenous immunoglobulins.

Pathobiology of Acquired Iron Overload

All iron-loading anemias are characterized by ineffective erythropoiesis and share with hemochromatosis an insufficient hepcidin production. The extent of ineffective erythropoiesis rather than the degree of anemia correlates with the suppression of hepcidin synthesis. The latter effect is due to erythroferron (ERFE), a tumor necrosis factor alpha (TNFα)-like protein produced and released by erythroblasts stimulated by erythropoietin (see Chapter 36). In non-transfusion-dependent βeta-thalassemia patients serum ERFE levels are high and hepcidin levels low despite iron overload, to ensure iron supply to the maturing erythroblasts (see Chapter 41). Since erythroblast differentiation is blocked, excess iron, unutilized for hemoglobin synthesis, interferes with erythroid maturation, aggravating ineffective erythropoiesis that in turn increases iron absorption in a vicious cycle. All iron-loading anemias share with beta-thalassemia variable levels of hepcidin inhibition by ERFE. The erythroblasts of MDS-RS have defective iron utilization in mitochondria and accumulate iron in these organelles, which cluster around the nucleus originating a ring appearance at Perls' staining (see Fig. 37.3). Patients with MDS-ringed sideroblasts usually have a somatic mutation in the spliceosome gene *SF3B1* at the heterozygous state (see Chapter 61). Among others the *ERFE* gene is abnormally spliced resulting in an elongated protein that suppresses hepcidin more efficiently than the wild type hormone.[25] Although iron overload is caused by hepcidin deficiency both in hemochromatosis and iron-loading anemias, molecular mechanisms are different: in the former hepcidin is low because of lack of BMP pathway activation in response to iron, in the latter hepcidin is low because of erythropoiesis-driven excessive inhibition.

Iron loading may be caused or aggravated by blood transfusions in patients with thalassemia major, Hb E/beta-thalassemia, Blackfan-Diamond anemia, and other congenital anemia requiring transfusions since infancy, or in selected patients with sickle cell disease transfused for preventing recurrent complications such as stroke and painful crises. At risk are patients with acquired aplastic or refractory anemia and survivors from successful allogeneic bone marrow transplantations, heavily transfused for the underlying disease and transplantation procedure. The iron content of each unit of packed red cells is calculated assuming that 450 mL of blood with a mean hemoglobin concentration of 13.5 g/dL contains approximately 200 mg of iron. After receiving 20 units of transfused blood patients accumulate about 4 g extra iron with the risk of organ damage.

The toxicity of iron in both hemochromatosis and other disorders is mediated by NTBI (see Chapter 36). This iron species appears in the circulation when saturation of transferrin outpaces 50% to 60%. NTBI is taken up in an uncontrolled way by a zinc transporter (ZIP14) in hepatocytes and acinar pancreatic cells and by other transporters like ZIP8, and low-voltage calcium channels in cardiomyocytes. NTBI favors the generation of ROS that damage proteins, lipids, DNA, and organelles, as lysosomes and mitochondria, causing cellular dysfunction, apoptosis, and death. In the liver, necrosis of the hepatocytes and the local inflammatory response mediate ROS-induced fibrogenesis by activating hepatic stellate and other mesenchymal cells to produce collagen. Liver fibrosis may progress over time to overt cirrhosis, while iron toxicity in other organs may lead to cardiomyopathy, diabetes mellitus, and other endocrine complications (Fig. 37.4). Iron-related damage of the hypothalamic-pituitary axis results in hypogonadotropic hypogonadism and low levels of gonadotropins and testosterone. The toxic manifestations are variable and depend not only on the iron burden, but also on the rate of iron accumulation and on the cellular pattern of deposition; the concomitant presence of chronic viral hepatitis or alcohol abuse are important cofactors of liver damage.[21] Hypoxia is a further damaging cofactor in iron-loading anemias.

Clinical Manifestations

Since iron accumulation is slow, HFE-hemochromatosis has late onset, usually in the 4th to 5th decades of life in males and after menopause in females. The clinical expression is low; the disease penetrance estimated in a large prospective Australian study was 28.4% in men and 1.2% in women.[26] Symptomless patients come to medical attention because of increased liver transaminases, abnormalities of iron tests, or both. Other patients may complain of vague symptoms such as fatigue and abdominal pain due to liver enlargement. Progressive iron accumulation may cause liver fibrosis/cirrhosis, diabetes and related complications, heart failure and arrhythmias, symptoms of gonadal insufficiency such as decreased libido and impotence, and rarely other endocrine complications (see Fig. 37.4). Hepatocellular carcinoma is an important cause of death in cirrhotic patients, even after iron depletion. Arthritis due to chondrocalcinosis with pyrophosphate deposition typically affects the second and third metacarpal-phalangeal joints but may also involve large joints such as hip and knee, requiring replacement prosthesis in severe cases. Skin pigmentation results from excess melanin deposition.[22] In C282Y/H63D

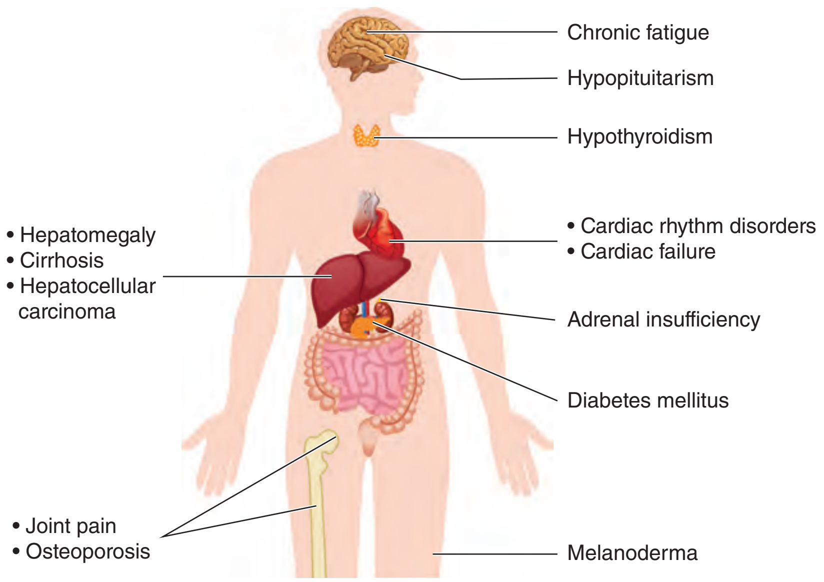


Figure 37.4 CLINICAL COMPLICATIONS OF IRON OVERLOAD. The most important complications that may occur in all types of iron overload are indicated. Liver disorders, chronic fatigue, and arthropathy are more frequent in classic hemochromatosis. Cardiac disease and hypopituitarism are more frequent in juvenile hemochromatosis and in beta-thalassemia patients under chronic blood transfusions.

compound heterozygotes, in the absence of contributory factors (alcohol, chronic hepatitis) iron accumulation is rare, when present is mild and liver fibrosis is exceptional. Individuals heterozygous for C282Y or H63D are not at risk of iron overload.[21]

Acquired and genetic factors may modify the course of the HFE disease. Menses and pregnancies explain the delayed onset of the disease in C282Y homozygous women and blood donations may delay the diagnosis in regular blood donors. Dietary habits like abundant meat consumption and excess alcohol intake may favor iron absorption; alcohol also contributes independently to liver damage. Besides environmental factors, genetic modifiers such as SNPs of iron regulating or other genes may influence the degree of iron overload and its complications, usually with a small effect.[21,22]

Clinical manifestations in non-HFE hemochromatosis are very similar to HFE disease but have earlier onset because hepcidin deficiency is more severe. In juvenile types the most common complications, cardiomyopathy and hypogonadism, affect both genders equally and may occur as early as in the second or third decade of life, mimicking complications encountered in transfused thalassemia patients with severe iron loading.[27] Growth and sexual maturation are delayed; secondary amenorrhea may occur in females a few years after menarche. Heart failure refractory to treatment, ventricular arrhythmias, or both may be life threatening in untreated patients. The phenotype of *TFR2* hemochromatosis is variable, usually less severe than juvenile hemochromatosis although the disease onset may be early as well.[28] Genetic and acquired modifiers have minor or no role in severe non-HFE disorders. Ferroportin disease is usually asymptomatic, in the presence of high ferritin and liver iron concentration (LIC) due to macrophage iron accumulation.

Patterns of iron deposition in non-transfusion-dependent iron-loading anemias are similar to hemochromatosis with overload of hepatocytes in adult life and iron-poor macrophages.[27] Liver fibrosis may occur while cardiac disease is unusual. Transfusion-dependent patients show a mixed pattern (hepatocytes and macrophages) of liver iron deposition. The clinical phenotype is related to the intensity of the transfusion regimen and the individual compliance to iron chelation therapy (see Chapter 41). The phenotype is similar to that described for juvenile hemochromatosis with hypogonadism and cardiac involvement manifesting as heart failure and arrhythmias, the latter being the major causes of death in untreated or inadequately chelated patients.

Osteoporosis is frequent in all types of severe iron loading and may predispose to bone pain and fractures. All iron-loaded patients have increased susceptibility to infections by rare siderophilic pathogens such as *Vibrio vulnificus* and *Yersinia enterocolitica*. The latter gram-negative bacteria cause a self-limited infection in normal subjects, while they may cause severe, systemic, in some cases fatal infections in iron-loaded patients, since NTBI favors their rapid growth.

Atransferrinemia and *DMT1* mutations lead to atypical microcytic anemia with iron overload of variable intensity. In aceruloplasminemia a late-onset clinical triad of diabetes, neurodegeneration (manifesting as dementia, dysarthria, dystonia), and retinal degeneration, often accompanied by iron overload and microcytic anemia, may suggest the diagnosis.

Laboratory Manifestations

Abnormalities of liver function or iron tests suggest hemochromatosis in asymptomatic patients. Increased transaminase and γglutamyltranspeptidase levels are common signs of initial liver damage. While the clinical penetrance is low, the biochemical penetrance (i.e., abnormalities of iron tests) is much higher.[22] High transferrin saturation is the hallmark of recessive hemochromatosis and an isolated finding in the early phases when ferritin is still normal. Transferrin saturation > 50% is present in almost all males and > 45% in about 50% of women. Progressive iron accumulation is paralleled by increased serum ferritin with levels > 200 μg/L in women and > 300 μg/L in males. Since ferritin concentration prevalently reflects the amount of macrophage iron the increase may be late, always accompanied by high transferrin saturation levels. An exception is ferroportin disease: owing to the early iron accumulation in macrophages ferritin is usually high, while, because of reduced recycling, transferrin saturation is normal/low; dominant inheritance may suggest the correct diagnosis. In non-HFE hemochromatosis, increased transferrin saturation and serum ferritin may appear during the first decade of life in both juvenile and *TFR2* diseases. In all types of hemochromatosis the complete blood count is normal; in a few cases Hb and MCV may be at the upper normal limits. Variable microcytic anemia and iron overload characterize the rare atypical microcytic anemias like atransferrinemia, DMT1 deficiency, and aceruloplasminemia.

Genetic testing for HFE (or other gene) mutations confirms the diagnosis of hemochromatosis in the presence of clinical or biochemical signs of iron overload. Genetic testing is recommended in family members of patients, except in children unless a juvenile form is suspected. An HFE C282Y homozygous genotype identified in an individual with normal iron tests (e.g., in a family member of the index

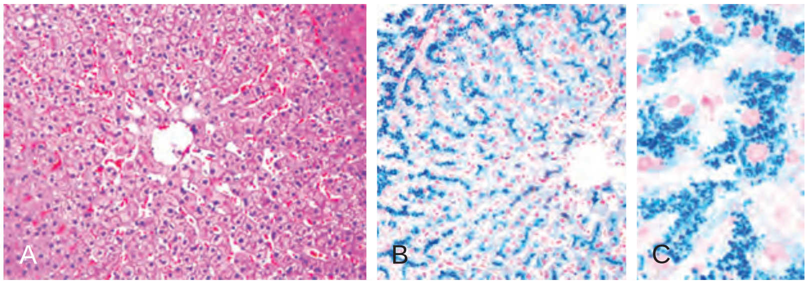


Figure 37.5 LIVER BIOPSY SAMPLE FROM A PATIENT WITH HFE HEMOCHROMATOSIS. Hematoxylin and eosin stain of the liver (A) shows intact hepatic architecture. Iron stain (B, C) shows marked diffuse iron deposits in the hepatocytes throughout the lobules. A normal liver would show essentially no iron in the hepatocytes.

case) does not diagnose hemochromatosis, but only a susceptibility to develop iron overload that should be monitored over time.

LIC provides important diagnostic information in all types of iron overload since it indirectly estimates total body iron content. Once measured by liver biopsy, nowadays it is non-invasively assessed by magnetic resonance imaging (MRI). Indications for liver biopsy persist only for prognostic purposes in C282Y homozygotes with ferritin concentration > 1000 µg/L, who have high risk of liver fibrosis. Besides directly assessing the histological damage, liver biopsy allows Perls' staining, information about iron distribution (Fig. 37.5), and LIC measurement on a dried specimen. Normal LIC ranges between 0.17 and 1.8 mg/g dry weight (or 3 to 33 µmol/g); its determination is useful to monitor iron excess. Studies in thalassemia major have clearly shown that LIC > 3.0 mg/g indicates significant iron overload and levels > 7.0 mg/g associate with clinical complications. Non-invasive tests combining LIC measurement by MRI and evaluation of liver fibrosis by fibroscan now replace liver biopsy in most cases of both genetic and acquired iron overload.[29] MRI may provide quantitative iron concentration also in spleen, pancreas, and heart and is an important tool for diagnosis, and monitoring both iron overload progression and regression after iron chelation in transfusion-dependent thalassemia (see Chapter 41). Assessment of cardiac function, glucose tolerance, and hormonal function is necessary in hemochromatosis patients with ferritin > 1000 µg/L at diagnosis as well as during follow-up of chronically transfused patients.

Differential Diagnosis

The hallmark of hemochromatosis is the high percentage of transferrin saturation in the absence of anemia. A normal blood count excludes iron-loading anemias. Occasionally, rare forms of sideroblastic or dyserythropoietic anemia are misdiagnosed as hemochromatosis because of extremely mild anemia. Bone marrow examination and/or genetic testing establish the correct diagnosis. Transfusion-dependent iron overload or, in rare cases, iron overload due to unintentional excessive intravenous iron administration, are easily excluded by patient history and records. Severity of iron overload, early presentation, presence of complications, and the absence of HFE mutations suggest non-HFE hemochromatosis that can be diagnosed by specific genetic testing. High ferritin levels and normal/low transferrin saturation in the absence of inflammation suggest ferroportin disease. In these cases LIC is increased but macrophages are more iron loaded than hepatocytes at liver biopsy (although rarely performed); at MRI the spleen is iron rich, at variance with the iron-poor spleen in recessive hemochromatosis.

Atransferrinemia is easily diagnosed by measuring serum transferrin (usually < 20 mg/mL) and aceruloplasminemia, by almost undetectable serum ceruloplasmin. Early diagnosis of the latter condition is challenging because the typical clinical triad occurs in midlife. DMT1 deficiency requires a great degree of suspicion and genetic testing for a precise diagnosis. The above three disorders are considered atypical microcytic anemias with features of iron overload.[24] Atypical iron deficiency may be observed in idiopathic pulmonary hemosiderosis, a rare condition of unknown cause, characterized by repeated episodes of bleeding into the lungs that lead to local iron accumulation and fibrosis. Symptoms include coughing, hemoptysis, and anemia. Ferritin may be increased in concomitance with iron deficiency.

Mild liver-restricted iron overload may be observed in individuals with chronic liver disease such as nonalcoholic fatty liver disease, chronic viral hepatitis C infection, alcohol-related liver disease, portacaval shunts, and porphyria cutanea tarda. Patient's history, results of liver function, and other tests may exclude these disorders. The diagnosis of porphyria cutanea tarda relies on its clinical manifestations with photosensitivity and skin lesions and the measurement of urinary porphyrins. The correct diagnosis is important since the disease manifestations improve with iron depletion.

In chronic inflammatory disorders ferritin, an acute phase reactant protein, is increased, transferrin saturation is normal or reduced, and anemia is present. Hyperferritinemia *without* iron overload may occur in malignancy, rheumatologic disorders such as Still disease, hemophagocytic lymphohistiocytosis/macrophage activation syndrome, and chronic infections. Rare cases of isolated high ferritin levels not associated with iron overload nor with inflammation are due to hyperferritinemia-cataract syndrome. This dominant disorder is characterized by an excess of L-ferritin in blood and in the lens, leading to congenital bilateral cataracts usually present in infancy or at an early age, easily diagnosed by slit lamp eye examination. Genetic testing identifies the responsible heterozygous mutation in the iron responsive element of the L-ferritin promoter that renders ferritin L refractory to translational suppression by Iron Regulatory Proteins (see Chapter 36). Transferrin saturation as well as LIC is normal.

Genetic testing is essential for the diagnosis of the different types of non-HFE hemochromatosis. Digenic inheritance (e.g., mutations in both *HFE* and *HJV* or *HFE* and *TFR2*) has been rarely reported. The detection of mutations in different genes will conceivably increase with the implementation of next-generation sequencing-based approaches in cases of unexplained iron overload.

Prognosis

Longitudinal studies have shown that the progression of iron accumulation monitored by increased levels of ferritin is slow in HFE hemochromatosis. In all cases early diagnosis and treatment allow the best outcome. The life expectancy of patients treated before the onset of cirrhosis and/or diabetes is normal. In C282Y homozygotes ferritin > 1000 µg/L is an important predictor of liver fibrosis that may develop even at lower values in the presence of cofactors such as alcohol. Early diagnosis is essential in juvenile hemochromatosis to prevent cardiac complications and the consequences of hypogonadism. In the past some patients died of refractory heart failure, while others have been rescued by heart transplantation.

Regular phlebotomy reduces disease morbidity and mortality. Fatigue, abdominal pain, transaminase increase, initial fibrosis, and skin pigmentation revert with iron depletion. Diabetes and arthralgia may improve, but cirrhosis and arthritis are irreversible and hypogonadism may require lifelong replacement therapy. Cardiomyopathy is of concern in juvenile hemochromatosis; it should be diagnosed early and intensively treated by iron chelation. Hepatocellular carcinoma accounts for a substantial proportion of deaths in patients with liver cirrhosis. For this reason, they need monitoring of serum α-fetoprotein and liver structure at ultrasound. Patients with end-stage liver disease or hepatocellular carcinoma may become candidates for orthotopic liver transplantation. Patients should undergo iron depletion before the procedure since the survival rate of iron-loaded patients is reduced because of iron-toxicity and increased prevalence of infectious complications.

The prognosis of iron-loading anemia is strongly dependent on the disease type, the degree of anemia, and available treatment. Current iron chelation therapy can control secondary iron loading (see Chapter 41).

Therapy

The goal of therapy for iron overload is reduction and maintenance of body iron at normal or near-normal levels. Treatment of hemochromatosis is regular phlebotomy. No evidence-based guidelines are available for this treatment. The recommendation is to phlebotomize all patients with evidence of iron overload since iron normalization decreases morbidity and mortality. However, when to start treatment is still controversial.[22] Phlebotomy is mandatory with ferritin > 1000 µg/L, to prevent/contrast liver fibrosis; it is usually started at lower ferritin thresholds (>500 µgL) or even for ferritin levels just above the normal range (>200 µg/L in females and >300 µg/L in males). An increased LIC detected by MRI is a further indication to start treatment. On the contrary, patients merely exhibiting increased transferrin saturation and normal ferritin are monitored and treated only when ferritin also increases.[30] Modalities to perform phlebotomy in hemochromatosis are described in the box Phlebotomy for Treatment of Hemochromatosis.

PHLEBOTOMY FOR TREATMENT OF HEMOCHROMATOSIS

Phlebotomy to remove excess body iron is the cardinal treatment of hemochromatosis. Removal of one (350–450 mL) unit of blood, according to body weight, removes approximately 200 mg iron. In the initial depletion phase venesection has to be performed weekly until iron stores are normalized, as indicated by reaching the target (50–100 µg/L) serum ferritin. A more intensive protocol is not recommended since strong iron depletion may further suppress hepcidin levels. The duration of this "depletion" phase is variable from a few months to >1 year, depending on the degree of iron overload at diagnosis. Hb levels are measured at each phlebotomy to avoid development of anemia and serum ferritin is checked monthly to monitor the treatment efficacy. If anemia develops the intensity of phlebotomy should be reduced. Concomitant saline infusions are useful to prevent hypotension. Plasma can be reinfused in selected cases, as in cirrhotic patients, to avoid further depletion of plasma proteins. Protocols based on red cell apheresis to remove more iron/session are expensive and had no significant impact on the disease treatment.

Quantitative phlebotomy is a tool to directly estimate iron mobilized from stores. The amount of iron removed may be calculated by weighing the blood bag before and after venesection (density of blood is 1.05 g/mL) and assuming that 450 mL of blood with a normal Hb concentration (13.5 g/dL) contains approximately 200 mg iron. With these assumptions, a patient who underwent 20 weekly venesections had about 4.0 g excess iron removed. This retrospectively provides a rough estimate of iron overload in the individual case.

With ferritin between 50 and 100 µg/L patients enter the "maintenance" phase of treatment to keep serum ferritin within these limits lifelong. Two to four phlebotomies/year usually prevent iron re-accumulation, but their number should be adapted to individual patients. In non-HFE hemochromatosis the protocol is similar, with some cases requiring a prolonged and more intensive treatment even in the maintenance phase (4 to 6 phlebotomy/year). The use of erythropoietin to increase the frequency of phlebotomy is anecdotal. Phlebotomy is not indicated in ferroportin disease due to *loss of function* mutations, while *gain of function* mutations should be treated with the same hemochromatosis protocols.

Phlebotomy is inexpensive, easily available, effective, and usually well tolerated. Hemochromatosis patients do not develop anemia, because low hepcidin, whose expression is further inhibited by phlebotomy, facilitates iron release from stores and erythropoiesis reconstitution. Notwithstanding the effort to set up novel targeted therapies the ancient practice of phlebotomy will likely continue to be key in the treatment of hemochromatosis. Iron chelators are the current alternative to phlebotomy when not tolerated or contraindicated because of anemia, cirrhosis, or cardiac disease. In early cardiac disease a cautious low-intensity phlebotomy may be combined with iron chelation. Advanced cardiomyopathy leading to chronic heart failure requires intensive iron chelation by high-dose intravenous or subcutaneous deferoxamine plus/minus oral chelators as deferiprone or deferasirox. These protocols pioneered in severely iron-loaded thalassemia patients (see Chapter 41) can be an option in advanced cardiac disease in juvenile hemochromatosis, although oral iron chelators are currently off label in non-transfusional iron overload and allowed for compassionate use. Iron chelation can be effective in cirrhotic patients and is mandatory in preparation for transplantation procedures.

In patients undergoing phlebotomy treatment diet recommendations are unnecessary except limiting alcohol intake. Patients with iron loading of whatever cause should avoid consuming raw shellfish, because high circulating NTBI make them susceptible to severe infections by *Vibrio vulnificus*. Depleted patients can be advised to avoid iron-rich food and to add tea at meals to reduce iron absorption.

Iron removal by phlebotomy is effective in porphyria cutanea tarda leading to clinical and biochemical remission of the disease.

Atransferrinemia may benefit from transferrin infusions or blood transfusions and iron chelation. No specific treatment is available for DMT1 deficiency: ESA may partially improve anemia and iron overload. No specific treatment is available for aceruloplasminemia: iron chelation may control systemic iron loading but has no effect on neurological symptoms and can worsen anemia.[24]

Phlebotomy is not an option in patients with iron-loading anemia. There is no correlation between the severity of anemia and the risk of iron loading. The necessity of iron chelation therapy based on determinations of ferritin levels and possibly LIC by MRI should be periodically evaluated even in patients with mild anemia. In non-transfused thalassemia intermedia iron chelation is suggested for ferritin >800 µg/L or LIC >5 mg/g and has to be discontinued for ferritin <300 µg/L or LIC < 3 mg/g.[27] In patients undergoing chronic transfusion regimens since infancy (e.g., thalassemia patients or in other congenital anemias) chelation therapy is usually started after transfusion of 10 to 20 units of red blood cells. In transfusion-dependent adults or elderly patients with anemia refractory to other treatments, indications may differ according to age, basal disorder, and life expectancy and chelation treatment should be individualized. However, the rule of starting therapy after 10 to 20 units of blood seems prudent. Iron overload in survivors of bone marrow transplantation for whatever disorder may be treated by iron chelation or in selected cases by phlebotomy. Three iron chelators are available and the largest experience with their use is for the treatment of transfusion-dependent thalassemia.[27] The time-honored deferoxamine (DFO) has a short half-life and is used either by continuous subcutaneous infusion (20 to 60 mg/Kg) for 8 to 12 hours with a portable pump for 5 to 7 days/week or in some cases intravenously at the highest dose. Side effects may be local hypersensitivity reactions, and at high doses growth defects in children and sensorineural hearing loss. Deferiprone is effective as well but requires divided doses (25 mg/Kg × 3/d) and monitoring of the CBC because of risk of neutropenia and, rarely, of agranulocytosis. Deferasirox (DFX) has a prolonged half-life, the most convenient schedule of administration (20 to 40 mg/Kg once daily), and limited side effects (rash, gastrointestinal disturbances, and transient abnormalities of renal function) and at present is the most widely adopted single agent for iron control in transfused patients. The choice of the regimen depends on the iron burden, the patient compliance, and the presence of comorbidities. Combination therapies may be useful in severe iron loading to accelerate depletion (see Chapter 41).

Future Directions

Diagnosis of HFE hemochromatosis is based on iron parameters, increased tissue iron, and genetic tests. Second-level genetic testing is required to diagnose non-HFE hemochromatosis or unexplained iron overload. Next-generation sequencing approaches are implemented in reference centers to precisely diagnose all hereditary iron disorders by panels of candidate genes.

New research developments include developing novel targeted treatments for iron overload as discussed in the box Targeted Therapies in Development for Iron Overload Disorders. These approaches are of special interest for iron-loading anemia, while their relevance is limited in hemochromatosis, considering both cost and the unknown risk of novel treatments compared to cheap, effective, and safe phlebotomy in most cases.

TARGETED THERAPIES IN DEVELOPMENT FOR IRON OVERLOAD DISORDERS

Hepcidin deficiency is central to iron overload of both hemochromatosis and iron loading anemias. Preclinical studies have shown that correcting the hepcidin deficit may prevent or at least ameliorate iron overload, redistributing iron to sites of safe storage. Hepcidin agonists are hepcidin analogues or inhibitors of the hepcidin suppressor as anti-*TMPRSS6* antisense oligonucleotides or small interfering RNA. Other compounds mimic the hepcidin function by blocking excess iron export by ferroportin. In animal models of thalassemia increasing hepcidin levels by whatever approach not only redistributes parenchymal iron to macrophages, but also improves ineffective erythropoiesis and partially corrects anemia by inducing iron restriction.

Combinatorial therapies targeting different molecular mechanisms may be more powerful than single agents. Results of preclinical studies indicate that increasing hepcidin might benefit even transfusion-dependent thalassemia, since hepcidin levels are rapidly increased by transfusions, and tend to decrease at a temporal distance from transfusions, in parallel with the decreased suppression of ineffective erythropoiesis.

Some molecules (anti-*TMPRSS6* compounds and ferroportin inhibitors), after positive results in preclinical studies, are now being tested in clinical trials. Other ways of limiting iron and improving erythropoiesis are being tested in preclinical studies including transferrin infusions, selective inactivation of bone marrow *Tfr2*, or ERFE neutralizing antibodies. Further novel approaches proposed to reduce iron overload are targeting the intestinal iron transporter *DMT1* by oral administration of specific siRNA in nanoparticles or blocking the HIF-2α essential function by specific inhibitors.

See the online chapter for Cases from Real Life with self-assessment questions.

REFERENCES

1. Camaschella C. Iron-deficiency anemia. *N Engl J Med*. 2015;372:1832–1843.
2. GBD 2016 Disease and Injury Incidence and Prevalence Collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet*. 2017;390:1211–1259.
3. Kassebaum NJ, Jasrasaria R, Naghavi M, et al. A systematic analysis of global anemia burden from 1990 to 2010. *Blood*. 2014;123:615–624.
4. Stauder R, Valent P, Theurl I. Anemia at older age: etiologies, clinical implications, and management. *Blood*. 2018;131:505–514.
5. Kiss JE, Vassallo RR. How do we manage iron deficiency after blood donation? *Br J Haematol*. 2018;181:590–603.
6. Weiss G, Ganz T, Goodnough LT. Anemia of inflammation. *Blood*. 2019;133:40–50.
7. Anand IS, Gupta P. Anemia and iron deficiency in heart failure: current concepts and emerging therapies. *Circulation*. 2018;138:80–98.
8. Heeney MM, Finberg KE. Iron-refractory iron deficiency anemia (IRIDA). *Hematol Oncol Clin North Am*. 2014;28:637–652.
9. Schwartz AJ, Das NK, Ramakrishnan SK, et al. Hepatic hepcidin/intestinal HIF-2alpha axis maintains iron absorption during iron deficiency and overload. *J Clin Invest*. 2019;129:336–348.
10. Das NK, Schwartz AJ, Barthel G, et al. Microbial metabolite signaling is required for systemic iron homeostasis. *Cell Metab*. 2020;31:115–130. e116.
11. Ganz T. Anemia of inflammation. *N Engl J Med*. 2019;381:1148–1157.
12. Girelli D, Nemeth E, Swinkels DW. Hepcidin in the diagnosis of iron disorders. *Blood*. 2016;127:2809–2813.
13. Tolkien Z, Stecher L, Mander AP, et al. Ferrous sulfate supplementation causes significant gastrointestinal side-effects in adults: a systematic review and meta-analysis. *PLoS One*. 2015;10:e0117383.
14. Stoffel NU, Cercamondi CI, Brittenham G, et al. Iron absorption from oral iron supplements given on consecutive versus alternate days and as single morning doses versus twice-daily split dosing in iron-depleted women: two open-label, randomised controlled trials. *Lancet Haematol*. 2017;4:e524–e533.
15. Hershko C, Camaschella C. How I treat unexplained refractory iron deficiency anemia. *Blood*. 2014;123:326–333.
16. Macdougall IC, White C, Anker SD, et al. Intravenous iron in patients undergoing maintenance hemodialysis. *N Engl J Med*. 2019;380:447–458.
17. Auerbach M, Gafter-Gvili A, Macdougall IC. Intravenous iron: a framework for changing the management of iron deficiency. *Lancet Haematol*. 2020;7:e342–e350.
18. Shander A, Kaufman M, Goodnough LT. How I treat anemia in the perisurgical setting. *Blood*. 2020;136(7):814-822.
19. Mueller MM, Van Remoortel H, Meybohm P, et al. Patient Blood management: recommendations from the 2018 Frankfurt Consensus conference. *JAMA*. 2019;321:983–997.
20. Adams PC, Reboussin DM, Barton JC, et al. Hemochromatosis and iron-overload screening in a racially diverse population. *N Engl J Med*. 2005;352:1769–1778.
21. Brissot P, Pietrangelo A, Adams PC, et al. Haemochromatosis. *Nat Rev Dis Primers*. 2018;4:18016.
22. Powell LW, Seckington RC, Deugnier Y. Haemochromatosis. *Lancet*. 2016;388:706–716.
23. Pietrangelo A. Ferroportin disease: pathogenesis, diagnosis and treatment. *Haematologica*. 2017;102:1972–1984.
24. Camaschella C. How I manage patients with atypical microcytic anaemia. *Br J Haematol*. 2013;160:12–24.
25. Bondu S, Alary AS, Lefevre C, et al. A variant erythroferrone disrupts iron homeostasis in SF3B1-mutated myelodysplastic syndrome. *Sci Transl Med*. 2019;11:eaav5467.
26. Allen KJ, Gurrin LC, Constantine CC, et al. Iron-overload-related disease in HFE hereditary hemochromatosis. *N Engl J Med*. 2008;358:221–230.
27. Taher AT, Weatherall DJ, Cappellini MD. Thalassaemia. *Lancet*. 2018;391:155–167.
28. Sandhu K, Flintoff K, Chatfield MD, et al. Phenotypic analysis of hemochromatosis subtypes reveals variations in severity of iron overload and clinical disease. *Blood*. 2018;132:101–110.
29. Kowdley KV, Brown KE, Ahn J, Sundaram V. ACG Clinical guideline: hereditary hemochromatosis. *Am J Gastroenterol*. 2019;114:1202–1218.
30. Adams P, Altes A, Brissot P, et al. Therapeutic recommendations in HFE hemochromatosis for p.Cys282Tyr (C282Y/C282Y) homozygous genotype. *Hepatol Int*. 2018;12:83–86.

Iron Challenge to Test Iron Absorption

Old literature relied on this simple test to discriminate between defective intestinal iron absorption and other causes of chronic iron deficiency. Though this test is rarely performed nowadays, it may still provide useful information. As an example it has been re-proposed to suspect reduced intestinal iron absorption in IRIDA (see reference 1 for further details).

Unfortunately there are no systematically validated, published procedures or expected response criteria for oral iron challenge test. The procedure is largely based on clinical experience and data published in individual case reports in old literature.

Performing the test. The test should be performed on a subject fasting for at least 6 hours.

Blood sample (1) is drawn for basal serum iron, transferrin (TIBC), and ferritin.

Ferrous sulfate (4 mg/kg elemental iron) is administered PO

Blood sample (2) is drawn for serum iron at 90 minutes post dose

Interpretation. If intestinal iron absorption is normal serum iron levels are expected to increase by at least 50 µg/dL 90 minutes after the oral iron challenge.

Failure to achieve serum iron increase (or a minimal increase) in a fasting subject is indicative of defective intestinal iron absorption.

In non-fasting subject's intermediate values cannot be interpreted clearly.

The test does not discriminate between reduced absorption caused by disorders of intestinal mucosa or by excessive hepcidin levels and should be interpreted in the context of clinical and laboratory data.

REFERENCE

1. Heeney MM, Finberg KE. Iron-refractory iron deficiency anemia (IRIDA). *Hematol Oncol Clin North Am.* 2014;28(4):637–652.

Intravenous Iron Treatment: How to Calculate the Correct Dose

The total iron dose needed with IV iron treatment is calculated by the Ganzoni formula:

$$\begin{aligned}&\text{Body weight}(\text{kg})\\&\quad\times\text{Hb deficit}(\text{target Hb}-\text{current Hb})(\text{g/L})\\&\quad\times 0.24*+\text{iron stores}(\text{mg})\end{aligned}$$

$$\begin{aligned}*0.24 &= \text{iron content of Hb}:0.0034\\&\quad\times 0.07(\text{blood volume}=7\%)\end{aligned}$$

Body weight: in obese patients use ideal weight; in patients underweight use current weight.

Examples

In a 50 kg female with Hb 80 g/L

$$50\times(140-80)\times 0.24\ +\ 500*\ =\ 1220\text{ mg iron}$$

In an 80 kg male with the Hb 80 g/L

$$80\times(150-80)\times 0.24\ +\ 500*\ =\ 1844\text{ mg iron}$$

*Iron stores are empirically calculated in 500 mg.

When using ferricarboxymaltose: in patient with Hb < 10 g/dL 1.5 to 2.0 g are infused according to body weight <70 or >70 kg respectively. In less severe anemia 1.0 or 1.5 g are advised according to the body weight as in the scheme below.

Hb g/L	Body weight >35 – <70 kg	Body weight >70
<100	1500 mg	2000 mg
>100	1000 mg	1500 mg

Examples

Thus a 50 kg female with Hb 80g/L will receive 1500 mg (two 750 mg doses 1 week apart)

An 80 kg male with Hb 80g/L will receive 2000 mg (two 1000 mg doses 1 week apart)

How to Manage Hypersensitivity Reactions to Intravenous Iron

Novel IV iron preparations have a better safety profile compared to the old HMW iron dextran that is no longer available. However, since reactions still occur, with severe reactions occasionally reported and a black box present on iron dextran and ferumoxytol, cautiousness is always needed when administering IV iron.

The personnel should be adequately trained. Although nowadays the anaphylactic reaction is exceptional the administration of IV iron has been suggested to be carried out in an environment equipped for resuscitation.[1]

Simple rules based on published recommendations[3–5] are the following:

1. The issue of test dose. A test dose is still advised in the case of iron dextran in United States and Canada: 25 mg of the saline diluted solution should be administered and if no adverse events occur within 1 hour the total dose can be infused. The value of the test dose is not universally accepted (e.g., in Europe it is not required). Further, the European Medicines Agency (EMA) suggests that the test dose should not be performed since reactions can occur even after a negative test that could lead to a false reassurance.[1,2]
2. The issue of premedication. History of previous hypersensitivity reactions to IV iron or of allergy in general should be assessed to ascertain the need of premedication. In all cases premedication with antihistamine preparations should be avoided. These drugs may cause a vasoactive reaction that could be erroneously interpreted as an iron-induced reaction. In patients with a positive history of allergy or undergoing rechallenge after a reaction to IV iron, corticosteroids are the correct indication.
3. Iron infusion The iron infusion should be started at a slower rate than recommended by the manufacturer in patients undergoing first infusion (e.g., 1 hour instead of 15 minutes). Personnel should monitor the patient and should be prepared to stop the iron infusion immediately in case of reaction. Vital signs must be monitored both at the beginning and 30 minutes after the end of the infusion. Reactions to IV iron can be *acute*, occurring during and up to 30 minutes after IV iron or *delayed*, occurring more than 30 minutes up to 24 hours after IV iron. Reactions could be mild, moderate, and severe.[3–5]

Acute Mild Reactions

Symptoms such as urticaria, transient flushing, mild back or chest pain, hypertension, arthralgia, may occur during IV iron infusion. Typically mild reactions are not accompanied by respiratory symptoms or hypotension.

Action: stop infusion and monitor vital signs. Wait and watch until symptoms abate. If symptoms recede, evaluate to resume the infusion at 50% slower rate. If symptoms persist, treat as moderate reactions.

Acute Moderate Reactions

Symptoms are as above plus myalgia, chest tightness, severe back pain, shortness of breath, nausea, tachycardia, mild hypotension.

Action: stop infusion and administer fluids and IV steroids (e.g., hydrocortisone 200 mg). If symptoms recede continue observation for few hours. Future iron therapy should be reconsidered at individual level.

If there is no response treat as a severe reaction.

Acute Severe Reactions

These reactions are potentially life-threatening with symptoms of *anaphylaxis*, shortness of breath, stridor, wheezing, angioedema, severe hypotension (drop of 30 mmHg SBP or SBP < 90 mmHg not corrected by fluids), cyanosis, loss of consciousness, cardiac/respiratory arrest.

Action: stop iron infusion followed by immediate administration of fluids IV and adrenaline (0.3 to 0.5 mL of 1/1000 IM), a dose that can be repeated, beta2 agonists, IV corticosteroids (methylprednisolone 100 mg), ACLS if needed. Evaluate transfer to ICU.

Risk of severe reactions is 1:200,000. Consider that this is much less than 1:20,000 with blood transfusions![8]

Delayed Reactions

The most common delayed symptoms are fever, myalgia, arthralgia, and headache, which are usually treated at home by acetaminophen.

All reactions to IV iron should be reported to the national pharmavigilance agency.

Re-challenge

Whether re-challenge with IV iron is worth it and safe in a patient who had a previous reaction is related to the reaction type. The decision to re-challenge should be based on the severity and nature of the previous reaction, individual characteristics and medical need, and patient preference. An open discussion with the patient is recommended.[5,6] In all cases of re-challenge the advice is to change the IV iron preparation. Re-challenge is not advised after a severe reaction. However, some cases were successfully treated after desensitization (for protocols see reference 8).

REFERENCES

1. European Medicines Agency. Assessment report for: iron containing intravenous (IV) medicinal products. EMEA/H/A-31/1322. European Medicines Agency, 2013. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Referrals_document/IV_iron_31/WC500150771.pdf.
2. Auerbach M, Deloughery T. Single-dose intravenous iron for iron deficiency: a new paradigm. *Hematol Am Soc Hematol Educ Prog*. 2016;2016(1):57–66.
3. Rampton D, Folkersen J, Fishbane S et al. Hypersensitivity reactions to intravenous iron: guidance for risk minimization and management. *Haematologica*. 2014;99:1671–1676.
4. Szebeni J, Fishbane S, Hedenus M, et al. Hypersensitivity to intravenous iron: classification, terminology, mechanisms and management. *Br J Pharmacol*. 2015;172(21):5025–5036.
5. Lim W et al, Canadian expert consensus: management of hypersensitivity reactions to intravenous iron in adults. *Vox Sanguinis*. 2019;114: 363–373.
6. Auerbach M. Treatment of iron deficiency anemia in adults. *UpToDate* (updated: May 05, 2020)
7. Avni T, Bieber A, Grossman A et al. The safety of intravenous iron preparations: systematic review and meta-analysis. *Mayo Clinic Proc*. 2015;90(1):12–23.
8. Chapman E, Leal D, Alvarez L, et al. Two case reports of desensitization in patients with hypersensitivity to iron. *World Allergy Organ J*. 2017;10:38.

CASES FROM REAL LIFE

Case 1

History

A young woman aged 28 complains of fatigue. Previous history is unremarkable. She has a balanced diet, does not drink alcohol, and does not smoke. Bowel movements are regular and she has no gastrointestinal symptoms. Menarche occurred at 12 years of age, menses are normal, and the patient has been on contraceptives for 3 years with regular follow-up. Previous CBC showed mild anemia that was not evaluated in depth except for search of fecal occult blood, which was repeatedly negative.
Physical examination is unremarkable.
A recent CBC showed:

Hb 9.6 g/dL, Hct 30 %, RBC 3.8×10^{12}/L, MCV 78 fL,
MCH 25.2 g/dL, MCHC 32%, WBC 4.2 ×10^{9}/L,
platelets 450.000/μL

A slight worsening of anemia was noted compared with the tests performed one year before:

Hb 10.2 g/dL, Ht 32%, RBC 4.0×10^{12}/L, MCV 80 fL,
MHC 25.5 pg, MCHC 31.8

Question #1. Which is the most important diagnostic test?

1. Hemoglobin electrophoresis to exclude alpha or beta-thalassemia trait
2. Serum ferritin to assess iron deficiency
3. Total iron binding capacity to assess iron deficiency
4. Serum soluble transferrin receptor to assess iron deficiency
5. CRP to exclude anemia secondary to an undiagnosed chronic inflammatory disorder

Comment. The correct answer is **2**.

Anemia is moderate, with worsening tendency, erythrocyte indexes are reduced compared to the previous borderline normal values, both the course and the values are suggestive of a slowly progressive iron deficiency anemia.

Low serum ferritin (<30 μg/L) provides evidence of iron deficiency even before anemia becomes overt. In the presence of anemia ferritin is usually <12 μg/L.

Even if alpha and beta thalassemia carriers (traits) have microcytic hypochromic RBC, anemia is usually mild and microcytosis is disproportionately severe for the degree of anemia. Erythrocytosis can also be observed.

Total iron binding capacity (TIBC) is usually increased in iron deficiency but it does not diagnose the condition if not related to serum iron. Soluble transferrin receptor levels are elevated in iron deficiency, however, the test is not a routine diagnostic test.

Anemia of inflammation is usually normocytic and associated with a chronic inflammatory disorder. Normal CRP excludes anemia of inflammation.

Making the Diagnosis

The results of the tests required by the general practitioner were as follows:

Serum iron 20 μg/dL, transferrin 310 mg/dL, transferrin saturation 8%.
Serum ferritin 8 μg/L, hemoglobin electrophoresis: Hb A_2 2.5% (normal level)

Glucose 92 mg/dL, creatinine 0.82 mg/dL, AST 45 U/L, ALT 75 U/L

Question #2. Which further test would you require to investigate a possible cause of anemia in this young woman?

1. Repeat the search for fecal occult blood
2. Repeat gynecological evaluation
3. Coagulation tests: INR and PTT
4. Gastroscopy and colonoscopy
5. Anti-transglutaminase antibodies to screen for celiac disease

Comment. The correct answer is **5**.

As for the more appropriate tests to be checked in a confirmed iron deficiency anemia the following should be considered.

Gastrointestinal blood losses are common causes of iron deficiency anemia in the elderly, but are exceptional in young females; moreover occult blood was previously tested in the patient and was negative. GI endoscopy is never a first-line exam in a young woman.

Gynecological examination is mandatory in the presence of heavy uterine bleeding, but it will unlikely reveal the cause of iron deficiency when menses are regular. In addition, since the patient is on contraceptives, she is on gynecologic follow-up.

Coagulation tests explore systemic bleeding tendency, but the patient had no evidence of susceptibility to bruising, epistaxis, or excess menstrual bleeding.

Celiac disease in adults may go undiagnosed because of unspecific symptoms and often can be revealed by iron deficiency. Mildly increased transaminases may be compatible with such a diagnosis. Final diagnosis requires endoscopy with histological examination of duodenal biopsy to demonstrate villus atrophy and lymphocyte infiltration.

Treatment

The patient was referred to a gastroenterologist who confirmed the diagnosis of celiac disease based on duodenoscopy and mucosal biopsy.

Question #3. Which treatment would you suggest as the most appropriate for an iron-deficient patient with celiac disease?

1. Start IV iron 750 mg in a single injection and gluten-free diet
2. Start gluten-free diet and oral iron (120 mg/day)
3. Prescribe iron sulfate 60 mg, vitamin B12, and folic acid plus vitamin C 1 g
4. Transfuse one unit of red blood cells followed by 60 mg oral iron sulfate
5. Gluten-free diet and oral iron sulfate: 60 mg at alternate day for 1 month

Comment. The correct answer is **1**.

Although oral iron may be used in mild celiac disease and has allowed healing of the duodenal mucosa after prolonged gluten-free diet, oral iron is not absorbed by damaged villi. For this reason IV iron (e.g., ferric-carboxymaltose) may be the best choice. A first 750 mg infusion can be adequate if the gluten-free diet and mucosal healing allows iron absorption. Whether a second injection is needed or oral iron can be prescribed should be evaluated based on further iron tests. The aim is to reach a ferritin level within normal range (40 to 100 µg/L).

The dose of 60 mg oral iron is insufficient in iron deficiency anemia. Moreover, a combination of oral iron with other vitamins is useless in isolated iron deficiency. This treatment increases costs without providing appreciable benefits, except for the addition of vitamin C, which facilitates iron absorption.

Transfusion of red blood cells is indicated only in severe symptomatic anemia. Young patients usually adapt to and tolerate anemia especially when moderate, as in the current patient whose only symptom was fatigue. The dosage of 60 mg oral iron at alternate days may be adequate in iron deficiency without anemia and will certainly help avoid side effects, but one-month oral treatment is insufficient to replete the iron-exhausted stores.

Patient Follow-Up

1 month after treatment with IV iron Hb was 11.5, ferritin 25 µg/L. Fatigue disappeared during treatment and the patient was feeling well. Iron sulfate was prescribed at 120 mg PO at alternate day for 2 further months. Hb and ferritin normalized (Hb 12.8, ferritin 40) 3 months later and anemia did not relapse.

Final diagnosis: Iron deficiency anemia of moderate degree in celiac disease, fully responsive to gluten-free diet and a combination of IV and oral iron.

Conclusion

This patient, who belongs to a risk group for iron deficiency, exemplifies how iron deficiency anemia may reveal a primary disorder that should be treated together with iron supplementation.

Case 2

History

A 45-year-old White male presents with fatigue and abdominal pain for a few months. He denies systemic and GI symptoms or recent infectious episodes.

The patient is an employee in a computer firm, is an occasional social drinker, and does not smoke. Past history was unremarkable until 2 years before when a modest increase of liver enzymes was discovered with negative serology for chronic hepatitis. At that time ferritin was 510 µg/L. Control after 6 months of alcohol withdrawal was advised, but patient did not comply with prescriptions.

At physical examination the liver was enlarged (about 2 cm from the costal margin) without splenomegaly or lymphadenopathy.

Reevaluation of blood tests showed:

Hb 15.4 g/dL, Ht 48%, RBC 5.4×10^{12}/L, MCV 88 fl,
MCH28.5 pg, MCHC 32%
WBC 5.6×10^9/L (N71 L24 M5%), Plat 180000/µL
AST 48 UI, ALT 86 UI, γGT 88 UI, Alk Ph 140 UI

Creatinine, glucose, uric acid, total bilirubin, CRP, total cholesterol, triglycerides, urinalysis were within normal ranges.

Liver US revealed an enlarged liver with normal structure and mild steatosis. No biliary stones, spleen of normal size and structure.

Question #1. Which other tests should be prescribed in this male with hepatomegaly and fatigue?

1. Liver biopsy
2. Anti-EBV and anti-cytomegalovirus antibodies
3. Neoplastic markers
4. Serum iron, TIBC, and serum ferritin
5. Antinuclear antibodies; anti-smooth muscle antibodies

Comment. The correct answer is **4**.

Fatigue and liver enlargement with modest alterations of liver function tests in a middle-aged male may be initial signs of hemochromatosis. Previous increased ferritin levels with normal CRP should be considered. Liver biopsy is not indicated as a first test considering the modest increase of liver enzymes. Lack of systemic symptoms and of lymph node enlargement are against the diagnosis of mononucleosis and normal CBC and differential against cytomegalovirus infection. There is no reason to suspect cancer and no symptoms of localization; in addition, neoplastic markers are never diagnostic. Autoimmune hepatitis is unlikely in the absence of other autoimmune manifestations and symptoms.

Results of iron tests were as follows:

Serum iron	195 µg/dL
TIBC	290 µg/dL
Tsat iron/TIBC	67 %
Serum ferritin	690 µg/L

Making the Diagnosis

The results of iron and liver function tests in a middle-aged White male without a history of significant alcohol intake suggest hemochromatosis. Hepatomegaly, fatigue, and abdominal pain are common initial symptoms. Transferrin saturation (serum iron/TIBC) in hemochromatosis is >50% and ferritin levels are high and increased as compared to 2 years before.
Question #2. Which is the most important diagnostic test?

1. Genetic test to search for *HFE* C282Y mutation
2. Reevaluation of iron tests and liver enzymes after changing lifestyle and decreasing meat consumption
3. Reevaluation of ferritin after alcohol withdrawal for three months
4. Liver biopsy to assess fibrosis and document increased LIC
5. Glucose tolerance test

Comment. The correct answer is **1**.

HFE homozygous C282Y mutation is the most common genotype in hereditary hemochromatosis in White males. Changing of lifestyle does not affect levels of iron, while withdrawing alcohol decreases ferritin when its excess is the cause of hyperferritinemia. Liver biopsy is not performed any longer for diagnostic purposes and rarely to assess fibrosis when ferritin is >1000. LIC and fibrosis can be both assessed in a non-invasive manner by MRI and fibroscan respectively, as a further evaluation and staging of the disease. Glucose tolerance test is useful in established diagnosis to assess one of iron overload complication.

Treatment

When a diagnosis of hemochromatosis is established in a symptomatic patient with high ferritin levels treatment should be considered.

Question #3. Which is the standard treatment of hemochromatosis?

1. Daily oral iron chelation with deferasirox
2. Weekly phlebotomy (350–450 mL) until iron depletion is reached
3. Cycles of iron chelation with subcutaneous deferoxamine
4. Blood donations
5. Plasmapheresis to remove circulating iron

Comment. The correct answer is **2**.

Weekly phlebotomy is the standard treatment of hemochromatosis. Iron chelation is indicated only in patients who cannot tolerate phlebotomy or when cardiac complications are present, but is never the first-line therapy. Individuals with hemochromatosis are accepted as blood donors (according to local rules in some countries) only in the absence of evidence of tissue damage. However, while blood donors may be protected by iron accumulation, the number of donations allowed per year would be insufficient to deplete iron in a patient with iron overload. Plasmapheresis has no common sense since iron in plasma is a minimal amount of total body iron and does not correlate with stored iron.

Patient Monitoring During Phlebotomy Treatment

After four venesections the patient feels better and more energetic. He tolerates well the treatment and Hb levels are normal. Phlebotomy is continued to reach iron depletion.

Question #4. Which test better indicates that iron depletion has been reached?

1. Normal serum hepcidin levels
2. Transferrin saturation <50%
3. Serum ferritin (50–100 µg/L)
4. Normal serum iron
5. Normal liver iron content (LIC) at MRI

Comment. The correct answer is **3**.

Serum ferritin provides guidance for phlebotomy treatment since its levels reflect iron stores. Serum hepcidin is not available as a clinical test; in addition, hepcidin levels are low in hemochromatosis and may be further decreased by phlebotomy.

Transferrin saturation is not a measure of iron stores and may persist elevated even at the end of phlebotomy treatment. The same holds true for serum iron.

LIC by MRI is a useful determination that can be done at the beginning (and eventually at the end) of treatment but is not indicated as a monitoring test.

Patient Follow-Up

Phlebotomy treatment was continued for 8 months before reaching ferritin 88 µg/L. Hb persisted normal; no adverse events were recorded and the patient continued his normal activity.

Control of liver function tests at the end of treatment showed normalization of AST, ALT, and γGT levels.

Final diagnosis: HFE hemochromatosis due to C282Y homozygous mutation with modest alterations of liver function tests and moderately increased ferritin (<1000 µg/L), successfully treated by phlebotomy.

Conclusion

This case exemplifies the classic presentation of HFE hemochromatosis with signs of initial liver damage. The diagnosis cannot be suspected until iron tests are performed. The clinical picture is fully reversible by iron depletion as shown by the liver function tests normalization and symptoms regression after treatment. Lifelong maintenance phlebotomy allows normal life and normal life expectancy.

CHAPTER 38

ANEMIA OF CHRONIC INFLAMMATION

Yelena Z. Ginzburg

Anemia of chronic inflammation (ACI) is also termed anemia of chronic disease and considered the second most frequent anemia in the world, after iron deficiency anemia (IDA).[1] ACI is also the most common anemia in hospitalized patients, found in conditions associated with an activated immune response, including chronic infections, autoimmune and inflammatory illnesses, and malignancy. The underlying cause of anemia in these diseases is multifactorial, including both direct and indirect inhibition of red blood cell (RBC) production in the bone marrow and mildly decreased RBC life span. The teleological argument for the presence of anemia in these conditions is presumed to be related to iron sequestration, providing an evolutionary advantage in light of the iron dependence of pathogens and rapidly replicating cells. Thus, iron sequestration restricts iron availability and serves to limit the growth of pathogens and malignant cells at the expense of hemoglobin synthesis. Previously considered a diagnosis of exclusion, with treatment mainly focused on the underlying disease, the identification of the peptide hormone hepcidin and its major role in the pathophysiology of ACI have enabled both an enhanced mechanistic understanding and development of novel therapeutics for ACI.

In addition to anemia associated with chronic infections, autoimmune and inflammatory illnesses and cancer, anemia in patients with chronic renal disease, congestive heart failure, and solid organ transplant is also categorized as ACI due to an inflammatory component in these conditions. In contrast, an inflammatory component in idiopathic anemia of the elderly, which often presents with typical features of ACI, remains presumptive. For the purposes of this chapter, we offer a perspective on ACI in its broadest definition. Despite this, the specific details may not apply identically to all diseases on this list (e.g., in chronic renal insufficiency, anemia is in large part driven by inadequate erythropoietin [Epo] or "Epo resistance," although therapeutic benefit in anemia management may be gained by suppressing hepcidin).

DIAGNOSIS OF ANEMIA OF CHRONIC INFLAMMATION

The traditional gold standard approach to identify iron deficiency anemia (IDA) in anemia of chronic inflammation (ACI) is Prussian-blue staining for iron within macrophages on a bone marrow biopsy specimen. However, this approach has been challenged in light of its invasiveness and high inter-observer variability. In a study of mostly older patients with idiopathic moderate anemia (i.e., hemoglobin 10 g/dL), a bone marrow aspirate with biopsy added little to physical examination and serology. Furthermore, iron-loaded macrophages in bone marrow biopsy specimens correlate poorly with the availability of this iron for erythropoiesis in high hepcidin states, as in ACI. Bone marrow examination may, however, be necessary in some situations to rule out other potential causes of anemia, including malignancy (e.g., myelodysplastic syndrome). Serum ferritin functions as an acute phase reactant. Hence, it alone is insufficiently specific to identify IDA in ACI, unless it is very low (i.e., serum ferritin <10 ng/mL) (Fig. 38.1). Serum ferritin is in equilibrium with cellular ferritin. Thus, serum ferritin increases when iron is sequestered in the liver and splenic macrophages where cellular ferritin expression is increased, as in ACI.[2] Despite this, some degree of elevated ferritin concentration does not exclude a possible response to parenteral iron therapy.[3]

While some investigators argue that a ferritin level >50 ng/mL excludes any component of iron deficiency even in inflammatory states, a meta-analysis of iron studies in clinical reports from 1842 subjects with serum ferritin levels >45 ng/mL showed a prevalence of iron deficiency of 6.7% and 3.5% of 1368 subjects with a serum ferritin level >100 ng/mL had iron deficiency. Other researchers have shown that in acute inflammation, even serum ferritin levels >3500 ng/mL can coexist with absent iron stores in bone marrow aspirates.

DEFINITION OF ANEMIA OF CHRONIC INFLAMMATION

Anemia of chronic inflammation (ACI) remains a diagnosis of exclusion and is characterized as a mild, normocytic normochromic hypoproliferative anemia with hemoglobin ranging from 8 to 10 g/dL (Fig. 38.1). ACI may present, however, with hemoglobin <8 g/dL and sometimes dramatic microcytosis in up to one-third of patients. Typically, elevated inflammatory markers (e.g., C-reactive protein [CRP], erythrocyte sedimentation rate [ESR], and interleukin 6 [IL-6]) and decreased circulating serum iron concentration and transferrin saturation despite ample iron stores (e.g., serum ferritin >100 ng/mL) help to diagnose ACI. ACI may be difficult to differentiate from iron deficiency anemia (IDA). IDA may also coexist with ACI as chronic inflammation, infection, and malignancy may coexist with nutritional deficiency, blood loss, hemolysis, renal failure, and primary bone marrow disorders. IDA in the setting of ACI is typically a consequence of blood loss, either gastrointestinal or genitourinary. For example, chronic inflammatory bowel diseases are frequently associated with both chronic gastrointestinal bleeding secondary to the underlying disease and its treatment (with nonsteroidal anti-inflammatory drugs [NSAIDs] and glucocorticoids). In such cases, localizing and halting the source of bleeding are both required in addition to potential iron replacement. In autoimmune gastritis and celiac disease, iron deficiency may also result from decreased iron absorption. Precise characterization of the diathesis underlying the anemia is thus essential to ascertaining whether administration of iron is likely to improve or resolve the anemia. Furthermore, the underlying pathophysiology of anemia can assist with the decision-making regarding whether enteral or only parenteral iron administration is expected to work.

ACI is independently associated with poor outcomes and a decrease in quality-of-life measures. Standard approaches, such as parenteral iron and erythropoiesis-stimulating agents (ESAs), are not consistently effective. Thus, despite its frequency and significant impact both on morbidity and mortality and new insight into the role of hepcidin, the pathophysiology of ACI remains incompletely understood and currently available therapies are not effectively optimized. The goals of this chapter are to (1) describe the clinical presentation and diagnosis of ACI, (2) elucidate our current understanding of inflammation and iron metabolism and their mechanistic relationship with erythropoiesis, and (3) discuss both the current standard and future potential therapeutic modalities.

Additional parameters have been proposed to differentiate ACI from IDA and provide evidence of concurrent IDA and ACI. Those include soluble transferrin receptor 1 (sTfR1) concentration and sTfR1/log ferritin (Fig. 38.1); these markers have been proposed as estimates of iron-deficient erythropoiesis. For example, circulating sTfR1 concentration is proportional to the cellular expression of the membrane-associated transferrin receptor 1 (TfR1), increases with increased cellular iron requirements and cellular proliferation[4–6] and is unaffected by inflammation. While a low ratio sTfR1/log ferritin (<1) suggests ACI, sTfR1/log ferritin greater than 2 is indicative of concurrent IDA and ACI. Despite this potential, sTfR1 assays have

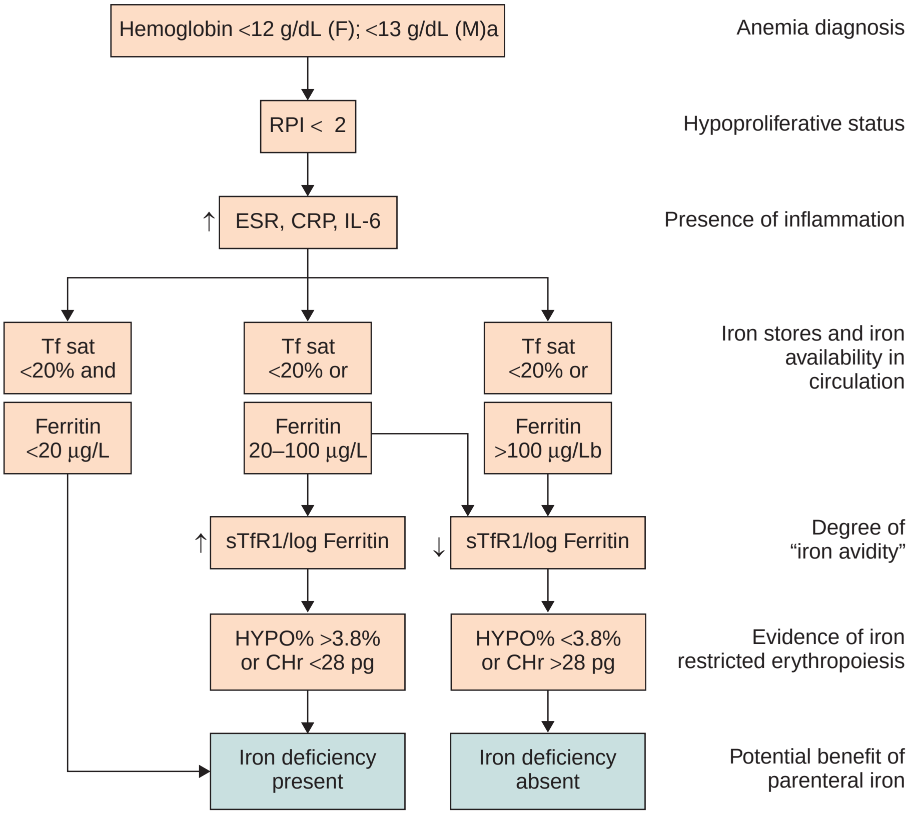

Hemoglobin <12 g/dL (F); <13 g/dL (M)a
Anemia diagnosis
RPI < 2
Hypoproliferative status
↑ ESR, CRP, IL-6
Presence of inflammation
Tf sat <20% and
Ferritin <20 µg/L
Tf sat <20% or
Ferritin 20–100 µg/L
Tf sat <20% or
Ferritin >100 µg/Lb
Iron stores and iron availability in circulation
↑ sTfR1/log Ferritin
↓ sTfR1/log Ferritin
Degree of "iron avidity"
HYPO% >3.8% or CHr <28 pg
HYPO% <3.8% or CHr >28 pg
Evidence of iron restricted erythropoiesis
Iron deficiency present
Iron deficiency absent
Potential benefit of parenteral iron

Figure 38.1 ALGORITHM FOR DIAGNOSIS OF ANEMIA OF CHRONIC INFLAMMATION AND IDENTIFYING CONCURRENT IRON DEFICIENCY. [a]Consider further diagnosis and treatment if hemoglobin less than 10 g/dL and patient is symptomatic. [b]In certain circumstances, patients with ferritin greater than 100 ug/L may be responsive to iron supplementation (e.g., chronic renal insufficiency and congestive heart failure). *CHr*, Reticulocyte corpuscular hemoglobin; *F*, female; *HYPO%*, percent hypochromic reticulocytes; *M*, male; *RPI*, reticulocyte production index; *sTfR1*, soluble transferrin receptor 1; *Tf sat*, transferrin saturation.

not been standardized or widely adopted in light of the lack of convincing data of their utility.

A CLIA-approved and CAP-accredited hepcidin assay is available,[7] and a potential standardized international hepcidin reference range is being developed.[8] An algorithm that incorporates hepcidin levels is under development to increase specificity for IDA in ACD. To date, studies reveal that relatively high hepcidin has been associated with lower mean corpuscular hemoglobin in anemic patients and impaired response to ESAs in an animal model of ACI,[9,10] providing some degree of promise for its utility. Finally, hemoglobin concentration in reticulocytes (CHr), distinct from indices of mature RBCs, can reflect the recent status of iron stores in normal or Epo-induced erythropoiesis. sTfR1-, CHr-, and hepcidin-based algorithms for iron-replete and iron-deficient ACI are being developed. A combination of these will likely be useful in diagnosing and differentiating ACI from ACI with concurrent IDA. For now, none of these additional parameters are fully characterized or yet widely incorporated into clinical practice, and the diagnosis of ACI relies on more standard measures (see Fig. 38.1).

CLINICAL OVERVIEW OF ANEMIA OF CHRONIC INFLAMMATION

ACI may co-occur with many different underlying diseases, including diseases not conventionally considered "inflammatory," such as coronary artery disease, congestive heart failure, obesity, renal failure, and diabetes; patients with these illnesses often exhibit cytokine abnormalities more typically associated with inflammatory conditions (e.g., elevated interleukin 6 [IL-6] and C-reactive protein [CRP] in kidney disease or CRP and fibrinogen in myocardial infarction). For example, 52% of hospitalized patients with unexplained anemia meet laboratory criteria for ACI, and of the 184 patients admitted to intensive care units, most patients with anemia are found to have laboratory findings consistent with ACI (while 13% also had nutritional causes of anemia [iron, folate, or vitamin B12 deficiency]). Patients older than age 60 years have a 24% 3-year incidence of ACI. The epidemiological studies of ACI are complicated by the heterogeneity of underlying diseases. ACI is also extremely common in cancer; 39% of cancer patients are found to be anemic. Chemotherapy with or without radiation therapy increases the incidence of anemia to 63% and 42%, respectively. Anemia is 56% prevalent in patients positive for human immunodeficiency virus (HIV), directly proportional to the burden of symptoms, and mild anemia is 33% to 60% prevalent in patients with rheumatoid arthritis (RA). Although the clinical setting in which anemia is found helps with the diagnosis of ACI, an underlying inflammatory process could not be identified in 30% of cases.

ETIOLOGY AND PATHOPHYSIOLOGY OF ANEMIA OF CHRONIC INFLAMMATION

The pathophysiology of ACI is multifactorial, resulting from the effects of inflammatory cytokines, particularly interleukin-1 (IL-1), IL-6, IL-10, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IFN-α, and IFN-β, all or some of which are increased in most inflammatory processes, i.e., infections, autoimmune disorders, and malignancies.[11] Cytokine levels frequently correlate inversely with hemoglobin, potentially implicating them in the etiology of ACI. Serum TNF-α levels correlate with both disease activity and the degree of anemia in RA. Transgenic mice with endogenously elevated IFN-γ exhibit preferential differentiation of non-erythroid lineage hematopoietic precursors and diminished erythroid burst-forming units (BFU-E). Furthermore, multiple clinical trials using cytokine antagonists in inflammatory diseases demonstrate effective reversal of anemia. For example, in patients with Crohn disease, anti-TNF-α therapy improved both disease activity and anemia. Anti-TNF-α therapy in vitro increased the growth of peripheral blood-derived erythroid progenitors from patients with active disease. Multiple lines of evidence suggest that elevated inflammatory cytokines lead to (1) decreased RBC life span, (2) increased iron sequestration and resultant decreases in iron availability for erythropoiesis and hemoglobin synthesis, (3) direct and indirect inhibition of erythroid progenitor differentiation, and (4) inadequate Epo response to anemia (Fig. 38.2).

Cytokine-Induced Decreases in Red Blood Cell Life Span

The underlying mechanism for the reduction in RBC life span in ACI is not fully elucidated, but studies support the absence of an intrinsic RBC defect. TNF-α, IFN-γ, and IL-1 specifically enhance erythrophagocytosis by splenic macrophages, leading to a shortened RBC life span (see Fig. 38.2). RBCs from ACI patients have a normal life span when infused into normal subjects, suggesting an abnormality extrinsic to the RBC itself. For example, mice with elevated IFN-γ levels (either as a transgene or by exogenous administration) exhibit reduced RBC survival because of RBC removal by cytokine-stimulated macrophages. In addition, chronic sub-lethal doses of TNF-α in rats results in a 25% decline in total RBC mass and decrease in RBC survival. Furthermore, alterations in RBC rheology (deformability and aggregation) are seen in intensive care unit patients, especially those with sepsis. In vivo and in vitro experiments suggest that fever itself can induce rheologic changes in RBCs, leading to increased destruction and up to a 15% decline in RBC mass. Similarly, increased cytokines, for example IL-1, in RA patients are associated with enhanced RBC-phagocytic ability of macrophages.

Cytokines Leading to Direct and Indirect Inhibition of Erythropoiesis

Inflammatory cytokines also directly suppress erythroid precursor proliferation and differentiation (Epo responsiveness is discussed in subsequent sections).[12–14] As a consequence, the bone marrow's ability to compensate for enhanced erythrophagocytosis is hampered. Studies support a role for TNF-α, IL-1, IFN-γ, and perhaps IL-6 in this direct inhibition.

First, IFN-γ modulates gene expression involved in the in vitro proliferation of hematopoietic stem cells, thus affecting self-renewal. Second, the addition of IFN-γ to in vitro cultured erythroid progenitors increases the expression of the transcription factor PU.1. Since PU.1 directly interacts with the major erythroid transcription factor GATA-1, it is postulated that IFN-γ treatment decreases proliferation and differentiation of erythroid progenitors by a PU.1 dependent mechanism.[15] Third, serum TNF-α levels inversely correlate with hemoglobin levels and BFU-E number in RA patients. Fourth, treatment with anti-TNF improves proliferation and decreases apoptosis of erythroid progenitor cells. The actual effect of TNF-α on erythropoiesis is likely indirect, mediated by the local release of other cytokines including IFN from accessory cells, since the inhibitory effect of TNF-α on CFU-E was completely abrogated by neutralizing antibodies against IFN-β but not by antibodies to IFN-γ or IL-1. Similarly, the effects of IL-1 also appear to be indirect since the growth of purified CFU-E is inhibited by IL-1 only in the presence of adherent T lymphocytes. This inhibition can be reversed by antibodies to IFN-γ, suggesting that IL-1 leads to lymphocyte secretion of IFN. Lastly, more recent data suggest that IL-6 may have a suppressive effect directly on erythroid precursors.[15]

In in vitro erythroid colony formation assays derived from bone marrows of patients with ACI have shed some light specifically on extrinsic effects on erythroblast suppression. The removal of bone marrow–adherent cells (mostly macrophages and monocytes) leads to increased erythroid colony formation. This effect is lost after co-culture with ACI-adherent cells but unaffected by co-culture with control bone marrow–derived adherent cells. Patients with RA have decreased BFU-E that are inversely proportional to circulating TNF

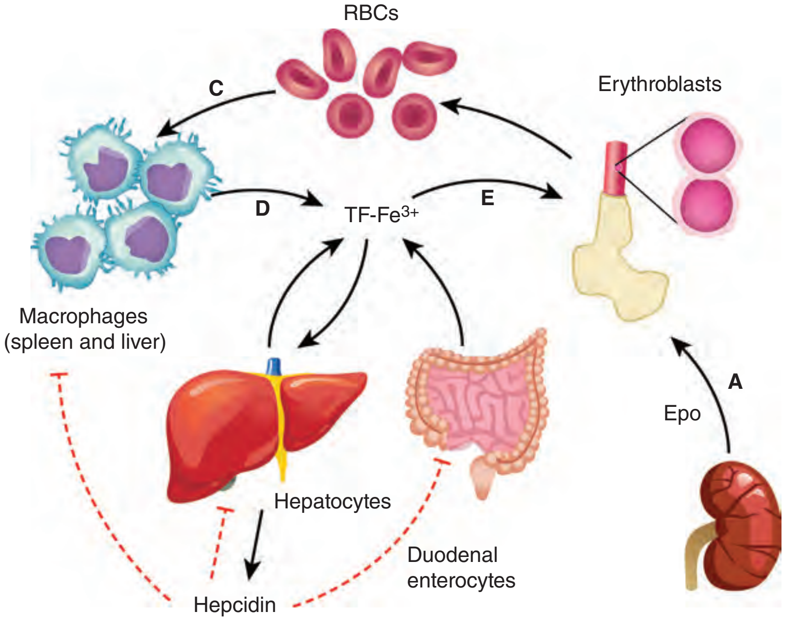


Figure 38.2 MECHANISTIC UNDERPINNINGS OF ANEMIA OF CHRONIC INFLAMMATION. Circulating inflammatory cytokines cause anemia in inflammatory states through several mechanisms. (A) Decreased erythropoietin production in the kidney. (B) Direct and indirect inhibition of erythropoiesis. (C) Decreased red blood cell life expectancy in circulation and increased macrophage erythrophagocytosis. (D) Iron sequestration in cells involved in iron flows (e.g., erythrophagocytosing macrophages, hepatocytes, duodenal enterocytes) in response to increased hepcidin expression, and consequently (E) decreased iron availability for erythropoiesis. *Epo*, Erythropoietin; *RBC*, red blood cell; *TF-Fe*$^{+3}$, transferrin-bound iron in circulation.

levels. In parallel, culture with serum from patients with RA and anemia inhibits BFU-E proliferation, while serum from non-anemic RA patients does not. While these are not definitive studies and do not specifically delineate the factors involved, they point to an extrinsic effect on erythropoiesis.

Together, these data provide evidence of the complex crosstalk between inflammation and erythropoiesis with significant work yet to be done to enhance clarity and provide a more comprehensive understanding of the underlying molecular mechanisms beyond the mostly correlative evidence thus far.

Cytokines Leading to Decreased Erythropoietin Secretion

Inflammatory cytokines may also influence Epo production.[16–18] Epo is the essential hormone that induces erythropoiesis and elevated serum Epo is a typical finding in all anemias. The exception to this rule is the anemia in renal insufficiency which is in large part the consequence of decreased Epo production as the underlying cause of anemia. The idea of whether Epo response is or is not appropriate relative to the degree of anemia is currently only measurable in relative clinical terms. In other words, serum Epo levels compared across different diseases with similar degrees of anemia (i.e., hemoglobin level) provide relative evidence of Epo responsiveness (e.g., high Epo with low hemoglobin) or insufficient Epo levels (e.g., low Epo with low hemoglobin) (Fig. 38.3). In a study of 81 patients with solid tumors and clinical ACI but without tumor infiltrating the bone marrow, Epo levels were higher than in controls without anemia but half that of control subjects with IDA. Similarly, serum Epo levels are inappropriately low in HIV-positive patients with anemia and in lung transplant recipients.

To understand how inflammation may cause decreased serum Epo, several hypotheses can be explored. First, both cytokine-mediated alterations in binding affinities of Epo-inducing transcription factors and damage of Epo-producing cells have been hypothesized as potential mechanisms. For example, lipopolysaccharide (LPS) injection to induce inflammation in mice results in decreased Epo mRNA expression in the kidney and decreased serum Epo levels. Second, IL-1α, IL-1β, and TNF-α significantly decrease Epo production in kidney cell cultures, and IL-1β inhibits Epo production in perfused rat kidneys. Third, autoantibodies to Epo have been detected in subjects with autoimmune diseases (e.g., systemic lupus erythematosus [SLE]) and may contribute to low circulating Epo levels even in the absence of renal involvement in these diseases. Fourth, Epo may not function optimally in the presence of inflammatory cytokines. Epo-resistant subjects with end-stage renal disease (ESRD) are more likely to have elevated inflammatory cytokines, and peripheral blood mononuclear cells isolated from Epo-refractory ESRD subjects are more likely to produce inflammatory cytokines than are those from non-Epo-refractory ESRD subjects.

The fact that exogenous Epo can often at least partially reverse ACI suggests strongly that insufficient Epo production is one of the underlying causes of ACI. In parallel, low-dose Epo administration in rats leads to more normal endothelial function, reverses vascular inflammation, and decreases oxidative stress, suggesting that Epo or its downstream effects may also decrease inflammation. Increasing Epo also indirectly decreases hepcidin via the induction of a recently recognized Epo-dependent erythroid suppressor of hepcidin, erythroferrone (ERFE), leading to reversal of iron sequestration and increasing iron availability for erythropoiesis to improve anemia.[19,20]

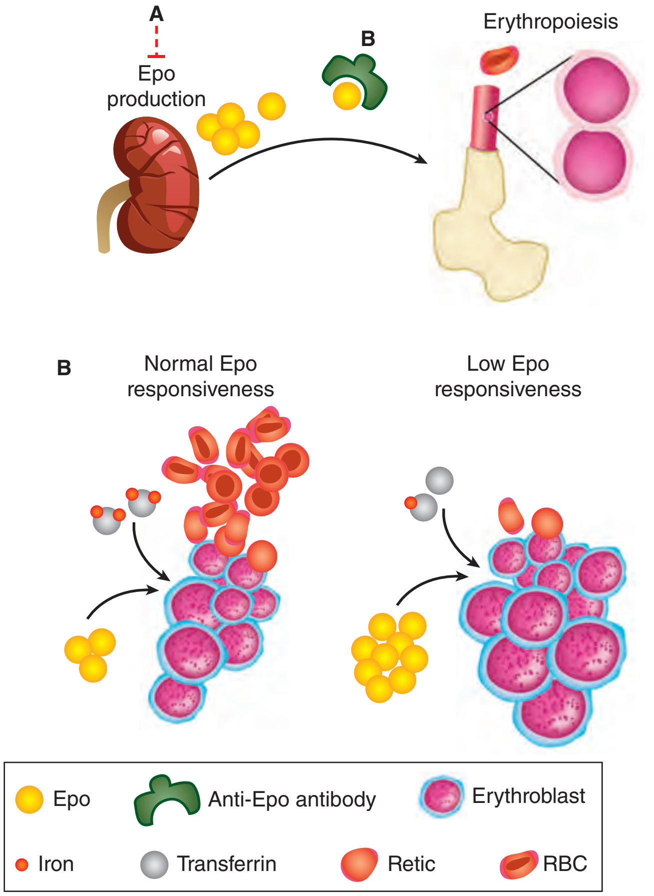


Figure 38.3 MECHANISMS UNDERLYING ERYTHROPOIETIN RESPONSIVENESS. Several mechanisms are responsible for anemia during inflammation. Inflammatory cytokine-mediated insufficient erythropoietin (Epo) production (A), anti-Epo antibodies (B), and decreased Epo responsiveness (C) are implicated. While decreased Epo production and anti-Epo antibodies lead to insufficient Epo stimulation of erythropoiesis, decreased Epo responsiveness correlates with higher than normal Epo levels, despite which a proportional increase in hemoglobin is not observed. Decreased Epo responsiveness may be a consequence of both direct cytokine-mediated suppression of erythroid differentiation despite increased Epo and iron restriction in high hepcidin conditions, e.g., anemia of chronic inflammation. In the iron-replete condition, small amounts of Epo induce proliferation and differentiation of erythroblasts that take up circulating transferrin-bound iron, enabling hemoglobin synthesis. During iron deficiency, circulating transferrin-bound iron is decreased, resulting in decreased hemoglobin synthesis per cell and decreased Epo responsiveness, modifying Epo receptor-dependent signaling to support cell viability in excess of erythroid proliferation and differentiation.

Cytokine-Induced Changes in Iron Metabolism

There is now a consensus view that ACI is in large part a consequence of altered iron metabolism. Specifically, production of inflammatory cytokines, such as IL-6, and possibly other cytokines leads to hepcidin-induced hypoferremia, resulting in iron sequestration within the reticuloendothelial system, thereby decreasing iron availability for erythropoiesis (Figs. 38.2 and 38.3). IL-6 induction of hepcidin expression is mediated through STAT3 signaling in hepatocytes (Fig. 38.4). Crosstalk between IL-6–induced STAT3 signaling and BMP/SMAD signaling to hepcidin provides the rationale for manipulating the BMP/SMAD pathway also in ACI. For example, the SMAD binding site on the hepcidin promoter remains essential for IL-6–mediated hepcidin expression and liver-specific Smad4 knockout mice demonstrate diminished hepcidin responsiveness to IL-6.[21] In vitro experiments reveal that blocking BMP receptor signaling inhibits IL-6-mediated hepcidin expression.

In support of the role of IL-6 and hepcidin in ACI, mice injected with IL-6 exhibit increased hepcidin levels and develop hypoferremia within 24 hours. LPS injection (which leads to IL-6 induction) did not induce hypoferremia in IL-6 knockout mice. In humans, LPS injection dramatically increases IL-6 levels in 3 hours and increased

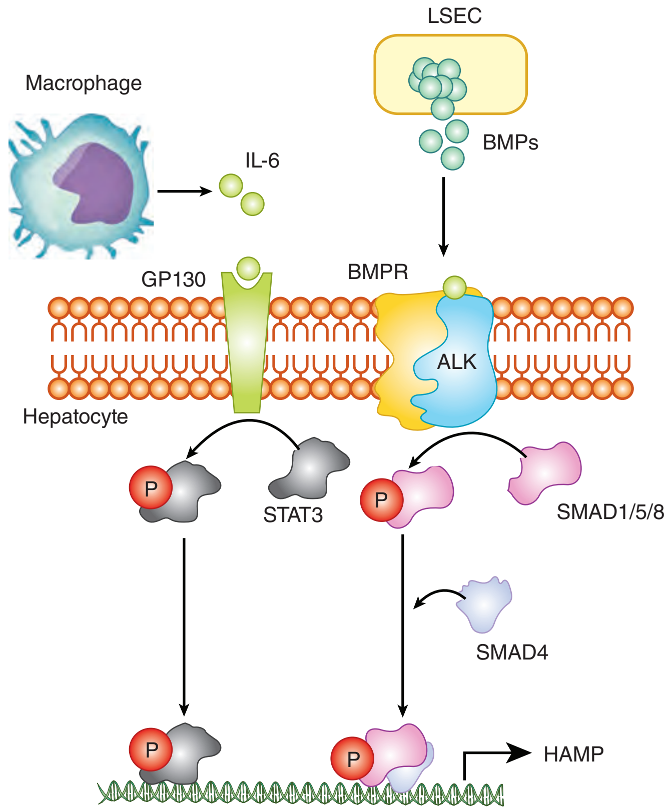


Figure 38.4 MULTIFACTORIAL REGULATION OF HEPCIDIN EXPRESSION IN HEPATOCYTES. Hepcidin is regulated via multiple pathways. In inflammatory states, macrophages release IL-6 which activates the STAT3 pathway in hepatocytes, leading to production of hepcidin. Local iron stores induce non-hepatocyte liver cells to produce BMPs (i.e., BMP2 and BMP6) that stimulate BMP receptor I, activating the SMAD1/5/8 pathway which, in concert with SMAD4, translocate to the nucleus to induce hepcidin transcription. *ALK*, Activin A receptor-like kinase; *BMP*, bone morphogenetic protein; *BMPR*, BMP receptor; *HAMP*, gene encoding hepcidin; *IL-6*, interleukin 6; *LSEC*, liver sinusoidal endothelial cell; *SMAD*, s-mothers against decapentaplegic; *STAT3*, signal transducer and activator 3.

urinary hepcidin in 6 hours. Finally, loss of both IL-6 and hepcidin in mouse models results in a milder anemia and more rapid recovery of hemoglobin in a well-established mouse model of ACI,[22] and IL-6 knockout animals exhibited faster bone marrow recovery relative to hepcidin knockout animals. Other pro-inflammatory cytokines, such as TNF and IL-1, also lead to increased iron content in macrophages (see Fig. 38.2). Rats injected with IL-1 or TNF experienced a 40% drop in serum iron levels, leading to a significant decrease in erythroblast iron uptake. Finally, in vitro studies demonstrate that the addition of TNF increases radiolabeled iron uptake by peritoneal macrophages without an increase in iron release, suggesting that macrophage sequestration of iron is cytokine-induced.

The peptide hormone hepcidin is the principal regulator of iron homeostasis, including dietary iron absorption, iron recycling by macrophages, and the release of iron from hepatic stores (see Fig. 38.2). Hepcidin down regulates iron release into plasma by binding to and functionally down regulating ferroportin 1 (FPN1), the sole iron exporter in enterocytes. When FPN1 is lost in the duodenum, absorption of dietary iron is reduced and serum iron levels are depressed (see Chapter 36); similarly, hepcidin inhibits FPN1 and iron export from macrophages, aggravating the low serum iron levels and enhancing iron retention in the macrophage. Iron retention within monocytes/macrophages induces ferritin expression and accounts for the increase in serum ferritin concentration in inflammatory states. The net result is serum hypoferremia and hyperferritinemia.

Hepcidin-independent mechanisms also contribute to altered iron homeostasis and are mainly orchestrated by cytokines (as well as hypoxia [see Chapter 36]). TNF-α, IL-1, IL-6, and IFN-γ all increase macrophage iron uptake by altering the expression of TfR1 and divalent metal transporter-1 (DMT1). In addition, many of these cytokines, including IL-4, IL-10, and IL-13, contribute to increased iron storage within the monocytes/macrophages by increasing the expression of ferritin both transcriptionally and post-transcriptionally. Transcription of FPN1 is also inhibited by IFN-γ and LPS, thereby contributing to iron sequestration in macrophages.

PATHOPHYSIOLOGY OF IRON SEQUESTRATION

Macrophages play a central role in iron homeostasis by recycling iron from senescent RBCs, providing more than 95% of the daily iron requirements for normal physiologic processes, including erythropoiesis. During inflammation, iron retention in the macrophage-phagocytic system and decreased delivery of iron for erythropoiesis results in ACI.[23] Studies using labeled iron show erythroblasts take in iron normally. This is consistent with the concept that under inflammatory conditions, iron availability, but not utilization, is primarily impaired.

The resultant increase in circulating hepcidin concentration leads to increased binding, internalization, and degradation of FPN1, and is expressed on cells involved in iron metabolism (e.g., duodenal enterocytes, macrophages, hepatocytes, and placental cells). FPN1 exports iron into the circulation. Its degradation thus traps iron within cells, leading to increased cellular ferritin and ultimately serum ferritin concentration (in equilibrium with ferritin inside cells). As a consequence, inflammation negatively affects erythropoiesis by sequestering iron away from erythroid precursors (i.e., within bone marrow macrophages), inducing iron-restricted erythropoiesis (see Fig. 38.3).

The term “functional iron deficiency” refers to insufficient iron availability at the site of erythroblast production, despite adequate body iron stores. It typically applies to the high hepcidin state in patients with renal insufficiency. However, broadly speaking, ACI, another high hepcidin condition, can also conceptually be referred to as functionally iron deficient. In response to this iron-restricted erythropoiesis, ERFE increases and possibly provides some counter-regulation of elevated hepcidin. In fact, ERFE knockout mice treated with heat-killed *Brucella abortus*, a well-accepted model of ACI, exhibit delayed hemoglobin recovery as a consequence of inadequate hepcidin suppression. Thus, increased Epo in iron-restricted erythropoiesis in ACI may enable counter-regulation to suppress hepcidin expression and increase iron availability for erythropoiesis.

Finally, the functional iron depletion mediated by increased hepcidin may itself inhibit erythropoiesis.[24] This “iron restriction response” employs multiple iron-sensitive molecular pathways to inhibit the differentiation and proliferation of erythroblasts and cause anemia in conditions of decreased iron availability (see Fig. 38.3). The proposed mechanisms involve mitochondrial aconitase enzymes, transferrin receptor 2 (TfR2), and scribble-mediated EpoR regulation, as well as effects on the erythroblast cell cycle. Strikingly, in these models, iron depletion uniquely sensitizes erythroid cells to suppression by inflammatory cytokines.

The Rationale and Methodology for Hepcidin-Lowering Agents

When treatment of the underlying disease is difficult or impractical, the limited options available for treating severe anemia (i.e., RBC transfusion in acute cases and ESAs and parenteral iron in more subacute and chronic cases) suffer from marginal efficiency and high risk. To address this unmet need, several translational studies are now focused on countering hepcidin overexpression. Hepcidin antagonists

TREATMENT OF ANEMIA OF CHRONIC INFLAMMATION

The goal of anemia management is to reverse patient symptoms attributable to decreased hemoglobin. Clinically, anemia has a broad and varied presentation including shortness of breath, lethargy, fatigue, skin pallor, palpitations, tachycardia, systolic flow murmurs, and angina, which can be difficult to distinguish from symptoms of the underlying disease, complicating the decision regarding treating anemia in addition to treating the cause of inflammation. Overall, resolution of anemia in anemia of chronic inflammation (ACI) parallels therapy and improvement of the underlying inflammatory condition, although treatment of anemia is associated with improved quality of life.

The anemia associated with chronic inflammation is often mild. For example, the annual incidence of more severe anemia (hemoglobin <10 g/dL) was only 1.5% in rheumatoid arthritis (RA) patients, with a lifetime prevalence of 13.7%. Similarly, in cancer patients referred for radiation therapy, only 16% had more severe anemia. Although over time the anemia can become more severe, the correction of ACI per se may be unnecessary, especially if the primary disorder contributing to the anemia can be treated and reversed. Certainly, treating ACI is not warranted if the patient is asymptomatic. There may also be teleological benefits in the pathophysiologic processes that contribute to ACI. As fever associated with infections inhibits bacterial growth, decreased iron availability (as seen in ACI) synergizes with pyrexia to inhibit bacterial growth. This “nutritional immunity” is postulated to be an adaptive factor that contributes to ACI.

ACI can occasionally be severe or symptomatic and warrant therapy. Epidemiologic studies provide evidence of improved signs and symptoms following anemia treatment in patients with congestive heart failure, HIV infection, cancer, or chronic renal disease. However, treatments need to be individualized because without clear benefit, the potential risks of red blood cell (RBC) transfusion, erythropoiesis-stimulating agents (ESAs), or iron therapy in non-iron-deficient subjects may not be insignificant and are in some cases high given the variety of underlying conditions that are incorporated under the rubric of chronic inflammatory diseases (Table 38.1).

The first priority in ACI would be to correct any reversible contributors to the anemia. Because the extent of ACI mirrors the activity of the underlying disease, all efforts should be made to treat the underlying disease. Furthermore, efforts to correct anemia should be modulated by the recognition that the “optimal” target hemoglobin for subjects with ACI is not known. Observations from profoundly anemic subjects without inflammation but religiously opposed to transfusions have suggested a physiologic cutoff for hemoglobin of 5 g/dL, below which increased mortality is seen.[25] In addition, randomized controlled trials in acutely ill patients do not demonstrate benefit from RBC transfusion for patients with hemoglobin >7 g/dL. Nonetheless, symptomatic improvement is seen in subjects with a range of chronic inflammatory diseases who are treated for anemia of a more modest degree. This benefit needs to be weighed against the known short- and longterm risks of RBC transfusion, including the potential for acute and chronic infections, iron overload, and human leukocyte antigen–sensitization risks.

ESAs have improved hemoglobin levels in patients with ACI, reducing the necessity for RBC transfusion, especially with endogenous Epo levels of <500 IU/L. The addition of typically parenteral administration of iron can be considered in ACI patients who are unresponsive to ESA alone. ESAs lead to increased hemoglobin levels in patients with a variety of non-hematologic malignancies (with or without chemotherapy), including squamous cell cancer, breast cancer, and colon cancer. However, despite the benefits of decreasing RBC transfusion requirements and improved quality of life, ESAs (both short- or long-acting forms) in patients with cancer and kidney disease increase the risk of stroke and thromboembolic events.[26] Studies have also indicated that critically ill patients and those with cancer undergoing chemotherapy may have an increased rate of thrombosis and overall death when treated with ESAs, particularly in patients with post-treatment hemoglobin >10–11 g/dL. In a large study, normalizing hemoglobin (12.0–15.0 g/dL) in ESA-treated ESRD patients with cardiac disease led to an increased number of cardiac events and deaths compared to partial hemoglobin normalization (10.5–11.5 g/dL). Although pretreatment hemoglobin levels in patients with breast cancer or head and neck cancer have been demonstrated repeatedly to predict good response to chemotherapy and radiation, patients treated with ESAs risk increased disease progression and decreased survival relative to placebo-treated patients. Based on these and other studies, the US Food and Drug Administration has warned that ESAs should be given only in the lowest possible doses necessary to avoid RBC transfusions. More recently, consensus recommendations for patients with cancer support the use of ESAs only in those receiving chemotherapy for palliative, rather than curative, intent. Thus, ESAs should be used sparingly, if at all, with active malignancy.

In the appropriate nonmalignant setting of symptomatic ACI, weekly subcutaneous ESA administration can be initiated. If a hemoglobin response is seen, the dose can be decreased and the interval prolonged to titrate the hemoglobin to a minimal asymptomatic level. Follow-up and dose titration are essential to minimize expense, injections, and potential deleterious effects from over- or under-treatment. The ESA response may take 4–8 weeks and should be monitored for objective symptomatic improvement (e.g., exercise tolerance). Approximately 35%–40% of patients demonstrate primary resistance. Predictors of ESA resistance include iron or vitamin deficiency, recent RBC transfusion requirement, and higher endogenous Epo levels before initiation of therapy. If, after 2 weeks of treatment, the serum Epo level is >100 mU/mL and the hemoglobin concentration has not increased by ≥0.5 g/dL, or if the serum ferritin level remains >400 ng/mL, a response is unlikely.[27] In a multivariate analysis of 80 patients with ACI in the context of cancer, only the hemoglobin, serum Epo, and serum ferritin levels, but not disease status, bone marrow involvement, or treatment status, were independent predictors of response to ESAs.

An important factor limiting response to ESAs is functional iron deficiency. Correction of functional iron deficiency with parenteral iron supplementation can decrease the required dosage while improving the effect of ESAs. Parenteral iron improves ESA responses and is cost-effective in ESRD patients, even those with elevated ferritin levels; however, the widespread applicability of this approach, and its long-term effects, are not known.

in development target either the hepcidin molecule or its regulators (e.g., BMP) in the hope that the potential therapeutic efficacy of hepcidin antagonists may extend to even mild anemias.

Via the Inflammatory Signal

Several diseases in the ACI category (e.g., Castleman disease and RA) have benefited from clinical trials with hepcidin-lowering agents (Table 38.2). Therapeutic monoclonal antibodies against IL-6 receptor (tocilizumab), IL-6 (siltuximab), and TNF-α (golimumab or infliximab) resulted in decreased hepcidin and improved anemia. In addition, downstream inhibition of STAT3 signaling (e.g., curcumin, AG490, and PpYLKTK) has demonstrated effectiveness in decreasing hepcidin in vitro and in pre-clinical studies in vivo. They are, however, unlikely to come to market as a consequence of their non-specific effects (all), competing iron-chelating properties (curcumin), and suboptimal pharmacokinetics (AG490 and PpYLKTK).

Via the Iron Signal

Heparin, a glycosaminoglycan and frequently used agent in the treatment and prophylaxis of blood clots, has a high affinity for BMPs. Thus, heparin inhibits hepcidin expression by binding to and sequestering BMPs.[28] Furthermore, heparins without anticoagulating effects have been developed, demonstrating reversal of anemia in mouse models of ACI and are specifically able to block hepcidin in vitro and in pre-clinical studies in vivo. These agents are poised for randomized clinical trials. Hemojuvulin (HJV) is a BMP coreceptor, enhancing hepcidin expression. Soluble HJV, resulting

TABLE 38.1 **Currently Available Therapeutic Options for Management of Anemia of Chronic Inflammation**

	Benefit	Risk
Treat underlying illness	• Most widely accepted as effective • Most disease-specific therapy	• Typical underlying illness often long term • 30% of ACI patients without identifiable illness
RBC transfusion	• Rapid increase in hemoglobin	• Requires a clinical setting for administration • Complications: hemolytic reactions, allo-immunization, infection, iron overload with repeat administration[a]
ESA	• Amenable to self-administration	• Relatively contraindicated in patients with cancer
Parenteral iron	• Multiple options for formulation available	• Requires a clinical setting for administration • Infusion reactions, albeit mild (e.g., self-limiting "Fishbein" reaction), may occur with all typically used formulations (unlike anaphylaxis with previously used high molecular weight iron dextran) • Does not benefit patients without "iron-restricted erythropoiesis" (see Fig. 38.3)

ACI, Anemia of chronic inflammation; *ESA*, erythropoietin stimulating agents; *RBC*, red blood cell.
[a] See Chapter 37 for a full list of complications of RBC transfusion.

from cleavage of cellular HJV, competes for binding of BMP to BMP receptor. Administration of either neutralizing anti-HJV antibodies (AbbVie Pharmaceuticals) or sHJV fused with IgG Fc fragment result in hepcidin suppression and correct anemia in murine ACI models.[29,30] Clinical studies using such agents in renal failure patients are underway.

Antibodies and small molecule inhibitors of BMP/SMAD have also demonstrated pre-clinical efficacy in decreasing hepcidin expression. For example, anti-BMP6 antibodies, dorsomorphin, an inhibitor of BMP/SMAD signaling, and dorsomorphin derivatives, e.g., LDN-193189, reduce hepcidin expression and increase serum iron concentration in mice. However, because dorsomorphin and its derivatives do not specifically target BMP/SMAD signaling to hepcidin, they are considered suboptimal candidates for therapeutic development. TP-0184 (Toledo Pharmaceuticals) and momelotinib (Gilead) are specific ALK2 inhibitors undergoing clinical trials in solid tumors and myelofibrosis, respectively. TP-0184 has recently been shown to suppress hepcidin in mouse models of ACI and cancer,[31] but further studies are required to evaluate its full translational potential in ACI.

Via Erythropoietic Signal

Novel hypoxia inducible factor (HIF) stabilizers provide promise in the treatment of ACI, enabling hepcidin blockade mediated via erythropoiesis, simultaneously stimulating erythropoiesis and iron availability. For example, propyl hydroxylase domain-2 (PDH2) inhibitors stabilize HIF-1 and HIF-2, stimulating endogenous Epo production to suppress hepcidin and enable greater iron availability for erythropoiesis.[32–34] In light of the recent discovery of ERFE and its negative regulation of hepcidin, modulation of this pathway is a clear area for future investigation.

Direct Hepcidin Inhibition

Direct hepcidin inhibitors, e.g., hepcidin neutralizing antibodies, have demonstrated efficacy in increasing iron availability for erythropoiesis, reversing anemia, and increasing responsiveness to ESAs in mouse models of ACI and in monkeys. LY2787106, a monoclonal anti-hepcidin antibody (Eli Lily), has recently demonstrated high tolerability, transient iron mobilization, and increased reticulocytosis in a Phase I study of cancer-related anemia patients.[35] PRS-080, a PEGylated anticalin with specific hepcidin binding and neutralizing capacity (Pieris Pharmaceuticals), demonstrated suppression of hepcidin activity in vitro and in mice[36] with decreased hepcidin and transiently increased transferrin saturation at doses above 0.4 mg/kg in a Phase I study in healthy volunteers,[37] warranting further investigations in anemic patients. Lastly, NOX-H94, a spiegelmer (PEGylated non-natural occurring mirror-image L-RNA aptamer) hepcidin antagonist (NOXXON Pharma AG), specifically binds hepcidin with high affinity. NOX-H94 improved ACI in monkeys and prevented hypoferremia in LPS-injected humans volunteers.[38] As a consequence, additional clinical trials in renal insufficiency, ACI, and cancer-related anemia are underway.

CONCLUSION

Anemia is a common consequence of many chronic inflammatory, infectious, and malignant conditions; these are characterized by disturbances in iron metabolism but are often multifactorial. Inflammatory cytokines are the most important causative factors in ACI. The challenges in ACI management are a consequence of (1) inadequate easy to use and standardized diagnostic tools, (2) incomplete appreciation of variability in therapeutic targets for hemoglobin, (3) insufficient guidelines to estimate the degree of functional iron deficit, and (4) incomplete understanding of how to tailor therapy with parenteral iron, ESAs, and RBC transfusion for individual patients. Cytokines such as IFN and TNF have wide-ranging effects, including both direct and indirect inhibition of erythropoiesis and on RBC life span in circulation. Direct inhibition of erythropoiesis is mediated by both a relative decrease in Epo as well as a decrease in iron available for hemoglobin synthesis. Anemia, iron deficiency, and hepcidin may also have independent antimicrobial and antitumor effects to be evaluated in the future. Furthermore, our recently enhanced understanding of the complexity of regulation of iron metabolism and crosstalk with erythropoiesis is starting to bear fruit with novel diagnostic contributions and therapeutics aiming to modulate hepcidin and increase iron availability for erythropoiesis being explored from multiple directions. Different therapeutic strategies for managing ACI associated with different underlying diseases may increase with the potential of hepcidin-targeted therapeutics to enable a greater degree of physiological self-regulation, preventing inadequate or excessing therapy in individual patients. With many novel therapeutic approaches already validated in pre-clinical and clinical settings and some currently being further explored in randomized clinical trials, ACI management is poised to benefit from our extensive additional knowledge regarding iron metabolism and its regulation in physiological and pathological conditions.

TABLE 38.2 Novel Hepcidin Suppressing Agents as Potential Therapy for Anemia of Chronic Inflammation

Mechanism	Pathway	Agent (Company)	Stage of Development
Suppressing inflammatory cytokines and inflammation-mediated signaling	Anti-IL-6 receptor antibody	Tocilizumab (Genentech)	FDA approved 2010; available therapy for multiple autoimmune diseases
	Anti-IL-6 antibody	Siltuximab (Janssen)	FDA approved 2014; available therapy for multicentric Castleman disease
	Anti-TNF-α antibody	Golimumab (Janssen) or infliximab (Janssen)	FDA approved 2013; available therapy for multiple autoimmune diseases
	STAT3 inhibitor	Curcumin, AG490, PpYLKTK	Not proceeding further
Suppressing iron-mediated signaling	BMP sequestration	Heparins (with or without anticoagulating effect) Anti-BMP6 antibody, dorsomorphin and derivatives, e.g., LDN- 193189	FDA approved 1939; available for anticoagulation Phase II, anemia in CKD (no further development)
	sHJV sequestration	Anti-HJV antibody (Abbvie Pharmaceuticals) sHJV-IgG Fc fusion (FerruMax Pharmaceuticals)	Phase II, anemia in CKD (no further development)
	BMP/SMAD inhibitor	ALK2 inhibitors momelotinib (Gilead) and TP-0184 (Toledo Pharmaceuticals)	Phase III, myelofibrosis (FDA fast track status); Phase I, solid tumors
Erythropoiesis- mediated signaling	HIF stabilizer	PHD inhibitor (multiple agents, e.g., roxadustat, daprodustat, vadadustat)	Phase III, anemia in CKD (pending FDA approval only for roxadustat)
Direct hepcidin inhibition	Anti-hepcidin antibody	LY2787106 (Eli Lily)	Phase I, anemia of cancer; Phase II, anemia in CKD (no further development)
	PEGylated anticalin	PRS-080 (Pieris Pharmaceuticals)	Phase IIa, anemia in CKD (completed 2019)
	Spiegelmer, PEGylated non-natural occurring L-RNA-aptamer	NOX-H94 (Noxxon Pharma AG)	Phase IIa, anemia in cancer (no further development)

ALK2, Activin A receptor-like kinase 2; *BMP*, bone morphogenetic protein; *CKD*, chronic kidney disease; *FDA*, (US) Food and Drug Administration; *HIF*, hypoxia inducible factor; *IL-6*, interleukin 6; *PHD*, prolyl hydroxylase domain; *sHJV*, soluble hemojuvulin; *SMAD*, s-mothers against decapentaplegic; *STAT3*, signal transducer and activator 3; *TNF*, tumor necrosis factor.

REFERENCES

1. Weiss G, Goodnough LT. Anemia of chronic disease. *N Engl J Med*. 2005;352:1011–1023.
2. Cohen LA, Gutierrez L, Weiss A, et al. Serum ferritin is derived primarily from macrophages through a nonclassical secretory pathway. *Blood*. 2010;116:1574–1584.
3. Cazzola M, Ponchio L, de Benedetti F, et al. Defective iron supply for erythropoiesis and adequate endogenous erythropoietin production in the anemia associated with systemic-onset juvenile chronic arthritis. *Blood*. 1996;87:4824–4830.
4. Cazzola M, Beguin Y. New tools for clinical evaluation of erythron function in man. *Br J Haematol*. 1992;80:278–284.
5. Skikne BS, Flowers CH, Cook JD. Serum transferrin receptor: a quantitative measure of tissue iron deficiency. *Blood*. 1990;75:1870–1876.
6. R'Zik S, Beguin Y. Serum soluble transferrin receptor concentration is an accurate estimate of the mass of tissue receptors. *Exp Hematol*. 2001;29:677–685.
7. Ganz T, Olbina G, Girelli D, et al. Immunoassay for human serum hepcidin. *Blood*. 2008;112:4292–4297.
8. Diepeveen LE, Laarakkers CMM, Martos G, et al. Provisional standardization of hepcidin assays: creating a traceability chain with a primary reference material, candidate reference method and a commutable secondary reference material. *Clin Chem Lab Med*. 2019;57:864–872.
9. Prentice AM, Doherty CP, Abrams SA, et al. Hepcidin is the major predictor of erythrocyte iron incorporation in anemic African children. *Blood*. 2012;119:1922–1928.
10. Theurl M, Nairz M, Schroll A, et al. Hepcidin as a predictive factor and therapeutic target in erythropoiesis-stimulating agent treatment for anemia of chronic disease in rats. *Haematologica*. 2014;99:1516–1524.
11. Raj DS. Role of interleukin-6 in the anemia of chronic disease. *Semin Arthritis Rheum*. 2009;38:382–388.
12. Wang CQ, Udupa KB, Lipschitz DA. Interferon-gamma exerts its negative regulatory effect primarily on the earliest stages of murine erythroid progenitor cell development. *J Cell Physiol*. 1995;162:134–138.
13. Grigorakaki C, Morceau F, Chateauvieux S, et al. Tumor necrosis factor α-mediated inhibition of erythropoiesis involves GATA-1/GATA-2 balance impairment and PU.1 over-expression. *Biochem Pharmacol*. 2011;82:156–166.
14. Means Jr. RT, Dessypris EN, Krantz SB. Inhibition of human erythroid colony-forming units by interleukin-1 is mediated by gamma interferon. *J Cell Physiol*. 1992;150:59–64.
15. McCranor BJ, Kim MJ, Cruz NM, et al. Interleukin-6 directly impairs the erythroid development of human TF-1 erythroleukemic cells. *Blood Cells Mol Dis*. 2014;52:126–133.
16. Krajewski J, Batmunkh C, Jelkmann W, Hellwig-Bürgel T. Interleukin-1beta inhibits the hypoxic inducibility of the erythropoietin enhancer by suppressing hepatocyte nuclear factor-4alpha. *Cell Mol Life Sci*. 2007;64:989–998.
17. Jelkmann W. Proinflammatory cytokines lowering erythropoietin production. *J Interferon Cytokine Res*. 1998;18:555–559.
18. Vannucchi AM, Grossi A, Rafanelli D, et al. Inhibition of erythropoietin production in vitro by human interferon gamma. *Br J Haematol*. 1994;87:18–23.
19. Ashby DR, Gale DP, Busbridge M, et al. Erythropoietin administration in humans causes a marked and prolonged reduction in circulating hepcidin. *Haematologica*. 2010;95:505–508.
20. Kautz L, Jung G, Valore EV, et al. Identification of erythroferrone as an erythroid regulator of iron metabolism. *Nat Genet*. 2014;46:678–684.
21. Wang R-H, Li C, Xu X, et al. A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression. *Cell Metab*. 2005;2:399–409.
22. Gardenghi S, Renaud TM, Meloni A, et al. Distinct roles for hepcidin and interleukin-6 in the recovery from anemia in mice injected with heat-killed *Brucella abortus*. *Blood*. 2014;123:1137–1145.

23. Langer AL, Ginzburg YZ. Role of hepcidin-ferroportin axis in the pathophysiology, diagnosis, and treatment of anemia of chronic inflammation. *Hemodial Int*. 2017;21(Suppl 1):S37–S46.
24. Khalil S, Delehanty L, Grado S, et al. Iron modulation of erythropoiesis is associated with Scribble-mediated control of the erythropoietin receptor. *J Exp Med*. 2018;215:661–679.
25. Viele MK, Weiskopf RB. What can we learn about the need for transfusion from patients who refuse blood? The experience with Jehovah's witnesses. *Transfusion*. 1994;34:396–401.
26. Samaras AT, Bennett CL. Risks of venous thromboembolism and mortality associated with erythropoiesis-stimulating agents for the treatment of cancer-associated anemia. *Am J Hematol Oncol*. 2008;7:327–332.
27. Sanz Ortiz J. Predictors of response to erythropoiesis-stimulating agents (ESA) in cancer patients: the role of baseline serum epoetin level. *Clin Transl Oncol*. 2008;10:486–492.
28. Poli M, Girelli D, Campostrini N, et al. Heparin: a potent inhibitor of hepcidin expression in vitro and in vivo. *Blood*. 2011;117:997–1004.
29. Kovac S, Böser P, Cui Y, et al. Anti-hemojuvelin antibody corrects anemia caused by inappropriately high hepcidin levels. *Haematologica*. 2016;101:e173–e176.
30. Theurl I, Schroll A, Sonnweber T, et al. Pharmacologic inhibition of hepcidin expression reverses anemia of chronic inflammation in rats. *Blood*. 2011;118:4977–4984.
31. Peterson P, Soh KK, Sol Lee Y, et al. ALK2 inhibition via TP-0184 abrogates inflammation-induced hepcidin expression and is a potential therapeutic for anemia of chronic disease. Annual Meeting of the American Society of Hematology, Orlando, FL, 2015.
32. Casper C, Chaturvedi S, Munshi N, et al. Analysis of inflammatory and anemia-related biomarkers in a randomized, double-blind, placebo-controlled study of siltuximab (anti-IL6 monoclonal antibody) in patients with multicentric Castleman disease. *Clin Cancer Res*. 2015;21:4294–4304.
33. Wilkinson N, Pantopoulos K. IRP1 regulates erythropoiesis and systemic iron homeostasis by controlling HIF2alpha mRNA translation. *Blood*. 2013;122:1658–1668.
34. Soni H. Prolyl hydroxylase domain-2 (PHD2) inhibition may be a better therapeutic strategy in renal anemia. *Med Hypotheses*. 2014;82:547–550.
35. Vadhan-Raj S, Abonour R, Goldman JW, et al. Phase 1 study of a hepcidin antagonist, LY2787106, in cancer-associated anemia. Annual Meeting of the American Society of Hematology 2015.
36. Hohlbaum AM, Trentman S, Gille H, et al. Discovery and preclinical characterization of a novel hepcidin antagonist with tunable PK/PD properties for the treatment of anemia in different patient populations. Annual Meeting of the American Society of Hematology San Diego, CA, 2011.
37. Moebius U, Feuerer W, Fenzl E, et al. A Phase I study investigating the safety, tolerability, pharmacokinetics and pharmacodynamic activity of the hepcidin antagonist PRS-080#022. results from a randomized, placebo controlled, double-blind study following single administration to healthy subjects. Annual Meeting of the American Society of Hematology Orlando, FL, 2015.
38. van Eijk LT, John ASE, Schwoebel F, et al. Effect of the antihepcidin Spiegelmer lexaptepid on inflammation-induced decrease in serum iron in humans. *Blood*. 2014;124:2643–2646.

CHAPTER 39

HEME BIOSYNTHESIS AND ITS DISORDERS: PORPHYRIAS AND SIDEROBLASTIC ANEMIAS

Stephen J. Fuller and James S. Wiley

The porphyrias and the sideroblastic anemias are metabolic disorders that involve defects in heme biosynthesis. Most forms of porphyria are inherited in a Mendelian autosomal dominant pattern, but some types are recessive, and others are acquired through exposure to porphyrinogenic drugs and chemicals. A linked group of diseases, the porphyrinurias, are not porphyrias but have in common alterations of heme biosynthesis following exposure to various toxins.[1] Porphyrins are tetrapyrroles, which are ubiquitous in nature and exhibit characteristic red fluorescence on exposure to ultraviolet light. The iron-porphyrin complex, called *heme*, is central to all biologic oxidation reactions. In plants, the porphyrin molecule is combined with magnesium to form chlorophyll. Porphyrin biosynthesis is one of the most essential biochemical processes in most life forms.

In humans, loss-of-function mutations affecting the first enzyme of the heme biosynthetic pathway, 5-aminolevulinate synthase (ALAS), produce sideroblastic anemia. Gain-of-function mutation of ALAS, and inborn errors that occur at subsequent sites in this pathway, usually result in metabolic disorders known as the *porphyrias* (Fig. 39.1). It has been hypothesized that Vincent van Gogh suffered from porphyria, most likely acute intermittent porphyria, based on the intermittent nature of gastrointestinal complaints, neurological disturbances, and exacerbations caused by inadequate nutrition and absinthe abuse.[2,3]

HEME BIOSYNTHESIS

Biosynthetic Pathways

Heme biosynthesis is an essential pathway and occurs in all metabolically active cells that contain mitochondria. It is most active in erythropoietic tissue, where it is required for hemoglobin synthesis, and in hepatic tissue, where the heme forms the basis of various heme-containing enzymes such as the cytochromes P450, catalase, cytochrome oxidase, and tryptophan pyrrolase. The synthetic pathway starts with the condensation of glycine and succinyl CoA to form 5-aminolevulinate (ALA) under the control of the mitochondrial enzyme ALAS. This enzyme requires pyridoxal phosphate as a cofactor. A series of enzymes then controls the conversion of ALA first to the monopyrrole porphobilinogen (PBG) and then to the various porphyrins. Iron is inserted into protoporphyrin by the enzyme ferrochelatase (FECH) to form heme (see Fig. 39.1). During the past 50 years, complementary deoxyribonucleic acid (cDNA) clones have been obtained for all the enzymes of heme biosynthesis, the structures of the corresponding genes have been determined, and molecular analyses have revealed numerous heterogeneous defects in all the porphyrias. These advances are certain to improve understanding of the pathogenesis of the porphyrias and methods for identification of carriers. The enzymes of the biosynthetic pathway have all been mapped to specific chromosomes (Table 39.1).[4] Heme synthesis and its disorders have been the subject of reviews,[5–7] and advances in our knowledge of mitochondrial iron trafficking and metabolism have recently been reviewed.[8,9]

Control of Heme Biosynthesis

The overproduction of porphyrins and their precursors in the different porphyrias is mainly hepatic or erythropoietic in origin. In the acute porphyrias and in porphyria cutanea tarda (PCT), the liver is the main source of overproduction; in congenital erythropoietic porphyria (CEP), the marrow is the main source; and in erythropoietic protoporphyria (EPP), porphyrins are overproduced by the liver and marrow.

Control of hepatic heme biosynthesis is regulated by the rate of the initial enzymatic step, ALAS1, a housekeeping form of ALAS that is expressed in all cell types, including erythroid cells, whereas ALAS2 encodes a red cell-specific form. ALAS1 is under negative-feedback control by heme, which occurs by more than one mechanism. Heme represses transcription of the ALAS1 gene and increases the rate of degradation of the messenger ribonucleic acid (mRNA) (Fig. 39.2A). At the posttranslational level, heme blocks the translocation of pre-ALAS1 into the mitochondrion.[10] In the mitochondrion, the molecular mass of ALAS1 is smaller than that of the cytosolic pre-ALAS1[10] because of the removal of the mitochondrial targeting sequence. Finally, heme regulates levels of mature ALAS1 by activation of a mitochondrial proteolysis system.[11]

The erythroid bone marrow is the major heme-forming tissue in the body, producing 85% of the daily heme requirement. Heme synthesis in erythroid cells varies from that in hepatocytes; it is linked to tissue differentiation, and the half-life of the same end product of the two is quite different. Heme complexed with globin is preserved in circulating red blood cells for approximately 120 days, whereas heme produced in liver for cytochromes and enzymes, such as catalase, is subject to much more rapid turnover, measurable in hours. Regulation in the liver is exquisitely sensitive to fluctuations in intracellular heme levels[12] and responds rapidly to the requirements for synthesis, as described in Fig. 39.2A. However, heme synthesis in the bone marrow shows a more leisurely response. This fundamental difference is explained by the finding of two different tissue-specific isoenzymes and two different cDNAs for human liver or “housekeeping” ALAS1 and erythroid specific ALAS2, which is expressed exclusively in erythroid cells.[13] The gene for ALAS2 has been mapped to the X chromosome and that for the hepatic enzyme to chromosome 3.[13] The ALAS2 gene has 12 exons; exons 4 to 11 encode the catalytic domain of the enzyme and include a lysine residue that forms a Schiff base with the pyridoxal phosphate cofactor. Exon 1 contributes to the 5′ untranslated region (UTR) whose structure allows iron to regulate ALAS2 mRNA translation, whereas exons 1 and 2 contribute the sequence that targets the enzyme to the mitochondria and is cleaved after import. Succinyl CoA synthetase associates specifically with ALAS2 within the mitochondrion, which helps promote the first step of heme synthesis.[14]

Enzyme levels of ubiquitous and erythroid isoenzymes of ALAS are controlled by different mechanisms. Ubiquitous ALAS1 levels in the liver are regulated by negative feedback by heme that inhibits its gene transcription and import of pre-ALAS1 (see Fig. 39.2A). More recently ALAS1 has been shown to be upregulated by peroxisome proliferator-activated receptor-gamma, coactivator 1, alpha (PPAR-γ coactivator 1-α), which regulates mitochondrial biogenesis and oxidative metabolism. Transcription of PPAR-γ coactivator 1-α is controlled by glucose availability. PPAR-γ coactivator 1-α production increases when glucose levels are low, leading to increased levels of ALAS1 and heme. These conditions are conducive to an acute attack of porphyria. However, the relative contribution of heme and

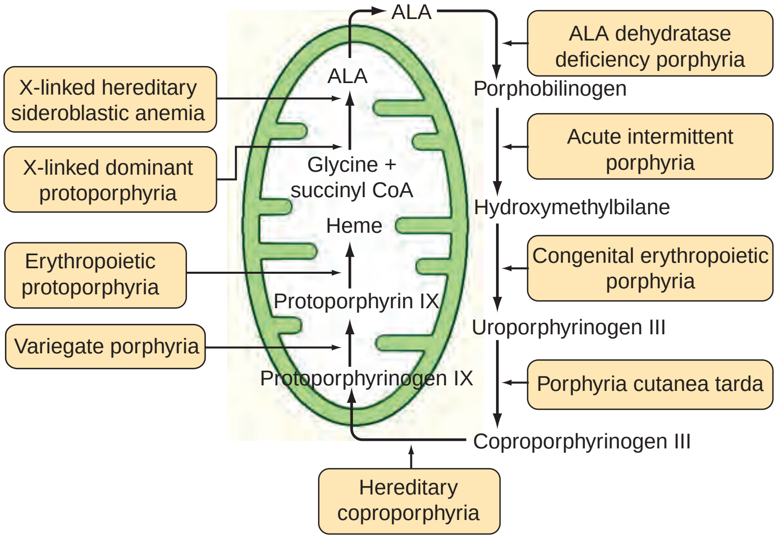

ALA
ALA dehydratase deficiency porphyria
X-linked hereditary sideroblastic anemia
ALA
Porphobilinogen
Acute intermittent porphyria
X-linked dominant protoporphyria
Glycine + succinyl CoA
Heme
Hydroxymethylbilane
Erythropoietic protoporphyria
Congenital erythropoietic porphyria
Protoporphyrin IX
Uroporphyrinogen III
Variegate porphyria
Protoporphyrinogen IX
Porphyria cutanea tarda
Coproporphyrinogen III
Hereditary coproporphyria

Figure 39.1 PATHWAY OF HEME BIOSYNTHESIS IN MAMMALIAN CELLS. The first step in the pathway is catalyzed by aminolevulinate synthase (ALAS) and occurs within the mitochondrion using pyridoxal 5′-phosphate as a cofactor. 5-Aminolevulinate (ALA) then leaves the mitochondrion and is converted by ALA dehydratase to give a monopyrrole, porphobilinogen. Four molecules of this compound are converted by porphobilinogen deaminase to a linear tetrapyrrole, hydroxymethylbilane. This molecule is then cyclized by uroporphyrinogen III synthase to uroporphyrinogen III, which is decarboxylated to coproporphyrinogen III. This molecule enters the mitochondrion and is oxidized in succession by coproporphyrinogen III oxidase and protoporphyrinogen oxidase. The product is protoporphyrin IX, a substrate for ferrochelatase, which catalyzes the insertion of Fe^{2+} to form heme. A mitochondrial heme exporter has been identified as feline leukemia virus subgroup C receptor 1b. The defective steps associated with specific porphyrias and X-linked hereditary sideroblastic anemia are shown.

TABLE 39.1 **Porphyrias: Clinical Involvement, Enzymatic Etiology, and Chromosomal Location**

Porphyria (Synonym)	Acute Attack, Skin and Organ Involvement	Enzyme of Heme Biosynthesis Affected (Gene)	Chromosome Location
—	—	Hepatic, 5-aminolevulinate synthase, nonspecific, mitochondrial (ALAS1)	3p21.2
X-linked sideroblastic anemia	Bone marrow	5-Aminolevulinate synthase, erythroid-specific, mitochondrial (ALAS2; low function mutation)	Xp11.21
X-linked dominant protoporphyria	Skin, red cells, liver	5-Aminolevulinate synthase, erythroid-specific, mitochondrial (ALAS2; high function mutation)	Xp11.21
ALA dehydratase deficiency porphyria (plumboporphyria)	Acute liver	ALA dehydratase (ALAD; porphobilinogen synthase)	9q32
Acute intermittent porphyria	Acute liver	Porphobilinogen deaminase (hydroxymethylbilane synthase; HMBS)	11q23.3
Congenital erythropoietic porphyria (Günther disease)	Skin, red cells, bone marrow	Uroporphyrinogen III synthase (UROS)	10q26.2
Porphyria cutanea tarda (symptomatic porphyria, cutaneous hepatic porphyria)	Skin, liver	Uroporphyrinogen decarboxylase (UROD)	1p34.1
Hereditary coproporphyria	Acute skin, liver	Coproporphyrinogen oxidase (CPOX)	3q11.2
Variegate porphyria (porphyria variegata)	Acute skin, liver	Protoporphyrinogen oxidase (PPOX)	1q23.3
Erythropoietic protoporphyria (erythrohepatic protoporphyria)	Skin, red cells, liver	Ferrochelatase (heme synthase; FECH)	18q21.3

ALA, 5-Aminolevulinate.

PPAR-γ coactivator 1-α in regulating ALAS1 expression remains to be resolved.[15,16] In contrast, heme does not affect transcription of the ALAS2 gene, which is under the control of erythroid-specific promoters such as GATA1, a globin transcription factor. Whether heme inhibits import of pre-ALAS2 into the mitochondrial matrix remains to be unequivocally established, although this is supported by in vitro experiments.[17] Heme also regulates the transcription of ferroportin, and H- and L-ferritin during erythroid differentiation, which may ensure adequate iron supply to mitochondria for heme synthesis (see Fig. 39.2B). In addition to transport of iron from plasma to the cytosol by the transferrin receptor, a second transport step is required for mitochondrial uptake of iron. The passage of iron across the outer mitochondrial membrane is ill-defined but is due largely to the presence of voltage-dependent anion channels. Iron import across the inner mitochondrial membrane is fulfilled by mitoferrin,[18] a member of the solute carrier 25 family of proteins located in the inner

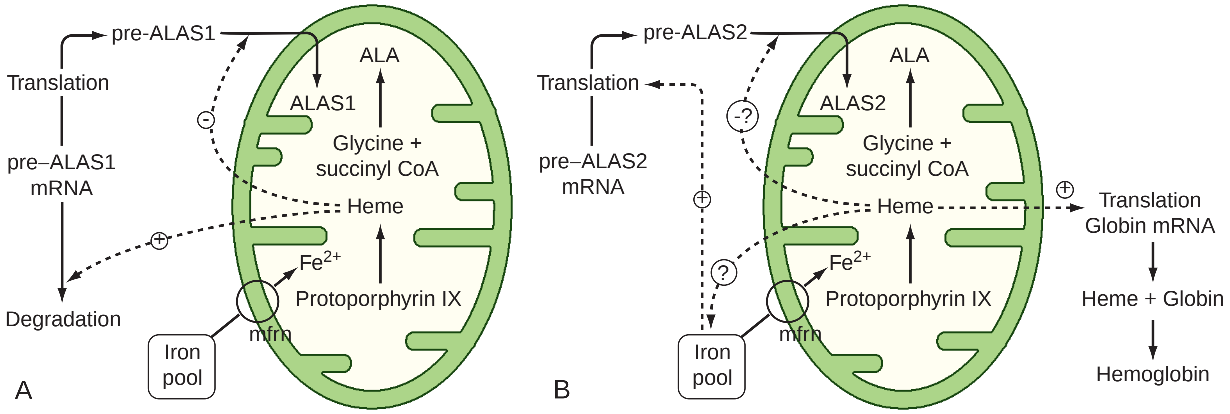


Figure 39.2 (A) Control of heme synthesis in hepatic and other tissues. The rate of heme synthesis depends on the first and rate-limiting enzymatic step catalyzed by 5-aminolevulinate synthase, nonspecific, mitochondrial (ALAS1). Heme represses transcription of the ALAS1 gene, increases the rate of degradation of its messenger ribonucleic acid (mRNA), and blocks the translocation of the ALAS1 isoenzyme into the mitochondrion. (B) Control of heme synthesis in erythroblasts. Cytosolic iron enhances the translation of mRNA of the pre-ALAS2 by inhibiting the interaction of a repressor protein with an iron-responsive element in the mRNA. The product of the last step, heme controls transcription of genes that may contribute to iron metabolism and ensure adequate supply of iron to the mitochondrion. Heme also may inhibit translocation of ALAS2 into the mitochondrion. The overall result is that the rate of heme synthesis is tightly linked to the availability of iron for the ferrochelatase reaction. Mitoferrin (mfrn) transports Fe^{2+} into the mitochondrial matrix. *ALA*, 5-Aminolevulinate.

mitochondrial membrane, which, to import iron into the mitochondrion, must interact both with FECH and with the adenosine triphosphate (ATP)-binding cassette transporter ABCB10.[19] Levels of intracellular iron regulate the translation of ALAS2 mRNA. Cellular iron homeostasis is maintained through a posttranscriptional regulatory mechanism, which is mediated by iron regulatory proteins that bind to iron-responsive elements (IRE) in mRNA of target genes to either increase or decrease translation.[20,21] The RNA binding activity of iron-responsive proteins (IRP) is regulated by mitochondrial iron-sulfur cluster synthesis and cytosolic iron levels.[22–24] When iron is available for heme synthesis, translation of ALAS2 is allowed to proceed as a result of decreased IRP binding to the 5′ UTR iron-responsive elements of ALAS2 mRNA. In contrast, under iron-depleted conditions, increased IRP binding to ALAS2 mRNA blocks translation and ensures that ALAS2 and protoporphyrin levels are not produced in excess of available iron. Furthermore, to prevent the cell from becoming iron deficient, increased translation of mRNA from genes that increase cellular iron, such as the transferrin 1 gene, results from stabilization of mRNA by binding of IRPs to mRNA (see Chapter 36). This effect ensures that protoporphyrin synthesis is coupled to iron availability.

A second rate-limiting step in the overall heme synthetic pathway lies at the level of porphobilinogen deaminase (PBGD), which has a low endogenous activity and is inhibited by protoporphyrinogen and coproporphyrinogen. There are also two forms of PBGD. The PBGD gene (hydroxymethylbilane synthase; HMBS) encodes two enzymes, which arise from alternative splicing of PBGD mRNA. One isoform is expressed in all cells, whereas a second is restricted to red cells.[25] Erythroid PBGD is stimulated by erythropoiesis in vitro and may play a regulatory role in heme biosynthesis during differentiation.[26]

HMBS, the human PBGD gene, has attracted extensive investigation because of the practical importance of detecting carriers of the gene for acute intermittent porphyria (AIP).[27] Studies of the genetic locus of PBGD on chromosome 11 show great molecular heterogeneity,[28] with over 380 mutations associated with AIP in the Human Gene Mutation Database (HGMD Professional 2019, www.hgmd.org). Most human mutations have been described in exons 10 and 12,[29] which is consistent with alteration of the binding sites for the dipyrromethane cofactor for the enzyme. The three-dimensional structure of PBGD has been defined by X-ray crystallography, which has allowed study of the structural and functional implications of mutations.[30]

PORPHYRIAS

Biologic and Molecular Aspects

The porphyrias are classified as acute or nonacute (cutaneous) according to their clinical and biochemical features (Table 39.2).

Each of the different types of porphyria is linked to a reduced activity or deficiency of a specific enzyme in the heme biosynthetic pathway, with the exception of X-linked dominant erythropoietic protoporphyria, which results from inheritance of a gain-of-function mutation in the ALAS2 gene (see Fig. 39.1).[31] When porphyria is caused by a loss-of-function mutation, the resulting enzyme deficiency impairs the production of the end-product heme, and there is overproduction and increased excretion of the heme precursors formed by the steps before the enzyme defect. There is also a compensatory increase in activity of the initial and rate-controlling enzyme ALAS. In the acute porphyrias, there is overproduction of all the porphyrins and porphyrin precursors (e.g., ALA, PBG) formed proximal to the enzyme defect. The increased excretion of porphyrin precursors in the acute porphyrias is caused by decreased activity of PBGD in these conditions. The decrease can be caused by genetic mutation of the enzyme (in AIP) or by inhibition of PBGD by protoporphyrinogen and coproporphyrinogen in variegate porphyria and hereditary coproporphyria, respectively.[32]

In the nonacute porphyrias, there is overproduction of all porphyrins formed before the enzyme defect but no overproduction of porphyrin precursors. The cause of this lack of overproduction of porphyrin precursors in the nonacute porphyrias is unclear, but it may result from a compensatory increase in the activity of the enzyme PBGD in addition to increased activity of ALAS and site-specific heme synthesis.[33] The pattern of overproduction and excretion of porphyrins and porphyrin precursors in the various porphyrias is shown in Table 39.3. A consequence is that each of the different porphyrias is characterized by a different excretion pattern. Quantitative studies of the different porphyrins and precursors in the urine and feces usually identify the particular type of porphyria. The porphyrin precursors ALA and PBG and the more water-soluble porphyrins (with multiple carboxyl groups) are excreted mainly in the urine. Other porphyrins are mainly excreted in the feces by way of bile (see box on Measurement of Porphyrins and Precursors).

TABLE 39.2 Classification of Porphyrias

Classification	Disease	Biochemistry	Clinical Features
Acute porphyria	Acute intermittent porphyria	Increased ALA and PBG	Acute attack
	Variegate porphyria	Increased ALA and PBG; increased porphyrin	Acute attack; photosensitivity
	Hereditary coproporphyria	Increased ALA and PBG; increased porphyrin	Acute attack; photosensitivity
	ALA dehydratase deficiency porphyria	Increased ALA; increased porphyrin	Acute and chronic neuropathy
Nonacute porphyria	Porphyria cutanea tarda	Increased porphyrin	Photosensitivity
	Erythropoietic protoporphyria	Increased porphyrin	Photosensitivity
	Congenital erythropoietic porphyria	Increased porphyrin	Photosensitivity
	X-linked dominant protoporphyria	Increased porphyrin	Photosensitivity
Porphyrinurias	Lead, alcohol, iron deficiency anemia, liver disease	Various biochemical manifestations	Various clinical presentations

ALA, 5-Aminolevulinate; *PBG*, porphobilinogen.

TABLE 39.3 Changes in Porphyrins and Their Precursors in the Porphyrias, Porphyrinurias, and Hereditary Sideroblastic Anemia

Porphyrias and Other Conditions	ALA	PBG	Urine Uroporphyrin	Urine Coproporphyrin	Feces Coproporphyrin	Feces Protoporphyrin	Erythrocyte Protoporphyrin
Acute Porphyrias							
Acute intermittent porphyria	Raised, very high in attack	Raised, very high in attack	Usually raised[a]	Sometimes raised	Sometimes raised	Sometimes raised	Normal
Variegate porphyria	Raised in attack	Raised in attack	Usually raised in attack	Usually raised in attack	Raised	Raised	Normal
Hereditary coproporphyria	Raised in attack	Raised in attack	Sometimes raised in attack	Usually raised, always in attack	Raised	Usually normal	Normal
ALA dehydratase–deficiency porphyria	Raised in attack	Normal	Normal	Usually raised in attack	Normal	Normal	Occasionally raised
Nonacute Porphyrias							
Porphyria cutanea tarda	Normal	Normal	Raised (7-/8- carboxylate porphyrin levels very high in attack)	Slightly raised	Isocoproporphyrin raised in remission	Raised in remission	Normal
Erythropoietic protoporphyria	Normal	Normal	Normal	Normal	Normal	Usually raised	Raised, usually very high
Congenital erythropoietic porphyria	Usually normal	Usually normal	Raised, isomer I	Raised, isomer I	Normal	Usually raised	Usually raised
X-linked dominant protoporphyria	Normal	Normal	Normal	Normal	Normal	Usually raised	Raised, usually very high
Other Conditions							
Hereditary sideroblastic anemia	Normal	Normal	Normal	Normal	Normal	Normal	Occasionally raised
Lead poisoning	Raised	Normal	Normal	Sometimes raised	Normal	Normal	Raised when blood lead level >2 μM
Hereditary tyrosinemia	Raised	Normal	Normal	Normal	Normal	Normal	Normal
Iron deficiency anemia	Normal	Normal	Normal	Normal	Normal	Normal	Raised

[a] PBG may cyclize to uroporphyrin nonenzymatically.
ALA, 5-Aminolevulinate; *PBG*, porphobilinogen.

MEASUREMENT OF PORPHYRINS AND PRECURSORS

PBG quantitation in a random urine sample collected during symptoms is recommended as the initial screening test for the acute porphyrias,[34] whereas plasma fluorescent spectroscopy is the best initial test for diagnosis of cutaneous porphyrias.[35] Diverse techniques such as high-pressure liquid chromatography,[36] quantitative extraction, and various forms of fluorometry are used to measure porphyrins and precursors.[34,37,38] The International Federation of Clinical Chemistry and Laboratory Medicine presents diagnostic information on its website (www.ifcc.org).

The clinical manifestations of an acute attack of porphyria can be explained by dysfunction of the central, peripheral, and autonomic nervous systems. The mechanism by which altered heme synthesis results in dysfunction is unknown, although porphyrin-induced protein aggregation may be a mechanism for both external and internal tissue damage in porphyrias that involve fluorescent porphyrin accumulation.[6]

Perhaps the most likely hypothesis is that the neurologic and muscular manifestations of acute porphyria arise as a result of heme deficiency within the nerve cells, which causes dysfunction of the energy-dependent Na^+/K^+ ATPase. The proposal that axonal dysfunction results from impaired energy metabolism is supported by the findings of axonal membrane depolarization during acute attacks of porphyric neuropathy and reduction in inward rectification between episodes.[39] However, this does not exclude the possibility that ALA may also act as a pharmacologic agent in these diseases, compounding the effects of heme deficiency.[40] ALA has a pro-oxidant effect on rat brain tissues and generates free radical species during its auto-oxidation, and this oxidant stress has been proposed to directly damage myelination by Schwann cells.[41,42] The concept of auto-oxidation or oxidative stress is supported by the hypothesis that manganese excess could contribute to induction of superoxide dismutase[43] and increased indicators of such stress in lead exposure.[44] There is evidence that ALA enters cells by a pathway common to it and γ-aminobutyric acid (GABA).[45]

Genetic Aspects

The enzymatic links and genetic loci in each of the hereditary porphyrias are shown in Table 39.1. Nearly all are inherited as autosomal dominant traits. The Chester porphyria family pedigree (Fig. 39.3) shows autosomal dominant inheritance of acute porphyria with attacks of neurovisceral dysfunction without cutaneous hypersensitivity. This was originally reported as a dual porphyria[46]; however, identification of a heterozygous truncating mutation in the HMBS gene and no mutations in other heme biosynthesis enzymes in affected individuals has confirmed that Chester porphyria is a variant of classic AIP.[47] HMBS is transcribed from two promoters to produce ubiquitous and erythroid specific isoforms. In classic AIP both isoforms

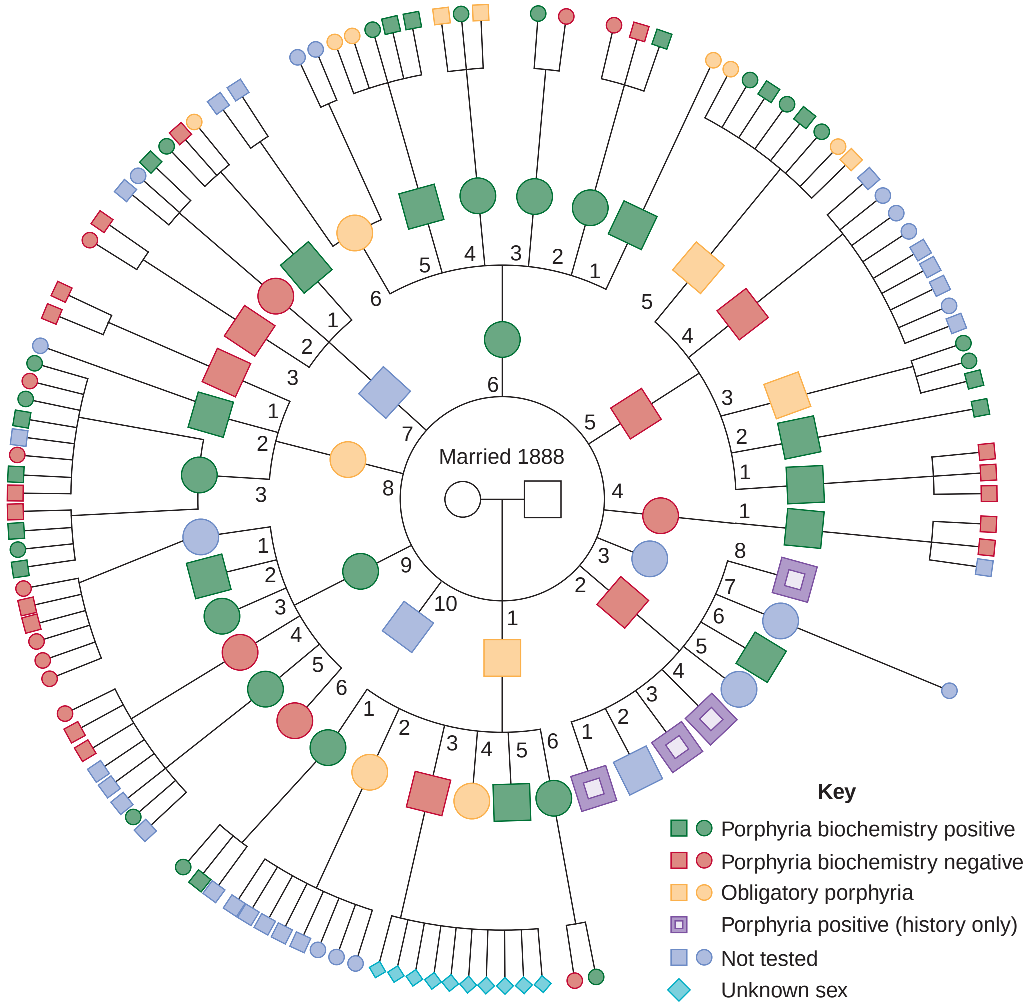

Married 1888
Key
🟩 ● Porphyria biochemistry positive
🟥 ● Porphyria biochemistry negative
🟧 ● Obligatory porphyria
▣ Porphyria positive (history only)
▢ ● Not tested
◆ Unknown sex

Figure 39.3 THE CHESTER FAMILY PEDIGREE. The propositus, Peter Dobson, was a salmon fisherman from a close-knit community living on the bank of the River Dee, which runs through the city of Chester, UK. Most of the 330 descendants of his marriage in 1888 still live in the city. Many suffered disabling illnesses and psychiatric upsets, which often went unrecognized as porphyria. The family called their illness *Dobson's complaint*. Chester porphyria has recently been confirmed as a variant of acute intermittent porphyria. *Squares* represent male subjects; circles represent females. *Courtesy Giles R. Youngs.*

are deficient; however, in the rare, nonerythroid variant, only the ubiquitous HMBS variant is defective. The rare CEP shows autosomal recessive inheritance. The mutations producing each of the acute porphyrias are heterogeneous at the molecular level and include complete or partial gene deletions, alterations of splicing or stability of mRNA, and missense mutations. In South Africa, the founder effect ensures a predominance of the Arg59Tryp mutation in protoporphyrinogen oxidase, although over 175 other mutations have been reported worldwide.[48–50] Homozygotic or compound heterozygotic inheritance has been found in a number of the porphyrias, as has concurrent inheritance of more than one defect. This may present as two types of porphyria in one family[51] or as two types in one patient.[52,53] Dual porphyrias most commonly arise from a combined deficiency of uroporphyrinogen decarboxylase with PBGD, coproporphyrinogen oxidase, or protoporphyrinogen oxidase.[54]

The prevalence of the different forms varies widely. For example, in northern Europe and North America, approximately 1 of 10,000 individuals carries the gene for AIP, although only about 10% of the affected persons will present with clinical features. It has been suggested that spontaneous mutation accounts for 3% of AIP cases.[55] Variegate porphyria occurs in 1 of 400 white South Africans. There is a reduction in gene frequency in variegate porphyria from generation to generation that suggests that the allele associated with it is selectively deleterious.[56] The same is probably true of the other porphyrias.

Acute Intermittent Porphyria

Clinical and Laboratory Manifestations

Acute intermittent porphyria is the most severe of the acute porphyrias. During an attack, patients display abdominal and neuropsychiatric or neurovisceral disturbances. Onset occurs in puberty; female patients exhibit a fourfold greater incidence of attacks than males. Attacks occur mainly in young adults and become less frequent after menopause. It is uncommon to see attacks in children.[57] Crises may vary in duration from several days to months. They are most commonly followed by complete remission, although deaths are still reported, especially with AIP (see box on Precipitating Factors in Acute Porphyria).[58] Late complications of AIP include renal impairment, hypertension, and hepatocellular carcinoma, and patients should be monitored for these complications.[59,60]

Gastrointestinal symptoms occur in 95% of cases; most patients present with acute colicky central abdominal pain. Examination reveals tenderness but little rigidity, and patients may also experience limb pain or generalized muscular aches. Severe vomiting may occur, and constipation is usual. Hyponatremia occurs in severe attacks.

Motor neuropathy complicates two-thirds of porphyric attacks and may be the presenting feature. Motor involvement is most common, but paresthesias may also occur. Paralysis usually starts peripherally and then spreads proximally; however, in some patients, shoulder girdle involvement may be the first manifestation. The neuropathy may progress rapidly, resulting in respiratory insufficiency. Weakness, usually symmetric, involves proximal and distal limb muscles more often than those of the trunk. Upper limbs and proximal muscles are often affected. Involvement of the wrists, ankles, and small muscles of the hand may lead to a permanent deformity (Fig. 39.4), and trunk muscle weakness can lead to respiratory embarrassment. Death is usually caused by respiratory paralysis. Progressive weakening of the voice may suggest this; treatment requires tracheotomy and intermittent positive pressure ventilation. Paresthesias, numbness, and objective evidence of sensory impairment may occur with loss of pinprick

PRECIPITATING FACTORS IN ACUTE PORPHYRIA

Most patients who have inherited acute porphyria enjoy normal health and go through life without any knowledge of their disorder or ever experiencing an acute attack. All porphyric patients, however, are at risk for developing an attack if exposed to various precipitating factors. Drugs are the most common precipitating agents. Other factors that may trigger attacks include alcohol ingestion, reduced caloric intake (from fasting or dieting), and infection. Smoking can cause more frequent attacks.

Hormonal status is also important. Attacks are more common in females, and they rarely occur before puberty or after menopause. Although generally uncomplicated, pregnancy in addition to oral contraceptives may precipitate attacks.[61] Some women experience regular attacks, commencing in the week before the onset of menstruation. These may require luteinizing hormone–releasing hormone (LH-RH) antagonists for control (Table 39.4).

Drugs for Porphyria

Before prescribing any medication to a porphyric patient, advice must be sought from an appropriate specialist. Full drug lists are available on the Internet at https://porphyriafoundation.org/ (in the United States) and www.porphyria-europe.com (in European Union countries, South Africa, and Canada), and a guide for drug prescription at www.drugs-porphyria.org. It should be borne in mind that such lists are far from encyclopedic, that new drugs are constantly being introduced to the pharmacopeia, and that any form of combined preparation must be viewed with suspicion, because little is known about metabolic interactions in these diseases.[62] More details of such use and side effects of drugs can be sought from the literature.[63]

TABLE 39.4 Precipitating Factors in Acute Porphyria

Drugs	Other Stimuli
Alcohol	Fasting or dieting
Barbiturates	Hormones, stress
Angiotensin-converting enzyme (ACE) inhibitors	Smoking
Anticonvulsants	
Antidepressants	
Calcium channel blockers	
Cephalosporins	
Ergot derivatives	
Erythromycin	
Steroids or anabolic steroids	
Contraceptives, hormone replacement therapy	
Sulfonamides	
Sulfonylureas	

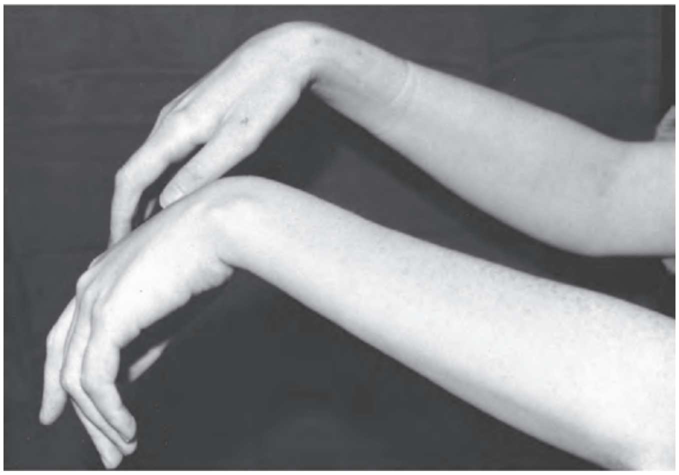


Figure 39.4 BILATERAL WRISTDROP CAUSED BY PERIPHERAL NEUROPATHY IN A PATIENT WITH ACUTE INTERMITTENT PORPHYRIA.

sensation, which is most marked around the shoulder and hip areas; generalized tonic-clonic seizures occasionally occur.

Severe anxiety, depression, and frank psychosis are the main psychiatric manifestations of porphyric attacks. These psychiatric manifestations may result in a patient being misdiagnosed as suffering from a primary psychiatric disorder. Agitation, mania, depression, hallucinations, and schizophrenic-like behavior may occur. Psychiatric manifestations may persist between attacks.[64] Quality of life is severely affected in those suffering from repeated attacks of acute porphyria.[65]

The cardiovascular system is involved in approximately 70% of attacks. Sinus tachycardia (to 160 beats/min) and hypertension can occur; these elevations usually revert to normal after an attack. There is evidence that hypertension may occasionally be permanent, even in latent cases of AIP (see box on Differential Diagnosis of Acute Intermittent Porphyria).

Other Acute Porphyrias

Hereditary Coproporphyria

Hereditary coproporphyria combines the clinical features of acute porphyria with photosensitive skin manifestations. It results from various mutations in the gene encoding coproporphyrinogen oxidase, the activity of which is decreased,[69,70] leading to overproduction of coproporphyrin.[71] A clinically distinct variant of hereditary coproporphyria, harderoporphyria, is associated with severe jaundice and hemolysis and is caused by specific mutations that lead to accumulation of harderoporphyrins.[72]

The porphyrin precursors ALA and PBG and the more water-soluble porphyrins (with multiple carboxyl groups) are excreted mainly in the urine. Other porphyrins are mainly excreted in the feces by way of bile.

Variegate Porphyria

Variegate porphyria is similar to hereditary coproporphyria, except that there are more severe skin lesions, sometimes with scarring (Fig. 39.5). Protoporphyrinogen oxidase is the affected enzyme, and protoporphyrin is the major circulating porphyrin. Conventionally, variegate porphyria is most readily diagnosed by measurement of fecal porphyrin concentrations. However, it has been reported[73] that biliary porphyrin levels may provide a better discriminator from normal patients in the asymptomatic phase. As in erythropoietic protoporphyria, there is a tendency toward cholelithiasis. The mechanism by which gallstones form is not certain, but some studies have suggested that porphyrins are cholestatic.[74] In hereditary coproporphyria and variegate porphyria, the pathway intermediates produced in excess, coproporphyrinogen and protoporphyrinogen, respectively, are inhibitors of the secondary rate-controlling enzyme PBGD.[75,76] Numerous mutations in the protoporphyrinogen oxidase gene have been described leading to 50% reduction in enzyme activity. The functional consequences of many of these mutations have been predicted based on the crystal structure of the enzyme.[77] In a few cases, homozygosity or compound heterozygosity has been described.[49,78]

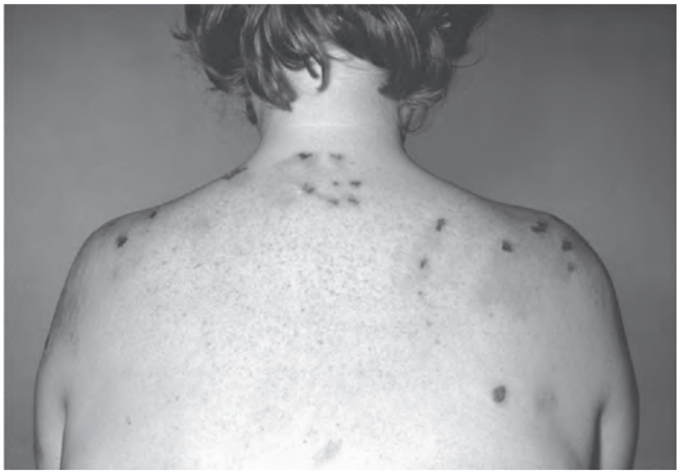


Figure 39.5 CUTANEOUS LESIONS AND SCARRING IN A PATIENT WITH VARIEGATE PORPHYRIA.

DIFFERENTIAL DIAGNOSIS OF ACUTE INTERMITTENT PORPHYRIA

Attacks of acute porphyria must be distinguished from other causes of acute abdominal pain or peripheral neuropathy sometimes associated with psychosis.[66,67] Heavy metal poisoning (i.e., lead or arsenic) and Guillain-Barré syndrome must be considered, as well as paroxysmal nocturnal hemoglobinuria with its characteristic early morning hemoglobinuria and abdominal pain.[7]

During an attack, all patients excrete a massive excess of the porphyrin precursors, 5-aminolevulinate (ALA) and porphobilinogen (PBG), in their urine. Urine, when first voided, is clear and darkens on exposure to light as the hexahydroporphyrins, the porphyrinogens, are oxidized to porphyrins.

A rapid screening test during an acute attack is to mix equal volumes of urine and Ehrlich aldehyde reagent and observe for the pink color of porphobilinogen; alternatively, the urine can be left standing in sunlight to observe darkening in color. The differential diagnosis of porphyrias uses qualitative and quantitative measurement of porphyrins and precursors, with subsequent use of enzymatic assay and identification of familial genetic alterations.[34,68]

5-Aminolevulinate Dehydratase Deficiency Porphyria

In this porphyria (also known as acute hepatic porphyria or plumboporphyria), the ALA dehydratase activity is depressed, such as seen with a low-function variant of the enzyme in a subject exposed to lead. The clinical picture resembles AIP, but very few cases have been described, and the disease only manifests in homozygous cases when there is a precipitating factor.[79,80]

Concurrent Porphyrias

The concurrent porphyrias are a rare group of conditions in which there is concurrent inheritance of two different defects within the heme biosynthetic pathway, although this requires confirmation from molecular evidence.[81] Previous descriptions[52–54] have shown the presence of concurrent porphyria within a family, and toxicologically there is good evidence that exposure to poisons such as lead can induce multiple changes within the pathway.[82] The first reported example of concurrent porphyria in a family combined the clinical features of acute and cutaneous porphyria, and biochemical analysis confirmed the segregation of variegate porphyria and PCT as independent inherited traits.[83] In another patient, dual genetic defects involving ALA dehydratase and coproporphyrinogen oxidase have been described (see box on Management of Acute Porphyria).[52]

Nonacute or Cutaneous Porphyrias

In all cutaneous porphyrias, porphyrins (which are photosensitizing) are deposited in the upper layers of the skin, and they are responsible for the characteristic skin lesions.[90] In the development of these lesions, reactive oxygen species and other radicals are formed and probably induce oxidative membrane damage, particularly to mast cells, which enables complement activation as one part of the inflammatory reaction (see box on Management of Nonacute Porphyria.[91]

MANAGEMENT OF ACUTE PORPHYRIA

Treatment of Acute Attack

A carbohydrate intake of 1500–2000 kcal/24 h should be maintained throughout the attack to reduce porphyrin synthesis; give this orally or, for more severe attacks, through a fine-bore Teflon nasogastric tube. If this cannot be tolerated, intravenous dextrose (e.g., 20% solution, 2 L/day) should be given. If early in the attack (2–4 days from onset), give intravenous hematin as heme arginate (Normosang, Recordati Rare Diseases, Lebanon, NJ) at a recommended daily dose of 3 mg/kg once daily for four days to further reduce the overproduction of porphyrin and precursors.[84,85] Hematin (Panhematin, Recordati Rare Diseases) in similar doses may also be used, although it should be reconstituted in human albumin solution to avoid phlebitis and mild transient prolongation of coagulation times. No renal complications have occurred with the standard recommended dosages, and even patients with renal insufficiency tolerate hematin well, although the dosage should be reduced slightly. Severe hepatic toxicity has been associated with rapid infusion of higher doses of heme arginate.[86] The action of heme therapy may be extended by heme oxygenase blockers such as tin protoporphyrin.[85] Liver transplantation may be an effective treatment for life-threatening acute intermittent porphyria that fails to respond to medical therapy.[87]

Prophylaxis

Many drugs are contraindicated, and the patient must be warned to avoid precipitating factors. Alcohol should be restricted and smoking discouraged. Dieting (<800 kcal/day) must be avoided. Pregnancy should also be avoided if the disease is active. If a patient requires an anesthetic, nitrous oxide, ether, and cyclopropane are safe, and suxamethonium appears to be a safe muscle relaxant. The opiates and belladonna derivatives can be used for premedication and propofol for maintenance of anesthesia. Infection can precipitate an attack and should therefore be sought and treated. Blood relatives of patients should be screened to see if they carry the gene.

Luteinizing hormone-releasing hormone (LH-RH) antagonists that suppress ovulation are a valuable form of prophylaxis in menstrually related attacks.[88] Estrogens and progestogens such as those in the contraceptive pill must be avoided in acute porphyria. The same applies to most steroids and receptor antagonists such as mifepristone.[89]

Porphyria Cutanea Tarda

Biologic and Molecular Aspects

PCT exists in inherited and acquired/sporadic forms. Type I, or sporadic PCT, comprises 70% to 80% of cases and is associated with 50% level of uroporphyrinogen decarboxylase activity. PCT type II or familial PCT results from heterozygous mutations in uroporphyrinogen decarboxylase, whereas a more severe form, hepatoerythropoietic porphyria (HEP), results from homozygous or compound heterozygous mutations.[106] Mutations in the uroporphyrinogen decarboxylase are found in around one-third of patients with PCT. In inherited and acquired forms, there is diminution in the activity of hepatic uroporphyrinogen decarboxylase, which converts uroporphyrinogen to coproporphyrinogen by the stepwise decarboxylation of the acetyl groups to methyl groups. The mechanism of enzyme inhibition has been elucidated, whereby iron-dependent oxidation of uroporphyrinogen generates uroporphomethene, a competitive inhibitor of uroporphyrinogen decarboxylase.[107] Most carriers of mutant uroporphyrinogen decarboxylase are not clinically evident unless precipitating factors are present. Iron alone or chlorinated hydrocarbons can diminish activity of uroporphyrinogen decarboxylase, and this effect is greatly potentiated when both are given together.[108,109] In a murine model, precipitation of uroporphyria by chlorinated hydrocarbons is dependent on hepatic cytochrome P450 Cyp 1A2 oxidase activity, and inherited variations in enzyme function may modulate susceptibility to PCT in humans.[108]

MANAGEMENT OF NONACUTE PORPHYRIA

Patients should avoid exposure to sunlight and use sunblock (to filter Soret band light) and physical barriers such as cotton gloves.

Porphyria Cutanea Tarda

The clinical features are reversed by removing any precipitating agent such as alcohol, halogenated hydrocarbons, and drugs. The patient should be screened for hepatic neoplasm and hepatitis C infection. The mainstay of treatment is to remove liver iron by venesection of 500 mL of blood weekly until clinical remission occurs or until the hemoglobin level falls below 12 g/dL. In patients with PCT where HCV is the primary risk factor, antiviral treatment with direct-acting antiviral agents such as simeprevir and sofosbuvir, is effective as a single-agent therapy.[92,93] Chloroquine, at low doses of 125 mg twice per week for several months, has also been helpful because it enhances urinary clearance of porphyrin. When this is not possible, oral cimetidine has been used.[94] When venesection is difficult, parenteral and orally active iron chelators can be used to reduce liver iron stores. It is of value to screen the patient and first-degree relatives for hereditary hemochromatosis.

Erythropoietic Protoporphyria

Oral β-carotene offers effective protection in erythropoietic protoporphyria (EPP) against solar sensitivity. It does so by quenching the radical formation that is a feature of the skin damage. Yellowing of the skin (i.e., carotenemia) is one side effect. Afamelanotide, an alpha-melanocyte-stimulating hormone that is now approved by the US Food and Drug Administration,[95] is effective for decreasing photosensitivity in EPP by increasing melanin production, and has shown efficacy and tolerability for the long-term treatment of EPP.[96,97] Interruption of the enterohepatic protoporphyrin circulation by bile salt–sequestering agents, such as cholestyramine, reduces plasma protoporphyrin levels and may retard the development of the liver disease. Liver transplantation has been reported to be an effective measure in preventing the progression of this disease.[98,99]

Congenital Erythropoietic Porphyria

The severity of hematologic manifestations are predictors of a poor prognosis, and patients with hemolytic anemia or thrombocytopenia should be considered for allogeneic hematopoietic stem cell transplantation (HSCT). The majority of patients treated with allogeneic HSCT have achieved long-term symptomatic cures.[100,101] Erythropoiesis should be reduced by means of erythrocyte hypertransfusion and by hematin or heme arginate infusion.[102] However, care should be taken when prescribing heme arginate, as overdosing may cause acute hepatic failure.[86] Splenectomy and chloroquine therapy (125 mg twice weekly) have an ameliorating effect, as does hypertransfusion, but life expectancy is usually severely shortened. Iron chelation with deferasirox to inhibit ALAS2 mRNA translation in a CEP patient has resulted in decreased porphyrin accumulation and reduced hemolysis and photosensitivity.[103] Therapeutic potential has been shown with the use of clinically approved proteasome inhibitors, such as bortezomib, which prevent enzymatically active uroporphyrinogen III synthase (UROS) mutants from early degradation in a murine model, and gene therapy using induced pluripotent stem cells.[104,105]

In sporadic (type I) PCT, there is no evident genetic basis for the condition, and low uroporphyrinogen decarboxylase activity is restricted to the liver. Patients may have clinical and biochemical evidence of liver disease. Hepatic siderosis invariably occurs, and iron is one of the causative agents in acquired PCT. An association of PCT with hereditary hemochromatosis has been documented, with about 20% of PCT patients being homozygous for the Cys282Tyr mutation in the HFE ("High Fe") gene, a defect that characterizes hereditary hemochromatosis.[110–112] These results strongly implicate the HFE gene as a genetic susceptibility factor in acquired PCT. An association between PCT and hepatitis C infection is well documented, and in countries with high prevalence rates, hepatitis C virus may be the dominant risk factor.[113] An apparent association has emerged between human immunodeficiency virus (HIV) infection and PCT.[114] It is possible that therapy for HIV with zidovudine precipitates the disease, but it is more likely that the association is merely coincidental or that the viral infection unmasks the preexisting uroporphyrinogen decarboxylase defect.[115]

Genetics

Whereas familial PCT is inherited in an autosomal-dominant mode, HEP is inherited in an autosomal-recessive pattern.[116] As in the other genetic lesions in the porphyrias, there is heterogeneity in the mutations causing PCT and HEP phenotypes.[117,118] In the inherited form, more than 120 different mutations (HGMD Professional 2019, www.hgmd.org) have been identified in uroporphyrinogen decarboxylase, many of which lie near the dimer interface, resulting in an unstable protein and reduced enzyme activity.[119] In different population groups, it is difficult to find the relative numbers of acquired and familial disease. In one analysis in Hungary, 77.5% of patients were found to suffer from the acquired form, and of the patients with the familial disease, females were affected more than males, suggesting that inheritance may predispose patients to estrogen-precipitated disease.[120]

Clinical Features

The most striking clinical feature of both forms of PCT is a bullous dermatosis on light-exposed areas. This starts as erythema and progresses to vesicles that become confluent to form bullae (Fig. 39.6), which may hemorrhage and leave scars; pruritus is often troublesome. Milia are common and may precede or follow vesicle formation. Facial hypertrichosis is common and may serve as a diagnostic clue. In less severe cases, increased fragility of the skin may be the only clinical sign. In severe cases, photomutilation can result, usually because of infection of slowly healing lesions.

The thickening and scarring with calcification have been described as pseudoscleroderma. Hyperpigmentation is common, and women often complain of hirsutism. Neurologic change is not observed. Patients may have clinical and biochemical evidence of chronic liver disease, sometimes with cirrhosis. There is an association with hepatocellular carcinoma. Hepatomegaly is particularly common when alcohol intake is excessive.

Precipitating Factors

Many patients with PCT have multiple precipitating factors, including mutations of the HFE gene, hepatitis C infection, or exposure to estrogen.[111,113] Excessive alcohol intake is an important precipitating agent, perhaps because of increased hepatic iron deposition in alcoholics. However, certain halogenated hydrocarbons are sometimes implicated. PCT may also develop in people treated with hemodialysis for kidney failure. An outbreak of cutaneous hepatic porphyria in southeast Turkey in 1956 was traced to seed wheat dressed with the fungicide hexachlorobenzene. A neoplastic subgroup has been identified in which PCT is associated with benign or malignant liver tumors.

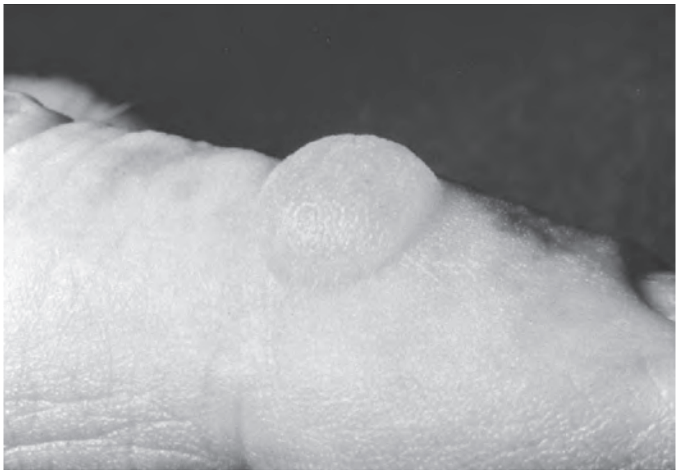


Figure 39.6 A BULLOUS SKIN LESION OF PORPHYRIA CUTANEA TARDA.

Differential Diagnosis

Other causes of bullous or vesicular skin lesions should be excluded, such as a drug reaction (see box on Pseudoporphyria and Renal Dialysis) or chronic renal failure. The distinction between PCT, variegate porphyria, and hereditary coproporphyria rests on biochemical testing of urine and feces, with the highest levels of urinary uroporphyrin found during attacks of PCT (see Table 39.3).

Erythropoietic Protoporphyria and X-Linked Protoporphyria

Biologic and Molecular Aspects

Although not described until 1961, EPP, also known as erythrohepatic protoporphyria, is much more common than CEP. FECH activity is reduced in peripheral blood, liver, bone marrow, and skin, and protoporphyrin is synthesized in excess.[124] Defects in the gene-encoding FECH are the usual cause of EPP; however, a gain-of-function variant in ALAS2, which causes X-linked protoporphyria (XLP),[31] and mutations in the CLPX gene that encodes a mitochondrial protein unfoldase,[125] both cause an identical EPP phenotype. The erythroid progenitor cells (i.e., burst-forming units-erythroid [BFU-E]) in EPP patients show intense fluorescence when viewed under 405-nm light. The gene mutation in EPP shows heterogeneity as in other porphyrias.[126] The last enzyme of the biosynthetic pathway, FECH, is important because its endogenous activity is relatively low, and it could act as a control point in the pathway. FECH ligates iron bound to three cysteine residues in an iron-sulfur cluster,[127] the mutation of which leads to decreased enzyme activity.[128] An oligomeric complex of FECH, ABCB10, and mitoferrin in the mitochondrial membrane would allow for channeling of iron from the cytoplasm to the mitochondrial matrix.[129] Immunologic studies on human protoporphyria show that immunologically reactive FECH is present, but that enzyme activity in three subjects was on average only 17% of normal.[130]

Genetics

EPP inheritance resembles an autosomal dominant disorder with incomplete penetrance; however, the disease usually results from

PSEUDOPORPHYRIA AND RENAL DIALYSIS

The term *pseudoporphyria* has been used to describe a bullous dermatosis associated with a number of dermatologic conditions that bear some resemblance to porphyria.[121] This photosensitivity is often induced by drugs such as the tetracyclines, naproxen, furosemide, voriconazole, oxaprozin, and many others. In these conditions, there is no alteration in porphyrin metabolism or excretion. It is therefore incorrect to name any of them porphyria; the term *pseudoporphyria* should not be applied to them, but only to conditions in which alterations of porphyrin metabolism can be found, such as the bullous dermatosis of hemodialysis

Patients with renal failure can present with many biochemical features of porphyria before hemodialysis. These abnormalities normalize after dialysis, especially when electrolyte abnormalities such as zinc deficiency are also corrected.[122] In a considerable proportion of patients with chronic renal failure, skin changes resembling porphyria cutanea tarda (PCT) develop some months to years after the onset of maintenance hemodialysis. In a minor proportion, genuine PCT can be diagnosed.[123] In such cases, there are elevated total porphyrin levels in plasma and in urine if the patient is not anuric. These patients present a therapeutic dilemma because they are normally anemic and unsuitable for phlebotomy therapy.

inheritance of a FECH null allele together with a low-expression mutation.[131] Haplotype segregation analysis has shown intronic nucleotide polymorphisms in the FECH gene that produces aberrant splicing of mRNA to a form that degrades more rapidly, resulting in enzyme deficiency.[131] Although these disease-associated polymorphisms are common,[132,133] there is heterogeneity of the molecular defect, including aberrant splicing and loss of function of the mitoferrin protein.[124,129,134,135] The erythroid-specific ALAS2 gene resides on the X chromosome, and mutations in exon 11 have been described in X-linked dominant erythropoietic protoporphyria. Recombinant mutants have been shown to increase enzyme activity by two- to threefold, which leads to protoporphyrin overproduction.[136] The XLP genetic variant accounts for 10% of patients with an EPP phenotype in the United States.[137] Five gain of function mutations have been identified in the ALAS2 gene, which is located on the X chromosome.[138] The penetrance and severity of the phenotype in heterozygous females varies due to the pattern of X-chromosome inactivation.[139] In one family, a dominant missense mutation in CLPX has been described that results in marked accumulation of zinc and free protoporphyrin.[125]

Clinical Features

The clinical features are mainly cutaneous on exposure to sunlight and can occur at any age, including infancy and childhood.[140] They include pruritic urticarial swelling and redness of the skin on exposure to sunlight. The most distressing symptom is an unbearable burning sensation on the affected parts. Remarkably, such features are ameliorated during pregnancy, which has been linked to lowered protoporphyrin levels.[141] Hepatic involvement, which occurs in later life, involves deposition of hepatotoxic protoporphyrin in the liver and can lead to fatal liver failure from an active chronic hepatitis with cirrhosis.[142] Such protoporphyrin deposition may also cause cholelithiasis; the gallstones contain high concentrations of protoporphyrin. The liver disease of EPP seems to correlate with erythrocyte protoporphyrin concentrations.[143] Mild microcytic anemia has been reported,[144,145] as well as mitochondrial iron accumulation and ring sideroblasts, in about 30% of patients.[146] Late onset of EPP has been reported in patients with myelodysplastic syndrome or overlap myelodysplasia/myeloproliferation. In one patient, EPP had been acquired as a result of expansion of hemopoietic cells containing only one allele of the FECH gene.[147] Inactivation of one allele by deletion involving chromosome 18 thus appears to be sufficient for overproduction of protoporphyrin.[146,148]

Differential Diagnosis

EPP should be distinguished from other causes of a photosensitive rash. The distinction can be made by demonstrating fluorescence in a proportion of red cells (i.e., fluorocytes) in the peripheral blood and confirmed by measurement of greatly increased erythrocyte and fecal protoporphyrin. Patients with EPP have a relatively high incidence of ring sideroblasts in the marrow.[146] This can lead to diagnostic difficulty because some patients with idiopathic sideroblastic anemia have increased levels of erythrocyte protoporphyrin.[149–152] However, EPP can be distinguished by the autosomal dominant inheritance pattern, dermal photosensitivity, normal or low serum levels of iron, and levels of protoporphyrin in red blood cells and feces.

Congenital Erythropoietic Porphyria (Günther Disease)

Biologic and Molecular Aspects

Congenital erythropoietic porphyria (CEP), or Günther disease, although extremely rare, was the first porphyria to be described in 1874.[153] Unlike the other porphyrias, it is inherited in a mendelian autosomal recessive pattern causing reduced activity of uroporphyrinogen III synthase. The onset of solar photosensitivity results from gross overproduction of porphyrins, caused by deficiency of uroporphyrinogen III synthase. Like other porphyrias, the defective enzyme results mainly from point mutations at multiple sites within the gene.[154] Other enzymes are largely normal, although mutations in the X-linked erythroid transcription factor GATA-binding protein 1 have been reported,[155] and there may be an increase in ALAS activity, which in some cases has been shown to result from gain-of-function mutations in the ALAS2 gene.[156] Excess porphyrins, particularly uroporphyrin-1, accumulate in the normoblasts of the bone marrow and are excreted in the urine and feces. They are also deposited in bones and teeth, resulting in a pink-brown discoloration that fluoresces bright red in light of wavelengths around 400 nm. Dental restoration has been used to correct the esthetic appearance of the teeth. There are frequently profound changes in bone structure in patients with CEP. This has been linked to vitamin D deficiency because of light avoidance.[157] However, bone changes can be seen when vitamin D levels are adequate, and it is reasonable to speculate that the porphyrins deposited in bone are cytotoxic because similar bone changes are features of homozygous variegate porphyria and HEP.[158]

Clinical Features

Typically the onset of CEP is from birth, but occasionally late-onset cases have been reported.[102] The skin reaction is severe and can be devastating, and the teeth become brownish pink because of their high porphyrin content. Severe cutaneous photosensitivity is manifested by blistering of light-exposed areas and fragility of the epidermis. Skin thickening occurs, and there is extensive scarring and hypertrichosis. The recurrent damage associated with scarring on the hand may produce a claw-shaped deformity and loss of digits. Dystrophic nails may curl up and drop off. Lenticular scarring may lead to blindness. Hemolytic anemia often occurs and is associated with increased erythrocyte fragility and splenomegaly. Dyserythropoiesis may contribute to the anemia.[159] One patient who underwent splenectomy at 5 years of age has been described with needle-like inclusions of porphyrin in the circulating red cells (Fig. 39.7).[160]

Differential Diagnosis

The most characteristic feature of CEP is the excess production of series 1 porphyrins rather than series 3 isomer produced in the other porphyrias. Red blood cells fluoresce in ultraviolet light, as do the brown-stained teeth, because of high porphyrin content (see box on Pseudoporphyria and Renal Dialysis).

SIDEROBLASTIC ANEMIAS

Sideroblastic anemias are a heterogeneous group of disorders characterized by anemia of varying severity and diagnosed by finding ring sideroblasts in the bone marrow aspirate. The peripheral blood shows hypochromic red cells, which are microcytic in the hereditary forms (Fig. 39.8A) but are often macrocytic in the acquired forms of the disease. The red blood cell parameters from automated cell counting may show bimodal volume distribution curves or widened range of cell sizes (see Fig. 39.8B); however, this dimorphic size distribution is not always present. Tiny inclusions may be visible in the red blood cells; these can be confirmed as iron-containing Pappenheimer bodies by Prussian blue staining of the blood smear (see Fig. 39.8C). The diagnostic test is bone marrow examination together with Prussian blue staining of the bone marrow smears.

The presence of ring sideroblasts (see Fig. 39.8D) is defined as erythroblasts containing five or more iron-positive (siderotic) granules arranged in a perinuclear collar distribution around one-third or more of the nucleus. Electron microscopic examination has shown that these siderotic granules are mitochondria containing amorphous deposits of ferric phosphate and ferric hydroxide. Iron is also bound to mitochondrial ferritin, a molecular form of ferritin that can be

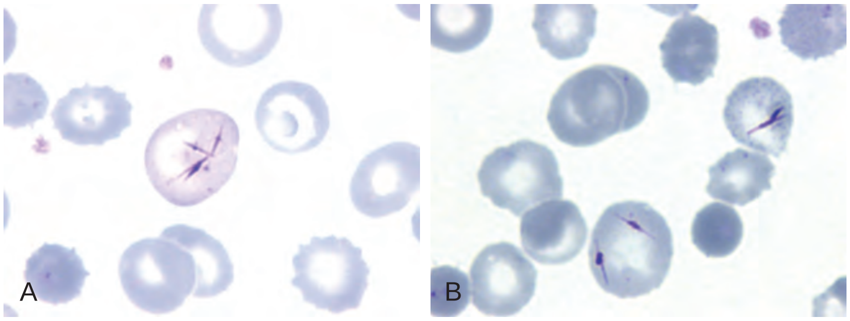


Figure 39.7 NEEDLE-LIKE INCLUSIONS OF PORPHYRIN IN THE CIRCULATING RED CELLS OF A PATIENT WITH CONGENITAL ERYTHROPOIETIC PORPHYRIA AFTER SPLENECTOMY. (A) The red cells contain straight or slightly curved needle-like inclusions that are usually present in a radial orientation but also (B) randomly aligned. *(From Merino A, To-Figueras J, Herrero C. Atypical red cell inclusions in congenital erythropoietic porphyria. Br J Haematol. 2006:132:124.)*

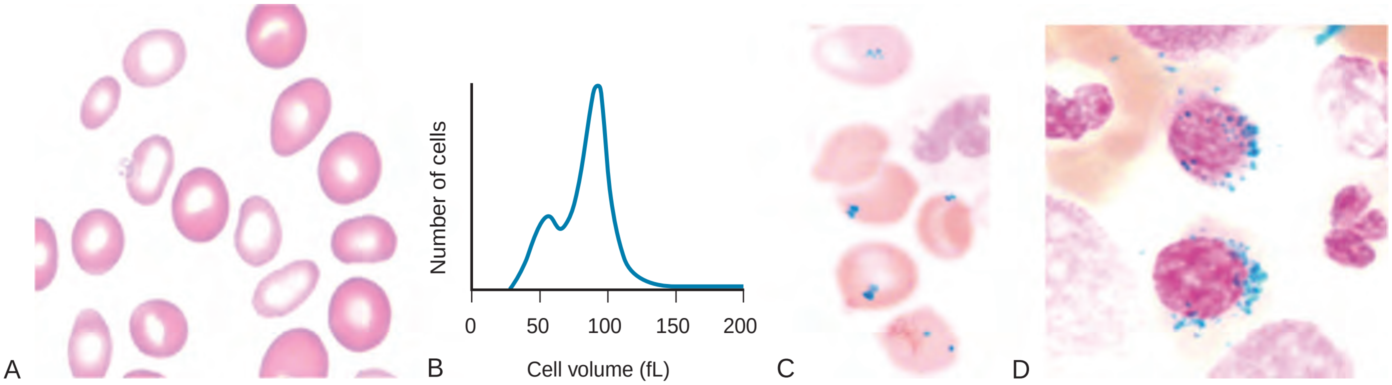


Figure 39.8 (A) Peripheral blood smear from a patient with hereditary sideroblastic anemia shows a population of hypochromic and microcytic erythrocytes. (B) Erythrocyte volume distribution curve of a patient with hereditary sideroblastic anemia. A dimorphic size distribution is evident. (C) Peripheral blood showing Pappenheimer bodies (Prussian blue stain). (D) The bone marrow smear stained with Prussian blue shows ring sideroblasts.

distinguished from cytoplasmic ferritin and that accumulates in large amounts in the erythroblasts of subjects with impaired heme synthesis.[161,162]

Iron overload is a common clinical feature of refractory sideroblastic anemia and, in severe cases, may lead to complications that characterize secondary hemosiderosis (e.g., diabetes, cardiac failure). Marrow examination shows prominent erythroid hyperplasia, which is a sign of the ineffective erythropoiesis and is responsible for increased iron absorption by a mechanism that may involve the erythroid factor erythroferrone suppressing hepcidin production.[163] The sideroblastic anemias have diverse causes but have in common an impaired biosynthesis of heme in the erythroid cells of the marrow. Most sideroblastic anemias are acquired as a clonal disorder of erythropoiesis, with various degrees of myelodysplastic features (Table 39.5). The inherited forms are uncommon and occur predominantly in males with an X-linked pattern of inheritance.[164] A number of drugs have been associated with reversible sideroblastic anemia, and ring sideroblasts may be found in patients who abuse alcohol (see Table 39.5). The first descriptions of ring sideroblasts in association with chronic refractory anemias appeared in the late 1950s,[165,166] after an earlier description of familial X-linked hypochromic microcytic anemia.[167]

Hereditary Sideroblastic Anemia

X-Linked Sideroblastic Anemia

Biologic and Molecular Aspects

Almost 40% of congenital sideroblastic anemias are molecularly unexplained.[164,168] Erythroid cells from patients with X-linked forms of hereditary sideroblastic anemia generally exhibit low activity of ALAS2[169]; however, for a minority of ALAS2 mutations this effect may be difficult to detect in vitro.[164] A defect in this enzyme is firmly established in patients whose anemia responds to pyridoxine therapy, because pyridoxal phosphate is an essential cofactor for ALAS2. However, even affected female patients with moderate anemia unresponsive to pyridoxine have been documented to have low levels of ALAS2 in bone marrow lysates. In some male patients with X-linked pyridoxine-responsive sideroblastic anemia, the low ALAS2 activity in bone marrow increased to levels above the normal range when the patient took pyridoxine supplements and recovered from the anemia.[170] There are several possible explanations for this enhancement of ALAS2 activity by dietary pyridoxine supplements. The most likely is that pyridoxine (or its phosphate) may stabilize the ALAS2 during folding of the mutant enzyme after its synthesis.[169] The gene for the ALAS2 isoenzyme has been localized to the X chromosome, and this gene is known to be the site of most mutations giving rise to X-linked pyridoxine-responsive sideroblastic anemia.[168,171] Approximately 100 different mutations have been identified in individuals or families with hereditary sideroblastic anemia, and nearly all have resulted from single base alterations in DNA.[164] A frequent mutation affects arginine at residue 452 of ALAS2, which occurs in a quarter of all pedigrees but does not affect enzyme activity measured in vitro.[172] All known mutations lie between exons 5 and 11 of ALAS2, the region that codes for the catalytic domain, with most lying within exon 9, which contains the lysine at which binding of pyridoxal 5′-phosphate occurs.[173] A mutation, Asp190Val, has been described in a pyridoxine-refractory patient and appears to affect the proteolytic processing of the ALAS2 during or after import into the mitochondrion.[174] The variety of different mutations in the erythroid ALAS2 gene responsible for X-linked sideroblastic anemia and their pyridoxine responsiveness were reviewed in 2019.[164,168]

TABLE 39.5 **Classification of Sideroblastic Anemias**

Hereditary (Nonsyndromic)
X-linked
Autosomal dominant or recessive
Hereditary (Syndromic)
X-linked sideroblastic anemia with ring sideroblasts and cerebellar ataxia
Myopathy, lactic acidosis, and sideroblastic anemia (MLASA1 and MLASA2)
Pearson syndrome
Mitochondrial respiratory protein mutations (thiamine-responsive megaloblastic anemia, MT-ATP6-SA, NDUFB11-SA)
Sideroblastic anemia with immunodeficiency, fevers, and developmental delay
Acquired
Idiopathic acquired[a] (myelodysplastic syndromes with ring sideroblasts)
Associated with previous chemotherapy, irradiation, or in transition myelodysplasia or myeloproliferative diseases
Drugs
Alcohol
Isoniazid
Chloramphenicol
Other drugs
Rare Causes
Erythropoietic protoporphyria
Copper deficiency or zinc overload
Hypothermia

[a] Trial of pyridoxine indicated.

Genetic Aspects

In most families with hereditary sideroblastic anemia, males are affected with an X-linked pattern of inheritance (Fig. 39.9). However, female carriers, although usually normal, can develop erythrocyte dimorphism or varying degrees of anemia. The assignment of the gene for erythroid ALAS2 to the X chromosome[171] and the many mutations documented in erythroid ALAS2 provide the genetic basis for this X-linked disease. In several families, coinheritance of other X-linked traits (e.g., glucose-6-phosphate dehydrogenase [G6PD] deficiency, ataxia with sideroblastic anemia) has been described.[175,176] Sporadic and familial cases have been described that affect only females, which has been shown to represent skewed X-chromosome inactivation ("unfortunate skewing") affecting the normal allele for the ALAS2 gene.[177] The absence of affected male members in these pedigrees suggests that the ALAS2 defects identified are lethal in hemizygous males.

Clinical and Laboratory Evaluation.

Typically the anemia of X-linked sideroblastic anemia manifests in infancy or childhood, but the milder forms of anemia may not be found until midlife. Even elderly patients have been diagnosed with this anemia.[178] Some cases may be discovered only during family surveys, which should always be undertaken when hereditary sideroblastic anemia is diagnosed. Still other patients may present with features of iron overload, such as diabetes or cardiac failure. Iron overload occurs commonly even with mild anemia and may occasionally be seen with female carriers. Enlargement of the liver and spleen may occur with mild abnormalities of liver function tests.

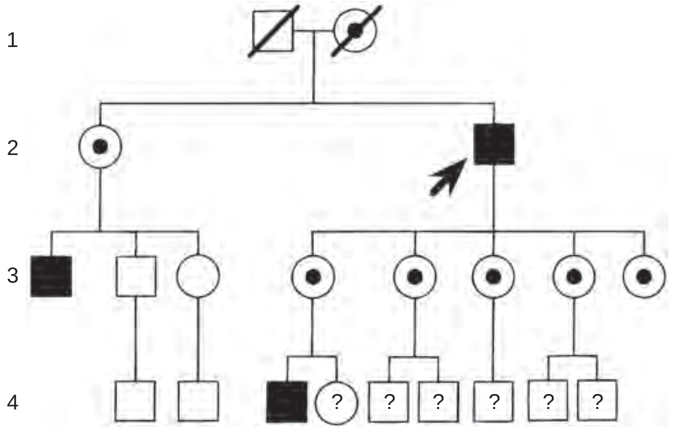


Figure 39.9 PEDIGREE OF A FAMILY WITH PYRIDOXINE-RESPONSIVE SIDEROBLASTIC ANEMIA SHOWING X-LINKED RECESSIVE INHERITANCE. Affected *(filled box)*, carrier *(filled circle within open circle)*, and unknown status *(question mark within circle or box)* are indicated. Diagonal lines indicate deceased members. This pedigree has been abbreviated to show only the affected branches of the family. The arrow indicates the proband.

Anemia is extremely variable, but even when little or no anemia is present, the mean corpuscular volume (MCV) is low, and the red cell volume distribution width may be increased. When anemia is severe, the MCV may be as low as 50 fL (50 μm^3). The blood smear shows a population of cells with hypochromic, microcytic morphology (see Fig. 39.8), which contrasts with the other normochromic, normocytic cells (i.e., dimorphism). Anisocytosis, poikilocytosis, elongated cells, and siderocytes may also be seen. The characteristic erythrocyte dimorphism is most prominent in patients with milder anemia, in female carriers, and in patients in whom pyridoxine has corrected the anemia but not restored the MCV to normal. In some pedigrees with only affected females, macrocytosis may be present, which contrasts with the typical microcytosis of male hemizygotes.[152,177] Leukocyte values are normal, whereas the platelet count is normal or increased.

Serum iron concentration is increased, and transferrin shows an increased percentage of saturation with iron. Serum ferritin levels are invariably increased. Ineffective erythropoiesis can be confirmed by ferrokinetic measurements showing that plasma iron clearance is rapid, with subnormal retention of the iron isotope in erythrocytes after 10 to 14 days. Other features of ineffective erythropoiesis may be variably present: a mild increase in bilirubin concentration, decrease in haptoglobin levels, mild increase in lactate dehydrogenase levels, and normal or slight increase in reticulocyte numbers. The magnitude of iron overload correlates poorly with the degree of anemia in patients who are not transfused. The degree of ineffective erythropoiesis is a better predictor of the amount of iron overload. When ferrokinetics are unavailable, the extent of erythroid hyperplasia relative to normal acts as a rough measure of the magnitude of ineffective erythropoiesis. Several studies have shown that the relative increase in erythroid activity multiplied by the patient's age shows a good correlation with the degree of iron overload as measured by plasma ferritin.[179,180] The iron overload does not result from mutations in the HFE gene (see box on Therapy for Hereditary Sideroblastic Anemia).[181]

Differential Diagnosis

Hereditary sideroblastic anemia should be distinguished from idiopathic hemochromatosis, because both have biochemical evidence of iron overload and a similar tissue pattern of iron deposition. Careful hematologic assessment of patient and family members should make the distinction, because the hemoglobin level and MCV are normal in idiopathic hemochromatosis.

THERAPY FOR HEREDITARY SIDEROBLASTIC ANEMIA

A trial of pyridoxine (100–200 mg/day taken orally) is indicated for 3 months for all patients with hereditary sideroblastic anemia. Response is variable and ranges from complete correction of hemoglobin levels to no effect. Even when pyridoxine completely corrects the anemia (Fig. 39.10), the increase in mean corpuscular volume (MCV) may not reach normal values, and a population of hypochromic, microcytic cells remains.

About 25%–50% of patients with hereditary sideroblastic anemia show a full or partial response to pyridoxine, and this vitamin should be continued on a lifelong basis in the responders. A lower maintenance dose should be determined for each responding patient by progressive dose reduction, because long-term therapy with pyridoxine at 100–200 mg/day has been associated with peripheral neuropathy.[182] The adult nutritional requirement for pyridoxine is 1–2 mg/day; some patients have been maintained on as little as 4 mg/day as a supplement.[170] The anemia of thiamine-responsive megaloblastic anemia usually improves with thiamine 25–75 mg/day.[183] Folic acid supplements should also be administered because the erythroid hyperplasia increases demand for this vitamin.

Allogeneic peripheral blood stem cell transplantation is rarely used in hereditary sideroblastic anemia but has been performed in younger patients.[184,185] Transfusions are the mainstay of treatment for severe anemia unresponsive to pyridoxine. Regular administration of packed red cells using white blood cell filters are given to relieve symptoms and permit normal childhood development. Iron overload and secondary hemosiderosis rapidly progress after transfusions begin; chelation therapy with desferrioxamine or oral deferasirox should be initiated from the onset.

Iron removal may be of great benefit for patients who have mild or moderate anemia and evidence of iron overload.[179,180] These patients can often tolerate intermittent phlebotomy, which in some patients may be preferable to chelation therapy for iron removal, and should be continued to reduce ferritin levels to less than 300 ng/mL. All patients with iron overload should avoid ingestion of ascorbic acid supplements, which enhance iron absorption and increase the tissue toxicity of elemental iron. Alcohol should also be avoided. Splenectomy is contraindicated in this disease.

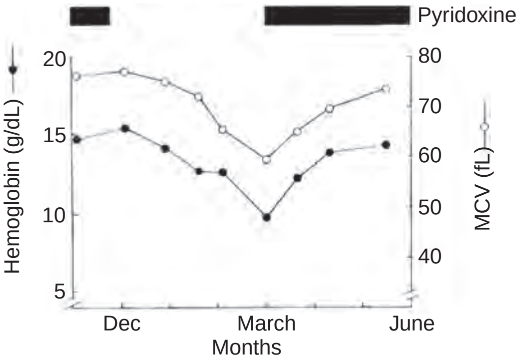


Figure 39.10 RESPONSE OF THE HEMOGLOBIN CONCENTRATION AND MEAN CORPUSCULAR VOLUME (MCV) TO WITHDRAWAL AND REINSTITUTION OF PYRIDOXINE IN A PATIENT WITH RESPONSIVE HEREDITARY SIDEROBLASTIC ANEMIA.

Other Nonsyndromic and Syndromic Hereditary Sideroblastic Anemias

X-linked sideroblastic anemia is considered the most common inherited sideroblastic anemia; however, a number of rare forms have been identified. These consist of nonsyndromic sideroblastic anemias, which cause defects in either heme synthesis or iron-sulfur cluster biogenesis and have similar phenotypes to X-linked sideroblastic anemia, and syndromic forms that cause defective mitochondrial protein synthesis or structure and affect a variety of other tissues in addition to red cells.[164]

Of the nonsyndromic forms, inherited mutations in SLC25A38, the iron-sulfur cluster biogenesis genes, GLRX5 and HSPA9, and FECH have been identified to cause autosomal recessive pyridoxine-refractory sideroblastic anemias.[146,186–188] SLC25A38 is located on chromosome 3p22.1 and encodes a mitochondrial carrier protein that may function to import glycine into the mitochondrion or exchange glycine for 5-aminolevulinate.[188] Homozygous or compound heterozygote mutations in SLC25A38 result in a similar phenotype to that seen in X-linked sideroblastic anemia, with onset in infancy of a severe microcytic anemia that is refractory to treatment with pyridoxine and folic acid. GLRX5 encodes a mitochondrial protein, glutaredoxin 5, which when deleted in the zebrafish mutant shiraz results in defective iron-sulfur cluster assembly and blocked synthesis of heme.[189] A late-onset pyridoxine-refractory sideroblastic anemia caused by homozygous mutation in GLRX5 has been described in a patient who in middle age developed symptoms of a microcytic hypochromic anemia, type 2 diabetes, cirrhosis, and liver iron overload.[187] HSPA9 is a mitochondrial heat shock protein 70 homolog that is involved in transfer of iron-sulfur complexes to mitochondrial glutaredoxin 5. Mutations in HSPA9 cause nonsyndromic microcytic to normocytic sideroblastic anemias.[186]

In addition to genetically defined forms of hereditary sideroblastic anemia, a number of syndromic types have been described, which present with anemia in combination with either muscle, neurologic, or pancreatic tissue involvement. The first of these disorders to be defined by molecular genetics, the Pearson syndrome, is a rare entity that manifests in early infancy with anemia and exocrine pancreatic dysfunction.[190,191] The anemia is normocytic or macrocytic, reticulocyte counts are low, and variable degrees of neutropenia and thrombocytopenia are present. The bone marrow shows striking vacuolation and ring sideroblasts.[191] Although usually fatal, milder forms of the anemia are consistent with survival into adult life. The syndrome, which is related to the Kearns-Sayre syndrome, results from deletions, mutations, or duplications of mitochondrial DNA, variably affecting multiple tissues of the body.[192,193]

A second syndromic congenital sideroblastic anemia, X-linked sideroblastic anemia with cerebellar ataxia (XLSA/A), is a rare mitochondrial disease caused by loss-of-function mutations in the ATP-binding cassette transporter ABCB7.[194–197] ABCB7 is localized to the inner mitochondrial membrane and has been proposed to function as an exporter of mitochondrial iron-sulfur clusters to the cytoplasm[198]; however, a direct role in heme synthesis may arise through an interaction with FECH.[199] Males affected with XLSA/A usually present in infancy with nonprogressive or slowly progressive ataxia and incoordination, which is accompanied by a mild to moderate hypochromic microcytic anemia and the presence of ring sideroblasts on bone marrow examination.

Mutations in the high-affinity thiamine transporter gene SLC19A2, located at 1q24.2, cause the thiamine-responsive megaloblastic anemia (TRMA) syndrome.[200] TRMA has the unusual bone marrow feature of megaloblastic erythroid maturation with ring sideroblasts. TRMA presents with early-onset megaloblastic anemia, diabetes mellitus, and sensorineural deafness, which respond variably to thiamine treatment. Mutations in other mitochondrial respiratory protein genes, including MT-ATP6 and NDUFB11, present in early childhood with lactic acidosis, myopathies, and in the case of MT-ATP6, neurological abnormalities.[201,202]

MLASA1 and MLASA2, are classically characterized by the triad of muscle weakness, lactic acidosis, and normo- to macrocytic anemia. Missense and nonsense mutations in the PUS1 gene coding for pseudouridine synthase-1 cause the rare autosomal recessive disease, myopathy, lactic acidosis, and sideroblastic anemia (MLASA1).[203–205] Mitochondrial and cytoplasmic transfer RNAs (tRNAs) from affected patients lack tRNA pseudouridylation at sites normally modified by PUS1; however, the mechanism by which this affects oxidative phosphorylation and iron metabolism in skeletal muscle and bone marrow are yet to be elucidated.[203] MLASA2 has an identical phenotype caused by a homozygous mutation in the mitochondrial tyrosyl-tRNA synthetase gene, YARS2.[206] The homozygous mutation in YARS2, identified in three patients from two consanguineous Lebanese families, causes defective mitochondrial synthesis and, similar to mutations in PUS1, results in defective oxidative phosphorylation.[206] MLASA1 and MLASA2 usually present with progressive exercise intolerance commencing in childhood followed by later development of sideroblastic anemia, basal lactic acidemia, and mitochondrial myopathy. A biallelic mutation has been described in another mitochondrial protein synthesis gene, LARS2, in a single patient with a related, lethal mitochondrial phenotype that included sideroblastic anemia.[207]

Over a dozen mutations in the TRNT1 gene, which encodes an essential enzyme that transfers the CCA nucleotide repeat to tRNA molecules, result in sideroblastic anemia with B-cell immunodeficiency, periodic fevers, and developmental delay (SIFD).[208,209] This autosomal recessive syndromic disorder manifests as a severe sideroblastic anemia in infancy, recurrent periodic fevers, B-cell lymphopenia, and hypogammaglobulinemia.

Acquired Sideroblastic Anemia

Acquired sideroblastic anemia is categorized within the myelodysplastic syndromes and may appear de novo or occur after chemotherapy or irradiation (see Table 39.5). The clonal nature of hemopoiesis in this condition was first suggested by Dacie et al.[166]

The World Health Organization (WHO) Classification collectively groups myelodysplastic syndromes with ring sideroblasts as “MDS-RS” and distinguishes three categories: MDS with ring sideroblasts and single-lineage dysplasia (MDS-RS-SLD), MDS with ring sideroblasts and multilineage dysplasia (MDS-RS-MLD), and MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T).[210] Many patients with refractory anemia with ring sideroblasts and thrombocytosis have a point mutation in the Janus kinase 2 gene (changing valine-617 to phenylalanine), which is a feature usually associated with the myeloproliferative disorders.[211] This latter group have a clinical phenotype that includes normal MCV, marrow fibrosis, and splenomegaly (see Chapter 61).[211]

Biologic and Molecular Aspects

Clonal hematopoiesis has been demonstrated in acquired idiopathic sideroblastic anemia and in the related myelodysplastic syndromes. Specific evidence was first provided by finding a single G6PD isoenzyme in erythrocytes, granulocytes, platelets, and B lymphocytes in a woman who was heterozygous for G6PD and carried two isoenzymes in her skin and T lymphocytes.[212] This technique is applicable only to the few women who have G6PD heterozygosity, but restriction fragment length polymorphism analysis can be applied to most women using probes directed at other X-chromosome genes, such as that for phosphoglycerate kinase or to an X-linked, variable-copy-number tandem repeat sequence.[213] The results show uniform monoclonality of hematopoiesis in acquired sideroblastic anemia with or without associated myelodysplastic features. Some indirect evidence exists for a primary mitochondrial lesion, perhaps in the mitochondrial respiratory chain, which impairs the reduction of Fe^{3+} because Fe^{2+} is essential for heme synthesis.[214–217] Recurrent mutations in the SF3B1 gene have been described in acquired sideroblastic anemia and are found in 70% to 90% of patients with MDS-RS.[218–220] The product of SF3B1 is associated with mRNA splicing, and mutations in this gene may influence a number of mitochondrial gene networks, including changes in the expression of the iron transporter ABCB7, resulting in iron-laden mitochondria during erythroid development.[221] MDS-RS-T cases have somatic SF3B1 mutations and gain-of-function hematopoietic receptor tyrosine kinase–signaling mutations, most commonly JAK2 valine-617 to phenylalanine, and less commonly other JAK2, MPL, or CALR gene mutations.[222] Genetic mutations in other spliceosome components, including SRSF2, ZRSR2, and PRPF8, are found in MDS-RS lacking SF3B1 mutations[223,224]; however, it is unclear whether these variants play any pathogenic roles in disease development or progression.

Etiology

Clonal chromosomal changes are found in bone marrow cells in approximately 60% of patients with acquired sideroblastic anemia. Characteristic changes are monosomy 7; trisomy 8; deletions involving chromosomes 5, 7, 11, or 20; and a number of balanced translocations.[225] When sideroblastic anemia is acquired after chemotherapy or irradiation, chromosomal changes are usually found and tend to be multiple.[225] Among these changes, the loss of an entire chromosome (5 or 7, or both), deletion of a long arm [del(5), del(7), or del(13)], and an unbalanced translocation are typical.[226,227] When karyotype shows loss of material from chromosomes 5 or 7, or both, a detailed occupational history may show exposure to potentially mutagenic chemical agents in a proportion of patients.[228] However, the development of visible chromosomal changes is probably a late event in acquired sideroblastic anemia and may be preceded by the expansion of a clone of genetically unstable stem cells. This concept is in accord with the view that multiple genetic events underlie the pathogenesis of other myelodysplastic syndromes and acute myeloid leukemia (see box on Clinical and Laboratory Evaluation of Sideroblastic Anemia).[212,229]

Differential Diagnosis

Ring sideroblasts are not limited to acquired sideroblastic anemia; they also occur in other myelodysplastic conditions, such as refractory

CLINICAL AND LABORATORY EVALUATION OF SIDEROBLASTIC ANEMIA

Typically, acquired sideroblastic anemia develops insidiously in a middle-aged or elderly patient with normal or increased mean corpuscular volume (MCV) and a blood smear showing a population of hypochromic red cells. Hepatosplenomegaly may be present. Leukocyte and platelet counts are usually normal, and the presence of a thrombocytosis should prompt investigating for the presence of the JAK2 V617F mutation and MDS-RS-T.[211] If leukopenia or thrombocytopenia is present, a careful search should be made for myelodysplastic features, which lead to the diagnosis of MDS-RS-MLD.[210] An iron stain of the bone marrow aspirate shows ring sideroblasts, which should total 15% or more of all erythroblasts to make the diagnosis of acquired sideroblastic anemia.[210] Iron cannot be assessed in the marrow trephine biopsy core because it may leach out during decalcification.

The bone marrow also shows erythroid hyperplasia. Although mild dyserythropoiesis (e.g., multinuclearity, nuclear budding) and megaloblastoid changes are present, myelopoiesis and megakaryopoiesis are usually normal. When changes are confined to dyserythropoiesis, the condition is defined as MDS-RS-SLD.[210] However, dysplasia of myelopoietic and megakaryopoietic elements may be present (i.e., refractory cytopenia with multilineage dysplasia) with the following features: Pelger-Huët–like anomaly, hypersegmentation or hypogranularity of neutrophils, micromegakaryocytes, large mononuclear megakaryocytes, and megakaryocytes with multiple small nuclei (see Chapter 61). Dysmegakaryopoiesis is more easily detected in trephine biopsies than in marrow smears, although the trephine may also show unsuspected islands of myeloblasts characteristic of myelodysplasia.[230] The overall blast count in marrow smears is, by definition, less than 5%, and the peripheral blood monocyte count is less than 1.0 × 10^9/L. Cytogenetic analysis of marrow aspirates provides important information, because a normal karyotype predicts long survival in any type of acquired sideroblastic anemia.[231]

anemia with excess blasts, in which the blast count is higher than 5%.[210,232] Careful examination of peripheral blood and bone marrow can distinguish acquired idiopathic sideroblastic anemia from these related myelodysplastic conditions. Family surveys are very useful in distinguishing acquired from hereditary forms of sideroblastic anemia, because the latter may present in late adult life.

Prognosis

Using MDS prognostic models, MDS-RS has the most favorable outlook among the myelodysplastic syndromes. The Revised International Prognostic Scoring System (R-IPSS) uses the bone marrow blast percentage, karyotypic analysis, and the presence of peripheral blood cytopenias to group newly diagnosed cases into one of five prognostic groups. Very low-risk patients have a median survival of 8.8 years, low-risk patients 5.3 years, intermediate-risk patients 3 years, high-risk patients 1.6 years, and very high-risk patients 0.8 years.[233] MDS-RS is generally stratified into low-risk groups. For example, in a Mayo Clinic cohort of 48 patients with MDS-RS-SLD using the R-IPSS, 34% were classified as very low, 64% as low, and 2% as intermediate.[234] The median OS for patients with MDS-RS-SLD ranges from 69 to 108 months, with a very low risk for leukemic transformation (<2%).[235] The survival of patients with MDS-RS-MLD is inferior to that of MDS-RS-SLD, but better than that of patients with MDS-MLD.[236] Karyotypic analysis of marrow aspirates provides valuable information, because a normal karyotype carries a more favorable prognosis. Conversely, chromosome 7 abnormalities impart a high probability of transformation to acute myeloid leukemia. Multiple chromosomal abnormalities and del(20q) are also associated with an increased risk for progression to leukemia; in contrast, trisomy 8 has no adverse prognostic significance (see box on Therapy for Acquired Sideroblastic Anemia).[225]

THERAPY FOR ACQUIRED SIDEROBLASTIC ANEMIA

Transfusions are indicated for relief of symptomatic anemia. Erythropoiesis-stimulating agents (ESAs), such as recombinant human erythropoietin (rh-EPO) and darbepoetin, can be used for the treatment of anemia and response rates in lower risk MDS range from 30% to 60%, with the median duration of response being 24 months.[237] Lenalidomide has preferential activity in MDS patients with isolated del(5q) but can also decrease transfusion requirements in MDS-RS-SLD, with 25%–35% of transfusion-dependent patients achieving transfusion independence.[238,239] Furthermore, there is evidence of a synergistic effect of combining lenalidomide with ESAs.[240]

A trial of pyridoxine at 100–200 mg/day for 3 months is worthwhile in patients who have anemia but who do not display neutropenia or thrombocytopenia. However, few patients with acquired idiopathic sideroblastic anemia respond to this vitamin. If any response is achieved, maintenance therapy with pyridoxine at lower dosage is indicated. It is unclear if iron chelation therapy with the oral iron-chelator, deferasirox is of benefit; and until this is established, its routine use is not recommended.

Sotatercept and luspatercept both inhibit molecules in the TGF-β superfamily and increase hemoglobin levels independently of the EPO pathway. Both agents have shown efficacy in reducing transfusion requirements in transfusion-dependent, low-risk MDS-RS patients, and the telomerase inhibitor imetelstat has also shown some activity.[234,241,242] Hypomethylating agents (HMA) such as 5-azacitidine and decitabine can be used in patients that have failed ESA or have developed disease progression, and azacitidine can achieve transfusion independence in up to 40% of patients with lower-risk MDS.[243,244]

Because of the risk of exacerbating anemia, cytoreductive therapy is avoided in MDS-RS-T unless there is a high risk for thrombosis. Hydroxyurea can be used to reduce the platelet count and has been shown to reduce the risk of thrombotic events.[245]

SIDEROBLASTIC ANEMIA AND PORPHYRINURIA CAUSED BY DRUGS

Alcohol

Ring sideroblasts may be found in the bone marrow of malnourished anemic alcoholics, usually in the presence of associated folate deficiency.[246–248] In contrast, binge drinking or chronic alcohol ingestion in subjects with good nutrition is not associated with sideroblastic abnormality. Sideroblastic change is never the sole cause for the anemia of alcoholism. Alcohol has a direct toxic effect on hematopoiesis.[249] An increased or high-normal MCV and vacuolation of red blood cell precursors is often seen in addition to the ring sideroblast abnormality. Red blood cells show dimorphic morphology; evidence in the marrow of folate deficiency is present in half of cases.[249] Transferrin saturation and marrow iron stores tend to be increased but may be low if gastrointestinal bleeding is present. The ring sideroblasts gradually disappear over 4 to 12 days when alcohol is withdrawn[246]; during this period, there may be a rebound erythroid hyperplasia, reticulocytosis, and thrombocytosis. Folic acid should be given for the associated megaloblastic changes after blood is taken for vitamin B_{12} and folate assays.

Alcohol consumption lowers the plasma concentration of pyridoxal phosphate, a cofactor for ALAS2, needed in the first step in heme synthesis.[250] Conversion of ethanol to acetaldehyde is necessary for this effect, and acetaldehyde acts by accelerating the degradation of intracellular pyridoxal phosphate in the liver, lowering plasma levels of this coenzyme.[251]

Chronic alcoholics have an altered heme metabolism with increased urinary excretion of coproporphyrin, mainly isomer III, but normal urinary excretion of uroporphyrin, ALA, and PBG. Acute and chronic ethanol ingestion markedly depresses the activity of ALA dehydratase in peripheral blood. Ethanol administration to normal subjects results in increased activity of leukocyte ALAS and erythrocyte PBGD, the two rate-controlling enzymes of the pathway. The activities of each of the other four enzymes are depressed. FECH, the enzyme that inserts iron into protoporphyrin to form heme, shows the most marked depression, and in alcoholism there is prolonged depression of uroporphyrinogen decarboxylase, which provides a rationale for the role of ethanol in the etiology of PCT.[252,253] As expressed earlier, ethanol is a major precipitating factor in acute porphyria.[254]

Isoniazid

Administration of the antituberculous drug isoniazid has occasionally been associated with development of a sideroblastic anemia after 1 to 10 months of therapy. The anemia is hypochromic and microcytic, with a dimorphic blood smear and ring sideroblasts in the marrow. This complication is thought to occur only in slow acetylators of isoniazid, allowing this drug to react nonenzymatically with pyridoxal and to form a hydrazone that is rapidly excreted in the urine. The anemia can be fully reversed by coadministration of pyridoxine (25 to 50 mg/day) with isoniazid or by withdrawing isoniazid.[255] Another antituberculous drug, pyrazinamide, may also cause a sideroblastic anemia, which is caused by inhibition of ALAS2 and responds to pyridoxine therapy.[256]

Chloramphenicol

Chloramphenicol is an antibiotic that produces a reversible suppression of erythropoiesis after several days of therapy (plasma levels of 10 to 15 µg/mL). This effect is predictable and separate from the rare idiosyncratic side effect of aplastic anemia in approximately 1 of 20,000 exposed persons. Nearly all patients given chloramphenicol (>2 g/day) develop vacuolation of the erythroid precursors and ring sideroblasts. These effects are thought to arise from suppression of mitochondrial respiration. Chloramphenicol inhibits mitochondrial protein synthesis and reduces cytochrome *a*, a_3, and *b* levels.[257] Serum iron concentrations are increased, and reticulocyte numbers are subnormal; these changes revert on stopping the antibiotic.

Other Drugs

A reversible acquired sideroblastic anemia has been described with penicillamine, linezolid, and with the use of triethylene tetramine hydrochloride, a copper-chelating agent used in the treatment of Wilson disease.[258,259] Acquired sideroblastic anemia has also been precipitated by progesterone given to a patient on two separate occasions 15 years apart, and this anemia promptly reversed on withdrawal of the drug.[260]

PRESENTATIONS ASSOCIATED WITH SIDEROBLASTIC ANEMIA OR PORPHYRINURIA

Copper Deficiency or Zinc Overload

The copper content of a Western diet averages 0.9 to 1.6 mg each day, which is only a few times greater than the amount needed to maintain homeostasis of this essential element.[261] Copper deficiency has been described in malnourished premature infants,[262] in patients receiving long-term parenteral or enteral hyperalimentation,[263] after gastrectomy,[264] with copper-chelating agents,[258] or on an idiopathic basis.[265] The syndrome of copper deficiency consists of sideroblastic anemia with hypochromic cells in the blood smear, accompanied by ring sideroblasts and vacuolated erythroid and myeloid precursors in the marrow, and of neutropenia with an absence of late myeloid forms in the marrow (Fig. 39.11). In some reports, patients present with neurologic symptoms such as paresthesias, weakness, or ataxia; and demyelination is seen on the magnetic resonance image of the brain.[265] In infants, additional features may be seen, such as osteoporosis and long bone changes, depigmentation of skin and hair, and central nervous system abnormalities. The platelet counts remain normal. Serum copper and ceruloplasmin levels are low, whereas serum iron and transferrin saturation levels are normal. The serum zinc concentration may be increased.[265] Prompt reversal of the hematologic changes follows therapy with 2 to 5 mg/day of copper sulfate taken orally or 100 to 500 μg/day of copper supplement to the intravenous alimentation formula.

Large quantities of ingested zinc interfere with copper absorption and produce the neutropenia and sideroblastic anemia characteristic of copper deficiency.[266] Zinc sulfate is freely available from health food stores, and as little as 450 mg/day for 2 years is sufficient for this effect. Sideroblastic anemia has also been ascribed to zinc toxicity arising from the ingestion of coins over a period of many years.[267] Serum zinc levels are high, whereas serum copper and ceruloplasmin levels are low. Zinc must be discontinued for 9 to 12 weeks for full reversal of the anemia and neutropenia.

Iron Deficiency Anemia

In iron deficiency anemia, there is an accumulation of protoporphyrin in erythrocytes that rarely reaches the level found in EPP. The zinc complex of protoporphyrin is produced because FECH uses Zn^{2+} during iron-deficient erythropoiesis.[268] Erythrocyte protoporphyrin may be raised before changes appear in peripheral blood and may be helpful in diagnosing iron deficiency when serum iron and ferritin levels are rising as a result of patients having started iron therapy. In iron-deficient erythropoiesis, erythroid ALAS activity is reduced below normal.[269]

Hypothermia

Thrombocytopenia, erythroid hypoplasia, and ring sideroblasts have been described in patients with hypothermia associated with neurologic disease.[270] These changes reverse slowly as body temperature returns to normal.

Other Conditions

In hereditary tyrosinemia, excess urinary ALA is excreted because ALA dehydratase is inhibited by succinyl acetone. Like acute porphyria and lead poisoning, this disease is associated with neurobehavioral disturbance (see box on Lead Poisoning). In liver disease, there may be

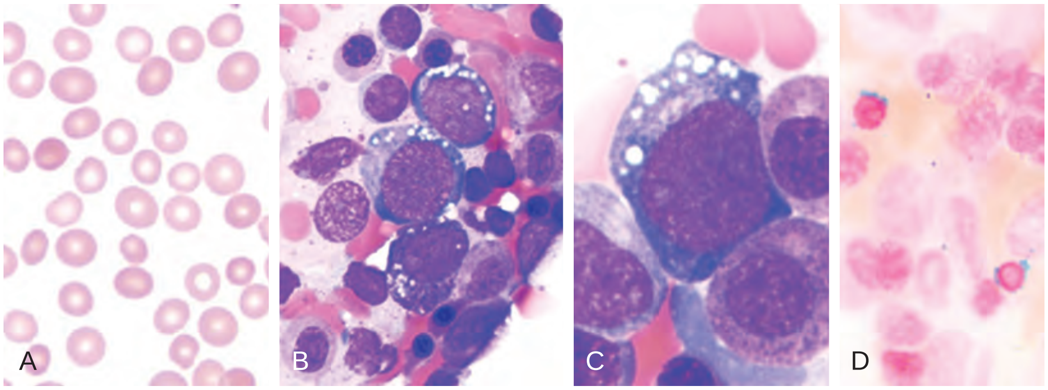


Figure 39.11 This 70-year-old man was being treated with zinc supplementation and was found to have anemia and neutropenia (hemoglobin, 8.3 g/dL; hematocrit, 23.9%; and white blood cell count, 1200/μL). His peripheral smear (A) showed a biphasic erythroid population with some small slightly hypochromic cells and increased anisocytosis (red blood cell distribution width [RDW], 23.9%). The bone marrow aspirate showed a left shift in granulopoiesis with vacuolization of immature granulocytic and erythroid precursors (B, C). A Prussian blue–stained aspirate revealed ring sideroblasts (D). The patient's copper level was less than 0.1 μg/mL (normal reference range, 0.75 to 1.45 μg/mL).

LEAD POISONING

It has been known for some time that patients suffering from lead poisoning have an accumulation of protoporphyrin in erythrocytes and increased urinary excretion of ALA and coproporphyrin.[274] There are sex-related differences in the porphyrin synthetic response to lead, with females showing a more profound coproporphyrinuria than men.[275] The elevated protoporphyrin chelated by zinc is retained in the erythrocyte, which may explain the absence of photosensitivity. This accumulation of porphyrins and precursors is caused by the inhibition by lead of the heme biosynthetic enzymes: 5-aminolevulinate (ALA) dehydratase, coproporphyrinogen oxidase, and ferrochelatase. An increase in the activity of the rate-controlling enzyme ALA synthase (ALAS) results.

Many of the clinical manifestations of lead poisoning may be the result of altered heme biosynthesis.[82] A mild to moderate anemia that can be hypochromic and microcytic occurs in a minority of patients, whereas basophilic stippling is prominent because of inhibition of pyrimidine 5′-nucleotidase in the maturing reticulocyte. Ring sideroblasts have not been reported. The abdominal pain, constipation, and peripheral neuropathy that occur in lead poisoning are also seen in acute attacks of hepatic porphyria. Neuropathy, seen in lead poisoning, may also be the result of disorders of heme biosynthesis, as in the porphyrias.[274] Alterations in porphyrin metabolism have provided a useful means of detecting and assessing the severity of lead exposure and poisoning. The diminution in activity of erythrocyte ALA dehydratase and elevated erythrocyte protoporphyrin levels are the most sensitive measures.

increased urinary excretion of coproporphyrin, predominantly isomer I. In the Dubin-Johnson syndrome, which is caused by mutations in the ABCC2 gene, the ratio of coproporphyrin isomer I to isomer III is markedly increased in the urine (>80%), possibly as a result of deficiency of hepatic uroporphyrinogen III cosynthase and increased activity of PBGD.[271] In Rotor syndrome, total urinary excretion of coproporphyrin is markedly increased and consists predominantly of coproporphyrin isomer I.[272] In the unconjugated hyperbilirubinemia of Gilbert syndrome, depressed activity of protoporphyrinogen oxidase and increased activity of ALAS has been found in peripheral leukocytes.[273]

Environmental Intolerances

It has been hypothesized that several otherwise-unexplained chemical-associated illnesses, such as multiple chemical sensitivity syndrome, may represent mild chronic cases of porphyria or other acquired abnormalities in heme synthesis. However, evidence for this concept is lacking.[275,276]

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Bergmann AK, Campagna DR, McLoughlin EM, et al. Systematic molecular genetic analysis of congenital sideroblastic anemia: evidence for genetic heterogeneity and identification of novel mutations. *Pediatr Blood Cancer*. 2010;54:273.

Bishop DF, Tchaikovskii V, Nazarenko I, et al. Molecular expression and characterization of erythroid-specific 5-aminolevulinate synthase gain-of-function mutations causing X-linked protoporphyria. *Mol Med*. 2013;19:18.

Bissell DM, Anderson KE, Bonkovsky HL. Porphyria. *N Engl J Med*. 2017;377:862.

Bottomley SS, Fleming MD. Sideroblastic anemia: diagnosis and management. *Hematol Oncol Clin North Am*. 2014;28:653–670.

Campagna DR, de Bie CI, Schmitz-Abe K, et al. X-linked sideroblastic anemia due to ALAS2 intron 1 enhancer element GATA-binding site mutations. *Am J Hematol*. 2014;89(3):315–319.

Chakraborty PK, Schmitz-Abe K, Kennedy EK, et al. Mutations in TRNT1 cause congenital sideroblastic anemia with immunodeficiency, fevers, and developmental delay (SIFD). *Blood*. 2014;124:2867.

Chen W, Dailey HA, Paw BH. Ferrochelatase forms an oligomeric complex with mitoferrin-1 and Abcb10 for erythroid heme biosynthesis. *Blood*. 2010;116:628.

Ducamp S, Fleming MD. The molecular genetics of sideroblastic anemia. *Blood*. 2019;133:59.

Ducamp S, Kannengiesser C, Touati M, et al. Sideroblastic anemia: molecular analysis of the ALAS2 gene in a series of 29 probands and functional studies of 10 missense mutations. *Hum Mutat*. 2011;32:590.

Donker AE, Raymakers RA, Nieuwenhuis HK, et al. Practice guidelines for the diagnosis and management of microcytic anemias due to genetic disorders of iron metabolism or heme synthesis. *Blood*. 2014;123:3873.

Furuyama K, Harigae H, Heller T, et al. Arg-452 substitution of the erythroid-specific 5-aminolaevulinate synthase, a hot spot mutation in X-linked sideroblastic anaemia, does not itself affect enzyme activity. *Eur J Haematol*. 2006;76:33.

Goodwin RG, Kell J, Laidler P, et al. Photosensitivity and acute liver injury in myeloproliferative disorder secondary to late-onset protoporphyria caused by deletion of a ferrochelatase gene in hematopoietic cells. *Blood*. 2006;107:60.

Guernsey DL, Jiang H, Campagna DR, et al. Mutations in mitochondrial carrier family gene SLC25A38 cause nonsyndromic autosomal recessive congenital sideroblastic anemia. *Nat Genet*. 2009;41:651.

Harigae H, Furuyama K. Hereditary sideroblastic anemia: pathophysiology and gene mutations. *Int J Hematol*. 2010;92(3):425–431.

Katugampola RP, Anstey AV, Finlay AY, et al. A management algorithm for congenital erythropoietic porphyria derived from a study of 29 cases. *Br J Dermatol*. 2012;167(4):888–900.

Lin CS, Krishnan AV, Lee MJ, et al. Nerve function and dysfunction in acute intermittent porphyria. *Brain*. 2008;131:2510.

Maitra D, Bragazzi Cunha J, Elenbaas JS, et al. Porphyrin-induced protein oxidation and aggregation as a mechanism of porphyria-associated cell injury. *Cell Mol Gastroenterol Hepatol*. 2019;8:535.

Malcovati L, Cazzola M. Refractory anemia with ring sideroblasts. *Best Pract Res Clin Haematol*. 2013;26:377.

Phillips JD, Bergonia HA, Reilly CA, et al. A porphomethene inhibitor of uroporphyrinogen decarboxylase causes porphyria cutanea tarda. *Proc Natl Acad Sci USA*. 2007;104:5079.

Philpott CC. The flux of iron through ferritin in erythrocyte development. *Curr Opin Hematol*. 2018;25:183.

Pondarre C, Campagna DR, Antioches B, et al. Abcb7, the gene responsible for X-linked sideroblastic anemia with ataxia, is essential for hematopoiesis. *Blood*. 2007;109:3567.

Puy H, Gouya L, Deybach JC. Porphyrias. *Lancet*. 2010;375:924.

Rand EB, Bunin N, Cochran W, et al. Sequential liver and bone marrow transplantation for treatment of erythropoietic protoporphyria. *Pediatrics*. 2006;118:1896.

Richardson DR, Lane DJ, Becker EM, et al. Mitochondrial iron trafficking and the integration of iron metabolism between the mitochondrion and cytosol. *Proc Natl Acad Sci USA*. 2010;107:10775.

Schultz IJ, Chen C, Paw BH, et al. Iron and porphyrin trafficking in heme biogenesis. *J Biol Chem*. 2010;285:26753.

Swenson SA, Moore CM, Marcero JR, et al. From synthesis to utilization: the ins and outs of mitochondrial heme. *Cells*. 2020:9.

Szpurka H, Tiu R, Murugesan G, et al. Refractory anemia with ringed sideroblasts associated with marked thrombocytosis (RARS-T), another myeloproliferative condition characterized by JAK2 V617F mutation. *Blood*. 2006;108:2173.

Thunell S, Pomp E, Brun A. Guide to drug porphyrogenicity prediction and drug prescription in the acute porphyrias. *Br J Clin Pharmacol*. 2007;64:668.

Yasuda M, Chen B, Desnick RJ. Recent advances on porphyria genetics: inheritance, penetrance and molecular heterogeneity, including new modifying/causative genes. *Mol Genet Metab*. 2019;128:320.

Ye H, Jeong SY, Ghosh MC, et al. Glutaredoxin 5 deficiency causes sideroblastic anemia by specifically impairing heme biosynthesis and depleting cytosolic iron in human erythroblasts. *J Clin Invest*. 2010;120:1749.

REFERENCES

1. Doss MO. Porphyrinurias and occupational disease. *Ann N Y Acad Sci*. 1987;514:204.
2. Arnold WN. The illness of Vincent van Gogh. *J Hist Neurosci*. 2004;13:22.
3. Loftus LS, Arnold WN. Vincent van Gogh's illness: acute intermittent porphyria? *Br J Haem*. 1991;303:1589.
4. Chiabrando D, Mercurio S, Tolosano E. Heme and erythropoieis: more than a structural role. *Haematologica*. 2014;99:973.
5. Yasuda M, Chen B, Desnick RJ. Recent advances on porphyria genetics: inheritance, penetrance and molecular heterogeneity, including new modifying/causative genes. *Mol Genet Metab*. 2019;128:320.
6. Maitra D, Bragazzi Cunha J, Elenbaas JS, et al. Porphyrin-induced protein oxidation and aggregation as a mechanism of porphyria-associated cell injury. *Cell Mol Gastroenterol Hepatol*. 2019;8:535.
7. Bissell DM, Anderson KE, Bonkovsky HL. Porphyria. *N Engl J Med*. 2017;377:862.
8. Philpott CC. The flux of iron through ferritin in erythrocyte development. *Curr Opin Hematol*. 2018;25:183.
9. Swenson SA, Moore CM, Marcero JR, et al. From synthesis to utilization: the ins and outs of mitochondrial heme. *Cells*. 2020;9:579.
10. Cox TC, Bawden MJ, Martin A, et al. Human erythroid 5-aminolevulinate synthase: promoter analysis and identification of an iron-responsive element in the mRNA. *EMBO J*. 1991;10:1891.
11. Tian Q, Li T, Hou W, et al. Lon peptidase 1 (LONP1)-dependent breakdown of mitochondrial 5-aminolevulinic acid synthase protein by heme in human liver cells. *J Biol Chem*. 2011;286:26424.
12. Granick JL, Sassa S. Hemin control of heme biosynthesis in mouse Friend virus-transformed erythroleukemia cells in culture. *J Biol Chem*. 1978;253:5402.
13. Conboy JG, Cox TC, Bottomley SS, et al. Human erythroid 5-aminolevulinate synthase. Gene structure and species-specific differences in alternative RNA splicing. *J Biol Chem*. 1992;267:18753.
14. Furuyama K, Sassa S. Interaction between succinyl CoA synthetase and the heme-biosynthetic enzyme ALAS-E is disrupted in sideroblastic anemia. *J Clin Invest*. 2000;105:757.
15. Handschin C, Lin J, Rhee J, et al. Nutritional regulation of hepatic heme biosynthesis and porphyria through PGC-1alpha. *Cell*. 2005;122:505.
16. Phillips JD, Kushner JP. Fast track to the porphyrias. *Nat Med*. 2005;11:1049.
17. Lathrop JT, Timko MP. Regulation by heme of mitochondrial protein transport through a conserved amino acid motif. *Science*. 1993;259:522.
18. Shaw GC, Cope JJ, Li L, et al. Mitoferrin is essential for erythroid iron assimilation. *Nature*. 2006;440:96.
19. Chen W, Paradkar PN, Li L, et al. ABCB10 physically interacts with mitoferrin-1 (SLC25a37) to enhance its stability and function in the erythroid mitochondria. *Proc Natl Acad Sci USA*. 2009;106:16263.
20. Hentze MW, Muckenthaler MU, Andrews NC. Balancing acts: molecular control of mammalian iron metabolism. *Cell*. 2004;117:285.
21. Muckenthaler M, Gray NK, Hentze MW. IRP-1 binding to ferritin mRNA prevents the recruitment of the small ribosomal subunit by the cap-binding complex eIF4F. *Mol Cell*. 1998;2:383.
22. Hentze MW, Kuhn LC. Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. *Proc Natl Acad Sci USA*. 1996;93:8175.
23. Salahudeen AA, Thompson JW, Ruiz JC, et al. An E3 ligase possessing an iron-responsive hemerythrin domain is a regulator of iron homeostasis. *Science*. 2009;326:722.
24. Vashisht AA, Zumbrennen KB, Huang X, et al. Control of iron homeostasis by an iron-regulated ubiquitin ligase. *Science*. 2009;326:718.
25. Grandchamp B, De Verneuil H, Beaumont C, et al. Tissue-specific expression of porphobilinogen deaminase. Two isoenzymes from a single gene. *Eur J Biochem*. 1987;162:105.
26. Sassa S. Hematologic aspects of the porphyrias. *Int J Hematol*. 2000;71:1.
27. Shoolingin-Jordan PM. Porphobilinogen deaminase and uroporphyrinogen III synthase: structure, molecular biology, and mechanism. *J Bioenerg Biomembr*. 1995;27:181.
28. Chen B, Solis-Villa C, Erwin AL, et al. Identification and characterization of 40 novel hydroxymethylbilane synthase mutations that cause acute intermittent porphyria. *J Inherit Metab Dis*. 2019;42:186.
29. Mgone CS, Lanyon WG, Moore MR, et al. Detection of a high mutation frequency in exon 12 of the porphobilinogen deaminase gene in patients with acute intermittent porphyria. *Hum Genet*. 1993;92:619.
30. Gill R, Kolstoe SE, Mohammed F, et al. Structure of human porphobilinogen deaminase at 2.8A: the molecular basis of acute intermittent porphyria. *Biochem J*. 2009;420:17.
31. Whatley SD, Ducamp S, Gouya L, et al. C-terminal deletions in the ALAS2 gene lead to gain of function and cause X-linked dominant protoporphyria without anemia or iron overload. *Am J Hum Genet*. 2008;83:408.
32. Meissner P. Enzyme studies in variegate porphyria [thesis], University of Cape Town, South Africa; 1990.
33. Brodie MJ, Moore MR, Goldberg A. Enzyme abnormalities in the porphyrias. *Lancet*. 1977;2:699.
34. Woolf J, Marsden JT, Degg T, et al. Best practice guidelines on first-line laboratory testing for porphyria. *Ann Clin Biochem*. 2017;54:188.
35. Hindmarsh JT, Oliveras L, Greenway DC. Plasma porphyrins in the porphyrias. *Clin Chem*. 1999;45:1070.
36. Macours P, Cotton F. Improvement in HPLC separation of porphyrin isomers and application to biochemical diagnosis of porphyrias. *Clin Chem Lab Med*. 2006;44:1433.
37. Lin CN, Huang YC, Ro LS, et al. Validation and evaluation of two porphobilinogen deaminase activity assays for diagnosis of acute intermittent porphyria. *Clin Chim Acta*. 2018;479:1.
38. Blake D, Poulos V, Rossi R. Diagnosis of porphyria-recommended methods for peripheral laboratories. *Clin Biochem Rev*. 1992;3:S1.
39. Lin CS, Krishnan AV, Lee MJ, et al. Nerve function and dysfunction in acute intermittent porphyria. *Brain*. 2008;131:2510.
40. Emanuelli T, Pagel FW, Porciuncula LO, et al. Effects of 5-aminolevulinic acid on the glutamatergic neurotransmission. *Neurochem Int*. 2003;42:115.
41. Felitsyn N, McLeod C, Shroads AL, et al. The heme precursor delta-aminolevulinate blocks peripheral myelin formation. *J Neurochem*. 2008;106:2068.
42. Monteiro HP, Bechara EJ, Abdalla DS. Free radicals involvement in neurological porphyrias and lead poisoning. *Mol Cell Biochem*. 1991;103:73.
43. Thunell S, Andersson C, Carlmark B, et al. Markers for vulnerability in acute porphyria. A hypothesis paper. *Eur J Clin Chem Clin Biochem*. 1995;33:179.
44. Costa CA, Trivelato GC, Pinto AM, et al. Correlation between plasma 5-aminolevulinic acid concentrations and indicators of oxidative stress in lead-exposed workers. *Clin Chem*. 1997;43:1196.
45. Bermudez Moretti M, Correa Garcia S, Ramos E, et al. delta-Aminolevulinic acid uptake is mediated by the gamma-aminobutyric acid-specific permease UGA4. *Cell Mol Biol (Noisy-Le-Grand)*. 1996;42:519.
46. McColl KE, Thompson GG, Moore MR, et al. Chester porphyria: biochemical studies of a new form of acute porphyria. *Lancet*. 1985;2:796.
47. Poblete-Gutierrez P, Wiederholt T, Martinez-Mir A, et al. Demystification of Chester porphyria: a nonsense mutation in the porphobilinogen deaminase gene. *Physiol Res*. 55(suppl 2):S137.
48. Meissner PN, Corrigall AV, Hift RJ. Fifty years of porphyria at the University of Cape Town. *S Afr Med J*. 2012;102:422.
49. Meissner PN, Dailey TA, Hift RJ, et al. A R59W mutation in human protoporphyrinogen oxidase results in decreased enzyme activity and is prevalent in South Africans with variegate porphyria. *Nat Genet*. 1996;13:95.
50. van Tuyll van Serooskerken AM, Drogemoller BI, Te Velde K, et al. Extended haplotype studies in South African and Dutch variegate porphyria families carrying the recurrent p.R59W mutation confirm a common ancestry. *Br J Dermatol*. 2012;166:261.
51. Day RS, Eales L, Meissner D. Coexistent variegate porphyria and porphyria cutanea tarda. *N Engl J Med*. 1982;307:36.
52. Akagi R, Inoue R, Muranaka S, et al. Dual gene defects involving delta-aminolaevulinate dehydratase and coproporphyrinogen oxidase in a porphyria patient. *Br J Haematol*. 2006;132:237.
53. Nordmann Y, Amram D, Deybach JC, et al. Coexistent hereditary coproporphyria and congenital erythropoietic porphyria (Gunther disease). *J Inherit Metab Dis*. 1990;13:687.
54. Poblete-Gutierrez P, Badeloe S, Wiederholt T, et al. Dual porphyrias revisited. *Exp Dermatol*. 2006;15:685.
55. Whatley SD, Roberts AG, Elder GH. De-novo mutation and sporadic presentation of acute intermittent porphyria. *Lancet*. 1995;346:1007.
56. Stine OC, Smith KD. The estimation of selection coefficients in Afrikaners: Huntington disease, porphyria variegata, and lipoid proteinosis. *Am J Hum Genet*. 1990;46:452.
57. Balwani M, Singh P, Seth A, et al. Acute intermittent porphyria in children: a case report and review of the literature. *Mol Genet Metab*. 2016;119:295.
58. Stein PE, Badminton MN, Barth JH, et al. Acute intermittent porphyria: fatal complications of treatment. *Clin Med*. 2012;12:293.
59. Stewart MF. Review of hepatocellular cancer, hypertension and renal impairment as late complications of acute porphyria and recommendations for patient follow-up. *J Clin Pathol*. 2012;65:976.
60. Baravelli CM, Sandberg S, Aarsand AK, et al. Acute hepatic porphyria and cancer risk: a nationwide cohort study. *J Intern Med*. 2017;282:229.
61. Marsden JT, Rees DC. A retrospective analysis of outcome of pregnancy in patients with acute porphyria. *J Inherit Metab Dis*. 2010;33:591.

62. Hift RJ, Meissner PN. Moore MR. Porphyria: a toxicogenetic disease. In: Kadish KM, Smith KM, Guilard R, eds. *The Porphyrin Handbook, Vol 14: Medical Aspects of Porphyrins*. San Diego: Academic Press; 2003.
63. Thunell S, Pomp E, Brun A. Guide to drug porphyrogenicity prediction and drug prescription in the acute porphyrias. *Br J Clin Pharmacol*. 2007;64:668.
64. Duque-Serrano L, Patarroyo-Rodriguez L, Gotlib D, et al. Psychiatric aspects of acute porphyria: a comprehensive review. *Curr Psychiatry Rep*. 2018;20:5.
65. Millward LM, Kelly P, Deacon A, et al. Self-rated psychosocial consequences and quality of life in the acute porphyrias. *J Inherit Metab Dis*. 2001;24:733.
66. Guerrero RB, Kloke KM, Salazar D. Inborn errors of metabolism and the gastrointestinal tract. *Gastroenterol Clin North Am*. 2019;48:183.
67. Nia S. Psychiatric signs and symptoms in treatable inborn errors of metabolism. *J Neurol*. 2014;261(suppl 2):S559.
68. Hindmarsh JT, Oliveras L, Greenway DC. Biochemical differentiation of the porphyrias. *Clin Biochem*. 1999;32:609.
69. Lamoril J, Puy H, Whatley SD, et al. Characterization of mutations in the CPO gene in British patients demonstrates absence of genotype-phenotype correlation and identifies relationship between hereditary coproporphyria and harderoporphyria. *Am J Hum Genet*. 2001;68:1130.
70. Cacheux V, Martasek P, Fougerousse F, et al. Localization of the human coproporphyrinogen oxidase gene to chromosome band 3q12. *Hum Genet*. 1994;94:557, 1994.
71. Allen KR, Whatley SD, Degg TJ, et al. Hereditary coproporphyria: comparison of molecular and biochemical investigations in a large family. *J Inherit Metab Dis*. 2005;28:779.
72. Schmitt C, Gouya L, Malonova E, et al. Mutations in human CPO gene predict clinical expression of either hepatic hereditary coproporphyria or erythropoietic harderoporphyria. *Hum Mol Genet*. 2005;14:3089.
73. Logan GM, Weimer MK, Ellefson M, et al. Bile porphyrin analysis in the evaluation of variegate porphyria. *N Engl J Med*. 1991;324:1408.
74. Herrick AL, Moore MR, Thompson GG, et al. Cholelithiasis in patients with variegate porphyria. *J Hepatol*. 1991;12:50.
75. Meissner P. Enzyme studies in variegate porphyria [thesis], University of Cape Town, South Africa; 1990.
76. Moore MR, Hift RJ. Drugs in the acute porphyrias–toxicogenetic diseases. *Cell Mol Biol (Noisy-Le-Grand)*. 1997;43:89.
77. Qin X, Tan Y, Wang L, et al. Structural insight into human variegate porphyria disease. *FASEB J*. 2011;25:653.
78. Wiman A, Harper P, Floderus Y. Nine novel mutations in the protoporphyrinogen oxidase gene in Swedish families with variegate porphyria. *Clin Genet*. 2003;64:122.
79. Gross U, Sassa S, Jacob K, et al. 5-Aminolevulinic acid dehydratase deficiency porphyria: a twenty-year clinical and biochemical follow-up. *Clin Chem*. 1998;44:1892.
80. Ishida N, Fujita H, Fukuda Y, et al. Cloning and expression of the defective genes from a patient with delta-aminolevulinate dehydratase porphyria. *J Clin Invest*. 1992;89:1431.
81. van Tuyll van Serooskerken AM, de Rooij FW, Edixhoven A, et al. Digenic inheritance of mutations in the coproporphyrinogen oxidase and protoporphyrinogen oxidase genes in a unique type of porphyria. *J Invest Dermatol*. 2011;131:2249.
82. Moore MR, Goldberg A, Yeung-Laiwah AA. Lead effects on the heme biosynthetic pathway. Relationship to toxicity. *Ann N Y Acad Sci*. 1987;514:191.
83. Watson CJ, Cardinal RA, Bossenmaier I, et al. Porphyria variegata and porphyria cutanea tarda in siblings: chemical and genetic aspects. *Proc Natl Acad Sci USA*. 1975;72:5126.
84. Anderson KE, Collins S. Open-label study of hemin for acute porphyria: clinical practice implications. *Am J Med*. 2006;119:801.
85. Dover SB, Moore MR, Fitzsimmons EJ, et al. Tin protoporphyrin prolongs the biochemical remission produced by heme arginate in acute hepatic porphyria. *Gastroenterology*. 1993;105:500.
86. Frei P, Minder EI, Corti N, et al. Liver transplantation because of acute liver failure due to heme arginate overdose in a patient with acute intermittent porphyria. *Case Rep Gastroenterol*. 2012;6:190.
87. Dowman JK, Gunson BK, Mirza DF, et al. Liver transplantation for acute intermittent porphyria is complicated by a high rate of hepatic artery thrombosis. *Liver Transpl*. 2012;18:195.
88. Herrick AL, McColl KE, Wallace AM, et al. LHRH analogue treatment for the prevention of premenstrual attacks of acute porphyria. *Q J Med*. 1990;75:355.
89. Cable EE, Pepe JA, Donohue SE, et al. Effects of mifepristone (RU-486) on heme metabolism and cytochromes P-450 in cultured chick embryo liver cells, possible implications for acute porphyria. *Eur J Biochem*. 1994;225:651.
90. Poh-Fitzpatrick MB. Porphyrin-sensitized cutaneous photosensitivity: pathogenesis and treatment. *Clin Dermatol*. 1985;3:41.
91. Lim HW. Pathophysiology of cutaneous lesions in porphyrias. *Semin Hematol*. 1989;26:114.
92. Singal AK, Venkata KVR, Jampana S, et al. Hepatitis C treatment in patients with porphyria cutanea tarda. *Am J Med Sci*. 2017;353:523.
93. Combalia A, To-Figueras J, Laguno M, et al. Direct-acting antivirals for hepatitis C virus induce a rapid clinical and biochemical remission of porphyria cutanea tarda. *Br J Dermatol*. 2017;177:e183.
94. Fujita Y, Sato-Matsumura KC. Effective treatment for porphyria cutanea tarda with oral cimetidine. *J Dermatol*. 2010;37:677.
95. Ratner M. Patients with porphyria bask in sunlight of FDA approval. *Nat Biotechnol*. 2019;37:1390.
96. Biolcati G, Marchesini E, Sorge F, et al. Long-term observational study of afamelanotide in 115 patients with erythropoietic protoporphyria. *Br J Dermatol*. 2015;172:1601–1612.
97. Harms J, Lautenschlager S, Minder CE, et al. An alpha-melanocyte-stimulating hormone analogue in erythropoietic protoporphyria. *N Engl J Med*. 2009;360:306.
98. Dowman JK, Gunson BK, Mirza DF, et al. UK experience of liver transplantation for erythropoietic protoporphyria. *J Inherit Metab Dis*. 2011;34:539.
99. Singal AK, Parker C, Bowden C, et al. Liver transplantation in the management of porphyria. *Hepatology*. 2014;60:1082.
100. Katugampola RP, Anstey AV, Finlay AY, et al. A management algorithm for congenital erythropoietic porphyria derived from a study of 29 cases. *Br J Dermatol*. 2012;167:888.
101. Katugampola RP, Badminton MN, Finlay AY, et al. Congenital erythropoietic porphyria: a single-observer clinical study of 29 cases. *Br J Dermatol*. 2012;167:901.
102. Rank JM, Straka JG, Weimer MK, et al. Hematin therapy in late onset congenital erythropoietic porphyria. *Br J Haematol*. 1990;75:617.
103. Egan DN, Yang Z, Phillips J, et al. Inducing iron deficiency improves erythropoiesis and photosensitivity in congenital erythropoietic porphyria. *Blood*. 2015;126:257.
104. Blouin JM, Duchartre Y, Costet P, et al. Therapeutic potential of proteasome inhibitors in congenital erythropoietic porphyria. *Proc Natl Acad Sci USA*. 2013;110:18238.
105. Bedel A, Taillepierre M, Guyonnet-Duperat V, et al. Metabolic correction of congenital erythropoietic porphyria with iPSCs free of reprogramming factors. *Am J Hum Genet*. 2012;91:109.
106. de Verneuil H, Beaumont C, Deybach JC, et al. Enzymatic and immunological studies of uroporphyrinogen decarboxylase in familial porphyria cutanea tarda and hepatoerythropoietic porphyria. *Am J Hum Genet*. 1984;36:613.
107. Phillips JD, Bergonia HA, Reilly CA, et al. A porphomethene inhibitor of uroporphyrinogen decarboxylase causes porphyria cutanea tarda. *Proc Natl Acad Sci USA*. 2007;104:5079.
108. Greaves P, Clothier B, Davies R, et al. Uroporphyria and hepatic carcinogenesis induced by polychlorinated biphenyls-iron interaction: absence in the Cyp1a2(−/−) knockout mouse. *Biochem Biophys Res Commun*. 2005;331:147.
109. Smith AG, Carthew P, Clothier B, et al. Synergy of iron in the toxicity and carcinogenicity of polychlorinated biphenyls (PCBs) and related chemicals. *Toxicol Lett*. 1995:82–83.
110. Bulaj ZJ, Phillips JD, Ajioka RS, et al. Hemochromatosis genes and other factors contributing to the pathogenesis of porphyria cutanea tarda. *Blood*. 2000;95:1565.
111. Roberts AG, Whatley SD, Morgan RR, et al. Increased frequency of the haemochromatosis Cys282Tyr mutation in sporadic porphyria cutanea tarda. *Lancet*. 1997;349:321.
112. Santos M, Clevers HC, Marx JJ. Mutations of the hereditary hemochromatosis candidate gene HLA-H in porphyria cutanea tarda. *N Engl J Med*. 1997;336:1327.
113. Munoz-Santos C, Guilabert A, Moreno N, et al. Familial and sporadic porphyria cutanea tarda: clinical and biochemical features and risk factors in 152 patients. *Medicine (Baltimore)*. 2010;89:69.
114. Blauvelt A. Hepatitis C virus and human immunodeficiency virus infection can alter porphyrin metabolism and lead to porphyria cutanea tarda. *Arch Dermatol*. 1996;132:1503.
115. Enriquez de Salamanca R, Sanchez-Perez J, Diaz-Mora F, et al. Porphyria cutanea tarda associated with HIV infection: are those conditions pathogenetically related or merely coincidental? *AIDS*. 1990;4:926.
116. Smith SG. Hepatoerythropoietic porphyria. *Semin Dermatol*. 1986;5:125.
117. McManus JF, Begley CG, Sassa S, et al. Five new mutations in the uroporphyrinogen decarboxylase gene identified in families with cutaneous porphyria. *Blood*. 1996;88:3589.

118. Moran-Jimenez MJ, Ged C, Romana M, et al. Uroporphyrinogen decarboxylase: complete human gene sequence and molecular study of three families with hepatoerythropoietic porphyria. *Am J Hum Genet*. 1996;58:712.
119. Phillips JD, Parker TL, Schubert HL, et al. Functional consequences of naturally occurring mutations in human uroporphyrinogen decarboxylase. *Blood*. 2001;98:3179.
120. Koszo F, Morvay M, Dobozy A, et al. Erythrocyte uroporphyrinogen decarboxylase activity in 80 unrelated patients with porphyria cutanea tarda. *Br J Dermatol*. 1992;126:446.
121. Poh-Fitzpatrick MB. Porphyria, pseudoporphyria, pseudopseudoporphyria …? *Arch Dermatol*. 1986;122:403.
122. Seubert S, Seubert A, Rumpf KW, et al. A porphyria cutanea tarda-like distribution pattern of porphyrins in plasma, hemodialysate, hemofiltrate, and urine of patients on chronic hemodialysis. *J Invest Dermatol*. 1985;85:107.
123. Guolo M, Stella AM, Melito V, et al. Altered 5-aminolevulinic acid metabolism leading to pseudoporphyria in hemodialysed patients. *Int J Biochem Cell Biol*. 1996;28:311.
124. Goerz G, Bunselmeyer S, Bolsen K, et al. Ferrochelatase activities in patients with erythropoietic protoporphyria and their families. *Br J Dermatol*. 1996;134:880.
125. Yien YY, Ducamp S, van der Vorm LN, et al. Mutation in human CLPX elevates levels of delta-aminolevulinate synthase and protoporphyrin IX to promote erythropoietic protoporphyria. *Proc Natl Acad Sci USA*. 2017;114:E8045.
126. Balwani M, Doheny D, Bishop DF, et al. Loss-of-function ferrochelatase and gain-of-function erythroid-specific 5-aminolevulinate synthase mutations causing erythropoietic protoporphyria and x-linked protoporphyria in North American patients reveal novel mutations and a high prevalence of X-linked protoporphyria. *Mol Med*. 2013;19:26.
127. Crouse BR, Sellers VM, Finnegan MG, et al. Site-directed mutagenesis and spectroscopic characterization of human ferrochelatase: identification of residues coordinating the [2Fe–2S] cluster. *Biochemistry*. 1996;35:16222.
128. Schneider-Yin X, Gouya L, Dorsey M, et al. Mutations in the iron-sulfur cluster ligands of the human ferrochelatase lead to erythropoietic protoporphyria. *Blood*. 2000;96:1545.
129. Chen W, Dailey HA, Paw BH. Ferrochelatase forms an oligomeric complex with mitoferrin-1 and Abcb10 for erythroid heme biosynthesis. *Blood*. 2010;116:628.
130. Straka JG, Hill HD, Krikava JM, et al. Immunochemical studies of ferrochelatase protein: characterization of the normal and mutant protein in bovine and human protoporphyria. *Am J Hum Genet*. 1991;48:72.
131. Gouya L, Puy H, Robreau AM, et al. The penetrance of dominant erythropoietic protoporphyria is modulated by expression of wildtype FECH. *Nat Genet*. 2002;30:27.
132. Bishop DF, Schneider-Yin X, Clavero S, et al. Congenital erythropoietic porphyria: a novel uroporphyrinogen III synthase branchpoint mutation reveals underlying wild-type alternatively spliced transcripts. *Blood*. 2010;115:1062.
133. Nakano H, Nakano A, Toyomaki Y, et al. Novel ferrochelatase mutations in Japanese patients with erythropoietic protoporphyria: high frequency of the splice site modulator IVS3–48C polymorphism in the Japanese population. *J Invest Dermatol*. 2006;126:2717.
134. Norris PG, Nunn AV, Hawk JL, et al. Genetic heterogeneity in erythropoietic protoporphyria: a study of the enzymatic defect in nine affected families. *J Invest Dermatol*. 1990;95:260.
135. Wang X, Poh-Fitzpatrick M, Taketani S, et al. Screening for ferrochelatase mutations: molecular heterogeneity of erythropoietic protoporphyria. *Biochim Biophys Acta*. 1994;1225:187.
136. Bishop DF, Tchaikovskii V, Nazarenko I, et al. Molecular expression and characterization of erythroid-specific 5-aminolevulinate synthase gain-of-function mutations causing X-linked protoporphyria. *Mol Med*. 2013;19:18.
137. Balwani M, Naik H, Anderson KE, et al. Clinical, biochemical, and genetic characterization of North American patients with erythropoietic protoporphyria and X-linked protoporphyria. *JAMA Dermatol*. 2017;153:789.
138. Fratz EJ, Clayton J, Hunter GA, et al. Human erythroid 5-aminolevulinate synthase mutations associated with X-linked protoporphyria disrupt the conformational equilibrium and enhance product release. *Biochemistry*. 2015;54:5617.
139. Brancaleoni V, Balwani M, Granata F, et al. X-chromosomal inactivation directly influences the phenotypic manifestation of X-linked protoporphyria. *Clin Genet*. 2016;89:20.
140. Todd DJ. Erythropoietic protoporphyria. *Br J Dermatol*. 1994;131:751.
141. Poh-Fitzpatrick MB. Human protoporphyria: reduced cutaneous photosensitivity and lower erythrocyte porphyrin levels during pregnancy. *J Am Acad Dermatol*. 1997;36:40.
142. Doss MO, Frank M, Sieg I. [Cholestatic erythrohepatic protoporphyria: porphyrin metabolism before and after liver transplantation]. *Z Gastroenterol*. 1993;31(suppl 2):85.
143. Bloomer JR. The liver in protoporphyria. *Hepatology*. 1988;8:402.
144. Bottomley SS, Tanaka M, Everett MA. Diminished erythroid ferrochelatase activity in protoporphyria. *J Lab Clin Med*. 1975;86:126.
145. Mathews-Roth MM. Letter: Anemia in erythropoietic protoporphyria. *JAMA*. 1974;230:824.
146. Rademakers LH, Koningsberger JC, Sorber CW, et al. Accumulation of iron in erythroblasts of patients with erythropoietic protoporphyria. *Eur J Clin Invest*. 1993;23:130.
147. Sarkany RP, Ross G, Willis F. Acquired erythropoietic protoporphyria as a result of myelodysplasia causing loss of chromosome 18. *Br J Dermatol*. 2006;155:464.
148. Goodwin RG, Kell WJ, Laidler P, et al. Photosensitivity and acute liver injury in myeloproliferative disorder secondary to late-onset protoporphyria caused by deletion of a ferrochelatase gene in hematopoietic cells. *Blood*. 2006;107:60.
149. Romslo I, Brun A, Sandberg S, et al. Sideroblastic anemia with markedly increased free erythrocyte protoporphyrin without dermal photosensitivity. *Blood*. 1982;59:628.
150. Rothstein G, Lee R, Cartwright GE. Sideroblastic anemia with dermal photosensitivity and greatly increased erythrocyte protoporphyrin. *N Engl J Med*. 1969;280:587.
151. Scott AJ, Ansford AJ, Webster BH, et al. Erythropoietic protoporphyria with features of a sideroblastic anaemia terminating in liver failure. *Am J Med*. 1973;54:251.
152. Tuckfield A, Ratnaike S, Hussein S, et al. A novel form of hereditary sideroblastic anaemia with macrocytosis. *Br J Haematol*. 1997;97:279.
153. Schultz JH. Ein Fall von Pemphigus Leprosus kompliziert durch Lepra Visceralis [inaugural dissertation]. Germany, Griefswald; 1874.
154. Di Pierro E, Brancaleoni V, Granata F. Advances in understanding the pathogenesis of congenital erythropoietic porphyria. *Br J Haematol*. 2016;173:365.
155. Di Pierro E, Russo R, Karakas Z, et al. Congenital erythropoietic porphyria linked to GATA1-R216W mutation: challenges for diagnosis. *Eur J Haematol*. 2015;94:491.
156. To-Figueras J, Ducamp S, Clayton J, et al. ALAS2 acts as a modifier gene in patients with congenital erythropoietic porphyria. *Blood*. 2011;118:1443.
157. Pullon HW, Bellingham AJ, Humphreys S, et al. The osteodystrophy of congenital erythropoietic porphyria. *Bone*. 1991;12:89.
158. Hift RJ, Meissner PN, Todd G, et al. Homozygous variegate porphyria: an evolving clinical syndrome. *Postgrad Med J*. 1993;69:781.
159. Pimstone NR, Gandhi SN, Mukerji SK. Therapeutic efficacy of oral charcoal in congenital erythropoietic porphyria. *N Engl J Med*. 1987;316:390.
160. Merino A, To-Figueras J, Herrero C. Atypical red cell inclusions in congenital erythropoietic porphyria. *Br J Haematol*. 2006;132:124.
161. Cazzola M, Invernizzi R, Bergamaschi G, et al. Mitochondrial ferritin expression in erythroid cells from patients with sideroblastic anemia. *Blood*. 2003;101:1996.
162. Levi S, Corsi B, Bosisio M, et al. A human mitochondrial ferritin encoded by an intronless gene. *J Biol Chem*. 2001;276:24437.
163. Kautz L, Jung G, Valore EV, et al. Identification of erythroferrone as an erythroid regulator of iron metabolism. *Nat Genet*. 2014;46:678.
164. Ducamp S, Fleming MD. The molecular genetics of sideroblastic anemia. *Blood*. 2019;133:59.
165. Bjorkman SE. Chronic refractory anemia with sideroblastic bone marrow; a study of four cases. *Blood*. 1956;11:250.
166. Dacie JV, Smith MD, White JC, et al. Refractory normoblastic anaemia: a clinical and haematological study of seven cases. *Br J Haematol*. 1959;5:26.
167. Cooley TB. A severe type of hereditary anemia with elliptocytosis. Interesting sequence of splenectomy. *Am J Med Sci*. 1945;209:561.
168. Fujiwara T, Harigae H. Molecular pathophysiology and genetic mutations in congenital sideroblastic anemia. *Free Radic Biol Med*. 2019;133:179.
169. Bottomley SS, Healy HM, Brandenburg MA, et al. 5-Aminolevulinate synthase in sideroblastic anemias: mRNA and enzyme activity levels in bone marrow cells. *Am J Hematol*. 1992;41:76.
170. Cox TC, Bottomley SS, Wiley JS, et al. X-linked pyridoxine-responsive sideroblastic anemia due to a Thr388-to-Ser substitution in erythroid 5-aminolevulinate synthase. *N Engl J Med*. 1994;330:675.
171. Cox TC, Bawden MJ, Abraham NG, et al. Erythroid 5-aminolevulinate synthase is located on the X chromosome. *Am J Hum Genet*. 1990;46:107.
172. Furuyama K, Harigae H, Heller T, et al. Arg452 substitution of the erythroid-specific 5-aminolaevulinate synthase, a hot spot mutation in X-linked sideroblastic anaemia, does not itself affect enzyme activity. *Eur J Haematol*. 2006;76:33.

173. Bottomley SS. Congenital sideroblastic anemias. *Curr Hematol Rep*. 2006;5:41.
174. Furuyama K, Fujita H, Nagai T, et al. Pyridoxine refractory X-linked sideroblastic anemia caused by a point mutation in the erythroid 5-aminolevulinate synthase gene. *Blood*. 1997;90:822.
175. Prasad AS, Tranchida L, Konno ET, et al. Hereditary sideroblastic anemia and glucose-6-phosphate dehydrogenase deficiency in a Negro family. *J Clin Invest*. 1968;47:1415.
176. Raskind WH, Wijsman E, Pagon RA, et al. X-linked sideroblastic anemia and ataxia: linkage to phosphoglycerate kinase at Xq13. *Am J Hum Genet*. 1991;48:335.
177. Aivado M, Gattermann N, Rong A, et al. X-linked sideroblastic anemia associated with a novel ALAS2 mutation and unfortunate skewed X-chromosome inactivation patterns. *Blood Cells Mol Dis*. 2006;37:40.
178. Cotter PD, May A, Fitzsimons EJ, et al. Late-onset X-linked sideroblastic anemia. Missense mutations in the erythroid delta-aminolevulinate synthase (ALAS2) gene in two pyridoxine-responsive patients initially diagnosed with acquired refractory anemia and ringed sideroblasts. *J Clin Invest*. 1995;96:2090.
179. Cazzola M, Barosi G, Bergamaschi G, et al. Iron loading in congenital dyserythropoietic anaemias and congenital sideroblastic anaemias. *Br J Haematol*. 1983;54:649.
180. Peto TE, Pippard MJ, Weatherall DJ. Iron overload in mild sideroblastic anaemias. *Lancet*. 1983;1:375.
181. Bottomley SS. The hemochromatosis of sideroblastic and other non-thalassemic anemias. In: Barton JC, Edwards CQ, eds. *Haemochromatosis: Genetics, Pathophysiology, Diagnosis and Treatment*. Cambridge, UK: Cambridge University Press; 2000.
182. Parry GJ, Bredesen DE. Sensory neuropathy with low-dose pyridoxine. *Neurology*. 1985;35:1466.
183. Bergmann AK, Sahai I, Falcone JF, et al. Thiamine-responsive megaloblastic anemia: identification of novel compound heterozygotes and mutation update. *J Pediatr*. 2009;155:888.
184. Imataki O, Uchida S, Uemura M, et al. Graft failure after reduced-intensity stem cell transplantation for congenital sideroblastic anemia. *Pediatr Hematol Oncol*. 2019;36:46.
185. Gonzalez MI, Caballero D, Vazquez L, et al. Allogeneic peripheral stem cell transplantation in a case of hereditary sideroblastic anaemia. *Br J Haematol*. 2000;109:658.
186. Schmitz-Abe K, Ciesielski SJ, Schmidt PJ, et al. Congenital sideroblastic anemia due to mutations in the mitochondrial HSP70 homologue HSPA9. *Blood*. 2015;126:2734.
187. Camaschella C, Campanella A, De Falco L, et al. The human counterpart of zebrafish shiraz shows sideroblastic-like microcytic anemia and iron overload. *Blood*. 2007;110:1353.
188. Guernsey DL, Jiang H, Campagna DR, et al. Mutations in mitochondrial carrier family gene SLC25A38 cause nonsyndromic autosomal recessive congenital sideroblastic anemia. *Nat Genet*. 2009;41:651.
189. Wingert RA, Galloway JL, Barut B, et al. Deficiency of glutaredoxin 5 reveals Fe–S clusters are required for vertebrate haem synthesis. *Nature*. 2005;436:1035.
190. Gagne KE, Ghazvinian R, Yuan D, et al. Pearson marrow pancreas syndrome in patients suspected to have Diamond-Blackfan anemia. *Blood*. 2014;124:437.
191. Pearson HA, Lobel JS, Kocoshis SA, et al. A new syndrome of refractory sideroblastic anemia with vacuolization of marrow precursors and exocrine pancreatic dysfunction. *J Pediatr*. 1979;95:976.
192. McShane MA, Hammans SR, Sweeney M, et al. Pearson syndrome and mitochondrial encephalomyopathy in a patient with a deletion of mtDNA. *Am J Hum Genet*. 1991;48:39.
193. Rotig A, Cormier V, Blanche S, et al. Pearson's marrow-pancreas syndrome. A multisystem mitochondrial disorder in infancy. *J Clin Invest*. 1990;86:1601.
194. Allikmets R, Raskind WH, Hutchinson A, et al. Mutation of a putative mitochondrial iron transporter gene (ABC7) in X-linked sideroblastic anemia and ataxia (XLSA/A). *Hum Mol Genet*. 1999;8:743.
195. Bekri S, Kispal G, Lange H, et al. Human ABC7 transporter: gene structure and mutation causing X-linked sideroblastic anemia with ataxia with disruption of cytosolic iron-sulfur protein maturation. *Blood*. 2000;96:3256.
196. Pondarre C, Antiochos BB, Campagna DR, et al. The mitochondrial ATP-binding cassette transporter ABCB7 is essential in mice and participates in cytosolic iron-sulfur cluster biogenesis. *Hum Mol Genet*. 2006;15:953.
197. D'Hooghe M, Selleslag D, Mortier G, et al. X-linked sideroblastic anemia and ataxia: a new family with identification of a fourth ABCB7 gene mutation. *Eur J Paediatr Neurol*. 2012;16:730.
198. Pondarre C, Campagna DR, Antiochos B, et al. ABCB7, the gene responsible for X-linked sideroblastic anemia with ataxia, is essential for hematopoiesis. *Blood*. 2007;109:3567.
199. Taketani S, Kakimoto K, Ueta H, et al. Involvement of ABC7 in the biosynthesis of heme in erythroid cells: interaction of ABC7 with ferrochelatase. *Blood*. 2003;101:3274.
200. Labay V, Raz T, Baron D, et al. Mutations in SLC19A2 cause thiamine-responsive megaloblastic anaemia associated with diabetes mellitus and deafness. *Nat Genet*. 1999;22:300.
201. Lichtenstein DA, Crispin AW, Sendamarai AK, et al. A recurring mutation in the respiratory complex 1 protein NDUFB11 is responsible for a novel form of X-linked sideroblastic anemia. *Blood*. 2016;128:1913.
202. Berhe S, Heeney MM, Campagna DR, et al. Recurrent heteroplasmy for the MT-ATP6 p.Ser148Asn (m.8969G>A) mutation in patients with syndromic congenital sideroblastic anemia of variable clinical severity. *Haematologica*. 2018;103:e561.
203. Patton JR, Bykhovskaya Y, Mengesha E, et al. Mitochondrial myopathy and sideroblastic anemia (MLASA): missense mutation in the pseudouridine synthase 1 (PUS1) gene is associated with the loss of tRNA pseudouridylation. *J Biol Chem*. 2005;280:19823.
204. Taylor RW, Pyle A, Griffin H, et al. Use of whole-exome sequencing to determine the genetic basis of multiple mitochondrial respiratory chain complex deficiencies. *JAMA*. 2014;312:68.
205. Bykhovskaya Y, Casas K, Mengesha E, et al. Missense mutation in pseudouridine synthase 1 (PUS1) causes mitochondrial myopathy and sideroblastic anemia (MLASA). *Am J Hum Genet*. 2004;74:1303.
206. Riley LG, Cooper S, Hickey P, et al. Mutation of the mitochondrial tyrosyl-tRNA synthetase gene, YARS2, causes myopathy, lactic acidosis, and sideroblastic anemia—MLASA syndrome. *Am J Hum Genet*. 2010;87:52.
207. Riley LG, Rudinger-Thirion J, Schmitz-Abe K, et al. LARS2 variants associated with hydrops, lactic acidosis, sideroblastic anemia, and multisystem failure. *JIMD Rep*. 2016;28:49.
208. Chakraborty PK, Schmitz-Abe K, Kennedy EK, et al. Mutations in TRNT1 cause congenital sideroblastic anemia with immunodeficiency, fevers, and developmental delay (SIFD). *Blood*. 2014;124:2867.
209. Wiseman DH, May A, Jolles S, et al. A novel syndrome of congenital sideroblastic anemia, B-cell immunodeficiency, periodic fevers, and developmental delay (SIFD). *Blood*. 2013;122:112.
210. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127:2391.
211. Szpurka H, Tiu R, Murugesan G, et al. Refractory anemia with ringed sideroblasts associated with marked thrombocytosis (RARS-T), another myeloproliferative condition characterized by JAK2 V617F mutation. *Blood*. 2006;108:2173.
212. Raskind WH, Tirumali N, Jacobson R, et al. Evidence for a multistep pathogenesis of a myelodysplastic syndrome. *Blood*. 1984;63:1318.
213. Young NS. The problem of clonality in aplastic anemia: Dr Dameshek's riddle, restated. *Blood*. 1992;79:1385.
214. Aoki Y. Multiple enzymatic defects in mitochondria in hematological cells of patients with primary sideroblastic anemia. *J Clin Invest*. 1980;66:43.
215. Gattermann N, Retzlaff S, Wang YL, et al. A heteroplasmic point mutation of mitochondrial tRNALeu(CUN) in non-lymphoid haemopoietic cell lineages from a patient with acquired idiopathic sideroblastic anaemia. *Br J Haematol*. 1996;93:845.
216. Gattermann N, Retzlaff S, Wang YL, et al. Heteroplasmic point mutations of mitochondrial DNA affecting subunit I of cytochrome c oxidase in two patients with acquired idiopathic sideroblastic anemia. *Blood*. 1997;90:4961.
217. Wulfert M, Kupper AC, Tapprich C, et al. Analysis of mitochondrial DNA in 104 patients with myelodysplastic syndromes. *Exp Hematol*. 2008;36:577.
218. Papaemmanuil E, Cazzola M, Boultwood J, et al. Somatic SF3B1 mutation in myelodysplasia with ring sideroblasts. *N Engl J Med*. 2011;365:1384.
219. Broseus J, Alpermann T, Wulfert M, et al. Age, JAK2(V617F) and SF3B1 mutations are the main predicting factors for survival in refractory anaemia with ring sideroblasts and marked thrombocytosis. *Leukemia*. 2013;27:1826.
220. Janusz K, Del Rey M, Abaigar M, et al. A two-step approach for sequencing spliceosome-related genes as a complementary diagnostic assay in MDS patients with ringed sideroblasts. *Leuk Res*. 2017;56:82.
221. Nikpour M, Scharenberg C, Liu A, et al. The transporter ABCB7 is a mediator of the phenotype of acquired refractory anemia with ring sideroblasts. *Leukemia*. 2013;27:889.
222. Patnaik MM, Tefferi A. Refractory anemia with ring sideroblasts (RARS) and RARS with thrombocytosis (RARS-T): 2017 update on diagnosis, risk-stratification, and management. *Am J Hematol*. 2017;92:297.
223. Yoshida K, Sanada M, Shiraishi Y, et al. Frequent pathway mutations of splicing machinery in myelodysplasia. *Nature*. 2011;478:64.
224. Kurtovic-Kozaric A, Przychodzen B, Singh J, et al. PRPF8 defects cause missplicing in myeloid malignancies. *Leukemia*. 2015;29:126.

225. Third MIC Cooperative Study Group. Recommendations for a morphologic, immunological, and cytogenetics (MIC) working classification of the primary and therapy-related myelodysplastic disorders. *Cancer Genet Cytogenet*. 1998;32.
226. Neuman WL, Rubin CM, Rios RB, et al. Chromosomal loss and deletion are the most common mechanisms for loss of heterozygosity from chromosomes 5 and 7 in malignant myeloid disorders. *Blood*. 1992;79:1501.
227. Pedersen-Bjergaard J, Pedersen M, Roulston D, et al. Different genetic pathways in leukemogenesis for patients presenting with therapy-related myelodysplasia and therapy-related acute myeloid leukemia. *Blood*. 1995;86:3542.
228. Golomb HM, Alimena G, Rowley JD, et al. Correlation of occupation and karyotype in adults with acute nonlymphocytic leukemia. *Blood*. 1982;60:404.
229. Gale RE, Wheadon H, Goldstone AH, et al. Frequency of clonal remission in acute myeloid leukaemia. *Lancet*. 1993;341:138.
230. Tricot G, De Wolf-Peeters C, Hendrickx B, et al. Bone marrow histology in myelodysplastic syndromes. I. Histological findings in myelodysplastic syndromes and comparison with bone marrow smears. *Br J Haematol*. 1984;57:423.
231. Malcovati L, Porta MG, Pascutto C, et al. Prognostic factors and life expectancy in myelodysplastic syndromes classified according to WHO criteria: a basis for clinical decision making. *J Clin Oncol*. 2005;23:7594.
232. Juneja SK, Imbert M, Sigaux F, et al. Prevalence and distribution of ringed sideroblasts in primary myelodysplastic syndromes. *J Clin Pathol*. 1983;36:566.
233. Greenberg PL, Tuechler H, Schanz J, et al. Revised international prognostic scoring system for myelodysplastic syndromes. *Blood*. 2012;120:2454.
234. Patnaik MM, Tefferi A. Refractory anemia with ring sideroblasts (RARS) and RARS with thrombocytosis: "2019 Update on Diagnosis, Risk-stratification, and Management." *Am J Hematol*. 2019;94:475.
235. Germing U, Strupp C, Kuendgen A, et al. Prospective validation of the WHO proposals for the classification of myelodysplastic syndromes. *Haematologica*. 2006;91:1596.
236. Patnaik MM, Lasho TL, Hodnefield JM, et al. SF3B1 mutations are prevalent in myelodysplastic syndromes with ring sideroblasts but do not hold independent prognostic value. *Blood*. 2012;119:569.
237. Jadersten M, Montgomery SM, Dybedal I, et al. Long-term outcome of treatment of anemia in MDS with erythropoietin and G-CSF. *Blood*. 2005;106:803.
238. Raza A, Reeves JA, Feldman EJ, et al. Phase 2 study of lenalidomide in transfusion-dependent, low-risk, and intermediate-1 risk myelodysplastic syndromes with karyotypes other than deletion 5q. *Blood*. 2008;111:86.
239. Santini V, Almeida A, Giagounidis A, et al. Randomized phase III study of lenalidomide versus placebo in RBC transfusion-dependent patients with lower-risk non-del(5q) myelodysplastic syndromes and ineligible for or refractory to erythropoiesis-stimulating agents. *J Clin Oncol*. 2016;34:2988.
240. Toma A, Kosmider O, Chevret S, et al. Lenalidomide with or without erythropoietin in transfusion-dependent erythropoiesis-stimulating agent-refractory lower-risk MDS without 5q deletion. *Leukemia*. 2016;30:897.
241. Tefferi A, Al-Kali A, Begna KH, et al. Imetelstat therapy in refractory anemia with ring sideroblasts with or without thrombocytosis. *Blood Cancer J*. 2016;6:e405.
242. Kubasch AS, Platzbecker U. Setting fire to ESA and EMA resistance: new targeted treatment options in lower risk myelodysplastic syndromes. *Int J Mol*. 2019;20:3853.
243. Silverman LR, Demakos EP, Peterson BL, et al. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. *J Clin Oncol*. 2002;20:2429.
244. Silverman LR, McKenzie DR, Peterson BL, et al. Further analysis of trials with azacitidine in patients with myelodysplastic syndrome: studies 8421, 8921, and 9221 by the Cancer and Leukemia Group B. *J Clin Oncol*. 2006;24:3895.
245. Cortelazzo S, Finazzi G, Ruggeri M, et al. Hydroxyurea for patients with essential thrombocythemia and a high risk of thrombosis. *N Engl J Med*. 332:1132.
246. Eichner ER, Hillman RS. The evolution of anemia in alcoholic patients. *Am J Med*. 1971;50:218.
247. Hines JD. Reversible megaloblastic and sideroblastic marrow abnormalities in alcoholic patients. *Br J Haematol*. 1969;16:87.
248. Pierce HI, McGuffin RG, Hillman RS. Clinical studies in alcoholic sideroblastosis. *Arch Intern Med*. 1976;136:283.
249. Savage D, Lindenbaum J. Anemia in alcoholics. *Medicine (Baltimore)*. 1986;65:322.
250. Hines JD, Cowan DH. Studies on the pathogenesis of alcohol-induced sideroblastic bone-marrow abnormalities. *N Engl J Med*. 1970;283:441.
251. Vech RL, Lumeng L, Li TK. Vitamin B6 metabolism in chronic alcohol abuse the effect of ethanol oxidation on hepatic pyridoxal 5′-phosphate metabolism. *J Clin Invest*. 1975;55:1026.
252. McColl KE, Moore MR, Thompson GG, et al. Abnormal haem biosynthesis in chronic alcoholics. *Eur J Clin Invest*. 1981;11:461.
253. McColl KE, Thompson GG, Moore MR, et al. Acute ethanol ingestion and haem biosynthesis in healthy subjects. *Eur J Clin Invest*. 1980;10:107.
254. Thunell S, Floderus Y, Henrichson A, et al. Alcoholic beverages in acute porphyria. *J Stud Alcohol*. 1992;53:272.
255. Haden HT. Pyridoxine-responsive sideroblastic anemia due to antituberculous drugs. *Arch Intern Med*. 1967;120:602.
256. Colucci G, Silzle T, Solenthaler M. Pyrazinamide-induced sideroblastic anemia. *Am J Hematol*. 2012;87:305.
257. Firkin FC. Mitochondrial lesions in reversible erythropoietic depression due to chloramphenicol. *J Clin Invest*. 1972;51:2085.
258. Kandola L, Swannell AJ, Hunter A. Acquired sideroblastic anaemia associated with penicillamine therapy for rheumatoid arthritis. *Ann Rheum Dis*. 1995;54:529.
259. Willekens C, Dumezy F, Boyer T, et al. Linezolid induces ring sideroblasts. *Haematologica*. 2013;98:e138.
260. Brodsky RA, Hasegawa S, Fibach E, et al. Acquired sideroblastic anaemia following progesterone therapy. *Br J Haematol*. 1994;87:859.
261. Dameron C, Moore MR, Imray P, et al. Copper: Environmental Health Criteria. Series no. 200. Geneva: World Health Organization; 1998.
262. Ashkenazi A, Levin S, Djaldetti M, et al. The syndrome of neonatal copper deficiency. *Pediatrics*. 1973;52:525.
263. Zidar BL, Shadduck RK, Zeigler Z, et al. Observations on the anemia and neutropenia of human copper deficiency. *Am J Hematol*. 1977;3:177.
264. Gregg XT, Reddy V, Prchal JT. Copper deficiency masquerading as myelodysplastic syndrome. *Blood*. 2002;100:1493.
265. Prodan CI, Holland NR, Wisdom PJ, et al. CNS demyelination associated with copper deficiency and hyperzincemia. *Neurology*. 2002;59:1453.
266. Fiske DN, McCoy HE 3rd, Kitchens CS. Zinc-induced sideroblastic anemia: report of a case, review of the literature, and description of the hematologic syndrome. *Am J Hematol*. 1994;46:147.
267. Kumar A, Jazieh AR. Case report of sideroblastic anemia caused by ingestion of coins. *Am J Hematol*. 2001;66:126.
268. Labbe RF, Rettmer RL. Zinc protoporphyrin: a product of iron-deficient erythropoiesis. *Semin Hematol*. 1989;26:40.
269. Houston T, Moore MR, McColl KE, et al. Erythroid 5-aminolaevulinate synthase activity during normal and iron deficient erythropoiesis. *Br J Haematol*. 1991;78:561.
270. O'Brien H, Amess JA, Mollin DL. Recurrent thrombocytopenia, erythroid hypoplasia and sideroblastic anaemia associated with hypothermia. *Br J Haematol*. 1982;51:451.
271. Devgun MS, El-Nujumi AM, O'Dowd GJ, et al. Novel mutations in the Dubin–Johnson syndrome gene ABCC2/MRP2 and associated biochemical changes. *Ann Clin Biochem*. 2012;49:609.
272. van de Steeg E, Stranecky V, Hartmannova H, et al. Complete OATP1B1 and OATP1B3 deficiency causes human Rotor syndrome by interrupting conjugated bilirubin reuptake into the liver. *J Clin Invest*. 2012;122:519.
273. McColl KE, Thompson GG, el Omar E, et al. Porphyrin metabolism and haem biosynthesis in Gilbert's syndrome. *Gut*. 1987;28:125.
274. Moore MR. Health implications of the hemopoietic effects of lead. In: Mahaffey KR, ed. *Dietary and Environmental Lead: Human Health Effects*. Amsterdam: Elsevier; 1985:261.
275. Oishi H, Nomiyama H, Nomiyama K, et al. Comparison between males and females with respect to the porphyrin metabolic disorders found in workers occupationally exposed to lead. *Int Arch Occup Environ Health*. 1996;68:298.
276. Daniell WE, Stockbridge HL, Labbe RF, et al. Environmental chemical exposures and disturbances of heme synthesis. *Environ Health Perspect*. 1997;105(suppl 1):37.

CHAPTER 40

MEGALOBLASTIC ANEMIAS

Aśok C. Antony

The term *megaloblastic anemia* is used to describe a group of disorders characterized by a distinct morphologic pattern in hematopoietic cells. A common feature is a defect in deoxyribonucleic acid (DNA) synthesis, with lesser alterations in ribonucleic acid (RNA) and protein synthesis, leading to a state of unbalanced cell growth and impaired cell division. Most megaloblastic cells are not resting but vainly engaged in attempting to double their DNA, with frequent arrest in the S phase and lesser degrees of arrest in other phases of the cell cycle. An increased percentage of these cells have DNA values between 2 N (where N is the amount of DNA in the haploid genome) and 4 N because of delayed cell division. This increased DNA content in megaloblastic cells is morphologically expressed as larger-than-normal "immature" nuclei with finely particulate chromatin, whereas the relatively unimpaired RNA and protein synthesis results in large cells with greater "mature" cytoplasm and cell volume. The net result of megaloblastosis is a cell whose nuclear maturation is arrested (immature) while its cytoplasmic maturation apparently proceeds normally independently of the nuclear events. The microscopic appearance of this nuclear-cytoplasmic asynchrony (or dissociation) is morphologically described as megaloblastic. Each cell lineage has a limited but unique repertoire of expression of defective DNA synthesis. This is significantly influenced by the normal patterns of maturation of the affected cell line. Additional variables that affect RNA and protein synthesis can lead to the attenuation or modification of megaloblastic expression (see Masked Megaloblastosis).

Megaloblastic hematopoiesis commonly manifests as anemia, but this feature is only a manifestation of a more global defect in DNA synthesis that affects all proliferating cells. The peripheral blood picture is characteristic and reflective of megaloblastic hematopoiesis within the bone marrow. The diagnosis is therefore usually straightforward, but because any condition that specifically perturbs DNA synthesis may lead to megaloblastosis, determination of the precise cause is necessary before institution of therapy. Inappropriate therapy can lead to disastrous consequences for the patient. The biochemical basis for megaloblastosis needs to be understood within the context of evaluation of potential and real variables affecting DNA, RNA, and protein synthesis in a given patient. The most common causes of megaloblastosis are true cellular deficiencies of vitamin B_{12} (cobalamin) or folate, vitamins that are essential for DNA synthesis.

Because of the imperative for conservation of cobalamin within the body, there is a finely tuned mechanism in place to ensure a sequential handover of this precious cargo from one protein to another—from the point of its entry into the mouth through the gut, across the enterocyte, into the circulation with specialized uptake into cells, passage through lysosomes into cytoplasm, and even into mitochondria. Throughout this odyssey, cobalamin is accompanied by several chaperones that sequentially bind, sequester, and thereby ensure that cobalamin does not participate in side reactions. This ensures its fitness for service for critical enzymes.

Despite the greater abundance of folate in the diet relative to cobalamin, there are also specialized means to ensure that the natural folates in food are first chopped and diced before being ushered across the enterocyte through specialized pathways. After passage from the portal blood into the general circulation, folate is extracted by cell surface folate receptors, undergoes endocytosis, and is then shunted together with a proton across another channel into the cytoplasm. It is then received by an overabundance of high-affinity multifunctional enzymes that channel the folate across set pathways to support critical synthesis of thymidine for DNA and other key cellular functions including methylation reactions.

A general principle is that the preexisting store of these vitamins will dictate the speed with which overt deficiency develops; this is particularly relevant to pregnant women and children in resource-limited countries with preexisting borderline stores of folate and cobalamin.

The pathophysiology of cellular cobalamin and folate deficiency is most readily discerned by the clinician who approaches megaloblastosis with a clear understanding of the physiology of these vitamins. A detailed discussion of cobalamin and folate therefore follows.

COBALAMIN

The term *cobalamin* refers to a family of compounds with the structure shown in Fig. 40.1. Details of the chemistry, nomenclature, and in vivo substitutions of cobalamin are shown in Figs. 40.1–40.3, and excellent reviews are available on the colorful history, chemistry, and biology of cobalamin.[1–4]

Nutrition

Cobalamin is produced in nature only by microorganisms, and humans receive cobalamin solely from the diet.[5–8] Cobalamin is synthesized and used by some microorganisms (e.g., bacteria, fungi). Some strains (such as *Pseudomonas denitrificans*) produce cobalamin during fermentation, making them excellent and cheap commercial sources for cobalamin used in therapy. Herbivores obtain their dietary quota of cobalamin from plants contaminated with cobalamin-producing soil bacteria (rhizobia) that grow in roots and nodules of legumes. Because rhizobia-related organisms are also found in the large intestine of animals (and humans), volitional or inadvertent coprophagy can lead to intake of cobalamin by herbivores; however, cobalamin from manure that contaminates plants is not a significant source for humans.[5] Nevertheless, colonic cobalamin-producing bacteria—like *Klebsiella pneumoniae* that are related to rhizobia—can be found in the small intestine of some individuals from which cobalamin can be absorbed. For all practical purposes, there is *no* unfortified plant food, including fermented soy products, tempeh/*tempe*, seaweed (which are actually multicellular algae, such as nori [red algae], chlorella [green algae], spirulina [blue-green algae]), or other organic produce that can consistently provide a sufficient amount of active cobalamin to support daily requirements.

Animal protein is the major dietary cobalamin source for nonvegetarians. Meats from parenchymal organs are richest in cobalamin (over 10 μg/100 g wet weight); fish and muscle meats, milk products, and egg yolk have 1 to 10 μg/100 g of wet weight. An average nonvegetarian Western diet contains 5 to 7 μg/day of cobalamin, which adequately sustains normal cobalamin equilibrium. A vegetarian diet only supplies between 0.25 and 0.5 μg/day of cobalamin, so all vegetarians *do not* receive adequate dietary cobalamin and are at risk for cobalamin deficiency.[7–9] Even a Mediterranean diet with modest intake of animal-source proteins places mothers and babies at risk for cobalamin deficiency.[10] The current recommended daily allowance is 2.4 μg for men and nonpregnant women, 2.6 μg for pregnant women, 2.8 μg for lactating women, and 1.5 to 2 μg for children 9 to 18 years old.[11] However, reevaluation of cobalamin requirements

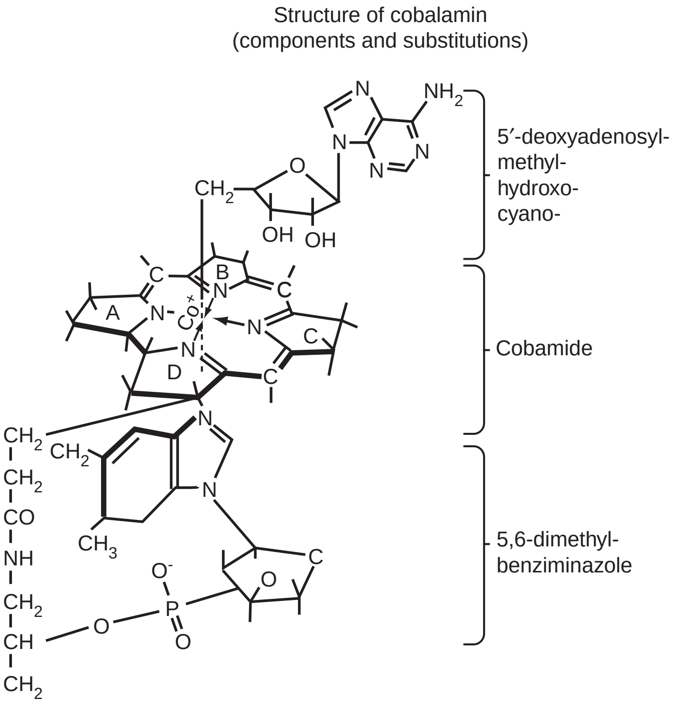


Figure 40.1 COBALAMIN (VITAMIN B_{12}) CHEMISTRY AND NOMENCLATURE. The central cobalt atom of cobalamin forms the focal point of this large complex organometallic molecule (approximately 1300 to 1500 Da). There are up to six ligands that can bind to this cobalt; of these, four involve nitrogen atoms of the planar corrin ring that surround the cobalt atom. The lower α-axial ligand, extending perpendicular below the corrin ring, links to nitrogen of a 5,6-dimethylbenzimidazole phosphoribosyl moiety that is also attached back to the corrin ring through one of its propionamide side chains (this is analogous to the hand guard on the handle on a saber that covers the knuckles of the hand). The upper or β-axial ligand varies and can exist in the fully oxidized Co^{3+} state, which is referred to as cob(III)alamin; in the Co^{2+} state, called cob(II)alamin (which can be used for the synthesis of methylcobalamin or adenosylcobalamin); or the fully reduced Co^{+} state (cob[I]alamin). These upper axial ligands include cyano- (cyanocobalamin), hydroxyl- (hydroxocobalamin), methyl- (methylcobalamin), or 5′-deoxyadenosyl- (adenosylcobalamin) and confer a distinct identity to cobalamin for participation in one-carbon metabolism. All these forms with substituted upper axial ligands are cob(III)alamins, which adopt a configuration in which the 5,6-dimethylbenzimidazole nitrogen base is coordinated to the cobalt in the lower axial position. This is referred to as the "base-on" position. However, when cobalamin is bound to the enzymes methionine synthase and methylmalonyl-CoA mutase, another conformational change results in the replacement of the 5,6-dimethylbenzimidazole by a histidine donated by the enzyme; this is the "His-on" position. Thus, shifts from "base-on/His-off" conformation to an alternative "base-off/His-on" conformation have an important bearing on the catalytic activity of these enzymes. Conversion of cyanocobalamin to its active cofactor forms requires a decyanation step. *(From Chanarin I. The Megaloblastic Anemias. Oxford, UK: Blackwell Scientific Publications; 1979.)*

within a university community (aged 18 to 50 years) from the USA suggests that a higher intake of 4 to 7 micrograms of cobalamin each day is optimum for adequate cobalamin status.[12] This is in line with earlier studies from the USA and Europe.[13–15]

Food cobalamin is stable to high-temperature cooking but is readily converted to inactive cobalamin analogues by ascorbic acid. Cobalamin is exceptionally well stored in tissues in its coenzyme forms. Of the total-body content of 2 to 5 mg in adults, about 1 mg is in the liver. There is an obligatory loss of 0.1% per day (1.3 μg) regardless of total-body cobalamin content. It takes about 3 to 4 years to deplete cobalamin stores when dietary cobalamin is abruptly malabsorbed, but it may take longer to develop nutritional cobalamin deficiency, because of an efficient enterohepatic circulation, which accounts for turnover of 5 to 10 μg/day of cobalamin.[16]

Absorption

Cobalamin in food is usually in coenzyme form (5′-deoxyadenosylcobalamin [adenosylcobalamin] and methylcobalamin), nonspecifically bound to proteins (see Fig. 40.2). In the stomach, peptic digestion at low pH is a prerequisite for cobalamin release from food protein.[16] Once released by proteolysis, cobalamin preferentially binds a high-affinity, 150-kDa, cobalamin-binding protein called *R protein* (a haptocorrin) from gastric juice and saliva that has an even higher affinity for cobalamin than gastric intrinsic factor (IF). The cobalamin-bound R protein complex (holo-R protein), along with excess unbound (apo)-R protein and IF, pass through into the second part of the duodenum, where pancreatic proteases degrade holo-R and apo-R proteins (but not IF). This results in the transfer of cobalamin to IF, a 45-kDa glycoprotein with high-affinity binding ($K_a = 1.5 \times 10^{10}\,M^{-1}$), 1 : 1 molar stoichiometry, stability, and resistance to proteolysis over a pH range of 3 to 9.[16] Failure to degrade holo-R proteins by pancreatic protease precludes the involvement of IF in cobalamin absorption because the downstream ileal IF-cobalamin receptors only interact with IF-bound cobalamin.[16] Although R proteins bind cobalamin and most cobalamin analogues with comparably high affinity, IF only binds cobalamin.

IF is produced in parietal (oxyntic) cells in the fundus and cardia of the stomach.[16] IF has two binding sites: one for cobalamin and another for the ileal IF-cobalamin receptor. IF is produced in far greater excess than is actually required for absorption; indeed, the IF in only 2 to 4 mL of normal gastric juice can reverse cobalamin deficiency in adults who lack IF.[16] In the absence of IF, less than 2% of ingested cobalamin is absorbed, whereas in its presence, approximately 70% is absorbed.

IF is secreted in response to food in the stomach in a manner analogous to secretion of acid (i.e., by vagal and hormonal stimulation). IF binds biliary cobalamin and newly ingested cobalamin following its transfer from R protein.[16] Because biliary cobalamin analogues are not transferred from R protein to IF, this is an efficient method for fecal excretion of cobalamin analogues while allowing for reabsorption of biliary cobalamin. The stable IF-cobalamin complex passes through the jejunum to the ileum, where specific membrane-associated IF-cobalamin receptors for IF-cobalamin are concentrated on microvilli of terminal ileal mucosal cells.[16]

The functional IF-cobalamin receptors are composed of a complex of two proteins collectively known as cubam, composed of *cub*ilin (CUBN)[17] and *am*nionless (AMN),[18] that is essential to complete transport of the IF-cobalamin complex from the intestinal lumen into the ileal enterocyte. CUBN is a large (400-kDa) peripheral membrane protein, which requires the smaller transmembrane protein, AMN, for its expression at the brush border, and vice versa. Dysfunction of cubam because of mutation in either CUBN or AMN is the basis for Imerslund-Gräsbeck syndrome. These IF-cobalamin receptors (cubam) require Ca^{2+} for binding at pH above 5.4; they do not bind free IF, cobalamin, or R protein–bound cobalamin; therefore these receptors are highly specific and have a high affinity for IF-cobalamin ($K_a = 1 \times 10^{9}\,M^{-1}$). The human ileum contains enough IF-cobalamin receptors [cubam] to bind up to 1 μg of IF-bound cobalamin; this is the rate-limiting factor in cobalamin absorption.[16]

After the cobalamin-IF complex is internalized by cubam for subsequent processing, these receptors are recycled back to the cell surface, whereas cobalamin enters the cytoplasm. Subsequent physiologic events are unclear. The multidrug resistance–associated protein (MRP) 1 mediates the cellular export of cobalamin across the basolateral membrane of intestinal epithelium.[19,20] Then cobalamin apparently binds transcobalamin II (TCII) within or at the basal surface of the ileal enterocyte.[16] TCII is abundant in the microvascular endothelium and cobalamin, which appears in portal blood largely (over 90%) bound to TCII, reaches peak levels in about 8 hours.[16]

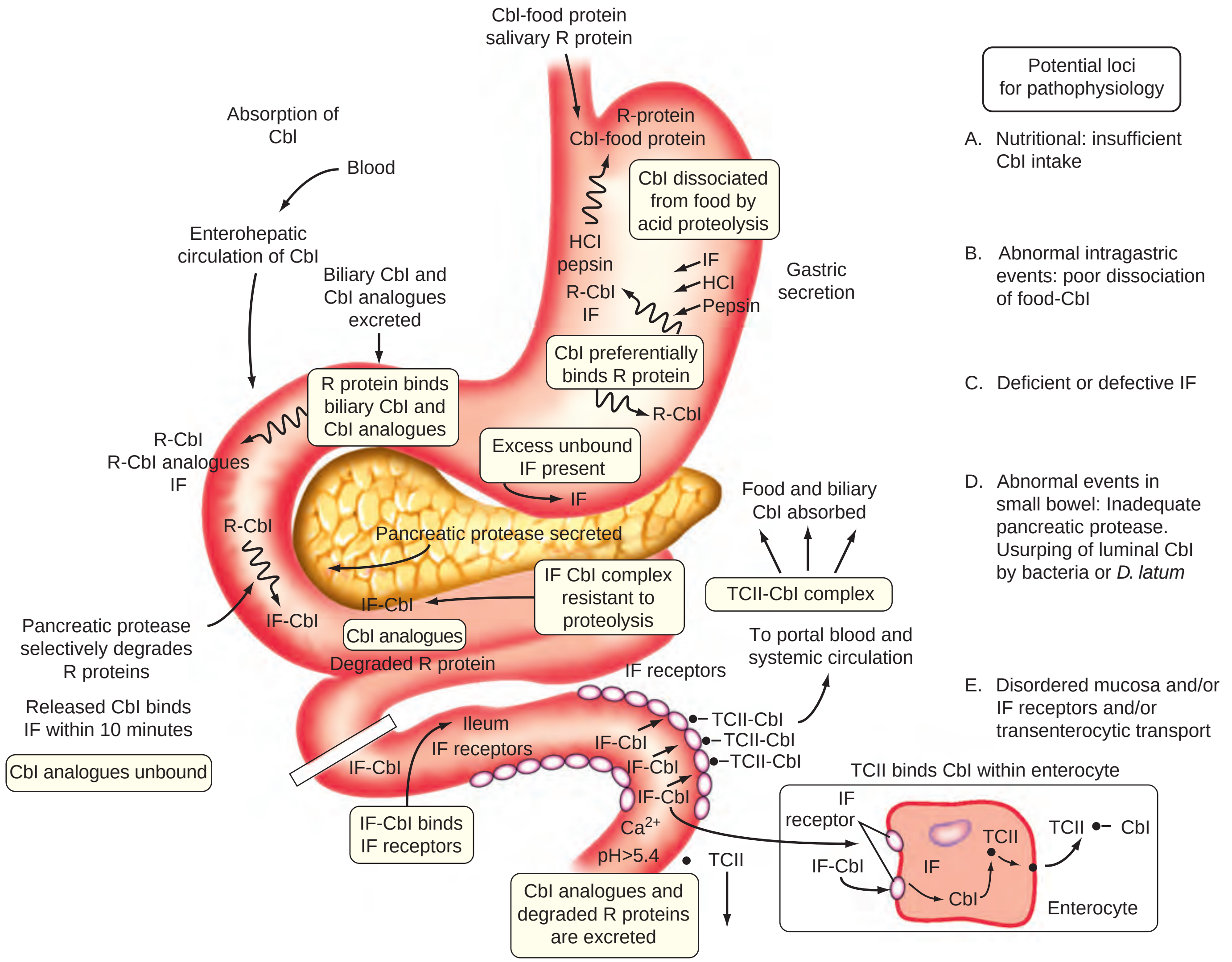

Cbl-food protein
salivary R protein
Potential loci for pathophysiology
A. Nutritional: insufficient Cbl intake
B. Abnormal intragastric events: poor dissociation of food-Cbl
C. Deficient or defective IF
D. Abnormal events in small bowel: Inadequate pancreatic protease. Usurping of luminal Cbl by bacteria or *D. latum*
E. Disordered mucosa and/or IF receptors and/or transenterocytic transport
Absorption of Cbl
Blood
Enterohepatic circulation of Cbl
R-protein
Cbl-food protein
Cbl dissociated from food by acid proteolysis
HCl
pepsin
IF
HCl
Pepsin
Gastric secretion
R-Cbl
IF
Biliary Cbl and Cbl analogues excreted
Cbl preferentially binds R protein
R-Cbl
R protein binds biliary Cbl and Cbl analogues
R-Cbl
R-Cbl analogues
IF
Excess unbound IF present
IF
Food and biliary Cbl absorbed
Pancreatic protease secreted
R-Cbl
IF Cbl complex resistant to proteolysis
TCII-Cbl complex
IF-Cbl
IF-Cbl
Cbl analogues
Degraded R protein
To portal blood and systemic circulation
Pancreatic protease selectively degrades R proteins
Released Cbl binds IF within 10 minutes
Cbl analogues unbound
IF receptors
TCII-Cbl
TCII-Cbl
TCII-Cbl
Ileum
IF receptors
IF-Cbl
IF-Cbl
IF-Cbl
IF-Cbl
TCII binds Cbl within enterocyte
IF-Cbl binds IF receptors
Ca^{2+}
pH>5.4
TCII
IF receptor
IF-Cbl
IF
TCII
Cbl
TCII
Cbl
Enterocyte
Cbl analogues and degraded R proteins are excreted

Figure 40.2 COMPONENTS AND MECHANISM OF COBALAMIN ABSORPTION. *Cbl*, Cobalamin; *D. latum, Diphyllobothrium latum; HCl*, hydrochloric acid; *IF*, intrinsic factor; *R-Cbl*, R-protein bound cobalamin; *TCII*, transcobalamin II.

By contrast, therapeutically administered cobalamin in large doses can also passively diffuse through nasal, buccal, gastric, and jejunal mucosa so that less than 1% of a large dose of oral cobalamin appears in the circulation in minutes. This property is used to advantage in individuals with cobalamin malabsorption in lieu of parenteral replacement (discussed later).[16]

Transport

More than 90% of recently absorbed or injected cobalamin is bound to TCII, which is the specific serum transport protein for delivery of cobalamin to tissues. TCII, a 38-kDa polypeptide synthesized in many tissues, preferentially binds cobalamin with 1:1 molar stoichiometry and high affinity ($K_a = 1 \times 10^{11}\,M^{-1}$).[16] The TCII-cobalamin complex is rapidly cleared from the circulation in less than an hour. TCII-bound cobalamin binds to specific cell surface 58-kDa TCII-cobalamin receptors (encoded by the *CD320* gene) present on several cells.[21,22] High-affinity TCII-cobalamin binding to TCII-cobalamin receptors is specific only for holo- and apo-TCII ($K_a = \sim 5 \times 10^{10}\,M^{-1}$). However, because some cobalamin analogues can bind TCII with high affinity, these also have the same potential for cellular uptake as cobalamin.[16]

Circulating cobalamin, which is predominantly in the form of methylcobalamin, is not found free in plasma. Binding to TCII accounts for only 10% to 30% of the total serum cobalamin, with the majority (approximately 75%) of remaining cobalamin being bound to another protein, transcobalamin I (TCI). TCI (another haptocorrin) binds biologically active cobalamins as well as biologically inactive cobalamin derivatives. Because TCI is not a cellular transport protein, it is best viewed as a plasma-storage form of cobalamin; indeed, cobalamin-bound TCI has a slow clearance rate (half-life of 9 to 12 days).[23] A third transport protein, transcobalamin III (TCIII), is closely related to TCI but has a half-life in minutes because it is an asialoglycoprotein. TCIII binds a wide spectrum of cobalamin analogues with high affinity and delivers them via hepatic asialoglycoprotein receptors to hepatic cells, and thence into bile for fecal excretion. Between 0.5 and 9 µg of cobalamin taken up by hepatic TCII receptors is secreted into bile, of which approximately 75% is reabsorbed, analogous to food cobalamin,[16] reflecting an efficient enterohepatic circulation of cobalamin.

Cellular Processing

Once bound to TCII receptors, the TCII-cobalamin complex is internalized by conventional receptor-mediated endocytosis (see Fig. 40.3).[16] At the low pH extant in lysosomes, TCII dissociates from cobalamin and is degraded, whereas transport of cobalamin across lysosomes into the cytosol requires two distinct membrane proteins,

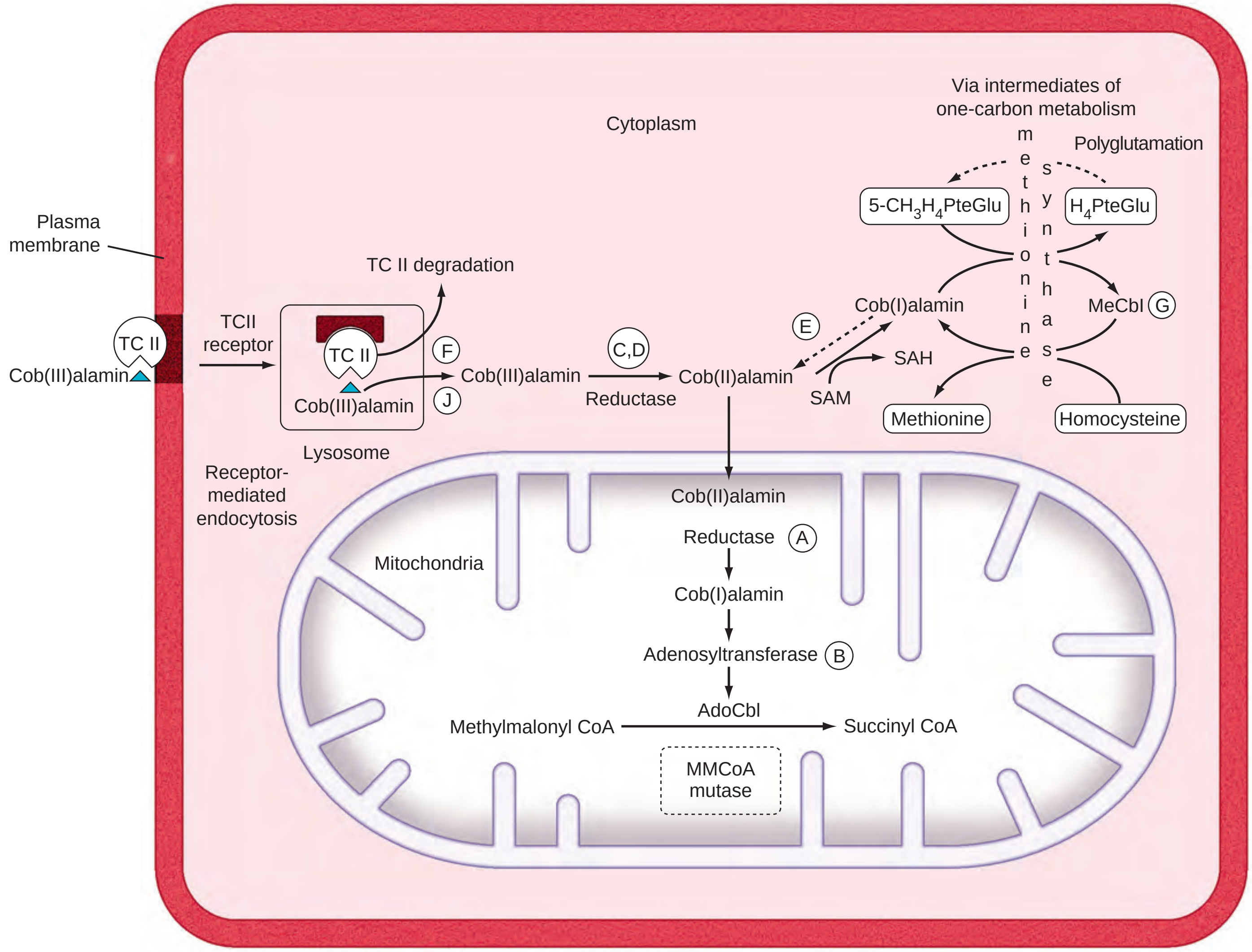

Via intermediates of
one-carbon metabolism
Cytoplasm
Polyglutamation
methionine synthase
$5\text{-}CH_3H_4PteGlu$
$H_4PteGlu$
Plasma membrane
TC II degradation
Cob(I)alamin
MeCbl
G
E
TC II
TCII receptor
Cob(III)alamin
TC II
F
C,D
SAH
Cob(III)alamin
Cob(II)alamin
Cob(III)alamin
J
Reductase
SAM
Methionine
Homocysteine
Lysosome
Receptor-mediated endocytosis
Cob(II)alamin
Reductase
A
Mitochondria
Cob(I)alamin
Adenosyltransferase
B
AdoCbl
Methylmalonyl CoA
Succinyl CoA
MMCoA mutase

Figure 40.3 CELLULAR UPTAKE AND INTRACELLULAR REACTIONS INVOLVING COBALAMIN. A large family of natural and synthetic cobalamins can be generated when the cyanide (CN) moiety (upper axial ligand in cyanocobalamin) is replaced. On exposure to light, CN is gradually lost from cyanocobalamin, with the production of hydroxocobalamin. In vivo substitutions include the replacement of hydroxocobalamin or cyanocobalamin by a 5′-deoxyadenosyl group attached by a covalent bond, giving rise to adenosylcobalamin (AdoCbl). Methylcobalamin (MeCbl) is the main form in plasma. In vivo, 5-methyltetrahydrofolate readily donates its methyl group to cob(I)alamin in a reaction involving methionine synthase to form methylcobalamin. Transport of cobalamin across lysosomes requires two distinct membrane proteins, LMBRD1 and ABCD4, which act synergistically and, when lacking, give rise to cblF and cblJ complementation groups, respectively. The approximate loci for defects in cobalamin mutants, cblA to cblJ, are shown. See text for details. *MMCoA mutase*, Methylmalonyl-CoA mutase; *SAH, S*-adenosylhomocysteine; *SAM, S*-adenosylmethionine.

LMBRD1 and ABCD4, which act synergistically (see Fig. 40.3).[20] Cob(III)alamin, the most "oxidized" form of cobalamin, must be converted to cob(II)alamin and cob(I)alamin by two sequential reductase steps (see Fig. 40.3). Once in the cytoplasm, cobalamin is bound to CblC, an enzyme that catalyzes removal of the cyano or methyl or adenosyl groups that are bound to cobalt in the cobalamin molecule.[20,24] From here, cobalamin is moved to target enzymes in the mitochondria or the cytoplasm.

Over 95% of intracellular cobalamin is bound to two intracellular enzymes, methylmalonyl-CoA mutase and methionine synthase.[16] When cobalamin interacts with its target enzymes, it exists in a "base-off/His-on" conformation, which reflects a very close relationship with these enzymes (see legend for Fig. 40.1).

In mitochondria, cob(I)alamin is converted to its coenzyme form, adenosylcobalamin, which acts as a coenzyme with methylmalonyl-CoA mutase to mediate the intramolecular exchange of a hydrogen atom attached to one carbon atom with a group attached to an adjacent carbon atom; in this way, methylmalonyl-CoA is converted to succinyl-CoA (methylmalonyl-CoA is normally generated during the catabolism of branched-chain amino acids, odd-chain fatty acids, and cholesterol). When formed, succinyl-CoA can then enter the Krebs tricarboxylic acid cycle.

In the cytoplasm, cobalamin, as methylcobalamin, functions as a coenzyme for methionine synthase, a critical enzyme for which both folates and cobalamin are required for normal one-carbon metabolism (see Fig. 40.3). Methionine synthase is a modular protein with four distinct and separate regions for binding homocysteine, 5-methyltetrahydrofolate (5-methyl-THF; 5-methyl-H_4PteGlu), the cobalamin prosthetic group, and *S*-adenosylmethionine (SAM).[23] The reaction proceeds by methyl transfer from 5-methyl-tetrahydrofolate to methionine synthase–bound cob(I)alamin to form methylcobalamin, followed by transfer of this methyl group to homocysteine to form methionine and regeneration of cob(I)alamin. In this process, 5-methyl-tetrahydrofolate is converted to tetrahydrofolate, which is subsequently polyglutamylated by folyl polyglutamate synthase; this addition of multiple glutamic acid moieties to tetrahydrofolate facilitates both its retention within cells and participation in one-carbon metabolism. During this reaction, spontaneous oxidation of cob(I)alamin (which has no axial ligand) to the catalytically inactive cob(II)alamin form requires reduction back to cob(I)alamin before it can accept a methyl group. There is a specific redox regulator known as methionine synthase reductase that restores enzyme activity in the presence of SAM and NADPH[25]; this enzyme is mutated in patients with cblE mutations.

The physiologic importance of the key cofactor roles of the two forms of cobalamin (i.e., adenosylcobalamin and methylcobalamin) in methylmalonyl-CoA mutase and methionine synthase, respectively, is that the products and by-products of these enzymatic reactions are critical for DNA, RNA, and protein biosynthesis.

FOLATES

Nutrition

Folates (the anionic forms of folic acid, also called vitamin B_9) are synthesized by microorganisms and plants, including leafy vegetables (spinach, lettuce, broccoli), beans, fruits (bananas, melons, lemons), yeast, and mushrooms, and are also found in animal meats[8,16]; see Fig. 40.4 for chemistry and nomenclature. Among natural folates, which are predominantly in polyglutamylated form, only one-half are bioavailable; by contrast, 85% of folic acid that is added to food or ingested as a supplement is bioavailable. Several factors can influence the bioavailability of folates. These include: (1) The stability of the food folate. Natural reduced folates are labile and susceptible to oxidative cleavage by nitrates or light exposure, but folic acid is much more stable. Prolonged boiling or cooking over 30 minutes reduces natural folates by 50% to 80%, whereas ascorbate increases bioavailability, and refrigeration of leafy foods exposed to fluorescent light in supermarkets can double the folate content.[26] (2) Pureed foods allow easier access to the glutamate carboxypeptidase II (also known as folate-polyglutamate hydrolase), which converts folate polyglutamates to simpler folate monoglutamates to facilitate absorption[27]; so perturbation of this enzyme by organic acids (orange juice) or drugs (sulfasalazine, ethanol) can preclude absorption; conversely, high-affinity folate-binding proteins in human or cow's milk can increase folate absorption for infants and women, respectively.[28] (3) Interference with the proton-coupled folate transporter (PCFT)-mediated folate absorption across the proximal jejunum from either intestinal diseases or drugs (proton-pump inhibitors, sulfasalazine, pyrimethamine)[29,30] will affect the bioavailability of food folate.

The recommended daily allowance of folate is as follows: adult men and nonpregnant women, 400 µg; pregnant women, 600 µg; lactating women, 500 µg; children 9 to 18 years, between 300 and 400 µg.[31] A balanced Western diet contains adequate amounts of folate, but the net dietary intake of folate in many resource-limited countries is often insufficient to sustain folate balance.[8,16,32–34]

Absorption

After dietary folate polyglutamates are converted to folate monoglutamates at the enterocyte brush border, they are transported through the duodenal and jejunal brush border by physiological, high-affinity, luminal surface–facing membrane PCFTs, which are most efficient in an acidic milieu. At pH 5.5, there is equivalent affinity for transport of physiologic reduced folates and folic acid, but at pH 6.5, reduced

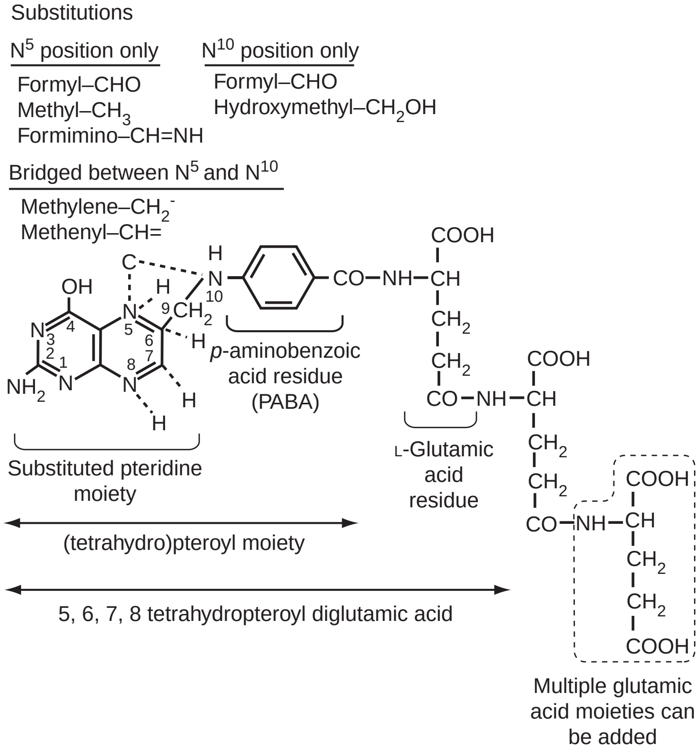


Figure 40.4 FOLATE CHEMISTRY AND NOMENCLATURE. Folic acid (pteroylmonoglutamate [PteGlu]) is the commercially available parent compound for more than 100 compounds collectively referred to as *folates*. PteGlu consists of three basic components: a pteridine derivative, a *p*-aminobenzoic acid residue, and an L-glutamic acid residue. Before PteGlu can play a role as a coenzyme, it must first be reduced at positions 7 and 8 to dihydrofolic acid (H_2PteGlu) and then to 5,6,7,8-tetrahydrofolic acid (THF; H_4PteGlu), and one to six additional glutamic acid residues must then be added by means of γ-peptide bonds to the L-glutamate moiety (for which the subscripted *n* in PteGlu_n denotes polyglutamation). Folate coenzymes donate or accept one-carbon units in numerous reactions in amino acid and nucleotide metabolism. The various substitutions in $H_4\text{PteGlu}_n$ occur at positions 5 or 10, or both; position 5 can be substituted by methyl (CH_3), formyl (CHO), or formimino (CHNH), and position 10 can be substituted by formyl or hydroxymethyl (CH_2OH). Positions 5 and 10 can be bridged by methylene ($–CH_2–$) or methenyl (–CH=). For an engaging account of the history of folic acid, see the article by Hoffbrand and Weir.[311]

5-methyl-tetrahydrofolate is transported more efficiently.[35] PCFT is a folate-hydrogen symporter, so with each folate molecule transported, there is a net translocation of positive charge. Loss-of-function mutations in PCFT in the enterocyte and choroid plexus result in hereditary folate malabsorption,[29,36] where there is an inability to transport folate across the intestine and the choroid plexus. Within the enterocyte, folates are reduced to tetrahydrofolate and then methylated before release into plasma as 5-methyl-tetrahydrofolate. Most of the folic acid taken up by the PCFT in the proximal small intestine is also converted within the enterocyte to 5-methyl-tetrahydrofolate. Folate production by bacteria in the small intestine can also enter the circulation.[37]

The flux of folate from the basolateral membrane of the enterocyte to the portal blood is mediated through MRP3.[29] MRP proteins have low affinity but high capacity and are best viewed as cellular "sump pumps" that eject excess folates (and antifolates) out of cells. Together with MRP2, which mediates folate transport into bile,[38] these MRPs maintain an efficient enterohepatic circulation, which helps retain folate.[29]

Some bacterially produced folates, especially those produced by probiotic bacteria (genus *Bifidobacterium*), can be absorbed across the large intestine,[39] sufficient to raise the serum folate levels,[40] but normally this accounts for no more than about 5% of the average folate requirement.

Passive diffusion of folic acid (which is formally referred to as pteroylmonoglutamate [and abbreviated as PteGlu]) is probably the primary mechanism of intestinal mucosal folate absorption at high pharmacologic concentrations.[16] The small intestine has a large capacity to absorb folate, with peak folate levels in plasma achieved 1 to 2 hours after oral administration.

Plasma Transport and Enterohepatic Circulation

The normal serum folate level is maintained by dietary folate and a substantial enterohepatic circulation that amounts to about 90 μg/day of folate.[16] Biliary drainage results in a dramatic fall in serum folate (to about 30% of basal levels in 6 hours), whereas abrupt interruption of dietary folate leads to a fall in serum folate levels in about 3 weeks. In the plasma, one-third of the folate is free, two-thirds are nonspecifically bound to serum proteins, and a small fraction binds soluble folate receptors. In contrast to cobalamin uptake, there is no specific *serum* transport protein that enhances cellular folate uptake.

Cellular Folate Uptake

Folate Receptors

Plasma 5-methyl-tetrahydrofolate and folic acid are rapidly transported into proliferating cells by specialized, high-affinity, glycosylphosphatidylinositol-anchored (membrane) folate receptor-α, which takes up these folates at physiologic (nanomolar) concentrations in serum.[41–43] The plasma membrane containing the folate–folate receptor complex then invaginates and forms an endosomal vesicle that moves into the cytoplasm along microtubules.[44] The perinuclear endosomal compartment then gets acidified to pH 6, which dissociates folate from folate receptors. The released folate then passes across the acidified endosome into the cytoplasm by a transendosomal pH gradient, which is mediated by either the PCFT, which closely colocalized with folate receptors, or a related moiety (Fig. 40.5).[36,45]

Folate receptor-α is critical to mediating the cellular uptake of folates in proliferating normal and malignant cells, in transport of folate across the placenta to the fetus,[42,46] across the choroid plexus into the brain,[47–49] and in renal conservation of folates.[45,50] Folate receptor-α is expressed in several types of cancer cells, whereas folate receptor-β is expressed most in monocytes and macrophages[51–53]; hence these two forms of folate receptors are under investigation for potential clinical use in detecting (and treating) occult malignancy and inflammation, respectively.[51–55]

The physiologic role of reduced-folate carriers is less clear; it is a low-affinity but high-capacity system that can also mediate the uptake of 5-methyl-tetrahydrofolate and pharmacologic folates (like methotrexate and folinic acid well, but folic acid poorly) into a variety of cells at physiologic pH.[36,45] Induction of the upregulation of reduced-folate carriers via the blood-brain barrier is being investigated as a means to improve folate transport into the brain when there is either hereditary or acquired perturbation of the physiological components of brain folate transport (folate receptors and PCFT).[56]

Folate Receptor Regulation and Cellular Folate Homeostasis

Cell surface folate receptor-α is upregulated in response to low extracellular and intracellular folate concentrations through transcriptional, translational, and posttranslational mechanisms.[57–62] During cellular folate deficiency the accumulation of intracellular homocysteine leads to its covalent binding to a cytoplasmic protein known as heterogeneous nuclear ribonucleoprotein-E1 (hnRNP-E1), which mediates translational upregulation of folate receptor-α.[58,63] Homocysteinylation of hnRNP-E1 at specific cysteine–cysteine disulfide bonds leads to the unmasking of an underlying messenger RNA (mRNA)-binding pocket for which folate receptor-α mRNA has a high affinity. This RNA-protein interaction then triggers the biosynthesis of folate receptors, which can then bind available folate and normalize cellular folate levels; in turn, this reduces the intracellular homocysteine concentration to baseline.[62] In this context, hnRNP-E1 fulfills criteria as a cellular sensor of physiologic folate deficiency (Fig. 40.6) because this protein is able to sense folate deficiency by interacting with homocysteine—a sensitive indicator of folate deficiency—and then respond by increasing RNA-protein interaction that triggers the biosynthesis and upregulation of folate receptors and restores folate status to normal.[62,64]

The broader significance of this mechanism is that *homocysteinylated hnRNP-E1 actually orchestrates a nutrition-sensitive posttranscriptional RNA operon during folate deficiency*. This is because the unmasked mRNA-binding pocket within homocysteinylated-hnRNP-E1, which is highly promiscuous, allows for binding of a variety of very diverse mRNAs (perhaps over 100), all of which have a common password composed of short RNA sequences; these RNA-protein interactions can, in turn, trigger the modulation (up or down) of a variety of several otherwise entirely unrelated proteins that likely contribute to the biologic features of reduced cell proliferation, differentiation, and apoptosis, which are a hallmark of folate deficiency.[62,65]

In the nucleus, folate receptors can also function as a transcription factor by binding to cis-regulatory elements at promoter regions of *Fgfr4* and *Hes1* to regulate their expression.[66]

Folate Receptors and Placental Folate Transport

Human placental folate receptors[67,68] that are abundant and polarized only on the maternal-facing microvillous membrane of the syncytiotrophoblast[69] are critical to transplacental maternal-to-fetal folate transport.[46] Physiologic transplacental folate transport relies on the continued provision of adequate dietary folate intake by the mother. Following capture of maternal folate by placental folate receptors,[43,70] the displacement of this pool by incoming dietary folates results in an intervillous blood concentration that is three times that of maternal blood. This allows for subsequent transfer of the folate to the fetal circulation along a downhill concentration gradient[46] and ensures continued unidirectional transplacental folate transport. Thus a suboptimum intake of folate by the mother can reduce maternal-to-fetal folate transfer and predispose the embryo/fetus to serious developmental defects.[71–73]

Because PCFT colocalizes with folate receptor-α, it is suggested that following binding and internalization of folate into low-pH endosomes, the folate dissociates from folate receptors and presumably passes via PCFT into the cytoplasm. However, because

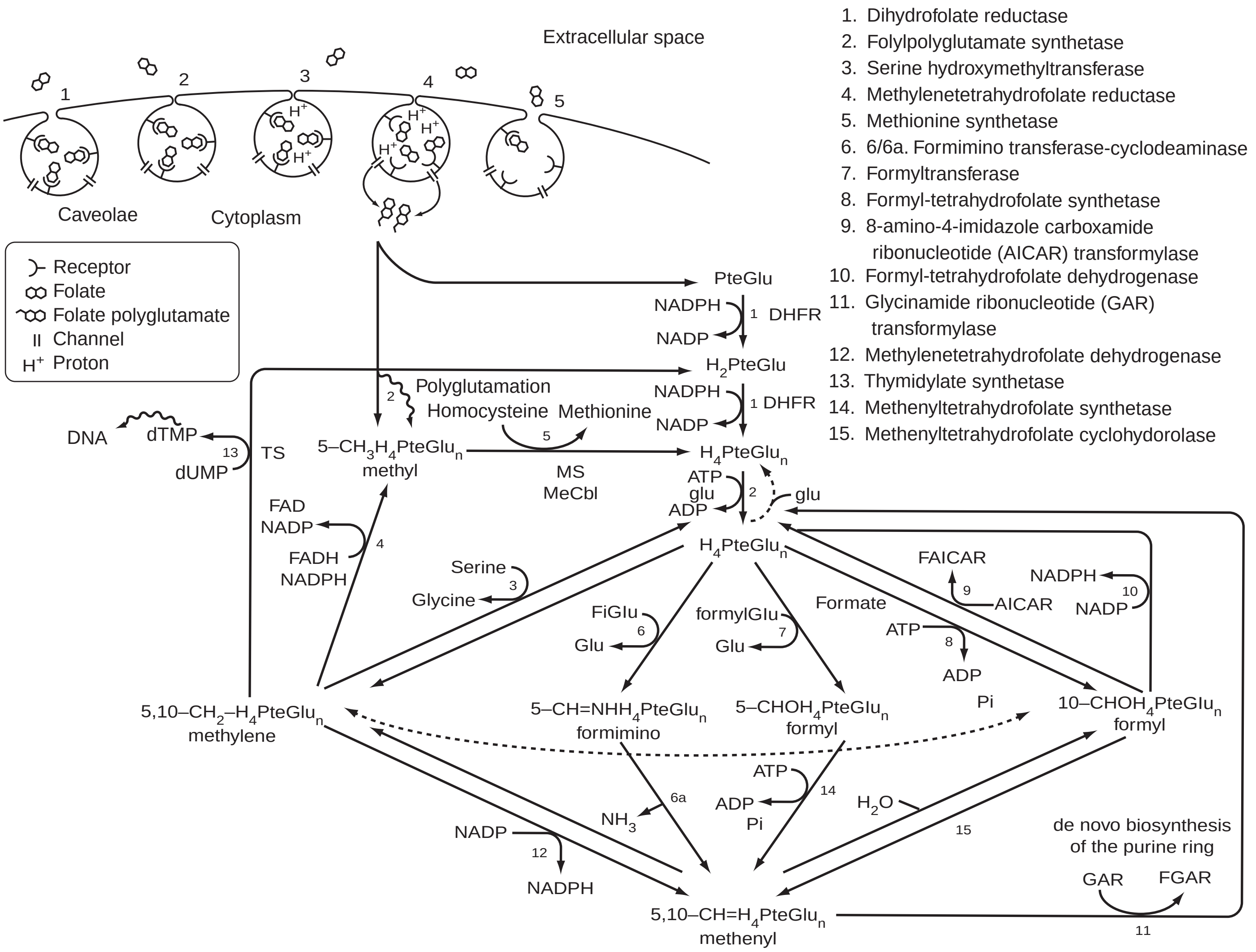

Extracellular space
1
2
3
4
5
H+
Caveolae
Cytoplasm
Receptor
Folate
Folate polyglutamate
Channel
H+ Proton
1. Dihydrofolate reductase
2. Folylpolyglutamate synthetase
3. Serine hydroxymethyltransferase
4. Methylenetetrahydrofolate reductase
5. Methionine synthetase
6. 6/6a. Formimino transferase-cyclodeaminase
7. Formyltransferase
8. Formyl-tetrahydrofolate synthetase
9. 8-amino-4-imidazole carboxamide ribonucleotide (AICAR) transformylase
10. Formyl-tetrahydrofolate dehydrogenase
11. Glycinamide ribonucleotide (GAR) transformylase
12. Methylenetetrahydrofolate dehydrogenase
13. Thymidylate synthetase
14. Methenyltetrahydrofolate synthetase
15. Methenyltetrahydrofolate cyclohydorolase
PteGlu
NADPH
NADP
1 DHFR
H_2PteGlu
Polyglutamation
Homocysteine
Methionine
DNA
dTMP
dUMP
13
TS
5–CH_3H_4PteGlu$_n$
methyl
MS
MeCbl
H_4PteGlu$_n$
ATP
glu
ADP
glu
FAD
NADP
FADH
NADPH
Serine
Glycine
FAICAR
AICAR
NADPH
NADP
FiGlu
Glu
formylGlu
Glu
Formate
ATP
ADP
Pi
5,10–CH_2–H_4PteGlu$_n$
methylene
5–CH=NHH_4PteGlu$_n$
formimino
5–$CHOH_4$PteGlu$_n$
formyl
10–$CHOH_4$PteGlu$_n$
formyl
6a
NH_3
ADP
Pi
H_2O
NADP
12
NADPH
de novo biosynthesis of the purine ring
GAR
FGAR
5,10–CH=H_4PteGlu$_n$
methenyl
11

Figure 40.5 FOLATE RECEPTOR–COUPLED FOLATE UPTAKE AND INTRACELLULAR ONE-CARBON METABOLISM INVOLVING FOLATES.[312] See text for details. The channel within the caveolae/endocytotic vesicle is related to the proton-coupled folate transporter (PCFT). The contribution of the reduced folate carrier–mediated transport and passive diffusion of folate into cells is not shown. *ADP*, Adenosine diphosphate; *ATP*, adenosine triphosphate; *DHFR*, dihydrofolate reductase; *DNA*, deoxyribonucleic acid; *dTMP*, deoxythymidine monophosphate; *dUMP*, deoxyuridine monophosphate; *FAD*, flavin adenine dinucleotide; *FADH*, the reduced form of flavin adenine dinucleotide; *FiGlu*, formimino glutamic acid; *Glu*, glutamic acid; *NADP*, nicotinamide adenine dinucleotide phosphate; *NADPH*, the reduced form of nicotinamide adenine dinucleotide phosphate. *(Modified from Shane B, Stokstad EL. Vitamin B12-folate interrelationships. Annu Rev Nutr. 1985;5:115; and Rothberg KG, Ying Y, Kolhouse JF, et al. The glycophospholipid-linked FR internalizes folate without entering the clathrin-coated pit endocytic pathway. J Cell Biol. 1990;110:637.)*

both PCFT and reduced-folate carriers are uniformly distributed in microvillous membrane, cytoplasm, and the fetal-facing basal plasma membrane,[69] the following events are all still unclear in human transplacental folate transport to the fetus: the precise handover of folate following endocytosis into the cytoplasm, the potential role of transcytosis of vesicles containing folate, the transport of folate across the syncytiotrophoblast basement membrane into the fetal vasculature, and the role of MRPs.

Folate Receptors in Embryonic and Fetal Development

Folate receptor-α is among the earliest genes activated in embryonic stem cells[74] when there is the need for increased folate requirements to support DNA synthesis during spectacular bursts of intense cell proliferation—ranging as short as 2 to 3 hours during brief windows within the proliferative zone in the epiblast.[16,72] Thus maternal folate deficiency compromises embryonic and fetal development—and probably even accounts for early miscarriages, as suggested by experimental studies in mice.[60]

Both early-stage neural tube cells and neural crest cells abundantly express folate receptor-α. Experimental perturbation of folate receptor-α[60,71,73,75–77] can lead to profound abnormalities in neural-tube closure leading to neural-tube defects (NTDs), as well as in heart, facial, and eye development which are collectively referred to as neurocristopathies. These neurocristopathies derive from abnormal proliferation and/or migration and/or differentiation of neural crest cells and result in cleft lip or cleft palate, endocardial cushion defects, and other midline defects.[71–73] Such studies are consistent with a physiologic role for folate receptor-α and the importance of its functioning normally to prevent the development of NTDs and neurocristopathies. Thus congenital heart defects, comprising approximately 1% of live births, and NTDs, among the most common congenital neurologic birth defects, are both dependent on the fidelity of folate receptor-α expression, precisely at the right time and in the right cells during early cardiac and neurologic development. The finding of a significant increase in blocking autoantibodies against placental

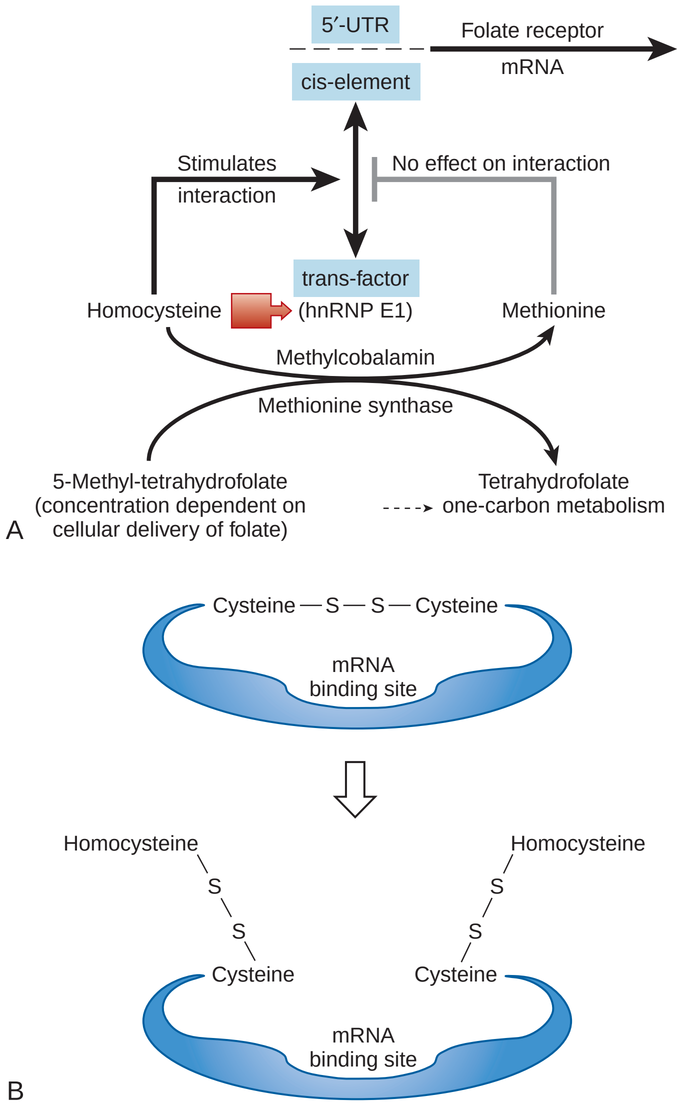


Figure 40.6 MODEL FOR HOW THE CELL SENSES FOLATE DEFICIENCY AND RESPONDS BY UPREGULATING FOLATE RECEPTORS. This model links perturbed folate metabolism, ribonucleic acid (RNA)–protein interaction, and coordinated translational regulation of folate receptors to optimize cellular folate uptake and restore folate homeostasis. The prominent *red arrow* highlights the critical role of heterogeneous nuclear ribonucleoprotein E1 (hnRNP-E1) as a sensor of cellular folate deficiency. (A) Reduced folate availability results in inactivation of methionine synthase and intracellular homocysteine build-up, which induces a direct posttranslational *S*-homocysteinylation of hnRNP-E1 via targeted homocysteine-*S-S*-cysteine mixed disulfide bonds; this results in the unmasking of a high-affinity folate receptor messenger RNA (mRNA) cis-element binding site and the RNA-protein interaction leads to increased translation of folate receptor-α. The net effect is a homeostatic response that aims to restore intracellular folate concentrations to normal by upregulating cell surface folate receptor. Folate repletion reactivates methionine synthase, which converts homocysteine to methionine, which has no effect on the RNA–protein interaction. As a result, the reduction in cellular homocysteine leads to reduced folate receptor-α synthesis and its downregulation. (Note: other metabolic pathways involving homocysteine are not included.) (B) A proposed mechanism for the unmasking of a cryptic mRNA binding site in hnRNP-E1 following the covalent binding of L-homocysteine, through the replacement of one (of many potential) cysteine disulfide bonds by protein-cysteine-*S-S*-homocysteine mixed disulfide bonds. *5′-UTR*, 5′ Untranslated region. *(From Tang YS, Khan RA, Zhang Y, et al. Incrimination of heterogeneous nuclear ribonucleoprotein E1 (hnRNP-E1) as a candidate sensor of physiological folate deficiency. J Biol Chem. 2011;286:39100.)*

folate receptor-α in women with pregnancy complicated by NTDs[78] is a striking human correlate of experimental studies in mice using antibodies, but the causal relationship warrants additional study.[76] Folate receptor-α also provides folate during neuronal regeneration and repair after injury where DNA methylation is also involved.[79]

Folate Receptors and Proton-Coupled Folate Transporter in Cerebral Folate Transport Across the Choroid Plexus

The pathway of folate receptor–α dependent basolateral-to-apical transcytosis within vesicles across the choroid plexus cells followed by exosome-mediated folate delivery across the cerebrospinal fluid (CSF) into the brain parenchyma has been characterized.[47] Briefly, 5-methyl-THF is bound by glycosyl-phosphatidyl inositol (GPI)-anchored folate receptor-α on the basolateral surface of choroid plexus cells. Following endocytosis there is sequential transfer of folate receptor-α-bound folate from an early- to a later-endosomal compartment called multivesicular bodies. The inward budding of the limiting membrane of multivesicular bodies leads to formation of several intraluminal vesicles that now contain outward-facing folate receptor-α. Following transcytosis of multivesicular bodies (containing their cargo of intraluminal vesicles) and eventual fusion with the apical membrane, there is discharge of these intraluminal vesicles as 40 to 100 nm exosomes into the CSF. These exosomes (containing outward oriented folate receptor-α-bound folates) then cross the ependymal cell layer and enter the brain parenchyma.[47]

Some of the endocytosed folate receptor-α-bound folate is transported out of acidified endosomal compartments via PCFTs into the choroid plexus cell cytoplasm.[47] However, more information on the cooperative function of folate receptor-α and PCFT in folate transport to the human brain is required, particularly because loss of PCFT-mediated function in hereditary folate malabsorption profoundly compromises choroid plexus transport of folates.[36,80]

Renal Retention of Folates (and Cobalamin)

After glomerular filtration, luminal folate binds folate receptor-α in the brush border membranes of proximal renal tubular cells and is internalized rapidly by folate receptor-α–mediated endocytosis; in the low pH of endocytotic vesicles, there is dissociation of folates and slow transport across basolateral membranes into the blood, with recycling of apo-folate receptor-α back to the luminal brush border membrane.[42,44] A large 550-kDa membrane protein called megalin, which interacts with CUBN and is found in renal proximal epithelial cells, functions as a multiligand receptor for a variety of macromolecules.[81,82] Megalin also binds to and mediates endocytosis of TCII-cobalamin complexes as well as filtered folate bound to soluble folate-binding proteins in kidney proximal tubules.[83]

INTRACELLULAR ONE-CARBON METABOLISM AND COBALAMIN–FOLATE RELATIONSHIPS

Cellular Folate Retention and One-Carbon Metabolism

Polyglutamylation of folate by the enzyme folylpolyglutamate synthase catalyzes the addition of multiple glutamate equivalents to their γ-carboxyl residue (see Fig. 40.4 and 40.5). In most eukaryotic cells, the pentaglutamate and hexaglutamate forms predominate. Polyglutamylation retains folates within cells; in addition, polyglutamylated folates are more efficient substrates for folate-dependent enzymes. In human erythrocytes (red blood cells [RBCs]), folate is accumulated at earlier stages within the marrow by folate receptors[84,85]; on maturation, more than 90% of $H_4PteGlu_{(n)}$ molecules interact with hemoglobin, which, because of its high capacity, assists in intracellular folate retention.[16]

Compartmentalization and Channeling of Folate Metabolism

In an elegant example of conservation of resources, metabolic pathways involving folate are compartmentalized within cells as multienzyme complexes that shuttle one-carbon units along set paths toward key reactions leading to pyrimidine and purine biosynthesis. The major form of folate transported into the cell is 5-methyl-tetrahydrofolate (5-methyl-THF; 5-methy-H_4PteGlu) (see Fig. 40.5); by contrast, folic acid (pteroylmonoglutamate (PteGlu) requires reduction to tetrahydrofolate by dihydrofolate reductase in a two-step reaction (PteGlu to *dihydro*pteroylglutamate [H_2PteGlu] to *tetrahydro*pteroylglutamate [H_4PteGlu, or THF]). After cellular uptake, 5-methyl-THF must first be converted to THF via methionine synthase (in the methylation cycle). This is a key reaction because THF is the preferred physiologic substrate for folylpolyglutamate synthase, which adds multiple glutamate moieties to THF (see Fig. 40.4). Only then can the polyglutamylated form of THF participate in one-carbon metabolism where it can be converted to either 10-formyl-THF—used in de novo biosynthesis of purines—or to 5,10-methylene-THF—used for synthesis of thymidylate. Moreover, 5,10-methylene-THF and 10-formyl-THF can be interconverted by intermediates (see Fig. 40.5).

Folate metabolism and folate-dependent enzymes are compartmentalized: approximately 40% are in the mitochondrial matrix, 50% in the cytoplasm, and 10% in the nucleus.[86] The methylation reaction occurs in the cytosol whereas thymidylate synthesis occurs in the nucleus. The mitochondrial compartment, which is *not* shown in Fig. 40.5, contains its complement of folate cofactors, and homologues of the major cytosolic enzymes. For example, cytoplasmic 5-methyl-THF and 5-formyl-THF can enter mitochondria by a mitochondria-specific reduced-folate carrier,[87] whereas SAM, which is also required for mitochondrial methylation reactions, enters mitochondria by a specific transporter.[88] Other one-carbon donors like serine, glycine, dimethylglycine, and sarcosine also enter mitochondria and ultimately generate formate that crosses back into the cytoplasm. In the cytoplasm, C1-THF synthase, a trifunctional enzyme, uses this mitochondria-derived formate with THF to form 10-formyl-THF, which is required for the de novo synthesis of purines (see Fig. 40.5); this enzyme can also catalyze the interconversion of THF, 10-formyl-THF, 5,10-methenyl-THF, and 5,10-methylene-THF. In this way, the continued delivery of mitochondrial formate helps perpetuate cytoplasmic one-carbon metabolism.[86] Another major entry point of one-carbon units into cytoplasmic folate metabolism is through the formation of 5,10-methylene-THF from serine (which is derived from glycolytic intermediates); here the enzyme serine hydroxymethyltransferase catalyzes the addition of carbon 3 from serine to THF to give rise to the key intermediate coenzyme 5,10-methylene-THF.[86] After 5,10-methylene-THF is converted to 5-methyl-THF by the enzyme methylenetetrahydrofolate reductase, it can be used in the methylation cycle, which involves methylation of homocysteine via methionine synthase to form methionine and tetrahydrofolate. After 5,10-methylene-THF is converted (via intermediates) to 10-formyl-THF, it can be used for purine nucleotide biosynthesis involving de novo synthesis of purine nucleotides for DNA and RNA. 5,10-Methylene-THF can also be used in the thymidylate cycle via the enzyme thymidylate synthase, which generates thymidylate (by converting deoxyuridine monophosphate [dUMP] to deoxythymidine monophosphate [dTMP]) for DNA synthesis (see Fig. 40.5). Parenthetically, this nuclear one-carbon metabolism compartment is activated during S phase of the cell cycle, following a posttranslational modification of folate-dependent enzymes like thymidylate synthase, serine-hydroxymethyltransferase, and dihydrofolate reductase by specialized small ubiquitin-like modifier (or SUMO) proteins; this modification allows the entry and enrichment of these sumoylated enzymes into the nucleus[89] to provide a buffer against the stress of imminent folate deficiency.[90]

Methylation

After methionine is generated, it can be converted to a methyl donor through its adenosylation to SAM. SAM is a universal donor of methyl groups for critically important biologic methylation reactions involving over 80 proteins, membrane phospholipids, the synthesis of neurotransmitters, RNA, DNA, and histones. Among these, DNA methylation is a major epigenetic mechanism that is central to the regulation of several cellular functions such as gene transcription, chromatin structure, imprinting, development, and genomic instability. DNA is highly methylated in CpG sequences (over 50%); here a methyl group is targeted to the DNA base cytosine in the context of a CpG dinucleotide by DNA methyltransferases. Methylation confers a condensed structure and transcriptional repression, whereas hypomethylation does the opposite. Altered patterns of DNA methylation, particularly hypomethylation involving growth-promoting genes, or hypermethylation of tumor suppressor genes, are popular contemporary themes in our understanding of the epigenetic changes in DNA and the genesis of cancer. In addition, histone hypomethylation can alter gene expression. After these transmethylation reactions that use SAM, the immediate product of these reactions is *S*-adenosylhomocysteine (SAH), which is converted to homocysteine by SAH hydrolase. The SAM to SAH ratio regulates the balance of such cellular methylation reactions.

Dietary folate deficiency can lead to hypomethylation in animals, whereas folate consumption supports normal patterns of methylation. However, analysis in humans has not yielded consistent results, nor has the correlation of folate status and DNA methylation been sufficiently rigorously studied.

Consequences of Perturbed One-Carbon Metabolism

Methyl-Folate Trapping

Because of the critical role of methylcobalamin for methionine synthase, a deficiency of cobalamin inactivates methionine synthase and results in the accumulation of the substrate 5-methyl-THF; this so-called methyl-folate trap results because the upstream enzyme reaction involving methylenetetrahydrofolate reductase (which converts 5,10-methylene THF to 5-methyl-THF in preparation for the methionine synthase reaction) is also irreversible. Therefore when 5-methyl-THF accumulates and cannot be converted to THF, it readily leaks out of the cell. (This explains why patients with cobalamin deficiency can have normal to high serum folate values even in the face of tissue folate deficiency). The ensuing intracellular THF deficiency compromises one-carbon metabolism and initiates the pathophysiologic cascade leading to perturbed DNA synthesis and megaloblastosis.

Thymidylate Deficiency and Perturbed DNA Synthesis

With either cobalamin or folate deficiency, there will be a net decrease in 5,10-methylene-THF that interrupts the thymidylate synthase–mediated conversion of dUMP to dTMP. (Although salvage pathways for purine synthesis can compensate for reduced generation of purines through one-carbon metabolism, salvage pathways cannot compensate for reduced thymidine.) This results in a high dUMP/dTMP ratio and an increase in deoxyuridine triphosphate (dUTP), which can get misincorporated into DNA. At this juncture, an editorial enzyme recognizes this faulty misincorporation and excises dUTP. However, with a continued inadequate supply of deoxythymidine triphosphate (dTTP) there is a continued cycle of uracil misincorporation into DNA in folate deficiency,[91–93] its removal by uracil-DNA glycosylase,[93] and refilling of the missing base by DNA polymerase β.[94] However, with repetition over several cycles, multiple single-strand nicks are introduced into DNA; this predisposes to chromosome breaks[95] that can contribute to an increased risk of cancer associated with folate deficiency.[92,96,97] In addition, folate deficiency can also lead to double-strand breaks in DNA, which are difficult to repair when the two nicks are close to one another (within 12 bp of each other) on opposite strands.[98] Collectively, such double-strand DNA breaks in folate-deficient cells predispose to the development of acentric chromosomes, DNA fragments, and micronuclei.[95,99] This can also render folate-deficient epithelial tissues more permissive to the genomic integration of HPV16 DNA, and trigger HPV16-induced cancer.[100,101]

Chromosome and Cell Cycle Defects

Defective DNA synthesis caused by folate deficiency is reflected by numerous chromosomal abnormalities, including abnormalities in telomeres, which correlate with biomarkers of chromosomal instability and mitotic dysfunction.[102] There is excessive chromosomal elongation with despiralization associated with random breaks and exaggerated centromere constriction, expression of folate-sensitive fragile sites in hematopoietic cells, and reduced biosynthesis, acetylation, and methylation of arginine-rich histone.[23] All this leads to perturbation of the cell cycle with an increased proportion of cells in prophase of the mitotic cycle and G_2 that leads to apoptosis of erythroid precursors and anemia.[23]

MORPHOLOGIC EXPRESSION OF MEGALOBLASTOSIS

There is widening disparity in nuclear-cytoplasmic asynchrony as a cobalamin- or folate-deficient cell divides, until the more mature generations of daughter cells die in the marrow or are arrested (as megaloblastic cells) at various stages of the cell cycle.[16] All proliferating cells exhibit megaloblastosis, including the luminal epithelial mucosal cells of the entire gastrointestinal tract, cervix, vagina, and uterus.[16] However, megaloblastic changes are most striking in the blood and bone marrow. Ineffective hematopoiesis extends into long bones, and the bone marrow aspirate—which is superior to the biopsy for observing megaloblastosis—exhibits trilineal hypercellularity, especially of the erythroid series. Indeed, the plethora of bone marrow morphologic changes can lead an untrained observer to the diagnosis of erythroleukemia. The appearance of exuberant cell proliferation with numerous mitotic figures is misleading because these cells are actually proliferating very slowly (see box on Morphology in Megaloblastosis From Cobalamin and Folate Deficiency).

Erythroid hyperplasia reduces the myeloid-to-erythroid ratio from 3:1 to 1:1. Proerythroblasts are not as obviously abnormal as later forms; they may simply be larger (promegaloblasts). Megaloblastic changes are most strikingly displayed in intermediate and orthochromatic stages, which are larger than their normoblastic counterparts. In contrast to the normally dense chromatin of comparable normoblasts, megaloblastic erythroid precursors have an open, finely stippled, reticular, and sieve-like pattern (Fig. 40.7). The orthochromatic megaloblast, with its hemoglobinized cytoplasm, continues to retain its large sieve-like immature nucleus, in sharp contrast to the clumped chromatin of orthochromatic normoblasts. The nucleus is often eccentrically placed in these large oval or oblong cells, and lobulation or indentation of nuclei with bizarre karyorrhexis is often seen. In cells destined for the circulation as macro-ovalocytes, the nucleus may occasionally not be completely extruded. Of the potential progeny of proerythroblasts that develop into later megaloblastic forms, 80% to 90% die in the bone marrow. Marrow macrophages effectively scavenge dead or partially disintegrated megaloblasts. This is the basis for ineffective erythropoiesis (intramedullary hemolysis).

Leukopoiesis is also abnormal. There is an absolute increase in these cells, which are large and have similar sieve-like chromatin. Spectacular giant (20 to 30 µm) metamyelocytes and “band” forms that are often seen are pathognomonic for megaloblastosis (see Fig. 40.7). There may be bizarre nucleoli with small cytoplasmic vacuoles. It is probable that giant metamyelocytes cannot easily traverse marrow sinuses, and their maturation into circulating hypersegmented polymorphonuclear neutrophils (PMNs) is unlikely. Granulation of the cytoplasm remains unaffected.

Megakaryocytes may be normal or increased in numbers and may exhibit additional complexities in megaloblastic expression (see Fig. 40.7). Complex hypersegmentation (i.e., pseudohyperdiploidy) is associated with liberation of fragments of cytoplasm and giant platelets into the circulation. The net output of platelets is decreased in severe megaloblastosis, and abnormal but reversible platelet dysfunction has been documented.[23]

In early cobalamin or folate deficiency, normoblasts may dominate the marrow with only a few megaloblasts seen. Complete transformation to megaloblastic hematopoiesis is observed in florid cases and is reflected by various degrees of pancytopenia.

The earliest manifestation of megaloblastosis is an increase in mean corpuscular volume (MCV) with macro-ovalocytes (up to 14 µm) (see Fig. 40.7). Because these cells have adequate hemoglobin, the central pallor, which normally occupies about one-third of the cell, is decreased. By contrast, thin macrocytes have larger than normal central pallor (Table 40.1). In severe anemia, poikilocytosis and anisocytosis are evident. Cells containing remnants of DNA (i.e., Howell-Jolly bodies), arginine-rich histone, and nonhemoglobin iron (i.e., Cabot rings) may be observed. Extramedullary megaloblastic hematopoiesis may also result in a leukoerythroblastic picture.

Nuclear hypersegmentation of DNA in PMNs strongly suggests megaloblastosis when associated with macro-ovalocytosis (see Fig. 40.7). Normally fewer than 5% of PMNs have more than five lobes, and no cells have more than six lobes in the peripheral blood; megaloblastosis is suggested when greater than 5% PMNs have more than five lobes or 1% PMN have more than six lobes. (See box on Diagnostic Issues: Information From the Peripheral Smear and Bone Marrow Aspirate.)

Ineffective use of iron results in an increased percentage of saturation of transferrin and increased iron stores. If there is associated iron deficiency, the MCV may be normal, and only iron therapy can unmask the megaloblastic manifestations in the peripheral blood. In thalassemia, the entire erythrocyte morphology normally expected in megaloblastosis is masked[16]; however, megaloblastic leukopoiesis is still observed. Significant intramedullary hemolysis (ineffective erythropoiesis) involving more than 90% of megaloblastic precursors is reflected by a lowered absolute reticulocyte count, increased bilirubin (up to 2 mg/dL), decreased haptoglobin, and increased lactate dehydrogenase (LDH) often above 1000 units/mL. There is also a modest decrease in the circulating RBC life span. (See box on Masked Megaloblastosis.)

Megaloblastosis in rapidly proliferating cells of the gastrointestinal tract leads to a variable degree of morphologic changes and atrophy of luminal epithelial cells. This leads to functional defects, which can include malabsorption of cobalamin and folate in some patients. A

MORPHOLOGY IN MEGALOBLASTOSIS FROM COBALAMIN AND FOLATE DEFICIENCY IS THE SAME

Peripheral Smear

- Increased mean corpuscular volume (MCV) with macro-ovalocytes (up to 14 µm), which is variously associated with anisocytosis and poikilocytosis
- Nuclear hypersegmentation of polymorphonuclear neutrophils (PMNs) (1% PMN with six lobes or 5% with five lobes)
- Thrombocytopenia (mild to moderate)
- Leukoerythroblastic morphology (from extramedullary hematopoiesis)

Bone Marrow Aspirate

- General increase in cellularity of all three major hematopoietic elements
- Abnormal erythropoiesis—orthochromatic megaloblasts
- Abnormal leukopoiesis—giant metamyelocytes and “band” forms (pathognomonic), hypersegmented PMNs
- Abnormal megakaryocytopoiesis—pseudo hyperdiploidy

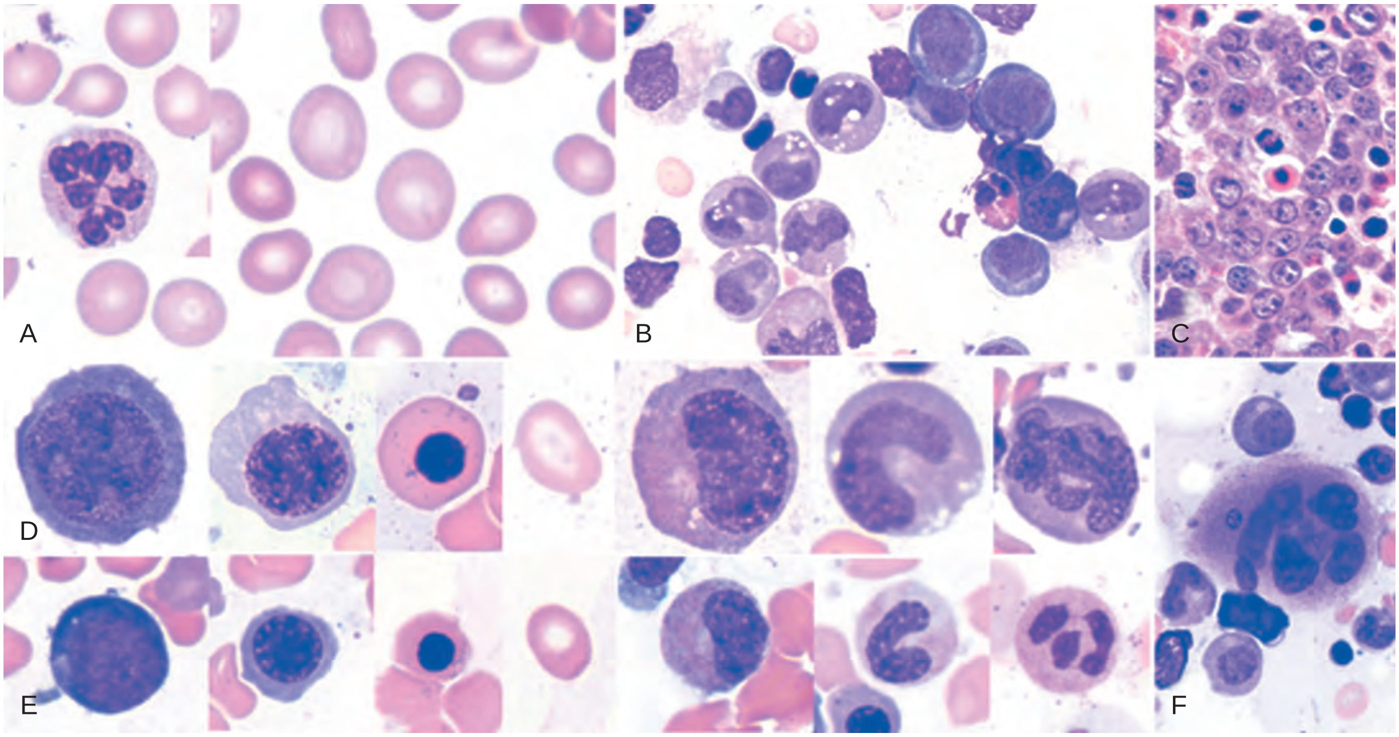


Figure 40.7 MEGALOBLASTIC ANEMIA. The peripheral smear (A) exhibits macro-ovalocytosis and a hypersegmented polymorphonuclear leukocyte *(inset)*. The bone marrow aspirate (B) shows megaloblastic changes in both granulopoiesis and erythropoiesis. The biopsy (C) is hypercellular and shows sheets of immature erythroid precursors with the appearance of a high mitotic rate. These can mimic acute erythroleukemia or even metastatic tumor cells. Details from the cells in the aspirate (D) compared with normal hematopoiesis at same magnification (E). Note the giant metamyelocyte and band form. In megaloblastic anemia, megakaryocytes also have nuclear atypia, including abnormal nuclear segmentation (F). Thus, the bone marrow aspiration and biopsy should be assessed for morphology, special stains, flow cytometry, and cytogenetic analysis. If the marrow is not obviously megaloblastic but the iron stain reveals absent stores, review the morphologic evaluation again with special emphasis on granulocytic precursors and promegaloblasts, and look for more subtle megaloblastic changes.

TABLE 40.1 Clinical Conditions Not to Be Confused With Megaloblastosis

Macrocytosis[a] Without Megaloblastosis[b]
Reticulocytosis
Liver disease
Aplastic anemia
Myelodysplastic syndromes (especially 5q–)
Multiple myeloma
Hypoxemia
Smokers
Spurious Increases in MCV Without Macro-Ovalocytosis[c]
Cold agglutinin disease
Marked hyperglycemia
Leukocytosis
Older individuals

[a] The central pallor that normally occupies about one-third of the normal red blood cell is decreased in macro-ovalocytes. This contrasts with the finding of thin macrocytes, in which the central pallor is increased.

[b] Although megaloblastosis implies that a bone marrow test has been performed, with the addition of highly sensitive tests for the specific diagnosis of cobalamin and folate deficiency, the need for a bone marrow test is often dictated by the urgency to make the diagnosis.

[c] When the Coulter counter readings of a high MCV are not confirmed by looking at the peripheral smear.

MCV, Mean corpuscular volume.

vicious cycle whereby megaloblastosis begets more megaloblastosis is established that can be interrupted only by specific therapy with cobalamin or folate.

NEUROLOGIC DYSFUNCTION WITH COBALAMIN DEFICIENCY

Because megaloblastosis caused by folate or cobalamin deficiency leads to a functional folate coenzyme deficiency, the morphologic manifestations of both deficiencies are understandably indistinguishable. However, only cobalamin deficiency results in a patchy demyelination process, which is expressed clinically as cerebral abnormalities and subacute combined degeneration of the spinal cord.[16] The precise role of cobalamin in maintaining the integrity of the central nervous system has not been completely defined (see box on Clues for Distinguishing Cobalamin and Folate Deficiencies).

The demyelinating process involves patchy swelling of the myelin sheath followed by its breakdown (demyelination), leading to axonal degeneration. Microscopic foci coalesce with one another, giving the surface of the spinal cord (on cross section) a spongy appearance; later there is secondary Wallerian degeneration of long tracts. Patchy demyelination usually begins in the dorsal columns in the thoracic segments of the spinal cord (Fig. 40.8) and then spreads contiguously to involve corticospinal tracts. These lesions spread throughout the length of the cord and ultimately involve spinothalamic and spinocerebellar tracts. There is also degeneration of the dorsal root ganglia, celiac ganglia, the Meissner plexus, and the Auerbach plexus.

DIAGNOSTIC ISSUES: INFORMATION FROM THE PERIPHERAL SMEAR AND BONE MARROW ASPIRATE

Although not specific for megaloblastic anemia, macro-ovalocytes are the hallmark of megaloblastosis (see Fig. 40.7). However, only one-half with MCV values greater than 105 fL may have vitamin deficiency. In almost one-half of all cases, macrocytosis per se is not associated with megaloblastosis, and additional tests are necessary for complete diagnosis.

The frequency of hypersegmented PMNs (5% with five lobes or 1% with six-lobed PMNs) in patients with megaloblastic hematopoiesis is 98%. The sensitivity decreases to 78% in alcoholics, although the specificity of this finding is approximately 95%. With a combination of hypersegmented PMNs and macro-ovalocytosis, the specificity is 96%–98%, and the positive predictive value of folate or cobalamin deficiency is about 94%.[23] Hypersegmentation of PMNs is insufficiently sensitive, when compared with metabolite levels, to be used as a clinical tool in the diagnosis of mild cobalamin deficiency.[23]

Diagnostic Bone Marrow Aspiration

With the addition of highly sensitive serum tests for the specific diagnosis of cobalamin and folate deficiency, the need for a bone marrow test is often dictated by the urgency to diagnose megaloblastosis (with results available in an hour). For example, in the case of florid hematologic disease with or without neurologic disease suggestive of cobalamin or folate deficiency, bone marrow aspiration carried out as soon as possible is invaluable in assisting the rapid diagnosis of megaloblastosis. However, in the outpatient setting, when the patient has a characteristic peripheral smear, or for a patient with a primary neuropsychiatric presentation, a case can be made to initiate the sequence of diagnostic tests without bone marrow aspiration by proceeding with measurement of serum levels of vitamins or metabolites (see Table 40.2). Conversely, in a pregnant patient with pancytopenia with macro-ovalocytes, hypersegmented polymorphonuclear neutrophils, and reticulocytopenia with a history of noncompliance with prenatal supplements (and no neurologic findings suggestive of cobalamin deficiency), bone marrow aspiration may not be necessary to initiate therapy for a strong presumptive diagnosis of folate deficiency. If there is no evidence of response within 10 days, bone marrow aspiration is indicated.

CLUES FOR DISTINGUISHING COBALAMIN AND FOLATE DEFICIENCIES

Although the megaloblastic manifestations of cobalamin and folate deficiencies are clinically indistinguishable, certain distinct patterns in mode of presentation provide clues to the type and cause of deficiency. In general, the cause of folate deficiency can be found in the patient's recent past (within 6 months), primarily discerned from the history and physical examination. In contrast, the cause of cobalamin deficiency can remain obscure until specific tests to define the cause are carried out. In the past, by the time anemia was symptomatic, more than 80% of patients had neurologic manifestations, and in 50% this led to some incapacity. Perhaps as a result of widespread use of multivitamins containing folic acid among patients and even in the food given livestock in the West and widespread folate-fortification of food, the hematologic expression of cobalamin deficiency is often substantially attenuated, leading to pure neurologic presentations. Studies highlight the apparent inverse correlation between hematologic and neurologic presentations such that in a third of patients with cobalamin deficiency, the earliest signs are often purely neurologic, and symptoms related to paresthesias and diminished proprioception may cause the patient to see the physician. Based on the multiple potential causes (see box on Etiopathophysiologic Classification of Cobalamin Deficiency and box on Etiopathophysiologic Classification of Folate Deficiency), the warning that "what the mind does not know, the eyes do not see" is a caveat that cannot be taken lightly; failure to recognize cobalamin deficiency as the cause of neurologic disease and treatment of cobalamin deficiency with folate, or misdiagnosis of megaloblastosis as erythroleukemia represent significant extremes of deviation from the dictum *primum non nocere*. Areas of overlap in the symptoms of cobalamin or folate deficiency are related to megaloblastosis (i.e., common cardiopulmonary and some gastrointestinal manifestations). Although pure folate deficiency in the alcoholic with thiamine deficiency (i.e., Wernicke encephalopathy) and peripheral neuropathy is almost indistinguishable from and may mimic cobalamin deficiency, the remaining neurologic manifestations are uniquely characteristic of cobalamin deficiency. Folate deficiency in *adults* has not been unequivocally shown to give rise to neurologic findings. Coexistence of folate deficiency with neurologic disease should prompt investigations to rule out cobalamin and other nutrient deficiencies arising from dietary insufficiency or malabsorption.

MASKED MEGALOBLASTOSIS

The term *masked megaloblastosis* is reserved for conditions in which true cobalamin or folate deficiency with anemia is not accompanied by classic findings of megaloblastosis in the peripheral blood and bone marrow. This occurs when there is a coexisting condition that neutralizes the tendency to generate megaloblastic cells (usually involving reduction in RBC hemoglobinization, as in iron deficiency or thalassemia).[23] A wide RBC distribution width (RDW) on the Coulter counter readout in the presence of a "normal" mean corpuscular hemoglobin or MCV may reflect megaloblastic anemia[23] or dimorphic anemia (macro-ovalocytes plus microcytic hypochromic RBCs). Because megaloblastic white blood cells and precursors are unaffected by deficient hemoglobinization, these pathognomonic findings (giant myelocytes and metamyelocytes, and hypersegmented PMNs) remain; the latter may persist for up to 2 weeks after replacement with cobalamin or folate.[16] The recognition of masked megaloblastosis should initiate investigations to rule out iron deficiency, anemia of chronic disease, or hemoglobinopathies. Appropriate replacement with cobalamin or folate elicits a maximal therapeutic benefit only when iron deficiency is corrected. Conversely, if combined iron and cobalamin deficiency (total gastrectomy or pernicious anemia) or iron and folate deficiency (pregnancy) is treated with iron alone, megaloblastosis will be unmasked.

Although demyelination may also extend to the white matter of the brain, it is unclear whether the peripheral neuropathy is caused by a distinct lesion or results from spinal cord disease; the clinical manifestations may be extremely varied.[16]

Vegetarians with cobalamin neuropathy in India[104] had cognitive impairment in nearly one-half of 36 patients; it was mostly global with impaired recall and "serial sevens" (which are useful bedside tests of attention); impaired naming was found among one-quarter of the patients. Patients can present with anxiety, apathy, aggression, irritability, hallucinations, delusions, and disinhibition. Nearly one-half had abnormal evoked potential (using the oddball auditory paradigm), which revealed P300 latency that was reversible in 3 months of cobalamin replacement; in one-fifth P300 was unrecordable. Objective tests to document cobalamin neuropathy[104] include nerve conduction studies and motor- and sensory-evoked potentials,[105,106] visual pathway abnormalities,[107] and magnetic resonance imaging (MRI) that shows T2 hyperintensity and atrophy. Myelopathy is present in nearly all patients. In 70% of patients, there is neuropathy which is mainly axonal with some demyelinating features[108]; but there may also be reversible frontal-subcortical neurobehavioral and cognitive abnormalities ascribed to cortical and subcortical dysfunction.[109,110]

Among another cohort of patients with cobalamin neuropathy from the United States, 65% had mild, about 25% had moderate, and about 10% had severe neurologic deficits.[23] Paresthesias or ataxia were most commonly the first symptoms, and diminished vibratory sensation and proprioception in the lower extremities were the most common objective early signs. Although multiple neurologic syndromes were often seen in the same patient, the spectrum of objective signs could include loss of fine or coarse touch, decreased or increased deep tendon reflexes with spasticity or muscle weakness, urinary or fecal incontinence, orthostatic hypotension, amaurosis, dementia, psychosis, or mood disturbances.[23] Overall, although the neurologic deficits were mild in most cases, the severity was judged related to the duration of symptoms before diagnosis; not unexpectedly, those with the shorter duration of symptoms responded most to appropriate replacement.

ETIOPATHOPHYSIOLOGIC CLASSIFICATION OF COBALAMIN DEFICIENCY

I. Nutritional cobalamin deficiency (insufficient cobalamin intake)—vegetarians, poverty-imposed near-vegetarians, breastfed infants of mothers with cobalamin deficiency
II. Abnormal intragastric events (inadequate proteolysis of food cobalamin)—atrophic gastritis, hypochlorhydria, proton pump inhibitors, H_2 blockers
III. Loss/atrophy of gastric oxyntic mucosa (deficient intrinsic factor [IF])—total or partial gastrectomy (bariatric surgery), adult and juvenile pernicious anemia, caustic destruction (lye ingestion)
IV. Abnormal events in the small bowel lumen
    A. Inadequate pancreatic protease (R factor–cobalamin not degraded, so cobalamin not transferred to IF)
        1. Insufficient pancreatic protease—pancreatic insufficiency
        2. Inactivation of pancreatic protease—Zollinger-Ellison syndrome
    B. Usurping of luminal cobalamin (inadequate binding of cobalamin to IF)
        1. By bacteria-stasis syndromes (blind loops, pouches of diverticulosis, strictures, fistulas, anastomosis), impaired bowel motility (scleroderma), hypogammaglobulinemia
        2. By *Diphyllobothrium latum* (fish tapeworm)
V. Disorders of ileal mucosa/IF-cobalamin receptors (IF-cobalamin not bound to IF-cobalamin receptors [cubam])
    A. Diminished or absent IF-cobalamin receptors (cubam)—ileal bypass/resection/fistula
    B. Abnormal mucosal architecture/function—tropical/nontropical sprue, Crohn disease, tuberculous ileitis, amyloidosis
    C. IF-cobalamin receptor (cubam) defects—Imerslund-Gräsbeck syndrome
    D. Drug-effects—metformin, cholestyramine, colchicine, neomycin
VI. Disorders of plasma cobalamin transport (transcobalamin [TCII]-cobalamin not delivered to TCII receptors)—congenital TCII deficiency, defective binding of TCII-cobalamin to TCII receptors (rare)
VII. Metabolic disorders (cobalamin unable to be utilized by cells)
    A. Inborn enzyme errors—cblA to cblJ disorders (rare)
    B. Acquired disorders (cobalamin inactivated by irreversible oxidation)—nitrous oxide

ETIOPATHOPHYSIOLOGIC CLASSIFICATION OF FOLATE DEFICIENCY

I. Nutritional causes
    A. Decreased dietary intake—poverty and famine, institutionalized individuals (psychiatric/nursing homes)/chronic debilitating disease, prolonged feeding of infants with goat's milk, special slimming diets or food fads (folate-rich foods not consumed), cultural/ethnic diets (devoid of folate-rich foods) or prolonged cooking techniques (food folate destroyed)
    B. Decreased diet and increased requirements
        1. Physiologic—pregnancy and lactation, prematurity, hyperemesis gravidarum, infancy
        2. Pathologic
            (a) Intrinsic hematologic diseases involving hemolysis with compensatory erythropoiesis, abnormal hematopoiesis, or bone marrow infiltration with malignant disease
            (b) Dermatologic disease—psoriasis
II. Folate malabsorption
    A. With normal intestinal mucosa
        1. Drugs—sulfasalazine, pyrimethamine, proton pump inhibitors (via inhibition of proton-coupled folate transporter [PCFT])
        2. Hereditary folate malabsorption (mutations in PCFTs) (rare)
    B. With mucosal abnormalities—tropical and nontropical sprue, regional enteritis
III. Defective folate transport across the choroid plexus into the cerebrospinal fluid—cerebral folate deficiency (mutation or autoantibodies to folate receptors) (rare)
IV. Inadequate cellular utilization
    A. Folate antagonists (methotrexate)
    B. Hereditary enzyme deficiencies involving folate
V. Drugs (multiple effects on folate metabolism)—alcohol, sulfasalazine, triamterene, pyrimethamine, trimethoprim-sulfamethoxazole, diphenylhydantoin, barbiturates, carbazepine.[103]

OTHER EFFECTS OF COBALAMIN AND FOLATE DEFICIENCY

Cobalamin deficiency more often than folate deficiency can also result in sterility from the effects on the gonads. An unexplained finding is generalized melanin pigmentation that is reversible by specific nutrient replenishment. Cobalamin deficiency negatively affects bone development and maintenance,[111] leading to reduced bone mineral density.[112–115] Folate is also important for maintenance of regulatory T cells[116]; this may explain findings of combined immunodeficiency in hereditary folate malabsorption.[117]

SPECTRUM OF CLINICAL PRESENTATIONS WITH COBALAMIN DEFICIENCY

The age-specific presentations with cobalamin deficiency are discussed in Nutritional Cobalamin Deficiency. Classic presentations of nutritional cobalamin deficiency in resource-limited countries are often accompanied by iron deficiency, and especially among malnourished populations, many will also have folate deficiency.[118] Among vegetarians in resource-limited countries, cases with nutritional cobalamin deficiency may present in the second and third decades with pancytopenia, mild hepatosplenomegaly, fever, and occasionally thrombocytopenic bleeding.[23] Alternatively, a neurologic and psychiatric syndrome may develop with or independent of anemia.[23] In over a quarter of patients with cobalamin neuropathy in the United States, there was no reduction in the hematocrit despite neurologic disease, and only a minority of patients had combined hematologic and neurologic disease. Indeed, the higher the hematocrit, the more severe the neurologic disorder! Conversely, anemic patients may have no neurologic deficits. Importantly, the level of cobalamin may have no correlation with either the existence or the severity of neurologic disease.

BIOCHEMICAL INDICATORS OF EVOLVING DEFICIENCY

Early manifestations of negative cobalamin balance are increased serum methylmalonic acid (MMA) and total homocysteine levels (Table 40.2).[23] *This can occur when the total cobalamin in serum is still in the low-normal range.* Continued negative cobalamin balance leads to an absolute decrease in serum cobalamin level below normal. Normal levels of MMA and homocysteine rule out clinically significant cobalamin deficiency with virtually 100% certainty.[23]

Likewise, metabolic evidence for folate deficiency (i.e., increased serum total homocysteine level) can be found when serum folates are still in the low-normal range.

Laboratory tests are more likely to be accurate when there is a high pretest probability of a particular disease. This can, however, be more vexing in the case of diagnosis of early cobalamin deficiency when symptoms are subtle, nonspecific, or not yet fully manifest. If there is macrocytosis and cobalamin levels are borderline or just below normal, an unequivocal elevation of MMA will support the diagnosis. However, in elderly patients with anemia, there may be several other causes for anemia, and depending on the population studied, cobalamin deficiency may be only one among several possible causes in the differential diagnosis. This is when a modified therapeutic trial—in which a patient is treated with full doses of both cobalamin and folate for 10 days—and the lack of objective response would effectively rule out cobalamin (and/or folate) deficiency as a cause (see box on

TABLE 40.2 Stepwise Approach to the Diagnosis of Cobalamin and Folate Deficiency

Megaloblastic Anemia or Neurologic-Psychiatric Manifestations Consistent with Cobalamin Deficiency *Plus* Test Results on Serum Cobalamin and Serum Folate

Cobalamin[a] (pg/mL)	Folate[b] (ng/mL)	Provisional Diagnosis	Proceed With Metabolites?[c]
>300	>4	Cobalamin or folate deficiency is unlikely	No
<200	>4	Consistent with cobalamin deficiency	No
200–300	>4	Rule out cobalamin deficiency	Yes
>300	<2	Consistent with folate deficiency	No
<200	<2	Consistent with combined cobalamin *plus* folate deficiency	Yes
>300	2–4	Consistent with (1) folate deficiency or (2) an anemia unrelated to vitamin deficiency	Yes

Test Results on Metabolites: Serum Methylmalonic Acid and Total Homocysteine

Methylmalonic Acid (Normal, 70–270 nM)	Total Homocysteine (Normal, 5–14 μM)	Diagnosis
Increased	Increased	Cobalamin deficiency confirmed; folate deficiency still possible (i.e., combined cobalamin plus folate deficiency possible)
Normal	Increased	Folate deficiency is likely
Normal	Normal	Cobalamin and folate deficiency is excluded

[a] Serum cobalamin levels: abnormally low, less than 200 pg/mL; clinically relevant low-normal range, 200–300 pg/mL.
[b] Serum folate levels: abnormally low, less than 2 ng/mL; clinically relevant low-normal range, 2–4 ng/mL.
[c] Any frozen-over sample from serum folate/cobalamin determination can be subjected to metabolite tests.

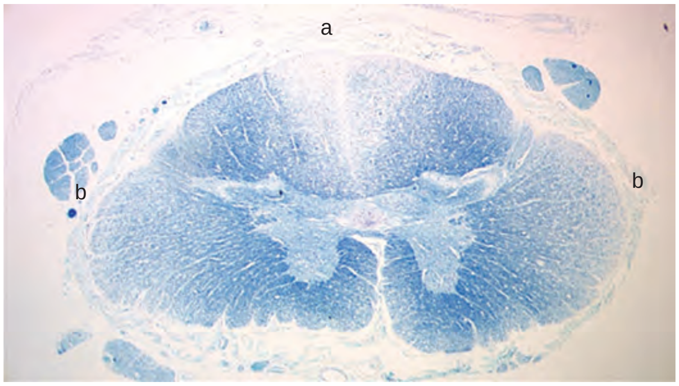


Figure 40.8 SPINAL CORD IN COBALAMIN DEFICIENCY. The cross section of the spinal cord stained with Luxol blue shows demyelination of the dorsal columns *(a)* and early demyelination of the lateral columns *(b)*.

Modified Therapeutic Trials). Alternatively, attribution of the cause of anemia to cobalamin (and/or folate) deficiency could be reasonably confirmed retrospectively if there was evidence for resolution of anemia following such therapy. Although reductions from high serum MMA and homocysteine values to baseline following therapy would also be confirmatory, this is impractical because of the expense of repeated testing.

BIOCHEMICAL EVALUATION OF COBALAMIN AND FOLATE DEFICIENCIES

Total Serum Homocysteine and Methylmalonic Acid Levels

Cellular nutrient *deficiency* of cobalamin or folate is reflected by decreased intracellular concentrations. Cobalamin deficiency perturbs methionine synthase activity; this results in substrate (homocysteine) buildup and elevated serum levels of homocysteine, which can be measured by a sensitive assay.[23] In addition, cobalamin deficiency perturbs the activity of methylmalonyl-CoA mutase, which leads to elevated serum MMA levels. Thus homocysteine and MMA are sensitive tests for cobalamin deficiency (see Table 40.2).

Folate deficiency also results in elevated levels of homocysteine because of reduced activity of the methionine synthase–catalyzed reaction.[23] Total homocysteine concentration, which comprises the sum of all homocysteine species in plasma/serum, including free and protein-bound forms, can be measured in plasma or serum.[23,120] In general, plasma levels are slightly lower. Thus an elevation of both homocysteine and MMA, while consistent with cobalamin deficiency, cannot rule out a combined cobalamin and folate deficiency (see Table 40.2).

Both homocysteine and MMA levels are elevated in patients with dehydration and renal failure. In addition, propionic acid derived from anaerobic fecal bacterial metabolism can also substantially contribute to methylmalonate production[6,121,122]; in this setting, the fraction of gut flora contribution to MMA can be reduced by treatment with metronidazole.

The normal value for serum homocysteine is 5.1 to 13.9 μM and serum MMA is 70 to 270 nM, and in general the higher the values, the more severe the clinical abnormalities.[23] However, there is a fairly wide range of “normalcy” in homocysteine values because of age-, creatinine-, gender-, diet-, and race-dependent variables.[120] Basal levels of MMA are usually less than 500 nM, and in renal failure, it rarely increases by more than 1000 nM.[23] If unseparated blood stands at room temperature, homocysteine levels will *increase* over 4 to 24 hours. Frozen serum (remaining from measurements of serum folate or cobalamin) can be used for serum MMA and homocysteine determinations.

Serum MMA levels are elevated in more than 95% of patients with clinically confirmed cobalamin deficiency (with median values of 3500 nM). Serum homocysteine concentrations are elevated in both cobalamin deficiency (median values of 70 μM) and folate deficiency (median values of 50 μM).

Serum Cobalamin Levels

For the most part, a low serum cobalamin level is an established biochemical indicator of cobalamin deficiency. In general, in patients with clinical cobalamin deficiency and megaloblastic anemia or neurologic disease consistent with cobalamin deficiency, the sensitivity of cobalamin concentration less than 200 pg/mL (or less than 148 pmol/L) exceeds 95%[123] when the pretest probability is high. However, up to 10% of adults with true cobalamin deficiency have cobalamin values in

MODIFIED THERAPEUTIC TRIALS

In a modified therapeutic trial, the intention is often to confirm the clinical suspicion that the patient does not have deficiency. This can be demonstrated by lack of response to full replacement doses of both vitamins (1 mg of folic acid orally for 10 days and 1 mg of cobalamin intramuscularly or subcutaneously daily for 10 days). Clinical scenarios in which such trials may be applicable (after drawing blood for serum cobalamin and folate levels) are as follows:

1. There is a clinical suspicion that the underlying disease is not caused by a vitamin deficiency, but this idea is not supported by results of clinical, morphologic, and biochemical evaluations. Such conditions include anemia with a megaloblastic bone marrow that may be due to underlying myelodysplastic syndromes, or acute leukemia, or the use of drugs that are known to induce megaloblastic anemia[119]; when time is of the essence in making the diagnosis; when the levels of cobalamin are likely to be falsely abnormal because of these diseases; or when there is underlying dehydration or renal dysfunction that predictably gives falsely high levels of metabolites, thereby precluding their diagnostic usefulness.
2. In other situations (i.e., pregnancy, acquired immunodeficiency syndrome, or alcoholism) with a multifactorial basis for anemia, the response or lack thereof to full replacement doses can eliminate cobalamin or folate deficiency and thereby narrow the (often extensive) differential diagnosis.
3. When severe anemia with megaloblastosis is clinically obvious and so serious that the physician cannot wait for the results of specific tests for deficiency, full doses of both vitamins are given, and if there is a response manifested by brisk reticulocytosis by days 5–7, retrospective assignment of the deficiency is based on the results of blood samples drawn before beginning the trial.

In all modified therapeutic trials, if there is no evidence of response within 10 days, bone marrow aspiration is indicated to identify another primary hematologic disease.

TABLE 40.3 Serum Cobalamin: False-Positive and False-Negative Test Results

Falsely Low Serum Cobalamin in the Absence of True Cobalamin Deficiency
Folate deficiency (one-third of patients)
Multiple myeloma
TCI deficiency
Megadose vitamin C therapy
Falsely Raised Cobalamin Levels in the Presence of a True Deficiency[a]
Cobalamin binders (TCI and II) increased (e.g., myeloproliferative states, hepatomas, and fibrolamellar hepatic tumors)
TCII-producing macrophages are activated (e.g., autoimmune diseases, monoblastic leukemias and lymphomas)
Release of cobalamin from hepatocytes (e.g., active liver disease)
High serum anti-IF antibody titer

[a] Although a low serum cobalamin level is not synonymous with cobalamin deficiency, 5% of patients with true cobalamin deficiency have low-normal cobalamin levels, a potentially serious problem because the patient's underlying cobalamin deficiency will progress if uncorrected.

IF, Intrinsic factor; *TC*, transcobalamin.

the low-normal (200 to 300 pg/mL) range and only metabolite testing with homocysteine and MMA will reveal the deficiency (see Table 40.2).

Thus a serum cobalamin concentration is less than 300 pg/mL in 99% of patients with clinical hematologic or neurologic manifestations of cobalamin deficiency,[23] and a cobalamin level of more than 300 pg/mL predicts folate deficiency or another hematologic or neurologic disease (see Table 40.2). However, a low serum cobalamin concentration is not synonymous with cobalamin deficiency, and several associated diseases and conditions can falsely raise or lower cobalamin levels (Table 40.3). Studies have also identified patients with true cobalamin deficiency who have cobalamin levels in the low-normal range. Among 173 unambiguously cobalamin-deficient patients[23] about 5% had normal cobalamin levels.

If the serum cobalamin test is broadly used as a screening test without clinical context, by virtue of the way normalcy is defined, 2.5% of nondeficient individuals will have low levels, which reflects our definition of the lower limit of normal for this test.[23] However, the finding in 2011 that the same blood sample can give different cobalamin results (one below normal versus one above normal) using different commercial assays has been of significant concern.[123] The more recent assays have periodically had such problems, apparently arising from a lack of transparency related to these tests, poor validation using low-cobalamin sera, and poor track record of continuous proficiency testing and tracking of assay performance.[123,124] It is a particularly serious issue when chemiluminescent tests for serum cobalamin give spuriously elevated levels and fail to detect clinically significant severe pernicious anemia. Such a false-negative test result has been attributed to the in vitro binding of anti-IF antibodies (present in the serum of patients with pernicious anemia) to the IF found in the manufacturer's reagent.[124,125] However, a reevaluation in 2014 of five different (currently used) automated cobalamin assays found that they are accurate and do not suffer from earlier problems.[126] Nevertheless, the principle that *"a clinical presentation which strongly suggests cobalamin deficiency should always lead to a therapeutic trial (with cobalamin replacement) even if the laboratory assay is nonconcordant"* must be upheld against future vagaries that can lead to dangerous false-negative errors in laboratory tests.

Therefore in the absence of availability of metabolite tests, if there are hematologic or neurologic findings that are consistent with clinical cobalamin deficiency, and the serum cobalamin level is normal or borderline low, it is entirely appropriate to empirically treat as for a cobalamin deficiency. If there is no improvement in hematologic parameters within a couple of months, provided there are no other conditions that limit a full response to cobalamin (e.g., iron deficiency or underlying thalassemia trait, hypothyroidism, renal disease, infection, alcoholism, or intrinsic hematologic disease in the bone marrow), cobalamin deficiency would be unlikely.

Cobalamin deficiency can falsely raise serum folate by 20% to 30% via methyl-folate trapping. This will seriously underestimate the prevalence of an associated folate deficiency among populations (particularly in resource-limited countries) where the dietary intake of both vitamins is consistently low.[118] Folate deficiency can also reduce serum cobalamin, but the mechanism is unclear. (See box on Summary of the Clinical Usefulness of Tests for Cobalamin and Folate Deficiencies.)

Serum Folate Levels

The serum folate level is clinically relevant and widely used. Indeed, when combined with a clinical picture of megaloblastic anemia and additional results of cobalamin levels, the serum folate concentration is the cheapest and most useful initial biochemical test to diagnose folate deficiency (see Table 40.2).[23] Microbiologic assays for

SUMMARY OF THE CLINICAL USEFULNESS OF TESTS FOR COBALAMIN AND FOLATE DEFICIENCIES

Within the clinical context of hematologic or neurologic features that suggest the diagnosis of cobalamin deficiency, if the cobalamin levels are suggestive but not definitive, then the methylmalonic acid (MMA) and homocysteine tests are an excellent gold standard test to confirm a clinical diagnosis. *Patients with clinical cobalamin deficiency usually have MMA values over 1000 nM and homocysteine values over 25 μM.* The MMA and homocysteine test results are much more sensitive than cobalamin levels and progressively increase much earlier than the drop in cobalamin levels; one or both metabolites was increased in 99.8% of more than 400 patients with proven cobalamin deficiency.[23]

folate, which measure all biologically active forms equally, have been replaced in the West by competitive folate-binding protein assays (from various commercial sources) that are indirect immunoassays, which rely on chemiluminescence methods. These tests are notorious for considerable lack of agreement with one another. Alignment with a new higher-order precision isotope-dilution liquid chromatography–tandem mass spectrometry assay, which demonstrates excellent agreement with the traditional *Lactobacillus casei* method,[127] will allow better standardization of the current competitive folate-binding protein assays.[128]

The serum folate level is highly sensitive to folate intake, and a single nutritious hospital meal may normalize it in a patient with true folate deficiency. Rapidly developing nutritional folate deficiency first leads to a decline in the serum folate level below normal (less than 2 ng/mL) in about 3 weeks because it is a sensitive indicator of negative folate balance.[16] However, in 25% to 50% of cases (predominantly alcoholics) with folate-deficient megaloblastosis, the serum folate levels may be below normal or borderline (2 to 4 ng/mL).[23] The serum folate level alone should never dictate therapy.

Based on the lower costs of serum cobalamin and folate compared with serum MMA and homocysteine levels, it is recommended (see Table 40.2) to first use the cheaper tests that can assist in the diagnosis of cobalamin and folate deficiency.[23] Clinicians should also restrict use of serum MMA and homocysteine *(a)* to patients with borderline cobalamin and folate levels; *(b)* to patients with existing clinical conditions known to be associated with difficulties in the interpretation of test results; *(c)* to situations in which cobalamin and folate levels are low, when a high MMA level is useful in confirming cobalamin deficiency (rather than attributing the condition to folate deficiency alone); and *(d)* to patients with clearly low serum levels but for whom there is an alternative explanation for the findings that caused an unusual serum cobalamin level to be obtained (e.g., a diabetic or alcoholic with peripheral neuropathy, an alcoholic with a high mean corpuscular volume and a low serum cobalamin without anemia). In these cases, serum levels of metabolites can assist in the diagnosis of vitamin deficiency. Diagnostic algorithms consistently stress the value of clinical data to improve the pretest probability of serum cobalamin and serum folate tests.[23] Without detailed clinical information, the combined test results for serum cobalamin, folate, and metabolite (homocysteine and MMA) are not sufficiently unambiguous to diagnose and distinguish cobalamin deficiency from combined cobalamin plus folate deficiency. In combined cobalamin plus folate deficiency, both vitamins would be needed to restore baseline values, particularly of homocysteine.[23]

When negative folate balance continues, hepatic folate stores are depleted in about 4 months.[16] This leads to tissue folate deficiency, which clinically correlates with a decrease in RBC folate (less than 150 ng/mL) by the microbiologic assay.[16] However, current RBC folate tests using different commercial kits have major limitations in sensitivity and specificity and are notoriously unreliable in alcoholics and in pregnancy; furthermore, a reduction of RBC folate also occurs in about 60% of patients with cobalamin deficiency.

The use of red-cell folates as a measure of long-term folate status is valid during clinical trials in which a single kit is used for a cohort of patients; however, it is *not* valuable for routine clinical diagnosis because of the significant variability of performance between different commercial kits and lack of clinical validation.[118,129] For these reasons, the serum folate level, although labile, is the preferred initial choice.[16,130–134]

However, there are important caveats to measuring serum folate levels in certain clinical settings (see box on Caveats Related to the Use of Laboratory Tests in Resource-Limited Countries).[118] First, the serum folate level can be artificially raised in a patient with either pure cobalamin deficiency or *combined* cobalamin and folate deficiency (Table 40.4). This is because cessation of the cobalamin-dependent methionine synthase reaction leads to a failure in utilization of intracellular folate for one-carbon metabolism. As a result, folate leaks out of cells into the plasma, thereby raising the patient's serum folate level; indeed, replacement of cobalamin alone will return the serum

CAVEATS RELATED TO THE USE OF LABORATORY TESTS IN RESOURCE-LIMITED COUNTRIES (SEE TABLE 40.4)[118]

Masking of Nutritional Folate Deficiency by Associated Cobalamin Deficiency and/or Malaria

In resource-limited countries, the majority of the population is either vegetarian or near-vegetarian; these diets are monotonous, low in fresh vegetables, fruits, and in animal-source foods. Such a diet predisposes to a combination of nutritional cobalamin and folate (and iron) deficiencies, particularly among women and children. With cobalamin deficiency, the failure in utilization of intracellular folate for one-carbon metabolism results in folate leaking out of cells, thereby raising the serum folate.[16] What this means is that the presence of cobalamin deficiency will consistently *mask* the coexistence of folate deficiency whenever there is an (undue) over-reliance by the clinician in using the biomarker of the serum folate level as a gold standard to diagnose folate deficiency. This is a surprisingly common error in the contemporary literature[118]—a fact that inadvertently downplays (with disastrous consequences) the seriousness of folate deficiency among millions of these particularly vulnerable women and children. Added to this is the fact that in many such regions, there is also the scourge of endemic malaria with its propensity for hemolysis (and release of the 30-fold higher red cell folate into plasma). Here too, the serum folate level will yield normal-to-high values and predictably underestimate the tissue folate status, thereby masking the diagnosis of mild-to-moderate folate deficiency in these very patients who desperately require folate to support compensatory hematopoiesis and for growth and development (see Table 40.4 and Summary of the Clinical Usefulness of Tests for Cobalamin and Folate Deficiencies).

The only solution to correctly identifying whether these patients are at risk for nutritional folate deficiency is to obtain a good dietary history and identify those at risk for nutritional insufficiency.[135] (Other formal assessment includes use of 24-h food recall, estimated/weighed record, or locally validated food-frequency questionnaires to evaluate the quality and quantity of nutrients consumed.)[135] *Indeed, knowledge of dietary folate and cobalamin intake should always take precedence over the results of conventional blood tests for folate deficiency, which are flawed in this clinical setting.* From the practical standpoint, all individuals at risk for nutritional anemia caused by deficiency of iron, folate, and cobalamin (including both adults[135] and children[136]) should be given prophylactic oral cobalamin, folate (and iron) replacement; and in malarious zones, these should be combined with antimalarial drugs and insecticide-treated bed nets.

Overreliance on Laboratory Data at the Expense of Clinical Information—A Case Study[118]

The majority of women and children in resource-limited countries suffer from combined nutritional iron, cobalamin, and folate deficiency—the three key causes of nutritional anemia. There are often additional congenital or acquired causes for hemolytic anemia in a single individual, such as malaria, bacteremia, hemoglobinopathy, or glucose-6-phosphate dehydrogenase deficiency. There is therefore potential for a clinician to make several critical interrelated errors: *First*, despite dire poverty and near-famine conditions, mere inattention and failure to obtain a good dietary assessment of (deficient) iron, cobalamin, and folate intake in these vulnerable patients will lead to an underestimation of the extent of deficiency of these nutrients.[118] *Second*, this can be coupled with a lack of appreciation of the potential for one deficient micronutrient to have on the blood test result of another. For example, cobalamin deficiency or hemolysis will invariably raise the serum folate level (from a below-normal level into the normal range) and thereby lead to an underestimation (and misdiagnosis) of the fact of *tissue* folate deficiency.[118] *Third*, a failure to replace all three deficient nutrients in a given individual will invariably lead to a failure in restoring hemoglobin values to optimum; indeed, replacing one or even two out of three key missing nutrients cannot possibly be expected to secure a complete resolution of anemia.

Continued

TABLE 40.4 **Serum Folates Are Misleadingly Elevated in Cobalamin (Vitamin B_{12}) Deficiency and/or Malaria Which Are Both Common in Resource-Limited Settings**[a,b]

	Serum Folates	Erythrocyte Folates	Serum Cobalamin
Pure folate deficiency	Low	Low	Normal/low[b]
Pure cobalamin deficiency	Normal/high[b,c]	Low[b,c]	Low
Folate *plus* cobalamin deficiency	Normal[b]	Low	Low
Pure malaria	Normal[b]/high[b,d]	High[b,e,f]	Normal
Malaria *plus* folate deficiency	Normal[b]	Normal[b]/high[b]/low	Normal
Malaria *plus* cobalamin deficiency	Normal[b]/high[b,d]	Normal[b]/high[b,f]	Low
Malaria *plus* folate *plus* cobalamin deficiency	Normal[b]/high[b,d]	Low/normal[b,f,g]	Low

[a] Both cobalamin deficiency and clinical malaria and other hemolytic states can complicate the diagnosis of folate deficiency using tests for serum- or erythrocyte-folate concentration.[118]

[b] Indicates misleading values in the clinical settings shown on the left. The information presented in this table has been synthesized from several sources (see text and references).[118]

[c] Cobalamin deficiency is accompanied by inability to use folates for one-carbon metabolism, so folates leak out of erythroid precursors into serum.

[d] Release of the 30-fold excess folate from infected erythroid precursors, reticulocytes, and mature erythrocytes during hemolysis raises serum folate levels. (An as-yet-unknown quantity of folate is released into serum when folate-rich hepatocytes are destroyed during the exoerythrocytic hepatic phase of malaria.)

[e] Hemolysis induces a compensatory reticulocytosis; these reticulocytes are richer in folate than mature erythrocytes.[16,118]

[f] *Plasmodium falciparum* can also synthesize folates in erythrocyte cultures in vitro and raises erythrocyte folates in animal models with high levels of parasitemia.

[g] Reticulocytopenia in severe *Plasmodium falciparum* malaria, caused by either combined cobalamin deficiency *plus* folate deficiency, which can trigger a reticulocytopenic (megaloblastic) crisis, or cytokine-induced inhibition of hematopoiesis, will negate an expected rise in erythrocyte folates.

From Antony AC. Megaloblastic anemias. In: Goldman L, Schafer A, eds. *Cecil Medicine*. Vol 1, 25th ed. Philadelphia, PA: Elsevier-Saunders; 2015:ch. 164, pp. 1104–1114.

folate level to baseline.[16,118] Thus associated nutritional cobalamin deficiency has the potential to consistently mask the coexistence of mild-to-moderate folate deficiency if the unwary clinician uses the serum folate level as a gold standard for diagnosing folate deficiency in this clinical setting. Second, such patients (with combined nutritional folate *and* cobalamin deficiency) often reside in malarious regions where there may be ongoing hemolysis from malaria per se as well as hemolysis from associated hemoglobinopathies that are common in these regions (e.g., thalassemia, sickle cell disease, glucose-6-phosphate dehydrogenase deficiency). In a patient with malaria during hemolysis of *Plasmodium falciparum* infected erythroid precursors, reticulocytes, and mature erythrocytes, there will be substantial release of the 30-fold more folate-rich intraerythrocyte contents *into* serum, thereby artificially raising the baseline serum folate level. Moreover, red cells normally contain substantial amounts of *various forms of folate*, that is, 5-methyltetrahydrofolate (monoglutamates) and folate-polyglutamates of different glutamate chain lengths[16,118]; whereas clearance of such released folate monoglutamates would be hindered with associated cobalamin deficiency,[118] we also know that the released folate polyglutamates are also inefficiently transported back into cells relative to monoglutamates,[118] thereby also resulting in poor clearance. In this clinical context, the current assays for serum folate may not consistently discriminate among these forms of folates. The net result is that a *high* serum folate could be reported in all such individuals with malaria, *even when the patient's tissue folates are significantly depleted*.[118] This predictable masking of tissue folate depletion *argues against* the use of serum tests for folate deficiency in this clinical setting,[118] where a dietary history of the intake of folate-rich foods in the diet is a better clinical method to assess folate status. (See box on Caveats Related to the Use of Laboratory Tests in Resource-Limited Countries and Table 40.4.)

Other Tests

The clinical use of low holo-TCII (holo-TCII) levels, to provide information on the extent of saturation of serum TCII as an early marker of cobalamin homeostasis[137] or to diagnose cobalamin deficiency in lieu of serum cobalamin values, is still unclear. This test has not yet been sufficiently clinically validated[123] to define sensitivity, specificity, and other clinical confounders that can alter the results; it has neither supplanted the use of the serum cobalamin test for diagnosis of deficiency, nor is it widely available or used in United States.[4,6,138,139]

PATHOGENESIS OF COBALAMIN DEFICIENCY

Nutritional Cobalamin Deficiency

Vegetarian diets can be classified as lactovegetarian, ovovegetarian, lacto-ovovegetarian, or vegan, respectively, if they include dairy products, eggs, dairy products and eggs, or no animal products at all.[7,8] However, all these vegetarian diets contain insufficient amounts of cobalamin. Therefore, *all* vegetarians are in various stages of progressive cobalamin depletion and moving inexorably toward cobalamin deficiency.[7–9] This warrants early supplementation with cobalamin.

In addition, the vast majority of those living in resource-limited countries subsist on a relatively monotonous diet that is intrinsically low in animal-source foods (that are more expensive than plant-based diets). Although not strictly considered vegetarians, such individuals are better classified as "near-vegetarians"[7,8] and should also be supplemented with cobalamin. Cobalamin deficiency has been widely reported among 45% of Northern Chinese women,[140] and in up to 85% of Indian adolescents[141] and 65% of newborns.[142] In Pakistan,[143] among those with megaloblastic anemia (hemoglobin $< 8\,g/dL$), nearly 80% had cobalamin deficiency. Surprisingly, even those consuming "Mediterranean diets" that are rich in fruits and vegetables but low in animal-source protein are at risk for cobalamin deficiency; among 180 pregnant women, cobalamin deficiency was found in 72% of mothers and 41% of babies.[144]

The fetus is dependent on the mother's cobalamin stores for a sufficient quota of cobalamin at birth; a close correlation exists between low maternal serum and breast milk cobalamin concentrations and cobalamin insufficiency in the infant.[4,145] Therefore when mothers do not consume sufficient amounts of animal-source foods, they themselves are at risk for nutritional cobalamin deficiency, and their infants will have smaller stores of the vitamin at birth.[16,146] Although the dictum is that infants should be exclusively breastfed for the first 6 months of life, a large percentage of mothers in resource-limited countries had evidence of cobalamin deficiency.[7,147,148] For example, three-quarters of a cross section of

366 pregnant urban women in South India had cobalamin deficiency.[135] So there is an imperative to raise these values in women and in babies. An important series of studies has shown that treatment of the mother during pregnancy[149] or even the infant shortly after delivery will promptly reverse preexisting cobalamin deficiency.[150]

Cobalamin-deficient infants can present with a spectrum of clinical findings, ranging from feeding difficulties and refusal of both breast milk and complementary food by regurgitation (which results in further failure to thrive) to motor and social retardation, reflecting a developmental delay. The child is persistently drowsy and rarely sits up or makes eye contact.[7] There may be lemon-tint jaundice with hypotonia, insufficient head control, and delayed spontaneous turning. There can be brownish-black areas of hyperpigmentation in the dorsal fingers and toes as well as over the medial thighs, arms, and axillae (which usually resolve within 3 months of therapy). Evidence of megaloblastic anemia may be masked with superimposed iron deficiency. If left untreated, there is growth retardation with reduced height and weight, and reduced head circumference with cranial MRI showing delayed myelination and frontoparietal cortical atrophy in affected infants[151]; these can also be reversed within 3 months of cobalamin replacement. Treatment results in a dramatic increase in alertness and responsiveness of the child, who is now miraculously transformed into a normal child who, within a few days, rolls over spontaneously, makes eye contact with its mother, and is much more interested in the surroundings. Any previous abnormal movements (tremors, chorea, or myoclonus) may regress but transiently return within a few days to affect the face or tongue; however, these will resolve in 2 to 3 months.[152] Cobalamin deficiency often resurfaces during wartime, which invariably leaves women and their infants malnourished.[153]

Infants in the West fed a macrobiotic diet (vegan-like with occasional servings of fish) must be rapidly replenished with full doses of cobalamin before switching to a cobalamin-rich diet. Otherwise, up to 20% continue to have low cobalamin status, which can lead to impaired psychomotor functioning well into youth and later adolescence with compromise in faculties related to reasoning, abstract thinking, and learning ability.[154]

When children in resource-limited settings grow up on the same monotonous diet as their parents, they are at risk for combined cobalamin, folate (and iron) deficiency.[7,155–158] *This problem of vertical intergenerational transfer of a deficient "bank balance" of minerals and micronutrients from one generation to another has been documented in all resource-limited countries*. These affected children with preexisting depleted stores of cobalamin, folate (and iron), and imminent deficiency, who are the unwitting victims of circumstance, limp on through life with cognitive dysfunction and lower intelligence quotient and emotional intelligence (when compared with their better nourished counterparts in resource-rich countries). When they move on into adolescence and (often premature) young motherhood, they pass on their deficient "bank balances" vertically to the next generation and so the cycle continues *ad infinitum*. The number of such affected individuals worldwide is probably in the hundreds of millions.

Longitudinal studies from the West among women who apparently consume a balanced nonvegetarian diet confirm that pregnancy places an additional stress on the mother's cobalamin stores and can lead to metabolic evidence of cobalamin deficiency.[159–161] This can negatively affect their breastfed infants' cobalamin status at 6 weeks; indeed, over two-thirds of Norwegian infants of otherwise healthy mothers had a metabolic profile consistent with cobalamin deficiency, which reverted to normal after cobalamin replenishment.[160,161] Among infants with only minor developmental delays and feeding difficulties (regurgitation) and biochemical evidence of cobalamin deficiency, those treated with cobalamin responded with significant clinical benefit, a fact that underscores the importance of cobalamin in postnatal neurodevelopment.[162] Thus it is likely that many more breastfed infants in the West probably need cobalamin supplements early in life, as do their mothers in preparation for pregnancy.[12]

Intragastric Events Leading to Cobalamin Malabsorption

Inadequate Dissociation of Cobalamin From Food Protein

Dietary cobalamin is bioavailable only after proteolytic digestion of food by gastric acid and pepsin. Failure to release cobalamin from food protein can lead to food-cobalamin malabsorption and frank cobalamin deficiency despite the presence of IF.[23]

Congenital Intrinsic Factor Deficiency[163]

Congenital IF deficiency arising from mutations in gastric IF,[163,164] resulting in complete loss of IF, can be transmitted as an autosomal recessive trait and expressed in homozygotes by the age of 2 years as severe megaloblastic anemia (less than 100 cases reported).[163] Dysfunctional IF may lead to only a mild abnormality in binding to cobalamin and result in a delayed presentation into the second decade.[23]

Loss or Atrophy of Gastric Oxyntic Mucosa

IF deficiency, which arises from atrophy of gastric parietal (oxyntic) mucosal cells, can be caused by total or partial gastrectomy (bariatric surgery); by autoimmune destruction, as observed in adult Addisonian pernicious anemia or, rarely, in a similar disease in children (juvenile pernicious anemia); and after destruction of gastric mucosa by caustic (lye) ingestion.

Total gastrectomy invariably leads to cobalamin deficiency in about 5 years (range, 2 to 10 years); indeed, longitudinal follow-up revealed that all 176 patients developed cobalamin deficiency within 4 years, with earlier clinical presentations occurring in those with lower cobalamin status preoperatively.[165] This condition is often associated with iron deficiency,[16] warranting routine prophylactic cobalamin and parenteral iron replacement.

By contrast, after partial gastrectomy, the degree of cobalamin deficiency depends on the size of the remaining gastric remnant. Cobalamin deficiency eventually develops in 10% to 20% of patients at 8 years with a minority (about 5%) developing overt clinical manifestations from a combination of decreased IF secretion, hypochlorhydria, intestinal bacterial overgrowth of cobalamin-consuming organisms, and iron deficiency. It is more common in Billroth II than in Billroth I surgery, and in subtotal than in partial gastrectomy. Morbidly obese patients treated surgically with gastric bypass also have more food-cobalamin malabsorption than patients treated with vertical banded gastroplasty.[23] Even after laparoscopic Roux-en-Y gastric bypass, and despite oral multivitamin supplementation, iron deficiency was seen in one-half of patients and cobalamin deficiency seen in one-quarter at 3 years[166]; therefore all such patients need prophylactic cobalamin and parenteral iron.

Absent Intrinsic Factor Secretion and Pernicious Anemia

A common cause of cobalamin malabsorption is pernicious anemia, an autoimmune disease in which the fundamental defect is atrophy of the gastric (parietal cell) oxyntic mucosa that eventually leads to the complete absence of IF and hydrochloric acid secretion (Fig. 40.9). The autoimmune gastritis (leading to chronic atrophic gastritis) associated with pernicious anemia involves the fundus and body of the stomach, and the histologic appearance of the gastric mucosa (infiltration with plasma cells and lymphocytes) is strongly reminiscent of autoimmune lesions.[6,167,168] Because cobalamin is absorbed only by binding to IF and uptake by ileal IF-cobalamin receptors, the net consequence is severe cobalamin malabsorption leading to cobalamin deficiency.

The annual incidence of pernicious anemia is approximately 25 new cases per 100,000 persons older than 40 years. Although the average age of onset is about 60 years, pernicious anemia is no respecter of age, race, or ethnic origin. The predisposition to developing pernicious anemia may have a genetic basis, but neither the

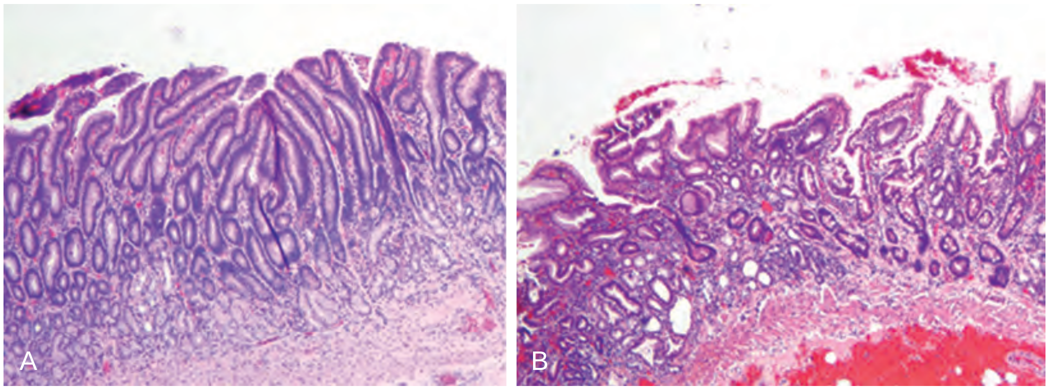


Figure 40.9 HISTOLOGIC FEATURES OF STOMACH IN PERNICIOUS ANEMIA COMPARED TO NORMAL. The normal gastric mucosa (A) is contrasted to that seen in pernicious anemia (B), in which there is atrophy of gastric glands, intestinal metaplasia with goblet cells, and loss of parietal cells (not visible at this magnification).

mode of inheritance nor the initiating events or primary mechanism is precisely understood. There is a positive family history for about 30% of patients, among whom the risk for familial pernicious anemia is 20 times as high as in the general population; about 20% of siblings of patients are projected to develop pernicious anemia by the age of 90 years, and pernicious anemia has developed concordantly in identical twins. There is a significant association of pernicious anemia with other autoimmune diseases,[16] including Graves disease (30%), Hashimoto thyroiditis (11%), vitiligo (8%), Addison disease, idiopathic hypoparathyroidism, primary ovarian failure, myasthenia gravis, type 1 diabetes mellitus, and adult hypogammaglobulinemia.[16,23]

Autoimmune gastritis progresses over decades to atrophic body gastritis and pernicious anemia. Serum anti-IF antibodies are highly specific (100%) for pernicious anemia, but the sensitivity is only about 50%. Earlier, the clinical use of anti-parietal cell antibodies was limited because of low specificity. This necessitated use of additional surrogate markers (high serum gastrin and low pepsinogen I levels) that reflected loss of acid- and IF-secreting parietal (oxyntic) cells. However, newer enzyme-linked immunosorbent assays (ELISA) for anti-parietal cell antibodies, which are directed against gastric H^+/K^+ ATPase, are 30% more sensitive than previous (immunofluorescence) assays. A reanalysis of the clinical utility of combining anti-IF and newer anti-parietal cell antibody tests to noninvasively diagnose pernicious anemia points to this approach as very promising.[169] Thus among 81 patients with biopsy-proven atrophic body gastritis and pernicious anemia, combining anti-IF antibodies (37% sensitivity; 100% specificity) with newer anti-parietal cell antibodies (sensitivity 91%; specificity 90%) significantly increased their diagnostic performance for pernicious anemia, yielding overall 73% sensitivity while maintaining 100% specificity.[169]

Juvenile pernicious anemia can manifest in the second decade with severe cobalamin deficiency in conjunction with many of the associated endocrinopathies and autoantibodies observed in adults.[16]

Undiagnosed pernicious anemia is common among free-living elderly persons (over 60 years of age) who have only minimal clinical manifestations of cobalamin deficiency (i.e., 1.9% of a Southern California survey population had unrecognized and untreated pernicious anemia).[170] The prevalence was 2.7% in women and 1.4% in men, but 4.3% of the African American women and 4.0% of the women of European descent had pernicious anemia.

Abnormal Events in the Small Bowel Lumen

Insufficient Pancreatic Protease

About 30% of patients with severe pancreatic insufficiency fail to degrade R proteins, which will lead to impaired transfer of cobalamin from R protein to IF. Pancreatic extract will normalize cobalamin malabsorption.[16]

Inactivation of Pancreatic Protease

Pancreatic protease can be inactivated by massive gastric hypersecretion arising from a gastrinoma in Zollinger-Ellison syndrome.[16] The continued low pH of the luminal contents reaching the ileum may also perturb interaction of the IF-cobalamin complex with IF-cobalamin receptors (which requires a pH above 5.4).

Usurpation of Luminal Cobalamin

The near-sterile condition of the small bowel is maintained by a combination of the mechanical cleansing action of peristalsis and the chemical action of gastric acid. Disorders conducive to relative stasis, impaired motility, and hypogammaglobulinemia are predisposing factors that favor colonization by bacteria. Many of these bacteria can take up free cobalamin, but not IF-bound cobalamin. However, if colonization extends proximally to the locus at which IF and cobalamin interact, significant cobalamin may be usurped before it can bind to IF.[16] This cobalamin malabsorption can be corrected to some extent by a 7- to 10-day course of antibiotic therapy.

Approximately 3% of individuals infested with the fish tapeworm *Diphyllobothrium latum*, which avidly usurps cobalamin for growth,[16] can develop frank cobalamin deficiency. Humans become infected when they eat partially cooked or raw fish containing plerocercoids, which develop into adult worms in the jejunum in about 6 weeks, growing to a length of 10 m, with up to 4000 proglottids[16]; when these worms lay eggs, the life cycle is repeated. After ova have been identified in the stools, expulsion of the worms by oral praziquantel (10 to 20 mg/kg as a single dose) and cobalamin replenishment is curative.

Disorders of Ileal Intrinsic Factor–Cobalamin Receptors or Mucosa

Absence of Intrinsic Factor–Cobalamin Receptors

The distal ileum has the greatest density of IF-cobalamin receptors. Disease or removal of only 1 to 2 feet of terminal ileum by resection or bypass reduces ileal IF-cobalamin receptor numbers for interaction with IF-cobalamin, resulting in cobalamin malabsorption.[16,23]

Defective Intrinsic Factor–Cobalamin Receptors or Post-Intrinsic Factor–Cobalamin Receptor Defects

Imerslund-Gräsbeck syndrome is a term used collectively for a heterogeneous group of congenital (autosomal recessive) disorders in children arising from biallelic mutations (in 80% of cases) involving either the *CUBN* or *AMN* genes that constitute the functional IF-cobalamin receptor (i.e., cubam).[171] This results in selective cobalamin malabsorption. Children present between 3 and 10 years of age with megaloblastic anemia and neurologic presentations with low serum cobalamin levels associated with mild, persistent, benign proteinuria (in 90% of cases). Because renal cubam also participates in renal tubular absorption of albumin, this explains the proteinuria in Imerslund-Gräsbeck syndrome. Diagnosis requires analysis of mutational status of gastric IF, *CUBN*, and *AMN* genes.[164,171]

Drug-Induced Defects

Long-term use of H_2 antagonists and/or proton pump inhibitors may interfere with the eventual handover of food-cobalamin to IF, especially in those with preexisting borderline cobalamin stores. Long-term treatment with metformin can interfere with IF-cobalamin binding to ileal IF-cobalamin receptors (cubam)[172] and increases risk for cobalamin deficiency over time,[173] warranting replacement therapy. Cholestyramine, colchicine, and neomycin probably also impair transepithelial transport of cobalamin.[16]

Disorders of Plasma Cobalamin Transport

Polymorphism or absence of TCI can be associated with low cobalamin levels, but the MMA and homocysteine levels are normal. By contrast, either deficiency or defective TCII can present with megaloblastic anemia in infancy; these conditions can nevertheless be associated with normal cobalamin levels (because TCI, which binds over 75% of serum cobalamin, is normal). However, there will be metabolic evidence of cobalamin deficiency that can be reversed by daily or biweekly injections of 1 mg of cobalamin, which ensures passive cobalamin delivery into cells. Rare mutations in the gene for the TCII receptor (*CD320*) have also been identified.[174]

Disorders of Intracellular Cobalamin Use

Congenital Metabolic Defects of Cobalamin Metabolism: Cobalamin Mutants A to J

Given the multitude of chaperones or transporters involved in escorting cobalamin intracellularly to their destination to function as coenzymes for methionine synthase and methylmalonyl-CoA mutase,[175] it is not difficult to envision that there would invariably be rare inborn errors of cobalamin metabolism where one of these escorts or transporters is missing. The combination of megaloblastic anemia with increased levels of homocysteine or MMA, or both, in serum and urine despite normal cobalamin and folate levels should suggest an inborn error of cobalamin metabolism.[176] The inherited defects of cobalamin use (see Fig. 40.3) are heterogeneous and are empirically defined as cobalamin mutations A to J (cblA to cblJ).[176] These infants must be differentiated from those with nutritional cobalamin deficiency who could have similar clinical features. Patients suspected of having an inborn error of metabolism should be evaluated by experts who have published extensively on this topic.[175]

Functional Cobalamin Deficiency After Nitrous Oxide Exposure

Nitrous oxide (N_2O) inactivates coenzyme forms of cobalamin by oxidizing the fully reduced cob(I)alamin to cob(III)alamin; this results in a state of functional intracellular cobalamin deficiency. This syndrome was first identified in patients with tetanus given nitrous oxide for up to 6 days.[16] Subsequently, persons exposed to nitrous oxide for open heart surgery and through chronic (recreational, accidental, or occupational) exposure have been recognized as being at high risk for developing megaloblastosis and cobalamin-deficient neuromyelopathy.[16,177] Megaloblastosis develops within 24 hours and lasts less than 1 week after a single exposure. Severe neurologic deficits have been reported after prolonged intraoperative exposure to nitrous oxide in patients with unsuspected or even borderline cobalamin deficiency.[23] The neurologic syndrome is usually seen with chronic intermittent exposure. (See box on Subclinical Cobalamin Deficiency.)

SUBCLINICAL COBALAMIN DEFICIENCY

The entity of subclinical cobalamin deficiency is present when there is biochemical evidence for cobalamin deficiency, reflected by a low cobalamin value (and increased methylmalonic acid and homocysteine) but without overt clinical manifestations. Although dependent on the population studied, the frequency of (silent) subclinical cobalamin deficiency in the United States is suspected to be 10 times higher than classic (overt) cobalamin deficiency that is found in 1%–2% of the population. Many elderly persons may have various symptoms consistent with aging (including fatigue, cognitive changes, lower quality-of-life measures, and subtle symptoms of neuropathy) that cannot be directly attributed to cobalamin deficiency, despite the fact that these very symptoms are often seen in symptomatic cobalamin deficiency.[178] Often this triggers testing with a serum cobalamin test, and a borderline result that can either spontaneously revert to normal, or minimally fluctuates above or below the cutoff value, or remains stable without change over many years; this in turn generates a new set of problems, including the need to label this entity and thereby make clinical decisions.

Although some experts do not feel obliged to treat, preferring to wait for overt symptoms, others feel ethically bound to treat even without overt clinical manifestations; the latter position is based on the fact that such clinical manifestations can be very subtle and are detected only by sophisticated neurophysiologic or imaging studies (which are both expensive and impractical in routine clinical practice). In support of earlier therapy in this clinical setting, there is compelling clinical evidence that combined B-vitamin supplementation to reduce homocysteine can reduce brain atrophy[178] and both cognitive and clinical decline (see section on Hyperhomocysteinemia for additional discussion).[179]

Therefore, after first replenishing potentially depleted cobalamin stores with parenteral cobalamin therapy, oral supplementation for up to 6 months on and 6 months off can normalize cobalamin status in most patients[180] as an alternative to continuous therapy. A key factor in such decisions is the benefit in avoiding progressive brain dysfunction and relatively low cost of cobalamin (and lack of side effects).

PATHOGENESIS OF FOLATE DEFICIENCY

Folate deficiency is usually recognized in the course of certain clinical presentations that predispose to negative folate balance and subsequent deficiency. It is instructive therefore to conceptualize cellular folate deficiency as arising from etiologic categories of decreased supply (i.e., reduced intake, absorption, transport, or use) or increased requirement (i.e., metabolic consumption, destruction, or excretion). However, in the same patient more than one mechanism may result in net folate deficiency. The precise contribution of one mechanism over the other is often not obvious, and specific tests to define each mechanism are not routinely available for clinical use. Thus the clinical context is especially important. Megaloblastic manifestations of folate deficiency are discussed within the context of the history and physical examination (discussed later). Cases of neuropathy in adults attributed to folate deficiency are rarely encountered; when they are, the possibility of alcoholism with thiamine deficiency must be considered. And every patient with neuropathy, myelopathy, or psychiatric manifestations associated with megaloblastosis must be investigated in detail to rule out cobalamin deficiency. Gastrointestinal megaloblastosis begets further folate malabsorption, which propagates a vicious cycle of folate deficiency in the short term and cobalamin deficiency in the long term. With the exception of drug-induced defects or inborn errors of folate metabolism that result in decreased use of intracellular folates, all causes, irrespective of mechanism, result in reduced net delivery of folates to normal proliferating cells.

Nutritional Causes of Folate Deficiency

The body stores of folate are adequate for only about 4 months[16] although those with higher folate stores could take longer to become frankly deficient.[181] Individuals who are chronically in negative folate balance may only require a brief "nudge"—from superimposition of an associated illness that leads to hemolysis, anorexia, or folate malabsorption—to "tip" them into frank folate deficiency. This is particularly common in resource-limited countries, where decreased seasonal availability of folate-rich foods, poverty, consumption of a poorly balanced, monotonous, cultural/ethnic diet (of wheat, maize, rice, and well-cooked lentils/beans and vegetables), is further compromised by prolonged cooking which destroys folate. Collectively, these factors contribute to the development of folate deficiency.[16,23]

Folate deficiency varies among countries and even within different regions of a country and is influenced by the economic status and net folate content of the ethnic diet consumed by the population under study. Before folate fortification of food, about 20% of the US population had low-folate status. In African countries, the prevalence of folate deficiency ranged from a low of 20% in Benin, to 30% in Zimbabwe, to nearly 60% in Sudan[182]; these differences are likely due to the variable masking of folate deficiency by associated cobalamin deficiency and/or hemolysis (see box on Caveats Related to the Use of Laboratory Tests in Resource-Limited Countries[118]). In Sri Lanka, one-half of schoolchildren had low-folate status, and in India 60% to 80% of women of childbearing age have nutritional folate deficiency.[16,118]

In Western countries, food faddism, alcoholism, or unbalanced slimming diets usually lead to decreased folate intake in young to middle-aged individuals.[16] Edentulous or infirm persons or neglected older adults who are too ill to prepare their meals, and patients with psychiatric problems, are at risk for nutritional folate deficiency (see box on Etiopathophysiologic Classification of Folate Deficiency).[16]

Folate fortification of foods in the West has led to widespread elimination of folate deficiency and related anemia,[183,184] leading to questions of whether testing for folate deficiency is even justified.[185,186] However, vigilance must be exercised particularly among women on low carbohydrate diets (not using folate supplements) and the elderly who are still at risk for both folate and cobalamin deficiency.[187,188]

Pregnancy and Infancy

Pregnancy and lactation are associated with significantly higher folate requirements (over 400 μg/day) for growth of the fetus, placenta, breast, and other maternal tissues.[189] Folate requirement increases throughout pregnancy and is maximal near term. There is also a three-fold increased urinary loss of folate during pregnancy because of a lower renal threshold. Poor preparation for pregnancy, with a poorly balanced diet and preexisting multifactorial nutritional anemia that remains unaddressed, is a major factor accounting for serious pregnancy complications and adverse birth outcomes. Therefore, additional folate during pregnancy is required to prevent both pregnancy complications (preeclampsia, placental abruption or infarctions, recurrent miscarriage) and poor pregnancy outcomes (preterm delivery, NTDs, congenital heart defects, and intrauterine growth retardation). Low-folate status associated with short interpregnancy intervals or twin pregnancies also predisposes to preterm births.[190] Despite this increased demand for folate, which must somehow be met by increased folate intake, the vast majority (over 90%) of pregnant women in resource-poor countries consume less than the estimated average requirement of folate[191]; in addition, a substantial number also consume less than optimum amounts of several other minerals, such as iron, and micronutrients, including cobalamin, as noted earlier. For example, studies on women from groups with low socioeconomic status from North India[32,33] have estimated that the daily intake of folic acid ranged between 75 and 167 μg, which is far lower than the 400 μg/day required to prevent birth defects.[68] This is simple to remedy by vigilant physicians. Indeed, when administered daily or even twice weekly, the combination of iron (100 mg elemental iron) and folic acid (0.5 mg) significantly improves several cognitive abilities of schoolgirls in India,[192] which renders them better prepared for pregnancy in the future. Not surprisingly, pregnancy with poor folate intake is the most common cause of megaloblastic anemia in resource-limited countries.

As noted earlier, the placenta has a large number of folate receptors,[68] which facilitate binding and transport of folates to the developing fetus. Preferential delivery of folate to the fetus can cause or aggravate folate deficiency in the mother.[16] This is observed clinically when a mother with severe folate deficiency gives birth to a baby who has normal folate stores.[67]

The rapidly proliferating tissues in children also have an absolute requirement for exogenously supplied folate. Although human milk of folate-replete mothers can maintain folate balance in breastfed infants, the breast milk content of folate is low when the mother's folate status is poor.

Before the advent of routine folate supplementation during pregnancy, the incidence of megaloblastic marrows in the United States, Canada, and the United Kingdom during late pregnancy was about 25%, but in South India, it was about 55%.[16] Folate deficiency is eight times as high in twin pregnancies. Multiparity (multiple frequent pregnancies with a prolonged state of negative folate balance) and hyperemesis gravidarum commonly lead to folate deficiency. When anemia of pregnancy is due to combined iron and folate deficiency, dimorphic anemia is the more frequent clinical presentation. Increased use of folates by the newborn leads to a drop in serum folate levels by about 6 weeks of age. This drop is exaggerated in premature infants (who have feeding difficulties, infection, or hemolytic disease leading to pure folate deficiency); hence supplementation is routine for them.[16]

Folates and Neurodevelopment

All inborn errors of folate metabolism, which result in reduced folate availability to the developing brain, give rise to mental retardation and related mental health problems. The fetal brain is dependent on sufficient provision of maternal folate during embryogenesis. Thus depending on the degree of maternal folate deficiency, there could be a spectrum of neurologic abnormalities; this could range from full-blown NTDs to more subtle changes that manifest in childhood as behavioral abnormalities.[60,72,193] Indeed, experimental studies in animals[60] indicate that folate deficiency significantly compromises early pregnancy outcomes (rates of pregnancy, rate of implantation, and effects on the number of live births). Even lesser degrees of folate deficiency to only one-third of optimum dietary folate—which mimics the extent of insufficient dietary folate availability among women in vast areas of Northern India[68]—results in increased apoptotic fetal brain loss (by 20%) and profound abnormalities in the white matter, reflecting perturbed neuronal development.[60] Surprisingly, despite postnatal folate replenishment, these mice exhibited an anxiety phenotype in adulthood[190]; in another study rodents exhibited learning problems.[194,195] In concordance with these studies in mice, human studies now consistently reveal that lower maternal folate status in early pregnancy is associated with psychological-neuropsychiatric disorders in childhood (hyperactivity/inattention and peer problems[196]; hearing loss[197]; learning deficits; children are emotionally reactive, anxious/depressed, have somatic complaints, withdrawn, with attention problems and aggressive behavior).[198–204] Thus women who take folic acid during the early stages of pregnancy provide an in utero environment that ultimately impacts positively on the child's intellect, emotional intelligence, cognition, language, and academic performance[200,201,205]; these observations are consistent with Barker's hypothesis on the developmental origins of disease.

As discussed above, the homocysteinylation of the mRNA-binding protein, hnRNP-E1, during folate deficiency orchestrates a nutrition-sensitive posttranscriptional RNA *operon* leading to

FOLATE-RESPONSIVE NEURAL-TUBE DEFECTS AND NEUROCRISTOPATHIES

NTDs are the most common major congenital malformation of the central nervous system. They arise from disturbances in neurulation that involve incomplete closure of neural tissues, leading to major midline defects. The neural tube, which begins as a tiny ribbon of tissue, normally folds inward to form a tube by the 28th day after conception. Thus NTDs originate in the first month of pregnancy (before many women know they are pregnant). Therefore, it is critical for a woman to have enough folate in her body before conception (periconceptionally) to ensure sufficient availability for the embryo. Anencephaly and spina bifida, the commonest NTDs, are important factors in fetal mortality (Fig. 40.10). Landmark studies have established the preventive role of periconceptional folates in both the *recurrence* of NTD (using folic acid 4000 μg/day) and the *first occurrence* of NTD (using a minimum of folic acid 400 μg/day).[206–208] Of significance, the greatest protection by folates occurs in those regions with the highest rates of NTDs.[209] Conversely, the use of folic acid antagonists (trimethoprim, triamterene, carbamazepine, phenytoin, phenobarbital, and primidone) during pregnancy increases the risk for these birth defects by twofold.[210]

Because 50% of pregnancies in the United States and elsewhere are unplanned and compliance with taking folic supplements to prevent NTD is only at about 50%, in January 1998, fortification of foods (i.e., rice, flour, pasta, macaroni, breads, and cake) with folic acid at 140 μg/100 g of food) was part of American law. This level was chosen to ensure that women of childbearing age would have an increase in folic acid intake of at least 100 μg a day, which is about 25% of the recommended daily intake. Subsequent evaluation has demonstrated that folate fortification of food has had multiple salutatory effects during human development and that major congenital abnormalities are prevented.[189] Table 40.5 shows several documented collateral benefits identified through population-based studies.

There remains concern about the effectiveness of the folic acid fortification program for women in the 15- to 35-year-old age group in preventing NTDs. This stems from an incomplete knowledge base[211] and their tendency to consume low-carbohydrate foods—the very foods that are fortified. This is the basis for recommendations to continue to educate women of childbearing age to take folic acid supplements at 400 μg/day (beyond what they receive through folate fortification of food).

Although regulations for mandatory fortification of wheat flour with folic acid are in place (in over 80 countries), they have not been uniformly implemented.[212] Moreover, folate-fortified foods do not reach all women of reproductive age adequately in resource-limited countries, particularly the most needy women in the lowest socioeconomic bracket.[213] As a result, over 90% of pregnant women worldwide still do not receive sufficient folate,[191] and over one-half of the world's population still does not have easy access to folate-fortified foods. As a consequence, in India, two population-based studies conducted a decade apart in the most populous state have identified that the incidence of NTDs was up to 8.21 and 7.48 per 1000 live births, respectively, which is still among the highest worldwide.[214,215]

Contrary to the American Dietetic Association's position paper on vegetarianism,[216] there is an insufficient body of evidence to confidently assert that women in the United States of childbearing age who consume a balanced vegetarian diet with abundant green leafy vegetables will obtain sufficient folate and therefore not be at risk for giving birth to babies with NTDs. Until robust evidence from clinical trials is available to support this Association's position paper,[216] a prudent approach is to recommend that all women who are vegetarians must take cobalamin and folate supplements.

TABLE 40.5 Beneficial Effects of Homocysteine-Lowering Therapy on Nonhematopoietic Systems

Using Folic Acid, Cobalamin, Pyridoxine Supplementation (Grade A Studies)
Reduction in hip fracture[293,a]
Reduction in the progression of carotid intima media thickness[294,a] (a surrogate marker of early subclinical arteriosclerosis)
Reduction in age-related macular degeneration[295,a]
Reduction in rate of brain atrophy[178,a]
Improvement in cognitive function[179,280,281,a]
Using Folic Acid Supplementation (Grade A Studies)
Reduction in stroke[291,a]
Reduction in the rate of cognitive decline among healthy older adults[279,a]
Reduction in age-related (sensorineural) hearing loss[292,a]
Reduction in first occurrence of NTDs[207,a]
Reduction in recurrence of NTDs[206,a]
Reduction in phenytoin-induced gingival hyperplasia[313,a]
Beneficial Effects of Folic Acid Fortification of Food (Population-Based Studies)
Reduction in NTDs[208,258] (anencephaly, spina bifida, encephalocele, meningocele, iniencephaly)
Reduction in cleft lip with or without cleft palate[314]
Reduction in severe congenital heart disease[258,314] (endocardial cushion defects, conotruncal defects)
Reduction in congenital pyloric stenosis, stenosis of the ureteropelvic junction, limb reduction defects[314]
Reduction in stroke mortality[315]
Decreased risk for preterm births, low birth weight, and small-for-gestational-age babies[316]

[a] Paper with randomized controlled trial data; grade A studies.
NTD, Neural-tube defect.

up- or down-regulation of several mRNA-derived proteins in murine brain.[62,64] There is now evidence that overexpression of one such mRNA member of this operon (encoding for neuronal intermediate neurofilament-middle molecular mass proteins) can perturb the integrity of myelin, whereas overexpression of another member, tyrosine hydroxylase mRNA, predicts for excessive generation of the key neurotransmitters (dopamine and norepinephrine) and disordered behavior.[62,64] (There may well be other proteins within the homocysteine-activated hnRNP-E1 operon that contribute to abnormal neurological dysfunction.) Such preclinical studies provide early mechanistic insight into how folate and cobalamin deficiency during pregnancy can influence fetal neurodevelopment and contribute to neuropsychological-neuropsychiatric disorders.[60,62,194] Indeed, it is possible that during folate deficiency, there may be a progressive homocysteine-mediated activation of many individual members of the wider hnRNP family within the developing brain. This, in turn, can trigger activation of the individual posttranscriptional RNA operons controlled by each of these hnRNPs, which, acting together in concert as a higher-order posttranscriptional RNA *regulon*, can profoundly affect fetal neurodevelopment.[62] Thus it appears that we are likely peering through the mist into an entirely new field, whereby nutritional folate insufficiency during fetal neurodevelopment can predispose to neuropsychiatric illness. (See box on Folate-Responsive Neural-Tube Defects and Neurocristopathies.)

Folates and Intrinsic Hematologic Disease

Because folate is necessary for hematopoiesis, folate requirements are increased when there is significant compensatory erythropoiesis in response to peripheral RBC destruction, abnormal hematopoiesis, or infiltration by abnormal cells in marrow. The recognition that folate deficiency developing in hemolytic disorders can lead to an acute aplastic crisis has long led to recommendations favoring routine prophylactic administration of folate. A meta-analysis of 19 studies has put to rest the concern of iron-folate and malarial progression[217] and a Cochrane analysis identified *no* evidence that this is a problem when combined with antimalarial drugs and insecticide-treated bed nets.[218,219] Sadly however, this dictum is not followed for most children living in malarial regions. An unexpected increase in transfusional requirement or a fall in platelets can also suggest folate deficiency.[16] The case of a patient with sickle cell disease on long-term folate who developed pernicious anemia and presented with neuropsychiatric dysfunction[220]

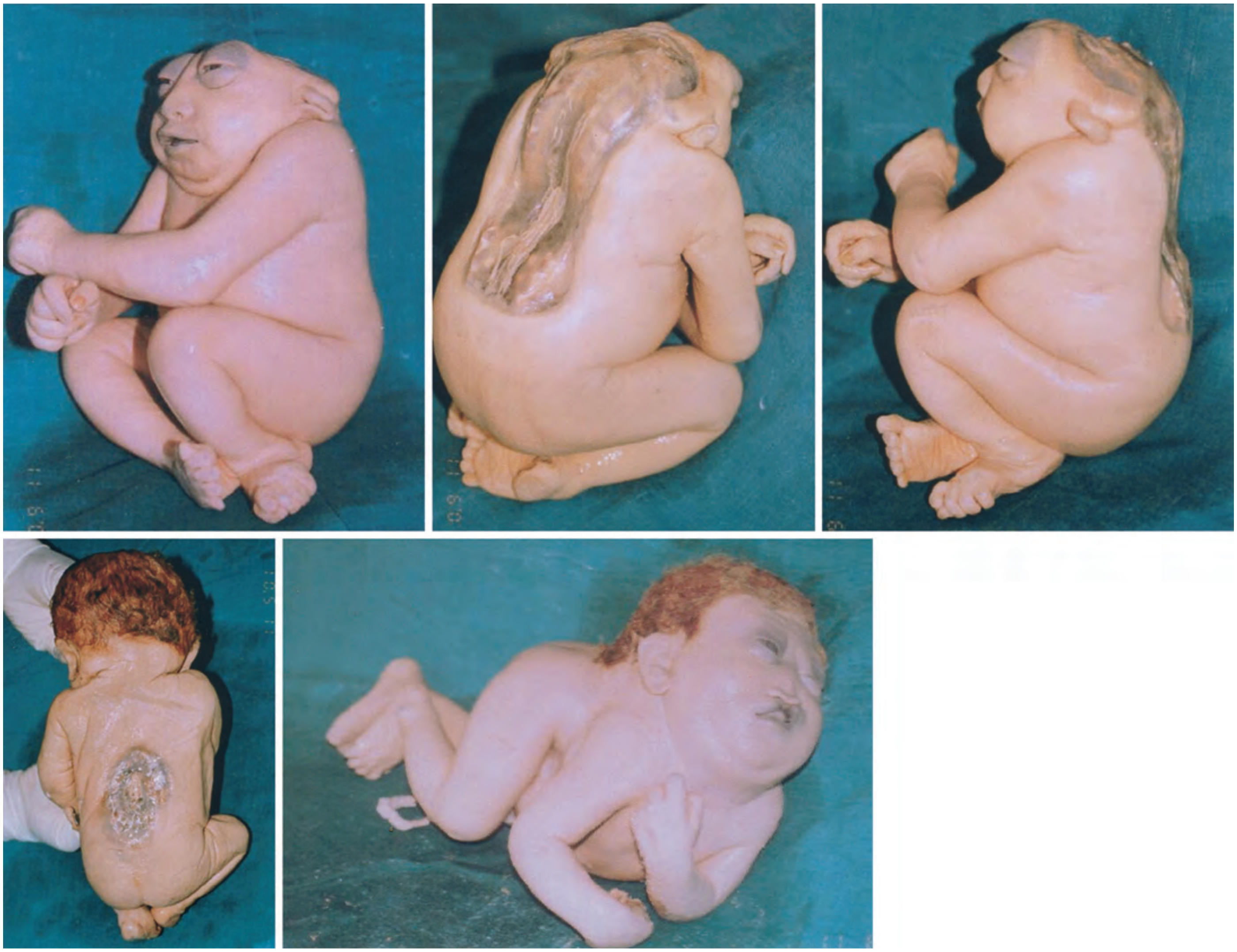


Figure 40.10 FOLATE-RESPONSIVE NEURAL-TUBE DEFECTS. Anencephaly with complete rachischisis *(top panel)*, open infected meningomyelocele *(bottom left)*, and iniencephaly with cleft lip *(bottom right*). *(Courtesy Prof. Molly Paul, Anatomy Department Museum, Christian Medical College and Brown Memorial Hospital, Ludhiana, Punjab, India.)*

is a valuable reminder that those on long-term folate prophylaxis need periodic follow-up for symptoms and signs of supervening cobalamin deficiency.

Folate Malabsorption With Normal Intestinal Mucosa

Hereditary Folate Malabsorption

Hereditary folate malabsorption, which is caused by loss of function mutations in the *PCFT* gene, was detected in approximately 35 different families (by 2015); in the USA, those of Puerto Rican descent share a common mutated allele.[221] Loss of PCFT is associated with inability to transport folate across the intestine and the choroid plexus and into the brain, resulting in low serum and CSF folate values with megaloblastic anemia, chronic diarrhea, and neurodevelopmental defects with seizures and mental retardation; this syndrome responds to high-dose parenteral folinic acid that bypasses and overcomes the transport defects. Affected patients can also present with a syndrome of reversible subacute combined immunodeficiency syndrome with hypogammaglobulinemia and recurrent infections.[222,223] If diagnosed early in infancy, hereditary folate malabsorption responds well to parenteral 5-formyltetrahydofolate and can allow normal development into adulthood.[218]

Folate Malabsorption With Intestinal Mucosal Abnormalities

Tropical Sprue

Residents of, and visitors to, endemic areas in the tropics can acquire a disorder characterized by small intestinal malabsorption.[16] Generalized, nonspecific small bowel malabsorption leads to a wide spectrum of clinical manifestations arising from defective absorption of fat, carbohydrate, albumin, calcium, folate, and in later stages, cobalamin.[16] There is abrupt onset of explosive, intermittent, or continuous diarrhea, abdominal distention, and pain, associated with anorexia, vomiting, and extreme fatigue. Stools are fluid or semisolid and frequently contain mucus and blood. This stage is followed weeks to months later by nutrient deficiency. Later, as steatorrhea continues, megaloblastosis dominates the clinical picture. In the short term, malabsorption leads to folate deficiency, but later in the chronic (longer than 3 years) phase of the disease, cobalamin malabsorption contributes additional clinical manifestations of cobalamin neuropathy.[16] There is some degree of villous atrophy and loss of intestinal functional surface. Although less severe than in nontropical sprue, it is more extensive, involving the entire small intestine.

After investigations for associated iron, cobalamin, and folate deficiencies, therapy with folate and a broad-spectrum antibiotic (e.g., tetracycline) is indicated together with symptomatic treatment of diarrhea and vomiting; fluid, mineral, and electrolyte imbalance; and other associated nutritional deficiencies.[16]

The dramatic response to folate and clinical response to antibiotics suggests a close interplay among a pathogenic infectious agent, endogenous flora, and the folate status of the enterocyte.

Nontropical Sprue

Nontropical sprue (i.e., celiac disease, gluten-induced enteropathy) is the most common cause of intestinal malabsorption in temperate zones. It results from a possibly inherited sensitivity to gluten (a glutamine-rich protein found in wheat, barley, rye, and other grains) and a related substance, gliadin.[16] The intestinal lesion (i.e., villous atrophy with hypertrophied crypts and lymphocytic and plasma cell infiltrate of the lamina propria) is more florid than that seen in tropical sprue but occurs to a greater extent in the proximal small intestine with relative ileal sparing; as a result, cobalamin malabsorption is less common. The consequences of malabsorption are otherwise the same. Patients present between the ages of 30 and 50 years with intermittent or persistent diarrhea (abrupt in 20%), weight loss, abdominal distention with discomfort, glossitis, and megaloblastic anemia. Iron deficiency is often prominent. Diagnosis is established by documenting the presence of sensitive and specific serum antiendomysial antibodies IgA type or anti-tissue transglutaminase antibodies, malabsorption, and jejunal biopsy. The megaloblastosis responds well to folate therapy.[16]

Regional Enteritis and Other Small Intestinal Disorders

The distal small intestine is involved in 80% of individuals with Crohn disease, so extensive ileal involvement or ileal bypass can result in malabsorption of cobalamin; the risk is twice as high with Crohn disease compared to ulcerative colitis.[224] However, a poor diet or extensive mucosal disease can increase requirements for folate[225] and the chronic use of sulfasalazine can interfere with the absorption of folates via interference with PCFT function.[45,226]

Cerebral Folate Deficiency

Cerebral folate deficiency is a broad syndrome whereby several diseases can be accompanied by neurologic findings with low CSF folate but normal serum folate levels.[227,228] The GPI-anchored folate receptor-α on the basolateral surface of choroid plexus cells is critical to the transport of folate from the blood across the CSF into the brain. Predictably, therefore, mutation of folate receptor-α, or the generation of antibodies to folate-binding proteins found in cow's milk that can cross-react with choroid plexus folate receptor-α, can perturb folate transport into the brain and lead to cerebral folate deficiency. Indeed, among a group of 14 children with extremely low (≤5 nmol/L) levels of 5-methyl-THF in the CSF, mutations in folate receptor-α, which led to a failure in expression of functional folate receptors, were identified in 10 of them (71%).[48,49] These children presented at less than 3 years with symptoms of developmental delay, movement disturbances, ataxia, myoclonic seizures, infantile spasms, and leukodystrophy that was reversed by folinic acid.[48,229] The finding of two older patients (aged 13 and 15 years) with severe polyneuropathy raises the possibility of other patients who may also have such defects that can present in young adulthood with unexplained neurologic disorders.

Acquired infantile-onset cerebral folate deficiency, which is associated with autoantibodies to folate receptor-α,[230,231] usually develops 4 to 6 months after birth and responds to high doses of folinic acid and a cow's milk–free diet.[232] Among 93 patients with autism spectrum disorders, 75% had autoantibodies to folate receptor in the serum; treatment with high-dose leucovorin led to improvement in one-third of those treated.[233] Of significance, an adult-onset presentation of cerebral folate deficiency with progressive memory loss and myoclonus caused by perturbation of folate receptor function by autoantibodies has also been described.[234] Finally, although anti-folate receptor antibodies are also associated with neural-tube defects,[78] infertility,[235] and orofacial clefts,[236] more information is required to confidently assign causality with these conditions.

Polymorphisms and Inborn Errors of Folate Metabolism

Several genetic polymorphisms involve genes that participate in one-carbon metabolism. These genetic polymorphisms merely reflect variants that are more frequent than the expected 1% allelic variation that could be found in any population. Whereas some polymorphisms impinge on normal physiology of folate and explain abnormal laboratory levels of folate, cobalamin, or metabolites, others may redistribute folate toward thymidylate synthesis and be "DNA protective." Predictably, two or more polymorphisms in the same individual (a combined heterozygote) can be associated with an increased risk for certain congenital diseases in offspring such as Down syndrome[237] or other birth defects. However, because disease association is not equivalent to disease causation, much more study is required to strengthen such relationships. A listing of the spectrum of these polymorphisms can be found in specialized texts.[238] Excellent reviews of inborn errors of folate metabolism are available.[163]

MEGALOBLASTIC ANEMIA NOT CAUSED BY FOLATE OR COBALAMIN DEFICIENCY

Several chemotherapeutic agents (e.g., antimetabolites, alkylating agents) kill malignant cells primarily by interfering with DNA synthesis; megaloblastosis is therefore an expected side effect. (See box on Miscellaneous Megaloblastic Anemias Not Caused by Cobalamin or Folate Deficiency.)

THERAPY

Routinely, treatment with full doses of parenteral cobalamin (1 mg/day) and oral folate (folic acid) (1 to 5 mg) before knowledge of the type of vitamin deficiency is established should be reserved for the severely ill patient. An appropriate regimen for conditions in which cobalamin replenishment can correct cellular cobalamin deficiency (but not correct the underlying problem that led to the deficiency, such as pernicious anemia) is 1 mg of intramuscular or subcutaneous cyanocobalamin per day (week 1), 1 mg twice weekly (week 2), 1 mg/week for 4 weeks, and then 1 mg per month for life (about 15%, or

MISCELLANEOUS MEGALOBLASTIC ANEMIAS NOT CAUSED BY COBALAMIN OR FOLATE DEFICIENCY

I. Congenital disorders of deoxyribonucleic acid (DNA) synthesis (rare)
  A. Orotic aciduria
  B. Lesch-Nyhan syndrome
  C. Congenital dyserythropoietic anemia
II. Acquired disorders of DNA synthesis
  A. Deficiency—thiamine-responsive megaloblastic anemia (thiamine transporter 1 mutation)
  B. Erythroleukemia, refractory sideroblastic anemias
  C. Drugs—any drug that inhibits DNA synthesis[119]
    - antinucleosides used as immunomodulators in autoimmune diseases
    - antineoplastics
    - antivirals against human immunodeficiency virus (HIV), hepatitis B/C, hantavirus, etc.
    - alcohol

DRUGS THAT PERTURB FOLATE METABOLISM[119]

Ethanol. Although beer has higher folate content than other alcoholic beverages, alcoholism may lead to neglect of healthy dietary practices in favor of alcohol. Alcohol consumption leads to a relatively rapid (2- to 4-day) fall in serum folate levels. Excess alcohol consumption is still a common cause of folate deficiency in the United States.[16]

Trimethoprim and *pyrimethamine* bind to bacterial and parasitic dihydrofolate reductase with much greater affinity than to human dihydrofolate reductase, but patients with underlying folate deficiency appear to be more susceptible to the effects of these drugs. The megaloblastosis can be reversed by folinic acid (5-formyl-tetrahydrofolate [5-formyl-THF]; leucovorin).[16]

Methotrexate binds with high affinity to human dihydrofolate reductase and leads to trapping of folate as a metabolically inert form (dihydrofolate). This leads to a true depletion of THF within hours and consequently to functional deficiency of 5,10-methylene-THF and reduced thymidylate synthesis. Although megaloblastosis can develop rapidly, the toxic effects of methotrexate can be avoided by rescue with 5-formyl-THF (leucovorin).[16]

Sulfasalazine produces megaloblastosis in up to two-thirds of patients taking full doses (over 2 g/day) by decreasing absorption of folates and induction of Heinz body hemolytic anemia thereby increasing requirements.[16]

Anticonvulsants can induce neural-tube defects (NTDs), and consensus guidelines have stressed the importance of ensuring that pregnant women[23] and children[239,240] with epilepsy be prescribed folates together with anticonvulsants. Whereas folates protect against spontaneous abortion,[241] folic acid supplementation of women receiving antiepileptic drugs also leads to a significant reduction of spontaneous abortion.[242] Phenytoin-induced gingival hyperplasia, which is a cosmetically undesirable side effect that affects a large percentage of patients, usually develops between 2 and 6 months of initiating therapy. A randomized controlled trial among children 6 to 15 years of age who were initiated on phenytoin has provided incontrovertible evidence that taking folic acid 0.5 mg daily can largely prevent phenytoin-induced gingival hyperplasia (whereas 88% in the placebo group developed gingival hyperplasia, only 21% in the folic acid group developed this side effect).[218] The data from this paper provide more “ammunition” to encourage young women on antiepileptic drugs to keep taking folic acid to prevent them from developing unsightly gingival hyperplasia—(particularly if reducing the risk for having a baby with NTD is too nebulous a concept for them). The only caveat is that before initiating long-term folic acid supplements, the cobalamin status must be normalized.

Although *antineoplastics, antiviral antinucleosides*, and *immunomodulators* can lead to megaloblastosis,[119] the temporal sequence and investigations to rule out cobalamin or folate deficiency should easily lead to a correct causal assignment.

CLINICAL PRESENTATIONS AND EVALUATION FOR FOLATE AND COBALAMIN DEFICIENCY

Clinical presentations and evaluations for folate and cobalamin deficiency are shown in Table 40.6.

The Interview

The patient’s general demeanor and answers to questions may reveal a blunted affect with evidence of depression, irritability, forgetfulness, and sleep deprivation (common in pure folate deficiency). Alternatively, cobalamin deficiency may present with paranoid ideation, dementia, cognitive dysfunction, delusions, or lack of energy manifested by slowed responses. Hallucinations or even obtundation may preclude obtaining an adequate history. The family may indicate the progressive evolution of a marked personality change and may be able to help trace the evolution of symptoms and deviations from the time when the patient was last well. Intermittent therapy with multivitamins, liver pills, or injections (often given by a well-meaning family member or unregistered practitioner) is a common quick fix in many cultures. Family members are a good source for details on the patient’s dietary habits (food faddism, vegetarianism, alcohol intake) and family history of medical problems (blood diseases, gluten sensitivity, autoimmune diseases).

A medical history of epilepsy or alcoholism with seizure disorder (anticonvulsant therapy) is important. Rarely, patients with autoimmune hemolytic anemias may be lost to follow-up and return with acute aplastic crises when they run out of folate. A surgical history of total or partial gastrectomy, anastomosis, fistula, or bowel resection can reveal the potential for perturbation of physiologic absorption (loss of intrinsic factor, bypassing or loss of absorptive surface, blind loop syndromes). Surreptitious or accidental inhalation of nitrous oxide in an occupational setting (dental or anesthesiology professionals) and deliberate inhalation of nitrous oxide using cartridges attached to whipped cream dispensers or visits to “houses of laughter,” where nitrous oxide can be inhaled for a small fee can be revealed only on direct questioning. Visits to tropical countries and the development of intermittent episodic diarrhea may give a clue to tropical sprue; prolonged (over 3 years) chronic gastrointestinal symptoms followed by insidious development of neurologic problems predicts a combined (folate followed by cobalamin) deficiency (see box on Drugs That Perturb Folate Metabolism).

Systemic review of symptoms may range from none (i.e., incidental increased mean corpuscular volume or PMN hypersegmentation) to severe (i.e., unstable angina from severe anemia). With slow development of anemia, the patient often does not develop cardiopulmonary symptoms until there is a 50% reduction in hemoglobin concentration, which leads to dyspnea on exertion, palpitation, and generalized fatigue or lethargy. Only when the hemoglobin concentration is below 5 g/dL does the patient develop dyspnea at rest and angina on modest exertion or even at rest. Congestive heart failure is heralded by pedal edema, nocturia, orthopnea, and tender hepatomegaly.

Upper gastrointestinal symptoms with anorexia associated with intrinsic gastrointestinal disease or anemia with heart failure must be distinguished from symptoms arising from aphthous ulcers or glossitis. The latter may lead to inability to wear dentures, or tolerate hot drinks or spicy foods because of burning, and even odynophagia, which may compromise further food intake (seen in cobalamin and folate deficiencies). The patient may volunteer that aphthous ulcers[243] or glossitis is relieved by vitamin B_{12} or multivitamin ingestion. Weight loss in cobalamin deficiency is not as severe as in folate deficiency arising from intrinsic gastrointestinal disease. Episodic or chronic diarrhea with steatorrhea is commonly caused by tropical sprue, although it may be brought on by gluten-containing foods. Although these symptoms may be accompanied by abdominal pain, pain in the absence of diarrhea could be caused by tabetic crisis (vomiting, abdominal rigidity, absence of leukocytosis, or fever) accompanying spinothalamic involvement in cobalamin-deficient myelopathy.

The patient with pernicious anemia may have two or three semisolid bowel movements per day; although this may be construed as a normal pattern, it may represent a change since the last time the patient was well. Constipation may be related to obstipation arising from involvement of the Meissner plexus and the Auerbach plexus within the gastrointestinal tract. Similarly, incipient loss of bladder or bowel control caused by cobalamin myelopathy may present with urgency or nocturia.

In contrast to musculoskeletal symptoms (arthralgia or frank arthritis) of autoimmune diseases, nocturnal cramps or pain in upper and lower extremities may indicate spinothalamic tract involvement. Hypoparathyroidism or systemic lupus erythematosus, alone or associated with pernicious anemia, leads to significant overlap of cerebral, musculoskeletal, and neurologic presentations.

Review of skin symptoms may elicit a history of increased diffuse or blotchy generalized brownish skin pigmentation, especially of nail beds and skin creases and knuckles. This is common in cobalamin deficiency; associated vitiligo suggests autoimmune disease.

Although symptoms related to neurologic dysfunction may be volunteered, a complete detailed questionnaire should be formulated during the interview. Questions should be directed to perversions in taste or smell, decreased visual acuity, changes in color vision, and eye pain (neuritis), tinnitus, or headache. Dizziness with orthostatic hypotension and “blacking out” may be related to severe anemia or cobalamin-deficient autonomic dysfunction, which can also be associated with night sweats.[244] Vertigo or difficulty in walking in the dark (loss of proprioception and position sense), difficulty in ambulation (which may feel like

CLINICAL PRESENTATIONS AND EVALUATION FOR FOLATE AND COBALAMIN DEFICIENCY—CONT'D

"walking on cotton wool"), stiffness of extremities (corticospinal tracts), or ataxia (spinocerebellar tracts) may be indicative of a serious cobalamin myelopathy. Early symptoms are symmetrical tingling ("pins and needles"), extending from the tips of the toes to a glove and stocking distribution in later stages. "Burning feet" syndrome, or more commonly, complaints of difficulty in performing simple tasks such as buttoning clothes, may also be a presenting symptom. When loss of bladder and bowel control brings the patient to the physician, advanced neurologic dysfunction is invariably present.

Genitourinary symptoms such as erectile dysfunction or recurrent cystitis from bladder dysfunction can suggest cobalamin neuropathy. Multiple pregnancies with short intervals between delivery and conception predispose to a high risk for overt folate deficiency and contribute to fetal growth restriction in babies (cobalamin deficiency is more often associated with infertility).

The Physical Examination

Physical examination may reveal different features in well-nourished patients (cobalamin-deficient vegetarians or pernicious anemia) and poorly nourished (folate-deficient) individuals. The latter show evidence of significant weight loss or other stigmata of multiple deficiencies because of either poor diet (malnutrition) or "broad-spectrum" malabsorption. Associated deficiency of vitamins A, D, and K and protein-calorie malnutrition may give rise to angular cheilosis, bleeding mucous membranes, dermatitis, osteomalacia, and chronic infections. Various degrees of pallor with lemon-tint icterus (i.e., a combination of pallor and icterus best observed in fair-skinned individuals) are common features of megaloblastosis.

When anemia is severe, the patient may have a low-grade fever. The skin may reveal a diffuse, brownish pigmentation or abnormal blotchy tanning.[245] A macular hyperpigmentation with follicular accentuation may be observed in the axilla and groin; hyperpigmentation can also involve the dorsal acral distal interphalangeal joints and a reticular pigmentation in the mid–upper back can develop slowly over a year (but resolves within 2 months of cobalamin replacement).[245] Special emphasis should be given to pigmentation of skin creases and nail beds and both palmar and dorsal aspects of hands and feet.[246] (Mucous membrane pigmentation is usually not observed, in contrast to Addison disease.) Premature graying, observed in light- and dark-haired individuals, is reversible within 6 months of cobalamin therapy.

A blunted mask-like facies is extremely common in folate deficiency. Alternatively, there may be evidence of classic hyperthyroid or hypothyroid facies (associated with pernicious anemia). Special attention should be given to the eyes and eyebrows for the well-known signs of thyroid dysfunction.

Examination of the mouth may reveal aphthous ulcers or glossitis with a smooth (depapillated), beefy red tongue with occasional ulceration of the lateral surface or gingival hyperplasia (antiepileptics). The neck may reveal thyromegaly (diffuse or with nodules) if there is associated disease. Increased jugular venous distention should alert the examiner to cardiovascular failure, with its attendant gallop, cardiomegaly (with or without pericardial effusions), pulmonary basal crepitations, pleural effusion, tender hepatomegaly, and pedal edema. Nontender hepatomegaly, but more often mild splenomegaly, may rarely be caused by extramedullary hematopoiesis in severe anemia, but a mid-epigastrium mass raises the ominous possibility of gastric carcinoma, which is up to seven times more likely[247] in patients with pernicious anemia.

In the West, an inverse correlation has been identified between the extent of anemia and neurologic dysfunction. Patients with normal complete blood count values often have neurologic signs and symptoms. In prolonged cobalamin deficiency, the neurologic examination reveals clear-cut evidence of involvement of posterior and pyramidal, spinocerebellar, and spinothalamic tracts. Among the earliest signs of posterior column dysfunction are loss of position sense in the index toes (before great toe involvement), which is elicited by passive movement, and loss of the ability to discern vibration of a high-pitched (256 cycles/s) tuning fork. This is a very early elicitable, objective sign, which invariably precedes by many months the loss of ability to sense the vibration of a lower-pitched (128 cycles/s) tuning fork. Usually the patient loses vibration sense to 256 cycles/s from toe to hip before loss of 128 cycles/s vibration sense even begins. Because of the slow coalescence of contiguous spinal cord lesions, a constellation of elicitable signs may be obtained. Upper motor neuron disease is indicated by weakness and progressive spasticity with increased muscle tone, exaggerated deep tendon reflexes with clonus, extensor plantar response, and incoordinate or scissor gait, which may progress to spastic paraplegia. The involvement of peripheral nerves may markedly modify these signs to include flaccidity and the absence of deep tendon reflexes. A positive Romberg sign is not uncommon, and a positive Lhermitte sign may be elicited. Loss of sphincter and bowel control, altered cranial nerve dysfunction with altered taste, smell, and visual acuity or color perception, and optic neuritis may be other physical signs indicating cobalamin deficiency. Inability to carry out serial subtraction of 7 from 100 is a valuable test to document reduced cerebral function (the electroencephalogram often reveals slow wave frequency) in pernicious anemia, which can progress over time to presentation with catatonia[248] or delirium (see box on Approach to Diagnosis and Therapy of Megaloblastosis and box on Practicing Classical Medicine Without the (Classical) Schilling Test.)[249]

APPROACH TO DIAGNOSIS AND THERAPY OF MEGALOBLASTOSIS

In general, there are three stages in approaching a patient: *recognizing* that megaloblastic anemia is present; *distinguishing* whether folate, cobalamin, or combined folate and cobalamin deficiencies have led to the anemia; and diagnosing the *underlying disease* and *mechanism* causing the deficiency. Establishing that the patient does have megaloblastosis is, in theory, straightforward. This is easily done by first evaluating the complete blood count, the mean corpuscular volume, and the peripheral smear; a bone marrow aspiration and biopsy is indicated if there is suspicion of another hematological disorder masquerading as a megaloblastic anemia (leukemia, myelodysplastic syndrome, aplastic anemia, etc.). Clues to whether cobalamin or folate deficiency is responsible for megaloblastosis can be obtained by serum cobalamin and serum folate levels; if these levels are borderline, additional testing of serum MMA and serum homocysteine can define the true nature of the deficiency.[23] However, this ideal and orderly workup is not always feasible in clinical practice, because the patient may present either for the first time with megaloblastosis with or without associated neurologic disease; or may be referred after a variable workup has already been initiated for possible megaloblastosis; or may present with symptoms primarily attributed to a disease predisposing to cobalamin or folate deficiency; or may present with a disease associated with hyperhomocysteinemia (discussed later), in which case anemia or neurologic dysfunction may only be a minor symptom; or may present with isolated neurologic disease in the absence of anemia; or may be referred after empirical therapy has been given for presumed cobalamin or folate deficiency. The immediate question therefore pertains to the overall status of the patient:

1. *If the patient is decompensated or decompensation is imminent*, obtain serum folate and cobalamin levels and freeze another serum sample in case metabolite testing is indicated later (i.e., if cobalamin and/or folate levels are equivocal). A bone marrow aspiration and biopsy should also be considered at this time (to confirm megaloblastosis or rule out another hematological disorder). Then proceed with transfusion of 1 unit of packed RBCs *slowly*, with vigorous diuretic therapy to obviate further congestive heart failure from fluid overload; this mandates the close monitoring and correction of fluid and electrolyte imbalance. Cobalamin and folate should also be administered simultaneously in full doses. Transfusion does not alter serum folate or cobalamin levels.
2. *If the patient is only mildly or moderately symptomatic* (but not likely to decompensate in the short term), proceed as above but withhold packed RBC transfusion. The strong likelihood of a dramatic response (in the sense of well-being and relief of sore tongue) within 2–3 days even before hematologic improvement argues against immediate blood transfusion.[23]
3. *If the patient is well compensated and is either in the hospital or in the outpatient setting*, the physician has time to develop an orderly

Continued

APPROACH TO DIAGNOSIS AND THERAPY OF MEGALOBLASTOSIS—CONT'D

sequence of diagnostic tests. First, check the peripheral smear and rule out other macrocytic anemias (thin macrocytes with a normoblastic marrow in contrast to macro-ovalocytes) (see Fig. 40.7). Draw blood for cobalamin and folate levels to sort out whether the problem is caused by a deficiency of folate or cobalamin, or both, or some other deficiency (see Table 40.2). The physician can elect to wait for the results of these tests before proceeding with the next test in the diagnostic workup.

If the serum cobalamin and folate levels are equivocal (i.e., in the low-normal range), then test for serum homocysteine and MMA. Integrating the results for serum MMA and homocysteine levels (which will be available after a week or more) with those for serum cobalamin and folate levels can help distinguish cobalamin and folate deficiencies (see Table 40.2). A normal MMA and homocysteine level eliminates cobalamin deficiency with 100% confidence, and normal homocysteine levels suggest that megaloblastic anemia is not caused by folate deficiency. These tests are particularly useful if the patient has pure neurologic disease or if there are associated conditions such as iron deficiency or thalassemia that can mask megaloblastosis. Administration of folate or cobalamin will reduce elevated serum homocysteine and MMA levels to basal values by 1 week,[48,175,221] so there is only a narrow window to clinch the diagnosis using metabolite tests.[23] In the rare situation when a hereditary defect in cobalamin or folate metabolism is suspected, early consultation with experts who have published in this area is advised.[20,48,175,176,221,250,251]

Additional supporting studies for megaloblastosis include reticulocytopenia, increased serum LDH, low haptoglobin, and increased bilirubin (evidence for intramedullary hemolysis).

Finally, try to determine the underlying mechanism of cobalamin or folate deficiency. The cause of folate deficiency is usually sorted out by this time from the history, physical examination, and the clinical setting. If pure folate deficiency has been prolonged, expect associated cobalamin deficiency to ensue (special emphasis should be given to identifying subtle manifestations of neurologic disease). If cobalamin deficiency is suspected, test for both serum anti-intrinsic factor antibodies and the newer more sensitive anti-parietal cell antibodies (see section on Pernicious Anemia.) Also see box on Practicing Classical Medicine Without the (Classical) Schilling Test.

TABLE 40.6 **Similarities of Clinical Manifestations and Megaloblastic Sequelae of Folate and Cobalamin Deficiency**[a]

System	Manifestations
Hematologic	Pancytopenia with megaloblastic marrow
Cardiopulmonary	Congestive heart failure
Gastrointestinal	Beefy-red tongue and added stigmata of broad-spectrum malabsorption in folate deficiency[b]
Dermatologic	Melanin pigmentation and premature graying
Genital	Cervical or uterine dysplasia
Reproductive	Infertility or sterility
Psychiatric	Depressed affect and cognitive dysfunction
Neuropsychiatric[c]	Unique to cobalamin deficiency with cerebral, myelopathic, or peripheral neuropathic disturbances, including optic and autonomic nerve dysfunction

[a] However, the neurologic spectrum of dysfunction in cobalamin deficiency is distinct. Inadequate hemoglobinization (from inadequate iron stores or globin synthesis) can mask the expected erythroid megaloblastic morphologic findings in the bone marrow and peripheral smear, and only specific therapy (i.e., iron) can unmask classic megaloblastic manifestations (i.e., masked megaloblastosis). Megaloblastic leukopoiesis is unchanged.

[b] If folate deficiency is uncorrected for 2–3 years, cobalamin deficiency will supervene.

[c] Dorsal tract involvement is earliest manifestation in more than 70% of patients with cobalamin deficiency. Neuropsychiatric manifestations are not associated with megaloblastosis in up to 30% of patients.

150 µg, is retained 48 hours after each 1 mg cobalamin injection).[16,23] *In principle, this protocol for rapid correction of cobalamin deficiency and complete replenishment of cobalamin stores should be used in the beginning for all patients with cobalamin deficiency, regardless of the etiology* (also see box above on Modified Therapeutic Trials).

Parenteral hydroxocobalamin is retained longer than cyanocobalamin and is widely used in Europe.[252] It is also reserved for all inborn errors of cobalamin metabolism. There is, however, no scientifically-proven major advantage of other preparations over generic cyanocobalamin in acquired cobalamin deficiency. There is equivalence between oral 2-mg cobalamin tablets consumed daily (where cobalamin is passively absorbed at high doses) and traditional monthly parenteral treatment with 1 mg of intramuscular/subcutaneous cobalamin among those requiring long-term cobalamin. So for patients who refuse monthly parenteral therapy, or prefer daily oral therapy, or in those with disorders of hemostasis, cobalamin (1 to 2 mg/day as tablets) can be recommended for all those patients with cobalamin malabsorption.[16,23,253] Alternative routes for cyanocobalamin delivery include nasal or sublingual preparations. The physician must ensure that the patient is compliant and demonstrates adequate cobalamin levels as well as resolution of hematologic and neurologic abnormalities on follow-up. For nutritional cobalamin deficiency (e.g., vegetarians) when the entire circuitry in cobalamin absorption is intact, after replenishing cobalamin stores with full doses, daily oral cobalamin of over 10 µg (found in conventional multivitamin tablets in the United States, or up to 50 to 150 µg tablets) taken for a lifetime of vegetarianism will suffice. However, if malabsorption of food-bound cobalamin is suspected (especially in the elderly with hypochlorhydria or achlorhydria), higher doses of daily oral cobalamin (equal to or greater than 1000 µg/day) is required.[254]

The bioavailability of oral cobalamin can be reduced by about 40% when it is taken with a meal; therefore, taking cobalamin on an empty stomach will lower losses in the stool. More than 98% of all the cobalamin in feces is in the form of cobalamin analogues, and about 80% of ingested food cobalamin is converted to analogues by microorganisms in the gut.[255]

Oral folate (folic acid) at doses of 1 to 5 mg/day results in adequate absorption (even where intestinal malabsorption of physiologic food folate is present). Therapy should be continued until complete hematologic recovery is documented. If the underlying cause leading to folate deficiency is not corrected, folate may be continued. Folinic acid (i.e., 5-formyl-THF [leucovorin]) should be reserved *only* for rescue protocols involving antifolates (methotrexate or trimethoprim-sulfamethoxazole), for 5-fluorouracil modulation protocols, after nitrous oxide toxicity, or in pediatric cases involving cerebral folate deficiency, hereditary folate malabsorption, or other inborn errors of folate metabolism. It is too expensive for conventional repletion in folate-deficient states in adults.

Response to Replenishment

The response of the patient to appropriate replacement is reversion of megaloblastic hematopoiesis to normal hematopoiesis within the first 12 hours; by 48 hours, normal hematopoiesis is reestablished, and the only evidence for a prior megaloblastic state may be the persistence of a few giant metamyelocytes. Clinically the first 36 to 48 hours are often highlighted by the awakening of an occasional semistuporous individual whose "chief complaint" is amazement at the remarkably improved sense of well-being experienced, with increased alertness and appetite, reduced soreness of the tongue, and healing aphthous ulcers.[243]

PRACTICING CLASSICAL MEDICINE WITHOUT THE (CLASSICAL) SCHILLING TEST

The Schilling test was used to identify the locus of cobalamin malabsorption and, in some instances, such as pernicious anemia or bacterial overgrowth, the cause of cobalamin deficiency. However, this test has been unavailable since 2003 in the United States. Although use of combined anti-IF antibodies and newer anti-parietal cell antibodies yields an overall 73% sensitivity while maintaining 100% specificity for pernicious anemia,[169] there remain difficulties in differentiating patients with all other causes of cobalamin malabsorption. A minimalist approach is to work around the unavailability of the Schilling test and use a classical clinical approach to rule out potential differential diagnoses of cobalamin deficiency (see box on Etiopathophysiologic Classification of Cobalamin Deficiency). For example, most conditions predisposing to cobalamin deficiency should be clinically manifest by the time cobalamin deficiency is evident. It should therefore be possible to identify several conditions through a detailed dietary history or past medical/surgical history, travel history, and drug history to suggest a nutritional deficiency, esophago-gastroduodenal disease, pancreatic insufficiency, impaired bowel motility, or other autoimmune diseases. The history and physical examination could provide further leads and suggest additional focused laboratory testing for rare conditions (stool for ova, anti–tissue transglutaminase antibodies, lipase, gastrin, intestinal biopsy, or radiographic contrast studies for stasis, strictures, fistulas). With no further leads, and therefore by default, one can assume that the diagnosis is either pernicious anemia or food-cobalamin malabsorption, which are both treated with similar replacement doses of cobalamin. For the younger patient with megaloblastic anemia, differentiating juvenile pernicious anemia and congenital IF deficiency would warrant measurement of gastric juice for IF and achlorhydria; DNA for gastric IF can identify hereditary megaloblastic anemia, and DNA for mutations in IF-cobalamin receptors (amnionless/cubilin genes) could identify Imerslund-Gräsbeck syndrome.

Thus the history, physical findings, and focused laboratory tests with careful clinical follow-up can potentially identify the cause of the majority of cases of cobalamin deficiency and bypass the need for a Schilling test.

Elevated serum MMA and homocysteine levels will return to normal in one week.

Accelerated turnover of normal DNA in erythroid precursors is associated with an increase in serum urate level, which usually peaks by the fourth day, and with increased cellular phosphate uptake for nucleotide synthesis. This may precipitate an attack of gout if the patient has a "gouty predisposition." The reticulocyte count increases by the second to third days and peaks by the fifth to eighth days (the peak reticulocyte count is directly proportional to the degree of preexisting anemia). This is followed by a rise in RBC count, hemoglobin, and hematocrit by the end of the first week, which normalizes in approximately 2 months, regardless of the initial degree of anemia. By the end of the third week, the RBC count should be above $3 \times 10^6/\text{mm}^3$; if it is not, additional causes of underlying iron deficiency, hemoglobinopathy, chronic disease, or hypothyroidism should be considered (Table 40.7).

Hypersegmented PMNs continue to remain in the blood for 10 to 14 days; however, the number of normal PMNs and platelets rises and normalizes within the first week. During this process, there may be a transient left shift to include myeloid precursors. The reduced intramedullary hemolysis (as a result of normalized hematopoiesis) leads to a gradual reduction in the serum bilirubin level by the end of the first week, and LDH levels will drop concomitantly.

In response to cobalamin, progression of neurologic damage and dysfunction is inhibited. In general, the degree of functional recovery is inversely related to the extent of disease and duration of signs and symptoms. As a rough estimate, signs and symptoms that have been present for less than 3 months are usually completely reversible; with longer duration, there is invariable residual neurologic dysfunction. The reversibility of neurologic damage is slow (a maximal response may take 6 months). Substantial increments (in recovery) are unlikely to be gained after the first 12 months of appropriate therapy. However, most neurologic abnormalities have improved in up to 90% of patients with documented subacute combined degeneration.

TABLE 40.7 Causes of Megaloblastosis Not Responding to Therapy With Cobalamin or Folate

Wrong Diagnosis
Combined folate and cobalamin deficiencies being treated with only one vitamin
Associated iron deficiency
Associated hemoglobinopathy (e.g., sickle cell disease, thalassemia)
Associated anemia of chronic disease
Associated hypothyroidism

Follow-Up

Patients with neurologic dysfunction from cobalamin deficiency have traditionally been given more frequent doses of cyanocobalamin (biweekly rather than monthly therapy for the first 6 months), despite the lack of evidence that this form of therapy is more beneficial. This approach nevertheless serves a purpose in that improvement in neurologic status can be carefully documented. Once maximal responses have been established, most patients can be treated with life-long cobalamin with a dose that is appropriate for the underlying cause of cobalamin deficiency. Follow-up outpatient visits every 6 months should be instituted to ensure adequate maintenance of hematopoiesis, as well as early diagnosis of other diseases commonly associated with the cobalamin- or folate-deficient state. Patients with pernicious anemia or food-cobalamin malabsorption are prone to developing iron deficiency that arises from poor iron absorption from achlorhydria and hypochlorhydria, respectively.[16,23] Therefore when iron deficiency is established, total dose replacement using parenteral iron, such as 1 gram of low-molecular-weight iron-dextran (INFeD) administered over an hour, is indicated.[256]

Patients with pernicious anemia have a twofold increase in proximal femur and vertebral fractures and a threefold increase in distal forearm fractures. The associated antral enterochromaffin cell hyperplasia (driven by hypergastrinemia that accompanies atrophic body gastritis) can be associated with dysplasia, a risk factor for development of neoplasia.[167] In addition, the loss of oxyntic glands and replacement by metaplastic pyloric or intestinal glands is a risk factor for gastric cancer.[167] Finally, three studies on nearly 15,000 patients with pernicious anemia have identified an excess risk for gastric cancers within the first few years of diagnosis,[257] so it is prudent to recommend upper endoscopy for these patients despite the lack of prospective data favoring this approach.

ROUTINE SUPPLEMENTATION OF COBALAMIN AND FOLATE

Routine periconceptional supplementation of folate for normal women[23] and in 10 times higher doses for women at risk for delivery of subsequent babies with NTDs[23] provides effective prophylaxis against the development of NTDs (Table 40.8); this also appears to reduce the risk for congenital heart disease,[258] and isolated cleft lip (with or without cleft palate) by about one-third.[259] Food fortification with folic acid (140 μg/100 g flour) has nearly eliminated folate deficiency[260]—the prevalence of low serum folate level decreased from 18.4% to 0.8% with a small (0.3 g/dL) increase in hemoglobin[261]; it has consistently reduced NTDs[208,209]; and is a cost-effective intervention,[262] particularly when additional supplementation is encouraged by physicians.[263] Despite fortification of wheat flour with folic acid in over 80 countries, in many instances, these are not rigorously

TABLE 40.8 **Indications for Prophylaxis With Cobalamin or Folate**

Prophylaxis With Cobalamin

Infants on specialized diets[a]

Premature infants

Infants of mothers with pernicious anemia[a]

Infants and children of mothers with nutritional cobalamin deficiency

Vegetarianism and poverty-imposed near-vegetarianism[a]

Total gastrectomy[b]

Prophylaxis With Folic Acid[c]

All women contemplating pregnancy (at least 400 μg/day)[d]

Pregnancy and lactation, premature infants

Mothers at risk for delivery of infants with neural-tube defects[e,f]

Hemolytic anemias/hyperproliferative hematologic states

Patients with rheumatoid arthritis or psoriasis on therapy with methotrexate[g]

Patients on antiepileptic drugs

Patients with ulcerative colitis

[a] For vegetarians, prophylaxis with cobalamin (5–10-μg tablet/day) orally should suffice. In all other conditions involving any abnormality of cobalamin absorption, cobalamin tablets of 1000 μg/day should be administered orally to ensure that cobalamin transport by passive diffusion across the intestine is sufficient to meet daily needs.

[b] Consider late development of cobalamin deficiency and iron malabsorption (prophylaxis with oral cobalamin and iron).

[c] Ensure that the patient does not have a cobalamin deficiency before initiating long-term folate prophylaxis.

[d] For prevention of first occurrence of neural tube defects.

[e] Previous delivery of a child with neural tube defects (e.g., anencephaly, spina bifida, meningocele) imparts a 10-fold greater risk for subsequent delivery of infant with neural tube defects.

[f] Folic acid (4 mg/day) administered periconceptionally and throughout the first trimester.

[g] To reduce toxicity of the antifolate.

implemented[212]; it is also not yet implemented in most highly populated resource-limited countries, resulting in over 90% of the world's women still vulnerable and without folate-fortified food.[191,213,264]

The administration of folate and cobalamin in addition to parenteral erythropoietin and iron to premature infants significantly improves hemoglobin values[16]; so this should be the standard of care.

Folate supplements during pregnancy also helps prevent premature delivery of low-birth-weight babies,[16] and routine supplementation for premature babies and lactating mothers is also recommended.

In addition to hematologic diseases leading to increased folate requirements (e.g., autoimmune hemolytic anemia, β-thalassemia, sickle cell disease), folic acid supplements reduce the hepatotoxicity and gastrointestinal intolerance of methotrexate in psoriasis[23,265] and rheumatoid arthritis[266] without impairing the efficacy of methotrexate.

For schoolchildren in resource-limited countries, simple community-level interventions, such as micronutrient fortification (using a premix added to school lunches), that build upon the infrastructure of an existing program have proven to be contextually acceptable and efficacious in improving folate and cobalamin (in addition to vitamin A and iron) status in Himalayan villages of India.[267] Table 40.8 summarizes conditions that warrant routine folate or cobalamin supplementation.

Following food fortification, the total folate intake of most US children 1 to 13 years of age does meet the estimated average requirement.[268] Whereas folic acid supplements consumed in excess of 1 mg/day can mask hematologic symptoms of cobalamin deficiency, it has been found that 94% of US adults who do not consume supplements, or who consume less than 400 μg of folic acid per day from supplements, do not exceed the upper limit in intake for folic acid.[269] There is still no unambiguous evidence that consumption of folate-fortified foods places individuals at risk for exacerbating any clinical manifestations of underlying cobalamin deficiency.[270] Indeed, a recent careful review of the historical record does not provide evidence that administration of folic acid can cause or aggravate neurological manifestations among subjects with cobalamin deficiency.[271]

In resource-limited countries, a strong case can be made to consider the fortification of flour (or other contextually relevant food vehicle) with small amounts of cobalamin and folate for the majority of the population whose dietary intake of animal-source foods is poor. This topic remains a work in progress. Likewise, immigrants from resource-limited countries will often continue to consume their native diet, which is usually low in animal-source foods, long after resettling in the United States[272]—as documented even among Indian physician trainees in New York![273] So these groups will also benefit from folate and cobalamin supplementation.

Although *Plasmodium falciparum* possesses folate transporter proteins that can facilitate membrane transport of folic acid and folinic acid,[274] the safety of using low-dose folic acid (1 mg/day) in 467 pregnant women with malaria has been affirmed[275]; however, high-dose folic acid (5 mg/day) can allow for *P. falciparum* resistance, with potential adverse outcomes in children.[276]

CHRONIC HYPERHOMOCYSTEINEMIA

Normally homocysteine is metabolized by the methylation reaction (discussed earlier) and by a second trans-sulfuration pathway, which essentially eliminates homocysteine as a potential source of methionine. In the trans-sulfuration pathway, cystathionine β-synthase catalyzes the condensation of homocysteine with serine in the presence of pyridoxyl phosphate (vitamin B_6) to form cystathionine, which is further cleaved by a vitamin B_6-dependent enzyme γ-cystathionase, to form cysteine and α-ketobutyrate. The cysteine that is formed can be used for synthesis of the antioxidant glutathione, a key component that defends against oxidative stress within cells. In some tissues like liver, homocysteine can also be remethylated to methionine by the transfer of a one-carbon moiety from betaine by the enzyme homocysteine methyltransferase, which is restricted to the liver and kidney. Thus the level of plasma homocysteine depends on genetically regulated levels of essential enzymes in one-carbon metabolism, the intake of folic acid or food folates, vitamin B_6, cobalamin, and other acquired conditions (dehydration, renal dysfunction, antifolates, and nitrous oxide exposure).

Chronic hyperhomocysteinemia is established as a major risk factor in occlusive vascular diseases, particularly increased small-vessel cerebrovascular disease–related strokes,[277,278] dementia, and Alzheimer disease.[279] Parenthetically, this is consistent with the observations that all inherited diseases involving a severe elevation of homocysteine are associated with cognitive deficit and poorer neurocognitive performance. And accelerated brain atrophy is often a characteristic among those with mild cognitive impairment who then go on to develop Alzheimer disease.[178,179,280,281] There have been three randomized controlled trials among elderly patients to suggest that homocysteine-lowering therapy can significantly slow down the accelerated rate of brain atrophy and improve cognition. Indeed, a recent consensus statement by a panel of experts concluded that elevated plasma total homocysteine is a modifiable risk factor for development of cognitive decline, dementia, and Alzheimer disease in older persons.[282] Chronic hyperhomocysteinemia is also a risk factor for reduced bone mineral density and increased incidence of fractures.[283,284]

Raised maternal plasma levels of homocysteine primarily from cobalamin and folate deficiency are associated with various pregnancy complications. These include preeclampsia and spontaneous pregnancy loss,[241,285–287] placental abruption,[285,286] recurrent pregnancy loss,[284–286] fetal growth restriction,[286,288] preterm birth,[289] and stillbirth.[288] The adverse outcomes for the baby include neural-tube defects and congenital malformations.[286,288] Normalization of maternal homocysteine level with cobalamin and folate and improvement of these pregnancy outcomes for some complications[284,286] indicate that some of these risks can be reduced with good nutrition.

FOLATE FORTIFICATION OF FOOD AND THE RISK FOR CANCER

Folate supplements protect the development of colorectal neoplasia in high-risk patients with ulcerative colitis.[23] Three large prospective studies[299–301] suggest that long-term folate intake actually *decreases the risk* for initiation or early development of colorectal cancer; in addition, there appears to be a diminished to nonexistent influence on (precancerous) adenomas. A meta-analysis of 13 randomized trials that compared the incidence of cancer of all types in ~50,000 individuals led to the conclusion that folic acid supplementation does not significantly increase or decrease the risk of cancer over a 5-year treatment period.[302] Collectively, these papers provide reassurance that the fortification of food to prevent neural-tube defects in women of childbearing age has not led to harm among the remaining "nontargeted" population of adults. There also could be long-term benefits in primary prevention (see Table 40.5). A cautious conclusion therefore is that clinical studies have still not unambiguously resolved whether or not folate supplements can actually prevent initiation of cancer or promote transformation of premalignant lesions to cancer.[303]

Homocysteine-Lowering Trials and Primary Versus Secondary Prevention

Most homocysteine-lowering intervention trials have been underpowered; they have looked at populations in which the serum homocysteine level has been borderline elevated rather than being elevated to the higher level (over 20 µM); and they have only had a follow-up of less than 5 years. Moreover, many of the intervention trials have been compromised by the advent of food fortification with folate or by enrolled patients who were consuming multivitamins containing folic acid. A review of this "homocysteine controversy"[290] has noted that the duration of follow-up in most studies has been too short (and likely dictated by the dramatic results from the use of antihypertensives and statins, which required only short follow-up to demonstrate positive effects). The point made is that such expectations are unrealistic for atherosclerosis because the atherosclerotic plaque commonly takes 30 to 40 years to develop into a full-blown clinical event. Moreover, there is a distinct difference between primary prevention of the earliest stages of a disease process and attempts to intervene after demonstrated vascular damage (secondary prevention). (Indeed, it was only longer follow-up for 10 to 15 years that established the primacy of blood glucose control in management of patients with diabetes.)

Thus despite the apparent failure of homocysteine-lowering therapy in several trials related to secondary prevention of atherosclerotic disease, this has *not* eliminated the possibility of a beneficial role of lowering homocysteine in primary prevention of diseases. Indeed, clinical trials have suggested that folate also has a role in reduction in strokes,[291] reduction in the rate of cognitive decline among healthy older adults,[280] and a reduction in age-related (sensorineural) hearing loss.[292] There is also Grade A evidence from randomized controlled trials for a role of folate in combination with cobalamin for reduction of hip fractures,[293] subclinical atherosclerosis,[294] and stroke prevention.[291] In addition, the value of a triple combination of folate, pyridoxine, and cobalamin to reduce age-related macular degeneration in women was shown by a relatively small study[295]; despite support by an observational epidemiology study,[296] larger studies are required (see Table 40.5).[297] *By inference, in addition to classical presentation with nutritional anemia, patients with long-standing hyperhomocysteinemia can also present with any of these additional clinical conditions, as well as a variety of pregnancy complications with or without poor pregnancy outcomes (affecting the newborn).*

One notable *exception* (to the value of homocysteine-lowering therapy) is in patients with diabetic nephropathy where such attempts *worsened* their kidney disease and placed them at greater risk for dying from serious vascular events.[298] (See box on Chronic Hyperhomocysteinemia and box on Folate Fortification of Food and Risk of Cancer.)

FUTURE DIRECTIONS

In this age of spiraling costs for health care delivery, and the ongoing debate on ways to reduce these costs, few instances in internal medicine and hematology yield more satisfying dividends than diagnosing and treating cobalamin and folate deficiency using generic vitamins that are "dirt cheap"—costing only a few cents a day. These conditions are devastating when undiagnosed or misdiagnosed or when cobalamin deficiency is treated with folate alone. Recognition of various populations at risk and the clinical scenarios in which folate and cobalamin deficiency are likely to be present, and the availability of sensitive and specific tests, should reduce uncertainty in diagnosis. The studies on folate supplementation during pregnancy that identified new folate-responsive NTDs and neurocristopathies are a paradigm for identification of hitherto unrecognized roles for other nutrients in human development. And the significant impact of supplemental folates in relieving human suffering consonant with reducing costs for intensive and long-term care of infants with prematurity or NTDs is a major achievement and an outstanding example of cost-effective preventive medicine. However, ensuring that *all* the world's women receive sufficient folate and cobalamin to break the cycle of vertical intergenerational deficiency in their progeny remains a critically important but still elusive goal. Other recent advances from randomized controlled studies indicate beneficial effects of supplemental folate and cobalamin in the prevention of diverse diseases. Folate receptor-α or -β targeting[304,305] using monoclonal antibodies against cancer or inflammation or various folate-tagged fluorescent probes,[306] newer radionuclides,[307] drugs, as well as ^{18}F-based folate radiotracers for positron emission tomography (PET) imaging,[308] are under intense scrutiny in the clinic. The influence of folates and cobalamin during development in utero—especially on behavioral neuroscience—allows us to peer into a new field where groundbreaking discoveries will be made in the coming decade. The new molecular links established between folate deficiency, homocysteinylated-hnRNP-E1, and its role in perturbing the HPV16 life cycle and HPV16-induced cancer, open the door to investigations into the potential role of these vitamins in the field of preventive oncology.[100,101] Finally, folate receptor-4 (renamed Juno after the Goddess of Fertility), which is found on the surface of eggs, is what the sperm protein, Izumo1 (named after a Japanese marriage shrine), recognizes and binds to gain entry and fuse to form a single diploid cell.[309] Further study of this pair of proteins raises the potential for identifying hitherto unrecognized fertility problems and the development of a new generation of contraceptives.[310] Collectively, these studies point to the profound spectrum of influence and the wealth of information that will be derived from continued study of these vitamins in physiology and medicine.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Antony AC. Evidence for potential underestimation of clinical folate deficiency in resource-limited countries using blood tests. *Nutr Rev*. 2017;75:600–615.

Antony AC. Vegetarianism and vitamin-B12 (cobalamin) deficiency. *Am J Clin Nutr*. 2003;78:3.

Bjorke-Monsen AL, Ueland PM. Cobalamin status in children. *J Inherit Metab Dis*. 2010;34:111.

Carmel R. Biomarkers of cobalamin (vitamin B-12) status in the epidemiologic setting: a critical overview of context, applications, and performance characteristics of cobalamin, methylmalonic acid, and holotranscobalamin II. *Am J Clin Nutr*. 2011;94:348S.

Grapp M, Just IA, Linnankivi T, et al. Molecular characterization of folate receptor 1 mutations delineates cerebral folate transport deficiency. *Brain*. 2012;135:2022–2031.

Grapp M, Wrede A, Schweizer M, et al. Choroid plexus transcytosis and exosome shuttling deliver folate into brain parenchyma. *Nat Commun*. 2013;4:2123.

Hesdorffer CS, Longo DL. Drug-induced megaloblastic anemia. *N Engl J Med*. 2015;373:1649–1658.

Hodis HN, Mack WJ, Dustin L, et al. High-dose B vitamin supplementation and progression of subclinical atherosclerosis: a randomized controlled trial. *Stroke*. 2009;40:730.

Kim YI. Folate and cancer: a tale of Dr. Jekyll and Mr. Hyde? *Am J Clin Nutr*. 2018;107:139–142.

Lahner E, Norman GL, Severi C, et al. Reassessment of intrinsic factor and parietal cell autoantibodies in atrophic gastritis with respect to cobalamin deficiency. *Am J Gastroenterol*. 2009;104:2071–2079.

McFarland R. Cerebral folate deficiency—mishaps and misdirection. *Brain*. 2012;135:2002–2003.

Quadros EV. Advances in the understanding of cobalamin assimilation and metabolism. *Br J Haematol*. 2010;148:195.

Smith AD, Refsum H, Bottiglieri T, et al. Homocysteine and dementia: an international consensus statement. *J Alzheimers Dis*. 2018;62:561–570.

Smulders YM, Blom HJ. The homocysteine controversy. *J Inherit Metab Dis*. 2011;34:93.

Stabler SP. Clinical practice. Vitamin B12 deficiency. *N Engl J Med*. 2013;368:149–160.

Toh BH. Diagnosis and classification of autoimmune gastritis. *Autoimmun Rev*. 2014;13:459–462.

Torheim LE, Ferguson EL, Penrose K, et al. Women in resource-poor settings are at risk of inadequate intakes of multiple micronutrients. *J Nutr*. 2010;140:2051S–2058S.

Torsvik I, Ueland PM, Markestad T, et al. Cobalamin supplementation improves motor development and regurgitations in infants: results from a randomized intervention study. *Am J Clin Nutr*. 2013;98:1233–1240.

Visentin M, Diop-Bove N, Zhao R, et al. The intestinal absorption of folates. *Annu Rev Physiol*. 2014;76:251–274.

Wang X, Qin X, Demirtas H, et al. Efficacy of folic acid supplementation in stroke prevention: a meta-analysis. *Lancet*. 2007;369:1876.

Watkins D, Rosenblatt DS. Inborn errors of cobalamin absorption and metabolism. *Am J Med Genet C Semin Med Genet*. 2011;157C:33–44.

Xiao S, Tang YS, Kusumanchi P, et al. Folate deficiency facilitates genomic integration of human papillomavirus type 16 DNA in vivo in a novel mouse model for rapid oncogenic transformation of human keratinocytes. *J Nutr*. 2018;148:389–400.

Yang Q, Botto LD, Erickson JD, et al. Improvement in stroke mortality in Canada and the United States, 1990 to 2002. *Circulation*. 2006;113:1335.

Zhao R, Matherly LH, Goldman ID. Membrane transporters and folate homeostasis: intestinal absorption and transport into systemic compartments and tissues. *Expert Rev Mol Med*. 2009;11:e4.

REFERENCES

Many of the “seed” references to original work published *before* 1989 can be found in reference 16, whereas seed references from 1990 to 2000 are found in reference 23.

1. Chanarin I. *The Megaloblastic Anemias*. Oxford, UK: Blackwell Scientific Publications; 1979.
2. Beck WS. Cobalamin as coenzyme: a twisting trail of research. *Am J Hematol*. 1990;34:83–89.
3. Banerjee R, Ragsdale SW. The many faces of vitamin B12: catalysis by cobalamin-dependent enzymes. *Annu Rev Biochem*. 2003;72:209–247.
4. Allen LH, Miller JW, de Groot L, et al. Biomarkers of nutrition for development (BOND): vitamin B-12 review. *J Nutr*. 2018;148:1995S–2027S.
5. Stabler SP, Allen RH. Vitamin B12 deficiency as a worldwide problem. *Annu Rev Nutr*. 2004;24:299–326.
6. Stabler SP. Clinical practice. Vitamin B12 deficiency. *N Engl J Med*. 2013;368:149–160.
7. Antony AC. Vegetarianism and vitamin B-12 (cobalamin) deficiency. *Am J Clin Nutr*. 2003;78:3–6.
8. Antony AC. Vegetarianism and other restricted diets. In: Robert T, Means J, eds. *Nutritional Anemia—Scientific Principles, Clinical Practice, and Public Health*. Cambridge, UK: Cambridge University Press; 2019:153–174.
9. Pawlak R, Parrott SJ, Raj S, et al. How prevalent is vitamin B(12) deficiency among vegetarians? *Nutr Rev*. 2013;71:110–117.
10. Balci YI, Ergin A, Karabulut A, Polat A, Dogan M, Kucuktasci K. Serum vitamin B12 and folate concentrations and the effect of the Mediterranean diet on vulnerable populations. *Pediatr Hematol Oncol*. 2014;31:62–67.
11. Dietary Reference Intakes (DRIs): Estimated Average Requirements. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B^{12}, pantothenic acid, biotin, and choline (1998); Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc (2001). These reports may be accessed via www.nap.edu; Table at https://fnic.nal.usda.gov/sites/fnic.nal.usda.gov/files/uploads/recommended_intakes_individuals.pdf. 1998, 2001.
12. Bor MV, von Castel-Roberts KM, Kauwell GP, et al. Daily intake of 4 to 7 microg dietary vitamin B-12 is associated with steady concentrations of vitamin B-12-related biomarkers in a healthy young population. *Am J Clin Nutr*. 2010;91:571–577.
13. Howard JM, Azen C, Jacobsen DW, et al. Dietary intake of cobalamin in elderly people who have abnormal serum cobalamin, methylmalonic acid and homocysteine levels. *Eur J Clin Nutr*. 1998;52:582–587.
14. van Asselt DZ, de Groot LC, van Staveren WA, et al. Role of cobalamin intake and atrophic gastritis in mild cobalamin deficiency in older Dutch subjects. *Am J Clin Nutr*. 1998;68:328–334.
15. Bor MV, Lydeking-Olsen E, Møller J, Nexo E. A daily intake of approximately 6 microg vitamin B-12 appears to saturate all the vitamin B-12-related variables in Danish postmenopausal women. *Am J Clin Nutr*. 2006;83:52–58.
16. Antony AC. Megaloblastic anemias. In: Hoffman R, Benz (Jr.) EJ, Shattil SJ, Furie B, Cohen HJ, eds. *Hematology: Basic Principles and Practice*. 1st ed. New York, NY: Churchill-Livingstone; 1991:392–422.
17. Kristiansen M, Aminoff M, Jacobsen C, et al. Cubilin P1297L mutation associated with hereditary megaloblastic anemia 1 causes impaired recognition of intrinsic factor-vitamin B(12) by cubilin.[comment]. *Blood*. 2000;96:405–409.
18. He Q, Madsen M, Kilkenney A, et al. Amnionless function is required for cubilin brush-border expression and intrinsic factor-cobalamin (vitamin B12) absorption in vivo. *Blood*. 2005;106:1447–1453.
19. Beedholm-Ebsen R, van de Wetering K, Hardlei T, et al. Identification of multidrug resistance protein 1 (MRP1/ABCC1) as a molecular gate for cellular export of cobalamin. *Blood*. 2010;115:1632–1639.
20. Gherasim C, Lofgren M, Banerjee R. Navigating the B(12) road: assimilation, delivery, and disorders of cobalamin. *J Biol Chem*. 2013;288:13186–13193.
21. Quadros EV, Nakayama Y, Sequeira JM. The protein and the gene encoding the receptor for the cellular uptake of transcobalamin-bound cobalamin. *Blood*. 2009;113:186–192.
22. Quadros EV. Advances in the understanding of cobalamin assimilation and metabolism. *Br J Haematol*. 2010;148:195–204.
23. Antony AC. Megaloblastic anemias. In: Hoffman R, Benz Jr EJ, Shattil SJ, eds. *Hematology Basic Principles and Practice*. 4th ed. Philadelphia, PA: Elsevier Churchill Livingstone; 2005:519–556.
24. Kim J, Gherasim C, Banerjee R. Decyanation of vitamin B12 by a trafficking chaperone. *Proc Natl Acad Sci U S A*. 2008;105:14551–14554.
25. Olteanu H, Banerjee R. Human methionine synthase reductase, a soluble P-450 reductase-like dual flavoprotein, is sufficient for NADPH-dependent methionine synthase activation. *J Biol Chem*. 2001;276:35558–35563.
26. Lester GE, Makus DJ, Hodges DM. Relationship between fresh-packaged spinach leaves exposed to continuous light or dark and bioactive contents: effects of cultivar, leaf size, and storage duration. *J Agric Food Chem*. 2010;58:2980–2987.
27. Castenmiller JJ, van de Poll CJ, West CE, et al. Bioavailability of folate from processed spinach in humans. Effect of food matrix and interaction with carotenoids. *Ann Nutr Metab*. 2000;44:163–169.
28. Picciano MF, West SG, Ruch AL, et al. Effect of cow milk on food folate bioavailability in young women. *Am J Clin Nutr*. 2004;80:1565–1569.
29. Zhao R, Matherly LH, Goldman ID. Membrane transporters and folate homeostasis: intestinal absorption and transport into systemic compartments and tissues. *Expert Rev Mol Med*. 2009;11:e4.
30. Urquhart BL, Gregor JC, Chande N, et al. The human proton-coupled folate transporter (hPCFT): modulation of intestinal expression and function by drugs. *Am J Physiol Gastrointest Liver Physiol*. 2010;298:G248–G254.
31. National-Academy-of-Sciences, Institute-of-Medicine, Food-and-Nutrition-Board. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. 1998. http://www.nap.edu/catalog/6015.html.
32. Chakravarty I, Sinha RK. Prevalence of micronutrient deficiency based on results obtained from the national pilot program on control of micronutrient malnutrition. *Nutr Rev*. 2002;60:S53–S58.
33. Misra A, Vikram NK, Pandey RM, et al. Hyperhomocysteinemia, and low intakes of folic acid and vitamin B12 in urban North India. *Eur J Nutr*. 2002;41:68–77.
34. Antony AC. Megaloblastic anemias. In: Goldman L, Schafer AI, eds. *Goldman-Cecil Medicine (Cecil's Textbook of Medicine)*. 25th ed. New York, NY: Elsevier Saunders; 2015:1104–1114.
35. Qiu A, Jansen M, Sakaris A, et al. Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. *Cell*. 2006;127:917–928.
36. Zhao R, Min SH, Wang Y, et al. A role for the proton-coupled folate transporter (PCFT-SLC46A1) in folate receptor-mediated endocytosis. *J Biol Chem*. 2009;284:4267–4274.
37. Camilo E, Zimmerman J, Mason JB, et al. Folate synthesized by bacteria in the human upper small intestine is assimilated by the host. *Gastroenterology*. 1996;110:991–998.
38. Masuda M, I'Izuka Y, Yamazaki M, et al. Methotrexate is excreted into the bile by canalicular multispecific organic anion transporter in rats. *Cancer Res*. 1997;57:3506–3510.
39. Aufreiter S, Gregory 3rd JF, Pfeiffer CM, et al. Folate is absorbed across the colon of adults: evidence from cecal infusion of (13)C-labeled [6S]-5-formyltetrahydrofolic acid. *Am J Clin Nutr*. 2009;90:116–123.
40. Valentini L, Pinto A, Bourdel-Marchasson I, et al. Impact of personalized diet and probiotic supplementation on inflammation, nutritional parameters and intestinal microbiota-The “RISTOMED project”: Randomized controlled trial in healthy older people. *Clin Nutr*. 2014;34(4):593–602.
41. Antony AC. The biological chemistry of folate receptors. *Blood*. 1992;79:2807–2820.
42. Antony AC. Folate receptors. *Annu Rev Nutr*. 1996;16:501–521.
43. Antony AC, Utley C, Van Horne KC, Kolhouse JF. Isolation and characterization of a folate receptor from human placenta. *J Biol Chem*. 1981;256:9684–9692.
44. Sabharanjak S, Mayor S. Folate receptor endocytosis and trafficking. *Adv Drug Deliv Rev*. 2004;56:1099–1109.
45. Zhao R, Diop-Bove N, Visentin M, Goldman ID. Mechanisms of membrane transport of folates into cells and across epithelia. *Annu Rev Nutr*. 2011;31:177–201.
46. Henderson GI, Perez T, Schenker S, et al. Maternal-to-fetal transfer of 5-methyltetrahydrofolate by the perfused human placental cotyledon: evidence for a concentrative role by placental folate receptors in fetal folate delivery. *J Lab Clin Med*. 1995;126:184–203.
47. Grapp M, Wrede A, Schweizer M, et al. Choroid plexus transcytosis and exosome shuttling deliver folate into brain parenchyma. *Nat Commun*. 2013;4:2123.
48. Grapp M, Just IA, Linnankivi T, et al. Molecular characterization of folate receptor 1 mutations delineates cerebral folate transport deficiency. *Brain*. 2012;135:2022–2031.
49. Steinfeld R, Grapp M, Kraetzner R, et al. Folate receptor alpha defect causes cerebral folate transport deficiency: a treatable neurodegenerative disorder associated with disturbed myelin metabolism. *Am J Hum Genet*. 2009;85:354–363.
50. Selhub J, Franklin WA. The folate-binding protein of rat kidney. Purification, properties, and cellular distribution. *J Biol Chem*. 1984;259:6601–6606.
51. Low PS, Antony AC. Folate receptor-targeted drugs for cancer and inflammatory diseases. *Adv Drug Deliv Rev*. 2004;56:1055–1058.
52. Puig-Kröger A, Sierra-Filardi E, Dominguez-Soto A, et al. Folate receptor beta is expressed by tumor-associated macrophages and constitutes a

marker for M2 anti-inflammatory/regulatory macrophages. *Cancer Res*. 2009;69:9395–9403.

53. van der Heijden JW, Oerlemans R, Dijkmans BAC, et al. Folate receptor beta as a potential delivery route for novel folate antagonists to macrophages in the synovial tissue of rheumatoid arthritis patients. *Arthritis Rheum*. 2009;60:12–21.
54. Han W, Zaynagetdinov R, Yull FE, et al. Molecular imaging of folate receptor beta positive macrophages during acute lung inflammation. *Am J Respir Cell Mol Biol*. 2014;53(1):50–59.
55. Shen J, Hilgenbrink AR, Xia W, et al. Folate receptor-beta constitutes a marker for human proinflammatory monocytes. *J Leukoc Biol*. 2014;96:563–570.
56. Alam C, Aufreiter S, Georgiou CJ, et al. Upregulation of reduced folate carrier by vitamin D enhances brain folate uptake in mice lacking folate receptor alpha. *Proc Natl Acad Sci U S A*. 2019;116:17531–17540.
57. Kane MA, Elwood PC, Portillo RM, et al. Influence on immunoreactive folate-binding proteins of extracellular folate concentration in cultured human cells. *J Clin Invest*. 1988;81:1398–1406.
58. Antony AC, Tang Y-S, Khan RA, et al. Translational upregulation of folate receptors is mediated by homocysteine via RNA-heterogeneous nuclear ribonucleoprotein E1 interactions. *J Clin Invest*. 2004;113:285–301.
59. Shatnawi A, Tran T, Ratnam M. R5020 and RU486 act as progesterone receptor agonists to enhance Sp1/Sp4-dependent gene transcription by an indirect mechanism. *Mol Endocrinol*. 2007;21:635–650.
60. Xiao S, Hansen DK, Horsley ET, et al. Maternal folate deficiency results in selective upregulation of folate receptors and heterogeneous nuclear ribonucleoprotein-E1 associated with multiple subtle aberrations in fetal tissues. *Birth Defects Res A Clin Mol Teratol*. 2005;73:6–28.
61. Salbaum JM, Finnell RH, Kappen C. Regulation of folate receptor 1 gene expression in the visceral endoderm. *Birth Defects Res A Clin Mol Teratol*. 2009;85:303–313.
62. Tang Y-S, Khan RA, Zhang Y, et al. Incrimination of heterogeneous nuclear ribonucleoprotein E1 (hnRNP-E1) as a candidate sensor of physiological folate deficiency. *J Biol Chem*. 2011;286:39100–39115.
63. Xiao X, Tang YS, Mackins JY, et al. Isolation and characterization of a folate receptor mRNA-binding trans-factor from human placenta. Evidence favoring identity with heterogeneous nuclear ribonucleoprotein E1. *J Biol Chem*. 2001;276:41510–41517.
64. Jakubowski H. Homocysteine modification in protein structure/function and human disease. *Physiol Rev*. 2019;99:555–604.
65. Tang Y-S, Khan RA, Xiao S, et al. Evidence favoring a positive feedback loop for physiologic auto upregulation of hnRNP-E1 during prolonged folate deficiency in human placental cells. *J Nutr*. 2017;147:482–498.
66. Boshnjaku V, Shim K-W, Tsurubuchi T, et al. Nuclear localization of folate receptor alpha: a new role as a transcription factor. *Sci Rep*. 2012;2:980.
67. Antony AC. Folate receptors: reflections on a personal odyssey and a perspective on unfolding truth. *Adv Drug Deliv Rev*. 2004;56:1059–1066.
68. Antony AC. In utero physiology: role of folic acid in nutrient delivery and fetal development. *Am J Clin Nutr*. 2007;85:598S–603S.
69. Solanky N, Requena Jimenez A, D'Souza SW, et al. Expression of folate transporters in human placenta and implications for homocysteine metabolism. *Placenta*. 2010;31:134–143.
70. Verma RS, Gullapalli S, Antony AC. Evidence that the hydrophobicity of isolated, in situ, and de novo-synthesized native human placental folate receptors is a function of glycosyl-phosphatidylinositol anchoring to membranes. *J Biol Chem*. 1992;267:4119–4127.
71. Piedrahita JA, Oetama B, Bennett GD, et al. Mice lacking the folic acid-binding protein Folbp1 are defective in early embryonic development. *Nat Genet*. 1999;23:228–232.
72. Antony AC, Hansen DK. Hypothesis: folate-responsive neural tube defects and neurocristopathies. *Teratology*. 2000;62:42–50.
73. Hansen DK, Streck RD, Antony AC. Antisense modulation of the coding or regulatory sequence of the folate receptor (folate binding protein-1) in mouse embryos leads to neural tube defects. *Birth Defects Res A Clin Mol Teratol*. 2003;67:475–487.
74. Bolton JA, Wood SA, Kennedy D, et al. Retinoic acid-dependent upregulation of mouse folate receptor-alpha expression in embryonic stem cells, and conservation of alternative splicing patterns. *Gene*. 1999;230:215–224.
75. Saitsu H, Ishibashi M, Nakano H, Shiota K. Spatial and temporal expression of folate-binding protein 1 (Fbp1) is closely associated with anterior neural tube closure in mice. *Dev Dyn*. 2003;226:112–117.
76. da Costa M, Sequeira JM, Rothenberg SP, Weedon J. Antibodies to folate receptors impair embryogenesis and fetal development in the rat. *Birth Defects Res A Clin Mol Teratol*. 2003;67:837–847.
77. Rosenquist TH, Chaudoin T, Finnell RH, Bennett GD. High-affinity folate receptor in cardiac neural crest migration: a gene knockdown model using siRNA. *Dev Dyn*. 2010;239:1136–1144.
78. Rothenberg SP, da Costa MP, Sequeira JM, et al. Autoantibodies against folate receptors in women with a pregnancy complicated by a neural-tube defect. *N Engl J Med*. 2004;350:134–142.
79. Iskandar BJ, Rizk E, Meier B, et al. Folate regulation of axonal regeneration in the rodent central nervous system through DNA methylation. *J Clin Invest*. 2010;120:1603–1616.
80. Zhao R, Min SH, Qiu A, et al. The spectrum of mutations in the PCFT gene, coding for an intestinal folate transporter, that are the basis for hereditary folate malabsorption. *Blood*. 2007;110:1147–1152.
81. Kozyraki R, Fyfe J, Verroust PJ, et al. Megalin-dependent cubilin-mediated endocytosis is a major pathway for the apical uptake of transferrin in polarized epithelia. *Proc Natl Acad Sci U S A*. 2001;98:12491–12496.
82. Moestrup SK, Verroust PJ. Megalin- and cubilin-mediated endocytosis of protein-bound vitamins, lipids, and hormones in polarized epithelia. *Annu Rev Nutr*. 2001;21:407–428.
83. Birn H, Zhai X, Holm J, et al. Megalin binds and mediates cellular internalization of folate binding protein. *FEBS J*. 2005;272:4423–4430.
84. Antony AC, Kincade RS, Verma RS, Krishnan SR. Identification of high affinity folate binding proteins in human erythrocyte membranes. *J Clin Invest*. 1987;80:711–723.
85. Antony AC, Bruno E, Briddell RA, et al. Effect of perturbation of specific folate receptors during in vitro erythropoiesis. *J Clin Invest*. 1987;80:1618–1623.
86. Tibbetts AS, Appling DR. Compartmentalization of mammalian folate-mediated one-carbon metabolism. *Annu Rev Nutr*. 2010;30:57–81.
87. Titus SA, Moran RG. Retrovirally mediated complementation of the glyB phenotype. Cloning of a human gene encoding the carrier for entry of folates into mitochondria. *J Biol Chem*. 2000;275:36811–36817.
88. Agrimi G, Di Noia MA, Marobbio CMT, et al. Identification of the human mitochondrial S-adenosylmethionine transporter: bacterial expression, reconstitution, functional characterization and tissue distribution. *Biochem J*. 2004;379:183–190.
89. Stover PJ. One-carbon metabolism-genome interactions in folate-associated pathologies. *J Nutr*. 2009;139:2402–2405.
90. Field MS, Kamynina E, Agunloye OC, et al. Nuclear enrichment of folate cofactors and methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) protect de novo thymidylate biosynthesis during folate deficiency. *J Biol Chem*. 2014;289:29642–29650.
91. Wickramasinghe SN, Fida S. Bone marrow cells from vitamin B12- and folate-deficient patients misincorporate uracil into DNA. *Blood*. 1994;83:1656–1661.
92. Duthie SJ, Hawdon A. DNA instability (strand breakage, uracil misincorporation, and defective repair) is increased by folic acid depletion in human lymphocytes in vitro. *FASEB J*. 1998;12:1491–1497.
93. Barnes DE, Lindahl T. Repair and genetic consequences of endogenous DNA base damage in mammalian cells. *Annu Rev Genet*. 2004;38:445–476.
94. Lindahl T. Suppression of spontaneous mutagenesis in human cells by DNA base excision-repair. *Mutat Res*. 2000;462:129–135.
95. Blount BC, Mack MM, Wehr CM, et al. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. *Proc Natl Acad Sci U S A*. 1997;94:3290–3295.
96. Ciappio E, Mason JB. Folate and carcinogenesis: basic mechanisms. In: Bailey LB, ed. *Folate in Health and Disease*. 2nd ed. Boca Raton, FL: CRC Press; 2010:235–262.
97. Ames BN, Wakimoto P. Are vitamin and mineral deficiencies a major cancer risk? *Nat Rev Cancer*. 2002;2:694–704.
98. Dianov GL, Timchenko TV, Sinitsina OI, et al. Repair of uracil residues closely spaced on the opposite strands of plasmid DNA results in double-strand break and deletion formation. *Mol Gen Genet*. 1991;225:448–452.
99. Kim YI, Pogribny IP, Basnakian AG, et al. Folate deficiency in rats induces DNA strand breaks and hypomethylation within the p53 tumor suppressor gene. *Am J Clin Nutr*. 1997;65:46–52.
100. Xiao S, Tang YS, Khan RA, et al. Influence of physiologic folate deficiency on human papillomavirus type 16 (HPV16)-harboring human keratinocytes in vitro and in vivo. *J Biol Chem*. 2012;287:12559–12577.
101. Xiao S, Tang Y-S, Kusumanchi P, et al. Folate deficiency facilitates genomic integration of human papillomavirus type 16 DNA in vivo in a novel mouse model for rapid oncogenic transformation of human keratinocytes. *J Nutr*. 2018;148:389–400.
102. Bull CF, Mayrhofer G, O'Callaghan NJ, et al. Folate deficiency induces dysfunctional long and short telomeres; both states are associated with hypomethylation and DNA damage in human WIL2-NS cells. *Cancer Prev Res (Phila)*. 2014;7:128–138.
103. Sharma TK, Vardey SK, Sitaraman S. Serum homocysteine, folate, and vitamin B12 levels in carbamazepine treated epileptic children. *Clin Lab*. 2016;62:1217–1224.

104. Kalita J, Misra UK. Vitamin B12 deficiency neurological syndromes: correlation of clinical, MRI and cognitive evoked potential. *J Neurol*. 2008;255:353–359.
105. Misra UK, Kalita J, Das A. Vitamin B12 deficiency neurological syndromes: a clinical, MRI and electrodiagnostic study. *Electromyogr Clin Neurophysiol*. 2003;43:57–64.
106. Hemmer B, Glocker FX, Schumacher M, et al. Subacute combined degeneration: clinical, electrophysiological, and magnetic resonance imaging findings. *J Neurol Neurosurg Psychiatry*. 1998;65:822–827.
107. Pandey S, Kalita J, Misra UK. A sequential study of visual evoked potential in patients with vitamin B12 deficiency neurological syndrome. *Clin Neurophysiol*. 2004;115:914–918.
108. Kalita J, Chandra S, Bhoi SK, et al. Clinical, nerve conduction and nerve biopsy study in vitamin B12 deficiency neurological syndrome with a short-term follow-up. *Nutr Neurosci*. 2014;17:156–163.
109. Kalita J, Agarwal R, Chandra S, Misra UK. A study of neurobehavioral, clinical psychometric, and P3 changes in vitamin B12 deficiency neurological syndrome. *Nutr Neurosci*. 2013;16:39–46.
110. Kalita J, Misra UK. Benefit of vitamin B-12 supplementation in asymptomatic elderly: a matter of endpoints. *Am J Clin Nutr*. 2015;102:529–530.
111. Clemens TL. Vitamin B12 deficiency and bone health. *N Engl J Med*. 2014;371:963–964.
112. Tucker KL, Hannan MT, Qiao N, et al. Low plasma vitamin B12 is associated with lower BMD: the Framingham osteoporosis study. *J Bone Miner Res*. 2005;20:152–158.
113. Cagnacci A, Bagni B, Zini A, et al. Relation of folates, vitamin B12 and homocysteine to vertebral bone mineral density change in postmenopausal women. A five-year longitudinal evaluation. *Bone*. 2008;42:314–320.
114. Goerss JB, Kim CH, Atkinson EJ, et al. Risk of fractures in patients with pernicious anemia. *J Bone Miner Res*. 1992;7:573–579.
115. Merriman NA, Putt ME, Metz DC, Yang YX. Hip fracture risk in patients with a diagnosis of pernicious anemia. *Gastroenterology*. 2010;138:1330–1337.
116. Kunisawa J, Hashimoto E, Ishikawa I, Kiyono H. A pivotal role of vitamin B9 in the maintenance of regulatory T cells in vitro and in vivo. *PLoS One*. 2012;7:e32094.
117. Kishimoto K, Kobayashi R, Sano H, et al. Impact of folate therapy on combined immunodeficiency secondary to hereditary folate malabsorption. *Clin Immunol*. 2014;153:17–22.
118. Antony AC. Evidence for potential underestimation of clinical folate deficiency in resource-limited countries using blood tests. *Nutr Rev*. 2017;75:600–615.
119. Hesdorffer CS, Longo DL. Drug-induced megaloblastic anemia. *N Engl J Med*. 2015;373:1649–1658.
120. Refsum H, Smith AD, Ueland PM, et al. Facts and recommendations about total homocysteine determinations: an expert opinion. *Clin Chem*. 2004;50:3–32.
121. Chitambar CR, Antony AC. Nutritional aspects of hematologic diseases. In: Shils ME, Shike M, Ross AC, Caballero C, Cousins RJ, eds. *Modern Nutrition in Health and Disease*. 10th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2005:1436–1461.
122. Chitambar CR, Antony AC. Nutritional aspects of hematologic diseases. In: Shils ME, Shike M, Ross AC, eds. *Modern Nutrition in Health and Disease (50th Anniversary Edition)*. 10th ed. Baltimore, MD: Lippincott Williams & Wilkins; 2006:1436–1461.
123. Carmel R. Biomarkers of cobalamin (vitamin B-12) status in the epidemiologic setting: a critical overview of context, applications, and performance characteristics of cobalamin, methylmalonic acid, and holotranscobalamin II. *Am J Clin Nutr*. 942011348S–358S.
124. Carmel R, Agrawal YP. Failures of cobalamin assays in pernicious anemia. *N Engl J Med*. 2012;367:385–386.
125. Yang DT, Cook RJ. Spurious elevations of vitamin B12 with pernicious anemia. *N Engl J Med*. 2012;366:1742–1743.
126. Merrigan SD, Yang DT, Straseski JA. Intrinsic factor blocking antibody interference is not detected in five automated cobalamin immunoassays. *Am J Clin Pathol*. 2014;141:702–705.
127. Fazili Z, Pfeiffer CM, Zhang M. Comparison of serum folate species analyzed by LC-MS/MS with total folate measured by microbiologic assay and Bio-Rad radioassay. *Clin Chem*. 2007;53:781–784.
128. Blackmore S, Pfeiffer CM, Lee A, et al. Isotope dilution-LC-MS/MS reference method assessment of serum folate assay accuracy and proficiency testing consensus mean. *Clin Chem*. 2011;57:986–994.
129. Antony AC. Megaloblastic anemias. In: Hoffman R, Benz EJJ, Shattil SJ, eds. *Hematology: Basic Principles and Practice*. 5th ed. Philadelphia, PA: Churchill Livingstone Elsevier; 2009:491–524.
130. Farrell CJ, Kirsch SH, Herrmann M. Red cell or serum folate: what to do in clinical practice? *Clin Chem Lab Med*. 2013;51:555–569.
131. Pfeiffer CM, Caudill SP, Gunter EW, Osterloh J, Sampson EJ. Biochemical indicators of B vitamin status in the US population after folic acid fortification: results from the National Health and Nutrition Examination Survey 1999–2000. *Am J Clin Nutr*. 2005;82:442–450.
132. Green R. Indicators for assessing folate and vitamin B12 status and for monitoring the efficacy of intervention strategies. *Food Nutr Bull*. 2008;29:S52–S63. discussion S4-6.
133. Yetley EA, Pfeiffer CM, Phinney KW, et al. Biomarkers of folate status in NHANES: a roundtable summary. *Am J Clin Nutr*. 2011;94:303S–312S.
134. Bailey LB, Stover PJ, McNulty H, et al. Biomarkers of nutrition for development-folate review. *J Nutr*. 2015;145:1636S–1680S.
135. Samuel TM, Duggan C, Thomas T, et al. Vitamin B(12) intake and status in early pregnancy among urban South Indian women. *Ann Nutr Metab*. 2013;62:113–122.
136. Chandelia S, Chandra J, Narayan S, et al. Addition of cobalamin to iron and folic acid improves hemoglobin rise in nutritional anemia. *Indian J Pediatr*. 2012;79:1592–1596.
137. Bor MV, Nexø E, Hvas A-M. Holo-transcobalamin concentration and transcobalamin saturation reflect recent vitamin B12 absorption better than does serum vitamin B12. *Clin Chem*. 2004;50:1043–1049.
138. Green R. Vitamin B_{12} deficiency from the perspective of a practicing hematologist. *Blood*. 2017;129:2603–2611.
139. Green R, Allen LH, Bjørke-Monsen A-L, et al. Vitamin B12 deficiency. *Nat Rev Dis Primers*. 2017;3:17040.
140. Dang S, Yan H, Zeng L, et al. The status of vitamin B12 and folate among Chinese women: a population-based cross-sectional study in northwest China. *PLoS One*. 2014;9:e112586.
141. Kapil U, Bhadoria AS. Prevalence of folate, ferritin and cobalamin deficiencies amongst adolescents in India. *J Family Med Prim Care*. 2014;3:247–249.
142. Sukla KK, Tiwari PK, Kumar A, Raman R. Low birthweight (LBW) and neonatal hyperbilirubinemia (NNH) in an Indian cohort: association of homocysteine, its metabolic pathway genes and micronutrients as risk factors. *PLoS One*. 2013;8:e71587.
143. Iqbal SP, Kakepoto GN, Iqbal SP. Vitamin B12 deficiency—a major cause of megaloblastic anaemia in patients attending a tertiary care hospital. *J Ayub Med Coll Abbottabad*. 2009;21:92–94.
144. Koc A, Kocyigit A, Soran M, et al. High frequency of maternal vitamin B12 deficiency as an important cause of infantile vitamin B12 deficiency in Sanliurfa province of Turkey. *Eur J Nutr*. 2006;45:291–297.
145. Bjørke Monsen A-L, Ueland PM, Vollset SE, et al. Determinants of cobalamin status in newborns. *Pediatrics*. 2001;108:624–630.
146. Hay G, Clausen T, Whitelaw A, et al. Maternal folate and cobalamin status predicts vitamin status in newborns and 6-month-old infants. *J Nutr*. 2010;140:557–564.
147. Taneja S, Bhandari N, Strand TA, et al. Cobalamin and folate status in infants and young children in a low-to-middle income community in India. *Am J Clin Nutr*. 2007;86:1302–1309.
148. Jones KM, Ramirez-Zea M, Zuleta C, Allen LH. Prevalent vitamin B-12 deficiency in twelve-month-old Guatemalan infants is predicted by maternal B-12 deficiency and infant diet. *J Nutr*. 2007;137:1307–1313.
149. Shahab-Ferdows S, Anaya-Loyola MA, Vergara-Castaneda H, et al. Vitamin B-12 supplementation of rural Mexican women changes biochemical vitamin B-12 status indicators but does not affect hematology or a bone turnover marker. *J Nutr*. 2012;142:1881–1887.
150. Duggan C, Srinivasan K, Thomas T, et al. Vitamin B-12 supplementation during pregnancy and early lactation increases maternal, breast milk, and infant measures of vitamin B-12 status. *J Nutr*. 2014;144:758–764.
151. Avci Z, Turul T, Aysun S, Unal I. Involuntary movements and magnetic resonance imaging findings in infantile cobalamine (vitamin B12) deficiency. *Pediatrics*. 2003;112:684–686.
152. Yavuz H. Vitamin B(12) deficiency, seizure, involuntary movements. *Seizure*. 2008;17:748–749.
153. Amin NMM, Zeki JM. Infantile tremor syndrome in Iraqi Kurdistan. *Indian J Pediatr*. 2005;72:839–842.
154. Louwman MW, van Dusseldorp M, van de Vijver FJ, et al. Signs of impaired cognitive function in adolescents with marginal cobalamin status. *Am J Clin Nutr*. 2000;72:762–769.
155. Antony AC. Prevalence of cobalamin (vitamin B-12) and folate deficiency in India—audi alteram partem. *Am J Clin Nutr*. 2001;74:157–159.
156. Whaley SE, Sigman M, Neumann C, et al. The impact of dietary intervention on the cognitive development of Kenyan school children. *J Nutr*. 2003;133:3965S–3971S.
157. Neumann CG, Bwibo NO, Murphy SP, et al. Animal source foods improve dietary quality, micronutrient status, growth and cognitive function in

Kenyan school children: background, study design and baseline findings. *J Nutr*. 2003;133:3941S–3949S.

158. Rogers LM, Boy E, Miller JW, Green R, Sabel JC, Allen LH. High prevalence of cobalamin deficiency in Guatemalan schoolchildren: associations with low plasma holotranscobalamin II and elevated serum methylmalonic acid and plasma homocysteine concentrations. *Am J Clin Nutr*. 2003;77:433–440.
159. Murphy MM, Molloy AM, Ueland PM, et al. Longitudinal study of the effect of pregnancy on maternal and fetal cobalamin status in healthy women and their offspring. *J Nutr*. 2007;137:1863–1867.
160. Bjørke-Monsen A-L, Torsvik I, Saetran H, Markestad T, Ueland PM. Common metabolic profile in infants indicating impaired cobalamin status responds to cobalamin supplementation. *Pediatrics*. 2008;122:83–91.
161. Bjørke-Monsen A-L, Ueland PM. Cobalamin status in children. *J Inherit Metab Dis*. 2010;34:111–119.
162. Torsvik I, Ueland PM, Markestad T, Bjørke-Monsen A-L. Cobalamin supplementation improves motor development and regurgitations in infants: results from a randomized intervention study. *Am J Clin Nutr*. 2013;98:1233–1240.
163. Whitehead VM. Acquired and inherited disorders of cobalamin and folate in children. *Br J Haematol*. 2006;134:125–136.
164. Tanner SM, Li Z, Perko JD, et al. Hereditary juvenile cobalamin deficiency caused by mutations in the intrinsic factor gene. *Proc Natl Acad Sci U S A*. 2005;102:4130–4133.
165. Hu Y, Kim H-I, Hyung WJ, et al. Vitamin B(12) deficiency after gastrectomy for gastric cancer: an analysis of clinical patterns and risk factors. *Ann Surg*. 2013;258:970–975.
166. Vargas-Ruiz AG, Hernandez-Rivera G, Herrera MF. Prevalence of iron, folate, and vitamin B12 deficiency anemia after laparoscopic Roux-en-Y gastric bypass. *Obes Surg*. 2008;18:288–293.
167. Toh BH. Diagnosis and classification of autoimmune gastritis. *Autoimmun Rev*. 2014;13:459–462.
168. Toh BH, van Driel IR, Gleeson PA. Pernicious anemia. *N Engl J Med*. 1997;337:1441–1448.
169. Lahner E, Norman GL, Severi C, et al. Reassessment of intrinsic factor and parietal cell autoantibodies in atrophic gastritis with respect to cobalamin deficiency. *Am J Gastroenterol*. 2009;104:2071–2079.
170. Carmel R. Prevalence of undiagnosed pernicious anemia in the elderly. *Arch Intern Med*. 1996;156:1097–1100.
171. Fyfe JC, Madsen M, Højrup P, et al. The functional cobalamin (vitamin B12)-intrinsic factor receptor is a novel complex of cubilin and amnionless. *Blood*. 2004;103:1573–1579.
172. Bauman WA, Shaw S, Jayatilleke E, et al. Increased intake of calcium reverses vitamin B12 malabsorption induced by metformin. *Diabetes Care*. 2000;23:1227–1231.
173. de Jager J, Kooy A, Lehert P, et al. Long term treatment with metformin in patients with type 2 diabetes and risk of vitamin B-12 deficiency: randomised placebo controlled trial. *BMJ*. 2010;340:c2181.
174. Quadros EV, Lai SC, Nakayama Y, et al. Positive newborn screen for methylmalonic aciduria identifies the first mutation in TCblR/CD320, the gene for cellular uptake of transcobalamin-bound vitamin B(12). *Hum Mutat*. 2010;31:924–929.
175. Watkins D, Rosenblatt DS. Inborn errors of cobalamin absorption and metabolism. *Am J Med Genet C Semin Med Genet*. 2011;157C:33–44.
176. Froese DS, Gravel RA. Genetic disorders of vitamin B12 metabolism: eight complementation groups–eight genes. *Expert Rev Mol Med*. 2010;12:e37.
177. Oussalah A, Julien M, Levy J, et al. Global burden related to nitrous oxide exposure in medical and recreational settings: a systematic review and individual patient data meta-analysis. *J Clin Med*. 2019:8.
178. Smith AD, Smith SM, de Jager CA, et al. Homocysteine-lowering by B vitamins slows the rate of accelerated brain atrophy in mild cognitive impairment: a randomized controlled trial. *PLoS One*. 2010;5:e12244.
179. de Jager CA, Oulhaj A, Jacoby R, et al. Cognitive and clinical outcomes of homocysteine-lowering B-vitamin treatment in mild cognitive impairment: a randomized controlled trial. *Int J Geriatr Psychiatry*. 2012;27:592–600.
180. Eussen SJPM, Ueland PM, Hiddink GJ, et al. Changes in markers of cobalamin status after cessation of oral B-vitamin supplements in elderly people with mild cobalamin deficiency. *Eur J Clin Nutr*. 2008;62:1248–1251.
181. Baggott JE, Tamura T. The second study of experimental human folate deficiency. *Springerplus*. 2014;3:719.
182. Abdelrahim II, Adam GK, Mohmmed AA, et al. Anaemia, folate and vitamin B12 deficiency among pregnant women in an area of unstable malaria transmission in eastern Sudan. *Trans R Soc Trop Med Hyg*. 2009;103:493–496.
183. Odewole OA, Williamson RS, Zakai NA, et al. Near-elimination of folate-deficiency anemia by mandatory folic acid fortification in older US adults: Reasons for Geographic and Racial Differences in Stroke study 2003–2007. *Am J Clin Nutr*. 2013;98:1042–1047.
184. McNamee T, Hyland T, Harrington J, et al. Haematinic deficiency and macrocytosis in middle-aged and older adults. *PLoS One*. 2013;8:e77743.
185. Gudgeon P, Cavalcanti R. Folate testing in hospital inpatients. *Am J Med*. 2015;128:56–59.
186. Gilfix BM. Utility of measuring serum or red blood cell folate in the era of folate fortification of flour. *Clin Biochem*. 2014;47:533–538.
187. Bach V, Schruckmayer G, Sam I, et al. Prevalence and possible causes of anemia in the elderly: a cross-sectional analysis of a large European university hospital cohort. *Clin Interv Aging*. 2014;9:1187–1196.
188. Meziere A, Audureau E, Vairelles S, et al. B12 deficiency increases with age in hospitalized patients: a study on 14,904 samples. *J Gerontol A Biol Sci Med Sci*. 2014;69:1576–1585.
189. Tamura T, Picciano MF. Folate and human reproduction. *Am J Clin Nutr*. 2006;83:993–1016.
190. van Eijsden M, Smits LJM, van der Wal MF, Bonsel GJ. Association between short interpregnancy intervals and term birth weight: the role of folate depletion. *Am J Clin Nutr*. 2008;88:147–153.
191. Torheim LE, Ferguson EL, Penrose K, Arimond M. Women in resource-poor settings are at risk of inadequate intakes of multiple micronutrients. *J Nutr*. 2010;140:2051S–2058S.
192. Sen A, Kanani SJ. Impact of iron-folic acid supplementation on cognitive abilities of school girls in Vadodara. *Indian Pediatr*. 2009;46:137–143.
193. Craciunescu CN, Brown EC, Mar-Heng M, et al. Folic acid deficiency during late gestation decreases progenitor cell proliferation and increases apoptosis in fetal mouse brain. *J Nutr*. 2004;134:162–166.
194. Ferguson SA, Berry KJ, Hansen DK, et al. Behavioral effects of prenatal folate deficiency in mice. *Birth Defects Res A Clin Mol Teratol*. 2005;73:249–252.
195. Berrocal-Zaragoza MI, Sequeira JM, Murphy MM, et al. Folate deficiency in rat pups during weaning causes learning and memory deficits. *Br J Nutr*. 2014;112:1323–1332.
196. Schlotz W, Jones A, Phillips DIW, Gale CR, Robinson SM, Godfrey KM. Lower maternal folate status in early pregnancy is associated with childhood hyperactivity and peer problems in offspring. *J Child Psychol Psychiatry*. 2010;51:594–602.
197. Martinez-Vega R, Garrido F, Partearroyo T, et al. Folic acid deficiency induces premature hearing loss through mechanisms involving cochlear oxidative stress and impairment of homocysteine metabolism. *FASEB J*. 2015;29:418–432.
198. Roza SJ, van Batenburg-Eddes T, Steegers EA, et al. Maternal folic acid supplement use in early pregnancy and child behavioural problems: the Generation R Study. *Br J Nutr*. 2010;103:445–452.
199. Suren P, Roth C, Bresnahan M, et al. Association between maternal use of folic acid supplements and risk of autism spectrum disorders in children. *JAMA*. 2013;309:570–577.
200. Christian P, Murray-Kolb LE, Khatry SK, et al. Prenatal micronutrient supplementation and intellectual and motor function in early school-aged children in Nepal. *JAMA*. 2010;304:2716–2723.
201. Roth C, Magnus P, Schjolberg S, et al. Folic acid supplements in pregnancy and severe language delay in children. *JAMA*. 2011;306:1566–1573.
202. Schmidt RJ, Tancredi DJ, Ozonoff S, et al. Maternal periconceptional folic acid intake and risk of autism spectrum disorders and developmental delay in the CHARGE (CHildhood Autism Risks from Genetics and Environment) case-control study. *Am J Clin Nutr*. 2012;96:80–89.
203. Veena SR, Krishnaveni GV, Srinivasan K, et al. Higher maternal plasma folate but not vitamin B-12 concentrations during pregnancy are associated with better cognitive function scores in 9- to 10- year-old children in South India. *J Nutr*. 2010;140:1014–1022.
204. Steenweg-de Graaff J, Roza SJ, Steegers EA, et al. Maternal folate status in early pregnancy and child emotional and behavioral problems: the generation R study. *Am J Clin Nutr*. 2012;95:1413–1421.
205. Nilsson TK, Yngve A, Böttiger AK, et al. High folate intake is related to better academic achievement in Swedish adolescents. *Pediatrics*. 2011;128:e358–e365.
206. MRC Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. MRC Vitamin Study Research Group. *Lancet*. 1991;338:131–137.
207. Czeizel AE, Dudas I. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. *N Engl J Med*. 1992;327:1832–1835.
208. Berry RJ, Li Z, Erickson JD, et al. Prevention of neural-tube defects with folic acid in China. China–U.S. collaborative project for neural tube defect prevention. *N Engl J Med*. 1999;341:1485–1490.
209. Crider KS, Bailey LB, Berry RJ. Folic acid food fortification-its history, effect, concerns, and future directions. *Nutrients*. 2011;3:370–384.

210. Hernandez-Diaz S, Werler MM, Walker AM, Mitchell AA. Folic acid antagonists during pregnancy and the risk of birth defects. *N Engl J Med*. 2000;343:1608–1614.
211. Feldkamp M, Friedrichs M, Marti K. Folic acid awareness, knowledge, and consumption among women of childbearing age in Utah, 1998–2000. *Am J Med Genet*. 2002;107:67–69.
212. Centers for Disease Control and Prevention (CDC) CDC Grand Rounds: additional opportunities to prevent neural tube defects with folic acid fortification. *MMWR Morb Mortal Wkly Rep*. 2010;59:980–984.
213. Imhoff-Kunsch B, Flores R, Dary O, Martorell R. Wheat flour fortification is unlikely to benefit the neediest in Guatemala. *J Nutr*. 2007;137:1017–1022.
214. Cherian A, Seena S, Bullock RK, Antony AC. Incidence of neural tube defects in the least-developed area of India: a population-based study. *Lancet*. 2005;366:930–931.
215. Rai SK, Singh R, Pandey S, et al. High incidence of neural tube defects in Northern part of India. *Asian J Neurosurg*. 2016;11:352–355.
216. Craig WJ, Mangels AR. Position of the American Dietetic Association: vegetarian diets. *J Am Diet Assoc*. 2009;109:1266–1282.
217. Titaley CR, Dibley MJ, Roberts CL, Agho K. Combined iron/folic acid supplements and malaria prophylaxis reduce neonatal mortality in 19 sub-Saharan African countries. *Am J Clin Nutr*. 2010;92:235–243.
218. Okebe JU, Yahav D, Shbita R, Paul M. Oral iron supplements for children in malaria-endemic areas. *Cochrane Database Syst Rev*. 2011;2:CD006589.
219. Neuberger A, Okebe J, Yahav D, Paul M. Oral iron supplements for children in malaria-endemic areas. *Cochrane Database Syst Rev*. 2016;2:CD006589.
220. Dhar M, Bellevue R, Carmel R. Pernicious anemia with neuropsychiatric dysfunction in a patient with sickle cell anemia treated with folate supplementation. *N Engl J Med*. 2003;348:2204–2207.
221. Mahadeo KM, Diop-Bove N, Ramirez SI, et al. Prevalence of a loss-of-function mutation in the proton-coupled folate transporter gene (PCFT-SLC46A1) causing hereditary folate malabsorption in Puerto Rico. *J Pediatr*. 2011;159 623-7 e1.
222. Borzutzky A, Crompton B, Bergmann AK, et al. Reversible severe combined immunodeficiency phenotype secondary to a mutation of the proton-coupled folate transporter. *Clin Immunol*. 2009;133:287–294.
223. Min SH, Oh SY, Karp GI, et al. The clinical course and genetic defect in the PCFT gene in a 27-year-old woman with hereditary folate malabsorption. *J Pediatr*. 2008;153:435–437.
224. Ward MG, Kariyawasam VC, Mogan SB, et al. Prevalence and risk factors for functional vitamin B12 deficiency in patients with Crohn's disease. *Inflamm Bowel Dis*. 2015;21:2839–2847.
225. Bermejo F, Algaba A, Guerra I, et al. Should we monitor vitamin B12 and folate levels in Crohn's disease patients? *Scand J Gastroenterol*. 2013;48:1272–1277.
226. Visentin M, Diop-Bove N, Zhao R, Goldman ID. The intestinal absorption of folates. *Annu Rev Physiol*. 2014;76:251–274.
227. McFarland R. Cerebral folate deficiency—mishaps and misdirection. *Brain*. 2012;135:2002–2003.
228. Perez-Duenas B, Ormazabal A, Toma C, et al. Cerebral folate deficiency syndromes in childhood: clinical, analytical, and etiologic aspects. *Arch Neurol*. 2011;68:615–621.
229. de Haan W. A folate receptor defect that causes treatable neurological disorder in children. *Clin Genet*. 2010;77:435–436.
230. Ramaekers VT, Rothenberg SP, Sequeira JM, et al. Autoantibodies to folate receptors in the cerebral folate deficiency syndrome. *N Engl J Med*. 2005;352:1985–1991.
231. Gordon N. Cerebral folate deficiency. *Dev Med Child Neurol*. 2009;51:180–182.
232. Ramaekers VT, Blau N, Sequeira JM, et al. Folate receptor autoimmunity and cerebral folate deficiency in low-functioning autism with neurological deficits. *Neuropediatrics*. 2007;38:276–281.
233. Frye RE, Sequeira JM, Quadros EV, et al. Cerebral folate receptor autoantibodies in autism spectrum disorder. *Mol Psychiatry*. 2013;18:369–381.
234. Sadighi Z, Butler IJ, Koenig MK. Adult-onset cerebral folate deficiency. *Arch Neurol*. 2012;69:778–779.
235. Berrocal-Zaragoza MI, Fernandez-Ballart JD, Murphy MM, et al. Association between blocking folate receptor autoantibodies and subfertility. *Fertil Steril*. 2009;91:1518–1521.
236. Bliek JB, Rothenberg SP, Steegers-Theunissen RP. Maternal folate receptor autoantibodies and cleft lip and/or palate. *Int J Gynaecol Obstet*. 2006;93:142–143.
237. O'Leary VB, Parle-McDermott A, Molloy AM, et al. MTRR and MTHFR polymorphism: link to Down syndrome? *Am J Med Genet*. 2002;107:151–155.
238. Christensen KE, Rozen R. Genetic variation: effect on folate metabolism and health. In: Bailey LB, ed. *Folate in Health and Disease.* 2nd ed. Boca Raton, FL: CRC Press; 2010.
239. Huemer M, Ausserer B, Graninger G, et al. Hyperhomocysteinemia in children treated with antiepileptic drugs is normalized by folic acid supplementation. *Epilepsia*. 2005;46:1677–1683.
240. Apeland T, Mansoor MA, Pentieva K, McNulty H, Seljeflot I, Strandjord RE. The effect of B-vitamins on hyperhomocysteinemia in patients on antiepileptic drugs. *Epilepsy Res*. 2002;51:237–247.
241. George L, Mills JL, Johansson AL, et al. Plasma folate levels and risk of spontaneous abortion. *JAMA*. 2002;288:1867–1873.
242. Pittschieler S, Brezinka C, Jahn B, et al. Spontaneous abortion and the prophylactic effect of folic acid supplementation in epileptic women undergoing antiepileptic therapy. *J Neurol*. 2008;255:1926–1931.
243. Volkov I, Rudoy I, Freud T, et al. Effectiveness of vitamin B12 in treating recurrent aphthous stomatitis: a randomized, double-blind, placebo-controlled trial. *J Am Board Fam Med*. 2009;22:9–16.
244. Rehman HU. Vitamin B12 deficiency causing night sweats. *Scott Med J*. 2014;59:e8–e11.
245. Sherley-Dale AC, Goulding JMR, Leithead J, Loft DE, Ahmed I. Pigmentary changes in pernicious anaemia: an important but under-recognized phenomenon. *Clin Exp Dermatol*. 2011;36:93–95.
246. Agrawala RK, Sahoo SK, Choudhury AK, et al. Pigmentation in vitamin B12 deficiency masquerading Addison's pigmentation: a rare presentation. *Indian J Endocrinol Metab*. 2013;17:S254–S256.
247. Vannella L, Lahner E, Osborn J, Annibale B. Systematic review: gastric cancer incidence in pernicious anaemia. *Aliment Pharmacol Ther*. 2013;37:375–382.
248. Jauhar S, Blackett A, Srireddy P, McKenna PJ. Pernicious anaemia presenting as catatonia without signs of anaemia or macrocytosis. *Br J Psychiatry*. 2010;197:244–245.
249. Mavrommati K, Sentissi O. Delirium as a result of vitamin B12 deficiency in a vegetarian female patient. *Eur J Clin Nutr*. 2013;67:996–997.
250. Field MS, Kamynina E, Watkins D, Rosenblatt DS, Stover PJ. Human mutations in methylenetetrahydrofolate dehydrogenase 1 impair nuclear de novo thymidylate biosynthesis. *Proc Natl Acad Sci U S A*. 2015;112:400–405.
251. Shah NP, Beech CM, Sturm AC, Tanner SM. Investigation of the ABC transporter MRP1 in selected patients with presumed defects in vitamin B12 absorption. *Blood*. 2011;117:4397–4398.
252. Devalia V, Hamilton MS, Molloy AM. British Committee for Standards in Haematology. Guidelines for the diagnosis and treatment of cobalamin and folate disorders. *Br J Haematol*. 2014;166:496–513.
253. Kuzminski AM, Del Giacco EJ, Allen RH, et al. Effective treatment of cobalamin deficiency with oral cobalamin. *Blood*. 1998;92:1191–1198.
254. Rajan S, Wallace JI, Brodkin KI, et al. Response of elevated methylmalonic acid to three dose levels of oral cobalamin in older adults. *J Am Geriatr. Soc*. 2002;50:1789–1795.
255. Allen RH, Stabler SP. Identification and quantitation of cobalamin and cobalamin analogues in human feces. *Am J Clin Nutr*. 2008;87:1324–1335.
256. Auerbach M, Pappadakis JA, Bahrain H, et al. Safety and efficacy of rapidly administered (one hour) one gram of low molecular weight iron dextran (INFeD) for the treatment of iron deficient anemia. *Am J Hematol*. 2011;86:860–862.
257. Mellemkjaer L, Gridley G, Møller H, et al. Pernicious anaemia and cancer risk in Denmark. *Br J Cancer*. 1996;73:998–1000.
258. Ionescu-Ittu R, Marelli AJ, Mackie AS, Pilote L. Prevalence of severe congenital heart disease after folic acid fortification of grain products: time trend analysis in Quebec, Canada. *BMJ*. 2009;338:b1673.
259. Wilcox AJ, Lie RT, Solvoll K, et al. Folic acid supplements and risk of facial clefts: national population based case-control study. *BMJ*. 2007;334:464.
260. Jacques PF, Selhub J, Bostom AG, et al. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. *N Engl J Med*. 1999;340:1449–1454.
261. Ganji V, Kafai MR. Hemoglobin and hematocrit values are higher and prevalence of anemia is lower in the post-folic acid fortification period than in the pre-folic acid fortification period in US adults. *Am J Clin Nutr*. 2009;89:363–371.
262. Yi Y, Lindemann M, Colligs A, Snowball C. Economic burden of neural tube defects and impact of prevention with folic acid: a literature review. *Eur J Pediatr*. 2011;170(11):1391–1400.
263. Robbins JM, Cleves MA, Collins HB, et al. Randomized trial of a physician-based intervention to increase the use of folic acid supplements among women. *Am J Obstet Gynecol*. 2005;192:1126–1132.
264. Paudel P, Wing K, Silpakar SK. Awareness of periconceptional folic acid supplementation among Nepalese women of childbearing age: a cross-sectional study. *Prev Med*. 2012;55:511–513.

265. Strober BE, Menon K. Folate supplementation during methotrexate therapy for patients with psoriasis. *J Am Acad Dermatol*. 2005;53:652–659.
266. Whittle SL, Hughes RA. Folate supplementation and methotrexate treatment in rheumatoid arthritis: a review. *Rheumatology (Oxford)*. 2004;43:267–271.
267. Osei AK, Rosenberg IH, Houser RF, et al. Community-level micronutrient fortification of school lunch meals improved vitamin A, folate, and iron status of schoolchildren in Himalayan villages of India. *J Nutr*. 2010;140:1146–1154.
268. Bailey RL, McDowell MA, Dodd KW, et al. Total folate and folic acid intakes from foods and dietary supplements of US children aged 1–13 y. *Am J Clin Nutr*. 2010;92:353–358.
269. Yang Q, Cogswell ME, Hamner HC, et al. Folic acid source, usual intake, and folate and vitamin B-12 status in US adults: National Health and Nutrition Examination Survey (NHANES) 2003–2006. *Am J Clin Nutr*. 2010;91:64–72.
270. Mills JL, Carter TC, Scott JM, et al. Do high blood folate concentrations exacerbate metabolic abnormalities in people with low vitamin B-12 status? *Am J Clin Nutr*. 2011;94:495–500.
271. Berry RJ. Lack of historical evidence to support folic acid exacerbation of the neuropathy caused by vitamin B12 deficiency. *Am J Clin Nutr*. 2019;110:554–561.
272. Cuffe K, Stauffer W, Painter J, et al. Update: vitamin B12 deficiency among Bhutanese refugees resettling in the United States, 2012. *MMWR Morb Mortal Wkly Rep*. 2014;63:607.
273. Carmel R, Mallidi PV, Vinarskiy S, et al. Hyperhomocysteinemia and cobalamin deficiency in young Asian Indians in the United States. *Am J Hematol*. 2002;70:107–114.
274. Salcedo-Sora JE, Ochong E, Beveridge S, et al. The molecular basis of folate salvage in *Plasmodium falciparum*: characterization of two folate transporters. *J Biol Chem*. 2011;286:44659–44668.
275. van Eijk AM, Ouma PO, Williamson J, et al. Plasma folate level and high-dose folate supplementation predict sulfadoxine-pyrimethamine treatment failure in pregnant women in western Kenya who have uncomplicated malaria. *J Infect Dis*. 2008;198:1550–1553.
276. Carter JY, Loolpapit MP, Lema OE, et al. Reduction of the efficacy of antifolate antimalarial therapy by folic acid supplementation. *Am J Trop Med Hyg*. 2005;73:166–170.
277. Spence JD. Homocysteine-lowering therapy: a role in stroke prevention? *Lancet Neurol*. 2007;6:830–838.
278. Khan U, Crossley C, Kalra L, et al. Homocysteine and its relationship to stroke subtypes in a UK black population: the South London Ethnicity and Stroke Study. *Stroke*. 2008;39:2943–2949.
279. Seshadri S, Beiser A, Selhub J, et al. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. *N Engl J Med*. 2002;346:476–483.
280. Durga J, van Boxtel MPJ, Schouten EG, et al. Effect of 3-year folic acid supplementation on cognitive function in older adults in the FACIT trial: a randomised, double blind, controlled trial. *Lancet*. 2007;369:208–216.
281. Walker JG, Batterham PJ, Mackinnon AJ, et al. Oral folic acid and vitamin B-12 supplementation to prevent cognitive decline in community-dwelling older adults with depressive symptoms—the Beyond Ageing Project: a randomized controlled trial. *Am J Clin Nutr*. 2012;95:194–203.
282. Smith AD, Refsum H, Bottiglieri T, et al. Homocysteine and dementia: an international consensus statement. *J Alzheimers Dis*. 2018;62:561–570.
283. McLean RR, Jacques PF, Selhub J, et al. Homocysteine as a predictive factor for hip fracture in older persons. *N Engl J Med*. 2004;350:2042–2049.
284. Quere I, Mercier E, Bellet H, et al. Vitamin supplementation and pregnancy outcome in women with recurrent early pregnancy loss and hyperhomocysteinemia. *Fertil Steril*. 2001;75:823–825.
285. Ray JG, Laskin CA. Folic acid and homocyst(e)ine metabolic defects and the risk of placental abruption, pre-eclampsia and spontaneous pregnancy loss: a systematic review. *Placenta*. 1999;20:519–529.
286. de la Calle M, Usandizaga R, Sancha M, et al. Homocysteine, folic acid and B-group vitamins in obstetrics and gynaecology. *Eur J Obstet Gynecol Reprod Biol*. 2003;107:125–134.
287. Bennett M. Vitamin B12 deficiency, infertility and recurrent fetal loss. *J Reprod Med*. 2001;46:209–212.
288. Vollset SE, Refsum H, Irgens LM, et al. Plasma total homocysteine, pregnancy complications, and adverse pregnancy outcomes: the Hordaland Homocysteine study. *Am J Clin Nutr*. 2000;71:962–968.
289. Bondevik GT, Schneede J, Refsum H, et al. Homocysteine and methylmalonic acid levels in pregnant Nepali women. Should cobalamin supplementation be considered? *Eur J Clin Nutr*. 2001;55:856–864.
290. Smulders YM, Blom HJ. The homocysteine controversy. *J Inherit Metab Dis*. 2011;34:93–99.
291. Wang X, Qin X, Demirtas H, et al. Efficacy of folic acid supplementation in stroke prevention: a meta-analysis. *Lancet*. 2007;369:1876–1882.
292. Durga J, Verhoef P, Anteunis LJC, et al. Effects of folic acid supplementation on hearing in older adults: a randomized, controlled trial. *Ann Intern Med*. 2007;146:1–9.
293. Sato Y, Honda Y, Iwamoto J, et al. Effect of folate and mecobalamin on hip fractures in patients with stroke: a randomized controlled trial. *JAMA*. 2005;293:1082–1088.
294. Hodis HN, Mack WJ, Dustin L, et al. High-dose B vitamin supplementation and progression of subclinical atherosclerosis: a randomized controlled trial. *Stroke*. 2009;40:730–736.
295. Christen WG, Glynn RJ, Chew EY, et al. Folic acid, pyridoxine, and cyanocobalamin combination treatment and age-related macular degeneration in women: the Women's Antioxidant and Folic Acid Cardiovascular Study. *Arch Intern Med*. 2009;169:335–341.
296. Gopinath B, Flood VM, Rochtchina E, et al. Homocysteine, folate, vitamin B-12, and 10-y incidence of age-related macular degeneration. *Am J Clin Nutr*. 2013;98:129–135.
297. Evans J. Should we be taking B vitamins to prevent age-related macular degeneration? Not yet, but worth doing more research. *Am J Clin Nutr*. 2013;98:4–5.
298. House AA, Eliasziw M, Cattran DC, et al. Effect of B-vitamin therapy on progression of diabetic nephropathy: a randomized controlled trial. *JAMA*. 2010;303:1603–1609.
299. Lee JE, Chan AT. Fruit, vegetables, and folate: cultivating the evidence for cancer prevention. *Gastroenterology*. 2011;141:16–20.
300. Lee JE, Willett WC, Fuchs CS, et al. Folate intake and risk of colorectal cancer and adenoma: modification by time. *Am J Clin Nutr*. 2011;93:817–825.
301. Stevens VL, McCullough ML, Sun J, et al. High levels of folate from supplements and fortification are not associated with increased risk of colorectal cancer. *Gastroenterology*. 2011;141 98-105, e1.
302. Vollset SE, Clarke R, Lewington S, et al. Effects of folic acid supplementation on overall and site-specific cancer incidence during the randomised trials: meta-analyses of data on 50,000 individuals. *Lancet*. 2013;381:1029–1036.
303. Kim Y-I. Folate and cancer: a tale of Dr. Jekyll and Mr. Hyde? *Am J Clin Nutr*. 2018;107:139–142.
304. Wibowo AS, Singh M, Reeder KM, et al. Structures of human folate receptors reveal biological trafficking states and diversity in folate and antifolate recognition. *Proc Natl Acad Sci U S A*. 2013;110:15180–15188.
305. Chen C, Ke J, Zhou XE, et al. Structural basis for molecular recognition of folic acid by folate receptors. *Nature*. 2013;500:486–489.
306. van Dam GM, Themelis G, Crane LM, et al. Intraoperative tumor-specific fluorescence imaging in ovarian cancer by folate receptor-α targeting: first in-human results. *Nat Med*. 2011;17:1315–1319.
307. Müller C, Reber J, Haller S, et al. Folate receptor targeted alpha-therapy using terbium-149 *Pharmaceuticals (Basel)*. 72014353–365.
308. Müller C. Folate-based radiotracers for PET imaging—update and perspectives. *Molecules*. 2013;18:5005–5031.
309. Bianchi E, Doe B, Goulding D, Wright GJ. Juno is the egg Izumo receptor and is essential for mammalian fertilization. *Nature*. 2014;508:483–487.
310. Bianchi E, Wright GJ. Izumo meets Juno: preventing polyspermy in fertilization. *Cell Cycle*. 2014;13:2019–2020.
311. Hoffbrand AV, Weir DG. The history of folic acid. *Br J Haematol*. 2001;113:579–589.
312. Stover PJ. Folate biochemical pathways and their regulation. In: Bailey LB, ed. *Folate in Health and Disease*. 2nd ed. Boca Raton, FL: CRC Press; 2010:49–74.
313. Arya R, Gulati S, Kabra M, et al. Folic acid supplementation prevents phenytoin-induced gingival overgrowth in children. *Neurology*. 2011;76:1338–1343.
314. Czeizel AE. Periconceptional folic acid and multivitamin supplementation for the prevention of neural tube defects and other congenital abnormalities. *Birth Defects Res A Clin Mol Teratol*. 2009;85:260–268.
315. Yang Q, Botto LD, Erickson JD, et al. Improvement in stroke mortality in Canada and the United States, 1990 to 2002. *Circulation*. 2006;113:1335–1343.
316. Bukowski R, Malone FD, Porter FT, et al. Preconceptional folate supplementation and the risk of spontaneous preterm birth: a cohort study. *PLoS Med*. 2009;6:e1000061.

CHAPTER 41

THALASSEMIA SYNDROMES

Sujit Sheth

The thalassemia syndromes are a heterogeneous group of inherited anemias which result from defects in the synthesis of one or more of the globin chain subunits of the hemoglobin (Hb) tetramer. The basic defect, decreased or absent production of a globin subunit, results in an imbalance between globins produced as a result of expression of genes from the alpha-globin gene cluster on chromosome 16, and those produced from expression of genes on the beta-globin gene cluster on chromosome 11. As a result of the imbalance, there is ineffective erythropoiesis (IE) and defective red cell production. The spectrum of disease severity is determined by the degree of imbalance in globin chain production and varies from mild, with few clinical manifestations, to severe anemia and transfusion dependence.

The disease was first described by Cooley and Lee in 1925,[1] who coined the name Cooley's anemia to describe a severe anemia with splenomegaly and bony changes. The term *thalassemia* is derived from a Greek term that roughly means "the sea" (Mediterranean) in the blood.[2] It was first applied to the anemias frequently encountered in people from the Italian and Greek coasts and nearby islands, likely beta-thalassemia.[3–5] The term thalassemia is now used to refer to a wide range of inherited defects in globin-chain biosynthesis. The inheritance of the disease, and its wide spectrum of severity was described progressively over the last century as our understanding of the disease and its pathophysiology has grown. Discovered to arise from abnormalities affecting expression of the autosomal globin genes, the homozygous condition was termed thalassemia major to describe the most severe form of the disease, the heterozygous condition was termed thalassemia minor or minima to describe a mostly asymptomatic carrier state, and the compound heterozygous states—somewhere in between—were called thalassemia intermedia.

The thalassemias are the commonest monogenic disorders in humans, and have a wide distribution across the globe. Approximately 1.5% of the world's population carries one of the globin gene mutations, the majority of these individuals living in Asia, in areas of the world where thalassemia is particularly prevalent. It comprises a major public health problem. The main thalassemia syndromes are broadly alpha (α)-thalassemia and beta (β)-thalassemia, the clinical manifestations and morbidity of which vary based on the genotype and other modifying factors such as access to care and therapy-related complications, particularly iron overload.

This chapter reviews the major features of these syndromes. Readers wanting more detailed information than can be included here are referred to more comprehensive monographs elsewhere.[2,6]

DEFINITIONS AND NOMENCLATURE

The most common forms of thalassemia arise from total absence of structurally normal globin chains or a partial reduction in their synthesis. In contrast to the "structural" hemoglobinopathies (e.g., sickle cell anemia), which are characterized by the production of normal amounts of mutant globin chains having abnormal physical or chemical properties, the thalassemias are quantitative disorders: the primary defects lie in the amount of globin produced. However, some forms of thalassemia are characterized by the production of structurally abnormal globin chains in reduced amounts, which are described in more detail in this chapter. These thalassemic hemoglobinopathies share features of thalassemia as well as those of structural hemoglobinopathies.[7]

Individual syndromes are named according to the globin chain whose synthesis is adversely affected. Thus, α-globin chains are absent or reduced in patients with α-thalassemia, β-globin chains in patients with β-thalassemia, δ-globin and β-globin chains in patients with δβ-thalassemia, and so forth. In some contexts, it is also useful to subclassify the syndromes according to whether synthesis of the affected globin chain is totally absent (e.g., β^0-thalassemia) or only partially reduced (e.g., β^+-thalassemia).

Some mutations alter the patterns of fetal to adult Hb switching. These conditions, called *hereditary persistence of fetal hemoglobin*, are not generally associated with clinical symptoms; nonetheless, they merit consideration in this chapter. Their importance lies in their role as modulating factors when coinherited with other hemoglobinopathies. They are also useful models for investigating the molecular basis for globin gene regulation during human development and as paradigms for rational therapy for the major β-chain hemoglobinopathies, namely, sickle cell anemia and β-thalassemia.

EPIDEMIOLOGY

Thalassemias have been encountered in virtually every ethnic group and geographic location. They are most common in the Mediterranean basin and tropical or subtropical regions of Asia and Africa. The "thalassemia belt" extends along the shores of the Mediterranean, through the Arabian peninsula, Turkey, Iran, India, and southeastern Asia, especially Thailand, Cambodia, and southern China.[8–11] The prevalence of carriers of thalassemia in these regions is in the range of 2.5% to 15%. Similar to sickle cell anemia, thalassemia is most common in areas historically affected by endemic malaria. Malaria seems to have conferred selective survival advantage to thalassemia heterozygotes in which infection with the malarial parasite is believed to result in milder disease and less impact on reproductive fitness.[2,6,12] Therefore, the gene frequency for thalassemia has become fixed and high in populations exposed to malaria over many centuries. Migration from these parts to the rest of the world has resulted in the thalassemias being found all over the world, including Australia and the Americas, though β-thalassemia is still somewhat uncommon in Africa, except parts of West Africa and North Africa bordering on the Mediterranean Sea. The most severe form of α-thalassemia is found mainly in the Oriental populations because of the higher prevalence of the two gene deletions in *cis* configuration in these areas.

PATHOPHYSIOLOGY: GENERAL PRINCIPLES

The classification, genetic basis, and pathophysiology of the thalassemia syndromes are based on a thorough understanding of the human hemoglobins, their biosynthesis, their encoding *globin* gene families, and their roles as soluble oxygen-carrying molecules. Therefore, readers of this chapter should first familiarize themselves with the material presented in Chapter 34. The material presented in this chapter is also substantially clarified by prior reading of Chapters 35, 36, and 37 because the principles underlying the pathophysiology of and therapy for thalassemia draw heavily on knowledge of iron metabolism.

The primary lesion in all forms of thalassemia is reduced or absent production of one or more globin chains. Red blood cells (RBCs) contain a mix of hemoglobin types, which vary through intrauterine development. These molecules have the same heme moieties but differ in globin chains. In the post-natal period, after infancy, the predominant hemoglobin is adult hemoglobin or HbA, a heterodimer of 2 alpha and 2 beta globins ($\alpha_2\beta_2$). For all practical purposes, the major impact on clinical well-being occurs only when these lesions affect the α- or β-globin chains necessary for the synthesis of adult Hb. Severe impairment of γ-, ε-, or ζ-globin production is presumably lethal in utero, and has not been observed in human biology.

One consequence of reduced globin-chain production is immediately apparent: reduced production of functioning Hb tetramers, and consequently anemia. Thus, hypochromia and microcytosis are characteristic of virtually all patients with thalassemia. In the mildest forms of the disease, this phenomenon may be barely detectable.

The second consequence of impaired globin biosynthesis is unbalanced synthesis of the individual α- and β-subunits. Hb tetramers are highly soluble and have reversible oxygen-carrying properties exquisitely adapted for oxygen transport and delivery under physiologic conditions. Free or "unpaired" α-, β-, and γ-globin chains are either highly insoluble ("unstable") or form homotetramers of alpha-globin (Fessas bodies), beta-globin (HbH), or gamma-globin (Hb Bart), that are incapable of releasing oxygen normally, and because they are relatively unstable, will precipitate as the cell ages. For poorly understood reasons, no compensatory regulatory mechanism exists whereby impaired synthesis of one globin subunit leads to a compensatory downward adjustment in the production of the other (partner) globin chain of the Hb tetramer. Thus, useless excess α-globin chains continue to accumulate and precipitate in red cell precursors in β-thalassemia, and excess β-globin chains form HbH in α-thalassemia. The abnormal solubility and oxygen-carrying properties of these chains lead to a variety of physiologic derangements. Indeed, in the severe forms of thalassemia, it is the behavior of the unpaired globin chains accumulating in relative excess that dominate the pathophysiology of the syndrome rather than the mere underproduction of functioning Hb tetramers. The precise complications of this pathophysiologic phenomenon are diverse and depend on the amount and the identity of the globin chain accumulating in excess.

The predominant pathophysiologic determinant in α-thalassemia is the production of HbH, with anemia and impaired oxygen carrying capacity, while in β-thalassemia it is apoptosis of the erythroid precursors in the bone marrow (BM) as a result of hemichrome formation from precipitation of alpha globin aggregates. Thus, IE, while part of both, is more severe when there is an excess of α-globin in β-thalassemia, compared with a similar degree of β-globin excess in α-thalassemia, making the pathophysiology of anemia quite different.

The predominant circulating Hb at the moment of birth is fetal hemoglobin (HbF, $\alpha^2\gamma^2$). Although the switch from γ- to β-globin biosynthesis begins before birth, the composition of Hb in the peripheral blood changes much later because of the long-life span of normal circulating RBCs. HbF is thus slowly replaced by HbA so that infants do not depend heavily on normal amounts and function of HbA until they are between 4 and 6 months old. The pathophysiologic consequences of these considerations are that whereas α-chain hemoglobinopathies tend to be symptomatic in utero and at birth, individuals with β-chain abnormalities are not very symptomatic until 4 to 6 months of age. These differences in the onset of phenotypic expression arise because α-chains are needed to form HbF and HbA, but β-chains are required only for HbA. Red cells which continue to produce gamma globin will have a selective survival advantage in β-thalassemia because they reduce the imbalance from excess α-globin.

BETA-THALASSEMIA SYNDROMES

Nomenclature

Approximately 350 mutations in the beta-globin gene cluster have been described. These mutations vary widely in severity, from complete non-production of beta-globin, called beta-zero (β^0), some expression and thus some production of beta-globin (β^+), to the production of abnormal beta-globins which result in abnormal hemoglobins such as HbE (β^E). There is, thus, considerable phenotypic variability in these syndromes. The most severe form was termed thalassemia major, and referred to a clinically severe disease with marked IE and its manifestations, severe anemia, lifelong transfusion dependence, complications mostly related to iron overload, and a shortened life expectancy. The genotype is typically, but not exclusively, β^0/β^0 or β^0/β^+. At the other end of the spectrum is thalassemia minor which refers to the trait, or carrier state, with one normal β-globin gene, either β/β^0 or β/β^+. In between is a broad spectrum of disease referred to as thalassemia intermedia, which is milder, with a moderate anemia which may not require regular transfusions, with more complications from IE and hemolysis than iron overload, mostly related to skeletal changes and vascular disease. Beta-globin genotypes which result in this clinical syndrome include, but are not limited to, β^0/β^+, β^+/β^+, β^0/β^E or β^+/β^E. A number of more complex genotypes may also be implicated, such as the delta-beta-thalassemias $(\delta\beta)^+$, or hemoglobin Lepore $(\delta\beta)^0$, or $({}^A\gamma\delta\beta)^0$, a single β-thalassemia defect and an excess of normal alpha-globin genes, or two β-thalassemia mutations coinherited with heterozygous α-thalassemia (in this last form, known as αβ-thalassemia, the α-thalassemia allele reduces the burden of unpaired α-chains).[13–15] Simple heterozygosity for certain forms of β-thalassemic hemoglobinopathies can also be associated with a thalassemia intermedia phenotype, sometimes called *dominant β-thalassemia*.[16,17]

Terminology based on the clinical spectrum of disease previously focused on the need for regular transfusions early in life, with those individuals being classified as thalassemia major. Those who did not require regular transfusions but had other manifestations of the disease were referred to as having thalassemia intermedia. However, in the course of their disease many individuals who were initially not regularly transfused could come to need regular transfusions, and thus move along the spectrum from intermedia to major. Currently, the trend has been to simply classify patients as having transfusion-dependent thalassemia (TDT) if they are regularly transfused (requiring 8 or more transfusions per year), or non-transfusion-dependent thalassemia (NTDT) who may or may not require intermittent transfusions.

Molecular Pathology

Forms of β-thalassemia arise from mutations that affect every step in the pathway of globin gene expression: transcription, processing of the messenger ribonucleic acid (mRNA) precursor, translation of mature mRNA, and posttranslational integrity of the β-polypeptide chain (Fig. 41.1 and Table 41.1).[18–20] Large deletions removing two or more non–β-genes are found in rare cases, as are smaller partial or total deletions of the β-gene alone (see Fig. 41.1). Most types of β-thalassemia are caused by point mutations affecting one or a few bases.[18–24] Of the more than 200 mutations causing β-thalassemia, approximately 15 account for the vast majority of affected patients, with the remainder responsible for the disorder in only relatively few patients. It has been determined that five or six mutations usually account for more than 90% of the cases of β-thalassemia in a given ethnic group or geographic area (see Table 41.1).[21]

Several mutations alter the promoter region upstream of the β-globin mRNA-encoding sequence, impairing mRNA synthesis. Mutations in the upstream "master switch" or "LCR" sequences that are needed to induce the high rate of β-globin expression also lead to inadequate production of globin mRNA, while mutations

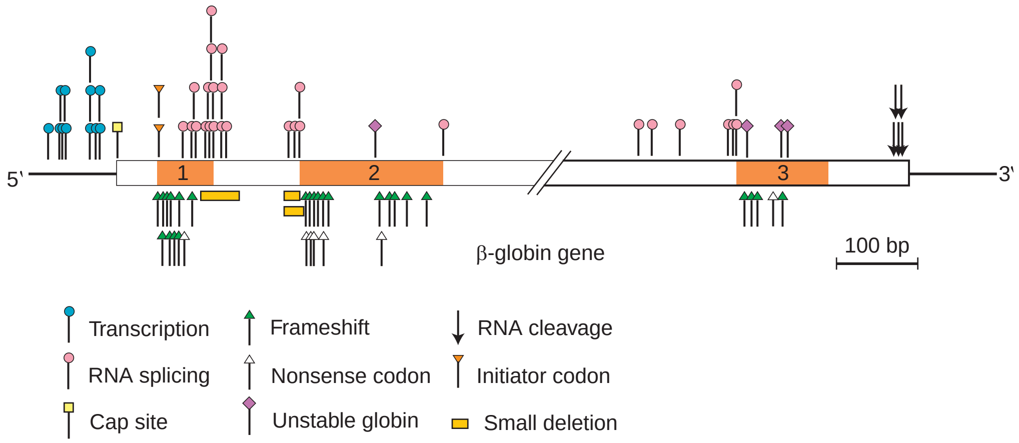

5'
1
2
3
3'
β-globin gene
100 bp
Transcription
RNA splicing
Cap site
Frameshift
Nonsense codon
Unstable globin
RNA cleavage
Initiator codon
Small deletion

Figure 41.1 MODEL OF THE HUMAN β-GLOBIN GENE SHOWING SITES AND TYPES OF VARIOUS MUTATIONS CAUSING β-THALASSEMIA. *(Adapted from Kazazian HH Jr. The thalassemia syndromes: molecular basis and prenatal diagnosis in 1990. Semin Hematol 1990;27:209.)*

TABLE 41.1 **Common β-Thalassemia Mutations in Different Racial Groups**

Racial Group	Description
Mediterranean	IVS-1, position 110 (G → A)
	Codon 39, nonsense (CAG → TAG)
	IVS-1, position 1 (G → A)
	IVS-2, position 745 (C → G)
	IVS-1, position 6 (T → C)
	IVS-2, position 1 (G → A)
African	−34 (A → G)
	−88 (C → T)
	Poly(A), (AATAAA → AACAAA)
Southeast Asian	Codons 41/42, frameshift (-CTTT)
	IVS-2, position 654 (C → T)
	−28 (A → T)
Asian Indian	IVS-1, position 5 (G → C)
	619-bp deletion
	Codons 8/9, frameshift (++G)
	Codons 41/42, frameshift (−CTTT)
	IVS-1, position 1 (G → T)

Data from Kazazian HH Jr, Boehm CD. Molecular basis and prenatal diagnosis of beta-thalassemia. *Blood*. 1988;72(4):1107. https://pubmed.ncbi.nlm.nih.gov/23637309/

that derange the sequence used as the signal for the addition of the poly-(A) tail of the mRNA polyadenylation signal have been shown to result in abnormal cleavage and polyadenylation of the nascent mRNA precursor, with resulting reduced accumulation of mature mRNA.[18–20]

Processing

Many forms of β-thalassemia are caused by mutations that impair splicing of the mRNA precursor into mature mRNA in the nucleus or that prevent translation of the mRNA in the cytoplasm. The molecular pathology of splicing mutations is complex (Fig. 41.2). Some base substitutions ablate the donor (GT) or acceptor (AG) dinucleotides, which are absolutely required at the intron–exon boundaries for normal splicing and thereby completely block production of mature

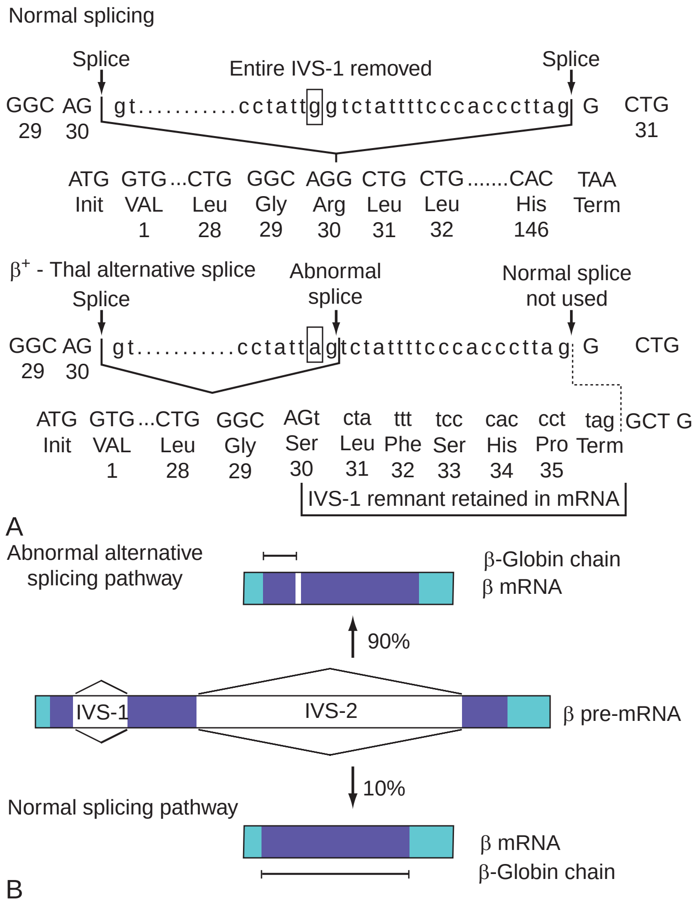

Normal splicing
Splice
Entire IVS-1 removed
Splice
GGC AG | gt...........cctattggtctattttcccaccttag | G CTG
29 30
31
ATG GTG...CTG GGC AGG CTG CTG.......CAC TAA
Init VAL Leu Gly Arg Leu Leu His Term
1 28 29 30 31 32 146
β+ - Thal alternative splice
Abnormal splice
Normal splice not used
Splice
GGC AG | gt...........cctattagtctattttcccaccttag G CTG
29 30
ATG GTG...CTG GGC AGt cta ttt tcc cac cct tag GCT G
Init VAL Leu Gly Ser Leu Phe Ser His Pro Term
1 28 29 30 31 32 33 34 35
IVS-1 remnant retained in mRNA
A
Abnormal alternative splicing pathway
β-Globin chain
β mRNA
90%
IVS-1
IVS-2
β pre-mRNA
10%
Normal splicing pathway
β mRNA
β-Globin chain
B

Figure 41.2 β+-THALASSEMIA ARISING FROM ALTERNATIVE MRNA SPLICING CAUSED BY A MUTATION ACTIVATING A CRYPTIC SPLICING SITE. (A) The G→A mutation is shown enclosed in *squares* located near the 3′ end of intron 1 (IVS-1); it creates a sequence motif closely mimicking a pre-mRNA acceptor splice site. The product of the alternative splicing event is also shown. Note that use of the activated cryptic site generates a mature mRNA that contains an in-frame termination codon and therefore does not encode a functional β-globin chain. (B) Diagram of the means by which use of the cryptic splice site 90% of the time results in only 10% of the mRNA precursor molecules to be spliced normally into translatable mature mRNA, thus causing β+-thalassemia. *mRNA*, Messenger ribonucleic acid. *((A) From Benz EJ Jr. The hemoglobinopathies. In: Kelly WN, DeVita VT, eds. Textbook of Internal Medicine. Philadelphia: JB Lippincott; 1988:1423. (B) Adapted from Bunn HF, Forget BG. Hemoglobin: Molecular, Genetic and Clinical Aspects. Philadelphia: WB Saunders; 1986.)*

functional messenger RNA. Thus, no β-globin can be synthesized (β^0-thalassemia). Other mutations alter the consensus sequences that surround the GT- and AG-invariant dinucleotides and decrease the efficiency of normal splicing signals by 70% to 95%, resulting in β$^+$-thalassemia; some consensus mutations even abolish splicing completely, causing β°-thalassemia. A third type of splicing aberration results from mutations that are not in the immediate vicinity of a normal splice site. These alter regions within the gene, called *cryptic splice sites*, which resemble consensus splicing sites but do not normally sustain splicing (see Fig. 41.2). The mutations activate the site by supplying a critical GT or AG nucleotide or by creating a sufficiently strong consensus signal to stimulate splicing at that site 60% to 100% of the time. The activated cryptic sites usually generate an abnormally spliced, untranslatable mRNA species. Only 10% to 40% of the mRNA precursors are thus spliced at the normal sites, which causes β$^+$-thalassemia of variable severity. The mutation responsible for the most common form of β-thalassemia among Greeks and Cypriots (Fig. 41.3) activates a cryptic splice site near the 3′ end of the first intron (position 110).[25,26] The determinants that dictate the degree to which each mutation alters splice site use remain largely unknown.

Translation

Mutations that abolish translation occur at several locations along the mature mRNA and are very common causes of β-thalassemia (see Fig. 41.1 and Table 41.1). The most common form of β°-thalassemia in Sardinians results from a base substitution in the gene that changes the codon encoding the 39th amino acid of the β-globin chain from CAG, which encodes glutamine to TAG, whose equivalent (UAG) in mRNA specifies termination of translation (see Fig. 41.3).[27,28] A premature termination codon totally abrogates the ability of the mRNA to be translated into normal β-globin. Premature translation termination also results indirectly from frameshift mutations (i.e., small insertions or deletions of a few bases, other than multiples of three, that alter the phase or frame in which the nucleotide sequence is read during translation).[28] An in-phase premature termination codon is usually encountered within the next 50 bases downstream from a frameshift.

Other Sites

Rare mutations that affect gene function by intriguing mechanisms have been described. An extremely large deletion of the β-globin gene cluster has been described that removes the ε-, γ-, and δ-genes.[29] The patient has a severe β-thalassemia phenotype, but the β-globin gene and 500 bases of adjacent 5′ and 3′ DNA have an entirely normal nucleotide sequence. The β-gene functions normally in surrogate cells. The important aspect of this deletion is that it removes the critical locus control region located thousands of bases upstream from the beginning of the globin gene cluster at the 5′ end of the ε-globin gene; loss of this region severely impairs β-gene expression.[18,30] A number of additional deletions involving the locus control region and various portions of the β-gene cluster, but sparing the β-gene itself, have the same phenotype.[2,6,18–20]

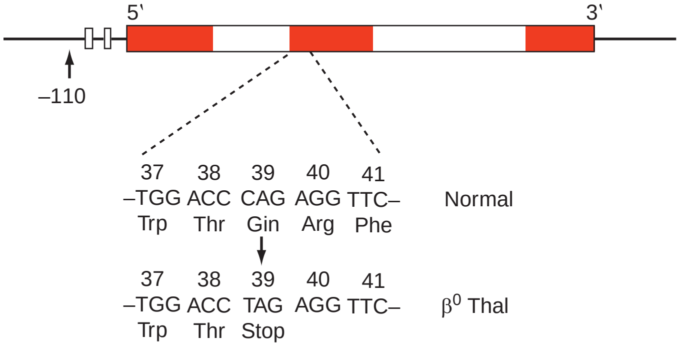


Figure 41.3 β^0-THALASSEMIA ARISING FROM A MUTATION CHANGING AN AMINO ACID CODON TO A TERMINATION CODON (NONSENSE MUTATION). *(Adapted from Takeshita K, Forget BG, Scarpa A, Benz EJ Jr. Intranuclear defects in β-globin mRNA accumulation due to a premature translation termination codon. Blood. 1984;64:13.)*

In other cases of β-thalassemia, the β-gene and adjacent DNA are structurally normal, and the basis of abnormal gene expression is unknown.[21]

Relationship Between Specific Mutations and Clinical Severity

The relationship between an individual mutation and the clinical severity of the β-thalassemia phenotype associated with that particular mutation is complex.[21] For example, the A to G mutation at position 34 of the β-gene promoter commonly encountered in patients of African origin is associated with a different clinical severity than that found in Chinese patients inheriting the same mutation.[31] Clearly, the genetic "context" of the mutation is different in the two populations. The mutant β-globin gene in the two different racial groups probably arose in different chromosome backgrounds that have different potentials for γ-gene expression. Multiple forms, or haplotypes, of normal non–α-globin gene clusters exist in various human populations. These were defined by the patterns of restriction fragment length polymorphisms detected when DNA is digested with restriction endonucleases and analyzed by Southern gene blotting for the fragments bearing the non–α-globin genes.[27] The gene cluster is now readily analyzed by direct genomic sequencing. Haplotypes differ according to whether each restriction site is present or absent along the gene cluster. More than 12 haplotypes have been defined by examination of several restriction sites located along the cluster that are present or absent in a polymorphic manner in normal individuals.[27] The clinical variability encountered in two different groups bearing identical primary mutations correlates best with the haplotype or chromosome background on which the mutation is inherited. The differences in physiologically important functions among haplotypes that modulate severity remain unknown, but a possible explanation lies in the variable abilities of the γ-globin genes on different chromosomes to respond to severe erythroid stress by increased expression during postnatal life. The β-globin genes carried on some haplotypes differ in the degree to which they can respond in this manner.[32] Because HbF synthesis reduces the severity of β-chain hemoglobinopathies,[2] the level of γ-gene expression from a given chromosome can play an important modulating role.

Pathophysiology

Erythropoiesis is essentially a two-phase process, erythropoietin (EPO) driving the initial proliferative phase, leading to stimulation of the stem cell toward the erythroid lineage, and proliferation through the BFU-E and CFU-E phases. The second stage is one of differentiation and maturation, with the proerythroblasts moving through the different erythroblast phases, into reticulocytes and eventually mature red cells (Fig. 41.4).

As described, the basic molecular abnormality in β-thalassemia is the imbalance between alpha and beta globin production, with reduction in beta globin production and thus a decrease in HbA ($\alpha_2\beta_2$) production. The ratio of β- to α-globin is typically close to 1. When abnormal, this ratio has a direct correlation with clinical severity in β-thalassemia patients.[33] In β-thalassemia heterozygotes, β-globin synthesis is approximately half-normal, with the ratio of β- to α-chain mRNA (β/α ratio) of 0.5 to 0.7 (normal = 1.0). In homozygotes for β^0-thalassemia, which account for approximately one-third of patients, β-globin synthesis is absent. β-globin synthesis is reduced

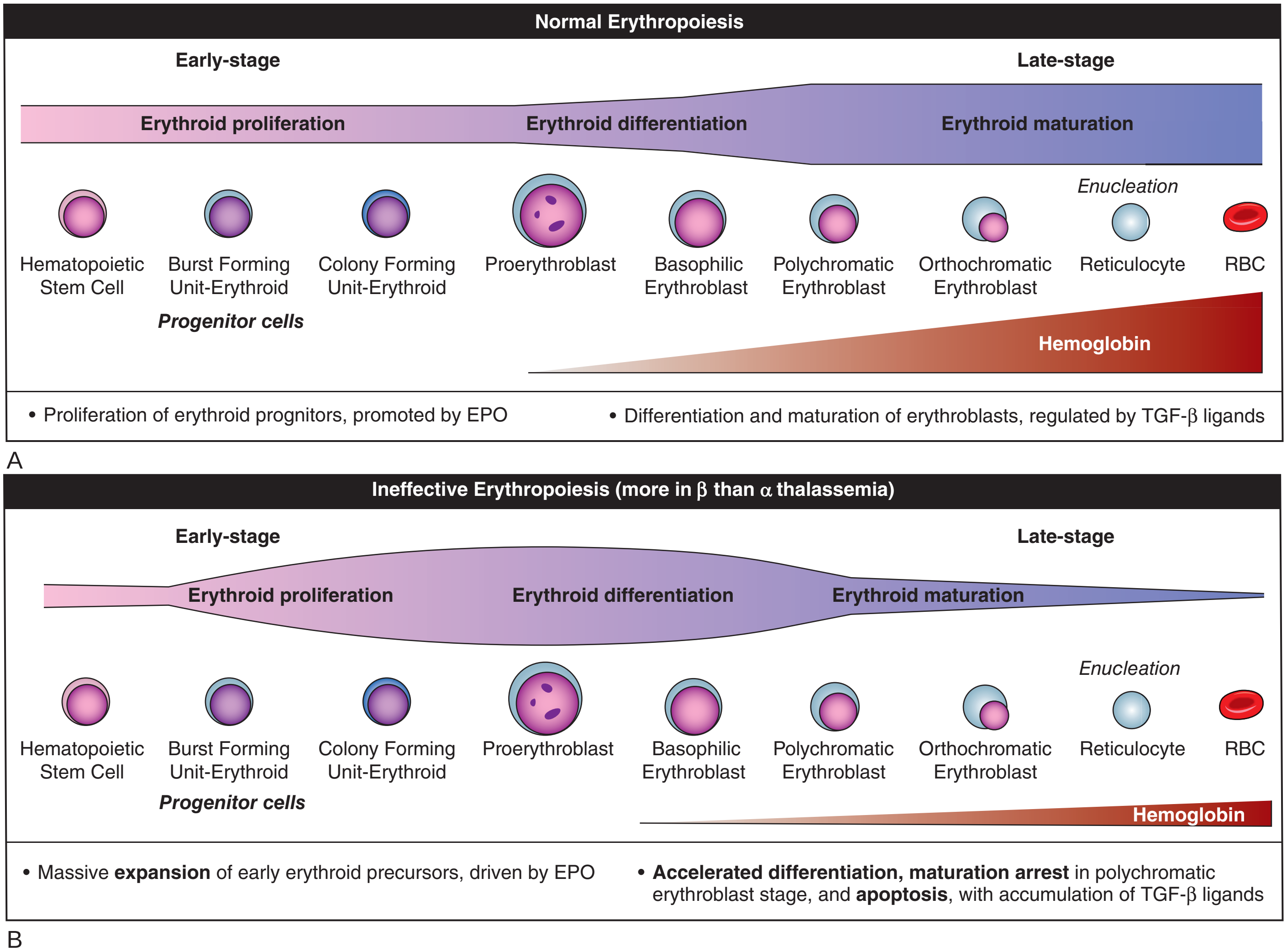

Normal Erythropoiesis
Early-stage
Late-stage
Erythroid proliferation
Erythroid differentiation
Erythroid maturation
Enucleation
Hematopoietic Stem Cell
Burst Forming Unit-Erythroid
Colony Forming Unit-Erythroid
Proerythroblast
Basophilic Erythroblast
Polychromatic Erythroblast
Orthochromatic Erythroblast
Reticulocyte
RBC
Progenitor cells
Hemoglobin
• Proliferation of erythroid prognitors, promoted by EPO
• Differentiation and maturation of erythroblasts, regulated by TGF-β ligands
A
Ineffective Erythropoiesis (more in β than α thalassemia)
Early-stage
Late-stage
Erythroid proliferation
Erythroid differentiation
Erythroid maturation
Enucleation
Hematopoietic Stem Cell
Burst Forming Unit-Erythroid
Colony Forming Unit-Erythroid
Proerythroblast
Basophilic Erythroblast
Polychromatic Erythroblast
Orthochromatic Erythroblast
Reticulocyte
RBC
Progenitor cells
Hemoglobin
• Massive expansion of early erythroid precursors, driven by EPO
• Accelerated differentiation, maturation arrest in polychromatic erythroblast stage, and apoptosis, with accumulation of TGF-β ligands
B

Figure 41.4 NORMAL AND INEFFECTIVE ERYTHROPOIESIS. (A) Schema of normal erythropoiesis, showing proliferation, differentiation, and maturation phases. (B) Schema of ineffective erythropoiesis, showing an expanded proliferative phase but a block of differentiation and maturation. *EPO*, Erythropoietin.

to 5% to 30% of normal levels in β^+-thalassemia homozygotes or β^+/β°-thalassemia compound heterozygotes, which together account for approximately two-thirds of cases.[2] The excess α-globin chains may be stabilized by alpha Hb stabilizing protein, which is a chaperone-like protein that assists in binding free α-globin chains. Higher levels of alpha Hb stabilizing protein result in more IE, and thus a more severe clinical phenotype.[33]

The decreased or absent synthesis of HbA ($\alpha_2\beta_2$) results in the production of smaller red cells and depending on the severity of the deficiency, hypochromia as well. RBCs are always microcytic in the thalassemias across the spectrum, and usually hypochromic though not so much in heterozygotes. γ-Chain synthesis is partially reactivated, and there is some upregulation of δ chain synthesis, though not enough to replace the deficiency of β-chains, and thus the red cells may contain relatively large proportions of HbF ($\alpha_2\gamma_2$) and some HbA2 ($\alpha_2\delta_2$).[2]

Individuals inheriting two β-thalassemic alleles experience a more profound deficit of β-chain production. Little or no HbA is produced, and importantly, the imbalance of α- and β-globin production is far more severe (Fig. 41.5). In the BM, the limited capacity of the red cell precursors to proteolyze the excess α-globin chains, a capacity that probably exerts a protective effect in heterozygous β-thalassemia, is overwhelmed in homozygotes. Free α-globin accumulates, and unpaired α-chains aggregate and precipitate to form inclusions called Fessas bodies and hemichromes with the addition of heme iron, which cause oxidative membrane damage leading to

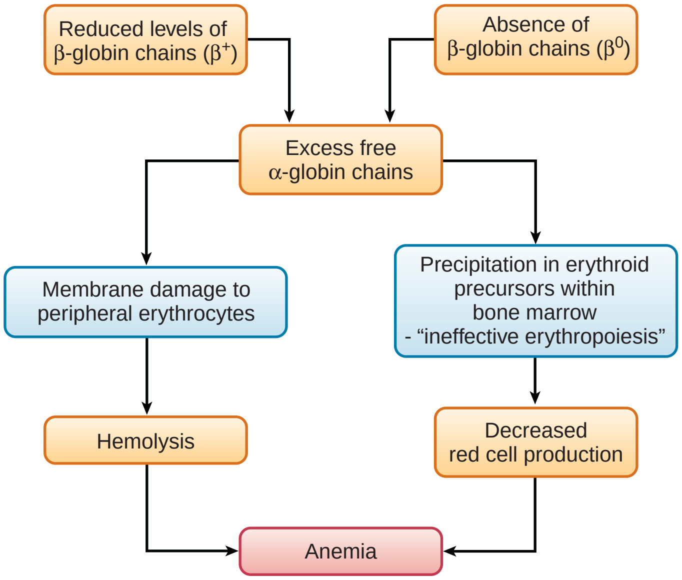

Reduced levels of β-globin chains (β+)
Absence of β-globin chains (β0)
Excess free α-globin chains
Membrane damage to peripheral erythrocytes
Precipitation in erythroid precursors within bone marrow - "ineffective erythropoiesis"
Hemolysis
Decreased red cell production
Anemia

Figure 41.5 FEATURES OF INEFFECTIVE ERYTHROPOIESIS (IE). Intramedullary apoptosis of abnormally maturing erythroid precursors and a reduction in the lifespan of red cells produced, lead to anemia, the hallmark of IE.

apoptosis and destruction of immature developing erythroblasts in the BM (intramedullary hemolysis).[34–36] Consequently, relatively few of the erythroid precursors undergoing erythroid maturation in the BM survive long enough to be released into the bloodstream as erythrocytes, leading to anemia. Thus, in β-thalassemia, the defect in the erythropoietic pathway is one of impaired differentiation and maturation of the developing erythroid precursors, beginning at the time globin genes begin expression in the proerythroblast-basophilic erythroblast stages. Erythropoiesis is *ineffective*, and the severity of this IE worsens as the imbalance between β- and α-globin synthesis deepens. The occasional erythrocytes that are formed during erythropoiesis bear a burden of inclusion bodies. The reticuloendothelial cells in the spleen, liver, and BM remove these abnormal cells prematurely, which reduces RBC survival as a consequence of this hemolytic anemia.

IE is thus the hallmark of β-thalassemia, triggering a cascade of compensatory mechanisms and resulting in clinical sequelae such as erythroid BM expansion, extramedullary hematopoiesis (EMH), and splenomegaly.[37] The master iron regulatory hormone hepcidin plays an integral and critical role in the pathophysiology of the disease as well (see Chapters 35–37).[38,39] Erythroferrone (ERFE) is a protein which is produced during erythropoiesis, which has the effect of reducing hepcidin production, likely through the BMP/SMAD signaling pathway.[40] During stress erythropoiesis, increased ERFE results in decreased hepcidin which in turn leads to increased iron absorption through the transporter ferroportin at the basolateral side of the intestinal enterocyte, the body mistakenly sensing anemia as being a result of iron deficiency. The greater the erythropoietic activity, the higher ERFE levels rise to, and the greater the suppression of hepcidin. In thalassemia, where IE is a significant feature, ERFE levels are high and therefore hepcidin levels are inappropriately low. Such patients become iron overloaded as a result of increased iron absorption even in the absence of transfusional iron loading. The critical role of hepcidin in iron trafficking is also a factor in iron toxicity in thalassemia. In the absence of IE, hepcidin levels are increased when there is iron overload, thus reducing iron absorption, and also safely sequestering iron in storage sites and preventing deposition in vulnerable tissues. The relatively lower levels of hepcidin driven by ERFE in ineffective erythropoiesis result in iron being able to be more easily redistributed, leading to organ toxicity. When hepcidin production is inappropriately low as a result of increased IE, tissues such as the heart and endocrine organs are exposed to more circulating iron and this results in uptake and organ dysfunction as a result of iron toxicity.

As a result of the anemia, there is increased secretion of EPO, and further proliferation, once again, without maturation. Accumulation of molecules belonging to the transforming growth factor-beta (TGF-β) superfamily of ligands has been described, which inhibits maturation and differentiation. Trapping these ligands has been the focus of a therapeutic intervention to try and improve IE by removing the block on maturation, and promoting more effective erythropoiesis.[41,42] Massive BM expansion does occur, but very few erythrocytes are actually supplied to the circulation. The BM becomes packed with immature erythroid precursors, which die from their burden of precipitated α-globin chains before they reach the reticulocyte stage. Profound anemia persists, driving erythroid hyperplasia to still higher levels. In some cases, erythropoiesis is so exuberant that masses of extramedullary erythropoietic tissue form in the chest, abdomen, or pelvis.

Clinical Manifestations

Anemia and IE are central to the pathophysiology and disease manifestations of thalassemia, and the clinical manifestations are a cascade that results from these (Fig. 41.6). However, there is tremendous heterogeneity in the spectrum of clinical features in the various thalassemia syndromes, determined by the genotype, genetic modifiers, age at presentation, and diagnostic and treatment modalities employed. Two siblings inheriting identical thalassemia mutations sometimes exhibit markedly different degrees of anemia and erythroid hyperplasia. Many factors contribute to this clinical heterogeneity. Individual alleles vary with respect to severity of the biosynthetic lesion. Other modifying factors ameliorate the burden of unpaired α-globin. Levels of γ-globin expression are widely variable in β-thalassemia, with greater production of γ-globin able to reduce the excess of

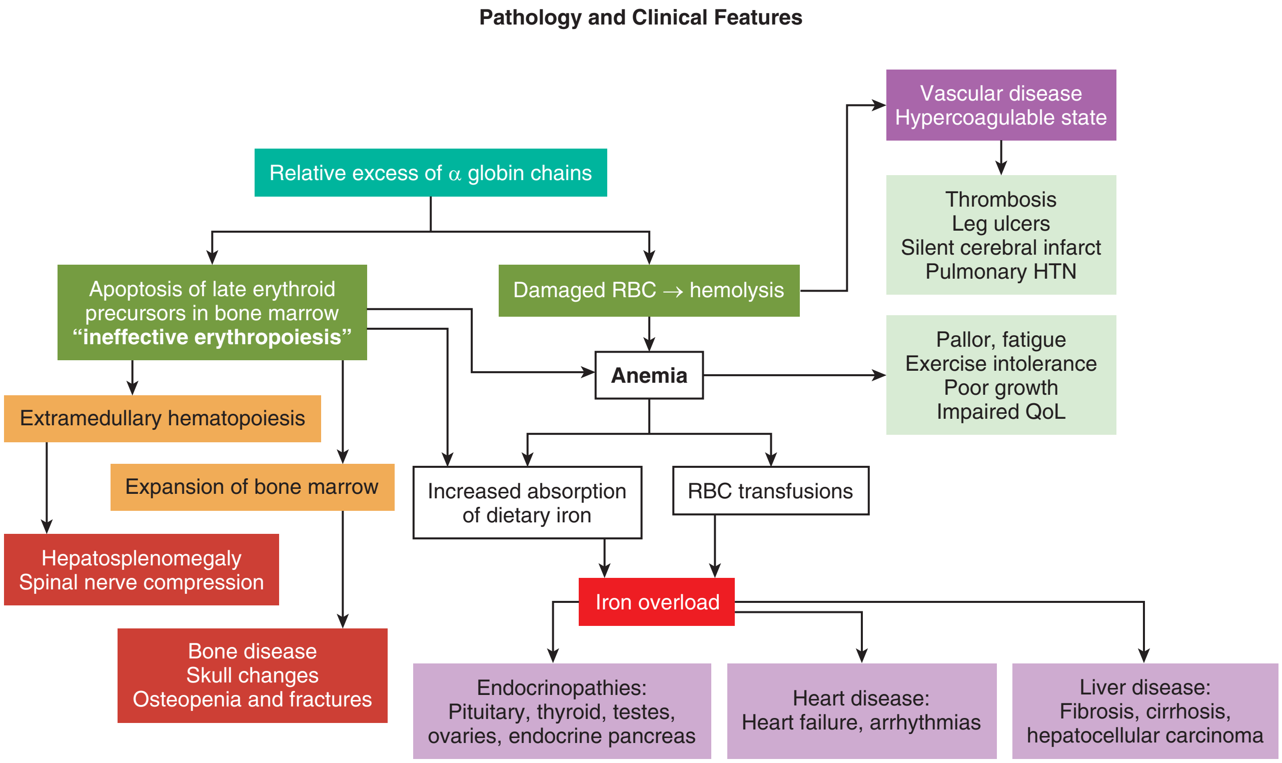

Pathology and Clinical Features
Relative excess of α globin chains
Apoptosis of late erythroid precursors in bone marrow "ineffective erythropoiesis"
Damaged RBC → hemolysis
Vascular disease Hypercoagulable state
Thrombosis Leg ulcers Silent cerebral infarct Pulmonary HTN
Anemia
Pallor, fatigue Exercise intolerance Poor growth Impaired QoL
Extramedullary hematopoiesis
Expansion of bone marrow
Increased absorption of dietary iron
RBC transfusions
Hepatosplenomegaly Spinal nerve compression
Iron overload
Bone disease Skull changes Osteopenia and fractures
Endocrinopathies: Pituitary, thyroid, testes, ovaries, endocrine pancreas
Heart disease: Heart failure, arrhythmias
Liver disease: Fibrosis, cirrhosis, hepatocellular carcinoma

Figure 41.6 CLINICAL FEATURES OF SEVERE THALASSEMIA AS A RESULT OF INEFFECTIVE ERYTHROPOIESIS AND ANEMIA. *HTN*, Hypertension; *QoL*, quality of life; *RBC*, red blood cell.

α-globin. Theoretically, patients may also vary in their ability to solubilize unpaired globin chains by proteolysis. Occasional heterozygous patients have had more severe anemia than expected, possibly because of defects in these proteolytic systems or because of the type of thalassemic mutation. Inheritance of more than the usual complement of α-globin genes may also increase with severity of β-thalassemia because of additional production of unpaired α-globin chains. All of these factors emphasize the essential role of α-globin inclusions in the pathophysiology of β-thalassemia.

In general, in patients who have not been transfused regularly, manifestations are mainly from the effects of IE, with bone changes and vasculopathy dominating, but iron overload remaining an important feature as well. In appropriately transfused individuals, the effects of the now suppressed IE are not prominent, and it is more the effects of iron overload that present as complications.

Beta-Thalassemia Major

Homozygotes or compound heterozygotes with the more severe genotypes are born, usually without prenatal complications, with mild anemia and microcytosis compared with newborns without these mutations. Nevertheless, deficient β-chain synthesis can be demonstrated at birth on the newborn screen, which would show an absence or marked reduction in HbA. As the Hb declines to the physiologic nadir, which is reached sooner as a result of the relative anemia at birth, erythropoiesis resumes under the drive of EPO. However, since the marrow is not able to produce adequate red cells, the Hb level continues to drop leading to further EPO production and BM hyperplasia, and persistent red marrow in bones which would otherwise have converted to yellow marrow. In addition, normally quiescent erythroid precursors in the liver and spleen are activated, with resulting EMH. Clinical manifestations usually emerge as the anemia becomes more pronounced. The diagnosis is almost always evident by 2 years of age.[43] Pallor, irritability, failure to thrive, growth retardation, abdominal swelling caused by enlargement of the liver and spleen, and jaundice are the usual presenting features.[44] Facial and skeletal changes caused by BM expansion develop as the anemia worsens, with frontal and parietal bossing of the skull bones, persistent marrow in the maxilla resulting in underdevelopment of normal sinuses (Fig. 41.7).

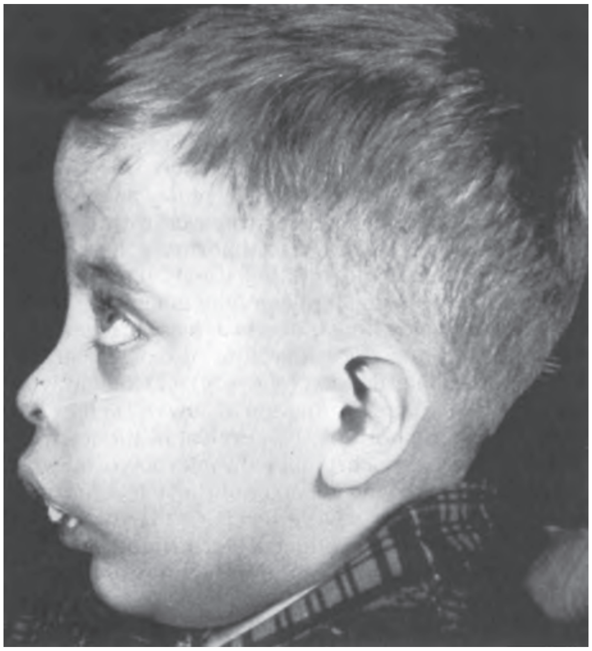


Figure 41.7 THALASSEMIC FACIES. See text for description. *(From Jurkiewicz MJ, Pearson HA, Furlow LT Jr. Reconstruction of the maxilla in thalassemia. Ann N Y Acad Sci. 1969;165:437.)*

Untreated patients die in late infancy or early childhood as a consequence of severe anemia. In a retrospective review from Italy, the average survival of children with untreated thalassemia major was less than 4 years; approximately 80% died in the first 5 years of life.[45] Patients who receive transfusions sporadically may live somewhat longer than untransfused patients, but their quality of life is extremely poor as a result of both the chronic anemia and the IE. The low Hb level and massive organomegaly are usually disabling, and the changes in the facial bones are disfiguring. After 10 to 20 years of weakness, stunted growth, and impaired activity, the undertransfused patients usually succumb to congestive heart failure.

This disastrous symptom constellation, so prevalent in the past, is now rare in most industrialized countries. Nonetheless, the clinical manifestations and complications of untreated or undertreated β-thalassemia major illustrate the principles of the pathophysiology. Furthermore, these descriptions accurately characterize the disease that is still prevalent in many underserved parts of the world.

Beta-Thalassemia Intermedia

Individuals with less severe genotypes have a milder course than that described above, but always have some pathologic features of the disease unlike heterozygotes who are mostly completely asymptomatic. However, this intermediate syndrome has a wide spectrum of severity based on the degree of IE and its consequences, and thus individuals may have mild symptoms to more complex presentations which may move them along the spectrum toward transfusion dependence. Approximately 10% of patients with homozygous β-thalassemia may exhibit a phenotype characterized by intermediate hematologic severity.[2–4,6] The balance of globin chain synthesis is better than in typical thalassemia major because of a less severe defect in β-globin chain synthesis, a decrease in α-globin chain synthesis along with decreased β-globin chain synthesis, or an increase in γ-globin chain synthesis. For example, homozygous β-thalassemia in African Americans, Portuguese, and other populations may be relatively mild, at least for the first two decades of life.[46–48] Homozygotes or mixed heterozygotes for forms of β-thalassemia associated with normal HbA_2 and normal HbF (so called "silent carrier" mutations) also tend to have mild to moderate disease.[49,50]

Unfortunately, many individuals with milder presentations are not appropriately diagnosed, being treated as chronic or refractory iron deficiency instead. More severely affected individuals have more marked IE, bone changes, and more severe anemia, with fatigue, abnormal bones of the skull, hepatosplenomegaly, and pathologic fractures. Individuals may present at a variable age, some in the first decade of life, some in the second, and some as late as the third. Patients do not remain static on the spectrum from thalassemia intermedia to thalassemia major. Complications in these individuals are primarily related to IE, and depending on the severity of the IE, may be mild or more severe. These complications will be described later in this chapter. Those with severe IE and anemia would likely benefit from regular transfusion. However, many such individuals either refuse regular transfusions for fear of transfusion-related infections or iron overload, or are not able to get them for logistic and economic reasons. These individuals may have growth retardation, chronic fatigue and malaise, bone pain, and worsening splenomegaly and still develop iron overload. Splenectomy is often performed when there is a component of hypersplenism contributing to worsening anemia. Gallbladder disease from increased red cell turnover and the development of pigment gallstones is quite common. Progressive EMH may result in paraspinal nodules as described previously. Particularly in splenectomized patients, vascular damage from fragmented and abnormal red cells, complicated by thrombocytosis may lead to clinical manifestations including the development of chronic, non-healing leg ulcers from poor perfusion and the effects of arterial endothelial damage and a possible hypercoagulable state leading to cerebral infarcts and development of pulmonary hypertension. Some individuals may remain in the intermedia spectrum until adulthood, receiving very few transfusions for stress situations such as severe infections or surgery, when they may have a drop in Hb and worsening symptoms related to anemia.

Clinically significant iron loading as a result of increased absorption is seen even in patients with infrequent transfusions (see Chapters 35 and 36). Iron overload may result in diabetes and endocrine disturbances, typically by fourth decade of life, though cardiac deposition is relatively rare in true thalassemia intermedia individuals.

Individuals with the most severe form of the disease will usually be placed on a regimen of regular transfusions. In appropriately transfused individuals who have suppressed IE, complications are now mostly related to those resulting from transfusional iron overload.

Beta-Thalassemia Minor

Heterozygotes for β-thalassemia are usually asymptomatic. Often the diagnosis is made as a result of their family history, a prenatal screen, as part of the workup for refractory iron deficiency anemia, or by the chance finding of the characteristic hematologic changes during a routine study. In general, thalassemia trait carries no direct clinical symptoms or pathologic consequences for the patient. Usually, they have adapted to a mild microcytic anemia, with no symptoms, no overt evidence of IE or EMH. A controlled study reported that individuals with the β-thalassemia trait suffer from fatigue and other symptoms indistinguishable from those with mild anemias from other causes. There was no difference in the frequency of palpable splenomegaly between the thalassemic and control groups.[51] There are some reports in the literature of individuals who have symptoms from anemia and may even have mild splenomegaly, but in the absence of genetic testing, it is not clear that these were all heterozygotes only. In situations in which there is an adaptive increase in Hb, such as endurance activities like running marathons, or living at high altitude, individuals may have some difficulty. The diagnosis of thalassemia trait assumes particular importance in women who are pregnant or considering pregnancy because of the potential for having a child with thalassemia major. During pregnancy, the anemia of thalassemia trait often becomes more severe, but transfusions are rarely necessary. Increased folic acid supplementation may improve Hb during this period. Because iron deficiency may occur during pregnancy, iron supplementation has been advised to avoid compounding the causes of anemia.[52,53] Studies have suggested there may be an increased tendency for gallstones and cholecystitis, but otherwise this condition should be largely asymptomatic.[54] Some β-thalassemia carriers have increased iron stores, although this is most often a result of inappropriate long-term iron therapy based on a misdiagnosis. In countries where there is a relatively high frequency of genetic determinants for hemochromatosis, the possibility of their coinheritance should be borne in mind if a patient with β-thalassemia trait with an unusually high plasma iron or serum ferritin level is encountered. Iron supplementation is only necessary if there is true iron deficiency, and routine supplementation to raise the Hb level is futile and should not be done. These individuals should seek genetic counseling when ready for childbearing.

Laboratory Findings

In order to make the diagnosis, the clinician must consider the ethnicity, family history, clinical presentation, physical exam as well as laboratory parameters. The laboratory diagnosis of the thalassemia syndromes has traditionally been based on careful interpretation of the complete blood count, including the red cell indices, the review of the peripheral blood smear, and characterization of the type of Hb produced. Hb levels vary based on the severity of the underlying genotype, but the anemia is always microcytic with low red cell indices. The smear shows variable hypochromia and the presence of abnormal shapes and sizes of cells, all more so in individuals with increasing severity of anemia. There is a reduction or absence of production of HbA, and thus an increase in HbF and HbA2 on Hb fractionation. Special staining, α-, β-, and γ-globin assessments are performed in some sophisticated laboratories, along with some degree of genotyping, especially for known mutations. In the developed world, it is now possible to characterize the disease more completely, including all of the possible genetic modifiers with the easy availability of sophisticated DNA testing, including whole exome or genome sequencing. While this is not routinely done for all patients, especially those with mild clinical manifestations, in more complex cases, it may be used to fully explain the clinical picture. There may also be some predictive value in genotyping for some of the newer therapies, as will be described later. Family members should also have testing if they have not been tested previously.

The diagnosis is usually fairly easy to make, though the following may be considered if it is not immediately clear:

- congenital sideroblastic anemia, one of the rarer instances when a BM exam or specific genetic testing may be needed to make the diagnosis
- juvenile chronic myelogenous leukemia may be superficially considered, because of the elevated HbF levels, but other testing is quite straightforward to make this diagnosis

Homozygous β^0 Thalassemia

Infants are born with relative anemia and microcytosis. The Hb level decreases progressively during the first months of life. When the child becomes symptomatic, the Hb level may be as low as 3 to 4 g/dL. RBC morphology is strikingly abnormal, with many microcytes, bizarre poikilocytes, teardrop cells, and target cells (Fig. 41.8). A characteristic finding is the presence of extraordinarily hypochromic, often wrinkled and folded cells (leptocytes) containing irregular inclusion bodies of precipitated α-globin chains. Nucleated RBCs are frequently present in peripheral circulation. The reticulocyte count is 2% to 8%, lower than would be expected in view of the extreme erythroid hyperplasia and hemolysis. The low count reflects the severity of intramedullary erythroblast destruction. The white blood cell count is elevated. A moderate polymorphonuclear leukocytosis and normal platelet count are typical unless hypersplenism has developed. The BM (not necessary for diagnosis) exhibits marked hypercellularity caused by erythroid hyperplasia. The RBC precursors show a maturational arrest with defective hemoglobinization and reduced amounts of cytoplasm.

The osmotic fragility is strikingly abnormal. The RBCs are so markedly resistant to hemolysis in hypotonic sodium chloride solution that some are not entirely hemolyzed even in distilled water. Even before transfusion therapy is initiated, the serum iron and

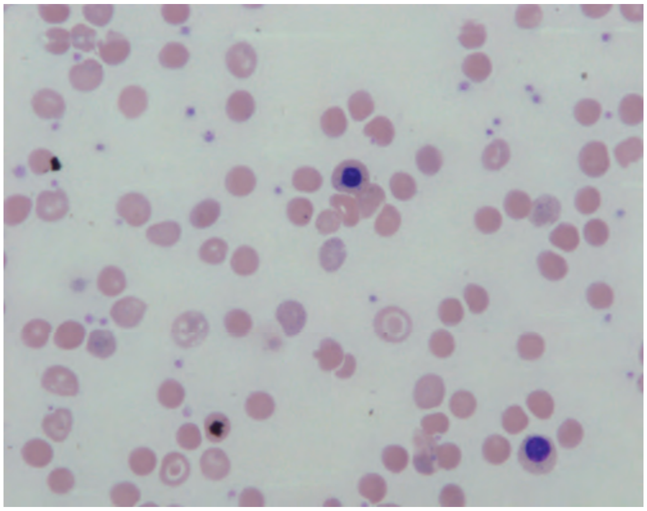


Figure 41.8 MORPHOLOGIC APPEARANCE OF THE PERIPHERAL BLOOD FILM IN A CASE OF SEVERE β-THALASSEMIA. Note the bizarre cells, the hypochromia, nucleated red blood cells, target cells, and polychromasia.

transferrin saturation and serum ferritin may already be increased as a result of increased iron absorption,[55] thus ruling out iron deficiency as an alternate diagnosis.

The Hb profile reveals predominantly HbF. In patients with homozygous β^0-thalassemia, HbA is absent throughout life, unless transfused. In β^+-thalassemia HbA may be very low or undetectable in the newborn but present in reduced amounts in later life. The levels of HbA2 in thalassemia major are variable, probably because of increased numbers of F cells that have a decreased HbA2 content.[2] Other biochemical abnormalities of the RBC in cases of thalassemia major include a postnatal persistence of the RBC in antigen and a decrease of RBC carbonic anhydrase; these findings are probably also caused by the elevated levels of circulating F cells.

The intraerythrocytic inclusions in the peripheral blood cells of patients with thalassemia, termed Fessas bodies,[56] are especially prominent after splenectomy. These inclusions, best seen by staining with supravital staining (Brilliant Cresyl Blue) or by phase microscopy, are aggregates of precipitated, denatured α-chains.[57] They are also found in large numbers within erythroid precursors in the BM.

RBC survival in cases of thalassemia major is variable but usually markedly decreased. The ^{53}Cr half-life ranges between 6.5 and 19.5 days compared with the normal half-life of 25 to 35 days,[35] but there is some variability with cells containing more fetal Hb surviving longer. Increased plasma iron turnover and poor use of radiolabeled iron provide further evidence of IE.[35]

Unconjugated bilirubin levels are elevated, in the range of 2.0 to 4.0 mg/dL at the time of diagnosis but may rise substantially as the anemia worsens in the absence of transfusion. Serum aspartate aminotransferase levels are frequently increased at diagnosis because of hemolysis. Alanine aminotransferase levels are usually normal before transfusion therapy but may rise subsequently because of iron-induced hepatic damage or viral hepatitis. Lactate dehydrogenase levels are markedly elevated as a consequence of IE. Haptoglobin and hemopexin are reduced or absent.[58]

Hypersplenic patients may have low white cell and platelet counts, and after splenectomy, leukocytosis and thrombocytosis are common. Asplenic individuals usually have large, flat "pseudo-macrocytes," or leptocytes that appear larger in two dimensions because they are so devoid of Hb that they collapse flat on the slide like a deflated balloon, and small, deformed microcytes, as well as markedly increased nucleated red cell counts, and the white cell count frequently has to be corrected for their presence. Staining of the blood with methyl violet, particularly in splenectomized subjects, reveals stippling or ragged inclusion bodies in the red cells.

When transfusions are initiated, the Hb level rises to the mild to moderate anemia range, being higher in patients on regular transfusions and generally lower in those receiving on-demand transfusions. The mean corpuscular volume (MCV) and Hb fractionation reflects transfused blood as well. Iron parameters are altered to reflect iron overload as transfusions continue. Serum iron and transferrin saturation, as well as ferritin levels are elevated, and remain so, with the ferritin level declining in response to chelation therapy.

Transfusion complications include the development of auto- or alloantibodies with a positive DAT and antibody screen, iron-related organ dysfunction, hepatic, and endocrine. All of these parameters must be regularly monitored in transfusion-dependent patients.

Homozygous or Compound Heterozygous β^+ Thalassemia

In these individuals, the Hb levels are generally higher, but otherwise the hematologic changes are similar. The more severe the anemia, with greater IE, the more the peripheral smear resembles that of a thalassemia major patient prior to starting transfusions, as noted above. Examination of the BM, if performed, would confirm IE to a variable degree, with more severe patients having the picture of untreated thalassemia major. Hb fractionation shows a variable amount of HbA depending on the specific genotype, and the rest is HbF and A2, or in cases of HbE-thalassemia, a proportion of HbE. Even in the absence of transfusions, iron studies will show a steady rise in serum iron, transferrin saturation, and ferritin as a result of increased intestinal absorption, the rate of rise determined by the degree of IE. With intermittent or regular transfusions, the iron levels will rise more rapidly, and follow the same course as individuals with thalassemia major.

Heterozygous β-Thalassemia

Inheritance of a single β-thalassemia allele usually results in a mild anemia. The classic picture of β-thalassemia minor is a mild microcytic anemia (Hb averages 1 or 2 g/dL lower than that seen in normal persons of the same age and gender, typically 9 to 11 g/dL, MCV 50 to 70 fL). The red cell count is usually normal or elevated. HbF levels decline more slowly than usual in the first year of life, and the diagnostic elevated HbA2 levels are established by approximately 6 months of age.[59–61] Strong intrafamilial correlations of both HbA2 and MCV are noted.[62,63] The HbA2 level is increased to 3.5% to 7%. The level of fetal Hb is elevated in approximately 50% of cases, usually to 1% to 3% and rarely to greater than 5%. The RBC count is increased or normal. The RBCs are characteristically hypochromic (mean corpuscular hemoglobin [MCH] <26 pg). Osmotic fragility is decreased; indeed, a one-tube osmotic fragility test has been used in the past for mass screening.[60] The smear shows varying numbers of target cells, poikilocytes, ovalocytes, and basophilic stippling (Fig. 41.9). The reticulocyte count is normal or slightly elevated. RBC survival is normal, iron utilization is decreased, and slight IE is present.[61] Iron studies are normal, unless concomitant iron deficiency is also present, in which case the transferrin saturation and ferritin are low. In this situation, the Hb is lower, as are the red cell indices, and

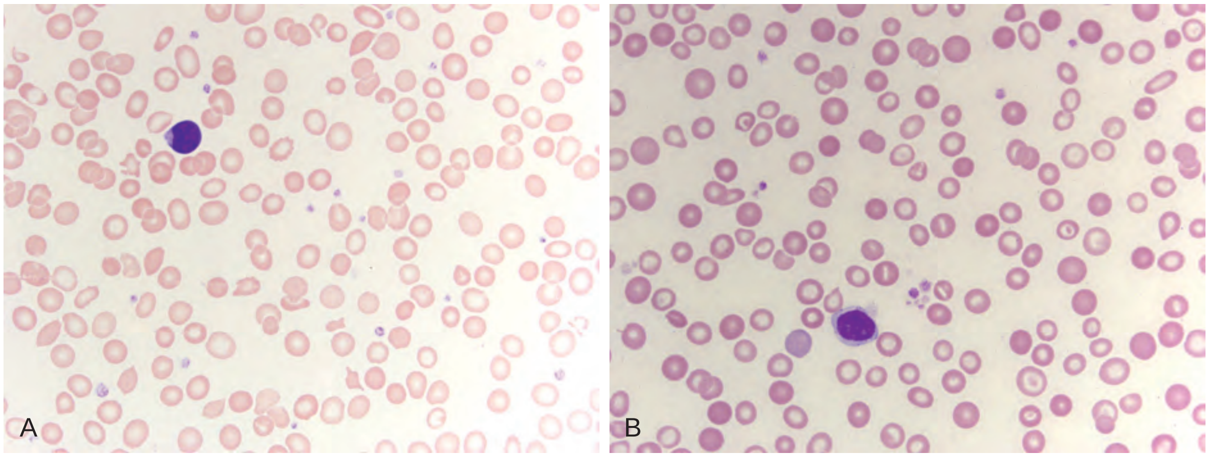


Figure 41.9 MORPHOLOGY OF THE PERIPHERAL BLOOD FILM IN A PATIENT WITH HETEROZYGOUS β-THALASSEMIA (A) AND A PATIENT WITH HETEROZYGOUS α-THALASSEMIA (B). Note the profound hypochromia and microcytosis and the many target cells. *(From Pearson HA, Benz EJ Jr. Thalassemia syndromes. In: Miller DR, Baehner RL, McMillan CW, eds. Smith's Blood Diseases of Infancy and Childhood. 5th ed. St. Louis: CV Mosby; 1984:439.)*

the red cell count. The BM (not usually done) in heterozygous β-thalassemia shows slight erythroid hyperplasia with rare red cell inclusions. Megaloblastic transformation as a result of folic acid deficiency occurs occasionally, particularly during pregnancy. A mild degree of IE is noted, but red cell survival is normal or nearly normal.

β-Thalassemia With Normal A2 Level

Rare forms of β-thalassemia are seen in which heterozygotes have a completely normal hemogram, or with mild microcytic anemia but with a completely normal Hb pattern on electrophoresis or high-pressure liquid chromatography (HPLC). This can be confused with the more common heterozygous α-thalassemia and may cause difficulties in genetic counseling and prenatal diagnosis. Based on hematologic studies, two main classes of "normal HbA2 β-thalassemia"—sometimes called types 1 and 2—are seen.[64]

Type 1, the "silent" form of β-thalassemia,[2] is characterized in heterozygotes by a normal hemogram—there is no anemia, and the MCV is normal. Although this condition can be partly identified by demonstrating a mild degree of globin-chain imbalance, with α-to-β synthesis ratios of approximately 1.5:1, it can only be diagnosed with certainty by DNA analysis. Compound heterozygotes for this condition and β^0-thalassemia have a mild form of β-thalassemia intermedia.

Normal HbA2 β-thalassemia type 2 in heterozygotes has a hematologic profile of mild microcytic anemia which is indistinguishable from typical β-thalassemia with elevated HbA2 levels.[64] The homozygous state has not been described. The compound heterozygous state for this gene and for β-thalassemia with raised HbA2 levels is characterized by a clinical picture of severe transfusion-dependent β-thalassemia.

Dominant β-Thalassemia

The clinical features of the dominant β-thalassemias resemble the features of thalassemia intermedia.[2] The blood picture shows the usual thalassemia changes, and there is moderate anemia and splenomegaly. The marrow shows erythroid hyperplasia with well-marked inclusion bodies in the red cell precursors, which may be seen in the peripheral blood after splenectomy. Hb analysis shows HbA and elevated HbA2, but the HbF level is not usually elevated much higher than that seen in β-thalassemia heterozygotes.

Other Laboratory Findings

Serum zinc levels may fall to abnormally low levels. A relationship between this finding and growth failure has been postulated but not established.[65,66] Low levels of plasma and leukocyte ascorbic acid are common in thalassemic patients because of increased metabolism of the vitamin to oxalic acid in the presence of iron overload.[67,68] Biochemical evidence of folic acid deficiency may occur as a result of excessive consumption secondary to increased requirements.[69,70] The serum levels of α-tocopherol are often reduced to less than 0.5 mg/dL, and increased RBC membrane lipid peroxidation has been described.[71–73]

Coagulation abnormalities consistent with liver disease (i.e., lowered levels of factors II, V, VII, IX, and X) may occur in older patients with hepatitis or iron-induced hepatic injury.[74] Only rarely are the abnormalities sufficient to require specific therapy. However, the combination of mild thrombocytopenia from hypersplenism and low coagulation factors and platelet dysfunction from liver disease may cause or aggravate bleeding.[75]

Numerous laboratory abnormalities reflect the accumulation of excessive iron and the consequences of iron-induced organ damage, and they are described in the following sections.

Treatment

The overall goals of management of the thalassemia syndromes are:

- To optimize the level of Hb, so as to facilitate growth and development in children, and overall activity levels in all, with or without transfusion.
- To minimize the complications of IE, by suppressing erythropoiesis, or facilitating erythroid maturation, particularly in those who are not regularly transfused.
- To prevent complications related to iron overload, especially in regularly transfused individuals.
- To provide a near-normal life expectancy with an optimized quality of life.

Transfusion and chelation therapy, described subsequently in detail, have ameliorated many of the most striking manifestations of the disease. Bone marrow transplantation (BMT) has allowed for the cure in some patients. Gene therapy and drugs mitigating the process by which ineffective erythropoiesis is aggravated by cytokines are emerging modalities. Current clinical management and associated clinical manifestations and complications have been reviewed in a number of publications.[2,76–81]

Transfusion Therapy

Transfusion therapy for thalassemia was once administered as a palliative measure only when patients became symptomatic. These periodic transfusion regimens were unsatisfactory even for those limited purposes; they did very little to address the underlying IE, and thus did not prevent complications such as bone disease, vascular disease, and overall quality of life. Consequently, several centers initiated transfusion programs in which patients received regular transfusions to keep their Hb levels high enough to ameliorate these symptoms,[82,83] but the median survival time of patients transfused to maintain Hb levels of 7 to 8 g/dL in the United States in the 1960s was only 17 years of age.[83,84] "Hypertransfusion" programs were designed initially to maintain Hb levels above 8 g/dL, but in their more modern application, which is in current use, Hb levels are usually maintained above 9.5 to 10.5 g/dL. From the model of physiologic anemia in the newborn, it appears that the renal threshold for increased EPO secretion is in that range. To suppress this EPO drive of mostly IE, maintaining the Hb above that range would adequately suppress IE, thus minimizing complications related to it. This is different than other congenital anemias such as Diamond-Blackfan anemia (DBA), where lower thresholds have been used since IE is not a component of the disease. This range is quite close to the Hb levels of heterozygous individuals who are quite asymptomatic, and is thus able to maintain normal growth and development, and a level of activity that optimizes quality of life. A review of transfusion guidelines may be found at https://www.thalassemia.org/thalassemia-management-checklists-now-available-download/, and https://thalassaemia.org.cy/publications/tif-publications/.

A more vigorous transfusion program (supertransfusion) aimed at keeping Hb levels above 12.0 g/dL is no longer recommended.[85] This approach rested on the assumption that the benefits of further suppression of erythropoiesis and gastrointestinal iron absorption will offset the increased need for RBCs. However, several studies have demonstrated that transfusion requirements (and therefore the rates of transfusional iron loading) increase as the Hb level is raised in both splenectomized and nonsplenectomized patients (Fig. 41.10).[86–88] As a result, the consistent maintenance of Hb levels above 11 or 12 g/dL results in excessive iron accumulation without proportional clinical benefit, and supertransfusion protocols should be reserved for patients with poor tolerance of lower Hb levels because of cardiac disease or other reasons.

Alternative approaches to conventional transfusion therapy have been proposed to reduce the rate of transfusion iron loading. These approaches have generally relied on the concept that younger RBCs (neocytes) will circulate longer in the recipient than older RBCs. Preclinical experiments based on the difference in density between younger and older RBCs established the validity of this approach.[89,90] However, in prospective clinical trials, blood requirements were reduced only by 13% to 20%.[91–93] This reduction in iron loading did not outweigh the disadvantages of neocyte transfusions that included increased cost, wastage of 50% of the donor RBCs, and increased

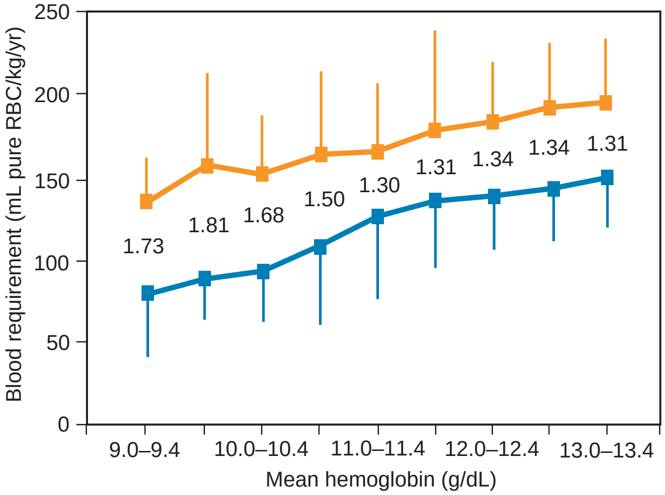


Figure 41.10 RELATIONSHIP BETWEEN TRANSFUSION REQUIREMENTS AND MEAN HEMOGLOBIN LEVEL MAINTAINED BY PATIENTS WITH THALASSEMIA MAJOR. *Blue*, Splenectomized patients, *yellow*, nonsplenectomized patients. *RBC*, Red blood cell. *(Adapted from Rebulla P, Modell B. Transfusion requirements and effects in patients with thalassemia major. Lancet. 1991;337:277.)*

TABLE 41.2 **Indications to Initiate Regular Transfusion Therapy in Nontransfusion-Dependent Thalassemia**

Declining hemoglobin with enlargement of the spleen
Growth failure
Poor school performance
Pregnancy
Surgery
Decreased exercise tolerance
Failure of secondary sexual development
Bony changes
Frequent hemolytic crisis (hemoglobin H disease)
Poor quality of life
Primary prevention and management of the following: 1. thrombotic or cerebrovascular disease 2. pulmonary hypertension/heart failure 3. extramedullary hematopoiesis 4. leg ulcers

donor exposures. The use of automated exchange transfusion has been proposed as another approach to reducing iron loading in patients with thalassemia.[94] With this method, RBCs are removed from the patient at the same time that new donor RBCs are transfused. This approach has been applied successfully to transfusion therapy for sickle cell disease. However, the goal of transfusion therapy in sickle cell disease is the replacement of HbS-containing RBCs with HbA-containing RBCs irrespective of the total Hb level. In contrast, the goal of transfusion therapy in patients with thalassemia is to maintain a specific total Hb level. Despite these different goals, studies of automated exchange transfusion in patients with thalassemia have demonstrated a reduction in net RBC requirements of 30% to 50%, either by reducing the amount of blood administered at the usual transfusion interval or by prolonging the interval between transfusions.[94,95] The benefits of this approach are probably attributable to the removal of previously transfused RBCs from the patients and replacement with younger, recently donated RBCs, reducing the overall age of the circulating RBC population.

The decision to initiate transfusion therapy should take into account the overall clinical condition of the patient as well as the Hb level. Patients with severe and persistent anemia (Hb <6 to 7 g/dL) usually also have failure to thrive, decreased activity level, and irritability. For these patients, transfusion therapy should begin after confirmation of the diagnosis of thalassemia and after demonstration that acute factors such as a febrile illness or folic acid deficiency are not confounding the assessment of the severity of anemia. For patients with higher Hb levels, the decision to begin transfusion depends on the careful assessment of the child's clinical findings. For example, some children with thalassemia have early and pronounced facial bone deformities caused by BM expansion despite a Hb level of 8 g/dL or higher. For such children, the benefits of transfusion therapy may outweigh the risks. In contrast, some patients with thalassemia have little or no clinical difficulty despite a persistent Hb level of 7 to 8 g/dL, and the benefits of transfusion therapy may be small. Determination of genotype may provide some guidance by distinguishing patients with more severe defects in β-globin production from those with less severe defects, but the overlap between genotype and phenotype in thalassemia still requires reliance on clinical assessment.

The ability to maintain a Hb level compatible with comfortable survival in the absence of regular transfusions is the generally accepted criterion for the diagnosis of thalassemia intermedia. In other words, distinguishing between thalassemia major and thalassemia intermedia, which in turn is the distinction between initiating a regular transfusion program or not, requires consideration of the Hb level and the quality of life (Table 41.2). These two parameters do not have a predictable relationship. Patients with thalassemia and Hb levels of 7 g/dL may be relatively symptom free, but patients with Hb levels of 9 g/dL may have numerous problems related to IE. Thus, the assessment of the patient rather than the Hb level alone is essential in deciding who does not require regular transfusions and therefore, by definition, has thalassemia intermedia. Some nontransfused patients have normal growth and sexual development, few medical problems, and normal or near-normal survival rates. However, others develop disfiguring facial changes, markedly delayed growth and sexual maturation, heart failure, severe osteoporosis, repeated fractures, arthritis, and massive splenomegaly. Calling the condition of this latter group "thalassemia intermedia" implies a milder disease than thalassemia major but, in fact, the patients' quality of life does not compare favorably with the quality of life of patients with thalassemia major. However, many such individuals either refuse regular transfusions for fear of transfusion related infections or iron overload, or are not able to get them for logistic and economic reasons. If the decision is made to manage the patient initially without regular transfusions, periodic reassessments of the patient's clinical condition, including appearance, growth, and development, and bone expansion are crucial.[96] Short-term transfusion therapy may be useful during pregnancy or in the management of cardiac and other serious complications. The need for regular transfusions often develops in adults with thalassemia intermedia because of a further decline in the Hb level or a growing intolerance of the anemia.[97]

In the most severe forms of the disease, transfusions are generally begun in the first or second year of life, and continued monthly. In order to minimize the risk of alloimmunization, it is recommended to have a limited extended phenotype of the recipient red cells, generally including the C, D, E, and Kell red cell antigens, but the standard may vary by region, and only involve the standard ABO and Rh antigens in areas where the donor and recipient pools are ethnically similar. There is no evidence that irradiation of the red cells is of specific benefit to patients with thalassemia. With the use of current additive solutions, the duration of storage of donor RBCs has only a small effect on the 24-hour recovery and the survival of the RBCs in each transfused unit. However, for patients with thalassemia major who undergo transfusion every 2 to 5 weeks, these small differences may have a significant impact on the annual consumption of blood. Consequently, the use of donor RBCs that have been stored for less than 7 to 10 days strikes a reasonable balance between the potential reduction in transfusion iron loading and the efficient use of the blood bank inventory. The use of volunteer blood donors remains the standard for patients with thalassemia. Although some families of children with thalassemia prefer directed donations, this approach has not reduced the rate of transfusion-transmitted infections among blood recipients in general and has not been shown to reduce the rate of alloimmunization in thalassemia. If directed donations are used, close relatives should be avoided because stem cell transplantation may be a later therapeutic option. Washed, filtered, or frozen red cells where the majority of the white cells and plasma-protein components are removed, are recommended only for individuals with severe or recurrent allergic transfusion reactions. The volume of packed red blood cells (PRBCs) transfused at each monthly visit is generally 15 mL/kg in children, to increase to a full unit when the child is about 15 kg in weight. Split units are not recommended since they do not afford the benefit of the full amount, and yet expose the recipient to another donor. Instead, the interval between transfusions may be shortened instead to 3 weeks or even 2 weeks, until the child reaches about 36 to 40 kg, when a second unit may be added to each transfusion visit. Most adults receive 2 units of PRBCs every 2 to 4 weeks. Occasionally, for logistical reasons, more blood may be administered at longer intervals, but this is not recommended because BM erythropoietic activity may be affected with wide variations in Hb levels (see box on Guidelines for Transfusion Therapy).

The clinical benefits of hypertransfusion programs are dramatic. The growth of younger children follows normal percentiles for height and weight.[98] Erythropoiesis is significantly suppressed as evidenced by decreased numbers of reticulocytes and normoblasts and TfR1 levels.[99,100] Hypertransfusion reduces or prevents the enlargement of the liver and spleen. Abnormal facies and bone fractures occur less frequently. The overall sense of well-being allows normal age-appropriate activities.[101,102]

There are many adverse effects of regular transfusions. Transfusion reactions may occur, ranging from non-hemolytic complications like febrile reactions, allergic reactions including urticarial reactions and rare anaphylaxis, to hemolytic complications like acute and delayed hemolysis. The development of autoantibodies and alloantibodies is well known in thalassemia patients. Alloantibodies make it harder to crossmatch blood for patients, though the incidence of development of new alloantibodies is declining with the institution of more standard extended typing.[103] Autoantibodies may also increase transfusion requirements, and should be appropriately treated when clinically significant. Other, longer-term complications of transfusions include transfusion-associated infections, the rates of which have gone down tremendously in the developed world, and iron overload, requiring chelation therapy.

GUIDELINES FOR TRANSFUSION THERAPY

Although some of the details of a transfusion program for patients with thalassemia major vary from center to center, the following guidelines are important for achieving the benefits while controlling the risks of a transfusion program. The rationales for these guidelines are discussed in the text.

1. Obtain a complete or limited extended (C, D, E, Kell) RBC antigen profile before the first transfusion. For patients who come to a new center after receiving transfusions elsewhere, contact the previous blood bank for information about alloantibodies and transfusion reactions.
2. Goal: maintain the pretransfusion hemoglobin level above 9.5–10.5 g/dL.
3. Administer 10–15 mL/kg of packed red blood cells (PRBCs) in children (in whole unit increments, avoid split units), 1–3 units PRBCs in adults at each visit.
4. Transfuse every 2–4 weeks.
5. Use leukoreduced packed RBCs that have been stored for less than 10–14 days.
6. Irradiation of units is not required.
7. Consider washed units only for individuals with frequent/severe allergic reactions.

Iron Toxicity

Transfusional iron overload or secondary hemochromatosis is the most consequential long-term complication that all patients receiving regular transfusions develop.[78] Irrespective of age at which transfusions are initiated, iron overload develops after about 15 transfusions because of the body's inability to physiologically excrete excess iron. If not treated, iron overload eventually results in deposition in the organs, particularly the heart and endocrine organs resulting in significant morbidity and even mortality from cardiac complications of myocardial siderosis.[76] Iron overload is discussed elsewhere, in Chapter 37, but in this section, the discussion will be focused specifically on the nuances related to thalassemia.[104]

Each unit of packed RBCs contains approximately 200 to 250 mg of iron. Based on usual blood requirements in patients with thalassemia major, the rate of transfusional iron accumulation is approximately 0.30 to 0.60 mg/kg/day (Fig. 41.11). The massive IE associated with the intermedia and major thalassemias leads to excessive gastrointestinal iron absorption that adds to the transfusional iron burden, although absorption is reduced when a Hb level above 9 g/dL is maintained.[105,106] Iron causes toxicity to cells as a result of the generation of free radicals via the Fenton reaction. These radicals cause oxidative damage to cellular components (including lipids and protein) and intracellular organelles (Fig. 41.12). Iron toxicity to tissues is a function of tissue iron burden, tissue iron sensitivity, and duration of exposure (see Fig. 41.11).

Phlebotomy, the most efficient method of removing iron in other situations, is precluded in severely anemic patients with thalassemia owing to transfusion dependence.

In general, maintaining normal ascorbic acid levels optimizes iron excretion because it increases tissue iron turnover in the plasma. A relationship between iron overload and ascorbic acid depletion, first suggested by the epidemiology of scurvy among the Bantu, exists in thalassemia major.[67,107] For thalassemia patients with low levels of ascorbic acid, daily supplementation with 100 to 200 mg of this vitamin increases urinary iron excretion in response to deferoxamine by approximately twofold.[68,108] Ascorbic acid may retard the conversion of ferritin to hemosiderin and therefore allow more iron to remain in the chelatable form.[109,110] However, it can also enhance iron-mediated peroxidation of membrane lipids[111,112] as well as membrane damage in cultured myocardial cells.[113] Cardiac toxicity manifested as arrhythmias and decreased ventricular contractility has been attributed to vitamin C therapy.[114] *Thus, ascorbic acid should only be used under continuous chelator coverage, and only in patients who are ascorbate depleted.*

Assessment of Iron Stores

Iron acquired by transfusions bypasses the body's normal gatekeeper of iron influx, the gastrointestinal tract, through which iron absorption is exquisitely regulated by hepcidin. This excess transfusional iron cannot be actively excreted, and it is taken up by normal storage organs, the liver and the macrophages of the reticuloendothelial system (RES). When the capacity for storage in these organs is overwhelmed, iron spills over into parenchymal tissue, generating free radical damage with cellular membrane lipid peroxidation and leading to end-organ dysfunction, particularly affecting the liver, endocrine system, and myocardium.

Iron stores were once almost exclusively assessed by measuring serum ferritin levels, with liver biopsy and quantitative iron measurement being used only in more advanced centers. The latter is a painful and invasive test, with a significant risk of complications, and thus not attractive to patients. Magnetic resonance techniques have been developed in the past two decades which allow more accurate assessment of tissue iron and allow the treatment regimen to be tailored. Magnetic resonance imaging (MRI) with proton transverse relaxation rates (R2) with spin-echo imaging, signal intensity ratios, and gradient-echo T2* are currently the preferred method to assess LIC.[115–119] Direct comparisons with hepatic tissue samples demonstrate significant correlations ($r = 0.97$) with biopsy-measured LICs.[116,118–122]

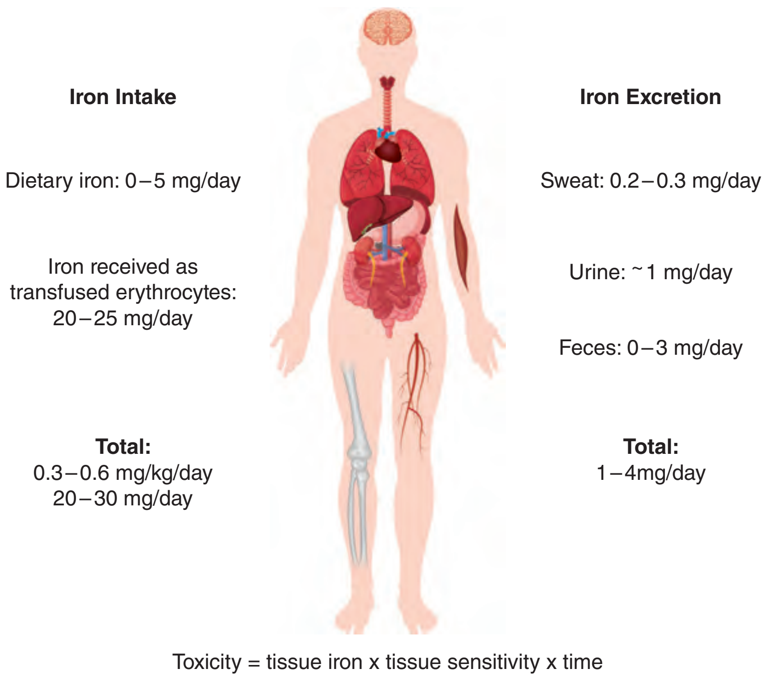

Iron Intake
Dietary iron: 0–5 mg/day
Iron received as transfused erythrocytes: 20–25 mg/day
Total:
0.3–0.6 mg/kg/day
20–30 mg/day
Iron Excretion
Sweat: 0.2–0.3 mg/day
Urine: ~1 mg/day
Feces: 0–3 mg/day
Total:
1–4mg/day
Toxicity = tissue iron x tissue sensitivity x time

Figure 41.11 Iron overload in thalassemia is an imbalance between iron loading from transfused red cells and increased absorption, and the innate inability to excrete excess iron.

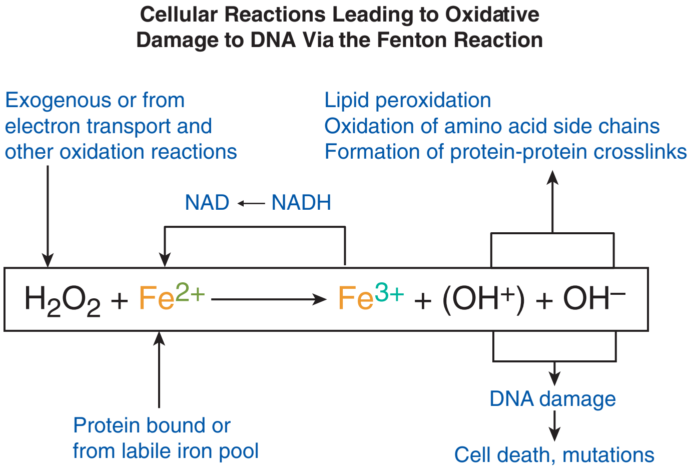

Cellular Reactions Leading to Oxidative Damage to DNA Via the Fenton Reaction
Exogenous or from electron transport and other oxidation reactions
Lipid peroxidation
Oxidation of amino acid side chains
Formation of protein-protein crosslinks
NAD ← NADH
$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + (OH^{+}) + OH^{-}$
Protein bound or from labile iron pool
DNA damage
Cell death, mutations

Figure 41.12 MECHANISMS OF IRON TOXICITY. *(Modified from Henle ES, Linn S. Formation, prevention, and repair of DNA damage by iron/hydrogen peroxide. J Biol Chem. 1997;272(31):19095–19098.)*

Strong evidence supports that with proper calibration, the measurement and mapping of liver iron concentrations (LIC) using MRI is an extremely effective approach for the regular assessment of the effectiveness of chelation therapy.[123] Myocardial T2* MRI has been able to quantify iron loading in the heart as well. However, it is now clear that there is a variable correlation between hepatic and cardiac iron concentrations.[124] Based on these assessments, it is now possible to track iron levels in two tissues to tailor chelation therapy, the LIC, which is normally less than 1.5 mg/g dry weight, and the myocardial iron level measures as a T2* value, normally more than 20 ms. LIC values between 3 and 7 mg Fe/g dry weight appear to be associated with minimal toxicity, while LIC levels greater than 15 mg Fe/g dry weight are associated with a greater risk of iron-induced heart disease.[125] Experience with cardiac MRI suggests that changes in T2* reflect levels of iron in the heart and may predict adverse changes in cardiac function. Cardiac T2* values are predictive of arrhythmias and cardiac failure over 1 year.[126] Cardiac MRI T2*-directed treatment results in improved chelation and iron stores.[127] MRI assessment of iron overload cannot be used in patients with pacemakers or those who are claustrophobic. Future MRI use may involve the quantification of iron concentration of endocrine glands to predict or monitor dysfunction.[128–130]

Measurements of LIC by magnetic susceptometry using a superconducting quantum interference device (SQUID) also correlate well with biochemical measurements of tissue iron.[131,132] At present, measurement of tissue iron by SQUID is limited to the liver, and the instruments are available in only a few sites in the United States of America and Europe. Importantly, the SQUID is not an enclosed space, so claustrophobia is not an issue.

Serum ferritin levels are safe, inexpensive, and readily available, and serial measurements may be predictive both of critical complications such as iron-induced heart disease and of adverse effects of chelation therapy such as impairment of vision and hearing. However, single ferritin levels may correlate poorly with LIC because it is an acute-phase reactant and may be influenced by inflammation, vitamin C deficiency, hepatitis, and other infectious states. Multiple studies correlating serum ferritin levels with liver iron measurements by both biopsy and MRI have shown a significant correlation, but there is marked variability between patients, confirming the low reliability of this measurement as a measure of total body iron. Transferrin saturation is not very useful in evaluating the severity of iron overload in patients with thalassemia because the massive IE usually results in a transferrin saturation greater than 60% even in the absence of iron overload.[133]

Previous standard practice was to initiate chelation in thalassemia patients when the serum ferritin reached 1000 mcg/dL. However, given the unreliable nature of this parameter, and since we can quantify the rate of iron loading, knowing the volume of blood transfused, and its iron content, this is not a reasonable starting point anymore. Chelation should be initiated after the patient has received about 15 transfusions (by which time the liver iron would be estimated to be approximately 6 to 8 mg/g DW.) A baseline MRI of the liver should be performed to quantify iron and provide a starting point for monitoring efficacy. Liver MRIs are recommended annually. In children who are chelated, other organs tend not to load iron since it is well sequestered in the liver and RES. For this reason and because it requires a fairly long breath-hold, cardiac imaging is usually not performed till after 10 years of age.[134] Adults with a longer-standing disease should have cardiac iron assessments along with liver iron assessments. Ferritin levels may still be of use to follow trends, and guide the use of interval MRI assessments or changes in chelation regimens. Ferritin levels are still used almost exclusively where MRI is not readily available but they should be used with caution.[135,136] In addition, regular monitoring of endocrine function should begin around age 10, including that of the pituitary, endocrine pancreas, thyroid, parathyroid, adrenals, and gonads, since these organs may also be severely affected by iron overload.

Chelation Therapy

A pharmacologic approach using specific iron-chelating agents remains the only strategy for removing excess iron in transfusion-dependent patients. Several drugs with chelating properties have been synthesized or recovered from microorganisms. Many lack iron specificity or are inefficient; others cause significant toxicity. To chelate iron, the chelating agent must complex with all of the iron atom's six available coordination sites. Three general classes of iron chelators occur or have been synthesized: hexadentate (deferoxamine), bidentate (deferiprone), and tridentate (deferasirox) (Fig. 41.13). Only one hexadentate molecule is necessary to bind one atom of iron, but three molecules of a bidentate iron chelator bind one iron atom and two molecules of a tridentate chelator are required to bind one atom of iron. Chelatable iron is thought to be derived from the intracellular "labile iron pool"[137,138] and from the circulating nontransferrin-bound plasma iron.[139,140]

Benefits of Iron Chelation Therapy

More than 30 years of experience using iron chelation therapy with deferoxamine in thalassemia major patients have demonstrated that:

1. LICs can be maintained at normal or mildly elevated levels.
2. Hepatic fibrosis is slowed or prevented.
3. The risk of iron-induced cardiac disease, including heart failure and serious arrhythmias, is markedly decreased.
4. Normal growth and sexual development are common but not universal.
5. Long-term survival is substantially improved. These benefits are directly related to compliance and generally require the prolonged administration of daily iron chelation therapy.
6. Long-term safety and efficacy studies of the oral iron chelators deferiprone and deferasirox are ongoing, and new formulations are in development.

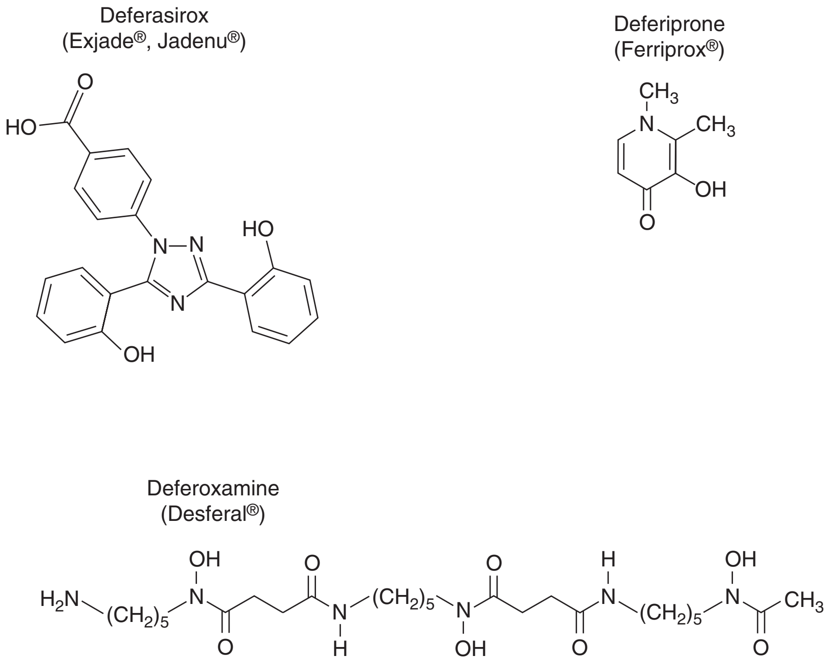
The excretion of excess iron must be facilitated by the use of iron chelation. There are three iron chelators in clinical use currently, one parenteral and the other two administered orally.

Deferoxamine

Deferoxamine mesylate is a naturally occurring hexadentate siderophore first isolated from cultures of *Streptomyces pilosus*, introduced as an iron chelator in 1960. Poorly absorbed from the gastrointestinal tract because of its high molecular weight, and with a short plasma half-life, it must be given parenterally by continuous infusion, either intravenously or subcutaneously. However, it is highly specific for iron and is associated with relatively low toxicity.[141] Deferoxamine enters cells, chelates iron, and appears in the serum and bile as the iron chelate, ferroxamine,[142] and is excreted through bile and in the urine.[143] Approximately one-half to two-thirds of the iron excreted in response to deferoxamine is in the stool, with the remainder in the urine.[144] These proportions vary from patient to patient and at different levels of iron overload, dose of deferoxamine, and endogenous erythropoietic activity.[145]

Iron excretion after the administration of deferoxamine is proportional to body iron stores. To achieve negative iron balance, the chelating agent must cause the daily excretion of 0.3 to 0.6 mg/kg of iron. Deferoxamine was initially administered by daily intramuscular injections of 0.5 g, which led to lower hepatic iron accumulation and fibrosis.[146,147] However, intramuscular injections proved to be too painful and, because of the limited dose administered, were insufficient to achieve negative iron balance. In the mid-1970s, it was demonstrated that iron excretion was markedly enhanced and negative iron balance was attained by continuous, prolonged 24-hour intravenous or 8- to 12-hour subcutaneous infusions of deferoxamine at 20 to 60 mg/kg/day.[148,149]

The typical regimen of subcutaneous infusions 5 to 6 nights a week was not only suboptimal because of the lack of 24-hour coverage, it was also extremely cumbersome. In addition, injection site pain, skin reactions, and occasionally abscesses often led to poor compliance. In patients who were poorly compliant with subcutaneous therapy, and with high iron burdens, intravenous continuous administration


Deferasirox
(Exjade®, Jadenu®)

Deferiprone
(Ferriprox®)

Deferoxamine
(Desferal®)

Figure 41.13 MOLECULAR STRUCTURE OF THE THREE IRON CHELATORS.

in normal or higher doses was accomplished through an indwelling line. Continuous intravenous administration of deferoxamine is particularly useful for rapidly lowering the total iron burden and is used for reversal of cardiac morbidity (e.g., cardiac arrhythmias or left ventricular dysfunction). Doses above 50 to 60 mg/kg/day are not associated with increased iron excretion. Complications of indwelling catheters include infection and thrombosis, and this route of administration is a last resort.

Patients receiving aggressive chelation therapy with lower iron burdens may be more susceptible to toxicity. Neurosensory toxicity of deferoxamine is dose related and inversely correlated with body iron burden. Impairments of visual and auditory acuity are associated with high doses of deferoxamine relative to the iron load.[150] The ototoxicity is characterized by bilateral high-frequency hearing loss. The retinal toxicity is characterized by the loss of night and color vision, retinal atrophy, and cataract formation.[151] Patients receiving deferoxamine should undergo baseline and annual audiograms and ophthalmologic examinations. Deferoxamine should be discontinued if such abnormalities arise, with cautious reinitiation at lower doses when abnormalities improve or resolve. The risk of visual and auditory side effects can be minimized by adjusting the daily deferoxamine dose to the patients' serum ferritin level.[150] Impaired growth associated with growth plate deformities or metaphyseal rickets-like changes in the long bones and histologic evidence of cartilage dysplasia may occur in young children receiving deferoxamine.[152–154] Other, less common complications of deferoxamine include anaphylaxis, hypotension, allergic reactions, acute pulmonary disease, impairment of renal function, and infection.[155–161] Severe allergic reactions are rare, and desensitization has been achieved successfully in some patients.[157,158] Acute pulmonary disease and renal failure have occurred in a few patients receiving unusually high doses of deferoxamine by intravenous infusion.[159] The mechanism of this toxicity is unclear. One of the most serious complications of deferoxamine is an increased risk of infection with *Yersinia*, and mucor, iron-avid organisms which use deferoxamine as a siderophore. Deferoxamine can enhance the growth and virulence of these organisms, leading to colitis, abdominal abscesses, or sepsis.[160,161] The safety of deferoxamine during pregnancy has been inferred from case reports rather than formal studies. A summary of these case reports identified 11 women who received deferoxamine beginning in the first trimester and 33 women who used the chelator beginning in the second or third trimester.[161] None of the infants showed evidence of drug-related toxicity.[162]

There are data confirming that endocrine dysfunction was improved and cardiac disease was delayed or prevented with standard deferoxamine regimens.[163] Cardiac arrhythmias and congestive heart failure have reversed in some patients with standard or aggressive deferoxamine regimens, and life expectancy was significantly prolonged.[164–166] Intense 24-hour intravenous deferoxamine regimens of no more than 15 mg/kg/h were used to reverse early cardiac hemosiderosis, but these doses were associated with pulmonary toxicity resembling ARDS. More conventional doses of 50–60 mg/kg/day have improved left ventricular ejection fractions and prevented death in most patients.[167,168]

However, though regular chelation with deferoxamine has proven remarkably effective in reducing the transfusional iron burden, primarily because of its route of administration, it is now a second-line agent, or in addition to another chelator in more severely iron-overloaded individuals.

Deferasirox

Deferasirox (ICL670) is an orally active iron chelator that was identified by computer technology at Novartis Pharmaceuticals in the 1990s. It is a tridentate compound known as 4-(3,5-bis[2-hydroxyphenyl]-1*H*-1,2,4-triazol-1-yl)-benzoic acid,[169] wherein two molecules of deferasirox are required to bind one atom of iron. It has a high affinity for iron and a much lower affinity for copper and zinc. Deferasirox is orally bioavailable and is well absorbed by the gastrointestinal tract. It has a dose-dependent plasma half-life of 12 to 18 hours that allows for once-daily oral administration after fasting on an empty stomach.[170] Defarasirox was initially provided as dispersible tablets (Exjade) and administered as a suspension in water or apple or orange juice.[171] It is now available as a film-coated tablet (Jadenu) which does not need suspension, as well as granules which can be sprinkled on yogurt or applesauce for young children. This formulation has a lower prevalence of GI side effects and is better tolerated. Dosing for the dispersible tablets is 20 to 40 mg/kg/day, while for the film-coated tablet and sprinkles is 14 to 28 mg/kg/day. Iron excretion in response to deferasirox is largely via the bile and hence in the stool (90%) and far less in the urine (10%).[171]

Deferasirox is generally well tolerated. Self-limited mild gastrointestinal complaints and maculopapular skin rashes are the most common adverse events. Discontinuation of deferasirox is rarely required, but may be necessary if the GI upset is debilitating or patients develop symptoms suggestive of colitis. Mild increases in serum aspartate transaminase (ALT), alanine aminotransferase (AST), and creatinine, and intermittent proteinuria may also occur and thus monitoring for these at least once a month is recommended. Severe renal complications may occur in patients with preexisting renal disease, and such patients may need to be treated with an alternate chelator. Dose reduction, interruption, or discontinuation should be considered for elevations in serum creatinine. Deferasirox-induced changes in creatinine and renal hemodynamics are usually mild and reversible without progression over time.[172] Reports of pancytopenia have occurred in postmarketing reports, but mostly in patients with preexisting hematologic disorders such as MDSs that are frequently associated with BM failure.

Deferiprone

Deferiprone, 1,2-dimethyl-3-hydroxypyrid-4-one, is an orally active bidentate iron chelator that requires three molecules to bind one iron atom. It was synthesized in the late 1980s and was first tested in uncontrolled clinical trials at the Royal Free Hospital in London, hence the eponym L1.[173,174] It is readily absorbed by the gastrointestinal tract and has a plasma half-life of approximately 2 hours, hence it was originally dosed three times a day to provide continuous chelation coverage. Chelated iron is excreted predominantly in the urine (90%) and far less in the stool (10%). A large observational study demonstrated improvement in cardiac iron deposition with deferiprone treatment.[175] At doses of 75 mg/kg/day, deferiprone reduces or maintains iron stores, thereby achieving negative iron balance or iron balance in many regularly transfused patients.[176–184] However, some patients remain in positive iron balance and continue to accumulate iron during long-term therapy with this dose of deferiprone,[176,180,185] and higher doses up to 100 mg/kg/day may be more effective.[186]

Agranulocytosis occurs in 1% of patients and, although rare, remains the principal concern for patients receiving deferiprone. Milder neutropenia (absolute neutrophil count <1500 but >500) is more common and occurs in approximately 6% to 8% of patients. Severely depressed neutrophil counts represent a significant risk of sepsis and hospitalizations, and in some cases, administration of granulocyte colony-stimulating factor is required. Some reported deaths have been related to deferiprone-induced agranulocytosis or neutropenia. Regular weekly monitoring of blood counts during deferiprone therapy is recommended to detect the rare but important deferiprone-induced complications of neutropenia and agranulocytosis.[180–182,187] In cases where mild neutropenia develops, it may be safe to continue deferiprone therapy with more frequent monitoring of blood counts and clinical symptoms.[188]

Other side effects of deferiprone include gastrointestinal complaints, mostly nausea and some vomiting that occur in approximately 33% of patients and usually resolve without specific intervention. Arthropathy with arthralgias and some joint effusions occur in approximately 15% of patients. The incidence of joint symptoms varies widely among various studies but may be severe enough to require reduction or interruption of chelation therapy.

Abnormal liver function tests may occur gradually or suddenly and in the absence of other causes of hepatic dysfunction. These elevations may return to baseline values with the interruption of deferiprone followed by reinitiation beginning with lower doses and close monitoring of liver function tests. Progressive liver disease attributed to deferiprone has not been reported, and initial concerns about drug-induced hepatic fibrosis have not been substantiated by subsequent studies.[177,179,189,190]

Given the need for three doses a day, and the impact on compliance, deferiprone is used mainly as a second-line agent, unless there is a dose-limiting toxicity with deferasirox. It is also used in combination with either of the other chelators. Combination regimens with deferoxamine also reduce iron stores or prove effective in restoring negative iron balance in some of these patients.[191–193] Enhanced urine and stool iron excretion in thalassemia patients using both deferoxamine and deferiprone have suggested an additive effect postulated by the shuttle hypothesis, that is, deferiprone may chelate intracellular labile iron and shuttle it to deferoxamine.[194] Some studies have also suggested that deferiprone alone or in combination with deferoxamine may be more effective than deferoxamine in removing iron from the heart, improving cardiac function, and preventing iron-induced cardiac disease.[122,184,195–198] A prospective randomized study comparing the combination of deferiprone at 75 mg/kg/day and deferoxamine at 40 to 50 mg/kg/day with deferoxamine alone demonstrated that the combination therapy more rapidly reduced hepatic and cardiac iron stores than deferoxamine alone.[199] Schedules for combination therapy vary but have usually included 5 to 7 days of deferiprone and 2 days of subcutaneous deferoxamine weekly. Intensive combined chelation therapy has been reported to reverse both cardiac and endocrine complications of thalassemia major.[200] Combination with deferasirox has also been reported in a number of case series with good efficacy overall.[201–204]

A twice daily preparation of deferiprone is now available.[205] This should improve compliance for those who were previously on three-times-a-day dosing and offer an alternative to deferasirox for those who continue to have some GI symptoms on that agent. A liquid formulation of deferiprone is also available and is particularly advantageous in pediatric patients.

Combination Chelation Therapy

Many different combination regimens of these chelators have been used for patients with severe iron overload, often tailored to whether the overload is more systemic or more cardiac. Deferiprone-containing combinations are more frequently used in cardiac overload than in systemic overload.

A review of chelation guidelines may be found at the Cooley's Anemia Foundation website[206] and at the Thalassaemia International Federation website.[207]

All of the iron chelators—deferoxamine, deferiprone, and deferasirox—have proved to be safe and effective in non-transfused thalassemia intermedia.[208–210]

Complications of Thalassemia and Their Management

The thalassemia syndromes have a myriad of complications both related to the disease as well as its treatment. The complications of the disease are all related to the underlying IE as a result of the imbalance in α- and β-globin production. Once appropriate treatment with regular transfusions is begun, and IE is adequately suppressed, those complications are now replaced by potential adverse effects of transfusion therapy, the most important long-term complications being related to transfusional iron overload. Individuals with thalassemia intermedia, who are not regularly transfused, that is, NTDT patients, will continue to have complications related to IE, which will be described in this section (Fig. 41.14).

Complications Related to Ineffective Erythropoiesis

Individuals with more severe IE have more significant anemia, and as a result may have growth retardation, chronic fatigue and malaise, bone pain, and worsening splenomegaly and still develop iron overload. Splenectomy is often performed when there is a component of hypersplenism contributing to worsening anemia. Gallbladder disease from increased red cell turnover and the development of

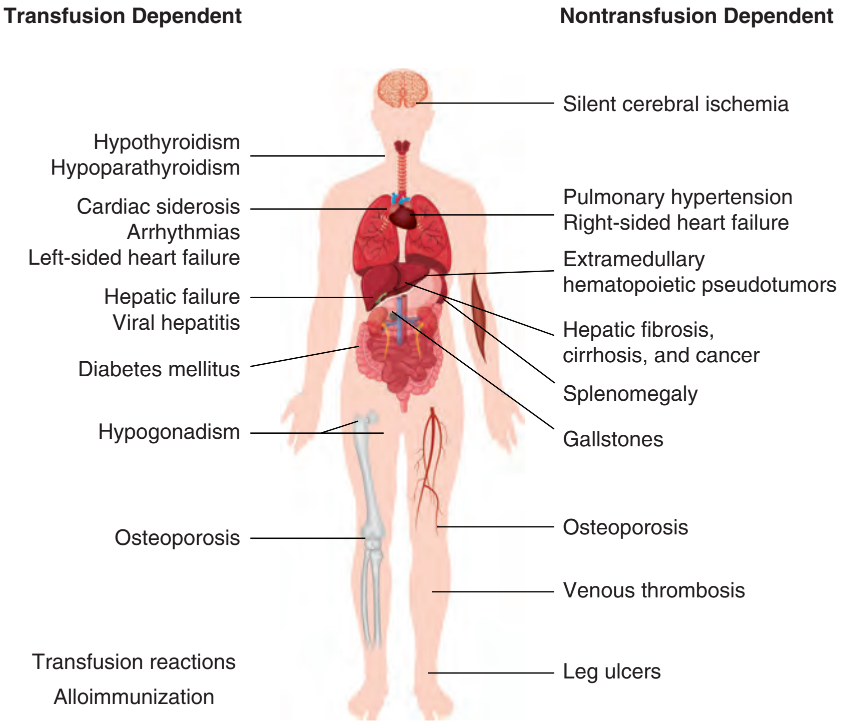


Figure 41.14 Complications of thalassemia—mainly related to iron overload in transfusion-dependent thalassemia, and related to the systemic effects of ineffective erythropoiesis in non-transfusion-dependent thalassemia.

pigment gallstones is quite common. Progressive EMH may result in paraspinal nodules as described previously. Thromboembolic events represent a major complication of thalassemia intermedia, occurring in 10% to 34% of patients.[211,212] These events include stroke, pulmonary embolism, portal vein thrombosis, and deep vein thrombosis of the legs. Splenectomy is a risk factor for thromboembolic events in patients with thalassemia intermedia, resulting in thrombocytosis and allowing the prolonged circulation of damaged RBCs that generate increased amounts of thrombin.[211] Clinical manifestations including the development of chronic, non-healing leg ulcers from poor perfusion and the effects of arterial endothelial damage. A possible hypercoagulable state may contribute to other manifestations of vascular disease such as cerebral infarcts and development of pulmonary hypertension, complications which commonly occur in patients with thalassemia intermedia with the latter being the primary cause of congestive heart failure.[213] Some investigators consider the risk of thromboembolic events after splenectomy for thalassemia intermedia to be sufficiently high to warrant short-term anticoagulation in the perioperative period and during pregnancy.[211] Oral contraceptives should be used with extreme caution, if at all. Interestingly, known genetic thrombophilias in other populations like factor V Leiden, the prothrombin gene mutation 20210, and MTHFR C677T mutations have not been associated with thrombotic risk in this population.[214]

Extension of hematopoietic tissue beyond the confines of the bones occurs in patients with thalassemia intermedia as a result of the intense erythropoiesis. This complication occurs less frequently in patients with thalassemia major because of the partial suppression of erythropoiesis by regular transfusions. Masses of extramedullary hematopoietic tissue develop in the spinal epidural space, thorax, cranium, pelvis, and elsewhere.[215–226] These masses may be detected as incidental findings on imaging studies of the chest or abdomen.[217–223] In other instances, the masses produce symptoms by compressing neighboring structures. For example, patients with extramedullary hematopoietic masses may develop paraplegia from spinal cord compression or loss of visual acuity or visual fields caused by optic nerve compression.[216,219,225,226] Additional clinical presentations of hematopoietic masses include pleural effusions and upper airway obstruction.[221,222,224] Initiation of regular transfusions for patients with thalassemia intermedia or intensification of the ongoing transfusion program for patients with thalassemia major reduces the size of extramedullary hematopoietic masses and helps to prevent recurrences (see Tables 41.4 and 41.5). Additional bone changes are described subsequently.

Even in the absence of regular transfusions, many patients develop progressive iron overload because of increased absorption of dietary iron induced by IE and, in many cases, the intermittent administration of RBC transfusions. By the third or fourth decade of life, and occasionally even the second, the total iron burden may attain the levels seen in transfusion-dependent patients.[227] Ferritin levels underestimate the degree of tissue iron loading in thalassemia intermedia, and assessment of LIC is more helpful in determining the need for chelation therapy.[135] Iron overload may result in diabetes and endocrine disturbances, typically by the fourth decade of life, though cardiac deposition is relatively rare in true thalassemia intermedia individuals.

Skeletal Changes

With improved survival in patients with thalassemia major, the problem of bone disease has assumed greater importance.[228] Approximately 50% to 80% of older thalassemia patients have an osteoporosis-osteopenia syndrome.[229,230] The pathogenesis of bone disease is multifactorial including expansion of the medullary cavity due to IE, with subsequent thinning of the cortices, endocrinopathy, and iron overload.[231] The widespread prevalence of osteoporosis in patients with thalassemia major was first observed across all ages in 1995.[232] Subsequently, others reported a high frequency of abnormal *Z* scores in pediatric, adolescent, and adult patients. Abnormal bone mineral density has been reported in older studies of pediatric and adolescent patients with thalassemia major,[233–235] though it is less likely that well transfused and chelated patients will have these complications at the same frequency. Vertebral fractures are associated with osteoporosis and cause significant morbidity in this population. Lumbar and thoracic vertebrae fractures are present in over 30% of thalassemia patients over the age of 30 years.[236,237] The Thalassemia Clinical Research Network noted the overall fracture prevalence of 12% in a contemporary sample of 702 patients with α - and β-thalassemia. The fractures occurred more frequently in thalassemia major (17%) and intermedia (12%) compared with β-E (7%) and α-thalassemia (2%). Fracture prevalence increased with age and with sex hormone replacement therapy.[238] An observational study by the Thalassemia Clinical Research Network has demonstrated a high prevalence of low bone mass across all the thalassemia syndromes, including β-thalassemia major, intermedia, β-E, HbH, H-Constant Spring, and homozygous α-thalassemia, which progresses with aging. In addition, increased serum and urine markers of bone turnover have been described in thalassemia patients with osteoporosis, which correlate with low dual-energy X-ray absorptiometry scores and improve with bisphosphonate therapy.[239] It remains unclear if different chelation therapies and transfusions can alter the progression of osteoporosis.[240] Bisphosphonates have been used with varying success to correct the osteopenia, not always being successful given the complex pathophysiology of the disease.[241–243] Zinc supplementation may also improve bone density in younger patients with thalassemia.[244]

Skeletal abnormalities are less common in patients receiving regular RBC transfusions but may still occur as a result of partially unchecked IE and expansion of the erythroid BM.[245] These cause widening of the BM space and thinning of the cortex, with consequent osteoporosis.[232,246] Changes in the skull and facial bones, including expansion of the frontal bone with prominent frontal bossing, may occur before the initiation of transfusion therapy. Radiographs reveal the diploic spaces to be widened. At first, the skull has a granular appearance, but later perpendicular bony trabeculae appear, giving the classic “hair on end” or “crewcut” appearance (Fig. 41.15). Marked overgrowth of the maxilla results in severe malocclusion, jumbling of the upper incisors, and prominence of the molar eminences.[247] These bone changes produce the classic thalassemic facies. Additional skeletal changes are observed in the metacarpals, metatarsals, and phalanges, where expanded medullary cavities produce a rectangular and then a convex shape. Irregular fusion of the epiphyses of the proximal humerus results in characteristic shortening of the upper arms.[248,249] Marked osteoporosis and cortical thinning may predispose to pathologic fractures of the extremities and compression fractures of the vertebrae (Fig. 41.16).

Several abnormalities in the ribs may occur, including notching and osteolytic lesions.[245,250] The ribs become very wide, especially at

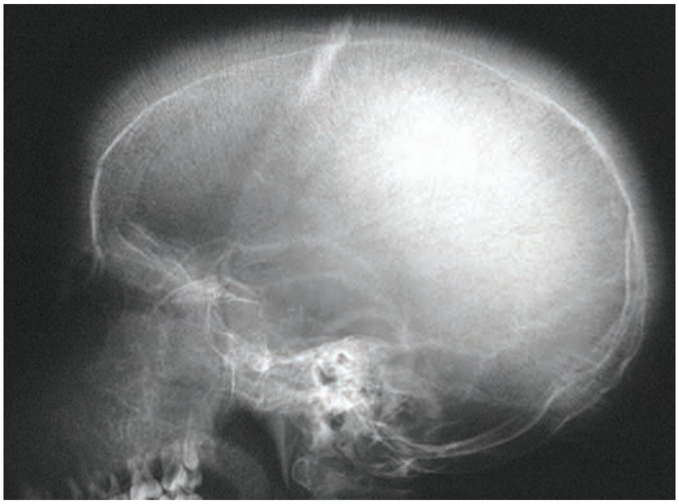


Figure 41.15 MARKED EXPANSION OF THE DIPLOEIC SPACE IN THE SKULL BONES WITH THE “HAIR ON END” OR “CREW CUT” APPEARANCE ON X-RAY.

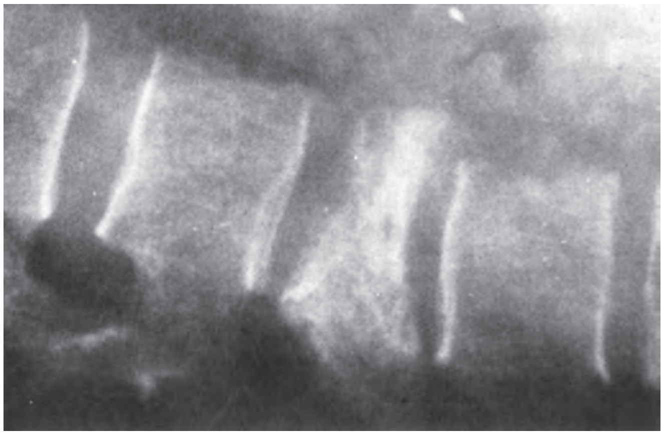


Figure 41.16 COMPRESSION FRACTURE OF L2 VERTEBRA IN A PATIENT WITH SEVERE β-THALASSEMIA. *(From Pearson HA, Benz EJ Jr. Thalassemia syndromes. In: Miller DR, Baehner RL, McMillan CW, eds. Smith's Blood Diseases of Infancy and Childhood. 5th ed. St. Louis: CV Mosby; 1984:439.)*

the points of their attachment to the vertebral column. BM masses may extrude from these sites, creating the appearance of paravertebral masses and compressing the spinal cord.[251]

Endocrinopathies

The endocrine organs are more susceptible to iron deposition and the resulting dysfunction can lead to significant morbidity. These complications are seen at higher frequencies in poorly chelated individuals and with improved chelation compliance with the oral agents it is anticipated that the incidence of these complications will decline. Regular monitoring for these complications is recommended, especially in those individuals with a history of more significant iron overload and poor chelation compliance.

Growth. Growth retardation, including skeletal and dental deformities,[252] was common even in young children until the use of hypertransfusion regimens was instituted. With the correction of the severe anemia, growth was initially restored to relatively normal during the first decade of life, unless heavy iron deposition in the pituitary resulted in growth hormone deficiency early. Without appropriate iron chelation therapy, the adolescent growth spurt is often delayed or absent; most patients, even those well maintained by transfusion, may not attain normal stature, partly because of iron-induced damage to the anterior pituitary.[43,253,254] Menarche is frequently delayed. Breast development may be poor, and many female patients have primary or secondary amenorrhea. Boys are frequently immature, with sparse facial and body hair. Although spermatogenesis may be normal at least initially, libido is often decreased as a result of decreased testosterone secretion. An older multicenter study of 250 adolescent patients in northern Italy showed that despite hypertransfusion and 7 to 10 years of deferoxamine iron chelation therapy, two-thirds of male patients and one-third of female patients older than 14 years of age were two standard deviations or more below the mean for height.[255] Many adolescents between 12 and 18 years of age lacked any secondary sexual changes of puberty. However, the mean serum ferritin level in the entire group was 3500 ng/mL, indicating persistence of a high level of excess iron burden in most of this group.

Regular chelation therapy started early in the first decade of life frequently allows a normal onset of puberty and development of secondary sexual changes.[256] If necessary, administration of recombinant human growth hormone in conventional doses increases height velocity in patients with growth hormone deficiency.[257,258] Normal or higher doses of recombinant human growth hormone increase growth velocity in patients with normal growth hormone reserve but low levels of insulin-like growth factor I.[259,260] For patients with a functional hypothalamic-pituitary axis, treatment with gonadotropin-releasing hormone may induce pubertal changes.[261] In others, administration of sex steroids is necessary to induce secondary sexual characteristics.

Diabetes. Abnormal carbohydrate metabolism is common in older patients with thalassemia major. Prepubertal children usually have normal glucose metabolism, but pubertal patients exhibit impaired responses to glucose load. Higher than normal insulin levels despite normal glucose levels are also encountered during puberty.[262] The defect in these patients appears to be related to insulin resistance, with insulin deficiency developing later in the progression to diabetes. Rates of diabetes are reported close to 6% to 8%.[263] Diabetes occurs more frequently in patients with hepatitis C and hepatic dysfunction.[264–266] Oral hypoglycemic agents have been used to regulate hyperglycemia and may reduce the rate of further deterioration of glucose metabolism.[267] It is important to keep in mind that the conventional HbA1C monitoring is not reliable in individuals who are regularly transfused since it measures levels in red cells which have been stored in high concentrations of glucose for variable periods of time. Hence an oral glucose tolerance test is recommended for monitoring for this complication in individuals at risk.

Thyroid and parathyroid disease. Laboratory findings of hypothyroidism and hypoparathyroidism are present in approximately 14% of patients with thalassemia major.[268–272] Though clinical findings associated with these deficiencies are uncommon, replacement therapy is recommended.[273]

Liver and Gallbladder

Hepatomegaly occurring before the initiation of transfusion therapy in severely affected patients is primarily a consequence of EMH. With the amelioration of the anemia, the liver diminishes in size. However, as transfusion therapy continues, iron accumulation provides a new reason for hepatomegaly and resultant liver injury. Iron deposition, first present in the Kupffer cells, ultimately engorges the parenchymal cells, resulting in an appearance that is indistinguishable from that of idiopathic hemochromatosis.[274–276] The hepatocellular injury of iron overload may be attributable to the liberation of hydrolases resulting from initiation by the ferrous form of iron and peroxidative damage of lysosomal membrane lipids.[277] Fibrosis is usually followed by cirrhosis and an increased risk of hepatocellular carcinoma. The risk of liver damage and the rate of progression may be increased by the concomitant presence of excessive iron with viral hepatitis, previously an issue in these patients receiving regular transfusions, but with improved screening of blood products it is not such an issue anymore in higher-income countries of the world.

Regular chelation therapy is the key to maintaining normal or near-normal hepatic iron concentrations and preventing iron-induced hepatic fibrosis and cirrhosis. Treatment with deferoxamine slows or prevents iron-induced liver damage and may reduce the severity of preexisting fibrosis in some cases.[146,278,279]

Pigmentary gallstones caused by high levels of bilirubin production from red cell turnover are found in an increasing number of patients older than 4 years of age. Two-thirds of patients have multiple calcified bilirubinate calculi after the age of 15 years.[280] Cholecystectomy is not routinely indicated unless biliary colic or obstructive jaundice has occurred.

Heart

Cardiac abnormalities are important causes of morbidity and mortality in patients with thalassemia major. Cardiomegaly secondary to anemia is almost always present in untransfused children. Before the availability of chelation therapy, myocardial hemosiderosis and serious iron-induced cardiac diseases were inevitable during the second decade, usually the cause of mortality. These problems still occur often in older patients with thalassemia who are poorly compliant with chelation therapy, and heart disease, usually in the form of cardiac failure or serious arrhythmias, remains the most common cause of death in patients with thalassemia major.[281,282]

Left-sided heart failure predominates in patients with thalassemia major and is characterized by dyspnea and orthopnea.[283] Right-sided heart failure is less common but may be the presenting cardiac finding in older patients with more severe iron overload. Diastolic dysfunction develops first, with few symptoms, but if this progresses to systolic dysfunction, the resulting heart failure is acute in onset and very difficult to treat.[284,285] Acute myocarditis, which occurs in approximately 5% of patients with thalassemia, is frequently followed by acute or chronic heart failure.[286]

Early electrocardiographic abnormalities include a prolonged P–R interval, first-degree heart block, and premature atrial contractions. Later, ST-segment depression and ventricular ectopic beats constitute ominous indicators of myocardial damage. Periodic evaluation of cardiac function is essential to detect iron-induced heart disease and to identify patients who will benefit from more intensive chelation therapy (see later discussion). Unfortunately, by the time cardiac results of studies such as echocardiography and 24-hour rhythm monitoring become abnormal, clinical heart disease is imminent. Whether assessment of cardiac iron by MRI using T2* or other measures can better anticipate the development of clinical heart disease is currently under investigation.

In the absence of intensified chelation therapy, ventricular dysfunction progresses rapidly to chronic refractory congestive heart failure, and arrhythmias become increasingly difficult to control. In the past, death usually occurred within 1 year of onset of heart failure. More recent data demonstrate a survival rate of 48% at 5 years.[283] Survival is notably poorer in patients with heart failure after myocarditis or with heart failure accompanied by arrhythmias.[286]

In addition to standard therapy for heart failure and arrhythmias, including angiotensin-converting enzyme inhibitors, β-blockers, diuretics, and antiarrhythmic agents, the pretransfusion Hb level should be maintained between 10 and 12 g/dL. The volume of transfused RBCs should be reduced as needed to prevent acute fluid overload. Because the iron-overloaded myocardium has little capacity to improve its performance unless excess iron is removed, intensive chelation therapy is a critical part of the management of heart disease in patients with thalassemia. Several studies have shown that heart failure can be reversed in many patients with the use of continuous treatment with deferoxamine.[164,287,288] The benefits of this approach may derive from the reduction in cardiac iron stores, the prevention of acute toxicity from nontransferrin-bound iron, or a combination of these two mechanisms. Data suggest that deferiprone may be more effective than deferoxamine in reducing the cardiac iron load and treating iron-induced cardiac disease, perhaps because of deferiprone's ability to enter cardiac cells more rapidly than deferoxamine.[122,195] On the other hand, it may not be as effective in reducing hepatic iron content.[122,195,289] Deferasirox treatment for 1 to 2 years has also been shown to reduce cardiac iron and improve cardiac MRI T2* in patients with transfusional iron overload.[290,291] In patients who have undergone hematopoietic stem cell transplantation (HSCT), improvements in left ventricular contractility and diastolic function accompany the removal of excess iron by phlebotomy.[292] Heart transplantation and combined heart-liver transplantation have been performed successfully in patients with end-stage cardiac disease.[293–295]

Sterile pericarditis occurs in some patients with massive iron overload.[296] Although pericarditis is most often attributed to hemosiderosis, an association with β-hemolytic streptococcal infection and other infectious agents has also been suggested.[297] Therapy usually consists of bed rest, treatment of infection, management of superimposed congestive heart failure, and the use of salicylates or corticosteroids. Occasionally, pericardiectomy may be indicated.

Lungs

Mild abnormalities of pulmonary function are common in patients with thalassemia but rarely cause clinical problems.[298,299] Some patients exhibit primarily restrictive defects[300,301]; others experience mild to moderate small airway obstruction and hyperinflation.[302–304] Most patients have a decreased maximal oxygen uptake and anaerobic threshold; these do not normalize after transfusion.[305] Postsplenectomy thrombocytosis and other prothrombotic changes can predispose to pulmonary vascular occlusion and pulmonary hypertension, more so in NTDT patients compared with well transfused TDT patients.[211,306–309] Treatment with high doses of the iron chelator deferoxamine may also be associated with acute deterioration of pulmonary function.[159,160]

Kidneys

The kidneys are frequently enlarged, partly because of EMH and partly because of marked dilation of the renal tubules.[310] The urine is often dark brown, reflecting the excretion of products of heme catabolism.[311] The urine also contains large amounts of urates and uric acid.

The Thalassemia Clinical Research Network studied the prevalence of renal abnormalities in patients with thalassemia major and thalassemia intermedia receiving deferoxamine chelation. One-third of thalassemia patients who were not regularly transfused had abnormally high creatinine clearance. Regular transfusions were associated with a decrease in clearance ($P = .004$). Almost one-third of patients with thalassemia had hypercalciuria, and regular transfusions were associated with an increase in the frequency and degree of hypercalciuria ($P < .0001$). Albuminuria was found in more than half of patients but was not consistently associated with transfusion therapy. In summary, renal hyperfiltration, hypercalciuria, and albuminuria are common in patients with thalassemia.[312] Higher transfusion intensity is associated with lower creatinine clearance but more frequent hypercalciuria.[313]

Deferasirox may also cause some renal dysfunction, ranging from mild proteinuria to a reduction in glomerular filtration rate (GFR) and increase in creatinine. Patients receiving this agent for chelation should be carefully monitored.[314]

Spleen and Splenectomy

Splenomegaly is common in thalassemia. It is initially a result of EMH, and once regular transfusions are initiated, will regress to near normal. However, in NTDT patients with moderate anemia, it may continue to enlarge in an attempt to compensate for the degree of anemia. Over time, very enlarged spleens may also become hypersplenic leading to the decision to splenectomize. After splenectomy, there may be some improvement in the Hb level, but this may come at a price—the development of vascular complications, as discussed later. In TDT, with continuing regular transfusions, mild or moderate splenomegaly may be associated with findings of hypersplenism, including thrombocytopenia, neutropenia, and increasing anemia. The usual indication for splenectomy in regularly transfused patients is a progressive increase in transfusion requirements caused by hypersplenism. The transfusion requirements, and therefore the rates of iron loading, of splenectomized patients are often considerably less than those of patients whose spleens are intact.[289,315–317] A transfusion requirement of more than 180 to 200 mL/kg/year of packed RBCs usually represents excessive RBC requirements.[316,317] For such patients, a 25% to 60% reduction in transfusion requirements after splenectomy is generally predictable. Before attributing increased transfusion requirements to hypersplenism, it is important to look for other causes, such as RBC alloimmunization or a change in the hematocrit of the units of donor blood. Surgery should be deferred until after 5 years of age whenever possible to maximize the development of the humoral immune system, because of the greater risk of postsplenectomy sepsis in younger patients. For well-transfused and well-chelated patients, splenectomy may have little benefit, and some centers have noted a significant decline in the number of patients undergoing splenectomy in recent years.

Laparoscopic splenectomy has proved safe for patients with thalassemia and has dramatically shortened the recovery time compared with open procedures.[318] Partial splenectomy and partial dearterialization of the spleen have been suggested as alternative approaches to reducing blood requirements without incurring the risk of sepsis.[319–321] The long-term benefits of this approach remain uncertain. Therapeutic embolization of the spleen avoids the need for surgery,[322–324] but this approach is frequently associated with postprocedure pain and fever and does not permit the removal of accessory spleens.

After splenectomy, striking thrombocytosis may occur, which may require thrombosis prophylaxis or platelet deaggregating agents.[325] Increased numbers of nucleated RBCs appear in the blood, and the presence of many RBCs containing inclusion bodies composed of precipitated α-globin chains can be demonstrated by staining with supravital staining. The release of abnormal red cells from the marrow as a result of IE, with the lack of a splenic filter, adds an additional risk. The latter, combined with thrombocytosis, results in a coagulopathy[326] which may lead to significant vascular disease, particularly the development of cerebral infarcts and pulmonary hypertension. This is more of an issue in NTDT intermedia patients than well transfused patients in whom the IE is adequately suppressed.[327–329] However, this is a major rationale for the declining use of splenectomy.

Splenectomy should generally be reserved for patients with excessive transfusion requirements from hypersplenism and difficulty controlling iron overload. A large spleen alone does not usually cause significant clinical problems and should rarely, if ever, be the sole reason for splenectomy. Patients with thalassemia major are at significant risk for the development of overwhelming, often fatal, infection after splenectomy (postsplenectomy sepsis syndrome).[330] The problem is most common in young children. *Streptococcus pneumoniae* causes two-thirds of cases; *Hemophilus influenzae* type B and *Neisseria meningitidis* account for most of the remaining infections. Typically, there is a fulminant clinical course, proceeding from mild fever and headache to hyperpyrexia, prostration, shock, and death within 6 to 12 hours. Before splenectomy, polyvalent pneumococcal, meningococcal, and *H. influenzae* vaccines should be administered if they have not been given earlier in life.[331,332] Prophylactic oral penicillin therapy, 250 mg twice daily, is generally used as prophylaxis against postsplenectomy infection in patients with thalassemia. However, the optimal duration of penicillin prophylaxis remains unknown, and compliance is frequently inadequate.[333] Although the risk of postsplenectomy sepsis decreases with age, it does not disappear, and fatal pneumococcal sepsis has occurred many years after removal of the spleen.[334]

Immunization against the most common pathogens before splenectomy, prophylaxis with antibiotics, and early assessment of fever after splenectomy have dramatically reduced the incidence of fatal postsplenectomy sepsis.

Survival in Patients With Thalassemia Major

Improved transfusion therapy and the consistent use of iron chelation therapy have extended the life span of patients with thalassemia major.[83,281,282,335] In a multicenter study of 1079 patients in Italy, the probability of survival to age 20 years was 96% for patients born between 1975 and 1979, the time at which chelation therapy became a regular part of the overall management of thalassemia major (Table 41.3).[281] In contrast, the probabilities of survival at 20 years of age were only 61% and 69% for those born in the periods of 1960 through 1964 and 1965 through 1969, respectively. Other investigators have shown that survival or prevention of life-threatening complications is strongly related to good chelation therapy, assessed either by compliance or by control of iron stores (Fig. 41.17).[125,168,282] The importance of good compliance with chelation therapy is further demonstrated by data from the United Kingdom showing that the probability of survival for the 1975 through 1984 birth cohort is to date not substantially different than the probability of survival for the 1965 through 1974 birth cohort.[335] The researchers attribute this poorer than expected survival rate, despite the availability of deferoxamine, to a lack of adherence to the recommended schedule of treatment with this chelator.

TABLE 41.3 **Survival by Birth Cohort at Different Ages of Patients With Transfusion-Dependent Thalassemia**

Patient Age (Years)	Cohort (%)		
	1970–1974	1975–1979	1980–1984
10	98 (96–99)	98 (96–99)	99 (95–100)
15	95 (92–97)	97 (94–98)	98 (93–100)
20	89 (85–92)	96 (93–98)	
25	82 (77–86)		

Data from Borgna Pignatti C, Rugolotto S, De Stefano X, et al. Survival and disease complications in thalassemia major. *Ann N Y Acad Sci.* 1998;850:227.

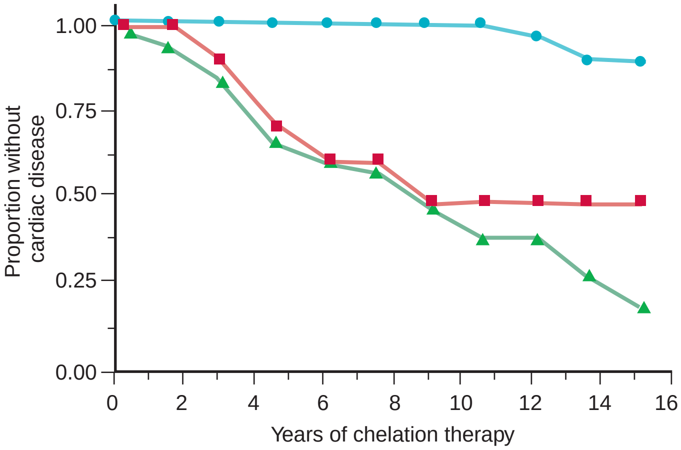


Figure 41.17 SURVIVAL WITHOUT CARDIAC DISEASE IN PATIENTS WITH THALASSEMIA MAJOR TREATED WITH DEFEROXAMINE ACCORDING TO THE PROPORTION OF SERUM FERRITIN MEASUREMENTS EXCEEDING 2500 ng/mL. The *circles* show cardiac disease-free survival among patients in whom less than 33% of ferritin measurements exceeded 2500 ng/mL; *squares* show survival among patients in whom 33% to 67% of ferritin measurements exceeded 2500 ng/mL; and *triangles* show survival among patients in whom more than 67% of ferritin measurements exceeded 2500 ng/mL. *(Adapted from Olivieri NF, Nathan DG, MacMillan JH, et al. Survival in medically treated patients with homozygous beta-thalassemia. N Engl J Med. 1994;331:574.)*

Consistent compliance with chelation to maintain a near-normal iron balance is undoubtedly the key to preventing complications in regularly transfused patients. Currently, with the advances in management of thalassemia, including a safer blood supply, lower rates of alloimmunization, oral chelators, improved methods of quantifying organ iron and tailoring chelation regimens, it may be fair to say that TDT individuals who are optimally managed with transfusions and chelation should anticipate near normal lifespans.

Chronic Care of the Adult Patient With Thalassemia

Adults with TDT syndromes are living longer into adulthood, with some approaching their seventh decade of life. It is likely in the coming decades that these patients will have improved life expectancies related to safer transfusion practices, improved screening of the blood supply, and better oral iron chelation medications. Therefore, the thalassemia patient born in the current era may expect to spend the majority of their life as an adult.

TABLE 41.4 Nontransfusion-Dependent Thalassemia Screening Recommendations

Test Name	Measurement	Frequency
MRI with T2* liver iron content	Liver iron	Every 1–2 years
MRI with T2* cardiac iron content	Cardiac iron	Every 1–2 years
Ferritin	Total body iron	Every 3-6 months
History and physical exam	General health, medication compliance	Every 3–4 months
Echocardiogram	Pulmonary hypertension TRV	Every 1–2 years
Liver function panel	Liver failure, hepatitis	Every 3-6 months
Liver ultrasound (if LIC >5 persistently)	Cirrhosis	Annually
AFP (if >40 or presence of clinical cirrhosis)	Hepatocellular carcinoma	Annually
Hepatitis B, C serologies (if receiving blood transfusions)	Hepatitis B and C viral infection/exposure	Annually
Free T4, TSH	Thyroid function	Annually
Calcium, phosphate, vitamin D	Parathyroid function	Annually
Fasting blood sugar/oral glucose tolerance test	Diabetes mellitus screening	Annually
DEXA	Bone mineral density	Annually

ACTH, Adrenocorticotropic hormone; *AFP*, α-fetoprotein; *DEXA*, dual-energy X-ray absorptiometry; *LIC*, liver iron concentration; *MRI*, magnetic resonance imaging; *TRV*, tricuspid regurgitant velocity; *TSH*, thyroid stimulating hormone.

TABLE 41.5 Transfusion-Dependent Thalassemia Screening Recommendations

MRI with T2* liver iron content	Liver iron	Every 1 year
MRI with T2* cardiac iron content	Cardiac iron	Every 1–2 years
Ferritin	Total body iron	Every 3 months
History and physical exam	General health, medication compliance	Every 3 months
Echocardiogram (if cine MRI not able to provide adequate information, or in years when cardiac MRI not done)	Pulmonary hypertension TRV	Every 1–2 years
Liver function panel	Liver failure, hepatitis, deferasirox toxicity	Every month
AFP (if > 40 or presence of clinical cirrhosis)	Hepatocellular carcinoma screening	Annually
Hepatitis B, C serologies	Hepatitis B and C viral infection/exposure	Annually
Free T4, TSH	Thyroid function	Annually
Calcium, phosphate, vitamin D	Parathyroid function	Annually
Fasting blood sugar/oral glucose tolerance test	Diabetes mellitus screening	Annually
DEXA	Bone mineral density	Annually
Dental evaluation	Maxillofacial disease, periodontal disease, caries	6–12 months

ACTH, Adrenocorticotropic hormone; *AFP*, α-fetoprotein; *AP*, alkaline phosphatase; *CTX*, collagen type 1 cross-linked C-telopeptide; *DEXA*, dual-energy X-ray absorptiometry; *LIC*, liver iron concentration; *MRI*, magnetic resonance imaging; *NTX*, N-terminal telopeptide; *TRV*, tricuspid regurgitant velocity; *TSH*, thyroid stimulating hormone.

There is currently limited expertise among adult hematologists in the management of adult thalassemia patients. Transition plans are essential for all pediatric patients as they enter adulthood, so that they may receive age-appropriate care. Transition is especially important in thalassemia, as they require the regular uninterrupted schedule of transfusions and chelation monitoring. Future efforts should be initiated to improve the care of adult patients with thalassemia, including understanding the pathology of the disease and training new generations of hematologists with an understanding of thalassemia syndromes.

Screening tests are also an important component of ongoing care in thalassemia. Currently, the most common cause of mortality in adults with thalassemia is related to cardiac disease and arrhythmias. These patients also have other chronic illnesses including cardiomyopathy, hepatic disease, endocrinopathies, osteoporosis, hypogonadism, pulmonary hypertension, and ongoing infectious risk from splenectomies. Recommended screening tests are shown in Tables 41.4 and 41.5, based on recommendations from the Thalassemia International Federation.[207] Regular follow-up with an internal medicine primary care physician is also recommended to make sure adults are aware of current screening and treatment guidelines applicable to the general population, for example age-appropriate cancer screening, an area where adult patients may not have previously been referred because of historically shorter lifespans.

Hematopoietic Stem Cell Transplantation

The only curative treatment available for thalassemia currently is HSCT. The current status of HSCT in general is discussed further in Chapters 105–107. There is now extensive experience with transplantation, with several thousand patients having undergone the treatment.[336] Historically, risk classification was based on three criteria: hepatomegaly, the degree of portal fibrosis, and the degree of iron overload.[337] In class 1 without adverse factors, the overall survival rate is 95%, and the event-free survival rate (without thalassemia) is 90%; class 2 patients with one or two risk factors have an 85% survival and an 81% event-free survival; and class 3 patients with all three risk factors have only 64% and 62% overall and event-free survival rates, respectively (Fig. 41.18).[337] Advances in conditioning regimens have considerably improved the outcome of class 3 patients who are younger than 17 years of age. Preparatory chemotherapeutic regimens to enhance immunosuppression and eradicate thalassemic clones using hydroxyurea, azathioprine, fludarabine, busulfan, and cyclophosphamide have increased the survival rate of class 3 patients to 93%; the rejection rate fell to 8%.[338] These favorable results have not been reproduced in the older, more heavily iron-overloaded patients who remain high risk for transplant-related mortality.[339]

While the criteria from the above classification are still relevant, currently, outcomes of HSCT in thalassemia are mostly dependent on two critical considerations: (a) the characteristics of the donor and (b) the preexisting morbidities in the recipient. The outcomes are superior when the donor is a matched relative, typically a sibling.[340] Although other donor types have been used in the clinical trial setting, outcomes are inferior from matched unrelated donors or any mismatched donor, including a haploidentical donor. However, over the years, results have improved quite significantly. In view of the available evidence, stem cell transplantation from a partially human leukocyte antigen (HLA)-matched relative is not routinely advisable, although it may be considered in extreme situations when transfusion support is impossible or life threatening when a patient is completely noncompliant with any type of iron chelation therapy. Additionally, alternative

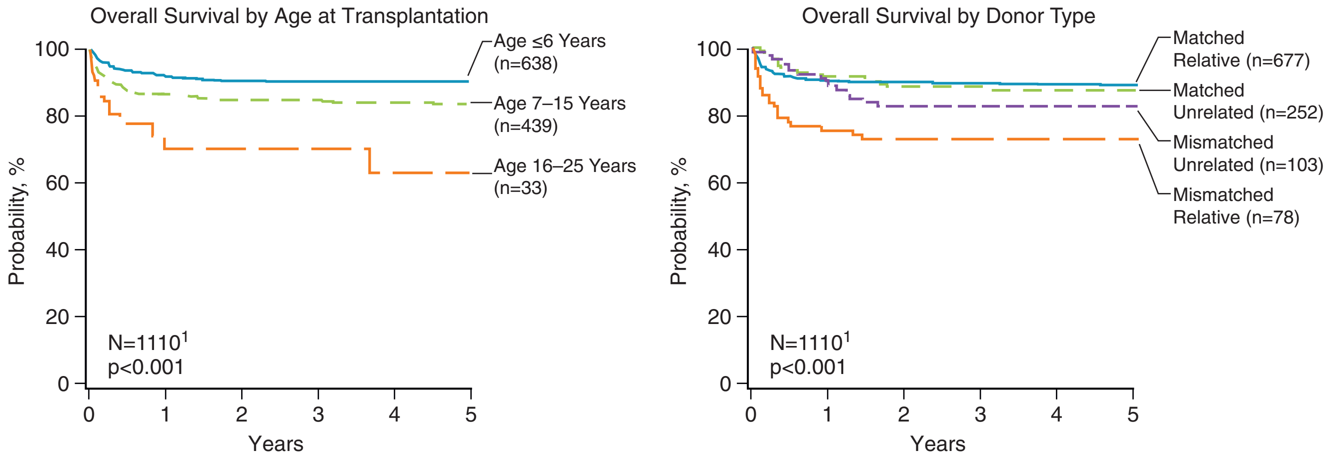


Figure 41.18 FAVORABLE OUTCOMES FOLLOWING ALLO-HSCT ARE ACHIEVED IN YOUNGER PATIENTS WITH HLA-MATCHED RELATED DONOR. The greatest 5-year probability of overall survival was observed in patients ≤ 6 years of age or who had an HLA-matched related donor.[1] Only a minority of patients with TDT undergo allo-HSCT with the majority of those being less than 14 years old with an HLA-matched sibling donor, well controlled iron, and lacking iron-related morbidities.[2–6] *Allo-HSCT*, Allogeneic hematopoietic stem cell transplantation; *HLA*, human leukocyte antigen; *TDT*, transfusion-dependent β-thalassemia.

References: [1]Li C, Mathews V, Kim S, et al. Related and unrelated donor transplantation for β-thalassemia major: results of an international survey. *Blood Adv*. 2019;3(17):2562–2570. [2]Baronciani D, Angelucci E, Potschger U, et al. Hemopoietic stem cell transplantation in thalassemia: a report from the European Society for Blood and Bone Marrow Transplantation Hemoglobinopathy Registry, 2000–2010. *Bone Marrow Transplant*. 2016:51(4):536–541. [3]Marziali M, Isgrò A, Gaziev J, Lucarelli G. Hematopoietic stem cell transplantation in thalassemia and sickle cell disease. Unicenter experience in a multi-ethnic population. *Mediterr J Hematol Infect Dis*. 2009;1(1):e2009027. [4]Angelucci E, Baronciani D. Allogeneic stem cell transplantation for thalassemia major. *Haematologica*. 2008;93:1780. [5]Shenoy S, Walters MC, Ngwube A, et al. Unrelated donor transplantation in children with thalassemia using reduced-intensity conditioning: The URTH Trial. *Biol Blood Marrow Transplant*. 2018;24:1216–1222. [6]See WSQ, Tung JYL, Cheuk DKL, et al. Endocrine complications in patients with transfusion-dependent thalassaemia after haemopoietic stem cell transplantation. *Bone Marrow Transplant*. 2018;53:356–360.

donor sources such as umbilical cord blood also have been used with some success, but because of the limited number of stem cells in each cord blood unit, this is limited to smaller children. Preexisting morbidities such as hepatomegaly, with or without fibrosis, and severe (inadequately treated) iron overload, increase the risk of failure.[336,337]

Stem cell transplantation can fail or be lethal owing to its immunologic complications. The complications related to the procedure are similar to those that would occur in other allogeneic transplants and are related to preparative myeloablative chemotherapy, the risk of infection during the neutropenic period prior to engraftment, the occurrence of graft-versus-host disease (GVHD), and loss of the graft. The overall incidence of acute GVHD is 17% to 32% depending on the prophylaxis regimen, and the incidence of chronic GVHD is 27% in patients receiving hematopoietic stem cells from an HLA-matched parent or sibling.[341] In addition, side effects, such as infertility and clonal disease from the conditioning chemotherapy, are sometimes deterrents for patients who are seeking a curative approach.

Novel Therapeutic Approaches

Major advances in the care of individuals with thalassemia have significantly increased life expectancy in this disease. However, the current paradigm of care remains very cumbersome and has a major impact on quality of life of these individuals and their families. Optimal care requires frequent visits to the hospital for transfusions, monitoring and doctor visits, daily intake of medications with attendant side effects, some persistent symptoms of chronic anemia even in transfused patients, and the potential for complications related to the treatment regimen. Individuals lose days at work or in school, have reduced family time, and suffer an ongoing drain on financial resources, not to mention the psychosocial impairment of dealing with a chronic disease. Knowing the inexorable course of the disease, parents and patients with the option of cure by HSCT most often avail of it, despite the known risk of complications described. However, many individuals do not have a matched related donor, and are thus left to manage a complex disease. Individuals with more severe thalassemia intermedia, while on a less demanding regimen, must cope with anemia and the complications related to IE. Therefore, there remains a significant unmet need for individuals with thalassemia—the need for therapies that would affect the course and provide relief from the burden of the disease and its management.

Over the past decade, major advances have been made thanks to technological developments which have opened a new era of targeted agents and gene therapy. Several approaches have been advanced, and many of these are already in advanced stages of clinical trials with impending regulatory approval for clinical application. These are summarized in Fig. 41.19). A brief description of each emerging modality follows.

Augmentation of fetal Hb production. In the absence of adequate β-globin production, if the excess α globin chains could be bound with γ globin, to produce HbF, this could increase the Hb level and ameliorate IE to a variable extent, thus modifying the pathophysiologic course of the disease.[342] Hypomethylation of the γ-globin genes can be induced by the drug 5-azacytidine; indeed, short-term administration of this drug produced the predicted effect in vivo.[343–345] Despite much subsequent experimental work, it remains unclear whether the effect was attributable to direct stimulation of fetal genes by demethylation or to recruitment and accelerated differentiation of primitive burst-forming unit-erythroid progenitor cells, which have greater potential to produce HbF.[346–348] Hydroxyurea has an effect on burst-forming unit-erythroid similar to that of 5-azacytidine and is a safer drug for long-term use.[347,349] Short-term as well as longer trials with hydroxyurea have been reported in a number of patients with thalassemia.[350–357] HbF levels frequently increased without a proportionate increase in total Hb level. A small improvement in total Hb level occurred in some patients with thalassemia intermedia but usually does not exceed 1 to 2 g/dL.[354,355] Most transfusion-dependent patients have shown no clinical benefit to hydroxyurea. Other approaches using butyrate analogues[358] also did not show sustained positive results. These pharmacological approaches have more or less been abandoned. Two novel strategies are now being employed to augment fetal Hb production.

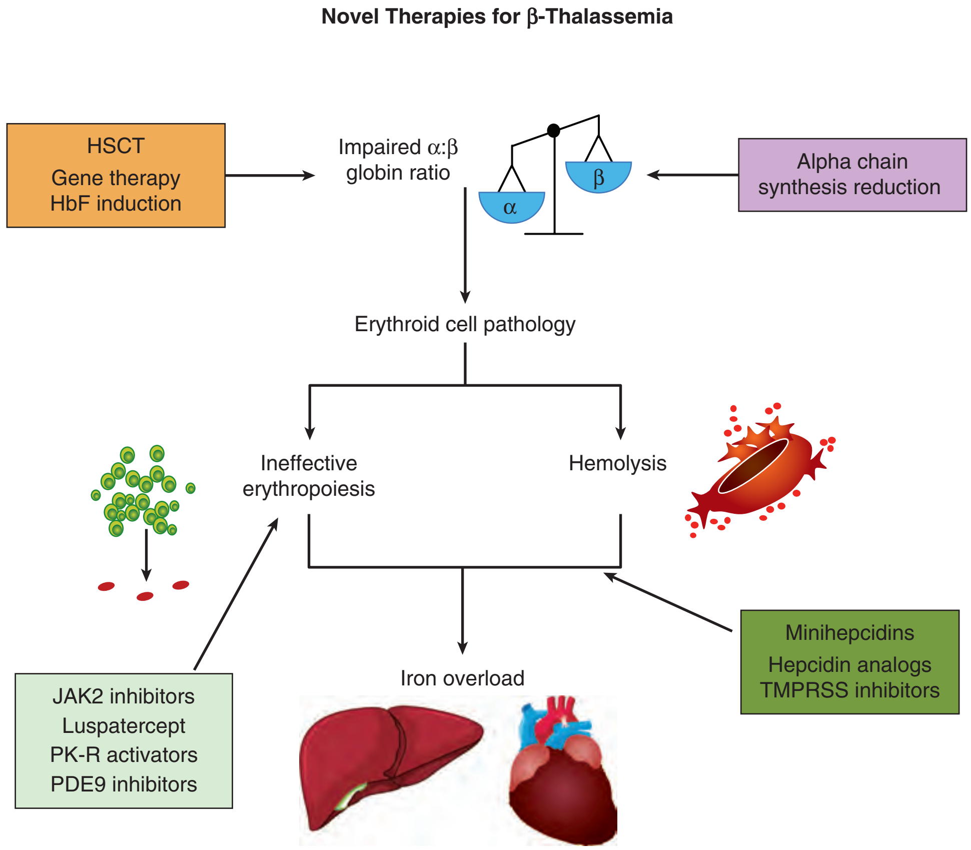


Figure 41.19 VARIED APPROACHES FOR NOVEL THERAPEUTIC INTERVENTIONS IN THE TREATMENT OF β-THALASSEMIA. *(Modified from Cappellini MD, Motta I. New therapeutic targets in transfusion-dependent and -independent thalassemia. Hematology Am Soc Hematol Educ Program. 2017[1]:278–283.)*

(i) Inhibition of phosphodiesterase 9 (PDE9) by the compound IMR-687. This has been shown in thalassemia mouse models to increase cGMP by inhibiting conversion of cGMP to GMP by PDE9. Early studies in patients with sickle cell disease showed increases in HbF and proportion of F cells. A clinical trial in thalassemia is in progress.[359]

(ii) Blocking the repression of γ-globin gene expression by BCL11A. Novel approaches based on our better understanding of the silencing of γ-globin gene expression by BCL11A[360] have led to the development of gene-editing strategies. BCL11A is a zinc-finger transcriptional repressor active in erythroid cells and in other hematopoietic lineages.[361] Several studies indicate that BCL11A silences γ-globin.[361–363] BCL11A does not bind the γ-globin promoter directly. Rather, it binds motifs in the LCR and different intergenic regions in the globin locus that were previously tied with γ-globin repression.[361,364–367] Knock down of BCL11A expression results in the reactivation of HbF expression.[368] Gene therapy or gene-editing techniques are used to modify the patient's own hematopoietic stem cells The corrected stem cells are transferred via an autologous hematopoietic stem cell transplant (Fig. 41.20). The patient's stem cells are collected by pheresis after mobilization from the marrow, purified, and sent to a central facility, where the cells undergo genetic modification. After myeloablative conditioning, the modified stem cells are reinfused. Once these engraft successfully, and begin to produce γ-globin, hemoglobin F containing 2 α-globin chains and 2 γ-globin chains is produced. Success depends on successful editing and engraftment of adequate numbers of stem cells. several approaches have been used and are in clinical trials: (a) introduction of a viral vector encoding an inhibitory RNA (RNAi) that interferes with the ability of BCL11a to interact with its cognate enhance sequence motifs; (b) targeting protein-DNA interactions using either transcription activator-like effector nucleases (TALENS) or zinc finger nucleases,[369] and (c) targeting RNA-DNA interactions using CRISPR-Cas-9.[370] Early results from this trial in 5 patients with thalassemia have shown successful engraftment of the edited stem cells with excellent expression of HbF, suggesting that this strategy is feasible and could result in durable continued expression of γ-globin, and thus production of HbF.

Targeted therapies aimed at ameliorating IE. Several molecules have been/are being developed with the aim of reducing IE and thus not only improving red cell production and reducing anemia, but also possibly preventing progressive bone disease as a result.[371] These include:

(i) Luspatercept: This is a recombinant fusion protein containing a modified extracellular domain of ActRIIB, which binds GDF11 and other TGF-β superfamily ligands, inhibits Smad2/3 signaling, and promotes RBC differentiation/maturation. As a result more proliferating erythroid precursors are able to mature, reducing intramedullary apoptosis, and ameliorating IE. Early studies in normal human volunteers showed a dose-dependent increase in Hb levels, and a good tolerability profile.[372] Phase II studies confirmed that IE was improved in patients with both TDT and NTDT, with clinically meaningful reductions in transfusion requirements.[373] The phase III 48-week BELIEVE study in adults with TDT met all of its primary and secondary endpoints for significant reduction in transfusion requirements by 33% to 50% compared with baseline. An extension phase is ongoing and will inform as to the durability of this therapy, which is now approved for clinical use.[374]

(ii) Ruxolitinib: This JAK2 inhibitor was developed based on the demonstration that JAK2 played a role mediating IE and its inhibition in the mouse model improved effective erythropoiesis and reduced splenomegaly.[375] However, the phase 2a study in individuals with TDT did not show a reduction in transfusion requirement, though there was significant reduction in spleen size.[376] No further studies using this agent are planned.

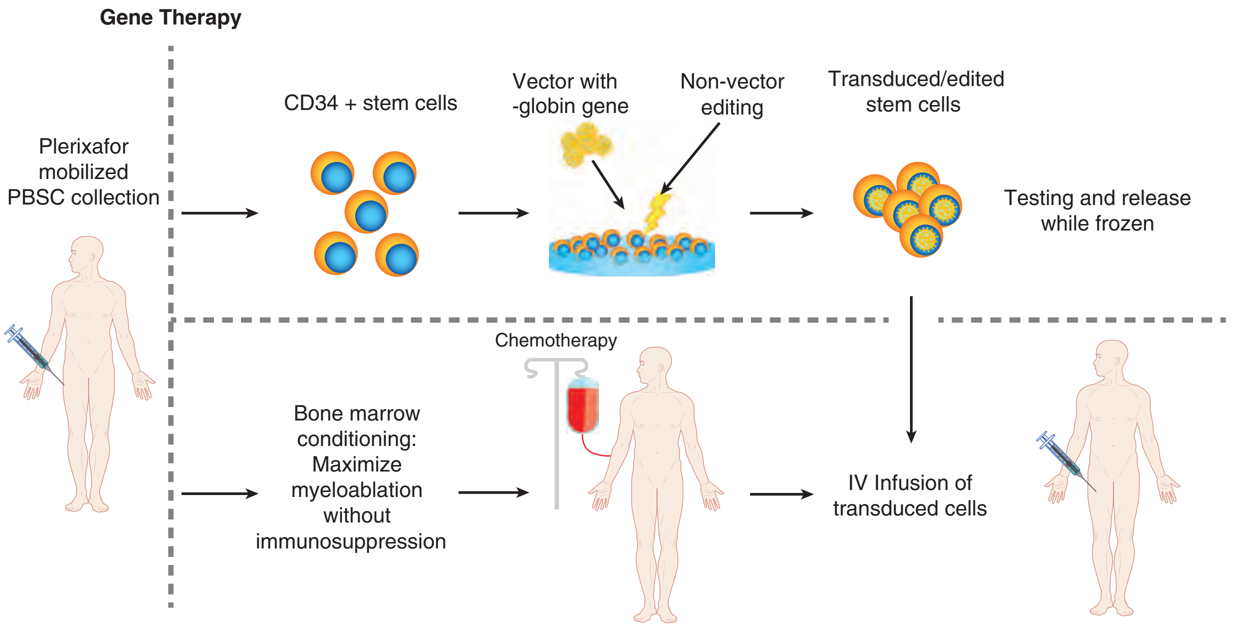

Gene Therapy
Plerixafor mobilized PBSC collection
CD34 + stem cells
Vector with -globin gene
Non-vector editing
Transduced/edited stem cells
Testing and release while frozen
Chemotherapy
Bone marrow conditioning: Maximize myeloablation without immunosuppression
IV Infusion of transduced cells

Figure 41.20 SCHEMA FOR AUTOLOGOUS TRANSPLANTATION OF STEM CELLS MODIFIED BY LENTIVIRAL GENE ADDITION OR CRISPR CAS9 GENE EDITING.

(iii) Hepcidin analogs: A clearer understanding of IE in thalassemia and the interaction between erythropoiesis and hepcidin via ERFE led to the use of hepcidin analogs in the mouse model of thalassemia.[377] Treated mice showed a reduction in spleen size and improvement of anemia.[378] A potential additional benefit would be to directly inhibit iron absorption and slow the development of iron overload. Further, increasing hepcidin would sequester iron in stores, prevent release, and therefore reduce iron exposure of the heart and endocrine organs. Clinical trials of these analogs have been initiated in humans, looking for reductions in transfusion requirement as the endpoint.[379] In a different study, patients with thalassemia and transfusional iron overload were given hepcidin to study its effect on myocardial iron.[380] However, this trial was terminated after the interim analysis did not show any beneficial effect.

(iv) AG-348 (Mitapivat): Improving aerobic glycolysis and reducing oxidative stress in developing erythroid precursors could ameliorate apoptosis and IE and increase production of red cells. Further, these red cells may also have a longer lifespan. Mitapivat is an oral, small molecule allosteric activator of wild-type and a variety of mutated pyruvate kinase-R (PKR) enzymes. In non-transfusion-dependent individuals with β thalassemia intermedia and HbH disease, early short-term phase II data show an increase in Hb levels and a reduction in markers of hemolysis. This agent is in clinical trials in NTD patients,[381] and trials in TDT are planned.

(v) FT-4202 is another pyruvate kinase activator which has shown benefit in sickle cell disease, with the same mechanism of action postulated as AG348. Clinical trials in thalassemia are also planned.

Correction of the defective globin gene. The most advanced and exciting curative strategy for the thalassemias is aimed at correcting the defective gene. The only reliable conventional curative treatment, allogeneic HSCT from a matched related donor, is available to a limited proportion of patients with such a donor. A gene therapy approach, being autologous, greatly expands the pool of patients who could be cured.[382–387]

This is done either by adding a new copy of the gene or possibly by correcting the defective gene through CRISPR/Cas-9 splicing. Gene addition strategies are in fairly advanced stages of clinical trials. The first successful gene therapy for β-thalassemia was reported in 2010, when a patient with HbE/β^0 thalassemia was cured using a lentiviral vector.[388] The corrected stem cells are transferred via an autologous hematopoietic stem cell transplant (see Fig. 41.20). The patient's stem cells are collected by pheresis after mobilization from the marrow, purified, and taken to the gene transfer facility. A functional copy of the β-globin gene (or possibly γ-globin gene) is introduced into the stem cells via transfection by a lentiviral vector. After myeloablative conditioning, the modified stem cells are reinfused. Once these engraft successfully, and begin to produce β-globin, hemoglobin A, containing 2 α-globin chains and 2 β-globin (normal or modified) chains is produced. The efficacy of the process depends on successful transfection of the stem cells in adequate numbers, the engraftment of these cells, and the maintenance of a high vector copy number (VCN) per cell to ensure adequate β-globin production. An added benefit of this approach is the virtual absence of graft-versus-host disease in the autologous setting.

Two vectors are currently in trials:

(i) BB305 vector: This vector (Fig. 41.21) encodes a β-globin gene which produces a β-globin chain with a single amino acid substitution—T87Q—resulting in the production of HbAT87Q, thus allowing easy measurement of the gene product as different from endogenous HbA production. Early clinical trials using this vector demonstrated proof of principle early, and a proportion of patients with both non-β^0/β^0 (and β^0/β^0 genotypes[389,390] showed robust production of the HbAT87Q, with transfusion independence, more in the non-β^0/β^0 patients. Patients who did not respond as well still experienced a reduction in transfusion requirements. Single agent busulfan myeloablative conditioning was used. The therapy was safe, with no deaths or graft failures during the trial, and all complications were expected and similar to those seen from myeloablative conditioning in allogeneic HSCT, including neutropenic fevers, mucositis, and veno-occlusive disease. No graft-versus-host disease was noted. Furthermore, vector integration was polyclonal, and there was no vector-mediated replication-competent lentivirus detected, thus providing reassurance that there was a very low risk for insertional mutagenesis. In addition, there was stable transfection, with a VCN in the 0.6 to 0.7 copies per cell, and stable production of HbAT87Q in most patients.[391] These patients have now been followed for approximately 5 years, and the graft appears durable.

Clinical trials using the same vector but manufactured with a refined process are currently underway in non-β^0/β^0 patients[392] and β^0/β^0 patients,[393] and preliminary results show improved VCNs in the 2 to 3 range, with a correspondingly greater production of

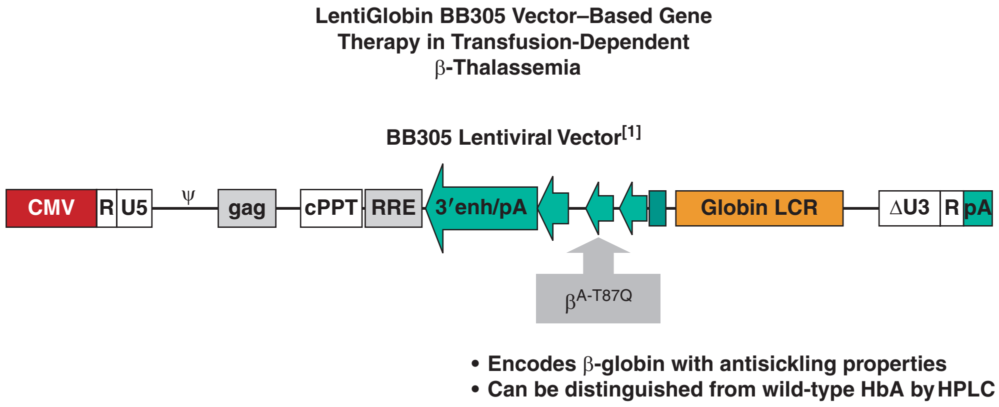

LentiGlobin BB305 Vector–Based Gene Therapy in Transfusion-Dependent β-Thalassemia
BB305 Lentiviral Vector[1]
CMV
R
U5
ψ
gag
cPPT
RRE
3′enh/pA
Globin LCR
ΔU3
R
pA
βA-T87Q
• Encodes β-globin with antisickling properties
• Can be distinguished from wild-type HbA by HPLC

Figure 41.21 CONSTRUCT OF THE BB305 VECTOR USED FOR LENTIVIRAL GENE ADDITION TO HEMATOPOIETIC STEM CELLS FOLLOWED BY AUTOLOGOUS TRANSPLANT.

HbAT87Q and thus a greater proportion of patients with transfusion independence. These data are being updated regularly, and so far, results are very promising, with success rates similar to those following allogeneic HSCT from a matched related donor.

(ii) GLOBE vector: Also a lentiviral vector, this contained a normal human β-globin gene with no modifications. Other differences from the BB305 trials were the use of thiotepa and treosulfan for myeloablative conditioning, and the introduction of the modified cells directly into the BM to avoid passage through, and possible clearance by the spleen. Though still early, the preliminary results from this phase I/II trial[394] have been positive.[395]

ALPHA-THALASSEMIA SYNDROMES

The α-thalassemias also occur widely throughout the world, including Africa, the Mediterranean countries, the Middle East, and Southeast Asia.[2,396,397]

As in β-thalassemia, there is some heterogeneity in presentation of the more severe forms of α thalassemia, but much less so. Genotype-phenotype correlation is consistent, and most of these individuals do not have a significant degree of IE, making them somewhat different than individuals with β-thalassemia. Since each individual normally has 2 α-globin genes on each chromosome 16, there are 4 genotypes of α-thalassemia, with 3, 2, 1, or no functioning α-globin genes.

The α-thalassemias are more difficult to diagnose because the characteristic elevations in HbA2 or HbF seen in β-thalassemia do not occur, precluding the use of hemoglobin fractionation for diagnosis. However, the gene deletions responsible for the most common varieties are readily detectable by molecular biology methods.[398]

Genetics

The four classic α-thalassemia syndromes are α^+-thalassemia trait, in which one of the four α-globin genes is non-functional; α^0-thalassemia trait, with two dysfunctional genes; HbH disease, with three affected genes; and hydrops fetalis with Hb Bart, in which all four genes are defective. In general, partial deletions are more deleterious and create a more severe phenotype than complete deletions.[399] In the older literature, α^0- and α^+-thalassemia are referred to as α-thalassemia-1 and α-thalassemia-2, respectively. Non-function of α-globin genes is usually deletional (Fig. 41.22). At the molecular level, α^+-thalassemia has been found to be associated with two common gene deletions resulting from different nonhomologous crossing-over events between the two linked α-globin genes: a 3.7-kb rightward deletion ($-\alpha^{3.7}$) resulting in a fused α2α1-globin gene and a 4.2-kb leftward deletion ($-\alpha^{4.2}$) resulting in loss of the 5′ (α2) gene.[400–402] One or two α gene deletions occur at a high frequency throughout West Africa, the Mediterranean, the Middle East, and Southeast Asia. In the United States, about 30% of Americans of African descent carry these defects. In some parts of Papua New Guinea up to 80% of the population are carriers. When two genes are deleted, this may occur in the *cis* or *trans* configuration and has implications for offspring should both parents have α-thalassemia trait. α^+-thalassemia trait inherited in *trans* configuration is very common in individuals of African or Middle Eastern ancestry, and since *cis* α^0-thalassemia deletion is rare in these individuals, HbH disease is rarely encountered, and hydrops fetalis has not yet been reported in individuals of African ethnicity.[403,404] α^0-thalassemia trait inherited in *cis* configuration is common in individuals of Asian ancestry, and hence HbH and hydrops are more commonly seen in these populations. α^0-thalassemia trait inherited in *cis* configuration is also found in Mediterranean populations, but less frequently than in Asians.

Nondeletional forms of α-thalassemia, which account for 15% to 20% of patients, arise from mutations similar to those described for β-thalassemia.[400,401] Fig. 41.23 illustrates the different α-thalassemia mutations and phenotypes. Structurally abnormal hemoglobins have also been associated with α-thalassemia. The Quong Sze α-globin chain ($\alpha^{125\text{Leu}\rightarrow\text{Pro}}$) is exceedingly labile and is destroyed so rapidly after its synthesis that no Hb tetramers containing the mutant α chain can be formed.[405] α^+-thalassemias also exhibit epistasis with haptoglobin variants that alters patterns in malaria protection.[406] How common the nondeletion forms of α^+-thalassemia are in any particular populations is uncertain, but they have been reported quite frequently in some of the Mediterranean island populations and in the Middle Eastern and Southeast Asian populations. The α-chain termination mutants, such as Hb Constant Spring, seem to be particularly common in Southeast Asia. Approximately 4% of the population in Thailand are carriers.

Previously, the diagnosis of α-thalassemia trait was usually one of exclusion, with a microcytic anemia and normal iron studies and Hb Fractionation. However, with the ready availability of globin gene analysis in the developed world, the diagnosis of α-thalassemia is now easier to make conclusively. These tests are also used for prenatal diagnosis of HbH or α-thalassemia major.

Molecular Pathology and Pathophysiology

As there is a relative excess of non-β-globins in β-thalassemia, there is an excess of non–α-chain production in the α-thalassemias, but the consequences are quite different. Alpha globin chains are required for HbF and HbA production, therefore, defective α-chain production is manifest in both fetal and adult life. In the fetus, excess γ-chain production results in formation of γ4 homotetramers or Hb Bart, while in the adult, excess of β chains forms β4 homotetramers or HbH. Hb Bart and HbH do not precipitate to any significant degree in the marrow, and therefore the α-thalassemias are not characterized by severe IE despite marked globin-chain imbalance in the severe α-thalassemias.[407] The erythroid marrow is more effective, and marrow

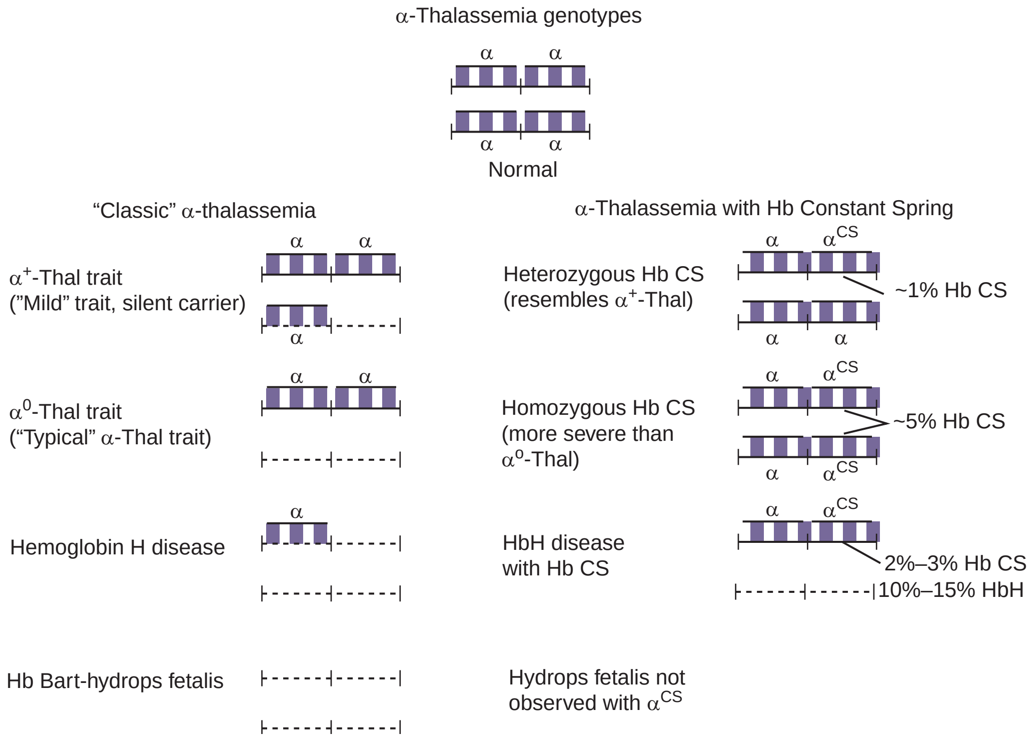

α-Thalassemia genotypes
α α
α α
Normal
"Classic" α-thalassemia
α-Thalassemia with Hb Constant Spring
α⁺-Thal trait
("Mild" trait, silent carrier)
α⁰-Thal trait
("Typical" α-Thal trait)
Hemoglobin H disease
Hb Bart-hydrops fetalis
Heterozygous Hb CS
(resembles α⁺-Thal)
~1% Hb CS
α αCS
Homozygous Hb CS
(more severe than
α⁰-Thal)
~5% Hb CS
HbH disease
with Hb CS
2%–3% Hb CS
10%–15% HbH
Hydrops fetalis not
observed with αCS

Figure 41.22 GENETIC ORIGINS OF THE "CLASSIC" α-THALASSEMIA SYNDROMES CAUSED BY GENE DELETIONS IN THE α-GLOBIN GENE CLUSTER. Hemoglobin Constant Spring is an α-globin-chain variant synthesized in such small amounts (1% to 2% of normal) that it has the phenotypic impact of a severe nondeletion α-thalassemia allele; however, the α^{cs} allele is always linked to a functioning α-globin gene, so it has never been associated with hydrops fetalis. *CS*, Constant Spring; *Hb*, hemoglobin.

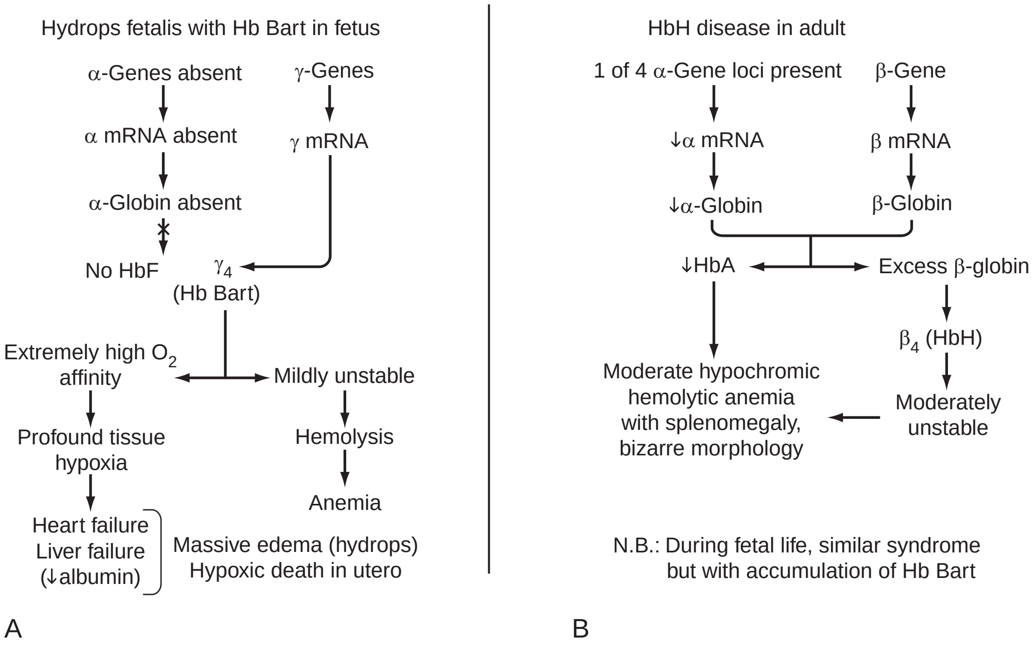

Hydrops fetalis with Hb Bart in fetus
α-Genes absent
γ-Genes
α mRNA absent
γ mRNA
α-Globin absent
No HbF
γ4
(Hb Bart)
Extremely high O2
affinity
Mildly unstable
Profound tissue
hypoxia
Hemolysis
Anemia
Heart failure
Liver failure
(↓albumin)
Massive edema (hydrops)
Hypoxic death in utero
A
HbH disease in adult
1 of 4 α-Gene loci present
β-Gene
↓α mRNA
β mRNA
↓α-Globin
β-Globin
↓HbA
Excess β-globin
β4 (HbH)
Moderate hypochromic
hemolytic anemia
with splenomegaly,
bizarre morphology
Moderately
unstable
N.B.: During fetal life, similar syndrome
but with accumulation of Hb Bart
B

Figure 41.23 PATHOPHYSIOLOGY OF HEMOGLOBIN H DISEASE AND HYDROPS FETALIS WITH HB BART. *Hb*, Hemoglobin; *mRNA*, messenger ribonucleic acid. *(Adapted from Benz EJ Jr. The hemoglobinopathies. In: Kelly WN, DeVita VT, eds. Textbook of Internal Medicine. Philadelphia: JB Lippincott; 1988:1423.)*

hyperplasia and EMH are not as significant. However, β4 tetramers precipitate as red cells age, with the formation of inclusion bodies which leads to a shortened survival of red cells from their damage in the microvasculature of the spleen. Therefore, anemia of the more severe forms of α-thalassemia in the adult results from hemolysis more than IE. Hb Bart is more stable than HbH and does not form large inclusions. Also, both Hb Bart and HbH show no heme-heme interaction and have almost hyperbolic oxygen dissociation curves with very high oxygen affinities. They are, therefore, not able to effectively release oxygen at physiologic tissue tensions making them very poor oxygen carriers, which exacerbates hypoxia from the anemia, with potentially severe consequences. Infants with high levels of Hb Bart in α-thalassemia major have severe intrauterine hypoxia, which is probably responsible for the enormously hypertrophied placentas, the severe erythroblastosis, the gross hydropic state of the fetus as a result of increased capillary permeability and possibly for the associated developmental abnormalities that occur with the severe forms of intrauterine α-thalassemia.

The two α-globin genes on each chromosome, α1 and α2, do not function identically. Studies of newborns from the archipelago of Vanuatu in the Southwest Pacific and from Papua New Guinea indicate that homozygotes for the rightward $-\alpha^{3.7III}$ deletion (where only a fused α*2*α1-globin gene, mostly of the α2 type, remains) have lower Hb Bart levels (3.5% ± 0.8%) than those of infants homozygous for the leftward $-\alpha^{4.2}$ deletion (in whom only the α1-globin gene remains) (6.0% ± 1.4%). These results suggest that the 5′ α2-globin gene has a higher output than the 3′ α1-globin gene, a conclusion supported by direct measurement of α2/α1 mRNA ratios.[408,409]

Clinical Manifestations and Diagnosis

Silent Carrier (α^+-Thalassemia Trait)

Individuals with α^+-thalassemia trait are typically completely asymptomatic and have no consistent hematologic manifestations. The Hb level is normal, and the RBCs are usually not microcytic. HbA2 and HbF are normal. During the newborn period, small amounts (≤3%) of Hb Bart (γ_4) may be seen. This condition is most often recognized when an apparently normal individual becomes the parent of a child with HbH disease after mating with a person with α^0-thalassemia trait in *cis* form. The mild excess of β-globin chains is probably removed in erythroblasts by proteolysis.[410] α^+-thalassemia is particularly common in Melanesia, as well as in Southeast Asia and in African Americans, reaching a prevalence of more than 80% in north coastal Papua New Guinea.

The level of α-globin gene expression differs in the two conditions, as discussed in the following section.

α^0-Thalassemia Trait (α-Thalassemia Trait)

These individuals typically present with a mild microcytic anemia, similar to those with β-thalassemia trait. They are usually asymptomatic and have no other clinical manifestations of thalassemia. Levels of HbA2 in the low to low-normal range (1.5% to 2.5%) and β/α synthetic ratios averaging 1.4:1 characterize α^0-thalassemia trait. During the perinatal period, elevated amounts of Hb Bart are noted (3% to 8%). Microcytosis is present in cord blood erythrocytes. The degree of anemia and amount of Hb Bart may be related to whether the α1 or α2 genes are deleted.

HbH is not detected in hemolysates of peripheral RBCs, probably because of rapid proteolysis of HbH or free β-globin chains. However, approximately 1% of erythroblasts and BM reticulocytes have inclusions. When an α-thalassemia gene occurs in persons who are also heterozygous for α-globin chain variant Hbs, such as HbS, HbC, or HbE, the proportion of the abnormal Hb is lower than that seen in simple heterozygotes.[411] The lower level of the abnormal Hb is attributable to posttranslational control because of higher affinity of β^{A} chains for a limited pool of α-globin chains coupled with proteolysis of the uncombined $\beta^{variant}$ chains.[412] In the nondeletion forms of α-thalassemia, there may be an extremely diverse series of phenotypes, with some individuals being more anemic, and having the clinical picture of HbH disease, while others may have only mild hypochromic anemia like the deletional form.

Hemoglobin H Disease

HbH disease is associated with a moderately severe but variable anemia resembling β-thalassemia intermedia, with osseous changes and splenomegaly.[413] It occurs predominantly in Asians and occasionally in persons of European (Mediterranean) descent but is rare in persons of African ancestry. Deletional HbH occurs when three deletional alpha mutations occur. Non-deletional HbH occurs when two deletional alpha mutations occur with an alpha (+) mutation (e.g., Constant Spring). Though all patients have anemia, the degree is variable, based on the expression of the single α-globin gene, and any other potential genetic disease modifiers. The clinical phenotype varies considerably, being considerably milder in some patients and severe enough to cause hydrops fetalis in others.[414,415] While a few may have severe anemia, and require regular or intermittent transfusions, most have a much milder course.[2,416] Some symptoms of the chronic microcytic anemia may be seen, such as fatigue and exercise intolerance, but these are usually not severe. Since HbH is unstable, it precipitates within the circulating RBCs and hemolysis occurs. The higher the proportion of HbH, the more hemolytic the clinical phenotype. Nondeletional HbH genotypes are more likely to have higher percentage HbH, more hemolysis, more splenomegaly, and more advanced disease.[406]

Since the degree of IE in α-thalassemia is mild, splenomegaly is somewhat variable, and the other clinical manifestations of the thalassemia intermedia syndrome, such as bone disease and iron overload are not frequently seen, though adults with deletional HbH may develop some of the same complications as β-thalassemia intermedia, including osteoporosis, cholelithiasis, and iron overload.[417] The degree of deletions in HbH is also relevant to clinical presentation.

Exacerbations of anemia during febrile illnesses are common and are usually characterized by increasing fatigue and jaundice.

HbH can be demonstrated by incubation of blood with supravital oxidizing stains such as 1% brilliant cresyl blue. Multiple small inclusions form in the RBCs. Electrophoresis of a freshly prepared hemolysate at alkaline or neutral pH demonstrates a fast-moving component amounting to 3% to 30% of the total Hb. Concomitant iron deficiency may reduce the amount of HbH in the patient's RBCs.[418] A syndrome of HbH disease associated with mental retardation, other congenital anomalies, and large deletions on chromosome 16 has been noted in several families of European origin.[419,420]

Hydrops Fetalis With Hb Bart

Alpha-thalassemia major (homozygous α^0 thalassemia) is found most commonly in Mediterranean and East Asian (Chinese, Cambodian, Thai, and Filipino) populations because of the higher prevalence of two gene deletions in *cis* configuration, but is rare in African and Middle Eastern populations, where two gene deletions are usually in *trans* configuration. Affected fetuses are usually born prematurely and are either stillborn or die shortly after birth.[2–4,6] Hydropic infants have marked anasarca and massive hepatosplenomegaly. Extreme extramedullary erythropoiesis occurs in response to the profound hypoxia and hemolytic anemia characteristic of this disease. The universal edema characteristic of the hydrops fetalis syndrome is a reflection of severe congestive heart failure and hypoalbuminemia in utero. This is partly a consequence of anemia, but the strikingly abnormal oxygen affinity of the tetrameric Hb Bart is probably the most important determinant of the severe tissue hypoxia. The oxygen dissociation curve of Hb Bart lacks the normal sigmoid form because of noncooperation during oxygen loading and unloading and is markedly shifted to the left. The shift is so great that little oxygen is released under conditions of low oxygen concentration in the tissues.

Infants with this syndrome do not die in an earlier trimester of pregnancy because of the presence of Hb Portland ($\zeta_2\gamma_2$). This Hb does display cooperativity in a manner similar to that of HbF and therefore has a much more favorable oxygen dissociation pattern than that of Hb Bart. A high incidence of toxemia of pregnancy has been described in women carrying severely affected infants, providing an increased rationale for prenatal diagnosis of this condition.

Severe anemia is usually present, with Hb levels of 3 to 10 g/dL. The RBCs are markedly microcytic and hypochromic and include target cells and large numbers of circulating nucleated RBCs. These morphologic abnormalities and a negative Coombs test result exclude hemolytic diseases caused by blood group incompatibility.

Hb electrophoresis reveals predominantly Hb Bart, with a smaller amount of HbH. A minor component identified as Hb Portland ($\zeta_2\gamma_2$) migrating in the position of HbA is also seen. Normal HbA and HbF are totally absent.[421]

Therapy

Fetuses with homozygous α^0-thalassemia usually die in utero because of severe hydrops fetalis and are stillborn. However, some infants have had successful blood exchange transfusion immediately after birth.[422–426] It is also possible to salvage affected fetuses by in utero blood transfusions.[427,428] Limb and urogenital defects are present in a substantial portion of infants with homozygous α^0-thalassemia who are rescued by these measures, and some infants have developmental delay or other neurologic abnormalities. Management after the perinatal period is similar to the management of patients with β-thalassemia major and includes transfusion and chelation therapy as well as the possibility of BMT.[429]

Most patients with HbH disease do not require RBC transfusions. For patients with more severe disease, characterized by lower Hb levels or frequent exacerbations of the anemia, splenectomy can be helpful. Oxidant drugs can accelerate precipitation of HbH and exacerbate hemolysis; they should therefore be avoided. Exchange transfusion can be used to decrease deleterious levels of HbH.

Infants with heterozygous α^0-thalassemia trait lose their Hb Bart during the first few months of life and are left with the hematologic findings of α-thalassemia trait, a mild hypochromic microcytosis that persists throughout life.[2] These individuals do not require any therapy, and the same guidelines are followed for their care as with β-thalassemia trait. Iron supplementation is only necessary if there is true iron deficiency, and routine supplementation to raise the hemoglobin level is futile and should not be done. These individuals should seek genetic counseling when ready for childbearing.

Novel Therapies

Several newer approaches are being studied for α-thalassemia:

A. In order to ameliorate anemia in individuals with HbH disease, AG-348 (Mitapivat) will be entering a phase II/III clinical trial in NTD HbH patients. Mitapivat is an oral, small molecule allosteric activator of wild-type and a variety of mutated PKR enzymes. In non-transfusion-dependent individuals with β-thalassemia intermedia and HbH disease, early short-term phase II data show an increase in Hb levels and a reduction in markers of hemolysis. This agent is in clinical trials in NTD patients.[430]

B. A phase I clinical trial to evaluate the safety of in utero HSCT in fetuses with α-thalassemia major is also underway.[431] Maternal BM would be infused at the time of the in utero red cell transfusion. The goal is to take advantage of the maternal-fetal tolerance that exists during pregnancy, thus no conditioning chemotherapy would be required.

De Novo and Acquired Forms of α-Thalassemia

Two distinct α-thalassemia syndromes have been described that are attributable to acquired or de novo mutations: (1) α-thalassemia associated with mental retardation and (2) HbH disease associated with MDS.

α-Thalassemia Associated With Mental Retardation

Alpha-thalassemia or HbH disease can occur as a de novo abnormality in a rare disorder called the α-thalassemia with mental retardation syndrome (ATR).[419,420] In this disorder, affected patients have mental retardation and a number of other developmental abnormalities in association with α-thalassemia trait or HbH disease that is inherited in a nontraditional manner. Two distinct types of the ATR syndrome have been identified. In some cases, there is the de novo appearance of large (2000 kb or so) deletions involving the entire α-globin gene cluster and adjacent DNA at the tip of chromosome 16, the so-called ATR-16 syndrome. In some of these patients, the deletion produces detectable cytogenetic abnormalities of chromosome 16, indicating that a very large segment of the chromosome is deleted, sometimes because of unbalanced chromosomal translocations involving the telomeres of the affected chromosomes. In some cases, one parent is heterozygous for α^+-thalassemia by various criteria and the other parent is completely normal; in such cases, the child has HbH disease (- -/- α). In other cases, both parents are normal and the affected child has the hematologic phenotype of heterozygous α^0-thalassemia (- -/αα) without HbH disease. In this form of ATR, the clinical findings, such as the degree of mental retardation and associated congenital abnormalities, are variable.

The second type of ATR syndrome is not associated with detectable deletions of the α-globin gene complex. The molecular basis of the disorder consists of mutations of a gene on the X chromosome, and the condition has been called the alpha-thalassemia/mental retardation, X-linked (ATR-X) syndrome.[432] In contrast to patients with the ATR-16 syndrome who have a varied phenotype of developmental abnormalities, patients with the ATR-X syndrome have a more uniform or consistent phenotype, particularly severe mental retardation (with IQs of 50 to 70) and a characteristic dysmorphic facial appearance.[420]

The affected gene in this syndrome encodes a *trans*-acting factor, called ATR-X, that is thought to influence the expression of the α-globin genes as well as that of other genes.[433,434] The structure of this large (280 kDa) DNA-binding protein is complex and contains two major functional domains: an *N*-terminal cysteine-rich zinc finger–containing domain, called the ADD domain, that has structural features similar to those of DNA methyl transferases, and a C-terminal helicase/ATPase domain. The majority of the mutations associated with the ATR-X syndrome are located in the ADD domain or the helicase domain. The ATRX protein is widely expressed in many different tissues and its intracellular localization is within three different nuclear subcompartments: heterochromatin, ribosomal DNA arrays, and promyelocytic leukemia (PML) bodies. It has been shown to interact with other proteins such as the heterochromatin-associated protein HP1 and Daxx, one of the proteins localized in PML bodies. The prevailing opinion is that the ATRX protein is part of a large chromatin-remodeling complex of the SWI2/SNF2 family. It also has ATPase activity and has translocase activity, that is, it can move along DNA as a "molecular motor." The precise mechanism(s) by which ATRX influences the expression of α-globin (and other) genes remains unknown.

Acquired HbH Disease Associated With Myelodysplastic Syndrome (ATMDS)

HbH disease has occasionally been observed to develop during the course of different types of MDS and more rarely in patients with other hematologic malignancies.[435] The disorder usually affects men over the age of 60 years. The degree of imbalance of globin chain synthesis and of α-globin mRNA deficiency in erythroid cells of affected patients is greater than that observed in the hereditary type of HbH disease. It is conceivable that erythroid cells of the abnormal clone synthesize no α-globin chains at all and that the expression of all four α-globin genes is suppressed or silenced,[436] but this phenomenon is difficult to quantify as long as some normal erythroid cells are being produced.

Cytogenetic, gene mapping, gene sequencing, and gene or chromosome transfer studies failed to detect any deletion or mutation in the α-globin gene cluster or functional abnormality of α-globin gene of affected patients. The results of all of these prior studies suggested that the defect responsible for this disorder probably involved the abnormal expression or function of a *trans*-acting factor capable of influencing α-globin gene expression and, indeed, such a factor was recently identified. The discovery of the factor responsible for HbH disease in MDS results from cDNA microarray analysis of RNA isolated from granulocytes of an affected patient. One of the genes that was found to be markedly underexpressed, compared with results obtained with RNA of normal granulocytes, was the *ATRX* gene,[437] the same gene that is mutated in the α-thalassemia with mental retardation syndrome of the ATR-X type. Sequence analysis of the *ATRX* gene in the DNA of blood cells of affected individuals has identified a number of different mutations. It is noteworthy that the mutations of the *ATRX* gene associated with acquired HbH disease associated with MDS (ATMDS) occur in the same regions of the gene as the mutations associated with the ATR-X syndrome, that is, in the ADD or helicase domains. In fact, some of the *ATRX* gene mutations identified in ATMDS are identical or similar in expected functional consequences to various mutations found in the ATR-X syndrome.

The hematologic features in the syndrome are characterized by the presence of a dimorphic RBC population on blood smear, one of which is hypochromic, microcytic, and poikilocytic. Incubation of the blood with the supravital stain brilliant cresyl blue results in the detection of typical HbH inclusions. Hb electrophoresis or HPLC detects the presence of HbH, usually in greater quantities than that typically observed in inherited HbH disease. In typical MDS, the MCV of the erythrocytes is normal or elevated, frequently higher than 100 fL. However, in ATMDS, the MCV and MCH are low: MCV usually less than 80 fL and MCH usually less than 26 pg.[435] The amount of HbH usually remains stable but may actually decrease during the course of the disease and no longer persists after transformation of MDS to acute leukemia. This finding suggests that the HbH-producing clone does not have a selective survival or growth advantage.

The hematologic phenotype, as reflected by the amount of HbH present in blood, of ATMDS is much more severe than that of the ATR-X syndrome.[433] Some of this difference in severity may be because of the nature of the ATMDS mutations, some of which are null mutations that are likely to be lethal when present in germline DNA of ATR-X embryos. However, the difference in severity is also observed in the case of mutations found in both syndromes that are identical or similar in expected functional consequences. This finding suggests that additional abnormalities in gene expression in ATMDS contribute to the severity of the deficit in α-globin gene expression observed in this syndrome. Perhaps the responsible defective cofactor(s) is one or more of the proteins that interact with the ATRX protein to produce a fully functional macromolecular complex that can act as a transcriptional cofactor or that can influence the epigenetic control of α-globin gene expression.

THALASSEMIC STRUCTURAL VARIANTS

Certain structural Hb variants are characterized by the presence of a biosynthetic defect as well as abnormal structure.[7] Thalassemic hemoglobinopathies are unusual forms of thalassemia caused by such structural variants.

Hemoglobin Lepore

Hb Lepore (α2δβ) is the prototype of a group of hemoglobinopathies characterized by fused globin chains.[2–4,6] The chains begin with a normal δ-chain sequence at their N-terminus and end with the normal β-chain sequence at their C-terminus. These hemoglobinopathies arise by unequal or nonhomologous crossover or recombination events that fuse the proximal end of one gene with the distal end of a closely linked structurally homologous gene (Fig. 41.24). During meiosis, mispairing and crossover of the highly homologous δ- and β-globin genes can occur, resulting in a Lepore chromosome, which contains (in addition to γ-globin genes) only the fused δβ gene, and an anti-Lepore chromosome, which contains the reciprocal fusion product (δβ), as well as intact δ- and β-globin genes.[2]

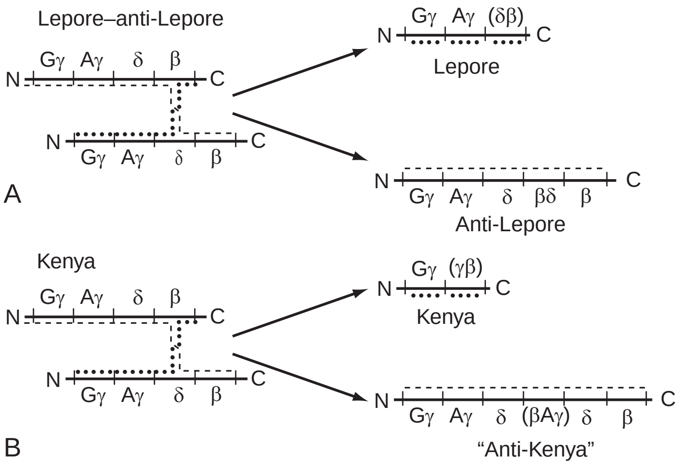


Figure 41.24 GENETIC ORIGINS OF HEMOGLOBIN (HB) LEPORE, ANTI-LEPORE HB, AND HB KENYA. *(Adapted from Benz EJ Jr. The hemoglobinopathies. In: Kelly WN, DeVita VT, eds. Textbook of Internal Medicine. Philadelphia: JB Lippincott; 1988:1423.)*

Lepore globin is synthesized in low amounts, presumably because it is under the control of the δ-globin gene promoter, which normally sustains transcription at only 2.5% the level of the β-globin gene.[438] Patients with Hb Lepore have the phenotype of β-thalassemia, distinguished by the added presence of 5% to 15% Hb Lepore. In contrast, the anti-Lepore globin (Miyada) is not associated with a β-thalassemia phenotype because of the presence of an intact and functionally normal β-globin gene on the same chromosome.

An analogous but rare variant, Hb Kenya (α2[Aγβ]2), arises from nonhomologous crossing over between the $^{A}\gamma$- and β-globin genes[439] and is associated with the phenotype of Gγ hereditary persistence of fetal Hb. A DNA sequence approximately 600 bases downstream from the β-globin gene acts as a strong enhancer, promoting the erythroid-specific expression of the β-globin genes in adult cells.[3,4,18] The fused $^{A}\gamma\beta$ gene as well as the linked upstream $^{G}\gamma$ gene are believed to come under the influence of the enhancer because of its abnormal proximity and thus are expressed at high levels in adult life.

Hemoglobin E

HbE ($\alpha_2\beta_2^{26Glu\rightarrow Lys}$) is a common variant (15% to 30% of the population) in Cambodia, Thailand, parts of China, and Vietnam. HbE is very mildly unstable, but this instability does not significantly alter the life span of RBCs. HbE trait resembles very mild β-thalassemia trait. Homozygotes exhibit more microcytosis but are still asymptomatic.[440] Compound heterozygotes for HbE and a β-thalassemia gene (HbE-β-thalassemia) resemble patients with β-thalassemia intermedia or β-thalassemia major. However, some problems such as infection and pulmonary hypertension may occur more commonly in HbE-β-thalassemia than in homozygous β-thalassemia.[441]

The only nucleotide sequence abnormality found in the β^E-gene is a base change in codon 26 that causes the amino acid substitution where glutamic acid is replaced by lysine. This mutation, which occurs in a potential cryptic RNA splice region, alters the consensus sequence surrounding a potential GT donor splice site and thus activates the cryptic site. Alternative splicing at this position occurs approximately 40% to 50% of the time, generating a structurally abnormal globin mRNA that cannot be translated appropriately.[442] The other mRNA precursors are spliced at the normal site, generating functionally normal mRNA, which is translated into β^E-globin because the mature mRNA retains the base change that encodes lysine at codon 26.

Hemoglobin Constant Spring

Hb Constant Spring (see Fig. 41.22) is an elongated α-globin variant resulting from a mutation that alters the normal translation termination codon.[443] Polyribosomes read through the usual translation stop site and incorporate an additional 31 amino acids until another in-phase termination codon is reached within the 3′ untranslated sequence. The amount of α^{cs} mRNA is markedly reduced, and α^{cs}-globin is synthesized in only minute amounts.[402,444] Six possible mutations of the normal translation termination codon (UAA) in α-globin mRNA could result in the generation of a "sense" codon.[445] Of these, five variants have been identified, each having a markedly underproduced abnormal variant, indicating that disruption of normal translation termination is in some way associated with abnormal mRNA accumulation, presumably because of instability of the mRNA.[402] The output of α-globin from the α^{cs} allele is only approximately 1% of normal, and the gene is thus rendered α-thalassemic. The α^{cs} allele has been identified only on chromosomes containing a *cis*-linked functionally normal α-globin gene.[2–4,6] Thus, α^{+}-thalassemia trait (α -/$\alpha^{cs}\alpha$) and HbH disease–(--/$\alpha^{cs}\alpha$) associated with Hb Constant Spring are common, but hydrops fetalis caused by four abnormal α-globin genes cannot occur in association with this variant. Homozygosity for the variant is associated with a relatively mild form of HbH disease.[2]

EXTRAORDINARILY UNSTABLE HEMOGLOBINS

Rare cases of α-thalassemia (e.g., Hb Quong Sze)[405] and β-thalassemia (e.g., Hb Indianapolis, recently renamed Hb Terre Haute)[446,447] arise from mutations that produce extremely labile globin chains. The chains fail to pair with the complementary chain, or they precipitate and are degraded so rapidly that they never form tetramers. These posttranslational lesions have the same pathophysiologic effects on Hb biogenesis as reduction of globin mRNA production or function. Another group of β-globin chain variants, usually caused by mutations in exon 3 of the β-globin gene, are associated with inclusion body formation and a phenotype of dominant β-thalassemia intermedia.[16,17,448]

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Baldini M, Forti S, Marcon A, et al. Endocrine and bone disease in appropriately treated adult patients with beta-thalassemia major. *Ann Hematol*. 2010;89(12):1207–1213.

Baronciani D, Angelucci E, Potschger U, et al. Hemopoietic stem cell transplantation in thalassemia: a report from the European Society for Blood and Bone Marrow Transplantation Hemoglobinopathy Registry, 2000–2010. *Bone Marrow Transplant*. 2016;51(4):536–541.

Belmont A, Kwiatkowski JL. Deferiprone for the treatment of transfusional iron overload in thalassemia. *Expert Rev Hematol*. 2017;10(6):493–503.

Borgna-Pignatti C, Cappellini MD, De Stefano P, et al. Survival and complications in thalassemia. *Ann N Y Acad Sci*. 2005;1054:40–47.

Cao A, Galanello R. Beta-thalassemia. *Genet Med*. 2010;12(2):61–76.

Cappellini MD, Motta I. New therapeutic targets in transfusion-dependent and -independent thalassemia. *Hematology Am Soc Hematol Educ Program*. 2017;2017(1):278–283.

Cappellini MD, Viprakasit V, Taher AT, et al. A phase 3 trial of luspatercept in patients with transfusion-dependent β-thalassemia. *N Engl J Med*. 2020; 382(13):1219–1231.

Hammond J, Thompson AA, Fogel MA, et al. Combination oral chelation in adult patients with transfusion-dependent thalassemia and high iron burden. *J Pediatr Hematol Oncol*. 2019;41(1):e47–e50.

Khandros E, Kwiatkowski JL. Beta Thalassemia: monitoring and new treatment approaches. *Hematol Oncol Clin North Am*. 2019;33(3):339–353.

Marcon A, Motta I, Taher AT, Cappellini MD. Clinical complications and their management. *Hematol Oncol Clin North Am*. 2018;32(2):223–236.

Musallam KM, Rivella S, Vichinsky E, Rachmilewitz EA. Non-transfusion-dependent thalassemias. *Haematologica*. 2013;98(6):833–844.

Noetzli LJ, Carson SM, Nord AS, et al. Longitudinal analysis of heart and liver iron in thalassemia major. *Blood*. 2008;112(7):2973–2978.

Porter JB, Cappellini MD, Kattamis A, et al. Iron overload across the spectrum of non-transfusion-dependent thalassaemias: role of erythropoiesis, splenectomy and transfusions. *Br J Haematol*. 2017;176(2):288–299.

Quek L, Thein SL. Molecular therapies in beta-thalassaemia. *Br J Haematol*. 2007;136(3):353–365.

Rachmilewitz EA, Giardina PJ. How I treat thalassemia. *Blood*. 2011;118(13): 3479–3488.

Sheth S. *Transfusional Iron Overload. Rossi's Principles of Transfusion Medicine*. 5th Edition. Oxford: Wiley Blackwell; 2016.

Sheth S. Strategies for managing transfusional iron overload: conventional treatments and novel strategies. *Curr Opin Hematol*. 2019;26(3):139–144.

Sheth S, Tang H, Jensen JH, et al. Methods for noninvasive measurement of tissue iron in Cooley's anemia. *Ann N Y Acad Sci*. 2005;1054:358–372.

Sobota A, Yamashita R, Xu Y, et al. Quality of life in thalassemia: a comparison of SF-36 results from the thalassemia longitudinal cohort to reported literature and the US norms. *Am J Hematol*. 2011;86(1):92–95.

Strocchio L, Locatelli F. Hematopoietic stem cell transplantation in thalassemia. *Hematol Oncol Clin North Am*. 2018;32(2):317–328.

Taher AT, Cappellini MD, Bou-Fakhredin R, et al. Hypercoagulability and vascular disease. *Hematol Oncol Clin North Am*. 2018;32(2):237–245.

Taher AT, Cappellini MD. How I manage medical complications of β-thalassemia in adults. *Blood*. 2018;132(17):1781–1791.

Taher AT, Musallam KM, Cappellini MD, Weatherall DJ. Optimal management of β thalassaemia intermedia. *Br J Haematol*. 2011;152(5):512–523.

Taher AT, Weatherall DJ, Cappellini MD. Thalassaemia. *Lancet*. 2018;391 (10116):155–167.

Thein SL. Molecular basis of β thalassemia and potential therapeutic targets. *Blood Cells Mol Dis*. 2018;70:54–65.

Thein SL. The molecular basis of β-thalassemia. *Cold Spring Harb Perspect Med*. 2013;3(5):a011700.

Thompson AA, Cunningham MJ, Singer ST, et al. Red cell alloimmunization in a diverse population of transfused patients with thalassaemia. *Br J Haematol*. 2011;153(1):121–128.

Thompson AA, Walters MC, Kwiatkowski J, et al. Gene therapy in patients with transfusion-dependent β-thalassemia. *N Engl J Med*. 2018;378(16): 1479–1493.

Trachtenberg FL, Gerstenberger E, Xu Y, et al. Relationship among chelator adherence, change in chelators, and quality of life in thalassemia. *Qual Life Res*. 2014;23(8):2277–2288.

Tubman VN, Fung EB, Vogiatzi M, et al. Guidelines for the standard monitoring of patients with thalassemia: report of the thalassemia longitudinal cohort. *J Pediatr Hematol Oncol*. 2015;37(3):e162–e169.

Vichinsky E, Cohen A, Thompson AA, et al. Epidemiologic and clinical characteristics of nontransfusion-dependent thalassemia in the United States. *Pediatr Blood Cancer*. 2018;65(7):e27067.

Vichinsky E, Neumayr L, Trimble S, et al. Transfusion complications in thalassemia patients: a report from the Centers for Disease Control and Prevention (CME). *Transfusion*. 2014;54(4):972–981. quiz 971.

Vogiatzi MG, Macklin EA, Fung EB, et al. Bone disease in thalassemia: a frequent and still unresolved problem. *J Bone Miner Res*. 2009;24(3): 543–557.

Weatherall DJ. *Thalassaemia. The Biography*. Oxford: Oxford University Press; 2010.

Weatherall DJ. The evolving spectrum of the epidemiology of thalassemia. *Hematol Oncol Clin North Am*. 2018;32(2):165–175.

Weatherall DJ, Clegg JB. *The Thalassaemia Syndromes*. 4th ed. Blackwell: Oxford; 2001.

Wood JC. Cardiac complications in thalassemia major. *Hemoglobin*. 2009;33 (Suppl 1):S81–S86. Suppl 1.

Wood JC, Enriquez C, Ghugre N, et al. Physiology and pathophysiology of iron cardiomyopathy in thalassemia. *Ann N Y Acad Sci*. 2005;1054:386–395.

Wood JC. Estimating tissue iron burden: current status and future prospects. *Br J Haematol*. 2015;170(1):15–28.

Wood JC. Use of magnetic resonance imaging to monitor iron overload. *Hematol Oncol Clin North Am*. 2014;28(4):747–764. vii.

REFERENCES

1. Cooley TB, Lee P. A series of cases of splenomegaly in children with anemia and peculiar bone changes. *Trans Am Pediatr Soc*. 1925;37:29.
2. Weatherall DJ, Clegg JB, eds. *The Thalassaemia Syndromes*. 4th ed. Oxford: Blackwell Scientific; 2001.
3. Cooley T, Lee P. A series of cases of splenomegaly in children with anemia and peculiar bone changes. *Trans Am Pediatr*. 1925;37:29.
4. Cooley T, Witwer E, Lee P. Anemia in children with splenomegaly and peculiar changes in bones: report of cases. *Am J Dis Child*. 1927;34:347.
5. Whipple G, Bradford W. Mediterranean disease-thalassemia (erythroblastic anemia of Cooley): associated pigment abnormalities simulating hemochromatosis. *J Pediatr*. 1932;9:279.
6. Steinberg MH, Forget BG, Higgs DR, eds. *Disorders of Hemoglobin*. New York: Cambridge University Press; 2001.
7. Adams 3rd JG, Coleman MB. Structural hemoglobin variants that produce the phenotype of thalassemia. *Semin Hematol*. 1990;27:229.
8. Barrai I, Rosito A, Cappellozza G, et al. Beta-thalassemia in the Po Delta: selection, geography, and population structure. *Am J Hum Genet*. 1984; 36:1121.
9. Livingston F, ed. *Abnormal Hemoglobins in Human Populations*. Chicago: Aldine; 1967.
10. Malamos B, Fessas P, Stamatoyannopoulos G. Types of thalassaemia-trait carriers as revealed by a study of their incidence in Greece. *Br J Haematol*. 1962;8:5.
11. Pearson HA, Guiliotis DK, O'Brien RT, et al. Thalassemia in Greek Americans. *J Pediatr*. 1975;86:917.
12. Nagel RL, Roth Jr EF. Malaria and red cell genetic defects. *Blood*. 1989; 74:1213.
13. Kanavakis E, Wainscoat JS, Wood WG, et al. The interaction of alpha thalassaemia with heterozygous beta thalassaemia. *Br J Haematol*. 1982; 52:465.
14. Rosatelli C, Falchi AM, Scalas MT, et al. Hematological phenotype of the double heterozygous state for alpha and beta thalassemia. *Hemoglobin*. 1984;8:25.
15. Wainscoat JS, Kanavakis E, Wood WG, et al. Thalassaemia intermedia in Cyprus: the interaction of alpha and beta thalassaemia. *Br J Haematol*. 1983;53:411.
16. Thein SL. Dominant beta thalassaemia: molecular basis and pathophysiology. *Br J Haematol*. 1992;80:273.
17. Kazazian Jr HH, Dowling CE, Hurwitz RL, et al. Dominant thalassemia-like phenotypes associated with mutations in exon 3 of the beta-globin gene. *Blood*. 1992;79:3014.
18. Forget BG. In: Steinberg MH, Forget BG, Higgs DR, eds. *Disorders of Hemoglobin*. New York: Cambridge University Press; 2001:117.
19. Weatherall DJ. The Thalassemias. In: Stamatoyannopoulos G, Majerus P, Perlmutter R, eds. *Molecular Basis of Blood Diseases*. 3rd ed Philadelphia: WB Saunders; 2001:183.
20. Forget BG, Olivieri NF. Hemoglobin synthesis and the thalassemias. In: Handin R, Lux S, Stossel T, eds. *Blood: Principles and Practice of Hematology*. 2nd ed Philadelphia: , JB Lippincott; 2003:1503.
21. Thein SL:. Molecular basis of β thalassemia and potential therapeutic targets. *Blood Cells, Molecules, and Diseases,*. 2018;70:54.
22. Patrinos GP, Giardine B, Riemer C, et al. Improvements in the HbVar database of human hemoglobin variants and thalassemia mutations for population and sequence variation studies. *Nucleic Acids Res*. 2004;32: D537.
23. Hardison RC, Chui DHK, Giardine B, et al. HbVar: A relational database of human hemoglobin variants and thalassemia mutations at the globin gene server. *Hum Mutat*. 2002;19:225.
24. Hardison RC, Chui DH, Riemer C, et al. Databases of human hemoglobin variants and other resources at the globin gene server. *Hemoglobin*. 2001; 25:183.
25. Westaway D, Williamson R. An intron nucleotide sequence variant in a cloned beta +-thalassaemia globin gene. *Nucleic Acids Res*. 1981;9: 1777.
26. Spritz RA, Jagadeeswaran P, Choudary PV, et al. Base substitution in an intervening sequence of a beta +-thalassemic human globin gene. *Proc Natl Acad Sci USA*. 1981;78:2455.
27. Antonarakis SE, Kazazian Jr HH, Orkin SH. DNA polymorphism and molecular pathology of the human globin gene clusters. *Hum Genet*. 1985;69:1.
28. Orkin SH, Goff SC. Nonsense and frameshift mutations in beta 0-thalassemia detected in cloned beta-globin genes. *J Biol Chem*. 1981; 256:9782.
29. Van der Ploeg LH, Konings A, Oort M, et al. gamma-beta-thalassaemia studies showing that deletion of the gamma- and delta-genes influences beta-globin gene expression in man. *Nature*. 1980;283:637.
30. Li Q, Peterson KR, Fang X, et al. Locus control regions. *Blood*. 2002; 100:3077.
31. Huang S, Wong C, Antonarakis SE, et al. The same "TATA" box beta-thalassemia mutation in Chinese and US blacks: another example of independent origins of mutation. *Hum Genet*. 1986;74:162.
32. Gilman JG, Huisman TH. DNA sequence variation associated with elevated fetal G gamma globin production. *Blood*. 1985;66:783.
33. Ranjbaran R, Okhovat MA, Mobarhanfard A, et al. Relationship between AHSP gene expression, β/α globin mRNA ratio, and clinical severity of the β-thalassemia patients. *Ann Clin Lab Sci*. 2014;44(2):189–193.
34. Shinar E, Rachmilewitz EA. Oxidative denaturation of red blood cells in thalassemia. *Semin Hematol*. 1990;27:70.
35. Finch CA, Sturgeon P. Erythrokinetics in Cooley's anemia. *Blood*. 1957; 12:64.
36. Pootrakul P, Sirankapracha P, Hemsorach S, et al. A correlation of erythrokinetics, ineffective erythropoiesis, and erythroid precursor apoptosis in Thai patients with thalassemia. *Blood*. 2000;96(7):2606–2612.
37. Rivella S. Ineffective erythropoiesis and thalassemias. *Curr Opin Hematol*. 2009;16:187.
38. Rivella S, Nemeth E, Miller JL. Crosstalk between erythropoiesis and iron metabolism. *Adv Hematol*. 2010;2010:317095.
39. Nemeth E. Hepcidin and β-thalassemia major. *Blood*. 2013;122(1): 3–4. https://doi.org/10.1182/blood-2013-05-502617.
40. Kautz L, Jung G, Valore EV, et al. Identification of erythroferrone as an erythroid regulator of iron metabolism. *Nat Genet*. 2014;46(7):678–684. https://doi.org/10.1038/ng.2996. [Epub 2014 Jun 1].
41. Dussiot M, Maciel TT, Fricot A, et al. An activin receptor IIA ligand trap corrects ineffective erythropoiesis in β-thalassemia. *Nat Med*. 2014;20: 398–407.
42. Suragani RNVS, Cadena SM, Cawley SM, et al. Transforming growth factor-β superfamily ligand trap ACE-536 corrects anemia by promoting late-stage erythropoiesis. *Nat Med*. 2014;20(4):408–414. https://doi.org/10.1038/nm.3512. Epub 2014 Mar 23.
43. Logothetis J, Loewenson RB, Augoustaki O, et al. Body growth in Cooley's anemia (homozygous beta-thalassemia) with a correlative study as to other aspects of the illness in 138 cases. *Pediatrics*. 1972;50:92.
44. Baty J, Blackfan K, Diamond L. Blood studies in infants and in children. I. Erythroblastic anemia: a clinical and pathologic study. *Am J Dis Child*. 1932;43:665.
45. Silvestroni E, Bianco I. Screening for microcytemia in Italy: analysis of data collected in the past 30 years. *Am J Hum Genet*. 1975;27:198.
46. Friedman S, Hamilton RW, Schwartz E. Beta-thalassemia in the American Negro. *J Clin Invest*. 1973;52:1453.
47. Tamagnini GP, Lopes MC, Castanheira ME, et al. Beta+ thalassemia–Portuguese type: clinical, haematological and molecular studies of a newly defined form of beta thalassaemia. *Br J Haematol*. 1983;54:189.
48. Rachmilewitz EA, Giardina PJ:. How I treat thalassemia. *Blood*. 2011;118: 3479.
49. Aksoy M, Bermek E, Almis G, et al. beta-Thalassemia intermedia homozygous for normal hemoglobin A2 beta-thalassemia. Study in four families. *Acta Haematol*. 1982;67:57.
50. Schwartz E. The silent carrier of beta thalassemia. *N Engl J Med*. 1969;281: 1327.
51. Premawardhena A, Arambepola M, Katugaha N, et al. Is the beta thalassaemia trait of clinical importance? *Br J Haematol*. 2008;141:407.
52. Hegde UM, Khunda S, Marsh GW, et al. Thalassaemia, iron, and pregnancy. *Br Med J*. 1975;3:509.
53. Schuman JE, Tanser CL, Peloquin R, et al. The erythropoietic response to pregnancy in beta-thalassaemia minor. *Br J Haematol*. 1973;25:249.
54. Borgna-Pignatti C, Rigon F, Merlo L, et al. Thalassemia minor, the Gilbert mutation, and the risk of gallstones. *Haematologica*. 2003;88(10):1106–1109.
55. Smith CH, Sisson TR, Floyd Jr WH, et al. Serum iron and iron-binding capacity of the serum in children with severe Mediterranean (Cooley's) anemia. *Pediatrics*. 1950;5:799.
56. Fessas P. Inclusions of hemoglobin erythroblasts and erythrocytes of thalassemia. *Blood*. 1963;21:21.
57. Fessas P, Loukopoulos D, Kaltsoya A. Peptide analysis of the inclusions of erythroid cells in beta-thalassemia. *Biochim Biophys Acta*. 1966;124:430.
58. Cutillo S, Meloni T. Serum concentrations of haptoglobin and hemopexin in favism and thalassemia. *Acta Haematol*. 1974;52:65.
59. Wood WG, Weatherall DJ, Hart GH, et al. Hematologic changes and hemoglobin analysis beta thalassemia heterozygotes during the first year of life. *Pediatr Res*. 1982;16:286.
60. Silvestroni E, Bianco I. A highly cost effective method of mass screening for thalassaemia. *Br Med J (Clin Res Ed)*. 1983;286:1007.
61. Pippard MJ, Wainscoat JS. Erythrokinetics and iron status in heterozygous beta thalassaemia, and the effect of interaction with alpha thalassaemia. *Br J Haematol*. 1987;66:123.

62. Berman BW, Ritchey AK, Jekel JF, et al. Hematology of beta-thalassemia trait–age-related developmental aspects and intrafamilial correlations. *J Pediatr*. 1980;97:901.
63. Pearson HA, O'Brien RT, McIntosh S. Screening for thalassemia trait by electronic measurement of mean corpuscular volume. *N Engl J Med*. 1973;288:351.
64. Kattamis C, Metaxatou-Mavromati A, Wood WG, et al. The heterogeneity of normal Hb A2-β thalassaemia in Greece. *Br J Haematol*. 1979;42:109.
65. Arcasoy A, Cavdar AO. Changes of trace minerals (serum iron, zinc, copper and magnesium) in thalassemia. *Acta Haematol*. 1975;53:341.
66. Prasad AS, Diwany M, Gabr M, et al. Biochemical studies in thalassemia. *Ann Intern Med*. 1965;62:87.
67. Wapnick AA, Lynch SR, Krawitz P, et al. Effects of iron overload on ascorbic acid metabolism. *Br Med J*. 1968;3:704.
68. Wapnick AA, Lynch SR, Charlton RW, et al. The effect of ascorbic acid deficiency on desferrioxamine-induced urinary iron excretion. *Br J Haematol*. 1969;17:563.
69. Jandl JH, Greenberg MS. Bonemarrow failure due to relative nutritional deficiency in Cooley's hemolytic anemia: painful erythropoietic crises in response to folic acid. *N Engl J Med*. 1959;260:461.
70. Luhby AL, Cooperman JM. Folic-acid deficiency in thalassaemia major. *Lancet*. 1961;2:490.
71. Hyman CB, Landing B, Alfin-Slater R, et al. Dl-alpha-tocopherol, iron, and lipofuscin in thalassemia. *Ann N Y Acad Sci*. 1974;232:211.
72. Rachmilewitz EA, Shohet SB, Lubin BH:. Lipid membrane peroxidation in beta-thalassemia major. *Blood*. 1976;47:495.
73. Miniero R, Piga A, Luzzatto L, et al. Vitamin E and beta-thalassaemia. *Haematologica*. 1983;68:562.
74. Hilgartner MW, Smith CH. Coagulation studies as a measure of liver function in Cooley's anemia. *Ann N Y Acad Sci*. 1964;119:631.
75. Stuart MJ:. Platelet dysfunction in homozygous beta-thalassemia. *Pediatr Res*. 1979;13:1345.
76. Olivieri N, Weatherall DJ. In: Steinberg MH, Forget BG, Higgs DR, Weatherall DJ, eds. Clinical aspects of β thalassemia and related disorders. In: Disorders of Hemoglobin. 2nd ed. Cambridge, UK: Cambridge University Press; 2009:357.
77. Olivieri NF, Brittenham GM. Management of the thalassemias. *Cold Spring Harb Perspect Med*. 2013;3:a011767.
78. Sheth S. Transfusional iron overload. In: Simon TL, et al. eds. 5th ed. Chichester: Wiley Blackwell; 2016.
79. Strocchio L, Locatelli F. Hematopoietic stem cell transplantation in thalassemia. *Hematol Oncol Clin North Am*. 2018;32(2):317–328.
80. Rachmilewitz EA, Giardina PJ. How I treat thalassemia. *Blood*. 2011;118(13):3479–3488.
81. Baronciani D, Angelucci E, Potschger U, et al. Hemopoietic stem cell transplantation in thalassemia: a report from the European Society for Blood and Bone Marrow Transplantation Hemoglobinopathy Registry, 2000–2010. *Bone Marrow Transplant*. 2016;51(4):536–541.
82. Wolman IJ. Transfusion therapy in Cooley's anemia: growth and health as related to long-range hemoglobin levels. a progress report. *Ann N Y Acad Sci*. 1964;119:736.
83. Ehlers KH, Giardina PJ, Lesser ML, et al. Prolonged survival in patients with beta-thalassemia major treated with deferoxamine. *J Pediatr*. 1991;118:540.
84. Giardina PJ, Grady RW, Ehlers KH, et al. Current therapy of Cooley's anemia. A decade of experience with subcutaneous desferrioxamine. *Ann N Y Acad Sci*. 1990;612:275.
85. Propper RD, Button LN, Nathan DG. New approaches to the transfusion management of thalassemia. *Blood*. 1980;55:55.
86. Kattamis C, Touliatos N, Haidas S, et al. Growth of children with thalassaemia: effect of different transfusion regimens. *Arch Dis Child*. 1970;45:502.
87. Gabutti V, Piga A, Nicola P, et al. Haemoglobin levels and blood requirement in thalassaemia. *Arch Dis Child*. 1982;57:156.
88. Rebulla P, Modell B. Transfusion requirements and effects in patients with thalassaemia major. Cooleycare Programme. *Lancet*. 1991;337:277.
89. Corash LM, Piomelli S, Chen HC, et al. Separation of erythrocytes according to age on a simplified density gradient. *J Lab Clin Med*. 1974;84:147.
90. Graziano JH, Piomelli S, Seaman C, et al. A simple technique for preparation of young red cells for transfusion from ordinary blood units. *Blood*. 1982;59:865.
91. Cohen AR, Schmidt JM, Martin MB, et al. Clinical trial of young red cell transfusions. *J Pediatr*. 1984;104:865.
92. Marcus RE, Wonke B, Bantock HM, et al. A prospective trial of young red cells in 48 patients with transfusion-dependent thalassaemia. *Br J Haematol*. 1985;60:153.
93. Spanos T, Ladis V, Palamidou F, et al. The impact of neocyte transfusion in the management of thalassaemia. *Vox Sang*. 1996;70:217.
94. Berdoukas VA, Kwan YL, Sansotta ML. A study on the value of red cell exchange transfusion in transfusion dependent anaemias. *Clin Lab Haematol*. 1986;8:209.
95. Cohen A, Freidman D, Larson P, et al. Erythrocytapheresis to reduce iron loading in thalassemia. *Blood*. 1998;92:532a.
96. Sbyrakis S, Karagiorga-Lagana M, Voskaki I, et al. A simple index for initiating transfusion treatment in thalassaemia intermedia. *Br J Haematol*. 1987;67:479.
97. Aessopos A, Kati M, Meletis J. Thalassemia intermedia today: should patients regularly receive transfusions? *Transfusion*. 2007;47:792.
98. Kattamis C, Touliatos N, Haidas S, et al. Growth of children with thalassaemia: effect of different transfusion regimens. *Arch Dis Child*. 1970;45:502.
99. Cazzola M, De Stefano P, Ponchio L, et al. Relationship between transfusion regimen and suppression of erythropoiesis in beta-thalassaemia major. *Br J Haematol*. 1995;89:473.
100. Cazzola M, Borgna-Pignatti C, Locatelli F, et al. A moderate transfusion regimen may reduce iron loading in beta-thalassemia major without producing excessive expansion of erythropoiesis. *Transfusion*. 1997;37:135.
101. Piomelli S, Danoff SJ, Becker MH, et al. Prevention of bone malformations and cardiomegaly in Cooley's anemia by early hypertransfusion regimen. *Ann N Y Acad Sci*. 1969;165:427.
102. Piomelli S, Karpatkin MH, Arzanian M, et al. Hypertransfusion regimen in patients with Cooley's anemia. *Ann N Y Acad Sci*. 1974;232:186.
103. Vichinsky E, Neumayr L, Trimble S, et al. CDC Thalassemia Investigators. Transfusion complications in thalassemia patients: a report from the Centers for Disease Control and Prevention (CME). *Transfusion*. 2014;54(4):972–981. quiz 971. doi: 10.1111/trf.12348. Epub 2013 Jul 25.
104. Sheth S. Strategies for managing transfusional iron overload: conventional treatments and novel strategies. *Curr Opin Hematol*. 2019;26(3):139–144. https://doi.org/10.1097/MOH.0000000000000499.
105. Erlandson ME, Walden B, Stern G, et al. Studies on congenital hemolytic syndromes, IV: gastrointestinal absorption of iron. *Blood*. 1962;19:359.
106. Pippard MJ, Weatherall DJ. Iron absorption in non-transfused iron loading anaemias: prediction of risk for iron loading, and response to iron chelation treatment, in beta thalassaemia intermedia and congenital sideroblastic anaemias. *Haematologia (Budap)*. 1984;17:17.
107. Cohen A, Cohen IJ, Schwartz E. Scurvy and altered iron stores in thalassemia major. *N Engl J Med*. 1981;304:158.
108. O'Brien RT. Ascorbic acid enhancement of desferrioxamine-induced urinary iron excretion in thalassemia major. *Ann N Y Acad Sci*. 1974;232:221.
109. Bridges KR, Hoffman KE. The effects of ascorbic acid on the intracellular metabolism of iron and ferritin. *J Biol Chem*. 1986;261:14273.
110. Bridges KR. Ascorbic acid inhibits lysosomal autophagy of ferritin. *J Biol Chem*. 1987;262:14773.
111. Miller DM, Aust SD. Studies of ascorbate-dependent, iron-catalyzed lipid peroxidation. *Arch Biochem Biophys*. 1989;271:113.
112. Burkitt MJ, Gilbert BC. The control of iron-induced oxidative damage in isolated rat-liver mitochondria by respiration state and ascorbate. *Free Radic Res Commun*. 1989;5:333.
113. Link G, Athias P, Grynberg A, et al. Effect of iron loading on transmembrane potential, contraction, and automaticity of rat ventricular muscle cells in culture. *J Lab Clin Med*. 1989;113:103.
114. Nienhuis AW. Safety of intensive chelation therapy. *N Engl J Med*. 1977;296:114.
115. Wang ZJ, Haselgrove JC, Martin MB, et al. Evaluation of iron overload by single voxel MRS measurement of liver T2. *J Magn Reson Imaging*. 2002;15:395.
116. Clark PR, Chua-Anusorn W, St Pierre TG. Proton transverse relaxation rate (R2) images of liver tissue: mapping local tissue iron concentrations with MRI [corrected]. *Magn Reson Med*. 2003;49:572.
117. Clark PR, St Pierre TG. Quantitative mapping of transverse relaxivity (1/T(2)) in hepatic iron overload: a single spin-echo imaging methodology. *Magn Reson Imaging*. 2000;18:431.
118. Wood JC, Enriquez C, Ghugre N, et al. MRI R2 and R2* mapping accurately estimates hepatic iron concentration in transfusion-dependent thalassemia and sickle cell disease patients. *Blood*. 2005;106:1460.
119. St Pierre TG, Clark PR, Chua-anusorn W, et al. Noninvasive measurement and imaging of liver iron concentrations using proton magnetic resonance. *Blood*. 2005;105:855.
120. Anderson LJ, Holden S, Davis B, et al. Cardiovascular T2-star (T2*) magnetic resonance for the early diagnosis of myocardial iron overload. *Eur Heart J*. 2001;22:2171.
121. Jensen PD, Jensen FT, Christensen T, et al. Evaluation of myocardial iron by magnetic resonance imaging during iron chelation therapy with deferrioxamine: indication of close relation between myocardial iron content and chelatable iron pool. *Blood*. 2003;101:4632.

122. Anderson LJ, Wonke B, Prescott E, et al. Comparison of effects of oral deferiprone and subcutaneous desferrioxamine on myocardial iron concentrations and ventricular function in beta-thalassaemia. *Lancet*. 2002;360:516.
123. Wood JC. Use of magnetic resonance imaging to monitor iron overload. *Hematol Oncol Clin North Am*. 2014;28(4):747–764. https://doi.org/10.1016/j.hoc.2014.04.002. vii.
124. Noetzli LJ, Carson SM, Nord AS, Coates TD, Wood JC. Longitudinal analysis of heart and liver iron in thalassemia major. *Blood*. 2008;112(7): 2973–2978. https://doi.org/10.1182/blood-2008-04-148767. Epub 2008 Jul 23.
125. Brittenham GM, Griffith PM, Nienhuis AW, et al. Efficacy of deferoxamine in preventing complications of iron overload in patients with thalassemia major. *N Engl J Med*. 1994;331:567.
126. Origa R, Danjou F, Cossa S, et al. Impact of heart magnetic resonance imaging on chelation choices, compliance with treatment and risk of heart disease in patients with thalassaemia major. *Br J Haematol*. 2013;163(3):400–403.
127. Nichols-Vinueza DX, White MT, Powell AJ, et al. MRI guided iron assessment and oral chelator use improve iron status in thalassemia major patients. *Am J Hematol*. 2014;89:684–688.
128. Wood JC. Impact of iron assessment by MRI. *Hematology Am Soc Hematol Educ Program*. 2011;2011:443.
129. Au WY, Lam WW, Chu W, et al. A T2* magnetic resonance imaging study of pancreatic iron overload in thalassemia major. *Haematologica*. 2008; 93:116.
130. Noetzli LJ, Papudesi J, Coates TD, et al. Pancreatic iron loading predicts cardiac iron loading in thalassemia major. *Blood*. 2009;114:4021.
131. Brittenham GM, Farrell DE, Harris JW, et al. Magnetic-susceptibility measurement of human iron stores. *N Engl J Med*. 1982;307:1671.
132. Fischer R, Tiemann CD, Engelhardt R, et al. Assessment of iron stores in children with transfusion siderosis by biomagnetic liver susceptometry. *Am J Hematol*. 1999;60:289.
133. Finch CA, Huebers H. Perspectives in iron metabolism. *N Engl J Med*. 1982;306:1520.
134. Borgna-Pignatti C, Meloni A, Guerrini G, et al. Myocardial iron overload in thalassaemia major. How early to check? *Br J Haematol*. 2014;164(4): 579–585. https://doi.org/10.1111/bjh.12643.
135. Taher AT, Porter JB, Viprakasit V, et al. Defining serum ferritin thresholds to predict clinically relevant liver iron concentrations for guiding deferasirox therapy when MRI is unavailable in patients with non-transfusion-dependent thalassaemia. *Br J Haematol*. 2015;168(2):284–290. https://doi.org/10.1111/bjh.13119. Epub 2014 Sep 12.
136. Krittayaphong R, Viprakasit V, Saiviroonporn P, Wangworatrakul W, Wood JC. Serum ferritin in the diagnosis of cardiac and liver iron overload in thalassaemia patients real-world practice: a multicentre study. *Br J Haematol*. 2018;182(2):301–305. https://doi.org/10.1111/bjh.14776. Epub 2017 May 23.
137. Jacobs A. Low molecular weight intracellular iron transport compounds. *Blood*. 1977;50:433.
138. White GP, Bailey-Wood R, Jacobs A. The effect of chelating agents on cellular iron metabolism. *Clin Sci Mol Med*. 1976;50:145.
139. Hershko C, Graham G, Bates GW, et al. Non-specific serum iron in thalassaemia: an abnormal serum iron fraction of potential toxicity. *Br J Haematol*. 1978;40:255.
140. Anuwatanakulchai M, Pootrakul P, Thuvasethakul P, et al. Non-transferrin plasma iron in beta-thalassaemia/Hb E and haemoglobin H diseases. *Scand J Haematol*. 1984;32:153.
141. Keberle H. The biochemistry of desferrioxamine and its relation to iron metabolism. *Ann N Y Acad Sci*. 1964;119:758.
142. Hershko C, Weatherall DJ. Iron-chelating therapy. *Crit Rev Clin Lab Sci*. 1988;26:303.
143. Hershko C, Rachmilewitz EA. Mechanism of desferrioxamine-induced iron excretion in thalassaemia. *Br J Haematol*. 1979;42:125.
144. Cumming RL, Millar JA, Smith JA, et al. Clinical and laboratory studies on the action of desferrioxamine. *Br J Haematol*. 1969;17:257.
145. Pippard MJ, Callender ST, Finch CA. Ferrioxamine excretion in iron-loaded man. *Blood*. 1982;60:288.
146. Barry M, Flynn DM, Letsky EA, et al. Long-term chelation therapy in thalassaemia major: effect on liver iron concentration, liver histology, and clinical progress. *Br Med J*. 1974;2:16.
147. Seshadri R, Colebatch JH, Gordon P, et al. Long-term administration of desferrioxamine in thalassaemia major. *Arch Dis Child*. 1974;49:621.
148. Propper RD, Cooper B, Rufo RR, et al. Continuous subcutaenous administration of deferoxamine in patients with iron overload. *N Engl J Med*. 1977;297:418.
149. Bing CM. Reference on the extended stability of injectable drugs for home care. *Am J Health Syst Pharm*. 2000;57:1312.
150. Porter JB, Jaswon MS, Huehns ER, et al. Desferrioxamine ototoxicity: evaluation of risk factors in thalassaemic patients and guidelines for safe dosage. *Br J Haematol*. 1989;73:403.
151. Olivieri NF, Buncic JR, Chew E, et al. Visual and auditory neurotoxicity in patients receiving subcutaneous deferoxamine infusions. *N Engl J Med*. 1986;314:869.
152. De Virgiliis S, Congia M, Frau F, et al. Deferoxamine-induced growth retardation in patients with thalassemia major. *J Pediatr*. 1988;113:661.
153. Olivieri NF, Koren G, Harris J, et al. Growth failure and bony changes induced by deferoxamine. *Am J Pediatr Hematol Oncol*. 1992;14:48.
154. Brill PW, Winchester P, Giardina PJ, et al. Deferoxamine-induced bone dysplasia in patients with thalassemia major. *AJR Am J Roentgenol*. 1991;156:561.
155. Athanasion A, Shepp MA, Necheles TF. Anaphylactic reaction to desferrioxamine. *Lancet*. 1977;2:616.
156. Batey R, Scott J, Jain S, et al. Acute renal insufficiency occurring during intravenous desferrioxamine therapy. *Scand J Haematol*. 1979;22:277.
157. Bousquet J, Navarro M, Robert G, et al. Rapid desensitisation for desferrioxamine anaphylactoid reaction. *Lancet*. 1983;2:859.
158. Miller KB, Rosenwasser LJ, Bessette JA, et al. Rapid desensitisation for desferrioxamine anaphylactic reaction. *Lancet*. 1981;1:1059.
159. Freedman MH, Grisaru D, Olivieri N, et al. Pulmonary syndrome in patients with thalassemia major receiving intravenous deferoxamine infusions. *Am J Dis Child*. 1990;144:565.
160. Gallant T, Freedman MH, Vellend H, et al. Yersinia sepsis in patients with iron overload treated with deferoxamine. *N Engl J Med*. 1986; 314:1643.
161. Green NS. Yersinia infections in patients with homozygous beta-thalassemia associated with iron overload and its treatment. *Pediatr Hematol Oncol*. 1992;9:247.
162. Cohen AR, Galanello R, Pennell DJ, et al.Thalassemia. *Hematology Am Soc Hematol Educ Program*. 2004;14–34.
163. De Sanctis V, D'Ascola G, Wonke B. The development of diabetes mellitus and chronic liver disease in long term chelated beta thalassaemic patients. *Postgrad Med J*. 1986;62:831.
164. Marcus RE, Davies SC, Bantock HM, et al. Desferrioxamine to improve cardiac function in iron-overloaded patients with thalassemia major. *Lancet*. 1984;1:392.
165. Engle MA, Erlandson M, Smith CH. Late cardiac complications of chronic, severe, refractory anemia with hemochromatosis. *Circulation*. 1964;30:698.
166. Olivieri NF, Nathan DG, MacMillan JH, et al. Survival in medically treated patients with homozygous beta-thalassemia. *N Engl J Med*. 1994;331:574.
167. Davis BA, O'Sullivan C, Jarritt PH, et al. Value of sequential monitoring of left ventricular ejection fraction in the management of thalassemia major. *Blood*. 2004;104:263.
168. Anderson LJ, Westwood MA, Holden S, et al. Myocardial iron clearance during reversal of siderotic cardiomyopathy with intravenous desferrioxamine: a prospective study using T2* cardiovascular magnetic resonance. *Br J Haematol*. 2004;127:348.
169. Nick H, Acklin P, Lattmann R, et al. Development of tridentate iron chelators: from desferrithiocin to ICL670. *Curr Med Chem*. 2003;10:1065.
170. Piga A, Galanello R, Cappellini MD, et al. Phase II study of ICL670, an oral chelator, in adult thalassemia patients with transfusional iron overload: efficay, safety pharmacokinetics (PK) and pharmacodymanics (PD) after 18 months of therapy. *Blood*. 2003;102:121a.
171. Nisbet-Brown E, Olivieri NF, Giardina PJ, et al. Effectiveness and safety of ICL670 in iron-loaded patients with thalassaemia: a randomised, double-blind, placebo-controlled, dose-escalation trial. *Lancet*. 2003;361:1597.
172. Piga A, Fracchia S, Lai ME, et al. Deferasirox effect on renal haemodynamic parameters in patients with transfusion-dependent β thalassaemia. *Br J Haematol*. 2015;168(6):882–890.
173. Hider RC, Singh S, Porter JB, et al. The development of hydroxypyridin-4-ones as orally active iron chelators. *Ann N Y Acad Sci*. 1990;612:327.
174. Kontoghiorghes GJ, Aldouri MA, Hoffbrand AV, et al. Effective chelation of iron in beta thalassaemia with the oral chelator 1,2-dimethyl-3-hydroxypyrid-4-one. *Br Med J (Clin Res Ed)*. 1987;295:1509.
175. Berdoukas V, Chouliaras G, Moraitis P, et al. The efficacy of iron chelator regimes in reducing cardiac and hepatic iron in patients with thalassaemia major: a clinical observational study. *J Cardiovasc Magn Reson*. 2009;11:20.
176. Olivieri NF, Brittenham GM, Matsui D, et al. Iron-chelation therapy with oral deferipronein patients with thalassemia major. *N Engl J Med*. 1995; 332:918.
177. Olivieri NF, Brittenham GM, McLaren CE, et al. Long-term safety and effectiveness of iron-chelation therapy with deferiprone for thalassemia major. *N Engl J Med*. 1998;339:417.
178. Hoffbrand AV, AL-Refaie F, Davis B, et al. Long-term trial of deferiprone in 51 transfusion-dependent iron overloaded patients. *Blood*. 1998;91: 295.

179. Hoffbrand AV, Cohen A, Hershko C. Role of deferiprone in chelation therapy for transfusional iron overload. *Blood*. 2003;102:17.
180. Cohen AR, Galanello R, Piga A, et al. Safety and effectiveness of long-term therapy with the oral iron chelator deferiprone. *Blood*. 2003;102:1583.
181. Cohen AR, Galanello R, Piga A, et al. Safety profile of the oral iron chelator deferiprone: a multicentre study. *Br J Haematol*. 2000;108:305.
182. Ceci A, Baiardi P, Felisi M, et al. The safety and effectiveness of deferiprone in a large-scale, 3-year study in Italian patients. *Br J Haematol*. 2002; 118:330.
183. Del Vecchio GC, Crollo E, Schettini F, et al. Factors influencing effectiveness of deferiprone in a thalassaemia major clinical setting. *Acta Haematol*. 2000;104:99.
184. Maggio A, D'Amico G, Morabito A, et al. Deferiprone versus deferoxamine in patients with thalassemia major: a randomized clinical trial. *Blood Cells Mol Dis*. 2002;28:196.
185. Mazza P, Amurri B, Lazzari G, et al. Oral iron chelating therapy. A single center interim report on deferiprone (L1) in thalassemia. *Haematologica*. 1998;83:496.
186. Taher A, Sheikh-Taha M, Sharara A, et al. Safety and effectiveness of 100mg/kg/day deferiprone in patients with thalassemia major: a two-year study. *Acta Haematol*. 2005;114:146.
187. Piga A, Roggero S, Vinciguerra T, et al. Deferiprone: new insight. *Ann N Y Acad Sci*. 2005;1054:169.
188. El-Beshlawy AM, El-Alfy MS, Sari TT, et al. Continuation of deferiprone therapy in patients with mild neutropenia may not lead to a more severe drop in neutrophil count. *Eur J Haematol*. 2014;92(4):337–340.
189. Kowdley KV, Kaplan MM. Iron-chelation therapy with oral deferiprone–toxicity or lack of efficacy? *N Engl J Med*. 1998;339:468.
190. Wanless IR, Sweeney G, Dhillon AP, et al. Lack of progressive hepatic fibrosis during long-term therapy with deferiprone in subjects with transfusion-dependent beta-thalassemia. *Blood*. 2002;100:1566.
191. Wonke B, Wright C, Hoffbrand AV. Combined therapy with deferiprone and desferrioxamine. *Br J Haematol*. 1998;103:361.
192. Mourad FH, Hoffbrand AV, Sheikh-Taha M, et al. Comparison between desferrioxamine and combined therapy with desferrioxamine and deferiprone in iron overloaded thalassaemia patients. *Br J Haematol*. 2003;121:187.
193. Kattamis A, Ladis V, Berdousi H, et al. Iron chelation treatment with combined therapy with deferiprone and deferioxamine: a 12-month trial. *Blood Cells Mol Dis*. 2006;36:21.
194. Grady RW, et al. Combination of desferrioxamine and deferiprone markedly enhances iron excretion. *Blood*. 2002;100:241.
195. Piga A, Gaglioti C, Fogliacco E, et al. Comparative effects of deferiprone and deferoxamine on survival and cardiac disease in patients with thalassemia major: a retrospective analysis. *Haematologica*. 2003;88:489.
196. Borgna-Pignatti C, Cappellini MD, De Stefano P, et al. Cardiac morbidity and mortality in deferoxamine- or deferiprone-treated patients with thalassemia major. *Blood*. 2006;107:3733.
197. Aydinok Y, Nisli G, Kavakli K. Alternate use of deferiprone and desferrioxamine in primary school children with thalassaemia major. *Br J Haematol*. 1999;106:252.
198. Pennell DJ, Berdoukas V, Karagiorga M, et al. Randomized controlled trial of deferiprone or deferoxamine in beta-thalassemia major patients with asymptomatic myocardial siderosis. *Blood*. 2006;107:3738.
199. Tanner MA, Galanello R, Dessi C, et al. A randomized placebo-controlled double-blind trial of the effect of combination therapy with deferoxamine and deferiprone on myocardial iron in thalassemia major using cardiovascular magnetic resonance. *Circulation*. 2007;115:1876.
200. Farmaki K, Tzoumari I, Pappa C, et al. Normalisation of total body iron load with very intensive combined chelation reverses cardiac and endocrine complications of thalassaemia major. *Br J Haematol*. 2010; 148:466.
201. Elalfy MS, Adly AM, Wali Y, Tony S, Samir A, Elhenawy YI. Efficacy and safety of a novel combination of two oral chelators deferasirox/deferiprone over deferoxamine/deferiprone in severely iron overloaded young beta thalassemia major patients. *Eur J Haematol*. 2015;95(5):411–420. https://doi.org/10.1111/ejh.12507. Epub 2015 Mar 27.
202. Totadri S, Bansal D, Bhatia P, Attri SV, Trehan A, Marwaha RK. The deferiprone and deferasirox combination is efficacious in iron overloaded patients with β-thalassemia major: a prospective, single center, open-label study. *Pediatr Blood Cancer*. 2015;62(9):1592–1596. https://doi.org/10.1002/pbc.25533. Epub 2015 Mar 28.
203. Karami H, Kosaryan M, Amree AH, Darvishi-Khezri H, Mousavi M. Combination iron chelation therapy with deferiprone and deferasirox in iron-overloaded patients with transfusion-dependent β-thalassemia major. *Clin Pract*. 2017;7(1):912. https://doi.org/10.4081/cp.2017.912.
204. Kwiatkowski JL. Current recommendations for chelation for transfusion-dependent thalassemia. *Ann N Y Acad Sci*. 2016;1368(1):107–114. https://doi.org/10.1111/nyas.13088.
205. US Food and Drug Administration Medication Guide for Ferriprox: https://www.accessdata.fda.gov/drugsatfda_docs/label/2020/212266s000lbl.pdf.
206. Chelation Guidelines on Cooley's Anemia Foundation website: https://www.thalassemia.org/thalassemia-management-checklists-now-available-download/).
207. Chelation Guidelines on Thalassemia International Federation website: https://www.thalassemia.org/thalassemia-management-checklists-now-available-download/).
208. Cossu P, Toccafondi C, Vardeu F, et al. Iron overload and desferrioxamine chelation therapy in beta-thalassemia intermedia. *Eur J Pediatr*. 1981;137:267.
209. Olivieri NF, Koren G, Matsui D, et al. Reduction of tissue iron stores and normalization of serum ferritin during treatment with the oral iron chelator L1 in thalassemia intermedia. *Blood*. 1992;79:2741.
210. Taher AT, Porter JB, Viprakasit V, et al. Approaching low liver iron burden in chelated patients with non-transfusion-dependent thalassemia: the safety profile of deferasirox. *Eur J Haematol*. 2014;92(6):521–526.
211. Cappellini MD, Robbiolo L, Bottasso BM, et al. Venous thromboembolism and hypercoagulability in splenectomized patients with thalassaemia intermedia. *Br J Haematol*. 2000;111:467.
212. Borgna Pignatti C, Carnelli V, Caruso V, et al. Thromboembolic events in beta thalassemia major: an Italian multicenter study. *Acta Haematol*. 1998;99:76.
213. Aessopos A, Farmakis D, Karagiorga M, et al. Cardiac involvement in thalassemia intermedia: a multicenter study. *Blood*. 2001;97:3411.
214. Zalloua PA, Shbaklo H, Mourad YA, et al. Incidence of thromboembolic events in Lebanese thalassemia intermedia patients. *Thromb Haemost*. 2003;89(4):767–768.
215. Cario H, Wegener M, Debatin KM, et al. Treatment with hydroxyurea in thalassemia intermedia with paravertebral pseudotumors of extramedullary hematopoiesis. *Ann Hematol*. 2002;81:478.
216. Issaragrisil S, Piankigagum A, Wasi P. Spinal cord compression in thalassemia. Report of 12 cases and recommendations for treatment. *Arch Intern Med*. 1981;141:1033.
217. Ma SK, Au WY. Images in haematology. Hepatic haemopoiesis in beta-thalassaemia trait. *Br J Haematol*. 1999;107:1.
218. Saisorn I, Leewansangtong S, Sukpanichnant S, et al. Intrarenal extramedullary hematopoiesis as a renal mass in a patient with thalassemia. *J Urol*. 2001;165:507.
219. Aarabi B, Haghshenas M, Rakeii V. Visual failure caused by suprasellar extramedullary hematopoiesis in beta thalassemia: case report. *Neurosurgery*. 1998;42:922 discussion 925–926.
220. Newton KL, McNeeley Jr SG, Novick M. Extramedullary hematopoiesis presenting as a pelvic mass in a patient with beta-thalassemia intermedia. *JAMA*. 1983;250:2178.
221. Ibabao J, Kassapidis S, Demetis S, et al. Bilateral pleural effusions in a beta-thalassemia intermedia patient with posterior mediastinal extramedullary hematopoietic masses. *Hemoglobin*. 1999;23:249.
222. Kapelushnik J, Shalev H, Schulman H, et al. Upper airway obstruction-related sleep apnea in a child with thalassemia intermedia. *J Pediatr Hematol Oncol*. 2001;23:525.
223. Porcaro AB, Novella G, Antoniolli SZ, et al. Adrenal extramedullary hematopoiesis: report on a pediatric case and update of the literature. *Int Urol Nephrol*. 2001;33:601.
224. Taher A, Skouri H, Jaber W, et al. Extramedullary hematopoiesis in a patient with beta-thalassemia intermedia manifesting as symptomatic pleural effusion. *Hemoglobin*. 2001;25:363.
225. Tan TC, Tsao J, Cheung FC. Extramedullary haemopoiesis in thalassemia intermedia presenting as paraplegia. *J Clin Neurosci*. 2002;9:721.
226. Sorcinelli R, Cacace E, Del Piano M. Optic nerve compression by extramedullary hematopoietic tissue in a patient suffering from beta-thalassemia intermedia. *Metab Pediatr Syst Ophthalmol*. 1999;22-23:5.
227. Pippard MJ, Callender ST, Warner GT, et al. Iron absorption and loading in beta-thalassaemia intermedia. *Lancet*. 1979;2:819.
228. Haidar R, Musallam KM, Taher AT. Bone disease and skeletal complications in patients with β thalassemia major. *Bone*. 2011;48(3):425–432. https://doi.org/10.1016/j.bone.2010.10.173. Epub 2010 Oct 28.
229. Cunningham MJ, Macklin EA, Neufeld EJ, et al. Complications of beta-thalassemia major in North America. *Blood*. 2004;104(1):34–39.
230. Voskaridou E, Terpos E. New insights into the pathophysiology and management of osteoporosis in patients with beta thalassaemia. *Br J Haematol*. 2004;127(2):127–139.
231. Voskaridou E, Terpos E. Pathogenesis and management of osteoporosis in thalassemia. *Pediatr Endocrinol Rev*. 2008;6(Suppl 1):86–93.

232. Giardina P, Schneider R, Lesser M, et al. Abnormal bone metabolism in thalassemia. In: Ando S, Brancati C, eds. *Endocrine Disorders in Thalassemia.* Berlin: Springer Verlag; 1995.
233. Jensen CE, Tuck SM, Agnew JE, et al. High prevalence of low bone mass in thalassaemia major. *Br J Haematol*. 1998;103:911.
234. Vogiatzi MG, Autio KA, Schneider R, et al. Low bone mass in prepubertal children with thalassemia major: insights into the pathogenesis of low bone mass in thalassemia. *J Pediatr Endocrinol Metab*. 2004;17:1415.
235. Vogiatzi MG, Autio KA, Mait JE, et al. Low bone mineral density in adolescents with beta-thalassemia. *Ann N Y Acad Sci*. 2005;1054:462.
236. Terpos E, Voskaridou E. Treatment options for thalassemia patients with osteoporosis. *Ann N Y Acad Sci*. 2010;1202:237–243.
237. Wong P, Fuller PJ, Gillespie MT, et al. Thalassemia bone disease: a 19-year longitudinal analysis. *J Bone Miner Res*. 2014;29(11):2468–2473.
238. Vogiatzi MG, Macklin EA, Fung EB, et al. Prevalence of fractures among the Thalassemia syndromes in North America. *Bone*. 2006;38:571.
239. Trachtenberg FL, Mednick L, Kwiatkowski JL, et al. Beliefs about chelation among thalassemia patients. *Health Qual Life Outcomes*. 2012; 10:148.
240. Wong P, Fuller PJ, Gillespie MT, et al. Thalassemia bone disease: a 19-year longitudinal analysis. *J Bone Miner Res*. 2014;29(11):2468–2473.
241. Giusti A, Pinto V, Forni GL, Pilotto A. Management of beta-thalassemia-associated osteoporosis. *Ann N Y Acad Sci*. 2016;1368(1):73–81. https://doi.org/10.1111/nyas.13041. Epub 2016 Apr 6.
242. Giusti A. Bisphosphonates in the management of thalassemia-associated osteoporosis: a systematic review of randomised controlled trials. *J Bone Miner Metab*. 2014;32(6):606–615. https://doi.org/10.1007/s00774-014-0584-8. Epub 2014 Apr 21.
243. Bhardwaj A, Swe KMM, Sinha NK, Osunkwo I. Treatment for osteoporosis in people with β-thalassaemia. *Cochrane Database Syst Rev*. 2016;3: CD010429. https://doi.org/10.1002/14651858.CD010429.pub2.
244. Fung EB, Kwiatkowski JL, Huang JN, et al. Zinc supplementation improves bone density in patients with thalassemia: a double-blind, randomized, placebo-controlled trial. *Am J Clin Nutr*. 2013;98(4):960–971.
245. Lawson JP, Ablow RC, Pearson HA. The ribs in thalassemia. I. The relationship to therapy. *Radiology*. 1981;140:663.
246. Caffey J. Cooley's anemia: a review of the roentgenographic findings in the skeleton: Hickey lecture, 1957. *Am J Roentgenol Radium Ther Nucl Med*. 1957;78:381.
247. Asbell MB. Orthodontic aspects of Cooley's anemia. *Ann N Y Acad Sci*. 1964;119:662.
248. Currarino G, Erlandson ME. Premature fusion of epiphyses in Cooley's anemia. *Radiology*. 1964;83:656.
249. Lawson JP, Ablow RC, Pearson HA. Premature fusion of the proximal humeral epiphyses in thalassemia. *AJR Am J Roentgenol*. 1983;140: 239.
250. Lawson JP, Ablow RC, Pearson HA. The ribs in thalassemia. II. The pathogenesis of the changes. *Radiology*. 1981;140:673.
251. Spinal cord compression in thalassemia [editorial]. *Lancet*. 1:664, 1982.
252. Laor E, Garfunkel A, Koyoumdjisky-Kaye E. Skeletal and dental retardation in beta-thalassemia major. *Hum Biol*. 1982;54:85.
253. Johnston FE, Krogman WM. Patterns of growth in children with thalassemia major. *Ann N Y Acad Sci*. 1964;119:667.
254. Aydinok Y, Darcan S, Polat A, et al. Endocrine complications in patients with beta-thalassemia major. *J Trop Pediatr*. 2002;48:50.
255. Borgna-Pignatti C, De Stefano P, Zonta L, et al. Growth and sexual maturation in thalassemia major. *J Pediatr*. 1985;106:150.
256. Bronspiegel-Weintrob N, Olivieri NF, Tyler B, et al. Effect of age at the start of iron chelation therapy on gonadal function in beta-thalassemia major. *N Engl J Med*. 1990;323:713.
257. Scacchi M, Danesi L, De Martin M, et al. Treatment with biosynthetic growth hormone of short thalassaemic patients with impaired growth hormone secretion. *Clin Endocrinol (Oxf)*. 1991;35:335.
258. Theodoridis C, Ladis V, Papatheodorou A, et al. Growth and management of short stature in thalassaemia major. *J Pediatr Endocrinol Metab*. 1998; 11:835.
259. Katzos G, Papakostantinou-Athanasiadou E, Athanasiou-Metaxa M, et al. Growth hormone treatment in short children with beta-thalassemia major. *J Pediatr Endocrinol Metab*. 2000;13:163.
260. Low LC, Kwan EY, Lim YJ, et al. Growth hormone treatment of short Chinese children with beta-thalassaemia major without GH deficiency. *Clin Endocrinol (Oxf)*. 1995;42:359.
261. Chatterjee R, Katz M. Reversible hypogonadotrophic hypogonadism in sexually infantile male thalassaemic patients with transfusional iron overload. *Clin Endocrinol (Oxf)*. 2000;53:33.
262. Merkel PA, Simonson DC, Amiel SA, et al. Insulin resistance and hyperinsulinemia in patients with thalassemia major treated by hypertransfusion. *N Engl J Med*. 1988;318:809.
263. Borgna-Pignatti C, Rugolotto S, De Stefano P, et al. Survival and complications in patients with thalassemia major treated with transfusion and deferoxamine. *Haematologica*. 2004;89:1187.
264. Labropoulou-Karatza C, Goritsas C, Fragopanagou H, et al. High prevalence of diabetes mellitus among adult beta-thalassaemic patients with chronic hepatitis C. *Eur J Gastroenterol Hepatol*. 1999;11:1033.
265. Chern JP, Lin KH, Lu MY, et al. Abnormal glucose tolerance in transfusion-dependent beta-thalassemic patients. *Diabetes Care*. 2001;24:850.
266. Pappas S, Donohue SM, Denver AE, et al. Glucose intolerance in thalassemia major is related to insulin resistance and hepatic dysfunction. *Metabolism*. 1996;45:652.
267. Ladis V, Theodorides C, Palamidou F, et al. Glucose disturbances and regulation with glibenclamide in thalassemia. *J Pediatr Endocrinol Metab*. 1998;11:871.
268. Gertner JM, Broadus AE, Anast CS, et al. Impaired parathyroid response to induced hypocalcemia in thalassemia major. *J Pediatr*. 1979;95:210.
269. Masala A, Meloni T, Gallisai D, et al. Endocrine functioning in multitransfused prepubertal patients with homozygous beta-thalassemia. *J Clin Endocrinol Metab*. 1984;58:667.
270. de Luca F, Melluso R, Sobbrio G, et al. Thyroid function in thalassaemia major. *Arch Dis Child*. 1980;55:389.
271. Zervas A, Katopodi A, Protonotariou A, et al. Assessment of thyroid function in two hundred patients with beta-thalassemia major. *Thyroid*. 2002;12:151.
272. Chern JP, Lin KH. Hypoparathyroidism in transfusion-dependent patients with beta-thalassemia. *J Pediatr Hematol Oncol*. 2002;24:291.
273. Angelopoulos NG, Goula A, Rombopoulos G, et al. Hypoparathyroidism in transfusion-dependent patients with beta-thalassemia. *J Bone Miner Metab*. 2006;24:138.
274. Ellis JT, Schulman I, Smith CH. Generalized siderosis with fibrosis of liver and pancreas in Cooley's (Mediterranean) anemia; with observations on the pathogenesis of the siderosis and fibrosis. *Am J Pathol*. 1954;30:287.
275. Fink H. Transfusion hemochromatosis in Cooley's anemia. *Ann N Y Acad Sci*. 1964;119:680.
276. De Virgiliis S, Cornacchia G, Sanna G, et al. Chronic liver disease in transfusion-dependent thalassemia: liver iron quantitation and distribution. *Acta Haematol*. 1981;65:32.
277. Mak IT, Weglicki WB. Characterization of iron-mediated peroxidative injury in isolated hepatic lysosomes. *J Clin Invest*. 1985;75:58.
278. Maurer HS, Lloyd-Still JD, Ingrisano C, et al. A prospective evaluation of iron chelation therapy in children with severe beta-thalassemia: A six-year study. *Am J Dis Child*. 1988;142:287.
279. Aldouri MA, Wonke B, Hoffbrand AV, et al. Iron state and hepatic disease in patients with thalassaemia major, treated with long term subcutaneous desferrioxamine. *J Clin Pathol*. 1987;40:1353.
280. Dewey KW, Grossman H, Canale VC. Cholelithiasis in thalassemia major. *Radiology*. 1970;96:385.
281. Borgna-Pignatti C, Rugolotto S, De Stefano P, et al. Survival and disease complications in thalassemia major. *Ann N Y Acad Sci*. 1998;850:227.
282. Gabutti V, Piga A. Results of long-term iron-chelating therapy. *Acta Haematol*. 1996;95:26.
283. Kremastinos DT, Tsetsos GA, Tsiapras DP, et al. Heart failure in beta thalassemia: a 5-year follow-up study. *Am J Med*. 2001;111:349.
284. Wood JC, Enriquez C, Ghugre N, et al. Physiology and pathophysiology of iron cardiomyopathy in thalassemia. *Ann N Y Acad Sci*. 2005;1054:386–395. https://doi.org/10.1196/annals.1345.047.
285. Wood JC. Cardiac complications in thalassemia major. *Hemoglobin*. 2009;33(Suppl 1):S81–S86. https://doi.org/10.3109/03630260903347526. Suppl 1.
286. Kremastinos DT, Tiniakos G, Theodorakis GN, et al. Myocarditis in beta-thalassemia major. A cause of heart failure. *Circulation*. 1995;91:66.
287. Cohen AR, Martin M, Schwartz E. Current treatment of Cooley's anemia. Intravenous chelation therapy. *Ann N Y Acad Sci*. 1990;612:286.
288. Davis BA, Porter JB. Long-term outcome of continuous 24-hour deferoxamine infusion via indwelling intravenous catheters in high-risk beta-thalassemia. *Blood*. 2000;95:1229.
289. Hershko C, Konijn AM, Nick HP, et al. ICL670A: a new synthetic oral chelator: evaluation in hypertransfused rats with selective radioiron probes of hepatocellular and reticuloendothelial iron stores and in iron-loaded rat heart cells in culture. *Blood*. 2001;97:1115.
290. Eleftheriou P, Tanner M, Pennell D, et al. Response of myocardial T2* to oral deferasirox monotherapy for 1 Year in 29 Patients with transfusion-dependent anaemias; A subgroup analysis. *Haematologica*. 2006;91:366.
291. Porter J, Tanner M, Pennell DJ, et al. Improved myocardial T2* in transfusion dependent anemias. *Blood*. 2005;106:3600.
292. Mariotti E, Angelucci E, Agostini A, et al. Evaluation of cardiac status in iron-loaded thalassaemia patients following bone marrow transplantation:

improvement in cardiac function during reduction in body iron burden. *Br J Haematol*. 1998;103:916.
293. Koerner MM, Tenderich G, Minami K, et al. Heart transplantation for end-stage heart failure caused by iron overload. *Br J Haematol*. 1997;97:293.
294. Perrimond H, Michel G, Orsini A, et al. First report of a cardiac transplantation in a patient with thalassaemia major. *Br J Haematol*. 1991;78:467.
295. Olivieri NF, Liu PP, Sher GD, et al. Brief report: combined liver and heart transplantation for end-stage iron-induced organ failure in an adult with homozygous beta-thalassemia. *N Engl J Med*. 1994;330:1125.
296. Stanfield J. Acute benign pericarditis in thalassemia major. *Proc R Soc Med*. 1962;55:236.
297. Wasi P. Streptococcal infection leading to cardiac and renal involvement in thalassaemia. *Lancet*. 1971;1:949.
298. Sohn EY, Noetzli LJ, Gera A, et al. Pulmonary function in thalassaemia major and its correlation with body iron stores. *Br J Haematol*. 2011;155(1):102–105. https://doi.org/10.1111/j.1365-2141.2011.08808.x. Epub 2011 Aug 2.
299. Piatti G, Allegra L, Ambrosetti U, Cappellini MD, Turati F, Fiorelli G. Beta-thalassemia and pulmonary function. *Haematologica*. 1999;84(9):804–808.
300. Cooper DM, Mansell AL, Weiner MA, et al. Low lung capacity and hypoxemia in children with thalassemia major. *Am Rev Respir Dis*. 1980;121:639.
301. Secchi GC, Scotti PG, Cambiaghi G, et al. [Respiratory function tests in adolescents with beta-thalassemia major (author's transl)]. *Haematologica*. 1982;67:23.
302. Hoyt RW, Scarpa N, Wilmott RW, et al. Pulmonary function abnormalities in homozygous beta-thalassemia. *J Pediatr*. 1986;109:452.
303. Keens TG, O'Neal MH, Ortega JA, et al. Pulmonary function abnormalities in thalassemia patients on a hypertransfusion program. *Pediatrics*. 1980;65:1013.
304. Ooi GC, Khong PL, Lam WK, et al. Pulmonary iron overload in thalassemia major presenting as small airway disease. *Acta Haematol*. 2002;108:43.
305. Cooper DM, Hyman CB, Weiler-Ravell D, et al. Gas exchange during exercise in children with thalassemia major and Diamond-Blackfan anemia. *Pediatr Res*. 1985;19:1215.
306. Eldor A, Maclouf J, Lellouche F, et al. A chronic hypercoagulable state and life-long platelet activation in beta thalassemia major. *Southeast Asian J Trop Med Public Health*. 1993;24:92.
307. Rostagno C, Prisco D, Abbate R, et al. Pulmonary hypertension associated with long-standing thrombocytosis. *Chest*. 1991;99:1303.
308. Fucharoen S, Youngchaiyud P, Wasi P. Hypoxaemia and the effect of aspirin in thalassaemia. *Southeast Asian J Trop Med Public Health*. 1981;12:90.
309. Meloni A, Detterich J, Pepe A, Harmatz P, Coates TD, Wood JC. Pulmonary hypertension in well-transfused thalassemia major patients. *Blood Cells Mol Dis*. 2015;54(2):189–194. https://doi.org/10.1016/j.bcmd.2014.11.003. Epub 2014 Nov 24.
310. Grossman H, Dische MR, Winchester PH, et al. Renal enlargement in thalassemia major. *Radiology*. 1971;100:645.
311. Kreimer-Birnbaum M, Rusnak PA, Bannerman RM, et al. Urinary pyrrole pigments in thalassemia and unstable hemoglobin diseases. *Ann N Y Acad Sci*. 1974;232:283.
312. Demosthenous C, Vlachaki E, Apostolou C, et al. Beta-thalassemia: renal complications and mechanisms: a narrative review. *Hematology*. 2019;24(1):426–438. https://doi.org/10.1080/16078454.2019.1599096.
313. Quinn CT, Johnson VL, Kim HY, et al. Renal dysfunction in patients with thalassaemia. *Br J Haematol*. 2011;153:111.
314. Thalassemia Management Checklists: Monitoring Deferasirox Therapy. https://www.thalassemia.org/boduw/wp-content/uploads/2018/05/Monitoring-Deferasirox-Therapy.pdf.
315. Wood JC, Kang BP, Thompson A, et al. The effect of deferasirox on cardiac iron in thalassemia major: impact of total body iron stores. *Blood*. 2010;116:537.
316. Cohen A, Markenson AL, Schwartz E. Transfusion requirements and splenectomy in thalassemia major. *J Pediatr*. 1980;97:100.
317. Modell B. Total management of thalassaemia major. *Arch Dis Child*. 1977;52:489.
318. Laopodis V, Kritikos E, Rizzoti L, et al. Laparoscopic splenectomy in beta-thalassemia major patients. Advantages and disadvantages. *Surg Endosc*. 1998;12:944.
319. de Montalembert M, Girot R, Revillon Y, et al. Partial splenectomy in homozygous beta thalassaemia. *Arch Dis Child*. 1990;65:304.
320. al-Salem AH, al-Dabbous I, Bhamidibati P. The role of partial splenectomy in children with thalassemia. *Eur J Pediatr Surg*. 1998;8:334.
321. Banani SA. Partial dearterialization of the spleen in thalassemia major. *J Pediatr Surg*. 1998;33:449.
322. Pringle KC, Spigos DG, Tan WS, et al. Partial splenic embolization in the management of thalassemia major. *J Pediatr Surg*. 1982;17:884.
323. Pinca A, Di Palma A, Soriani S, et al. Effectiveness of partial splenic embolization as treatment for hypersplenism in thalassaemia major: a 7-year follow up. *Eur J Haematol*. 1992;49:49.
324. Stanley P, Shen TC. Partial embolization of the spleen in patients with thalassemia. *J Vasc Interv Radiol*. 1995;6:137.
325. Ikeda M, Sekimoto M, Takiguchi S, et al. High incidence of thrombosis of the portal venous system after laparoscopic splenectomy: a prospective study with contrast-enhanced CT scan. *Ann Surg*. 2005;241:208.
326. Taher AT, Otrock ZK, Uthman I, Cappellini MD. Thalassemia and hypercoagulability. *Blood Rev*. 2008;22(5):283–292. https://doi.org/10.1016/j.blre.2008.04.001. Epub 2008 Jun 3.
327. Meloni A, Detterich J, Pepe A, Harmatz P, Coates TD, Wood JC. Pulmonary hypertension in well-transfused thalassemia major patients . *Blood Cells Mol Dis*. 2015;54(2):189–194. https://doi.org/10.1016/j.bcmd.2014.11.003. Epub 2014 Nov 24.
328. Cappellini MD, Grespi E, Cassinerio E, Bignamini D, Fiorelli G. Coagulation and splenectomy: an overview. *Ann N Y Acad Sci*. 2005;1054:317–324. https://doi.org/10.1196/annals.1345.039.
329. Taher AT, Musallam KM, Karimi M, et al. Splenectomy and thrombosis: the case of thalassemia intermedia. *J Thromb Haemost*. 2010;8(10):2152–2158. https://doi.org/10.1111/j.1538-7836.2010.03940.x.
330. Singer DB. Postsplenectomy sepsis. *Perspect Pediatr Pathol*. 1973;1:285.
331. Ammann AJ, Addiego J, Wara DW, et al. Polyvalent pneumococcal-polysaccharide immunization of patients with sickle-cell anemia and patients with splenectomy. *N Engl J Med*. 1977;297:897.
332. Ambrosino DM, Molrine DC. Critical appraisal of immunization strategies for prevention of infection in the compromised host. *Hematol Oncol Clin North Am*. 1993;7:1027.
333. Borgna-Pignatti C, De Stefano P, Barone F, et al. Penicillin compliance in splenectomized thalassemics. *Eur J Pediatr*. 1984;142:83.
334. Linet MS, Nyren O, Gridley G, et al. Causes of death among patients surviving at least one year following splenectomy. *Am J Surg*. 1996;172:320.
335. Modell B, Khan M, Darlison M. Survival in beta-thalassaemia major in the UK: data from the UK Thalassaemia Register. *Lancet*. 2000;355:2051.
336. Angelucci E, Baronciani D. Allogeneic stem cell transplantation for thalassemia major. *Haematologica*. 2008;93:1780.
337. Lucarelli G, Galimberti M, Giardini C, et al. Bone marrow transplantation in thalassemia. The experience of Pesaro. *Ann N Y Acad Sci*. 1998;850:270.
338. Sodani P, Gaziev D, Polchi P, et al. New approach for bone marrow transplantation in patients with class 3 thalassemia aged younger than 17 years. *Blood*. 2004;104:1201.
339. Gaziev J, Sodani P, Polchi P, et al. Bone marrow transplantation in adults with thalassemia: Treatment and long-term follow-up. *Ann N Y Acad Sci*. 2005;1054:196.
340. Li C, Mathews V, Kim S, et al. Related and unrelated donor transplantation for β-thalassemia major: results of an international survey. *Blood Adv*. 2019;3(17):2562–2570. https://doi.org/10.1182/bloodadvances.2019000291.
341. Gaziev D, Polchi P, Galimberti M, et al. Graft-versus-host disease after bone marrow transplantation for thalassemia: an analysis of incidence and risk factors. *Transplantation*. 1997;63:854.
342. Perrine SP, Castaneda SA, Boosalis MS, White GL, Jones BM, Bohacek R. Induction of fetal globin in beta-thalassemia: cellular obstacles and molecular progress. *Ann N Y Acad Sci*. 2005;1054:257–265. https://doi.org/10.1196/annals.1345.033.
343. Ley TJ, DeSimone J, Anagnou NP, et al. 5-azacytidine selectively increases gamma-globin synthesis in a patient with beta+ thalassemia. *N Engl J Med*. 1982;307:1469.
344. Dunbar C, Travis W, Kan YW, et al. 5-Azacytidine treatment in a beta (0)-thalassaemic patient unable to be transfused due to multiple alloantibodies. *Br J Haematol*. 1989;72:467.
345. Lowrey CH, Nienhuis AW. Brief report: treatment with azacitidine of patients with end-stage beta-thalassemia. *N Engl J Med*. 1993;329:845.
346. Ley TJ. The pharmacology of hemoglobin switching: of mice and men. *Blood*. 1991;77:1146.
347. Olivieri NF. Reactivation of fetal hemoglobin in patients with beta-thalassemia. *Semin Hematol*. 1996;33:24.
348. Stamatoyannopoulos G, Veith R, al-Khatti A, et al. Induction of fetal hemoglobin by cell-cycle-specific drugs and recombinant erythropoietin. *Am J Pediatr Hematol Oncol*. 1990;12:21.
349. Olivieri NF, Rees DC, Ginder GD, et al. Treatment of thalassaemia major with phenylbutyrate and hydroxyurea. *Lancet*. 1997;350:491.
350. Zeng YT, Huang SZ, Ren ZR, et al. Hydroxyurea therapy in beta-thalassaemia intermedia: improvement in haematological parameters due to enhanced beta-globin synthesis. *Br J Haematol*. 1995;90:557.

351. Fucharoen S, Siritanaratkul N, Winichagoon P, et al. Hydroxyurea increases hemoglobin F levels and improves the effectiveness of erythropoiesis in beta-thalassemia/hemoglobin E disease. *Blood*. 1996;87:887.
352. Hajjar FM, Pearson HA. Pharmacologic treatment of thalassemia intermedia with hydroxyurea. *J Pediatr*. 1994;125:490.
353. Voskaridou E, Kalotychou V, Loukopoulos D. Clinical and laboratory effects of long-term administration of hydroxyurea to patients with sickle-cell/beta-thalassaemia. *Br J Haematol*. 1995;89:479.
354. Loukopoulos D, Voskaridou E, Stamoulakatou A, et al. Hydroxyurea therapy in thalassemia. *Ann N Y Acad Sci*. 1998;850:120.
355. Hoppe C, Vichinsky E, Lewis B, et al. Hydroxyurea and sodium phenylbutyrate therapy in thalassemia intermedia. *Am J Hematol*. 1999;62:221.
356. de Paula EV, Lima CS, Arruda VR, et al. Long-term hydroxyurea therapy in beta-thalassaemia patients. *Eur J Haematol*. 2003;70:151.
357. Steinberg MH, Rodgers GP. Pharmacologic modulation of fetal hemoglobin. *Medicine (Baltimore)*. 2001;80:328.
358. Perrine SP, Ginder GD, Faller DV, et al. A short-term trial of butyrate to stimulate fetal-globin-gene expression in the beta-globin disorders. *N Engl J Med*. 1993;328(2):81–86. https://doi.org/10.1056/NEJM199301143280202.
359. A Study of IMR-687 in Subjects With Beta Thalassemia. ClinicalTrials.gov. https://www.clinicaltrials.gov/ct2/show/NCT04411082.
360. Sankaran VG, Orkin SH. The switch from fetal to adult hemoglobin. *Cold Spring Harb Perspect Med*. 2013;3(1):a011643.
361. Sankaran VG, Menne TF, Xu J, et al. Human fetal hemoglobin expression is regulated by the developmental stage-specific repressor BCL11A. *Science*. 2008;322:1839.
362. Sankaran VG, Xu J, Ragoczy T, et al. Developmental and species-divergent globin switching are driven by BCL11A. *Nature*. 2009;460:1093.
363. Xu J, Peng C, Sankaran VG, et al. Correction of sickle cell disease in adult mice by interference with fetal hemoglobin silencing. *Science*. 2011;334:993.
364. Bank A. Regulation of human fetal hemoglobin: new players, new complexities. *Blood*. 2006;107:435.
365. Chakalova L, Osborne CS, Dai YF, et al. The Corfu deltabeta thalassemia deletion disrupts gamma-globin gene silencing and reveals post-transcriptional regulation of HbF expression. *Blood*. 2005;105:2154.
366. Xu J, Sankaran VG, Ni M, et al. Transcriptional silencing of γ-globin by BCL11A involves long-range interactions and cooperation with SOX6. *Genes Dev*. 2010;24:783.
367. Jawaid K, Wahlberg K, Thein SL, et al. Binding patterns of BCL11A in the globin and GATA1 loci and characterization of the BCL11A fetal hemoglobin locus. *Blood Cells Mol Dis*. 2010;45:140.
368. Sankaran VG, Xu J, Orkin SH. Advances in the understanding of haemoglobin switching. *Br J Haematol*. 2010;149:181.
369. A Study to Assess the Safety, Tolerability, and Efficacy of ST-400 for Treatment of Transfusion-Dependent Beta-thalassemia (TDT). ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT03432364.
370. A Safety and Efficacy Study Evaluating CTX001 in Subjects With Transfusion-Dependent β-Thalassemia. ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT03655678.
371. Breda L, Rivella S. Modulators of erythropoiesis: emerging therapies for hemoglobinopathies and disorders of red cell production. *Hematol Oncol Clin North Am*. 2014;28(2):375–386. https://doi.org/10.1016/j.hoc.2013.12.001. Epub 2014 Jan 18.
372. Attie KM, Allison MJ, McClure T, et al. A phase 1 study of ACE-536, a regulator of erythroid differentiation, in healthy volunteers. *Am J Hematol*. 2014;89(7):766–770. https://doi.org/10.1002/ajh.23732. Epub 2014 Apr 26.
373. Piga A, Perrotta S, Gamberini MR, et al. Luspatercept improves hemoglobin levels and blood transfusion requirements in a study of patients with β-thalassemia. *Blood*. 2019;133(12):1279–1289. https://doi.org/10.1182/blood-2018-10-879247. Epub 2019 Jan 7.
374. Markham A. Luspatercept: First Approval. *Drugs*. 2020;80(1):85–90. https://doi.org/10.1007/s40265-019-01251-5.
375. Casu C, Presti VL, Oikonomidou PR, et al. Short-term administration of JAK2 inhibitors reduces splenomegaly in mouse models of β-thalassemia intermedia and major. *Haematologica*. 2018;103(2):e46–e49. https://doi.org/10.3324/haematol.2017.181511. Epub 2017 Nov 2.
376. Taher AT, Karakas Z, Cassinerio E, et al. Efficacy and safety of ruxolitinib in regularly transfused patients with thalassemia: results from a phase 2a study. *Blood*. 2018;131(2):263–265. https://doi.org/10.1182/blood-2017-06-790121. Epub 2017 Nov 2.
377. Casu C, Nemeth E, Rivella S. Hepcidin agonists as therapeutic tools. *Blood*. 2018;131(16):1790–1794. https://doi.org/10.1182/blood-2017-11-737411. Epub 2018 Mar 9.
378. Casu C, Oikonomidou PR, Chen H, et al. Minihepcidin peptides as disease modifiers in mice affected by β-thalassemia and polycythemia vera. *Blood*. 2016;128(2):265–276. https://doi.org/10.1182/blood-2015-10-676742. Epub 2016 May 6.
379. Study of PTG-300 in Non-Transfusion Dependent and Transfusion-Dependent Beta-Thalassemia Subjects With Chronic Anemia (TRANSCEND). ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT03802201.
380. A Study With LJPC-401 for the Treatment of Myocardial Iron Overload in Adult Patients With Transfusion-Dependent Beta Thalassemia. ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT03381833.
381. A Study to Determine the Efficacy, Safety, Pharmacokinetics, and Pharmacodynamics of AG-348 in Adult Participants With Non-transfusion-dependent Thalassemia. ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT03692052.
382. Sadelain M. Genetic treatment of the haemoglobinopathies: recombinations and new combinations. *Br J Haematol*. 1997;98:248.
383. Dominski Z, Kole R. Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides. *Proc Natl Acad Sci U S A*. 1993;90:8673.
384. Lan N, Howrey RP, Lee S-W, et al. Ribozyme-mediated repair of sickle β-globin mRNAs in erythrocyte precursors. *Science*. 1998;280:1593.
385. Rivella S, Sadelain M. Therapeutic globin gene delivery using lentiviral vectors. *Curr Opin Mol Ther*. 2002;4:505.
386. Persons DA, Nienhuis AW. Gene therapy for the hemoglobin disorders. *Curr Hematol Rep*. 2003;2:348.
387. Nienhuis AW, Persons DA. Development of gene therapy for thalassemia. *Cold Spring Harb Perspect Med*. 2012;2(11):a011833.
388. Cavazzana-Calvo M, Payen E, Negre O, et al. Transfusion independence and HMGA2 activation after gene therapy of human β-thalassaemia. *Nature*. 2010;467(7313):318–322. https://doi.org/10.1038/nature09328.
389. A Study Evaluating the Safety and Efficacy of the LentiGlobin BB305 Drug Product in β-Thalassemia Major Participants. ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT01745120.
390. A Study Evaluating the Safety and Efficacy of LentiGlobin BB305 Drug Product in β-Thalassemia Major (Also Referred to as Transfusion-dependent β-Thalassemia [TDT]) and Sickle Cell Disease. ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT02151526.
391. Thompson AA, Walters MC, Kwiatkowski J, et al. Gene Therapy in Patients with Transfusion-Dependent β-Thalassemia. *N Engl J Med*. 2018;378(16):1479–1493. https://doi.org/10.1056/NEJMoa1705342.
392. A Study Evaluating the Efficacy and Safety of the LentiGlobin® BB305 Drug Product in Subjects With Transfusion-Dependent β-Thalassemia, Who do Not Have a β0/β0 Genotype. ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT02906202.
393. A Study Evaluating the Efficacy and Safety of the LentiGlobin® BB305 Drug Product in Subjects With Transfusion-Dependent β-Thalassemia. ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT03207009.
394. Gene Therapy for Transfusion Dependent Beta-thalassemia (TIGET-BTHAL). ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT02453477.
395. Marktel S, Scaramuzza S, Cicalese MP, et al. Intrabone hematopoietic stem cell gene therapy for adult and pediatric patients affected by transfusion-dependent ß-thalassemia. *Nat Med*. 2019;25(2):234–241. https://doi.org/10.1038/s41591-018-0301-6. Epub 2019 Jan 21.
396. Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: An increasing global health problem. *Bull World Health Organ*. 2001;79:704.
397. Christianson A, Howson CP, Modell B. *March of Dimes Global Report on Birth Defects*. New York: March of Dimes Birth Defects Foundation; 2006.
398. Higgs DR. alpha-Thalassaemia. *Baillieres Clin Haematol*. 1993;6:117.
399. Vichinsky EP. Clinical manifestations of α-thalassemia. *Cold Spring Harb Perspect Med*. 2013;3(5):a011742.
400. Higgs DR, Vickers MA, Wilkie AO, et al. A review of the molecular genetics of the human alpha-globin gene cluster. *Blood*. 1989;73:1081.
401. Higgs DR. Molecular mechanisms of alpha thalassemia. In: Steinberg MH, Forget BG, Higgs DR, eds. *Disorders of Hemoglobin*. New York: Cambridge University Press; 2001:405.
402. Bowden DK, Hill AV, Higgs DR, et al. Different hematologic phenotypes are associated with the leftward (-alpha 4.2) and rightward (-alpha 3.7) alpha+-thalassemia deletions. *J Clin Invest*. 1987;79:39.
403. Dozy AM, Kan YW, Embury SH, et al. alpha-Globin gene organisation in blacks precludes the severe form of alpha-thalassaemia. *Nature*. 1979;280:605.
404. Schwartz E, Atwater J. -thalassemia in the American Negro. *J Clin Invest*. 1972;51:412.
405. Liebhaber SA, Kan YW. alpha-Thalassemia caused by an unstable alpha-globin mutant. *J Clin Invest*. 1983;71:461.

406. Atkinson SH, Uyoga SM, Nyatichi E, et al. Epistasis between the haptoglobin common variant and α+thalassemia influences risk of severe malaria in Kenyan children. *Blood*. 2014;123(13):2008–2016.
407. Weatherall DJ, Clegg JB, Naughton MA. Globin synthesis in thalassemia: An in vitro study. *Nature*. 1965;208:1061.
408. Liebhaber SA, Kan YW. Differentiation of the mRNA transcripts originating from the alpha 1- and alpha 2-globin loci in normals and alpha-thalassemics. *J Clin Invest*. 1981;68:439.
409. Orkin SH, Goff SC. The duplicated human alpha-globin genes: their relative expression as measured by RNA analysis. *Cell*. 1981;24:345.
410. Wickramasinghe SN, Hughes M, Fucharoen S, et al. The fate of excess beta-globin chains within erythropoietic cells in alpha-thalassaemia 2 trait, alpha-thalassaemia 1 trait, haemoglobin H disease and haemoglobin Q-H disease: an electron microscope study. *Br J Haematol*. 1984;56:473.
411. Weatherall DJ. The genetics of the thalassaemias. *Br Med Bull*. 1969;25:24.
412. Liebhaber SA, Cash FE, Cornfield DB. Evidence for posttranslational control of Hb C synthesis in an individual with Hb C trait and alpha-thalassemia. *Blood*. 1988;71:502.
413. Koler RD, Rigas DA. Genetics of haemoglobin H. *Ann Hum Genet*. 1961;25:95.
414. Chui DH, Fucharoen S, Chan V. Hemoglobin H disease: not necessarily a benign disorder. *Blood*. 2003;101:791.
415. Lorey F, Charoenkwan P, Witkowska HE, et al. Hb H hydrops foetalis syndrome: a case report and review of literature. *Br J Haematol*. 2001;115:72.
416. Wasi P. In: Bowman JE, ed. Hemoglobinopathies in Southeast Asia. In: *Distribution and Evolution of the Hemoglobin and Globin Loci*. New York: Elsevier; 1983:179.
417. Sheeran C, Weekes K, Shaw J, et al. Complications of HbH disease in adulthood. *Br J Haematol*. 2014;167(1):136–139.
418. O'Brien RT. The effect of iron deficiency on the expression of hemoglobin H. *Blood*. 1973;41:853.
419. Weatherall DJ, Higgs DR, Clegg JB, et al. The significance of haemoglobin H in patients with mental retardation or myeloproliferative disease. *Br J Haematol*. 1982;52:351.
420. Gibbons RJ, Brueton L, Buckle VJ, et al. Clinical and hematologic aspects of the X-linked alpha-thalassemia/mental retardation syndrome (ATR-X). *Am J Med Genet*. 1995;55:288.
421. Weatherall DJ, Clegg JB, Boon WH. The haemoglobin constitution of infants with the haemoglobin Bart's hydrops foetalis syndrome. *Br J Haematol*. 1970;18:357.
422. Beaudry MA, Ferguson DJ, Pearse K, et al. Survival of a hydropic infant with homozygous alpha-thalassemia-1. *J Pediatr*. 1986;108:713.
423. Bianchi DW, Beyer EC, Stark AR, et al. Normal long-term survival with alpha-thalassemia. *J Pediatr*. 1986;108:716.
424. Li CK, Chan H, Chiu W, et al. Survival of an infant with homozygous alpha thalassemia [abstract]. *Pediatr Res*. 1990;27:266a.
425. Lam TK, Chan V, Fok TF, et al. Long-term survival of a baby with homozygous alpha-thalassemia-1. *Acta Haematol*. 1992;88:198.
426. Singer ST, Styles L, Bojanowski J, et al. Changing outcome of homozygous alpha-thalassemia: cautious optimism. *J Pediatr Hematol Oncol*. 2000;22:539.
427. Carr S, Rubin L, Dixon D, et al. Intrauterine therapy for homozygous alpha-thalassemia. *Obstet Gynecol*. 1995;85:876.
428. Naqvi J, Morrow W, Nisbet-Brown E, et al. Normal development of an infant with homozygous alpha thalassemia [abstract]. *Blood*. 1997;90:132a.
429. Chik KW, Shing MM, Li CK, et al. Treatment of hemoglobin Bart's hydrops with bone marrow transplantation. *J Pediatr*. 1998;132:1039.
430. A Study to Determine the Efficacy, Safety, Pharmacokinetics, and Pharmacodynamics of AG-348 in Adult Participants With Non-transfusion-dependent Thalassemia. ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT03692052.
431. In Utero Hematopoietic Stem Cell Transplantation for Alpha-thalassemia Major (ATM). ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT02986698.
432. Gibbons RJ, Picketts DJ, Villard L, et al. Mutations in a putative global transcriptional regulator cause X-linked mental retardation with alpha-thalassemia (ATR-X syndrome). *Cell*. 1995;80:837.
433. Steensma DP, Higgs DR, Fisher CA, et al. Acquired somatic ATRX mutations in myelodysplastic syndrome associated with alpha thalassemia (ATMDS) convey a more severe hematologic phenotype than germline ATRX mutations. *Blood*. 2004;103:2019.
434. Higgs DR. Gene regulation in hematopoiesis: new lessons from thalassemia. *Hematology Am Soc Hematol Educ Program*. 2004;1
435. Steensma DP, Gibbons RJ, Higgs DR. Acquired alpha-thalassemia in association with myelodysplastic syndrome and other hematologic malignancies. *Blood*. 2005;105:443.
436. Anagnou NP, Ley TJ, Chesbro B, et al. Acquired alpha-thalassemia in preleukemia is due to decreased expression of all four alpha-globin genes. *Proc Natl Acad Sci USA*. 1983;80:6051.
437. Gibbons RJ, Pellagatti A, Garrick D, et al. Identification of acquired somatic mutations in the gene encoding chromatin-remodeling factor ATRX in the alpha-thalassemia myelodysplasia syndrome (ATMDS). *Nat Genet*. 2003;34:446.
438. Humphries RK, Ley T, Turner P, et al. Differences in human alpha-, beta- and delta-globin gene expression in monkey kidney cells. *Cell*. 1982;30:173.
439. Ojwang PJ, Nakatsuji T, Gardiner MB, et al. Gene deletion as the molecular basis for the Kenya-G gamma-HPFH condition. *Hemoglobin*. 1983;7:115.
440. Fairbanks VF, Oliveros R, Brandabur JH, et al. Homozygous hemoglobin E mimics beta-thalassemia minor without anemia or hemolysis: hematologic, functional, and biosynthetic studies of first North American cases. *Am J Hematol*. 1980;8:109.
441. Fucharoen S, Winichagoon P. Clinical and hematologic aspects of hemoglobin E beta-thalassemia. *Curr Opin Hematol*. 2000;7:106.
442. Orkin SH, Kazazian Jr HH, Antonarakis SE. Abnormal RNA processing due to the exon mutation of beta E-globin gene. *Nature*. 1982;300:768.
443. Milner PF, Clegg JB, Weatherall DJ. Haemoglobin-H disease due to a unique haemoglobin variant with an elongated alpha-chain. *Lancet*. 1971;1:729.
444. Kan YW, Todd D, Dozy AM. Haemoglobin Constant Spring synthesis in red cell precursors. *Br J Haematol*. 1974;28:103.
445. Weatherall DJ, Clegg JB. The alpha-chain–termination mutants and their relation to the alpha-thalassaemias. *Philos Trans R Soc Lond B Biol Sci*. 1975;271:411.
446. Adams 3rd JG, Boxer LA, Baehner RL, et al. Hemoglobin Indianapolis (beta 112[G14] arginine). An unstable beta-chain variant producing the phenotype of severe beta-thalassemia. *J Clin Invest*. 1979;63:931.
447. Coleman MB, Steinberg MH, Adams JG 3rd. Hemoglobin Terre Haute arginine beta 106. A posthumous correction to the original structure of hemoglobin Indianapolis. *J Biol Chem*. 1991;266:5798.
448. Thein SL. Structural variants with a beta thalassemia phenotype. In: Steinberg MH, Forget BG, Higgs DR, eds. *Disorders of Hemoglobin*. New York: Cambridge University Press; 2001:342.

CHAPTER 42

PATHOBIOLOGY OF SICKLE CELL DISEASE

Robert P. Hebbel and Gregory M. Vercellotti

Since it was recognized as the "first molecular disease," sickle cell anemia (HbSS) caused by homozygosity for the mutant sickle beta-globin gene has provided the classic paradigm for single-gene disorders. Predominant clinical features include hemolytic anemia, episodic painful vasoocclusive events, an inflammatory vasculopathy, chronic organ deterioration, and a foreshortened life span. The genesis of clinical HbSS is complicated, and an understanding of its pathophysiology integrates concepts from multiple scientific disciplines, includes contributions from the red blood cell (RBC) membrane and the vascular wall endothelium, and recognizes the likely participation of multiple genetic influences. This chapter addresses the fundamental pathophysiology that underlies the sickle cell disease syndromes described in Chapter 43.

EARLY YEARS OF SICKLE CELL DISEASE RESEARCH

Sickle disease syndromes were known in folk medicine for centuries in parts of Africa, but the eponymous RBC was first reported in the medical literature in 1910 when Herrick described a young Grenadian man with recurrent pain, anemia, and sickle-shaped red corpuscles in the blood (Fig. 42.1). In 1940, Ham and Castle presciently postulated that sickle disease pathophysiology resulted from a "vicious cycle" involving mutually promotive erythrostasis and RBC sickling with adverse viscosity changes. In 1949, Neel validated the Mendelian inheritance of HbSS, and Pauling demonstrated the presence of an abnormal hemoglobin (Hb) in patients and carriers. This was followed by observation of the poor solubility of deoxygenated sickle Hb (HbS) and the reversible sol-gel transformation of HbS solutions. In 1957, Ingram identified the underlying amino acid substitution. Thereafter, increasingly detailed investigations began to reveal the striking complexities of sickle cell disease pathobiology.

GENETIC CONSIDERATIONS

Molecular Context

The sickle mutation in the *HBB* gene is a GAG→GTG conversion that creates a $\beta^{6\text{Glu}\rightarrow\text{Val}}$ substitution and thereby forms β^S globin chains. Genes for other β-globin variants are allelic to the β^S gene and have a codominant impact. Examples include genes for the normal β chain (β^A), other β mutants (e.g., β^C, β°, or β^+ thalassemia), and deletional hereditary persistence of fetal Hb (HPFH). Compound heterozygosity for β^S and each one of these results in well-defined clinical syndromes, such as HbAS (sickle trait), HbSC disease, HbS–β-thalassemia, and HbS-HPFH. Eight percent of African Americans have a β^S gene, 3% have β^C, 1.5% have β-thalassemia, and 0.1% have HPFH. Among African Americans, about 1 in 600 births results in the homozygous state, HbSS, and about 1 in 400 results in some form of sickle cell disease, which additionally includes the compound heterozygous variants other than HbAS. Worldwide, about 78% of HbSS births now occur in sub-Saharan Africa, 14% in India, 4% in the Americans, 3% in the Eastern Mediterranean, and 1% in Europe.

The *HBB* gene resides in a β-like gene cluster within which are various non-exonic polymorphic sites. Different combinations of these define discrete β-locus backgrounds, referred to as the Senegal, Benin, Bantu, Cameroon, and Arab-India haplotypes. Each refers to an ethnographic region in which the sickle mutation arose and achieved high gene frequency, typically peaking at 0.15 to 0.18. In most cases, the sickle gene resides on one of these five major haplotypes. Recent DNA sequence analysis of these haplotypes has suggested that, in fact, the sickle allele arose just once about 7300 years ago in central Africa and spread to these 5 haplotypes as the result of migration and continued selective pressure from malaria.

Origin, Selection, and Dispersion of the Sickle Gene

The residence of both β^A and β^S alleles on the distinct regional β cluster haplotypes suggests that the sickle mutation was selected for independently in the five regions. The β^C mutation arose only once. Historical and biological data argue that the frequency of the β^S gene greatly expanded in South Asia about 4000 years ago and in Africa about 3000 years ago, following the introduction of iron tools in each case. That led to the adoption of an agricultural system that promoted both increased human habitation density and favorable breeding conditions for the *Anopheles* mosquito, which in turn enabled the development of endemic *Plasmodium falciparum* malaria. In this context, high fixed β^S gene frequencies were reached because of a balanced polymorphism, such that heterozygotes (HbAS) have an adaptive advantage over either homozygote. Thus, the Old World geographic distributions of the sickle gene and historical endemic malaria are notably concordant, suggesting that the sickle gene represents "a biologic solution to a cultural problem."

In hyperendemic areas, *falciparum* malaria uniformly infects the young and is the primary cause of death for children with HbSS. However, those with HbAS are less likely to develop severe malaria, a protective effect appearing early in childhood and possibly increasingly until age 10. At the level of the RBC, this protection reflects steps after the initial parasite invasion. One proposed mechanism links protection to the instability of HbS, immune status, and splenic function. That is, infection of HbAS RBCs with *P. falciparum* leads sequentially to augmented Hb denaturation, clustering of membrane protein band 3, attraction of band 3 autoantibody, complement binding, and enhanced erythrophagocytosis even of the early ring forms. Thereby, an accelerated clearance of parasitized RBC by the functional spleen could protect those with HbAS, while HbS homozygotes would lose this protection as they acquire functional asplenia. In synergy with this scenario, the presence of HbS impairs endothelial cytoadherence of infected RBC, thereby diminishing cerebral symptoms and impeding the RBC sequestration that would otherwise protect them from splenic exposure. An alternative (or complementary) immune mechanism may be developing antibodies to *falciparum* antigens on the RBC surface.

Additionally, the blunted malarial susceptibility in HbAS reflects complex interrelationships between the sickle gene, other globin genes, host biology, and environmental factors. For example, the protective benefit of HbAS is lost if there is concurrent α thalassemia (which lowers the proportion of HbS). Malarial severity is affected by polymorphisms in non-globin genes such as *CR1* (complement receptor 1), *CD36*, *TGFB1* (transforming growth factor β), *HMOX1* (heme oxygenase 1), and *TLR4* (toll-like receptor 4). Both carbon monoxide (CO) and nitric oxide (NO) blunt the severity of experimental malaria. And certain micro RNAs, enriched in HbS-containing RBC, can inhibit *P. falciparum* growth.

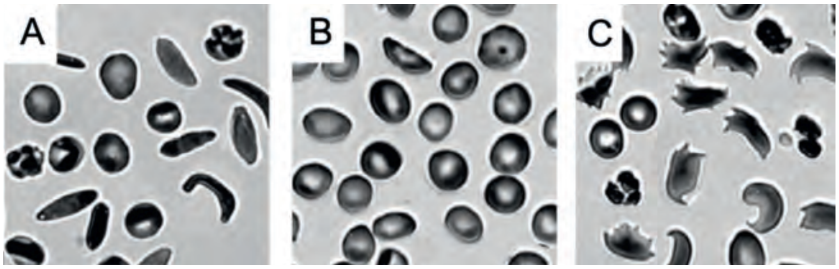


Figure 42.1 SICKLE RED BLOOD CELL (RBC) MORPHOLOGIES. (A) A sickle cell anemia blood smear made directly from venous blood not exposed to oxygen. Smoothly elongated, raisin-shaped, and normal-appearing sickle RBC are present. (B) The same blood fully oxygenated reveals morphology that is largely normal, except for a single irreversibly sickled cell (ISC). (C) Cells from panel B that have now been fully deoxygenated, causing most RBCs to become sickled. The physical–chemical basis for these shapes is presented in Fig. 42.4. *(Reproduced with permission from Obata K, Mattiello J, Asakura K, et al. Exposure of blood from patients with sickle cell disease to air changes the morphological, oxygen-binding, and sickling properties of sickled erythrocytes. Am J Hematol. 2006;81:26.)*

Eventually, the sickle gene spread geographically by means of commerce, migration, and the slave trade. This dispersion has been tracked by analyses of the regional β haplotypes, a biological marker that largely corroborates predictions of gene flow derived from historical records. As a generalization, it spread on the Benin haplotype to North Africa and then across the Mediterranean. All three major African haplotypes are present in the western Arabian Peninsula; but on the eastern side, the sickle gene tends to be on the Arab-India haplotype. This is also true in India, although sub-Saharan haplotypes are represented as well. In the Americas, the β^S gene is mostly found on the Benin, Senegal, and Bantu haplotypes.

ABNORMAL MOLECULAR BEHAVIORS OF SICKLE HEMOGLOBIN

Because the $\beta^{6Glu \rightarrow Val}$ substitution entails a loss of negative charge and gain in hydrophobicity, HbS exhibits three abnormal molecular behaviors of direct relevance to pathophysiology. (See box on Relationship of HbS Molecular Behaviors to Disease Features.)

RELATIONSHIP OF HbS MOLECULAR BEHAVIORS TO DISEASE FEATURES

- Altered dimer assembly → RBC Hb composition
  - Hb phenotype and diagnosis
  - Polymerization risk
- HbS instability → Membrane defects
  - RBC dehydration
  - Hemolysis
  - Malaria resistance
- HbS polymerization → Sickling
  - Vasoocclusion
  - Hemolysis

Hemoglobin S Charge and Tetramer Assembly

The formation of Hb tetramers requires the proximate assembly of stable dimers from unlike monomers (e.g., $\alpha + \beta \rightarrow \alpha\beta$), an event governed by electrostatic attraction. The normal α and β chains are positively and negatively charged, respectively. In heterozygous states for β-globin mutants, β-chain competition for dimer assembly is a determinant of the relative proportions of the Hb variants.[1] Mutant β chains with less negative charge form αβ dimers more slowly; the relative rates for dimer association are $\alpha\beta^A > \alpha\beta^S > \alpha\beta^C$, with $\alpha\beta^A$ dimers formed about twice as rapidly as $\alpha\beta^S$ dimers. This explains why those with HbAS typically have only 40% HbS and why the proportion of HbS exceeds this in HbSC disease. It also explains the effect of concurrent α-thalassemia on the proportion of HbS in sickle trait—availability of α chains becomes limiting, the percentage of HbS typically drops from 40% to 35% (one α deletion), 30% (two α deletions), or less than 25% (three α deletions).

Hemoglobin S Stability and Oxidant Formation

HbS is modestly unstable, observed in vitro as instability to various applied stresses. Two stresses that are most clearly physiologic involve Hb oxidation.[2] HbS has an abnormal redox potential compared with HbA that may underlie its modestly (~40%) increased autooxidation rate. Yet, HbS exhibits markedly (~340%) augmented instability and oxidation upon interaction with aminophospholipids of the membrane's inner leaflet. This instability leads to the accumulation of various Hb and iron forms at the cytosol–membrane interface.[2] The resulting localization of abnormal oxidative biochemistry promotes a number of prominent defects of the sickle RBC membrane. Other contributors to oxidant stress within sickle RBC are addressed later.

Hemoglobin S and Polymerization

OxyHbS, oxyHbA, and deoxyHbA have very high solubilities, but deoxyHbS aggregates into densely packed polymers, a process that is fully reversible upon reoxygenation.[3, 4] This abnormal property, the fundamental pathogenesis of the sickling disorders, causes the eponymous RBC shape to change because of polymer-mediated distortion (see Fig. 42.1).

Polymer Structure

Deoxygenation transforms soluble HbS into a highly viscous and semisolid gel that behaves thermodynamically similar to a crystal in equilibrium with a solution of individual tetrameric Hb molecules. Even complete deoxygenation does not convert all deoxyHbS to polymers. The insoluble phase is a collection of domains of aligned polymers, the basic unit of which is a double-strand in which two strings of deoxyHb tetramers make multiple contacts with each other (Fig. 42.2).

Each HbS tetramer has two β^S chains, the β_1 and β_2. DeoxyHbS undergoes a slight structural shift so that the A helix β^{6Val} "donor" site of the β_2 chain in one tetramer can contact an EF helix "acceptor" site (formed mainly by β^{85Phe}, β^{88Leu}, and β^{70Ala}) in the β_1 chain of a tetramer in the neighboring single string. This critical, lateral association can be made only when HbS is in its deoxy conformation. In HbA this EF helix hydrophobic pocket is not a favorable acceptor site for the charged β^{6Glu} of the β^A. In HbS, the β^{6Val} in the β_1 subunit is located so it cannot participate in such contacts. However, the β_2 chain of the second single string can form chemically similar β^{6Val}-dependent contacts with the β_1 chain of the first single string. There are multiple additional axial and lateral contacts, but these are largely the same for deoxyHbA and deoxyHbS and are not themselves sufficient to stabilize a polymeric structure.

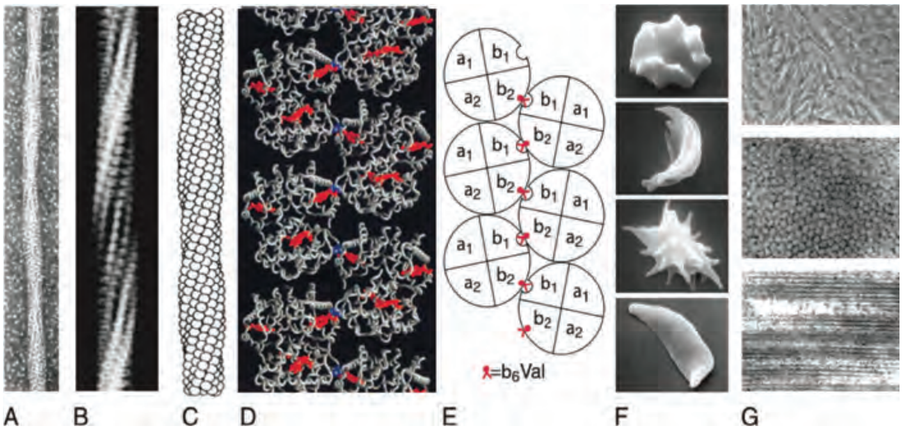


Figure 42.2 DEOXYGENATED HEMOGLOBIN S (HBS) POLYMER. (A) Electron micrograph of a fiber of polymerized HbS. (B) Electron density surface map, modeled from authentic HbS fibers, shows pairings that create double strands plus a helical twist. (C) Model of the HbS fiber, with Hb tetramers rendered as solid spheres. (D) Protein backbone shows tetramer staggering in the HbS crystal. Heme in red, and β^6 valines in blue. (E) Schematic representation of a double-strand (without the twist), emphasizing that only one of the two β^6 valines residing (in red) in each HbS tetramer participates in critical lateral contacts. (F) Sickled red blood cells, showing various morphologies *(top to bottom):* granular, holly leaf-shaped, classically sickled, and irreversibly sickled. (G) Electron microscopy of sickled RBC cytoplasm reveals highly ordered polymer domains, as seen from the side *(bottom)* and on the end *(middle)*, or highly disorganized domains from rapid polymerization *(top)*. *(A and C, Reproduced with permission from Dykes G, Crepeau RH, Edelstein SJ. Three-dimensional reconstruction of the fibres of sickle cell hemoglobin. Nature. 1978;272:506. B, Reproduced with permission from Carragher B, Bluemke DA, Becker M, et al. Structural analysis of polymers of sickle cell hemoglobin. J Mol Biol. 1988;199:315. D, Reproduced with permission from Harrington DJ, Adachi K, Royer WE, Jr. The high-resolution crystal structure of deoxyhemoglobin S. J Mol Biol. 1997;272:398. F and G, Courtesy Dr. James G. White and reproduced with permission from White JG. Ultrastructural features of erythrocyte and hemoglobin sickling. Arch Intern Med. 1974;133:545.)*

In the physiologic form of the polymer, the component strings of HbS molecules in a double-strand are half-staggered and have a slight twist, creating a fiber that is approximately 21 nM in diameter and is composed of one central and six peripheral double strands. The crystal formed in vitro lacks the twist, but its molecular structure is known in great detail.

HbS Solubility

The RBC's hydration state dominates the physical-chemical behavior of HbS. The solubility of deoxyHbS (approximately 16 g/dL, measured under laboratory conditions) is much lower than the RBC mean cell Hb concentration (MCHC, ~34 g/dL). So, even partial cellular deoxygenation can raise deoxyHbS concentration above its solubility limit, allowing polymerization to occur. The biophysical effect of macromolecular crowding (boosting a protein's activity far above that predicted from concentration alone) confers nonideal behavior upon cytoplasmic constituents, augmenting the likelihood for polymerization at any given degree of deoxygenation.

Equilibrium and Polymerization

In vitro studies carried out under (nonphysiologic) equilibrium conditions of stable oxygen tension and long-time scale corroborate crystallographic identification of critical amino acids involved in atomic contacts by revealing the influence of other Hbs on HbS solubility (Fig. 42.3).[3] When different Hbs are mixed together, the tetramers dissociate into dimers that intermix and randomly reassemble in a binomial distribution to reform tetramers. This clarifies the impact of naturally occurring, intracellular Hb mixtures. In a mixture of HbS and HbA, overall solubility is improved because the hybrid $\alpha\beta^S/\alpha\beta^A$ tetramer integrates into polymer only one half as well as the $\alpha\beta^S/\alpha\beta^S$ tetramer (see Fig. 42.3A). The addition of HbF to HbS has a greater sparing effect because neither the $\alpha\gamma/\alpha\gamma$ nor the hybrid $\alpha\beta^S/\alpha\gamma$ tetramer can be incorporated into polymers. In this regard, HbC has the same effect as HbA, and HbA_2 has the same effect as HbF. This sparing effect of HbA is such that much lower Hb oxygen saturation is required for a polymer to form in HbAS than in HbSS RBCs (see Fig. 42.3B).

Kinetics and Polymerization

Laboratory measurements of polymerization kinetics, enabled by inducing (nonphysiologic) near-instantaneous and complete conversion of HbS from R (oxy) to T (deoxy) state, reveal a delay until polymer forms explosively.[4] This inherent delay time is inversely related to an extraordinarily high power of the initial Hb concentration. For example, the delay is 10 ms at HbS 40 g/dL, but it is 100,000 seconds at HbS 20 g/dL (Fig. 42.4A). HbS solutions and sickle RBCs behave similarly in this regard. Delay times must vary enormously from cell to cell because they are dominated by the marked heterogeneity in MCHC (i.e., shorter delay for more dehydrated cells) and are influenced by the presence of any non-S Hb (i.e., longer delay for the presence of HbA, C, or F) (see Fig. 42.4E). Admixture of 20% to 30% HbA with HbS (simulating HbS-β^+-thalassemia) increases the delay time 10- to 100-fold, and admixture of 20% to 30% HbF with HbS increases it by 10^3- to 10^4-fold.

The mechanism of such polymer formation is understood to proceed by a two-step, double-nucleation process (see Fig. 42.4F). Accordingly, the initial homogeneous nucleation takes place in bulk solution, during which small numbers of tetramers associate, with accumulation not favored until a critical nucleus size, develops (estimated to be 30 to 50 tetramers). Only then can new tetramers be added lengthwise to form a large polymer. After this occurs, heterogeneous nucleation causes explosive, autocatalytic polymer formation as new fibers form and extend on the surface of the preexisting polymer. It is the time until this explosive formation occurs that laboratory experiments detect as the *inherent* "delay time."

The striking irreproducibility of long delay times reflects the underlying stochastic formation of a single (or very few) homogeneous nucleation event(s) in cells that form polymer slowly—and thus assume a sickled shape reflecting membrane deformation by highly elongated polymer (see Fig. 42.4B). In contrast, short delay times are highly reproducible and reflect the simultaneous formation of multiple nucleation sites in cells that polymerize rapidly—and thus assume a less dramatic, irregular shape reflecting membrane deformation by multiple short polymer domains (see Fig. 42.4C and D).

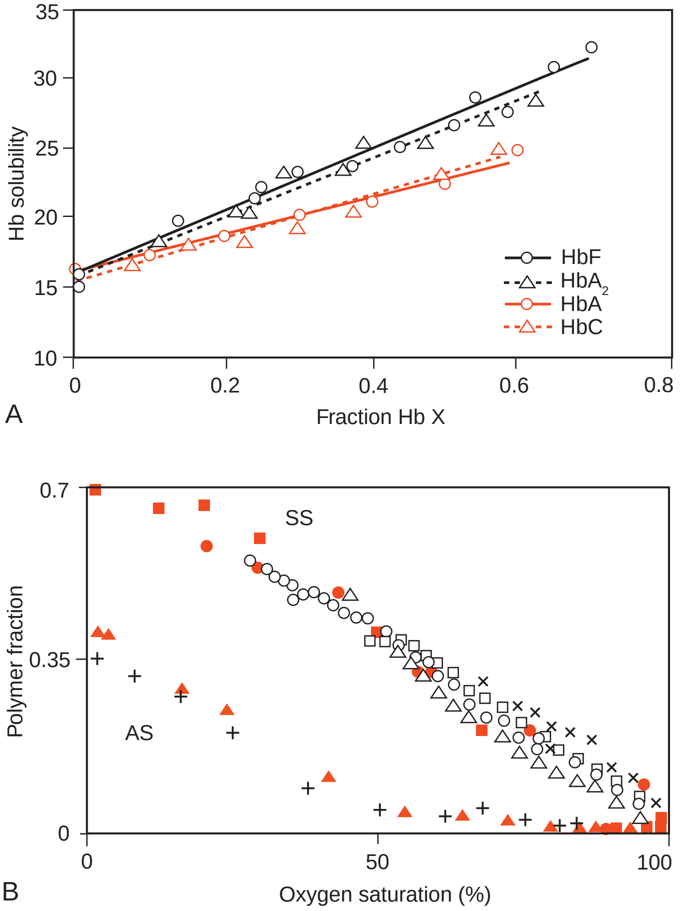


Figure 42.3 DEOXYHEMOGLOBIN S SOLUBILITY, DEFINED BY EQUILIBRIUM STUDIES. (A) Under laboratory conditions, solubility of 100% deoxyHbS is ~16 g/dL. An admixture of other hemoglobins with HbS raises overall solubility in absence of oxygen. The x-axis indicates the proportion of admixed non-sickle Hb. (B) The hemoglobin oxygen saturation required to initiate intracellular polymer formation (i.e., polymer fraction) is much lower for HbAS RBC than for HbSS RBC. *(A, Reproduced with permission from Poillon WN, Kim BC, Rodgers GP, et al. Sparing effect of hemoglobin F and hemoglobin A_2 on the polymerization of hemoglobin S at physiologic ligand saturations. Proc Natl Acad Sci U S A. 1993;90:5039. B, Reproduced with permission from Schechter AN, Noguchi CT. Sickle hemoglobin polymer: structure-function correlates. In: Embury SH, Hebbel RP, Mohandas N, Steinberg MH, eds. Sickle Cell Disease: Basic Principles and Clinical Practice. New York: Raven Press; 1994.)*

Polymerization Under Physiologic Conditions

In physiologic conditions, however, sickle RBCs are neither at equilibrium with constant oxygen tension nor undergoing instantaneous complete deoxygenation. Rather, irrespective of the inherent delay time addressed above, the rate of deoxy-HbS polymer growth in vivo is limited by the rate at which RBC deoxygenation develops during the microvascular passage. Since this transit time is on the order of ~1 second, it effectively renders irrelevant any inherent delay times of more than ~1 second (see Fig. 42.4G).[4] Thus kinetic considerations argue that most RBCs in patients with HbSS are unlikely to sickle during their passage through the microcirculation unless something, such as RBC–endothelial adhesion, slows their transit.

Predictability is complicated by the marked heterogeneity among sickle RBCs in both MCHC and HbF content, as well as the natural biologic variability in capillary transit times. A good qualitative correspondence between polymerization in solution and within RBCs argues that the fundamental polymerization mechanism is not altered by RBC membranes. Yet, emerging evidence suggests that the notably abnormal sickle RBC membrane can accelerate nucleation, in essence eliminating the inherent delay time. A similar effect would be exerted by any preexisting polymer not completely melted during prior pulmonary transit (expected for fewer than 1% of RBCs). However, neither of these effects would alter the physiologic constraint that bulk polymer growth rate can only parallel RBC deoxygenation rate.

In vitro, sickle RBC can become classically sickled or assume holly leaf or granular forms, depending on deoxygenation rate (slow to rapid, respectively), which determines the number of nucleation domains created (see Fig. 42.4B–D). In the microcirculation, granular forms are probably most likely to occur; in contrast, frankly sickled forms are probably more likely to develop in veins. The RBC shape per se is not a determinant of RBC deformability, but rigidification caused by polymer does and, therefore, affects microvascular passage.

HbF and Its Protective Effect

In HbSS, HbF in RBC lysates averages 5% to 8% (range 1% to 25%). However, this HbF is not distributed evenly amongst RBCs.[5] Rather, its heterocellular expression is evident in the presence of F cells (RBC particularly enriched in HbF) that comprise anywhere between 2% and 80% of all RBCs. For most patients, only the small proportion of their F cells that contain at least ~10 pg HbF (roughly one-third of RBC Hb content) are predicted to be protected from polymerization under physiologic conditions.[5] On average, F cells remain better hydrated and exhibit better survival.

Alternative Ligands: CO and NO

Patients with HbSS can have nontrivial elevations of CO-Hb levels (reportedly as high as 7.6% in children) because of hemolysis. Hb that is partially liganded with CO is shifted to the R state conformation but has lost a portion of its oxygen-carrying capacity. RBC and Hb appear to participate in NO transport to the microcirculation, although both magnitude of the effect and mechanisms involved are debated. NO is asserted to improve RBC deformability and impair HbS polymerization. The reaction of NO with oxyHb causes Hb oxidation to metHb and reciprocal consumption of NO.

ABNORMALITIES OF SICKLE RED BLOOD CELLS

Even oxygenated sickle RBCs exhibit a variety of cellular and membrane abnormalities that contribute directly to pathophysiology. Some are the direct consequence of polymer formation, some result from oxidative biochemistry, and some even reflect RBC response to its environment. An overarching theme in sickle disease pathobiology is that individual sickle RBC exhibit remarkable heterogeneity in various cellular characteristics. Their striking variabilities in hydration status (MCHC) and HbF content are particularly important. (See box on Major Sickle RBC Membrane Defects.)

Membrane Iron and Oxidant Generation

An abnormal oxidative biochemistry takes place at the cytosol–membrane interface of the sickle RBC.[2] The avidity of HbS for bilayer lipid, and perhaps its modestly enhanced auto-oxidation in solution, resulting in the augmented formation of superoxide and met-Hb. This, in turn, can readily lose its heme to the lipid bilayer, where it is easily destroyed by lipid hydroperoxides to liberate “free” iron. Forms of iron on the sickle RBC membrane are catalytically active, generating highly reactive oxidants. Also, membrane “free” iron can form a redox couple with soluble oxyHb to promote further Hb oxidation, denaturation, and deposition. The sickle RBC membrane thereby

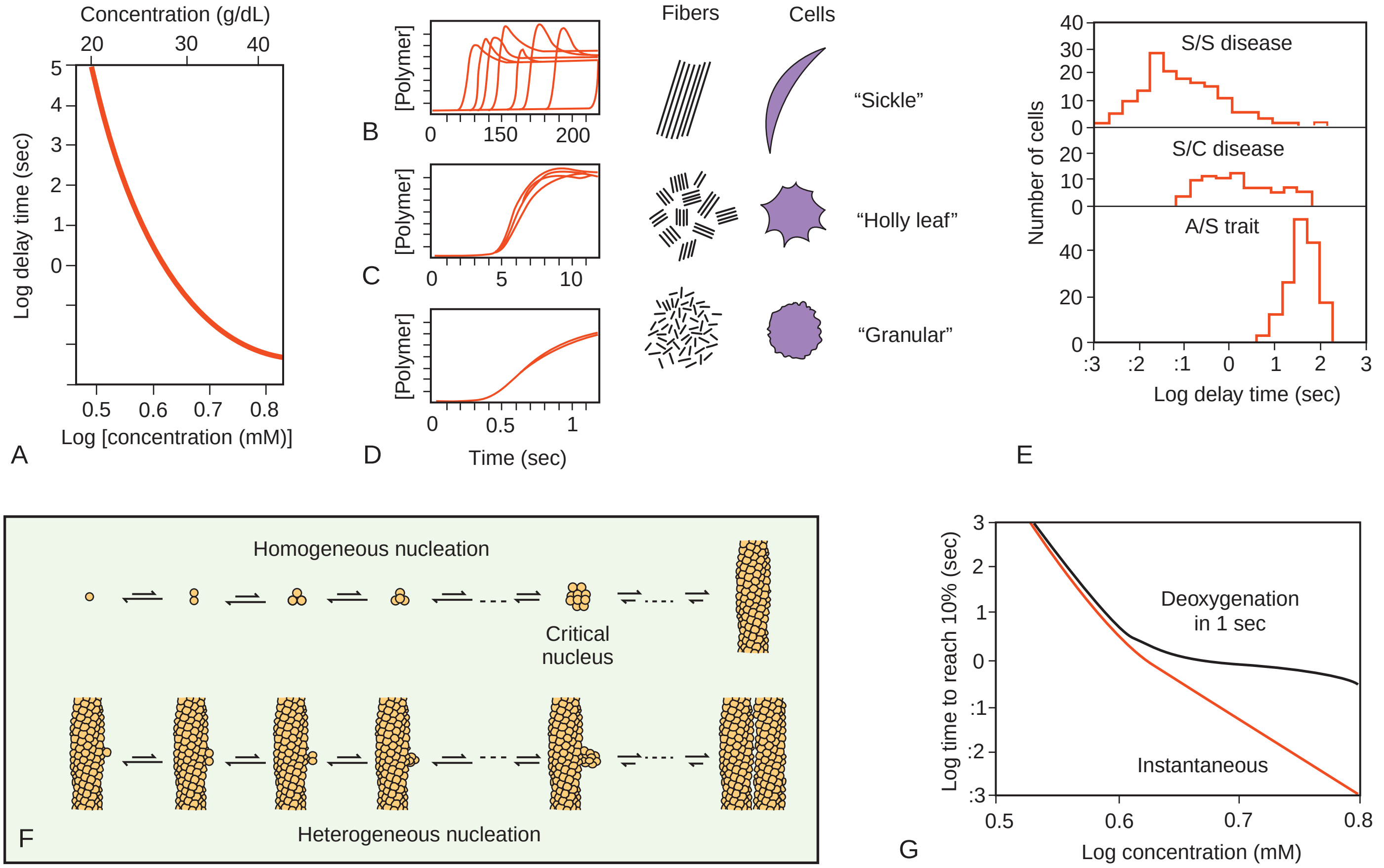


Figure 42.4 KINETICS OF HEMOGLOBIN S POLYMERIZATION AFTER NEAR-INSTANTANEOUS AND COMPLETE DEOXYGENATION. (A) Extreme dependence of delay time on hemoglobin concentration. (B–D) Kinetic progress curves for HbS polymer formation. (B) Long delay times are highly variable, tend to involve a single elongated polymer domain and result in sickle-shaped RBC. (C and D) Short delay times are far more reproducible, tend to involve multiple polymer domains and result in holly leaf or granular shaped RBC. (E) Delay times for individual RBCs are influenced by substituent hemoglobins. (F) A double nucleation process underlies polymer formation, with unfavored homogeneous nucleation *(top)* followed by explosive heterologous nucleation *(bottom)*. (G) Delay times that are short relative to the physiological rate of deoxygenation rate (i.e., ~1 second) are pathophysiologically irrelevant. *(A–E, Reproduced with permission from Eaton WA, Hofrichter J. Hemoglobin S gelation and sickle cell disease. Blood. 1987;70:1245. F, Reproduced with permission from Ferrone FA, Hofrichter J, Eaton WA. Kinetics of sickle hemoglobin polymerization II. A double nucleation mechanism. J Mol Biol. 1985;183:611. G, Reproduced with permission from Ferrone FA. Oxygen transits and transports. In Embury S, Hebbel RP, Mohandas N, Steinberg MH, eds. Sickle Cell Disease: Basic Principles and Clinical Practice. New York: Raven Press; 1994.)*

acquires abnormal amounts of various iron forms: Hb, denatured hemichrome, free heme, and nonheme iron.[2]

The membrane association of catalytic iron establishes in sickle RBC a unique oxidant risk (not present in normal RBC) because it effectively targets oxidative damage to membrane components. Further, the juxtaposition of iron with bilayer lipid allows re-initiation of peroxidative chain reactions, effectively bypassing protection by vitamin E. Deficient levels of antioxidants (e.g., vitamin E, glutathione, ascorbic acid) in sickle RBC, caused by oxidative consumption and dietary insufficiencies, contribute. The result is abnormal oxidation of membrane protein thiols and peroxidation of membrane lipids. Among the many sickle membrane defects, evidence for an oxidative origin or contribution is strongest for Band 3 clustering, abnormal stiffness, formation of irreversibly sickled cells (ISCs), aberrant cation homeostasis, microvesiculation tendency, abnormal mechanosensitivity, and erythrophagocytosis.[2]

Other sources of oxidant generation by sickle RBC include the reaction of H_2O_2 with metHbS to form membrane-damaging ferryl-HbS. Enhanced activity of sickle RBC nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase exhibits responsiveness to certain plasma substances, for example, endothelin-1 (ET-1). And some oxidant generation derives from retained mitochondria. within sickle RBC.

Cation Homeostasis and Dehydrated Cells

For normal RBCs, MCHC averages ~32 g/dL and varies from 27 to 38 g/dL, with fewer than 1% of cells having MCHC greater than 38 g/dL. In contrast, the MCHC of sickle RBCs averages ~34 g/dL and ranges from 23 to 50 g/dL, with up to 40% of cells having MCHC greater than 38 g/dL. This extreme density heterogeneity results from reticulocytosis (low-density, low-MCHC cells) and dehydrating mechanisms (higher density, high-MCHC cells) (Fig. 42.5).

The most dramatic ion-handling abnormality of the sickle RBC is sickling-induced permeabilization of the RBC membrane to cations (Na^+, K^+, Ca^{2+}).[6] Since this depends on cell deformation, it probably partly reflects, in part, the sickle RBC's exaggerated leak susceptibility to deformation (mechanosensitivity).[2] Sickling induces calcium influx and slight acidification, occurring stochastically and only in some cells at any one time. This results in net potassium and water loss mediated mostly by activation of a Ca^{2+} activated (Gardos) K^+ channel and potassium chloride (KCl) cotransport. The latter can be activated by lowered pH, ET-1, thiol oxidation, and a membrane interaction effect of Hbs that are relatively positively charged (HbC > HbS > HbA). It is influenced by macromolecular crowding of cytosolic proteins caused by the high MCHC. Via its greater membrane association, HbC promotes greater cation loss via KCl cotransport than HbS does, probably the reason HbSC is more severe than HbAS. Even at a steady state, sickle RBCs can contain increased Ca^{2+} because it can be sequestered in cytoplasmic inside-out membrane vesicles, a footprint of prior cytosolic Ca^{2+} transients.

These aberrancies lead to decrements in RBC hydration and deformability.[7] Consequently, hyperdense RBCs—mostly ISCs—are not necessarily older cells with longer histories of sickling and unsickling. Rather, they can develop via a rapid reticulocyte-to-ISC transformation, with those RBC having lower HbF levels being particularly susceptible. It is unclear whether this rapid induction of cation loss or the gradualism of classic interpretations is the dominant mechanism underlying sickle RBC dehydration. Dehydrated RBCs have diminished deformability and an increased propensity for polymerization, the mutually promotive effects of dehydration and sickling comprising a vicious cycle. RBC dehydration is particularly likely to be exaggerated by the renal medullary environment and possibly by nocturnal arterial desaturation accompanying disordered sleep.

MAJOR SICKLE RBC MEMBRANE DEFECTS

- Membrane Hb/iron deposits →
  - Band 3 clumping →
  - Ig attraction →
    - Erythrophagocytosis
  - Oxidative reactions targeted at membrane →
    - Thiol oxidation →
      - ISC formation
      - ↓ Deformability
      - ↑ Fragility
      - PS externalization
      - Cation leak
      - Microvesiculation
    - Lipid peroxidation →
      - Enhanced mechanosensitivity →
        - ↑ Leakiness from deformation
      - Erythrophagocytosis
- Abnormal cation homeostasis →
  - RBC dehydration →
    - ↓ Deformability
- Abnormal microrheology →
  - ↓ Deformability
- PS externalization →
  - Coagulation acceleration
  - Erythrophagocytosis
- Adhesion to endothelium →
  - Endothelial activation/injury
  - Vasoocclusion

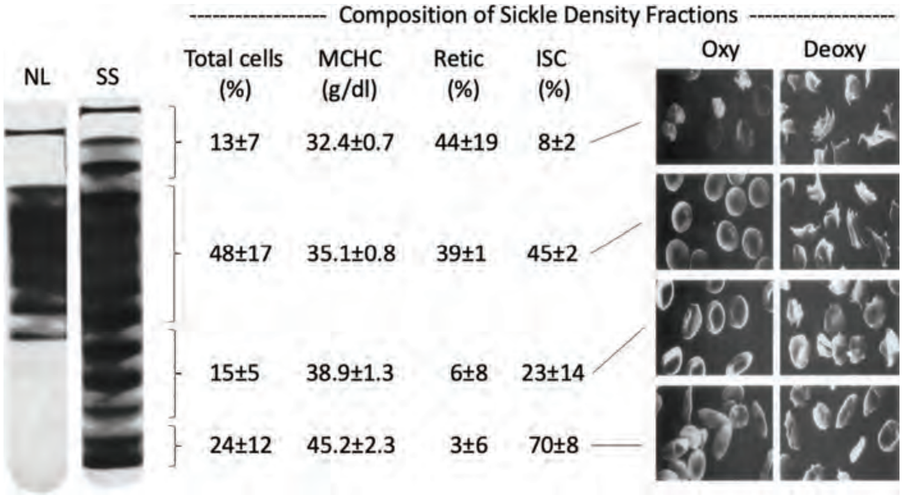
Deformability, Fragility, and Vesiculation

Even oxygenated sickle RBCs are poorly deformable.[7] The dominant cause of this is the abnormally high cytoplasmic viscosity of dehydrated cells. Additional factors include abnormal stiffness of the RBC membrane caused, in part, by protein thiol oxidation and, in part, by a poorly understood direct rigidifying effect of HbS upon the membrane. Upon RBC deoxygenation in vitro, there is a temporal correspondence between the appearance of polymer-induced shape change and deterioration of deformability, as measured by micropipette and laser diffractometer. On the other hand, filtration studies found decreased deformability before the morphologic change, and viscometry reveals a large increase in bulk viscosity caused by deoxygenated dense discocytes that show little shape change.

Sickle RBCs are somewhat mechanically fragile, which may be a consequence of dehydration and a weakening of critical skeletal associations caused by oxidative protein damage. The tendency of sickled RBCs to lose membrane microvesicles reflects the separation of the bilayer from the underlying skeleton due to spicules of polymerized Hb, with enhanced susceptibility caused by protein thiol oxidation.

Membrane Proteins and Lipids

Sickle RBC membrane protein function is adversely affected by excess thiol oxidation and possibly other oxidative protein modifications.[2] Ankyrin interactions with spectrin and Band 3 are abnormal, glycophorin and Band 3 exhibit decreased mobility, and thiol-oxidized β-actin displays abnormal associations in the spectrin–actin-4.1 complex. Band 3 is abnormally clumped from the binding of denatured



Composition of Sickle Density Fractions

Total cells (%)	MCHC (g/dl)	Retic (%)	ISC (%)
13±7	32.4±0.7	44±19	8±2
48±17	35.1±0.8	39±1	45±2
15±5	38.9±1.3	6±8	23±14
24±12	45.2±2.3	3±6	70±8

Figure 42.5 MARKED HETEROGENEITY IN SICKLE RED BLOOD CELL HYDRATION. *Left:* Density gradient separations of normal (NL) and sickle (SS) RBCs illustrate that many sickle RBC are abnormally dense *(at bottom)*, and an increased number are low-density RBCs *(at top)*. The brackets indicate the (approximate) subpopulations for which data are presented. Note that most sickle RBCs have abnormally elevated MCHC. *Right:* Morphology of these sickle RBC density-separated subpopulations, under oxygenated versus deoxygenated conditions. *(Density gradients reproduced with permission from Ballas SK and Mohandas N: Sickle red cell rheology and sickle blood rheology. Microcirculation. 2004;11:209. The displayed data and RBC images are extracted with permission from Kaul DK, Fabry ME, Windisch P, Baez S, Nagel RL. Erythrocytes in sickle cell anemia are heterogeneous in their rheological and hemodynamic characteristics. J Clin Invest. 1983;72:22.)*

HbS, which enables the attraction of naturally occurring anti-Band 3 immunoglobulin (Fig. 42.6). In turn, this promotes engulfment of sickle RBC by macrophages.

Normal enforcement of bilayer phospholipid asymmetry is impaired in sickle RBCs. Sickling promotes PS externalization, especially in ISCs, but also in some reticulocytes. A scramblase that moves phosphatidylserine (PS) outward is activated by calcium transits, and a translocase that restores PS inwardly can be inhibited by thiol oxidation.

Other changes include the presence of peroxidation byproducts such as malondialdehyde (MDA) that can cross-link proteins and promote erythrophagocytosis. Notably, the increased presence of bilayer lipid hydroperoxides appears to account for the sickle RBC membrane's abnormal mechanosensitivity, evident in its enhanced cation leak response to a deforming stress. Perhaps this abnormal deformation susceptibility augments other aberrancies, for example, microvesiculation tendency.

Irreversibly Sickled Cells

The elongated RBCs seen on a typically-obtained blood smear are mostly partially deoxygenated RBCs and ISCs (see Fig. 42.1). The latter's permanent shape abnormality is caused not by persistent polymer but rather by membrane retention of an elongated shape, explained by thiol oxidation of β-actin such that the spectrin–actin-4.1 complex exhibits abnormally slow dissociation. Otherwise, ISCs are similar to other equally dense RBC in having high MCHC, poor deformability, externalized PS, and low HbF content (Fig. 42.5). ISC counts on average are higher in male patients, perhaps reflecting their average lower levels of HbF. The fundamental requirements for ISC formation seem to be RBC dehydration, prolonged deoxygenation, and the assumption of a fixed membrane shape. We suspect that the latter is fostered by a prior deforming and "conditioning" residence in the microcirculation.

The clinical importance of ISCs lies in their ability to prompt diagnosis of a sickling disorder when seen on blood smear, their short life span contributing to overall hemolytic rate, and their participation in the RBC logjam involved in occlusion. Although still adhesive to endothelium, ISCs are less so than other sickle subpopulations, but they exhibit greater adherence to macrophages.

Endothelial Adhesivity

Sickle RBCs are abnormally adhesive to vascular endothelial cells (Fig. 42.7). In vitro studies have implicated about two dozen candidate mechanisms, most involving adhesion molecules on endothelium and sickle RBCs, with or without intermediate bridging by adhesogenic plasma proteins.[8] Some mechanisms require RBC signaling responses to plasma factors, for example, epinephrine, for activation. Some described mechanisms are most evident for reticulocytes and are probably high affinity, identified using flowing conditions. Yet, microcirculatory blood flow can be intermittent and involves constraining vascular diameters that enable greater contact surface area between RBC and endothelium. So it seems plausible that low-affinity adhesive mechanisms could gain relevance in capillaries.[8] To date, only sickle RBC/endothelial adhesion mediated by $\alpha_v\beta_3$ and P-selectin and ICAM-4 (LW) has been verified under flow in vivo in the sickle mouse. An unanswered question is whether or not RBC adhesion in vivo occurs via a single dominant mechanism—or via different mechanisms that can vary amongst patients, locations, and clinical circumstances.

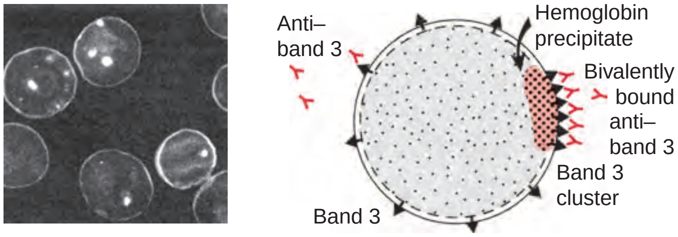


Figure 42.6 BAND 3 AND IMMUNOGLOBULIN COCLUSTERING. Denatured Hb on the RBC membrane causes clumping of Band 3 and opsonization by naturally occurring anti-Band 3 antibodies. Clusters (white spots) of band 3 colocalized with immunoglobulin on the membranes of sickle RBC *(left)*. The drawing shows the colocalization scheme *(right)*. Not shown here is that these changes attract complement and promote erythrophagocytosis by macrophages. *(Reproduced with permission from Schluter K, Drenckhahn D: Co-clustering of denatured hemoglobin with band 3: its role in binding of autoantibodies against band 3 to abnormal and aged erythrocytes. Proc Natl Acad Sci U S A. 1986;83:6137.)*

Macrophage Interaction

Sickle RBCs adhere to and are readily phagocytosed by macrophages because of RBC membrane modifications by malondialdehyde, PS externalization, and opsonization by immunoglobulin. The latter process is triggered by abnormal clustering of membrane protein Band 3 (see Fig. 42.6) and possibly by modification induced by malondialdehyde. The densest cells have the most surface immunoglobulin and higher PS externalization, and they exhibit the greatest interaction with macrophages and the potential for erythrophagocytosis.

DISEASE PATHOGENESIS

Vascular Occlusion

Notwithstanding the conceptual simplicity of the sickling phenomenon, when acute microvascular occlusion causes an acute painful episode it is a complex and evolving process. It seems virtually certain that similar events, but of a less severe degree and remaining at a subclinical level, are a recurrent or even near-constant feature of sickle vascular pathobiology.[9]

Insofar as sickling is responsible, risk factors include anything that can prolong microvascular transit time (e.g., inflammatory milieu, RBC stiffness), increase RBC dehydration and MCHC (e.g., clinical

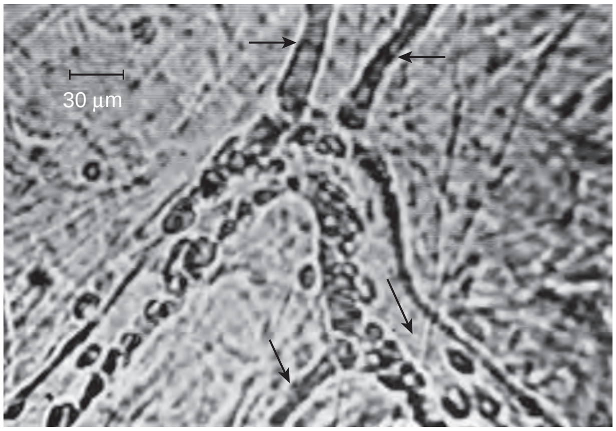


Figure 42.7 SICKLE RBC ADHESION TO ENDOTHELIUM. RBCs adhere to the vascular wall endothelium under flowing conditions in the microcirculation of a rat infused with human sickle RBC. Immobile RBCs are on walls of the postcapillary venule that still is flowing *(long arrow)*. The smaller feeder microvessels *(small arrows)* have no flow because of the logjam of RBC. *(Reproduced with permission from Kaul DK, Fabry ME, Nagel RL. Microvascular sites and characteristics of sickle cell adhesion to vascular endothelium in shear flow conditions: pathophysiological implications. Proc Natl Acad Sci U S A. 1989;86:3356.)*

dehydration, injudicious use of diuretics), foster arterial oxygen desaturation (e.g., lung disease, sleep apnea), increase blood viscosity (e.g., transfusion, inflammation, clinical dehydration), right-shift the oxygen binding curve (e.g., acidosis), or disturb vascular dynamics (e.g., cold, neurochemical responses).

The extraordinary heterogeneity amongst sickle RBCs undoubtedly confers considerable behavioral variability as they lose oxygen while traversing the microcirculation single-file. Such heterogeneity is perhaps the most perplexing feature of sickle disease pathobiology.

RBC Deformability

The diminished deformability of dehydrated (high MCHC) sickle RBC undoubtedly contributes to the impairment of microvascular flow. Some dense cells (especially ISCs) can have difficulty entering the microvasculature, for example, at bifurcations. RBC with lower HbF content will transit with a higher risk of sickling, and HbF level is inversely related to the frequency of vasoocclusive painful crises.[5,10] Low HbF RBCs tend to become more dehydrated (have higher MCHC) and are less deformable.[7] They, therefore, pass within the microcirculation less easily. Nonetheless, among sickle patients vasoocclusive severity correlates positively with preserved deformability, rather than impairment thereof. This probably reflects the higher endothelial adhesivity of more deformable RBC. Whether RBC adhesivity and poor deformability exert synergistic effects within the smallest vessels has not been studied, nor has any role for dynamic change of rigidity during deoxygenation on the timescale of microvascular transit.

Adhesive RBC

The tendency of sickle RBC to abnormally adhere to endothelium slows the microvascular flow. Indeed, in transgenic sickle mice, vascular occlusion is a two-step process: adhesion of less dense (reticulocyte-enriched) sickle RBC to endothelium in the postcapillary venule slows the flow and initiates vasoocclusion via retrograde log-jamming by dense, poorly deformable, and sickling cells (see Fig. 42.7).[11] Consistent with this, among patients clinical vasoocclusive severity correlates positively with the endothelial adhesiveness displayed by sickle RBCs in vitro.[8] To be sure, participation of sickle RBC adhesion in pathophysiology is governed by endothelial activation states. In the sickle context, a multitude of biologic mediators can influence endothelial surface features (see box on The Complex Sickle Milieu).

Adhesive WBC

Consistent with their inflammatory endothelial activation state, sickle mice also exhibit abnormal leucoadhesion to endothelium even at baseline; and when they are exposed to hypoxia/reoxygenation (which triggers a sickling/unsickling response) this is enormously augmented.[12] Just as for adherent sickle RBC, leucoadhesion in post-capillary venules slows microvascular transit of RBC, and blocking this with antibody to P-selectin (but not to E-selectin) greatly improves microvascular flow.[12] On the other hand, in sickle disease WBC, participate in intravascular heterocellular interactions, just as they do in another arteriopathy, atherosclerosis. Also, in sickle mice, it has been observed that when adherent WBC engages E selectin, an activating wave causes them to secondarily capture sickle RBC—but only after massive tumor necrosis factor (TNF) injections.

Hemolytic Anemia

RBC life span in HbSS averages about 15 days, but with marked interindividual variability (from ~7 to ~30 days); in HbSC disease, the average is about 30 days.[13] All four fundamental mechanisms that can underlie premature RBC removal in hematologic disease—erythrophagocytosis, fragmentation, trapping, and osmotic lysis—probably contribute (Fig. 42.8, bottom). These mechanisms derive from the multiple aberrant properties of sickle RBC (see Fig. 42.8, middle) that are consequences of the pathogenic molecular behaviors of the mutant HbS (see Fig. 42.8, top).

This model suggests that the routes to accelerated RBC removal resolve into two contributory mechanistic cascades. One flows from

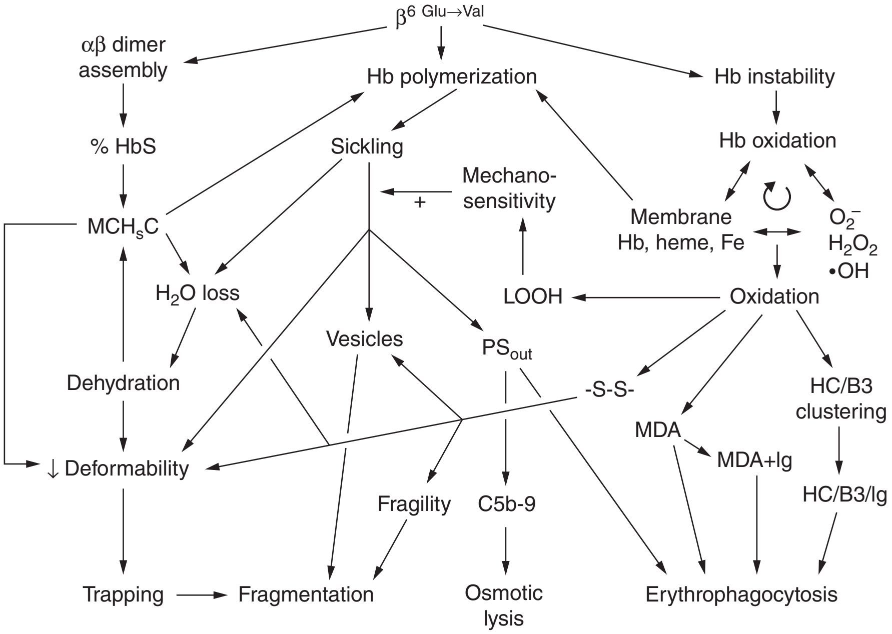


Figure 42.8 MECHANISMS LEADING TO HEMOLYSIS IN SICKLE CELL DISEASE. This integrated synthesis proposes how the distinct molecular behaviors of HbS (*top*) cause the development of multiple sickle RBC abnormalities (*middle*) that, in turn, lead to the four mechanisms of accelerated RBC destruction (*bottom*). *(Modified with permission from Hebbel RP: Reconstructing sickle cell disease: A data-based analysis of the "hyperhemolysis paradigm" for pulmonary hypertension from the perspective of evidence-based medicine. Am J Hematol. 2011:86:123–154.)*

polymer formation and underlies RBC trapping, fragmentation, and osmotic lysis, causes of intravascular hemolysis. The other cascade flows from HbS instability and underlies erythrophagocytosis, the cause of extravascular hemolysis. Although speculative, the illustrated integrated synthesis of extant research data presents a plausible mechanistic blueprint.[13] The proportionate contributions of intravascular versus extravascular hemolysis to the overall hemolytic rate have never been determined. These may vary amongst individuals and with clinical context, just as the net hemolytic rate does. For example, sickle RBC survival drops substantially during acute painful episodes, but whether this precedes or follows vasoocclusion onset is not known.

The shortest survival is exhibited by sickle RBCs that are most dehydrated and that have the lowest amounts of HbF,[14] consistent with the polymerization-based abnormalities (see Fig. 42.8, left side). Yet, it is not known whether these two RBC features fully explain the very wide range of hemolytic rates. Presumably, the sickle RBCs' fragility is related, and sickled RBCs do lose Hb via microvesiculation. Improved RBC hydration caused by concurrent α-globin gene deletion improves RBC survival.[15]

The only biomarkers so far documented to correlate significantly with *measured* RBC life span in HbSS are the (uncorrected) reticulocyte percentage and HbF level.[13] As for lactate dehydrogenase (LDH) as a putative hemolysis severity biomarker, the only available data show that LDH level does *not* correlate with measured sickle RBC survival. Despite this, associations with LDH are what the "hyperhemolysis" hypothesis is based on (see section on Concept of Hyperhemolysis, later).

THE UNIQUE SYSTEMS BIOLOGY OF SICKLE CELL ANEMIA

HbSS is unique amongst human diseases in its extraordinary complexity with a highly aberrant milieu (see box on The Complex Sickle Milieu). Remarkably, this state derives from only two proximal events that are interrelated and mutually promotive, vasoocclusion/ischemia-reperfusion (I/R) and hemolysis. Although it is not possible to identify their proportionate contributions to pathogenesis, our view is that ischemia/reperfusion and hemolysis likely exert co-dominant impacts. Indeed, HMGB1 (high mobility group box 1) from ischemia/reperfusion and free heme from hemolysis biologically converge at TLR4 inflammatory signaling (Fig. 42.9).[9]

Ischemia-Reperfusion Physiology

Sickle transgenic mice reveal that proximal instigation of endothelial activation derives from I/R injury (Fig. 41.10).[9] The complicated biology of I/R can occur when vascular ischemia is followed by reperfusion that reintroduces oxygen to the formerly ischemic area. In the unique sickle context, the initiating occlusive events are likely to be microvascular and multifocal, and they occur recurrently, possibly incessantly. Consequently, sickle transgenic mice exhibit the reperfusion-dependent, classical, early I/R processes: localized and rapid onset of oxygen radical generation, complement activation, nuclear factor kappa-B (NFκB) activation, the release of HMGB1, production of TNF-α, and local activation of leukocytes and mast cells. This results in local I/R injury, i.e., extension of tissue injury beyond the volume affected by the inciting ischemia itself. In HbSS, this most clearly participates in the genesis of stroke, damage to the kidney and liver, and post-priapism damage, for example.[9]

Thereafter, I/R pathobiology can explosively arborize, seen in HbSS as the chronic, systemic inflammatory state that is unique in its cyclicity, complexity, instability, and perpetuity. Specifically, inflammation begets adhesion/occlusion begets inflammation (Fig. 42.10). This robust systematization of inflammation promotes the multitude of vascular biologic abnormalities in HbSS, including endothelial dysfunction and arterial vasculopathy. In addition, remote organ injury can develop at a site remote from a triggering I/R injury site, the most common manifestation being an acute lung injury syndrome, such as the acute chest syndrome (ACS) of sickle disease.

THE COMPLEX SICKLE MILIEU

Anemia (altered wall shear stress, tissue hypoxia, HIF-1 signaling)
RBC abnormalities (rigidity, sickling, fragility, adhesivity)
Hemolysis (plasma Hb and heme, RBC microparticles, arginase, oxidized lipid)
Ischemia/reperfusion physiology
Blood cell activation (signaling and adhesive interactions with endothelium)
Systemic inflammation:
    TLR4 activators (HMGB1, free heme)
    Other mediators (TNF, IL-1β, MCP-1, PAF, thrombin, leukotrienes, and many others)
    Endothelial glycocalyx degradation
    Activated monocytes, granulocytes, and lymphocyte subsets
    Microparticles (endothelial, monocyte, platelet)
    Mast cell activation (neuroinflammatory mediators)
    NETs
Oxidant stress (Hb oxidation, activated WBC, xanthine oxidase, NADPH oxidase, mitochondrial, others)
Growth factors (VEGF, PIGF, erythropoietin, others)
Dehydration (viscosity, vasopressin)
Hemostatic system abnormalities:
    Coagulation activation (thrombin, others)
    Fibrinolysis (D dimer, others)
    Platelet activation (platelet microparticles, plasma TSP, others)
Vasoactive agents (hypoxia, ET-1, thromboxane, vasopressin, prostaglandins, adrenergic agonists, peroxides, CO, NO, and others)
Adhesogenic proteins in plasma (TSP, ultra-large vWF)
Endothelial cell activation (pro-inflammatory, pro-coagulant phenotype)
Endothelial dysfunction (vasoregulatory aberrancy, pro-inflammatory/coagulant phenotype, barrier leakiness)
Endothelial cell injury
Inadvertent effects of therapies (iron overload, opioid-enhanced angiogenesis signaling)

ET, Endothelin; *Hb*, hemoglobin; *HIF*, hypoxia-inducible factor; *HMGB1*, high-mobility group box 1; *IL*, interleukin; *MCIP*, monocyte chemotactic protein; *NETs*, neutrophil extracellular traps; *PAF*, platelet-activating factor; *PIGF*, placenta growth factor; *RCB*, red blood cell; *TLR4*, Toll-like receptor 4; *TNF*, tumor necrosis factor; *TSP*, thrombospondin; *VEGF*, vascular endothelial growth factor; *vWF*, von Willebrand *factor*; *WBC*, white blood cell.

Hemolysis

Hemolysis exerts plethoric adverse effects, both indirect and direct. The accompanying expanded erythropoiesis enhances the production of adhesive reticulocytes and augments the elaboration of placental growth factors. This mediator activates blood monocytes to promote the production of ET-1 that, in turn, can induce vasoconstriction, cause nociceptive hypersensitization, activate RBC NADPH oxidase activity, and prompt the release of inflammatory mediators. Released cell-free oxyHbS is rapidly converted to met HbS that easily loses its heme,[2] which is then able to initiate inflammatory injury responses such as oxidization of blood and membrane lipids, injuring endothelial cells, activating blood monocytes to produce TNF-α and express tissue factor (TF), and triggering disgorgement of neutrophil extracellular traps, among other effects. Some pathogenic responses to heme are mediated by its binding to TLR4; for example, in sickle mice, this causes enhanced endothelial surface expression of P-selectin and triggers vascular stasis.[16] Released RBC arginase lowers plasma arginine somewhat, hypothesized—but unlikely—to impede endothelial nitric oxide synthase (eNOS). Released PS-positive RBC microparticles can exert an inflammatory signaling impact upon endothelial cells, accelerate thrombin generation, and promote pulmonary sequestration of WBCs.

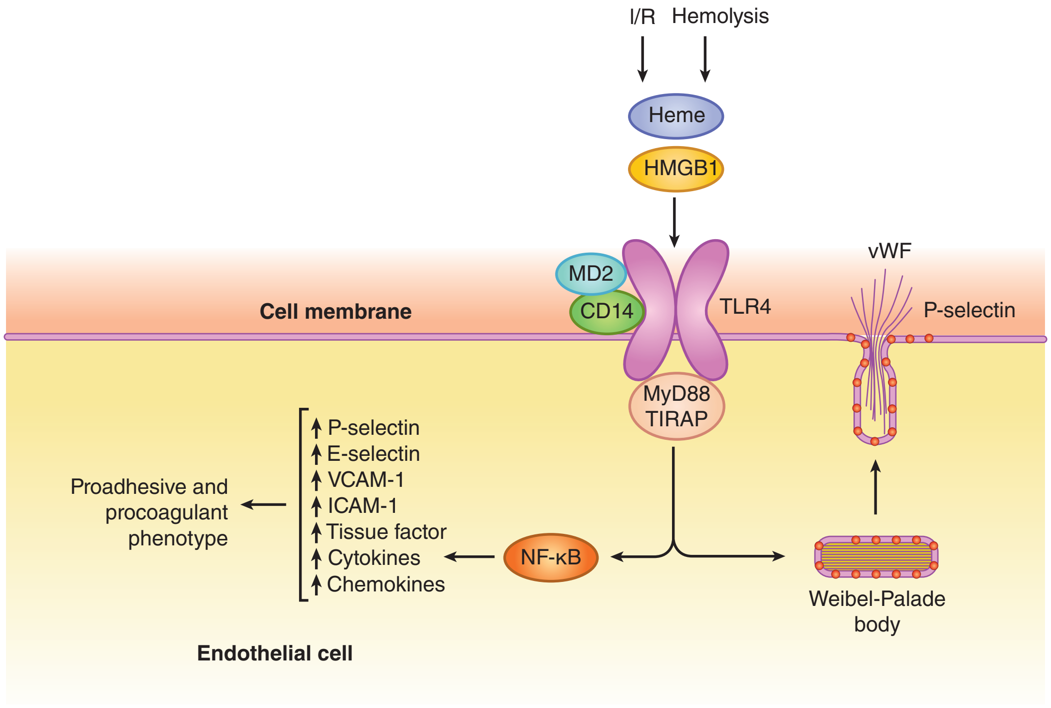

I/R
Hemolysis
Heme
HMGB1
MD2
CD14
TLR4
vWF
P-selectin
Cell membrane
MyD88
TIRAP
P-selectin
E-selectin
VCAM-1
ICAM-1
Tissue factor
Cytokines
Chemokines
Proadhesive and procoagulant phenotype
NF-κB
Weibel-Palade body
Endothelial cell

Figure 42.9 BIOLOGICAL CONVERGENCE. Free heme from hemolysis and HMGB1 from ischemia/reperfusion biologically converge at sterile inflammatory signaling by Toll-like receptor 4 (TLR4). *(Reproduced with permission from Hebbel RP, Belcher JD, Vercellotti GM. The multi-faceted role of ischemia/reperfusion in sickle cell anemia. J Clin Invest, 2020;130[3]:1062–1072.)*

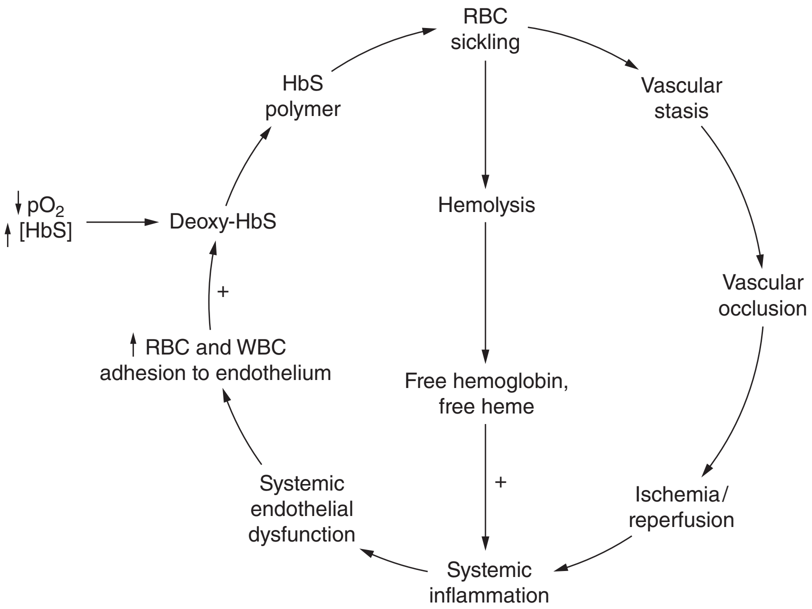

RBC sickling
Vascular stasis
Vascular occlusion
Ischemia/ reperfusion
Systemic inflammation
Systemic endothelial dysfunction
RBC and WBC adhesion to endothelium
+
Deoxy-HbS
pO2
[HbS]
HbS polymer
Hemolysis
Free hemoglobin, free heme
+

Figure 42.10 ISCHEMIA/REPERFUSION AS A CORE DRIVING FORCE IN SICKLE CELL ANEMIA. As the consequence of vasoocclusion, ischemia-reperfusion comprises an incessant driving force causing systemic inflammation with microvascular dysfunction. The adhesion biology resulting from activated/dysfunctional endothelial cells slows microvascular transit of sickle RBC and WBC, thereby establishing a positive feedback loop that slows microvascular transit, increasing the likelihood of polymer formation. *(Reproduced with permission from Hebbel RP, Belcher JD, Vercellotti GM. The multi-faceted role of ischemia/reperfusion in sickle cell anemia. J Clin Invest. 2020;130(3):1062–1072.)*

Cell-free Hb derived from lysing sickle RBC can consume NO.[17] This contributes to the NO biodeficiency of sickle disease, and this specific process can *mimic* some features of endothelial dysfunction; for example, it limits NO's vasodilatory and superoxide buffering functions, and it weakens NO's normal braking effects on platelet activation and inflammation. In the sickle context, any potential effects of liberated cell-free Hb/heme are substantially augmented due to the markedly low levels of scavenging proteins, haptoglobin and hemopexin.

Concept of Hyperhemolysis

The "hyperhemolysis" hypothesis argues that HbSS patients having the highest hemolytic rates develop a specific subset of complications (stroke, leg ulcer, priapism, and pulmonary hypertension) caused by a unique mechanism, the accompanying greater consumption of NO.[18] However, support for this concept is limited to an association between these complications and the level of LDH. So, it is notably problematic that correlative association says nothing about causality and that the sole relevant study found that LDH level does *not* correlate with the *measured* hemolytic rate in sickle subjects. Therefore, in our view, the artifice linking higher hemolysis to NO biodeficiency to certain specific complications is, rather, more parsimoniously explained by the well-known, simple fact that greater hemolysis-driven anemia lowers blood viscosity, thereby lessening the likelihood of vasoocclusion-instigated disease in high-hemolysis patients.

INFLAMMATION

Characteristic footprints of the chronic, systemic inflammatory state in HbSS[19] include leukocytosis; activation of granulocytes, monocytes, lymphocyte subsets, and mast cells; elevated levels of inflammatory mediators, acute-phase reactants, and distal effectors; abundance of soluble adhesion molecules; co-activation of coagulation and platelets; the presence of microparticles from multiple activated cell types; and generation of excess oxidant via multiple mechanisms. Importantly, in humans with HbSS, footprints of inflammatory activity are apparent and fluctuate chaotically, even when far removed from any acute clinical events – further evidence that the sickle inflammatory state is perpetual and subject to repeated re-incitement.

The complex sickle milieu, fashioned by effects of I/R and hemolysis, includes many factors that directly or indirectly or interactively promote inflammation and its consequences (see box on The Complex Sickle Milieu). It is impossible to identify proportionate contributions of the innumerable specific inflammatory inputs, and in any case, their relative influences undoubtedly change over time, and they are highly interactive. As but one example, the growth factor angiopoietin-2, released from Weibel-Palade bodies, sensitizes the endothelial cell toward TNF and consequent adhesion molecule expression. Interestingly, a recent study suggests that part of the pro-inflammatory thrust of sickle disease is a failure of normal inflammation resolution mechanisms.

Clinically, leukocytosis in HbSS is a risk factor for mortality, clinical and silent stroke, and ACS, and it helps predict which babies will develop a severe clinical course. Conversely, several clinical complications are themselves overtly inflammatory in nature, in particular acute painful episodes and ACS. But other consequences of chronic inflammation, for example, promotion of arterial vasculopathy, are far more insidious.

Endothelial Activation and Dysfunction

The vascular endothelial cell receives and integrates disparate inputs, both sensing and modifying its environment, normally in an adaptive fashion. In the sickle context, the extreme complexity of the sickle milieu (see box on The Complex Sickle Milieu) molds endothelial cell function, leading to a maladaptive state. The core inflammatory and oxidative inputs cause a high level of endothelial activation and dysregulation, with adverse consequences such as the assumption of a pro-adhesive and pro-coagulant phenotype, vasoregulatory dysfunction, and probable degradation of the endothelial glycocalyx (a critical determinant of endothelial cell homeostasis).

An often-overlooked principle predicts that endothelial dysfunction in the sickle context creates a unique risk (see Mortality and Sudden Death, later).

Aberrant Vasoregulation

HbSS involves deficient endothelial-dependent, flow-mediated conduit artery dilation.[20] NO bioavailability seems to be higher for females than males, who exhibit substantial non-NO regulation of flow. In the context of I/R and inflammation-induced endothelial dysfunction, there undoubtedly are multiple contributing processes that are known to induce dysfunction of eNOS.[9, 13] In sickle disease, these include the following.

TNF-α induces endothelial L-arginase, limiting the availability of intracellular L-arginine for eNOS function. NADPH-oxidase derived excess intracellular superoxide depletes an essential eNOS cofactor, tetrahydrobiopterin. An endogenous eNOS inhibitor, asymmetric dimethylarginine, is elevated. Multiple other dysfunction-inducing factors are extant: oxidized lipids, activated platelets, thrombin, abnormal shear stress, microparticles.[13] Although unstudied in the specific sickle context, a highly likely contributor to eNOS dysfunction is the glycocalyx degradation that invariably accompanies inflammation generally and I/R specifically. Indeed, the normal integrity of the endothelial glycocalyx is an absolute requirement for the mechanosensing of shear stress that adaptively activates eNOS.[9] Since each of these factors individually can cause eNOS dysfunction, their proportionate contributions to endothelial dysfunction in sickle disease are unknowable—and their impacts undoubtedly vary with time and amongst patients.

Perfusion patterns are complex in HbSS but can manifest as impaired microvascular flow concurrent with the augmented macrovascular flow.[21] Aberrancy of both endothelial-dependent and endothelial-independent vasoregulation is apparent, and the milieu is replete with a multitude of vasoactive substances. A state of vascular instability may derive from tonic upregulation of both vasoconstrictive and vasodilatory systems, in addition to exaggerated α_1-adrenergic vessel wall responsiveness. Sickle humans exhibit disruption of autonomic regulation (e.g., with augmented risk for hypoxia-induced perfusion decrements).[22] Systemic blood pressure in patients with HbSS is lower than in nonanemic controls, yet is higher than in comparably anemic β-thalassemics.

Coagulation Activation

The well-known interplay between inflammation and coagulation is evident in HbSS, with chronic activation of coagulation, fibrinolysis, and platelets.[23] This is accompanied by increased whole blood TF and its increased expression on endothelial cells and blood monocytes. Multiple stimuli can contribute to this, including signaling via TLR4. The presence of PS on released microparticles and some sickle RBC promotes accelerated thrombin generation, which appears to be ongoing. Levels of endogenous anticoagulants are deficient. The oxidation appears to create resistance to cleavage of ultra-large forms of von Willebrand factor by ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13). There is excessive consumption of antithrombotic proteins. The biodeficiency of NO would contribute to TF expression and augmented platelet activation because it normally inhibits these. Products of platelet and coagulation activation undoubtedly exert signaling and perturbing effects on endothelium.

Genesis of Clinical Disease

The spectrum of clinical involvements in HbSS is remarkable (see Chapter 43). For the individual patient, *evident* clinical disease may not parallel—or accurately predict—the extent of the underlying overall disease burden. That different processes may contribute to different degrees to specific clinical complications is possible,[18] but our view is that both I/R and hemolysis contribute to everything. In any case, the dominant pathogenic influence is the robust systemic inflammatory state, fashioned by the complex milieu such that multiple biologic systems are aberrantly and concurrently activated. Consequently, the vascular endothelium is exposed lifelong to a uniquely perturbing milieu. As the interface between blood and tissues, the endothelium undoubtedly participates significantly, if not dominantly, in the genesis of all clinical complications.

Pain Syndromes

In adults, a higher frequency of acute painful episodes is associated with increased mortality,[9] presumably reflecting derivation of both from the severity of underlying disease activity. The immense further disturbance of multiple biologic processes during acute painful episodes probably augments the risk for associated complications like ACS and sudden death. Pain frequency is higher in association with higher hematocrit and lower HbF levels, consistent with the concept that acute painful episodes involve ischemic pain from vasoocclusion. However, pain in sickle disease appears to be multimodal and complex. In particular, there is a substantial inflammatory contribution to both acute and chronic sickle pain via neuropeptides and mast cell products.[24] Many sickle patients exist in a state of nociceptive hypersensitization, a known potential complication of I/R pathobiology. The chronic, daily pain affecting many patients seems to be both inflammatory and neuropathic in origin.

Chronic Vasculopathy

A prominent feature of the sickle disease is macrovascular in nature, a chronic inflammatory arteriopathy.[19] Most supporting histopathology is from the large and medium vessels at the circle of Willis. Pathologic features are those of intimal hyperplasia, the vessel wall's typical response to inflamed endothelium. In sickle disease, this arteriopathy affects the spleen, umbilical cord, lung, bowel, kidney, and penis. The specific generative stressors for vasculopathy may vary somewhat from organ to organ, but it seems likely that universal contributors include perpetual inflammation and anemic hyperemia causing aberrant (i.e., "atheropromotive") wall shear stress.

Thrombosis

The sickle hypercoagulable state is accompanied by a clinical thrombotic diathesis.[23] In situ arterial thrombosis occurs in areas of vasculopathy in the circle of Willis and lungs exhibiting pulmonary hypertension. Incidence of venous thromboembolism is increased, and HbSS is a complicating condition for pregnancy. Downstream consequences of the coagulation system activation may play a greater role than is currently appreciated. For example, thrombin activates endothelium via protease-activated receptor-1, triggering exocytosis of vWF and P-selectin that underlies abnormal adhesion of sickle RBC and WBC to endothelium.

Stroke and Cerebrovascular Disease

Several stroke syndromes occur in sickle disease and may have somewhat differing pathogeneses. Ischemic stroke with clinical deficit develops in 8% of children with HbSS, in some patients accompanied by prior occlusive vasculopathy at the circle of Willis. The case has been made for causation derived from the combination of anemic hyperemia (a known inducer of middle cerebral arteriopathy), chronic vascular wall inflammation, and vascular anatomy at that particular site.[9] Evolution of this stroke type undoubtedly involves an I/R component, a classical aspect of ischemic stroke pathobiology. Completion of the actual stroke sometimes involves thrombosis at the site of vessel wall disease. A deficiency of clinical research on this problem has been looking for associations with "stroke" rather than associations with its likely underlying generative factors. Concurrent α-thalassemia or HbSC disease lowers the risk of this stroke type, and HbF level is not protective. It can be suspected that elevated growth factors are participants in arterial wall disease and the sometimes-associated moya moya.

In a seemingly separate syndrome, "silent" strokes accumulate throughout childhood and into adulthood. They derive from inadequate microvascular blood flow, typically in transitional white/grey matter locations. Indeed, systemic endothelial dysfunction extends to the brain, rendering deficient the sickle patient's cerebrovascular reserve. Such strokes are not truly silent; rather, they establish lifelong cognitive deficits. Interestingly, silent stroke, hemorrhagic transformation of ischemic stroke, and cognitive deficits all are fostered by endothelial dysfunction at the blood-brain barrier causing hyperpermeability. This is a known aspect of I/R[9] and is a feature of the brain in both sickle mice and humans.

Young adults have an increased risk for hemorrhagic stroke. Underlying causes can be vessel fragility associated with moya moya, endothelial hyperpermeability, and aneurysm.

Pulmonary Disease

The spectrum of sickle pulmonary complications includes chronic restrictive lung disease, pulmonary hypertension, asthma, infection, in situ and embolic thrombosis, and ACS.

The inflammatory ACS is associated with infection in children and sometimes with fat embolism in adults, but additional presumptive triggers are similarly implicated via association. Most notably, the nature and occurrence and timing of ACS during acute vasoocclusive episodes perfectly matches the acute lung injury syndromes that present as a remote organ injury complicating I/R physiology.[9] In the sickle context, a predisposition for this syndrome likely derives from chronic inflammation, abnormal adhesion biology, NO deficiency, TNF-driven endothelial permeability, I/R-driven mast cell activation, and especially the multiple unique features of the pulmonary microvasculature.[9] Chronic pulmonary mast cell activation is a candidate contributor to the asthma prevalence in sickle disease.

Pulmonary hypertension occurs in about 6% of adults with HbSS,[25] in half from pulmonary arterial disease and half from pulmonary venous disease. Genesis is undoubtedly multifactorial, as the sickle context involves multiple features strongly associated with pulmonary hypertension in the general population. These include the absence of splenic function, presence of activated platelets, a chronic systemic inflammatory state, endothelial dysfunction, and a surfeit of growth factor signaling. Indeed, the pulmonary arterial wall in HbSS is awash with suspect mediators, and the NO biodeficiency emasculates a normal braking mechanism on inflammation, proliferation, and vasoconstriction. The pulmonary artery also is uniquely buffeted by the pre-sickled RBC in venous blood; experimental sickle RBC contact with endothelium induces an injury response.

Sleep apnea with nocturnal hypoxia is not uncommon in sickle patients, and of course, hypoxia is a classical pulmonary vasoconstrictor. We suggest this comprises a mechanism that can explain sudden death in sickle disease (see below).

Kidney Disease

In HbSS, renal hyposthenuria is a nearly universal problem arising from medullary hypoperfusion resulting from its harsh, polymerization-promoting renal medullary environment (hypoxia, acidosis, hyperosmolarity). Indeed, this occurs in HbAS as well. The renal cortex, on the other hand, exhibits hyperperfusion, believed to underlie glomerular dysfunction with albuminuria.[21] Patients can also develop an acute renal injury, papillary necrosis, and chronic renal disease, the latter being a significant contributor to mortality and a known consequence of I/R in general medicine. Hematuria from papillary damage is very common.

Mortality and Sudden Death

The mortality rate is higher in HbSS patients having higher rates of acute painful episodes,[26] with notable risk indicators including low HbF, high WBC count, chronic renal failure, and elevated tricuspid regurgitant jet velocity. Among apparent causes of death at autopsy, cardiopulmonary disease is most prominent.[27] Prophylactic penicillin for children has greatly improved the average life span in sickle. Further improvement has derived from chronic use of hydroxyurea, an agent that boosts HbF level, lowers WBC count, improves RBC hydration and survival, and blunts inflammatory biology and RBC adhesivity. Unfortunately, survival remains much worse in parts of the world with less access to medical care and abundant comorbidities (e.g., malaria, diarrheal disease).

HbSS entails an abnormal risk for sudden death, tending to occur during painful episodes. We have hypothesized that this is a direct consequence of the pre-existing, underlying endothelial dysfunction of sickle disease.[13] In that context, arterioles can exhibit exaggerated vasoconstrictive responses if potential constrictors are superimposed, for example, hypoxia, augmented NO consumption, or inflammatory signaling. Each can fluctuate on a short time scale, for example, during acute painful episodes, and thereby might create a risk for sudden death by suddenly elevating right heart pressure.

BASIS OF PHENOTYPIC DIVERSITY

Factors aside from HbS itself undoubtedly contribute to the remarkable inter-individual diversity of clinical phenotype in HbSS, but only a few have been identified thus far. Phenotypes could be significantly influenced by the environment, nutrition, socioeconomic status, endemic infectious agents, and availability of medical care. It is believed that phenotypic variability is most evident in countries where the survival of sickle patients is longer. The innumerable questions regarding phenotypic diversity are of great importance. In HbSS, why do some children, but not others, develop stroke? Why do only a small fraction of adults develop pulmonary hypertension, even though all have ongoing hemolysis? Why does pain severity vary so remarkably irrespective of globin genotype? And so on.

Level of Hemoglobin F

HbF level varies amongst individuals as a quantitative trait and is determined by approximately 80% by genetics.[5] After its decline over the first 6 months of life, most of the anti-sickling protection from its high level at birth is lost. HbF level varies among sickle adults over a 20-fold range, and this reflects the number of F cells, the amount of HbF per F cell, and the preferential survival of F cells.[14] It is estimated that a given sickle RBC requires >10 pg HbF for polymerization to be effectively inhibited.[5]

Known HbF determinants account for perhaps one-half of its variance: a polymorphic *XmnI* site (11p) upstream of the Gγ gene; the *HBSIL-MYB* intergenic region (6q23); SNPs in *TOX* (8q12.1); and polymorphisms of *BCL11A* (2p16), a transcriptional silencer of the *HBG* gene.[5] HbF is somewhat higher levels in females, hinting at a contributing locus on the X chromosome. Polymorphisms in trans-acting enhancers of *BCL11A* account for some, but not all, of the regional variations in the HbF level. Among the African autochthonous haplotypes, the Benin haplotype is associated with higher HbF levels; however, it is twice again as high among those with the Arab-India haplotype.

α-Thalassemia

The normal genotype is αα/αα, but about 30% of African Americans have a single α deletion (-α/αα), so concordance with sickle cell disease is common; and homozygosity for the allele is seen (–α/–α). Its prevalence elsewhere varies regionally. An α-gene deletion has minimal effect on the HbF level but results in improved RBC hydration, a lower ISC count, improved RBC survival, and less severe anemia.[15] Perhaps the loss of one α gene nudges α/β chain balance toward normal, given the mild instability of β^S globin.[2] Yet there is no amelioration of pain severity, and some complications (osteonecrosis, retinopathy) increase, possibly because of the higher blood viscosity.

β-Globin Alleles

Compound heterozygosity for the sickle gene and another β allele affects clinical phenotype, for example, in the direction of amelioration from admixing β^C or β^{+thal} chains with β^S. The resulting HbSC disease, however, presents a unique case. Although HbC impedes HbS polymer formation as well as HbA (see Fig. 42.3A), HbSC disease is more similar to HbSS than it is to HbAS. The reason is that HbC interacts more with the RBC membrane than HbA does, and it thereby stimulates RBC KCl cotransport, promoting RBC dehydration that increases HbS concentration.[28] Although pain and anemia are lessened a bit in HbSC disease, there is an increased propensity for retinopathy and osteonecrosis. It has been assumed that this derives from the somewhat higher blood viscosity when anemia is less severe. HbSC blood reveals footprints of greater pro-angiogenic tone compared to HbSS blood, consistent with the higher propensity for retinopathy. Other less common β gene alleles can likewise interact with HbS and affect clinical phenotype via impact on polymerization.

HbAS is typically associated with renal hyposthenuria, not surprising given the several polymer-promoting influences of the renal milieu. More seriously, trait comprises risk for exertional sudden death, probably precipitated by effects of dehydration on RBC and blood viscosity. At an epidemiologic level, the trait also involves a heightened risk for thromboembolism and chronic renal disease, and it predisposes toward ischemic stroke. It is relevant that, unlike Hb tetramers that include either aγ or a β^{A2} globin chain, tetramers that include a β^A or a β^C globin chain are only partially inhibited from participating in polymerization (see Fig. 42.3A).

Unexplained Phenotypic Diversity

Beyond these well-defined influences, there is still enormous unexplained variability in the clinical phenotype of HbSS. The disparate biologic processes that participate in SCA pathophysiology highlight the certainty that phenotypic heterogeneity will be influenced by underlying genetic variations affecting adhesion biology, cation homeostasis, inflammatory signaling, vasoregulation, and so on. Indeed, the spectrum of potential foci at which genetic variation might exert effects and be relevant to sickle disease phenotypic diversity is as vast and complex as human biology itself.

The single-nucleotide polymorphisms (SNPs) that have been detected in association with specific clinical complications are far too numerous to describe here.[29] Of course, much work is still needed to discern whether such associations are actually informative vis à vis pathogenic specifics. However, several SNPs seem particularly interesting. A TNF (-308) promoter polymorphism is associated with large vessel stroke in sickle children. Polymorphisms affecting the HO-1 promoter create heterogeneity in GT repeat lengths (the shorter of which enable greater HO-1 responsiveness, e.g., to heme) and are described as being associated with a lower hospitalization rate for ACS in children. Interestingly, a TLR4 polymorphic haplotype prevalent only in sub-Saharan Africa leads to greater inflammatory responsiveness to TLR4 ligands; it is protective in malaria, but it could well adversely impact sickle biology. Similarly, the Duffy null phenotype prevalent in Sub-Saharan Africa appears to confer heightened severity for acute lung injury; its presence may likewise promote ACS in sickle disease.

A wholly different approach to this general problem was provided by examination of gene expression and inflammatory response of endothelial cells derived from living subjects. Those from sickle children with circle of Willis disease predicted an augmented inflammatory response, that was experimentally verified as an exaggerated NFκB activation response to stimulation with TNF/IL-1.[30] Certainly, the contribution of various non-globin genetic influences will continue to be identified as modern and creative approaches are being applied to the problem of phenotypic diversity in HbSS.

REFERENCES

1. Bunn HF:. Subunit assembly of hemoglobin: an important determinant of hematologic phenotype. *Blood*. 1987;69:1.
2. Browne P, Shalev O, Hebbel RP:. The molecular pathobiology of cell membrane iron: the sickle red cell as a model. *Free Radic Biol Med*. 1998; 24:1040.
3. Noguchi CT, Schechter AN:. The intracellular polymerization of sickle hemoglobin and its relevance to sickle cell disease. *Blood*. 1981;58:1057.
4. Ferrone FA:. Polymerization and sickle cell disease: a molecular view. *Microcirculation*. 2004;11:115.
5. Steinberg MH, Chui DHK, Dover GJ, et al. Fetal hemoglobin in sickle cell anemia: a glass half full? *Blood*. 2014;123:481.
6. Joiner CH. Cation transport and volume regulation in sickle red blood cells. *Am J Physiol*. 1993;264:C251.
7. Ballas SK, Mohandas N. Sickle red cell microrheology and sickle blood rheology. *Microcirculation*. 2004;11:209.
8. Hebbel RP. Adhesive interactions of sickle erythrocytes with endothelium. *J Clin Invest*. 1997;99:2561.
9. Hebbel RP, Belcher JD, Vercellotti GM. The multi-faceted role of ischemia/reperfusion in sickle cell anemia. *J Clin Invest*. 2020;130(3):1062–1072.
10. Platt OS, Thorington BD, Brambilla DJ, et al. Pain in sickle cell disease. Rates and risk factors. *N Engl J Med*. 1991;325:11.
11. Kaul DK, Fabry ME:. In vivo studies of sickle red blood cells. Sickle red cell-endothelium interactions. *Microcirculation*. 2004;11:153.
12. Kaul DK, Hebbel RP:. Hypoxia/reoxygenation causes inflammatory response in transgenic sickle mice but not normal mice. *J Clin Invest*. 2000;106:411.
13. Hebbel RP:. Reconstructing sickle cell disease: a data-based analysis of the "hyperhemolysis paradigm" for pulmonary hypertension from the perspective of evidence-based medicine. *Am J Hematol*. 2011;86:123.
14. Franco RS, Yasin Z, Palascak MB, et al. The effect of fetal hemoglobin on the survival characteristics of sickle cells. *Blood*. 2006;108:1073.
15. Embury SH, Clark MR, Monroy G, et al. Concurrent sickle cell anemia and α-thalassemia. Effect on pathological properties of sickle erythrocytes. *J Clin Invest*. 1984;73:116.
16. Belcher JD, Chen C, Nguyen J, et al. Heme triggers TLR4 signaling leading to endothelial cell activation and vaso–occlusion in murine sickle cell disease. *Blood*. 2014;123:377.
17. Reiter CD, Wang X, Tanus-Santos JE, et al. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat Med*. 2002;8:1383.
18. Kato GJ, Steinberg MH, Gladwin MT. Intravascular hemolysis and the pathophysiology of sickle cell disease. *J Clin Invest*. 2017;127:750.
19. Hebbel RP, Osarogiagbon R, Kaul D. The endothelial biology of sickle cell disease: inflammation and a chronic vasculopathy. *Microcirculation*. 2004;11:129.
20. Gladwin MT, Schechter AN, Ognibene FP, et al. Divergent nitric oxide bioavailability in men and women with sickle cell disease. *Circulation*. 2003;107:271.
21. Nath KA, Katusic ZS, Gladwin MT:. The perfusion paradox and vascular instability in sickle cell disease. *Microcirculation*. 2004;11:179.
22. Sangkatumvong S, Khoo MCK, Kato R, et al. Peripheral vasoconstriction and abnormal parasympathetic response to sighs and transient hypoxia in sickle cell disease. *Am J Respir Crit Care Med*. 2011;184:474.
23. Sparkenbaugh E, Pawlinski R. Interplay between coagulation and vascular inflammation in sickle cell disease. *Brit J Haematol*. 2013;162:3.
24. Vincent L, Vang D, Nguyen J, et al. Mast cell activation contributes to sickle cell pathobiology and pain in mice. *Blood*. 2013;122:1853.
25. Parent F, Bachir D, Inamo J, et al. A hemodynamic study of pulmonary hypertension in sickle cell disease. *N Engl J Med*. 2011;365:44.
26. Platt OS, Brambilla DJ, Rosse WF, et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. *N Engl J Med*. 1994;330:1639.
27. Fitzhugh CD, Lauder N, Jonassaint JC, et al. Cardiopulmonary complications leading to premature deaths in adult patients with sickle cell disease. *Am J Hematol*. 2010;85:36.
28. Bunn HF, Noguchi CT, Hofrichter J, et al. Molecular and cellular pathogenesis of hemoglobin SC disease. *Proc Natl Acad Sci U S A*. 1982; 79:7527.
29. Fertrin KY, Costa FF:. Genomic polymorphisms in sickle cell disease: implications for clinical diversity and treatment. *Expert Rev Hematol*. 2010; 3:443.
30. Milbauer LC, Wei P, Enenstein J, et al. Genetic endothelial systems biology of sickle stroke risk. *Blood*. 2008;111:3872.

CHAPTER 43

CLINICAL ASPECTS OF SICKLE CELL DISEASE

Laurel A. Menapace and Swee Lay Thein

Hemoglobinopathies are the most common genetic diseases in humans. In sickle cell disease (SCD), a single nucleotide substitution (GTG for GAG, *rs334*) in the sixth codon of the β-globin gene produces an abnormal sickle hemoglobin (HbS). Due to a change in the convention for numbering amino acid residues in the human β-hemoglobin chain, the HbS mutation becomes E7V rather than E6V and the current numbering puts the HbS mutation in codon 7 rather than 6. In addition to homozygosity for the HbS allele (HbSS), the phenotype of SCD encompasses other genotypes including HbSC (compound heterozygosity of HbS with HbC), and HbSβ-thalassemia (HbSβ^0 or HbS β^+-thalassemia, depending on the type of the β-thalassemia mutation) and rarer genotypes of HbS with hereditary persistence of fetal Hb (S/HPHH), HbS/HbE syndrome, as well as compound heterozygosity of HbS with HbD Los Angeles, HbO Arab, G-Philadelphia, and others.[1]

All of these SCD genotypes have in common at least 50% HbS, the level of which is a key determinant of the SCD severity. Hence, HbSS and HbSβ^0 thalassemia, which have the highest proportion of intracellular HbS fraction (80% to 96% of total Hb), commonly referred to as sickle cell anemia (SCA), generally have a more severe disease course compared to the other SCD genotypes but even within each genotypic and ethnic group, a spectrum of clinical variability exists. Key contributors to observed clinical variability include environmental factors (weather, nutritional status, and access to social and medical support) and genetic background. Co-inheritance of α-thalassemia and genetic determinants of persistent fetal hemoglobin (HbF) production are established major genetic modifiers of SCD.[2]

The key mechanism underlying SCD is polymerization of HbS under deoxygenated conditions altering the shape and function of red blood cells (RBCs). These "sickled" red cells initiate a series of events and the clinical hallmarks of the disease that include chronic hemolytic anemia and recurrent vaso-occlusive (VOC) episodes causing pain, acute chest syndrome (ACS), hepatic or splenic sequestration, and priapism. The end result is chronic organ damage from both microvascular and macrovascular vaso-occlusion. Once a disease with a high childhood mortality, improved childhood survival in well-resourced countries[3,4] has transformed SCD into a chronic degenerative disease in adults with increasing morbidity due to complications in multiple organs, all contributing to the reduction in life expectancy by 20 to 30 years compared to the general population.[5,6]

This chapter presents the diagnosis and natural history of SCD and describes overall clinical management as well as specific management by organ complications. Clinical interventions are founded on an understanding of underlying pathophysiologic processes. The exigency of living with a painful, life-threatening chronic disease in an ethnically diverse society adds complexity to the psychosocial aspects of this illness. A comprehensive management approach directed at preventing pain crises, chronic organ damage, and early mortality while effectively managing acute complications is recommended. For a full discussion of the history and molecular pathology of this disease, refer to Chapter 42. Normal Hb synthesis, structure, and function are described in Chapter 34, and the thalassemias are described in depth in Chapter 41.

GENETIC EPIDEMIOLOGY OF THE SICKLE GENE

Heterozygotes for the sickle gene (sickle trait or HbAS) who are generally asymptomatic have a substantial survival advantage in malaria-endemic environments. HbAS individuals are not only protected from effects of severe *Plasmodium falciparum* malaria but also from consequences of malaria infection, including malnutrition and bacterial infections,[7] a result of which is the very high population frequencies of 10% to 20% in historically malaria-endemic regions.[8] However, with the increased premature death rate of patients with SCD, the sickle cell gene represents a balanced polymorphism.[9] Early genetic studies based on β^S haplotypes generated by restriction fragment length polymorphisms in the globin-gene cluster on chromosome 11, hypothesized that the β^S mutation arose and amplified separately in at least four African locations between 2000 and 4000 years ago and once in the Middle East or India.[10] Recent studies based on whole-genome sequence data and phased single nucleotide polymorphisms (SNPs), however, suggest that *rs334* arose once approximately 7300 years ago in West Africa on HAP1 haplotype from which the other haplotypes were then derived.[11] Another study based on sequencing of the β-globin gene (*HBB*) cluster supported a single origin of *rs334* but estimated an earlier origin about 22,000 years ago.[12]

VARIANT SICKLE CELL SYNDROMES

The sickle cell syndromes that result from inheritance of the sickle cell gene in simple heterozygosity or in compound heterozygosity with other mutant β-globin genes are sickle cell trait, HbSC disease, and HbSβ-thalassemia. These and other less common compound heterozygosity syndromes are reviewed.

PREVALENCE

The distribution and frequency of the sickle cell gene in geographic regions of the world have been influenced by natural selection and gene transmission via trade routes including the slave trade.[10,13,14] In recent years, further expansion of the distribution of the sickle cell allele resulted from globalization. Among African Americans,[15] the prevalence of sickle cell trait is 8% to 10% among newborns,[16] and in this population, the frequencies of the sickle cell (0.045), HbC (0.015), and β-thalassemia (0.004) genes[16] indicate that there are 4000 to 5000 pregnancies a year at risk for SCD.

Based on data from the Centers for Disease Control and Prevention, there are approximately 100,000 individuals in the United States (US) with SCD.[17] Overall, it is estimated that approximately 1 out of every 365 African American and 1 out of every 16,300 Hispanic American births in the US are affected by SCD.[18] Approximately 3 million people (majority of African descent) and 8% of Black or African American individuals in the United States are sickle carriers.[19] It is worth noting that due to both the presence of the HbS mutation in the Mediterranean region and to population admixture, sickle cell disorders can occur in individuals of European origin who do not report African ancestry.

The burden of this disease in the US is dwarfed by that in the rest of the world, evidenced by an estimated 300 million sickle carriers (~5% of the world's population) globally, with 5.5 million AS births annually.[20] Globally, it is estimated that more than 300,000 children are born with SCA every year, and this burden is expected to increase to 400,000 by 2050 largely due to reductions in infant mortality[21,22]; more than 50% of the births occur in Nigeria, Democratic Republic of Congo, and India.

CLINICAL LABORATORY FEATURES AND DIAGNOSIS

The chronic hemolytic anemia of SCD presents with mild to moderately low Hb and hematocrit (Hct) levels and a reticulocytosis of approximately 3% to 15%. Additional laboratory features of hemolysis include unconjugated hyperbilirubinemia with relative elevations of both indirect (unconjugated) and total bilirubin, elevated lactate dehydrogenase (LDH), and low to absent haptoglobin levels. During vaso-occlusive episodes (VOEs), hemolytic laboratory parameters may become more pronounced with significant elevations in bilirubin, LDH, and reticulocyte counts above steady-state levels in addition to worsening of anemia. Baseline compensatory reticulocytosis in the setting of chronic hemolysis accounts for high or high-normal mean corpuscular volume (MCV). If the age-adjusted MCV is not elevated, the possibility of sickle cell–β-thalassemia, coincident α-thalassemia, or iron deficiency must be considered in the diagnostic work up.

In the peripheral smear (Fig. 43.1), there may be sickled RBC forms, target cells, polychromasia indicative of reticulocytosis, and Howell-Jolly bodies in the setting of functional hyposplenia or asplenia. RBCs are typically normochromic unless there is coexistent thalassemia or iron deficiency. Sickled forms (irreversibly sickled cells [ISCs]) occur in the peripheral smear only in SCD and not in sickle cell trait. In HbSS disease, ISCs predominate, and target cells may be few; in HbSC disease, target cells predominate, and ISCs are rare; in sickle cell–β-thalassemia, ISCs, target cells, and hypochromic microcytic discocytes are prominent.

White blood cell (WBC) counts are higher than normal in HbSS and HbSβ^0-thalassemia disease, particularly in patients under age 10 years. Mean WBC counts tend not to be elevated in HbSC disease or HbSβ^+-thalassemia. Mean platelet counts are elevated in SCA, particularly in patients younger than age 18 years, but are usually normal in those with HbSC disease and HbSβ^+-thalassemia.

Solubility Tests and Hemoglobin Electrophoresis

Solubility test results (e.g., Sickledex) are positive in both SCD and sickle cell trait. All patients require definitive diagnosis with Hb electrophoresis (which separates Hb species according to amino acid composition) (Fig. 43.2) or high-performance liquid chromatography (HPLC).[23] Cellulose acetate electrophoresis at a pH of 8.4 is a standard method of separating HbS from other variants. However, HbS, G, and D have the same electrophoretic mobility with this method. Using citrate agar electrophoresis at pH 6.2, HbS has a different mobility than HbD and HbG, which comigrate with HbA in this system.

Results from electrophoresis or thin-layer isoelectric focusing are similar in HbSS disease and HbSβ^0-thalassemia: nearly all of the Hb consists of HbS. Although differences in the HbF (see Variant Sickle Cell Syndromes) and HbA$_2$ levels may be useful in distinguishing these syndromes, the presence of microcytosis of one parent without sickle cell trait is a more useful indicator of HbSβ^0-thalassemia or HbS–β^+-thalassemia. The diagnosis of HbSC disease is straightforward; nearly equal amounts of HbS and HbC are detected. HbSβ^+-thalassemia and sickle cell trait both have substantial amounts of HbA and HbS; whereas sickle cell trait is associated with neither anemia nor microcytosis and has an HbA fraction more than 50%,[24] sickle cell–β^+-thalassemia is associated with anemia, microcytosis, and an HbA fraction less than HbS, ranging from 5% to 30%.

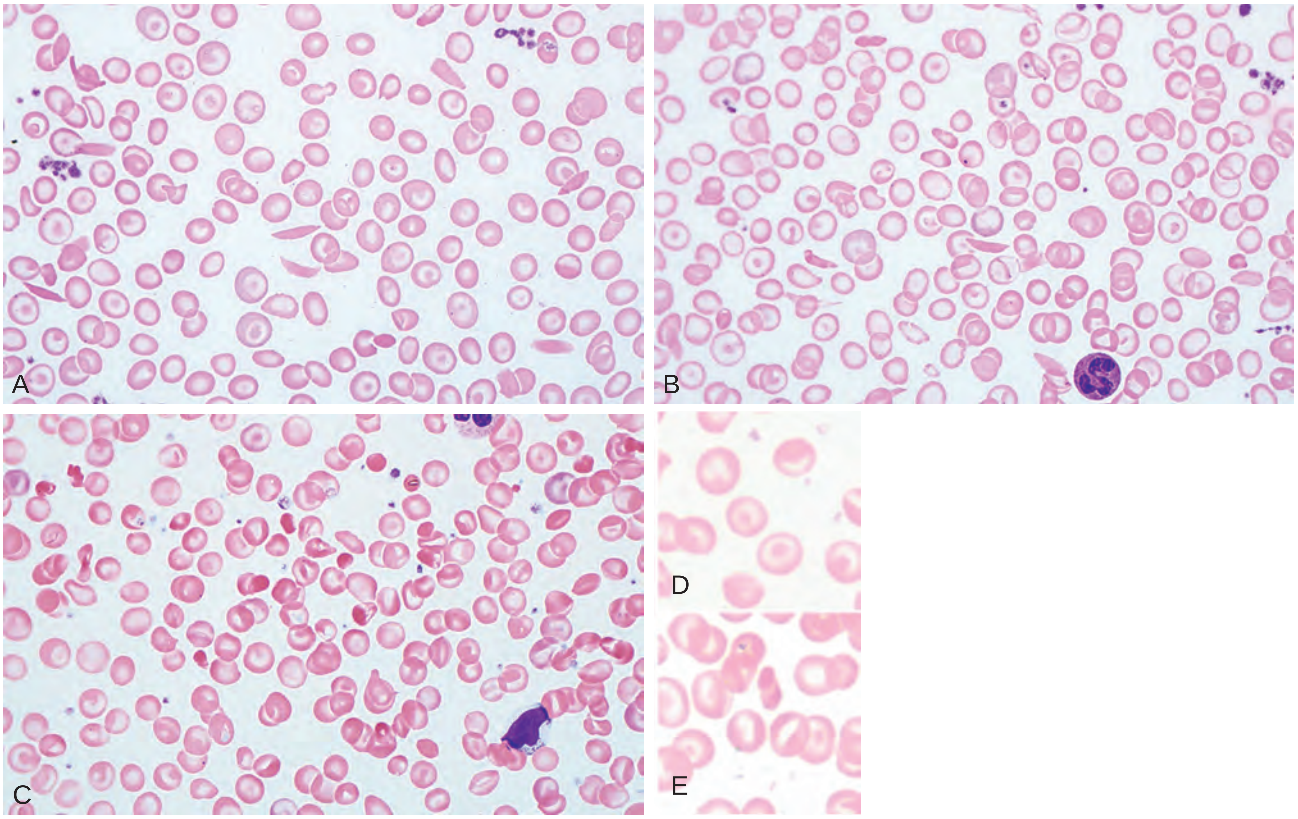


Figure 43.1 SICKLE CELL DISEASE AND HEMOGLOBIN SC PERIPHERAL BLOOD SMEARS. The peripheral smear in sickle cell disease (A) shows sickle cells that are mostly irreversibly sickled and sometimes referred to as "cigar forms." Higher power detail (B) shows a sickle cell *(upper left)*, red blood cell containing a Howell-Jolly body *(middle right)*, and polychromatophilic cell *(lower center)*. These indicate sickle cell anemia and splenic dysfunction but marrow response with reticulocytosis, respectively. A peripheral smear of a patient with HbSC (C) shows no sickled cell, but there are target forms (D) and occasional cells (E) with hemoglobin condensed at each pole of the cell.

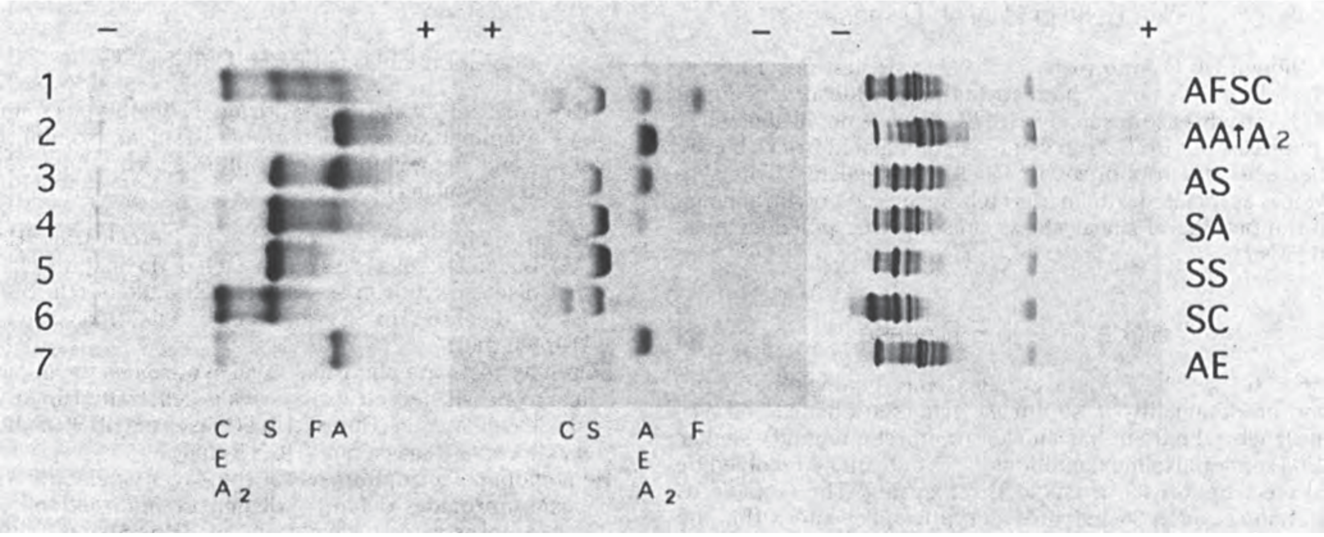


Figure 43.2 COMPARATIVE ANALYSES OF SEVERAL MUTANT HEMOGLOBINS USING ALKALINE ELECTROPHORESIS, ACID ELECTROPHORESIS, AND THIN-LAYER ISOELECTRIC FOCUSING. On the *right* are shown the components of the standard *(top)* and the phenotypes of the other six samples. Their analyses are shown by alkaline hemoglobin electrophoresis in the *left panel*, acid electrophoresis in the *center panel*, and thin-layer isoelectric focusing in the *right panel*. Locations of the various hemoglobin bands are shown below the left and center panels. *A*, Hemoglobin A; *A2*, hemoglobin A2; *C*, hemoglobin C; *E*, hemoglobin E; *F*, hemoglobin F; *S*, hemoglobin S. (*Courtesy M. H. Steinberg.*)

Coinheritance of alpha-thalassemia (silent carrier or trait) is difficult to ascertain from standard hematology and requires DNA sequencing of the alpha-globin gene which can be performed as a send out test to specialized laboratories.

The HbF level is usually slightly to moderately elevated although there is significant variability among patients. The amount of HbF present is a function of the number of reticulocytes that contain HbF, the extent of selective survival of HbF-containing reticulocytes that become mature HbF-containing erythrocytes (F cells), the amount of HbF per F cell, and co-inheritance of HbF genetic variants, including the β^S-haplotypes.[25] The Arab–Indian and Senegal haplotypes are associated with higher levels of HbF than the others.[26] HbF is also influenced by SCD-directed therapies, specifically hydroxyurea (HU), which will be discussed later in this chapter.

Newborn Screening

The use of prophylactic penicillin[27] and the provision of comprehensive medical care during the first 5 years of life have reduced the mortality rate from approximately 25% to less than 3%, thereby underlining the importance of early identification of infants with SCD. Based on its economy and superiority of detection, universal screening of all newborns is preferred over ethnically targeted approaches.[28,29] This screening approach was endorsed by a consensus panel convened by the National Institutes of Health.[28] Since 2008, SCD screening in newborns is mandated in all 50 states of the US and the District of Columbia.[30] Blood samples for testing are obtained by heel stick and spotted onto filter paper for stable transport and subsequent HPLC. It should be noted that HPLC screening in very premature newborns can result in misdiagnosis given that the switch from fetal to adult Hb has not completed and the level of HbS may still be below the threshold for solubility testing and the results may not be valid.

As HbS increases and HbF declines in the first months of life (Fig. 43.3), the clinical manifestations of SCD, including anemia, emerge.[31] ISCs can be seen on the peripheral blood smear (Fig. 43.4) of children with SCA at 3 months of age, and by 4 months of age, moderately severe hemolytic anemia is evident.

Tests used in newborn screening must be capable of distinguishing between HbF, S, A, and C. The Hb distribution pattern is described in descending order according to the quantities detected. Therefore, a newborn with SCA who has predominantly HbF with a small amount of HbS and no HbA is described as having an FS pattern. An FS pattern is obtained also in newborns who have HbSβ°-thalassemia, HbS–hereditary persistence of HbF (HPFH), and HbSD or HbSG (since HbD and HbG have the same electrophoretic mobility as HbS). A newborn with sickle cell trait will have HbF, HbA, and HbS (FAS pattern). The quantity of HbA is greater than that of HbS. If the quantity of HbS exceeds that of HbA, the presumptive diagnosis is HbS/β^+-thalassemia (FSA pattern). It may not be possible to distinguish FAS and FSA patterns in newborns, so DNA-based testing or repeat Hb testing at age 3 to 6 months is recommended. Several alternative, point-of-care approaches are currently under development and a commercially available kit utilizing an antibody-based lateral-flow assay is marketed under the name of Sickle SCAN.[32]

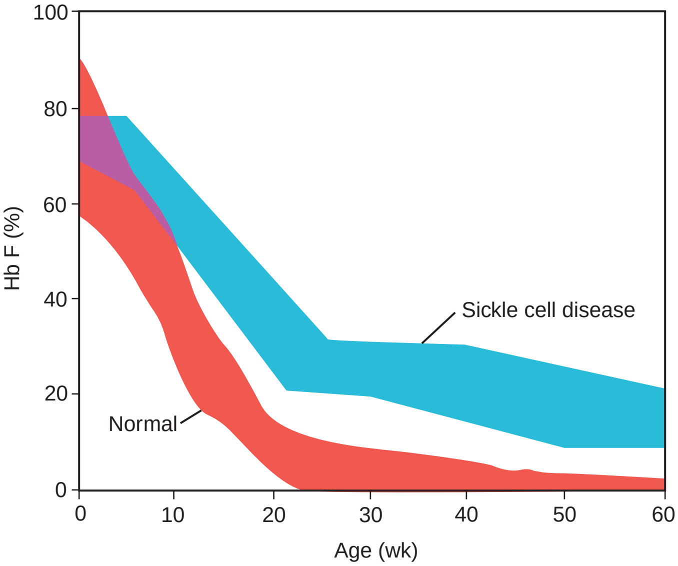


Figure 43.3 FETAL HEMOGLOBIN DECLINE IN CHILDREN WITH HEMOGLOBINS AA AND SS. HbF, fetal hemoglobin. *(Data from O'Brien, Mclatosh S, Aspnes AT, et al. Prospective study of sickle cell anemia in infancy. J Pediatr. 1976;89(2):205, 1976.)*

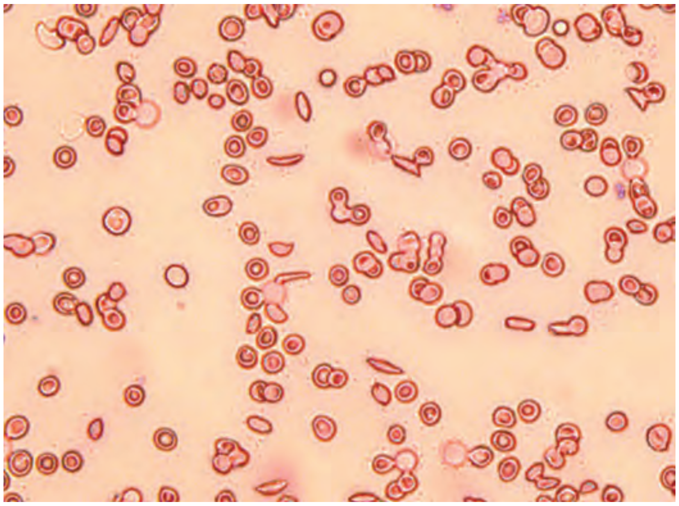


Figure 43.4 The peculiar elongated shapes of the erythrocytes is what Herrick's intern Ernest E. Irons noted, and together with a report from the German literature of *sichel formen* blood cells, inspired the name by which this condition is now known.

CLINICAL PRESENTATION AND MANAGEMENT

The clinical manifestations of SCD include chronic hemolytic anemia, recurrent VOEs, and chronic end organ damage, particularly of the spleen, bones, brain, kidneys, lungs, skin, and heart. It is important to recognize that chronic organ damage is insidious and all SCD patients have decreased survival, even those with infrequent pain episodes or with a seemingly mild disease phenotype. The pattern of disease manifestation varies among the major genotypes of HbSS, HbSC, and HbSβ-thalassemia but also within the same genotype. Some of this variability is the result of coinherited genetic variants, for example, α-thalassemia or HPFH (refer to the end of this chapter).

This section begins with a brief overview of the natural history of SCD and survival followed by a discussion of disease-modifying therapies and then a discussion of organ-specific complications and their respective management.

Natural History and Life Expectancy

The manifestations of disease begin after the first few months of life as HbF levels decline and HbS levels increase. Certain complications predominate in particular age groups. Between the ages of 1 and 3 years, affected individuals develop splenomegaly and splenic sequestration (Fig. 43.5), pneumonia, and meningitis from *Streptococcus pneumoniae* and other encapsulated organisms because of functional hyposplenism, and hand–foot syndrome (dactylitis); in early childhood, ACS, osteonecrosis, and stroke predominate; in mid childhood, pain crises, osteonecrosis, and ACS are again common presentations; between ages 12 and 20 years, patients may develop strokes, priapism, and recurrent pain episodes.[33]

As patients with SCD age, chronic end organ damage begins to manifest thereby complicating their clinical management and disease course. By the ages of 20 to 30, patients with SCD may develop sickle cell nephropathy with albuminuria, sickle retinopathy, sickle related liver disease, disabling avascular necrosis of multiple joints, recalcitrant leg ulcerations, and chronic pain. Pain becomes the most common complication, frequently acute-on-chronic; chronic pain is usually multifactorial secondary to ongoing inflammation, central and peripheral neural sensitization, and avascular necrosis.[34]

Verbal and motor neurologic deficits secondary to prior overt strokes and neurocognitive deficits are common in adult SCD cohorts. In addition, complications related to SCD treatment such as transfusional iron overload and alloimmunization occur although these may develop in much younger individuals depending on clinical course and transfusion history.

Beyond age 30, patients demonstrate progressive worsening of end organ complications and may also develop age-related medical conditions such as osteoarthritis and diabetes mellitus. Aging patients with SCD are at high risk for developing pulmonary complications including pulmonary hypertension (PH) related to the underlying pathophysiology of the disease and/or recurrent thromboembolic events, termed chronic thromboembolic pulmonary hypertension (CTEPH). PH affects 6% to 11% of SCD adults and is a leading cause of morbidity and early mortality.[35] Other pulmonary complications include reductions in vital capacity from pulmonary fibrosis due to parenchymal damage, manifested as impaired exercise tolerance, worsening heart failure, and impaired pulmonary function with evidence of restrictive physiology. Sickle cell chronic lung disease is a term used for these pulmonary lung complications but should be discouraged as these pulmonary complications are not unique to SCD.[36] Exercise intolerance can also arise from associated high output and left ventricular heart failure in the setting of chronic anemia.[37]

Albuminuria and Stage I–II chronic kidney disease (CKD) may progress to late-stage renal disease and overt renal failure requiring hemodialysis (HD) in approximately 4% to 12% of patients with SCD.[38] A multidisciplinary approach integrating the expertise of hematology, cardiology, pulmonology, and nephrology is necessary in the medically complicated aging patient with SCD.

Life expectancy for individuals with SCD is decreased as compared to the general population without SCD, although over the past 30 years this has dramatically improved for patients in Western countries with access to comprehensive medical care. In 1973, Diggs[39] reported that the mean survival was 14.3 years of age. In 1994, Platt et al.[40] reported that life expectancy was 42 years for men and 48 years for women with SCD (Fig. 43.6). Early improvements in survival trends were most likely the result of improved general medical care, including prophylactic penicillin therapy and vaccination against *S. pneumoniae*.[27]

In a cohort of 542 adult patients with SCD followed at tertiary care medical centers in the Southeastern United States from 2002, the observed median survival for patients with HbSS and HbSβ^0 genotypes was 58 years, and median survival for HbSC and HbSβ$^+$ genotypes was 66 years.[6] In a 2016 study involving 712 patients followed at King's College Hospital in London, the median survival for patients with HbSS and HbSβ^0 disease was 67 years.[5] This improved survival was attributed to expert care delivered at a specialized hematology clinic, comprehensive multidisciplinary inpatient management, involvement of specialists, availability of RBC exchange, and an established program to facilitate the safe and effective transition from pediatric to adult care. It is important to note that disease-modifying agents, such as HU, can improve survival in SCD cohorts. Because of its overall safety profile and efficacy, guidelines from both the US and UK recommend HU as standard care treatment for all individuals with HbSS and HbS/HbSβ^0-thalassemia regardless of clinical severity as prevention/reduction of sickle-related complications.[41–43] HU was US Food and Drug Administration (FDA) approved for use in adults in 1998 and in children in 2017.

Predictors of Disease Severity

The ability to predict clinical course allows for more rational tailoring of therapy to individual patients (e.g., selection of patients for high-risk but effective options such as stem cell transplant). While some clear predictors have been identified, the challenge is to predict "severe disease" before end organ complications manifest as reflected by commonly used clinical, laboratory, and imaging markers.[44] Higher baseline HbF levels and the coinheritance of an α-thalassemia trait have been identified as favorable disease modifiers in multiple studies (Table 43.1).[49–51] The severity of chronic anemia is of considerable predictive value. Patients with more severe anemia are more likely to develop infarctive and

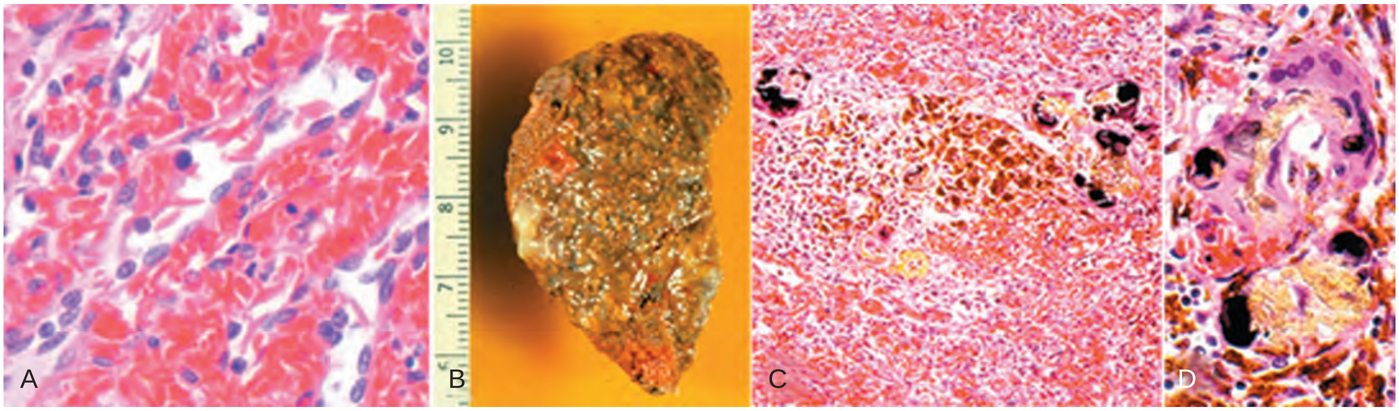


Figure 43.5 THE SPLEEN IN SICKLE CELL DISEASE. Histologic section (A) of the splenic red pulp shows engorgement of the splenic cords with sickled cells. In infants, excessive pooling in cords can lead to a splenic sequestration crisis. Later in life, the spleen undergoes autoinfarction. The gross pathology (B) shows a tiny 4.5-cm spleen with rough external surface caused by scarring from repeated infarcts. Histologic section reveals classic Gamna-Gandy bodies (C and D) also caused by repeated infarction. These are composed of hemosiderin-laden macrophages, calcium deposits, and foreign body giant cells.

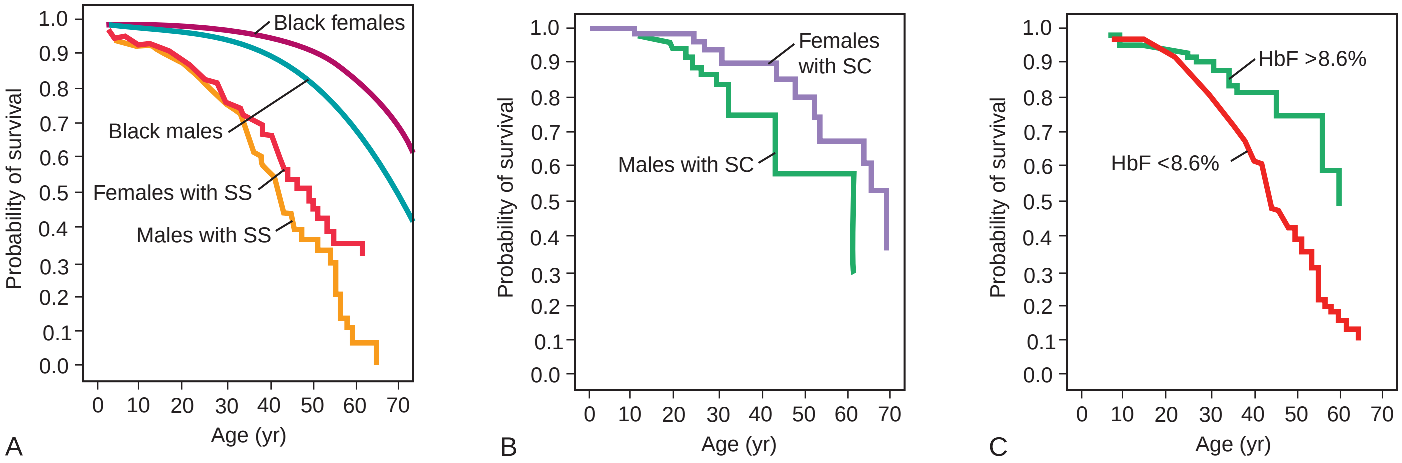


Figure 43.6 Life expectancy in patients with sickle cell disease for patients with HbSS disease (A), HbSC disease (B), and with different levels of fetal hemoglobin (HbF) (C). *(From Platt OS, Brambilla DJ, Rosse WF, et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. N Engl J Med. 1994;330:1639–1644.)*

TABLE 43.1 **Effect of α-Thalassemia on the Level of Anemia in Sickle Cell Anemia**

Reference	αα/αα[a]	−α/αα	−α/−α
Embury et al.[45]	7.8[b] (*n* = 25)[c]	9.7 (*n* = 18)	9.2 (*n* = 4)
Higgs et al.[46]	7.8 (*n* = 88)	8.1 (*n* = 44)	8.8 (*n* = 44)
Steinberg et al.[47]	8.0 (*n* = 73)	9.0 (*n* = 39)	9.5 (*n* = 13)
Felice et al.; age 5 years[48]	8.6 (*n* = 88)	8.4 (*n* = 52)	8.3 (*n* = 50)
Felice et al.; age 11 years[48]	7.9 (*n* = 40)	8.5 (*n* = 34)	9.6 (*n* = 2)

[a] The different α-globin genotypes indicate the presence of four (αα/αα), three (−α/αα), or two (−α/−α) α-*globin* genes.
[b] The mean hemoglobin level (g/dL) for each group is shown.
[c] The number of subjects in each group is denoted by *n*.

hemorrhagic stroke,[52] to have glomerular dysfunction,[53,54] and are more likely to give birth to low-birthweight babies.[55,56] Conversely, they have fewer episodes of ACS[57] and after age 20 years, a lower mortality rate.[57] Progressive anemia from renal, endocrine deficiency or a decrease in bone marrow function from vaso-occlusion is associated with early death.[58,59]

An early study aimed at identifying risk factors for mortality in adults utilizing algorithmic modeling in a cohort of 964 individuals with SCD identified ACS, renal failure, baseline white blood count greater than 15,000/μL, and a HbF of 8.6% or less to be associated with early death.[40]

Risk factors for death in two contemporary adult SCD cohorts treated at tertiary medical centers included high frequency of hospitalizations, presence of iron overload, elevated tricuspid regurgitant jet velocity (TRJV) on transthoracic echocardiography (>2.5 m/s), history of any cerebrovascular event, lower estimated GFR, and at least one pain episode in the preceding year.[5,6] Laboratory findings including low hemoglobin, high baseline WBC count, low baseline HbF, elevated LDH, high C-reactive protein (CRP), and elevated NT-proBNP were also predictive of early mortality in these patient cohorts. A more recent approach utilized novel machine learning methods to select variables of importance without any pre-conceived assumptions leading to the selection of nine key parameters and a simple risk score that reflects the summation of cardio-pulmonary, renal, and liver end-organ damage. An online tool was created to calculate this individual risk score and predicted survival in SCD patients.[60]

In genome-wide association studies (GWAS), millions of genetic variants, typically SNPs, are queried for possible association with clinical subphenotype in patients with SCD. GWAS have been employed in SCD to study the genetic associations of HbF, bilirubin, cholelithiasis, hemolysis, HbA2 level, tricuspid regurgitation velocity, stroke, and systemic blood pressure.[61] The *UGT1A1* gene promoter variant which underlies Gilbert syndrome is a genetic modifier of bilirubin levels and prevalence of gallstones in patients with SCD. Bilirubin levels increase with hemolysis, and the intensity of hemolytic anemia is a marker of severity in SCD. Another validated result in SCD populations as a result of GWAS is the association of an SNP in *NPRL3* with hemolysis.[61] A number of other genetic polymorphisms may be relevant to disease severity and phenotype. However, the number of validated markers is small and at a stage that cannot be used to guide decision-making.[50,62]

Principles of Clinical Management

The pillars of therapy are disease modification (prevention of crises, complications, chronic end organ damage, and early mortality) and compassionate, prompt, effective, and safe relief of acute crises, including pain episodes.[63] Therefore, outpatient clinic management is mostly directed at initiating measures to prevent pain crises, prevent organ complications, and improve survival. This effort should include identification of existing organ complications and initiation of measures to prevent further deterioration. Outpatient management can thus be divided into baseline evaluations, basic treatment or disease modification, and additional treatment dictated by the organ complications that are identified. The suggested treatments are based on current understanding of SCD pathophysiology. As shown in Fig. 43.7, some treatments address only one aspect of pathophysiology, but others may have a broader impact.

Baseline Evaluations

Baseline blood, urine, and other evaluations are directed at quantifying chronic hemolytic anemia and organ-specific complications (Table 43.2). They also provide baseline parameters that can be followed to assess response to therapeutic interventions.

In pediatric patients with HbSS disease and HbSβ°-thalassemia, at least annual assessment of cerebral blood flow in the internal carotid artery and the middle or anterior cerebral artery using transcranial Doppler ultrasonography (TCD) is recommended. TCD screening is initiated at age 2; it is not recommended in patients greater than 16 years of age. This evaluation is a validated predictor of stroke risk. Primary prevention with chronic transfusion to maintain the maximum hemoglobin S level at less than 30% is effective in such patients.[64] Children with evidence of cognitive or neurologic dysfunction (e.g., poor school performance, concerns expressed by family members) should be screened for silent infarcts using magnetic resonance imaging (MRI). In adults, MRI or magnetic resonance angiography (MRA) of the brain can be used instead of TCD[65] to assess thrombotic or hemorrhagic stroke risk, especially in those with a history of prior stroke or seizure.

The recognition of cardiopulmonary complications as a cause of early mortality in SCD warrants evaluation for this condition with either echocardiogram or brain natriuretic peptide (BNP) levels. In adult asymptomatic patients, baseline transthoracic echocardiogram should be considered for screening. If PH is suspected based on clinical symptoms or echocardiogram findings including borderline or elevated TRJV in an adult, a referral to a pulmonologist with consideration of right-heart catheterization is recommended.

The 2019 American Society of Hematology (ASH) Guidelines for Sickle Cell Disease advise aggressive blood pressure management in patients with a history of pre-hypertension or hypertension. Early treatment of systemic hypertension is critical; mild elevations in blood pressure are associated with an increased risk of overt stroke and silent cerebral infarct in patients with SCD.[66] The ASH 2019 Guidelines strongly recommended a blood pressure goal of less than 130/80 to prevent progression of renal damage which results in chronic insufficiency and in later stages, renal failure.

Urinalysis and metabolic panel to screen for albuminuria and CKD should be assessed at baseline and at least annually. The

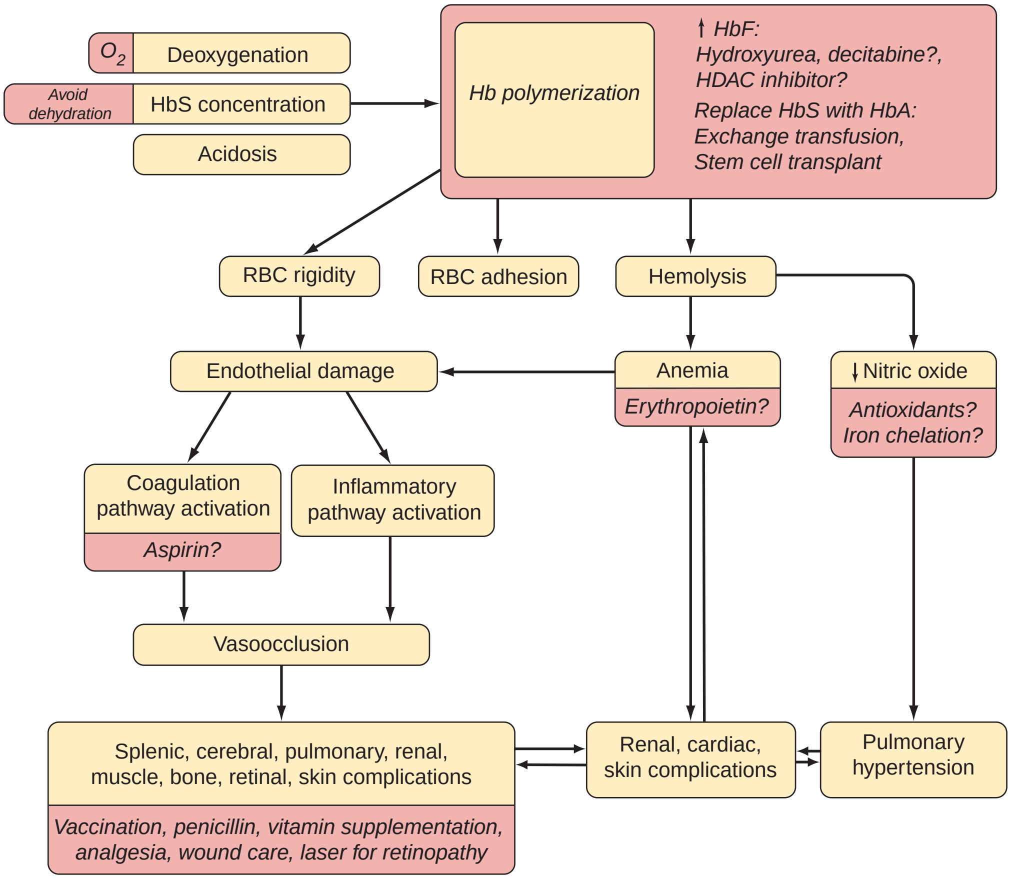

O_2
Deoxygenation
Avoid dehydration
HbS concentration
Acidosis
Hb polymerization
↑ HbF:
Hydroxyurea, decitabine?,
HDAC inhibitor?
Replace HbS with HbA:
Exchange transfusion,
Stem cell transplant
RBC rigidity
RBC adhesion
Hemolysis
Endothelial damage
Anemia
Erythropoietin?
↓ Nitric oxide
Antioxidants?
Iron chelation?
Coagulation pathway activation
Aspirin?
Inflammatory pathway activation
Vasoocclusion
Splenic, cerebral, pulmonary, renal, muscle, bone, retinal, skin complications
Vaccination, penicillin, vitamin supplementation, analgesia, wound care, laser for retinopathy
Renal, cardiac, skin complications
Pulmonary hypertension

Figure 43.7 WHERE THERAPEUTICS INTERVENE IN THE PATHOPHYSIOLOGIC CASCADE. *Hb*, Hemoglobin; *HDAC*, histone deacetylase; *RBC*, red blood cell.

TABLE 43.2 Baseline Evaluations to Consider

	Tests
Blood tests	CBC with differential
	Reticulocyte count
	Hemoglobin HPLC or electrophoresis
	LDH
	Renal function tests
	Liver function tests
	Mineral panel
	Serum iron, ferritin, TIBC
	Vitamin D level
	Hepatitis B sAg
	Hepatitis C antibody
	RBC alloantibody screen
	RBC typing
	D-dimer[a]
	C-reactive protein[a]
	Brain natriuretic peptide
Urine and kidney tests	Urinalysis
	Renal ultrasonography[b]
Radiology	MRI or MRA brain (adults)[c] or transcranial Doppler ultrasonography starting at age 2 years (children)
	Chest radiography[d]
	Hip or shoulder radiograph or MRI (or both)[c]
	Bone density in teenagers and adults
Cardiology and pulmonary	Echocardiogram
Neurocognitive	Neurocognitive testing[d]

[a] Consider following as surrogate markers after initiation of disease-modifying intervention.
[b] If hematuria with red blood cells in urine.
[c] As clinically indicated.
[d] If the patient has poor school performance, an abnormal memory, or abnormal MRI findings.
CBC, Complete blood count; *HPLC*, high performance liquid chromatography; *LDH*, lactate dehydrogenase; *MRA*, magnetic resonance angiography; *MRI*, magnetic resonance imaging; *RBC*, red blood cell; *sAg*, surface antigen; *TIBC*, total iron-binding capacity.

TABLE 43.3 Disease-Modifying Treatments to Consider[a]

Robust clinical data	Penicillin prophylaxis
	Streptococcus pneumoniae vaccination
	Hydroxyurea
	Chronic exchange transfusion
	Iron chelation for chronic iron overload[b]
Limited clinical data	Daily multivitamin without iron or Folate supplementation AND vitamin D replacement[c]
	Haemophilus influenzae vaccination
	Influenza vaccination
	Erythropoietin
	Phlebotomy
Experimental	HbF reactivation with decitabine, histone deacetylase inhibitors, or IMiDs
	Erythropoietin for chronic relative reticulocytopenia
	Nutritional supplements and antioxidants (e.g., glutamine, zinc, multivitamins)
	N-acetylcysteine

[a] See text for specific indications and limitations.
[b] Best data from thalassemia patient experience.
[c] Risks minimal, therefore it is generally done.
HbF, Fetal hemoglobin; IMiDs, immunomodulatory drugs.

National Heart, Lung, and Blood Institute (NHLBI) 2014 expert panel report stated that screening for albuminuria should occur annually beginning at 10 years of age for patients with SCD. In select patients with albuminuria, the use of angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor blockers (ARBs) may be considered although caution must be exercised during initiation of therapy, particularly in the setting of concomitant renal insufficiency. While prospective data is limited, there is increasing evidence that early initiation of ACE inhibitors or ARB therapy may prevent progression of albuminuria and medical renal disease in patients with SCD.

Retinal evaluation is begun at age 10 and continued on an annual basis. More frequent retinal evaluations and ophthalmologic interventions are necessary if proliferative sickle cell retinopathy is noted.

In chronically transfused patients, ferritin levels should be serially measured at least every 3 to 4 months and must be drawn in the steady state (i.e., in the absence of acute pain crisis or VOC).

Chelation therapy is typically started after two years of transfusion in chronically transfused individuals, or after approximately 120 mL of transfused RBCs per kilogram, and when the serum ferritin exceeds at least 1000 μg/L or the liver iron concentration (LIC) is greater than 7 mg/g dry weight.[67] Quantification of tissue iron stores should be undertaken to determine optimal timing of iron chelation therapy. At specialized centers, both hepatic and cardiac iron burden can be quantified non-invasively with MRI; although liver biopsy to assess LIC remains an alternative it is less desirable method. Patients receiving iron chelation should have serial iron indices and ferritin levels performed regularly and chelation regimen adjusted as needed.

Basic Management and Disease Modification

Sufficient evidence suggests that a number of treatments should be considered in all patients. These treatments have been demonstrated to decrease symptoms and complications, increase survival, or both (Table 43.3) (disease modification). There are other treatments for which there are sufficient scientific grounds or clinical data to suggest a potential impact on disease natural history. However, there is presently insufficient clinical data to make firm recommendations (see Fig. 43.7 and Table 43.3). Although treatments such as vaccination and penicillin prophylaxis do not directly affect the sickling process or vasculopathy, they have had an impact on survival and therefore are included under the umbrella of disease-modifying therapies.

Therapeutic options are further discussed in the sections describing organ-specific complications.

Nutritional Supplementation

Chronic hemolysis results in increased utilization of folic acid stores. Megaloblastic crises from folic acid deficiency have been reported.[68,69] Pediatric patients with SCD have higher homocysteine levels than age-matched control African American patients.[70] Folic acid, 1 mg/day orally, is administered as a standard of care.[71] Vitamin B_{12} deficiency can also be seen in patients with SCD. Folate replacement can mask and possibly exacerbate vitamin B_{12} deficiency.[72]

A growing body of research indicates that sickle cell patients have multiple mineral and vitamin deficiencies, including zinc, vitamin C, vitamin E, acetylcysteine, calcium, vitamin D, and vitamin A.[73] Fifty percent of children with SCD have evidence of osteoporosis or osteopenia that is associated with inadequate calcium and vitamin D intake.[74–76] Recently, zinc supplementation in a prospective trial documented significant improvement in linear growth and weight gain in children with SCD.[77] Therefore daily supplementation with a multivitamin without iron may be of value, and in pediatric and adult individuals with vitamin D deficiency (which is often severe[78]), vitamin D replacement therapy is warranted.

Despite increased intestinal absorption of iron in SCD, the combination of nutritional deficiency and urinary iron losses results in iron deficiency in 20% of children with SCD.[79] The diagnosis of iron deficiency may be obscured by the elevated serum iron levels associated with chronic hemolysis, necessitating the detection of a low serum ferritin level or an elevated serum transferrin level for the diagnosis. The treatment of iron deficiency in children must be balanced with the potential risk for transfusion iron overload in the setting of RBC transfusions. In contrast, there are also reports that suggest iron-restricted erythropoiesis may reduce the frequency of VOCs due to the reduction of intracellular hemoglobin S concentration[80] but to date, there have been no clinical trials to test this hypothesis.

Vaccination and Penicillin Prophylaxis

Children with SCD should be immunized against *Neisseria meningitidis*, *S. pneumoniae*, *Haemophilus influenzae* type B, seasonal influenza, and hepatitis B in addition to all other standard vaccinations.[81] Vaccination decreases the incidence of invasive pneumococcal disease in children with SCD.[26,82] In the United States, a 13-valent pneumococcal conjugate vaccine (PCV13) and a 23-valent pneumococcal polysaccharide vaccine (PPSV23) are available. All children with SCD should be immunized with both PCV13 and PPSV23 as per American Academy of Pediatrics (AAP) guidelines. In adults, the Pneumovax should be re-administered every 5 years.

Penicillin prophylaxis begins at 3 months. The dose from age 3 months to 3 years is 125 mg penicillin V orally twice daily, and at age 3 years this should be increased to 250 mg twice daily until age 5 years. After the age of 5 years, penicillin prophylaxis can be stopped unless the patient has been splenectomized or there is a previous history of pneumococcal sepsis.[81,81a] Randomized, double-blind, placebo-controlled studies of prophylactic penicillin beginning in infancy, including the prophylactic penicillin or placebo study (PROPS), have found that this therapy reduced the incidence of *S. pneumoniae* bacteremia by 84% in children younger than 3 years.[26,82] A randomized, double-blind, placebo-controlled study, the PROPS II study, concluded that it is safe to stop prophylactic penicillin therapy at age 5 years in children who have not had prior severe pneumococcal infection or splenectomy and are receiving regular follow up care.[83] However, the power of the study was restricted by the limited number of *S. pneumoniae* systemic infection events. The decision of whether to continue antibiotic prophylaxis after age 5 must be made on a case-by-case basis. In an analysis of a patient population receiving penicillin prophylaxis and the Pneumovax, the rate of severe *S. pneumoniae* infections was 2.4 per 100 patient-years. This was favorable compared with the historic pre-penicillin prophylaxis rate of 3.2 to 6.9 per 100 patient-years.[84] These measures reduce risk but do not remove it. The risk of recurrent *S. pneumoniae* sepsis and death in patients who have had previous sepsis is significantly increased; all patients with a history of pneumococcal sepsis should remain on penicillin prophylaxis indefinitely and are not candidates for outpatient management of febrile episodes.[85] Parents must be aggressively counseled to seek medical attention for all febrile events.

Hydroxyurea and Fetal Hemoglobin Reactivation

The level of HbF in erythrocytes plays a critical role in determining patient outcomes. Individuals who have SCD and HPFH have 70% HbS in their RBCs but are neither anemic nor symptomatic.[86] The uniform distribution of HbF among their RBCs interferes with HbS polymerization, increases its solubility, and prevents RBC sickling.[43,87] Even at lower levels of HbF seen in patients without HPFH, crisis rate and mortality are inversely proportional to HbF level.[51]

HU is an inhibitor of ribonucleotide reductase and a cytotoxic agent that elevates HbF levels via unknown pathways. Long-term use of HU in non-randomized studies has been associated with increased survival in patients with HbSS disease.[88]

A double-blind, placebo-controlled, intention-to-treat multicenter study of HU as treatment of pain crisis in SCD found that HU produced definite hematologic changes. HU was initiated at 15 mg/kg/day and escalated to 0.30 mg/kg/day as tolerated and to maintain an absolute neutrophil count no lower than $2000 \times 10^9 L^{-1}$. There were significant increases in the levels of Hb, HbF, F cells, F reticulocytes, packed cell volume (PCV), and MCV and declines in the mean level of leukocytes, polymorphonuclear leukocytes, reticulocytes, and dense sickle cells (Table 43.4).[89] The significant clinical changes were decreased rate of acute painful episodes, longer interval to first and second acute painful episodes, fewer episodes of ACS, and diminished numbers of PRBC units transfused (Table 43.5).[90] In follow-up analysis, higher pre- or post-treatment HbF levels were associated with a reduction in mortality rate although no

TABLE 43.4 Hematologic Effects of Hydroxyurea Therapy

Variable	Hydroxyurea	Placebo	*P*
Leukocytes (10^3 cells/μL)	9.9	12.2	.0001
PMNs (10^3 cells/μL)	4.9	6.4	.0001
Reticulocytes (10^3 cells/μL)	231	300	.0001
Hemoglobin (g/dL)	9.1	8.5	.0009
PCV (%)	27.0	25.1	.0007
MCV (fl)	103	93	.0001
HbF (%)	8.6	4.7	.0001
F cells (%)	48	35	.0001
Dense sickle cells (%)	11	13	.004

Shown are mean values after 2 years of study. Baseline values, which were not significantly different, are not shown.
HbF, Fetal hemoglobin; *MCV*, mean corpuscular volume; *PCV*, packed cell volume; *PMN*, polymorphonuclear leukocyte.
Adapted from Charache S, Terrin ML, Moore RD, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. *N Engl J Med*. 1995;332(20):1317, with permission.

TABLE 43.5 Clinical Effects of Hydroxyurea Therapy

Variable	Hydroxyurea	Placebo	*P*
Acute pain crisis rate	2.5/year	4.5/year	<.001
Hospitalization rate for acute pain crisis	1.0/year	2.4/year	<.001
Interval to first pain crisis	3.0 months	1.5 months	<.001
Interval to second pain crisis	8.8 months	4.6 months	<.001
Acute chest syndrome	25	51	<.001
Subjects transfused	48	73	.001
Blood units transfused	336	586	.004

Adapted from data in Charache S, Barton FB, Moore RD, et al. Hydroxyurea and sickle cell anemia. Clinical utility of a myelosuppressive "switching" agent. The multicenter study of hydroxyurea in sickle cell anemia. *Medicine (Baltimore)*. 1996;75(6):300.

significant changes were observed in the incidence of stroke, hepatic sequestration, or death in the initial study.[58] No short-term toxicity caused by HU was observed. One child born to a patient taking HU and two born to partners of patients taking HU were normal at birth. Although follow-up analyses suggest the importance of HbF to better outcomes, it is possible that some HU-induced changes in sickle cell erythrocytes, such as increased water content and decreased HbS concentration,[91] may be independent of HbF.

In the original study, only patients with two or more pain crises per year requiring hospitalization were eligible. However, other at-risk patients should be considered for HU therapy. These include patients with evidence of chronic organ damage, patients with severe anemia (unless the reticulocyte count is $<250{,}000\ \mu L^{-1}$, in which case consider erythropoietin [EPO] deficiency from renal damage or bone marrow suppression that may require alternative treatment), and patients with indications for chronic transfusion but who have alloantibodies. After obtaining the baseline evaluations per Table 43.2, HU is usually started at 500 to 1000 mg/day with monitoring of the CBC every 4 to 8 weeks to ensure that neutropenia (absolute neutrophil count $<2 \times 10^9\ L^{-1}$) is not produced. Lower doses may be required in patients with renal insufficiency and/or relative reticulocytopenia. The dose may be increased to a stable maximum HbF response or neutropenia, but the majority of patients receive between 500 and 2000 mg/day. Response is defined by clinical symptoms, by a persistent and significant (>0.5 g/dL) increase in total Hb or HbF, and a decrease in LDH. These improvements in symptomatology and hematologic indices may require at least 3 to 4 months of therapy but can be seen as soon as week 6 of treatment.

In studies of HU as a therapy for children with SCD, the drug was well tolerated and produced favorable hematologic changes similar to those seen in the adult population.[92] In approximately 10% of the children treated, the increase in HbF was less than 2%. Baseline HbF levels, baseline total Hb levels, and compliance were associated with the final HbF level.[93] Other studies in children have documented a decrease in the number of days of hospitalization and suggest a decreased incidence of vaso-occlusive crises (VOCs).[94] The favorable changes in hematologic indices suggest that HU therapy might be an alternative to blood transfusions for the prevention of recurrent stroke in children with SCD.[95,96] HU therapy appears to lower transcranial Doppler velocities in children with SCD.[97] Studies in the United States and in Belgium support the potential role of HU in the prevention of cerebrovascular accidents (CVAs).[96,98,99] HU was found to improve, but not correct, the abnormal cerebral oxygen saturation associated with SCD.[100] A persistent concern pertaining to the use of HU in SCD is its putative leukemogenic effect. This concern derives from reports on HU treatment of myeloproliferative diseases, conditions associated with an inherent propensity for leukemic conversion. Although the use of HU combined with 32 P or alkylating agents is associated with increased leukemic conversion in patients with myeloproliferative disease,[101] reports claiming a leukemogenic effect for HU alone in polycythemia vera either lacked control subjects[102] or were not designed to assess this issue.[103] In children with the nonmalignant underlying condition of erythrocytosis secondary to inoperable cyanotic congenital heart disease, no leukemic conversion was observed.[104]

Alternatives to Hydroxyurea for Fetal Hemoglobin Induction

Alternatives to HU for pharmacologic induction of HbF that have been studied in clinical trials include the methyltransferase inhibitor 5-aza-2-deoxycytidine (decitabine) and histone deacetylase inhibitors.[105] These classes of agents act on chromatin processes that regulate gene transcription.[106]

The methyltransferase inhibitors 5-azacytidine and 5-aza-2′-deoxycytidine have produced the largest increases in HbF of any of the pharmacologic reactivators of HbF that have been tested.[107,108] Responding patients included those who did not respond to HU, consistent with a different mechanism of action. Although improvements in a number of surrogate clinical endpoints have been demonstrated, larger studies to confirm safety and clinical effectiveness with chronic use are required. In the United States, 5-azacytidine and decitabine have been approved by the FDA for the treatment of myelodysplastic syndrome (MDS). Decitabine, an analogue of 5-azacytidine, is also a potent DNA methyltransferase inhibitor and has a more favorable safety profile but decitabine is rapidly deaminated and inactivated by cytosine deaminase, if taken orally. To overcome this limitation, a clinical study combining decitabine and tetrahydrouridine (THU), a cytosine deaminase inhibitor, as a therapeutic strategy for inducing HbF, is currently under evaluation for efficacy in a phase II study, following promising results in a phase I study.[109] The efficacy of the class of agents known as histone deacetylase inhibitors in HbF reactivation has been reviewed and includes Panobinostat, a pan histone deacetylase inhibitor.[108,110,111] Other classes of drugs being evaluated for potential HbF reactivation are the “imid” class of drugs (analogues of thalidomide such as pomalidomide) and inhibitors of lysine demethylase (LSD1/KDM1A).[112]

Voxelotor

Voxelotor was approved by the FDA in November 2019 for the treatment of SCD in patients aged 12 years and older. Voxelotor inhibits sickling by preferentially binding to the high-oxygen affinity, nonpolymerizing R conformation of hemoglobin, thereby reducing the concentration of the polymerizing T conformation and stabilizing the oxygenated hemoglobin state.[113] Although there is a modest increase in hemoglobin that is accompanied by decreased markers of hemolysis, suggestive of reduced sickling, to date, there is no evidence that it reduces frequency of pain crises, prevents organ damage, or prolongs survival. Approval was based on the surrogate endpoint of Hb increase of at least 1.0 g/dL or greater from baseline at Week 24 of treatment. There is concern that Hb molecules with the drug bound are in a conformation that delivers very little oxygen, i.e., drug-induced reduction of functional oxygen content (FOC), which is particularly detrimental in a disease characterized by decreased oxygen delivery.[114] Hopefully, these concerns will be addressed in current multicenter phase III clinical studies in both adults and children.

It is important to note that voxelotor interferes with determination of Hb fractions as per HPLC and may make routine HbF monitoring in patients receiving concomitant HU challenging.[115,116] As such, it is recommended that baseline HPLC be obtained in patients receiving HU prior to initiating voxelotor therapy.

Crizanlizumab

Crizanlizumab-tmca was approved by the FDA in November 2019, and represents the first in its class approved for reducing the frequency of VOCs in adult and pediatric patients aged 16 years and older.[117] Crizanlizumab is a humanized monoclonal antibody to P-selectin and its mechanism of action is to block the abnormal adhesion of activated erythrocytes, neutrophils, and platelets to the endothelium. A phase II, multicenter, randomized, placebo controlled double-blind study of crizanlizumab with or without HU (SUSTAIN study) (ClinicalTrials.gov Identifier: NCT01895361) demonstrated that patients on the treatment arm had a significantly lower rate of sickle-related pain crises compared to placebo with a lower incidence of adverse events.[118]

The primary efficacy endpoint in the SUSTAIN trial was the annualized rate of VOC episodes, which was a composite endpoint comprised of pain crisis, ACS, hepatic sequestration, splenic sequestration, and priapism. Post hoc analyses showed that more patients were VOC event-free in the crizanlizumab arm than in the placebo arm, and that crizanlizumab also significantly increased time-to-first VOC compared to placebo.[119] A phase 3 interventional, multicenter, randomized, double-blind clinical trial is ongoing to assess safety and efficacy of crizanlizumab with or without HU in patients with SCD and history of VOC (ClinicalTrials.gov Identifier: NCT03814716). Infusion-related reactions were observed in 2 (3%) patients treated with the 5 mg/kg dose level in the SUSTAIN trial. Crizanlizumab can induce pseudothrombocytopenia on automated platelet counts when ethylenediaminetetraacetic acid (EDTA) tubes are implemented.[120]

L-Glutamine

L-glutamine (an amino acid) taken twice daily in powder form was FDA-approved for preventing acute VOC events in children aged 5 years and older and adults with SCD in July 2017.[121]

In a phase III study, L-glutamine demonstrated a 25% reduction in the median number of pain crisis, 30% less hospitalizations, and reduced acute chest episodes in children and adults with SCD with or without HU over a 48-week period.[122] Interpretation of the results is complicated by high dropout rates that were observed with approximately 36% in the L-glutamine arm and 24% in placebo arm discontinuing the study early. It should be noted that one of the exclusion criteria for the study was renal insufficiency, a concern for SCD patients, many of whom have renal impairment (see box on Role of Novel Therapeutic Agents in Sickle Cell Disease).[123]

ROLE OF NOVEL THERAPEUTIC AGENTS IN SICKLE CELL DISEASE

In 2017, L-glutamine (Endari), and in 2019, voxelotor and crizanlizumab were approved by the US FDA for the treatment of SCD. Voxelotor increases Hb levels whereas Endari and crizanlizumab were demonstrated to reduce the frequency of VOC events, including pain crises, as compared to placebo. There are no consensus-based algorithms to guide hematologists on how these drugs should be implemented in the clinical setting. Notably, all three drugs can be used concomitantly with hydroxyurea therapy and the majority of patients in the pivotal trials had been stabilized on hydroxyurea therapy prior to enrollment. So, when do we initiate these new agents in the management of SCD?

HU is an efficacious anti-sickling agent and has an established safety profile and therapeutic benefits for greater 30 years. HU is now considered standard of care for all individuals with sickle cell anemia, regardless of severity of disease, in both adults and children. Thus, before considering these newer agents, a trial of hydroxyurea therapy should initially be considered and dosage titrated for maximal benefit. Consider adding second-line agents only when response to HU is minimal despite stabilization on maximum tolerated dose (MTD). Choice of second agents depends on an individual's clinical phenotype and what we are trying to achieve—improving baseline Hb levels or reducing the frequency of acute pain episodes.

If the main aim is to improve oxygen-carrying capacity, i.e., hemoglobin, consider supplementing HU with an ESA but some patients may be resistant to a subcutaneous route of administration. Long-term follow-up from final analysis of 72-week voxelotor treatment data is not yet available. Voxelotor can be considered in combination with HU, and also in patients intolerant to hydroxyurea secondary to significant cytopenias or adverse effects. There is some concern, however, that Hb molecules bound with voxelotor are in a conformation that delivers very little oxygen, which can be especially detrimental in a disease characterized by decreased oxygen delivery. Hence, special consideration must be paid to patients with prior stroke and silent cerebral infarcts. It should also be noted that HbS-voxelotor complexes, while useful in monitoring voxelotor therapy, cause interference with determination of HbS and HbF fractions in routine HPLC laboratory techniques.

HU has a proven efficacy in reducing frequency of VOCs and also ACS events, but some patients continue to experience recurrent acute pain episodes while on a MTD of HU. In such patients, crizanlizumab or Endari can be considered in addition to hydroxyurea therapy. While no improvements in baseline hemoglobin or reductions in hemolytic parameters can be anticipated based on the mechanism of the drug, a secondary effect in reduction of VOCs will be stabilization of patient's Hb as VOC events are often accompanied by a drop in Hb. Patients initiating crizanlizumab need to be closely monitored for infusion-related reactions during the initiation of drug. As L-glutamine is an amino acid, one should be cautious in its use among SCD patients in whom renal and hepatic dysfunction are not uncommon.

Stem Cell Transplantation

At this time, allogeneic hematopoietic stem cell transplantation (HSCT) from an HLA-matched sibling donor (MSD) remains the only curative option for SCD, and improvements in conditioning have made this approach feasible in both pediatric and adult populations.[124] Between 1986 and 2013, 1000 SCD patients received HLA-identical MSD HSCTs; the outcomes were excellent with 93% overall survival.[125] Eighty-seven percent of the patients received myeloablative chemotherapy and the rest (13%), mainly adults, received reduced intensity chemotherapy. It is important to note that patients 16 years or older had worse overall survival (81% vs. 95% $P = 001$) and a higher probability of graft-versus-host disease (GVHD)-free survival (77% vs. 86% $P = 001$). As patients can be cured of their SCD without complete replacement of their bone marrow, siblings with sickle cell trait are acceptable as donors as stable mixed hemopoietic chimerism after BMT can be achieved.[126]

Although myeloablative conditioning has achieved high rates of overall and event-free survival, the conditioning is too toxic for adult patients with pre-existing organ dysfunction. Reversal of the sickle hematology without complete replacement of the patient's bone marrow led to the development of less intense conditioning regimens expanding allogeneic transplantation in adult patients[127,128] who otherwise would not be able to tolerate intense myeloablative conditioning. Donors can be HbAA or HbAS, and in order to reverse the sickle hematological genotype, myeloid donor chimerism must exceed 20%.[129] Recent data reported at least 95% cure in 234 children and young adults (<30 years of age) with SCA who received HSCT from an MSD, with no increase in mortality compared to the disease itself and with a better quality of life. The data also demonstrated that myeloablative HSCT can be a safe option for patients less than 15 years old if an MSD is available unless there is a clear and strong recommendation not to undergo transplant.[130,131]

However, in the US, less than 15% of patients with SCD have HLA-matched siblings as donors, but a promising alternative donor source is haplo-identical family members. Studies are now underway in several centers to find a balance of a conditioning regimen that provides adequate immunosuppression without rejection and minimal GVHD.[132] Other sources of hematopoietic stem cells (HSCs) include related umbilical cord blood[133] and matched unrelated donors, the latter source of HSCs as an approach is limited by the probability of finding suitable donors for African and African American populations as per the National Marrow Donor Program. HLA-haploidentical HSCT following reduced-intensity conditioning has been reported to show promising results with prolonged and stable engraftment, but rejection remains a major obstacle as also reported for umbilical cord blood as donor source.[134–138]

The issue of the cost-effectiveness of bone marrow transplantation (BMT) gains perspective from the comparative costs in the United States of $150,000 to $200,000 for an uncomplicated BMT versus up to $112,000 annually for conventional medical care of a chronically transfused, iron-overloaded patient.[139]

Transfusion Therapy

Transfusions in SCD improve the oxygen-carrying capacity of blood and concomitantly dilute circulating sickled erythrocytes to improve microvascular circulation; they also suppress endogenous production of RBCs containing HbS.[140,141]

Blood transfusions as a therapeutic option are used in both the acute and chronic setting. Apart from chronic RBC transfusion for stroke prevention or treatment in children, its use in many other medical situations, such as prevention of recurrent acute pain, priapism, leg ulcers, renal dysfunction, and PH, remains pragmatic based on observations and clinical experience.[141,142]

The two main approaches to transfusion in SCD are simple transfusion and exchange transfusion. These transfusions can be administered in an acute/episodic fashion or on a chronic basis. Therefore, transfusion therapy in SCD is of the following types: episodic simple, episodic partial exchange, or chronic partial exchange. Exchange transfusion can also be manual or automated. In both simple and exchange transfusion, the target Hb level is 10 to 11 g/dL (hematocrit, 30%).[143,144] Transfusing to a higher Hb or hematocrit level is avoided because a hematocrit level greater than 30% is associated with hyperviscosity if there is a substantial proportion of HbS in the blood. The aim of chronic transfusion (simple or exchange) is to maintain a relatively low concentration of HbS. The HbS% target will vary according to indication, HbS% of less than 30% for primary or secondary stroke prevention but when prescribed for other indications (e.g., recalcitrant leg ulceration), a relatively higher concentration of HbS% less than 40% to 50% may suffice. In partial-exchange transfusion, a proportion of the patient's RBCs are removed before transfusion of normal donor RBCs; this can be done manually through phlebotomy followed by transfusion or concurrently using an automated device. In patients who need chronic transfusions, partial exchange is recommended because of the reduced iron burden of this approach. Partial exchange is also indicated if the baseline Hb level is more than 10 g/dL. Simple transfusion in this instance risks exacerbating the clinical condition through increased viscosity. For critical illness, exchange transfusion is also preferred. Although the target HbS level should be less than 30% in exchange transfusion, decreasing HbS levels to less than 50% may suffice depending on the severity of the complication being treated.

The volumes required for simple and exchange transfusions (Table 43.6) are particularly important for transfusing children. For normal-size adults, the general rule is that each unit of RBCs infused should approximately increase the Hb level by 1 g/dL.[142]

Episodic simple transfusion should be considered for blood volume replacement in aplastic crisis and splenic sequestration crises and for protection when there is a more than 20% decrease in Hb from baseline from severe illness such as septicemia, severe VOCs, or Hb levels of less than 5 g/dL. Episodic simple or exchange transfusion should be considered for ACS, multi-organ failure, priapism, and preoperatively. The choice of simple versus exchange transfusion is determined by the pre-transfusion total Hb level and the severity of the illness.

In the preoperative setting, there is randomized data to support the use of preoperative transfusion to decrease the risk of complications, including ACS.[145,146] Preoperative transfusions can be categorized as follows. (1) Top-up transfusions to a target Hb level of 10 g/dL. This is an appropriate intervention for patients with baseline Hb between 6.5 and 9 g/dL undergoing low- or medium-risk surgeries (e.g., cholecystectomy, joint replacement). (2) Partial exchange transfusion to achieve a target Hb level of 10 g/dL with an estimated HbS percentage of less than 60%. This is an appropriate intervention for patients with baseline Hb levels of greater than 9.5 g/dL undergoing low- or medium-risk surgeries as per above. (3) Exchange transfusion to achieve a target Hb level of 10 g/dL with an estimated HbS percentage of less than 30%. This procedure should be considered for patients with SCD undergoing high-risk surgeries (e.g., cardiothoracic surgery). In short, surgery, even low- or medium-risk surgery, is a substantial physiologic stressor for patients with SCD, even those who do not have high-risk characteristics at the time of surgery, and preoperative transfusion should be considered. A caveat is that extended phenotyping of transfused red cells should be performed, as discussed later in this chapter.

TABLE 43.6 **Transfusion Formulas**

Dilutional effects of transfusion on HbS: PRBC volume (PRBCV) (mL) = $(\text{Hct}_d - \text{Hct}_i) \times \text{TBV} \times \text{Hct}_{rp}\text{B}$

Manual partial-exchange transfusion:[a] $\text{HbSf} = 1 - (\text{PRBCV} \times \text{Hct}_{rp})$ $(\text{TBV} \times \text{Hct}_i) + (\text{PRBCV} \times \text{Hct}_{rp}) \times \text{HbS}_i\text{C}$

Automated exchange transfusion: Exchange volume (mL) = $(\text{Hct}_d - \text{Hct}_i) \times \text{TBVHct}_{rp} - (\text{Hct}_i + \text{Hct}_d)\text{2D}$

RBC volume (mL) = $\text{Hct}_i \times \text{TBV}$

[a] In these formulas, Hct and HbS are fractions (e.g., 40% = 0.4).
Hct_d, Desired hematocrit; Hct_i, initial hematocrit; Hct_{rp}, hematocrit of replacement cells (usually 0.75); HbS_i, initial HbS; HbS_f, final HbS; *PRBC*, packed red blood cells; *TBV*, estimated total blood volume in milliliters (children, 80 mL/kg; adults, 65 mL/kg; nomograms are available).
From Linderkamp et al., 1977, Springer-Verlag. From Nieburg and Stockman, 1977, American Medical Association.

Chronic partial-exchange transfusion is indicated in primary and secondary prevention of cerebral thrombosis as discussed in the Neurologic Complications section.

Transfusion complications include alloimmunization, delayed hemolytic transfusion reactions (DHTR; discussed in Exacerbations of Anemia), iron overload, and transmission of blood-borne diseases, including hepatitis C. The incidence of alloimmunization in SCD is between 19% and 30% and usually occurs with fewer than 15 transfusions.[147] Some patients seem to tolerate multiple transfusions without developing alloantibodies, but others are readily allosensitized. Alloimmunization to multiple RBC antigens often results in the development of autoantibodies which poses challenges in sourcing appropriately matched blood products.[148] The high rate of alloimmunization in transfused sickle cell patients is partly attributable to minor blood group incompatibilities between the recipient and donor pool, which often differ in ethnicity.[149,150] Ethnic mismatch of RBC antigens is not the only reason, however, as molecular analyses have revealed significant RH allelic diversity with mismatch between serological Rh phenotype and RHD or RHCE genotype in patients with SCD and African American blood donors.[151,152] Due to the prevalence of *RH* genetic diversity in patients with SCD, matching of the donor and recipient RBC antigens at the molecular level may be the only way forward to avoid alloimmunization and a recent study has demonstrated that this is feasible.[152] Antibodies against the C and E antigens of the Rh group, Kell (K) and Lewis, Duffy (Fya, Fyb), and Kidd (Jk) are common.[148] In the Stroke Prevention Trial in Sickle Cell Anemia, the routine use of WBC-reduced RBCs matched for E, C, and Kell decreased the allosensitization rate compared with historical data from 3% to 0.5% per unit transfused and decreased the rate of hemolytic transfusion reactions by 90%.[153] Therefore the recommended approach to preventing alloimmunization is to reduce leukocytes and perform extended phenotype matching for all patients (ABO, C, D, E, Kell, Kidd, Duffy, and S and s).[154] Many blood transfusion centers in the US have also adopted molecular genotyping using microarray chips for RBC phenotype prediction. The management of a DHTR and transfusional iron overload are discussed under Exacerbations of Anemia later in this chapter.

Transmission of human immunodeficiency virus, hepatitis B and C, and human T-cell leukemia/lymphoma virus-1 has diminished with improved screening of banked units but remains an issue. In addition to better screening programs, the use of leukocyte-depleted RBC transfusions can reduce this hazard.[154]

Phlebotomy

As mentioned, an Hb level of greater than 10 to 11 g/dL (hematocrit 30%) in the presence of substantial amounts of HbS (>30%) is associated with hyperviscosity. Symptoms of hyperviscosity are relatively non-specific and related to the affected vascular bed. Central nervous system hyperviscosity can be associated with central venous thrombosis or cerebral infarcts in patients with SCD. Some data indicate that phlebotomy to reduce the hematocrit and viscosity (and which may also address iron overload) can decrease the frequency of crises in HbSC or $\text{HbS}\beta^{+}$ disease.[155] In HbSS disease, phlebotomy has successfully been used in combination with HU (which increases the Hb level) in secondary stroke prevention in patients previously treated with chronic transfusion.[156] Phlebotomy alone has also been used in HbSS disease with baseline Hb levels of more than 9.5 g/dL with favorable results on the frequency and duration of pain crises. This benefit may have resulted from decreased hematocrit and viscosity and from a decrease in intracellular Hb concentration from iron deficiency.[157] One approach to phlebotomy is to remove approximately

10 mL/kg of blood over 20 to 30 minutes followed by infusion of an equal volume of normal saline. This is repeated every 2 weeks until the target Hb level of 9 to 9.5 g/dL is achieved.

Erythropoietin or Darbepoetin

The chronic hemolytic anemia of SCD is partially compensated by vigorous reticulocytosis. A decrease in compensatory reticulocytosis will exacerbate already existent anemia and can be expected to increase clinical risk. Accordingly, chronic relative reticulocytopenia (defined as Hb <9 g/dL and absolute reticulocyte count $<250{,}000 \times 10^9\,L^{-1}$) was identified as a significant risk factor for early mortality in a prospective cohort study of patients with SCD.[158]

In the general population, evaluation of EPO levels is usually prompted by the combination of anemia and abnormal serum creatinine level. EPO levels are then interpreted in relationship to the Hb level to assess for the possibility of EPO deficiency. In patients with SCD, this approach to diagnosis has pitfalls. Patients with SCD are already anemic; therefore, gradual anemia exacerbation is easily missed, and clinicians must weigh many possible causes in the context of multisystem SCD pathology. Furthermore, patients with SCD have low serum creatinine levels at baseline and serum creatinine is a poor measure of renal function due to tubular secretion even when renal function is abnormal. Therefore, a substantial increase in serum creatinine from baseline may nonetheless remain below the threshold defined as abnormal for the general population, potentially disguising the presence of renal damage that is sufficient to decrease renal endocrine function. Furthermore, EPO levels are not readily interpretable and generally inappropriately low in the individual patient with SCD as the EPO-producing cells in the renal medulla are unable to properly sense hypoxia due to tubular dysfunction.[159] One contributing factor could be increased uptake by the massive compensatory reticulocytosis. Further, EPO levels are lower in SCD adults than in children,[159] and EPO levels are inappropriately lower in patients with chronic relative reticulocytopenia.[59] Hence, EPO deficiency should be considered as a possible cause of progressive anemia in patients with absolute reticulocyte counts below $250{,}000 \times 10^9\,L^{-1}$ even if their serum creatinine levels are in the normal range. The cumulative published experience of EPO use in SCD is limited (52 patients).[160] Although EPO by itself has been reported to increase HbF levels, the most important role for EPO may be as replacement therapy for EPO deficiency that causes relative reticulocytopenia and progressive anemia. EPO replacement can also facilitate enhanced HU dosing and HbF augmentation.[160] In using recombinant human EPO, caution must be exercised not to elevate the hematocrit to levels that result in hyperviscosity. Also, the reticulocyte fraction is the most adhesive, and it is possible that EPO could exacerbate or trigger sickle cell crises.[160] Patients with SCD may be relatively resistant to EPO and require doses higher than those used in other patients with chronic renal failure. The reasons for EPO resistance are unclear but may include increased inflammation-mediated suppression of erythropoiesis which could explain favorable responses in some patients when initiation of EPO is accompanied by a dose of IV iron despite adequate iron stores as demonstrated by ferritin levels.[161]

EPO therapy is probably not indicated in patients receiving chronic transfusion therapy in whom encouraging endogenous HbS containing erythropoiesis may be counterproductive.

Iron Chelation

Early death is well described in association with iron overload in patients with β-thalassemia and hereditary hemochromatosis.[162,163] Similarly, iron overload is a problem in chronically transfused patients with SCD, although the clinical significance depends on the degree and duration of overload. Excess iron is a cause of significant morbidity and mortality in patients with SCD and one mechanism relates to hepatic fibrosis and hemosiderosis-related cirrhosis of the liver.[164] While individuals with SCD accumulate cardiac iron at a lower rate than thalassemia cohorts, they still remain at risk for cardiac hemosiderosis and cardiac iron deposition cannot be predicted by LIC. Cardiac iron monitoring utilizing non-invasive T2* MRI of the heart should be considered in patients who have undergone 5 to 8 years of transfusion therapy, or earlier in patients with markedly elevated ferritin or LIC.[165]

Chelation guidelines for patients with SCD are similar to those for other chronically transfused, iron-overloaded patients.[164,166] Steady state serum ferritin values should be no greater than 1000 to 1500 ng/mL (1000 to 1500 mcg/L). Notably, the serum ferritin level is not a good indicator of clinically significant iron overload.[167,168] When cardiac iron overload is present, an aggressive chelation regimen is warranted, given the potential risk for fatal cardiac arrythmias.

Iron chelation options in the United States include deferoxamine (via continuous intravenous or subcutaneous infusion), deferasirox (orally), and deferiprone. Optimal dosing of a chelating agent is determined by age, iron stores, frequency of transfusions, and the presence of organ dysfunction. There is some data to support greater effectiveness of the oral agents, in particular deferiprone, in cardiac iron unloading, and together with the oral route of administration and toxicity profile, deferasirox or deferiprone are favored over deferoxamine.[169–171] The side effect profile of deferasirox includes gastrointestinal symptoms; reversible, dose-dependent increases in serum creatinine; and liver dysfunction. Deferiprone carries a rare but significant risk of agranulocytosis in addition to hepatoxicity.

Newer FDA-approved methods of quantitating iron burden by FerriScan of the liver[172] can avoid the need for liver biopsies.

In addition to monitoring iron stores, formal audiograms should be performed prior to initiation of chelation therapy and annually thereafter. Hearing loss is most frequently reported with deferoxamine treatment, especially at higher doses. Annual evaluation by an ophthalmologist should be performed to screen for visual complications. Serum metabolic panel, urine protein, and creatinine should be monitored monthly in individuals on deferasirox, and every 3 months in those receiving deferoxamine. Liver function tests should also be closely monitored in patients on chelation therapy.

Anticoagulation or Antiplatelet Therapy

Although there is clear evidence of activation of the coagulation system in SCD, the role of thrombogenesis in VOCs remains unclear.[173] Similarly, there have been no thorough evaluations of the role of antiplatelet or antithrombotic agents for the treatment of SCD. D-dimer levels (a degradation product of cross-linked fibrin) increase during acute VOCs.[174]

Minidose heparin, 5000 to 7500 units every 12 hours, administered to four patients for 2 to 6 years reduced hospitalization and emergency department time by 75%, and pretreatment pain frequency recurred after heparin was discontinued.[175] Larger clinical studies will be required to better understand the risks and benefits of heparin therapy for acute VOCs in SCD. Heparin has not been studied for acute arterial stroke in patients with SCD but has a role in SCD-associated dural venous sinus thrombosis.[176] The management of stroke is fully discussed under Specific Complications and Their Management.

Acenocoumarol was administered in low doses that achieved a mean international normalized ratio (INR) of 1.64 and reduced the elevated levels of prothrombin activation fragment (fragment 1 +2) to 50% of pretreatment levels.[177] Clinical endpoints were not measured. In a crossover study, 29 patients were treated with acenocoumarol to target an INR of 1.6 to 2.0. No effect on crisis frequency was noted, although again, there were significant reductions in markers of coagulation system activation.[178] In 37 acutely ill sickle cell patients with elevated D-dimers, the effect of low-dose warfarin therapy (1 mg without a target INR) in 12 patients was examined. In multivariate analysis, low-dose warfarin was the only variable associated with a significant decrease in D-dimer levels, suggesting a warfarin-induced decrease in thrombin activity.[177] Therefore oral anticoagulation, even at low doses, is associated with a decrease in laboratory markers of coagulation pathway activation in SCD;

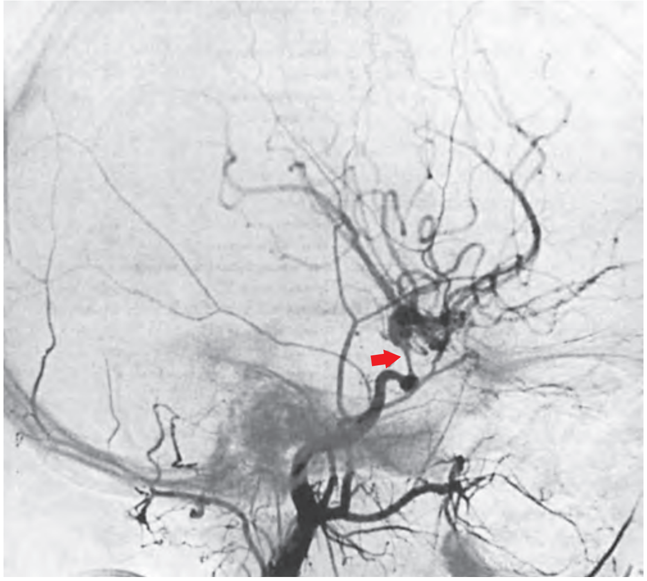


Figure 43.8 Right common carotid arteriogram taken in anteroposterior projection demonstrating complete occlusion of the origin of the right anterior cerebral artery *(arrowhead). (From Stockman JA, Nigro MA, Mishkin MM, Oski FA. Occlusion of large cerebral vessels in sickle-cell anemia. N Engl J Med. 1972;287:846.)*

however, further clinical trials are required to understand the clinical risks and benefits.

Platelet counts are often elevated and activated in patients with SCD.[179] Anti-platelet agents as potential anti-sickling agents, however, have been disappointing. Aspirin was the first anti-platelet drug to be considered; one study compared aspirin with placebo in 49 pediatric patients with SCD in a double-blind crossover study. The frequency and severity of crises were not affected by aspirin therapy, and aspirin did not significantly affect Hb levels.[180] Cerebral thrombosis, which accounts for 70% to 80% of all CVAs in SCD, results from large-vessel occlusion (Fig. 43.8) rather than the more typical microvascular occlusion of SCD. In the United States, a clinical trial testing the safety and efficacy of aspirin in diminishing the incidence and progression of cognitive defects and overt or silent stroke in pediatric patients (START) was completed in 2009 but to date, the results of this study have not appeared in print. Antiplatelet therapy with clopidogrel in patients with SCD, unfortunately, was also disappointing. New, third-generation P2Y12 inhibitors such as ticagrelor and prasugrel have also been studied in patients with SCD. Although prasugrel showed appropriate levels of anti-platelet aggregation compared to healthy patients in ex vivo studies, and was well tolerated by patients, in a phase III (DOVE) study involving 341 children and adolescents, there was no significant difference in the rate of VOCs between the treatment group and placebo, and the study was terminated early.[181,182] Ticagrelor, in a phase IIB study, was well tolerated, but failed to show effect in the frequency of VOC.[183] While aspirin as an anticoagulant therapy did not provide benefit over placebo, it continues to be used as an analgesic in many parts of Africa.[184]

The management of stroke risk and stroke is fully discussed under Specific Complications and Their Management.

Experimental Therapies

Gene-based studies using different therapeutic strategies are currently ongoing, some in their early stages.[185,186] Studies directed at the *BCL11A* gene for therapeutic reactivation of fetal hemoglobin show great promise and include two different experimental approaches: (1) addition of a lentiviral vector encoding a short hairpin RNA (shRNA) targeting erythroid-specific expression of BCL11A, thus encouraging endogenous HbF production[187]; (2) using CRISPR/Cas9-mediated gene editing to disrupt the erythroid-specific enhancer of the *BCL11A* gene.[188] Precise correction of the sickle mutation converting the sickle allele to a normal β-globin allele is the most direct approach but the approach is still fraught with challenges.

Specific Complications and Their Management

Pain Crisis

Acute Pain Episode or Crisis

Acute pain is the first symptom of disease in more than 25% of patients and is the most frequent symptom after age 2 years.[189] Pain is the complication for which patients with SCD most commonly seek medical attention.[190] An episode of acute pain was originally called a “sickle cell crisis” by Diggs, who used the expression “crisis” to refer to any new rapidly developing syndrome in the life of a patient with SCD.[191] Pain crises result from vaso-occlusion of the bone marrow vasculature causing bone infarction, which in turn releases inflammatory mediators that activate afferent nociceptors.[192]

Although a general correlation of vaso-occlusive severity and genotype has been posited,[193] there is tremendous variability within genotypes and in the same patient over time. In one large study of patients with HbSS disease, one-third rarely had pain, one-third were hospitalized for pain approximately two to six times per year, and one-third had more than six pain-related hospitalizations per year.[194] Over a 5-year period in the National Cooperative Study of SCD, 40% of patients had no painful episodes, and 5% of patients accounted for one-third of the emergency department visits. Pain is more frequent with the HbSS genotype, low levels of HbF, higher Hb levels,[57] and sleep apnea.[195] The frequency of pain peaks between ages 19 and 39 years. After the age of 19 years, more frequent pain correlates with a higher mortality rate.[57] Medical personnel who see patients only in the emergency department gain a biased view of SCD skewed by a frequently affected minority with severe disease.[196,197]

Pain may be precipitated by events such as cold weather and extreme heat which may induce rapid cooling of the skin via sweating,[198] dehydration, infection, stress, menses, and alcohol consumption. Underlying etiologies should be searched for and corrected, but the majority of painful episodes have no identifiable cause. Pain can affect any area of the body, most commonly the back, chest, extremities, and abdomen; may vary from trivial to excruciating; and is usually endured at home without a visit to the emergency department. There may be premonitory symptoms.[199] In adults, the average length of hospital admission is 7.5 days in contrast to only 4.4 days in children.[199] Painful episodes are biopsychosocial events caused by vaso-occlusion in an area of the body having nociceptors and nerves.[200] Pain is an effect and, as such, consists of sensory, perceptual, cognitive, and emotional components. Frequent pain generates feelings of despair, depression, and apathy that interfere with everyday life and promote an existence that revolves around pain. This scenario may lead to a chronic debilitating pain syndrome in some patients.

There is no specific clinical or laboratory finding pathognomonic of pain crisis. The diagnosis is established by history and physical examination. Changes in steady-state Hb values, hemolytic parameters, sickled cells on blood smear, and WBC counts are not reliable indicators. Numerous laboratory tests, leukocytosis, D-dimer, fragments of fibrin, and markers of platelet activation have been found to lack specificity as indicators of acute vaso-occlusion. Often patients can tell if they are having a typical pain crisis or something more sinister. It is thus good practice to ask the patient if symptoms are representative of their usual pain crisis.

Initial medical assessment should focus on detection of triggers or medical complications requiring specific therapy, which include viral or bacterial infection, dehydration, ACS (fever, tachypnea, chest pain, hypoxia, and chest signs), severe anemia, cholecystitis, splenic enlargement, neurologic events, and priapism.[200,201] Pain management should be aggressive to make the pain tolerable and enable patients to attain maximum functional ability. To make the patient pain free is an unrealistic goal and risks oversedation and hypoventilation, which must be avoided. A pain chart should be started and analgesia titrated against the patient's reported pain together with medical assessment of the patient's overall clinical status.[201,202] When clinicians consistently observe a disparity between patients' verbal self-report of their pain and their ability to function, further assessment should be performed to ascertain the reason for disparity. Patients are often undertreated for pain because many physicians and healthcare providers are overly concerned with the potential for addiction. Undertreatment of pain is no more desirable than overtreatment and oversedation; undertreatment can prolong the duration of a painful episode and can weaken the relationship between a patient and their healthcare providers. The benefit of early and aggressive treatment of pain was demonstrated in a study that incorporated rapid administration of intravenous morphine with the addition of long acting opioid therapies in 50 patients with SCD; overall, emergency department visits decreased and hospital admissions and length of stay dramatically declined in patients with the greatest frequency of acute pain episodes.[203] Many centers have incorporated protocols and guidelines for rapid treatment of acute pain in day hospital settings which results in a striking reduction in emergency room visits and admission rates, as well as healthcare associated costs.

The pain pathway should be targeted at different points with different agents, avoiding toxicity with any one class (Table 43.7). The mainstays are nonsteroidal anti-inflammatory drugs (NSAIDs), acetaminophen, and opioids. NSAIDs can be used to control mild to moderate pain and may have an additive role in combination with opioids for severe pain. The most potent NSAID is ketorolac. NSAIDs should be used with caution in those with a history of peptic ulcer disease, renal insufficiency, asthma, or bleeding tendencies. Within limits, use the agents that the patients know work for them and avoid meperidine (Demerol), which should only be used under very exceptional circumstances. Sedatives and anxiolytics alone should not be used to manage pain because they can mask the behavioral response to pain without providing analgesia.

Treatment of persistent or moderate to severe pain should be based on increasing the opioid strength or dose.[201] In assessing patient responses to conventional doses of analgesia, it must be remembered that individuals with SCD metabolize narcotics rapidly.[201] One approach is to administer morphine 0.1mg/kg SLT intravenously or subcutaneously every 20 minutes until pain is controlled. The patient should be checked at 20-minute intervals for pain; respiratory rate, depth, and quality; and sedation until the patient is stable with adequate pain control. Subsequently, the patient should receive a maintenance dose of 0.05 to 0.15 mg/kg SLT intravenously or subcutaneously every 2 to 4 hours. A rescue dose of 50% of the maintenance dose can be considered on an as-needed basis every 30 minutes for breakthrough pain.

During maintenance with opioids, pain control, respiratory rate, depth, and quality, and oxygen saturation should be monitored approximately every 2 hours. If respiratory depression is noted, omit the maintenance dose of morphine. For severe respiratory depression or oxygen desaturation, administer naloxone. Incentive spirometry and mandatory time out of bed are helpful in patients with chest pain to decrease the risk for hypoventilation. Severe or intractable pruritus is a common problem encountered in patients requiring high doses of opioids necessitating non-sedating antihistamine therapy or the

TABLE 43.7 Recommended Dose and Interval of Analgesics Necessary to Obtain Adequate Pain Control in Patients With Sickle Cell Disease

	Dose/Rate	Comments
Severe to Moderate Pain		
Morphine	Parenteral: 0.1–0.15 mg/kg every 3–4 h Recommended maximum single dose, 10 mg PO: 0.3–0.6 mg/kg every 4 h	Drug of choice for pain; lower doses in elderly adults and infants and in patients with liver failure or impaired ventilation
Meperidine	Parenteral: 0.75–1.5 mg/kg every 2–4 h Recommended maximum dose, 100 mg PO: 1.5 mg/kg every 4 h	Increased incidence of seizures; avoid in patients with renal or neurologic disease and those who receive MAOIs
Hydromorphone	Parenteral: 0.01–0.02 mg/kg every 3–4 h PO: 0.04–0.06 mg/kg every 4 h	
Oxycodone	PO: 0.15 mg/kg/dose every 4 h	
Ketorolac	IM: Adults: 30 or 60 mg initial dose followed by 15–30 mg; children: 1 mg/kg load followed by 0.5 mg/kg every 6 h	Equal efficacy to 6 mg MS; helps narcotic-sparing effect; not to exceed 5 days; maximum, 150 mg first day, 120 mg maximum on subsequent days; may cause gastric irritation
Butorphanol	Parenteral: Adults: 2 mg every 3–4 h	Agonist–antagonist; can precipitate withdrawal if given to patients who are being treated with agonists
Mild Pain		
Codeine	PO: 0.5–1 mg/kg every 4 h Maximum dose, 60 mg	Mild to moderate pain not relieved by aspirin or acetaminophen; can cause nausea and vomiting
Aspirin	PO: Adults: 0.3–6 mg every 4–6 h; children: 10 mg/kg every 4 h	Often given with a narcotic to enhance analgesia; can cause gastric irritation; avoid in febrile children
Acetaminophen	PO: Adults: 0.3–0.6 g every 4 h; children: 10 mg/kg	Often given with a narcotic to enhance analgesia
Ibuprofen	PO: Adults: 300–400 mg every 4 h; children: 5–10 mg/kg every 6–8 h	Can cause gastric irritation
Naproxen	PO: Adults: 500 mg/dose initially and then 250 every 8–12 h; children: 10 mg/kg/day (5 mg/kg every 12 h)	Long duration of action; can cause gastric irritation
Indomethacin	PO: Adults: 25 mg every 8 h; children: 1–3 mg/kg/day given 3 or 4 times	Contraindicated in psychiatric, neurologic, renal diseases; high incidence of gastric irritation; useful in gout

IM, Intramuscular; *MAOI*, monoamine oxidase inhibitor; *MS*, morphine sulphate; *PO*, oral.
Adapted from Charache S, Terrin ML, Moore RD, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. *N Engl J Med*. 1995;332:1317.

addition of a 5-HT3 receptor antagonist. Laxatives or stool softeners should be prescribed to prevent opioid-induced constipation.

When intravenous dosing of opioids is roughly equivalent to home doses of oral medications, consider gradually tapering the dose by 10% to 20% at a time and subsequently switching from parenteral to oral administration of opioids once pain symptoms have stabilized. In general, opioids should be tapered by decreasing the dose rather than by increasing the interval between doses. For adult patients whose pain requires several or many days to resolve, a sustained-release opioid preparation is appropriate and provides more consistent analgesia.

Hydration is a critical part of management. However, cardiac function may be significantly impaired, especially in adult patients, and caution should be exercised with intravenous fluid management to avoid iatrogenic fluid overload. Patients with SCD cannot concentrate their urine and are at risk for dehydration when not taking adequate fluids (60 mL/kg/24 h in adults). Intravenous hydration is indicated when the patient is not taking oral fluids adequately. Ideally, the urine specific gravity should be kept under 1.010 daily. Hb may decrease by 1 to 2 g/dL in an uncomplicated pain crisis; blood transfusion is not routinely indicated for an uncomplicated pain crisis.

Equianalgesic doses of oral opioids should be prescribed for home use when necessary to maintain the relief achieved in the emergency department or hospital. Care should be taken to appropriately taper opioids in patients who have received daily opioids over many days. In these patients, there may be physical opiate dependence, which is characterized by the onset of acute withdrawal symptoms upon cessation of opioid administration. For patients at risk for physical dependence, opiates should be titrated downward by 15% to 20% per day. All patients with SCD should receive pain prescriptions from the hospital pharmacy prior to discharge; discharge without opioid medications is associated with higher 30-day readmission rates.[204] A follow-up visit in the outpatient clinic should be scheduled within one to two weeks of discharge.

If the patient is not taking a disease-modifying agent such as HU, consideration should be given to initiating such therapy either as an inpatient or during follow up in the outpatient setting (see Case 1).

Chronic Pain

Chronic pain occurs in at least 29% of adults with SCD and is most frequently reported in those 25 to 44 years of age.[205] The mechanisms of chronic pain in SCD remain poorly understood and are multifactorial secondary to a number of biopsychosocial variables. In the post-hematopoietic stem cell transplantation setting, a subgroup of patients with SCD have persistence of chronic pain despite reversal of the underlying disease process, suggesting that individual and environmental factors play a role in pain phenotype.[206] Chronic pain can be challenging to treat and involvement of Palliative Care specialists early in the disease course is highly recommended.

Adequate analgesia with both short- and long-acting opiates is important to maintain the psychosocial functioning of patients who suffer from chronic pain. Underlying identifiable etiologies including avascular necrosis, early degenerative changes, or osteoarthritis should be addressed with orthopedic evaluation and potentially joint replacement in the case of femoral head necrosis. Agents including gabapentin, amitriptyline, and some antiseizure medications[207] can address neuropathic components and decrease the sleep impairment that can occur with chronic pain. Given the high incidence of comorbid depression, all individuals should be screened for depressive symptoms and be considered for pharmacotherapy if major depressive disorder is diagnosed. Several antidepressants, including serotonin and norepinephrine reuptake inhibitors (SNRIs), are effective in the treatment of chronic pain and can be considered after consulting with mental health professionals and considering side effect profiles.

Chronic Anemia

Chronic hemolytic anemia is one of the hallmarks of SCD. Sickle erythrocytes are destroyed randomly, with a mean life span of about 17 days.[208] The overall hemolytic rate reflects the number of ISCs.[209] The degree of anemia is most severe in SCA (HbSS), and HbSβ°-thalassemia, milder in HbSβ$^+$-thalassemia and HbSC disease,[210] and, among patients with SCA, less severe in those who have coexistent α-thalassemia (Table 43.8 and see Table 43.1).[211]

CASE 1

A 40-year-old African American female with sickle cell anemia complicated by frequent VOC, and sickle cell nephropathy, presented with acute pain and was subsequently hospitalized for IV analgesia and supportive care. Admission hemoglobin was 7.9 g/dL with elevated hemolytic parameters from baseline consistent with acute pain crisis. She was initiated on IV hydromorphone, parenteral NSAIDs for the first 24 hours, and subsequently transitioned to a hydromorphone PCA on Day 2 of hospitalization for improved pain control. She stabilized with conservative management but on Day 5 of hospitalization developed chest pain associated with mild dyspnea, non-productive cough, and low-grade fever.

Physical evaluation was notable for a new onset hypoxia (SpO$_2$ 88% on room air), tachypnea, fever, and the patient was also mildly distressed. Apart from decreased breath sounds in the right lower lung base, the rest of the physical exam was unremarkable. The patient's hypoxia resolved on 2 L nasal cannula and the patient was encouraged to use incentive spirometry. Chest radiography (CXR) and contrast-enhanced chest computed tomography (CT) demonstrated basilar airspace opacities involving the right lower and middle lobes. Repeat CBC showed that the Hb had dropped to 7.3 g/dL.

Question 1: What is your provisional diagnosis and what are your next steps in management?

Answer 1: Acute chest syndrome complicating acute pain crisis; obtain stat blood cultures and initiate broad-spectrum empiric antibiotic therapy. A simple top-up blood transfusion of 1–2 units pRBC to Hb below 10 g/dL is recommended.

The patient defervesced and stabilized after initiation of antibiotic therapy, however, after 24 h, fever recurred with worsening hypoxia. Arterial blood gas demonstrated hypoxemia with an arterial oxygen partial pressure to fractional inspired oxygen ratio of 89 mm Hg. In the setting of worsening tachypnea with concern for impending respiratory failure, the patient was transferred to the ICU, endotracheal intubation was performed, and the patient was placed on mechanical ventilation. Total Hb was 7.9 g/dL with 48.6% HbS.

Question 2: What is your next step in management?

Urgent exchange transfusion to reduce HbS fraction, continue intravenous hydration at maintenance rate and broad-spectrum empiric IV antibiotics.

Blood culture data remained negative. Her condition remarkably improved after 36 hours. She had an uneventful extubation and was safely discharged home after a total 13-day hospital stay.

ACS is often preceded by febrile episodes in children and by vaso-occlusive pain crisis in adults. Rapid recognition of ACS and prompt clinical intervention are necessary to prevent adverse health outcomes, especially in adults. Mainstays of treatment include judicious use of opioid analgesics to prevent respiratory compromise, supplemental oxygen therapy and arterial blood gas assessment, frequent use of bedside incentive spirometry in conjunction with respiratory therapy and empiric antibiotics, typically a cephalosporin and macrolide. Simple or exchange transfusion are indicated for the acute treatment of ACS; the decision to pursue exchange transfusion depends on the clinical severity and progression of ACS as well as the level of hemoglobin. If the patient is not on hydroxyurea (HU), initiate HU therapy at outpatient follow-up and encourage adherence to HU if the patient is on therapy to reduce/prevent future ACS episodes.

As already noted, EPO deficiency from otherwise subclinical chronic renal damage may also contribute to a decline in Hb levels below baseline. The level of chronic anemia is a significant prognostic marker in SCD.[59]

The treatment options for the chronic anemia of SCD have already been discussed earlier in this chapter.

TABLE 43.8 **Bacteria and Viruses That Most Frequently Cause Serious Infection in Patients With Sickle Cell Disease**

Microorganism	Type of Infection	Comments
Streptococcus pneumoniae	Septicemia	Common despite prophylactic penicillin and pneumococcal vaccine
	Meningitis	Less frequent than in years past
	Pneumonia	Rarely documented except in infants and young children
	Septic arthritis	Uncommon
Haemophilus influenzae type b	Septicemia	
Meningitis		
Pneumonia	Much less common in recent years because of immunization with conjugate vaccine	
Salmonella species	Osteomyelitis	
Septicemia	Most common cause of bone and joint infection	
Escherichia coli and other gram-negative enteric pathogens	Septicemia	
Urinary tract infection		
Osteomyelitis	Focus sometimes not apparent	
Staphylococcus aureus	Osteomyelitis	Uncommon
Mycoplasma pneumoniae	Pneumonia	Pleural effusions; multilobe involvement
Chlamydia pneumoniae	Pneumonia	
Parvovirus B19	Bone marrow suppression (aplastic crisis)	High fever common; rash and other organ involvement infrequent
Hepatitis viruses (A, B, and C)	Hepatitis	Marked hyperbilirubinemia

Data from Buchanan GR, Glader BE: Benign course of extreme hyperbilirubinemia in sickle cell anemia: Analysis of six cases. *J Pediatr.* 1977;91:21.

Exacerbations of Anemia

The constant level of hemolytic anemia may be exacerbated by additional events including aplastic crises, acute splenic or hepatic sequestration, chronic renal disease, acute kidney failure on a background of chronic renal disease, renal endocrine deficiency that may be present without overt renal failure, bone marrow necrosis, deficiency of folic acid or iron, DHTR, autoimmune hemolytic anemia, or hyperhemolysis. Laboratory evaluations that are very useful in the evaluation of a patient with an abrupt decline in baseline Hb level are the reticulocyte count, liver aspartate transaminase (AST), LDH, alloantibody screening, direct antiglobulin (Coombs) test, and EPO levels.

Aplastic Crises

Aplastic crises are transient arrests of erythropoiesis characterized by abrupt falls in Hb levels, reticulocyte number (typically, reticulocytes <1.0%, absolute reticulocyte count <10,000 per μL), and RBC precursors in the bone marrow without necessarily an increase in the LDH. Although these episodes typically last only a few days, severe anemia may develop in the setting of continued hemolysis in the absence of RBC production. Significant cardiac decompensation can occur if anemia and reticulocytopenia are not promptly recognized and treated. Although the mechanisms that impair erythropoiesis in inflammation are operative in infections of all types (see Chapter 36), human parvovirus B19 specifically invades proliferating erythroid progenitors resulting in cytotoxicity, which accounts for its importance in SCD (see Chapters 33 and 35).[212] Reticulocytopenia secondary to parvovirus B19 infection lasts approximately 7 to 10 days. Parvovirus B19 (Fig. 43.9) accounts for 68% of aplastic crises in children with SCD,[213] but the high incidence of protective antibodies in adults makes parvovirus a less frequent cause of aplasia in this age group (see also Infections, later in this chapter). Other reported causes of transient aplasia are infections by *S. pneumoniae*, *Salmonella*, streptococci, and Epstein-Barr virus. Bone marrow necrosis, which also may be the result of parvovirus infection, characterized by fever, bone pain, reticulocytopenia, and a leukoerythroblastic response, also causes aplastic crisis.[214,215]

Inhaled oxygen therapy also causes transient RBC hypoproduction; supraphysiologic oxygen tensions curtail EPO production promptly and suppress reticulocytosis within 2 days.[216]

The mainstay of treating aplastic crises is RBC transfusion. When transfusion is necessitated by the degree of anemia or cardiopulmonary symptoms, a single PRBC transfusion may suffice because reticulocytosis may resume spontaneously within a few days. Transfusion may be avoided by keeping severely anemic patients on bed rest to prevent symptoms and by avoiding supraphysiologic oxygen tensions. A useful guideline for transfusion in the context of an aplastic crisis is the reticulocyte count. A patient having an aplastic crisis with a reticulocyte count that is recovering is less likely to require urgent transfusion than one with a normal or low absolute reticulocyte count.

Sequestration Crisis (Spleen or Liver)

Acute splenic sequestration of blood is characterized by acute exacerbation of anemia, persistent reticulocytosis, a tender, enlarging spleen, and is sometimes accompanied by hypovolemia.[217] The LDH level may remain stable or increase and thrombocytopenia may also be present. Patients susceptible to this complication are those whose spleens have not undergone fibrosis including young patients with HbSS disease and adults with HbSC disease or sickle cell–β^+-thalassemia. Sequestration may occur as early as a few weeks of age and may cause death before SCD is diagnosed. In one study, 30% of children had splenic sequestration over a 10-year period and 15% of the attacks were fatal.[218]

The basis of therapy is to restore blood volume and RBC mass, thereby preventing evolution to hypovolemic shock. Because splenic sequestration recurs in 50% of cases, follow up management is imperative. For children older than age 2 to 3 years, splenectomy is recommended shortly after the acute event has abated. Alternatively, chronic transfusion therapy is used in young children to delay splenectomy until it can be tolerated safely but such practices have only been partly successful in the prevention of recurrence. Age of the first episode is predictive of recurrence—risk is lower when the first episode occurs after 2 years compared to before 1 year of age.[219] Because recurrence is possible during transfusion therapy, parents should be trained to detect a rapidly enlarging spleen and to seek immediate medical attention in this event. Elective splenectomy should be considered in older patients with chronic hypersplenism. Less common sites of acute sequestration include the liver and possibly the lung.[220,221]

Delayed Hemolytic Transfusion Reaction and Autoimmune Hemolytic Anemia

DHTR are potentially life-threatening complications. They are under-recognized in patients with SCD in particular because the symptoms mimic those of acute VOCs, and serological markers of new alloantibodies may be equivocal.[222,223] HTRs occur primarily

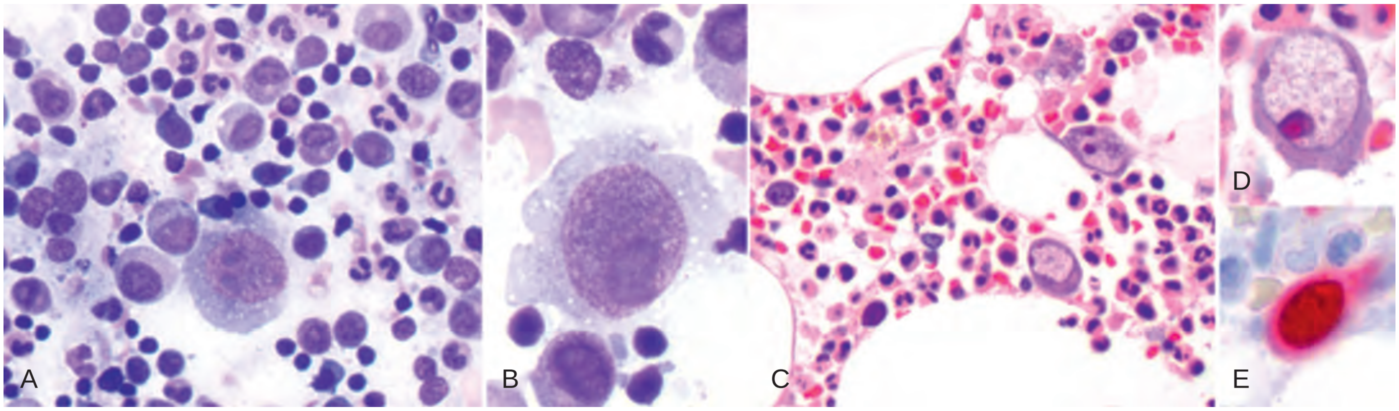


Figure 43.9 PARVOVIRUS. Bone marrow aspirate in a patient with sickle cell disease and aplastic crisis (A). Note the absence of red blood cell precursors except for the single, large degenerating pronormoblast *(lower center)*. Such pronormoblasts contain large nuclear inclusions (B) as a result of replication of parvovirus B19. The same can be seen in the tissue sections of a bone core biopsy (C and D). The parvovirus can now be recognized immunohistochemically with an immunostain (E).

in RBC alloimmunized patients, and approximately 30% of patients with SCD are predisposed to develop alloantibodies, in part because of minor blood group incompatibilities in racially mismatched blood.[149,150] Mismatch of RBC antigens, however, does not fully explain observed rates of alloimmunization given that a significant percentage of patients who receive phenotypically matched blood exclusively from ethnically matched donors still become alloimmunized.[152,224] Molecular analyses have demonstrated significant RH allelic diversity with mismatch between serological Rh phenotype and RHD or RHCE genotype in patients with SCD and African American blood donors.[150,152]

After alloimmunization, there is a subsequent decrease in antibody titer that can fall below serologically detectable levels. Therefore antigen-positive RBCs appear compatible in cross-matching and are transfused. This can result in a DHTR produced by the amnestic response of the immune system as opposed to the immediate hemolytic reaction that occurs with preformed antibody. The DHTR consists of an unexplained acute fall in Hb, elevated LDH level, elevated bilirubin levels above baseline, and hemoglobinuria, typically occurring between 4 and 10 days after the RBC transfusion.[223,224] Delayed hemolytic reactions and hyperhemolysis have been shown to occur in 11% of pediatric patients with SCD and a history of alloantibodies.[225] In a recent prospective analysis of an adult SCD cohort, DHTRs occurred following 4.2% of episodic transfusions and were associated with significant mortality—approximately 11% of patients who experienced DHTR died.[226]

In SCD, the DHTR can be particularly devastating because it can be accompanied by reticulocytopenia, which together with a bystander effect of destruction of recipient blood (not just donor blood) can result in unanticipated worsening of anemia to levels below that seen before transfusion.[223,227] In addition to the manifestations of a DHTR as listed, patients may develop acute congestive heart failure, acute renal failure, or ACS accompanied by vaso-occlusive pain crisis. Subsequent transfusions may further exacerbate ongoing hemolysis and worsen anemia.

Resolution of severe anemia may only occur after withholding further transfusions with subsequent reticulocyte count recovery. Corticosteroids at high doses (e.g., intravenous methylprednisolone 500mg/day for 2 to 3 days) should be considered if the anemia is life threatening or if further transfusion is deemed necessary to save the patient's life. Intravenous immunoglobulin can also be considered, with proper attention paid to avoiding iatrogenic fluid overload. Intravenous iron and EPO are often administered concurrently to accelerate endogenous RBC production in the setting of severe anemia.[228] Cases of severe DHTR and hyperhemolysis are driven by extensive complement activation. Treatment with the anti-C5 complement-blocking antibody, eculizumab, has been shown to reduce hemolysis and result in rapid clinical improvement.[229,230] If emergency transfusion of least compatible blood is deemed necessary in the setting of severe anemia or multi-organ failure, rituximab prophylaxis has demonstrated efficacy in several case reports.

Approaches to minimizing this complication include transfusing extended-matched (see Basic Management and Disease Modification), phenotypically compatible blood.[148,150,153,223,228] Extended RBC antigen molecular typing in recipients of chronic or episodic transfusions has been implemented in many centers across the US. Blood banks maintain records of previously identified alloantibodies to reduce the risk of re-exposure to a particular alloantigen once an alloantibody has been detected. Effective communication with other healthcare systems which have previously transfused the patient is paramount. Complex alloantibody profiles obtained from other institutions should be honored when sourcing compatible blood products even when in-house serological testing does not detect alloantibodies. Unfortunately, limited or inadequate alloimmunization histories may result from multiple patient encounters in various healthcare systems; there is a great need for a universal platform in which laboratory information exchange between institutions can occur rapidly.

Recognition of DHTR is vital; not only is DHTR itself potentially life-threatening, but giving a further transfusion in this setting can prove fatal. Diagnosis of DHTR is best made by simply being aware of DHTR when patients present with acute pain following a recent transfusion. This syndrome may or may not recur with further transfusions after a recovery period (see Case 2).[231]

Hyperhemolytic Crisis

Hyperhemolytic crisis is the sudden exacerbation of anemia with increased reticulocytosis and bilirubin level. If suspected, the approach to management should first be to look for an underlying etiology, which may be one of the events listed earlier: aplastic crisis (during the recovery phase when the reticulocyte count may not be decreased), sequestration crisis, DHTR, or autoimmune hemolysis. Another possible cause is glucose-6-phosphate dehydrogenase deficiency.[232]

Hypothyroidism

Iron overload in SCD can result in hypothyroidism.[233] Therefore hypothyroidism is another etiology to consider in a patient with SCD with an otherwise unexplained decrease in Hb below baseline.

Infections

Immune Deficit

The propensity of children with SCD to contract *S. pneumoniae* infection is related to impaired splenic function[234] and diminished serum opsonizing activity. Even before the anatomic autoinfarction of the spleen in patients with SCA, defective splenic function is demonstrable by Howell-Jolly bodies on the peripheral blood smear, visible

CASE 2: DELAYED HEMOLYTIC TRANSFUSION REACTION AND HYPERHEMOLYSIS

A 30-year-old female with HbSS disease on hydroxyurea therapy, congenital atrophic right kidney, and proteinuria was evaluated for acute pain and the onset of dark, "Coca-Cola"-colored urine. On further questioning, the patient endorsed a recent hospitalization at an outside hospital for VOC accompanied by ACS and reportedly received 2 units of packed RBCs, 8 days prior to current presentation. She had no known history of alloimmunization. At this visit, her Hb had decreased below her baseline to 7.7 g/dL accompanied by marked elevations in LDH (1500) and total and indirect bilirubin levels and acute kidney injury (AKI) with a serum creatinine of 1.49 mg/dL. Urinalysis demonstrated positive bilirubin, 3 plus hemoglobin, and 34 RBCs.

Question 1: What is your provisional diagnosis and what tests would you perform at this point?

Acute sickle pain triggered by a delayed hemolytic transfusion reaction (DHTR) and initiate full evaluation of DHTR.

Further investigations showed a positive direct antiglobulin (Coombs) test (DAT) for IgG and negative for complement, but all clinically significant alloantibodies were ruled out.

By the following morning, her Hb had declined further to 5.5 g/dL in the setting of persistent AKI; the patient remained hemodynamically stable and relatively asymptomatic.

Question 2: What is the next step in management?

Initiate IV steroids and IVIG in addition to a single dose of IV iron and ESA therapy to support endogenous RBC production.

Despite appropriate management for DHTR, the patient's anemia continued to worsen with marked elevations in LDH and persistent hemoglobinuria. On the fourth day of hospitalization, the Hb level decreased to 3.6 g/dL.

Question 3: What is the next step in management?

It is clear from the drop in hemoglobin to below baseline that the patient's own red cells are also being destroyed, a situation referred to as "hyperhemolysis." The patient requires a life-saving blood transfusion to prevent end-organ damage, specifically, worsening renal injury. Given concern for DHTR-associated hyperhemolysis and lack of response to steroids and IVIG, eculizumab, a C5 complement inhibitor which impedes complement activation, was administered. She subsequently received 2 units of pRBCs. Given the known risk for invasive meningococcal disease in patients treated with eculizumab, the meningococcal vaccine was administered to the patient. The majority of patients with SCD receive meningococcal vaccination in childhood. The patient's Hb levels gradually stabilized over the ensuing days without additional blood transfusion; she achieved a Hb level to 6.5 g/dL prior to discharge. Hydroxyurea therapy was resumed with resolution of AKI.

Delayed hemolytic transfusion reactions are under-recognized in patients with SCD because symptoms mimic those of acute vaso-occlusive crises and serological markers of new alloantibodies may be equivocal. Recognition of DHTR is vital; not only is DHTR itself potentially life-threatening, but administering further transfusions in this setting can prove fatal. It is important to continue screening for allo-antibodies.

"pits" on the surface of RBCs, and abnormal results of radionuclide spleen scanning.[235] Specific syndromes exhibiting greater rates of hemolysis cause loss of splenic function at earlier ages—HbSS disease earlier than HbSC disease earlier than HbSβ^+-thalassemia.

Infectious complications of SCD are a major cause of morbidity and mortality[236] even with current vaccination and prophylactic antibiotic regimens. The infections caused by particular organisms are shown in Table 43.8, and the specific organisms affecting different target organs are shown in Table 43.9. By 5 years of age, almost all patients are functionally asplenic, contributing to infectious susceptibility. Historically, pneumococcal sepsis has been the predominant cause of death in those younger than 20 years of age.[237]

Evaluation

The most critical aspect of infectious illness in SCD is the evaluation and treatment of febrile episodes. Routine evaluation includes a history for localizing symptoms, physical examination with assessment of vital signs for hemodynamic instability and/or hypoxia, CBC with differential and reticulocyte count, urinalysis, peripheral blood and urine cultures, a lumbar puncture if meningitis is suspected, and chest radiography or computed tomography (CT) to evaluate for pneumonia. Results of the CBC are compared with baseline values. A left shift in the differential count suggests bacterial infection. Patients presenting with acute fever should promptly receive empiric parenteral antibiotics, ideally within 60 minutes from triage.

Streptococcus pneumoniae, Haemophilus influenzae, Atypical Mycobacteria, and Acute Febrile Illness

S. pneumoniae bacteremia is accompanied by leukocytosis, a left shift, aplastic crisis, sometimes disseminated intravascular coagulation, and a 20% to 50% mortality rate.[237] Although concerns about *S. pneumoniae* sepsis are largely for young children, this complication also occurs in adults, often with devastating results.[238] *S. pneumoniae* is the major cause of meningitis in infants and young children with SCD, and it occurs in the setting of bacteremia.

The second most common organism responsible for bacteremia in these children, *H. influenzae* type b, accounts for 10% to 25% of episodes. *H. influenzae* bacteremia affects older children and is less fulminant than *S. pneumoniae* bacteremia, but it may be fatal.[237] Conjugated *H. influenzae* type b vaccines produce excellent antibody responses in children with SCD and are now administered in early infancy (http://www.cdc.gov/vaccines/recs/schedules/child-schedule.htm).

Owing to the high mortality rate of bacteremia, hospitalization and parenteral antibiotics have been the standard of care for children with fevers higher than 38.5°C. Prompt attention to fever can reduce the risk of severe pneumococcal sepsis. Rapid administration of antibiotics has resulted in a lower incidence of meningitis among patients with bacteremia than 20 years ago when the incidence was 50%.[239] The efficacy of ceftriaxone therapy for *S. pneumoniae* and *H. influenzae* infection[240] has led to new treatment algorithms that recommend outpatient therapy for most patients. However, resistant *S. pneumoniae* have emerged, necessitating a thorough knowledge of local resistance patterns to guide the choice of alternate antibiotics (particularly vancomycin, to which resistance has not been observed).

See the section on Pulmonary Complications for further discussions regarding pneumonia and ACS.

Meningitis

Meningitis therapy should cover *S. pneumoniae* and probably *H. influenzae* type b and should be continued for at least 2 weeks.

Salmonella and Osteomyelitis

In this patient population, osteomyelitis is commonly caused by *Salmonella* spp.[241] *Staphylococcus aureus*, the most common etiology in patients without SCD, accounts for less than 25% of SCD cases. These complications are more frequent in children due to the vasculature of immature bone that is conducive to hematogenous spread, but adults can also be affected. Infection usually affects long bones, often at multiple sites. Clinical presentations of acute and chronic osteomyelitis can vary widely and may be difficult to discern from acute vaso-occlusive pain episodes. Patients may report bone pain, difficulty ambulating, edema, skin erythema, and point tenderness to palpation. In chronic osteomyelitis, draining sinus tracts can develop. Providers should have a high index of suspicion and consider dedicated MRI imaging for further evaluation. In a meta-analysis focused on management of septic arthritis in children with SCD, no single laboratory value was able to predict septic arthritis.[242] The only laboratory value that may be helpful in diagnosing osteomyelitis is elevated CRP. However, caution should be exercised when interpreting CRP results given that SCD is an inflammatory disease.

The diagnosis is confirmed by culture of blood or infected bone. Parenteral antibiotics that cover *Salmonella* spp. and *S. aureus* are

TABLE 43.9 **Organ-Related Infection in Sickle Cell Disease**

Primary Sites of Infection	Most Common Pathogen(s)	Other Pathogens	Pathophysiology	Prevention	Management
Septicemia	*Streptococcus pneumonia*	*Haemophilus influenza* type b *Escherichia coli* *Salmonella* spp.	Defective splenic function; deficiency of opsonic antibody	Vaccines[a] Prophylactic penicillin	Empiric intravenous antibiotics for fever
Meningitis	*S. pneumoniae*			Same as for septicemia	
Osteomyelitis and septic arthritis	*Salmonella* spp. *S. pneumonia*	*E. coli* *Proteus* spp. *Staphylococcus aureus*		–	Surgical drainage, intravenous antibiotics
Pneumonia	*Mycoplasma pneumonia* Respiratory viruses	*Chlamydia pneumonia* *S. pneumoniae*		Vaccines[a]	See pulmonary and therapy sections for management of acute chest syndrome

[a]Against *Streptococcus pneumoniae* and *Haemophilus influenzae* type b.
Data from Buchanan GR, Glader BE. Benign course of extreme hyperbilirubinemia in sickle cell anemia: analysis of six cases. *J Pediatr*. 1977;91(1):21.

given, and tailored antibiotic therapy is based on culture results. Parenteral antibiotics are continued for 2 to 6 weeks.[242] Surgical drainage or sequestrectomy may be required. Most patients are cured by this approach, but there may be recurrences.[242]

Articular infection is less common and is often caused by *S. pneumoniae*.[241]

Parvovirus B19

The specificity of the parvovirus B19 (see also Aplastic Crises) for erythroid precursor cells, coupled with the accelerated erythropoiesis in hemolytic anemias, leaves sickle cell patients vulnerable to infection by this agent.[243] In SCD, parvovirus infection is a common cause of aplastic crisis, especially in children. It has been reported to cause bone marrow necrosis, ACS, pulmonary fat embolism, hepatic sequestration, glomerulonephritis, and acute nephrotic syndrome.[212–215,244]

Urinary Tract Infections

Patients with SCD are at a higher risk for urinary tract infections and pyelonephritis than the general population. Higher rates of volume depletion and urinary tract obstruction related to infarction or papillary necrosis may contribute to observed rates of infection. Evaluation should include urinalysis, urine culture with sensitivities, renal ultrasonography to assess for ureteral obstruction, as well as pregnancy testing in women of reproductive age. Children notably may lack typical urinary tract symptoms such as dysuria or urinary frequency. *Escherichia coli* is the most common uropathogen and can cause septicemia in these patients. Persistent urinary tract infections may be secondary to renal papillary necrosis. Recent literature has suggested that patients with SCD may have higher rates of asymptomatic bacteriuria although the clinical relevance of this finding remains unclear.[245] All urinary tract infections in this patient population should be considered complicated and duration of antibiotic therapy may range from 10 to 21 days.

Neurologic Complications

Neurologic complications occur in 25% or more of patients with SCD.[246] Neurologic complications include CVAs (consisting of transient ischemic attacks [TIAs], overt and silent cerebral infarction, cerebral hemorrhage), seizures (which can be a presenting feature of CVA), unexplained coma, spinal cord infarction or compression, central nervous system infections, vestibular dysfunction, and sensory hearing loss.[247–249]

Cerebrovascular Accidents, Pathophysiology, Incidence, Risk Factors, and Presentation[250]

Histopathologic evaluation of large-vessel involvement in SCD shows a pattern of smooth muscle proliferation with overlying endothelial damage and fibrosis. Smaller arterioles and capillaries demonstrate distension, thrombosis, and vessel-wall necrosis.[251,252] Aneurysmal dilatation associated with hemorrhagic stroke occurs at regions of intimal hyperplasia.[252] The vessel wall changes are likely multifactorial in origin related to endothelial injury from high and turbulent flow, RBC adherence, and hypoxia; but in addition, it has been speculated that depletion of NO by the free Hb released through intravascular hemolysis may also play a role.[254] The age-specific pattern of stroke risk in SCD may be related to the higher cerebral flow rates in early childhood.[254] Cerebral thrombosis, which accounts for 70% to 80% of all CVAs in patients with SCD, results from large-vessel occlusion (see Fig. 43.8) rather than the more typical microvascular occlusion of SCD.[253,254] Silent infarcts are thought to result from microvascular vaso-occlusion or thrombosis or chronic hypoxia in the periphery stemming from large-vessel disease.[255] In 30% of patients with SCD, major vessel stenosis results in the formation of friable collateral vessels that appear as puffs of smoke (*moyamoya* in Japanese) on angiography.[256] Moyamoya disease predisposes to thrombotic and hemorrhagic strokes, seizures, and cognitive disability.[257]

The relative risk for stroke is 200 to 400 times higher in children with SCD compared with children without SCD. The prevalence of clinically overt stroke is 11%. Clinically silent infarction detectable by MRI affects 17% to 20% of patients by age 20 years and 53% of adults (21 to 42 years, median age 30 years).[258] Silent infarcts are lesions visible in at least two planes of T2-weighted MRI images and are at least 3 mm in greatest linear dimension.[259] Such lesions are not truly silent and are associated with both neurocognitive and behavioral impairments in patients with SCD. Even in patients without silent or overt cerebral infarction, cognitive functioning can be impaired.[260] Almost 50% of children with “silent” infarcts eventually require lifelong support or custodial care because of neuropsychologic deficits.[261] Whereas infarctive strokes are more common in children and those older than 30 years of age, hemorrhagic stroke tends to occur between ages 20 and 30 years.[52] Intracranial aneurysms are reported in 3% of children and 11% to 15% of adults with SCD. Affected patients will often have aneurysms at multiple sites.[262,263] Aneurysms may require surgical intervention or endovascular coiling if they are rapidly enlarging or result in subarachnoid hemorrhage.

Sickle cell–specific risk factors for CVA include increased cerebral blood flow velocity[264] (discussed further under Primary Prevention of Cerebrovascular Accidents), a history of overt or silent cerebral infarction,[265] nocturnal hypoxemia or sleep apnea,[266] severe anemia, higher reticulocyte counts, lower HbF levels, higher WBC counts, HbSS genotype (rather than HbSC disease or sickle cell–β-thalassemia), prior ACS, migraines, elevated homocysteine levels, as well as "relative" systolic hypertension (i.e., those at the high end of the lower-than-normal range characteristic of SCD).[52,66] Genetic markers of increased risk are the Central African Republic (CAR) haplotype and the absence of α-thalassemia.[253,267] Both small- and large-vessel thrombosis can occur. Increased stroke risk in siblings with SCD suggests that additional genetic factors might be involved. For instance, specific HLA alleles separately correlate with small- versus large-vessel stroke risk as well as VCAM-1 variants.[253]

In addition to these sickle cell–specific predictors of stroke, one must also consider the well-documented modifiable risk factors for stroke that are operational in the general population; these are hypertension, exposure to cigarette smoke (active smoking or passive exposure), diabetes, atrial fibrillation, dyslipidemia, carotid artery stenosis, postmenopausal hormone therapy, poor diet, physical inactivity, obesity, and fat distribution. Less well-documented but potentially modifiable risk factors include alcohol or drug use, oral contraceptive use (especially those containing estrogen), and sleep-disordered breathing.[268]

CVAs are heralded by focal seizures in 10% to 33% of cases and by TIAs in 10%. CVAs are fatal in approximately 20% of initial cases, recur within 3 years in nearly 70%, and are the cause of motor and cognitive impairment in the majority. Intracranial hemorrhage results in the same signs as thrombosis, but in addition, neck stiffness, photophobia, severe headache, vomiting, and altered consciousness may occur. Coma suggests hemorrhage rather than thrombosis. A typical presentation is coma and seizures without hemiparesis. Although the mortality rate may be as high as 50%, the morbidity of survivors is low. Hemorrhage may be subarachnoid, intraparenchymal, or intraventricular, which can be differentiated by angiography. The favorable neurosurgical outcome in subarachnoid hemorrhage caused by ruptured aneurysm justifies an aggressive approach to diagnosis, transfusion, vasodilatory therapy, and surgery.

Primary Prevention of Cerebrovascular Accidents

The overall risk of stroke in pediatric patients with SCD is 1% per year; however, in the subset of patients with transcranial Doppler evidence of a high (>200 cm/s) cerebral blood flow velocity in the internal carotid artery or middle cerebral artery, the stroke risk is in excess of 10% per year (although this is still much lower than the risk of recurrent stroke in a sickle cell patient after a first event, which is approximately 70%). In the Stroke Prevention Trial in Sickle Cell Disease (STOP), 130 children (mean age 8 years) diagnosed as having clinically silent cerebral artery stenosis on the basis of high cerebral flow rates were randomized to receive chronic transfusion therapy with a goal HbS of less than 30% versus observation. Over a period of more than 2 years, the risk of stroke was reduced to less than 1% per year in the transfused group[264] (a risk reduction of >90%). The ability of transfusion to curtail progression of large-vessel stenosis has also been proven with angiography.[269]

Because of the risks of iron overload and allosensitization with chronic transfusion, a randomized controlled trial of withdrawal of transfusion was conducted (STOP 2). This trial evaluated discontinuation of transfusion after at least 30 months in children who had not had an overt stroke and in whom the cerebral flow rates decreased to low risk (<170 cm/s) with transfusion. This study was terminated early because of a high rate of reversion to high-risk TCD flow rates (34%) and stroke (5%) in the patients taken off transfusion compared with the group who continued transfusion.[268]

In children, MRI can also be used to assess stroke risk: 8.1% of children with an asymptomatic MRI lesion versus 0.5% of those with a normal MRI had a stroke during the ensuing 5 years.[265] A randomized trial of MRI-guided prophylactic transfusion (Silent Infarct Treatment Trial) concluded that regular blood transfusion significantly reduced the incidence of cerebral infarction in children with SCA.[270] In adults, MRI or MRA of the brain should be used[65] to assess thrombotic or hemorrhagic stroke risk.

Chronic transfusion is associated with a significant complication rate including alloimmunization and iron overload. Therefore there is a need for alternatives, especially because some patients and physicians believe that the 10% annual stroke risk does not warrant the risks and burdens of chronic transfusion.[250]

The use of HU as upfront therapy for primary prevention of stroke has not been evaluated in randomized trials. However, select children and adolescents may be able to transition to HU following a period of chronic transfusion. In the TWiTCH trial, 121 children on chronic transfusion therapy for at least 1 year were randomized to transition gradually from transfusions to HU or to continue transfusion therapy.[271] The primary endpoint was non-inferiority for TCD velocity at 24 months. The trial was terminated early because the first interim analysis revealed non-inferiority for the primary endpoint. No strokes or silent infarcts were reported. There were 3 TIAs in each treatment arm (5%). Iron levels in the TWiTCH trial were significantly reduced in the HU arm. In addition, liver iron increased from 8.5 to 11.3 mg/g liver weight in the transfusion arm and decreased from 11.3 to 9.5 mg/g liver weight in the HU arm. There are multiple caveats to consider including the relatively short duration of follow up and adherence rates to HU regimens in the study; caution should be exercised when selecting patients for this approach.

The role of aspirin in ischemic stroke prevention in SCD is being evaluated (see Basic Management and Disease Modification).

Other modifiable risk factors for stroke (see Cerebrovascular Accidents, Pathophysiology, Incidence, Risk Factors, and Presentation) should be identified and treated. Notably, in the general population, hypertension is particularly associated with a risk for hemorrhagic stroke, and effective treatment of hypertension can produce a relative risk reduction of 26% for ischemic stroke and 49% for hemorrhagic stroke.[272] In patients with SCD followed through the Cooperative Study of Sickle Cell Disease, both diastolic and systolic blood pressures were noted to be lower than for matched control participants. Patients with systolic pressures in the higher range for the sickle cell group, even with systolic pressures less than 140 mmHg, had an increased risk of first ischemic stroke (there were insufficient events to make firm conclusions regarding hemorrhagic stroke).[52] Therefore, at a minimum, it seems reasonable to follow population-wide recommendations for blood pressure control in patients with SCD.

Evaluation and Management of Acute Cerebrovascular Accidents

Patients with symptoms and signs of CVA should be evaluated immediately using CT scanning or MRI to distinguish among TIA, cerebral thrombosis, and hemorrhage. In those with hemorrhage, angiography or MRA is indicated after partial-exchange transfusion is performed to avoid complications associated with the injected contrast material. In both thrombosis and hemorrhage, prompt partial-exchange transfusion is performed, and chronic direct transfusion to maintain the HbS level below 30% is instituted to prevent recurrent events (see also Basic Management and Disease Modification) and promote resolution of arterial stenoses.[269]

Secondary Prevention of Cerebrovascular Accidents

The risk of recurrent stroke is approximately 70%, a risk that is reduced to around 13% with chronic transfusion.[273] Although recurrent CVAs during chronic transfusion have been reported, this therapeutic modality provides the best means of preventing recurrence (Fig. 43.10). This treatment also provides incidental protection against pain crises, bacterial infections, ACS, and hospitalization.

Based on the data from STOP 2 (see Primary Prevention of Cerebrovascular Accidents), chronic transfusion is continued indefinitely. This may not be feasible for administrative reasons or because of allosensitization or iron overload for which the patient is unable or unwilling to undergo treatment. In patients with a history of CVA transitioned from chronic transfusion to HU, the recurrent stroke rate remained stable and in the range seen with continued transfusion.[273a]

Per primary prevention, all other identified modifiable risk factors for stroke should be identified and treated.

In patients with moyamoya disease, surgical approaches to therapy, such as extracranial-intracranial bypass, have been useful in improving the perfusion of affected regions of the brain.

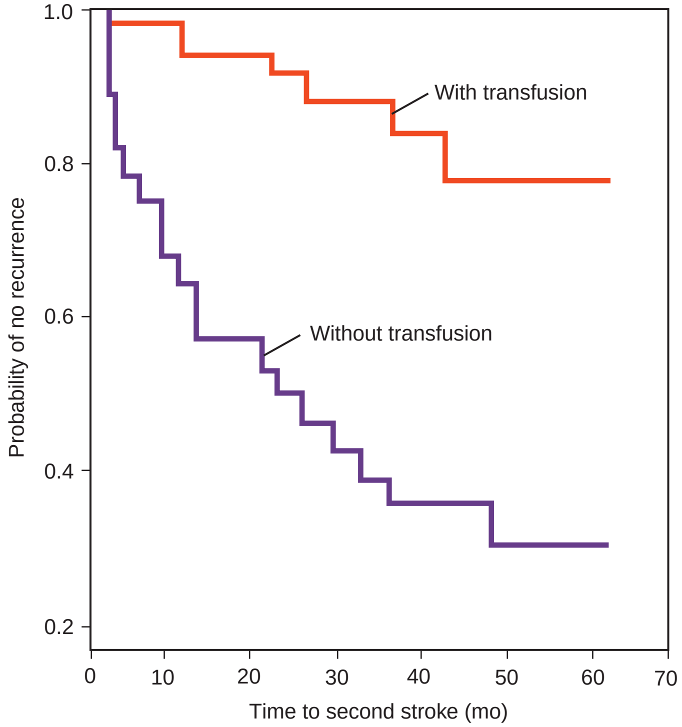


Figure 43.10 COMPARISON OF STROKE RECURRENCE OVER 62 MONTHS IN A TRANSFUSED GROUP AND IN UNTRANSFUSED HISTORICAL CONTROL GROUPS. *(Adapted with permission from Pegelow CH, Adams RJ, McKie V, et al. Risk of recurrent stroke in patients with sickle cell disease treated with erythrocyte transfusions. J Pediatr. 1995;126:896–899.)*

Stem cell transplantation has resulted in stabilization of cerebral vasculopathy,[274] but the risk of a second neurologic event is higher in the peritransplant period, and the mortality rate with this procedure is between 6% and 10%.[274]

Seizures

Seizures occur more commonly among patients with SCD. In one study, 21 of 152 patients in a pediatric clinic had seizures, four of which were related to meperidine therapy. Most had non-focal CT and MRI studies but focal electroencephalographic changes.[275] CVAs are heralded by focal seizures in 10% to 33% of cases. Therefore, seizures in SCD ultimately may be related to the underlying vasculopathy.

Pulmonary Complications

Pulmonary disease is the leading cause of death in patients with SCD.[40] Both acute and chronic pulmonary complications are common; a unifying clinical manifestation is hypoxia.[276] Acute complications include pneumonia and ACS and chronic complications range from PH to chronic thromboembolic disease.

Pneumonia

Pneumonia is defined as chest infiltrates on chest radiography or chest CT scan associated with fever and an identified infectious etiology. The risk for and increased frequency of *S. pneumoniae* infections is discussed under Infections earlier. In addition, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella* spp. are also relatively common causes of pneumonia in patients with SCD. Antibiotic therapy for pneumonia or ACS should cover these agents in addition to pneumococcus and *H. influenzae*. When antibiotics are used to treat the ACS, they should cover *S. pneumoniae*, *H. influenzae* type b, *M. pneumoniae*, and *C. pneumoniae*.

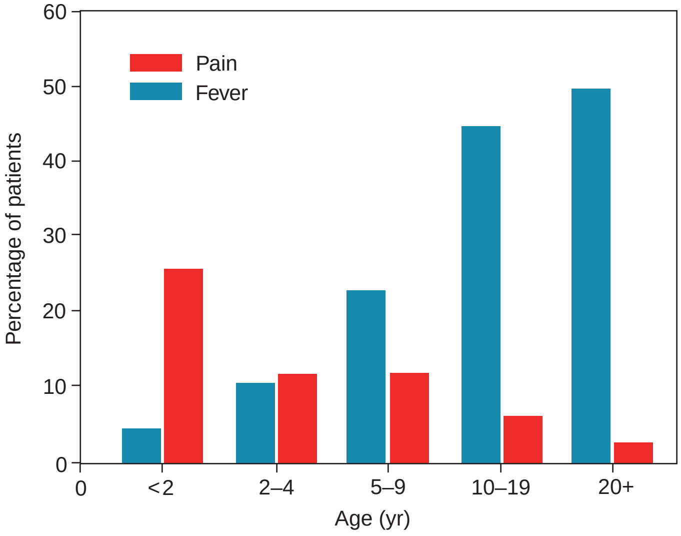


Figure 43.11 AGE-SPECIFIC ASSOCIATED EVENTS WITHIN 2 WEEKS PRECEDING ACUTE CHEST SYNDROME. *(Adapted with permission from Vichinsky EP, Styles LA, Colangelo LH, Wright EC, Castro O, Nickerson B. Acute chest syndrome in sickle cell disease: clinical presentation and course. Cooperative Study of Sickle Cell Disease. Blood. 1997;89:1787–1792.)*

Acute Chest Syndrome

ACS occurs in approximately 30% of patients.[277] ACS is defined as a new segmental (involving at least one complete segment) radiographic pulmonary infiltrate identified on chest radiography or chest CT scan associated with one or more new symptoms, which include fever, chest pain, cough, sputum production, dyspnea, tachypnea, and hypoxia (>2% decrease in SpO_2 [O_2 saturation]) from a documented steady-state value on room air). Risk factors include HbSS genotype, low HbF concentrations, and high steady state Hb and leukocyte values.[277] This entity is included in discussions of SCD because processes other than infection, such as vaso-occlusion, could also lead to pulmonary symptoms, signs, and chest radiographic changes. However, it should be borne in mind that the usual etiology might be both vaso-occlusion and infection simultaneously, and in all cases of ACS, antibiotics should be administered. Many episodes in which common pathogens are not cultured are caused by "atypical" agents (*Mycoplasma*, *Legionella*, and *Chlamydia* spp.), suggesting that antibiotic therapy include agents directed at atypical agents. Pulmonary fat embolus, evidenced by stainable fat in pulmonary macrophages obtained by bronchoalveolar lavage or sputum induction, is found in 44% to 60% of cases of ACS.[278] ACS caused by pulmonary fat embolus is associated with more severe hematologic and clinical abnormalities. A decrease in Hb values and a platelet count less than 200,000 cells per μL in association with neurological symptoms including confusion or sternal pain is highly suggestive of marrow infarction with fat embolism. In adults, the ACS mortality rate is four times higher than in children, largely due to a higher incidence of bone marrow and fat emboli and the presence of other comborbidities.[15,279]

ACS is often preceded by febrile episodes in children and by vaso-occlusive pain crisis in adults (Fig. 43.11).[279] Elevation of serum phospholipase A2 was detected in patients admitted with vaso-occlusive pain crisis 24 to 48 hours before ACS was clinically diagnosed.[280] Pulmonary fat embolus is often preceded by an acute painful episode. ACS should prompt aggressive inpatient management including judicious use of opioid analgesics to prevent respiratory compromise, supplemental oxygen therapy with pulse oximetry monitoring, frequent use of bedside incentive spirometry in conjunction with respiratory therapy and empiric antibiotics, typically a cephalosporin and macrolide. All adult patients should receive thromboprophylaxis with unfractionated heparin or low-molecular-weight heparin (LMWH) as per institutional guidelines unless contraindicated. Simple or exchange transfusion are the mainstays of acute treatment of ACS; the decision to pursue exchange transfusion depends on the clinical severity and progression of ACS as well as hemoglobin levels. Some patients have a rapidly progressive course associated with a precipitous decrease in arterial oxygen tension within 24 hours of developing respiratory symptoms and multiorgan failure; they may require intensive care treatment. Repeat chest imaging with plain films and serial blood gas measurements to assess arterial oxygenation status may be necessary in cases of rapid respiratory decline. If there are clinical signs of respiratory distress or when arterial oxygen tension cannot be maintained above 70 mmHg with inhaled oxygen, partial-exchange transfusion is indicated and mechanical ventilation may be required.

In patients with pneumonia or chest syndrome, antibiotics should cover *S. pneumoniae*, *M. pneumoniae*, *C. pneumoniae*, *H. influenzae*, and *Legionella* spp.

Pulmonary Hypertension

PH affects approximately 6% to 11% of patients with SCD and is defined as a mean pulmonary artery pressure ≥25 mmHg at rest diagnosed by right heart catheterization.[281–284] Sickle-related PH is an independent risk factor for mortality with an approximate 40% six-year mortality according to one study of 531 patients.[35,285] PH in SCD is classified as multifactorial; some patients have hemodynamics consistent with pulmonary arterial hypertension, while others have features of PH related to chronic thromboembolic disease or left-sided heart disease. There does not appear to be an association with the occurrence of ACS,[282] emphasizing that the pathophysiology of these two conditions may differ in some key features. The pathophysiology may involve thrombi in large and small arteries,[286,287] cardiac decompensation from progressive anemia, and potentially reversible increases in vascular tone from NO depletion and medial and intimal hypertrophy.[287] The recognition that PH is a feature of hemolytic syndromes other than sickle cell and that markers of hemolysis correlate with the risk of PH supports the idea that depletion of NO through scavenging by free Hb may have an important role in the pathophysiology of this disease.[283,288]

The association with early death and the emerging availability of candidate treatments suggest that efforts should be made to diagnose this condition in all patients with SCD.[283,288] The diagnosis is challenging in patients with SCD because of the many potential causes of dyspnea, exercise intolerance, and fatigue.[289] The feasibility of an echocardiogram-determined TRJV measurement of 2.5 m/s to make the diagnosis was suggested by selective cardiac catheterization in a cohort of 195 patients[281]; however, the sensitivity and specificity of this test may have limitations.[288] Transthoracic echocardiographic estimates of pulmonary pressures are substantially less accurate than right heart catheterization, with a positive predictive value of approximately 31%.[290] However, risk stratification based on TRJV helps to guide clinical decision-making in patients with SCD. In adult patients aged 18 and older, echocardiography screening should be considered in patients with respiratory symptoms including exertional dyspnea, TRJV of 2.5 m/s or greater on prior echocardiogram, thromboembolic disease, or severe hemolytic anemia. Additional testing including ventilation perfusion scanning and the 6-minute walk test (6MWT) can aid in the diagnostic work-up of suspected PH. Elevations in BNP levels have been correlated with an increased TRJV and risk of early death in SCD cohorts. All patients with suspected PH should be referred to a pulmonologist for further evaluation and consideration of right heart catheterization.

The optimal therapy for PH secondary to SCD is not known.[289] Management of adults with sickle-related PH includes supplemental oxygen therapy for those with low oxygen saturation, diuretics to treat right ventricular overload, long-term anticoagulation in patients with evidence of prior or current thromboembolic events with low bleeding risk, as well as HU and/or transfusions to maintain adequate Hb levels, decrease hemolysis, and reduce the frequency of VOC. Targeted agents including endothelin receptor and prostanoid pathway agonists may be considered in select patients.

In a 16-week, double-blind, placebo-controlled trial of sildenafil to treat patients with SCD with increased TR-jet velocity and a low exercise capacity, sildenafil increased hospitalization rates for pain without evidence of improvement in TR-jet velocity or BNP levels.[291] As such, sildenafil is relatively contraindicated in patients with sickle-related PH.

Other Pulmonary Complications

Other findings include restrictive and obstructive lung disease and hypoxemia.[292] High-resolution, thin-section CT scanning of the lungs may show chronic interstitial fibrosis. Airway hyperreactivity occurs in nearly two-thirds of children with SCD not diagnosed as having asthma. Thirty-six percent of 53 children with SCD were found to have sleep-related upper airway obstruction, 16% had hypoxemia, and all 15 who underwent adenotonsillectomy improved symptomatically and had improved hypoxemia.[293] Sleep apnea may be associated with exacerbations of painful episodes and strokes. Pulmonary function measurements should be obtained as baseline data and patients with signs or symptoms suggestive of sleep apnea should undergo formal full-night polysomnography (PSG). This is especially important in patients who chronically require opiate therapy and/or sedative medications which can induce central apnea.

Hepatobiliary Complications

"Sickle hepatopathy" is an umbrella term that encompasses the broad clinical spectrum of liver disease that is observed in patients with SCD. It occurs predominantly in patients with HbSS disease but can also be seen in HbSC or HbSβ-thalassemia disease. Hepatobiliary complications in patients with SCD include cholelithiasis, cholecystitis, acute hepatic cell crisis, acute hepatic sequestration crisis, and sickle cell intrahepatic cholestasis.[294] Chronic transfusion places patients at risk for hepatitis infection and transfusional hemosiderosis. Nontransfusion-related hepatic hemosiderosis can also be seen. The prevalence of cirrhosis has been reported to be as high as 30% in SCD autopsy case series.[295] In rare instances, patients with SCD may present with decompensated chronic liver failure necessitating liver transplantation.[296]

The serum bilirubin level is higher in SCA (HbSS or HbSβ^0-thalassemia) than in HbSC disease or HbSβ$^+$-thalassemia as a result of a greater hemolytic rate. The level rises after the first decade, possibly as a result of chronic hepatobiliary dysfunction. Total bilirubin concentrations are usually less than 6 mg/dL (102.6 μmol/L) but may increase beyond this threshold during sickle hepatic crises.[297] Total bilirubin levels are also influenced by co-inheritance of the promoter variant in the *UGT1A1* gene, also referred to as Gilbert syndrome.[2] The AST and alanine aminotransferase (ALT) levels are often elevated, particularly in adult patients with SCA. Serum AST increases during hemolysis and correlates with LDH whereas acute increases in ALT levels may reflect hepatocyte injury. Alkaline phosphatase levels are elevated in all genotypes. Minor abnormalities in liver function tests may reflect ongoing hemolysis but significant increases in conjugated bilirubin, decreased synthetic function, and low albumin levels are suggestive of intrinsic liver disease. Concurrent autoimmune liver disease has been observed in SCD cohorts and a liver autoantibody screen (antinuclear antibodies, anti-smooth muscle antibodies, anti-liver kidney microsomal antibodies) should be performed in select cases. MRI imaging of the abdomen which can quantify hepatic iron (R2-MRI or T2*) should be performed.[296] Percutaneous liver biopsy is associated with a high risk of severe complications and death in patients with acute hepatic syndromes and in general should be avoided.[298]

Cholelithiasis and Cholecystitis

The prevalence of pigmented gallstones in SCD is directly related to the rate of hemolysis.[299] In SCA, gallstones occur in children as young as 3 to 4 years of age and are eventually found in approximately 70% of adult patients. Co-inheritance of a genetic variant in the promoter of *UGT1A1* gene greatly influences level of bilirubin and age of onset of gallstones. Some have recommended the surgical removal of asymptomatic gallstones to avoid subsequent difficulty in distinguishing gallbladder pain from acute painful episodes. The management of acute cholecystitis and ascending cholangitis requires broad-spectrum antibiotic therapy covering *Salmonella* and anaerobic organisms; elective cholecystectomy should be performed once acute complications resolve. This approach has become more feasible with the availability of laparoscopic cholecystectomy.[300] Laparoscopic cholecystectomy is associated with a shorter hospital length of stay and lower rates of postoperative complications, although it does not reduce the risk of sickle cell–related complications compared with open cholecystectomy. The role of cholecystectomy in patients with asymptomatic cholelithiasis remains controversial.[295]

Acute Hepatic Cell Crisis

Acute hepatic cell crisis occurs in approximately 10% of patients and presents with tender hepatomegaly, worsening jaundice, and fever.[294] The likely etiology is hepatocellular cell ischemia. The AST and bilirubin are elevated, but rarely above 300 IU/L and 255 μM, respectively. The serum total bilirubin concentration is usually less than 15 mg/dL. This syndrome usually resolves within 3 to 14 days with supportive care alone but can progress to liver failure and fatal outcomes, therefore patients should be monitored closely and exchange transfusion initiated if they show signs of progressive liver dysfunction (e.g., increasing AST).

Acute Hepatic Sequestration Crisis

The pathophysiology of acute hepatic sequestration involves sequestration of sickled erythrocytes in the liver. Patients present with right hypochondrial pain, acute hepatic enlargement, and a dramatic fall in Hb concentration. Management includes supportive care and conservative transfusions in the setting of acute anemia. Providers should target the minimal acceptable Hb level with transfusion to prevent hypovolemia or organ dysfunction; rare cases of auto-transfusion have been reported with resultant hyperviscosity.[301]

Intrahepatic Cholestasis

The most severe hepatic complication encountered in SCD patients is acute sickle cell intrahepatic cholestasis which is rare but fatal. Widespread sickling within sinusoids induces hepatic ischemia and injury. This results in severe hyperbilirubinemia (predominantly conjugated) with fever, right upper quadrant pain, and leukocytosis.[302] Total serum bilirubin levels may be markedly elevated and levels of 273 mg/dL have been reported in the literature; transaminase levels can be in the thousands. Intrahepatic cholestasis is accompanied by renal failure and coagulopathy.[303] Evidence of progressive liver dysfunction should prompt consideration of acute hepatic cell crisis and exchange transfusion to maintain the HbS percentage less than 30%.

Hepatitis C Infection

Chronic hepatitis C virus (HCV) infection in SCD occurs with a prevalence that is related to the number of transfusions received; it may be a leading cause of cirrhosis. With improved screening, the likelihood of acquiring new HCV infection with blood transfusion in the United States is now extremely rare (approximately 1:1,900,000 units). There have been marked improvements in therapies for chronic hepatitis C infection demonstrating viral eradication rates of over 95% and most patients can receive ribavirin-free drug regimens.[304,305] Nucleotide/nucleoside analogs can be implemented to treat chronic hepatitis B although the prevalence of this infection in SCD cohorts remains low.[306] Liver transplantation has been used successfully as therapy for this complication.[307] Experience with liver transplantation in SCD patients is increasing although early studies demonstrated poor outcomes, with mortality rates of approximately 60% at 1 year post-transplant.[295,296] Later studies have shown decreases in transplant-related mortality as a result of improved patient selection and perioperative management practices.

Obstetric and Gynecologic Issues

Gynecologic complications (delayed menarche, dysmenorrhea, ovarian cysts, pelvic infection, and fibrocystic disease of the breast) are more common in women with SCD. Pregnancy entails increased risks to the mother and child compared with the general population.[308]

Pregnancy

Pregnancy in patients with SCD is associated with increased risks to both the mother and fetus, although these risks are not so great as to prohibit continuation of pregnancy.[309,310] The fetal complications of pregnancy, most of which are related to compromised placental blood flow, are the increased incidence of spontaneous early abortion, intrauterine growth retardation, low birthweight, and fetal death. Maternal complications include increased rates of painful episodes, severe anemia caused by iron or folate deficiencies, exaggeration of the physiologic "anemia of pregnancy," increased infections (urinary tract infections, pneumonias, endometritis), venous thromboembolism, preeclampsia and eclampsia, and death.[310] It is controversial whether the degree of anemia predicts the birth of babies with low birthweight. The occurrence of a perinatal death in a previous pregnancy and the presence of twins in the present pregnancy are two major risk factors for an unfavorable outcome. The course of pregnancy is more benign in HbSC disease than in SCA, the latter is more likely to have painful crises and blood transfusions, and the overall rate of maternal and fetal complications in mothers with SCD is higher compared to mothers without SCD.[311]

Better fetal and maternal outcomes in recent years are largely attributable to generally improved antenatal and obstetric care. Patients should be followed in a high-risk obstetric clinic in addition to the hematology clinic and receive the usual vitamin, mineral, and folate supplements. A high-calorie, high-protein diet can be considered. There is no specific therapeutic or preventive treatment for intrauterine growth retardation. Some experts recommend prophylactic transfusion, but a large controlled study showed no improvement in fetal outcomes from this management option, although maternal symptoms are reduced.

Women with SCD may be receiving disease-modifying therapies and current guidelines recommend discontinuation of HU several months before conception or at time of pregnancy. Exposure to HU has been reported in less than 100 human pregnancies and an increase in major congenital defects was not observed but has been reported in animal studies.[312] Fetal risks from L-glutamine, voxelotor, and crizanlizumab are unknown and as such these agents should be avoided during pregnancy. Folic acid supplementation should be continued. Medications to manage renal medical disease and hypertension, including ACE inhibitors and ARBs, should be discontinued given known teratogenic effects and alternative antihypertensive agents should be instituted. Chelation should be discontinued once pregnancy occurs unless there are extenuating circumstances with severe iron overload given the potential risk for congenital abnormalities. Close monitoring for VTE is necessary throughout pregnancy; women should receive low-molecular-weight heparin for thromboprophylaxis when hospitalized.[313]

An individualized transfusion plan should be developed for pregnant patients highlighting indications for acute and chronic transfusion regimens. In addition to the usual indications for transfusion therapy in SCD, transfusion therapy is indicated for patients with cardiac or respiratory compromise, in preparation for cesarean section, preeclampsia, twin pregnancy, ACS, Hb levels more than 20% below steady state or less than 5 g/dL, and previous history of perinatal mortality. If the Hb is between 8 and 10 g/dL and transfusion is indicated for any of the reasons above, partial exchange should be performed (e.g., phlebotomy of 400 to 500 mL and transfusion of 2 units of packed RBCs).[309] The type of delivery does not appear to represent a problem, and both spontaneous delivery and cesarean section are well tolerated.

Some experts advise that hypertonic saline injections are contraindicated for elective termination of pregnancy because of the risk of sickling-induced vaso-occlusion. There are anecdotal reports of a higher incidence of acute painful episodes after therapeutic abortion; inpatient intravenous hydration before and for the 24 hours after the procedure is recommended.

Prenatal Diagnosis

All pregnant women with SCD should be offered prenatal counseling, including hemoglobinopathy testing for their partner. The genotype of the mother should be established and testing of the partner should

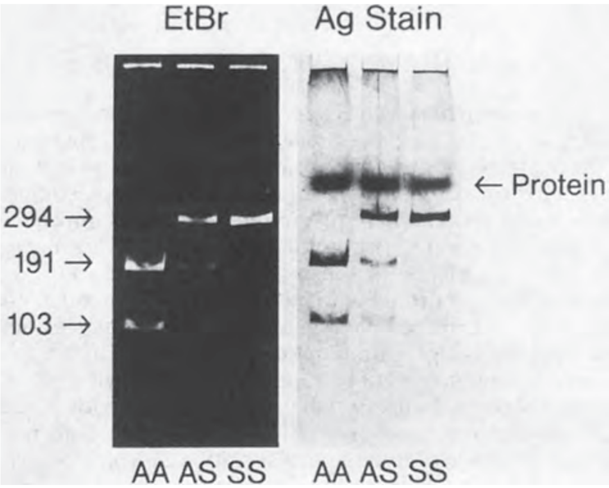


Figure 43.12 POLYMERASE CHAIN REACTION (PCR)–BASED RESTRICTION ANALYSIS FOR THE SICKLE CELL GENE. The genotypes of the DNA samples tested are shown *below*. The size in base pairs for the undigested PCR product and the products resulting from Oxa Nl are shown at the *left* in base pairs. The fragments from normal β-globin DNA (AA) shows complete Oxa Nl cleavage, from sickle cell trait DNA (AS) shows partial cleavage, and from sickle cell anemia (SS) shows no cleavage. *Ag stain*, Silver stain; *EtBr*, ethidium bromide.

include a CBC and hemoglobin electrophoresis.[313] One large survey found that parents at risk for having a child with SCD were interested in prenatal diagnosis and would consider termination of pregnancy for an affected fetus.[314] Community acceptance of reproductive genetic services depends on the effectiveness of education and counseling but currently the ability to predict severity of disease is very limited.

Fetal DNA samples are obtained by chorionic villus sampling at 8 to 10 weeks' gestation. Polymerase chain reaction (PCR)–based methods for detecting the sickle gene include restriction analysis (Fig. 43.12), allele-specific hybridization, reverse dot blotting, and allele-specific fluorescence PCR. PCR-based diagnosis for HbSC disease is possible using specific molecular methods for detecting the HbC gene, and the diagnosis of HbSβ-thalassemia can be made using reverse dot-blot methodology to screen the many African American β-thalassemia mutations, as well as the HbS and HbC mutations, in a single hybridization reaction. Given current DNA technology, it is far more efficient to directly sequence the beta globin genes. Currently, fetal DNA is obtained from amniocentesis or chorionic villus sampling; although these techniques increase the risk of fetal miscarriage, a meta-analysis concluded that the procedure-related risks are much lower than quoted and less than 0.5%.[315] Non-invasive prenatal diagnosis avoids this risk using cell-free fetal DNA in maternal plasma but the accuracy of this technique is not at a clinically applicable stage. Some couples want to avoid having a fetus with SCD but find PND and selective termination unacceptable. Preimplantation genetic diagnosis involves the use of in vitro fertilization techniques to generate 5 to 15 embryos; at the eight-cell stage, one embryonic cell can be removed and tested for sickle alleles; it is then possible to implant only embryos without SCD. It is currently, however, a difficult, stressful, and expensive procedure.

Reproductive Health

Reproductive healthcare should be provided to all adolescent and adult females with SCD. All women should undergo age-appropriate gynecologic and cancer screening on an annual or semi-annual basis, including breast and pelvic examination with pap smear testing. Family planning should be discussed with patients who desire to conceive children with early referral to reproductive endocrinology given the high incidence of infertility. HU and chronic transfusion regimens may support fertility by reducing sickled erythrocytes and resultant organ dysfunction.[313]

CASE 3: VENOUS THROMBOEMBOLISM IN SICKLE CELL DISEASE

A 28-year-old female with HbSS disease and previous history of unprovoked bilateral pulmonary emboli (PE) presented with the acute complaint of worsening dyspnea after recent plane travel. At the time of the initial PE event several years prior, evaluation with compression ultrasonography of the lower extremities was negative for deep venous thrombosis (DVT). Transthoracic echocardiogram demonstrated a borderline TRJV of 2.8 m/s without evidence of right heart strain physiology. After the standard period of anti coagulation, the patient discontinued chronic anticoagulation with warfarin due to inconvenience and cost of INR laboratory monitoring. She also desired contraception and was placed on an oral hormonal contraceptive containing estrogen. On evaluation, intake vitals including oxygen saturation on room air were within normal limits. Cardiopulmonary auscultation was unrevealing. Physical assessment of the lower extremities did not demonstrate stigmata of acute DVT.

Question 1: What is your provisional diagnosis and what tests would you perform?

Exclude pulmonary embolism and/or DVT given the history of recent plane travel. A CTPA was performed which revealed a subsegmental filling defect in the right upper lobe. Lower extremity duplex ultrasonography was negative for DVT.

Question 2: What is the next step in evaluation and management?

Given the patient's prior history of venous thromboembolism (VTE), it was recommended to resume therapeutic anticoagulation on an indefinite basis. Initiate DOAC therapy and counsel patient on the risks of estrogen-containing contraceptives in relation to VTE.

She discontinued her oral birth control pill and was advised to consider alternative birth control methods in the future, including an intrauterine device (IUD).

VTE is a frequent and important clinical complication encountered in adults with SCD. SCD is a known hypercoagulable state associated with both coagulation and platelet function abnormalities. The diagnostic value of D-dimer screening for VTE in SCD is limited given baseline elevations secondary to chronic inflammation. Other screening tools, including the modified Geneva score, are of limited clinical utility in SCD. Heparin, vitamin K antagonists, and DOACs are all effective agents in the treatment of VTE in SCD patients. The optimal treatment duration of confirmed pulmonary thrombosis in SCD is not known, but the ongoing thrombotic risk and high rate of recurrence has led to the practice of lifelong anticoagulation after a first-time PE in SCD patients without contraindications or increased bleeding risk. Recurrent PE is a significant risk factor for chronic thromboembolic pulmonary hypertension (CTEPH).

In patients who desire contraceptive methods to prevent pregnancy, non-estrogen-containing intrauterine devices should be considered. Although depot injections of medroxyprogesterone (Depo-Provera) given every 3 months may be safe with regard to stroke risk,[316] there is a risk of bone loss, a consideration in patients with SCD given their propensity to skeletal complications. Oral contraceptives containing low doses of estrogen can be considered with no clear evidence of increased stroke demonstrated to date, although the patients' overall stroke risk should be taken into consideration. Another caution with low-dose estrogen oral contraception is the risk of contraceptive failure with less than excellent compliance. There may be risks to contraception, but this must be weighed against the risks of unintended pregnancy (see Case 3).

Renal Complications

Renal dysfunction attributed to SCD is generally referred to as sickle cell nephropathy and is a diagnosis of exclusion. CKD occurs in as many as one-fourth to one-third of adults with SCD.[317] The development can be insidious and a screening program incorporating blood pressure monitoring, urinalysis with examination of the urine sediment, spot urine albumin-to-creatinine ratio performed annually, and metabolic panels performed at routine intervals should be instituted by no later than 10 years of age.[318,319] Renal imaging with ultrasound should be performed in patients with a significant unexplained decline in renal function, hematuria, or symptoms suggestive of urinary obstruction. Patients receiving iron chelation may require more frequent monitoring of renal function as deferasirox and deferoxamine are associated with nephrotoxicity.

Hypertension, proteinuria, hematuria, worsening anemia, and nephrotic syndrome reliably predict progression to renal failure, which are clinical indices to pay attention to because the serum creatinine may be misleading. In patients with SCA, serum creatinine is a poor measure of renal function due to an increased proximal tubular secretion of creatinine, and if available, Cystatin C should be used as it is freely filtered by the glomerulus, and not secreted by the renal tubules. Thus, patients may have a significant decline in renal function before it is detectable by measuring creatinine clearance.[320] The mean age at presentation with end-stage renal disease is 41 years.[321]

In infants and young children, there is increased glomerular pressure with compensatory renal hypertrophy, glomerular hyperfiltration, and impaired urinary concentrating ability.[322] Glomerular hyperfiltration is defined as an estimated glomerular filtration rate (eGFR) above gender-specific thresholds (e.g., >130 mL/min/1.73 m^2 for females and >140 mL/min/ 1.73 m^2 for males). These changes often persist into young adulthood but glomerular hyperfiltration is subsequently followed by a decline in GFR over the ensuing years. Serum creatinine levels are low in all genotypes until age 18 years, when young men experience a rise, apparently related to increasing muscle mass. Creatinine levels increase with age in all genotypes, presumably because of declining renal function. Cystatin-based methods for estimating GFR may be more accurate when assessing renal function although such an approach has yet to be validated for routine testing.

Other risk factors for the development of chronic renal failure include the presence of albuminuria, low HbF, and chronic NSAID treatment.[323] The severity of hemolysis impacts renal impairment as measured by proteinuria or eGFR.[324] Thus, it is not surprising that co-inheritance of alpha-thalassemia protects against albuminuria.[325] Variants in the *APOL1* gene, which encodes apolipoprotein L1, have also been associated with renal disease in individuals with SCD.[326]

Episodes of acute renal failure requiring intermittent HD may occur in the setting of vigorous hemolysis, hypovolemia, sepsis, hepatorenal syndrome, cardiac failure, renal vein thrombosis, and rhabdomyolysis. These patients typically recover renal function although cumulative insults result in progressive renal insufficiency. In general, nephrotoxic medications such as NSAIDs, aminoglycoside antibiotics, and radiocontrast used for contrast-enhanced imaging exams should be administered judiciously. Patients should be counseled to avoid chronic use of NSAIDs in over-the-counter medications for pain.

It is important to remember that the SCD population at risk of developing sickle nephropathy (SCN) is also at risk of other diseases that affect the kidneys such as lupus nephritis, blood-borne viruses, and various forms of glomerulonephritis, and that there is no pathognomonic sickle renal phenotype. Not only may these diseases be managed differently to SCN, but they may also influence the prognosis of SCN when they co-exist, and thus need to be excluded. Nephropathies that affect patients with disease include nephrotic syndrome, focal segmental glomerulonephritis (FSGS), and SLE glomerulonephritis which disproportionally affect those of African American ancestry, all of which contributes to end stage renal failure. Once eGFR falls below 40 mL/min/1.73 m^2, the subsequent rate of decline can be rapid, and patients should be offered peritoneal or HD and renal transplantation if eligible.[318]

The renal medulla with its unique hyperosmolar and acidotic environment, together with the low oxygen tension and low-flow state in medullary vasa recta, create optimal conditions for HbS polymerization. Individuals with sickle cell trait have a defect in concentrating urine (hyposthenuria); other renal manifestations encountered in sickle cell trait individuals include albuminuria and hematuria.[327]

Renal Endocrine (Erythropoietin) Deficiency

This is discussed under Basic Management and Disease Modification.

Gross Hematuria

Hematuria may result from microthrombi formation in the peritubular capillaries of the renal medulla or from frank papillary necrosis. Episodes of hematuria occur in approximately 6% of children with SCD and in individuals with sickle cell trait.[328] For gross hematuria, diagnostic evaluation includes urinalysis, culture, and renal ultrasound to evaluate for papillary necrosis, renal stones, and other renal pathology. A much rarer cause of hematuria is renal medullary carcinoma (RMC), an aggressive malignancy found almost exclusively in young Black patients with sickle cell trait.[329] Significant hematuria may resolve with high urinary flow through oral hydration and bed rest. Hematuria that lasts longer than 1 to 2 weeks and results in need for transfusion may require maintenance of high urinary flow using a combination of hypotonic fluids, loop diuretics, and urinary alkalinization using sodium bicarbonate and acetazolamide. These therapies are aimed at changing the acidic, hypertonic environment of the renal medulla that favors erythrocyte dehydration, increased HbS concentrations, and HbS polymerization. If bleeding persists for 72 hours despite these measures, then alternative treatment should be considered. These may include oral urea,[330] ε-aminocaproic acid, and vasopressin. Embolization or nephrectomy should be reserved for prolonged, life-threatening cases of hematuria that require multiple transfusions.

Increased hematuria can also be seen as a consequence of DHTR (discussed in Exacerbations of Anemia).

Papillary Necrosis

Papillary necrosis is most often detected incidentally by imaging or microscopic examination of urine in asymptomatic patients. Renal ultrasound typically demonstrates increased echogenicity of the inner medulla and, in more advanced cases, a filling defect in the area of the medullary tip.[328] CT of the abdomen may be pursued for definitive diagnosis. Sloughed papilla may cause ureteral obstruction and urinary tract infection. In addition to broad-spectrum antibiotics, this occurrence requires emergent relief of the obstruction with a retrograde ureteral stent or placement of a percutaneous nephrostomy tube. NSAIDs should be avoided in patients with papillary necrosis. Otherwise, treatment is as for hematuria.

Proteinuria

Proteinuria can be detected in some patients from late childhood; it is much more prevalent in patients with SCA (HbSS and HbSβ^0-thalassemia), it increases with age and in one cohort, proteinuria was identified in greater than 60% of SCA patients over 45 years of age.[324] Severity of hemolysis correlates with proteinuria, hence co-inheritance of alpha-thalassemia which is associated with reduced hemolysis protects against albuminuria.[324,325] Proteinuria can progress to nephrotic syndrome when proteinuria is accompanied by hypoalbuminemia, edema, and hyperlipidemia. Treatment in adults should be initiated with an ACE inhibitor or ARB when there is persistent urine protein ≥300 mg/day and when the urine protein to creatinine ratio (uPCR) is 50 to 100 mg/mmol (>884 mg/g), which is equivalent to urine protein ≥1000 mg/day. Close monitoring of the blood pressure is essential to avoid hypotension.[318] ACE inhibitors or angiotensin receptor blockers (ARBs) slow the progression of proteinuria in sickle proteinuria, and there is increasing evidence that ACE inhibitors and ARBs may slow or halt the progression of proteinuria to nephrotic syndrome and renal failure.[331,332] Angiotensin II inhibitors are being studied for a potential role in decreasing sickle cell–related proteinuria and renal function deterioration.

Hyposthenuria and Other Abnormalities of Tubular Function

The inability to maximally concentrate urine (hyposthenuria) in response to water deprivation is an early finding of sickle cell

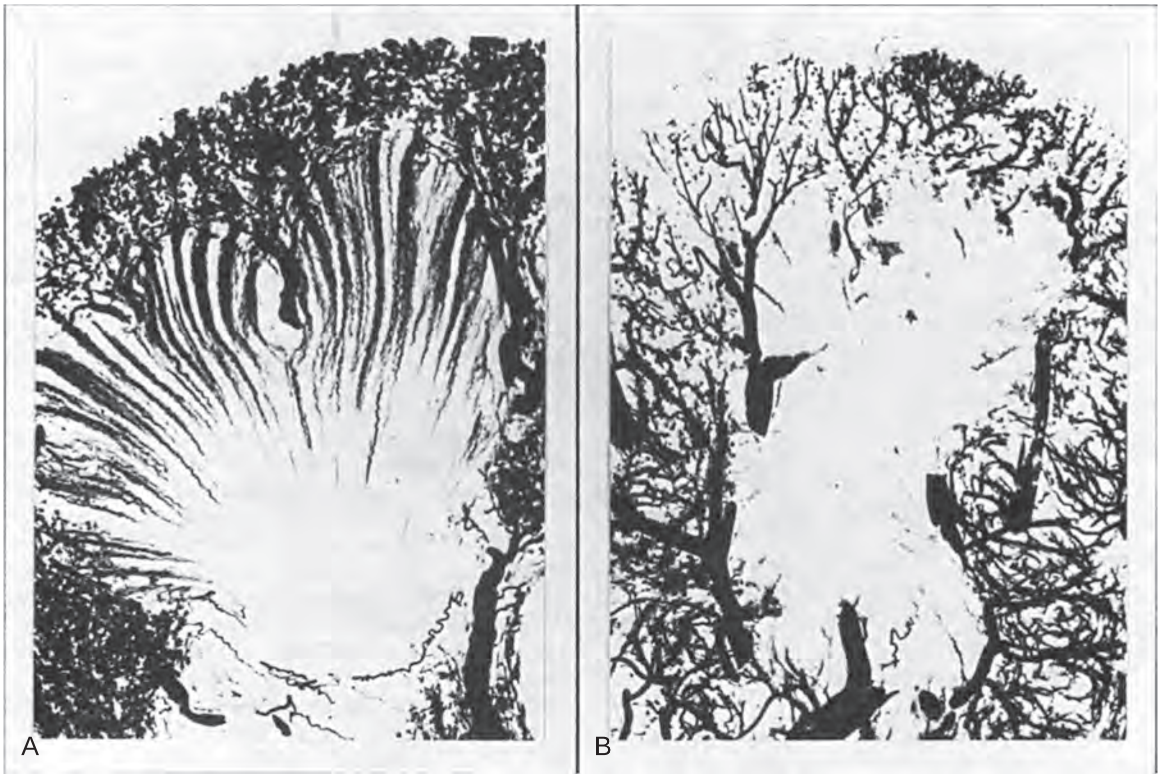


Figure 43.13 Postmortem microangiographic studies of the vasa recta in a normal individual (A) and a patient with sickle cell anemia (B). *(Reproduced with permission from Elsevier. From Statius van Eps LW, Pinedo-Veels C, de Vries GH, de Koning J. Nature of concentrating defect in sickle-cell nephropathy. Microradioangiographic studies. Lancet. 1970;1:450–452.)*

nephropathy. Both sickle cell trait and SCD patients may be affected. Hyposthenuria is the cumulative result of recurrent microinfarcts in the vasa recta caused by sickling (Fig. 43.13). When water deprived, these patients cannot maximally concentrate their urine and develop hypovolemia and dehydration.

Other abnormalities of renal tubular dysfunction found in SCA include an incomplete form of distal renal tubular acidosis with hyperchloremic metabolic acidosis and hyperkalemia.[333] The hyperkalemia may respond to oral sodium bicarbonate.

Enuresis

Enuresis, or nighttime involuntary bedwetting, is common in pediatric SCD patients and may persist into young adulthood. Hyposthenuria, sleep-disordered breathing, and psychological stress may contribute. The evaluation and treatment of this disorder is similar to that of non-SCD patients; behavioral techniques including bed alarms may be helpful is some cases.

Urinary Tract Infections

Urinary tract infections and pyelonephritis are discussed under infectious complications.

Renal Medullary Carcinoma

Sickle cell trait has been reported to be associated with RMC, which is a rare, highly aggressive non-clear-cell renal cancer.[334] It is almost twice as common in men compared to women and the onset is typically in early adulthood.[329] The clinical presentation may include gross hematuria, abdominal or flank pain, or significant weight loss. The disease may be metastatic at diagnosis, and the overall prognosis is poor. There is a weak association of RMC with HbSC disease but no identified association with SCA. The reason for these patterns of disease remain unknown.

Priapism

Priapism is a condition that is characterized by a sustained erection that does not result from sexual desire and is not relieved by sexual activity.[335] Stuttering priapism is a separate entity that is characterized by multiple, brief episodes of sustained unwanted erection. It has been reported to affect 6.4% to 42% of boys and men with SCD[336] and can also occur in sickle cell trait. Its peak frequencies are between ages 5 and 13 years and 21 and 29 years. Priapism is most likely to develop in patients with lower HbF levels and reticulocyte counts, increased platelet counts, and the HbSS genotype. Priapism caused by SCD is usually ischemic, or low-flow, priapism. High-flow priapism is caused by unregulated arterial flow and can be distinguished from low-flow priapism by a blood gas obtained from the corpora. Most likely, priapism begins with a physiologic erection. The relative stasis of blood within the corpora leads to a decrease in oxygen tension and development of acidosis, predisposing to HbS polymerization in the corporal sinusoids, venous occlusion, and low-flow priapism. Pain develops as the corpora become increasingly ischemic after approximately 4 hours of erection. In a minority of patients, usually postpubertal, the engorgement also affects the corpus spongiosum and glans. The mild acidosis that accompanies hypoventilation during sleep may also contribute to the pathophysiology. Impotence is the primary complication of priapism, although some patients with a history of multiple episodes of priapism and significant corporeal fibrosis may report adequate erections and maintain active sex lives. Corporeal ischemia during priapism may induce local inflammation, causing the fibrosis responsible for impotence.

The goal of treatment is to relieve priapism and maintain potency. Patients should be educated to seek medical attention for unrelenting erection of more than 2 hours' duration. Detumescence within 12 hours is optimal to retain potency. After 72 hours, impotence is more likely. The strategy is prompt initiation of supportive medical therapy with intravenous hydration and analgesia and involvement by a urology consultant if the priapism persists for more than 4 hours; aspiration of blood from the corpora with or without irrigation, and injection of an α-adrenergic agonist should be considered (Guidelines of the American Urological Association, https://www.auanet.org/education/guidelines/priapism.cfm). If priapism persists 12 hours, options include partial-exchange transfusion to reduce the HbS level to less than 30% with a total Hb of less than 10 g/dL or irrigation as outlined earlier. The latter procedure is less effective after 36 hours of priapism. Irrigation should be performed using penile anesthesia (dorsal nerve block; circumferential penile block; or subcutaneous, local, penile shaft block). All irrigation in boys should be performed under conscious sedation. There is anecdotal evidence for the use of hydralazine to treat acute priapism.[337]

Preventing recurrent priapism is an important component of management. Strategies include HU administration; chronic transfusion; the antiandrogen bicalutamide[335,338]; self-administration of the α-adrenergic agent etilefrine orally and for episodes lasting over 1 hour, by intracavernous injection; and monthly administration of intramuscular gonadotropin-releasing hormone analogue.[339,340] Prophylactic pseudoephedrine (Sudafed) appears beneficial in preventing recurrent mild episodes. Nocturnal desaturation due to sleep disorder breathing can contribute to priapism, and patients with a history of priapism should undergo an overnight sleep study and be managed accordingly.

Surgical creation of shunts is reserved for severe cases resistant to the above interventions. As many as 45% of patients who have priapism develop some degree of impotence.[341] When impotence persists for 12 months, a semirigid penile prosthesis may be implanted.

Ocular Complications[342]

The retina is particularly vulnerable to vaso-occlusion, and annual retinal examination is part of routine health care maintenance for patients with SCD. Superficial retinal hemorrhages have a pink "salmon patch" appearance. Deeper retinal hemorrhages have a "black sunburst" appearance. Other manifestations of sickle cell retinopathy include iridescent spots, retinal neovascularization, and retinal detachment. More subtle signs of sickle cell retinopathy are optic nerve head vascular changes, vascular tortuosity, macular changes (e.g., microaneurysms and vascular loops), and peripheral arteriovenous anastomoses. Other ophthalmologic complications are anterior chamber ischemia, tortuosity of conjunctival vessels, retinal artery occlusion, and angioid streaks. Sickle cell retinopathy is best seen by fluorescein angiography (Fig. 43.14). The earlier onset and greater frequency of proliferative retinopathy in HbSC disease and HbSβ^+-thalassemia compared with HbSS and HbSβ^0-thalassemia suggest that retinal vessels are more susceptible to occlusion by more viscous blood than by more rigid individual cells. Peripheral sickle retinopathy may require vision-saving therapy with laser photocoagulation. Orbital compression syndrome caused by vaso-occlusion of the periorbital marrow space and subperiosteal hemorrhage has been observed to result in headache, fever, and palpebral edema. In this situation, culture, CT scan, and MRI should be used to rule out infectious, neoplastic, and other hemorrhagic etiologies. Conservative therapy, including local measures, analgesia, fluids, transfusion, and careful ophthalmologic surveillance, is recommended unless compression of the optic nerve ensues, in which case surgical decompression should be considered.

Bone Complications

Chronic tower skull, bossing of the forehead, and fish-mouth deformity of the vertebrae are the result of extended hematopoietic bone marrow, causing widening of the medullary space, thinning of the trabeculae and cortices, and osteoporosis. Osteonecrosis may cause a steplike depression of the vertebrae, selected shortening of the cuboidal bones of the hands and feet, and acute aseptic or avascular necrosis. The excruciating pain of bone infarction in the "hand–foot syndrome" that occurs around 2 years of age is often the first symptom of SCD (Fig. 43.15).[343] This dactylitis resolves spontaneously and is treated with hydration and analgesia. Bone infarcts are demonstrable using nuclear medicine scintigraphy or MRI. Serial scans specific for bone osteoclasts, bone marrow macrophages, and inflammatory cells may be useful adjuncts for distinguishing bone marrow infarction from osteomyelitis, but it is essential to obtain cultures directly from the affected tissue before starting antibiotics. Treatment of osteomyelitis is addressed in the Infection sections.

Osteonecrosis (avascular necrosis) is a prevalent skeletal complication attributed to repetitive ischemia-reperfusion injury in vulnerable articular surfaces. Recurrent painful VOC episodes result in compromised blood flow to all joints and increase the risk for multifocal osteonecrosis. Bone necrosis occurs with equal frequency in the femoral and humeral heads, but the femoral heads more commonly undergo progressive joint destruction as a result of lack of collateral blood flow and chronic weight bearing. Approximately 50% of adult patients with HbSS disease will develop osteonecrosis of the femoral heads by the age of 35.[344] Risk factors include male gender, high body mass index, leukopenia, concomitant alpha thalassemia trait with higher Hb, frequent VOC, and multiple episodes of ACS.[345] All children and adults with intermittent hip pain should undergo screening with plain films and/or MRI as per 2014 evidence-based NHLBI guidelines. The process is associated with increased intraosseous pressure and is most sensitively detected by MRI. Aggressive physical therapy appears to prevent progression in mild cases and should be considered in the therapy of avascular necrosis. Core decompression surgery to relieve increased intraosseous pressure can be used in early-stage osteonecrosis (i.e., no radiographic evidence of bone collapse) to prevent disease progression. In more advanced disease, joint replacement should be considered. There is a 30% likelihood that a

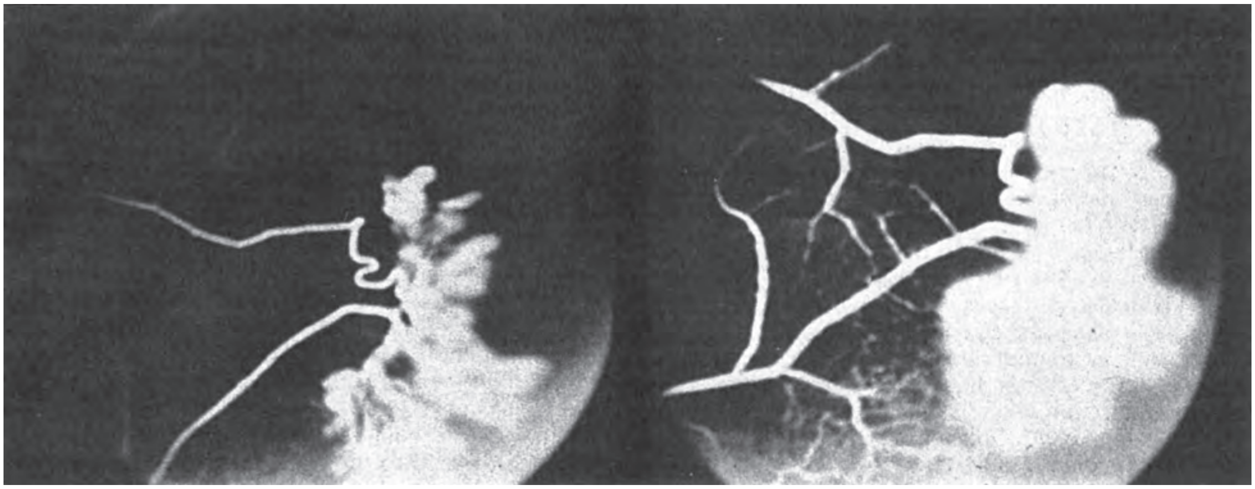


Figure 43.14 FLUOROSCEIN ANGIOGRAPHY DEMONSTRATING A "SEA FAN" APPEARANCE OF SICKLE PROLIFERATIVE RETINOPATHY. *(Courtesy W. C. Mentzer.)*

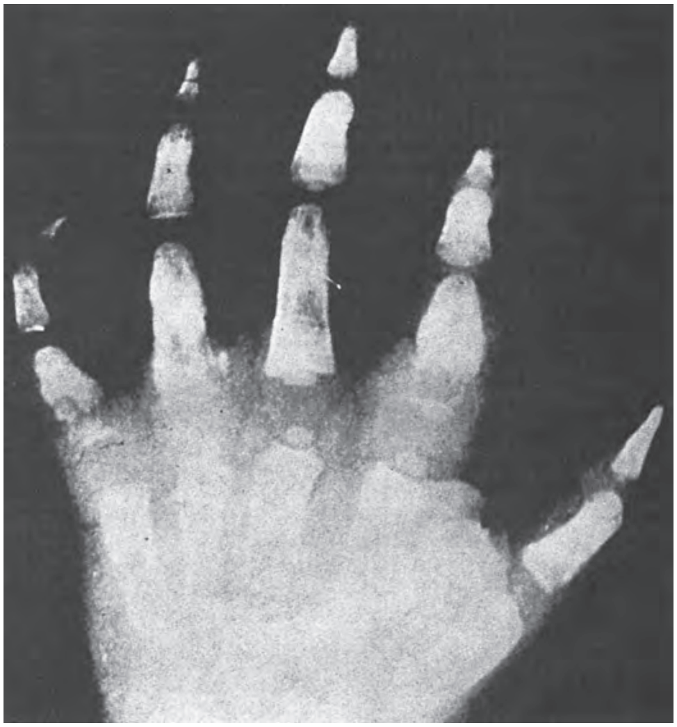


Figure 43.15 RADIOGRAM SHOWING THE BONE INFARCTIONS IN THE HANDS OF A CHILD WITH THE "HAND–FOOT SYNDROME" DACTYLITIS. *(Courtesy W. C. Mentzer.)*

second hip revision will be required within 4 to 5 years of prosthetic hip placement in patients with SCD.[346] However, the outlook for total hip replacement in SCD has greatly improved with cementless ceramic prosthetic devices and joint management by an experienced surgeon and hematologist, prompting earlier intervention.[347,348]

Arthritic pain, swelling, and effusion may be related to periarticular infarction or gouty arthritis.

Bone marrow infarction causes reticulocytopenia, exacerbation of anemia, a leukoerythroblastic picture, and sometimes pancytopenia.[214,215] Pulmonary fat embolism is a rare complication of bone marrow infarction.[349] It is associated with fat globules in the sputum and refractile bodies visible in the optic fundi. It is a life-threatening event that may require prompt exchange transfusion and perhaps the use of heparin and corticosteroids.[349]

Dermatologic Complications

Leg ulcers are major causes of morbidity in SCD as a result of their frequency, chronicity, and resistance to therapy. Most occur near the medial or lateral malleolus (Fig. 43.16), may be associated with venous hypertension[350] and hemolytic rate,[351] and are frequently bilateral.[352] They may begin spontaneously or as a result of trauma and may become infected, most commonly by *S. aureus, Pseudomonas* spp., streptococci, or *Bacteroides* spp. Systemic infection, osteomyelitis, and tetanus are rare complications. Ulcers are resistant to healing and tend to be recurrent in well over half of cases. Their incidence has been reported to vary from 25% to 75%.[353] Ulcers rarely occur in patients younger than age 10 years and are most common in SCA (HbSS and HbSβ^0-thalassemia), and almost nonexistent in HbSC disease and HbSβ^+-thalassemia.[352] The incidence in SCA patients declines substantially in those who have coexistent α-thalassemia.[354] Low steady-state Hb levels and low HbF levels are associated with an increased risk of leg ulceration.[354] Males have a threefold greater risk for developing leg ulcers than females. Treatment of leg ulcers requires persistence and patience; healing usually takes weeks. Therapy[355] begins with gentle debridement to remove nonviable, superficial tissue from more vital areas. Wet-to-dry dressings and DuoDerm hydrocolloid dressings facilitate devitalization. When debridement is complete, zinc oxide–impregnated Unna boots are used to promote healing. Bed rest speeds healing,[356] and topical antibiotics may be required. It may be necessary to use elastic wraps or leg elevation to control edema. Rapid healing of leg ulcers has been reported in patients treated with intravenous arginine butyrate.[357] Oral zinc, local hyperbaric oxygen, chronic transfusion, recombinant EPO, propionyl-L-carnitine, skin grafts, pentoxifylline, and becaplermin (platelet-derived growth factor) may have therapeutic roles and should be considered in individual cases but have not been formally tested for their effectiveness in accelerating resolution of sickle cell–associated leg ulcers.

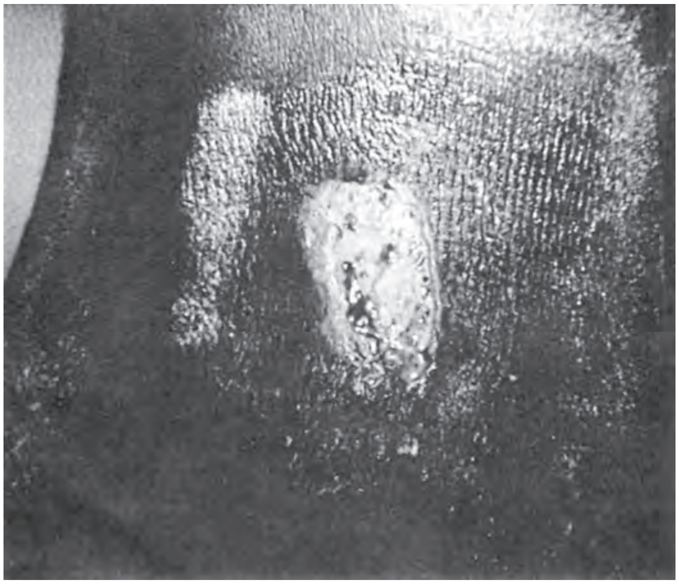


Figure 43.16 CHRONIC LEG ULCER NEAR THE MEDIAL MALLEOLUS. *(Courtesy W. C. Mentzer.)*

Myofascial syndromes consist of soft tissue swelling in subcutaneous edema that may have a *peau d'orange* appearance. These may be large or discrete lesions a few centimeters in diameter. These lesions are probably the result of dermal or subdermal vaso-occlusion. Treatment is symptomatic.

Cardiac Complications

The chronic anemia of SCD is compensated by high cardiac output, which results in chronic chamber enlargement and cardiomegaly, and mild to moderate mitral and tricuspid regurgitation even in young children. The electrocardiogram shows evidence of left ventricular hypertrophy and less often first-degree block and nonspecific ST-T wave changes. Left ventricular dilation correlates with age and inversely correlates with total Hb.[358] An age-dependent loss of cardiac reserve may predispose to heart failure in adult patients stressed by fluid overload, transfusion, exacerbation of anemia, hypoxia, or hypertension.[359] Cardiac function can be improved by chronic transfusion regimens.[360] Acute myocardial infarction in the absence of coronary disease has been reported, and in one autopsy series, 9.7% of 72 consecutive patients with SCD had myocardial infarction.[361] It appears that myocardial infarction may occur with normal coronary arteries as a result of increased oxygen demand exceeding limited oxygen-carrying capacity or as a result of microcirculatory impairment. As discussed earlier, PH is a relatively common finding in patients with SCD and can be associated with right ventricular hypertrophy. It has been suggested that the increased rate of sudden death observed in SCD may be related to cardiac autonomic dysfunction, as detected by abnormal heart rate variability in response to selected postural maneuvers.[362]

Multiorgan Failure

This disastrous acute event involves multiple organ systems, including the lungs, brain, kidneys, liver, hematologic system, and heart, and usually leads to death.[363] It may be precipitated by infection, vaso-occlusion, or fat embolus and consists of a constellation of life-threatening processes, including hypoxemia, acidosis, inflammation, vascular permeability, severe anemia, disseminated intravascular coagulation, renal failure, and hepatic failure. In addition to therapy specific for these processes, exchange transfusion, plasma infusion or exchange, and corticosteroids should be considered.

Psychosocial Issues

Modern insights into the psychosocial adjustment of patients with SCD have provided a level of understanding that allows interventional therapy. Although most patients with SCD are generally well adjusted,[364] there are risks of depression, low self-esteem, social isolation, poor family relationships, and withdrawal from normal daily living.[364] Particular stressors are recurrent and unpredictable pain and the response to it, curtailed activity because of pain, misinterpretation of the meaning of pain, and depression leading to learned helplessness. Although some patients with SCD become addicted to narcotics, this is uncommon and usually is the result of social influences rather than pain therapy. Active coping strategies, family support, and support from the extended family unit are paramount. Interventional approaches should emphasize recognizing and reinforcing individual strengths; confronting pathologic behavior; and establishing coping skills through reinterpreting pain, diverting attention from pain, and using support systems.[365] Attention to psychosocial concerns is vital to the psychosocial well-being of patients with SCD (see Chapter 98).

Growth and Development

By age 2 years, children with SCD have detectable growth retardation that affects weight more than height and has no clear gender difference.[366] By adulthood, normal height is achieved, but weight remains lower than that of control participants. More severe growth delay is noted in children with HbSS and HbSβ^0-thalassemia; HbSC disease is associated with a less severe growth delay. Girls with SCD have retarded sexual maturation that is greater in those with HbSS and HbSβ^0-thalassemia than those with HbSC disease and HbSβ^+-thalassemia[366]; it is associated with elevated gonadotropin levels for the stage of sexual development and delayed menarche. Boys also have delayed sexual maturation, which is more severe in those with HbSS than those with HbSC disease.[366] Retarded sexual maturation in boys can be attributable to primary hypogonadism, hypopituitarism, or hypothalamic insufficiency. The etiology of these multiple endocrine deficiencies may relate to underlying sickle cell pathophysiology or iron overload and emphasizes the importance of a comprehensive basic management and disease modification approach as outlined earlier in this chapter. Improved growth has been reported with HU,[367] transfusion,[367] folic acid supplementation, zinc supplementation,[368] and nutritional supplementation.[369] When children have both SCD and hypersplenism, splenectomy may result in improved protein turnover, metabolic rate, and growth parameters.[370] Although most SCD patients worldwide have low BMI, in the better-resourced countries obesity is increasing among SCD patients, and was noted to be as high as 50% in a small US cohort.[371] Obesity is correlated with hypertension, sleep apnea, and cardiovascular complications in SCD patients and has been proposed as the "sixth vital sign."[372]

Sickle Cell Trait

The prevalence of sickle cell trait is approximately 8% to 10% in African Americans and as high as 25% to 30% in certain areas of western Africa.[16] An estimated 2.5 million people in the United States and 300 million in the world (~5% of the world's population) are heterozygous for the sickle cell gene.[373] Sickle cell trait is largely a benign carrier condition without obvious laboratory hematologic manifestations under basal conditions: RBC morphology, RBC indices, and the reticulocyte count are normal, and ISCs are not seen on the peripheral blood smear. The usual partition of HbA and HbS in sickle cell trait is 60:40 owing to a greater posttranslational affinity of α chains for β^A than for β^S chains.[23] When α-thalassemia is coinherited with sickle cell trait, the preferential affinity results in a decreased percentage of HbS relative to the number of α-globin genes deleted (i.e., $\alpha\alpha/\alpha\alpha$ 40% HbS; $-\alpha/\alpha\alpha$ 35% HbS; $-\alpha/-\alpha$ 29% HbS; $--/-\alpha$ 21% HbS).[210]

Life expectancy is not reduced by heterozygosity for the sickle cell mutation.[374] There are several clinical complications associated with sickle cell trait. Splenic infarction can occur at high altitudes[375] and presenting symptoms may include left upper quadrant pain and elevated bilirubin levels. Therapy is primarily supportive including changing altitude although splenectomy may be required in a small percentage of cases. Rare cases of splenic sequestration reported in the literature have been triggered by dehydration, rigorous physical exercise, high altitudes, or increased pressure associated with scuba diving. People with sickle cell trait also may develop hematuria and hyposthenuria which are generally attributed to renal papillary infarcts.[376] There is preliminary evidence suggesting that individuals with sickle cell trait are at greater risk for developing proteinuria and CKD, but further research is required to clarify these findings.[377] The frequency of urinary tract infection may be increased. There is an association with RMC and individuals with unexplained hematuria should undergo prompt evaluation with CT imaging.[334] There is an increased risk for venous thrombosis with an approximately twofold increase in risk and sickle trait explaining 7% of thrombotic episodes in African Americans.[377] Armed forces recruits in basic training with the sickle cell trait have a substantially increased, age-dependent risk of exercise-related sudden death.[327]

Despite the known complications, past experiences with discrimination in the employment market and health insurance industry provide reminders that the rare clinical events in sickle cell trait provide no real justification for regarding it as anything but a benign carrier condition.[317] Newborn screening programs detect a large number of infants with sickle cell trait; for these parents, genetic counseling is essential. Parents should understand that their child has a hereditary condition with some risks as above and that there is a risk for subsequent offspring to be born with SCD.

In individuals who appear to have sickle cell trait but are symptomatic, the laboratory diagnosis must be verified, and detailed family history is very important.[317] Heterozygotes for double mutants, HbS Antilles[378] and Hb Jamaica Plain[379] can present with symptoms of moderate to severe SCD. Compound heterozygotes of HbS with Hb Arlington Park and HbS with Hb Quebec-CHORI produce a severe sickling phenotype. In both cases, since the variant Hbs migrate like HbA on electrophoresis, compound heterozygosity with HbS can be mistaken for sickle cell trait on electrophoresis. In other cases, co-inheritance of non-hemoglobin red cell disorders (e.g., Red cell membrane and enzyme) can have important modifying effects on the clinical outcome of sickle cell carriers. An example is the concomitant inheritance of pyruvate kinase deficiency, which lowers hemoglobin oxygen affinity due to an accumulation of the upstream glycolytic intermediate, 2,3-DPG, thus increasing HbS polymerization.[380,381]

HbSC Disease

The gene for HbC ($\alpha_2\beta_2{}^6$Glu→Lys) is approximately one-fourth as frequent among African Americans as the sickle cell gene.[16] Hemoglobin analysis demonstrates approximately equal amounts of HbS and HbC (or slightly more HbS than HbC) and no HbA is present. Although oxygenated HbC forms crystals, HbC does not participate in polymerization with deoxy-HbS. However, HbC sustains potassium chloride cotransport and RBC dehydration, raising the intraerythrocytic concentration of HbS to levels that support polymerization, sickling, and clinical symptoms. As a result of a longer circulatory survival of HbSC RBCs compared with HbSS cells (i.e., 27 vs. 17 days),[208] the degree of anemia and reticulocytosis is frequently mild: 75% of the patients have a milder level of anemia (hematocrit level >28%) than is usually seen in SCA. The predominant RBC abnormality on the peripheral smear is an abundance of target cells; folded ("pita bread") cells, ISCs, "billiard ball" cells, and crystal-containing cells may also be seen.

Splenomegaly may be the only physical finding, and the frequency of acute painful episodes is approximately half that in HbSS disease, with a life expectancy two decades longer.[40] HU therapy may be beneficial in selected patients with frequent VOC episodes and appears safe. Patients are at risk for hyperviscosity syndrome secondary to increased Hb levels, and may benefit from therapeutic phlebotomy.[382] Nonetheless, significant morbidity can occur. The incidence of fatal bacterial infection is less than in SCA, but there is still an increased risk of *S. pneumoniae* and *H. influenzae* infection. Osteonecrosis occurs in approximately 15% of patients.[383] There is a higher incidence of peripheral retinopathy in HbSC disease, in some cohorts as high as 70%,[383] than in SCA. Coexistent α-thalassemia reduces risk of chronic organ complications.[384] There is an association between RMC and HbSC disease.

Sickle Cell–β-Thalassemia

Beta-thalassemia results in impaired production or absence of beta-globin chains. In compound heterozygotes of HbS with β-thalassemia, HbS comprises the majority of the total hemoglobin with some (HbS/β^+-thalassemia), or no HbA (HbSβ^0-thalassemia), and the outcome is dependent of the severity of the beta-thalassemia mutation. The gene frequency of β-thalassemia among African Americans is 0.004, one-tenth that of the sickle cell gene,[16] and hence there is one-tenth the prevalence of compound heterozygous sickle cell–β-thalassemia in this population. Individuals of Hispanic ethnicity are also at risk for sickle beta-thalassemia. HbS–β-thalassemia is divided into HbSβ^+-thalassemia and HbSβ^0-thalassemia, which have, respectively, reduced or no amounts of Hb A present. Most β-thalassemia mutations among African Americans result in mild β^+-thalassemia; 80% of African American β-thalassemia mutations are attributable to the promoter region mutations (−88 [C to T] and −29[A to G]) that result in a minor deficit of HbA production, ranging from 18% to 25%. Compound heterozygous HbSβ^0-thalassemia occurs infrequently.

In HbSβ-thalassemia, the RBCs are hypochromic and microcytic; HbA2 is higher than normal (3.5% to 4.2%) in SCD and thus is not a good diagnostic guide. The ISCs present on the peripheral blood smear are more numerous in HbSβ^0-thalassemia than in HbSβ^+-thalassemia. The hematologic and clinical severity is a function of the amount of HbA inherited (Table 43.10), thus the majority of HbSβ^+-thalassemia in African Americans tend to be mild unlike those of Mediterranean descent where β^+-thalassemia mutations result in a severe deficit of HbA production.[387,390] Additional mitigating influences in HbSβ-thalassemia are elevated levels of Hb A_2.

In HbSβ^+-thalassemia, levels of Hb A range up to 30%. These affect both the solubility and polymerization of HbS. HbF is a more active inhibitor of polymerization than HbA, as shown by HbS solutions with 15% to 30% Hb A (HbSβ^+-thalassemia) having delay times 10 to 100 times longer than pure HbS solutions, and HbS solutions with 20% to 30% HbF (resembling HbS/HPFH) having delay times 1000 to 1,000,000 times longer. A further influence mitigating the polymerization, sickling, and clinical aspects of HbSβ-thalassemia is the reduced MCHC, which retards HbS polymerization. Hematologic values for SCA, the HbSβ-thalassemias, and HbS/HPFH are found in Table 43.10.

For patients with sickle cell β^0-thalassemia who do not have any HbA, the clinical outcome is similar to that of HbSS disease with similar incidence of acute pain crises and ACS.[57,386] In addition, rates of PH appear similar between sickle cell–β^0-thalassemia and HbSS disease cohorts. Sickle cell β^0-thalassemia patients develop functional asplenia during infancy and have an increased rate of sepsis, including pneumococcal sepsis as for HbSS patients.

In general, sickle cell β^+-thalassemia is associated with a more mild disease phenotype, with a later age of presentation and less frequent pain crises and episodes of ACS. There is a higher incidence of proliferative retinopathy given that higher Hb levels and blood viscosity are associated with ocular complications However, the genotype-phenotype correlations in sickle cell β^+-thalassemia are variable; a key factor here is the type and severity of β^+-thalassemia mutations.

Sickle Cell–Hemoglobin Lepore Disease

The Hb Lepore gene is a crossover fusion product of the δ- and β-globin genes, the product of which, in the case of Hb Lepore Boston, has the same alkaline electrophoretic mobility as HbS. Therefore patients with the Hb Lepore trait can appear to have sickle cell trait but with only 12% HbS from thalassemic expression of the abnormal fusion gene. Again, because of the electrophoretic similarity with HbS, compound heterozygous HbSHb Lepore Boston resembles SCA or sickle cell–β^0-thalassemia electrophoretically but clinically has less severe anemia, resembling that of sickle cell–β^+-thalassemia. The diagnosis is also suggested by the low to low-normal Hb A_2 levels that result from the incapacitation of one δ-globin gene by the crossover. HbF levels vary. The peripheral smear shows microcytosis, hypochromia, and ISCs. Vaso-occlusive complications occur, and splenomegaly is common.

TABLE 43.10 **Hematologic Variables Associated With Sickle Cell Anemia and the Different Sickle Cell–β-Thalassemia Syndromes**

Genotype	Hb[a]	%HbA[b]	%HbF[b]	%HbA2[a]	MCV[a]	Reticulocytes[a]	*n*
HbSS[c]	7.83	0	4.56	2.87	85.9	10.18	≈123
HbSβ^0-thalassemia[c]	8.85	0	5.86	5.02	69.3	7.2	≈41
HbSβ^+-thalassemia, type I[d]	8.37	3-5	6.8	4.90	63.7	9.7	3
HbSβ^+-thalassemia, type II[d]	10.28	8-14	5.2	4.68	70.0	6.6	14
HbSβ^+-thalassemia, type III[e]	11.55	18-25	5.1	4.66	73.3	1.27	76
HbS/HPFH[f]	14.6	0	25.8	1.95	81.7	2.4	4

[a] The mean data for each variable are shown. Units of measure are grams per deciliter for Hb, percentage of total hemoglobin for HbF and A2, fl for MCV, and percentage of total red blood cells for reticulocytes.

[b] Percentage HbA that defines the HbS–β^+-thalassemia type.[385]

[c] Data from Serjeant et al.[386]

[d] Data from Christakis et al.[387]

[e] Data from Serjeant et al.[390]

[f] Data from Friedman et al.[389]

Hb, Hemoglobin; *HPFH*, persistence of fetal hemoglobin; *MCV*, mean corpuscular volume

Sickle Cell–Hemoglobin D Disease[384]

Because HbD Punjab or HbD Los Angeles ($\alpha_2\beta_2{}^{127}$Glu→Gln) has a similar electrophoretic mobility to HbS under alkali conditions. HbSD disease was first reported as an unusual case of SCA. HbD can be distinguished from HbS by acid electrophoresis or isoelectric focusing. There is moderately severe hemolytic anemia, and the peripheral smear shows marked anisocytosis and poikilocytosis, target cells, and ISCs. The clinical manifestations of this syndrome are similar to those of SCA.

Sickle Cell–Hemoglobin O Arab Disease[385]

Although HbO Arab ($\alpha_2\beta_2{}^{127}$Glu→Lys) was first described in an Israeli Arab family, its distribution is widespread. Sickle cell–O Arab disease resembles HbSC disease on alkaline electrophoresis, but HbO Arab can be distinguished from HbC by acid electrophoresis or isoelectric focusing. This syndrome is associated with moderately severe hemolytic anemia, and the peripheral smear shows anisocytosis, poikilocytosis, and ISCs. Oxygen affinity is reduced in sickle cell-HbO Arab as compared to SCD. Clinical manifestations are severe and resemble that of homozygous HbSS disease.[385]

Sickle Cell–Hemoglobin E Disease[1]

HbE ($\alpha_2\beta_2{}^{26}$Glu→Lys) is a β-thalassemic hemoglobinopathy found predominantly in Southeast Asia (see Chapter 41). The structural mutant has an electrophoretic mobility similar to HbC under alkaline conditions but can be resolved by acid electrophoresis or isoelectric focusing. The GAG→AAG mutation in codon 26 activates a cryptic splice site within the first intron of the β^E gene, causing alternate splicing and decreased expression of the structural mutant, resulting in a mild beta thalassemia phenotype. As a result, HbE makes up only 30% of the total Hb in compound heterozygosity for the sickle cell and HbE genes. HbSE disease is essentially benign in at least 50% and possibly most patients, with only mild hemolysis, no vaso-occlusive complications, and no remarkable abnormality of RBC morphology. However, vaso-occlusive complications and manifestations of chronic hemolytic anemia such as pain crisis, splenic infarction, recurrent pneumonia, and frontal bossing have been reported.

Coinherited Hemoglobin Abnormalities That Interact With Sickle Cell Disease: Hereditary Persistence of Fetal Hemoglobin and α-Thalassemia Trait

Sickle Cell–Hereditary Persistence of Fetal Hemoglobin

Adult Hb (or in the case of SCA, HbS) replaces HbF as a result of the switch from γ- to β-globin synthesis that occurs in fetuses. Because of the inhibitory effect of HbF on HbS polymerization and cellular sickling (see Chapter 42), the high fraction of HbF at birth masks the expression of SCD until HbS levels increase to 75% at approximately 6 months of age (see Fig. 43.3). Conditions that preserve elevated levels of HbF into adulthood similarly modulate the course of SCD, and this persistence of HbF production into adulthood is referred to as HPFH.[389] Common forms of variable HPFH are associated with co-inheritance of genetic variants on the β^S-haplotype, *BCL11A* gene, and the *HBS1L-MYB* intergenic region on chromosome 6q. The level of HbF in HbSS and HbSβ^0-thalassemia depends on the number of HbF boosting alleles inherited,[388] in some case the HbF can be as high as 25% to 30% but the distribution of HbF among the red cells is not uniform, i.e. heterocellular. Rarely, individuals are compound heterozygotes for HbS and HPFH caused by deletions of the beta-globin gene cluster or point mutation changes in the promoter of the gamma-globin genes. The most common form of gene deletion HPFH is the Ghanaian HPFH. In HbS/HPFH, the HbF is 30% to 40% and the rest is HbS, the concentration of HbF is homogeneous in every red cell, and individuals with HbS/HPFH have minimal or no sickle-related complications. It is likely that many cases of apparent SCA with unexplained elevations of HbF are the result of a heterocellular HPFH related to co-inheritance of genetic variants boosting HbF production on a background of stress erythropoiesis.

The gene frequency of the deletional HPFH locus is 0.0005 among African Americans, resulting in a calculated incidence for compound heterozygous sickle cell–deletional HPFH of 1/100 that of SCA. HbS/deletional HPFH provided the first evidence that HbF was a potent inhibitor of HbS polymerization: individuals with pancellular distribution of 25% HbF were generally neither anemic nor affected with vaso-occlusive manifestations (see Table 43.10).[389] Hb electrophoresis revealed only HbS, F, and A_2, which resembles HbSS, HbSβ°-thalassemia, and HbSδβ^0-thalassemia. Notable differences, however, are the pancellular distribution of 15% to 35% HbF, HbA_2 levels less than 2.5%, and the absence of anemia.[391,392] The generally benign course of HbS/deletional HPFH is uncommonly associated with vaso-occlusive complication.

Sickle Cell Anemia with Coexistent α-Thalassemia

Prevalences of the silent carrier of α-thalassemia syndrome (genotype −α/αα) and α-thalassemia trait (genotype −α/−α) among African Americans are approximately 30% and 2%, respectively.[393] The peripheral blood smear contains less polychromasia and fewer sickle forms and more hypochromia and microcytosis, commensurate with the numbers of α-globin genes deleted. Increased Hb A_2 levels are associated with increasing α-globin gene deletions; the HbF levels are not consistently affected.

Clinically, the impact of α-globin gene deletions on sickle cell is not as consistent as that of high HbF.[45,149] Because of the powerful effect of HbS concentration on the kinetics and extent of HbS polymerization (see Chapter 42), the lower MCHC from α-globin gene deletions decreases the hemolytic rate, and anemia is milder in subjects with both α-thalassemia syndrome (genotype −α/αα) and trait (genotype −α/−α) (see Table 43.1). In one series, hemoglobin concentration was 9.8 g/dL in patients with SCD and the (-a/aa) genotype compared with 7.9 g/dL in those with HbSS disease.[45] There is a decreased incidence of leg ulcers but an increased incidence of osteonecrosis. The frequency of retinal vessel closure is higher but not the incidence of retinopathy. Complications related to hemolysis (e.g., leg ulcers, chronic renal damage) may be decreased, but the heterogeneity of the patients in previous studies and mixed results make conclusions difficult. Similarly, the influence of α-gene deletions on survival in patients with SCD is not well understood.[50]

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Eaton WA. Hemoglobin S polymerization and sickle cell disease: A retrospective on the occasion of the 70th anniversary of Pauling's Science paper. *Am J Hematol.* 2020;95:205–211.

Strasser BJ. Perspectives: molecular medicine. Sickle cell anemia, a molecular disease. *Science.* 1999;286:1488.

Tisdale JF, Thein SL, Eaton WA. Treating sickle cell disease. *Science.* 2020;367:1198–1199.

Williams TN, Thein SL. Sickle cell anemia and its phenotypes. *Annu Rev Genomics Hum Genet.* 2018;19:113–147.

Wong TE, Brandow AM, Lim W, et al. Update on the use of hydroxyurea therapy in sickle cell disease. *Blood.* 2014;124:3850.

Yawn BP, Buchanan GR, Afenyi-Annan AN, et al. Management of sickle cell disease: summary of the 2014 evidence-based report by expert panel members. *JAMA.* 2014;312:1033.

REFERENCES

1. Rees DC, Williams TN, Gladwin MT. Sickle-cell disease. *Lancet*. 2010;376: 2018–2031.
2. Williams TN, Thein SL. Sickle cell anemia and its phenotypes. *Annu Rev Genomic Hum Genet*. 2018;19:113–147.
3. Telfer P, Coen P, Chakravorty S, Wilkey O, Evans J, Newell H, et al. Clinical outcomes in children with sickle cell disease living in England: a neonatal cohort in East London. *Haematologica*. 2007;92:905–912.
4. Quinn CT, Rogers ZR, McCavit TL, Buchanan GR. Improved survival of children and adolescents with sickle cell disease. *Blood*. 2010;115:3447–3452.
5. Gardner K, Douiri A, Drasar E, Allman M, Mwirigi A, Awogbade M, Thein SL. Survival in adults with sickle cell disease in a high-income setting. *Blood*. 2016;128(10):1436.
6. Elmariah H, Garrett ME, De Castro LM, Jonassaint JC, Ataga KI, Eckman JR, Ashley-Koch AE, Telen MJ. Factors associated with survival in a contemporary adult sickle cell disease cohort. *Am J Hematol*. 2014; 89(5):530–535.
7. Scott JA, Berkley JA, Mwangi I, Ochola L, Uyoga S, Macharia A, et al. Relation between falciparum malaria and bacteraemia in Kenyan children: a population-based, case-control study and a longitudinal study. *Lancet*. 2011;378:1316–1323.
8. Piel FB, Patil AP, Howes RE, Nyangiri OA, Gething PW, Williams TN, et al. Global distribution of the sickle cell gene and geographical confirmation of the malaria hypothesis. *Nat Commun*. 2010;1:104.
9. Nagel RL. Malaria and Hemoglobinopathies. In: Steinberg MH, Forget BG, Higgs DR, Nagel RL, eds. *Disorders of Hemoglobin: Genetics, Pathophysiology, Clinical Management*. Cambridge, UK: Cambridge University Press; 2001.
10. Pagnier J, Mears JG, Dunda-Belkhodja O, et al. Evidence for the multicentric origin of the sickle cell hemoglobin gene in Africa. *Proc Natl Acad Sci USA*. 1984;81:1771.
11. Shriner D, Rotimi CN. Whole-genome-sequence-based haplotype reveal single origin of the sickle allele during the holocene wet phase. *Am J Hum Genet*. 2018;102:547–556.
12. Laval G, Peyregne S, Zidane N, Harmant C, Renaud F, Patin E, et al. Recent adaptive acquisition by African rainforest hunter-gatherers of the late Pleistocene sickle-cell mutation suggests past differences in malaria exposure. *Am J Hum Genet*. 2019;104:553–561.
13. Micheletti SJ, Bryc K, Ancona Esselmann SG, Freyman WA, Moreno ME, Poznik GD, et al. Genetic consequences of the transatlantic slave trade in the Americas. *Am J Hum Genet*. 2020;107:265–277.
14. Fleming AF. The presentation, management and prevention of crisis in sickle cell disease in Africa. *Blood Rev*. 1989;3:18.
15. Nagel RL, Fleming AF. Genetic epidemiology of the beta s gene. *Baillieres Clin Haematol*. 1992;5:331.
16. Motulsky AG. Frequency of sickling disorders in U.S. blacks. *N Engl J Med*. 1973;288:31.
17. Hassell LK. Population estimates of sickle cell disease in the U.S. *Am J Prev Med*. 2010 Apr;38(4 Suppl):S512–521.
18. Hassell LK. Sickle cell disease: a continued call to action. *Am J Prev Med*. 2016;51(1 Suppl 1):S1–S2.
19. Key NS, Derebail VK. Sickle-cell trait: novel clinical significance. *Hematology Am Soc Hematol Educ Program*. 2010;418–422.
20. Tsaras G, Owusu-Ansah A, Boateng FO, Amoateng-Adjepong Y. Complications associated with sickle cell trait: a brief narrative review. *Am J Med*. 2009;122:507–512.
21. Piel FB, Patil AP, Howes RE, Nyangiri OA, Gething PW, Dewi M, et al. Global epidemiology of sickle haemoglobin in neonates: a contemporary geostatistical model-based map and population estimates. *Lancet*. 2013;381: 142–151.
22. Piel FB, Hay SI, Gupta S, Weatherall DJ, Williams TN. Global burden of sickle cell anaemia in children under five, 2010–2050: modelling based on demographics, excess mortality, and interventions. *PloS Med*. 2013;10: e1001484.
23. Lorey F, Cunningham G, Shafer F, et al. Universal screening for hemoglobinopathies using high-performance liquid chromatography: clinical results of 2.2 million screens. *Eur J Hum Genet*. 1994;2:262.
24. Bunn HF. Subunit assembly of hemoglobin: an important determinant of hematologic phenotype. *Blood*. 1987;69:1.
25. Dover GJ, Boyer SH, Charache S, et al. Individual variation in the production and survival of F cells in sickle-cell disease. *N Engl J Med*. 1978;299:1428.
26. Nagel RL, Ranney HM. Genetic epidemiology of structural mutations of the beta-globin gene. *Semin Hematol*. 1990;27:342.
27. Gaston MH, Verter JI, Woods G, et al. Prophylaxis with oral penicillin in children with sickle cell anemia. A randomized trial. *N Engl J Med*. 1986;314:1593.
28. sickle cell disease and other hemoglobinopathies. *JAMA*. 258:1205, 1987.
29. Tsevat J, Wong JB, Pauker SG, et al. Neonatal screening for sickle cell disease: a cost-effectiveness analysis. *J Pediatr*. 1991;118:546.
30. Task Force. Screening for sickle cell disease in newborns: recommendation statement. *Am Fam Physician*. 2008;77(9):1300.
31. O'Brien RT, McIntosh S, Aspnes GT, et al. Prospective study of sickle cell anemia in infancy. *J Pediatr*. 1976;89:205.
32. Kanter J, Telen MJ, Hoppe C, Roberts CL, Kim JS, Yang X. Validation of a novel point of care testing device for sickle cell disease. *BMC Med*. 2015;13:225.
33. Powars DR, Chan LS, Hiti A, et al. Outcome of sickle cell anemia: a 4-decade observational study of 1056 patients. *Medicine (Baltimore)*. 2005;84:363.
34. Lanzkron S, Haywood Jr. C. The five key things you need to know to manage adult patients with sickle cell disease. *Hematol Am Soc Hematology Educ Program*. 2015;2015:420–425.
35. Mehari A, Gladwin MT, Tian X, et al. Mortality in adults with sickle cell disease and pulmonary hypertension. *JAMA*. 2012;307(12):1254–1256.
36. Howard J, Thein SL. Optimal disease management and health monitoring in adults with sickle cell disease. *Hematology Am Soc Hematol Educ Program*. 2019;1:505–512.
37. Sachdev V, Rosing DR, Thein SL. Cardiovascular complications of sickle cell disease. *Trends Cardiovasc Med*. 2020;31:187–193.
38. Drawz P, Ayyappan S, Nouraie M, et al. Kidney disease among patients with sickle cell disease, hemoglobin SS and SC. *Clin J Am Soc Nephrol*. 2016;11:207–215.
39. Diggs LW. Bone and joint lesions in sickle-cell disease. *Clin Orthop Relat Res*. 1967;52:119.
40. Platt OS, Brambilla DJ, Rosse WF, et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. *N Engl J Med*. 1994;330:1639.
41. Yawn BP, Buchanan GR, Afenyi-Annan AN, et al. Management of sickle cell disease: summary of the 2014 evidence-based report by expert panel members. *JAMA*. 2014;312(10):1033–1048.
42. Qureshi A, Kaya B, Pancham S, et al. Guidelines for the use of hydroxycarbamide in children and adults with sickle cell disease: A British Society for Haematology Guideline. *Br J Haematol*. 2018;181(4):460–475.
43. Goldberg MA, Husson MA, Bunn HF. Participation of hemoglobins A and F in polymerization of sickle hemoglobin. *J Biol Chem*. 1977;252:3414.
44. Quinn CT. Minireview: clinical severity in sickle cell disease: the challenges of definition and prognostication. *Exp Biol Med (Maywood)*. 2016;241: 679–688.
45. Embury SH, Dozy AM, Miller J, et al. Concurrent sickle-cell anemia and alpha-thalassemia: effect on severity of anemia. *N Engl J Med*. 1982;306(5):270.
46. Higgs DR, Aldridge BE, Lamb J, et al. The interaction of α-thalassemia and homozygous sickle-cell disease. *N Engl J Med*. 1982;306:1441.
47. Steinberg MH, Rosenstock W, Coleman MB, et al. The cooperative study of sickle cell disease: effects of thalassemia and microcytosis on the hematologic and vasoocclusive severity of sickle cell anemia. *Blood*. 1984;63:1353.
48. Felice AE, McKie KM, Cleek MP, et al. Effects of α-thalassemia-2 on the developmental changes of hematological values in children with sickle cell disease from Georgia. *Am J Hematol*. 1987;25:389.
49. Thomas PW, Higgs DR, Serjeant GR. Benign clinical course in homozygous sickle cell disease: a search for predictors. *J Clin Epidemiol*. 1997;50:121.
50. Steinberg MH. Predicting clinical severity in sickle cell anaemia. *Br J Haematol*. 2005;129:465.
51. Platt OS, Brambilla DJ, Rosse WF, et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. *N Engl J Med*. 1994;330:1639.
52. Ohene-Frempong K, Weiner SJ, Sleeper LA, et al. Cerebrovascular accidents in sickle cell disease: rates and risk factors. *Blood*. 1998;91:288.
53. Guasch A, Cua M, Mitch WE. Early detection and the course of glomerular injury in patients with sickle cell anemia. *Kidney Int*. 1996;49:786.
54. Faulkner M, Turner EA, Deus J, et al. Severe anemia: a risk factor for glomerular injury in sickle cell disease. *J Natl Med Assoc*. 1995;87:209.
55. Morris JS, Dunn DT, Poddar D, et al. Haematological risk factors for pregnancy outcome in Jamaican women with homozygous sickle cell disease. *Br J Obstet Gynaecol*. 1994;101:770.
56. Smith JA, Espeland M, Bellevue R, et al. Pregnancy in sickle cell disease: experience of the Cooperative Study of Sickle Cell Disease. *Obstet Gynecol*. 1996;87:199.
57. Platt OS, Thorington BD, Brambilla DJ, et al. Pain in sickle cell disease. Rates and risk factors. *N Engl J Med*. 1991;325:11.
58. Steinberg MH, Barton F, Castro O, et al. Effect of hydroxyurea on mortality and morbidity in adult sickle cell anemia: risks and benefits up to 9 years of treatment. *JAMA*. 2003;289:1645.
59. Saraf S, Farooqui M, Infusino G, et al. Standard clinical practice underestimates the role and significance of erythropoietin deficiency in sickle cell disease. *Br J Haematol*. 2011;153:386.
60. Sachdev V, Tian X, Gu Y, Nichols J, Sidenko S, Li W, et al. A phenotypic risk score for predicting mortality in sickle cell disease. *Br J Haematol*. 2021;192:932–941.

61. Habara A, Steinberg MH. Minireview: genetic basis and heterogeneity and severity in sickle cell disease. *Exp Biol Med (Maywood)*. 2016;241(7): 689–696.
62. Steinberg MH, Thein SL. Genetic basis of sickle cell disease. In: Gladwin MT, Kato GJ, Novelli EM, eds. *Sickle Cell Disease*. New York, NY: McGraw Hill; 2021:11–32.
63. Claster S, Vichinsky EP. Managing sickle cell disease. *BMJ*. 2003;327:1151.
64. Kwiatkowski JL, Granger S, Brambilla DJ, et al. Elevated blood flow velocity in the anterior cerebral artery and stroke risk in sickle cell disease: extended analysis from the STOP trial. *Br J Haematol*. 2006;134:333.
65. Valadi N, Silva GS, Bowman LS, et al. Transcranial doppler ultrasonography in adults with sickle cell disease. *Neurology*. 2006;67:572.
66. Pegelow CH, Colangelo L, Steinberg M, et al. Natural history of blood pressure in sickle cell disease: risks for stroke and death associated with relative hypertension in sickle cell anemia. *Am J Med*. 1997;102:171.
67. Porter J, Garbowski M. Consequences and management of iron overload in sickle cell disease. *Hematology Am Soc Hematol Educ Program (2013)*. 2013(1):447–456.
68. Jonsson U, Roath OS, Kirkpatrick CI. Nutritional megaloblastic anemia associated with sickle cell states. *Blood*. 1959;14:535.
69. Lopez R, Shimizu N, Cooperman JM. Recurrent folic acid deficiency in sickle cell disease. *Am J Dis Child*. 1973;125:544.
70. van der Dijs FP, Schnog JJ, Brouwer DA, et al. Elevated homocysteine levels indicate suboptimal folate status in pediatric sickle cell patients. *Am J Hematol*. 1998;59:192.
71. Pearson HA, Cobb WT. Folic acid studies in sickle-cell anemia. *J Lab Clin Med*. 1964;64:913.
72. Dhar M, Bellevue R, Carmel R. Pernicious anemia with neuropsychiatric dysfunction in a patient with sickle cell anemia treated with folate supplementation. *N Engl J Med*. 2003;348:2204.
73. Amer J, Ghoti H, Rachmilewitz E, et al. Red blood cells, platelets and polymorphonuclear neutrophils of patients with sickle cell disease exhibit oxidative stress that can be ameliorated by antioxidants. *Br J Haematol*. 2006;132:108.
74. Lal A, Fung EB, Pakbaz Z, et al. Bone mineral density in children with sickle cell anemia. *Pediatr Blood Cancer*. 2006;47:901.
75. Buison AM, Kawchak DA, Schall J, et al. Low vitamin D status in children with sickle cell disease. *J Pediatr*. 2004;145:622.
76. Schall JI, Zemel BS, Kawchak DA, et al. Vitamin A status, hospitalizations, and other outcomes in young children with sickle cell disease. *J Pediatr*. 2004;145:99.
77. Zemel BS, Kawchak DA, Fung EB, et al. Effect of zinc supplementation on growth and body composition in children with sickle cell disease. *Am J Clin Nutr*. 2002;75:300.
78. Nolan VG, Nottage KA, Cole EW, et al. Prevalence of vitamin D deficiency in sickle cell disease: a systematic review. *PLoS One*. 2015;10(3):e0119908.
79. Vichinsky E, Kleman K, Embury S, et al. The diagnosis of iron deficiency anemia in sickle cell disease. *Blood*. 1981;58:963.
80. Castro O, Poillon WN, Finke H, et al. Improvement of sickle cell anemia by iron-limited erythropoiesis. *Am J Hematol*. 1994;47:74.
81. Wong WY, Overturf GD, Powars DR. Infection caused by Streptococcus pneumoniae in children with sickle cell disease: epidemiology, immunologic mechanisms, prophylaxis, and vaccination. *Clin Infect Dis*. 1992;14:1124.
81a. Pasternack MS. Prevention of infection in patients with impaired splenic function. *UpToDate*. Jul 05, 2022.
82. John AB, Ramlal A, Jackson H, et al. Prevention of pneumococcal infection in children with homozygous sickle cell disease. *Br Med J (Clin Res Ed)*. 1984;288:1567.
83. Falletta JM, Woods GM, Verter JI, et al. Discontinuing penicillin prophylaxis in children with sickle cell anemia. Prophylactic Penicillin Study II. *J Pediatr*. 1995;127:685.
84. Hord J, Byrd R, Stowe L, et al. Streptococcus pneumoniae sepsis and meningitis during the penicillin prophylaxis era in children with sickle cell disease. *J Pediatr Hematol Oncol*. 2002;24:470.
85. Hongeng S, Wilimas JA, Harris S, et al. Recurrent *Streptococcus pneumoniae* sepsis in children with sickle cell disease. *J Pediatr*. 1997;130:814.
86. Perrine RP, Pembrey ME, John P, et al. Natural history of sickle cell anemia in Saudi Arabs. A study of 270 subjects. *Ann Intern Med*. 1978;88:1.
87. Nagel RL, Bookchin RM, Johnson J, et al. Structural bases of the inhibitory effects of hemoglobin F and hemoglobin A2 on the polymerization of hemoglobin S. *Proc Natl Acad Sci USA*. 1979;76:670.
88. Steinberg MH, McCarthy WF, Castro O, et al. The risks and benefits of long-term use of hydroxyurea in sickle cell anemia: a 17.5 year follow-up. *Am J Hematol*. 2010;85(6):403–408.
89. Charache S, Barton FB, Moore RD, et al. Hydroxyurea and sickle cell anemia. clinical utility of a myelosuppressive "switching" agent. The multicenter study of hydroxyurea in sickle cell anemia. *Medicine (Baltimore)*. 1996;75:300.
90. Charache S, Terrin ML, Moore RD, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. *N Engl J Med*. 1995;332:1317.
91. Orringer EP, Blythe DS, Johnson AE, et al. Effects of hydroxyurea on hemoglobin F and water content in the red blood cells of dogs and of patients with sickle cell anemia. *Blood*. 1991;78:212.
92. Kinney TR, Helms RW, O'branski EE, et al. Safety of hydroxyurea in children with sickle cell anemia: results of the HUG-KIDS study, a phase I/II trial. Pediatric Hydroxyurea Group. *Blood*. 1999;94:1550.
93. Ware RE, Eggleston B, Redding-Lallinger R, et al. Predictors of fetal hemoglobin response in children with sickle cell anemia receiving hydroxyurea therapy. *Blood*. 2002;99:10.
94. Ferster A, Tahriri P, Vermylen C, et al. Five years of experience with hydroxyurea in children and young adults with sickle cell disease. *Blood*. 2001;97:3628.
95. Ware RE, Zimmerman SA, Schultz WH. Hydroxyurea as an alternative to blood transfusions for the prevention of recurrent stroke in children with sickle cell disease. *Blood*. 1999;94:3022.
96. Zimmerman SA, Schultz WH, Burgett S, et al. Hydroxyurea therapy lowers transcranial Doppler flow velocities in children with sickle cell anemia. *Blood*. 2007;110:1043.
97. Kratovil T, Bulas D, Driscoll MC, et al. Hydroxyurea therapy lowers TCD velocities in children with sickle cell disease. *Pediatr Blood Cancer*. 2006; 47:894.
98. Gulbis B, Haberman D, Dufour D, et al. Hydroxyurea for sickle cell disease in children and for prevention of cerebrovascular events: the Belgian experience. *Blood*. 2005;105:2685.
99. Ware RE, Zimmerman SA, Sylvestre PB, et al. Prevention of secondary stroke and resolution of transfusional iron overload in children with sickle cell anemia using hydroxyurea and phlebotomy. *J Pediatr*. 2004;145:346.
100. Tavakkoli F, Nahavandi M, Wyche MQ, et al. Effects of hydroxyurea treatment on cerebral oxygenation in adult patients with sickle cell disease: an open-label pilot study. *Clin Ther*. 2005;27:1083.
101. Najean Y, Rain JD, Dresch C, et al. Risk of leukaemia, carcinoma, and myelofibrosis in 32P- or chemotherapy-treated patients with polycythaemia vera: a prospective analysis of 682 cases. The "French Cooperative Group for the Study of Polycythaemias. *Leuk Lymphoma*. 1996;22:111.
102. Weinfeld A, Swolin B, Westin J. Acute leukaemia after hydroxyurea therapy in polycythaemia vera and allied disorders: prospective study of efficacy and leukaemogenicity with therapeutic implications. *Eur J Haematol*. 1994;52:134.
103. Fruchtman SM, Mack K, Kaplan ME, et al. From efficacy to safety: a Polycythemia Vera Study Group report on hydroxyurea in patients with polycythemia vera. *Semin Hematol*. 1997;34:17.
104. Triadou P, Maier-Redelsperger M, Krishnamoorty R, et al. Fetal haemoglobin variations following hydroxyurea treatment in patients with cyanotic congenital heart disease. *Nouv Rev Fr Hematol*. 1994;36:367.
105. Atweh GF, Sutton M, Nassif I, et al. Sustained induction of fetal hemoglobin by pulse butyrate therapy in sickle cell disease. *Blood*. 1999;93:1790.
106. Lavelle D, Engel JD, Saunthararajah Y. Fetal hemoglobin induction by epigenetic drugs. *Semin Hematol*. 2018;55:60–67.
107. Ley TJ, DeSimone J, Noguchi CT, et al. 5-Azacytidine increases gamma-globin synthesis and reduces the proportion of dense cells in patients with sickle cell anemia. *Blood*. 1983;62:370.
108. Saunthararajah Y, Hillery CA, Lavelle D, et al. Effects of 5-aza-2′-deoxycytidine on fetal hemoglobin levels, red cell adhesion, and hematopoietic differentiation in patients with sickle cell disease. *Blood*. 2003;102:3865.
109. Molokie R, Lavelle D, Gowhari M, Pacini M, Krauz L, Hassan J, et al. Oral tetrahydrouridine and decitabine for non-cytotoxic epigenetic gene regulation in sickle cell disease: a randomized phase 1 study. *PLoS Med*. 2017;14:e1002382.
110. Perrine SP. Fetal globin induction—Can it cure beta thalassemia? *Hematology Am Soc Hematol Educ Program*. 2005:38–44.
111. Atweh GF, Schechter AN. Pharmacologic induction of fetal hemoglobin: raising the therapeutic bar in sickle cell disease. *Curr Opin Hematol*. 2001;8:123.
112. Moutouh-de Parseval LA, Verhelle D, Glezer E, et al. Pomalidomide and lenalidomide regulate erythropoiesis and fetal hemoglobin production in human CD34+ cells. *J Clin Invest*. 2008;118:248.
113. Vichinsky E, Hoppe CC, Ataga KI, Ware RE, et al. A phase 3 randomized trial of voxelotor in sickle cell disease. *N Engl J Med*. 2019;381(6): 509–519.
114. Hebbel RP, Hedlund BE. Sickle hemoglobin oxygen affinity-shifting strategies have unequal cerebrovascular risks. *Am J Hematol*. 2018;93:321–325.
115. Rutherford NJ, Thoren KL, Shajani-Yi Z, Colby JM. Voxelotor (GBT440) produces interference in measurements of hemoglobin S. *Clin Chim Acta*. 2018;482:57–579.
116. Global Blood Therapeutics. Oxbryta™ (voxelotor) tablets, for oral use: US prescribing information. 2019. http://www.accessdata.fda.gov.

117. US Food and Drug Administration. FDA approves first targeted therapy to treat patients with painful complication of sickle cell disease [media release]. November 15, 2019. http://www.fda.gov
118. Ataga KI, Kutlar A, Kanter J, Liles D, Cancado R, Friedrisch J, et al. Crizanlizumab for the prevention of pain crises in sickle cell disease. *N Engl J Med*. 2017;376(5):429–439.
119. Kutlar A, Kanter J, Liles DK, Alvarez OA, Cancado RD, Friedrisch JR, et al. Effect of crizanlizumab on pain crises in subgroups of patients with sickle cell disease: A SUSTAIN study analysis. *Am J Hematol*. 2019;94:55–61.
120. ADAKVEO® (crizanlizumab-tmca) injection, for intravenous use: US prescribing information. 2019. http://www.accessdata.fda.gov.
121. US Food and Drug Administration. Approved drugs—FDA D.I.S.C.O. L-glutamine for sickle cell disease. https://www.fda.gov/drugs/informationondrugs/approveddrugs/ucm572213.htm.
122. Niihara YM, Miller ST, Kanter J, Lanzkron S, et al. A phase 3 trial of l-glutamine in sickle cell disease. *N Engl J Med*. 2018;379:226–235.
123. Quinn CT. L-Glutamine for sickle cell anemia: more questions than answers. *Blood*. 2018;132:689–693.
124. Bernaudin F, Souillet G, Vannier JP, et al. Bone marrow transplantation (BMT) in 14 children with severe sickle cell disease (SCD): the French experience. GEGMO. *Bone Marrow Transplant*. 1993;12:118.
125. Gluckman E, Cappelli B, Bernaudin F, et al. Sickle cell disease: an international survey of results of HLA-identical sibling hematopoietic stem cell transplantation. *Blood*. 2017;129(11):1548.
126. Walters MC, Patience M, Leisenring W, Rogers ZR, Aquino VM, Buchanan GR, et al. Stable mixed hematopoietic chimerism after bone marrow transplantation for sickle cell anemia. *Biol Blood Marrow Transplant*. 2001;7:665–673.
127. Hsieh MM, Kang EM, Fitzhugh CD, Link MB, Bolan CD, Kurlander R, et al. Allogeneic hematopoietic stem-cell transplantation for sickle cell disease. *N Engl J Med*. 2009;361:2309–2317.
128. Hsieh MM, Fitzhugh CD, Weitzel RP, Link ME, Coles WA, Zhao X, et al. Nonmyeloablative HLA-matched sibling allogeneic hematopoietic stem cell transplantation for severe sickle cell phenotype. *JAMA*. 2014;312:48–56.
129. Fitzhugh CD, Cordes S, Taylor T, Coles W, Roskom K, Link M, et al. At least 20% donor myeloid chimerism is necessary to reverse the sickle phenotype after allogeneic HSCT. *Blood*. 2017;130:1946–1948.
130. Bernaudin F, Dalle JH, Bories D, de Latour RP, Robin M, Bertrand Y, et al. Long-term event-free survival, chimerism and fertility outcomes in 234 patients with sickle-cell anemia younger than 30 years after myeloablative conditioning and matched-sibling transplantation in France. *Haematologica*. 2020;105:91–101.
131. Hsieh MM, Fitzhugh CD, Tisdale JF. Allogeneic hematopoietic stem cell transplantation for sickle cell disease: the time is now. *Blood*. 2011;118:1197.
132. Joseph JJ, Abraham AA, Fitzhugh CD. When there is no match, the game is not over: Alternative donor options for hematopoietic stem cell transplantation in sickle cell disease. *Semin Hematol*. 2018;55:94–101.
133. Locatelli F, Rocha V, Reed W, et al. Related umbilical cord blood transplantation in patients with thalassemia and sickle cell disease. *Blood*. 2003;101:2137.
134. Fitzhugh CD, Abraham AA, Tisdale JF, Hsieh MM. Hematopoietic stem cell transplantation for patients with sickle cell disease: progress and future directions. *Hematol Oncol Clin North Am*. 2014;28:1171–1185.
135. Bolanos-Meade J, Fuchs EJ, Luznik L, Lanzkron SM, Gamper CJ, Jones RJ, et al. HLA-haploidentical bone marrow transplantation with post-transplant cyclophosphamide expands the donor pool for patients with sickle cell disease. *Blood*. 2012;120:4285–4291.
136. Saraf SL, Oh AL, Patel PR, Sweiss K, Koshy M, Campbell-Lee S, et al. Haploidentical peripheral blood stem cell transplantation demonstrates stable engraftment in adults with sickle cell disease. *Biol Blood Marrow Transplant*. 2018;24:1759–1765.
137. Angelucci E, Matthes-Martin S, Baronciani D, Bernaudin F, Bonanomi S, Cappellini MD, et al. Hematopoietic stem cell transplantation in thalassemia major and sickle cell disease: indications and management recommendations from an international expert panel. *Haematologica*. 2014;99:811–820.
138. Bolanos-Meade J, Cooke KR, Gamper CJ, Ali SA, Ambinder RF, Borrello IM, et al. Effect of increased dose of total body irradiation on graft failure associated with HLA-haploidentical transplantation in patients with severe haemoglobinopathies: a prospective clinical trial. *Lancet Haematol*. 2019;Vol. 6(Issue 4):e183–e193.
139. Kauf TL, Coates TD, Huazhi L, Mody-Patel N, Hartzema AG. The cost of health care for children and adults with sickle cell disease. *Am J Hematol*. 2009;84:323–327.
140. Chou ST, Fasano RM. Management of patients with sickle cell disease using transfusion therapy: guidelines and complications. *Hematol Oncol Clin North Am*. 2016;30:591–608.
141. Howard J. Sickle cell disease: when and how to transfuse. *Hematology Am Soc Hematol Educ Program*. 2016:625–631.
142. Lottenberg R, Hassell KL. An evidence-based approach to the treatment of adults with sickle cell disease. *Hematology Am Soc Hematol Educ Program*. 2005:58–65.
143. Alexy T, Pais E, Armstrong JK, et al. Rheologic behavior of sickle and normal red blood cell mixtures in sickle plasma: implications for transfusion therapy. *Transfusion*. 2006;46:912.
144. Charache S. Treatment of sickle cell anemia. *Annu Rev Med*. 1981;32:195.
145. Vichinsky EP, Haberkern CM, Neumayr L, et al. A comparison of conservative and aggressive transfusion regimens in the perioperative management of sickle cell disease. The preoperative transfusion in sickle cell disease study group. *N Engl J Med*. 1995;333:206.
146. Howard J, Malfroy M, Llewelyn C, et al. The transfusion alternatives preoperatively in sickle cell disease (TAPS) study: a randomised, controlled, multicentre clinical trial. *Lancet*. 2013;381(9870):930–938.
147. Rosse WF, Gallagher D, Kinney TR, et al. Transfusion and alloimmunization in sickle cell disease. The cooperative study of sickle cell disease. *Blood*. 1990;76:1431.
148. Chou ST, Alsawas M, Fasano RM, et al. American Society of Hematology 2020 guidelines for sickle cell disease: transfusion support. *Blood Adv*. 2020;4:327–355.
149. Vichinsky EP, Earles A, Johnson RA, et al. Alloimmunization in sickle cell anemia and transfusion of racially unmatched blood. *N Engl J Med*. 1990;322:1617.
150. Chou ST, Jackson T, Vege S, Smith-Whitley K, Friedman DF, Westhoff CM, et al. High prevalence of red blood cell alloimmunization in sickle cell disease despite transfusion from Rh-matched minority donors. *Blood*. 2013;122:1062–1071.
151. Dean CL, Maier CL, Chonat S, et al. Challenges in the treatment and prevention of delayed hemolytic transfusion reactions with hyperhemolysis in sickle cell disease patients. *Transfusion*. 2019;59(5):1698–1705.
152. Chou ST, Evans P, Vege S, Coleman SL, Friedman DF, Keller M, et al. RH genotype matching for transfusion support in sickle cell disease. *Blood*. 2018;132:1198–1207.
153. Vichinsky EP, Luban NL, Wright E, et al. Prospective RBC phenotype matching in a stroke-prevention trial in sickle cell anemia: a multicenter transfusion trial. *Transfusion*. 2001;41:1086.
154. Loos JA, Wautier JL. Leukocyte depletion: a biotechnical transfusion story. *Transfus Clin Biol*. 1998;5:64.
155. Markham MJ, Lottenberg R, Zumberg M. Role of phlebotomy in the management of hemoglobin SC disease: case report and review of the literature. *Am J Hematol*. 2003;73:121.
156. Ware RE, Zimmerman SA, Sylvestre PB, et al. Prevention of secondary stroke and resolution of transfusional iron overload in children with sickle cell anemia using hydroxyurea and phlebotomy. *J Pediatr*. 2004;145:346.
157. Bouchair N, Manigne P, Kanfer A, et al. Prevention of sickle cell crises with multiple phlebotomies. *Arch Pediatr*. 2000;7:249.
158. Steinberg MH, Barton F, Castro O, et al. Effect of hydroxyurea on mortality and morbidity in adult sickle cell anemia: risks and benefits up to 9 years of treatment. *JAMA*. 2003;289:1645.
159. Sherwood JB, Goldwasser E, Chilcote R, et al. Sickle cell anemia patients have low erythropoietin levels for their degree of anemia. *Blood*. 1986;67:46.
160. Little JA, McGowan VR, Kato GJ, et al. Combination erythropoietin-hydroxyurea therapy in sickle cell disease: experience from the National Institutes of Health and a literature review. *Haematologica*. 2006;91:1076.
161. Breen CP, Macdougall IC. Improvement of erythropoietin-resistant anaemia after renal transplantation in patients with homozygous sickle-cell disease. *Nephrol Dial Transplant*. 1998;13:2949.
162. Niederau C, Fischer R, Purschel A, et al. Long-term survival in patients with hereditary hemochromatosis. *Gastroenterology*. 1996;110:1107.
163. Brittenham GM, Cohen AR, McLaren CE, et al. Hepatic iron stores and plasma ferritin concentration in patients with sickle cell anemia and thalassemia major. *Am J Hematol*. 1993;42:81.
164. Porter JB, Shah FT. Iron overload in thalassemia and related conditions: therapeutic goals and assessment of response to chelation therapies. *Hematol Oncol Clin North Am*. 2010;24(6):1109.
165. Meloni A, Puliyel M, Pepe A, Berdoukas V, Coates TD, Wood JC. Cardiac iron overload in sickle-cell disease. *Am J Hematol*. 2014;89(7):678.
166. Porter JB, Huehns ER. Transfusion and exchange transfusion in sickle cell anaemias, with particular reference to iron metabolism. *Acta Haematol*. 1987;78:198.
167. Karam LB, Disco D, Jackson SM, et al. Liver biopsy results in patients with sickle cell disease on chronic transfusions: poor correlation with ferritin levels. *Pediatr Blood Cancer*. 2008;50:62.
168. Pakbaz Z, Fischer R, Fung E, et al. Serum ferritin underestimates liver iron concentration in transfusion independent thalassemia patients as compared to regularly transfused thalassemia and sickle cell patients. *Pediatr Blood Cancer*. 2007;49:329.

169. Maggio A. Light and shadows in the iron chelation treatment of haematological diseases. *Br J Haematol*. 2007;138:407.
170. Vichinsky E, Onyekwere O, Porter J, et al. A randomised comparison of deferasirox versus deferoxamine for the treatment of transfusional iron overload in sickle cell disease. *Br J Haematol*. 2007;136:501.
171. Neufeld EJ. Oral chelators deferasirox and deferiprone for transfusional iron overload in thalassemia major: new data, new questions. *Blood*. 2006;107(9): 3436–3441.
172. St Pierre TG, Clark PR, Chua-Anusorn W. Measurement and mapping of liver iron concentrations using magnetic resonance imaging. *Ann N Y Acad Sci*. 2005;1054:379.
173. Francis Jr RB, Johnson CS. Vascular occlusion in sickle cell disease: current concepts and unanswered questions. *Blood*. 1991;77:1405.
174. Ahmed S, Siddiqui AK, Iqbal U, et al. Effect of low-dose warfarin on D-dimer levels during sickle cell vaso-occlusive crisis: a brief report. *Eur J Haematol*. 2004;72:213.
175. Chaplin Jr H, Monroe MC, Malecek AC, et al. Preliminary trial of minidose heparin prophylaxis for painful sickle cell crises. *East Afr Med J*. 1989;66:574.
176. Ciurea SO, Thulborn KR, Gowhari M. Dural venous sinus thrombosis in a patient with sickle cell disease: case report and literature review. *Am J Hematol*. 2006;81:290.
177. Wolters HJ, ten Cate H, Thomas LL, et al. Low-intensity oral anticoagulation in sickle-cell disease reverses the prethrombotic state: promises for treatment? *Br J Haematol*. 1995;90:715.
178. Schnog JB, Kater AP, Mac Gillavry MR, et al. Low adjusted-dose acenocoumarol therapy in sickle cell disease: a pilot study. *Am J Hematol*. 2001;68:179.
179. Wun T, Paglieroni T, Tablin F, Welborn J, Nelson K, Cheung A. Platelet activation and platelet-erythrocyte aggregates in patients with sickle cell anemia. *J Lab Clin Med*. 1997;129:507–516.
180. Greenberg J, Ohene-Frempong K, Halus J, et al. Trial of low doses of aspirin as prophylaxis in sickle cell disease. *J Pediatr*. 1983;102:781.
181. Conran N, Rees DC. Prasugrel hydrochloride for the treatment of sickle cell disease. *Expert Opin Investig Drugs*. 2017;26:865–872.
182. Heeney MM, Hoppe CC, Abboud MR, Inusa B, Kanter J, Ogutu B, et al. A multinational trial of prasugrel for sickle cell vaso-occlusive events. *N Engl J Med*. 2016;374:625–635.
183. Kanter J, Abboud MR, Kaya B, Nduba V, Amilon C, Gottfridsson C, et al. Ticagrelor does not impact patient-reported pain in young adults with sickle cell disease: a multicentre, randomised phase IIb study. *Br J Haematol*. 2019;184:269–278.
184. Sins JWR, Mager DJ, Davis S, Biemond BJ, Fijnvandraat K, et al. Pharmacotherapeutical strategies in the prevention of acute, vaso-occlusive pain in sickle cell disease: a systematic review. *Blood Adv*. 2017;1:1598–1616.
185. Hoban MD, Cost GJ, Mendel MC, et al. Correction of the sickle cell disease mutation in human hematopoietic stem/progenitor cells. *Blood*. 2015;125(17):2597–2604.
186. Orkin SH, Bauer DE. Emerging genetic therapy for sickle cell disease. *Annu Rev Med*. 2019;70:257–271.
187. Esrick EB, Lehmann LE, Biffi A, Achebe M, Brendel C, Ciuculescu MF, et al. Post-transcriptional genetic silencing of BCL11A to treat sickle cell disease. *N Engl J Med*. 2021;384:205–215.
188. Frangoul H, Altshuler D, Cappellini MD, Chen YS, Domm J, Eustace BK, et al. CRISPR-cas9 gene editing for sickle cell disease and beta-thalassemia. *New Engl J Med*. 2021;384:252–260.
189. Bainbridge R, Higgs DR, Maude GH, et al. Clinical presentation of homozygous sickle cell disease. *J Pediatr*. 1985;106:881.
190. Brozovic M, Davies SC, Brownell AI. Acute admissions of patients with sickle cell disease who live in Britain. *Br Med J (Clin Res Ed)*. 1987;294:1206.
191. Diggs LW. The crisis in sickle cell anemia: hematologic studies. *Am J Clin Pathol*. 1956;26:1109.
192. Bunn HF. Pathogenesis and treatment of sickle cell disease. *N Engl J Med*. 1997;337:762.
193. Brittenham GM, Schechter AN, Noguchi CT. Hemoglobin S polymerization: primary determinant of the hemolytic and clinical severity of the sickling syndromes. *Blood*. 1985;65:183.
194. Powars DR. Natural history of sickle cell disease—The first ten years. *Semin Hematol*. 1975;12:267.
195. Sidman JD, Fry TL. Exacerbation of sickle cell disease by obstructive sleep apnea. *Arch Otolaryngol Head Neck Surg*. 1988;114:916.
196. Baum KF, Dunn DT, Maude GH, et al. The painful crisis of homozygous sickle cell disease. A study of the risk factors. *Arch Intern Med*. 1987;147:1231.
197. Murray N, May A. Painful crises in sickle cell disease—Patients' perspectives. *BMJ*. 1988;297:452.
198. Tewari Piel FBS, Brousse V, Analitis A, Font A, Menzel S, et al. Associations between environmental factors and hospital admissions for sickle cell disease. *Haematologica*. 2017;102:666–675.
199. Panepinto JA, Brousseau DC, Hillery CA, et al. Variation in hospitalizations and hospital length of stay in children with vaso-occlusive crises in sickle cell disease. *Pediatr Blood Cancer*. 2005;44:182–186.
200. Rees DC, Olujohungbe AD, Parker NE, et al. Guidelines for the management of the acute painful crisis in sickle cell disease. *Br J Haematol*. 2003;120:744.
201. Brandow AM, Carroll CP, Creary S, et al. American Society of Hematology 2020 guidelines for sickle cell disease: management of acute and chronic pain. *Blood Adv*. 2020;4:2656–2701.
202. Brookoff D, Polomano R. Treating sickle cell pain like cancer pain. *Ann Intern Med*. 1992;116(5):364.
203. Dampier CD, Setty BN, Logan J, et al. Intravenous morphine pharmacokinetics in pediatric patients with sickle cell disease. *J Pediatr*. 1995;126:461.
204. Brodsky MA, Rodeghier M, Sanger M, et al. Risk factors for 30-day readmission in adults with sickle cell disease. *Am J Med*. 2017;130(5): 601 e9-601.e15.
205. Taylor LE, Stotts NA, Humphreys J. A review of the literature on the multiple dimensions of chronic pain in adults with sickle cell disease. *J Pain Symptom Manage*. 2010;40:416–435.
206. Darbari DS, Liljencrantz J, Ikechi A. Pain and opioid use after reversal of sickle cell disease following HLA-matched sibling haematopoietic stem cell transplant. *Br J Haematol*. 2019;184:690–693.
207. Ballas SK. Pain management of sickle cell disease. *Hematol Oncol Clin North Am*. 2005;19:785.
208. McCurdy PR. 32-DFP and 51-Cr for measurement of red cell life span in abnormal hemoglobin syndromes. *Blood*. 1969;33:214.
209. Serjeant GR, Serjeant BE, Milner PF. The irreversibly sickled cell; a determinant of haemolysis in sickle cell anaemia. *Br J Haematol*. 1969;17:527.
210. West MS, Wethers D, Smith J, et al. Laboratory profile of sickle cell disease: a cross-sectional analysis. The Cooperative Study of Sickle Cell Disease. *J Clin Epidemiol*. 1992;45:893.
211. Steinberg MH, Embury SH. Alpha-thalassemia in blacks: genetic and clinical aspects and interactions with the sickle hemoglobin gene. *Blood*. 1986;68:985.
212. Pattison JR, Jones SE, Hodgson J, et al. Parvovirus infections and hypoplastic crisis in sickle-cell anaemia. *Lancet*. 1981;1:664.
213. Rao SP, Miller ST, Cohen BJ. Transient aplastic crisis in patients with sickle cell disease. B19 parvovirus studies during a 7-year period. *Am J Dis Child*. 1992;146:1328.
214. Charache S, Page DL. Infarction of bone marrow in the sickle cell disorders. *Ann Intern Med*. 1967;67:1195.
215. Pardoll DM, Rodeheffer RJ, Smith RR, et al. Aplastic crisis due to extensive bone marrow necrosis in sickle cell disease. *Arch Intern Med*. 1982;142:2223.
216. Embury SH, Garcia JF, Mohandas N, et al. Effects of oxygen inhalation on endogenous erythropoietin kinetics, erythropoiesis, and properties of blood cells in sickle-cell anemia. *N Engl J Med*. 1984;311:291.
217. Topley JM, Rogers DW, Stevens MC, et al. Acute splenic sequestration and hypersplenism in the first five years in homozygous sickle cell disease. *Arch Dis Child*. 1981;56:765.
218. Emond AM, Collis R, Darvill D, et al. Acute splenic sequestration in homozygous sickle cell disease: natural history and management. *J Pediatr*. 1985;107:201.
219. Brousse V, Elie C, Benkerrou M, Odievre MH, Lesprit E, Bernaudin F, et al. Acute splenic sequestration crisis in sickle cell disease: cohort study of 190 paediatric patients. *Br J Hematol*. 2012;156:643–648.
220. Hatton CS, Bunch C, Weatherall DJ. Hepatic sequestration in sickle cell anaemia. *Br Med J (Clin Res Ed)*. 1985;290:744.
221. Davies SC, Luce PJ, Win AA, et al. Acute chest syndrome in sickle-cell disease. *Lancet*. 1984;1:36.
222. Vidler JB, Gardner K, Amenyah K, Mijovic A, Thein SL. Delayed haemolytic transfusion reaction in adults with sickle cell disease: a 5-year experience. *Br J Haematol*. 2015;169(5):746–753.
223. Thein SL, Pirenne F, Fasano RM, Habibi A, Bartolucci P, Chonat S, et al. Hemolytic transfusion reactions in sickle cell disease: underappreciated and potentially fatal. *Haematologica*. 2020;105:539–544.
224. Pirenne F, Yazdanbakhsh K. How I safely transfuse patients with sickle-cell disease and manage delayed hemolytic transfusion reactions. *Blood*. 2018;131(25):2773–2781.
225. Talano JA, Hillery CA, Gottschall JL, et al. Delayed hemolytic transfusion reaction/hyperhemolysis syndrome in children with sickle cell disease. *Pediatrics*. 2003;111:e661.
226. Narbey D, Habibi A, Chadebech P, et al. Incidence and predictive score for delayed hemolytic transfusion reaction in adult patients with sickle cell disease. *Am J Hematol*. 2017;92(12):1340–1348.
227. King KE, Shirey RS, Lankiewicz MW, et al. Delayed hemolytic transfusion reactions in sickle cell disease: simultaneous destruction of recipients' red cells. *Transfusion*. 1997;37:376.

228. Gardner K, Hoppe C, Mijovic A, Thein SL. How we treat delayed haemolytic transfusion reactions in patients with sickle cell disease. *Br J Haematol*. 2015;170:745–756.
229. Dumas G, Habibi A, Onimus T, et al. Eculizumab salvage therapy for delayed hemolysis transfusion reaction in sickle cell disease patients. *Blood*. 2016;127(8):1062–1064.
230. Chonat S, Mener A, Verkerke H, et al. Role of complement in alloimmunization and hyperhemolysis. *Curr Opin Hematol*. 2020;27: 406–414.
231. Petz LD, Calhoun L, Shulman IA, et al. The sickle cell hemolytic transfusion reaction syndrome. *Transfusion*. 1997;37:382.
232. Smits HL, Oski FA, Brody JI. The hemolytic crisis of sickle cell disease: the role of glucose-6-phosphate dehydrogenase deficiency. *J Pediatr*. 1969;74:544.
233. Phillips Jr G, Becker B, Keller VA, et al. Hypothyroidism in adults with sickle cell anemia. *Am J Med*. 1992;92:567.
234. Pearson HA, Spencer RP, Cornelius EA. Functional asplenia in sickle-cell anemia. *N Engl J Med*. 1969;281:923.
235. Pearson HA, Gallagher D, Chilcote R, et al. Developmental pattern of splenic dysfunction in sickle cell disorders. *Pediatrics*. 1985;76:392.
236. Zarkowsky HS, Gallagher D, Gill FM, et al. Bacteremia in sickle hemoglobinopathies. *J Pediatr*. 1986;109:579.
237. Leikin SL, Gallagher D, Kinney TR, et al. Mortality in children and adolescents with sickle cell disease. Cooperative Study of Sickle Cell Disease. *Pediatrics*. 1989;84:500.
238. Greene JR, Polk OD, Castro O. Fulminant pneumococcal sepsis in an adult with sickle-cell anemia. *N Engl J Med*. 1984;311:674.
239. Robinson MG, Watson RJ. Pneumococcal meningitis in sickle-cell anemia. *N Engl J Med*. 1966;274:1006.
240. Wilimas JA, Flynn PM, Harris S, et al. A randomized study of outpatient treatment with ceftriaxone for selected febrile children with sickle cell disease. *N Engl J Med*. 1993;329:472.
241. Syrogiannopoulos GA, McCracken Jr GH, Nelson JD. Osteoarticular infections in children with sickle cell disease. *Pediatrics*. 1986;78:1090.
242. Kang SN, Sanghera T, Mangwani J, Paterson JM, Ramachandran M. The management of septic arthritis in children: systematic review of the English language literature. *J Bone Joint Surg Br*. 2009;91(9):1127.
243. Kurtzman GJ, Gascon P, Caras M, et al. B19 parvovirus replicates in circulating cells of acutely infected patients. *Blood*. 1988;71:1448.
244. Quek L, Sharpe C, Dutt N, Height S, Allman M, Awogbade M, et al. Acute human parvovirus B19 infection and nephrotic syndrome in patients with sickle cell disease. *Br J Haematol*. 2010;149:289–291.
245. Cumming V, Ali S, Forrester T. Asymptomatic bacteriuria in sickle cell disease: a cross-sectional study. *BMC Infect Dis*. 2006;6:46.
246. Powars D, Wilson B, Imbus C, et al. The natural history of stroke in sickle cell disease. *Am J Med*. 1978;65:461.
247. Sarnaik SA, Lusher JM. Neurological complications of sickle cell anemia. *Am J Pediatr Hematol Oncol*. 1982;4:386.
248. Debaun MR, Kirkham FJ. Central nervous system complications and management in sickle cell disease. *Blood*. 2016;127:829–838.
249. Debaun American Society of Hematology 2020 guidelines for sickle cell disease: prevention, diagnosis, and treatment of cerebrovascular disease in children and adults. *Blood Adv*. 2020;4:1554–1588.
250. Switzer JA, Hess DC, Nichols FT, et al. Pathophysiology and treatment of stroke in sickle-cell disease: present and future. *Lancet Neurol*. 2006;5:501.
251. Koshy M, Thomas C, Goodwin J. Vascular lesions in the central nervous system in sickle cell disease (neuropathology). *J Assoc Acad Minor Phys*. 1990;1:71.
252. Tuohy AM, McKie V, Manci EA, et al. Internal carotid artery occlusion in a child with sickle cell disease: case report and immunohistochemical study. *J Pediatr Hematol Oncol*. 1997;19:455.
253. Hoppe C, Klitz W, Noble J, et al. Distinct HLA associations by stroke subtype in children with sickle cell anemia. *Blood*. 2003;101:2865.
254. Stockman JA, Nigro MA, Mishkin MM, et al. Occlusion of large cerebral vessels in sickle-cell anemia. *N Engl J Med*. 1972;287:846.
255. Hoppe C, Klitz W, Cheng S, et al. Gene interactions and stroke risk in children with sickle cell anemia. *Blood*. 2004;103:2391.
256. Suzuki J, Takaku A. Cerebrovascular “moyamoya” disease. Disease showing abnormal net-like vessels in base of brain. *Arch Neurol*. 1969;20:288.
257. Ueki K, Meyer FB, Mellinger JF. Moyamoya disease: the disorder and surgical treatment. *Mayo Clin Proc*. 1994;69:749.
258. Kassim AA, Pruthi S, Day M, Rodeghier M, Gindville MC, Brodsky MA, et al. Silent cerebral infarcts and cerebral aneurysms are prevalent in adults with sickle cell anemia. *Blood*. 2016;127:2038–2040.
259. Goldstein LB, Adams R, Alberts MJ, et al. Primary prevention of ischemic stroke: a guideline from the American Heart Association/American Stroke Association Stroke Council: cosponsored by the Atherosclerotic Peripheral Vascular Disease Interdisciplinary Working Group; Cardiovascular Nursing Council; Clinical Cardiology Council; Nutrition, Physical Activity, and Metabolism Council; and the Quality of Care and Outcomes Research Interdisciplinary Working Group. *Circulation*. 2006;113:e873.
260. Schatz J, Finke RL, Kellett JM, et al. Cognitive functioning in children with sickle cell disease: a meta-analysis. *J Pediatr Psychol*. 2002;27:739.
261. Schatz J, White DA, Moinuddin A, et al. Lesion burden and cognitive morbidity in children with sickle cell disease. *J Child Neurol*. 2002;17:891.
262. Nabavizadeh SA, Vossough A, Ichord RN, et al. Intracranial aneurysms in sickle cell anemia: clinical and imaging findings. *J Neurointerv Surg*. 2016; 8(4):434.
263. Birkeland P, Gardner K, Kesse-Adu R, Davies J, Lauritsen J, Rom F. Poulsen, et al. Intracranial aneurysms in sickle-cell disease are associated with the hemoglobin SS genotype but not with moyamoya syndrome. *Stroke*. 2016;47:1710–1713.
264. Adams RJ, McKie VC, Hsu L, et al. Prevention of a first stroke by transfusions in children with sickle cell anemia and abnormal results on transcranial Doppler ultrasonography. *N Engl J Med*. 1998;339:5.
265. Miller ST, Macklin EA, Pegelow CH, et al. Silent infarction as a risk factor for overt stroke in children with sickle cell anemia: a report from the Cooperative Study of Sickle Cell Disease. *J Pediatr*. 2001;139:385.
266. Kirkham FJ, Hewes DK, Prengler M, et al. Nocturnal hypoxaemia and central-nervous-system events in sickle-cell disease. *Lancet*. 2001;357:1656.
267. Powars DR. Sickle cell anemia: beta s-gene-cluster haplotypes as prognostic indicators of vital organ failure. *Semin Hematol*. 1991;28:202.
268. Russell MO, Goldberg HI, Hodson A, et al. Effect of transfusion therapy on arteriographic abnormalities and on recurrence of stroke in sickle cell disease. *Blood*. 1984;63:162.
269. Adams RJ, Brambilla D. Discontinuing prophylactic transfusions used to prevent stroke in sickle cell disease. *N Engl J Med*. 2005;353:2769.
270. DeBaun MR, Gordon M, McKinstry RC, et al. Controlled trial of transfusions for silent cerebral infarcts in sickle cell anemia. *N Engl J Med*. 2014;371:699–710.
271. Ware RE, Davis BR, Schultz WH, et al. Hydroxycarbamide versus chronic transfusion for maintenance of transcranial doppler flow velocities in children with sickle cell anemia-TCD With Transfusions Changing to Hydroxyurea (TWiTCH): a multicentre, open-label, phase 3, non-inferiority trial. *Lancet*. 2016;387(10019):661.
272. Chapman N, Huxley R, Anderson C, et al. Effects of a perindopril-based blood pressure-lowering regimen on the risk of recurrent stroke according to stroke subtype and medical history: the PROGRESS Trial. *Stroke*. 2004;35:116.
273. Pegelow CH, Adams RJ, McKie V, et al. Risk of recurrent stroke in patients with sickle cell disease treated with erythrocyte transfusions. *J Pediatr*. 1995;126:896.
273a. Ware RE, Helms RW. Stroke with transfusions changing to hydroxyurea (SWiTCH). *Blood*. 2012;119:3925–3932.
274. Walters MC, Storb R, Patience M, et al. Impact of bone marrow transplantation for symptomatic sickle cell disease: an interim report. Multicenter investigation of bone marrow transplantation for sickle cell disease. *Blood*. 2000;95:1918.
275. Liu JE, Gzesh DJ, Ballas SK. The spectrum of epilepsy in sickle cell anemia. *J Neurol Sci*. 1994;123:6.
276. Machogu EM, Machado RF. How I treat hypoxia in adults with hemoglobinopathies and hemolytic disorders. *Blood*. 2018;132:170–1780.
277. Castro O, Brambilla DJ, Thorington B, et al. The acute chest syndrome in sickle cell disease: incidence and risk factors. The Cooperative Study of Sickle Cell Disease. *Blood*. 1994;84:643.
278. Godeau B, Schaeffer A, Bachir D, et al. Bronchoalveolar lavage in adult sickle cell patients with acute chest syndrome: value for diagnostic assessment of fat embolism. *Am J Respir Crit Care Med*. 1996;153:1691.
279. Vichinsky EP, Styles LA, Colangelo LH, et al. Acute chest syndrome in sickle cell disease: clinical presentation and course. Cooperative Study of Sickle Cell Disease. *Blood*. 1997;89:1787.
280. Styles LA, Aarsman AJ, Vichinsky EP, et al. Secretory phospholipase A(2) predicts impending acute chest syndrome in sickle cell disease. *Blood*. 2000;96:3276.
281. Gladwin MT, Sachdev V, Jison ML, et al. Pulmonary hypertension as a risk factor for death in patients with sickle cell disease. *N Engl J Med*. 2004;350: 886.
282. Vichinsky EP. Pulmonary hypertension in sickle cell disease. *N Engl J Med*. 2004;350:857.
283. Collins FS, Orringer EP. Pulmonary hypertension and cor pulmonale in the sickle hemoglobinopathies. *Am J Med*. 1982;73:814.
284. Gordeuk VR, Castro OL, Machado RF. Pathophysiology and treatment of pulmonary hypertension in sickle cell disease. *Blood*. 2016;127:820–828.
285. Castro O, Hoque M, Brown BD. Pulmonary hypertension in sickle cell disease: cardiac catheterization results and survival. *Blood*. 2003;101:1257.
286. Adedeji MO, Cespedes J, Allen K, et al. Pulmonary thrombotic arteriopathy in patients with sickle cell disease. *Arch Pathol Lab Med*. 2001;125:1436.

287. Haque AK, Gokhale S, Rampy BA, et al. Pulmonary hypertension in sickle cell hemoglobinopathy: a clinicopathologic study of 20 cases. *Hum Pathol*. 2002;33:1037.
288. Machado RF, Anthi A, Steinberg MH, et al. N-terminal pro-brain natriuretic peptide levels and risk of death in sickle cell disease. *JAMA*. 2006;296:310.
289. Klings An official American Thoracic Society clinical practice guideline: diagnosis, risk stratification, and management of pulmonary hypertension of sickle cell disease. *Am J Respir Crit Care Med*. 2014;189:727–740.
290. Niss O, Quinn CT, Lane A, Daily J, et al. Cardiomyopathy with restrictive physiology in sickle cell disease. *JACC Cardiovasc Imaging*. 2016;9(3):243.
291. Machado RF, Barst RJ, Yovetich NA, et al. Hospitalization for pain in patients with SCD treated with sildenafil for elevated TRV and low exercise capacity. *Blood*. 2011;118:855.
292. Powars D, Weidman JA, Odom-Maryon T, et al. Sickle cell chronic lung disease: prior morbidity and the risk of pulmonary failure. *Medicine (Baltimore)*. 1988;67:66.
293. Samuels MP, Stebbens VA, Davies SC, et al. Sleep related upper airway obstruction and hypoxaemia in sickle cell disease. *Arch Dis Child*. 1992;67: 925.
294. Banerjee S, Owen C, Chopra S. Sickle cell hepatopathy. *Hepatology*. 2001;33:1021.
295. Suddle AR. Management of liver complications in sickle cell disease. *Hematology Am Soc Hematol Educ Program*. 2019;1:345–350.
296. Gardner K, Suddle A, Kane P, O'Grady J, Heaton N, Bomford A, et al. How we treat sickle hepatopathy and liver transplantation in adults. *Blood*. 2014;123:2302–2307.
297. Sheehy TW. Sickle cell hepatopathy. *South Med J*. 1977;70(5):533.
298. Zakaria N, Knisely A, Portmann B, et al. Acute sickle cell hepatopathy represents a potential contraindication for percutaneous liver biopsy. *Blood*. 2003;101:101.
299. Rennels MB, Dunne MG, Grossman NJ, et al. Cholelithiasis in patients with major sickle hemoglobinopathies. *Am J Dis Child*. 1984;138:66.
300. Ware RE, Kinney TR, Casey JR, et al. Laparoscopic cholecystectomy in young patients with sickle hemoglobinopathies. *J Pediatr*. 1992;120:58.
301. Norris WE. Acute hepatic sequestration in sickle cell disease. *J Natl Med Assoc*. 2004;96(9):1235–1239.
302. Buchanan GR, Glader BE. Benign course of extreme hyperbilirubinemia in sickle cell anemia: analysis of six cases. *J Pediatr*. 1977;91:21.
303. Malik A, Merchant C, Rao M, Fiore RP. Rare but lethal hepatopathy-sickle cell intrahepatic cholestasis and management strategies. *Am J Case Rep*. 2015;16:840–843.
304. Hézode C, Colombo M, Bourliére M, et al. C-EDGE IBLD Study Investigators. Elbasvir/grazoprevir for patients with hepatitis C virus infection and inherited blood disorders: a phase III study. *Hepatology*. 2017;66(3):736–745.
305. Swaim MW, Agarwal S, Rosse WF. Successful treatment of hepatitis C in sickle-cell disease. *Ann Intern Med*. 2000;133:750.
306. European Association for the Study of the Liver. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol*. 2017;67(2):370-398.
307. Kindscher JD, Laurin J, Delcore R, et al. Liver transplantation in a patient with sickle cell anemia. *Transplantation*. 1995;60:762.
308. Oteng-Ntim E, Meeks D, Seed PT, et al. Adverse maternal and perinatal outcomes in pregnant women with sickle cell disease: systematic review and meta-analysis. *Blood*. 2015;125:3316–3325.
309. Koshy M. Sickle cell disease and pregnancy. *Blood Rev*. 1995;9:157.
310. Hassell K. Pregnancy and sickle cell disease. *Hematol Oncol Clin North Am*. 2005;19:903.
311. Oteng-Nim E, Ayensah B, Knight M, Howard J. Pregnancy outcome in patients with sickle cell disease in the UK—a national cohort study comparing sickle cell anaemia (HbSS) with HbSC disease. *Br J Haematol*. 2015;169:129–137.
312. Sickle Cell Society. Pregnancy, contraception and fertility. In: Standards for the Clinical Care of Adults with Sickle Cell Disease in the UK. London: Sickle Cell Society; 2008. p.59c
313. Smith-Whitley K. Complications in pregnant women with sickle cell disease. *Hematology Am Soc Hematol Educ Program*. 2019;1:359–366.
314. Jones S, Shickle DA, Goldstein AR, et al. Acceptability of antenatal diagnosis for sickle-cell disease among Jamaican mothers and female patients. *West Indian Med J*. 1988;37:12.
315. Akolekar R, Beta J, Picciarelli G, Ogilvie C, D'Antonio F. Procedure-related risk of miscarriage following amniocentesis and chorionic villus sampling: a systematic review and meta-analysis. *Ultrasound Obstet Gynecol*. 2015;45: 16–26.
316. Manchikanti A, Grimes DA, Lopez LM, et al. Steroid hormones for contraception in women with sickle cell disease. *Cochrane Database Syst Rev*. 2007(2):CD006261.
317. Powars DR, Elliott-Mills DD, Chan L, et al. Chronic renal failure in sickle cell disease: risk factors, clinical course, and mortality. *Ann Intern Med*. 1991;115:614.
318. Sharpe CC, Thein SL. How I treat renal complications in sickle cell disease. *Blood*. 2014;123:3720–3726.
319. Vichinsky E. Chronic organ failure in adult sickle cell disease. *Hematology Am Soc Hematol Educ Program*. 2017(1):435.
320. Molitierno Jr JA, Carson CC. III: Urologic manifestations of hematologic disease sickle cell, leukemia, and thromboembolic disease. *Urol Clin North Am*. 2003;30:49.
321. Abbott KC, Hypolite IO, Agodoa LY. Sickle cell nephropathy at end-stage renal disease in the United States: patient characteristics and survival. *Clin Nephrol*. 2002;58:9.
322. Wesson DE. The initiation and progression of sickle cell nephropathy. *Kidney Int*. 2002;61(6):2277.
323. Perneger TV, Whelton PK, Klag MJ. Risk of kidney failure associated with the use of acetaminophen, aspirin, and nonsteroidal antiinflammatory drugs. *N Engl J Med*. 1994;331:1675.
324. Day TG, Drašar ER, Fulford T, Sharpe CC, Thein SL. Association between hemolysis and albuminuria in adults with sickle cell anemia. *Haematologica*. 2012;97:201–205.
325. Nebor D, Broquere C, Brudey K, Mougenel D, Tarer V, Connes P, et al. Alpha-thalassemia is associated with a decreased occurrence and a delayed age-at-onset of albuminuria in sickle cell anemia patients. *Bld Cells Mol Dis*. 2010;45:154–158.
326. Kruzel-Davila E, Wasser WG, Skorecki K. APOL1 nephropathy: a population genetics and evolutionary medicine detective story. *Semin Nephrol*. 2017;37(6):490.
327. Xu JZ, Thein SL. The carrier state for sickle cell disease is not completely harmless. *Haematologica*. 2019;104:1106–1111.
328. Stallworth JR, Tripathi A, Jerrell JM. Prevalence, treatment, and outcomes of renal conditions in pediatric sickle cell disease. *South Med J*. 2011;104(11):752.
329. Alvarez Renal medullary carcinoma and sickle cell trait: a systematic review. *Pediatr Blood Cancer*. 2015;62(10):1694-–1699.
330. Pariser S, Katz A. Treatment of sickle cell trait hematuria with oral urea. *J Urol*. 1994;151:401.
331. Falk RJ, Scheinman J, Phillips G, et al. Prevalence and pathologic features of sickle cell nephropathy and response to inhibition of angiotensin-converting enzyme. *N Engl J Med*. 1992;326:910.
332. Foucan L, Bourhis V, Bangou J, et al. A randomized trial of captopril for microalbuminuria in normotensive adults with sickle cell anemia. *Am J Med*. 1998;104:339.
333. Batlle D, Itsarayoungyuen K, Arruda JA, et al. Hyperkalemic hyperchloremic metabolic acidosis in sickle cell hemoglobinopathies. *Am J Med*. 1982;72:188.
334. Davis Jr CJ, Mostofi FK, Sesterhenn IA. Renal medullary carcinoma. The seventh sickle cell nephropathy. *Am J Surg Pathol*. 1995;19:1.
335. Anele UA, Le BV, Resar LM, Burnett AL. How I treat priapism. *Blood*. 2015;125:3551–3558.
336. Adeyoju AB, Olujohungbe AB, Morris J, et al. Priapism in sickle-cell disease; incidence, risk factors and complications—An international multicentre study. *BJU Int*. 2002;90:898.
337. Baruchel S, Rees J, Bernstein ML, et al. Relief of sickle cell priapism by hydralazine. Report of a case. *Am J Pediatr Hematol Oncol*. 1993;15:115.
338. Dahm P, Rao DS, Donatucci CF. Antiandrogens in the treatment of priapism. *Urology*. 2002;59:138.
339. Levine LA, Guss SP. Gonadotropin-releasing hormone analogues in the treatment of sickle cell anemia-associated priapism. *J Urol*. 1993;150:475.
340. Rogers ZR. Priapism in sickle cell disease. *Hematol Oncol Clin North Am*. 2005;19:917 viii.
341. Fowler Jr JE, Koshy M, Strub M, et al. Priapism associated with the sickle cell hemoglobinopathies: prevalence, natural history and sequelae. *J Urol*. 1991;145:65.
342. Emerson GG, Lutty GA. Effects of sickle cell disease on the eye: clinical features and treatment. *Hematol Oncol Clin North Am*. 2005;19:957 ix.
343. Stevens MC, Padwick M, Serjeant GR. Observations on the natural history of dactylitis in homozygous sickle cell disease. *Clin Pediatr (Phila)*. 1981;20:311.
344. Milner PF, Kraus AP, Sebes JI, et al. Sickle cell disease as a cause of osteonecrosis of the femoral head. *N Engl J Med*. 1999;325(21):1476–1481.
345. Adesina O, Brunson A, Keegan THM, Wun T. Osteonecrosis of the femoral head in sickle cell disease: prevalence, comorbidities, and surgical outcomes in California. *Blood Adv*. 2017;1(16):1287–1295.
346. Aguilar C, Vichinsky E, Neumayr L. Bone and joint disease in sickle cell disease. *Hematol Oncol Clin North Am*. 2005;19:929 viii.
347. Jack CM, Howard J, Aziz ES, Kesse-Adu R, Bankes MJ. Cementless total hip replacements in sickle cell disease. *Hip Int*. 2016;26(2):186–192.
348. Ilyas I, Alrumaih HA, Rabbani S. Noncemented total hip arthroplasty in sickle-cell disease: long-term results. *J Arthroplasty*. 2018;33(2):477–481.
349. Hutchinson RM, Merrick MV, White JM. Fat embolism in sickle cell disease. *J Clin Pathol*. 1973;26:620.

350. Clare A, FitzHenley M, Harris J, et al. Chronic leg ulceration in homozygous sickle cell disease: the role of venous incompetence. *Br J Haematol*. 2002;119:567.
351. Kato GJ, McGowan V, Machado RF, et al. Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension, and death in patients with sickle cell disease. *Blood*. 2006;107:2279.
352. Koshy M, Entsuah R, Koranda A, et al. Leg ulcers in patients with sickle cell disease. *Blood*. 1989;74:1403.
353. Cackovic M, Chung C, Bolton LL, et al. Leg ulceration in the sickle cell patient. *J Am Coll Surg*. 1998;187:307.
354. Koshy M, Entsuah R, Koranda A, et al. Leg ulcers in patients with sickle cell disease. *Blood*. 1989;74:1403.
355. Schleucher R, Gaessler M, Knobloch J. Rapid healing of a late diagnosed sickle cell leg ulcer using a new combination of treatment methods. *J Wound Care*. 2007;16:197.
356. Keidan AJ, Stuart J. Rheological effects of bed rest in sickle cell disease. *J Clin Pathol*. 1987;40:1187.
357. Sher GD, Olivieri NF. Rapid healing of chronic leg ulcers during arginine butyrate therapy in patients with sickle cell disease and thalassemia. *Blood*. 1994;84:2378.
358. Covitz W, Espeland M, Gallagher D, et al. The heart in sickle cell anemia. The Cooperative Study of Sickle Cell Disease (CSSCD). *Chest*. 1995;108:1214.
359. Sachdev V, Rosing DR, Thein SL. Cardiovascular complications of sickle cell disease. *Trends Cardiovasc Med.* 20201; 31: 187-193.
360. Miller DM, Winslow RM, Klein HG, et al. Improved exercise performance after exchange transfusion in subjects with sickle cell anemia. *Blood*. 1980;56:1127.
361. Martin CR, Johnson CS, Cobb C, et al. Myocardial infarction in sickle cell disease. *J Natl Med Assoc*. 1996;88:428.
362. Romero Mestre JC, Hernandez A, Agramonte O, et al. Cardiovascular autonomic dysfunction in sickle cell anemia: a possible risk factor for sudden death? *Clin Auton Res*. 1997;7:121.
363. Hassell KL, Eckman JR, Lane PA. Acute multiorgan failure syndrome: a potentially catastrophic complication of severe sickle cell pain episodes. *Am J Med*. 1994;96:155.
364. Kumar S, Powars D, Allen J, et al. Anxiety, self-concept, and personal and social adjustments in children with sickle cell anemia. *J Pediatr*. 1976;88:859.
365. Vichinsky EP, Johnson R, Lubin BH. Multidisciplinary approach to pain management in sickle cell disease. *Am J Pediatr Hematol Oncol*. 1982;4:328.
366. Platt OS, Rosenstock W, Espeland MA. Influence of sickle hemoglobinopathies on growth and development. *N Engl J Med*. 1984;311:7.
367. Hankins JS, Ware RE, Rogers ZR, et al. Long-term hydroxyurea therapy for infants with sickle cell anemia: the HUSOFT extension study. *Blood*. 2005;106:2269.
368. Prasad AS, Cossack ZT. Zinc supplementation and growth in sickle cell disease. *Ann Intern Med*. 1984;100:367.
369. Heyman MB, Vichinsky E, Katz R, et al. Growth retardation in sickle-cell disease treated by nutritional support. *Lancet*. 1985;1:903.
370. Badaloo AV, Singhal A, Forrester TE, et al. The effect of splenectomy for hypersplenism on whole body protein turnover, resting metabolic rate and growth in sickle cell disease. *Eur J Clin Nutr*. 1996;50:672.
371. Ogunsile FJ, Bediako SM, Nelson J, Cichowitz C, Yu T, Patrick Carroll C, et al. Metabolic syndrome among adults living with sickle cell disease. *Blood Cells Mol Dis*. 2019;74:25–29.
372. Ballas SK. The sixth vital sign: body mass index in patients with sickle cell disease. *J Clin Med Res*. 2017;9:889–890.
373. Tsaras G, Owusu-Ansah A, Boateng FO, Amoateng-Adjepong Y. Complications associated with sickle cell trait: a brief narrative review. *Am J Med*. 2009;122:507–512.
374. Castro O, Rana SR, Bang KM, Scott RB. Age and prevalence of sickle-cell trait in a large ambulatory population. *Genet Epidemiol*. 1987;4(4):307.
375. Lane PA, Githens JH. Splenic syndrome at mountain altitudes in sickle cell trait. *Its occurrence in nonblack persons. JAMA*. 1985;253:2251.
376. Diggs LW. The sickle cell trait in relation to the training and assignment of duties in the armed forces: III. Hyposthenuria, hematuria, sudden death, rhabdomyolysis, and acute tubular necrosis. *Aviat Space Environ Med*. 1984;55:358.
377. Naik RP, Smith-Whitley K, Hassell KL, et al. Clinical outcomes associated with sickle cell trait: a systematic review. *Ann Intern Med*. 2018;169(9): 619.
378. Monplaisir N, Merault G, Poyart C, Rhoda MD, Craescu C, Vidaud M, et al. Hemoglobin S antilles: a variant with lower solubility than hemoglobin S and producing sickle cell disease in heterozygotes. *Proc Natl Acad Sci U S A*. 1986; 83:9363–9367.
379. Geva A, Clark JJ, Zhang Y, Popowicz A, Manning JM, Neufeld EJ. Hemoglobin Jamaica plain—a sickling hemoglobin with reduced oxygen affinity. *N Engl J Med*. 2004;351:1532–1538.
380. Cohen-Solal M, Préhu C, Wajcman H, et al. A new sickle cell disease phenotype associating HbS trait, severe pyruvate kinase deficiency and an alpha2 globin gene variant. *Br J Haematol*. 1998;103(4):950.
381. Alli N, Coetzee M, Louw V, van Rensburg B, Rossouw G, Thompson L, et al. Sickle cell disease in a carrier with pyruvate kinase deficiency. *Hematology*. 2008;13:369–372.
382. Summarell CC, Sheehan VA. Original research: use of hydroxyurea and phlebotomy in pediatric patients with hemoglobin SC disease. *Exp Biol Med*. 2016;241(7):737.
383. Lionnet F, Hammoudi N, Stojanovic KS, Avellino V, Grateau G, Girot R, et al. Hemoglobin SC disease complications: a clinical study of 179 cases. *Haematologica*. 2012;97:1136–1141.
384. Gonzalez-Redondo JM, Kutlar A, Kutlar F, et al. Molecular characterization of HbS(C) β-thalassemia in American Blacks. *Am J Hematol*. 1991;38:9.
385. Zimmerman SA, O'Branski EE, Rosse WF, Ware RE. Hemoglobin S/O(Arab): thirteen new cases and review of the literature. *Am J Hematol*. 1999;60(4):279.
386. Serjeant GR, Sommereux AM, Stevenson M, et al. Comparison of sickle cell-β° thalassemia and homozygous sickle cell disease. *Br J Haematol*. 1979;41:83.
387. Christakis J, Vavatsi N, Hassapopoulou H, et al. A comparison of sickle cell syndromes in northern Greece. *Br J Haematol*. 1991;77:386.
388. Gardner K, Fulford T, Silver N, Rooks H, Angelis N, Allman M, et al. g(HbF): a genetic model of fetal hemoglobin in sickle cell disease. *Blood Adv*. 2018;2:235–239.
389. Friedman S, Schwartz E, Ahern E, et al. Variations in globin chain synthesis in hereditary persistence of fetal hemoglobin. *Br J Haematol*. 1976;32:357.
390. Serjeant GR, Serjeant BE. Comparison of sickle cell-β° thalassemia and sickle cell-β+ thalassemia in Black populations. In: Cao A, Carcassi U, Rowley PT, eds. *Thalassemia: Advances in Detection and Treatment. Birth Defects Original Article Series*. New York: Alan R. Liss; 1982:233.
391. Embury SH. The clinical pathophysiology of sickle cell disease. *Annu Rev Med*. 1986;37:361.
392. Wood WG. Hereditary persistence of fetal hemoglobin and delta beta thalassemia. In: Steinberg MH, Forget BG, Higgs DR, Nagel RL, eds. *Disorders of Hemoglobin: Genetics, Pathophysiology, and Clinical Management*. Cambridge, UK: Cambridge University Press; 2001.
393. Dozy AM, Kan YW, Embury SH, et al. Alpha-globin gene organisation in blacks precludes the severe form of alpha-thalassaemia. *Nature*. 1979;280: 605.

CHAPTER 44

HEMOGLOBIN VARIANTS ASSOCIATED WITH HEMOLYTIC ANEMIA, ALTERED OXYGEN AFFINITY, AND METHEMOGLOBINEMIAS

Edward J. Benz, Jr. and Benjamin L. Ebert

Hemoglobinopathies are inherited diseases caused primarily by mutations affecting the globin genes. Nearly 1000 mutations are known to alter the structure, expression, or developmental regulation of individual globin genes and the hemoglobins that they encode. Of these, only a few produce clinical disease. Many are highly instructive for students of gene structure, function, and regulation, but further consideration of most is not warranted in a clinically oriented textbook. The gene mutations that cause sickle cell anemia and the thalassemia syndromes are by far the most important mutations that cause clinical morbidity, in terms of both the complexity of the clinical syndromes they cause and the number of patients affected. These conditions are considered in detail in other chapters (see Chapters 41, 42, and 43). This chapter reviews additional abnormalities of the hemoglobin molecule that produce clinical syndromes. Even though each variant is uncommon, these hemoglobinopathies represent, in the aggregate, important problems for hematologists because they must be considered as possible causes for conditions about which hematologists are often consulted: hemolytic anemia, cyanosis, polycythemia, jaundice, premature gallstones, rubor, hepatosplenomegaly, and reticulocytosis. In some patients, secondary hematologic complications such as hypercoagulable states are also encountered.

The major inherited hemoglobinopathies producing clinical symptoms (other than sickle cell anemia and thalassemias) can be classified as those hemoglobins exhibiting altered solubility (unstable hemoglobins), hemoglobins with increased oxygen affinity, hemoglobins with decreased oxygen affinity, and methemoglobins (Table 44.1). A few acquired conditions in which toxic modifications of the hemoglobin molecule are important (e.g., acquired methemoglobinemia and carbon monoxide poisoning) also merit consideration.

The sections that follow emphasize hemoglobinopathies that produce the most severe or dramatic alterations in clinical phenotype and those in which a single clinical abnormality (e.g., hemoglobin precipitation) predominates. However, it is important to emphasize at the outset that, although more than 100 mutations affect solubility or affinity, only a few are clinically important. The abnormal functional properties of most mutant hemoglobins can be readily detected in sophisticated research laboratories, but only a few mutant hemoglobins produce laboratory or clinical abnormalities relevant to clinical practice. Moreover, many mutations are pleiotropic, affecting several functional properties of the hemoglobin molecule. Thus a single mutation can increase oxygen affinity and reduce solubility or produce methemoglobinemia and reduce solubility.

Table 44.2 summarizes the major forms of structurally abnormal hemoglobin, with examples. This table serves as a point of reference for the remaining sections of the chapter.

UNSTABLE HEMOGLOBINS

Unstable hemoglobins are hemoglobins exhibiting reduced solubility or higher susceptibility to oxidation of amino acid residues within the individual globin chains. More than 100 unique unstable hemoglobin mutants have been documented. Most exhibit only mild instability in in vitro laboratory tests and are associated with minimal clinical manifestations. Both α- and β-globin variants can cause this condition. However, approximately 75% of the mutations described are β-globin variants. This probably reflects the potential for α-globin variants to exert pathologic effects in utero. Clinical symptoms of unstable hemoglobins also depend, in part, on the quantitative proportion of the abnormal hemoglobin. Because the α-globin genes are duplicated, mutations in an individual locus generally produce only 25% to 35% abnormal globin. By contrast, a simple heterozygote at the single β-globin locus usually produces approximately 50% of the abnormal variant.

The mutations that impair hemoglobin solubility usually disrupt hydrogen bonding or the hydrophobic interactions that either retain the heme moiety within the heme-binding pockets or hold the tetramer together (Fig. 44.1). Some alter the helical segments (e.g., hemoglobin [Hb] Geneva [$\beta^{28\text{Leu}\rightarrow\text{Pro}}$]), others weaken contact points between the α and β subunits (e.g., Hb Philadelphia [$\beta^{35\text{Tyr}\rightarrow\text{Phe}}$]), and still others derange interactions of the hydrophobic pockets of the globin subunits with heme (e.g., Hb Köln [$\beta^{98\text{Val}\rightarrow\text{Met}}$]). The common pathway to reduced solubility invariably involves weakening of the binding of heme to globin. Actual loss of heme groups can occur; for example, in Hb Gun Hill, five amino acids, including the F8 histidine that is crucial for heme binding, are deleted. In other cases, mutations that introduce prolines into helical segments disrupt the helices and interfere with normal folding of the polypeptide around the heme group. Another feature of these mutations is disruption of the integrity of the tetrameric structure of globin chains. Only the intact hemoglobin tetramer can remain dissolved at the high concentrations that must be achieved within the circulating red blood cell (see Chapters 34 and 41).

Pathophysiology of Unstable Hemoglobin Disorders

The mechanisms by which unstable hemoglobin mutations produce hemoglobin precipitation remain incompletely understood; however, the major outlines of the process have been described (Fig. 44.2). The fundamental step in pathogenesis appears to be derangement of the normal linkages between heme and globin. Loss of appropriate globin chain folding and interaction ultimately destabilizes the heme–globin linkage and likely leads to partial proteolysis of the chain, thereby releasing heme from that linkage. Once freed from its cleft, heme probably binds nonspecifically to other regions of the globin molecule, forming precipitated hemichromes, these lead to further denaturation and aggregation of the globin subunits. These form a precipitate containing α- and β-globin chains, globin fragments, and heme, called the *Heinz body*.

Heinz bodies interact with delicate red blood cell membrane components (see Chapter 46), thereby reducing red blood cell deformability. These rigid cells tend to be detained in the splenic microcirculation and “pitted,” reflecting attempts by the splenic macrophages to remove the Heinz bodies. Red blood cell damage can be

TABLE 44.1 Classification of Hemoglobinopathies

Structural hemoglobinopathies: hemoglobins with altered amino acid sequences that result in deranged function or altered physical or chemical properties

Abnormal Hemoglobin Polymerization: HbS

Altered Oxygen Affinity

High affinity: polycythemia
Low affinity: cyanosis, pseudoanemia

Hemoglobins That Oxidize Readily

Unstable hemoglobins, hemolytic anemia, jaundice
M hemoglobins: methemoglobinemia, cyanosis

Thalassemias: Defective Production of Globin Chains

α-Thalassemias
β-Thalassemias
δβ-, γδβ-, αβ-Thalassemias
Structural hemoglobinopathies: structurally abnormal Hb associated with coinherited thalassemia phenotype
HbE
Hb Constant Spring
Hb Lepore

Hereditary Persistence of Fetal Hemoglobin: Persistence of High Levels of HbF Into Adult Life

Pancellular: all red blood cells contain elevated HbF levels
Nondeletion forms
Deletion forms
Hb Kenya
Heterocellular: only specific subpopulation of red blood cells contains elevated levels of HbF

"Acquired Hemoglobinopathies"

Methemoglobin due to toxic exposures
Sulfhemoglobin due to toxic exposures
Carboxyhemoglobin
HbH in erythroleukemia
Elevated HbF in states of erythroid stress and bone marrow dysplasia, usually heterocellular

Hb, Hemoglobin.

TABLE 44.2 Mutations Producing Abnormal Hemoglobin Molecules[a]

Residue	Mutation	Common Name(s)	Molecular Pathology
Abnormal Solubility			
β6	Glu→Val	S	Polymerization
β6	Glu→Lys	C	Crystallization
β121	Glu→Gln	D-Los Angeles, D-Punjab	Increases polymer in S/D heterozygote
β121	Glu→Lys	O-Arab	Increases polymer in S/O heterozygote
Increased Oxygen Affinity			
α92	Arg→Gln	J-Capetown	Stabilizes R state
α141	Arg→His	Suresnes	Eliminates bond to Asn 126 in T state
β89	Ser→Asn	Creteil	Weakens bonds in T state
β99	Asp→Asn	Kempsey	Breaks T state intersubunit bonds
Decreased Oxygen Affinity			
α94	Asp→Asn	Titusville	Alters R state intersubunit bonds
β102	Asn→Thr	Kansas	Breaks R state intersubunit bonds
β102	Asn→Ser	Beth Israel	Breaks R state intersubunit bonds
Methemoglobin			
α58	His→Tyr	M-Boston, M-Osaka	Heme liganded to Tyr not His
α87	His→Tyr	M-Iwate	Heme liganded to both His and Tyr
β28	Leu→Gln	St Louis	Opens heme pocket
β63	His→Tyr	M-Saskatoon	Tyr ligand stabilizes ferriheme
β67	Val→Glu	M-Milwaukee-I	Negative charge stabilizes ferriheme
β92	His→Tyr	M-Hyde Park	Bond of His to heme disrupted
Unstable			
α43	Phe→Val	Torino	Loss of heme contact
v94	Asp→Tyr	Setif	Alters subunit contacts
β28	Leu→Gln	St Louis	Polar group in heme pocket
β35	Tyr→Phe	Philadelphia	Loss of dimer bond favors precipitation
β42	Phe→Ser	Hammersmith	Loss of heme
β63	His→Arg	Zurich	Opens heme pocket
β88	Leu→Pro	Santa Ana	Disrupts helix
β91	Leu→Pro	Sabine	Disrupts helix
β91-95	Deletion	Gun Hill	Shortens F helix
β98	Val→Met	Köln	Alters heme contact

[a]Partial list includes some of the most widely studied hemoglobin structural mutations.
Modified from Dickerson RE, Geis I. *Hemoglobin: Structure, Function, Evolution, and Pathology.* Menlo Park, CA: Benjamin-Cummings; 1983. Copyright Irving Geis.

aggravated by the release of free heme into the red blood cell. Several biochemical perturbations correlate with the presence of free heme, such as generation of reactive oxidants (i.e., hydrogen peroxide, superoxide, and hydroxyl radicals). The end result of this process is premature destruction of the red blood cell, producing hemolytic anemia.

Individual unstable hemoglobins vary in their propensity to generate Heinz bodies and hemolysis. For example, Hb Zurich exhibits relatively mild insolubility. Hemolysis is minimal in nonstressed patients with this variant and becomes clinically apparent only in the presence of additional oxidant stresses, such as infection, fever, or the ingestion of oxidant agents. Because of the propensity of unstable hemoglobins to be hypersensitive to oxidation, some patients with unstable hemoglobins can exhibit episodic appearance or aggravation hemolysis in response to many of the same oxidative stressors as those exacerbating the clinical phenotype of glucose-6-phosphate dehydrogenase (G6PD)-deficient patients (see Chapters 45 and 48).

Patterns of Inheritance and Clinical Manifestations

Unstable hemoglobins are usually inherited as autosomal dominant disorders. However, the rate of spontaneous mutation appears to be high, so the absence of affected parents or siblings does not rule

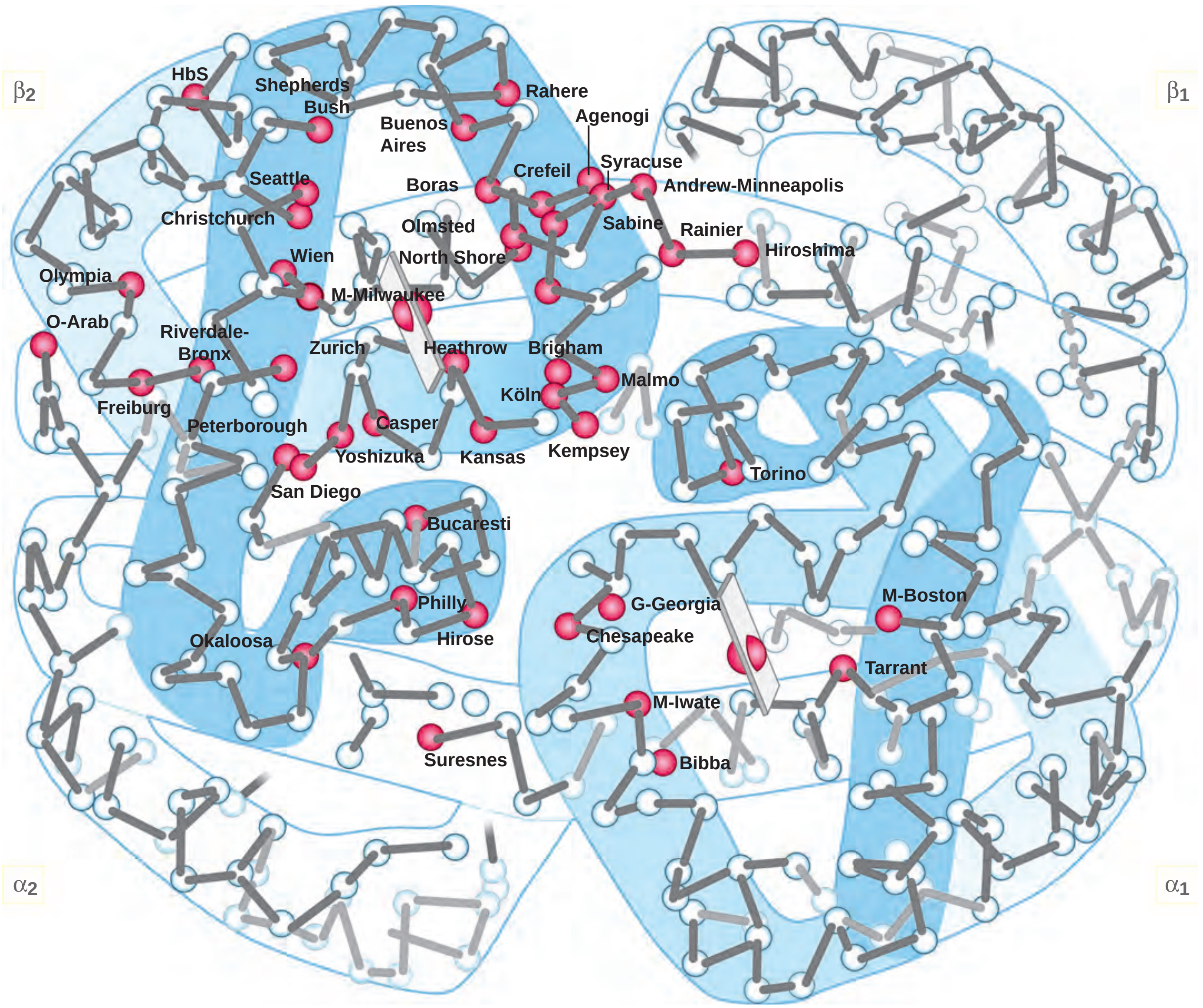

β2
HbS
Shepherds Bush
Rahere
β1
Agenogi
Buenos Aires
Crefeil
Syracuse
Boras
Andrew-Minneapolis
Seattle
Christchurch
Olmsted
Sabine
Rainier
Wien
North Shore
Hiroshima
Olympia
M-Milwaukee
O-Arab
Riverdale-Bronx
Zurich
Heathrow
Brigham
Malmo
Köln
Freiburg
Casper
Peterborough
Kempsey
Yoshizuka
Kansas
Torino
San Diego
Bucaresti
M-Boston
G-Georgia
Philly
Okaloosa
Hirose
Chesapeake
Tarrant
M-Iwate
Suresnes
Bibba
α2
α1

Figure 44.1 HEMOGLOBIN TETRAMER SHOWING THE POSITION OF THE MORE COMMON, CLINICALLY SIGNIFICANT HEMOGLOBIN MUTANTS. Most of those that have been described occur on the β-chain at invariant residue sites, near critical intermolecular contacts, or in proximity to the prosthetic heme-binding site. *(Modified from Dickerson RE, Geis I. Hemoglobin: Structure, Function, Evolution, and Pathology. Menlo Park, CA: Benjamin-Cummings; 1983. Copyright Irving Geis.)*

out the presence of an unstable hemoglobin in an individual family. Nonetheless, the presence of a positive family history can be a useful adjunct to diagnosis and should provoke consideration of an unstable hemoglobin as the cause of the familial hemolytic diathesis.

The clinical syndrome associated with unstable hemoglobin disorders is often called *congenital Heinz body hemolytic anemia*. This term derives from the fact that only the most severe cases were detected before the widespread availability of sophisticated methods for detecting and characterizing abnormal hemoglobins. Clinical manifestations are highly variable, ranging from a virtually asymptomatic state in the absence of environmental stressors to severe hemolytic anemia manifesting at birth. Patients with chronic hemolysis present with variable degrees of typical symptoms, including anemia, reticulocytosis, hepatosplenomegaly, jaundice, leg ulcers, and a propensity toward premature biliary tract disease.

For Hb variants with a given degree of reduced solubility, the degree of anemia may fluctuate because some of these variants also exhibit altered oxygen affinity. Thus Hb Köln has increased oxygen affinity, resulting in relatively higher levels of tissue hypoxia and erythropoietin stimulation at any given level of hematocrit (see Diagnosis); therefore patients with Hb Köln tend to have higher hematocrit levels than expected on the basis of hemolytic severity because of increased erythropoietin stimulation. By contrast, Hb Hammersmith exhibits decreased oxygen affinity, improving oxygen delivery and allowing patients to function at a lower hematocrit level (see low affinity variants later in this chapter). Hb Zurich possesses, for complex molecular reasons, a higher-than-normal affinity for carbon monoxide. A high carbon monoxy–Hb level develops in patients with Hb Zurich who also smoke. Binding of carbon monoxide protects Hb Zurich from denaturation, thus reducing hemolysis, so these people tend to exhibit lesser degrees of hemolytic anemia than do nonsmoking relatives.

Diagnosis

The presence of an unstable hemoglobin should be suspected in patients with one or more stigmata of accelerated red blood cell

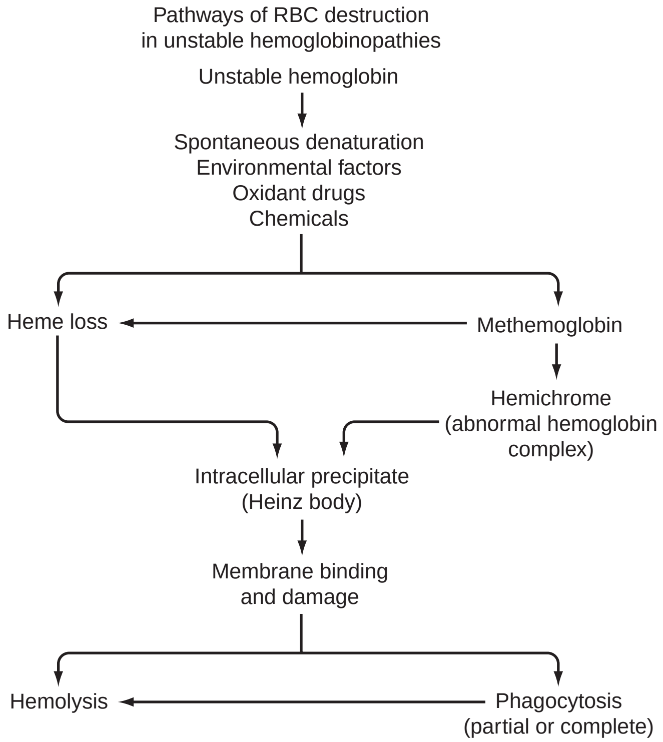


Figure 44.2 PRESUMED MECHANISMS BY WHICH DENATURATION OF HEMOGLOBIN LEADS TO ERYTHROCYTE DESTRUCTION. The rate of travel through the various pathways probably differs for the different hemoglobin variants and for a variety of stresses to which the protein is subjected. *RBC*, Red blood cell. *(From Wynngaarden JB, Smith LH Jr, Bennett JC, eds. Cecil Textbook of Medicine. Philadelphia: WB Saunders; 1992.)*

destruction: chronic or intermittent hemolytic anemia or jaundice, premature development of bilirubin gallstones or biliary tract disease (as a result of accelerated red blood cell turnover), unexplained reticulocytosis, or bouts of intermittent symptoms that can be related to exposure to oxidant drugs or infections. Other suggestive symptoms include dark urine, transient jaundice, and leg ulcers. It is important to remember that significant anemia may or may not be present despite hemolysis brisk enough to produce the other signs and symptoms of accelerated red cell destruction, because of compensatory erythroid hyperplasia, especially in younger otherwise healthy patients.

Laboratory diagnosis depends on identification of a mutant hemoglobin that precipitates more easily than normal hemoglobin. The peripheral blood smear may or may not show evidence of hemolysis (i.e., poikilocytosis, polychromasia, or shift cells; Fig. 44.3A). The morphologic evidence for precipitated hemoglobin is the Heinz body, the intraerythrocytic inclusion body detected by staining the peripheral blood smear with a supravital dye, such as brilliant cresyl blue or new methylene blue (see Fig. 44.3B and C). The spleen removes Heinz bodies efficiently, especially if hemolysis is not particularly acute or brisk. Thus Heinz bodies may not be demonstrable at all times. Two provocative laboratory maneuvers are used to aid detection, both of which unmask the tendency of unstable hemoglobins to precipitate: the heat instability test (heating of a hemoglobin solution to 50°C) or the isopropanol instability test (insolubility in 17% isopropanol).

Hemoglobin electrophoresis should be performed but *should not be relied on as the major diagnostic criterion for ruling in or ruling out a hemoglobinopathy*. Many amino acid substitutions that have a profound effect on solubility do not change the overall charge on the hemoglobin molecule. For example, Hb Köln, the most common of the unstable hemoglobin mutations, arises from a mutation changing the valine at position 98 to a methionine. This mutation is electrically neutral; it does not alter electrophoretic mobility. Therefore these variants do not form an abnormal band on an electrophoresis gel. Demonstration of an abnormal band would clearly add strong evidence in support of the diagnosis. However, a normal electrophoretogram should never be regarded as strong evidence against the presence of a mutant hemoglobin, especially if the clinical picture or family history otherwise supports the diagnosis. Mass spectrometry analysis of hemoglobin and direct globin gene sequencing are supplanting electrophoresis as diagnostic strategies. They usually provide unambiguous identification of a sequence abnormality. However, electrophoresis is still in use in many clinical settings. Thus the aforementioned precautions in interpretation are still worth noting.

Additional sophisticated analyses of hemoglobin can be obtained from reference laboratories if detailed characterization seems warranted. For example, abnormal hemoglobin or globin bands migrating to novel positions on an isoelectric focusing gel can result from hemoglobin or globin moieties lacking heme in groups. When heme is added to the sample and the proteins are reanalyzed, these bands disappear. This behavior is nearly diagnostic of an unstable variant.

Detection of unstable hemoglobins is occasionally compromised by the selective precipitation of the unstable variant into Heinz bodies. Because most patients are heterozygotes, this phenomenon greatly reduces the apparent percentage of the variant in soluble form. Thus even a variant possessing altered electrophoretic mobility may be very difficult to detect. Indeed, some unstable hemoglobins, such as Hb Geneva or Hb Terre Haute, are so unstable that no mutant gene product can be detected in the steady state. These abnormal hemoglobins actually produce a thalassemic phenotype. They may be detectable only by isotope labeling studies or direct analysis of the globin genes.

The amino acid sequence predicted from genetic sequencing may rarely be inaccurate because of posttranslational conversion of the substituted residue into one causing an unstable hemoglobin. For example, in the first reported case of congenital Heinz body hemolytic anemia due to Hb Bristol, the DNA sequence predicts a valine-to-methionine substitution at β67. Through posttranslational

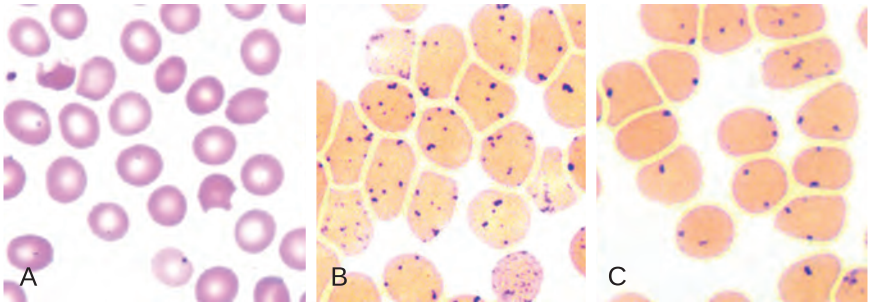


Figure 44.3 UNSTABLE HEMOGLOBINS; PERIPHERAL BLOOD SMEAR AND HEINZ BODY PREPARATION. The peripheral smear (A) shows "bite" cells with pitted-out semicircular areas of the red blood cell membrane as a result of removal of Heinz bodies by macrophages in the spleen. The Heinz body preparation (B) shows increased Heinz bodies in the same specimen, when compared with a control (C).

modification, the methionine is altered to aspartate, a hydrophilic residue that disrupts the heme pocket.

The differential diagnosis of unstable hemoglobin variants is usually straightforward if this general category of hemolytic disorders is suspected. The most common form of G6PD deficiency can also manifest with bouts of intermittent or chronic hemolysis exacerbated by oxidant drugs or infection (see Chapter 45). This diagnosis should be considered, as should other causes of chronic or intermittent hemolytic anemia, including red blood cell membrane disorders (e.g., hereditary spherocytosis) or immune hemolytic anemias (see Chapters 46 and 47). Spherocytes are relatively rare in patients with unstable hemoglobin disorders; this is sometimes a useful discriminant.

Management

The severity of the clinical complications of unstable hemoglobins varies enormously. Many patients can be managed adequately by observation and education to avoid agents that provoke hemolysis. Some patients require transfusions during bouts of severe acute hemolytic anemia. Patients who have significant morbidity because of chronic anemia or repeated episodes of severe hemolysis should be considered candidates for splenectomy, especially if hypersplenism has developed. Children with severe hemolysis may require transfusion support until they are old enough (at least 3 or 4 years of age) to undergo splenectomy without unacceptable immunologic compromise. Splenectomy is usually effective for abolition or reduction of anemia. However, splenectomy should be used only as a last resort because of the long-term risks of overwhelming sepsis and thrombosis. Infection often exacerbates hemolysis. Fever should therefore prompt close monitoring of patients for evidence of hemolysis or infection. Postsplenectomy patients with a hemolytic diathesis are also afflicted by a hypercoagulable state, probably due to the deranged membrane architecture resulting from oxidative damage to red cells no longer being cleared by the spleen. They thus require monitoring for thrombotic events and may need intermittent or long-term anticoagulant therapy.

HEMOGLOBINS WITH INCREASED OXYGEN AFFINITY

Efficient oxygen delivery by hemoglobin depends on the sigmoid shape of the hemoglobin–oxygen affinity curve. During the transition from the fully deoxygenated to the fully oxygenated state, the initial oxygenation steps occur with difficulty. In fact, the act of binding the first oxygen molecule increases the affinity of the molecule for subsequent oxygen-binding events, thus creating the sigmoid shape of the curve. The necessary intramolecular reorganization occurs only when there are the precise arrangements of hydrogen bonds, hydrophobic interactions, and the breaking and formation of salt bridges in the proper sequence (see Chapter 34).

Some mutant hemoglobins exhibiting altered oxygen affinity arise from amino acid substitutions at the interface between α- and β-chains or in regions affecting the hydrogen bonds, hydrophobic interactions, or salt bridges that influence the interaction of heme with oxygen. A second major class of mutations alters binding to 2,3-diphosphoglycerate (2,3-DPG, also known as 2,3-bisphosphoglycerate or 2,3-BPG), which in turn alters oxygen affinity when bound to hemoglobin (see Chapter 34).

Pathogenesis and Pathophysiology

High-affinity hemoglobins exhibit higher avidity for oxygen, causing the oxygen dissociation curve to shift to the left; an example is Hb Kempsey ($\beta^{99\text{Asp}\rightarrow\text{Asn}}$) (Fig. 44.4). These hemoglobins bind oxygen more readily than normal and retain more oxygen at lower partial pressure of oxygen ($P\mathrm{o}_2$) levels. They thus deliver less oxygen to tissues at normal capillary oxygen pressures. The $P\mathrm{o}_2$ in the normal lung (90 to 100 mmHg) is well above that needed to saturate hemoglobin fully with oxygen (60 mmHg). These variant hemoglobins cannot acquire any additional oxygen in the lung despite their higher affinity. However, at capillary $P\mathrm{o}_2$ (35 to 45 mmHg), high-affinity hemoglobins deliver *less* oxygen. At normal hematocrit levels, a mild tissue hypoxia results, triggering increased production of erythropoietin and red blood cells, thus resulting in polycythemia. In extreme cases, hematocrit levels of 60% to 65% can be encountered.

Many types of mutations can increase oxygen affinity. Some alter interactions within the heme pocket, others disrupt the Bohr effect or the salt-bond site, and still others impair the binding of HbA to 2,3-DPG. Loss of 2,3-DPG binding results in increases in oxygen affinity (see Chapter 34). These and numerous other examples that have been analyzed at the molecular level have greatly aided our understanding of the molecular basis for reversible oxygen binding.

Diagnosis

High-affinity hemoglobins are a cause of familial unexplained erythrocytosis (see Chapters 27, 33, and 70). Functional testing of the hemoglobin is the key to diagnosis. Oxygen affinity is usually measured as P_{50}, the $P\mathrm{o}_2$ at which hemoglobin is 50% saturated

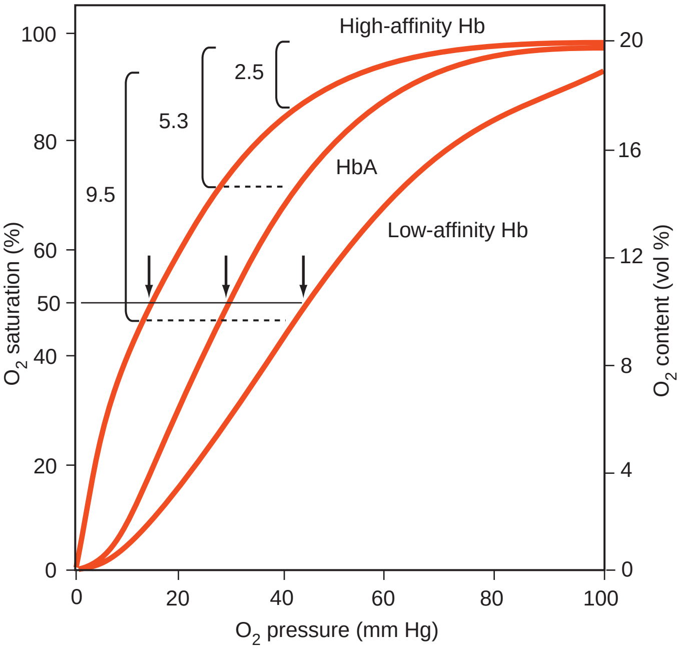


Figure 44.4 HEMOGLOBIN–OXYGEN DISSOCIATION CURVES ARE ILLUSTRATED FOR NORMAL HEMOGLOBIN (HBA) AND FOR MODEL ABNORMAL HEMOGLOBINS WITH HIGH AND LOW OXYGEN AFFINITIES. On the abscissa, the partial pressure of oxygen *(*$P\mathrm{o}_2$*)* is indicated in millimeters of mercury. On the left ordinate, the saturation of hemoglobin with oxygen is indicated as a percentage; on the right ordinate, the oxygen content of the hemoglobin is expressed as volume percent. The three inverted arrows show the $P\mathrm{o}_2$ at which the hemoglobin is 50% saturated (P_{50}) for the three hemoglobins. This value is lowest for the high-affinity hemoglobin. As the $P\mathrm{o}_2$ drops from 100 (arterial) to 40 (tissues) mmHg, hemoglobin desaturates, giving up a portion of its bound oxygen; the numbers on the brackets indicate the amount of oxygen unloaded by the three hemoglobin types expressed as volume percent. Note that the high-affinity hemoglobin delivers less than one-half the oxygen that HbA gives to the tissues, resulting in tissue anoxia, increased erythropoietin secretion, and erythrocytosis. Conversely, the low-affinity hemoglobin is even more efficient than HbA in supplying tissues with oxygen, resulting in diminished erythropoietin production and anemia. *(From Wynngaarden JB, Smith Jr LH, Bennett JC, eds. Cecil Textbook of Medicine. Philadelphia: WB Saunders; 1992.)*

with oxygen (see Fig. 44.4). The hemoglobin preparation is exposed to increasing oxygen pressures, and the relative percentages of oxyhemoglobin and deoxyhemoglobin are determined. The values are plotted on a curve, and the 50% saturation point is determined. A shift to the left means that the hemoglobin reaches 50% saturation at a lower $P\text{O}_2$. High-affinity variants are thus associated with a lower-than-normal P_{50} value. Hemoglobin electrophoresis can, but may not, reveal an abnormal band. High-performance liquid chromatography (HPLC) and DNA sequencing are better diagnostic options. In contrast to patients with polycythemia vera rubra, splenomegaly is usually absent, unless the high-affinity hemoglobin is also unstable enough to produce brisk hemolysis, in which case anemia will usually predominate, rather than polycythemia.

The most common cause of a low P_{50} value is carbon monoxide. Carbon monoxide stabilizes hemoglobin in the R "oxy" state without the need for oxygen binding. The oxygen affinity curve is therefore extremely left-shifted and is hyperbolic, rather than sigmoidal, in shape. The clinical consequences of mild chronic carbon monoxide poisoning can resemble those seen with high-affinity hemoglobin variants. The most common cause of carbon monoxide toxicity is cigarette smoking, although chronic environmental carbon monoxide exposure can elevate the hematocrit level in people such as caisson workers or tunnel toll collectors. Severe acute carbon monoxide poisoning can cause rapid death as a result of tissue hypoxia.

Management

Most patients with high-affinity hemoglobins have mild erythrocytosis; they do not require intervention. Very rarely, the hematocrit level is very high (>55% to 60%). The blood viscosity is then sufficiently elevated to require therapeutic phlebotomy. Carbon monoxide poisoning is treated with supplemental oxygen. When a patient breathes room air, the half-life of carboxyhemoglobin is 4 to 6 hours, but the half-life is 40 to 80 minutes with the use of normobaric oxygen and 15 to 30 minutes with the use of hyperbaric oxygen. Carbon monoxide detectors, designed to detect occult carbon monoxide poisoning, are now required in many municipalities and are predicted to prevent numerous fatalities from occult carbon monoxide poisoning.

HEMOGLOBINS WITH DECREASED OXYGEN AFFINITY

Pathogenesis

Low-affinity hemoglobin variants, such as Hb Kansas ($\beta^{102\text{Asn}\rightarrow\text{Thr}}$), arise from mutations that impair hemoglobin-oxygen binding or reduce cooperativity. In cases of Hb Kansas, the threonine position, β^{102}, cannot form a hydrogen bond with aspartic acid at position α^{94}. Because this aspartate residue stabilizes the R (oxy) state, Hb Kansas binds oxygen less well and exhibits a right-shifted P_{50} value (see Fig. 44.4).

Most low-affinity variants possess enough oxygen affinity to become fully saturated in the normal lung. At the low capillary $P\text{O}_2$ in other tissues, these hemoglobins deliver *higher* than normal amounts of oxygen. They become more desaturated than normal hemoglobins. Two abnormalities result from this high level of oxygen delivery. First, because tissue oxygen delivery is so "overly" efficient, normal oxygen requirements can be met by lower-than-normal hematocrit levels. This situation produces a state of "pseudoanemia," in which the low hematocrit level is deceiving because both oxygen delivery and the patients are completely normal. Second, the amount of desaturated hemoglobin circulating in capillaries and veins can be greater than 5 g/dL. Cyanosis may thus be associated with these variants. This usually ominous finding is entirely misleading in these individuals because it reflects no morbidity.

Diagnosis

Patients with unexplained anemia or cyanosis who appear to be entirely well in all other respects should be evaluated, especially if there is a positive family history. Testing for the abnormal variant follows the same reasoning as that just described for high-affinity variants. The oxygen dissociation curve will be shifted to the right, and the numeric value of the P_{50} will be higher than normal.

Management

Patients with low-affinity hemoglobins are usually asymptomatic. No treatment is required. It is important to document that a low-affinity hemoglobin is the cause of an apparent anemia or cyanosis to preempt inappropriate work-ups and provide reassurance to the patient. Cyanosis in some patients can pose a cosmetic problem, but correction with transfusions is rarely justified.

Methemoglobinemias

Methemoglobin results from oxidation of the iron moieties in hemoglobin from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state. Normal oxygenation of hemoglobin causes a partial transfer of an electron from the iron to the bound oxygen. Iron in this state thus resembles ferric iron and the oxygen resembles superoxide (O_2^-). Deoxygenation returns the electron to the iron, with release of oxygen. Methemoglobin forms if the electron is not returned. Methemoglobin constitutes 3% or less of the total hemoglobin in normal humans. Under normal circumstances, these levels in humans are maintained at 1% or less by the methemoglobin reductase enzyme system (the reduced form of nicotinamide adenine dinucleotide [NADH]–dehydratase, [NADH]-diaphorase, erythrocyte cytochrome b_5).

Pathogenesis and Clinical Manifestations

Methemoglobinemias of clinical interest arise by one of three distinct mechanisms: (1) globin chain mutations that result in increased formation of methemoglobin, (2) deficiencies of methemoglobin reductase, and (3) "toxic" methemoglobinemia, in which normal red blood cells are exposed to substances that oxidize hemoglobin iron to such a degree that normal reducing mechanisms are subverted or overwhelmed (see Chapter 45; Table 44.3).

Abnormal hemoglobins producing methemoglobinemia (M hemoglobins) arise from mutations that stabilize the heme iron in the ferric state. Classically a histidine in the vicinity of the heme pocket is replaced by a tyrosine (e.g., Hb M-Iwate, → β87 (F8) His → Tyr); the hydroxyl group of the tyrosine forms a complex that stabilizes the iron in the ferric state (Fig. 44.5). The oxidized heme iron is relatively resistant to reduction by the methemoglobin reductase system.

Methemoglobin has a brownish to blue color that does not revert to red on exposure to oxygen. Patients with methemoglobinemia thus

TABLE 44.3 Types of Methemoglobinemia

Congenital
Defective enzymatic reduction of Fe^{3+}-hemoglobin to Fe^{2+}-hemoglobin NADH-methemoglobin reductase (cytochrome-b_5 reductase) deficiency Cytochrome b_3 deficiency Abnormal hemoglobins resistant to enzymatic reduction (M hemoglobins)
Acquired
Excessive (toxic) oxidation of Fe^{2+}-hemoglobin Environmental chemicals
Drugs

NADH, Reduced form of nicotinamide adenine dinucleotide.

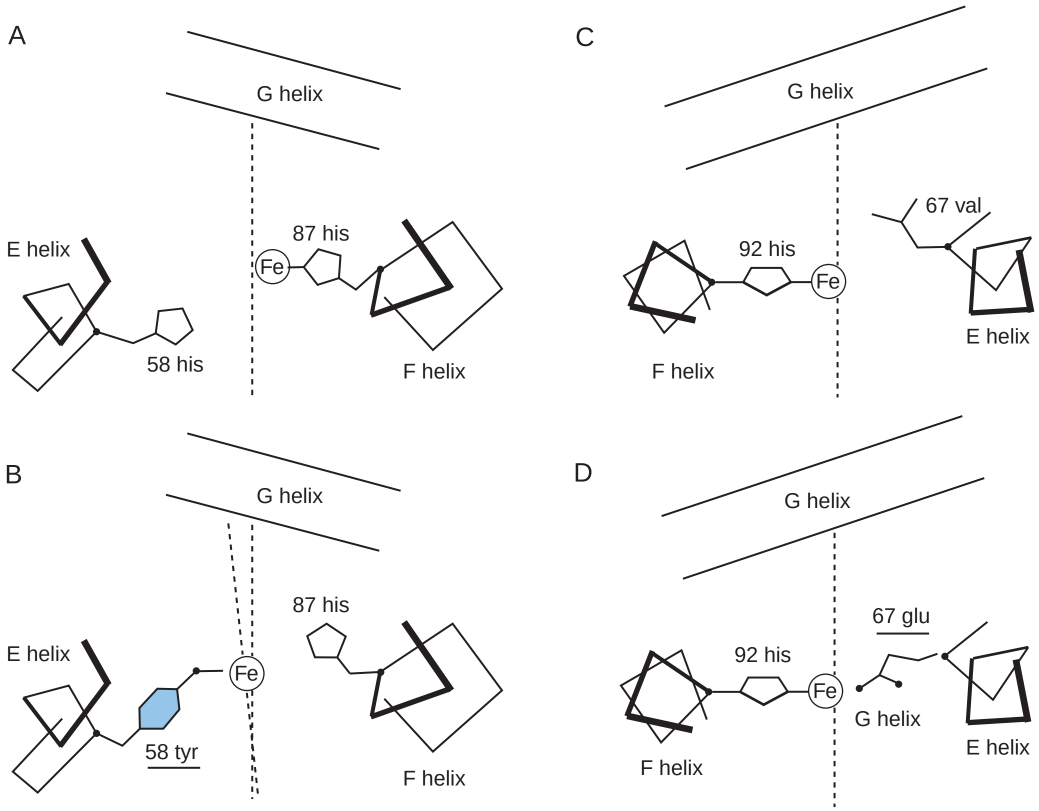


Figure 44.5 MODIFICATIONS OF THE HEME AND ITS ENVIRONMENT THAT ACCOUNT FOR TWO COMMON M HEMOGLOBINS. (A) Hemoglobin A has a His residue at the α58(E7) position. (B) In hemoglobin M-Boston, the histidine is replaced by a tyrosine, the phenolic side chain of which is capable of covalently binding to the heme iron, resulting in stabilization in the oxidized form. (C) HbA has a Val residue at position β67(E11). (D) Hb M-Milwaukee has a glutamic acid substitution for the β67 valine. The carboxylic side chain of the Glu forms a bond with iron, shifting the equilibrium toward the ferric state. *(Modified from Dickerson RE, Geis I. Hemoglobin: Structure, Function, Evolution, and Pathology. Menlo Park, CA: Benjamin-Cummings; 1983. Copyright Irving Geis.)*

appear to be cyanotic. In contrast to truly cyanotic people, however, arterial partial pressure of oxygen (PaO_2) values are usually normal. Pulse oximeters will produce ambiguous results. Patients with these hemoglobins are otherwise asymptomatic because methemoglobin is usually less than 30% to 50%, the levels at which symptoms become apparent.

Hereditary methemoglobinemia resulting from methemoglobin reductase deficiency (cytochrome-b_5 reductase deficiency) is very rare. Mutations in the b_5 reductase gene cause two distinct phenotypes (see Chapter 45 for details). In cases of type I methemoglobin reductase deficiency, patients suffer solely from cyanosis; in cases of type II disease, patients manifest both cyanosis and severe mental retardation. One isoform of the b_5 reductase gene is expressed in diverse tissues for participation in a variety of cellular processes. A second isoform, produced by alternative splicing, is expressed in erythrocytes, producing a soluble protein that reduces methemoglobin. Mutations causing type I methemoglobin reductase deficiency occur throughout the gene and result in an unstable protein. Such mutations are primarily significant in erythrocytes that, without nuclei, cannot replace the degraded protein. Mutations causing type II disease occur in the critical NADH or flavin adenine dinucleotide (FAD)-binding domains, causing inactivation of the protein in all tissues and the more severe clinical phenotype.

Like patients with M hemoglobins, patients with methemoglobin reductase deficiency exhibit slate-gray “pseudocyanosis.” However, even homozygotes rarely accumulate more than 25% methemoglobin, a level compatible with minimal symptoms. Heterozygotes can have normal methemoglobin levels but are especially sensitive to agents causing methemoglobinemia.

A third toxic form of methemoglobinemia is caused by exposure to certain chemical agents and drugs that accelerate the oxidation of hemoglobin to methemoglobin (Table 44.4). Some compounds directly oxidize hemoglobin, whereas other compounds produce reactive oxygen intermediates that oxidize hemoglobin. Nitrite compounds are especially notorious and common. Some of these compounds also have a propensity to exacerbate G6PD deficiency (see Chapter 45) and the precipitation of unstable hemoglobins.

Nitrates are a frequent environmental cause of toxic methemoglobinemia. Nitrates do not directly interact with either hemoglobin or the reductase pathway but are converted to nitrites in the gut. Well water is a frequently encountered source of excessive nitrates. In general, substantial intake of these agents is required before significant amounts of methemoglobin are generated. Very young infants have lower levels of methemoglobin reductase in erythrocytes and are therefore more susceptible to these agents than are adults. However, all age groups are at risk, given sufficient exposure. Systemic acidosis, particularly in young infants suffering from diarrhea and dehydration, can also cause clinically significant methemoglobinemia.

Acquired methemoglobinemia is virtually the only situation in which life-threatening amounts of methemoglobin accumulate. In general, the only symptom produced when methemoglobin constitutes less than 30% of total hemoglobin is the cosmetic effect of cyanosis. However, as levels of methemoglobin rise to greater than 30%, patients begin to exhibit symptoms of oxygen deprivation, such as malaise, giddiness, and other alterations of mental status. The symptoms reflect a true lack of oxygen availability at the tissue level. Methemoglobin is a markedly left-shifted hemoglobin that delivers little oxygen to the tissues. When methemoglobin accounts for more than 50% of total hemoglobin, loss of consciousness, coma, and death can rapidly ensue. At this level the blood is chocolate brown.

TABLE 44.4 **Drugs and Chemicals Having Toxic Effects on Hemoglobin Molecule**

Agent	Observed Hemoglobin Derivative	
	Methemoglobin	Sulfhemoglobin
Acetanilid, phenacetin	+	+
Nitrites (ferric, amyl, sodium, potassium, nitroglycerin)	+	+
Trinitrotoluene, nitrobenzene	+	+
Aniline, hydroxylamine dimethylamine	+	+
Sulfanilamide	+	+
p-Aminosalicylic acid	+	
Dapsone	+	
Primaquine, chloroquine	+	
Prilocaine, benzocaine, lidocaine	+	
Menadione, naphthoquinone	+	
Naphthalene	+	
Resorcinol	+	
Phenylhydrazine	+	+

Diagnosis

Methemoglobinemia should be suspected in patients with unexplained cyanosis. It is obviously a medical emergency when any patient has cyanosis and altered mental status; a $P\text{ao}_2$ more normal than expected on the basis of the O_2 saturation should trigger a consideration of methemoglobinemia. The ingestion of nitrites as a suicidal gesture, especially in people knowledgeable with respect to chemistry, medicine, or pharmacology, should be considered. Exposure to nitrate-containing therapeutic compounds (e.g., in the setting of the intensive care unit) should also raise suspicion. Methemoglobinemia can be suspected from the brownish color of blood when it is drawn. Laboratory detection is simple; methemoglobin exhibits characteristic peaks of absorption at 630 and 502 nm, rendering it easily distinguishable from normal hemoglobin. Pulse oximetry, using a ratio of absorption at 660 nm and 940 nm, gives an inaccurate reading of 85% oxygen saturation for blood with 100% methemoglobin. The inherited M hemoglobin mutants are frequently detectable by altered electrophoretic mobility, especially if ferricyanide treatment in vitro is used to convert all the hemoglobin solution to methemoglobin.

In the case of toxic methemoglobinemia, recognition of exposure to an appropriate agent provides the most important historical clue. Acute poisoning can represent a life-threatening emergency; therefore laboratory evaluation for methemoglobin should be requested for any person displaying atypical cyanosis or cyanosis occurring along with more normal than anticipated blood gas values. Methemoglobin due to deficiencies of the reductase system can be further evaluated in reference laboratories by direct analysis of these enzymes.

Management

Patients with M hemoglobins are usually asymptomatic and require no management. The secondary cyanosis can present a cosmetic problem. The cyanosis is not reversible because ascorbic acid and methylene blue are usually ineffective.

Patients with deficiency of the reductase system usually do not require treatment. Cyanosis in these cases can be improved by treatment with oral methylene blue, 100 to 300 mg/day, or 500 mg/day of oral ascorbic acid. Riboflavin (20 mg/day) has also been reported to be effective and may be the preferred agent, because methylene blue produces discolored *(blue)* urine and ascorbic acid can cause sodium oxalate stones (see Chapter 45).

Emergency treatment of high levels of toxic methemoglobinemia begins with 1 to 2 mg/kg of intravenous methylene blue as a 1% solution in saline. It is usually infused rapidly (over 3 to 5 minutes); the dose can be repeated at 1 mg/kg after 30 minutes if necessary. This treatment is usually effective. Methylene blue acts through the reduced form of nicotinamide adenine dinucleotide (NADPH) reductase system, which in turn requires G6PD activity. The method is therefore ineffective in patients who also have G6PD deficiency. These patients or patients who are severely affected may require exchange transfusion or hyperbaric oxygen therapy. Oral ascorbic acid is not useful for emergency situations because it acts too slowly. However, follow-up maintenance management can be accomplished with either ascorbic acid or oral methylene blue. See Chapter 45 for cautionary comments about patients with pharmacologically induced serotonin deficiency.

Mild cases of methemoglobin intoxication do not require treatment. The patient can be monitored for 1 to 3 days, during which time methemoglobin levels gradually return to normal if the offending agent is eliminated. The most important follow-up therapy for patients with toxic methemoglobinemia involves a thorough search for the offending agent and its removal from the environment.

Hemoglobin Variants That Modify the Clinical Phenotypes of Thalassemia and Sickle Cell Anemia

There are a number of rare structural variants that, when coinherited with a thalassemia or sickle hemoglobin allele, create doubly heterozygous states that modify the thalassemia or sickle cell presentation. These are discussed in Chapters 41 and 42.

SUGGESTED READINGS

Bunn HF. Sickle hemoglobin and other hemoglobin mutants. In: Stamatoyannopoulos G, Nienhuis AW, Majerus PO, eds. *The Molecular Basis of Blood Disease*. 2nd ed. Philadelphia: WB Saunders; 1993.

Bunn HF, Forget BG. *Hemoglobin: Molecular, Cellular and Clinical Aspects*. Philadelphia: WB Saunders; 1985.

Dickerson RE, Geis I. *Hemoglobin: Structure, Function, Evolution, and Pathology*. Menlo Park, CA: Benjamin-Cummings; 1983.

Ernst A, Zibrak J. Carbon monoxide poisoning. *N Engl J Med*. 1998;339:1603.

Fermi G, Perutz MF. *Atlas of Molecular Structures in Biology*. Vol. 2: *Hemoglobin and Myoglobin*. Oxford: Oxford University Press; 1981.

Ho C, ed. *Hemoglobin and Oxygen Binding*. New York: Elsevier Biomedical; 1982.

Park CM, Nagel RL. Sulfhemoglobinemia: clinical and molecular aspects. *N Engl J Med*. 1984;310:1579.

Perutz MF. Molecular anatomy, physiology, and pathology of hemoglobin. In: Stamatoyannopoulos G, Nienhuis AW, Leder P, eds. *The Molecular Basis of Blood Diseases*. Philadelphia: WB Saunders; 1987:127.

Smith RP, Olson MV. Drug-induced methemoglobinemia. *Semin Hematol*. 1973;10:253.

Wishner BC, Ward KB, Lattman EE, et al. Crystal structure of sickle-cell deoxyhemoglobin at 5Å resolution. *J Mol Biol*. 1975;98:179.

Wright RO, Lewander WJ, Woolf AD. Methemoglobinemia: etiology, pharmacology, and clinical management. *Ann Emerg Med*. 1999;34:646.

Wynngaarden JB, Smith Jr LH, Bennett JC, eds. *Cecil Textbook of Medicine*. Philadelphia: WB Saunders; 1992.

CHAPTER 45

RED BLOOD CELL ENZYMOPATHIES

Xylina T. Gregg and Josef T. Prchal

Red blood cells (RBCs) are highly specialized cells with the sole function of delivering oxygen to the tissues. During oxygen delivery, a highly destructive reactive oxygen species (ROS)—such as superoxide—is generated from a small fraction of the oxygen released from hemoglobin. ROS oxidizes hydroxylated sulfur groups (SH), leading to profound alterations in protein structures. This is most marked by the transformation of the hemoglobin present in high concentrations in the RBC cytoplasm to an insoluble product (i.e., Heinz bodies. ROS also damages proteins in the RBC membrane), leading to increased membrane rigidity and decreased RBC survival. In addition, ROS also oxidizes ferrous (Fe^{2+}) to ferric (Fe^{3+}) iron, converting hemoglobin to methemoglobin, which cannot bind oxygen. In order to repair this damage and allow oxygen delivery, RBC metabolism must generate reduced nicotinamide adenine dinucleotide phosphate (NADPH) for antioxidant protection, nicotinamide adenine dinucleotide phosphate (NADH) for continuous reduction of methemoglobin to hemoglobin, 2,3-bisphosphoglycerate (2,3-BPG) to augment oxygen tissue release, and adenosine triphosphate (ATP) to fuel RBC membrane integrity and transport pathways.

During oxygen delivery, the 7-micron-sized erythrocytes must negotiate through a much smaller diameter of capillaries. The red-cell membrane accomplishes this because it is flexible yet retains its integrity and RBCs lose their intracellular organelles, including the nucleus, mitochondria, and ribosomes. However, the lack of a nucleus, ribosomes, and mitochondria means that RBCs cannot make proteins and lack more efficient ways of energy production by oxidative phosphorylation in the mitochondria. RBC enzymes allow RBCs to accomplish their tasks by supporting glycolysis and the pentose shunt and by providing protection against ROS by maintaining a high ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Other RBC enzymes facilitate nucleotide degradation and salvage to remove toxic nucleotides from RBCs.

In non-erythroid tissues some of these same enzymes may have additional diverse functions, including roles in cell movement, cancer metastasis, apoptosis, oncogene modulation, and neurotropism. These other roles of red-cell enzymes may explain some of the non-erythroid effects of their mutations. In addition, RBCs contain enzymes, such as glutamine-oxaloacetic transaminase, erythrocyte lactate dehydrogenase (LDH), that do not have an apparent disease phenotype or any obvious physiologic function.

The activities of some RBC enzymes rapidly decrease with aging, but the activities of others decrease slowly or not at all. These age-related changes in enzyme activity impact the phenotype and biochemical testing in some enzyme deficiencies. Clinically significant abnormalities of RBC enzymes cause various hematologic phenotypes, principally acute and chronic hemolytic anemia, erythrocytosis, and methemoglobinemia. However, in rarer enzyme defects, the phenotype may be due not only to the mutant enzyme inherited, but also to other genetic, epigenetic, or environmental factors. Disorders of some RBC enzymes have no RBC phenotype, but their dysfunction in non-erythroid tissues causes glycogen storage disorders and other systemic disorders such as galactosemia. The presence of these enzymes in easily accessible RBCs serves as a convenient approach for the diagnosis of these disorders.

RBC-enzyme disorders are predominantly autosomal recessively inherited, either as homozygotes or compound heterozygotes. Two enzymes, glucose-6-phosphate dehydrogenase (G6PD), and phosphoglycerokinase are X-linked. Two others, adenosine deaminase and prolyl hydroxylase 2, are inherited in an autosomal dominant manner.

Confirmatory diagnosis of an enzyme deficiency increasingly relies on next generation sequencing and other molecular approaches of all potential genes, accounting for respective phenotypes: hemolysis, erythrocytosis, methemoglobinemia, or other typical non-erythroid phenotypes. The potential shortcomings of this testing approach and the role of biochemical testing are discussed below under specific disorders.

METABOLIC PATHWAYS

Glucose, the energy source for RBC metabolism, is used in two different pathways: glycolysis and the pentose shunt (Fig. 45.1). Glycolysis is the primary means of obtaining energy and provides up to two molecules of ATP. Glycolysis also reduces NAD to NADH, which is needed for protection of heme ferro-iron to oxidized ferri-iron (methemoglobin). The energy shunt (Rapoport-Luebering pathway) in glycolysis also generates 2,3-BPG. 2,3-BPG is an important glycolytic intermediate that facilitates hemoglobin oxygen delivery. If 2,3-BPG is formed, only one ATP molecule is generated.

Some glucose is also metabolized in the pentose shunt, which generates five and seven carbon carbohydrates and reduces NADP to NADPH, the sole source of NADPH in red cells. Under physiologic conditions, approximately 90% of glucose is consumed in the glycolytic pathway and about 10% is used in the pentose shunt. In conditions of increased oxidant stress, however, the contribution of the pentose shunt may be significantly increased.

RBCs also have an active glutathione metabolism comprised of its synthesis and reduction (Fig. 45.2). Maintaining a high ratio of GSH to its oxidized form GSSG, utilizing NADPH conversion to NADP, is the principal mechanism for protecting RBCs from ROS generated from the release of oxygen. Other lines of defense against endogenous ROS in RBCs are catalase, superoxide dismutase (SOD1), peroxiredoxin, and the active efflux of GSSG. These defense mechanisms protect the RBCs not only from ROS generated as a byproduct of released oxygen from hemoglobin but also from ROS generated by NADPH oxidase and, in reticulocytes, from mitochondria-derived ROS during oxidative phosphorylation. Non-quenched ROS is detrimental to RBCs by affecting membrane deformability and hemoglobin solubility.

ENZYMOPATHIES ASSOCIATED WITH HEMOLYTIC ANEMIA

This section discusses the deficiencies in the four RBC enzymes that account for most cases of enzymopathies associated with hemolytic anemia. The most common is G6PD deficiency, which is an enzyme essential for glutathione metabolism. G6PD deficiency may cause acute—or rarely—chronic hemolysis, depending on the mutation. Deficiencies of the three other enzymes are all associated with chronic hemolysis. Pyruvate kinase (PK) is the most common enzymopathic cause of chronic hemolysis, followed by glucose phosphate isomerase

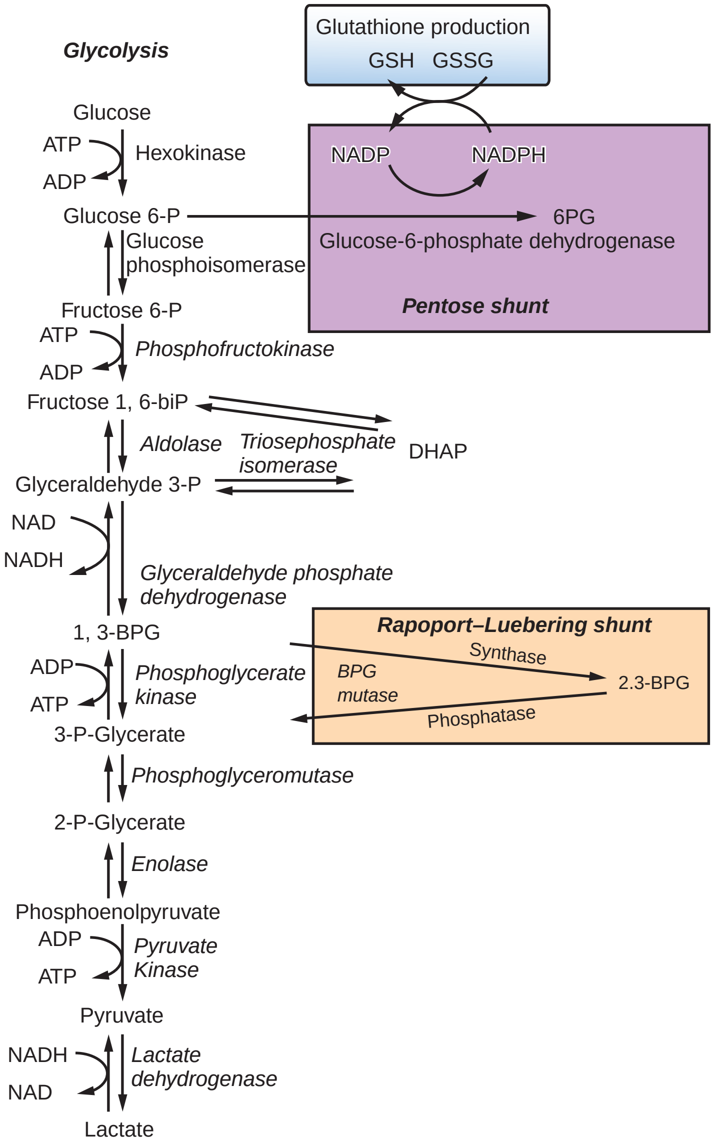

Glycolysis
Glutathione production
GSH GSSG
Glucose
ATP
ADP
Hexokinase
NADP
NADPH
Glucose 6-P
6PG
Glucose phosphoisomerase
Glucose-6-phosphate dehydrogenase
Fructose 6-P
Pentose shunt
ATP
ADP
Phosphofructokinase
Fructose 1, 6-biP
Aldolase
Triosephosphate isomerase
DHAP
Glyceraldehyde 3-P
NAD
NADH
Glyceraldehyde phosphate dehydrogenase
1, 3-BPG
Rapoport–Luebering shunt
Synthase
BPG mutase
2.3-BPG
Phosphatase
ADP
ATP
Phosphoglycerate kinase
3-P-Glycerate
Phosphoglyceromutase
2-P-Glycerate
Enolase
Phosphoenolpyruvate
ADP
ATP
Pyruvate Kinase
Pyruvate
NADH
NAD
Lactate dehydrogenase
Lactate

Figure 45.1 PRINCIPAL COMPONENTS OF THE ERYTHROCYTE METABOLISM WITH CLINICAL RELEVANCE. Shown are glycolysis, glutathione production *(purple shaded area)*, pentose shunt *(orange shaded area)*, and Rapoport-Luebering shunt *(blue shaded area)*. *1,3-BPG*, 1,3-bisphosphoglycerate; *2,3-BPG*, 2,3-bisphosphoglycerate; *6PG*, 6-phosphogluconate; *ADP*, adenosine diphosphate; *ATP*, adenosine triphosphate; *DHAP*, dihydroxyacetone phosphate; *GSH*, reduced glutathione; *GSSG*, oxidized glutathione; *NAD*, oxidized form of nicotinamide adenine dinucleotide; *NADH*, reduced form of nicotinamide adenine dinucleotide; *NADP*, oxidized form of nicotinamide adenine dinucleotide phosphate; *NADPH*, reduced form of nicotinamide adenine dinucleotide phosphate.

deficiency (GPI), and pyrimidine 5′ nucleotidase-1 (P5′N1), which is essential for removal of toxic nucleotide precursors. Deficiencies of other enzymes are rarer and have highly variable clinical phenotypes that may be specific for each enzymatic defect.

Enzyme disorders affecting glutathione metabolism can cause either chronic or acute intermittent hemolysis. Heinz bodies (precipitated denatured hemoglobin) may be seen in RBCs during an acute hemolytic episode. The glycolytic enzyme deficiencies result in chronic hemolysis by a poorly understood mechanism. These patients are not subject to hemolytic crises after exposure to oxidants and their RBCs do not have any characteristic morphologic abnormalities. In contrast, marked basophilic stippling of RBCs is a hallmark of P5′N1 deficiency, which also causes chronic hemolysis. The chronic hemolytic anemia that occurs in enzyme deficiencies has been termed: hereditary non-spherocytic hemolytic anemia.

Glucose-6-Phosphate Dehydrogenase Deficiency

Introduction

G6PD deficiency was the first described and is the most common and best-studied RBC enzyme deficiency. G6PD deficiency is more common where *Plasmodium falciparum* malaria is or has been endemic. It was discovered in the 1950s as a result of investigations into a self-limited hemolysis that occurred after administration of the antimalarial drug primaquine, most commonly in individuals of African or Mediterranean ethnic origin. These early studies also determined that G6PD deficiency is X-linked. Subsequent studies in carrier females led to the discovery of X-inactivation, a phenomenon that has been exploited to study the hierarchy of hematopoiesis and the clonality of malignant neoplasms.

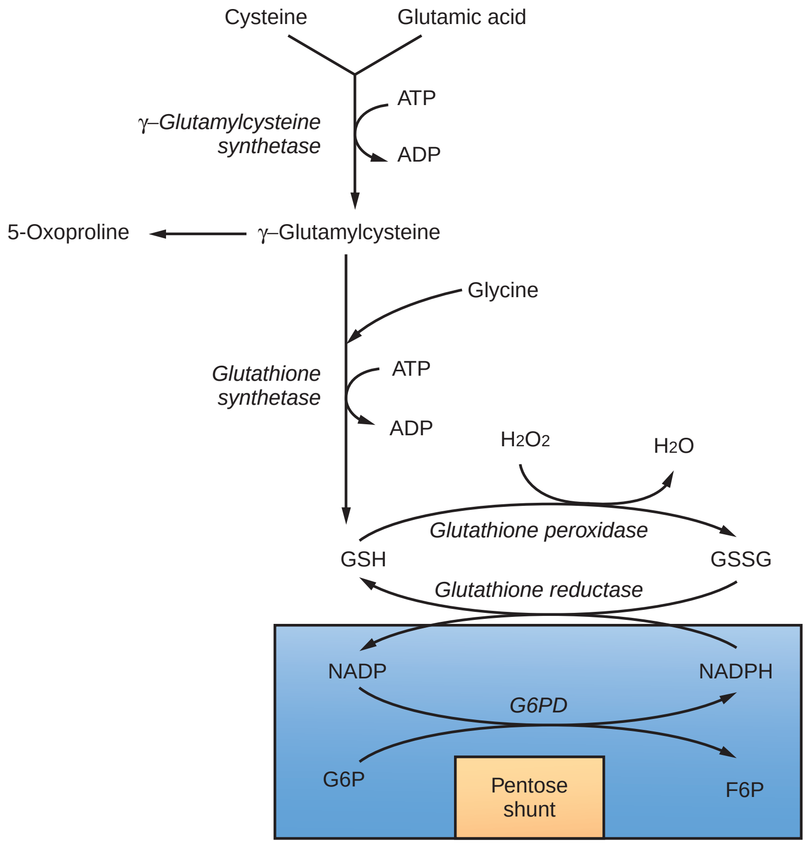


Figure 45.2 GLUTATHIONE PATHWAY. *ADP*, Adenosine diphosphate; *ATP*, adenosine triphosphate; *F6P*, fructose-6-phosphate; *G6P*, glucose-6-phosphate; *G6PD*, glucose-6-phosphate dehydrogenase; *GSH*, reduced glutathione; *GSSG*, oxidized glutathione; *NADP*, oxidized form of nicotinamide adenine dinucleotide phosphate; *NADPH*, reduced form of nicotinamide adenine dinucleotide phosphate.

Epidemiology

G6PD deficiency is the most prevalent human enzyme deficiency in the world, affecting about 500 million people, although the vast majority of affected individuals never become symptomatic. Although most prevalent in individuals of African, Mediterranean, and southeast Asian ethnic origins, it has been found in almost every population. The highest prevalence is in the tropical belt of sub-Saharan Africa (>32%) and the Arabian Peninsula. In other populations, its prevalence ranges from less than 1 in 1000 among northern European populations to 50% of Kurdish Jews. The distribution across Asia is heterogeneous. Nearly 20% of males in Thailand are affected by one of the five prevalent variants. It is common in southern China (~5% in Hong Kong) and rare in other parts of China, while in India the prevalence varies from 0% to 27% in different regions, caste, ethnic, and linguistic groups. In the United States, G6PD deficiency affects about 10% of African American males, a lower proportion of individuals of Italian, Greek, Spanish, Corsican, and Sardinian ancestries and a variable proportion of Middle-Eastern and recent Asian immigrants. G6PD deficiency is virtually nonexistent among indigenous peoples of the Americas and Asian highlanders. The variable geographic distribution of G6PD deficiency implies that it confers a selective advantage and, as it coincides with the geographic distribution of endemic malaria, suggests protection from lethal malaria, although the exact mechanism has not been fully elucidated.

The wild-type enzyme is designated G6PD B. The most common G6PD low activity allelic variants in the United States are G6PD A– and G6PD Mediterranean; however, Asian variants are being encountered in the US population with increasing frequency. G6PD A– accounts for approximately 90% of G6PD deficient variants in Africa but is also prevalent in North and South America, the West Indies, Italy, the Canary Islands, Spain, Portugal, and the Middle East. The *G6PD A–* mutation (G202A; c.202 C>T) arose on a *G6PD A+* chromosome (A376G, c.*C376C>T*); these two missense changes are in *cis* orientation. G6PD A+ has no obvious hematologic phenotype and has a gene frequency similar to that of G6PD A– among African Americans. G6PD Mediterranean is found in highest frequencies in the southern part of Italy, Greece, Spain, Sardinia and Corsica, as well as the Middle East, Iran, and the Arabian Peninsula, India, and Indonesia. G6PD Mediterranean is not homogeneous, but is composed of several distinct mutations, which *G6PD* Mediterranean (c.C563T) predominates. Several G6PD variants are pandemic in Asia. There are more than 100 different mutations in various Asian populations.

The high frequency of the most common *G6PD* variants and the diversity of the variants suggest selection of the variants, presumably because of protection from malaria, given the geographical distribution of these variants in areas of the world where malaria is or has been endemic. However, data on which genotype confers protection from malaria and the mechanism of the protection has been conflicting. The conflicting epidemiological data may be in part due to the large number of variants and phenotypical heterogeneity. The most common African variant *G6PD A–* (c.202 C>T) and possibly other variants associated with more severe G6PD deficiency is associated with a decreased risk of cerebral malaria in male hemizygotes and female heterozygotes, but an increased risk of severe malarial anemia in male hemizygotes and female homozygotes. The mechanism of malarial protection remains to be established. However, deficient cells infested with malaria parasites appear to be phagocytized more efficiently than normal cells. The host RBCs' impaired ability to restore intracellular NADPH to maintain a high GSH/GSSG ratio may also mean that malarial parasites in G6PD-deficient RBCs are more vulnerable to ROS.

Pathobiology

G6PD is a housekeeping enzyme that catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconate in the first reaction of a pentose shunt, which reduces $NADP^+$ to NADPH (see Figs. 45.1 and 45.2). In RBCs, the pentose shunt is the only source of NADPH, which is crucial to maintaining high cellular levels of GSH to protect the cell from oxidative, stress-induced damage. Within the RBCs, oxidant injury leads to the oxidation of sulfhydryl (SH) groups on the hemoglobin molecule. This results in the formation of disulfide bridges (-S-S-), which in turn leads to decreased hemoglobin solubility and ultimately the irreversible precipitation of oxidized hemoglobin. Under normal conditions these oxidized -S-S- groups of hemoglobin and other RBC proteins are reduced by GSH to SH-groups by glutathione peroxidase, which in turn is oxidized to GSSG that is restored back to GSH by glutathione reductase in a reaction requiring NADPH. NADPH levels are maintained by G6PD, meaning in G6PD-deficient RBCs, GSH is not restored to adequate levels under oxidative stress, leading to a buildup of free radicals and insoluble hemoglobin within the cell. Precipitated hemoglobin is disruptive to the structure and function of the RBC membrane and leads to increased membrane permeability, osmotic fragility, and cell rigidity. This compromised integrity of the RBC membrane in G6PD-deficient RBCs results in extravascular hemolysis from the rapid removal of these cells within the splenic pulp and also in varying degrees of intravascular hemolysis.

The *G6PD* gene localizes to Xq28, spans 18 kb, and contains 13 exons. The active G6PD enzyme exists as a tetramer or dimer. Stability of the multimeric structures is crucial for optimal G6PD activity. G6PD activity decreases significantly as RBCs age, with a half-life of about 60 days. Reticulocytes have five times higher enzyme activity than the oldest RBC subpopulation. An even greater decrease in G6PD activity with aging is present in some mutant variants and is particularly pronounced in the African G6PD A– mutant.

G6PD variants can be divided into three categories based on the type of hemolysis they cause: acute intermittent (most common), chronic (rare), or none. The World Health Organization also classifies the different G6PD variants according to the degree of enzyme deficiency and severity of hemolysis. Class I deficiencies are the most severe and cause chronic hemolysis. Less severe G6PD Mediterranean is a class II deficiency. The even less deficient G6PD A– is a class III deficiency. Classes IV and V do not cause hemolysis and are of no clinical significance.

Over 400 variants of the G6PD enzyme have been identified by biochemical methods. However, this is likely an overestimate, as some previously described different mutations were found to be caused by the same mutation through molecular genetic studies. Currently, 237 *G6PD* mutations have been characterized by DNA sequencing in the Human Gene Mutation Database (HGMD, http://www.hgmd.org; accessed June 23, 2020); 212 of these cause disease. The majority are missense mutations but small deletions (13), splicing mutations (4), and small indels and gross deletions (4) also exist. Only one regulatory domain substitution has so far been identified. Of these mutations, at least 100 have the prevalence of a polymorphism (a rate of at least 1% in tested population). Mutations associated with chronic hemolysis tend to cluster in the vicinity of the NADP-binding domain of the *G6PD* gene and cause more severe deficiency, whereas those associated with acute intermittent hemolysis or no hemolysis are scattered throughout the gene. Unlike disease-causing mutations of other genes, total gene deletions and insertions causing frameshift and stop codon mutations are not observed. These events would be expected to be fatal, since *G6PD* is a housekeeping gene essential for basic cellular functions.

Clinical Manifestations

Acute Hemolysis

Individuals with the most common forms of G6PD deficiency have no anemia or other clinical manifestations, unless they are exposed to triggers of acute hemolysis such as oxidant drugs, infection, or ingestion of fava beans. Exposure of red cells to certain drugs results in the formation of low levels of hydrogen peroxide as the drug interacts with hemoglobin; other drugs may form other ROS that oxidize GSH without the formation of peroxide as an intermediate. Hemolysis begins in hours to 1 to 3 days after exposure to the offending drug. However, many drugs implicated in acute hemolysis in G6PD deficiency may not be true culprits because infection and other stressors can also provoke hemolysis in these people; this was elegantly reviewed by Beutler in *Blood* in 1994. In 2010 Youngster and colleagues critically evaluated extensive literature from the Cochrane database, MEDLINE, and PubMed, as well as leading pediatric, adult, pharmacology, and hematology textbooks. They concluded that solid evidence for drug-induced hemolysis in G6PD-deficient individuals exists only for these seven drugs: dapsone, methylthioninium chloride (methylene blue), nitrofurantoin, phenazopyridine, primaquine, rasburicase, and tolonium chloride (toluidine blue).

The mechanism of hemolysis induced by infection is not well understood, but the generation of hydrogen peroxide by phagocytizing leukocytes or the diffusion of oxidants from neutrophils undergoing oxidative bursts, leading to the formation of disulfide bridges of hemoglobin may be causative factors.

Depending on the G6PD variant, the duration of hemolysis can be brief or protracted. The RBCs of G6PD A- contain only 5% to 15% of the normal amount of enzyme activity and the age-dependent decline of the activity renders old RBCs severely deficient and susceptible to hemolysis. As this subpopulation is eliminated, younger RBCs and reticulocytes produced in response to hemolysis have higher G6PD activity and are typically not hemolyzed. Thus, the hemolytic process is self-limited, even when the offending agent is continued. In contrast, in G6PD Mediterranean the enzyme activity of the young RBCs is lower than in G6PD A– and hemolysis continues longer, even a few days after discontinuation of the culprit drug, albeit it is also self-limiting.

Favism

Fava beans are a staple food in many parts of the world where G6PD deficiency is found at a high gene frequency. The hemolysis precipitated by fava bean ingestion known as favism, occurs only in people who are G6PD-deficient. It is most frequently associated with the more severe G6PD Mediterranean and G6PD Cairo variants, but is also seen with lower frequency with G6PD A–. Not all individuals with G6PD Mediterranean are susceptible to favism and a tendency toward familial occurrence suggests that additional genetic factors may be important. Favism is more common in children undiagnosed with G6PD deficiency than in adults, perhaps because persons who have experienced favism are likely to avoid this exposure. Hemolysis usually occurs one to several days after fava bean consumption, but onset within the first hours after exposure has been reported.

Chronic Hemolysis

The rare G6PD variants causing chronic hemolytic anemia occur sporadically. The severity of the hemolysis ranges from mild to transfusion-dependent. Exposure to the oxidants that cause hemolysis in the acute hemolytic G6PD variants may further exacerbate hemolysis.

Neonatal Jaundice

Neonatal jaundice, which may result in kernicterus, is the most common and often the most serious consequence of G6PD deficiency. The icterus is not only caused by hemolysis but also to inadequate processing of bilirubin by the immature liver of the G6PD-deficient infant. Further, the genetically unrelated coinheritance of polymorphic UDP-glucuronosyltransferase 1 (UGT1A1) promoter alleles (Gilbert syndrome) exacerbates the icterus. Neonatal screening for G6PD deficiency and early phototherapy treatment in endemic areas has been associated with a decreased incidence of kernicterus.

Non-Erythroid Effect of Glucose-6-Phosphate Dehydrogenase Deficiency

Although patients with the common endemic G6PD variants are not at an increased risk for infections, neutrophil dysfunction has been described in some patients with rare severely deficient G6PD variants. Occasionally, cataracts have been observed in patients with some rare variants of G6PD that produce chronic hemolytic anemia. Small studies from the Middle East that remain to be confirmed suggest that decreased G6PD activity may predispose to the development of diabetes. Splenomegaly is generally not seen in G6PD-deficient individuals.

Laboratory Manifestations

Under normal conditions, most G6PD-deficient individuals are not anemic and have no laboratory evidence of hemolysis. In the setting of oxidative stress, laboratory findings indicative of acute hemolysis including anemia and reticulocytosis are seen. As the hemolysis is mainly extravascular with a variable intravascular component, variable degrees of hyperbilirubinemia, increased LDH, and decreased haptoglobin occur. Heinz bodies may be visible in the erythrocytes during an acute hemolytic episode, but not under normal circumstances. “Bite cells” have been described and purported to be indicative of G6PD deficiency, but they are not specific for G6PD deficiency and these cells are usually not present in acute hemolytic states of patients with common G6PD variants or in G6PD-deficient patients with chronic hemolysis.

Individuals with the chronic hemolytic G6PD variants have varying degrees of anemia and reticulocytosis.

Diagnosis

A biochemical diagnosis of G6PD deficiency can be made using quantitative spectrophotometric analysis to measure the generation of NADPH from NADP in RBC hemolysates. A more convenient rapid fluorescent screening test can be used to test at-risk populations. False-negative results are not unusual, however, especially if enzymatic analysis is performed during or shortly after resolution of acute hemolytic episodes, or in heterozygous females. After acute hemolysis, reticulocytes and young RBCs, which have much higher enzymatic activity, predominate. These false-negative test results are more likely to occur when a screening test rather than a quantitative spectrophotometric analysis of the enzyme activity is used. However, this obstacle can be overcome when hexokinase (HK) activity, another glycolytic enzyme with higher enzymatic activity in reticulocytes and young RBCs than in aged RBCs, is concomitantly measured; the HK/G6PD enzyme activity ratio can then be used to diagnose or rule out G6PD deficiency.

Females heterozygous for G6PD are particularly difficult to diagnose because of their mosaicism for this X-chromosome enzyme and may have total RBC enzymatic activity ranging anywhere from hemizygote to normal. However, these females have a variable mixture of deficient and nondeficient RBCs and their deficient RBCs are subject to same hemolytic destruction as those of males. Since the nucleotide substitutions of most G6PD-deficient isoenzymes have been identified, molecular diagnostic methods are more reliable for the accurate diagnosis of females who are heterozygous for G6PD deficiency and also in males with ongoing or recent acute hemolysis, or after transfusion.

Severe sporadic variants causing chronic hemolytic anemia may be considered for prenatal diagnosis in some circumstances.

Prognosis

Most patients with G6PD deficiency have normal life spans with no clinical sequelae. Neonatal icterus with resultant kernicterus that may lead to neurological deficits and mental retardation has the gravest consequences.

Therapy

Treatment of an acute hemolytic crisis includes withdrawal of any offending agent and supportive care, which in severe cases includes RBC transfusions. Folic acid supplementation is advocated for patients with chronic hemolysis, but even without folic acid supplementation, significant folate deficiency rarely occurs. Neonatal icterus associated with G6PD deficiency is treated in the same manner as neonatal icterus arising from other causes; G6PD deficiency should be considered in any neonate with hyperbilirubinemia, especially those of high-risk ethnic descent. The chronic hemolytic anemia in some exceedingly rare G6PD variants may be severe enough to require chronic transfusions and iron chelation.

Future Directions

In certain areas of the world where G6PD deficiency reaches epidemic proportions and ingestion of fava beans is staple, screening for endemic G6PD-deficient variants and avoiding fava beans in those that are G6PD deficient reduces hospitalizations and RBC transfusions. Screening of neonates in endemic areas can also reduce mortality (from kernicterus).

Pyruvate Kinase Deficiency

Introduction

PK deficiency is the most common enzyme deficiency causing hemolysis. Although this disorder is far less common than G6PD deficiency, the vast majority of patients with G6PD deficiency never suffer a hemolytic episode, while PK deficiency has a high penetrance, although also a highly variable phenotype.

Genetics

PK enzymes consist of several isoforms. They are products of two distinct genes, *PKLR* and *PKM* located on different chromosomes. *PKLR* (encoding L, liver, and R, RBC isoenzymes) is located on chromosome 1q21. The R isoform, unique to RBCs, is 33 amino acids larger than the L isoform, which is unique to hepatocytes. Expression in RBCs versus liver is due to differential use of tissue-specific promoters, which drive expression as well as tissue-specific exon usage (use of exon 1 but not exon 2 in RBCs and exon 2 but not exon 1 in liver). Regulatory elements in *PKLR*-gene expression are not fully defined, but one is a key erythroid transcription factor, Kruppel Like Factor 1 (KLF1).

The *PKM* gene encodes the M (muscle) enzymes. There are two isoforms, M1 and M2, which are different splicing products of the *PKM*-gene's single transcript. The M1 isoform is expressed in muscle, heart, brain. The M2 isoform initially predominates in fetal erythropoiesis but is progressively replaced in RBCs with the R form during fetal development. In contrast, M2 persists in leukocytes and platelets. M2 is overexpressed in many tumors and is present in other cells such as lung, fat, retina, and pancreatic islets. PKM proteins have co-stimulatory activity with hypoxia-inducible transcription factor 1 (HIF-1). The *PKM* gene is located on chromosome 15q22.

Thus far, 290 *PKLR* mutations have been characterized by DNA sequencing (Human Gene Mutation Database [HGMD, http://www.hgmd.org; accessed June 23, 2020]). Of these mutations, 276 are disease-causing, most of which are missense mutations.

Epidemiology

PK deficiency is distributed worldwide but is more common among people of northern European extraction. PK deficiency is an autosomal recessive disease, and affected patients are typically double heterozygotes, or, less commonly, homozygous for the same mutation. Homozygous mutations are usually seen in groups with marked consanguinity, and homozygous PK deficiency has been well studied in the Amish populations of Pennsylvania and Ohio and also in an isolated fundamental Mormon settlement at the Utah/Arizona border. Common mutations have well-defined geographic associations. *PKLR* c.1529 G>A mutation is the most common mutation in the United States, northern and central Europe with a population prevalence in the US of ~50/million, while c.1456 C>T is the most common mutation in southern Europe, and c.1468 C>T in Asia.

PK deficiency does not localize to geographic areas of malarial endemicity. However, there is in vitro evidence that PK deficiency provides protection against infection and replication of *Plasmodium falciparum* in human RBCs, an effect possibly mediated by reduced ATP levels in PK-deficient RBCs. PK deficiency was also shown to be protective in a mouse model of infection with *Plasmodium chabaudi*.

Pathobiology

PK enzymes catalyze the final step in glycolysis: the irreversible transfer of phosphate from phosphoenolpyruvate to adenosine diphosphate (ADP), yielding one molecule of pyruvate and one molecule of ATP (see Fig. 45.1). PK is allosterically activated by the binding of fructose 1,6-diphosphate (FDP); that is, FDP binds to a site other than the active site for substrate binding, causing a conformational change in the PK enzyme. FDP is an intermediate in the glycolytic pathway, and higher concentrations of FDP increase PK activation (a process known as feedforward stimulation). PK deficiency results in decreased glycolytic ATP production and an accumulation of glycolytic intermediates, including 2,3-BPG.

PKLR mutations affecting the active catalytic site are associated with more severe hemolytic anemia. However, the phenotypical expression of identical mutations can be strikingly different, even within the same family. Clinical PK deficiency with hemolytic anemia is largely limited to mutations of the *PKLR* gene. However, a mutation in the transcription factor *KLF1* that regulates *PKLR* transcription is a rare cause of PK deficiency (see below). Since *PKM* encoded proteins are present on leukocytes and platelets, using whole blood to analyze for PK deficiency may result in false-negative results.

PK-R exists as a heterotetramer. Since most PK-deficient patients are compound heterozygous for two different mutations, rather than homozygous for one, several different tetrameric forms of PK-R may be present, each with distinct structural and kinetic properties. This complicates genotype-to-phenotype correlations in these individuals, as it is difficult to infer which mutation is primarily responsible for deficient enzyme function and the clinical phenotype. There are even cases in which the activity of PK as measured in vitro is higher than normal, but a kinetically abnormal enzyme with low in vivo activity in erythrocytes is responsible for the hemolytic anemia. These mutations can only be detected if PK-enzyme activity is measured with and without FDP and at several concentrations of PK's substrate phosphoenolpyruvate.

PK deficiency may be also caused by mutations not directly involving the *PKLR* gene. Mutations in the erythroid transcription factor *KLF1* caused severe congenital hemolytic anemia because of a deficiency of PK. In this instance, the *PKLR* gene was intact and thus sequencing of the *PKLR* gene would miss the diagnosis of PK deficiency.

The mechanism of hemolysis in PK deficiency is not clear. The defect in ATP generation is unlikely to be the sole cause, as ATP deficiency is difficult to demonstrate in many patients and other disorders with more severe ATP deficiency are not associated with significant hemolysis. Increased apoptosis and ineffective erythropoiesis may be features of PK deficiency, although this has only been studied in splenic erythroid progenitors. Following splenectomy, despite decreased hemolysis and improved anemia, patients paradoxically have a higher number of reticulocytes, sometimes reaching over 50%. The reasons for this phenomenon is as yet unexplained.

Tolerance of Anemia

The anemia of PK deficiency is better tolerated than a comparable level of anemia seen in patients with hexokinase deficiency, since the block in glycolysis occurs after the Rapoport-Leubering shunt (see Fig. 45.1); (see section on 2,3-BPG deficiency). Levels of 2,3-BPG may be elevated up to two times normal in PK-deficient individuals, resulting in decreased hemoglobin oxygen affinity. This accumulation of 2,3-BPG shifts the oxyhemoglobin dissociation curve to the right, leading to better oxygen delivery to the tissue and improved tolerance of anemia than would be otherwise expected.

Clinical and Laboratory Manifestations

Hemolysis and Anemia

Hemolysis in PK deficiency is mainly extravascular (i.e., due to phagocytosis of cells by reticuloendothelial macrophages). However, if the hemolysis is severe, there may be spillover to intravascular hemolysis. Thus, many affected patients have normal lactate dehydrogenase (LDH) levels, but most have elevated bilirubin levels and clinical jaundice, as well as reticulocytosis. The reticulocyte count invariably increases after splenectomy.

The severity of hemolysis in PK-deficient patients is highly variable, ranging from a mild, fully compensated chronic hemolytic process without anemia, to life-threatening transfusion-requiring hemolytic anemia present at birth, to rare hydrops fetalis because of homozygosity for *PKLR* null mutations. The disease severity is often but not always similar among siblings of a given family. In most cases the degree of hemolysis declines after infancy, by a not fully understood pathophysiologic mechanism. Splenomegaly is often but not invariably present. Patients with severe hemolysis may be chronically jaundiced and may develop the clinical complications of chronic hemolytic states, including gallstones, transient aplastic anemia crises (caused by parvovirus infection), folate deficiency (rarely), extramedullary hematopoiesis and infrequently, skin ulcers. Pregnancy may precipitate hemolysis.

Iron Overload

As in any patient with ineffective erythropoiesis and hyperactive production of early RBC precursors, iron absorption is increased and iron retention in macrophages is decreased, due to erythroferrone-mediated decreases in the levels of hepcidin (see Chapters 36 and 37). Iron overload can occur, not only in transfusion-dependent patients but also in nontransfused patients, and may be severe. In some patients, clinically significant iron overload is detected in adulthood before the diagnosis of PK deficiency and chronic hemolysis.

Neonatal Icterus

Neonatal icterus may occur and is augmented by coincidental heterozygosity or homozygosity for the *UGT1A1* polymorphism.

Diagnosis

There are no characteristic RBC morphologic findings in PK deficiency; however, nonspecific changes such as echinocytes (burr cells), anisocytes, or poikilocytes may be present. PK deficiency should be suspected in patients with Coombs-negative hemolytic anemia, no RBC morphologic features of membrane disorders, and a history consistent with congenital hemolysis.

Biochemical Testing

A rapid screening test using crude hemolysate with a single concentration of substrate has been used for the detection of pyruvate deficiency but should be avoided unless leukocytes and platelets are first filtered out. PK-enzymatic screening tests also miss some *PKLR* variants.

The gold standard test for diagnosing PK deficiency is *spectrophotometric testing* of PK activity and estimation of enzyme kinetics in

RBCs free of contaminating leukocytes and platelets. In this assay, testing enzyme activity at different concentrations of the allosteric activator FDP and the substrate phosphoenolpyruvate permits detection of high Km–low-affinity mutants. However, this assay is not available at most commercial laboratories.

Genetic Testing

DNA testing for PK deficiency is increasingly available, including testing performed by commercial laboratories using heterogeneous genomic sequencing methods including next generation sequencing (NGS) of whole *PKLR* exomes or targeted sequencing. However, if a previously undescribed mutation is found, it may be challenging to determine if it is disease-causing or a rare polymorphism. If the familial genetic variant is not known, and no mutation found by NGS covering all exons, additional sequencing of flanking regions, the *PKLR* promotor, and in some instances the whole genome, may be needed. Some patients have large deletions and intronic mutations at cryptic splice sites that may not be detected by routine sequencing methods. In individuals with more than one *PKLR* mutation (compound heterozygotes), it is also important that parent samples be obtained, in order to determine whether the two mutations are present in *cis* (both from the same parent) or in *trans* (one from each parent). Identification of a deleterious mutation can facilitate testing of potentially affected family members, including prenatal testing. As the severity of hemolysis may be variable even among relatives with the same *PKLR* mutation, it is likely that in large families genetic testing will identify some affected, yet relatively asymptomatic patients, who otherwise would never be diagnosed.

However, as noted above, sequencing the *PKLR* gene will not diagnose the rare mutations of genes other than *PKLR*, such as KLF1, that have been shown to reduce PK enzymatic activity.

Therapy

Many patients do not require therapy. Some require RBC transfusions only in transient settings of increased stress, such as the perioperative period, coexistent infections, or pregnancies. However, others require chronic transfusions. Iron status should be monitored even in patients who have never been transfused. If iron overload is detected in those who are not severely anemic phlebotomy therapy, then iron chelation should be instituted.

Splenectomy has documented benefits in severe cases; the degree of hemolysis and anemia is ameliorated and the transfusion requirement is abolished or markedly decreased. The increase in hemoglobin concentration in non-transfusion-requiring patients after splenectomy ranges from 1 to 3 g/dL. It is recommended to delay splenectomy, if possible, until after the age of 3 years when the risk of infections with encapsulated organisms declines, and because in most cases the degree of hemolysis declines after infancy, by a not fully understood pathophysiologic mechanism. One PK-deficient boy with severe hemolysis was apparently cured by an allogeneic marrow transplant. Gene therapy is also actively being explored.

Mitapivat (previously called AG-348) is an orally available small molecule (a quinolone sulfonamide). It was developed using biochemical assays for compounds that could allosterically activate the PK-R enzyme similarly to FDP, but with greater efficacy. In a study in 52 patients, mitapivat increased the hemoglobin level by more than 1.0 g/dL in 26 (50% response). Responses to mitapivat correlated with the PKLR genotype and were best in individuals with missense mutations in *PKLR*. Responses did not occur (or were lower) in individuals who were homozygous for *PKLR*$^{\text{R479H}}$ or for two non-missense mutations. Of 20 individuals with a good response who continued therapy in an extension phase, 19 had a sustained increase in hemoglobin. Therapy was well-tolerated with most adverse events being grade 1 or 2 in severity. In responding patients, mitapivat was also shown to restore glycolytic pathway activity and normalize previously low ATP and previously elevated 2,3-BPG levels.

Prognosis

The clinical course is highly variable ranging from fatal hydrops fetalis, transfusion dependency from birth, and a high risk of kernicterus to normal childhood development with no or rare transfusions, or to compensated hemolysis without anemia. However, hemolysis that is fully compensated because of excessive erythropoiesis may be deceptively benign. In addition to the usual complications of chronic hemolysis such as gallstones, extramedullary hematopoiesis, and parvovirus-induced aplastic crisis, the excessive number of erythroblasts in these patients produces erythroferrone, which mediates low hepcidin and may result in typical hemochromatosis with cardiac and hepatic dysfunction.

Future Directions

New gene therapeutic techniques are being evaluated in severe PK deficiency. These include the zinc finger nucleases and *CRISPR/Cas9* technology (clustered regularly interspaced short palindromic repeats) that use noncoding RNAs to guide the Cas9 nuclease to induce site-specific DNA cleavage. Preclinical gene therapy has also been reported for PK deficiency based on a lentiviral vector harboring the promoter that drives the expression of the PKLR cDNA.

Glucose Phosphate Isomerase Deficiency

Introduction

GPI deficiency is one of the three most common RBC enzyme defects causing chronic hemolytic anemia (the other two are PK and P5’N1) with approximately 100 affected families described. Most patients are compound heterozygous for mutations that partially inactivate the enzyme.

Pathobiology

GPI converts glucose-6-phosphate to fructose-6-phosphate in the second step of glycolysis. The GPI enzyme is a homodimer and only the dimeric form has enzyme activity. The monomeric form of GPI has non-erythroid functions as a neuroleukin, i.e. a neurotrophic factor for spinal and sensory neurons and as an autocrine motility factor, which is also secreted by cancer cells and stimulates cancer metastasis. GPI also has a role in glycerolipid biosynthesis. The *GPI* gene is large (50 kb) with 18 exons and cDNA of 1.9 kb. Approximately 50 missense mutations altering the catalytic site have been reported. In some families, impaired granulocyte and neuromuscular function occurs, likely due to the non-erythroid functions of GPI.

Clinical and Laboratory Features

Most patients are compound heterozygotes, although some are homozygotes, usually as the consequence of a consanguineous union. Splenomegaly is often present. The severity of the hemolysis is variable. Many affected patients do not require therapy, other than blood transfusions during hemolytic periods, but some are transfusion dependent. In many splenectomized patients, reticulocytes increase after splenectomy, but not as strikingly as in PK deficiency; the pathophysiology of this phenomenon remains unclear.

Like any other chronic hemolytic anemia, patients are at risk for iron overload, gallstones, and for parvoviral-induced pure red-cell aplasia. Severe enzyme deficiency can be associated with hydrops fetalis, neonatal death, and neurological impairment.

In isolated GPI-deficient families, severe neuromuscular symptoms, mental retardation, and granulocyte dysfunction have been documented and dominate the disease phenotype.

Therapy

Patients may be transfused as needed. In those who are transfusion-dependent, splenectomy should be considered. Anemia significantly improves in the majority, but not all, GPI-deficient patients following splenectomy. Iron status should be monitored in all patients, even in those who have never been transfused. If iron overload is detected, iron chelation should be instituted.

Pyrimidine 5′ Nucleotidase-1 Deficiency

Introduction

P5′N1 deficiency is one of the three most common causes of chronic hemolytic anemia caused by a red cell enzymopathy. It is less common than PK deficiency but has a similar frequency as GPI deficiency. It is inherited in an autosomal recessive manner.

Pathobiology

As the reticulocyte matures, ribosomes and RNA are degraded. P5′N1 assists in the process by catalyzing the dephosphorylation of pyrimidine nucleoside monophosphates into cytidine and uridine, which can diffuse across the cell membrane. P5′N1 activity is specific for pyrimidines and is much higher in reticulocytes than mature red cells. P5′N1 activity rapidly declines during the first few days of red-cell maturation. P5′N1 activity is dependent on Mg^{2+} and is inhibited by lead (Pb^{2+}). Lead intoxication causes acquired P5′N1 deficiency with a hemolytic phenotype similar to hereditary P5′N1 deficiency.

Thus far, more than 50 different mutations in over 100 patients have been reported in P5′N1 deficiency. Most patients are homozygous and consanguinity is common.

The accumulation of pyrimidines in RBCs because of a deficiency of P5′N1 is presumed to be toxic, although the exact mechanism by which P5′N1 causes hemolysis is unknown. Deficiency of P5′N1 is at least partly compensated in vivo by other nucleosidases or other nucleotide metabolic pathways.

Clinical and Laboratory Features

P5′N1 deficiency causes chronic hemolytic anemia, with severity ranging from compensated hemolysis without anemia to transfusion-dependent anemia. Ribosomal aggregates are visible in RBCs as coarse basophilic stippling. This basophilic stippling of RBCs is a laboratory hallmark of P5′N1 deficiency, and thus morphologic examination of the peripheral blood smear provides simple and inexpensive screening. However basophilic stippling is not a specific finding, as it is also found in hemolytic anemia caused by acute lead toxicity and in some sideroblastic anemias. The nucleotides of normal red cells consist largely of purine derivatives (which have an absorption maximum at approximately 260 nm) with very low levels of pyrimidine nucleotides (absorption at 280 nm). In P5′N1 deficiency, high levels of pyrimidine nucleotides accumulate in erythrocytes, resulting in a decrease in the OD260:OD280 absorbance ratio. Confirmation of the diagnosis requires demonstration of decreased P5′N1 activity, normal blood lead levels and, if feasible, high concentrations of pyrimidine nucleotides in red cells.

Therapy

Chronic transfusion support may be necessary for severe cases. Milder cases may require transfusion only periodically during pregnancy, infection, or other stressors. Iron overload may occur, as in any chronic hemolytic condition, and iron chelation may be needed. Splenectomy was reported to be beneficial in several cases.

OTHER ENZYMOPATHIES OF THE GLUTATHIONE PATHWAY

γ-Glutamylcysteine Synthase Deficiency

γ-Glutamylcysteine synthase (GCL) catalyzes the first metabolic step of glutathione synthesis. Deficiency of this enzyme is rare. Only a few cases, usually in consanguineous families, have been reported. All patients had hemolytic anemia and approximately half also had neurologic defects, including spinocerebellar degeneration, peripheral neuropathy, and mental retardation. Some patients have been characterized at the molecular level and in all these cases the causative mutation affected the catalytic domain of GCL.

Glutathione Synthetase Deficiency

Glutathione synthetase (GS) deficiency is a slightly more common enzymopathy of glutathione metabolism than GCL deficiency. Inheritance is autosomal recessive. The clinical severity is variable, but approximately 25% of the patients die in childhood. Mild GS deficiency can manifest as only mild chronic hemolysis. Moderately affected individuals present in the neonatal period with both hemolytic anemia and severe metabolic acidosis caused by the accumulation of the γ-glutamylcysteine metabolite 5-oxoproline, which results from decreased feedback inhibition of GCL by the decreased levels of GSH. Severely affected patients have progressive neurologic defects and animal experiments suggest that 5-oxoproline has a direct neurotoxic effect. This defect may also impair skin pigmentation and one affected African American child was an albino. The different clinical manifestations are likely compounded by environmental factors as phenotypical variability among siblings with the same mutation has been encountered.

High levels of 5-oxyproline in the urine associated with metabolic acidosis (found in all severely and moderately affected but in only some of the mildly affected patients) suggest the diagnosis, which is confirmed by documenting deficiency of the enzyme or by demonstrating mutations in the *GS* gene. Correction of the metabolic acidosis and early supplementation with antioxidants such as vitamins C and E was reported to improve survival and long-term outcome in some but not all affected patients.

Patients with glutathione synthetase deficiency should avoid the same agents known to precipitate acute hemolysis in patients with G6PD deficiency.

Glutathione Reductase Deficiency

Glutathione reductase (GR) restores intracellular GSH by reducing GSSG in the presence of NADPH and flavine adenine dinucleotide (FAD), a derivative of the water-soluble vitamin riboflavin. Hereditary GR deficiency has only been reported in two Dutch families. In one consanguineous family, acute hemolytic anemia after ingestion of fava beans occurred; GR deficiency was caused by a large homozygous GR-gene deletion. The second family had a compound heterozygous mutation and a clinical phenotype of neonatal jaundice.

Riboflavin is a cofactor of GR. Mild acquired GR deficiency is common and occurs in malnourished individuals because of riboflavin deficiency but has no hematologic phenotype.

Glutathione Peroxidase Deficiency

Glutathione peroxidase is a selenium-containing enzyme that reduces hydrogen peroxide to water. Although a polymorphism affecting the activity of glutathione peroxidase and acquired decreased activity because of selenium deficiency have been described, neither was associated with a clinical phenotype.

OTHER ENZYMOPATHIES OF THE GLYCOLYTIC PATHWAY

Hexokinase Deficiency

HK catalyzes the initial step in the utilization of glucose and thus is required for both glycolysis and the pentose shunt. HK produces glucose 6-phosphate. The two main HK isoenzymes in red cells, HK1 and HKR, are the product of a single gene *HK-1* by the use of an alternate translation initiation site. HKR is unique to RBCs and reticulocytes. HKR activity is maximum in reticulocytes, while HK1 activity declines gradually. HK deficiency is a rare cause of hereditary chronic nonspherocytic hemolytic anemia with about 30 patients reported. Most patients are of northern European extraction, although one case was reported in a Chinese individual; most are compound heterozygotes. Missense mutations are most common, but deletions, splice site mutations and one mutation located in the erythroid specific promotor have been reported. These mutations were associated with variable clinical severity, ranging from mild to severe hemolytic anemia and to fetal death. A single nucleotide polymorphism in *HK-1* is associated with reduced hemoglobin and hematocrit levels in a European population. The diagnosis of HK deficiency requires demonstration of decreased HK activity; however, the biphasic decline in HK activity adds to the complexity of the assay. HK is proximal to the generation of 2,3-BPG and thus decreased levels of 2,3-BPG in HK deficiency lead to a left-shifted hemoglobin-oxygen dissociation curve and a more symptomatic anemia than comparable levels of anemia seen in patients with other RBC-enzyme deficiencies. Splenectomy may be beneficial. In a mouse model of HK deficiency, severe hemolytic anemia with extensive tissue iron deposition and marked reticulocytosis was present.

Phosphofructokinase Deficiency

Phosphofructokinase (PFK) catalyzes the rate-limiting phosphorylation of fructose-6-phosphate by ATP to FDP. PFK is a tetrameric enzyme with three types of subunits: PFKL (liver), PFKM (muscle), and PFKP (platelet). Red cells contain both M and L subunits of PFK. PFK deficiency is an autosomal recessive disorder and, in the United States, predominantly occurs in Ashkenazi Jews. Approximately 100 patients have been described, all with mutations of *PFKM*. These mutations lead to an almost complete loss of PFK activity in muscle, but only partial loss of activity in erythrocytes. Two common mutations, an intronic splice site mutation, c.237+1 G>A, and single base pair deletion, c.2003delC, are present in Ashkenazi Jews; thus, genomic screening for these mutations should facilitate diagnosis in this patient population. The most common manifestation of PFK deficiency is type VII glycogen storage disease (Tarui disease), which causes muscle weakness and exercise intolerance; a mild chronic hemolytic anemia may also be present. In some patients, hemolysis is present without muscle manifestations.

Studies of PFK deficiency in both dogs and mice demonstrated exercise intolerance, reduced lifespan, and progressive cardiac hypertrophy.

Aldolase Deficiency

Aldolase reversibly cleaves FDP into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Aldolase deficiency is a very rare cause of chronic non-spherocytic hemolytic anemia; myopathy and mental retardation may also occur.

Phosphoglycerokinase Deficiency

Phosphoglycerokinase catalyzes the reversible conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate, forming ATP in the process. This reaction can be bypassed by the Rappaport-Luebering shunt where 2,3 BPG is formed. Deficiency of this X-chromosome encoded enzyme is variably associated with hemolytic anemia, myopathy and myoglobinuria, mental retardation, seizures, and progressive neurological deterioration; it is suspected that other environmental, genetic, or epigenetic factors contribute to the wide range of clinical manifestations. Marrow transplant with resulting stabilization of neurological symptoms has been reported in one patient.

Triosephosphate Isomerase Deficiency

Triosephosphate isomerase (TPI) catalyzes the reversible interconversion of the triose phosphate isomers, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. TPI deficiency is a rare autosomal recessive disorder characterized by chronic hemolytic anemia, increased susceptibility to bacterial infections, cardiomyopathy, and progressive neuromuscular disease, although the phenotype can vary even in family members with the same mutation. Neonatal jaundice may also occur. The neuromuscular disease is likely caused by the formation of toxic protein aggregates of glycated proteins formed by elevated byproducts of dihydroxyacetone phosphate.

Approximately 50 patients with several different mutations have been reported. Most patients have the same Glu104Asp mutation and are descendants from a common British/French ancestor about 1000 years ago, although the same Glu104Asp mutation was described on a different haplotype in one Spanish family. There is no effective therapy and most patients die in childhood, although there are rare exceptions.

OTHER ENZYMOPATHIES

Transaldolase Deficiency

Transaldolase catalyzes the conversion of seduhepulose-7-phosphate and glyceraldehyde-3-phosphate into erythrose-4-phosphate and fructose-6-phosphate. Transaldolase deficiency presents in the neonatal period and clinical manifestations include dysmorphic features, hepatosplenomegaly, cirrhosis, cardiac and renal abnormalities, skin manifestations, and thrombocytopenia. Hemolysis has been described in some subjects, although the phenotype is highly variable.

Adenosine Deaminase Hyperactivity

Adenosine deaminase (ADA) helps regulate the concentration of adenosine ribonucleotides in red cells by irreversibly deaminating them to inosine. Hyperactivity of ADA is a rare cause of hereditary nonspherocytic hemolytic anemia and is one of the two red-cell enzyme disorders inherited in an autosomal dominant disorder. However, the molecular mechanism of this disorder has not been identified. Deficiency of ADA is associated with severe combined immunodeficiency (see Chapter 23).

ENZYMOPATHIES ASSOCIATED WITH ERYTHROCYTOSIS

Bisphosphoglycerate Mutase Deficiency

Bisphosphoglycerate mutase (BPGM) regulates the concentration of 2,3-BPG in RBCs. 2,3-BPG is an important modifier of RBC oxygen delivery. 2,3-BPG binds to the hemoglobin tetramer and allosterically converts hemoglobin to a low oxygen affinity state, resulting in a rightward shift of the oxygen dissociation curve. BPGM is a multifunctional enzyme with both synthase and phosphatase activity. The synthase activity of BPGM converts 1,3-bisphosphoglycerate to 2,3-BPG, which is then metabolized to 3-phosphoglycerate (3-PGA), an intermediate of the glycolytic pathway, by BPGM-phosphatase (see Fig. 45.1). Deficiency of BPGM results in decreased levels of 2,3-BPG.

The consequent left shift of the oxygen dissociation curve increases hemoglobin affinity for oxygen, thus resulting in decreased delivery of oxygen into the peripheral tissues and compensatory erythrocytosis.

BPGM deficiency is rare and only two families have been comprehensively studied. In a family in France, four siblings were compound heterozygotes for two different BPGM mutations and had erythrocytosis, markedly decreased 2,3-BPG levels and undetectable BPGM activity. Some of their heterozygote relatives had a milder decrease of 2,3-BPG levels and a mild erythrocytosis. Other affected individuals have also been reported, although they have not have been as well characterized.

Cytochrome b5 Reductase Deficiency

b5R deficiency can cause erythrocytosis in association with chronic methemoglobinemia; this entity is described below.

Prolyl Hydroxylase 2 Deficiency

Prolyl hydroxylase 2 is part of the oxygen sensing pathway regulating HIFs. This enzyme is the principal component of hypoxia sensing, binds to alpha subunits of HIFs and initiates their degradation, and thus down regulates HIF-regulated genes including erythropoietin. Loss-of-function mutations of the gene encoding prolyl hydroxylase 2, *EGLN1*, cause dominantly inherited erythrocytosis. Mutations of other genes in this pathway including von Hippel-Lindau *(VHL)* and *EPAS1* mutations (encodes HIF2α) also cause congenital erythrocytosis (see Chapters 27 and 70).

ENZYMOPATHIES ASSOCIATED WITH METHEMOGLOBINEMIA

Introduction

Methemoglobinemia occurs when an imbalance arising from either increased methemoglobin production or decreased methemoglobin reduction is present. Methemoglobin is a derivative of hemoglobin in which the ferrous (Fe^{2+}) irons are oxidized to the ferric (Fe^{3+}) state. Methemoglobin is formed spontaneously at a slow rate by the autoxidation of hemoglobin. In the release of oxygen from Fe^{2+}-oxyhemoglobin, one electron is partially transferred to a small portion of released oxygen, generating superoxide and eventually forming other ROS, which convert the iron to the ferric state and form Fe^{3+}-hemoglobin, that is, methemoglobin (Fig. 45.3). Methemoglobin may also be formed from the oxidation of hemoglobin in other reactions with endogenous and exogenous compounds. The ferric hemes of methemoglobin are unable to bind oxygen and, additionally, if a ferriheme subunit is a part of a hemoglobin tetramer, the oxygen affinity of the accompanying ferrous hemes in the hemoglobin tetramer is increased. As a result, the oxygen dissociation curve is left-shifted and oxygen tissue delivery is impaired. Methemoglobin is formed continuously, but reducing mechanisms keep the methemoglobin level at about 1% of the total hemoglobin. The only physiologically important mechanism is the NADH-dependent cytochrome *b5* reductase (b5R). b5R contains a prosthetic FAD group that acts as an electron acceptor. NADH reduces FAD to FADH2, which then reduces the heme protein cytochrome *b5*. Electrons from the reduced cytochrome *b5* are in turn transferred to methemoglobin, reducing iron back to the ferrous state (see Fig. 45.3)

An alternative pathway mediated by NADPH-diaphorase uses NADPH generated by G6PD as a source of electrons to reduce redox dyes, such as methylene blue and flavin. Reduced methylene blue then reduces methemoglobin. Since these electron acceptors are not physiologic, this pathway is only of importance as it is the mechanism by which methylene blue treats acute toxic methemoglobinemia.

Epidemiology

Most cases of methemoglobinemia are acquired, resulting from increased methemoglobin formation by various exogenous agents. Acute or toxic methemoglobinemia may occur in the setting of overdose or poisoning, but also at standard doses of drugs. Acute methemoglobinemia occurs equally between males and females and over a wide range of ages; however, infants are more susceptible because their erythrocyte b5R activity is normally ~50% of adult activity.

Hereditary methemoglobinemia is most commonly caused by deficiency of b5R. b5R deficiency is an autosomal recessive condition and occurs in all racial and ethnic groups, but is endemic in certain

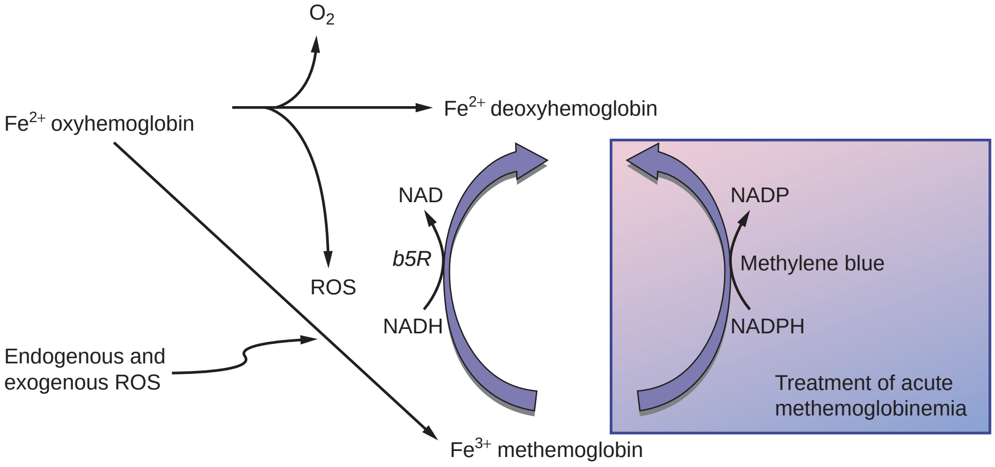

O_2
Fe^{2+} oxyhemoglobin
Fe^{2+} deoxyhemoglobin
NAD
b5R
NADH
ROS
Endogenous and exogenous ROS
Fe^{3+} methemoglobin
NADP
Methylene blue
NADPH
Treatment of acute methemoglobinemia

Figure 45.3 METHEMOGLOBINEMIA. Formation of methemoglobin and its physiologic *(open space)* and therapeutic *(shaded space)* reduction. Iron is in the ferrous state (Fe^{2+}) in oxygenated and deoxy-hemoglobin (deoxyHb). When oxygen is released, a small proportion of oxygen released from hemoglobin is converted to ROS. ROS then oxidizes bivalent ferrous iron (Fe^{2+}); to the ferric state (Fe^{3+}) (i.e., methemoglobin). NADH generated in glycolysis is a cofactor for methemoglobin reduction mediated by cytochrome b5 reductase *(b5R)* depicted in the *open space*, keeping methemoglobin at low levels (<1%). When this physiologic reduction of NADH-dependent methemoglobin reduction is either insufficient because of excessive ROS or decreased b5R activity, methemoglobin reduction can be achieved therapeutically. Exogenously administered methylene blue utilizing NADPH produced by G6PD in the pentose shunt can nonenzymatically convert methemoglobin to Fe^{2+} hemoglobin *(shaded space)*. *b5R*, Cytochrome b5 reductase; *NAD*, oxidized form of nicotinamide adenine dinucleotide phosphate; *NADH*, reduced form of NAD; *NADP*, oxidized form of nicotinamide adenine dinucleotide phosphate; *NADPH*, reduced form of NADP; *ROS*, reactive oxygen species.

populations, including Navajo and Athabasca Native Americans and natives of Yakutsk, Siberia and is also common among Puerto Ricans. Other causes of hereditary methemoglobinemia are the autosomal dominant inheritance of an abnormal hemoglobin in hemoglobin M disease (see Chapter 44) and, very rarely, deficiency of cytochrome b5.

Pathobiology

Acute Methemoglobinemia

Many drugs and toxins have been implicated in acute methemoglobinemia. More common culprits include dapsone and local anesthetics (benzocaine, lidocaine, prilocaine). Exposure to nitrates and nitrites, widely used as food preservatives and found in well water, can also cause methemoglobinemia. Infants less than 6 months of age may have increased susceptibility to methemoglobinemia at least in part because of their lower b5R activity.

b5 Reductase Deficiency

In RBCs, b5R transfers electrons to methemoglobin to reduce it to hemoglobin. In other cells, b5R transfers electrons from cytochrome *b5* to stearoyl-CoA in the endoplasmic reticulum, a reaction that has an important role in cholesterol biosynthesis, fatty acid elongation, desaturation, and drug metabolism. There are two types of b5R deficiency. The more common type I b5R deficiency is usually caused by missense mutations leading to decreased stability of the enzyme. Thus, although b5R is abnormal in all cells, only mature RBCs, which cannot synthesize proteins and replace the enzyme, are significantly affected in patients with type I b5R deficiency. Type II b5R mutations affect the catalytic site or lead to marked structural changes and all cells have decreased b5R activity.

Clinical Manifestations

Methemoglobinemia greater than 1.5 g/dL causes clinically discernible cyanosis. Methemoglobinemia should be clinically suspected when "cyanosis," occurs in the presence of a normal arterial $P\mathrm{ao}_2$. However, cyanosis is not specific for methemoglobinemia as it can be caused by high levels of deoxygenated hemoglobin (above approximately 5 g/dL) and sulfhemoglobin (above approximately 0.5 g/dL). The hemoglobin level is also a factor in the development of cyanosis. For example, a person with an Hb of 15 g/dL and 10% methemoglobin would be cyanotic, but a person with an Hb of 10 g/dL would need at least 15% methemoglobin to become cyanotic.

Acute Methemoglobinemia

Acute methemoglobinemia is an acquired disorder caused by certain oxidant drugs and chemicals; some patients who have baseline normal methemoglobin levels but who are heterozygous for b5R deficiency (see above) are more likely develop acute methemoglobinemia. Symptoms develop in acute methemoglobinemia due to abruptly impaired tissue oxygenation. Early symptoms include headache, fatigue, dyspnea, and lethargy. At higher levels, respiratory depression, altered consciousness, shock, seizures, and death may occur. As methemoglobin levels rise above 20% to 30%, patients can experience progressive respiratory compromise, myocardial ischemia, seizures, and coma. Death typically ensues at methemoglobin levels above 70% but can occur at lower levels.

Chronic Methemoglobinemia

Individuals with *type I b5R deficiency*, which is limited to RBCs, have methemoglobin concentrations of 10% to 35% and may or may not be cyanotic but are usually asymptomatic, even with methemoglobin levels up to 40%. Life expectancy is not shortened and pregnancies occur normally. Compensatory erythrocytosis is at times observed.

In addition to methemoglobinemia and cyanosis, patients with the less common *type II b5R deficiency* exhibit mental retardation and developmental delay. Other neurologic symptoms may be present, including microcephaly, opisthotonus, athetoid movements, strabismus, seizures, and spastic quadriparesis. Life expectancy is significantly shortened and death in infancy is typical.

Laboratory Manifestations

The laboratory diagnosis of methemoglobinemia is based on analysis of its absorption spectra. A fresh specimen should always be obtained because methemoglobin levels tend to increase with storage. Traditional pulse oximetry is unreliable in the presence of methemoglobinemia because of its light absorbance properties; however, arterial or venous blood co-oximetry can determine the methemoglobin fraction along with all other substances with the optical density at 630 nm.

Methemoglobin detected by co-oximeter is optimally confirmed by the specific Evelyn-Malloy method if available. This method involves direct spectrophotometric analysis and should be used when methemoglobinemia is suspected. In the Evelyn-Malloy method, blood is lysed in a slightly acidic buffer and the optical density is measured at 630 nm before and after adding a small amount of neutralized cyanide. Absorption of methemoglobin at this wavelength disappears when it is converted to cyanmethemoglobin. This method remains the most accurate technique for the estimation of methemoglobin concentration. It also rules out spurious methemoglobinemia from extrinsic substances with an absorbance at 630 nm.

An eight-wavelength pulse oximeter, Masimo Rad-57 (the Rainbow-SET Rad-57 Pulse CO-Oximeter, Masimo Inc, Irvine, CA) has been approved by the US Food and Drug Administration and appears to be accurate for the measurement of both carboxyhemoglobin and methemoglobin.

Distinguishing the hereditary forms of congenital methemoglobinemia requires interpretation of family pedigrees as well as biochemical analyses. Cyanosis in successive generations suggests autosomal dominant hemoglobin (Hb) M disease, whereas normal parents but possibly affected siblings implies autosomal recessive b5R deficiency. Incubation of blood with methylene blue also distinguishes b5R deficiency from Hb M disease, because this treatment results in the rapid reduction of methemoglobin through the NADPH-flavin reductase pathway in cases of b5R deficiency, but not in cases of Hb M disease. Types I and II b5R deficiency are distinguished by their clinical phenotype and by analysis of enzymatic activity in erythroid and non-erythroid cells. Because the enzyme defect is found in fibroblasts, analysis of b5R activity in cultured amniotic cells for prenatal diagnosis is possible.

Differential Diagnosis

Sulfhemoglobin in concentrations greater than 0.5 gm% also causes "cyanosis," with a normal $P\mathrm{ao}_2$ and may be erroneously measured as methemoglobin. Some drugs and other pigments, including methylene blue, may also produce false-positive results when methemoglobin is measured by co-oximetry.

Prognosis

Mild acute methemoglobinemia usually resolves spontaneously in a few days. Acute methemoglobinemia that is more symptomatic, including those with respiratory or other significant compromises, generally resolves with treatment, provided if the offending cause is discontinued; however, severe cases may be fatal. Patients with type I b5R deficiency have cyanosis but a normal life expectancy, whereas the pan-deficient type II b5R patients usually succumb in childhood.

Therapy

Offending agents in cases of acquired methemoglobinemia should be discontinued. No other therapy may be required in an asymptomatic patient. However, if the patient is symptomatic or if methemoglobin levels are greater than 20%, specific therapy is indicated. Methylene blue, 1 to 2 mg/kg intravenously over 5 minutes, is an effective treatment for patients with methemoglobinemia because NADPH formed in the pentose shunt can rapidly reduce this dye to leukomethylene blue in a reaction catalyzed by NADPH diaphorase. Leukomethylene blue, in turn, nonenzymatically reduces methemoglobin to hemoglobin.

There are two exceptions to the efficacy of this treatment. In patients who are G6PD deficient, methylene blue would not only fail to give the desired effect on methemoglobin levels but might compound the impaired tissue oxygen delivery by inducing an acute hemolytic episode. Methylene blue also has monoamine oxidase inhibitor properties and thus can precipitate a potentially life-threatening serotonin syndrome in individuals receiving serotonergic agents such as selective serotonin reuptake inhibitors (SSRIs) and other serotonergic antidepressants. If methylene blue is contraindicated, ascorbic acid can be given. Patients with G6PD deficiency and acute methemoglobinemia have been successfully treated with exchange transfusion. Hyperbaric oxygen has also been used in severe cases of methemoglobinemia.

The cyanosis in hereditary b5R type I deficiency is of cosmetic significance only but can be treated with methylene blue or ascorbic acid, both of which facilitate the reduction of methemoglobin through alternate pathways. However, this therapy has no effect on the neurologic and other systemic defects seen in type II b5R deficiency.

SUGGESTED READINGS

Balasubramaniam S, Duley JA, Christodoulou J. Inborn errors of purine metabolism: clinical update and therapies. *J Inherit Met ab Dis*. 2014;37:687–698.

Purine metabolism reviewed with implications for erythrocyte toxicity.

Bartels M, van Beers EJ, van Wijk R. Erythrocyte enzyme disorders. In: Kaushansky K, Lichtman MA, Prchal JT, eds. *Williams Hematology*. 10th ed. New York: McGraw Hill; 2022. p. 259–294.

Review of red cell enzymes with extensive bibliography of original and recent articles.

Beutler E. G6PD deficiency (review). *Blood*. 1994;84:3613–3636.

Succinct, yet comprehensive, review of G6PD pathophysiology, genetics, and clinical features by a major contributor to the field; continues to be relevant.

Clarke GM, Rockett K, Kivinen K, et al. Characterisation of the opposing effects of G6PD deficiency on cerebral malaria and severe malarial anaemia. *Elife*. 2017;2(6):e15085.

Critical review of G6PD deficiency and its beneficial and detrimental roles in complications of malaria.

Fermo E, Vercellati C, Marcello AP, et al. Clinical and molecular spectrum of glucose-6-phosphate isomerase deficiency. Report of 12 new cases. *Front Physiol*. 2019;10:467.

A critical review of GPI deficiency.

Grace RF, Mark Layton D, Barcellini W. How we manage patients with pyruvate kinase deficiency. *Br J Haematol*. 2019;184(5):721–734.

A summary of current understanding and management of clinical and metabolic features of pyruvate kinase deficiency.

Koralkova P, van Solinge WW, van Wijk R. Rare hereditary red blood cell enzymopathies associated with hemolytic anemia—pathophysiology, clinical aspects, and laboratory diagnosis. *Int J Lab Hematol*. 2014;36:388–397.

A critical review of rarer red cell enzyme enzymopathies.

Luzzatto L, Seneca E. G6PD deficiency: a classic example of pharmacogenetics with on-going clinical implications. *Br J Haematol*. 2014;164:133–201.

Critical review of G6PD deficiency.

UpToDate: Hematology chapters. Prchal JT. Methemoglobinemias, edited by Stan Schrier. First Edition 1999. Published on line, renewed yearly, last August 2020.

Critical review of common causes and management of acute toxic methemoglobinemia.

UpToDate: Hematology chapters. Prchal JT. Molecular pathogenesis of congenital erythrocytoses and polycythemia vera, edited by Stan Schrier. First Edition 1999. Published on line, renewed yearly, last August 2019.

A continuous update of defects and phenotypes of inherited erythrocytoses.

UpToDate: Hematology chapters. Prchal JT. Pyruvate kinase deficiency. First Edition 1999. Published on line, renewed yearly, last April 2020.

Continuous update of the phenotype and evolving therapy of pyruvate kinase deficiency.

van Wijk R, van Solinge WW. The energy-less red blood cell is lost: erythrocyte enzyme abnormalities of glycolysis. *Blood*. 2005;106:4034–4042.

This review focuses on the impact of energy metabolism of erythrocyte and its pathophysiology.

van Zwieten R, Verhoeven AJ, Roos D. Inborn defects in the antioxidant systems of human red blood cells. *Free Radic Biol Med*. 2014;67:377–386.

A critical review of defects in the antioxidant system of red cells.

Viprakasit V, Ekwattanakit S, Riolueang S, et al. Mutations in Kruppel-like factor 1 cause transfusion-dependent hemolytic anemia and persistence of embryonic globin gene expression. *Blood*. 2014;123:1586–1595.

An unusual cause of pyruvate kinase deficiency.

Youngster I, Arcavi L, Schechmaster R, et al. Drugs implicated to cause hemolysis in G6PD deficient subjects. *Drug Saf*. 2010;33:713–726.

Extensive and critical literature review and categorization of drugs and medications alleged to cause hemolysis in G6PD deficient subjects.

CHAPTER 46

RED BLOOD CELL MEMBRANE DISORDERS

Patrick G. Gallagher

Characterization of the structure and function of red blood cell (RBC) membrane proteins and their genes (Fig. 46.1) has led to considerable advances in our understanding of the molecular pathology of membrane-associated disorders, including the definition and characterization of mutations of membrane proteins as a well-defined cause of hereditary hemolytic disease. Likewise, knowledge of the molecular mechanisms underlying changes in RBC deformability, structural integrity, and shape has advanced. RBC shape abnormalities often provide a clue to the pathobiology and diagnosis of the underlying disorder. This chapter categorizes RBC membrane disorders according to the following morphologic and clinical phenotypes: (1) hereditary spherocytosis (HS); (2) hereditary elliptocytosis (HE), hereditary pyropoikilocytosis (HPP), and related disorders; (3) Southeast Asian ovalocytosis (SAO); (4) hereditary and acquired acanthocytosis; and (5) hereditary and acquired stomatocytosis (Tables 46.1 and 46.2).

VERTICAL AND HORIZONTAL INTERACTIONS OF MEMBRANE PROTEINS AND DISORDERS OF RED BLOOD CELL SHAPE

For a better understanding of the pathobiology of membrane disorders, membrane protein-protein and protein-lipid interactions are classified into two categories, vertical and horizontal interactions. Vertical interactions, which are perpendicular to the plane of the membrane, stabilize the lipid bilayer. These interactions include spectrin-ankyrin–band 3 interactions, spectrin-protein 4.1R–junctional complex proteins linkage, spectrin-ankyrin–Rh multiprotein complex linkage, and the weak interactions between the skeletal proteins and the negatively charged lipids of the inner half of the membrane lipid bilayer. Horizontal interactions, which are parallel to the plane of the membrane, support the structural integrity of erythrocytes after their exposure to shear stress. Horizontal interactions involve the spectrin heterodimer association site, where spectrin heterodimers assemble into tetramers, the principal building blocks of the membrane skeleton, and the contacts of the distal ends of spectrin heterodimers with actin and protein 4.1R within the junctional complex. Although interactions between proteins of the erythrocyte membrane are significantly more complex than can be classified by horizontal and vertical interactions, the model serves as a useful starting place for understanding erythrocyte membrane protein interactions, particularly in reference to membrane-related disorders.

According to the vertical/horizontal model, HS is considered a disorder of vertical interactions. Although the primary molecular defects in HS are heterogeneous (including deficiencies or dysfunctions of α- and β-spectrin, ankyrin, band 3, and protein 4.2), one common feature of HS RBCs is a weakening of the vertical contacts between the skeleton and the overlying lipid bilayer membrane together with its integral proteins. Consequently, the lipid bilayer membrane is destabilized, leading to the release of bilayer lipids from the cells in the form of skeleton-free lipid vesicles. This lipid loss, in turn, results in membrane surface area deficiency and spherocytosis.

In most patients with HE and the related disorder HPP (see Hereditary Elliptocytosis and Related Disorders), the principal lesion involves horizontal membrane-protein associations, primarily spectrin dimer-dimer interactions. In a subset of HE patients with a deficiency or a dysfunction of protein 4.1R or glycophorin C (GPC), the horizontal defect resides in the junctional complex, where the distal ends of spectrin tetramers connect to actin, in conjunction with protein 4.1R. In patients with severely dysfunctional spectrin mutations, the weakened spectrin dimer-dimer self-association disrupts the skeletal lattice, leading to a marked skeletal instability and cell fragments. In patients with mildly dysfunctional spectrins, the RBC shape is that of biconcave elliptocytes. It is speculated that elliptocytes are permanently deformed cells because the weakened horizontal interactions facilitate a shear stress-induced rearrangement of skeletal proteins, precluding recovery of the normal biconcave shape. This hypothesis is not applicable to all forms of elliptocytosis. For example, in SAO, the elliptocytic/ovalocytic cells containing mutant band 3 protein are rigid and "hyperstable" rather than unstable.

Acanthocytosis, Stomatocytosis, and the Bilayer Couple Hypothesis

The mechanism of acanthocytosis and stomatocytosis associated with defects of membrane proteins is much less clear. Most forms of acanthocytosis are associated with either acquired or inherited abnormalities of membrane lipids (e.g., acanthocytosis in end-stage liver disease or abetalipoproteinemia). In rare cases with acanthocytosis, membrane protein abnormalities have been detected, but the associated mechanisms leading to acanthocyte formation are unknown. These abnormalities occur in the McLeod phenotype, the chorea-acanthocytosis syndrome, and other rare disorders. In acanthocytosis erythrocytes, agents that interact with the lipids of the inner lipid bilayer leaflet normalize the shape. These studies suggest that the shape abnormalities reflect an asymmetry in the distribution of membrane lipids between the two halves of the RBC lipid bilayer as predicted by the bilayer couple hypothesis. According to the bilayer hypothesis, the shape of the RBC reflects the ratio of the surface areas of the two hemileaflets of the bilayer. The preferential expansion of the outer leaflet leads to RBC crenation (echinocytosis or acanthocytosis), whereas expansion of the inner lipid bilayer produces a cup shape (stomatocytosis) and surface invaginations.

Hereditary Spherocytosis

Introduction and Epidemiology

The typical features of HS include a dominantly inherited hemolytic anemia of mild to moderate severity, spherocytosis on the peripheral blood film, and a favorable response to splenectomy. The clinical spectrum of HS is variable and includes both mild and asymptomatic forms, as well as severe forms that appear in infancy. The previously reported HS prevalence in Western populations of 1 in 4000 persons is an underestimation because milder forms of HS might be asymptomatic, suggesting a prevalence of 1 in 2000 individuals. HS has been reported worldwide, particularly in Northern European and Japanese populations, but its prevalence in other ethnic groups is unknown.

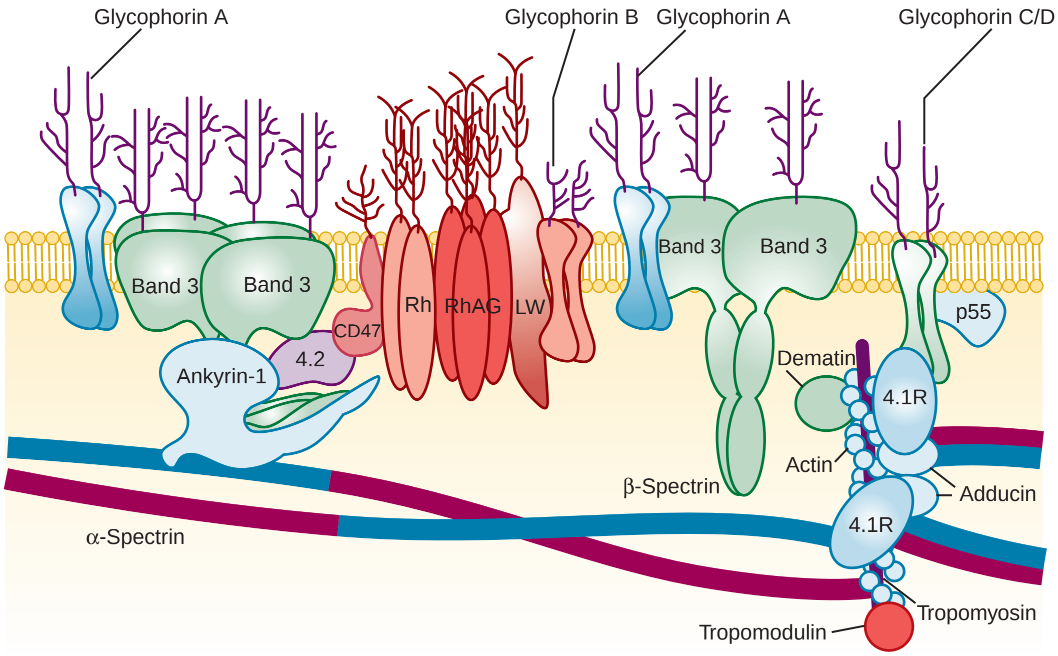


Figure 46.1 A SIMPLIFIED CROSS-SECTION OF THE ERYTHROCYTE MEMBRANE. The lipid bilayer forms the equator of the cross-section with its polar heads *(small circles)* turned outward. *4.1R*, Protein 4.1R; *4.2*, protein 4.2; *Rh*, Rhesus polypeptide; *RhAG*, Rh-associated glycoprotein; *LW*, Landsteiner-Wiener glycoprotein. *(Reproduced with permission from Perrotta S, Gallagher PG, Mohandas N. Hereditary spherocytosis. Lancet. 2008:372:1411.)*

TABLE 46.1 **Erythrocyte Membrane Abnormalities in Hereditary Spherocytosis, Hereditary Elliptocytosis, and Related Disorders**

Gene	Disorder	Comment
α-Spectrin	HS, HE, HPP, NIHF	Location of mutation determines clinical phenotype. α-Spectrin mutations are most common cause of typical HE
Ankyrin	HS	Most common cause of typical dominant HS
Band 3	HS, SAO, NIHF	In HS "pincer-like" spherocytes on smear presplenectomy. SAO erythrocytes have transverse ridge or longitudinal slit
β-Spectrin	HS, HE, HPP, NIHF	Location of mutation determines clinical phenotype. In HS, acanthrocytic spherocytes on smear presplenectomy
Protein 4.2	HS	Common in Japanese HS
Protein 4.1	HE	
GPC	HE	Concomitant protein 4.1 deficiency is basis of HE in GPC defects

GPC, Glycophorin C; *HE,* hereditary elliptocytosis; *HPP,* hereditary pyropoikilocytosis; *HS,* hereditary spherocytosis; *NIHF,* nonimmune hydrops fetalis; *SAO,* Southeast Asian ovalocytosis.

Pathobiology

Two major factors are involved in HS pathophysiology: (1) an intrinsic RBC defect and (2) an intact spleen that selectively retains and damages abnormal HS erythrocytes. An inherited deficiency or dysfunction of proteins of the erythrocyte membrane leads to a multistep process of accelerated HS RBC destruction. Destabilization of the lipid bilayer facilitates a release of lipids from the membrane, leading to surface area deficiency and the formation of poorly deformable spherocytes that are selectively retained and damaged in the spleen.

Molecular Pathology

The molecular basis of HS is heterogeneous. Based on densitometric quantitation of membrane proteins separated by polyacrylamide gel electrophoresis, HS can be divided into the following subsets: (1) isolated deficiency of spectrin, (2) combined deficiencies of spectrin and ankyrin, (3) deficiency of band 3 protein, (4) deficiency of protein 4.2, and (5) no abnormality identified.

Isolated Spectrin Deficiency

The reported mutations of isolated spectrin deficiency include defects of both α- and β-spectrin. Mutations of the β-spectrin gene have been identified in a number of patients with dominantly inherited HS associated with spectrin deficiency. A few cases have been associated with de novo β-spectrin gene mutations. With a few exceptions, these mutations are private and may be associated with decreased β-spectrin messenger ribonucleic acid (mRNA) accumulation. Mutations in the highly conserved region of β-spectrin involved in the interaction with protein 4.1R likely lead to dysfunctional binding to protein 4.1R and thereby the linkage of spectrin to actin.

In nondominantly inherited HS associated with isolated spectrin deficiency, the defect involves α-spectrin. In normal erythroid cells, α-spectrin is synthesized in large excess of β-spectrin. Thus, patients with one normal and one defective α-spectrin allele are asymptomatic because α-spectrin production remains in excess of β-spectrin synthesis, allowing normal amounts of spectrin heterodimers to be assembled on the membrane. Patients who are homozygotes or compound heterozygotes for α-spectrin defects suffer from moderate to severe HS.

Combined Deficiency of Spectrin and Ankyrin

The biochemical phenotype of combined spectrin and ankyrin deficiency is the most common abnormality found in the erythrocytes of HS patients. Ankyrin represents the principal binding site for spectrin on the membrane; thus, it is not surprising that ankyrin deficiency is accompanied by a proportional decrease in spectrin assembly on

TABLE 46.2 Peripheral Blood Film Evaluation in a Patient With Red Cell Membrane Disorder

Shape	Pathobiology	Diagnosis
Microspherocytes	Loss of membrane lipids leading to a reduction of surface area resulting from deficiencies of spectrin, ankyrin, or band 3 and protein 4.2 Removal of membrane material from antibody-coated red cells by macrophages Removal of membrane-associated Heinz bodies, with the adjacent membrane lipids, by the spleen	HS Immunohemolytic anemias Heinz body hemolytic anemias
Elliptocytes	Permanent red cell deformation resulting from a weakening of skeletal protein interactions (such as the spectrin dimer-dimer contact). This facilitates disruption of existing protein contacts during shear stress-induced elliptical deformation. Subsequently, new protein contacts are formed that stabilize elliptical shape Unknown	Mild common HE Iron deficiency, megaloblastic anemias, myelofibrosis, myelophthisic anemias, myelodysplastic syndrome, thalassemias
Poikilocytes/fragments	Weakening of skeletal protein contacts resulting from skeletal protein mutations Unknown	Hemolytic HE/HPP Iron deficiency, megaloblastic anemias, myelofibrosis, myelophthisic anemias, myelodysplastic syndrome, thalassemias
Schistocytes, fragmented red cells	Red cells “torn” by mechanic trauma (fibrin strands, turbulent flow)	“Microangiopathic” hemolytic anemia associated with disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, vasculitis, heart valve prostheses
Acanthocytes	Uptake of cholesterol and its preferential accumulation in the outer leaflet of the lipid bilayer Selective accumulation of sphingomyelin in the outer lipid leaflet Unknown	Spur cell hemolytic anemia in severe liver disease Abetalipoproteinemia Chorea-acanthocytosis syndrome, malnutrition, hypothyroidism, McLeod phenotype
Echinocytes	Expansion of the surface area of the outer hemileaflet of lipid bilayer relative to the inner hemileaflet Unknown	Hemolytic anemia associated with hypomagnesemia and hypophosphatemia in malnourished patients, pyruvate kinase deficiency; in vitro artifact of low blood storage (ATP depletion), contact with glass or elevated pH Hemolysis in long-distance runners, renal failure
Stomatocytes	Expansion of the surface area of the inner hemileaflet of the bilayer relative to the outer leaflet Unknown	Exposure of red cells to cationic anesthetics in vitro; in vivo the drug concentrations may not be sufficient to produce similar effect Alcoholism, inherited disorders of membrane permeability (hereditary stomatocytosis)
Target cells	Absolute excess of membrane lipids (both cholesterol and phospholipids: “symmetric” lipid gain), followed by an increase of cell surface area Relative excess of surface area because of a decrease in cell volume	Obstructive jaundice, liver disease with intrahepatic cholestasis Thalassemias and some hemoglobinopathies (C, D, E)

ATP, Adenosine triphosphate; *HE*, hereditary elliptocytosis; *HPP*, hereditary pyropoikilocytosis; *HS*, hereditary spherocytosis.

the membrane despite normal spectrin synthesis. Similar to HS associated with β-spectrin mutations, most ankyrin defects are private point mutations associated with decreased mRNA accumulation. In some cases, mutations of the ankyrin promoter leading to decreased ankyrin expression have been found. Approximately 15% to 20% of ankyrin gene mutations reported are de novo mutations.

A number of patients with atypical HS associated with karyotypic abnormalities involving deletions or translocations of the ankyrin gene locus on chromosome 8p have been described. Ankyrin deletions may be part of a contiguous gene syndrome with manifestations of spherocytosis, mental retardation, typical facies, and hypogonadism.

Deficiency of Band 3 Protein

Deficiency of band 3 protein is found in a subset of HS patients who present with a phenotype of a mild to moderate dominantly inherited HS. Most, if not all, of these patients also have concomitant protein 4.2 deficiency. Numerous band 3 mutations associated with HS have been reported, spread throughout both the cytoplasmic and the membrane-spanning domains.

A number of band 3 mutations clustered in the membrane-spanning domain that replace highly conserved arginines have been described. These arginines, which are all located at the cytoplasmic end of a predicted transmembrane helix, exhibit defective cellular trafficking from the endoplasmic reticulum to the plasma membrane.

Alleles have been identified that influence band 3 expression and that, when inherited in trans to a band 3 mutation, aggravate band 3 deficiency and worsen the clinical severity of the disease.

Deficiency of Protein 4.2

Recessively inherited HS caused by mutations in protein 4.2 is relatively common in Japan. In these cases, an almost total absence of protein 4.2 from the erythrocyte membranes of homozygous patients

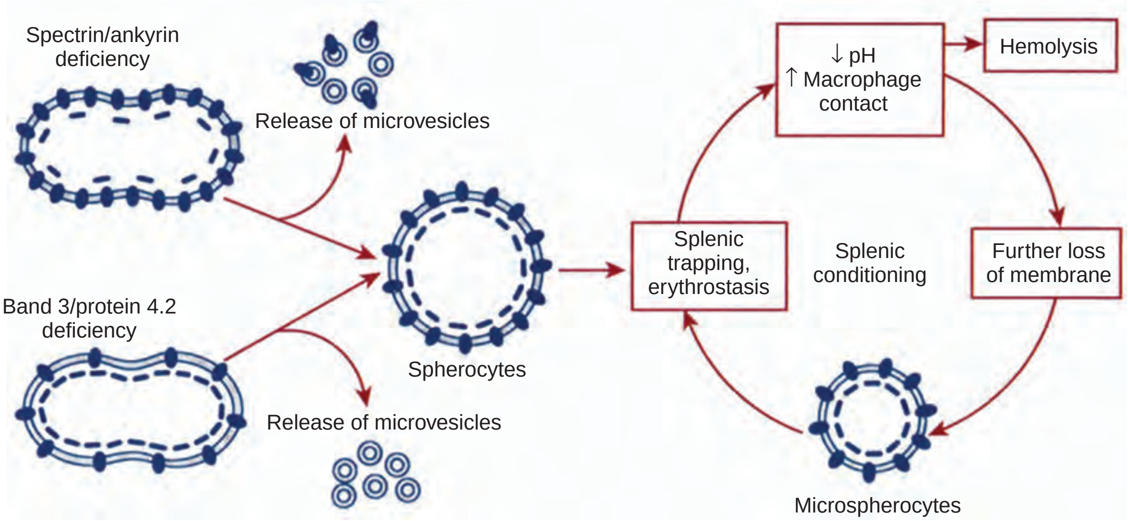


Figure 46.2 PATHOPHYSIOLOGY OF HEREDITARY SPHEROCYTOSIS. The primary defect in hereditary spherocytosis is a deficiency of membrane surface area. Decreased surface area may be produced by two different mechanisms: (1) Defects of spectrin, ankyrin, or protein 4.2 lead to reduced density of the membrane skeleton, destabilizing the overlying lipid bilayer and releasing band 3-containing microvesicles. (2) Defects of band 3 lead to band 3 deficiency and loss of its lipid-stabilizing effect. This results in the loss of band 3-free microvesicles. Both pathways result in membrane loss, decreased surface area, and formation of spherocytes with decreased deformability. These deformed erythrocytes become trapped in the hostile environment of the spleen where splenic conditioning inflicts further membrane damage, amplifying the cycle of red cell membrane injury.

is detected. Protein 4.2–deficient erythrocytes can also have a decreased content of ankyrin and band 3. Protein 4.2 deficiency also occurs in association with band 3 mutations, probably as a result of abnormal binding of protein 4.2 to the cytoplasmic domain of band 3.

A detailed listing of HS mutations is available in mutation databases maintained by the Human Gene Mutation Database (HGMD, http://www.hgmd.org).

Molecular Basis of Surface Area Deficiency

Hereditary spherocytes are intrinsically unstable, releasing lipids under a variety of in vitro conditions, including adenosine triphosphate (ATP) depletion or exposure of cells to shear stress. The loss of membrane material occurs through the release of vesicles containing integral proteins devoid of spectrin. During in vitro incubation, the loss of membrane material is sufficient to augment the surface area deficiency, as evidenced by increased osmotic fragility of the cells after incubation. It is assumed but not proved that a similar process takes place in vivo.

The molecular basis of HS is heterogeneous; thus, it is likely that surface area deficiency is a consequence of several distinct molecular mechanisms whose common denominator is either a weakening of the vertical connections between the skeleton and the lipid bilayer membrane or a weakening of the stabilizing effect of transmembrane proteins on adjacent lipid molecules of the plasma membrane. Hypothetic pathways that can lead to surface area deficiency are depicted in Fig. 46.2. In patients with isolated spectrin deficiency or a combined deficiency of spectrin and ankyrin, the loss of RBC surface may be caused by an uncoupling of the lipid bilayer membrane from the underlying skeleton. In normal RBCs, the skeleton forms a nearly monomolecular submembrane layer occupying more than one-half of the inner surface of the membrane. Consequently, spectrin deficiency leads to a decreased density of this network. As a result, areas of the lipid bilayer membrane that are not directly supported by the skeleton are susceptible to release from the cells in the form of microvesicles.

In HS associated with a deficiency of band 3 protein, two hypothetic pathways may lead to a loss of surface area. One mechanism may involve a loss of band 3 protein from the cells. Because band 3 protein spans the lipid bilayer membrane many times, it is likely that a substantial amount of "boundary" lipids are released together with the band 3 protein, thus leading to surface area deficiency. Another possible mechanism may involve a formation of band 3-free domains in the membrane, followed by the formation of membrane blebs, which are subsequently released from the cells as microvesicles. Such a hypothesis is based on the observation that aggregation of intramembrane particles (composed principally of band 3) in ghosts leads to the formation of particle-depleted domains from which membrane lipids bleb off as microvesicles. Additional evidence supporting the latter model comes from the band 3 knock-out mouse model and from human, cow, and zebrafish cases of complete band 3 deficiency. Erythrocytes lacking band 3 spontaneously shed membrane vesicles, leading to spherocytosis and hemolysis.

Alterations in Cation Content and Permeability

HS RBCs, particularly those collected from the spleen, are somewhat dehydrated and abnormally permeable to monovalent cations, presumably as a consequence of the underlying membrane defect. The cellular dehydration may be caused by activation of pathways causing a selective loss of potassium and water or a hyperactive Na^+/K^+ pump.

Entrapment of Nondeformable Spherocytes in the Spleen

The importance of the spleen in the pathophysiology of hemolysis in HS was appreciated in the original description of the disease and has been substantiated by subsequent studies. HS cells are selectively destroyed in the spleen because of their poor deformability and because of the unique anatomy of the splenic vasculature that acts as a microcirculation filter.

The poor RBC deformability is principally a consequence of a decreased cell surface/cell volume ratio resulting from the loss of surface material. Normal discocytes have an excess surface, allowing them to deform and pass through narrow microcirculation openings. In contrast, HS RBCs lack this extra surface, and their poor deformability may be further impaired by cellular dehydration.

The principal sites of RBC entrapment in the spleen are fenestrations in the wall of splenic sinuses, where blood from the splenic cords of the red pulp enters the venous circulation. In rat spleen, the length and width of these fenestrations, 2 to 3 μm and 0.2 to 0.5 μm, respectively, are approximately half the RBC diameter. Electron micrographs show that very few HS RBCs traverse these slits. Consequently, the nondeformable spherocytes accumulate in the red pulp, which becomes grossly engorged.

Splenic Conditioning and Destruction

Once trapped in the spleen, HS erythrocytes undergo additional damage or conditioning with further loss of surface area and an increase in cell density, as is evident in cells removed from the spleen at splenectomy. Some of these conditioned RBCs reenter the systemic circulation, as revealed by the "tail" of the osmotic fragility (OF) curve, indicating the presence of a subpopulation of cells with a markedly reduced surface area. After splenectomy, this RBC population disappears.

Contributions to conditioning may include a relatively low pH in the spleen as well as in the sequestered RBCs that may further compromise the poor HS RBC deformability and contact of RBCs with macrophages that may inflict additional damage on the RBC membrane. The conditioning effect of the spleen appears to represent a cumulative injury. The average residence time of HS RBCs in the splenic cords is between 10 and 100 minutes compared with 30 to 40 seconds for normal RBCs, and only 1% to 10% of blood entering the spleen is temporarily sequestered in the congested cords, whereas the remaining 90% of blood flow is rapidly shunted into the venous circulation.

Inheritance

The HS genes are assigned to several chromosomes, including chromosome 1 (α-spectrin), chromosome 8 (ankyrin), chromosome 14 (β-spectrin), chromosome 15 (protein 4.2), and chromosome 17 (band 3). In approximately two-thirds of HS patients, inheritance is autosomal dominant. In the remaining patients, inheritance is nondominant. In many of these patients, HS is caused by a de novo mutation, which is inherited in an autosomal recessive fashion in subsequent generations. Recessively inherited HS cases manifesting with severe hemolytic anemia have been reported. The majority of the affected patients were found to be severely deficient in RBC spectrin, associated with α-spectrin defects. The remaining cases characterized by a recessive inheritance pattern are caused by a defect in protein 4.2, a deficiency that is associated with relatively mild hemolysis.

Only a few cases of homozygous or "double dominant" HS have been reported. These patients have a severe hemolytic anemia, whereas their mostly consanguineous parents have a mild to moderate form of the disease or are asymptomatic.

Although the clinical severity of HS is highly variable among different kindred, in general, it is relatively uniform within a given family, in which HS is typically inherited as an autosomal dominant disorder. However, HS kindred have been described in which there was great variability in the clinical severity of affected family members. Several explanations might account for these observations, including variable penetrance of the genetic defect, a de novo mutation, presence of a mild recessive HS in the kindred, presence of a modifier allele that influences the expression of a membrane protein, or a tissue-specific mosaicism of the defect.

Clinical Manifestations

Typical Forms

The typical HS patient is relatively asymptomatic. As noted in the earliest descriptions of HS, mild jaundice can be the only symptom of the disease. Anemia is usually mild to moderate but may be absent because of compensatory bone marrow hyperplasia manifest by reticulocytosis. Splenomegaly gradually develops in most patients, with the spleen occasionally reaching large dimensions.

Mild Forms and Carrier State

In some families, anemia is absent, the reticulocyte count is normal or only minimally elevated, laboratory evidence of hemolysis is minimal or absent, and the changes in RBC shape can be mild, escaping detection on the peripheral blood film. The presence of HS is detected only by laboratory testing or during evaluation of a relative with a more symptomatic form of the disease. Some patients are first diagnosed during transient viral infections such as infectious mononucleosis or parvovirus infection, during pregnancy, or even in the 7th to 9th decades of life as the bone marrow's ability to compensate for hemolysis wanes.

Severe and Atypical Forms

The relatively uncommon patients with nondominant forms of HS can present with a severe life-threatening hemolysis early in life. Some patients can be transfusion dependent during early infancy and childhood. The underlying molecular defects include severe spectrin or band 3 deficiency.

Hereditary Spherocytosis and Nonerythroid Manifestations

In most HS cases, the clinical manifestations are confined to the erythroid lineage, probably because many of the nonerythroid counterparts of the RBC membrane proteins (e.g., spectrin and ankyrin) are encoded by separate genes or because some proteins (e.g., protein 4.1R, β-spectrin, ankyrin) are subject to tissue-specific alternative splicing. However, several HS kindred have been reported with a cosegregating neurologic or muscular abnormality, such as a degenerative disorder of the spinal cord, cardiomyopathy, or mental retardation. The observation that both erythrocyte ankyrin and β-spectrin are also expressed in muscles and the brain, particularly the cerebellum, and spinal cord raises the possibility that these HS patients may have a defect in one of these proteins. This hypothesis is further supported by studies of *nb/nb* mice, a mouse model of HS caused by an ankyrin mutation. These mice develop a neurologic syndrome with a progression that coincides with the loss of ankyrin from the Purkinje cells of the cerebellum.

Mutations of band 3 without HS have been described in patients with distal renal tubular acidosis. With a few rare exceptions, most patients with heterozygous mutations of band 3 and HS have normal renal acidification.

Laboratory Manifestations

Most HS patients have mild to moderate anemia or no anemia at all, reflecting the fact that the hemolytic rate can be very mild and that the hemolysis is fully compensated for by increased RBC production, as evidenced by reticulocytosis. Some patients, however, particularly those with nondominantly inherited HS, are severely anemic, with hemoglobin concentrations as low as 4 to 6 g/dL.

Despite the increased percentage of reticulocytes with a larger volume than mature RBCs, the mean corpuscular volume (MCV) of HS RBCs is often low normal or even slightly decreased, and the mean corpuscular hemoglobin concentration (MCHC) is usually increased (>35 g/dL), together reflecting mild cellular dehydration.

The finding of an MCHC greater than 35.4 g/dL combined with an RBC distribution width (RDW) <14% has been found to be an excellent screening test for HS. Another screening method measures MCV by light scattering and provides a histogram of hyperdense erythrocytes (MCHC > 40 g/dL) claimed to identify nearly all HS patients. These hyperdense erythrocytes can be detected with newer laser-based blood counters or using aperture impedance analysis available in many clinical laboratories.

Evidence of accelerated RBC destruction, as indicated by increased lactate dehydrogenase and unconjugated bilirubin levels and by decreased haptoglobin, as well as by reticulocytosis, is present in typical HS patients. However, these abnormalities can be absent in individuals with a mild form of the disease.

Blood Film

In a typical case of HS, spherocytes are readily identified by their characteristic shape on the peripheral blood film (Fig. 46.3). They lack central pallor, their mean cell diameter is decreased, and they appear more intensely hemoglobinized, reflecting both altered RBC geometry and increased cell density. In a three-dimensional view, some spherocytes have a spherostomatocytic shape that is occasionally appreciated on the peripheral blood film. In mild forms of the disease, the peripheral blood smear can appear normal because the loss of surface area can be too small to be appreciated by blood smear evaluation; the cells appear as “fat” disks rather than as true spherocytes.

Additional morphologic features have been described in some HS patients (see Fig. 46.3). A subset of HS patients whose RBCs are deficient in band 3 protein have some pincer-like RBCs on the peripheral blood film, a finding that is both sensitive and specific for this HS subset. These pincer-like cells disappear after splenectomy. Surface spiculations or acanthocytic spherocytes have been described in cases of HS associated with defects in β-spectrin. Frequent spherovalocytes and stomatocytes have been reported in Japanese patients with protein 4.2 deficiency.

Osmotic Fragility and Eosin-5′-Maleimide Binding

Both OF testing and eosin-5′-maleimide (EMA) binding are used in evaluating HS patients.

The OF test (Fig. 46.4) measures the in vitro lysis of RBCs suspended in solutions of decreasing osmolarity. The normal RBC membrane is unstretchable and is virtually freely permeable to water. Thus, the cell behaves as a nearly perfect osmometer in that it increases its volume in hypotonic solutions progressively until a “critical hemolytic volume” is reached. At this point, the RBC membrane ruptures, and hemoglobin escapes into the supernatant solution. As a result of the loss of membrane and the ensuing surface area deficiency, the critical hemolytic volume of spherocytes is considerably lower than that of normal RBCs. Consequently, these cells hemolyze more than normal RBCs when suspended in hypotonic sodium chloride solutions. However, a finding of increased osmotic fragility is not unique to HS and is also present in other conditions associated with spherocytosis on the peripheral blood film, such as autoimmune hemolytic anemia.

The OF curve often reveals uniformly increased osmotic fragility. A “tail” of the OF curve can be present in nonsplenectomized HS patients, indicating a subpopulation of particularly fragile RBCs conditioned by splenic stasis. This subpopulation of cells disappears after splenectomy. In patients with mild HS, osmotic fragility can be normal and abnormalities can be found only after incubation that further augments the loss of surface area; however, the sensitivity of the incubated OF test can be outweighed by a loss of its specificity. The relative contributions of cell dehydration and surface area deficiency can be accurately determined by osmotic gradient ektacytometry, available in specialized laboratories.

OF testing is unreliable in patients who have small numbers of spherocytes, including those who have been recently transfused, and it is abnormal in other conditions where spherocytes are present.

The binding of EMA to band 3 and Rh-related proteins with a 1 : 1 stoichiometry in the erythrocyte membrane is the basis for the EMA binding test. After binding of fluorescently labeled EMA to erythrocyte membranes, the relative amount of fluorescence, reflecting the amount of EMA binding, is analyzed by flow cytometry. The intensity of EMA binding is decreased in HS erythrocytes (see Fig. 46.4). Although defects of band 3 protein are only found in ~25% of typical HS patients, decreased EMA fluorescence is also observed in HS erythrocytes with primary defects in ankyrin and spectrin, thought to be caused by the transmission of long-range effects of varying protein defects across the membrane, influencing EMA binding. EMA binding has high sensitivity and specificity. In laboratories with the ability to perform fluorescence-activated cell sorting-based studies, it is simple and rapidly performed, even on samples after shipment or storage.

Like osmotic fragility, EMA binding struggles in the diagnosis of mild HS where results may be normal or indeterminate. In addition, normal EMA binding has been reported in some cases of spectrin-deficient HS. Other erythrocyte abnormalities such as defects of erythrocyte hydration and variants of dyserythropoietic anemia can also yield abnormal results.

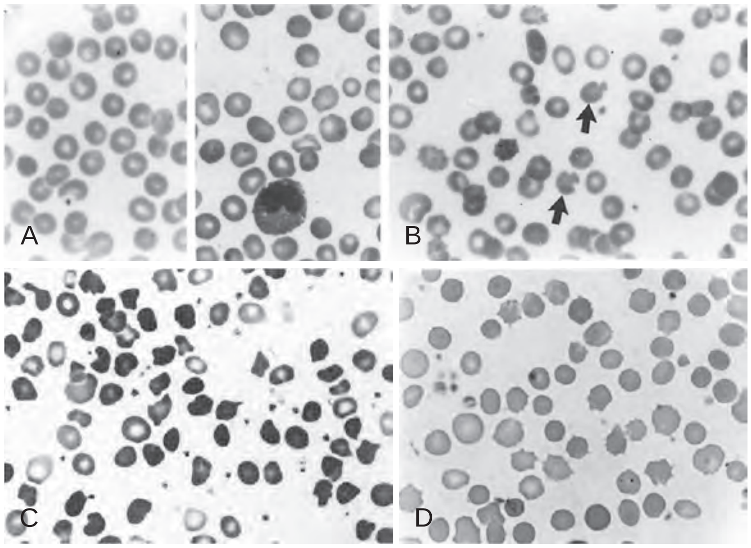


Figure 46.3 BLOOD FILMS FROM PATIENTS WITH HEREDITARY SPHEROCYTOSIS (HS) OF VARYING SEVERITY. (A) Two blood films of typical moderately severe HS with a mild deficiency of red blood cell (RBC) spectrin and ankyrin. Although many cells have a spheroidal shape, some retain a central concavity. (B) HS with pincer-like RBCs *(arrows)*, as typically seen in HS associated with band 3 deficiency. Occasional spiculated RBCs are also present. (C) Severe atypical HS caused by a severe combined spectrin and ankyrin deficiency. In addition to spherocytes, many cells have irregular contour. (D) HS with isolated spectrin deficiency caused by a β-spectrin mutation. Some of the spherocytes have prominent surface projections resembling sphero-acanthocytes.

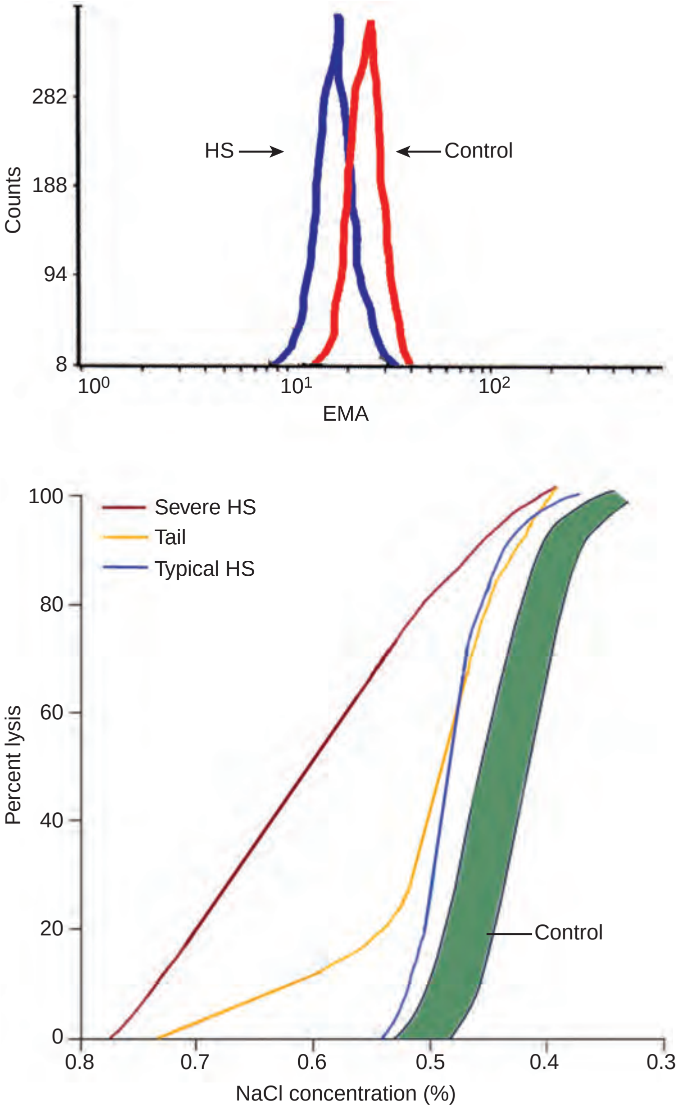


Figure 46.4 TESTING IN HEREDITARY SPHEROCYTOSIS (HS). *(Top)* Eosin-5′-maleimide (EMA) binding. EMA binding, a flow cytometric test that measures the fluorescence intensity of intact red blood cells labeled with the dye eosin-5′-maleimide that reacts with membrane band 3 protein, has been shown to be useful as a first-line test for the diagnosis of hereditary spherocytosis. Histogram of fluorescence of EMA-labeled erythrocytes from normal controls *(red)* and a patient with typical hereditary spherocytosis (HS) *(blue)*. Decreased fluorescence is observed from HS erythrocytes. *(Bottom)* Osmotic fragility (OF) curves in hereditary spherocytosis. The shaded region is the normal range. Results representative of both typical and severe spherocytosis are shown. A tail, representing fragile erythrocytes conditioned by the spleen, is common in spherocytosis patients before splenectomy. *(Reproduced with permission from Gallagher PG. Abnormalities of the erythrocyte membrane. Pediat Clin North Am. 2013;60:1349.)*

Autohemolysis and Other Tests

RBC autohemolysis, the spontaneous hemolysis of RBCs incubated under sterile conditions without glucose, was previously advocated as a sensitive test for the detection of HS. This test is being used less frequently and is probably no more sensitive than the incubated OF test. Other tests described in the literature such as the glycerol lysis test, the pink test, hypertonic cryohemolysis, and the skeleton gelation test are infrequently performed in diagnostic laboratories in the United States. The former two tests, which use glycerol to retard the osmotic swelling of RBCs, are preferred by some laboratories because they are easy to perform and can be adapted to microsamples. Cryohemolysis testing in particular remains popular in Europe.

Detection of the Underlying Molecular Defect

Because the most common finding in erythrocytes of patients with HS is a deficiency of one or more of the membrane proteins, molecular studies often include sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) solubilized RBC membrane proteins followed by densitometric quantitation. The results are expressed as ratios of individual red cell membrane proteins to band 3. This technique reveals abnormalities in approximately 70% to 80% of patients, defining the distinct biochemical phenotypes discussed previously. Direct quantitation of membrane proteins by radioimmunoassay is superior to densitometric quantitation and permits accurate measurement of the copy number of the individual proteins per RBC.

Application of molecular genetic analyses including DNA sequencing and other molecular studies complement clinical and laboratory screening and provide definitive diagnosis in most cases. Mutation detection in the major erythrocyte membrane protein genes is now available commercially. Gene-based studies are of use in diagnosing difficult cases and in cases in which a molecular diagnosis is desired. Some practitioners perform genetic diagnostic studies prior to splenectomy to ensure the correct diagnosis. Molecular analyses have potential pitfalls. In some cases, variants of unknown significance are detected, making genetic diagnosis uncertain. Mutations not detected by a study of coding regions and splice junctions may be causative, such as in distant regulatory elements, deep intronic splicing mutations, and intragenic deletions. In these cases, diagnosis is assigned based on clinical, laboratory, and biochemical findings.

Complications

Gallstones

Bilirubin stones are found in approximately 50% of patients with HS, often even in those with a very mild form of the disease. Gallstones have occasionally been detected during infancy, but they are most likely to occur in older children and young adults. The coinheritance of Gilbert syndrome increases the risk for gallstones in HS patients. Because of the high incidence of gallstones, HS patients should be periodically examined by ultrasonography for the presence of gallstones, beginning in childhood.

Crises

True hemolytic crises are relatively rare and only occasionally reported in association with infections. Aplastic crises during viral infections are largely attributable to infection by parvovirus B19. This infection (erythema infectiosum, fifth disease) manifests with fever, chills, lethargy, malaise, nausea, vomiting, abdominal pain with occasional diarrhea, respiratory symptoms, muscle and joint pains, and a maculopapular rash on the face (slapped cheek appearance), trunk, and extremities. The virus selectively infects erythroid precursors and inhibits their growth. The ensuing anemia, often profound, can be the first manifestation of HS. Multiple family members with undiagnosed HS who are infected with parvovirus have developed aplastic crises at the same time. Infection with parvovirus is a particular danger to susceptible pregnant women because it can infect the fetus, leading to fetal anemia, hydrops fetalis, and fetal demise.

Rarely, at least in developed countries, patients present with megaloblastic crises caused by folate deficiency. This typically occurs in patients with increased folate demands, such as those recovering from an aplastic crisis, pregnant women, and older adults. Megaloblastic crisis in pregnancy has been reported as the first manifestation of HS. Folate supplementation is recommended for patients with moderate to severe HS.

Other Complications

In patients with more severe forms of HS, other complications include gout, leg ulcers, or chronic dermatitis of the legs that heal after splenectomy. Symptoms of expanded erythroid space, including paravertebral or renal pelvic masses of extramedullary hematopoiesis, which can mimic an underlying neoplasm, may occur. Several cases of hemochromatosis in HS patients have been reported. In some, iron overload resulted from repeated transfusions; in others, the patients had two genetic defects, one involving HS and the other involving a hemochromatosis carrier state. Other rare complications include thrombosis, pulmonary hypertension, spinocerebellar degenerative syndromes, movement disorders, myopathy, and hypertrophic cardiomyopathy.

More than a dozen cases of HS and hematologic malignancy, including myeloproliferative disorders, multiple myeloma, and leukemia, have been reported. It is unknown if long-standing hematopoietic stress predisposes to the development of these secondary disorders or if they occurred randomly.

DIFFERENTIAL DIAGNOSIS

Because of the relatively asymptomatic presentation of HS, this diagnosis should be considered during an evaluation for unexplained splenomegaly, unconjugated hyperbilirubinemia of unknown cause, gallstones at a young age, severe anemia during pregnancy, or transient anemia during acute infections. The diagnosis of HS can be missed in mild forms of the disease because spherocytosis might not be apparent on the peripheral blood film. Autoimmune hemolytic anemia should be ruled out by negative results of a Coombs test.

More typical forms of HS, characterized by relatively uniform spherocytosis with increased MCHC, are usually easily distinguished from other disorders manifesting with spherocytosis, such as immune hemolytic anemias and unstable hemoglobins. In some patients, the spherostomatocytes in the rare Rh-null syndrome and the intermediate syndromes of hereditary stomatocytosis can be confused with HS RBCs.

Spherocytosis is transiently improved, and both the osmotic fragility and hemolysis are normalized in patients with obstructive jaundice. This is because of an expansion of RBC surface area that follows an increased uptake of phospholipids and cholesterol from the abnormal plasma lipoproteins. In normal RBCs, this leads to target cell formation; in HS, spherocytes are transformed to discocytes. Spherocytosis and the increased osmotic fragility of HS red cells are likewise improved by iron deficiency, but the RBC life span remains shortened. In addition, the coexistence of β-thalassemia trait and HS partially corrects the HS phenotype.

THERAPY AND PROGNOSIS

Splenectomy

Splenectomy is curative in almost all patients with typical forms of HS because RBC survival is normalized, and anemia and hyperbilirubinemia are corrected. Spherocytosis and the increase in osmotic fragility persist, but the tail of the OF curve, indicating the presence of a subpopulation of cells conditioned by the spleen, disappears. In patients with severe, nondominantly inherited HS, splenectomy produces a dramatic clinical improvement, but hemolysis is only partially corrected.

Several weeks to months before splenectomy, patients should be immunized with polyvalent vaccine against pneumococcus as well as vaccines against *Haemophilus influenzae* type b and meningococcus.

Indications for Splenectomy

Risks and benefits should be considered carefully in HS patients before splenectomy is performed (see box on Splenectomy for Hereditary Spherocytosis). A multitude of factors influence the decision for splenectomy in HS patients, including the risk for overwhelming postsplenectomy sepsis, the emergence of penicillin-resistant pneumococci, and the potentially increased risk for cardiovascular disease and pulmonary hypertension later in life. Indications for splenectomy include growth retardation, skeletal changes, symptomatic hemolytic disease, anemia-induced compromise of vital organs, the development of leg ulcers, or the appearance of extramedullary hematopoietic tumors. Whether to perform splenectomy in patients with moderate HS without any of these factors remains controversial.

SPLENECTOMY FOR HEREDITARY SPHEROCYTOSIS

Splenectomy is a permanently curative treatment in most cases of HS. Thus, for years, splenectomy was recommended for all HS patients regardless of the severity of anemia, gallbladder disease, or other symptoms. However, increasing concerns regarding overwhelming postsplenectomy infection (OPSI), the emergence of penicillin-resistant pneumococci, and increased risk for cardiovascular diseases have tempered these recommendations. When considering splenectomy, health care providers, the patient, and the patient's family should review and consider the risks and benefits. Individual factors that may pose additional risk, such as the distance from medical care in case of febrile illness and residence in or travel to areas where parasitic diseases such as malaria or babesiosis occur, should be considered. Expert opinions vary on indications for splenectomy. There are no studies to guide practice. However, because the risk for OPSI is highest in infancy and childhood, most agree it is best to avoid total splenectomy in early childhood.

Benefits

Anemia ameliorated
Risk for hemolysis-associated gallbladder disease eliminated
Risk for aplastic crisis eliminated

Risks

OPSI—especially with encapsulated organisms (*Streptococcus pneumoniae, Neisseria meningitides,* and *Haemophilus influenzae*)
Increased risk for thrombotic complications
Increased risk for vascular complications (pulmonary hypertension, cardiovascular disease)

Indications for Splenectomy

Expert opinions vary
Severe, symptomatic spherocytosis
Growth failure, skeletal changes, leg ulcers, and extramedullary hematopoietic tumors (signs of severe anemia)
Older patients with vascular compromise of vital organs
Moderate HS with compensated, asymptomatic anemia: controversial; decision should be individualized

Because of an increased frequency of postsplenectomy infection in young children, most practitioners avoid splenectomy in infancy and early childhood.

Operative Considerations

When splenectomy is warranted, laparoscopic splenectomy has become the procedure of choice in many centers. Laparoscopic splenectomy has been associated with less postoperative discomfort, a quicker return to preoperative diet and activities, a shorter hospitalization time, decreased costs, and smaller scars. Early complications of splenectomy include local infection, bleeding, and pancreatitis. In general, the morbidity rate of splenectomy is lower in patients with HS than with other hematologic diseases. However, the benefits of surgery must be weighed against possible complications, such as postsplenectomy infections. Although these complications are rare and their frequency is likely to diminish further with appropriate vaccinations, the indiscriminate performance of splenectomy in all HS patients with splenomegaly is unwarranted.

Subtotal (Partial) Splenectomy

Partial splenectomy was developed for infants and young children with severe HS, anemia, and poor growth with the goals of palliating anemia and decreasing transfusion requirements while preserving splenic function. More recently, some have advocated expanding the procedure to all patients being splenectomized for HS. The goal of the operation is to decrease hemolysis while maintaining splenic phagocytic function. In initial cohorts of patients treated with partial splenectomy, stable increases in hemoglobin levels with decreased reticulocyte counts have been observed. The volume of splenic tissue left behind ranges from 10 to 30 mL. Although initial data are promising, it is not clear whether the remaining splenic tissue will effectively prevent postsplenectomy sepsis. In follow-up studies, regrowth of the splenic remnant leading to increased severity of HS and necessitating total splenectomy occurs about a third of the time.

There are no specific data to support the use of prophylactic antibiotics post splenectomy. Some practitioners avoid the prescription of prophylactic antibiotics, others recommend prophylactic antibiotics for at least 5 years post splenectomy, and others recommend their use for life.

Postsplenectomy Failures

Postsplenectomy failures are caused either by the presence of an accessory spleen missed during surgery (accessory spleens were found in 17% to 39% of all patients) or by the presence of another superimposed RBC disorder such as pyruvate kinase deficiency. The recurrence of hemolytic anemia several years after splenectomy should raise the suspicion of development of splenunculi, resulting from autotransplantation of splenic tissue during surgery. The presence of an accessory spleen or splenunculus is suggested by the absence of both Howell-Jolly bodies and the "pitted" cells with crater-like surface indentations readily seen by interference contrast microscopy. A definitive confirmation of splenosis is made by a radiocolloid liver-spleen scan or by a scan using chromium (Cr)-labeled heated RBCs, which are taken up by the ectopic splenic tissue.

Genetic Counseling

After a patient is diagnosed with HS, family members should be examined for the presence of HS. A history, physical examination for splenomegaly, complete blood count with indices, reticulocyte count, examination of the peripheral blood smear for spherocytes, and biochemical evaluation including bilirubin and haptoglobin levels should be obtained for available close relatives.

Future Directions

Advances in high-throughput DNA sequencing methodology have greatly facilitated the precise genetic diagnosis in many cases of suspected inherited membrane-associated disorders. Both targeted gene capture and whole exome sequencing have rapidly developed into effective tools for the identification of disease-causing variants in genetic disease, particularly monogenic disorders. Because they can be applied to disorders with recessive or dominant inheritance or de novo occurrence, like HS, they obviate the need for cumbersome biochemical assays and for linkage analysis in large numbers of individuals. As more patients are studied, it is likely that new HS-associated loci and modifying genes will be discovered. Identification of novel mutations will provide insight into the structure and function of associated membrane protein genes, and our understanding of genotype-phenotype relationships, particularly those with variable influence on disease severity, will be extended.

HEREDITARY ELLIPTOCYTOSIS AND RELATED DISORDERS

Introduction and Epidemiology

Hereditary elliptocytosis designates a group of inherited disorders that have in common the presence of elliptical RBCs on peripheral blood films. Elliptocytosis was first described by Dresbach in 1904, and its heritability was firmly established by Hunter. Subsequent reports have revealed a considerable heterogeneity of clinical expression and have defined several distinct syndromes, including HPP and SAO.

HE is common in people of African and Mediterranean ancestries. In the US population, the prevalence of HE is approximately 3 to 5 per 10,000. The true incidence of HE is unknown because its clinical severity is heterogeneous and most patients are asymptomatic without anemia. HE is considerably more frequent in areas of endemic malaria. In equatorial Africa, the prevalence of common HE has been estimated at between 0.6% and 1.6%. Worldwide, HE appears to be more common among people of African origin. In Southeast Asian populations, the prevalence of SAO, a variant of HE, is as high as 30%.

The molecular basis of HE remained obscure until a defect of membrane skeletal proteins was suggested. Subsequently, defects in the erythrocyte membrane proteins α-spectrin, β-spectrin, protein 4.1R, GPC, and band 3 were described.

On the basis of RBC morphologic characteristics, HE can be divided into three major groups. Common HE, a dominantly inherited condition, is morphologically characterized by biconcave elliptocytes and, in some patients, rod-shaped cells. The clinical severity of common HE is highly variable, ranging from an asymptomatic condition to a severe recessively inherited hemolytic anemia, including hemolytic HE and HPP, in which the blood film reveals numerous RBC fragments, microspherocytes, and poikilocytes. Spherocytic HE, also called hemolytic ovalocytosis, is a much less common condition in which both round "fat" ovalocytes and spherocytes are present on the blood film. SAO, a disorder highly prevalent in the malaria belt of Southeast Asia and the Pacific, is characterized by rigid, spoon-shaped cells that have either a longitudinal slit or a transverse ridge.

Pathobiology of Common Hereditary Elliptocytosis

The possibility that the primary lesion of HE and HPP erythrocytes resides in the proteins of the RBC membrane skeleton was first raised by the findings of thermal instability of HPP spectrin, retention of the elliptical shape in HE membrane skeletons, disintegration of membrane skeletons after exposure to shear stress, defective self-association of spectrin dimers to tetramers, altered susceptibility of spectrin to tryptic digestion, and a deficiency of the membrane skeleton proteins spectrin and protein 4.1R. Gene cloning and determination of the primary structure of these proteins was soon followed by reports of mutations in the genes encoding erythrocyte membrane proteins.

Spectrin Mutations

The most common defects in HE, found in approximately two-thirds to three-quarters of all patients, are mutations of α- or β-spectrin. Both α- and β-spectrin are elongated flexible molecules consisting of triple-helical repeats connected by nonhelical segments. These polypeptides are associated side to side in an antiparallel position, forming a flexible, rod-like αβ heterodimer in which the NH_2-terminal of α-spectrin and the COOH-terminal of β-spectrin form the head region of the heterodimer. Spectrin heterodimers associate head to head to form spectrin tetramers, the major structural subunits of the membrane skeleton. Spectrin tetramers in turn are interconnected into a highly ordered two-dimensional lattice through binding, at their distal ends, to actin oligomers with the aid of protein 4.1R.

The contact site between the α- and β-spectrin chains of the opposed heterodimers is a combined "atypical" triple-helical repetitive segment in which the first two helices are contributed by the COOH-terminal of β-spectrin, whereas helix 3 is the first helical segment of α-spectrin. Spectrin dimer-tetramer interconversion is governed by a simple thermodynamic equilibrium that under physiologic conditions strongly favors spectrin tetramers. Most α-spectrin defects are at or near the NH_2-terminal of α-spectrin, which is involved in the heterodimer contact (the αI domain defined by limited tryptic peptide mapping; see the discussion under Laboratory Manifestations), and impair the self-association of spectrin into tetramers. Most α-spectrin mutations are point mutations. These mutations create abnormal proteolytic cleavage sites that typically reside in the third helix of a repetitive segment and give rise to abnormal tryptic peptides on two-dimensional tryptic peptide maps of spectrin.

Elliptocytogenic β-spectrin mutations are COOH-terminal point mutations or truncations that disrupt the formation of the combined β triple-helical repetitive segment and consequently the self-association of spectrin heterodimers to tetramers. All of these mutations open a proteolytic cleavage site residing in the third helix of the combined repetitive segment, giving rise to a 74-kDa αI peptide.

Although most spectrin mutations reside in the vicinity of the αβ-spectrin self-association site, a few mutations remote from the self-association site have been described. These mutations are asymptomatic in the simple heterozygous state but cause hemolytic anemia, which can be severe, in homozygous patients. Unlike mutations located in the self-association contact site, which are predicted to disrupt the conformation of the local protein structure, mutations outside this region are predicted to perturb long-range protein-protein interactions, disrupting the positively coupled, cooperative interactions of αβ spectrin self-association, spectrin-ankyrin interactions, and ankyrin–band 3 interactions. One HE-associated mutation in a linker region remote from the self-association contact site disrupted the stability propagated from one spectrin repeat to the next.

Protein 4.1R Mutations

Another group of elliptocytogenic mutations, although much less common than spectrin mutations, are quantitative or qualitative defects of protein 4.1R. Protein 4.1R is a multifunctional protein that contains several important sites of protein interactions, including the spectrin-binding domain, where 4.1R binds to the distal end of the spectrin αβ heterodimer, markedly increasing the binding of spectrin to oligomeric actin, and the basic NH_2-terminal domain, where 4.1R interacts with GPC, phosphatidylinositol, and phosphatidylserine, facilitating the attachment of the distal end of spectrin to the membrane.

Studies of 4.1R mRNA from normal RBCs revealed 4.1R isoforms resulting from complex tissue- and developmental stage-specific patterns of alternate mRNA splicing. Alternate translation initiation sites are present in the protein 4.1R mRNA. When an upstream initiator methionine is used, isoforms greater than 80kDa are synthesized. During erythropoiesis, this upstream initiator methionine is spliced out and a downstream initiator methionine is used, leading to the production of the 80-kDa mature erythroid protein 4.1R isoform. On SDS-PAGE, protein 4.1R is resolved into two bands of different sizes: 4.1a and 4.1b. The larger band, 4.1a, is typically found in normal RBCs, whereas the shorter one, 4.1b, represents the major isoform of reticulocytes. The 4.1b isoform is converted into the 4.1a isoform by deamidation of Asn 502.

A partial deficiency of protein 4.1R is associated with mild, dominantly inherited HE, whereas a complete deficiency (a homozygous state) leads to a severe hemolytic disease. Homozygous protein 4.1R(−) erythrocytes fragment more rapidly than normal at moderate shear stresses, an indication of their intrinsic instability. Membrane mechanic stability can be restored by reconstituting the deficient RBCs with protein 4.1R or the protein 4.1R/spectrin/actin-binding site. Homozygous protein 4.1R(−) erythrocytes also lack p55 and have only 30% of the normal content of GPC. These homozygous protein 4.1R(−) erythrocytes, as well as GPC-deficient Leach erythrocytes, demonstrate decreased invasion and growth of *Plasmodium falciparum* in vitro.

Mutations associated with protein 4.1R deficiency have included deletions that include the exon encoding the erythroid transcription start size and mutations of the transcription initiation codon. Qualitative defects of protein 4.1R protein include deletions and duplications of the exons encoding the spectrin-binding domain, leading either to truncated or elongated forms of protein 4.1R. Electron microscopic studies of homozygous protein 4.1R(−) erythrocyte membranes revealed a markedly disrupted skeletal network with disruption of the intramembrane particles, suggesting that protein 4.1R plays an important role in maintenance not only of the skeletal network but also of the integral proteins of the membrane structure.

Glycophorin C Deficiency

GPC has been found absent because of a variety of molecular defects. In contrast to other forms of HE, which are dominantly inherited, heterozygous carriers are asymptomatic, with normal RBC morphology, and homozygous patients have no anemia and only mild elliptocytosis apparent on the peripheral blood film.

GPC deficiency with elliptocytosis, the so-called Leach phenotype, caused by reduced expression of GPC, should be distinguished from the immunochemically defined phenotypes Gerbich and Yus, in which abnormal glycoproteins are formed that can functionally substitute for normal GPC and preserve the normal RBC shape. The Leach phenotype is usually caused by a large deletion of genomic DNA (~7 kb) that removes exons 3 and 4 from the GPC/glycophorin D locus. In one patient, the Leach phenotype was caused by a frameshift mutation.

GPC-deficient patients are also partially deficient in protein 4.1R and lack p55, presumably because these proteins form a complex and recruit or stabilize each other on the membrane. It has been speculated that the protein 4.1R deficiency in Leach erythrocytes is the cause of the elliptocytic shape. In contrast, patients deficient in glycophorin A, the major transmembrane glycoprotein, are asymptomatic.

Membrane Effects

Most of the elliptocytogenic mutations of spectrin reside within, or in the vicinity of, the spectrin heterodimer self-association site, disrupting this region and consequently disrupting the two-dimensional integrity of the membrane skeleton (Fig. 46.5). These defects are detected by ultrastructural examination of the membrane skeleton, revealing disruption of a normally uniform hexagonal lattice. Consequently, membrane skeletons are mechanically unstable, as are whole cell membranes and the cells. In patients with severely dysfunctional spectrin mutations or patients homozygous or doubly heterozygous for spectrin mutations, the membrane instability is sufficient to cause RBC fragmentation with hemolytic anemia under conditions of normal circulatory shear stress.

The pathobiology of the elliptocytic shape is less clear. RBC precursors in common HE are round and the cells become progressively more elliptical as they age in vivo. RBCs subjected to shear stress in vitro, or RBCs flowing through microcirculation in vivo, have an elliptical or parachute-like shape, respectively. It is possible that elliptocytes and poikilocytes are permanently stabilized in their abnormal shape because the weakened spectrin heterodimer contacts facilitate skeletal reorganization, which follows axial deformation of cells resulting from application of a prolonged or excessive shear stress. This reorganization is likely to involve breakage of the unidirectionally stretched protein connections followed by the formation of new protein contacts that preclude the recovery of a normal biconcave shape. This process has been shown to account for the permanent deformation of irreversibly sickled cells.

In HPP, the recessively inherited form of HE characterized by severe hemolysis, RBCs have two abnormalities. They contain a mutant spectrin that characteristically disrupts spectrin heterodimer self-association, and they are also partially deficient in spectrin, as evidenced by a decreased spectrin/band 3 ratio. In some HPP cases, this biochemical phenotype is a consequence of a double heterozygous state for an elliptocytogenic α-spectrin mutation and a defect involving reduced α-spectrin synthesis. Such synthetic defect of α-spectrin is fully asymptomatic in the heterozygous carrier because under normal conditions, the synthesis of α-spectrin is approximately three to four times greater than that of β-spectrin.

When present in conjunction with an elliptocytogenic mutation of α-spectrin, such a synthetic defect augments the expression of the

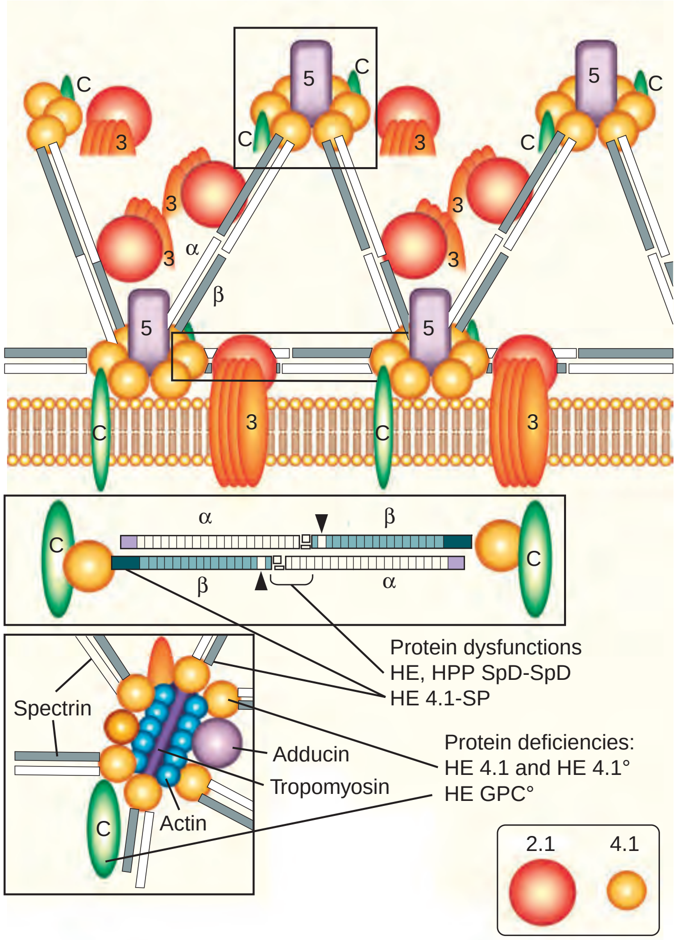


Figure 46.5 SCHEMATIC REPRESENTATION OF THE MOLECULAR ASSEMBLY OF THE MEMBRANE SKELETON AND THE MOLECULAR DEFECTS IN HEREDITARY ELLIPTOCYTOSIS (HE) AND HEREDITARY PYROPOIKILOCYTOSIS (HPP). Spectrin is composed of α- and β-spectrin heterodimers (SpD) that associate in their head regions into tetramers. At their distal ends, SpD bind to the junctional complexes of oligomeric actin (band 5 *[5]*) and protein 4.1. Additional proteins found in the junctional complex, such as adducin and tropomyosin, are shown in the lower enlarged area. The membrane skeleton is attached to transmembrane proteins by interactions of β-spectrin with ankyrin (protein 2.1; *[black arrowhead]* designates the ankyrin-binding site in β-spectrin), which in turn binds to the cytoplasmic domain of band 3 *(3)*, and by linkage of protein 4.1 to glycophorin C (GPC). The known protein dysfunctions in HE and HPP include (1) defects of the SpD head region because of a mutation of either α- or β-spectrin, causing impaired assembly of SpD into tetramers, and (2) defects of proteins of the junctional complex such as a qualitative or quantitative defect of protein 4.1R or GPC. *SP*, Spectrin.

mutant spectrin. Because the elliptocytogenic α-spectrin mutants are often unstable, the combination of the two defects leads to spectrin deficiency in the cells. Other HPP patients are homozygous or doubly heterozygous for one or two elliptocytogenic spectrin mutations, respectively. In such cases, the spectrin deficiency may be a consequence of spectrin instability that reduces the amount of spectrin available for membrane assembly. Furthermore, in RBCs containing a high fraction of unassembled dimeric spectrin, the spectrin deficiency may in part be related to the stoichiometric ratio of one ankyrin copy per one spectrin tetramer (i.e., two spectrin heterodimers). Consequently, only approximately one-half of spectrin heterodimers succeed in attaching to the ankyrin-binding sites. The phenotype of HPP, characterized by the presence of fragments and elliptocytes, together with evidence of RBC surface area deficiency (as reflected by the presence of microspherocytes on the peripheral blood film), suggests that the membrane dysfunction involves both vertical interactions (a consequence of spectrin deficiency) and horizontal interactions involving the elliptocytogenic spectrin mutation.

The RBC lesion in protein 4.1R deficiency shows similarities in regard to cell shape and membrane stability to the elliptocytogenic mutations of spectrin, suggesting that the deficiency principally affects the spectrin-actin contact (see Fig. 46.5) rather than the skeleton attachment to GPC via protein 4.1R (a vertical interaction).

The molecular basis of elliptocytosis and the mechanical instability of GPC-deficient RBCs are not fully understood. However, recent studies suggest that the deficiency of GPC is not directly responsible for the altered mechanical properties. Instead, the mechanical instability appears to be related to a concomitant partial deficiency of protein 4.1R, as evidenced by a full correction of membrane instability by introduction into the cells of protein 4.1R or its spectrin-binding peptide, which facilitates the contact of β-spectrin to actin. The superimposed deficiency of protein 4.1R is likely to be related to the fact that GPC serves as an attachment site for protein 4.1R to the membrane, recruiting protein 4.1R to the RBC membrane. The effects of these defects on the mechanical stability of GPC-deficient cells appear to be relatively minor because GPC-deficient patients have no detectable hemolytic anemia and the mechanical properties of the RBCs are normal when tested by micropipette aspiration.

Inheritance

In most patients, HE is inherited as an autosomal dominant disorder. The clinical severity is highly variable among different kindred (reflecting heterogeneous molecular lesions) and, to a lesser extent, within a given kindred, presumably because of other genetic or acquired defects that modify disease expression. Occasionally HE is inherited as an autosomal recessive condition from an asymptomatic parent who carries the same molecular defect of spectrin as the HE offspring. In one kindred with a submicroscopic chromosome X deletion, inheritance was X-linked.

The inheritance of the related disorder HPP is autosomal recessive: one of the parents carries the α-spectrin mutation and either is asymptomatic or has mild HE, whereas the other parent is fully asymptomatic and has no abnormalities detectable by current biochemical approaches. However, several HPP patients have recently been studied who were doubly heterozygous for two α-spectrin mutations; in the heterozygous parents, these mutations were either silent or expressed as mild HE.

Clinical Manifestations

In view of the striking molecular heterogeneity of common HE, it is not surprising that the clinical spectrum of this disorder is variable, ranging from an asymptomatic trait without hemolysis to a life-threatening hemolytic anemia.

Mild Hereditary Elliptocytosis and Asymptomatic Carrier State

In most of these patients, HE is found accidentally during the evaluation of the peripheral blood film. Although some HE patients have a mild compensated hemolytic anemia, others do not have any evidence of hemolysis, their RBC survival is normal, and the peripheral blood film may reveal only modest (≥15%) elliptocytosis. The molecular basis of mild HE is heterogeneous, and the reported molecular defects include both α- and β-spectrin mutations, partial deficiency of the 4.1R protein, and the absence of GPC. Some individuals carrying the spectrin mutation are completely asymptomatic, including normal RBC morphologic features; this is often the case in one of the parents of a patient with HPP.

Hereditary Elliptocytosis With Sporadic Hemolysis

Worsening of hemolysis together with the appearance of poikilocytes on the peripheral blood film has been reported in patients with hypersplenism, infections, or vitamin B_{12} deficiency, as well as in those with microangiopathic hemolysis such as disseminated intravascular coagulation or thrombotic thrombocytopenic purpura. In the latter two conditions, worsening hemolysis can be caused by microcirculatory damage superimposed on the underlying mechanical instability of the RBCs.

Hereditary Elliptocytosis With Neonatal Poikilocytosis

Neonatal offspring of parents with mild HE present with symptomatic hemolytic anemia and a marked poikilocytosis. During the first year of life, the hemolysis and poikilocytosis abate, and the clinical picture transforms into that of mild HE. Such patients typically carry one mutant α-spectrin allele. The severity of the molecular defect, in terms of the percentage of spectrin dimers and the amount of mutant spectrin in the cells, is the same in the neonatal period as it is later in life. The worsening of hemolysis in the neonatal period has been attributed to the presence of fetal hemoglobin, which binds poorly to 2,3-diphosphoglycerate (2,3-DPG, also known as 2,3-bisphosphoglycerate or 2,3-BPG). The ensuing elevation of free 2,3-DPG levels has a marked destabilizing effect on the spectrin–protein 4.1R–actin interaction, thereby further destabilizing the membrane skeleton.

Hereditary Elliptocytosis With Chronic Hemolysis

Patients with HE with chronic hemolysis present with moderate to severe hemolytic anemia with elliptocytes and poikilocytes on peripheral blood film; some require splenectomy. In some of the kindred, the hemolytic HE has been transmitted through several generations. In some kindred, not all of the HE patients have chronic hemolysis; some have a mild hemolysis only, presumably because of another genetic factor modifying the disease expression.

Homozygous and Compound Heterozygous Hereditary Elliptocytosis

Several HE individuals have been described who were apparent homozygotes for the HE gene. These individuals were found to be either homozygotes or compound (double) heterozygotes for one or two α- or β-spectrin mutations. The clinical severity is variable, from a relatively mild hemolytic anemia to a severe, life-threatening disease, depending on the severity of the underlying molecular defect, and in some cases is indistinguishable from HPP.

Hereditary Pyropoikilocytosis

It is now established that HPP represents a subtype of common HE, as evidenced by the coexistence of both HE and HPP in the same family and by the presence of the same molecular defect of spectrin. Unlike HE patients carrying the spectrin mutation, the RBCs of HPP patients are also partially deficient in spectrin. Typically, one parent of the HPP offspring carries an α-spectrin mutation, whereas the other parent is fully asymptomatic and has no detectable biochemical abnormality. In many such patients, the asymptomatic parent carries a silent "thalassemia-like" defect of spectrin synthesis, enhancing the relative expression of the spectrin mutant and leading to a superimposed spectrin deficiency in the HPP offspring. Subsequent studies of the original HPP kindred revealed that defective spectrin synthesis from the null allele was caused by a splicing mutation of the α-spectrin gene. Some HPP patients inherited two α-spectrin mutations; either their parents were hematologically normal or one had mild HE. In these HPP patients, spectrin deficiency may be related to the instability of the mutant spectrin. The thermal instability of spectrin originally reported as diagnostic of HPP is not unique for this disorder; it is also found in HE patients carrying this α-spectrin mutation and in the homozygous and in the heterozygous states. HPP is seen predominantly in Black patients, but it has also been diagnosed in Arabs and Whites.

Molecular Determinants of Clinical Severity

The severity of hemolysis in common HE often varies not only among different kindred but within a given family as well. The two principal determinants of severity of hemolysis are the spectrin content of the cells and the percentage of dimeric spectrin in the crude spectrin extract. The fraction of dimeric spectrin in such extracts in turn depends on several factors. The first of them is the degree of dysfunction of the mutant spectrin. Typically, mutations that are either within or near the combined αβ triple-helical repetitive segment representing the spectrin heterodimer self-association site produce a more severe clinical phenotype and a more severe defect of spectrin function than those seen with point mutations in the more distant triple-helical repeats. Second, the percentage of the dimeric spectrin depends on the fraction of the mutant spectrin in the cells, which in turn is determined by the gene dose (e.g., simple heterozygote versus homozygote or double heterozygote) or the presence of other genetic defects such as the presence, in trans, of a defect leading to a reduced α-spectrin synthesis in some patients with HPP.

The low-expression α-spectrin allele α^{LELY} is the best-characterized abnormality affecting spectrin content and clinical severity. Initially, a polymorphism of the αV domain, $\alpha^{V/41}$ was identified in HE patients who, when they inherited $\alpha^{V/41}$ in trans, had more severe HE than expected. Subsequently, an amino acid substitution of exon 46, Leu1857Val, and partial skipping of exon 46, linked to the $\alpha^{V/41}$ polymorphism, were identified as the characteristics of the α^{LELY} allele. These abnormalities are located within the site at which spectrin monomers assemble into heterodimers (the spectrin heterodimer nucleation site). In vitro studies suggest that the inability of α-spectrin chains to assemble into the mature membrane skeleton is because of a combination of decreased αβ dimer-binding affinity and increased proteolytic cleavage of the mutant α-spectrin chains. The presence of α^{LELY} in trans diminishes the propensity of the otherwise normal allele to associate with the corresponding β-chain, favoring the attachment of the elliptocytogenic α-spectrin allele. Conversely, the coexistence of the α-spectrin mutation in cis and the mutation involving the α-spectrin nucleation site diminishes the propensity of the mutant allele to be incorporated into the spectrin heterodimer, thereby ameliorating the clinical severity of this mutation. The α^{LELY} allele is clinically silent by itself, even when inherited in the homozygous state, probably because α-spectrin is normally synthesized in threefold to fourfold excess.

Laboratory Manifestations

Blood Film and Laboratory Evidence of Hemolysis

A careful blood smear evaluation is essential for the diagnosis of HE and for the classification of the disorder into the three major subtypes outlined previously. In patients in whom elliptocytosis is the only morphologic abnormality, hemolysis is characteristically minimal or absent, with the exception of spherocytic elliptocytosis, in which the presence of round "fat" ovalocytes is associated with accelerated RBC destruction. In patients with hemolytic forms of common HE, poikilocytosis is characteristically found on the blood film. In severe forms of HE, particularly in homozygous HE, many RBCs circulate as cell fragments, producing a marked decrease in MCV. The finding of RBC fragments together with a striking microspherocytosis and often only occasional elliptocytes is characteristic of HPP (Fig. 46.6).

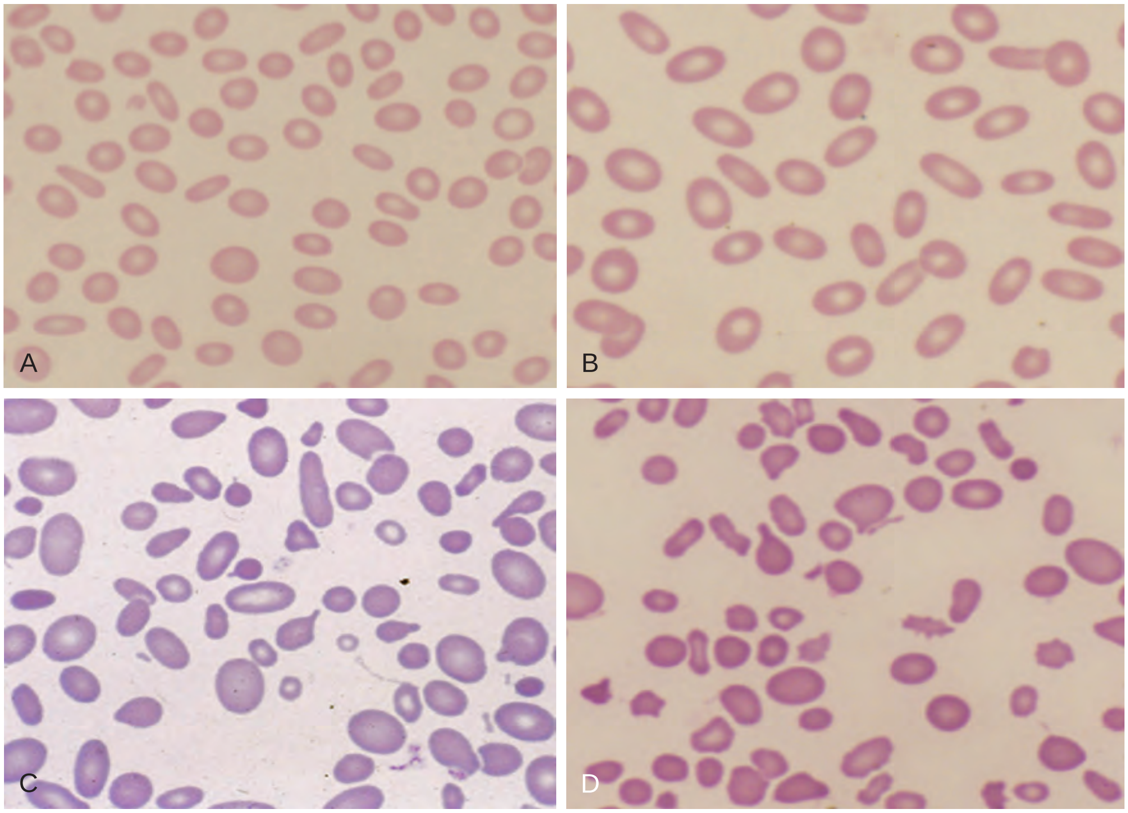


Figure 46.6 BLOOD FILMS OF PATIENTS WITH VARIOUS FORMS OF HEREDITARY ELLIPTOCYTOSIS (HE). (A) Simple heterozygote with mild common HE. Note the predominant elliptocytosis with some rod-shaped cells *(arrow)* and virtual absence of poikilocytes. (B) Simple heterozygote with severe common hemolytic elliptocytosis. Note the numerous small fragments and poikilocytes. (C) "Homozygous" common HE because of doubly heterozygous state for two mutant α-spectrins. Both parents have mild HE. Note the many elliptocytes, spherocytes, as well as numerous fragments and poikilocytes. (D) Hereditary pyropoikilocytosis. The patient is a double heterozygote for a structural α-spectrin mutant and a presumed α-spectrin synthetic defect. Note the prominent microspherocytosis, micropoikilocytosis, and fragmentation. Only a few elliptocytes are present. Some poikilocytes are in the process of budding.

Osmotic and Thermal Fragility

Osmotic fragility is increased in HPP, in spherocytic elliptocytosis, and in HE patients with poikilocytosis apparent on the peripheral blood film. In patients with a mild common HE without poikilocytosis on the peripheral blood film, osmotic fragility is normal.

Thermal instability of RBCs was originally reported as a characteristic feature of HPP. It reflects thermal instability of the mutant spectrin: In normal RBCs, spectrin is denatured and RBCs fragment at 50°C. HPP RBCs fragment and their spectrin denatures at 41°C. However, the diagnostic value of this test is limited because the thermal instability of RBCs is also noted in HE RBCs containing mutant spectrin. In contrast, an occasional patient with otherwise typical HPP may have normal thermal stability of RBCs and spectrin. RBCs in common HE have unstable membranes and membrane skeletons when subjected to shear stress.

Electrophoretic Separation of Solubilized Membrane Proteins

In HE and HPP, SDS-PAGE can reveal proteins of abnormal mobility, and the origin of which can be subsequently identified by Western blotting (e.g., truncated α- or β-spectrins in HE and HPP, or elongated or truncated forms of the 4.1R protein, and a partial or, rarely, complete deficiency of the 4.1R protein in HE). In HPP, SDS-PAGE reveals a partial deficiency of spectrin, as indicated by a decreased spectrin/band 3 ratio. Spectrin deficiency, in conjunction with an elliptocytogenic spectrin mutation affecting the spectrin heterodimer contact, is invariably found in cases of HPP.

Nondenaturing Gel Electrophoresis of Low-Ionic-Strength Spectrin Extract

Analysis of the ratio of tetrameric and dimeric spectrin in the low-ionic-strength extracts reveals the most common functional abnormality in HE (i.e., weakened self-association of spectrin heterodimers into tetramers). Because the spectrin dimer-tetramer interconversion has a high activation energy, it is kinetically immobilized at near 0°C. Consequently, the percentage of spectrin dimers and tetramers in the 0°C crude spectrin extract reflects the relative distribution of these species in the RBC membrane in situ. Mutations of α- or α-spectrin residing within or near the αβ-spectrin heterodimer self-association site invariably lead to an increase in the fraction of dimeric spectrin in the crude 0°C spectrin extract.

Tryptic Peptide Mapping of Spectrin and the Detection of the Underlying DNA Defect

Tryptic digestion of spectrin followed by electrophoretic separation gives rise to highly reproducible tryptic peptide patterns. Among these peptides, the 80-kDa αI domain peptide representing the self-association site of the normal α-spectrin is among the most prominent. Nearly all α- or β-spectrin mutations reported are associated with a formation of tryptic peptides of abnormal size and mobility that are generated from the normal 80-kDa αI domain peptide. The cleavage sites of the most common abnormal tryptic peptides are found in the third helix of a given triple-helical repetitive segment. The reported mutations reside in the vicinity of these cleavage sites either in the same helix or, less commonly, in helix 1 or 2 of a given repetitive segment. Consequently, tryptic peptide mapping remains a powerful tool with which to map the site of the underlying spectrin mutation, which can be subsequently defined by polymerase chain reaction amplification and sequencing of the respective region of the genomic DNA or complementary DNA (cDNA).

Differential Diagnosis

Various acquired and inherited conditions can be associated with elliptocytosis and poikilocytosis, including iron deficiency, thalassemias, megaloblastic anemias, myelofibrosis, myelophthisic anemias, myelodysplastic syndromes, and pyruvate kinase deficiency. The percentage of elliptocytes in these conditions is seldom greater than 60%. However, this is not diagnostically useful because some HE patients can have a relatively low percentage of elliptocytes. In normal patients, the percentage of elliptocytes is not greater than 5%, although in earlier reports it was listed as high as 15%. Previous diagnostic criteria of HE, based on the percentage of elliptocytes, such as 25%, 33%, or 40%, and their axial ratio, do not appear useful. The most reliable differentiation of HE from the other conditions mentioned is based on a positive family history rather than on the percentage of elliptocytes.

Therapy and Prognosis

As in the case of HS, RBCs from patients with more severe forms of HE are retained by the spleen, producing a marked engorgement of splenic pulp. Consequently, patients with symptomatic hemolysis benefit from splenectomy. This procedure is virtually never indicated in heterozygotes with autosomal dominant HE because most do not have clinically significant hemolytic anemia. If hemolysis is still active after splenectomy, folate should be administered daily. Recommendations for antibiotic prophylaxis, immunizations, and monitoring for intercurrent illnesses are similar to those noted earlier for HS patients before and after splenectomy. Serial interval ultrasonographic investigations to detect gallstones should be performed in patients with significant hemolysis.

Spherocytic Elliptocytosis

Spherocytic elliptocytosis, which shares features of HS and HE, has been designated spherocytic HE, HE with spherocytosis, or hereditary hemolytic ovalocytosis. The diagnosis is based on the simultaneous presence of elliptical RBCs and spherocytes or “fat,” round sphero-ovalocytes in the peripheral blood film. In contrast to common HE, cells of other shapes, such as rod-shaped cells, poikilocytes, and fragments, are absent. Importantly, hemolysis, despite relatively mild alterations in RBC morphologic features, and increased osmotic fragility are the main diagnostic features distinguishing this disorder from common HE.

The molecular basis of spherocytic HE is unknown. However, patients with mutations, particularly truncations at the C-terminal of β-spectrin, have many of the clinical features of spherocytic HE and probably represent an example of this disorder. Patients who lack GPC have rounded, smooth elliptocytes and could be classified as having a mild, recessively inherited variant of spherocytic HE. Finally, some patients with recessively inherited defects of protein 4.2 can display some features of spherocytic HE, particularly mild ovalostomatocytosis.

Southeast Asian Ovalocytosis

SAO is characterized by the presence of oval RBCs, many containing one or two transverse ridges or a longitudinal slit. The condition is widespread in certain ethnic groups of Malaysia, Papua New Guinea, the Philippines, and Indonesia. Numerous functional abnormalities of ovalocytes have been reported, including increased RBC rigidity, decreased osmotic fragility, increased thermal stability, resistance to shape change by echinocytogenic agents, and a reduced expression of many RBC antigens. A remarkable feature of ovalocytes is their resistance to in vitro invasion by several strains of malaria parasites, including *P. falciparum* and *Plasmodium knowlesi*. In areas of endemic malaria, SAO individuals have reduced numbers of intracellular parasites in vivo, with decreased prevalence and disease severity of malaria in SAO patients compared with controls.

SAO individuals are heterozygotes for two band 3 gene mutations in cis: the deletion of nine codons encoding amino acids 400 through 408 from the boundary of the cytoplasmic and membrane domains of band 3, and the 56 Lys to Glu substitution. The 56 Lys to Glu substitution represents an asymptomatic polymorphism known as band 3 Memphis. The SAO phenotype is associated with a tighter binding of band 3 to ankyrin, increased tyrosine phosphorylation of the band 3 protein, inability to transport sulfate anions, and a markedly restricted lateral and rotational mobility of band 3 protein in the membrane.

Laboratory Manifestations

The finding of 30% or greater of oval RBCs on the peripheral blood film, some containing a central slit or a transverse ridge, in the context of a notable absence of clinical and laboratory evidence of hemolysis in a patient from the ethnic groups noted earlier is highly suggestive of the diagnosis. A useful screening test is the demonstration of the resistance of ovalocytes or their ghosts to changes in shape produced by treatments that produce spiculation in normal cells, such as metabolic depletion or exposure of ghosts to salt solutions. In contrast to normal RBCs, which form spicules in response to such stimuli, SAO RBCs or ghosts do not change shape after these treatments. The mechanism of this resistance to changes in shape is not clear, and it may reflect the high rigidity of the RBC membrane.

Because the underlying cause of SAO is the deletion of 27 bases from the band 3 gene, isolation of genomic DNA or reticulocyte cDNA with subsequent amplification of the deletion-containing region appears to be the most specific test for establishing the diagnosis of SAO. A single, severely affected homozygous SAO individual who was transfusion dependent has been described.

Molecular Basis of Southeast Asian Ovalocytosis Membrane Rigidity and Malaria Resistance

The RBCs of SAO are unique among axially deformed cells in that they are rigid and hyperstable rather than unstable. The SAO mutation is the first example of a defect of an integral membrane protein leading to RBC membrane rigidity, an observation previously attributed to properties of the membrane skeleton. The basis of the increased rigidity is unclear.

The molecular basis of malaria resistance of SAO RBCs is likely related to altered properties of the band 3 protein, which serves as one of the malaria receptors, as evidenced by the inhibition of in vitro invasion by band 3-containing liposomes. In normal RBCs, the invasion process is associated with a marked membrane remodeling that involves the redistribution of intramembrane particles that contain band 3 protein. Such particles cluster at the site of parasite invasion, forming a ring around the orifice through which the parasite enters the cell. The invaginated RBC membrane, which surrounds the invading parasite, is free of intramembrane particles. The reduced lateral mobility of band 3 protein in SAO RBCs may preclude band 3 receptor clustering, thereby preventing the attachment of the parasites to the cells. Decreased exchange of anions across the RBC membrane has also been proposed to contribute to the resistance of ovalocytes to malaria invasion. In addition, SAO

RBCs consume ATP at a higher rate than normal cells, and the partial depletion of ATP levels in ovalocytes has been suggested to account, at least in part, for the resistance of these cells to malaria invasion in vitro.

Acanthocytosis and Related Disorders

Acanthocytes (from the Greek *acantha*, "thorn") or spur cells are RBCs with prominent thorn-like surface protrusions that vary in width, length, and surface distribution. Spur cells must be distinguished from echinocytes (Greek *echinos*, "sea urchin") or burr cells, characterized by multiple small projections that are uniformly distributed throughout the cell surface (Fig. 46.7). Acanthocytes should also be distinguished from keratocytes ("horn" red cells) that have few massive protuberances.

Acanthocytosis was first described in cases of abetalipoproteinemia and subsequently in severe liver disease, the chorea-acanthocytosis syndrome, the McLeod blood group phenotype, and other conditions. The molecular mechanisms leading to acanthocytosis in abetalipoproteinemia and severe liver disease have been extensively studied and have been attributed to changes in composition of membrane lipids and their altered distribution between the two hemileaflets of the lipid bilayer.

Spur Cell Hemolytic Anemia of Severe Liver Disease

Spur cell hemolytic anemia is an uncommon ominous complication of severe liver disease that is manifested by rapidly progressive hemolytic anemia and acanthocytes on the peripheral blood smear.

Pathobiology

The human RBC membrane contains nearly equal amounts of free (unesterified) cholesterol and phospholipids. The free cholesterol in the plasma readily equilibrates with the RBC membrane cholesterol pool. This is in contrast to esterified cholesterol, which cannot be transferred from plasma into the RBC membrane. The plasma of patients with severe liver disease contains abnormal lipoproteins that have a high free cholesterol/phospholipid ratio. The excess free cholesterol readily partitions into the RBC membrane, leading to a marked increase in free cholesterol in the cells. Consequently, normal cells can develop a spur cell shape after their transfusion into a patient with severe liver disease or after incubation with the liver disease patient's plasma or cholesterol-enriched liposomes.

Spur cell formation involves two steps. The first step is evident in RBCs of splenectomized patients with spur cell hemolytic anemia: RBCs have an expanded surface area with irregular contour and targeting, reflecting the accumulation of free cholesterol in the membrane (Fig. 46.8). This extracholesterol accumulates preferentially in the outer bilayer leaflet, as suggested by findings of increased accessibility of cholesterol to cholesterol oxidase and a selective decrease in lipid fluidity of the outer hemileaflet of the lipid bilayer.

The second step in acanthocyte formation involves RBC remodeling by the spleen. As a result, RBCs become spheroidal, and the surface projections are considerably longer and more irregular. The end result of these processes is poorly deformable RBCs with long bizarre projections that are readily trapped in the spleen, which is often markedly enlarged because of passive congestion as a result of underlying portal hypertension. Cholesterol also alters membrane permeability and interacts with several membrane skeletal proteins, but the role of these changes in spur cell lesions is unclear.

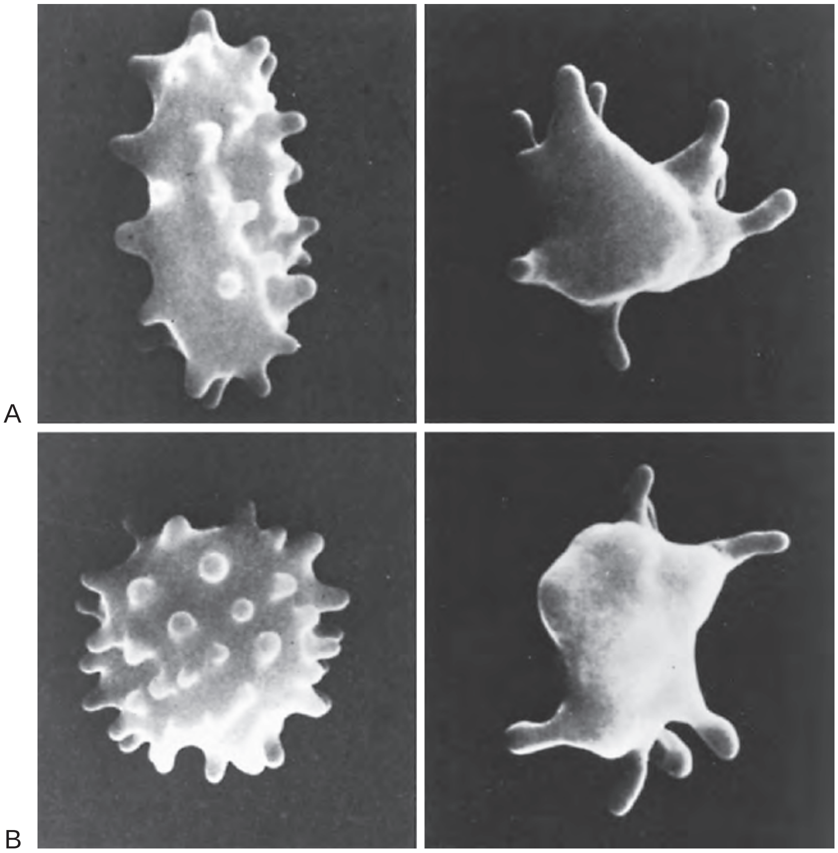


Figure 46.7 MORPHOLOGIC DIFFERENCES BETWEEN ACANTHOCYTES (A) AND ECHINOCYTES (B) AS DEMONSTRATED BY SCANNING ELECTRON MICROSCOPY. *(Modified from Bessis M. Red cell shapes: an illustrated classification and its rationale. In: Bessis M, Weed RI, Leblond PF, eds. Red Cell Shape Physiology, Pathology, Ultrastructure. New York: Springer-Verlag; 1973.)*

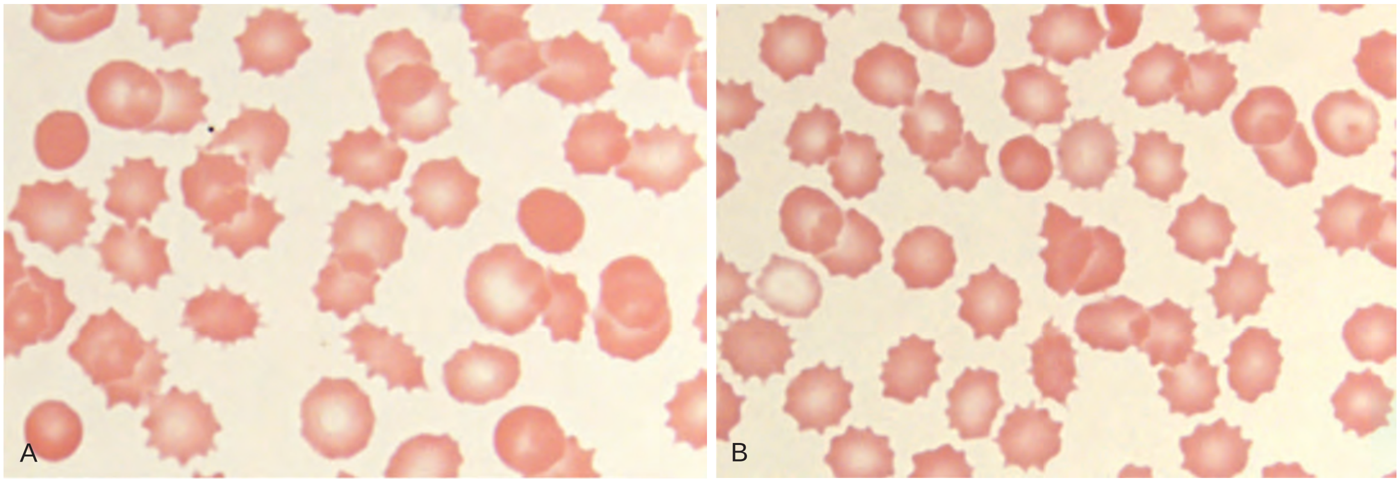


Figure 46.8 BLOOD FILM OF A PATIENT WITH LIVER CIRRHOSIS AND SPUR CELL ANEMIA. Erythrocytes have an expanded surface area with irregular contour and targeting, reflecting the accumulation of free cholesterol in the membrane, preferentially in the outer bilayer leaflet. Splenic remodeling leads to increasing spheroidicity with longer and more irregular surface projections.

Clinical Manifestations

Most patients with chronic liver disease have a mild to moderate anemia related to gastrointestinal blood loss, iron and folic acid deficiencies, or hemodilution or as a direct effect of alcohol on RBC precursors. Peripheral blood smears from these patients often reveal target cells that are particularly prominent in obstructive jaundice.

In some patients, particularly those with end-stage liver disease, anemia rapidly worsens and spur cells appear in high percentage in the peripheral blood. This is accompanied by worsening jaundice, rapid deterioration of liver function, hepatic encephalopathy, and hemorrhagic diatheses. A similar clinical syndrome has been described in patients with advanced metastatic liver disease, cardiac cirrhosis, Wilson disease, fulminant hepatitis, and infantile cholestatic liver disease. The development of spur cell hemolytic anemia is an ominous sign in most patients, predicting a survival seldom exceeding weeks to months. In theory, splenectomy could provide a marked improvement because the spleen is the major sequestration site of nondeformable acanthocytes; in reality, splenectomy is seldom considered because of the severity of the underlying liver disease.

Abetalipoproteinemia

Bassen and Kornzweig first described an association of acanthocytosis with atypical retinitis pigmentosa, progressive ataxic neurologic disease, and a “celiac disease” later attributed to fat malabsorption. Subsequently, several investigators reported a congenital absence of β-lipoprotein, accounting for the diverse manifestations of the disorder.

Pathobiology

Abetalipoproteinemia is an autosomal recessive disorder found in people of diverse ethnic backgrounds. The primary molecular defect involves a congenital absence of β-apolipoprotein in plasma. The B apoproteins (B100 and B48) are generated by alternate transcription of a single gene residing on the short arm of chromosome 2. Their deficiency is secondary to the defective cellular secretion of the apoprotein by liver cells, caused either by aberrant posttranslational processing or by defective aposecretion. In some patients, this is because of qualitative or quantitative defects in the microsomal triglyceride transfer protein, which catalyzes the transport of triglyceride, cholesterol ester, and phospholipid from phospholipid surfaces. Microsomal triglyceride transfer protein is the only tissue-specific component, other than apolipoprotein B, required for the secretion of apolipoprotein B-containing lipoproteins. As a result, apoprotein B is absent in plasma, as are the individual lipoprotein fractions that contain this apoprotein. These lipoprotein fractions include chylomicrons and very-low-density lipoproteins that transport triglycerides, as well as the low-density lipoproteins that are products of very-low-density lipoproteins and transport cholesterol. Consequently, preformed triglycerides are not transported from the intestinal mucosa, and they are nearly absent in the plasma. Plasma cholesterol and phospholipids are markedly reduced, with a relative increase in sphingomyelin at the expense of lecithin.

As is the case in acanthocytosis of liver disease, the acanthocytic lesion is acquired from the plasma. Erythrocyte precursors are of normal shape, and the acanthocytic lesion develops as the cells mature and age in the circulation. Normal cells acquire this shape when transfused into the recipient.

The most striking abnormality of RBC membrane lipids involves a net increase in sphingomyelin. Because plasma lipids readily exchange with the lipids of the RBC membrane, it is likely that this change simply mirrors the alterations in plasma lipid composition. In contrast to RBCs in spur cell anemia of severe liver disease, the content of membrane cholesterol is normal or only slightly increased.

The role of membrane lipids in the acanthocyte shape transformation was first established by findings of restoration of biconcave shape after extraction of lipids from the cell membrane by detergents. The molecular basis of the acanthocytic shape is unknown, but several indirect observations suggest that it is related to an increase of the surface area of the outer hemileaflet of the lipid bilayer relative to the inner leaflet. Several other abnormalities have been noted in abetalipoproteinemia, including a decrease in plasma lecithin-cholesterol transferase activity and an increased susceptibility of membrane and plasma lipids to oxidation as a result of malabsorption-induced deficiency of vitamin E. The contributions of these abnormalities to the acanthocyte red cell lesions are unknown.

Clinical Manifestations

This autosomal recessive disease can become evident in the first few months of life, manifested by fat malabsorption with normal absorption of other nutrients. Intestinal biopsy is diagnostic, revealing engorgement of mucosal cells with lipid droplets. Other features include retinitis pigmentosa and a progressive ataxia with intention tremors that usually develops at 5 to 10 years of age, progressing to death in the second or third decade of life. The hematologic manifestations are mild and include mild normocytic anemia with acanthocytosis (50% to 90%) and normal or slightly elevated reticulocyte counts. Occasional patients can have more severe anemia resulting from the nutritional deficiencies (iron and folate) that accompany fat malabsorption. The treatment includes dietary restriction of triglycerides and supplementation with the lipid-soluble vitamins A, K, D, and E. Vitamin E can stabilize or even improve both the retinal and neuromuscular abnormalities.

Autosomal recessive abetalipoproteinemia should be distinguished from the homozygous form of familial hypobetalipoproteinemia. Although the clinical presentation of both disorders is similar, the latter disorder is milder, and the parents have occasional acanthocytes on the peripheral blood film, and their plasma low-density lipoprotein levels are decreased. The molecular lesions in familial hypobetalipoproteinemia involve a variety of apoprotein B gene mutations, leading to aberrant apoprotein B gene transcription or translation.

Varying degrees of acanthocytosis without anemia have also been described with isolated deficiency of apoprotein B100.

Neuroacanthocytosis Syndromes

The neuroacanthocytosis syndromes are a group of degenerative neurologic disorders with phenotypic and genetic heterogeneity that share the feature of acanthocytes on peripheral blood smear. These disorders include chorea-acanthocytosis, the X-linked McLeod syndrome (see McLeod Phenotype), and several other neurodegenerative diseases, including Huntington disease-like 2 caused by mutations in junctophilin-3 and pantothenate kinase-associated neurodegeneration (formerly known as Hallervorden-Spatz syndrome and its allelic variant syndrome—hypobetalipoproteinemia, acanthocytosis, retinitis pigmentosa, pallidal degeneration [HARP]) caused by mutations in pantothenate kinase 2.

Chorea-acanthocytosis syndrome is an autosomal recessive syndrome of adult onset that is manifest by multiple neurologic abnormalities, including limb chorea, progressive orofacial dyskinesia with tics, tongue-biting neurogenic muscle hypotonia, and atrophy. The hematologic manifestations are minimal and include a variable percentage of acanthocytes on the peripheral blood film without anemia and normal or only slightly decreased RBC survival. The mechanism of acanthocytosis in this syndrome is unknown. Studies of plasma and RBC membrane lipids have revealed a high content of unsaturated fatty acids, presumably accounting for reduced RBC membrane fluidity. Additional abnormalities of uncertain significance include an uneven distribution of intramembrane particles, impaired phosphorylation of the erythrocyte actin-bundling protein dematin, abnormal accumulation of transglutaminase products, and altered function and structure of band 3.

Mutations have been identified in the chorein gene (also known as *CHAC* or *VPS13A*—vacuolar protein sorting 13 homolog A) in many affected patients. Chorein does not belong to any known human gene family, and computer searches have not identified any known structural motifs or domains. The function of the chorein gene product remains unknown in either erythrocytes or the brain. In yeast, a chorein homologue is involved in protein sorting and transport and in the regulation of levels of phosphatidylinositol-4-phosphate in cell membranes.

McLeod Phenotype

The McLeod syndrome is characterized by a mild compensated hemolytic anemia with a variable percentage of acanthocytes on the peripheral blood film and, in some patients, late-onset myopathy or chorea. The McLeod blood group phenotype is an X-linked anomaly of the Kell blood group system in which RBCs, white blood cells, or both react poorly with Kell antisera. The affected cells lack Kx, the product of the *XK* gene, which appears to be a membrane precursor of the Kell antigens. The *XK* gene encodes a novel 444-amino acid integral membrane transporter. In most patients, pathogenic nonsense or deletion mutations lead to absent or shortened XK protein that lacks the Kell protein binding site. Male hemizygotes who lack Kx have variable acanthocytosis (8% to 85%) and mild, compensated hemolysis. Because of the RBC mosaicism predicted by the Lyon hypothesis of X chromosome inactivation, female heterozygote carriers can have occasional acanthocytes on the peripheral blood film. Lyonized women with more severe symptoms have been described. Diagnosis may be challenging. CK levels are almost always elevated. In affected males, erythrocytes demonstrate absent Kx antigen and reduced Kell antigens. Because of the susceptibility to alloimmunization, it is important to diagnose affected patients because if they are transfused, they can develop antibodies compatible only with McLeod red cells.

The McLeod syndrome has been reported in association with chronic granulomatous disease of childhood, retinitis pigmentosa, and Duchenne muscular dystrophy. This association is caused by the close proximity of the genetic loci for these disorders in the p21 region of the X chromosome (Xp21), suggesting the occurrence of various manifestations because of contiguous gene syndromes. This may explain the occasional findings of either echinocytes or stomatocytes in Duchenne dystrophy or a choreiform disorder in some patients with the McLeod phenotype.

The Kell antigen consists of two protein components: a 37-kDa protein that carries the Kx antigen, a precursor molecule necessary for the Kell antigen expression, and a 93-kDa protein that carries the Kell blood group antigen. RBCs with the McLeod phenotype have no detectable Kx antigen, and they have a marked deficiency of the 93-kDa protein that carries the Kell antigen. McLeod RBCs should be distinguished from Kell null (K_0) RBCs, which have a normal shape. In K_0 cells, only the Kell antigen carrying the 93-kDa glycoprotein is absent, whereas these cells have twice the amount of the Kx antigen. As in the other acanthocytic disorders, the surface projections of acanthocytes may be related to the asymmetry of the surface area of the two lipid bilayer hemileaflets, as indicated by correction of the acanthocytosis by agents that expand the inner lipid layer, as well as the finding of an increased rate of exchange of phosphatidylcholine (localized preferentially in the outer lipid hemileaflet) with an exogenous source.

Acanthocytosis in Other Conditions

Acanthocytes have also been noted in malnourished patients, including those with anorexia nervosa and cystic fibrosis. In these patients, RBC shape normalizes after restoration of the nutritional status. Likewise, a small number of cells with long spicules resembling acanthocytes are found in patients with hypothyroidism, after splenectomy, and with myelodysplasia.

Differentiation of Acanthocytes From Other Spiculated Red Blood Cells

Echinocytes (Burr Cells)

In contrast to acanthocytes, echinocytes, also called burr cells, have rather uniform surface projections. Although early echinocytic forms have a regularly scalloped cell contour, advanced forms of echinocytes have a spheroidal shape and the surface projections appear as short, narrow spikes (see Fig. 46.8). Although the finding of echinocytes on a peripheral blood film is often an artifact related to blood storage, contact with glass, or an elevated pH, several hemolytic anemias have been reported in association with echinocytosis on peripheral blood films. These conditions include mild hemolytic anemia in long-distance runners and in patients with hypomagnesemia and hypophosphatemia (presumably because of decreased intracellular ATP stores), uremia because of an unknown plasma factor, and pyruvate kinase deficiency.

Inspection of wet blood preparations (but not dried blood films) reveals echinocytosis in most patients with liver disease. In contrast to spur cells in patients with severe liver disease, these echinocytes have a normal cholesterol content, and the molecular abnormality may be related to the binding of abnormal echinocytogenic high-density lipoproteins to the RBC surface.

The mechanisms of echinocytosis in these diverse disorders are likely to be heterogeneous, as suggested by findings that many diverse factors, such as exposure of RBCs to certain drugs, calcium loading, or ATP depletion, can induce the transformation of discocytes to echinocytes in vitro. However, in vitro studies of the discocyte-echinocyte-stomatocyte equilibrium have suggested a possible common denominator. As discussed earlier, the lipid bilayer of normal RBCs is asymmetric in lipid composition: The outer half of the lipid bilayer is relatively enriched in sphingomyelin and phosphatidylcholine, whereas the inner half is preferentially enriched in the negatively charged phosphatidylserine and phosphatidylethanolamine. Agents that preferentially bind to one or another class of these phospholipids dramatically influence RBC shape. Consequently, agents that preferentially accumulate in the outer half of the RBC lipid bilayer, expanding this lipid bilayer, produce an echinocytic shape, presumably by creating an asymmetry between the two surface areas of the two halves of the lipid bilayer. Conversely, agents that asymmetrically expand the inner half of the lipid bilayer, such as chlorpromazine, lead to stomatocytic shape transformation. In the case of echinocytes produced by ATP depletion or calcium loading, the altered phospholipid distribution between the two bilayer hemileaflets may be a consequence of calcium-induced phospholipid scrambling or a decrease in the activity of aminophospholipid translocase, an ATP-dependent enzyme that actively translocates aminophospholipids from the outer leaflet to the inner hemileaflet.

Keratocytes, Bizarre Poikilocytes, and Schistocytes

Mechanical trauma of circulating RBCs has occasionally produced bizarre shapes resembling acanthocytes, such as cells with horny projections (keratocytes). Some acanthocyte-like cells are also seen in splenectomized HE and HS patients. Similar shape changes are seen in heated RBCs, in which spectrin has been damaged by thermal denaturation, suggesting that these cells are bizarre poikilocytes rather than true acanthocytes.

RED BLOOD CELL MEMBRANE DISORDERS MANIFESTED BY TARGET CELL FORMATION

The common feature of target cells is an increase in the ratio of the cell surface area to cell volume. In microcytic RBCs of patients with various forms of thalassemia and hemoglobinopathies, the increased surface to volume ratio, and consequently the target cell shape, reflect at least in part the relative abundance of cell surface area. In liver disease and other disorders discussed subsequently, the target cell formation reflects an absolute expansion of the cell surface area because of a net accumulation of membrane phospholipids and cholesterol.

Liver Disease

The presence of target cells in association with either normal or slightly increased cell volume is characteristically found in patients with obstructive jaundice, including various forms of liver disease associated with intrahepatic cholestasis. These target cells have a normal survival in the peripheral circulation and do not typically account for the anemias often encountered in patients with liver disease.

In these patients, target cell formation is a consequence of a net uptake of both free cholesterol and phospholipids into the RBC membrane from the plasma because of abnormalities in the cholesterol/phospholipid/protein ratios of low-density lipoproteins. Target cells have a decreased osmotic fragility because the excess of membrane surface area leads to an increase in the critical hemolytic volume.

Lecithin-Cholesterol Acyltransferase Deficiency

The lecithin-cholesterol acyltransferase (LCAT) enzyme catalyzes the formation of cholesterol esters in lipoproteins. It circulates in plasma as a complex with components of high-density lipoproteins. LCAT deficiency, caused by mutations in the *LCAT* gene, is a rare autosomal dominant disorder manifested by hyperlipidemia, premature atherosclerosis, corneal opacities, chronic nephritis, proteinuria, mild anemia, and the presence of target cells on the blood film. The anemia is caused by mild hemolysis together with a diminished compensatory erythropoiesis. As in obstructive jaundice, the target cells in LCAT deficiency have a marked increase in both cholesterol and phospholipids. In addition, the membrane phosphatidylcholine is increased at the expense of sphingomyelin and phosphatidylethanolamine. Bone marrow aspiration and biopsy reveal the presence of sea-blue histiocytes. Analysis of plasma lipoproteins reveals multiple abnormalities secondary to the underlying enzyme deficiency. Inherited LCAT deficiency should be distinguished from an acquired deficiency of this enzyme, which is found in patients with severe liver disease.

Stomatocytosis and Related Disorders

Stomatocytes were first described in a girl with dominantly inherited hemolytic anemia. On blood films, her RBCs contained a wide transverse slit or stoma (Fig. 46.9). In a three-dimensional view, these cells have a shape of a cup or a bowl. The slit-like appearance is an artifact that results from folding of the cells during blood smear preparation.

Stomatocytes are seen in a variety of acquired and inherited disorders. The latter are often associated with abnormalities in RBC cation permeability that lead to changes in RBC volume, which can be either increased (hence the designation hydrocytosis or overhydrated stomatocytosis) or decreased (xerocytosis or desiccytosis [dessicate]), or in some cases near normal.

There is no unifying theory to explain this morphologic abnormality. In vitro, stomatocytes can be produced by drugs that preferentially intercalate into the inner half of the asymmetric lipid bilayer, expanding its surface area relative to that of the outer half of the bilayer.

Hereditary Stomatocytosis-Hydrocytosis

Hereditary hydrocytosis designates a heterogeneous group of hereditary hemolytic anemias that are transmitted in an autosomal dominant manner. The disorder is characterized by a moderate to severe hemolytic anemia with 10% to 30% stomatocytes (see Fig. 46.9), an elevated MCV, and a reduced MCHC. Osmotic fragility of RBCs is markedly increased, as some of the swollen RBCs approach their critical hemolytic volume. For unexplained reasons, RBC membrane lipids and consequently membrane surface area are also increased, but this increase in surface area is insufficient to correct the osmotic fragility of the RBCs. RBC deformability is decreased.

The principal cellular lesion involves a marked increase in intracellular sodium and water content with a mild decrease in intracellular potassium as a result of a marked sodium influx into the RBCs. Despite a marked compensatory increase in active transport of sodium (Na) and potassium by the Na^+/K^+-ATPase (which normally maintains the low sodium and high potassium concentrations in the cells) and an ensuing increase in glycolysis, the pump hyperactivity is unable to compensate for the vastly increased sodium leak. Stomatin (also known as band 7.2b), an integral membrane protein, is decreased or absent from the erythrocyte membranes of most affected patients. This deficiency appears to be a maturational loss in the bone marrow and in the circulation, perhaps because of a defect in cellular trafficking. Stomatin gene mutations have not been found in unrelated stomatocytosis patients deficient in this protein.

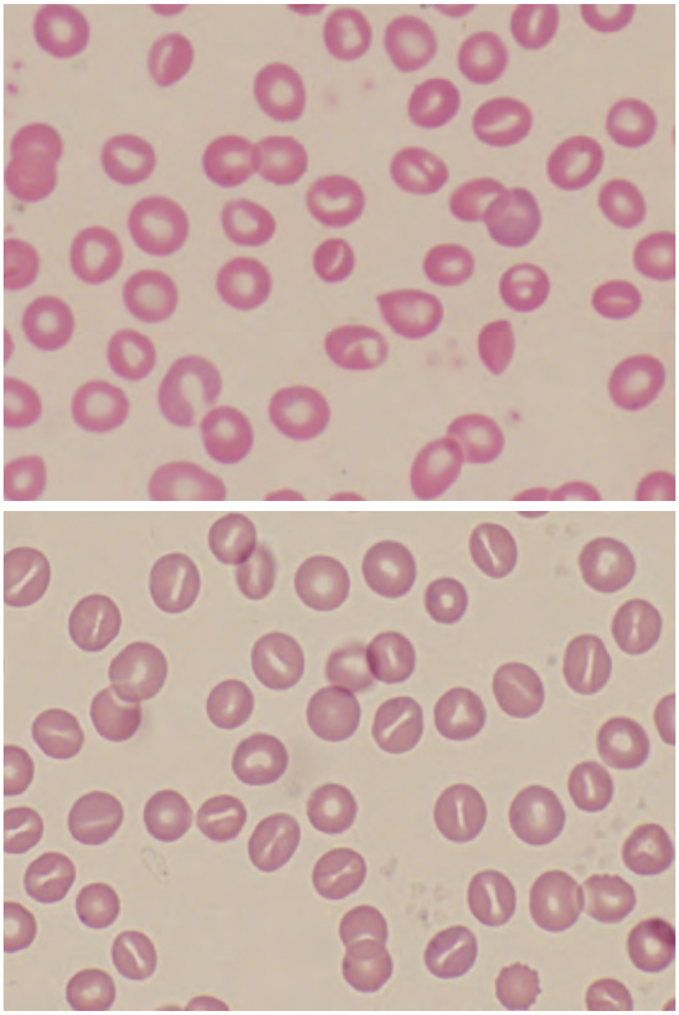


Figure 46.9 PERIPHERAL BLOOD SMEARS FROM PATIENTS WITH HEREDITARY XEROCYTOSIS (DESICCYTOSIS) AND STOMATOCYTOSIS (HYDROCYTOSIS). *(Top)* A Wright-stained peripheral blood smear from a patient with hereditary xerocytosis caused by a PIEZO1 mutation showing rare stomatocytes, occasional dessicytes—dense, abnormal erythrocyte forms where hemoglobin appears puddled at the periphery, and rare target cells. *(Bottom)* A Wright-stained peripheral blood smear from a patient with hereditary hydrocytosis is shown. Numerous stomatocytes, erythrocytes with a central mouth-like "stoma" are seen.

In some patients with hereditary hydrocytosis, missense mutations in RhAG, I61R, or F65S, have been found. In oocytes, these mutations induce a monovalent cation leak, possibly opening the pore of an ammonium transporter. Additional studies suggest that the F65S mutation exhibits a gain-of-function phenotype with increased cation conductance/permeability.

Splenectomy can improve, but not fully correct, the hemolysis. In some patients, splenectomy can be deleterious or even contraindicated (see later), perhaps because of altered endothelial cell adherence and membrane phospholipid asymmetry.

Hereditary Xerocytosis and the Intermediate Syndromes

Hereditary xerocytosis or desiccytosis describes an autosomal dominant hemolytic anemia characterized by RBC dehydration and decreased osmotic fragility. Affected individuals have characteristically moderate to severe hemolysis with an increased MCHC, reflecting cellular dehydration. Hydrops fetalis with fetal anemia or fetal ascites or the presence of pseudohyperkalemia have been reported in a number of xerocytosis kindred. Frequently, the MCV is mildly increased. In Coulter-type electronic counters, the conversion of pulse height (from the resistance of a cell passing through an electric field) to a cellular volume is dependent on cell shape. Xerocytes do not deform to the same degree as normal cells, causing the MCV to be approximately 10% too high. The peripheral blood film (see Fig. 46.9) does not always reveal stomatocytes (which are more prominent on wet films), but frequently, target cells, dessicytosis (dessicate), and spiculated cells are seen. In some of the cells, hemoglobin is concentrated ("puddled") in discrete areas on the cell periphery.

The mechanism of cellular dehydration is unclear and complex, involving a net potassium loss from the cells that is not accompanied by a proportional gain of sodium. Consequently, the net intracellular cation content and cell water are decreased. In some reports, a decrease in RBC 2,3-DPG has also been noted.

Most HX patients have heterozygous missense mutations in PIEZO1. In vitro studies of HX-associated PIEZO1 mutations demonstrate a gain-of-function phenotype, with many mutants demonstrating delayed channel inactivation, indicating increased cation permeability may lead to dehydration of HX erythrocytes. Piezo proteins are putative ion channels mediating mechanosensory transduction in mammalian cells. Animal models suggest mechanically activated Piezo1 plays a critical role in erythrocyte volume homeostasis. In a small subset of HX patients, defects of the Gardos channel encoded by the *KCNN4* gene have been described. Not surprisingly, when PIEZO1 mutations are co-inherited with other inherited anemias manifesting erythrocyte dehydration, such as sickle cell disease, marked erythrocyte dehydration worsens clinical severity.

Some of the reported cases of hereditary stomatocytosis share features of both hereditary stomatocytosis and xerocytosis categorized as "intermediate" syndromes. These patients characteristically have both stomatocytes and some target cells on the peripheral blood smear. Osmotic fragility is either normal or slightly increased. Sodium and potassium permeability is somewhat increased, but the intracellular cation concentration and the RBC volume are either normal or slightly reduced. These cells were reported to have subnormal glutathione content. In some patients, RBCs undergo in vitro hemolysis at 5°C, hence the designation *cryohydrocytosis*. A similar susceptibility to cold-induced cation permeability in which potassium and water loss predominates and xerocytes instead of hydrocytes are present has also been described.

A study of stomatocytosis, spherocytosis, and spherostomatocytosis patients whose erythrocytes demonstrated significant cation leaks at 0°C and in some cases band 3-deficient membranes revealed a series of missense mutations located in an intramembrane domain of band 3. In vitro studies suggest that these mutations convert band 3 from an anion exchanger to a nonselective cation leak channel.

Several investigators have also reported a dominantly inherited hemolytic anemia with stomatocytosis, occasional target cells, spherocytes, and a decreased osmotic fragility in which the main RBC membrane abnormality involved a nearly 50% increase in phosphatidylcholine and a corresponding decrease in phosphatidylethanolamine. Because abnormalities in membrane phospholipid composition have not been systematically investigated, it is uncertain whether the disorder represents a distinct disease entity.

The results of splenectomy in this group of disorders are variable. In some patients, the hemolytic anemia is improved, although often not fully corrected, by splenectomy, whereas in others, the severity of the hemolysis is unchanged. Splenectomy should be carefully considered in patients with hereditary stomatocytosis. Several patients with stomatocytosis (both hydrocytosis and xerocytosis) have developed hypercoagulability after splenectomy, leading to catastrophic thrombotic episodes or chronic pulmonary hypertension. Fortunately, the majority of persons with hereditary stomatocytosis are able to maintain an adequate hemoglobin level so that splenectomy is not required.

Rh Deficiency Syndrome

Rh deficiency syndrome designates rare individuals who have either absent (Rh_{null}) or markedly reduced (Rh_{mod}) Rh antigen expression, mild to moderate hemolytic anemia associated with the presence of stomatocytes, and occasional spherocytes on the peripheral blood film. Hemolytic anemia is improved by splenectomy.

The Rh antigens are present in ~20,000 to 30,000 copies per cell and reside on minor transmembrane proteins with an electrophoretic mobility of 28 to 33 kDa on SDS-PAGE. The Rh gene locus encodes two closely linked genes, one encoding the D polypeptide and the other encoding the CcEe proteins, and the antigenic expression of which is a consequence of alternate splicing of their pre-mRNA.

Rh proteins are part of a multiprotein complex that includes two Rh proteins and two Rh-associated glycoproteins (RhAG). Other proteins that associate with this complex include CD47, LW, glycophorin B, and protein 4.2. The Rh-RhAG complex interacts with ankyrin to link the membrane skeleton to the lipid bilayer. The Rh proteins share sequence homology to the Mep/Amt family of ammonium transporters in lower organisms and may participate in ammonium transport.

Rh_{null} erythrocytes have no Rh antigen and have reduced or absent LW, Fy5, Ss, U, and Duclos antigens. Rh, RhAG, LW, glycophorin B, CD47, and protein 4.2 are also reduced or absent. Rh_{null} erythrocytes have increased osmotic fragility, reflecting a marked reduction in membrane surface area. These cells are also dehydrated, as indicated by decreased cell cation and water content and increased cell density. The potassium transport and the Na^+/K^+ pump activity are increased, possibly because of reticulocytosis. Phospholipid asymmetry is also altered.

Although the clinical syndromes are the same, the genetic basis of the Rh deficiency syndrome is heterogeneous, and at least two groups have been defined. The amorph type is caused by defects involving the RH30 locus encoding the RhD and RhE polypeptides. The regulatory type of Rh_{null} and Rh_{mod} phenotypes results from suppressor or modifier mutations at the RH50 locus. When one chain of the Rh-RhAG complex is absent, the complex either is not transported to or is assembled at the membrane.

Familial Deficiency of High-Density Lipoproteins

Familial deficiency or absence of high-density lipoproteins (Tangier disease) because of mutations in ABCA1, a protein involved in cellular export of cholesterol, leads to accumulation of cholesterol esters in many tissues. Clinical manifestations include large orange tonsils, hepatosplenomegaly, lymphadenopathy, cloudy corneas, and peripheral neuropathy. Reported hematologic manifestations include a moderately severe hemolytic anemia with stomatocytosis and thrombocytopenia. Erythrocyte membrane lipid analyses reveal a low free cholesterol content, leading to a decreased cholesterol/phospholipid ratio and a relative increase in phosphatidylcholine at the expense of sphingomyelin.

Sitosterolemia

Sitosterolemia or phytosterolemia is a recessive disorder associated with elevated plasma levels of plant sterols. Affected patients exhibit xanthomatosis and early-onset premature cardiovascular disease. Reported hematologic manifestations include hemolytic anemia with stomatocytosis and macrothrombocytopenia. Mutations in the transporters ABCG5 or ABCG8 lead to gastrointestinal hyperabsorption and decreased biliary elimination of plant sterols as well as altered cholesterol metabolism. Plant sterols are not synthesized endogenously in humans but are passively absorbed in the intestine. ABCG5 and ABCG8 actively pump plant sterols out of the intestinal cells back into the intestine and out of liver cells into bile ducts. It has been hypothesized that the stomatocytic phenotype is caused by the intercalation of plant sterols into the inner leaflet of the lipid bilayer.

Acquired Stomatocytosis

Stomatocytes have been noted in diverse acquired conditions, including neoplasms, cardiovascular and hepatobiliary disease, alcoholism, and therapy with drugs, some of which are known to be stomatocytogenic in vitro. In some of these conditions, the percentage of stomatocytes on the peripheral blood smear can approach 100%. However, the clinical significance of this observation is unclear because stomatocytes are absent in most patients with the conditions listed. Furthermore, some stomatocytes can be found in normal individuals (3% to 5%). The most consistent association is that of stomatocytosis and heavy alcohol consumption.

RED CELL MEMBRANE VARIANTS AND INFECTIOUS DISEASE

Viral, bacterial, and parasitic infections can all cause anemia. Multiple mechanisms leading to hemolysis have been described. As mentioned earlier in this chapter, parvovirus B19 selectively infects erythroblasts through interaction with globoside, which encodes the P blood group antigen and temporarily shuts down erythropoiesis. Although this infection is tolerated well by healthy patients, it can lead to severe, at times life-threatening, aplastic crises in patients with anemias because of premature erythrocyte destruction. As one might predict, parvovirus cannot invade erythroblasts of the rare P-negative individuals.

Most infections cause hemolytic anemias triggered by several distinct, and at times overlapping, mechanisms. *Plasmodium, Babesia*, and *Bartonella* species directly attack the membrane and lyse the red cells. Some bacteria, such as *Clostridium perfringens*, elaborate hemolytic toxins or phospholipases that damage the membrane. Other infectious agents trigger the occasional production of autoantibodies against red cell membrane components, which in turn leads to autoimmune hemolytic anemia. Finally, many sepsis syndromes are associated with anemia because of disseminated intravascular coagulation.

Malaria and the Erythrocyte Membrane

The red cell membrane defects described earlier in this chapter cause mild to severe hemolytic anemias. At the same time, many red cell membrane alterations have developed as a defense against microorganisms and parasites invading and lysing red cells. This is especially true for malarial parasites. Although four different species of the malaria parasite *Plasmodium*, including *P. falciparum, Plasmodium ovale, Plasmodium vivax*, and *Plasmodium malariae*, infect humans, almost all of the 1.5 to 2 million annual deaths caused by malaria are attributable to *P. falciparum*.

Because malaria coexisted with humans over the course of human evolution, it comes as no surprise that multiple erythroid genotypes were selected that confer some level of resistance to infection or mitigate disease severity. The ensuing heritable phenotypes include, among others, resistance to red cell adhesion and/or invasion, slower intraerythrocytic growth, decreased or increased adhesion of infected red cells to vascular endothelium, and increased phagocytosis of parasitized red cells.

Malaria and other infections causing hemolytic anemias are described in more detail in Chapter 158, which also discusses hemoglobinopathies and red cell enzyme variants that reduce invasion and/or retard parasite growth. Consequently, we focus here on the heritable erythrocyte membrane alterations that developed as a defense against malaria.

Erythrocyte Preference

Two parasites, *P. vivax* and *P. ovale*, selectively infect reticulocytes, whereas *P. malariae* infects older erythrocytes. In contrast, *P. falciparum* infects red cells of all ages. This fact and the tendency of *P. falciparum*-infected erythrocytes to sequester in circulation explain the markedly higher severity of *P. falciparum* malaria.

Attachment and Invasion

Duffy Antigen

The *P. vivax* merozoite is completely dependent on attachment to the Duffy blood group antigen (also known as the *Duffy antigen receptor for chemokines [DARC]*) for erythrocyte invasion, and consequently, it cannot invade Duffy-negative RBCs. It has been hypothesized that this is why the Duffy-negative phenotype is common in large areas of Africa. The Duffy-negative phenotype is caused by mutation in a *GATA1* motif in the Duffy antigen gene promoter, preventing its expression in erythroid cells, leaving its expression in other tissues intact. Elucidation of this mutation explained a longstanding conundrum of transfusion medicine: why individuals with the Duffy-negative phenotype never develop antibodies against the Duffy antigen.

Glycophorins

All major erythrocyte glycophorins, A, B, and C/D, are involved in the attachment of *P. falciparum* to the RBC membrane. Consequently, invasion of *P. falciparum* into red cells from patients lacking glycophorin A (En[a−]), glycophorin B (S-s-U−), or glycophorins C and D (Gerbich negative, Ge−) is diminished. As noted earlier, the Gerbich-negative phenotype is associated with mild, asymptomatic ovalocytosis.

Protein 4.1R and Spectrin

Deficiency of protein 4.1R or self-association defects of spectrin are associated with elliptocytosis of varying severity. Both phenotypes appear to reduce the burden of RBC invasion.

Band 3 and Southeast Asian Ovalocytosis

Conflicting explanations of the basis of the protective phenotype of SAO (described earlier) from malaria have been described. Initial reports suggested that SAO erythrocytes were resistant to malarial invasion. These results were repeatedly questioned until recent studies demonstrated SAO cells to be resistant to invasion by the more virulent *P. falciparum* strains. This may explain the apparent contradiction with the reports of comparable parasitemias in SAO carriers and patients with a normal red cell phenotype from Papua New Guinea.

The protection from cerebral malaria afforded by SAO erythrocytes is likely because of the reduced cytoadherence of SAO red cells to the cerebral vasculature. Under conditions of flow, *P. falciparum*-infected ovalocytes adhere more strongly than normal infected red cells to the endothelial receptor CD36. Because this receptor is not expressed in the brain, this raises a possibility that ovalocytosis protects from cerebral malaria by diminishing the number of parasitized red cells available for adhesion to the cerebral vasculature via alternative receptors. Moreover, ovalocytes appeared resistant to invasion by parasite strains that tend to bind to intracellular adhesion molecule I (ICAM1), the likely receptor for cytoadherence in the brain, but the exact mechanism is not yet known.

Knops Blood Group System

Severe malaria, particularly cerebral malaria, has been associated with the formation of rosettes, clumps of cells formed by the adhesion of malaria-infected erythrocytes to complement receptor 1 (CR1) on uninfected erythrocytes. Identification of the Knops blood group antigens on CR1, followed by observations that frequencies of various Knops antigens varied significantly in Whites and individuals of African ancestry, led to the hypothesis that some Knops group antigens might be protective from rosetting and severe malaria. Case-control studies with genotyping and/or flow cytometry have yielded conflicting results, but several have linked low-expression CR1 alleles with malaria resistance. Further studies have shown that the expression of CR1 and other complement proteins increases with age. Together these data suggest that genetic and age-related differences in complement protein expression contribute to the variability observed in individuals with severe malaria.

Although these erythrocyte membrane polymorphisms offer fascinating insight into natural defenses against one of the most serious diseases affecting humans, the mechanism of resistance to malaria has not been fully elucidated for any of them. Malaria has clearly had a profound impact on the genetic makeup of populations living in endemic areas and provided us with multiple clues about the host-parasite relationship. Better understanding of these natural defenses might eventually be converted into effective therapeutic interventions.

SUGGESTED READINGS

Andolfo I, Russo R, Gambale A, Iolascon A. New insights on hereditary erythrocyte membrane defects. *Haematologica*. 2016;101(11):1284–1294.

Andolfo I, Russo R, Rosato BE, et al. Genotype-phenotype correlation and risk stratification in a cohort of 123 hereditary stomatocytosis patients. *Am J Hematol*. 2018;93(12):1509–1517.

Cahalan SM, Lukacs V, Ranade SS, et al. Piezo1 links mechanical forces to red blood cell volume. *eLife*. 2015;4:e07370.

Chonat S, Risinger M, Sakthivel H, et al. The spectrum of SPTA1-associated hereditary spherocytosis. *Front Physiol*. 2019;10:815.

Englum BR, Rothman J, Leonard S, et al. Splenectomy in Congenital Hemolytic Anemia Consortium. Hematologic outcomes after total splenectomy and partial splenectomy for congenital hemolytic anemia. *J Pediatr Surg*. 2016;51(1):122–127.

Feng Z, Waugh RE, Peng Z. Constitutive model of erythrocyte membranes with distributions of spectrin orientations and lengths. *Biophys J*. 2020;119(11):2190–2204.

Gallagher PG, Maksimova Y, Lezon-Geyda K, et al. Aberrant splicing contributes to severe alpha-spectrin-linked congenital hemolytic anemia. *J Clin Invest*. 2019;129(7):2878–2887.

Gautier EF, Leduc M, Cochet S, et al. Absolute proteome quantification of highly purified populations of circulating reticulocytes and mature erythrocytes. *Blood Adv*. 2018;2(20):2646–2657.

Glogowska E, Schneider ER, Maksimova Y, et al. Novel mechanisms of PIEZO1 dysfunction in hereditary xerocytosis. *Blood*. 2017;130(16):1845–1856.

Haw A, Palevsky HI. Pulmonary hypertension in chronic hemolytic anemias: pathophysiology and treatment. *Respir Med*. 2018;137:191–200.

Ilboudo Y, Bartolucci P, Garrett ME, et al. A common functional PIEZO1 deletion allele associates with red blood cell density in sickle cell disease patients. *Am J Hematol*. 2018;93(11):E362–E365.

Iolascon A, Andolfo I, Barcellini W, et al. Recommendations regarding splenectomy in hereditary hemolytic anemias. *Haematologica*. 2017;102(8):1304–1313.

Iolascon A, Andolfo I, Russo R. Advances in understanding the pathogenesis of red cell membrane disorders. *Br J Haematol*. 2019;187(1):13–24.

Kaufman HW, Niles JK, Gallagher DR, et al. Revised prevalence estimate of possible hereditary xerocytosis as derived from a large U.S. Laboratory database. *Am J Hematol*. 2018;93(1):E9–E12.

King MJ, Garçon L, Hoyer JD, et al. International Council for Standardization in Haematology. ICSH guidelines for the laboratory diagnosis of nonimmune hereditary red cell membrane disorders. *Int J Lab Hematol*. 2015;37(3):304–325.

Ma S, Cahalan S, LaMonte G, et al. Common PIEZO1 allele in African populations causes RBC dehydration and attenuates plasmodium infection. *Cell*. 2018;173(2):443–455. e12.

Peikert K, Danek A, Hermann A. Current state of knowledge in chorea-acanthocytosis as core neuroacanthocytosis syndrome. *Eur J Med Genet*. 2018;61(11):699–705.

Picard V, Guitton C, Thuret I, et al. Clinical and biological features in PIEZO1-hereditary xerocytosis and Gardos channelopathy: a retrospective series of 126 patients. *Haematologica*. 2019;104(8):1554–1564.

Pincez T, Guitton C, Gauthier F, et al. Long-term follow-up of subtotal splenectomy for hereditary spherocytosis: a single-center study. *Blood*. 2016;127(12):1616–1618.

Pincez T, Guitton C, Landman-Parker J, et al. Subtotal and total splenectomy for hereditary pyropoikilocytosis: benefits and outcomes. *Am J Hematol*. 2018;93(10):E340–E342.

Reid ME, Niss O, Chonat S, et al. Genotype-phenotype correlations in hereditary elliptocytosis and hereditary pyropoikilocytosis. *Blood Cells Mol Dis*. 2016;61:4–9.

Rice HE, Englum BR, Rothman J, et al. Splenectomy in Congenital Hemolytic Anemia (SICHA) Consortium. Clinical outcomes of splenectomy in children: report of the splenectomy in congenital hemolytic anemia registry. *Am J Hematol*. 2015;90(3):187–192.

Risinger M, Glogowska E, Chonat S, et al. Hereditary xerocytosis: diagnostic considerations. *Am J Hematol*. 2018;93(3):E67–E69.

Risinger M, Kalfa TA. Red cell membrane disorders: structure meets function. *Blood*. 2020;136(11):1250–1261.

Roulis E, Hyland C, Flower R, et al. Molecular basis and clinical overview of McLeod syndrome compared with other neuroacanthocytosis syndromes: a review. *JAMA Neurol*. 2018;75(12):1554–1562.

Tole S, Dhir P, Pugi J, et al. Genotype-phenotype correlation in children with hereditary spherocytosis. *Br J Haematol*. 2020;191(3):486–496.

van Vuren A, van der Zwaag B, Huisjes R, et al. The complexity of genotype-phenotype correlations in hereditary spherocytosis: a cohort of 95 patients: genotype-phenotype correlation in hereditary spherocytosis. *Hemasphere*. 2019;3(4):e276.

Vives-Corrons JL, Krishnevskaya E, Rodriguez IH, Ancochea A. Characterization of hereditary red blood cell membranopathies using combined targeted next-generation sequencing and osmotic gradient ektacytometry. *Int J Hematol*. 2021;113(2):163–174.

CHAPTER 47

AUTOIMMUNE HEMOLYTIC ANEMIA

Marc Michel and Ulrich Jäger

Autoimmune hemolytic anemia (AIHA) is a rare autoimmune disease caused by autoimmune-mediated destruction of red blood cells (RBCs) by autoantibodies with various properties and target specificities. Exact laboratory diagnosis may sometimes be difficult; therefore, experienced diagnostic reference centers play an important role. The disease can be primary (idiopathic) or caused by an underlying condition (secondary), including systemic autoimmune diseases, infections, primary immunodeficiencies, drugs, or neoplasms (mostly lymphomas). The clinical course of the disease as well as treatment decisions are influenced by the type of antibody involved. Currently, treatments for AIHA are still mainly based on experience and opinion as opposed to robust evidence and there is still no therapy licensed for AIHA. Several guidelines have been proposed. Recommendations for diagnosis and treatment by an international group of experts were recently published. Management of the disease still requires general hematologic skills as well as knowledge of diagnostic criteria and treatment options. In the last years, new promising treatment approaches have been developed.

HISTORY

The history of diagnostic and therapeutic progress in AIHA has been described by Dacie,[1] one of the great pioneers in this field. Milestones were the discovery of the first RBC autoantibody (Donath–Landsteiner [DL] antibody) in 1904, the introduction of the Coombs test in 1945, the establishment of splenectomy as an effective treatment of AIHA in the 1950s, and the finding that rituximab was an effective treatment in the past decade. The diagnosis and treatment of patients with AIHA were reviewed by several authors.[2–8]

EPIDEMIOLOGY

Based on the characteristics and thermal amplitude of autoantibodies, AIHAs are usually classified as either warm (wAIHA) or cold (cAIHA). A low proportion of patients achieve spontaneous or treatment-induced, long-term remissions; overall mortality is relatively low[9–11] and the prevalence of AIHA has been estimated as 17 in 100,000 in Denmark.[12] The incidence of AIHA in children and teenagers is 0.2 to 1.0 per million per year.[9] There is some evidence of a genetic predisposition and/or familial clustering of AIHA/Evans syndrome in children, but no clear genetic background has been identified for the majority of patients.[13]

Primary AIHA and Evans syndrome are slightly more prevalent in adult females and in children.[10,14] In secondary AIHA, the female-to-male ratio is very high in systemic lupus erythematosus (SLE), but close to 1 in chronic lymphocytic leukemia (CLL)-associated AIHA. The incidence of chronic cold agglutinin disease (CAD) is estimated to be approximately 1 to 2 per million per year with a female prevalence.[15] Geographical differences have been suggested with a higher incidence of CAD in Northern climates, potentially explained by a higher number of symptomatic patients in these areas.

PATHOBIOLOGY

Hemolysis is initiated when an autoantibody binds to the one or several RBC membrane antigens and promotes antibody-dependent, cell-mediated cytotoxicity, and/or recruits and activates complement. Destruction of the RBC can occur directly in the circulation (intravascular hemolysis) and/or by removal of the cell by macrophages in the spleen, liver, or both (extravascular hemolysis) (Fig. 47.1). Several immunoglobulin (Ig) subclasses can fix complement and activate the classical complement (Ct) pathway: IgG3 activates Ct more efficiently than does IgG1, whereas IgM, which is a potent Ct activator, is usually eluted ex vivo and therefore not found on RBC membrane by the direct antiglobulin test (DAT). Macrophages recognize opsonized erythrocytes via receptors specific for the Fc fragment of IgG, namely Fcγ receptors (FcγRs) and for C3d. RBCs coated with IgG or complement alone are destroyed in the spleen and liver, IgG-coated cells in the spleen, and IgM-coated cells in the liver.[16,17] This has major implications for treatment, particularly for the effect of steroids and splenectomy.

Warm Antibody Hemolytic Anemia

wAIHAs represent 60% to 80% of all AIHAs. The RBC antibodies in wAIHAs are mostly polyclonal IgG1 or IgG3, which are able to activate the complement system (Table 47.1). The DAT in wAIHAs is positive either with IgG (37%) or IgG + C3d (43%). Rarely, the DAT is only positive with C3d (when the amount of IgG on the RBCs is very small or in case of “warm,” IgM autoantibody). Patients with IgG antibodies may have also IgA antibodies, but IgA antibodies without IgG antibodies are a very rare cause of wAIHAs. wAIHAs are often directed against Rh antigens, but also against other RBC’s membrane antigens (non-Rh–related autoantibodies) such as band 3 protein or glycophorin A. The antibodies fix complement and bind tightly to the RBCs at 37°C. Therefore, only a small amount of antibody is detectable in the serum. The antibody-coated RBCs are removed from the circulation by splenic (to a lesser degree also by hepatic) macrophages via FcγRIII receptors. IgG_3 and IgG_1 have the highest affinity for the Fc receptors of macrophages. Erythrocytes that are only partially phagocytosed by macrophages become spherocytes, which are removed in the splenic cords because of their rigid structure and can be easily detected on peripheral blood smear. Destruction of RBCs may also be caused by other mechanisms such as antibody-dependent cellular cytotoxicity as well as complement-dependent cytotoxicity when complement activation is involved.[18]

IgM wAIHAs are a very rare cause of AIHA. This type of AIHA can be suspected if RBC autoagglutination occurs at room temperature. The DAT result is positive with C3d alone (65%) or with IgG (24%), there are no detectable cold agglutinins (CA) in the serum. Using sensitive methods, IgM on the RBCs can be detected in 71%.[19] Non-Hodgkin lymphoma (NHL) is the underlying disease in some of these cases. This AIHA is often life-threatening and refractory to steroids and splenectomy.

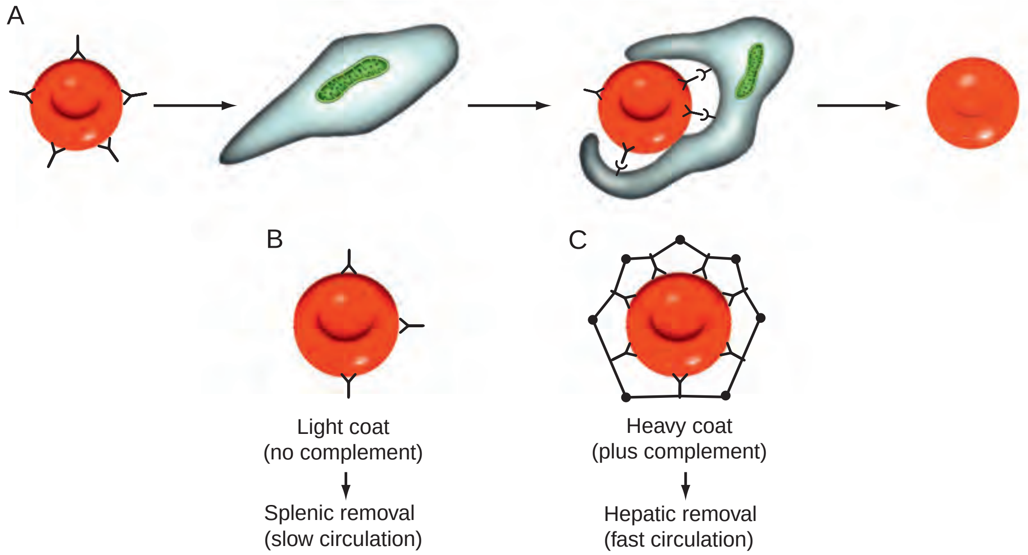


Figure 47.1 MECHANISM OF EXTRAVASCULAR HEMOLYSIS IN AUTOIMMUNE HEMOLYTIC ANEMIA. (A) Macrophage encounters an IgG-coated erythrocyte and binds to it via its Fc receptors. Thus entrapped, the red blood cell (RBC) loses bits of its membrane as a result of digestion by the macrophage's ectoenzymes. The discoid erythrocyte transforms into a sphere. (B) RBCs lightly coated with IgG (and therefore incapable of activating the complement cascade) are preferentially removed in the sluggish circulation of the spleen. (C) RBCs with a heavy coat of IgG; thus, C3b *(black circles)* can be removed both by the spleen and the liver. (*Courtesy Cunningham MJ, Silberstein LE. Autoimmune hemolytic anemia. In: Hoffman R, Benz EJ Jr., Shattil SJ, et al., eds. Hematology: Basic Principles and Practice. 4th ed. Philadelphia, PA: Elsevier; 2005.*)

TABLE 47.1 **Properties and Specificities of Red Blood Cell Autoantibodies**

	Immunoglobulin (Subclass)	Type of Antibody	Clonality	Specificity	Hemolysis	Predominant Site of Removal of Red Blood Cells
wAIHAs	$IgG_{(1–4)}$, IgA	Incomplete	Mostly polyclonal	Rh-antigens and non-Rh-antigens	Extravascular	Spleen (liver)
cAIHAs	IgM	Complete	Mostly clonal	Anti-i, -I^T, -I, -Pr	Intravascular	Liver
Donath–Landsteiner antibody	IgG	—	Polyclonal	P antigen	Intravascular	—

cAIHA, Cold autoimmune hemolytic anemia; *Ig,* immunoglobulin; *wAIHA,* warm autoimmune hemolytic anemia.

Cold Antibody Hemolytic Anemia

Cold antibodies in primary or secondary chronic forms of cAIHA (known as cold agglutinin disease or CAD) are usually monoclonal IgM. The IgM has two binding sites for C1q and fixes complement easily. The targets are polysaccharides (I, I^T, I, or Pr antigens). The "i" antigen is a nonbranched polysaccharide in the cord blood and the "I" antigen is a similar but a branched molecule expressed in the RBCs of adults. Cold antibodies, more commonly known as "CAs," bind to the RBCs at low temperatures and cause their lysis at temperatures above 22°C. The DAT is typically positive with C3d alone. When CAs are present at high titers, they may activate the complement system directly, produce a membrane attack complex, and produce intravascular hemolysis with hemoglobinuria. Usually complement-coated RBCs are sequestered by liver macrophages.

Some forms of transient cAIHA triggered by an infection may rarely occur, they can classically be associated with a *Mycoplasma pneumoniae* infection or yet with some viral infections (mostly EBV or CMV). The onset is usually abrupt and the degree of anemia may be severe with transfusion requirement, but the outcome is most often good within few weeks.

Lastly, paroxysmal cold hemoglobinuria (PCH) is a very rare form of AIHA caused by the DL antibody. This is a rare, usually polyclonal IgG cold antibody to P antigen (glycosphingolipid globoside), which binds to the RBCs at 4°C. The cells are lysed at higher temperatures. The DAT is positive with C3d, no CAs are present, and the diagnosis can only be ascertained by means of the DL test in a specialized lab. In this test, normal RBCs and patient and normal serum are incubated at 4°C. Agglutination occurs after warming to 37°C. The prominent clinical feature of PCH is a brisk, immediate but sometimes also delayed hemoglobinuria after cold exposure even in patients with low antibody titers. In the past, it has been associated with viral infections in children and secondary or tertiary syphilis in adults. Now, two types can be distinguished clinically[20]: (1) an acute, severe form (often associated with hemoglobinuria) but self-limiting AIHA after (respiratory) infections in children, and (2) a rare, chronic AIHA in nonsyphilitic persons with various underlying conditions, including NHL. Patients with chronic PCH respond poorly to steroids and splenectomy.

Mixed Warm and Cold Autoimmune Hemolytic Anemia

A small number of patients have an AIHA mixed with a positive DAT for both IgG and C3d, indicating coexistence of warm IgG autoantibodies and high-titer CAs (titer >1/64).[21] Mixed AIHAs have a severe course of disease and lower median hemoglobin (Hb) values (5.8 g/dL) that frequently require multiple therapy.[10]

Atypical Autoimmune Hemolytic Anemia

The term characterizes DAT-negative, IgA-driven, or warm-IgM forms. They represent a diagnostic challenge, resulting in delayed treatment.[22,23]

ETIOLOGY AND PATHOPHYSIOLOGY

Our knowledge about the etiology of AIHA is still limited. Factors that may play a role are antigen mimicry; immune deficiency; and, to a lesser extent, probably genetic factors. AIHA, similar to other autoimmune diseases, is a consequence of the loss of immunologic (self-) tolerance against antigens expressed on the erythrocyte surface. Production of RBC antibodies is a result of the interaction of T and B cells, as well as regulatory factors (e.g., T-regulatory cells, cytokines[24]). Disturbances of the Th1/2 T-cell subset balance with IL-10/IL-12 imbalance toward Th2 cells as well as the occurrence of clonal regulatory T cells specific for a RBC autoantigen have been described.[25–27] Moreover, BAFF-dependent B-cell and plasma-cell activation plays a role with the possible emergence of long-lived autoreactive plasma cells.[28]

This may be linked to the fact that AIHA does not only occur in immunocompetent individuals but frequently occurs in patients with acquired B or T-cell defects such as CLL, common variable immunodeficiency (CVID), HIV infection, or immunosuppressive therapy, particularly after stem-cell or solid-organ transplantation. Polymorphisms or altered expression of negative regulators of T-cell responses such as cytotoxic T lymphocyte antigen 4 (CTLA4) or interleukin-10 may also play a role. Mouse models (New Zealand black mice) have revealed an association of genetic loci with antierythrocyte antibody production or cold agglutinin escape tolerance after *Mycoplasma* infection.

Various target antigens have been described, with Rhesus polypeptides, glycophorin A, and erythrocyte band 3 being the most prominent in wAIHA. Cold reactive antibodies frequently target the I or i blood group-specific antigens. Events linked to the development of secondary AIHA by induction of cross-tolerance (molecular mimicry) are infections (*M. pneumoniae* [I antigen target], parvovirus B19, herpes viruses), neoplastic diseases (paraneoplasia), and drugs by various mechanisms. Important differences exist in the pathogenesis of wAIHA and cAIHA. The pathogenesis of primary wAIHA is largely unknown. Secondary wAIHA is a complication of several congenital or acquired immune deficiencies. Both moderate (e.g., in CLL) and severe (HIV, post-transplant, congenital severe T-cell deficiencies) T-cell and humoral immune deficiency predispose to wAIHA, but no correlation has been established between the type and severity of immune deficiency and the risk of AIHA. One poorly understood phenomenon is the lack of a clear relationship between the presence of RBC antibodies and anemia. In many instances, no anemia is present despite a strongly positive DAT or high titers of cAIHAs. There is also only a poor correlation between antibody titers and severity of anemia. Another unexplained finding in secondary AIHA is the occurrence of both wAIHAs and cAIHAs in the same condition, for example, in lymphomas or infections.

Antibodies in primary AIHA are frequently polyreactive and polyclonal (no clonal B cells detected by polymerase chain reaction [PCR]). Antibodies in CAD are mostly produced by PCR-detectable, oligoclonal, or monoclonal B-cell populations. The nature of these antibodies has been extensively studied in CAD. However, in only a few cases has it been established that the RBC antibody is clonal. In most reports, clonality of RBC antibodies was assumed if the patient had a paraproteinemia.

B-cell neoplasms, expressing IgMκ antibodies, directed against RBC antigens have few somatic mutations, which seem to be fairly restricted to certain Igheavy and light-chain families (VH4–34, VκIV).[29] Moreover, a VH4–34 CLL confounding subclone was shown to arise from a pre-existing CAD-producing, B-cell population. The restricted clonality of CAD-producing B cells is further corroborated by the detection of clonal Ig rearrangements and recurrent chromosomal aberrations (trisomy 3).[30,31] CLL cells may also drive AIHA by presenting the autoantigen (e.g., erythrocyte protein band 3) to T cells.

SYMPTOMS, CLINICAL FINDINGS, AND RISKS

The symptoms of AIHA depend on the type of antibody, the mode of onset, and the severity of anemia. In patients with wAIHA, the onset is mostly gradual or subacute and the symptoms (i.e., tiredness, reduction of physical activity, jaundice, and shortness of breath in elderly patients) are attributable only to anemia. However, patients with postinfectious, drug-induced AIHA or patients with DL or Pr antibodies often present with acute severe symptoms such as malaise, fever, jaundice, abdominal pain, shortness of breath, and hemoglobinuria. The course in such patients may be fulminant and even fatal. Patients with chronic cAIHA (CAD) often have a rather indolent course. Symptoms suggestive of cAIHA are cold sensitivity, cold-dependent acrocyanosis, acral numbness, and rarely livedo reticularis. Anemia worsens after cold exposure or after conditions associated with an acute phase reaction such as infections.

During clinical examination, a subicterus may be seen. Lymphadenopathy, important splenomegaly, or any organomegaly is rare in patients with primary AIHA. Its presence suggests secondary AIHA.

Patients with wAIHA are at an increased risk of venous thromboembolism, sometimes associated with a lupus anticoagulant (LA).[10,32] Older patients with AIHA are at an increased risk of cardiovascular complications, which may also be partly caused by the treatment.

LABORATORY DIAGNOSIS OF AUTOIMMUNE HEMOLYTIC ANEMIA

AIHA is essentially a laboratory diagnosis. The diagnostic pathway of AIHA should proceed in a stepwise fashion answering the following questions.

FOUR IMPORTANT QUESTIONS FOR THE DIAGNOSIS AND MANAGEMENT OF AUTOIMMUNE HEMOLYTIC ANEMIA

It is of utmost importance to differentiate between various types of autoimmune hemolytic anemia (AIHA). A step-wise approach helps in making the right decisions:

Question 1:

Hemolytic anemia? The basic features of hemolytic anemia are low haptoglobin levels, elevated indirect bilirubin, and elevated lactate dehydrogenase. Reticulocytosis is a common feature (≈80% of the cases) of AIHA reflecting the increase of erythropoiesis in response to accelerated destruction of red blood cells (RBCs).

Question 2:

Autoimmune hemolytic anemia? A direct antiglobulin test (DAT) is initially performed with a polyspecific antibody to detect IgG or complement C3d bound to RBCs. If the DAT result is positive, the diagnosis of AIHA is established in the absence of any other cause of hemolysis and/or any recent transfusion. In parallel, the peripheral blood smear is very helpful because it may show the presence of spherocytes (wAIHA) or agglutinates (cAIHA) and show the absence of schizocytes.

Question 3:

Warm or cold autoimmune hemolytic anemia? The DAT is further elaborated with monospecific antibodies to IgG and complement (C3d). If the DAT result is positive with IgG alone or with IgG + C3d, the AIHA is most probably caused by a warm antibody (wAIHA). If the DAT is positive with C3d only, the AIHA is most probably caused by a cold antibody (cAIHA).

Question 4:

Primary or secondary AIHA? More than half of wAIHAs are secondary to underlying diseases. Secondary AIHA should be suspected in patients with additional findings or who are refractory to initial steroid treatment. In this case, the underlying disease has to be diagnosed (e.g., by serologic tests, computed tomography (CT) scan, or bone marrow biopsy).

Diagnostic Steps

The first step is to establish the diagnosis of hemolytic anemia (see box on Four Important Questions for the Diagnosis and Management of Autoimmune Hemolytic Anemia). This diagnosis is established by the presence of the following pentad of findings: normocytic or macrocytic anemia (male Hb <13.0 to 14.0 g/dL; female <12.0 g/dL), reticulocytosis (corrected reticulocyte count >2% or absolute reticulocyte count >100,000/μL to 120,000/μL), low haptoglobin, elevated lactate dehydrogenase (LDH), and elevated unconjugated (indirect) bilirubin. Haptoglobin is an α2-globulin that binds Hb. This Hb–haptoglobin complex is degraded in the liver. Hemopexin is another plasma protein with a very high binding affinity to Hb. It scavenges heme released from RBCs and protects the organisms from the adverse effects of circulating Hb. The determination of hemopexin is not essential for the diagnosis of AIHA. Indirect bilirubin is usually not more than 5 mg/dL except in associated liver disease (Epstein-Barr [EBV]-associated AIHA). Additional findings are increased urobilinogen in the urine and spherocytes in the blood smear (Fig. 47.2). Leukoerythroblastosis occurs only in peracute AIHA, but microangiopathic hemolytic anemia should always be suspected in such cases. Bone marrow examination is usually not necessary except in patients in whom secondary AIHA, in particular lymphoma, is suspected. RBC survival is shortened, but its measurement with radioisotopes has no diagnostic value, not even for the prediction of the efficacy of splenectomy.

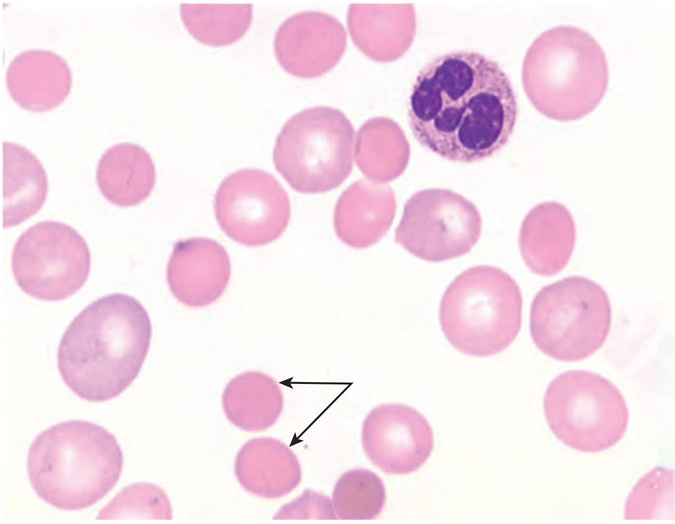


Figure 47.2 BLOOD SMEAR SHOWING NUMEROUS SPHEROCYTES *(ARROWS)*.

Step 1: Hemolytic anemia?

Among methodologic diagnostic problems, reticulocyte counting is the biggest because in many laboratories low-precision microscopic counts are still performed. Automatic-flow cytometric methods are more precise, reliable, and convenient. With flow cytometry, the number of highly fluorescent reticulocytes can also be measured. Falsely very high mean corpuscular volume (MCV) and mean corpuscular Hb concentration occur in some cases of cAIHA because RBC counts are falsely low because of agglutination of RBC at room temperature (Fig. 47.3). If a cAIHA is suspected, blood samples should be sent as quickly as possible to the laboratory in warmed containers.

All of the findings of the pentad are not always present. Reticulocytosis is often (in ≈20%) not present at the onset of AIHA. This is mostly because of a delayed initial bone marrow response of erythropoiesis. After 1 week, most of these patients have reticulocytosis. In other patients (particularly in secondary cases), absence of reticulocytosis may be attributable to impairment of erythropoiesis caused by bone marrow infiltration, vitamin B_9 or B_{12} deficiency or blunted erythropoiesis caused by an acute-phase reaction. If the reticulocyte count is very low, pure RBC aplasia (PRCA), either immune mediated or induced by a parvovirus (or HHV6) infection, should be suspected. Haptoglobin may be falsely normal or even slightly increased, particularly in patients with malignant or immune diseases, because haptoglobin is an acute-phase protein. Haptoglobin may be falsely low in patients with a haplotype H_0H_0 and in patients with severe liver disease. Both increased bilirubin and elevated LDH have a limited specificity for AIHA.

Step 2: Autoimmune Hemolytic Anemia?

The next step is to find out whether the hemolytic anemia is an AIHA. This is best done by the DAT (Fig. 47.4). In this test, washed RBCs of the patient (obtained from an ethylenediaminetetraacetic acid [EDTA] blood sample) are incubated in a tube with a

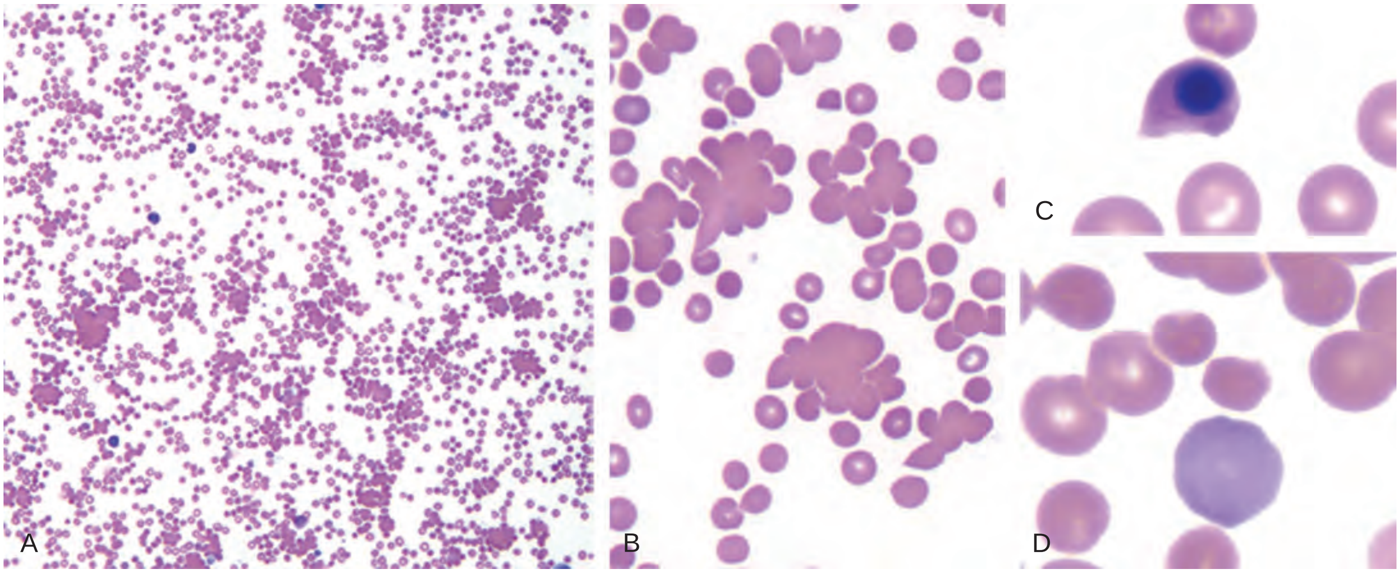


Figure 47.3 PERIPHERAL SMEAR IN COLD AGGLUTININ DISEASE. Low-power scan shows uneven distribution of red blood cells (RBCs) (A), which at slightly higher power (B) shows the RBCs to be clumped together or agglutinated. This must be distinguished from rouleaux formation. High-power scan shows nucleated RBCs (C), polychromatophilia, and microspherocytes (which are mostly seen in wAIHA) (D).

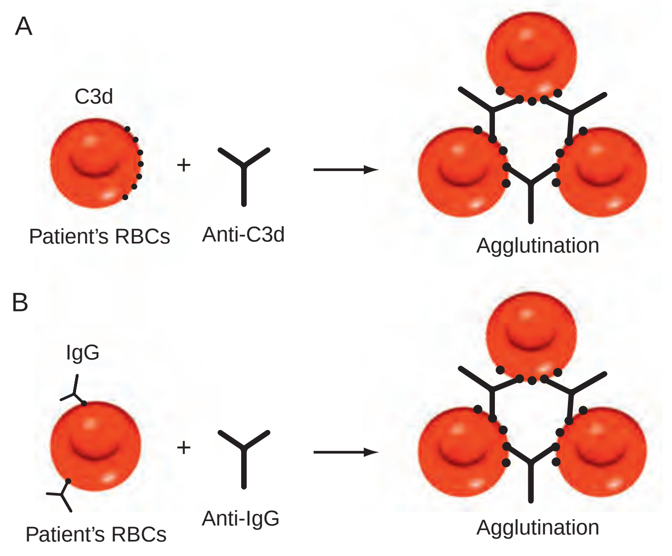


Figure 47.4 DIRECT ANTIGLOBULIN TEST FOR DETECTION OF (A) ERYTHROCYTE-BOUND C3d OR (B) IgG. Hemagglutination occurs when anti-C3d or anti-IgG can create a lattice structure by bridging sensitized RBCs. *IgG*, Immunoglobulin G; *RBC*, red blood cell. *(Courtesy Cunningham MJ, Silberstein LE. Autoimmune hemolytic anemia. In: Hoffman R, Benz EJ Jr, Shattil SJ, et al., eds. Hematology: Basic Principles and Practice. 4th ed. Philadelphia, PA: Elsevier; 2005.)*

polyspecific antibody to IgG and complement (C3d). If the RBCs agglutinate, the test result is positive. In many laboratories, the tube test has been replaced by the tube-gel test, which is easier to perform, more reliable, and probably more sensitive (Fig. 47.5). In the indirect antiglobulin test (IAT), patient plasma or serum is incubated with test RBCs and (after washing) RBC-bound IgG is detected with the DAT. IAT is usually not required for the diagnosis of AIHA except when a drug-dependent antibody is suspected. For the differentiation of drug-dependent antibodies and autoantibodies, an acid eluate of the patient's RBCs should be made and tested in the IAT. If the IAT result is positive, the patient has autoantibodies. The severity of AIHA does not correlate with the strength of the DAT, but rather with the Ig subclass of the antibody (IgG_1 or IgG_3). The result of the DAT is not a reliable marker of treatment success because patients with a complete hematologic remission may remain DAT positive, and DAT positivity or negativity has only limited value to predict the duration of hematologic remission.

Falsely Negative and Positive Direct Antiglobulin Test Results Without Hemolysis or Anemia

If the conventional DAT test result is negative, hemolytic anemia is defined as DAT-negative. This implies that positive DAT with anti-IgA has been excluded as well as other causes or hereditary of acquired hemolytic anemia—in particular hereditary spherocytosis and paroxysmal nocturnal hemoglobinuria. However, AIHA cannot be definitely excluded because about 5% (2% to 11%) of AIHA patients are DAT negative. If AIHA is suspected for clinical grounds despite a negative DAT result, more sensitive quantitative tests are required to determine the amount of IgG on the RBCs. The threshold of positivity of the conventional DAT is 100 to 200 IgG molecules per RBC, but in some AIHA patients the RBC IgG is less than this amount. In about one-third of DAT-negative cases, one of the more sensitive test results (e.g., immunoradiometric tests) will be positive. However, the relationship between the amount of RBC IgG and hemolysis is not clear cut, and there is no "hemolysis threshold."[33] The reasons for these discrepancies between the in vitro and in vivo activity of RBC antibodies are largely unknown. Differences in macrophage activity may be one possible explanation. A search for antibodies in the RBC eluate in which antibodies are more concentrated is also useful. IgA antibodies are rare and sometimes not included in the analysis. Finally, there is the possibility of low-affinity antibodies. Such antibodies are washed out when the washes are made with 37°C saline. A high rate of DAT-negative AIHA has been observed in AIHA induced by nucleoside analogues, but also in other secondary AIHA.

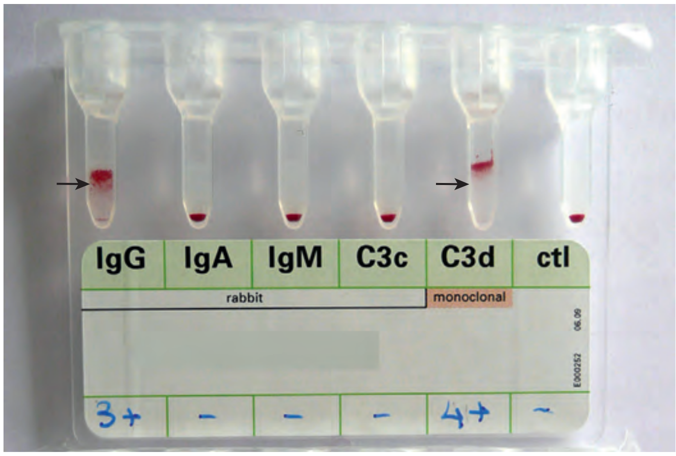


Figure 47.5 RESULT OF A DIRECT ANTIGLOBULIN TEST PERFOMED ON GEL COLUMNS, WITH A POSITIVE RESULT SHOWN WITH AN ANTI-IgG AND ANTI-C3d. *ctl*, Control; *IgA*, immunoglobulin A; *IgG*, immunoglobulin G; *IgM*, immunoglobulin M.

The DAT result is positive in 1 in 10,000 to 3 in 10,000 normal persons and 10% of hospital patients without anemia or signs of hemolysis.[34]

Step 3: Warm or Cold Autoimmune Hemolytic Anemia?

In a further step, the DAT is carried out with monospecific antibodies to IgG and complement (C3d) to find out whether a warm or cold antibody is the cause of hemolysis. If the DAT result is positive with IgG alone or with IgG plus C3d, the AIHA is most probably a wAIHA. If the DAT result is positive with only C3d, the AIHA is most probably a cAIHA. The differentiation between wAIHAs and cAIHAs is extremely important for the choice of treatment.

Some special diagnostic problems exist when it comes to cAIHAs. If cAIHA is suspected, it must be taken care that the blood sent to the laboratory is kept at 37°C to get reliable results. Patients with only RBC C3d may (rarely) have wAIHAs when the amount of RBC IgG is very small. In patients with C3d positivity, the cold agglutinin titer should be determined. If it is equal or greater than 1:64 the diagnosis of a CAD is established. There is no consensual threshold titer that separates normal from abnormal. If the titer is less than 1:64, the thermal amplitude of the antibody must be determined. If the thermal amplitude is above 22°C, the diagnosis is CAD. Mixed type AIHA is rare. Frequently used criteria are a positive IgG DAT result and a positive eluate result plus a C3d-positive DAT result and the presence of CAs with a thermal amplitude of greater than 30°C or high titer Cas (>1:40). Many published cases of mixed-type antibodies do not fulfill these criteria.[35,36]

Step 4: Primary or Secondary Autoimmune Hemolytic Anemia?

In the last diagnostic step, it must be determined whether the AIHA is primary or a complication of an underlying disease (secondary). About half of cases of AIHA (or probably even more) are secondary. The main causes are systemic immune diseases and malignancies and mostly B-cell lymphomas. Other underlying conditions are infections, primary immunodeficiencies, drugs, transplantation, and congenital defects. The decision regarding which diagnostic procedures should be used for this purpose depends mainly on the type of AIHA (wAIHAs or cAIHAs) and should include history, physical examination, laboratory tests, and imaging procedures if indicated. In children and younger patients with wAIHA, evidence for an infection should be sought. A list of all recent medication should be made. A history of weight loss, fever, or poor general condition, and arthritis points to a malignancy or systemic immune disease as the underlying condition. Palpable lymphadenopathy and important splenomegaly do not belong to the usual clinical picture of primary wAIHA. In this case, lymphoma (particularly splenic marginal zone lymphoma [SMZL]) should be suspected. Laboratory tests should include acute-phase proteins, LDH, quantitative determination of Igs, antinuclear antibodies, and other tests guided by the clinical history. A bone marrow examination is not obligatory except when lymphoma is suspected. Abdominal ultrasonography or a CT scan is reasonable in all cases to look for a disproportionate splenomegaly, the remote (but important) possibility of an ovarian teratoma (in women), another solid tumor, lymphadenopathy, or solitary extranodal lymphomas. An activated partial thromboplastin time with a lupus-sensitive reagent may reveal an LA. Fluorescence-activated cell sorting or PCR for the detection of monotypic/monoclonal lymphocytes may be done, but the clinical usefulness of such tests to guide treatment decisions is uncertain.

In cAIHA, a history of a febrile illness should prompt a thoracic radiography, and if results are positive, a serologic test for mycoplasma pneumoniae is required. The quantitative determination of serum Igs and a search for a clonal Ig by immune fixation are important. If immune fixation findings are positive, a search for lymphoma is necessary, which may include bone marrow biopsy even when there is no lymphadenopathy. In addition to classical CAD, CAs occurring in association with other cancers, aggressive lymphomas, or infection should be termed *cold agglutinin syndrome*.[37]

Other tests, including blood glucose, Hba_{1c}, renal and hepatic function tests, HIV serology, hepatitis B antigen, and exclusion of latent tuberculosis are necessary to avoid complications of steroids (in wAIHAs) or rituximab (in cAIHAs and wAIHAs).

IMMUNOLOGIC PHENOMENA ASSOCIATED WITH AUTOIMMUNE HEMOLYTIC ANEMIA

Evans syndrome is the simultaneous or sequential combination of wAIHAs with autoimmune thrombocytopenia (ITP) ± autoimmune neutropenia, a typical but rare association. Platelet antibodies are usually directed against glycoprotein IIB/IIIA. Autoimmune neutropenia occurs in 25% of patients. There is no sex predilection; about half of the cases are secondary. Evans syndrome is relatively more common in patients with SLE, antiphospholipid antibodies (APAs), CVID, and autoimmune lymphoproliferative syndrome (ALPS).[38] A search for an ALPS is mandatory in younger patients with Evans syndrome. In roughly 50% of the cases, AIHA and ITP occur simultaneously; in the others disease starts with either ITP or AIHA. There may be an interval of several years between the onset of AIHA and ITP.[39]

wAIHA, cAIHA, or a positive DAT result without anemia may be associated with PRCA. PRCA may be either immune mediated such as in T-cell neoplasms (particularly T-cell large granulocytic leukemia or thymoma) or may be caused by an infection with parvovirus B19. Patients with parvovirus-associated AIHA often present with PRCA.

AIHA is definitely associated with APAs and LA. In one single-center, prospective study, LA was found in 30% predominantly idiopathic AIHA. AIHA (4%) and Evans syndrome (10%) are frequent in patients with APAs. It is well established that patients with AIHA have a significantly (2.8-fold to 3.8-fold) elevated risk of venous thromboembolism, especially during the active phases of the disease.[32] It is likely, but not proven, that patients with AIHA associated with LA are at a higher risk.

In lymphomas, some cases of AIHA are associated with C1-esterase inhibitor deficiency and mixed cryoglobulinemia.

Temporal Relationship of Secondary Autoimmune Hemolytic Anemia to the Underlying Condition

The temporal relationship of AIHA to an underlying condition is complex. AIHA antedates the diagnosis of malignancy in many cases—including NHL—sometimes for years. In population-based studies, clonal B cells have been detected in some patients with seemingly "idiopathic," AIHA. In most instances, AIHA occurs concurrently with the malignancy. In some other cases, AIHA occurred only at the recurrence and rarely in complete remission (CR) after successful treatment. In CLL, AIHA occurs preferentially in a late and active phase of the disease. The patients have poor prognostic factors and often have been treated with various agents.

In immune diseases, particularly SLE, AIHA is a complication of the early phase of the disease. Infection-related AIHA occurs shortly after the onset of symptoms, except in HIV infection in which AIHA is a complication in the late advanced stage of the disease.

In CIVD, AIHA or Evans syndrome preceded or revealed CVID in more than half of the cases.

Serologic Type of Autoimmune Hemolytic Anemia in Secondary Autoimmune Hemolytic Anemia

It is an interesting phenomenon that in underlying diseases associated with AIHA, all serologic types of AIHA may occur, but usually one serologic type (wAIHA, cAIHA, or DL antibody) prevails. The only exception is ovarian teratoma, which is only associated with wAIHA.

Autoimmune Hemolytic Anemia in Immune Diseases

Autoimmune Hemolytic Anemia in Systemic Lupus Erythematosus and Primary Antiphospholipid Syndrome

The prevalence of AIHA in patients with SLE is 7.5% (average of seven studies; range: 5.2% to 12.5%). AIHA may occur at any time during the course of SLE, but two-thirds of cases of AIHA occur at diagnosis or soon thereafter. Almost half of the patients are already taking steroids. Most patients are women and have wAIHA. Evans syndrome is common. Compared with SLE patients without AIHA, those with AIHA are younger; have a higher prevalence of thrombocytopenia, APAs, renal disease, serositis, and central nervous system involvement; and have a higher risk of venous thrombosis.[40]

The prevalence of AIHA in primary antiphospholipid syndrome (PAPS) is about 10%. AIHA precedes PAPS in 25% of cases, but in others occurred after a median time of 4.4 years after the diagnosis of PAPS. Patients with PAPS and AIHA have an increased risk for the development of SLE.[41]

Autoimmune Hemolytic Anemia in Inflammatory Bowel Disease

In the only larger study, the prevalence of AIHA in ulcerative colitis was 1.7%.[42] The mean time from diagnosis of colitis to AIHA was 17 months. Three-quarters of these patients had total colitis. AIHA may also be associated with Crohn disease, but the prevalence is lower than in ulcerative colitis.

Autoimmune Hemolytic Anemia in Other Immune Diseases

A few or single cases of an association of wAIHA with Sjögren syndrome, dermatomyositis, biliary cirrhosis, Graves disease, Churg-Strauss syndrome, crescentic glomerulonephritis, polymyalgia rheumatica, scleroderma, autoimmune pancreatitis, and cAIHA with rheumatoid arthritis have been reported. A high prevalence of AIHA was found in small children with giant-cell hepatitis.

Autoimmune Hemolytic Anemia in Transplanted Patients

AIHA is a rare but important and dangerous complication of allogeneic hematopoietic stem-cell transplantation (HSCT). Aside from AIHA, causes of hemolytic anemia in transplanted patients may be lymphocyte passenger syndrome and ABO incompatibility. AIHA may be caused by severe immunosuppression by drugs to prevent rejection, graft-versus-host disease (GVHD), viral infections, EBV lymphoproliferative disorder (LPD), or the recurrence of an immune disorder after transplantation (biliary cirrhosis).

The highest rate of AIHA occurred in small children after unrelated cord HSCT for inborn metabolic defects (44%) and in children with severe combined immune deficiency after haploidentical HSCT with T-cell–depleted stem cell grafts.

In adults, the incidence of AIHA ranges from 3.0% to 4.4% after allogeneic HSCT.[43] The median time from HSCT to AIHA is 4 to 10 months. Most patients have wAIHAs. Risk factors for AIHA are unrelated donors, T-cell depletion, and extensive GVHD. In most cases, AIHA occurred in patients in CR of the underlying disease. However, in one study AIHA was associated with a relapse of chronic myeloid leukemia in the majority of patients.

After transplantation of solid organs, the highest risk of AIHA was in patients with pancreatic transplantation. A number of patients with AIHA or cAIHA were observed after liver transplantation, but only a small number were observed after cardiac, lung, intestinal, and renal transplantation. The most likely cause of AIHA in these patients is severe drug-induced immunosuppression.

Autoimmune Hemolytic Anemia in Pregnancy and After Blood Transfusion

Very few cases of AIHA and Evans syndrome—with wAIHAs—occurred during pregnancy. In these patients, there seems to be a higher risk for pre-eclampsia. AIHA responds well to steroids and resolves in most cases after delivery. In two cases, the newborns had mild hemolysis.

Development of RBC autoantibodies is common in multitransfused patients and is associated with the presence of alloantibodies. However, anemia is rare.

Autoimmune Hemolytic Anemia in Malignancies

Autoimmune Hemolytic Anemia in Lymphoproliferative Disorders

AIHA is a typical, relatively common immune-mediated, paraneoplastic syndrome in LPD. It occurs in almost all histologic subtypes of NHL, but there is no correlation between the frequency of lymphoma and the risk of AIHA. AIHA is relatively most common in SMZL and angioimmunoblastic T-cell lymphoma (Table 47.2). In most cases of LPD, wAIHAs is predominant. Most wAIHAs, particularly in CLL, seem to be polyclonal. The proportion of patients with wAIHAs is highest in CLL and Hodgkin lymphoma; in other malignancies, the ratio of wAIHAs to cAIHAs is 2:1. In lymphoplasmacytic lymphoma (LPL), almost all antibodies are clonal cAIHAs.

Autoimmune Hemolytic Anemia as a Risk Factor for Lymphoproliferative Disorder

In various population-based studies, AIHA emerged as a risk factor for subsequent development of diffuse, large B-cell lymphoma, LPL, CLL, monoclonal gammopathy with unknown significance (MGUS), and multiple myeloma. Data regarding the type of AIHA and a possible influence of treatment were not available in these studies. It is uncertain whether autoimmune disorder per se is the causal factor or whether these patients had a clinically silent clonal disorder at the time of AIHA.

TABLE 47.2 Secondary Autoimmune Hemolytic Anemia in Malignancies

Malignancy	Prevalence	wAIHAs	cAIHAs
MGUS	Very low	None	All
All NHL	0.23%–2.6%		
CLL	4.3%–9%	90%	10%
SMZL	10%	2/3	1/3
LPL	3%–5%	None	Most
Angioimmunoblastic T-cell lymphoma	13%	One-third	Two-thirds
Hodgkin lymphoma	0.19%–1.7%	All	None
Ovarian teratoma	Very low	All	None
Solid tumors	Very low	Two-thirds	One-third

cAIHA, Cold autoimmune hemolytic anemia; *CLL,* chronic lymphocytic leukemia; *LPL,* lymphoplasmacytic lymphoma; *MGUS,* monoclonal gammopathy with unknown significance; *NHL,* non-Hodgkin's lymphoma; *SMZL,* splenic marginal zone lymphoma; *wAIHA,* warm autoimmune hemolytic anemia.

Autoimmune Hemolytic Anemia in Chronic Lymphocytic Leukemia

The prevalence of AIHA is highest in CLL, ranging from 4.3% to 9%. The prevalence is highest in poor-risk patients (Binet stage B or C, increased ZAP70 expression, unmutated IgVH status, and CD38 positivity) and patients who had already been treated. However, AIHA may also occur in very early stages of CLL, including B-cell monoclonal lymphocytosis. In a large group of non-treated CLL patients, the prevalence of positive DAT result (with or without anemia) was 14%.[44] Most CLL patients with AIHA are men. The vast majority of patients have (presumably) polyclonal wAIHAs, but in all studies, there is small number of cases with cAIHA (specificity anti-I), often with IgM paraproteinemia.

Autoimmune Hemolytic Anemia in Monoclonal Gammopathy With Unknown Significance and Lymphoplasmacytic Lymphoma

The clinical picture of primary chronic CAD suggests the presence of "idiopathic," or primary cAIHAs, but in fact, most of these patients have a clonal disease with either only clonal IgMk (IgM-MGUS) or LPL with IgM paraproteinemia, MYD88 mutation and bone marrow infiltration (or Waldenström macroglobulinemia). Traditionally, the latter category would be classified as secondary AIHA (CAS). More than 90% of patients with CAD have a monoclonal IgMκ; 7% had IgG or IgA monoclonal immune globulin with λ chains. The course of CAD is usually indolent. Fewer than half of patients require transfusions and the risk for progression to highly malignant lymphomas is small.

Autoimmune Hemolytic Anemia in Other Lymphoproliferative Diseases and Myeloma

The prevalence of AIHA is low in NHL, ranging from 0.23% to 2.6%. AIHA has been described in all histologic subtypes of NHL. Based on the prevalence of NHL, the association with AIHA is highest with SMZL, LPL, angioimmunoblastic T-cell lymphoma, and γ heavy-chain disease. The antibody may be either a warm (two-thirds) or a cold (one-third) antibody. In NHL, there seems to be a relatively frequent association of AIHA with LA, C1-esterase deficiency, or essential cryoglobulinemia.

A number of predominant wAIHAs have been described in IgG and IgA myelomas.

Autoimmune Hemolytic Anemia in Myeloid Disorders

Generally, AIHA is rare in patients with myeloid malignancies. A number of cases have been reported in myelodysplastic syndromes, particularly chronic myelomonocytic leukemia. Very few cases were described in acute myelogenous and lymphoblastic leukemia, myelofibrosis, and polycythemia.

Autoimmune Hemolytic Anemia in Solid Tumors

A special, rare, but highly interesting cause of wAIHA is an ovarian dermoid cyst. These patients respond very poorly to drug therapies, but the AIHA resolves completely and the DAT result becomes negative a few weeks after ovariectomy. The same behavior has been described in microcystic adenoma of the pancreas and dermoid cyst of the mesentery, both associated with AIHA.

AIHA is rarely but definitely associated with solid tumors. It is a very rare complication of lung, renal cell, and ovarian cancer. In some cases of renal-cell cancer, AIHA resolved after curative surgery.

Infection-Related Autoimmune Hemolytic Anemia

In immunocompetent patients, AIHA may occur after viral, bacterial, or parasitic infections (Table 47.3). Virus-associated AIHA occurs mostly in newborns and children; bacterial AIHA occurs more often in adults. The onset of AIHA occurs shortly after signs of infection, but sometimes after a latency time up to weeks. After some specific infections, patients may preferentially develop a warm or a cold antibody (sometimes with specific targets such as I, i, P, or Pr antigen. In almost all instances beside the dominant antibody (cold or warm), a few cases exist of another antibody (see Table 47.3). Of particular interest is varicella-zoster, virus-associated, and rubella-associated cAIHAs, because the cold antibody is mostly directed to the Pr antigen and the AIHA is clinically severe. In *Mycoplasma*-associated AIHA, the antibody target is almost always "I." After *Mycoplasma* infection, the DAT result is positive in 50% to 60% of cases, but anemia is rare. In some specific infections, the antibody was a DL antibody, but in a large study most DL antibodies were found after unspecific respiratory infections.[18] In many cases, the course of AIHA is short, uncomplicated, and self-limited, but some cases with a severe, even fatal, course, particularly in patients with Pr antibodies and in *Mycoplasma*-associated AIHA, have been reported. In AIHA associated with bacterial infections or in leishmaniasis, treatment of the infection seemed to be beneficial.

AIHA has been described in a few cases of acute hepatitis A, B, C, or E and in a number of cases of untreated chronic hepatitis C. However, in a large study, an increased prevalence of AIHA has only been found in interferon (IFN)-treated hepatitis C patients.[45]

Anecdotal reports have described patients in whom wAIHAs were associated with measles, *Chlamydia pneumoniae*, or miliary tuberculosis and cAIHAs with adenovirus, measles, leptospiral pneumonia, *Escherichia coli* infection (all anti-I), pneumococcal pneumonia (anti-Pr), *Haemophilus influenzae* (DL), babesiosis, or *Bartonella henselae* (DAT negative).

Some cases of AIHA have been described after vaccination against hepatitis B, influenza (MF59 adjuvanted), diphtheria–tetanus–pertussis, or rubella. Such associations could not be confirmed in systematic studies.

Recently, both warm and cold autoantibody associated AIHA has been reported in patients with SARS-Cov-2.[46]

TABLE 47.3 **Autoimmune Hemolytic Anemia After Infections**

	Infection	wAIHA	cAIHA (Specificities)
Respiratory tract infections (unspecified)	—	—	+ (DL)/PCH
Viral infections (specific)	EBV	±	+ (anti-i)
	CMV	+	± (anti-i)
	Parvovirus (B19)	+ (often with PRCA)	± (DL)
	Varicella	±	+ (anti-Pr, anti-I, anti-DL)
	Rubella	—	+ (anti-Pr1) Monotypic IgM
	HIV	+	+ (anti-I, anti-i, anti-Pr)
	SARS-Cov-2	+	+
Bacterial infections (specific)	Mycoplasma	±	+ (anti-I, anti-Pr)
	Brucellosis	±	+ (anti-I)
	Haemophilus influenzae		+ (DL)
Parasitic infections (specific)	Visceral leishmaniosis	+	—

—, Not reported; +, predominant type of autoimmune hemolytic anemia; ±, single or few cases reported; *cAIHA,* cold autoimmune hemolytic anemia; *CMV,* cytomegalovirus; *DL,* Donath–Landsteiner antibody; *EBV,* Epstein-Barr virus; *PCH,* paroxysmal cold hemoglobinuria; *PRCA,* pure red blood cell aplasia; *wAIHA,* warm autoimmune hemolytic anemia.

Drug-Induced Autoimmune Hemolytic Anemia

Historically, methyldopa, a antihypertensive drug, was the first known drug to induce AIHA. The prevalence of methyldopa-induced AIHA is 1% with 10% to 20% DAT-positive patients without anemia, indicating that DAT positivity is not always followed by overt disease. Currently, IFN-α and purine nucleoside analogues are the most common causes of drug-induced AIHA (Table 47.4). The diagnosis of drug-induced AIHA in a patient with AIHA after drug exposure can only be made if the *indirect* DAT result is positive and the RBC eluate contains an RBC antibody. Such tests have not been performed in all patients in whom drug-induced AIHA was claimed.

The temporal relationship between drug exposure and AIHA is complex. In some instances, AIHA may occur after long-term exposure to a drug (IFN or targeted antibodies). In AIHA caused by purine analogues, AIHA occurs typically after a few cycles of therapy of CLL and is probably dose related (lower incidence and severity at reduced doses of fludarabine). Chlorambucil is said to induce AIHA, particularly after the end of treatment. This has not been found in all studies.

Interferon-induced AIHA has been described in various diseases (particularly hepatitis C and malignant diseases). It is caused by wAIHAs, occurs usually after long exposure to the drug, and disappears spontaneously after cessation of the drug within weeks. AIHA induced by purine analogues (fludarabine, cladribine) occurs mostly during or immediately after the end of drug treatment, but the onset is delayed in some cases. The antibodies are warm or mixed antibodies. This complication occurs predominantly in patients with CLL but seems to be very rare in lymphoma patients treated with these drugs. The incidence of AIHA in patients with CLL after fludarabine or cladribine monotherapy ranges from 2% to 11%. When fludarabine is combined with cyclophosphamide (and rituximab), AIHA is less frequent and probably less severe, but this may be attributable to the lower fludarabine dose in these protocols. Patients with a positive DAT result before treatment have a higher risk of purine analogue-induced AIHA. With steroids alone, about half of the patients achieve

TABLE 47.4 **Drug-Induced Autoimmune Hemolytic Anemia**

Drug	Risk Factors	AIHA Onset	Type of AIHA	Response to Treatment	Diseases Treated
Methyldopa	Not known	Delayed	wAIHA	Resolution after withdrawal	Hypertension
IFN-α	Pretherapeutic positive DAT	Delayed (8–11 months)	wAIHA	Resolution spontaneous or after steroids	Hepatitis C Hematologic malignancies
Efazulimab	Not known	Many months	wAIHA	Resolution after withdrawal	Arthritis (rare)
Etanercept	Not known	Delayed	cAIHA	Resolution after rituximab	Rheumatoid arthritis (rare)
Fludarabine Cladribine Pentostatin	CLL Pretherapeutic positive DAT result	Early (median, 3–4 cycle) or delayed	wAIHA Mixed AIHA	Half of AIHA resolve after steroids	CLL Lymphomas[a] AML[a]
Bendamustin	CLL	No or only very low risk of AIHA			CLL Lymphomas
Chlorambucil	CLL	Delayed onset	wAIHA		CLL
Eculizumab	Patients with incomplete response	After treatment	cAIHA		PNH
Lenalidomide		During treatment	wAIHA	Resolution after withdrawal	One case treated for lymphoma
Checkpoint inhibitors (anti-CTLA4, anti-PD1/PD1L		During treatment	wAIHA		Solid tumors, Hodgkin lymphoma

[a] No or very low risk.

AML, Acute myeloid leukemia; *cAIHA*, cold autoimmune hemolytic anemia; *CLL*, chronic lymphocytic leukemia; *DAT*, direct antiglobulin test; *IFN*, interferon; *PNH*, paroxysmal nocturnal hemoglobinuria; *wAIHA*, warm autoimmune hemolytic anemia.

a lasting CR. So far no cases of AIHA have been reported after treatment of CLL with bendamustine.

An interesting and as yet unexplained phenomenon is the fact that some patients with CLL-associated AIHA may achieve remissions after fludarabine or cladribine treatment. However, it is general practice to avoid purine analogues in patients with AIHA. Other drugs that have been associated with wAIHAs (in single case reports) were IFN-β, the monoclonal anti-CD11 antibody efalizumab, and the recently developed checkpoint inhibitors, namely anti-CTLA4 and anti-PD1/PD-L1 antibodies, and lenalidomide.

A peculiar type of cAIHA has been described in some patients with paroxysmal nocturnal hemoglobinuria who had been treated with the anti-C5 antibody ecuzulimab. These patients had an incomplete response to ecuzulimab. The RBCs of these patients accumulate C3d on their surfaces.

Autoimmune Hemolytic Anemia in Immune Deficiency States

AIHA is one of the classical complications of CVID. The prevalence of AIHA was 4% to 5.5% in large studies. AIHA preceded the diagnosis of CVID in more than half of the cases (median time from diagnosis of AIHA to CVID: 5.5 years). Evans syndrome is common.

AIHA (often combined with thrombocytopenia) occurs in 23% to 51% of patients with ALPS. The diagnosis of ALPS is made by the demonstration of a high number of circulating TCRαβ$^+$ double-negative (CD4$^-$ CD8$^-$) T lymphocytes. The basic defect of ALPS is a disturbance of apoptosis.[38]

AIHA is also common in deficiency of purine nucleoside phosphorylase (a T-cell deficiency with almost normal B cells). At least 15% of patients with Wiskott-Aldrich syndrome have AIHA. AIHA occurs before the age of 5 years and is associated with high serum IgM levels. These patients are candidates for allogeneic stem cell transplantation.

DIFFERENTIAL DIAGNOSIS

In patients with DAT-positive hemolytic anemia, the distinction from drug-dependent immune hemolytic anemia is important. In recipients of ABO- or D-incompatible allografts, anti-RBC antibodies are probably produced by immunocompetent memory B cells from the donor. This so-called *passenger lymphocyte syndrome* (PLS) is frequent in heart and lung transplantation (≤68%). A difficult problem is differential diagnosis of DAT-negative AIHA or Evans syndrome. In younger patients with isolated hemolytic anemia with or without symptoms, hereditary diseases such as hereditary spherocytosis, sickle cell anemia, and thalassemia must be considered. Family history and RBC morphology are most helpful in these cases. In patients with drug-induced hemolytic anemia, non-immune hemolysis caused by glucose-6-phosphate-dehydrogenase deficiency should be considered. Babesiosis or other infections may cause non-immune hemolytic anemia. In severely ill patients with hemolytic anemia and thrombocytopenia, idiopathic or cancer-related thrombotic thrombocytopenic purpura, hemolytic uremic syndrome, or disseminated intravascular coagulation should prompt a search for schistocytes in the blood smear.

Paroxysmal nocturnal hemoglobinuria is diagnosed by a deficiency of glycosylphosphatidylinositol anchor proteins on the cell surface caused by a mutation of the *PIG-A* gene. Type II mixed cryoglobulinemia is a condition caused by a monoclonal IgM autoantibody with anti-IgG activity and rheumatoid factor properties frequently associated with hepatitis C. The clinical picture is characterized by a systemic vasculitis syndrome but not by anemia.

TREATMENT

Recommendations for therapy are frequently based on retrospective analyses with small numbers of patients, registries, or expert experience. A few randomized studies have been performed. However, successful phase I and II studies with novel drugs have introduced additional options. The treatment recommendations in this chapter are largely based on published guidelines and the First International Consensus.[8,11,36,47,48]

Goals of Treatment

As in all diseases, the goal of treatment of AIHA is the achievement of a complete clinical and laboratory sustained remission without any residual signs of the disease. Such results can be obtained not only in primary AIHA, but also in a substantial number of secondary cases when the underlying disease disappears spontaneously (infection), when the causative drug is withdrawn, or after (curative) treatment (surgery or chemoimmunotherapy) of an underlying malignancy. In most patients with primary wAIHAs, the expectations for success must be tempered. Therefore, a practical goal is the achievement of a good clinical response with freedom from symptoms in the absence of side effects of treatment. Remission of AIHA after treatment is often defined by laboratory values, but there is no consensus on the definition of CR or partial remission (PR) regarding Hb concentration.[49] Formally, one could define CR as absence of transfusion requirement, a normal Hb value (according to the age and sex of the patient), and absence of signs of hemolysis (normal reticulocyte counts, haptoglobin, and LDH; negative DAT result). Such CR is sometimes seen in secondary AIHA. In primary AIHA, CR is often defined as Hb above 11.0 g/dL without sign of hemolysis, but the DAT result may remain positive. A minimal requirement for PR is the absence of transfusion requirement and a satisfactory clinical condition (usually Hb >9 to 10 g/dL). Thus, in AIHA, the treatment goals must be defined individually and tailored to the patient's needs.[50] Asymptomatic patients with mild anemia (Hb > 10 g/dL) may even be monitored without immediate treatment.

Blood Transfusions

Blood transfusions may be necessary for emergency treatment of AIHA. The problem is to find well-matched RBC concentrates. In critical cases, transfusions should not be avoided or delayed because of uncertainty in matching (see box on Transfusion Therapy in Selected Patients With Severe Autoimmune Hemolytic Anemia). The decision is made on an individual basis depending on the speed of development and severity of anemia, the type and cause of hemolytic anemia (the highest acute death rates were observed in patients with fludarabine-associated AIHA, IgM wAIHAs, and DL antibodies), and the age and clinical condition of the patient. Because the antibody in wAIHAs is directed against blood group antigens, no truly matched blood transfusions are possible, but RBCs can be safely given if alloantibodies are excluded.[3] However, some precautions have to be taken. In patients without a history of previous transfusions or pregnancy the risk of alloantibodies is low, allowing for transfusion of only ABO- and RhD-matched RBCs. In all other patients, extended phenotyping with respect to Rh subgroups (C, c, E, e), Kell, Kidd, and S/s using monoclonal IgM antibodies should be performed for selection of compatible RBC concentrates. Warm autoadsorption or allogeneic adsorption procedures for detection of alloantibodies may be used in exceptional cases. In patients with cAIHAs, transfused blood must be prewarmed using commercial warming coils. In all instances, an important precaution is a biologic in vivo compatibility test, which includes rapid infusion of 20 mL of blood; 20 minutes of observation; and, if there is no reaction, further transfusion at usual speed.

TRANSFUSION THERAPY IN SELECTED PATIENTS WITH SEVERE AUTOIMMUNE HEMOLYTIC ANEMIA

Immediate blood transfusion should not be withheld from patients with severe anemia. Small amounts of red blood cells may be lifesaving in patients with acute cardiac or cerebral dysfunction caused by anemia. However, precautions have to be taken to avoid transfusion reactions. This requires close cooperation between clinicians and the blood bank. The exclusion of alloantibodies is most important. Patients with a low risk for severe reactions caused by an alloantibody are those without a history of previous transfusions or pregnancy. In high-risk patients, extended RBC phenotyping should be performed for selection of compatible RBC concentrates. In all instances, a biologic in vivo compatibility test has to be performed at the beginning of the transfusion.

For patients with warm or cold AIHA with an Hb level greater than 10 g/dL, an inadequate reticulocytosis (which can be assessed by means of the bone marrow reticulocytes index), and the transient (wAIHA) or sometimes long-term (CAD) use of recombinant erythropoietin (RhEpo) can be helpful in about 70% of patients and can decrease transfusion-dependency.[51]

Treatment of Primary Warm Antibody Hemolytic Anemia

First-Line Treatment With Steroids

Newly diagnosed severe wAIHAs should be treated immediately with glucocorticoids (steroids) (Fig. 47.6 and Table 47.5). Therapy starts with an initial dose of 1 mg/kg/day of predniso(lo)ne orally or an equivalent dose of methylprednisone intravenously. It is recommended to continue with this dose until a hematocrit of greater than 30% or a Hb level greater than 10 g/dL is achieved. CR or PR is obtained in approximately 80% of patients. Failure to achieve this goal after 3 weeks should result in a switch to second-line therapy. In responding patients, prednisone dose is gradually reduced to 20 to 30 mg/day within a few weeks. Subsequently, the dose is tapered slowly by 2.5 to 5 mg/day per month guided by Hb and reticulocyte counts. If the patient is still in remission after 3 to 4 months at a dose of 5 mg/day, withdrawal of steroids can be attempted. The exact rate of patients remaining in CR after the end of steroid therapy is not known but is estimated to be around 20% to 40%.[52] Most responders require maintenance steroids to keep the Hb above 9 to 10 g/dL. About 40% to 50% of patients need 15 mg/day or less prednisone (regarded as the highest tolerable dose for long-term treatment). However, 15% to 20% need higher maintenance prednisone doses. Two prospective randomized studies combining prednisone with rituximab have shown higher response rates at 12 months (75% vs. 31% to 36%) and a longer relapse-free survival (70% vs. 45% at 3 years).[53,54] This combination provides a good alternative to initial steroid monotherapy, particularly if the goal is to spare steroids as for elderly patients and/or patients with comorbidities.

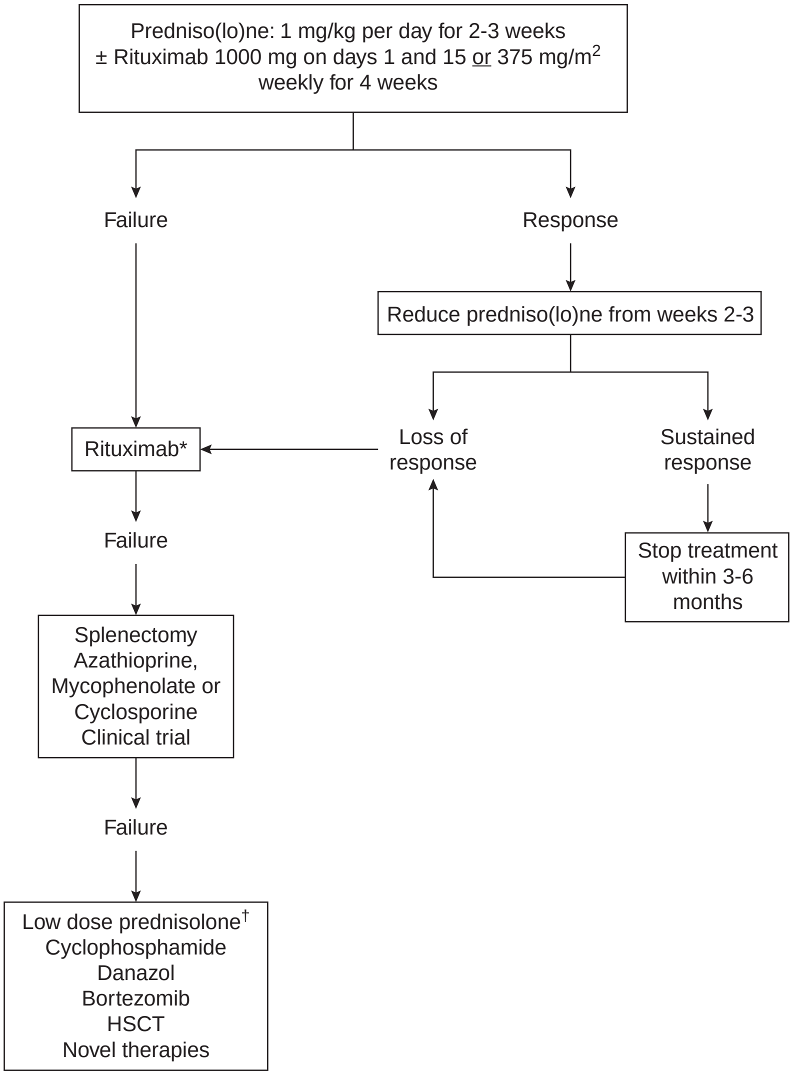
Therapeutic management of steroid treatment should include blood glucose monitoring, prophylaxis against osteoporosis (commence early), supplementation with folic acid, and thromboprophylaxis treatment in selected cases. When rituximab and corticosteroids are combined, a prophylaxis towards *Pneumocystis jirovecii* pneumonia is strongly advised.

Great concerns are osteoporosis, osteonecrosis, and bone fracture particularly of the lumbar spine. About 30% to 50% of patients on long-term steroid treatment experience fractures. The highest loss of bone density occurs early, even at smaller steroid doses, and the risk of fracture increases by 75% during the first months of treatment.

Thus, patients on steroid therapy should receive bisphosphonates, vitamin D, and calcium from the beginning. Folic acid is also recommended. Steroid-induced diabetes is a major risk factor for treatment-related deaths from infections. Although heparin treatment is not recommended for all patients, the possibility of pulmonary embolism must be considered, particularly in patients with AIHA and LA or recurrent AIHA after splenectomy. Previous studies have also found a beneficial effect of standard heparin in AIHA.[10,36]

Second-Line Treatment

Second-line treatment is considered in patients (1) refractory to initial steroids as defined earlier, (2) in need of a maintenance dose of more than 15 mg/day of prednisone (absolute indications), or (3) who need between 15 mg/day and 0.1 mg/kg/day (relative indication; Fig. 47.6). Patients with prednisone requirement of 0.1 mg/kg/day


Predniso(lo)ne: 1 mg/kg per day for 2-3 weeks
± Rituximab 1000 mg on days 1 and 15 or 375 mg/m2 weekly for 4 weeks
Failure
Response
Reduce predniso(lo)ne from weeks 2-3
Loss of response
Sustained response
Rituximab*
Stop treatment within 3-6 months
Failure
Splenectomy
Azathioprine, Mycophenolate or Cyclosporine
Clinical trial
Failure
Low dose prednisolone†
Cyclophosphamide
Danazol
Bortezomib
HSCT
Novel therapies

*If rituximab given first line, re-treatment may be considered if a sustained response was achieved. Otherwise, move to third-line options.

† Prednisolone 10 mg daily ± a steroid-sparing agent
HSCT; haematopoietic stem cell transplantation

Figure 47.6 PROPOSED ALGORITHM FOR THE TREATMENT OF PRIMARY WARM ANTIBODY AUTOIMMUNE HEMOLYTIC ANEMIA IN ADULTS. *IV*, Intravenous; *IVIg*, intravenous immunoglobulin; *PDN*, prednisone. *(Modified from Lechner K, Jäger U. How I treat autoimmune haemolytic anemias in adults. Blood. 2010;116:1831.)*

or less are potential candidates for long-term, low-dose prednisone. Patients refractory to steroid treatment should be re-evaluated for underlying diseases or warm IgM antibodies (Table 47.6).

Rituximab

Rituximab off-label is currently the best available drug option for second-line treatment).[55–57] It can be given either at a standard induction dose of 375 mg/m^2 intravenously on days 1, 8, 15, and 22 or at a fixed dose of 1000 mg 2 weeks apart. In a meta-analysis of 21 studies, the ORR for wAIHAs after rituximab was 79% (67% for primary and 72% for secondary).[57] The CR rate was 42% (32% primary and 46% secondary). The median time to response was 3 to 6 weeks with the highest CR rate after 2 to 4 months. Approximately half of the patients had concomitant steroids. Rituximab therapy results in good long-term response rates with only 30% of initial responders relapsing at 3 years. Low-dose rituximab at 100 mg/m^2 for 4 consecutive weeks also produced high response rates.[58,59]

Patients taking steroids before initiation of rituximab should continue on a low dose of steroids until response to the CD20 antibody. Predictors of long-term response are an achievement of a CR, conversion to a negative DAT result, and previous splenectomy.

Re-treatment of relapsing patients with rituximab is feasible and results in similar response rates. Rituximab has been given to children without apparent safety problems.

The caveats of rituximab therapy are that the drug is not licensed for the indication and its well-known side effects including infectious complications also apply for AIHA patients. Whenever possible, in a non-splenectomized patient, anti-pneumococcal vaccination should be performed at least 2 weeks before the first administration of rituximab.

Third-Line Treatment

Azathioprine

Efficacy has been reported in small series with response rates of 56% to 71%.[10] Azathioprine is dosed at 2 to 2.5 mg/kg/day (if thiopurine methyltransferase testing is available) or at a starting dose of 50 mg daily.

TABLE 47.5 Treatment Options for Primary and Secondary Warm Autoimmune Hemolytic Anemia and Cold Autoimmune Hemolytic Anemia

Disease or Condition	First Line	Second Line	Beyond Second Line	Last Resort
Primary AIHA	Steroids (+ rituximab)	Splenectomy Rituximab	Azathioprine, MMF, cyclosporine, cyclophosphamide	High-dose cyclophosphamide, bortezomib, alemtuzumab
B- and T-cell NHL	Steroids	Chemotherapy ± rituximab (splenectomy in SMZL)	Other anti-CD20 antibodies, ibrutinib	
Hodgkin lymphoma	Steroids	Chemotherapy	Rituximab if HL is in remission	
Solid tumors	Steroids Surgery			
Ovarian dermoid cyst	Ovariectomy			
SLE	Steroids	Rituximab	MMF Azathioprine	Autologous SCT
Ulcerative colitis	Steroids	Azathioprine		Total colectomy
CVID	Steroids + IgG replacement			
ALPS	Steroids	MMF	Sirolimus	
Wiskott-Aldrich syndrome	Steroids	Allogeneic SCT		
Allogeneic SCT	Steroids	Rituximab[a]	Splenectomy T-cell infusion	
Organ transplantation	Reduction of immunosuppression, steroids			
Drug induced	Withdrawal	Steroids		
Primary CAD	Protection from cold exposure	Rituximab	Bendamustine + rituximab	Sutimlimab, ecuzulimab[b] bortezomib[b]
PCH	Supportive treatment (postinfectious)	Rituximab[a] (chronic)		

[a] Early second-line treatment because of known poor response to steroids.
[b] Off-label use in single cases.
AIHA, Autoimmune hemolytic anemia; *ALPD*, autoimmune lymphoproliferative disorders; *ALPS*, autoimmune lymphoproliferative syndrome; *CAD*, cold agglutinin disease; *CVID*, common variable immune deficiency; *IgG*, immunoglobulin G; *MMF*, mycophenolate mofetil; *NHL*, non-Hodgkin lymphoma; *PCH*, paroxysmal cold hemoglobinuria; *SCT*, stem cell transplantation; *SLE*, systemic lupus erythematosus; *SMZL*, splenic marginal zone lymphoma.

Cyclosporine

At an initial dose of 2.5 mg/kg twice daily the response rate was 58% in all AIHA types.[60] Repeated cycles of high-dose cyclophosphamide (50 mg/kg/day for 4 days) remain an option in selected, highly refractory patients. In a pilot study, six out of nine patients received a CR with a median duration of 15 months or more.

Mycophenolate mofetil

At a starting dose of 500 mg twice daily with escalation to 1 g, varying response rates from 25% to 100% were observed in rather small series.[61]

Splenectomy

Splenectomy has been a good second-line option before the establishment of rituximab. CR or PR is achieved in two-thirds of patients (38% to 82%), depending on the percentage of secondary cases. In addition, there is good evidence that a substantial number of patients will remain in remission without the need for medical intervention for years. In the initial series by Chertkow and Dacie,[62] only 2 out of 28 patients were in remission for more than 5 years, but 6 patients remained in a stable PR for up to 7 years. In 52 splenectomized patients (percentage of primary AIHA unknown), Coon[63] found that 63% had a hematocrit of 30% or greater without steroids after a mean follow-up of 33 months, and 21% had a hematocrit of 30% or greater with a prednisone requirement of 15 mg/day or less after a mean follow-up of 73 months. In a study by Allgood and Chaplin,[64] 44% of patients were in CR after more than 1 year after splenectomy.

Splenectomy can safely be performed laparoscopically in almost all patients with primary AIHA with normal-sized spleens. The mortality rate of laparoscopic splenectomy was 0.5% in a large national study.

There is a lifelong increased risk of infections and venous thrombosis and a very small risk of pulmonary hypertension. The most serious, often fatal, but rare infectious complication is overwhelming pneumococcal septicemia. The risk of infections is highest shortly after splenectomy and decreases after 2 years. Although fatal postsplenectomy infections seem to be less common in recent years, it is mandatory to take all measures to reduce this risk. Vaccination for pneumococci, meningococci, and *Haemophilus influenzae* should be done before splenectomy. Pneumococcal vaccination should be repeated regularly. Long-term antibiotic prophylaxis is probably not required in adults, but patients should be informed about this risk and should take antibiotics immediately in case of unexplained fever. Risk in children may be reduced by subtotal splenectomy.

Treatment Options Beyond Third Line.

The proteasome inhibitor *bortezomib*, given in combination with dexamethasone, induced responses in children and adult patients with refractory wAIHA, although numbers are small.[65,66]

TABLE 47.6 **Second-Line Treatment Options After Steroids**

Treatment	Dosing and Application	Side Effects	Precautions
Rituximab	375 mg/m^2 on days 1, 8, 15, and 22 IV or 1000 mg fixed dose 2 weeks apart	Infusional reactions Serum sickness disease Late-onset neutropenia Infections	Premedication with antihistamines (and steroids) Consider prophylaxis against pneumocystis jiroveci pneumonia
Splenectomy (acute)	Preferentially laparoscopic	Infections, thrombosis	Postoperative thromboprophylaxis
Splenectomy (long term)	—	Infections Venous thrombosis	Vaccination, patient information
Danazol	200–400/day PO	Hepatotoxicity, hirsutism	None
Cyclophosphamide	PO or IV Dose adjusted to neutrophil count	Neutropenia Mutagenesis	Neutrophil count monitoring, bladder protection after high doses
Azathioprine	2.0–3 mg/kg/day PO Dose adjusted to neutrophil count	Neutropenia	Neutrophil count monitoring; avoid interaction with other drugs (e.g., allopurinol)
MMF	1–2 × 1 g/day PO	Gastrointestinal	
Cyclosporine	PO Dose adjusted to blood levels of CyA Target level, 200–400 ng/mL	Nephrotoxicity Gum hyperplasia hypertension	Monitoring of CyA levels and creatinine
Alemtuzumab	SC (variable doses)	Neutropenia	Anti-infectious prophylaxis
Complement inhibition with sutimlimab (CAD)	60 mg/kg after test dose of 10 mg/kg	Infections	Vaccination

CyA, Cyclosporine A; *IV*, intravenous; *MMF*, mycophenolate mofetil; *PO*, oral; *SC*, subcutaneous.

Danazol has shown some activity but seems to be less effective in refractory wAIHA.[67,68]

Response to *intravenous immunoglobulin* was reported in 12 of 37 patients at doses of 0.4 to 0.5 hg/kg in one retrospective study, but robust evidence of their efficacy in this setting are lacking and their use may enhance the risk of thrombosis.[69]

The results of *autologous stem-cell transplantation* were disappointing, but allogeneic transplantation may be used as a last-resort treatment in patients with Evans syndrome.

High-dose methyl-prednisolone, emergency splenectomy, partial splenic embolization, or plasma exchange may be used in emergency situations, but data are limited.

Novel agents in clinical studies. The tyrosine kinase inhibitors fostamatinib (syk) and INCB050465(PI-3kinase) have shown promising activity and are investigated in clinical studies.[36] Therapeutic antibodies against the neonatal FC-receptor (e.g., orilanolimab) showed promising effects.

With the advent of more novel agents, patients in need for third-line treatment should be referred to clinical studies.

Treatment of Secondary Warm Autoimmune Hemolytic Anemia

In secondary wAIHA, the treatment goal depends on the type and severity of the underlying disease. The treatment goal may vary from palliative treatment of AIHA, to cure by eradication of the underlying disease. Preferred treatment options are listed in Table 47.6.

Warm Autoimmune Hemolytic Anemia Associated With Systemic Lupus Erythematosus

Given its activity in both diseases, rituximab seems to be a reasonable option.[70] Increased side effects should be considered in patients with previous or concomitant immunosuppressive therapies.

Steroids used at a similar dosage as in primary wAIHAs induce high response rates. Concomitant treatment with hydroxychloroquine is advised, although its efficacy for AIHA as a steroid-sparing agent has not been proven. Maintenance treatment with azathioprine, cyclophosphamide may be required. Mycophenolate mofetil is an alternative. Splenectomy should be avoided due to a high infection and thrombosis rate especially if antiphospholipid antibodies are present.

Warm Autoimmune Hemolytic Anemia Associated With Chronic Lymphocytic Leukemia

In previously untreated CLL with isolated DAT-positive anemia but no other treatment indication, treatment with prednisone as in primary wAIHAs is the first choice.[71,72] Rituximab monotherapy has response rates of up to 71% but responses may not be sustained.[73,74] Combination of rituximab in combination with cyclophosphamide and dexamethasone produces response rates of over 80%[73] Cyclosporine also has good activity. In refractory AIHA or in AIHA with advanced or progressive CLL, treatment of the underlying disease with a rituximab-containing regimen—according to International

Workshop on Chronic Lymphocytic Leukemia guidelines—is recommended.[74] In general, fludarabine-containing regimens should be used with caution in the presence of a positive DAT result or overt AIHA. In this case, bendamustine with (or without) rituximab is a good alternative. There is increasing evidence that obinutuzumab, ibrutinib, or venetoclax are effective even in refractory wAIHA associated with CLL.[36,75] On the other hand, immune cytopenias may also occur during inhibitor treatment.[76]

Warm Autoimmune Hemolytic Anemia in Other Non-Hodgkin Lymphomas

AIHA in NHLs does not respond well to steroids in general and splenectomy is only effective in SMZLs. Sustained responses in most other lymphoma subtypes have been obtained with lymphoma-specific treatment with or without rituximab.

Drug-Related Warm Autoimmune Hemolytic Anemia

Cessation of the drug may be effective in many cases and further use of the drug should be avoided. Fludarabine-induced AIHA may be life threatening but responds to steroid therapy.

Treatment of Warm Autoimmune Hemolytic Anemia in Congenital and Acquired Immune Deficiency States and Special Serologic Types

The treatment of wAIHAs in patients with CVID is very similar to that of those with primary wAIHA. Additional regular prophylactic treatment with subcutaneous Ig reduces the risk of infection especially if rituximab is considered. Splenectomy is effective, but the risk of serious infections is increased in this setting.

In ALPS, treatment should be started with steroids and then switched to MMF, but sirolimus has the highest efficacy in improving not only AIHA but also lymphadenopathy. Both rituximab and splenectomy should be avoided as the risk of severe infections is much higher in this context. AIHA associated with IgM hypergammaglobulinemia in Wiskott-Aldrich syndrome is an indication for allogeneic stem cell transplantation.

Patients with AIHA after allogeneic stem cell transplantation respond poorly to steroids but often do respond to rituximab. An early switch to rituximab may be reasonable in these patients. AIHA in drug-related severely immunosuppressed patients after transplantation of solid organs responds best to reduction of immune suppression. Patients with IgM wAIHAs respond poorly to steroids. These patients are candidates for early rituximab treatment. The same is true for chronic AIHA caused by DL antibodies.

Treatment of Cold Autoimmune Hemolytic Anemia

Primary Chronic Cold Agglutinin Disease

Primary CAD is defined as a cAIHAs in patients with IgM-MGUS or lymphoma without overt clinical signs but with bone marrow infiltration. All patients should be advised to avoid cold exposure. Drug treatment is required in 30% to 50% of the patients.[77] Watchful waiting is indicated, but thromboprophylaxis is recommended in episodes of exacerbations or risk situations.

First-Line Therapy

Treatment is initiated in symptomatic patients or when Hb levels drop below 10 g/dL (Fig. 47.7). Because IgM-coated RBCs are mainly destroyed in the liver, cAIHA does not respond to splenectomy and responds poorly to steroids. The most effective and best evaluated treatment is rituximab in a standard lymphoma dose. Two studies have provided similar results. In a prospective phase II study, 20 out of 27 patients responded, but most responses (n=19) were partial. The median response duration was 11 months, but most patients responded to retreatment with rituximab. In a meta-analysis, the ORR for rituximab in CAD was 57% with a CR rate of 21%.[57] The Nordic group has reported excellent results for combinations of rituximab with fludarabine, but even better with bendamustine for CAD.[78–80]

Response to rituximab with bendamustine may take time but lasts for years in the majority of patients.

Second-Line Therapy

A combination of rituximab and oral fludarabine induced higher ORRs (76%) with a longer duration (median: 66 months). A prospective trial with single agent bortezomib showed the activity of this drug with 6 of 19 patients responding to a single cycle.[81]

While all previously mentioned treatments intend to eradicate or suppress the causative B-cell clone, complement plays a decisive role in the execution of hemolysis and can also be targeted. Initial studies with the terminal complement inhibitor ecuzulimab showed moderate responses.[82,83] The most striking results have been obtained with the C1s antibody sutimlimab.[84,85] Seven of 10 patients responded an increase in Hb of >2 g/dL. Responses with normalization of bilirubin, reticulocyte counts occurred within a few days, which makes this treatment also attractive for emergency situations. The disadvantage of complement inhibition is that this therapy is symptomatic and needs maintenance or rechallenge.[86] No major infectious complications have been observed in vaccinated patients. Other complement antibodies and inhibitors are being developed.

Emergency Situations and Further Treatment Lines

Blood transfusions may be necessary, particularly during the initial phase of treatment. The use of IVIg or plasma exchange can be considered. Given their efficacy in B-cell malignancies, inhibitors like ibrutinib (*BTK*) or venetoclax (*BCL2*) may not only be effective in secondary cold agglutinin syndrome.

Secondary Cold Agglutinin Syndrome

In cAIHAs associated with lymphomas or solid tumors, treatment of the underlying disease by chemo(immuno)therapy or curative resection results in good responses. Cases of infection-related cAIHAs usually resolve spontaneously, but antibiotic therapy may accelerate the process.

FUTURE DIRECTIONS

Future research should mainly be directed toward generation of better treatment options. The generation of international guidelines is still hampered by the lack of evidence. Every effort should be made to initiate valid comparisons of major treatment options such as steroids, splenectomy, and rituximab, potentially through registries or randomized trials.

Novel therapeutic options may be provided by next-generation anti-CD20 antibodies or antibodies against other targets such as complement factors. Insights into the way T cells drive and control the immune reaction could lead to novel immunotherapeutic approaches. Synthetic peptides modulating Th1 responses via regulatory T cells may be developed into therapeutic tools. Another option would be to target structures on macrophages responsible for RBC destruction (e.g., the CD47–SIRP-α interaction). After years of small studies and case reporting, a number of novel agents are now being studied prospectively in well powered phase II or even randomized phase III trial.

There is hope that novel targeted therapies will replace the current treatment of patients with AIHA.

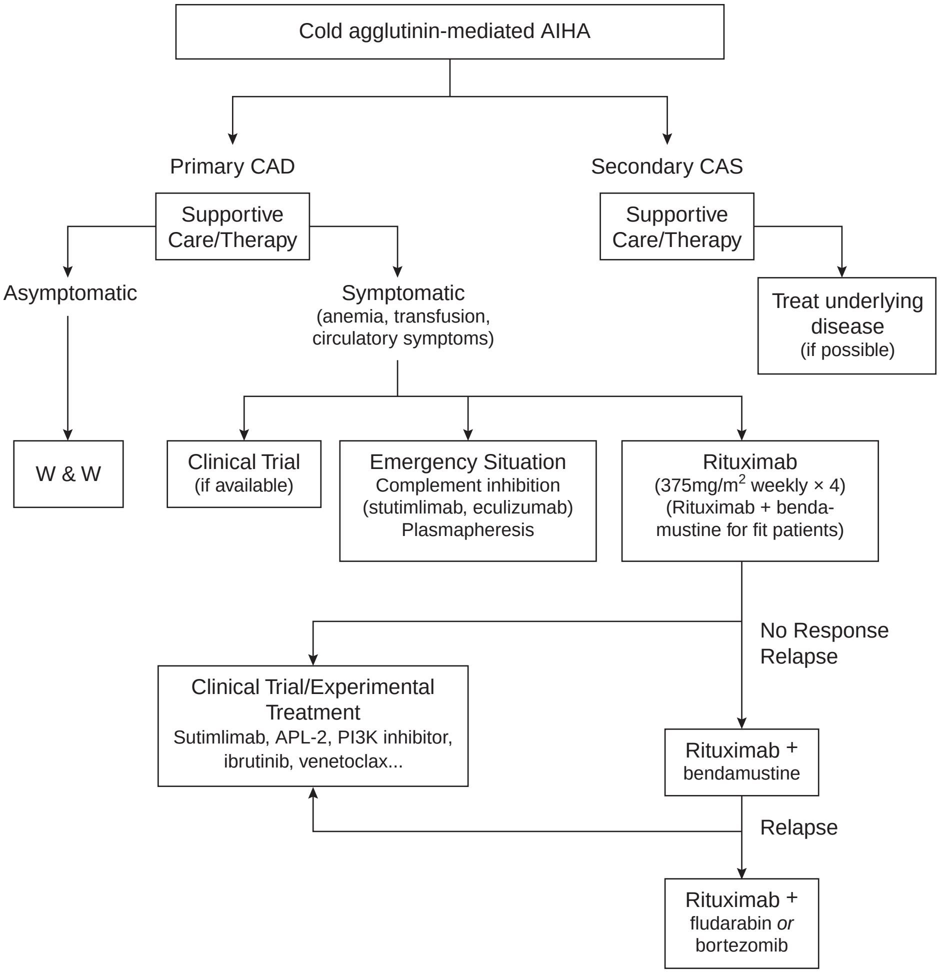

Cold agglutinin-mediated AIHA
Primary CAD
Secondary CAS
Supportive Care/Therapy
Supportive Care/Therapy
Asymptomatic
Symptomatic (anemia, transfusion, circulatory symptoms)
Treat underlying disease (if possible)
W & W
Clinical Trial (if available)
Emergency Situation Complement inhibition (stutimlimab, eculizumab) Plasmapheresis
Rituximab (375mg/m2 weekly × 4) (Rituximab + benda-mustine for fit patients)
No Response Relapse
Clinical Trial/Experimental Treatment Sutimlimab, APL-2, PI3K inhibitor, ibrutinib, venetoclax...
Rituximab + bendamustine
Relapse
Rituximab + fludarabin or bortezomib

Figure 47.7 PROPOSED ALGORITHM FOR THE TREATMENT OF PRIMARY COLD AGGLUTININ DISEASE. *W & W, Watch and wait. (Modified from Jäger U, et al. Diagnosis and treatment of autoimmune hemolytic anemia in adults: Recommendations from the First International Consensus Meeting. Blood Rev. 2020;41:100648.)*

ACKNOWLEDGMENTS

We are greatly indebted to our colleagues from the First International Consensus Meeting on Diagnosis and Treatment of AIHA: Barcellini W, Broome CM, Gertz MA, Hill A, Hill QA, Jilma B, Kuter DJ, Montillo M, Röth A, Zeerleder SS, and Berentsen S. Expert technical assistance by Michaela Bronhagl is much appreciated.

This chapter is in memory of Professor Klaus Lechner, author of the chapter in the sixth edition.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Aladjidi N, Leverger G, Leblanc T, et al. New insights into childhood autoimmune hemolytic anemia: a French national observational study of 265 children. *Haematologica*. 2011;96:655.

Barcellini W. New insights in the pathogenesis of autoimmune hemolytic anemia. *Transfus Med Hemother*. 2015;42:287.

Barcellini W, Fattizzo B, Zaninoni A, et al. Clinical heterogeneity and predictors of outcome in primary autoimmune hemolytic anemia: a GIMEMA study of 308 patients. *Blood*. 2014;124:2930.

Barcellini W, Zaninoni A, Fattizzo B, et al. Predictors of refractoriness to therapy and healthcare resource utilization in 378 patients with primary autoimmune hemolytic anemia from eight Italian reference centers. *Am J Hematol*. 2018;93:E243.

Barros MM, FBlajchman MA, Bordin JO Warm autoimmune haemolytic anemia: recent progress in understanding the immunobiology and the treatment. *Transfus Med Rev*. 2010;24:195.

Berentsen S. Role of complement in autoimmune hemolytic anemia. *Transfus Med Hemother*. 2015;42(5):303–310.

Berentsen S, Barcellini W, D'Sa S, et al. Cold agglutinin disease revisited: a multinational, observational study of 232 patients. *Blood*. 2020;136(4): 480–488.

Berentsen S, Randen U, Oksman M, et al. Bendamustine plus rituximab for chronic cold agglutinin disease: results of a Nordic prospective multicenter trial. *Blood*. 2017;130:537.

Berentsen S, Ulvestad E, Langholm R, et al. Primary chronic cold agglutinin disease: a population based clinical study of 86 patients. *Haematologica*. 2006;91:460.

Birgens H, Frederiksen H, Hasselbalch HC, et al. A phase III randomized trial comparing glucocorticoid monotherapy versus glucocorticoid and rituximab in patients with autoimmune haemolytic anaemia. *Br J Haematol*. 2013;163:393–399.

Brodsky RA. Complement in hemolytic anemia. *Blood*. 2015;126:2459.

Coon WW. Splenectomy in the treatment of hemolytic anemia. *Arch Surg*. 1985;120:625.

Dacie SJ. The immune haemolytic anaemias: a century of exciting progress in understanding. *Br J Haematol*. 2001;114:770.

D'Arena G, Laurenti L, Capalbo S, et al. Rituximab therapy for chronic lymphocytic leukemia-associated autoimmune hemolytic anemia. *Am J Hematol*. 2006;81:598.

Dearden C, Wade R, Else M, et al. The prognostic significance of a positive direct antiglobulin test in chronic lymphocytic leukemia: a beneficial effect of the combination of fludarabine and cyclophosphamide on the incidence of hemolytic anemia. *Blood*. 1820;2007:111.

Eaton WW, Rose NR, Kalaydjian A, et al. Epidemiology of autoimmune diseases in Denmark. *J Autoimmun*. 2007;29:1.

Garratty G. Immune hemolytic anemia associated with negative routine serology. *Semin Hematol*. 2005;42:156.

Garratty G. The James Blundell Award Lecture 2007: Do we really understand immune red cell destruction? *Transfusion Med*. 2008;18:321.

Go RS, Winters JL, Kay NE. How I treat autoimmune hemolytic anemia. *Blood*. 2017;129:2971.

Hill QA, Hill A, Berentsen S. Defining autoimmune hemolytic anemia: a systematic review of the terminology used for diagnosis and treatment. *Blood Adv*. 2019;3:1897.

Hill QA, Stamps R, Massey E, et al. The diagnosis and management of primary autoimmune haemolytic anaemia. *Br J Haematol*. 2017;176:395.

Jäger U, Barcellini W, Broome CM, et al. Diagnosis and treatment of autoimmune hemolytic anemia in adults: recommendations from the First International Consensus Meeting. *Blood Rev*. 2020;41:100648.

Jäger U, D'Sa S, Schörgenhofer C, et al. Inhibition of complement C1s improves severe hemolytic anemia in cold agglutinin disease: a first-in-human trial. *Blood*. 2019;133:893.

Lechner K, Jäger U. How I treat autoimmune haemolytic anemias in adults. *Blood*. 2010;116:1831.

Michel M, Chanet V, Dechartres A, et al. The spectrum of Evans syndrome in adults: new insight into the disease based on the analysis of 68 cases. *Blood*. 2009;114:3167.

Michel M, Terriou L, Roudot-Thoraval F, et al. A randomized and double-blind controlled trial evaluating the safety and efficacy of rituximab for warm auto-immune hemolytic anemia in adults (the RAIHA study). *Am J Hematol*. 2017;92:23.

Montillo M, O'Brien S, Tedeschi A, et al. RESONATE^{TM} study of ibrutinib vs ofatumumab in relapsed/refractory chronic lymphocytic leukemia/small lymphocytic lymphoma, including a case report. *Blood*. 2014;124:5654.

Mullins M, Jiang X, Bylsma LC, et al. Cold agglutinin disease burden: a longitudinal analysis of anemia, medications, transfusions, and health care utilization. *Blood Adv*. 2017;1:839.

Porpaczy E, Jäger U, How I manage autoimmune cytopenias in patients with lymphoid cancer. *Blood*. 2022;139(10):1479.

Protocole National de Diagnostic et de Soins (PNDS). Anémie Hémolytique Auto-Immune [French guidelines for AHAI in adults and children]. February 2017. Available at: www.has-sante.fr >jcms> anemie hemolytique auto-immune.

Reynaud Q, Durieu I, Dutertre M, et al. Efficacy and safety of rituximab in auto-immune hemolytic anemia: a meta-analysis of 21 studies. *Autoimmun Rev*. 2015;14:304.

Silberstein LE, Litwin S, Carmack CE. Relationship of variable region genes expressed by a human B cell lymphoma secreting pathologic anti-Pr2 erythrocyte autoantibodies. *J Exp Med*. 1989;169:1631.

Visco C, Barcellini W, Maura F, et al. Autoimmune cytopenias in chronic lymphocytic leukemia. *Am J Hematol*. 2014;89:1055.

REFERENCES

1. Dacie SJ. The immune haemolytic anaemias: a century of exciting progress in understanding. *Br J Haematol*. 2001;114:770.
2. Packman CH:. Hemolytic anemia due to warm autoantibodies. *Blood Rev*. 2008;22:17.
3. Garratty G. Immune hemolytic anemia—a primer. *Semin Hematol*. 2005;42:119.
4. Petz LD. Immune hemolysis associated with transplantation. *Semin Hematol*. 2005;42:145.
5. Pirofsky B. Clinical aspects of autoimmune hemolytic anemia. *Semin Hematol*. 1976;13:251.
6. Berentsen S, Ulvestad E, Langholm R, et al. Primary chronic cold agglutinin disease: a population based clinical study of 86 patients. *Haematologica*. 2006;91:460.
7. King KE, Ness PM:. Treatment of autoimmune hemolytic anemia. *Semin Hematol*. 2005;42:131.
8. Lechner K, Jäger U. How I treat autoimmune haemolytic anemias in adults. *Blood*. 2010;116:1831.
9. Aladjidi N, Leverger G, Leblanc T, et al. New insights into childhood autoimmune hemolytic anemia: a French national observational study of 265 children. *Haematologica*. 2011;96:655.
10. Barcellini W, Fattizzo B, Zaninoni A, et al. Clinical heterogeneity and predictors of outcome in primary autoimmune hemolytic anemia: a GIMEMA study of 308 patients. *Blood*. 2014;124:2930.
11. Hill QA, Stamps R, Massey E, et al. The diagnosis and management of primary autoimmune haemolytic anaemia. *Br J Haematol*. 2017;176:395.
12. Eaton WW, Rose NR, Kalaydjian A, et al. Epidemiology of autoimmune diseases in Denmark. *J Autoimmun*. 2007;29:1.
13. Hadjadj J, Aladjidi N, Fernandes H, et al. Pediatric Evans syndrome is associated with a high frequency of potentially damaging variants in immune genes. *Blood*. 2019;134(1):9.
14. Michel M, Chanet V, Dechartres A, et al. The spectrum of Evans syndrome in adults: new insight into the disease based on the analysis of 68 cases. *Blood*. 2009;114:3167.
15. Bylsma LC, Gulbech Ording A, Rosenthal A, et al. Occurrence, thromboembolic risk, and mortality in Danish patients with cold agglutinin disease. *Blood Adv*. 2019;3(20):2980–2985.
16. Brodsky RA. Complement in hemolytic anemia. *Blood*. 2015;126:2459.
17. Berentsen S. Role of complement in autoimmune hemolytic anemia. *Transfus Med Hemother*. 2015;42:303.
18. Garratty G. The James Blundell Award Lecture 2007: Do we really understand immune red cell destruction? *Transfusion Med*. 2008;18:321.
19. Arndt PA, Leger RM, Garratty G. Serologic findings in autoimmune hemolytic anemia associated with immunoglobulin M warm autoantibodies. *Transfusion*. 2009;49:2352.
20. Sokol RJ, Booker DJ, Stamps R, et al. Autoimmune hemolytic anemia caused by warm-reacting IgM-class antibodies. *Immunohematol*. 1998;14:53.
21. Barcellini W. New insights in the pathogenesis of autoimmune hemolytic anemia. *Transfus Med Hemother*. 2015;42:287.
22. Aladjidi N, Jutand MA, Beaubois C, Fernandes H, Jeanpetit J, Coureau G, et al. Reliable assessment of the incidence of childhood autoimmune hemolytic anemia. *Pediatr Blood Cancer*. 2017;64(12).
23. Barcellini W, Zaja F, Zaninoni A, et al. Low-dose rituximab in adult patients with idiopathic autoimmune hemolytic anemia: clinical efficacy and biologic studies. *Blood*. 2012;119:3691–3697.
24. Calkins CE. Regulatory T cells essential to prevent the loss of self-tolerance in murine models of erythrocyte-specific autoantibody responses. *Immunol Res*. 2011;51(134):25.
25. Barros MM, Blajchman MA, Bordin JO. Warm autoimmune haemolytic anemia: recent progress in understanding the immunobiology and the treatment. *Transfus Med Rev*. 2010;24:195.
26. Fagiolo E, Toriani-Terenzi C. Th1 and Th2 cytokine modulation by IL-10/IL-12 imbalance in autoimmune haemolytic anaemia (AIHA). *Autoimmunity*. 2002;35:39.
27. Hall AM, Zamzami OM, Whibley N, et al. Production of the effector cytokine interleukin-17, rather than interferon-γ, is more strongly associated with autoimmune hemolytic anemia. *Haematologica*. 2012;97:1494.
28. Mahévas M, Michel M, Vingert B, et al. Emergence of long-lived autoreactive plasma cells in the spleen of primary warm auto-immune hemolytic anemia patients treated with rituximab. *J Autoimmun*. 2015;62:22.
29. Silberstein LE, Litwin S, Carmack CE. Relationship of variable region genes expressed by a human B cell lymphoma secreting pathologic anti-Pr2 erythrocyte autoantibodies. *J Exp Med*. 1989;169:1631.
30. Arthold C, Skrabs C, Mitterbauer-Hohendanner G, et al. Cold antibody autoimmune hemolytic anemia and lymphoproliferative disorders: a retrospective study of 20 patients including clinical, hematological and molecular findings. *Wien Klin Wochenschr*. 2014;126:376.
31. Michaux L, Dierlamm J, Wlodarska L, et al. Trisomy 3q11–q29 is recurrently observed in B-cell non-Hodgkin's lymphomas associated with cold agglutinin syndrome. *Ann Hematol*. 1998;76:201.
32. Ramagopalan SV, Wotton CJ, Handel AE, et al. Risk of venous thromboembolism in people admitted to hospital with selected immune-mediated diseases: record-linkage study. *BMC Med*. 2011;9:1.
33. Garratty G. Immune hemolytic anemia associated with negative routine serology. *Semin Hematol*. 2005;42:156.
34. Petz LD, Garraty G. *Immune Haemolytic Anemias*. 2nd ed. Philadelphia, PA: Churchill Livingstone; 2004.
35. Mayer B, Yürek S, Kiesewetter H, et al. Mixed-type autoimmune hemolytic anemia: differential diagnosis and a critical review of reported cases. *Transfusion*. 2008;48:2229.
36. Jäger U, Barcellini W, Broome CM, et al. Diagnosis and treatment of autoimmune hemolytic anemia in adults: recommendations from the First International Consensus Meeting. *Blood Rev*. 2020;41:100648.
37. Berentsen S. Role of complement in autoimmune hemolytic anemia. *Transfus Med Hemother*. 2015;42(5):303–310.
38. Teachey DT, Seif AE, Grupps SA. Advances in the management and understanding of autoimmune lymphoproliferative syndrome (ALPS). *Br J Haematol*. 2010;148:205.
39. Kokori SI, Ioannidis JP, Voulgarelis M, et al. Autoimmune hemolytic anemia in patients with systemic lupus erythematosus. *Am J Med*. 2000;108:198.
40. Gómez-Puerta JA, Martín H, Amigo MC, et al. Long-term follow-up in 128 patients with primary antiphospholipid syndrome: do they develop lupus? *Medicine (Baltimore)*. 2005;84:225.
41. Giannadaki E, Potamianos S, Roussomoustakaki M, et al. Autoimmune hemolytic anemia and positive Coombs test associated with ulcerative colitis. *Am J Gastroenterol*. 1997;92(10):1872–1874.
42. Uzzan M, Galicier L, Gornet JM, et al. Autoimmune cytopenias associated with inflammatory bowel diseases: insights from a multicenter retrospective cohort. *Dig Liver Dis*. 2017;49:397.
43. Sanz J, Arriaga F, Montesinos P, et al. Autoimmune haemolytic anemia following allogeneic hematopoietic stem cell transplantation in adult patients. *Bone Marrow Transplant*. 2007;39:555.
44. Dearden C, Wade R, Else M, et al. The prognostic significance of a positive direct antiglobulin test in chronic lymphocytic leukemia: a beneficial effect of the combination of fludarabine and cyclophosphamide on the incidence of hemolytic anemia. *Blood*. 2007;111:1820.
45. Chao TC, Chen CY, Yang YH, et al. Chronic hepatitis C virus infection associated with primary warm-type autoimmune hemolytic anemia. *J Clin Gastroenterol*. 2001;33:232.
46. Lazarian G, Quinquenel A, Bellal M, et al. Autoimmune haemolytic anaemia associated with COVID-19 infection. *Br J Haematol*. 2020;190(1):29–31.
47. Go RS, Winters JL, Kay NE. How I treat autoimmune hemolytic anemia. *Blood*. 2017;129:2971.
48. Protocole National de Diagnostic et de Soins (PNDS). Anémie Hémolytique Auto-Immune. [French guidelines for AHAI in adults and children.] February 2017. Available at: www.has-sante.fr >jcms> anemie hemolytique auto-immune.
49. Hill QA, Hill A, Berentsen S. Defining autoimmune hemolytic anemia: a systematic review of the terminology used for diagnosis and treatment. *Blood Adv*. 2019;3:1897.
50. Barcellini W, Zaninoni A, Fattizzo B, et al. Predictors of refractoriness to therapy and healthcare resource utilization in 378 patients with primary autoimmune hemolytic anemia from eight Italian reference centers. *Am J Hematol*. 2018;93:E243.
51. Fattizzo B, Michel M, Zaninoni A, et al. Efficacy of recombinant erythropoietin in autoimmune haemolytic anaemia: a multicentre international study. *Haematologica*. 2021;106(2):622–625.
52. Kulpa J, Skrabs C, Simanek R, et al. Probability of remaining in unsustained complete remission after steroid therapy withdrawal in patients with primary warm-antibody reactive autoimmune hemolytic anemia. *Wien Klin Wochenschr*. 2016;128:234.
53. Birgens H, Frederiksen H, Hasselbalch HC, et al. A phase III randomized trial comparing glucocorticoid monotherapy versus glucocorticoid and rituximab in patients with autoimmune haemolytic anaemia. *Br J Haematol*. 2013;163:393–399.
54. Michel M, Terriou L, Roudot-Thoraval F, et al. A randomized and double-blind controlled trial evaluating the safety and efficacy of rituximab for

warm auto-immune hemolytic anemia in adults (the RAIHA study). *Am J Hematol*. 2017;92:23.

55. Dierickx D, Kentos A, Delannoy A. The role of rituximab in adults with warm antibody autoimmune hemolytic anemia. *Blood*. 2015;125:3223.
56. Reynaud Q, Durieu I, Dutertre M, et al. Efficacy and safety of rituximab in auto-immune hemolytic anemia: a meta-analysis of 21 studies. *Autoimmun Rev*. 2015;14:304.
57. D'Arena G, Laurenti L, Capalbo S, et al. Rituximab therapy for chronic lymphocytic leukemia-associated autoimmune hemolytic anemia. *Am J Hematol*. 2006;81:598.
58. Fattizzo B, Zaninoni A, Pettine L, et al. Low-dose rituximab in autoimmune hemolytic anemia: 10 years after. *Blood*. 2019;133:996.
59. Roumier M, Loustau V, Guillaud C, et al. Characteristics and outcome of warm autoimmune haemolytic anemia in adults: new insights based on a single-center experience with 60 patients. *Am J Hematol*. 2014;89:E150.
60. Emilia G, Messora C, Longo G, Bertesi M. Long-term salvage treatment by cyclosporin in refractory autoimmune haematological disorders. *Br J Haematol*. 1996;93:341.
61. Howard J, Hoffbrand AV, Prentice HG, Mehta A. Mycophenolate mofetil for the treatment of refractory auto-immune haemolytic anaemia and auto-immune thrombocytopenia purpura. *Br J Haematol*. 2002;117:712.
62. Chertkow G, Dacie JV. Results of splenectomy in auto-immune haemolytic anaemia. *Br J Haematol*. 1956;2:237.
63. Coon WW. Splenectomy in the treatment of hemolytic anemia. *Arch Surg*. 1985;120:625.
64. Allgood JW, Chaplin Jr. H. Idiopathic acquired autoimmune hemolytic anemia. A review of forty-seven cases treated from 1955 through 1965. *Am J Med*. 1967;43:254.
65. Ratnasingam S, Walker PA, Tran H, et al. Bortezomib-based antibody depletion for refractory autoimmune hematological diseases. *Blood Adv*. 2016;1:31.
66. Fadlallah J, Michel M, Crickx E, et al. Bortezomib and dexamethasone, an original approach for treating multi-refractory warm autoimmune haemolytic anaemia. *Br J Haematol*. 2019;187:124.
67. Ahn YS, Harrington WJ, Mylvaganam R, et al. Danazol therapy for autoimmune hemolytic anemia. *Ann Intern Med*. 1985;102:298.
68. Ahn YS. Efficacy of danazol in hematologic disorders. *Acta Haematol*. 1990;84:122.
69. Flores G, Cunningham-Rundles C, Newland AC, Bussel JB. Efficacy of intravenous immunoglobulin in the treatment of autoimmune hemolytic anemia: results in 73 patients. *Am J Hematol*. 1993;44:237.
70. Serris A, Amoura Z, Canoui-Poitrine F, et al. Efficacy and safety of rituximab for systemic lupus erythematosus-associated immune cytopenias: a multicenter retrospective cohort study of 71 adults. *Am J Hematol*. 2018;93:424.
71. Rogers KA, Woyach JA. Secondary autoimmune cytopenias in chronic lymphocytic leukemia. *Semin Oncol*. 2016;43:300.
72. Visco C, Barcellini W, Maura F, et al. Autoimmune cytopenias in chronic lymphocytic leukemia. *Am J Hematol*. 2014;89:1055.
73. Rossignol J, Michallet AS, Oberic L, et al. Rituximab-cyclophosphamide-dexamethasone combination in the management of autoimmune cytopenias associated with chronic lymphocytic leukemia. *Leukemia*. 2011;25:473.
74. Hallek M, Cheson BD, Catovsky D, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood*. 2008;111:5446.
75. Montillo M, O'Brien S, Tedeschi A, et al. RESONATE™ study of ibrutinib vs ofatumumab in relapsed/refractory chronic lymphocytic leukemia/small lymphocytic lymphoma, including a case report. *Blood*. 2014;124:5654.
76. Rogers KA, Ruppert AS, Bingman A, et al. Incidence and description of autoimmune cytopenias during treatment with ibrutinib for chronic lymphocytic leukemia. *Leukemia*. 2016;30:346.
77. Mullins M, Jiang X, Bylsma LC, et al. Cold agglutinin disease burden: a longitudinal analysis of anemia, medications, transfusions, and health care utilization. *Blood Adv*. 2017;1:839.
78. Berentsen S, Randen U, Vågan AM, et al. High response rate and durable remissions following fludarabine and rituximab combination therapy for chronic cold agglutinin disease. *Blood*. 2010;2010:3180.
79. Berentsen S, Randen U, Oksman M, et al. Bendamustine plus rituximab for chronic cold agglutinin disease: results of a Nordic prospective multicenter trial. *Blood*. 2017;130:537.
80. Berentsen S, Barcellini W, D'Sa S, et al. Cold agglutinin disease revisited: a multinational, observational study of 232 patients. *Blood*. 2020;136(4): 480–488.
81. Rossi G, Gramegna D, Paoloni F, et al. Short course of bortezomib in anemic patients with relapsed cold agglutinin disease: a phase 2 prospective GIMEMA study. *Blood*. 2018;132:547.
82. Gupta N, Wang ES. Long-term response of refractory primary cold agglutinin disease to eculizumab therapy. *Ann Hematol*. 2014;93:343.
83. Röth A, Bommer M, Hüttmann A, et al. Complement inhibition with eculizumab in patients with cold agglutinin disease (CAD): results from a prospective phase II trial (DECADE trial). *Blood*. 2015;126:274.
84. Shi J, Rose EL, Singh A, et al. TNT003, an inhibitor of the serine protease C1s, prevents complement activation induced by cold agglutinins. *Blood*. 2014;123:4015.
85. Jäger U, D'Sa S, Schörgenhofer C, et al. Inhibition of complement C1s improves severe hemolytic anemia in cold agglutinin disease: a first-in-human trial. *Blood*. 2019;133:893.
86. Gelbenegger G, Schoergenhofer C, Derhaschnig U, et al. Inhibition of complement C1s in patients with cold agglutinin disease: lessons learned from a named patient program. *Blood Adv*. 2020;4:997.

CHAPTER 48

EXTRINSIC NONIMMUNE HEMOLYTIC ANEMIAS

William C. Mentzer and Stanley L. Schrier†

By definition, extrinsic causes of hemolysis are abnormalities in the environment in which the red blood cells (RBCs), usually normal themselves, circulate. These abnormalities can be acute or chronic in nature. They can arise from congenital lesions but usually result from acquired insults. Inherited anomalies of glucose-6-phosphate dehydrogenase (G6PD) deficiency, which reduces the RBCs' ability to deal with oxidative stressors, can leave RBCs more vulnerable to a variety of environmental insults. Determination of hemolysis with various levels of compensation as the cause of an anemia is accomplished using the approaches described in Chapter 34. Signs of extrinsic hemolysis with minimal or no anemia can be valuable clues to diseases of other organ systems. Among the most important forms of extrinsic hemolytic anemia are those caused by immune mechanisms; these are discussed in Chapter 46.

Extrinsic triggers of red cell destruction can lead to either intravascular or extravascular hemolysis or both. In general, only the most devastating damage leads to direct intravascular destruction. Usually, the initial insult leads to an eventual change in the external portion of the RBC membrane, which causes macrophages to retard, hold, remove, or otherwise modify RBCs. Infection or inflammation can activate these macrophages. Some RBC changes are accompanied by a decrease in RBC deformability, which retards flow and thereby facilitates the action of macrophages on the affected RBC. All of these changes lead to extravascular hemolysis.

FRAGMENTATION HEMOLYSIS: MICROANGIOPATHY

Clinical Manifestations

Patients present with various degrees of hemolytic anemia and compensation, with evidence of RBC fragmentation on smear (Fig. 48.1; see the box on Differential Diagnosis of Extrinsic Nonimmune Hemolytic Anemias). RBC removal is generally extravascular, with minimal or moderately decreased levels of haptoglobin. If RBC damage is sufficiently severe, signs of intravascular hemolysis may be present. Because of the underlying pathology, some of these syndromes show evidence of platelet removal, leading to thrombocytopenia. Occasionally, the underlying cause produces activation and depletion of procoagulant factors with consequent activation of the fibrinolytic system, consistent with disseminated intravascular coagulation (DIC; see the box on Causes of Red Blood Cell Fragmentation Hemolysis).

DIFFERENTIAL DIAGNOSIS OF EXTRINSIC NONIMMUNE HEMOLYTIC ANEMIAS

There is no simple approach to the differential diagnosis of hemolysis caused by extrinsic nonimmune hemolytic anemia. The physician must pay close attention to the clinical finding. Useful clues come from a determination of whether red blood cell (RBC) breakdown is predominantly extravascular or intravascular, but most important in the analysis is the observation of RBC morphology, which can focus the differential diagnosis. Unhelpful terms such as *aniso* and *poik* should be discarded. RBCs are spherocytic, stomatocytic, fragmented, echinocytic, acanthocytic, spurred, or bite cells, or can be mixtures of these types.

CAUSES OF RED BLOOD CELL FRAGMENTATION HEMOLYSIS

- Damaged microvasculature
- Thrombotic thrombocytopenic purpura–hemolytic uremic syndrome (TTP–HUS)
- Associated with pregnancy: preeclampsia or eclampsia; hemolysis plus elevated liver enzymes plus low platelets (HELLP syndrome)
- Associated with malignancy, with or without mitomycin C treatment
- Vasculitis: polyarteritis, Wegener granulomatosis, acute glomerulonephritis, or *Rickettsia*-like infections
- Systemic lupus erythematosus
- Abnormalities of renal vasculature: malignant hypertension, acute glomerulonephritis, scleroderma, or allograft rejection with or without cyclosporine treatment
- Disseminated intravascular coagulation
- Malignant hypertension
- Catastrophic antiphospholipid antibody syndrome
- Atrioventricular malformations
- Kasabach-Merritt syndrome
- Hemangioendotheliomas
- Atrioventricular shunts for congenital and acquired conditions (e.g., stents, coils, transjugular intrahepatic portosystemic shunt, Levine shunts)
- Cardiac abnormalities
- Replaced valve, prosthesis, graft, or patch
- Aortic stenosis or regurgitant jets (e.g., in ruptured sinus of Valsalva)
- Drugs: cyclosporine, mitomycin, ticlopidine, clopidogrel, tacrolimus, or cocaine
- Systemic infection: bacterial endocarditis, brucellosis, cytomegalovirus, HIV, ehrlichiosis, Rocky Mountain spotted fever.

HELLP, Hemolysis, elevated liver enzymes, and low platelet count; *HIV*, human immunodeficiency virus.

Pathophysiology

Fragmentation hemolysis occurs when mechanical forces disrupt the physical integrity of the RBC membrane. In vitro shear stresses in excess of 3000 dynes/cm^2 cause RBC fragmentation. For example, in vivo studies in patients with mitral prosthetic regurgitation and hemolysis show high peak shear stresses of 4500 dynes/cm^2, very rapid acceleration or deceleration, or both. Valvular abnormalities or prosthetic vales can thus cause microangiopathic changes.

Research suggests additional mechanisms of producing microangiopathic hemolysis that involve platelets and small vessel thrombi. The platelet-rich, fibrin-poor microvascular thrombi found in many patients with thrombotic thrombocytopenic purpura (TTP) (see Chapter 134) now are thought to be caused by abnormally decreased ADAMTS 13 activity.[1,2] This metalloprotease is responsible for converting the highly thrombogenic ultra-large multimers of von Willebrand factor made by platelets and endothelial cells into the smaller forms normally found in circulation. Mutations in or antibodies against ADAMTS 13 result in unusually large multimers of von Willebrand factor attached to endothelial cell surfaces, where platelets may excessively aggregate, leading to the formation of microvascular thrombi even in the absence of endothelial damage. In the case of disseminated cancer, the cause of microangiopathy may be microvascular tumor emboli.

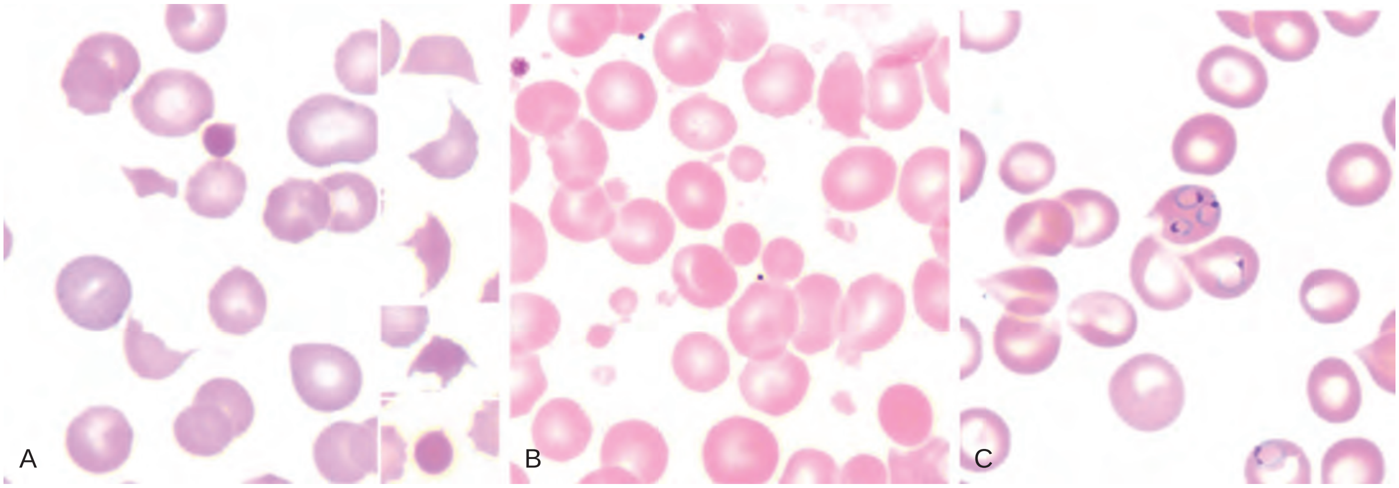


Figure 48.1 PERIPHERAL BLOOD SMEARS FROM EXAMPLES OF EXTRINSIC NONIMMUNE HEMOLYTIC ANEMIA. (A) Microangiopathic hemolytic anemia. Note the schistocytes, fragmented cells, spherocyte, and polychromasia. More examples of damaged red blood cells (RBCs), including classic "helmet cell" *(top)*, are seen to the immediate *right insert*. (B) Thermal injury from a burn. Thermally damaged RBCs form numerous microspherocytes and tiny RBC fragments. (C) Malaria infestation. RBCs containing *Plasmodium falciparum* malaria. Note the high rate of infestation, the presence of only ringed forms, and the multiply infested RBCs *(center)*.

Whatever the mechanism of mechanical trauma, the RBC membrane is viscoelastic and has self-sealing properties (see Chapter 45) so that little hemoglobin leaks out as the cell is being cut. However, prolonged distortion of the membrane produces a plastic change; therefore, the smaller RBC fragments usually do not become microspheres or microdisks but continue to display evidence of the shearing event or distortion in the form of typical irregular shapes. These irregular shapes and the rigidity that they reflect subsequently interfere with the ability of RBCs to fold, elongate, and deform sufficiently to pass through 3-μm capillaries and even smaller slits in the walls of the sinusoids of the reticuloendothelial system. This sequence leads to their destruction.

Differential Diagnosis

Generally, the differential diagnosis of fragmentation hemolysis can be deduced from the clinical findings. The presence of a prosthetic heart valve or a regurgitant jet that fragments or accelerates (i.e., Waring blender syndrome) can be readily discerned.[3] The clinical picture of thrombotic thrombocytopenic purpura and hemolytic uremic syndrome (TTP–HUS) is generally dramatic and acute (see Chapter 134). Atrioventricular malformations may be associated with disseminated intravascular coagulation (DIC) and platelet removal; the diagnosis requires a high index of suspicion and imaging studies. The presence of preeclampsia in a pregnant woman with microangiopathic hemolysis usually is obvious, but hemolysis plus elevated liver enzymes plus low platelet count (HELLP) syndrome is a serious complication of pregnancy that can occur without other signs of preeclampsia or hypertension. This syndrome can produce hepatic rupture, visual failure, DIC, seizures, and congestive heart failure and requires treatment by prompt delivery of the fetus. Cancer can be an underlying cause of microangiopathy. Vessels supplying malignant tumors are thought to be structurally abnormal. They exhibit the same sort of fibrin stranding that produces fragmentation hemolysis in DIC and TTP–HUS.

Continued use of invasive diagnostic and therapeutic procedures with insertion of foreign bodies into the circulation has been complicated by microangiopathic hemolysis. A transjugular intrahepatic portosystemic shunt can cause the syndrome in approximately 10% of patients. The hemolysis usually disappears after 12 to 15 weeks. Similarly, the use of coil embolization to seal off a patent ductus arteriosus may also cause significant hemolytic anemia. Vasculitis has also been implicated as a cause.

Multiple drugs are associated with microangiopathic hemolysis, most commonly quinine.[3,4] A recent review found that in only 22 of 78 drugs reported to produce drug-induced thrombotic microangiopathy was a definite association found.[4] Cyclosporine, tacrolimus, and mitomycin C have been implicated as causing a HUS picture that typically develops within weeks to months of exposure. Total body irradiation and bone marrow transplantation also are associated with microangiopathic hemolysis. Both chemotherapeutic agents and targeted cancer agents, including immunotoxins, monoclonal antibodies, and tyrosine kinase inhibitors, are associated with thrombotic microangiopathy.[5] The thienopyridines ticlodipine and clopidogrel are both capable of producing a significant thrombotic microangiopathy that differs somewhat in presentation. Ticlodipine-associated TTP typically occurs between 2 and 12 weeks after initiation of therapy and presents with severe thrombocytopenia, microangiopathic hemolytic anemia, highly elevated lactate dehydrogenase, and normal renal function and is associated with severe deficiency of plasma ADAMTS13 activity.[6] In contrast, clopidogrel-associated TTP usually presents within 2 weeks of drug initiation and is associated with mild thrombocytopenia, microangiopathic hemolytic anemia, mildly elevated lactate dehydrogenase levels, marked renal insufficiency, and near-normal levels of ADAMTS13 activity. Other reported exposures associated with microangiopathic hemolytic anemia include the use of cocaine and the herb Echinacea. The mechanisms of drug-induced thrombotic microangiopathy are not well understood but include immune-mediated causes (as in the case of quinine) and direct toxicity to the endothelium.[4]

Thrombotic microangiopathic hemolytic anemia can also be the presenting feature of severe, systemic infection, including viral (cytomegalovirus [CMV], HIV), fungal, and bacterial infections.[7] Whether infection "triggers" the development of TTP or instead the presentation remains debatable.

In one large series, 10 of 351 (2.8%) patients diagnosed with TTP were subsequently diagnosed with disseminated malignancy.[8] Symptoms suggesting an underlying malignancy include dyspnea, cough, atypical pain, and poor response to plasma exchange. The diagnosis was made by bone marrow biopsy in six of the 10 patients, and all patients died shortly after the diagnosis of malignancy was made.

A current review highlights four hereditary and four acquired disorders that lead to thrombotic microangiopathy.[2] TTP may be hereditary, due to mutations in ADAMTS13, or acquired, due to autoantibody inhibition of ADAMTS13 activity. In addition, complement mutations causing uncontrolled activation of the alternative pathway, mutations in components of cobalamin metabolism, and mutations in a protein kinase C-associated protein, diacylglycerol kinase, are other hereditary causes of thrombotic microangiopathy. Acquired causes in addition to autoantibodies to ADAMTS13 include shiga toxin (hemolytic uremic syndrome), drug mediated on an immune basis (i.e., quinine), drug mediated on a toxic, dose-related basis (i.e., gemcitabine, cyclosporine), and acquired antibodies to complement factor H.

Therapy

Management is primarily directed toward the underlying disease or event. Compensation of RBC production should be optimized by replacing iron or folic acid if the patient is deficient in these nutrients. Occasionally, removal or repair of a damaged native or prosthetic heart valve is necessary when the hemolysis produces a disabling transfusion requirement. Treatment of TTP with plasma exchange is highly effective in classic TTP with very low ADAMTS13 levels but not indicated in other settings of thrombotic microangiopathy such as cancer where plasma exchange may be ineffective. Where autoantibodies to ADAMTS13 are central to pathogenesis, immunosuppressive treatment with steroids and rituximab is important adjuvant therapy.[9]

OTHER FORMS OF MECHANICAL DAMAGE TO RED BLOOD CELLS

Heat Denaturation

Normal RBCs undergo budding and fragmentation when exposed to a temperature of 49°C (120°F) in vitro (see Fig. 48.1B). In some of the hereditary hemolytic anemias, this process occurs at temperatures as low as 46°C (115°F; see Chapter 45). Under some clinical circumstances, temperatures sufficient to cause heat denaturation of RBCs have been generated. Occasionally, cell warmers used with transfusions in cold agglutinin disease have malfunctioned and cooked the RBCs about to be transfused. In one case, a patient's mother warmed the RBCs with a hot water bottle, reasoning that such cells would cause less vein irritation to her child. Such transfusion was followed by evidence of intravascular and extravascular hemolysis, and the peripheral smear showed RBC budding and fragmentation (Fig. 48.2). Presumably, similar events can lead to hemolysis in patients who have sustained very extensive burns. In patients with heat stroke, the temperature usually is below 42°C (108°F), a temperature at which little RBC denaturation occurs.

Mechanical Trauma

The classic example of RBC damage caused by mechanical trauma is march hemoglobinuria, which occurs in soldiers after a long march, in joggers after running on a hard road, or in karate or conga drumming enthusiasts after practice. Anemia is rare, and reticulocytosis is uncommon. Evidence of typical intravascular RBC destruction is present and is thought to be caused by direct trauma to RBCs in the vessels of the feet or hands. Switching jogging paths or wearing better footwear often relieves the problem. Some cases show evidence of an underlying RBC membrane abnormality. Strenuous exercise may induce oxidant stress, as evidenced by increased levels of malonyldialdehyde, a marker of lipid peroxidation, in marathon runners after a race. Occasionally, malfunction of the cell savers used during abdominal or thoracic surgery mechanically injures RBCs.

Cardiopulmonary Bypass

Postperfusion syndrome occurs in some patients after cardiopulmonary bypass. The syndrome includes acute intravascular hemolysis and leukopenia as part of a febrile, inflammatory clinical picture. Affected patients may develop pulmonary distress and even adult or acute respiratory distress syndrome. Visible hemoglobinemia occurs, with rising plasma hemoglobin levels, and is associated with an increase in lysed RBC ghosts seen in the whole blood and plasma. These ghosts are coated with the complement complex C5bC9 (see Chapter 24). Presumably, the complement pathway is activated as the blood passes through the oxygenator. The reason why complement activation results in lytic attack on RBCs (and granulocytes) is unknown. Free hemoglobin released into the plasma secondary to intravascular hemolysis may contribute to acute kidney injury after cardiopulmonary bypass. Treatment involves knowledge of the process and requisite support until the situation corrects itself.

Osmotic Attack

Abrupt changes in osmolality can cause hemolysis. Freshwater drowning may be associated with so much water in the lungs that the RBCs swell as they undergo an in vivo osmotic fragility test in the pulmonary vasculature. Conversely, saltwater drowning can cause profound dehydration of RBCs, producing a situation analogous to xerocytosis (see Chapter 45). Rarely, acute hemolysis occurs from the mistaken infusion of or exposure to concentrated hypertonic solutions such as those used in hemodialysis. To manage such an event, the physician must recognize its cause, appreciate the shrunken RBCs on a peripheral smear, and restore isotonicity as quickly as possible. In these cases, the use of a hemodialysis device, if available, may be helpful.

Hypersplenism

In all organs of the monocyte–macrophage system (i.e., reticuloendothelial system), blood cells leaving the arterial bed are generally unloaded into channels such that the RBCs must pass through the

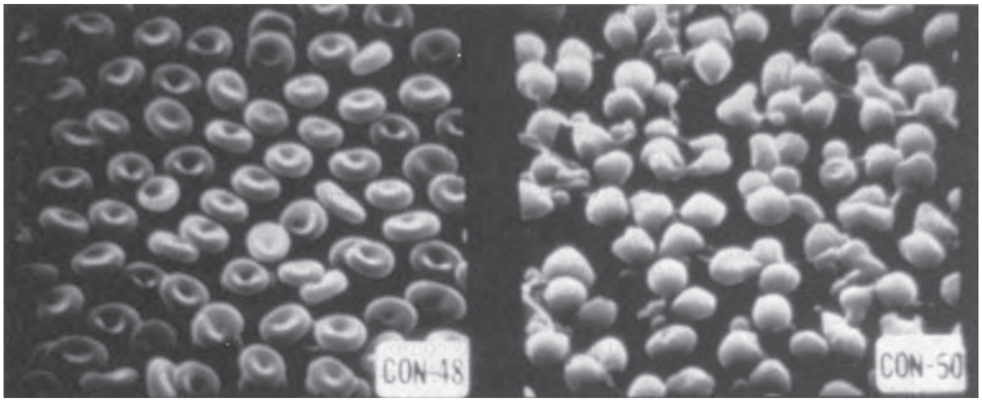


Figure 48.2 MORPHOLOGIC CHANGES ARE PRODUCED BY HEATING NORMAL RED BLOOD CELLS AT THE INDICATED TEMPERATURES. Budding begins abruptly at 50°C (122°F) and eventually leads to spherocytosis.

wall of the sinus to reenter the circulation. The sinusoidal wall in the spleen has slits 2 to 3 μm long and usually is endothelialized on one side and has a macrophagic lining on the other side. The normal human adult RBC is a discocyte with a surface area 40% larger than a sphere of that volume (see Chapter 33). This excess surface area allows an RBC with a diameter of approximately 8 μm to twist, elongate, and deform sufficiently to squeeze through these 2 to 3-μm slits. The excess surface area, occasionally referred to as the *ratio of surface area to volume* (SA:V), is critical and is normally approximately 1.4. Any condition that reduces SA:V reduces the ability of RBCs to traverse these sinusoidal slits because plump spheres cannot deform sufficiently.

Factors that interfere with interaction of the cytosol and the membrane also impair the ability of the RBC to deform. Oxidant attack may produce Heinz bodies that come to lie adjacent to the membrane. They interfere with the smooth movement of the membrane over the cytosol, a process called *tank treading*. Such cells are selectively blocked from leaving the splenic cords and entering the sinuses. Inflammation or infection may enhance the ability of splenic macrophages to attack and ingest RBCs. Although not strictly a mechanism of hypersplenism, Kupffer cell erythrophagocytosis is a prominent finding in patients undergoing graft-versus-host hemolysis seen after liver transplantation.

The spleen is more complicated than other reticuloendothelial organs in that the afferent arterioles pass through lymphoid nodules (i.e., white pulp) and then terminate in the cords of Billroth (i.e., red pulp), into which blood cells are discharged. In the slow flow of the cords of Billroth, blood cells are selectively attacked by macrophages and are in direct contact with several classes of lymphocytes. The blood cells must then pass through the cordal walls before they can approach the sinus wall, which they must pass through to reenter the circulation. The spleen provides a double filter, and the blood cells must be remarkably deformable to pass through it. This slow passage permits highly selective action by macrophages, which have receptors that can detect several sorts of alterations in these blood cells. These receptors include the Fc receptor for the appropriate portion of the Ig molecule, receptors for complement components such as C3b, and perhaps receptors that detect alterations in the outer portion of the phospholipid bilayer or in the externally oriented glycopeptides. The macrophage then holds, retards, modifies (i.e., pitting function), or removes (i.e., culling function) the blood cells identified. Normally, the pitting function of the spleen allows it to remove Howell–Jolly bodies and normally occurring endocytic vacuoles (called *pocks* because of their appearance on phase interference or Nomarski microscopy). The normal culling function of the spleen is exemplified by its removal of senescent RBCs.

All the activities of the spleen presumably are markedly accentuated in a large spleen, and if the increased activity is sufficiently extensive, hypersplenism ensues. The size of the spleen, not the portal pressure, is important in determining the degree of RBC sequestration. Other factors that may play a role are the state of activation of the splenic macrophages and the size of the small slits between the splenic cords and sinuses. The macrophages and slits seem to be under a degree of control, as evidenced by variations in splenic removal of RBCs in patients with malaria.

The clinical picture of hypersplenic hemolysis is dominated by the specific cause of the splenomegaly. Although the causes of splenomegaly are legion, there are several general mechanisms (Table 48.1). Usually some degree of anemia is seen, with evidence of a compensatory increase in RBC production. Because stasis and trapping in the spleen are associated with macrophagic attack and remodeling of the RBC surface, the reduction in SA:V leads to spherocytosis. If the RBCs undergo a prolonged period of distortion when traversing the cordal–sinus barrier, tailed RBCs will be present as the RBC membranes undergo a plastic change (see Chapter 45). Because the enlarged spleen can trap and remove platelets and white blood cells, variable thrombocytopenia and leukopenia may occur. The bone marrow may show normal to increased cellularity with erythroid hyperplasia.

Management depends on the cause of splenic enlargement. The anemia or pancytopenia usually is not profound; however, splenectomy may be contemplated if the anemia is severe. Alternatives to splenectomy include splenic embolization and high-intensity focused ultrasound ablation. In most situations, recognition of the possibility of hypersplenism is most important in guiding the approach to the diagnosis of an unexplained anemia. Massive splenomegaly is frequently associated with the expansion of the plasma compartment, and measurement of hemoglobin, hematocrit, or RBC levels may give a falsely low value of the RBC mass present. In that circumstance, the true RBC mass can be determined by ^{51}Cr assay.

TABLE 48.1 **Causes of Splenomegaly**

Cause	Example
Neoplasia	Lymphoma, hairy cell leukemia
Infection	Bacterial endocarditis, malaria, schistosomiasis, tuberculosis
Portal bed obstruction	Alcoholic cirrhosis, splenic vein thrombosis
Collagen vascular disease	Systemic lupus erythematosus, malignant phase of rheumatoid arthritis
Chronic inflammatory disease	Rheumatoid arthritis
Chronic hereditary or acquired hemolytic anemia	Severe β-thalassemia, autoimmune hemolytic anemia
Lipidosis	Gaucher disease
Amyloidosis	AL and AA types
Tropical splenomegaly syndrome	Hyperreactive malarial splenomegaly syndrome

A good example of massive splenomegaly causing plasma volume expansion is tropical splenomegaly syndrome, also known as *hyperreactive malarial splenomegaly syndrome*. Diagnostic criteria include massive splenomegaly more than 10 cm below the costal margin with no other cause identified; immunity to malaria; elevated serum IgM levels; and clinical response to treatment with antimalarial drugs such as chloroquine, proguanil, or pyrimethamine and folic acid. The pathophysiology of the splenomegaly seems to be poorly controlled B-lymphocytic production of antibodies, and IgM stimulation may be a response to malarial antigens or an unidentified mitogen. Malarial parasites are almost never found. The apparent anemia is in large part caused by plasma volume expansion, although RBC survival is reported to be slightly attenuated. Antimalarial therapy for several months reduces spleen size, so splenectomy is unnecessary.

Infection

Infection can cause hemolytic anemia via several pathophysiologic mechanisms (Table 48.2).

TABLE 48.2 **Mechanisms by Which Infection Can Cause Hemolysis**

Mechanism	Example
Direct parasitization of red cells	Malaria, babesiosis
Immune mechanisms	Cold agglutinin hemolysis after infectious mononucleosis or mycoplasmal pneumonia (see Chapter 46)
Induction of hypersplenism	Malaria, schistosomiasis
Altered red cell surface topology	*Haemophilus influenzae* infection
Release of toxins and enzymes	Clostridial infection causing thrombotic thrombocytopenic purpura–hemolytic uremic syndrome, *Escherichia coli* 0197, HIV infection

Parasite Infections

The classic example of direct parasitization is infection by *Plasmodium falciparum* (see Fig. 48.1C), *Plasmodium vivax*, or *Plasmodium malariae*. Infection with malaria, primarily *P. falciparum*, is a major health problem in the developing world, causing an estimated 300 to 500 million infections and 1 to 3 million deaths annually.[10] The burden of disease rests most heavily on young children and pregnant women. *Falciparum* malaria can cause a life-threatening anemia, with severe anemia defined as hemoglobin less than 5 g/dL associated with parasitemia and a normocytic blood film.[10] Malaria is primarily a disease of the tropical developing world but is still seen in the United States and its territories, primarily as an import from outside the United States. Of the 1298 cases reported in the United States in 2008, 117 (9%) were classified as severe, two of which were fatal. In each of the malarias, sporozoites injected by the mosquito in its saliva make their way to liver cells. After 1 to 2 weeks, they become merozoites, which burst out of the liver cells and into the bloodstream. Then, in a remarkable process, the parasite, by means of its apical end and related organelles called *rhoptries*, attaches to a specific receptor on the RBC surface. For *P. vivax*, the Duffy blood group antigen appears to be involved.

P. falciparum binds to sialic acid residues on the RBC surface that are on glycophorin A. After specific attachment, a convulsive movement occurs during which the RBC engulfs the parasite by a process resembling receptor-mediated endocytosis. Upon invasion of the RBC, the malarial parasite starts digesting the hemoglobin, depositing the undigested heme in the form of hemozoin. Knobs appear on the RBC surface, and the RBC becomes a sphere. Many proteins of parasitic origin are inserted into the RBC membrane, and some appear to cluster underneath these knobs. A parasite protein called *mature parasite-infected erythrocyte surface antigen* binds to membrane protein 4.1, and another parasite protein called *ring-infected erythrocyte surface antigen* binds to β spectrin. Both spectrin and protein 4.1 are integral components of the membrane skeleton, and the consequence of the binding of malarial proteins is RBC membrane stabilization. Thus stabilized, the parasitized RBC can continue to survive while the parasite continues to digest its contents.[11]

The parasite recruits the RBCs metabolic machinery, degrades and ingests hemoglobin, and grows, eventually bursting out of the RBC, and the cycle begins again. The RBCs are lysed intravascularly as a consequence of direct parasitic destruction and extravascularly as a consequence of changes in the splenic microvasculature and in the activation state of the monocyte–macrophage system. Treatment consists of the use of appropriate antimalarials and the support of erythropoiesis, including the use of RBC transfusion or exchange (or both) when indicated.

The anemia of malarial infections also involves mechanisms distinct from the lysis of parasitized RBCs. Erythropoiesis is suppressed, leading to the suppression of erythroid precursors and inadequate reticulocytosis during acute infection.[12] This inadequate hematopoietic response may be exacerbated by underlying iron deficiency, hemoglobinopathies, or concomitant infection (e.g., HIV), all potential contributors to the severity of anemia. In addition, loss of uninfected erythrocytes plays a major role in the development of anemia, with an 8- to 10-fold greater loss of unparasitized RBCs compared with parasitized cells. Uninfected RBCs have reduced deformability, and the degree of reduced deformability correlates with the severity of infection. Poorly deformable RBCs likely are cleared by the spleen. A recent study in rats indicates that CD8 T-cell–dependent parasite clearance in the spleen is involved in the damage of uninfected RBCs and their subsequent destruction.[13] Evidence of in vivo removal of immature malarial forms prompts the question of whether "uninfected" cells that are lost represent previously infected cells that nevertheless remain abnormal. Insertion of a merozoite rhoptry protein, ring surface protein 2, on RBCs in which infection was aborted has been implicated in the clearance of unparasitized erythrocytes and erythroid progenitors. In addition, uninfected RBCs demonstrate increased binding of Igs that are probably nonspecific immune complexes. Acute malarial infection, particularly with *P. falciparum*, also leads to alteration in the splenic function that incites premature destruction of uninfected RBCs.[14]

The lifespan of transfused RBCs is likewise decreased. ^{51}Cr-labeled normal RBCs infused into patients infected with malaria demonstrate a shorter lifespan than in normal control participants; this effect may persist after clearance of the parasitemia. Alternatively, parasites can be removed from RBCs along with the RBC membrane by the process of pitting, producing parasite-free spherocytes. This mechanism potentially explains the observed disparity between anemia and parasitemia.

Other infections that have somewhat similar pathophysiologies include Carrión disease (i.e., bartonellosis), in which a bite from the sandfly injects *Bartonella bacilliformis*, which attaches to the RBC surface of up to 80% of erythrocytes and causes lysis, leading to the massive hemolysis that characterizes acute infection. It appears that the invasion of RBCs partly depends on the flagella of *Bartonella* spp. Incubation with antiflagellin antiserum reduces invasion of RBCs. The bacteria also secrete deformin, a factor that leads to deep pitting on the surface of RBCs, presumably providing a portal of entry into the erythrocyte. There also may be a role for splenic clearance of infected cells, and prior splenectomy appeared to protect a patient from hemolysis during acute infection.

Babesia organisms also directly invade RBCs by mechanisms somewhat resembling those seen is in malaria,[15] producing fever and hemolytic anemia. The parasite is transmitted by ticks and transfusions of infected blood products[16] and can be transmitted vertically. Most tick-borne cases occur on the West Coast, particularly in Washington and California, and in the northern portion of the Midwest; cases occurring on the East Coast are concentrated in Massachusetts and Nantucket Island. However, transfusion-associated cases have been reported throughout the United States.[16] In one report of transfusion-associated babesiosis, the median interval from transfusion to onset of clinical manifestations was 37 days. The organisms can be seen invading RBCs on smear examination, somewhat like *P. falciparum* malaria, but these organisms produce no pigment. The highest risk of death from babesiosis occurs in individuals who are older than 50 years or are immunocompromised because of acquired immunodeficiency syndrome (AIDS), drugs, transplantation, or asplenism. Sporadic reports indicate that acquired chronic toxoplasmosis is occasionally associated with hemolytic anemia.

Alteration of the Red Blood Cell Surface by Bacterial Products

Infection can produce hemolysis by altering the RBC surface. An example is the hemolysis caused by *Haemophilus influenzae* type b. Severely affected patients, particularly those with meningitis, have developed hemolytic anemias requiring RBC transfusions. The capsular polysaccharide of the bacterium, composed of polyribosyl ribitol phosphate (PRP), is released during infection and binds to the RBC surface. Infected patients develop antibodies to PRP. When the balance between PRP-coated RBCs and anti-PRP antibodies is correct, an immune-type hemolysis occurs and requires a complement. RBC destruction is thought to be both intravascular and extravascular.

Bacterial Products Causing Hemolysis by Direct Damage to Red Blood Cells

The most dramatic example of hemolysis caused by bacterial action is clostridial infection, during which the organism releases enzymes that acutely degrade the phospholipids of the membrane bilayer and the structural membrane proteins. The resulting spherocytes are extremely sensitive to osmotic lysis. The setting can be any infection, but our experience is limited to acute cholecystitis, surgery of the biliary tree, and infections surrounding an obstetric event, including criminal or self-induced abortion, or other infection of the gravid uterus. Patients may also have an underlying gastrointestinal, genitourinary, neuroendocrine, or hematologic malignancy.[17] The signs of infections may be obvious, but fever may be unimpressive. Signs of collapse appear acutely, and the clue is profound intravascular hemolysis, with a spherocytic anemia developing with shocking suddenness. The blood smear characteristically has numerous spherocytes with little evidence of microangiopathy, may be tinged red because of marked hemoglobinemia, and may have ghost cells. A clue to the severity of the process may be the inability of the laboratory to perform chemical determinations or to type and cross-match the blood because the sample is hemolyzed. With even the slightest suspicion of hemolysis caused by bacterial action, the physician immediately starts full doses of penicillin and clindamycin; evaluates the patient for DIC (see Chapter 137); and prepares to support the patient for shock, DIC, acute renal failure, and hemolytic anemia. Whether hysterectomy is lifesaving in the case of septic abortion is unclear.

Hemolysis Caused by Less Well Understood Infections

HIV infection can cause Coombs-positive autoimmune hemolytic anemia, a TTP-like syndrome, and microangiopathic hemolysis. CMV infection has been reported to cause severe Coombs-negative hemolytic anemia in immunocompetent adults.[18] Case reports of autoimmune hemolytic anemia and HUS associated with CMV infection have emerged. The hemolytic anemia in visceral leishmaniasis may be caused in part by the generation of oxidative metabolic products. Severe microangiopathic hemolytic anemia has been described in cases of cutaneous anthrax.

Hemolysis Associated With Liver Disease

Hemolysis in liver disease by itself usually is not of overwhelming clinical importance, but it may contribute to the severity of anemia when coupled with defects in RBC production and the type of gastrointestinal blood loss that occurs in several forms of liver disease. Hemolysis in patients with liver disease has several causes. The spleen may be enlarged as a consequence of portal hypertension and produce a hypersplenic picture, a phenomenon seen commonly in hepatic cirrhosis.

A classic red cell manifestation of liver disease is the appearance of target cells. The literature on RBC shape change in liver disease is considerable. The target cell in cirrhosis has an increased SA:V that appears to be a consequence of increased cholesterol and phospholipid content of the membrane bilayer. The cholesterol increase is usually proportionately greater, resulting in an increased cholesterol-to-phospholipid ratio. This increase in lipid probably accounts for the increased RBC surface area, such that more membrane than usual is present in relation to cellular contents. These RBCs probably circulate as bell-shaped RBCs called *codocytes*. However, on dried blood films, they assume the appearance of target cells. Target cells do not have a shortened survival. Their volume (MCV) is in the normocytic range, in contrast to the microcytic target cells seen in thalassemia (Chapter 40) and in advanced iron deficiency anemia (Chapter 36). The RBCs of patients with liver disease frequently are echinocytes when wet preparations are examined, but these echinocytes are not easily apparent on dried blood smears. The echinocytes seem to be produced by a material in the patient's plasma that causes normal RBCs to become echinocytic; this material is an abnormal echinocytogenic high-density lipoprotein. Echinocytes do not necessarily have a shortened survival. Some forms of echinocytic RBCs are normally deformable when studied in the ektacytometer or rheoscope.

A brisk, clinically important hemolysis can occur in some patients with severe liver disease. The peripheral smear in these individuals usually shows acanthocytes (i.e., distorted RBCs). Extreme forms are called *spur cells*, which are probably acanthocytes additionally remodeled by an enlarged spleen and are considerably enriched in cholesterol. They are rapidly removed in the spleen, which is usually enlarged.

Increased RBC membrane proteolytic activity may be a partial explanation for the differences between acanthocytosis and spur cells, and additional pathophysiologic mechanisms may be involved. Although the adult RBC cannot synthesize phospholipids de novo, it can identify and remove peroxidized fatty acid chains that interfere with normal membrane lipid fluidity. When the fatty acid is removed, a lytic lysoderivative remains; therefore, the missing fatty acid chain must be replaced. A store of acyl groups in the form of acylcarnitine exists in RBC membranes. When needed, the fatty acid (i.e., acyl group) is transferred to acyl-coenzyme A and then inserted into the potentially lytic lysophospholipid by the enzyme lysophosphocholine acyltransferase. Lysophosphocholine acyltransferase is inhibited in spur RBCs, and the same inhibition can be produced by heavily loading RBCs with cholesterol in vitro.

In spur cell anemia, the RBCs have an abnormal membrane SA:V ratio, their membrane fluidity is impaired, and they are unable to remove and repair peroxidatively damaged fatty acids. Occasionally, spur cell hemolytic anemia is severe enough to necessitate the consideration of splenectomy. Operative morbidity in such cases is considerable because the underlying liver disease usually produces problems with thrombocytopenia and leukopenia, as well as with procoagulants and intolerance to anesthesia. Spur cell anemia is typically associated with alcoholic cirrhosis, but it can also be seen in patients with nonalcoholic cirrhosis. The anemia tends to be severe and portends a poor prognosis. Transfusions are of limited efficacy because the membrane abnormalities are acquired by transfused RBCs.[19]

Acute alcoholism can be associated with hypophosphatemia, defined as levels less than 0.2 mg/dL. Such hypophosphatemia presumably interferes with RBC intermediary metabolism (see Chapters 33 and 44), and RBC adenosine triphosphate (ATP) levels fall. Very low ATP levels are associated with RBC rigidity, which leads to fragmentation, loss of surface area, and spheroidicity. The RBCs then are further trapped in the spleen. This hypophosphatemia syndrome can also cause neuromuscular disorders, including weakness, paresthesias, tremors, and seizures. It should be treated aggressively with orally and intravenously administered phosphate supplements. Hypophosphatemia also occurs in patients with cirrhosis, patients receiving total parenteral nutrition whose phosphate intake is not carefully monitored, and patients taking large amounts of phosphate-binding antacids.

Stomatocytosis can occur in severe liver disease and is thought to be a sign of acute alcoholic intoxication. The change in RBC shape can also be seen in acute pancreatitis. The stomatocyte is a cell well on its way to becoming a spherocyte. The reduction in SA:V leads to trapping in the microvasculature of the spleen and other organs of the monocyte–macrophage system, producing various degrees of hemolysis.

Hemolysis in Renal Disease

The anemia in renal disease is multifactorial. A major component is impaired RBC production, which can be well controlled with erythropoietin. Renal disease also impairs platelet function, which may lead to occult blood loss. However, hemolysis also can occur and is multifactorial. Disease of the small renal arterioles can produce fragmentation hemolysis of the sort seen in TTP–HUS, preeclampsia, and malignant hypertension (see the box on Causes of Red Blood Cell Fragmentation Hemolysis). Otherwise, whether uremia produces significant shortening of RBC survival is not clear. Patients with chronic renal failure who are undergoing hemodialysis may be particularly susceptible to oxidative damage to their RBCs. RBC glutathione is reduced in some patients, and the activity of the enzymes G6PD and glutathione peroxidase is relatively low. The ability of these RBCs to deal with the generation of peroxides probably is impaired.

Venoms, Bites, Stings, and Toxins

The best-known example of toxin-caused hemolysis is discussed in the earlier section, Bacterial Products Causing Hemolysis by Direct Damage to Red Blood Cells.

Insect, Spider, and Snake Bites

Hemolysis occurs after bee and wasp stings, snake bites, and spider bites. Isolated cases of acute intravascular hemolysis after bee and wasp stings have been reported. Two kinds of dangerous spiders live in the United States: the southern black widow and the brown recluse spider. Both sexes of the black widow produce the venom, but only the female has fangs capable of penetrating human skin. Black widow spider bites produce generalized muscle pain and muscular rigidity. Hemolysis is not common. Brown recluse spider bites cause a considerable local reaction, called the *volcano lesion*. DIC and hemolysis may occur after a lag of 24 to 48 hours. Envenomation results in cleavage of RBC glycophorins, presumably making the RBCs more susceptible.[20] Corticosteroids may be beneficial. The hemolysis appears to be self-limiting, but RBC transfusion support may be needed.

In some parts of the world, cobra bites can cause intravascular hemolysis because the venom contains phospholipases. In the United States, the two classes of venomous snakes are pit vipers (e.g., rattlesnakes, cottonmouths, moccasins, and copperheads) and coral snakes. Pit viper venom affects hemostasis and may produce DIC with bleeding but rarely hemolysis. Coral snake venom produces severe neurologic impairment. Therapy consists of support and use of the appropriate antivenin and prophylactic antimicrobials and tetanus injections.

Drugs and Chemicals Exclusive of Those Producing Oxidative Hemolysis

Potassium Chlorate

Potassium chlorate ingestion is listed as a cause of hemolysis, but this compound is no longer available in hospital pharmacies and has no currently recognized medical use. Arsine gas is generated in industrial plants that engage in lead plating, galvanizing, etching, and soldering. Inhalation of a toxic amount produces a severe intravascular hemolysis of unknown pathogenesis and may require urgent RBC and plasma exchange.

Copper

The idea that copper can produce human hemolytic disease is best supported by observations of episodes of severe hemolysis and acute liver failure in patients with Wilson disease. The patient usually is a child, adolescent, or young adult for whom the diagnosis of Wilson disease has not yet been made. The initial clinical presentation is usually dominated by Coombs-negative hemolytic anemia accompanied by weakness and dark urine. Associated findings include coagulopathy, a rapid progression to renal failure, relatively modest rises in serum aminotransferases, and a low alkaline phosphatase level. In addition to the presence of a brisk reticulocytosis, the typical findings of intravascular hemolysis may be present, including elevated lactate dehydrogenase, low haptoglobin, and markedly elevated bilirubin levels. Review of the peripheral smear may not reveal any specific morphologic findings, although both stomatocytosis and blister cells consistent with oxidant injury have been described. In one reported patient with concomitant transfusion-dependent hemoglobin E/β thalassemia, the acute hemolysis led to a severe unexpected drop in the posttransfusion hemoglobin level. Because of the hereditary deficiency in the copper-binding protein ceruloplasmin, urine and serum nonceruloplasmin–bound copper levels in patients with hemolysis are very high.

Free copper can interfere with glucose metabolism by hexokinase inhibition and alternatively can generate oxidative hemolysis, perhaps by acting as a Fenton reagent. It is important to establish the diagnosis promptly. When this condition is suspected, the practitioner should look for Kayser–Fleischer rings on physical examination and measure serum and urine copper and ceruloplasmin levels. Treatment with penicillamine or trientine plus zinc reduces the serum copper level and stops the hemolysis.[21] In the case of acute liver failure, the treatment is urgent liver transplantation. Plasmapheresis and hemofiltration may be beneficial in reducing the copper level and can serve as a bridge to transplant. In some cases, plasmapheresis in combination with chelation therapy or the use of a fractionated plasma separation and adsorption dialysis system may avert the need for transplant in impending acute liver failure. Other forms of copper poisoning may cause hemolysis in patients who do not have underlying Wilson disease. The amount of copper ingested would have to exceed the copper-binding capacity of normal ceruloplasmin levels.

Lead

There are at least two general forms of lead intoxication. One type is chronic, slow cumulative poisoning (i.e., saturnism). An example is occupational exposure. Symptoms are predominantly neurologic and nephrologic, with variable degrees of anemia, which may be caused by a production defect combined with hemolysis. Relatively acute poisoning occurs when lead inadvertently finds its way into a food source or is consumed as part of an exotic medication. Subacute lead poisoning leads to central nervous system symptoms, hepatitis, nephrotoxicity, hypertension, and abdominal colic along with seizures and severe hemolytic anemia. Physical examination may reveal a lead line on the gums. Peripheral smear shows extensive coarse basophilic stippling and reticulocytosis; however, RBC morphology is not otherwise characteristic. Some researchers state that intravascular destruction occurs, but no proof has been provided. Bilirubin levels are not significantly elevated.

The diagnosis of lead-related hemolysis can be made from the history and findings on physical examination, including a lead line on the gingiva and coarse basophilic stippling on RBCs, reflecting the pathologic aggregation of ribosomes. The diagnosis is confirmed by measuring blood and urine lead levels. The level of acuity determines the therapy.

The cause of the anemia is complex. Lead interferes with several steps in heme synthesis, particularly those involving heme synthetase and δ-aminolevulinic acid dehydratase (see Chapter 38). The inhibition of heme synthetase probably accounts for the elevation in free erythrocyte protoporphyrin, providing a useful corroborative diagnostic test for lead toxicity. Inhibition of heme synthesis also probably accounts for the elevated urinary levels of δ-aminolevulinic acid and coproporphyrin. Lead poisoning mimics the basophilic stippling and accumulation of pyrimidines seen in hereditary deficiency of the enzyme pyrimidine 5′-nucleotidase, probably because lead attacks the enzyme (see Chapter 44).

Ribavirin

A current treatment for chronic hepatitis C virus (HCV) infection may consist of combination therapy with pegylated interferon (IFN) and ribavirin, a nucleoside analog. Ribavirin's activity against HCV includes inhibition of inosine monophosphate dehydrogenase, a key step in de novo guanine synthesis. Treatment with ribavirin may produce dose-dependent hemolytic anemia, which is typically reversible 1 to 2 months after discontinuing treatment.[22] The hemoglobin drops by an average of 2 to 3 g/dL and may fall below 11 g/dL in one-third or more of patients. The anemia may necessitate a dose reduction of ribavirin or may be treated with recombinant erythropoietin at 40,000 units weekly. A decrease in the total cumulative dose of ribavirin may be associated with decreased sustained virologic response, which would suggest that dose reduction secondary to anemia would have an adverse impact on treatment efficacy. However, in one retrospective study, a drop in hemoglobin of greater than 3 g/dL was instead associated with improved sustained virologic response rate compared with those with a drop in hemoglobin 3 g/dL or less,[23] suggesting conversely that the degree of hemolytic anemia may serve as a biomarker of efficacy. Ribavirin is transported into the erythrocytes and accumulates as ribavirin monophosphates, diphosphates, and triphosphates.[22] The steady-state concentration of ribavirin in erythrocytes is approximately 100-fold higher than that of plasma, and higher erythrocyte ribavirin levels correlate with worsened anemia during therapy. The accumulation of phosphates leads to a decrease in ATP levels compared with control erythrocytes. Because ATP is required to generate the glucose-6-phosphate needed for glycolysis and the hexose monophosphate shunt, reduced levels of ATP may lead to oxidative damage, as evidenced by increased aggregates of band 3, which bind anti-band 3 IgG and complement. The ribavirin prodrug viramidine induces less anemia than ribavirin, although efficacy was decreased when used at fixed doses compared with a ribavirin-containing regimen. Fellay et al.[24] have detected two polymorphisms in the inosine triphosphatase (*ITPA*) gene, which encodes a protein that hydrolyzes inosine triphosphate, that were protective against severe anemia in patients treated with HCV. These two polymorphisms were associated with reduced *ITPA* activity and accumulation of inosine triphosphate in RBCs. The mechanism of this protective effect is not yet fully understood. The anemia in patients treated with ribavirin may be exacerbated by concomitant treatment with pegylated IFN, which suppresses hematopoiesis, and may also be associated with autoimmune hemolytic anemia. Anemia may also be exacerbated when ribavirin and IFN are used in combination with the protease inhibitor, a regimen that is efficacious in the treatment of genotype 1 disease.

DRUG-INDUCED OXIDATIVE HEMOLYSIS

General Concepts

The potential for normal RBCs to undergo auto-oxidative destruction is great because the cell is loaded with 20-mM hemoglobin, most of which is bonded to oxygen at the iron(Fe^{++}) atom in heme. The bond that allows the reversible association and dissociation of oxygen from the heme moiety of hemoglobin involves the partial transfer of an electron from iron(Fe^{++}) to oxygen. Consequently, oxygen then has an extra electron, making it a superoxide radical. Ordinarily, when oxygen leaves hemoglobin, it returns the electron. If it does not, a highly reactive superoxide ion is released, leaving behind it an iron(Fe^{+++}) moiety called *methemoglobin*.

$$HbFe^{2+}O_2 \rightarrow HbFe^{3+} + O_2^{-1}$$

Methemoglobin cannot reversibly bind oxygen. Methemoglobin in itself is not harmful to RBCs, but if the oxidative assault persists, methemoglobin is converted to hemichromes, which are variably denatured hemoglobin intermediates in which the distal histidine unit binds to the oxidized heme. This step is associated with conversion from a high to a low spin state, as measured by electron spin resonance. Continued oxidation leads to the irreversibility of hemichrome oxidation, precipitation, and eventually the formation of Heinz bodies. Hemichromes and Heinz bodies can destroy membrane function directly or by causing the oxidation of membrane proteins and lipids.[25] Approximately 3% of hemoglobin is converted to methemoglobin each day, but the finding that only 1% of hemoglobin normally is in the form of methemoglobin indicates that mechanisms preventing oxidation in RBCs are in effect. These mechanisms are limited because RBCs lack the ability to either efficiently generate ATP or synthesize enzymes. The primary means for preventing or addressing oxidant injury are the generation of the reduced form of nicotinamide adenine dinucleotide (NADH) via the Embden–Meyerhof glycolytic pathway and the generation of nicotinamide adenine dinucleotide phosphate (NADPH) via the hexose monophosphate shunt. NADH is used to reduce methemoglobin by cytochrome b5 reductase, and NADPH is used to reduce glutathione and for catalase activity. Defects in this defense system against oxidation lead to an enhanced tendency to oxidative hemolysis. Examples are G6PD deficiency states.[26] G6PD catalyzes the initial rate-limiting step in the hexose monophosphate shunt. Deficiencies lead to a reduced ability to generate NADPH in response to oxidant stress. Any agent or event that interferes with the smooth offloading of oxygen enhances the generation of O_2^{-1} and methemoglobin, as indicated in the equation. If the reducing power of the RBC is inadequate, hemichromes and Heinz bodies are generated. Many agents appear to cause oxidative hemolysis by interfering with the smooth functioning of the heme cleft.

REDUCTION OF DANGEROUS METHEMOGLOBIN LEVELS

Levels of methemoglobin in excess of 20%–30% can be dangerous, but they can be easily treated with methylene blue (1–2 mg/kg) infused intravenously over 5 min as 0.1–0.2 mL/kg of a 1% solution. In the presence of a functioning, intact reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)–methemoglobin reductase system, methylene blue is reduced to leukomethylene blue, which reduces methemoglobin to hemoglobin.

Pathophysiology

After the oxidative attack has been initiated, the sequence proceeds along a recognizable track. The oxidative attack is directed at hemoglobin and the RBC membrane. However, these structures are not clearly separable because the precipitated hemichrome and Heinz bodies come to lie against the cytosolic face of the membrane. Methemoglobin may be detectably elevated, with levels as high as 50% to 60% of total hemoglobin. The hemichromes, by themselves or with their iron portions acting as a Fenton reagent, mediate the generation of hydroxyl free radicals, which add their effect to that of superoxide and hydrogen peroxide. Lipid peroxidation may take place, leading to membrane blebbing and cell lysis, as well as the loss of asymmetry of the phospholipid membrane bilayer. Movement of phosphatidylserine and phosphatidylethanolamine to the outer bilayer of the membrane results in increased recognition by macrophages in the reticuloendothelial system. Membrane proteins may be crosslinked, with binding of denatured, oxidized hemoglobin to the membrane cytoskeleton, which may increase splenic macrophage recognition. In addition, the RBCs are rigid and susceptible to trapping in sinusoidal structures, whether or not they have Heinz bodies lying against the membrane. In vitro evidence suggests that oxidized RBCs are increasingly susceptible to phagocytosis by macrophages. These features may account for extravascular destruction. The oxidative lesions can be severe enough to cause intravascular destruction as well, producing hemoglobinemia and hemoglobinuria.

The smear may show bite cells, which look as if a macrophage had taken a bite, removing a Heinz body-containing segment of the membrane. RBC rigidity may result in irregularly shaped cells because these undeformable cells are unable to undergo elastic recoil after fighting their way through the sinus wall. Recurrent loss of membrane material may produce spherocytes. Severe hemolysis may produce the kind of circulating ghost or hemighost called a *blister cell* or *bite cell*. These RBCs have an empty veil of membrane on one side and puddled hemoglobin on the other. A Heinz body preparation may be positive. However, the absence of bite cells does not rule out the diagnosis.

The clinical picture is determined by the specific agent used. Screening for G6PD deficiency or a related disorder using an enzyme assay or the ascorbate cyanide test may be useful (Chapter 44). Although any defect in the antioxidant defense mechanisms, such as G6PD deficiency, considerably increases the susceptibility to hemolysis, many agents can produce oxidant hemolysis even in persons with normal defense mechanisms (see the box on Agents That Cause Oxidative Hemolysis).

Paraquat ingestion has occurred inadvertently and in suicide attempts. Profound cyanosis with methemoglobinemia can occur within hours, with levels of 120% or higher. The condition may be succeeded by hemolysis, with Heinz bodies seen in appropriate preparations of RBCs.

AGENTS THAT CAUSE OXIDATIVE HEMOLYSIS

- Therapeutic agents
  - Nitrofurantoin (Furadantin)
  - Sulfasalazine (Azulfidine)
  - *p*-Aminosalicylic acid
  - Phenazopyridine (Pyridium)
  - Clotrimoxazole
  - Quinolones
  - Phenacetin
  - Rasburicase
  - Dapsone and other sulfones
  - Primaquine
- Recreational drugs
  - Isobutyl nitrate
  - Amyl nitrite
- Miscellaneous agents
  - Naphthalene mothballs
  - Methylene blue
  - Paraquat
  - Hydrogen peroxide

Toxic ingestion or inhalation of nitrites may occur in suicide attempts from industrial exposures; via diets high in pickled or smoked foods; through intentional recreational use; or in infants from formulas prepared using well water high in nitrates, which are reduced to nitrites in the infant gut.[27] Nitrites bind to hemoglobin, producing methemoglobinemia, which may be so profound as to produce coma. If methylene blue infusion does not quickly turn the chocolate color of blood back to normal, the physician must consider the possibility that the patient is G6PD deficient and therefore unable to generate adequate amounts of NADPH (discussed earlier in the section Drug-Induced Oxidative Hemolysis: General Concepts). In that case, exchange transfusion may be lifesaving. Benzocaine topical anesthesia in the form of a spray or cream can cause severe methemoglobinemia, with cyanosis and dyspnea requiring methylene blue treatment.

In a case of almost fatal oxidative hemolysis, hydrogen peroxide was injected directly into the Hickman catheter of a patient with AIDS because some persons infected with HIV had circulated a pamphlet suggesting that hydrogen peroxide could be used therapeutically to control HIV infection. We now are seeing AIDS patients with dapsone-induced methemoglobinemia and hemolytic anemia (see Chapter 44). Methemoglobinemia, if severe, is treated as described in the preceding paragraph and in Chapter 44.

Pyridium (phenazopyridine) can cause oxidative hemolysis even in the absence of renal disease. This agent is commonly used for the treatment of bladder irritation. The *Physician's Desk Reference* recommends maximum therapy of 2 days. However, patients not uncommonly are given a prescription for 1 to 4 weeks of therapy.

It has been recognized for more than 130 years that therapy with dapsone causes oxidative hemolysis. In the past, dapsone was used primarily to treat leprosy and dermatitis herpetiformis and was not often encountered as a cause of oxidative hemolysis. Dapsone has come into more widespread use in some communities as a very effective prophylactic agent against *Pneumocystis carinii* pneumonia in patients with AIDS. The reduced levels of glutathione reported in patients with AIDS may enhance dapsone toxicity. Dapsone is also used in the treatment of malaria where it regularly causes hemolytic anemia in G6PD-deficient patients.[28] Some clinics screen potential recipients for G6PD deficiency (see Chapter 44) and, if results are negative, proceed with dapsone therapy. However, dapsone can cause oxidative attack on normal RBCs, leading sequentially to methemoglobinemia, Heinz bodies, and hemolysis, all occurring at generally accepted standard doses. Dapsone is metabolized to a hydroxylamine derivative that is directly toxic to RBCs.

MISCELLANEOUS, POORLY CHARACTERIZED CAUSES OF EXTRINSIC HEMOLYTIC ANEMIAS

Interferon-α as a Cause of Hemolytic Anemia

Both microangiopathic and autoimmune hemolysis have been reported with IFN-α use. Zuber et al.[29] reported eight patients with chronic myeloid leukemia who developed thrombotic microangiopathy confirmed by renal biopsy. Seven of these patients had identifiable hemolysis, and three had thrombocytopenia. They also reviewed 13 other cases of microangiopathy associated with IFN-α reported in the literature and observed that most cases occurred in the setting of prolonged therapy in chronic myeloid leukemia. Two cases of chronic hepatitis with microangiopathy were notable for having received unusually high doses of IFN for this indication. Cases of autoimmune hemolysis have been reported with IFN-α therapy in the setting of chronic myeloid leukemia or chronic hepatitis C.

Hemolysis With Intravenous Immunoglobulin G

Strictly speaking, hemolysis with intravenous IgG is a form of immune hemolysis. However, preparations of IgG contain anti-A and anti-B antibodies and rarely cause an alloimmune hemolytic anemia, as described in two young women undergoing treatment for idiopathic thrombocytopenic purpura. If this situation occurs and more intravenous IgG is needed, performing a minor cross-match and choosing a preparation of intravenous IgG that gives no reaction is recommended. In addition to isoantibody production, anemia has been reported with intravenous IgG because of immune complex-mediated complement activation.

For hemolytic anemia with large granular lymphocyte leukemia, see the box on Hemolytic Anemia in Chronic Large Granular Lymphocytic Leukemia.

HEMOLYTIC ANEMIA IN CHRONIC LARGE GRANULAR LYMPHOCYTIC LEUKEMIA

Although large granular lymphocytic leukemia usually manifests as neutropenia and a rheumatoid-like picture, several patients with a severe Coombs-negative hemolytic anemia in the absence of splenomegaly have been described. Large granular lymphocytic leukemia has occurred in a splenectomized patient. The mechanism is unknown, but one report identified direct cytotoxicity against red blood cells (RBCs) by the large granular lymphocytic cell lines.

There have been no formal studies on therapy for large granular lymphocytic leukemia-related hemolysis, although immunosuppressive therapy with prednisone or methotrexate has been reported as being partially or wholly successful.

ACKNOWLEDGMENT

Dr Stanley Schrier, the author of the first version of this chapter, died in 2019. His contribution to this present chapter is gratefully acknowledged.

REFERENCES

1. Moake J. Thrombotic microangiopathies: multimers, metalloprotease, and beyond. *Clin Transl Sci*. 2009;2:366.
2. George JN, Nester CM. Syndromes of thrombotic microangiopathy. *N Engl J Med*. 2014;371:654.
3. Alkhouli M, Farooq A, Go RS, Balla S. Cardiac prostheses-related hemolytic anemia. *Clin Cardiology*. 2019;42:692.
4. Al-Nouri ZL, Reese JA, Terrell DR, et al. Drug-induced thrombotic microangiopathy: a systematic review of published reports. *Blood*. 2015;125:616.
5. Weitz IC. Thrombotic microangiopathy in cancer. *Semin Thromb Hemost*. 2019;45:348.
6. Zakarija A, Kwaan HC, Moake JL, et al. Ticlopidine- and clopidogrel-associated thrombotic thrombocytopenic purpura (TTP): review of clinical, laboratory, epidemiological, and pharmacovigilance findings (1989–2008). *Kidney Int Suppl*. 2009(112):S20.
7. Booth KK, Terrell DR, Vesely SK, et al. Systemic infections mimicking thrombotic thrombocytopenic purpura. *Am J Hematol*. 2011;86:743.
8. Francis KK, Kalyanam N, Terrell DR, et al. Disseminated malignancy misdiagnosed as thrombotic thrombocytopenic purpura: a report of 10 patients and a systematic review of published cases. *Oncologist*. 2007;12:11.
9. Dane K, Chatarvedi S. Beyond plasma exchange: novel therapies for thrombotic thrombocytopenic purpura. *Hematol Am Soc Hematol Educ Program*. 2018(1):539.
10. Casals-Pascual C, Roberts DJ. Severe malarial anaemia. *Curr Mol Med*. 2006;6:155.
11. An X, Mohandas N. Red cell membrane and malaria. *Transfus Clin Biol*. 2010;17:197.
12. Chang K-H, Tam M, Stevenson MM. Inappropriately low reticulocytosis in severe malarial anemia correlates with suppression in the development of late erythroid precursors. *Blood*. 2004;103:3727.
13. Safeukul I, Gomez ND, Adelani AA, et al. Malaria induces anemia through CD8$^+$ T cell-dependent parasite clearance and erythrocyte removal in the spleen. *mBio*. 2015;6:1.
14. Looareesuwan S, Ho M, Wattanagoon Y, et al. Dynamic alteration in splenic function during acute falciparum malaria. *N Engl J Med*. 1987;317:675.
15. Lobo CA, Rodriguez M, Cursino-Santos JR. Babesia and red cell invasion. *Curr Opin Hematol*. 2012;19:170.
16. Herwaldt BL, Linden JV, Bosserman E, et al. Transfusion-associated babesiosis in the United States: a description of cases. *Ann Intern Med*. 2011;155:509.
17. McArthur HL, Dalal BI, Kollmannsberger C. Intravascular hemolysis as a complication of clostridium perfringens sepsis. *J Clin Oncol*. 2006;24:2387.
18. Veldhuis W, Janssen M, Kortlandt W, et al. Coombs-negative severe haemolytic anaemia in an immunocompetent adult following cytomegalovirus infection. *Eur J Clin Microbiol Infect Dis*. 2004;23:844.
19. Cooper RA, Kimball DB, Durocher JR. Role of the spleen in membrane conditioning and hemolysis of spur cells in liver disease. *N Engl J Med*. 1974;290:1279.
20. Tambourgi DV, Morgan BP, de Andrade RM, et al. Loxosceles intermedia spider envenomation induces activation of an endogenous metalloproteinase, resulting in cleavage of glycophorins from the erythrocyte surface and facilitating complement-mediated lysis. *Blood*. 2000;95:683.
21. Roberts EA. Update on the diagnosis and management of Wilson disease. *Curr Gastroenterol Rep*. 2018;20:56.
22. McHutchison JG, Manns MP, Longo DL. Definition and management of anemia in patients infected with hepatitis C virus. *Liver Int*. 2006;26:389.
23. Sulkowski MS, Shiffman ML, Afdhal NH, et al. Hepatitis C virus treatment-related anemia is associated with higher sustained virologic response rate. *Gastroenterology*. 2010;139:1602.
24. Fellay J, Thompson AJ, Ge D, et al. ITPA gene variants protect against anaemia in patients treated for chronic hepatitis C. *Nature*. 2010;464:405.
25. Hebbel RP, Eaton JW. Pathobiology of heme interaction with the erythrocyte membrane. *Semin Hematol*. 1989;26:136.
26. Luzzatto L, Seneca E. G6PD deficiency: a classic example of pharmacogenetics with on-going clinical implications. *Br J Haematol*. 2014;164:469.
27. Greer FR, Shannon M. Infant methemoglobinemia: the role of dietary nitrate in food and water. *Pediatrics*. 2005;116:784.
28. Pamba A, Richardson ND, Carter N, et al. Clinical spectrum and severity of hemolytic anemia in glucose 6-phosphate dehydrogenase-deficient children receiving dapsone. *Blood*. 2012;120:4123.
29. Zuber J, Martinez F, Droz D, et al. Alpha-interferon-associated thrombotic microangiopathy: a clinicopathologic study of 8 patients and review of the literature. *Med (Baltimore)*. 2002;81:321.

CHAPTER 49

NEUTROPHILIC LEUKOCYTOSIS, NEUTROPENIA, MONOCYTOSIS, AND MONOCYTOPENIA

Lawrence Rice, Arthur W. Zieske, and Moonjung Jung

Abnormalities of leukocyte number are commonly encountered in medical practice. The clinical significance of leukocytosis or leukopenia varies from none at all to being an early clue to a life-threatening process, whether that is a primary hematologic or secondary reactive process. Potential causes of leukocytosis or leukopenia are myriad. This chapter considers disorders faced by adult practitioners in hospital and outpatient clinics where the predominant hematologic abnormality is neutrophilic leukocytosis, neutropenia, monocytosis, or monocytopenia; other chapters consider lymphocytosis, lymphopenia, eosinophilia, pancytopenia, and hematologic neoplasms.

The normal range for leukocyte count in most laboratories is from about 4500/mm^3 to 11,000/mm^3. Neutrophils (and band forms) comprise the majority of circulating leukocytes (1800 mm^3 to 7700/mm^3). Monocytes are about 4% of cells (mean absolute count: 300/mm^3). The physician should always think in terms of absolute counts of leukocyte subpopulations (total leukocyte count multiplied by the differential percentage). Thus, in a patient presenting with a normal white blood cell (WBC) count of 5000/mm^3 and an elevated lymphocyte percentage of 65%, the differential diagnosis to be considered is that of neutropenia, not lymphocytosis, because the absolute neutrophil count (ANC) is decreased but absolute lymphocytes are normal (only relatively increased).

When approaching a patient with abnormal leukocyte number, several factors impact heavily on the differential diagnosis and the vigor with which diagnosis and therapy should be pursued. Diagnostic considerations are vastly different when the abnormality first manifests **in the hospital versus in the outpatient clinic**. Also crucial is the **degree of the abnormality**, providing guidance to its likely cause and consequence. For example, agranulocytosis (Fig. 49.1) is a life-threatening disorder in which neutrophils are at or near zero, has a limited spectrum of underlying causes (drug reactions being paramount), and demands immediate interventions. **Duration** has major implications; determining the onset of changes and whether they are stable or progressive informs as to etiology and significance. Whether the abnormality is **symptomatic**—for example, whether a neutropenic or monocytopenic patient has or has had infectious complications—bears on likely etiologies and need for therapy. If there are known or suspected **comorbid conditions**, such as autoimmune or inflammatory disorders, this can crystallize the approach; occasionally, the leukocyte abnormality may be the first sign of a previously unrecognized disorder or may provide important confirmation (e.g., neutropenia in a patient with systemic lupus erythematosus [SLE]). If the leukocyte abnormality is accompanied by **additional hematologic abnormalities** (unexplained abnormalities of red blood cells [RBCs], platelets, or cell morphology), this would point away from disorders considered in this chapter and often toward a primary hematologic disease. Beyond history and physical examination, the peripheral blood smear is key to establish the direction of further evaluation.

NEUTROPHILIC LEUKOCYTOSIS (NEUTROPHILIA)

A high WBC count, particularly a high neutrophil count, is common with any infectious or inflammatory disorder. In the emergency department, leukocytosis is often equated with significant bacterial infection or is at least a sign of illness severe enough to warrant hospital admission rather than outpatient management. Leukocytosis can also be a prominent presenting feature of leukemias and myeloproliferative neoplasms (MPNs). The presence of increased neutrophils assures that acute leukemia is not present. When leukocytosis is extreme, it indicates chronic myeloid leukemia (CML), other MPNs, or a leukemoid reaction.

Leukemoid reaction has been defined as a reactive (nonclonal) neutrophilic leukocytosis with WBC count above 50,000/mm^3. This must be differentiated from a neoplastic proliferation.

Leukoerythroblastosis (Fig. 49.2) refers to the presence in the peripheral blood of immature myeloid cells (generally myelocytes) and nucleated RBCs, often with giant platelets as well. This is always abnormal. Patients with leukoerythroblastosis do not necessarily have leukocytosis, but they usually do. Most patients (two-thirds) with leukoerythroblastosis have an underlying myelophthisic process, such as primary or secondary myelofibrosis, metastatic tumor, necrosis, or granulomas in the bone marrow (BM). Therefore, BM examination is indicated when leukoerythroblastosis is unexplained. Teardrop poikilocytes and elliptocytes on blood smear would strengthen concerns for myelophthisis. In 20% of patients with leukoerythroblastosis, the cause is hemolytic anemia, and miscellaneous other causes consist mainly of those with shock (septic, hemorrhagic, cardiogenic, anaphylactic) when hypoperfusion of areas of BM disrupt the microenvironment and permit disorderly egress of precursor cells.

Left-shifted neutrophils refer to relative immaturity of circulating cells, often manifest as an increased percentage of band neutrophils. Marked left-shift includes less mature precursor forms, myelocytes and metamyelocytes. Left-shift is nonspecific and may occur with infection or any cause of marked neutrophilia.

Detailed directed history and physical examination are indispensable to the evaluation of neutrophilia (Table 49.1). Fever and chills suggest infection (or inflammation), mandating a search for more specific symptoms that could pinpoint the focus. Examples include a sore throat, pharyngeal erythema, and exudate in pharyngitis; productive cough and abnormal lung auscultation in pneumonia; and dysuria and flank tenderness in urinary tract infection. Medication history mainly explores glucocorticoid use. With mild chronic neutrophilia, smoking habits and obesity become considerations. Recent vigorous exercise, emotional stress, burns, shock, or trauma can increase circulating neutrophils because of catecholamine-induced demargination. A positive family history may suggest hereditary neutrophilia. Often neglected are attempts to delineate the time course of the leukocyte abnormality by seeking prior medical contacts and blood count results at the time. On physical examination, care should be directed to lymph node palpation because this can be an important clue for infection or malignancy. Palpable splenomegaly may not only direct the evaluation toward hematologic disorders but can be a cardinal sign of a variety of infectious and inflammatory disorders.

Blood smear should always be a part of initial evaluation when there are abnormalities of blood counts. BM aspirate or biopsy morphology may be helpful when pathophysiology and diagnosis are unclear. When appropriate, essential information can be gained by sending BM for microbiologic cultures, cytogenetic or molecular, or other ancillary studies.

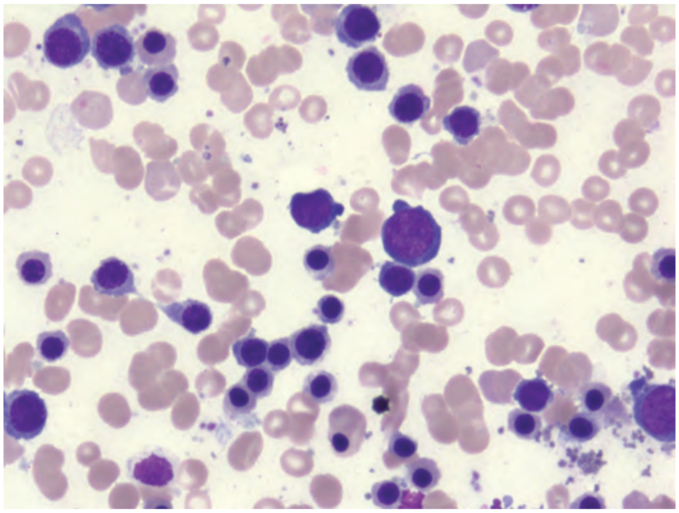


Figure 49.1 AGRANULOCYTOSIS (PURE WHITE CELL APLASIA) (bone marrow aspirate). Virtual absence of myeloid precursors. Most often due to drug reactions, but can be due to immunologic disorders.

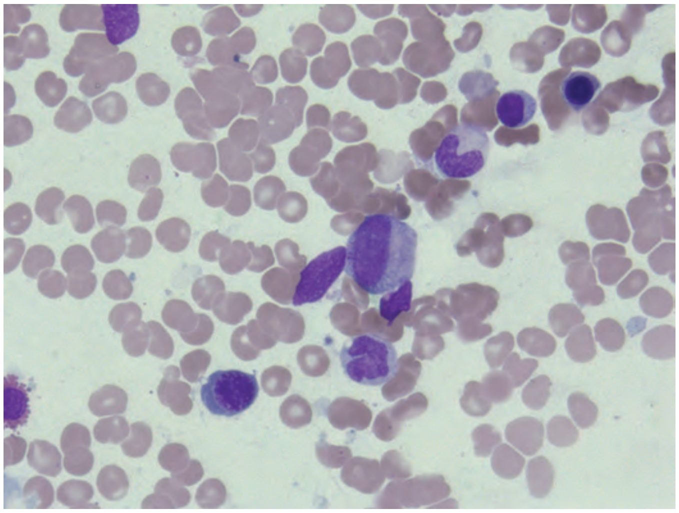


Figure 49.2 LEUKOERYTHROBLASTIC SMEAR (peripheral blood). The presence of immature myeloid cells (myelocytes) and nucleated red blood cells, often with giant platelets. Most often due to myelophthisis (marrow invasion by tumor, fibrosis, granulomas), but also due to hemolytic anemia or shock.

TABLE 49.1 **Causes of Neutrophilia**

1. Hematologic malignancy (CML, CNL, CMML)
2. Infection
3. Inflammation, physiologic stress, hemorrhage, hemolysis
4. Hereditary or congenital neutrophilias
5. Smoking
6. Drugs: colony-stimulating factors, glucocorticoids, epinephrine, lithium
7. Nonhematologic malignancy
8. Asplenia
9. Obesity
10. Recovery from neutropenia
11. Chronic binge drinking

CML, Chronic myeloid leukemia; *CMML*, chronic myelomonocytic leukemia; *CNL*, Chronic neutrophilic leukemia.

Leukemoid Reaction Versus Chronic Leukemia

A relatively common reason for hematologic consultation is for very high WBC count. CML is reviewed elsewhere in this text book, as are chronic myelomonocytic leukemia (CMML) and the very rare chronic neutrophilic leukemia. Marked neutrophilic leukocytosis or overt leukemoid reaction (WBC count >50,000/mm^3) can represent an overly exuberant reaction to any stimulus associated with neutrophilia.[1] Infection is the most common non-hematologic cause of leukemoid reaction. In patients with leukemoid reaction, a disproportionate number have infection with *Clostridioides* (formerly *Clostridium) difficile (C. difficile)*, an organism that elicits a vigorous neutrophil response. Leukemoid reactions may be associated with solid tumors, sometimes due to paraneoplastic production of colony-stimulating factor (e.g., granulocyte colony-stimulating factor [G-CSF] or granulocyte-macrophage colony-stimulating factor [GM-CSF]) or other cytokines (e.g., interleukin [IL]-6 or IL-17), or due to particularly aggressive tumors with necrotic areas. The course of neutrophilia usually correlates with the course of solid cancer. The use of hematopoietic growth factors in cancer patients, cardiopulmonary distress, tissue ischemia such as bowel obstruction, gastrointestinal bleeding, major operations, pancreatitis, or splenectomy are other causes of leukemoid reaction.[1] It is important to quickly differentiate a reactive neutrophilic leukocytosis from a clonal leukemic proliferation. The history and clinical context are usually quite different between leukemoid reaction and CML. Most patients with leukemoid reaction are encountered very ill in the hospital with obvious underlying illnesses (e.g., sepsis, organ rejection). Prior WBC counts, which are often available, demonstrate normal WBC counts until the recent onset of acute illness. This contrasts with CML, typically presenting in outpatients with hypermetabolism (weight loss, sweats, low-grade fever), symptoms referable to splenomegaly, or frequently asymptomatic. On physical examination, the spleen is palpable (occasionally massive) in the great majority of patients with CML, but splenomegaly is unusual with leukemoid reaction in the absence of comorbidities such as liver disease.

Laboratory findings reliably differentiate CML from leukemoid reaction. The total leukocyte count is commonly extremely high with CML (median 100,000/mm^3 in past series), but counts above 100,000/mm^3 are rare and above 150,000/mm^3 virtually unheard of with leukemoid reaction. Circulating myelocytes and even a few blasts are more typical of CML, but may be seen in both disorders. Similarly, changes in platelet number and morphology can be seen with both but are more characteristic of CML (especially when changes are extreme). RBC changes do not reliably separate the disorders except in a few cases with prominent teardrops, which point toward MPN. More helpful is the leukocyte differential: patients with CML almost always have some degree of absolute basophilia and eosinophilia, but infection and glucocorticoid excess induce eosinopenia. (When the leukocyte count is 100,000/mm^3, realize that 2% to 3% basophils is a substantial absolute increase.)

The leukocyte alkaline phosphatase (LAP) score, high with leukemoid reaction and classically low with CML, has limited utility now that there are more sensitive and specific tests for CML. When CML is reasonably considered, testing should be done for the Philadelphia chromosome, t(9;22)(q34;q11.2) by chromosome G-banding, or *BCR–ABL1* fusion product by fluorescence in situ hybridization or reverse-transcriptase polymerase chain reaction. When other MPNs are judged reasonably possible, *JAK2* V617F mutational status may be informative. In cases with very high suspicion for MPNs but negative *JAK2* V617F mutation, *JAK2* exon 12, *MPL*, and *CALR* mutations can be assessed in a serial manner.

Infection

To protect against the ever-present threats to health and longevity, evolution has armed us with reactant cytokine cascades designed to increase the number of phagocytes and dispatch them to threatened locales. Neutrophilia is classically seen as a response to bacterial infection, responding to such cytokines as IL-6, tumor necrosis factor (TNF), and G-CSF. Neutrophilia is also a frequent response to other types of infections, such as fungal, parasitic, mycobacterial, and sometimes viral.

C. difficile infection should be suspected when unexplained neutrophilia is encountered in a patient with diarrhea and abdominal distension, especially with a history of recent use of antibiotics. Two large clostridial glucosylating toxins, toxin A (TcdA) and toxin B (TcdB), are primarily responsible for the colonic epithelial damage and death.[2] These toxins also stimulate epithelial cells and tissue-resident immune cells (macrophages, dendritic cells, and mast cells) to secrete proinflammatory cytokines and neutrophil chemoattractants which recruit neutrophils and result in inflammation.

Changes in neutrophil morphology may be useful in predicting whether bacterial or other infection underlie a neutrophilic response. The authors have confirmed some published reports that prominent neutrophil vacuolization (Fig. 49.3) is highly specific and moderately sensitive for serious bacterial infection, as are prominent Döhle bodies (in the absence of a primary hematologic disorder). The authors blindly assessed 125 blood smears from 50 from patients with serious bacterial infection (half bacteremic), 25 with influenza, 25 with noninfectious fever, and 25 controls. Toxic granulation of neutrophils, touted as a sign of bacterial infection, was found useless in distinguishing infections from other febrile illnesses.[3]

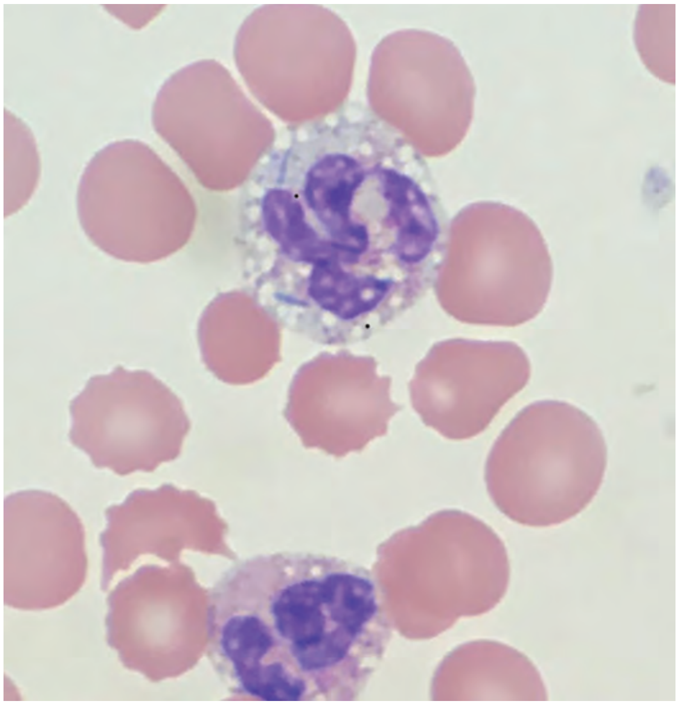


Figure 49.3 VACUOLATED NEUTROPHILS. Strong association with severe bacterial infection/bacteremia.

Inflammation and Stress

Acute or chronic inflammation can cause neutrophilia by mechanisms similar to infection, mediated by the proinflammatory cytokines G-CSF, GM-CSF, TNF, IL-1, IL-6, IL-8, and others. Diseases such as rheumatoid arthritis (RA), vasculitis, inflammatory bowel disease, thyrotoxicosis, eclampsia, and many others are commonly accompanied by neutrophilia. Another rare but notable example is Familial Mediterranean Fever, in which an inherited *MEFV* mutation leads to dysfunctional pyrin and an autoinflammatory disorder with recurrent inflammatory serositis and secondary (AA) amyloidosis.

Physiologic stresses, including exercise and emotional stress, lead to endogenous catecholamine and glucocorticoid release in addition to inflammatory cytokines. This causes a rapid doubling of circulating neutrophils caused by demargination and by more rapid BM egress of maturing neutrophils. Paulsen found early peaks in M-CSF, growth hormone, and cortisol after exercise followed by increases in G-CSF, IL-6, and monocyte chemoattractant protein 1.[4] Acute hemorrhage and hemolytic anemia are other physiologic stresses. This contributes to increased steady-state neutrophil counts recorded in patients with sickle cell anemia, and the degree of elevation correlates with pain crisis frequency, other complications, and mortality; leukocyte reduction has been postulated to be one mechanism of hydroxyurea's beneficial actions. The stress of acute myocardial infarction is commonly accompanied by mild neutrophilia, and the early magnitude of rise has correlated with poor outcomes.

Patients with severe asthma have neutrophil-predominant lung inflammation due to formation of neutrophil extracellular traps (NETosis) and enucleated neutrophil cytoplasts, which in turn lead to T helper 17-mediated neutrophilic inflammation.[5]

Hereditary and Congenital Neutrophilias

In newborns, neutrophilia and leukoerythroblastosis are among the hematologic abnormalities associated with trisomy 13, trisomy 18, and trisomy 21 (Down syndrome). A transient clonal MPN can be seen in children with Down syndrome and is usually self-limited, but it does put patients at increased risk for later acute megakaryoblastic leukemia.

Very rare hereditary neutrophilias are sometimes first appreciated in adults. In 1971, Herring reported a mother and three of her four children with lifelong neutrophilia (WBC: 14,000/mm^3 to 164,000/mm^3; granulocytes: 9000/mm^3 to 62,000/mm^3), unusual bleeding, thickened calvariae, and hepatosplenomegaly. They had no increase in infections. The LAP score was high, BM revealed few Gaucher-like histiocytes, karyotype was normal by routine G-banding, but chromatid breaks and gaps were increased. In 2009, French investigators identified a mutation in the *CSF3R* gene in a kindred with hereditary chronic neutrophilia.[6] The point mutation led to constitutive activation of the G-CSF receptor, driving neutrophil proliferation and differentiation. One of 12 affected individuals in this kindred developed overt myelodysplastic syndrome (MDS). The frequency of this and other mutations are unknown with hereditary neutrophilias.

Smoking

Smoking has been well associated with mild neutrophilia in epidemiologic and animal studies. Perry et al. found a 27% higher WBC count in smokers. Sunyer et al. correlated the degree of leukocytosis and percentage of neutrophils with the number of cigarettes smoked. The effect can persist up to 5 years in those who stop smoking. A suggested mechanism is chronic inflammation because inflammatory markers, including C-reactive protein (CRP) and fibrinogen, are elevated. In a rodent model, cigarette smoke caused overexpression of hematopoietic growth factor genes IL-6, G-CSF, and GM-CSF. Thus, mild neutrophilia without other symptoms in a smoker could be attributed to this practice without further evaluation.

Drugs

Systemic glucocorticoids cause neutrophilia mainly by interfering with neutrophil adhesion to the capillary wall and decreasing neutrophil turnover rate. Maximal neutrophil counts occur 4 to 6 hours after dexamethasone use in normal volunteers. G-CSF increases circulating neutrophils by increasing BM production and mobilization. Epinephrine increases proinflammatory cytokines and demargination. Suggested mechanisms for lithium-induced neutrophilia are induction of G-CSF and downregulation of CXCR4 chemokine receptor, thus facilitating egress from the BM.

Malignancy

Leukocytosis is frequently associated with solid tumors without direct BM involvement. Some malignant tumors have been reported to produce G-CSF or GM-CSF, occasionally producing leukocytosis in the range of a leukemoid reaction. Recent epidemiological studies suggested that the presence of pre-treatment neutrophilia is an independent adverse prognostic factor in patients with advanced solid cancers.[7]

Asplenia

Whereas neutrophilia is classically observed early after splenectomy, lymphocytosis predominates in the long run. Nevertheless, chronic mild neutrophilia can also be seen in functionally asplenic individuals. Furthermore, there may be an exaggerated neutrophil response to infections or other stresses. On the blood smear, Howell–Jolly bodies are a very sensitive and specific (when numerous) sign of functional asplenia.

Obesity

Chronic mild neutrophilia has been observed with obesity.[8] Fat tissue can release inflammatory cytokines, creating a state of low-grade inflammation manifest by elevated CRP. Leptin, a hormone released by adipocytes, may act on CD34$^+$ cells to promote neutrophil differentiation. Chronically inflamed visceral adipose tissue in obesity has been shown to stimulate BM hematopoietic progenitor cells to proliferate and expand, causing both neutrophilia and monocytosis via IL-1β.[9] In an obese patient with chronic mild neutrophilia and no obvious other underlying disorder, this can be assumed to be the cause.

Alcoholic Liver Disease

While excessive alcohol use has been classically associated with neutropenia due to BM suppression, Cai et al. found that some patients with chronic excessive alcohol use may develop neutrophilia and hepatic neutrophil infiltration.[10] Alcohol consumption may cause increased levels of circulating and tissue neutrophil chemotaxin, such as IL-8 or CXCL1, which in turn recruits neutrophils to liver and increases circulating neutrophils. Alcohol also induces hepatic endoplasmic reticulum stress and release of mitochondrial DNA-enriched microparticle into circulation, leading to systematic inflammatory response. In these patients, signs of liver injury, such as elevated serum transaminases, are frequently observed. Therefore, history of excessive alcohol use with elevated liver function tests should raise a suspicion for alcohol being a contributing factor for neutrophilia.

NEUTROPENIA (AND AGRANULOCYTOSIS)

The risk of neutropenia-related infection begins to rise at an ANC near 1000/mm^3, rising dramatically below 500/mm^3 and more so below 100/mm^3; thus, an ANC of 500/mm^3 to 1000/mm^3 is considered moderate neutropenia, and below 500/mm^3 is considered severe. This corresponds to National Cancer Institute criteria for medication adverse hematologic event reporting: grade 1 toxicity is 1500/mm^3 to lower limit of normal, grade 2 is 1000/mm^3 to 1500/mm^3, grade 3 is 500/mm^3 to 1000/mm^3, and grade 4 is less than 500/mm^3. Beyond the ANC, the risk for infection is greatly influenced by the nature of the underlying problem, the BM myeloid reserve, and whether other risk factors for infection are present (e.g., immunoglobulin deficiency or breaks in mucosal barriers). The course with mild and moderate neutropenia is often benign.

The history focuses on the severity and duration of neutropenia, whether infectious complications have occurred (including stomatitis or gingivitis), and whether prior blood counts can be obtained. A history of drug exposures and their timing is especially relevant, not only for prescribed medications but also for over-the-counter, herbal, and illicit drugs. Symptoms of systemic inflammatory illness, such as arthritis, skin rash, and photosensitivity, can bear on the etiology and significance of the low WBC count. Fevers, weight loss, and sweats could be clues to many disorders, including malignancy. Liver disorders commonly present with cytopenias, so a history of hepatitis, jaundice, and HIV risk factors should be specifically sought. Symptoms of anemia or bleeding could be clues to more general hematologic disorders. As with most hematologic problems, physical examination should devote careful attention to lymph node areas and the spleen. Oropharynx and skin examination also take on added importance.

Peripheral blood smear review is irreplaceable to direct the workup. MDS does not characteristically present with isolated neutropenia, but this occasionally is the predominant presenting feature in this relatively common syndrome affecting older patients. Specific blood smear findings which could suggest MDS include pseudo-Pelger–Huet neutrophils (Fig. 49.4), hypogranularity, Döhle bodies (Fig. 49.5), and macrocytic and/or dimorphic RBCs with a hypochromic population. Megaloblastic processes, such as vitamin B_{12} and folic acid deficiency, similarly do not characteristically present with isolated neutropenia, but this occasionally predominates (especially in the presence of acute infection). Hypersegmentation of neutrophils is always present with megaloblastic processes, including those that are drug-induced (e.g., methotrexate, hydroxyurea). Hypersegmented neutrophils (Fig. 49.6) are also seen with uremia, MDS, and MPN. In a neutropenic patient, one should always specifically look for large granular lymphocytes (LGLs) (Fig. 49.7). A modest increase could indicate a reactive T-cell or natural killer (NK) cell process, and a more dramatic increase could alert to clonal T-NK or NK cell proliferations (LGL leukemia). Hairy cells (Fig. 49.8), other morphologic types of circulating lymphoma

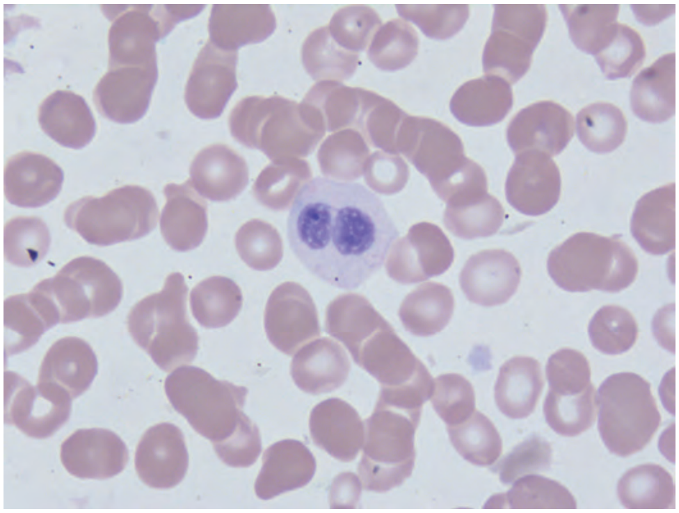


Figure 49.4 PELGER-HUET ANOMALY. (Two symmetric round or oval nuclear lobes connected by a thin strand of chromatin.) Hereditary form (involving almost all neutrophils) is benign. Acquired form suggests MDS (as in this hypogranular example), but may be seen transiently with infection, advanced MPNs, myxedema, others. *MPNs*, Myeloproliferative neoplasms; *MDS*, myelodysplastic syndrome.

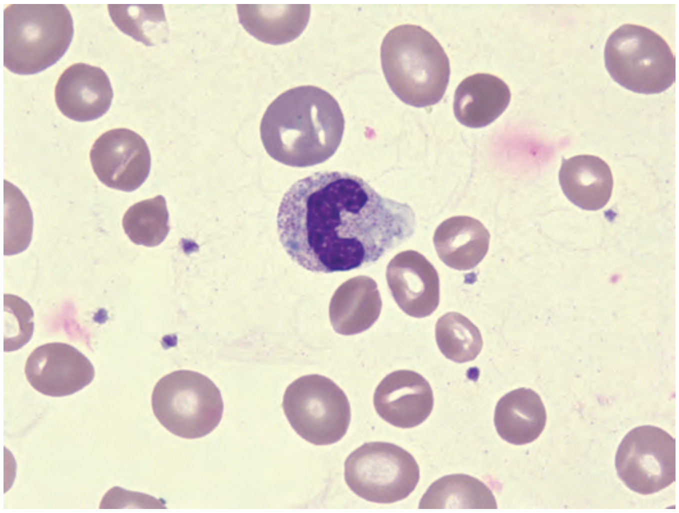


Figure 49.5 DÖHLE BODIES. Most commonly due to infection; myriad other causes include drug-induced (including growth factors), congenital (including MYH9 disorders), MDS. *MDS*, Myelodysplastic syndrome.

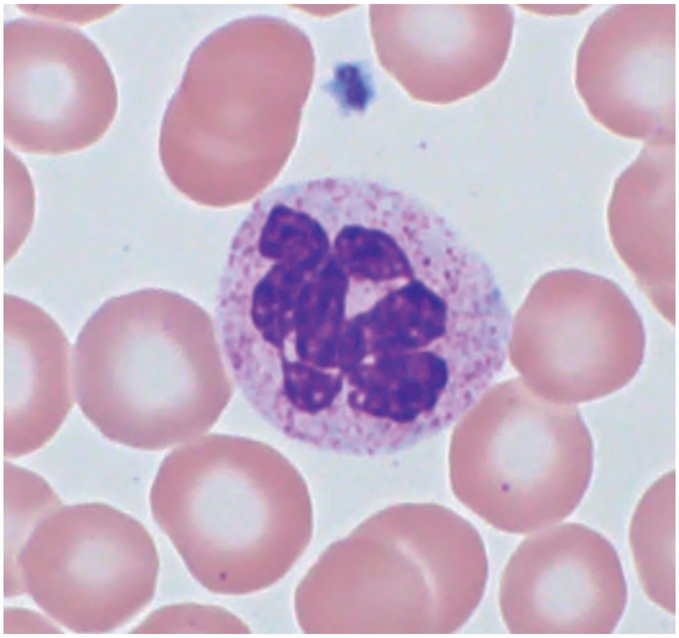


Figure 49.6 HYPERSEGMENTED NEUTROPHIL. Victor Herbert established that hypersegmentation exists if 5% of neutrophils have five lobes or if one has six lobes. This is a sensitive finding for megaloblastic processes (including drug-induced). It is commonly seen with uremia and can be a congenital polymorphism.

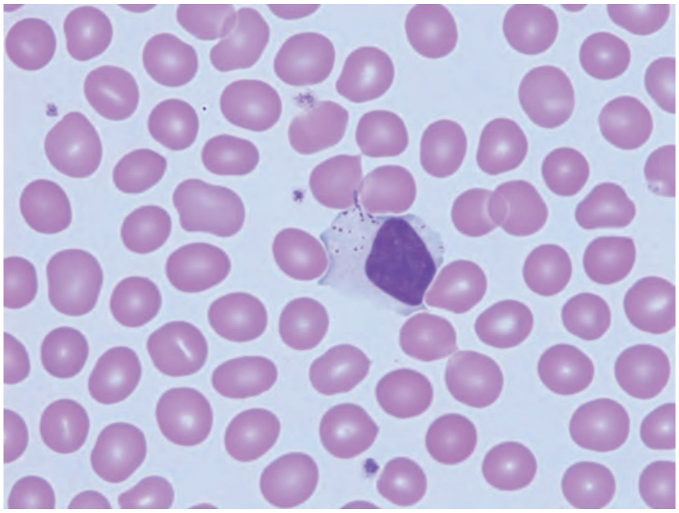


Figure 49.7 LARGE GRANULAR LYMPHOCYTES. (See Chapter 50.) T-NK lymphocytosis can be reactive or clonal, and are associated with cytopenias, especially neutropenia. *NK*, Natural killer.

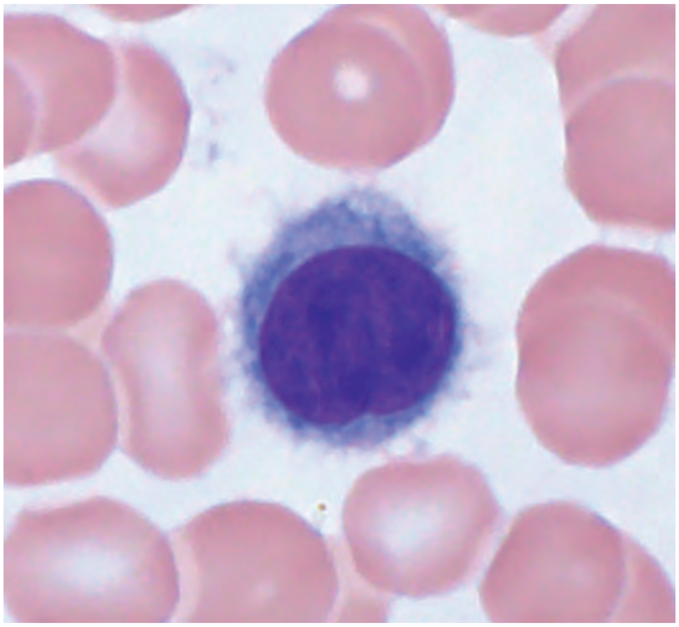


Figure 49.8 HAIRY CELLS. (See Chapter 77.) Hairy cell leukemia is associated with cytopenias, including monocytopenia (in the typical form).

cells, and blasts are obvious indicators of hematologic malignancy. Morphologic suspicions for such processes could be confirmed by flow cytometry. Reactive lymphocytes may suggest viral infection but also occur with drug reactions and other processes.

Further laboratory testing in patients with neutropenia may often be unnecessary. Especially in mild or moderate cases, the lack of specific diagnostic tests has created some overlap and confusion among what have been called *chronic idiopathic neutropenia*, *chronic benign neutropenia*, *ethnic neutropenia*, and *autoimmune neutropenia*. Autoimmune neutropenia is a relatively common cause of both mild and more severe neutropenia, but tests for antineutrophil antibodies are not clinically reliable (see later). Serologic tests for antinuclear antibodies and rheumatoid factor can be helpful when autoimmune neutropenia is considered, as positive results may raise suspicion for an undiagnosed collagen vascular disorder or may just support a less specific autoimmune problem. Direct antiglobulin test and antiphospholipid antibodies can also be supportive when there is suspicion for an autoimmune process. Quantitative immunoglobulin levels may reveal an underlying immunodeficiency when there is evidence of autoimmunity or when infectious complications are disproportionate. Serologic tests for some viral infections (HIV, hepatitis, Epstein-Barr virus [EBV]) may sometimes be appropriate. The main utility of flow cytometry occurs when peripheral blood smear suggests an abnormal lymphocyte population (e.g., LGLs or hairy cells). Because LGL leukemia is a relatively common cause of significant neutropenia and because this is a diagnosis frequently initially missed, one should have a low threshold to obtain flow cytometry, assuring that proper markers are analyzed (see later). BM examination may not be helpful or warranted with mild or even moderate isolated neutropenia or in straightforward cases of severe neutropenia (e.g., after drug exposure), but can be essential when the diagnosis is in doubt and especially if other cell lines are compromised.

Severe Congenital Neutropenias

It is beyond the scope of this chapter to review in detail the heterogeneous genetic disorders that manifest in early childhood as severe congenital neutropenia (SCN); nevertheless, consultant hematologists should have some familiarity with this problem both because modern therapy has extended survival for many into adulthood, and because milder forms of these disorders may go undiagnosed until young adulthood. More severe forms of SCN present in infancy with severe stomatitis and recurrent bacterial infections. The molecular bases have been greatly elucidated, among them: (1) an autosomal dominant mutation of the neutrophil elastase gene *ELANE* in 50% to 60% of patients, (2) an autosomal recessive mutation of *HAX1*, which is associated with mental retardation and other congenital defects found in Kostmann syndrome, (3) the other genes account for only a small proportion of all cases: *G6PC3*, *GF11*, *CSF3R*, *WAS* (X-linked), *CXCR4*, *VPS45A* and *JAGN1*, and (4) about one-third of patients do not have identifiable mutations in the above-mentioned genes.[11]

In young children, the differential diagnosis of SCN includes transient postinfectious neutropenia, alloimmune neutropenia, or hematologic malignancy. BM examination often shows a pattern of maturation arrest (Fig. 49.9). The majority of affected patients are responsive to G-CSF, which has favorably impacted infectious morbidity and mortality. Allogeneic stem cell transplant is another therapeutic option. An intrinsic risk of acute myeloid leukemic transformation, 15% at 20 years, accompanies these disorders, with higher risk in those requiring higher doses and/or responding poorly to G-CSF.

Cyclic neutropenia is a rare autosomal dominant disorder with variable expression, also caused by autosomal dominant mutations of *ELANE* or rarely its transcription regulator. Neutrophil counts vary from mildly to severely low with predictable periodicity, usually about 21 days. The great majority of cases are responsive to G-CSF at low dosage.

Warts, Hypogammaglobulinemia, Infections, and Myelokathexis Syndrome

WHIM syndrome is a rare autosomal dominant immunodeficiency with **W**arts, **H**ypogammaglobulinemia, **I**nfections, and **M**yelokathexis. It is due to heterozygous gain-of-function mutations in the chemokine receptor, *CXCR4*.[12] CXCR4 is expressed on hematopoietic progenitor cells and regulates homing to BM where the ligand of CXCR4, CXCR12 (also known as SDF-1), is abundantly expressed. In WHIM syndrome, mutant *CXCR4* prolongs interaction with CXCR12 and causes excessive marrow retention of neutrophils and other leukocytes. The diagnosis is made based on DNA sequencing for relevant mutations in *CXCR4*. Treatment includes G-CSF (titrate to the lowest possible dose to achieve an ANC between 1000 and 2000/mm^3) and more recently, CXCR4 antagonists, such as plerixafor or mavorixafor, have been used with success.

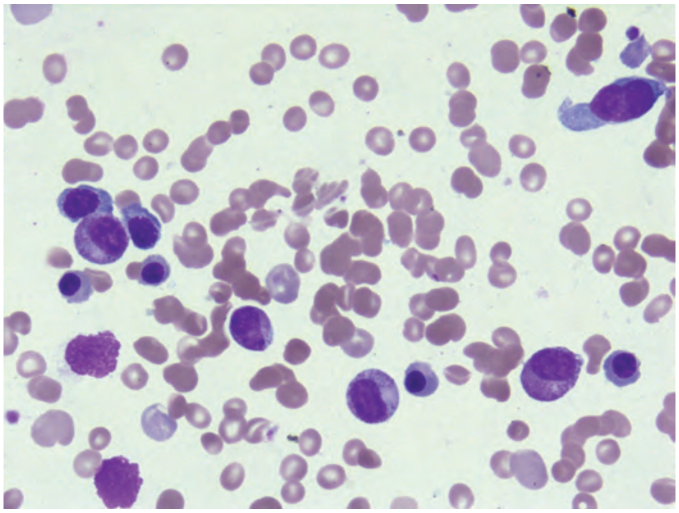


Figure 49.9 MATURATION ARREST (bone marrow aspirate). (Almost no myeloid precursors beyond myelocyte [sometimes promyelocyte] stage.) Non-specific: can be seen with early precursor release, early recovery from myelosuppression, peripheral destruction (including antibody-mediated), and stem cell disorders.

Shwachman-Diamond syndrome is an inherited BM failure disorder due to biallelic pathogenic variants in *SBDS*, *EFL1*, or *SBDS*, or a heterozygous pathogenic variant in *DNAJC21*.[13] Neutropenia is the most common presenting cytopenia, but bilineage or trilineage cytopenia can occur. Patients have hypocellular BM and characteristic exocrine pancreatic dysfunction manifesting as diarrhea or failure to thrive. Positive family history and other constitutional abnormalities, such as short stature and skeletal malformation, can point toward this diagnosis. About 15% to 20% of patients progress to MDS/AML by the age of 20. Hematopoietic stem cell transplantation should be considered for progressive BM failure or MDS/AML.

Lower Neutrophil Count With Fy(a-b-) Status (Previously Known as Benign Ethnic Neutropenia)

Individuals of African descent may have chronic mild neutropenia not associated with infectious complications. The 1999 to 2004 National Health and Nutrition Survey found that Black individuals had mean leukocyte counts 900/mm^3 lower than White individuals, with ANCs below 1500/mm^3 in 4.5% (compared with 0.8% of White individuals).[14] Low ANC is correlated with homozygosity of a single nucleotide polymorphism in the gene Duffy antigen receptor for chemokines (*DARC*; also known as *ACKR1*; rs2814778), which results in the absence of Duffy antigen from RBCs (termed Duffy null or Fy(a-b-)). The Fy(a-b-) phenotype is commonly found in individuals from sub-Saharan African and Arab, but rare in White European or Asian ancestry.[15] BM biopsies performed for isolated low ANC in the setting of the Fy(a-b-) genotype rarely reveal abnormalities.[16] Thus, an asymptomatic mildly neutropenic person without abnormalities on blood smear who carries the Fy(a-b-) genotype may require no further diagnostic workup. It may be reassuring to the patient (and the doctor) if prior blood counts can be retrieved to document the chronic, nonprogressive, and complication-free course of the neutropenia.

Autoimmune Neutropenia (Primary and Secondary)

Autoimmune neutropenia can be primary or secondary to an autoimmune disorder such as SLE or RA.[17] Neutropenia can be mild or severe. Classically, it is caused by antineutrophil autoantibodies, although autoreactive cytotoxic lymphocytes may also cause this problem. When performed, BM examination often is normocellular or hypercellular, with a late "maturation arrest" picture. Severe cases can display pure WBC aplasia (similar to drug-induced agranulocytosis, which may sometimes also be antibody-mediated). There are several challenges to making the diagnosis. One is that the maturation arrest BM picture is very nonspecific with regard to mechanism: it could indicate a true stem cell differentiation defect, or the early release of later precursors (as with sepsis or splenic sequestration), or an immune attack aimed at antigens on later myeloid precursors, or early recovery from a toxic insult. Another problem is the lack of clinically reliable neutrophil antibody tests. Experimentally, several methods have been used, generally suffering from high false-negative and false-positive rates in detecting antibodies against neutrophil antigens or immune complexes that bind to neutrophil Fc receptors. With no definitive test, diagnosing autoimmune neutropenia rests largely on clinical context and judgment.

Neutropenia caused by antineutrophil antibodies has been seen in infants about 1 year old, usually running a benign clinical course with spontaneous remission in 95% within 2 years. Newborns are at risk of alloimmune neutropenia in the first months of life, but this is not generally accompanied by infectious complications. Secondary autoimmune neutropenias are most common in adults, related to disorders such as SLE, RA, Sjögren syndrome, thymoma, and common variable immunodeficiency. In SLE, neutrophil counts correlate with disease activity and have been related to the induction of TNF–related apoptosis-inducing ligand (TRAIL), a ligand that increases myeloid apoptosis and killing by autologous T cells. Immunosuppressive therapy of the SLE or the use of G-CSF can improve the WBC count.

Felty syndrome was classically described as severe neutropenia and splenomegaly complicating RA, and has been attributed to antineutrophil antibodies, but the distinction between this syndrome and other causes of neutropenia with RA have become blurred. In particular, classic Felty syndrome and LGL syndrome appear to represent points on a disease continuum. About 1% of patients with RA develop Felty syndrome, and it predisposes many to serious infections. Effective therapies have often included methotrexate, gold salts, and rituximab. Because of some risk of exacerbating underlying inflammatory problems, G-CSF should be used with caution.

Large Granular Lymphocyte Leukemia

LGL leukemias are covered elsewhere, but mention is required here because these are common considerations in adults with neutropenia. LGL leukemia is a chronic lymphoproliferative disorder due to clonal expansion of cytotoxic T or NK LGLs. The expansion of cytotoxic effector cells are due to chronic antigen-driven immune stimulation and therefore treatment is based on immunosuppression. These proliferations are frequently associated with autoimmune disorders (especially RA), variable degrees of neutropenia, anemia, commonly splenomegaly, and increased infectious risks. The diagnosis is suggested on blood smear by large lymphocytes with mature chromatin, excessive cytoplasm, with or sometimes without prominent cytoplasmic granules. Flow cytometry demonstrates increased $CD3^+CD57^+$ lymphocytes with the more common T-LGL proliferations or may show $CD3^-$ $CD56^+$ clones with "pure" NK cell LGL proliferation. Clonality can be established by T cell receptor (TCR) repertoire analysis by PCR. Killer cell immunoglobulin-like receptor (KIR) repertoire analysis by flow cytometry is used to determine clonality in NK-LGL. Somatic activating mutations in the signal transducer and activator of transcription 3 gene (STAT3) were found in 40% of patients with LGL leukemia, suggesting aberrant STAT3 signaling pathway is an underlying mechanism of this disease.[18] Somatic activating STAT5b mutations were also found in a minority of patients.[19]

Neutropenia With Infectious Diseases

Leukocytosis is the expected response to most bacterial infections, but leukopenia also occurs and is characteristic of infection by certain organisms. Viral infections often cause transient mild leukopenia, so restraint is wise in the diagnostic approach to a newly recognized moderate leukopenia in a febrile, modestly ill patient. Nonbacterial infections in which neutropenia is frequent or even characteristic include HIV, EBV, cytomegalovirus (CMV), hepatitis A and B, measles, rubella, varicella, rickettsia, and anaplasmosis (ehrlichiosis). Bacterial infections with characteristic leukopenia include typhoid fever and brucellosis, and granulomatous infections (tuberculosis, histoplasmosis [Fig. 49.10] also commonly cause neutropenia, especially when the BM is directly involved. In patients with poor BM reserve (e.g., prior chemotherapy, malnutrition, myelodysplasia), an acute infection very often lowers, rather than raises, the neutrophil count. Profound neutropenia is a known consequence of overwhelming sepsis and has been associated with poor outcomes.

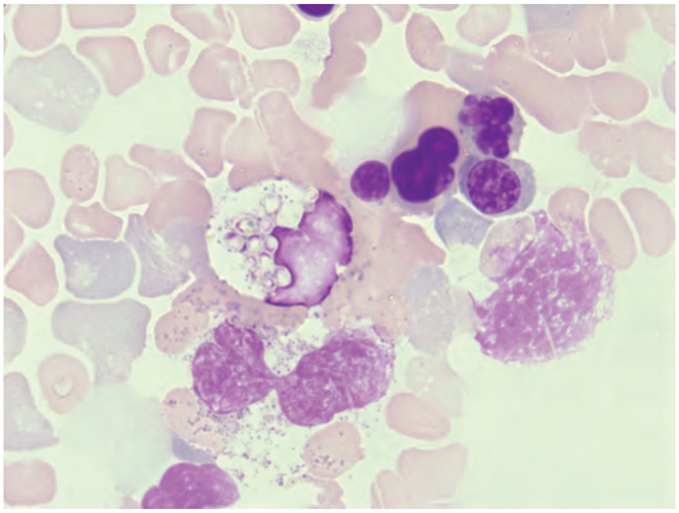


Figure 49.10 HISTOPLASMOSIS. In disseminated histoplasmosis, organisms can frequently be seen in leukocytes and histiocytes in bone marrow and sometimes peripheral blood.

Hypersplenism

Hematologists are frequently consulted for cytopenias, only to find an unappreciated large spleen as the etiology (this is most commonly due to liver disease with portal hypertension). Splenic enlargement can lead to sequestration and reduction of circulating WBCs, RBCs, platelets, or any combination of these. The degree of cytopenia is somewhat proportional to the degree of splenic enlargement. Substantial neutropenia out of proportion to the depletion of other cell lines and to the degree of splenomegaly suggests an autoimmune component (which can be seen with hepatitis C or autoimmune hepatitis) or medication effect (e.g., interferon).

Chemotherapy-Induced Neutropenia

The major dose-limiting toxicity of most cancer chemotherapy regimens is neutropenia. This results in hospitalizations, antibiotic costs, reduced quality of life, infectious morbidity, dose reductions, and treatment delays that compromise efficacy and outcomes, and result in some deaths: 75% of chemotherapy-related mortality. Febrile neutropenia is defined as an ANC less than $500/mm^3$ (or expected to decrease to $<500/mm^3$ over the next 48 hours) with fever ($>38.3°C$ or sustained $>38°C$).

Principles of empiric antibiotic coverage for acute febrile episodes in severely neutropenic patients are covered in detail elsewhere. Basically, there is a high risk of sepsis (even when the source is occult), and a favorable outcome depends on prompt effective antimicrobial coverage, so empiric administration of broad-spectrum antibacterial agents is considered medically emergent. There must be coverage of the traditional aerobic gram-negative rod culprits (particularly *Pseudomonas*), yet vigilance must be maintained for the Gram-positive organisms that have emerged as major pathogens. With prolonged or repeated episodes of neutropenia, invasive fungi become common threats to survival, particularly *Aspergillus*, *Candida*, *Mucor*, and *Fusarium* spp. Published guidelines can aid the choice of empiric antimicrobials. Because of difficulty demonstrating favorable impact, enthusiasm for granulocyte transfusions has waned in the past 30 years.

The utility of prophylactic antibiotics is problematic because of the wide array of potential pathogens and because of concerns of inducing antibiotic resistance. Prophylactic fluoroquinolones, antifungals, and antivirals have had some favorable impact in very high-risk patients after consolidation therapy for acute leukemia or allogeneic stem cell transplant.[20]

G-CSF or pegfilgrastim is widely used for primary or secondary prevention of neutropenia to decrease morbidity and mortality of febrile neutropenia. Primary prophylaxis is recommended by various guidelines if the risk of developing febrile neutropenia is greater than 20%.[21] This risk is calculated from age, extent of primary cancer, comorbidities, and the known myelotoxicity of the chemotherapy regimen. G-CSF is begun 24 to 72 hours after the completion of myelotoxic chemotherapy.

Drug-Induced Neutropenia

Drug reactions account for a high percentage of acquired neutropenia, both mild but, more so, severe cases (agranulocytosis; Table 49.2). Neutropenia often develops abruptly, within 4 weeks of initiation of a causative agent. Pathophysiology may involve immune mechanisms or more direct toxicity such as enhancement of reactive oxygen species made by nicotinamide adenine dinucleotide phosphate oxidase or myeloperoxidase on neutrophil precursors.[22] They can be strongly suspected from an accurate history that plots leukocyte numbers against the time course of drug exposure; confirmation is leukocyte recovery after drug withdrawal. More specific confirmatory testing, such as drug-dependent antibodies, is mainly an area of research laboratory investigation. BM examination is not usually required but may be helpful when pathophysiology and diagnosis are in doubt, when the course is atypical, and to provide prognostic information (earlier recovery may be anticipated with a picture of "maturation arrest" than that of myeloid aplasia). Treatment demands discontinuation of suspected offending drugs. Although the efficacy of myeloid growth factors (G-CSF) has been questioned, most recommend this therapy because even a modest shortening of severe neutropenia can occasionally be lifesaving. Mortality of drug-induced agranulocytosis is generally reported to be 10%.

The most commonly implicated drugs are listed in Table 49.2. Some merit additional comment. Some drugs have a direct myelotoxic effect rather than induce an idiosyncratic immune-based reaction. Neutropenia will have more gradual onset related to dose and duration, and may be accompanied by suppression of other hematologic cell lines. Linezolid and most instances of chloramphenicol myelosuppression are examples.

Beginning 2007, an epidemic of agranulocytosis was found among intravenous cocaine users. The adulterant levamisole was found in 71% of confiscated cocaine by the Drug Enforcement Agency, with the incidence of agranulocytosis 2.5% to 13% in those exposed. Nadir BM showed severe myeloid hypoplasia. Levamisole had long been linked to agranulocytosis, having been used as an antihelminth and as an immune adjuvant in patients with autoimmune disorders and colorectal cancer.

Severe neutropenia is seen in patients treated with rituximab.[23] This neutropenia is unusual for its late onset, generally about 3 months after the last rituximab dose. It occurs with underlying autoimmune disorders, B-cell malignancies, and stem cell transplants—basically in all situations in which rituximab is used—often occurring while the underlying illness is in complete remission. Severe neutropenia has been reported in about 5% of rituximab-treated patients, much higher in some series. Most patients recover quickly, usually after G-CSF therapy, but there have been protracted cases. The authors and others have reported a high risk of relapse (100% in our series) if rituximab is reinitiated. The mechanism is not definitively established, but an intriguing report implicates imbalanced recovery of B-cell clones with a deficiency of stromal-derived factor 1.

Immunotherapy-induced severe neutropenia has been reported in individuals with pre-existing autoimmune disorders.

MONOCYTOSIS

Monocytosis is extremely nonspecific, usually not requiring investigation per se. Monocytosis has been defined as a sustained absolute increase in monocyte count greater than $800/mm^3$ to $1000/mm^3$ (Table 49.3). Transient monocytosis, relative or absolute, is common with recovery from myelosuppression, such as after chemotherapy. Relative or absolute monocytosis may occur in other myelosuppressed states, such as aplastic anemia. This is a favorable factor associated with a lower risk of neutropenic infection, perhaps because some phagocytic capacity is maintained. On the other hand, monocytosis is also very common with hematologic malignancies. In a patient with unexplained cytopenia, monocytosis can be an important clue to an underlying MDS/MPN.

Infectious Diseases

Mycobacterial infection is a common cause of monocytosis worldwide, related to its propensity for intracellular infection and tissue granuloma formation. Brucellosis and subacute bacterial endocarditis have also been associated with monocytosis. Certain viral infections, including influenza, varicella-zoster, CMV, and dengue, have been reported to cause monocytosis.

TABLE 49.2 **Drugs Commonly Associated With Neutropenia**

1. Antibiotics: vancomycin, semisynthetic penicillins, chloramphenicol, sulfa, linezolid
2. Antithyroid drugs: methimazole, propylthiouracil
3. Cardiovascular: ticlopidine, procainamide
4. Antipsychotics: clozapine, olanzapine, chlorpromazine
5. Anticonvulsants: phenytoin, carbamazepine, valproic acid
6. Antiinflammatory agents: indomethacin, sulfasalazine, phenylbutazone
7. H2 blockers: cimetidine, ranitidine
8. Analgesics: dipyrone
9. Antineoplastic: rituximab
10. Anthelmintic: levamisole

TABLE 49.3 **Changes in Monocyte Number**

Monocytosis

Infections: tuberculosis, granulomatous infection, brucellosis, subacute bacterial endocarditis
Connective tissue disorder
Recovery from myelosuppression
Hematologic malignancies:
1. MDS, MPN, MDS–MPN overlap (e.g., CMML)
2. Acute and chronic monocytic leukemia, myelomonocytic leukemia
3. Hodgkin and non-Hodgkin lymphomas

Obesity

Monocytopenia

Hairy cell leukemia
GATA2 deficiency (also known as MonoMAC syndrome, DCML, Emberger syndrome, familial MDS/AML)
Aplastic anemia
Drugs: chemotherapy, IFN-α, glucocorticoids (transient)
Radiation therapy

AML, Acute myeloid leukemia; *CMML*, chronic myelomonocytic leukemia; *DCML*, dendritic cell, monocyte and lymphoid deficiency; *IFN*, interferon; *MDS*, myelodysplastic syndrome; *monoMAC*, monocytopenia and mycobacterium avium complex syndrome; *MPD*, myeloproliferative disorder; *MPNs*, myeloproliferative neoplasms.

Connective Tissue Disorder

Connective tissue disorders, such as SLE and RA, have been associated with monocytosis in the context of chronic inflammation.

Hematopoietic Malignancies

Monocytosis is common with MDS/MPN. CMML is defined as persistent peripheral blood absolute monocytosis greater than 1000/mm^3 and relative monocytosis in peripheral blood (≥10%), absent Philadelphia chromosome, absent *PDGFRA* and *PDGFRB* rearrangements, less than 20% blasts in peripheral blood and BM, and evidence of dysplasia in one or more hematopoietic cell lineages. Juvenile myelomonocytic leukemia, a disease of children that shares pathologic features with CMML, results from defective RAS signaling. Acute myeloid leukemias (AMLs) involving the monocyte line (acute myelomonocytic and acute monoblastic leukemias) may release substantial amounts of lysozyme (muramidase), which is toxic to renal tubules. Serum lysozyme was used to aid in the diagnosis of these leukemias. Monocytosis can result from other myeloid leukemias, MPNs, and lymphomas, particularly Hodgkin lymphoma.

MONOCYTOPENIA

Monocytopenia frequently accompanies granulocytopenia with chemotherapy, aplastic anemia, or other BM-suppressive insults. Isolated or disproportionate monocytopenia is rarely recognized, but observations suggest that this can have serious clinical sequelae when it occurs. In hairy cell leukemia and monoMAC syndrome (GATA2 deficiency), there is a clear association with severe opportunistic infections, particularly those normally engendering a granulomatous response.

Hairy Cell Leukemia

This disorder, considered in detail elsewhere, classically presents with pancytopenia and splenomegaly, often in middle-aged men. The WBC count is sometimes high because of hairy cell proliferation, but monocytes are invariably severely depressed. (Monocytopenia is not seen with “variant hairy cell leukemia.”) Neutropenia only partly explains the very high infectious morbidity and mortality. An extraordinary incidence of opportunistic granulomatous infections has been encountered, including atypical mycobacteria, tuberculosis, histoplasmosis, and other fungi.[24] These are linked to monocytopenia, with impaired granuloma formation. With remission induced by modern therapies (e.g., cladribine, pentostatin or moxetumomab pasudotox), monocytopenia and infectious risks have been mitigated.

GATA2 Deficiency

In 2010, investigators at the National Institutes of Health and in the UK simultaneously described an immunodeficiency syndrome characterized by decreased or absent monocytes, NK cells, B cells, and dendritic cells; this clinical syndrome was named *MonoMAC* or *dendritic cell, monocyte and lymphoid deficiency*.[25] This was largely diagnosed in young adults (median age: 33 years). All patients have opportunistic infections, particularly *Mycobacterium avium* complex and other mycobacteria, opportunistic fungi, and viruses, particularly human papillomavirus. Both autosomal dominant and sporadic cases are described, all linked to mutations in the hematopoietic stem cell regulator *GATA2*. Heterozygous mutations in *GATA2* are also responsible for Emberger syndrome (primary lymphedema and a predisposition to AML) and familial MDS/AML. BM examination findings are almost always abnormal, usually hypocellular, with increased reticulin fibrosis, multilineage dysplasia with characteristic separated nuclei in megakaryocytes, and absent B and NK precursors. Clonal cytogenetic abnormalities are seen in most patients, particularly monosomy 7 in 16% and trisomy 8 in 24%. Because leukemic progression with or without death from infection is high, stem cell transplantation is recommended when feasible. When a family member is used as a BM donor, *GATA2* sequencing must be performed to rule out asymptomatic carrier status before donating. When monocytopenia out of proportion to neutropenia is detected in a patient with or without human papillomavirus-associated warts or MAC infection, examination of B- and NK-cell count by flow cytometry will be informative. Detailed family history may reveal additional patients with infectious complications or MDS/AML.

CHRONIC MILD NEUTROPHILIC LEUKOCYTOSIS

A 46-year-old man was referred to the hematology clinic for evaluation of neutrophilia that was noted by his primary care physician on a routine blood test. He reported no fevers, chills, or night sweats. He had good energy and was working full time as a lawyer. He denies fatigue, weight loss, or rashes. His past medical history included hypertension and obesity, but he was otherwise healthy. He has been smoking cigarettes for 10 years. Family history was negative for blood diseases. Vital signs were normal. Body mass index was 40.5 kg/m^2. Physical examination revealed only an obese abdomen. Laboratory values showed white blood cell (WBC) 12,500/mm^3, absolute neutrophil count (ANC) 8500/mm^3, Hb 14 g/dL, hematocrit (Hct) 42%, platelets 400 K/mm^3. Chemistries were unremarkable. Peripheral blood smear showed only increased neutrophils, with unremarkable red blood cells (RBCs) and platelets. A complete blood cell count 2 years earlier showed WBC 11,800/mm^3, ANC 8100/mm^3, Hb 14.2 g/dL, platelets 385 K/mm^3. What is the next step in evaluation?

This patient has mild neutrophilia of at least 2 years of duration. Obesity and smoking are common causes of neutrophilia. There is no evidence of chronic infection, inflammation, or hematologic malignancy. No further hematologic evaluation is warranted. We recommend only follow-up and counseling about lifestyle changes.

ACUTE SEVERE NEUTROPHILIC LEUKOCYTOSIS

A 78-year-old woman who was a nursing home resident presented to the emergency department with altered mental status, diarrhea, and abdominal pain. She was recently treated with ciprofloxacin for urinary tract infection. On physical examination, she is difficult to arouse. Vital signs show blood pressure 105/66 mmHg, heart rate 98/min, respiratory rate 18/min, temperature of 100.4°F. Abdominal examination reveals distension, active bowel sounds, no organomegaly. Laboratory findings show white blood cell (WBC) 88,000/mm^3, Hb 10.5 g/dL, platelets 200/mm^3, neutrophils 65%, lymphocytes 5%, monocytes 4%, eosinophils <1%, basophils <1%, bands 25%. Hematology is consulted for the high WBC. Peripheral blood smear shows increased neutrophils, with a few metamyelocytes, and myelocytes. Red blood cells (RBCs) are normocytic and normochromic. Platelets are unremarkable. Records from one month earlier show normal blood counts except mild anemia with a Hb of 10.9 g/dL.

The almost certain cause of the extreme leukocytosis in this patient is leukemoid reaction, perhaps due to urosepsis or *Clostridioides difficile* infection. *C. difficile* is classically associated with severe neutrophilia and recent antibiotic use with diarrhea and abdominal distension. The absence of splenomegaly, eosinophilia, and basophilia, and near normal blood counts a month ago makes chronic myeloid leukemia (CML) or other myeloproliferative neoplasm (MPN) very unlikely. We would recommend appropriate tests, antibiotics, clinical follow-up, but no molecular testing for CML at this time.

REFERENCES

1. Potasman I, Grupper M. Leukemoid reaction: spectrum and prognosis of 173 adult patients. *Clin Infect Dis*. 2013;57:e177.
2. Chandrasekaran R, Lacy DB. The role of toxins in *Clostridium difficile* infection. *FEMS Microbiol Rev*. 2017;41(6):723–750.
3. Rice L, Harris RL, Lynch EC, et al. Utility of peripheral blood changes in discriminating causes of fever and infection. *Blood*. 1987;70:94A.
4. Paulsen G, Benestad HB, Strøm-Gundersen I, et al. Delayed leukocytosis and cytokine response to high-force eccentric exercise. *Med Sci Sports Exerc*. 2005;37(11):1877–1883.
5. Krishnamoorthy N, Douda DN, Brüggemann TR, et al. Neutrophil cytoplasts induce Th17 differentiation and skew inflammation toward neutrophilia in severe asthma. *Sci Immunol*. 2018;3:26.
6. Plo I, Zhang Y, Couedic JL, et al. An activating mutation in the CSF3R gene induces a hereditary chronic neutrophilia. *J Exp Med*. 2009;206:1701.
7. Shaul ME, Fridlender ZG. Tumour-associated neutrophils in patients with cancer. *Nat Rev Clin Oncol*. 2019;16:601.
8. Herishanu Y, Rogowski O, Polliack A, et al. Leukocytosis in obese individuals: possible link in patients with unexplained persistent neutrophilia. *Eur J Haematol*. 2006;76:516.
9. Nagareddy PR, Kraakman M, Masters SL, et al. Adipose tissue macrophage promote myelopoiesis and monocytosis in obesity. *Cell Metab*. 2014;6:19.
10. Cai Y, Xu MJ, Koritzinsky EH, et al. Mitochondrial DNA-enriched microparticles promote acute-on-chronic alcoholic neutrophilia and hepatotoxicity. *JCI Insight*. 2017;2:14.
11. Skokowa J, Dale DC, Touw IP, et al. Severe congenital neutropenias. *Nat Rev Dis Primers*. 2017;3:17032.
12. McDermott DH, Murphy PM. WHIM syndrome: immunopathogenesis, treatment and cure strategies. *Immunol Rev*. 2019;287:91.
13. Kennedy AL, Shimamura A. Genetic predisposition to MDS: clinical features and clonal evolution. *Blood*. 2019;133(10):1071–1085.
14. Hsieh MM, Everhart JE, Byrd-Holt DD, et al. Prevalence of neutropenia in the U.S. population: age, sex, smoking status, and ethnic differences. *Ann Intern Med*. 2007;146:486.
15. Merz LE, Achebe M. When non-Whiteness becomes a condition. *Blood*. 2021;137(1):13–15.
16. Van Driest SL, Abul-Husn NS, Glessner JT, et al. Association between a common, benign genotype and unnecessary bone marrow biopsies among African American patients. *JAMA Intern Med*. 2021;181:1100–1105.
17. Newburger PE. Autoimmune and other acquired neutropenias. *Am Soc Hematol Educ Program*. 2016;1:38.
18. Koskela HL, Eldfors S, Ellonen P, et al. Somatic STAT3 mutations in large granular lymphocytic leukemia. *N Engl J Med*. 2012;366:20.
19. Rajala HL, Eldfors S, Kuusanmäki H, et al. Discovery of somatic STAT5b mutations in large granular lymphocytic leukemia. *Blood*. 2013;121:4541.
20. Tplitz RA, Kennedy EB, Bow EJ, et al. Antimicrobial prophylaxis for adult patients with cancer-related immunosuppression: ASCO and IDSA Clinical Practice Guideline Update. *J Clin Oncol*. 2018;36:3043.
21. Smith TJ, Bohlke K, Lyman GH, et al. Recommendations for the use of WBC growth factors: American Society of Clinical Oncology Clinical Practice Guideline Update. *J Clin Oncol*. 2015;33:3199.
22. Curtis BR. Non-chemotherapy drug-induced neutropenia: key points to manage the challenges. *Hematol Am Soc Hematol Educ Prog*. 2017;1:187.
23. Wolach O, Bairey O, Lahav M. Late-onset neutropenia after rituximab treatment: case series and comprehensive review of the literature. *Med. (Baltimore)*. 2010;89:308.
24. Rice L, Shenkenberg T, Wheeler T, et al. Opportunistic granulomatous infections in hairy cell leukemia. *Cancer*. 1982;49:1924.
25. Spinner MA, Sanchez LA, Hsu AP, et al. GATA2 deficiency: a protein disorder of hematopoiesis, lymphtics, and immunity. *Blood*. 2014;123:6.

CHAPTER 50

LYMPHOCYTOSIS, LYMPHOCYTOPENIA, HYPERGAMMAGLOBULINEMIA, AND HYPOGAMMAGLOBULINEMIA

Sravanti P. Teegavarapu and Martha P. Mims

Any discussion of quantitative abnormalities of lymphocytes and immunoglobulins is necessarily linked because the B-cell compartment is responsible for immunoglobulin production and the T-cell compartment helps to provide the stimulus. Nevertheless, clinicians are often consulted when a quantitative disorder of one or the other is recognized. Thus, although overlapping, the defects are presented separately.

QUANTITATIVE DISORDERS OF LYMPHOCYTES

The normal number and distribution of lymphocyte subtypes in the peripheral blood vary with age, but no careful study has demonstrated gender or ethnic differences in lymphocyte count. In general, circulating T cells exceed B cells by a ratio of approximately four to one, with that ratio increasing slightly with age (Table 50.1). Natural killer (NK) cells are grouped with lymphocytes but comprise only approximately 10% of the lymphocyte population. Infants have total lymphocyte counts between 5500/μL and 7000/μL, but this number declines beginning at approximately 1 year of age to reach 2000/μL to 2400/μL in adults. At birth, the total number of circulating B cells is approximately 1000/μL but decreases over the first 10 years of life to approximately 200/μL to 300/μL by the age of 18 years. A slow decline in circulating B cells continues throughout adulthood and is primarily accounted for by a decline in transitional and naive B cells with a stable or mild increase in circulating memory B-cell numbers with age. Circulating naive B cells represent about two thirds of the entire naive B-cell pool, and circulating memory B cells represent only about one-third of the entire memory B-cell pool.[1]

T cells dominate the circulating lymphocyte population with approximately 3500/μL in infancy, declining to 1500/μL on average in young adults, with even lower numbers (approximately 1200/μL) in elderly adults. In both children and adults, CD4 cells outnumber CD8 cells. Naive CD4 and CD8 T cells decline with age by twofold to fourfold. In one large study, CD4 memory T cells significantly increased with age, but no such trend was seen for CD8 memory T cells.[2]

Against this background, *lymphocytosis* is defined as a lymphocyte count greater than 8000/μL in young children and greater than 4000/μL in teenagers and adults. Lymphocytopenia has been defined as a total lymphocyte count of less than 1000/μL in older children and adults and less than 1500/μL in infants and young children. Given the composition of the circulating lymphocyte pool, it is critically important to define which lymphocyte subsets are overrepresented or underrepresented when there is a quantitative disorder.[2]

Lymphocytosis

As in any clinical disorder, a careful history is critical to defining the origin of lymphocytosis. Inherited causes of lymphocytosis are rare. One recently identified inherited lymphocytosis is BENTA disease (B-cell expansion with nuclear factor kappa-B [NF-κB] and T-cell anergy). Germline gain-of-function mutations in caspase-activating recruitment domain 11 (*CARD11*) drive this disorder, which is characterized by polyclonal lymphocytosis and splenomegaly beginning in infancy. However, in most cases of lymphocytosis, the issue is to determine whether a lymphocyte disorder is clonal/malignant or benign and related to infection, drugs, or physiologic stress. Rarely, neither a malignancy nor an underlying condition can be identified, in which case the lymphocytosis is termed *persistent polyclonal B-cell lymphocytosis* (PPBL). When an excess of B cells exists, clonality can usually be defined by examining cell surface expression of κ- and λ-light chains using antibody techniques; with T-cell proliferation, it may be important to define clonality by examining T-cell receptor gene rearrangement using molecular procedures. In the case of NK cell disorders, clonality can be difficult to determine and often only provided by analysis of chromosomal abnormalities of restriction fragment length polymorphisms of X-linked genes. In general, a restricted flow cytometry pattern of killer immunoglobulin-like receptor (KIR) expression on NK cells is accepted as a surrogate marker of clonality.

Clonal Disorders

Malignant causes of peripheral blood lymphocytosis are covered in Chapters 75, 76, 81, 82, 85, and 88 and include chronic lymphocytic leukemia (CLL), hairy cell leukemia, splenic marginal zone lymphoma, lymphoplasmacytic lymphoma, follicular lymphoma, mantle cell lymphoma, adult T-cell leukemia or lymphoma, and Sézary syndrome. Occasionally, precursor T-cell or precursor B-cell leukemia presents with circulating small cells, which morphologically appear more similar to mature lymphocytes than blasts.

A recently identified clonal disorder causing lymphocytosis is monoclonal B-cell lymphocytosis (MBL), an accumulation of clonal B lymphocytes in the blood that does not meet the criteria for CLL (greater than 5000 clonal B cells/μL) but is nevertheless defined by the presence of a clonal population of B cells. Data demonstrate that up to 10% to 15% of patients with lymphocytosis have MBL. Three major subtypes of MBL exist: the CLL immunophenotype ($CD5^+$, $CD23^+$), which is by far the most common; the atypical CLL type ($CD5^+$, $CD20^+$, $CD23^{+/-}$); and non-CLL lymphoproliferative disorder ($CD5^-$). In a systematic study of blood donors more than 45 years of age, 7.1% had detectable MBL. Of these, the majority had the CLL immunophenotype and more than 93% were low-count MBL (B-cell clonal count less than 500 cells/μL). Similar to the relationship between monoclonal gammopathy of uncertain significance and multiple myeloma, only a small proportion of patients with CLL-type MBL (1% to 4% per year) go on to develop progressive disease requiring treatment. Epidemiologic studies of MBL patients have demonstrated strong familial risk for CLL, and the first reports of MBL came from studies of "unaffected" CLL family members. Studies of CLL families suggest that there is an inherited abnormality that increases the risk of developing CLL, and some families exhibit anticipation, with the disease occurring earlier in successive generations. The supposition that MBL is a progenitor lesion for CLL is strengthened by the observation that both low-count and high-count MBL carry the same cytogenetic aberrations observed in good-prognosis CLL. MBL mutations including SF3B1, BIRC3, DDX3X, CHD2, or NOTCH1 had shorter time-to-treatment and expression of CD38, ZAP-70, and CD49d increased the risk of progression. Only del17p and trisomy 12 were independent predictors of need for treatment. MBL has been identified in up to 30% of individuals infected

TABLE 50.1 **Normal Lymphocyte Subsets With Age**[a]

	Cord Blood	2 Days–11 Months	1–6 Years	7–17 Years	18–70 Years
Total lymphocyte count (×10^3 cells/mL)	5.4 (4.2–6)	4.1 (2.7–5.4)	3.6 (2.9–5.1)	2.4 (2.0–2.7)	2.1 (1.6–2.4)
CD4$^+$ T cells (%)	35 (28–42)	41 (38–50)	37 (30–40)	37 (33–41)	42 (38–46)
CD45Ra$^+$ in CD4$^+$ (naive T cells)[b]	91 (82–97)	81 (66–88)	71 (66–77)	61 (55–67)	40 (32–49)
CD8$^+$ T cells (%)	29 (26–33)	21 (18–25)	29 (25–32)	30 (27–35)	35 (31–40)
B cells (%)	20 (14–23)	23 (19–31)	24 (21–28)	16 (12–22)	13 (11–16)
NK cells (%)	20 (14–30)	11 (8–17)	11 (8–15)	12 (9–16)	14 (10–19)

[a] Values are median, with ranges from the 25th to 75th percentiles.
[b] Naive T cells expressed as a percentage of CD4$^+$ T cells.
NK, Natural killer.

with hepatitis C virus (HCV), and up to 50% of the HCV-associated cases demonstrate the atypical CLL immunophenotype. The presence of MBL correlates with more advanced liver disease, suggesting that the persistence of viral infection is crucial to development of the B-cell clone. Follow-up for MBL patients is not clearly defined, although it is probably reasonable to have all but low-count CLL-type MBL patients seen by a hematologist at yearly intervals. For high-count CLL-type MBL, CLL-related risk factors including CD38 and CD49D expression, CLL fluorescence in situ hybridization (FISH) markers, and immunoglobulin heavy chain variable region (IGHV) mutation should be evaluated. Cyclin D1 overexpression or t(11;14) should be examined in atypical CLL type MBL. Conventional karyotyping should be considered in both atypical CLL type MBL and non-CLL type MBL. In addition to standard labs, immunoglobulin titers should be followed in all MBL patients, and live vaccinations should be avoided.[3]

Infectious Causes

The most common infections causing lymphocytosis are Epstein-Barr virus (EBV) and cytomegalovirus (CMV). In young children, EBV infection frequently presents as an upper respiratory infection, but in adolescents and young adults, it can result in acute glandular fever with pharyngitis, splenomegaly, lymphadenitis, and profound reactive lymphocytosis. Lymphocytosis is less prominent in older adults. Although the B cells are targeted by EBV, the lymphocytosis consists of CD8$^+$ lymphocytes reacting to neoantigens expressed on the surface of infected B cells. The massive T-cell response usually clears the infection in a matter of days to 1 week, and the lymphocytosis resolves. CMV infection can produce a similar lymphocytosis. However, in the case of CMV, the macrophages are the target of infection, and the T-cell lymphocytosis results from a response to the macrophage neoantigen. CMV infection and lymphocytosis are more common in older adults. In both viral infections, lymphocytosis can be profound, with 50% or more of the circulating white blood cells (WBCs) identified as lymphocytes. Examination of the peripheral smear reveals that up to 10% of the circulating lymphocytes are atypical and larger than normal lymphocytes with open chromatin and increased cytoplasm. It is particularly important to recognize lymphocytosis caused by these two viruses in pregnant women because congenital infection can cause fetal death and birth defects. In addition to EBV and CMV, primary infection with human immunodeficiency virus (HIV) can cause lymphocytosis and should be suspected in the presence of a viral syndrome in the appropriate clinical circumstance. In children, infection with Coxsackie A and B6 viruses, echovirus, and adenovirus can cause a brief but profound lymphocytosis. Infections with other viruses, including human herpesvirus 6 (HHV-6) and HHV-8 as well as rubella virus, varicella, human T-lymphotropic virus type 1 (HTLV-1), and hepatitis viruses, can cause a lymphocytosis, although much less frequently than CMV and EBV.

Important nonviral infections causing lymphocytosis include *Toxoplasma gondii* and *Bordetella pertussis*. In an immune-competent host, *Toxoplasma* infection is often asymptomatic, but patients can have fever, chills, and lymphadenopathy. Mild lymphocytosis with atypical lymphocytes can be observed. As in the case of EBV and CMV, infections during pregnancy can lead to adverse effects on the fetus. In children and adults, infection with *B. pertussis* can lead to lymphocytosis, with the absolute lymphocyte count frequently greater than 10,000/μL. In more severe cases, lymphocytosis is more pronounced.[4] Unlike viral infections and *Toxoplasma* infection, the lymphocytosis observed in *B. pertussis* infection is caused by increases in all lymphocyte subsets, and it appears that the pertussis toxin blocks migration of the lymphocytes from the bloodstream into lymph nodes.[4] Tuberculosis, rickettsial infection, brucellosis, and shigellosis may also cause lymphocytosis.

Physiologic Stress

Lymphocytosis related to physiologic stress is a poorly studied phenomenon. After strenuous physical exercise, subjects develop lymphocytosis, which returns to preexercise levels within 15 minutes to 1 hour of ceasing the activity. The exercise-induced rise is thought to be attributable to catecholamine and steroid hormones and their effect on expression of cell adhesion molecules and on cardiac output and shear stress.[5] Exposure to catecholamines increases the expression of β_2-adrenergic receptors on lymphocytes influencing cell trafficking. Reports suggest that a number of other physiologic stresses, including surgery, trauma, cardiac conditions, sickle cell crises, abdominal pain, and obstetric emergencies, increase lymphocyte counts. In these cases, all lymphocyte subsets appear to increase, but the increase is most profound for CD4 and CD8 memory T cells. Neutrophil counts also rise in these patients, but in most cases, the lymphocytosis resolves before the peak of the neutrophil count.[6]

Drug Reactions

Drug-induced lymphocytosis can occur as part of a hypersensitivity syndrome. In these cases, the lymphocytosis is usually part of a systemic condition that includes a fever, rash, and lymphadenopathy (drug reaction with eosinophilia and systemic symptoms [DRESS]). Elevation in other WBC counts, including eosinophils and monocytes, is common, and atypical lymphocytes are seen. The period between drug introduction and the syndrome is usually approximately 3 weeks, with the most common implicated drugs being aromatic anticonvulsants and sulfonamides.[7] Some studies differentiate this syndrome from drug-induced cutaneous pseudolymphomas in which collections of nonclonal lymphocytes appear in the skin after longer periods of drug exposure, but there is no peripheral lymphocytosis.

Polyclonal B-Cell Lymphocytosis

A final entity causing lymphocytosis is PPBL. This rare disorder is seen primarily in young to middle-aged women who smoke and results in mild polyclonal lymphocytosis. The lymphocytes are medium sized with abundant cytoplasm and a variable proportion is binucleate. A polyclonal increase in serum immunoglobulin M (IgM) is also observed, and there is an association with the human leukocyte antigen (HLA) antigen D-related 7 (DR7) phenotype. Examination of the B cells reveals that most are CD19$^+$, CD5$^-$, and CD23$^-$, with a normal kappa-to-lambda ratio and a variety of heavy chain rearrangements. Adenopathy, hepatomegaly, or splenomegaly has been observed in some, but not all, patients. Genetic analysis has demonstrated the presence of isochromosome 3q in a proportion of B cells, as well as the presence of multiple B-cell lymphoma immunoglobulin *(BCL2-Ig)* gene rearrangements. Similar gene rearrangements have been identified in family members of PPBL patients, along with increases in serum IgM, suggesting that there may be an underlying genetic defect. In vitro studies have shown that PPBL cells proliferate in a CD40-CD154 culture system and secrete both IgM and IgG (isotype switching). This suggests that PPBL may arise from deregulation of the microenvironment or from a defect in a different B-cell activation pathway resulting in extensive proliferation. Overall, the clinical course of this disorder is benign, and the lymphocytosis is not usually progressive; however, clonal B-cell disorders have been seen in a few patients with the disorder, suggesting that it may represent a preneoplastic state.[8]

Lymphocytopenia

Inherited Disorders

Although there are few inherited causes of lymphocytosis, such is not the case for lymphocytopenia, in which the genetic bases of a number of inherited immunodeficiency disorders have been identified. Chief among these disorders is severe combined immunodeficiency (SCID), which is characterized by the absence of functional T lymphocytes. T lymphocytes, B lymphocytes, and NK cells share progenitors, signaling pathways in development and function, and metabolic pathways; thus B lymphocytes, NK cells, or both are often severely affected in SCID. Moreover, in the absence of functional CD4$^+$ T-helper lymphocytes, B lymphocytes cannot function properly, and hypogammaglobulinemia or agammaglobulinemia is observed. In most cases of SCID, the absence of T lymphocytes leads to an extremely low absolute lymphocyte count. In general, SCID is characterized by complete loss of function of the affected gene, whereas hypomorphic mutations of the same genes lead to quite different phenotypes, including Omenn syndrome and "leaky SCID" in which there is some development of T cells but with limited function and repertoire. Defects in more than 30 genes are known to lead to SCID. Inheritance is primarily X-linked or autosomal recessive, but in a few cases, such as the DiGeorge anomaly and some cases of Hoyeraal-Hreidarsson syndrome (defects in telomerase), inheritance is autosomal dominant. In the past, it was useful to characterize SCID syndromes as $T^-B^+NK^+$, $T^-B^-NK^+$, $T^-B^+NK^-$, or $T^-B^-NK^-$ based on the presence or absence of defects affecting B and/or NK cells. SCID can be classified based on the cellular function which is lacking, including deficiency in cytokine-mediated signaling, defects in V(D)J recombination, absent signaling through the T-cell receptor, defects in antigen presentation, and defects in basic cellular processes; the genetic defects for a number of syndromes are now known. Defects in the interleukin (IL)-7 receptor alpha chain *(IL7RA)*, actin-regulating protein coronin 1A (*CORO1A*), CD3 chain components (*CD3D*, *CD3E*, *CD247*), and CD45 (*PTPRC*) lead to $T^-B^+NK^+$ SCID. $T^-B^+NK^-$ SCID defects include deficiencies in cytokine-mediated signaling (*IL2RG*, *JAK3*). Defects in V(D)J recombination (*RAG1*, *RAG2*) or in nonhomologous end joining for repair of double-strand DNA breaks (*DCLRE1C*, *PRKDC*, *LIG4*, and *NHEJ1* mutations) lead to $T^-B^-NK^+$ SCID. Defects that lead to increased lymphocyte apoptosis (*AK2*, *ADA* gene mutations) lead to $T^-B^-NK^-$ SCID and are often associated with anomalies outside of the immune system. Defects in thymic embryogenesis and calcium flux, as well as a collection of other abnormalities, including defects in telomerase activity, can also lead to SCID and are usually associated with abnormalities of other organ systems.[9]

In addition to SCID, other inherited disorders can also perturb T- and B-cell numbers. Patients with classic Wiskott-Aldrich syndrome, caused by mutations in *WAS* (which encodes a cytoplasmic protein responsible for transducing cell surface signals to the actin cytoskeleton), can present with low T-cell counts early in life and become profoundly lymphopenic over time. Abnormalities in immunoglobulins are also noted in this syndrome, with low levels of IgM and high levels of IgA and IgE.[10] X-linked thrombocytopenia is also due to mutations in *WAS*, although clinical presentation is milder probably due to preservation of partial WAS protein expression. Immunodeficiency affects more than half of all patients with ataxia telangiectasia. Patients with ataxia telangiectasia have homozygous or compound heterozygous mutations in the ataxia-telangiectasia–mutated (*ATM*) gene, which encodes a protein kinase with functions in the cellular response to DNA damage. Lymphopenia, especially of naive CD4 cells, is observed in approximately half of patients with ataxia telangiectasia, with mutations leading to absent expression of ATM kinase activity.[11] Heterozygous germline mutations in *GATA2* lead to a spectrum of clinical syndromes characterized by dendritic cell, monocyte, B cell, and NK lymphoid deficiency (DCML deficiency) with elevated Fms-related tyrosine kinase 3 ligand (Flt3L). Mononuclear cytopenia appears to evolve in diverse clinical groups of *GATA2* mutation, including monoMAC syndrome (monocytopenia, B-cell and NK-cell lymphopenia, mycobacterial, fungal, and viral infections and alveolar proteinosis), Emberger syndrome (lymphedema, deafness, and myelodysplastic syndrome [MDS]), and familial (MDS)/acute myeloid leukemia (AML).[12] *GATA2* mutation appears to cause loss of progenitor cells, clonal hematopoiesis, and elevation of Flt3L, but the molecular mechanisms of marrow failure and transformation to MDS or AML are as yet unclear.

Infections

A variety of viral and nonviral infections can lead to lymphopenia. HIV is the most common virus associated with lymphopenia. The target of HIV is the CD4 receptor, and the virus selectively targets and infects activated expanding CD4 T cells. Large studies in HIV-infected patients have shown that peripheral blood CD4 T-cell counts decrease most rapidly in the year after seroconversion (from approximately 1000/µL before seroconversion to 670/µL at 1 year after infection) and then decline more slowly by approximately 50/µL per year.[13] In a subset of untreated patients, viremia is absent or well controlled, and lymphocytopenia develops very slowly if at all; HLA class I alleles, in particular HLA-B, are overrepresented in this group as are rare variants in genes involved in innate immune sensing, CD4-dependent infectivity, HIV trafficking, and HIV transcription. Lymphopenia is an early and reliable laboratory observation in adult influenza infection and is also detected in infections caused by swine influenza (H1N1) and the highly pathogenic avian influenza (H5N1).[14] Lymphopenia has been reported in patients with severe acute respiratory syndrome (SARS) caused by the SARS-coronavirus. Mild illness due to SARS-CoV-2 infection produces lymphopenia in approximately a third of patients, but early reports from Wuhan, China demonstrate that lymphopenia occurred in more than 80% of critically ill patients. In children, respiratory syncytial virus (RSV) infection is associated with a reduction in lymphocyte count, which is most extreme in the sickest patients; similar effects on lymphocyte counts are seen in measles infections (also a paramyxovirus). In West Nile virus encephalitis, lymphopenia is profound and prolonged, and the initial degree of lymphopenia is predictive of outcome. A variety of other viruses can also cause lymphopenia, including herpes viruses (herpes simplex, HHV-6, HHV-8), parvovirus B19, and Dengue virus. In many viral infections, the degree of lymphopenia is correlated with the severity of the disease.[14]

A variety of nonviral infections causes lymphopenia. Infections with *Ehrlichia* (a tick-borne obligate intracellular gram-negative bacteria), *Salmonella typhi*, and *Leptospira* have all been reported to cause lymphocytopenia during the acute illness. CD4$^+$ T-cell depletion has been described in a subset of HIV-negative patients with tuberculosis and low albumin levels, low body weight, and more extensive disease. Recovery of CD4 count after treatment of tuberculosis suggests that the lymphopenia is caused by the tuberculosis infection.[15] Lymphocytopenia is often observed in sepsis and is thought to occur as a result of cytokine-mediated apoptosis of B cells, CD4 and CD8 T cells, and follicular dendritic cells. In autopsy series, most deaths from sepsis occur during the prolonged hypoimmune state, and the more prolonged the sepsis, the more profound the loss of splenic lymphocytes. In one large retrospective study, severe persistent lymphopenia (defined as an absolute lymphocyte count of less than 600 cells/μL) on the fourth day following a diagnosis of sepsis was predictive of development of secondary infections, as well as short- and long-term survival.[16]

Collagen Vascular Disorders

Autoimmune diseases frequently exhibit decreases in circulating lymphocytes. In systemic lupus erythematosus (SLE), lymphopenia (usually decreases in T cells but occasionally in B cells as well) is not only one of the diagnostic criteria but also a parameter used to assess disease activity. Lymphopenia was observed in more than 60% of patients at diagnosis, with the cumulative incidence over the course of the disease reaching more than 90%. Lymphopenia in SLE seems to be more frequent in patients of African descent, and in one study more than half of patients with lymphopenia demonstrated antilymphocyte antibodies. Antigalectin 8 antibodies have been described in patients with SLE, rheumatoid arthritis, and sepsis. In SLE, these autoantibodies are associated with lymphopenia. Apoptosis may also play a role in lymphopenia in SLE, possibly by upregulation of fas antigen on naive peripheral T cells.[17] CD4 T cells may also be decreased in rheumatoid arthritis, and increasing evidence suggests that deficiencies in DNA repair enzymes such as ATM render rheumatoid arthritis T cells sensitive to apoptosis.[18] Apoptotic loss of naive T cells results in lymphopenia-induced proliferation to preserve T-cell homeostasis; this proliferation is now thought to lead to both premature immune aging and an autoimmune-biased T-cell repertoire. In Sjögren syndrome, a minority of patients has been noted to have deficient CD4 counts, and this has been correlated with the presence of anti-CD4 antibodies. Similarly, low lymphocyte counts have been observed in patients with primary vasculitides, type 1 diabetes, and Crohn disease.[17]

Malignancies

Lymphopenia is found in a variety of systemic illnesses; chief among them are cancers. In hematologic malignancies, including Hodgkin lymphoma, diffuse large B-cell lymphoma, and peripheral T-cell lymphoma, patients with lymphopenia have a worse prognosis. Lymphopenia has also been observed in solid tumors, including breast and colon cancer, and soft tissue sarcomas, in which its presence before treatment predicts decreased overall survival. Which specific lymphocyte subsets are involved and the cause(s) of lymphopenia in these tumor types have not been described.

Systemic Disorders

End-stage renal disease (ESRD) has also been associated with lymphopenia, an observation not thought to be exclusively attributable to an effect of dialysis alone. Naive and central memory CD4 and CD8 T cells are significantly reduced in the blood of ESRD patients, apparently because of increased susceptibility of these cells to apoptosis. Lymphopenia occurs in more than 50% of sarcoidosis patients and is associated with chronic disease. Sarcoidosis patients with severe organ system involvement, including neurologic, cardiac, ocular, and advanced pulmonary disease, have lower lymphocyte counts than patients with less severe manifestations (see E-Slide VM03953). Older studies suggest that burn victims have profound decreases in T-cell counts, which may contribute to the infection risk in these patients. Interestingly, lymphopenia is a characteristic of both protein-energy malnutrition and zinc deficiency. Both of these deficiencies perturb the hypothalamic-pituitary-adrenocorticoid axis and increase glucocorticoid levels, which results in increased apoptosis of B and T cells.[19] Intestinal lymphangiectasia, which may be either congenital or secondary to processes that obstruct lymphatic drainage of the gastrointestinal tract, can cause lymphopenia as a result of loss of lymph fluid into the gut along with lymphocytes.

A rare cause of lymphopenia is idiopathic CD4$^+$ lymphocytopenia (ICL), which is defined as a CD4 count less than 300/μL or less than 20% of the T-cell count on two occasions that is not caused by HIV or HTLV infection, drug therapy, or a known immunodeficiency. Patients usually come to clinical notice when they present with opportunistic infections. CD4 T-cell counts typically remain low, but counts do not continue to drop or do not decrease rapidly after diagnosis. In one large series, only approximately 20% of patients recovered from lymphopenia within 3 years of diagnosis. Opportunistic infections plague these patients, and autoimmune diseases occur both before and after the diagnosis of ICL. In ICL, unlike HIV infection, increased activation and turnover are observed in CD4 but not CD8 T lymphocytes.[20]

Drug Effects

A number of drugs are known to cause lymphopenia. Glucocorticoids inhibit production of a number of cytokines and rapidly deplete circulating T cells by enhancing emigration from the circulation, inducing apoptosis, interfering with growth signaling, and inhibiting release from lymphoid tissues. B cells are less affected acutely by glucocorticoid administration, but prolonged administration may result in decreased IgG levels. In clinical trials for multiple sclerosis, delayed-release dimethyl fumarate resulted in a decrease in mean lymphocyte count of approximately 30% in the first year, but approximately 2% of patients had lymphocyte counts less than 500 cells/μL, which persisted for 6 months or longer. Antimetabolite chemotherapeutic agents such as methotrexate, azathioprine, and 6-mercaptopurine lower lymphocyte as well as neutrophil counts, and alkylating agents such as cyclophosphamide have more profound effects on lymphocytes. Purine nucleoside analogs, including cladribine, fludarabine, pentostatin, nelarabine, clofarabine, and others, inhibit DNA synthesis and repair and cause accumulation of DNA strand breaks. All of these agents are associated with profound lymphopenia, which may persist for several years after completion of treatment. In general, T cells are more affected than B cells. Nelarabine in particular is a prodrug of ara-G and is converted to arabinosylguanine triphosphate (ara-GTP), which accumulates at higher levels in T cells.[21]

Monoclonal and polyclonal antibodies directed against lymphocytes are useful in clinical practice and can induce profound and long-lasting lymphopenia. Antithymocyte globulin and alemtuzumab produce depletion of both T and B cells, whereas the monoclonal antibodies OKT3, daclizumab, and basiliximab produce a more pronounced decrease in T cells. The drugs rituximab and ofatumumab are monoclonal antibodies directed against distinct epitopes on B cells. These drugs deplete peripheral B cells but do not routinely produce lymphopenia.

Finally, radiation exposure commonly results in lymphopenia, which occurs before depression of other cell counts. In fact, lymphopenia can develop within the first 24 hours of exposure if the dose of radiation is great enough. Irradiation of the bone marrow, spleen, lymph nodes, and the tumor can all produce lymphopenia. The spleen and lymph nodes are lymphocyte reservoirs and sites for clonal expansion of lymphocytes to specific antigens. Lymphocytes circulating through the radiation portal receive a dose of radiation with each fraction which can result in lymphopenia, particularly when one considers that the D_{50} (dose required for inactivation of 50% of cells) for lymphocytes is only approximately 2 gray. Some patients never recover their lymphocyte count, and this is thought to be due both to the radiation-related destruction of lymphocytes and lack of a surge in compensatory cytokines IL-7 and IL-15 to produce clonal expansion of lymphocytes, maturation, and formation of memory cells.[22]

QUANTITATIVE DISORDERS OF IMMUNOGLOBULINS

In practice, as there are several methods for determining serum immunoglobulin levels, it is critical that age-adjusted normal reference ranges are provided by the laboratory when evaluating immunoglobulin level results. Children first achieve adult levels of IgM around 2 years of age, IgG by 6 years of age, and IgA during puberty. Hypogammaglobulinemia is defined as an IgG more than two standard deviations less than the normal mean or less than 450 to 500 mg/dL, and agammaglobulinemia is usually defined as an IgG level less than 100 mg/dL. Low levels of IgA, IgG, and IgM are characteristic of most forms of SCID.

Hypogammaglobulinemia

Causes of hypogammaglobulinemia can be divided into primary causes related to genetic deficiencies and secondary causes related to malignancies or their treatment, infections, medications, and protein-losing states that deplete antibody.

Secondary Causes

Before embarking on a search for a primary disorder causing hypogammaglobulinemia, it is important to rule out secondary causes. Malnutrition, malabsorption, and any disease state in which large amounts of protein are lost, such as nephrotic range proteinuria, severe burns, lymphangiectasia, or protein-losing enteropathy, can overwhelm the capacity of the B cells to provide adequate immunoglobulin to maintain normal serum levels. Aside from chemotherapeutic agents, a number of medications can produce hypogammaglobulinemia; these include captopril, antiseizure medications (carbamazepine, phenytoin), gold salts, antimalarials, fenoclofenac, penicillamine, and sulfasalazine, as well as glucocorticoids. Infection with HIV and EBV, and congenital infection with rubella, CMV, and *T. gondii* can produce very low immunoglobulin levels and should be ruled out as appropriate. A host of lymphoid malignancies produce hypogammaglobulinemia, but the defect is most profound in CLL, in which up to 70% of patients are said to possess hypogammaglobulinemia even in the absence of treatment, and several studies suggest that this hypogammaglobulinemia at diagnosis is an independent predictor of poor survival.[23]

Primary Immunodeficiencies

The International Union of Immunological Societies has classified the primary immunodeficiency diseases into nine groups, including one class called *predominantly antibody deficiencies*.[9] These disorders present in both adults and children, although some are very rare. Table 50.2 provides a simple grouping of these antibody disorders.

The first group is characterized by a severe reduction in all serum immunoglobulin isotypes, with absence or profound reduction in B cells. Patients with these disorders, particularly those with a well-defined genetic basis, present with severe bacterial infections, most commonly in the respiratory tract (pneumonia, sinusitis, and otitis media), as well as diarrhea caused by bacteria, parasites, and viruses. The prototypic member of this group is X-linked agammaglobulinemia (XLA) caused by a mutation in the Bruton tyrosine kinase (*Btk*) gene, which produces a block in B-cell maturation. Female carriers of a *Btk* mutation are generally asymptomatic, but most boys with XLA come to clinical attention by the age of 1 year. In addition to low-serum immunoglobulins, a clue to the diagnosis is the absence of lymphoid tissue, including tonsils. Autosomal recessive mutations in the μ heavy chain as well as defects in *λ5*, *Igα*, *Igβ*, and *BLNK* produce a similar phenotype. Phosphatidylinositol 3-kinase deficiency (*PIK3R1*) and heterozygous mutation of the E47 transcription factor deficiency (*TCF3*) can cause severe reduction in all serum immunoglobulin isotypes. In a small percentage of patients, no clear molecular defect can be identified. Also included in this group are thymoma with immunodeficiency (Good syndrome) and myelodysplasia. Good syndrome is a poorly understood disorder that presents primarily in middle-aged adults. Immunodeficiency can precede or follow the diagnosis of thymoma and does not resolve with thymectomy. In addition to infections, patients experience autoimmune phenomena, including myasthenia gravis, immune thrombocytopenia purpura, pure red blood cell aplasia, and pernicious anemia. MDSs can also mimic XLA and generally present with low B cells and pancytopenia with monosomy 7, trisomy 8, or dyskeratosis congenita.[24]

A second group is characterized by severe reduction of at least two serum immunoglobulin isotypes with normal or low numbers of B cells. Most patients in this group can be categorized as having common variable immune deficiency (CVID), a heterogeneous disorder characterized by recurrent infection and failure to make antibody to vaccine antigens. Both males and females are affected, and patients can have autoimmune and gastrointestinal disease as well as lymphoproliferative disorders. Autoimmune disease can precede the hypogammaglobulinemia. Approximately 10% of patients with a CVID phenotype have a family history of immunodeficiency. Homozygous or compound heterozygous mutations in *ICOS* (inducible costimulator), which is expressed on activated T cells and plays a role in activating T-helper cells and providing B-cell help, and *CD19*, a B-cell surface molecule that participates in signaling after antigen binding to the B-cell receptor, have been shown to result in recurrent infections in childhood and hypogammaglobulinemia. Mutations in *TACI* (transmembrane activator and CAML-interactor) and *BAFF-R* (B-cell–activating factor receptor) members of the tumor necrosis factor (TNF) receptor superfamily, which play roles in B-cell survival and antibody production, and *MSH5*, a mismatch repair gene, are thought to predispose to CVID

TABLE 50.2 **Predominant Antibody Deficiencies**

	Disease	Mode of Inheritance/ Genetic Locus	Clinical Features
Severe reduction in all serum immunoglobulin isotypes with absent B cells	Bruton tyrosine kinase deficiency	XL/Xq21.3–22	Severe bacterial infections (especially of the respiratory tract), absent lymphoid tissue
	μ heavy chain deficiency	AR/14q32.3	Severe bacterial infections
	λ5 deficiency	AR/22q11.21	Severe bacterial infections
	Igα deficiency	AR/19q13.2	Severe bacterial infections
	Igβ deficiency	AR/17q23	Severe bacterial infections
	BLNK deficiency	AR/10q23.2	Severe bacterial infections
	PIK3R1	AR/5q13.1	Recurrent bacterial infection
	E47 transcription factor deficiency	AD/19p13.3	Recurrent bacterial deficiencies

Continued

TABLE 50.2 **Predominant Antibody Deficiencies—cont'd**

	Disease	Mode of Inheritance/ Genetic Locus	Clinical Features
Severe reduction in at least two serum immunoglobulin isotypes with low or normal B-cell numbers	Common variable immunodeficiency syndromes	≈10% with family history AR or AD	Recurrent respiratory tract infections leading to chronic sinusitis, hearing loss, bronchiectasis, autoimmune disease, lymphoproliferation, malignancy (especially non-Hodgkin lymphoma and gastric carcinoma)
	TACI alterations	AD and AR/17p11.2	
	BAFFR alterations	AR/22q13	
	CD19 deficiency	AR/16p11.2	Recurrent infections
	CD81 deficiency	AR/11p15.5	Recurrent infections
	CD20 deficiency	AR/11q12.2	Recurrent infections
	CD21 deficiency	AR/1q32.2	Recurrent infections
	TNSF12 deficiency	AD/17p13.1	Recurrent bacterial infections, thrombocytopenia, neutropenia
	NFKB1 deficiency	AD/4q24	Recurrent infections, COPD, immune cytopenias
	NFKB2 deficiency	AD/10q24.32	Recurrent infections
	PIK3CD gain of function	AD/1p36.22	Severe bacterial infections
	PIK3R1 deficiency	AD/5q13.1	Severe bacterial infections
	PTEN deficiency	AD/10q23.31	Lymphoproliferations, autoimmune disease.
	MOGS deficiency	AR/2p13.1	Recurrent bacterial and viral infections, severe neurologic disease
	TRNT1 deficiency	AR/3p26.2	Congenital sideroblastic anemia, deafness, developmental delays
	TTC37 deficiency	AR/5q15	Recurrent bacterial and viral infections, abnormal hair
	IKAROS deficiency	AD/7p12.2	Recurrent infections
	IRF2BP2 deficiency	AD/1q42.3	Recurrent infections, autoimmune disease
	ATP6AP1 deficiency	X-linked	Liver disease, leukopenia, low copper
Severe reduction in serum IgG and IgA with increased IgM and normal B-cell numbers (disorders of immunoglobulin class switching)	AID deficiency	AR/12p13	Recurrent bacterial infections and diarrhea, marked enlargement of lymphoid organs
	UNG deficiency	AR/12q23–q24.1	Recurrent bacterial infections and diarrhea, marked enlargement of lymphoid organs
	INO80	AR/15q15.1	Bacterial infections
	MSH6	AR/2p16.3	Cancer history in patient or family
Isotype or light-chain deficiencies with normal B-cell numbers	Ig heavy-chain deficiency	AR/14q32	Most patients are healthy
	κ-chain deficiency	AR/2p11.2	Most patients are healthy
	Isolated IgG subclass deficiency	Variable/unknown	Most patients are healthy
	IgA deficiency associated with IgG subclass deficiency	Variable/unknown	Most patients are healthy
	Selective IgA deficiency	Variable/unknown	Most patients asymptomatic, but increased prevalence of infections, autoimmune disease, atopy, and celiac disease
	Specific antibody deficiency with normal immunoglobulin level and B-cell number	Variable/unknown	Recurrent sinopulmonary infection, bronchiectasis, diarrhea, autoimmune disease
	Inability to make antibodies to specific antigens		
	Transient hypogammaglobulinemia of infancy IgG and IgA deficiency	Variable/unknown	IgG and IgA deficiency. More likely to be male (60%–80%), mild infections and diarrhea, atopy
	CARD11 gain of function	AD/7p22.2	Large spleen, lymphadenopathy, poor response to vaccines
	Selective IgM deficiency	Unknown	Bacterial infections

λ5, Immunoglobulin lambda-like polypeptide (a surrogate light chain subunit that is part of the pre–B-cell receptor); *AID*, activation-induced cytidine deaminase (thought to be essential for initiation of the DNA cleavage required for class-switch recombination and somatic hypermutation); *ATP6AP1*, ATPase, proton transporting, lysosomal, accessory protein 1 (required for luminal acidification of secretory vesicles); *BAFF-R*, B-cell–activating factor receptor; *BLNK*, B linker (a cytoplasmic linker or adaptor protein that plays a critical role in B-cell development); *CARD11*, caspase recruitment domain-containing protein 11; *COPD*, chronic obstructive pulmonary disease; *Ig*, immunoglobulin; *Igα*, immunoglobulin-associated α (necessary for expression and function of the B-cell antigen receptor); *Igβ*, immunoglobulin-associated β (necessary for expression and function of the B-cell antigen receptor); *IKAROS*, a hematopoietic zinc finger transcription factor; *INO80*, the catalytic ATPase subunit of the INO80 chromatin remodeling complex; *IRF2BP2*, interferon regulatory factor 2-binding protein 2; *MOGS*, Mannosyl-oligosaccharide glucosidase; *MSH6*, member of heterodimer required for mismatch-specific binding; *NFκB*, nuclear factor kappa-B; *PI3K-γ*, protein kinase C family member (critical for regulation of cell survival, proliferation, and apoptosis); *PTEN*, phosphatase and tensin homolog (tumor suppressor that antagonizes the PI3K pathway); *TACI*, transmembrane activator and CAML-interactor; *TNSF12*, tumor necrosis factor ligand superfamily, member 12 (weak inducer of apoptosis); *TRNT1*, tRNA nucleotidyltransferase, *CCA-adding*, 1 (adds the CCA terminus to the 3-prime end of tRNA precursors); *TTC37*, tetratricopetide repeat domain-containing protein 37; *UNG*, uracil *DNA* glycosylase (allows creation of single-stranded breaks essential to class switch recombination and somatic hypermutation).

and IgA deficiency but are not sufficient to independently cause their onset. X-linked lymphoproliferative syndrome caused by mutation in the signaling lymphocyte activation molecular-associated protein SAP (gene, *SH2D1A*) can present atypically or later in life with a CVID phenotype.[25] Single-gene mutations of *TNSF12* (*TWEAK*), *NFKB2*, and *CXCR4* have also been demonstrated to cause low levels of immunoglobulins. Autosomal dominant mutations in *PTEN, NFKB1, IKZF1, and IRF2BP2*, and X-lined mutations in *ATP6AP1* have been shown to result in a CVID phenotype and have been added to Table 50.2. Unlike most primary immune deficiencies for which a single gene defect has been identified, CVID is heterogeneous and no genetic defect has been identified in 90% of CVID patients. Class-switch recombination defects encompass a third group of antibody deficiencies and result in hyper-IgM syndrome characterized by reductions in serum IgG and IgA with normal or elevated IgM. X-linked hyper-IgM syndrome due to mutations in the CD40 ligand (*CD40LG*) gene are no longer included in this category because this disease has defects in cellular immunity that now place it into the category of combined immunodeficiency. Defects in the activation-induced cytidine deaminase gene (*AID*) produce hypogammaglobulinemia with recurrent bacterial infections and diarrhea, as well as enlarged lymphoid organs filled with proliferating B cells. A similar clinical picture is produced by homozygous defects in uracil-DNA glycosylase (*UNG*). AID is thought to deaminate cytosine to uracil, and UNG subsequently deglycosylates and removes the uracil residue, creating an abasic site, which allows for creation of single-stranded DNA breaks. Deficiencies in these enzymes result in defective class switching and somatic hypermutation. Patients with similar phenotypes but without defects in *AID* or *UNG* have been described and include mutations in *INO80*, a member of the chromatin remodeling complex and *MSH6* involved in both the induction and repair of DNA double-strand breaks in switch regions.[26]

Additional groups of antibody deficiencies exist in which overall antibody levels are normal, but there are defects in specific isotypes, light chains, or specific antibodies with normal numbers of B cells; the majority of patients with these deficiencies are healthy. The most common member of these groups is selective IgA deficiency, which occurs in approximately 1 in 500 white individuals. The molecular mechanisms underlying this deficiency are unknown, but there is an association with CVID. Finally, an entity termed *transient hypogammaglobulinemia of infancy* has been described in which the normal decline in immunoglobulins after maternal transfer is prolonged. This entity is poorly understood and usually affects boys, who have mild infections and diarrhea. Recovery usually, but not always, occurs by 3 years of age.[27]

Hypergammaglobulinemia

Hypergammaglobulinemia results from an overproduction of immunoglobulins by plasma cells, either monoclonal and reflective of a plasma cell or lymphoproliferative disorder, or polyclonal and accompanying other disease states. The distinction between polyclonal and monoclonal disorders is made by inspection of the serum protein electrophoretic pattern. Detection of one or several monoclonal bands within a polyclonal background is not unusual, and the literature suggests that small bands frequently disappear and do not become clinically relevant. No data are available to suggest that polyclonal gammopathy drives development of monoclonal gammopathy; however, if a clonal plasmaproliferative disorder is suspected, immunofixation or immunoelectrophoresis should be performed.

Disorders Producing Polyclonal Gammopathy

Polyclonal gammopathy usually reflects one of five major disorders, including liver disease, connective tissue disorders, infections, hematologic disorders, and solid tumors.[28] IL-6 and IL-10 have been implicated in polyclonal gammopathy, as have defects in T cells and chronic antigenic stimulation, but the exact sequence of events leading to polyclonal B-cell activation is not known. In general, treatment is directed at the underlying disease, but there are reports of polyclonal gammopathy leading to symptomatic hyperviscosity. In these cases, plasmapheresis and/or corticosteroids seem to be effective. It should also be noted that polyclonal elevations in serum immunoglobulins can sometimes interfere with the direct Coombs test, possibly via nonspecific antibody binding to red blood cells. The degree of gamma globulin elevation does not seem to be helpful in defining the underlying disease state.

In the largest recent review of polyclonal gammopathy, the majority of patients had liver disease, which covered the spectrum from autoimmune disorders (autoimmune hepatitis, primary biliary cirrhosis, and primary sclerosing cholangitis) to viral hepatitis, and alcoholic liver disease. In fact, elevation of serum gamma globulins is a distinguishing characteristic of autoimmune hepatitis, and the levels usually correlate with activity of disease. The most common etiology for polyclonal gammopathy related to liver disease in the United States is likely infection with HCV, but in any particular clinical setting, the exact distribution likely depends on the population demographic. Other diseases affecting the liver, such as α_1-antitrypsin deficiency and hemochromatosis, are also accompanied by increases in serum immunoglobulins.

Connective tissue diseases, including Sjögren syndrome, SLE, ankylosing spondylitis, and rheumatoid arthritis, are also accompanied by polyclonal gammopathy. In many of these diseases, the degree of gamma globulin elevation may reflect disease activity, although a causative link between the autoimmune phenomena and immunoglobulin levels has not been determined. Several of the periodic fever syndromes, which are sometimes classified with the connective tissue disorders, have elevated immunoglobulin levels as a part of their manifestations. Hyperimmunoglobulinemia D is one such syndrome and is linked to mutations in the mevalonate kinase gene. Patients with this disorder present in the first year of life with febrile attacks, lymphadenopathy, abdominal symptoms, arthritis, and oral and genital ulcers. Most, but not all, patients have elevated levels of polyclonal IgD during and between episodes often accompanied by elevated IgA levels. TNF receptor 1-associated periodic syndrome (TRAPS) results from mutation in the gene (*TNRFRSF1A*) for the TNF receptor 1 and can cause prolonged febrile attacks with abdominal pain, arthralgias, and myalgias. During attacks, polyclonal elevation of immunoglobulins (primarily IgA) is observed.[29]

Infections, usually chronic in nature, are frequently accompanied by polyclonal gammopathy. HIV infection is a common cause, and immunoglobulin levels tend to increase slowly until the diagnosis of acquired immunodeficiency syndrome (AIDS) and then decline over the ensuing 6 to 18 months. Polyclonal gammopathy can be a clue to occult infections such as subacute bacterial endocarditis, tuberculosis, perinephric abscess, Lyme disease, and a variety of parasitic infections.

Malignant B- and T-cell disorders can cause polyclonal hypergammaglobulinemia. These diseases include CLL; large granular lymphocytic leukemia; hairy cell leukemia; and angioimmunoblastic T-cell lymphoma (AITL), a rare disease characterized by rash, widespread lymphadenopathy and extranodal involvement, autoimmune phenomena, and polyclonal gammopathy. Interestingly, despite the elevated levels of immunoglobulins observed in AITL, patients exhibit immunodeficiency and a propensity to develop opportunistic infections. Patients with myeloid disorders can also have polyclonal gammopathies; in one large series, nearly 40% of patients with MDS had hypergammaglobulinemia, and patients with immunologic abnormalities had inferior survival.[30] The incidence of hypergammaglobulinemia is reported to approach 50% in chronic myelomonocytic leukemia (CMML). Hypergammaglobulinemia has been reported in AML both in adults and children, but it appears to be a rare phenomenon. Among solid tumors, ovarian and hepatocellular cancers are most commonly associated with polyclonal gammopathy. There are case reports of cancers, particularly lung and breast tumors, producing and releasing the secretory component (SC) of IgA into the bloodstream with the binding of SC to polyclonal IgA, producing hypergammaglobulinemia of serum sIgA.

A variety of other diseases, including asbestos exposure and several subtypes of hypersensitivity pneumonitis and idiopathic interstitial pneumonia, are associated with polyclonal gammopathy. In general, these disorders represent diffuse activation of B cells. Of note, there are reports of polyclonal gammopathy resulting in symptomatic hyperviscosity and these cases have been treated successfully with corticosteroids or plasmapheresis.

Disorders Producing Monoclonal Gammopathy

The differential diagnosis of monoclonal gammopathy includes monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma, solitary plasmacytoma of bone or extramedullary plasmacytoma, Waldenström macroglobulinemia, lymphoma, CLL, and primary systemic amyloidosis. These individual disorders are described elsewhere in this text; however, there are a few points to be made about the M protein itself and MGUS. These disorders can produce intact immunoglobulins (IgG, IgM, IgD, or IgE), κ-, or λ-light chains alone or in combination with intact immunoglobulins, and rarely heavy chains only. The monoclonal protein is usually detected as a discrete band in the γ or β region in serum or urine protein electrophoresis (M spike) and then characterized and confirmed by immunofixation electrophoresis (IFE). Monoclonal antibodies have been associated with a wide variety of bacterial antigens as well as various other antigens, including thyroglobulin, von Willebrand factor, and lactate dehydrogenase; however, for most M proteins, the antigen is not recognized. A variety of other disorders are also associated with an M protein (Table 50.3), including connective tissue

TABLE 50.3 Diseases Associated With Monoclonal Gammopathy

Plasma cell and related disorders	MGUS	See Chapters 89–92
	Solitary plasmacytoma:	
	Bone	
	Soft tissue	
	Multiple myeloma	
	Waldenström macroglobulinemia	
	Primary amyloidosis	
Lymphoid disorders	Non-Hodgkin lymphoma	Monoclonal protein observed in CLL (>20% of cases with IgM, ≈50% with IgG, light chains also observed), extranodal marginal zone lymphomas (>30% of cases and correlated with BM involvement), follicular, mantle cell, and diffuse large B-cell lymphomas also reported with serum M proteins as has AITL
	Hodgkin lymphoma	Rare but reported
	Castleman disease	<2% with monoclonal gammopathy
Other hematologic disorders	Acquired von Willebrand disease	IVIG more effective than factor concentrate in increasing factor VIII coagulant and VWF levels
	Gaucher disease	Observed in 25% in one study; M protein declined after splenectomy
	Pernicious anemia, pure RBC aplasia, hereditary spherocytosis, MPD, MDS	
Connective tissue disorders	SLE	IgG, IgM, and IgA have been observed, no difference in disease activity or outcome
	Inclusion body myositis	80% with IgG M protein
	Polymyositis, RA, scleroderma	
Neurologic disorders	POEMS syndrome	Most have M-protein of λ light chain
	Peripheral neuropathy	Most common is IgM followed by IgG and IgA
		In half, IgM protein binds to myelin-associated glycoprotein
		Size of M protein not correlated with severity of neuropathy
		Some benefit from plasma exchange for those with IgG and IgA
		Fludarabine and rituximab with some benefit for IgM
	Myasthenia gravis, ALS, Alzheimer disease	
Dermatologic disorders	Schnitzler syndrome	Neutrophilic urticarial dermatitis, monoclonal IgM protein, and two of: lymphadenopathy, fever, hepatosplenomegaly, joint pain, increased ESR, increased neutrophils, or abnormal bone imaging
	Scleroderma	
	Pyoderma gangrenosum	Frequently an IgA protein
Infections	HIV	Both IgG and IgM M proteins observed
	HCV	M protein present in up to 10% of patients
Immunosuppression	Renal transplant	In children CMV infection associated with M protein
	Liver and heart transplant	Most patients with posttransplant lymphoproliferative disorders have M proteins
	BM transplant	Observed in both autologous and allogeneic transplants
		Appearance of M protein correlated with GVHD

AITL, Angioimmunoblastic T-cell lymphoma; *ALS*, amyotrophic lateral sclerosis; *BM*, bone marrow; *CLL*, chronic lymphocytic leukemia; *CMV*, cytomegalovirus; *ESR*, erythrocyte sedimentation rate; *GVHD*, graft-versus-host disease; *HCV*, hepatitis C virus; *HIV*, human immunodeficiency virus; *Ig*, immunoglobulin; *IVIG*, intravenous immunoglobulin; *MGUS*, monoclonal gammopathy of uncertain significance; *MDS*, myelodysplastic syndrome; *MPD*, myeloproliferative disorder; *POEMS*, polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes; *RA*, rheumatoid arthritis; *RBC*, red blood cell; *SLE*, systemic lupus erythematosus; *VWF*, von Willebrand factor.

disorders, neurologic disorders (including POEMS [polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes] syndrome), renal disorders, and some infections such as HCV and HIV. Patients undergoing bone marrow and solid-organ transplants in which there is immune suppression are also occasionally observed to have M proteins, but these are usually transient and disappear with recovery of the immune system. Acquired immune disorders such as acquired C1 inhibitor deficiency, type 2 acquired angioedema, and acquired von Willebrand syndrome have also been associated with M proteins.

REFERENCES

1. Morbach H, Eichhorn EM, Liese JG, et al. Reference values for B cell subpopulations from infancy to adulthood. *Clin Exp Immunol*. 2010;162(2):271–279.
2. Erkeller-Yuksel FM, Deneys V, Hulstaert F, et al. Age-related changes in human blood lymphocyte subpopulations. *J Pediatr*. 1992;120(2 Pt 1):216–222.
3. Maitre E, Troussard X. Monoclonal B-cell lymphocytosis. *Best Pract Res Clin Haematol*. 2019;32(3):229–238.
4. Hudnall SD, Molina CP. Marked increase in L-selectin-negative T cells in neonatal pertussis: the lymphocytosis explained? *Am J Clin Pathol*. 2000;114(1):35–40.
5. Walsh NP, Gleeson M, Shephard RJ, et al. Position statement. Part one: immune function and exercise. *Exerc Immunol Rev*. 2011;17:6–63.
6. Karandikar NJ, Hotchkiss EC, McKenna RW, et al. Transient stress lymphocytosis: an immunophenotypic characterization of the most common cause of newly identified adult lymphocytosis in a tertiary hospital. *Am J Clin Pathol*. 2002;117(5):819–825.
7. Phillips EJ, Chung WH, Mockenhaupt M, et al. Drug hypersensitivity: pharmacogenetics and clinical syndromes. *J Allergy Clin Immunol*. 2011; 127(3 Suppl):S60.
8. Tesson B, Huet S, Grange B, et al. Absence of driver mutations in persistent polyclonal B-cell lymphocytosis with binucleated lymphocytes. *Blood*. 2017;130(10):1267–1269.
9. Picard C, Bobby Gaspar H, Al-Herz W, et al. International Union of Immunological Societies: 2017 Primary Immunodeficiency Diseases Committee Report on Inborn Errors of Immunity. *J Clin Immunol*. 2018;348(1):96–128.
10. Rivers E, Worth A, Thrasher AJ, Burns SO. How I manage patients with Wiskott-Aldrich syndrome. *Br J Haematol*. 2019;185(4):647–655.
11. Amirifar P, Ranjouri MR, Yazdani R, et al. Ataxia-telangiectasia: a review of clinical features and molecular pathology. *Pediatr Allergy Immunol*. 2019;30(3):277–288.
12. Dickinson RE, Milne P, Jardine L, et al. The evolution of cellular deficiency in GATA2 mutation. *Blood*. 2014;123(6):863–874.
13. Stein DS, Korvick JA, Vermund SH:. CD4$^+$ lymphocyte cell enumeration for prediction of clinical course of human immunodeficiency virus disease: a review. *J Infect Dis*. 1992;165(2):352–363.
14. Yuen KY, Chan PK, Peiris M, et al. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. *Lancet*. 1998;351(9101):467–471.
15. Chedid C, Kokhreidze E, Tukvadze N, et al. Association of baseline white blood cell counts with tuberculosis treatment outcome: a prospective multicentered cohort study. *Int J Infect Dis*. 2020;100:199–206.
16. Drewry AM, Samra N, Skrupky LP, et al. Persistent lymphopenia after diagnosis of sepsis predicts mortality. *Shock*. 2014;42(5):383–391.
17. Fayaz A, Igoe A, Kurien BT, et al. Haematological manifestations of lupus. *Lupus Sci Med*. 2015;2(1):e000078.
18. Shao L, Fujii H, Colmegna I, et al. Deficiency of the DNA repair enzyme ATM in rheumatoid arthritis. *J Exp Med*. 2009;206(6):1435–1449.
19. Prasad AS. Zinc in human health: effect of zinc on immune cells. *Mol Med*. 2008;14(5–6):353–357.
20. Zonios DI, Falloon J, Bennett JE, et al. Idiopathic CD4$^+$ lymphocytopenia: natural history and prognostic factors. *Blood*. 2008;112(2):287–294.
21. Robak T, Lech-Maranda E, Korycka A, et al. Purine nucleoside analogs as immunosuppressive and antineoplastic agents: mechanism of action and clinical activity. *Curr Med Chem*. 2006;13(26):3165–3189.
22. Venkatesulu BP, Mallick S, Lin SH, Krishnan S. A systematic review of the influence of radiation-induced lymphopenia on survival outcomes in solid tumors. *Crit Rev Oncol Hematol*. 2018;123:42–51.
23. Andersen MA, Vojdeman FJ, Andersen MK, et al. Hypogammaglobulinemia in newly diagnosed chronic lymphocytic leukemia is a predictor of early death. *Leuk Lymphoma*. 2016;57(7):1592–1599.
24. Conley ME, Dobbs AK, Farmer DM, et al. Primary B cell immunodeficiencies: comparisons and contrasts. *Annu Rev Immunol*. 2009;27:199–227.
25. Aggarwal V, Banday AZ, Jindal AK, et al. Recent advances in elucidating the genetics of common variable immunodeficiency. *Genes Dis*. 2019;7(1): 26–37.
26. Qamar N, Fuleihan RL. The hyper IgM syndromes. *Clin Rev Allergy Immunol*. 2014;46(2):120–130.
27. Moschese V, Graziani S, Avanzini MA, et al. A prospective study on children with initial diagnosis of transient hypogammaglobulinemia of infancy: results from the Italian Primary Immunodeficiency Network. *Int J Immunopathol Pharmacol*. 2008;21(2):343–352.
28. Dispensieri A, Gertz M, Therneau T, et al. Retrospective cohort study of 148 patients with polyclonal gammopathy. *Mayo Clin Proc*. 2001;76(5):476–487.
29. Ozen S, Bilginer Y. A clinical guide to autoinflammatory diseases: familial Mediterranean fever and next-of-kin. *Nat Rev Rheumatol*. 2014;10(3): 135–147.
30. Mosalpuria K, Bociek RG, Vose JM. Angioimmunoblastic T-cell lymphoma management. *Semin Hematol*. 2014;51(1):52–58.

CHAPTER 51

DISORDERS OF PHAGOCYTE FUNCTION

Mary C. Dinauer and Thomas D. Coates

Phagocytic leukocytes are an essential component of the innate immune system that has evolved to rapidly respond to the presence of invading bacteria, fungi, and parasites. This first line of host defense also includes natural killer (NK) lymphocytes, complement, and other plasma proteins. As reviewed in Chapters 28 and 49, phagocytes are responsible for ingesting, killing, and digesting pathogens. Granulocytic phagocytes (neutrophils and eosinophils) circulate in the bloodstream until they sense chemotactic signals from infected tissues, resulting in adhesion to the vascular endothelium and subsequent migration into the site of infection.[1] In contrast, mononuclear phagocytes (macrophages and their circulating precursor, the monocyte) function primarily as resident cells in a variety of tissues such as the lungs, liver, peritoneal cavity, and spleen, where they perform a surveillance role and also interact closely with lymphocytes to promote specific immune responses. Microbial killing is accomplished by two types of mechanisms: (1) de novo synthesis of highly toxic and often unstable derivatives of molecular oxygen by an enzyme known as *respiratory burst oxidase* and (2) preformed polypeptide "antibiotics" and proteases stored within several types of lysosomal granules that are delivered into phagocytic vacuoles containing the ingested microbes.

This chapter reviews the major congenital and acquired disorders of phagocyte function, which from the clinical standpoint largely involve neutrophils. As would be predicted, these disorders manifest clinically by recurrent bacterial and fungal infections, often with atypical pathogens or unusual presentations. Interestingly, the converse of this is only rarely observed. Most patients with recurrent infections do not have any identifiable abnormality in their phagocytes. There are at least two explanations for the clinical rarity of phagocyte disorders. First, given their critical role in host defense, nature may be quite intolerant of major abnormalities in phagocytes. Before the modern antibiotic era, patients with severe disorders probably did not survive into their childbearing years. Second, there is a remarkable redundancy in the antimicrobial machinery of the phagocytes that permits one system to compensate for a defect in another. For example, the host does not rely on a single chemotactic signal or neutrophil membrane receptor to ensure that phagocytes accumulate at sites of infection. Instead, multiple chemotactic signals and receptors are used. A similar phenomenon is seen in the reactions that kill microbes, as both oxidative and nonoxidative systems are used. It is also important to note that phagocytic leukocytes synthesize and secrete multiple inflammatory mediators, including leukotrienes, chemokines, and other cytokines that amplify and regulate the inflammatory response and initiate cross-talk with adaptive immune cells. Disorders resulting from functional phagocyte defects thus can display not only recurrent, severe bacterial and fungal infections but also aberrant inflammation that is not always related to infection.

This chapter is organized according to the aforementioned cellular functions: disorders of the respiratory burst microbicidal pathway, abnormalities of phagocyte adhesion and chemotaxis, and defects in the structure and function of lysosomal granules. This chapter is not meant to be an encyclopedic review of the numerous papers published on phagocyte abnormalities. It is important to note that many of these reports describe marginal in vitro defects, with little evidence that they are responsible for a clinical problem. Comprehensive reviews offering additional information on phagocyte disorders are available.[2,3]

APPROACH TO DIAGNOSIS OF PHAGOCYTE FUNCTION DISORDERS

Inherited and acquired clinical disorders of phagocyte function result from defects in one or more of the major steps leading to microbial killing—adhesion, chemotaxis, ingestion, degranulation, and production of microbicidal oxidants (Fig. 51.1). Patients with inherited disorders typically present in infancy or childhood with recurrent, unusual, or recalcitrant bacterial and fungal infections, and it is usually not difficult to determine that these are outside the range of normal. The presentation of these different inherited disorders can overlap, so that a specific diagnosis cannot be made on clinical grounds alone. Infections commonly seen include those of skin or mucosa, lung, lymph node, deep tissue abscesses, or childhood periodontitis. These can often have an indolent presentation with only low-grade fevers. Bacterial sepsis is an unusual initial symptom and usually reflects dissemination from an infected site. Inherited defects in phagocyte function are rare and represent only approximately 20% of the primary immunodeficiencies.[2] Thus children with suspected disorders of host defense should also be screened for defects in humoral, cellular, and complement-mediated immunity (see Chapters 23, 50 and 52). An approach to evaluating the patient with significant recurrent infections is shown in Fig. 51.2. Patients in whom a defect is identified should be referred to a center specialized in care of such patients.

In clinical practice, although nearly all patients with well-characterized phagocyte abnormalities have recurrent or unusual infections, the majority of individuals with histories of persistent or recurrent infections do not have identifiable phagocyte disorders or other immune defects. In some cases, these reflect another underlying medical condition or nonimmunologic problem related to an anatomic or obstructive defect. This chapter focuses largely on disorders in which a good correlation exists between the clinical condition and an identifiable defect in phagocyte function.

DISORDERS OF THE RESPIRATORY BURST PATHWAY

Reactive oxygen species generated by the phagocyte respiratory burst are critical for microbial killing. The enzyme responsible for the initial reaction in this pathway is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase found in plasma and phagolysosomal membranes. Upon activation by inflammatory stimuli, NADPH oxidase catalyzes the transfer of an electron from NADPH to molecular oxygen, thereby forming superoxide (as the O_2^- ion; Fig. 51.3, reaction 1).[3–5] This NADPH oxidase, along with enzymes and reactions that are directly involved in the production or metabolism of O_2^-, constitutes the respiratory burst pathway as depicted in Fig. 51.3. Superoxide is the precursor to numerous microbicidal oxidants, including hydrogen peroxide and hypochlorous acid. Five clinically significant defects have been identified in the respiratory burst, involving the following enzymes: NADPH oxidase (reaction 1), leukocyte glucose-6-phosphate dehydrogenase (G6PD; reaction 8), myeloperoxidase (MPO; reaction 4), glutathione reductase, and glutathione synthetase (reaction 9). These reactions are involved in the production of O_2^- (reactions 8 and 1) in the conversion of O_2^- and hydrogen peroxide to other toxic derivatives (reaction 4) or in

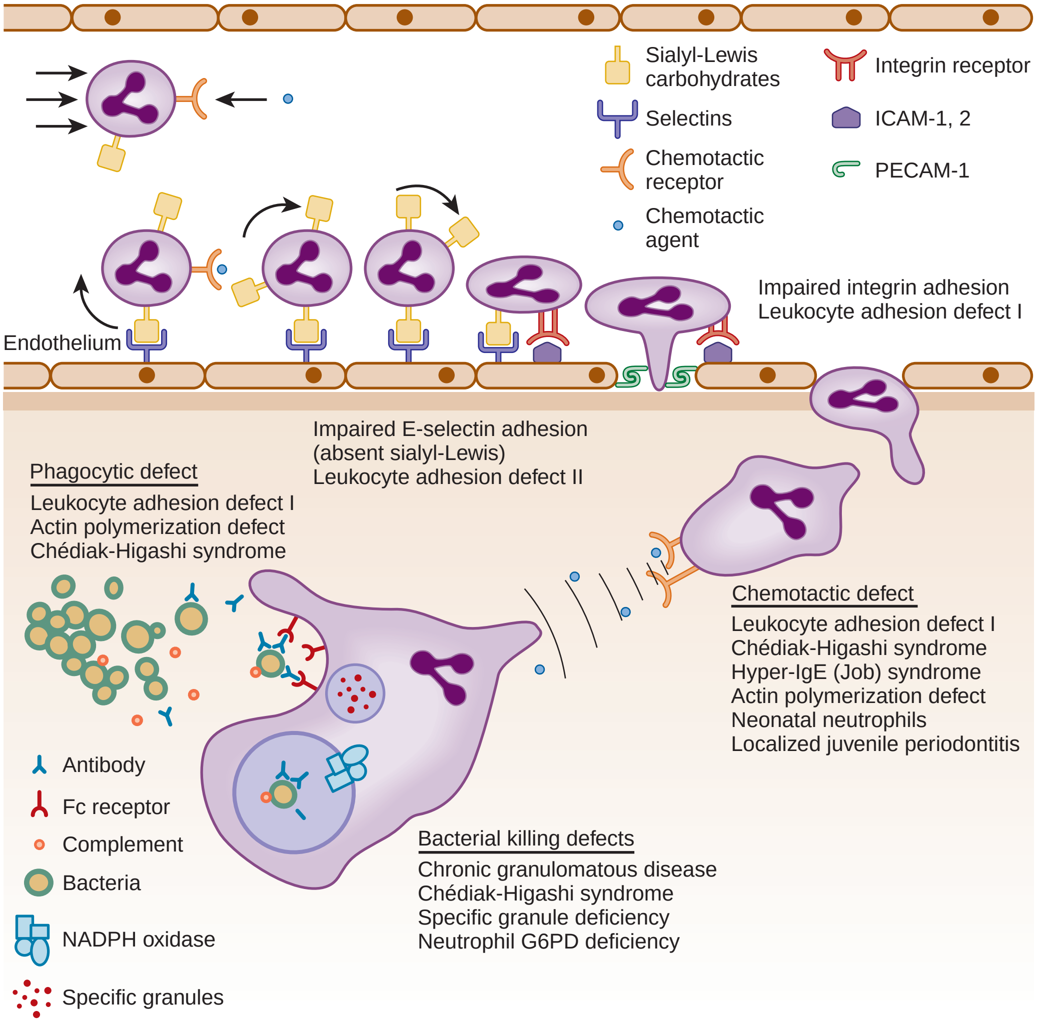

Sialyl-Lewis carbohydrates
Selectins
Chemotactic receptor
Chemotactic agent
Integrin receptor
ICAM-1, 2
PECAM-1
Endothelium
Impaired integrin adhesion
Leukocyte adhesion defect I
Impaired E-selectin adhesion
(absent sialyl-Lewis)
Leukocyte adhesion defect II
Phagocytic defect
Leukocyte adhesion defect I
Actin polymerization defect
Chédiak-Higashi syndrome
Chemotactic defect
Leukocyte adhesion defect I
Chédiak-Higashi syndrome
Hyper-IgE (Job) syndrome
Actin polymerization defect
Neonatal neutrophils
Localized juvenile periodontitis
Antibody
Fc receptor
Complement
Bacteria
NADPH oxidase
Specific granules
Bacterial killing defects
Chronic granulomatous disease
Chédiak-Higashi syndrome
Specific granule deficiency
Neutrophil G6PD deficiency

Figure 51.1 STEPS IN THE RESPONSE OF CIRCULATION NEUTROPHILS TO INFECTION. The adhesion molecule E-selectin is upregulated on endothelial cells in response to inflammatory mediators (interleukin-1 *[IL-1]*, endotoxin, tumor necrosis factor-α), resulting in rolling attachment and margination through interaction with sialyl-Lewis carbohydrates on its surface. Chemoattractants such as IL-8 cause upregulation of neutrophil β2 integrins that, in turn, mediate tight adhesion to ICAM-1 and PECAM-1 on endothelial cells. Activated neutrophils can detect as little as a 2% change in the chemoattractant gradient and move to the site of infection. Neutrophils phagocytize bacteria opsonized by antibody and complement. Both oxidative and nonoxidative antimicrobial mechanisms are then used to kill bacteria. Disorders of phagocyte function associated with each of these steps are noted. *G6PD*, Glucose-6-phosphate dehydrogenase; *ICAM-1*, intercellular adhesion molecule 1; *NADPH*, nicotinamide adenine dinucleotide phosphate; *PECAM-1*, platelet endothelial cell adhesion molecule 1. *(Courtesy Kyoto W, Coates TD. A practical approach to neutrophil disorders. Pediatr Clin North Am. 2002;49:929, with permission.)*

the detoxification of excess hydrogen peroxide needed to protect the phagocyte during the respiratory burst (reactions 7 and 9). Of note, NADPH oxidase (NOX) homologs to the leukocyte NADPH oxidase are present in the gut, vascular cells, and other tissues, which may generate oxidants for local host defense or for regulation of other cellular functions.[6]

Chronic Granulomatous Disease

Chronic granulomatous disease (CGD) is a genetically heterogeneous group of defects that share in common the failure of neutrophils, monocytes, macrophages, dendritic cells, and eosinophils to undergo a respiratory burst and generate O_2^- due to genetic deficiency of the NADPH oxidase.[3,7] CGD is relatively rare, having an estimated incidence of between 1 in 200,000 and 1 in 250,000 live births based on data from the United States CGD Registry,[7] although it is still the most common inherited phagocyte disorder of clinical significance (MPO deficiency is more common, but affected patients are rarely symptomatic). The absence of respiratory burst–derived oxidants results in recurrent, often life-threatening bacterial and fungal infections and is also associated with formation of inflammatory granulomas and other manifestations of aberrant inflammation.[3,7–10] Dysregulated inflammatory responses, which are not always related to infection, reflect a broad impact of NADPH oxidase–derived reactive oxygen species on cellular pathways involved in innate and adaptive immunity.

CGD was first described in 1957 as a syndrome characterized by severe recurrent infections in boys who also had visceral granulomas containing pigmented histiocytes. The disease was termed *fatal granulomatous disease* owing to this distinguishing histologic feature and the grim clinical course in most patients. It was not until the late 1960s and early 1970s that the defect in oxygen consumption and O_2^- production was identified and a convenient diagnostic assay, the nitroblue tetrazolium (NBT) test, was developed. In the 1980s a combination of biochemical and molecular genetic approaches led to the identification of four critical subunits of NADPH oxidase and the recognition that mutations in the corresponding genes are responsible for four different genetic subgroups of CGD (Fig. 51.4).

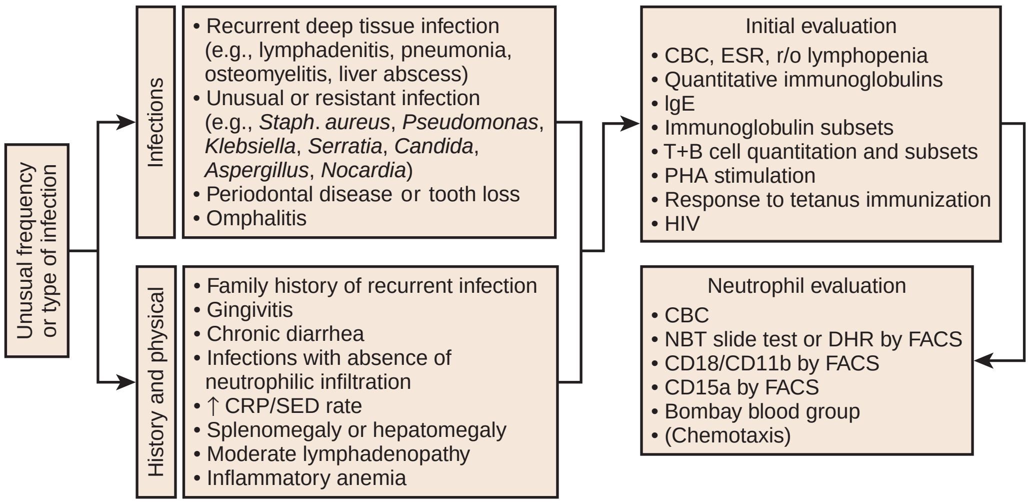

Unusual frequency or type of infection
Infections
• Recurrent deep tissue infection (e.g., lymphadenitis, pneumonia, osteomyelitis, liver abscess)
• Unusual or resistant infection (e.g., *Staph. aureus*, *Pseudomonas*, *Klebsiella*, *Serratia*, *Candida*, *Aspergillus*, *Nocardia*)
• Periodontal disease or tooth loss
• Omphalitis
History and physical
• Family history of recurrent infection
• Gingivitis
• Chronic diarrhea
• Infections with absence of neutrophilic infiltration
• ↑ CRP/SED rate
• Splenomegaly or hepatomegaly
• Moderate lymphadenopathy
• Inflammatory anemia
Initial evaluation
• CBC, ESR, r/o lymphopenia
• Quantitative immunoglobulins
• IgE
• Immunoglobulin subsets
• T+B cell quantitation and subsets
• PHA stimulation
• Response to tetanus immunization
• HIV
Neutrophil evaluation
• CBC
• NBT slide test or DHR by FACS
• CD18/CD11b by FACS
• CD15a by FACS
• Bombay blood group
• (Chemotaxis)

Figure 51.2 EVALUATION OF PATIENTS WITH RECURRENT BACTERIAL OR FUNGAL INFECTIONS. The history, physical examination, and infections episodes in patients with a possible primary neutrophil dysfunction syndrome are noted. The initial evaluation can be done in most clinical laboratories. A qualified reference laboratory with special expertise in this area should carry out the neutrophil evaluations. Chemotaxis is very difficult to evaluate clinically and should be attempted only in a qualified research laboratory with extensive experience. *CBC*, Complete blood count; *CRP*, C-reactive protein; *DHR*, dihydrorhodamine; *ESR*, erythrocyte sedimentation rate; *FACS*, fluorescence-activated cell sorting; *HIV*, human immunodeficiency virus; *IgE*, immunoglobulin E; *NBT*, nitroblue tetrazolium; *PHA*, phytohemagglutinin; *r/o*, rule out; *SED*, sedimentation.

CLINICAL APPROACH TO PATIENTS WITH DISORDERS OF PHAGOCYTE FUNCTION

Index of Suspicion

Patients with disorders of phagocyte function usually present at a young age with recurrent, deep-seated bacterial and fungal infections. Unlike patients with severe neutropenia caused by bone marrow (BM) failure, these patients usually do not have sepsis. Blood cultures are often negative. The major diagnostic problem faced by the clinician is to determine if the history of infection is unusual enough to warrant consideration of an underlying neutrophil dysfunction defect. The first point to remember is that primary immunodeficiency disorders are rare and primary neutrophil dysfunction syndromes form only a small percentage of all primary immunodeficiency syndromes. The patient is more likely to have recurrent community-acquired *Staphylococcus* infection than chronic granulomatous disease (CGD).

Specific features that may suggest a phagocytic defect are shown in Fig. 51.2. Excellent discussions of this problem have been published (see Kyono and Coates[2] and Dinauer, Newburger and Borregaard[3]). Four aspects of each patient's infection history should be considered: frequency, severity, location, and responsible pathogen. Patients with unusual features in at least one of these aspects should alert the clinician to a possible underlying phagocyte disorder. When considering frequency, the patient's age and associated medical conditions must be taken into account. For example, recurrent otitis media in a 2-year-old patient is far less worrisome than a similar history in a 40-year-old patient. The more unusual or severe the infections, the less frequently these have to occur before a phagocyte evaluation is indicated. Infections in unexpected anatomic locations, such as hepatic, pulmonary, and rectal abscesses, may indicate an underlying phagocyte defect. Childhood periodontal disease or gingivitis is normally very uncommon, and in the absence of neutropenic conditions, strongly suggests underlying neutrophil dysfunction. The identification of certain pathogens (e.g., *Serratia marcescens*, *Klebsiella* spp., *Aspergillus* spp., *Nocardia* spp., *Burkholderia cepacia*, invasive candidiasis) in children and young adults can provide the strongest indications for pursuing further studies. A history of delayed separation of the umbilical cord is often mentioned as a sign of phagocytic defect. This is fairly common as an isolated finding and is usually of no significance. However, this in conjunction with omphalitis or other pyogenic infections raises the possibility of leukocyte adhesion deficiency (LAD) or chemotactic defects. A child with nystagmus, fair skin, and recurrent staphylococcal infections should be evaluated for Chédiak-Higashi syndrome (CHS). Finally, the family history may provide clues suggesting an inherited disorder.

Evaluation

Performing a good history and physical examination to eliminate common causes of recurrent infection is important before looking for rare syndromes. For example, is the recurrent pneumonia caused by an aspirated foreign body in the bronchus? In general, patients should first be evaluated for lymphocyte or complement defects. A useful algorithm is presented in Fig. 51.2. Note that testing described in this algorithm is not exhaustive, and patients with truly striking histories of unusual kinds of infections should be referred for unbiased whole-exome sequencing and further evaluation by specialized research laboratories.

Recently, a fifth genetic subgroup due to mutations in a specialized NADPH oxidase subunit was identified, first in a single patient and subsequently in additional families.[11] Another new and uncommon form of CGD results from defects in a protein important for assembling the NADPH oxidase flavocytochrome.[9] Databases for the main four genetic subgroups of CGD have been developed that are accessible through the websites http://structure.bmc.lu.se/idbase/and http://www.hgmd.cf.ac.uk/ac/index.php. Recent publications on disease manifestations and genotypes in large patient registries are also available and support earlier reports.[3,7,8,10,12,13] Interestingly, common hypomorphic variants in NADPH oxidase genes are linked to autoimmune disorders, particularly systemic lupus erythematosus.[9]

Molecular Genetics of Chronic Granulomatous Disease

CGD results from mutations in any of the five genes encoding subunits of the NADPH oxidase or a gene encoding a chaperone protein (see Fig. 51.4; Table 51.1).[3,9,12,13] These genes are expressed primarily in myeloid cells and in B and perhaps T lymphocytes, although here its function is not well understood. The biochemical and genetic analysis of CGD has been instrumental in characterizing this complex enzyme. The oxidase subunits are referred to by their apparent molecular mass (kDa) and have been given the designation *phox*, for *ph*agocyte *ox*idase. A b-type cytochrome known as flavocytochrome b_{558}, a membrane-bound heterodimer composed of gp91^{phox} and p22^{phox}, is the redox center of the oxidase. In North American, Latin American, and European registries, approximately two-thirds of CGD cases result from defects in the X-linked gene encoding the gp91^{phox} subunit, which contains both the flavoprotein and heme-binding domains responsible for electron transport. X-linked recessive CGD accounts for a smaller percentage of CGD in areas with higher rates of consanguinity, where autosomal recessive (AR) forms are more frequent. One AR form of CGD is caused by mutations in the gene encoding p22^{phox}, the smaller subunit of flavocytochrome b_{558}, which provides a critical docking site for a regulatory subunit, p47^{phox}. Defects in EROS, an endoplasmic reticulum protein that facilitates flavocytochrome assembly, are found in a rare new subgroup of AR CGD. Other cases of AR CGD involve genetic defects in p47^{phox}, p67^{phox}, or p40^{phox}, three regulatory proteins associated with each other in the cytosol of unstimulated cells but which rapidly move to the membrane to activate flavocytochrome b_{558} and superoxide formation upon cell activation by inflammatory or phagocytic stimuli. The p40^{phox} subunit plays a selective role in stimulating high-level superoxide production within phagosomes or endosomes via membrane-bound phosphatidylinositol-3-phosphate. Formation of the active NADPH oxidase complex also involves the activation of the small guanosine triphosphate (GTP)-binding protein Rac, which then binds to the plasma membrane and p67^{phox}.[3] No cases of CGD have been identified resulting from genetic defects in Rac, although a mutation in the blood cell–specific Rac2 isoform was found in an infant with recurrent infections and abnormal neutrophil adhesion, motility, and partial NADPH oxidase defects.[3]

The gene for gp91^{phox}, termed *CYBB*, spans approximately 30 kb in the Xp21.1 region of the X chromosome. More than 600 distinct mutations have been identified in the gp91^{phox} gene in X-linked CGD (MIM306400), which include deletions, frameshifts, splice site, nonsense, and missense mutations that are distributed throughout the gene (Table 51.2).[12] Approximately 10% to 15% of cases of X-linked CGD are caused by new germline mutations. In most X-linked CGD, gp91*phox* is completely absent, and there is no measurable flavocytochrome *b* or superoxide production (the X91° subtype). In about 5% of X-linked cases, gp91*phox* can be present in normal levels but be nonfunctional (X91^{+}), mutated in such a way that gp91*phox* is poorly functional (X91^{-}), or expressed in only a small fraction of phagocytes (X91^{-}). The first two "variant" forms of X-linked CGD result from coding sequence mutations, and the latter are caused by mutations in the regulatory portion of the gp91*phox* gene. Some X-linked CGD patients have large deletions that affect not only *CYBB* but also portions of or all the flanking gene loci for McLeod hemolytic anemia syndrome (absence of the Kell erythrocyte antigen, Kx), Duchenne

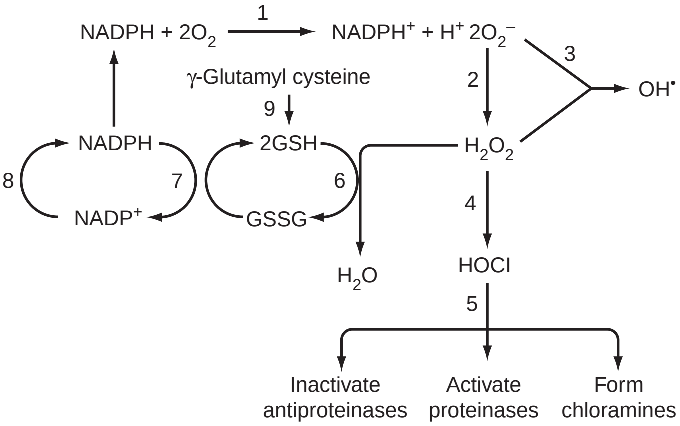


Figure 51.3 REACTIONS OF RESPIRATORY BURST PATHWAY. *GSH*, Glutathione; *GSSG*, oxidized glutathione; *HOCl*, hypochlorous acid; *NADPH*, nicotinamide adenine dinucleotide phosphate.

DIAGNOSIS OF CHRONIC GRANULOMATOUS DISEASE

The diagnosis of chronic granulomatous disease (CGD) is easily established by doing a nitroblue tetrazolium (NBT) slide test or flow cytometry of dihydroxyrhodamine (DHR) 123 fluorescence to detect neutrophil nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity. The NBT slide test is very easy to set up, as is DHR flow cytometry. However, because the probability of getting an abnormal result is very low, there may be confusion in interpretation because of a lack of experience. In the authors' experience, incorrect positive and negative results have been reported for both assays. Thus, if the index of suspicion is high, consultation should be obtained from a center with extensive experience with the test and with the disorder.

Neutrophil respiratory burst activity is preserved in anticoagulated blood maintained at room temperature for several days; thus DHR testing can be done 1–2 days later after shipping to a commercial laboratory. A normal blood sample should always be shipped with the patient specimen as a control for problems in specimen handling during transport. NBT tests are best performed on the same day the blood sample is obtained.

Nitroblue Tetrazolium Slide Test

- No NBT reduction (absence of cells with dark blue formazan deposits) in both X-linked and autosomal recessive (AR) forms of CGD (see Fig. 51.5B).
- Usually no reduction in 50% of cells and normal in 50% for X-linked carrier. The percent positive cells can vary if there is unequal X inactivation and may appear normal or like CGD with extreme lyonization (see Fig. 51.5C).
- False-positive results can occur (i.e., apparent failure to reduce NBT supporting the diagnosis of CGD) if the neutrophils do not adhere to the slide. This happens with greasy slides or with some cases of leukocyte adhesion deficiency (LAD). Using phorbol myristate acetate to stimulate the cells will avoid this.

Dihydroxyrhodamine Flow Cytometry

- This approach has replaced the NBT slide test in many laboratories. It has the advantage of assessing large numbers of cells and can give quantitation of the amount of oxidant production.
- The change in fluorescence channel number with stimulation is the critical number and not the percent positive cells.
- X-linked CGD patients will not respond at all and show no increase in fluorescence with stimulation (see Fig. 51.5F).
- X-linked carriers will show approximately 50% of the cells that respond with a normal increase in fluorescence, and the other half will have no response. Degrees of unequal X inactivation are much more accurately quantified by this assay (see Fig. 51.5G).
- AR patients, particularly those with absent p47^{phox}, have some response to stimulation and show a small increase in fluorescence (see Fig. 51.5H). This level of oxidant production is usually not visible on the NBT test.
- AR carriers have a good response, but the histogram may be broader than normal and may even appear bimodal with a weakly fluorescent peak and a strongly fluorescent peak. This is not distinguishable on the NBT slide test.
- Falsely negative results not supporting the diagnosis of CGD have been reported in specimens that have been run a few days after phlebotomy.
- Falsely abnormal results suggesting CGD can be seen in patients with myeloperoxidase (MPO) deficiency because MPO is required to generate strong DHR fluorescence.

Genetic Analysis

- Genetic analysis for X-linked and AR CGD is clinically available and should be performed on at least the proband in each kindred.

Those with fewer than 5% oxidase-positive cells have full-blown CGD.

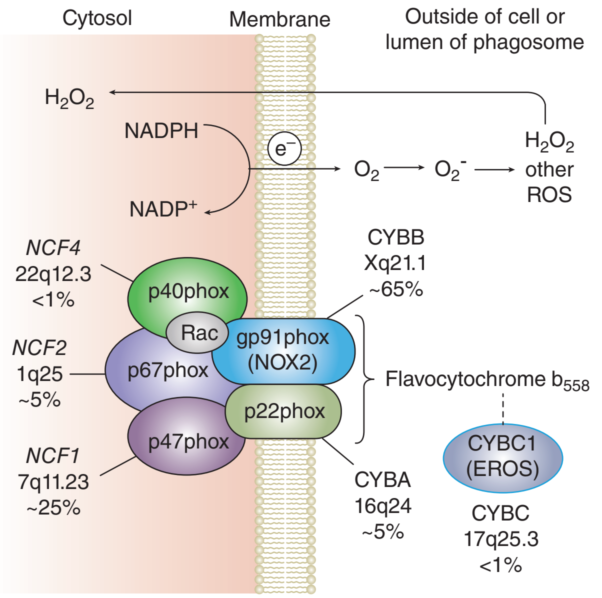


Figure 51.4 NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE (NADPH) OXIDASE AND MOLECULAR GENETICS OF CHRONIC GRANULOMATOUS DISEASE. The leukocyte NADPH oxidase enzyme complex is composed of membrane and cytosolic subunits, referred to by their molecular mass (kDa) and the designation "*phox,*" for ***ph***agocyte ***ox***idase. Chronic granulomatous disease (CGD) results from inactivating recessive mutations in any one of the five *phox* subunits or *CYBC1*, as indicated with the approximate incidence, gene, and chromosomal location. *CYBB* and *CYBA* refer to cytochrome b-245 β chain and cytochrome b-245 α chain, the large and small subunits of flavocytochrome b_{558}, whereas *NCF* refers to neutrophil cytosolic factor, used to designate the cytosolic regulatory subunits of the oxidase. Flavocytochrome b_{558} is the electron transferase and is located in plasma, specific granule (in neutrophils), and phagosome and some endosome membranes. This heterodimer is composed of gp91^{phox} and p22^{phox}, which are affected in X-linked and an autosomal recessive (AR) form of CGD, respectively. The gp91^{phox} subunit, which contains the flavine adenine dinucleotide (FAD) and heme redox centers, is sometimes referred to as NOX2 (**N**ADPH **ox**idase 2). CYBC1, also known as EROS (essential for reactive oxygen species), is an endoplasmic reticulum protein important for expression of the flavocytochrome b_{558} heterodimer and is affected in a rare AR form of CGD. Mutations in the genes encoding p47^{phox}, p67^{phox}, and p40^{phox} account for other AR subgroups. These soluble regulatory proteins form a complex in the cytosol, and upon leukocyte activation, phosphorylation-induced conformational changes lead to their binding to flavocytochrome b_{558} and, for p40^{phox}, to phosphatidylinositol 3-phosphate on phagosome and endosome membranes. The small GTPase, Rac, is also essential for NADPH oxidase enzymatic activity, which in its active GTP-bound state, becomes membrane-bound and activates p67^{phox}. Mutations in Rac do not result in a CGD phenotype. Together, these regulatory proteins activate flavocytochrome b_{558}-mediated transfer of electrons from cytosolic NADPH across the membrane to molecular oxygen, thereby forming superoxide in the extracellular space or within phagosomes or endosomes. Superoxide is converted into H_2O_2, which can diffuse across membranes, and other reactive oxygen species (ROS). *(Originally published in Blood. Dinauer MC. Inflammatory consequences of inherited disorders affecting neutrophil function. Blood. 2019;133(20):2130–2139. © the American Society of Hematology.)*

muscular dystrophy, and X-linked retinitis pigmentosa. Rare missense point mutations in *CYBB* lead to markedly impaired flavocytochrome b expression and activity in macrophages, with much less effects in neutrophils.[14] Affected patients were susceptible to mycobacterial infections but did not have other bacterial and fungal infections characteristic of CGD, highlighting the importance of macrophages for controlling mycobacteria.

AR CGD involving p22^{phox} (MIM 233690) occurs in approximately 5% of CGD patients and usually involves the complete absence of cytochrome *b* (A22°), encoded by *CYBA*. Mutations in A22 CGD are heterogeneous and range from large interstitial gene deletions to point mutations associated with missense, frameshift, or RNA splicing defects.[13] Because the full expression of flavocytochrome *b* in the membrane requires the production of both subunits, a primary deficiency of either component leads to a secondary loss of the other. Thus neither subunit can be detected on immunoblot analysis in either X91° or A22° CGD. A patient with A22$^+$ CGD has been described with a missense mutation disrupting the binding site for p47^{phox}.

AR patients with p47^{phox}-deficient CGD (MIM 233700) account for approximately one-fourth of cases in the United States and Europe, but only approximately 7% of cases in Japan. The p47^{phox} subunit is encoded by the *NCF1* gene. A limited number of mutations have been identified in *NCF1*.[13] Virtually all patients are either homozygotes or compound heterozygotes for a mutant allele with a GT deletion at the beginning of exon 2 that predicts a premature stop codon following the amino acid residue and results in absence of the p47^{phox} protein. The high frequency of the p47^{phox} GT deletion mutation appears to reflect the existence of at least one closely linked highly conserved p47^{phox} pseudogene(s) that contains this GT deletion. This close physical proximity leads to recombination events between the wild-type gene and pseudogene(s).

A heterogeneous group of mutations in the p67^{phox} gene, *NCF2*, are responsible for A67 CGD, a rare AR form of CGD (MIM233710) accounting for approximately 5% of cases overall.[13] Almost all mutations identified to date in A67 CGD lead to absent expression of the p67^{phox} protein. However, one A67$^+$ patient has been reported in which a nonfunctional form of p67^{phox} with an amino acid deletion is expressed but is unable to translocate to the membrane or bind to Rac.

Two additional forms of AR CGD, both with partial loss of oxidase activity, have been described. Affected patients have a different phenotype than "classic" CGD, with more inflammatory manifestations, including granulomatous gastrointestinal disease and lupus-like skin lesions and few if any severe deep-seated infections. Following an initial report in a boy with mutations in *NCF4*, which encodes p40^{phox}, other patients with defects in *NCF4* (MIM613960) were identified in another dozen families.[11] Although NADPH oxidase activity on the plasma membrane is typically normal in p40^{phox}-deficient CGD, intracellular oxidant production is markedly impaired. Another uncommon form of AR CGD results from AR mutations in *CYBC1* (MIM 618935), which encodes the endoplasmic reticulum-resident protein EROS (essential for reactive oxygen species [ROS]) that helps mediate assembly of the flavocytochrome b_{558} heterodimer.[9] Most patients were from Iceland and homozygous for the same founder mutation. *CYBC1* defects seem to affect flavocytochrome b_{558} expression in monocytes and macrophages much more substantially than in neutrophils. Although several patients with *CYBC1* defects developed mycobacterial disease, invasive bacterial and opportunistic fungal infections have not been reported.

Even though more than 90% of patients with CGD have respiratory burst defects that result in undetectable levels of O_2^- production, there is a surprising heterogeneity in the clinical manifestations of the disease.[7] At one end of the spectrum are patients who begin to have severe bacterial and fungal infections during infancy and who rarely have more than 4 to 12 months between such serious infections. At the other end of the spectrum are patients who are well for many years and then unexpectedly develop a serious infection typical of CGD, such as a staphylococcal hepatic abscess or *Aspergillus* pneumonia. After their first major infection, some of these patients may be relatively healthy again for another 3 to 10 years before the next severe infection occurs. As a group, patients with X-linked CGD, A22 CGD, and A67 CGD seem to have a more severe clinical course compared with patients with A47 CGD,[7,8] who have a small amount of detectable oxidant production even in the complete absence of this subunit (Fig. 51.5G). Individuals with partial respiratory burst activity but

TABLE 51.1 Classification of Chronic Granulomatous Disease

Component Affected	Gene Symbol	Gene Locus	Inheritance	Subtype[a]	NBT Score (% Positive)	O_2^- Production (% Normal)	Flavocytochrome *b* Spectrum (% Normal)	Defect in Cell-Free NADPH Oxidase Assay	Frequency (% of Cases)[b]
gp91^{phox}	*CYBB*	Xp21.1	X	X91°	0	0	0	Membrane	68
				X91^{-}	80–100 (weak)	3–30	Low	Membrane	5
				X91^{-}	5–10	5–10	Low	Membrane	<1
				X91^{+}	0	0	N	Membrane	1
p22^{phox}	*CYBA*	16p24.3	AR	A22°	0	0	0	Membrane	4
				A22^{+}	0	0	N	Membrane	<1
p47^{phox}	*NCF1*	7q11.23	AR	A47°	0	0–1	N	Cytosol	17
p67^{phox}	*NCF2*	1q25.3	AR	A67°	0	0	N	Cytosol	5
p40^{phox}	*NCF4*	22q13.1	AR	A40	100	<20 (intracellular)	N	n/a	<1
EROS	*CYBC1*	17q25.3	AR	—	100	5–20	Low	n/a	<1

[a] In this nomenclature, the first letter represents the mode of inheritance (X-linked *[X]* or autosomal recessive *[A]*), and the number indicates the *phox* component that is genetically affected. The superscript symbols indicate whether the level of protein of the affected component is undetectable (°), diminished (–), or normal (+) as measured by immunoblot analysis.

[b] Combined data from 209 kindreds evaluated at the Scripps Research Institute/Stanford University CGD Clinic and a cooperative European study representing 57 kindreds and 63 patients. (Courtesy Casimir C, Chetty M, Bohler MC, et al. Identification of the defective NADPH-oxidase component in chronic granulomatous disease: a study of 57 European families. *Eur J Clin Invest*. 1992;22:403; and Curnutte JT. Chronic granulomatous disease: the solving of a clinical riddle at the molecular level. *Clin Immunol Immunopathol*. 1993;67:S2.) These frequencies remain similar to those in more recent reports from Europe and the United States.

AR, Autosomal recessive inheritance; *N*, normal; *NADPH*, nicotinamide adenine dinucleotide phosphate; *n/a*, not applicable; *NBT*, nitroblue tetrazolium; *X*, X-linked inheritance.

TABLE 51.2 Summary of Mutations in the *CYBB* Gene Encoding gp91^{phox} in 261 Kindreds With X-linked Chronic Granulomatous Disease

Type of Mutation	Number of Kindreds	Frequency (%)	Phenotype
Deletions	63	24.2	X91°
Insertions	27	10.3	X91°
Splice-site mutations	42	16.1	X91°
Missense mutations	59	22.6	X91°, X91^{-}, X91^{+}
Nonsense mutations	70	26.8	X91°

Data from Roos D, Curnutte J, Hossle JP, et al. X-CGDbase: a database of X-CGD-causing mutations. *Immunol Today*. 1996;17:517.

less than 10% of normal (most X91^{-} patients; see Table 51.1) also tend to have disease of intermediate severity. Polymorphisms in oxygen-independent antimicrobial systems or other components regulating the innate immune response are also likely to play an important role in modifying disease severity.[8] Specific polymorphisms in the MPO, mannose-binding lectin, and FcγRIIa genes are associated with a higher risk for granulomatous or autoimmune or rheumatologic complications.[3,9] Finally, as already mentioned, in contrast to "classic" CGD, the clinical spectrum of disease in patients with mutations involving p40^{phox} or *CYBC1* resemble an atypical form of CGD, with inflammatory manifestations much more prominent than infections.[9,11] Because of this heterogeneity, the diagnosis of CGD should be entertained, not only in young children with recurrent severe infections but also in adolescents and young adults who experience exceptionally severe or unusual infections or granulomatous intestinal inflammation.

Clinical Manifestations

In approximately two-thirds of patients, the first symptoms of CGD appear during the first year of life, with the onset of recurrent, purulent bacterial, and fungal infections.[7] Table 51.3 summarizes the types of infections and infecting organisms most frequently encountered in CGD. The most common types of infections are those that involve sites in contact with the outside world, which is consistent with the role of neutrophils as a first line of defense against infection. *Staphylococcus aureus*, enteric gram-negatives, *Serratia marcescens*, *Burkholderia cepacia*, *Nocardia* spp., and *Aspergillus* spp. represent the most frequently encountered pathogens in North American patients, but *Burkholderia* and *Nocardia* spp. are less frequently seen in Europe. *S. aureus* is the most frequently isolated organism overall. CGD patients have increased risk from *Mycobacterium tuberculosis* in endemic areas and can develop severe local or systemic disease with Bacille Calmette Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, following BCG vaccination. The most common causes of death have been pneumonia or sepsis caused by *B. cepacia* and *Aspergillus* spp., although use of newer azole antifungals has markedly improved the outcome of the latter in recent years.[3,7,10]

Most CGD pathogens share the property of being catalase negative, and as such inadvertently "lend" H_2O_2 secreted from the pathogen to the peroxide-starved CGD phagocyte, which in turn uses it (after being converted to hypochlorous acid [HOCl] by MPO; see Fig. 51.3) to kill the microbe. It also appears that at least some of the CGD pathogens are resistant to the nonoxidative killing mechanisms of the phagocyte. It is somewhat surprising how often one fails to identify the infecting organism in CGD—perhaps greater than half the time despite aggressive culturing. In this situation, one treats empirically with the antibiotic that should work and if it fails, one then aggressively pursues more invasive diagnostic procedures looking for one (or more) of the less commonly seen microbes such as *Nocardia* spp., *Candida* spp., mycobacteria, and a host of other bacteria and fungi (see Table 51.3). Other unusual organisms that cause infection in CGD include other members of the *Burkholderia* family, including *Burkholderia*

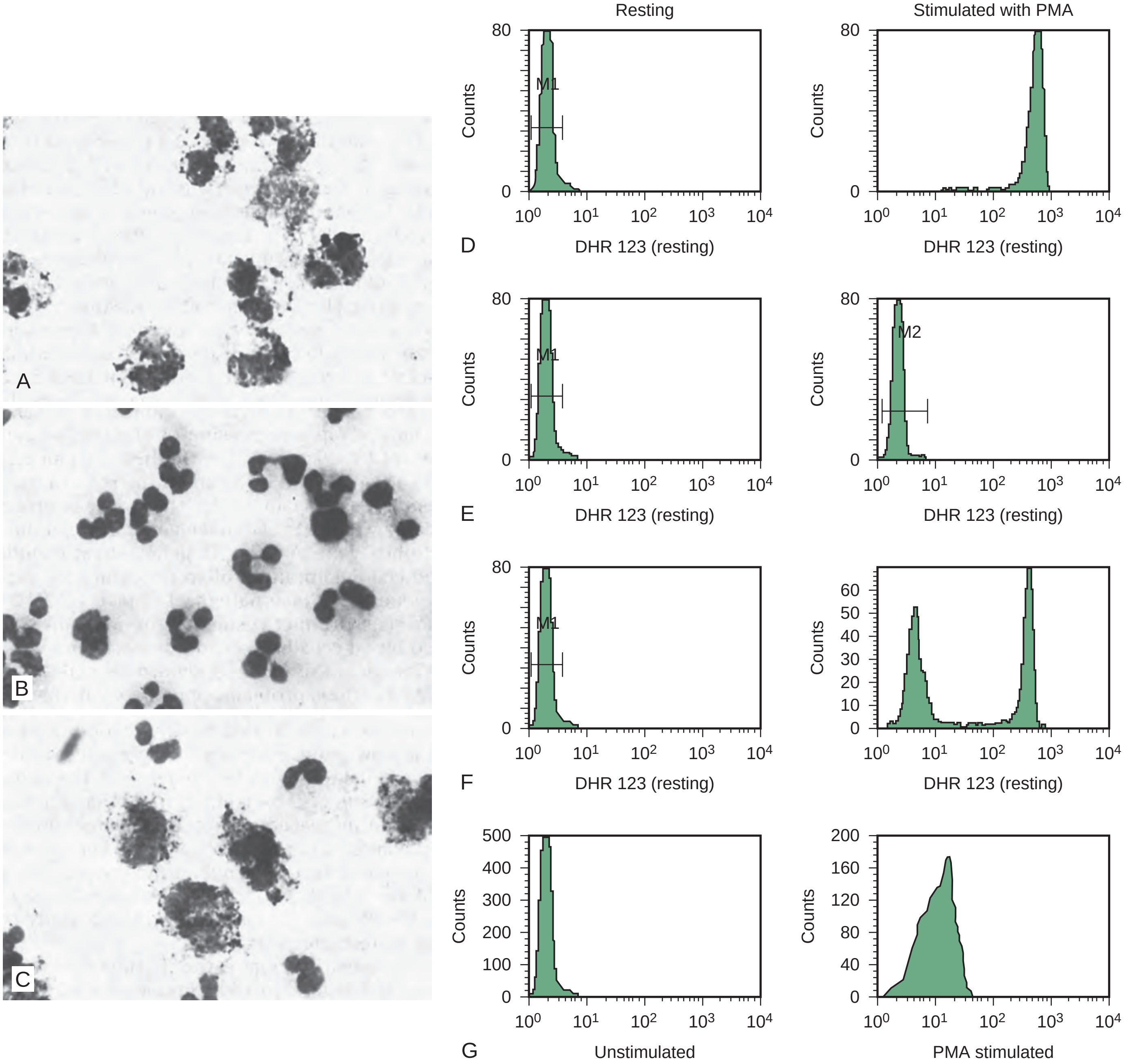


Figure 51.5 ANALYSIS OF NEUTROPHIL NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE OXIDASE ACTIVITY FOR THE DIAGNOSIS OF CHRONIC GRANULOMATOUS DISEASE. (A–C) Nitroblue tetrazolium (NBT) slide test. Peripheral blood neutrophils and monocytes from a drop of fresh whole blood were made adherent to glass slides and stimulated with phorbol myristate acetate. (A) Normal neutrophils and monocytes, all of which are NBT-positive. (B) Neutrophils and monocytes from an X-linked chronic granulomatous disease (CGD) patient, which are all NBT-negative. (C) A mixture of NBT-positive and NBT-negative neutrophils from the X-linked carrier mother of the patient in (B). (D–G) DHR 123 flow cytometry test. Nonfluorescent DHR 123 is taken up by neutrophils, which become fluorescent after reaction with reactive oxygen species produced in the respiratory burst. (D) Normal neutrophils. (E) Neutrophils from an X-linked CGD patient, which do not fluoresce after stimulation. (F) A mixture of nonfluorescent and fluorescent neutrophils from an X-linked CGD carrier. (G) Neutrophils from a p47^{phox}-deficient patient, which show weak fluorescence after stimulation. *DHR*, Dihydrorhodamine; *PMA*, phorbol myristate acetate.

cenocepacia, *Burkholderia gladioli*, and *Burkholderia mallei* (the causative agent in melioidosis, a septic illness common in East Asia), and *Chromobacterium violaceum*, found in brackish fresh water and which can cause a febrile illness with bacteremia in CGD. A previously unknown gram-negative bacteria, *Granulobacter bethesdensis*, was identified in a CGD patient with recurrent fevers associated with chronic necrotizing deep lymphatic infection. This organism is a member of the *Acetobacteraceae* family, which has previously not been linked to invasive human disease.

Pneumonia is the most common type of infection seen in CGD with *S. aureus*, *Aspergillus* spp., *B. cepacia*, and enteric gram-negative organisms as the major pathogens. It is noteworthy that *B. cepacia* has emerged as a particularly lethal organism in CGD. This organism often is not covered with the first line of antibiotics used for *S. aureus* and most gram-negative organisms and can quietly proliferate (with persistent fevers) to the point of quick, explosive collapse caused by endotoxic shock. Intravenous trimethoprim-sulfamethoxazole (TMP-SMX) has been most effective in treating patients if given

TABLE 51.3 Infections in Chronic Granulomatous Disease

Infections	Infections (%)	Infecting Organisms
Pneumonia	70–80	*Aspergillus*, *Staphylococcus*, *Burkholderia cepacia*, *Pseudomonas*, *Nocardia*, *Mycobacterium* (including atypical), *Serratia*, *Candida*, *Klebsiella*, *Paecilomyces*
Lymphadenitis	50–60	*Staphylococcus*, *Serratia*, *Klebsiella*, *B. cepacia, Candida*, *Nocardia, Mycobacteria*
Cutaneous infections/impetigo	50–60	
Hepatic or perihepatic abscesses	20–30	*Staphylococcus*, *Serratia*, *Streptococcus viridans*, *Nocardia*, *Aspergillus*
Osteomyelitis	20–30	*Serratia*, *Aspergillus*, *Paecilomyces*, *Staphylococcus*, *B. cepacia*, *Pseudomonas*, *Nocardia*
Perirectal abscesses or fistulae	15–30	Enteric gram-negative organisms, *Staphylococcus*
Septicemia	10–20	*B. cepacia*, *Pseudomonas*, *Salmonella*, *Staphylococcus*, *Serratia*, *Klebsiella*
Urinary tract infections or pyelonephritis	5–15	Enteric gram-negative organisms
Brain abscesses	<5	*Aspergillus*, *Staphylococcus*
Meningitis	<5	*Candida lusitaniae*, *Haemophilus influenzae*, *B. cepacia*

The relative frequencies of different types of infections in chronic granulomatous disease are estimated from data pooled from mainly several large series of patients in the United States, Europe, and Japan: (1) Mouy R, Fischer A, Vilmer E, et al. Incidence, severity, and prevention of infections in chronic granulomatous disease. *J Pediatr.* 1989;114:555; (2) Bemiller LS, Roberts DH, Starko KM, et al. Safety and effectiveness of long-term interferon gamma therapy in patients with chronic granulomatous disease. *Blood Cells Mol Dis.* 1995;21:239.; (3) Forrest CB, Forehand JR, Axtell RA, et al. Clinical features and current management of chronic granulomatous disease. *Hematol Oncol Clin North Am.* 1988;2:253; (4) Hitzig WH, Seger RA. Chronic granulomatous disease, a heterogeneous syndrome. *Hum Genet.* 1983;64:207; (5) Tauber AI, Borregaard N, Simons E, et al. Chronic granulomatous disease: a syndrome of phagocyte oxidase deficiencies. *Medicine (Baltimore).* 1986;62:286; (6) Cohen MS, Isturiz RE, Malech HL, et al. Fungal infection in chronic granulomatous disease. The importance of the phagocyte in defense against fungi. *Am J Med.* 1981;71:59; (7) Hayakawa H, Kobayashi N, Yata J. Chronic granulomatous disease in Japan: a summary of the clinical features of 84 registered patients. *Acta Paediatr Jpn.* 1985;27:501; and (8) Johnston RB, Newman SL. Chronic granulomatous disease. *Pediatr Clin North Am.* 1977;24:365. These series encompass approximately 550 patients with chronic granulomatous disease (CGD) after accounting for overlap between reports. Unpublished data from the United States CGD Registry encompassing 368 patients was also used to estimate the relative frequencies of infections and the responsible organisms. The infecting organisms are arranged in approximate order of frequency for each type of infection. Note: *B. cepacia* was previously classified as *Pseudomonas cepacia*.

before widespread dissemination of the infection. Proven or suspected *Aspergillus* infections were previously treated with amphotericin B therapy, but azole antifungal agents are now typically used.

Lymphadenitis is the second most common infection and is usually caused by gram-negative organisms, *S. aureus*, or *S. marcescens*. Incision and drainage should not be delayed if the lesion fails to respond to parenteral antibiotics. Cutaneous abscesses should be similarly managed. Recurrent perinatal impetigo is almost a signature infection in CGD and often requires months of therapy (mostly oral antibiotics) to clear. Hepatic (and perihepatic) abscesses are also common in CGD and are usually, but not always, caused by *S. aureus*. Lesions often require drainage (needle or surgical) to permit efficient healing to occur. Bone infections, most commonly caused by *Serratia* spp. or *Aspergillus* spp., are particularly problematic in CGD and arise from either hematogenous or contiguous spread (as often is the case with *Aspergillus* infections in the lung invading the ribs, vertebral bodies, or the diaphragm). Perirectal abscesses are difficult to treat, even with months of therapy, and can lead to fistula formations.

Chronic inflammation with granuloma formation is a distinctive hallmark of CGD and contributes to some of its more problematic complications.[3,9,10] In some cases, this results from imperfectly controlled infections in which stalemates develop between the pathogen and the patient's leukocytes. These lesions become granulomas as the host uses lymphocytes and activated macrophages to assist in containing the pathogens. However, this complication is not always clearly linked to persistent infection and, in these cases, is speculated to involve a dysregulated inflammatory response. Inflammatory complications in CGD are thought to reflect the importance of oxidants generated from the NADPH oxidase to downregulate the inflammatory response through redox-mediated effects, promote efficient degradation of debris, or both. In the absence of oxidant production, excessive production of cytokines and delayed neutrophil apoptosis and clearance at inflammatory sites appear to contribute as underlying mechanisms. Subtle defects in the absence of NADPH oxidase in memory B or T cells may also play a role.

As a result of persistent inflammatory stimulation, CGD patients can have a variety of chronic complications (Table 51.4). Lymphadenopathy, hepatosplenomegaly, eczematoid dermatitis, and anemia of chronic disease (hemoglobin levels usually 8 to 10 g/dL) are common manifestations of this process and are most prominent in the first 5 to 10 years of life in those with CGD. Throughout the body, granuloma formation can lead to dysfunction and obstruction in the esophagus, urinary bladder, and kidneys. In the stomach, the gastric antral narrowing can be severe enough in infants and children to resemble pyloric stenosis. Inflammatory involvement of the gastrointestinal tract can be seen in many CGD patients. A chronic ileocolitis resembling Crohn disease occurs in up to 30% of patients and can range from mild diarrhea to a debilitating syndrome of bloody diarrhea and malabsorption that can necessitate a colectomy. Interestingly, antigliadin antibodies suggesting Crohn disease are positive in more than 50% of CGD patients. Inflammatory lung disease with interstitial and/or micronodular infiltrates, often seen better on computed tomography (CT) scan rather than regular X-ray, can develop in many older patients and lead to reduced diffusion capacity and progressive hypoxia. Other types of chronic inflammation include gingivitis, chorioretinitis, destructive white matter lesions in the brain, and glomerulonephritis. Discoid lupus has been reported in 10% to 20% of patients, and occasional patients may develop systemic lupus erythematosus, sarcoidosis, or rheumatoid arthritis. The underlying mechanisms are poorly defined, although recent studies suggest that these manifestations may be partly related to subtle defects in the absence of NADPH oxidase in memory B or T cells. Finally, infections in CGD due to fungi, bacteria (especially *B. cepacia*), or *Leishmania* in endemic areas can trigger the macrophage activation syndrome (MAS) and hemophagocytic lymphohistiocytosis (HLH) in CGD.[9]

Carriers of CGD, whether the X-linked form or any one of the AR forms, are usually asymptomatic, with two important exceptions. First, approximately one-fourth of X-linked carriers are at risk of developing mild to moderately severe discoid lupus erythematosus characterized by discoid skin lesions and photosensitivity.[3,9]

TABLE 51.4 **Chronic Conditions Associated With Chronic Granulomatous Disease**[a]

Condition	Relative Frequency (%)
Lymphadenopathy	98
Hypergammaglobulinemia	60–90
Hepatomegaly	50–90
Splenomegaly	60–80
Anemia of chronic disease	Common
Underweight	70
Chronic diarrhea	20–60
Short stature	50
Gingivitis	50
Dermatitis	35
Hydronephrosis	10–25
Granulomatous ileocolitis	10–15
Gastric antral narrowing	10–15
Ulcerative stomatitis	5–15
Granulomatous cystitis	5–10[b]
Chronic lung disease	10–50[c]
Esophagitis	<10[b]
Granulomatous cystitis	<10
Chorioretinitis	<10
Glomerulonephritis	<10
Discoid lupus erythematosus	<10

[a] The relative frequencies of chronic conditions associated with chronic granulomatous disease (CGD) were estimated from the series of reports listed in Table 50.3.
[b] The incidence is estimated from the 50 cases of CGD followed at Scripps Research Institute and Stanford University (unpublished data).
[c] Chronic lung disease can be especially problematic in adults with CGD; see Campos LC, Di Colo G, Dattani V, et al. Long-term outcomes for adults with chronic granulomatous disease in the United Kingdom. *J Allergy Clin Immunol*. 2021;147:1104 and Dunogué B, Pilmis B, Mahlaoui N, et al. Chronic granulomatous disease in patients reaching adulthood: a nationwide study in France. *Clin Infect Disease*. 2017;64, 767.

The onset is usually in the second decade of life. The disease does not progress to systemic lupus nor does one find serologic evidence of even subclinical disease. Those with severe discoid lupus can be treated with hydroxychloroquine. Recurrent stomatitis, significant gingivitis, or both have also been noted in as many as half of X-CGD carriers. A few also have arthralgias, polyarthritis, and Raynaud phenomenon. The second important complication of the X-linked CGD carrier state is serious infection in women who have an unusually high degree of inactivation of the normal X chromosome in their myeloid cells. If the circulating neutrophil population is skewed to the point that fewer than 10% to 15% of the cells function, then the carrier has an increased risk of bacterial infections that in some cases have been severe.[3]

Diagnosis

The diagnosis of CGD is usually suggested by the unusual clinical histories outlined earlier or by a family history of CGD. The NBT slide test on fresh blood is the classic diagnostic test. A typical result is shown in Fig. 51.5. Fig. 51.5A shows the normal positive staining of a group of seven neutrophils and one monocyte. Fig. 51.5B shows the complete absence of NBT staining in a patient with X91° CGD, the classic X-linked form of the disease. Fig. 51.5C shows the mixed population of NBT-positive and NBT-negative cells observed in that patient's mother, reflecting random X chromosome inactivation. Because nearly 100% of the normal cells in this test are positive, the carrier state in X-linked CGD can be detected when as few as 5% of the cells are NBT-negative. This test also permits detection of diffuse populations of weakly positive cells such as those seen in X91^{-} CGD, which are characterized by a partial deficiency of flavocytochrome *b*. Because X-linked CGD can arise by new mutations in the maternal germline, one does not always see NBT-negative cells in the mother. Flow cytometric assays of oxidase activity, such as those based on the conversion of dihydroxyrhodamine (DHR) 123 to rhodamine 123, can also provide both quantitative measurements of oxidant generation and the cell-by-cell distribution of activity (see Fig. 51.5D–G). The DHR 123 assay for oxidase activity is now available in many referral centers and reference laboratories. In addition to X91^{-} CGD neutrophils, weak staining in the NBT test or a small but measurable level of DHR fluorescence can be seen in A47° cells (see Fig. 51.5H) because of a small amount of residual oxidant production. Regardless of diagnostic assay used, it is important to have these tests performed on appropriately handled blood samples and by experienced laboratories to avoid inconclusive or false-normal results.

Genetic testing is useful to solidify the diagnosis and for genetic counseling and is commercially available for the five genetic subgroups involving NADPH oxidase subunits (see Table 51.1). The analysis of mutations in *NCF1* can require specialized polymerase chain reaction (PCR) testing due to the presence of adjacent pseudogenes. Laboratories specializing in neutrophil biochemistry can also perform immunoblot analysis of neutrophil extracts, flavocytochrome *b* spectroscopy, or functional analysis of membrane and cytosol fractions in the cell-free oxidase assay.

Testing for the McLeod red cell phenotype should be done in patients diagnosed with X-linked CGD who harbor large *CYBB* deletions that potentially involve the locus encoding the Kell erythrocyte antigen. Loss of Kell causes a mild hemolytic anemia. More importantly, there can be serious problems with development of hemolytic antibodies if these patients are transfused with Kell-positive blood.

Prognosis and Treatment

The cornerstones of therapy in CGD are currently (1) prevention and early treatment of infections, (2) aggressive use of parenteral antibiotics for most infections, (3) use of prophylactic TMP-SMX (5 mg/kg/day of trimethoprim) or dicloxacillin (25 to 50 mg/kg/day) for sulfa-allergic patients, (4) prophylactic itraconazole (200 mg/day if 13 years of age or older or if weighing at least 50 kg, or 100 mg daily if younger than 13 years of age or weighing less than 50 kg), and (5) use of prophylactic recombinant human interferon-γ (rIFN-γ; 0.05 mg/m^2 or 0.0015 mg/kg if less than 0.5 m^2 three times per week).[3,15] Using these guidelines, the prognosis for patients with CGD has improved dramatically since the disorder was first described in the 1950s, when almost all patients died in childhood. In a large study based on data collected by a CGD registry in the United States in the 1990s, the overall mortality rate was estimated to be 5% per year for X-CGD and 2% per year for AR CGD,[7] and a more recent single-institutional study of 76 patients reported an overall mortality rate of 1.5% per year.[3]

There is a general consensus that a large majority of newly diagnosed children should survive well into their adult years with aggressive and careful management. While survival is improved, many of these adults continue to have significant complications due to chronic inflammation.[10] The quality of their survival is further affected by the paucity of providers with sufficient experience in the management of this very rare disorder. As already noted, patients with deficiency of p47^{phox} have a tendency for milder disease compared with those with flavocytochrome-negative CGD.[8] On the other hand, some patients (usually X-linked) prove to have more frequent serious infections or inflammatory complications (or both), likely because of the effects of modifier genes; these patients may warrant more aggressive treatment such as bone marrow transplantation (BMT; see later).

Several approaches can be used to prevent infections. Patients with CGD should receive all their routine immunizations on schedule (including live virus vaccines), with influenza vaccine administered each year as well. Cuts and skin abrasions should be cleansed promptly with soap and water and a topical antiseptic applied (2% hydrogen peroxide, Betadine ointment, or both). Frequent brushing, flossing, use of antibacterial mouthwash, and professional cleaning of teeth can help prevent gingivitis. Constipation should be avoided because it can lead to rectal or anal fissures and abscesses. Early anal infections can be treated with soaking in soapy water (with or without betadine). The frequency of pulmonary infections can be reduced by not using commercially available bedside humidifiers; avoiding smoking (cigarettes and marijuana); and refraining from handling decaying plant materials (e.g., hay, mulch, rotting sawdust), which often contain numerous *Aspergillus* spp. Avoidance of construction sites, especially demolition of old buildings that may harbor fungi, is recommended. There have been clear outbreaks of *Aspergillus* pneumonias in immunosuppressed children visiting hospitals undergoing renovation.

There is clear evidence that chronic prophylactic TMP-SMX can decrease the number of bacterial infections in CGD patients by more than half without a concomitant increased risk of fungal infection. In addition, itraconazole is an effective agent for prophylaxis for fungal infections in CGD. Liver function tests should be monitored in patients receiving itraconazole.

Prophylactic rIFN-γ has been another mainstay of current management of CGD.[3] The clinical benefit of rIFN-γ is probably related to generally enhanced phagocyte function and killing by nonoxidative mechanisms because its use is not accompanied by any measurable improvement in NADPH oxidase activity in the vast majority of CGD patients. In the original multicenter trial, patients were randomized in a double-blind fashion to receive either placebo or rIFN-γ ($0.05\,\text{mg/m}^2$ three times per week). As summarized in Table 51.5, there was a substantial decrease in the number of serious infections in the rIFN-γ arm. Side effects were observed in some of the patients but typically were restricted to mild fever and flu-like symptoms. Additional adverse reactions, including any increased incidence of chronic inflammatory complications, have not been noted with more prolonged courses of prophylactic rIFN-γ (more than 10 years), and the patients continued to have a substantial benefit, with fivefold fewer serious infections compared with the placebo group in the phase III study in Table 51.5. This group of patients averaged one serious infection per patient every 4 to 5 years. However, rIFN-γ is used less frequently in Europe because nonrandomized trial data did not suggest much benefit. The use in a cohort followed at the National Institutes of Health was recently reported to be only 36% of patients because of either lack of access or because of side effects (fever, myalgia); the availability of more potent antifungals and oral antibiotics may be a mitigating factor for reducing serious infectious complications in the absence of prophylactic rIFN-γ.

One of the most frequent errors in the management of CGD patients is the failure to treat potentially serious infections promptly and aggressively with appropriate parenteral antibiotics. Even the best antibiotics can be rendered ineffective if given too late in the course of an infection in CGD. Therefore early intervention is advisable. Although many of the minor infections and low-grade fevers in CGD patients can be managed on an outpatient basis, episodes of consistently high fever over a 24-hour period or clearly established infections (e.g., pneumonia or lymphadenitis) should be treated with parenteral antibiotics that cover, at least initially, *S. aureus* and enteric gram-negative organisms. Reasonable attempts to define the source of the infection and the responsible microbe should also begin promptly. The use of PCR to supplement culture-based methods to detect microbial pathogens may be very helpful. A few organisms can cause a large inflammatory response, so often an organism cannot be found and empiric treatment must be administered without specific identification of an organism. Monitoring markers of inflammation such as the erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) can be very useful, both as a clue to the presence of a significant infection as well as following the patient's response to therapy. If the patient fails to respond, then more aggressive diagnostic procedures should be instituted (CT, bone, and gallium scans; open biopsies if indicated) and empirical changes in the antibiotics used to broaden coverage to *Pseudomonas cepacia*. If fungus is identified or strongly suspected, amphotericin B has been the drug of choice in the past, but newer azole antifungal agents such as voriconazole are supplanting its use.

Even with appropriate antibiotics, certain types of infections respond slowly and may require many months of therapy. This point about duration of treatment is critical. Especially deep tissue infections such as pneumonia require continued treatment until they are clear radiographically, and the sedimentation rate has normalized. This can take many months of intensive treatment. Otherwise, the infection will recur and long-term complications such as emphysema develop. In this sense, treatment of infection in CGD is very different than the normal population. Surgical drainage or resection can sometimes play a key role in accelerating healing of certain types of infection such as lymphadenitis, osteomyelitis, and abscesses of visceral organs such as the liver or lung. Corticosteroids in combination with high-dose antibiotics can help to avoid surgical intervention for liver abscesses. Finally, granulocyte transfusions may be of benefit in the treatment of stubborn or life-threatening infections.[3,7,10,15]

Recurrent fever in CGD always raises the possibility of infection in these patients; however, the MAS-HLH spectrum of disorders should be considered, especially if the patient has splenomegaly, leukopenia, or thrombocytopenia. If significant MAS/HLH is suspected, IFN-γ should be stopped. As in inflammatory disorders such as rheumatoid arthritis, secondary MAS-HLH has been reported in CGD, often in association with bacterial or fungal infection, and is probably often overlooked (see Chapter 53). Specific treatment may be indicated,

TABLE 51.5 **Efficacy of Interferon-γ in Preventing Serious Infections in Chronic Granulomatous Disease**

	Clinical Study				
Variable	**Phase III Placebo[a]**	**Phase III IFN-γ[a]**	**Phase IV (US) IFN-γ[b]**	**Phase IV (Europe) IFN-γ[c]**	**Phase IV IFN-γ[d]**
Patients (*n*)	65	63	30	28	76
Average duration of therapy on study (years)	0	0.83	1.03	2.4	4.3
Patient-years in study	50.9	52.1	31.10	67.2	328
Serious infections per patient-year	1.1	0.38	0.13	0.4	0.30
Number of hospital days per patient-year	28.2	8.6	2.2	15.0	Not reported

[a] Results from The International Chronic Granulomatous Disease Cooperative Study Group: A controlled trial of interferon gamma to prevent infection in chronic granulomatous disease. *N Engl J Med.* 1991;324:509.

[b] Results from Weening RS, Leitz GJ, Seger RA. Recombinant human interferon-gamma in patients with chronic granulomatous disease—European follow up study. *Eur J Pediatr.* 154:295, 1995.

[c] Results from Bemiller LS, Roberts DH, Starko KM, et al. Safety and effectiveness of long-term interferon gamma therapy in patients with chronic granulomatous disease. *Blood Cells Mol Dis.* 21:239, 1995.

[d] Results from Marciano BE, Wesley R, De Carlo ES, et al. Long-term interferon-gamma therapy for patients with chronic granulomatous disease. *Clin Infect Dis.* 39:692, 2004.

IFN, Interferon.

especially if the patient has significant cytopenias or evidence of hepatic dysfunction.

Inflammatory complications of CGD can occur at all ages but can be an increased burden in adults. These can be challenging to treat in CGD because use of agents to suppress the inflammatory response can increase the risk of fungal or bacterial infections.[3,9,10,15] Judicious use of corticosteroids can be indicated, including in cases of severe asthma, esophageal strictures, gastric antral narrowing, granulomatous cystitis, inflammatory bowel disease, or certain cases of pneumonia. Clear evidence shows that corticosteroids are beneficial in these clinical settings because the steroids induce rapid regression of obstructive symptoms at low oral doses (e.g., 1 mg/kg/day of prednisone). Steroids can be lifesaving in young children with airway obstruction because of inflammation. Because of the exaggerated inflammatory reaction seen in CGD, there can be significant swelling in the airway and compression by pulmonary nodes that can block air movement and impede drainage. In these cases, the physician and patient should be aware of the risks of the additional immunosuppression caused by corticosteroids, particularly for fungal infection. Corticosteroids have also emerged as important component of treating *Staphylococcal* liver abscesses in combination with antibiotics, with improved outcomes and often avoidance of invasive procedures for abscess drainage. In colitis, steroid-sparing agents such as sulfasalazine and azathioprine for colitis can be helpful. Biologic agents such as TNF-α inhibitors or those blocking IL-1 are also occasionally used in patients with difficult-to-manage inflammatory disease, but with caution due to the increased risk of infection. The chronic lung disease that can develop in older patients with CGD can be difficult to treat and may be an indication for stem cell transplantation.

Rare patients with X91° CGD have genomic deletions that span the gp91^{phox} gene and the Xk gene, which encodes a membrane protein necessary for expression of the Kell genes.[3] Absence of the Xk gene product results in the McLeod syndrome, in which red blood cells have weak Kell antigens and variable acanthocytosis along with nerve and muscle disorders related to its expression in nonerythroid tissues. Transfusion of patients with McLeod syndrome poses a serious problem because they can develop alloantibodies of wide specificity that can preclude any further transfusions except with Kell-negative blood products. McLeod-matched blood is extremely rare, and patients with this syndrome should have their own blood frozen in case it is needed. Note that use of maternal blood does not solve the problem because only 50% of the mother's blood will match. Because of the difficulty in finding blood, transfusion with non-McLeod blood is likely to occur. Although management is difficult and use of steroids is necessary, the hemolytic anemia can be managed successfully.

Allogeneic hematopoietic stem cell transplant (HSCT) can be used to treat CGD, including using matched unrelated donors.[10,16] Because of risks associated with this procedure, HSCT was previously considered only for patients who have a fully human leukocyte antigen (HLA)-matched sibling and frequent and severe infections or refractory gastrointestinal disease despite aggressive medical management. However, reduced-intensity conditioning regimens for allogeneic transplantation with sibling or nonsibling donors have now been successfully used for HSCT in CGD and has a high curative rate with low mortality.[10,16] Which patients with CGD should undergo transplantation is an individualized decision, particularly for those with residual NADPH oxidase activity, for adults, or when well-matched donor marrow is not available. The best results are in young patients without preexisting complications. Because it is currently not possible to predict which patients will develop significant inflammatory complications such as gastrointestinal or lung disease later in life, transplantation in the first decade of life should be strongly considered for any patient with a fully HLA-matched healthy sibling or fully matched high-resolution HLA-typed unrelated donor, regardless of infection history. The indications for transplant in adults should be carefully assessed in consultation with an experienced HSCT center, although results in recent years have been encouraging. While the resolution of gastrointestinal disease in CGD following transplant has been appreciated for some time, the problematic inflammatory lung disease with progressive dyspnea that can develop in older CGD patients also appears to be reversible to a remarkable degree with successful HSCT.

Finally, gene therapies aimed at correcting the defective gene in bone marrow (BM) stem cells hold promise for the future if obstacles can be solved to achieve effective and safe gene delivery and their transplantation (see Chapter 104).[10] Observations on female carriers of X-linked CGD with skewed X-inactivation and preclinical studies in murine CGD models suggest that complete correction of NADPH oxidase activity in 10% of circulating neutrophils will lead to clinically relevant improvements in host defense, although inflammatory complications may still be problematic if only partial correction is achieved.

Neutrophil Glucose-6-Phosphate Dehydrogenase Deficiency

NADPH, the primary substrate for the respiratory burst oxidase, is generated by the first two reactions of the hexose monophosphate shunt pathway, which are catalyzed by G6PD (see Fig. 51.3, reaction 8) and 6-phosphogluconate dehydrogenase (6PGD).[17] The leukocyte and erythrocyte G6PD are encoded by the same gene. Thus a severe deficiency of G6PD in neutrophils can result in a greatly attenuated respiratory burst because of low levels of NADPH. However, the vast majority of individuals with inherited G6PD deficiency do not have problems with a decreased respiratory burst or recurrent infections. A CGD-like syndrome has very rarely been observed in G6PD-deficient patients who have congenital nonspherocytic hemolytic anemia (CNSHA), in whom hemolysis occurs in the absence of redox stress.[3,17] Even in CNSHA, most G6PD mutations cause the enzyme to decay over a period of days and weeks, so that levels in the short-lived neutrophil usually do not become critically low even in some of the most unstable G6PD variants (see Chapter 45). A few rare and poorly understood G6PD mutations that cause CNSHA are associated with extremely low (<5% of normal) levels of G6PD in the neutrophil, resulting in a deficient respiratory burst and CGD-like symptoms. The combination of chronic, severe hemolytic anemia, recurrent infections, and the laboratory demonstration of extremely low G6PD levels in neutrophils and erythrocytes serves to distinguish this disease from CGD. The treatment for neutrophil G6PD deficiency is the same as for CGD, except that the efficacy of rIFN-γ has not been demonstrated in the former. The chronic hemolytic anemia is treated by supportive means, including transfusions.

Disorders of Glutathione Metabolism

As depicted in Fig. 51.3 (reaction 6), the reduced form of glutathione (GSH) serves to protect the neutrophil from the harmful effects of hydrogen peroxide on NADPH oxidase and other neutrophil proteins. Adequate intracellular levels of GSH are maintained by recycling oxidized glutathione (GSSG) to GSH by glutathione reductase (see Fig. 51.3, reaction 7), as well as by de novo synthesis of glutathione by glutathione synthetase (see Fig. 51.3, reaction 9). Severe deficiencies in either of these enzymes are extremely rare and are apparently inherited in an AR manner.[3] In the case of glutathione reductase deficiency, the respiratory burst terminates prematurely, presumably owing to the toxic effects of accumulating hydrogen peroxide on NADPH oxidase. However, this brief burst of O_2^- appears to be sufficient for adequate microbial killing because the few patients reported have not had problems with recurrent infections. However, they do have congenital hemolytic anemia during periods of oxidant stress caused by diminished levels of glutathione reductase in erythrocytes. In glutathione synthetase deficiency, the respiratory burst proceeds normally. Patients have severe metabolic acidosis caused by elevated levels of

5-oxoproline, which is the product of the first step in glutathione synthesis and is present in increased levels because of a lack of feedback of GSH on the synthetic pathway. Patients with glutathione synthetase deficiency also have intermittent neutropenia (perhaps caused by the acidosis), as well as oxidant-induced hemolysis. There are mild problems with recurrent infections. Therapy with vitamin E (400 IU/day) has been found to be beneficial in patients with severe glutathione synthetase deficiency with hemolysis and infections.

Myeloperoxidase Deficiency

MPO deficiency is the most common inherited disorder of phagocytes but is almost always asymptomatic.[18] MPO is present in azurophilic granules of neutrophils and monocytes and catalyzes the production of a potent antimicrobial agent, HOCl, from chloride and hydrogen peroxide (see Fig. 51.3, reaction 4).[4,5] HOCl in turn reacts with a variety of primary and secondary amines to form chloramines, some of which can be toxic. Moreover, HOCl is capable of activating latent metalloproteinases (e.g., collagenase) and inactivating antiproteinases.

Complete MPO deficiency is seen in approximately 1 in 4000 individuals, and partial deficiency is even more common (1 in 2000 persons). The key features of MPO deficiency are summarized in Table 51.6. The disorder is inherited in an AR manner. In the few cases reported, several different mutations have been identified, which generally appear to affect the posttranslational processing of a precursor polypeptide for MPO. Acquired forms of MPO deficiency are also seen. The gene that encodes for MPO is located on chromosome 17 at q22–q23 near the breakpoint for the 15-to-17 translocation of promyelocytic leukemia. Subpopulations of MPO-deficient cells can be seen not only in the M3 (promyelocytic) form of acute myeloid leukemia but also in the M2 and M4 forms. MPO-deficient cells are also seen in approximately 25% of patients with chronic myeloid leukemia and myelodysplastic syndromes.

One of the most curious features of MPO deficiency is the remarkable lack of clinical symptoms in affected persons, given the prediction that severe MPO deficiency would cripple important antimicrobial reactions catalyzed by **hypochlorous** acid (HOCl). In vitro an impressive defect in killing *Candida albicans* and hyphal forms of *Aspergillus fumigatus* is observed.[18] Bacterial killing in vitro is also abnormal in being somewhat slower than normal, but eventually it is complete. MPO-deficient mice also exhibit abnormalities in host defense against *Candida* and *Klebsiella* spp. However, excessive or unusual infections in MPO-deficient patients are uncommon, except for rare individuals who also have diabetes mellitus.[18] In these individuals, disseminated fungal infections (usually candidiasis) are seen.

The discrepancy between the in vitro and in vivo manifestations of MPO deficiency in most patients can be explained in several ways. First, the respiratory burst in MPO-deficient neutrophils is substantially augmented, presumably from the absence of HOCl-mediated toxic effects on the NADPH oxidase. Second, other products of the respiratory burst, together with the oxygen-independent antibacterial proteins, appear to have sufficient potency to compensate for the loss of MPO-dependent reactions. Finally, residual amounts of MPO coupled with the normal levels of eosinophil peroxidase may provide at least some degree of peroxidative activity at the sites of infection.

Treatment is usually not required for MPO deficiency except in those individuals with fungal infections. In these patients, aggressive use of antifungal antibiotics is indicated. The prognosis is excellent in the majority of patients with MPO deficiency.

TABLE 51.6 Summary of Myeloperoxidase Deficiency

Incidence	1 in 2000 (partial deficiency) 1 in 4000 (total deficiency)
Inheritance	Autosomal recessive with variable expression; MPO gene on chromosome 17 at q22–q23
Molecular defect	Defective posttranslational processing of an abnormal MPO precursor polypeptide; eosinophil peroxidase encoded by different gene and levels normal
Pathogenesis	Partial or complete MPO deficiency leads to diminished production of HOCl and HOCl-derived chloramines; MPO products are necessary for rapid killing of microbes (especially *Candida* spp.) but not absolutely required
Clinical manifestations	Usually clinically silent Disseminated candidiasis or fungal disease (rare; usually in conjunction with diabetes mellitus) Acquired deficiency in M2, M3, and M4 AMLs and myelodysplasia
Laboratory evaluation	Deficiency of neutrophil and monocyte peroxidase by histochemical analysis (eosinophil peroxidase normal) Delayed, but eventually normal, killing of bacteria in vitro Failure to kill *Candida albicans* and hyphal forms of *Aspergillus fumigatus* in vitro
Differential diagnosis	Acquired partial MPO deficiency seen in M2, M3, and M4 AML; MDS; and Batten disease
Therapy	None in asymptomatic patients Aggressive treatment of fungal infections when they occur Control of blood glucose levels in diabetics
Prognosis	Usually excellent

AML, Acute myeloid leukemia; *HOCl*, hypochlorous acid; *MDSs*, myelodysplastic syndromes; *MPO*, myeloperoxidase.

DISORDERS OF PHAGOCYTE ADHESION AND CHEMOTAXIS

Numerous investigators have found in vitro chemotactic abnormalities in neutrophils from patients with a wide variety of clinical disorders associated with increased susceptibility to bacterial and fungal infections.[3] In most circumstances, the chemotactic abnormality identified was only marginal and not always clearly related to the clinical status of the patient. In other instances, clear and major defects were identified in vitro that correlated with the in vivo propensity for infection. Extensive classification systems have been devised to categorize the numerous acquired defects in chemotaxis. The problem in many of these reports is that it is unclear whether the infections were caused by the in vitro chemotactic abnormality or by the medical complications of the underlying disorder (e.g., acidosis, malnutrition, or exposure to nosocomial infections). A further complicating factor is that there are inherent limitations in the in vitro chemotaxis assay, which is subject to laboratory artifacts both as a result of neutrophil purification procedures as well as the assay itself. Furthermore, the extent to which these in vitro chemotactic assay systems faithfully reflect prevailing in vivo conditions is not known. Our understanding of chemotactic disorders has been hampered by the limitations of these assays, just as the elucidation of respiratory burst defects was obscured when the major available assay was in vitro bacterial killing. In this section, the most important and best characterized of the chemotactic disorders, leukocyte adhesion deficiency (LAD), is discussed in detail. A brief discussion of several other clinically significant chemotactic disorders is also provided.

DIAGNOSIS OF CHEMOTACTIC DISORDERS

The direct measurement of neutrophil chemotaxis in a clinical setting is challenging and requires a specialized research laboratory. Because the assays are biologic assays, the laboratory must run the tests at least monthly to maintain competence and have acceptable normal ranges. Neutrophil chemotaxis is significantly affected by inflammation, complement activation, and medications, making it very difficult, especially in a patient with infection, to determine if the infection is attributable to a chemotactic defect or if the defect is attributable to the infection. This is further complicated by the fact that inflammation activates the neutrophils and affects which populations actually come off of the density gradients required to separate the cells for assay. Unlike the respiratory burst, chemotaxis must be done on fresh cells, so samples cannot be reliably shipped.

The neutrophils from patients with primary chemotactic defects have almost no motility in standard biologic assay systems. Some chemotactic disorders can be diagnosed by assays of other characteristic features.

Leukocyte Adhesion Deficiency I (LAD I)

- LAD I has a significant chemotactic defect as well as phagocytic defect and is characterized by leukocytosis. The diagnosis can be made by flow cytometry of the CD11b complex on the surface.
- Fig. 51.6 indicates surface expression of C3b and CD11b on neutrophils. C3b is used as a positive control and is normal in LAD1. With stimulation, CD11b increases *(top panel)*.
- The results in this assay are expressed as percent of normal stimulated control mean channel number. It is important to note that this is not the percent positive cell, as is the case for most flow cytometry assays.
- Severe LAD1 has no increase with stimulation.
- Moderate LAD1 shows some shift of fluorescence with stimulation.
- Genetic analysis for this disorder is clinically available.

Other chemotactic disorders: Genetic analysis is available for several primary neutrophil defects, and this approach should be pursued before attempting assay of chemotaxis in a clinical setting.

No other primary chemotactic defects are readily diagnosed by a routine clinical laboratory. Measurement of neutrophil chemotaxis itself to look for secondary defects for any kind of clinical decision making is difficult if not impossible to interpret.

TABLE 51.7 **Summary of Leukocyte Adhesion Deficiency Type 1**

Incidence	More than 300 patients described in literature
Inheritance	AR
Molecular defect	An absent, diminished, or structurally abnormal β subunit (CD18) caused by one of several types of mutations in the β gene; in the absence of a normal β subunit, the three types of α chains in the β2 integrin subfamily (CD11a, b, c) cannot assemble into normal α–β heterodimers
Pathogenesis	All three β2 integrins (CD11a/CD18, CD11b/CD18, and CD11c/CD18) are deficient on all leukocytes, causing multiple abnormalities in cell function: adherence; chemotaxis; and C3bi-mediated ingestion, degranulation, and respiratory burst
Clinical manifestation	Persistent granulocytosis (neutrophil count: 12,000–100,000/mm^3) Severe or moderate phenotypes depending on severity of deficiency Recurrent pyogenic infections with absent neutrophil infiltration Delayed umbilical cord separation Severe gingivitis or periodontitis
Laboratory evaluation	Flow cytometric measurement of surface CD11b in stimulated neutrophils with monoclonal anti-CD11b
Differential diagnosis	CGD May be associated with severe neutrophil actin dysfunction
Therapy	Hematopoietic stem cell transplant in clinically severe patients (CD11b <0.3% of normal) Aggressive use of parenteral antibiotics Possible benefit of prophylactic TMP-SMX
Prognosis	Severe: high incidence of death before 2 years of age unless transplantation is performed Moderate: can survive into 20s and 30s but with recurrent infections

AR, Autosomal recessive; *CGD*, chronic granulomatous disease; *TMP-SMX*, trimethoprim–sulfamethoxazole.

Leukocyte Adhesion Deficiency Type I

LAD type I (LAD I) is a rare AR disorder of leukocyte adhesion, chemotaxis, and ingestion of C3bi-opsonized microbes as a result of decreased or absent expression of the leukocyte β_2 integrins (Table 51.7).[19,20] The hallmark of LAD I is the occurrence of repeated, often severe bacterial and fungal infections without the accumulation of pus despite persistent granulocytosis (see Table 51.7). The molecular basis for LAD was first suggested by Crowley and colleagues, who found that neutrophils from a patient with this clinical syndrome lacked a high-molecular-weight membrane glycoprotein (see Dinauer, Newburger, and Borregaard[3]). The patient's neutrophils could not be made to adhere to plastic surfaces or to respond to serum-opsonized particles in terms of ingestion and respiratory burst activity.

The molecular basis of LAD I is now known to result from mutations in the gene for the common CD18 β2 subunit for these three leukocyte glycoproteins, now termed *β2 integrins*, that belong to the integrin superfamily of adhesion molecules (see Chapter 15). Integrins are noncovalently linked heterodimeric glycoproteins consisting of an α and a β subunit. Within each of the eight known integrin subfamilies, the β subunit is identical (and defines the subfamily), but the α subunit varies and confers the functional specificity on the integrin. The molecular defect in LAD involves all members of the β2 integrin subfamily: αL β2 (CD11a/CD18), αm β2 (CD11b/CD18), and α× β2 (CD11c/CD18). CD11a/CD18 is often referred to as *LFA-1*, and CD11b/CD18 is also called *Mac-1*, *Mo1*, or *CR3*. LAD I patients have an absent, diminished, or structurally abnormal β2 subunit (CD18; see later), and as a result, the three types of α chains in the β2 integrin subfamily cannot assemble into normal α–β heterodimers. Thus all three β2 integrins are moderately to severely deficient on all leukocytes in LAD.

The β2 integrins serve as receptors for the opsonic complement fragment C3bi, the intercellular adhesion molecules 1 and 2 (ICAM-1 and ICAM-2) that are expressed on endothelial cells and leukocytes, and fibrinogen. The diminished or absent expression of β2 integrins in LAD I leukocytes results in the failure of phagocytes to emigrate from the bloodstream to sites of infection. The early interactions with the endothelium, termed *rolling*, are normal in LAD I because these are mediated by a different family of adhesion molecules known as *selectins*. However, β2 integrins are responsible for the subsequent tight binding of neutrophils and monocytes to ICAMs on cytokine-activated endothelium, and this step is therefore severely defective in LAD I. Transendothelial migration is also impaired. A second major functional defect in LAD is the failure of phagocytes to bind C3bi-opsonized microbes. Because CD11b/CD18 is the predominant phagocyte receptor for this complement fragment, C3bi-mediated ingestion, degranulation, and respiratory burst activity are severely affected in LAD. Finally, β2 integrin–dependent signals play a key role in activating neutrophils for enhanced migration, phagocytosis of antibody-opsonized microbes, and degranulation.

Despite in vitro defects in lymphocyte responses dependent on LFA-1 (CD11a/CD18), patients with LAD I rarely have clinical manifestations related to impaired lymphocyte function. It is believed that the role CD11a/CD18 plays in lymphoid cell function can be compensated by other adhesion proteins (CD2, CD4, CD8, and so on).

Molecular Genetics of Leukocyte Adhesion Deficiency Type I

The fact that LAD I involves a deficiency of all leukocyte β2 integrins focused attention on the common β2 chain (CD18), and mutations in the corresponding gene, *ITGB2*, have been identified in all LAD I patients who have been analyzed at the molecular level to date.[19,20] Although expression of the leukocyte integrin α subunits is normal in LAD I, these are not transported to the cell surface because the β2 chain is absent or contains mutations that disrupt its structure or its interaction with the α subunit. Mutations in the α subunits have not been found thus far in patients with LAD I. The CD18 glycoprotein has a large extracellular domain at the N terminus, a single transmembrane domain, and a 46-residue cytoplasmic tail. CD18 mutations in LAD I are heterogeneous in nature and family specific and can lead to either undetectable or low (9% to 20% of normal) levels of α–β dimer expression that correlates with the clinical severity of the disease. More than 50 different mutations have now been characterized in more than 100 families.[20] These include missense mutations, messenger RNA (mRNA) splicing defects, small deletions, and a premature termination signal. Many patients are compound heterozygotes and have two different mutant alleles for CD18. About half of patients with LAD I in whom the genetic defect has been identified have point mutations in a stretch of 250 amino acids in the extracellular domain of CD18. This region is highly conserved among all β subunits and appears to be important for interaction with the α subunit.

Clinical Features

The key features of LAD I are summarized in Table 51.7. The clinical presentation of LAD is heterogeneous and is related to the severity of the deficiency of the β2 integrins. The severe clinical phenotype is associated with less than 0.3% of the normal amount of these glycoproteins on the leukocyte surface; the moderate phenotype has 2.5% to 6% of normal levels. In both the severe and moderate forms of the disease, persistent granulocytosis (neutrophil count of 12,000 to 100,000/mm^3) is a constant finding, as are recurrent cutaneous abscesses and aggressive periodontitis and gingivitis. Recent studies suggest that impaired migration and clearance of neutrophils in periodontal tissues results in a dysregulated interleukin (IL)-23 to IL-17 inflammatory axis, which drives the severe periodontal disease typical of LAD I rather than impaired control of gum bacteria.[21] Additional clinical features seen more often in the severe clinical phenotype include delayed umbilical cord separation, omphalitis, perirectal cellulitis, severe ulcerative stomatitis, and bacterial sepsis. A striking finding in LAD I is that abscesses and other sites of infections are devoid of pus despite the marked neutrophilia because neutrophils are unable to emigrate to tissues. *S. aureus* and gram-negative enteric bacteria cause the majority of infections in LAD I. Fungal infections can also occur, particularly from *C. albicans* and *Aspergillus* spp.

Note that infants with delayed separation of the umbilical cord who are healthy and have normal blood counts are very unlikely to have LAD I. Although the mean age of cord separation ranges from 7 to 15 days, 10% of healthy infants can have cord separation at 3 weeks of age or later.

Diagnosis

The diagnosis of LAD I is made by flow cytometric measurement of surface CD11b (Mac1; or the shared CD18 subunit) in unstimulated and stimulated neutrophils using commercially available monoclonal antibodies directed against CD11b or CD18 (Fig. 51.6). Neutrophils contain an intracellular pool of CD11b/CD18 in their secondary (specific) and tertiary granules, which can be mobilized to the cell surface during stimulation. Therefore the deficiency of CD11b can be more dramatically demonstrated by using stimulated neutrophils. Carriers of LAD I can be identified by this method because they have been found to express approximately 50% of normal levels of CD11b on the surface of their stimulated neutrophils (see Fig. 51.6).

Prognosis and Treatment

Treatment of LAD I depends on the clinical severity of the disorder. In patients with the moderate clinical phenotype, cutaneous and oral infections can be managed as they occur. The use of prophylactic antibiotics such as TMP-SMX appears to be beneficial, as does aggressive prophylactic treatment of periodontal disease, which could also potentially benefit from agents targeting the IL-23 axis. It is important to note that even patients with the moderate phenotype can die of overwhelming infection. In patients with severe LAD I, aggressive management is indicated because of the high incidence of death before the age of 2 years, and HSCT is recommended. LAD I should also be amenable to gene-replacement therapy in the future.

Leukocyte Adhesion Deficiency Types II and III

LAD type II is a very rare clinical syndrome closely related to LAD I but caused by a defect in selectin-mediated adhesion events from a deficiency in leukocyte sialyl-Lewis X ligands.[3,19] It was first reported by Etzioni et al. in two unrelated boys of Muslim Arab origin and has since been described in a total of five individuals (four Arab and one Turkish). The disease is inherited in an AR manner. Patients

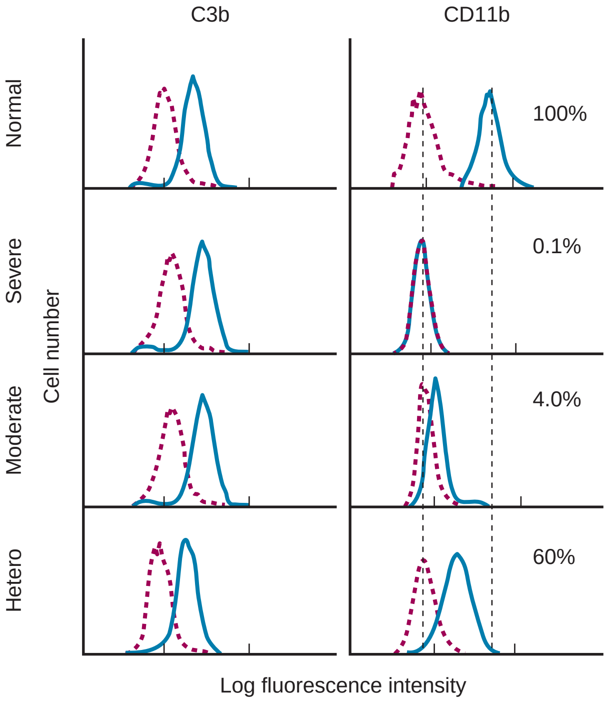


Figure 51.6 EVALUATION OF ADHESION MOLECULE EXPRESSION FOR DIAGNOSIS OF LEUKOCYTE ADHESION DEFICIENCY. Fluorescence of C3b-specific antibody labeled neutrophils *(solid line)* increases compared with a nonspecific control *(dashed line)* after 30-minute exposure to 10-nM N-formylmethione-leucyl-phenylalanine (fMLP) in healthy donors, patients with severe and moderate forms of leukocyte adhesion deficiency (LAD) I, and a heterozygous LAD I carrier. However, the increase in CD11b fluorescence *(solid line)* is markedly diminished in LAD I patients. The respective percent of normal stimulated mean channel number is shown in the *right column*. Note that these results are expressed as percent of normal fluorescence intensity, not as percent of positive cells, as is the case in most flow cytometry assays.

present with neutrophilia, recurrent bacterial infections, and periodontitis, similar to LAD I, although these symptoms were generally not as severe. In addition, LAD II is associated with dysmorphic features and psychomotor retardation. LAD II neutrophils express normal levels of CD18. A clue as to the molecular cause of LAD II came from the observation that LAD II red cells were Lewis antigen–negative and also had the rare Bombay (hh) erythrocyte phenotype, in which red blood cells express a nonfucosylated variant of the H antigen. These antigenic defects share in common the failure to form certain fucose carbohydrate linkages. The defect in fucose metabolism in LAD II has now been shown to result from mutations in the Golgi guanosine diphosphate–fucose membrane transporter. This leads to a generalized loss of expression of fucosylated glycans on the surface of cells, particularly the sialylated and fucosylated tetrasaccharide, SleX (CD15a), on the neutrophil surface. As a result, LAD II neutrophils are unable to bind to E- and P-selectin receptors on endothelium and therefore have an impairment in the early steps of rolling and loose binding to blood vessel walls before tight adhesion and emigration into infected tissues. Fucose supplementation has been partially successful in increasing expression of SLeX and decreasing clinical problems.

LAD type III has been described in a handful of patients with severe, recurrent infections similar to LAD I, as well as a bleeding tendency similar to Glanzmann thrombasthenia (a β3 integrin–related disorder).[3,19] This disorder results from AR defects in KINDLIN-3, a regulatory protein required for "inside-out" activation of multiple classes of integrins in blood cells. Because the clinical manifestations are typically severe, early BMT may be indicated for patients with LAD III (see Etzioni[19]).

Hyperimmunoglobulin E Syndrome

Hyperimmunoglobulin E syndrome (HIES) is a complex disorder characterized by markedly elevated serum immunoglobulin E (IgE) levels, serious recurrent staphylococcal infections, mucocutaneous candidiasis, chronic dermatitis, and skeletal and dental abnormalities.[22] Although not a primary phagocyte defect, neutrophils from patients with this syndrome exhibit a variable and at times profound chemotactic defect. HIES was first described in 1966 and was called *Job syndrome*, in reference to the biblical description of Job as being affected by "sore boils from the soles of his feet unto his crown." The skin abscesses in patients with HIES lack the erythema that is typical of such lesions and are referred to as *cold abscesses*. The key features of HIES are described in Table 51.8. The disorder is rare, affecting fewer than 1 per million population.

Most cases of HIES result from inherited or sporadic autosomal dominant negative (DN) mutations in signal transducer and activator of transcription 3 (STAT3), a Janus kinase (JAK)-activated transcription factor activated in response to many cytokines and growth factors. These mutations, which are usually a result of missense or in-frame deletions and located primarily in regions of the protein that interact with other proteins or with DNA, inhibit the activity of the wild-type STAT3 allele and result in a complex pattern of altered cytokine signaling and impaired T helper type 17 (Th17) cell differentiation. Mutations in *DOCK8*, a guanine nucleotide exchange factor, or *TYK2*, a JAK family member, result in an AR immunodeficiency with some overlap with HIES features but have more profound impairment in lymphocyte functions, leading to a broader risk of infection and other immune dysregulation. DOCK8 deficiency has recently been recategorized from HIES to combined immunodeficiency[22] and will not be further discussed in this chapter (see Chapter 52).

Insights into how STAT3 signaling defects lead to the clinical syndrome characteristic of DN STAT3-HIES are now emerging. Defective responses to IL-6 may account for the minimal inflammatory responses characteristic of HIES, and the enhanced IgE production may reflect dysregulated immune responses secondary to impaired signaling by IL-10, a negative regulator. Th17 cells are important for control of mucocutaneous *Candida* infection, which is problematic in many patients. In addition, keratinocytes and bronchial epithelial cells are particularly dependent on Th17 cytokines to produce chemokines and antimicrobial peptides, which may explain the predilection for skin and lung infections. The dental and skeletal abnormalities may be related to defective STAT3 signaling in osteoblasts and osteoclasts.

MANAGEMENT OF INFECTIONS

The management of infections in patients with primary neutrophil dysfunction syndromes is quite different than in the normal population and for the most part different from other patients with neutropenia.

- Patients tend to present with relatively low fevers and chronic inflammatory processes associated with marked elevation of erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). Unless they have untreated abscesses or inflammatory masses, they tend not to present with frank sepsis and positive blood cultures.
- The frequency of infections decreases somewhat with age in children as their normal T cell– and B cell–mediated immunity develops.
- Although one should always attempt to obtain culture proof of an infection, more often than not, it is not possible to identify an organism, and it is necessary to treat empirically.
- Because these patients tend to develop deep-seated tissue infections, the ESR can be of great value even though it is quite nonspecific. Elevation in the ESR suggests deep tissue inflammation; CRP is more acute and suggests monocyte activation. Persistent significant elevation of the ESR (>15–20 mm/h) even in the absence of fever or other symptoms may warrant radiologic search for deep-seated infection.
- The authors advocate an "antibiotic sensitivity by ESR response" approach to empiric therapy in stable patients. One can start at parenteral anti-*Staphylococcus* and gram-negative therapy and monitor the ESR daily. A monotonic decrease in the ESR within several days that is clear-cut suggests the process is sensitive to the antibiotic selected. Although complete resolution of inflammatory response may take many weeks, usually there is some clear change in the ESR within 1 week. If there is worsening or no clear response, then an antifungal can be added and the ESR monitored in the same fashion. Return of an elevated ESR can be a sign of development of organism resistance.
- If a patient with chronic granulomatous disease (CGD) is particularly ill appearing or febrile, it is important to make sure that *Burkholderia cepacia* complex bacteria are covered.
- There is no fixed duration of therapy for any infections in these patients, but antibiotic treatment must often be prolonged. The duration of therapy is critical, and if the infections are not completely extinguished, they will return and will contribute to development of chronic pulmonary and hepatic fibrosis. Parenteral antibiotics or antibiotics that can deliver very high tissue levels should be continued significantly past normalization of the ESR and disappearance of any radiographic evidence of deep tissue infection. This can take many months for some pneumonias and liver abscesses.
- Short pulses of steroids (4–6 days) can be lifesaving, particularly for pulmonary infections in young children with CGD. They reduce airway inflammation and promote drainage. Steroids can also be useful for management of liver abscesses in CGD.
- Young children are susceptible to infections with routine childhood viruses and infections and tend to do well with standard therapeutic approaches and courses of treatment that are 2 to 3 times longer than the usual recommended course. Again, monitoring with the ESR can be a guide.

All standard childhood immunizations and influenza vaccinations are strongly recommended. Prophylactic antibiotics may be appropriate (see text).

Clinical Manifestations

The clinical manifestations of STAT3-HIES are at times dramatic. Onset is generally in the first 2 months of life and is manifested

TABLE 51.8 Summary of Signal Transducer and Activator of Transcription 3–Hyperimmunoglobulin E Syndrome

Incidence	More than 300 patients reviewed in the literature
Inheritance	AD with incomplete penetrance; sporadic forms with de novo mutations
Molecular defect	Dominant negative mutations in STAT3 (AD inheritance or de novo mutations)
Clinical manifestations	Staphylococcal pneumonia Pneumatoceles Fungal superinfection of lung cysts "Cold" cutaneous skin abscesses and furuncles Chronic eczematoid dermatitis Mucocutaneous candidiasis Coarse facies, growth retardation Osteopenia, recurrent fractures Sinusitis, keratoconjunctivitis Scoliosis Hyperextensible joints Delayed shedding of primary teeth Vascular disease
Laboratory evaluation	Serum IgE >2500 IU/mL Peripheral blood eosinophilia Immunophenotyping for Th17 cells
Differential diagnosis	Atopic dermatitis Wiskott-Aldrich syndrome, DiGeorge syndrome Hypergammaglobulinemia Chronic granulomatous disease
Therapy	Prophylactic anti-*Staphylococcus aureus* antibiotics Aggressive treatment of acute infections with parenteral antibiotics Surgical drainage of deep infections and resection of lung cysts Monitor for scoliosis, fractures, vascular disease
Prognosis	Generally good if managed aggressively

AD, Autosomal dominant; *HIES*, hyperimmunoglobulin E syndrome; *IgE*, immunoglobulin E; *STAT3*, signal transducer and activator of transcription 3; *Th17*, T helper type 17.

by chronic dermatitis. By 5 years of age, patients have a history of recurrent skin abscesses, pneumonias, chronic otitis media, and sinusitis. As patients grow older, recurrent staphylococcal pneumonia is a common problem and can be complicated by the formation of pneumatoceles that can become colonized with *Pseudomonas aeruginosa* or *Aspergillus*. Septic arthritis, cellulitis, and osteomyelitis are also observed and are usually caused by *S. aureus*, although other bacterial pathogens have also been found. Patients can have chronic mucocutaneous candidiasis and occasionally exhibit keratoconjunctivitis, sometimes complicated by corneal scarring. One feature noted in the majority of patients by the time they reach the teenage years is the presence of coarse facial features (broad nasal bridge, prominent nose). Dental and bone abnormalities are also common features of HIES. Delayed or failure to shed primary teeth occur in the majority. Hyperextensible joints and scoliosis are frequent. Osteopenia of unknown etiology is observed in most patients, and there is an increased risk of fractures to the long bones and vertebral bodies even in the absence of osteopenia. Vascular disease, including aneurysms, tortuosity of middle-sized arteries, and hypertension, is also frequently seen.

Diagnosis

The diagnosis of STAT3-HIES should be entertained in any child or young adult who has the above-described clinical picture or simply a history of recurrent infections. The hallmark laboratory finding is a marked elevation of serum IgE, almost always greater than 2500 IU/mL. Levels can be as high as 150,000 IU/mL. Most patients also have peripheral eosinophilia. However, there is no correlation of clinical disease activity with the level of either IgE or peripheral eosinophilia. Atopic dermatitis is the major differential diagnosis because comparably high serum levels of IgE can be seen in patients in this disorder, as well as superficial skin infections. The severe and recurrent nature of the staphylococcal furuncles and pneumonias usually seen in HIES can help distinguish these patients from those with atopic dermatitis. Patients with other primary immunodeficiency syndromes may also manifest elevated IgE levels. Scoring criteria predictive of STAT3 mutations, including recurrent pneumonia, pathologic bone fractures, and lack of Th17 cells, are helpful.[22] DNA testing of STAT3 should be used to make a definitive diagnosis; there are also rare patients who phenocopy STAT3-HIES but have mutations in pathway-related genes.

Therapy

The therapy for STAT3-HIES is largely supportive.[22] Treatment emphasizes prevention of end-organ complications by using antimicrobial prophylaxis and aggressive treatment of infections. Prophylactic antibiotics (e.g., dicloxacillin or TMP-SMX) can be effective in preventing *S. aureus* infections. Dermatitis can be treated with topical steroids. Bathing in diluted bleach can diminish colonization by *S. aureus*. Prophylactic antifungals can be helpful in patients with chronic mucocutaneous candidiasis. Intravenous antibiotics are used for deep-seated infections or for resistant cutaneous infections. Surgical resection of persistent pneumatoceles is sometimes indicated to prevent superinfection by fungal and gram-negative organisms. Intravenous Ig infusions have shown some success in the management of HIES. Attention should also be paid to blood pressure and other vascular complications. Although a role for HSCT in STAT3-HIES is unclear, two children with STAT3 mutations who underwent transplantation for non-Hodgkin lymphoma are alive 10 and 14 years later with resolution of all immunologic and nonimmunologic features of HIES.[22]

Miscellaneous Chemotactic Disorders

It is extremely rare to have primary defects in neutrophil actin polymerization as a cause of abnormal chemotaxis and recurrent infections.[2,3] An apparent AR disorder of actin polymerization has been described in a male infant of Tongan descent who presented with severe skin infections, recurrent pulmonary infiltrates, thrombocytopenia, and invasive *Candida tropicalis* infection (MIM %257150). Neutrophil actin polymerization was markedly abnormal and associated with increased expression of the actin-binding protein LSP-1. In another single case, a heterozygous point mutation in β-actin affecting binding to actin-regulatory proteins was discovered in a female patient with recurrent infections, photosensitivity, and mental retardation. Neutrophils had a marked impairment in chemotaxis and in the formyl peptide-induced respiratory burst. Finally, the use of unbiased exome sequencing has identified the genetic basis for several "actinopathies" due to defects in various actin regulatory proteins, resulting in impaired chemotaxis of neutrophils and other immune cells.[23] Affected patients suffer from recurrent bacterial and other infections, but each disorder also has distinctive laboratory and clinical features. For example, in patients with genetic defects in the actin-binding protein WDR1, which is important for depolymerizing actin via cofilin, about 50% of neutrophils show herniation of the nuclear lobes, which colocalize with increased F-actin staining. These patients also often have severe stomatitis resulting in acquired microstomia and oral stenosis.

A syndrome of severe neutrophil dysfunction caused by a DN mutation in Rac2, a small GTPase expressed in blood cells that acts in many signal transduction pathways, was described in an infant boy born to unrelated parents (see Dinauer and Newburger[3]). This baby presented with rapidly progressive and deep-seated soft tissue infections, along with neutrophilia and poor formation of pus. The clinical phenotype resembles LAD, but there is normal expression of β2 integrins and fucosylated proteins. Neutrophils had marked defects in actin polymerization, chemotaxis, degranulation, and the respiratory burst in response to chemoattractants. Neutrophil responses to other agonists were normal, suggesting that the DN Rac2 mutation produces a selective intracellular signaling defect.

Localized juvenile periodontitis (LJP) is a heterogeneous disorder of unknown etiology characterized by chronic and recurrent periodontal infections and severe alveolar bone loss with onset at the time of puberty.[24] Many patients with LJP have been reported to have defective neutrophil chemotaxis in vitro. At present, it appears that LJP is an acquired disorder in some patients and a genetic disorder in others. It may also be a combination of both in certain patients because they may inherit an unusual sensitivity to the chemotactic inhibitors released by certain periodontal microorganisms. The diagnosis of the disorder is made on the basis of severe periodontal disease and destructive alveolar bone loss involving the first molars and incisors developing during adolescence. It is important to note that many qualitative and quantitative neutrophil disorders are also associated with severe periodontal disease. Therefore the differential diagnosis should include neutropenia (both chronic and cyclic), LAD, CGD, and Chédiak-Higashi syndrome (CHS).

One of the most consistently observed chemotactic abnormalities is seen in neonatal neutrophils.[25] These cells exhibit impaired chemotaxis in vitro in response to a wide variety of chemotactic factors. It appears as though this abnormality is caused, at least in part, by defects in cellular adhesion as a result of diminished mobilization of intracellular adhesion-promoting molecules to the cell surface. Defective neutrophil chemotaxis can be seen in normal neonates between birth and 5 days of age. In severely ill infants, the defect may persist for a longer time.

DEFECTS IN THE STRUCTURE AND FUNCTION OF LYSOSOMAL GRANULES

Two major disorders of neutrophil granules have been described, CHS and specific granule deficiency (SGD). A great deal has been learned about the structural and functional abnormalities of neutrophils from patients with these conditions. Although rare, these disorders are also obligatory components in the differential diagnosis for any patient with recurrent bacterial and fungal infections.

Chédiak-Higashi Syndrome

CHS is a rare AR, multisystem disease resulting from widespread defects in granule morphogenesis, with giant lysosomes in leukocytes and other cells throughout the body.[26] The disorder is characterized by partial oculocutaneous albinism, frequent (and sometimes fatal) bacterial infections, a mild bleeding diathesis, and peripheral as well as cranial neuropathies associated with defects at the optic chiasm. Those who survive the recurrent infections develop an "accelerated phase" of the disease; one of the hereditary forms of HLH (see Chapter 53), which, if untreated, is eventually fatal because of a profound pancytopenia that develops.

The most dramatic granule defects are manifested in the various blood cells. Neutrophils contain a highly inhomogeneous population of huge granules derived from coalescence of azurophilic (primary) granules. The giant granules are often more prominent in the BM than in the peripheral blood because many of the abnormal myeloid precursors are apparently destroyed before they leave the BM, resulting in moderate neutropenia with absolute neutrophil counts ranging from 500 to 2000 cells/mm^3. Granules are also markedly deficient in antimicrobial granule enzymes such as cathepsin G and elastase, consistent with a defect in granule morphogenesis. Degranulation is delayed and incomplete in Chédiak-Higashi neutrophils, resulting in impaired bacterial killing. Chemotaxis is also defective, perhaps related to poor deformability because of the presence of the large granules. Monocytes and macrophages exhibit similar giant cytoplasmic granules, with resultant abnormalities in their phagocytic functions. Giant granules are also seen in lymphocytes and are associated with defects in cytotoxic T-lymphocyte and NK cell function. Eosinophils contain large granules, the functional significance of which is not known. Platelets in this disorder have a storage pool deficiency of adenosine diphosphate and serotonin, presumably caused by the abnormal granule morphogenesis in megakaryocytes, leading to a defect in platelet aggregation.

Abnormal giant granules are also present in other cell types. These include melanocytes, which contain abnormal melanosomes that cannot transfer their contents to adjacent keratinocytes; Schwann cells; astrocytes; and certain cells in the liver, spleen, pancreas, gastric mucosa, kidney, adrenal gland, and pituitary gland.

Molecular Genetics

A gene termed *CHS1* or *LYST* (for its presumed function as a lysosomal trafficking regulatory protein) is affected in the majority of CHS cases and is on the long arm of chromosome 1.[26] The encoded protein is very large (3801 amino acids); its specific function is unknown, but recent studies suggest that it may inhibit lysosome fusion with other intracellular membrane vesicles. A variety of frameshift and nonsense mutations that predict synthesis of truncated forms of the protein have been identified in most, but not all, CHS patients studied to date. There is no correlation between the length of the truncated CHS1 protein and the severity of the disease. Disorders similar to human CHS have also been described in many mammalian species, including Aleutian mink, *beige* mice, blue foxes, cats, killer whales, and Hereford cattle. Identification of the human CHS1 gene was aided by positional cloning of the mouse *lyst* homolog affected in *beige* mice.

Clinical Manifestations

The key features of CHS are summarized in Table 51.9. The disease usually presents in infancy or early childhood, with infections involving the lungs, skin, and mucous membranes being most commonly encountered. Dental caries and periodontal disease are also common.[21] The most frequent offending organism is *S. aureus*. Gram-negative bacteria, *Aspergillus* spp., and *Candida* spp. also are responsible for many infections. Platelet granule defects result in easy bruising and epistaxis. There is partial oculocutaneous albinism and photosensitivity. Patients may have a white forelock or an ashen or grayish silver sheen to the hair, which can vary from blond to dark brown. In younger patients, there may be a cartwheel distribution of pigment in the iris and an abnormal red reflex. Neurologic manifestations include peripheral or cranial neuropathies, gait abnormalities, muscle weakness, sensory loss, seizures, or spinocerebellar degeneration. Neurologic symptoms worsen with age.

Approximately 85% of children surviving into the second decade of life develop an accelerated phase of the disease, with fever, lymphadenopathy, and progressive pancytopenia, that is now recognized to be a genetic form of HLH caused by impaired lymphocyte and NK cell function.[27] The development of HLH can sometimes be precipitated by Epstein-Barr virus infection. A reactive-appearing lymphohistiocytic proliferation occurs in the liver, spleen, lymph nodes, and BM, and the prognosis is uniformly fatal unless patients undergo BMT.

Diagnosis

The diagnosis of CHS is made on the basis of the giant peroxidase-positive lysosomal granules in the peripheral blood granulocytes or in BM myeloid cells. Identification of large, acid phosphatase–positive lysosomes in amniocytes and chorionic villus cells has been used to diagnose CHS prenatally. Other clinical features characteristic of

TABLE 51.9 **Summary of Chédiak-Higashi Syndrome**

Incidence	More than 200 cases described
Inheritance	AR
Molecular defect	A defect in granule morphogenesis in multiple tissues resulting from mutations in the *CHS1 (LYST)* gene encoding a lysosomal trafficking regulator protein
Pathogenesis	Giant coalesced azurophil granules in neutrophils, resulting in ineffective granulopoiesis and neutropenia, delayed and incomplete degranulation, and defective chemotaxis; abnormal granules in other cells (NK cells, cytotoxic T cells, platelets, melanocytes, neurons)
Clinical manifestations	Partial oculocutaneous albinism Recurrent severe bacterial infections (usually *Staphylococcus aureus*) Cranial and peripheral neuropathies (muscle weakness, ataxia, sensory loss) HLH (accelerated phase)
Laboratory evaluation	Giant granules in peripheral blood granulocytes and in BM myeloid progenitor cells Widespread lymphohistiocytic infiltrates in accelerated phase
Differential diagnosis	Other genetic forms of partial albinism Giant granules can be seen in acute and CMLs
Therapy	Prophylactic TMP-SMX Parenteral antibiotics for acute infections Ascorbic acid (200 mg/day for infants; 6 g/day for adults) HSCT before or at beginning of HLH
Prognosis	Most patients die from infection or complications of HLH during the first or second decade of life unless transplantation is performed

AR, Autosomal recessive; *BM*, bone marrow; *CML*, chronic myeloid leukemia; *HLH*, hemophagocytic lymphohistiocytosis; *HSCT*, hematopoietic stem cell transplant; *NK*, natural killer; *TMP-SMX*, trimethoprim-sulfamethoxazole.

CHS can support the diagnosis, including mild oculocutaneous albinism; silvery hair, in which microscopic examination reveals giant melanin granules; and a bleeding diathesis. The development of HLH is characterized by diffuse infiltrates of lymphohistiocytic cells seen on biopsy and by pancytopenia (see Chapter 53). Occasionally, giant granules that resemble those of CHS can be seen in both acute and chronic myelogenous leukemias.

Therapy

The treatment for the stable phase of CHS is similar to that for other neutrophil disorders. Prophylactic antibiotics such as TMP-SMX appear to be beneficial. Parenteral antibiotics are indicated for acute infections, and responses are often slow. Treatment with high-dose ascorbic acid (200 mg/day for infants; 6 g/day for adults) has been found to improve the clinical status of some patients. Although there is some controversy regarding the efficacy of ascorbic acid, given the safety of this medication, it seems prudent to administer it to all patients. The treatment of HLH (accelerated phase) includes combinations of drugs that suppress the function of activated macrophages and T cells followed by allogeneic HSCT.[27] Indeed, transplantation is ideally performed before or at the beginning of the accelerated phase. Note that transplantation does not prevent the progressive neuropathy of CHS.

Specific Granule Deficiency

Neutrophil SGD is an extremely rare congenital disorder characterized by recurrent bacterial and fungal infections, primarily involving the skin, ears, and lungs.[3,28] Infections are often indolent and smoldering, and *S. aureus*, *P. aeruginosa*, enteric gram-negative bacteria, and *C. albicans* are the major pathogens. Family studies suggest that it is inherited in an AR manner. Neutrophils from patients with SGD have atypical bilobed nuclei, absent specific granules, and multiple deficiencies of secondary and tertiary granule mRNAs and proteins, including lactoferrin, vitamin B_{12}–binding protein, and gelatinase B; although azurophil granules in this disorder are present and contain MPO and lysozyme, they are markedly deficient in defensins. Monocytes display cell surface and functional defects, eosinophils lack eosinophil-specific granule proteins such as eosinophil cationic protein, and platelets have abnormal α granules, suggesting that the underlying defect may be related to regulation of the synthesis of certain granule and membrane proteins. This defect in synthesis is confined to BM-derived cells because lactoferrin secretion is normal in the glandular epithelia of SGD patients despite the severe deficiency in the neutrophils.

The recurrent skin and pulmonary infections characteristic of SGD appear to be caused by two fundamental defects in the neutrophils. One defect is the marked deficiency of at least two important microbicidal granule proteins, lactoferrin and defensins. The other defect is a relatively severe chemotactic abnormality presumably caused by the absence of the intracellular pool of leukocyte adhesion molecules that normally resides in the specific granules. As discussed earlier, these β2 integrins play a key role in phagocyte chemotaxis.

The molecular defect responsible for most cases of SGD has recently been shown to involve a myeloid transcription factor known as C/EBPε, which regulates expression of certain genes activated during granulocyte differentiation.[3,28] This came about serendipitously when it was recognized that mice with a targeted deletion in the C/EBPε gene displayed characteristics similar to those of SGD patients, including neutrophils with bilobed nuclei, absent secondary granules, and impaired chemotaxis along with increased susceptibility to bacterial infections. To date, mutations in the C/EBPε gene have been identified in two SGD patients that encode truncated, nonfunctional proteins. However, DNA sequencing of the C/EBPε gene of several other SGD patients has not revealed abnormalities, suggesting that SGD is a genetically heterogeneous disorder.

The diagnosis of SGD can be readily made by microscopic examination. Wright-stained neutrophils are devoid of specific granules but contain normal numbers of azurophilic granules. Electron microscopy reveals small peroxidase-negative vesicles, which presumably represent empty specific granules. The diagnosis of SGD can also be established by demonstrating a severe deficiency in either lactoferrin or vitamin B_{12}–binding protein. An acquired form of SGD can be seen in burn patients or in individuals with various myeloproliferative neoplasms. The treatment for SGD is similar to that for other neutrophil disorders. If medical management is aggressive, the prognosis appears quite good, with patients surviving into their adult years.

MISCELLANEOUS INHERITED AND ACQUIRED DISORDERS OF PHAGOCYTE FUNCTION

Rare inherited defects in phagocyte production or response to inflammatory cytokines are manifested as recurrent infections.[14,29] HIES also falls into this category (see earlier discussion). A distinctive group of disorders referred to as *mendelian susceptibility to mycobacterial diseases* (MSMD) involves inherited defects in macrophage IL-12/23–dependent IFN-γ–mediated immunity. Macrophage production of IL-12 and IL-23 after ingestion of mycobacteria triggers IFN-γ production by T and NK lymphocytes, which in turn activates crucial genes in macrophages. MSMD (MIM 20990) can be caused by inherited AR, autosomal dominant (AD), or X-linked defects leading to impaired T-cell production of IFN-γ, expression of the IFN-γ receptor, or downstream signaling, which results in a spectrum of recurrent and severe atypical mycobacterial infections, as well as susceptibility to *Salmonella* and, in some subgroups, viral infections.[14,29] As mentioned earlier, recessive point mutations in X-linked *CYBB* that selectively impair expression of the NADPH oxidase subunit gp91^{phox} in macrophages but not neutrophils are also considered a subgroup of MSMD.[14] A variety of genetic defects in a scaffolding protein known as IKK-γ or NEMO that is important for activation of the nuclear factor kappa-B (NF-κB) signaling pathway have been reported in patients with anhydrotic ectodermal dysplasia and immunodeficiency; affected children have recurrent pyogenic infections with a minimal systemic inflammatory response and can also develop opportunistic infections with atypical mycobacteria or *Pneumocystis carinii*.[14] Several other kindred have been described with genetic deficiency of another protein, IRAK-4, important for NF-κB activation by pyogenic bacteria via the Toll-like receptor/IL-I receptor superfamily. Affected children also had recurrent infections with *S. pneumonia* and *S. aureus* and a poor inflammatory response but no dysmorphic features or opportunistic infections. Finally, with the increasing use of low-cost gene sequencing, it is likely that new inherited disorders of phagocyte function will continue to be identified in the coming years.[29]

Patients with glycogen storage disease type Ib (GSD-Ib), which results from absence of a glucose-6-phosphate transporter, have impaired neutrophil functions and neutropenia (see Chapter 54).[30] Neutrophil defects include depressed chemotaxis, phagocytosis, and NADPH oxidase activity. Although neutropenia appears to result from increased neutrophil apoptosis, how impaired glucose homeostasis leads to abnormal neutrophil function is uncertain. Interestingly, the BM in the face of neutropenia shows marked hypercellularity with increased mature neutrophils in some patients. The neutrophil count increases within hours of granulocyte colony-stimulating factor (G-CSF) administration, suggesting it is acting in part by releasing neutrophils from the BM. GSD-Ib patients can have infections as well as inflammatory bowel disease. Therefore, in addition to management of the metabolic disruption, these patients are also often treated with G-CSF to improve neutropenia.

There are a variety of noncongenital defects in phagocyte function that can be associated with an increased risk of bacterial or fungal infection.[2,3] Several have already been mentioned, including neonatal neutrophil dysfunction manifested as poor adhesion and chemotaxis. Patients with myelodysplastic syndromes and acute nonlymphoblastic lymphoma can have subpopulations of neutrophils variably defective in adhesion, migration, and production of reactive oxidants, and microbicidal activity that has correlated with an increased risk of infection. Similar defects have been described in diabetes mellitus, Gaucher disease, and renal failure. Severe bacterial infections, surgical trauma, and severe burns can also result in transient depression of a variety of neutrophil functions. The underlying mechanism(s) are incompletely defined and may be related to effects of high levels of inflammatory mediators produced in response to infection or trauma or to products released by bacteria.

REFERENCES

1. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol*. 2013;13(3):159–175.
2. Kyono W, Coates TD. A practical approach to neutrophil disorders. *Pediatr Clin North Am*. 2002;49(5):929–971.
3. Dinauer MC, Newburger PE, Borregaard N
. Phagocyte system and disorders of granulopoiesis and granulocyte function. In: Nathan DG, Orkin SH, Ginsburg D, Thomas Look A, Fisher D, Lux S, eds. *Nathan and Oski's Hematology of Infancy and Childhood*. 8th ed. Philadelphia: WB Saunders Company; 2014:773–847.
4. Klebanoff SJ, Kettle AJ, Rosen H, et al. Myeloperoxidase: a front-line defender against phagocytosed microorganisms. *J Leukoc Biol*. 2013;93(2): 185–198.
5. Nauseef WM. How human neutrophils kill and degrade microbes: an integrated view. *Immunol Rev*. 2007;219:88–102.
6. Al Ghouleh I, Khoo NK, Knaus UG, et al. Oxidases and peroxidases in cardiovascular and lung disease: new concepts in reactive oxygen species signaling. *Free Radic Biol Med*. 2011;51(7):1271–1288.
7. Winkelstein JA, Marino MC, Johnston Jr RB, et al. Chronic granulomatous disease. report on a national registry of 368 patients. *Medicine (Baltimore)*. 2000;79(3):155–169.
8. Kuhns DB, Alvord WG, Heller T, et al. Residual NADPH oxidase and survival in chronic granulomatous disease. *N Engl J Med*. 2010;363(27):2600–2610.
9. Dinauer MC. Inflammatory consequences of inherited disorders affecting neutrophil function. *Blood*. 2019;133(20):2130–2139.
10. Gennery AR. Progress in treating chronic granulomatous disease. *Br J Haematol*. 2021;192(2):251–264.
11. van de Geer A, Nieto-Patlan A, Kuhns DB, et al. Inherited P40phox deficiency differs from classic chronic granulomatous disease. *J Clin Invest*. 2018;128(9):3957–3975.
12. Roos D, Kuhns DB, Maddalena A, et al. Hematologically important mutations: X-linked chronic granulomatous disease (third update). *Blood Cells Mol Dis*. 2010;45(3):246–265.
13. Roos D, Kuhns DB, Maddalena A, et al. Hematologically important mutations: the autosomal recessive forms of chronic granulomatous disease (second update). *Blood Cells Mol Dis*. 2010;44(4):291–299.
14. Bustamante J, Boisson-Dupuis S, Abel L, Casanova JL. Mendelian susceptibility to mycobacterial disease: genetic, immunological, and clinical features of inborn errors of ifn-gamma immunity. *Semin Immunol*. 2014;26(6):454–470.
15. Thomsen IP, Smith MA, Holland SM, Creech CB. A comprehensive approach to the management of children and adults with chronic granulomatous disease. *J Allergy Clin Immunol Pract*. 2016;4(6):1082–1088.
16. Gungor T, Chiesa R. Cellular therapies in chronic granulomatous disease. *Front Pediatr*. 2020;8:327.
17. Beutler E. G6PD deficiency. *Blood*. 1994;84(11):3613–3636.
18. Lanza F. Clinical manifestation of myeloperoxidase deficiency. *J Mol Med (Berl)*. 1998;76(10):676–681.
19. Etzioni A. Genetic etiologies of leukocyte adhesion defects. *Curr Opin Immunol*. 2009;21(5):481–486.
20. Almarza Novoa E, Kasbekar S, Thrasher AJ, et al. Leukocyte adhesion deficiency-I: a comprehensive review of all published cases. *J Allergy Clin Immunol Pract*. 2018;6(4):1418–1420. e1410.
21. Silva LM, Brenchley L, Moutsopoulos NM. Primary immunodeficiencies reveal the essential role of tissue neutrophils in periodontitis. *Immunol Rev*. 2019;287(1):226–235.
22. Tsilifis C, Freeman AF, Gennery AR. STAT3 Hyper-Ige Syndrome-an Update and Unanswered Questions. *J Clin Immunol*. 2021;41:864–880.
23. Sprenkeler EGG, Guenther C, Faisal I, et al. Molecular mechanisms of leukocyte migration and its potential targeting-lessons learned from Mkl1/Srf-related primary immunodeficiency diseases. *Front Immunol*. 2021;12:615477.
24. Oh TJ, Eber R, Wang HL. Periodontal diseases in the child and adolescent. *J Clin Periodontol*. 2002;29(5):400–410.
25. Lawrence SM, Corriden R, Nizet V. Age-appropriate functions and dysfunctions of the neonatal neutrophil. *Front Pediatr*. 2017;5:23.
26. Toro C, Nicoli ER, Malicdan MC, et al. Chediak-Higashi syndrome. In: Adam MP, Ardinger HH, Pagon RA, et al., eds. *GeneReviews®*. Seattle, WA: University of Washington; 2009. [Updated 2018 Jul 5].
27. Canna SW, Marsh RA. Pediatric hemophagocytic lymphohistiocytosis. *Blood*. 2020;135(16):1332–1343.
28. Gombart AF, Koeffler HP. Neutrophil specific granule deficiency and mutations in the gene encoding transcription factor C/Ebpε. *Curr Opin Hematol*. 2002;9(1):36–42.
29. Milner JD, Holland SM. The cup runneth over: lessons from the ever-expanding pool of primary immunodeficiency diseases. *Nat Rev Immunol*. 2013;13(9):635–648.
30. Chou JY, Jun HS, Mansfield BC. Type I glycogen storage diseases: disorders of the glucose-6-phosphatase/glucose-6-phosphate transporter complexes. *J Inherit Metab Dis*. 2015;38(3):511–519.

CHAPTER 52

CONGENITAL DISORDERS OF LYMPHOCYTE FUNCTION

Sung-Yun Pai and Luigi D. Notarangelo

Over 350 molecular defects that result in primary immune deficiency are known to date.[1,2] Many of these gene defects have been identified in recent years with the advent of whole-exome and whole-genome sequencing. The study of patients with primary immune deficiencies has unraveled fundamental mechanisms that govern lymphocyte development and function. Importantly, characterization of the molecular basis of these diseases has revealed unanticipated heterogeneity of the clinical and immunological phenotype. This chapter reviews disorders of thymus organogenesis, severe combined immune deficiency (SCID), other combined immunodeficiencies, disorders with immune dysregulation, and defects of humoral immunity.

DEFECTS OF THYMUS ORGANOGENESIS

The thymus is the primary organ where T lymphocytes are generated and educated. Endoderm-derived thymic stem cells derived from the third pharyngeal pouch differentiate into cortical and medullary epithelial cells, which in turn induce the differentiation of hematopoietic precursors into T cells. Thus, defects of thymus organogenesis have important consequences on immune function.

DiGeorge Syndrome

DiGeorge syndrome (DGS) is caused by developmental anomalies of the third and fourth pharyngeal pouches, and is characterized by thymic hypoplasia, hypoparathyroidism, conotruncal heart malformations, which are a group of congenital cardiac outflow tract anomalies that include such defects as tetralogy of Fallot, pulmonary atresia with ventricular septal defect, double-outlet right ventricle, double-outlet left ventricle, truncus arteriosus and transposition of the great arteries, interrupted aortic arch type B among others, and facial dysmorphisms (micrognathia, hypertelorism, antimongoloid slant of the eyes, cleft palate, and ear malformations).[3] Hypocalcemic seizures are common. Feeding problems, microcephaly, speech delay, neurobehavioral problems (including bipolar disorders, autistic spectrum disorders, and schizophrenia later in life), and scoliosis are frequently observed.

Between 50% and 90% of patients with DGS carry a hemizygous deletion of chromosome 22q11, which occurs in ~1:3000 newborns, most arising de novo. Fluorescent in situ hybridization (FISH) and multiplex ligation-dependent probe amplification (MLPA) readily identify the 22q11del in most cases. More rarely, DGS is associated with mutations of the *TBX1* gene, contained within the 22q11 interval, or with 10p deletion.

Most patients have "partial DGS," manifested by mild T-cell lymphopenia and immunodeficiency. Such patients may be asymptomatic, may have oral thrush and recurrent infections, or may develop autoimmune disease such as juvenile idiopathic arthritis, immune thrombocytopenic purpura, and Raynaud phenomenon. Approximately 1% of DGS patients have "complete DGS," with absence of circulating T cells. Some DGS patients may develop low numbers of oligoclonal T lymphocytes that undergo activation in vivo and infiltrate target tissues, causing skin rash, liver dysfunction, and lymphadenopathy. This condition is known as complete atypical DGS.

Treatment of DGS includes correction of severe heart defects, and supplementation with calcium and vitamin D to correct the hypocalcemia.[3] If a significant immune defect is present, prophylaxis of *Pneumocystis jiroveci* pneumonia with trimethoprim-sulfamethoxazole (TMP-SMZ) is indicated. Live-attenuated vaccines can be safely administered to patients with partial DGS who have good cellular immunity (with CD8$^+$ count >300 cells/μL); however, these vaccines are contraindicated in patients with complete DGS. Use of immunosuppressive drugs is indicated in patients with complete atypical form of the disease.

Ultimately, survival in patients with complete DGS requires immune reconstitution. Stem cell transplantation from human leukocyte antigen (HLA)-identical donors may allow engraftment of mature T cells contained in the graft. However, because of the absence of thymic tissue, newly developed T cells are not generated, and the patient may remain susceptible to pathogens that are not recognized by donor-derived T cells contained in the graft.

By contrast, thymic transplantation represents the treatment of choice for patients with complete DGS, including the atypical variant.[4,5] The thymus obtained as discarded tissue from unrelated infants undergoing heart surgery is sliced and cultured in vitro, then implanted in the quadriceps muscles of the infant. In most cases, naïve T cells appear at around 4 to 7 months of age; these cells are tolerant to donor thymic cells, display a polyclonal repertoire, and have normal proliferative capacity. Although the number of CD3+ T cells in transplanted patients often remains lower than normal, their diversity and function are sufficient to prevent life-threatening infections. The success of this treatment has recently led to approval of this therapy by the Food and Drug Administration in the United States.

FOXN1 Deficiency

The transcription factor FOXN1 (whose gene is mutated in the *nude* mouse) plays a critical role in thymus and eccrine glands development. *FOXN1* mutations in humans cause athymia, profound T-cell lymphopenia, alopecia totalis, and nail dystrophy.[6] Similar to DGS, reconstitution of T-cell immunity can be achieved with thymic transplantation.[4,5] FOXN1 haploinsufficiency may also cause T-cell lymphopenia at birth, followed by gradual and partial recovery of T-cell count.

CHARGE Syndrome

This syndrome is characterized by the association of coloboma (a hole in one of the structures of the eye, such as the iris, retina, choroid, or optic disc), heart anomalies, choanal atresia, mental retardation, genital and ear anomalies, and immunodeficiency. Most cases are sporadic and represent de novo mutations of the *CHD7*[7] or of the *SEMA3E* genes.[8] The immunodeficiency is secondary to defects of thymic development. There is a variable degree of T-cell lymphopenia, which in some cases is very severe, resembling SCID.

PAX1 Deficiency

Paired Box 1 (PAX1) is a transcription factor involved in early stages of pharyngeal pouch differentiation and thymus development. Autosomal recessive PAX1 deficiency is characterized by T-cell lymphopenia associated with facial anomalies, hearing loss, vertebral anomalies and neurodevelopmental problems.[9]

SEVERE COMBINED IMMUNE DEFICIENCY DUE TO EARLY DEFECTS IN T-LYMPHOCYTE DEVELOPMENT

SCID, the most severe form of congenital immunodeficiency, is caused by defects that completely abrogate the development of T lymphocytes, and in some cases also B and/or natural killer (NK) lymphocytes. Advances in the genes responsible, newborn screening, and gene therapy have had a strong impact on the diagnosis and therapy of SCID.

Pathobiology and Genetics

Genetic defects that cause SCID affect various stages in T-cell development (Fig. 52.1), and can be grouped into three major categories: (1) defects in cytokine receptor signaling; (2) defects in lymphocyte survival; and (3) defects of expression and function of the pre–T-cell receptor

Cytokine Receptor Signaling Defects

The most common form of SCID in humans is the X-linked form due to mutations of the *IL2RG* gene, which encodes for the common gamma chain (γc).[10,11] This protein is shared by receptors for interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15, and IL-21, and signals through the intracellular kinase Janus-activated kinase (JAK)3. Patients with mutations in *IL2RG* or *JAK3* lack both T and NK cells, since development of these subsets depends on IL-7- and IL-15-mediated signaling, respectively. B lymphocytes are present but antibody production is impaired because of the lack of T cells and defective signaling through the IL-21R.[12]

Defects in Lymphocyte Survival

Proliferation and survival of lymphoid progenitor cells are essential to permit generation of a normal number of mature lymphocytes. Some forms of SCID are associated with increased apoptosis. Adenosine deaminase (ADA) converts adenosine to inosine (and deoxyadenosine to deoxyinosine). In patients with ADA deficiency, accumulation of toxic phosphorylated derivatives of deoxyadenosine causes cell death and results in extreme lymphopenia, with virtual absence of T, B, and NK lymphocytes.[13,14] Reticular dysgenesis (RD) is a form of SCID characterized by the association of severe lymphopenia, agranulocytosis, and sensorineural deafness (see Chapter 30). RD is caused by defects in adenylate kinase 2 (AK2), resulting in increased sensitivity to reactive oxygen species and increased apoptosis.[15,16]

Defects of Expression and Signaling Through the Pre–T-Cell Receptor and the T-Cell Receptor

Rearrangement of the T-cell receptor (TCR) genes by means of variable diversity joining (V(D)J) recombination allows expression of the pre-TCR (composed of the pre-Tα and the TCRβ chain), and of mature forms (TCRαβ and TCRγδ) of the TCR.

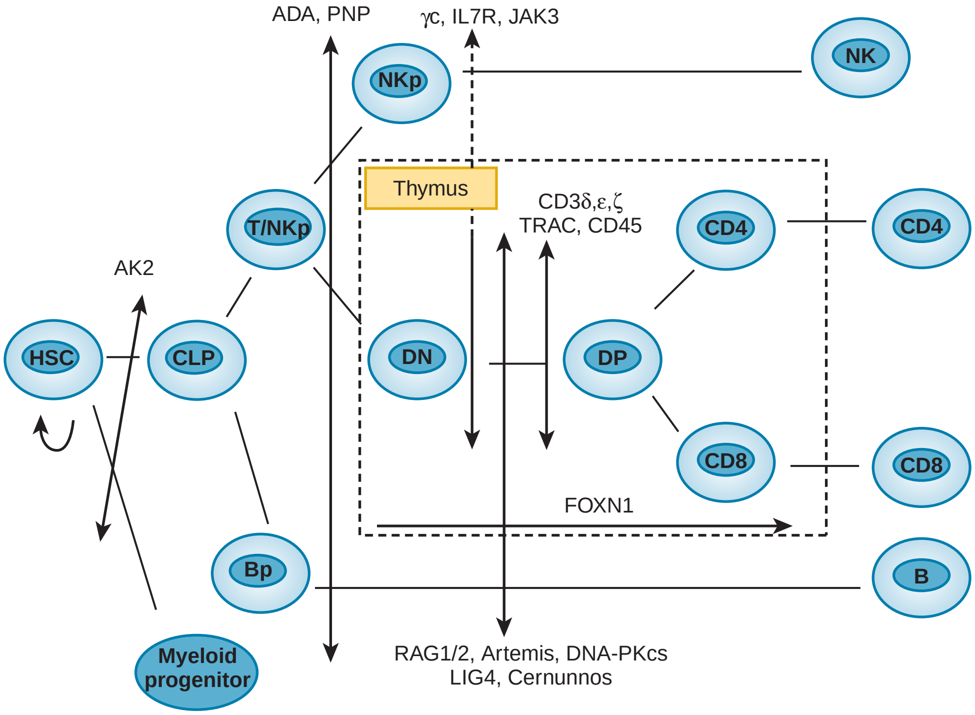


Figure 52.1 GENETIC DEFECTS ASSOCIATED WITH SEVERE COMBINED IMMUNE DEFICIENCY (SCID). Schematic representation of blocks (*arrows*) in lymphoid development associated with genetic defects responsible for SCID. Dashed line indicates that the generation of NK lymphocytes is compromised in γc and JAK3 deficiency, but not in IL7R deficiency. *ADA*, Adenosine deaminase; *AK2*, adenylate kinase 2; *B*, B-cell; *Bp*, B-cell progenitor; *CLP*, common lymphoid progenitor; *DN*, double-negative thymocyte; *DNA-PKcs*, DNA protein kinase catalytic subunit; *DP*, double-positive thymocyte; *FOXN1*, forkhead box N1; *γc*, common gamma chain; *HSC*, hematopoietic stem cell; *IL7R*, interleukin-7 receptor; *JAK3*, Janus-associated kinase 3; *LIG4*, DNA ligase IV; *NK*, natural killer cell; *NKp*, natural killer cell progenitor cell; *PNP*, purine nucleoside phosphorylase; *RAG*, recombinase activating gene; *T/NKp*, common progenitor of T and natural killer lymphocytes; *TRAC*, T-cell receptor alpha constant chain.

The lymphoid-specific recombinase-activating genes (*RAG1* and *RAG2*) proteins initiate V(D)J recombination by recognizing recombination-specific sequences that flank the V(D)J elements of the TCR and of immunoglobulin genes, introducing DNA double-strand breaks. These are then repaired through the ubiquitously expressed nonhomologous end-joining (NHEJ) pathway. Mutations of the *RAG1* and *RAG2* genes, and genes that encode for Artemis, DNA ligase IV, and DNA-protein kinase catalytic subunit (all components of the NHEJ pathway), result in SCID with a lack of T and B lymphocytes (see Figs. 52.1 and 52.2), but normal numbers of NK lymphocytes.[17–20] Mutations of the *NHEJ1* gene (encoding Cernunnos/XLF, another component of the NHEJ pathway) severely impair, but do not completely abrogate, T- and B-cell development.[21] Because NHEJ is involved in general mechanisms of DNA repair involving nonlymphoid cells, patients with defects of this pathway also show increased radiation sensitivity, are at a higher risk of tumors. In addition, neurologic problems are common, except in patients with Artemis deficiency.

Signaling through the pre-TCR/CD3 complex is essential to promote progression from $CD4^-CD8^-$ double-negative (DN) to $CD4^+CD8^+$ double-positive (DP) stage of thymocyte development. Mutations of the CD3δ (*CD3D*), CD3ε (*CD3E*), and CD3ζ (*CD3Z*) chains interfere with this process and result in SCID.[22–24] In contrast, mutations of CD3γ (*CD3G*) are more often associated with a milder phenotype that includes autoimmunity.[25] Downstream of the TCR/CD3 complex, mutations of the Linker for Activation of T cells (LAT), also perturb signaling and cause $T^- B^+$ SCID.[23] Finally, mutations of the CD45 phosphatase, also involved in cell signaling, cause $T^- B^+$ SCID.[26]

Clinical and Laboratory Manifestations

Typical clinical features of SCID include early-onset severe infections caused by bacteria, viruses, fungi, and opportunistic pathogens (including *P. jiroveci* pneumonia), protracted diarrhea, candidiasis, and failure to thrive. Engraftment of maternally derived T lymphocytes is common in SCID.[27] The patient may be asymptomatic or may present with symptoms similar to graft-versus-host disease (GVHD): skin rash, elevation of liver enzymes, diarrhea, and cytopenias. Hypomorphic mutations in SCID-causing genes may lead to residual development of T cells that undergo peripheral expansion and infiltrate target organs, causing various symptoms (erythroderma, diarrhea, hepatosplenomegaly, lymphadenopathy, diarrhea). This clinical phenotype is also known as *Omenn syndrome*.[28]

Some forms of SCID may present with additional clinical features (see earlier section on Defects in Thymic Development). In patients with ADA deficiency, accumulation of toxic metabolites may cause cupping and flaring of the ribs, liver dysfunction, sensorineural deafness and neurobehavioral problems. Microcephaly is often seen in forms of SCID associated with radiosensitivity and impairment of DNA double-strand break repair. Sensorineural deafness is observed in RD.

A family history (including consanguinity, deaths in infancy, and the gender of other affected family members) may be suggestive of X-linked versus autosomal recessive inheritance patterns. Measurement of absolute lymphocyte count (ALC) and the absolute number of $CD3^+$ T cells, $CD4^+$ and $CD8^+$ T-cell subsets, $CD19^+$ B cells, and NK cells ($CD16^+/CD56^+$ NK lymphocytes) confirms the diagnosis and may direct the work-up towards specific gene defects. The presence of $CD3^+$ cells in a child with clear clinical manifestations of SCID may indicate maternal T-cell engraftment or may be due to hypomorphic mutations that allow residual T-cell development. In both of these situations, the T cells have an activated/memory ($CD45RO^+$), whereas T cells in normal infants are predominantly naive ($CD45RA^+$). In vitro proliferative responses to mitogens are drastically reduced in patients with SCID, but may be partially preserved in infants with Omenn syndrome. A chest X-ray with a lack of a thymic shadow supports the diagnosis of SCID. Serum IgA and IgM levels are low to undetectable, but IgG levels may be normal early in life, reflecting the transplacental passage of maternally derived antibodies. (See box on Diagnostic Approach to Severe Combined Immune Deficiency.)

DIAGNOSTIC APPROACH TO SEVERE COMBINED IMMUNE DEFICIENCY

- SCID presents early in life with severe infections of bacterial, viral, or fungal origin.
- Opportunistic infections are common in infants with SCID.
- Respiratory infections, protracted diarrhea, and failure to thrive are typical signs at presentation.
- Lymphopenia is present in 50%–70% of infants with SCID. Age-specific norms must be used in evaluating the ALC since infants and children have much higher ALCs than adults (3500–13,000 in very young infants versus 1000–2800 in adults).
- T-cell lymphopenia is the hallmark of the disease; abnormalities of the absolute number of B and NK lymphocytes are observed in some forms of SCID. However, T lymphocytes may be present in SCID infants with maternal T-cell engraftment or with hypomorphic mutations in SCID-associated genes that allow residual T-cell development. Thus, a normal ALC does not rule out SCID.
- Maternally engrafted T cells proliferate in the infant with SCID in vivo, but the vast majority do not proliferate in vitro when stimulated with traditionally used mitogens such as concanavalin A and phytohemagglutinin, as measured by thymidine incorporation. Thus, if SCID is suspected, but T cells are detectable, maternal engraftment studies and proliferation to mitogens must be evaluated.
- Universal newborn screening for SCID, based on enumeration of TRECs by quantitative PCR, has been implemented in the United States and in several other countries worldwide. TRECs are high in newly generated T cells and low when T cells are absent or when maternally engrafted T cells are present.
- SCID is genetically heterogeneous. The most common form in Western countries is inherited as an X-linked trait and is $T^-B^+NK^-$.
- The lack of all lymphocytes ($T^-B^-NK^-$ SCID) is highly suspicious for ADA deficiency, in which accumulation of toxic metabolites results in death of all lymphocytes. The diagnosis can be confirmed by testing of ADA enzymatic activity and of intracellular levels of phosphorylated derivatives of deoxyadenosine.

Diagnosis by Universal Newborn Screening

SCID can be diagnosed at birth by measuring levels of TCR excision circles (TRECs). TRECs are a byproduct of V(D)J recombination and are present as circularized DNA fragments in newly generated, naïve T lymphocytes that express the αβ form of the TCR. Levels of TRECs in circulating lymphocytes are particularly high in newborns and infants, and can be detected by polymerase chain reaction (PCR) amplification of DNA extracted from the Guthrie card.[29] Newborn screening for SCID was recommended to be added to the standard panel in the United States in 2010, and as of December 2018 all 50 states were screening for SCID. Based on screening of ~3,000,000 infants in the United States, the incidence of SCID is now estimated to be 1 in 58,000 births, higher than with clinical screening alone.[30] Many other countries in the world are also screening.[29]

SCID is not the only cause for low TREC levels at birth; other conditions include prematurity, loss of lymphocytes in the third space (such as in babies with chylothorax) as well as various other syndromes. Enumeration of the absolute lymphocyte count and of lymphocyte subsets (including naïve and memory T cells) should be part of the laboratory work-up following a positive newborn screening concerning for SCID. Ultimately, genetic tests may reveal the molecular basis of the disease.

Prognosis, Therapy, and Future Directions

Supportive Management

Management of SCID includes observance of strict hygiene measures, prevention of *P. jiroveci* pneumonia with TMP-SMZ, prompt investigation and aggressive treatment of infections, immunoglobulin replacement, and adequate support with enteral or parenteral nutrition. Infections caused by cytomegalovirus (CMV; causing interstitial pneumonia, hepatitis and/or gastroenteritis) and Epstein-Barr virus (EBV; causing lymphoproliferative disease) require active surveillance and preemptive therapy. To prevent transmission of viral infections, blood products from CMV-seronegative donors or leukofiltered products should be used and must be irradiated to prevent transfusion-associated GVHD. Immunosuppression with steroids and cyclosporine A may be needed to treat GVHD-like manifestations associated with Omenn syndrome or maternal T-cell engraftment. Administration of live vaccines must be avoided in infants with SCID. In spite of these measures, SCID is inevitably fatal within the first few years of life, unless immune reconstitution is achieved with treatment.

In most cases, definitive treatment of SCID requires hematopoietic cell transplantation (HCT). Enzyme-replacement therapy (ERT), with weekly intramuscular injection of pegylated recombinant ADA which allows conversion of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively, thus preventing accumulation of toxic phosphorylated derivatives. This treatment leads to improvement of lymphocyte counts and of immune function. Disadvantages of ERT include expense, as it must be continued indefinitely, waning of therapeutic effect over time, and the development in some patients of neutralizing antibodies to polyethylene glycol-modified adenosine deaminase (PEG-ADA).

General Principles of Stem Cell Transplantation for Severe Combined Immune Deficiency

Stem cell transplantation (SCT) is the standard treatment that promotes long-term immune reconstitution in infants with SCID. SCT for other conditions is generally performed with chemotherapy or radiation conditioning, to prevent graft rejection and eliminate or reduce host hematopoietic stem cells (HSCs), favoring donor hematopoiesis.[31] Because of the lack of T lymphocytes, infants with SCID are considered to have an inherent inability to reject the graft, and may therefore receive SCT from an HLA-identical related donor (sibling) without conditioning and with no need for GVHD prophylaxis.[32] With this approach, expansion of mature T cells present in the donor bone marrow allows rapid improvement of T-cell count; newly generated naïve T cells derived from donor HSCs appear on average 3 to 4 months after transplant.

Unconditioned SCT may also be performed from mismatched related donors (parent), with T-cell depletion of the graft and post-transplant prophylaxis to prevent GVHD. In this case, the appearance of peripheral T cells is entirely dependent on intra-thymic differentiation of donor-derived progenitor cells and can take 4 to 6 months.[33]

Following unconditioned SCT for SCID, donor-derived stem cells have a selective advantage over genetically defective autologous HSCs in their capacity to differentiate to T cells. However, failure to obtain robust and durable engraftment of donor HSCs may occur if no conditioning is used. In such cases, after an initial period in which donor-derived committed lymphoid progenitor cells contained in the graft differentiate to T cells, lack of sustained seeding of HSC-derived progenitor cells in the thymus may cause reappearance of severe T-cell lymphopenia and affect the long-term durability and quality of immune reconstitution. For this reason, several centers prefer to perform haploidentical SCT with conditioning. A similar approach is typically followed when performing SCT from matched unrelated adult or cord blood.

While use of conditioning improves the rate of HSC engraftment, it is associated with short-term and long-term toxicity and an increased risk of GVHD, especially when myeloablative regimens are used. These risks may be attenuated by using reduced-intensity conditioning regimens. Finally, non-genotoxic approaches to conditioning with use of monoclonal antibodies (such as anti-CD117)[34] to target the autologous HSCs are currently being evaluated.

Survival and Long-Term Outcomes After Stem Cell Transplantation for Severe Combined Immune Deficiency

Survival after SCT for SCID has improved with time due to advances in early diagnosis and supportive care for infants with SCID.[35–37] Current figures indicate a 5-year survival that exceeds 90% in the case of SCT from matched sibling donors, and >80% in other cases. It has been demonstrated that age and clinical status at transplantation are the major determinants of survival.[36,38] In this regard, implementation of newborn screening may facilitate early identification of infants with SCID, prompting adoption of adequate measures to prevent infections and early referral to SCT. Rates of acute and chronic GVHD (aGvHD, cGvHD) are generally low and largely limited to recipients of SCT from donors other than HLA-matched siblings.

The durability and quality of immune reconstitution after SCT depend on multiple factors, including: (1) HLA matching between donor and recipient; (2) use of conditioning regimen; and, (3) the nature of the genetic defect causing SCID.[36] Survivors of SCT performed with a conditioning regimen tend to have higher counts of total and of naïve T-cells than those treated with SCT without conditioning.[36,38] Among recipients of SCT without conditioning, patients with RAG deficiency have an inferior long-term T cell reconstitution when compared to those with X-linked SCID or JAK3 deficiency or IL7R deficiency. This difference may be due to the fact that RAG deficiency is characterized by the presence of NK cells (which can mediate graft rejection and are absent in patients with X-linked SCID and JAK3 deficiency) and by a later block in intra-thymic T cell development, so that competition between donor-derived and autologous cells occurs during the initial stages of T-cell development. The nature of the genetic defect accounting for SCID may also determine whether use of conditioning regimen may be needed to obtain optimal reconstitution of humoral immunity. Forms of SCID where the genetic defect has no impact on B-cell development and function (such as IL7R deficiency) do not require conditioning, whereas chemotherapy-based preparative regimens that facilitate replacement of autologous HSCs with donor derived cells improve humoral immunity in patients with defects that affect B cell development (RAG deficiency). In the absence of conditioning, inadequate B-cell function (requiring immunoglobulin-replacement therapy) is often observed also in patients with γc and JAK3 deficiency, due to intrinsic defects in the host B-cell response to γc-dependent cytokines such as IL-4 and IL-21.[36] (See box on Therapeutic Approach to Severe Combined Immune Deficiency.)

Patients with poor T-cell reconstitution following SCT are at risk for viral and opportunistic infections; autoimmunity (especially cytopenias and hypothyroidism) has been reported in 10% to 20% of these patients, particularly in those with cGVHD.[39] Other long-term complications include nutritional problems, poor growth and development, and neurologic complications (mental retardation, motor dysfunction, sensorineural hearing deficits). Some of these complications are more common in certain subtypes, particularly growth in patients with Artemis-SCID and neurologic problems in patients with ADA deficiency.

Gene Therapy for Severe Combined Immune Deficiency

Gene therapy, in which the gene of interest is introduced into the patient's own cells, is an attractive therapeutic option for SCID, in particular for infants who do not have an HLA-identical related donor (see Chapter 104). Expression of the normal copy of the gene in $CD34^+$ stem cells confers a selective advantage to the gene-corrected cells during T-cell differentiation. Furthermore, there is no risk of GVHD. Gene therapy has been used successfully to correct SCID in patients with ADA deficiency and with X-linked SCID.[40]

Since the year 2000, more than 70 patients with ADA-deficient SCID have been treated with gene therapy worldwide. Initial studies have been conducted with transduction of the patients' $CD34^+$ cells

THERAPEUTIC APPROACH TO SEVERE COMBINED IMMUNE DEFICIENCY

- HSC transplantation is the mainstay of treatment. Optimal survival is achieved when the transplant is performed early in life. This is now possible with newborn screening.
- Transplantation for SCID can be performed without conditioning, due to the profound absence of T cells and inability to reject. Thus, the bone marrow of a fully HLA-matched sibling can be infused without manipulation and without giving any conditioning to the baby. GVHD prophylaxis is also not necessary. Haploidentical bone marrow from a parent can also be infused without conditioning but generally must be T-cell depleted either ex vivo or in vivo with agents such as cyclophosphamide.
- Such transplants without conditioning can result in T-cell reconstitution that lasts for decades. In the case of a matched sibling graft, initial T-cell reconstitution is rapid, generally within the first 1–3 months, due to proliferation of mature T cells. In the case of a haploidentical graft, mature T cells are removed and thus 4–6 months is required for HSC to develop and emerge from the thymus as mature T cells.
- Without conditioning, a small number of donor-derived long-lived HSCs engraft, and this may lead to lack of donor B-cell reconstitution and lack of humoral immunity. With conditioning, donor HSCs are more likely to engraft and give rise to donor B cells. Currently, matched unrelated donor transplants are performed with conditioning.
- Enzyme replacement may be used in patients with adenosine deaminase deficiency. Gene therapy has offered promising results; however, it is also associated with increased risk of developing leukemia due to insertional mutagenesis.

with gammaretroviral vectors expressing the ADA cDNA.[41–43] One such vector has received approval as orphan drug by the European Medical Agency. More recently, clinical trials of gene therapy for ADA deficiency have been conducted with use of lentiviral vectors, with great success.[44] Altogether, these experiences have been very successful, leading to sustained production of gene marked T, B and myeloid cells. The majority of patients have been able to stop ERT. Engraftment of gene-corrected stem cells has been facilitated by the use of a reduced-intensity chemotherapy regimen with low-dose busulfan. A single case of T-cell leukemia due to insertional mutagenesis of the retroviral vector has been reported.[45]

Twenty patients with X-linked SCID were treated by gene therapy using a gammaretroviral vector in Paris and London at the turn of the century, without chemotherapy conditioning.[46,47] Eighteen patients are alive, and 17 of them show normalization of T-cell count and function, with sustained thymic output, diversified T-cell repertoire, and ability to mount antigen-specific T-cell responses.[48,49] In a few cases, improvement of humoral immunity has also been observed. However, leukemic T-cell proliferation due to insertional mutagenesis was observed in six of these patients, with fatal outcome in one.[50,51] These serious events have led to the design of new vectors, including self-inactivating gammaretroviral[47] and more recently lentiviral vectors.[52,53] With these new vectors, successful immune reconstitution has been achieved in treated patients, including older patients who had shown declining immune function after previous SCT.[52] Improved B-cell reconstitution has been achieved in several patients after inclusion of conditioning with low-dose busulfan in the treatment protocol. Importantly, no cases of leukemia have been observed after gene therapy with self-inactivating viral vectors.

OTHER COMBINED IMMUNODEFICIENCIES

Defects of T-Cell Receptor Signaling

Several forms of combined immune deficiency are caused by genetic defects that affect the following molecules involved in TCR signaling.[54–58] In particular, the T lymphocyte-specific protein tyrosine kinase (Lck) associates with CD4 and CD8 molecules and mediates phosphorylation of CD3 chains upon TCR engagement. The Zeta-associated protein of 70 kDa (ZAP-70) tyrosine kinase phosphorylates the CD3 chains, thus promoting downstream TCR signaling. RHOH is an atypical Rho GTPase that participates in receptor-induced signaling in hematopoietic cells. The IL-2-inducible tyrosine kinase (ITK) is a member of the family of nonreceptor tyrosine kinases, and modulates the strength of the TCR-induced signal. The serine-threonine protein kinase 4 (STK4) protein induces the transcriptional coactivation of target genes involved in cell proliferation and survival and is involved in the control of cell death.

At variance with typical forms of SCID, genetic defects of TCR signaling are characterized by residual numbers and/or function of T cells, consistent with partially preserved thymic architecture and function. Since the thymus of these patients is not empty, the use of chemotherapy is required to facilitate engraftment and T-cell differentiation with donor-derived cells. The immunological phenotype of ZAP-70 deficiency in humans is characterized by a virtual lack of $CD8^+$ lymphocytes; the number of $CD4^+$ cells is preserved, but they are unable to proliferate in response to mitogens and antigens. In contrast, both $CD4^+$ and $CD8^+$ lymphocytes are absent in *Zap70*$^{-/-}$ mice.

Both Ras homolog family member H (RHOH) and STK4 deficiencies are characterized by a reduced number of naïve T cells, an increased proportion of effector memory T-cells (including $CD8^+$ cells with an “exhausted” $CD45RA^+CCR7^-$ phenotype), and defective lymphocyte proliferation. CD4 lymphopenia and low levels of CD4 and CD8 surface expression have been reported in LCK deficiency. Progressive CD4 lymphopenia is also commonly seen in patients with ITK deficiency.

Each of these forms of impaired TCR signaling are inherited as autosomal recessive traits. Clinically, patients with impaired T-cell signaling present with recurrent, often severe, infections. Cutaneous viral infections are especially common in patients with RHOH and STK4 deficiency. The clinical phenotype of ZAP-70 deficiency is often undistinguishable from SCID; in a few cases, hypomorphic *ZAP70* mutations have been associated with Omenn syndrome. Severe lung disease and a high risk of EBV-driven lymphoproliferation are a hallmark of ITK deficiency. Autoimmune manifestations have been observed in several patients with these disorders. Prevention of infections is based on antimicrobial prophylaxis and use of intravenous immunoglobulins; however, ultimate treatment requires SCT.

Coronin-1A Deficiency

Coronin-1A is involved in reorganization of the actin cytoskeleton that allows egress of thymocytes to the periphery. While complete deficiency of coronin-1A in humans is a cause of SCID, hypomorphic mutations have been associated with severe naive T-cell lymphopenia, oligoclonal expansion of activated T cells, and increased risk of EBV-driven lymphoproliferation.[59]

Human Leukocyte Antigen Class II Deficiency

Expression of HLA class II molecules on the surface of thymic epithelial cells (TECs) and dendritic cells is essential to promote positive selection of $CD4^+$ lymphocytes. HLA class II deficiency includes a group of autosomal recessive disorders in which mutations in transcription factors (*CIITA*, *RFXAP*, *RFX5*, and *RFXANK*) that regulate HLA class II gene expression result in profoundly reduced numbers of $CD4^+$ lymphocytes.[60] The clinical phenotype consists of recurrent infections since infancy. Treatment is based on antimicrobial prophylaxis, regular use of immunoglobulins, and SCT. Persistence of CD4 lymphopenia is often observed after SCT, because transplantation of HSCs does not correct defective expression of HLA class II molecules on the surface of TECs, causing defective positive selection of $CD4^+$ lymphocytes.

Human Leukocyte Antigen Class I Deficiency

Presentation of antigens in association with MHC class I molecules is important for thymic development of CD8$^+$ lymphocytes and for the development of antigen-specific cytotoxic T-cell responses. In addition, HLA class I molecules also modulate the function of NK cells that express several HLA class I-binding molecules. HLA class I molecules are assembled and loaded with peptides in the endoplasmic reticulum by transporter associated with antigen presentation (TAP) proteins (TAP1 and TAP2) and the TAP binding or Tapasin protein (TAPBP). Mutations of *TAP1*, *TAP2*, or *TAPBP* cause defective expression of HLA class I molecules, which is associated with a severe reduction in the number of circulating CD8$^+$ TCRαβ$^+$ lymphocytes.[60] NK cells expressing killer inhibitory receptors may not receive HLA class I-dependent inhibitory signals, and hence may mount cytotoxic responses to uninfected cells, causing inflammatory lesions in the lungs and in the skin. There is no definitive treatment for this disease, and management is based on supportive care.

CD40 Ligand and CD40 Deficiencies

CD40 ligand (CD40LG) is expressed by activated CD4$^+$ cells. Interaction of CD40LG-expressing CD4$^+$ cells with B lymphocytes that constitutively express CD40 delivers a signal for B-cell proliferation and class-switch recombination in the presence of appropriate cytokines (see Fig. 52.2). Furthermore, interaction between CD40LG-expressing CD4$^+$ lymphocytes and CD40$^+$ dendritic cells and macrophages promotes secretion of IL-12, and thus enables the development of Th1 responses against intracellular pathogens.

The *CD40LG* gene is located on the X chromosome (at Xq26). Males with *CD40LG* mutations suffer from a combined immunodeficiency characterized by recurrent infections, often caused by opportunistic pathogens.[61] *P. jiroveci* pneumonia classically occurs in the first year of life, but may occur at any age. *Cryptosporidium parvum* infection may cause chronic watery diarrhea and lead to sclerosing cholangitis. Chronic or intermittent neutropenia has been frequently observed. Patients with CD40LG deficiency are also uniquely prone to tumors of the liver and biliary tract.

Levels of serum IgG and IgA are markedly reduced or undetectable; IgM may be normal or increased, and for this reason the disease is also known as *X-linked hyper-IgM syndrome*. The number of memory B cells is also markedly reduced.

In vitro activation of T cells with phorbol myristate acetate and ionomycin fails to induce expression of CD40LG on the surface of CD4$^+$ cells, whereas other activation markers (e.g., CD69) are normally expressed.

The prognosis is severe. Death may occur early in life secondary to infections, or during late childhood and young adulthood due to severe liver disease and tumors.[62] Clinical management consists of continuous prophylaxis of *Pneumocystis* infection with TMP-SMZ, regular use of intravenous immunoglobulins, and hygiene measures to limit the risk of exposure to *Cryptosporidium*. SCT is the only definitive cure, with overall and disease-free survival of ~70% to 78% reported in Europe.[63] Preexisting pulmonary disease and *Cryptosporidium* infection are risk factors for less favorable outcomes.

CD40 deficiency is a very rare autosomal recessive disorder, whose clinical and immunological phenotype is indistinguishable from CD40LG deficiency (see Fig. 52.2).[64] The diagnosis is based on the lack of expression of CD40 on the surface of circulating B lymphocytes. Management is similar to that for CD40LG deficiency.

Defects of Nuclear Factor κB Signaling

The nuclear factor κ-B (NF-κB) signaling pathway is ubiquitous to all tissues and stages of development, playing critical roles in cell proliferation, oncogenesis, cell survival, and inflammatory responses. Several

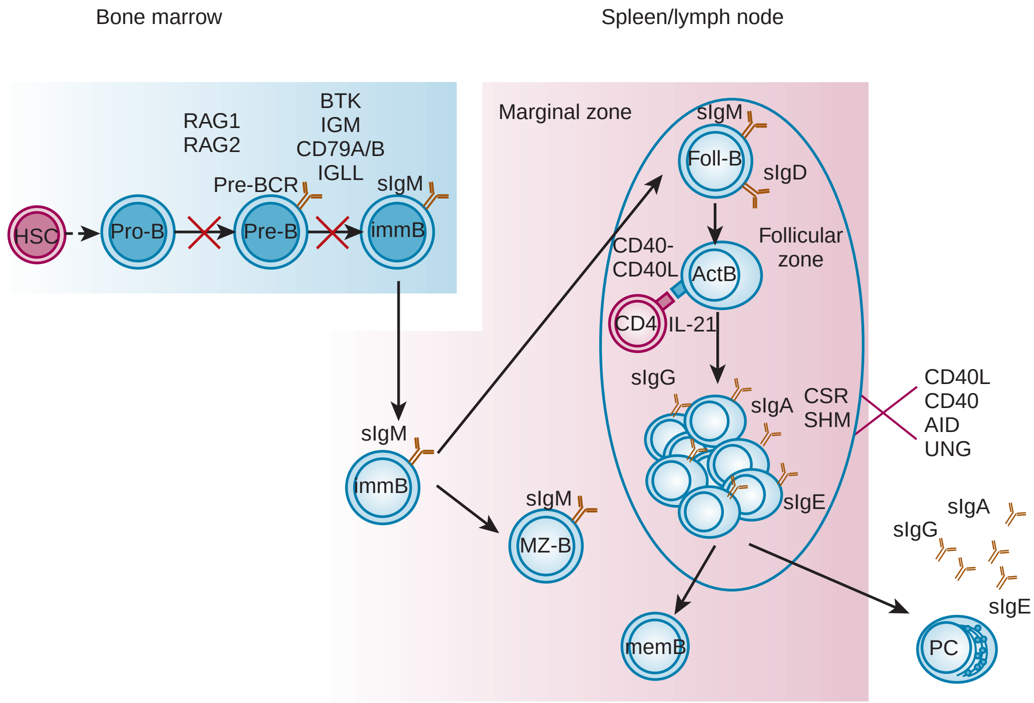


Figure 52.2 GENETIC DEFECTS ASSOCIATED WITH HYPOGAMMAGLOBULINEMIA. Schematic of B-cell development in bone marrow and secondary lymphoid organs, including migration of B cells into the follicular zone where they undergo activation, class-switch recombination (CSR), and somatic hypermutation (SHM). "X" denotes maturation steps at which the genes indicated are required, resulting in a block in differentiation at that stage when the gene is deficient. *ActB*, Activated B cell; *AID*, activation-induced cytidine deaminase; *BTK*, Bruton's tyrosine kinase; *Foll-B*, follicular B cell; *HSC*, hematopoietic stem cell; *IGLL*, immunoglobulin light-like chain; *IL-21*, interleukin-21; *immB*, immature B cell (also termed *transitional B cell*); *memB*, memory B cell; *MZ-B*, marginal zone B cell; *PC*, plasma cell; *Pre-B*, precursor B cell; *Pre-BCR*, pre–B-cell receptor; *Pro-B*, progenitor B cell; *RAG1*, recombination activating gene 1; *RAG2*, recombination-activating gene 2; *sIgA*, surface IgA; *sIgD*, surface IgD; *sIgG*, surface IgG; *sIgM*, surface immunoglobulin M; *UNG*, uracil-DNA glycosylase.

genetic defects affecting activation of NF-κB have been described that result in combined immune deficiency variably associated with extra-immune manifestations. Hypomorphic mutations in the *IKBKG* gene, which encodes for the NF-κB essential modulator (NEMO; also known as *IKK-γ*), the regulatory subunit of the IκB kinase complex, are inherited as an X-linked trait and represent the prototype of disorders of NF-κB signaling.[65] Complete lack of NEMO expression is embryonically lethal in males; females who are heterozygous for null NEMO mutations suffer from incontinentia pigmenti. Typically, males with NEMO deficiency present with ectodermal dysplasia and immunodeficiency (EDA-ID). The phenotypic manifestations of EDA-ID are largely restricted to epithelial tissues and the immune system, with sparse hair, shiny skin, lack of sweat glands and other appendages, and rare conical-shaped teeth. There is often frontal bossing and a characteristic facies. Typical infections are caused by bacteria, including sepsis and meningitis, especially *Streptococcus pneumoniae*, *Staphylococcus*, and *Haemophilus influenzae*; DNA viral infections such as CMV and herpes simplex virus (HSV); and atypical mycobacterial infection. Pneumocystis and other opportunistic infections may also occur. Inflammatory colitis is a frequent manifestation of immune dysregulation. Lymphedema and osteopetrosis have been reported in some patients. Occasionally, manifestations of ectodermal dysplasia may be modest or even absent, thus adding to phenotypic heterogeneity. Immunological abnormalities include variable defects in T-cell proliferation to mitogen and antigens, hypogammaglobulinemia with normal or high IgM, defective antibody production (especially against polysaccharides), and impairment of NK-cell cytotoxicity. Finally, monocytes from patients with NEMO deficiency fail to elaborate inflammatory cytokines such as tumor necrosis factor (TNF)α in response to stimulation by a variety of Toll-like receptor ligands. A similar clinical and immunological phenotype is observed in patients with autosomal dominant EDA-ID caused by gain-of-function (GOF) mutations of the *IKBA* gene. In these patients, the mutated IκB-α protein cannot be phosphorylated at residues that are critical for its degradation. This prevents release of the cytoplasmic components of NF-κB, which cannot translocate to the nucleus and mediate transcription of target genes.

Aside from supportive therapy with intravenous immunoglobulin replacement, antibiotic prophylaxis and treatment of infections, SCT for these disorders has been used with variable success. Because of the role of NF-κB signaling in nonhematopoietic tissues, certain manifestations of the disease, such as colitis, may not be uniformly ameliorated by SCT.[66]

Mutations of the *IKBKB* gene are responsible for an autosomal recessive disease, characterized by a SCID-like phenotype without ectodermal dystrophy.[67] Patients lack regulatory T cells and TCRγδ$^+$ T cells. The *MAP3K14* gene encodes for NIK, a component of the noncanonical NF-κB signaling pathway. Patients with NIK deficiency suffer from recurrent infections. Immunological abnormalities include reduced T-cell proliferation, hypogammaglobulinemia with reduced number of total and switched memory B cells, and NK deficiency.[68,69] Haploinsufficiency of *RELA*, encoding for the p65 subunit of NF-κB, is associated with impaired NF-κB activation and mucocutaneous ulcers.[70] Finally, autosomal recessive RelB deficiency is associated with normal numbers of T cells but reduced proliferation to mitogens and antigens, and restricted T cell repertoire. B cells are present in increased number; immunoglobulin levels are normal, but specific antibody production is impaired.

The CARD11–BCL10–MALT1 complex regulates NF-κB signaling. Deficiency of any of these proteins causes combined immune deficiency with increased susceptibility to infections. T-cell proliferation is impaired.[71] Defective antibody responses are observed in patients with BCL10 and CARD11 deficiency; the latter condition is also characterized by an increase in the proportion of transitional B cells.

FCHO1 Deficiency and Transferrin Receptor Deficiency

FCH and Mu Domain Containing Endocytic Adaptor 1 (FCHO1) is an adaptor protein involved in clathrin mediated endocytosis. Autosomal recessive FCHO1 deficiency is characterized by numerical and functional defects of T cells and impaired clathrin-mediated endocytosis, including defective transferrin and iron intake.[72,73] Patients suffer from severe infections early in life. An autosomal recessive missense mutation (Tyr20His) in the transferrin receptor (*TFRC*) gene causing impaired internalization of the transferrin receptor causes recurrent infections, neutropenia and thrombocytopenia associated with reduced T cell proliferation and low number of switched memory B cells.[74] Both FCHO1 and TFRC deficiency can be corrected by SCT.

Moesin Deficiency

Moesin is a member of family of proteins (also including ezrin and radixin) involved in reorganization of the actin cytoskeleton. X-linked moesin deficiency is a combined immunodeficiency disorder characterized by recurrent bacterial and viral infections (especially, varicella), molluscum contagiosum and eczema.[75] Immunological abnormalities include profound lymphopenia, fluctuating neutropenia and antibody deficiency.

Dedicator of Cytokinesis 2 Deficiency

The dedicator of cytokinesis (DOCK) proteins are atypical guanine exchange factors containing a phosphatidylinositol (3,4,5)-triphosphate (PIP3) binding domain and a Rho-family guanosine triphosphate phosphohydrolases (GTPase) binding domain with guanine exchange factor activity. DOCK2 is predominantly expressed in various hematopoietic cell lineages and is activated in response to engagement of the TCR, BCR, and chemokine receptors. DOCK2 activation allows formation of GTP Ras-related C3 botulinum toxin substrate 1 (GTP-Rac1), thereby promoting reorganization of the cellular cytoskeleton, and intracellular signaling. DOCK2 deficiency is an autosomal recessive combined immunodeficiency characterized by increased susceptibility to severe infections starting early in life.[76] Severe varicella has been reported in multiple patients. T-cell lymphopenia, with marked reduction of naïve T cells and impaired T-cell proliferation, defective antibody responses, reduced NK cell function, poor migration of T and B lymphocytes in response to chemokines, NKT-cell deficiency, and markedly reduced secretion of interferon-alpha (IFN-α) by plasmacytoid dendritic cells represent the major immunological abnormalities. Low TREC levels at birth have been reported in two patients. The prognosis is very poor, but definitive cure may be achieved with SCT. Use of systemic IFN-α may help in the treatment of severe viral infections.

Dedicator of Cytokinesis 8 Deficiency

DOCK8 deficiency is an autosomal recessive combined immunodeficiency characterized by severe and recurrent infections, particularly systemic and cutaneous viral infections (molluscum contagiosum, HSV-1 and HSV-2, human papillomavirus), allergic manifestations (early-onset eczema, food allergies) with elevated serum IgE, impaired humoral immunity (with frequent sino-pulmonary infections), and increased risk for squamous cell carcinoma and lymphoma.[77,78] Large intragenic deletions account for the majority of the mutations. Affected patients have low numbers of naive CD8 T cells, and accumulation of CD8 memory cells with an “exhausted phenotype”. A profound impairment in the generation of CD8$^+$ memory T cells in response to influenza or other viral infections has been reported. Humoral immunity is also compromised, with loss of germinal center B cells and defective immunoglobulin affinity maturation. The disease has a severe prognosis, with considerable mortality.[79] SCT represents the only definitive treatment. Use of systemic IFN-α may ameliorate manifestations of severe cutaneous viral infection.

Activated RAC2 Defect

RAC2 is a small GTPase. Heterozygous GOF mutations of *RAC2* cause a profound immunodeficiency with low number of T and B cells, poor antibody production and neutropenia, associated with increased susceptibility to infections and lymphoproliferation.[80–82]

Wiskott-Aldrich Syndrome

Wiskott-Aldrich syndrome (WAS) is an X-linked disorder, characterized by thrombocytopenia with small-sized platelets, eczema, and immunodeficiency.[79] Autoimmune manifestations are common, and there is an increased risk of developing hematologic malignancies (primarily non-Hodgkin lymphoma, often EBV-driven). The estimated incidence is 1 in 100,000 births. The defect is due to mutations in the *WAS* gene, which encodes an intracytoplasmic protein (WAS protein [WASp]) expressed in hematopoietic cells and involved in cytoskeleton reorganization and immune synapse formation.[83] Deficiency of WASp results in impaired T-cell activation, poor production of antibodies, particularly to carbohydrate antigens, defective NK cell function, impaired phagocytic cell migration, and defective antigen presentation, thus explaining the immunodeficiency associated with the disease.

The clinical phenotype may range from typical and severe WAS to isolated thrombocytopenia; milder phenotypes are often associated with residual expression of WASp.[84] Rarely, mutations occurring in the GTPase binding domain of the protein results in a hyperactive form of WASp, resulting in X-linked neutropenia and myelodysplasia without thrombocytopenia, eczema, or immunodeficiency.[85–87]

Boys with WAS typically present in infancy with bleeding manifestations that can range from petechiae and bruising, to severe gastrointestinal and intracranial hemorrhages.[79] Eczema can be mild to severe. Common infections in WAS patients include otitis, sinusitis, pneumonia, and cellulitis. Viral infections (especially from herpesviruses, including CMV, EBV, and HSV-1) and opportunistic infections, such as *P. jiroveci* pneumonia, are also frequent. Approximately 40% of patients with WAS develop autoimmunity, including autoimmune hemolytic anemia, autoimmune thrombocytopenia, thyroiditis, colitis, vasculitis, nephropathy, and arthritis.[88] In addition to thrombocytopenia, typical laboratory findings include inability to mount responses to polysaccharide antigens, low or absent isohemagglutinin titers, high IgE, and poor proliferation of T cells when stimulated with anti-CD3 antibody. Other immune findings are more variable, including progressive T cell lymphopenia with age, low IgM, and high IgA. Analysis of WASp expression and demonstration of *WAS* gene mutations confirm the diagnosis.

Supportive treatment for WAS consists of monitoring and treatment for bleeding, antimicrobial prophylaxis, immunoglobulin-replacement therapy, and treatment of eczema and autoimmune manifestations with immunosuppressive drugs. Splenectomy can be effective in many to correct thrombocytopenia, but leads to an increased risk of sepsis.

Allogeneic SCT (typically with myeloablative regimens) for WAS has become standard at many institutions, and may be indicated early in life, especially in patients with severe mutations that abrogate protein expression.[89,90]

SCT is usually associated with robust T-cell engraftment, whereas some patients develop mixed chimerism in the B and myeloid lineages, indicating differential requirement for normal WAS protein to promote lineage-specific development or survival.[89,90]

Survival after SCT for WAS is influenced by donor type and age at transplantation, but continuous improvement of outcome has been reported during the last decades. However, mixed chimerism may be associated with increased risk of autoimmunity, and low myeloid chimerism with persistence of thrombocytopenia.[89,90]

Gene therapy represents another treatment option for patients with WAS (see Chapter 104). In a first trial, 10 boys with WAS were treated with gene therapy by transduction of CD34$^+$-selected peripheral blood stem cells using a murine leukemia virus-based gammaretroviral vector after low-dose busulfan conditioning.[91] Immune reconstitution was attained in nine patients; however, seven of these developed leukemia due to insertional mutagenesis.[92] More recently, gene therapy for WAS has been performed using a self-inactivating lentiviral vector. Results show good immune reconstitution and improved safety (with no leukemic events reported); however, the platelet count, although increased, remains low in most patients.[93–95]

COMBINED IMMUNODEFICIENCIES WITH OSSEOUS DYSPLASIA

Cartilage hair hypoplasia (CHH) is an autosomal recessive disorder characterized by short-limbed dwarfism, hair abnormalities, increased risk of bone marrow dysplasia, malignancies and Hirschsprung disease, and a variable degree of immunodeficiency.[96] The majority of patients show susceptibility to bacterial and viral infections; however, some may present with SCID, Omenn syndrome, or selective deficiency of CD8$^+$ cells. The disease is more common among certain nationalities and communities (the Amish populations and the Finns), and is caused by mutations of the gene encoding for the untranslated RNA component of the ribonuclease mitochondrial RNA processing (*RMRP*) complex, which is involved in cleavage of ribosomal RNA, processing of mitochondrial RNA, and cell cycle control. Management of the immunologic problems in CHH depends on their severity. SCT may be indicated in cases with severe T lymphocyte defects.

Schimke immunoosseous dysplasia is an autosomal recessive disorder that also combines immunologic and skeletal abnormalities. These children have short stature, skeletal dysplasia, renal dysfunction and T-cell immunodeficiency caused by mutations in the *SMARCAL1* gene.[97]

Autosomal recessive EXTL3 deficiency due defects of heparan sulfate biosynthesis causes severe T-cell lymphopenia, short stature, cervical spine stenosis and neurodevelopmental delay.[98]

OTHER COMBINED IMMUNODEFICIENCIES

Mutations of ICOS, a molecule expressed by activated T cells, cause recurrent infections of the respiratory and gastrointestinal tracts and autoimmunity.[99]

Heterozygous dominant-negative mutations of the *IKZF1* gene, encoding for the transcription factor IKAROS, cause a combined immunodeficiency with recurrent infections (including a high rate of *P. jiroveci* pneumonia). Generation of memory T and B cells is severely impaired.[100]

Hepatic venoocclusive disease with immunodeficiency is an autosomal recessive condition caused by mutations of the *SP110* gene.[101] In addition to liver disease, patients are highly prone to infections that are often sustained by opportunistic pathogens (CMV, *Candida*, *P. jiroveci*). Cerebrospinal leukodystrophy has been observed in several cases. The disease is rapidly fatal, unless treated by SCT.

Autosomal recessive combined immune deficiency with intestinal atresia is caused by mutations in the *TTC7A* gene, which encodes for a protein involved in maintaining cell polarity in epithelial cells.[102,103] Variable, but often severe, lymphopenia and panhypogammaglobulinemia are typical immunological abnormalities. The prognosis is generally severe and is determined by the severity of the gastrointestinal involvement and of immune deficiency. Small-bowel transplantation is often necessary. In patients with profound immune deficiency SCT may be required and does not in general improve the intestinal manifestations[104]; however, limited data are available on long-term outcome.

The cytidine triphosphate synthase 1 (CTPS1) protein is essential for lymphocyte proliferation. Patients with CTPS1 deficiency have a high risk of recurrent and chronic infections (especially those due to herpesviruses), and of EBV-related lymphoma.[105]

A very high risk of EBV-induced lymphoproliferative disease (see Chapter 87) is also observed in patients with CD27 and with CD70 deficiency. These patients lack memory B cells and often develop hypogammaglobulinemia after EBV infection.[106–108]

EBV-associated lymphoma (see Chapter 87) is a common manifestation in patients with magnesium transporter type 1 (MAGT1) deficiency.[109] This condition is inherited as an X-linked trait. The number of naive $CD4^+$ T cells is reduced, and expression of NKG2D, an important activating molecule, on the surface of $CD8^+$ cytotoxic T cells and NK lymphocytes, is markedly decreased. SCT can correct the disease.[110]

CD137 (4-1BB) is a costimulatory molecule, whose deficiency causes an increased risk of EBV-lymphoproliferative disease, chronic active EBV infection and B-cell lymphoma.[111]

IL-21 and IL-21R deficiencies are inherited as autosomal recessive traits.[112] Gastrointestinal manifestations with colitis and *Cryptosporidium*-associated cholangitis have been reported. A single patient with Kaposi sarcoma and OX40 deficiency has been reported.[113]

Autosomal recessive deficiency of phosphoglucomutase 3 (PGM3) is a disorder of glycosylation characterized by severe atopy, skeletal problems, cutaneous viral infections, respiratory tract infections and increased risk of lymphoma. Immunological abnormalities include impaired T-cell activation, elevated serum IgE and defective antibody production.[114,115]

DISORDERS WITH T-CELL-MEDIATED IMMUNE DYSREGULATION

Along with generation of a diversified repertoire of T and B lymphocytes, purging of self-reactive cells is essential to maintain immune homeostasis and prevent autoimmune diseases. Some forms of primary immunodeficiency manifest with immune dysregulation due to genetic defects of central or peripheral T-cell tolerance.

Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy Syndrome

Also known as *autoimmune polyglandular syndrome type 1* (APS1), autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) is an autosomal recessive disorder characterized by autoimmune endocrine manifestations (hypoparathyroidism, Addison disease), candidiasis, and nail dystrophy.[116,117] The disease is caused by mutations of the autoimmune regulator (*AIRE*) gene,[118,119] which encodes for a transcription factor expressed by medullary TECs (mTECs) where it regulates expression of tissue specific antigens that are presented to nascent T cells, thereby promoting clonal deletion of self-reactive T lymphocytes, or their conversion to natural regulatory T cells (nTreg cells).

In patients with APECED, mucocutaneous candidiasis usually develops first (in infancy or early childhood), followed by autoimmune hypoparathyroidism with hypocalcemia and adrenal failure. Around 15% to 20% of patients may develop insulin-dependent diabetes. Gastrointestinal and pulmonary involvement, reflecting autoimmune damage, are frequently observed. Autoimmune hepatitis and ovarian failure are also common. Candidiasis may reflect elevated levels of antibodies to IL-17 and IL-22, which play an important role in the defense against *Candida* spp.

Management of patients with APECED is based on regular follow-up and searching for autoantibodies, which should be repeated every 6 months. Hormone-replacement therapy is indicated for the endocrinopathies. Aggressive management and prevention of candidiasis is important to avoid persistent or recurrent infections that may favor development of squamous cell carcinoma.

Immune Dysregulation Polyendocrinopathy Enteropathy X-Linked Syndrome

Immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) is an X-linked disorder of systemic autoimmunity.[120] Males with IPEX syndrome present early in life with skin rash, intractable watery diarrhea, insulin-dependent diabetes mellitus, lymphadenopathy, splenomegaly, and failure to thrive. Other autoimmune manifestations include thyroid disease, nephropathy, cytopenias, and vasculitis. In addition, patients with IPEX show elevated levels of IgE.

IPEX is caused by mutations of the *FOXP3* gene, which encodes for a transcription factor that plays a critical role in the development and function of regulatory T (Treg) lymphocytes.[121] These cells have suppressive functions; they have a distinctive phenotype ($CD4^+$ $CD25^{hi}$ $FOXP3^+$) and normally represent 5% to 15% of $CD4^+$ circulating lymphocytes.

IPEX has a poor prognosis. Death often occurs within the first few years of life due to severe malabsorption, failure to thrive, metabolic derangement, or because of severe infections secondary to immune suppression. Use of immunosuppressive drugs (steroids, sirolimus, tacrolimus, rituximab) is necessary to treat the disease. However, the only definitive treatment is allogeneic SCT.[122]

CD25 and STAT5B Deficiencies

IL-2 plays an important role in T lymphocyte proliferation and in immune homeostasis. Upon binding to the high-affinity IL-2 receptor, IL-2 promotes phosphorylation and nuclear translocation of the transcription factor signal transducer and activator of transcription (STAT)5, which drives expression of IL-2 target genes, including *FOXP3*. Autosomal recessive mutations of the *IL2RA* (*CD25*) gene, which encodes for the α chain of the IL-2 receptor, cause a disease that is associated with IPEX-like manifestations (lymphadenopathy, hepatosplenomegaly, autoimmune cytopenias, inflammatory bowel disease) and increased susceptibility to severe viral infections.[123] A similar phenotype may also be caused by mutations of the *IL2RB* gene, encoding for the IL-2Rβ chain.[124] HCT represents the only definitive treatment.

Autosomal recessive mutations of the *STAT5B* gene cause a syndrome characterized by autoimmunity (especially cytopenias and autoimmune hepatitis), eczema, severe and/or recurrent infections, lymphocytic interstitial pneumonia, and growth hormone (GH)-insensitive dwarfism.[125] The latter reflects the critical role played by STAT5 in the cellular response to GH. Affected patients have a low number of circulating T and NK lymphocytes and Treg cells, and in vitro proliferative responses to mitogens are reduced. In contrast, IgG levels are often elevated. Aggressive treatment of infections, use of immunosuppressive drugs to treat autoimmune manifestations, and regular monitoring of pulmonary function are the mainstay of treatment.

Cytotoxic T Lymphocyte Antigen 4 Deficiency

Cytotoxic T lymphocyte antigen 4 (CTLA4) is a surface molecule expressed by conventional and regulatory T cells. It competes with CD28 for binding to CD80/CD86 molecules expressed by antigen-presenting cells. However, in contrast to CD28, CTLA4 delivers a suppressive signal that limits T-cell activation. Heterozygous loss-of-function mutations of CTLA4 cause a condition characterized by lymphoid infiltrates in the lung, gastrointestinal tract, and the brain (simulating lymphoma), autoimmune cytopenias, hypogammaglobulinemia, lymphopenia, accumulation of activated T cells, and decreased Treg-cell function. The disease is inherited as an autosomal dominant trait with incomplete penetrance. Treatment is based on use of CTLA4-Ig (abatacept) and sirolimus. SCT may be considered in severe cases.

Lipopolysaccharide Responsive Beige-Like Anchor Protein Deficiency

Deficiency of the lipopolysaccharide responsive beige-like anchor protein (LRBA) is inherited as an autosomal recessive trait and causes a combined immunodeficiency with prominent immune dysregulation affecting mostly the gastrointestinal tract (with severe chronic diarrhea and failure to thrive) and the lungs.[126–128] Autoimmune cytopenias are also common. Typical immunological abnormalities include hypogammaglobulinemia and a variable degree of lymphopenia, with increased proportion of activated T cells. The disease shares some clinical and immunologic manifestation similarities with CTLA4 deficiency.[129] Indeed, LRBA binds to CTLA4 and allows endosomal trafficking of the latter to the cell membrane. In the absence of LRBA, CTLA4 is targeted to the lysosomal compartment where it is degraded. Consistent with this, Treg cells from patients with LRBA deficiency have low levels of expression of CTLA4 and FOXP3, and the actual number and function of Treg lymphocytes are impaired. Treatment with CTLA4-Ig (alone or in combination with sirolimus) may be beneficial.

DEF6 deficiency

Differentially expressed in FDCP 6 homolog (DEF6) is a guanine nucleotide exchange factor involved in TCR signaling. DEF6 deficiency affects availability of CTLA-4 at the cell surface and Treg function.[130] Clinical manifestations include recurrent infections, enteropathy, cardiomyopathy and lymphoproliferation with hepatosplenomegaly.

Defects of Calcium Flux

T lymphocyte activation and homeostasis depend on calcium mobilization. In particular, TCR-induced activation results in release of Ca^{2+} from the endoplasmic reticulum stores, followed by Ca^{2+} entry through the Ca^{2+} release-activated channels (CRAC) located in the cell membrane. Mutations of *STIM1* (which encodes for a sensor of endoplasmic reticulum calcium stores) and of *ORAI1* (which encodes for CRAC) cause severe immunodeficiency in which T-lymphocyte generation in the thymus is not affected, but peripheral T cells are functionally impaired.[131,132] Autoimmunity and nonprogressive myopathy are distinguishing features of these disorders.

Autoimmune Lymphoproliferative Syndrome

Interaction between FAS (CD95) on activated T and B lymphocytes, and Fas ligand (FASLG) on activated T cells triggers the extrinsic pathway to activation of the caspase cascade, culminating in apoptosis. In addition, cellular stress (including cytokine deprivation) may activate the intrinsic pathway of apoptosis, leading to disruption of mitochondrial membrane permeability, release of cytochrome c, activation of caspase 9 and apoptosis. Defects of FAS- or mitochondrial-dependent apoptosis are responsible for autoimmune lymphoproliferative syndrome (ALPS), characterized by early-onset, chronic (>6 months), nonmalignant lymphoproliferation, autoimmunity, and increased risk of lymphoma.[133]

The majority of patients with ALPS carry heterozygous dominant-negative mutations in the *FAS* (*CD95*, *TNFRSF6*) gene (ALPS-FAS).[134,135] Somatic mutations of the *FAS* gene are the second most common cause of the disease (ALPS-sFAS).[136] More rarely, ALPS is caused by mutations of the *FASLG* gene (ALPS-FASLG), of caspase 8, caspase 10, Fas-associated death domain (FADD) protein, or by somatic activating mutations of N-RAS or K-RAS.[137] Finally, autosomal recessive deficiency of protein kinase C (PRKCD), which plays an important role in regulating cell survival and apoptosis, also causes an ALPS-like disease with B-cell lymphoproliferative disease.[138,139]

Lymphoproliferation, manifesting as lymphadenopathy, splenomegaly, or hepatomegaly, is the most common clinical manifestation, with a trend toward progressive remission in adulthood. More than 70% of ALPS patients develop autoimmune disease—most commonly immune cytopenias, less frequently organ-specific autoimmunity. There is an increased risk of malignancies, especially EBV-associated non-Hodgkin lymphoma. Patients with PRKCD deficiency often present with SLE-like manifestations, with nephropathy and antiphospholipid antibody syndrome.[138,139]

An immunologic signature of ALPS is the increased number of DN circulating T lymphocytes that express the αβ form of the TCR, but do not express CD4 or CD8 molecules.[133] There is defective in vitro apoptosis of lymphocytes through the FAS-mediated or intrinsic pathway (depending on the genetic variant). Elevated levels of soluble FAS ligand, vitamin B_{12}, and IL-10, hypergammaglobulinemia, and anti-phospholipid antibodies are often present in patients with ALPS-FAS. By contrast, patients with PRKCD deficiency have low levels of IgG and an increased proportion of $CD5^+$ B cells. Patients with caspase 8 deficiency, FADD deficiency, and PRKCD deficiency have recurrent infections. The number of DN T cells may be normal in patients with *NRAS* or *KRAS* mutations.

Treatment is based on immunosuppression (with steroids, rituximab, mycophenolate mofetil, or sirolimus) and surveillance for lymphoma.[140] Splenectomy may improve cytopenias; however, relapse of autoimmunity has been observed in 50% of the splenectomized patients. Furthermore, severe, invasive bacterial infections have been reported in 30% of splenectomized patients, and the mortality rate for invasive bacterial infection after splenectomy is as high as 13.3%.

Other Conditions With Immune Dysregulation

***STAT1* GOF** mutations cause a variable clinical phenotype ranging from chronic mucocutaneous candidiasis to increased occurrence of viral, fungal, and bacterial infections, to autoimmunity.[141]

GOF mutations of *STAT3* cause increased STAT3 signaling and enhanced Th17 differentiation, with lymphoproliferation and solid-organ autoimmunity. The number of Treg cells is reduced.[142–144]

GOF mutations of *JAK1* cause an autosomal dominant condition with hypereosinophilia, hepatosplenomegaly, eosinophilic enteritis, poor growth and viral infections.[145]

Deficiency of ITCH, an E3 ubiquitin ligase that regulates activation of phospholipase Cγ1 (PLCγ1), causes multiple autoimmune manifestations (enteropathy, thyroiditis, type 1 diabetes, hepatitis), interstitial lung disease, dysmorphic features, and developmental delay.[146]

Defects of IL-10 and IL-10R are responsible for very early onset inflammatory bowel disease.[147]

Mutations of the tripeptidyl-peptidase II (*TPP2*) gene cause severe autoimmune hemolytic anemia, variable lymphoproliferation, and recurrent infections. Accelerated senescence of lymphoid cells and hypergammaglobulinemia are observed.[148]

DEFECTS OF CELL-MEDIATED CYTOTOXICITY

Mechanisms of immune defense against viral infections are largely dependent on responses mediated by $CD8^+$ cytotoxic T lymphocytes (CTLs) and NK lymphocytes. Multimers of perforin, a protein contained in cytolytic granules, form pores through which CTL and NK cells release cytotoxic proteins (granzyme B, granulolysin) into virus-infected target cells, causing activation of caspases and apoptosis. In resting CTLs and NK lymphocytes, cytotoxic proteins are contained in endosomal secretory lytic granules. Following interaction of CTLs or NK cells with the target cell, vesicles containing lytic granules are transported and dock and fuse with the cell membrane, permitting release of cytotoxic proteins into the target cells.

Hemophagocytic Lymphohistiocytosis

Defects in the intracellular transport and release of cytolytic granules are responsible for various forms of familial HLH, including deficiency of Munc13-4 (required to prime vesicles), syntaxin 11 and Munc18-2 (both of which promote vesicle fusion with the cell membrane), RHOG and perforin.[149] In these disorders, infections (mostly caused by viruses) trigger an uncontrolled immune response mediated by CD8$^+$ T lymphocytes and NK cells that secrete large amounts of IFN-γ, which activates macrophages. Fever, liver, and splenic enlargement, lymphadenopathy, profound cytopenias, hypoalbuminemia, coagulopathy, high levels of ferritin and triglycerides, and immune activation (with increased levels of soluble CD25) characterize these acute episodes, which may lead to multiple-organ failure and death. The diagnosis of these familial forms of HLH is facilitated by flow cytometric analysis of surface expression of CD107a (a protein normally contained in endosomal vesicles) upon in vitro activation of cytotoxic T cells and NK cells. Defective expression of CD107a is observed in patients with Munc13-4, Munc18-2, and syntaxin 11 deficiencies. By contrast, this test is normal in patients with perforin deficiency, in which reduced or absent expression of perforin can be easily demonstrated by flow cytometry.

Zinc finger NFX1-type containing 1 (ZNFX1) is an interferon-stimulated double-strand RNA sensor. Autosomal recessive **ZNFX1 deficiency** causes increased susceptibility to viral infections, HLH-like manifestations, early-onset seizures, as well as renal and lung disease.[150,151]

Human Leukocyte Antigen Associated With Pigmentary Dilution Disorders

Typical features of HLH are also observed in some pigmentary dilution disorders.[149] In particular, Chediak-Higashi syndrome (CHS) (see Chapter 51) is an autosomal recessive disease due to mutations of the *LYST* gene, which encodes for a protein involved in the sorting of proteins to secretory endosomes. This defect affects not only cytotoxic lymphocytes (accounting for FHL-like clinical features), but also melanocytes (that are unable to transfer melanin to keratinocytes and other epithelial cells) and peripheral neurons. Clinical features of CHS include partial albinism (with silvery hair), progressive peripheral neuropathy, recurrent bacterial infections, and a mild bleeding tendency. Neutropenia is often present and may cause bacterial infections. Giant lysosomes can be identified in circulating leukocytes.

Griscelli syndrome type 2 (GS2) is caused by mutations of the *RAB27A* gene, which encodes for a protein involved in docking of secretory granules of cytotoxic lymphocytes and melanocytes to the cell membrane. Accordingly, patients with GS2 present with HLH and hypopigmentation. The diagnosis is facilitated by the demonstration of large clumps of pigment distributed irregularly along the hair shaft, and by reduced expression of CD107a on the surface of CD8$^+$ and NK lymphocytes upon in vitro activation.

Hermansky-Pudlak syndrome type 2 (HPS2) and HPS10 are autosomal recessive disorders caused by mutations of the *AP3B1* and the *AP3D* genes, which encodes for the β and the δ components of the AP-3 complex, respectively, involved in sorting of transmembrane proteins to secretory lysosomes. Both disorders are characterized by partial albinism, nystagmus, recurrent bacterial and viral infections, neutropenia and defective cytotoxic activity account for increased risk of HLH. In addition, HPS2 patients have tendency to bleeding due to platelet dysfunction (with absence of dense granules and reduced platelet aggregation), whereas patients with HPS10 have hearing loss and developmental delay.

Irrespective of the nature of the genetic defect, HLH therapy is based on the prompt and aggressive treatment of underlying infections and immunosuppression to curtail macrophage activation. Administration of humanized anti-IFN-γ monoclonal antibodies may block continuous activation of the immune system and induce remission.[152] Ultimately, definitive treatment is based on allogeneic SCT from a matched related or unrelated donor; while survival is better with reduced-intensity conditioning, a high incidence of graft failure favors the use of moderate intensity regimens.[153] Mixed chimerism is sufficient to correct the immunologic abnormalities. (See box on Diagnostic and Therapeutic Approach to Cytotoxicity Defects.)

X-Linked Lymphoproliferative Disease

In normal individuals, primary infection with EBV causes infectious mononucleosis, a self-limiting disease. EBV establishes latency in B lymphocytes, salivary glands, and some epithelial cells, and is maintained under control by CD8$^+$ CTLs, and NK lymphocytes. Males with X-linked lymphoproliferative disease type 1 (XLP1) are uniquely susceptible to life-threatening complications of EBV infections.[154] XLP1 is caused by mutations of the *SH2D1A* gene, which encodes for a small adaptor molecule, SLAM-associated protein (SAP).[155–157] In the absence of SAP, cytotoxic responses to EBV-infected cells are markedly reduced. Persistence of EBV triggers continuous activation of CD8$^+$ CTLs that release high amounts of IFN-γ, ultimately resulting in a macrophage activation syndrome. In addition to HLH, patients with XLP1 are also at high risk of B-cell lymphoma. SAP is also important for the function of follicular helper T cells (T_{FH}), which promote maturation of antibody responses. Consistent with this, XLP1 males often develop hypogammaglobulinemia with a lack of memory B lymphocytes. Finally, XLP1 is also associated with impaired development of NK T (NKT) lymphocytes. Flow cytometric analysis of SAP expression and mutation analysis at the *SH2D1A* locus confirms the diagnosis.

A minority of patients with XLP carry defects in another gene (*BIRC4*) that encodes for the X-linked inhibitor of apoptosis (XIAP).[158] Consistent with this, lymphocytes from patients with this disease (XLP2) show increased susceptibility to activation-induced apoptosis. Compared to XLP1, patients with XLP2 have a higher incidence of HLH (with or without EBV infection), but do have an increased risk of lymphoma. Severe inflammatory bowel disease has been reported in several patients. Hypogammaglobulinemia is often present. Flow cytometric analysis of XIAP expression and mutation analysis at the *BIRC4* locus are used to confirm the diagnosis.

DIAGNOSTIC AND THERAPEUTIC APPROACH TO CYTOTOXICITY DEFECTS

- Primary immunodeficiency with defects of cell-mediated cytotoxicity include a heterogeneous group of disorders characterized by increased susceptibility to severe viral infections.
- Typical clinical features include high fever, liver and splenic enlargement, lymphadenopathy, coagulation defects, abnormalities of liver function, and cytopenias after viral infections.
- Two major groups of cell-mediated cytotoxicity defects include familial forms of HLH (fHLH) and X-linked lymphoproliferative disease. In the former, diagnosis is based on demonstration of abnormal cytotoxic function of T and NK lymphocytes. Defective expression of CD107a on activated lymphocytes or reduced expression of perforin may help define the nature of the underlying disorder causing fHLH.
- Prompt recognition and treatment of the underlying infection is essential. Immunosuppression is also required to block exaggerated inflammatory responses. However, the only curative approach to defects of cell-mediated cytotoxicity is represented by SCT. Mixed chimerism is sufficient to allow immune reconstitution. This goal can be achieved with reduced intensity conditioning, with a lower risk of treatment-related toxicity but also a risk of graft failure.

Both XLP1 and XLP2 are life-threatening disorders. Administration of immunoglobulins may be beneficial; however, the only curative approach is SCT.

DEFECTS OF B-CELL DEVELOPMENT AND FUNCTION

The prior section covered defects that predominantly affect T lymphocyte development and/or function. This section covers primary immunodeficiencies in which abnormalities of B-cell development and/or function predominate (see Figs. 52.2 and 52.3).

X-Linked Agammaglobulinemia

X-linked agammaglobulinemia (XLA), also known as *Bruton's agammaglobulinemia*, is the most common monogenic cause of failure of B-cell development. Males with XLA lack circulating B cells, caused by mutations of the Bruton's tyrosine kinase or *BTK* gene, which encodes for a tyrosine kinase involved in signaling through various receptors, including the pre–B-cell receptor (pre-BCR) and the BCR.[159,160] Impaired signaling through the pre-BCR causes a severe, but incomplete, block at the pre–B-cell stage in the bone marrow (see Fig. 52.2).

Clinical manifestations of XLA include recurrent sinopulmonary infections (pneumonia, bronchitis, sinusitis, otitis), particularly with encapsulated organisms such as pneumococcus and *Haemophilus influenza*, bacterial skin infections (cellulitis, impetigo, pyoderma gangrenosum, perirectal abscess), and sepsis with *Pseudomonas* or *Staphylococcus*.[161] Symptoms typically begin after 3 to 6 months of age when maternally derived antibodies disappear. The proportion of circulating B cells is markedly reduced (typically 0.05% to 0.3% of lymphocytes) and there is profound deficiency of all immunoglobulin isotypes. Neutropenia, secondary to severe infections, is observed in approximately 10% to 25% of patients; typically, it resolves with antibiotics and immunoglobulin-replacement therapy.

In addition to bacterial infections, patients with XLA are uniquely susceptible to enteroviral infections (including chronic meningoencephalitis) caused by poliovirus, coxsackie virus, and others. However, such complications have become rare after the introduction of optimal doses of immunoglobulin-replacement therapy. Patients with XLA are also at higher risk of infections caused by *Mycoplasma* and *Ureaplasma* species, which affect the joints, prostate, or lungs; and *Giardia lamblia*, causing protracted diarrhea and malabsorption. An increased incidence of lymphoma, colorectal cancer, and gastric adenocarcinoma has been reported in XLA.

Therapy is based on life-long regular administration of immunoglobulins intravenously or subcutaneously, and on prompt and aggressive treatment of infections. With this regimen, survival approaches that of the general population. Antibiotic prophylaxis may be beneficial, but its role has not been firmly established. Patients on immunoglobulin-replacement therapy should be monitored for side effects and adverse reactions, and for liver and renal function.

Autosomal Recessive Agammaglobulinemia

While the majority of agammaglobulinemia cases are caused by the X-linked form, about 15% of cases are presumed to be autosomal recessive (see Fig. 52.2).[161,162] Mutations of the immunoglobulin μ heavy chain gene (*IGHM*) are the second most common cause of agammaglobulinemia. Other cases are caused by defects in other components of the pre-BCR/BCR complex including the signaling moieties Igα (CD79A) and Igβ (CD79B), and λ5 (IGLL1), the surrogate light chain that pairs with Vpre-B, and the scaffold protein BLNK, which brings BTK into the signaling complex. Finally, congenital agammaglobulinemia may also be caused by mutations of the *PIK3CD* and *PIK3R1* genes, causing complete loss of the p110α and p85α subunits, respectively, of phosphatidylinositol 3-kinase (PI3K), and to heterozygous, dominant-negative mutations of the *TCF3* gene encoding for the transcription factor E47.

Common Variable Immunodeficiency

Common variable immunodeficiency (CVID) is the most common primary immunodeficiency severe enough to require treatment, with a prevalence estimated to be 1 in 25,000 to 1 in 30,000 Caucasians, and accounting for approximately 10% to 20% of humoral immunodeficiencies. A growing number of genetic defects have been found to be associated with a CVID phenotype, but most cases remain genetically undefined.[163] Mutations in transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) can also be found in asymptomatic individuals,[164,165] and more likely represents a predisposition to CVIDs than primary cause.

Mutations in CD19, CD81, and CD21, which are all components of the B-cell coreceptor, have been reported in few individuals. These defects do not affect B-cell development but impair signaling, causing hypogammaglobulinemia, low number of switched memory B cells, and poor antibody response to T-independent antigens. In addition to recurrent infections, patients with CD19 and CD81 deficiency may also develop glomerulonephritis.

CD20 is a surface molecule involved in B-cell development and plasma cell differentiation. Autosomal recessive CD20 deficiency is characterized by recurrent infections, low serum IgG, with normal or elevated IgA and/or IgM.

BAFF-R deficiency is very rare. It has a variable clinical presentation, with CVID-like features or even absence of symptoms. Laboratory findings include hypogammaglobulinemia, low numbers of circulating B cells, a defective proportion of memory B cells, and normal antibody response to T-dependent antigen but a defective response to carbohydrate antigens.

A variable clinical phenotype has been also reported in patients with haploinsufficiency of the p50 subunit of NF-κB (encoded by the *NFKB1* gene), causing defective signaling through the canonical NF-κB signaling pathway. In addition to recurrent infections and progressive lung disease, patients often present with autoimmunity (hemolytic anemia, thyroiditis, alopecia) and lymphadenopathy. However, genotypically affected individuals may also remain asymptomatic even through adulthood. Heterozygous mutations of the *NFKB2* gene (affecting mostly the noncanonical NF-κB signaling pathway) cause recurrent infections (especially due to HSV, varicella-zoster virus [VZV], and *Giardia*), central adrenal insufficiency, hypopituitarism, alopecia, trachyonychia, and growth deficiency. Hypogammaglobulinemia, a low number of memory B cells, Treg lymphocytes, T follicular helper (Tfh) cells, and NK lymphocytes have been demonstrated.

Haploinsufficiency of the *IKZF1* gene, causes recurrent infections and autoimmunity, associated with progressive B-cell lymphopenia and hypogammaglobulinemia. These patients are at significantly increased risk of acute lymphoblastic leukemia.

Autosomal recessive RAC2 deficiency causes recurrent infections, post-streptococcal glomerulonephritis and urticaria, along with hypogammaglobulinemia and poor antibody production.

Diagnostic criteria for CVIDs include the presence of hypogammaglobulinemia (<2 standard deviations below the mean for age of IgG, IgA, and/or IgM) and the lack of specific antibody responses in individuals with onset of symptoms >2 years of age, for whom other causes of humoral immunodeficiency have been ruled out.[166] The number of circulating B cells detected is variable. Specific antibody responses should be assessed for more than one antigen.

Consistent with the antibody deficiency, CVID patients have recurrent bacterial infections of the respiratory tract, sepsis with encapsulated organisms, and infections from *Ureaplasma uraelyticum*, *Giardia* species and enteroviruses. However, variable derangement of T-cell numbers and function often results in noninfectious manifestations, including autoimmunity, lymphoid infiltration or proliferation, and malignancy. In one survey only 26% of CVID patients had infections as the only manifestation of disease.[167] Autoimmune cytopenias are quite common (~12%) and may be the presenting feature. Other autoimmune complications include rheumatoid arthritis, systemic lupus erythematosus, hypothyroidism, vitiligo, psoriasis, diabetes, and autoimmune gastritis with pernicious anemia. Polyclonal lymphocytic granulomatous infiltrates are common and may involve the lungs, liver, or gut (causing enteropathy that is resistant to gluten withdrawal). Finally, both lymphoid (non-Hodgkin lymphoma, chronic lymphocytic leukemia) and nonlymphoid (gastric cancer) malignancies, as well as nonmalignant lymphoproliferation,[168] are more frequent in CVID, although the gastric cancers may be related to *Helicobacter pylori* infection.

Replacement of immunoglobulins and treatment and prevention of infection remains the mainstay of management of CVID.[162] Immunosuppression may be needed for inflammatory and autoimmune manifestations. Disease manifestations such as autoimmunity, polyclonal lymphocytic infiltrative disease, enteropathy, and malignancy have differential and increasing impact on survival (relative risk of death 2.5, 3.0, 4.0, and 5.5, respectively), while patients with solely infectious manifestations have equivalent survival to the general population. Treatment with HCT has been performed with limited success, and often in the context of consolidative treatment for lymphoproliferation. (See box on Diagnostic and Therapeutic Approach to Defects in Humoral Immunity.)

B-CELL–INTRINSIC DEFECTS OF CLASS-SWITCH RECOMBINATION

Maturation of the antibody response is marked by class-switch recombination (CSR) and by somatic hypermutation (SHM). With CSR, the μ heavy chain is replaced by other Ig heavy chains. With SHM, mutations are introduced in the Ig V region, allowing affinity maturation. Following CD40LG–CD40 interaction and activation of the NF-κB signaling pathway, expression of activation-induced cytidine deaminase (AID) and of uracil-DNA glycosylase (UNG) occurs in germinal center B lymphocytes (see Fig. 52.3). AID acts on the DNA of Ig heavy chain switch regions and converts cytidine to uridine, which is recognized and removed by UNG. The abasic sites are cleaved by a DNA endonuclease. DNA repair then brings together two different switch regions, allowing CSR.

Mutations in AID and UNG are inherited as autosomal recessive traits.[169] Patients suffer from recurrent bacterial infections. Autoimmune manifestations have been observed in several patients. Circulating B cells are present, but there is a severe reduction of all Ig isotypes with the exception of IgM, which is often elevated. Lymphadenopathy is common and is associated with expansion of germinal centers. Somatic hypermutation is severely compromised in patients with AID deficiency, and follows an abnormal pattern in patients with UNG deficiency.

Treatment is based on regular administration of Igs, as well as prompt recognition and therapy of infections.

Defects in CSR have also been reported in patients with mutations in *INO80* (encoding for a chromatin remodeling complex) and *MSH6* (involved in the DNA mismatch repair pathway). The latter group of patients is at increased risk of cancer.

Other Immunoglobulin Defects

Gain-of-function mutations of the *PIK3CD* gene (encoding for the p110δ catalytic component of PI3K) cause an immunodeficiency characterized by nodular lymphoid hyperplasia, progressive lung disease with bronchiectasis, chronic or recurrent herpesvirus viremia (CMV, EBV, VZV, HSV), and hepatosplenomegaly.[170,171] There is an increased risk of lymphomas, which are not necessarily associated with EBV infection. The immunologic phenotype includes CD4

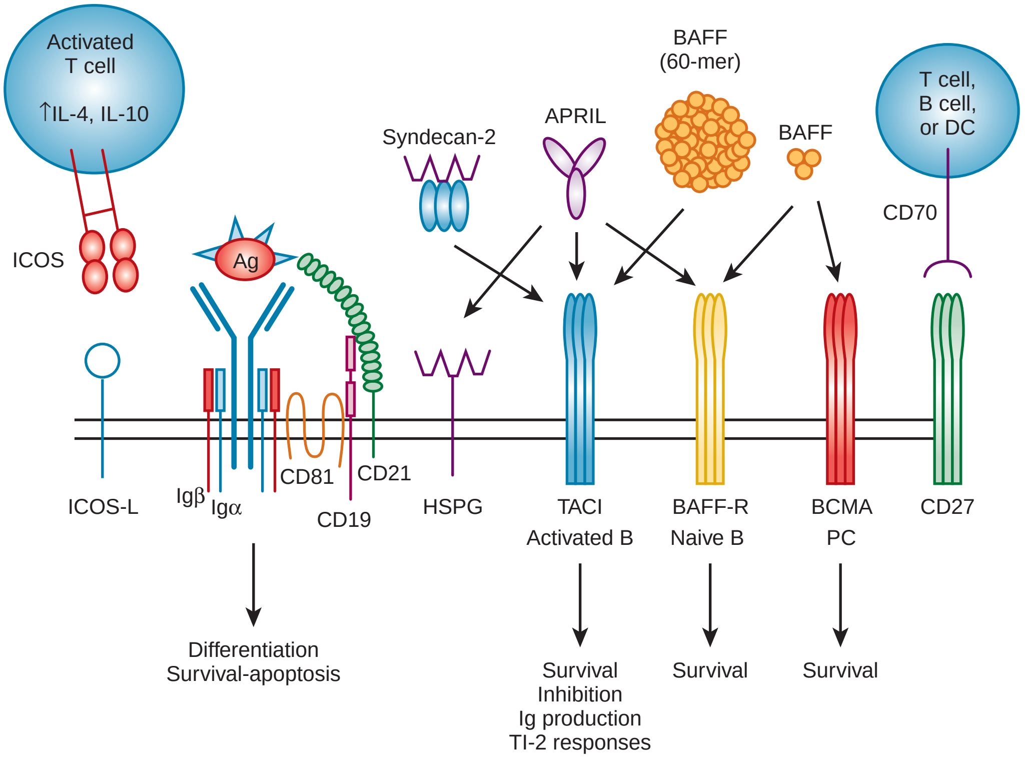


Figure 52.3 GENETIC DEFECTS ASSOCIATED WITH COMMON VARIABLE IMMUNODEFICIENCY (CVID). Schematic of cell surface molecules expressed on B cells and their ligands, highlighting those in which defects have been shown to be associated with CVID. *Ag*, Antigen; *APRIL*, a proliferation-inducing ligand; *BAFF*, B-cell–activating factor; *BAFFR*, B-cell–activating factor receptor; *BCMA*, B-cell–maturation antigen; *DC*, dendritic cell; *HSPG*, heparin sulfate proteoglycan; *ICOS*, inducible T-cell costimulator; *ICOS-L*, inducible T-cell costimulator ligand; *Ig*, immunoglobulin; *IL-4*, interleukin 4; *IL-10*, interleukin 10; *PC*, plasma cell; *TACI*, transmembrane activator and calcium modulator and cyclophilin ligand interactor; *TI-2*, T-cell–independent type 2.

DIAGNOSTIC AND THERAPEUTIC APPROACH TO DEFECTS IN HUMORAL IMMUNITY

- Defects in humoral immunity may be caused by intrinsic defects in B-cell development and function, or caused by combined immunodeficiency disorders where lack of T-cell help is the primary cause of B-cell dysfunction.
- Age-specific norms must be used for children. Immunoglobulin (Ig) G levels at birth reflect the maternally transferred antibody, which nadir between 2 and 6 months, then progressively rise with age. IgA and IgM levels rise continually with age until the teenage years.
- Based on enumeration of T, B and natural killer cells, X-linked agammaglobulinemia (XLA; absence of B cells) can be rapidly ruled out.
- The finding of hyper-IgM with low IgG should prompt a work-up for CD40L deficiency, CD40 deficiency, activation-induced cytidine deaminase (AID), and uracil-DNA glycosylase (UNG). The likelihood of each of these is of course different depending on gender and family history. It is important to note that CD40L deficiency may present with a normal IgM.
- The diagnosis of common variable immunodeficiency disorders (CVIDs) should only be made once other causes have been ruled out, and should be made with caution, if at all, in children under 4 years of age. Disorders such as XLA, autosomal recessive agammaglobulinemia, various hyper-IgM syndromes, and XLP should be specifically considered and ruled out. Other diseases that may masquerade as CVID include other combined immunodeficiencies, cystic fibrosis, HIV, and conditions leading to Ig loss such as protein-losing enteropathy. Medications may mimic the symptoms of CVID. Finally, in adults hypogammaglobulinemia may herald the development of thymoma or lymphoid malignancies, and therefore may be secondary to evolving neoplasia.
- Treatment of XLA and the hyper-IgM syndromes other than CD40LG deficiency is long-term intravenous Ig replacement, which is very effective.
- Experts disagree on whether CD40LG deficiency should be treated with early SCT, and currently there are no clear predictors to determine who is likely to suffer from opportunistic infections. A patient with CD40LG deficiency who has developed cryptosporidium infection and sclerosing cholangitis generally has a much worse outlook with SCT. However, outcomes have generally improved in recent years.
- Treatment of CVID with SCT is generally reserved for those with another requirement for SCT such as developing a lymphoid malignancy or lymphoproliferation, which requires continual immunosuppression.

cell lymphopenia, a reduced proportion of naïve T cells, expansion of effector memory and “exhausted” $CD8^+$ T cells, defective T-cell proliferation, variable Ig levels (often with low IgG2), reduced proportion of memory B cells, and poor antibody responses. In these patients, increased PI3K signaling causes hyperactivation of the AKT–mammalian target of rapamycin (mTOR) pathway, shifting the intracellular metabolism of lymphoid cells towards glycolysis and thereby deranging lymphocyte function and differentiation.

A very similar clinical and laboratory phenotype has been observed in patients with heterozygous mutations of the *PIK3R1* gene (encoding for the p85α regulatory subunit of PI3K), which permit expression of a mutated p85α protein lacking the p110δ-interacting domain.[172–174] Treatment with rapamycin, to reduce mTOR activation, may be beneficial in both conditions. An alternative strategy, currently under investigation, is based on the use of PI3K inhibitors.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Ameratunga R, Allan C, Woon ST. Defining common variable immunodeficiency disorders in 2020. *Immunol Allergy Clin North Am*. 2020;40(3):403–420.

Bacchetta R, Barzaghi F, Roncarolo MG. From IPEX syndrome to FOXP3 mutation: a lesson on immune dysregulation. *Ann NY Acad Sci*. 2018;1417(1):5–22.

Buckley RH, Schiff SE, Schiff RI, et al. Hematopoietic stem-cell transplantation for the treatment of severe combined immunodeficiency. *N Engl J Med*. 1999;340(7):508–516.

Czechowicz A, Kraft D, Weissman IL, Bhattacharya D. Efficient transplantation via antibody-based clearance of hematopoietic stem cell niches. *Science*. 2007;318(5854):1296–1299.

Davies EG, Cheung M, Gilmour K, et al. Thymus transplantation for complete DiGeorge syndrome: European experience. *J Allergy Clin Immunol*. 2017;140(6):1660–1670. e1616.

de la Morena MT, Leonard D, Torgerson TR, et al. Long-term outcomes of 176 patients with X-linked hyper-IgM syndrome treated with or without hematopoietic cell transplantation. *J Allergy Clin Immunol*. 2017;139(4):1282–1292.

Demirdag YY, Gupta S. Update on infections in primary antibody deficiencies. *Front Immunol*. 2021;12:634181.

Ferre EMN, Rose SR, Rosenzweig SD, et al. Redefined clinical features and diagnostic criteria in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. *JCI Insight*. 2016;1(13):e88782.

Ferrua F, Galimberti S, Courteille V, et al. Hematopoietic stem cell transplantation for CD40 ligand deficiency: results from an EBMT/ESID-IEWP-SCETIDE-PIDTC study. *J Allergy Clin Immunol*. 2019;143(6):2238–2253.

Gatti RA, Meuwissen HJ, Allen HD, et al. Immunological reconstitution of sex-linked lymphopenic immunological deficiency. *Lancet*. 1968;2(7583):1366–1369.

Gennery AR, Slatter MA, Grandin L, et al. Transplantation of hematopoietic stem cells and long-term survival for primary immunodeficiencies in Europe: entering a new century, do we do better? *J Allergy Clin Immunol*. 2010;126(3):602–610.

Haddad E, Logan BR, Griffith LM, et al. SCID genotype and 6-month posttransplant CD4 count predict survival and immune recovery. *Blood*. 2018;132(17):1737–1749.

Kohn LA, Kohn DB. Gene therapies for primary immune deficiencies. *Front Immunol*. 2021;12:648951.

Kwan A, Abraham RS, Currier R, et al. Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. *JAMA*. 2014;312(7):729–738.

Locatelli F, Jordan MB, Allen C, et al. Emapalumab in children with primary hemophagocytic lymphohistiocytosis. *N Engl J Med*. 2020;382(19):1811–1822.

Lu HY, Bauman BM, Arjunaraja S, et al. The CBM-opathies—a rapidly expanding spectrum of human inborn errors of immunity caused by mutations in the CARD11-BCL10-MALT1 complex. *Front Immunol*. 2018;9:2078.

Markert ML, Gupton SE, McCarthy EA. Experience with cultured thymus tissue in 105 children. *J Allergy Clin Immunol*. 2021;149(2):747–757.

Oliveira JB, Bleesing JJ, Dianzani U, et al. Revised diagnostic criteria and classification for the autoimmune lymphoproliferative syndrome (ALPS): report from the 2009 NIH International Workshop. *Blood*. 2010;116(14):e35–e40.

Pai SY. Treatment of primary immunodeficiency with allogeneic transplant and gene therapy. *Hematology*. 2019;2019(1):457–465.

Ramirez NJ, Posadas-Cantera S, Caballero-Oteyza A, et al. There is no gene for CVID—novel monogenetic causes for primary antibody deficiency. *Curr Opin Immunol*. 2021;72:176–185.

Risma KA, Marsh RA. Hemophagocytic lymphohistiocytosis: clinical presentations and diagnosis. *J Allergy Clin Immunol Pract*. 2019;7(3):824–832.

Shouval DS, Ouahed J, Biswas A, et al. Interleukin 10 receptor signaling master regulator of intestinal mucosal homeostasis in mice and humans. *Adv Immunol*. 2014;122:177–210.

Sullivan KE. Chromosome 22q11.2 deletion syndrome and DiGeorge syndrome. *Immunol Rev*. 2019;287(1):186–201.

Tangye SG, Al-Herz W, Bousfiha A, et al. Human inborn errors of immunity: 2019 update on the classification from the international union of immunological societies expert committee. *J Clin Immunol*. 2020;40(1):24–64.

Tangye SG, Al-Herz W, Bousfiha A, et al. The ever-increasing array of novel inborn errors of immunity: an interim update by the IUIS committee. *J Clin Immunol*. 2021;41(3):666–679.

van der Burg M, Mahlaoui N, Gaspar HB, Pai SY. Universal newborn screening for severe combined immunodeficiency (SCID). *Front Pediatr*. 2019;7:373.

Yakaboski E, Fuleihan RL, Sullivan KE, et al. Lymphoproliferative disease in CVID: a report of types and frequencies from a US patient registry. *J Clin Immunol*. 2020;40(3):524–530.

REFERENCES

1. Tangye SG, Al-Herz W, Bousfiha A, et al. Human inborn errors of immunity: 2019 update on the classification from the international union of immunological societies expert committee. *J Clin Immunol*. 2020;40(1):24–64.
2. Tangye SG, Al-Herz W, Bousfiha A, et al. The ever-increasing array of novel inborn errors of immunity: an interim update by the IUIS committee. *J Clin Immunol*. 2021;41(3):666–679.
3. Sullivan KE. Chromosome 22q11.2 deletion syndrome and DiGeorge syndrome. *Immunol Rev*. 2019;287(1):186–201.
4. Davies EG, Cheung M, Gilmour K, et al. Thymus transplantation for complete DiGeorge syndrome: European experience. *J Allergy Clin Immunol*. 2017;140(6):1660–1670. e1616.
5. Markert ML, Gupton SE, McCarthy EA. Experience with cultured thymus tissue in 105 children. *J Allergy Clin Immunol*. 2021;149(2):747–757.
6. Frank J, Pignata C, Panteleyev AA, et al. Exposing the human nude phenotype. *Nature*. 1999;398(6727):473–474.
7. Vissers LELM, Ravenswaaij CMAv, Admiraal R, et al. Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. *Nat Gen*. 2004;36(9):955–957.
8. Lalani SR, Safiullah AM, Molinari LM, Fernbach SD, Martin DM, Belmont JW. SEMA3E mutation in a patient with CHARGE syndrome. *J Med Genet*. 2004;41(7):e94.
9. Yamazaki Y, Urrutia R, Franco LM, et al. PAX1 is essential for development and function of the human thymus. *Sci Immunol*. 2020;5(44):eaax1036.
10. Noguchi M, Yi H, Rosenblatt HM, et al. Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell*. 1993;73(1):147–157.
11. Puck JM, Deschênes SM, Porter JC, et al. The interleukin-2 receptor gamma chain maps to Xq13.1 and is mutated in X-linked severe combined immunodeficiency, SCIDX1. *Hum Mol Genet*. 1993;2(8):1099–1104.
12. Miggelbrink AM, Logan BR, Buckley RH, et al. B-cell differentiation and IL-21 response in IL2RG/JAK3 SCID patients after hematopoietic stem cell transplantation. *Blood*. 2018;131(26):2967–2977.
13. Giblett E, Anderson J, Cohen F, Pollara B, Meuwissen H. Adenosine-deaminase deficiency in two patients with severely impaired cellular immunity. *Lancet*. 1972;300(7786):1067–1069.
14. Bradford KL, Moretti FA, Carbonaro-Sarracino DA, Gaspar HB, Kohn DB. Adenosine deaminase (ADA)-deficient severe combined immune deficiency (SCID): molecular pathogenesis and clinical manifestations. *J Clin Immunol*. 2017;37(7):626–637.
15. Lagresle-Peyrou C, Six EM, Picard C, et al. Human adenylate kinase 2 deficiency causes a profound hematopoietic defect associated with sensorineural deafness. *Nat Genet*. 2009;41(1):106–111.
16. Pannicke U, Hönig M, Hess I, et al. Reticular dysgenesis (aleukocytosis) is caused by mutations in the gene encoding mitochondrial adenylate kinase 2. *Nat Genet*. 2009;41(1):101–105.
17. Schwarz K, Gauss GH, Ludwig L, et al. RAG mutations in human B cell-negative SCID. *Science*. 1996;274(5284):97–99.
18. Moshous D, Callebaut I, Chasseval Rd, et al. Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency. *Cell*. 2001;105(2):177–186.
19. O'Driscoll M, Cerosaletti KM, Girard P-M, et al. DNA ligase IV mutations identified in patients exhibiting developmental delay and immunodeficiency. *Mol Cell*. 2001;8(6):1175–1185.
20. van der Burg M, Ijspeert H, Verkaik NS, et al. A DNA-PKcs mutation in a radiosensitive T-B-SCID patient inhibits Artemis activation and nonhomologous end-joining. *J Clin Invest*. 2009;119(1):91–98.
21. Buck D, Malivert L, Chasseval Rd, et al. Cernunnos, a novel nonhomologous end-joining factor, is mutated in human immunodeficiency with microcephaly. *Cell*. 2006;124(2):287–299.
22. Dadi HK, Simon AJ, Roifman CM. Effect of CD3δ Deficiency on maturation of α/β and γ/δ T-cell lineages in severe combined immunodeficiency. *N Engl J Med*. 2003;349(19):1821–1828.
23. Soudais C, Villartay J-Pd, Deist FL, Fischer A, Lisowska-Grospierre B. Independent mutations of the human CD3–ε gene resulting in a T cell receptor/CD3 complex immunodeficiency. *Nat Genet*. 1993;3(1):77–81.
24. Rieux-Laucat F, Hivroz C, Lim A, et al. Inherited and Somatic CD3 ζ mutations in a patient with T-cell deficiency. *N Engl J Med*. 2006;354(18):1913–1921.
25. Arnaiz-Villena A, Timon M, Corell A, Perez-Aciego P, Martin-Villa JM, Regueiro JR. Primary immunodeficiency caused by mutations in the gene encoding the CD3-γ Subunit of the T-lymphocyte receptor. *N Engl J Med*. 1992;327(8):529–533.
26. Kung C, Pingel JT, Heikinheimo M, et al. Mutations in the tyrosine phosphatase CD45 gene in a child with severe combined immunodeficiency disease. *Nat Med*. 2000;6(3):343–345.
27. Müller SM, Ege M, Pottharst A, Schulz AS, Schwarz K, Friedrich W. Transplacentally acquired maternal T lymphocytes in severe combined immunodeficiency: a study of 121 patients. *Blood*. 2001;98(6):1847–1851.
28. Villa A, Santagata S, Bozzi F, et al. Partial V(D)J recombination activity leads to Omenn syndrome. *Cell*. 1998;93(5):885–896.
29. van der Burg M, Mahlaoui N, Gaspar HB, Pai SY. Universal newborn screening for severe combined immunodeficiency (SCID). *Front Pediatrics*. 2019;7:373.
30. Kwan A, Abraham RS, Currier R, et al. Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. *JAMA*. 2014;312(7):729–738.
31. Pai S-Y. Treatment of primary immunodeficiency with allogeneic transplant and gene therapy. *Hematology*. 2019;2019(1):457–465.
32. Gatti RA, Meuwissen HJ, Allen HD, Hong R, Good RA. Immunological reconstitution of sex-linked lymphopenic immunological deficiency. *Lancet*. 1968;2(7583):1366–1369.
33. Buckley RH, Schiff SE, Schiff RI, et al. Hematopoietic stem-cell transplantation for the treatment of severe combined immunodeficiency. *N Engl J Med*. 1999;340(7):508–516.
34. Czechowicz A, Kraft D, Weissman IL, Bhattacharya D. Efficient transplantation via antibody-based clearance of hematopoietic stem cell niches. *Science*. 2007;318(5854):1296–1299.
35. Gennery AR, Slatter MA, Grandin L, et al. Transplantation of hematopoietic stem cells and long-term survival for primary immunodeficiencies in Europe: entering a new century, do we do better? *J Allergy Clin Immunol*. 2010;126(3):602–610.
36. Haddad E, Logan BR, Griffith LM, et al. SCID genotype and 6-month posttransplant CD4 count predict survival and immune recovery. *Blood*. 2018;132(17):1737–1749.
37. Heimall J, Logan BR, Cowan MJ, et al. Immune reconstitution and survival of 100 SCID patients post hematopoietic cell transplant: a PIDTC natural history study. *Blood*. 2017
38. Pai S-Y, Logan BR, Griffith LM, et al. Transplantation outcomes for severe combined immunodeficiency, 2000-2009. *N Engl J Med*. 2014;371(5):434–446.
39. Neven B, Leroy S, Decaluwe H, et al. Long-term outcome after haematopoietic stem cell transplantation of a single-centre cohort of 90 patients with severe combined immunodeficiency: long-term outcome of HSCT in SCID. *Blood*. 2009;113(17):4114–4124.
40. Kohn LA, Kohn DB. Gene therapies for primary immune deficiencies. *Front Immunol*. 2021;12:648951.
41. Aiuti A, Cattaneo F, Galimberti S, et al. Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *N Engl J Med*. 2009;360(5):447–458.
42. Candotti F, Shaw KL, Muul L, et al. Gene therapy for adenosine deaminase-deficient severe combined immune deficiency: clinical comparison of retroviral vectors and treatment plans. *Blood*. 2012;120(18):3635–3646.
43. Gaspar HB, Bjorkegren E, Parsley K, et al. Successful reconstitution of immunity in ADA-SCID by stem cell gene therapy following cessation of PEG-ADA and use of mild preconditioning. *Mol Ther*. 2006;14(4):505–513.
44. Kohn DB, Booth C, Shaw KL, et al. Autologous ex vivo lentiviral gene therapy for adenosine deaminase deficiency. *N Engl J Med*. 2021;384(21):2002–2013.
45. EMA. Strimvelis. https://www.ema.europa.eu/medicines/human/EPAR/strimvelis. Published 2016. Updated 5/9/2018. Accessed November 4, 2018.
46. Gaspar HB, Parsley KL, Howe S, et al. Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet*. 2004;364(9452):2181–2187.
47. Hacein-Bey-Abina S, Le Deist F, Carlier F, et al. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N Engl J Med*. 2002;346(16):1185–1193.
48. Gaspar HB, Cooray S, Gilmour KC, et al. Long-term persistence of a polyclonal T cell repertoire after gene therapy for X-linked severe combined immunodeficiency. *Sci Transl Med*. 2011;3(97):97ra79.
49. Hacein-Bey-Abina S, Hauer J, Lim A, et al. Efficacy of gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med*. 2010;363(4):355–364.
50. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science*. 2003;302(5644):415–419.
51. Howe SJ, Mansour MR, Schwarzwaelder K, et al. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest*. 2008;118(9):3143–3150.

52. De Ravin SS, Wu X, Moir S, et al. Lentiviral hematopoietic stem cell gene therapy for X-linked severe combined immunodeficiency. *Sci Transl Med*. 2016;8(335):335ra357.
53. Mamcarz E, Zhou S, Lockey T, et al. Lentiviral gene therapy combined with low-dose busulfan in infants with SCID-X1. *N Engl J Med*. 2019;380(16):1525–1534.
54. Arpaia E, Shahar M, Dadi H, Cohen A, Rolfman CM. Defective T cell receptor signaling and CD8+ thymic selection in humans lacking Zap-70 kinase. *Cell*. 1994;76(5):947–958.
55. Elder ME, Lin D, Clever J, et al. Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase. *Science*. 1994;264(5165):1596–1599.
56. Crequer A, Troeger A, Patin E, et al. Human RHOH deficiency causes T cell defects and susceptibility to EV-HPV infections. *J Clin Invest*. 2012;122(9):3239–3247.
57. Huck K, Feyen O, Niehues T, et al. Girls homozygous for an IL-2–inducible T cell kinase mutation that leads to protein deficiency develop fatal EBV-associated lymphoproliferation. *J Clin Invest*. 2009;119(5):1350–1358.
58. Crequer A, Picard C, Patin E, et al. Inherited MST1 deficiency underlies susceptibility to EV-HPV infections. *PLoS One*. 2012;7(8):e44010.
59. Shiow LR, Roadcap DW, Paris K, et al. The actin regulator coronin 1A is mutant in a thymic egress–deficient mouse strain and in a patient with severe combined immunodeficiency. *Nat Immunol*. 2008;9(11):1307–1315.
60. Hanna S, Etzioni A. MHC class I and II deficiencies. *J Allergy Clin Immunol*. 2014;134(2):269–275.
61. Korthäuer U, Graf D, Mages HW, et al. Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. *Nature*. 1993;361(6412):539–541.
62. de la Morena MT, Leonard D, Torgerson TR, et al. Long-term outcomes of 176 patients with X-linked hyper-IgM syndrome treated with or without hematopoietic cell transplantation. *J Allergy Clin Immunol*. 2017;139(4):1282–1292.
63. Ferrua F, Galimberti S, Courteille V, et al. Hematopoietic stem cell transplantation for CD40 ligand deficiency: results from an EBMT/ESID-IEWP-SCETIDE-PIDTC study. *J Allergy Clin Immunol*. 2019;143(6):2238–2253.
64. Ferrari S, Giliani S, Insalaco A, et al. Mutations of CD40 gene cause an autosomal recessive form of immunodeficiency with hyper IgM. *Proc Natl Acad Sci*. 2001;98(22):12614–12619.
65. Zonana J, Elder ME, Schneider LC, et al. A novel X-linked disorder of immune deficiency and hypohidrotic ectodermal dysplasia is allelic to incontinentia pigmenti and due to mutations in IKK-gamma (NEMO). *Am J Hum Genet*. 2000;67(6):1555–1562.
66. Miot C, Imai K, Imai C, et al. Hematopoietic stem cell transplantation in 29 patients hemizygous for hypomorphic IKBKG/NEMO mutations. *Blood*. 2017;130(12):1456–1467.
67. Pannicke U, Baumann B, Fuchs S, et al. Deficiency of innate and acquired immunity caused by an IKBKB mutation. *N Engl J Med*. 2013;369(26):2504–2514.
68. Schlechter N, Glanzmann B, Hoal EG, et al. Exome sequencing identifies a novel MAP3K14 mutation in recessive atypical combined immunodeficiency. *Front Immunol*. 2017;8:1624.
69. Willmann KL, Klaver S, Doğu F, et al. Biallelic loss-of-function mutation in NIK causes a primary immunodeficiency with multifaceted aberrant lymphoid immunity. *Nat Commun*. 2014;5(1):5360.
70. Badran YR, Dedeoglu F, Castillo JML, et al. Human RELA haploinsufficiency results in autosomal-dominant chronic mucocutaneous ulceration. *J Exp Med*. 2017;214(7):1937–1947.
71. Lu HY, Bauman BM, Arjunaraja S, et al. The CBM-opathies—a rapidly expanding spectrum of human inborn errors of immunity caused by mutations in the CARD11-BCL10-MALT1 complex. *Front Immunol*. 2018;9:2078.
72. Łyszkiewicz M, Ziętara N, Frey L, et al. Human FCHO1 deficiency reveals role for clathrin-mediated endocytosis in development and function of T cells. *Nat Commun*. 2020;11(1):1031.
73. Calzoni E, Platt CD, Keles S, et al. F-BAR domain only protein 1 (FCHO1) deficiency is a novel cause of combined immune deficiency in humans. *J Allergy Clin Immunol*. 2019;143(6):2317–2321. e2312.
74. Jabara HH, Boyden SE, Chou J, et al. A missense mutation in TFRC, encoding transferrin receptor 1, causes combined immunodeficiency. *Nat Genet*. 2016;48(1):74–78.
75. Lagresle-Peyrou C, Luce S, Ouchani F, et al. X-linked primary immunodeficiency associated with hemizygous mutations in the moesin (MSN) gene. *J Allergy Clin Immunol*. 2016;138(6):1681–1689. e1688.
76. Dobbs K, Dominguez Conde C, Zhang SY, et al. Inherited DOCK2 deficiency in patients with early-onset invasive infections. *N Engl J Med*. 2015;372(25):2409–2422.
77. Engelhardt KR, McGhee S, Winkler S, et al. Large deletions and point mutations involving the dedicator of cytokinesis 8 (DOCK8) in the autosomal-recessive form of hyper-IgE syndrome. *J Allergy Clin Immunol*. 2009;124(6):1289–1302. e1284.
78. Zhang Q, Davis J, Lamborn I, et al. Combined immunodeficiency associated with DOCK8 mutations. *N Engl J Med*. 2009;361(21):2046–2055.
79. Albert MH, Freeman AF. Wiskott-Aldrich syndrome (WAS) and dedicator of cytokinesis 8- (DOCK8) deficiency. *Front Pediat*. 2019;7:451.
80. Hsu AP, Donkó A, Arrington ME, et al. Dominant activating RAC2 mutation with lymphopenia, immunodeficiency, and cytoskeletal defects. *Blood*. 2019;133(18):1977–1988.
81. Lagresle-Peyrou C, Olichon A, Sadek H, et al. A gain-of-function RAC2 mutation is associated with bone-marrow hypoplasia and an autosomal dominant form of severe combined immunodeficiency. *Haematologica*. 2020;106(2):230250 haematol.2019.
82. Smits BM, Lelieveld PHC, Ververs FA, et al. A dominant activating RAC2 variant associated with immunodeficiency and pulmonary disease. *Clin Immunology*. 2020;212(18):108248.
83. Derry JM, Ochs HD, Francke U. Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. *Cell*. 1994;78(4):635–644.
84. Imai K, Morio T, Zhu Y, et al. Clinical course of patients with WASP gene mutations. *Blood*. 2004;103(2):456–464.
85. Devriendt K, Kim AS, Mathijs G, et al. Constitutively activating mutation in WASP causes X-linked severe congenital neutropenia. *Nat Genet*. 2001;27(3):313–317.
86. Ancliff PJ, Blundell MP, Cory GO, et al. Two novel activating mutations in the Wiskott-Aldrich syndrome protein result in congenital neutropenia. *Blood*. 2006;108(7):2182–2189.
87. Beel K, Cotter MM, Blatny J, et al. A large kindred with X-linked neutropenia with an I294T mutation of the Wiskott-Aldrich syndrome gene. *Br J Haematology*. 2009;144(1):120–126.
88. Dupuis-Girod S, Medioni J, Haddad E, et al. Autoimmunity in Wiskott-Aldrich syndrome: risk factors, clinical features, and outcome in a single-center cohort of 55 patients. *Pediatrics*. 2003;111(5 Pt 1):e622–e627.
89. Burroughs LM, Petrovic A, Brazauskas R, et al. Excellent outcomes following hematopoietic cell transplantation for Wiskott-Aldrich syndrome: a PIDTC report. *Blood*. 2020;135(23):2094–2105.
90. Moratto D, Giliani S, Bonfim C, et al. Long-term outcome and lineage-specific chimerism in 194 patients with Wiskott-Aldrich syndrome treated by hematopoietic cell transplantation in the period 1980-2009: an international collaborative study. *Blood*. 2011;118(6):1675–1684.
91. Boztug K, Schmidt M, Schwarzer A, et al. Stem-cell gene therapy for the Wiskott–Aldrich syndrome. *N Engl J Med*. 2010;363(20):1918–1927.
92. Braun CJ, Boztug K, Paruzynski A, et al. Gene therapy for Wiskott-Aldrich syndrome–long-term efficacy and genotoxicity. *Sci Transl Med*. 2014;6(227):227ra233.
93. Aiuti A, Biasco L, Scaramuzza S, et al. Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. *Science*. 2013;341(6148):1233151.
94. Ferrua F, Cicalese MP, Galimberti S, et al. Lentiviral haemopoietic stem/progenitor cell gene therapy for treatment of Wiskott-Aldrich syndrome: interim results of a non-randomised, open-label, phase 1/2 clinical study. *Lancet Haematol*. 2019;6(5):e239–e253.
95. Hacein-Bey Abina S, Gaspar HB, Blondeau J, et al. Outcomes following gene therapy in patients with severe Wiskott-Aldrich syndrome. *JAMA*. 2015;313(15):1550–1563.
96. Vakkilainen S, Taskinen M, Mäkitie O. Immunodeficiency in cartilage-hair hypoplasia: pathogenesis, clinical course and management. *Scand J Immunology*. 2020;92(4):e12913.
97. Boerkoel CF, Takashima H, John J, et al. Mutant chromatin remodeling protein SMARCAL1 causes Schimke immuno-osseous dysplasia. *Nat Genet*. 2002;30(2):215–220.
98. Volpi S, Yamazaki Y, Brauer PM, et al. EXTL3 mutations cause skeletal dysplasia, immune deficiency, and developmental delay. *J Exp Med*. 2017;214(3):623–637.
99. Grimbacher B, Hutloff A, Schlesier M, et al. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. *Nat immunology*. 2003;4(3):261–268.
100. Boutboul D, Kuehn HS, Wyngaert ZVd, et al. Dominant-negative IKZF1 mutations cause a T, B, and myeloid cell combined immunodeficiency. *J Clin Invest*. 2018;128(7):3071–3087.
101. Roscioli T, Cliffe ST, Bloch DB, et al. Mutations in the gene encoding the PML nuclear body protein Sp110 are associated with immunodeficiency and hepatic veno-occlusive disease. *Nat Genet*. 2006;38(6):620–622.
102. Chen R, Giliani S, Lanzi G, et al. Whole-exome sequencing identifies tetratricopeptide repeat domain 7A (TTC7A) mutations for combined

immunodeficiency with intestinal atresias. *J Allergy Clin Immunol*. 2013;132(3):656–664. e617.

103. Samuels ME, Majewski J, Alirezaie N, et al. Exome sequencing identifies mutations in the gene TTC7A in French-Canadian cases with hereditary multiple intestinal atresia. *J Med Genet*. 2013;50(5):324.
104. Kammermeier J, Lucchini G, Pai S-Y, et al. Stem cell transplantation for tetratricopeptide repeat domain 7A deficiency: long-term follow-up. *Blood*. 2016;128(9):1306–1308.
105. Martin E, Palmic N, Sanquer S, et al. CTP synthase 1 deficiency in humans reveals its central role in lymphocyte proliferation. *Nature*. 2014;510(7504):288–292.
106. Salzer E, Daschkey S, Choo S, et al. Combined immunodeficiency with life-threatening EBV-associated lymphoproliferative disorder in patients lacking functional CD27. *Haematologica*. 2013;98(3):473–478.
107. Abolhassani H, Edwards ESJ, Ikinciogullari A, et al. Combined immunodeficiency and Epstein-Barr virus–induced B cell malignancy in humans with inherited CD70 deficiency. *J Exp Med*. 2017;214(1):91–106.
108. Izawa K, Martin E, Soudais C, et al. Inherited CD70 deficiency in humans reveals a critical role for the CD70–CD27 pathway in immunity to Epstein-Barr virus infection. *J Exp Med*. 2017;214(1):73–89.
109. Li F-Y, Chaigne-Delalande B, Kanellopoulou C, et al. Second messenger role for Mg2+ revealed by human T-cell immunodeficiency. *Nature*. 2011;475(7357):471–476.
110. Dimitrova D, Rose JJ, Uzel G, et al. Successful bone marrow transplantation for XMEN: hemorrhagic risk uncovered. *J Clin Immunol*. 2019;39(1):1–3.
111. Somekh I, Thian M, Medgyesi D, et al. CD137 deficiency causes immune dysregulation with predisposition to lymphomagenesis. *Blood*. 2019;134(18):1510–1516.
112. Cagdas D, Mayr D, Baris S, et al. Genomic spectrum and phenotypic heterogeneity of human IL-21 receptor deficiency. *J Clin Immunol*. 2021;41(6):1272–1290.
113. Byun M, Ma CS, Akçay A, et al. Inherited human OX40 deficiency underlying classic Kaposi sarcoma of childhood. *J Exp Med*. 2013;210(9): 1743–1759.
114. Sassi A, Lazaroski S, Wu G, et al. Hypomorphic homozygous mutations in phosphoglucomutase 3 (PGM3) impair immunity and increase serum IgE levels. *J Allergy Clin Immunol*. 2014;133(5):1410–1419. e1413.
115. Zhang Y, Yu X, Ichikawa M, et al. Autosomal recessive phosphoglucomutase 3 (PGM3) mutations link glycosylation defects to atopy, immune deficiency, autoimmunity, and neurocognitive impairment. *J Allergy Clin Immunol*. 2014;133(5):1400–1409. e1405.
116. Ferre EMN, Rose SR, Rosenzweig SD, et al. Redefined clinical features and diagnostic criteria in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. *JCI Insight*. 2016;1(13):e88782.
117. Kisand K, Peterson P. Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy. *J Clin Immunol*. 2015;35(5):463–478.
118. Nagamine K, Peterson P, Scott HS, et al. Positional cloning of the APECED gene. *Nat Genet*. 1997;17(4):393–398.
119. Aaltonen J, Björses P, Perheentupa J, et al. An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. *Nat Genet*. 1997;17(4):399–403.
120. Bacchetta R, Barzaghi F, Roncarolo MG. From IPEX syndrome to FOXP3 mutation: a lesson on immune dysregulation. *Ann N Y Acad Sci*. 2018;1417(1):5–22.
121. Bennett CL, Christie J, Ramsdell F, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet*. 2001;27(1):20–21.
122. Barzaghi F, Amaya Hernandez LC, Neven B, et al. Long-term follow-up of IPEX syndrome patients after different therapeutic strategies: An international multicenter retrospective study. *J Allergy Clin Immunol*. 2018;141(3):1036–1049. e1035.
123. Sharfe N, Dadi HK, Shahar M, Roifman CM. Human immune disorder arising from mutation of the α chain of the interleukin-2 receptor. *Proc Natl Acad Sci*. 1997;94(7):3168–3171.
124. Zhang Z, Gothe F, Pennamen P, et al. Human interleukin-2 receptor β mutations associated with defects in immunity and peripheral tolerance. *J Exp Med*. 2019;216(6):1311–1327.
125. Nadeau K, Hwa V, Rosenfeld RG. STAT5b deficiency: an unsuspected cause of growth failure, immunodeficiency, and severe pulmonary disease. *J Pediatrics*. 2011;158(5):701–708.
126. Alangari A, Alsultan A, Adly N, et al. LPS-responsive beige-like anchor (LRBA) gene mutation in a family with inflammatory bowel disease and combined immunodeficiency. *J Allergy Clin Immunol*. 2012;130(2):481–488. e482.
127. Lopez-Herrera G, Tampella G, Pan-Hammarström Q, et al. Deleterious mutations in LRBA are associated with a syndrome of immune deficiency and autoimmunity. *Am J Hum Genet*. 2012;90(6):986–1001.
128. Burns SO, Zenner HL, Plagnol V, et al. LRBA gene deletion in a patient presenting with autoimmunity without hypogammaglobulinemia. *J Allergy Clin Immunol*. 2012;130(6):1428–1432.
129. Lo B, Zhang K, Lu W, et al. Patients with LRBA deficiency show CTLA4 loss and immune dysregulation responsive to abatacept therapy. *Science*. 2015;349(6246):436–440.
130. Serwas NK, Hoeger B, Ardy RC, et al. Human DEF6 deficiency underlies an immunodeficiency syndrome with systemic autoimmunity and aberrant CTLA-4 homeostasis. *Nat Commun*. 2019;10(1):3106.
131. Feske S, Gwack Y, Prakriya M, et al. A mutation in orai1 causes immune deficiency by abrogating CRAC channel function. *Nature*. 2006;441(7090):179–185.
132. Picard C, McCarl C-A, Papolos A, et al. STIM1 mutation associated with a syndrome of immunodeficiency and autoimmunity. *N Engl J Med*. 2009;360(19):1971–1980.
133. Oliveira JB, Bleesing JJ, Dianzani U, et al. Revised diagnostic criteria and classification for the autoimmune lymphoproliferative syndrome (ALPS): report from the 2009 NIH International Workshop. *Blood*. 2010;116(14):e35–e40.
134. Fisher GH, Rosenberg FJ, Straus SE, et al. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell*. 1995;81(6):935–946.
135. Rieux-Laucat F, Deist FL, Hivroz C, et al. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science*. 1995;268(5215):1347–1349.
136. Holzelova E, Vonarbourg C, Stolzenberg M-C, et al. Autoimmune lymphoproliferative syndrome with somatic Fas mutations. *N Engl J Med*. 2004;351(14):1409–1418.
137. Rieux-Laucat F, Magérus-Chatinet A, Neven B. The autoimmune lymphoproliferative syndrome with defective FAS or FAS-ligand functions. *J Clin Immunol*. 2018;38(5):558–568.
138. Salzer E, Santos-Valente E, Klaver S, et al. B-cell deficiency and severe autoimmunity caused by deficiency of protein kinase Cδ. *Blood*. 2013;121(16):3112–3116.
139. Belot A, Kasher PR, Trotter EW, et al. Protein kinase Cδ deficiency causes mendelian systemic lupus erythematosus with b cell-defective apoptosis and hyperproliferation. *Arthritis Rheum*. 2013;65(8):2161–2171.
140. Price S, Shaw PA, Seitz A, et al. Natural history of autoimmune lymphoproliferative syndrome associated with FAS gene mutations. *Blood*. 2014;123(13):1989–1999.
141. Liu L, Okada S, Kong X-F, et al. Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis Human STAT1 activating mutations impair IL-17 immunity. *J Exp Med*. 2011;208(8):1635–1648.
142. Flanagan SE, Haapaniemi E, Russell MA, et al. Activating germline mutations in STAT3 cause early-onset multi-organ autoimmune disease. *Nat Genet*. 2014;46(8):812–814.
143. Haapaniemi EM, Kaustio M, Rajala HLM, et al. Autoimmunity, hypogammaglobulinemia, lymphoproliferation, and mycobacterial disease in patients with activating mutations in STAT3. *Blood*. 2015;125(4):639–648.
144. Milner JD, Vogel TP, Forbes L, et al. Early-onset lymphoproliferation and autoimmunity caused by germline STAT3 gain-of-function mutations. *Blood*. 2015;125(4):591–599.
145. Bel KLD, Ragotte RJ, Saferali A, et al. JAK1 gain-of-function causes an autosomal dominant immune dysregulatory and hypereosinophilic syndrome. *J Allergy Clin Immunol*. 2017;139(6):2016–2020. e2015.
146. Lohr NJ, Molleston JP, Strauss KA, et al. Human ITCH E3 ubiquitin ligase deficiency causes syndromic multisystem autoimmune disease. *Am J Hum Genet*. 2010;86(3):447–453.
147. Shouval DS, Ouahed J, Biswas A, et al. Interleukin 10 receptor signaling master regulator of intestinal mucosal homeostasis in mice and humans. *Adv Immunol*. 2014;122:177–210.
148. Stepensky P, Rensing-Ehl A, Gather R, et al. Early-onset Evans syndrome, immunodeficiency, and premature immunosenescence associated with tripeptidyl-peptidase II deficiency. *Blood*. 2015;125(5):753–761.
149. Risma KA, Marsh RA. Hemophagocytic lymphohistiocytosis: clinical presentations and diagnosis. *J Allergy Clin Immunol Pract*. 2019;7(3): 824–832.
150. Vavassori S, Chou J, Faletti LE, et al. Multisystem inflammation and susceptibility to viral infections in human ZNFX1 deficiency. *J Allergy Clin Immunol*. 2021;148(2):381–393.
151. Voyer TL, Neehus A-L, Yang R, et al. Inherited deficiency of stress granule ZNFX1 in patients with monocytosis and mycobacterial disease. *Proc Natl Acad Sci USA*. 2021;118(15). e2102804118.
152. Locatelli F, Jordan MB, Allen C, et al. Emapalumab in children with primary hemophagocytic lymphohistiocytosis. *N Engl J Med*. 2020;382(19):1811–1822.

153. Marsh RA, Hebert K, Kim S, et al. A comparison of hematopoietic cell transplant conditioning regimens for hemophagocytic lymphohistiocytosis disorders. *J Allergy Clin Immunol*. 2021
154. Panchal N, Booth C, Cannons JL, Schwartzberg PL. X-linked lymphoproliferative disease type 1: a clinical and molecular perspective. *Front Immunol*. 2018;9:666.
155. Coffey AJ, Brooksbank RA, Brandau O, et al. Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. *Nat Genet*. 1998;20(2):129–135.
156. Nichols KE, Harkin DP, Levitz S, et al. Inactivating mutations in an SH2 domain-encoding gene in X-linked lymphoproliferative syndrome. *Proc Natl Acad Sci USA*. 1998;95(23):13765–13770.
157. Sayos J, Wu C, Morra M, et al. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature*. 1998;395(6701):462–469.
158. Rigaud S, Fondanèche M-C, Lambert N, et al. XIAP deficiency in humans causes an X-linked lymphoproliferative syndrome. *Nature*. 2006;444(7115):110–114.
159. Tsukada S, Saffran DC, Rawlings DJ, et al. Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell*. 1993;72(2):279–290.
160. Vetrie D, Vořechovský I, Sideras P, et al. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. *Nature*. 1993;361(6409):226–233.
161. Conley ME, Dobbs AK, Farmer DM, et al. Primary B cell immunodeficiencies: comparisons and contrasts. *Ann Rev Immunol*. 2009;27(1):199–227.
162. Demirdag YY, Gupta S. Update on infections in primary antibody deficiencies. *Front Immunol*. 2021;12:634181.
163. Ramirez NJ, Posadas-Cantera S, Caballero-Oteyza A, Camacho-Ordonez N, Grimbacher B. There is no gene for CVID—novel monogenetic causes for primary antibody deficiency. *Curr Opin Immunol*. 2021;72:176–185.
164. Castigli E, Wilson SA, Garibyan L, et al. TACI is mutant in common variable immunodeficiency and IgA deficiency. *Nat Genet*. 2005;37(8):829–834.
165. Salzer U, Chapel HM, Webster ADB, et al. Mutations in TNFRSF13B encoding TACI are associated with common variable immunodeficiency in humans. *Nat Genet*. 2005;37(8):820–828.
166. Ameratunga R, Allan C, Woon ST. Defining common variable immunodeficiency disorders in 2020. *Immunol Allergy Clin North Am*. 2020;40(3):403–420.
167. Chapel H, Cunningham-Rundles C. Update in understanding common variable immunodeficiency disorders (CVIDs) and the management of patients with these conditions. *Br J Haematol*. 2009;145(6):709–727.
168. Yakaboski E, Fuleihan RL, Sullivan KE, Cunningham-Rundles C, Feuille E. Lymphoproliferative disease in CVID: a report of types and frequencies from a US patient registry. *J Clin Immunol*. 2020;40(3):524–530.
169. Kracker S, Gardes P, Mazerolles F, Durandy A. Immunoglobulin class switch recombination deficiencies. *Clin Immunol*. 2010;135(2):193–203.
170. Angulo I, Vadas O, Garçon F, et al. Phosphoinositide 3-kinase δ gene mutation predisposes to respiratory infection and airway damage. *Science*. 2013;342(6160):866–871.
171. Crank MC, Grossman JK, Moir S, et al. Mutations in PIK3CD can cause hyper IgM syndrome (HIGM) associated with increased cancer susceptibility. *J Clin Immunol*. 2014;34(3):272–276.
172. Lucas CL, Kuehn HS, Zhao F, et al. Dominant-activating germline mutations in the gene encoding the PI(3)K catalytic subunit p110 result in T cell senescence and human immunodeficiency. *Nat Immunol*. 2014;15(1):88–97.
173. Deau MC, Heurtier L, Frange P, et al. A human immunodeficiency caused by mutations in the PIK3R1 gene. *J Clin Invest*. 2014;124(9):3923–3928.
174. Lucas CL, Zhang Y, Venida A, et al. Heterozygous splice mutation in PIK3R1 causes human immunodeficiency with lymphoproliferation due to dominant activation of PI3K. *J Exp Med*. 2014;211(13):2537–2547.

CHAPTER 53

PEDIATRIC AND ADULT HISTIOCYTIC DISORDERS

Adi Zoref Lorenz, Olive S. Eckstein, Nitya Gulati, Michael B. Jordan, and Carl E. Allen

The histiocytic disorders comprise a broad grouping of hematologic and immunologic diseases united by aberrant function, differentiation, and/or proliferation of cells of the mononuclear phagocyte system. The term *histiocyte* historically referred to tissue phagocytes but now more precisely defines cells of the monocyte/macrophage lineage (Fig. 53.1). The ontogeny of these cells and their role(s) in pathogenesis continue to be studied and refined. In the 1970s, Steinman and Cohn distinguished dendritic cells (DCs) from macrophages based on specific morphologic features of DC and superior antigen-presenting capacity.[1,2] The characteristic cells seen in various histiocytic lesions can, in general, be differentiated by a variety of functional and phenotypic markers.[3,4] The current classification of the histiocytoses is based on histological comparisons to physiologic "histiocytes" (Table 53.1; Fig. 53.2).[5] Updated classification including features of differentiation, genetic lesions, and tissue involvement has been proposed (Table 53.2).[6] Although malignant disorders involving monocyte/macrophages or dendritic cells are included in this classification, this chapter focuses solely on non-malignant histiocytic disorders.

Of the dendritic cell-related histiocytoses, the most clinically common and prominent one is Langerhans cell histiocytosis (LCH). This chapter also briefly discusses related histiocytic disorders, including juvenile xanthogranuloma (JXG); Erdheim-Chester disease (ECD); and sinus histiocytosis with massive lymphadenopathy (SHML), also referred to as *Rosai-Dorfman disease* (RDD). These conditions are now recognized as myeloid neoplastic disorders characterized by recurrent mitogen-activated protein kinase (MAPK) pathway–activating mutations.

Unlike LCH and related disorders, hemophagocytic lymphohistiocytosis (HLH) is a reactive immune disorder where activated macrophages observed in tissue biopsies reflect coordinated immune dysfunction of interactive partners including activated T cells and defective natural killer (NK) cells.

Table 53.1 lists a currently accepted classification of histiocytic disorders, and Table 53.2 outlines a proposed revision. These disorders are a diverse grouping ranging from benign skin lesions to rapidly life-threatening systemic disorders. Tables 53.3 and 53.4 outline clinical features and commonly used histologic markers or pathologic features that may be used to help distinguish some of these disorders. The clinical diversity of histiocytic disorders is underscored by recent discoveries related to their pathogenesis.

LANGERHANS CELL HISTIOCYTOSIS

LCH is the most common histiocytic disorder.[7] The physiologic epidermal Langerhans cell was first described in 1868 by 21-year-old medical student Paul Langerhans, though he initially described it as a nerve cell.[8] In the early 1900s, Hand, Schüller, and Christian described the various presentations of LCH, which include lytic bone lesions, skin rash, and central diabetes insipidus (DI).[9–11] Letterer and Siwe described more severe aggressive and rapidly fatal cases with liver and spleen involvement in young children.[12,13] These disorders were later grouped under the term *histiocytosis X* by Lichtenstein based on common histologic appearance and uncertain cell of origin.[14] Nezelof subsequently theorized that histiocytosis X was caused by a proliferation of pathologic Langerhans cells (LC) due to a common feature of Birbeck granules identified by electron microscopy.[15] At present, *"Langerhans cell histiocytosis"* is typically accepted as the universal terminology replacing all previous nomenclature to refer to all varied clinical presentations of this protean disorder, though recent discoveries identify cells of origin as myeloid precursor cells rather than differentiated epidermal LC.[7] LCH can present at any time in life from the neonatal period to old age. LCH lesions may spontaneously regress or repeatedly "reactivate," contributing to long-term disabilities such as endocrinopathies or a neurodegenerative disease.

Epidemiology

The incidence of LCH has been estimated to be between 5 and 10 cases per million children, per year.[16] While the incidence of LCH in adults is thought to be lower and estimated at 1 case per million adults per year, it is likely underdiagnosed in adults due to the under-recognition of LCH in adult medicine.[17,18] Recent registry studies suggest an increased incidence in Hispanic populations and a significantly lower incidence in African Americans.[19] Further, a genome-wide association study (GWAS) trio study identified an increased risk of LCH in patients with a *SMAD6* variant that is enriched in Hispanic populations.[20] Although families with multiple cases of LCH have been reported, these events are extremely rare. LCH may rarely arise as a shared clone with other hematologic malignancies, most notably in adults with myeloid histiocytic disorders as part of myeloid neoplasias associated with clonal hematopoiesis.[21]

Pathophysiology

Over the past decade, there has been a significant evolution in the scientific understanding of the biologic mechanisms leading to LCH, driven by new insights in both normal epidermal and disease-associated LC.[7,22] Prior to these more recent discoveries, LCH was variably classified as a neoplasm, a reactive disorder, or an aberrant immune response and thought to arise from transformed or pathologically activated epidermal LC. Like epidermal LC, LCH lesion LC express CD1a and CD207 (langerin) surface markers. The frequency of LC within LCH lesions is highly variable, with a median of about 8%. The balance of this granulomatous lesion includes lymphocytes (primarily T cells), eosinophils, and macrophages.

Two studies demonstrated that pathologic LCH cells have clonal properties based on clonal X-chromosome inactivation.[23,24] Additionally, expression studies of pathologic LCH cells from lesions and epidermal Langerhans cells found in skin have demonstrated that pathologic LCH cells have relatively increased expression of immature myeloid dendritic-cell precursors and are therefore likely to derive from abnormal bone-marrow precursor cells or their differentiated ontogeny.[25] Further, several recent studies demonstrated that the expression of *langerin/CD207* is not exclusive to epidermal LC but rather that it can be induced in cells from multiple hematopoietic lineages.[22]

The discovery of recurrent *BRAF*V600E somatic mutations in LCH lesions supports a neoplastic etiology for LCH.[26] BRAFV600E is observed in ~8% of all human cancers and also in benign neoplastic conditions such as melanocytic nevi. Constitutive activation of a MAPK pathway leads to activation of the terminal extracellular signal-related

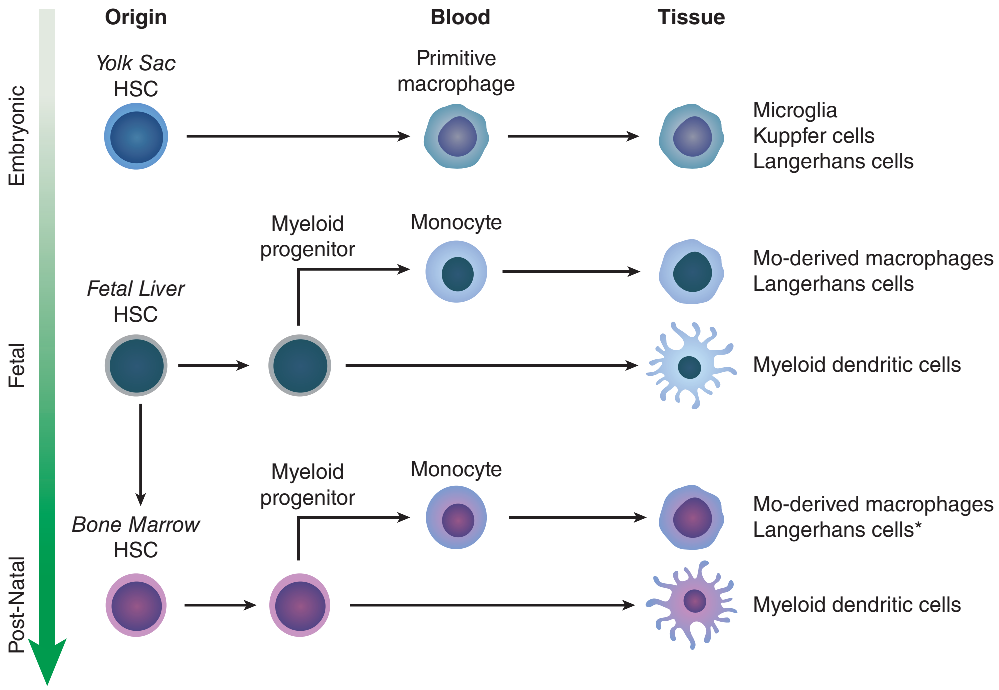


Figure 53.1 ONTOGENY OF TISSUE "HISTIOCYTES." Tissue macrophages and dendritic cells seed tissues at various stages of development. Microglia, Kupffer cells, and Langerhans cells (LCs) arise from yolk sac-derived progenitors, followed by fetal liver, and then adult hematopoiesis. *Langerhans cells may be generated from adult HSC monocyte-derived LCs following inflammation or tissue damage.

TABLE 53.1 **Historical Classification of Histiocytic Disorders**

A. Dendritic cell–related
  Langerhans cell histiocytosis
  Juvenile xanthogranuloma and related disorders including:
  - Erdheim-Chester disease
  - Solitary histiocytomas with juvenile xanthogranuloma phenotype
  - Secondary dendritic cell disorders

B. Monocyte/macrophage–related
  Hemophagocytic lymphohistiocytosis: familial and sporadic
  Secondary hemophagocytic syndromes
  - Infection-associated
  - Malignancy-associated
  - Autoimmune-associated
    Sinus histiocytosis with massive lymphadenopathy (Rosai-Dorfman disease)
    Solitary histiocytoma of macrophage phenotype

Adapted from Jaffe R. Histiocytic and dendritic cell neoplasms. In: Swerdlow SH, ed. *WHO Calssification of Tumours of Hematopoietic and Lymphoid Tissues.* Lyon: IARC Press; 2008;353–357.

kinase (ERK). Several groups have confirmed recurrent *BRAF*V600E in 50% to 64% of LCH cases as well as mutually exclusive activating mutations in MAPK pathway genes (primarily alternative BRAF mutations or mutations in *MAP2K1* [encodes MEK1]) (Fig. 53.3).[27]

Taking advantage of the *BRAF*V600E discovery in LCH lesions, subsequent studies identified *BRAF*V600E in hematopoietic stem cells from bone-marrow aspirate and myeloid precursors from peripheral blood of patients with disseminated LCH.[28] Interestingly, BRAFV600E+ peripheral blood mononuclear cells were rarely observed in patients with more limited "low risk" LCH. A pathogenic role for BRAFV600E in myeloid precursors is further supported by recapitulation of an LCH-like phenotype in mice in which *BRAF*V600E expression was enforced in CD11c+ cells. The "Misguided Myeloid Differentiation" model suggests that the stage of myeloid precursor in which this mutation develops is related to the differentiation potential of the pathologic cell that determines the extent and severity of disease.[7] These observations support the reclassification of LCH as an inflammatory myeloid neoplastic disorder.

Clinical Presentations

The clinical manifestations of LCH can be highly variable, with bony lesions (~80% of cases) as the most common site of disease (Fig. 53.4).[29] Skin involvement is highly pleiotropic and can mimic many common infant skin rashes. Soft tissue swelling, often in proximity to bony lesions, external ear drainage, enlargement of lymph nodes and thymus, and gum hypertrophy with premature eruption of baby teeth are also typical manifestations. More serious systemic involvement occurs when there is hepatosplenomegaly, liver dysfunction, and hematopoietic failure, typical of the widely disseminated form of LCH seen in young infants, and is associated with a high mortality rate.

Bony Involvement

Solitary or multifocal bone lesions are found predominantly in older children and young adults, usually within the first three decades of life. Such lytic bone lesions have commonly been referred to as *eosinophilic granuloma* because of their pathologic appearance, though we prefer consistent nomenclature of *isolated*, *multifocal*, or *multisystem* LCH of bone. The incidence of bone lesions peaks between 5 and 10 years of age. Solitary or multifocal LCH bone lesions (with or without the involvement of other organ systems) represent approximately 60% to 80% of all instances of LCH. These bone lesions often cause abnormal gait or inability to bear weight and may have tender swelling caused by soft tissue infiltrates overlying the bone lesions.

The most frequent sites of skeletal involvement include the flat bones of the skull, ribs, pelvis, and scapula (see Fig. 53.3H). There may be extensive involvement of the skull, with multiple irregularly shaped, lucent lesions giving rise to the so-called *geographic skull*. Involvement of the vertebral bodies may lead to collapse (*vertebra plana*) as the principal or only presenting manifestation. In such cases, the diagnosis may be problematic, although biopsy is typically not

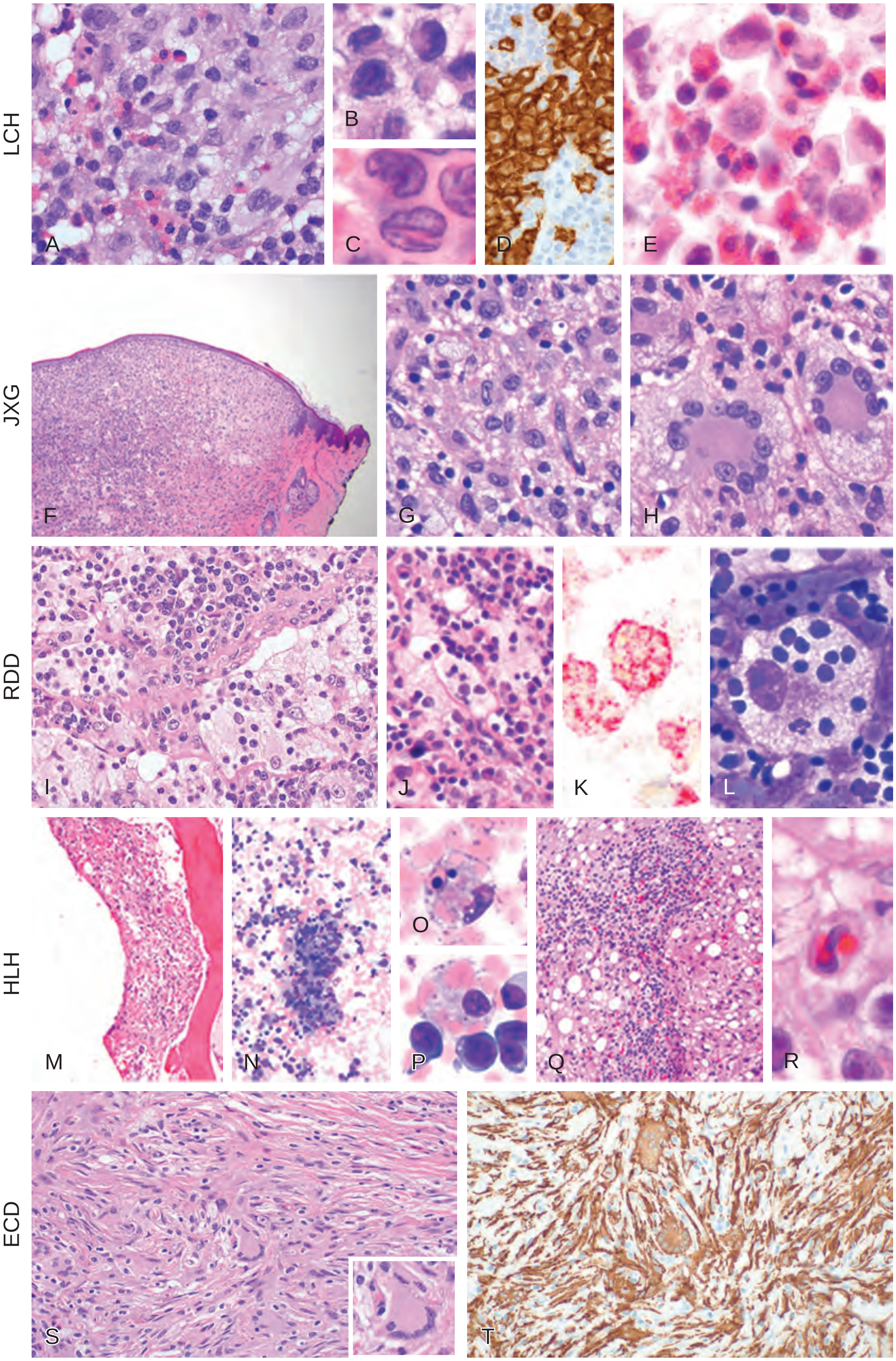


Figure 53.2 HISTOLOGY OF LANGERHANS CELL HISTIOCYTOSIS (LCH), JUVENILE XANTHOGRANULOMA (JXG), HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS (HLH), ERDHEIM-CHESTER DISEASE (ECD), AND ROSAI-DORFMAN DISEASE (RDD). ***LCH:*** (A) Biopsy sample showing sheets of histiocytes with abundant pink cytoplasm and folded nuclei with prominent nuclear grooves. (B and C) Cells with a central longitudinal nuclear groove, giving the cell a coffee bean appearance. (D) Immunohistochemical stain for CD1A showing the histiocytic cells are positive. (E) Some cases of Langerhans histiocytosis are associated with prominent eosinophilia. ***JXG:*** Histologic features of juvenile xanthogranuloma vary. (F) In this case, low-power magnification shows a dome-shaped lesion with an attenuated epidermis. (G) At higher power, the bulk of the lesion is composed of a proliferation of histiocytes with abundant pink cytoplasm. Sometimes these histiocytes show more vacuolization or xanthomatization. (H) Scattered Touton-type giant cells are present. ***RDD:*** Low-power magnification of the biopsy sample (I) shows a mottled appearance of the lesion caused by dark areas containing small lymphocytes and lighter areas

(*Continued*)

TABLE 53.2 **Proposed Revised Classification of Histiocytoses**

L Group	LCH
	Intermediate-cell histiocytosis (ICH)
	Erdheim-Chester disease (ECD)
	Mixed LCH/ECD
C Group	Cutaneous non-LCH
	Xanthomatous granuloma (XG) family: includes JXG
	Non-XG family: includes cutaneous RDD
	Cutaneous non-LCH with major systemic component
	XG family: xanthoma disseminatum
	Non-XG: multicentric reticulohistiocytosis
R Group	Familial RDD
	Sporadic RDD
	Classic RDD
	Extranodal RDD
	RDD with neoplasia or immune disease
	Unclassified
M Group	Primary malignant histiocytosis
	Secondary malignant histiocytosis
H Group	Primary HLH
	Secondary HLH (non-Mendelian)
	HLH of unknown/uncertain origin

ECD, Erdheim-Chester disease; *HLH*, hemophagocytic lymphohistiocytosis; *JXG*, juvenile xanthogranuloma; *LCH*, Langerhans cell histiocytosis; *RDD*, Rosai-Dorfman disease.
Adapted from Emile JF, Abla O, Fraitag S, et al. Revised classification of histiocytoses and neoplasms of the macrophage-dendritic cell lineages. *Blood*. 2016;127(22):2672–2681.

advisable unless a soft tissue mass is present. In long bones, growth of lesions in the medullary cavity leads to pressure that may result in erosion through the cortex, stimulating the formation of periosteal new bone accompanied by soft tissue extension. The differential diagnosis includes Ewing and osteogenic sarcoma, bone lymphoma, benign bone tumor and cyst, and infection. Involvement of distal bones such as the wrists, hands, or feet is less common. Orbital involvement may result in vision loss or strabismus caused by optic nerve or orbital muscle involvement, respectively, and may mimic pre-septal cellulitis. Signs and symptoms may include bony defects with exophthalmos, with a characteristic tumor mass in the orbital cavity related to disease arising from the roof and lateral wall of the orbital bone. Oral involvement commonly affects the gums, palate, or both. In addition, teeth may be lost due to maxillary or mandibular involvement with or without oral mucosal gum infiltration. Erosion of the lamina dura gives rise to the characteristic "floating tooth" seen on dental radiographs. Erosion of gingival tissue causes premature eruption, decay, and tooth loss. Parents of affected children, particularly infants, frequently report precocious eruption of teeth when, in fact, the gums are receding, leading to the exposure of immature dentition. Chronic otitis media is caused by involvement of the mastoid and petrous portion of the temporal bone, leading to otitis externa manifesting as chronic otorrhea is common (see Fig. 53.3J).

Cutaneous Involvement

Cutaneous involvement by LCH is both common (occurring in 20% to 40% of patients) and highly variable and can mimic more common skin conditions of children. The rash is typically a scaly seborrheic, eczematoid, sometimes purpuric rash involving the scalp, ear canals, abdomen, and intertriginous areas of the neck, face, trunk, and groin (see Fig. 53.4C–F). Ulceration may result, especially in intertriginous areas, and may be painful. Mild, isolated cutaneous involvement is relatively common in young infants. Rarely, LCH may present only as subcutaneous skin nodules (formerly described as *Hashimoto–Pritzker syndrome*), most often in young infants. In many cases of skin-limited neonatal LCH, the lesions may spontaneously resolve.

Involvement of Other Organ Systems

A subset of infants and toddlers with LCH have spleen, liver, or bone-marrow (BM) involvement, often in addition to prominent skin involvement and variable bony disease. This potentially fatal clinical phenotype is the most severe manifestation of LCH. Liver involvement may be clinically problematic, leading to significant cholestasis, hypoproteinemia, diminished synthesis of clotting factors, and ultimately sclerosing cholangitis.

LCH can rarely present with lymph node-limited disease that may be difficult to distinguish SHML, also known as RDD. This presentation is characterized by significant enlargement of multiple lymph node groups with little or no other signs of disease. Thymic involvement is relatively common in children with multisystem involvement but can also be isolated. Gastrointestinal tract disease is rare but is sometimes associated with severe symptoms of diarrhea, hematochezia, malabsorption, and hypoproteinemia. Pancreatic, thyroid, and thymus disease has also been reported.

Isolated pulmonary involvement is usually seen in young adults in their third or fourth decades of life, and occasionally in children and adolescents. It may follow a severe and often chronic debilitating course and patients may often present with spontaneous pneumothorax. Cigarette smoking has been strongly implicated in primary pulmonary histiocytosis. Findings on chest radiographs vary from a diffuse infiltrate consistent with bilateral interstitial pneumonia to a "honeycomb lung" appearance with nodules and cysts (see Fig. 53.4G).

Other Clinical Features

Central DI affects 5% to 40% of patients with LCH.[30] Most instances of DI occur in children who present with systemic disease and involvement of the orbit and skull. DI is caused by infiltration by LC and immune infiltrate into the hypothalamus with or without the involvement of the posterior pituitary gland (see Fig. 53.4K). Most cases of DI present within the first 4 years of diagnosis, although it may occur at any time during the course of LCH. Definitive documentation of DI with measurement of serum and urine electrolytes and osmolality before and after a several-hour water deprivation period should be performed, usually in consultation with an endocrinologist. Antidiuretic hormone (ADH) levels can also be measured to document deficiency, although they are not required for diagnosis. DI

---

containing histiocytes. At higher magnification (J), the histiocytes have abundant pale cytoplasm with scattered cells within. This is emperipolesis, a process of cells traveling through the cytoplasm but not apparently becoming phagocytized or degraded. Note the plasma cells in the background. The emperipolesis can be better visualized with a CD68 stain for histiocytes (K). This process delineates the cell boundary and the cells within the histiocyte cytoplasm. The emperipolesis can also be seen on a Wright-stained touch preparation (L). ***HLH*** Illustrations from a 3-month-old girl who presented with diarrhea, pancytopenia, hepatomegaly, and liver failure. Bone marrow (M and N) showed left-shifted granulopoiesis and increased histiocytes, which at high power (O and P) were undergoing prominent phagocytosis of erythrocytes, platelets, and other cells. Liver biopsy sample (Q) showed a lymphohistiocytic infiltrate also associated with hemophagocytosis (R). The patient was shown to harbor a mutation of the perforin gene in exon 2. ***ECD:*** Lower magnification biopsy of skin from a patient involved by ECD characterize by dermal infiltrate (S) of bland appearing histiocytes characterized by abundant foamy (xanthomatous) cytoplasm with surrounding fibrosis. Note the multinucleated Touton giant cell in the inset. The cells are positive by histiocytic marker CD163 (T).

TABLE 53.3 Clinical Features of the Non-Langerhans Cell Histiocytosis, Non-Hemophagocytic Lymphohistiocytoses

Diagnosis	Ages	Lesions (*n*)	Appearance	Common Sites	Natural History
JXG	0–18 years (median, 2 years)	Single: multiple 9 : 1 (disseminated in <6 months)	Reddish progressing to yellow brown	Head and neck	Gradual involution
Giant JXG	Young	Single	>2 cm	Upper extremity or back	Involution
Systemic JXG	(3 months[a])	Single-multiple	Almost 50% have no skin lesions	Subcutis, liver, spleen, lung, CNS, iris	May involute (4%–10% fatal)
Adult XG	18–80 (35 years[a])	Single	Same as JXG	Upper body	No involution
BCH	Young child	Few-multiple	Reddish-tan papules	Head and neck	Involution or progression to XG
GEH	Young adult	Disseminated	Reddish-tan papules in crops	Face, trunk, arms	Involution or progression
XD	Young adult	Disseminated	Yellow/reddish-brown plaques and nodules	Eyelids, mucosae, viscerae, CNS	Slow involution or progression
PNH	40–60 years	Disseminated	Xanthoma, nodules	Skin, subcutis	Progression to disfigurement
MRH	>40 years	Multiple	Pink/reddish-brown or yellow	Head, extremities with erosive polyarthritis	Progression
SHML	Wide age range (20 years[a])	Mainly systemic	Firm indurated papules	Cervical adenopathy, 80% “B” symptoms, extranodal (43%)	Exacerbations and remissions (5%–11% fatal)
ECD	7–84 years (53 years[a])	Mainly systemic	Xanthelasma, xanthoma	Long-bone sclerosis, retroperitoneal fibrosis	Highly fatal

[a] Approximate median age of presentation.

BCH, Benign cephalic histiocytosis; *CNS*, central nervous system; *DI*, diabetes insipidus; *ECD*, Erdheim-Chester disease; *GEH*, generalized eruptive histiocytosis; *JXG*, juvenile xanthogranuloma; *MRH*, multicentric reticulohistiocytosis; *PNH*, progressive nodular histiocytosis; *SHML*, sinus histiocytosis with massive lymphadenopathy; *XD*, xanthoma disseminatum.

Adapted from Weitzman S, Jaffe R. Uncommon histiocytic disorders: the non-Langerhans cell histiocytoses. *Pediatr Blood Cancer*. 2005;45:256–264.[7]

TABLE 53.4 Histologic Features of Histiocytic Disorders

Clinical Entity	LCH	ECD/JXG	HLH	SHML
CD1a	++	–	–	–
CD14	–	++	++	++
CD68	+/–	++	++	++
CD163	–	++	++	++
Factor XIIIa	–	++	–	–
Langerin	++	–	–	–
Fascin	–	++	+/–	+
S100	+	–	+/–	+
Lysozyme	–	–	++	++
Birbeck granules	+	–	–	–
Hemophagocytosis			+/–	
Emperipolesis				+

ECD, Erdheim-Chester disease; *HLH*, hemophagocytic lymphohistiocytosis; *JXG*, juvenile xanthogranuloma; *LCH*, Langerhans cell histiocytosis; *SHML*, sinus histiocytosis with massive lymphadenopathy.

Adapted from Weitzman S, Egeler RM. *Histiocytic Disorders of Children and Adults*. Cambridge: Cambridge University Press; 2005.

typically persists beyond successful LCH therapy, requiring life-long management with desmopressin.

Short stature has been found in up to 40% of children with systemic LCH. Chronic illness and steroid therapy are believed to play an important role in this phenomenon. However, short stature also may be a consequence of anterior pituitary involvement and growth hormone deficiency, which can occur in up to half of patients with initial anterior pituitary dysfunction. Other endocrine manifestations associated with LCH may include adrenal insufficiency, hyperprolactinemia, or hypogonadism caused by hypothalamic infiltration.

One of the most severe complications of LCH is the development of a progressive central nervous system (CNS) neurodegenerative syndrome (LCH-ND).[30] This syndrome may develop with the onset of LCH or more typically years after the patient is presumed to be cured. Increased risk of LCH-ND has been associated with “CNS-risk” bone lesions (orbit, mastoid, maxilla, temporal, sphenoid, zygomatic, clivus), pituitary lesions, and *BRAF*V600E mutations. Delayed CNS involvement is typically diagnosed after a prolonged, sometimes insidious, decrease in cognitive abilities/school function. Magnetic resonance imaging (MRI) characteristically reveals diffuse or polymorphic lesions involving the white matter of the cerebellum, pons, basal ganglia, and less often the cerebral hemispheres with T2 hyperintensity (see Fig. 53.4L). Limited biopsy studies have previously revealed an inflammatory infiltrate, dominated by $CD8^+$ T cells, initially suggestive of a paraneoplastic or autoimmune phenomenon. More recently, analysis of brain biopsy and autopsy specimens showed perivascular infiltrates of BRAFV600E+ monocytes and microglia-like cells at sites of neurodegeneration, suggesting that neurodegenerative disease likely represents an active form of LCH rather than an autoimmune or paraneoplastic phenomenon.[31] Of note, although abnormal MRI findings (with white matter lesions) may precede clinical manifestations, such findings do not always correlate with clinical disease (even in retrospect) and do not always progress. Therapy for LCH-ND is controversial. Reports suggest the potential for responses in early disease with LCH-directed chemotherapy and/or MAPK pathway inhibitors.[32,33]

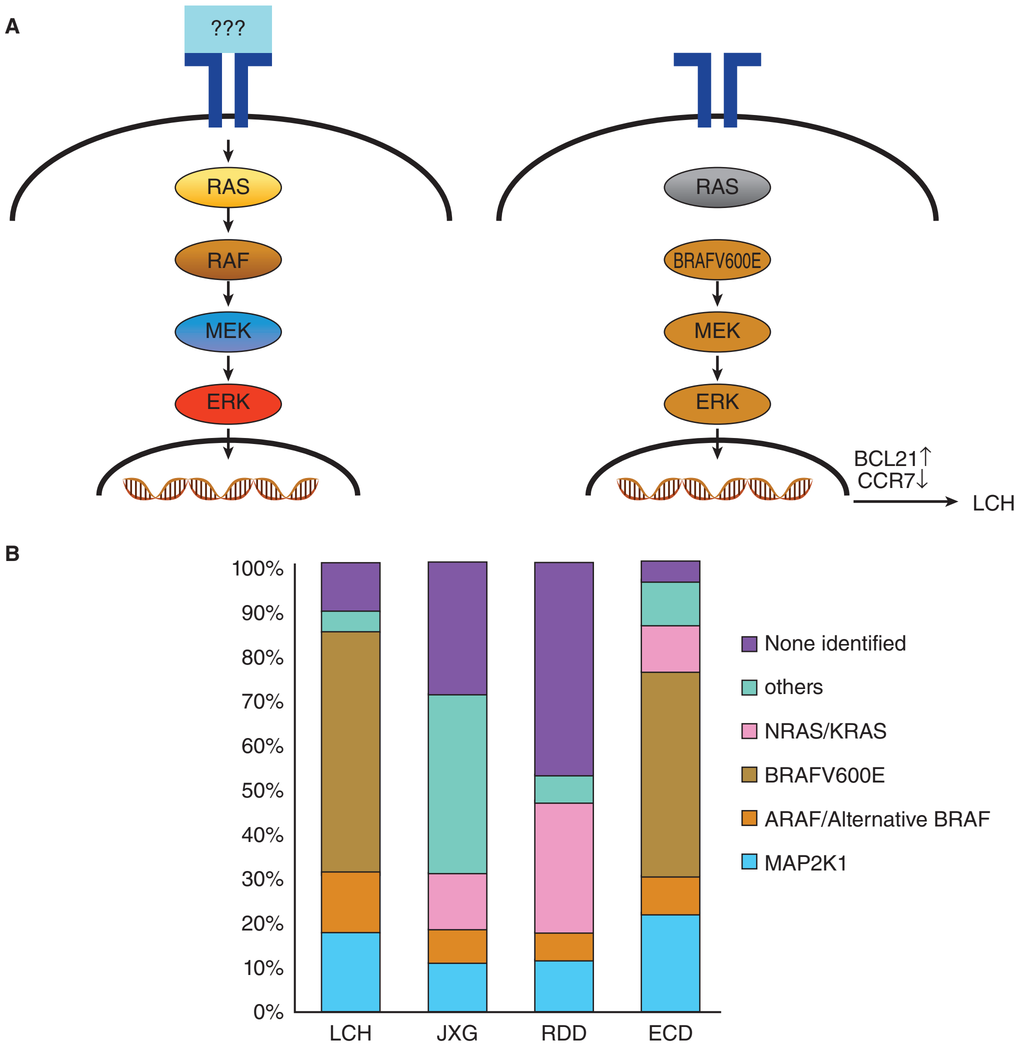


Figure 53.3 MAPK PATHWAY MUTATIONS IN HISTIOCYTIC DISORDERS. (A) (*Left*) Schematic of MAPK pathway. Under physiologic conditions, growth factor (*gray* box) engages tyrosine kinase receptor that transduces signal to nucleus. (*Right*) Activating mutations (such as BRAF-V600E) drive constitutive ERK activation. In the case of Langerhans cell histiocytosis (LCH), this drives expression of anti-apoptosis BCL2L1 *(BCL-xL)* and inhibits CCR7. (B) Stacked bar graphs represent percentages of MAPK pathway mutations in each histologic subtype of histiocytosis. *ECD*, Erdheim-Chester disease; *JXG*, juvenile xanthogranuloma; *LCH*, Langerhans cell histiocytosis; *RDD*, Rosai-Dorfman disease.

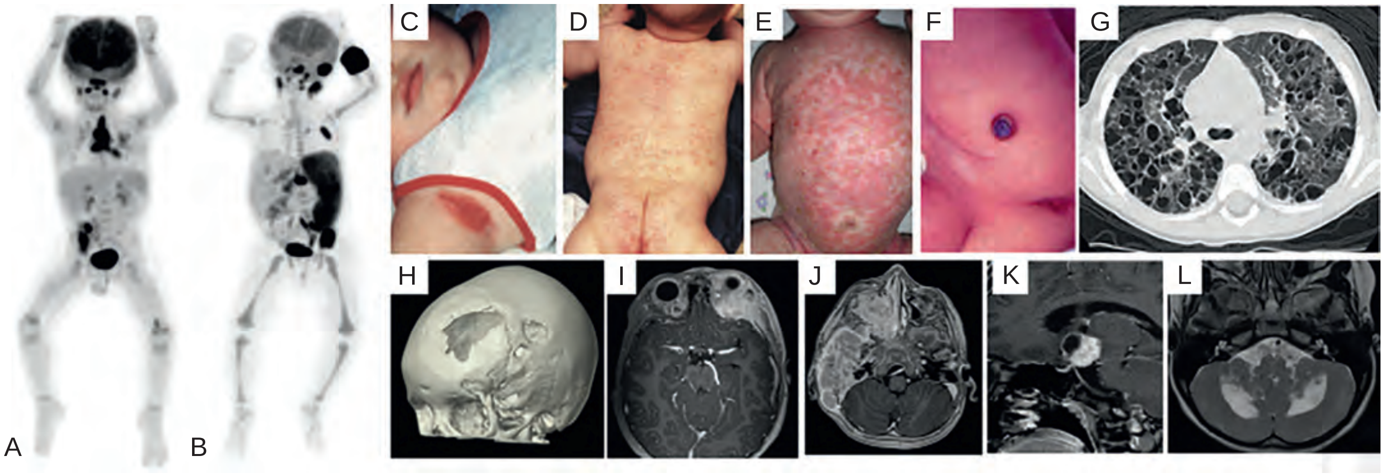


Figure 53.4 CLINICAL PRESENTATIONS OF LANGERHANS CELL HISTIOCYTOSIS (LCH). (A) PET-CT of patient with low-risk LCH including thymus, pelvis, and femur lesions; (B) PET-CT of patient with high-risk LCH including spleen, LN, and multifocal bone lesions; (C–F) various LCH skin lesions; (G) CT scan of pulmonary LCH; (H) CT demonstrating typical multifocal skull lesions; (I) orbital lesion in patient presenting with proptosis; (J) extensive skull/mastoid lesions; (K) pituitary lesion; and (L) LCH-associated neurodegenerative disease with typical T2-hyperintensity of cerebellum and pons on MRI.

Laboratory Manifestations

Tables 53.3 and 53.4 list the clinical and pathologic features that help to describe and distinguish LCH from other, much rarer, histiocytic disorders. The CD1a+/CD207+ LC is the essential diagnostic feature in the histology of LCH. Mitoses are rare. Multinucleated giant cells may be present. Other inflammatory cells, such as granulocytes, eosinophils, macrophages, and lymphocytes, are also present in variable numbers. The diagnosis of LCH relies on the immunohistochemical identification of the presence of LC by characteristic appearance with reniform nuclei and CD1a+/CD207+ by immunohistochemistry (see Fig. 53.2A–E).[34,35] Electron microscopy can identify Birbeck granules but is currently rarely used. Notably, CD207 expression levels may be variable within lesions and may be absent from liver, brain, or BM lesions. There are no predictive pathologic features that may define "favorable" or "unfavorable" histology. Immunohistochemistry, qualitative or quantitative PCR, and/or targeted sequencing may be used to identify *BRAF*V600E to support the diagnosis of LCH and also for risk-stratification. Additionally, high-sensitivity PCR of peripheral blood or BM mononuclear cells can identify BRAFV600E+ hematopoietic precursors, and the presence of which is associated with disseminated disease.[28]

Patients who are suspected to have LCH or who have a new biopsy-proven diagnosis of LCH should have imaging to determine the extent of disease. Positron emission (PET) scanning is highly sensitive for LCH.[36] Initial studies including a complete blood count, chemistries including liver function tests, coagulation workup, and urine osmolality are also warranted. Dental examination and radiographs should also be considered depending on clinical history and exam. The occurrence of cytopenias, particularly thrombocytopenia, in the presence of liver or spleen involvement may suggest BM involvement. We typically perform BM studies for all patients under the age of two years old and patients with liver or spleen disease, regardless of complete blood count (CBC) results.

Differential Diagnosis

The differential diagnosis of LCH depends on the clinical presentation and is typically elucidated with a tissue biopsy. Skin involvement may mimic seborrheic dermatitis, albeit with a severe or refractory course. Immunodeficiency syndromes or viral infection must be considered as well. The differential diagnosis of bony lesions, although typically quite distinctive, may include bone cyst, non-ossifying fibroma, lymphoma, sarcoma, or metastatic solid tumor. Chronic otorrhea due to temporal bone involvement is often initially misdiagnosed as chronic otitis media. Liver and spleen involvement must be distinguished from leukemia, infection, and metabolic storage diseases. The new onset of DI may also arise from germinoma or pituitary hypophysitis.

Prognosis

The prognosis of patients with LCH is largely determined by the extent of disease and response to initial therapies. In general, the population with the highest risk of mortality includes patients with visceral, or so-called "high risk organ" involvement (e.g., liver, spleen, and/or BM). Furthermore, the International Histiocyte Society conducted a clinical trial (LCH-II) that identified the response after an initial 6 weeks of therapy with weekly vinblastine and daily prednisolone as a risk factor in predicting mortality in patients with risk organ involvement.[37] Of the approximately 79% of patients who responded to initial therapy, 94% were alive at 5 years, but only 11% of the non-responders survived. These important data suggest that alternative therapies should be tested early during the course of therapy for patients with poor early responses. Additionally, *BRAF*V600E mutation is associated with increased risk of refractory disease/relapse and LCH-ND.[28,33,38]

Therapy

A generally accepted standard for the initial treatment of patients with LCH is the use of an appropriate amount of the least toxic therapy to treat the disease. Isolated skin lesions may spontaneously resolve or respond to topical therapies. Single bone lesions may be treated with curettage and/or steroid injection, with exceptions for functionally critical lesions that are not amenable to surgery and sites of disease that are considered CNS-risk. Patients with multifocal and multisystem disease typically require systemic therapy.[39,40]

Surgery and Radiotherapy

Patients with disease involving a single bone can usually be managed with local therapy in consultation with surgical subspecialists. This most often involves surgical curettage or intralesional steroid injection for patients whose lesions are in easily accessible and non-CNS risk locations. Overly aggressive complete "cancer operation" resections are contraindicated and may impair remodeling that typically occurs if margins remain intact. Limited local radiotherapy may be indicated for rare circumstances where an isolated lesion is inaccessible.

Chemotherapy

Historically, systemic treatment of LCH has used chemotherapy drugs classically used for malignant diseases. Therapeutic advances for LCH in recent years have largely come from international cooperative trials testing empiric chemotherapeutic strategies conducted by the Histiocyte Society. LCH-III demonstrated lower relapse rates in patients treated with 1 year versus 6 months of vinblastine/prednisone. Further, the addition of methotrexate to patients with high-risk LCH was not beneficial.[41] Therefore, the current standard of care for patients requiring chemotherapy for LCH includes vinblastine/prednisone for 1 year (with mercaptopurine added for high-risk LCH). The Histiocyte Society is currently testing the impact of further treatment prolongation (2 vs. 1 year) with vinblastine/prednisone/(mercaptopurine for high-risk) for frontline therapy (clinicaltrials.gov: NCT02205762). Based on responses to cytarabine monotherapy in an institutional series, another phase III trial is currently testing 1 year of vinblastine/prednisone/mercaptopurine versus cytarabine for frontline LCH (clinicaltrials.gov: NCT00276757).[42]

Alternative treatment has not been standardized for patients with recurrent or refractory disease. Patients with recurrent disease (i.e., disease that reappears after a period of remission) may respond to reinitiation of the drugs with which they were initially treated. Several studies, including an international phase II trial, have demonstrated significant activity of nucleoside analogs (2-chlorodeoxyadenosine [2-CdA], cytarabine, and clofarabine) against recurrent and refractory LCH. The combination of 2-CdA and high-dose cytarabine has high response rates, but also notable treatment-related mortality.[43] Moderate dose cytarabine and clofarabine have been reported as effective in institutional series.[42,44] Prospective trials are required to determine the optimal agents and doses. Hematopoietic cell transplant has also been reported as successful in some extreme cases of disseminated LCH.[45]

Since the discovery of the central pathologic role of MAPK pathway activation, BRAF-V600E and MEK inhibitors have been utilized primarily as salvage therapies with extremely high response rates.[32,46–49] However, disease typically recurs with cessation of therapy and the toxicity profiles are not ideal for life-long use. Pre-clinical studies and clinical trials are required to determine optimal timing, duration, and potential combination therapies with MAPK inhibitors.

Long-Term Follow-Up

Over 50% of LCH survivors experience at least one permanent consequence, with more frequent long-term effects in patients with multisystem disease and multiple relapses.[51] The most commonly reported late effects are DI and orthopedic abnormalities. Complications of developing DI include a significant incidence of anterior pituitary hormone deficiencies and an associated risk for later developing neurodegenerative syndrome. Patients with LCH-ND typically present with ataxia, dysarthria, dysmetria, and learning and behavior difficulties. The diagnosis may be aided by brain MRI, in which T2 hyperintense signals in the cerebellum, basal ganglia, or pons may be present, although such abnormalities do not always correlate with clinical disease and vice versa is also true (see Fig. 53.4). Treatment with low-dose cytarabine or MAPK inhibition has anecdotally resulted in stabilization or improvement of these symptoms.[30,31,33]

The small but real risk of secondary malignancy in patients with LCH undergoing radiation and chemotherapy is well documented. Thus, judicious use of radiotherapy, avoidance of potentially carcinogenic chemotherapeutic agents, and good supportive care are recommended. Because etoposide was not shown to be any more effective than vinblastine in both the LCH-I and LCH-II trials for patients without risk of organ involvement, there does not appear to be compelling reasons to include this leukemogenic agent in the treatment of patients with newly diagnosed LCH.[37,51] Rarely LCH can occur along with other hematologic malignancies that share a common origin.[52]

Another serious late effect of LCH is sclerosing cholangitis, which may lead to secondary biliary cirrhosis and liver failure. Sclerosing cholangitis may develop years after successful therapy for LCH and does not typically signify disease recurrence. The only successful treatment of sclerosing cholangitis has been liver transplantation, although anecdotal reports with clofarabine and MAPK inhibitors have also shown promise in at least stabilizing this process.

Other late complications of LCH include pulmonary cyst formation, fibrosis, and chronic pneumothoraces, and progression to cor pulmonale and respiratory failure may occur. Lung transplantation has been used for the treatment of such patients. Consequently, all patients with LCH require long-term follow-up.[53]

Future Directions

Frontline vinblastine/prednisone, the current standard of care for systemic LCH, cures fewer than 50% of patients. More effective therapy that minimizes toxicity and the risks of late consequences is clearly needed. With the recent insights into the pathophysiology of LCH, including a central role for MAPK activation in myeloid precursors, new possibilities for rationale, targeted therapies being explored. Ideally, future trials will lead to precision strategies to determine optimal therapy for patients based on features of the patient and disease.

JUVENILE XANTHOGRANULOMATOUS DISEASE

JXG (or more broadly, the full spectrum of juvenile xanthogranulomatous diseases) is a histiocytic disorder with features of macrophage histology. JXG most commonly affects infants and young children, with a slight male predominance and presents as a solitary or a few “fleshy nodules.” The most common sites are the skin of the frontal trunk, head, and neck, but they can occur at any site on the body. These red-yellowish, benign-appearing lesions are sometimes mistaken for molluscum. However, when biopsied, these lesions reveal a distinctive pathology (see Fig. 53.2F–H). Multinucleated, Touton giant cells are usually found, and lesional histiocytes are positive for CD14, CD68, CD163, factor XIIIa, and fascin, historically compared to dermal dendrocytes.[54] The cells are usually negative for CD1a, CD207, S100, plasmacytoid monocyte antigen CD123, and absent Birbeck granules. Lesions may vary significantly in size and number but are often several millimeters to 1 cm in size and solitary. However, in some patients, the lesions become widespread and quite disfiguring (Fig. 53.5). Furthermore, JXG may be systemic (<5% pediatric cases) involving multiple organs, including deeper soft tissue, central nervous system, bone, lung, liver, spleen, pancreas, adrenal glands, intestines, kidneys, lymph nodes, BM, orbit, and heart.[55] CNS involvement can present with seizures, hemiplegia, and increased intracranial pressure. Patients diagnosed with JXG, particularly multifocal JXG, may benefit from screening computed tomography (CT) or MRI scans to rule out disseminated involvement. Ophthalmologic examination may be helpful to rule out anterior chamber involvement and prevent potentially blinding complications.

The etiology of JXG and the basis for prevalence in children and tissue distribution is not well defined. Several cases suggesting an association between JXG, neurofibromatosis (types 1 and 2), and juvenile chronic myelogenous leukemia that potentially implicates MAPK pathway hyperactivity as shared pathogenesis have been reported. Additionally, molecular alteration in the MAPK pathway such as *CSF1R*, *KRAS, NRAS*, and *MAP2K1* (rarely *BRAF*V600E) and PI3K pathway mutations have been identified (see Fig. 53.3).[56]

Cutaneous JXG lesions usually resolve over several months and require no treatment. Of note, residual pigmented areas may persist indefinitely even after lesions have regressed. In patients in whom JXG becomes systemic and involves multiple organs, LCH-based systemic chemotherapy regimens such as vinblastine and steroids, cladribine, and clofarabine has led to responses in some case reports and series.[44,55]

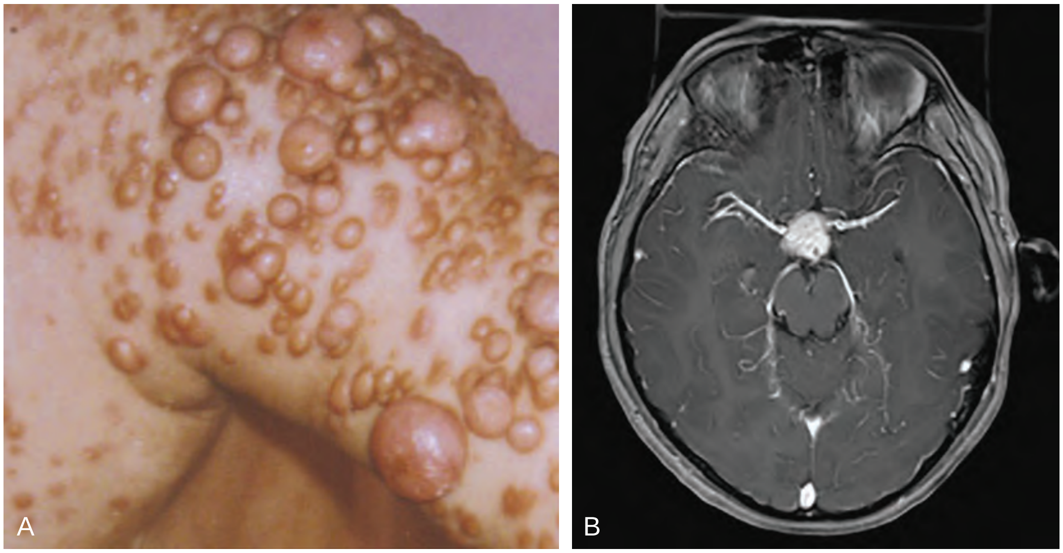


Figure 53.5 JUVENILE XANTHOGRANULOMA. (A) Extensive skin disease; (B) parenchymal brain lesion in patient presenting with diplopia.

ERDHEIM-CHESTER DISEASE

ECD is a rare, non-Langerhans form of histiocytosis first described in 1930 with a wide range of manifestations. While still extremely uncommon, the number of new cases has increased over the past 10 years because of the better recognition of this condition. The natural evolution is variable, but the prognosis in the absence of effective therapy is poor.[57]

ECD is seen most commonly in patients between ages of 40 to 70 years old, with a male predominance. It usually presents with xanthelasma lesions on the upper eyelids or yellow/red-brown plaques skin rash and bilateral lower limb bone pain. In fact, the consensus diagnostic criteria for ECD require presence of (1) foamy CD68+/CD1a− histiocytes, often with admixed inflammation and fibrosis, and (2) skeletal findings of bilateral and symmetric abnormalities in the diaphyseal and metaphyseal regions of the long bones of the legs. Patients with more disseminated disease may have cardiopulmonary insufficiency; renal failure caused by characteristic retroperitoneal and perinephric infiltrative or constrictive changes; and CNS involvement manifested by cerebellar symptoms such as ataxia, dysarthria, dysphagia or endocrinopathies such as DI, or cognitive decline. They may also have periorbital involvement with exophthalmos and impingement on the optic nerves. Untreated, this disease may be progressive and fatal.[58]

There has been significant advancement in understanding the pathophysiology of ECD over the last decade. ECD is now considered to be a clonal hematopoietic disorder driven by recurrent alterations in the MAPK pathway (*BRAF*V600E being the most common in approximately 50% of patients), with clinical manifestations thought to be due to the neoplastic histiocytic infiltrates and uncontrolled inflammation. A unique inflammatory cytokine signature consisting of elevated interferon-α (IFN-α), interleukin-12 (IL-12), and monocyte chemotactic protein-1 and decreased IL-4 and IL-7 in these patients suggests a systemic immune perturbation associated with ECD pathogenesis.[59]

First-line treatment for ECD has consisted of IFN-α. However, early reports of near-universal metabolic responses to MAPK inhibition led to FDA approval of vemurafenib for BRAFV600E+ ECD, and studies evaluating MEK inhibition are ongoing (NCT04079179, NCT02649972).[47,48,55,60,61] Other effective treatments have been limited, although responses have been observed with steroids, vinblastine plus steroids, anakinra, tocilizumab, methotrexate, 2-CdA, and bisphosphonates. Autologous hematopoietic BMT has also been reported as a therapeutic modality.[57]

ROSAI-DORFMAN-DESTOMBES DISEASE (OR SINUS HISTIOCYTOSIS WITH MASSIVE LYMPHADENOPATHY)

Pierre Pail Lois Lucien Destombes first reported four patients with lymphadenopathy and sinus histiocytosis on histology in 1965. In 1969, Juan Rosai and Ronald Dorfman analyzed 34 similar cases that they described as sinus histiocytosis with massive lymphadenopathy.[62,63] SHML, or RDD, can have a widely variable clinical presentation and can occur as an isolated disorder or in association with other autoimmune, malignant, or hereditary diseases. The most common clinical presentation is that of chronic, painless massive lymphadenopathy usually involving cervical lymph nodes and, less frequently, axillary, hilar, peri-tracheal, and inguinal nodes with or without intermittent fevers, night sweats, and weight loss (Fig. 53.6).[64]

Extranodal disease is present in approximately 43% of patients, with multisystem involvement present in 19% of cases. Skin involvement (painless, nonpruritic nodules, plaques, or papules) is present in 10% of extranodal RDD cases, though it is rarely isolated. The upper respiratory mucosa (sino-nasal, oral cavity including salivary glands, parotid gland, larynx, pharynx, thymus or thyroid gland) may also be involved. Ocular manifestations, such as an orbital soft tissue mass, mass involving the eyelids or lacrimal gland, uveitis and compressive optic neuropathy have also been reported. Extranodal bone involvement, typically in the metaphysis or diaphysis manifesting as osteolytic or mixed lytic/sclerotic lesion on imaging is seen in 5% to 10% of cases, and it is usually associated with nodal disease. Occasionally, there is involvement of the CNS (intracranial, spinal cord, meninges), lung, heart, gastrointestinal tract, liver, and kidney. Overall prognosis has been found to be correlated to both the number of nodal groups and extranodal systems involved. Significant morbidity and death have been associated with massive tissue invasion of the liver, kidney, lung, brain, and other critical structures.[65]

RDD is more common in children and young adults (median age 20.6 years), though it has also been reported in older adults. RDD arises with increased frequency in children of African descent, with a slight male predominance.

Laboratory evaluation frequently reveals an elevated erythrocyte sedimentation rate (ESR), moderate polyclonal hypergammaglobulinemia, anemia, and granulocytosis. Subtle abnormalities of immune indices may also be seen. Involved lymph nodes (see Fig. 53.2I–L) show marked sinusoidal dilation with proliferation of foamy histiocytes within the sinuses. Eosinophils are usually low in number, but

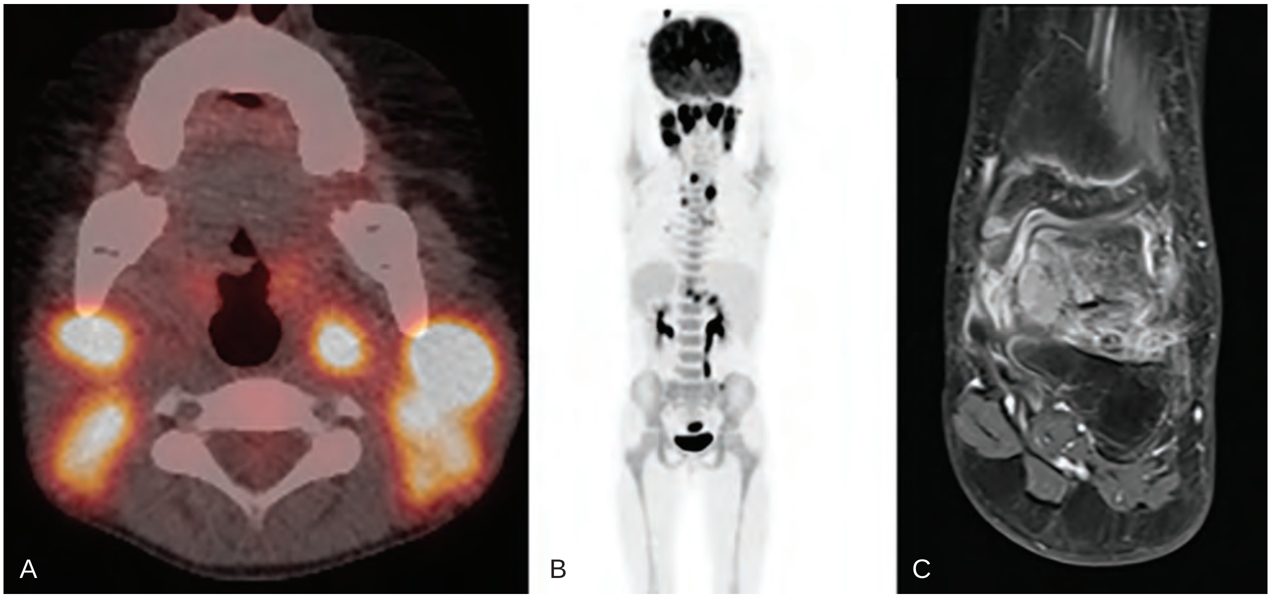


Figure 53.6 ROSAI-DORFMAN DISEASE. (A and B) Typical hypermetabolic cervical lymphadenopathy demonstrated in PET-CT scan; (C) extramedullary talus lesion.

plasma cells are abundant. A characteristic finding, referred to as *emperipolesis*, demonstrates intact leucocytes trafficking through histiocytes as observed best by electron microscopy. The proliferating histiocytes appear to be macrophages. These large, pale cells show variable expression of cytoplasmic and nuclear S100. They can be distinguished from Langerhans cells found in the lymph nodes of patients with classic LCH by CD163+ and CD1a–/CD207– immunohistochemistry.[54]

The etiology of RDD is not well defined and is thought to be variable across the varied phenotypic presentation. Activating MAPK pathway mutations have been reported in RDD lesions, but not with the same frequency as other myeloid neoplastic histiocytic disorders discussed above.[56]

The prognosis of RDD is variable. Overall, the outcomes are usually favorable with disease manifestations that may spontaneously subside over several months to years, especially in the cases of cutaneous and nodal RDD in children. For other patients, the course can be unpredictable with periods of remission and relapse. As the disease resolves, extranodal disease typically regresses before nodal disease. Mortality related to RDD or as a result of direct complication of the disease has been reported to be approximately 10%. No standard of care exists and treatment options vary on a case-to-case basis, depending on the site(s) of disease, associated morbidities, etc. Treatment options range from observation only, corticosteroids, surgical resection, radiotherapy, sirolimus or other immunomodulatory agents, and/or chemotherapy with variable responses and based largely on individual case reports or small case series.[64] Lesions may respond to corticosteroids but typically rebound when steroids are weaned. Case reports and series note potential for responses to vinca alkaloids or low-dose methotrexate/6MP. However, the results with these agents have been inconsistent. Recent reports have nucleoside analogs, especially single-agent clofarabine has the potential to achieve cure in patients with SHML who require systemic therapy. Targeted therapies targeting the MAPK pathway have shown robust activity in histiocytic disorders and are being explored in RDD in patients with proven activating MAPK pathway mutations.[47] Attempts at treatment should generally be reserved for cases with multifocal/extranodal RDD, progressive disease, or special circumstances such as tracheal or epidural compression, invasion of other vital structures, or significant cosmetic disfigurement.

HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

HLH is a life threatening, hyperinflammatory syndrome that can occur as a familial or sporadic disorder. In the broad classification of histiocytic disorders (see Table 53.1), HLH is categorized as a monocyte/macrophage-related histiocytic disorder. HLH derives its name from its sometimes distinctive pathology (hemophagocytosis), in which macrophages appear to be widely infiltrating tissues and engulfing blood and BM cells in a nonspecific fashion. However, HLH is best conceptualized as an immune regulatory disorder, which is characterized by clinical signs and symptoms of extreme inflammation and the development of cytopenias, hepatitis, and CNS dysfunction, which are severe and life threatening.[66] Indeed, it has been proposed that the name of this disorder should be changed to "hyperinflammatory lymphohistiocytosis" (also HLH).

HLH was first described as a familial disease by Farquhar and Claireux in 1952, which they named "familial hemophagocytic reticulosis." Through the years, the syndrome of HLH has been recognized as both a sporadic and familial disorder and in various clinical contexts. Although we prefer to lump each of these clinical variations into a single syndrome (HLH), the medical literature has used a plethora of names: familial erythrophagocytic lymphohistiocytosis, viral-associated hemophagocytic syndrome, and malignancy-associated hemophagocytic syndrome, among others. Furthermore, macrophage activation syndrome (MAS), the systemic inflammatory syndrome observed in association with rheumatologic disorders, is likely a variant of HLH syndrome because both "classic" HLH and MAS have similar clinical phenotypes and appear to share some underlying mechanisms. The International Histiocyte Society formally adopted the name of HLH in 1998 and defined criteria for its diagnosis, which were updated in 2004 (Table 53.5).[67] Despite clinical and scientific advances, uncertainty remains about how to conceptualize and recognize HLH. Due to the complexity of diagnostic criteria and similarity to other inflammatory disorders, diagnosis is often delayed. Moreover, with increasing awareness, the syndrome misdiagnosis has become a major issue of concern.

TABLE 53.5 **Diagnostic Criteria for Hemophagocytic Lymphohistiocytosis**

A. A molecular diagnosis consistent with HLH: Pathologic mutations of *PRF1*, *UNC13D*, *Munc18-2*, *Rab27a*, *STX11*, *SH2D1A*, or *BIRC4*

-OR-

B. **Five** out of the **eight** criteria listed below are fulfilled:

1. Fever ≥38.3°C
2. Splenomegaly
3. Cytopenias (affecting at least 2 of 3 lineages in the peripheral blood):
   Hemoglobin <9 g/dL (in infants <4 weeks: hemoglobin <10 g/dL)
   Platelets <100 × 10^3/mL
   Neutrophils <1 × 10^3/mL
4. Hypertriglyceridemia (fasting, ≥265 mg/dL) and/or hypofibrinogenemia (≤150 mg/dL)
5. Hemophagocytosis in bone marrow or spleen or lymph nodes or liver
6. Low or absent NK-cell activity
7. Ferritin >500 ng/mL[a]
8. Elevated soluble CD25 (soluble IL-2 receptor alpha)[b]

[a] While the HLH-2004 protocol uses ferritin >500 ng/mL, we generally view ferritin>3000 ng/mL as concerning for HLH, and ferritin >10,000 as highly suspicious.[7]

[b] Elevations above age-adjusted, laboratory-specific normal levels (defined as >2 SD from the mean).

Adapted from Jordan MB, Allen CE, Weitzman S, et al. How I treat hemophagocytic lymphohistiocytosis. *Blood.* 2011;118:4041–4052; and Henter JI, Horne A, Aricó M, et al. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer.* 2007;48:124–131.

Epidemiology

The true incidence and prevalence of HLH are unknown and remain difficult to ascertain accurately. The diagnosis of HLH is challenging because of its variable presentation and the many nonspecific clinical features it shares with other disease processes. HLH is considered to be rare, but increasing awareness and recognition of the syndrome is leading to more frequent diagnoses. Currently, it is estimated that the autosomal recessive forms of familial HLH have a prevalence of 1 in 50,000 live births. A recent report estimated the incidence of HLH of 1 in 100,000 children, and a review of HLH cases from the largest pediatric hospital in Texas revealed 1 in 3,000 inpatients admissions associated with HLH diagnosis.[68,69] Because of the typically autosomal recessive nature of familial HLH (F-HLH), this disorder is reported to occur more frequently in isolated populations or kindreds with consanguinity. Although HLH was thought to be a primarily pediatric disease, it is diagnosed in all ages, and in a large Japanese cohort, 40% of the patients were adults.[70] The incidence may be as high as 1 in 2000 adult admissions at tertiary centers.[71]

Pathophysiology

HLH is a syndrome of excessive inflammation and tissue destruction due to abnormal immune activation. Different forms of HLH probably share the same terminal pathway though each has a different pathogenic root.

The immunologic basis of F-HLH, the prototype of disease, was long suspected because of its inflammatory nature and the finding of cytotoxic deficiencies and other immune abnormalities in patients with HLH. The first confirmation that HLH is an immunodeficiency came in 1999 with the discovery of perforin mutations in affected patients. However, unlike other immunodeficiencies, the principal clinical characteristic of HLH is one of intense, prolonged systemic inflammation rather than unusual or severe infections. Although this inflammation may be triggered by infection or vaccination, the inflammation itself (and not apparently the sometimes benign or transient infection) appears to drive the clinical features of HLH. Thus, F-HLH appears to be a deficiency of immune regulation. Animal studies have detailed how this immune regulation functions and how deficiencies may lead to HLH. In brief, cytotoxic lymphocytes kill not only infected cells but also antigen-presenting cells (APCs). These APCs promote T-cell activation during infection. This persistence of activating signals leads to excessive or prolonged acute T-cell activation. Abnormal T-cell activation, in turn, leads to the activation of macrophages and the development of disease pathology. In animal models, IFN-γ appears to be the critical nexus between T-cell activation and disease development.[72] Furthermore, clinical testing of antibodies against IFN-γ (emapalumab) has proven IFN-γ is a key driver of the disease.[73] An underlying lesion of immune regulation and the associated pathophysiology is less clear in apparently sporadic cases of HLH. These cases were historically referred to as *secondary HLH*. However, the traditional dichotomy between primary (familial) HLH and secondary HLH (associated with infections, malignancy, or autoimmunity) is becoming increasingly murky.[74] Further obscuring this dichotomy, a recent report by Zhang et al. described a large series of adults with HLH who were found to have genetic mutations typically seen in children with familial HLH.[75]

Recently, the North American Consortium for Histiocytosis (NACHO) has defined the following terminology to help differentiate the different clinical scenarios answering the diagnostic criteria (Fig. 53.7)[76]:

1. HLH syndrome: A condition that describes patients displaying sufficient diagnostic features (from the HLH-2004 diagnostic criteria presented in Table 53.5) to fulfill the pattern of HLH.
2. HLH disease: A condition characterized by distinct immune dysregulation in patients who are likely to benefit from immunosuppression. Only a subset of patients with "HLH syndrome" have "HLH disease."
3. HLH disease mimics: Disorders leading to the HLH pattern but which are not likely to benefit from immune suppression should be viewed as "disease mimics."

HLH may present in a variety of clinical contexts and with a variety of etiologic associations. Patients with F-HLH are those with clear familial inheritance or known genetic causes, are usually infants or younger children, and are thought to have fixed defects of cytotoxic function (although this is not always the case). These patients have a clear risk of HLH recurrence and will generally not survive long term without hematopoietic cell transplantation (HCT). Although HLH in these patients can be associated with infections (e.g., cytomegalovirus [CMV] or Epstein-Barr virus [EBV]) or vaccination, the immunologic trigger is often not apparent.

However, some of the patients with HLH disease are older children (or adults) who present without a family history or known genetic cause for their HLH. These patients typically have concurrent infections or medical conditions that appear to trigger their HLH, such as EBV infection, malignancy, or rheumatologic disorders. The list of triggering stimuli for both familial and apparently nonfamilial HLH is extensive. Patients with nonfamilial HLH are sometimes reported as having immune studies, including NK cell function, that normalize with disease resolution, although in the authors' experience this is variable or unclear. Although the mortality rate from HLH may be significant, the risk of recurrence in cases of secondary HLH is poorly defined. Recurrence of HLH in the absence of autoimmune disease or malignancy is generally considered to be good evidence that a patient has F-HLH, regardless of the other clinical features. In the absence of a known genetic defect or family history, it is often not possible to make an initial diagnosis of F-HLH.

A variety of genetic causes of F-HLH have been identified, all either autosomal recessive or X-linked (Table 53.6). Most of these

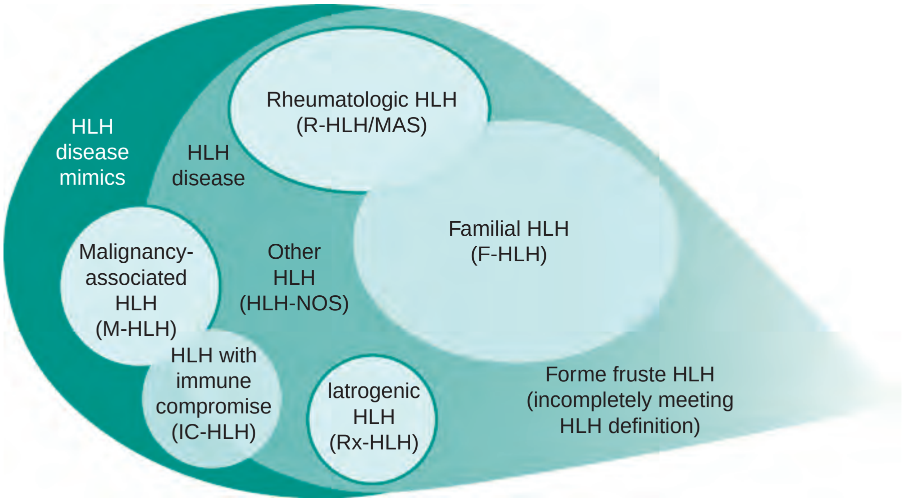

HLH disease mimics
HLH disease
Rheumatologic HLH (R-HLH/MAS)
Familial HLH (F-HLH)
Malignancy-associated HLH (M-HLH)
Other HLH (HLH-NOS)
HLH with immune compromise (IC-HLH)
Iatrogenic HLH (Rx-HLH)
Forme fruste HLH (incompletely meeting HLH definition)

Figure 53.7 CATEGORIZING HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS (HLH). The HLH syndrome includes all conditions meeting consensus diagnostic criteria. This syndrome includes conditions that would benefit from HLH-directed immunosuppressive therapies, which are termed "HLH disease," and those conditions that would not benefit from such therapy or require entirely different treatments, termed "HLH disease mimics." HLH disease includes recognizable subgroups: familial HLH with clear genetic etiology, HLH associated with malignancy, HLH associated with rheumatologic conditions (also called MAS), HLH observed after immune-activating therapies (iatrogenic HLH, also called cytokine release syndrome), HLH associated with immune compromise (either primary immune deficiency or treatment-related immune suppression), and HLH not associated with other specific conditions. Recognition of these subcategories is valuable as this may alter treatment, though some categories overlap with each other or have indistinct borders (as indicated above) and may include examples of both HLH disease and HLH disease mimics. Use of these category-specific terms is preferred over the historical terms of "primary" and "secondary" because the older concepts are ambiguous due to increasing understanding of genetic complexity, involvement of infection in triggering multiple distinct variations of HLH, and varied application. Incomplete, forme fruste episodes of HLH (similar, but not completely fulfilling diagnostic criteria) are also well recognized in patients with familial HLH and may occur in others, such as "mild MAS" in soJIA patients. *(Adapted from Jordan MB, Allen CE, Greenberg J, et al. Challenges in the diagnosis of hemophagocytic lymphohistiocytosis: recommendations from the North American Consortium for Histiocytosis (NACHO). Pediatr Blood Cancer. 2019;66[11]:e27929.)*

TABLE 53.6 **Hemophagocytic Lymphohistiocytosis-Associated Gene Mutations**

Gene	Disease	Apparent Mechanism of HLH Predisposition
PRF1	FHL2	Defective granule-mediated cytotoxicity
UNC13D	FHL3	
STX11	FHL4	
STXBP2	FHL5	
RAB27A	Griscelli syndrome	
LYST	Chediak-Higashi syndrome	
AP3B1	Hermanski-Pudlak syndrome	
XIAP	XLP2/X-linked HLH	Disorders of inflammasome activation
NLRC4	Auto inflammation with infantile enterocolitis	
CDC42	NOCARH syndrome	
SH2D1A	Lymphoproliferative syndrome, X-linked, 1 (XLP1)	Disorders of T cell signaling
ITK	Lymphoproliferative syndrome 1	
CD27	Lymphoproliferative syndrome 2	
MAGT1	Immunodeficiency, X-linked, with magnesium defect, Epstein-Barr virus infection, and neoplasia (X-MEN)	
SLC7A7	Lysinuric protein intolerance	Disorders of macrophage inflammatory signaling
HMOX1	Heme oxygenase deficiency	

FHL, Familial HLH; *HLH*, hemophagocytic lymphohistiocytosis.

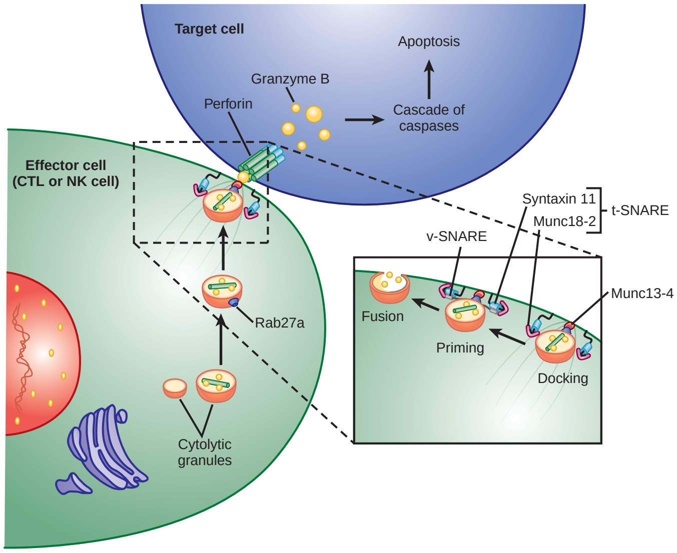


Figure 53.8 MECHANICS OF CYTOTOXIC FUNCTION REVEALED BY HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS (HLH)–ASSOCIATED GENE MUTATIONS. Hemophagocytic lymphohistiocytosis-associated genetic abnormalities (in the indicated genes) may affect granule-dependent lymphocyte cytotoxicity by impairing trafficking, docking, priming for exocytosis, or membrane fusion of cytolytic granules. The function of this pathway may also be severely impaired by loss of functional perforin, the key delivery molecule for proapoptotic granzymes. Diverse mutations in this pathway all give rise to similar clinical phenotypes (albeit of variable severity). *LYST* (the gene affected in Chediak-Higashi syndrome) is not portrayed because its function is not entirely clear, although it appears to play an important role in the maintenance of normally sized (and functional) cytolytic granules. *CTL*, Cytotoxic T lymphocyte; *NK*, natural killer, *SNARE*, soluble *N*-ethylmaleimide-sensitive fusion attachment receptor protein. *(Adapted from Jordan MB, Allen CE, Weitzman S, et al. How I treat hemophagocytic lymphohistiocytosis. Blood. 2011;118:4041–4052.)*

genetic lesions affect a biologic pathway referred to as *granule-dependent*, or *perforin-dependent, cytotoxicity* (Fig. 53.8).[66] This pathway is used by T cells and NK cells to kill target cells, typically those infected by viruses. When triggered, specialized lysosomal granules containing perforin, granzymes, and other proteins are released, leading to apoptotic death of target cells. The first genetic lesions identified in patients with HLH were mutations in *prf1*, the gene encoding the protein perforin. *Prf1* mutations account for about 15% to 20% of HLH in certain geographic areas and are known as familial HLH 2 (FHL2). FHL2 has mild and severe phenotypes that correlate with the degree of mature perforin protein that is produced. Abnormalities of granule formation, mobilization, and extrusion are also identified as causes of HLH. Munc13-4, a protein essential to the exocytotic process, is mutated in FHL3. FHL3 has a worldwide distribution and accounts for 15% to 20% of all hereditable HLH. However, recent discoveries of previously unappreciated intronic mutations in this gene may expand the proportion of patients with FHL3. FHL4 is caused by mutations in the protein syntaxin 11, a member of the soluble *N*-ethylmaleimide-sensitive fusion attachment receptor protein family of proteins, which is necessary for the fusion of cytotoxic vesicles with the plasma membrane to release their granules. Mutations in the syntaxin-binding protein 2, also important for this process, have been designated FHL5. The genetic defect responsible for FHL1 has not been identified, and no mutations in granzyme proteins have been associated with HLH.

Two X-linked causes of HLH are known. The first, called "X-linked lymphoproliferative syndrome 1" (or XLP1), is caused by defects in SH2D1A or SAP (signaling-lymphocytic-activation-molecule-associated protein). This disorder is a complex one characterized by lymphoproliferation, hypogammaglobulinemia, excess risk of lymphoma, and development of HLH. It appears to share some of the pathophysiology with classic familial HLH, although other abnormalities (of B cells and other cells) make this disorder distinctive. The second X-linked cause of HLH, sometimes called "XLP2" (although this is disputed), is abnormalities of a gene/protein called baculoviral inhibitor of apoptosis protein repeat-containing protein 4 (BIRC4)/X-linked inhibitor of apoptosis protein (XIAP). The apparently unique pathophysiology of HLH caused by XIAP deficiency is not understood. Other immunodeficiency syndromes caused by defects in lysosomal trafficking have been linked to life-threatening episodes of HLH. These include Chediak-Higashi syndrome, Griscelli syndrome, and Hermansky-Pudlak syndrome, type II.

Taken together, all the genetic disorders described previously still account for fewer than half of the diagnosed cases of HLH in children, including many familial cases still awaiting molecular definition. Until recently, it was widely believed that symptoms of HLH triggered by genetic causes arose during infancy and early childhood. With the more widespread availability of genetic testing, it is apparent that the first significant episode of HLH can occur throughout life from prenatal presentations through the seventh decade of life. Distinctions between primary (genetically determined) and secondary (acquired) forms of HLH become increasingly blurred together as new genetic causes are identified and patients who develop HLH beyond early childhood or in the contexts of EBV infection or autoimmune disease are being found to share some of the same genetic etiologies.

Diagnosis

Because of the severe nature of this disorder and the existence of disease-altering therapies, it is crucial to identify patients early in their course.

The Histiocyte Society established diagnostic criteria for the HLH-2004 trial, which is widely used for patients not treated in this trial, using both clinical and laboratory findings (see Table 53.5). Although imperfect, these criteria are widely accepted for the diagnosis of HLH.

Laboratory verification of a known genetic defect confirms the diagnosis independent of the presence or absence of any clinical signs or symptoms. However, genetic diagnosis is not usually available in a timely fashion for patients with acute clinical presentations. In the absence of a genetic diagnosis, five of the eight clinical criteria must be met to make a diagnosis. It is important to note that NK cell dysfunction is only present in about 50% of patients with HLH. Additionally, hemophagocytosis may not be present early in the HLH disease process. The HLH-probability score (HScore) was developed as a diagnostic tool for reactive HLH in adults.[77]

Initial symptoms of HLH are nonspecific and may overlap with other inflammatory or hematopoietic diseases. No single sign or symptom has sensitivity and specificity to allow an unambiguous HLH diagnosis. Close clinical observation with repeated laboratory assessments are mandatory for diagnosis. Thus, conditions fulfilling this syndromic definition may be conceptually split into *HLH disease* (F-HLH and others) and *mimickers* of HLH disease. Fig. 53.9 displays a practical algorithm from the NACHO consortium to aid clinicians make a timely diagnosis while excluding "HLH mimics." Why is this concept important? HLH typically requires prompt recognition and treatment, often before all data can be gathered. However, this aggressive stance should be balanced by a cautious and careful exclusion of conditions that look like HLH but would not benefit from or even be harmed by immune suppression and may require different treatments. HLH triggers/activators should be persistently sought, despite ongoing HLH treatment. Undiagnosed malignancy is especially problematic as HLH-directed therapy often obscures diagnosis. Moreover, the cases should be divided by the HLH activator because each has its characteristics and treatment. Specific etiologies for HLH are discussed at the end of this chapter.

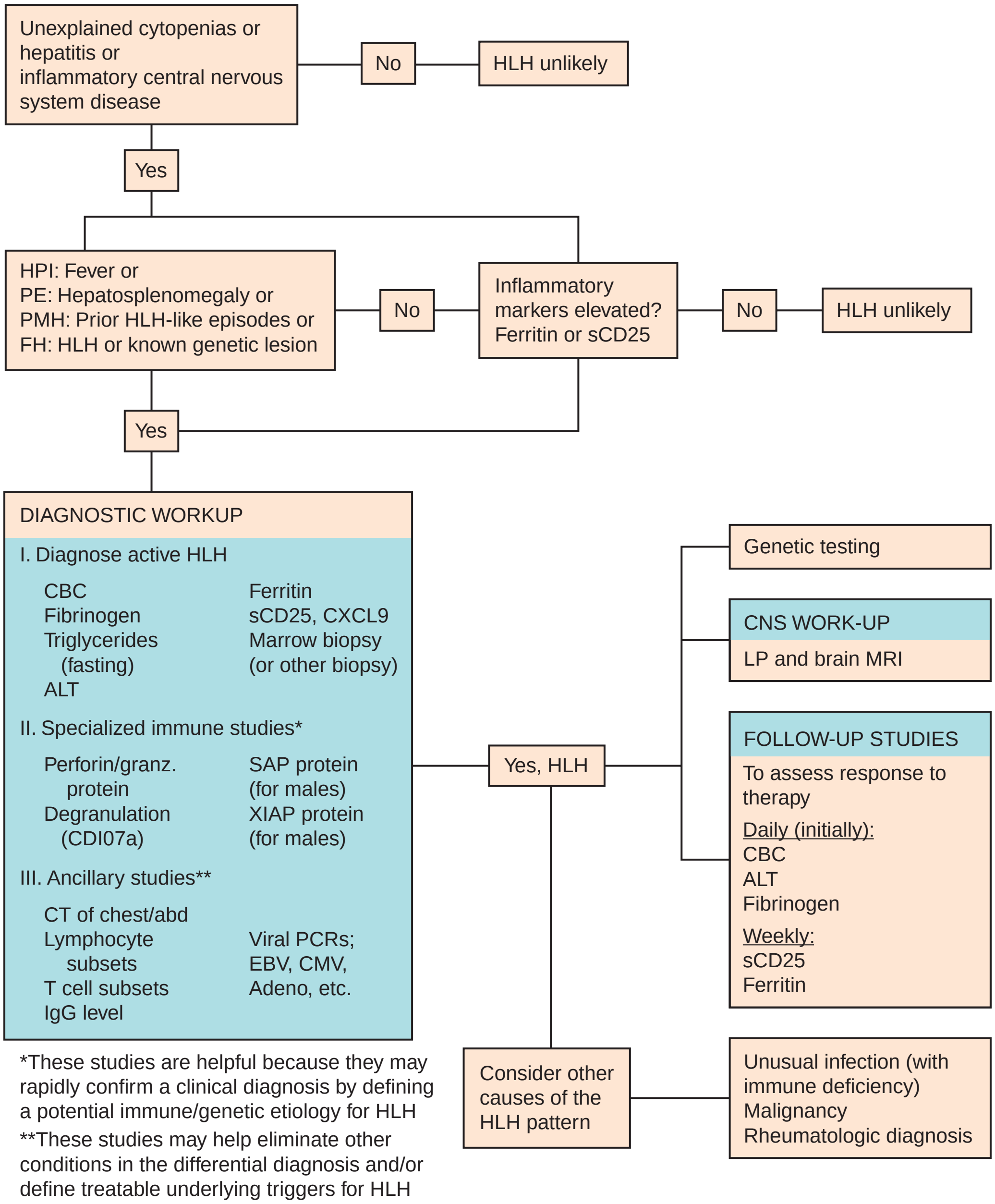

Unexplained cytopenias or hepatitis or inflammatory central nervous system disease
No
HLH unlikely
Yes
HPI: Fever or
PE: Hepatosplenomegaly or
PMH: Prior HLH-like episodes or
FH: HLH or known genetic lesion
No
Inflammatory markers elevated? Ferritin or sCD25
No
HLH unlikely
Yes
DIAGNOSTIC WORKUP
I. Diagnose active HLH
CBC
Fibrinogen
Triglycerides (fasting)
ALT
Ferritin
sCD25, CXCL9
Marrow biopsy (or other biopsy)
II. Specialized immune studies*
Perforin/granz. protein
Degranulation (CDI07a)
SAP protein (for males)
XIAP protein (for males)
III. Ancillary studies**
CT of chest/abd
Lymphocyte subsets
T cell subsets
IgG level
Viral PCRs; EBV, CMV, Adeno, etc.
Yes, HLH
Genetic testing
CNS WORK-UP
LP and brain MRI
FOLLOW-UP STUDIES
To assess response to therapy
Daily (initially):
CBC
ALT
Fibrinogen
Weekly:
sCD25
Ferritin
Consider other causes of the HLH pattern
Unusual infection (with immune deficiency)
Malignancy
Rheumatologic diagnosis
*These studies are helpful because they may rapidly confirm a clinical diagnosis by defining a potential immune/genetic etiology for HLH
**These studies may help eliminate other conditions in the differential diagnosis and/or define treatable underlying triggers for HLH

Figure 53.9 THE DIAGNOSTIC ALGORITHM FOR HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS. *(Adapted from Jordan MB, Allen CE, Greenberg J, et al. Challenges in the diagnosis of hemophagocytic lymphohistiocytosis: recommendations from the North American Consortium for Histiocytosis (NACHO). 2019. Pediatr Blood Cancer. 2019;66(11):e27929.)*

Clinical Manifestations

The classic presentation of HLH consists of prolonged, hectic fevers (usually present for 1 to 2 weeks before diagnosis), hepatosplenomegaly, and cytopenias. Neurologic symptoms are common and distinctive features of these patients; However, HLH may present in many forms, including fever of unknown origin (FUO); hepatitis or acute liver failure; and sepsis-like, Kawasaki-like, and primary neurologic abnormalities. Not all of the HLH diagnostic criteria may be present initially, so it is important to follow clinical signs and laboratory markers of pathologic inflammation repeatedly to identify the trends. Typical clinical features seen in patients with HLH, grouped by organ system, are described in the following text.

Prolonged Fever

FUO is a very common diagnosis in general pediatric wards, and differentiating HLH from other causes of FUO may be challenging. In one series, patients ultimately diagnosed with HLH presented with fevers above 102°F (38.8°C) for a median of 19 days (range: 4 to 41 days). In patients with FUO, cytopenias, highly elevated ferritin (>3000 g/dL), or sCD25 significantly above age-adjusted normal ranges generally suggest that a complete HLH diagnostic evaluation should be pursued.

Liver Disease and Coagulopathy

Most patients with HLH have variable evidence of hepatitis at presentation. HLH should be considered in the differential diagnosis of acute liver failure, especially if lymphocytic infiltrates are noted on biopsy. Autopsy evaluation of the liver has shown chronic persistent hepatitis with periportal lymphocytic infiltration in the majority of patients. Neonates with HLH may present with hydrops fetalis and liver failure. Most patients have evidence of disseminated intravascular coagulation (DIC) and are at high risk for acute bleeding. Furthermore, patients with HLH caused by degranulation defects may have intrinsic platelet dysfunction.

Bone Marrow Failure

Anemia and thrombocytopenia occur in more than 80% of patients at the time of presentation with HLH. The cellularity of BM aspirates varies from normocellular to hypocellular or hypercellular. The prevalence of hemophagocytosis in association with HLH diagnosis ranges from 25% to 100%. Although hemophagocytosis in BM is associated with HLH, the morphologic phenomenon may also be induced by more common events, including blood transfusions, infection, autoimmune disease, and other forms of BM failure or causes of red blood cell destruction. Despite the nomenclature of HLH, diagnosis of HLH should never be made or excluded solely on the presence or absence of hemophagocytosis. Infiltration of BM or liver by CD163$^+$ macrophages, along with global clinical evaluation, may distinguish HLH from other causes of hemophagocytosis.

Skin Manifestations

Patients may have a variety of skin manifestations, including generalized maculopapular erythematous rashes, generalized erythroderma, edema, panniculitis, inflamed papular lesions, petechiae, and purpura. The incidence of skin manifestations ranges from 6% to 65% in published series with highly pleomorphic presentations. Some patients may present with features suggestive of Kawasaki disease, including erythematous rashes, conjunctivitis, red lips, and enlarged cervical lymph nodes. Rashes may correlate with lymphocyte infiltration on skin biopsy, and hemophagocytosis may also be found.

Pulmonary Dysfunction

Patients may develop pulmonary dysfunction that leads to urgent admission to the intensive care unit. In a review of the radiographic abnormalities in 25 patients, 17 had acute respiratory failure with alveolar or interstitial opacities, with fatal outcomes in 88% of those cases.

Brain, Ophthalmic, and Neuromuscular Symptoms

More than one-third of patients present with neurologic symptoms, including seizures, meningismus, decreased level of consciousness, cranial nerve palsy, psychomotor retardation, ataxia, irritability, or hypotonia. The cerebrospinal fluid (CSF) is abnormal in more than 50% of HLH patients with findings of pleocytosis, elevated protein, or hemophagocytosis. MRI findings are highly variable and include discrete lesions, leptomeningeal enhancement, or global edema, and images correlate with neurologic symptoms. Retinal hemorrhages, swelling of the optic nerve, and infiltration of the choroid have been reported in infants with HLH. Diffuse peripheral neuropathy with pain and weakness secondary to myelin destruction by macrophages may also occur.

Laboratory Manifestations

Laboratory manifestations are a critical component of diagnosing HLH. Biochemical abnormalities noted on clinical laboratory assessment include anemia, thrombocytopenia, neutropenia, elevated liver transaminases, hyperbilirubinemia, hypofibrinogenemia, coagulation abnormalities, hypoalbuminemia, hyponatremia, hypertriglyceridemia, elevated soluble CD163, elevated soluble CD25 (sCD25; also called "soluble IL-2 receptor"), and hyperferritinemia. Specialized immunologic testing may reveal low or absent NK cell or cytotoxic T lymphocyte function (although this is not always seen), low levels of perforin or other disease-associated proteins (e.g., SAP or XIAP), and decreased degranulation (observed with mutations affecting granule trafficking). Additionally, pathologic examination of BM biopsy, liver biopsy, CSF, spleen or lymph node, or even occasionally peripheral blood may reveal hemophagocytosis and infiltration with CD163$^+$ macrophages and activated T cells (see Fig. 53.2M–Q). Examination of CSF reveals pleocytosis, elevated protein, and elevated neopterin levels with CNS involvement. Brain MRI often reveals polymorphic white or grey matter abnormalities in such cases.

Elevations in sCD25 are a particularly useful and notable laboratory feature of HLH. In the authors' experience, this marker is dynamically associated with "active" HLH, and patients with active HLH rarely, if ever, have normal levels of sCD25. Of note, the interpretation of soluble IL-2 receptor levels must be undertaken with care because normal levels change with age and the method of analysis. Despite a few small reports of increased soluble IL-2 receptor levels in sepsis and hematologic malignancies, significant elevations of this marker are rarely observed outside the context of HLH. However, the clinical utility of sCD25 is often compromised by delays because most institutions must send patient samples to referral laboratories. Similarly, though not as well established, because HLH appears to be primarily driven by IFN-γ, elevations of CXCL9 (a sensitive indicator of IFN-γ bioactivity) should be seen in untreated cases of HLH disease. Because of its ready availability in most hospitals, the serum ferritin level can serve as an important adjunct to the decision-making process. The HLH diagnostic guidelines define a cutoff at greater than 500 μg/L, which may be observed in sepsis or other hyperinflammatory conditions. Ferritin levels in HLH are usually dramatically higher, with some series finding mean levels near 45,000 μg/L. A review of elevated ferritin values at a large pediatric academic tertiary care hospital demonstrated that a ferritin level greater than 10,000 was 90% sensitive and 96% specific for HLH.

While specialized immunologic testing may facilitate diagnosis, if a diagnosis can be made without them, then treatment should not be delayed pending these results. Likewise, treatment should not be delayed for assessment of CNS involvement, though this should always be conducted (once a lumbar puncture may be safely performed).

Differential Diagnosis of Hemophagocytic Lymphohistiocytosis

Because HLH presents as an inflammatory syndrome, the differential diagnosis of this disorder is a broad one. Infection must be considered as either a mimic of HLH or as an underlying trigger of the disorder. The list of infections reported as associated with HLH is extensive. If the patient presents with acute multiorgan failure, then sepsis is generally considered first before a diagnosis of HLH is considered. Viral infections, including EBV, CMV, dengue, and severe influenza, should always be considered and treated. Protozoan infections, including malaria, toxoplasmosis, and leishmaniasis, may be a consideration in endemic areas. Visceral leishmaniasis, in particular, may be clinically indistinguishable from primary HLH. In addition to infectious disorders, rheumatologic disorders, including systemic onset juvenile idiopathic arthritis (soJIA) and Kawasaki syndrome, should be considered. Drug reactions such as Drug Rash with Eosinophilia and Systemic Symptoms (DRESS) syndrome may present as HLH as both a trigger and mimic as treatment requires both withdrawal of the offending agent and prolonged corticosteroids.

Storage diseases including Gaucher disease can mimic HLH (e.g., splenomegaly, cytopenias).

Finally, as either a mimic or trigger, malignancy should be considered, particularly lymphoma and leukemia.

Therapy

Without therapy, survival of patients with active F-HLH is historically reported to be approximately 2 months. Often, the principal challenge for treating patients with HLH is making a timely and accurate diagnosis. Rapid treatment of HLH is essential, especially for those who are acutely ill or clinically deteriorating. Currently, the standard of care for HLH should be considered to be treatment with etoposide and dexamethasone, the HLH-94 protocol, as illustrated in Fig. 53.10. The protocol is based on an 8-week induction therapy with dexamethasone, etoposide, and intrathecal methotrexate. The principal goal of induction therapy is to suppress the life-threatening inflammatory process that underlies HLH. At the end of 8 weeks, patients are either weaned off therapy or transitioned to continuation therapy, which is intended only as a bridge to transplantation. HLH-94 treatment components are highly effective in treating hyper inflammation in adults with HLH. However, due to the heterogeneity of HLH disease in adults, the protocol is not suitable for all patients. Each adult with HLH should be treated based on the trigger/activator as discussed at the end of this chapter.

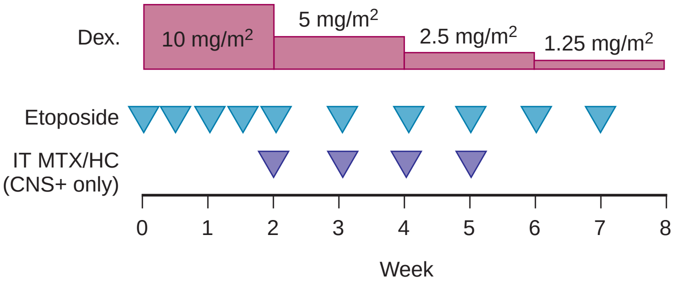


Figure 53.10 INDUCTION THERAPY FOR HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS (HLH). Based on the HLH-94 study, this approach should be considered standard of care for all patients not enrolled in clinical trials based on published evidence of efficacy. Etoposide is dosed as 150 mg/m^2 per dose. Alternatively, for patients weighing less than 10 kg, consideration may be given to dosing etoposide as 5 mg/kg per dose. Dexamethasone *(Dex.)* is dosed as indicated and may be given orally or intravenously, although the latter is preferred at therapy initiation. IT MTX/HC should be given to patients with evidence of CNS involvement as early as lumbar puncture may be safely performed (which may vary from the diagram) and dosed as follows: age younger than 1 year, 6/8 mg *(MTX/HC)*; age 1 to 2 years, 8/10 mg; age 2 to 3 years, 10/12 mg; and age older than 3 years, 12/15 mg. Weekly intrathecal therapy is generally continued until at least 1 week after resolution of CNS involvement (both clinical and cerebrospinal fluid indices). *CNS*, Central nervous system; *IT MTX/HC*, intrathecal methotrexate and hydrocortisone. *(Adapted from Jordan MB, Allen CE, Weitzman S, et al. How I treat hemophagocytic lymphohistiocytosis. Blood. 2011;118:4041–4052.)*

It is critical to search for and treat underlying triggers of HLH and institute specific antimicrobial therapy. Rituximab is often helpful in controlling EBV infection. Intravenous immunoglobulin (IVIG) is an appropriate adjunct for most viral infections. If the patient is stable and not severely ill, consideration can be given to treating the underlying trigger with disease-specific therapy with or without corticosteroids and close follow-up. However, in most cases, an aggressive therapeutic approach is warranted and may reasonably be initiated before obtaining final results for all diagnostic studies. Specifically, HLH therapy should not be withheld while awaiting results of genetic testing because our understanding of HLH-associated gene defects remains incomplete and testing typically takes weeks to complete. With the exception of autoimmune disease and malignancy, initial therapy for patients with suspected familial or reactive HLH does not differ.

Induction Therapy

The current standard of care consists of a decrescendo course of etoposide and dexamethasone with or without intrathecal therapy (see Fig. 53.10). Ideally, critically ill patients should be treated at facilities familiar with care of cancer and BMT patients. It is important to initiate therapy promptly even in the face of unresolved infections, cytopenias, or organ dysfunction. After starting therapy, patients should be monitored closely for signs of improvement as well as potential complications and toxicities, and therapy may need to be customized. Adult patients and especially the elderly ones may have comorbidities, making them more vulnerable to the toxicities of etoposide. A reduced etoposide frequency, from twice weekly to once a week, with or without a reduction on dose from 150 mg/m^2 to 50 to 100 mg/m^2 should be considered.[78] In the HLH-2004 study, cyclosporine was administered upfront instead of after 8 weeks, as in HLH-94, and pre-HCT mortality was slightly reduced. Because the improvement was not significant and does not justify the toxicities of the treatment, HLH-94 remains the recommended standard of care. For patients who respond well, with a resolution of symptoms and normalization of inflammatory markers, therapy may be weaned per protocol. However, dexamethasone doses and etoposide frequency may need to be increased in response to disease reactivation (see the Salvage Therapy section). Deterioration of liver function and blood counts as well as steady increases in serum ferritin, sCD25, and sCD163 tests may signal relapse of HLH disease activity. If patients do not display at least a partial response within 2 to 3 weeks of therapy initiation, salvage therapy should be considered. Recurrence of fever and increased inflammatory markers after an apparent response should also prompt a careful search for opportunistic infection. Most non-F-HLH adult patients may continue the full course of induction (8 weeks) if no major toxicity is limiting the treatment. However, many patients require less than 8 weeks.

Central Nervous System Disease

Patients may present with CNS involvement or may have recurrent disease as treatment doses are being tapered. All patients should receive a careful neurologic examination, lumbar puncture, and brain MRI, even if they are asymptomatic, as soon as they can be safely performed. Changes in mental status at any time during therapy should be investigated urgently. Patients with proven CNS involvement should be treated with weekly intrathecal methotrexate and hydrocortisone until CSF abnormalities and symptoms normalize. The risk of posterior reversible encephalopathy syndrome (PRES) appears to be significant during induction therapy. Although the etiology of PRES is incompletely understood, it is more frequent in settings of hypertension and is also associated with cyclosporine use. Blood pressure should be aggressively managed during induction. Because CNS involvement suggests a familial etiology and because this disease feature is associated with substantial risks for long-term morbidity, HCT should be considered for patients with this complication.

Supportive Care

Supportive care guidelines for patients on therapy for HLH should be similar to standard practice for patients undergoing HCT, including acute care nursing, *Pneumocystis jiroveci* prophylaxis, fungal prophylaxis, IVIG supplementation, and neutropenic precautions. Any new fever should be evaluated for HLH reactivation, as well as opportunistic infection, and empiric broad-spectrum antibiotic therapy should be initiated. Because of inflammation, consumptive coagulopathy, and intrinsic platelet defects in some patients, they are at a very high risk of spontaneous bleeding. The authors aim to maintain platelet count greater than 50×10^9/L and do not recommend prophylactic heparin, which is sometimes used in acutely ill patients. Platelets, fresh-frozen plasma, cryoprecipitate, and occasionally activated factor VII are required for acute bleeding.

Continuation Therapy

Patients who can be weaned off of dexamethasone and etoposide without recurrence, recover normal immune function, and have no identified HLH-associated gene defects may stop therapy after the 8-week induction course. HCT is generally recommended in patients with age younger than 2 years, CNS involvement, recurrent or refractory disease, persistent NK cell dysfunction, or proven familial or genetic disease. Continuation according to HLH-94 consists of pulses of dexamethasone and etoposide (etoposide,150 mg/m^2 every 2 weeks alternating with dexamethasone 10 mg/m^2/day for 3 days every 2 weeks). Cyclosporine may be added in patients with stable blood pressure and adequate liver and kidney function. Patients on continuation therapy should proceed to HCT as quickly as possible because of the ongoing risks of infection, disease reactivation, or leukemia or myelodysplastic syndrome related to prolonged use of etoposide.

Salvage or Second-Line Therapy

A significant number of patients with HLH either fail to respond adequately to current therapies or relapse before HCT. Approximately 50% of patients treated in the HLH-94 study experienced a complete resolution of HLH, 30% experienced a partial resolution, and approximately 20% died before HCT. Notably, most deaths occurred during the first few weeks of treatment and may reflect either preexisting morbidities or primary refractory disease. Although it is hoped that some patients will fare better with more prompt diagnosis of HLH, others remain unresponsive to standard therapy. Emapalumab, an IFN-γ blocking monoclonal antibody, was recently approved by the US Food and Drug administration for refractory or recurrent HLH after a successful clinical trial.[73] Alemtuzumab, anti-CD52, was reported to have significant activity against refractory HLH in a retrospective series of patients and can be used as a bridge for HCT. Although refractory HLH appears to have a dismal prognosis, approximately 70% of patients in this series survived. Because of its immunoablative qualities, alemtuzumab should be used with caution and by those with experience caring for profoundly immune-compromised patients. CMV reactivation and adenoviremia were frequent complications of this therapy. In contrast to refractory patients, those patients who initially respond well to standard therapy but then relapse as treatment is tapered or withdrawn often respond to the reintensification of therapy with standard agents. Because of the variability in patient responses, a critical aspect of initial or salvage therapy is close monitoring of the patients for improvement and potential toxicities such as BM suppression or infection.

Hematopoietic Cell Transplantation

Transplantation is performed to prevent potentially fatal HLH disease recurrence. Because the time to transplant is a factor in morbidity and mortality from the disease, a donor search should begin at the time of diagnosis even though the precise etiology of HLH (e.g., genetic defect) has not yet been defined. Generally, HCT is recommended in cases of documented familial HLH, recurrent or progressive disease despite intensive therapy, and CNS involvement. Moreover, patients with a hematologic malignancy that cannot be cured with conventional agents (but may be treated with HCT) should also proceed to transplant.[79] Long-term disease-free survival after HCT was approximately 50% to 65% before the year 2000, regardless of whether a matched sibling or closely matched unrelated donor was used. Most patients transplanted during that era succumbed to “transplant-related” complications during the first 100 days after infusion. A significant proportion of fatal complications involved inflammatory conditions termed *acute respiratory distress syndrome*, *veno-occlusive disease*, and *multisystem organ failure*, *unspecified*. In rare cases, residual HLH was identified at autopsy despite the use of myeloablative conditioning therapy.

During the past decade, the use of reduced-intensity conditioning (RIC) regimens before HCT has been investigated after encouraging results from an institutional series.[80,81] Most cases of RIC pretreatment have included alemtuzumab and demonstrated superior early post-transplant survival. In a single-center analysis directly comparing HCT outcomes after myeloablative conditioning versus RIC, a statistically significant improvement was observed after RIC conditioning, with all patients surviving at 6 months after transplant. Published data regarding outcome of RIC transplants using umbilical cord blood are not sufficient to draw conclusions regarding safety or efficacy. A retrospective European study did not show superiority of RIC over myeloablative conditioning (MAC) in adults. Donor choice should also take into account the possibility of an occult predisposition to HLH in siblings of patients without identified gene defects. Much remains to be learned and refined regarding the optimal application of alemtuzumab as well as other agents used before HCT. The timing of pretransplant alemtuzumab impacts the probability of graft-versus-host disease, mixed chimerism, and, in rare cases, rejection. Other factors, such as donor source, human leukocyte antigen match, cell dose, and patient condition with regard to HLH disease activity at the time of conditioning may all play roles in determining the likelihood of success after RIC HCT. The pretransplant conditioning regimen in malignancy-associated HLH may be guided by disease-specific protocols.

Patients with CNS HLH need close post-transplant follow-up. The authors recommend examination of CSF within 100 days of HCT even in asymptomatic patients. Follow-up MRIs are recommended if pretransplant abnormalities were present. In some patients with mixed or full hematopoietic donor chimerism, HLH disease activity in the CSF can be effectively treated with intrathecal therapy during the early post-transplant months. CNS disease is subsequently controlled as donor immune reconstitution progresses.

Prognosis

Significant strides have been made in the treatment of HLH, with survival now generally ranging from 50% to 70%. In children with nonfamilial HLH, overall survival has been reported at 72%, but only 20% of the patients did not require HCT. Survival is increased in children, irrespective of genetic status, who receive HSCT from matched rather than unmatched donors. RIC pretransplant regimens appear to further decrease mortality. The best outcomes in HSCT are seen in children who have a rapid and complete response to pretransplant

TABLE 53.7 **Distinctive Clinical Contexts in Which Hemophagocytic Lymphohistiocytosis May Arise**

F-HLH	M-HLH	R-HLH	Rx-HLH	IC-HLH
Defects in granule-mediated cytotoxicity	Malignancy triggered HLH	R-HLH triggered by soJIA	Cytokine storm associated with CAR-T-cell therapy	**Primary**[a]
Disorders of inflammasome activation	HLH during chemotherapy	R-HLH triggered by lupus	Cytokine storm associated with checkpoint blockade	SCID
Disorders of T cell signaling	EBV associated lymphoproliferative disease	R-HLH triggered by vasculitis		Omenn syndrome
Disorders of macrophages inflammatory signaling				Wiskott-Aldrich syndrome
				X-linked agammaglobulinemia
				Autoimmune lymphoproliferative syndrome
				Secondary
				Patients receiving immunosuppressive therapy in the context of immunosuppression
All HLH subtypes can be triggered by viruses, mainly EBV and CMV				

[a] Some primary immune deficiency disorders are only a disease mimic and would not benefit from immunosuppression.
CAR, chimeric antigen receptor; *CGD*, chronic granulomatous disease; *CMV*, cytomegalovirus; *EBV*, Epstein-Barr virus; *F-HLH*, familial hemophagocytic lymphohistiocytosis; *HLH*, hemophagocytic lymphohistiocytosis; *IC-HLH*, HLH in the context of immune compromise; *M-HLH*, malignancy associated HLH; *R-HLH*, rheumatologic disease associated HLH; *Rx-HLH*, HLH in the context of immune-activating therapies; *SCID*, severe combined immunodeficiency.

therapies and who do not exhibit significant neurologic involvement. Prompt initiation of HSCT in familial patients after disease remission is obtained is also likely to increase survival. Patients with significant neurologic involvement may experience severe and permanent sequelae even if they survive.

DISTINCTIVE CLINICAL CONTEXTS

HLH, especially in adults can arise in different clinical contexts, summarized in Table 53.7.

HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS ASSOCIATED WITH MALIGNANCIES

Malignancy may present with HLH at all ages (including infancy), but is increasingly likely at older ages, and is associated with the majority of cases in adults. About 40% to 70% of HLH cases in adults are malignancy-associated (M-HLH). The association of HLH with malignancy has been recognized for decades. Patients may present with the clinical syndrome of HLH associated with undiagnosed underlying malignancy or transformation of an indolent hematologic malignancy, or they may develop HLH during treatment for known malignancy, usually in the context of infection.[82] The pathophysiology of HLH associated with malignancies is not well defined, and the tumor itself may “mimic” the HLH diagnostic criteria. However, the prognosis of these patients is abysmal and the overall survival of patients can be as low as 10% to 20% 5 years overall survival. Lymphoma deserves special mention as it is the most common malignancy associated with HLH at its initial presentation.

Diagnosis and Treatment

Because of the difficulty of distinguishing lymphoma from F-HLH or R-HLH, thorough history and imaging, adults with unexplained HLH should have a thorough evaluation for lymphoma. sCD25 may be disproportionately elevated compared to other features of HLH in patients with occult lymphoma. A ratio of Scd25/ferritin that is >2 strongly supports the presence of lymphoma in the context of HLH syndrome. A tissue biopsy, often guided by PET-CT, should be pursued or at least considered before starting corticosteroids and other therapies that may obscure diagnosis. In patients with HLH of unknown etiology and splenomegaly, splenectomy may be considered to detect lymphomas hiding in the spleen. Treatment of M-HLH should balance HLH-specific and tumor-specific therapy. Corticosteroids are commonly used as a first-line treatment to combat inflammation. In highly active HLH, etoposide (50 to 100 mg/m^2) may be added prior to tumor-specific treatment. Etoposide may be added to CHOP or CHOP-like protocols (cyclophosphamide, doxorubicin, vincristine, etoposide, prednisone) or dose-adapted etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin [DA-EPOCH]). Patients in remission who are eligible for treatment intensification may be candidates for autologous SCT, using high-dose etoposide-containing high-dose chemotherapy as primary consolidation. In patients with a malignancy that can only be cured with allogeneic HCT, early HLA phenotyping and decision-making regarding transplant should be made in a timely manner.[83]

When considering M-HLH, it is essential to note that the presence of EBV viremia does not rule out malignancy (including B or T cell lymphomas). On the contrary, EBV by itself can signify the presence of a lymphoproliferative disorder. A weekly treatment with rituximab should be administered (375 mg/m^2 once weekly for 2 to 4 times or until PCR level goes down to 0). If the PCR levels increase with a worsening of HLH signs and symptoms, attempts should be made to identify the affected cell lineage (by sorted PCR of peripheral blood mononuclear cells/ BM biopsy). Patients from Asian and Hispanic countries tend to develop lymphoproliferative disease. These patients should be considered as having EBV lymphoproliferative disorder (EBV-LPD). The treatment of EBV-LPD should start with HLH therapy, followed by CHOP chemotherapy (cyclophosphamide, hydroxydaunorubicin, vincristine, and prednisone) and plan for HCT. Recent reports have shown an impressive response of HLH associated with EBV-LPD to nivolumab (PD-1 checkpoint inhibitors).

HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS IN THE CONTEXT OF A RHEUMATOLOGIC DISEASE (R-HLH)

MAS is the name commonly given to a severe, potentially fatal inflammatory condition seen in the context of rheumatologic disorders such as soJIA or systemic lupus erythematosus (SLE). The syndrome of MAS shares many similarities with classic HLH, and many investigators view it as a special form of HLH, suggesting that the name may be changed to rheumatologic HLH (or R-HLH). The main manifestations of MAS include fever, hepatosplenomegaly, lymphadenopathy, severe cytopenias, serious liver disease, and coagulopathy consistent with DIC. Hemophagocytosis is often (although not always) seen in the BM of patients with MAS.

The true incidence of MAS may be underestimated because relatively mild cases of MAS often remained unrecognized. Recent evidence suggests that mild subclinical MAS occurs in as many as one-third of patients with active soJIA and may be the first manifestation of soJIA. Infections or change in medications may precede the diagnosis of MAS. However, in most patients, MAS is triggered by a flare-up of the underlying rheumatologic disease.[84] Published observations suggest that as in HLH, MAS patients have profoundly depressed NK cell function[85] sometimes associated with abnormal perforin expression, and these abnormalities are associated with specific perforin and Munc13-4 polymorphisms.

Diagnosis and Treatment

There are no validated diagnostic criteria for MAS, and early diagnosis is often difficult. A recent consensus conference has developed expert-based *classification* criteria for MAS, although these have not been validated as *diagnostic* criteria in clinical trials. Thus, in a patient with persistently active underlying rheumatologic disease, a fall in the ESR and platelet count, particularly in combination with persistently high C-reactive protein and increasing levels of ferritin, should raise a suspicion of impending MAS. The diagnosis of MAS is usually confirmed by the demonstration of hemophagocytosis in the BM. Assessment of the levels of sCD25 and sCD163 in serum may help with the timely diagnosis of MAS. Although mild elevation of sCD25 has been reported in many rheumatic diseases, including JIA and SLE, a several-fold increase in the levels of sIL2Rα in these diseases is highly suggestive of MAS. The application of the HLH diagnostic criteria to systemic JIA patients with suspected MAS is problematic. Some of the HLH markers, such as lymphadenopathy, splenomegaly, and hyperferritinemia, are common features of active systemic JIA itself and therefore do not distinguish MAS from a conventional systemic JIA flare. Patients with systemic JIA often have increased white blood cell and platelet counts, as well as serum levels of fibrinogen as a part of the inflammatory response seen in this disease. Therefore, when they develop MAS, they reach the degree of cytopenias and hypofibrinogenemia seen in HLH only at the late stages of the syndrome when medical management becomes challenging. This is even more problematic for the diagnosis of MAS in patients with SLE in whom autoimmune cytopenias are common and difficult to distinguish from those caused by MAS.

Early recognition of this syndrome and immediate therapeutic intervention to produce a rapid response are critical. Prompt administration of more aggressive treatment in these patients may, in fact, prevent development of the full-blown syndrome. To achieve rapid reversal of coagulation abnormalities and cytopenias, most clinicians start with intravenous methylprednisolone pulse therapy (30 mg/kg for 3 consecutive days) followed by 2 to 3 mg/kg/day divided into four doses. After normalization of hematologic abnormalities and resolution of coagulopathy, steroids are tapered slowly to avoid relapses of MAS. Commonly, however, MAS appears to be corticosteroid-resistant, with deaths being reported even among patients treated with massive doses of steroids. Parenteral administration of cyclosporine A has been shown to be highly effective in patients with corticosteroid-resistant MAS. The utility of biologic drugs in MAS treatment remains unclear. Although tumor necrosis factor-inhibiting agents, biologics that neutralize IL-1 and IL-6, have been reported to be effective in occasional MAS patients, other reports describe patients in whom MAS occurred while they were receiving these agents. Recently, there have been reports on chronic complications with these agents such as lung disease. Based on some success with IVIG administration in virus-associated HLH, this treatment might be effective in MAS triggered by viral infection. If MAS, however, is driven by EBV infection, rituximab, a monoclonal antibody that depletes B lymphocytes (the main type of cells harboring the EBV virus), may be considered.

HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS IN THE CONTEXT OF IMMUNE-ACTIVATING THERAPIES (RX-HLH)

The HLH syndrome develops in some patients receiving immune-activating therapies, such as T cell–engaging antibodies, chimeric antigen receptor (CAR) T cells, or immune checkpoint inhibitors. In this context, this syndrome is usually referred to as cytokine release syndrome (CRS). However, its pathophysiology appears quite similar to F-HLH and should be recognized as iatrogenic HLH, or Rx-HLH.

Diagnosis and Treatment

The anti-IL-6 antibody tocilizumav has been used with rapid resolution of CRS in patients after CAR T cell immunotherapies.[86] A CART cell associated toxicity working group suggests that CRS should be managed with anti-IL-6 and corticosteroids for organ toxicities higher than grade 3. If the patient has no clinical or laboratory improvement within 48 hours, additional therapy with etoposide (75 to 100 mg/m^2 should be considered). Treatment interruption or corticosteroids alone have been used with successful outcomes in CRS induced by immune checkpoint inhibitors.

HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS IN THE CONTEXT OF IMMUNE COMPROMISE (IC-HLH)

HLH can occur in a variety of immune-compromised patients including primary immune deficiency (PID) or patients receiving immunosuppressive therapy, mostly in the context of unresolved infection. For instance, patients with inflammatory bowel disease (IBD), usually treated with azathioprine or mercaptopurine, have been reported to develop (usually relatively mild) HLH after infection with EBV or CMV. In these contexts, the pathophysiology is not well understood. While it may be related to the underlying disease, HLH appears to be a dysregulated response to infection in the context of immunosuppression.

A variety of PIDs have been reported to present with HLH (detailed in Table 53.7). Patients with PID and HLH often have unresolved, severe infections. Patients with severe combined immunodeficiency (SCID) most often have viral infections, while those with chronic granulomatous disease (CGD) present with bacterial infections. Thus, the presence of HLH associated with unusual or unusually severe infection should suggest undiagnosed immune deficiency.[87]

Diagnosis and Treatment

The diagnosis is based on a history of immunosuppression (primary or secondary to drug use) or unusually severe infection as the initial presentation of a PID. Some of the IC-HLH can be included in the HLH disease category and as thus, will gain from immunosuppression while others should be considered as HLH mimics (some PIDs). IBD patients should be treated with drug withdrawal, treatment of infection, and a moderate dose of corticosteroids +/- etoposide.

Future Directions

Although HLH appears to be a disease of excessive immune activation, the ideal form of immune suppression and anti-inflammatory therapy remains unknown. Although somewhat responsive to corticosteroids and clearly responsive to etoposide or anti-T-cell serotherapy, such as ATG or alemtuzumab, HLH remains difficult to treat. However, newer, targeted therapies, based on insights from animal models, were developed and are being tested in clinical trials. Anti-IFN-γ, emapalumab, is the first targeted therapy approved and is now being examined for frontline use in HLH and in specific subsets (R-HLH, M-HLH). A JAK1/2 inhibitor (ruxolitinib) is being investigated currently in several clinical trials. Anti-cytokine treatment is common in R-HLH (IL-1 or IL-6 blocking agents), though these patients are increasingly diagnosed with chronic complications such as lung disease. The blockade of IL-18 is being investigated for patients with HLH driven by the inflammasome. It is likely that such targeted therapies will eventually supersede current approaches.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Allen CE, Ladisch S, McClain KL. How I treat Langerhans cell histiocytosis. *Blood*. 2015;126:26–35.

Allen CE, Li L, Peters TL, et al. Cell-specific gene expression in Langerhans cell histiocytosis lesions reveals a distinct profile compared with epidermal Langerhans cells. *J Immunol*. 2010;184:4557–4567.

Allen CE, Merad M, McClain KL. Langerhans-cell histiocytosis. *N Engl J Med*. 2018;379:856–868.

Badalian-Very G, Vergilio JA, Degar BA, et al. Recurrent BRAF mutations in Langerhans cell histiocytosis. *Blood*. 2010;116:1919–1923.

Berres ML, Lim KP, Peters T, et al. BRAF-V600E expression in precursor versus differentiated dendritic cells defines clinically distinct LCH risk groups. *J Exp Med*. 2014;211:669–683.

Diamond EL, Durham BH, Ulaner GA, et al. Efficacy of MEK inhibition in patients with histiocytic neoplasms. *Nature*. 2019;567:521–524.

Emile JF, Abla O, Fraitag S, et al. Revised classification of histiocytoses and neoplasms of the macrophage-dendritic cell lineages. *Blood*. 2016;127(22):2672–2681.

Favara BE, Feller AC, Pauli M, et al. Contemporary classification of histiocytic disorders. The WHO committee on histiocytic/reticulum cell proliferations. Reclassification Working Group of the Histiocyte Society. *Med Pediatr Oncol*. 1997;29:157–166.

Ginhoux F, Schultze JL, Murray PJ, et al. New insights into the multidimensional concept of macrophage ontogeny, activation and function. *Nat Immunol*. 2016;17:34–40.

Henter JI, Horne A, Aricó M, et al. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer*. 2007;48:124–131.

Jordan MB, Allen CE, Weitzman S, et al. How I treat hemophagocytic lymphohistiocytosis. *Blood*. 2011;118:4041–4052.

Locatelli F, Jordan MB, Allen C, et al. Emapalumab in children with primary hemophagocytic lymphohistiocytosis. *N Engl J Med*. 2020;382:1811–1822.

McClain KL, Picarsic J, Chakraborty R, et al. CNS Langerhans cell histiocytosis: common hematopoietic origin for LCH-associated neurodegeneration and mass lesions. *Cancer*. 2018;124(12):2607–2620.

Merad M, Ginhoux F, Collin M. Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. *Nat Rev Immunol*. 2008;8:935–947.

Merad M, Sathe P, Helft J, et al. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol*. 2013;31:563–604.

Rodriguez-Galindo C, Allen CE. Langerhans cell histiocytosis. *Blood*. 2020;135:1319–1331.

Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. II. *Functional properties in vitro. J Exp Med*. 1974;139:380–397.

Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med*. 1973;137:1142–1162.

Willman CL, Busque L, Griffith BB, et al. Langerhans'-cell histiocytosis (histiocytosis X)—a clonal proliferative disease. *N Engl J Med*. 1994;331:154–160.

Yeh EA, Greenberg J, Abla O, et al. Evaluation and treatment of Langerhans cell histiocytosis patients with central nervous system abnormalities: current views and new vistas. *Pediatr Blood Cancer*. 2018;65(1). Jan.

REFERENCES

1. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med*. 1973;137:1142–1162.
2. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. J Exp Med. 1974;139:380–397.
3. Ginhoux F, Schultze JL, Murray PJ, et al. New insights into the multidimensional concept of macrophage ontogeny, activation and function. *Nat Immunol*. 2016;17:34–40.
4. Merad M, Sathe P, Helft J, et al. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol*. 2013;31:563–604.
5. Favara BE, Feller AC, Pauli M, et al. Contemporary classification of histiocytic disorders. The WHO committee on histiocytic/reticulum cell proliferations. reclassification working group of the histiocyte society. *Med Pediatr Oncol*. 1997;29:157–166.
6. Emile JF, Abla O, Fraitag S, et al. Revised classification of histiocytoses and neoplasms of the macrophage-dendritic cell lineages. *Blood*. 2016;127(22):2672–2681.
7. Allen CE, Merad M, McClain KL. Langerhans-cell histiocytosis. *N Engl J Med*. 2018;379:856–868.
8. Langerhans P. Ueber die nerven der menschlichen haut. *Arch pathol Anat*. 1868:325.
9. Christian HA. Defects in membranous bones, exophthalmos and diabetes insipidus; an unusual syndrome of dyspituitarism. *Med Clin North Am*. 1919;3:849.
10. Hand AJ. Polyuria and tuberculosis. *Arch Pediatr*. 1893;10:673.
11. Schüller A. Über eigenartige schädeldefekte im jugendalter. *Fortschri Geb Roentgenstrahlen*. 1915;23:12.
12. Letterer E. Aleukämische retikulose (Ein Beitrag zu den proliferativen Erkankungen des Retikuloendothelialapparetes). *Frankf Z Pathol*. 1924:377.
13. Siwe SA. Reituloendotheliose-ein neues krankheitsbild under den Hepatosplenomegalien. *Z Kinderheilk*. 1933:377.
14. Lichtenstein L, Histiocytosis X. Integration of eosinophilic granuloma of bone, letterer-siwe disease, and schuller-christian disease as related manifestations of a single nosologic entity. *AMA Arch Pathol*. 1953;56: 84–102.
15. Nezelof C, Basset F, Rousseau MF. Histiocytosis X histogenetic arguments for a Langerhans cell origin. *Biomedicine*. 1973;18:365–371.
16. Stalemark H, Laurencikas E, Karis J, et al. Incidence of Langerhans cell histiocytosis in children: a population-based study. *Pediatr Blood Cancer*. 2008;51:76–81.
17. Girschikofsky M, Arico M, Castillo D, et al. Management of adult patients with Langerhans cell histiocytosis: recommendations from an expert panel on behalf of Euro-Histio-Net. *Orphanet J Rare Dis*. 2013;8:72.
18. Arico M, Girschikofsky M, Genereau T, et al. Langerhans cell histiocytosis in adults. Report from the international registry of the histiocyte society. *Eur J Cancer*. 2003;39:2341–2348.
19. Ribeiro KB, Degar B, Antoneli CB, et al. Ethnicity, race, and socioeconomic status influence incidence of Langerhans cell histiocytosis. *Pediatr Blood Cancer*. 2015;62:982–987.
20. Peckham-Gregory EC, Chakraborty R, Scheurer ME, et al. A genome-wide association study of LCH identifies a variant in SMAD6 associated with susceptibility. *Blood*. 2017;130:2229–2232.
21. Cohen AF, Roos-Weil D, Armand M, et al. High frequency of clonal hematopoiesis in Erdheim-Chester disease. *Blood*. 2020
22. Merad M, Ginhoux F, Collin M. Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. *Nat Rev Immunol*. 2008;8:935–947.
23. Willman CL, Busque L, Griffith BB, et al. Langerhans'-cell histiocytosis (histiocytosis X)—a clonal proliferative disease. *N Engl J Med*. 1994;331:154–160.
24. Yu RC, Chu C, Buluwela L, Chu AC. Clonal proliferation of Langerhans cells in Langerhans cell histiocytosis. *Lancet*. 1994;343:767–768.
25. Allen CE, Li L, Peters TL, et al. Cell-specific gene expression in Langerhans cell histiocytosis lesions reveals a distinct profile compared with epidermal Langerhans cells. *J Immunol*. 2010;184:4557–4567.
26. Badalian-Very G, Vergilio JA, Degar BA, et al. Recurrent BRAF mutations in Langerhans cell histiocytosis. *Blood*. 2010;116:1919–1923.
27. Allen CE, Parsons DW. Biological and clinical significance of somatic mutations in Langerhans cell histiocytosis and related histiocytic neoplastic disorders. *Hematology Am Soc Hematol Educ Program*. 2015;2015:559–564.
28. Berres ML, Lim KP, Peters T, et al. BRAF-V600E expression in precursor versus differentiated dendritic cells defines clinically distinct LCH risk groups. *J Exp Med*. 2014;211:669–683.
29. Allen CE, Ladisch S, McClain KL. How we treat Langerhans cell histiocytosis. *Blood*. 2015
30. Yeh EA, Greenberg J, Abla O, et al. Evaluation and treatment of Langerhans cell histiocytosis patients with central nervous system abnormalities: Current views and new vistas. *Pediatr Blood Cancer*. 2018;65(1).
31. McClain KL, Picarsic J, Chakraborty R, et al. CNS Langerhans cell histiocytosis: common hematopoietic origin for LCH-associated neurodegeneration and mass lesions. *Cancer*. 2018;124(12):2607–2620.
32. Eckstein OS, Visser J, Rodriguez-Galindo C, Allen CE. Clinical responses and persistent BRAF V600E+ blood cells in children with LCH treated with MAPK pathway inhibition. *Blood*. 2019;133(15):1691–1694.
33. Allen CE, Flores R, Rauch R, et al. Neurodegenerative central nervous system Langerhans cell histiocytosis and coincident hydrocephalus treated with vincristine/cytosine arabinoside. *Pediatr Blood Cancer*. 2010;54: 416–423.
34. Hicks J, Flaitz CM. Langerhans cell histiocytosis: current insights in a molecular age with emphasis on clinical oral and maxillofacial pathology practice. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2005;100: S42–S66.
35. Chikwava K, Jaffe R. Langerin (CD207) staining in normal pediatric tissues, reactive lymph nodes, and childhood histiocytic disorders. *Pediatr Dev Pathol*. 2004;7:607–614.
36. Phillips M, Allen C, Gerson P, McClain K. Comparison of FDG-PET scans to conventional radiography and bone scans in management of Langerhans cell histiocytosis. *Pediatr Blood Cancer*. 2009;52:97–101.
37. Gadner H, Grois N, Potschger U, et al. Improved outcome in multisystem Langerhans cell histiocytosis is associated with therapy intensification. *Blood*. 2008;111:2556–2562.
38. Heritier S, Emile JF, Barkaoui MA, et al. BRAF mutation correlates with high-risk Langerhans cell histiocytosis and increased resistance to first-line therapy. *J Clin Oncol*. 2016;34:3023–3030.
39. Allen CE, Ladisch S, McClain KL. How I treat Langerhans cell histiocytosis. *Blood*. 2015;126:26–35.
40. Rodriguez-Galindo C, Allen CE. Langerhans cell histiocytosis. *Blood*. 2020;135:1319–1331.
41. Gadner H, Minkov M, Grois N, et al. Therapy prolongation improves outcome in multi-system Langerhans cell histiocytosis. *Blood*. 2013
42. Simko SJ, McClain KL, Allen CE. Up-front therapy for LCH: is it time to test an alternative to vinblastine/prednisone? *Br J Haematol*. 2014
43. Donadieu J, Bernard F, van NM, et al. Cladribine and cytarabine in refractory multisystem Langerhans cell histiocytosis: results of an international phase 2 study. *Blood*. 2015;126:1415–1423.
44. Simko SJ, Tran HD, Jones J, et al. Clofarabine salvage therapy in refractory multifocal histiocytic disorders, including Langerhans cell histiocytosis, juvenile xanthogranuloma and Rosai-Dorfman disease. *Pediatr Blood Cancer*. 2014;61:479–487.
45. Veys PA, Nanduri V, Baker KS, et al. Haematopoietic stem cell transplantation for refractory Langerhans cell histiocytosis: outcome by intensity of conditioning. *Br J Haematol*. 2015;169:711–718.
46. Donadieu J, Larabi IA, Tardieu M, et al. Vemurafenib for refractory multisystem Langerhans cell histiocytosis in children: an international observational study. *J Clin Oncol*. 2019:JCO1900456.
47. Diamond EL, Durham BH, Ulaner GA, et al. Efficacy of MEK inhibition in patients with histiocytic neoplasms. *Nature*. 2019;567:521–524.
48. Diamond EL, Subbiah V, Lockhart AC, et al. Vemurafenib for BRAF V600-mutant erdheim-chester disease and Langerhans cell histiocytosis: analysis of data from the histology-independent, phase 2, open-label VE-BASKET study. *JAMA Oncol*. 2017
49. Haroche J, Cohen-Aubart F, Emile JF, et al. Dramatic efficacy of vemurafenib in both multisystemic and refractory Erdheim-Chester disease and Langerhans cell histiocytosis harboring the BRAF V600E mutation. *Blood*. 2013;121:1495–1500.
50. Haupt R, Nanduri V, Calevo MG, et al. Permanent consequences in Langerhans cell histiocytosis patients: a pilot study from the Histiocyte Society-Late Effects Study Group. *Pediatr Blood Cancer*. 2004;42:438–444.
51. Gadner H, Grois N, Arico M, et al. A randomized trial of treatment for multisystem Langerhans' cell histiocytosis. *J Pediatr*. 2001;138:728–734.
52. Rodig SJ, Payne EG, Degar BA, et al. Aggressive Langerhans cell histiocytosis following T-ALL: clonally related neoplasms with persistent expression of constitutively active NOTCH1. *Am J Hematol*. 2008;83:116–121.
53. Eckstein OS, Nuchtern JG, Mallory GB, et al. Management of severe pulmonary Langerhans cell histiocytosis in children. *Pediatr Pulmonol*. 2020;55:2074–2081.

54. Jaffe R. Histiocytic and dendritic cell neoplasms. In: Swerdlow SH, ed. *WHO Calssification of Tumours of Hematopoietic and Lymphoid Tissues*. Lyon: IARC Press; 2008:353–357.
55. Stover DG, Alapati S, Regueira O, et al. Treatment of juvenile xanthogranuloma. *Pediatr Blood Cancer*. 2008;51:130–133.
56. Durham BH, Lopez RE, Picarsic J, et al. Activating mutations in CSF1R and additional receptor tyrosine kinases in histiocytic neoplasms. *Nat Med*. 2019;25:1839–1842.
57. Haroche J, Cohen-Aubart F, Amoura Z. Erdheim-Chester disease. *Blood*. 2020;135:1311–1318.
58. Arnaud L, Hervier B, Neel A, et al. CNS involvement and treatment with interferon-alpha are independent prognostic factors in Erdheim-Chester disease: a multicenter survival analysis of 53 patients. *Blood*. 2011;117:2778–2782.
59. Arnaud L, Gorochov G, Charlotte F, et al. Systemic perturbation of cytokine and chemokine networks in Erdheim-Chester disease: a single-center series of 37 patients. *Blood*. 2011;117:2783–2790.
60. Cohen AF, Emile JF, Carrat F, et al. Targeted therapies in 54 patients with Erdheim-Chester disease, including follow-up after interruption (the LOVE study). *Blood*. 2017;130:1377–1380.
61. Haroche J, Cohen-Aubart F, Emile JF, et al. Reproducible and sustained efficacy of targeted therapy with vemurafenib in patients with BRAF(V600E)-mutated Erdheim-Chester disease. *J Clin Oncol*. 2015;33:411–418.
62. Destombes P. Adenitis with lipid excess, in children or young adults, seen in the Antilles and in Mali. (4 cases). *Bull Soc Pathol Exot Filiales*. 1965;58:1169–1175.
63. Rosai J, Dorfman RF. Sinus histiocytosis with massive lymphadenopathy. A newly recognized benign clinicopathological entity. *Arch Pathol*. 1969;87:63–70.
64. Abla O, Jacobsen E, Picarsic J, et al. Consensus recommendations for the diagnosis and clinical management of Rosai-Dorfman-Destombes disease. *Blood*. 2018;131:2877–2890.
65. Foucar E, Rosai J, Dorfman R. Sinus histiocytosis with massive lymphadenopathy (Rosai-Dorfman disease): review of the entity. *Semin Diagn Pathol*. 1990;7:19–73.
66. Jordan MB, Allen CE, Weitzman S, et al. How I treat hemophagocytic lymphohistiocytosis. *Blood*. 2011;118:4041–4052.
67. Henter JI, Horne A, Arico M, et al. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer*. 2007;48:124–131.
68. Allen CE, Yu X, Kozinetz CA, McClain KL. Highly elevated ferritin levels and the diagnosis of hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer*. 2008;50:1227–1235.
69. Meeths M, Horne A, Sabel M, et al. Incidence and clinical presentation of primary hemophagocytic lymphohistiocytosis in Sweden. *Pediatr Blood Cancer*. 2014
70. Ishii E. Hemophagocytic lymphohistiocytosis in children: pathogenesis and treatment. *Front Pediatr*. 2016;4:47.
71. Ramos-Casals M, Brito-Zeron P, Lopez-Guillermo A, et al. Adult haemophagocytic syndrome. *Lancet*. 2014;383:1503–1516.
72. Jordan MB, Hildeman D, Kappler J, Marrack P. An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8+ T cells and interferon gamma are essential for the disorder. *Blood*. 2004;104:735–743.
73. Locatelli F, Jordan MB, Allen C, et al. Emapalumab in children with primary hemophagocytic lymphohistiocytosis. *N Engl J Med*. 2020;382:1811–1822.
74. Risma K, Jordan MB. Hemophagocytic lymphohistiocytosis: updates and evolving concepts. *Curr Opin Pediatr*. 2012;24:9–15.
75. Zhang K, Jordan MB, Marsh RA, et al. Hypomorphic mutations in PRF1, MUNC13-4, and STXBP2 are associated with adult-onset familial HLH. *Blood*. 2011;118:5794–5798.
76. Jordan MB. Challenges in the diagnosis of hemophagocytic lymphohistiocytosis: recommendations from the North American Consortium for Histiocytosis (NACHO). 2019. *Pediatr Blood Cancer*. 2019;66(11):e27929.
77. Fardet L, Galicier L, Lambotte O, et al. Development and validation of the HScore, a score for the diagnosis of reactive hemophagocytic syndrome. *Arthritis Rheumatol*. 2014;66(9):2613–2620. Sep.
78. Schram AM, Berliner N. How I treat hemophagocytic lymphohistiocytosis in the adult patient. *Blood*. 2015;125:2908–2914.
79. Ehl S, Astigarraga I, von Bahr GT, et al. Recommendations for the use of etoposide-based therapy and bone marrow transplantation for the treatment of HLH: consensus statements by the HLH Steering Committee of the Histiocyte Society. *J Allergy Clin Immunol Pract*. 2018;6:1508–1517.
80. Allen CE, Marsh R, Dawson P, et al. Reduced-intensity conditioning for hematopoietic cell transplant for HLH and primary immune deficiencies. *Blood*. 2018;132:1438–1451.
81. Marsh RA, Vaughn G, Kim MO, et al. Reduced-intensity conditioning significantly improves survival of patients with hemophagocytic lymphohistiocytosis undergoing allogeneic hematopoietic cell transplantation. *Blood*. 2010;116:5824–5831.
82. Daver N, McClain K, Allen CE, et al. A consensus review on malignancy-associated hemophagocytic lymphohistiocytosis in adults. *Cancer*. 2017;123:3229–3240.
83. La RP, Horne A, Hines M, et al. Recommendations for the management of hemophagocytic lymphohistiocytosis in adults. *Blood*. 2019;133:2465–2477.
84. Ravelli A, Grom AA, Behrens EM, Cron RQ. Macrophage activation syndrome as part of systemic juvenile idiopathic arthritis: diagnosis, genetics, pathophysiology and treatment. *Genes Immun*. 2012;13:289–298.
85. Grom AA, Villanueva J, Lee S, et al. Natural killer cell dysfunction in patients with systemic-onset juvenile rheumatoid arthritis and macrophage activation syndrome. *J Pediatr*. 2003;142:292–296.
86. Teachey DT, Rheingold SR, Maude SL, et al. Cytokine release syndrome after blinatumomab treatment related to abnormal macrophage activation and ameliorated with cytokine-directed therapy. *Blood*. 2013;121:5154–5157.
87. Bode SF, Ammann S, Al-Herz W, et al. The syndrome of hemophagocytic lymphohistiocytosis in primary immunodeficiencies: implications for differential diagnosis and pathogenesis. *Haematologica*. 2015;100:978–988.

CHAPTER 54

LYSOSOMAL STORAGE DISEASES, FOCUSING ON GAUCHER DISEASE: PERSPECTIVES AND PRINCIPLES

Atul Mehta, Mia Horowitz, Joaquin Carrillo-Farga, and Ari Zimran

LYSOSOMAL STORAGE DISEASES

Lysosomal storage diseases (LSDs) are genetic disorders caused by deficiencies in single lysosomal hydrolases. Deficiencies result in cellular and organ damage due to subsequent accumulation of a specific substrate for that particular enzyme, hence the term “storage” disease. However, the diverse role of the lysosome in cellular metabolism means that the pathological consequences of enzyme deficiency extend beyond substrate accumulation.

Although individually rare, the true prevalence of these disorders is likely to be much higher. Overall, 60 or so monogenic disorders have a combined frequency of approximately 1:7000 live births, and are probably increased due to consanguinity rates among different populations. All are autosomally inherited except for Fabry, Danon and mucopolysaccharidoses II (MPS II), which are sex-linked. The classification is in accordance with the biochemical nature of the primary storage material and pathogenic mechanisms (Table 54.1).[1,2]

Hers was the first to describe enlarged and abnormally shaped lysosomes resulting from primary accumulation of storage material in a patient with Pompe disease (α-glucosidase deficiency), thus delineating the first LSD.[3] Many LSDs have an early age of onset, associated with genetic mutations which lead to absent, or very low, enzyme levels and a correspondingly severe clinical course. However, several also have an attenuated form with later age of onset, genetic mutations associated with reduced or mutant enzyme production and reduced (but not absent) catalytic activity. LSDs are characterized by broad systemic involvement of multiple tissues and organs (typically involving bone, viscera, connective tissue, skin, kidney, and heart). Crucially, the majority have extensive neurological involvement manifesting as intellectual disability, dementia, seizures, movement disorders and sensory organ abnormalities.

Accumulation of storage material compromises the functional capacity of the lysosome and initiates a cascade of secondary downstream pathologic events. These include impaired autophagy (see Chapter 10), apoptosis and signal transduction, which precipitate cellular death and neurodegeneration. There is deficient supply, recycling and transport of key membrane and intracellular receptors, and macromolecules leading to synaptic failure and energy depletion. Cellular damage induces inflammation as an initial protective response but chronic induction is deleterious. Abnormal calcium homeostasis disturbs intracellular fusional and signaling events, and alters the potential of hydrogen (pH) to cause trafficking defects affecting storage and egress of cholesterol, sphingomyelin, and glycosphingolipids (GSLs). Macrophage activation, possibly mediated by cytokine release, has been demonstrated in Gaucher disease (GD) and other LSDs.[4] Accumulation of lysosphingolipids (i.e., GSLs that do not have N-acylated fatty acids) may be of pathogenic significance in Krabbe, Gaucher, and Fabry diseases.

Gaucher disease is important for hematologists because the presenting signs are splenomegaly and bleeding tendency (mainly due to thrombocytopenia but also platelet dysfunction and coagulopathies), because early diagnosis is important so therapy begins before irreversible bony complications occur, and because comorbidities include MGUS with increased risk of myeloma, lymphoma and MDS. Gene therapy trials are ongoing and adding relevance.

The biochemical composition of the accumulated macromolecules distinguishes the main categories of LSD: the glycosphingolipidoses (GPS), the mucopolysaccharidoses (MPS) and carbohydrate storage diseases. The pattern of storage of the accumulated lipid substrate in LSDs often mirrors the normal distribution of the molecule in the body, giving rise to organ-specific pathology. Thus, accumulation of GSLs in the central nervous system (CNS) leads to neurodegeneration, whereas storage in visceral cells leads to organomegaly, skeletal abnormalities, pulmonary infiltration, and other manifestations. In the MPS disorders, the accumulating lipid substrates (also known as glycosaminoglycans [GAGs]) are found predominantly within connective tissues to cause cartilage and bone abnormalities. The CNS may also be affected, particularly in diseases (e.g., MPS type I) where the GAG heparan sulfate accumulates. In contrast, in Maroteaux-Lamy disease (MPS type VI), only dermatan sulfate, which is not usually found in the brain, accumulates, and CNS manifestations do not occur; while in MPS IV, the accumulation of keratan sulfate leads to a predominant skeletal phenotype. (See box on Illustrative Mucopolysaccharidoses Case History.)

Lysosomal Protein Biosynthesis and Sorting

All lysosomal enzymes are translated on the endoplasmic reticulum (ER)-bound polyribosomes and contain a leader sequence. They enter the ER through the ER-specific translocons, and then lose the leader sequence. During translation the protein sequence, already within the ER is glycosylated on asparagine residues, which are part of the consensus sequence: Asparagine-X-Serine/Threonine (N-X-S/T; “X”

ILLUSTRATIVE MUCOPOLYSACCHARIDOSES CASE HISTORY

A male infant whose parents were first cousins was born healthy at full term. He presented at age 2 months with an inguinal hernia and a congenital hydrocoele. He developed four episodes of bronchiolitis and chest infection requiring antibiotics in the first 6 months of life. At 7 months hepato-splenomegaly was incidentally noted but initial general investigations did not yield a diagnosis. He was reviewed by a specialist pediatrician at 17 months when delay in attainment of motor, language and cognitive milestones was noted. He had coarse facial features with hepatosplenomegaly, corneal clouding, lower thoracic and lumbar kyphosis, umbilical hernia ultimately leading to a suspicion of an MPS disorder. Specialist testing and enzymology showed zero activity for alpha iduronidase, and a diagnosis of MPS 1 (Hurler syndrome) was made. Enzyme replacement therapy (ERT) was commenced immediately. Allogeneic stem cell transplant (SCT) from a fully matched sibling donor was performed at age 24 months.

Comment: If specialist referral had been undertaken at 7 months the diagnosis would have been made earlier, specific treatment including ERT and SCT would have been undertaken earlier and there would have been earlier supply of enzyme to the developing brain, CNS and systemic tissues leading to amelioration of the damage caused by the enzyme deficiency.

TABLE 54.1 Classification of Lysosomal Storage Disorders[1,2]

Disease	Prevalence	Deficient Enzyme	Subtypes
Sphingolipidoses, sphingolipid activator defects	>1/20,000 people		
Gaucher disease	1:40,000	beta glucocerebrosidase Saposin C	Types 1, 2, 3
Fabry disease	>1:20,000	alpha galactosidase A	Classic late onset Heterozygous
Tay-Sachs	<1:100,000	beta hexosaminidase A	Infantile and adult onset types
Sandhoff		beta hexosaminidase B	Infantile and adult onset types
Krabbe		beta galactosylceramidase	
Niemann-Pick types A and B		Acid sphingomyelinase deficiency	
Metachromatic leukodystrophy	<1:200.000	Arylsulfatase A Saposin B	
GM1 gangliosidosis		beta galactosidase	Types I, II, and III
Mucopolysaccharidosis (MPS)			
MPS I	>1:100,000	alpha-L-iduronidase	Severe (Hurler), milder (Scheie)
MPS II	<1:100,000	Iduronate-2-sulfatase	Hunter, and milder phenotypes
MPS III		Heparan-N-sulfatase and other enzymes	
MPS IV		N-acetylgalactosamine 6-sulfatase and beta galactosidase	Sanfilippo 4 subtypes: Morquio 2 subtypes
MPS VI		N-acetylgalactosamine 4-sulfatase	Maroteaux-Lamy
MPS VII	<1:300,000	beta glucuronidase	Sly
MPS IX		Hyaluronidase	Natowicz
Other enzyme deficiencies			
Glycogen storage disease type II	<1:100,000	Acid maltase	Pompe milder adult forms
Glycoproteinoses (e.g., aspartylglucosaminuria, fucosidosis, alpha/beta mannosidosis, Schindler disease)			
Lipidoses		Acid lipase	Wolman cholesterol ester storage disease
Defective post-translational processing of lysosomal enzymes			
Multiple sulfatase	<1:300,000	Formyl glycine generating enzyme	
Mucolipidosis II and III		N-acetylglucosamine-1-phosphotransferase	
Lysosomal membrane and transport defects			
Cystinosis	<1:300,000	Cystine transporter	
Sialic acid storage disease		Sialic acid transporter	
Cobalamin F disease		Cobalamin transporter	
Danon		Lysosome associated membrane protein 2 (LAMP-2)	
LIMP-2 deficiency[a] Action myoclonus renal failure syndrome		Lysosomal integral membrane protein 2	
Niemann-Pick types C1 and C2		Niemann-Pick types C1/C2 protein	
(CLN) Types 1–10; Batten = CLN3			
Defects in lysosome biogenesis			
Chediak-Higashi syndrome	<1:300,000	LYST Lysosomal traffic regulator	
Griscelli syndrome types 1–3			
Hermansky-Pudlak syndrome types 1–9			

[a] LIMP 2 (lysosomal integral membrane protein 2) aids trafficking of GCase from the ER to lysosomes; is involved in the pathogenesis of hand, foot, and mouth disease, caused by enterovirus-71 and possibly by coxsackievirus A16; mutations in the *LIMP2* gene can serve as a modifier in Gaucher disease (GD).

can be any amino-acid except proline). These N-linked glycan trees (dubbed N-glycans) consist of nine mannose residues and[5] three glucose residues. The enzymes are then folded, and, if recognized by the ER quality control machinery as correctly folded, they exit the ER in specific trafficking vesicles (called COPII vesicles) toward the Golgi network. They undergo further modifications on their N-linked glycan trees, which lose the glucose residues and three to four mannose residues. Most lysosomal enzymes are phosphorylated by the enzyme N-acetylglucosaminyl-1-phosphotransferase on a terminal mannose and as such are recognized by the mannose 6-phosphate receptor, which shuttles them, on specific late endosomal vesicles to the lysosomes. Mutations in the gene encoding this enzyme lead to severe LSD (I-cell disease), characterized by the abnormal secretion of all lysosomal enzymes whose lysosomal targeting depend on the M6P recognition signal.

If recognized as misfolded by the ER quality control machinery, lysosomal proteins undergo several folding attempts. If unsuccessful, the misfolded protein is translocated from the ER to undergo polyubiquitination and proteasomal degradation. This is known as ER-associated degradation (ERAD; Fig. 54.1).[6] Persistent retention of misfolded molecules in the ER provokes ER stress and induces the Unfolded Protein Response, known as UPR.[7] UPR monitors the conditions in the ER by sensing insufficiency in protein folding capacity and translating this information into gene expression by a signal transduction.

Several lysosomal enzymes and proteins (including the hexosaminidase A alpha and beta chains, mutated in Tay-Sachs disease and in Sandhoff disease, respectively) undergo proteolytic cleavage in the lysosomes as the last stage of their maturation process.

A large number of mutant lysosomal enzymes undergo ERAD and, therefore, lysosomal diseases may be regarded as misfolding or conformational diseases.

Genetics of Lysosomal Storage Diseases

Lysosomal genes are regarded as "housekeeping," that is, they are expressed in the majority of cell types and are essential for cellular function, such that complete absence of most of the enzymes is incompatible with life; Fabry disease is an important exception. All LSDs are inherited in an autosomal recessive fashion except for Fabry, MPS II and Danon, which are X-linked. One lysosomal gene, SMPD1 encoding acid sphingomyelinase (ASM), is known to be "paternally imprinted" (i.e., preferentially expressed from the maternal chromosome).[8] Diverse mutations underly individual LSDs, and the majority of these are unique ("private") to individual pedigrees. Broad genotype-phenotype relationships have been established. GD is a good example—major organizational disruptions of the gene are associated with a more severe phenotype.[9,10]

In general, heterozygous "carriers" of single mutations in a lysosomal gene do not develop clinical symptoms of the disorder, except in the X-linked disorders. For example, in Fabry disease X-inactivation patterns can lead to clusters of cells without enzyme activity, and heterozygous females often develop disease-related pathology primarily as a result of unbalanced X chromosome inactivation.[11] Heterozygous carriers of glucocerebrosidase gene (*GBA1*) mutations have a much increased risk of developing Parkinson disease (PD).

Some LSD-specific populations have recurrent mutations caused by founder effects and/or consanguinity. This facilitates the use of DNA-based screening methods for their detection. This has been translated into clinical use in the Ashkenazi Jewish population and has led to the establishment of a DNA-based "Jewish Genetic Disease" screening panel. Evolving cost-effective, high-throughput sequencing methods are likely to open other populations and disorders to these screening approaches. However, DNA-based methods will generate large numbers of GVUS (genetic variants of uncertain significance) and should not be used to predict clinical outcomes in patients unless the biochemical consequences of the mutations are fully established. An example of a mutation which was formerly a GVUS, but has recently been demonstrated to undoubtedly be associated with late-onset hypertophic cardiomyopathy in Fabry disease, is c.472 G>A; however, detailed studies have shown that although this variant is a necessary requirement for Fabry cardiomyopathy in affected individuals, it is not sufficient and other disease mechanisms are important for disease manifestations to develop.[12]

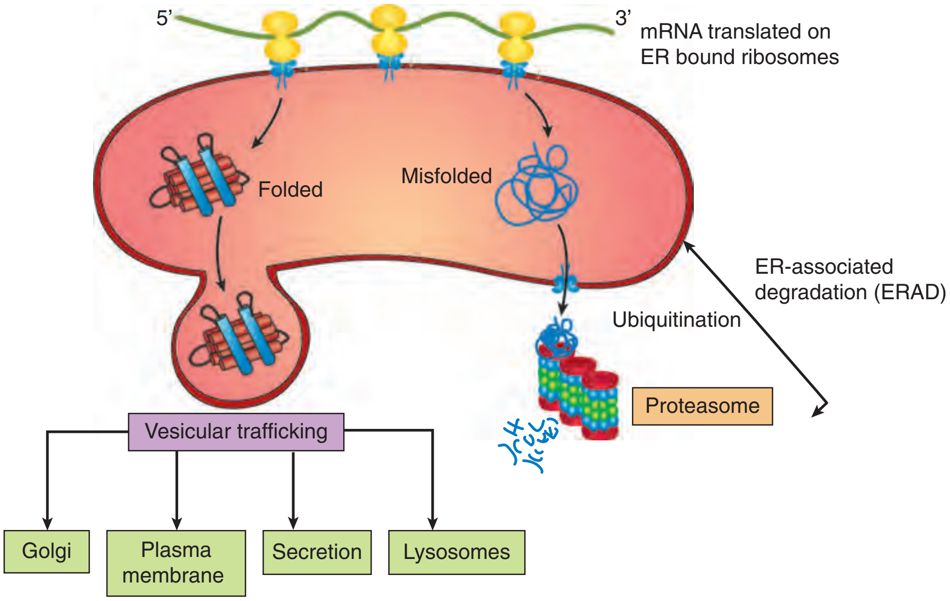


Figure 54.1 THE ER-ASSOCIATED DEGRADATION (ERAD) PROCESS. Proteins in the secretory pathway are synthesized on ER bound ribosomes. Following their entry to the ER and loss of their leader peptide, they undergo glycosylation on asparagines. Recognition of the peptide as correctly folded allows its shuttling to the secretory pathway via the Golgi apparatus. When recognized as misfolded, the proteins undergo several chaperone-mediated attempts to refold them. If unsuccessful, the misfolded molecules are retro-translocated from the ER to the cytoplasm, where they are polyubiquitinated and undergo proteasomal degradation, known as ERAD.

Diagnosis of Lysosomal Storage Diseases

The key to diagnosis is clinical suspicion of the disease. Clinical and laboratory diagnosis should be coordinated—once there is clinical suspicion, laboratory diagnosis is usually straightforward. These are multi-system disorders with few signature manifestations which occur among all ethnicities. The carrier rate among Ashkenazi Jews is about 1 in 15 for GD, 1 in 30 for Tay-Sachs, and 1 in 80 to 100 for Niemann-Pick type A.

Accurate clinical history and physical examination are crucial. The history must include a detailed family history and inquiry about consanguinity within the pedigree. Delay in diagnosis is a common problem and often leads to inappropriate investigations, delay in instituting specific treatment, and avoidable complications. A particular pitfall in diagnosis for pediatric and adult hematologists is that LSDs may have features that are shared by hematologic conditions (e.g., anemia, thrombocytopenia, hepatosplenomegaly, bone lesions). A survey of hematologists in the USA who have a clinical history of classic type 1 GD revealed that the overwhelming majority were initially considered to have a hematologic malignancy. The majority of Gaucher patients make their initial presentation to a hematologist and report a delay in diagnosis.[13]

Screening Programs

Screening programs aim to reduce diagnostic delay by institution of coordinated community diagnosis. Newborn screening (NBS) is already performed worldwide for certain metabolic diseases where early intervention is widely available and effective at preventing long term complications (e.g., dried blood spot [DBS] tests for phenylketonuria and hypothyroidism). Many states in the USA and countries in Europe and worldwide have embarked on these programs. Most of the laboratories engaged in LSD NBS carry out first-tier testing by measurement of lysosomal enzymatic activities in DBS on NBS cards using either tandem mass spectrometry (MS/MS) or fluorimetry (either digital microfluidics [DMF-F] or a standard plate reader).[14] Confirmatory enzymatic and DNA analysis is performed on positive samples. An emerging problem is that the programs are generally associated with a significant false positive rate. Furthermore, for some conditions (e.g., Fabry) neonatal screening based on gene sequencing has revealed prevalence rates which are far higher than previously suspected (e.g., 1:3200 for Fabry), but the majority of detected variants are of uncertain significance (GVUS), raising important ethical and procedural issues.

Targeted screening is the approach whereby clinical algorithms are used to identify subjects and family members considered to be at increased risk of suffering an LSD who can then be offered accelerated laboratory diagnosis. Such clinical algorithms have been devised for GD based on expert guidance.[15] A multi-national multi-center targeted screening study of 500 subjects with splenomegaly attending hematologists in Europe yielded a diagnosis of GD among 15 subjects.[16] Other examples of successful targeted screening programs include screening for Fabry disease among subjects with left ventricular hypertrophy and premature “cryptogenic” stroke.

Laboratory Diagnosis

DBS samples are increasingly used, replacing anticoagulated whole blood and, in some cases urine, and referred to specialty laboratories so that common pitfalls in laboratory diagnosis are avoided. Full clinical details and family history should be provided. Tissue biopsy is not indicated. Blood film morphology may give important clues (see below). Specific enzyme activity will then be measured in white blood cells, lymphocytes (such as T cells), plasma or DBS. Cultured fibroblasts are required to measure the activity of certain enzymes, for example, neuraminidase. Mass spectrometric assays allow simultaneous analysis of activity of several enzymes from DBS. Positive and negative controls are often included. Deficient activity in a clinically normal individual may also indicate “pseudodeficiency” due to the presence of a polymorphism—for example, as is commonly found with arylsulfatase A. A false-negative result may indicate a laboratory error but could rarely occur due to deficiency of a sphingolipid activator protein (SAP, e.g., the GM-2 activator protein or saposins A–D). Enzymatic activity from a heterozygous female will be normal or only marginally reduced if the condition is X-linked. Confirmatory DNA analysis should always be performed Laboratory diagnosis should also include confirmatory tests, for example, measurement of substrate level and relevant biomarkers.[17]

Baseline and Sequential Assessment

A genetic counselor can help with the interpretation of the results, the organization of pedigree analysis, and guidance with respect to treatment. A biomarker is a laboratory analyte that reflects the presence and/or extent of a biological process, and should be directly linked to the clinical manifestations and treatment outcome. Quantifiable biomarkers, which are specific, sensitive, reliable, and reproducible, are of great value in the management of several LSDs.[18,19]

The diversity of clinical manifestations means that multidisciplinary clinical assessment should be conducted to document structure, function, and patient-reported elements at diagnosis and at specified time intervals thereafter, irrespective of whether or not specific treatments are applied. Specific therapy should improve the disease manifestations, yet in many LSDs the impact of the intervention is much more limited, and may only amount to structural or functional reduction in the natural rate of decline. Reliable data on untreated individuals is crucial. Regulatory and funding authorities increasingly regard patient-reported outcome (PRO) as good measures reflecting the quality of life (QOL) and the overlay of psychological, social, societal concerns and stressors which are unique to LSD patients.

Specific Treatment

General Principles

The fundamental underlying principle behind the treatment of most LSDs is to replace the missing or defective protein, or to augment its activity, for example, by stem cell or organ transplantation, (protein) enzyme replacement therapy (ERT), gene therapy, substrate reduction therapy (SRT), or chaperone therapy (CT). “Proof-of-principle” was first documented by Neufeld and colleagues, who showed in an elegant series of studies that metabolic cross-correction of MPS cells could be achieved by co-culture of these cells with normal cells, or by replacing the media in MPS cells with “conditioned” media obtained from normal cells.[20] The identification of the mannose 6-phosphate (M6P)-targeting system for lysosomal enzymes and the finding that the secreted forms of many lysosomal enzymes could be rapidly internalized by cell surface M6P receptors and delivered into the lysosomal compartment further raised the prospect of ERT.[21]

A small biotechnology company (Genzyme, Cambridge, MA) began to commercially prepare β-glucocerebrosidase, the enzyme deficient in GD, from human placentas. The purified enzyme was chemically modified to expose terminal mannose residues, leading to preferential uptake by macrophages, the primary cellular site of pathology in type 1 disease (GD1). The results of biweekly intravenous ERT in GD1 included dramatically reduced organomegaly and improved hematologic findings, and substantially improved QOL.[22] Human recombinant enzymes for different LSDs are now produced in various sources, including Chinese Hamster Ovary (CHO) cell lines, transformed human cells, plants, and chicken eggs. Generic and bio-engineered preparations are increasingly being manufactured.

The physiological state requires that a small amount of protein is delivered on a constant basis. ERT has several drawbacks, among them the need for repeated intravenous infusions because of a short half-life, the inability to cross the blood-brain barrier (BBB), immunogenicity and excessive cost. The two major different approaches are cell therapy and the use of small molecules.

Cellular Therapy, Including Gene Therapy

Cell therapy is aimed at engraftment of cells (autologous or allogeneic) which can produce endogenous enzymes (see Chapter 103). Advantages of hemopoietic stem cells (HSCs) include their potential to self-propagate and to differentiate into multiple stem and progenitor cell lineages, which could potentially repopulate diverse organs and tissues. Furthermore, the application of this technology in subjects with non-malignant metabolic diseases does not impose a need to eliminate host hematopoiesis; and advances in molecular techniques increasingly offer ways of bioengineering the grafted cells. However, intensive immunosuppressive pre-conditioning with chemotherapeutic agents, with its associated high morbidity and potential mortality, is still required to achieve effective engraftment; and an allogeneic donor raises the specter of graft-versus-host disease. Donor availability is improved with the availability of cord blood banks, and haploidentical and even sibling carrier donors; but despite improved transplantation techniques which have reduced the risks of stem cell transplantation, the possibility of confounding the Hippocratic injunction to "do no harm" remains high.

Results of hematopoietic stem-cell transplantation (HSCT) for LSDs have been variable. The capacity of HSCs to repopulate different tissues is not uniform throughout the body, and is limited with respect to the skeleton (cartilage and bone) and the CNS. Permanent tissue damage is not reversible and transplantation is best undertaken early in the course of the disease. Liver transplantation has offered interesting perspectives which may have lessons for future gene therapy strategies using adenoviral constructs which infect hepatocytes. The procedure has been undertaken to treat end-stage cirrhosis complicating GD and long-term results show excellent recovery of liver function and continued absence of substrate accumulation in the liver. However, skeletal remodeling does not occur, and subjects continue to require ERT to treat systemic disease outside the liver. Ex vivo gene therapy approaches (see Chapter 104) for a range of LSDs are being investigated whereby the genetic construct is introduced into autologous HSCs which are then transplanted. A pilot study (prematurely closed to recruitment in January 2022) has reported autologous infusion of lentivirus transduced HSCs in five male patients with Fabry disease with up to 3 years of follow-up; all patients produced α-gal A to near normal levels within one week and three patients have discontinued ERT. However, safety concerns have led to discontinuation of the trial in January 2022. (Also see box on Fabry Disease: Specific Treatment).

The long-term results of this and other studies are awaited with interest, amid continuing concern about future risk of side-effects related to the chemotherapeutic conditioning regimen. Exciting developments have also been reported with regard to the potential for cellular therapy with induced neural stem cells.[23]

Small Molecule Therapy

Small molecules have important advantages over ERT. They can be taken orally, they have the potential to cross the BBB and access the CNS, antibody formation is not anticipated, production costs are lower, and small molecules are likely to have a higher volume of distribution in the body and to thereby access tissues such as bone, kidney, lungs, and the myocardium.

Two important approaches are SRT and PC. Eliglustat (see below) is established as an important alternative to ERT for many patients with type 1 GD. Neurologic pathology in Niemann-Pick disease (NPD), more specifically NPD type C (NPDC), is associated with the secondary accumulation of a particular glycolipid, GM2 ganglioside. Miglustat inhibits glycolipid biosynthesis and results in reduction of GM2 storage and partial neurologic improvements.

FABRY DISEASE: SPECIFIC TREATMENT

1. Team meetings—Essential to gather all relevant baseline data and for lead physician to record it. Cardiac (including echo, MRI, LV mass, 24 h ECG), renal (GFR, 24 h proteinuria, albumin/creatinine ratio, ultrasound) and pain (including pain scores, nerve conduction if relevant) data essential; also record baseline assessment of GI, skin, ophthalmology, hearing/audiometry and quality of life.
2. Whom to treat—Males and females with demonstrable organ damage; or severe pain/reduced quality of life unresponsive to other interventions. ERT is safe in children.
3. When to treat—Commence treatment early before there is established or irreversible organ damage.
4. How to treat—a. Usual to start with ERT as it will treat all mutations. Agalsidase beta IV 1 g/kg given over 2–4 h every 2 weeks (Fabrazyme, Sanofi—available world-wide); agalsidase alfa IV 0.2 mg/kg given over 40 min every 2 weeks (Replagal, Takeda/Shire—not licensed in the US); penunigalsidase alfa IV every 2 weeks (Protalix/Chiesis, Israel—License pending)
   b. Adults—Chaperone therapy—Migalastat (Amicus therapeutics) 123 mg orally once daily on alternate days is equally effective as ERT if the mutation is amenable. Compliance is essential.
5. Where to treat—ERT must be commenced in the clinic under supervision. Infusion reactions are commoner with agalsidase beta and usually easily managed with hydrocortisone and anti-histamine. Home treatment by a nurse or self-infusion is commonly practiced throughout Europe.
6. How to monitor—Clinical review with full data set of blood tests including assessment for neutralizing antibodies every 6 months. Complete assessment of organ function (must include cardiac, renal, pain and quality of life assessment) every 12 months.
7. Investigational treatments—Substrate reduction therapy—Venglustat (Sanofi), Lucerastat (N-butyldeoxygalactonojirimycin, Idorsia, Switzerland)
   - Gene therapy—Sangamo, Freeline
   - Apabetalone (RVX-208, Resverlogix), a selective BET (bromodomain and extra-terminal) inhibitor that can regulate disease-causing genes.
8. Who should be considered for investigational therapy?
   All patients should be considered, as there are significant unmet needs with all available treatments.
   Particularly consider those:
   - unresponsive to or allergic to licensed therapies;
   - unable to access them;
   - special considerations—e.g., severe disease phenotype;
   - strong preference for oral treatment but with a mutation unamenable to Migalastat;
   - strong preference for a single as opposed to repeated treatments;
   - personal choice.

Pharmacological chaperones (PCs) are usually specific inhibitors of the corresponding misfolded mutant proteins, and facilitate their delivery to lysosomes by enhancing residual activity; they are currently undergoing clinical trials for several LSDs. Migalastat is licensed for use in Fabry patients with "amenable" mutations. It is a PC which stabilizes and facilitates trafficking of amenable mutant forms of alpha galactosidase A from the endoplasmic reticulum to lysosomes and increases its lysosomal activity. Oral migalastat is the first pharmacological chaperone approved for treating patients ≥ 18 years in the US and Canada or ≥ 16 years in other countries with Fabry disease who have a migalastat-amenable alpha galactosidase A mutation. In patients with migalastat-amenable alpha galactosidase A mutations, relative to placebo, migalastat treatment significantly reduced the mean number of GL-3 inclusions/KIC and plasma lyso-globotriaosylsphingosine levels at 6 months. Among evaluable patients, migalastat maintained renal function and reduced cardiac mass after ≤24 months' therapy. Renal function was maintained during 18 months of migalastat or ERT; however, migalastat significantly reduced cardiac mass compared with ERT. Migalastat is generally well tolerated. Given that it can be administered orally and that limited therapeutic options are available, migalastat is an important treatment option for Fabry disease in patients with migalastat-amenable alpha galactosidase A mutations. Numerous other small molecule approaches are being evaluated in the various LSD animal models, including targeting inflammatory pathways and stem cell-based therapies. A combination of approaches, targeted at individual diseases and organs, may well be optimal.

General Aspects of Treatment

The advent of treatment for LSDs is a recent phenomenon. Specialty centers are best able to assess patients and families in a multidisciplinary fashion.[21] Each patient should have a designated "lead" specialist, who not only provides specialist input into their own discipline (e.g., a hematologist working within a center for GD; pediatricians trained in metabolic medicine), but will also coordinate care across a team of specialist physicians and health care professionals. Centers of excellence focus on providing holistic care, not only to the index patient, but to families and care providers. Specialty centers should work alongside patient advocacy groups and the pharmaceutical industry to organize active research programs, extending beyond the clinical trials. It is a mandatory requirement for manufacturers of many LSD drugs to provide post-marketing information regarding the long-term safety and efficacy of their products, and this is often done through Registries.

HEMATOLOGIC ASPECTS OF INDIVIDUAL LYSOSOMAL STORAGE DISORDERS

The immense clinical diversity of LSDs means that hematologists across the entire spectrum of the specialty (pediatric, malignant, transplantation, coagulation and hemostasis, transfusion medicine, general and laboratory) are likely to encounter these conditions. In large teaching hospitals, hematologists often practice clinical and laboratory medicine, and may be consulted about appropriate investigations in patients with a systemic disease presenting as a diagnostic conundrum.

The next section considers selected presenting features in more detail; and then focuses on one disease in particular, GD, which is the province of the hematologists.

Splenomegaly

The spleen is an integral part of the reticuloendothelial, immunological, and hepatobiliary systems. Splenomegaly will lead to anemia, thrombocytopenia, and leukopenia, which raise concerns about hematologic malignancy; hence hematologists and hepatologists will encounter affected patients. In the neonatal period and during childhood, LSDs (particularly the MPS disorders and some GSLs including GD) must be considered as part of the differential. Among adult and adolescent patients, splenomegaly in the absence of cirrhosis leads to suspicion of an infiltrative or storage disorder, and hematologists must be aware of rare causes, including LSDs. GD is discussed in more detail below. Splenectomy will improve peripheral blood counts but will accelerate deposition of substrate in other organs and tissues (e.g., liver, bone), leading to further complications.

Niemann-Pick disease types A and B (NPDA/B) present with thrombocytopenia, anemia, leukopenia, and splenomegaly. Some patients with NPDB have excessive bleeding without significant thrombocytopenia or coagulopathy. Frequent and prolonged epistaxis can be particularly problematic, and in some extreme cases may require cauterization, packing, and blood transfusions.

As with GD, a primary cellular site of pathology in NPDA/B is the monocyte-macrophage system, and the characteristic pathological cells are referred to as Niemann-Pick cells. These can be distinguished from Gaucher cells by an experienced pathologist, but are frequently misclassified. NPDC is caused by primary defects in cholesterol transport, and many patients present with mild hematologic findings including splenomegaly, albeit less severe than in NPDA/B (see Fig. 54.2B).

Wolman disease (cholesterol ester storage disease) presents in adults with liver disease (classically as non-alcoholic fatty liver disease) and splenomegaly. It is under-diagnosed, results from acid lipase deficiency, and is supremely responsive to ERT.

Excessive Bruising/Bleeding

Thrombocytopenia due to splenomegaly occurs in several childhood and adult LSDs. However, splenomegaly usually causes asymptomatic thrombocytopenia, with platelet counts ranging from 50 to 120 × 10^9/L, and, if unexplained, should alert the physician to seek a definitive diagnosis. Massive splenomegaly, for example in GD, can cause thrombocytopenia and may be associated with spontaneous bruising, and excessive bleeding from mucous membranes (menorrhagia, epistaxis), or in response to trauma, after surgery or delivery. Marrow infiltration, or disturbed function of hemopoietic stem cells, is reported in some LSDs (e.g., GD), and may be a contributory factor. Excessive bleeding with moderate to severe thrombocytopenia should raise suspicion of platelet dysfunction. Platelets release lysomal granules containing glycohydrolases capable of degrading glycoproteins, glycolipids and glycosaminoglycans upon activation. Hermansky-Pudlak syndrome (see Table 54.1) is an autosomal recessive disorder characterized by prolonged bleeding with oculocutaneous albinism, a storage pool deficiency of platelets, and lysosomal accumulation of ceroid lipofuscin; it is rare worldwide but is one of the commonest inherited disorders in Puerto Rico.

Thrombosis

Thrombosis and fibrinolysis with excessive consumption of platelets and coagulation factors is an important pathophysiologic component of LSDs. Tissue infarction may result in osteonecrosis in GD; sub-clinical thrombotic events, in part induced by inflammation, are likely to be involved in the neurologic pathology of many LSDs. Fabry disease warrants particular mention. Adults with mild missense mutations develop late-onset organ damage often restricted to a single organ, for example, the heart, kidney, or the CNS. Stroke, both thrombotic and hemorrhagic, often in the posterior fossa, is a frequent presenting feature among adults with Fabry disease. Structural abnormalities of blood vessels and disturbed regulation of blood flow are likely to be important contributory factors, but Fabry patients often have renal and cardiac disease, which will independently increase the risk of stroke.

Abnormal Morphologic Appearances: Bone Marrow and Peripheral Blood

It is frequently quoted, in articles and textbooks, that in lysosomal diseases "vacuoles in leukocytes" can be found. This assertion suggests that these changes are non-specific or of little diagnostic value. In a study of more than 1000 cases of LSDs, in the past 50 years, the *Journal of Cardiac Failure (JCF)* has found distinctive morphologic changes in peripheral blood or bone-marrow cells that, in many cases, suggest the specific diagnosis and, in some cases, are completely diagnostic.

The morphologic changes consist of macro-molecules that have not been degraded in the lysosome due to specific enzyme deficiencies. Fig. 54.2 depicts 16 of the most characteristic cells. The diagnosis, in all cases, has been confirmed by enzyme and molecular methods. Romanowsky-stained blood or bone-marrow smears are

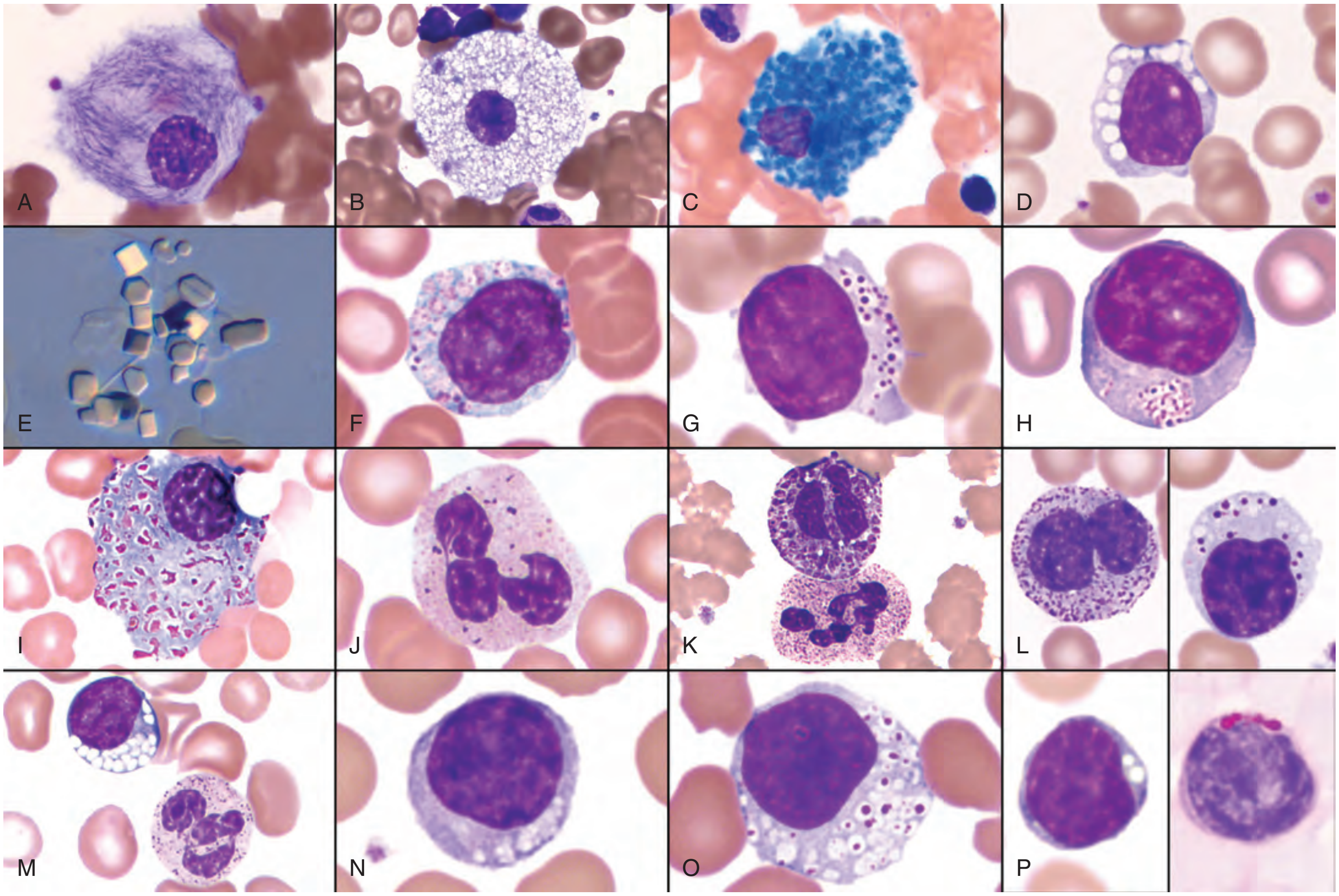


Figure 54.2 (A) Macrophage, bone marrow, Gaucher disease. (B) Macrophage, bone marrow, Niemann-Pick B. (C) Macrophage, bone marrow, Neuronal Ceroid Lipofucsinosis 1. (D) Lymphocyte, peripheral blood, Neuronal Ceroid Lipofucsinosis 3 (Batten disease). (E) Macrophage, bone marrow (unstained; DIK microscopy), Cystinosis. (F) B lymphocyte, peripheral blood, mucolipidosis II ("I cell" disease). (G) B lymphocyte, peripheral blood, MPS I (Hurler disease). (H) B lymphocyte, peripheral blood, MPS II (Hunter disease). (I) Plasma cell, bone marrow, MPS IIIA (Sanfilippo disease A). (J) Neutrophil, peripheral blood, MPS IVA (Morquio A disease). (K) Eosinophil and neutrophil, peripheral blood, MPS VI (Maroteaux-Lamy disease). (L) Monocyte and lymphocyte, peripheral blood, MPS VII (Sly disease). (M) T-lymphocyte and neutrophil, peripheral blood, generalized gangliosidosis GM1 type I (Landing disease). (N) Lymphocyte, peripheral blood, Fucosidosis. (O) Lymphocyte, peripheral blood, Sialidosis. (P) B lymphocyte, peripheral blood (Wright and PAShiff stains), Pompe disease.

an easy, cheap and rapid method for the initial evaluation of many LSDs. Detailed explanation of the findings in each of the different LSDs is complex and beyond the scope of this chapter.

GAUCHER DISEASE

GD is an autosomal recessive disease resulting from decreased activity of the lysosomal enzyme beta-glucocerebrosidase, leading to accumulation of glucosylceramide mainly in macrophages throughout the body. It is important for hematologists because the presenting symptoms are typically hematological (including splenomegaly, anemia, thrombocytopenia, hyperferritinemia, gammopathy). The diagnosis is frequently made by finding Gaucher cells in bone marrow aspiration (albeit a suboptimal way to diagnose GD—see below), because of the need for hematological consultations before surgery and delivery, and because of an increased incidence of hematological malignancies (particularly multiple myeloma) and idiopathic thrombocytopenic purpura (ITP). Recent ex vivo gene therapy trials (see Chapter 104), involving autologous HSCT, have further engaged hematologists with GD.

GD has been traditionally classified to three clinical forms, based on absence (type 1; GD1) or presence (types 2 and 3) of neurological signs (see box on The Three Clinical Forms of Gaucher Disease). While these sub-types may be regarded as a continuum, specific differences between them are useful in genetic counseling. Therapy is available only for the systemic complications of GD1 and GD3 and not for the neuropathic forms of GD (nGD).

Genetics and Biochemistry

Glucocerebrosidase is encoded by the *GBA1* gene, located on chromosome 1q21, close to a pseudogene, *GBA1P*, which shares 96% identity with the *GBA1* active gene.[9] The consequent occurrence (fortunately rare) of complex gene/pseudogene rearrangements may create pitfalls in molecular diagnosis, particularly when performed by next-generation sequencing (NGS) analysis.[24] More than 860

THE THREE CLINICAL FORMS OF GAUCHER DISEASE

• Type 1:	Non-neuronopathic, adult, chronic
• Type 2:	Acute neuronopathic, infantile: classic/neonatal
• Type 3:	Sub-acute (juvenile) neuronopathic 3a: neurological > visceral 3b: neurological < visceral 3c: cardiac variant

different mutations have been identified in the *GBA1* gene, including exonic missense, splice-junction, complex/recombinant alleles, deletions, insertions, and termination mutations. These explain, in part, the great phenotypic heterogeneity which is a hallmark of GD. A mild mutation on one of the alleles (such as N370S; according to the new nomenclature it would be termed: c.1226 A>G p.Asn409Ser, but we keep the traditional or "legendary" one throughout the chapter) will ensure GD1, which will be milder when the second allele also has a mild mutation. The combination of two severe mutations (any mutation that has been detected in a patient with nGD) will cause GD3 or GD2; two null alleles are incompatible with life. These basic genotype-phenotype correlations are important for genetic counseling and management.

In addition to the GC storage in macrophages, there are many other pathophysiological processes (partially delineated in the first part of this chapter) including inflammation, complement activation, protein misfolding, impaired autophagy, and other processes leading to lysosomal dysfunction and together[25] contributing to the diversity of disease manifestations.

Epidemiology

With a prevalence of ~1:40,000 to 1:150,000 (based on different NBS studies), GD is often considered the commonest LSD (although late-onset Fabry is probably commoner). GD1 is particularly common among Ashkenazi Jews who have a carrier rate of approximately 1:15 and population prevalence of 1:850, the majority of them are homozygous for the N370S mutation. Two additional ethnical predilections are a subset of patients with GD3b in Northern Sweden ("the Norbotnian type") all homozygous for the L444P mutation, and GD3c mainly in Palestinian Arabs near Jenin ("the cardiac variant") homozygous for the D409H allele.

Clinical Manifestations

Type 1 (GD1)

GD1, also known as "adult type" or chronic, non-neuronopathic form, usually presents with splenomegaly (from minimal enlargement detected incidentally to massive enlargement extending across the midline to the right lower quadrant) and bleeding tendencies, mainly mucosal bleeding (i.e., epistaxis, ecchymosis, menorrhagia) or bleeding after surgery, dental procedures, or postpartum. Massive hepatomegaly may occur in splenectomized patients; but predominant hepatomegaly suggests a primary hepatic comorbidity. Hypersplenism manifests as thrombocytopenia followed by anemia and then leukopenia. An elevated platelet count is seen in splenectomized patients. The younger the age, the more severe the clinical course, and in young children, there is often linear growth retardation. The bleeding tendency is exacerbated by platelet dysfunction, a coagulopathy (probably due to low level activation of the coagulation pathway); and in Ashkenazi Jewish patients, an association with partial deficiency of Factor XI is common.

Bone involvement is common and may be asymptomatic or extensive and debilitating; the skeletal complications tend to develop later in the course of the disease, and they are far more severe in splenectomized patients or among untreated patients with severe mutations, who do not have access to GD-specific therapies. Bone crisis is an acute onset of excruciating pain, usually above or below the knee, commonly accompanied by swelling and redness, with or without systemic signs of inflammation including fever, leukocytosis and elevated erythrocyte sedimentation rate (ESR) or c-reactive protein (CRP). These bone crises are commonly mistaken for acute osteomyelitis, particularly before GD has been diagnosed, and many patients undergo surgical drainage to exclude infection, which, not infrequently, leads to bacterial contamination. Bone crises were traditionally related to reduction in blood flow ("aseptic infarction"), and hence to lack or reduced tracer uptake in bone scintigraphy, when performed within a few hours from the onset of pain, unlike the expected increased uptake during infection). However, magnetic resonance imaging (MRI) studies done during the acute events reveal that at least in some cases there is evidence of subperiosteal bleeding. Bone crises by themselves are predictors for osteonecrosis (avascular necrosis; AVN) in the future[26]; AVN usually develops in the hip joints (unilateral or bilateral; Fig. 54.3) but also in other large joints including the knees and shoulders. Pathological fractures may occur in the ribs and vertebrae due to osteoporosis. Devastating bone complications have become much rarer since the introduction of ERT, in part due to the fact that splenectomy is no longer indicated.

Lung involvement usually arises due to interstitial infiltration by pathological/alveolar macrophages, but intravascular shunts and pulmonary hypertension may occur. While symptomatic lung disease is rare (mainly splenectomized previously untreated patients, and in GD3), pulmonary function tests commonly show airways obstruction, with reduced expiratory flow, reduction in lung volumes and alveolar-capillary diffusion abnormality.

Fatigue is a frequent symptom which does not necessarily correlate with overall disease severity nor with anemia, but still impacts significantly on QOL. It has been related to underlying inflammation, possibly ER-stress, and it is not always responsive to ERT or SRT, but theoretically might improve with PCs.

Types 2 (GD2) and 3 (GD3)

Neuronopathic GD (nGD) includes GD2, known as the "acute infantile" type, and GD3—the sub-acute juvenile neuronopathic type, represent the more severe GD forms, which also demonstrate diverse phenotypes. The distinction between "milder" GD2 and very severe GD3 may be challenging in young children. A common and pathognomonic sign is oculomotor apraxia, or supra-nuclear gaze palsy, predominantly horizontal, which in GD2 becomes a fixed convergent squint early in the course of the disease. Its universal absence in GD1 supports our preference to keep the classification of GD to three distinct forms.

GD2 is subdivided into a classic form (a fatal disease presenting during early infancy with survival of up to 2 to 3 years without complete life support), and a neonatal form (presenting at birth with hydrops fetalis, ichthyosis and death within 48 hours). GD3 has three sub-types: GD3a with a dominant neurological disease and relatively minor visceral involvement, GD3b with minimal neurological symptoms and massive visceral and skeletal disease, and more recently GD3c, the cardiac variant, with severe calcifications of the left heart valves and aorta, leading to death during late childhood to early adulthood, without significant visceral or neurological abnormalities.

The definitions of the exact form of nGD are based on clinical symptoms, and may not be based on the molecular diagnosis. With the launch of clinical trials specific for nGD, it became important to establish well-defined criteria to allow uniform patients' recruitment.[27]

GD2

Patients with GD2 typically present with severe, progressive neurological deficits in the first weeks of life. Stridor and apnea are common, necessitating tracheostomy by age 2 years; swallowing dysfunction, aspiration (requiring percutaneous endoscopic gastronomy [PEG] feeding), opisthotonos, progressive myoclonic epilepsy, and failure to achieve gait are other symptoms.

GD3

This nGD form is more heterogeneous; the gaze palsy (sometimes hard to notice at an early age) may be the isolated neurological finding

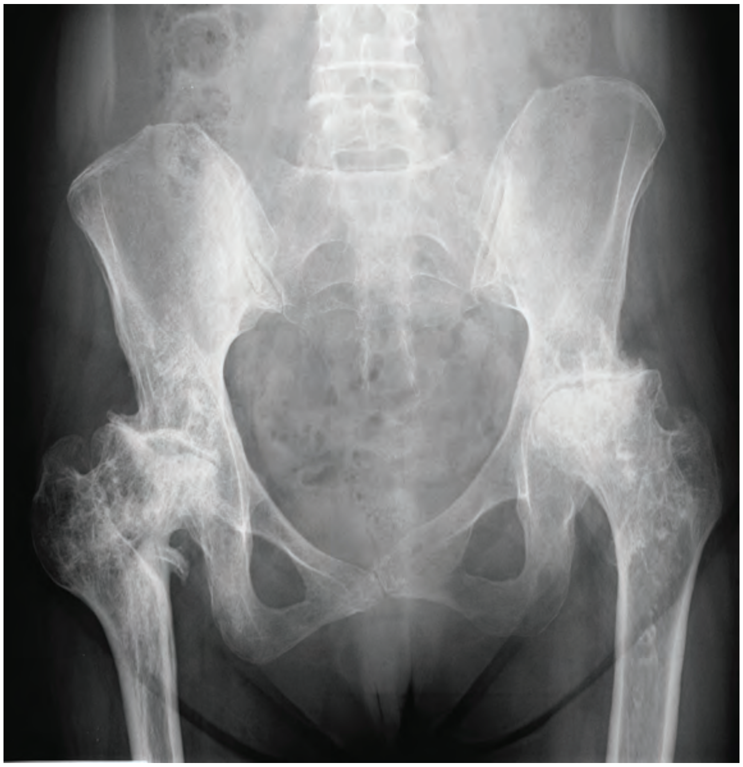


Figure 54.3 BILATERAL OSTEONECROSIS OF THE HIP JOINT. Bilateral osteonecrosis (AVN) in a 48-year-old woman with Gaucher disease (GD)1; the AVN occurred sequentially within 3 years after splenectomy, two decades earlier, in the pre-ERT era.

(GD3b) or be part of dominantly neurological abnormalities including motor and coordination deficits (ataxia), epilepsy (generalized seizures or myoclonic), stridor, dysarthria, cognitive impairments and more—GD3a. Most GD3 patients do not deteriorate rapidly. The most common genotype is homozygosity of L444P, and it is also the most frequent type of GD in Asia. A rather typical and very debilitating symptom is thoracic kyphosis (usually without scoliosis) and with no evidence of vertebral body collapse, usually developing between the age of 6 to 16 years. In GD3b, all the visceral and hematological manifestations that were described in GD1 are also present but usually with more severe prevalence of lung involvement.

Laboratory Findings

The diagnosis of GD is based primarily on the enzymatic assay demonstrating reduced glucocerebrosidase activity combined with the detection of DNA mutations, preferably by a whole *GBA1* gene sequence. There is no need to perform a bone marrow examination to diagnose GD.

Complete blood count (CBC) typically shows various degrees of thrombocytopenia (most patients), anemia (normocytic, normochromic found in ~30%) and less commonly leukopenia. The WBC differential count is normal, but lymphocytosis is frequently found in splenectomized patients. Platelet function and neutrophil function may be abnormal. Other causes of anemia (such as iron deficiency, that may be related to bleeding tendency, or vitamin B12 deficiency) should be excluded.

The biochemical panel is frequently normal; there may be a slight elevation of liver enzymes, as well as low levels of high-density lipoprotein (HDL) cholesterol, a non-specific GD biomarker, as are elevated serum ferritin levels (occasionally GD patients have been wrongly suspected of having hemochromatosis), angiotensin-converting enzyme (ACE) (not part of routine biochemistry panel), and tartrate-resistant acid phosphatase (no longer in use). GD-specific biomarkers are performed in referral centers and include chitotriosidase, CCL18, and glucosylsphingosine (LysoGb1); since the latter is the most sensitive, specific, reproducible and can easily be done via DBS[19], we rely on it, and perform the other two mainly during clinical trials.[28]

Both polyclonal and monoclonal gammopathies are frequently detected; monoclonal gammopathies are related to increased risk of multiple myeloma (see Chapter 91), and recent studies have identified that the clonal immunoglobulin in GD is actually reactive against the lysolipids.[29] There is also an increased prevalence of autoantibodies, some of which may trigger autoimmune diseases (see below).

Imaging Findings

Imaging is important at diagnosis for baseline assessment of the disease burden and for the decision on whether to initiate therapy. Imaging is used mainly to assess organ volume and skeletal involvement. Organ volume can be measured by ultrasonography (widely available, no radiation, no need for sedation in children and inexpensive; but operator-dependent and not as accurate as an MRI, which is the preferred methodology in clinical trials). Intrasplenic and less commonly intrahepatic focal lesions, may be detected and mimic malignancies.

Plain X-ray imaging has largely been replaced by MRIs, but is still done for specific indications (e.g., exclusion of fracture or assessing bone age in children). Frequent radiological features include the famous Erlenmeyer flask deformities of the distal femurs, osteopenia or osteoporosis and lytic lesions within long bones, even in the absence of bone pain. Lytic lesions are also relatively common in panoramic x-rays of the mandible. The bone marrow burden score is an MRI-based semi-quantitative assessment of the lumbar spine and entire femora, allowing prediction of skeletal complications when done before specific therapy, and for sequential monitoring.

Differential Diagnosis

Delayed awareness is a major concern in GD, and therefore GD must be included in the differential diagnosis (DD) of any patient at any age group who presents with splenomegaly and/or thrombocytopenia. Massive splenomegaly increases the likelihood of GD, particularly when imaging identifies intrasplenic focal lesions, as are any combination of findings (i.e., low platelets plus bone pains, splenomegaly plus elevated ferritin levels), medical history of gall-stones and family history of PD.[15] (See box on Gaucher Disease: Diagnosis and Specific Treatment.)

GD should be considered in the DD of patients presenting with bone crisis (DD from bacterial osteomyelitis), osteoporosis (especially in adult male or young subjects), history of pathological fractures, and short stature in the pediatric age group.

Myoclonic epilepsy and gaze palsy must include nGD (usually GD3a) in the DD, as well as those with severe kyphosis without scoliosis (GD3b); hydrops fetalis and ichthyosis should bring neonatal GD2 to the DD.

GAUCHER DISEASE: DIAGNOSIS AND SPECIFIC TREATMENT

1. Presenting disease features—Typically include bleeding tendency, fatigue, splenomegaly, bone pains and in children linear growth retardation. Common laboratory findings—thrombocytopenia, anemia, elevated serum ferritin, low HDL-cholesterol, abnormal liver function tests and (in adults) polyclonal or monoclonal gammopathies. Less common features—history of cholelithiasis, delayed menarche, family history of Parkinsons, intrasplenic lesions and osteoporosis/osteopenia. In young children you must exclude neurological involvement, particularly anormal eye movements.
2. Specific diagnosis—Document reduced enzymatic/glucocerebrosidase activity, mutant genotype by whole gene (GBA) sequence, and elevated biomarker—LysoGb1 (glucosylsphigosine). All three tests can be done on dry-blood-spot; avoid bone-marrow examination.
3. When to treat—Start before the development of irreversible organ damage (mainly bones). Selected asymptomatic GD1 patients can be monitored and treated upon symptom onset or progressive disease parameters.
4. How to treat—In all children and most adults, bi-weekly infusions of ERT should be the first specific therapy. Dose—varies between countries and centers; most US doctors begin with 60 units/kg, whereas 15–30 units/kg are typically prescribed in Israel, UK, and Australia. The three enzymes are imiglucerase (Sanofi/Genzyme), Velaglucerase-alfa (Takeda), and taliglucerase-alfa (Pfizer/Protalix).

   Oral therapy with SRT, eliglustat 84 mg (Sanofi), is approved as first-line therapy for adult GD1 patients who are CYP2D6 extensive, intermediate or poor metabolizers (twice daily or once-a-day accordingly). Contraindications are cardiac, renal, or hepatic problems and current or contemplated pregnancy. Compliance is important, and drug-drug interactions requires attention. Another SRT, miglustat (Actelion) is approved for patients with mild to moderate GD1 for whom ERT is not suitable, but it is not widely used.

   Investigational treatments include gene therapy (in vivo and ex vivo) and pharmacological chaperones. Ambroxol, a cough medicine available over-the-counter, acts as a GBA-chaperone, may improve myoclonic epilepsy and other neuronopathic features in nGD, and is in clinical trials for GBA-related Parkinson's.
5. Where to treat—Commence ERT in the clinic under supervision. Infusion reactions are commoner with taliglucerase-alfa and least common with velaglucerase-alfa. Home therapy by a nurse or self-infusion is commonly practiced (less popular in the US).
6. How to monitor—Comprehensive assessment in a referral center is recommended. Perform blood tests including biomarkers and imaging studies every 6 months for treated patients and every 12 months for untreated, stable patients.
7. Who should be considered for investigational therapy?
   - Patients who cannot receive or are unresponsive to ERT/SRT
   - Patients with neuronopathic GD
   - Altruistic patients wishing to advance medical knowledge

The ultra-rare GD3c should not be forgotten in the consideration of rheumatic heart disease, particularly in patients of Palestinian descent, who present with mitral stenosis and/or aortic stenosis and insufficiency.

Symptomatic Therapy

Patients who have undergone splenectomy and suffered bony complications before the era of ERT often require pain management and surgical (mainly orthopedic) procedures. When indicated (i.e., massive splenomegaly and refractory hypersplenism, splenic torsion, concern about malignancy) hand-assisted laparoscopic splenectomy is safe and feasible.[30] Arthroplasties, particularly total hip replacement in patients with osteonecrosis of the femoral head, have been shown to be safe, and achieve long-term implant survival, improve function, and QOL.[31]

Specific Treatments

Not all patients with GD, particularly N370S homozygous, require treatment.[32] The most common indications for commencing treatment include marked splenomegaly, significant cytopenias, bony manifestations, impaired QOL, and growth retardation. The currently available treatments do not cross the BBB, and improvement of the neurological symptoms of nGD is not achievable. New treatment approaches are being intensively investigated, including PCs, new SRTs, and gene therapy.

Enzyme Replacement Therapy

Background

ERT for LSDs was first suggested by Christian de Duve in 1964, a year before the identification of glucocerebrosidase deficiency as the etiology of GD by Roscoe Brady, who a year later presented the concept of ERT.[22] It took more than 25 years before Brady's first ERT was approved for GD1 in 1991, following a seminal clinical trial of 9 months in 12 patients.[33] Historically, the introduction of the first placental-derived ERT, alglucerase, was a dual success: Medically, its safety and efficacy has revolutionized the management of patients with GD1, and led to the development of ERT for several other LSDs; and financially, the great marketing success, opened the doors for making drugs available for patients with rare diseases in general.

ERT does not only stop the deterioration of GD, but actually reverses many of the disease's manifestations (such as organomegaly, hypersplenism, bone pains, growth retardation, etc.), so when given to patients before they develop irreversible bony complications, their morbidity is pretty much restricted to the need for bi-weekly intravenous infusions. Alglucerase was replaced in 1994 by the CHO-derived human recombinant imiglucerase, which was for 16 years the single ERT available. Imiglucerase has one amino-acid substitution (Arg495His) due to a cloning artifact in the original cDNA used for its production. In 2010 and 2012, two additional ERTs became commercially available: velaglucerase-alfa, produced in a human cell-line using proprietary gene-activation technology, has an amino-acid sequence identical to the wild-type enzyme, and a higher alpha-mannosyl content than imiglucerase (which is important for targeting to macrophages); and taliglucerase alfa, the first human enzyme produced from transduced carrot cells, that contains the same amino-acid substitution as imiglucerase. These ERTs were developed as novel proteins, whereas at least three "non-comparable biotherapeutics"[34] are currently available in a few countries: Abcertin™, Asbroder™ and Glurazyme™ (from Korea, Mexico and Russia, respectively), all sharing the same generic name "imiglucerase biosimilar," none of which has been approved by Food and Drug Administration (FDA)/European Medicines Agency (EMA) and are not discussed in this chapter.

Results of Treatment

Despite minor differences, the three FDA-approved ERTs can be discussed as a class, and in the majority of the patients, the impact on the key disease's symptoms is similar: improvement of hematological parameters, reduction in organomegaly, even if huge at baseline, amelioration of bone pain, prevention of osteonecrosis, growth spurts in children, and general improvement in QOL (currently expressed as GD-specific patient reported outcome; PRO; 34). The response to ERT tends to be more dramatic when the degree of abnormality is more prominent; children tend to respond faster than adults, and in the majority of the patients the detection of anti-ERT antibodies does not change the response. For female patients who may conceive while on ERT, the risk of postpartum hemorrhage decreases, and ERT, even if detected in breast milk, does not harm the babies.

There is still debate about the optimal dosage regimen; the majority of centers in the US begin with the high-dose of 60 units/kgBW/EOW, and may later reduce the dose upon stabilization, typically after 2 to 3 years. In Israel, UK, and a few other countries, most of the patients begin with lower doses (15 to 30 units/kg/EOW) and increase the dose if the response is sub-optimal. Because the human-derived velaglucerase-alfa has the wild-type sequence, and has demonstrated better in vitro internalization into macrophages, when the "mutant" ERTs were used (imiglucerase or taliglucerase alfa), we usually switch these patients to the same-dose of velaglucerase alfa before increasing the dose (usually doubling to 60), as we prefer the minimal effective dose to the maximally tolerated one.

Most patients in Europe and Israel receive their ERT at home, either by home care nurses or by self-administration. This practice has become particularly important during the COVID-19 pandemic.

The overall safety profile of ERT is excellent; most of the adverse events (AEs) are mild and transient; anaphylaxis and severe allergic reactions are rare (their risk is higher with taliglucerase alfa, probably due to the existence of D-xylose and fucose, two sugars derived from the plant cells); mild hypersensitivity reactions are often treated with premedication, but here too, we have found that a switch to the human-derived ERT usually resolves the need for premedication.

Unfortunately, the high cost of ERT has not changed dramatically with the introduction of additional enzymes products, and many patients in underdeveloped countries are still unable to receive this standard of care, except a few lucky ones who receive compassionate treatment.[35] None of the ERTs are capable of crossing the BBB, and hence they only impact the non-neuronopathic symptoms in nGD; there seems to be no impact on GD-related comorbidities, and usually not on "gaucheromas" (masses composed mainly of Gaucher cells, at times causing massive lymphadenopathy with protein losing enteropathy), or on pulmonary involvement.

Substrate Reduction Therapy

SRT was first proposed as a treatment for GD by Radin in 1975. The technique exploits the fact that all patients with GD have a degree of residual enzyme activity and the partial inhibition of substrate production would lead to its reduced accumulation.

Miglustat (N-butyl-deoxynojirimycin) is a low-molecular-weight glucose analogue that inhibits glucosylceramide synthase, the enzyme that catalyzes the first step in the biosynthesis of glucosylceramide and other GSLs. Miglustat was the first oral SRT to receive market authorization in 2000; its use resulted in decreased organomegaly and improvement in hematologic parameters in GD1, but not to the extent achieved with ERT, and with significantly more AEs, particularly gastrointestinal and peripheral neuropathy; hence, its use has been limited to patients with mild to moderate GD1 for whom ERT is not suitable.

Eliglustat, a ceramide analogue with a better tolerability and efficacy profiles, was the second SRT approved in 2014. In contrast to miglustat, eliglustat was approved as first-line oral treatment for certain adult patients with GD1, who have a suitable CYP2D6 status (extensive, intermediate or poor metabolizers) due to its metabolism in the liver. Although it has been claimed to be as good as ERT, the findings from the clinical trials suggest inferiority to ERT and a more problematic safety profile, including cardiac AEs (in at least 5% of the patients), palpitations and syncope.[36] Drug-drug interactions, several contraindications/precautions (hepatic, renal and cardiac diseases, pregnancy, and suitability for children and older patients), and the need for good compliance are additional disadvantages. Taking into consideration also the partial inhibition of hundreds of different glycolipids (SRT class effect), our policy is to use eliglustat for patients unwilling or unable to receive ERT.[37] Eliglustat is particularly suitable for stabilized patients (young adults, excluding females planning a family) without underlying diseases and co-medications, who wish to receive an oral alternative to ERT.

Associated Diseases: Parkinson and Malignancies

When a patient with a chronic disorder develops an additional disease, one should distinguish between two independently sorting diseases (GD-unrelated) and those that occur in significantly higher incidence compared to the general population (GD-related), but all are considered comorbidities. There may be a mutual impact of two unrelated diseases due to common symptoms, such as bleeding tendency due to the GD-unrelated partial deficiency of Factor XI, also common among Ashkenazi Jews, and there may be common symptoms of a GD-related diseases such as bone pains and/or anemia in multiple myeloma, which contributes to delayed diagnosis of the other disorder (Fig. 54.4). Both related and unrelated comorbidities should be differentiated from atypical manifestations or complications of GD, as well as delayed occurrence of drug-related medical problems, such as peripheral neuropathy with SRT or metabolic syndrome with ERT.[38]

When GD-related, specific therapy may or may not ameliorate the comorbidity, when GD-unrelated there will be no effect; if drug-related, it has not been present prior to advent of specific therapy, and it will be sustained (and possibly worsen over time) while on therapy but may ameliorate or disappear upon withdrawal of therapy.

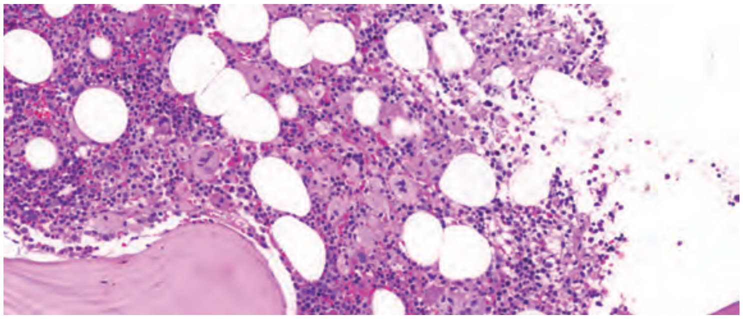


Figure 54.4 GAUCHER DISEASE (GD)1 AND MULTIPLE MYELOMA. Bone marrow biopsy from a 62-year-old female with GD1. She was noted to develop a progressive rise in her paraprotein and had developed new onset vertebral collapse. A diagnosis of myeloma was confirmed.

CONSIDER THE EXISTENCE OF A COMORBIDITY

In the untreated patient:

1. when hemoglobin level is significantly lower than the platelet counts
2. when the degree of hepatomegaly is greater than that of the splenomegaly

In the treated patient:

3. when there is no response to specific therapy (ERT/SRT)
4. when there is a change in the pattern of response

GD-related comorbidities include cholelithiasis, several autoimmune diseases (i.e., ITP, uveitis, Hashimoto thyroiditis and celiac disease), but the most important ones are plasma-cell dyscrasias (MGUS and multiple myeloma) and PD.[39,40] (See box on Consider the Existence of a Comorbidity.)

Prognosis

The majority of the patients with GD1 are expected to have a normal lifespan, especially those who are mildly affected, non-splenectomized, and those who receive ERT/SRT prior to the development of irreversible complications. A single paper from 2008, based on a drug company registry, claimed that patients with GD1 live on the average 9 years less compared to the expected survival of the American population[41]; this biased paper suffers major pitfalls including the comparison of the GD global population (including under-developed countries) to the US general population, the inclusion of children with nGD who died at ages 0 to 10 years, and the lack of molecular diagnosis in 46% of the 102 deceased patients. Importantly, malignancies did not contribute to the shortened lifespan.

Patients with GD2 have a grave prognosis with death usually by the age of 2 years, whereas no data are available regarding life expectancy of patients with GD3, which is shorter and inferior for GD3a and GD3c. Sudden death has been reported in a large cohort of GD3 patients from Egypt.

New Therapeutic Modalities, Including Gene Therapy

New treatment options address nGD, improved treatments for GD1 and the prevention of GD-related comorbidities. PCs include ambroxol, which is already being used off-label as well as in different trials for patients with nGD or *GBA1*-related PD. New PCs are being investigated, ideally non-inhibitory drugs, and several candidate molecules are in early stages of research.[42] Arimoclomol is a heat-shock-protein (HSP70) amplifier, stimulating the endogenous correction of misfolded proteins into a functional conformation; it has potential activity in several disorders including ALS, NPC, GD1, GD3, and more.

Gene therapy clinical trials, both in vivo and ex vivo, are already ongoing in humans; one-time in vivo injection of AAV9-based gene therapy to the cisterna magna in nGD or intravenously for patients with GD1, and ex vivo lentivirus-based gene therapy also for GD1, with a potential also to impact the CNS via CD34+ derived microglia cells that can migrate into the brain. If successful, these pioneering modalities will improve the prognosis and QOL of patients with GD.[43] (See box on Illustrative Case of Gaucher Disease.)

ILLUSTRATIVE CASE OF GAUCHER DISEASE

A 25-year-old female, ultra-orthodox Ashkenazi Jew, mother of two, was referred to our clinic at the 20th week of her third pregnancy, to rule out GD. She is known to have low platelet counts since age 8, but has never been evaluated. Splenomegaly has never been mentioned; menarche age 14 years, with no story of bleeding tendency, early satiety or bone pains. Low platelet count was noted upon arrival to delivery room at her first pregnancy; the delivery was complicated by massive bleeding and required blood transfusions. At her second pregnancy the thrombocytopenia (lowest counts were around 50,000/μL) was related to ITP, and treatment with steroids was given with no effect; platelet infusions and intravenous iron were administered during and after delivery, respectively. In her discharge report she was advised to consult a hematologist. Routine gynecological ultrasound during her third pregnancy revealed an enlarged spleen, when she was referred to our clinic. The diagnosis of GD was confirmed by DBS demonstrating reduced glucocerebrosidase activity, elevated LysoGb1 and the N370S/N370S genotype. Intravenous administration of ERT was initiated immediately, and at this time, her platelet count increased to 95,000/μL; the delivery was uneventful. At the last follow-up visit, at 14 weeks of her fourth pregnancy, the platelet counts normalized, and hopefully she will enjoy for the first time an epidural analgesia during labor.

Comment: Delayed diagnosis is common even among Ashkenazi Jews in Israel; an earlier diagnosis of this rather typical patient should have led to earlier initiation of treatment, and to the prevention of unnecessary blood transfusions or steroid therapy, which may increase the risk for osteonecrosis. A recent evaluation of the effect of ERT on the pregnancy and obstetric outcome in a unique group of multiparous women with GD1 who had pregnancies with and without ERT, has demonstrated that early PPH and RBC transfusions were significantly lower when ERT was used.[44]

CONCLUSIONS AND FUTURE DIRECTIONS

There have been huge advances in our understanding of LSDs and GD in particular, which have been translated into groundbreaking treatments. There remain, however, very significant unmet needs for patients—including the need to access the CNS, more convenient treatments with greater activity, and the need to improve access to these treatments for major populations worldwide who cannot afford the current options. There is a universal need for hematologists to raise awareness of these diseases and reduce delays in diagnosis. The fundamental role of lysosomes in cellular homeostasis implies that a better understanding of the physiology will lead to identification of the downstream consequences of enzyme deficiencies and substrate accumulation and elucidation of the underlying pathological processes. These include inflammation, complement activation, abnormal autophagy, the unfolded protein response, aberrant intra- and extra-cellular signaling, calcium metabolism and many other processes, many of which are amenable to therapeutic intervention. NBS will uncover many new patients and focus energies on the need to better define genotype-phenotype relationships, which in turn will allow us to improve targeting of existing and new treatments. Hematologists know too well that this is essential if we are to successfully introduce treatments with significant treatment-related morbidities (e.g., gene and cellular therapies); while all physicians as well as society as a whole should require that patients with these rare diseases receive cost-effective and ethical care, which is on a par with that provided to patients with more common ailments.

REFERENCES

1. Winchester B. Classification of lysosomal storage diseases. In: Mehta A, Winchester B, eds. *Lysosomal Storage Disorders. A Practical Guide*. 1st ed. Oxford: Wiley-Blackwell; 2012:37–47. chap 5. (2nd ed. in press, 2022.)
2. Mehta AB. Inherited common hereditary lysosomal storage diseases. *British Medical Journal Best Practice*. 2022.
3. Hers HG. Inborn lysosomal diseases. *Gastroenterology*. 1965;48:625.
4. Platt FM, d'Azzo A, Davidson BL, Neufeld EF, Tifft CJ. Lysosomal storage diseases. *Nat Rev Dis Primers*. 2018;4:27.
5. Bendikov-Bar I, Maor G, Horowitz M. Processing and maturation of human glucocerebrosidase. *Future Medicine*. August 2013:140–157.
6. Brodsky JL. Cleaning up: ER-associated degradation to the rescue. *Cell*. 2012;151:1163.
7. Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science*. 2011;334:1081.

8. Simonaro CM, Park JH, Eliyahu E, et al. Imprinting at the SMPD1 locus: implications for acid sphingomyelinase-deficient Niemann-Pick disease. *Am J Hum Genet*. 2006;78:865.
9. Horowitz M, Wilder S, Horowitz Z, et al. The human glucocerebrosidase gene and pseudogene: structure and evolution. *Genomics*. 1989;4:87.
10. Grabowski GA. Gaucher disease: gene frequencies and genotype/phenotype correlations. *Genet Test*. 1997;1:5.
11. Mehta A, Hughes DA. Fabry dsease. 2002, Aug 5 [Updated 2022, Jan 27]. In: Adam MP, Mirzaa GM, Pagon RA, et al., eds. *GeneReviews* [Internet]. Seattle, WA: University of Washington, Seattle; 1993–2022. https://www.ncbi.nlm.nih.gov/books/NBK1292/.
12. Fuller M, Mehta A. Fabry cardiomyopathy: missing links from genotype to phenotype. *Heart*. 2020;106:553.
13. Mehta A, Belmatoug N, Bembi B, et al. Exploring the patient journey to diagnosis of Gaucher disease from the perspective of 212 patients with Gaucher disease and 16 Gaucher expert physicians. *Mol Genet Metab*. 2017;122:122.
14. Gelb MH. Newborn screening for lysosomal storage diseases: methodologies, screen positive rates, normalization of datasets, second-tier tests, and post-analysis tools. *Int J Neonatal Screen*. 2018;4:23.
15. Mehta A, Kuter DJ, Salek SS, et al. Presenting signs and patient co-variables in Gaucher disease: outcome of the Gaucher Earlier Diagnosis Consensus (GED-C) Delphi initiative. *Intern Med J*. 2019;49:578.
16. Motta I, Consonni D, Stroppiano M, et al. Predicting the probability of Gaucher disease in subjects with splenomegaly and thrombocytopenia. *Sci Rep*. 2021;11:2594.
17. Winchester B. Laboratory diagnosis of LSDs. In: Mehta A, Winchester B, eds. *Lysosomal Storage Disorders. A Practical Guide*. 1st ed. Oxford: Wiley-Blackwell; 2012. chap 2.
18. Mashima R, Okuyama T, Ohira M. Biomarkers for lysosomal storage disorders with an emphasis on mass spectrometry. *Int J Mol Sci*. 2020;21:2704.
19. Cozma C, Cullufi P, Kramp G, et al. Treatment efficiency in Gaucher patients can reliably be monitored by quantification of lyso-Gb1 concentrations in dried blood spots. *Int J Mol Sci*. 2020;21:4577.
20. Neufeld EF. The uptake of enzyme into lysosomes: an overview. *Birth Defects Orig Artic Ser*. 1980;16:77.
21. Sly WS, Fischer HD, Gonzalez-Moriega A, et al. Role of the 6-phosphomannosyl-enzyme receptor in intracellular transport and adsorptive pinocytosis of lysosomal enzymes. *Methods Cell Biol*. 1981;23:191.
22. Brady RO. Enzyme replacement for lysosomal diseases. *Annu Rev Med*. 2006;57:283.
23. Peng Y, et al. Intravenous infusion of iPSC-derived neural precursor cells increases acid -glucosidase function in the brain and lessens the neuronopathic phenotype in a mouse model of Gaucher disease. *Hum Mol Genet*. 2019;28:3406.
24. Zampieri S, Cattarossi S, Bembi B, Dardis A. GBA analysis in next-generation era: pitfalls, challenges, and possible solutions. *J Mol Diagn*. 2017;19:733.
25. Myerowitz R, Puertollano R, Raben N. Impaired autophagy: the collateral damage of lysosomal storage disorders. *EBioMedicine*. 2021;63:103166.
26. Degnan AJ, Ho-Fung VM, Ahrens-Nicklas RC, et al. Imaging of non-neuronopathic Gaucher disease: recent advances in quantitative imaging and comprehensive assessment of disease involvement. *Insights Imaging*. 2019;10:70.
27. Schiffmann R, Sevigny J, Rolfs A, et al. The definition of neuronopathic Gaucher disease. *J Inherit Metab Dis*. 2020;43:1056.
28. Revel-Vilk S, Fuller M, Zimran A. Value of glucosylsphingosine (Lyso-Gb1) as a biomarker in Gaucher disease: a systematic literature review. *Int J Mol Sci*. 2020 Sep 28;21(19):7159.
29. Nair S, Bar N, Xu ML, et al. Glucosylsphingosine but not Saposin C, is the target antigen in Gaucher disease-associated gammopathy. *Mol Genet Metab*. 2020;129:286.
30. Freund MR, Reissman P, Zimran A, Alberton J. Splenectomy in Gaucher disease: a call for minimally invasive surgery. *Surg Laparosc Endosc Percutan Tech*. 2018 Apr;28(2):86–89.
31. Cohen D, Kogan D, Rubin A, et al. Longevity of total hip arthroplasty implants in patients with Gaucher disease. *Hip Int*. 2020;30:147.
32. Dinur T, Zimran A, Becker-Cohen M, et al. Long term follow-up of 103 untreated adult patients with type 1 Gaucher disease. *J Clin Med*. 2019;8:1662.
33. Barton NW, Brady RO, Dambrosia JM, et al. Replacement therapy for inherited enzyme deficiency—macrophage targeted glucocerebrosidase for Gaucher's disease. *N Engl J Med*. 1991;324:1464.
34. Drelichman G, Castañeda-Hernández G, et al. The road to biosimilars in rare diseases—ongoing lessons from Gaucher disease. *Am J Hematol*. 2020;95:233.
35. Mehta A, Ramaswami U, Muenzer J, et al. A charitable access program for patients with lysosomal storage disorders in underserved communities worldwide. *Orphanet J Rare Dis*. 2021;16(1):8.
36. Peterschmitt MJ, Cox GF, Ibrahim J, et al. A pooled analysis of adverse events in 393 adults with Gaucher disease type 1 from four clinical trials of oral eliglustat: Evaluation of frequency, timing, and duration. *Blood Cells Mol Dis*. 2018;68:185.
37. Revel-Vilk S, Szer J, Mehta A, Zimran A. How we manage Gaucher disease in the era of choices. *Br J Haematol*. 2018;182:467.
38. Langeveld M, Elstein D, Szer J, et al. Classifying the additional morbidities of Gaucher disease. *Blood Cells Mol Dis*. 2018;68:209.
39. Zimran A, Ruchlemer R, Revel-Vilk S. A patient with Gaucher disease and plasma cell dyscrasia: bidirectional impact. *Hematology Am Soc Hematol Educ Program*. 2020;4:389.
40. Ryan E, Seehra G, Sharma P, Sidransky E. GBA1-associated parkinsonism: new insights and therapeutic opportunities. *Curr Opin Neurol*. 2019;32:589.
41. Weinreb NJ, Deegan P, Kacena KA, et al. Life expectancy in Gaucher disease type 1. *Am J Hematol*. 2008;83:896.
42. Han TU, Sam R, Sidransky E. Small molecule chaperones for the treatment of Gaucher disease and GBA1-associated Parkinson disease. *Front Cell Dev Biol*. 2020;8:271.
43. Cring MR, Sheffield VC. Gene therapy and gene correction: targets, progress, and challenges for treating human diseases. *Gene Ther*. 2022;29:3.
44. Cohen Y, Frydman D, Rotem R, et al. Risk of postpartum hemorrhage in multiparous women with Gaucher disease: a call for reconsidering enzyme replacement therapy in all pregnant patients. *J Inherit Metab Dis*. 2021;44(5):1165–1173.

CHAPTER 55

EPSTEIN-BARR VIRUS AND ASSOCIATED LYMPHOPROLIFERATIVE CONDITIONS

Nader Kim El-Mallawany, Lisa R. Forbes, Rayne H. Rouce, and Carl E. Allen

The initial clinical descriptions of primary Epstein-Barr virus (EBV) infections are credited to Filatov and Pfeiffer at the end of the 19th century. Pfeiffer coined the term *glandular fever*, which described an illness consisting of fever, malaise, sore throat, and lymphadenopathy. In 1920, Sprunt and Evans introduced the term *infectious mononucleosis (IM)* to describe a series of patients with fatigue, fever, lymphadenopathy, and prominent mononuclear lymphocytosis (Fig. 55.1). Serologic diagnosis of IM became available in the 1930s with the heterophile agglutination test developed by Paul and Bunnel and later modified by Davidson.

The identification of EBV as the causative agent of IM was impeded for many years by the inability to transmit the disease to animals or to grow the virus ex vivo. In 1958, Denis Burkitt described a lymphoma in Ugandan children, and investigators suspected an infectious etiology because the geographic distribution of disease coincided with the malaria belt of equatorial Africa.[1] In 1964 Epstein, Achong, and Barr described herpesvirus-like particles in tumor biopsies from Ugandan children with Burkitt lymphoma (BL). Werner and Gertrude Henle developed an indirect immunofluorescent antibody assay to this new virus, now called *Epstein-Barr virus*, and showed that African children with BL, as well as 90% of American adults, had antibodies against EBV. In 1965 the Henles documented seroconversion to EBV of an individual who presented with clinical symptoms of IM. This initial observation was corroborated by larger studies confirming the association of EBV and IM.

EBV has subsequently been linked to a heterogeneous group of diseases.[2] EBV was the first human virus implicated in oncogenesis, and the biology of the virus has been studied extensively on a cellular and molecular level.[2] Because primary EBV infection is a self-limiting disease in almost all immune-competent individuals, clinical strategies have focused on the diagnosis and treatment of rare, potentially fatal EBV-associated diseases. Over the past two decades, successful immunotherapeutic approaches have been developed for EBV-associated malignancies, using either monoclonal antibodies or the adoptive transfer of EBV-specific T cells.[3–7]

BIOLOGY OF EPSTEIN-BARR VIRUS

EBV belongs to the family of herpesviruses, which has almost 100 members. Membership is based on the architecture of the virion that is 120 to 300 nm in size and contains (1) a core of linear, double-stranded DNA, (2) an icosadeltahedral capsid with 162 capsomers, (3) an amorphous material between the capsid and envelope designated tegument, and (4) an envelope containing viral glycoproteins. Besides EBV, designated human herpesvirus 4 (HHV-4), seven other herpesviruses have been isolated from humans: herpes simplex viruses 1 and 2, cytomegalovirus (CMV), varicella-zoster virus, HHV-6, HHV-7, and the Kaposi sarcoma–associated herpesvirus (KSHV; HHV-8). Herpesviruses are further divided into subfamilies to reflect evolutionary relatedness and similar biologic properties. EBV and KSHV belong to the human gamma herpesvirus subgroup and have a limited tissue tropism to B and T lymphocytes and certain types of epithelial cells. Several variants of EBV have been identified by genomic polymorphisms. Initially, two EBV types were distinguished by sequence changes in EBV nuclear antigens 2 and 3 (EBNA2 and EBNA3). However, using polymorphisms in the latent membrane protein 1 (LMP1), further subtypes have been described. EBV strains vary by geography and have not been linked to a particular EBV-associated disease.

PRIMARY EPSTEIN-BARR VIRUS INFECTION

Primary EBV infection usually occurs through the oropharynx, where mucosal epithelial cells and/or B cells become infected (Fig. 55.2). Infection of B cells by EBV is initiated by binding of the dominant viral glycoprotein gp350/220 to CD21, the C3d complement receptor; subsequent cell entry is mediated by a complex of three viral glycoproteins, gH, gL, and gp42. Gp42 binds to human leukocyte antigen (HLA) class II, which functions as a coreceptor, and gH is most likely involved in virus-cell fusion. The entry of EBV into epithelial cells may occur through multiple mechanisms because the majority of epithelial cells are CD21 negative. After viral entry, the capsid is dissolved and the EBV genome is transported into the nucleus, where it circularizes. Infection of epithelial cells results in lytic or abortive infection. In contrast, B-cell infection results predominantly in latency; however, lytic infection also occurs, resulting in the release of infectious virus into the saliva and other secretions. During primary infection, EBV establishes lifelong latency in B cells, and it is estimated that 1 to 50 cells per 1×10^6 B cells in the peripheral circulation are infected with EBV. The number of latently infected B cells within a person remains stable over years; however, intermittent reactivation of EBV in B cells into the lytic cycle at mucosal sites is probably responsible for the observed shedding of infectious virus into the saliva of asymptomatic carriers (see Fig. 55.2).

Although EBV can infect any B cell and express the full spectrum of latency proteins, typically only infection of naïve B cells results in persistent infection (see Fig. 55.2). EBV infection pushes the naïve B cell into a memory state independent of an antigen-dependent germinal center (GC) reaction by upregulation of cytosine deaminase, which induces both class switching and somatic hypermutation. The former also requires the expression of EBV-encoded LMP1, a constitutively activated CD40 molecule, or CD40 ligation, most likely provided by GC T helper (Th)3 cells that can provide T-cell help for B-cell differentiation by provision of CD40 ligand, interleukin (IL)-4, and IL-10 while preventing antigen-dependent effector T cell–mediated B-cell elimination by expression of transforming growth factor (TGF)-β. This reaction occurs within the lymph node and also involves downregulation of latency proteins and expression of latency type II. On exit from the lymph node, expression of latency proteins is completely inhibited. In this way, infected B cells can evade immune elimination. By contrast, primarily infected memory B cells enter and remain in latency type III and are rapidly eliminated by effector T cells and therefore do not contribute to virus persistence.

LATENT EPSTEIN-BARR VIRUS INFECTION

During latent infection, EBV persists episomally in resting memory B cells. Initially, it was thought that EBNA1 and LMP2 were expressed in memory B cells; however, more recent studies indicate that the

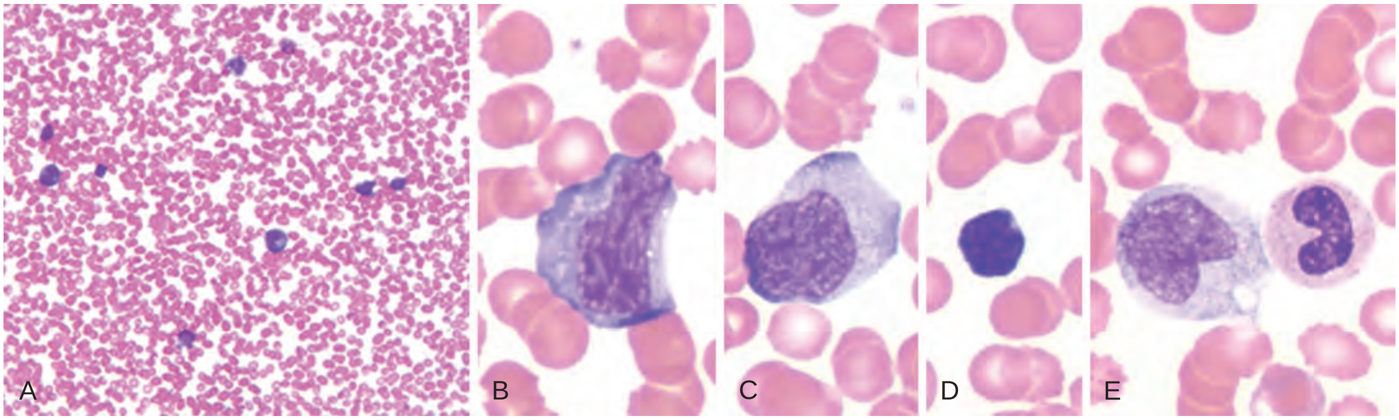


Figure 55.1 PROMINENT MONONUCLEAR LYMPHOCYTOSIS IN MONONUCLEOSIS. Low power (A) illustrates the leukocytosis, mainly due to activated lymphocytes (B and C), which are contrasted with a normal small lymphocyte (D) and a monocyte and granulocyte (E). The large reactive lymphocytes are frequently confused with monocytes because of their morphologic resemblance and the term *mononucleosis*. Monocytes usually have a finer, lacy chromatin and a gray cytoplasm with small granules and vacuoles when compared with the large activated lymphocytes.

majority of infected cells do not express viral proteins and of the almost 100 viral proteins, only EBNA1 is expressed during memory B-cell division (see Fig. 55.2).[2] This extremely limited expression of viral proteins allows EBV to persist long term despite a robust cellular EBV-specific immune response.

Three other distinct types of EBV latency have been characterized in a heterogeneous group of malignancies (Fig. 55.3). Latency type III, which can be readily produced by infecting B cells in vitro with EBV, is expressed in lymphoblastoid cell lines (LCLs). These cells express the entire array of nine EBV latency proteins: EBNA1, -2, -3A, -3B, and -3C, EBNA leader protein (LP), and the two viral membrane proteins LMP1 and LMP2. This pattern of EBV gene expression characterizes the EBV-associated lymphoproliferative disorders (EBV-LPDs) that occur in individuals severely immunocompromised by solid organ or hematopoietic stem cell transplantation (SOT, HSCT), congenital immunodeficiency, or human immunodeficiency virus (HIV) infection. Latency type II is the hallmark of EBV-positive Hodgkin lymphoma (HL), various EBV-associated non-Hodgkin lymphomas (NHLs), as well as nasopharyngeal carcinoma (NPC), which express EBNA1, LMP1, and LMP2. In addition, BARF1 is expressed in subsets of latency type II–associated malignancies. In latency type I, classically found in EBV-positive BL,

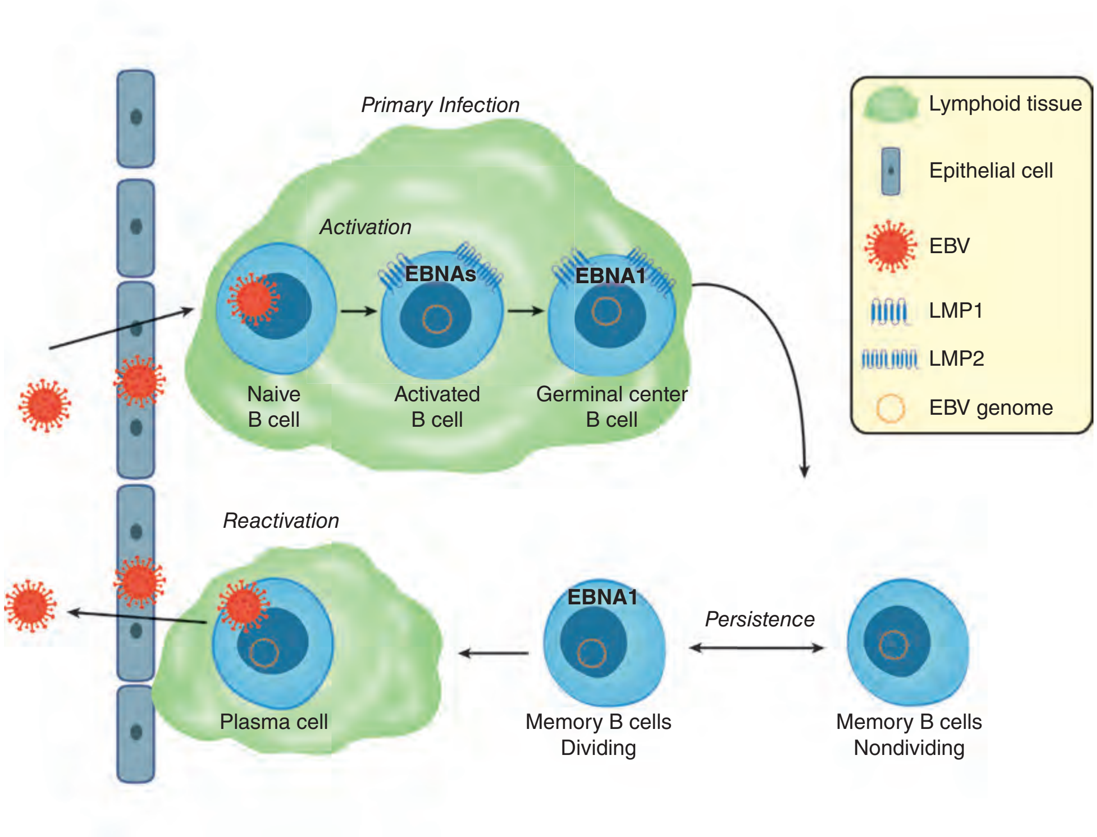

Primary Infection
Activation
EBNAs
EBNA1
Naive B cell
Activated B cell
Germinal center B cell
Reactivation
EBNA1
Persistence
Plasma cell
Memory B cells Dividing
Memory B cells Nondividing
Lymphoid tissue
Epithelial cell
EBV
LMP1
LMP2
EBV genome

Figure 55.2 INFECTIOUS LIFE CYCLE OF EPSTEIN-BARR VIRUS. *EBV*, Epstein-Barr virus; *EBNA*, Epstein-Barr nuclear antigen; *LMP*, latent membrane protein.

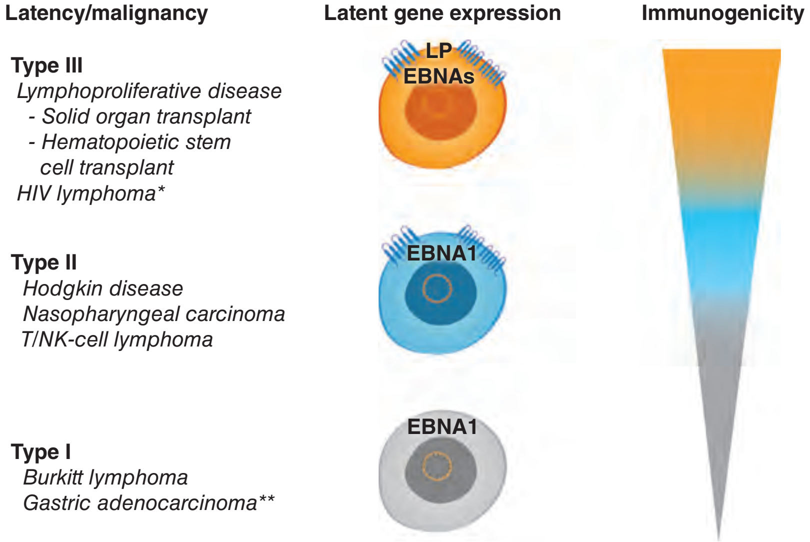


Figure 55.3 EPSTEIN-BARR VIRUS (EBV)–LATENT GENE EXPRESSION AND IMMUNOGENICITY OF COMMON EBV-ASSOCIATED MALIGNANCIES. *EBNA*, Epstein-Barr nuclear antigen; *LP*, leader protein; *NK*, natural killer. For an explanation of symbols, see Fig. 55.2. *Not all lymphomas are latency type III. **Gastric adenocarcinoma can also express latency type II genes.

only EBNA1 is expressed. However, variants in which all EBNAs are expressed in the absence of LMP1 have also been described. Latency type I or latency type II is found in EBV-associated gastric adenocarcinoma. Although grouping EBV-associated malignancies according to their dominant gene expression profile provided a useful framework for understanding EBV-driven oncogenesis, more recent studies using comprehensive gene expression array profiling have demonstrated expression of lytic cycle genes in BL or expression of lytic cycle and latency III genes in NPC.[8,9]

The EBV proteins expressed during type III latency are involved in the transformation and growth of EBV-infected B cells. EBNA1 binds to the origin of replication of the latent viral genome and is responsible for the maintenance of the EBV episome in host B cells. EBNA2 upregulates the expression of the viral proteins LMP1 and LMP2 and cellular proteins that contribute to transformation. EBNA3A and EBNA3C are essential for EBV-induced B-cell transformation, and although EBNA3B is not essential for transformation, it is highly conserved and therefore must provide a survival function in vivo. EBNA-LP cooperates with EBNA2 in the induction of viral and cellular genes. LMP1, a viral oncogene, behaves like a constitutively activated CD40 molecule and is essential for EBV-mediated B-cell transformation. LMP2 mimics an activated B-cell receptor (BCR), allowing for long-term B-cell survival in the absence of antigen. In addition, it prevents the reactivation of EBV into the lytic phase of infection.

Besides EBV proteins, small nonpolyadenylated viral RNAs termed *EBERs 1* and *2* and the BamHI-A rightward transcripts microRNAs (BART-miRs) are expressed in all forms of latency. In addition, the expression of at least 17 distinct EBV-derived microRNAs has been reported. The EBERs are the most abundant viral RNAs in latently infected cells. They enhance the oncogenic phenotype of EBV-transformed cells but are nonessential for EBV-mediated transformation. The expression pattern of the microRNAs depends on the latency type, and it is therefore likely that they play an important role during the life cycle of the virus.

IMMUNE RESPONSE TO EPSTEIN-BARR VIRUS

Healthy individuals mount vigorous humoral and cellular immune responses to primary EBV infection.[10] Although antibodies to the viral membrane proteins neutralize virus infectivity, the cellular immune response is essential for controlling virus-infected cells during both lytic and latent phases.

Humoral Immune Responses

Heterophile Antibodies

Heterophile antibodies, originally described by Paul and Bunnell, are present in 90% to 95% of EBV infections at some point during the illness. However, in infants and children younger than the age of 4 years with primary EBV infection, heterophile antibody responses may not be detected. Heterophile antibodies are immunoglobulin M (IgM) antibodies, which agglutinate erythrocytes from different species including bovine, camel, horse, goat, and sheep. EBV-induced heterophile antibodies have no reactivity against guinea pig kidney cells in contrast to naturally occurring antibodies (Forssman antibodies) or antibodies present in patients with serum sickness and other conditions.

In addition to heterophile antibodies, cold agglutinins directed preferentially against the anti-I antigen on red cell membranes are frequently detected in the sera of patients with IM; however, hemolytic anemia is rare. Other antibodies (including anti-I, anti-N, Donath-Landsteiner antibodies, platelet antibodies, and anti–smooth muscle antibodies) have been described in association with EBV infection

Epstein-Barr Virus–Specific Antibodies

EBV-specific antibody responses are detected with immunofluorescence assays developed in the first decades of EBV research. EBV antibodies are directed against (1) EBNA, (2) early antigen (EA), (3) the membrane antigen expressed on the surface of cells late in the lytic cycle, and (4) the viral capsid antigen (VCA) expressed within cells late in the lytic cycle. Each antigen is a composite of several distinct viral proteins, and attempts have been made to replace the aforementioned assays with tests using specific viral proteins; however, no single test has attracted widespread use.

VCA-IgM and IgG antibodies are usually present at the onset of clinical symptoms because of the prolonged viral incubation period (Table 55.1). VCA-IgM antibodies are a good marker for an acute infection because they rapidly disappear within 4 to 8 weeks.

TABLE 55.1 Frequently Determined Epstein-Barr Virus–Specific Antibodies

Antibody Specificity	Positive in IM (%)	Time of Appearance in IM	Persistence	Comments
Viral Capsid Antigen (VCA)				
VCA-IgM	100	At clinical presentation	4–8 weeks	Highly sensitive and specific; of major diagnostic utility
VCA-IgG	100	At clinical presentation	Lifelong	Useful for documentation of past EBV infection
Early Antigen (EA)				
Anti-D	70	Peaks 3–4 weeks after onset	3–6 months	Correlates with disease severity; seen in patients with NPC
Anti-R	Low	2 weeks to several months after onset	2 months to >3 years	Occasionally seen with unusually severe cases; seen in patients with African Burkitt lymphoma
EBNA	100	3–4 weeks after onset	Lifelong	Presence excludes primary EBV infection

EBV, Epstein-Barr virus; *IM*, infectious mononucleosis; *NPC*, nasopharyngeal carcinoma.
Modified from Schooley RT. Epstein-Barr virus (infectious mononucleosis). In: Mandell GL, Bennett JE, Dolin R, eds. *Principles and Practice of Infectious Diseases*. Philadelphia: Churchill Livingstone; 2000:1599.

VCA-IgG antibodies persist for life and are commonly used to document prior EBV infection. IgG antibodies against EA are present at the onset of the clinical illness in approximately 70% of patients. EA antibodies are divided into methanol-sensitive (anti-D) and methanol-resistant (anti-R) antibodies, and the majority of EA antibodies detected are anti-D antibodies. The presence of anti-D antibodies is consistent with recent infection, because titers disappear after recovery. IgG antibodies to EBNA appear late in the course of almost all cases of EBV infection and persist throughout life; their presence early in a suspected case of primary EBV infection excludes the diagnosis. Aberrations in this pattern of serum reactivity are observed in many EBV-associated diseases and will be discussed under the specific disease sections. For example, the absence of EBNA antibodies despite previous EBV infection is one of the serologic markers suggestive for chronic active EBV (CAEBV) infection.

Cellular Immune Responses

In normal individuals, primary EBV infection often results in a massive expansion of activated, antigen-specific T cells. Using tetramer technology to enumerate antigen-specific T cells, it has been documented that the $CD8^+$ T-cell response may be dominated by T cells specific for a limited number of epitopes, as seen with T-cell responses against other herpesviruses. T cells specific for epitopes derived from immediate early and several early EBV proteins of the lytic cycle are dominant during the acute phase of IM, and long-term persistence of EBV-specific, $CD8^+$ T cells has been documented after primary EBV infection. As many as 5.5% of the circulating $CD8^+$ T cells in a healthy virus carrier may be positive for a single EBV epitope, illustrating how persistent EBV infection can influence the composition of the host's T-cell pool. Besides EBV-specific $CD8^+$ T cells, EBV-specific $CD4^+$ T cells play an important role in the control of EBV infections, and EBNA1-specific $CD4^+$ T cells have been implicated in the control of newly infected B cells. As for $CD8^+$ T-cell responses, there is a marked hierarchy of immunodominance, with the majority of $CD4^+$ T cells being specific for EBNA1 and to less extent EBNA3C.

EPSTEIN-BARR VIRUS VACCINE DEVELOPMENT

At present there is only limited experience with human EBV vaccines.[11] Vaccine studies have been conducted in healthy donors in the prophylactic setting using recombinant vaccinia virus encoding the major viral glycoprotein gp350 (1), recombinant gp350 protein (2), or an EBNA3A peptide. While vaccination with gp350 induced neutralizing antibodies and in one study prevented the clinical picture of IM, these vaccines did not significantly reduce the incidence of EBV infection. Also, no reduction in the incidence of EBV infection was observed with the EBNA3A peptide vaccine. In addition, a recombinant gp350 protein was evaluated in patients with chronic kidney disease prior to kidney transplantation. Although the vaccine-induced transient neutralizing antibodies, it did not reduce the incidence of EBV-LPD post transplant. Newer vaccines consisting of gp350-ferritin complexes that induce significantly higher titers of neutralizing antibodies in preclinical models have been developed but require testing in humans.[12]

Vaccine strategies for EBV-associated malignancies should seek to elicit or boost the EBV-specific cellular immune response against EBV latency. Individuals likely to benefit from this approach are EBV-seronegative patients scheduled to undergo SOT or patients who have an EBV-associated malignancy with a low tumor burden or are in remission.[12] A variety of vaccine studies have been conducted in patients with EBV^+ NPC.[13] In two studies patients with advanced disease were vaccinated with either dendritic cells (DCs) loaded with peptides derived from LMP2 or DC transduced with an adenoviral vector encoding full-length LMP2 and an inactive form of LMP1. Administration of DC vaccines was safe, and a transient increase in the frequency of LMP2-specific T cells was observed on the DC/peptide vaccine trial. However, the clinical benefit in both studies was limited. Thus future studies will need to explore DC vaccines with greater potency administered to subjects with less tumor burden.[12] In another phase I clinical study, after frontline therapy, patients with NPC were vaccinated with a modified vaccinia virus Ankara encoding the c-terminal portion of EBNA1 and LMP2 (MVA-EL). Induction of $CD4^+$ and $CD8^+$ antigen-specific T-cell responses was observed in the majority of patients after vaccination, and a phase II clinical study is in progress.[13] Lastly, a recombinant adenovirus vaccine encoding LMP2 was evaluated in patients with NPC. Although vaccine administration was safe, no data are available if the vaccine boosted LMP2-specific T-cell responses.[12] Ultimately, although development of an effective EBV vaccine has remained an elusive achievement, numerous studies have been undertaken in pursuit of this goal.[14–24]

Although the aggregate number of patients around the world with EBV-associated lymphomas and lymphoproliferative disorders (LPDs) is modest, when EBV-associated gastric carcinoma and NPC are considered, global estimates of EBV-associated malignancies approach 200,000 cases per year.[25] Acknowledging the burden of these malignant complications from EBV, in addition to the toll of IM in adolescents and young adults infected by the virus, a case for vaccine development gains traction. As precedent for effective vaccination against other oncogenic viruses such as human papillomavirus and hepatitis B has been established, EBV should be considered a target for wide-scale vaccine development.[11,26,27]

INFECTIOUS MONONUCLEOSIS

Epidemiology

EBV infections occur worldwide, and, in most populations, 90% to 95% of adults have antibodies against EBV. Depending on geographic and socioeconomic factors, there is a wide variation in the age of primary EBV infections. Early, asymptomatic primary EBV infection occurs more often in individuals from lower socioeconomic groups, particularly those living in low- and middle-income countries. In higher socioeconomic groups in high-income countries, the age of primary infection is more often delayed until the second decade of life and clinically apparent IM is more prevalent.

Humans are the only source of EBV, which is present in the saliva of patients with IM. A majority of EBV-positive adults shed virus into their saliva, and this percentage is increased in immunocompromised patients such as SOT recipients. EBV is viable outside the body for 2 weeks at 4°C but is susceptible to drying; the virus has not been recovered from environmental sources, suggesting that close contact is needed for viral spread. The incubation period of EBV is estimated to be 30 to 50 days.

Clinical Manifestations

Primary EBV infection in infants and young children is either asymptomatic or accompanied by mild, nonspecific symptoms and signs such as fever, upper respiratory tract infection, pharyngitis with or without tonsillitis, and cervical lymphadenopathy. In contrast, approximately 30% to 50% of adolescents and young adults present with the clinical picture of IM.[28] Frequently, a prodrome consisting of fatigue, malaise, and low-grade fever is present for 1 to 2 weeks. Prominent pharyngitis with exudative tonsillitis is often the cardinal sign of IM; other signs and symptoms are listed in Table 55.2. The adenopathy in IM most commonly affects the posterior cervical lymph nodes, although diffuse adenopathy can occur. The enlarged lymph nodes are not fixed, may be tender to palpation, and lack overlying skin erythema. Hepatomegaly is uncommon; however, splenomegaly develops in 15% to 65% of patients and is more prominent in the second to fourth week of the illness. Skin manifestations include a faint, morbilliform rash reminiscent of rubella and less commonly erythema multiforme and erythema nodosum. Most patients with primary EBV infection have symptoms for 2 to 4 weeks and recover without significant complications or sequelae.

Complications of Infectious Mononucleosis

Although the majority of patients with IM fully recover without severe complications, any organ system can be affected in this clinical syndrome of primary EBV infection.

TABLE 55.2 Clinical Manifestations of Infectious Mononucleosis

Manifestation	Percentage (Range)
Symptoms	
Sore throat	82 (70—88)
Malaise	57 (43–76)
Headache	51 (37–55)
Anorexia	21 (10–27)
Myalgias	20 (12–22)
Chills	16 (9–18)
Abdominal discomfort	9 (2–14)
Signs	
Lymphadenopathy	94 (93–100)
Pharyngitis	84 (69–91)
Fever	76 (63–100)
Splenomegaly	52 (50–63)
Hepatomegaly	12 (6–14)
Palatal enanthem	11 (5–13)
Rash	10 (0–15)
Jaundice	9 (4–10)

Modified from Schooley RT. Epstein-Barr virus (infectious mononucleosis). In: Mandell GL, Bennett JE, Dolin R, eds. *Principles and Practice of Infectious Diseases*. Philadelphia: Churchill Livingstone; 2000:1599.

Hematologic Complications

Patients with IM may present with a wide range of hematologic findings besides the atypical lymphocytosis (Fig. 55.4). These include anemia, neutropenia, thrombocytopenia, and rare cases of aplastic anemia.

Anemia

Autoimmune hemolytic anemia occurs in approximately 3% of patients with IM. It presents in the first 2 weeks of the illness, and the majority of patients recover within 1 to 2 months. Patients usually have a positive direct Coombs test. Most common anti-I antibodies are present; however, anti-I, anti-N, and Donath-Landsteiner antibodies have also been reported. In addition to hemolysis, IM-associated anemia can be caused by transient erythroblastopenia.

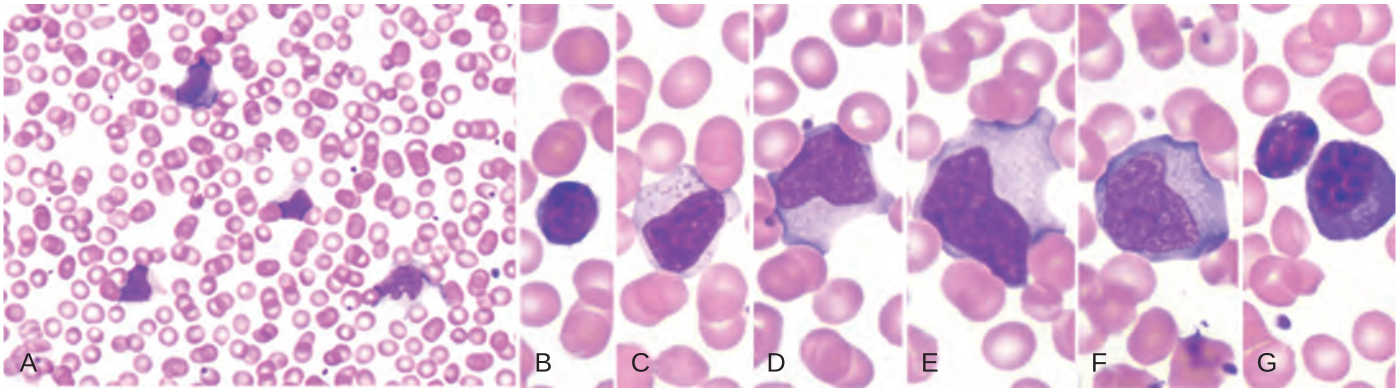


Figure 55.4 PERIPHERAL BLOOD SMEAR IN INFECTIOUS MONONUCLEOSIS. (A) Low power shows moderately high white blood cell count and high number of reactive, or "atypical" lymphocytes. (B–G) Higher power illustrates spectrum of lymphoid morphology, including small resting lymphocyte (B) for comparison, large granular lymphocyte (C), atypical forms (D–F), also referred to as "reactive" lymphs, and circulating plasma cell (G).

Neutropenia

Mild, self-limiting neutropenia is a common finding during the first 4 weeks of the disease. However, severe neutropenia associated with fatal bacterial infections has been reported.

Thrombocytopenia

Mild thrombocytopenia (50,000 to 150,000/mm^3) is a common finding in patients with IM. It usually occurs within the first 2 weeks of presentation and resolves within 2 months. Severe thrombocytopenia with overt bleeding is rare; however, death from intracranial hemorrhage has been described. The etiology of the thrombocytopenia is not completely understood, and a variety of explanations have been suggested. Because bone marrow examination shows normal or increased numbers of megakaryocytes, peripheral platelet consumption is most likely due to the presence of antiplatelet antibodies or platelet pooling and destruction within an enlarged spleen.

Splenic Rupture

Splenic rupture occurs predominately in males, with an incidence of 1/1000 to 1/3000. The incidence of rupture is highest in the second and third week of illness and can be the first sign of IM. Clinical symptoms include abdominal pain or pain referred to either shoulder. Because abdominal pain is an unusual symptom of uncomplicated IM, a splenic rupture should be strongly considered in IM cases when abdominal pain is reported. Although it is a life-threatening complication, with current management the mortality rate is very low.

Neurologic Complications

Neurologic complications develop usually during the first 2 weeks of IM and may be the only manifestation of IM. EBV infection can cause a wide spectrum of neurologic diseases, including encephalitis, meningitis, Guillain-Barré syndrome, acute transverse myelitis, and peripheral neuritis. Most patients with neurologic complications recover completely.

Other Organ Involvement

Although symptomatic heart disease with IM is uncommon, in one cohort of patients, nonspecific ST- and T-wave abnormalities were found in 6% of patients. Renal involvement manifested as microscopic hematuria, and proteinuria is seen in 10% to 15% of patients; however, significant renal dysfunction is rare. Airway compromise due to hypertrophy of the adenoids and tonsils or mucosal inflammation and edema is uncommon but potentially fatal.

Diagnosis

Atypical lymphocytosis is the cardinal hematologic finding in IM (see Fig. 55.4). It develops during the first week of the illness and peaks between the second and third week. Atypical lymphocytes represent 60% to 70% of the total white cell count, which usually ranges between 12,000/mm^3 and 18,000/mm^3. In general, the atypical lymphocytes are large and vary in size. Nuclei are large and eccentrically placed; the cytoplasm is basophilic, and vacuoles are often present. The variable morphologic pattern of atypical lymphocytes in IM distinguishes them from the monotonous appearance of immature leukemic blasts. Atypical lymphocytosis is not pathognomonic for IM and is associated with other diseases such as acute viral hepatitis, CMV infections, mumps, toxoplasmosis, rubella, roseola, and drug reactions.

The diagnosis of EBV infection depends on serologic testing. Tests for heterophile antibodies, including the monospot test and slide agglutination tests, are routinely available. The results of these tests are often negative in children younger than monopod 4 years of age, but they identify 90% of cases in older children and adults. Of the available EBV-specific serologic tests, VCA-IgM antibodies are most commonly determined to diagnose primary EBV infection in heterophile-negative IM cases; determining antibodies against EA may also be helpful (see Table 55.1). VCA-IgG antibodies are positive during acute infections as well as the convalescent period. The presence of anti-EBNA antibodies excludes an acute infection. Isolation of EBV from throat washings is feasible; however, it is of little diagnostic value because 10% to 20% of healthy adult EBV carriers may shed the virus.

Differential Diagnosis

In the majority of cases, the diagnosis of IM is straightforward. The differential diagnosis includes streptococcal and nonstreptococcal pharyngitis, acute infections with CMV, HHV-6, hepatitis viruses, and toxoplasma. Depending on the presentation, other diseases may be considered such as HIV, rubella, and leukemia or lymphoma.

Treatment

Supportive therapy should include rest and analgesia in the acute stage of IM. Contact sports should be avoided until the patient has fully recovered and the spleen is no longer palpable. The use of corticosteroids is not indicated for uncomplicated IM; however, a trial of corticosteroids may be warranted in patients with marked tonsillar inflammation and hypertrophy resulting in impending airway obstruction. Although acyclovir reduces EBV shedding into oral secretions, treatment of IM with acyclovir has resulted in no clinical benefit.

INHERITED IMMUNODEFICIENCY DISORDERS AND PREDISPOSITION TO EPSTEIN-BARR VIRUS–ASSOCIATED NEOPLASMS

A variety of inherited immunodeficiency disorders have been associated with a genetic predisposition to EBV-associated complications. Patients with dysfunctional cytotoxic T-cell function are particularly susceptible to EBV-related lymphoproliferation and subsequent lymphoma due to inability to "prune" EBV$^+$ B cells. The clinical syndromes driven by EBV are heterogeneous and include non-malignant B-cell hyperplasia, CAEBV infection, hemophagocytic lymphohistiocytosis (HLH), and overt lymphomas. X-linked lymphoproliferative disease (XLP) was historically the classic immunodeficiency disorder associated with vulnerability to life-threatening complications of EBV infection, but since the turn of the century a number of novel immunodeficiency disorders have been identified through technological advances in genetic testing and the enhanced capacity to identify clinically relevant gene mutations.[29,30] XLP is notable in that patients are specifically susceptible to EBV and typically are unaffected by other microorganisms. Other immunodeficiency disorders (e.g., X-MEN disease, ITK, coronin 1A, CD27, CD16, CD70, and MCM4 deficiencies) are characterized by susceptibility to viral infections in general, and patients frequently experience severe complications from EBV. EBV-related complications can also occur in immunodeficiency disorders in which patients are generally susceptible to both viral and nonviral infections (e.g., activated PI3Kδ syndrome, Chédiak-Higashi syndrome, and deficiencies of STK4, ZAP70, CTPS1, and GATA2). Another group of immunodeficiency disorders notable for predisposition to EBV-associated complications is characterized specifically by susceptibility to developing lymphoma (e.g., Wiskott-Aldrich syndrome, ataxia telangiectasia, and autoimmune lymphoproliferative syndrome). This section will highlight XLP1 and XLP2 and discuss the ever-expanding range of uncommon and even rare immune deficiency disorders that have been associated with lymphoproliferative complications of EBV infection (Table 55.3).

TABLE 55.3 **Primary Immunodeficiencies Associated With Lymphoproliferative Complications of Epstein-Barr Virus Infection**

Immune Deficiency Syndrome	Affected Gene	Immune Defect Related to EBV Immunity
Impaired T-Cell and NK–Cell Cytotoxicity		
XLP1	*SH2D1A*	Impaired T/NK-cell cytotoxicity
XLP2	*BIRC4*	Impaired T/NK-cell cytotoxicity, BIRC4 encodes XIAP
Chédiak-Higashi syndrome	*LYST*	Impaired T/NK-cell cytotoxicity
Griscelli syndrome	*RAB27A*	Impaired T/NK-cell cytotoxicity
Familial HLH-2	*PRF1*	Impaired T/NK-cell cytotoxicity
Familial HLH-3	*UNC13D*	Impaired T/NK-cell cytotoxicity
Familial HLH-5	*STXBP2*	Impaired T/NK-cell cytotoxicity
CD16 deficiency	*FCGR3A*	Impaired spontaneous NK-cell cytotoxicity
Defects in Cell Proliferation, Differentiation, and Signaling		
Coronin1A deficiency	*CORO1A*	Defective actin polymerization and T-cell activation
Activated PI3Kδ syndrome	*PIK3CD*	Impaired CD8$^+$ T-cell and NK-cell effector function
Activated PI3Kδ syndrome	*PIK3R1*	Impaired CD8$^+$ T-cell and NK-cell effector function
IL-2 inducible T-cell kinase deficiency	*ITK*	Impaired T-cell function
X-linked immunodeficiency with magnesium defect, EBV, and neoplasia	*MAGT-1*	Defective TCR glycosylation and impaired cytolytic function
CD27 deficiency	*CD27*	Impaired T/NK-cell function
CD70 deficiency	*CD70*	Impaired B-cell signaling
NF-κB1 haploinsufficiency with common variable immunodeficiency	*NFκB1*	Impaired T- and B-cell function
STAT1 gain of function	*STAT1*	Impaired T-, B-, and NK-cell function
STIM1 deficiency	*ORAI1*	Defective Ca^{2+} sensing and T-cell activation
RASGRP1 immunodeficiency	*RASGRP1*	Defective T-cell development and B-cell differentiation
CTPS1 immunodeficiency	*CTPS1*	Impaired lymphocyte function and differentiation
STK4 deficiency	*STK4 (MST1)*	T-cell lymphopenia and defective antigen-induced T-cell stimulation and increased apoptosis
DOCK8 deficiency	*DOCK8*	Impaired cytoskeletal rearrangement, immune cell trafficking and survival
ALPS	*TNFRSF6*	Impaired T-cell stimulation
CD137 deficiency	*TNFRSF9*	Impaired T-cell stimulation
CARMIL2 deficiency	*CARMIL2*	Impaired T-cell stimulation and proliferation
LRBA deficiency	*LRBA*	Defective T-cell signaling
B-cell expansion with NF-κB and T-cell anergy (BENTA) disease	*CARD11*	Defective B-cell and T-cell signaling
Defective T-Cell and NK-Cell Development		
SCID	*RAG1* and *RAG2*	Defective V(D)J recombination and impaired T- and B-cell development
SCID	*DCLRE1C*	Defective V(D)J recombination and DNA DSB repair impair T- and B-cell development
SCID	*IL2RG*	Defective T/NK-cell development
SCID	*JAK3*	Defective T/NK-cell development
SCID	*IL7RA*	Defective T/NK-cell development
Combined immunodeficiency syndrome	*ZAP70*	Defective CD8$^+$ T-cell development
Wiskott-Aldrich syndrome	*WAS*	Defective T/NK-cell development and function
GATA2 deficiency	*GATA2*	Defective T/NK-cell development
MCM4 deficiency	*MCM4*	Defective T/NK-cell development and function
Defective DNA Repair		
Ataxia-telangiectasia	*ATM*	Defective DNA repair
Nijmegen breakage syndrome	*NBN*	Defective DNA repair
Ligase 4 syndrome	*LIG4*	Defective DNA repair, defective T/NK-cell development

ALPS, Autoimmune lymphoproliferative syndrome; *DSB*, double-strand break; *EBV*, Epstein-Barr virus; *HLH*, hemophagocytic lymphohistiocytosis; *IL*, Interleukin; *NK*, natural killer; *SCID*, severe combined immunodeficiency syndrome; *TCR*, T-cell receptor; *XLP1*, X-linked lymphoproliferative disease 1.

X-Linked Lymphoproliferative Diseases

X-Linked Lymphoproliferative Disease 1

Mutations or deletions in *SH2D1A* (Src homology 2 domain protein 1A) result in X-linked lymphoproliferative disease 1 (XLP1; Duncan disease), an immunodeficiency characterized by potentially fatal IM meeting the diagnostic criteria for HLH, agammaglobulinemia, or B-cell lymphoma. SH2D1A interacts with SLAM (signaling lymphocyte activation molecule), which plays a central role in the stimulation of B and T cells. SH2D1A controls several distinct key T-cell signaling pathways, and mutant SH2D1A does not bind SLAM, suggesting that it is a natural SLAM inhibitor. SAP association with SLAM receptors is crucial for development of normal natural killer (NK)/T cells, formation of normal GCs, and NK- and T-cell killing of EBV-infected B cells. T cells from patients with XLP1 are also resistant to apoptosis by radiation-induced cell death. Immune hyperactivation induced by primary EBV infection may be due to specific defects in NK and CD8$^+$ T-cell cytotoxicity rather than from decreased or absent cytotoxic proteins. Resistance to apoptosis may exacerbate the inflammatory response due to persistence of ineffective activated NK and T cells.

Following infection with EBV, patients with XLP1 mount a vigorous, uncontrolled polyclonal expansion of T and B cells. Infiltrating T cells cause extensive tissue destruction of the liver and bone marrow, resulting in death in 50% of XLP1 patients during primary EBV infection. Approximately 30% of patients have acquired hypogammaglobulinemia, and 25% of patients develop malignant B-cell lymphomas that are often extranodal, involving the intestinal ileocecal region. It is important to realize that some patients with *SH2D1A* mutations may present with only hypogammaglobulinemia, mimicking common variable immunodeficiency, and a diagnosis of XLP1 should be considered when more than one male patient with hypogammaglobulinemia is encountered in the same family. Patients with fulminant immunologic responses to primary EBV infection may be treated with HLH treatment strategies (glucocorticoids and etoposide) and/or rituximab. However, the only curative therapy for immune dysregulation associated with dysfunctional XLP1 is allogeneic HSCT.

X-Linked Lymphoproliferative Disease 2

A second X-linked immune deficiency characterized by recurrent HLH (with or without EBV infection) is XLP2, caused by *BIRC4* mutations and XIAP deficiency.[31] Unlike patients with XLP1, those with XLP2 have less pleotropic clinical manifestations. It rarely results in lymphoproliferation or lymphoma and may be more accurately characterized as "X-linked familial HLH." XIAP is a ubiquitously expressed member of a family of proteins defined by baculovirus IAP repeat (BIR) domains that inhibit apoptosis through inhibition of caspases. The mechanism of XIAP-induced HLH remains uncertain. Paradoxically, unlike in cases of XLP1, in which lymphocytes are resistant to apoptosis, XIAP deficiency in XLP2 confers increased sensitivity to radiation-induced cell death. The clinical manifestations of HLH in XLP2 patients with primary EBV infections appear less severe than in patients with XLP1. However, data remain insufficient to make specific therapy recommendations for XLP1 versus XLP2 or other forms of familial HLH.

Other Inherited Immunodeficiency Disorders Associated with Epstein-Barr Virus Disease

Most individuals recover from the acute phase of primary EBV infection with no long-term sequelae, including some patients with primary immunodeficiency disorders. However, a minority of patients with intrinsic defects in immune function may be vulnerable to severe complications from primary EBV infection (see Table 55.3). Impairment of T/NK-cell cytotoxicity primarily is associated with risk for potentially lethal uncontrolled pathologic inflammation driven by EBV infection, manifest by fever and multisystem organ failure, typically meeting diagnostic criteria for HLH. Others, with immune deficiencies derived from defective T/NK-cell development, proliferation, differentiation, and signaling pathways may develop CAEBV or even EBV-associated LPD or malignancies.[32,33] In addition to primary immunodeficiencies that result in failure to control EBV infection, there is an increasing list of gene defects associated with complex immune dysfunction that predispose to complications of EBV infection.[29,30,32,33]

Immune regulation of EBV in primary immune deficiency (PID) or regulation syndromes depends on the particular cellular defect. Table 55.3 categorizes immune deficiency syndromes associated with lymphoproliferative complications of EBV infection based on type of immune defect. For example, similar to XLP1 and XLP2, there are numerous other disorders characterized by impairment of T/NK-cell cytotoxicity are associated with EBV-driven HLH.[29,30,32,33] Primary T-cell deficiencies with defective T-, NK-, and/or B-cell development, such as severe combined immunodeficiency (SCID) lead to absence of T cells which render patients vulnerable to not only EBV, but a broad array of infectious diseases.[32] Immune disorders characterized by improper T-cell differentiation, antigen-induced expansion, and signaling also leave patients not only susceptible to EBV, but a broad range of herpesviral diseases.[29,30,32,33] Lastly, impaired T-cell immune regulation and poor B-cell control of EBV are associated with a risk of EBV-driven lymphoma in combined immunodeficiencies such as Wiskott-Aldrich syndrome, ataxia telangiectasia, as well as other DNA repair defects including Nijmegen breakage syndrome. Ultimately, although many of these immune deficiency syndromes are extremely rare, it is critical to evaluate patients with EBV-driven lymphoproliferation for potential underlying genetic alterations; identification of inherited predisposition not only sheds light on the disease etiology but has important implications in future therapeutic strategies. Such patients typically require restoration of immune function to provide curative intervention, be it through novel immunotherapeutic strategies or allogeneic HSCT.[33]

EPSTEIN-BARR VIRUS–ASSOCIATED NEOPLASIA

Over the past decades, EBV has been associated with a variety of different malignancies.[25] Each year it is estimated that 200,000 cases of EBV-associated malignancy are diagnosed worldwide. Nonlymphoid cancers—gastric carcinoma and NPC specifically—account for more than three quarters of these, while lymphoid neoplasms of B-, T-, and NK-cell origin plus the exceedingly rare EBV-associated leiomyosarcoma make up the remainder (Figs. 55.5 and 55.6).[25] The following sections focus on lymphoid neoplasms associated with EBV, categorized by the underlying cell of origin (i.e., B vs. T/NK cell, Table 55.4). Characteristics of EBV-associated lymphoid neoplasms occurring in patients with acquired immune deficiency will be discussed separately in an ensuing section.

The diverse spectrum of EBV-associated neoplasia is characterized by complex nuances. Although some neoplasms occur primarily in the context of immune deficiency, others typically occur in patients without underlying immune dysfunction, whereas others can occur in either scenario (Table 55.5). Although there is strong circumstantial evidence linking EBV to these malignancies, the potential causative relationship between EBV and these tumors remains to be firmly established. In addition, the frequency of EBV involvement in this spectrum of EBV-associated neoplasia ranges from virtually always to sporadic (Table 55.6). Nonetheless, all EBV-associated malignancies are associated with viral latency, and spontaneous viral replication occurs at a very low frequency. Because antiviral agents, like acyclovir, prevent only viral replication and do not affect latency, these agents are of limited therapeutic value for EBV-associated malignancies.

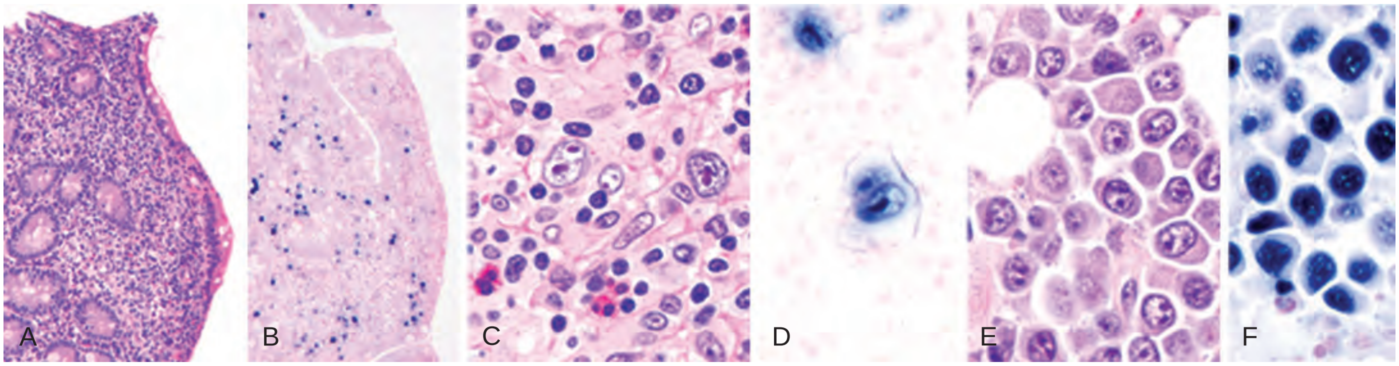


Figure 55.5 EXAMPLES OF EPSTEIN-BARR VIRUS–POSITIVE LYMPHOID MALIGNANCIES. Posttransplant lymphoproliferative disorder (PTLD), Hodgkin lymphoma, and large B-cell lymphoma. PTLD in the duodenum of a 15-month-old (A) with history of liver transplant. (B) The PTLD was classified as a polymorphic type and was Epstein-Barr virus (EBV)-positive. Hodgkin lymphoma (C) and EBV-positive Reed-Sternberg cells (D). Large B-cell lymphoma (plasmablastic type) in a human immunodeficiency virus–positive patient (E), diffusely EBV-positive (F). Note, all EBV studies are in situ hybridizations for EBV messenger RNA, EBER.

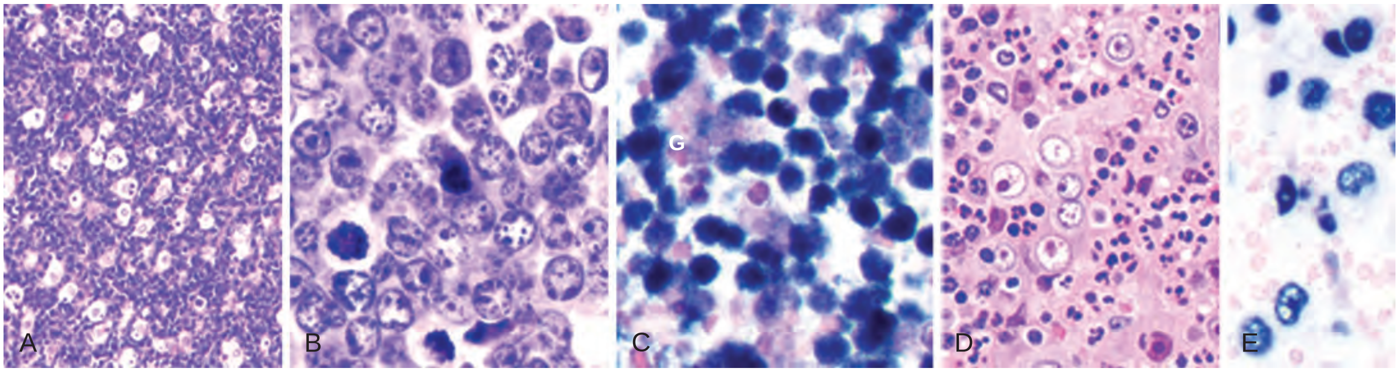


Figure 55.6 FURTHER EXAMPLES OF EPSTEIN-BARR VIRUS (EBV)-POSITIVE MALIGNANCIES. (A–E) Burkitt lymphoma and nasopharyngeal carcinoma. Low power of Burkitt lymphoma showing the classic "starry sky" appearance (A) and higher power illustrating the highly proliferative lymphoma cells (B), which are uniformly EBV-positive (C). Nasopharyngeal carcinoma (D) with EBV-positive cells (E) demonstrated by in situ hybridization for EBV messenger RNA.

TABLE 55.4 Epstein-Barr Virus–Associated Lymphoid Neoplasms Categorized by Cell of Origin

B Lymphocyte	T/NK Lymphocyte
Burkitt lymphoma	Systemic chronic active EBV
Diffuse large B-cell lymphoma	Hydroavacciniforme-like T-cell LPD
Classic Hodgkin lymphoma	Severe mosquito bite allergy
Plasmablastic lymphoma	Systemic EBV$^+$ TCL of childhood
Primary effusion lymphoma	Aggressive NK-cell leukemia
Primary CNS lymphoma	Extranodal NK/T-cell lymphoma, nasal type
Lymphomatoid granulomatosis	Primary EBV$^+$ nodal T/NK-cell lymphoma
Nondestructive and polymorphic PTLD	Angioimmunoblastic lymphoma
Monomorphic PTLD	Monomorphic PTLD (rare)
Other immunodeficiency-associated LPD	EBV-associated HLH
Systemic chronic active EBV (rare)	

CNS, Central nervous system; *EBV*, Epstein-Barr virus; *HLH*, hemophagocytic lymphohistiocytosis; *LPD*, lymphoproliferative disorder; *NK*, natural killer; *PTLD*, posttransplant lymphoproliferative disorder; *TCL*, T-cell lymphoma.

TABLE 55.5 Epstein-Barr Virus–Associated Lymphoid Neoplasms Categorized by Occurrence With or Without Identified Immunodeficiency

Primarily With Immune Deficiency	With or Without Immune Dysfunction	Primarily Without Immunodeficiency
PTLD	EBV-associated HLH	Burkitt lymphoma
Other immunodeficiency-associated LPD	EBV$^+$ DLBCL, NOS	Classic Hodgkin lymphoma
Primary effusion lymphoma	Lymphomatoid granulomatosis	Chronic active EBV (systemic and cutaneous)
Plasmablastic lymphoma		Systemic EBV$^+$ TCL of childhood
Primary CNS lymphoma		Aggressive NK-cell leukemia
		Extranodal NK/TCL, nasal type
		Primary EBV$^+$ nodal T/NK-cell lymphoma
		Angioimmunoblastic TCL

CNS, Central nervous system; *DLBCL*, diffuse large B-cell lymphoma; *EBV*, Epstein-Barr virus; *HLH*, hemophagocytic lymphohistiocytosis; *LPD*, lymphoproliferative disorder; *NOS*, not otherwise specified; *PTLD*, post-transplant lymphoproliferative disorder; *TCL*, T-cell lymphoma.

TABLE 55.6 **Neoplasms Categorized by Frequency of their Association With Epstein-Barr Virus**

Almost Always EBV$^+$ (>90%)	Usually EBV$^+$ (>50%)
Endemic Burkitt lymphoma	Plasmablastic lymphoma
Lymphomatoid granulomatosis	Primary effusion lymphoma
Nondestructive and polymorphic PTLD	Primary CNS lymphoma
Other immunodeficiency-associated LPD	Monomorphic PTLD
Chronic active EBV	Angioimmunoblastic lymphoma
Hydroavacciniforme-like T-cell LPD	Nasopharyngeal carcinoma
Severe mosquito bite allergy	**Sporadically EBV$^+$ (<50%)**
Systemic EBV$^+$ TCL of childhood	Sporadic Burkitt lymphoma
Aggressive NK-cell leukemia	Classic Hodgkin lymphoma
Extranodal NK/T-cell lymphoma, nasal type	Diffuse large B-cell lymphoma
Primary EBV$^+$ nodal T/NK-cell lymphoma	Gastric carcinoma

CNS, Central nervous system, *EBV*, Epstein-Barr virus; *LPD*, lymphoproliferative disorder, *PTLD*, posttransplant lymphoproliferative disorder; *TCL*, T-cell lymphoma.

Lymphoproliferative Disorders and Lymphomas of B-Cell Origin

EBV-driven B-cell LPDs and lymphomas can develop in patients with congenital or acquired immunodeficiencies, including HIV infection and iatrogenic immunosuppression in SOT or HSCT recipients (see Fig. 55.6).[34] The model of immunodeficiency-associated lymphoproliferative disorders (IA-LPDs) is based on the direct proliferative effects that EBV exerts on the B-cell compartment in patients who fail to amount an effective T-cell response to EBV infection. This model is exemplified by EBV-transformed LCL through in vitro models. EBV-driven B-cell LPD and LCL express the full spectrum of EBV latent proteins and are characterized as latency program III. Because the latency III profile of antigen expression is characteristically immunogenic, in immune-competent individuals, these EBV-transformed cells would be immediately eradicated. This highlights the necessary role that T-cell dysfunction plays in enabling the development of EBV-associated B-cell LPD.

The spectrum of proliferative effects caused by EBV on the B-cell compartment ranges from non-malignant B-cell hyperplasia, to polymorphic but monoclonal B-cell lymphoproliferation, to malignant transformation into monomorphic lymphomas of B-cell origin. These EBV-associated B-cell neoplasms are heterogeneous in many aspects—from the pathogenic role of EBV, to the clinical manifestations, all the way to the expected treatment response. Although non-malignant B-cell LPD is characteristically seen in patients with established T-cell dysfunction, overt EBV-associated B-cell lymphomas can occur in patients with or without known immune deficiency (see Table 55.5). Such lymphomas are characterized by the more restricted latency I and II profiles, and their pathophysiology is thought to be more complex in nature. These malignancies are not solely dependent on EBV-driven proliferation but rather on a multitude of factors including genetic and epigenetic alterations that seem to collaborate with EBV to drive the underlying oncogenesis.[25] The majority of immunocompetent patients with EBV-associated lymphomas are latency type II and express EBNA1, LMP1, and LMP2, except for BL, which is typically latency type I and only expresses EBNA1.

Immunodeficiency-Associated Lymphoproliferative Disorders

IA-LPDs are typically driven by an oncogenic virus in patients with underlying T-cell dysfunction. Although HHV-8 can also cause IA-LPDs, the majority of cases are associated with EBV infection and will be highlighted here. IA-LPD as a category includes the spectrum of neoplastic transformation ranging from non-malignant B-cell hyperplasia to polymorphic B-cell LPD to overt B-cell lymphomas.[35] This section will focus on the overarching characteristics of B-cell hyperplasia and polymorphic LPD, and subsequent sections will discuss various monomorphic lymphomas of B-cell origin as well as posttransplant lymphoproliferative disorders (PTLDs) in more detail.

EBV is a driver of B-cell hyperplasia and polymorphic LPD in patients with IA-LPDs of B-cell origin.[36] Whether the underlying immunodeficiency is inherited through genetic mutations in the immune system, acquired through infection with HIV, or iatrogenically imposed by immunosuppressive medications, the pathophysiology of EBV-associated IA-LPDs remains similar.[35] EBV-driven B-cell hyperplasia is characterized by nondestructive lymphocytic infiltrates with varying morphological patterns that virtually always stain positive for EBV on histologic evaluation. These lymphoproliferative changes may regress with restoration of T-cell function and carry low potential for transformation into lymphoma.

Polymorphic LPD is generally associated with EBV in patients with underlying immunodeficiency. Histologically, the disease pathology is characterized by EBV-infected lymphocytic infiltrates that are destructive to the tissue architecture of the affected lymphoid organ. Morphology of these lesions is characterized by infiltrates spanning the spectrum of B-cell development, thus rendering a “polymorphic” appearance. Despite this, the majority exhibits monoclonal *BCR* gene rearrangements and risk for transformation to a monomorphic lymphoma exists. While restoration of immune function can successfully induce remission in polymorphic LPD, some patients will require more aggressive therapeutic intervention with anti-CD20 humoral immunotherapy plus/minus chemotherapy.

EBV$^+$ mucocutaneous ulcer is a distinct form of EBV-driven IA-LPD that characteristically presents with ulcerating lesions confined to the oral mucosa or skin.[37] These ulcers are well-circumscribed and painful and, similar to polymorphic LPD, typically respond favorably to restoration of immune function through reduction of immune suppression. EBV$^+$ mucocutaneous ulcer can also occur in elderly patients who have undergone immune senescence. Differentiation from EBV$^+$ lymphomas is critical to avoid overaggressive treatment.[37]

These characteristics of EBV-associated B-cell hyperplasia and polymorphic LPD have historically been defined through experience in patients with PTLD. However, it has become apparent that similar features are shared by patients with EBV-associated LPD across a variety of immunodeficiency states.[35] Such patients are additionally at risk for developing monomorphic lymphomas as well. The following sections will discuss the most common lymphomas of B-cell origin associated with EBV; these occur in both patients with underlying immunodeficiency disorders and those that appear to be immune competent.

Burkitt Lymphoma

BL is a high-grade mature B-cell lymphoma. Typical histology reveals a “starry-sky” pattern resulting from numerous benign macrophages that have ingested apoptotic tumor cells (see Fig. 55.6A–C, Chapters 84 and 87). Although most cases of endemic BL in equatorial Africa are associated with EBV, the virus has been implicated less often in sporadic cases, and in the United States only 20% of BL are EBV-positive. In some low- and middle-income countries, an intermediate association with EBV has been described, which varies from 25% to 80%.[38] It is notable that EBV was first discovered in a patient sample from a Ugandan child with a jaw tumor through collaborative work between Denis Burkitt and Anthony Epstein.[39,40]

The EBV genome is clonal in BL as in other EBV-associated malignancies and is characterized by the latency I EBV antigen profile, although some subtle variants have been described.[25] BL is defined by its characteristic translocation of the *C-MYC* oncogene. The majority of cases carry a translocation between the long arm of chromosome 8, the site of *C-MYC* (8q24), and the Ig heavy chain region on chromosome 14, t(8;14). Although the t(8;14) translocation is seen in endemic as well as sporadic BL, the exact location of the chromosomal breakpoints on chromosome 8 and 14 can differ.[41] Other chromosomal translocations seen in BL are between *C-MYC* and the κ-light chain locus on chromosome 2, t(2;8), or the λ-light chain locus on chromosome 22, t(8;22).[42] Recent genomic studies comparing endemic and sporadic BL as well as EBV$^+$ and EBV-negative disease have shed light on some novel biological findings with distinctions and similarities shared between the epidemiological variants.[43–51] Compared with EBV-negative BL, EBV$^+$ BL has been associated with fewer driver mutations but higher mutation burden. The precise pathogenic role of EBV in BL and its contribution to the distinct epidemiological pattern observed in equatorial Africa remain elusive.

Current treatment strategies rely on intensive chemotherapy, and event-free survival in high-income countries in the contemporary era after treatment with rituximab plus combination chemotherapy now exceeds 90%, even for patients with advanced-stage disease.[52,53] The prospect for the development of an EBV-specific immunotherapy for BL is problematic because lymphoma cells evade the immune system by downregulating the expression of EBV latency antigens, cell adhesion molecules, and MHC class I molecules. EBNA1, the only EBV protein expressed in BL, autoregulates its own translation and inhibits its HLA class I presentation because of an internal glycine-alanine repeat region. The relatively dismal outcomes for African children with BL remain dismal and expose the injustice of global health disparities rooted in unequal access to healthcare resources,[54] highlighting the critical importance in improving healthcare infrastructure across the globe, establishing equal access to healthcare resources to all of humankind, and capacity building for healthcare professionals throughout low- and middle-income countries.

Hodgkin Lymphoma

HL is a malignant neoplasm of lymphoreticular cell origin, and 20% to 40% of cases in immunocompetent individuals are associated with expression of EBV-derived antigens in malignant Hodgkin and Reed-Sternberg (HRS) cells and their variants (see Fig. 55.5C and D).[55,56] The frequency of EBV association with HL differs by histologic subtype and is highest with the mixed-cellularity subtype. Evidence linking EBV to the pathogenesis of HL includes the findings that (1) every HRS cell in an EBV-positive tumor mass carries the virus, and (2) the EBV genome is clonal, indicating that the malignant HRS cells originated from a single EBV-infected cell. In addition, LMP1, one of the EBV proteins expressed in HRS cells, activates the transcription factor nuclear factor κB (NF-κB), which is thought to play an important role in HL pathogenesis. EBV-positive HL patients also differ in their antibody response to the major EBV-associated antigens in comparison with healthy controls. Ultimately, while the precise etiological role of EBV remains unknown, alterations in the NF-κB and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways and disruptions in apoptotic mechanisms have been implicated.[56]

The overall outcome of EBV-positive and EBV-negative HL is similar; with combination chemotherapy and radiation, the prognosis is excellent for low-stage disease, and overall survival rate for advanced-stage disease exceeds 85% (see Chapters 77–79). Depending on age and histologic subtype, the presence of EBV might be associated with better survival. As with BL, mechanistic role for EBV in pathogenesis of HL remains uncertain. Studies evaluating the role of EBV viral load and its utility as a prognostic biomarker have produced interesting preliminary results. Adult data from a large cooperative group trial in North America demonstrated inferior failure-free survival for patients with plasma EBV viremia at baseline and those with persistent EBV viremia at the 6-month time point as well.[57] However, there was no correlation with survival and histologic evidence of EBV on tumor biopsy. The pediatric HL experience was evaluated similarly and demonstrated inferior event-free survival if EBV viremia was detected on day 8 of induction in cell-free blood samples. However, baseline EBV viremia was not prognostic.[58] These studies highlight the potential utility of leveraging EBV viral load as a prognostic biomarker for lymphoma, both at baseline and as gauge for treatment response, similar to the precedent established for NPC.[59]

Interesting patterns of association between EBV and HL have been long been observed but without precise explanation. Nearly 50 years ago, descriptions of increased risk for HL in individuals with a previous diagnosis of IM were published. More recently, onset of EBV-associated HL occurring after episodes of IM was confirmed in children as well.[55] The association between HL and EBV is more commonly seen in children and in low- and middle-income countries.[60] However, connecting the dots between such epidemiologic observations is complex because IM occurs much less frequently in low- and middle-income countries, despite HL having a much higher frequency of association with EBV.[55]

Other B-Cell Neoplasms Associated With Epstein-Barr Virus

A variety of other B-cell neoplasms is associated with EBV across a spectrum of clinical contexts including diffuse large B-cell lymphoma (DLBCL), lymphomatoid granulomatosis, plasmablastic lymphoma, primary effusion lymphoma (PEL), and primary central nervous system (CNS) lymphoma (PCNSL). The association of these lymphomas with EBV varies, ranging from 10% to 95%, and all of them occur in both immune competent individuals and those living with underlying immunodeficiency.

EBV$^+$ DLBCL, not otherwise specified (NOS) was previously known as EBV$^+$ DLBCL of the elderly prior to the 2016 version of the World Health Organization (WHO) classification of lymphoid neoplasms.[61] While it had previously been described as occurring in elderly patients who presumably had undergone immune senescence, it is now accepted that this disease entity can occur in individuals of any age and in patients with or without defined immunodeficiency.[62] Younger patients with EBV$^+$ DLBCL, NOS typically present with nodal involvement. The neoplastic cells express both latency II (in younger patients) and III (in elderly patients) EBV antigen profiles and most often are categorized as the activated B-cell–like subtype of DLBCL. The diagnosis represents a heterogeneous group, and the histology can display a variety of patterns including the T-cell/histiocyte-rich large B-cell lymphoma, B-cell lymphoma unclassifiable with features intermediate between DLBCL and classic HL (also known as gray zone lymphoma), and DLBCL, NOS.[62,63] In addition, variants with very specific clinical phenotypes have been described such as EBV$^+$ DLBCL associated with chronic inflammation (e.g., chronic pyothorax) and fibrin-associated DLBCL (in patients with localized chronic fibrin deposition).[25] Younger patients tend respond favorably to rituximab plus combination chemotherapy, and long-term remission has been reported as high 80%.[63]

Lymphomatoid granulomatosis is an EBV-driven B-cell LPD associated with impaired cytotoxic function, clinically characterized by a unique predilection for the lungs.[64,65] The most common extrapulmonary sites of involvement are the CNS, skin, and kidneys.[66] Lymphomatoid granulomatosis shares significant overlap in the clinical and histological characteristics between EBV$^+$ DLBCL, NOS, which also can present with lung involvement. The typical histological pattern of lymphomatoid granulomatosis is described as angioinvasive and angiodestructive lymphoproliferative lesions with numerous EBV$^+$ B cells amid a polymorphous background including T-cell infiltrates.[67] Lesions contain EBV$^+$ large atypical B cells, and the density of these determine a histologic grade ranging from 1 to 3; grade 3 lesions share significant morphologic overlap with EBV$^+$ DLBCL, NOS. Unlike the majority of other EBV-driven lymphoid neoplasms (including EBV$^+$ DLBCL, NOS), peripheral blood EBV viral load is often undetectable or displays low-level viremia in patients with lymphomatoid granulomatosis.[64] Patients with grade 3 disease are treated similarly as patients with EBV$^+$ DLBCL, NOS; however, the response to therapy is rarely sustained, and relapse is common.[64,65] Allogeneic HSCT is often a consideration in patients with lymphomatoid granulomatosis based on patient status, treatment response, and available donor options.

Plasmablastic lymphoma and PEL are relatively rare B-cell lymphomas with an overlapping malignant cell immunophenotype demonstrating plasmablastic cell differentiation; they are associated with EBV, HIV, and, in the case of PEL, HHV-8 as well.[68] PCNSL is another acquired immunodeficiency syndrome (AIDS)-defining lymphoma that is etiologically linked with EBV; the underlying histology in HIV-infected patients is typically a GC-derived DLBCL.[68] Although the incidence of these three lymphomas is dramatically increased in HIV-infected patients, they can occur rarely in HIV-negative, immune-competent individuals; in such scenarios, they are not necessarily EBV associated.

Plasmablastic lymphoma typically presents with masses in the oral cavity or other extranodal sites, is EBV associated in approximately 75% of HIV$^+$ and 50% of HIV-negative cases, expresses either a latency I or III EBV antigen profile, and often carries *C-MYC* gene rearrangements.[69] PEL is distinguished from plasmablastic lymphoma by a unique clinical presentation and its characteristic association with HHV-8. Large effusions in the pleural, pericardial, or peritoneal cavities, in the absence of a solid mass, characterize the typical presentation of PEL, although an extracavitary variant has also been described. Approximately 80% of PEL cases are EBV-associated, and latency profile I is most commonly displayed.[70,71] PCNSL is a heterogenous diagnostic entity; as noted earlier, in the context of HIV infection, it is frequently EBV$^+$ and with a DLBCL morphology. However, it is important to note that PCNSL also refers to other NHLs that present primarily with CNS tumors, and the clinical characteristics and treatment strategies are typically derived based on the underlying histopathology, which can include anaplastic large cell lymphoma, BL, and lymphoblastic lymphoma, among others. In these scenarios, PCNSL is often EBV negative.[72] All three of these rare lymphomas have generally poor prognoses; however, successful treatment with intensive multiagent chemoimmunotherapeutic regimens has been reported.[68]

Lymphoproliferative Disorders and Lymphomas of T/Natural Killer–Cell Origin

Seeming to defy convention, EBV-associated T- and NK-cell lymphoid neoplasms also occur despite B cells serving as the typical host target for EBV infection. The mechanism by which EBV extends its tropism to the T- or NK-cell compartment remains unknown and has been the subject of investigation. Theories postulating a virological synapse to transfer from B to T/NK cells have yet to be validated, and recent evidence supports EBV infection of precursor lymphoid cells that extend to all three lymphocyte lineages.[73] Regardless of infectious entry point in lymphocyte differentiation, EBV-associated LPD and lymphomas of T/NK-cell origin are a rare but critical group of diseases that are often characterized by poor survival.

The most recent WHO classification of lymphoid neoplasms stratifies diagnostic entities based on systemic versus localized clinical presentation. It has been proposed that chronic active T/NK-cell EBV infection (T/NK CAEBV) as well as EBV-associated HLH (EBV-HLH) be included in this classification (see Table 55.4).[67,74–76] Localized disease specifically refers to two forms of CAEBV that present with primarily cutaneous manifestations—hydroavacciniforme-like lymphoproliferative disease (HV-LPD) and severe mosquito bite allergy (SMBA). Systemic EBV$^+$ T/NK cell lymphoid neoplasms include the systemic form of T/NK CAEBV, EBV-HLH, and four overt lymphomas—systemic EBV$^+$ T-cell lymphoma of childhood (sEBV$^+$ T-NHL), primary EBV$^+$ nodal T/NK-cell lymphoma (EBV$^+$ PTCL), aggressive NK-cell leukemia (ANKL), and extranodal NK/T-cell lymphoma, nasal type (ENKTL). This heterogeneous spectrum of EBV-associated diseases of T/NK-cell origin shares some common characteristics. There is a unique susceptibility in individuals of East Asian or indigenous ancestry of Central and South America, the EBV latency profile pattern varies between type I and II, and although there has yet to be an identified immunodeficiency associated with them, the patterns of genetic predisposition in distinct populations are evident.[25]

EBV-associated lymphoproliferation in T/NK cells does not appear to mimic the model established by the spectrum of disease entities observed in the B-cell compartment. Although a gradation of EBV-driven lymphoproliferation is well described in B cells, ranging from premalignant B-cell hyperplasia, to lymphoproliferative states of varied malignant potential and ultimately to overt B-cell lymphomas, the occurrence of EBV-associated T/NK-cell LPD and lymphomas is seemingly idiosyncratic. It typically arises in patients without obvious immune dysfunction, and reasons for ethnic predisposition in Asians and indigenous populations of the Americas are not known. EBV$^+$ T/NK-cell diseases range from indolent (e.g., CAEBV) to acutely fulminant (e.g., all the systemic disorders). There does not appear to exist an initial state of polymorphic T-cell lymphoproliferation that is observed in B-cell PTLD.[77,78] Nonetheless, there has been significant progress achieved over the past two decades, shedding light on the biologic and clinical characteristics of this enigmatic spectrum of disease. Recently discovered primary immunodeficiency disorders have been associated with EBV-HLH and a clinical phenotype of T/NK CAEBV. Meanwhile, preliminary data demonstrating efficacy of immune checkpoint blockade in a case series of patients with relapsed/refractory EBV-HLH suggest that some form of immune restoration may indeed have a role to play in the therapeutic approach for EBV$^+$ T/NK-cell LPD.[79]

Epstein-Barr Virus–Associated Hemophagocytic Lymphohistiocytosis

HLH is a syndrome characterized by uncontrolled inflammation (see Chapter 53). Diagnostic criteria defined by the Histiocyte Society reflect the clinical features of pathologic immune activation, including persistent fever, splenomegaly, cytopenias, hyperferritinemia, decreased fibrinogen or increased triglycerides, hemophagocytosis (in bone marrow, spleen, or lymph nodes), increased soluble IL-2 receptor α, and decreased or absent NK-cell function. The diagnosis of HLH can also be established by a pattern of familial inheritance or proven gene defects. HLH may be associated with EBV in a variety of clinical scenarios including: (1) primary EBV infection as an inciting factor in a patient with inherited predisposition to familial/primary HLH, (2) primary EBV infection provoking a secondary HLH syndrome in a patient without genetic predisposition to HLH, and (3) HLH occurring secondary to an EBV-associated T/NK LPD/lymphoma (including CAEBV).

Gene defects resulting in impaired cytotoxic NK- and T-cell function have been associated with autosomal recessive inheritance of HLH, including *PRF1* (familial [f]HLH-2), *UNC13D* (fHLH-3), *STX11* (fHLH-4), *STXPB2* (fHLH-5), *Munc18-2, RAB27A* (Griscelli syndrome, type 2), *LYST* (Chédiak-Higashi syndrome), *AP3B1* (Hermansky-Pudlak syndrome, type II), *ITK*, *CD27*, and *MAGT1* (X-MEN syndrome). The X-linked lymphoproliferative diseases (XLP1 *(SH2D1A)* and XLP2 *(BIRC4)* are also associated with HLH.[80] EBV infection may trigger HLH in patients with any form of familial disease. Gene defects are identified in only 50% of patients with family history consistent with inherited HLH. Therefore, although a diagnosis "primary" or "familial" HLH may be proven, it is impossible to exclude. Familial HLH commonly presents in young children, but there are reports of new onset of HLH in adults as old as 62 years with hypomorphic mutations in HLH-associated genes. Patients with familial HLH typically require prompt treatment with chemotherapy and immune suppression (etoposide/dexamethasone) followed by HSCT.[81] Recently, a phase 2 to 3 study of interferon(IFN)-γ inhibition led to US Food and Drug Administration (FDA) approval of emapalumab for recurrent or refractory primary.[82] Without therapy, survival of patients with active familial HLH is approximately 2 months. The first international treatment protocol for HLH organized by the Histiocyte Society in 1994 reported long-term survival of greater than 50%. For those who are able to undergo allogeneic HSCT, reduced-intensity condition has contributed to improved survival but with suboptimal engraftment.[83,84]

The term "secondary" HLH generally refers to older children (or adults) who present without a family history or known genetic cause for their HLH. EBV is a common trigger for "secondary HLH," ranging from inflammation that resolves spontaneously to unrelenting disease requiring allogeneic HSCT. Differentiating IM from EBV-HLH can be challenging in patients who are systemically ill. Clinical criteria for HLH should be evaluated in patients with persistently high cell-free EBV genome copy numbers in plasma or whole blood, persistent symptoms of inflammation, or severe symptoms. Patients who initially meet clinical criteria for HLH may occasionally improve spontaneously. Patients with less severe presentations may also respond to corticosteroids, intravenous Igs, or cyclosporine. However, in patients with progressive or severe disease, early initiation (within 4 weeks of onset of symptoms) of etoposide is associated with significant improvement in survival.[85] Because it can eliminate EBV-infected B cells, rituximab may be a beneficial addition to other therapies in patients with HLH secondary to EBV infection.[86] However, one must take caution, recognizing that an underlying lymphoproliferative process within the T- or NK-cell compartment characterizes the vast majority of patients with EBV-HLH.[87,88] In such cases, rituximab will counteract only EBV-infected B cells, while the aberrant T/NK-cell population will proliferate unmitigated.

Ultimately, it is extremely challenging to differentiate non-malignant EBV-HLH from HLH secondary to an underlying EBV$^+$ T/NK-cell LPD/lymphoma. This umbrella of EBV$^+$ T/NK-cell LPD/lymphoma includes the following diagnoses that are discussed in further detail later: systemic T/NK CAEBV, sEBV$^+$ T-NHL, EBV$^+$ PTCL, ANKL, and ENKTL.[67,74,75] Attempts to establish clinical criteria to differentiate EBV$^+$ T/NK-cell LPD/lymphoma from non-malignant EBV-HLH have failed to definitively identify patients with EBV$^+$ malignancy in prospective fashion. The inability to effectively do so prevents appropriate risk-stratification and intensification to multiagent chemotherapy and allogeneic HSCT, which would be needed to avoid the otherwise fatal clinical course for patients with EBV$^+$ T/NK-cell LPD/lymphoma. A few clinical factors, including hyperbilirubinemia, hyperferritinemia, younger age, higher levels of EBV viremia, and presence of cytogenetic abnormalities, have been identified that increase diagnostic suspicion for EBV$^+$ T/NK-cell LPD/lymphoma.[87,89,90] It is important to recognize that histopathology features of non-malignant EBV-HLH and EBV$^+$ T/NK-cell LPD/lymphoma share considerable overlap, and pathologists often struggle to definitively determine the diagnosis as well.[67,75,76] For some patients, an underlying EBV$^+$ T/NK-cell LPD/lymphoma declares itself only after an initial clinical improvement with HLH-directed therapy, to only later develop recurrent HLH requiring immunochemotherapy and HSCT. Therefore it remains important to follow patients beyond resolution of initial symptoms until they successfully establish viral suppression of EBV, a process which can take up to 12 months.[91] Patients with persistent or recurrent symptoms and or persistent high-level EBV viremia may in fact have EBV$^+$ T/NK-cell LPD/lymphoma and intensification of therapy would be indicated.[91]

Chronic Active Epstein-Barr Virus

CAEBV represents a range of clinical manifestations resulting from persistent, uncontrolled infection of T or NK cells by EBV.[91–93] Inability to control infection is likely due to defects in cytotoxic immune function. The systemic form of CAEBV has considerable pathologic and clinical overlap with HLH, and immune dysfunction is likely due to a variety of causes. Early descriptions of CAEBV primarily reported disease in Asian patients, and almost all cases were due to proliferation of EBV-infected T or NK cells.[94] A review of several centers in the United States found a predominance of B cell–associated CAEBV in the Western hemisphere; however, B-cell CAEBV is considered extremely rare.[93] Therefore the focus of this section will be on the predominant phenotype, T/NK CAEBV. CAEBV is further categorized as systemic versus cutaneous (localized) disease.

To establish the diagnosis of CAEBV, patients must have (1) sustained or recurrent IM-like signs and symptoms for 3 to 6 months, (2) elevated EBV viral load in the peripheral blood or diagnostic tissue biopsy, (3) EBV infection of T or NK cells in the blood or affected tissues, and (4) exclusion of other possible diagnoses.[95] The systemic and life-threatening form of T/NK CAEBV is characterized by high fevers, hepatosplenomegaly, and extensive lymphadenopathy, followed by hepatic, cardiac, or pulmonary dysfunction.[91] These patients have very high EBV DNA levels (viral loads) in their whole blood or peripheral blood mononuclear cells and can also demonstrate elevated EBV VCA titers. Although B cells are the typical host target for EBV, in severe CAEBV, either T or NK cells are infected, predisposing the patient to transformation to lethal T-cell or NK-cell lymphomas. Severe, often fatal CAEBV is more common in East Asia but has recently been described throughout the world.[91] Case series from the United States have included a predominance of patients with ancestry originating from Central and South America; however, cases have also been reported in children of European or African ancestry.[96–98]

Milder, more indolent forms of CAEBV primarily demonstrate cutaneous lesions. The two clinical phenotypes of cutaneous CAEBV are HV-LPD and SMBA, which have similarly been described predominantly in East Asia and Central/South America. HV-LPD skin lesions typically occur in sun-exposed areas of skin and most often involve CD8$^+$ T cells, whereas lesions in SMBA, as the name suggests, are characterized by exaggerated hypersensitivity reactions to mosquito bites and most often involve CD56$^+$ NK cells. These cutaneous forms of T/NK CAEBV tend to run indolent courses of remitting and relapsing disease; however, the severe form of HV-LPD includes systemic symptoms and carries a risk of progression to overt lymphoma.[76]

Patients with CAEBV do not have XLP-associated *SH2D1A* or *BIRC4* mutations, and although the etiology of CAEBV remains poorly understood, a recent study demonstrated that GATA2 deficiency can be associated with CAEBV and HV-LPD.[74] While the initial diagnostic criteria for CAEBV described seemingly immune competent patients, increased access to comprehensive genetic diagnostic tools has discovered some underlying mutations in patients with CAEBV.[30,91] Whether an underlying immune deficiency is identified, it remains clear that patients with CAEBV exhibit impaired T- and NK-cell cytotoxic activity.[94]

Although sporadic clinical improvements of mild/moderate CAEBV have been reported after infusion of IL-2, high-dose Ig, antiviral drugs, tumor necrosis factor (TNF)-α antibodies, or steroids, the only curative option for severe CAEBV is HSCT. Survival rates vary between 50% and 95%, with better outcomes for patients who (A) are transplanted early after diagnosis, (B) have fewer complications before transplant, and (C) have received reduced intensity conditioning regimens.[99–101] The largest clinical experience with CAEBV comes from Japan, where many of the contemporary therapeutic strategies originated. Expert opinion from Osaka advocates for pretransplant cytoreductive chemotherapy using CHOP-like regimens (CHOP = cyclophosphamide, doxorubicin hydrochloride, vincristine (oncovorin), prednisone) to decrease the lymphoproliferative burden of T/NK CAEBV infection. They argue that this approach provides a safe bridge as patients are prepared for allogeneic HSCT, improves engraftment of donor cells, and decreases the risk for post-HSCT relapse. However, this approach has not yet been validated in a randomized clinical trial.[100,101] Besides HSCT, the adoptive transfer of autologous EBV-specific T cells has been explored in five patients with mild or moderate CAEBV. Infusion of EBV-specific T cells resulted in resolution of fatigue, malaise, fever, lymphadenopathy, and splenomegaly lasting for 6 to 36 months. For severe CAEBV in which EBV resides in the T- or NK-cell compartment, the use of EBV-specific T cells has been investigated anecdotally. For example, the Center for Cell and Gene Therapy (CAGT) at Baylor College of Medicine in Houston, Texas, has infused donor-derived LMP2-specific T cells with a good partial response as judged by decreasing EBV-DNA load.[4,102]

Epstein-Barr Virus–Associated T/Natural Killer–Cell Lymphomas

NK/T-cell lymphomas that are always associated with EBV include sEBV$^+$ T-NHL, EBV$^+$ nodal PTCL, ANKL, and ENKTL.[74,75] This group of malignancies is distinct in its predilection to occur more commonly in persons of Asian and indigenous Central and South American ancestry. They tend to occur with overlapping yet distinct clinical patterns differentiated by the phenotype of the clinical presentation and its predilection to occur in either children or adults. Angioimmunoblastic lymphoma is a nodal peripheral T-cell lymphoma that is also often EBV-associated but does not share the epidemiological pattern as the other EBV-associated NK/T-cell lymphomas. The overall outcome of EBV-associated T/NK-cell NHL depends on histologic subtype and risk factors present at diagnosis, but most are high-grade malignancies with an unfavorable prognosis.

One of the most deadly diagnoses encountered in pediatric oncology, sEBV$^+$ T-NHL of childhood, is a rare but aggressive malignancy characterized by a fulminant clinical course and high case fatality rates.[96] Patients virtually always present with systemic features consistent with HLH in the setting of extreme EBV viremia. High fevers, pancytopenia, hepatosplenomegaly, and multiorgan failure are commonly seen. Both non-malignant EBV-HLH and sEBV$^+$ T-NHL are characterized by proliferation of EBV$^+$ CD8$^+$ cytotoxic T cells, most often identified by bone marrow aspirate/biopsy. However, because sEBV$^+$ T-NHL cells may lack overt atypia on histopathology, these two diagnoses are extremely difficult to differentiate on initial evaluation. This presents a major clinical dilemma because patients with non-malignant EBV-HLH may be cured with well-tolerated HLH-directed therapy (etoposide plus dexamethasone), whereas patients with sEBV$^+$ T-NHL require intensive multiagent chemotherapy followed by allogeneic HSCT. Ultimately, the overlap between non-malignant EBV-HLH and sEBV$^+$ T-NHL presents one of the most acute challenges in contemporary pediatric oncology.[75,98]

ANKL is the analogous NK-cell version of sEBV$^+$ T-NHL. Characterized by a virtually identical clinical presentation, it differs in that the lymphoproliferative population is an aberrant EBV$^+$ NK cell. It shares the same clinical challenges as sEBV$^+$ T-NHL because, although less common, non-malignant EBV-HLH can also be characterized by an EBV$^+$ NK-cell lymphoproliferation. Thus differentiation between non-malignant EBV-HLH and ANKL can be extremely challenging as well. ANKL shares immunophenotypic overlap with ENKTL; however, the clinical phenotypes differ because ANKL typically presents with severe systemic and/or leukemic disease and ENKTL often presents with extranodal masses.[74,75]

ENKTL and EBV$^+$ nodal PTCL essentially represent the extranodal and primary nodal clinical presentations of lymphoma and occur most commonly in adults. Nasal-type ENKTL represents 70% to 80% of ENKTL cases, with patients presenting with a mass in the upper aerodigestive tract. However, extranasal presentations may also occur in other extranodal sites, most commonly in the skin, gastrointestinal (GI) tract, soft tissue, and testis. Although the majority of cases are characterized by an NK-cell immunophenotype, T-cell variants also occur.[103,104] In contrast, primary EBV$^+$ nodal PTCL is represented by a mature T-cell immunophenotype in the majority of cases, although a subset of patients can have NK-cell disease. Although the clinical presentation is primarily limited to lymph node involvement, extranodal disease may also occur.[105] These two diagnoses are not as fulminant as sEBV$^+$ T-NHL or ANKL; however, they both still carry guarded prognoses with high rates of relapse.[75]

ACQUIRED IMMUNODEFICIENCY AND EPSTEIN-BARR VIRUS–ASSOCIATED NEOPLASMS

Posttransplant Lymphoproliferative Disorders

PTLDs constitute a heterogeneous group of diagnostic entities that mirror the spectrum described in the earlier section on IA-LPD. This umbrella of lymphoproliferative disorders ranges from early lesion/nondestructive PTLD (reactive EBV-associated B-cell hyperplasia) to polymorphic, destructive PTLD (polymorphic but invasive and often clonal lymphocytic infiltrates) all the way to monomorphic, destructive PTLD (overt lymphoma). PTLD is the specific category of IA-LPD that occurs in patients who are iatrogenically immunosuppressed post-allogeneic HSCT (to prevent graft-versus-host disease [GVHD]) or post SOT (to prevent graft rejection). In the setting of SOT, the reported incidence of EBV$^+$ PTLD ranges from 1% to 25%, with the highest risk in seronegative recipients, patients receiving intensive immunosuppressive therapy, and patients receiving grafts with a high lymphoid content. Host factors such as younger age (and higher chance of being EBV-naïve at time of transplant) and recipients of heart, lung, and intestinal transplants carry a higher risk of PTLD.

After allogeneic HSCT, the incidence of EBV-PTLD varies with the transplant regimen and may be as high as 25%. Risk factors for the development of EBV-PTLD post HSCT include the use of stem cells from an HLA-mismatched family member (including haploidentical donors) or HLA-matched unrelated donor, T-cell depletion of the donor cells, intensive immunosuppression, and an underlying diagnosis of primary immunodeficiency. The incidence is much lower when methods that also deplete B cells are used in the conditioning regimen (i.e., receiving alemtuzumab instead of antithymocyte globulin).

The clinical presentation of PTLD is variable. Patients can manifest with systemic symptoms (fevers, night sweats, weight loss), involvement of lymphoid (lymphadenopathy, hepatosplenomegaly, tonsillar/adenoidal hypertrophy) or extranodal tissue (GI symptoms such as malabsorption, nausea/vomiting, or bloody stool), or with dysfunction of the allograft. Fulminant PTLD is a relatively rare phenomenon but is severe and characterized by rapid progression of multiorgan failure with severe cytopenias. Careful history taking and physical exam are critical tools in evaluating patients post transplant, particularly in the setting of EBV viremia.

The onset of EBV-PTLD seems to be preceded by a large increase in viral load as well as the proliferation of EBV-infected B cells. Frequent monitoring of the EBV DNA load in peripheral blood is a valuable screening test for early detection of EBV-PTLD after HSCT or SOT. However, it remains a subject of debate which is the optimal sample (whole blood, isolated peripheral blood mononuclear cells, plasma) for EBV DNA quantitation. Lastly, the threshold level of EBV-DNA suggestive of impending EBV-PTLD varies according to the polymerase chain reaction (PCR) method of quantifying viral DNA. However, it should be emphasized that not all patients with high EBV-DNA levels, especially those with an SOT, develop EBV-PTLD. Several distinct patterns of EBV latent gene expression have been identified in the memory B cells of high-load EBV carriers, with type III latency conferring the highest risk for EBV-LPD development. Besides EBV-DNA levels, determining the frequency of EBV-specific T cells or the functionality of T cells in patients with high EBV-DNA load might also assist in identifying patients who are at increased risk for developing EBV-PTLD. In addition, host factors such as polymorphisms in the promoter regions of cytokines have been implicated in increasing the risk for developing EBV-PTLD. Thus an elevated EBV-DNA load can lead to early diagnosis of EBV-PTLD, with consequent reductions in mortality and treatment-related morbidity, although additional results such as clinical signs and symptoms, as well as radiographic findings, must be taken into account before therapy is initiated.[106,107] Besides EBV and a dysfunctional cellular immune system, genetic alterations in B cells have also been implicated in the pathogenesis of PTLD, especially in SOT recipients. These alterations can be characterized by microsatellite instability, DNA hypermethylation, aberrant somatic hypermutation, and mutations in specific genes such as *MYCC, BCL-6, N-ras*, and *p53*.

Treatment of Posttransplant Lymphoproliferative Disorders

The treatment of PTLD will depend on the specific diagnostic histology and the involvement of EBV, with a variety of therapeutic considerations available (Fig. 55.7). Treatment also differs for recipients of SOT versus HSCT.

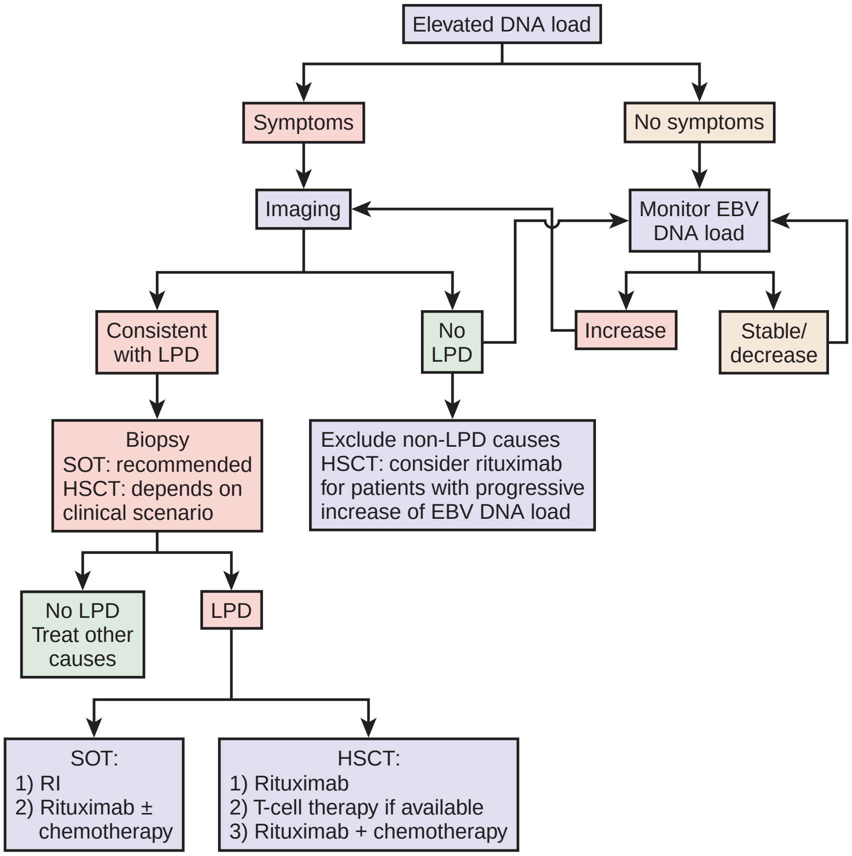


Figure 55.7 ALGORITHM FOR FOLLOWING PATIENTS WITH INCREASED EPSTEIN-BARR VIRUS (EBV)-DNA LOAD. With the caveat that many individualized factors contribute to clinical decisions, a general diagnostic and clinical approach is outlined. *HSCT*, Hematopoietic stem cell transplant; *LPD*, lymphoproliferative diseases; *RI*, reduction of immunosuppression; *SOT*, solid organ transplant.

For patients who are post allogeneic HSCT, strict monitoring of EBV viral loads is performed in the posttransplant period to monitor for viral reactivation manifest by persistent EBV viremia. For asymptomatic patients with a reassuring radiographic work-up, PTLD can essentially be ruled out and patients would be categorized as posttransplant EBV viremia. It is commonly accepted to preemptively treat HSCT recipients with rituximab, not only to prevent potential development of PTLD, but just as importantly to avoid graft failure caused by EBV reactivation.[108,109] For those patients who do develop PTLD, treatment would be similar to the approach outlined later for SOT recipients with PTLD.

Preemptive rituximab for patients post SOT with persistent EBV viremia is not a universally standard practice. Weaning of immunosuppression, when feasible, is typically the first intervention upon recognition of post-SOT EBV viremia. If it persists, there are some institutions that prefer to give preemptive rituximab with the goal of suppressing the EBV viral load. Although definitive evidence that this practice is effective in preventing PTLD is lacking, it is a widely accepted practice. For those clinicians who opt to use preemptive rituximab, it is typically based on the rationale that it is well tolerated, and any potential mitigation of risk for PTLD may have benefit, particularly if it helps to avoid future exposure to multiagent chemotherapy. Conversely, it is also evident that many patients post SOT have persistent EBV viremia and never develop PTLD. Therefore the counterargument to preemptive therapy is that patients may unnecessarily be exposed to the adverse effects of rituximab without definitive clinical benefit.

For SOT or HSCT recipients who develop PTLD, the therapeutic approach depends heavily on the underlying histology. Patients with early lesion/nondestructive PTLD are almost always EBV associated and typically improve after reduction of immunosuppression. This diagnostic entity has three morphologic variants, including IM-like, plasmacytic, and florid follicular hyperplasia. These reactive B-cell hyperplasias stain positive for EBV on histology, with only rare exceptional cases of florid follicular hyperplasia having been described as EBV negative. Typically, weaning of immune suppression, when feasible without compromising the allograft, is sufficient to restore cellular immune function enough to suppress this EBV-driven benign lymphoproliferation. However, this approach is limited by the risk for graft rejection, and it is not useful after HSCT because of the profound immunosuppression and the risk for inducing GVHD. In such cases, treatment with rituximab monotherapy is indicated and often successful in treating early lesion/nondestructive PTLD.

Polymorphic PTLD is more aggressive than early lesion PTLD; however, it is similarly responsive to reductions of immunosuppression and/or treatment with rituximab. Unlike early lesion PTLD, polymorphic disease is a destructive lymphoproliferative lesion that effaces the affected lymphoid organ tissue. It is usually EBV$^+$ and often responsive to weaning of immunosuppression. Especially in SOT patients for whom consequences of allograft rejection can be dire, it is important to balance risk of weaning immune suppression versus relative risks of rituximab monotherapy, which is generally well tolerated and effective. This is of particular importance in recipients of allografts for whom the regenerative potential is less robust and the risk of graft rejection is severe. Patients with polymorphic PTLD tend to have excellent responses to rituximab monotherapy, and their overall prognosis is favorable. In both EBV$^+$ polymorphic and early lesion PTLD, EBV viral load monitoring can be useful as a biomarker to monitor treatment response and potential for future relapse.

For patients with monomorphic PTLD, the therapeutic approach hinges on the specific underlying histology. The majority of cases of monomorphic PTLD are DLBCL histology. Occasionally, these get more generically classified as an aggressive, mature B-cell lymphoma, NOS. DLBCL PTLD is often EBV-associated but is more likely to be EBV-negative than polymorphic PTLD. The treatment approach to DLBCL PTLD is similar to that of polymorphic PTLD; however, the chance of improvement simply by weaning immunosuppression is low. Treatment approaches combining rituximab with low-dose cyclophosphamide and prednisone, or even rituximab monotherapy, have resulted in approximately 70% event-free survival.[110–114] Treatment for those patients with DLBCL PTLD who relapse or do not achieve complete remission is a bigger challenge. In such scenarios, conventional multiagent chemotherapy, radiation for localized disease, and adoptive transfer of T cells or EBV-specific T cells are all considerations. Recently, traction has gained on adoptive cellular therapies for EBV^+ PTLD, and this will be discussed in depth in the following section on immunotherapy.[113]

Finally, for those patients with non-DLBCL monomorphic disease, the treatment approach must be tailored to the specific histological diagnosis. For patients with BL PTLD (sometimes EBV^+) or HL PTLD (usually EBV^+), disease-specific multiagent chemotherapy is indicated. On rare occasion, peripheral T-cell lymphomas or plasma cell neoplasms present as monomorphic PTLD. These histologies are less frequently EBV^+ and also require disease-specific treatment plans. The aforementioned strategy of rituximab monotherapy or rituximab with low-dose chemotherapy will not be successful for these non-DLBCL monomorphic PTLD diagnoses.[112–114]

Epstein-Barr Virus–Associated Non-Hodgkin Lymphoma in Human Immunodeficiency Virus Patients

Patients infected with HIV are at high risk to develop NHL. The incidence increases with age, and the male-to-female ratio is approximately 2:1. Depending on specific histologies, the EBV association ranges from 30% in systemic HIV-related BL, to 70% to 80% in HIV-related DLBCL, and virtually all cases of HIV-related PCNSL are EBV-positive.[115] In biopsies of EBV-associated HIV-NHL, there is considerable variation in the number of EBV-positive cells, and the pattern of EBV latent gene expression varies among tumor types as in immunocompetent individuals (see Fig. 55.5E and F). As discussed in the section on EBV-associated B-cell lymphomas, other specific diagnoses that are more prominent in HIV-infected patients include PEL and plasmablastic lymphoma. In addition, HL is EBV-positive in upwards of 80% of HIV-related cases.[115]

In HIV-infected patients, the development of EBV-associated HIV-NHL is preceded by a loss of functional EBV-specific T cells, suggesting that strategies to boost the endogenous EBV-specific T-cell response might prevent lymphomas. Additional factors known to predispose HIV-infected patients to EBV-associated lymphomas include underlying cytokine dysregulation and proinflammatory state.[115] Restoring $CD4^+$ T-cell counts in patients with HIV with highly active antiretroviral therapy (ART) has decreased the incidence of PCNSL and HIV-related DLBCL.[116] The incidence of HIV-related BL, which tends to occur in patients with higher CD4 counts and with less HIV viremia, has remained stable over time despite improved access to ART.[116]

Oral hairy leukoplakia (OHL) develops frequently, although not exclusively, in patients who are HIV-positive. It is a non-malignant hyperplasia of epithelial cells, and most patients present with white, corrugated lesions on the tongue. Besides IM, OHL is the only EBV-associated disease in which active viral replication is apparent, and multiple strains are often present within the same lesion. Inhibiting EBV replication in vivo with antivirals such as valacyclovir results in resolution of OHL. However, after valacyclovir treatment, EBV replication recurs in normal tongue epithelial cells, indicating that productive EBV replication is necessary but not sufficient to induce OHL.[25,117]

IMMUNOTHERAPY FOR EPSTEIN-BARR VIRUS–ASSOCIATED NEOPLASMS

Monoclonal Antibody Therapy for Lymphoproliferative Disease

The CD20 monoclonal antibody rituximab is currently widely used as prophylaxis and as therapy for EBV-LPD. A comprehensive review of the literature that included articles and abstracts published between 1999 and 2008 reported that the use of rituximab as preemptive therapy prevented the development of EBV-LPD in 90% of 341 HSCT recipients, whereas therapy was associated with a response rate of 63% in 126 HSCT patients.[108,109] However, response rates varied widely, likely reflecting heterogeneity in patient populations and the fact that early diagnosis and treatment lead to better outcomes. For EBV-LPD after SOT, a recent multicenter analysis of 80 patients reported that rituximab-based therapy had a 3-year progression-free survival of 70% compared with 21% for patients treated without rituximab.[3] Half of the patients treated with rituximab also received chemotherapy, most of them having bulky disease and a high International Prognostic Index. A recent prospective multicenter study suggests that sequential therapy of rituximab followed by chemotherapy results in excellent disease control and overall survival. However, at present it remains unclear for which group of patients rituximab monotherapy is sufficient. Retrospective studies have identified risk factors including extra-lymphatic disease, high LDH, low albumin, and poor performance status, but these risk factors need to be validated in prospective studies.[118] Rituximab has led to a dramatic improvement in outcome of EBV-LPD but does not restore the cellular immune response to EBV, which may be crucial for the long-term control of EBV-mediated B-cell proliferation. EBV-infected B cells may therefore increase with B-cell recovery, and EBV-LPD may recur. Although rare, the recurrence of CD20-negative lymphomas with the use of rituximab has been reported. Patients who fail to respond to chemoimmunotherapy strategies may respond to high-dose chemotherapy followed by autologous HSCT or adoptive T-cell therapies.

Epstein-Barr Virus–Directed T-Cell Therapies

Donor lymphocyte infusions were first used to treat post-HSCT EBV-LPD over 30 years ago.[119,120] Although clinical responses were observed in the vast majority of patients treated, the risk of acute GVHD posed by these nonspecific T cells made this an untenable option.[121,122] Ex vivo–manufactured polyclonal EBV-specific T cells (EBVSTs) have emerged as a cellular therapeutic strategy able to treat EBV lymphoproliferation while reducing the risk of GVHD.[123] Initially, HSCT donor–derived EBVSTs were generated ex vivo by repeated stimulation with autologous LCLs. Clinical studies in HSCT recipients have shown that these EBVSTs are safe, do not cause significant GVHD, reconstitute EBV-specific cellular immunity, and are effective as prophylaxis *and* therapy for EBV-LPD.[4,5,124] In SOT recipients who develop EBV-LPD, donor-derived T cells are of limited value because the tumor almost always arises in recipient B cells, and donor T cells are unlikely to survive in the recipient's hematopoietic system.

Although donor-derived EBVSTs have potent clinical activity in EBV-LPD, their generation using LCLs as antigen-presenting cells (APCs) is a lengthy process, requiring a minimum of 6 to 8 weeks to first establish autologous LCLs, then at least 4 weeks for T-cell expansion, followed by 2 weeks for quality testing. Thus several groups have developed strategies to rapidly select or generate EBVSTs.[125–128] Rapid selection strategies rely on the use of HLA-peptide multimers or streptamers, or mechanisms such as IFN-α capture, in which T cells that secrete IFN-α in response to antigen stimulation are selected.[127,129] Although these selection procedures require limited (<48 hours) ex vivo culture, they require substantial amounts of donor blood to generate a clinical product; thus leukopheresis is sometimes required. In addition, HLA-peptide multimer or streptamer selection requires knowledge of a particular epitope and is restricted to the selection of CD8$^+$ T cells. Our group has therefore focused on rapid expansion protocols in which we have replaced LCLs with APCs stimulated with overlapping peptide libraries (pepmixes) spanning the protein sequence of selected viral antigens to stimulate and expand EBVSTs in the presence of cytokines. Resulting cell lines are polyclonal and contain CD4$^+$ and CD8$^+$ T cells recognizing MHC class I as well as class II restricted viral antigens. This “rapid pepmix” method has shortened EBVST manufacture to as few as 10 days and is now widely used in clinical trials, with promising results. More recently, generation of EBVSTs as part of multivirus-specific T-cell products using the rapid pepmix method has been effective in treating patients with EBV-LPD or EBV viremia in the post-HSCT setting.[130]

Adoptive Immunotherapy for Epstein-Barr Virus$^+$ Lymphomas in Immunocompetent Individuals

In contrast to EBV-LPD, only a limited number of EBV-derived antigens (EBNA1, LMP1, and LMP2) are present in EBV-positive HL and NHL. Initial studies with patient-derived, LCL-generated EBVSTs demonstrated their safety, but infused T cells had limited antitumor activity against HL and NHL in comparison with EBV-LPD.[131] This lack of efficacy was postulated to be due in part to immunosuppressive factors secreted by lymphoma cells (leading to T-cell anergy) or dominance of clones reactive to viral proteins not expressed in latency type II lymphoma. In an effort to improve T-cell efficacy, we generated patient-derived T cells specific for EBV proteins LMP1 and LMP2 expressed in latency type II lymphoma using LCLs as APCs and recombinant adenovirus vectors encoding LMP1 and LMP2. Although these EBVSTs homed to tumor sites and some patients experienced transient clinical benefit, only a small percentage of these T cells were specific for the type II latency antigens.[132]

Clinical studies with LMP1/2 (LMP)-specific T cells demonstrate efficacy in patients with EBV-positive HL and NHL.[133,134] One trial targeted LMP2 alone and the subsequent LMP1 and LMP2. Combined with a follow-up study, 50 patients received LMP-specific T cells. Of 29 patients who received these cells as adjuvant therapy, 28 remained in remission for a median of 3.1 years. Thirteen out of 21 patients infused with active disease had objective clinical responses, with 11 patients being complete. Although LMP-specific T cells had clinical activity, generation required not only LCLs but also recombinant adenovirus vectors encoding LMP1 and LMP2, which was lengthy and added regulatory complexity. This strategy has been extended to donor-derived EBVSTs with promising results.[102] More recent clinical trials have used the rapid pepmix method to specifically target type II latency proteins using overlapping peptide libraries and cytokine stimulation (NCT01555892; NCT02287311).

Immediately Available “Off-the-Shelf” Epstein-Barr Virus–Specific T-Cell Options

Despite the clinical responses observed with use of stem cell donor-derived EBVSTs as treatment for EBV LPD, there are several limitations to this approach. For one, this treatment is restricted to patients in the post-HSCT setting with available and willing EBV-seropositive donors. In addition, even when using rapid methods, the shortened manufacture time frame is still too long for patients with fulminant, aggressive disease. In an effort to create an immediately available EBVST product that can be used in patients with post-HSCT or post-SOT EBV LPD as well as in pretransplant immunocompetent patients, several groups, including ours, have created healthy donor-derived third-party EBVST banks.[135] These cryopreserved “off-the-shelf” banks of EBVSTs are generated from the peripheral blood of blood-bank eligible healthy EBV-seropositive donors and can be manufactured using a variety of techniques. Importantly, third-party banks allow selection of donors with highest in vitro EBV antigen-specific activity (thus predicted to have greatest clinical activity) as well as selection of the EBVST product with highest degree of HLA match with confirmed HLA-restricted cytotoxicity through shared HLA alleles with the recipient, which corresponds with clinical response. To date, banks with as few as 25 EBVST lines have been able to cover the majority (>90%) of referred patients.[136–139]

To date, third-party or “off-the-shelf” EBVSTs have been administered to patients with EBV LPD in the post-HSCT, post-SOT, and pretransplant settings. Importantly, no reports of de novo acute or chronic GVHD or flares of preexisting GVHD have occurred (likely related to the manufacturing processes which reduce alloreactive T cells and the limited persistence of these partially HLA-matched allogeneic EBVSTs). Banked EBVSTs have also shown remarkable clinical responses and possess the potential for use as a preemptive strategy in patients with EBV viremia prior to development of overt LPD or PTLD. Larger trials of third-party EBVSTs continue, and ongoing research is aimed at enhancing persistence of these allogeneic cells as well as incorporating them earlier into therapy.[140] Lastly, combination strategies combining adoptive transfer of EBVSTs with immune checkpoint blockade such as PD-1/PD-L1 (programmed death 1/programmed death ligand 1) inhibition are currently being explored.[141]

OTHER EPSTEIN-BARR VIRUS–ASSOCIATED DISEASES

Nasopharyngeal Carcinoma

NPC arises from the epithelial cells of the nasopharynx. The WHO classifies NPC into keratinizing squamous cell carcinoma (type 1), nonkeratinizing carcinoma (type 2), and undifferentiated carcinoma (type 3, most common). Type 2 and 3 carcinomas are associated with EBV (see Fig. 55.6D and E); however, environmental and genetic factors play an important role in oncogenesis, because the incidence of NPC varies 50- to 100-fold from Southern China to Western countries. EBV was initially linked to NPC by the observation that patients had elevated levels of VCA-IgG, VCA-IgA, and EA-IgG antibodies. Further studies showed that EBV-DNA is present in every tumor cell of type 2 and 3 carcinomas with remarkable consistency. As in HL, the EBV episome in an individual tumor is clonal.

EBV antibody responses have been used to follow tumor burden in patients with NPC; in addition, VCA-IgA antibodies and antibodies against EBV DNase are predictive for NPC in high-risk populations. More recently, detection of EBV-DNA in serum by different PCR methods has shown to be useful for the diagnosis, prognosis, and monitoring of patients with NPC. Most patients with NPC are treated with radiation with or without chemotherapy. Other modalities such as surgery and combined approaches may be appropriate in selected circumstances, but a detailed discussion is beyond the scope of this chapter. In NPC, as for EBV-positive lymphomas, only a limited number of EBV latent antigens are expressed. Therapeutic vaccine studies targeting LMP2, LMP1, and LMP2, or EBNA1 and LMP2 have been conducted, and the results of these trials were discussed in the earlier section EBV Vaccine Development.

The adoptive transfer of autologous EBV-specific T cells has shown promise for NPC, using manufacture strategies similar to those mentioned in previous section. Several groups have reported that the infusion of T cells is safe and has resulted in clinical responses, especially in patients with loco-regional disease. In addition, EBV-specific T cells, given as adjuvant after chemotherapy, resulted in a significant increase in 3-year overall survival in comparison with historical controls, who only received chemotherapy.[142] A pivotal, randomized clinical study to validate these findings is in progress.

Multiple Sclerosis

Multiple sclerosis (MS) is a rare inflammatory demyelinating disease of the CNS. MS is triggered by a combination of genetic as well as environmental factors. The role of EBV in the pathogenesis of MS has been studied for the past 30 years.[143] Although the vast majority of

EBV-seropositive individuals do not develop MS, thereby questioning an association with EBV, the incidence of EBV seropositivity is higher in MS patients than healthy controls. In addition, individuals who have IM as their clinical presentation of primary EBV infection have a higher incidence of MS. Lastly, increased or decreased cellular immune responses to EBV antigens have been reported in MS patients. If this dysregulation of EBV-specific cellular immune responses contributes to the pathogenesis of MS is the subject of active investigation. Based on findings from these studies, EBV-targeted approaches might be explored in the future.

FUTURE DIRECTIONS

Since its discovery in 1964, EBV has been linked to a heterogeneous group of diseases. EBV was the first human virus implicated in oncogenesis, and the biology of the virus has been studied extensively on a cellular and molecular level. Over the past two decades immunotherapeutic approaches targeting EBV have been developed for EBV-associated malignancies, and later-phase clinical studies are currently in progress. Although adoptive transfer of T cells so far has been more successful than vaccines, novel, more potent vaccines have been developed that await testing in humans. Combinatorial therapies, for example combining EBV-targeted immunotherapy with antibodies that block immune checkpoints, are currently being evaluated in clinical studies and hold the promise to improve outcomes. Lastly, whole-exome sequencing will continue to identify genes that are associated with life-threatening complications of EBV infections, providing clues into their pathogenesis and novel therapeutic interventions. There is much potential to leverage EBV as a diagnostic, prognostic, and therapeutic biomarker in EBV-associated lymphoproliferative disorders and lymphomas, and current translational and clinical research appear poised to improve outcomes for patients.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Bollard CM, Cohen JI. How I treat T cell chronic active Epstein-Barr virus disease. *Blood*. 2018;131(26):2899–2905.

Carbone A, Gloghini A, Caruso A, et al. The impact of EBV and HIV infection on the microenvironmental niche underlying Hodgkin lymphoma pathogenesis. *Int J Cancer*. 2017;140(6):1233–1245.

Castillo JJ, Bibas M, Miranda RN. The biology and treatment of plasmablastic lymphoma. *Blood*. 2015;125(15):2323–2330.

Cohen JI. Epstein-Barr virus infection. *N Engl J Med*. 2000;343(7):481–492.

Cohen JI. Primary immunodeficiencies associated with EBV disease. *Curr Top Microbiol Immunol*. 2015;390(Pt 1):241–265.

Cohen JI. Vaccine development for Epstein-Barr virus. *Adv Exp Med Biol*. 2018;1045:477–493.

Cohen JI, Iwatsuki K, Ko YH, Kimura H, Manoli I, Ohshima K, et al. Epstein-Barr virus NK and T cell lymphoproliferative disease: report of a 2018 international meeting. *Leuk Lymphoma*. 2019;61(4):808–819.

Grande BM, Gerhard DS, Jiang A, et al. Genome-wide discovery of somatic coding and noncoding mutations in pediatric endemic and sporadic Burkitt lymphoma. *Blood*. 2019;133(12):1313–1324.

Heslop HE, Sharma S, Rooney CM. Adoptive T-cell therapy for Epstein-Barr virus-related lymphomas. *J Clin Oncol*. 2021;39(5):514–524.

Heslop HE, Slobod KS, Pule MA, et al. Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood*. 2010;115(5):925–935.

Kato S, Yamashita D, Nakamura S. Nodal EBV+ cytotoxic T-cell lymphoma: a literature review based on the 2017 WHO classification. *J Clin Exp Hematop*. 2020;60(2):30–36.

Kim WY, Montes-Mojarro IA, Fend F, Quintanilla-Martinez L. Epstein-Barr virus-associated T and NK-cell lymphoproliferative diseases. *Front Pediatr*. 2019;7:71.

Kogawa K, Sato H, Asano T, et al. Prognostic factors of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in children: report of the Japan Histiocytosis Study Group. *Pediatr Blood Cancer*. 2014;61(7):1257–1262.

Latour S, Fischer A. Signaling pathways involved in the T-cell-mediated immunity against Epstein-Barr virus: lessons from genetic diseases. *Immunol Rev*. 2019;291(1):174–189.

Luzuriaga K, Sullivan JL. Infectious mononucleosis. *N Engl J Med*. 2010;362(21):1993–2000.

Magrath I. Epidemiology: clues to the pathogenesis of Burkitt lymphoma. *Br J Haematol*. 2012;156(6):744–756.

Marsh RA. Epstein-Barr virus and hemophagocytic lymphohistiocytosis. *Front Immunol*. 2017;8:1902.

Melani C, Jaffe ES, Wilson WH. Pathobiology and treatment of lymphomatoid granulomatosis, a rare EBV-driven disorder. *Blood*. 2020;135(16):1344–1352.

Natkunam Y, Gratzinger D, Chadburn A, et al. Immunodeficiency-associated lymphoproliferative disorders: time for reappraisal? *Blood*. 2018;132(18):1871–1878.

Nicolae A, Pittaluga S, Abdullah S, et al. EBV-positive large B-cell lymphomas in young patients: a nodal lymphoma with evidence for a tolerogenic immune environment. *Blood*. 2015;126(7):863–872.

Palendira U, Rickinson AB. Primary immunodeficiencies and the control of Epstein-Barr virus infection. *Ann NY Acad Sci*. 2015;1356:22–44.

Quintanilla-Martinez L, Kumar S, Fend F, et al. Fulminant EBV(+) T-cell lymphoproliferative disorder following acute/chronic EBV infection: a distinct clinicopathologic syndrome. *Blood*. 2000;96(2):443–451.

Sawada A, Inoue M. Hematopoietic stem cell transplantation for the treatment of Epstein-Barr virus-associated T- or NK-cell lymphoproliferative diseases and associated disorders. *Front Pediatr*. 2018;6:334.

Shannon-Lowe C, Rickinson A. The global landscape of EBV-associated tumors. *Front Oncol*. 2019;9:713.

Shimada K, Hayakawa F, Kiyoi H. Biology and management of primary effusion lymphoma. *Blood*. 2018;132(18):1879–1888.

Styczynski J, van der Velden W, Fox CP, et al. Management of Epstein-Barr virus infections and post-transplant lymphoproliferative disorders in patients after allogeneic hematopoietic stem cell transplantation: Sixth European Conference on Infections in Leukemia (ECIL-6) guidelines. *Haematologica*. 2016;101(7):803–811.

Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127(20):2375–2390.

Tangye SG, Palendira U, Edwards ES. Human immunity against EBV-lessons from the clinic. *J Exp Med*. 2017;214(2):269–283.

Wistinghausen B, Gross TG, Bollard C. Post-transplant lymphoproliferative disease in pediatric solid organ transplant recipients. *Pediatr Hematol Oncol*. 2013;30(6):520–531.

Yamaguchi M, Suzuki R, Oguchi M. Advances in the treatment of extranodal NK/T-cell lymphoma, nasal type. *Blood*. 2018;131(23):2528–2540.

REFERENCES

1. Lieberman PM. Virology. Epstein-Barr virus turns 50. *Science*. 2014;343(6177):1323–1325.
2. Longnecker RM, Kieff E, Cohen JI. *Epstein-Barr Virus*. 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:1898–1959.
3. Evens AM, David KA, Helenowski I, Nelson B, Kaufman D, Kircher SM, et al. Multicenter analysis of 80 solid organ transplantation recipients with post-transplantation lymphoproliferative disease: outcomes and prognostic factors in the modern era. *J Clin Oncol*. 2010;28(6):1038–1046.
4. Heslop HE, Slobod KS, Pule MA, Hale GA, Rousseau A, Smith CA, et al. Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood*. 2010;115(5):925–935.
5. Rooney CM, Smith CA, Ng CY, Loftin SK, Sixbey JW, Gan Y, et al. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood*. 1998;92(5):1549–1555.
6. Heslop HE, Ng CY, Li C, Smith CA, Loftin SK, Krance RA, et al. Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. *Nat Med*. 1996;2(5):551–555.
7. Rooney CM, Smith CA, Ng CY, Loftin S, Li C, Krance RA, et al. Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. *Lancet*. 1995;345(8941):9–13.
8. Tierney RJ, Shannon-Lowe CD, Fitzsimmons L, Bell AI, Rowe M. Unexpected patterns of Epstein-Barr virus transcription revealed by a high throughput PCR array for absolute quantification of viral mRNA. *Virology*. 2015;474:117–130.
9. Hu L, Lin Z, Wu Y, Dong J, Zhao B, Cheng Y, et al. Comprehensive profiling of EBV gene expression in nasopharyngeal carcinoma through paired-end transcriptome sequencing. *Front Med*. 2016;10(1):61–75.
10. Taylor GS, Long HM, Brooks JM, Rickinson AB, Hislop AD. The immunology of Epstein-Barr virus-induced disease. *Annu Rev Immunol*. 2015;33:787–821.
11. Cohen JI. Epstein-barr virus vaccines. *Clin Transl Immunology*. 2015;4(1):e32.
12. Sharma S, Rouce RH. Are we there yet? The never-ending quest for an Epstein-Barr virus vaccine. *J Clin Invest*. 2019;129(5):1836–1838.
13. Chow JC, Ngan RK, Cheung KM, Cho WC. Immunotherapeutic approaches in nasopharyngeal carcinoma. *Expert Opin Biol Ther*. 2019; 19(11):1165–1172.
14. Elliott SL, Suhrbier A, Miles JJ, Lawrence G, Pye SJ, Le TT, et al. Phase I trial of a CD8+ T-cell peptide epitope-based vaccine for infectious mononucleosis. *J Virol*. 2008;82(3):1448–1457.
15. Gurer C, Strowig T, Brilot F, Pack M, Trumpfheller C, Arrey F, et al. Targeting the nuclear antigen 1 of Epstein-Barr virus to the human endocytic receptor DEC-205 stimulates protective T-cell responses. *Blood*. 2008;112(4):1231–1239.
16. Yu X, Ilecka M, Bartlett EJ, Schneidt V, Bhat R, Mautner J, et al. Antigen-armed antibodies targeting B lymphoma cells effectively activate antigen-specific CD4+ T cells. *Blood*. 2015;125(10):1601–1610.
17. Schneidt V, Ilecka M, Dreger P, van Zyl DG, Fink S, Mautner J, et al. Antibodies conjugated with viral antigens elicit a cytotoxic T cell response against primary CLL ex vivo. *Leukemia*. 2019;33(1):88–98.
18. Cui X, Cao Z, Sen G, Chattopadhyay G, Fuller DH, Fuller JT, et al. A novel tetrameric gp350 1-470 as a potential Epstein-Barr virus vaccine. *Vaccine*. 2013;31(30):3039–3045.
19. Cui X, Cao Z, Chen Q, Arjunaraja S, Snow AL, Snapper CM. Rabbits immunized with Epstein-Barr virus gH/gL or gB recombinant proteins elicit higher serum virus neutralizing activity than gp350. *Vaccine*. 2016;34(34):4050–4055.
20. Kanekiyo M, Bu W, Joyce MG, Meng G, Whittle JR, Baxa U, et al. Rational design of an Epstein-Barr Virus vaccine targeting the receptor-binding site. *Cell*. 2015;162(5):1090–1100.
21. Ogembo JG, Muraswki MR, McGinnes LW, Parcharidou A, Sutiwisesak R, Tison T, et al. A chimeric EBV gp350/220-based VLP replicates the virion B-cell attachment mechanism and elicits long-lasting neutralizing antibodies in mice. *J Transl Med*. 2015;13:50.
22. Perez EM, Foley J, Tison T, Silva R, Ogembo JG. Novel Epstein-Barr virus-like particles incorporating gH/gL-EBNA1 or gB-LMP2 induce high neutralizing antibody titers and EBV-specific T-cell responses in immunized mice. *Oncotarget*. 2017;8(12):19255–19273.
23. Ruiss R, Jochum S, Wanner G, Reisbach G, Hammerschmidt W, Zeidler R. A virus-like particle-based Epstein-Barr virus vaccine. *J Virol*. 2011;85(24):13105–13113.
24. van Zyl DG, Tsai MH, Shumilov A, Schneidt V, Poirey R, Schlehe B, et al. Immunogenic particles with a broad antigenic spectrum stimulate cytolytic T cells and offer increased protection against EBV infection ex vivo and in mice. *PLoS Pathog*. 2018;14(12):e1007464.
25. Shannon-Lowe C, Rickinson A. The global landscape of EBV-associated tumors. *Front Oncol*. 2019;9:713.
26. Cohen JI, Fauci AS, Varmus H, Nabel GJ. Epstein-Barr virus: an important vaccine target for cancer prevention. *Sci Transl Med*. 2011;3(107):107fs7.
27. Cohen JI. Vaccine development for Epstein-Barr virus. *Adv Exp Med Biol*. 2018;1045:477–493.
28. Luzuriaga K, Sullivan JL. Infectious mononucleosis. *N Engl J Med*. 2010;362(21):1993–2000.
29. Palendira U, Rickinson AB. Primary immunodeficiencies and the control of Epstein-Barr virus infection. *Ann N Y Acad Sci*. 2015;1356:22–44.
30. Cohen JI. Primary immunodeficiencies associated with EBV disease. *Curr Top Microbiol Immunol*. 2015;390(Pt 1):241–265.
31. Filipovich AH, Zhang K, Snow AL, Marsh RA. X-linked lymphoproliferative syndromes: brothers or distant cousins? *Blood*. 2010;116(18):3398–3408.
32. Latour S, Fischer A. Signaling pathways involved in the T-cell-mediated immunity against Epstein-Barr virus: lessons from genetic diseases. *Immunol Rev*. 2019;291(1):174–189.
33. Tangye SG, Palendira U, Edwards ES. Human immunity against EBV-lessons from the clinic. *J Exp Med*. 2017;214(2):269–283.
34. Gottschalk S, Rooney CM, Heslop HE. Post-transplant lymphoproliferative disorders. *Annu Rev Med*. 2005;56:29–44.
35. Natkunam Y, Gratzinger D, Chadburn A, Goodlad JR, Chan JKC, Said J, et al. Immunodeficiency-associated lymphoproliferative disorders: time for reappraisal? *Blood*. 2018;132(18):1871–1878.
36. Natkunam Y, Goodlad JR, Chadburn A, de Jong D, Gratzinger D, Chan JK, et al. EBV-positive B-cell proliferations of varied malignant potential: 2015 SH/EAHP workshop report-part 1. *Am J Clin Pathol*. 2017;147(2):129–152.
37. Ikeda T, Gion Y, Sakamoto M, Tachibana T, Nishikori A, Nishimura MF, et al. Clinicopathological analysis of 34 Japanese patients with EBV-positive mucocutaneous ulcer. *Mod Pathol*. 2020;33(12):2437–2448.
38. Magrath I. Epidemiology: clues to the pathogenesis of Burkitt lymphoma. *Br J Haematol*. 2012;156(6):744–756.
39. Epstein A. Burkitt lymphoma and the discovery of Epstein-Barr virus. *Br J Haematol*. 2012;156(6):777–779.
40. Epstein MA, Achong BG, Barr YM. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. *Lancet*. 1964;1(7335):702–703.
41. Shiramizu B, Barriga F, Neequaye J, Jafri A, Dalla-Favera R, Neri A, et al. Patterns of chromosomal breakpoint locations in Burkitt's lymphoma: relevance to geography and Epstein-Barr virus association. *Blood*. 1991;77(7):1516–1526.
42. El-Mallawany NK, Cairo MS. Advances in the diagnosis and treatment of childhood and adolescent B-cell non-Hodgkin lymphoma. *Clin Adv Hematol Oncol*. 2015;13(2):113–123.
43. Piccaluga PP, De Falco G, Kustagi M, Gazzola A, Agostinelli C, Tripodo C, et al. Gene expression analysis uncovers similarity and differences among Burkitt lymphoma subtypes. *Blood*. 2011;117(13):3596–3608.
44. Abate F, Ambrosio MR, Mundo L, Laginestra MA, Fuligni F, Rossi M, et al. Distinct viral and mutational spectrum of endemic Burkitt lymphoma. *PLoS Pathog*. 2015;11(10):e1005158.
45. Amato T, Abate F, Piccaluga P, Iacono M, Fallerini C, Renieri A, et al. Clonality analysis of immunoglobulin gene rearrangement by next-generation sequencing in endemic Burkitt lymphoma suggests antigen drive activation of BCR as opposed to sporadic Burkitt lymphoma. *Am J Clin Pathol*. 2016;145(1):116–127.
46. Piccaluga PP, Navari M, De Falco G, Ambrosio MR, Lazzi S, Fuligni F, et al. Virus-encoded microRNA contributes to the molecular profile of EBV-positive Burkitt lymphomas. *Oncotarget*. 2016;7(1):224–240.
47. Lenze D, Leoncini L, Hummel M, Volinia S, Liu CG, Amato T, et al. The different epidemiologic subtypes of Burkitt lymphoma share a homogenous micro RNA profile distinct from diffuse large B-cell lymphoma. *Leukemia*. 2011;25(12):1869–1876.
48. Love C, Sun Z, Jima D, Li G, Zhang J, Miles R, et al. The genetic landscape of mutations in Burkitt lymphoma. *Nat Genet*. 2012;44(12):1321–1325.
49. Panea RI, Love CL, Shingleton JR, Reddy A, Bailey JA, Moormann AM, et al. The whole-genome landscape of Burkitt lymphoma subtypes. *Blood*. 2019;134(19):1598–1607.
50. Kaymaz Y, Oduor CI, Yu H, Otieno JA, Ong'echa JM, Moormann AM, et al. Comprehensive transcriptome and mutational profiling of endemic Burkitt lymphoma reveals EBV type-specific differences. *Mol Cancer Res*. 2017;15(5):563–576.

51. Grande BM, Gerhard DS, Jiang A, Griner NB, Abramson JS, Alexander TB, et al. Genome-wide discovery of somatic coding and noncoding mutations in pediatric endemic and sporadic Burkitt lymphoma. *Blood*. 2019;133(12):1313–1324.
52. Cairo MS, Sposto R, Gerrard M, Auperin A, Goldman SC, Harrison L, et al. Advanced stage, increased lactate dehydrogenase, and primary site, but not adolescent age (≥15 years), are associated with an increased risk of treatment failure in children and adolescents with mature B-cell non-Hodgkin's lymphoma: results of the FAB LMB 96 study. *J Clin Oncol*. 2012;30(4):387–393.
53. Minard-Colin V, Auperin A, Pillon M, Burke GAA, Barkauskas DA, Wheatley K, et al. Rituximab for high-risk, mature B-cell non-Hodgkin's lymphoma in children. *N Engl J Med*. 2020;382(23):2207–2219.
54. Ozuah NW, Lubega J, Allen CE, El-Mallawany NK. Five decades of low intensity and low survival: adapting intensified regimens to cure pediatric Burkitt lymphoma in Africa. *Blood Adv*. 2020;4(16):4007–4019.
55. Murray PG, Young LS. An etiological role for the Epstein-Barr virus in the pathogenesis of classical Hodgkin lymphoma. *Blood*. 2019;134(7): 591–596.
56. Carbone A, Gloghini A, Caruso A, De Paoli P, Dolcetti R. The impact of EBV and HIV infection on the microenvironmental niche underlying Hodgkin lymphoma pathogenesis. *Int J Cancer*. 2017;140(6):1233–1245.
57. Kanakry JA, Li H, Gellert LL, Lemas MV, Hsieh WS, Hong F, et al. Plasma Epstein-Barr virus DNA predicts outcome in advanced Hodgkin lymphoma: correlative analysis from a large North American cooperative group trial. *Blood*. 2013;121(18):3547–3553.
58. Welch JJG, Schwartz CL, Higman M, Chen L, Buxton A, Kanakry JA, et al. Epstein-Barr virus DNA in serum as an early prognostic marker in children and adolescents with Hodgkin lymphoma. *Blood Adv*. 2017;1(11):681–684.
59. Wang WY, Twu CW, Chen HH, Jan JS, Jiang RS, Chao JY, et al. Plasma EBV DNA clearance rate as a novel prognostic marker for metastatic/recurrent nasopharyngeal carcinoma. *Clin Cancer Res*. 2010;16(3):1016–1024.
60. Lee JH, Kim Y, Choi JW, Kim YS. Prevalence and prognostic significance of Epstein-Barr virus infection in classical Hodgkin's lymphoma: a meta-analysis. *Arch Med Res*. 2014;45(5):417–431.
61. Swerdlow SH, Campo E, Pileri SA, Harris NL, Stein H, Siebert R, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127(20):2375–2390.
62. Beltran BE, Castro D, Paredes S, Miranda RN, Castillo JJ. EBV-positive diffuse large B-cell lymphoma, not otherwise specified: 2020 update on diagnosis, risk-stratification and management. *Am J Hematol*. 2020;95(4):435–445.
63. Nicolae A, Pittaluga S, Abdullah S, Steinberg SM, Pham TA, Davies-Hill T, et al. EBV-positive large B-cell lymphomas in young patients: a nodal lymphoma with evidence for a tolerogenic immune environment. *Blood*. 2015;126(7):863–872.
64. Song JY, Pittaluga S, Dunleavy K, Grant N, White T, Jiang L, et al. Lymphomatoid granulomatosis—a single institute experience: pathologic findings and clinical correlations. *Am J Surg Pathol*. 2015;39(2):141–156.
65. Melani C, Jaffe ES, Wilson WH. Pathobiology and treatment of lymphomatoid granulomatosis, a rare EBV-driven disorder. *Blood*. 2020; 135(16):1344–1352.
66. Roschewski M, Wilson WH. Lymphomatoid granulomatosis. *Cancer J*. 2012;18(5):469–474.
67. Dojcinov SD, Fend F, Quintanilla-Martinez L. EBV-positive lymphoproliferations of B- T- and NK-cell derivation in non-immunocompromised hosts. *Pathogens*. 2018;7(1).
68. Yarchoan R, Uldrick TS. HIV-associated cancers and related diseases. *N Engl J Med*. 2018;378(11):1029–1041.
69. Castillo JJ, Bibas M, Miranda RN. The biology and treatment of plasmablastic lymphoma. *Blood*. 2015;125(15):2323–2330.
70. Shimada K, Hayakawa F, Kiyoi H. Biology and management of primary effusion lymphoma. *Blood*. 2018;132(18):1879–1888.
71. Arora N, Gupta A, Sadeghi N. Primary effusion lymphoma: current concepts and management. *Curr Opin Pulm Med*. 2017;23(4):365–370.
72. Hochberg J, El-Mallawany NK, Abla O. Adolescent and young adult non-Hodgkin lymphoma. *Br J Haematol*. 2016;173(4):637–650.
73. Okuno Y, Murata T, Sato Y, Muramatsu H, Ito Y, Watanabe T, et al. Defective Epstein-Barr virus in chronic active infection and haematological malignancy. *Nat Microbiol*. 2019;4(3):404–413.
74. Cohen JI, Iwatsuki K, Ko YH, Kimura H, Manoli I, Ohshima K, et al. Epstein-Barr virus NK and T cell lymphoproliferative disease: report of a 2018 international meeting. *Leuk Lymphoma*. 2019:1–12.
75. Kim WY, Montes-Mojarro IA, Fend F, Quintanilla-Martinez L. Epstein-Barr virus-associated T and NK-cell lymphoproliferative diseases. *Front Pediatr*. 2019;7:71.
76. Montes-Mojarro IA, Kim WY, Fend F, Quintanilla-Martinez L. Epstein-Barr virus positive T and NK-cell lymphoproliferations: morphological features and differential diagnosis. *Semin Diagn Pathol*. 2020;37(1):32–46.
77. Gratzinger D, de Jong D, Jaffe ES, Chadburn A, Chan JK, Goodlad JR, et al. T- and NK-cell lymphomas and systemic lymphoproliferative disorders and the immunodeficiency setting: 2015 SH/EAHP workshop report – part 4. *Am J Clin Pathol*. 2017;147(2):188–203.
78. Swerdlow SH. T-cell and NK-cell posttransplantation lymphoproliferative disorders. *Am J Clin Pathol*. 2007;127(6):887–895.
79. Liu P, Pan X, Chen C, Niu T, Shuai X, Wang J, et al. Nivolumab treatment for relapsed/refractory Epstein-Barr virus-associated hemophagocytic lymphohistocytosis in adults. *Blood*. 2020;135(11):826–833.
80. Allen CE, McClain KL. Pathophysiology and epidemiology of hemophagocytic lymphohistiocytosis. *Hematology Am Soc Hematol Educ Program*. 2015;2015:177–182.
81. Jordan MB, Allen CE, Weitzman S, Filipovich AH, McClain KL. How I treat hemophagocytic lymphohistiocytosis. *Blood*. 2011;118(15):4041–4052.
82. Locatelli F, Jordan MB, Allen C, Cesaro S, Rizzari C, Rao A, et al. Emapalumab in Children with primary hemophagocytic lymphohistiocytosis. *N Engl J Med*. 2020;382(19):1811–1822.
83. Marsh RA, Vaughn G, Kim MO, Li D, Jodele S, Joshi S, et al. Reduced-intensity conditioning significantly improves survival of patients with hemophagocytic lymphohistiocytosis undergoing allogeneic hematopoietic cell transplantation. *Blood*. 2010;116(26):5824–5831.
84. Allen CE, Marsh R, Dawson P, Bollard CM, Shenoy S, Roehrs P, et al. Reduced-intensity conditioning for hematopoietic cell transplant for HLH and primary immune deficiencies. *Blood*. 2018;132(13):1438–1451.
85. Imashuku S. Treatment of Epstein-Barr virus-related hemophagocytic lymphohistiocytosis (EBV-HLH); update 2010. *J Pediatr Hematol Oncol*. 2011;33(1):35–39.
86. Chellapandian D, Das R, Zelley K, Wiener SJ, Zhao H, Teachey DT, et al. Treatment of Epstein Barr virus-induced haemophagocytic lymphohistiocytosis with rituximab-containing chemo-immunotherapeutic regimens. *Br J Haematol*. 2013;162(3):376–382.
87. Kogawa K, Sato H, Asano T, Ohga S, Kudo K, Morimoto A, et al. Prognostic factors of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in children: report of the Japan histiocytosis study group. *Pediatr Blood Cancer*. 2014;61(7):1257–1262.
88. Marsh RA. Epstein-Barr virus and hemophagocytic lymphohistiocytosis. *Front Immunol*. 2017;8:1902.
89. Smith MC, Cohen DN, Greig B, Yenamandra A, Vnencak-Jones C, Thompson MA, et al. The ambiguous boundary between EBV-related hemophagocytic lymphohistiocytosis and systemic EBV-driven T cell lymphoproliferative disorder. *Int J Clin Exp Pathol*. 2014;7(9):5738–5749.
90. Yanagaisawa R, Matsuda K, Ohga S, Kanegane H, Morimoto A, Okamoto Y, et al. Factors predicting the recurrence of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis in children after treatment using the HLH-2004 protocol. *Int J Hematol*. 2019;109(5):612–617.
91. Bollard CM, Cohen JI. How I treat T cell chronic active Epstein-Barr virus disease. *Blood*. 2018;131(26):2899–2905.
92. Cohen JI, Kimura H, Nakamura S, Ko YH, Jaffe ES. Epstein-Barr virus-associated lymphoproliferative disease in non-immunocompromised hosts: a status report and summary of an international meeting, 8–9 September 2008. *Ann Oncol*. 2009;20(9):1472–1482.
93. Cohen JI, Jaffe ES, Dale JK, Pittaluga S, Heslop HE, Rooney CM, et al. Characterization and treatment of chronic active Epstein-Barr virus disease: a 28-year experience in the United States. *Blood*. 2011;117(22): 5835–5849.
94. Kimura H, Cohen JI. Chronic active Epstein-Barr virus disease. *Front Immunol*. 2017;8:1867.
95. Arai A. Advances in the study of chronic active Epstein-Barr virus infection: clinical features under the 2016 WHO classification and mechanisms of development. *Front Pediatr*. 2019;7:14.
96. Quintanilla-Martinez L, Kumar S, Fend F, Reyes E, Teruya-Feldstein J, Kingma DW, et al. Fulminant EBV(+) T-cell lymphoproliferative disorder following acute/chronic EBV infection: a distinct clinicopathologic syndrome. *Blood*. 2000;96(2):443–451.
97. Sevilla DW, El-Mallawany NK, Emmons FN, Alexander S, Bhagat G, Alobeid B. Spectrum of childhood Epstein-Barr virus-associated T-cell proliferations and bone marrow findings. *Pediatr Dev Pathol*. 2011;14(1):28–37.
98. Coffey AM, Lewis A, Marcogliese AN, Elghetany MT, Punia JN, Chang CC, et al. A clinicopathologic study of the spectrum of systemic forms of EBV-associated T-cell lymphoproliferative disorders of childhood: a single tertiary care pediatric institution experience in North America. *Pediatr Blood Cancer*. 2019;66(8):e27798.

99. Kawa K, Sawada A, Sato M, Okamura T, Sakata N, Kondo O, et al. Excellent outcome of allogeneic hematopoietic SCT with reduced-intensity conditioning for the treatment of chronic active EBV infection. *Bone Marrow Transplant*. 2011;46(1):77–83.
100. Sawada A, Inoue M, Kawa K. How we treat chronic active Epstein-Barr virus infection. *Int J Hematol*. 2017;105(4):406–418.
101. Sawada A, Inoue M. Hematopoietic stem cell transplantation for the treatment of Epstein-Barr virus-associated T- or NK-cell lymphoproliferative diseases and associated disorders. *Front Pediatr*. 2018;6:334.
102. McLaughlin LP, Rouce R, Gottschalk S, Torrano V, Carrum G, Wu MF, et al. EBV/LMP-specific T cells maintain remissions of T- and B-cell EBV lymphomas after allogeneic bone marrow transplantation. *Blood*. 2018;132(22):2351–2361.
103. Haverkos BM, Pan Z, Gru AA, Freud AG, Rabinovitch R, Xu-Welliver M, et al. Extranodal NK/T cell lymphoma, nasal type (ENKTL-NT): an update on epidemiology, clinical presentation, and natural history in North American and European cases. *Curr Hematol Malig Rep*. 2016;11(6):514–527.
104. Yamaguchi M, Suzuki R, Oguchi M. Advances in the treatment of extranodal NK/T-cell lymphoma, nasal type. *Blood*. 2018;131(23):2528–2540.
105. Kato S, Yamashita D, Nakamura S. Nodal EBV+ cytotoxic T-cell lymphoma: a literature review based on the 2017 WHO classification. *J Clin Exp Hematop*. 2020;60(2):30–36.
106. Bollard CM, Heslop HE. T cells for viral infections after allogeneic hematopoietic stem cell transplant. *Blood*. 2016;127(26):3331–3340.
107. Weinstock DM, Ambrossi GG, Brennan C, Kiehn TE, Jakubowski A. Preemptive diagnosis and treatment of Epstein-Barr virus-associated post transplant lymphoproliferative disorder after hematopoietic stem cell transplant: an approach in development. *Bone Marrow Transplant*. 2006;37(6):539–546.
108. Styczynski J, Einsele H, Gil L, Ljungman P. Outcome of treatment of Epstein-Barr virus-related post-transplant lymphoproliferative disorder in hematopoietic stem cell recipients: a comprehensive review of reported cases. *Transpl Infect Dis*. 2009;11(5):383–392.
109. Styczynski J, van der Velden W, Fox CP, Engelhard D, de la Camara R, Cordonnier C, et al. Management of Epstein-Barr virus infections and post-transplant lymphoproliferative disorders in patients after allogeneic hematopoietic stem cell transplantation: Sixth European Conference on Infections in Leukemia (ECIL-6) guidelines. *Haematologica*. 2016;101(7):803–811.
110. Gross TG, Bucuvalas JC, Park JR, Greiner TC, Hinrich SH, Kaufman SS, et al. Low-dose chemotherapy for Epstein-Barr virus-positive post-transplantation lymphoproliferative disease in children after solid organ transplantation. *J Clin Oncol*. 2005;23(27):6481–6488.
111. Gross TG, Orjuela MA, Perkins SL, Park JR, Lynch JC, Cairo MS, et al. Low-dose chemotherapy and rituximab for posttransplant lymphoproliferative disease (PTLD): a Children's Oncology Group report. *Am J Transplant*. 2012;12(11):3069–3075.
112. Maecker B, Jack T, Zimmermann M, Abdul-Khaliq H, Burdelski M, Fuchs A, et al. CNS or bone marrow involvement as risk factors for poor survival in post-transplantation lymphoproliferative disorders in children after solid organ transplantation. *J Clin Oncol*. 2007;25(31):4902–4908.
113. Wistinghausen B, Gross TG, Bollard C. Post-transplant lymphoproliferative disease in pediatric solid organ transplant recipients. *Pediatr Hematol Oncol*. 2013;30(6):520–531.
114. Mynarek M, Schober T, Behrends U, Maecker-Kolhoff B. Posttransplant lymphoproliferative disease after pediatric solid organ transplantation. *Clin Dev Immunol*. 2013;2013:814973.
115. Shindiapina P, Ahmed EH, Mozhenkova A, Abebe T, Baiocchi RA. Immunology of EBV-related lymphoproliferative disease in HIV-positive individuals. *Front Oncol*. 2020;10:1723.
116. Gopal S, Patel MR, Yanik EL, Cole SR, Achenbach CJ, Napravnik S, et al. Temporal trends in presentation and survival for HIV-associated lymphoma in the antiretroviral therapy era. *J Natl Cancer Inst*. 2013;105(16):1221–1229.
117. Cohen JI. Epstein-Barr virus infection. *N Engl J Med*. 2000;343(7):481–492.
118. Knight JS, Tsodikov A, Cibrik DM, Ross CW, Kaminski MS, Blayney DW. Lymphoma after solid organ transplantation: risk, response to therapy, and survival at a transplantation center. *J Clin Oncol*. 2009;27(20):3354–3362.
119. Heslop HE, Brenner MK, Rooney CM. Donor T cells to treat EBV-associated lymphoma. *N Engl J Med*. 1994;331(10):679–680.
120. O'Reilly RJ, Small TN, Papadopoulos E, Lucas K, Lacerda J, Koulova L. Biology and adoptive cell therapy of Epstein-Barr virus-associated lymphoproliferative disorders in recipients of marrow allografts. *Immunol Rev*. 1997;157:195–216.
121. Papadopoulos EB, Ladanyi M, Emanuel D, Mackinnon S, Boulad F, Carabasi MH, et al. Infusions of donor leukocytes to treat Epstein-Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. *N Engl J Med*. 1994;330(17):1185–1191.
122. Doubrovina E, Oflaz-Sozmen B, Prockop SE, Kernan NA, Abramson S, Teruya-Feldstein J, et al. Adoptive immunotherapy with unselected or EBV-specific T cells for biopsy-proven EBV+ lymphomas after allogeneic hematopoietic cell transplantation. *Blood*. 2012;119(11):2644–2656.
123. Heslop HE, Sharma S, Rooney CM. Adoptive T-cell therapy for Epstein-Barr virus-related lymphomas. *J Clin Oncol*. 2021;39(5):514–524.
124. Leen AM, Myers GD, Sili U, Huls MH, Weiss H, Leung KS, et al. Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. *Nat Med*. 2006;12(10):1160–1166.
125. Papadopoulou A, Gerdemann U, Katari UL, Tzannou I, Liu H, Martinez C, et al. Activity of broad-spectrum T cells as treatment for AdV, EBV, CMV, BKV, and HHV6 infections after HSCT. *Sci Transl Med*. 2014;6(242). 242ra83.
126. Leen AM, Bollard CM, Mendizabal AM, Shpall EJ, Szabolcs P, Antin JH, et al. Multicenter study of banked third-party virus-specific T cells to treat severe viral infections after hematopoietic stem cell transplantation. *Blood*. 2013;121(26):5113–5123.
127. Icheva V, Kayser S, Wolff D, Tuve S, Kyzirakos C, Bethge W, et al. Adoptive transfer of epstein-barr virus (EBV) nuclear antigen 1-specific t cells as treatment for EBV reactivation and lymphoproliferative disorders after allogeneic stem-cell transplantation. *J Clin Oncol*. 2013;31(1):39–48.
128. Uhlin M, Gertow J, Uzunel M, Okas M, Berglund S, Watz E, et al. Rapid salvage treatment with virus-specific T cells for therapy-resistant disease. *Clin Infect Dis*. 2012;55(8):1064–1073.
129. Neudorfer J, Schmidt B, Huster KM, Anderl F, Schiemann M, Holzapfel G, et al. Reversible HLA multimers (Streptamers) for the isolation of human cytotoxic T lymphocytes functionally active against tumor- and virus-derived antigens. *J Immunol Methods*. 2007;320(1-2):119–131.
130. Tzannou I, Papadopoulou A, Naik S, Leung K, Martinez CA, Ramos CA, et al. Off-the-shelf virus-specific T cells to treat BK virus, human herpesvirus 6, cytomegalovirus, Epstein-Barr virus, and adenovirus infections after allogeneic hematopoietic stem-cell transplantation. *J Clin Oncol*. 2017;35(31):3547–3557.
131. Roskrow MA, Suzuki N, Gan Y, Sixbey JW, Ng CY, Kimbrough S, et al. Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes for the treatment of patients with EBV-positive relapsed Hodgkin's disease. *Blood*. 1998;91(8):2925–2934.
132. Bollard CM, Aguilar L, Straathof KC, Gahn B, Huls MH, Rousseau A, et al. Cytotoxic T lymphocyte therapy for Epstein-Barr virus+ Hodgkin's disease. *J Exp Med*. 2004;200(12):1623–1633.
133. Bollard CM, Gottschalk S, Torrano V, Diouf O, Ku S, Hazrat Y, et al. Sustained complete responses in patients with lymphoma receiving autologous cytotoxic T lymphocytes targeting Epstein-Barr virus latent membrane proteins. *J Clin Oncol*. 2014;32(8):798–808.
134. Bollard CM, Gottschalk S, Leen AM, Weiss H, Straathof KC, Carrum G, et al. Complete responses of relapsed lymphoma following genetic modification of tumor-antigen presenting cells and T-lymphocyte transfer. *Blood*. 2007;110(8):2838–2845.
135. Rouce RH, Louis CU, Heslop HE. Epstein-Barr virus lymphoproliferative disease after hematopoietic stem cell transplant. *Curr Opin Hematol*. 2014;21(6):476–481.
136. Haque T, Wilkie GM, Jones MM, Higgins CD, Urquhart G, Wingate P, et al. Allogeneic cytotoxic T-cell therapy for EBV-positive posttransplantation lymphoproliferative disease: results of a phase 2 multicenter clinical trial. *Blood*. 2007;110(4):1123–1131.
137. Eiz-Vesper B, Maecker-Kolhoff B, Blasczyk R. Adoptive T-cell immunotherapy from third-party donors: characterization of donors and set up of a T-cell donor registry. *Front Immunol*. 2012;3:410.
138. Vickers MA, Wilkie GM, Robinson N, Rivera N, Haque T, Crawford DH, et al. Establishment and operation of a good manufacturing practice-compliant allogeneic Epstein-Barr virus (EBV)-specific cytotoxic cell bank for the treatment of EBV-associated lymphoproliferative disease. *Br J Haematol*. 2014;167(3):402–410.
139. Rouce RH, Shah N, Thakkar A, Sharma S, Reyna A, Zhang H, et al. Use of highly characterized EBV-specific T cells for treatment of EBV+ lymphoma outside of the HSCT setting. *Cytotherapy*. 2017;19(5):S43.
140. Quach DH, Becerra-Dominguez L, Rouce RH, Rooney CM. A strategy to protect off-the-shelf cell therapy products using virus-specific T-cells engineered to eliminate alloreactive T-cells. *J Transl Med*. 2019;17(1):240.
141. Lulla P, Heslop HE. Checkpoint inhibition and cellular immunotherapy in lymphoma. *Hematology Am Soc Hematol Educ Program*. 2016;2016(1):390–396.
142. Chia WK, Teo M, Wang WW, Lee B, Ang SF, Tai WM, et al. Adoptive T-cell transfer and chemotherapy in the first-line treatment of metastatic and/or locally recurrent nasopharyngeal carcinoma. *Mol Ther*. 2014;22(1):132–139.
143. Fernandez-Menendez S, Fernandez-Moran M, Fernandez-Vega I, Perez-Alvarez A, Villafani-Echazu J. Epstein-Barr virus and multiple sclerosis. From evidence to therapeutic strategies. *J Neurol Sci*. 2016;361:213–219.

PART VII HEMATOLOGIC MALIGNANCIES

CHAPTER 56

PROGRESS IN THE CLASSIFICATION OF HEMATOPOIETIC AND LYMPHOID NEOPLASMS: CLINICAL IMPLICATIONS

Mohamed E. Salama and Ronald Hoffman

Defining and classifying tumors of the hematopoietic and lymphoid tissue accurately is critical for providing optimal treatment to patients with hematologic malignancies. Explicit definitions and terminologies are prerequisites for the precise classification of hematologic malignancies. A reproducible classification that is based on consensus definitions and terminologies is fundamentally essential for proper medical practice and the advancement of medical knowledge. Generally, such classifications should be based upon clinically distinct disease entities that can be grouped together based on shared distinguishable clinical features, as well as phenotypic and molecular markers which allow for reproducible laboratory testing, clinical outcomes, and responses to therapeutic agents. Traditionally, preliminary attempts at disease classification were initiated by the personal experience of experts, and then evolved after many years or even decades of controversy and exhaustive debate. Subsequently, classification schemes change when new scientific discoveries are validated.

Following many years of controversy, a collaborative project of members of the European Association for Haematopathology and the Society for Hematopathology, along with advice from clinical hematologists and oncologists, resulted in the World Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues.[1,2] This WHO classification, which is the first true worldwide consensus classification of hematologic malignancies, is based on the principles initially defined in the "Revised European-American Classification of Lymphoid Neoplasms" (REAL) from the International Lymphoma Study Group (ILSG).[3] The most recent WHO classification (4th edition) was formulated in a series of meetings by two clinically advisory committees: one for myeloid neoplasms, and another for acute leukemias and lymphoid neoplasms.[4] The current WHO classification, which addresses new developments related to disease definitions, nomenclature, grading, and clinical relevance, provides the basis for an approach to hematopoietic and lymphoid neoplasm classification that employs clinical features, morphology, immune phenotype, and genetic features to define diseases.[4] The relative importance of each of these components varies among diseases and is dependent on the current state of knowledge. This process of classification is an ever changing process which evolves upon new scientific and clinical advances. These classifications should be used as ever changing tools, not as groupings of disease entities that are fixed in academic concrete. Morphologic assessment remains a key element of the diagnostic evaluation and classification of hematologic malignancies, as many diseases have characteristic diagnostic features. Morphologic evaluation also remains a decisive step for prioritization of ancillary testing choices that may include phenotyping, as well as molecular or genetic testing. Immunophenotypic studies such as flow cytometry and immunohistochemistry (IHC) are used routinely to identify the lineage of the benign or malignant process, and often are necessary to reach a definitive diagnosis. In several lymphoid and myeloid neoplasms, a specific diagnosis can be facilitated by using genetic or molecular abnormalities (e.g., *CCND1* in suspected mantle cell lymphoma [MCL] or *BCR/ABL* for chronic myeloid leukemia [CML]). In addition, some abnormalities can serve as prognostic markers in several diseases (e.g., *TP53 deletion or mutation*). However, many entities still lack defining genetic or molecular abnormalities.

In some conditions, rendering an accurate diagnosis requires knowledge of the patient's clinical features, such as age and prior therapy, as well as the anatomic site and/or extent of disease. Although most of the disease entities described in the WHO classification represent distinct entities, some categories are not as clearly defined and are regarded as provisional entities. In addition, borderline categories have been created for cases that do not clearly fit into one category; thus well-defined categories can remain homogeneous while borderline cases require further study and characterization. Furthermore, the WHO classification stratifies hematologic neoplasms according to lineage into three main groups: myeloid, lymphoid, and histiocytic/dendritic cells. In this chapter we provide a summary of neoplasms in these three groups, emphasizing changes that have had an effect on practice guidelines along with highlights to significant findings that have been identified in these entities since the publication of the most recent WHO classification in 2016.

PROGRESS IN THE DIAGNOSIS AND CLASSIFICATION OF MYELOID NEOPLASMS

Clinical Implications

The myeloid neoplasms are a heterogeneous group of diseases. In general terms, they are clonal hematopoietic malignancies that can arise in or affect a single myeloid lineage (e.g., monocytic). Alternatively, they can be derived from a pluripotent stem cell and can affect multiple or even all myeloid cell types (erythroid, megakaryocytic, monocytic, neutrophilic, basophilic, eosinophilic, and mast cells). The myeloid neoplasms include chronic and acute diseases, along with diseases that evolve from an indolent process to a more aggressive state. As such, they can manifest as proliferative disorders, mostly involving primary mature hematopoietic elements; they can predominately affect immature or blastic cells; or sometimes they can be a combination of these two cell types. The diseases can result in excess production of blood cells, or they can proliferate in the bone marrow (BM) and exhibit ineffective hematopoiesis, resulting in peripheral cytopenias. Because the diseases range from indolent to acute, they exhibit a wide variation in prognosis. Some disorders are fairly indolent and require only supportive care and transfusional support; others slowly progress but have no successful treatment option. Acute and life-threatening disorders are often curable, even within days or weeks after presentation, if appropriately managed. Accurate, timely diagnosis and correct classification has a tremendous impact on patient outcomes. Treatment of a particular hematological malignancy should only proceed once the nature of the blood cancer is known, which allows for the selection of rational choices of treatment options.

The diagnosis of the myeloid neoplasms typically requires a multifaceted team approach that relies on cooperation among the clinician, laboratory personnel, and a skilled pathologist. The precise diagnosis requires compiling accurate historical data, clinical information, general laboratory findings, and carefully interpreted observations from the peripheral blood smear, bone core biopsy, and aspirate, merged with information gained from immunophenotyping, cytogenetic analysis, and molecular analyses (Table 56.1). Although in some cases a diagnosis can be made with a quick examination of the blood smear, with a single molecular test, or with a simple immunophenotype, in general, careful assessment of the total clinical picture provides the most accurate and clinically relevant diagnosis. Future routine diagnostic modalities may include next-generation sequencing, single-nucleotide polymorphism array karyotyping, gene expression arrays, and genome-wide epigenetic studies.

The myeloid neoplasms are grouped as different categories of diseases. These categories seem to be changing constantly, both in name and in the components of the disorders. However, refinements have led to an increased understanding of the clinical, pathologic, and genetic basis of the diseases and ultimately serve, or at least strive to, improve diagnosis, treatment, and outcome. The current classification of myeloid neoplasms includes the categories of myeloproliferative neoplasms (MPNs), acute myeloid leukemias (AMLs), myelodysplastic syndromes (MDS), and MDS/MPNs, which is a category with features that are intermediate between MDS and MPN (Table 56.2). This generally accepted classification system was derived from a number of earlier classification systems[5] and represents the working system of the WHO as of 2008.[4,6] The recent 2016 update of the WHO classification is based on the additional information gained from molecular testing modalities that further impact the classification and categorization of these neoplasms.[7,8]

TABLE 56.1 Components in the Routine Clinical Evaluation of Myeloid Malignancies

Current

- Accurate clinical history, including family history and physical examination findings
- General laboratory findings, including CBC, and other specific tests (e.g., EPO) when appropriate
- Evaluation of well-prepared and stained peripheral blood smear with 200 cell differential count
- Review of BM aspirate, including iron stain, and 500 cell differential count
- Evaluation of H&E sections of BM biopsy of sufficient length and reticulin stain
- Phenotyping studies, including cytochemical reactions (nonspecific and specific esterase reactions and myeloperoxidase) and flow cytometric analysis of peripheral blood or BM for phenotype of blasts or other cells when appropriate
- Cytogenetic analysis, including karyotype, and FISH for specific abnormalities when appropriate
- Single-nucleotide polymorphism array karyotyping
- Genetic analysis for particular genetic rearrangements or mutations, including gene sequencing when appropriate
- Next-generation sequencing

Potential Future Clinical Studies

- Gene expression arrays,
- RNA, sequencing
- Genome-wide epigenetic studies

BM, Bone marrow; *CBC*, complete blood count; *EPO*, erythropoietin; *FISH*, fluorescence in situ hybridization; *H&E*, hematoxylin and eosin.

TABLE 56.2 World Health Organization Classification of Myeloid Neoplasms

The myeloproliferative neoplasms (MPNs), including the myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB*, and *FGFRA*

Acute myeloid leukemia (AML)

The myelodysplastic syndromes (MDS)

The MDS/MPN ("overlap") syndromes

AML, Acute myelogenous leukemia; *MDS*, myelodysplastic syndromes; *MPN*, myeloproliferative neoplasm.

The Myeloproliferative Neoplasms

The MPNs, formerly referred to as *myeloproliferative disorders* (see Chapters 69–74), are a group of clonal multipotential hematopoietic stem cell disorders that have a proliferative nature, presenting frequently with hypercellular BMs, and an elevation of one or more myeloid cell types in the blood. The MPNs are insidious in onset and chronic in course but have a variable tendency to terminate in BM failure or acute leukemia.[9] The MPNs include CML (Table 56.3), which illustrates how the elucidation of pathways involved in the molecular pathogenesis (i.e., dysregulation of *ABL1* tyrosine kinase [TK] signaling) can successfully lead to the rational targeted therapy (i.e., imatinib and other TK inhibitors).[10]

The MPNs also include *BCR-ABL1*–negative entities, essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (MF) (see Chapters 70–72). These MPNs share common features, including multipotential hematopoietic stem cell origin, clonal proliferation, and chronic nature. Although these disorders seem to be distinctive, they can present a diagnostic challenge, and they can have subtle and overlapping presentations. Recent discoveries in the underlying molecular pathology of these entities have demonstrated that, similar to CML and eosinophilic disorders, they too share TK

TABLE 56.3 Tyrosine Kinase Involvement in Myeloproliferative Neoplasms

Disease	Tyrosine Kinase Involvement
Chronic myeloid leukemia, *BCR-ABL1+*	*ABL1* (100%)
Myeloid and lymphoid neoplasms with eosinophilia TK abnormalities	*PDGFRA, PDGFRB*, or *FGFRA*
Polycythemia vera	*JAK2* V617F (≈95%), *JAK* exon 12 (≈4%)
Primary myelofibrosis	*JAK2* V6174 (≈50%), *MPL* W515 K/L (5%–9%), *CALR* (35%)
Essential thrombocythemia	*JAK2* V617F (≈50%), *MPL* W515 K/L (≈1%), *CALR* (35%)
Chronic neutrophilic leukemia	*CSF3R* (83%–90%)
Chronic eosinophilic leukemia, not otherwise specified	—
Mastocytosis	*KIT* D816V (≈95%)
MPN, unclassified	—

MPN, Myeloproliferative neoplasm; *TK*, tyrosine kinase.

signaling dysregulation, at least to some degree. This is attributable to an associated mutation in the TK, *JAK2* (*JAK2* V617F), which is present in about 50% to 95% of cases of Ph-negative MPNs. Variants of *JAK2* mutations involving exon 12 are also observed in 2% to 3% of PV patients. This association has led to significant revisions in the 2008 WHO criteria for diagnosis, but it also has raised questions as to how a single mutation can be associated with such heterogeneous phenotypic characteristics. Proposed revisions in 2016 WHO classifications for the diagnosis of the Ph-negative MPNs include suggested modifications of lower hemoglobin thresholds for PV. BM biopsy results and the presence of *JAK2* mutations now represents a major criterion. A subnormal erythropoietin level became a minor criterion, and the endogenous erythroid colony assay is no longer included in the current WHO diagnostic criteria for MPNs.[11] These changes are intended to capture patients with subtle presentations, such as those with masked PV. Experts also have recommended inclusion of mutations in calreticulin (*CLAR*) and the thrombopoietin receptor, MPL, which is identified in 20% to 30% of ET and MF cases, as well as the colony-stimulating factor 3 receptor mutations in the next revision.[11–13]

All three (*JAK2*, *CLAR*, and *MPL*) driver mutations are negative in approximately 10% of cases (so-called "triple negative" [TN] cases). In these TN cases, the non-clonal differential diagnostic considerations pose significant diagnostic challenges. As such large-scale next generation sequencing is increasingly being used to establish not only the clonal nature of the disease, but also to guide molecular risk stratification and management decisions, including clinical trial selection for these patients when evaluated for MF.

JAK2 V617F inhibitors are among several agents in use to treat patients with MPNs. In 2011 the US Food and Drug Administration (FDA) approved ruxolitinib for the treatment of patients with intermediate or high-risk MF. Subsequently, the FDA also designated ruxolitinib as an orphan product, as it demonstrated potentially significant improvement in safety and efficacy over other available therapies for PV. A number of ruxolitinib-based combination therapies, as well as novel agents that target various pathways, are currently being investigated and will be further discussed in detail later (see Chapters 70–72).

Chronic neutrophilic leukemia (CNL) (see Chapter 72) and mast cell disease (see Chapter 75) are currently included in the category of MPNs. CNL is now better defined with the integration of *CSF3R* mutation as a diagnostic tool, and is distinct from atypical CML.[14]

Mast cell disease is yet another disease related to abnormal TK signaling—in this case, the *KIT* gene.[15] Its classification system generally considers systemic mastocytosis and cutaneous mastocytosis as main entities but also includes a mast cell proliferation associated with clonal non-mast cell hematopoietic malignancies; this can be another myeloid malignancy or, less commonly, a lymphoid malignancy. With the advances of comprehensive mutational profiling, it is proposed that additional mutations in *SRSF2*, *ASXL1*, and/or *RUNX1* identify a high-risk group of patients with *KIT* D816V^{+} advanced systemic mastocytosis.[16] A more recently improved understanding of the cellular and molecular basis of eosinophilic disorders has translated into more biologically oriented classification schemes that carry therapeutic implications. Thus, in 2008, the WHO established a semi-molecular classification scheme of separately listed disease subgroups including "myeloid and lymphoid neoplasms with eosinophilia and abnormalities of platelet-derived growth factor receptors (PDGFRA, PDGFRB), or fibroblast growth factor receptor 1(FGFR1)," chronic eosinophilic leukemia (CEL), not otherwise specified (NOS), lymphocyte-variant hypereosinophilia, and idiopathic hypereosinophilic syndrome (see Chapter 74). Although quite uncommon, these are noteworthy because, similar to CML, they have also been found to be caused by dysregulation of TK signaling (caused by mutations in *PDGFRA*, *PDGFRB*, or *FGFRA*) and at least partially successfully treated with TK inhibitors.[17]

It is important to emphasize that the modern diagnosis of the MPNs does not rest solely with the routine microscopic examination. The diagnostic work-up is more far-reaching and must include reviewing the clinical history and pertinent physical findings, as well as obtaining and assessing laboratory values, including recent complete blood cell counts. Examination of a well-prepared peripheral blood smear and both BM aspirate and biopsy specimens are still crucial. However, ancillary studies, such as cytogenetic and molecular analysis, as well as other more specific laboratory evaluations, are just as important in formulating the correct diagnosis, and in particular, in distinguishing MPNs from reactive myeloid proliferations. This is particularly true, for example, regarding the MPN driver mutations, and the impact not only on deciding the diagnosis but also on the prognosis and treatment of MPN patients.

Acute Myeloid Leukemia

The historical and emerging classifications systems for AML dramatically illustrate the marked heterogeneity of these diseases (see Chapters 59 and 60). Despite this heterogeneity, however, to date a major breakthrough has not been made in the development of specific initial treatment for AML as an entity apart from acute promyelocytic leukemia. However, to a large extent this concept is changing as newer agents targeting molecularly or genetically defined forms of AML have become available (e.g., FMS-like tyrosine kinase-3 [FLT3] inhibitors for FLT3-mutated cases).[18,19] It is anticipated that the gap between the growing number of unique or genetically defined acute leukemia types and the somewhat limited treatment options may begin to close.

The French–American–British (FAB) classification of AML, introduced in 1976 with its subsequent revision in 1985, provided the first real framework for classifying the AMLs. It also provided fairly reproducible definitions of diseases. The classification was mainly based on morphologic and cytochemical features of the leukemic blasts in the BM. In general, the FAB scheme required that 30% of the BM-nucleated elements be blasts; then, it further defined AML subtypes based on the presence of maturation in the granulocytic series, the presence of a monocytic component, and the presence of an erythroid component. Later, as immunophenotyping allowed for improved identification of myeloid precursors, acute megakaryoblastic leukemia and AML with minimal differentiation were added. The types of AML noted in the revised FAB scheme included one with minimal differentiation (M0), AML without maturation (M1), AML with maturation (M2), and acute promyelocytic leukemia (M3). Also included was a type with combined monocytic and myeloid (neutrophilic) components (M4), a pure monocytic or monoblastic type (M5A, M5B), a type with a prominent erythroid component (M6), and a megakaryoblastic type (M7) (Table 56.4).

The FAB classification provided a framework and defined criteria for different types of acute leukemia. However, leukemia-associated genetic changes, first recognized in the 1980s, seemed to provide more important prognostic information and did not always correlate well with the FAB-defined entities. As the cytogenetic abnormalities became more widely appreciated and their prognostic implications better understood, classification schemes based solely on the "favorable," "intermediate," and "adverse" prognostic cytogenetic findings were introduced and used along with, or as an alternative to, the FAB scheme (see Table 56.4).

The AML WHO classification published in 2001 provided a different strategy from that used by the FAB in two important ways: it redefined AML as requiring only 20% blasts in the BM or blood, and it emphasized the importance of the associated cytogenetic abnormalities. The change in blast percentage came from the recognition that patients with 20% to 30% blasts, who previously were classified as having MDS (refractory anemia [RA] with excess of blasts in transformation [RAEB-T] in the FAB MDS classification), often had outcomes similar to those with AML and frequently required treatment as if they had been diagnosed with AML. Furthermore, the inclusion of karyotypic abnormalities recognized the important prognostic information associated with these abnormalities, as well as the fact that a karyotypic abnormality could point to the underlying molecular pathogenesis. As we learned from our experience with CML, this was critical in developing new drugs and treatment strategies.

TABLE 56.4 **Classification Schemes for the Acute Myeloid Leukemias**

French–American–British (FAB, 1985)	Cytogenetic	WHO (2001)	WHO (2008)	WHO (2016)
M0: AML with minimal differentiation	**Favorable**	**Genetic**	**With recurrent genetic abnormalities**	**With recurrent genetic abnormalities**
M1: AML without maturation	t(8;21)	t(8;21)	With t(8;21)(q22;q22)	AML with t (8;21)(q22;q22.1);RUNX1-RUNX1T1
M2: AML with maturation	inv(16)/t(16;16)	t(inv(16)/t(16;16)	With inv(16)/t(16;16)(p13.1;q22)	AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);CBFB-MYH11
M3: APL	t(15;17)	t(15;17)	With t(15;17)(q22;q12)	APL with PML-RARA
M3v: microgranular variant	**Intermediate**	t(v;11q23)	With t(9;11)(p22;q23)	AML with t(9;11)(p21.3;q23.3);MLLT3-KMT2A
M4: AMML	Normal	**AML with dysplasia therapy–related AML**	With t(6;9)(p23;q34)	AML with t(6;9)(p23;q34.1);DEK-NUP214
M4eo: AMML with abnormal eosinophils	+8	Etoposide	With inv(3)/t(3;3)(q21;q26.2)	AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM
	t(v;11q23)	Cytotoxic or radiation	With t(1;22)(p13;q13)	AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);RBM15-MKL1
M5A: acute monoblastic leukemia	del(7q)	**Not otherwise specified**	With mutated NPM1 (provisional)	Provisional entity: AML with BCR-ABL1
M5B: acute monocytic Leukemia	+21	With minimal differentiation	With mutated CEBPA (provisional)	AML with mutated NPM1
M6: erythroleukemia	+22	Without maturation	**With myelodysplasia related changes**	AML with biallelic mutations of CEBPA
M7: acute megakaryoblastic leukemia	Other	With maturation	Previous history of MDS	Provisional entity: AML with mutated RUNX1
	Adverse	Myelomonocytic leukemia	Multilineage dysplasia	**AML with myelodysplasia-related changes**
	del(5q)	Monoblastic leukemia	MDS-related cytogenetic abnormalities	**Therapy-related myeloid neoplasms**
	−5	Monocytic leukemia	**Therapy-related myeloid neoplasms**	**AML, NOS**
	−7	Erythroleukemia	t-AML/t-MDS (t-MN)	AML with minimal differentiation
	Complex	Erythroid myeloid type	**Not otherwise specified**	AML without maturation
	abn (3q)	Pure erythroid	With minimal differentiation	AML with maturation
		Megakaryoblastic	Without maturation	Acute myelomonocytic leukemia
			With maturation	Acute monoblastic/monocytic leukemia
			Myelomonocytic leukemia	Pure erythroid leukemia
			Monoblastic leukemia	Acute megakaryoblastic leukemia
			Monocytic leukemia	Acute basophilic leukemia
			Erythroleukemia	Acute panmyelosis with myelofibrosis
			Erythroid myeloid type	**Myeloid sarcoma**
			Pure erythroid	**Myeloid proliferations related to Down syndrome**
			Acute megakaryoblastic leukemia	Transient abnormal myelopoiesis (TAM)
			Acute basophilic leukemia	Myeloid leukemia associated with Down syndrome
			Acute panmyelosis with myelofibrosis	
			Myeloid sarcoma	
			Myeloid leukemia associated with Down syndrome	
			Blastic plasmacytoid dendritic cell tumor	

AML, Acute myeloid leukemia; *AMML*, acute myelomonocytic leukemia; *APL*, acute promyelocytic leukemia; *WHO*, World Health Organization.

However, the WHO classification recognized that not all acute leukemias could be defined by cytogenetic abnormalities and that some needed to be defined either clinically or still based on morphologic findings. In this regard, the 2001 classification recognized four major subclasses of AML: AML with recurring cytogenetic abnormalities; therapy-related AML (subclassified further as those following etoposide treatment and those with a history of cytotoxic drug therapy or radiation); AML with multilineage dysplasia; and cases that did not fit into the other subclasses were referred to as AML not otherwise categorized (see Table 56.4).

A 2008 revision of the 2001 classification scheme emphasized risk-based stratification and made a number of changes (see Table 56.4).[4] In particular, it expanded the cytogenetic abnormality-associated cases to those with some less common translocations. It added provisional entities defined by mutations often occurring in cytogenetically normal cases, namely those with *NPM1* and *CEBPA* mutations. It combined the different types of therapy-related MDS/AML and renamed this category *therapy-related myeloid neoplasm* (t-MN). It also redefined the dysplasia-associated cases by allowing the cases to be identified by history (of previous MDS), morphology (with multilineage dysplasia), or by cytogenetics (with defined chromosomal changes associated with dysplasia). Additional categories were also added, including AML associated with Down syndrome, acute panmyelosis with MF, granulocytic sarcoma, and blastic plasmacytoid dendritic cell tumor (see Chapter 64). The 2008 classification eventually defined more than 25 different types of AML. The advent of newer technologies, such as single-nucleotide polymorphism array karyotyping and next-generation sequencing, facilitated the recognition of several relevant driver mutations.[20] Molecular analysis for leukemic driver mutations, particularly in the cytogenetically normal AML subgroup (40% to 50% of AML patients), has been incorporated into routine clinical practice to assess disease progression and prognosis. Important mutations involve *FLT3*, *NPM1*, *KIT*, *CEBPα*, *TET2*, *DNMT3A*, and *IDH1*. Although the relevance of many of these mutations to prognosis is defined, some are still debated and their coexisting consequences are yet to be determined.

With the development of more sensitive analysis, the subclassification of AML patients has become more detailed. The revisions to AML classification in 2016 remain largely unchanged for the entities of AML and NOS, with the exception of including the erythroid/myeloid subtype of acute erythroid leukemia with MDS. The 2016 revisions include recognition of new cytogenetic subgroups, such as a provisional entity of AML with BCR-ABL and accompanied cryptic deletions of antigen receptors, particularly immunoglobulin heavy chain (IGH) gene rearrangement; this was recently shown to be specific for de novo disease.[21,22] This update also included new and revised subgroups based on the presence of myeloid gene mutations. For example, AML with *RUNX1* mutations include only de novo cases. AML with *CEBPα* now are heterozygous/double mutations (Fig. 56.1). In addition, *NPM1* and double mutations in *CEBPα* trump multilineage dysplasia in de novo disease without MDS-related cytogenetic abnormalities other than del(9q). There is also a greater emphasis on the recognition of familial myeloid neoplasms, with an added classifications section.20 Overall, the 2016 WHO classification of AML has limited changes but recognizes the importance of myeloid gene mutation studies without making the classification overly complex. Cytogenetic studies, screening for gene rearrangements, and assessment of gene panels, including *NPM1, CEBPA, RUNX1*, are currently required to assign AML to the recurrent genetic abnormalities category.

The Myelodysplastic Syndromes

The earliest recognition of myelodysplastic disorders as a clinical entity came with the identification of long-standing and treatment-refractory anemia as occasionally representing a preleukemic disorder (see Chapters 61 and 65). MDS patients classically present with peripheral cytopenias, a hypercellular BM with ineffective hematopoiesis and uni- or multi-lineage dysplasia, with or without an increase in blast cell numbers. Although this is fairly typical, it belies the wide pathologic spectrum of MDS, which includes cases that are diagnostically challenging, and which can be difficult to distinguish on one hand from benign causes of cytopenias in older adult patients, and on the other hand from AML and other more aggressive clonal myeloid neoplasms.

The FAB group proposed the first formal classification of MDS in 1982.[5] In this classification scheme, the myelodysplastic disorders

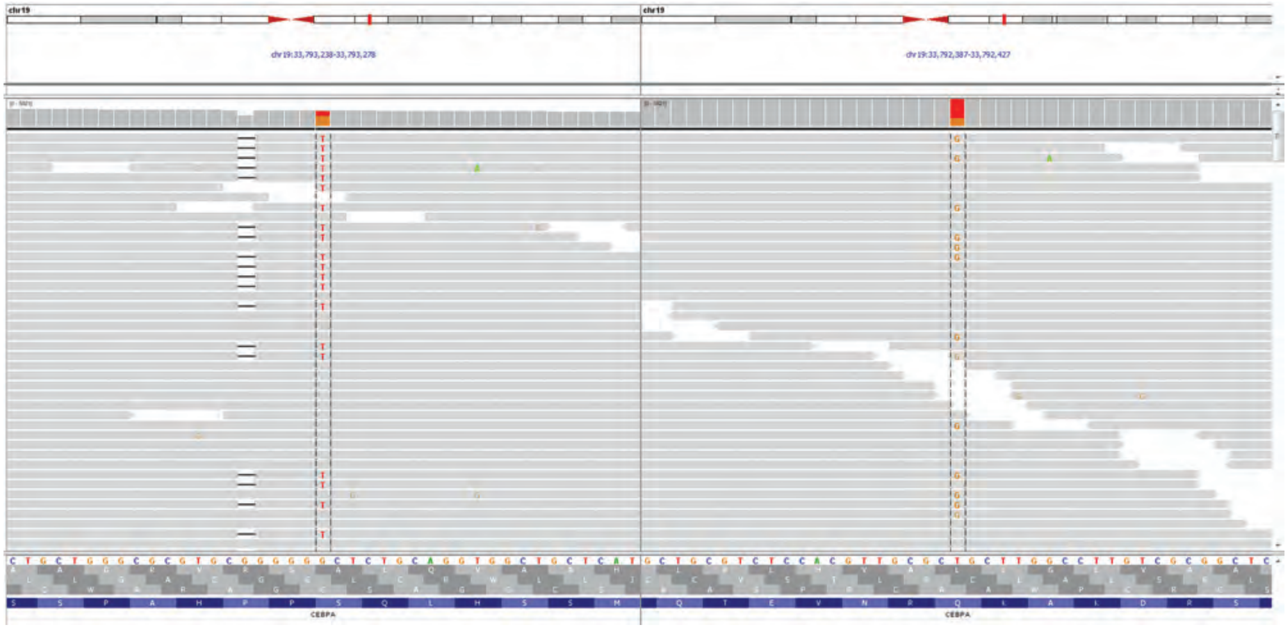


Figure 56.1 ALIGNMENT DATA OF INTEGRATIVE GENOMICS VIEWER FOR DOUBLE CEBPA MUTATIONS IDENTIFIED IN AN AML PATIENT. The *left* side shows c.63_64delinsA, p.Ser21fs, the *right* side shows c.914 A>C, p.Gln305Pro. The *CEBPα* N-terminal frame-shift mutation (p.Ser21fs) is predicted to disrupt the normal function of *CEBPα*. The other *CEBPA* mutation (c.914 A>C, p.Gln305Pro) is a C-terminal missense mutation that has been reported in acute myeloid leukemia (AML) patients. Double-*CEBPα* mutations (as seen in this patient) are found in 5% of patients with AML and are associated with a favorable outcome.[23] *CEBPα*-double mutant AML patients also had a significantly better overall survival at 8 years.

were divided into four subtypes based on increasing blast cell numbers or as chronic myelomonocytic leukemia (CMML). The four entities included RA, refractory anemia with ring sideroblasts (RARS), refractory anemia with excess of blasts (RAEB), and refractory anemia with excess of blasts in transformation (RAEB-T). These entities were differentiated mainly by the percentage of blasts seen in the BM (Table 56.5). As noted earlier, in the FAB scheme AML was defined by the presence of 30% or more blasts in the blood or BM.

The FAB included CMML in the MDS category, although it was recognized that CMML differed in that it had a proliferative component with increased numbers of circulating monocytes. At times, leukocytosis was a predominant feature of the so-called *myeloproliferative type* of CMML, but this was considered to be a dysplastic form and was classified as a type of MDS when the white blood cell count was less than 13,000/μL.

The 2001 WHO classification of MDS included significant changes to the classification of both MDS (see Table 56.5) and AML (as discussed earlier). As mentioned previously, the most notable change was the reduction in the blast percentage required for a diagnosis of AML from 30% to 20%, leading to the elimination of the RAEB-T category. The 2001 classification also included a new subtype of MDS that, despite the lack of increased blasts (less than 5%), had a more aggressive course, probably owing to the presence of more pronounced multilineage dysplasia. This category was called *refractory cytopenia with multilineage dysplasia* (RCMD), and it included a substantial proportion of cases previously grouped in the low-grade RA and RARS categories. Although the recognition of RCMD served to deemphasize the importance of the blast percentage when determining prognosis, these classifications subdivided the RAEB category into two types, with 5% to 9% blasts (RAEB-1) and 10% to 19% blasts (RAEB-2), paradoxically emphasizing the prognostic significance of blast percentage in this category.

TABLE 56.5 Evolving Classifications of the Myelodysplastic Syndromes

FAB 1982	WHO 2001	WHO 2008	WHO 2016
RA	RA	RCUD RA RN RT	MDS with single lineage dysplasia (MDS-SLD)
RARS	RARS	RARS	MDS-RS with single lineage dysplasia (MDS-RS-SLD)
	RCMD RCMD-RS	RCMD (-RS)	MDS with multilineage dysplasia (MDS-MLD)
			MDS-RS with multilineage dysplasia (MDS-RS-MLD)
RAEB	RAEB-1	RAEB-1	MDS with excess blasts (MDS-EB-1)
	RAEB-2	RAEB-2	MDS with excess blasts (MDS-EB2)
		MDS-U	MDS, unclassifiable (MDS-U)
		MDS with 5q-	MDS with isolated del(5q)
		RCC (provisional)	Refractory cytopenia of childhood
RAEB-T			
CMML			

CMML, chronic myelomonocytic leukemia; *FAB*, French–American–British classification; *MDS-U*, myelodysplastic syndrome, unclassified; *RA*, refractory anemia; *RARS*, refractory anemia with ring sideroblasts; *RAEB*, refractory anemia with excess blasts; *RAEB-T*, refractory anemia with excess blasts in transformation; *RCC*, refractory cytopenia of childhood; *RCMD*, refractory cytopenia with multilineage dysplasia; *RCUD*, refractory cytopenia with unilineage dysplasia; *RN*, refractory neutropenia; *RS* ring sideroblasts (% indicates percent RS of total nucleated erythroid precursors); *RT*, refractory thrombocytopenia; *WHO*, World Health Organization classification.

An additional significant change in the WHO 2001 MDS classification scheme included the exclusion of CMML from the MDS category, and the development of a separate nosologic group for CMML and other diseases in which there were features of both myelodysplasia and myeloproliferation at the time of diagnosis. These "overlap" disorders are mentioned briefly later.

Further refinements in the WHO classification scheme for MDS were made subsequently in 2008 (see Table 56.5). These included expanding low-grade MDS from RA to refractory cytopenia with unilineage dysplasia (RCUD), thereby recognizing that the megakaryocyte or granulocyte lineage could be equally affected. The 2008 WHO classification scheme also emphasized the key role of cytogenetic analysis in the diagnosis of MDS, particularly in cases with otherwise insufficient morphologic evidence to substantiate a diagnosis of MDS. This is reflected in the inclusion of the subtype MDS unclassified (MDS-U), defined by the presence of cytopenias, less than 1% peripheral blasts, less than 10% dysplastic cells in any lineage, and less than 5% BM blasts with the presence of specific cytogenetic abnormalities commonly associated with MDS. In addition, the WHO 2008 classification now includes "MDS with an isolated del(5q)" including the "5q minus syndrome." This syndrome had been recognized for some time and is characterized typically by its presentation in middle-aged women with macrocytic anemia, splenomegaly, normal-to-elevated platelet counts, hypolobated megakaryocytes in the BM, and an isolated del(5q). The specific types of MDS have been increased and there are more than 10 different entities.

The 2016 WHO revision introduced nomenclature changes including replacement of the terminology "refractory anemia" and "refractory cytopenia" with "myelodysplastic syndrome with single lineage dysplasia." Other changes in the 2016 revision include the considerations of the prognostic significance of myeloid gene mutations in MDS, revising the diagnostic criteria for MDS entities with ring sideroblasts based on the detection of *SF3B1* mutations, slightly modifying the cytogenetic criteria for MDS with isolated del(5q), reclassifying most cases of the erythroid/myeloid type of acute erythroleukemia, and recognizing the familial link in some cases of MDS.[24] Flow cytometric immunophenotyping is recognized as a useful ancillary technique in the evaluation of MDS.[24]

The Overlap Myelodysplastic/Myeloproliferative Neoplasms

In 2001 WHO introduced the overlap syndromes—that is, the MDS/MPNs (Table 56.6)—because at the time there was disagreement among committee members as to whether CMML was an MDS, as the FAB suggested, or an MPN, as a number of investigators suggested. This group of diseases was defined to include disorders that share features of the MPNs and of MDS at the time of initial presentation but do not fit well into either group. Some of the entities in the MPD/MPN category still are not well understood and

TABLE 56.6 The Myelodysplastic/Myeloproliferative Neoplasms

- CMML
- "aCML," *BCR-ABL1*-negative
- JMML
- RARS-T (provisional)

aCML, "Atypical" chronic myeloid leukemia; *CMML*, chronic myelomonocytic leukemia; *JMML*, juvenile myelomonocytic leukemia; *RARS-T*, refractory anemia with ring sideroblasts and thrombocytosis.

may represent a disease in transition from MDS or MPN, although a patient should not be placed in this category if initially diagnosed as MDS or MPN. However, without complete knowledge of the historical pathology of each individual patient, it is useful to have this category in order to construct a more reasonable classification. The overlap syndromes include CMML, including the juvenile type and juvenile myelomonocytic leukemia (JMML; see Chapter 65), in addition to "atypical" CML (atypical CML, *BCR-ABL1* negative), and an "unclassifiable" category that includes refractory anemia with ring sideroblasts and thrombocytosis (RARS-T) (see Chapter 73). The MDS/MPNs share proliferative features in some cell lineages but also have dysplastic features, including ineffective hematopoiesis, in others. Similar to the MPNs, the overlap syndromes require a full evaluation of clinical and morphologic findings and evaluation of ancillary studies before a firm diagnosis can be rendered.

In the 2008 WHO classification, RARS-T was proposed as a provisional entity; however, the strong association with *SF3B1* mutation and MPN-driver mutations (JAK2, MPL and CALR) supported its inclusion as a full entity in the 2016 revision.

The most common mutations reported with CMML include *TET2, SRSF2, ASXL1*, and genes of the *RAS* pathway. In fact, the coexistence of *SRSF2* and *TET2* mutations is particularly helpful in the appropriate clinical setting and can provide support to the diagnosis of CMML. Despite the progress in the molecular characterization of MPN/MDS neoplasm, significant molecular overlap with abnormalities have been noted in the spectrum of other myeloid neoplasms. As such, no disease-defining molecular abnormalities have been identified in this group, with the notable exception of JMML, which is caused by either somatic or germline mutations in one of the *RAS* pathway genes (*PTPN11, NF-1, NRAS, KRAS, CBL*). The diagnosis of MPN/MDS neoplasms requires an integrated approach to incorporate clinicopathologic findings along with cytogenetic and molecular findings.[25]

EVOLVING CONCEPTS IN CLASSIFICATION OF LYMPHOID NEOPLASMS

Hodgkin lymphoma was first described by Thomas Hodgkin in 1932 and is a distinct entity (Table 56.7) that can be distinguished from the majority of lymphomas that are designated non-Hodgkin lymphomas (NHL) (see Chapters 78 and 79).[4] Historically, different classification systems have been proposed for NHL (Table 56.8). Henry Rappaport utilized the histologic features and the architectural arrangement of the neoplastic cells and their cytology when developing a classification system in 1956, which became widely accepted. This system was created prior to the advent of modern immunology; as such, the Lukes–Collins classification in 1975 attempted to relate cell morphology to immunologic function. Subsequently in 1982, the Working Formulation for classifying NHL replaced the Rappaport and Lukes–Collins classification. This system had three groups based on patient prognosis: low, intermediate, and high grade. In 1994, the REAL classification implemented a new approach for classifying NHL, taking into account immunologic, genetic, and clinical features, and not solely relying on histopathologic characteristics of the tumor cells.[3] In 2001, the WHO classification successfully provided a common language and was adopted as the standard for clinicians and investigators worldwide.[1] The modifications made in the 2008 classification are the result of a successful coordination between pathologists, clinicians, and biologists.[4]

TABLE 56.7 Hodgkin Lymphoma

- Nodular lymphocyte-predominant Hodgkin lymphoma
- Classic Hodgkin lymphoma
  - Nodular sclerosis classic Hodgkin lymphoma
  - Lymphocyte-rich classic Hodgkin lymphoma
  - Mixed cellularity classic Hodgkin lymphoma
  - Lymphocyte-depleted classic Hodgkin lymphoma

TABLE 56.8 Historic Reflection of Lymphoma Classification

1832	Hodgkin	A report of seven lymphoma cases
1966	Rappaport	Rappaport Classification
1974	Lukes–Collins	Lukes–Collins Classification
1978	Lennert	Keil Classification
1982	National Cancer Institute	Working Formulation of Non-Hodgkin Lymphoma
1988	Stansfeld et al.	Updated Keil Classification
1994	Harris et al.	REAL Classification
2001	Jaffe et al.	2001 WHO Classification
2008	Swerdlow et al.	2008 WHO Classification

REAL, Revised European-American Classification of Lymphoid Neoplasms; *WHO*, World Health Organization.

B-cell, T-cell, and natural killer (NK)-cell neoplasms often represent clonal expansion of these cells at certain developmental stages. Although B-cell neoplasms tend to mimic stages of normal B-cell development, some common B-cell neoplasms, such as hairy cell leukemia, do not conform to a normal B-cell differentiation stage. Additionally, some lymphomas show overt heterogeneity or lineage plasticity; consequently, the normal counterpart of neoplastic cells cannot be used as the sole basis for developing a classification system. The 2008 WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues schema routinely employs a multiple-parameter approach that is based on clinical, morphologic, and biologic features, keeping in mind that a precise separation between entities is not possible in certain cases. Thus, the WHO recognized "gray zones" in which tumor cells may cross boundaries between currently used categories, such as the boundaries between classic Hodgkin lymphoma (CHL) and primary mediastinal large B-cell lymphoma.[4]

In 2008 WHO expanded the classification of lymphoid neoplasms, with more consideration being given to disease definitions, nomenclature, grading, and clinical relevance. Since then, disease definitions have continued to evolve and expand, with new entities and variants being recognized.[26,27] The most recent 2016 WHO revisions developed a focus on early or in situ lesions, as well as definition of the earlier steps of neoplastic transformation, age as a disease-defining feature (e.g., diffuse large-cell lymphoma of the older adult; Table 56.9), and site-specific impact on disease definition. In addition, there was an emphasis on overlapping or borderline entities, with fuzzy demarcation of morphologic, molecular, and genetic characteristics as areas of diagnostic challenge.[8,26]

Early Events in Lymphoid Neoplasms

Recent studies have identified additional clonal lymphoid lesions that share genetic and/or phenotypic properties with well-defined neoplasms such as chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL),[28,29] multiple myeloma (MM), follicular lymphoma (FL), and MCL (see Chapter 81). However, these entities do not fulfill the diagnostic criteria for these well-defined neoplasms, and many appear to have a limited potential for progression. Monoclonal gammopathy of undetermined significance (MGUS), monoclonal B-cell lymphocytosis (MBL), FL in situ, and MCL in situ (see Chapter 86) are examples of such entities.

MGUS is considered an early form of its malignant counterpart MM, with an age-related increased incidence and a small but definitive risk of progression to MM at an annual rate of 1% (see Chapter 90). Recent reports have emphasized the significance of genetic profiling in MGUS for risk stratification, and support the view that progression from MGUS to MM results from the selection and expansion of multiple aberrant clones rather than a linear step-wise acquisition of specific genetic abnormalities.[30,31] The International Myeloma Working Group (IMWG) 2010 guidelines recommend a MGUS risk stratification system, with periodic follow-up with serum electrophoresis for low-risk MGUS patients. However, patients with intermediate-risk and high-risk MGUS are suggested to undergo a baseline BM examination including cytogenetics and skeletal survey, and to be followed with serum electrophoresis studies twice in the first year following diagnosis and annually thereafter.[32]

TABLE 56.9 **World Health Organization Classification of Mature B-Cell Neoplasms**

- Chronic lymphocytic leukemia/small lymphocytic lymphoma
- B-cell prolymphocytic leukemia
- Splenic marginal zone lymphoma
- Hairy cell leukemia
- Splenic lymphoma/leukemia, unclassifiable
- Splenic diffuse red pulp small B-cell lymphoma
- Hairy cell leukemia variant
- Lymphoplasmacytic lymphoma
- Waldenström macroglobulinemia
- Heavy chain diseases
  - μHeavy chain disease
  - γHeavy chain disease
  - αHeavy chain disease
- Plasma cell myeloma
- Solitary plasmacytoma of bone
- Extraosseous plasmacytoma
- Extranodal marginal zone lymphoma of MALT lymphoma
- Nodal marginal zone lymphoma
- Pediatric nodal marginal zone lymphoma
- Follicular lymphoma
- Pediatric follicular lymphoma
- Primary cutaneous follicle center lymphoma
- Mantle cell lymphoma
- DLBCL, NOS
- T-cell/histiocyte–rich large B-cell lymphoma
- Primary DLBCL of the CNS
- Primary cutaneous DLBCL, leg type
- EBV-positive DLBCL of the elderly
- DLBCL associated with chronic inflammation
- Lymphomatoid granulomatosis
- Primary mediastinal (thymic) large B-cell lymphoma
- Intravascular large B-cell lymphoma
- ALK-positive large B-cell lymphoma
- Plasmablastic lymphoma
- Large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease
- Primary effusion lymphoma
- Burkitt lymphoma
- B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma
- B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classic Hodgkin lymphoma

ALK, Anaplastic lymphoma kinase; *CNS*, central nervous system; *DLBCL*, diffuse large B-cell lymphoma; *NOS*, not otherwise specified; *EBV*, Epstein-Barr virus; *HHV8*, human herpesvirus-8; *MALT*, mucosa-associated lymphoid tissue.

MBL has been diagnosed more frequently than previously reported; this is thought to be related to high sensitivity of flow cytometric analyses (see Chapter 76). MBL is defined as the presence of a circulating monoclonal B-cell population below 5×10^9/L, persisting for at least 3 months, in otherwise asymptomatic individuals. Based on clonal B-cell counts and clinical significance, MBL is now subdivided into high-count MBL ($0.5–5.0 \times 10^9$/L) and low-count MBL (less than 0.5×10^9/L).[33] High-count MBL progresses to CLL at an annual rate of 1% to 2%, with the clonal B-cell count at presentation being the greatest risk factor. The presence of palpable lymphadenopathy, organomegaly, and/or infiltration of lymphocytes within the BM (greater than 30% of nucleated cells) fulfills the International Workshop on Chronic Lymphocytic Leukemia (IWCLL) criteria for CLL, even in the absence of clonal lymphocytosis, while the WHO diagnosis of CLL/SLL is based upon the presence of extramedullary involvement. Current recommendations for management of CLL-like MBL include yearly monitoring with therapeutic intervention if clinically indicated. Low-count MBL, based on current data, is unlikely to progress to CLL and does not warrant clinical monitoring. MBL with the understanding that an atypical CLL-like phenotype ($CD5^+$, bright CD20, $CD23^-$) could represent an early leukemic manifestation of MCL, and a thorough staging workup, including fluorescence in situ hybridization (FISH) testing for t(11;14) translocation, is warranted.

FL in situ (see Chapter 82) was initially described[34] as the localization of atypical B cells that have t(14;18) (IgH-B-cell lymphoma [BCL2]) translocation in the germinal centers of reactive-appearing lymph nodes with strong expression of CD10 and BCL-2. The lymph node architecture is generally intact. The majority of cases of FL in situ do not progress to overt lymphoma, thus the alternate term “follicular lymphoma-like B cells of undetermined significance” was recently proposed. In the 2016 WHO classification revision, an alternative term for in situ FL neoplasia is used in order to reflect the uncertain clinical significance of this entity. In situ FL neoplasia must be differentiated from partial involvement by low-grade FL, which usually shows partially effaced nodal architecture with enlarged, crowded follicles, and attenuated mantle zones. This distinction is clinically relevant, as partial involvement by low-grade FL is more likely to be associated with or progress towards overt FL.

Analogous to in situ FL neoplasia, incidental diagnosis of colonization of the mantle cuffs of reactive follicles by cyclin D1-positive B cells has been termed in situ mantle cell neoplasia (MCLIS) (see Chapter 86). In these cases,[35] cyclinD1 highlighted a small subset of B cells within the mantle zone, indicating low-level involvement by lymphoma. This peculiar pattern of cyclinD1 positivity encircling follicles is characteristic of an early or in situ MCL. The in situ variant of MCL is rare and exhibits cyclinD1 positivity within the innermost mantle zone B lymphocytes, and often is reserved for only partial encircling of follicles by MCL cells. In contrast, it has been proposed that full encircling of follicles by cyclin D1-positive cells should be interpreted as an early sign of involvement by MCL. This distinction is important as partial MCL is more likely than MCLIS to progress or coexist with advanced disease. Current management recommendations for MCLIS include whole-body imaging and unilateral BM biopsy (if indicated) to rule out a concomitant overt lymphoma. In the absence of overt disease, no treatment is required and careful follow-up is advised.

Age as a Disease-Defining Feature

The 2008 WHO classification introduced the concept of age as a defining feature in several disease categories.[4] The entities of FL and nodal marginal zone lymphomas (MZLs) that present in the pediatric age group differ from their adult counterparts clinically and biologically (see Chapter 88). The pediatric variant of FL usually presents with localized disease and is of high histologic grade. These lymphomas lack translocations t(14;18) and do not express BCL2 and have good prognosis, although the optimal management remains to be determined. Nodal MZLs in children also appear to have a low risk of progression. Pediatric nodal MZLs are clonal diseases characterized by marginal zone expansion with fragmentation of germinal centers and progressive transformation of germinal center-like changes. Presentation with isolated cervical lymphadenopathy is common in pediatric nodal MZL. The WHO classification also recognizes two rare Epstein-Barr virus (EBV)–associated T-cell diseases: systemic EBV$^{\text{-positive}}$ T-cell lymphoproliferative disease of childhood and hydroa vacciniforme-like lymphoma (see Chapters 87–89). These diseases occur almost entirely in children, primarily in those of Asian origin.

On the opposite spectrum of age-related lymphoproliferative disorders (LPDs) is EBV-positive diffuse large B-cell lymphoma (EBV-positive DLBCL) of the older adult, which is among the newly included provisional entities in the 2008 WHO classification (see Chapter 85). The underlying immunologic deficit in this setting is believed to be immunosenescence, which is defined as the natural decay of the immune system as a consequence of aging. This entity also is defined histologically as malignant B-cell lymphoproliferation in people older than 50 years of age, without any known immunodeficiency or prior lymphoma diagnosis, and is associated with an aggressive clinical behavior pattern. Initial studies reported some overlap with classic Hodgkin lymphoma encountered in the older adult.[26] At present, there is a broader understanding than previously appreciated of the wide clinicopathologic spectrum of age-related EBV-positive LPD. This spectrum includes reactive lymphoid hyperplasia, polymorphic extranodal LPD (76% have EBV mucocutaneous ulcer), polymorphic nodal LPD and EBV-positive DLBCL. Both polymorphic nodal lesions and EBV-positive DLBCL are characterized by poor survival. In this clinicopathologic spectrum, histologic types are highly predictive of outcome, and small-volume disease, particularly in mucosal sites and skin (EBV mucocutaneous ulcer), which are associated with a good prognosis. It is important to keep in mind that although these lymphomas tend to cluster in the very young or the older adult age groups, they are not age restricted. Similarly, EBV-positive DLBCL of the older adult can occur in a younger population.[36]

Aggressive B-Cell Lymphoma, Borderline Entities, and Site-Specific Categories

The 2008 WHO classification identified several subtypes of DLBCLs (see Table 56.9). However, many DLBCLs lack pathologic, clinical, or other defining features that can be used to stratify them. Thus, these are designated DLBCL, NOS in the WHO classification. Stratification according to gene expression profiling as germinal center B-cell (GCB) versus activated B-cell (ABC) types has proven to have prognostic value. The GCB and ABC subtypes are now formally recognized in the 2016 WHO classification, despite the absence of a reproducible routine diagnostic test and the imperfect correlation of immunohistochemical surrogate markers with genomic studies.[37] Digital multiplexed gene expression utilizing formalin-fixed paraffin-embedded–derived RNA at a reasonable cost and turnaround time to classify B-cell lymphomas according to cell of origin is currently being validated as a routine clinical assay.[38,39] The clinical need and utility of this designation is rising, with more studies suggesting that ABC versus GCB lymphomas exhibit differential sensitivity to certain drugs and therapeutic modalities.[37]

In addition to the designation of DLBCL to ABC or GCB subtypes, next-generation sequencing technologies have unveiled the remarkable complexity of DLBCL (see Chapters 81 and 84). More importantly, it has become increasingly clear that these complexities represent lymphoma subtypes which are driven by very different intracellular oncologic signaling pathways, and which can be differentially exploited for therapeutic benefit.[40] Recent studies have demonstrated the high frequency of abnormalities (i.e., noted in greater than 50% of DLBCLs) affecting histone/chromatin-modification enzymes, such as *CREBBP*, *EP300*, *MLL2*, and *EZH2*.[41] The role of therapeutic agents that target the epigenome has been suggested in DLBCL subgroups due to the variability in DNA methylation and its association with outcome.[42,43]

Currently, the role of MYC and BCL2 proteins in DLBCL is an area of active investigation. It has been proposed that individuals with DLBCL be evaluated for concurrent MYC and BCL2 dysregulation at diagnosis, in order to determine the presence of translocation/protein overexpression. Patients with dual overexpression of MYC and BCL2 have a significantly poorer outcome compared with patients who express only one or neither protein. In addition, concurrent MYC and BCL2 translocation, known as *double-hit lymphoma*, indicates a subgroup of patients who are refractory to treatment and have a median survival of approximately 8 months.

The 2008 WHO classification system emphasizes the importance of integrating morphologic, immunophenotypic, and molecular data to make a final diagnosis. This integration has refined our ability to diagnose several entities such as DLBCL and Burkitt lymphoma (BL). The 2008 WHO classification eliminated the variant category of atypical BL, which had been included in the 2001 WHO classification. Thus, a case with the typical BL phenotype (CD20$^+$, CD10$^+$, BCL2$^-$) and genotype (so-called *MYC-simple* or *MYC/IG* in the absence of other major cytogenetic anomalies) may be classified as BL even if there is some variability in the morphology of the neoplastic cells. In addition, the 2008 WHO classification recognizes a group of high-grade B-cell lymphomas that are not readily classified as either BL or DLBCL. This provisional category is termed *B-cell lymphoma, unclassifiable*, with features that are intermediate between DLBCL and BL, including double-hit lymphomas.[4] In the 2016 WHO classification, DLBCL with MYC and BCL2 and/or BCL6 rearrangements are designated high-grade B cell lymphoma (HGBL), with MYC and BCL2 and/or BCL6 rearrangements (see Chapter 85). The category of BCLU was eliminated. Cases that appear blastoid or cases intermediate between DLBCL and BL, but lacking an MYC and BCL2 and/or BCL6 rearrangement, are placed in the category of HGBL, NOS. The 2008 WHO classification recognized another provisional category of B-cell neoplasms with features that are intermediate between DLBCL and CHL. These tumors occur predominantly in young men and appear to be more aggressive than either primary mediastinal large B-cell lymphoma or nodular sclerosis CHL. There are other settings in which the distinction between DLBCL and CHL is challenging. For example, some EBV-associated B-cell lymphomas may exhibit features that closely resemble or mimic CHL. The borderline category should be used sparingly but is appropriate when a distinction between CHL and DLBCL is not possible.[4]

Several aggressive B-cell lymphomas have a distinct immunoprofile or resemble a cell-specific stage of differentiation. These include plasmablastic lymphoma, ALK$^+$ large B-cell lymphoma, human herpesvirus 8-associated malignancies, primary effusion lymphoma, and large B-cell lymphoma associated with multicentric Castleman disease. All of these entities resemble a stage of plasma cell differentiation. Other site-specific categories are primary DLBCL of the central nervous system and primary cutaneous DLBCL, leg type (see Chapters 81 and 84). Both primary central nervous system DLBCL and other DLBCLs arising in privileged sites, such as the testis, may exhibit distinctive biologic feature (see Chapter 84). However, clinical features remain important in clinical management. Interestingly, primary central nervous system DLBCL has a distinctive gene expression signature that may continue to justify it as a separate entity.

Follicular Lymphoma Grading

Revisions to the 2008 WHO classification incorporated changes related to the grading of FL (see Chapter 82). Both grade 1 and grade 2 were combined in one category and designated "low-grade FL." This revision was the result of questioning the clinical significance of separating grade 1 from grade 2 despite minimal differences in long-term outcome.[4] In addition, several studies identified biologic differences between grades 3A and 3B, with the latter being more closely related to DLBCL. The separation of FL grade 3A from 3B is based on the absence of centrocytes in 3B; however, in routine practice this distinction is difficult. Further studies in this area are needed, as they may be helpful in increasing reproducibility of this distinction. According to the 2008 WHO classification, diffuse areas in grade 3 FL should be designated DLBCL, along with FL, as the bottom-line diagnosis.[4]

Mature T-Cell Neoplasms

In the 2016 WHO classification, over 30 defined or provisional entities were named under mature and T-cell neoplasms. However, since then, there have been significant findings impacting diagnosis, prognostication and management of T-cell NHL (see Chapter 89). The genetic basis of ALK-positive, ALK-negative, and breast implant-associated forms of anaplastic large cell lymphoma are further elucidated. In addition, molecular alterations of gastrointestinal and peripheral T-cell (PTC) lymphoma have been characterized in depth. For instance, the proposed molecular GATA3 and TXB21 subtypes of PTCL, NOS demonstrated distinct genetic aberrations.

Peripheral T-cell lymphomas (PTCLs) comprise 10% to 15% of all NHLs and encompass numerous entities (Table 56.10) that are characterized by a poor prognosis, with the exception of histologic subtype "ALK-positive anaplastic large-cell lymphoma." Most PTCLs lack distinct genetic or biologic features, and the mechanisms underlying the pathogenesis of these lymphomas are not yet fully understood (see Chapter 89). However, development of genomic high-throughput profiling techniques now allows us to extensively identify the molecular abnormalities present in these entities.

Anaplastic Large Cell Lymphoma

ALCLs share a common pathologic feature that include expression of CD30 and the presence of characteristic cells, designated "hallmark cells" (see Chapter 89). ALCL classifications depend on clinical presentation (systemic or localized) and the expression of anaplastic lymphoma kinase (ALK). Systemic ALCLs include ALK-positive ALCL and ALK-negative ALCL, while the localized form includes primary cutaneous ALCL. Although ALK-positive ALCL are associated with a better outcome compared to ALK-negative ALCL, recent reports highlighted genetic heterogeneity where cases with *RNF213-ALK, ATIC-ALK*, and *TPM3-ALK* fusions have been shown to have copy number gains of the rearranged ALK gene, whereas only one copy of NPM1-ALK was present. It was also shown that pediatric ALK-positive ALCLs with NPM1-ALK fusions could be distinguished into two groups based on the expression levels of the NPM1-ALK fusion transcript (see Chapter 89).[44]

TABLE 56.10 **World Health Organization Classification of Mature T-Cell and Natural Killer Cell Neoplasms**

- T-cell prolymphocytic leukemia
- T-cell large granular lymphocytic leukemia
- Chronic lymphoproliferative disorder of NK cells
- Aggressive NK-cell leukemia
- Systemic EBV-positive T-cell lymphoproliferative disease of childhood
- Hydroa vacciniforme-like lymphoma
- Adult T-cell leukemia/lymphoma
- Extranodal NK/T-cell lymphoma, nasal type
- Enteropathy-associated T-cell lymphoma
- Hepatosplenic T-cell lymphoma
- Subcutaneous panniculitis-like T-cell lymphoma
- Mycosis fungoides
- Sézary syndrome
- Primary cutaneous $CD30^-$ T-cell lymphoproliferative disorders
- Lymphomatoid papulosis
- Primary cutaneous anaplastic large-cell lymphoma
- Primary cutaneous T-cell lymphoma
- Primary cutaneous aggressive epidermotropic $CD8^+$ cytotoxic T-cell lymphoma
- Primary cutaneous small/medium $CD4^+$ T-cell lymphoma
- Peripheral T-cell lymphoma, NOS
- Angioimmunoblastic T-cell lymphoma
- Anaplastic large-cell lymphoma, ALK-positive
- Anaplastic large-cell lymphoma, ALK-negative

ALK, Anaplastic lymphoma kinase; *EBV*, Epstein-Barr virus; *NK*, natural killer; *NOS*, not otherwise specified.

ALK-negative ALCL is recognized as definite entities in the 2016 WHO revisions. Although noted in only 5% of the cases, recurrent *FRK* rearrangements were recently reported exclusively in ALK-negative ALCL. Chromosomal rearrangements of the DUSP22/IRF4 locus on chromosome 6p25.3 are noted in 30% of ALK-negative ALCL and are associated with favorable outcome similar to that of ALK-positive ALCL. DUSP22-rearrangement is not specific to ALK-negative ALCL cases and can be seen in a subset of primary cutaneous ALCLs and occasional cases of lymphomatoid papulosis.[44] An additional group of ALK-negative ALCL shows rearrangement of *P63* gene; a finding consistently associated with aggressive clinical course. IHC for P63 is a sensitive method to screen for *P63* fusion; however, it is worth mentioning that it is not specific as ALK IHC, and a positive P63 IHC result needs to be confirmed by another method, such as FISH.

T-Cell Lymphomas of T-Follicular Helper Cell Origin

WHO introduced nodal lymphomas of T-follicular helper (TFH) as a new category in the 2017 classification based on common phenotype of the postulated TFH cell of origin, and, in some cases, underlying genetic features among a subset of tumors (see Chapter 89). This category encompasses angioimmunoblastic T-cell lymphoma (AITL) as the classic form and the new provisional entities, including nodal PTCL with TFH phenotype and follicular T-cell lymphoma. AITL and approximately 20% of PTCL-NOS[4] that show TFH phenotyping share a spectrum of genetic abnormalities, such as *TET2* and *DNMT3A*, as well as mutations in the motility and adhesion gene *RHOA*.[45]

Peripheral T-Cell Lymphoma, Not Otherwise Specified

The diagnosis of many T-cell lymphoma cases is challenging even for expert hematopathologists, and about one-third of the cases cannot be further classified and consequently are relegated to a "waste basket" category PTCL-NOS (see Chapter 88). Despite its intentional heterogeneity, gene expression profiling identified two subgroups based on differential expression of the transcription factor gene *TBX21* and GATA3 along with their Target genes. The GATA3 subgroup of PTCL-NOS has an inferior prognosis.

Cutaneous T-Cell Lymphoma

Mycosis fungoides and Sézary syndrome are the most common cutaneous T-cell lymphomas, and the diagnostic criteria for both entities did not change significantly in the 2016 WHO classification; however, their association with a variety of genetic abnormalities, including mutations and loss of tumor suppressor genes, including *TP53, RB1, PTEN, CDKN2A*, and *CDKN1B*, have been reported (see Chapter 89). Three variants of primary cutaneous PTCL were introduced in the 2008 WHO classification: primary cutaneous gamma-delta T-cell lymphoma, primary cutaneous $CD4^+$ small/medium T-cell lymphoma as a provisional entity, and primary cutaneous aggressive epidermotropic $CD8^+$ cytotoxic T-cell lymphoma. Cutaneous gamma-delta T-cell lymphomas have a diverse histologic and clinical spectrum and may display a panniculitis-like pattern. However, this disease has a much poorer prognosis than subcutaneous panniculitis-like T-cell lymphoma, which is defined as a lymphoma exclusively of alpha-beta phenotype in the 2008 WHO classification. Primary cutaneous small/medium CD4^{+} T-cell lymphoma is another lymphoma with T-cell origin that presents commonly as an isolated lesion in the head and neck region.[46] In the 2016 WHO classification, a change in the terminology of this entity is proposed—to primary cutaneous small/medium CD4^{+} T-cell lymphoproliferative disease instead of lymphoma.

T-Cell Lymphoma of the Gastrointestinal Tract

The WHO classification required more stringent criteria to establish a diagnosis of enteropathy-associated T-cell lymphoma (EATL), previously designated type I EATL—a highly aggressive disease that is associated with celiac disease with most cases showing TCRαβ phenotype (see Chapter 89). Specifically, in order to make the diagnosis of EATL, evidence of celiac disease was required either at the genetic level, with the appropriate HLA-DQ2 or DQ8 phenotype, or intraepithelial T cells characterized by varying degrees of cellular pleomorphism histologically. The monomorphic epitheliotropic intestinal T-cell lymphoma, formerly known as *type II EATL*, is not associated with celiac disease and exhibits some distinctive immunophenotypic and genotypic features. Most cases show a T-cell receptor γδ (TCRγδ) phenotype with CD8^{+} and CD56^{+} expression.[4]

Epstein-Barr Virus–Associated Neoplasms of T and Natural Killer Cells

Extranodal NK/T-cell lymphoma, nasal type is the most common of these types of disorders with a usual upper aerodigestive tract presentation and high prevalence in Asia and some areas of Central and South America (see Chapter 86). In the pediatric population, EBV-positive T-cell and NK-cell LPDs can be categorized into two major groups in the 2016 WHO classification: systemic EBV-positive T-cell lymphoma of childhood and chronic active EBV infection. Systemic EBV-positive T-cell lymphoma of childhood is a clonal proliferation of EBV-infected cytotoxic T cells with a characteristic fulminant and aggressive clinical course usually associated with hemophagocytic syndrome, multiorgan failure, and sepsis. The disease most often occurs in the context of chronic active EBV infection, though it can occur shortly after primary acute EBV infection (see Chapter 88). Chronic active EBV infection is a systemic EBV-positive polyclonal, oligoclonal, or monoclonal LPD that is characterized by persistent infectious mononucleosis-like symptoms. Patients may show a predisposition to T-/NK-cell lymphoma or aggressive NK-cell leukemia.

Precursor Lymphoid Neoplasms

The lymphoblastic neoplasms are derived from precursor cells or blasts, most of which are precursor B- and T-cell neoplasms that present as leukemia. However, the designation of lymphoblastic lymphoma is used when the neoplasm is confined to a mass lesion with or without minimal blood or BM involvement. The WHO classification retains the convention that precursor neoplasms are designated *leukemia/lymphoma*. When distinction between leukemia and lymphoma is required for clinical protocol eligibility at presentation with a patient mass lesion and increased blasts in the BM, a threshold of 25% blasts is used as the defining feature of leukemia.[4]

The 2008 classification recognizes genetic features in the definition of some forms of B-lymphoblastic leukemia (B-ALL) (see Chapters 66–68). One such example is Philadelphia chromosome-positive (Ph^{+}) B-ALL, associated with *BCR-ABL1*, which is more common in adults than in children and is considered very high risk, regardless of other factors. Deletions and other alterations in the *IKZF1* (Ikaros) gene are adverse prognostic indicators in both Ph= and Ph^{-} patients with B-ALL. Another variant with distinctive clinical features at presentation is B-ALL/lymphoma with t(5;15)(q31;q32) (*IL3-IGH*). These patients present with a marked increase in eosinophils, which may mask a relatively small number of blasts in the BM—a diagnostic pitfall worthy of note. The ongoing and increasing complexity highlights the importance of clinicopathologic correlation and the value of ancillary studies in the classification and workup of patients with B-ALL.[47]

T-lymphoblastic leukemia (T-ALL) is also associated with considerable genetic variability. Routine histopathology, flow cytometry immunophenotyping, conventional cytogenetic analysis, FISH, and/or clonality testing are usually adequate to establish the diagnosis. The most commonly involved genes include the HOX transcription factors. However, genotyping is recommended in the workup of the disease, although at this time it is not used as a criterion to define distinct entities.

Hodgkin Lymphoma

Hodgkin lymphomas are lymphoid neoplasms characterized histologically by large neoplastic cells that constitute only a small proportion of the tumor tissue in a background of nonneoplastic cells that account for the majority of the cellularity (see Chapters 79 and 80). Hodgkin lymphomas include two main types: nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) and CHL.

CHL is divided into four histological subtypes: lymphocyte rich, nodular sclerosis, mixed cellularity, and lymphocyte depleted. NF- κB is constitutively activated in the neoplastic cells of CHL (Hodgkin/Reed–Sternberg cells) and contributes to their proliferation and survival. In contrast, six distinct histologic patterns of NLPHL have been described: classic nodular B-cell rich (A), serpiginous nodular (B), nodular with prominent extranodular tumor cells (C), T-cell-rich nodular (D), diffuse T-cell-rich (E), and diffuse B-cell-rich (F). Pattern C through F are associated with advanced disease and higher relapse rate than patterns A and B.

NLPHL and T-cell/histiocyte-rich large B-cell lymphoma (THRLBCL) share a number of features, and it has been suggested that NLPHL with a diffuse pattern overlaps with THRLBCL. In the 2016 WHO classification, NLPHL cases that progress to a diffuse T-cell-rich pattern are designated 'THRLBCL-like transformation of NLPHL.' Both NLPHL and THRLBCL have recently been shown to share recurrent mutations, including those involving JUNB, DUSP2, and SGK1, further supporting the close relationship between these entities.

DENDRITIC CELL AND HISTIOCYTIC NEOPLASMS

Dendritic and histiocytic neoplasms are hematologic malignancies that have distinct yet variable clinical presentations, and together they make up less than 1% of the neoplastic process involving the lymph nodes or soft tissues .[4] However, the true prevalence of these disorders remains uncertain because many have been recognized only recently. Several entities were recognized in the 2008 WHO classification of histiocytic and dendritic cell neoplasms (Table 56.11). Traditionally, these tumors are placed into two categories based on either BM precursors or mesenchymal cells being the cell of origin. Histiocytic sarcoma (HS), Langerhans cell histiocytosis (LCH), and interdigitating dendritic cell sarcoma (IDCS) are derived from BM precursors, while follicular dendritic cell sarcoma (FDCS), indeterminate dendritic cell sarcoma, fibroblastic reticular cell tumors, and disseminated juvenile xanthogranuloma (JXG) are derived from stromal-derived dendritic cells or are mesenchymal in origin. Erdheim-Chester disease (ECD) was added to the 2016 WHO classification to emphasize the need to distinguish it from other members of the juvenile JXG family based on clinical features. Irrespective of myeloid or mesenchymal origin, these tumors may occur subsequent to or concurrent with mature or precursor B- or T-cell neoplasms, with which they may share immunoglobulin or TCR gene rearrangements and chromosomal aberrations,

TABLE 56.11 World Health Organization Classification of Histiocytic and Dendritic Cell Neoplasms

- Histiocytic sarcoma
- Langerhans cell histiocytosis
- Langerhans cell sarcoma
- Interdigitating dendritic cell sarcoma
- Follicular dendritic cell sarcoma
- Fibroblastic reticular cell tumor
- Intermediate dendritic cell tumor
- Disseminated juvenile xanthogranuloma

a phenomenon known as trans-differentiation. Concomitant myeloid neoplasms was recently reported in ~10% of adults with non-Langerhans cell histiocytoses including ECD. Significant findings relating to HDC neoplasms since the 2016 WHO classification in this group includes the identification of recurrent mutations activating Ras-MAPK and PI3K pathways and frequent mutations of CDKN2A in HS, and the in-frame BRAF deletions in 25% of LCH without BRAF or MAP2K1 mutations.[44]

Excisional biopsies are often required to render the diagnosis of these disorders. Consultation with an experienced hematopathologist is often needed, as morphologic review and an adequate battery of immunohistochemical stains are the most important tools in making an accurate diagnosis of these entities and in differentiating them from other, often-mistaken categories, most commonly NHLs. The rarity of these disorders is the major factor that makes this group of diseases difficult to accurately diagnose and challenging to treat. Advances in IHC have contributed to an enhanced understanding of the biology of dendritic and histiocytic neoplasms and have improved our ability to classify and diagnose these disorders. For example, in contrast to LCH, IDCS are usually positive for S100 but negative for CD1a and langerin (CD207). Unlike FDCS, IDCS do not express follicular dendritic cell markers such as CD21 or CD35.[4]

These entities can involve various organs, although most occur in the lymph nodes and skin, with a unifocal or solitary presentation, and are associated with a good prognosis with surgical resection. On the other hand, patients with disseminated disease have shown a poor outcome, although data on treatment options are limited. Nonetheless, chemotherapy and referral to a tertiary-care center should be considered for patients with these diagnoses.

REFERENCES

1. Jaffe ES, Harris NL, Stein H. *Pathology and Genetics of Tumours of the Haematopoietic and Lymphoid Tissue*. Lyon: IARC Press; 2001.
2. Harris NL, Jaffe ES, Diebold J, et al. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the clinical advisory committee meeting-airlie house, Virginia, November 1997. *J Clin Oncol*. 1999;17:3835–3849.
3. Harris NL, Jaffe ES, Stein H, et al. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. *Blood*. 1994;84:1361–1392.
4. Swerdlow SH, Campo E, Harris NL. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon: IARC Press. 2008.
5. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of the myelodysplastic syndromes. *Br J Haematol*. 1982;51:189–199.
6. Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*. 2009;114:937–951.
7. Maciejewski JP, Haferlach T. Introduction: molecular pathogenesis of hematologic malignancies. *Semin Oncol*. 2012;39:9–12.
8. Swerdlow S, Campo E, Harris N, et al. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Revised 4th ed. Lyon: IARC Press; 2017.
9. Anastasi J. The myeloproliferative and overlap, myeloproliferative/myelodysplastic neoplasms. In: Hsi E, ed. *The Myeloproliferative and Overlap, Myeloproliferative/Myelodysplastic Neoplasms*. St Louis: Elsevier; 2012:479.
10. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med*. 2001;344:1031–1037.
11. Tefferi A, Thiele J, Vannucchi AM, et al. An overview on CALR and CSF3R mutations and a proposal for revision of WHO diagnostic criteria for myeloproliferative neoplasms. *Leukemia*. 2014;28:1407–1413.
12. Klampfl T, Gisslinger H, Harutyunyan AS, et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med*. 2013;369:2379–2390.
13. Nangalia J, Massie CE, Baxter EJ, et al. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *N Engl J Med*. 2013;369:2391–2405.
14. Elliott MA, Tefferi A. Chronic neutrophilic leukemia 2014: update on diagnosis, molecular genetics, and management. *Am J Hematol*. 2014;89:651–658.
15. Chiu A, Orazi A. Mastocytosis and related disorders. *Semin Diagn Pathol*. 2012;29:19–30.
16. Jawhar M, Schwaab J, Schnittger S, et al. Additional mutations in SRSF2, ASXL1 and/or RUNX1 identify a high-risk group of patients with KIT D816V(+) advanced systemic mastocytosis. *Leukemia*. 2016;30:136–143.
17. Gotlib J. World Health Organization-defined eosinophilic disorders: 2011 update on diagnosis, risk stratification, and management. *Am J Hematol*. 2011;86:677–688.
18. Stone RM, Mandrekar S, Sanford BL, et al. The multi-kinase inhibitor midostaurin (M) prolongs survival compared with placebo (P) in combination with Daunorubicin (D)/Cytarabine (C) induction (ind), high-dose C consolidation (consol), and as maintenance (maint) therapy in newly diagnosed acute Myeloid Leukemia (AML) patients (pts) age 18–60 with FLT3 mutations (muts): an international prospective randomized (rand) P-controlled double-blind Trial (CALGB 10603/RATIFY [Alliance]). *Blood*. 2015;126(23):6.
19. Pratz K, Levis M. Incorporating FLT3 inhibitors into acute myeloid leukemia treatment regimens. *Leuk Lymphoma*. 2008;49:852–863.
20. West AH, Godley LA, Churpek JE. Familial myelodysplastic syndrome/acute leukemia syndromes: a review and utility for translational investigations. *Ann NY Acad Sci*. 2014;1310:111–118.
21. Nacheva EP, Grace CD, Brazma D, et al. Does BCR/ABL1 positive acute myeloid leukaemia exist? *Br J Haematol*. 2013;161:541–550.
22. Konoplev S, Yin CC, Kornblau SM, et al. Molecular characterization of de novo Philadelphia chromosome-positive acute myeloid leukemia. *Leuk Lymphoma*. 2013;54:138–144.
23. Fasan A, Haferlach C, Alpermann T, et al. The role of different genetic subtypes of CEBPA mutated AML. *Leukemia*. 2014;28:794–803.
24. Arber DA, Hasserjian RP. Reclassifying myelodysplastic syndromes: what's where in the new WHO and why. *Hematol Am Soc Hematol Educ Program*. 2015;2015:294–298.
25. Sangiorgio VFI, Orazi A, Arber DA. Myelodysplastic/myeloproliferative neoplasms: are morphology and immunophenotyping still relevant? *Best Pract Res Clin Haematol*. 2020;33(2):101139.
26. Xie Y, Pittaluga S, Jaffe ES. The histological classification of diffuse large B-cell lymphomas. *Semin Hematol*. 2015;52:57–66.
27. Tirado CA, Chen W, Garcia R, et al. Genomic profiling using array comparative genomic hybridization define distinct subtypes of diffuse large B-cell lymphoma: a review of the literature. *J Hematol Oncol*. 2012;5:54.
28. Henopp T, Quintanilla-Martinez L, Fend F, et al. Prevalence of follicular lymphoma in situ in consecutively analysed reactive lymph nodes. *Histopathology*. 2011;59:139–142.
29. Fend F, Cabecadas J, Gaulard P, et al. Early lesions in lymphoid neoplasia: conclusions based on the Workshop of the XV. Meeting of the European Association of Hematopathology and the Society of Hematopathology, in Uppsala, Sweden. *J Hematop*. 2012;5:169–199.
30. Dhodapkar MV, Sexton R, Waheed S, et al. Clinical, genomic, and imaging predictors of myeloma progression from asymptomatic monoclonal gammopathies (SWOG S0120). *Blood*. 2014;123:78–85.
31. Morgan GJ, Walker BA, Davies FE. The genetic architecture of multiple myeloma. *Nat Rev Cancer*. 2012;12:335–348.
32. Kyle RA, Durie BG, Rajkumar SV, et al. Monoclonal gammopathy of undetermined significance (MGUS) and smoldering (asymptomatic) multiple myeloma: IMWG consensus perspectives risk factors for progression and guidelines for monitoring and management. *Leukemia*. 2010;24:1121–1127.
33. Shim YK, Rachel JM, Ghia P, et al. Monoclonal B-cell lymphocytosis in healthy blood donors: an unexpectedly common finding. *Blood*. 2014;123:1319–1326.
34. Cong P, Raffeld M, Teruya-Feldstein J, et al. In situ localization of follicular lymphoma: description and analysis by laser capture microdissection. *Blood*. 2002;99:3376–3382.

35. Carvajal-Cuenca A, Sua LF, Silva NM, et al. In situ mantle cell lymphoma: clinical implications of an incidental finding with indolent clinical behavior. *Haematologica*. 2012;97:270–278.
36. Beltran BE, Morales D, Quinones P, et al. EBV-positive diffuse large b-cell lymphoma in young immunocompetent individuals. *Clin Lymphoma Myeloma Leuk*. 2011;11:512–516.
37. Sehn LH, Gascoyne RD. Diffuse large B-cell lymphoma: optimizing outcome in the context of clinical and biologic heterogeneity. *Blood*. 2015;125:22–32.
38. Scott DW, Wright GW, Williams PM, et al. Determining cell-of-origin subtypes of diffuse large B-cell lymphoma using gene expression in formalin-fixed paraffin-embedded tissue. *Blood*. 2014;123:1214–1217.
39. Masque-Soler N, Szczepanowski M, Kohler CW, et al. Molecular classification of mature aggressive B-cell lymphoma using digital multiplexed gene expression on formalin-fixed paraffin-embedded biopsy specimens. *Blood*. 2013;122:1985–1986.
40. Zhang J, Grubor V, Love CL, et al. Genetic heterogeneity of diffuse large B-cell lymphoma. *Proc Natl Acad Sci USA*. 2013;110:1398–1403.
41. Pasqualucci L, Dominguez-Sola D, Chiarenza A, et al. Inactivating mutations of acetyltransferase genes in B-cell lymphoma. *Nature*. 2011;471:189–195.
42. Chambwe N, Kormaksson M, Geng H, et al. Variability in DNA methylation defines novel epigenetic subgroups of DLBCL associated with different clinical outcomes. *Blood*. 2014;123:1699–1708.
43. Cerchietti L, Leonard JP. Targeting the epigenome and other new strategies in diffuse large B-cell lymphoma: beyond R-CHOP. *Hematol Am Soc Hematol Educ Program*. 2013;2013:591–595.
44. Satou A, Bennani NN, Feldman AL. Update on the classification of T-cell lymphomas, Hodgkin lymphomas, and histiocytic/dendritic cell neoplasms. *Expert Rev Hematol*. 2019;12(10):833–843.
45. Ahearne MJ, Allchin RL, Fox CP, et al. Follicular helper T-cells: expanding roles in T-cell lymphoma and targets for treatment. *Br J Haematol*. 2014;166:326–335.
46. Rodriguez Pinilla SM, Roncador G, Rodriguez-Peralto JL, et al. Primary cutaneous CD4+ small/medium-sized pleomorphic T-cell lymphoma expresses follicular T-cell markers. *Am J Surg Pathol*. 2009;33:81–90.
47. Loghavi S, Kutok JL, Jorgensen JL. B-acute lymphoblastic leukemia/lymphoblastic lymphoma. *Am J Clin Pathol*. 2015;144:393–410.

CHAPTER 57

CONVENTIONAL AND MOLECULAR CYTOGENOMIC BASIS OF HEMATOLOGIC MALIGNANCIES

Vesna Najfeld

Dedicated to the loving memory of Eta Najfeld, MD, a Holocaust survivor and an amazing mother.

Over the past 66 years the cytogenetic analysis of hematologic malignancies has been an area of prolific growth. Chromosome studies and karyotype analysis provide information of both biologic and clinical significance. Refinements in cell culture methods and the application of chromosome banding techniques have advanced our understanding of disease-specific abnormalities, and molecular cytogenetic and cytogenomic methods now have made possible the identification of genes involved at translocation breakpoints in specific chromosomal rearrangements. These advances in molecular cytogenetic methods permit mapping of structural rearrangements within a single gene and have fundamentally contributed to our knowledge of the biology of many hematologic malignancies. This evolution in our understanding of cancer genetics has resulted in a distinct terminology (Table 57.1). Application of conventional and molecular cytogenetic methods has identified over 100 fusion genes involving over 500 different genes and approximately 1000 recurrent balanced translocations in human cancers. Relevance of these methods has played a pivotal role in the diagnosis, treatment, and prognostications of the hematologic malignancies. This chapter discusses specific cytogenetic events and delineates molecular phenotypes that are important to understand the molecular pathogenesis of hematologic malignancies and provides several genetic testing algorithms. The remarkable hypothesis put forward by Boveri at the turn of the 20th century—namely, that an abnormal chromosome pattern is intimately associated with the malignant phenotype of a tumor cell—has proven correct. Knowledge of the molecular cytogenetic genotype of hematologic malignancies has led to innovative and targeted treatments. The first example of such gene-targeted therapy has already been successfully applied to chronic myeloid leukemia (CML) (Chapter 69).

METHODS

Fig. 57.1 shows the current cytogenomic methods and their resolution in detecting clonal chromosomal, gene, or other genomic rearrangements and abnormalities in hematologic malignancies.

Cytogenetic Analysis

Cells arrested in metaphase are obtained by exposing marrow cells sequentially to mitotic inhibitors, hypotonic potassium chloride, and fixative. Chromosomes obtained from leukemic marrow are then subjected to trypsin-Giemsa banding (Fig. 57.2). The criteria used to define clonal abnormalities are listed in Table 57.1 and described in the International System for Human Cytogenetic Nomenclature, 2021. Table 57.2 describes the optimal logarithm and evolving new testing strategies. These technologies are currently either stand alone or are used in combination.

Fluorescence In Situ Hybridization Methods

Fluorescence in situ hybridization (FISH) is a molecular method that allows detection of the number, size, and location of DNA and RNA segments within individual cells in a tissue sample. It is based on the ability of single-stranded DNA to anneal to complementary DNA. In hematologic disorders, the target DNA is the marrow or peripheral blood DNA present in interphase cells or the DNA of metaphase chromosomes that is fixed on a microscope slide.

Fig. 57.3 shows four types of FISH probes that are used alone or in combination to determine both numeric and structural rearrangements: (a) centromere enumeration probes, (b) whole chromosome painting probes, and (c) subtelomeric probes, while Fig. 57.4 shows the four FISH probe strategies used in probe design for detection of chromosomal translocations in hematologic malignancies. The first application of FISH technology for detection of chromosomal translocations in hematologic malignancies occurred when the *BCR-ABL1* hybrid gene was identified using two-color FISH in interphase cells as well as in metaphase marrow-derived CML cells. In the standard strategy for interphase evaluation of chromosomal translocation, a DNA probe comprising sequences mapped proximal to the breakpoint in one of the chromosomes involved in reciprocal translocations is combined with a differentially labeled DNA probe that includes sequences mapped distal to the breakpoint in the other chromosome. Nuclei positive for the translocation display one dual-color fusion signal, representing one of the derivative chromosomes generated by the translocation, and two single-color signals, one for each of the normal alleles. This standard FISH strategy has been used for detection of translocations in hematologic disorders at diagnosis.

One of the most significant advances in diagnostic leukemia cytogenetics has been the application of interphase FISH. Interphase cytogenetics is the term used to describe detection of chromosomal abnormalities in non-dividing, interphase nuclei (Fig. 57.5). Five aspects of interphase FISH are particularly useful: (1) Interphase cytogenetics allows screening of a large number of cells. This permits investigation of hematologic malignancies with a low mitotic yield, such as chronic lymphocytic leukemia (CLL) and multiple myeloma (MM). (2) Interphase FISH permits detection of chromosomal rearrangements in peripheral blood samples, thus obviating the need for marrow aspiration. For instance, in CML, which rarely yields a large number of dividing cells in peripheral blood, conventional cytogenetics usually is uninformative. However, detection of *BCR-ABL1*, the molecular equivalent of the Philadelphia chromosome (Ph), in peripheral blood using interphase FISH provides reliable, fast, quantitative results. (3) Interphase FISH offers a quantitative assay for monitoring disease progression or detection of minimal residual disease (MRD) following chemotherapy or hematopoietic stem cell transplantation (HSCT). (4) Use of specific probe sets allows detection of specific disease-associated abnormalities such as t(8;21), which denotes the M2 subtype of acute myeloid leukemia (AML), or t(15;17), which is associated with acute promyelocytic leukemia (APL), within 4 hours, allowing for timely and appropriate therapy. (5) FISH nomenclature is described in the International System for Human Cytogenetic Nomenclature.

Multicolor karyotyping permits examination of the entire genome in a single analysis (Figs. 57.1 and 57.6). In 1996 it became possible

TABLE 57.1 Glossary of Cytogenetic, Fluorescence in Situ Hybridization and Genomic Terminology

Aneuploidy	Abnormal chromosome number, either gain or loss.
Array CGH	A higher-level CGH technology that provides gene copy information.
Balanced translocation	Exchange of chromosomal material that creates no extra or missing DNA.
Banding	Set of dark and pale segments along the length of chromosomes, resulting from treatment with enzyme before staining. Each chromosome identified by its unique set of bands.
Breakpoint	Specific site on a chromosome containing a break in the DNA that is involved in chromosomal structural rearrangement, such as translocation or deletion.
Centromere	Constriction on the chromosome at the spindle site attachment. During cell mitosis two copies of the DNA in each chromosome are separated by shortening of the spindle fibers attached to opposite sides of the dividing cell.
Centromere enumeration probe (CEP)	Highly repetitive α (or β) satellite DNA, located in the heterochromatin of the centromeric area of chromosomes. CEP targets repetitive α (or β) sequences produces bright compact fluorescent signals.
Comparative genomic hybridization (CGH)	Molecular cytogenomic technique that provides a copy-number karyotype at the chromosome and band level.
Chromosomal rearrangement	Aberration in which chromosomes are broken and rejoined.
Clonal abnormality	In cytogenetic analysis, two cells showing the same additional or structural abnormality or three cells with loss of the same chromosome. In FISH analysis, any abnormality present after the probe has been validated and normal reference range established, above the normal reference range.
Chromothripsis	A catastrophic DNA damage occurring during a single mitotic division the application of molecular biology to determine genomic copy number.
Deletion	Segment of chromosome that is missing (terminal) or segment of chromosome missing between two breakpoints (interstitial).
DNA sequence	Order of nucleotides in a DNA segment, usually displayed from the 5′-triphosphate (5′ end) to the 3′-hydroxyl (3′ end) nucleotides.
Driver mutation	This mutation affects the biology of cell.
Enhancer	DNA sequence that increases the rate of transcription.
Exon	Portion of gene that encodes protein.
Fiber FISH	Application of FISH technology to extended DNA or free DNA fibers.
FISH	Fluorescence in situ hybridization, a method for detection of the number and location of DNA sequences (genes) in tissue section or cell population.
Fluorochrome	Fluorescence molecule that, when conjugated to a molecule, binds to a hapten to facilitate detection of the chromosomal probe. By definition, a fluorochrome is a molecule that will become excited by the light of one wavelength.
Gene construct	Recombinant DNA containing a gene of interest surrounded by sequences engineered to promote a measure of its expression.
Gene map	Order of genes within a chromosome or entire genome.
Genotype	Genetic constitution, usually with reference to particular alleles at a locus.
Haploid	Half of a normal complement (i.e., 23 chromosomes).
Haploinsufficiency	Deletion or inactivation of one allele producing disease caused by inadequate activity of the remaining allele.
Hybrid gene	Fusion of two different genes as a result of a structural chromosomal rearrangement that functions as one transcriptional unit.
Hybridization	Method for rejoining (reannealing) complementary DNA or RNA strands.
Hyperdiploid	Additional chromosomes (e.g., 47 or 48 chromosomes).
Hypermetaphase FISH	Application of FISH to accumulated large number of metaphase cells.
Hypodiploid	Loss of chromosomes (e.g., 45 or 44 chromosomes).
I-FISH	Interphase fluorescence in situ hybridization, application of FISH to nondividing (resting) cells.
Interphase	Stage of mitosis in which the cell is not dividing.
Inversion	Structural chromosomal rearrangement as a result of two breaks occurring in the same chromosome. Paracentric inversion refers to both breaks occurring on the same side of the centromere.Pericentric inversion refers to breaks occurring on the opposite side of the centromere.
Isochromosome	Structural chromosomal rearrangement that consists of doubling of one of the two chromosome arms (connected by the centromere) and loss of the other arm.
Karyotype	Arrangement of metaphase chromosomes from a particular cell according to size and banding so that the largest chromosome is placed first and the smallest one last (see Fig. 56.2).
kb (kilobase)	Unit of DNA/RNA length = 1000 base pairs of DNA.

TABLE 57.1 **Glossary of Cytogenetic, Fluorescence in Situ Hybridization and Genomic Terminology—cont'd**

Kataegis	Kataegis is a recently discovered phenomenon in which multiple mutations cluster in a few hotspots in a genome. This region is often colocalized with regions of somatic genome rearrangements.
Locus	Unique location of a gene on a chromosome.
Locus (sequence)-specific probe (LSI)	Probe targeted to unique sequence region of the chromosome. Useful for localization of genes on normal chromosomes (gene mapping) and for detection of gene amplification, deletion, inversion, or translocation.
Marker chromosome	Chromosome whose morphology cannot be identified using banding method. Marker chromosomes are frequent in hematologic neoplasms.
M-FISH	Multicolor FISH karyotyping, which allows identification of 24 different human chromosomes (22 autosomes, and the X and Y chromosomes) (see text for details).
NGS	Next generation sequencing.
Nonsilent mutations	Mutations that alter the protein sequences.
Oncogene	Locus that is activated in association with tumor growth. One abnormal allele is sufficient to cause tumor formation or cancer.
SNP	Single nucleotide polymorphism.
Passenger mutation	Is usually a subclone and does not affect the biology of cell.
PCR	Polymerase chain reaction, by which individual gene segments are amplified through sequential cycles of polymerization, heat denaturation, and reannealing.
Pseudodiploid	Diploid number of chromosomes (46) accompanied by structural rearrangement.
Recurrent abnormality	Structural or numerical abnormality observed in multiple patients with the same or similar disease. Recurrent chromosome abnormalities in hematologic neoplasms have prognostic significance.
Telomeric probe	Used in FISH to detect repeated DNA sequences present at the end of the chromosome, which is called the telomere. Telomeric DNA contains 10–15 kb of TTAGGG repeats.
Translocation	Structural chromosome abnormality resulting from a break in at least two chromosomes with an exchange of material. In reciprocal or balanced translocation, no loss of chromosomal material occurs. In unbalanced translocation, loss of chromosomal DNA occurs.
Tumor suppressor gene	Locus that prevents tumor growth when at least one allele is functional. Loss of both alleles, first through constitutional and then through somatic mutation, is associated with tumor formation or cancer.
Whole chromosome painting probe (WCP)	Spans the entire length of chromosomal DNA sequences and, as the name implies, targets the entire length of DNA sequences.
Nomenclature	
p	= Short arm
q	= Long arm
+	When placed before the chromosome, denotes a gain of a whole chromosome (e.g., +8)
−	When placed before the chromosome, indicates a loss of a whole chromosome (e.g., −7); in rare situations, when placed after the chromosome, as in 5q−, indicates loss of a part of the long arms of chromosome 5
t	translocation
del	deletion
der	derivative
inv	inversion
i	isochromosome
mar	marker chromosome
con	connected
nuc ish	nuclear in situ hybridization
nuc ish 21q22 (D21S65X2)	two copies of D21S65 DNA segment on chromosome 21
nuc ish 9q34 (*ABL1* x2), 22q11.2 (*BCRx2*) (*ABL1* con *BCRx1*)	two *ABL* and two *BCR* loci, but one of each locus is juxtaposed on one chromosome as a result of t(9;22)

FISH, Fluorescence in situ hybridization.

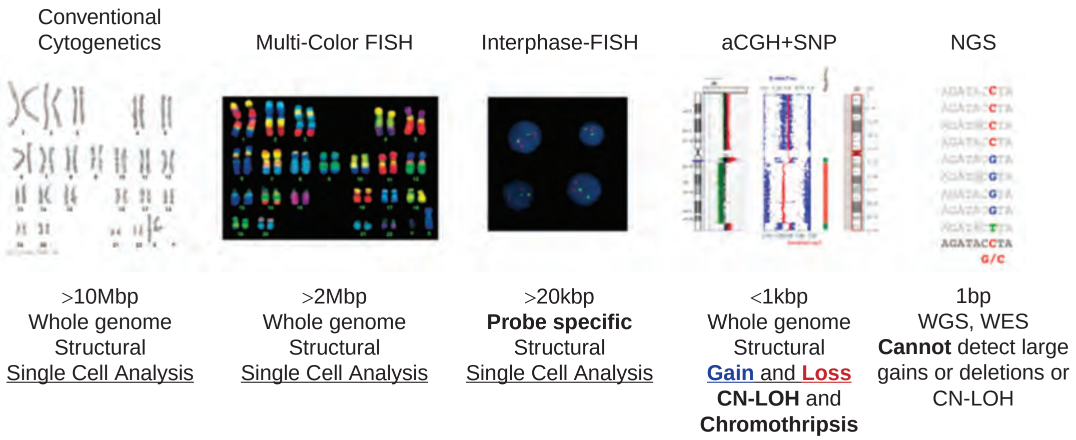

Conventional Cytogenetics
Multi-Color FISH
Interphase-FISH
aCGH+SNP
NGS
>10Mbp
Whole genome
Structural
Single Cell Analysis
>2Mbp
Whole genome
Structural
Single Cell Analysis
>20kbp
Probe specific
Structural
Single Cell Analysis
<1kbp
Whole genome
Structural
Gain and Loss
CN-LOH and
Chromothripsis
1bp
WGS, WES
Cannot detect large
gains or deletions or
CN-LOH

Figure 57.1 COMPLEMENTARY METHODOLOGIES OF IDENTIFYING VARIOUS GENOMIC ALTERATIONS AND THEIR RESOLUTIONS. See text for detailed descriptions of how these methodologies are applied for detection of genomic abnormalities in hematologic malignancies. Conventional cytogenetic methods detect clonal numerical and structural chromosomal abnormalities on a single cell level, at the resolution of 5 to 7 Mb. Multicolor FISH method with 24 different colors is specifically useful to identify the origin of marker chromosomes, complex 3- or more-way translocations, origin of ring chromosomes and other chromosomal abnormalities present in a complex karyotype such as those in MDS, lymphoma, and multiple myeloma. As mentioned in the text, there are at least five different reasons to perform interphase FISH in non-dividing cells from specimens of patients with hematologic malignancies. Interphase FISH is specifically useful for initial screening of the most recurrent rearrangements associated with CML, AML, ALL, and multiple myeloma as well as for detection of minimal residual disease with a diagnostic abnormality originally determined by conventional cytogenetics. In the lab of the author interphase FISH for *PML-RARA* fusion for the diagnosis of APL is a stat test with results obtained within 4 hours. Array CGH+SNP is a molecular method for detection of small and cryptic DNA changes at the exon-level resolution to 1 Mb. SNP platform is particularly useful for detection of acquired copy-number neutral chromosomal regions as well as detection of chromothripsis (see text). Next-generation sequencing (NGS) is the most powerful method for detection of acquired somatic mutation at the single nucleotide level. Although not yet used routinely in clinical laboratories, the application of NGS to hematologic malignancies has revolutionized the current knowledge of many leukemic entities. *aCGH*, array comparative genomic hybridization; *ALL*, acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *APL*, acute promyelocytic leukemia; *CML*, chronic myeloid leukemia; *FISH*, fluorescence in situ hybridization; *MDS*, myelodysplastic syndrome; *SNP*, single-nucleotide polymorphism.

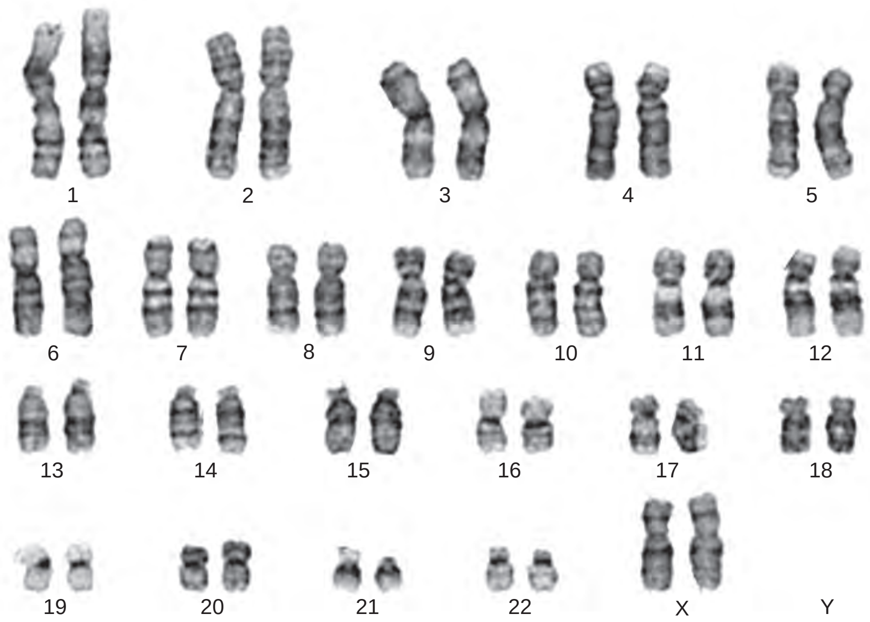

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y

Figure 57.2 NORMAL ARRANGEMENT OF CHROMOSOMES IN A KARYOTYPE FROM A NORMAL BONE MARROW METAPHASE SHOWING A SLIGHTLY FUZZY MORPHOLOGY COMPARED WITH A NORMAL KARYOTYPE OBTAINED FROM PHYTOHEMAGGLUTININ-STIMULATED PERIPHERAL BLOOD CELLS.

to identify 24 different human chromosomes (22 autosomes and the X and Y sex chromosomes), each with a unique color, with the help of fluorochrome-specific optical filters. This method is called *multicolor FISH (M-FISH)*. When interferometer-based spectral imaging is used, the method is called *spectral karyotyping*. The starting point in both methodologies is the use of whole chromosome painting probes for each chromosome. Thus each chromosome is labeled with a different combination of fluorescent dyes and images are sequentially obtained using five different fluorochrome-specific optical filters. A computer program combines the data and displays each chromosome

TABLE 57.2 Recommended Testing for Different Hematologic Malignancies

Disease	Test	Requirement	Suggested Methodology
CML	Karyotype	Mandatory	Chromosome banding
	BCR-ABL1 gene fusion	Mandatory	FISH or molecular methods/NGS
	ABL1 mutation when resistance to therapy	Mandatory	Molecular methods
MPN	*JAK2, CALR, MPL* mutations depending on referral reason	Mandatory	Molecular methods
	Karyotype, if normal	Highly recommended	Chromosome banding/aCGH+SNP
Myeloid/lymphoid neoplasms with eosinophilia	Recurrent gene fusions involving *PDGFRA, PDGFRB, FGFR1, PCM1-JAK2*	Strongly recommended for most patients	FISH, molecular methods
	Karyotype	Recommended in absence of recurrent gene fusion	Chromosome banding
MDS	Karyotype	Mandatory	Chromosome banding
	Targeted chromosome abnormalities −5/del(5q),−7/del(7q), *MECOM* (extended panel +8,del 20q, de(*TP53*)	Recommended	FISH/aCGH+SNP
	High resolution chromosome analysis	Recommended	SNP array
	Mutation analysis of candidate genes	Recommended	Molecular methods, NGS
AML	Karyotype	Mandatory	Chromosome banding
	Gene mutations: *NMP1,CEBPA, RUNX1, FLT3,TP53, ASXL1*	Mandatory	Molecular methods
	Recurrent gene fusions: *PML-RARA, CBFB-MYH11, RUNX1- RUNXT1*. Gene rearrangements of *KMT2A, MECOM, TP53*	Mandatory	FISH or molecular methods
Treatment-related MDS/AML	Karyotype/FISH: −5/del(5q),−7/del(7q), *KMT2A, RUNX1*	Mandatory	Chromosome banding/FISH
ALL	Karyotype	Mandatory	Chromosome banding
	Recurrent gene fusions	Strongly Recommended	FISH
	Infants: K*MT2A;* Pediatric: *ETV1-RUNX1, BCR-ABL, TCF3* Adults: *KMT2A, ETV1-RUNX1, BCR-ABL1,*		
	Hyperdiploidy	Recommended in pediatric	aCGH+SNP
	Recurrent microdeletions/AMP/9p21, *RUNX1*	Mandatory	FISH/aCGH+SNP
CLL	Deletion 13q14, *ATM, TP53*, trisomy 12	Mandatory	FISH, aCGH+SNP or molecular methods
	TP53 mutation/IGHV mutational status	Mandatory	Molecular methods
	Karyotype	Recommended for clinical trials	Chromosome banding
Multiple myeloma	t(4;14), t(14;16), deletion *TP53* gain 1q/del(1p)	Recommended	FISH for gene rearrangements
	t(11;14), t(14;20), ploidy status (extended panel)		FISH or Array, MLPA for copy number gains and losses
Other mature B-cell neoplasms	Recurrent gene rearrangements depending on differential diagnosis		FISH
	MYC rearrangements for prognostic testing/IgH		FISH

aCGH, Array comparative genomic hybridization; *AML*, acute myeloid leukemia; *CML*, chronic myeloid leukemia; *FISH*, fluorescence in situ hybridization; *MDS*, myelodysplastic syndrome; *MLPA*, multiplex ligation-dependent probe amplification; *MPN*, myeloproliferative neoplasm; *NGS*, next generation sequencing; *SNP*, single-nucleotide polymorphism.

Modified from Rack KA, van den Berg E, Haferlach C, et al. European recommendations and quality assurance for cytogenomic analysis of haematological neoplasms. *Leukemia*. 2019;33:1851–1867.

as if it were stained with a distinct color. Spectral karyotyping is based on the use of an interferometer (used by astronomers to measure the light spectra of distant stars) to determine the full spectrum of light emitted by each stained chromosome. A computer program then displays all the chromosomes simultaneously, each with its own unique color. These methods are applied with increasing frequency to resolve complex karyotypes and to resolve the origin of the marker chromosome.

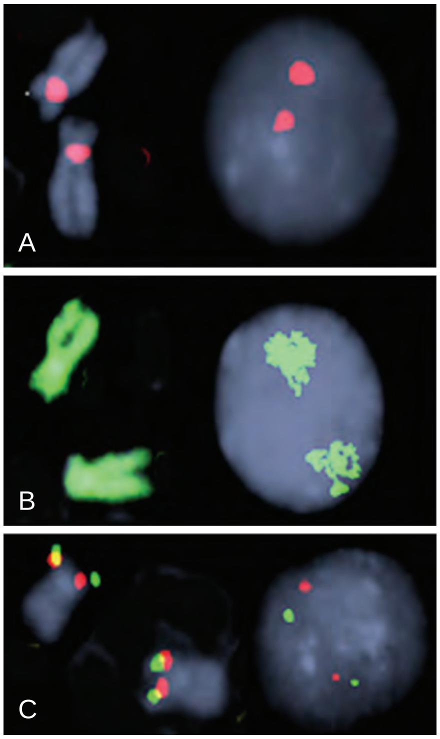


Figure 57.3 TYPES OF CHROMOSOMAL PROBES (SEE TEXT FOR DETAILS). (A) Pair of chromosome 12 *(left)* and interphase cell *(right)* after fluorescence in situ hybridization (FISH) study with centromere enumeration probe (CEP) showing two hybridization signals *(red)* in the centromeric area of chromosome and two tight signals in interphase cell consistent with disomy (normal copy number). CEP probes are most useful for detection of numerical abnormalities. (B) Hybridization with a whole chromosome 8 painting probe showing the hybridization signal *(green)* along the length of the entire chromosome 8 *(left)* and hybridization domains in interphase cell *(right)*. Whole chromosome painting probes are useful for identifying unknown chromosomes in metaphase cells. (C) Target of locus-specific indicators are specific gene sequences such as P53 seen after hybridization as two small signals *(red)* on chromosome 17, band p13. The main applications of locus-specific indicator (LSI) probes are gene mapping, numerical enumeration in interphase cells, and detection of translocations. Telomeric probe, shown in green for the short arms of chromosome 17, are repetitive probes and are useful for detection of cryptic translocations involving ends of chromosomes. Chromosomes and nuclei are counterstained with DAPI *(blue)*.

Comparative Genomic Hybridization and Next Generation Sequencing

Another powerful method used for identifying the location of chromosomal gains, losses, deletions, or amplifications, without prior knowledge of the chromosomal target that may be altered, is comparative genomic hybridization (CGH) (see Fig. 57.1). Briefly, isolated DNA from a patient's marrow or tumor tissue is labeled with a one-color fluorochrome (e.g., red), whereas DNA isolated from normal control tissue is labeled with a different color (e.g., green). These differently labeled DNAs are hybridized against each other in a competitive hybridization reaction onto normal metaphase spreads. Computer-assisted image analysis detects colors generated after hybridization, which indicate equal hybridization, relative excess, or deficiency of the target DNA (relative to control). The ratio of color intensity provides a "copy number" karyotype.

A particularly useful investigational approach is a "microchip array" in which labeled DNA or RNA from the sample of interest is hybridized with defined target sequences immobilized on a solid support system. This method can screen for genes that are gained/amplified or deleted from the genome on a large scale or, in the case of RNA, to learn whether such genes are expressed at a particular stage of disease. The first example of successful application of the RNA microchip array technique was the discrimination of AML from acute lymphoblastic leukemia (ALL) based solely on gene expression. As shown in Fig. 57.1, in the array CGH (aCGH) procedure, large-insert genomic clones, oligonucleotides, or single-nucleotide polymorphisms (SNPs) have replaced metaphase chromosomes used in routine CGH. Array CGH is a higher-resolution CGH technology of approximately 5 to 50 kb and provides diagnostic information for diseases associated with DNA dosage. It can also be used to discover previously unexpected sites of altered gene dosage associated with specific hematologic malignancies. The concept of obtaining gene copy number from multiple genome locations in a single measurement has been used to characterize numerous hematologic malignancies over the last 17 years, and its clinical utility is demonstrated throughout this chapter. Nowadays, SNP arrays are also used to genotype a few hundred to millions of SNPs in order to detect rare and common genomic rearrangements (see Fig. 57.1). These arrays require hybridization of only the test sample onto the array, unlike aCGH, which relies on co-hybridization of test and reference DNA. At this time aCGH+SNP arrays are used together in one study and they provide exon-level resolution of genomic changes.

The most advanced genomic technologies currently known is next generation sequencing (Chapter 3) or NGS (see Fig. 57.1). These approaches use a range of techniques that enable sequencing of hundreds of thousands of nucleic acids simultaneously. In order of complexity, these approaches include sequencing of gene panels, exome sequencing (protein-coding genes), transcriptome (expressed RNA), and sequencing the whole-genome sequencing (WGS). Implementation of these techniques requires several arrays of several hundred thousand sequencing templates in parallel generating up to several hundred million short reads of DNA sequence per lane. The basic principle of NGS involves a process of DNA fragmentation, adapter ligation, and immobilization of the fragments via the adapters to create libraries. The libraries then undergo a process of amplification, generating multiple copies of each DNA fragment which are then sequenced in parallel by a fluorescence- or chemiluminescence-based method, yielding billions of short sequence reads (Chapter 3). These reads are then aligned to the human reference genome and highly efficient algorithms are used to map these complex genomes. Because of its cost effectiveness and enormous sequencing capacity, NGS is revolutionizing hematologic malignancy research by facilitating the discovery of disease-initiating mutations, identifying novel drug targets, and allowing for the first time personalized treatment strategies for hematologic malignancies. Exome sequencing is a relatively inexpensive approach to identify protein-coding mutations, but is incapable of identifying structural genetic rearrangements, deletions, and insertions of DNA, a hallmark of many hematologic malignancies. Exome sequencing is performed at a depth of 100- to 200-fold coverage of the haploid genome, which enables detection of mutations present in leukemic subclones, which is important in the study of relapse. Transcriptome sequencing involves sequencing of genes actively expressed and can be adjusted to selectively study RNA transcripts that encode proteins (mRNA), transcripts regardless of coding potential (RNA), or a variety of small and noncoding RNA transcripts. RNA sequencing is a highly informative approach which enables identification of chromosomal rearrangements that result in the expression of chimeric fusion genes and sequence mutation detection. WGS usually involves sequencing leukemic and nonleukemic DNA from an individual. Although it is the most comprehensive modality, WGS may not identify all genetic alterations caused by variations in sequence coverage and difficulties in sequencing complex and GC-rich regions of the genome (including gene promoters). These modern cytogenomic methods have increased the resolution at which chromosomal and gene rearrangements can be identified. Conventional cytogenetics, FISH, and aCGH+SNP along with NGS are complementary. Each has its own pros and cons in investigating genomic rearrangements of malignant cells.

Although conventional cytogenetics is the comprehensive study of all chromosomes, it requires a large number of dividing cells, which, in some diseases, such as myelofibrosis, is difficult to obtain

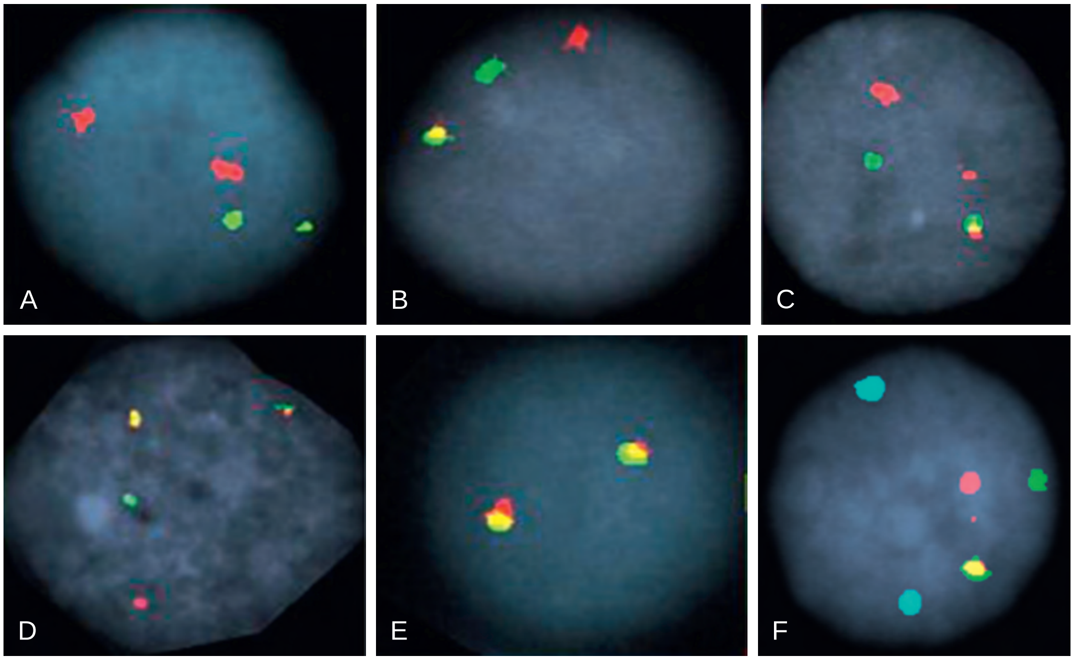


Figure 57.4 FOUR DIFFERENT PROBE STRATEGIES FOR DETECTION OF CHROMOSOMAL TRANSLOCATIONS (SEE TEXT FOR DETAILS). (A) Normal cell after in situ hybridization with breakpoint cluster region *(green)* and *ABL* (Abelson) *(red)* showing a normal distribution of two red and two green single signals. (B) Conventional fusion strategy after in situ hybridization shows one fusion *(yellow)* signal representing derivative chromosome generated by the translocation and one single-color signal, red and green, for normal homologues in positive nuclei. (C) An extra-sensitive fusion approach generates an extra small *(red)* signal, as well as a fusion signal *(yellow)* and one signal in single color *(green and red)* on normal homologues. (D) Dual-fusion strategy generates two fusion signals *(yellow)* on two derivative chromosomes and one single-color signal on each of two normal chromosomes. (E) Breakapart approach in a normal cell appears as two fusion signals *(yellow)*. In this strategy, the 3′ end and the 5′ part of the gene are labeled in two colors. (F) When the rearrangement occurs, the normal chromosomes show co-localization of red and green *(yellow)* as a result of the proximity of the sequences on the chromosome, whereas abnormal derivative chromosomes each have one single red and single green signal, indicating that the rearrangement occurred between the two ends of the gene separating the green and red signals on two different chromosomes. The third-color probe *(blue)* can be used as an internal control (usually centromere enumeration probe) to determine the disomic number of chromosomes.

(see Table 57.2). Furthermore, many small deletions or structural rearrangements are beyond the microscopic level of detection. FISH should be used in conjunction with conventional cytogenetics with both interphase and metaphase cells. It is a more sensitive method and detects rearrangements smaller than 1 kb. The main disadvantage of interphase FISH is that it cannot be used unless a known abnormality is suspected. When the abnormality is known, interphase FISH identifies the clonal aberration at the single-cell level. aCGH+SNP provides genome-wide higher resolution analysis and important information concerning genomic changes in patients with a normal karyotype as well as acquired loss of heterozygosity, and chromothripsis (chromosome shattering), which are important prognostic and predictive tools. With the introduction of high throughput genomic technologies such as NGS, FISH-based chromosome level detection has gradually changed focus to genome-wide detection of single nucleotide and copy number variants that are common in leukemia. It is evident that identification of chromosomal aberrations by molecular cytogenomic techniques is important in detecting novel chromosomal rearrangements and genes involved in leukemogenesis. Understanding the basis of these techniques and their application is critical in the accurate diagnosis of hematologic malignancies. Table 57.2 describes the optimal algorithm and evolving new testing strategies. New technologies, capable of simultaneously detecting copy number changes, structural variants, and mutations, are expected to be used in future in a diagnostic setting.

Genome sequencing has rapidly become routine practice. However, the intimate relationship between DNA sequencing, chromosome structure, and the position of chromosomes in the nucleus is not fully understood. The term “*chromosomics*” was recently proposed in order to integrate the original definition of cytogenetics (chromosomes and cytology) with genomics (gene content, structure and function) to ensure a closer integration of these fields. This term chromosomics is encompassing the integration of the latest advances in cytogenetics, genome sequencing, epigenomics, and cell biology. *Chromosomics* approaches in future will lead to additional success in answering fundamental biological questions in hematologic malignancies.

Next-Generation Karyotyping

In early 2021 three studies used novel *next-generation karyotyping* which employed WGS as a potential replacement for conventional cytogenetics (Chapter 3). These initial studies focused on patients with AML and myelodysplastic syndrome (MDS) and the genetic profiles and sequencing approaches were tested against the backbone of orthogonal results obtained at the same time using cytogenetics, FISH, and polymerase chain reaction (PCR) and/or NGS for detection of acquired somatic mutation. In a study reported by the Washington University group, in 235 patients who had undergone successful cytogenetic analysis, WGS provided rapid and accurate genomic profiling in patients with AML or MDS after modifications of sample preparation, sequencing, and analysis to detect mutations to be used for risk stratification using existing European Leukemia Network (ELN) guidelines.[1] The WGS detected all 40 recurrent translocations and 91 copy-number alterations that had been identified by cytogenetic analysis. In addition, next-generation karyotyping identified new previously unrecognized genomic events in 17% of patients. In 117 consecutive patients analyzed prospectively WGS provided results within a median of 5 days and additional genetic information was identified in almost 25%, which changed the ELN and Revised International Prognostic Scoring System (IPSS-R) genetic prognostic risk categories in 16% of patients. Standard AML risk groups, as defined by sequencing results instead of cytogenetic analysis, correlated with clinical outcomes. WGS was also used to stratify patients who had inconclusive results by cytogenetic analysis into risk groups in which clinical

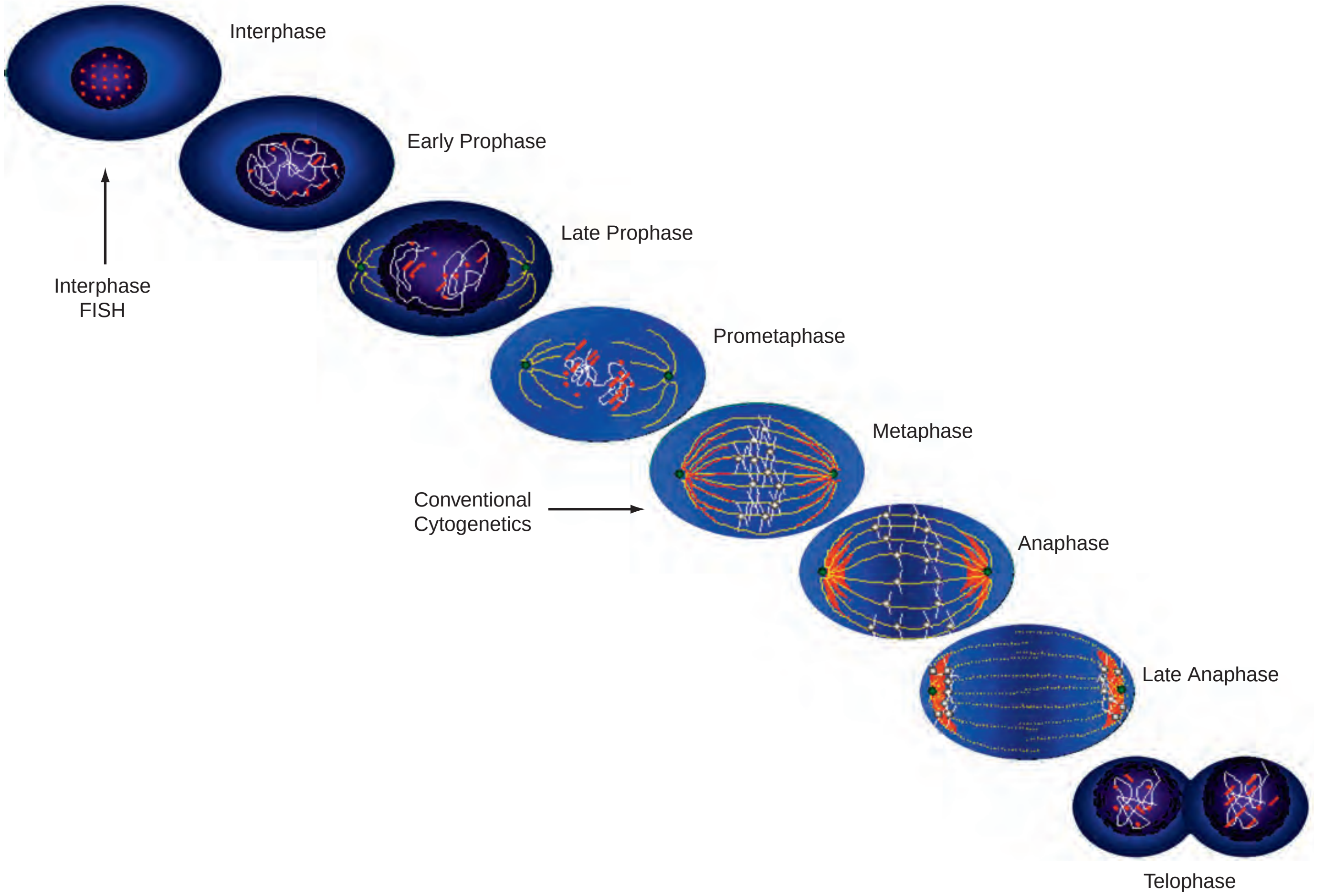


Figure 57.5 SCHEMATIC REPRESENTATION OF CELL DIVISION. Most clinical FISH studies are performed on non-dividing interphase cells, whereas conventional cytogenetics is performed at the metaphase stage of cell division. *FISH*, fluorescence in situ hybridization. *(Courtesy Dr. Ari Melnik, Cornell Medical Center, New York.)*

outcomes were measurably different.[1] These initial data are highly promising because they provided a greater diagnostic yield than conventional cytogenetic analysis and allowed for more efficient risk stratification on the basis of standard risk categories. Moreover, the speed by which the clinically relevant genomic profiles were obtained allowed reports to be generated in as little as 3 days.

Clonal Origin of Leukemia

The question of whether cell proliferation is monoclonal or polyclonal is fundamental to understanding the underlying origins of hematologic malignancies. Markers of clonality are used to determine the origin of disease; to differentiate malignant from nonmalignant populations; to establish hematopoietic hierarchy, clonal evolution, and clonal remission; and to delineate steps involved in the multistep pathogenesis of hematologic malignancies.

The clonal origin of leukemias and lymphomas can be assessed by either intrinsic or extrinsic cellular markers. Intrinsic cellular markers are specific for a cell population, arising either during normal differentiation or as a part of disease process. For instance, cell surface-associated immunoglobulin (Ig) markers such as the λ or κ light chain or idiotypes and T-cell receptors (TCRs) can be useful for evaluating lymphoid malignancies. Application of *IgH* markers demonstrated for the first time that MM was of clonal origin. Somatic cytogenetic alterations are useful intrinsic markers for identifying abnormal clones and following disease progression. Thus the observation of identical chromosome anomalies in different cells of the same tumor is evidence of clonality. Since the discovery of the Ph in 1960 it has been well established that nonrandom, recurrent chromosomal abnormalities characterize many hematologic malignancies. The finding of the Ph in different CML-derived hematopoietic cell lineages led to the hypothesis that CML originates in a single precursor cell that has a clonal development pattern. Moreover, the presence of additional recurrent chromosomal abnormalities in the Ph-positive clone (such as trisomy 8, duplication of the Ph, or trisomy 19) not only indicates the progression of the disease to accelerated phase or blast crisis, but also demonstrates the subclonal evolution of the Ph-positive clone. Currently, disease-associated somatic genomic mutations, such as rearrangements of *KMT2A (MLL)*, runt-related transcription factor gene *(RUNX1), ETV6, PML-RARA*, and many others, can be identified by PCR-based assays, FISH assay, and novel aCGH and NGS, and may serve, with or without conventional cytogenetics, as intrinsic markers of disease processes.

On the other hand, extrinsic marker systems use cellular mosaicism that is completely independent of the disease being studied and is not restricted to the cell lineages. Individuals with Turner or Klinefelter syndrome are mosaic for XX or XY and monosomy X cells or XXY and XY cells, respectively. The mosaicism created by X-chromosome inactivation in females is much more widely applicable, and initially provided fundamental insights into the pathogenesis of hematologic malignancies. Original studies with X-linked glucose-6-phosphate dehydrogenase (*G6PD*) as a marker of clonality were based on the Lyon hypothesis, which asserts that early in embryogenesis, one X chromosome in females is inactivated in somatic cells and the activation status is stably transmitted to daughter cells during mitosis (Fig. 57.7). The choice of maternal versus paternal X-chromosome inactivation is random; however, once it occurs, it is maintained in all daughter cells. Random X inactivation occurs by embryonic day 6.5

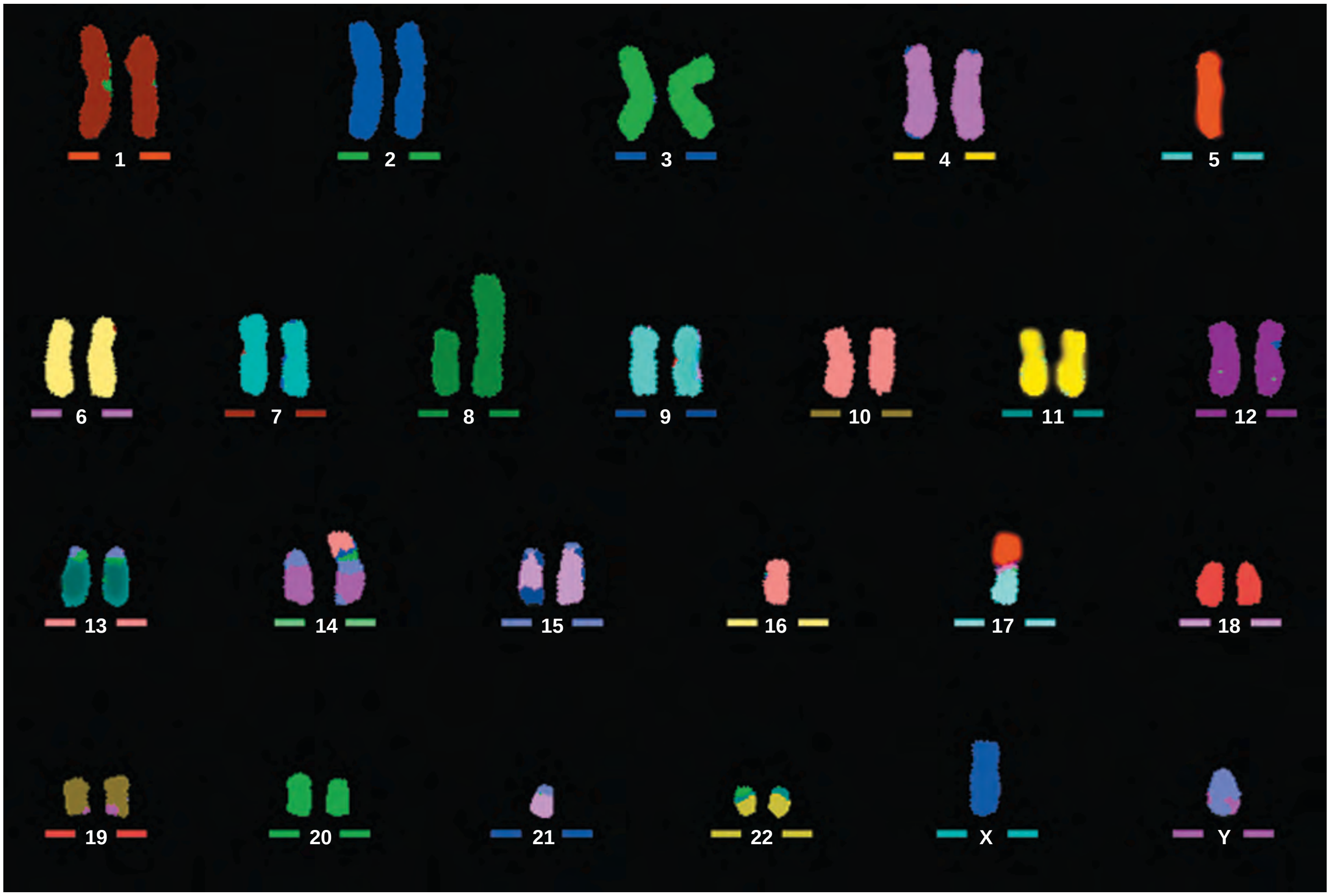


Figure 57.6 Multicolor metaphase FISH of a bone marrow cell from a patient with myelodysplastic syndrome documenting 43,XY, −5, der(8)t(8;8)(p23; q11.2), der(14;16)(p12;p11.1), inv(15)(q21;q24), der(17)t(5;17)(p13;p13), −21 karyotype. The origin of t(8;8) and der(14;16) could not have been determined by conventional cytogenetic study alone. *FISH*, fluorescence in situ hybridization.

around the start of gastrulation and results in a mosaic pattern that characterizes adult females. Therefore an adult female is a mosaic for two-cell populations, one expressing genes from an active X chromosome and the other expressing genes from the inactive X chromosome. Incidentally, mammalian X-chromosome inactivation is a mechanism that equalizes the dosage of X-linked genes between sexes. Although the exact mechanism of X-chromosome inactivation remains to be elucidated, the process of X inactivation starts with methylation of CpG islands. The inactivation process is believed to occur before differentiation of the embryonic stem cell into various cell lineages. Hematopoietic cells do not originate from a single embryonic stem cell but from several hematopoietic stem cells, thereby allowing for mosaic expression from both X chromosomes.

The observation that human females were heterozygous for the G6PD variant A and A^{-} and that two mosaic cell populations may be distinguishable by electrophoretic mobility was reported in the 1960s. The X-inactivation *G6PD* mosaic system was then applied to the study of clonality in human tumors (uterine leiomyomas) in 1964 by Gartler and Linden. In females who were heterozygous for the *G6PD* polymorphism and had malignant hematologic disorders such as CML, the finding of a single *G6PD* type in marrow or blood cells and both the A and B type *G6PD* in tissues not involved by the malignant process first demonstrated that CML was of clonal origin and provided evidence that the malignant transformation occurred at the level of a stem cell common to most hematopoietic cell lineages. Additional studies with heterozygous *G6PD* females who had CML demonstrated that some CML-derived B lymphocytes had a single G6PD type, but these clonal cells were Ph-negative; thus leukemic transformation might predate development of the chromosomal abnormality. This observation provided evidence that CML has a multistep pathogenesis. Application of this approach to hematologic malignancies demonstrated the clonal and stem cell origin for AML, ALL, Ph-negative myeloproliferative neoplasm (MPN), MDS, and CLL.[2] Studies using *G6PD* were particularly useful in the investigation of red blood cells and platelets in hematologic malignancies because the absence of nuclei in these cells did not allow them to be studied with cytogenetics or DNA analysis. Although it is now considered common knowledge that hematologic malignancies are characterized by clonal development, this concept was largely developed by Dr Phillip Fialkow.

Despite the importance of the *G6PD* approach, it is limited by the rarity of females who are heterozygous for the G6PD isoenzymes. An alternative and more extensive DNA-based X-chromosome clonal assay used common polymorphic markers that are caused by changes in DNA methylation patterns that accompany inactivation of the X chromosome. These X-linked loci such as phosphoglycerine kinase, hypoxanthine phosphoribosyltransferase, DXS25 (M27β), and human androgen receptor (HUMARA), have been extensively used in assessment of clonality, and can be used to identify clonal cell populations in virtually all females. DNA-based marker systems rely on a sequence polymorphism that has adjacent differences in methylation on the active and inactive X chromosomes. The inactive X chromosome is more highly methylated than its active homologue, but this is only true for certain regions of genes as 10% to 20% of X-linked genes escape inactivation and can be found both in clusters and in isolation. The most widely used HUMARA assay appears to maintain stringent methylation differences. The number of CAG tandem repeats differentiates the maternal from the paternal X chromosome.

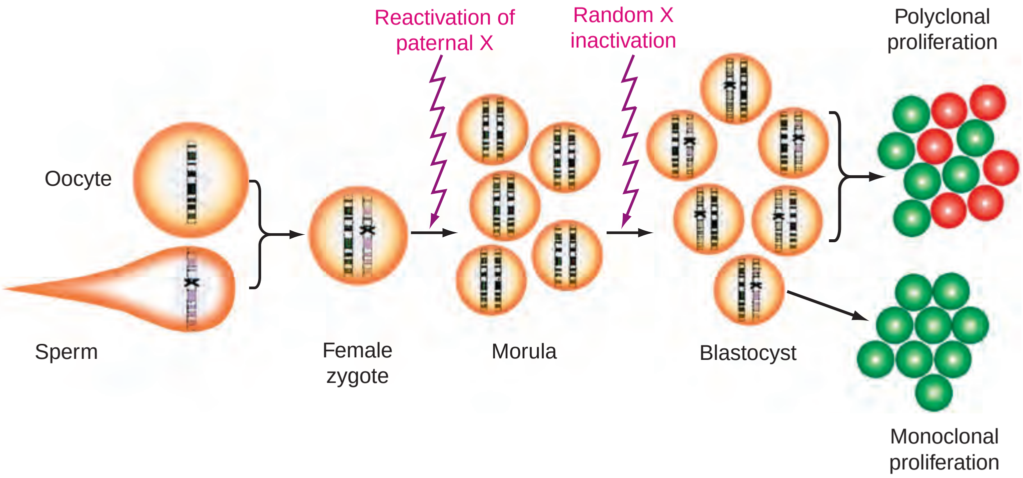


Figure 57.7 X-CHROMOSOME–LINKED ENZYME GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD) AS A MARKER TO INVESTIGATE CLONAL DEVELOPMENT OF HUMAN HEMATOPOIETIC DISORDERS. Early in embryogenesis, regions of all but one X chromosome are inactivated in each cell containing two or more X chromosomes. The choice of maternal versus paternal X chromosome for inactivation is random. Once the inactivation occurs, it is fixed and is stably transmitted to daughter cells during mitosis (Lyon hypothesis). Females who are heterozygous for the common B type and the less frequent A type, G6PD (localized on Xq27), are mosaic. This cellular mosaicism is used to study monoclonal versus polyclonal cell proliferation and development of malignant hematopoietic diseases. *(Courtesy Dr. W Raskind, University of Washington, Seattle.)*

The utility of the DNA-based X-chromosome clonal assay is limited to females younger than 60 years because they usually have 1:1 distribution of two-mosaic–cell population. A ratio greater than 3:1 is found in women older than 60 years, probably as a result of stem cell kinetics influenced by X-linked genetic factors. When the ratio of two-cell populations is greater than 3:1, this phenomenon is called a *skewed X-inactivation pattern*. With the HUMARA assay, acquired unequal or skewed X-chromosome inactivation (excessive lyonization) is found in 35% to 40% of women older than 60 years. Thus X-chromosome–based clonality studies must incorporate age-matched controls. More recently acquired somatic mutations using NGS have been useful in documenting clonal hematologic malignancies.

In Utero Mutations and Clonal Origin of Hematologic Malignancies

The observation that monozygotic twins share identical but nonconstitutive and clone-specific fusion gene sequences (e.g., *ETV6-RUNX1*) in pediatric ALL provided the first unambiguous evidence (in 2003) that genetic lesions, generated by chromosomal translocation, arise in utero.[3] These studies initiated by Mel Greaves and his colleagues contributed a wealth of knowledge about founder mutations, subclonal development, clonal origin, and the evolution of disease. The initiating lesion and premalignant clone is shared by the twins as a consequence of intraplacental vascular anastomoses and blood cell chimerism. The twin data were endorsed by backtracking of prenatal-initiating genetic lesions in the archived blood spots, or Guthrie cards, of patients with ALL. These data were interpreted to suggest that *ETV6-RUNX1* was likely a critical initiating lesion for *ETV6-RUNX1*-positive ALL. However, such fusions were detectable in cord blood from newborn infants at rates approximately 100-fold higher than the incidence of ALL, suggesting an obligatory requirement for additional mutations for leukemia development to occur. Over a period of 10 years these results were confirmed and the in utero origin of *MLL*, *BCR-ABL1*, and *RUNX1-RUNXT1* fusion rearrangements further documented, providing direct evidence for a prenatal origin of many childhood leukemias.

Results from genome sequencing of *ETV6-RUNX1* fusion region suggests it arises as a consequence of nonhomologous end-joining in the pro-B-cell stage with possible self-renewal capacity to downstream B-cell precursors. Additional evidence was provided by screening for trisomies in stored cord blood and the data indicated that ~6% of enriched CD34+/CD19+ B progenitor cells carry trisomies frequently seen in hyperdiploid childhood ALL. Using novel SNP as well as other technologies, it has become apparent that ALL has multiple genome copy number variations (CNVs), mostly deletions, and these CNVs are distinctive between a pair of twins, indicating a secondary, postnatal origin.

When genotyping sequencing combined with other molecular technologies of five pairs of monozygotic twins with concordant *ETV6-RUNX1*-positive ALL, Greaves and his colleagues demonstrated that all recurrent CNVs (32 in total) were different within twin pairs providing strong evidence that they are probably secondary events and postnatal in origin in both twins as well as in non-twins with ALL. Another two twin pairs who shared a monochorionic placenta and had Ph-positive ALL, were also studied by Greaves and his colleagues.[3] These studies provided confirmation of the previous observation that *BCR-ABL1* is not sufficient to cause Ph-positive leukemia. Twin A presented with ALL at age 3.8 years and twin B presented at age 4.1 years. Both had an identical *BCR-ABL1* fusion transcript and both received an allogeneic stem cell transplantation from the same matched sibling donor but 7 months later twin B died, whereas twin A remained in good health 8 years following the transplantation. SNP analysis revealed that twin A had a subclone with trisomies for chromosomes 4, 6, 9, 14, 17, and X, tetrasomy 21, and a gain of 22q11.1–q11.23 region, whereas twin B had deletions of *EBF1* and *IKZ1*. These results provide direct evidence that rearrangements additional to *BCR-ABL1* are postnatal in origin, they represent subclonal evolution, and *BCR-ABL1* by itself is not sufficient for development of Ph-positive ALL. Recently two groups have reported, using mathematical models, that some patients' MPNs acquire the *JAK2* mutation in utero.[4]

These mutation-driven natural history studies provide evidence for sequential multistep pathogenesis of hematologic malignancies with sequential accumulation of genetic changes, which may be linear through clonal succession but that clonal evolution in most leukemias is complex and may represent a branching structure, as predicted by Darwin.

Clonal Hematopoiesis of Indeterminate Potential (CHIP) and Age-Related Clonal Hematopoiesis

High-throughput sequencing has provided novel observations which have added to our current understanding of the initial mutations that occur in premalignant stem cells and their clonal development that eventually leads to myeloid malignancies (Chapter 157).

Clonal mosaicism for large chromosomal anomalies (duplications, deletions, and copy number neutral (CN-LOH)), using SNP microarray data from 50,000 subjects in the GENEVA study, indicated that CH is infrequent (<0.5%) from birth until 50 years of age after which its frequency rapidly increases to 2% to 3% of the elderly. It has been estimated that individuals with clonal hematopoiesis (CH) have 10-fold higher risk of developing a subsequent hematologic malignancy. These age-related mutations were confirmed by analysis of mutation acquisition in hematopoietic stem cells, over time, through whole-exome sequencing of single hematopoietic stem cell-derived colonies, which showed that the total number of mutations in healthy individual stem cells increases with age. Therefore, the term CH describes the acquisition of somatic mutations in hematopoietic stem and progenitor cells resulting in clonal expansion in individuals with no overt hematologic disease. Moreover, sequencing of multiple elderly women provided evidence of CH based on X inactivation pattern and recurrent somatic mutation in *TET2* gene. Subsequent analysis of 182 additional elderly women with CH determined that more than 5% of these individuals had mutations in *TET2* (Chapter 157).

Investigation of 82 paired bone marrow and blood samples from carefully selected healthy adult volunteers using a panel of 41 genes known to be mutated in myeloid malignancies, with 1% threshold of detection revealed that bone marrow clones were found in almost 40% of healthy volunteers over 50 years of age. The most frequent mutations included *DNMT3A* and *TET2*. Variant allele frequencies were highly concordant between blood and bone marrow samples.[5]

However, individuals with CH may live for many years and decades without developing hematologic malignancies though they are at increased risk as compared with those without mutations. These studies may explain the higher frequency of myeloid malignancies in the elderly population (Chapter 157).

Loss of Y chromosome

Loss of Y chromosome (LOY) was first described in 1963 in cultured blood leukocytes as a sole cytogenetic abnormality in bone marrow cells which represents one of the most common acquired, post zygotic somatic genomic alterations in elderly men. Approximately 6% of male bone marrow karyotypes from normal individuals and 16% of bone marrow karyotypes with hematologic malignancies show a LOY. The incidence of LOY in bone marrow cells increases proportionally with aging with up to 20% of healthy men over 80 years of age showing LOY using standard G-banding techniques. Mosaic LOY in blood has been reported to be associated with higher risk of all-cause mortality and overall lower life expectancy. LOY has been described most frequently in patients with myelodysplastic disorders and has been reported in MPN, AML, and other myeloid diseases as well as in various solid tumors. Since MDS and MPN are primarily diseases of elderly individuals it is often difficult to separate age-associated LOY from disease-associated LOY. Although the incidence of LOY in MDS is up to 30%, the sole LOY is lower, ranging from 4% to 10%; the WHO does not recognize LOY by itself to be useful in cytogenetically identifying a case of MDS unlike other abnormalities such as del(5q), del(7q), +8, and del(20q).

Recent observations that a higher LOY burden is associated with a higher likelihood for progression to a myeloid malignancy represents the basis for a proposal to include LOY as another form of CH with striking similarities with CHIP. Among 73 studied male patients with sole LOY and a median age of 75 (range 29 to 90), those who had equal or greater than 75% of marrow metaphase cells with LOY had a significantly greater chance of developing a myeloid neoplasm and a higher lifetime incidence of diagnosis of MDS ($P < .0001$). Male individuals with greater than 75% of metaphase cells with LOY also had greater likelihood of having somatic mutations ($P = .0009$) and greater numbers of these mutations ($P = .0002$).[6] The presence of metaphase cells with equal or less than 25% LOY was associated with normal bone marrow morphology and a lower likelihood of progression to MDS. Multivariate analysis showed that these observations are consistent with samples showing 75% or more metaphase cells with LOY are myeloid disease-associated and not related due to aging. Therefore, it has been proposed that LOY represent a marker of CH and as such should be included in the definitions of CHIP in clinical and research settings.

Loss of X chromosome

The relationship between isolated loss of X chromosome (LOX) and aging was first documented in 1995 using interphase FISH method applying to peripheral blood cells of 90 healthy females (age: 1 week to 93 years) with a frequency of 3.2% below 51 years to 5.1% at age 51 to 93 years. This observation was confirmed a few years later in a cohort of 655 females and the frequency of LOX is ranging from 0.07% in patients older than 16 years to 7.3% in individuals older than 65 years. LOX was occasionally reported in BM cells at the frequency of 0.1% of females with the median age of 71.

Acquired trisomy 15 as an isolated abnormality is rarely detected. Approximately 0.1% to 0.2% of elderly patients show +15, frequently in association with LOY. In bone marrow cells, when I-FISH is used, +15 may represent a small clone of up to 24% of nondividing cells but none of these patients developed a myeloid neoplasm after a median follow-up of 28 months (range 0 to 149 months). These observations suggest that +15, like LOY, when present as a small clone is not disease-related but represents an abnormality associated with CH and is most likely age-related.

Chronic Myeloproliferative Neoplasms

The World Health Organization (WHO) characterizes MPNs as clonal stem cell disorders. CML has a unique place among hematologic malignancies and is described separately from the other Ph-negative MPNs.

Chronic Myeloid Leukemia (see Chapter 69)

Knowledge of the origins of CML has accumulated over the last 61 years and serves as a classic example of molecular medicine at its best (Table 57.3). The Ph chromosome is the first example of a specific chromosomal abnormality associated with a malignant disease.[2] *ABL1* and *BCR* genes are the first oncogenes localized at the site of a chromosomal breakpoint in t(9;22)(q34;q11.2). The *BCR-ABL1* fusion leads to a "hybrid" gene, resulting in the production of a dysregulated tyrosine kinase protein. Finally, imatinib mesylate, a specific tyrosine kinase inhibitor (TKI), was the first rationally designed targeted form of cancer therapy.

The Ph chromosome, named in honor of Philadelphia, the city of its discovery, was described for the first time in 1960. It represents a signature genomic rearrangement occurring in more than 95% of patients with CML. Approximately 3% of all pediatric leukemias are Ph-positive CML. The incidence in children increases with age and it is exceptionally rare in infants. The Ph chromosome results from a balanced translocation t(9;22)(q34;q11.2) (Fig. 57.8A–B). The Ph chromosome arises postzygotically, being found only in hematopoietic tissue. The finding of the Ph chromosome in myeloid cells, erythroid

TABLE 57.3 History of Discovery of Philadelphia Chromosome and BCR-ABL1 Fusion

1960	Philadelphia chromosome (Ph) is identified.
1973	Ph is t(9;22)(q34;q11.2).
1983	*ABL1* is translocated from chromosome 9 to chromosome 22.
1984	*BCR* is localized to 22q11.
1987	Ph′ is *BCR-ABL1* fusion.

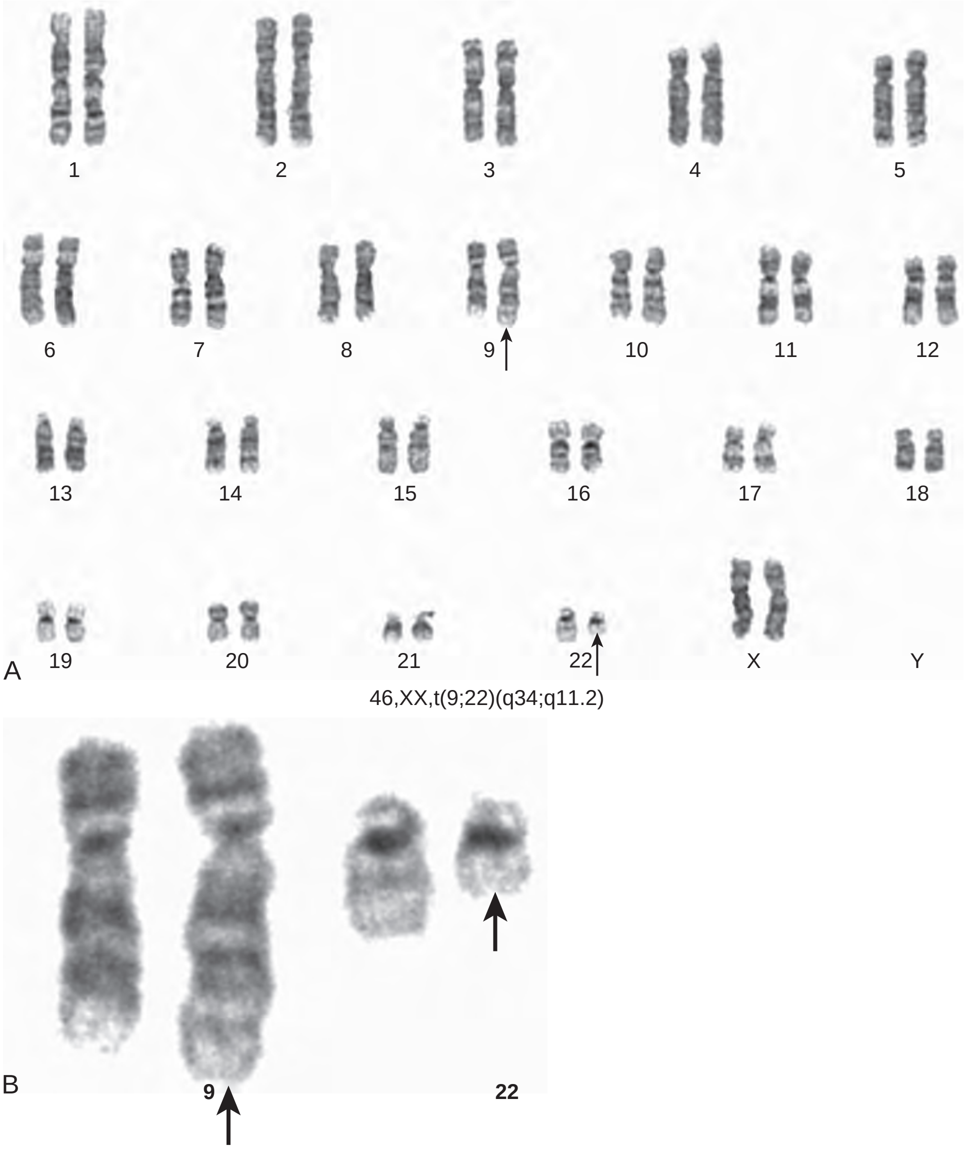


Figure 57.8 (A) The karyotype of a female patient showing a balanced t(9;22)(q34;q11.2), also known as the Ph chromosome. (B) Isolated t(9;22) showing two-thirds of the long arms of chromosome 22 translocated to one homologue of chromosome 9.

cells, eosinophils, monocytes/macrophages, basophils, and B lymphocytes, along with the absence of the Ph chromosome in cultured marrow fibroblasts, supports the concept that the Ph chromosome results from a specific rearrangement in a multipotent hematopoietic stem cell and that it is an acquired rather than an inherited abnormality.[7] Of interest, the Ph chromosome is rarely identified in T cells. T lymphocytes are long-lived cells and may antedate the development of CML. These observations combined with studies exploiting G6PD heterozygosity provide further evidence for the concept that CML is a clonal disease arising in a stem cell capable of differentiation into all hematopoietic cell lineages.

In a review of 1129 Ph-positive patients, the 9;22 translocation was identified in 1036 (92%) cases. Karyotypic analysis of marrow cells in patients with CML is a time-consuming task. However, it demonstrates not only the presence of the Ph chromosome but also the presence of other chromosomal rearrangements (clonal evolution) of clinical significance.

The reciprocal nature of the Ph-positive translocation was confirmed when studies showed that it is the molecular consequence of the translocation of the *ABL1* gene from chromosome 9, band region q34, and subsequent fusion to the breakpoint cluster region (*BCR*) gene on chromosome 22, band q11.2 (Fig. 57.9). This creates a hybrid *BCR-ABL1* gene that is transcribed into a chimeric BCR-ABL1 messenger RNA (mRNA) and translated into a specific chimeric protein.

Three major breakpoint locations along the *BCR* gene on chromosome 22 result in three chimeric proteins. They include $\text{P210}^{\text{BCR-ABL1}}$, $\text{P190}^{\text{BCR-ABL1}}$, and $\text{P230}^{\text{BCR-ABL1}}$ which are associated with three distinct types of leukemia. $\text{P210}^{\text{BCR-ABL}}$ is found in the majority of patients with classic Ph-positive, *BCR-ABL1* fusion-positive CML and approximately 30% of patients with Ph-positive ALL. Expression of $\text{P190}^{\text{BCR-ABL1}}$ is seen in 20% to 30% of adults and 80% of children with Ph-positive ALL. Expression of $\text{P230}^{\text{BCR-ABL1}}$ is associated with a rare indolent chronic neutrophilic leukemia variant and up to 1.6% of CML (approximately 50 patients in the worldwide literature have been described). The most common *BCR-ABL1* isoform results from a fusion of *BCR* exon 13 or 14 (e13/e14) with *ABL1* exon 2 (a2) producing a fusion gene referred to as e13a2 (b2a2) or e14a2(bra2) producing above-mentioned p210 protein. The coiled-coil domain of the *BCR* N terminus facilitates dimerization and constitutive autophosphorylation of the *ABL1* tyrosine kinase domain resulting in subsequent phosphorylation of numerous substrates including the JAK2/STAT CRKL MAPK and P13K/AKT pathways.

Approximately 1% to 2% of Ph-positive patients with CML express both $\text{P210}^{\text{BCR-ABL1}}$ and $\text{P190}^{\text{BCR-ABL1}}$ and their response to TKI therapy

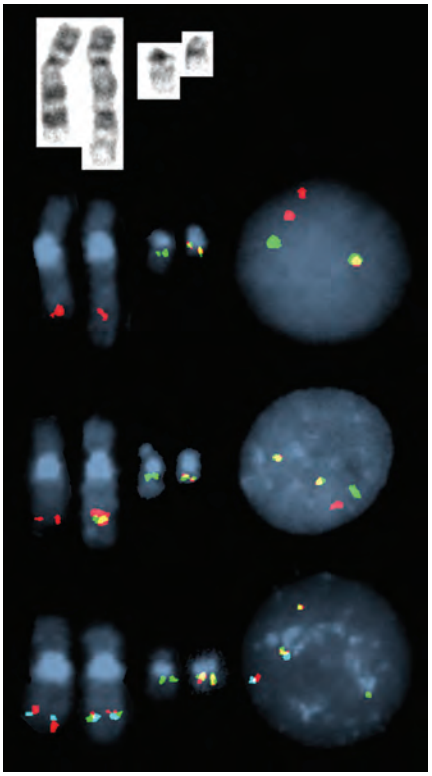


Figure 57.9 CURRENT FISH PROBE STRATEGIES FOR DETECTING *BCR-ABL1* FUSION. Partial karyotype showing the Philadelphia chromosome as a result of t(9;22)(q34;q11.2) *(top row)*. The same chromosomes 9 and 22, as well as a bone marrow nucleus, after hybridization with BCR *(green)* and ABL1 *(red)* using an extra-sensitive dual-color, single-fusion FISH strategy *(second row)*. Chromosomes and a nucleus are counterstained with DAPI *(blue)*. In the extra-sensitive strategy, a part of ABL1 *(red)* remains on der(9) and is shown in interphase nucleus as a smaller red signal, whereas the normal-size ABL1 hybridization signal is seen on normal homolog 9. The other part of ABL1 is on the Ph chromosome and is seen co-localized with BCR as yellow in interphase nucleus. Therefore interphase nuclei will have one normal-size red signal, one smaller-size red signal, one normal-size BCR signal, and co-localization of BCR and ABL1, producing a yellow signal both in interphase cells and metaphase chromosomes. In the dual-color, dual-fusion strategy *(third row)*, there are two co-localized signals on both der(9) and the Ph chromosome, as well as one red signal of ABL1 on chromosome 9 and one green signal of BCR on normal chromosome 22. In the triple-color, dual-fusion strategy, *ASS* gene *(aqua)* is added. It is used to determine whether sequences from der(9) are deleted at the time of the Ph formation. As shown in the fourth row, ASS is localized centromeric from ABL1 on 9q34, and in patients without deletion, ASS is present as two signals in nucleus and on the chromosome 9.

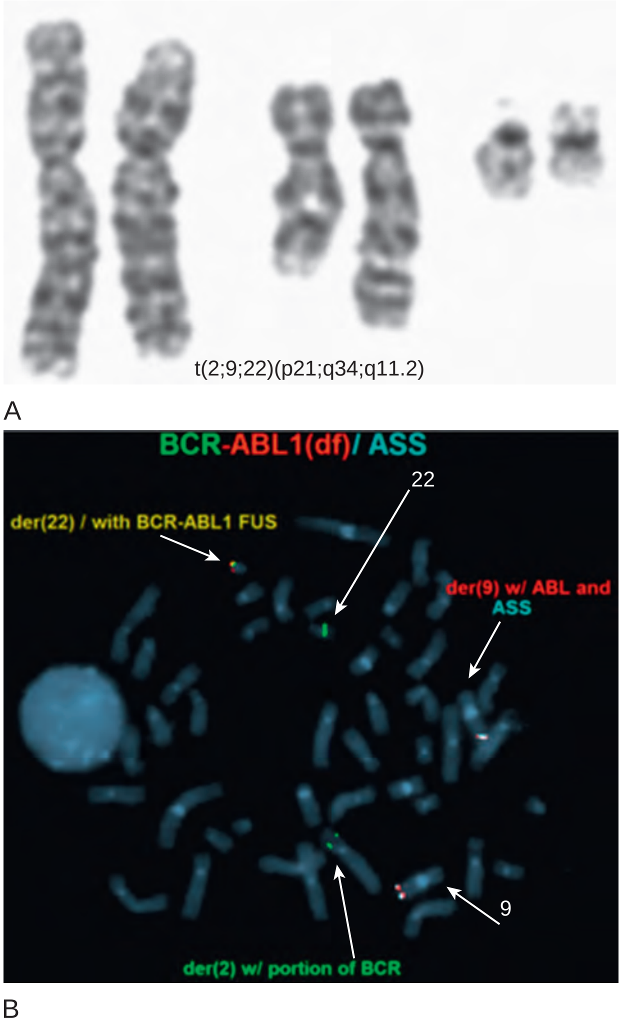


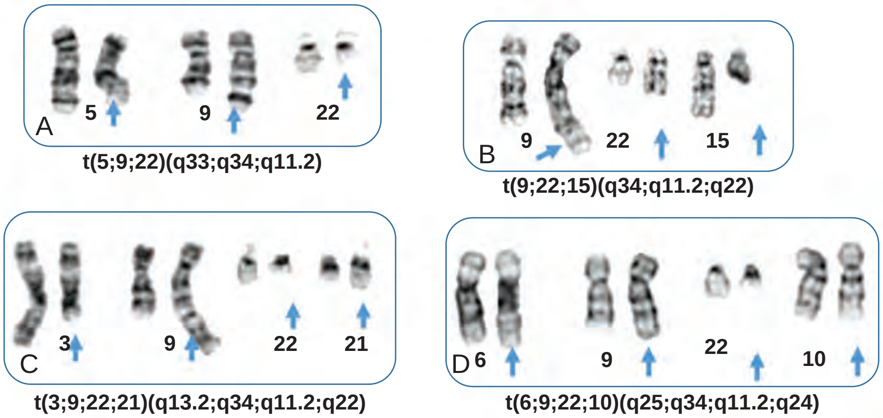
Figure 57.10 (A) Complex Ph translocation. *Top:* A partial karyotype from a patient showing a three-way translocation t(2;9;22) and (B) metaphase FISH *(bottom)* indicating that even in complex karyotype the fusion of *BCR* and *ABL1* takes place on the Ph chromosome or der(22) indicated in *yellow*.

is inferior to patients showing only P210$^{BCR\text{-}ABL1}$. The *BCR-ABL1* fusion is present in both standard and variant forms, in cases where chromosome 9 involvement is cytogenetically not detectable, and when a masked Ph is present. In the majority of patients, the fusion of *ABL1* and *BCR* takes place on chromosome 22 (Fig. 57.10A–B). The *BCR-ABL1* fusion transcript is present in neutrophils, monocytes, eosinophils, erythrocytes, B cells, rarely in T cells, and in CD34+ cells and is associated with increased proliferation of CD34+ myeloid progenitor cells but not of other more mature myeloid precursors.

These observations confirm the hypothesis that CML originates in a multipotent stem cell capable of differentiating to all hematopoietic cell lineages with the exception of T cells. These and other studies provide evidence for the existence of clonal *BCR-ABL1* fusion-negative stage. The formation of *BCR-ABL1* and the Ph chromosome occurs in an already abnormal and genetically unstable clone of pluripotent hematopoietic stem cells. Thus it is the preexisting genetic instability that predisposes to the formation of the Ph chromosome. Once Ph chromosome formation occurs, it confers a further selective growth advantage over normal cells, resulting in overwhelming *BCR-ABL1*-positive, Ph-positive marrow cells at the time of diagnosis of CML.

In 5% to 8% of newly diagnosed patients with CML, the Ph chromosome is present as a 3- or 4-way complex derivative Ph chromosome, always involving chromosomes 9 and 22 as well as an additional one or two chromosomes. In our own laboratory we have observed over many years at least 25 different complex Ph translocations. Three-way translocations are more frequent than 4-way translocations. Some of the examples are the following translocations (Fig. 57.11): t(2;9;22)(p13;q34;q11.2), (9;22;7)((q34;q11.2;p15), t(9;19;22)(q34;p12;q11.2), t(5;9;22) (q35;q34;q11.2), t(9;22;8)(q34;q11.2;p21), t(9;11;22)(q34;p15;p11.2), t(9;22;22) (q34;q11.2;p11.2), t(6;9;22;10)(q25,q34;q11.2;q24), t(7;9;22;22)(q22;q34;q11.2;q13.2), t(6;9;22;11)(q25,q34;q11.2;q13). Most of these complex structural rearrangements are unique but some of them such as t(2;9;22) are recurrent. Moreover, there are less than 1% of CML patients in chronic phase who acquire the t(9;22) clone abnormalities as well as t(8;21), inv(16), t(11q23), t(15;17), and t(3;3) that are associated with AML. In one recent retrospective study among 1382 Ph-positive patients 0.36% of patients had co-occurrence of these abnormalities and most of them progressed to accelerated or myeloid blast crisis.

In about 3% to 5% of patients with CML who are Ph-negative by cytogenetic studies, the clonal and stem cell origin of these hematologic malignancies can still be demonstrated, and molecular analysis reveals the *BCR-ABL1* fusion in approximately 1%



t(5;9;22)(q33;q34;q11.2)

t(9;22;15)(q34;q11.2;q22)

t(3;9;22;21)(q13.2;q34;q11.2;q22)

t(6;9;22;10)(q25;q34;q11.2;q24)

Figure 57.11 PARTIAL KARYOTYPES FROM 4 PATIENTS SHOWING 3- AND 4-WAY Ph TRANSLOCATIONS IN CHRONIC MYELOID LEUKEMIA. *Blue arrows* show abnormal chromosomes. (A) In this t(5;9;22) 3-way translocation a distal segment of the Ph chromosome from 22q was first translocated to chromosome 5, band q33 and the subsequent translocation was between the 5q33 and 9q34 segment. (B) In the t(9;22;15) abnormality there was a traditional first translocation between 9 and 22, followed by the second rearrangement involving 15q22 segment translocated to chromosome 9. (C) In the 4-way translocation involving chromosomes 3, 9, 22, and 21, long arms of chromosome 3 moved to the long arms of chromosome 9 while part of chromosome 22 moved to the long arms of chromosome 21. (D) t(6;9;22;10) has been rarely but recently observed in which a part of 6q moved to the already t(9;22) while part of 10q moved to the long arms of chromosome 6.

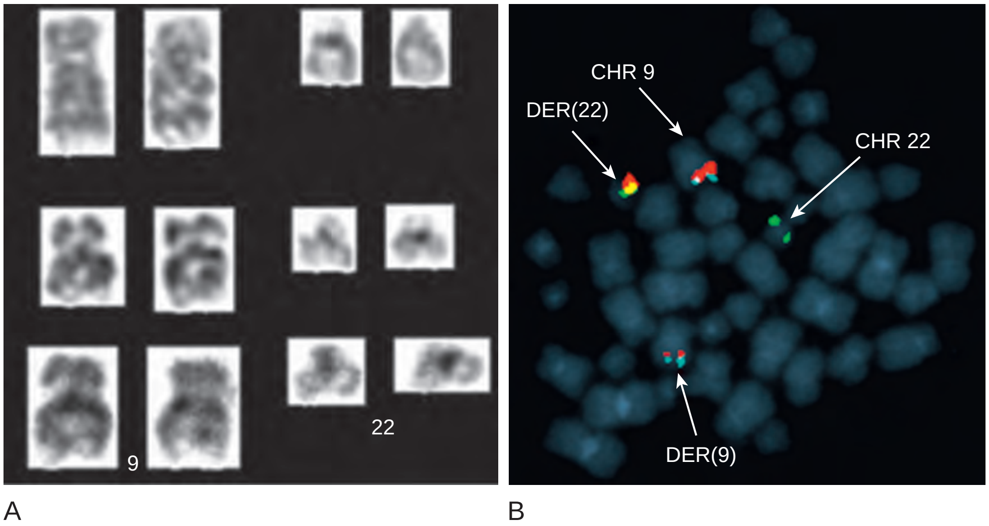


Figure 57.12 *BCR-ABL1* FUSION IN A PATIENT WITH THE Ph-NEGATIVE, NORMAL KARYOTYPE. *Left panel* shows a partial karyotype from three cells showing normal homologs 9 and 22. The *right panel* shows the *BCR-ABL1* fusion *(yellow)* on der (22) and diminished *ABL (red)* on der(9). The fusion has occurred as a result of *ABL1* insertion into *BCR* without apparent chromosomal translocation. Note an intact ASS *(aqua)* on both chromosomes 9.

(Fig. 57.12). These patients have chromosomal insertions resulting in *BCR-ABL1* fusion which can occur in multiple ways. Approximately 70% of cases exhibit *BCR-ABL1* fusion located on a chromosome 22, which was either a cryptic der(22), or "cryptic Ph" or non-cryptic der(22) or "masked Ph." The remaining cases have *BCR-ABL1* fusion located on a cryptic or non-cryptic der(9). Of the 119 such cases reported in literature 77 patients (64.7%) had an insertion-derived *BCR-ABL1* fusion as a cryptic or a masked Ph. This type of *BCR-ABL1* fusion does not influence OS of these CML patients. In the majority of Ph-negative patients, an *ABL1* insertion from chromosome 9 to 22q11.2 results in a *BCR-ABL1* fusion product without reciprocal translocation of sequences from chromosome 22 to chromosome 9. Sizes of the inserts can vary, which may be closely related to the morphological appearance of the chromosomes with the *BCR-ABL1* or those involved chromosomes without the *BCR-ABL1*. Intensive FISH mapping using many probes targeting *ABL1*, *BCR*, and their flanking regions determined that a classic t(9;22) usually involves an exchange of approximately 10 MB of 9q (from the *ABL1* to the 9q telomere) and ~25 MB of 22q (from *BCR* to the 22q telomere).

Approximately 2% of patients truly are Ph-negative and *BCR-ABL* fusion-negative. These patients may not have CML but rather another MPN (Chapter 71). The concept that the *BCR-ABL1* fusion plays a central role in the pathogenesis of CML is strongly supported by two lines of evidence: (a) retroviral transduction experiments in which P210$^{BCR-ABL1}$ is expressed in murine marrow cells, resulting in an MPN resembling CML, and (b) the fact that imatinib, a TKI, selectively inhibits the BCR-ABL1 fusion protein in mice and specifically inhibits the growth of human Ph-positive cells in vitro and in vivo. Although considered necessary, *BCR-ABL1* may not be the initial event or be sufficient to cause the malignant transformation resulting in CML (see earlier section on clonal origin).

PCR analysis can determine the exact breakpoints of DNA fusion products. Reverse transcriptase PCR (RT-PCR) and Northern blot analysis allow detection of *BCR-ABL1* transcripts at the RNA level. Current FISH studies can detect *BCR-ABL1* fusion at diagnosis

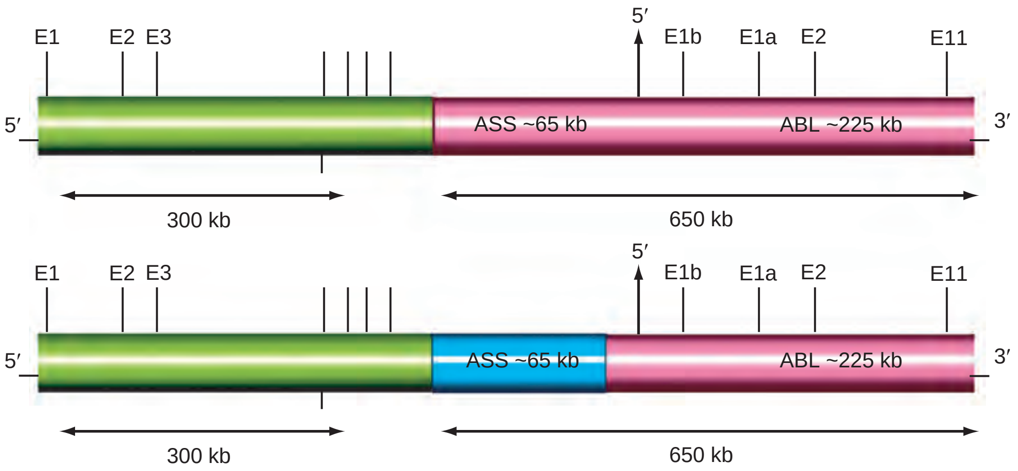


Figure 57.13 SCHEMATIC REPRESENTATION OF THE MOST FREQUENTLY USED BCR-ABL PROBES. Dual-color/single-fusion extra-sensitive probe strategy, as indicated in text, uses a 650-kb probe in which two loci, *ABL* and *ASS*, both are labeled in red. *BCR-ABL* fusion-positive nuclei show three red signals: one small red signal on der(9), one red signal on normal homologue 9, and a third red signal in fusion with *BCR*. When a triple-color probe is applied, the *ASS* locus usually is labeled in aqua and the BCR-ABL fusion-positive cells show two aqua signals, unless there is deletion of der(9). The most useful application of triple-color probe is documentation of deletion of derivative chromosome 9.

using a dual-color *BCR-ABL1* ES probe or *BCR-ABL1* dual-fusion probe (see Fig. 57.12, see also Fig. 57.9). A triple-color *BCR-ABL1-ASS* probe is used for detection of deletions on both chromosomes 9 and 22 (Fig. 57.13). Approximately 12% to 15% of patients with CML have large deletions adjacent to the Ph chromosome translocation breakpoint on the derivative 9 chromosome. These deletions are heterogeneous and may involve both chromosomes 9 and 22 (majority of cases), only chromosome 9 (8% of patients with deletions), or only chromosome 22 (4% of cases with deletions). Moreover, deletion size is variable, ranging from 0.5 Mb to greater than 10 Mb. A more refined study applying SNP microarrays revealed three common deleted regions: (1) a 162-kb loss at 9q34, (2) a 138-kb deletion at 22q11.2, and (3) a 102-kb deletion at 22q11.2. It appears that the partial deletion of the *ABL1-BCR* fusion on derivative 9 chromosome occurs as a part of the same process as the formation of the *BCR-ABL1* translocation. Before imatinib therapy, these deletions were associated with an adverse prognosis; however, in a study of 521 patients with CML, the cumulative incidence of complete cytogenetic responses and major molecular responses and the overall survival (OS) were comparable after 5 years of follow-up between CML patients with and without the del(9q).

At diagnosis, conventional cytogenetics remains the gold standard because the chromosome analysis will identify not only the t(9;22) but also other chromosomal abnormalities that may indicate accelerated or blast phase of the disease or clonal proliferation of Ph-negative cells.

Imatinib mesylate (Gleevec) has revolutionized therapy for CML and, for most patients, has transformed a deadly disease into a chronic disorder that is compatible with normal life. The standard method for monitoring a patient's response to therapy is conventional cytogenetic analysis of cells obtained from a marrow aspirate. In the phase III international Randomized and STI-571 (IRIS) study, 89% of patients had Ph-negative marrow aspirates as determined by conventional cytogenetics after 5 years of treatment. However, conventional cytogenetics has limited sensitivity. The degree of MRD is determined to be an important prognostic factor for patients with CML on therapy. Patients with CML without a discernible Ph chromosome detected by conventional cytogenetics analysis may still harbor up to 10^{10} leukemic cells.

Interphase FISH does not depend on the cycling status of cells, and use of double-fusion probes has reduced false-positive results to approximately 1%. However, if peripheral blood cells rather than marrow aspirate cells are used to monitor MRD, the high percentage of *BCR-ABL1* fusion-negative lymphoid cells may underrepresent the actual residual tumor load. In most direct comparison studies, interphase FISH of peripheral blood compared with conventional cytogenetics of marrow in patients who are treated with imatinib showed good correlations ($r = 0.91$ to 0.97). Real-time quantitative PCR (RQ-PCR) is by far the most sensitive method for detecting MRD. It provides an accurate measure of the total leukemia cell mass and the degree to which *BCR-ABL1* transcripts are reduced by therapy, and it correlates with progression-free survival.

The goal of therapy in CML is to achieve a molecular remission as measured by the reduction or elimination of *BCR-ABL1* transcripts. In the IRIS study, at 5-year follow-up, complete cytogenetic response combined with major molecular response at 12 months was associated with a 97% progression-free survival rate. This compares with an 89% progression-free survival for those with complete cytogenetic response but without a major molecular response. Current international recommendations for optimal molecular monitoring of patients receiving imatinib treatment includes an RQ-PCR assay expressing the *BCR-ABL1* transcript levels on an internationally agreed upon scale. The term major molecular response corresponds to ≤0.0% BCR-ABL1, whereas the designation complete molecular response should be used only for patients with undetectable *BCR-ABL1*, where the limit of detection is confirmed to be at least a 4.5 log reduction from baseline levels. The two major obstacles to successful imatinib-based therapy for patients with Ph-positive, *BCR-ABL1* fusion-positive CML are the persistence of *BCR-ABL1* fusion-positive cells and relapse of the disease because of emergence of resistance to imatinib. Acquired resistance to imatinib treatment can be due to two events: amplification of *BCR-ABL1* fusion product (Fig. 57.14) and mutations in the ABL kinase domain. Currently, 100 different ABL1 kinase domain mutations have been described, although only about 15 are common and account for more than 85% of all mutations detected.

Patients with CML who cannot tolerate or are resistant to imatinib may benefit from the second and now third generation of TKIs (Chapter 69) such as dasatinib, nilotinib, bosutinib, and ponatinib. These agents bind to the ABL kinase domain in a matter distinct from that of imatinib and thereby retain activity against nearly all imatinib-resistant mutations. Recent meta-analysis revealed ABL mutation rates with imatinib 9.7%, dasatinib 1.7%, nilotinib 3.3%. The most common specific mutations were T315I, E255k, and M315I. T315I mutations constitute 58% of dasatinib-related mutations and 13% of imatinib-related mutations. These mutations inhibit the binding of the TKIs, hindering the treatment of patients with CML. The introduction of ponatinib, a new formulation of TKI, has been shown to overcome resistance incurred by the T315I mutation (see Chapter 69).

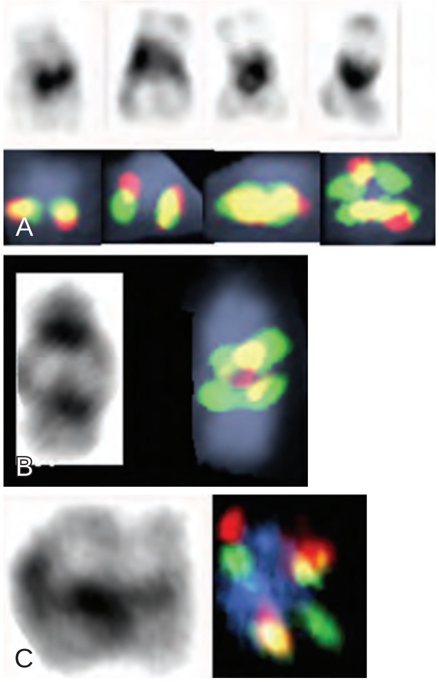


Figure 57.14 EXAMPLES OF UNUSUAL Ph CHROMOSOME ASSOCIATED WITH IMATINIB RESISTANCE AND BLAST CRISIS OF CHRONIC MYELOGENOUS LEUKEMIA. (A) Amplification of the Ph chromosome *(top row)* and the BCR-ABL fusion in a patient treated for 3 months with imatinib. The patient developed five copies of the Ph chromosome and five copies of the BCR-ABL fusion *(yellow)*. (B) G-banding of dicentric Ph chromosome *(left)* dic der(22)t(9;22)(q34;q11.2) and after FISH studies *(right)* showing two copies of BCR-ABL fusion. (C) G-banding of isoderivative Ph, ider(22)t(9;22)(q34;q11.2) *(left)* and after FISH studies showing two copies of BCR-ABL fusion *(yellow)* on the end of both arms. *FISH*, Fluorescence in situ hybridization.

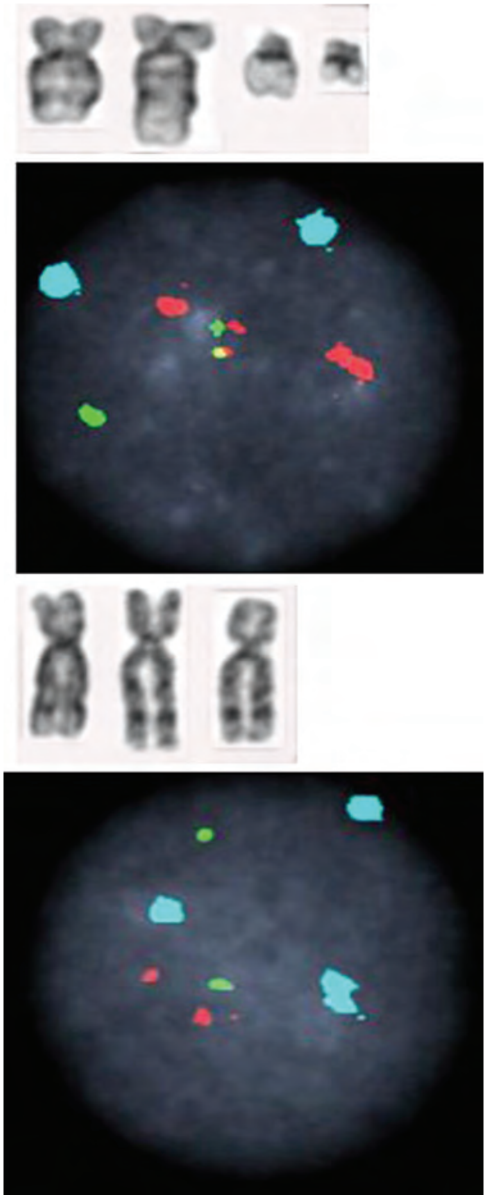


Figure 57.15 TWO DIFFERENT CELL POPULATIONS FROM A PATIENT TREATED WITH IMATINIB. One population shows t(9;22) (*top row*, partial G-banded karyotype) and the BCR-ABL fusion signal *(yellow, second row)* as well as disomy 8 *(aqua)*. In contrast, the second population shows trisomy 8 (*third row*, partial G-banded karyotype) in the BCR-ABL fusion-negative cells *(fourth row)*, showing disomy for the *BCR (green)*, ABL *(red)*, as well as trisomy for chromosome 8 *(aqua)* (see text for details).

Between 5% and 8% of patients undergoing treatment with imatinib will develop additional chromosomal abnormalities (ACAs) such as trisomy 8, monosomy 7, del(20q), and other anomalies in *BCR-ABL1* fusion-negative cells. Imatinib may induce chromosomal abnormalities in BCR-*ABL1*$^{-}$ cells. Alternatively, imatinib may uncover chromosomal abnormalities present before therapy after significant reduction of Ph-positive cells has occurred (Fig. 57.15). Presence of +8 and other chromosomal anomalies in Ph-negative cells in patients treated with imatinib suggests that CML has a multistep pathogenesis and that clonal Ph-negative cells precede the development of the Ph-positive clone (Fig. 57.16). This important observation about the pathogenesis of CML demonstrates the power of conventional cytogenetics, even in the era of molecular assays, and should be used at least annually while patients are undergoing TKI treatment. This hypothesis that clonal development occurs in Ph-negative cells before development of the Ph chromosome was recently confirmed using NGS by targeting 25 genes frequently mutated in myeloid disorders. Ph-negative clones were analyzed in 14 patients who developed clonal cytogenetic abnormalities during TKI treatment. Mutations affecting *DNMT3A*, *EZH2*, *RUNX1*, *TET2*, *TP53*, *U2AF1*, and *ZRSR2* were detected in 43% of these patients. In two patients, the mutations were also found in corresponding Ph-positive diagnostic samples. Moreover, somatic mutations in addition to *BCR-ABL1* affecting *ASXL1*, *DNMT3A*, *RUNX1*, and *TET2* genes are found in 33% of patients. When individual hematopoietic colonies were analyzed from patients with CML at diagnosis, most mutations were present in the Ph-positive cells. By contrast, NGS of subsequent samples during TKI treatment revealed that one *DNMT3A* mutation occurred in Ph-negative cells that was also present in Ph-positive cells at diagnosis, strongly implying that *DNMT3A* preceded the appearance of the *BCR-ABL1* rearrangement which provides support for the initial hypothesis that *BCR-ABL1* and the formation of the Ph chromosome were not the initiating events in the pathogenesis of CML. Another example of clonal evolution in MPNs evolving over time is the co-occurrence of the *JAK2V617F* mutation and *BCR-ABL1*. These rare patients usually have a long (10 to 20 years) history of *JAK2V617F*+ polycythemia vera (PV) before *BCR-ABL1* and the Ph chromosome is acquired. Single cell genotyping revealed that both *JAK2V617F* and *BCR-ABL1* can occur concurrently in hematopoietic stem cells and that *JAK2V617F*+ occurs before the acquisition of *BCR-ABL1*. The contribution of *BCR-ABL1* to disease progression appears to be greater than that of *JAK2V617F*, since these patients display a clinical phenotype that is consistent with CML rather than PV. Recent studies have demonstrated the presence of somatic mutations in *TET2*, *DMNT3A*, and *ASXL1* in both diagnostic and/or follow up remission CML specimens implying evolution within the Ph-negative clone and indicating that these mutations are early events

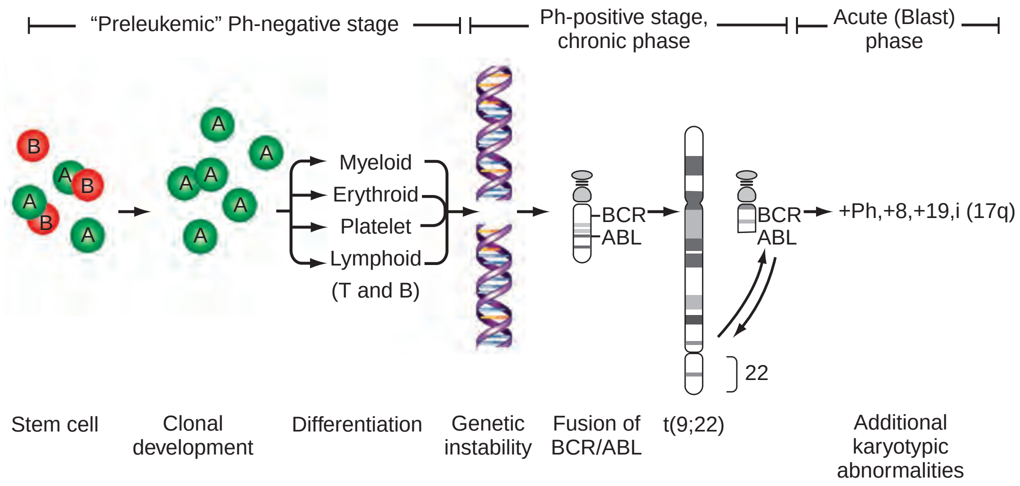


Figure 57.16 HYPOTHETICAL MODEL OF MULTISTEP PATHOGENESIS OF Ph-POSITIVE CHRONIC MYELOGENOUS LEUKEMIA. The first detectable event is a clonal proliferation of cells that are capable of differentiating to all hematopoietic lineages. These cells are genetically unstable and give rise to BCR-ABL fusion and the Ph chromosome. The blast crisis is characterized by nonrandom abnormalities occurring in a genetically unstable Ph-positive clone. At least six events can be delineated. *(Courtesy Dr. W Raskind, University of Washington, Seattle.)*

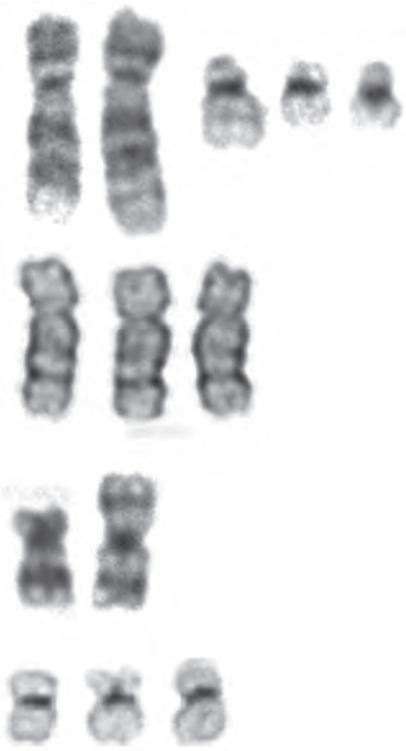


Figure 57.17 THE FOUR MOST FREQUENT ABNORMALITIES ASSOCIATED WITH THE BLAST CRISIS OF CHRONIC MYELOGENOUS LEUKEMIA. Duplication of the Ph chromosome *(top row)* is identified in about 30% of patients, trisomy 8 *(second row)* is found in 30%, isochromosome of the long arms of chromosome 17 *(third row)* is found in 20%, and gain of chromosome 19 *(fourth row)* is seen in approximately 12% of patients with blast crisis of chronic myeloid leukemia.

which preceded formation of the *BCR-ABL1* gene. The mechanism of transformation to advanced-phase CML is complex and varied and remains poorly understood.[8] In myeloid blast crisis (70%) of CML patients show karyotypic evolution, that is, new chromosomal abnormalities in very distinct patterns in addition to the Ph chromosome. The most common abnormalities include gain of chromosome 8 (33%) or +19 (12%), gain of a second Ph chromosome (30%), i(17q)(20%), alone or in combination, to produce modal chromosome numbers of 47 to 50 (Figs. 57.17 and 57.18). In males, LOY is frequently observed. Isochromosome 17q occurs almost exclusively in myeloid blast crisis. Other less frequently observed abnormalities include monosomy of chromosomes 7 and 17, and trisomy of chromosomes 17 and 21. In addition to these common karyotypic evolutions, additional chromosomal aberrations specific for AML—for example, t(8;21) (Fig. 57.19), inv(16), t(3;21)(q26;q22), and most frequently, inv(3q)/t(3;3), resulting in the overexpression of *MECOM (EVI1)*—have been observed. Simultaneous presence of t(9;22) and inv(3) at diagnosis or following TKI therapy appears to be associated with a lack of response to treatment and a clinical course is similar to that of cases with inv(3) without any other chromosomal abnormality. In patients treated with TKI, these ACAs have been classified into two groups based on their impact on survival. ACAs in the Good prognostic group include trisomy 8, LOY, and an extra copy of the Ph chromosome. The second group includes chromosomal abnormalities that are associated with a relatively poor prognosis including i(17q), −7/del(7q), and 3q26.2 rearrangements. The concurrent presence of two or more ACAs was associated with an inferior survival. A prospective study of 1551 imatinib-treated chronic phase patients (German CML-study IV) defined more precisely a high-risk and a low-risk group based on the presence of ACAs. High-risk chromosome abnormalities and their impact on survival included +9,+Ph, i(17q),+17,+19, 3q26.2, 11q23, −7/del(7q), and a complex karyotype, while the low-risk group included all other karyotypic changes. High-risk abnormalities were observed in 6% of patient during chronic phase but in 61% of patients who progressed to blast crisis while low-risk chromosome abnormalities were observed in 21% during CP and in 2.8% of patients who progressed. Most importantly, this study demonstrated that the combination of high-risk cytogenetic abnormalities and a low-level blast cell count (1% to 5%) was associated with disease progression and death from CML. Therefore, additional high-risk chromosomal abnormalities are an early marker of CML progression even when present in patients with low numbers of blast cells.

Complete cytogenetic remission for patients in accelerated phase or blast crisis CML treated with imatinib is rarely accompanied by a normal karyotype; however, 6% to 17% of these patients may have some cytogenetic response (see box on Genetic Testing for Chronic Myeloid Leukemia).

High-resolution cytogenomic studies have demonstrated that in contrast to the chronic phase CML (which is characterized only by genomic imbalances in or around *BCR* and *ABL1*, specifically deletions), during the blast phase, many additional genomic imbalances

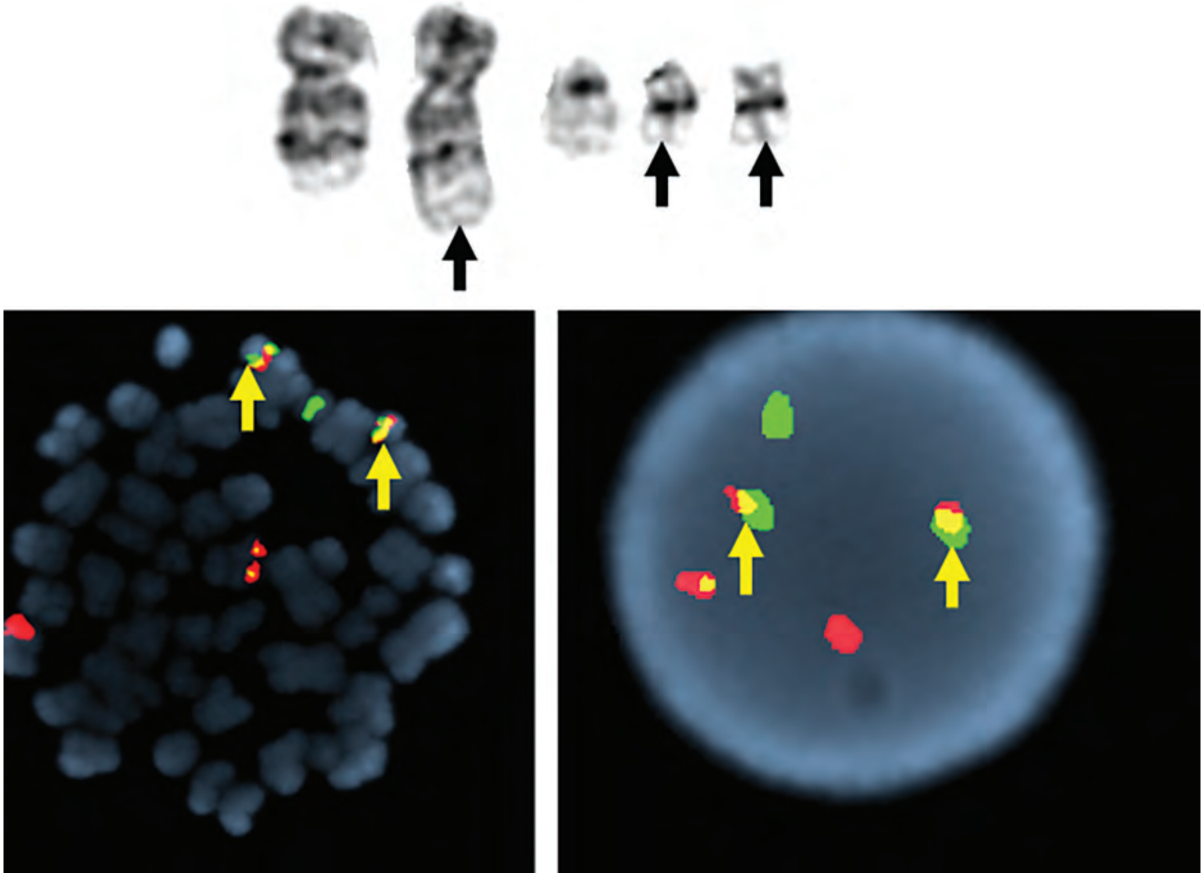


Figure 57.18 BLAST CRISIS OF CHRONIC MYELOID LEUKEMIA. A partial karyotype at the diagnosis of the blast stage of CML was 47,XX,t(9;22)(q34;q11.2 +der(22)t(9;22). The partial karyotype shows two copies of the Ph chromosome as well as one copy on 9q+ *(black arrows)*. Bottom left panel shows two copies of the *BCR-ABL1* fusion on the two Ph chromosomes after FISH hybridization with the dual-color extra sensitive BCR-ABL FISH probe *(yellow arrows)*. The bottom right panel shows bone marrow interphase cells with two fusion signals *(yellow) (yellow arrows)*. Note a tiny bit of yellow signal at the edge of a big red (ABL) signal, consistent with the ES probe to reduce the number of false positive cells. *CML*, Chronic myeloid leukemia; *FISH*, fluorescence in situ hybridization.

GENETIC TESTING FOR CHRONIC MYELOID LEUKEMIA

At diagnosis of CML, perform quantitative cytogenetic analysis using the bone marrow aspirate, which is the sine qua non because peripheral blood cells rarely contain sufficient numbers of mitotic cells at the time of presentation. If the bone marrow aspirate is a "dry tap," perform interphase FISH using a BCR-ABL1 extra-sensitive dual-fusion color probe. According to the ELN 2020 and British Society of Haematology 2020 recommendations, a full bone marrow karyotype is important also to determine the phase of the disease. Their recommendations also include mandatory molecular work to identify the BCR-ABL1 fusion transcript type for molecular monitoring to guide future management. To monitor patients with CML during therapy, use FISH of blood or bone marrow to track changes in the percentage of cells with BCR-ABL1 fusion at 3-month intervals. If the molecular assay demonstrates BCR-ABL1, but the Ph cannot be identified by cytogenetics, a FISH test is required. Once the patient is in complete cytogenetic and FISH remission, the consensus recommendation is to perform real-time quantitative polymerase chain reaction and to follow the patient at 3-month intervals until molecular remission is achieved. According to ELN 2020 recommendations, greater than 10% BCR-ABL1 at 3 months indicates treatment failure. BCR-ABL1 transcript level at less than 0.1% is defined as major molecular response remission. Early indicators of progression are the appearance of additional chromosomal abnormalities and somatic mutations. At relapse, perform a cytogenetic analysis to assess the karyotype of the malignant clone and to determine if a new chromosomally abnormal clone has developed or a new subclone has evolved from the Philadelphia chromosome–positive clone.

occur. Using aCGH, 44 patients in chronic phase CML as well as 11 in myeloid and in lymphoid blast crisis were investigated, and a spectrum of recurrent genomic imbalances was associated with disease progression, including losses at 1p36, 5q21, 9p21, and 9q34 and gains at 1q, 8q24, 9q34, 16p, and 22q11. The most common mutations in myeloid blast phase involve tumor suppressor gene *TP53* (about 25% of cases) and the *RUNX1* in about 40% of cases. Moreover, the *MYC* oncogene has been recently linked to *BCR-ABL1* activity and CML progression to myeloid blast crisis. Recently, mutations in genes frequently found in Ph-negative MPNs such as *CBL*, *TET2*, *ASXL11*, and *IDH1/2* were also identified in patients in accelerated/blast phase. Additionally, deletions of *RB1*, gain of function mutations in *GATA-2* and *RAS* are just some of the spectrum of cytogenomic changes characterizing progression of CML.

Patients with CML may present initially in lymphoid blast crisis mimicking Ph-positive B-cell ALL. Although both the CML and Ph-positive ALL (see later) are driven by the constitutively active *BCR-ABL1* tyrosine kinase, their prognoses differ significantly and it is important to differentiate these two entities. Cytogenomic analysis of 78 CML patients in lymphoid blast crisis demonstrated a unique signature of genomic deletions within the Ig heavy-chain and *TCR* genes, indicating that their presence is essential for the development of a malignant clone with a lymphoid phenotype. Moreover, 50% of patients in lymphoid blast crisis had mutations in cyclin-dependent kinase inhibitor *CDKN2A*, 25% to 35% in *RUNX1*, and ~15% to 25% have mutation in the *BCOR* gene, which interacts with histone deacetylates, a known apoptosis regulator. One of the most important

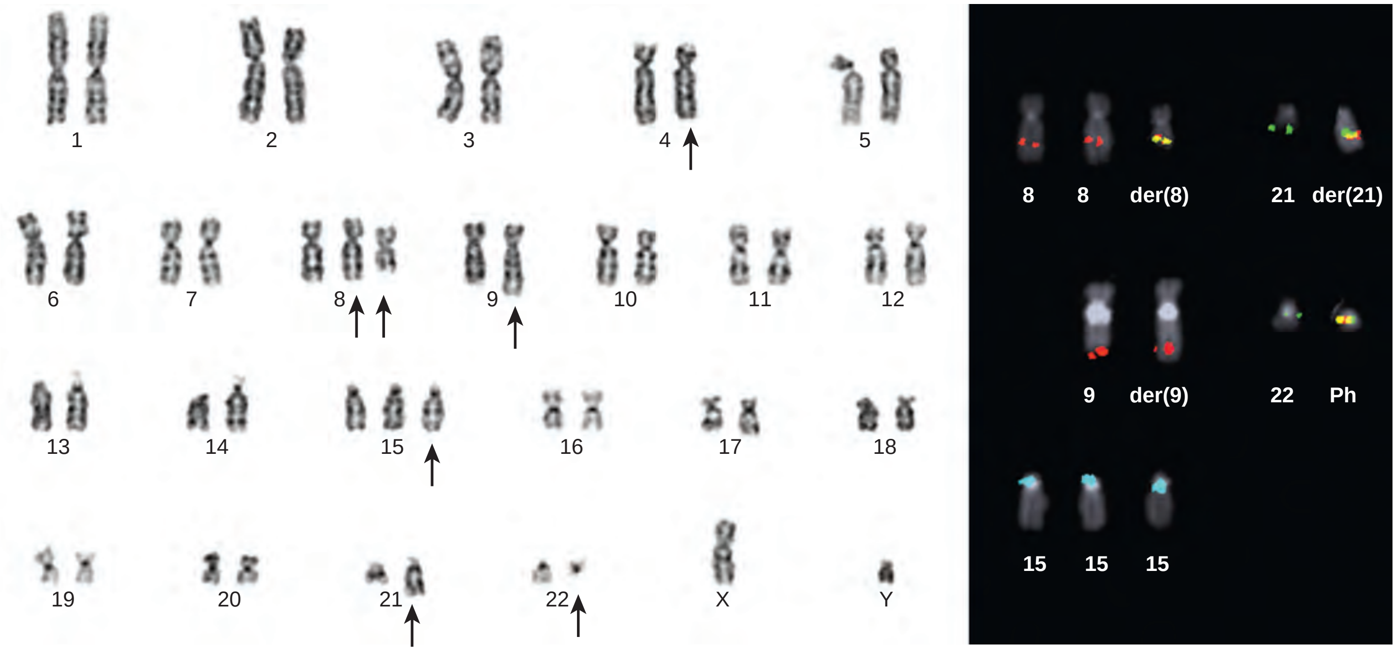


Figure 57.19 SIMULTANEOUS DETECTION OF t(9;22) AND t(8;21) IN A PATIENT WITH BC-CML. A karyotype *(left)* from a patient with chronic myelogenous leukemia who failed three tyrosine kinase inhibitor treatments and progressed into blast crisis showing 46,XY, del(4)(q31q33), t(8;21)(q22;q22), +8, t(9;22)(q34;q11.2), +15. Metaphase FISH studies *(right panel)* confirmed that the BCR-ABL1 fusion-positive cells *(middle row, yellow* co-localization), the patient had RUN1-RUNXT1 fusion as a result of t(8;21) [*yellow* signals on der(8) and der(21)] and a gain of chromosome 8 *(top row)* as well as trisomy 15 *(bottom row)* as shown using centromeric FISH probe specific for chromosome 15 *(aqua)*. *CML*, Chronic myeloid leukemia; *FISH*, fluorescence in situ hybridization.

differences between lymphoid and myeloid blast crisis is the exclusive interaction with STAT proteins. STAT1 is activated mainly by p190, while STAT3 and STAT5 are preferentially activated by the P210 isoform.

The precise causes and mechanisms responsible for CML progression are still elusive and current thinking is that progression of CML is the result of increased and complex genomic instability combined with heterogeneity of numerous coexisting mutations and signaling disruptions causing defective or an insufficient ability to repair DNA.

Ph-Negative Chronic Myeloproliferative Neoplasm

Ph-negative MPNs are disorders arising in a single clone of multipotent precursor cells in which one or all myeloid lineages are abnormally amplified. Classic and more frequently encountered Ph-negative MPNs include PV, primary myelofibrosis, and essential thrombocytopenia (ET). Their cytogenetic and genomic profiles are described in Chapters 70–73. However, included in this chapter are Fig. 57.20A and B and 57.21 in order to illustrate the most frequent abnormalities associated with the Ph-negative MPN. Different 9p chromosomal abnormalities result in *JAK2* numerical gain (see Fig. 57.20) while nine examples of 1q gain, associated with disease progression, are shown in Fig. 57.21.

Less frequently encountered similar disorders include chronic neutrophilic leukemia (Chapter 72), hypereosinophilic syndrome/chronic eosinophilic leukemia (Chapter 74), systemic mastocytosis (Chapter 75), atypical CML, and unclassifiable MPNs (Chapters 72–75). Rare recurrent balanced abnormalities in atypical Ph-negative MPNs can involve the *PDGFRB* gene localized at 5q33, *FGFR1* gene on 8p11, and *PDGFRA* gene on 4q12. The best described are t(5;12)(q33;p13), resulting in *ETV6-PDGFRB* fusion protein, and t(8;13)(p11;q12), resulting in the *ZNF198-FGFR1* fusion gene. The disorder known as *8p11 myeloproliferative syndrome* has been classified by WHO as belonging to the group of myeloid/lymphoid neoplasms associated with eosinophilia and gene rearrangements. Some of these diseases are related to fusion genes between *FGFR1* located on 8p11 and various translocations fusing at least 14 different 5′ partner genes to the 3′ part of the *FGFR1* gene that encodes tyrosine kinase domain (Table 57.4). They include *TPR* (1q25), *LRRFIP1* (2q37), *SQSTM1* (5q35) *FGFR10P* (6q27), *TRIM24* (7q34), *CUX1* (7q22), *PLAG1* (8q12), *CEP110* (9q33), *NUP98* (11p15*), FGFR1OP2* (12p11), *CPSF6* (12q15), *ZMYM2* (13q12), *MYO18A* (17q23), *HERVK* (19q13), and *BCR* (22q11) (Fig. 57.22). The most frequent translocation is t(8;13), and cases with the submicroscopic deletion of the 5′ part of the *FGFR1* gene have also been reported, similar to the formation of t(9;22) with deleted sequences from der(9q) and/or 22q identified in CML. The presence of 8p11 abnormalities in both myeloid and lymphoid cells suggests its origin from a common stem cell.

In patients with chronic eosinophilic leukemia/hypereosinophilic syndrome (Chapter 74) the most frequent recurrent abnormality is the cryptic deletion on 4q12 as a result of *FIP1L1-PDGFRA* fusion gene reported to occur in from 3% to 56% of cases. The disparity in frequencies reflects differing levels of stringency criteria in the diagnosis of disorders with hypereosinophilia as well as different technologies used for detection of *FIP1L1-PDGFRA* fusion. An investigation of 376 patients with persistent unexplained hypereosinophilia revealed an 11% incidence of the *FIP1L1-PDGFRA* fusion gene detected using highly sensitive RQ-PCR. Patients with *FIP1L1-PDGFRA* fusion are characterized by a male predominance, marrow fibrosis, increased number of mast cells, elevated serum tryptase levels, and a favorable response to low doses of imatinib. Most of these patients have a normal karyotype because a cryptic deletion of *CHIC2* locus on 4q12 which is only 800 kb in size. This abnormality is detectable using a more sensitive FISH technology and RQ-PCR in the majority of cases. Moreover, the commercially available tricolor FISH probe will detect not only deletion 4q12 as a result of *FIP1L1-PDGFRA* but also a rare *BCR-PDGFRA* fusion resulting from the t(4;22)(q12;q11.2) rearrangement (Fig. 57.23). Serial monitoring with RQ-PCR demonstrates exquisite sensitivity of *FIP1L1-PDGFRA*-positive patients to low-dose imatinib treatment (Chapter 74). The *FIP1L1-PDGFRA* has also been detected in histopathologically defined cases of systemic mastocytosis with associated eosinophilia, and in cases of AML and T-cell lymphoblastic lymphoma associated with eosinophilia. In an Italian prospective cohort of 27 patients with *FIP1L1-PDGFRA* who were treated with imatinib, a complete hematologic response was achieved within 1 month and all patients became PCR-negative for *FIP1L1-PDGFRA* after a median of 3 months (range 1 to 10 months). In contrast to CML, very few cases of acquired imatinib resistance have been reported. Those rare reported cases had a mutation in T674I within the ATP-binding domain of PDGFRA, analogous to the T315I mutation in CML.

Although translocations involving *PDGFRB* at 5q32 region have been identified with 22 different fusion partners, they are indeed very rare. Any patient with a 5q31–q33 chromosomal abnormality with or without eosinophilia should be investigated for *PDGFRB* rearrangements by FISH testing (Fig. 57.24).

Once the *PDGFRA, PDGFRB*, and *FGFR1* rearrangements are excluded, the only other recurrent secondary abnormalities include trisomy 8 and trisomy 21 (see box on Genetic Testing for Ph-Negative Myeloproliferative Neoplasms).

GENETIC TESTING FOR Ph-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS

At diagnosis, perform a real-time alleles-specific polymerase chain reaction for JAK2V617F, as well as mutational studies for CALR and MPL. Cytogenetic analysis of marrow cells is recommended for patients with essential thrombocytopenia and polycythemia vera. Unstimulated peripheral blood can be used instead of marrow aspirate for patients with primary myelofibrosis patients. Perform FISH studies with BCR-ABL1 for patients with essential thrombocythemia to exclude the diagnosis of chronic myeloid leukemia. FISH studies can be performed when cytogenetics is uninformative for detection of the most frequent abnormalities: +1q, +8, +9, +9p, del(13)(q14), del(20)(q11q13). A panel of five probes includes CEP9, CDKN2A/B at 9p21, D8Z2 as a centromeric probe for chromosome 8, RB1 for deletion 13q, and D20S108 for deletion of 20q12. For patients with a normal karyotype perform aCGH+SNP testing because 68% of these patients have copy number abnormalities including cnLOH. For diagnostic purposes and to monitor treatment response in patients with hypereosinophilic syndrome, perform FISH using triple-color FIP1L1 probe. For 5q32 rearrangements perform FISH with a PDGFRB FISH probe.

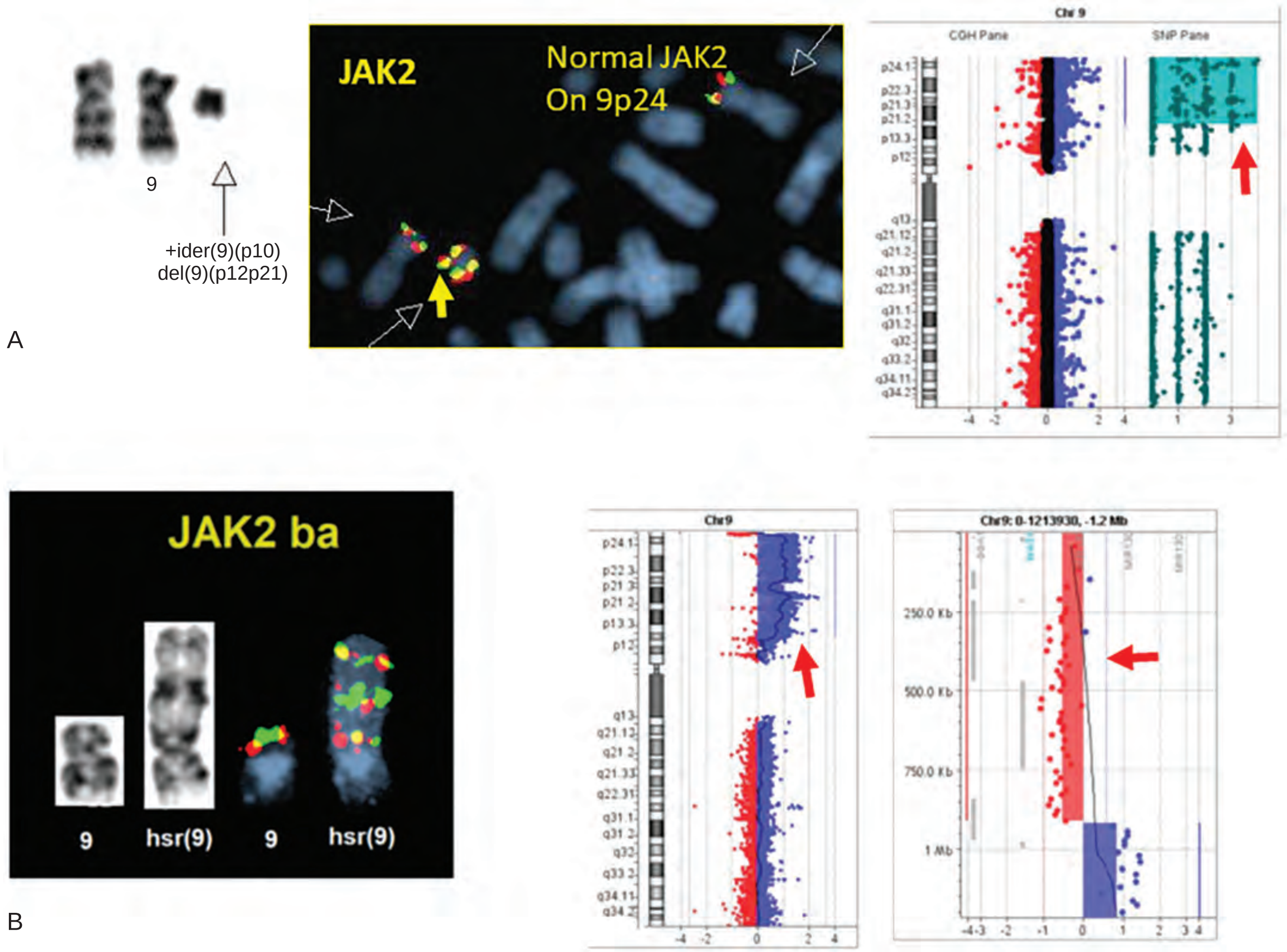


Figure 57.20 TWO DIFFERENT TYPES OF 9P CHROMOSOMAL ABNORMALITIES AND SUBSEQUENT *JAK2* COPY NUMBER ALTERATIONS IN Ph-NEGATIVE MYELOPROLIFERATIVE NEOPLASM. (A) Tetrasomy of *JAK2* in a newly diagnosed patient with myelofibrosis. The initial full karyotype at diagnosis was 47,XX,+mar. Following metaphase FISH evaluation using JAK2 breakapart probe, the karyotype was revised to 47,XX,+ider(9)(p10)del(9)(p12p21) resulting in tetrasomy of *JAK2*. The panel on the right shows the results obtained from array CGH+SNP indicating a gain of 24.7 Mb on the short arms of chromosome 9 including the *JAK2*. (B) Homogenous staining region (HSR) and amplification of *JAK2* in a patient with myelofibrosis. The cytogenetic results showed 20% of cells with sole del(20q) and 80% of cells had a complex karyotype including intrachromosomal complexity such as chromothripsis detected within chromosomes 1, 15, and 20 (not shown) and amplification of 9p (right panel), including *JAK2* and cn LOH of 17p involving TP53 (not shown). *CGH*, Comparative genomic hybridization; *FISH*, fluorescence in situ hybridization; *SNP*, single-nucleotide polymorphism

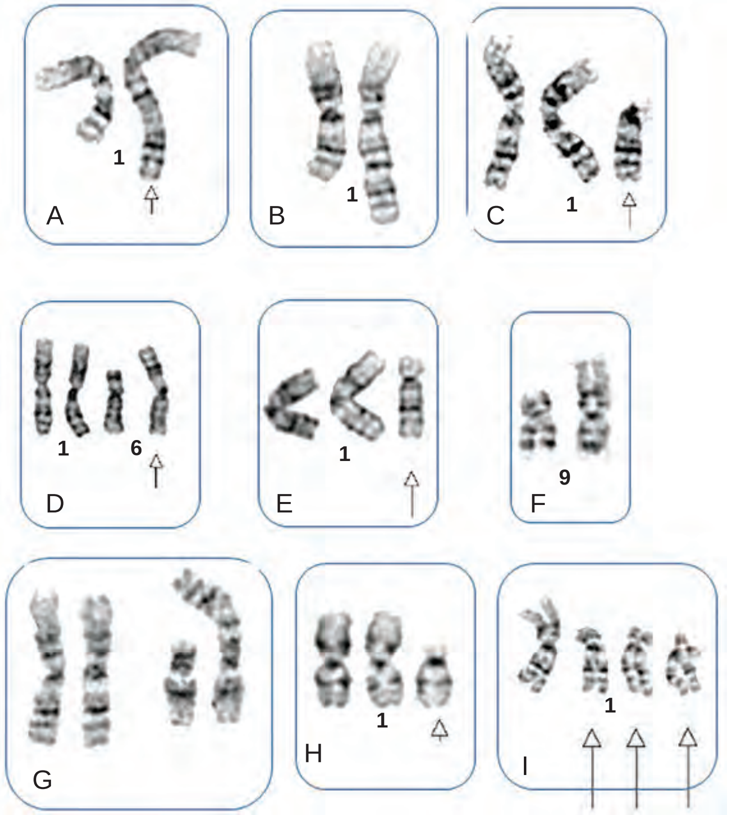


Figure 57.21 NINE TYPES OF 1q GAINS IN PATIENTS WITH Ph-NEGATIVE MYELOPROLIFERATIVE NEOPLASM WHO EITHER HAD +1q AT DIAGNOSIS OR PROGRESSED TO MYELOFIBROSIS AND DEVELOPED +1q. (A and B) Shows different sizes of duplicated 1q segment in two patients: 1q24 shown in (A) is a smaller 1q segment and breakpoint at 1q32 shown in (B) a larger duplicated gain of 1q. (C) Almost an entire long arms of chromosome 1 are translocated to the short arms of chromosome 19 resulting in gain of 1q and loss of 19q. (D) This der(6)t(1;6)(q21;p21.3) rearrangement is a recurrent abnormality both in Ph-negative MPN and MDS and most frequently observed in patients characterized with jumping 1q phenomenon. Often the extra copy of 1q is translocated to the short arms of chromosome 6 and very rarely to the long arm. (E) Another recurrent gain of 1q is the translocation to acrocentric chromosomes such as 22 without apparent loss of any genetic material and is described as +1,der((1;22)(q10;q10). (F) der(9)t(1;9)(p24;p21) is rarely observed in Ph-negative MPN and more frequently in MDS. In this unbalanced translocation there is a loss of terminal band from 9p and a gain of distal 1q segment. (G) der(12)(t(1;12)(q21;p13) is a recurrent abnormality in the (H) +1,del(1)(p13p36.1), (I) der(1)t(1;9)(p13;p13), + der(1)t(1;9)(p13;p13) x2 resulting in tetrasomy for 9p and 1q. *MDS*, Myelodysplastic syndrome.

In the WHO 2016 classification, *mastocytosis* is no longer listed as a Ph-negative MPN (Chapters 56 and 75) but rather as a distinct category, "Mastocytosis." Mastocytosis includes a group of rare and heterogeneous diseases characterized by accumulation of clonal mast cells in one or multiple organs. Over 80% of patients with systemic mastocytosis (Chapter 75) are characterized by *KIT* mutations, specifically in the activation loop of *KIT*, *KIT* D816V. *KIT* is encoded by a 21-exon-containing gene located on the long arms of chromosome 4, 4q12. Other rare *KIT* mutations have been described in adult and pediatric patients. The knowledge of the type and structure of *KIT* mutation is important because the A-loop mutations D816V/H/Y/N disrupt the structure of the receptor, leading permanently to an active conformation and resistance to imatinib. In patients with advanced systemic mastocytosis additional mutations have been observed including *TET*2 (up to 39%), *SRSF2* (up to 36%), *ASXL1* (up to 21%), and *RUNX1* (23%). At diagnosis of mastocytosis patients testing for *KIT* D816V in peripheral blood leukocytes, using highly sensitive and specific PCR-based assays, is necessary to determine the *KIT* allelic burden rapidly, which is now used as a gold standard for assessing disease burden.

MYELODYSPLASTIC SYNDROMES

The MDSs (see Chapters 61 and 65) are a clinically heterogeneous group of hematologic neoplasms with differing biology and clinical manifestations. They have in common a clonal origin, dysplastic cellular morphology, cytopenias, abnormalities of cellular maturation, and an increased propensity to develop acute leukemia (20% to 40%). They predominantly occur in elderly people. Cytogenetic studies can provide both diagnostic and profound prognostic information regarding OS and the risk of transformation to AML. A chromosomally abnormal clone can be detected in 50% to 60% of patients with de novo MDS and in approximately 90% of patients with therapy-related MDS (Table 57.5). There appears to be a correlation between the frequency of chromosomal abnormalities and the severity of disease. Approximately 35% of patients with less aggressive MDS, such as refractory anemia and refractory anemia with ring sideroblasts, have clonal chromosomal rearrangements, whereas approximately 60% to 70% of patients with refractory anemia with excess blasts in transformation have such chromosomal abnormalities. A single or complex chromosomal abnormality may be present initially, and cytogenetic progression may occur during the course of the disease. Even at diagnosis, complex genomic lesions involving five or more different chromosomes are not unusual. Despite the heterogeneity of chromosomal defects (gain, loss, deletion, amplification, rare balanced translocations, transcriptional silencing via methylation, or point mutation), the unifying concept of genetic instability in MDS is hemizygosity of specific genes or chromosomal regions. The heterogeneity of chromosomal abnormalities is overwhelming. However, the most common chromosomal anomalies observed in MDS include gain of 1q, del(5q)/−5, del(7q)/−7, trisomy 8, del(11)(q23), del(12p), +13/del(13q), t(11q23), del(17p), del(20)(q11q13), +21, and idic(X)(q13).

TABLE 57.4 Chromosomal Translocations in Ph-Negative Myeloproliferative Neoplasms

Chromosomal Abnormality	Genes Involved	MPD Entity
t(9;12)(q34;p13)	*ETV6-ABL*	CML-like, T-ALL
5q32	*PDGFRB*	Associated translocations
t(1;5)(q25;q32)	*TPM3-PDGFRB*	Atypical MPN
t(2;5)(p21;q32)	*SPTBN1-PDGFRB*	Atypical MPN
t(4;5)(q21;q32)	*PRKG2-PDGFRB*	Atypical MPN
t(5;7)(q32;q11)	*HIP1-PDGFRB*	CMML-like
t(5;10)(q32;q21)	*CCDC6-PDGFRB*	Ph-negative MPN
t(5;12)(q32;p13)	*ETV6-PDGFRB*	CEL, CMML
t(5;12)(q32;p13.3)	*ERC1-PDGFRB*	Atypical MPN
t(5;14)(q33;q24)	*NIN-PDGFRB*	Atypical MPN
t(5;14)(q32;q32)	*KIA1509-PDGFRB*	Atypical MPN
t(5;14)(q32;q32)	*TRIP11-PDGFRB*	AML
t(5;15)(q23;q15)	*TP53BP1-PDGFRB*	CEL
t(5;15)(q32;q22)	*TPS3BP1-PDGFRB*	Atypical MPN
t(5;16)(q32;p13)	*NDE1-PDGFRB*	Atypical MPN
t(5;17)(q32;p11)	*HCMOGT1-PDGFRB*	Juvenile CMML
t(5;17)(q32;p11,2)	*MYO18A-PDGFRB*	Atypical MPN
t(5;17)(q32;p13)	*RABEP1-PDGFRB*	CMML
t(5;17)(q32;q21)	*COL1A1-PDGFRB*	Atypical MPN
8p11	*FGFR1*	Associated translocations
t(2;8)(q37;8p11)	*LRRFIP1-FGFR1*	MPN syndrome
t(6;8)(q27;p11)	*FGFR1OP-FGFR1*	Stem cell MPD
t(7;8)(q32;p11)	*TRIM24-FGFR1*	MPN
t(7;8)(q22;p11)	*CUX1-FGFR1*	MPN syndrome
t(8;9)(p11;q22)	*CEP110-FGFR1*	MPN syndrome
t(8;11)(p11;p15)	*Nup98-FGFR1*	MPN syndrome
t(8;12)(p11;p11)	*FGFR1OP-FGFR1*	MPN syndrome
t(8;12)(p11;q12)	*CPSF6-FGFR1*	MPN syndrome
t(8;13)(p11;q12)	*ZMYM2-FGFR1*	MPN syndrome, leukemia, lymphoma
t(8;17)(p11;q11)	*MYO18A-FGFR1*	MPN syndrome
t(8;19)(p11;q13)	*HERVK-FGFR1*	MPN syndrome
t(8;22)(p11;q11.2)	*BCR-FGFR1*	Lymphoproliferative disorder
4q12	*PDGFRA*	
del(4)(q12;q12)	*FIP1L1-PDGFRA*	CEL
t(4;22)(q12;q11.2)	*BCR-FGFR1*	Atypical CML
4q12	*KIT*	SM
9p24	*JAK2*	
t(9;22)(p24;q11.2)	*BCR-JAK2*	CML-like
t(9;12)(p24;p13)	*JAK2-ETV6*	CML-like
der(9)t(9;12)(p24;q13)	*JAK-NF-E2*	MDS
t(8;9)(p22;p24)	*PCM1-JAK2*	CMPD, AL
der(9;18)t(p13;p11)	Not reported	PV, PV→MF
der(9;18)(p10;q10)	Not reported	PV, PV→MF, ET→AML
der(9)t(1;9)(q12;q12)	Not reported	PV, MF
der(1;7)(q10;p10)	Not reported	ET
t(12)(q21 or q21)	Not reported	MF

AL, Acute leukemia; *ALL*, acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *CEL*, chronic eosinophilic leukemia; *CML*, chronic myelogenous leukemia; *CMML*, chronic myelomonocytic leukemia; *CMPD*, chronic myeloproliferative disorder; *ET*, essential thrombocytopenia; *MF*, myelofibrosis; *MPD*, myeloproliferative disorder; *MPN*, myeloproliferative neoplasm; *PV*, polycythemia vera; *SM*, systemic mastocytosis.

Until recently guidelines-based indicators (GBI) for MDS patient management did not receive adequate attention. Several population-based studies reported that approximately 25% of MDS patients do receive bone marrow cytogenetic analyses at diagnosis and the lack of chromosome analysis impeded appropriate risk stratification by IPSS-R. GBI indicators represent a standardized best practice performance, outcome, and clinically relevant guidelines for a patient with MDS. According to the diagnostic guidelines the initial work-up must include cytogenetic analysis and molecular diagnostics/NGS/*TP53* evaluation (among other parameters).

In 2012 a new cytogenetic classification of MDS was established that includes five different risk groups (see Chapter 61).

The clinical relevance and the power of conventional cytogenetics in MDS were recognized by the WHO, which documented a strong association between del(5)(q13q33) and 5q− syndrome. The 5q− syndrome is a unique subtype of low-risk MDS with a favorable prognosis, lack of other cytogenetic abnormalities, low risk of leukemic transformation, and more commonly occurs in older females (Fig. 57.25, *second row*). Patients with isolated del(5q) remains the only MDS subtype defined by the genetic abnormality.

Among patients who do not have 5q− syndrome (with or without other chromosomal abnormalities), interstitial deletion of the long arms of chromosome 5 occur in 10% to 15% of patients and are among the most frequent chromosomal abnormalities in MDS (see Fig. 57.25, *second row*). The finding of del(5q) in CD34CD38− stem cells indicates its origin in a stem cell capable of differentiating into myeloid and lymphoid cell lineages. Data on 1432 patients with del(5q) showed a significant amount of heterogeneity in breakpoints. FISH studies delineated a commonly deleted segment that is currently estimated to be 1.5 Mb in size, on 5q31.1. The clustering of genes responsible for growth and differentiation of hematopoietic cells at this site and recurrent nature of −5/del(5q) in MDS caused many investigators to speculate that (a) tumor suppressor gene(s) was/were located in the 5q31 or 5q22–23 band region. To date, the tumor suppressor gene responsible for MDS on 5q has yet to be identified. Because the mechanism causing the interstitial del(5q) is elusive, haploinsufficiency or inactivation caused by methylation, rather than a typical tumor suppressor gene, has been speculated to be involved in this process.

Patients with isolated del(5q) have a more favorable prognosis and have a more prolonged survival than patients with ACAs. Specifically, patients with del(5)(q13q31) live longer than patients with other 5q deletions, indicating that the type of 5q deletion may significantly affect prognosis and response to therapy. Indeed, lenalidomide

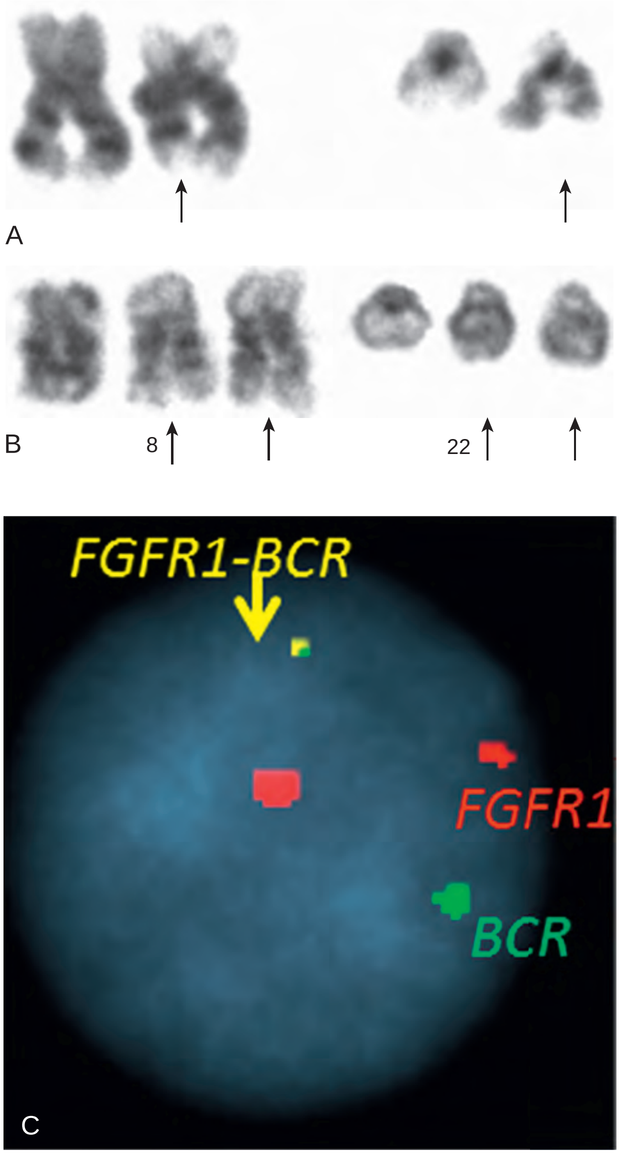


Figure 57.22 (A) A partial karyotype from a patient diagnosed with Ph-negative myeloproliferative neoplasm showing t(8;22)(p11;q11.2). (B) Six months later the patient developed hyperdiploid karyotype, acute lymphoblastic leukemia, and two copies of t(8;22). (C) Interphase FISH confirmed a FGFR1 *(red)*-BCR *(green)* fusion *(yellow)*.

therapy leads to a normal karyotype in 44% of 148 patients with interstitial del(5q) (Chapter 61). Isolated monosomy 7 or 7q deletion occurs in 20% of patients. Frequently, chromosome 7 abnormalities occur with ACA, most commonly rearrangements of 3q or del(12p) (see Fig. 57.25, *third* and *fourth row*). Monosomy 7 is present in all MDS subtypes and is seen predominantly in males. In pediatric patients with constitutional disorders associated with a predisposition to develop AML including Fanconi anemia (FA), congenital neutropenia, neurofibromatosis type 1, Down syndrome (DS), or Kostmann syndrome, −7/del(7q) may be seen as an isolated abnormality (Chapter 30). Therefore the question remains to whether these patients have genetic imprinting and preferentially lose chromosome 7. Unequivocal evidence exists that preferential parental origin of the missing chromosome 7 does not occur; approximately half of the patients have loss of either the maternal or paternal homologue, excluding the genomic imprinting hypothesis. Embryonic origin of partial chromosome 7 deletion in monozygotic twins with juvenile chronic myelomonocytic leukemia has been reported. As shown in Fig. 57.26, acquired cnLOH of 7q is a known recurrent genomic rearrangement in MDS patients not detected cytogenetically but identified using aCGH+SNP, and often detected in patients showing either a normal karyotype or other abnormalities.

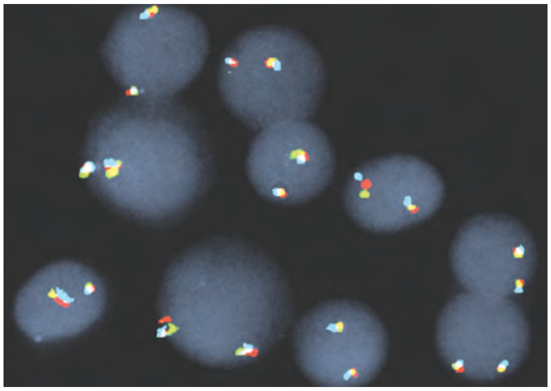


Figure 57.23 BONE MARROW INTERPHASE NUCLEI AFTER FLUORESCENCE IN SITU HYBRIDIZATION STUDIES USING TRICOLOR PROBE FOR CHROMOSOME 4, BAND REGION q12. The *green* color covers an approximately 750-kb region centromeric from FIP1L1. The *red* probe is telomeric of the FIP1L1 gene. The *aqua* color probe begins between exons 15 and 16 of the *PDGFRA* gene and extends toward the 4q telomere. In normal nuclei, as shown here, the probe appears as two tricolor fusions because of close proximity of probes in interphase DNA. Patients with hypereosinophilic syndrome have fusion of FIP1L1 and PDGFRA genes by interstitial deletion and produce one signal with *green-aqua* fusion and a missing orange signal. If the translocation involves the *PDGFRA* gene with loci on other chromosomes, the expected signal pattern is one *orange–green* fusion and one separate aqua signal.

MDS patients with del(7q) as a single abnormality have a distinct clinicopathologic profile with an overall better prognosis than seen in patients with an isolated monosomy 7. Isolated del(7q) is more frequent in patients with less advanced forms of MDS according to the WHO classification or the IPSS-R. They have fewer blasts in the bone marrow than other cytogenetic groups and display a significantly superior survival when compared with patients with isolated monosomy 7. The presence of ACA in patients with del(7q) is associated with shortened OS (see Fig. 57.25, *third row*). Allele typing studies implicated three regions that are most frequently deleted: 7q22, 7q31.1, and 7q31.3. Isolated 7q deletion is a common finding in a variety of myeloid malignancies (MDS, AML, MPN, and others) and as a sole abnormality is present in less than 5% of patients. High-resolution genomic analysis has revealed that among 81 patients with a sole 7q deletion 67 cases had an interstitial deletion while 14 cases showed a terminal 7q deletion. In this cohort, according to the SNP array karyotyping, 85% of patients with interstitial 7q deletion had a commonly deleted regions located at 7q22.1 to 7q31.31. Consequently, there is speculation that a putative myeloid suppressor gene(s) is located in the regions that are frequently deleted. Because prototypic tumor suppressor genes have not been identified in patients with 7q deletions, an alternative explanation may be haploinsufficiency whereby the level of protein is critical, or a complex of two cooperating proteins is affected as a result of inactivation caused by methylation.

Trisomy 8 (see Fig. 57.25, *third row*) is the third most frequent chromosomal abnormality in MDS patients. As a sole abnormality, it is found in 11% of patients with MDS and overall is found in 17% of patients. A significantly higher incidence of trisomy 8 occurs in males than in females. Trisomy 8 is present in all age groups of patients with MDS. Although trisomy 8 is detected in the hematopoietic stem cells of patients with MDS, a sizable fraction of stem cells are disomic but functionally abnormal, suggesting that the acquisition of trisomy 8 is a secondary event. These findings provide evidence for the multistep pathogenesis of MDS, where gain of chromosome 8 is not an early event in the stepwise disease evolution. Although trisomy 8 serves as an intermediate risk factor when detected at diagnosis,

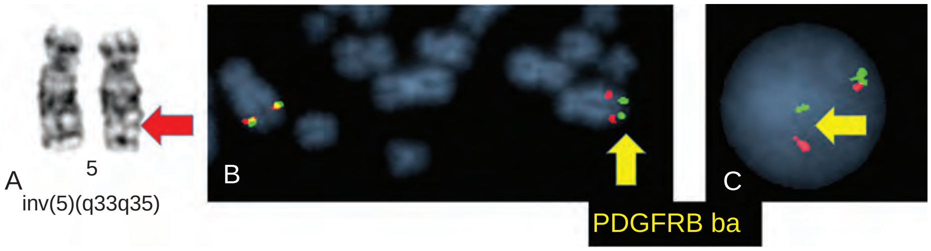


Figure 57.24 A partial karyotype from a patient with Ph-negative MPN showing in (A) panel an inversion of chromosome 5 *(red arrow)* identified cytogenetically in 90% of marrow cells. In panel (B) both chromosomes 5 are shown, after metaphase FISH: the left chromosome 5 has a normal *PDGFRB* while the other homolog 5, with an invasion *(yellow arrow)*, shows the split of the 5′ and 3′ part of the PDGFRB probe, consistent with the rearrangement. The panel (C) shows a bone marrow interphase cell and the yellow arrow points to the split of the red and green signals that reflect rearrangement of the *PDGFRB* gene detected in 28% of non-dividing cells, indicating that this rearrangement in Ph-negative MPN has a higher proliferative capacity than nondividing cells. *FISH*, fluorescence in situ hybridization; *MPN*, myeloproliferative neoplasm.

TABLE 57.5 Recurrent Chromosomal Abnormalities in Primary Myelodysplastic Syndrome

Abnormality	Frequency (%)
−5 or del 5q	10–15
−7 or del 7q	10
trisomy 8	10–17
i(17q) or t(17p)	2–3
del(12p) or t(12p)	1–2
del(11q)	1–2
−13 or del(13q)	1–2
del(9q)	1–2
idic(X)	1
inv(3)(q21q26.2)	1
t(6;9)(p23;q34)	1
t(3;21)(q26.2;q22.1)	<1
t(1;3)(p36.3;q21.2)	<1
t(11;16)(q23;p13.3)	<1
t(2;11) (p21;q23)	<1

Modified from Malcovati L, Hellström-Lindberg E, Bowen D, et al. Diagnosis and treatment of primary myelodysplastic syndromes in adults: recommendations from the European LeukemiaNet. *Blood.* 2013;122:2943.

patients with MDS with trisomy 8 treated with the hypomethylating agent 5-azacitidine have a significantly better survival than patients with other chromosomal abnormalities. Tetrasomy, pentasomy, and hexasomy 8 (polysomy 8) are relatively rare compared to trisomy 8. They have been reported in MDS, AML, or MPN. Tetrasomy 8 was the most common presentation of polysomy 8. In 60.7% of patients, polysomy 8 occurred as part of complex changes (16.2% with 11q23 rearrangements). No cryptic *KMT2A* rearrangements were found in cases when polysomy 8 was the only karyotypic change. Therefore polysomy 8 is a subtype of AML, MDS, and MPN characterized by a high incidence of prior diseases, and poor OS (6 months). Age significantly reduced median survival but associated cytogenetic abnormalities did not modify it. Cytogenetic results further demonstrate an in vitro preferential growth of the cells with a high level of aneuploidy suggesting a selective advantage for polysomy 8 cells.

MDS or MDS/MPN associated with trisomy 11 as a sole abnormality or as a part of noncomplex karyotype has been reported. This rare recurrent abnormality has an overall frequency of approximately 0.3% of MDS patients and is associated with a significantly inferior survival in patients with IPSS intermediate-risk disease ($P = .0002$) but comparable to the poor-risk group ($P = .97$). Trisomy 11 is associated with clinical aggressiveness and represents a high-risk cytogenetic abnormality.

Deletions of 17p are seen in 3% to 4% of MDS and AML patients. These patients often display several other chromosomal rearrangements, including monosomy 17, isochromosome 17q (see Fig. 57.25, *third row*), and unbalanced translocations between chromosome 17 and another chromosome. Approximately 30% of these deletions are related to therapy. The extent of 17p deletion in all cases involves the *TP53* gene. There appears to be a close correlation between dysgranulopoiesis (e.g., pseudo–Pelger-Huet abnormalities) and small vacuoles in neutrophils with 17p abnormalities and *TP53* deletion. The median survival of these patients is poor.

Besides deletion of *TP53*, molecular characterization of MDS with NGS has shown that *TP53* mutant MDS and therapy-related AML have emerged as molecular cohorts with profoundly inferior outcomes regardless of treatment, with a median OS of only 6 to 12 months. In MDS, mutations of *TP53* are commonly seen in patients with complex karyotypes and have a significant negative impact on prognosis. *TP53* mutations are strongly associated with thrombocytopenia, increased blasts, and chromosome 5 abnormalities, isolated del(5q), and complex karyotypes. Presence of *TP53* mutations in patients with a complex karyotype identifies a patient group with an extremely poor prognosis and median OS of 6 months highlighting the unmet need for novel therapeutic approaches in these patients. Recent analysis of 3324 patients with MDS delineated two subsets of patients with distinct phenotypes and outcomes.[9] One-third of *TP53*-mutated patients had monoallelic mutations whereas two-thirds had multiple hits (multi-hit) consistent with biallelic targeting. Established associations with complex karyotype, few co-occurring mutations, high-risk presentation, and poor outcomes were specific for multi-hit patients only. The *TP53* multi-hit state predicted risk of death and leukemic transformation independently of the IPSS-R. Surprisingly, monoallelic patients did not differ from *TP53* wild-type patients in outcomes and response to therapy. These observations show that consideration of *TP53* allelic state is critical for prognostication of MDS patients.

An isodicentric X chromosome in Xq13-idic(X) is a rare but recurrent abnormality that has been associated with refractory anemia with ringed sideroblasts. All MDS patients with idic(X)(q13) are females, most likely because the formation of idic(X) would result in nullisomy for Xq13-qter in males. The median age at the time of diagnosis is 73.5 years. The outcome of idic(X)-positive cases is variable; some investigators report aggressive and rapidly fatal disease and others a relatively favorable clinical course with survival for several years. The fact that idic(X) most often occurs as the sole cytogenetic abnormality suggests that it may be sufficient for leukemogenesis. Using a high-resolution SNP array, the breakpoints on idic(X)(q13) were mapped to two distinct breakpoint clusters, at approximately 70.9 Mb (five cases) and 72.1 Mb (seven cases) on the X chromosome. None of the 11 breakpoints occurred in a gene, strongly indicating that the idic(X)(q13) does not result in a fusion gene. Instead, the functional

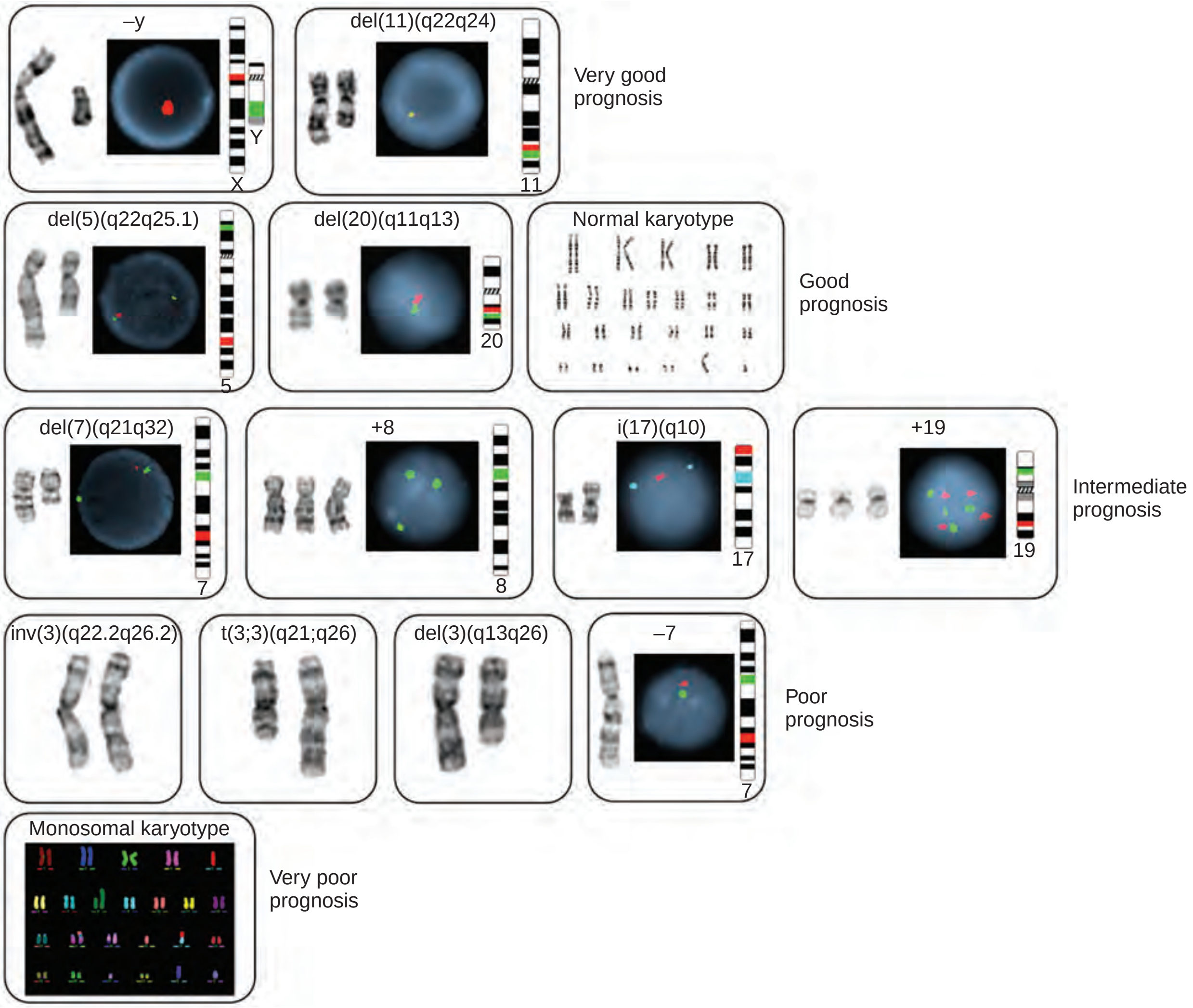


Figure 57.25 PROGNOSTIC SIGNIFICANCE OF THE RECURRENT CHROMOSOMAL ABNORMALITIES IN MYELODYSPLASTIC SYNDROME, ACCORDING TO THE NEW COMPREHENSIVE CYTOGENETIC SCORING SYSTEM.

outcome of the abnormality confers a gene dosage effect because of the concurrent gain of Xpter-q13 and loss of Xq13-qter. This region of the X chromosome is enriched for repeated sequences and most likely, these repeats may facilitate the formation of idic(X). The isodicentric X chromosome was inactive in some patients and active in other patients; hence idic(X) appears to be leukemogenic regardless of X_a or X_i involvement.

Gain of 1q, usually in the form of an unbalanced translocation, is a recurrent abnormality in MDS and appears to be a marker of disease progression. Specifically, the gain of 1q in the form of jumping translocations either at diagnosis or the subsequent acquisition of jumping 1q translocations appears to be associated with imminent transformation to AML in patients after an average of 9 months (Figs. 57.27 and 57.21). As mentioned earlier in the Ph-negative MPN section (Chapters 70 and 72), gain of 1q is also associated with disease progression and is most frequently found at the diagnosis of PV. Once acquired, the patient's prognosis tended to be dependent on the copy number of 1q, indicating that gain of jumping 1q translocations was associated with both disease progression and poor prognosis. Jumping translocations of 1q has also been described in donor cell derived MDS after cord blood transplantation; the average time to developing jumping 1q was less than 2 years. These findings did not provide any common pattern as to the role of the partner chromosomes in unbalanced jumping 1q translocations. However, 81% of recipient breakpoints were localized in pericentric regions whereas the remaining 19% were telomeric fusions. Decondensation of pericentromeric heterochromatin of chromosome 1 together with centromere and repeat DNA sequences interspersed with histones and acetylated sequences may favor illegitimate recombinations leading to jumping 1q translocations. Moreover, exposure to azacitidine has been shown to be associated with alterations of pericentromeric heterochromatin of chromosome 1, as well as an increase in Alu DNA repeats. Hypomethylation of 1q12–21 pericentromeric region of chromosome 1 appears to be at least one aspect of copy number gains of 1q. The mechanism underlying formation of trisomy 1q in MDS has not been investigated but in patients with myelofibrosis we reported that +1q was associated with increased transcripts of *MDM4*, likely leading to further downregulation of *TP53* activity. Elevated *MDM4* levels have been previously found in other hematologic malignancies, including de novo AML. There are numerous other oncogenes that are present on 1q, which could also contribute to disease progression. The overexpression of *CKS1B*, involved in cell cycle regulation, located at band 1q21 has been proposed as the culprit responsible for disease progression in patients with myeloma and gain of 1q (Chapter 91). Therefore, it is possible that in MDS, similar to patients with myelofibrosis with +1q, leads to further dysregulation of the wild-type *TP53* pathway by increasing *MDM4* activity.

The most frequent rearrangement of chromosome 3 involves two bands on chromosome 3—band 3q21 and 3q26 simultaneously—which produces either t(3;3)(q21;q26) or inv(3)(q21q26)

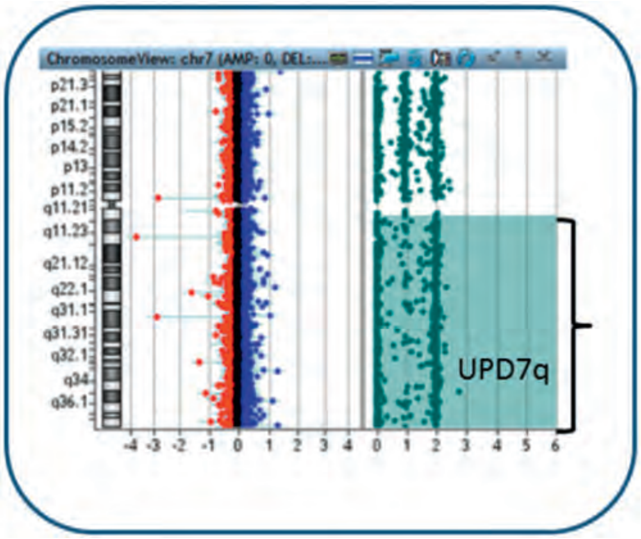
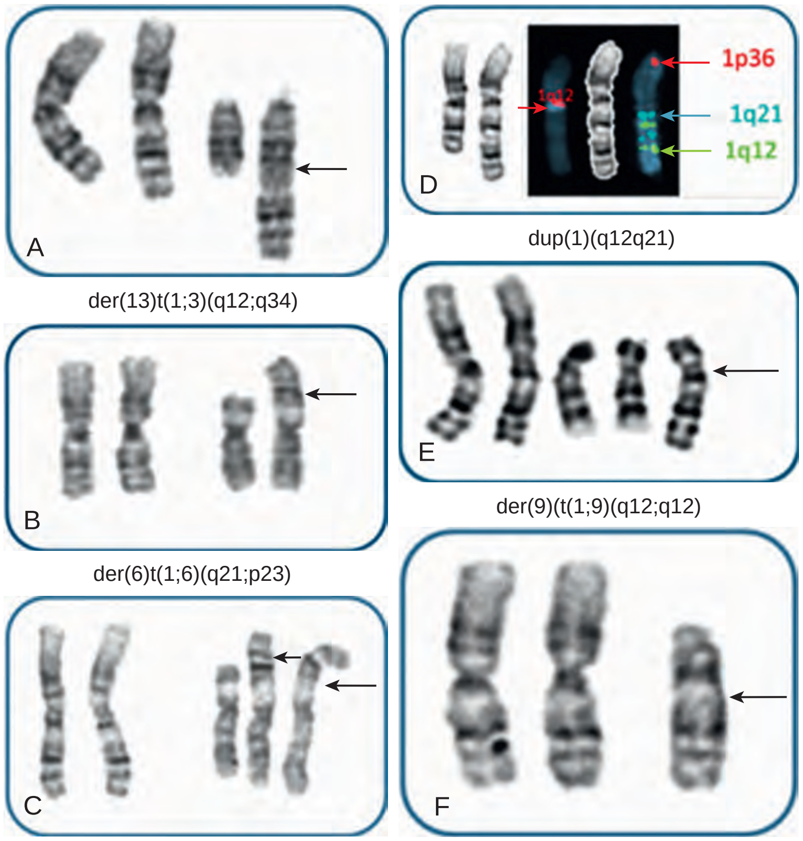


Figure 57.26 ARRAY COMPARATIVE GENOMIC HYBRIDIZATION PLUS SINGLE-NUCLEOTIDE POLYMORPHISM FROM A PATIENT WITH MYELODYSPLASTIC SYNDROME AND NORMAL CHROMOSOME 7. Note acquired uniparental disomy (UPD)/copy-number neutral loss of heterozygosity (cnLOH) of the long arms of chromosome 7, known to be associated with worse prognosis.

(Fig. 57.28A and B). These chromosomal rearrangements are present in de novo and therapy-related MDS, as well as in AML and megakaryoblastic crisis of CML. The incidence of the 3q rearrangements is 2% to 5%. Characteristic clinical features include an elevated platelet count, marked hyperplasia with dysplasia of megakaryocytes, and a poor prognosis with minimal or no response to chemotherapy and a short survival. In addition to similar clinicopathologic features, patients with 3q21q26 share molecular heterogeneity in both the breakpoints and the expression pattern of the genes near these breakpoints (see Fig. 57.28, also Fig. 57.30A.) The chromosomal breakpoints, defined by FISH, in 3q26 are scattered over several hundred kB in either the 5′ or the 3′ region of the *MECOM* (*EVI1* complex locus) gene, whereas the breakpoints in the 3q21 region are restricted to two different smaller genomic clusters approximately 100 kb downstream of the *RPN1* gene. *EVI1* overexpression is observed in the majority of patients, but some patients with the 3q21q26 rearrangement do not have detectable *EVI1* expression, and at least 9% of patients with AML without 3q26 abnormalities overexpress *EVI1*. Therefore the poor prognosis of these patients may be independent of *EVI1* expression, despite the fact that extensive 3q26 breakpoint FISH mapping suggests *EVI1* involvement in numerous novel sporadic and recurrent 3q26 rearrangements. A fusion transcript of *RPN1-EVI1* is rarely observed in patients with 3q21q26 rearrangements. Interestingly, functional genomic studies and allelic-specific analysis revealed experimentally that inv(3)/t(3q) results simultaneously in haploinsufficiency of *GATA2* and upregulation of *MEECOM* as a result of rearrangements in noncoding regulatory sequences of



der(13)t(1;3)(q12;q34)

der(6)t(1;6)(q21;p23)

der(6)t(1;6)(q25;p25)×2

dup(1)(q12q21)

der(9)(t(1;9)(q12;q12)

der(Y)(t(Y;1)(q12;q21)

Figure 57.27 GAIN OF LONG ARMS OF CHROMOSOME 1 IN MYELODYSPLASTIC SYNDROME. Partial karyotypes in these six cases represent examples of gain or duplication of 1q. Gain of 1q in MDS has overlapping features with +1q in Ph-negative MPN and both are associated with rapid progression to AML. (A) Trisomy 1q in the form of der(13) with an extra 1q is translocated to the terminal portion of the long arms of chromosome 13. (B) Gain of 1q in the form of der(6) whereby the entire long arms of 1 are translocated to the short arms of chromosome 6. (C) Two copies of der(6) resulting in tetrasomy 1q in a patient with an advanced myelodysplastic syndrome. (D) Duplication of region 1q12–q21 in a patient with essential thrombocytopenia. (E) Trisomy 1q translocated to the short arms of chromosome 9 resulting in a gain of 9p and 1q in a patient with polycythemia vera (PV) who transformed to myelofibrosis. This is a PV-specific and recurrent abnormality. (F) Trisomy 1q translocated to the terminal portion of the Y chromosome. *AML*, acute myeloid leukemia; *MDS*, myelodysplastic syndrome; *MPN*, myeloproliferative neoplasm.

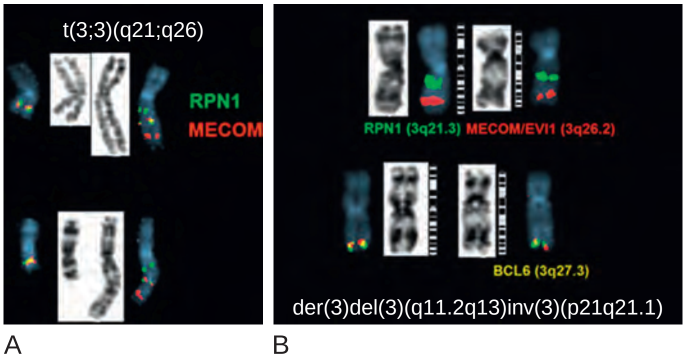

t(3;3)(q21;q26)
RPN1
MECOM
RPN1 (3q21.3) MECOM/EVI1 (3q26.2)
BCL6 (3q27.3)
der(3)del(3)(q11.2q13)inv(3)(p21q21.1)
A
B

Figure 57.28 CHROMOSOME 3 ABNORMALITIES IN MYELODYSPLASTIC SYNDROME. (A) A partial karyotype from a patient with MDS showing a recurrent t(3;3)(q21;q26). In this rearrangement metaphase FISH shown on the left, *RPN1 (green)* and a part of *MECOM (red)* gene on 3q21 giving an impression of a "yellow" signal because of their proximity on a metaphase chromosome. The right chromosome 3 shows *RPN1 (green)* at 3q21, part of *RPN1 (green)* and *MECOM (red)* on 3q26 as well as unrearranged *MECOM* translocated from the left chromosome 3 homolog. (B) An unusual chromosome 3 rearrangement in MDS showing a deletion of 3q11–q13 region, followed by inversion with two breakpoints at p21 and q21.2. All three genes *RPN1 (green), MECOM (red)*, and *BCL6 (yellow)* remained in their normal loci. *FISH*, fluorescence in situ hybridization; *MDS*, myelodysplastic syndrome.

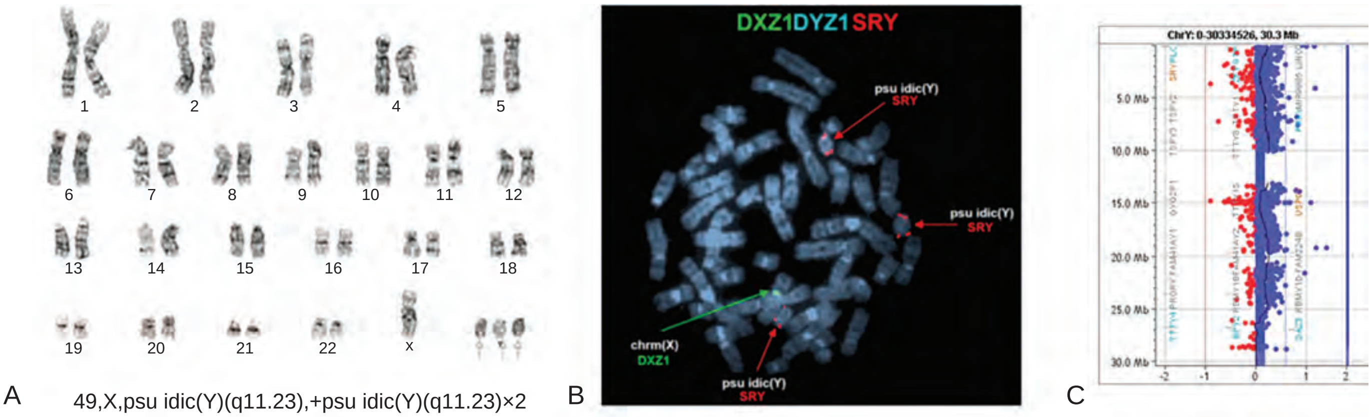


Figure 57.29 PSEUDO ISODICENTRIC Y CHROMOSOME IN MYELODYSPLASTIC SYNDROME. The patient was cytogenetically normal at diagnosis and continued to have a normal karyotype for 4 years during 24 consecutive cytogenetic studies. (A) After 4 years he developed psu idic Y chromosome present in 1 to 3 copies and a loss of normal Y chromosome. (B) Metaphase FISH showed loss of centromere of Y chromosome *(aqua)*, one X chromosome *(green)*, and 2 to 10 copies of SRY gene *(red) (red arrows)* at the telomere of each copy of the psu idic Y chromosome structure. (C) Array CGH+FISH confirmed low copy gain (6%) of Yp11.32 –q12) *(blue)*. *CGH*, Comparative genomic hybridization; *FISH*, fluorescence in situ hybridization.

these genes, indicating the importance of mutations residing outside the coding exome.

A monosomal karyotype (MK), a new cytogenetic category, is defined as a karyotype showing two or more distinct autosomal chromosome monosomies or one single autosomal monosomy (excluding isolated loss of X or Y) in the presence of a structural abnormality (see Fig. 57.25, *bottom row*). Initial reports indicated that MDS with a MK, with or without monosomy for chromosomes 5 and 7, was associated with a poorer prognosis than other complex karyotypes, although later reports could not confirm these results. The reason for this discrepancy may be due to azacitidine treatment with reducing the negative impact of MK in high-risk patients with MDS.

The clinical significance of LOY present in 10% of patients with MDS and approximately 7% of older adult males without MDS was recently elucidated (see section above on LOY in clonal hematopoiesis). Mosaic LOY in blood has been reported to be associated with higher risk of all-cause mortality and overall lower life expectancy. In bone marrow samples containing more than 75% of metaphase cells with a LOY, the observation is associated with MDS. Structural Y chromosome abnormalities are also present in patients with MDS (Fig. 57.29). Older adult males with MDS and LOY who achieve complete hematologic remission regain the Y chromosome in their marrow cells.

The prognosis of patients with MDS is very heterogeneous. In 1997, based on the cytogenetic abnormalities identified in 816 patients with MDS, as well as percentage of blasts and number of cytopenia, an IPSS was proposed. According to the IPSS, 86% of all cytogenetic findings can be explicitly classified according to their prognostic impact. The system is highly reproducible and very simple to use, but it has certain limitations. Moreover, in the remaining 14% of patients the cytogenetic abnormalities have unknown prognostic significance (Fig. 57.30). This limitation underscores two major limitations of cytogenetic classifications of MDS: the profound heterogeneity of acquired cytogenetic aberrations in MDS and the associated challenge of designing a comprehensive cytogenetic scoring system that predicts the prognostic impact of rare abnormalities (Fig. 57.31). A new and comprehensive cytogenetic scoring system based on an international data collection of 2902 patients was reported in 2012 (see Fig. 57.25). Patients included were from the German-Austrian MDS Study Group *(n = 1193)*, the International MDS Risk Analysis Workshop *(n = 816)*, the Spanish Hematological Cytogenetics Working Group *(n = 849)*, and the International Working Group on MDS Cytogenetics *(n = 44)* databases. In total, cytogenetic categories were defined, providing clear prognostic classification for 91% of all patients. All abnormalities were arranged according to OS and development of AML to classify their prognostic impact. The abnormalities were classified into five prognostic subgroups: very good (*n* = 81) included del(11q) and LOY (median OS, 61 months); good *(n = 1809)* included normal karyotype, del(5q), del(12p), and del(20q) (all as single anomaly) and double abnormalities including del(5q) (median OS 49 months); intermediate *(n = 529)* included del(7q), +8, i(17q)(q10), +19, +21, any other single abnormality, independent clones, and double abnormalities not harboring del(5q) or −7/del(7q) (median OS 26 months); poor *(n = 148)* included inv(3)/t(3q)/del(3q), −7, and double abnormalities including −7/del(7q) and complex (three abnormalities; OS of 16 months); and very poor *(n = 197)* included complex karyotypes with more than three abnormalities (OS of 6 months) (see Fig. 57.25. This new scoring system proposed should be viewed as a dynamic model, open to further refinement as the knowledge in karyotypic abnormalities of MDS evolves.

Cytogenetics and FISH studies have relatively similar sensitivities in detecting an abnormal clone among patients with MDS. A FISH test for targeted recurrent abnormalities in MDS should use probes to detect numerical and structural anomalies of chromosome regions 1q, 3q, 5q, 7, 7q31, 8, 11q, 12p, 13q, 17p, 20q, and 21 (see box on Genetic Testing for Myelodysplastic Syndrome and Fig. 57.25).

GENETIC TESTING FOR MYELODYSPLASTIC SYNDROME

Due to the tremendous chromosomal and genomic heterogeneity the best genetic test at diagnosis is conventional cytogenetic studies. It is also required for the IPSS-R prognosis. FISH for detection of P53 single or biallelic deletion will identify a group of patients with very poor prognosis. In patients with a normal karyotype a reflex test is aCGH+SNP for detection of cnLOH, specifically on 7q, allowing greater confidence in detection of genomic change fostering improved patient-specific management.

Detection rate of submicroscopic or cryptic aberrations in MDS patients with normal karyotype is 32.8%. The chromosomal abnormalities detected by SNP-A can influence the prognosis of patients with MDSs, especially in the low-risk group. The patients with multiple chromosomal abnormalities detected by SNP karyotyping had an inferior prognosis, and those with SNP abnormalities (≥3 per patient) were found to be an independent predictor of poor prognosis in patients with MDS. Moreover, SNP karyotyping in MDS revealed chromothripsis (chromosomal shattering) as recurrent genomic abnormality (1.2%), specifically in high-risk MDS cases, and the presence of chromosome 13 chromothripsis is a novel recurrent genomic change in high-risk MDS.

Familial MDS is very rare (Chapters 30 and 61). MDS presenting in children and younger adults is more frequently associated with germline genetic predisposition (Chapter 65). In recognition of the growing subgroup of MDS or acute leukemia associated with germline mutations or familial cases, the revised WHO of myeloid neoplasms now includes a new category: myeloid neoplasms with germline predisposition (Chapter 30). The diagnosis of a germline MDS predisposition disorder carries profound implications for medical management and treatment. Although there is a whole spectrum

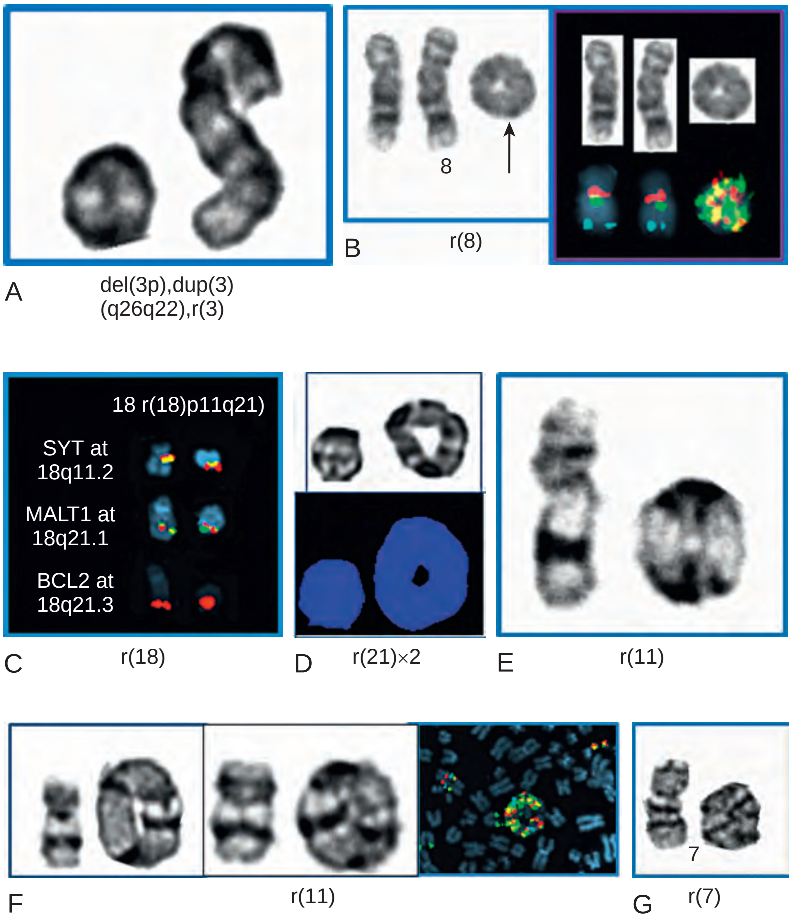


Figure 57.30 RING CHROMOSOMES IN MYELODYSPLASTIC SYNDROME AND OTHER HEMATOLOGIC MALIGNANCIES. (A) Ring 3 of one homolog and deletion 3p and duplication 3q of another homolog 3 in a patient with MDS and a complex karyotype. (B) A gain of ring of chromosome 8 in a patient with acute lymphoblastic leukemia. Metaphase FISH revealed amplification of *FGFR1 (red)* and *D8Z2 (green)* and loss of chromosome 8 region telomeric from 8p12 and below centromere *(green)* resulting in almost total loss of 8p and 8q because *MYC (aqua)* at 8q24 was deleted in r(8). r(18) with breakpoints at p11 and q21 in a patient with acute myeloid leukemia at diagnosis. (C) Metaphase FISH revealed that *SYT*, *MALT1*, and *BCL2* were retained in the ring 18. (D) Two different ring 21, as revealed by multicolor FISH in a patient with hairy cell leukemia and a complex karyotype. (E) Ring 11 in a patient with MDS. (F) Two different ring 11 in a patient with multiple myeloma. Metaphase FISH revealed *MLL* amplification *(yellow)* but not amplification of *CCND1 (red), IGH (green)*, or centromere 11 *(aqua)*. (G) Ring of chromosome 7 in a patient with T-cell leukemia. *TCRB* and *TCRG* are localized at 7p14 and 7q34, respectively. *FISH*, Fluorescence in situ hybridization; *MDS*, myelodysplastic syndrome.

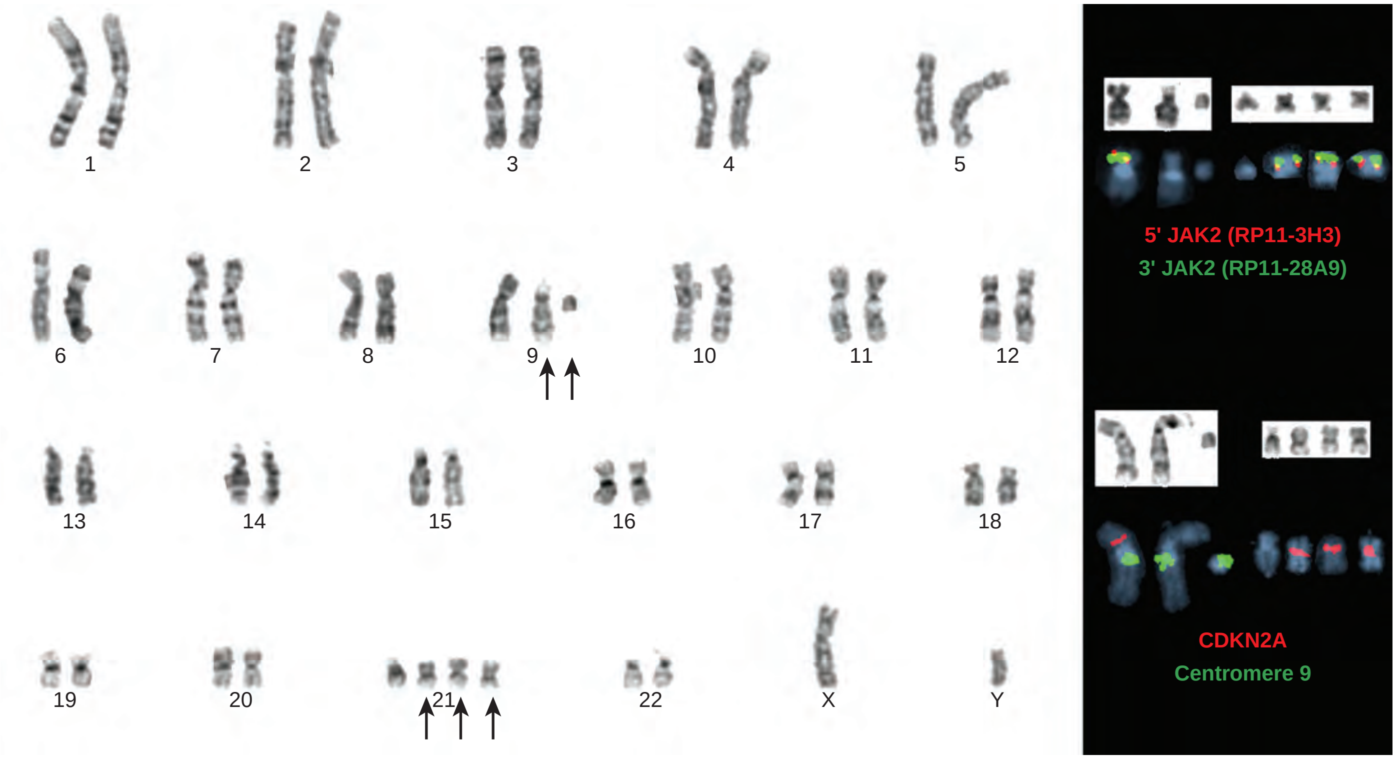


Figure 57.31 A GAIN OF JAK2 IN A PATIENT WITH MYELODYSPLASTIC SYNDROME. A bone marrow karyotype *(left panel)* from a patient with MDS showing a 49,XY add(9)(p12), +der(9)del(9)(p12p24)del(9)(q12q34)der(21)t(9;21)p21p24;p11), +der(21)t(9;21)(p21p24;p11)×2. FISH testing with JAK2 (top part) and CDKN2 and centromere 9 *(bottom panel)* revealed 70% of cells to have a deletion of 9p, including *CDKN2* and four copies of unrearranged *JAK2*, suggesting that in some patients with MDS the underlying mechanism may be a gain of *JAK2*. *FISH*, Fluorescence in situ hybridization; *MDS*, myelodysplastic syndrome.

of germline MDS predisposition conditions a few of those associated with acquired chromosomal abnormalities will be listed here. MDS with germline *GATA2* mutations is frequently associated with monosomy 7/del7q (−7) or trisomy 8, particularly in children and young adults. A study of 426 cases of pediatric MDS patients identified germline *GATA2* mutations in 37% of patients with primary MDS with −7 and 16% of MDS cases with trisomy 8 (Chapter 65). Germline *GATA2* mutations were identified in 72% of adolescents with −7 MDS. In contrast, germline *GATA2* mutations were absent in therapy-related MDS. Germline mutations in *SAMD9* and *SAMD9L* genes are frequently present in patients with bone marrow failure and a high risk for monosomy 7 MDS at a young age and these patients present either with MDS or AML. FA is characterized by genomic instability, hypersensitivity to DNA cross-linking agents, and predisposition to marrow failure, as well as hematologic malignancies and solid tumors (Chapter 30). The cumulative incidence of patients with FA to develop MDS is 40% by age 50 years and 20% by age 40 years for AML (Chapter 30). The incidence of leukemia is even higher in the FANCD1/BRCA2 subtype of FA, with many cases presenting at ages less than 5 years. In the registry of patients with Diamond-Blackfan anemia (DBA) of North America, 2% of DBA patients developed AML by age 45 years. Dyskeratosis congenita (DC) or telomere biology disorders (TBDs) encompass genetically heterogeneous disorders related to impaired telomere maintenance (Chapter 30). These patients often present associated with hematologic complications including bone marrow failure, MDS, and AML. The cumulative incidence of MDS in patients with DC has been estimated to be 2% by age 50 years. *RUNX1* deficiency is the cause of an autosomal dominant familial platelet disorder with predisposition to myeloid leukemia. Familial platelet disorder with predisposition to developing MDS, AML, or lymphoid malignancies such as T-cell leukemia or B-cell malignancies (Chapter 30). Another constitutional abnormality that appears to predispose to MDS/AML is a deletion of band region q22.1 to q22.2 of chromosome 21 (where *RUNX1* gene is located) with a complex phenotype (dysmorphic features, organ malformations, growth delay, and mental retardation), germline *RUNX1* deletion, and congenital thrombocytopenia. Although longitudinal data are limited, lifetime risk for developing a myeloid malignancy in patients with germline *RUNX1* mutations appears to be highly variable and has been estimated to be around 44%, ranging from 11% to 100%. Tables 57.6 and 57.7 shows the acquired recurrent somatic mutations observed in patients with MDS. According to the European Leukemia Net report 52% to 90% of patients with a normal karyotype have at least one genomic point mutation or MDS-related copy number change as detected by combing three technologies: NGS, cytogenetics, and a CGH+SNP. Using SNP array data from 50,000 subjects recruited for genome-wide association studies demonstrated that 0.5% of healthy individuals possessed genomic lesions. In contrast, recent whole-exome sequencing data of DNA from the peripheral blood of 17,182 persons showed clonal mutations leading to clonal outgrowth of hematopoietic cells in 10% of persons over the age of 70 years and in 18.4% among people aged 90 to 108 years. The most commonly mutated such genes were *DMT3A*, *TET2*, and *ASXL1*, which are frequent mutations in MDS and AML. These elderly people with clonal mutations in their peripheral blood did not have MDS or any other hematologic malignancy, indicating that age-related CH is a common premalignant condition and individuals with clonal mutations have an increased risk of developing hematologic malignancy (Chapters 13 and 19).

The widespread use of NGS has identified more than 50 recurrent somatic mutations in patients with MDS leading to the development of a rich mosaic detailing the mutational landscape and genetic determinants of prognostic significance and survival in MDS (Chapters 3, 13, 19, and 61). The only mutation that has diagnostic validity is an acquired mutation in *SF3B1* gene which was recognized by WHO for its potential to establish diagnosis of MDS with ring sideroblasts.

TABLE 57.6 **Recurrently Mutated Genes in Myelodysplastic Syndrome**

Gene	Chromosomal Location	Frequency (%)	Prognosis
SFRB1	2q33.1	25–30	Favorable?
TET2	4q24	20–25	Neutral
RUNX1	21q22.12	10–20	Unfavorable
ASXL1	20q11.21	10–15	Unfavorable
SRSF2	17q25.1	10–15	Unfavorable
TP53	17p13	5–10	Unfavorable
U2AF1	19q13.42	5–10	Unfavorable
NRAS/KRAS	1p13.2/12p12.1	5–10	Unfavorable
DNMT3A	2p23.3	5	Unfavorable
ZRSR2	Xp22.2	5	Neutral?
EZH2	7q35-36	5	Unfavorable
IDH1/IDH2	2q33.3/15q26.1	2–3	Unfavorable
ETV6	12p13	2	Unfavorable
CBL	11q23.3	1–2	Unfavorable
NPM1	5q35.1	1–2	?
JAK2	9p24	1–2	Unfavorable
SETBP1	18q12.3	1–2	?
SF3A1	22q12.2	1–2	?
SF1	11q13.1	1–2	?
U2AF65	19q13.42	1–2	Unfavorable
PRPF40B	2q23.3	1–2	?

Modified from Malcovati L, Hellström-Lindberg E, Bowen D, et al. Diagnosis and treatment of primary myelodysplastic syndromes in adults: recommendations from the European LeukemiaNet. *Blood*. 2013; 122:2943.

TABLE 57.7 **Incidence of Common Gene Mutations in Myelodysplastic Syndrome Organized by Gene Function**

RNA Splicing	
SF3B1	20%–35%
SRSF2	10%–15%
U2AF1	5%–10%
ZRSR2	<5%
DNA Methylation	
TET2	11%–26%
DNMT3A	7%–10%
Histone Modification	
ASXL1	~20%
EZH2	~5%
DNA Transcription	
RUNX1	~10%
TP53[a]	~5%
Signal Transduction	
NRAS	<5%
KRAS	<5%
PTPN11	<5%
Chromatid Cohesion	
STAG2	<10%
RAD21	<5%
SMC1A	<5%
SMC3	<5%

Modified from Spaulding TP, Stockton SS, Savona MR, et al. The evolving role of next generation sequencing in myelodysplastic syndromes. *Br J Haematol*. 2019;188(2):224.

Four lines of evidence support recognition of somatic *SFRB1* mutation as a disease-defining genomic event: (1) its most often an initial (founding) genetic event occurring in the most primitive hematopoietic stem cells (CD34+CD38−CD90+CD45RA-cells), (2) it is a major determinant of the disease phenotype, (3) it has an independent prognostic parameter which influences survival and risk of progression to AML, and (4) it may predict response to specific agents. With the widespread adoption of targeted sequencing panels it is now recognized that more than 80% of MDS patients harbor one or more known recurrent mutation. *TP53* VAF (variable allele frequency) may inform distinct risk subgroups because of lower-risk patients with *TP53* mutations at the time of diagnosis, more than 6% VAF were linked to shorter OS and progression-free survival compared to patients with less than 6% VAF.

ACUTE MYELOID LEUKEMIA

AML refers to a group of heterogeneous diseases with respect to clonality, molecular lesions, chromosomal aberrations, and response to treatment (see Chapters 59, 60, and 63). Initially, using *G6PD* as a marker of clonality, it was determined that AML originates from a single clone and has a multistep pathogenesis. In adults, at the time of diagnosis all hematopoietic cell lineages are clonal. In children younger than 16 years, erythroid cells and platelets often are not part of the leukemic clone.

The application of nucleotide-polymorphism technologies has confirmed the clonal origin of AML and further revealed that most, if not all, patients with AML evolve through a process of constrained clonal evolution. Multicolor metaphase analysis, FISH, CGH arrays, SNP arrays, PCR, and NGS have all greatly improved detection of subclones and established the concept of a clonal hierarchy in AML. To understand the branching evolution of AML development, each mutation must be placed in the context of whether it is a "driver" versus "passenger" and an "initiation" versus "progression" event (Chapter 59). Driver mutations directly affect the biology of the cell, whereas passenger mutations do not. Initiation mutations are present within the founding clone and are found in all AML cells. Progression mutations emerge during leukemic evolution, leading to subclones, and exist in only a fraction of AML cells. Selection pressure, such as chemotherapy, can favor the elimination or outgrowth of different branches within the AML evolutionary hierarchy.

Modern genomic technologies have provided a better understanding of normal and leukemic hematopoiesis (Chapter 3). A subset of AML cases evolve from a preceding clinically overt phase such as MDS by subclonal evolution with an increased number of genomic changes. The founder mutations present in preleukemic cells are retained in AML blasts, implicating them as putative initiating events and establishing clonal expansion as the first step in leukemogenesis. However, some recurrent leukemia-associated somatic mutations, such as *TET2*, have also been linked to multilineage CH in aging healthy individuals. For the majority of de novo AML without any prior clinical symptoms, the cell of origin, biologic consequences of initiating mutations, and order of subsequent mutations remain poorly understood. Today, state-of-the-art AML diagnostics relies on integration of different disciplines including cytogenetics and molecular genetics to provide most optimal diagnosis and management. Initial observations by the International Workshop on Chromosomes in Leukemia asserted that cytogenetic findings may serve as the single most important prognostic marker in AML; these observations were subsequently validated through studies conducted by the Medical Research Council (MRC), the Cancer and Leukemia Group B (CALGB), and the Southwest Oncology Group. Finally, in 2008, the WHO incorporated cytogenetic findings along with morphology, immunologic markers, and molecular genetics into their classification system (Chapter 56). Cytogenetics remains the most powerful independent prognostic factor and provides the framework for risk stratification schemes that have been generally adopted to guide treatment approaches for AML patients. Based on karyotype status, two major groups of AML can be distinguished: (a) those with an abnormal karyotype, which accounts for approximately 52% of patients, and (b) those with a normal karyotype by conventional cytogenetics, which accounts for 48% of patients with AML.

AML is generally considered a medical emergency implying an immediate start of treatment as soon as the diagnosis is made. However, a report from the German Study Alliance Leukemia-Acute Myeloid Leukemia registry investigated 2263 patients and analyzed the relationship between the time from diagnosis to treatment start (TDT) and concluded that the TDT had no prognostic significance on remission rates and OS. They suggested that it may be safe to wait for genetic and other laboratory test results in order to assign patients to the best available treatment option before the start of intensive therapy for AML.

Acute Myeloid Leukemia With a Normal Karyotype

Patients with AML and a normal karyotype who present between the ages of 16 and 60 years carry an intermediate prognosis. However, cytogenetically normal AML is highly heterogeneous at the molecular level, both mutations and overexpression of single genes have been identified, and their complex interactions are frequently utilized to provide more accurate risk stratification schemas. Within the normal cytogenetic category, 45% to 62% of patients with AML have nucleophosmin 1 (*NPM1*) mutations, 25% to 35% have *FLT3* mutations, 5% to 10% have *MLL* tandem duplications, and 8% to 15% have *CEBPA* mutations. The prognosis of patients with a normal karyotype differs depending on the presence of each of these mutations (Table 57.8). Patients with *NPM1* mutations alone have a favorable prognosis, with 60% of patients living longer than 11 years. Even in patients age 60 to 74, the presence of an *NPM1* mutation is an independent predictor of a more favorable outcome. More than 20 different mutations have been described in the C-terminal portion of the protein that lead to loss of tryptophan residues and generation of a nuclear export signal that acts in concert to cause delocalization of *NPM1* from the nucleus to the cytoplasm. Because the functional integrity of *NPM1* is dependent on its ability to shuttle between the nucleus and cytoplasm, this ability is severely compromised in *NPM1*-mutated AML. *NPM1*-mutated AML is associated with distinctive biologic and clinical features including older age, female predominance, multi-lineage involvement, extramedullary disease, high blast percentages and increased white blood cell counts, and lack of CD34 expression. As an isolated molecular abnormality, *NPM1*-mutated AML is associated with a highly favorable prognosis, with complete remission (CR) rates with modern chemotherapy being between 70% and 80%. The most common mutation (type A, accounting for 75% of all mutations) generates an aberrant extra nuclear export signal. Although *NPM1* mutations are heterozygous, hetero/homodimerization with wild-type *NPM1* results in cytoplasmic mislocalization of both mutant and wild-type protein. This alteration in subcellular location perturbs normal *NPM1* function, including the mislocalization and stabilization of critical proteins such as the *TP53* regulator p14ARF. This process also generates a distinct transcriptional signature. *NPM1* mutations are considered to be early events in the pathogenesis of AML. In childhood AML with normal cytogenetics, *NPM1* mutations are relatively uncommon, occurring at a frequency of 8% of cases (Chapter 63).

TABLE 57.8 Gene Mutations in Patients With Acute Myeloid Leukemia and a Normal Karyotype

Gene	Chromosomal Location	Frequency (%) of Patients With Normal Karyotype	Prognosis
NPM1	5q35	45–62	Favorable, increased CR rates and prolonged OS in the absence of FLT3 mutation
FLT3-IDT	13q12	25–35	Unfavorable, specifically when the mutant to WT allelic ratio is high
FLT3-TKD	13q12	7–10	Controversial
CEBPA	19q13.1	15	Favorable
MLL-PTD	11q23	10	Not known
IDH1	2q33.3	15–20	Most likely unfavorable
IDH2	15q26.1	20	Most likely unfavorable
DNMT3A	2p23.2	36	Most likely unfavorable
TET2	4q24	5	Likely unfavorable
ASXL1	20q11.21	9–10	Inferior outcome

CR, Complete remission; *OS*, overall survival.

In contrast, the presence of *FLT3* and *MLL* mutations are associated with an adverse prognosis, and coexistence of *FLT3* and *NPM1* does not improve the prognosis. Internal tandem duplications (ITDs) of the *FLT3* gene confer an increased risk for relapse and death when compared with patients without *FLT3*-ITD. Three types of *FLT3* gene changes are present, ITD of the juxtamembrane domain-coding sequence, a point mutation within the activation loop domain, and the copy-neutral loss of heterozygosity. Although both mutations lead to the constitutive activation of the receptor, only presence of *FLT3*-ITD with high allelic ratio has been associated with an inferior outcome in young patients. This mutation initially develops as a heterozygous mutation and over time the *FLT3*-IDT blasts acquire copy-neutral loss of heterozygosity resulting in homozygous mutated allele and high *FLT3*-ITD allelic ratio. OS of patients with *FLT3*-ITD differs significantly when categorized by the 2017 ELN risk stratification criteria.[10] Therefore, at diagnosis of AML a complete work-up according to ELN recommendations, including assessment of ITD allelic burden is mandatory to select patients eligible for intensive therapy

Intact functioning of the CCAAT/enhancer binding protein (*CEBPA*) gene is essential for normal granulocytic differentiation. It is a lineage-specific transcription factor that is required for the formation of committed myeloid progenitors from multipotent progenitor cells. *CEBPA* executes this function by coupling the direct transcriptional activation of myeloid-specific genes with the arrest of cell proliferation. *CEBPA* is an intron-less gene with mRNA that can be translated into two different AUG codons to give rise to two distinct isoforms, p42 and p30. Only the p42 isoform of *CEBPA* can promote proliferation arrest. *CEBPA* mutations, leading to arrest of differentiation, are seen in 5% to 10% of de novo AML and in up to 15% of normal karyotype AML. Only mutations affecting both alleles, double mutations, confer superior survival as compared to single mutations. Concomitant *GATA1* and *WT1* mutations are seen more often in double mutants while *FLT3-ITD, NPM1, ASXL1*, and *RUNX1* mutations are more commonly associated with single mutations, possibly contributing to their poorer prognosis.

A rare but specific subset of adult AML can be defined by the cytogenetically cryptic *NUP98-NSD1* fusion gene that involves the nucleoporin gene 98 (*NUP98*) on chromosome1, 1p15, and the nonhomeobox gene *NSD1* present on chromosomal band 5q35. *NUP98* encodes a 98-kDa protein of the nuclear pore complex, which is known to fuse with at least 21 different fusion gene partners in chromosomal rearrangements in various hematologic malignancies. NSD1 is thought to function both as a transcriptional co-activator and a co-repressor. Among 293 pediatric and 808 adult cytogenetically normal cases of AML the *NUP98-NSD1* fusion gene was described in 16.1% of pediatric and 2.3% of adult AML cases. Apparently *NUP98-NSD1* has been shown to correlate with *FLT3*-ITD, which is associated with increased blast cell percentages.

TET2 proteins are necessary for the conversion of 5-methylcytosine to 5-hydromethylcytosine and play a role in demethylation processes within the cell. *TET2* mutations have been reported in 10% to 23% of patients with AML with a normal karyotype. The impact of *TET2* mutations on the prognosis and outcome remains poorly defined.

DNMT3A mutations, which affect the DNA methyltransferase enzymes and subsequent epigenetic modulation, have been described in about 27% to 33% of normal karyotype AML and are associated with a worse OS (see earlier section on clonal origin and early mutations).[11]

TABLE 57.9 **The European Leukemia Net 2017 Risk Stratification of Acute Myeloid Leukemia**

Prognostic Group	Subset	CR Rate; Relapse With 3+7 Without HCT	Comments
Favorable	inv(16)/t(16;16) NK and *NPM1+/ FLT3* ITD-NK and *CEBPA* +/+	>80%–90%; 35%–40%	Outcome worse in older patients, with t-AML, KIT mutations, and poor response to initial therapy
Intermediate	Mutated NPM1 and FLIT3-ITD (without adverse-risk genetic lesion), t(9;11)(p21.3;q23.3)/ MLL-KMT2A	50%–80%; 50%–60%	Outcome worse in older patients, with t-AML, and poor initial response
	Cytogenetic abnormalities Not in favorable or adverse group		Outcome worse in older patients, with t-AML, and poor initial response
	FLT3 ITD+		
Adverse	−5, −7, 5q abn, 3q, 17p, 11q (other than 9;11), t(6;9), complex, monosomal karyotype, wild type NMP1 and FLT3-ITD, Mutated RUNX1, Mutated ASXL1 and mutated TP53, insufficient metaphases for analysis	<50%; >90%	Outcome worse in older patients, with t-AML, and poor initial response

AML, Acute myeloid leukemia; *CR*, complete remission; *HCT*, hematopoietic cell transplant; *NK*, normal karyotype, +/+, double mutation.

Currently, a newly diagnosed patient with AML and a normal karyotype should be checked for common mutations such as *FLT3, NPM1, CEBPA, RUNX1, MLL-PTD*, or *EVI1, TET2*, and *DNMT3A*, which may have prognostic and possibly therapeutic implications (Table 57.9).

Integration of NGS and high-density SNP array in a study of 252 patients with de novo AML and normal karyotype showed co-occurrence of mutations and cryptic cytogenetic abnormalities in 58% of which 49% harbored copy-number abnormalities or copy neutral loss of heterozygosity (CN-LOH).[7] Of the 282 cryptic acquired abnormalities, genomic losses were more frequent than gains or CH-LOH (54%, 27%, and 19%, respectively). Losses were distributed virtually across all chromosomes, differently from CN-LOH and gains. Most important, the majority of lesions were not recurrent with the exception of those located on 13q. Losses and CN-LOH were concentrated in chromosomes 1, 2, 5q, 7, 11q, and 13q. Due to their location, and general implications of losses and CN-LOH, these genomic cryptic abnormalities could be acting as a second hit in the leukemogenesis process due to the loss of the wild-type alleles. Initial results are consistent that the presence of two or more genomic lesions has a negative impact on patient outcomes.

Acute Myeloid Leukemia with an Abnormal Karyotype

Among patients with an abnormal karyotype, 25% have balanced translocations and 27% showed unbalanced abnormalities or a complex karyotype.

The pretreatment AML karyotype constitutes an independent prognostic determinant for attainment of CR and risk for relapse and survival. Four broad cytogenetic risk categories of AML are useful in clinical practice: favorable, intermediate, unfavorable, and unknown (Figs. 57.32, 57.33 and Table 57.9). It is important to perform appropriate cytogenetic and FISH studies to establish the correct cytogenetic risk category. Table 57.10 lists 72 of approximately 250 recurrent chromosomal translocations observed in leukemias, including AML and excluding *MLL* translocations (see later) (see Fig. 57.40). Because of their specific association with distinct subtypes of leukemia, some AML-specific translocations have been incorporated into the WHO classification as criteria for subclassification of AML, including t(8;21) (Fig. 57.34), t(15;17), inv(16) (Fig. 57.35), and 11q23 rearrangements, regardless of the morphology or percentage of blast cells.

Two specific cytogenetic types of AML—t(8;21)(q22;q22) and inv(16)(p13q22) or t(16;16)(p13;q22) (see Fig. 57.35)—are called *core-binding factor (CBF)* AMLs and are usually grouped together due to similarities in their molecular and prognostic features. However, the morphologic features of these two AML subtypes are very different, suggesting that individual approaches may enhance clinical benefits in future studies. Patients with t(8;21) exhibit large myeloid blasts with abundant basophilic cytoplasm, numerous azurophilic granules, and occasional Auer rods. Patients with 16q22 abnormalities show increased numbers of eosinophils to varying degrees, and at all stages of maturation. The immature eosinophilic granules are larger than normal and may contain purple-violet cytoplasmic granules. The t(8;21) subtype may be present alone, although 30% to 35% of patients also display LOY in males and LOX in females. Another 20% of patients with t(8;21) have a deletion of 9q12–23, including a commonly deleted segment that spans 7 to 8 Mb. Trisomies for chromosomes 4 and 8 together with t(8;21) are observed in 6% to 10% of patients. Virtually all patients with t(8;21) achieve CR with induction chemotherapy. Additional cytogenetic abnormalities, irrespective of their nature or complexity, do not have a deleterious effect on rates of remission, relative risk of relapse, and OS. t(8;21) interrupts two genes—*RUNX1* (*CBFA2*) on chromosome 21, band q22, in intron 5, and *RUNX1T1 (ETO)* [eight, twenty-one gene], on chromosome 8, band q22—and joins them to form a new chimeric gene on the abnormal der(8) chromosome. *RUNX1* is a gene on chromosome 21, also known as *CBFA2* because it encodes for a DNA-binding component of CBF and binds DNA through a specific sequence called the *runt domain*. The *RUNX1* gene locus on chromosome 21 spans 120 kb; the *RUNX1T1* gene on chromosome 8 is distributed over 87 kb.

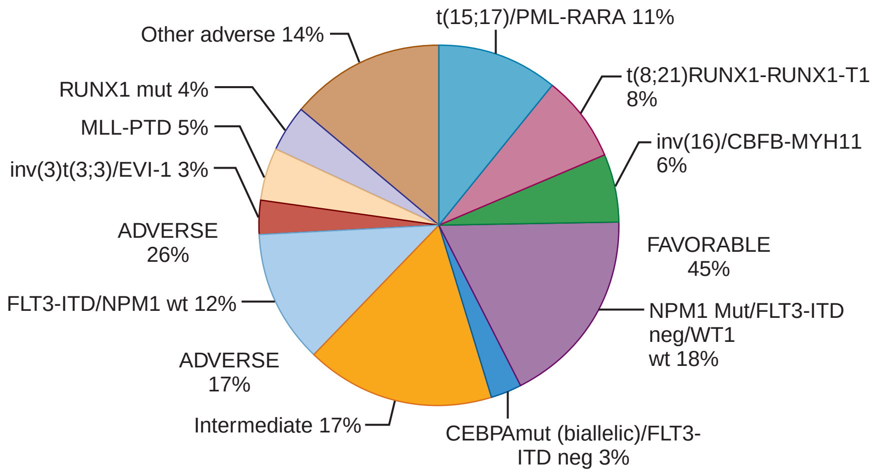


Figure 57.32 INTEGRATION OF CYTOGENETIC ABNORMALITIES AND MOLECULAR MARKERS TO REFINE RISK GROUPS IN ACUTE MYELOID LEUKEMIA. *(Reprinted from Smith ML, Hills RK, Grimwade D. Independent prognostic variables in acute myeloid leukaemia. Blood Rev. 2001;25:39; with permission.)*

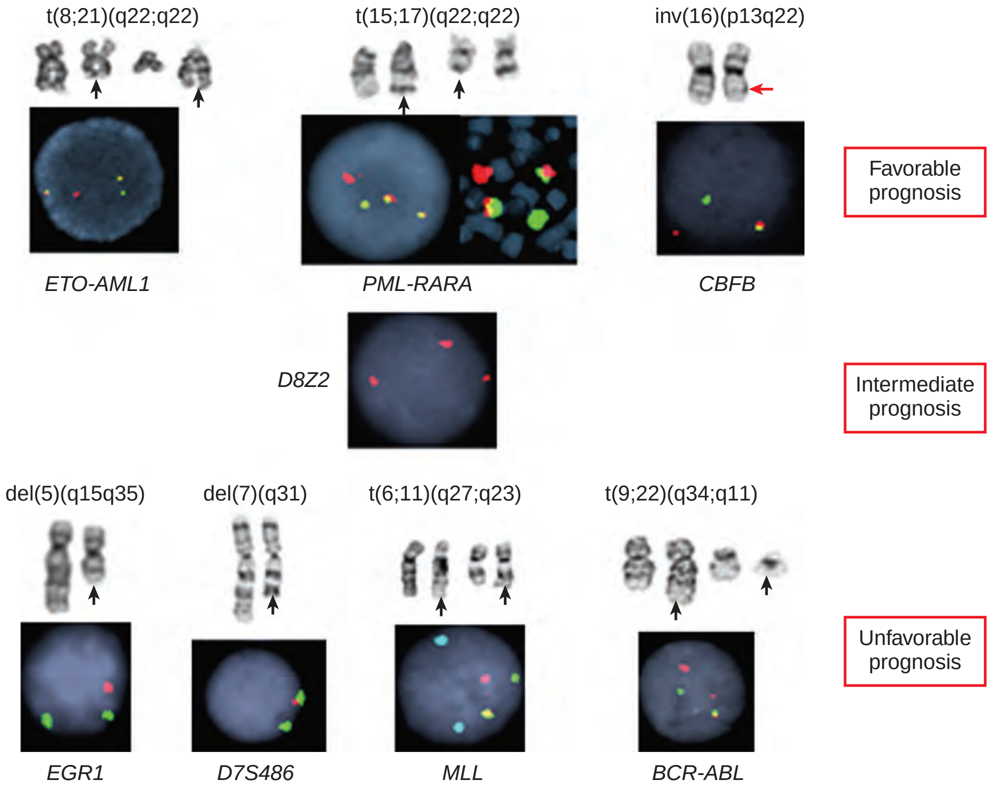


Figure 57.33 PROGNOSTIC CYTOGENETIC RISK CATEGORIES IN ACUTE MYELOID LEUKEMIA. Favorable prognosis includes t(8;21) and ETO-AML1 fusion, t(15;17) and PML-RARA fusion, and inv(16) and rearrangements of *CBFB* on 16q22. Trisomy 8 is associated with intermediate prognosis. Unfavorable cytogenetic risk categories include monosomy 5/del(5q), −7/del(7q), and translocations of 11q23 and *MLL*, represented here by t(6;11), and the Philadelphia chromosome.

The *RUNX1* gene has been identified in more than 39 chromosomal translocations in leukemia and plays a critical role during hematopoiesis (see different *RUNX1* chromosomal partners: http://atlasgeneticsoncology.org/Genes/AML1ID52.html). The fusion gene is located on the partner chromosome in the majority of *RUNX1* translocations. Its disruption is associated with the development of myeloid and lymphoid leukemias. Therefore *RUNX1* is viewed as a master regulatory switch that controls development of a definitive hematopoietic lineage. Moreover, the *RUNX1* transcription factor is critical for proliferation and differentiation of hematopoietic stem cells. Haploinsufficiency of *RUNX1* has been linked to a propensity to develop AML, and biallelic nonsense mutations in the *RUNX1* gene have been identified with primitive forms of AML(M0 subtype). The RUNX1T1 protein belongs to the ETO family of proteins involved in protein–protein interactions but not in protein-DNA interactions.

In the hybrid RUNX1-RUNXT1 protein, the C-terminus of RUNX1 is replaced by the entire RUNXT1 protein. The main functional characteristic of RUNX1-RUNX1T1 chimeric protein is its

TABLE 57.10 Recurring Chromosome Translocations in Leukemia and the Involved Genes

Translocations	Genes Involved	Associated Diseases
CBF (AML1/CBFA and CBFB)- and TEL/ETV6-Associated Translocation/Inversion		
t(X;21)(p22;q22)	*PRDX4-AML1*	AML
t(3;21)(q26;q22)	*EVI1-MDS1-EAP-AML1*	t-AML/CML-ACC/BC
t(8;21)(q22;q22)	*ETO-AML1*	AML
t(8;21)(q23q22)	*FOG2-AML1*	MDS
t(8;21)(q24q22)	*TRPS1-AML1*	ALL/AML
t(16;21)(q24;q22)	*MTG16-AML1*	t-AML
t(19;21)(q13;q22)	*AMP19-AML1*	t-AML
t(12;21)(p12;22)	*ETV6-AML1*	ALL
t(21;21)(q11;q22)	*UPS25-AML1*	MDS
inv(16)/t(16;16)(p13;q22)	*MYH11-CBFB*	AML-M4
t(1;12)(p36;p13)	*MDS2-ETV6*	CML/MDS
t(1;12)(q21;p13)	*ARNT-ETV6*	AML
t(1;12)(q25;p13)	*ARG-ETV6*	AML
t(3;12)(q26;p13)	*MDS1-EV1-ETV6*	MPD
t(4;12)(p11;p13)	*BTL-TV6*	AML
t(5;12)(pq31;p13)	*ACS2-ETV6*	AML
t(5;12)(q33;p13)	*PDGFRB-ETV6*	CMML
t(6;12)(q23;p13)	*STL-ETV6*	ALL
t(7;12)(q36;p13)	*HLXB9-ETV6*	AML
t(9;12)(p24;p13)	*JAK2-ETV6*	ALL, aCML
t(9;12)(q22;p13)	*SYK-ETV6*	MDS
t(9;12)(p34;p13)	*ABL-ETV6*	CMML
t(12;13)(p13;q12)	*ETV6-CSX2*	AML
t(12;13)(p13;q14)	*ETV6-TTL*	ALL
t(12;15)(p13;q25)	*ETV6-NTRK3*	AML
t(12;17)(p13;p12)	*ETV6-PER1*	AML
t(12;21)(p13;q11)	*ETV6-MN1*	AML
t(12;16)(p13;p11)	*CHOP-TLS/FUS*	AML
t(16;21)(p11;q22)	*TLS-FUS/-RG*	AML, MLS
RARA-Associated Translocation		
t(15;17)(q22;21)	*PML-RARA*	APL
t(5;17)(q32;q21)	*NPM-RARA*	APL
t(2;17)(q32.3;q21)	*NABP1-RARA*	APL
t(4;17)(q12;q21)	*FIP1L1-RARA*	APL
t(11;17)(q23;q21)	*ZBTB16-RARA*	APL
t(11;17)(q13;q21)	*NuMA-RARA*	APL
t(11;17)(q23;q21)	*MLL-RARA*	APL
t(17;17)(q21.2.;q21.2)/del(17)(q21.2	*STATB-RARA*	APL
t(X;17)(p11.2;q21)	*BCOr-RARA*	APL
t(3;5)(q25;q35)	*MLF1-NPM*	AML/MDS
E2A-Associated Translocations		
t(1;19)(q23;p13)	*PBX1-E2A*	B-ALL
t(17;19)(q23;p13)	*HLF-E2A*	B-ALL
Tyrosine Kinase–Associated Translocations		
del(4)(q12q12)	*FIP1L1-PDGFRA*	HES
t(4;22)(q12;q11)	*PDGFRA-BCR*	MPN
t(3;5)(q25;q35)	*MLF1-NPM*	AML/MDS
t(1;5)(q23;q33)	*Myomegalin-PDGFRB*	MPD
t(5;7)(q33;q11.2)	*PDGFRB-HIP1*	CMML
t(5:10)(q33;q21)	*PDGFRB-H4*	MPD
t(5;12)(q33;p13)	*PDGFRB-ETV6*	CMML, CEL
t(5;14)(q33;q32)	*PDGFRB/-AV14*	AML
t(5;14)(q33;q24)	*PDGFRB/-IN*	MPD
t(5;15)(q33;q15)	*PDGFRB/-P53BP1*	MPD
t(5;17)(q33;p13)	*PDGFRB-RABEPI*	CMML
t(5;17)(q33;p11.2)	*PDGFRB-HCMOGT*	JMML
t(q;22)(p24;q11.2)	*BCR-JAK2*	CML-
t(9;12)(p24;q13)	*JAK2-ETV6*	CML-
t(8;9)(p22;p24)	*PCMI-JAK2*	MPD, AL
NUP98/NUP214-Associated Translocations		
t(1;11)(q23;p15)	*PMX1/NUP98*	AML
t(2;11)(q31;p15)	*HOXD13/NUP98*	t-AML
t(4;11)(q21;p15)	*RAP1GDS1/NUP98*	T-ALL
t(5;11)(q35;p15)	*NSD/NUP98*	AML
t(7:11)(p15;p15)	*HOXA9/NUP98*	AML
t(9;11)(p22;p15)	*LEDGF/NUP98*	AML
inv(11)(p15q22)	*NUP98/DDX10*	t-AML
t(11;20)(p23;q34)	*NUP98/TOP1*	t-MDS
t(6;9)(p23;q34)	*DEK/NUP214(CAN)*	AML
Normal karyotype	*SET/NUP214(CAN)*	AML
FGFR1-Associated Translocations		
t(8;13)(p11;q12)	*ZNF198-FGFR1*	MPN
t(7;8)(q32;P11)	*TRIM24-FGFR1*	MPN
t(6;8)(q27;P11)	*FGFR1OP-FGFR1*	MPN
ins(12;8)(p11;p11p22)	*FGFR1OP2-FGFR1*	MPN
t(8;17)(p11;q11)	*MYO18A-FGFR1*	MPN
t(8;22)(p11;q11,2)	*BCR-FGFR1*	MPN

ACC/BC, Accelerated blast crisis; *AL*, acute leukemia; *aCML*, atypical CML; *ALL*, acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *APL*, acute promyelocytic leukemia; *CBF*, core-binding factor; *CEL*, chronic eosinophilic leukemia; *CML*, chronic myelogenous leukemia; *CMML*, chronic myelomonocytic leukemia; *HES*, hypereosinophilic syndrome; *JMML*, juvenile myelomonocytic leukemia; *MDS*, myelodysplastic syndrome; *MLS*, myelodysplastic syndrome; *MPD*, myeloproliferative disorder; *MPN*, myeloproliferative neoplasm; *T-ALL*, T-cell ALL; *t-AML*, therapy-related AML; *t-MDS*, therapy-related MDS.

ability to bind DNA containing RUNX1 binding sites and thereby exert a dominant negative inhibition of the endogenous RUNX1 protein. The exact role of the RUNX1-RUNX1T1 fusion protein in determining the onset and progression of AML is not fully understood; however, accumulating evidence indicates that even a point mutation in the RUNX1 protein, responsible for assembling and organizing the machinery for hematopoietic gene expression at multiple sites in target genes, results in a block of differentiation of myeloid progenitors to granulocytes. *RUNX1* mutations are present in 46% of patients with AML with the M0 subtype and in 80% of patients with trisomy 13 AML. As mentioned earlier, the *FLT3* gene is localized on chromosome 13, and *RUNX1* mutations and trisomy 13 are associated with a fivefold increase in *FLT3* transcript levels as compared with patients without trisomy 13. The exact relationship

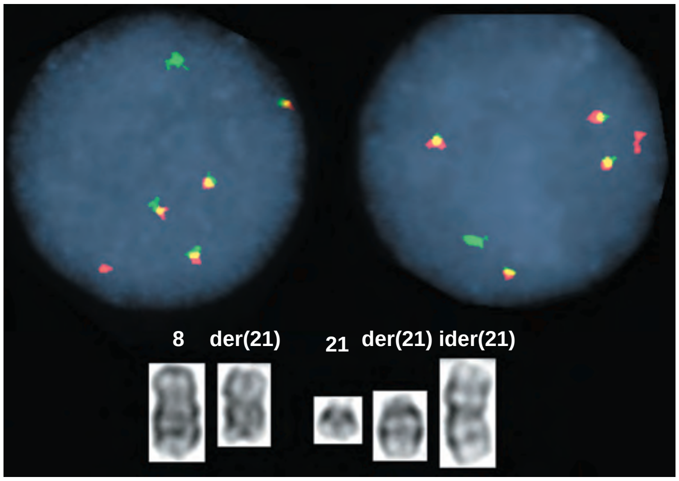


Figure 57.34 DUPLICATION OF T(8;21). Four copies of ETO-AML1 (RUNX) fusion *(yellow)* shown in two interphase cells *(top)* from a patient with acute myeloid leukemia and t(8;21) *(bottom)* karyotype, as well as ider(21). This formation is equivalent to the Ph duplication in the blast crisis of chronic myelogenous leukemia because of duplication of der(21) without accompanying t(8;21).

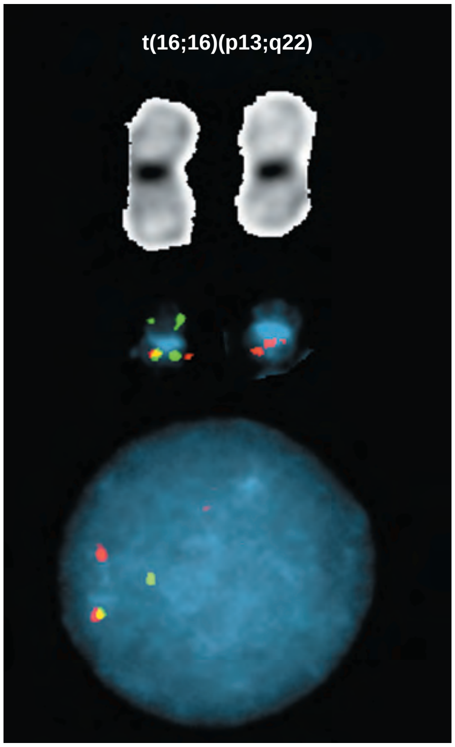


Figure 57.35 Partial G-banded karyotype from a patient with M4 acute myeloid leukemia showing t(16;16) *(top)*, after FISH study using a break-apart CBFB probe (at 16q22), demonstrating that the 5′ end *(red)* of the gene remains on 16q of one chromosome 16, whereas the 3′ end *(green)* translocated to the short arms of the other chromosome 16. Separation of 5′ and 3′ ends as single signals is indicated in the bone marrow nucleus *(bottom)*. *FISH*, Fluorescence in situ hybridization.

between *FLT3* and *RUNX1* in leukemogenesis remains unknown. Multiple copies of *RUNX1/RUNXT1* fusion have been demonstrated (see Fig. 57.34). The t(8;21) is the most common translocation in pediatric patients with AML (10% to 20%) (Chapter 63). Prenatal origin of t(8;21) AML has been established for approximately 50% of pediatric AML patients using Guthrie card analysis.

Although 60% to 70% of patients with t(8;21) achieve complete and long-term remission with modern chemotherapy regimens, monitoring MRD using t(8;21) marker has proven to be useful in identifying patients at a high risk for relapse. Multiparametric approaches, such as flow cytometry, RQ-PCR, and interphase FISH, are complementary methods and provide useful information concerning MRD and relapse kinetics. It should be noted that 18% of healthy individuals have *RUN1-RUNXT1* transcripts detectable by PCR and the fusion transcripts have been detected in 40% of cord blood samples, suggesting that *RUNX1-RUNXT1* by itself may not have overt leukemia-initiating properties.

In patients with 16q22 abnormalities such as inv(16)(p13;q22) and t(16;16)(p13;q22), the marrow contains an increased percentage of abnormal eosinophils (see Fig. 57.35). Combined May-Grünwald-Giemsa staining with FISH demonstrates that the abnormal eosinophils possess inv(16) and therefore belong to the leukemic clone. Trisomy 22 is a frequent accompanying abnormality. Both inv(16) (see Fig. 57.35, *top right*) and t(16;16) (see Fig. 57.35, middle) are abnormalities of the *CBFβ* gene at 16q22 and are associated with M4Eo subtype, as was characterized previously by the FAB AML classification system. Both rearrangements result in fusion of *CBFβ* and *MYH11 (myosin heavy-chain) gene of 16p13. The exact role of the resulting hybrid protein, CBFβ- SMMHC* (smooth muscle myosin heavy chain), is unknown, but is likely involved in impaired hematopoietic differentiation. Both t(8;21) and inv(16) result in abnormal repression of CBF target genes. CBF is a heterodimeric transcription factor complex that consists of three distinct DNA-binding CBFα subunits—*RUNX1, RUNX2*, and *RUNX3*—and a common *CBFβ* subunit, which is non-DNA-binding. The binding affinity of *RUNX1* subunit to the DNA promoter sequences is significantly increased by association with CBFβ, which does not directly interact with DNA and protects the *RUNX1* subunit from proteolysis. The breakpoints in t(8;21) affect *RUNX1* exon 5 and R*UNX1T1* exon 2. The breakpoint in *MYH11* involved in inv(16) and t(16;16) is variable and gives rise to at least 10 different fusion variants. In contrast, the breakpoints in CBFβ at 16q22 are at intron 5. Both translocations are associated with a favorable prognosis, but they exhibit different leukemic cell morphology.

Approximately 4% of patients with *CBFβ-MYH11* rearrangement lack a cytogenetically detectable inv(16) or t(16;16). Cytogenetic detection of inv(16) may be difficult, and interphase FISH with a dual-color CBFβ probe at diagnosis is a crucial genetic test. *CBFβ-MYH11* fusion can be found by either RT-PCR or FISH in AML patients without eosinophilia; therefore *CBFβ* testing should be included in the standard AML testing panel. The presence of additional abnormalities, such as trisomy 8, do not adversely affect clinical outcomes.

The *CBFβ-MYH11* chimeric fusion can be detected in utero approximately 10 years before development of childhood leukemia. This observation suggests that formation of *CBFβ-MYH11* alone is not sufficient to cause leukemia and that subsequent genetic events must occur before clinically recognizable AML. CBF AML is associated with a favorable CR rate, OS, and lower relapse risk than patients with cytogenetically normal AML. However, a retrospective analysis of 113 patients with CBF AML demonstrated that patients with inv(16) at diagnosis were less likely to have any normal metaphases when compared with patients with t(8;21). Moreover, the identification of an increasing number of normal metaphase cells increased the risk for relapse and negatively affected the survival of patients with inv(16); identifying at least one normal metaphase at diagnosis and 19 with inv(16) had a significant impact on 5-year survival (60% versus 14%, $P = .00005$). These factors, along with age, were the only independent variables associated with refractory disease and higher relapse. Nevertheless, CBF AML has been defined as a favorable genetic group by National Comprehensive Cancer Network (NCCN) and ELN guidelines. NCCN guidelines have classified t(8;21) and inv(16) AMLs with c-*KIT* mutations as having intermediate risk disease, whereas the ELN has provided no further recommendations for those with c-*KIT* mutations. Mutations in the *KIT* gene are the most recurrent molecular abnormalities, occurring in 15% to 46% of CBF

AML, and are associated with a higher risk for relapse. The mutation frequency was similar for pediatric and adult patients with t(8;21) whereas pediatric patients with inv(16) AML tend to have a higher frequency of *KIT* mutations than adult patients. Approximately 5% to 10% of CBF AML patients, mostly those with t(8;21), have concurrent systemic mastocytosis with *KIT* mutations and a good prognosis (Chapter 75).

The cKIT mutations are clustered within exon 17 which encodes the *KIT* activation loop (A-loop) in the kinase domain, and in exon 8, which encodes an evolutionarily highly conserved region in the extracellular portion of the *KIT* receptor. KIT17 mutations occur almost exclusively at codon D816 in patients with inv(16), and at codons D816 or N8822 in patients with t(8;21). *KIT* mutations represent not only a prognostic indicator but also a potential therapeutic target for TKI therapy. In adults the mutation frequency in exon 17 is significantly higher in t(8;21) than in inv(16) patients with AML (35.6% vs. 6.9%, respectively, $P < .0001$), whereas the mutation frequency in exon 8 is significantly lower in t(8;21) than inv(16) patients with AML (4.4% vs. 18.8%, respectively, $P = .0003$). The prognostic value of *KIT* mutations in CBF AML remains debatable. The incidence, characteristics, and prognostic effects of *KIT* mutations are different for each subgroup. It appears that mutations in exon 17 have a strong adverse impact on the relapse and survival of adult patients with t(8;21) AML.

Other activating mutations associated with CBF AML include *FLT3*, *CBL*, *NRAS* or *KRAS*, and *ASXL2*. *FLT3* point mutations (most common D835) are frequently associated with inv(16) (6% to 24%) and 6% of patients have *CBL* mutations associated with improved OS. *NRAS*- and *KRAS*-activating mutations are preferentially observed in inv(16) and do not impact prognosis. *ASXL2* mutations (11.5%) are exclusively associated with t(8;21) and were not observed in inv(16) patients. The ASXL2 protein is involved in epigenetic regulation of gene transcription and seemingly confers a worse prognosis but these results need more extensive validation. *RUNX1* mutations present in about 5% to 15% of patients with M0 AML, are mutually exclusive when t(8;21) and inv(16) are present. As mentioned earlier, there are 47 recurrent abnormalities involving the 21q22 chromosomal site where *RUNX1* is localized. Some of the more frequent rearrangements are discussed here.

The t(16;21)(q24;q22) is a rare but recurrent chromosomal abnormality associated with therapy-related AML. FISH and RT-PCR methods have demonstrated fusion of *RUNX1* on 21q22 and *CBFA2T3* on chromosome 16, which produces an *RUNX1/CBFA2T3* fusion gene on chromosome 16. The breakpoints of both t(8;21) and t(16;21) occur within the same intron of the *RUNX1* gene. *RUNX1/CBFA2T3* fusion results in the production of a protein that is very similar to the RUNX1/RUNX1T1 protein in t(8;21).

The (16;21)(p11;q22) chromosomal rearrangement is rare and associated with M1-M2 AML. A proportion of these patients may have additional abnormalities. This translocation fuses the *FUS/ERG TLS/FUS* gene on chromosome 16, band p11, to the *ERG* gene on chromosome 22, band q22. The *ERG* gene is a member of the E twenty-six (ETS) family of transcription factors and is a sequence-specific transcriptional activator. The presence of a fusion transcript is detected by RT-PCR, at the time of diagnosis, at relapse, and during remission. These observations are consistent with the impression that patients with t(16;21) have a poor prognosis and may benefit from early detection of this chimeric gene to determine the need for more aggressive therapy.

AML with intrachromosomal amplification of chromosome 21 (iAMP21) is a rare entity (~0.1%). It occurs in pediatric B-cell ALL (~2%) (see below ALL) and as such was recognized by the current WHO classification as a provisional entity in 2017. In adult AML, iAMP21 is most often identified using FISH technology. Interphase cells showing 5 or more copies of *RUNX1* gene, that are localized on chromosome 21q22, are considered positive. Most of the adult AML patients with iAMP21 have a complex karyotype and the most frequent additional abnormalities include −5/del(5q), −7(del7q), −17/del(17p). High copy numbers of *RUNX1* are identified as homogenous staining of region 21(q22), extra copies of chromosome 21 and/or i(21q) insertion of 21q22, double minutes (DM), and ring or marker chromosomes. AML iAMP21 differs in several ways from B-cell ALL: (a) AML patients with iAMP21 are almost all adults (median age 65 years old), (b) they are almost always associated with a complex karyotype, (c) they show a high frequency of *TP53* deletion/mutation, (d) often they present with pancytopenia, and (e) these patients are highly refractory to standard AML-induction therapy. The regions predominantly amplified span chromosome 21q22.1q22.3 and these chromosomal segments range in size from 11.8 to 40 Mb. Since most patients with AML iAMP21 have a deletion/mutation of *TP53*, a very complex karyotype, and genomic instability chromothripsis appears to be an underlying mechanism of pathogenesis.

The t(15;17)(q22;q21) involves the promyelocytic leukemia (*PML*) gene on chromosome 15, band q22, and *RARA* on chromosome 17, band q21. This abnormality constitutes the genetic basis for approximately 95% of all cases of APL (Fig. 57.36).[12] The remaining 5% of cases possess nine rare variant translocations: t(11;17)(q23;q21)/*ZBTB16-RARA* and *MLL-RARA*, t(11;17)

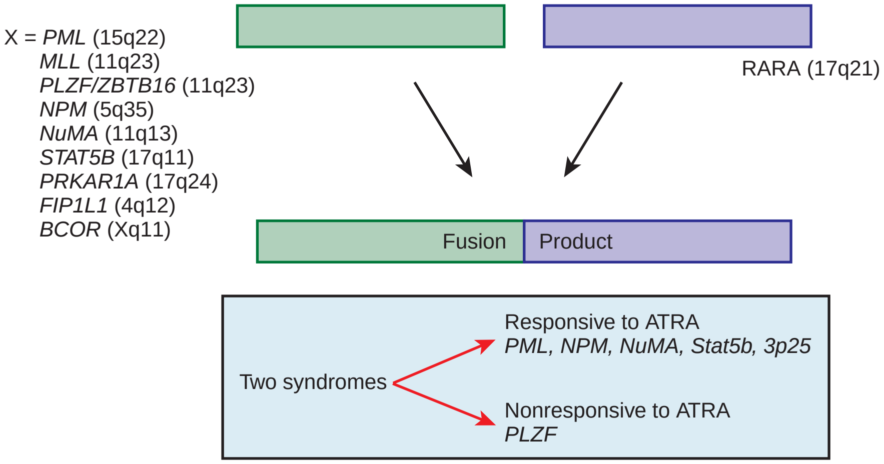


Figure 57.36 MOLECULAR CYTOGENETIC DEFECTS IN ACUTE PROMYELOCYTIC LEUKEMIA ARE RESPONSIBLE FOR DIFFERENT RESPONSE TO ALL-*TRANS* RETINOIC ACID DIFFERENTIATION THERAPY. Patients with t(11;17)(q23(q22) and PLZF(ZBTB16)-RARA fusion do not respond to all-*trans* retinoic acid (ATRA) differentiation therapy, whereas patients with classic t(15;17) and other four cytogenetic variants have exquisite sensitivity to differentiate in response to all-trans retinoic acid. *(From Cheng L, Zhang DY, eds. Molecular Genetic Pathology. Totowa, NJ: Humana Press, a part of Springer Science; 2008.)*

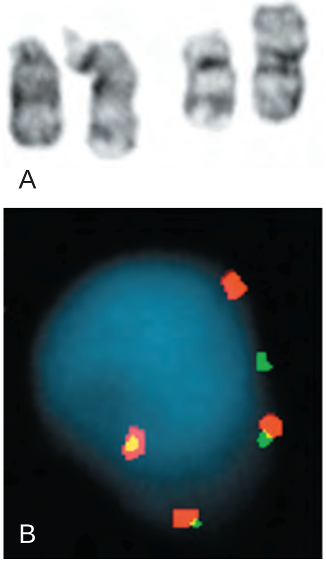


Figure 57.37 ISOCHROMOSOME 17 IN ACUTE PROMYELOCYTIC LEUKEMIA WITH A GAIN OF PML-RARA FUSION. (A) Partial karyotype from a patient with acute promyelocytic leukemia, showing ider(17) t(15;17)(q22;q21). The karyotype shows isochromosome for the long arms of chromosome 17 and deletion of the short arms. (B) FISH studies revealed three copies of PML-RARA fusion in bone marrow nucleus, confirming that the first event in the pathogenesis was PML-RARA fusion and the subsequent event was a structural rearrangement of isochromosome. *FISH*, Fluorescence in situ hybridization.

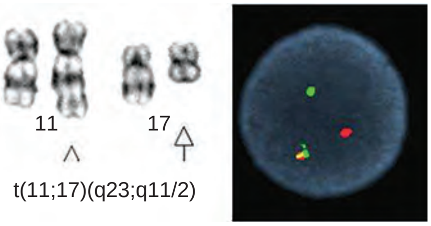


Figure 57.38 A variant of APL showing in 90% of marrow cells a balanced translocation between the long arms of chromosomes 11 and 17. This is a variant APL translocation involving *KMT2A* and *STATB* at 17q11 chromosomal site. Interphase bone marrow nuclei after FISH showed 78% of cells with a split in KMT2A dual color breakapart probe, between the 5′ *(green)* and 3′ *(red)*. *APL*, acute promyelocytic leukemia; *FISH*, Fluorescence in situ hybridization.

(q23;q21)/*NUMA1-RARA*, t(5;17)(q35;q21)/*NPM1-RARA*, t(2;17)(q32.3;q21)/*NABP1-RARA*, t(17;17)(q21.2;q21.2)/*STATB-RARA*) (Fig. 57.37) or del(17)(q21.2q21), iso (17q) (Fig. 57.38) t(4;17)(q12;q21) *FIP1L1/RARA*, t(X;17)(p11.2;q21)/*BCOR-RARA*, and cryptic *PRKAR1A-RARA* detected in a patient with a normal karyotype. Therefore APL, which accounts for 10% to 15% of AML cases, is associated with several different genetic rearrangements fusing the *RARA* gene with a different partner gene. Based on these genomic rearrangements, a FISH assay, using a breakapart probe strategy with dual-color *RARA*, can identify two different clinical syndromes: those responsive to all-*trans* retinoic acid (ATRA) (more than 99%) therapy and those carrying a *ZBTB16-RARA* or *STAT5B-RARA* fusion gene, which are not responsive to ATRA and are naturally resistant to arsenic trioxide (ATO) therapy. Patients with promyelocytes that have exquisite sensitivity to differentiate in response to ATRA treatment have one of the other six RARA fusion rearrangements (see Fig. 57.36). ATRA resistance is heterogeneous and commonly involves mutations in the RARA ligand-biding domain of the fusion protein. FISH studies have also identified patients with cryptic translocations and unusual chromosomal variants (see Fig. 57.36). It is important to recognize patients who will or will not respond to ATRA so that appropriate therapy can be administered.

The t(15;17) is often the only chromosomal abnormality present in 70% to 100% of APL bone marrow metaphase cells. The most frequent additional abnormality is trisomy 8, but this abnormality does not influence the rate of CR. The *RARA* gene is a nuclear hormone receptor, spanning 7.5 kb, and contains nine exons. The breakpoint occurs almost exclusively in intron 2 of the *RARA* gene. The *PML* gene locus spans 35 kb and contains nine exons coding for mRNA of 4.6, 3.0, and 2.1 kb. The breakpoints in *PML* occur in three distinct clusters leading to mRNAs of different length. The existence of different breakpoint regions in the *PML* gene and the presence of alternative splicing of PML transcripts are responsible for the heterogeneity of *PML RARA* junctions and variants.

APL is a disease characterized by accumulation of cells blocked at the promyelocytic stage of granulocytic differentiation. APL accounts for only 5% to 8% of pediatric AML, and 95% of children with APL show a classic t(15;17). However, almost 35% of pediatric APL have *FLT3-ITD* mutations. The clinical signs of these additional mutations on relapse rate and OS is not yet apparent.

Our current understanding of the molecular pathogenesis of APL is that *PML-RARA* behaves as a potent transcriptional repressor and that supra physiologic doses of retinoic acid can overcome this repression. In the presence of retinoic acid, *PML-RARA* behaves as a transcriptional activator. In patients with variant *ZBTB16-RARA*, an additional co-repressor complex binding site is present, and histone deacetylase inhibitors are used to restore sensitivity to retinoic acid. It appears that *PML-RARA* fusion-induced differentiation arrest of leukemic blasts and increased self-renewal of progenitors are two distinct features of APL and are probably driven by two different gene programs. The *RARA* fusion protein has a dual action, activating transcription of a number of genes and repressing transcription of others. *PML-RARA* and *ZBTB16-RARA* repress several DNA repair genes and activate the Wnt signaling and NOTCH pathways leading to increased stem cell renewal. Many of the genes targeted by retinoid acid are known to play a key role in regulating myeloid cell proliferation and differentiation. Therefore it is possible that inhibition of their expression by RARA fusion proteins may lead to differentiation blockade. A rare translocation (11;12)(p15;q13) has been identified in ∼10 patients, with morphological and immunophenotypic features of APL. This chromosomal translocation results in a fusion between *nucleoporin* (*NUP*)*98 and RARG* gene (RAR gamma) and these patients do not respond to treatment with ATRA and ATO. The common characteristic of these very rare patients is the presence of *RARG* rearrangement. Some partner proteins can bind to different DNA-binding domains of *RARG* to form fusion proteins such *as PML–RARG*, *CPSF6–RARG*, and *NPM1–RARG–NPM1*.

Rapid confirmation of APL diagnosis is mandatory and should be performed, if possible, on a bone marrow specimen, using ELN recommendations.[12] Accurate identification of the APL-associated genomic lesion at diagnosis is important because APL is a medical emergency that is frequently associated with an abrupt onset, high risk for early death (10% to 20%), and a potential for high cure rate (>80%) if appropriate treatment is rapidly initiated, based on its definitive diagnosis.

Although the *PML-RARA* rearrangement is the main pathogenetic event in APL other recurrent somatic mutations in genes including *FLT3*, *WT1*, *NRAS*, *KRAS*, *ARID1B*, and *ARID1A* have been observed. Recent data indicate that FLT3-ITD mutations do not confer a worse prognosis in patients receiving ATRA plus ATO. Comparing the initial mutational status of patients who relapsed during the course of disease and those in CR the number of concomitant mutations per patient was significantly higher in the group of patients who relapsed ($P < .0001$) indicating an accumulation of genetic alterations during disease progression. The treatment outcome in patients with newly diagnosed APL improved dramatically with the introduction of ATRA and ATO. ATRA activates transcription of blocked genes and degrades PML-RARA while ATO degrades PML-RARA by promoting apoptosis and has a pro-oxidant effect. Despite remarkable scientific advances over the past two decades high-risk patients and patients exhibiting uncharacteristic translocations remain problematic. The initial workup should include conventional karyotyping, FISH studies, RT-PCR, and anti-PML antibodies. Longitudinal monitoring of disease with RQ-PCR and FISH is currently recommended to provide assessment of MRD and early intervention if relapse should occur.

Less than 1% of patients with AML have a Ph chromosome (see Fig. 57.33, *bottom right*) and it remains a controversial entity as it is not listed in the WHO 2016 classification as a separate entity. Ph-positive (Ph+) AML may be cytogenetically distinguished from the myeloid blast crisis of CML by monosomy 7 (also frequently found in Ph+ ALL) and inv(16). Moreover, mutations found in 40% to 60% of AML as well as in Ph+ AML have not been described in Ph+ CML. All Ph+ AML examined to date showed a unique loss of Ig genes with a specific genomic signature, suggesting a distinct biologic entity. Both P210$^{BCR\text{-}ABL1}$ and P190$^{BCR\text{-}ABL1}$ were observed, but P190 appears to be prevalent. Before TKI therapy, the median survival of the Ph+ AML was 9 months; this has improved to a median survival of 18 months (range 6 to 71) with the use of imatinib therapy. In rare patients with AML, late appearance of a Ph chromosome either as a sole abnormality or in a clone showing t(8;21) is taken as evidence that the Ph chromosome in these patients is a secondary event. The late-appearing Ph chromosome in AML is characterized by P190$^{BCR\text{-}ABL1}$ protein. The coexistence of the Ph chromosome and inv(16) is a rare but recurrent finding in both CML and AML. In CML the fusion transcript is P210$^{BCR\text{-}ABL1}$, whereas in AML the fusion transcript is P190$^{BCR\text{-}ABL1}$. The presence of both the Ph chromosome and inv(16) in AML is associated with a favorable prognosis, whereas in CML, the coexistence of *BCR-ABL1* and *CBFB-MYH11* is predictive of rapid evolution to blast crisis. Rare AML patients may present with inv(3)(q21;q26.6)/*GATA2-MECOM* fusion and a subclone showing the Ph chromosome/*BCR-ABL1*.

Using extra-sensitive *BCR-ABL1* FISH probe Ph+ AML expressing P190$^{BCR\text{-}ABL1}$ protein may be recognized, and distinguished from the Ph+ CML because it is characterized by a second co-localized “fusion” signal, since the breakpoint on chromosome 22 in AML is more centromeric with two signals being closer to each other giving an impression of two fusion signals.

The *KMT2A* [lysine (K)-specific methyltransferase 2A], (*MLL*) gene at 11q23.3 is responsible for 95% of all 11q23 translocations, including both those in patients with AML and ALL (Figs. 57.39 and 57.40 and Table 57.11). *KMT2A* (previously known as *MLL*) is a large, 90-kb gene containing 36 exons coding for a 431-kDa protein. The protein contains AT hooks, which are known to regulate transcription by inducing changes in DNA conformation that permit the association of transcription factors with regulatory regions of DNA. *KMT2A* rearrangements occur in approximately 70% of infant and 10% of adult therapy-related leukemias. As of January 2021 102 direct *KMT2A*/*MLL*-gene fusions, 11 *KMT2A* rearrangements but not in-frame translocations, an additional 11 cases where the partner

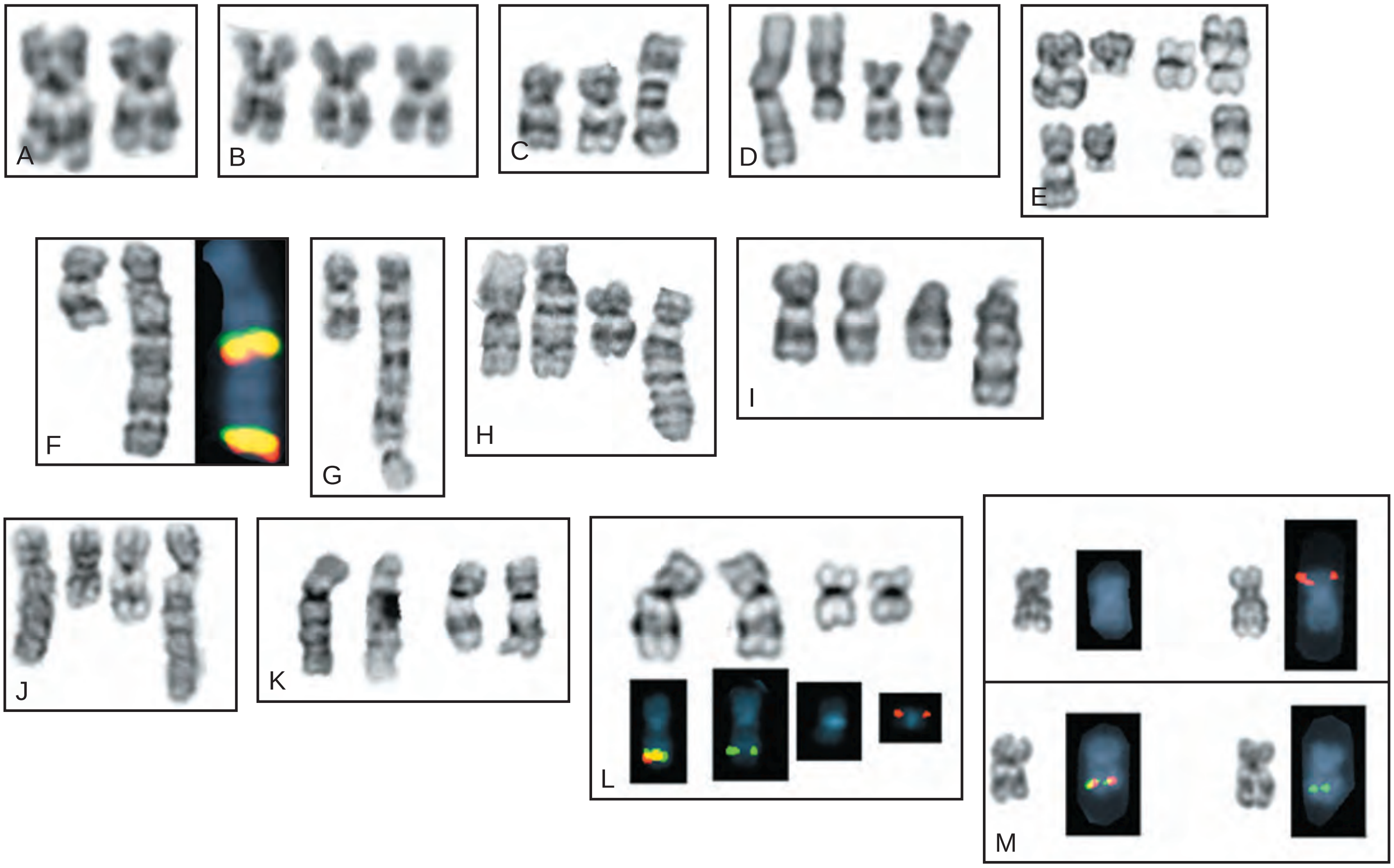


Figure 57.39 EXAMPLES OF CHROMOSOME 11 ABNORMALITIES. (A) Deletion of chromosome 11 at band q23. (B) Gain (trisomy) of chromosome 11. (C) Gain of isodicentric, idic(q11) in myelodysplastic syndrome (MDS). (D) Balanced t(1;11)(q13;p15) in MDS. (E) t(11;19)(q13;p13) in a pediatric patient with acute myeloid leukemia. (F) Duplication of the long arms of chromosome 11 and FISH image of *MLL* duplication. (G) Duplication (11;22) in the form of dicder(11;22)dup(11)(q13q14)t(11;22)(q23;p11) in a patient with MDS. (H) der(11)dic(1;11)(q12;q23) in a patient with myelofibrosis transforming to acute myeloid leukemia. (I) der(14)t(11;14)(q23;q23), resulting in trisomy for part of the long arms of chromosome 11. (J) t(4;11)(q23;q23) in pediatric acute lymphoblastic leukemia. (K) t(6;11)(q27;q23) in pediatric acute lymphoblastic leukemia. (L) t(11;19)(q23;p13) in pediatric acute lymphoblastic leukemia and after FISH study, showing separation of *MLL* breakapart probe where the 5′ end of the *MLL (green)* remains on der(11) and the 3′ end *(red)* is translocated to 19p. (M) t(9;11)(p22;q23) in pediatric acute myeloid leukemia and after metaphase FISH study *(right)*, showing that the 3′ end of the *MLL (red)* is translocated to 9p but the 5′ end *(green)* remains on der(11). *FISH*, fluorescence in situ hybridization.

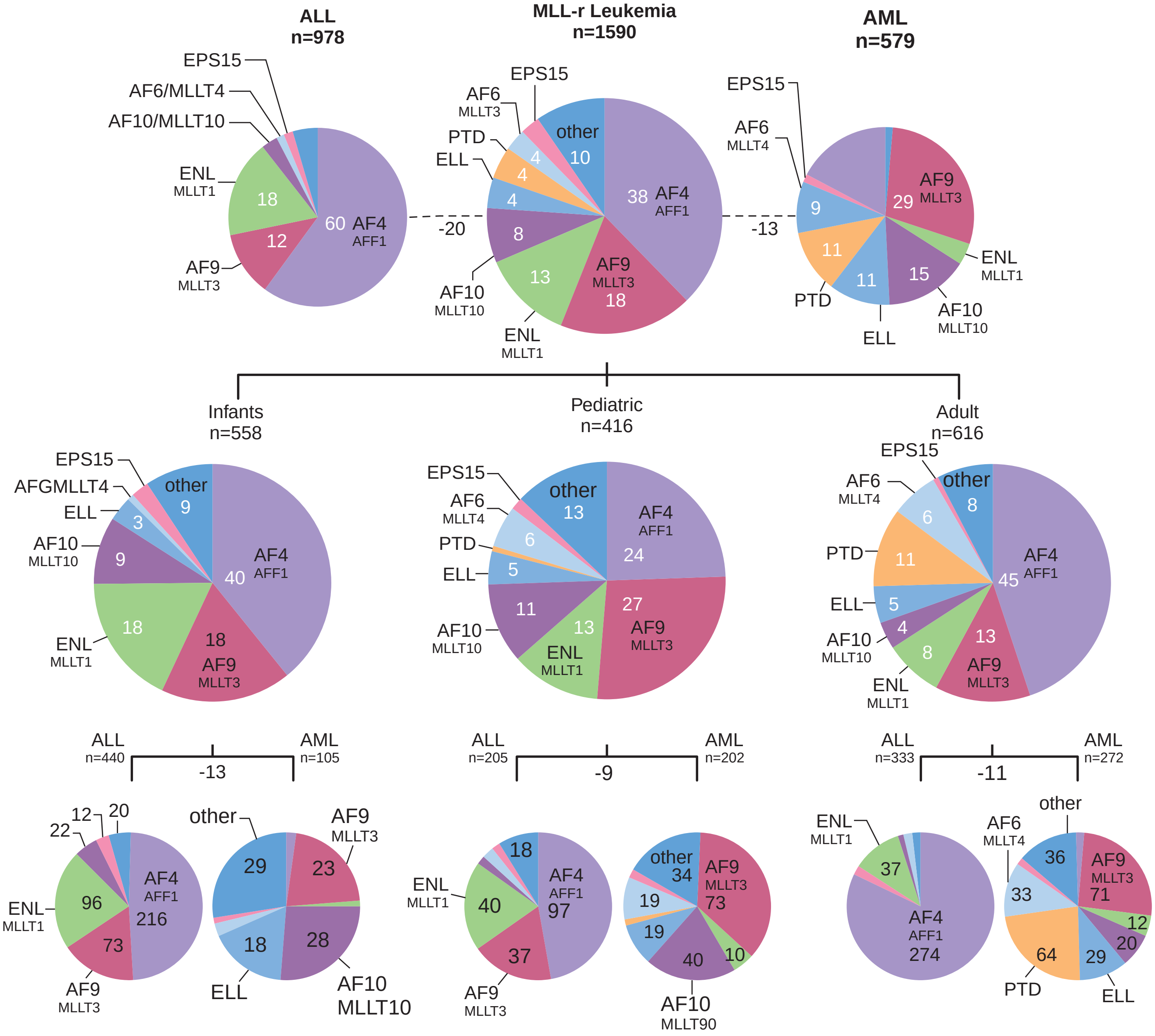


Figure 57.40 THE MLL RECOMBINOME IN ACUTE LEUKEMIA. Classification of patients according to age classes and disease type. (*Top*) Frequency of most frequent translocation partner groups in the investigated patient cohort of *MLL*-rearranged acute leukemia patients ($n = 1590$). This patient cohort was divided into ALL *(left)* and AML patients *(right)*. Gene names are written in black, and percentages are indicated as white numbers. Thirty-three patients could not be classified into the ALL or the AML disease types, respectively. *(Middle)* TPG frequencies for the infant, pediatric, and adult patient group. *(Bottom)* Subdivision of all three age groups into ALL and AML patients. Negative numbers refer again to the number of patients who were neither classified to the ALL nor to the AML subgroup. *ALL*, Acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *TPG*, translocation partner group. *(Reproduced with permission from Meyer C, Hofmann J, Burmeister T, et al. The MLL recombinome of acute leukemias in 2013. Leukemia. 27:2165, 2013.)*

gene is not directly fused, and 35 additional cases where the partner gene is not characterized at the molecular level have been identified (personal communication with Dr. Claus Meyer in January 2021).[13] In nearly all direct and reciprocal *KMT2A* recombinomes, the 3′ portion of the *KMT2A* gene is retained causing a direct fusion of the 5′ *KMT2A* gene portion with a portion localized telomeric to *KMT2A*. Of note, an abnormal FISH signal pattern, using dual-color, break-apart FISH, shows an abnormal signal pattern arising from 3′ *KMT2A* deletions in up to 28% of cases, and very rarely a gain of 3′ *KMT2A* portion of the gene, usually as a result of an additional derivative partner chromosome.

The five most frequent rearrangements of the 11q23/*KMT2A* gene in adult AML occur either with a translocation partner gene such as t(9;11)/KMT2A-*MLLT3 (MLL-AF9)* occurring in 24% of cases (see Figs. 57.40), (6;11)(q27;q23)/*KMT2A-MLLT4*, in 10% of cases, t(11;19)(q23;p13.3)/*KMT2A-MLLT1* in 4%, t(11;19)(q23;p13.1/*KMT2A-ELL* (*MLL-ELL*) in 12%, t(10;11)((p12;q23)/*KMT2A-MLLT10* in 9% of patents, or derived from *KMT2A* gene internal duplication *(KMT2A-PTDs)* of the amino-terminus region of the gene (see Fig. 57.39).

The BCR for about 96% of patients is localized between *KMT2A* exon 8 and 14. A novel, second (minor) BCR was reported in 2019, and was localized between *MLL* intron 21 and exon 24 (with a clear preference for *MLL* intron 21-23. -23, and includes a novel *KMT2A/MLL* fusion with *USP2. USP2* is localized about 1 Mpb telomeric to *KMT2A/MLL* at 11q23.3 and transcriptionally oriented in the direction of the chromosome 11 centromere and thereby classifying all as fusions ($n = 17$) and intrachromosomal inversions. Additionally 4 novel translocations include *USP8* gene as a partner. *KMT2/MLL-USP2* and *KMT2A/MLL-USP8* alleles seems to be restricted to the minor BCR. A breakpoint in *MLL* intron 11 is associated with a worse prognosis than those patients with upstream breakpoints. The

TABLE 57.11 Mixed Lineage Leukemia Translocations and Rearrangements 2021

Characterized on the molecular level:

#	Cytogenetic abnormality	Breakpoint	HUGO Name	Leukemia-Type
1	t(1;11)(p32.3;q23.3)	1p32.3	EPS15	ALL, AML, CML, MDS
2	t(1;11)(q21.3;q23.3)	1q21.3	MLLT11	AML, ALL
3	ins(1;11)(q42.12;q23.3)	1q42.12	ENAH	AML
4	t(1;11)(q43;q23.3)	1q43	ACTN2	ALL
5	t(2;11)(p23.3;q23.3)	2p23.3	ASXL2	t-AML
6	ins(2;11)(q11.2;q23.3)	2q11.2	AFF3	ALL, T-ALL
7	t(2;11)(q33.2;q23.3)	2q33.2	ABI2	AML
8	t(2;11)(q37.3;q23.3)	2q37.3	SEPTIN2	t-AML, AML, t-MDS
9	t(2;11)(q37.1;q23.3)	2q37.1	GIGYF2	ALL
10	t(3;11)(p21.31;q23.3)	3p21.31	NCKIPSD	t-AML
11	t(3;11)(p21.1;q23.3)	3p21.1	DCP1A	ALL
12	t(3;11)(q13.13;q23)	3q13.13	CIP2A	AML
13	t(3;11)(q25.31;q23.3)	3q25.31	GMPS	t-AML
14	t(3;11)(q27.3-q28;q23)	3q27.3-q28	LPP	t-AML
15	t(4;11)(p14;q23.3)	4p14	PDS5A	t-ALL, AML
16	t(4;11)(p11;q23.3)	4p11	FRYL	t-ALL, t-AML,t-MDS
17	t(4;11)(q21.1;q23.3)	4q21.1	USO1	t-AML
18	t(4;11)(q21.1;q23.3)	4q21.1	SEPTIN11	CML, t-ALL, t-AML
19	t(4;11)(q21.3-q22.1;q23.3)	4q21.3-q22.1	AFF1	ALL, t-ALL, BAL,(AML)
20	ins(4;11)(q22.1;q23.3)	4q22.1	FAM13A	ALL
21	t(4;11)(q35.1;q23.3)	4q35.1	SORBS2	AML
22	complex chromosomal abnormalities	5q12.3	CENPK	AML
23	t(5;11)(q12.1;q23.3)	5q12.1	KIF2A	ALL
24	t(5;11)(q23.2;q23.3)	5q23.2	PRRC1	t-ALL
25	ins(5;11)(q31.1;23.3)	5q31.1	AFF4	ALL
26	t(5;11)(q31.3;q23.3)	5q31.3	ARHGAP26	JMML, AML
27	t(5;11)(q35.3;q23.3)	5q35.3	MAML1	ALL
28	t(6;11)(q13;q23.3)	6q13	SMAP1	AML
29	t(6;11)(q15;q23.3)	6q15	CASP8AP2	AML
30	t(6;11)(q21;q23.3)	6q21	FOXO3	t-AML, t-ALL
31	t(6;11)(q25.3;q23.3)	6q25.3	SNX9	t-AML
32	t(6;11)(q27;q23.3)	6q27	AFDN	AML, t-AML, ALL, T-ALL
33	t(7;11)(p22.1;q23.3)	7p22.1	TNRC18	ALL, T-ALL
34	t(7;11)(p11.2;q23.3)	7p11.2	SEPTIN14	AML
35	t(7;11)(q11.23;q23.3)	7q11.23	CLIP2	B-ALL
36	t(7;11)(q21.12;q23.3)	7q21.12	RUNDC3B	T-ALL
37	t(7;11)(q32.1;q23.3)	7q32.1	FLNC	t-AML
38	t(7;11)(q36.1;q23.3)	7q36.1	GIMAP8	AUL
39	t(9;11)(p13.3;q23.3)	9p13.3	CLTA	ALL
40	t(9;11)(p21.3;q23.3)	9p21.3	MLLT3	AML,t-AML, ALL, BAL
41	t(9;11)(q33.2;q23.3);	9q33.2	DAB2IP	AML
42	t(9;11)(33.2-q33.3;q23.3)	9q33.2-q33.3	RABGAP1	ALL
43	ins(9;11)(q34.11;q23)	9q34.11	FNBP1	AML
44	t(9;11)(q34.12;q23.3)	9q34.12	LAMC3	t-AML
45	t(10;11)(p12.31;q23.3)	10p12.31	NEBL	AML
46	ins(10;11)(p12.31;q23.3)	10p12.31	MLLT10	AML, t-AML, ALL, T-ALL

TABLE 57.11 Mixed Lineage Leukemia Translocations and Rearrangements 2021—cont'd

	Characterized on the molecular level:			
#	Cytogenetic abnormality	Breakpoint	HUGO Name	Leukemia-Type
47	t(10;11)(p12.1;q23.3)	10p12.1	ABI1	AML
48	t(10;11)(q21.3;q23.3)	10q21.3	TET1	t-AML, AML, ALL
49	inv(11)(p15.4q23.3)	11p15.4	NRIP3	AML
50	INV(11)(q12.1q23.3)	11q12.1	BTBD18	ALL
51	INV(11)(q12.2q23.3)	11q12.2	PRPF19	ALL
52	t(11;11)(q13.4;q23.3)	11q13.4	ARHGEF17	AML
53	inv(11)(q13.4q23.3)	11q13.4	C2CD3	AML
54	inv(11)(q14.2q23.3)	11q14.2	PICALM	AML, ALL
55	inv(11)(q21q23.3)	11q21	MAML2	t-ALL, t-T-ALL, t-AML, t-MDS
56	del(11)(q23.3q23.3)	11q23.3	CBL	AML, t-AML, T-ALL, ALL
57	INV(11)(q23.3q23.3)	11q23.3	USP2	MPAL, ALL, AML
58	del(11)(q23.3q23.3)	11q23.3	ARHGEF12	AML, t-AML, t-ALL
59	del(11)(q23.3q24.2)	11q24.2	DCPS	AML
60	del(11)(q23.3q24.3)	11q24.3	ARHGAP32	ALL
61	t(11;12)(q23.3;p11.23)	12p11.23	ITPR2	t-MDS
62	t(11;12)(q23.3;q13.2)	12q13.2	SARNP	AML
63	t(11;14)(q23.3;q23.3-q24.1)	14q23.3-q24.1	GPHN	AML, t-AML
64	t(11;14)(q23.3;q32.33)	14q32.33	CEP170B	t-AML
65	t(11;15)(q23.3;q15.1)	15q15.1	KNL1	AML, t-MDS, ALL, T-ALL
66	t(11;15)(q23.3;q15.1)	15q15.1	ZFYVE19	AML
67	t(11;15)(q23.3;q21.3)	15q21.3	TCF12	t-AML
68	t(11;15)(q23.3;q21.2)	15q21.2	USP8	ALL, BAL
69	t(11;15)(q23.3;q25.3)	15q25.3	AKAP13	t-AML
70	t(11;16)(q23.3;p13.3)	16p13.3	CREBBP	t-MDS, t-AML,AML, t-ALL,t-CML, t-T-NHL
71	t(11;16)(q23.3;p13.11)	16p13.11	MYH11	AML
72	t(11;16)(q23.3;q22.1)	16q22.1	EDC4	AML
73	t(11;16)(q23.3;q24.1)	16q24.1	USP10	AML
74	t(11;17)(q23.3;p13.1)	17p13.1	GAS7	AML, t-AML, ALL
75	t(11;17)(q23.3;p11.2)	17p11.2	TOP3A	AML
76	ins(11;17)(q23.3;q12)	17q12	ACACA	AML
77	t(11;17)(q23.3;q12)	17q12	MLLT6	AML, ALL
78	t(11;17)(q23.3;q12)	17q12	LASP1	AML
79	t(11;17)(q23.3;q23.1)	17q23.1	CLTC	AML, ALL, T-ALL
80	t(11;17)(q23.3;q25.3)	17q25.3	SEPTIN9	t-AML, AML, MDS
81	t(11;18)(q23.3;q21.2)	18q21.2	ME2	t-AML,
82	t(11;19)q23.3;p13.3)	19p13.3	SH3GL1	AML
83	t(11;19)q23.3;p13.3)	19p13.3	RANBP3	ALL
84	t(11;19)(q23.3;p13.3)	19p13.3	MLLT1	ALL, T-ALL, AML,t-AL
85	t(11;19)q23.3;p13.3)	19p13.3	ACER1	ALL
86	ins(11;19)q23.3;p13.3)	19p13.3	VAV1	AML
87	t(11;19)q23.3;p13.2)	19p13.2	MYO1F	AML
88	t(11;19)q23.3;p13.11)	19p13.11	ELL	AML, t-AML, ALL, BAL
89	t(11;19)q23.3;q13.2)	19q13.2	ACTN4	t-ALL, t-AML
90	t(11;20)q23.3;q11.21)	20q11.21	MAPRE1	ALL
91	t(11;20)q23.3;q13.12)	20q13.12	STK4	T-ALL
92	t(11;20)q23.3;q13.13)	20q13.13	BCAS4	ALL
93	t(11;20)q23.3;q13.2)	20q13.2	PFDN4	T-ALL

(*Continued*)

TABLE 57.11 Mixed Lineage Leukemia Translocations and Rearrangements 2021—cont'd

	Characterized on the molecular level:			
#	**Cytogenetic abnormality**	**Breakpoint**	**HUGO Name**	**Leukemia-Type**
94	t(11;22)(q23.3;q11.21)	22q11.21	SEPTIN5	AML, T-ALL, ALL
95	t(11;22)(q23.3;q13.1-13.2)	22q13.1-13.2	MKL1	ALL
96	t(11;22)(q23.1;q13.2)	22q13.2	EP300	t-AML
97	t(11;22)(q23;q13.2)	22q13.2	SEPTIN3	t-AML
98	t(X;11)(p11.23;q23.3)	Xp11.23	TFE3	ALL
99	t(X;11)(q13.1;q23)	Xq13.1	FOXO4	ALL, t-ALL, AML
100	ins(X;11)(q24;q23)	Xq24	SEPTIN6	AML
101	ins(X;11)(q26.3;q23.3)	Xq26.3	CT45A2	BAL
102	ins(11;X)(q23q28q13.1)	Xq28	FLNA	AML
	Not in frame fusion			
1	t(3;11)(p21.31;q23.3)	3p21.31	SACM1L	N/A
2	t(3;11)(q21.3;q23.3)	3q21.3	EEFSEC	ALL
3	t(6;11)(p22.3;q23.3)	6p22.3	NUP153	ALL
4	inv(11)(p15.5q23.3)	11p15.5	AP2A2	AML
5	complex	11q14.3	NOX4	AML
6	inv(11)(q23.3q23.3)	11q23.3	BUD13	AML
7	t(11;15)(q23.3q;q21)inv(11)(q23.3q23.3)	11q23.3	LOC100131626	MDS
8	del(11)(q23.3q23.3)	11q23.3	CEP164	t-ALL
9	del(11)(q23.3q23.3)	11q23.3	DDX6	ALL
10	del(11)(q23.3q23.3)	11q23.3	BCL9L	ALL
11	t(2;11;19)(p23.3;q23.3;p13.3)	19p13.3	RANBP3-DT	AML
	No partner gene is directly fused to the MLL gene			
1	t(1;11)(p13.1;q23.3)	1p13.1	N/A	PMF Ostemyelofibrosis
2	t(6;11)(q27;q23.3)	6q27	N/A	AML
3	t(9;11)(p13.3;q23.3)	9p13.3	N/A	t-ALL
4	del(11)(q23q23.3)	11q23.3	N/A	ALL
5	t(11;21)(q23.3;q11.21)	21q22	N/A	t-ALL
6	INV(11)(q23.3q23.3)	11q23.3	N/A	AML
7	del(11)(q23.3q23.3)	11q23.3	N/A	ALL
8	del(11)(q23.3q23.3)	11q23.3	N/A	BAL
9	del(11)(q23.3q23.3)	11q23.3	N/A	t-AML
10	INV(11)(p12q23.3)	11p12	N/A	t-AML
11	complex case	20p13	N/A	?
1	**Not characterized on the molecular level**			
1	t(1;11)(p36;q23)			
2	t(1;11)(q31;q23)			
3	t(1;11)(q32;q23)			
4	t(2;11)(p21;q23)			
5	t(2;11)(q37;q23)			
6	t(3;11)(p13;q23)			
7	t(4;11)(p11;q23)			
8	t(6;11)(q13;q23)			
9	t(7;11)(p15;q23)			
10	t(7;11)(q22;q23)			
11	t(7;11)(q32;q23)			

TABLE 57.11 Mixed Lineage Leukemia Translocations and Rearrangements 2021—cont'd

1	Not characterized on the molecular level
12	t(8;11)(q11;q23)
13	t(8;11)(q21;q23)
14	t(8;11)(q24;q23)
15	t(9;11)(p11;q23)
16	t(9;11)(q33;q23)
17	t(10;11)(q25;q23)
18	t(11;11)(q11;q23)
19	t(11;11)(q13;q23)
20	t(11;11)(q21;q23)
21	t(11;12)(q23;p13)
22	t(11;12)(q23;q13)
23	t(11;12)(q23;q24)
24	t(4;13;11)(q21;q34;q23)
25	t(11;14)(q23;q11)
26	t(11;14)(q23;q32)
27	t(11;15)q23;q15)
28	t(11;17)(q23;q11)
29	t(11;17)(q23;q23)
30	t(11;18)(q23;q12)
31	t(11;18)(q23;q23)
32	t(11;20)(q23;q13)
33	t(11;21)(q23;q11)
34	t(Y;11)(p11;q23)
35	t(X;11)(q22;q23)

AL, Acute leukemia; *ALL*, acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *AUL*, acute undifferentiated leukemia; *B-ALL*, B-cell ALL; *BAL*, biphenotypic acute leukemia; *CML*, chronic myelogenous leukemia; *HUGO*, Human Genome Organisation; *JMML*, juvenile myelomonocytic leukemia; *MDS*, myelodysplastic syndrome; *MPAL*, mixed-phenotype acute leukemia; *PMF*, primary myelofibrosis; *t-AL*, therapy-related AL; *t-ALL*, therapy-related ALL; *T-ALL*, T-cell ALL; *t-AML*, therapy-related AML; *t-CML*, therapy-related CML; *t-MDS*, therapy-related MDS; *t-T-ALL*, therapy-related T-cell ALL; *t-T-NHL*, therapy-related T-cell non-Hodgkin lymphoma.
Courtesy C. Meyer, Patrizia Larghero, and Rolf Marschalek, Diagnostic Center of Acute Leukemia (DCAL), Institute for Pharmaceutical Biology, Goethe University, Frankfurt, Germany, January, 2021.

breakpoint in *MLL* intron 11 causes a *cis to trans* conversion and switches the MLL protein from a transcriptional activator/maintenance factor to a transcriptional repressor.

KMT2A rearrangements are initiated by DNA damage, which induces DNA repair via the nonhomologous-end-joining DNA repair mechanism. These genetic recombination events produce five different rearrangements: (1) reciprocal chromosomal translocations which fuse the 5′ –*MLL* gene portion with the corresponding gene; (2) partial tandem repeats, frequently in AML; (3) inversions or deletions on 11p and 11q in which inversions lead to reciprocal *KMT2A* fusions whereas deletions cause fusion of the 5′ KMT2A directly to another gene located further downstream (*ARHGEF12, BCL9L, CBL* and *CEP164*); and (4) complex *KMT2A* rearrangements involving three- or four-way translocations resulting in more than two fusion alleles as well as in *KMT2A* amplification (Fig. 57.41). About 15% of *KMT2A* recombinations represent in-frame fusions that can be readily expressed into a fusion protein and 85% are out-of-frame fusions and express a 5′ truncated MLL protein.

The t(9;11), which has been associated with a more favorable outcome in adult and pediatric AML, is distinguished as a separate entity in the latest WHO classification and ELN risk stratification recommendation (Fig. 57.42). Other frequent *KMT2A* translocations are t(6;11)(q27;q23) involving *MLLT4(AF6)* and t(11;19)(q23;p13.3) involving *MLLT1 (ENL)*. A partial tandem duplication of the amino-terminus region of the *KMT2A* gene is associated in patients with or without trisomy 11 (see Fig. 57.39B, C, and F).

The *KMT2A* gene is encoded by 37 exons. It is recruited to the promoters of select cell-cycle regulatory genes, suggesting its role in cell-cycle control. KMT2A protein regulates gene expression and cell cycle control via chromatin modification. Translocation of 11q23 cluster within an 8.3-kb region that encompasses exons 8 to 14 of *KMT2A* fuses the N-terminal portion of *KMT2A*, which contains the AT hook and methyltransferase domains, to numerous different proteins. In infant and in therapy-related AML, the *KMT2A* genomic breakpoints cluster at the 3′ end, near exon 12. In childhood and adult de novo AML, the breakpoints usually occur in the 5′ end, between exons 9 and 10. The most frequent *KMT2A* rearrangements in pediatric and adult patients as well as in ALL and AML are summarized in Fig. 57.39.

Patients with AML and *KMT2A* rearrangements have a poor prognosis despite treatment with aggressive chemotherapy. As mentioned earlier in this chapter, identical *KMT2A* rearrangements have been detected in three pairs of monozygotic infant twins, indicating in utero *KMT2A* rearrangement that results in clinical manifestations developing sometime during the first year of life. The contribution of various *KMT2A* fusion partners to transformation has been recently clarified for some fusion partners. Survival associated with the rare

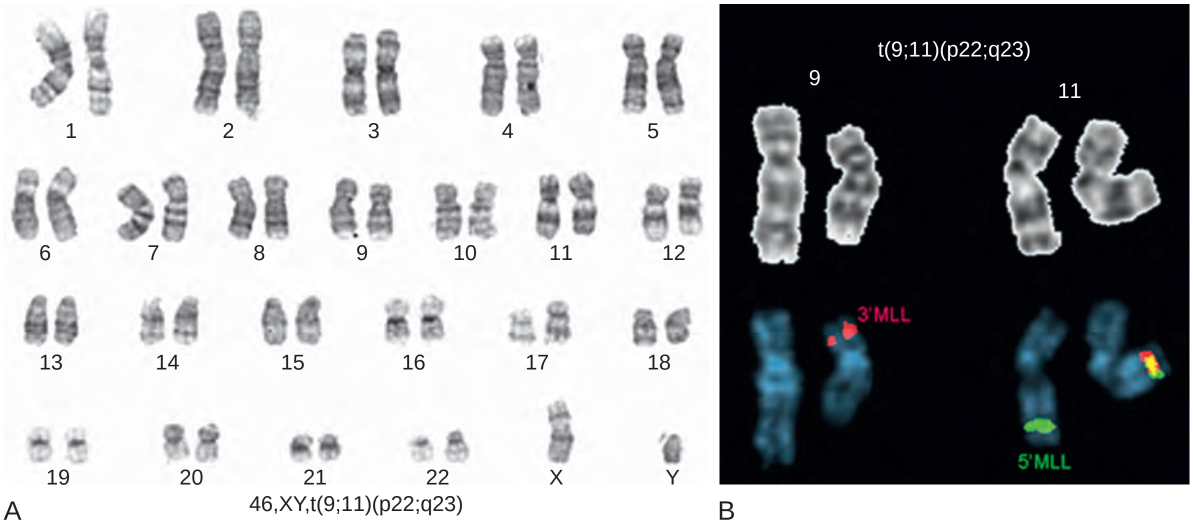


Figure 57.41 THE MOST FREQUENT *MLL* TRANSLOCATION IS t(9;11)(p22;q23). (A) The G-banded karyotype and (B) a partial karyotype, from another metaphase, after hybridization with dual color "breakapart" MLL probe showing the 5′ *MLL (green)* being retained on 11q23 and the 3′ *MLL (red)* moved as a result of the translocation to 9p22. This translocation results in a fusion of *MLLT3-AF9*.

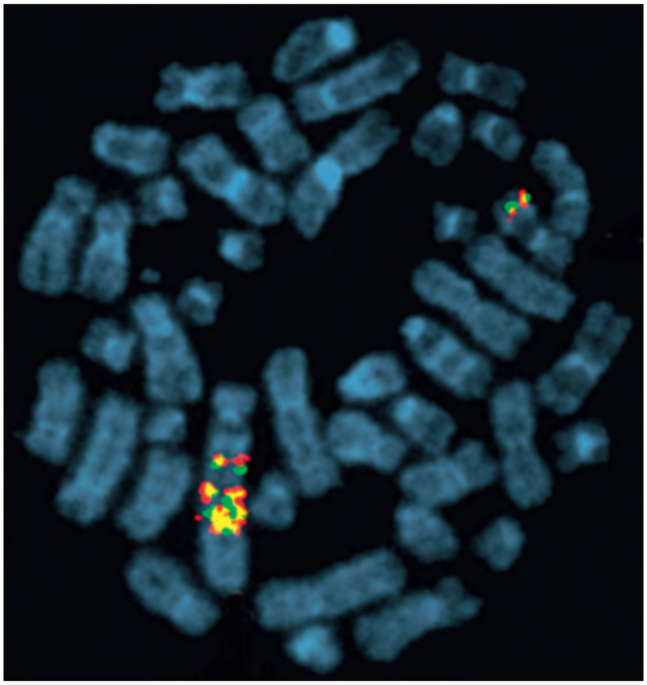


Figure 57.42 MLL AMPLIFICATION. Metaphase cell from a patient with acute myeloid leukemia after DAPI staining *(blue)* showing *MLL* amplification *(yellow)* along the abnormal chromosome 11. At least 15 copies of *MLL* amplicon were inserted into an abnormal chromosome 11. A normal copy of chromosome 11 with one copy of *MLL* is shown on the right side of the metaphase.

t(1;11)(q21;q23) translocation was favorable, in contrast to very poor outcomes with the more frequent t(4;11), t(10;11), and t(6;11) translocations. Even now, novel translocations, such as the one shown in Fig. 57.43, are still being identified and their prognostic implications are currently unknown.

ACAs occur in about 46% of adult AML patients with *KMT2A* rearrangements and most frequently involve trisomy 8 (23%), trisomy 21 (14%), and greater than 3 or more abnormalities (12%), but other additional chromosomal changes such as abnormalities of chromosomes 7 and 5 are not uncommon.

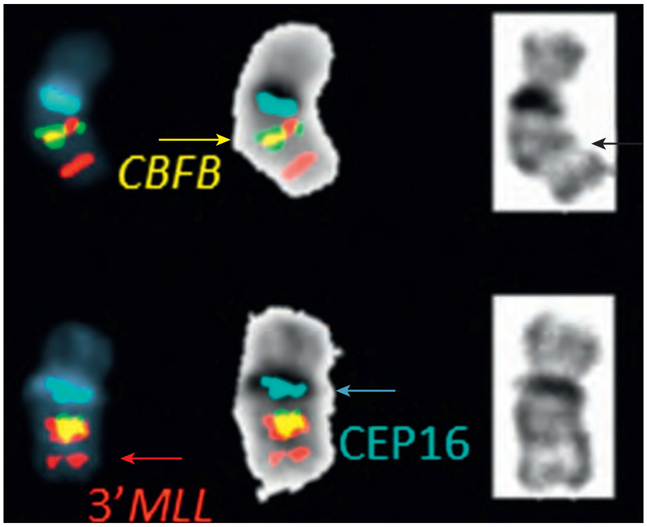


Figure 57.43 A NOVEL MLL PARTNER. A partial karyotype showing an abnormal chromosome 16 from two different metaphase cells from a patient with acute myeloid leukemia showing t(11;16)(q23;q23) karyotype as a sole abnormality. On the *left* is chromosome 16 stained with DAPI *(blue)* after metaphase FISH with three probes: centromere 16 in *aqua, CBFB* "breakapart" probe in *yellow*, and 3′ *MLL* in *red*. *Middle panel* is an "inverted" DAPI image and on the *right* is the G-banding of an abnormal chromosome 16. As a result of t(11;16) a part of 11q23 was translocated to 16 and the 3′ moved at 16q23.

In neonatal leukemia, defined as occurring within the first 28 days of life, most, if not all are congenital and very rare. In two thirds of patients the disease manifests as an AML and the remainder include ALL, specifically of B lineage, some with mixed phenotype or blastic plasmacytoid dendritic cell neoplasms (BPDCN). The most frequently observed cytogenomic abnormalities include t(4;11)(q21.3;q23.3)/*KMT2A-AFF1*, t(1;22)(p13.3;q13.1)/*RBM15-MKL1*, and t(8;16)((p11.2;p13.3)/ *KAT6A-CREBBP*. Intrauterine origin with transmission from one twin to the other via inter-uterine placental sharing has been reported in cases of AML with t(9;11) abnormality as well as in cases of ALL with t(11;19), (4;11) and t(1;11).

The rarity of these patients suggests that fetal and progenitor cell hematopoiesis are not inherently susceptible to leukemic transformation. This is supported by the observation that NGS shows a relatively "silent" mutational landscape and the key molecular event, *KMT2A* rearrangement alone, may be sufficient for development of leukemia in some cases.

Gene expression studies have demonstrated clear differences in the expression of lineage-associated genes, between *KMT2A* rearranged AML and ALL but there appears to be a core gene expression profile found in all *MLL*-rearranged leukemias, independent of the lineage.

AML patients with the partial tandem duplication within *KMT2A* (*KMT2A*-PTD) show internal duplication spanning exon 3 to 9, exon 3 to 10, or exon 3 to 11. *KMT2A*-PTD in AML occurs in 3% to 11% of adult de novo AML and is more frequently present in AML, and as mentioned above, with normal cytogenetics (57%) and AML with trisomy 11 (7%) as a sole cytogenetic abnormality. The most frequently mutated genes which accompany *KMT2A*-PTD AML include *DNMT3A* (39%), *FLT3*-ITD (35%), *IDH2* (21%), and RUNX1 (20%). The prognostic impact of *KMT2A*-PTD on OS and event-free survival (EFS) is similar to wild-type *KMT2A* AML. However, concomitant presence of *DNMT3* and *NRAS* mutations appears to be associated with an adverse clinical outcome.

t(8;16)(p11;p13)/MYST3-CREBBP rearrangements are a distinct pediatric subtype of AML with 97% of cases showing FAB subgroup M4 or M5 (Fig. 57.44). This specific association has also been observed in adult AML. Gene expression analysis revealed that t(8;16) cases clustered strongly together with, but separate from, *MLL* rearranged AML.

t(6;9)(p23.3;q34.1) is a rare cytogenetic abnormality, found in approximately 1% of AML cases, and subsequently reported to be associated with AML and marrow basophilia (Fig. 57.45). Basophilic leukemia is now recognized by the WHO classification as a separate entity. In addition to t(6;9), other chromosomal abnormalities such as t(8;21)(q22;q22), del(12)(p11–13), t(X;6),(p11;q23), and t(2;6)(q23;p22) may be associated with basophilic leukemia. As a result of t(6;9), the 3′ end of the *NUP214*, nuclear pore complex protein 214 kDa, gene located on chromosome 9, band q34, is fused to the 5′ end of the DEK gene located on chromosome 6, band p23. The resulting *DEK-NUP214* fusion gene is a derivative of chromosome 6. The translocation breakpoints occur in exon 6 in the *NUP214* gene and in exon 2 in the *DEK* gene. As a result, the presence of the *DEK-NUP214* fusion can be identified by PCR but not by dual-color FISH probes. Approximately 70% of patients with *DEK-NUP214* have an *FLT3* tandem duplication.

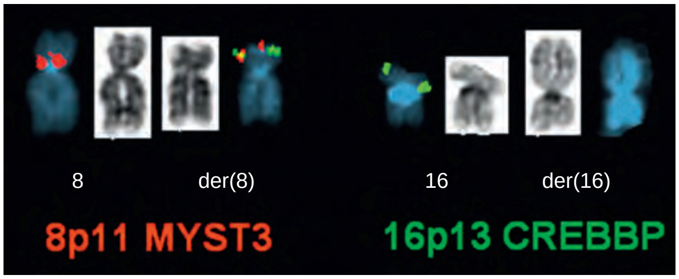


Figure 57.44 THE t(8;16). A partial karyotype from a patient with acute myeloid leukemia showing a t(8;16)(p11;p13) which results in fusion of *MYST3 (red)* and *CREBBP (green)*.

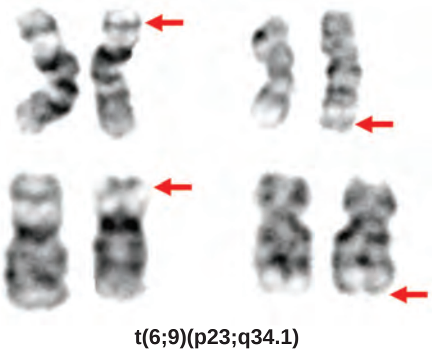


Figure 57.45 MARROW BASOPHILIA WITH CHARACTERISTIC t(6;9). t(6;9)(p23;q24). Shown as partial karyotype from 2 metaphase cells from a patient with acute myeloid leukemia and marrow basophilia. This translocation results in a fusion between *NUP214* gene on 9p34 (chromosomes on the right) t and *DEK* gene on 6p23, chromosome pair shown on the left *(red arrows)*.

inv(3)(q21q26.2) or t(3;3)(q21;q26.2) (see Fig. 57.28) is a distinct subtype of AML with recurrent chromosome abnormalities according to 2016 WHO classification, occurring in about 1% to 2.5% of all AML cases. Each rearrangement is associated with the juxtaposition of the *RPN1* (ribophosphorin 1 located on 3q21) gene with the transcription factor MECOM (*EVI1*, located in the 3q26.2 band). Two alternative forms exist, one generated from *EVI1*, the other *MECOM* (*MDS1* and *EVI1* complex locus) through intergenic splicing with *MDS1*, a gene located 140 kb upstream of *EVI1*. Both rearrangements may present as de novo or secondary AML and are characterized by normal or increased platelet counts and abnormal megakaryocytopoiesis. These abnormalities are also observed in MDS, blast crisis of CML, as well as in Ph-negative MPNs. De novo AML associated with t(3;3)/inv(3) is an aggressive form of leukemia with minimal responses to chemotherapy and poor clinical outcomes. Rare patients with an inversion on both chromosome 3 have been described and the second inv(3) appears to be a secondary event that carries an even worse prognosis. Patients with double inv(3)(q21q26.2) are associated with monosomy7/del(7q) or complex karyotype. Moreover, the second inv(3) is a result of an acquired copy neutral loss of heterozygosity, a somatic event occurring as a part of mitotic recombination of 3q. These patients have a rapidly progressive disease. Breakpoints in band 3q21 are distributed in a 235-kb region centromeric to and including the *RPN1* locus, whereas those in band 3q26.2 are scattered over a 900-kb region located on each side of and including the MECOM (*EVI1)* locus. There are a few cluster breakpoints within 3q21 region. The first BCR of about 30 kb is located 15 kb centromeric of the *RPN1* gene and the other breakpoints are centromeric to the first one, located up to 60 kb. In contrast to most of the translocations and inversions associated with de novo AML, that lead to the fusion genes, inv(3)/t(3;3) does not generate a chimeric gene, but rather induces gene overexpression. Approximately 68% of reported patients with inv(3)/t(3;3) have one ACA, including −7/del(7q) present in 75% of cases.

Resistance to therapy in patients with inv(3)/t(3;3) is linked to the inappropriate activation of the *EVI1* as a consequence of the 3q structural rearrangement. *EVI1* is a hematopoietic stemness factor and is a transcription factor with chromatin-remodeling activity. *EVI1* is also expressed in approximately 11% of patients with AML in the absence of 3q aberrations and represents an independent adverse prognostic factor. *EVI1* is activated as a consequence of inv(3)/t(3;3) via structural repositioning of a distal *GATA2* enhancer from 3q21 to the *EVI1* locus at 3q26. Relocation of the enhancer additionally confers reduced and monoallelic *GATA2* expression. Besides deregulated expression of *EVI1*, a molecular hallmark of patients with 3q structural rearrangements, 98% of these patients harbor mutations in genes activating RAS/receptor tyrosine kinase signaling pathways including hemizygous mutations in *GATA2*, heterozygous alterations in *RUNX1*, *SF3B1*, and genes encoding epigenetic modifiers. Chromosomal rearrangements involving 3q26.2 often lead to *EVI1/MECOM* rearrangements and induce *EVI*1 overexpression. More than 30 *MECOM* partner genes have been identified with the following abnormalities being the most common: inv(3)(q21q26.2 or t(3;3)(q21;q26.2)/EVI1*(MECOM)-GATA2* ~60%, t(3;21)(q26.2;q22)/*MECOM-RUNX1*(~15%) (associated with therapy-related MDS/AML), t(3;12)(q26.2;p13)/*MECOM-ETV6* (6%), and t(2;3)(p15–23;q26.2)/?-*MECOM* (6%), t(3;8)(q26.2;q24) often leads to *MECOM-MYC* rearrangement and is commonly associated with disease progression or therapy-related myeloid neoplasms with a dismal outcome.

t(3;5)(q25;q35*), NPM1/MLF1:* translocation (3;5)(q25;q35) is present in approximately 0.5% cases of AML and is associated with an intermediate prognosis, it is observed in all age groups, but more commonly in younger patients. These patients have shown to have a 34% survival rate after 10 years. In younger patients, this translocation is usually the sole karyotypic abnormality, whereas older patients may demonstrate a more complex karyotype. This translocation generates an *NPM1/MLF1* chimeric gene, which includes approximately half of the 24-kb *NPM1* gene, localized on 5q35, extending from exons 1 to 6, juxtaposed to virtually the entire 35-kb *MLF1* gene, localized on 3q25, starting at exon 2. The chimeric *NPM1/MLF1* fusion protein totals 426 amino acids with 175 amino acids from *NPM1* fused to 251 amino acids from *MLF1*, which excludes only the initial 16 amino acids from *MLF1* in this chimeric protein. In approximately 88% of patients with t(3;5) the translocation results in *NPM1/MLF1* fusion. In some patients with balanced t(3;5) the fusion is not apparent because of the variant 3;5 translocations that may include multiple genes at 3q21–25 and 5q31–35.

The presence of a hyperdiploid karyotype in *acute erythroleukemia* occurs in 47% to 56% of patients, along with a loss of genetic material in chromosomes 5, 7, and 18. An MK was identified in 43% of cases in one series. Balanced translocations are rare in erythroleukemia, although rare cases of *KMT2A* rearrangements have been reported. The frequent occurrence of a complex karyotype with abnormalities of chromosomes 5, 7, and 17 may be one reason for the poor prognosis associated with acute erythroleukemia. Mutations frequently seen in other subgroups of AML (such as *FLT3*, *KIT*, or *RAS* mutations) have not been reported in acute erythroleukemia. In pediatric patients, acute erythroleukemia is very rare. Congenital erythroleukemia is exceedingly rare with only six cases reported in the literature. The mutational spectrum of adult and childhood acute erythroleukemia allows classification of this entity into 5 distinct transcriptional profiles which are the strongest predictors of outcome: in adults *TP53*-mutated, *NPM1*-mutated, *KMT2A*-mutated and rearranged, and *DDX41*-mutated; and pediatric *NUP98* rearranged. Patients with *NPM1* mutations and *HOXB9* overexpression are associated with a favorable prognosis, and *TP53*, *FLT3*, and *RB1* alterations are associated with poor survival.

Pure erythroid leukemia represents a neoplastic proliferation of immature cells committed exclusively to the erythroid lineage with no evidence of a significant myeloblastic component. Pure erythroid leukemia is extremely rare and is frequently associated with complex cytogenetic abnormalities. The uncontrolled proliferation of immature erythroid precursors comprises at least 80% of the marrow. A complex karyotype is present in 83% of cases and the median OS is 2.9 months. Compared with AML with more than 50% erythroblasts, PEL demonstrate a higher incidence of poor-risk chromosomal abnormalities. Within the complex karyotype monosomy 7 appears to be the most frequent abnormality.

The M7 or megakaryocytic subtype of AML is a rare clonal disease, with an estimated frequency of 1% among AMLs, arising in a multipotent stem cell capable of differentiating along the megakaryocytic and granulocytic pathways. This acute leukemia subtype has a variety of genetic and morphologic characteristics. The M7 subtype is more frequent in children than in adults. In adults, megakaryocytic leukemia is frequently observed as a secondary leukemia after chemotherapy or leukemic transformation of several chronic MPNs, including CML. Approximately 65% of acute megakaryocytic leukemias are associated with marrow fibrosis. After multivariate analysis, the AML megakaryocytic subtype is an independent predictor of increased mortality. Approximately 50% of patients with M7 AML have chromosomal abnormalities at diagnosis. No specific chromosomal abnormality is associated with the adult form but observed abnormalities include 3q21 to 3q26 rearrangements, partial or total deletion of chromosomes 5 and 7, gain of chromosomes 8 and 19, and t(9;22). Adult patients with megakaryocytic leukemia are also characterized by mutations in *TP53*, *DMNT3A, RB1*, and genes in cohesion, and *ASXL* family.

Two manifestations of childhood megakaryocytic leukemia have been observed. First is constitutional trisomy 21 association with *GATA1* (located on X chromosome) mutation (Chapters 29, 30, 63). Young children with constitutional trisomy 21, median age at diagnosis 6.5 days, are more than 100 times more likely to develop megakaryocytic leukemia than normal children. However, children with constitutional trisomy 21 and megakaryocytic leukemia have a more favorable prognosis as compared with patients without constitutional trisomy 21. In these patients with constitutional trisomy 21, a second event includes somatic mutations of the transcription factor *GATA1* which leads to the third event of malignant transformation. The presence of +21 is sufficient to cause an increased number of megakaryocytes *in utero* but not sufficient to cause leukemia. However, the mutations in *GATA1* lead to production of the shortened GATA1 protein that lacks the first 83 amino acids and together with constitutional +21 leads to transient myeloproliferative disorder (TMD). TMD is a self-limiting preleukemic condition seen at birth in up to 30% of constitutional +21 neonates. Although the majority of TMD cases undergo a spontaneous remission, 10% to 20% of clinically significant cases progress to megakaryocytic leukemia within 4 years. This progression is associated with acquisition of additional mutations resulting in malignant transformation of the residual *GATA1* mutant clone. The second form of childhood acute megakaryocytic leukemia involves t(1;22)(p13;q13) encoding the OTT-MAL (*RBM15-MKL1*) fusion protein in infants without constitutional trisomy 21 (Fig. 57.46). It is a very rare abnormality, described in about 40 cases worldwide (about 5% of infant AML cases), and associated with infantile M7 and with children younger than 3 years of age. Detection of t(1;22) is diagnostic. Another fusion protein often seen in pediatric non-constitutional +21 patients is inv(16)(p13.3q24.3) leading to the chimeric protein *CBFA2T3-GLIS2*. This translocation is observed in 18.6% of pediatric acute megakaryocytic leukemia patients and associated with a worse overall and reduced EFS. It has not been detected in adult patients but it has been identified in other pediatric patients with AML. The translocation (11;15)(p15;q35) /*NUP98-JARIDIA (KDM5a)* is observed in 8% to 12% of pediatric megakaryocytic leukemia patients and does not carry any statistically significant prognostic risk although these patients tend to show lower survival rates than those with other chromosomal fusions. Patients expressing *NUP98-KDM5a* also have frequently mutated *RB1*, *KMT2A* rearrangements, and *RAS* mutations. These children

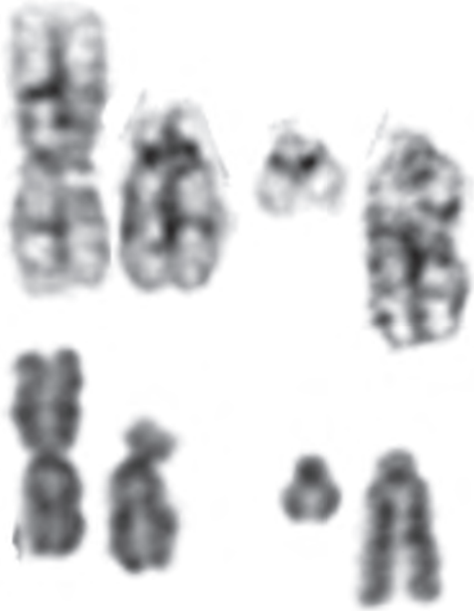


Figure 57.46 MEGAKARYOCYTIC LEUKEMIA WITH T(1;22). Partial karyotype from two metaphase cells from a 4-week-old baby with M7 megakaryocytic leukemia showing a diagnostic t(1;22)(p13;q13) abnormality.

acquire diverse chromosomal abnormalities, most notably tetrasomy 21 and trisomy 8 and *KMT2A* rearrangements (17.4%). Pediatric non-constitutional +21 acute megakaryocytic leukemia is a heterogenous malignancy comprised of distinct subsets of subgroups with varying outcome.

The t(1;22) rearrangement has been observed in a set of monozygotic twins, suggesting an *in utero* origin in some cases. To date there are 16 cases of *GATA1* mutation-related transient MPDs and M7 AML in phenotypically and cytogenetically normal children. Thirteen of the 16 children were diagnosed during the first few months of life and in six cases, the blasts disappeared spontaneously without chemotherapy and the patients did not develop M7 AML. Gene expression profiling has provided the insight into the molecular pathogenesis of M7 leukemia in children with and without constitutional trisomy 21. These patients have distinct molecular phenotypes, with increased expression of chromosome 21 genes in patients with constitutional trisomy 21 as compared with M7 leukemia patients without constitutional trisomy 21. The *RUNX1* gene, localized on chromosome 21 which is essential for normal megakaryocytopoiesis, is expressed at lower levels in children with constitutional trisomy 21 and M7 leukemia, indicating a mechanism that may contribute to a block in differentiation.

Down Syndrome–Associated Acute Myeloid Leukemia

The myeloid leukemia of DS (Chapters 29, 30, and 63) was given a special WHO subclassification (ML-DS) because of its unique clinical and biologic features. These erythromegakaryoblastic leukemias are diagnosed before the age of 5 years and often present with thrombocytopenia and/or myelodysplasia. ML-DS is always preceded by the neonatal preleukemic syndrome, transient abnormal myelopoiesis (TAM; also known as transient MPD) that may, or may not, be clinically apparent. Unlike acute megakaryoblastic leukemias in patients without DS, these patients usually respond well to therapy with most patients being cured. Genetically, ML-DS is characterized by an acquired mutation in the *GATA1* gene (90%). The mutation in *GATA1* is necessary but insufficient for development of ML-DS. Virtually all cases of TAM and ML-DS have N-terminal truncating *GATA1* mutations. *GATA1* mutations are present at birth in both neonates with DS with TAM and, through retrospective analysis of neonatal blood spots, also in children with ML-DS without a previous history of TAM. It is not clear at what stage in fetal development *GATA1* mutations arise; the earliest point in gestation at which mutations have been identified is 21 weeks. *GATA1* mutations disappear when TAM (or ML-DS) enters remission, indicating that they are acquired.

Monosomal Karyotype

MK is defined by the presence of at least two separate autosomal monosomies or one monosomy plus one or more structural abnormalities. The overall frequency of MK in AML varies between 6% and 20%. In a study of 1058 patients with AML and an abnormal karyotype, 30% had MK (see Fig. 57.33, *bottom row*). AML with MK is frequently associated with other adverse risk cytogenetic abnormalities, such as inv(3), −5 or del(5q), −7 or del(7q), abnormal (12p), −18/del(18q), abnormal (17p), and a complex karyotype. The frequency of these recurrent monosomies are: −7 and −17 in 6%, −18 in 5%, −5 and −21 in 4%, −20 in 3%, −3, −12, and 22 in 2%, and loss of chromosomes 2, 4, 9, 13, and 19 in 1% of cases. MK is a strong prognostic predictor of poor outcome compared with a traditionally defined complex karyotype. Responses to induction therapy and OS of patients who have AML with MK are dismal: CR rates of 32% and a 4-year survival of 9%. MK in a study of 248 patients with AML was found to be associated in more than 50% of patients with deletion of P53, whereas in another study of newly diagnosed 369 patients with AML, MK predicted an adverse treatment outcome and was associated with multidrug-resistance. A low frequency of mutations such as *FLT3* and *NPM1* are found in patients with MK AML.

Gain or Loss of Chromosomes in Acute Myeloid Leukemia

Approximately 15% to 20% of patients with AML have a numerical gain or loss of a single chromosome as the sole primary karyotypic abnormality. Each of the autosomes and sex chromosomes can contribute to the numerical changes. The most common trisomies in decreasing order of frequency are gain of chromosome 8 (Figs. 57.47

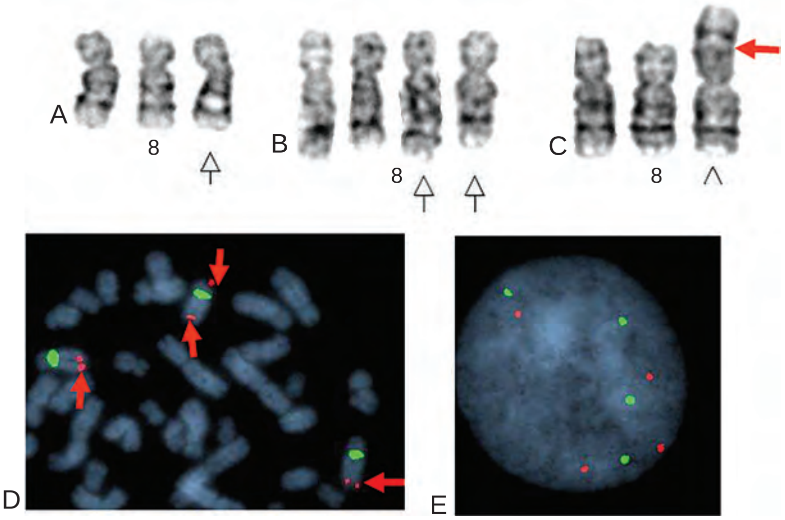


Figure 57.47 GAIN OF CHROMOSOME 8 DETECTED AT RELAPSE OF ACUTE MYELOID LEUKEMIA. All evaluated cells had a gain of chromosome 8. Four related cell populations evolved: (A) 35% of cells had +8, (B) 25% of cells had tetrasomy 8, (C) 35% of cells had a gain of an isochromosome of 8q and a deleted 17p and loss of TP53 (not shown). The i(8q) is a novel abnormality not previously observed in multiple testing from the diagnosis (a year earlier) but del (17p) was observed in 11% of cells at diagnosis, indicating genomic instability as a result of TP53 deletion. (D) Shows a metaphase cell after FISH with two homolog 8 and the presence of i(8q) *(two red arrows)*. (E) Shows an interphase FISH with 4 red signals (MYC at 8q24) and 4 centromeres *(green)* indicating a cell with tetrasomy 8 detected in 58% of cells while 11% had 3 copies of #8 whereas 24% of cells had 4 copies of MYC and 3 copies of centromere 8 consistent with i(8q).

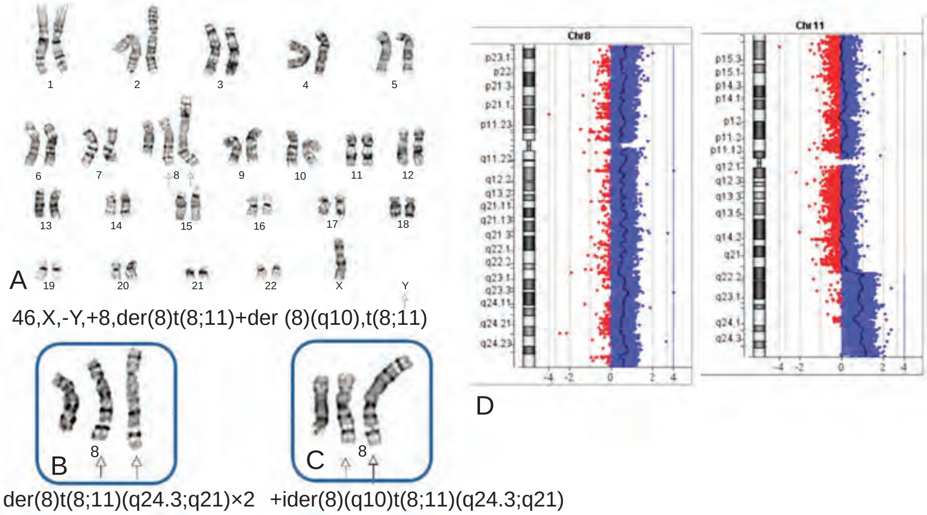

A
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y
46,X,-Y,+8,der(8)t(8;11)+der (8)(q10),t(8;11)
B
8
C
8
der(8)t(8;11)(q24.3;q21)×2
+ider(8)(q10)t(8;11)(q24.3;q21)
Chr8
Chr11
D
-4
-2
0
2
4

Figure 57.48 STRUCTURALLY ABERRANT CHROMOSOMES 8 AND 11 IN A NEWLY DIAGNOSED PATIENT WITH ACUTE MYELOID LEUKEMIA. (A) 90% of cells had a loss of Y chromosome and a gain of an aberrant chromosome 8 in two copies. The derivative chromosome 8 first resulted from an unbalanced translocation between the long arms of chr(8) and chr(11) which entirely replicated itself leading to a gain of chr(8) and four copies of 11q. (B) A partial karyotype from another cell showing two copies of der(8)t(8;11). (C) Additionally, 10% of cells showed an alternative aberrant chromosome 8 present in one copy with a gain of an additional aberrant copy in the form of an isoderivative chr(8). Ider(8)t(8;11) was produced from an unbalanced translocation between 8q and 11q and a subsequent replication of the proximal region leading to two identical arms of the aberrant chromosome, resulting in four copies of 8q, and five copies of 11q. (D) Partial gains of 8q and 11q are rare recurrent abnormalities found in myeloid malignancies. According to the 2017 ELN recommendations, these results are associated with an intermediate-adverse risk category. (D) Array testing detected trisomy and tetrasomy of chromosome 8 (a ~146.4 Mb gain 8p23.3q24.3) and partial gain of the long arm of chromosome 11q (~35.2 Mb 11q22.1q25) *(blue)*.

and 57.48), 11, 13, 22, 21, 14, and 9 and most common monosomies include −5, −17, and −7. The gain of chromosome 8, the most frequent abnormality seen in AML, is a sole abnormality in 6.3% of cases and overall occurs in 16% of cases. The incidence of +8 detected by FISH varies between 19% and 25% of AML cases. The prognosis of AML with +8 depends on whether +8 occurs as an isolated abnormality or accompanies other cytogenetic aberrations. In the latter situation, +8 does not appear to adversely affect the favorable outcome of patients with t(15;17), inv(16)t(16;16), and t(8,21). By contrast, patients with +8 and a complex karyotype and/or an unfavorable aberration such as del(5q) or −7 usually have a very poor outcome. Isolated +8 has been considered to be associated with either intermediate or unfavorable prognosis.

MYC Amplicon in Acute Myeloid Leukemia

In normal cells, *MYC* gene is localized on chromosome 8, band region q24.21. Less than 1% of hematologic malignancies have genomic amplification and they are usually associated with an elderly age and poor prognosis. Genomic amplifications are cytogenetically identified as homogeneous staining regions (HSR), DM, and ring chromosomes. Gene amplification(s) in hematologic malignancies is a rare entity (<1%). Using a comprehensive and high-resolution genomic method, analysis of 23 patients with *MYC*-amplification revealed amplicon heterogeneity in 65% of these patients and in 48% deletions of 8q24 segment was observed. Moreover, amplification of 8q24 region has been observed in a ring structure suggesting that 8q24 amplicon architecture is shared between different chromosomal structures. Discovery of neocentromere in these patients may represent scattered acentric DM as well as HSR resulting in mitotic stabilization, and providing an adaptive advantage of leukemia cells harboring 8q24 amplicon.

Deletion of 17p often results in the loss of tumor suppressor *TP53* gene on band p13.1, which has been reported in 5% to 9% of adult AML patients. The abnormalities of 17p are often associated with other chromosomal aberrations such as del(5q), −5, −7, but is an independent poor-risk prognostic factor.

TP53 mutations are more common in older patients and those who have previously received alkylating agents. These patients have an increased number of CD34+ cells, suggesting that the loss of *TP53* function could cause cell cycle arrest. Based on genomic classification of 1540 AML patients treated in different intensive chemotherapy trials, the *TP53*-mutation-positive subgroup of patients consisted of complex cytogenetic alterations or isolated chromosome losses or gains and were associated with an adverse prognosis. Therefore, it has been proposed that testing for deletion and mutation of *TP53* should be included in the ELN and WHO classification system of AML (Figs. 57.49 and 57.50).

Acute Myeloid Leukemia With Complex Karyotype

Any karyotype with at least three chromosomal aberrations, regardless of their type and the individual chromosomes involved, is designated as “complex” in the absence of the WHO-designated recurrent genetic abnormalities. Several studies have shown that patients with t(8;21), t(16;16)/inv(16) and t(15;17) constitute a separate biologic and clinical entity even if they contain additional abnormalities, because these additional cytogenetic abnormalities do not adversely affect the clinical outcome. Therefore the category of AML patients with a complex karyotype exclude patients with t(8;21),inv(16)/t(16;16) and t (15;17). Approximately, 10% to 12% of AML patients have three or more chromosome abnormalities whereas 8% to 9% have five or more abnormalities. The incidence of a complex karyotype increases with age. In patients with AML, age 18 to 60, 6% to 8% have three or more chromosomal abnormalities whereas 17% to 19% of patients older than 60 have a complex karyotype. In three large series of AML patients with a complex karyotype analyzed using multicolor FISH, more than 90% of patients had at least five abnormalities, with a median number of chromosomal aberrations being between 6 and 10. Approximately 80% of all patients with a complex karyotype have deletion 5q, followed by deletions 17p and 7q, occurring in approximately 50% of cases. At least 85% of all patients with AML with a complex karyotype showed one of these three deletions. The prognosis of patients with a complex karyotype is generally very poor. Among

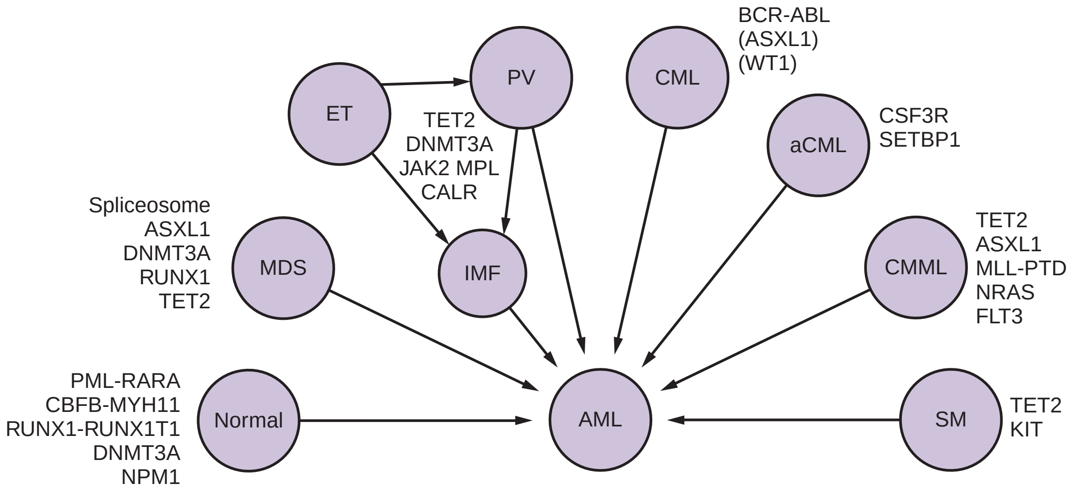

BCR-ABL
(ASXL1)
(WT1)
PV
CML
ET
TET2
DNMT3A
JAK2 MPL
CALR
aCML
CSF3R
SETBP1
Spliceosome
ASXL1
DNMT3A
RUNX1
TET2
MDS
IMF
CMML
TET2
ASXL1
MLL-PTD
NRAS
FLT3
PML-RARA
CBFB-MYH11
RUNX1-RUNX1T1
DNMT3A
NPM1
Normal
AML
SM
TET2
KIT

Figure 57.49 COMMON MUTATIONS IN DE NOVO AND SECONDARY ACUTE MYELOID LEUKEMIA. A number of clonal blood disorders with a myeloid phenotype are represented. Each of these disorders is characterized by recurrent mutations in specific genes, some of which are shared between several different phenotypes (e.g., *TET2*). All of these disorders can transform to secondary AML upon acquisition of additional somatic mutations. When AML arises in the absence of an antecedent clonal blood disorder, it is known as primary AML. *aCML*, Atypical CML; *AML*, acute myeloid leukemia; *CML*, chronic myeloid leukemia; *ET*, essential thrombocytopenia; *IMF*, idiopathic myelofibrosis; *PV*, polycythemia vera; *SM*, systemic mastocytosis. *(Reprinted with permission from Grove CS, Vassiliou GS. Acute myeloid leukaemia: a paradigm for the clonal evolution of cancer? Dis Model Mech. 2014;7:941.)*

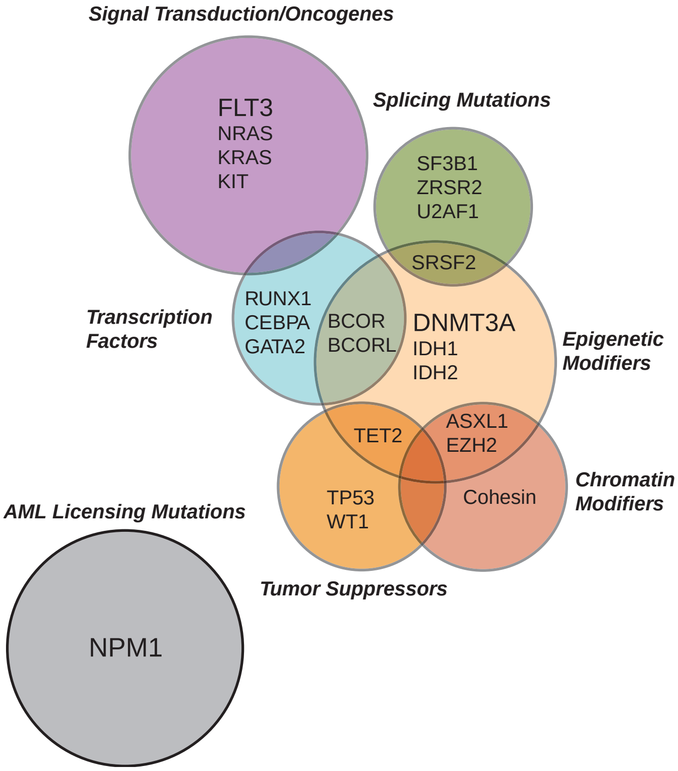

Signal Transduction/Oncogenes
FLT3
NRAS
KRAS
KIT
Splicing Mutations
SF3B1
ZRSR2
U2AF1
SRSF2
Transcription Factors
RUNX1
CEBPA
GATA2
BCOR
BCORL
DNMT3A
IDH1
IDH2
Epigenetic Modifiers
TET2
ASXL1
EZH2
TP53
WT1
Cohesin
Chromatin Modifiers
AML Licensing Mutations
Tumor Suppressors
NPM1

Figure 57.50 FUNCTIONAL OVERLAP OF ACUTE MYELOID LEUKEMIA DRIVER MUTATIONS. Driver mutations in AML stratified mechanistic consequences. Size of circles reflect mutational frequency with three most common mutations in larger type. *(Reprinted with permission from Kihstagari A, Levine RL, Viny AD. Driver mutations in acute myeloid leukemia. Curr Opin. 2020;27:49.)*

patients with AML above the age of 60, which constitute the majority of patients with complex karyotype, only 10% to 44% of those who harbor three or more chromosomal abnormalities achieve a CR, which usually is of very short duration (median 6 to 8 months). CR rates of karyotypically complex patients are slightly higher in younger patients. The number of chromosomal abnormalities is central to the definition of a complex karyotype. Adult patients with *hyperdiploidy* with 49 to 65 chromosomes is a rare abnormality in AML, less than 2%, and by definition is included in a subgroup of patients with complex karyotypes, irrespective of the structural abnormalities involved. Patients with only numerical gains should be classified as belonging to the intermediate risk group of patients with AML.

Complex karyotype AML occurs in a heterogeneous group of patients that almost never is associated with mutations in *NPM1* nor with biallelic *CEBPA* genes and infrequently with mutations in *FLT3*, *IDH1/IDH2*, *DNMT3A*, and *TET2*. In sharp contrast, this subgroup of AML patients are characterized by chromosomal aneuploidy, by structural rearrangements and ~70% demonstrate point mutations in *TP53* gene. Most of the patients with *TP53* deletions are accompanied by *TP53* mutations in the remaining allele leading to the complete loss of the *TP53* activity. Heterozygous *TP53* mutations are also common.

Acute Myeloid Leukemia in Children (Chapter 63)

The Children Oncology Group (COG)–National Cancer Institute (NCI) TARGET AML Initiative has characterized the genomic landscape of almost 1000 pediatric AML patients.[14] This extensive effort revealed similarities and unique differences between adult and pediatric AML. The most important observation was that the overall frequency of myeloid malignancy gene mutations is lower in pediatric AML than that in adult patients. The mutational burden increases with age, with fusions and copy number alterations being more common in younger patients while smaller sequence variants are more frequent in older patients. Mutations in *DNMT3A*, *IDH1/2*, *NPM1*, and *TP53* are less common in pediatric patients while co-occurring *NUP98* and *FLT3* or *WT1* mutations were demonstrated to affect pediatric AML outcomes. The TARGET initiative also revealed some "novel" pediatric-specific chromosomal copy number changes including focal deletions of *MBNL1* gene (3q25.2) and *ELF1* (13q14.11). Although the TARGET pediatric study did not report *PML-RARA* fusion-positive children, about 5% to 10% of pediatric AML patients harbor *PML-RARA* fusion. However, in about 10% of these patients who are morphologically diagnosed as APL, RARA rearrangements could not be identified which suggests that these children should be classified as a subclass of AML rather than APL. The impact of cytogenetics on outcome in a pediatric group of patients with AML (excluding APL) demonstrated about 80% OS at 10 years in patients with *CBFA2T3* AML. Within these groups, t(16;21)(q24;q22) resulting in *CBFA2T3-GLIS2* fusion is an exclusive pediatric cytogenetic abnormality which leads to a strong downregulation of *GATA1*. It is currently thought that pediatric acute megakaryocytic leukemia associated with DS (+21 constitutional) targets *ERG* and *GATA1* genes but that the *CBFA2T3-GLIS2* fusion is able alone to maintain both high ERG and low GATA1.

In contrast to *CBFA2T3* AML, pediatric patients with *MLL(KMT2A)* abnormalities had an intermediate prognosis (61% OS at 10 years) with no evidence of heterogeneity according to the translocation partner of *MLL*. Additional abnormalities among the 11q23 leukemias have diverse prognoses; trisomy 8 is an independent favorable, whereas trisomy 19 is considered an adverse prognostic factor in the *MLL* rearranged group of pediatric patients. Approximately 10% to 15% of children and adolescents and 30% to 60% of infants have *KMT2A* AML. Most of the *KMT2A* fusion-positive AMLs are considered an aggressive form of AML, although the outcome is overall neutral (fusion specific impact on prognosis) and notably t(9;11)(p22;q23)/ and ins(10;11)(p12;q23q13) are more prevalent in children than adults. *MLL10* (10p12.31) is the fourth common gene fusion partner and is predominantly observed in pediatric AML, although it has been reported in B- and T-cell ALL. In infant patients with t(9;11) the patients present with ALL rather than AML, and the phenotype usually changes into typical myelomonocytic AML with increasing age of patients. *FLT3*-ITD alterations are found in about 10% to 20% of newly diagnosed pediatric AML patients and is an independent prognostic factor for poor outcome particularly for patients with high ITD allelic ratios and/or loss of the wild-type allele leading to copy number-neutral ITD homozygosity.

Recurrent fusions involving *NUP98* (11p15) with over 30 different partner chromosomes have been identified in approximately 4% to 9% of pediatric AML patients. *NUP98-KMT5A* fusions are more prevalent in children below 3 years of age. Clinically *NUP98* rearrangements are associated with normal karyotype AML and most frequently occur in the myelomonocytic subtype with the exception of *NUP98-KDM5A* fusions [t(11;12)(p15.4;p13–33)] which are enriched in acute megakaryocytic leukemia occurring in approximately 10% of such cases. The presence of a *NUP98-KDM5A* fusions in megakaryocytic leukemia is associated with a poor prognosis with an EFS of 25%. The *NUP98* fusions are associated with a poor prognosis in acute erythroleukemia, with 20% of children with this rare form of AML. Isolated *NUP98* rearrangements are not independently prognostic but the combination of *NSD1-NUP98*/ [t(5;11)(q35.2;p15.4)] fusion and concomitant *FLT3*ITD mutation is strongly associated with a poor outcome.

Acute Myeloid Leukemia in the Elderly

The biology of AML changes with age (Chapters 19, 59, 60, and 157). The spectrum of cytogenetic abnormalities in older adults includes a higher percentage of abnormalities involving −5/del(5q), −7/del(7q), and 17p and a lower incidence of translocations associated with a favorable prognosis. Older patients with a complex karyotype have an extremely poor prognosis, with only 26% achieving CR owing to high rates of resistant disease. Multidrug resistance is present in 57% of patients over 75 years of age and in 33% of patients with AML younger than 56 years of age. The OS rate in older adult patients with AML is only 2% at 5 years. The biology of AML in the elderly patients may be a consequence of the age of hematopoietic stem cells, shortened telomere length (associated with older cells), presence of fewer normal stem cells to compete with the malignant clones and repopulate marrow following chemotherapy and as mentioned earlier, the increased frequency of age-related somatic mutations present in over 10% of healthy individuals over 70 years of age (Chapters 19, 59, 60, and 157).

Chromothripsis in Acute Myeloid Leukemia

Chromothripsis describes a process whereby hundreds of genomic rearrangements have been acquired as a result of a single catastrophic event. A chromosomal region or a chromosome or telomere belonging to other chromosomes is shattered into hundreds of pieces; some but not all are stitched together by the DNA repair machinery in a mosaic patchwork of genomic fragments. Cells not only survive this crisis but emerge with a genomic landscape that confers a selective advantage, thereby promoting further malignant evolution. In addition to the clustering of structural variants, multiple base-pair mutations can also be acquired in a single mitotic explosion, called *kataegis*. This process drives cytosine-specific mutagenesis in regions flanking sites of genomic rearrangement, and can result in the rapid occurrence of up to 20 base-pair substitutions. The best methodology to identify chromothripsis is high-resolution array SNP karyotyping using at least three of five criteria: (1) Regularity of oscillating copy-number states. In chromothripsis, the copy number value should typically oscillate between two or occasionally three states; (2) the presence of "interspersed regions with loss and retention of heterozygosity," which means that the segments in the highest copy number state should retain heterozygosity; (3) clustering of breakpoints, 5 to 10 breakpoints should be observed within 50 kb genomic interval; (4) more than 10 breakpoints within a minimum segment size of 10 kb; and (5) 0.3 kb as signal distance between adjacent segments. Using these criteria, chromothripsis has been identified in 6.5% of 395 newly diagnosed AML and these patients are older, higher risk, lower WBC, *TP53* loss and/or mutations, while *FLT3* and *NPM1* mutations were mutually exclusive with chromothripsis. Chromothripsis-positive patients with AML (and MDS) have a poor OS, particularly those with deletion 5q31.1 to 5q33.1, containing key genes involved in DNA damage and histone modifications, as well as *P53* deletion and/or mutation.

Therapy-Related Acute Myeloid Leukemia and Therapy-Related Myelodysplastic Syndrome

Therapy-related AML and MDS are distinct clinical syndromes occurring as late complications following high-dose chemotherapy, radiation therapy, or autologous stem cell transplantation. An abnormal karyotype is detected in 92% of this patient population. From the cytogenetic point of view, two different categories of therapy-related AML and MDS can be identified. The first group includes patients who develop AML/MDS following exposure to alkylating agents approximately 5 years after therapy. These leukemias are associated with the presence of monosomy 5/del(5q) or monosomy 7/del(7q) (see Fig. 57.31, *third row*). Many of these patients initially develop MDS features before transforming into frank AML. Recurrent abnormalities of chromosomes 5, 7, or both account for 70% of all abnormalities observed in therapy-related leukemias. These patients respond poorly to therapy and have a poor OS. A second group of patients develop therapy-related AML without prior MDS and exhibit 11q23 (3%) and 21q22 (3%) balanced rearrangements, which have been attributed to the late effects of topoisomerase II inhibitors combined with alkylating agents and radiation see Fig. 57.39). These forms of AML may develop within a few months to 3 years after topoisomerase II inhibitor therapy. Polysomy (tetrasomy, pentasomy, hexasomy) of chromosome 8 defines a clinical-cytogenetic entity associated with therapy-related myeloid malignancies and poor OS (see Figs. 57.47 and 57.48).

A rare but recurrent chromosomal translocation, t(3;8)(q26.2;q24) in therapy-related AML and/or in disease progression often leads to *MECOM-MYC* fusion product. Since only 30+ patients are reported, the median survival of these patients is estimated to be ~6 months. Similar to patients with t(3;3)/inv(3), patients with myeloid disease and t(3;8) often show small dysplastic megakaryocytes in the BM and monosomy 7 is the most frequent additional abnormality (50%). Unlike patients with t(3;3)/inv(3) who often have either normal platelet count or thrombocytosis, patients with t(3;8) commonly are thrombocytopenic. Since *MYC* is consistently upregulated in these patients investigators currently speculate that there is a cooperation between

MYC and *PVT1* whereby inactivation/disruption of the *PVT1* promoter enhancer leads to an increase in MYC in expression. In other words, the *PVT1* promoter partially insulates downstream enhancer elements from regulating MYC expression. As mentioned earlier, variant t(3;21)/*MECOM/RUNX1* is also common in therapy-related MDS/AML. When the genome of 22 patients with therapy-related AML was sequenced and compared to whole genome sequence data from patients with de novo AML and secondary AML arising from MDS, the mutational burden of therapy-related AML genomes was found to be similar to that of de novo AML indicating that prior chemotherapy does not induce genome-wide DNA damage.

In a study of 509 older (60 to 85 years old) patients with secondary AML (disease evolving, 28% from MDS), 53% had an abnormal karyotype and 48% had t-AML-like gene mutations including *ASXL1, SRSF2, STAG2, BCOR, U2AF1, EZH2, SF3B1, and ZRSR2*. These mutations were associated with a shorter EFS within ELN 2017 intermediate risk group (which exclude mutated cases by definition). However, a distinct subset of mutated genes are present in therapy-related AML/MDS. The whole genome sequence data revealed that *TP53* mutations and mutations in ABC transporter genes (a subset of which have been implicated in chemotherapy resistance) are significantly increased in therapy-related AML/MDS compared with de novo AML/MDS. *TP53* mutations are present in approximately 33% of patients with therapy-related AML/MDS and are associated with poor risk cytogenetics and a poor prognosis. More recent genomic data suggests that cytotoxic therapy does not directly induce *TP53* mutations. Rather, these studies support a model in which rare stem cells carrying age-related *TP53* mutations that are resistant to chemotherapy expand preferentially following treatment.

The Pathway Forward

A novel hierarchical prognostic model of AML solely based on molecular mutations was proposed by a German group in 2012. This model was based on 1000 patients with AML at diagnosis after a median follow-up of 23.7 months. Molecular screening for the recurrent balanced rearrangements (*PML-RARA, RUNX1-RUNX1T1, CBFB-MYH11*) and mutations in *CEBPA*, *NPM1*, *RUNX1, ASXL1*, and *TP53* as well as for *FLT3-IDT* and *MLL-PTD* can be used to create a prognostic classification system in AML that is more effective than any prognostic model based on cytogenetics alone[15] (see earlier section on next generation karyotyping[1]). Five distinct prognostic subgroups have been identified: (1) very favorable: *PML-RARA* rearrangement or *CEPBA* double mutations with OS at 3 years of 82.9%; (2) favorable: *RUNX1-RUNXT1*, *CBFB-MYH11* or *NPM1* mutations without *FLT3*-ITD, OS at 3 years of 62.6%; (3) intermediate: none of the mutations leading to assignment into groups 1, 2, 4, or 5; OS at 3 years 44.2%; (4) unfavorable: *MLL*-PTD and/or *RUNX1* mutation and/or *ASXL1* mutation: OS at 3 years 21.9%; and (5) unfavorable *TP53* mutation: OS at 3 years 0%. However, using whole-genome or exome sequencing from 71 patients with AML treated with standard induction therapy demonstrated that for risk stratification the clearance of somatic mutations after chemotherapy is more important than the identification of specific mutations at the time of diagnosis. In this study, the detection of persistent AML-associated mutations in at least 5% of bone marrow cells in day-30 remission samples was associated with significantly increased risk of relapse and reduced OS (see box on Genetic Testing for Acute Myeloid Leukemia).

GENETIC TESTING FOR ACUTE MYELOID LEUKEMIA

At diagnosis, conventional cytogenetics and I-FISH with AML-specific panel of FISH probes, for detection of PML-RARA, RUNX1-RUNXT1, CBFB, BCR-ABL1 fusions, and rearrangements of KMT2A and TP53 is mandatory. Patients with CBF AML should be tested for KIT exon 17 mutations. For patients who have AML with a normal karyotype, mutation studies for DNMT3, NPM1, FLT3, CEBPA, and TP53 are recommended as well as aCGH+SNP.

ACUTE LYMPHOBLASTIC LEUKEMIA

ALL accounts for at least 85% of acute leukemias in children and 20% of acute leukemias in adults (Chapters 66–68). The susceptibility to ALL varies with age; the first peak is between 2 and 5 years of age after birth, followed by a gradual decrease during adulthood but an increase in incidence again in individuals older than age 70 years, suggesting that different environmental and genetic factors contribute to leukemogenesis at different ages. Most published series of patients with ALL indicate that 70% to 75% have an abnormal clone by conventional cytogenetic studies (Figs. 57.51 and 57.52). Genomic rearrangements detected with intensive interphase FISH screening have been found in up to 91% of cases. The application of contemporary genome-wide molecular analysis continues to reveal many additional genetic rearrangements that are not detectable with chromosome studies.[16] At least one clonal aberration has been detected in 60% to 79% of adults and 57% to 82% of children with ALL. Today, cytogenetic analyses combined with FISH and/or array CGH+SNP are mandatory in most ALL clinical trials, and genetic findings play a pivotal role in proper risk stratification and identifying treatment options. Several of the ALL-specific chromosome abnormalities and their molecular counterparts have been included in the 2016 WHO classification. Biology of B-cell ALL is determined mainly by the first genetic hit, which is usually a change in chromosome numbers (hyperdiploidy or hypodiploidy) or a chromosomal translocation (e.g., t(12;21)/*ETV6-RUNX1*). In pediatric patients, these genetic abnormalities can first occur in utero. Accumulated evidence suggests that additional genetic insults are required for leukemia development and these will be described below.

Pretreatment cytogenetics is an independent prognostic factor in children and adults presenting with ALL and is important in determining risk categories.

As shown in Fig. 57.52 and Table 57.12, the risk categories in children include (a) low risk: high hyperdiploidy (trisomies for chromosomes 4, 10, and 17) and t(12;21)/*TEL-RUNX1*; (b) high risk: t(1;19)/*TCF3-PBX1*; and (c) very high risk: t(9;22)/*BCR-ABL1*, *BCR-ABL1*-like, 11q23/KMT2A rearrangements, and iAMP21. In adults the low-risk category includes high hyperdiploidy and del(9p), whereas the high-risk category includes hypodiploidy/near triploidy, t(9;22)(q34;q11), t(4;11)(q21;q23), t(8;14)(q24;q32), and a complex karyotype (five or more chromosomal abnormalities).

Approximately 20% to 30% of children and 3% of adults with B-cell ALL are *hyperdiploid*. Two groups can be distinguished based on cytogenetics (a) those with 56 to 67 chromosomes whose prognosis is excellent and (b) those with 51 to 55 chromosomes whose prognosis is less favorable. Both groups have a more favorable prognosis than do children with hypodiploidy or near-haploid ALL. In a study of 1880 children with ALL, patients with 45 chromosomes have an outcome similar to that of pseudodiploid or low hyperdiploid patients with ALL (47 to 50 chromosomes). Children and adolescents with ALL and hypodiploidy with fewer than 44 chromosomes have a poor outcome. The distribution of specific chromosome gains is not random, with the most-involved chromosomes being X, 4, 6, 10, 14, 17, 18, and 21 with individual trisomies or tetrasomies being seen in over 75% of cases each of which is gained in more than 50% of hyperdiploid patients with ALL, followed by gains in chromosomes 8, 5, 11, and 12, that occur more often in patients with 57 or more chromosomes. The prognosis of children with high hyperdiploidy is excellent with a 5-year EFS rate of between 71% and 83% and a 5-year OS rate of approximately 90%. Pretreatment cytogenetic analyses of more than 5400 children with ALL unequivocally shows that simultaneous trisomies for chromosomes 4, 10, and 17 are associated with a higher long-term EFS (see Fig. 57.52D). By contrast a hyperdiploid karyotype is not associated with a favorable outcome in adult ALL patients. The reason for this discordance may be that adults often have poor-risk chromosomal translocations, such as the Ph chromosome. Approximately 50% of the high hyperdiploid patients harbor other structural abnormalities, such as gains of 1q, del(6q), which do not appear to influence prognosis, with a possible exception of prognostically adverse isochromosome 17q. Frequently,

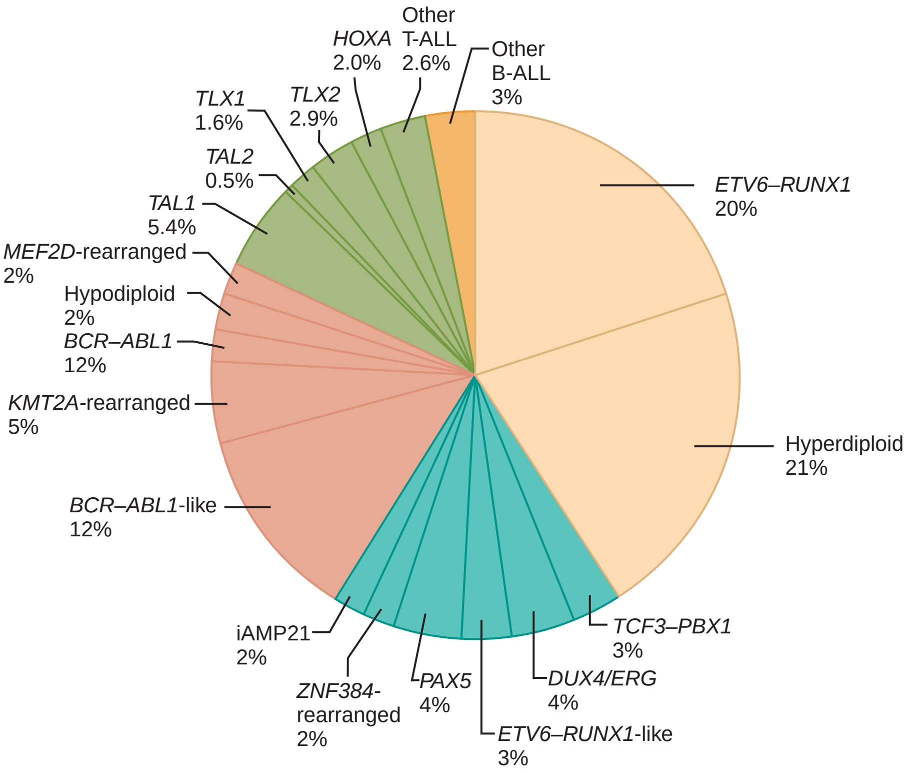


Figure 57.51 ESTIMATED FREQUENCY OF SPECIFIC GENETIC SUBTYPES IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA. The pie chart depicts the estimated frequencies of each subtype of acute lymphoblastic leukemia (ALL) among patients treated in St. Jude Total Therapy Study, with modifications to account for discoveries of novel genetic abnormalities. T cell ALL (T-ALL) subtypes are depicted in blue. Among the B cell precursor ALL (B-ALL) subgroup, low-risk subtypes are in yellow, intermediate-risk subtypes are in green, and high-risk subtypes are in red; however, the prognosis of genetic subtypes depends on treatment efficacy and, hence, might differ between various clinical trials. Moreover, the prognosis of the 3% of patients with B-ALL who cannot be classified into one of these subgroups *(orange segment)* cannot be predicted at present. *DUX4/ERG, DUX4* rearranged and *ERG* deregulated; iAMP21, intrachromosomal amplification of chromosome 21. *(Reproduced with permission from Pui C-H, et al. Somatic and germline genomics in paediatric acute lymphoblastic leukaemia. Nat Rev Clin Oncol. 2019;16(4):227–240.)*

hyperdiploid leukemic cells fail to proliferate in vitro; therefore the use of an ALL FISH panel of probes is strongly recommended. A significant proportion of hyperdiploid ALL patients harbors mutations in *FLT3* (~25%), *NRAS/KRAS* (~15%), or *PTPNI1* (~10%) genes. These mutations are mutually exclusive. Deletions of *ETV6, CDKN2A, PAX5*, and *PAN3* are also frequently deleted loci in these patients. Recurrent somatic hemizygous 13q12.2 microdeletions have been found in 27 of 1418 pediatric patients with B-ALL with high hyperdiploidy and are associated with *FLT3* mutations and deletion of *PAN3*. The size of deletion varies from 39 to 160 kb, with the median size of 129 kb. A novel mechanism leads to simultaneous dysregulation of *FLT3* and *PAN3* deletion in these patients.[17] The interstitial deletions of 13q12.1 are occurring at the 5′ to the *FLT3* promoter and since they are focal deletions, there is a loss of a *PAN3* gene and co-occurring *FLT3* gene overexpression. These two events are changing promoter-enhancer-interactions resulting in an increased expression of the *FLT3* allele encoded on the same chromosome homolog. These observations suggest a novel mechanism of chromatin remodeling whereby enhancer hijacking represents an alternative pathway of *FLT3* activation. High *FLT3* expression has been reported to be a poor prognostic parameter in both AML and ALL.

Modal chromosome numbers of 45 or less occur in 1% to 2% of cases (specifically, the near-haploid numbers of 24 to 36) that confer a poor prognosis. Similarly, ALL in adults presenting with low hyperdiploid/near-triploidy have poor outcomes. Loss of chromosome 7 is frequently a secondary abnormality in adult patients with ALL and the majority of these patients also have t(9;22). Hypodiploid ALL can be further divided into multiple subgroups according to chromosome number. Genomic profiling has shown that near haploid (24 to 31 chromosomes) and low-hypodiploid (32 to 39 chromosomes) childhood ALL are distinct subtypes: near-haploid-ALL is associated with a high frequency of *RAS*-activating mutations including focal deletions of *NF*-1(71%) whereas low-hypodiploid ALL has biallelic alterations of the *TP53* locus, deletions of *CDKN2A/B, IKZF2*, and/or *Rb1(41%). TP53* mutations are also present in non-hematopoietic cells in half of childhood patients with a low-hypodiploid karyotype, indicating that this disease is a manifestation of Li-Fraumeni syndrome (Chapter 30). Diagnosis of low hypodiploid ALL requires *TP53* testing and genetic counseling.

In childhood ALL, *t(12;21)* is difficult to detect with conventional cytogenetics because the translocated portions of 12p13 and 21q22 have virtually identical G-banding patterns. In contrast, the *ETV6-RUNX1* fusion product of t(12;21) is detected using PCR or FISH in 17% to 30% of pediatric patients with ALL (see Fig. 57.52A–C). The *ETV6-RUNX1* fusion, found almost exclusively in children 1 to 15 years old with B-precursor ALL, represents the most frequent molecular rearrangement in childhood cancer. Children with the *ETV6-RUNX1* fusion gene have significantly lower rates of relapse than do *ETV6-RUNX1*-negative patients. *ETV6-RUNX1*-positive B-precursor ALL is characterized by a prolonged duration of first remission and excellent cure rates. Prospective analyses have demonstrated that the survival rate in t(12;21)-positive patients is significantly better when compared with cases lacking this abnormality; however, this abnormality in multivariate analysis was not found to be an independent predictor of outcome. The *ETV6-RUNX1* fusion is rare in adult ALL. t(12;21)(p13;q22) fuses the helix-loop-helix domain of the *ETV6* gene, located on chromosome 12, band p13, to the DNA-binding and transactivation domain of the *RUNX1* gene, located on 21q22. FISH studies allow visualization of the fusion gene on 21q22. Fusion with *ETV6* converts *RUNX1* from an activator to a repressor of transcription. The *ETV6-RUNX1* fusion is accompanied in 55% to 70% of patients by the

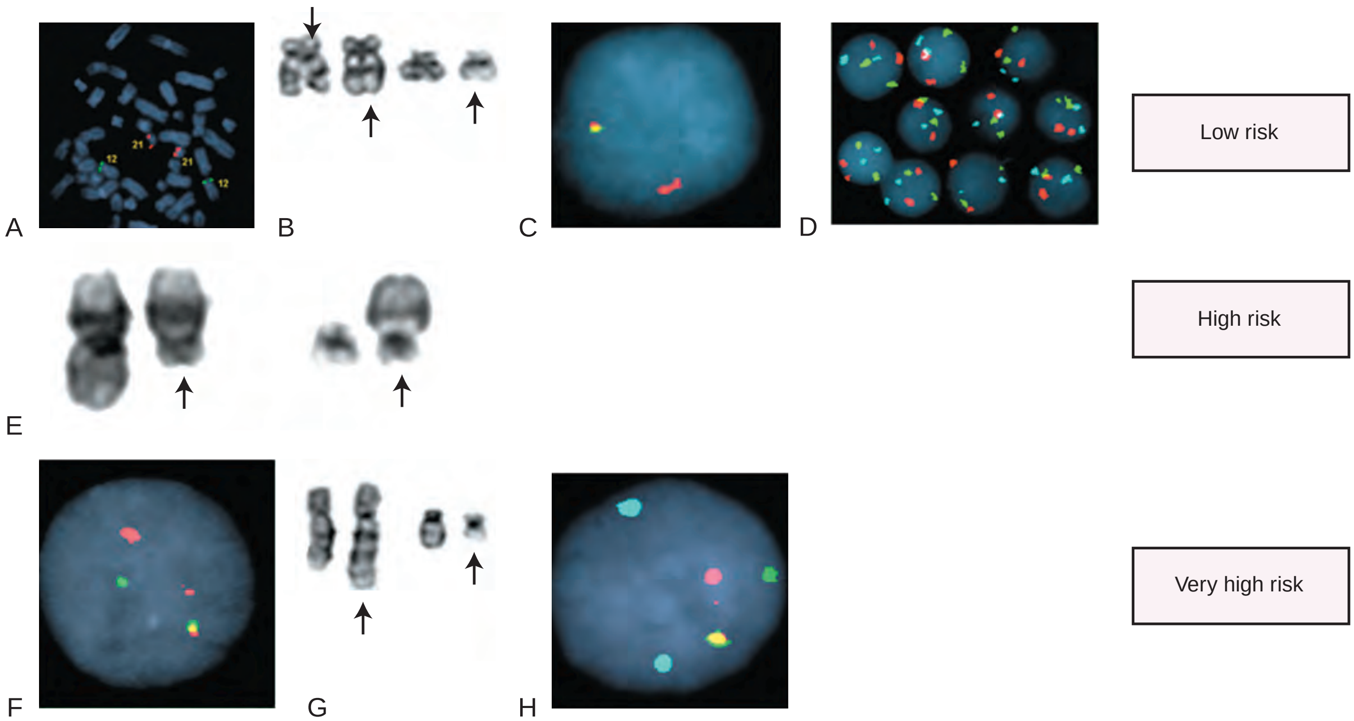


Figure 57.52 PROGNOSTIC CYTOGENETIC CATEGORIES IN ACUTE LYMPHOBLASTIC LEUKEMIA. (A) Localization of *TEL/ETV6* and *AML1* fluorescence probes to chromosomes from a normal bone marrow metaphase cell. *TEL/ETV6* is on 12p13 *(green)* and *AML1* is on 21q *(red)*. (B) Partial karyotype showing t(12;21)(p13;q22) *(arrows)*. The short arrow at 12p indicates a possible *TEL* deletion from normal chromosome 12. (C) FISH study showing loss of *TEL (green)* from normal 12 homologue in interphase nucleus, a frequent subclonal evolution in patients with t(12;21). (D) Hyperdiploidy, specifically trisomies for chromosomes 4 *(green)*, 10 *(red)*, and 17 *(aqua)*, is associated with low-risk cytogenetic category (see text for details) and is present in disomy in interphase cells *(top left)*. (E) Partial G-banded karyotype showing t(1;19)(q23;p13.3), which occurs in approximately 6% of patients with B-cell precursor childhood acute lymphoblastic leukemia. (F) FISH hybridization to bone marrow nucleus showing BCR-ABL fusion *(yellow)*. (G) As a consequence of t(9;22), occurring in 5% of children and 20% to 25% of adults with acute lymphoblastic leukemia. (H) Interphase nucleus after FISH study with tricolor probe. Dual-color/breakapart *MLL* shows separation of the 3′ end and the 5′ end as a result of 11q23 rearrangement. CEP11 *(aqua)* indicates disomy of chromosome 11, used as internal control. *MLL* rearrangements in acute lymphoblastic leukemia are associated with unfavorable prognosis. *FISH*, fluorescence in situ hybridization.

loss of the other *ETV6* allele. This deletion represents subclonal evolution. *ETV6-RUNX1* has been detected in utero, probably in a committed B-cell progenitor, and is present in normal cord blood and peripheral blood samples at frequencies 100-fold greater than the risk for corresponding leukemia. The current view of development of *ETV6-RUNX1*-positive leukemia is that these early events are followed by a long “preleukemic” phase followed by loss of the normal *ETV6* homologue, which appears to be an important event in the multistep pathogenesis of this form of leukemia. Currently, of the 397 children with *ETV6-RUNX1*-positive leukemia reported, approximately 60% harbor additional karyotypic abnormalities that contribute to their pseudodiploid or near-diploid karyotypes. The most common secondary change, which occurs in approximately 50% of cases with additional abnormalities, is trisomy 21.

A rare group of patients with B-precursor ALL lack fusion of *ETV6* and *RUNX1* but have 3 to 15 copies of the q22 band of chromosome 21, including the *RUNX1* locus. Intrachromosomal amplification of the 21q22 band of chromosome 21 is a clonal marker of the leukemic cells that defines a distinct ALL subgroup (Fig. 57.53A). The British Childhood Leukemia Working Party prospectively screened 1630 patients with childhood ALL and identified 28 children with intrachromosomal amplification of chromosome 21 (iAMP21) (see Fig. 57.53D). Approximately 2% of children and less than 0.5% of adults display iAMP21. Children with iAMP21 have a common or pre–B-cell immunophenotype, a median age of 9 years at presentation, and a significantly inferior event-free and OS at 5 years as compared with children exhibiting other cytogenetic subgroups. Even children with Ph-positive ALL have a better 5-year EFS as compared with children with iAMP21. These children have a threefold increased risk for relapse and are twice as likely to die than are their counterparts without iAMP21. The complexity and variability of the iAMP21 includes multiple regions of gains, amplification, inversion, and deletions. In spite of their differences in genomic profiles, the consistent features of patients with ALL and iAMP21 include a common region of highest-level amplification spanning 5.1 Mb from 32.8 to 37 Mb, within which the *RUNX1* gene is located. These observations confirmed that FISH, using probes directed to *RUNX1* locus, to determine the number of copies of the most amplified region, provides a reliable detection method. Thus the finding of three or more extra copies of *RUNX1* on a single abnormal chromosome 21 (a total of five or more *RUNX1* signals per interphase cell) is currently used as the international definition of iAMP21.

Recent genomic, cytogenetic, and transcriptional analyses coupled with novel bioinformatics approaches revealed that individuals born with a rare constitutional Robertsonian translocation, t(15;21)(q10;q10)c, have an approximately 2700-fold increased risk of developing iAMP21ALL compared with the general population. In these patients, amplification is initiated by a chromothripsis event (a process whereby localized genomic regions are shattered and rearranged in one catastrophic event) that affects both sister chromatids of the Robertsonian chromosome. In sporadic iAMP21, breakage-fusion-bridge (BFB) cycles are typically the initiating event, often followed by chromothripsis. In both sporadic and rob(15;21)c-associated iAMP21, the final steps frequently involve duplications of the entire abnormal chromosome. The end-product is a derivative of chromosome 21 or the rob(15;21)c chromosome with gene dosage optimized for leukemic potential, showing constrained copy number levels over multiple linked genes.

TABLE 57.12 Frequency of Cytogenetic Aberrations in Adult and Childhood Acute Lymphoblastic Leukemia and Their Prognostic Relevance

		Adults		Children	
Cytogenetic Abnormality	**Genes Involved**	**Frequency (%)**	**Prognosis**	**Frequency (%)**	**Prognosis**
Normal karyotype	NA	15–34	Good	31–42	Favorable
High hyperdiploidy (>55)	NA	7–8	Good-intermediate	23–30	Good
Low hyperdiploidy (>50)	NA	10–15	Poor	10–11	Intermediate
Near haploidy (<35)	NA	Rare	NA	1–4	Poor
Pseudodiploidy	NA	31–50	Poor	18–26	Intermediate
Hypodiploidy (35–44)	NA	4–9	Poor	6	Poor to intermediate
t(9;22)(q34;q11.2)	*BCR-ABL1*	11–29	Poor	1–3	Intermediate
t(4;11)(q21;q23)	*MLL-AFF1*[a]	4–9	Poor	2	Poor
t(1;19)(q23;p13.3)	*TCF3-PBX1*	1–3	Poor, intermediate favorable	1–6	Intermediate, favorable
t(12;21)(p12;q22)	*ETV6 (TEL)-RUNX1*	0–3	Not known	22–26	Good
t(8;14)(q24;q32)	*MYC-IGH*	5–15	Poor	5	Poor
Abnormal 9p	*CDKN2A*	7–13	Poor	7–11	Adverse
Abnormal 9p13	*PAX5*	2	?Intermediate	2–5	Poor
Abnormal 12p	*ETV6*	0–3	Favorable/unfavorable	3–9	Not prognostic
del(6q)	Not known	3–6	Not prognostic	6–9	Adverse
del(7p)/del(7q)/–7	Not known	6–11	Not prognostic	1	Adverse
del(5q)	Not known	<2	Not prognostic	2	Not known
Trisomy 8	NA	10–12	Poor	3–4 (17%–22% in T-ALL)	Not prognostic
14q11	*TCRα*	5–7 (26% in T-ALL)	Excellent	Rare	Poor
t(10;14)(q24;q11)	*TRD-TLX1*	1–3	Excellent, intermediate		
BCR-ABL1-like	*ABL, JAK, EPOR CRLF2, IKZF del*	17	High risk	15	High risk
iAMP21	*RUNX1*	0.5	Poor	2	Poor, unless treated on intense protocol
Monosomal karyotype	*NA*	9.2	Treated on high-risk protocol	12.8	Treated on high-risk protocol

NA, Not applicable; *T-ALL*, T-cell acute lymphoblastic leukemia.

[a] See Table 57.11 for all other mixed lineage leukemia rearrangements.

Patients with iAMP21 display a unique spectrum of secondary chromosomal abnormalities and they include gain of X chromosome, 10 or 14, or monosomy 7/deletion 7q, deletions of 11q, including *ATM* and *MLL* genes, deletions of *ETV6* and *RB1*.

Both the UK and US Children Oncology groups demonstrated that patients with ALL and iAMP21 when considered a high-risk group and treated on the most intensive treatment arm have a 5-year EFS (from 29% to 78%), a risk of relapse (reduced from 70% vs. 16%), and OS from 67% versus 89%.

The *ETV6* transcription factor gene was first identified as a part of a *TEL*–platelet-derived growth factor receptor β fusion [TEL (ETV6)-PDGFRB] created by t(5;12)(q33;p13) in chronic myelomonocytic leukemia (Chapter 73). It was detected using FISH owing to difficulties in detecting cytogenetic rearrangements at the 12p13 site. As a result of this translocation, the helix-loop-helix domain of *ETV6* is fused in frame to the *PDGFRB* transmembrane and tyrosine kinase domain. Fusion of *ETV6* to a tyrosine kinase also occurs as a result of t(9;12)(q34;p13), leading to the *ABL1-ETV6* fusion that has been observed in patients with ALL, AML, and atypical CML. More than 39 partner genes are known to participate in fusions with *ETV6*, primarily in ALL and less frequently in myeloid malignancies.

Other rearrangements involving 12p include deletions, duplications, and translocations and are observed most often as part of a complex karyotype, frequently associated with chromosome 5 and/or 7 abnormalities. Deletion of 12p13 is much more frequent in children than in adults with ALL and is observed in patients with myeloid disorders, specifically in adults with MDS. Two other genes residing on 12p are also rearranged: *CCND2* is frequently amplified, and *CDKN1B* is often deleted.

Less than 5% of children and 20% to 25% of adults with ALL have a Ph chromosome, making it the most common structural rearrangement in adult ALL (Fig. 57.54). The breakpoint on the Ph chromosome is more centromeric than in CML (see Chronic Myeloid Leukemia, earlier). It includes the 5′ breakpoint on chromosome 22, distal to the first exon (falling between exon e1 and 32) of the *BCR* gene, resulting in the P190$^{\text{BCR-ABL1}}$ variant, providing a diagnostic distinction between the lymphoid blast crisis of CML and de novo ALL. Ph-positive ALL is characterized by a smaller BCR-ABL1 (P190) protein, containing less BCR than the P210 and P230 fusion proteins. Among Ph-positive patients with ALL, approximately 30% to 50% of adults and 85% of children have this breakpoint variant. Up to 19% of Ph-positive patients with ALL may express both the P190 and the P210$^{\text{BCR-ABL1}}$ proteins. ALL has been classified as a stem cell disorder because of the presence of the *BCR-ABL1* transcript in both the myeloid and lymphoid cells.

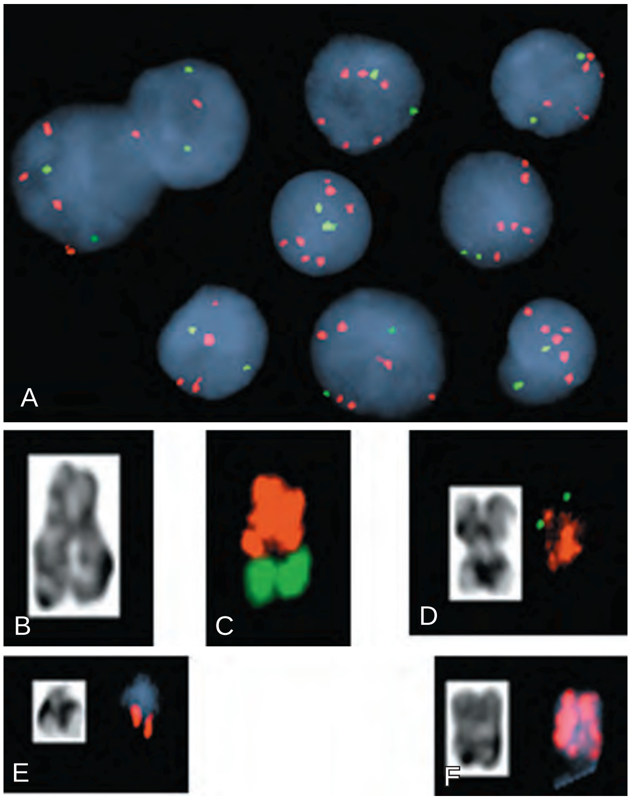


Figure 57.53 DETECTION OF *RUNX1* AND 21q22 AMPLIFICATION. (A) Intrachromosomal amplification of *RUNX1 (red)* (*ETV6 [green]*, present in disomy) in a pediatric patient with pre–B-cell acute lymphoblastic leukemia. This finding is associated with poor prognosis. (B) G-banding of der(18) chromosome and after FISH study (C) with WCP 18 *(green)* and LSI 21q22.13 *(red)*, documenting der(18)t(18;21) and amplification of 21q22 region in a patient with myelodysplastic syndrome/acute myeloid leukemia. (D) Amplification of 21q22 region in myelodysplastic syndrome may be present in another formation: der(21) (G-banding, *left*). FISH study shows amplification of 21q22.13 locus *(red)* and identified der(21) as t(5;21) with 5p15.2 probe *(green)*. (E) Localization of 21q22.13 LSI probe on normal chromosome 21. (F) Homogeneous staining region (hsr)(21), G-banding *(left)*, and after FISH study *(right)* with locus-specific probe for 21q22.13 *(red)* consistent with 21q22.13 band amplification in a patient with myelodysplastic syndrome. *FISH*, fluorescence in situ hybridization; *LSI*, locus-specific indicator; *WCP*, whole chromosome painting probe.

The *BCR-ABL1* fusion variants are easily detectable by FISH using an extra-sensitive probe strategy, and *BCR-ABL1* leukemia can be identified in up to 10% of Ph-negative cases using conventional cytogenetics. Moreover, FISH will identify *ABL* rearrangements in *BCR-ABL1* patients with fusion-negative ALL.

Reactivation of BCR-ABL1 kinase activity is most commonly associated with the emergence of a point mutation in the ABL1 kinase domain implicated in imatinib resistance (see Chronic Myeloid Leukemia, earlier). Recent studies have indicated that kinase domain mutations are present in 70% of imatinib-resistant patients with T315I (37%), E255K (18%), and Y253H (18%) mutations accounting for 75% of these cases. Moreover, 78% of patients resistant to second-generation TKIs were positive for such mutations, and 58% of them had multiple mutations.

More than 60% of adult patients with Ph-positive ALL show ACAs in the Ph-positive clone. Most frequent are gain of Ph chromosome, monosomy 7, +8, +X, del(9p), and high triploidy. The ACAs have no effect on survival of these patients. High hyperdiploidy is detected in approximately 15% of Ph-positive ALL. According to the WHO classification, 1% of all leukemias are mixed phenotype acute leukemias with t(9;22)(q34;q11.2) and leukemic blast cells expressing B-cell and myeloid phenotypic markers. Occasional T-cell phenotype and trilineage *BCR-ABL1* mixed phenotype leukemia have been reported.

B-cell ALL display a differentiation arrest at the pro-B to pre-B stage and is associated with ongoing *IGH* rearrangements. The IL7 receptor (IL7R) is crucially involved in lymphoid development and mutations in IL7R in pediatric B- and T-cell ALL resulting in the constitutive activation of the receptor and cytokine independent growth of progenitor lymphoid cells have recently been reported. Once activated IL7R interacts with JAK/STAT pathway which is often activated in the blast cells of Ph-positive pre–B ALL which may explain the sensitivity of Ph-positive leukemic cells to *JAK2* inhibitors.

“*BCR-ABL1-like*” Acute Lymphoblastic Leukemia

Retrospective studies have demonstrated that GEP can be used effectively to classify ALL into prognostically important subtypes. *BCR-ABL1*-like ALL is a newly described ALL subtype lacking *BCR-ABL1*, but with a similar expression profile to *BCR-ABL1*-positive leukemia (Chapter 68). This new “*BCR-ABL1*-like” (“Ph-chromosome-like”) ALL includes approximately 15% of all precursor B-cell ALL in children and 30% to 40% of adults with ALL and is associated with higher relapse rate and lower EFS (see Fig. 57.51 and Table 57.13). The prevalence of Ph-like ALL increases with age ranging from 10% to 15% of children to 20% to 25% of young adults (21 to 39 years) and adults (>40 years) with ALL. The WHO (2017) endorsed *BCR-ABL1*-like B-ALL as a provisional entity with the gene expression pattern “very similar” to that seen in Ph-positive/*BCR-ABL1* fusion-positive ALL. Both entities commonly harbor genetic alterations targeting B –lymphoid transcription factors, including *IKZF1*. Large-scale genomic profiling and sequencing studies of over 1700 childhood and young adult patients with ALL has revealed rearrangements of *ABL*-class genes, rearrangements of *JAK2*, *EPOR*, and *CRLF2*. *ABL*-class rearrangements result in the expression of fusion genes that activate *ABL1, ABL2, CSFR1*, and *PDGFRB*. In this group of patients *IKZF-1* deletions occur in 40% of pediatric and in 46% of adult patients. *CRLF2* (Xp22.33/p11.3) rearrangements are observed in approximately 50% of patients with *BCR-ABL1*-like ALL with frequent concomitant mutations of *JAK2*, and as mentioned above, are potentially amenable to treatment with *JAK2* inhibitors. Two forms of *CRLF2* rearrangements have been identified which occur either as translocations, *CRLF2-IGH*, or through focal deletion of the *PAR1*, a pseudo-autosomal region of chromosome X/Y resulting in *P2RY8-CRLF2* fusion. The gene encodes for cytokine receptor-like factor 2, also known as thymic-stromal–derived lymphopoietin receptor which in combination with the interleukin-7 receptor forms the receptor for the thymic stromal lymphopoietin. *CRLF2*-rearranged leukemic cells have activated JAK-STAT and PI3K signaling pathways. Detection of rearrangements involving *ABL1, ABL2, CRLF2, EPOR, PDGFRB, and CSF1R* using FISH analysis is very useful to identify *BCR-ABL1*-like ALL.

t(1;19)(q23;p13.3)/E2A-*PBX1* occurs in 5% to 6% of patients with B-cell precursor childhood and adult ALL. However, among patients with a pre-B (cytoplasmic Ig-positive) t(1;19) is found in approximately 25% of cases (see Fig. 57.52D). Cytogenetically, two forms of t(1;19) have been identified: 25% of cases have a balanced reciprocal t(1;19), whereas 75% have a rearrangement of unbalanced der(19)t(1;19)(q23;p13.3). The unbalanced der(19)t(1;19) arises from the initial trisomy of chromosome 1 followed by the t(1;19) translocation, with subsequent loss of the derivative chromosome 1. More than 95% of t(1;19) are associated with the *E2A–PBX1* chimeric gene protein product which arrests cell differentiation. The *E2A* gene (originally identified by the binding of *E2A* proteins to the kE2DNA sequence motif contained in the Ig κ light-chain enhancer) on chromosome 19, band p13.3, is fused to the *PBX1* (homeobox) gene on chromosome 1, band q23. Approximately 1% of pediatric patients with B-cell-ALL have a variant t(17;19)(q21–q22;p13)/*E2A-HLF* translocation resulting with two different genomic rearrangements. The first is the fusion between the *HLF* gene (breakpoint in intron 3) on chromosome 17 and the *E2A* gene (within intron 13) on chromosome 19, associated with disseminated intravascular

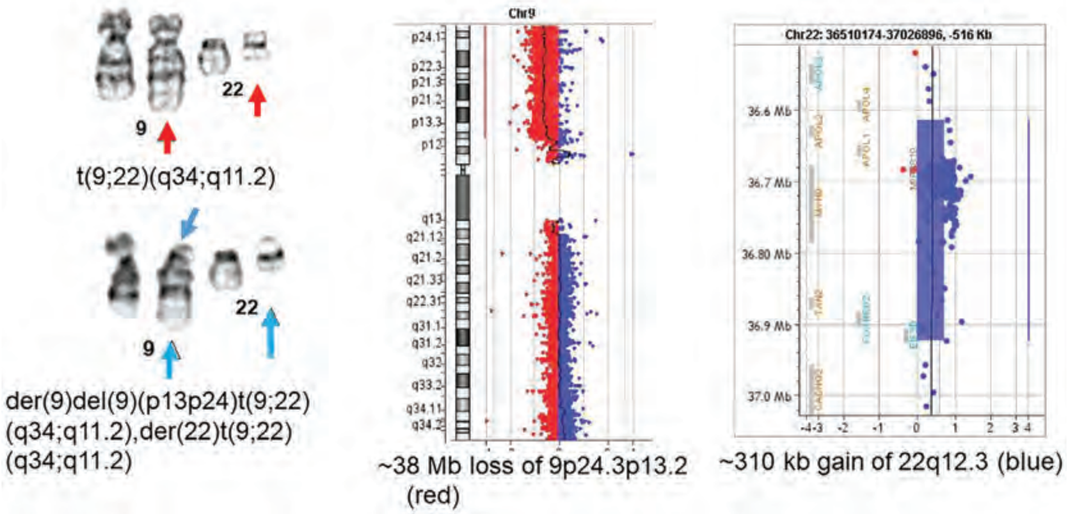


Figure 57.54 THE Ph CHROMOSOME IN ACUTE LYMPHOBLASTIC LEUKEMIA. Partial karyotype of two cells from a 29-year-old patient with B-cell ALL. The t(9;22)(q34;q11.2 *(top)* was detected in 15% of cells at diagnosis *(red arrows)*. The other 85% of cells *(bottom)* had der(9)del(9)(p13p24)t(9;22)(q34;q11.2),der(22)t(9;22)(q34;q11.2) *(blue arrows)*. Note that the 9q+ has loss of almost entire 9p. Using array CGH+SNP it was determined *(right panels)* that loss is ~38 Mb large (as shown in *red*) between 9p24.3 and p13.2. This is a frequent finding in both adult and pediatric patients with ALL. Array results also uncovered a cryptic gain of ~310 kb of chromosome 22 (22q12.3) that was not cytogenetically visible. *ALL*, acute lymphoblastic leukemia; *CGH*, comparative genomic hybridization; *SNP*, single-nucleotide polymorphism.

coagulopathy. The second is the breakpoint in intron 12 of *E2A* and intron 3 of *HLF*, which is associated with hypercalcemia.

Currently, it is thought that an unbalanced der(19) in pediatric ALL is associated with significantly improved outcome as compared with patients with balanced t(1;19). In adults as the sole abnormality, t(1;19)/der(19)t(1;1.9) is associated with an intermediate prognosis; however, within the context of a hyperdiploid karyotype, it is associated with a poor prognosis. Patients with *E2A-PBX1* fusion also display *PAX5* (19p13.2) haploinsufficiency, detectable both by conventional and molecular cytogenetics. A variant t(17;19) rearrangement is associated with a poor prognosis. A recent study of adult patients with ALL has suggested that prognosis of patients with t(1;19) can be substantially improved by the treatment with the hyper-CVAD regimen. Both t(1;19) and t(17;19) are easily identifiable by conventional cytogenetics, FISH, and RT-PCR; the latter two methodologies are particularly useful in post-treatment specimens that are cytogenetically normal. Similar to the *ETV6-RUNX1* fusion, in utero origin of *E2A-PBX1* (see later) was recently documented as well as its presence in approximately 0.6% of healthy newly born and therefore they either convert to overt leukemia or, in most cases, die out later in life. Recent molecular studies underlying leukemogenesis of t(1;19) proposed the following model by which *E2A-PBX1* directly interacts, through a region spanning the *PBX1* homeodomain, with *RUNX1* gene that specifically targets a subset of *RUNX1*-occupied genes and 2 enhancers in the *RUNX1* locus. Gene-expression analysis confirmed that *E2A-PBX1* directly controls *RUNX1* activation in pre-B ALL. Therefore *RUNX1* acts as a master regulator in the establishment of hematopoietic stem cells. It is the physical interaction of *E2A-PBX1* with *RUNX1* but not the DNA binding activity that plays the crucial event in the activation of *RUNX*1-related gene programs both by direct activation of *RUNX1* targets and by reinforcing *RUNX1* transcriptional autoregulatory loop and thereby contributing to ALL pathogenesis.

In ALL, the most frequent *KMT2A* translocations include t(4;11) (1% to 2% incidence in children and two-thirds of *KMT2A*-positive adults) (Fig. 57.55) leading to an *MLL-AFF1* (AF4) fusion and t(11;19)(q23;p13.3) (see Fig. 57.39L) resulting in *KMT2A*/*MLL-MLLT1 (ENL)* fusion. These abnormalities are present in more than 80% of patients with infant leukemia and 10% of childhood and adult *KMT2A*-positive leukemia. *KMT2A*r is associated with a poor outcome in both children and adults. One-third of patients with t(4;11) have secondary abnormalities; the most frequent are +X, i(7q), abnormalities of 9p, including i(9q), and +8. The outcomes of patients with t(11;19) is generally poor, especially in children younger than 1 year of age. The most frequent additional abnormalities in patients with t(11;19) are +X, +8, and del(6q). Other less common *KMT2A*/*MLL* translocations include t(9;11)(p22;q23)/*KMT2A*/*MLL-MLLT3*, t(10;11)(p13–15;q23)/*KMT2A-MLLT10*, and others. A large multi-institutional study has determined that secondary aberrations do not affect the prognosis of children with ALL showing t(4;11), t(11;19) or other *KMT2A* translocations.

Secondary or therapy-related forms of ALL are rarely reported but the majority of them are associated with *MLL* rearrangements, the most frequent being t(4;11). Most of these patients have received topoisomerase II inhibitors which are known to cause double-strand DNA breaks.

t(8;14)(q24;q32) is seen in fewer than 5% of all patients with ALL (children and adults) (Fig. 57.56). Variant translocations t(8;22)(q24;q11) and t(2;8)(p12;q24) are seen in less than 1% of children and adults. These cells express CD10, CD19, CD20, and surface IgM immunophenotype. This form of ALL has an extremely poor prognosis. The same translocation is found in Burkitt lymphoma (BL), and both entities likely represent the same disease with different manifestations. The majority of adult patients with t(8;14) die within 1 year of diagnosis. Approximately 4% of children and 11% of adult pre–B-cell precursor ALL have recurrent *IGH* translocations usually identified by cytogenetics or FISH. The *CRLF2* gene, which maps to the pseudo-autosomal region 1 of the sex chromosomes, is the gene that is most frequently targeted in 20% to 26% of *IGH* translocations in pre–B-cell precursor ALL. *IGH-CRLF2* rearrangements result from the cryptic t(Y;14)(p11;q32) or t(X;14)(p22;q32). The second most frequent *IGH* translocation is t(14;19)(q32;q13) and *IGH-CEBP* chimeric fusion. The third most frequent translocation partner involves *CEBPD* gene (8q11) resulting in t(8;14)(q11;q32) and *IGH-CEBPD* fusion, which is primarily found in children and young adults and is strongly associated with DS (about 30% of cases). This subgroup frequently has a gain of X chromosome, trisomy 21 as an acquired abnormality, and the Ph chromosome/t(9;22)(q34;q11). The t(14;19)(q32;q13)/*IGH-EPOR* appears to be causally related to pre–B-cell precursor ALL because it always appears as the single abnormality. The inv(14) (q11q32)/ins(14;14)(q11;q32) leads to *IGH-TRA/D*, which represent a rearrangement mediated by an

TABLE 57.13 **Clinical and Biological Characteristics of Newly Identified Molecular Subtypes in Childhood Acute Lymphoblastic Leukemia**

Molecular Subtype	Frequency	Characteristics
Ph-like	• 12% in children • 21% in adolescents	• Dysregulation of cytokine receptor and kinase signaling pathways owing to genetic aberrations including *CRLF2* rearrangement (in ~50% of patients) and translocation of non-receptor tyrosine kinases (predominantly ABL class and Janus kinases) • Generally unfavorable
iAMP21	2%	• ≥3 extra copies of *RUNX1* on a single abnormal chromosome 21 (total of ≥5 copies of *RUNX1*per cell) • Requires intensified treatment to reduce risk of relapse
DUNX4-rearranged	4%–5%	• Overexpression of *DUX4* and transcriptional deregulation of *ERG*, resulting in loss of function of the transcriptional regulator ERG • Favorable prognosis
MEF2D-rearranged	2%–3%	• Increased *MEF2D* transcriptional activity, activation of *HDAC9* expression and increased sensitivity to histone deacetylase inhibitor treatment • Low or absent expression of CD10 and high expression of CD38 • Older age at diagnosis (median of 12 years) • Unfavorable prognosis
ZNF384-rearranged	2%–3%	• Enrichment of gene expression for hematopoietic stem cell signatures • Low CD10 expression and aberrant CD13 and/or CD33 expression • intermediate prognosis
ETV6-RUNX1-like	2%–3%	• Gene expression profile similar to that of *ETV6-RUNX1*-postive ALL with coexisting *ETV6* and *IKZF1* aberrations • Expression of CD27, and no or low expression of CD44 • Intermediate of favorable prognosis

Favorable, intermediate, and unfavorable prognosis acute lymphoblastic leukemia (ALL) subtypes are associated with 5-year overall survival of >90%, 70%–90%, and <70%, respectively.
iAMP21, Intrachromosomal amplification of chromosome 21; *Ph*, Philadelphia chromosome.
Modified from Pui C-H, Nichols KE, Yang JJ, et al. Somatic and germline genomics in paediatric acute lymphoblastic leukaemia. *Nat Rev.* 2019;16(4):227.

interlocus site-specific recombination event between *IGH* and the *TRA* and *TRB* genes. The t(14;20)(q32;q13) results in *IGH-CEBPB*. Collectively, patients with *IGH* translocations may be included in a subgroup of pre–B-cell precursor ALL.

Abnormalities of the short arms of chromosome 9 (p21–22) occur at a frequency of 7% to 13%. In adults the presence of del(9p) appears to be associated with a favorable outcome, whereas in children with ALL, del(9p) is associated with poor outcome. The most frequent abnormalities are co-deletions of two genes, *CDKN2A* and *CDKN2B*, as well as the interferon α and β genes found in many cases. Among the structural rearrangements involving the short arms of chromosome 9, t/dic(9;12)(p11–12;p11–13) is a rare recurrent abnormality associated with L1 morphology (FAB classification), a pre–B-cell phenotype, and an excellent prognosis.

The *IKZF1* gene at 7p12.2 codes for *IKAROS*, an essential transcription factor in hematopoiesis primarily involved in lymphoid differentiation and is an essential player in the regulation of both T- and B-cell lineage specification (Chapter 9). The gene is composed of eight exons, spanning a total of 6.2 kb and coding for a 519-amino acid protein. Deletions of *IKZF1* have been reported in 15% of pediatric and 30% to 50% of adult patients with ALL, and in 75% of Ph-positive B-cell ALL. Both focal and nonfocal *IKZF1* deletions have been shown to be associated with an increased risk of relapse and decreased EFS in both pediatric and adult ALL. Most laboratories use SNP array technique to detect *IKZF1* deletion. Recent genomic studies of patients with ALL have confirmed a higher hazard of relapse in adult ALL with focal *IKZF1* deletions.

Other chromosomal abnormalities detected in nonrandom fashion in adult patients with ALL include deletions, both terminal and interstitial, of the long arm of chromosome 6, and isochromosomes of 7q, 9q, 17q, and 21q. Adult patients with ALL harboring t(9;22), t(4;11), t(8;14), -7, +8 chromosomal aberrations have a poorer prognosis and significantly lower probability of long-term CR and survival than do patients with a normal karyotype or patients with other chromosomal rearrangements.

Complex and Monosomal Karyotype

Complex karyotype (≥3 or ≥5 chromosomal abnormalities) as well as patients with near triploidy defined as 60 to 78 chromosomes and high hyperdiploidy, as well as the presence of 51 to 65 chromosomes are adverse prognostic factors in adults with ALL independent of MRD status. In a more recent study 9.2% of adult patients with ALL had a complex karyotype and 12.8% had a MK and neither was associated with a worse prognosis when treated with risk-adapted or subtype-oriented protocols. MK did not have an impact on prognosis in Ph-positive ALL irrespective of imatinib treatment.

The lymphoid leukemias associated with Down syndrome (DS-ALL) are almost exclusively B-precursor ALLs. In a recent large series of DS-ALLs, there were only five cases of T-ALL among 700 patients. Also, in a sharp contrast to the myeloid neoplasms, they almost never occur in infants. Clinically the outcome of DS-ALL is significantly worse than sporadic childhood B-cell precursor ALLs because of intrinsic resistance to therapy and increased treatment-related mortality. Although all known cytogenetic subgroups of childhood B-cell ALLs have been observed, the common abnormalities such as *ETV6/RUNX1* fusion and hyperdiploidy are less frequent.

Up to 60% of DS-ALLs have aberrant expression of the cytokine receptor *CRLF2* that is often associated with additional mutations activating JAK-STAT signaling. The aberrant expression of this receptor is caused by genomic rearrangements consisting either of a translocation into the *IgH* locus control region or a microdeletion upstream to the *CRLF2* gene, located on the pseudoautosomal component of the sex chromosomes. This deletion fuses *CRLF2* with the promoter of an upstream constitutively expressed *P2RY8* gene. Analysis of the breakpoint sequences suggest that this rearrangement is mediated by *RAG1* or *RAG2* in early B-cell precursors.

Application of high-resolution RNA sequencing, FISH, and CGH+SNP array to 117 consecutive pediatric patients with B-ALL demonstrated that all genetic changes necessary for risk stratification were reliably identified. Specifically, array CGH+SNP proved to be more sensitive in detecting cryptic hyperdiploidy. Hyperdiploid leukemic cells frequently fail to proliferate in culture resulting in

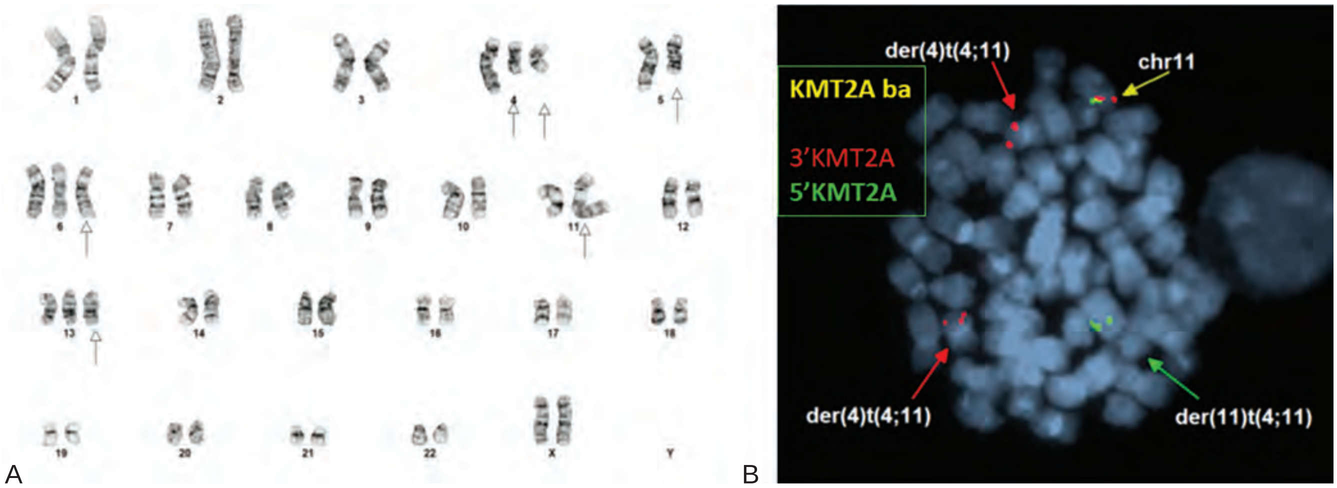


Figure 57.55 A HYPERDIPLOID B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA WITH TWO COPIES OF t(4;11). A diagnostic karyotype, in 100% of examined bone marrow metaphase cells, had 49,XX, +der(4)t(4;11) (q21;q23),t(4;11), del(5)(q15q34),+6,+13 karyogram. These findings are consistent with a hyperdiploid karyotype including trisomies of chromosomes 6 and 13. The patient also had a balanced translocation between the long arms of chromosomes 4 and 11 followed by a replication of the abnormal derivative chromosome 4. (B) A second abnormal chromosome der(4) is shown within the metaphase cell after FISH probing using a KMT2A breakapart FISH probe. *Red arrows* point to a gain of 3′ *KMT2A* localized on two der(4) chromosomes while the green arrow indicates that the 5′ of KMT2a remained on the abnormal 11. In adult ALL, a hyperdiploid karyotype involving t(4;11) is considered a high-risk category. Of note, del(5q) is typically observed in myeloid disorders but it has been reported rarely in lymphoid diseases as well. *ALL*, acute lymphoblastic leukemia; *FISH*, fluorescence in situ hybridization.

an absence of aneuploidy. Moreover, copy-number analysis appears to be a crucial and central diagnostic tool and should be included into a standard workflow for B-ALL patients enrolled in the clinical trials.

Somatic Mutations

The most commonly altered pathways in ALL, involving the transcriptional regulation of lymphoid development, such as *PAX5* (9p13.2), *IKZF1* (7p12.2), and *EBF1* (5q33.3), are frequently mutated (Chapter 66). *PAX5* is required for B-lymphoid lineage commitment and maturation. *PAX5* genetic alterations include deletions, sequence mutations, and chimeric genes with at least 16 different chromosomal partners (http://atlasgeneticsoncology.org/Genes/PAX5ID62.html). *IKZF1* is required for the development of all lymphoid lineages and its genetic lesions are implicated in resistance to chemotherapy and TKIs.

Other commonly mutated pathways in ALL include genes involved in tumor suppression and cell cycle regulation (*TP53*, *Rb1*, and *CDKN2A*), cytokine receptor, RAS-signaling, lymphoid signaling, and epigenetic modification such as *EZH2*. An integrated approach including NGS and array studies has contributed to an increased number of novel molecular entities such as *DUX4/ERG*, *ETV6-RUNX1-like* ALL, and *MEF2D/ZNF384* ALL. Rearrangements of *DUX4*, a homeobox transcription factor (4q35.2) such as overexpression, or transcription binding causing a loss of function of *ERG* (21q22.2) is present in 4% to 7% of B-cell-ALL. Aside from ERG deletion, *DUX4* fusions are frequently accompanied by *IKZF1, PAX5*, and 9p deletions and by activating mutations in *NRAS*, *KRAS*, and *MYC*. Another novel form, *ETV6-RUNX1-like ALL*, is characterized by *ETV6* and *IKZF1* aberrations and approximately 3% to 5% of all childhood B-cell ALL belong to this new biological category. This integrated approach revealed that *MEF2D* (1q1.3) and *ZNF38* (12p13.1) fusions as recurrent events both in adult (~7%) and pediatric (3.6%) ALL and that they should be considered as two distinct expression subgroups. *MEF2D* fusions identify a group of patients with upregulation of *HDAC9* both in children and adults which is associated with a poor outcome. The expression subgroup of patients with *ZNF384* fusions is observed in patients with hyper-expression of JAK-STAT signaling pathways genes, and of *GATA3* and *CEBPA/B*.

Prenatal Origin and Multistep Pathogenesis of Acute Lymphoblastic Leukemia

Epidemiologic, genomic, and twin studies indicate a multistep pathogenesis of B-cell ALL in infants and children, with an initial leukemogenic event(s) occurring in utero and subsequent genomic changes occurring postnatally. Most of the current thinking of the natural history of childhood ALL was based on the exquisite work of Greaves and co-workers who studied the clonal origin of leukemia in monozygotic twins 30 years ago. Greaves found that most common chromosomal translocations and their resultant gene fusions were present on neonatal blood spots or Guthrie cards. If a unique gene fusion sequence is present in at least one cell per 30,000 in the peripheral blood, it can be detected by a sensitive PCR assay. The first observations of an in utero origin of an acute leukemia were demonstrated with *KMT2A* rearrangements in three children with ALL, in monozygotic twins who shared the identical fusion, and in a pair of twins diagnosed at age 3 with B-precursor cell ALL. Subsequently in utero occurrence of *ETV6-RUNX1, BCR-ABL1, E2A-PBX1*, and loss of *IKZ1, PAX5*, biallelic loss of *CDKN2A*, and hyperdiploidy were documented prenatally on Guthrie cards. The concordance rate of twins who share a monochorionic placenta and develop leukemia is nearly 100%, whereas older twins have a discordance rate of 90%, indicating that additional postnatal leukemic events are needed. The frequency of an in utero origin of a B-cell precursor ALL in patients who are not twins as measured by clonal *IGH* rearrangements is reported to be 71%, supporting the notion that in the majority of infants older than 1 year, the development of ALL occurs during fetal development because they share a single placenta resulting in blood cell chimerism. The prenatal mutation occurs commonly, exceeding the actual rate of developing leukemia by some 100-fold, indicating a low rate of evolution. The acquisition of the additional genetic lesions, such as copy number alterations of *ETV6*, *PAX5* and *CDKN2A* (25% to 75% of cases) are recurrent and contribute to leukemogenesis. After testing five pairs of monozygotic twins with concordant *ETV6-RUNX1*-positive

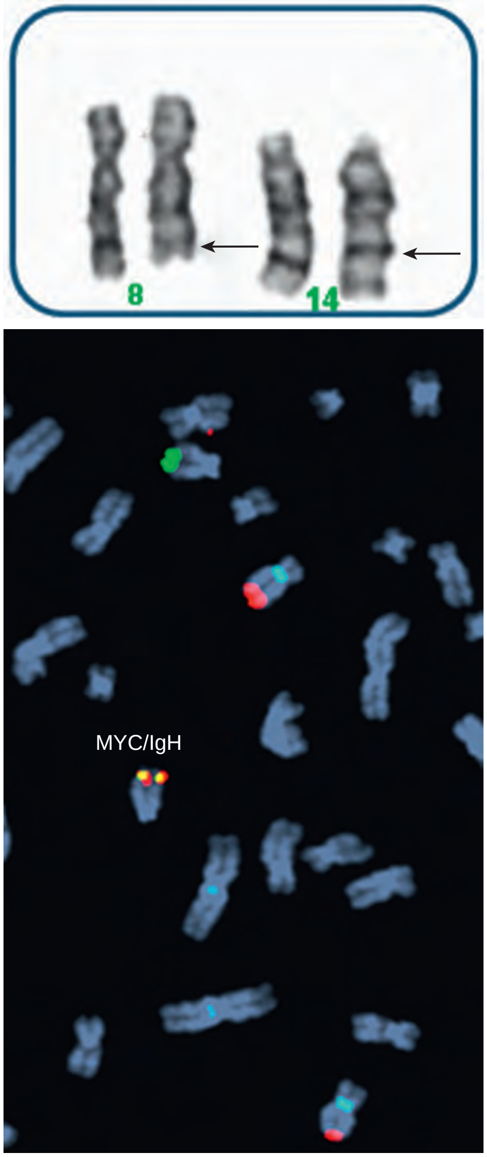


Figure 57.56 t(8;14)(q24;q32) IN ACUTE LYMPHOBLASTIC LEUKEMIA. *Top panel* shows a partial G-banded karyotype of chromosomes 8 and 14 with *arrows* indicating the breakpoints on each chromosome. The *bottom panel* is a partial metaphase (chromosomes are stained blue with DAPI) after FISH testing using three probes: *IGH* signal *(green)* is seen on normal chromosome 14, *aqua* signals for centromere 8 are observed on both chromosomes 8, *MYC (red)* is seen on both chromosomes 8 but note a more bold signal on one chromosome 8 when compared to the other. Because *MYC* is broken as a result of t(8;14), the third copy of *MYC* is on chromosome 14 where it is fused to *IGH* and seen as a *yellow* signal.

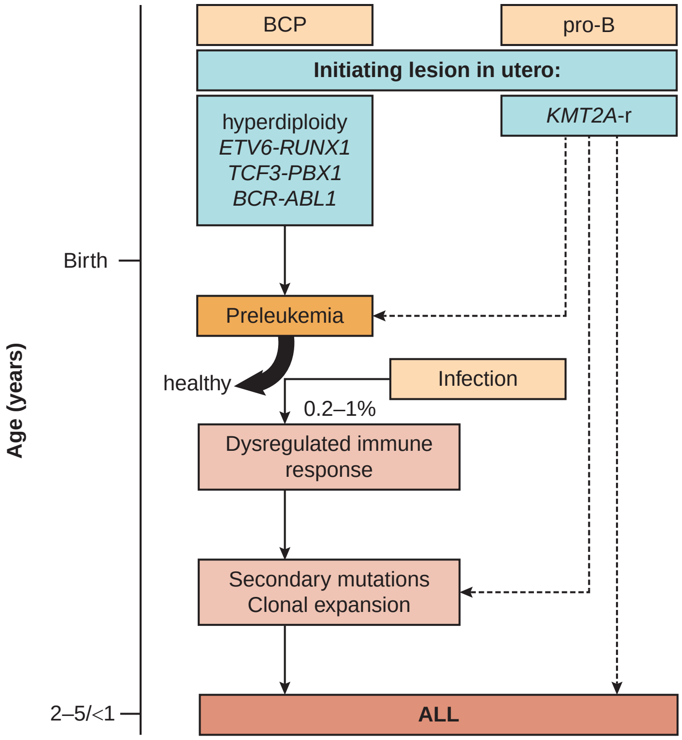


Figure 57.57 TIMELINE OF ALL DEVELOPMENT. The initiating lesions *(green)* occur in utero and lead to a state of preleukemia after birth. Exposure to infection leads to a dysregulated immune response in a small fraction (about 0.2% to 1%) of preleukemic children. Most preleukemic children remain healthy.[6,7] The children develop ALL by acquiring secondary mutations, eventually leading to clonal expansion. In the case of *KMT2A* rearrangements (*KMT2A*-r, right), it is not completely clear whether the *KMT2A*-r are sufficient for ALL development *(right dashed arrow)*. It is also possible that the *KMT2A*-r directly trigger secondary mutations *(central dashed arrow)*. One case of a healthy *KMT2A*-r carrier has been reported leading to the possibility of a preleukemic state for those cases *(left dashed arrow)*. The given median ages of ALL onset are 2–5 years of age for B-cell precursor subtypes (BCP, left) and below 1 year of age for pro-B-cell subtype (pro-B, right). *ALL*, acute lymphoblastic leukemia. *(Reproduced with permission from Hein D, Borkhardt A, Fischer U. Cancer and Metastasis Review, 2020;39:161–171.)*

ALL, Greaves and his colleagues demonstrated that all recurrent driver mutations detected (32 in total) were different within twin pairs, indicating that they are postnatal in origin in both twins and most likely represent secondary mutations. Single cell analyses have revealed considerable complexity within a tree-like or branching structure of genetically distinct subclones, as Greaves and colleagues first suggested. Current molecular data suggest that *KMT2A* fusions may be sufficient to cause leukemia since they are found in aborted fetal tissues (Fig. 57.57).[4] However, formation of *ETV6-RUNX1* fusion in utero is not sufficient to cause leukemia and postnatal mutations are required, such as a loss of a normal *ETV6* from the other homologue. Formation of *ETV6-RUNX1* has been shown, using qRT-PCR and FISH, to be present in ∼1% of newborns. Current evidence suggests the frequency of *ETV6-RUNX1* incidence in a newly born is estimated to be very low, about 10^{-4}. Prenatal origin of *E2A-PBX1*, as mentioned above, was also traced to Guthrie cards and was detected in cord blood of healthy newborns at the frequency of 0.6% (see box on Genetic Testing for Acute Lymphoblastic Leukemia).

GENETIC TESTING FOR ACUTE LYMPHOBLASTIC LEUKEMIA

At diagnosis it is mandatory to perform conventional cytogenetics especially for pediatric patients in whom solely numerical abnormalities occur in 25%, while in adults complex and near triploid karyotype are associated with adverse prognosis. The panel of FISH probes useful at diagnosis of pediatric ALL include ETV6-RUNX1, MLL, BCR-ABL1, E2A-PBX1, and CDKN2A and CDKN2B. Array CGH+SNP is recommended as a reflex test for patients with a normal karyotype.

CHRONIC B-CELL LYMPHOCYTIC LEUKEMIA (CHAPTER 76)

Historically, classic metaphase cytogenetic analyses of CLL were difficult because of the low mitotic rate of neoplastic B cells despite the use of polyclonal B-cell mitogens. FISH analysis of nondividing interphase cells was applied for the first time to hematologic malignancy and was used to create a risk stratification tool for CLL. Introduced in 2000 by Dohner, the FISH hierarchical prognostic model stratifies patients into good [normal cytogenetics, del(13q)], intermediate (trisomy 12), and poor [del(11q) and del(17p)] prognostic groups and remains the most accepted and validated genomic prognostic model. Moreover, current guidelines still recommend FISH analysis before treatment initiation and following relapse or lack of response to therapy.

Cytogenetically CLL is characterized by relatively stable genome harboring a gain or loss of chromosomal regions; balanced translocations are rare in CLL. With improved detection rate of aberrant karyotypes in CLL using cultivation with CD40 ligand (DSP30) and interleukin-2 and the application of FISH, aCGH+SNP, as well as NGS, the detection rate of genomic changes has risen to 80% to 90%. Clinically relevant chromosomal loci, combined with immunophenotyping, mutational analyses of the Ig heavy-chain variable region (IgV_H), and ZAP-70 overexpression, has led to advances in prognostication of CLL patients (Tables 57.14 and 57.15 and Fig. 57.58; see also box on Genetic Testing for B-Cell Chronic Lymphocytic Leukemia). Microarray and high-density SNP array studies are helpful in clarifying the heterogeneous nature of CLL, which is a single disease entity with a common genetic phenotype resembling memory B lymphocytes. Genetic testing is strongly recommended for all patients with CLL, particularly in the context of novel therapeutic trials for. Moreover, a complex conventional karyotype remains an independent prognostic indicator associated with a poor outcome. Chromosome abnormalities in CLL detected by FISH are of prognostic significance (Fig. 57.59). Four genomic aberrations, as well as normal findings, are independent predictors of disease progression and survival. Genomic aberrations of prognostic significance include 17p deletion, 11q deletion, trisomy 12, and 13q deletion. Survival in these groups is 32, 79, 114, and 133 months, respectively, and the treatment-free intervals are 8, 12, 33, and 92 months, respectively. Survival of patients with a normal karyotype is 111 months, and the treatment-free interval is 49 months. In 80% to 90% of the cases, a deletion of 17p tumor suppressor gene (*TP53)* is associated with mutated *TP53* on the remaining copy. *TP53* mutations have been found in 4% to 15% of patients with early-stage CLL and are associated with poorer outcomes. Deletion of 11q and deletion of the *ATM* gene on 11q23.1 are found in 18% of patients with CLL. In about one-third of patients with deleted 11q, a simultaneous mutation of *ATM* has been found.

TABLE 57.14 **Most Frequent Clinically Relevant Chromosomal Abnormalities and Copy Number Alterations in Chronic Lymphocytic Leukemia**

Chromosomal Abnormality	Frequency %	Likely Gene Target	Clinical Associations and Consequence	miRNAs	Prognosis
amp(2p)	7	*REL, XPO1, BCL11A*	Unknown		Poor
amp(3q26.32)	6	*PIK3CA*	Unknown		Poor
del(6q)	~6	Unknown	Associated with prominent lymphocytosis		Poor
			Atypical morphology		
			Splenomegaly		
			Higher rates of CD38 positivity		
del(8p)	5	Unknown	Unknown		Poor
amp(8)(q24.21)	5	MYC			Poor
del(10)(q24)	2	*NFkB2*	Unknown		Unknown
del(11)(q22.3)	10–20	*ATM, BIRC3*	Defect in DNA repair	↑miR-29b	Poor
			Deregulation of P53	↑miR-155	
			Deregulation of cell cycle	miR-29a	
				miR-34b	
			Extensive lymphadenopathy	miR-34c	
Trisomy 12	10–23	Unknown	Atypical morphology	↑miR-148a	Poor
			Aggressive clinical phenotype	↑miR-146a	
del(13)(q14)	57–61	miR15a/16 in an intron of DLEU2	BCL2 expression	↑miR-155	Good
		Its deletion leads to the release of BCL2		↑miR-7–1	
			Resistance to apoptosis	↑miR-154	
				↓miR-220	
				↓miR-221	
del(15)(q15.1)	4	*MGA*	Unknown		None
del(17)(p13.1)	6–8	TP53	Defect in DNA repair	↑miR-151	Poor
			Deregulation of cell cycle	↓miR29C	
			Aggressive clinical phenotype	↓miR34a	
				↓miR148a	
				↓miR-181	

TABLE 57.15 Genomic Risk Stratification Based on Array Comparative Genomic Hybridization[a]

Outcome	Frequency	Gain	Loss
Good[b]	32.5	None	13q14
Intermediate	53.1	1p,7p,12,18p,18q,19	4p,5p,6q,7p
Adverse	20.6	2p,3q,[b]8q,[b]17q	7q,8p,11q,17p,[b]18p

[a] 223 naive patients with chronic lymphocytic leukemia.
[b] Gain of 3q and 8q and loss of 17p are independent unfavorable prognostic biomarkers.

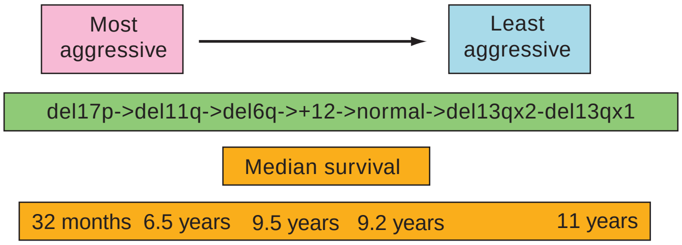

Most aggressive
Least aggressive
del17p->del11q->del6q->+12->normal->del13qx2-del13qx1
Median survival
32 months 6.5 years 9.5 years 9.2 years 11 years

Figure 57.58 SURVIVAL OF PATIENTS WITH SPECIFIC GENOMIC DEFECTS IN CHRONIC LYMPHOCYTIC LEUKEMIA.

GENETIC TESTING FOR B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

At diagnosis, interphase FISH is used in lieu of karyotype studies because FISH detection of abnormalities in CLL correlates with clinical risk groups and prognosis. FISH studies should be performed on blood for detection of trisomy 12, deletions of 11q22.3, 13q14.3, and the *P53* loci, as well as rearrangement of 14q32.3, *IGH* locus. The FISH test can distinguish between patients with B-cell CLL and those with the leukemic phase of certain lymphomas, such as mantle cell lymphoma and follicular lymphoma. If FISH for these loci is negative then somatic mutations for *ATM, BIRC3, NOTCH 1*, and *TP53* should be searched for using NGS. As per *iw*CLL guidelines, it is absolutely critical for the optimal therapy and clinical trials to perform conventional cytogenetics using the standard procedure of stimulating CLL lymphocytes in the presence of DSP30 plus interleukin 2.

FISH-detected anomalies are frequent in B-CLL cases with Rai stages 0 to 1 disease, but are more frequent among patients with progressive disease (88%) than in those with stable disease (66%). Two risk groups have been recognized: (a) low-risk disease includes patients with a normal karyotype or isolated del(13q); (b) high-risk includes patients with del(11q) and del(17p). Patients with +12 are high risk, but in contrast to del(11q) and del(17p), they respond to fludarabine-based therapies. A comparison of quantitative PCR method with FISH for assessment of the four most frequent aneuploidies revealed a tight correlation in 103 of 110 patients examined, with FISH being more sensitive in detecting subclonal genetic evolution (Fig. 57.60).

The frequency of chromosome 13 abnormalities in CLL detected by conventional cytogenetics is 10% to 15%. However, with the use of FISH, smaller or larger deletions of band q14.3 are detected in up to 60% of patients over time. When multiple DNA probes are used, D13S319 and D13S25 DNA markers are deleted more frequently than is *RB1*. Molecular analyses have detected deletions of 13q in cells that are cytogenetically normal as well as abnormal. These deletions can either be heterozygous (76%) or homozygous (24%). Heterozygous deletions most frequently occur in the early stages of the disease and homozygous deletions occur in the more advanced stages. In a study examining loss of heterozygosity and subchromosomal copy losses of chromosome 13, two types of deletions were defined: type 1 aberrations occurred in 60% of cases and were associated with loss of *RB1* and breaks close to the *miR16/15a* locus; and type 2 aberrations that included *RB1* occurred in 40% of cases. The 13q14.3-deleted segment contains micro-RNA (miRNA) genes (Chapters 4 and 76). All patients with homozygous 13q deletion have a dramatic downregulation of miRNA15a, whereas patients with hemizygous 13q deletions are indistinguishable from controls, providing the first molecular clues for pathogenesis of CLL. The percentage of CLL cells with del(13q) is predictive of survival. A high percentage (>80%) of del(13q) cells is correlated with a higher lymphocyte count, a tendency to exhibit a diffuse bone marrow infiltration pattern, and splenomegaly. Trisomy 12 or 13q rearrangements are found separately in a substantial proportion of patients with CLL. They co-exist in only 2% to 5% of patients, suggesting that each change may have a distinct pathogenetic route.

Among the first-degree relatives of patients, population studies have demonstrated a sevenfold increased risk for developing CLL and a twofold increased risk for developing other lymphoproliferative disorders (Chapter 76). More than 80 families with CLL affecting multiple family members have been reported. Linkage studies suggest a region of interest in band q22.1 of chromosome 13 (marker D13S156). Fine FISH mapping of six CLL-prone families (63 individuals) reveals deletion of 13q14 in 85% of patients with familial CLL, and four CLL families shared a 3.6-Mb minimal region in 13q21.33–q22.2. This region included 12 candidate genes, but thus far informative candidates have not been identified.

Trisomy 12 was the first recurrent abnormality reported in CLL patients. It is detected by classic cytogenetics in 7% to 15% of all cases. FISH detects +12 in 15% to 20% of patients with CLL. Trisomy 12 may be present as a sole abnormality or in combination with other chromosomal rearrangements. Because only a proportion of cells are trisomic, normal cells or disomic neoplastic cells may also be present. Follow-up analysis over a 4-year period demonstrated clonal expansion of cells with trisomy 12 as the disease progresses. The observation that trisomy 12 is present in B cells and is absent from T lymphocytes and CD34 cells in the majority of CLL patients is consistent with the hypothesis that CLL is of clonal origin and that trisomy 12 arises in a progenitor cell already committed to the B-cell pathway. The exact mechanism by which trisomy 12 contributes to the pathogenesis of CLL remains unknown.

The chronic lymphocytic leukemia upregulated gene-1 (CLLU1) has been shown to be the first highly specific CLL gene. Six transcripts have been identified, two of which contain a putative coding sequence with low similarity to human interleukin-4. Overexpression of the transcript cDNA1 appears to be specific for CLL, and is of prognostic significance and can be used for MRD detection.

Deletions of the long arm of chromosome 11 in CLL as detected by conventional cytogenetics have been reported in 5% to 8% of cases. An interphase FISH study identified deletion of 11q22.3–23.1 in 10% to 20% of cases. FISH characterization of aberrations involved in 11q21–q23 demonstrate a minimal consensus deletion segment of 2 to 3 Mb, containing a number of genes including *ATM* at 11q22.3. Up to 12% of patients have simultaneous deletions of *ATM* and *MLL* at 11q23. Mutations in *ATM* gene are responsible for the ataxia-telangiectasia syndrome. *ATM* functions as a cell-cycle checkpoint regulator. Somatic disruptions of both *ATM* alleles by deletion or point mutation are detected in 25% to 34% of cases. This finding strongly suggests the pathologic role of *ATM* in some patients with

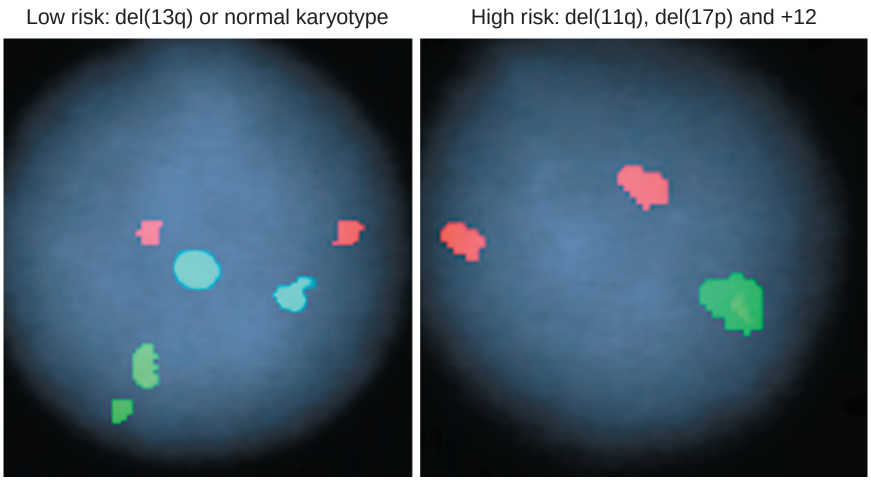

Low risk: del(13q) or normal karyotype
High risk: del(11q), del(17p) and +12

Figure 57.59 PANEL OF CHROMOSOMAL PROBES USED FOR DETECTION OF GENOMIC DEFECTS IN CHRONIC LYMPHOCYTIC LEUKEMIA. They include 13q14.3 *(red)*, 13q34 *(aqua)*, and CEP12 (all present in disomy; *left*) and 11q22.3 (ATM, *green*) and 17p13.1 (P53*; red, right*). Note there is only one copy of the *ATM* gene *(green, right)* consistent with deletion of sequences from the 11q22.3 band region, which is associated with unfavorable prognosis.

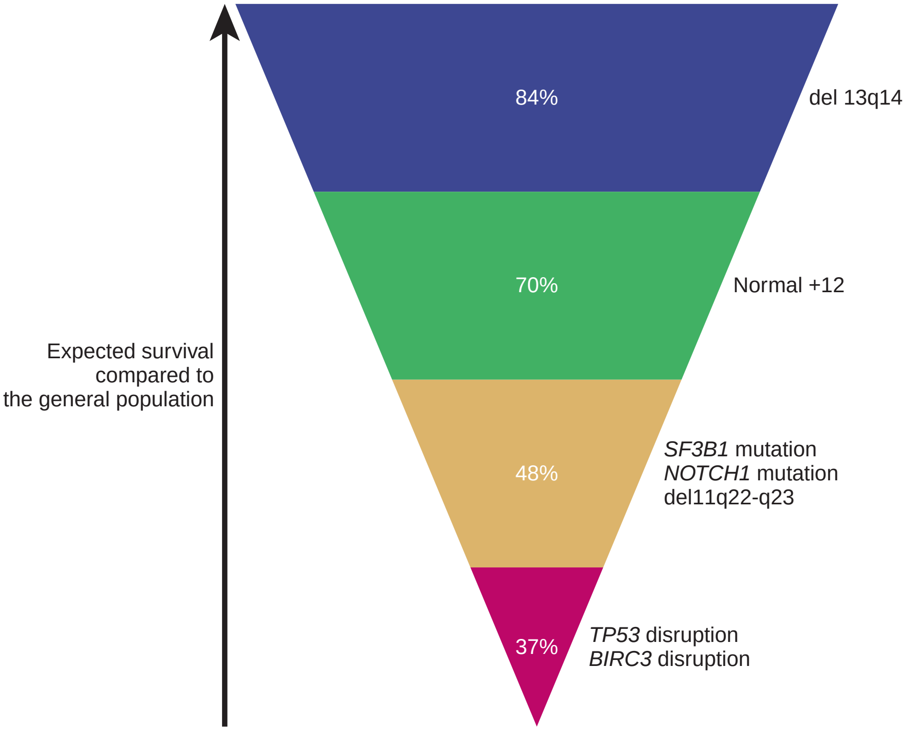


Figure 57.60 SURVIVAL OF PATIENTS WITH SPECIFIC GENOMIC DEFECTS IN CHRONIC LYMPHOCYTIC LEUKEMIA ACCORDING TO GENETIC DEFECTS. Expected survival of patients with chronic lymphocytic leukemia stratified according to the integrated mutational and cytogenetic model and compared to the matched general population. Expected survival is calculated at 10 years. *(Reprinted with permission from Foà R, Del Giudice I, Guarini A, et al. Clinical implications of the molecular genetics of chronic lymphocytic leukemia. Haematologica. 2013;98:675.)*

B-cell-CLL. Moreover, a discontinuous deletion between 11q23.1 and 11q23.3 segments indicates that genes in this region may have pathogenic significance because they constitute independent targets for amplification or deletion in cases of B-CLL. Most of the patients with del(11q) are relatively young. The appearance of this deletion is clinically associated with lymphadenopathy, rapid disease progression, poor response to treatment, and a shorter OS.

Structural abnormalities of chromosome 17 have been observed in 4% of cytogenetically evaluable B-cell CLL cases. This abnormality frequently affects the short arm of chromosome 17, the site of the *TP53* tumor suppressor gene. Monoallelic deletions of *TP53*, detected by FISH, are present in 7% to 20% of patients and represent the strongest predictor of poor survival. The median survival of these patients is only 32 months. *TP53* mutations are associated with aggressive disease and a lack of response to conventional therapy. Deletion of *TP53* and mutations of *TP53* are independent processes and at diagnosis ~about 10% of CLL patients harbor the *TP53* gene mutation. Up to 50% of patients may have *TP53* defects as the disease progresses, or becomes treatment-refractory. Of all patients with *TP53* gene abnormalities, mutations and deletions occur together in the majority of patients with monoallelic *TP53* mutations accounting for another 40% of CLL patients, while monoallelic deletions and also mutations with copy neutral loss of heterozygosity account for a minority of patients. A novel recurrent dic(8;17)(p11;p11) abnormality also results in loss of *TP53*, and low copy repeats in 17p12 and 8p11 may represent the origin of the translocation by nonallelic homologous recombination on a single chromosome 17. B-CLL in patients with deletion of the *TP53* gene is associated with progressive disease, resistance to treatment, and shortened survival. Patients with 17p deletions or *TP53* mutations are frequently resistant to purine analogs.

Deletion 6q is a relatively rare chromosomal change occurring in approximately 6% of CLL cases and is associated with marked lymphocytosis, abnormal morphology, splenomegaly, overexpression of CD38, and unmutated *IGH* heavy chain variable region.

Chromosome 14q32 rearrangements/translocations involving *IGH* was considered a rare abnormality affecting fewer than 4% of CLL patients. With the emergence of novel molecular approaches a higher incidence of *IGH* rearrangements has been reported in recent years (5% to 15%). The genes most frequently rearranged with IGH are *BCL2*/t(14;18)(q32;q21)(~2% of cases) and *BCL3*/t(14;19)(q32q13) or *BCL11*/t(2;14)(p16;q32) (less than 1%). Because the leukemic phase of certain lymphomas can clinically mimic B-CLL, FISH for translocations using an *IGH* probe is important. Fewer than 7% of patients with CLL/prolymphocytic leukemia patients have t(11;14)(q13;q32), and these cases usually evolve into prolymphocytic leukemia. Moreover, *IGH* testing in CLL is important for detecting patients with recurrent del(14)(q24.1) associated with unmutated IgV_H status (66%) and trisomy 12 (47%). Other rare chromosomal aberrations in CLL include trisomy 3q27 (3%), trisomy 8q24 (5%), gains of 15q15-qter, trisomy 18, and trisomy 19. Other recurrent rearrangements involving loss of 8p21-pter and del(9)(q11) have been reported.

Based on the recent CGH results, CLL may be classified into three groups: those with poor outcome (20.6%) exhibit at least one aberration: gain of 2p, 3q, 8q, 17q, and loss of 7q, 8p, 11q, 17p, and 18p; good outcome (32.5%) includes 13q14 loss without any of the other 10 aberrations (gain: 1p, 7p, 12, 18p, 18q, 19, loss: 4p, 5p, 6q, 7p); and the third, intermediate outcome are all other abnormalities. The three groups are significantly separated with respect to time to first treatment and OS ($P < .001$). Gain of 3 and 8q and 17p loss are independent unfavorable prognostic biomarkers.

According to the recently updated *iw*CLL recommendations, genetic risk stratification in CLL requires not only FISH analysis but must be complemented by mutational screening for the *TP53* gene. FISH offers only a partial view (5 loci are tested) of the CLL-genetic landscape whereas classical cytogenetics present a global view of the single cell analysis. Therefore, *iw*CLL guidelines indicate that conventional cytogenetics should be performed and is "desirable" before treatment initiation as well as in the context of clinical trials. Cytogenetic complexity of CLL cells was brought to light when 5082 patients from 17 European institutions on the behalf of European Research Initiative were retrospectively evaluated. This large study defined complex karyotypes in CLL according to the presence of ≥3 numerical and/ or structural abnormalities which was considered to be an unfavorable risk factor.[18] Based on these findings CLL should be considered a heterogeneous group of B-cell malignancies with a clinical course, ranging from remarkably indolent to extremely aggressive. A high-complex karyotype, defined as the presence of at least 5 abnormalities, was associated with a dismal clinical outcome, independently of clinical stage and *TP53* status. In contrast, low-complex and intermediate-complex, defined according to the presence of 3 or 4 aberrations, respectively, appears to be clinically relevant only in the presence of *TP53* abnormalities. At the other end of the spectrum, patients with CLL with complex karyotype and +12, +19 displayed an indolent profile.[19]

Gene Mutations as Diagnostic and Prognostic Biomarkers

Approximately 60% to 65% of patients with CLL have somatic mutations in the *IGHV*. The remaining 35% to 40% lack *IGHV* mutations. *IGHV* gene mutations (M-CLL) are markers for a favorable prognosis, whereas an unmutated *IGHV* gene (U-CLL) in CLL is associated with clinically more aggressive disease. The OS rate for U-CLL is 8 to 9 years whereas that for M-CLL is more than 24 years. The frequency of other somatic mutations is shown in Table 57.16. The somatic mutational landscape of CLL includes more than 50 different genes and the most common mutations are the following genes: *SF3B1, DDX3X, BIRC3, ATM, NOTCH1, EGR2,TP53*, and *XPO1*. As mentioned above, mutations in *ATM* may occur in patients with and without a del(11q) in 10% and 25% of patients, respectively. Thus far the *ATM* mutation remains the most important marker of poor outcome in del(11q) CLL patients. CLL patients carrying *IGHV4-39*, *NOTCH1*, or *TP53* mutations and a complex karyotype are at higher risk of transformation. Patients with Richter syndrome (Chapter 76) (see later) frequently have additional *CDKN2A* deletions and *MYC* translocations or amplifications on top of genomic alterations already present in the original CLL. As mentioned earlier, in addition to del(17p) resulting in deletion of *TP53*, 17p may be targeted by acquired copy neutral loss of heterozygosity (cnLOH) which results from a somatic recombination event that duplicates a single mutated *TP53* allele. Sanger sequencing has identified *TP53* mutations without loss or cnLOH of the other *TP53* allele, and these patients exhibit poor survival. Most CLL patients with *TP53* mutations are refractory to chemotherapy. Most *TP53* somatic mutations are missense and located within the DNA binding domain of *TP53* encoded by exons 5 to 8, and six "hot spots" are mutated in approximately 20% of patients.

In the CLL-IPI scoring system, the presence of either a 17p deletion or a *TP53* mutation confers the same prognostic significance. These two genetic events are usually detected by different methodologies measuring two different abnormalities. Interphase FISH is used to detect deletion of 17p13.1 chromosomal segment on the short arms of chromosome 17, containing the *TP53* locus. The breakpoints, and therefore the size of deletion, may vary between patients. *TP53* mutation usually occurs on one chromosome 17 but only biallelic *TP53* mutations result in the loss of *TP53* function. In practice homozygous 17p13.1 deletion by FISH is very rare and monosomy of 17p or monosomy of entire chromosome 17 may represent an indirect measure of biallelic loss of *TP53* function. Approximately 80% of patients with del 17p also have mutations in the remaining TP53 allele which may explain the strong association between heterozygous 17p deletion with poor prognosis. Recently, mutations of *NOTCH1*, *BRAF*, *SF3B1*, *NFKBIE*, and *EGR2* have been detected in CD34+ cells and CD14+ (myeloid) progenitors in patients with CLL suggesting involvement of early hematopoietic cells in the origins of CLL. These observations may indicate that CLL might develop from preleukemic multipotent hematopoietic progenitors as a result of deregulation of B-cell receptor intracellular signaling and that CLL is preceded by a preleukemic phase.

Genome Complexity in Chronic Lymphocytic Leukemia

Array CGH and NGS studies have effectively clarified the level of genomic complexity in CLL and revealed that the average number of mutations in CLL cells at diagnosis lies between 10 and 20, which is one of the lowest among the adult cancers, confirming previous cytogenetic observations. The recent studies also revealed that no single unifying mutation is responsible for CLL. Array-based genomic profiling has extended previous cytogenetic data demonstrating that a subset of patients with CLL have complex genomic profiles pointing to the interplay between chromosomal abnormalities and somatic mutations that are associated with reduced OS. Moreover, telomere dysfunction in CLL and acute telomere attrition results in fusion events that contribute to genomic complexity such as *chromothripsis*, which is a high level of DNA damage that may occur during a single mitotic division Fig. 57.61. Chromothripsis has been shown to occur in approximately 5% of patients, primarily in patients with unmutated *IGHV* status (74%) and high-risk genomic aberrations (79%). The presence of chromothripsis and kataegis in CLL implies that multiple cancer genes can be disrupted in a single step, providing a "quantum leap" for the malignant potential of the initial CLL clone.

TABLE 57.16 Most Frequent Somatic Mutations in Chronic Lymphocytic Leukemia

Chromosomal Location	Gene	Pathway	Co-Segregation	Frequency
Alterations Associated With M-IGHV				
15q26	*CDH2*	Chromatin modification		5
3p22	*MYD88*	Inflammatory pathway	del(13q)	3–5
Alterations Associated With U-IGHV				
11q22.3	*ATM*	DNA damage response	del(11q)	9–14
11q22	*BIRC3*	Cell cycle control	del(11q)	1–5
7q31.33	*POT1*	Cell cycle control	*SF3B1*	5
17p13.1	*TP53*	Cell cycle control	del(17p)	5–27
2q33.1	*SF3B1*	mRNA processing	del(11q)	10–19
9q34.3	*NOTCH*	NOTCH signaling	Trisomy 12, XPO1, TP53	12–24
4q31.3	*FBXW7*	NOTCH signaling	Trisomy 12	4
Other Recurrent Mutations				
	NFKB1E			10
	FAT1			10
	EGR2			1–8
	LRP1B			5
	ZMYM3			4
	DDX3X			3
	MAPK1			3
	HIST1H1E			3
	BCOR			3
	RIPK1			3
	SAMHDI			3
	SI			3
	PO1X			2.5
	LHL6K			2
	RAFB			2
	RASK			2
	ED12M			2
	RASN			1–3
	IRF4			1.5

M-IGHV, Mutated immunoglobulin heavy chain variable; *U-IGHV*, unmutated *IGHV* gene.

Richter Syndrome

Approximately 15% of patients with CLL transform eventually to Richter syndrome (RS), a highly aggressive phase of CLL, which morphologically mimics diffuse large B-cell lymphoma (DLBCL) and frequently has a dismal outcome (Chapter 84). RS is characterized by a complex karyotype, *TP53* defects (50% to 60%), *NOTCH1* activation (30%), and *MYC* abnormalities (30%). The majority of RS cases are derived from the original CLL clone with an average of 20 acquired molecular lesions during the evolution to RS (Fig. 57.62). There appears to be considerable heterogeneity regarding the number and type of genomic abnormalities.

Several clinical and biologic risk factors may predict future RS development. Patients with CLL with an unmutated *IGHV* are four times more likely to develop RS as compared to those with mutated *IGHV*. High ZAP70 expression and CD49d have also been associated with an increased risk of RS in some studies.

Recently, two genetic pathways that lead to transformation of RS from CLL have been delineated. Approximately 50% of patients with RS have cell-cycle deregulation via inactivation of *TP53*, loss of *CDKN2A* (9p21.3), gains or translocations of *MYC*, and loss of 13q14.3 region. These patients had a high cell proliferation rate (Ki-67 >70%) and a worse OS than patients with wild-type *TP53* and *CDKN2A*. The loss of *CDKN2A* function appears to be acquired at the time of transformation, as evidenced by its presence in the RS but not CLL cells. The second group of patients with RS (30%) is characterized almost exclusively by trisomy 12 and *NOTCH1* mutations. The remaining 20% represent a heterogeneous group, and do not exhibit either *TP53/CDKN2A* inactivation or trisomy 12 and individual cases are characterized by del(11q), del(14q), *MYC* activation, *IGH* translocations, and *NOTCH1* mutations. Using paired-sample analysis CLL-specific lesions are almost always present in the RS cells along with RS-specific abnormalities. These observations imply that RS develops through a linear model of clonal evolution from the underlying CLL. Genome-wide DNA analysis confirmed that RS DNA profile remains clearly distinct from that of primary DLBCL.

Newly diagnosed RS is an oncologic emergency requiring assessment of a karyotype and at least FISH detection of *TP53, MYC*, and *NOTCH1* mutations.

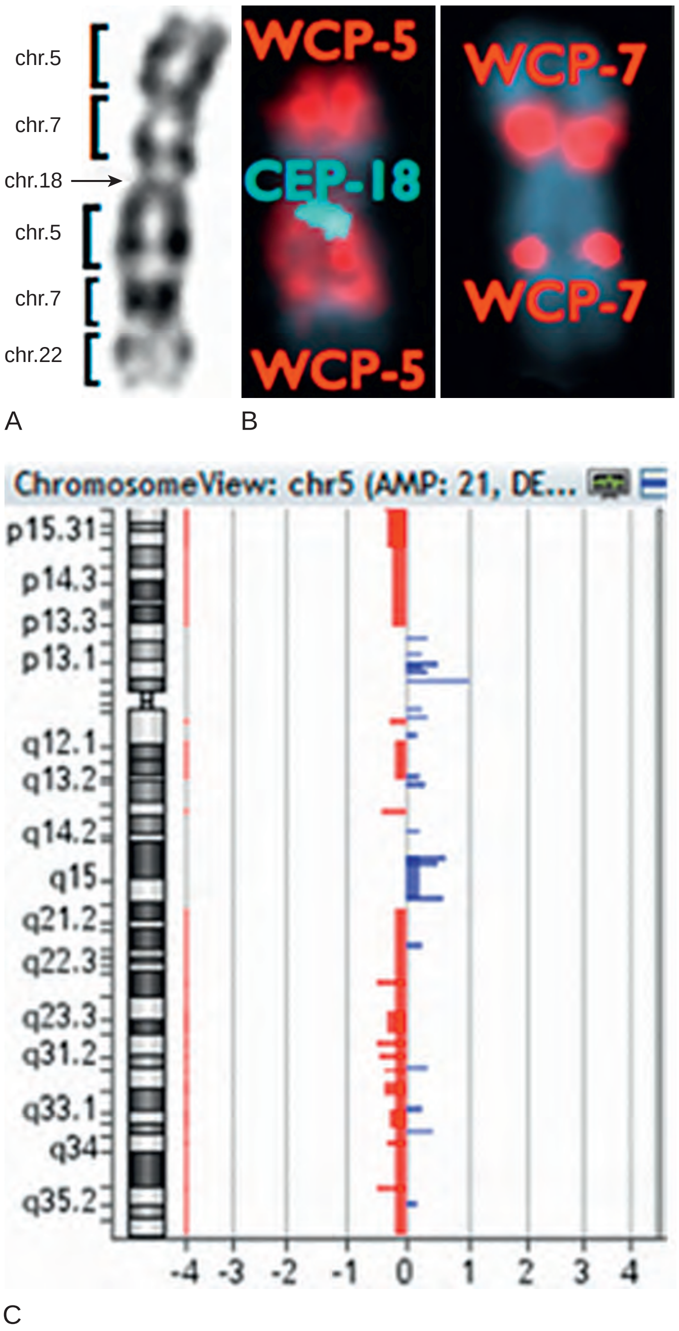


Figure 57.61 CHROMOTHRIPSIS. (A) Conventional G-banded cytogenetic analysis showed a derivative chromosome 18 composed of additional chromosomal segments. (B) Various FISH probes confirmed that the derivative chromosome 18 is composed of segments of chromosome 5, 7, 18, and 22, whereby the fragments of multiple chromosomes are stitched together by paired end joining. (C) Array comparative genomic hybridization (aCGH) of chromosome 5 shows segments within both the p arm and q arm with gains *(blue bars)* and loss *(red bars)* of genomic DNA illustrating that there are frequent copy number changes in a localized region of a chromosome as a result of chromothripsis.

MULTIPLE MYELOMA

MM is a malignancy of terminally differentiated B cells (see Chapters 90 and 91). MM plasma cells, CD138-positive cells, have a very low proliferation rate, which has limited cytogenetic studies. Historically, conventional karyotyping reveals chromosomal abnormalities in 30 to 40% of newly diagnosed patients, especially in cases with an exceptionally high plasma cell proliferative rate. Karyotypes obtained from these cells usually are complex and exhibit more than 20 aberrations in approximately 10% of cases. Currently, many laboratories have adopted a long-term culture, 72 to 120 hours, using a variety of cytokines or growth factors including L2, IL4, DSP30, and others. Despite a low number of chromosomally abnormal cases, cytogenetics is still the only clinically available test that can clearly delineate independent clones and definitively ascertain clonal evolution. Its use has been recommended according to the NCCN 2018 guidelines.

To obtain accurate FISH results with MM cells plasma cells (CD138+) enrichment prior to using FISH for detection of genomic abnormalities is recommended. Magnetic microbeads conjugated with anti-138 antibodies is a quick and rapid way to increase sensitivity of FISH as well as NGS and array CGH-SNP because enrichment of CD138+ cells greatly diminishes the number of non-plasma cells and therefore provides increased test accuracy.

When reassessed by FISH, CGH, and multicolor karyotyping using a large panel of centromere-specific and translocation-specific probes, interphase plasma cell nuclei in MM and other clonal plasma cells including monoclonal gammopathy of unknown significance (MGUS), smoldering myeloma (Chapter 90), and monoclonal immunoglobulin deposition disease (amyloidosis) (Chapter 93) have been shown to be characterized by chromosomal aneuploidy. Array CGH indicates that 100% of patients with newly diagnosed MM have copy number alterations and frequent homozygous deletions of genes including *TRAF3*, *BIRC1/BIRC2*, *RB1*, and *CDKN2C*. Even after achieving CR, after therapy, 12% to 71% of plasma cells may still have a numerical gain or loss of chromosomes 3, 7, 8, 9, 11, 13, 15, 21, and X.

Genomic abnormalities in MM are complex and heterogenous both at diagnosis and even more so with disease progression with several subclones evolving within the same individual. There is no identifiable single genomic abnormality that is MM-specific or that can distinguish MM from other clonal plasma cell entities.

Current recommendation requires that plasma cells are enriched and FISH analysis performed on CD138-positive cells. Table 57.17 lists the most frequent chromosomal rearrangements that occur in patients with MM. Analysis of numerical abnormalities reveals two groups of MM patients: those who are hyperdiploid and those who are not hyperdiploid (hypodiploid, pseudodiploid, or near-tetraploid) and contain immunoglobulin heavy-chain locus (*IGH*) 14q32, translocations (see box on Genetic Testing for Multiple Myeloma.)

GENETIC TESTING FOR MULTIPLE MYELOMA

In practice at the time of this writing (2021) it is recommended that genomic testing be performed on enriched plasma cells (CD138-positive) for FISH and array CGH analysis or NGS. FISH testing of CD138+ purified plasma cells should include, at minimum, t(4;14)(p16;q32), t(14;16)(q32;q23), t(14;20)(q32;q11), gain of 1q, and loss of 17p. In our laboratory we also include testing for the t(11;14) and an IGH breakapart probe because if the t(11;14) as well as other IGH lesions mentioned earlier are not detected, IGH translocations without known partners are associated with adverse prognosis. Testing for gains of 1q 12-q44 is an independent marker associated with disease progression and deletion of TP53 defines a group of patients with ultra-high-risk MM (see Figs. 57.65 and 57.66). If IGH rearrangements are detected using a breakapart IGH locus and CCND-IGH fusion is not identified, refine the translocation partner and use the following set of probes: MAF-IGH for detection of t(14;16) and FGFR3-IGH for detection of t(4;14). Cytogenetics is very useful in determining primary vs secondary chromosomal abnormalities and the clonal evolution.

Hyperdiploid Multiple Myeloma

Approximately 55% to 60% of patients with newly diagnosed MM are characterized by a hyperdiploid karyotype with the number of chromosomes ranging from 48 to 74 and trisomies of odd-numbered

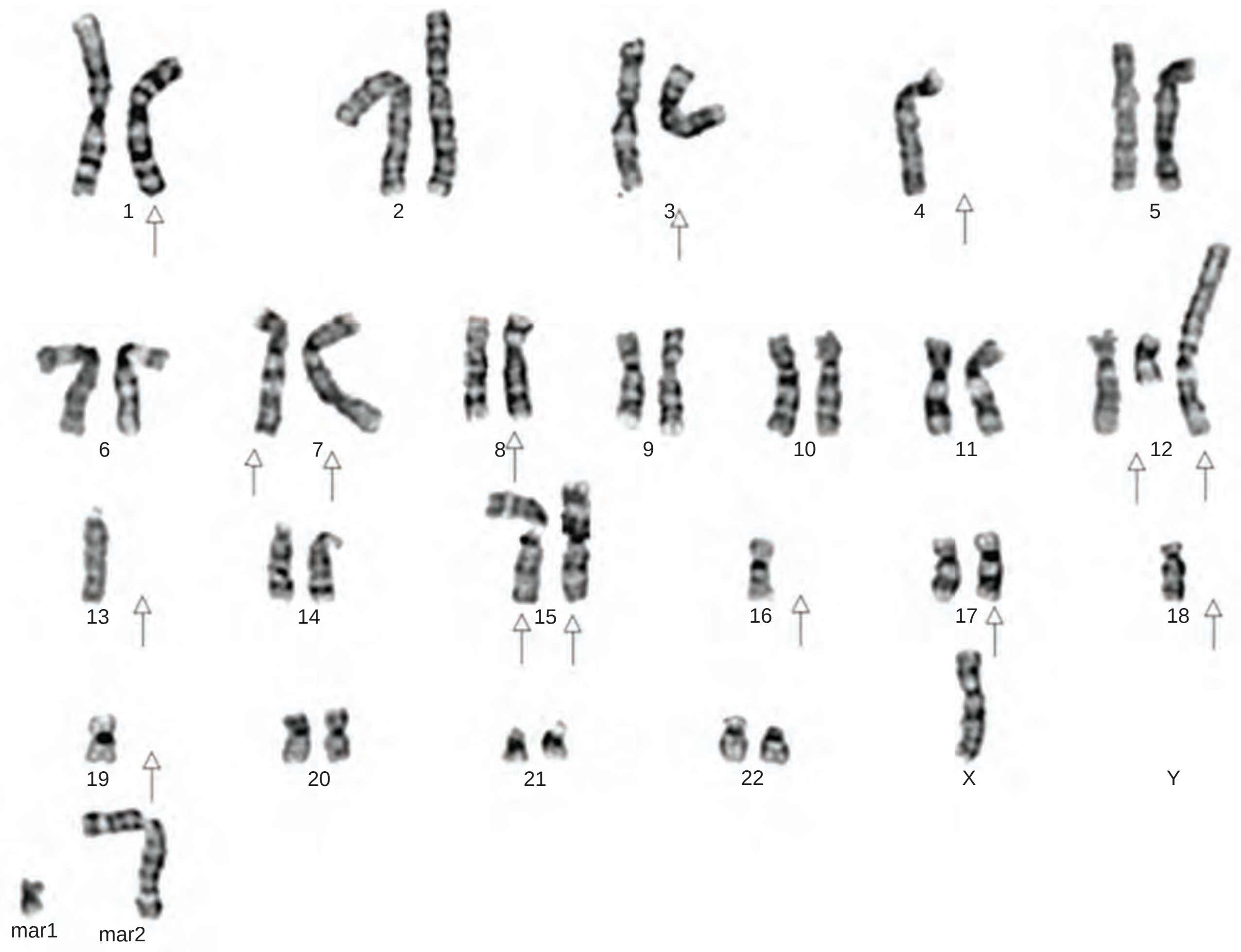


Figure 57.62 COMPLEX KARYOTYPE IN RICHTER SYNDROME. This karyotype is from a patient who progressed from CLL to Richter syndrome. Ninety percent of cells showed a complex modal karyotype 42~46,X,-Y,t(1;7)(p22;q22),del(3)(p21p25),-4,add(7) (q35),+8,der(8)t(8;17)(p11.2;q21.3) x2,+12,der(12)add(12)(p12)add(12)(q12),der(12)t(4;12)(q13;p13),-13,add(15)(p11.2),der(15)t(15;17)(p11.2;q21.3),-16,add(17)(p11.2),-18,-19,-21,add(22)(p11.2),add(22)(p11.2),+mar1,+mar2,+mar3. Abnormalities of 14 chromosomes were identified and the origin of two markers could not be deciphered using conventional cytogenetics. Array CGH+SNP detected additional 31 copy number alterations, including 20 losses and 13 gains. There were 3 to 4 CNAs per 14 abnormal chromosomes including a loss of *TP53* which may explain genomic imbalance of the patient. *CGH*, Comparative genomic hybridization; *SNP*, single-nucleotide polymorphism.

chromosomes including 3, 5, 7, 9, 11, 15, 19, and 21 and few *IGH* translocations (Figs. 57.63 and 57.64). Among these gains, chromosomes 9, 15, 19, and 21 are usually acquired first, and may be followed by trisomy of chromosomes 3, 5, and 7. These chromosomal abnormalities are considered primary genomic changes in the early pre-malignant stage and theoretically involved in disease pathogenesis. Therefore, the prognostic relevance of numerical abnormalities using classic cytogenetics is unknown, although the presence of abnormal metaphases cells is an indicator of poor prognosis. However, hyperdiploid MM detected by FISH tend to have a better prognosis than do those with nonhyperdiploid disease. This effect is lost in cases where hyperdiploidy is associated with other genetic markers of progression such as gain of 1q21 and deletion of 17p13. Many studies have shown a median OS of 60.8 months for hyperdiploid patients with MM with no adverse cytogenetic lesions as compared to 33.7 months for those hyperdiploid patients with MM who had one or more of the adverse cytogenetic abnormalities such as t(4;14), t(14;16), t(14;20), del(17p), and +1q (see Figs. 57.65 and 57.66). The exact origins of hyperdiploidy remain unknown, although four processes are proposed: (1) a near-haploid cell doubles all chromosomes; (2) a tetraploid cell experiences subsequent loss of chromosomes; (3) a diploid cell undergoes sequential gains of chromosomes during clonal evolution; and (4) a diploid cell suffers a single mitotic catastrophe resulting in simultaneous gain of additional chromosomes. The data suggest that a single mitotic catastrophe, such as failed mitotic event, may be the most likely mechanism of acquisition of hyperdiploidy, which may be followed by the secondary gain of extra chromosomes during subclonal evolution. In some patients hyperdiploidy may precede primary translocations affecting *IGH* as revealed by studies using single cell sequencing analysis. In general, hyperdiploidy is associated with more favorable survival than hypodiploidy; however, adverse cytogenetic features, such as +1q, del(17p), and t(4;14) predict worse survival than hyperdiploidy alone.

Nonhyperdiploid Multiple Myeloma

Approximately 45% to 50% of patients with MM have nonhyperdiploid MMs that include patients with hypodiploid, near diploid, pseudodiploid, or near-tetraploid chromosome numbers (fewer than 48 or more than 74 chromosomes), which are characterized by a high frequency of *IGH* translocations (>85%). With the exception of t(11;14)(q13;q32), most of the nonhyperdiploid patients have an aggressive disease course characterized by a short time to relapse and limited survival. Table 57.18 outlines a risk stratification system of

TABLE 57.17 Most Frequent Chromosomal Abnormalities in Multiple Myeloma and Their Frequencies

Genetic Lesion	Frequency (%)	Oncogenes/Fusion Genes
Hyperdiploid	~50	Gain of odd-number chromosomes
Nonhyperdiploid		IGH translocations
Translocations		
t(4;14)(p16.3;q32.3)	15	*FGFR3, MMSET-IGH*
t(11;14)(q13;q32.3)	15–20	*CCND1-IGH*
t(14;16)(q13;q23)	5–10	*MAF-IGH*
t(8;14)(q24;q32.3)	<10	*MYC-IGH*
t(14;20)(q32.3;q11)	5	*IGH-MAFB*
Gain of Chromosomal Region		**Candidate Oncogenes**
1q21–q22	55	*BCL9, IL6R*
3q27.1–3q27.2	47	*POLR2H, EIF4G1*
5p12	44	*?*
7p11.2	44	*?*
9q34.11–9q34.3	54	*ABL1, ANAPC2*
11q13.4–11q14.1	52	*SPCS2*
15q24.2	44	*IMP3*
19q13.1	47	*PDCD5*
21q22.3	37	*MCM3AP, HRMT1L1*
Loss of Chromosomal Region		**Candidate Tumor Suppressor Gene**
1p13.1–1p12	41	*DENND2D*
8p23.3–8p21.3	28	*DLC1*
10q26,2–10q26.3	18	*PTPRE*
13q34	49	*RFP2, micro RNA 15/16*
1432.13–13q32.2	33	*?*
16q11.2–16q12.3	31	*CYLD*

Modified from Anderson KC, Carrasco RD. Pathogenesis of myeloma. *Ann Rev Pathol.* 2011;6:249.

patients with MM based upon chromosomal abnormalities. These patients are at risk of acquiring genetic events such as deletion of chromosomes 13 and 14, chromosome 17 abnormalities, as well as 1q amplification and 1p deletion.

Both hyperdiploidy and nonhyperdiploidy are also present in patients with MGUS, suggesting that such abnormalities occur early in the course of disease. More detailed analyses using genome-wide CNAs have revealed numerical aberrations in 98% of MM cases and identified amplification of 1q and deletions of 1p, 12p, 14q, 16q, and 20p to be associated with a poor prognosis, whereas gains of chromosomes 5, 9, 11, 15, and 19 are associated with a more favorable outcome.

Hypodiploid karyotype (<44 chromosomes) in MM accounts for one-fifth of all patients with MM and represents an aggressive subtype. In these patients, abnormalities such as monosomy of chromosomes 13, 14, and 22 as well as deletions for 1p, 12p, 16q, and 17p are common. These genotypes are associated with a poor outcome and disease progression. Hypodiploid chromosomal status is an independent risk factor for a poor survival.

Translocations in Multiple Myeloma

Primary translocations appear to occur early during the course of MM, whereas secondary translocations occur later on and are involved in tumor progression. Most primary translocations are simple balanced translocations that juxtapose an oncogene and one of the Ig enhancers. An *IGH* rearrangement on 14q32.3 is found in most patients with MM. This rearrangement consists of complex and heterogeneous translocations with the breakpoint involving either the switch region of IGH or the *V, D*, or *J* gene. The primary translocations are caused by somatic hypermutation or errors in the V(D)J portion of the switch recombination region. The translocations include a promiscuous array of at least 20 nonrandom chromosomal partners.14q32 rearrangements are altered in 45% to 50% of MM patients. The characterization of these translocations has led to the identification of critical dysregulated oncogenes (e.g., *BCL2, cyclin D*). In each translocation, a potent enhancer is juxtaposed to dysregulated oncogenes. The five most frequent *IGH* translocations are t(4;14)(p16.3;q32.3), t(6;14)(p21;q32.3), t(11;14)(q13;q32.3), t(14;16)(q32.3;q23), and t(14;20)(q32.3;q12) (see Table 57.17).

A recurrent *CCND1-IGH* rearrangement is characterized by overexpression of cyclin D1. In contrast to mantle cell lymphoma (MCL), breakpoints on 11q13 with MM are not clustered but are scattered over a relatively large genomic region. Virtually all MM and MGUS cells have cyclin D dysregulation, suggesting that this abnormality is an early and unifying pathogenetic event. In contrast to other abnormalities involving the *IGH* locus, t(11;14) MM cells tend to be diploid. There appears to be an association of t(11;14) with an oligosecretory or light chain form of MM with CD20 expression, and a lymphoplasmacytic cell morphology. This chromosomal rearrangement is the most common genetic lesion in MM (15% to 20%) but it has also been observed in MGUS, primary plasma cell leukemia, and light chain amyloidosis. Two groups of patients with t(11;14) are recognized: one with a rather indolent course and the other associated with a more aggressive course.

Approximately 15% of patients have a recurrent t(4;14)(p16.3;q32.3) abnormality associated with aggressive disease. This cytogenetic abnormality is associated with IgAλ form of MM with immature cell morphology, and a poor response to therapy. This abnormality is cytogenetically cryptic and is detected by FISH. Two genes on chromosome 4p16.3 and the *IGH* switch region on 14q32.3 are involved. The fibroblast growth factor receptor 3 gene (*FGFR3*) is detected on der(14), where it is overexpressed along with a fusion of the MM set domain *NSD2* (*MMSET*) gene, which is located on 4p16.3. This is the first example of a translocation that simultaneously deregulates two genes with oncogenic potential: the *FGFR3* gene detected on der(14) and the NSD2 gene detected on der(4). *FGFR3* is 50 to 100 kb telomeric to NSD2. Loss of *FGFR3* on der(4) is detected in approximately 20% of cases. A significant number of these patients have del(1p32) and del(13q) within the same clone and are hypodiploid.

Translocation (14;16)(q32.3;q23) can only be detected with FISH studies. The incidence of t(14;16) has been estimated to be approximately 5% of cases. This translocation results in the relocation of the *MAF* proto-oncogene from its position on 16q23 to chromosome 14, band q32.3, and it is overexpressed. Survival of patients with t(14;16) is significantly shorter as compared with patients without t(14;16).

t(6;14)(p21;q32) is a rare translocation and present in approximately 1% to 2% of patients with MM. This rearrangement results in juxtaposition of *CCND3* to the *IGH* enhancer and causes direct upregulation of *CCND3* expression. The breakpoints are all located in the 3′ end of *CCND3*. The prognostic implication is neutral. Of the five recurrent translocations in MM the rarest translocation is t(14;20)(q32.3;q12)/*IGH-MAFB*, present in approximately 1%, and also results in the upregulation of *MAFB* gene. *MAFB* is also mutated in about 25% of patients harboring t(14;20). These MM patients have a poor prognosis, however, when t(14;20)(q32.3;q12)/*IGH-MAFB* is detected in the MGUS or in smoldering MM stage of disease, it is associated with long-term stable disease.

A large number of *secondary chromosomal aberrations* are found during disease progression. Disease progression is accompanied by four main abnormalities including translocations of *MYC*, the loss or deletion of chromosome 13, deletions of short arms and amplification of long arms of chromosome 1, as well as the deletion of short arms of

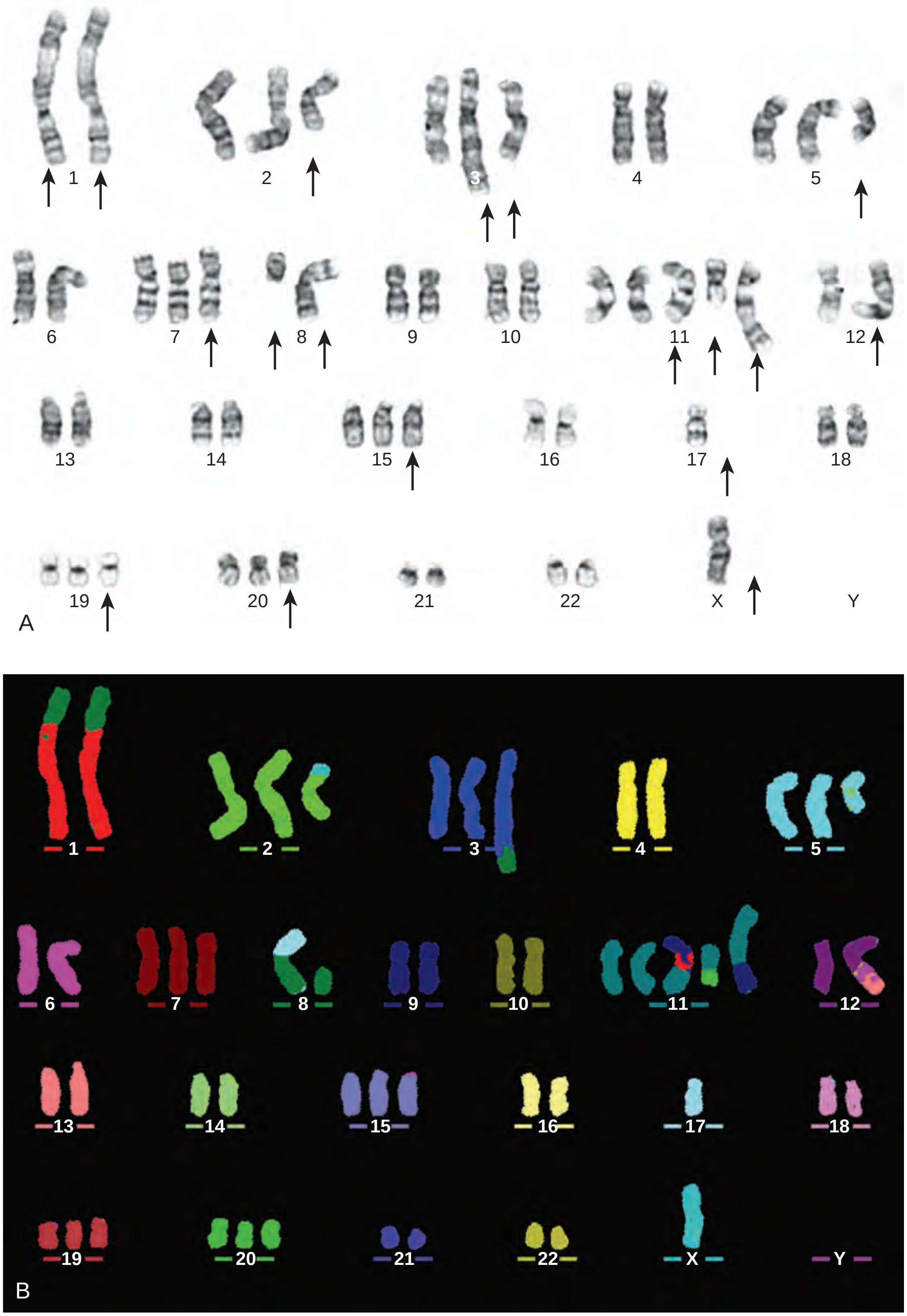


Figure 57.63 A complex hyperdiploid karyotype from a patient with multiple myeloma (A) and after multicolor FISH (B), which was used to resolve a number of complex derivative chromosomes: 54, X,−X, der(1)t(1;8)(q34;q21q24)x2, der(2) del(2)(p13p25)t(X;2)(p21p22.3;p13), t(3;8)(q27;q22q24), +i(5)(p13p15), +7, der(8;17)(p23;q11.2q25), +der(11)t(1;9;11(?p36;q21q31;p15), t(11;20)(q13;q11.2, der(11)t(9;11)(q13q34;q25), t(12;13)9(p13;q14q34), +15, +19, +20.

chromosome 17. Translocations and/or amplification of *MYC* (8q24) may involve up to 45% of patients with an advanced MM, and then cytogenetic studies frequently involve very complex nonreciprocal rearrangements, duplications, and amplifications. More recent data based on SNP-array technologies has revealed homozygous deletions such as *BIRC2/3* on chromosome 11, deletions of *TRAF3* on chromosome 14, and deletions of *CYLD* on chromosome 16. Another recurrent double deletion that has been observed in a limited number of the patients occurs on chromosome 1, targeting the tumor suppressor *CDKN2C* gene at 1q21 chromosomal site.

Deletion of chromosome 13 is detected in 10% to 20% of patients using conventional cytogenetics and in 50% using interphase FISH. The minimally deleted region on chromosome 13, between bands q14.11 to q14.3 contains over 68 genes including *RB1*, DNA marker

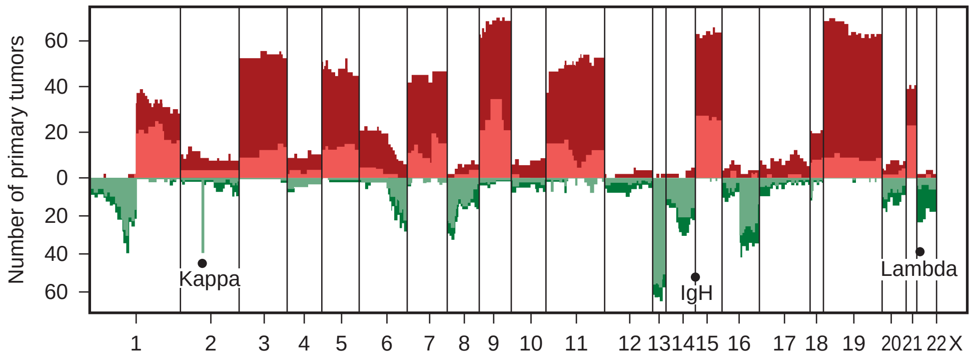


Figure 57.64 CYTOGENOMIC PROFILES IN MULTIPLE MYELOMA. Summary of genomic profiles and recurrence of chromosomal alterations in primary tumors demonstrated by array comparative genomic hybridization. The recurrence plot mirrors the frequencies of previously reported chromosomal gains and losses, including the deletions of 1p and amplifications of 1q. Integer-value recurrence of copy number aberrations across the samples in segmented data is plotted on the y-axis. The x-axis is in chromosomal order. *Dark red* or *green* bands denote the number of samples with gain or loss of chromosome material, and *bright red* or *green* bars represent the number of samples showing amplification or deletion. *Black dots* show focal deletions of the kappa (2p12), IgH (14q32), and lambda (22q11) loci physiologic in B-cell postgerminal center neoplasms. *(Reprinted with permission from Carasco DR, Tonon G, Huang Y, et al. High-resolution genomic profiles define distinct clinico-pathogenetic subgroups of multiple myeloma patients. Cancer Cell. 2006;4:313.)*

D13S319, and micro RNAs *mir16. -1* and *mir15*). Loss of the entire chromosome 13 (82%) is more frequent than is deletion of the long arms of chromosome 13. The median percentage of plasma cells carrying del(13q), as identified by FISH, ranges from 75% to 90%. Deletion of 13q is associated with specific clinical-pathologic features, including a higher frequency of λ-type MM, high plasma cell-labeling index, female predominance, and inferior survival after standard chemotherapy. t del(13q), however, no longer has adverse prognostic significance in patients treated with bortezomib. Molecular cytogenetics analyses have revealed that del(13q) is present in 90% of patients with t(4;14) or t(14;16); therefore the apparent adverse impact of del(13q) may be related to translocation events.

Deletion of *TP53* at 17p13.1, which occurs in 15% of patients with MM, is a powerful independent predictor of shortened survival and is associated with clonal evolution, drug resistance, and genetic instability This deletion is not found in patients with other high-risk abnormalities such as t(4;14) or t(14;16), and it appears to be mutually exclusive. Patients with *TP53* gene deletion have a significantly shorter OS regardless of therapy. The 17p deletion, identified by FISH, is considered the most important molecular cytogenetic factor when determining prognosis. In newly diagnosed MM patients del(17p) is observed in 8% of patients. Biallelic alteration of *TP53* (deletion and mutation, a.k.a. "double hit") is present in 3.7% of newly diagnosed MM and is associated with very poor outcome. Biallelic inactivation of *TP53* has been reported to occur more frequently at relapse, having been reported at 21% to 26% of patients. Another study provides the most compelling evidence that del(17p) is an important prognostic factor even in the absence of *TP53* mutation and should remain a high-risk feature in MM patients.

The gain of 1q21 locus has been identified in 45% of cases of MGUS, about a third of newly diagnosed MM, and 72% of relapsed MM, and represents one of the most frequent recurrent chromosomal abnormalities in MM (see Fig. 57.65). Chromosome 1 abnormalities include both short and long arms of chromosome 1, gains, deletions or jumping translocations. Gain of 1q is observed cytogenetically as an isochromosome, duplications, or jumping translocations and is detected with a 1q21-specific FISH probe. Among 479 patients with newly diagnosed MM, 43% with amp lq21 with either a hypodiploid and hyperdiploid karyotype and del(13q) was associated with poor prognosis as compared with patients lacking amp1q21 (see Fig. 57.65). In a study of 92 patients treated with lenalidomide and dexamethasone, del(17p) and gain of 1q21 was associated with a dismal OS. In the recent retrospective study of 3578 patients with MM 24% had documented chromosome 1 abnormalities or high-risk chromosomal abnormalities within 90 days of diagnosis. Median OS was lower for patients with chromosome 1 abnormalities and these abnormalities were independently associated with an inferior OS, and were older and had high-risk cytogenetic abnormalities despite the use of state-of-the-art therapies. Similar observations were obtained when 1376 patients were examined at diagnosis for the presence of gains of 1q only; FISH analysis confirmed that 28% of patients with +1q, along with other high-risk chromosome abnormalities as well as chromosome 13 abnormalities had decreased OS.[20]

In the most comprehensive expression profiling survey of patients with MM, reported by the Arkansas Multiple Myeloma Group using GEP70 (gene expression profiling of 70 genes), 30% of these genes were located on chromosome 1, with most of the downregulated genes located on the short arms of chromosome 1 and most of the upregulated genes on 1q. The mechanism for the amplification of 1q is believed to involve 1q12 pericentromeric instability, which most commonly increases the copy number of 1q by a direct and/or inverted duplication. Further instability can result in adding a whole-arm segment of 1q to nonhomologous chromosomes by jumping translocations of 1q. The 1q12–q23 amplicon has been reported to contain the 1q12 pericentromeric heterochromatin and the adjacent bands of 1q, resulting in an inverted repeat pattern of amplification of the 1q12–q23 region. Copies of the 1q12–q23 amplicon can become integrated into complex multichromosome translocations during tumor progression. In vitro studies have shown that treatment with hypomethylating agents apparently amplify any 1q region juxtaposed to 1q12 chromosome band producing copy number aberrations in the bone marrow of these patients. Frequent additional deletions detected by conventional cytogenetics include regions within 6q, 8p, 12p, 14q, 16q, or 20p.

The median number of mutations per MM genome is about 55 to 60, with a very large range (21 to 488). Moreover, in contrast to leukemia, "good-risk" cytogenomic abnormalities have not been described. The current data indicate that genetic abnormalities have a major role in determining a patient's prognosis. The current thinking is that development of subclones is a very early event in MM, probably occurring soon after the cell undergoes transformation.

Frequent mutations in MM include *KRAS* (particularly in previously treated patients), *NRAS*, *BRAF*, *FAM46C* (hyperdiploid subgroup), *TP53*, and *DIS3* (in nonhyperdiploid MM with *IGH* rearrangement, *TRAF3*, *CYLD*, *RB1*, and *PRDM1*. Subclonal *KRAS*, *NRAS*, and *BRAF* mutations are observed in about one-third of patients with MM. The most recent exome sequencing study identified germline variant in *DIS3* gene in 4 unrelated families with familial MM. The *DIS3* gene, located on chromosome 13, band q22.1, and as mentioned above, chromosome 13 is recurrently deleted and somatically mutated in MM. This is the first description of germline variant with potential involvement in MM etiology and extend

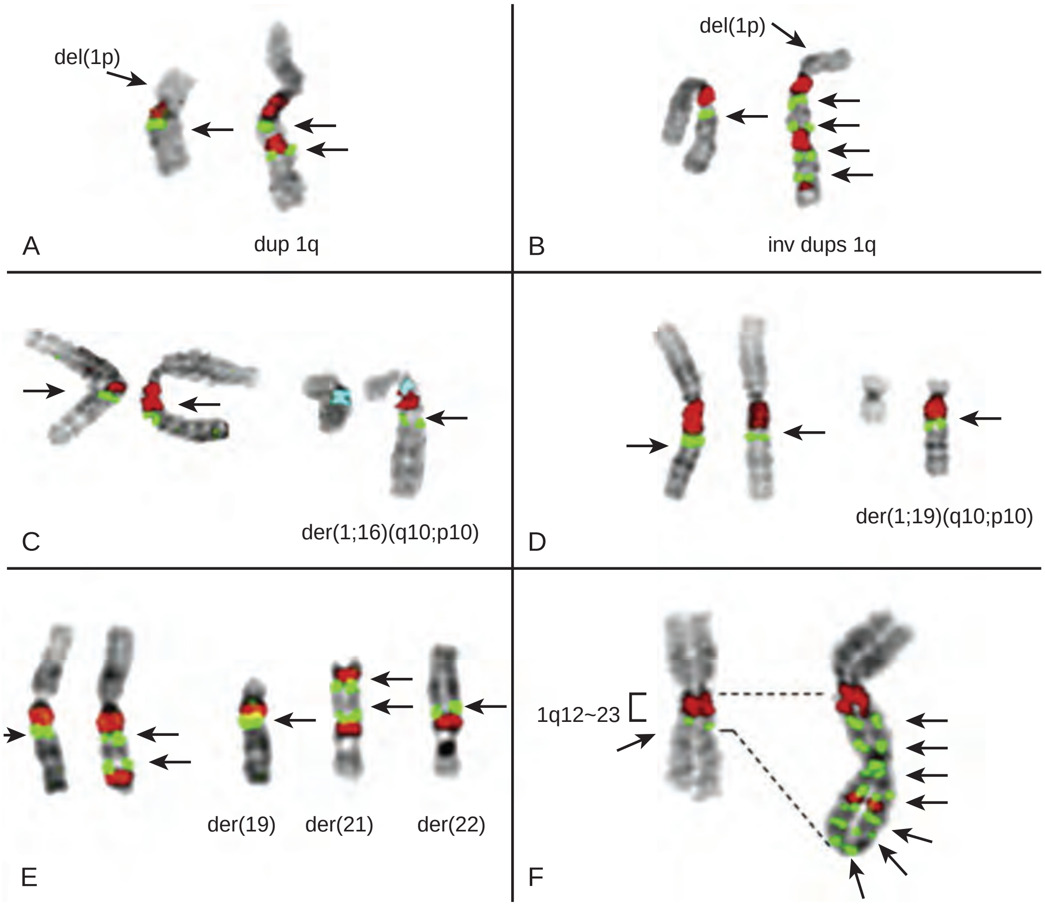


Figure 57.65 REPRESENTATIVE PARTIAL KARYOTYPES OF METAPHASE CHROMOSOMES DEMONSTRATING THE DIFFERENT TYPES AND DEGREE OF AMPLIFICATION OF CHROMOSOME 1. FISH probes for 1q12 *(red)*, 1q21 *(green)*, and 16q11 *(aqua)* are shown on inverted DAPI images of chromosomes. (A) Interstitial deletion of 1p *(arrow)* in the homolog on the left and a direct dup1q12–q23 on chromosome on the right. (B) Normal homolog 1 on the left and the abnormal homolog on the right, demonstrating both an interstitial deletion of 1p and the amplification of 1q in the same chromosome. Note four copies of 1q21 *(arrows)* in an inverted duplication pattern. (C) Examples of an unbalanced whole-arm translocation of 1q to chromosome 16q. Chromosomes 1 are on the left, and chromosomes 16 are on the right. Aqua probe denotes 16q11 heterochromatin. Note the loss of 16q distal to the aqua probe on the der(1;16)(q10;p10). The entire long arm 1q is translocated to the pericentromeric region of 16q, and a total of three copies of 1q21 *(arrows)* are present. (D) Examples of an unbalanced whole-arm translocation of 1q to 19q. Note that the result of this translocation is the der(1;19)(q10;p10) chromosome, which shows an extra copy of 1q21 *(arrows)* and loss of the entire 19q. (E) Examples of jumping 1q, in which all or a part of 1q is translocated to three copies of 1q *(arrows)* on the three different nonhomologous chromosomes. The whole-arm der(19)(q10;p10) in this case is the same type seen in patient in (D). The der(21) results from the segmental translocation of the inverted dup of 1q to the short arm of 21. The der(22) results from the whole-arm 1q translocated to the short arm of 22. (F) Homologous of chromosome 1 demonstrating amplification of 1q12–q23 by breakage-fusion-bridge cycles. Note multiple copies of 1q21 *(arrows)* on the abnormal homologue on the right. The copies of the 1q12–q23 amplicon occur in an inverted repeated pattern, with a deletion of the 1q distal to the amplified region. Dotted lines between normal homologue 1 *(left)* and abnormal homologue denote the size of expansion of the 1q12–q23 region by break-fusion-bridge cycles. *FISH*, fluorescence in situ hybridization. *(Reprinted from Sawyer JR. The prognostic significance of cytogenetics and molecular profiling in multiple myeloma. Cancer Genet. 2011;204:3, with permission.)*

beyond somatic alterations to germline susceptibility. The frequency reported is about 2.6% among four families with multiple cases of MM and MGUS.

Finally it's important to mention that until recently, classification of MM patients and risk stratification has relied on either biochemical parameters, cytogenetic markers, or gene expression analysis. Novel MM-PSN, the first Patient Similarity Network (PSN) of newly diagnosed patients has enabled the identification of 3 broad patient groups and 12 distinct sub-groups defined by 5 data types generated from genomic and transcriptomic patient profiling of 655 patients.[21] PSNs have been proposed as a potential powerful integrative approach to combine different omics data, to create a comprehensive view of a given disease and identify disease subtypes. The MM-PSN classification uncovered novel associations between distinct MM hallmarks with significant prognostic implications and allowed further refinement of risk stratification. Most important, MM-PSN confirmed that gain of 1q is the most important single lesion conferring high risk of relapse, and its association with an MMSET translocation as the most significant determinant of poor outcome. These findings suggest, as previously demonstrated in many studies, that gain of 1q should be incorporated in routine staging systems and risk assessment tools.

Combining novel single cell methodologies, such as sc RNA seq and sci CNV, a tool for inferring CNV from sc RNAseq allows dual profiling of DNA and RNA in a single cell. When MM cells with +1q were evaluated, using this dual profiling, it was recently uncovered that +1q cells in MM cause multiple effects on MM cells including a reduction in the unfolded protein response, enhanced oncogenic growth, oxidative phosphorylation, and *MCL* gene increased expression (anti-apoptotic gene located at 1q21.2). In contrast, there was no increase in *CKS1B* gene expression (located at 1q21.3) indicating that an alternative mechanism likely drives cell cycling in MM cells with a gain of 1q. Another gene, inflammation-responsive RNA editase gene adenosine deaminase acting on RNA1(*ADAR1*), also located at 1q21, has been recently implicated in the possible mechanism underlying 1q21 formation. ADAR1 is an RNA-editing enzyme

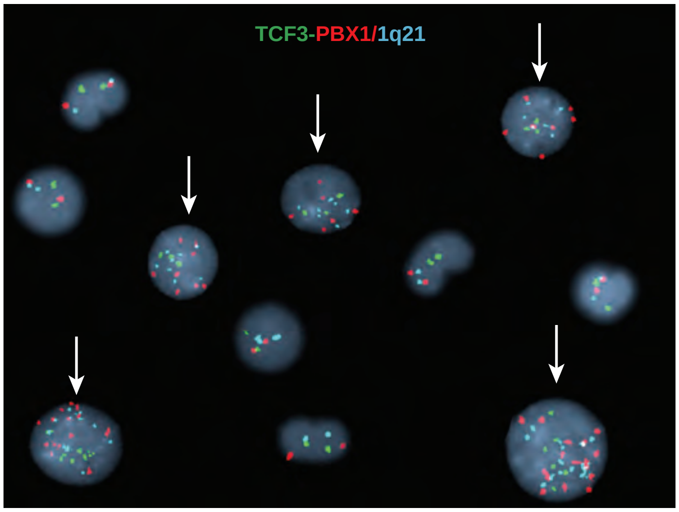


Figure 57.66 Bone marrow nuclei from a patient with multiple myeloma after hybridization using three probes: 1q21 *(aqua)*, PBX1 *(red)* localized on 1q25, and TCF3 *(green)* localized on 19q13. Note amplification (up to 17 copies) of 1q21 an2 1q25 *(white arrows)*, as well as multiple copies of 19q loci. Amplification of 1q21–q25 is associated with disease progression and very poor prognosis in multiple myeloma despite novel therapies.

TABLE 57.18 Risk Stratification of Myeloma

A. Standard Risk
  - Trisomies (hyperdiploidy)
  - t(11;14)
  - t(6;14)

B. Intermediate Risk
  - t(4;14)

C. High Risk
  - 17p deletion
  - t(14;16)
  - t(14;20)
  - High-risk gene expression profiling signature

that edits adenosine-to-inosine (A-to-I) nucleotides within double stranded RNA loops formed by primate-specific *Alu* repeats. ADAR1 has emerged as a vital contributor to transcriptomic diversity and is abundantly expressed in MM suggesting that MM cells with +1q transcriptome are in hyperediting state. In vitro and other functional studies suggests that *ADAR1* can enhance the growth and proliferation of MM cells in an editing-dependent manner and sometimes independently of 1q amplification status implying that a disturbed transcriptome mediated by *ADAR1* overexpression is both clinically and functionally crucial in myeloma. The interplay between the gain of 1q, IL6R (localized also on 1q21.3), and *ADAR1* provides yet another possible mechanism for the adverse outcome of patients with MM and 1q abnormalities.

LYMPHOMA

Non-Hodgkin lymphoma (NHL) is a heterogeneous group of disorders characterized by a localized proliferation of lymphocytes (see Chapters 78, 79, and 81). Analogous to other hematopoietic neoplasms, the pathogenesis of NHL is attributable to a multistep process involving progressive and clonal accumulation of genetic lesions (Chapter 81). The majority of NHLs are of B-cell origin and involve translocations of Ig loci (Table 57.19 and Fig. 57.67). *IGH* translocations usually are detected by cytogenetics, often in conjunction with FISH probes that span the *IGH* loci and/or PCR-based technologies. These molecular abnormalities exhibit multiple and complex translocations, deletions, and amplifications within one clone. Genetic anomalies represent one of the most reliable criteria for classification of malignant lymphomas. The most common associations between chromosome anomalies and specific lymphomas include t(14;18)(q32;q21) and FL (Chapter 82); t(8;14)(q24;q32) and BL (Chapter 85); t(11;14)(q13;q32) and MCL; and t(11;18)(q21;q21) and mucosa-associated lymphoid tissue (MALT) lymphoma (Chapter 83) (see box on Genetic Testing for Non-Hodgkin Lymphoma and Fig. 57.66). However, identification of a specific translocation is not diagnostic of a specific lymphoma subtype. Chromosomal studies are difficult and expensive for the study of lymphomas. Thus many investigators use FISH and/or molecular genetic methods to study touch-cell preparations or paraffin-embedded biopsy material to detect genetic anomalies.

GENETIC TESTING FOR NON-HODGKIN LYMPHOMA

FISH testing can be performed on touch preps from surgically excised nodes, paraffin-embedded tissue, or bone marrow cells if involved by the disease. Peripheral blood cells are not appropriate for genetic testing of NHL. Bone marrow cytogenetics at diagnosis is not mandatory but is important for staging in some cases. Fig. 57.67 lists the most useful FISH probes for detection of genomic defects in NHL. They include CCND1-IGH, IGH-BCL2, MYC-IGH, BCL6, MYC for detection of Burkitt variant not associated with t(8;14), and MALT1 for detection of t(11;18). For patients with suspected anaplastic large cell lymphoma, ALK probe should be informative.

Approximately 85% to 90% of patients with FL and some patients with DLBCL exhibit t(14;18)(q32.3;q21.3), resulting in fusion of *BCL2* on 18q21 and *IGH* on 14q32. This translocation is one of the most common chromosomal abnormalities in NHL (see Fig. 57.67D). This somatic rearrangement places *BCL2* gene under the influence of transcriptional enhancers associated with *IGH*, resulting in overexpression of the antiapoptotic BCL2 protein. Translocation 14;18 appears to originate from an erroneous *IGH* rearrangement, during B lymphopoiesis in the bone marrow (pre-B cells) and therefore represents a very early genomic event. About 75% of the *BCL2* breakpoints occur within a remarkably narrow region of 15 to 20 bp at the 3′ end of the *BCL2* gene, whereas the breakpoints in *IGH* fall within D_H and J_H regions. With routine FISH methods using *BCL2* breakpoint flanking probes, individual 5′ and 3′ breakpoints can be detected. With disease progression, 100% of patients have BCL2 protein overexpression. Variant translocations, such as t(2;18)(p12;q21) and t(18;22)(q21;q11) involving the *IGK* or *IGL* gene, respectively, rather than *IGH*, are also associated with overexpression of *BCL2*.

Although t(14;18) is most likely an early genetic event in the pathogenesis of FL, it is not sufficient for malignant transformation. Approximately 50% of healthy individuals harbor low levels of circulating t(14;18) cells but do not develop FL, indicating that ectopic *BCL2* expression is necessary but not sufficient for lymphomagenesis. Recent studies of 520,000 healthy participants enrolled in the European Prospective Investigation into Cancer and Nutrition study determined that approximately 20% of healthy individuals with t(14;18) will subsequently develop FL up to 20 years later. Progression to FL was significantly associated with elevated t(14;18) burdens in peripheral blood as measured by Q-PCR. They determined that individuals with t(14;18) frequency reaching one in every 10,000 blood cells had a 23-fold greater incidence of progression to FL.

Numerous secondary chromosomal abnormalities have been identified, and at least five recurrent anomalies, each occurring in at least 20% of FL, can be used to distinguish two subgroups of patients with

TABLE 57.19 **Recurrent Chromosomal Translocations in B-Cell Lymphoproliferative Disorders**

Translocations	Genes Involved	Associated Diseases
Immunoglobulin (Ig)-Related Translocations		
t(1;14)(p22;q32)	*CNN3-IGH*	B-cell ALL, NHL
t(1;14)(q21;q320)	*BCL9-IGH*	Pre–B-ALL
t(1;14)(q21;q32)	*MUC1-IGH*	Multiple myeloma
t(1;14)(q24;q32)	*LHX4-IGH*	
t(2;14)(p13;q32)	*BCL11A-IGH*	CLL/SLL, ALL, NHL
t(2;8)(p12;q32)	*IGK-cMYC*	ALL (Burkitt)
t(4;14)(p16.3;q32.3)	*FGFR3-IqH*	Multiple myeloma
t(5;14)(q31;q32)	*IL3-IGH*	B-CLL
t(6;14)(p25;q32)	*IRF-IGH*	Multiple myeloma
t(6;14)(p22;q32)	*ID4-IGH*	Plasma cell leukemia
t(6;14)(p21;q32)	*CCND3-IGH*	B-ALL
t(7;14)(q21;q32)	*IGH/-CDK6*	B-CLL
t(8;14)(q24;q32)	*IGH/-MYC*	ALL (Burkitt)
t(8;22)(q24;q11)	*IGL-MYC*	ALL (Burkitt)
t(10;14)(q24;q34)	*NFKB2-IGH*	T-cell ALL, CLL, NHL
t(11;14)(q13;q32.3)	*CCND1-IGH*	Multiple myeloma
t(11;14)(q23;q32)	*DDX6-IGH*	
t(11;14)(q23;q32)	*PAFAH1B2-IGH*	
t(11;14)(q23;q32)	*PCSK7-IGH*	
t(12;14)(p13;q32)	*ETV6-IGH*	Pre–B-ALL
t(14;14)((q11;q32)	*TCRA-IGH*	T-PLL
t(14;16)(q32.3;q23)	*IGH-MAF*	Multiple myeloma
t(14;19)(q32;p13)	*IGH-BCL-3*	B-CLL
t(14;20)(q32;q11)	*IGH-MAFb*	Multiple myeloma
t(14;20)(q32;q13)	*IGH-CEPBP B-ALL*	
Lymphoma-Associated Translocations		
t(1;14)(p22;q32)	*BCL10-IGH*	MALT
t(3;14)(q14;q32)	*FOXP1-IGH*	MALT
t(5;14)(q35;q32)	*ODZ2-IGH*	MALT
t(11;14)(q13;q32.3)	*CCNDI-IGH*	Mantle cell
t(14;18)(q32.3;q21)	*IGH-BCL2*	Follicular
t(3;14)(q27;q32)	*BCL6-IGH*	Follicular
t(11;18)(q21;q21)	*API2-MALTI*	MALT
t(14;18)(q32.3;q21)	*IGM-MALT*	MALT
t(1;14)(p22;q32.3)	*BCL10-IGH*	MALT
3q27 rearrangements	*BCL 6*	Diffuse large B cell
t(14;15)(q32.3;q11–13)	*IGH-BCL8*	Diffuse large B cell
t(3;14)(p14;q32.3)	*FOXPIF-IGH*	MALT
t(9;14)(p13;q32.3)	*PAX5-IGH*	LPL

ALL, Acute lymphoblastic leukemia; *B-ALL*, B-cell ALL; *B-CLL*, B-cell CLL; *CLL*, chronic lymphocytic leukemia; *LPL*, lymphoplasmacytoid lymphoma; *MALT*, mucosa-associated lymphoid tissue; *NHL*, non-Hodgkin lymphoma; *PLL*, prolymphocytic leukemia; *SLL*, small lymphocytic leukemia; *T-PLL*, T-cell PLL.

FL. Patients with t(14;18) and additional trisomy for chromosome 2, 7, or 8 are associated with a more favorable clinical course as compared with patients with del(1p), del(1q), del(6q), der(18), del(22q), or gain of chromosome 12 and X. Interstitial del(6)(q25–q27) is the strongest predictor of a poor prognosis and a shorter survival time. Rearrangements of chromosome 1, such as del(1)(p32–36), + 1(p11–q44), and unbalanced translocations of der(1)(1;1)(p36;q11–23) are among the most frequent secondary chromosomal abnormalities in FL. Progression of FL to DLBCL is accompanied by the accumulation of secondary abnormalities, including homozygous del(9p).

Mutations in the *BCL2* gene are found in 12% of patients with FL at diagnosis and in 53% at the time of progression/transformation. The presence of *BCL2* mutations at the time of diagnosis correlates with increased risk of transformation and an increased risk of death due to lymphoma (the median survival of patients with *BCL2* mutations is 9.5 years vs. 20.4 years without).

As mentioned earlier, the *IGH-BCL2* fusion represents a very early event in the pathogenesis of FL. FL-like cells (FLLC) reside in the germinal center of normal lymph nodes. In a study of 85 reactive lymph nodes in healthy individuals 14% were found to contain high levels of t(14;18) as detected by Q-PCR. These studies provide evidence that FLLCs consistently accumulate within the germinal center, as non-proliferating cells that have not acquired sufficient genetic events to result in transformation to FL.

GEP has identified a distinct pattern associated with an indolent form of FL (median survival 11.1 years) and a more aggressive form (median survival 3.9 years). Detailed genetic analyses have confirmed that a single mechanism driving the transformation of FL to DLBCL does not exist, rather several discrete events are operational, including alterations of cell-cycle control (mutations and deletions of cyclin-dependent kinase, *CDKN2A/B*), *MYC* rearrangements, and impairment of the DNA damage response (loss of *TP53* and/or *CDKN2A/B*). NGS has shown that each is associated typically with between 20 and 200 small somatic mutations. At least 21 different tumor suppressor genes are altered in FL at frequencies between 2% and 86%, suggesting that FL requires multiple collaborative genomic events acquired over many years. Mutations of chromatin modifying genes (*KMT2D*, *CREBBP*, and *EZH2*) are common features of FL. Normal T-cell function within the malignant microenvironment plays a role in the pathology of the disease and includes altered expression and upregulation of *PMCH*, *ETV1*, and *TNFRSF9* genes.

BL is associated with three translocations, all affecting the *MYC* gene at 8q24 (Chapter 85). In 80% of patients a reciprocal translocation t(8;14)(q24; q32) is observed between the *MYC* gene and the *IGH* locus (Figs. 57.68 and 57.69). In the remainder of patients, the reciprocal translocations t(8;22)(q24;q11) and t(2;8)(p12;q24) occur juxtaposing MYC to one of the light-chain loci (κ on 2p12 and λ on 22q11). The t(8;14) translocation was originally described in Epstein-Barr virus (EBV) tumor cells obtained from BL patients in Africa (see Fig. 57.67B–C). Variant translocations involving *MYC* with a variety of other non-*IG* loci subsequently have been reported. In most patients with sporadic BL, the breakpoints on 8q24.1 are located on 5′ end of the coding region of *MYC* gene. By contrast, in most cases of endemic BL and in cases with non-IG translocations, the *MYC* breakpoints are at the 3′ end. Due to the translocation, control of *MYC* is lost, and the intact protein is constitutively expressed throughout the cell cycle.

Although *MYC-IGH* translocations are the *sine qua non* of BL, they are not restricted to BL since they can also appear in DLBCL, plasma cell myeloma, ALL, MCL, and CLL.

MYC regulates almost 15% of human genes. The *MYC* gene can function as both a transcriptional activator and a transcriptional repressor. MYC forms a heterodimer with MYC associated factor X (MAX). MYC-MAX heterodimer binds to the E-box consensus sequence (CACATG) and serves as a platform for other proteins involved in chromatin remodeling and transcriptional regulation. *MYC* represses genes when it is not bound directly to DNA, but instead is recruited by the *MIZ1* transcription factor, which in turn recruits DNA methyltransferase (*DNMT3*) and histone deacetylase 3 (*HDAC3*) which alter chromatin making it less accessible.

An increased level of constitutive *MYC* synthesis is found not only due to a translocation but also as a result of mutations and amplification of *MYC* (see Fig. 57.68). Approximately 65% of BL are associated with *MYC* point mutations. *MYC* both directly and indirectly

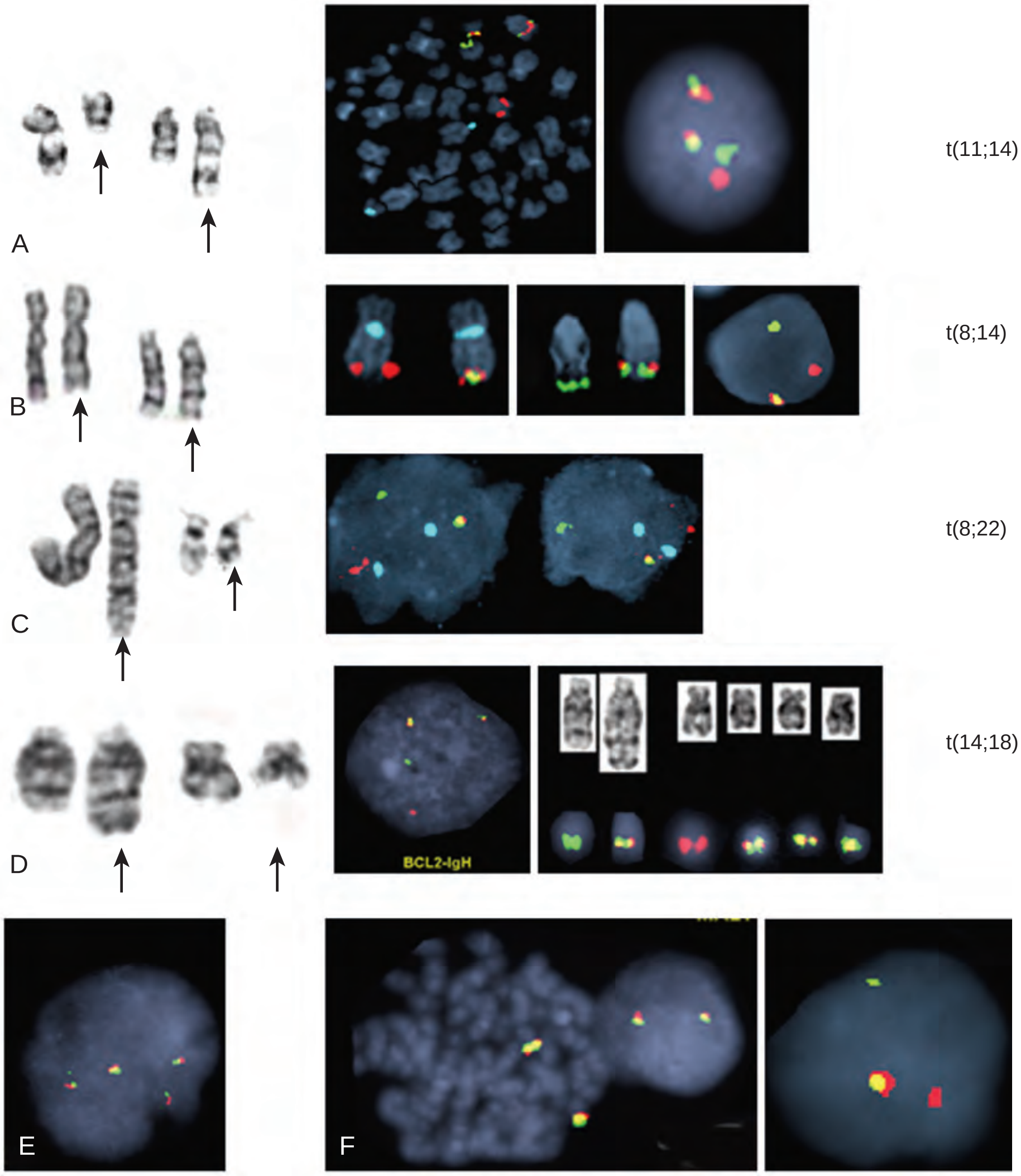


Figure 57.67 MOST FREQUENT CHROMOSOMAL ABNORMALITIES IN NON-HODGKIN LYMPHOMA. (A) Partial G-banded karyotype of t(11;14)(q13;q32.3) *(left)*, resulting in CCND1-IGH fusion *(yellow)* on metaphase *(middle)* and in interphase cell *(right)* present in the majority of patients with mantle cell lymphoma. (B) Partial G-banded karyotype of t(8;14)(q24;q32.3) *(left)*, resulting in *MYC-IGH* fusion [*yellow* on der(8) on isolated chromosomes]. Application of *MYC* breakapart probe shows separation of red and green signals consistent with *MYC* rearrangement. Approximately 80% of Burkitt lymphomas are characterized by t(8;14). (C) Partial G-banded karyotype of t(8;22)(q24;q11), a variant of Burkitt lymphoma *(left)*. Two interphase lymph node cells after hybridization with *MYC* breakapart probe and CEP8 *(aqua)*. Separation of green and red signals is consistent with *MYC* relocation from 8q24 to 22q11. (D) Partial G-banded karyotype of t(14;18)(q32.3;q21.3) *(left)*, resulting in IGH-BCL fusion (two yellow signals, *middle*). A composite image on the *right* shows both a partial karyotype and FISH study of triplicated der(18)t(14;18) and three copies of *IGH-BCL2* fusion *(yellow)*, as well as normal chromosome 14 and 18. Multiple copies of abnormal der(18) chromosome are associated with progressive disease similar to a duplication of the Philadelphia chromosome in the blast crisis of chronic myelogenous leukemia. (E) Lymph node cell from a patient with diffuse large B-cell lymphoma, showing four copies of *BCL6* (breakapart probe). *BCL6* is localized at 3q27 and is numerically or structurally rearranged in 35% of diffuse large B-cell lymphoma. (F) Bone marrow metaphase and interphase cell hybridized with breakapart *MALT1* gene at 18q21 *(left)* indicating two fusion *(yellow)* signals when *MALT1* gene is intact. In contrast, rearrangement of the *MALT1* gene in MALT lymphoma usually is the consequence of t(11;18)(q21;q21.1), as shown in the cell *(right)* with clear separation of the 3′ end *(green)* and the 5′ end *(red)*.

activates *CCND2* and cyclin-dependent kinases and downregulates cell cycle inhibitors, promoting the transition from G0 to S phase. MYC plays a role in the formation and maintenance of germinal centers.

The most common secondary change associated with t(8;14) in BL is duplication of the long arms of chromosome 1, which includes the *MDM4* locus, which is associated with disease progression. A common duplicated region of 93 kb at 1q21.2 has been observed using FISH. A rare complex BL karyotype is shown in Fig. 57.68. Based on the incidence of the *TP53* mutations (40%) and frequent gain of 1q in BL one of the mechanisms involved may include a widespread suppression of *TP53* activity to overcome p53-mediated cell cycle arrest and apoptosis caused by *MYC* overexpression. Rearrangements of *MYC* can now be detected in nondividing cells as well as in paraffin-embedded lymph node biopsies using a very sensitive interphase FISH assay for t(8;14).

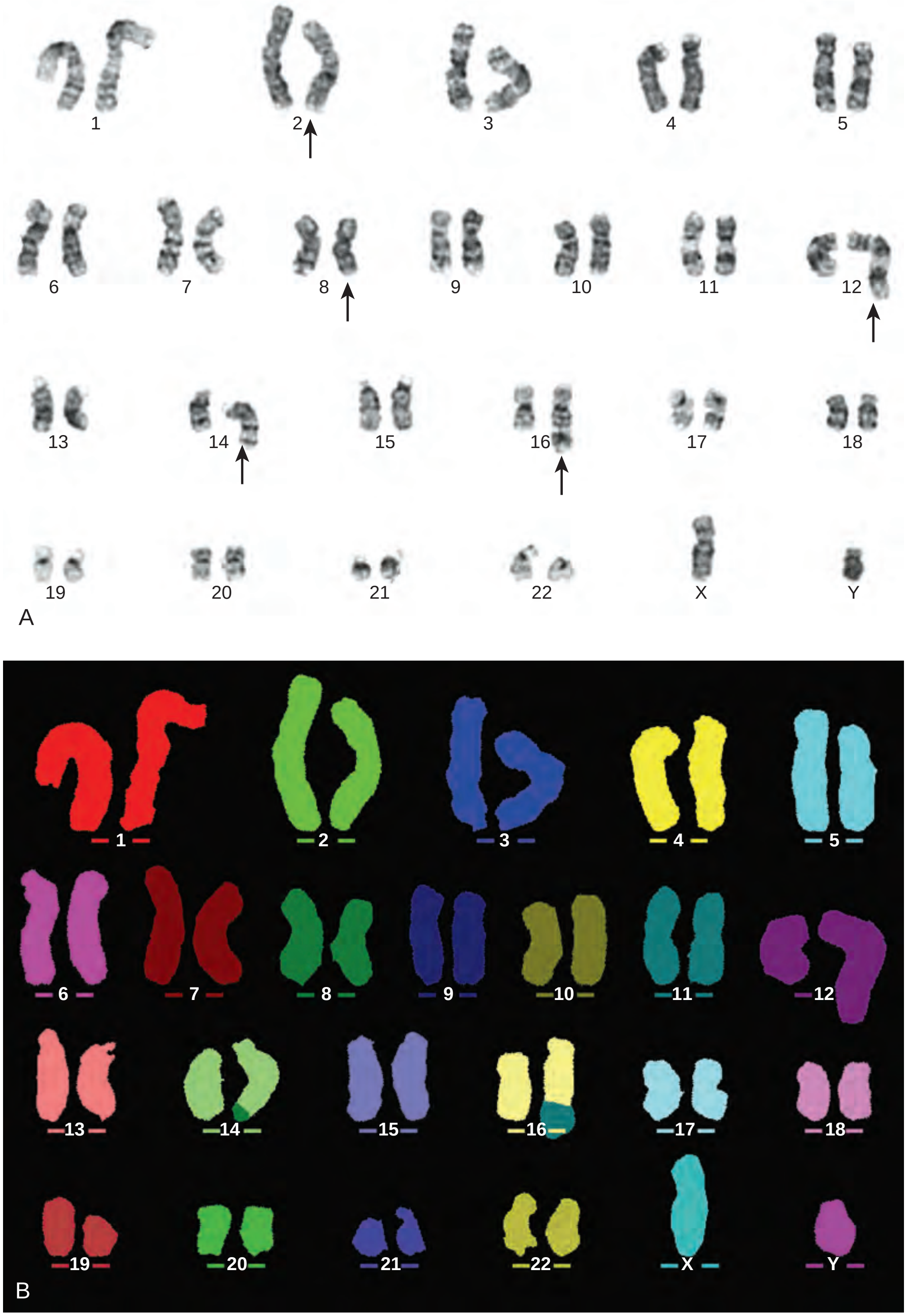


Figure 57.68 COMPLEX KARYOTYPE OF BURKITT LYMPHOMA. Bone marrow karyotype of a patient diagnosed in an advanced stage of Burkitt lymphoma (A) and after application of the multicolor FISH (B) to resolve complex derivative chromosomes, showing 46, XY, del(2)(p21p23), t(8;14)(q24;q32), dup(12)(q13q24.3), der(16)(t(11;16)(p11.2;q24) karyotype. *Arrows* indicate abnormal chromosomes. In addition to the classic t(8;14), both duplications of 11q and 12q were identified with multicolor FISH technology. *FISH*, fluorescence in situ hybridization.

According to the WHO classification, three BL variants are recognized: endemic, sporadic (1% to 2% of all lymphomas and 30% to 50% of childhood lymphoma in the USA), and BL with immunodeficiency disorders such as HIV infections (Chapter 152). In contrast, many cases of Burkitt-like lymphoma (a separate WHO entity) lack BL translocations but exhibit increased MYC expression.

Through an integrated analysis of whole genome and novel transcriptome data EBV-positive tumors have a striking genome-wide increase in aberrant somatic hypermutation supporting a link between the EBV and activation-induced cytidine deaminase (AICDA) activity.[22] High-throughput RNA sequencing has revealed acquired somatic mutations in the *CCND3* gene in 38% of sporadic BL cases.

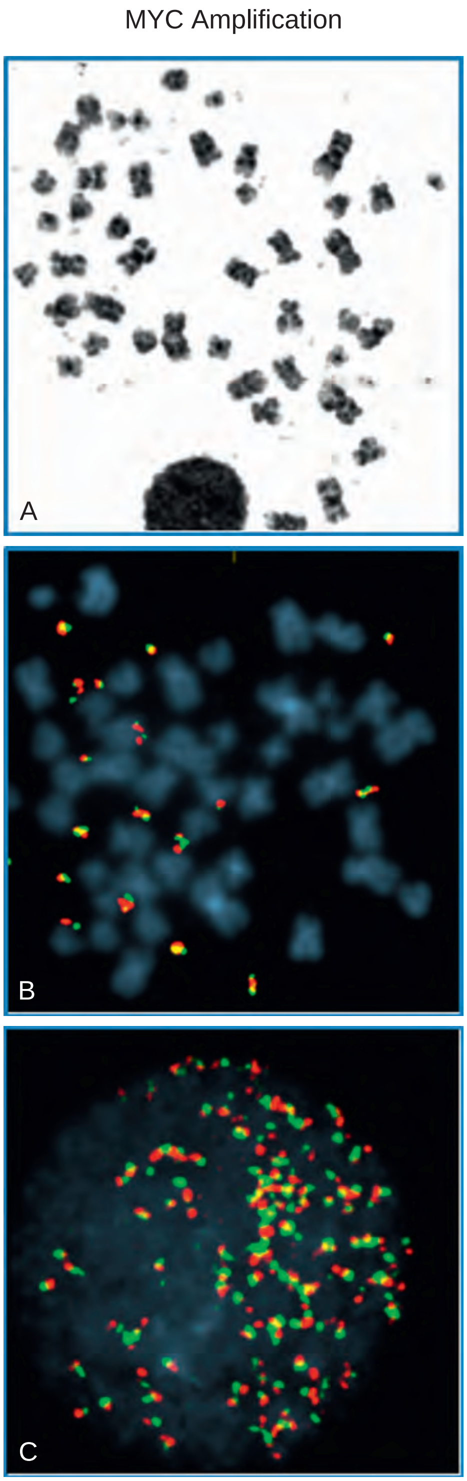


Figure 57.69 MYC AMPLIFICATION. (A) A conventional G-banded bone marrow metaphase from a patient with non-Hodgkin lymphoma. Note very pale doublets of chromatin material all over metaphase cells. They are termed double minutes. (B) All double minutes after hybridization with *MYC* breakapart probe were hybridized to *MYC* consistent with *MYC* amplification. (C) Interphase, nondividing bone marrow cell showing a very high number of *MYC* amplicon. Note that *MYC* gene is not rearranged, only amplified.

This is an important gene in cell cycle progression and 70% of these patients acquire mutations affecting the transcription factor *TCF3* or its negative regulator *ID3*. Mutations in the *ID3* gene have been detected in 50% of all *IGH-MYC* translocation-positive sporadic BL and these mutations were rare in other *IG-MYC*-positive lymphomas. These findings suggest that disruption of *ID3* function may be a key mechanism in the pathogenesis of BL. Moreover, recurrent mutations in *TP53*, *SMARCCA4* (chromatin modulating complex gene), and *DDX3X* (chromatin modulating complex gene) may contribute to BL evolution.

Approximately 35% of patients with DLBCL and approximately 5% to 10% of patients with FL have rearrangements in the *BCL6* gene which resides at 3q27. Translocation and dysregulation of *BCL6* gene represents a key mechanism of the development of DLBCL. Chromosomal rearrangements of the *BCL6* locus occur in about 35% of patients with DLBCL, and at least 33 different partners have been described (http://atlasgeneticsoncology.org/) (see Fig. 57.67). The most frequent chromosomal band partners are 2p13, 4p13, 6p22, 7p12, 8q24, 13q14, 14q32, 18p11.2, and 22q11. These translocations juxtapose the coding domain of *BCL6* downstream to heterologous promoters derived from different chromosomal partners, leading to dysregulated expression of an intact protein by preventing its downregulation during postgerminal center differentiation. The *BCL6* 5′ sequences are targeted by multiple point mutations in over 70% of cases. Most of the breakpoints at 3q27 occur within a 10-kb region. The fact that the 3q27 region is affected in different lymphomas, irrespective of the translocation partner chromosomes, strongly suggests that alterations of *BCL6*, and not the reciprocal loci, are important in lymphomagenesis. The alterations in 3q27 are small so most can be detected with PCR-based assays, or FISH. *BCL6* functions as a transcriptional repressor of genes containing its binding sites; therefore the mechanism responsible for the malignant phenotype is transcriptional deregulation.

The 2016 WHO classification for lymphoma included a new category termed “high-grade B-cell lymphoma” (HGBL) with translocations involving *MYC* and *BCL2* or *BCL6* comprising ~10% of cases of high-grade DLBC. However, a number of reports have appeared describing patients with DLBCL demonstrating, with FISH technology, to have a gain of copy numbers of *MYC*, *BCL2*, and *BCL6* with or without translocations of these genes. Overall, extra copies of these three genes were not associated with a poor outcome, however, there are reports indicating that extra copies of *MYC*, three or more copies, were significantly associated with an inferior prognosis. Moreover, co-occurrence of *MYC* dysregulation and *TP53* mutation indicates ultra-high risk and has an unfavorable impact on survival.

t(14;15)(q32;q11–13) occur in 3% to 4% of DLBCL, which results in fusion of the *BCL8* gene on chromosome 15 to the V_H segment of the *IGH* locus. The most common secondary abnormalities are trisomies of chromosomes 3, 5, 7q, 11, 12p, 18q, and Xq, which are observed in greater than 10% of cases. Amplification of *REL*, *MYC*, *BCL2*, *GLI*, *CDK4*, and *MDM2* genes are most frequently associated with advanced disease. The most frequent monosomies include chromosomes 13, 14, and 15. A complex karyotype may have an adverse impact on prognosis.

The use of modern molecular studies identified two major subgroups in DLBCL: activated B cell–like DLBCL (ABC-DLBCL) and germinal center B (GCB)–like DLBCL (Chapter 79 and 81). According to the WHO 2016 classification, these two subgroups of DLBCL, the GCB and ABC subgroups, differ in their chromosomal alterations, activation of signaling pathways, and clinical outcomes. Patients with ABC-DLBCL frequently exhibit trisomy of 3/+3q and 18q21–23, and loss of the 6q21–23 region. Patients with GCB-DLBCL have frequent gains of the 12q12 chromosomal region. Patients with primary mediastinal lymphoma have gains of 2p14–p16 and 9p21-pter. Apparently, gains in several regions of chromosome 3 are associated with inferior survival. Moreover, genomic gains involving the 3p11–p12 region have an independent impact on prognosis based on previously defined optimal gene expression–based models. Somatic point mutations of the polycomb-group oncogene *EZH2* have been reported in 22% of patients with GCB-DLBCL. The differentiation of GCB B cells into plasma cells requires *PRDM1*, a sequence-specific transcriptional repressor that is upregulated; 25% of ABC-DLBCL have deletions of *PRDM1* as a result of truncated mutations, or genomic deletions, while an additional fraction of patients lack the PRDM1 protein due to transcriptional repression by a constitutively active translocated *BCL6* gene.[23]

Patients with non-BL may have *MYC* translocations without additional mutations in *BCL2* or *BCL6* and they are described as having single hit lymphomas. Although the most recent studies suggested that patients with *MYC*+, but *BCL2*− and *BCL6*− *DLBVL* do not have an inferior outcome when compared to those who are *MYC* negative, the clinical relevance of *MYC* rearrangements as a sole abnormality in DLBCL remains controversial with some studies indicating an adverse prognosis.

Double hit lymphoma (DHL) refers to B-cell lymphoma with multiple activating oncogenes, one of them being *MYC*. Among DHL, those with alterations in *MYC* and *BCL2* are the most common by far, occurring in 87% of patients, followed by *MYC* and *BCL6* rearrangements, found in 5% of patients. Triple hit lymphoma (THL) involves rearrangements of *MYC*, *BCL6*, and *BCL2* in a single cell and is observed in about 8% of patients with DLBCL (Fig. 57.70).

DHL can be detected cytogenetically but cryptic gene rearrangements may be missed. FISH therefore should be used for the detection of DHL and THL. FISH studies can be successfully performed using formalin-fixed paraffin embedded tissue sections as well as fresh smears or touch-cell imprints of an involved tissue.

Because one of the translocations may be cryptic, a combination of three FISH probes, labeled with three colors, is especially useful in identifying these very complex translocations (see Fig. 57.70). Non-*IG* genes also act as *MYC* translocation partners in 35% to 53% of *MYC* rearranged DLBCL. The t(8;9)(q24;p13), which results in the juxtaposition of *MYC* to *PAX5*, has been frequently reported, accounting for 20% of non-*IG*/*MYC* rearrangement. Both the DHL and THL have complex karyotypes. Complex karyotypes are not seen in BL, which by definition always has a single abnormality.

The detection of increased number of cells expressing *MYC* correlates well with the presence of gene rearrangements, but the relationship varies. Tumors with more than 70% of *MYC*-positive cells usually are associated with gene rearrangements, but *MYC* translocations can be present in up to 17% of cases with less than 30% *MYC*-positive cells. The current recommendation is that all patients with DLBCL have FISH for *MYC* rearrangements because combined alterations in *MYC*, *BCL2*, and *BCL6* confers a more aggressive disease phenotype. Targeted sequencing of 928 patients with DLBCL, using a 293-gene panel, by Haematological Malignancy Research Network, reported in 2020 five molecular subtypes: *MYD 88*, *BCL2*, *SOCKS1/2*, *TET2/SGK1*, and *NOTCH2* along with an unclassifiable group. The subtypes characterized by genetic alterations of *BCL2*, *NOTCH2*, and *MYD88* indicated a good, intermediate, and poor prognosis, respectively. The *SOCK1/SGK1* subtype showed biological overlap with primary mediastinal B-cell lymphoma. Although not identified as a distinct cluster, a mutation *of NOTCH2* was associated

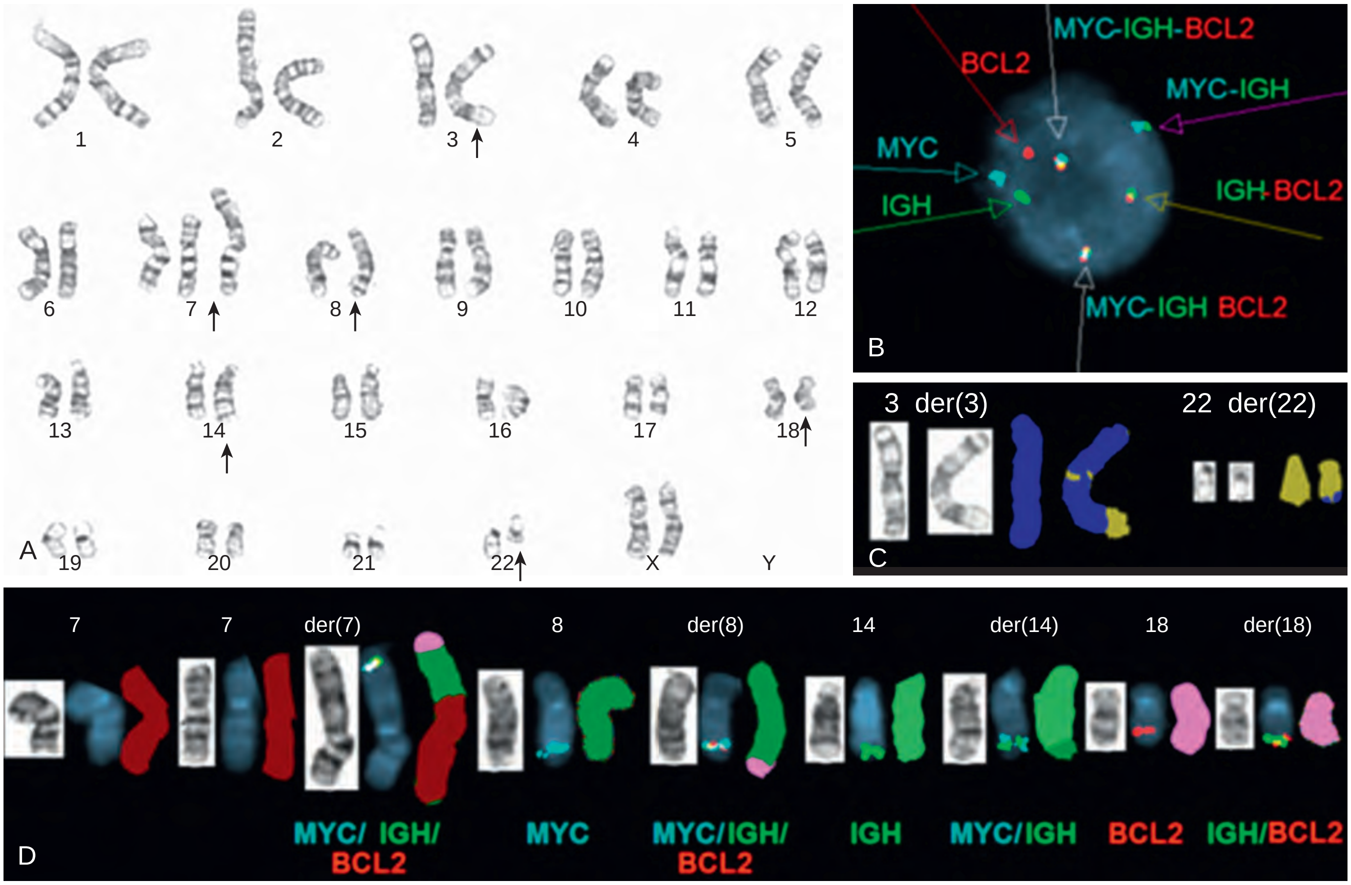


Figure 57.70 TRIPLE HIT LYMPHOMA. (A) A karyotype of patient with triple hit lymphoma with *arrows* pointing to the abnormal chromosomes. Note a gain of der(7;8) resulting in three copies of 7q and three copies of rearranged 8q, and the three-way translocation between chromosomes 8, 14, and 18 as well as a translocation (3;22). (B) A bone marrow nucleus showing normal *MYC (aqua)*, *IGH (green)*, and *BCL2 (red)* as well as two copies of *MYC-IGH-BCL2* fusion, *MYC-IGH*, and *IGH-BCL2* fusion. (C) A partial karyotype after multicolor metaphase FISH study, using chromosome paining probes, showing a t(3;22) resulting in *BCL6* rearrangement. (D) A partial karyotype of chromosomes 7, 8, 14, and 18. G-banded chromosomes are on the left, DAPI-stained chromosomes with *MYC (aqua)*, *IGH (green)*, and *BCL2* are in the middle, and multicolor stained chromosomes are on the right. Note der(7) is composed of 7q *(brown)*, gain of 8q *(dark green)*, and the third copy of *MYC-IGH-BCL2* fusion is on the tip of the 8q. The patients had three copies of *IGH2-BCL2* although cytogenetically this was manifested as a gain of 8q. *FISH*, Fluorescence in situ hybridization. *(Reprinted with permission from McFarland DC, Brody J, Tripodi J, et al. Two cases of triple hit lymphoma: a call for imperative MYC, BCL2 and BCL6 testing by FISH in aggressive lymphomas. Personal Med Oncol. 2015; 4:16–22. Copyright 2015. Green Hill Healthcare, LLC.)*

with a poor prognosis. The impact of *TP53* mutations varied with genomic subtypes conferring no effect on prognosis in the NOTCH2 subgroup and an inferior prognosis in the MYD88 subtype.

All patients with MCL exhibit t(11;14)(q13;q32) abnormality as detected by FISH (see Fig. 57.67, *top panel*) (Chapter 86). t(11;14)(q13;q32) is also found in a variety of other B-cell malignancies, including MM, splenic lymphoma with villous lymphocytes, and B-cell prolymphocytic leukemia. The WHO recognizes two molecular subtypes that differ in their clinical and biological features. The most common MCL is derived from naïve-like mature B cell, expresses the oncogenic transcription factor *SOX11*, and accumulates large numbers of genomic alterations. In contrast, the other subtype, leukemic, non-nodal MCL originates from memory-like B cells, is *SOX11*-negative, and is genetically stable.

Cyclin D1 overexpression is the hallmark of MCL and it is associated with t(11;14). Most breakpoints on chromosome 11, band q13, occur over a region of approximately 130 kb centromeric to the *cyclin D1* (*CCND1*) gene. At the molecular level, the *BCL1* locus (*CCND1*) on chromosome 11q13 is juxtaposed to an enhancer sequence within the *IGH* gene on 14q32, leading to overexpression of the *cyclin D* gene (involved in cell cycle control), which is not expressed in normal B and T cells or in other malignant lymphomas.

Cyclin D1 activates cyclin-dependent kinases 4 and 6 which in turn phosphorylate and inactivate *RB1* and promote the transition from the G1 to the S phase, resulting in rapid cell proliferation. *SOX11* overexpression is observed in many patients with MCL which leads to many events, including blocking B-cell maturation to plasma cells.

Because the breakpoints within 11q13 are scattered along a 130-kb distance, dual-color FISH has proved to be the most sensitive assay for detection of *IGH-CCND1* fusion in MCL. Furthermore, the *CCND1* fusion rearrangement can also be detected in formalin-fixed, paraffin-embedded samples, making this a rapid and reliable method (see Fig. 57.67).

The secondary chromosomal alterations include gains of 3q, 8q, and 15q and losses of 1p, 8p23-pter, 9p21-pter, 11q21–23, and 13q. Some of the genes residing in these chromosomal regions are dysregulated and are involved in cell proliferation, DNA repair, cellular homeostasis, and apoptosis. In MCL, DNA amplification of several chromosomal regions appears to be associated with a blastoid variant. Loss of 9p21-pter, inactivation of *TP53*, gain of 3q, and high cyclin D expression are biomarkers of shorter survival and a more aggressive clinical phenotype. Common MCL is enriched for *ATM* mutations while non nodal MCL is slightly enriched for *TP53* and *TERT* alterations. *ATM* mutations are present in 64% of cases with the common subtype of MCL and represents an early event in pathogenesis with some suggestions that it may even occur in the germline of some patients. Although a number of other somatic mutations were described in both groups of MCL, such as *CDKN1B*, *SAMDHD1*, *BCOR*, *SYNE1*, *SMARCB1*, *CCND1* only *TP53*, and *MYC* alterations, provide MCL cells a higher proliferative capacity and have independent prognostic value.

Two types of aggressive histology of MCL, according to the WHO, can be differentiated as variants of MCL, blastoid and pleomorphic. They may arise de novo or transform from a prior classic MCL. The first and the largest genomic profile of these two variants of MCL included 183 patients with aggressive histology and in general, they had inferior outcomes when compared to patients with classic MCL, and the pleomorphic subtype had a trend for a worse OS compared to patients with blastoid subtype.[24] WES analysis demonstrated that a *CCND1* gene mutation was enriched in both aggressive subtypes and there were predominance of *NOTHC1/2*, *NSD2*, and *KMT2A* gene mutations most likely related to the previous therapy these patients had received before disease transformation.

Extranodal marginal zone lymphoma and MALT lymphoma (Chapter 83) are considered the third most frequent subtypes of NHL. An etiologic link between low-grade gastric MALT lymphoma and a lymphoid reaction associated with *Helicobacter pylori* infection has been well established. Growing evidence suggests that chronic antigenic stimulation caused by autoimmune diseases, such as Hashimoto thyroiditis, also contributes to an increased risk for developing MALT lymphomas.

The most frequent and specific aberration occurring in MALT lymphomas is t(11;18)(q21;q21.1) (see Fig. 57.67F). Although this abnormality has been described in other B-cell lymphomas, t(11;18) in MALT lymphoma it is usually the sole lesion. It is the only recurrent translocation that does not involve *IG* genes, even though it presents in a B-cell lymphoma. As a consequence of t(11;18), *API2* gene on chromosome 11, band q21, which encodes an inhibitor of apoptosis, and a novel gene *MALT1* on chromosome 18, band q21, characterized by several Ig-like C2-type domains, are often rearranged. The resultant chimeric transcript consists of *5′-API2 and 3′-MALT* located on der(18). More than 90% of breakpoints in the *API2* locus occur in intron 7, whereas the breakpoints within *MALT1* are variable and occur in four different introns. The *API2-MALT1* fusion can be identified using a dual-color *API2-MALT1* FISH probe or the breakapart dual-color *MALT1* probe on lymph node biopsy specimens (see Fig. 57.67F). However, detection of deletions and duplications occurring at high frequencies in both the *API2* and *MALT1* genomic sequences require more precise molecular cytogenetic methods.

t(1;14)(p22;q32) and its variant t(1;2)(p22;q12) occur in less than 5% of MALT lymphomas, and are associated with an advanced disease. This translocation relocates the entire *BCL10* gene from 1p22 to chromosome 14, bringing it under the control of an *IGH* enhancer. Currently, a specific probe for detecting *BCL10-IGH* fusion is not commercially available, but an *IGH* dual-color breakapart FISH probe may determine *IGH* rearrangements without identifying the partner chromosome. The recurrent t(3;14)(p13;q32) abnormality, which results in fusion of the *FOXP1F* gene on chromosome 3 to *IGH*, is a rare rearrangement that causes *FOXP1F* overexpression. Its significance in lymphoma remains unknown. The t(14;18)(q32;q21), which occurs in 2% to 18% of all MALT lymphomas, results in fusion of *IGH-MALT*. The 18q21 breakpoint involving the *MALT1* gene is 5 Mb centromeric from the *BCL2* breakpoint on chromosome 18, which is associated with FL. In contrast to *API2-MALT1* cases, patients with an *IGH-MALT1* fusion gene have disease outside the gastrointestinal tract, usually presenting with ocular, skin, liver, or salivary gland tumors. Trisomy of chromosomes 3, 7, 12, and 18 are recurrent numerical changes associated with MALT lymphomas. FISH studies are useful for detecting the *IGH-MALT* fusion in paraffin-embedded lymph node biopsies. Compelling evidence links these translocations to constitutive activation of the NF-κB pathway. In those MALT lymphomas lacking the aforementioned aberrations, inactivating mutations of *TNFAIP3* (tumor necrosis factor alpha-induced protein 3) are frequent. Remarkably, the majority of mutagenic events in MALT lymphomas, at least two of the three trisomies, three of the translocations and *TNFAIP3* inactivation, all result in activation of the NF-κB pathway.

Splenic marginal zone lymphoma (SMZL) (Chapter 83) lacks recurrent chromosome translocations although approximately 30% of patients have heterozygous 7q deletions. Studies utilizing whole-exome sequencing have identified novel and recurrent inactivating mutations in Kruppel-like factor 2 (*KLF2*) in 42% of patients. These mutations are rarely observed in other B-cell lymphomas. Different *KLF2* mutations compromise the ability of *KLF2* gene to suppress *NF-κB* activation, leading to an altered gene expression pattern favoring B-cell homing to the marginal zone. *KLF2* inactivation alone is insufficient for malignant transformation and requires cooperating genetic events. Other common mutations found in SMZL include *NOTCH2*, *TRAF3*, *TNFAIP3*, and *CARD11*. These mutations with and without *KLF2* mutations indicate that *KLF2* mutation identifies a subset of patients with SMZL with distinct genotype. NGS has demonstrated that 40% of patients with SMZL have mutations in fours genes: *TP53*, *KLF2*, *NOTCH2*, and *TRAF3*, of which *TP53* mutations were associated with a shorter survival whereas mutations in *MYB* gene were predictors of a longer OS.

Lymphoplasmacytoid lymphoma (LPL) (Chapter 92) is a small lymphocytic lymphoma with plasmacytoid differentiation (CD5− CD10−) characterized by t(9;14)(p13;q32) in approximately 50% of cases. As a result of this translocation, the paired homeobox 5 (*PAX5*) gene on 9p13 moves to the *IGH* locus on der(14), causing dysregulation of *PAX5*. Molecular characterization of t(9;14) has revealed that the coding region of the *PAX5* gene remains intact in some patients. t(9;14) should be considered a regulatory alteration in which the *PAX5* gene is brought under the control of the *IGH* locus. In other cases, molecular studies of t(9;14) have revealed that the breakpoint occurs upstream of the *PAX5* promoter, leading to insertion of the *IGH* enhancer upstream of the *PAX5* gene. Recurrent mutations in *MYD88*, specifically L265P mutation, have been identified in over 96% of patients with LPL whereas mutations in chemokine receptor, *CXCR4*, have been observed in about 36% of cases. These mutations identify a subgroup of LPL with aggressive disease.

Waldenström macroglobulinemia (WM) (see Chapter 92) is characterized by a lymphoplasmacytic clonal expansion in the marrow. Historically recurrent chromosomal abnormalities including deletion of the long arms of chromosome 6 in 21% to 55% of cases and trisomy 4 in approximately 20% of cases have been reported. In contrast to other B-cell disorders, abnormalities of *IGH* at 14q32 as detected by FISH are rarely observed. WGS of WM lymphoplasmacytic cells has led to the identification of a recurring sequence variant at position 38182641 in chromosome 3p22.2. A single-nucleotide change from T to C in the *MYD88* gene results in a leucine-to-proline change at amino acid position 265. *MYD88* (3p22 locus) mutations can be detected by PCR in the peripheral blood of patients at presentation. The *MYB88* L265P mutation is highly specific and is the most frequent mutation in WM. It is found in over 91% of cases, including those with a gain of chromosome 3 (approximately 12%). This mutation may be considered as the first detectable genetic hit in WM that promotes NF-κB and JAK-STAT signaling pathways. *MYD88* L265P mutation is also seen in SMZL and MALT lymphoma. *MYD88* L265P cannot be used to differentiate between WM and IgM MGUS. Patients with mutated *MYD88* have a shorter survival than patients with unmutated *MYD88*. The *CXCR4* (2q22.1 locus), one of the main regulators of B-cell homing, is mutated in almost 30% of patients with WM and 20% of patients with IgM MGUS. Collectively these results indicate that *CXCR4* is an activating mutation in WM and has a critical role in WM pathogenesis.

Until recently, little progress has been made in delineating recurrent chromosomal abnormalities in Hodgkin lymphoma (HL) (see Chapters 78–80) because of the rarity of Reed-Sternberg cells (less than 1% of the cells in HL are Reed-Sternberg cells-HRS) in the involved lymph nodes. Until about 5 years ago, the most specific chromosomal abnormalities in HL were hyperdiploidy/tetraploidy with tremendous variations in chromosome number, indicating heterogeneity from patient to patient. Even with use of nine different centromeric probes, a specific numerical chromosomal abnormality was not identified. Deletions of 1p, 4q, 6q, and 7q were recurrent, and *JAK2*, located on 9p21, frequently was found amplified in patients with HL (see Table 57.19). Another gene, *REL*, on chromosome 2, band region p14–p15, was amplified in ~70% of patients. Of note, *REL* is under the influence of NF-κB and constitutive NF-κB activation is a critical prerequisite for Reed-Sternberg cell survival and proliferation. Progress in microdissection, whole genome amplification (WGA), and FISH led to clarifying the genetic origins of HRS cells. Laser microdissection of HRS cells ($n = 1200$ to 1800 cells per case) from 34 biopsies revealed that ~90% of patients had a variety of JAK-STAT pathway gene alterations (Tables 57.20 and 57.21). These include *JAK2* copy gains mutational disruption of the *SOCS1* and *PTPN1* genes as well as *JAK2* activating mutations along with recurrent mutations of STAT6 (32% of cases). Another study using full-exome deep sequencing of HRS cells in 10 patients revealed inactivating mutations in β2-microglobulin gene (*B2M*, localized at 15q21.1) in 7 of 10 patients. These mutations lead to the loss of major histocompatibility complex class I (*MHC1*) expression. A lack of *MHC1* expression in HRS cells was previously reported as an independent adverse prognostic factor. Therefore one of the molecular mechanisms leading to MHC1 downregulation in HL is through inactivating mutations in *B2M* gene.

TABLE 57.20 Currently Known Recurrent Copy Number Alterations in Classic Hodgkin Lymphoma[a]

Location	Frequency	Affected Genes, Pathways, and Biological Processes
2p-Gain	28%–54%	
4q-Loss	7%–25%	*REL* (NF-KB pathway)
6q-Loss	5%–30%	
9p-Gain	24%–40%	*JAK2* (JAK/STAT pathway), *PD-L1, PD-L2* (immune evasion)
11q-Loss	3%–25%	
12q-Gain	37%–40%	*MDM2* (cell cycle, apoptosis)
13q-Loss	22%–35%	
14q-Gain	7%–30%	
16p-Gain	24%–30%	*ABCC1* (multidrug resistance)
17p-Gain	25%–40%	
17q-Gain	20%–70%	*MAP3K14* (NF-KB pathway)
19q-Gain	25%	
20q-Gain	15%–23 15%	*CD40* (NF-KB pathway)

[a] Locations, frequencies in three major studies and possibly affected genes and pathways are given in the respective columns.

Modified from Borchmann S, Engert A. The genetics of Hodgkin lymphoma: an overview and clinical implications. *Curr Opin Oncol.* 2017;29(5):307.

TABLE 57.21 Reported Frequencies of Genetic Alterations in Major Hallmarks of Classic HD Pathology

Pathway/Gene	Genomic Alteration	Frequency (%)
NF-KB		
NFKBIA	SNV	25–40
NFKBIE	SNV	17–27
REL	CNV gain/amp	28–70
MAP3K14	CNV gain/amp	25
TNFAIP3	SNV	40–45
	CNV loss	21–60
BCL3	CNV gain/amp	15–70
	SR	10
JAK-STAT		
JAK2	CNV gain/amp	35–40
SOCS1	SNV	41
PTPN1	SNV	20
STAT6	SNV	31–45
Immune Escape		
CIITA	SR	15
CD274/PDC1LG2	CNV gain/amp	38–75
B2M	SNV	21–70
CD58	CNV loss	23
TNFRSF14	CNV loss	13–42

amp, Amplification; *CNV*, copy number variation; *SNV*, single-nucleotide variants (including small deletions); *SR*, structural rearrangement.

Modified from Mottok A, Steidl C. Biology of classical Hodgkin lymphoma: implications for prognosis and novel therapies. *Blood.* 2018;131(15):1654.

Blastic Plasmacytoid Dendritic Cell Neoplasms (Chapter 64)

BPNDCN is a rare and clinically aggressive hematopoietic malignancy derived from precursor plasmacytoid dendritic cells. In 2016, for the first time, WHO classified this malignancy as a separate entity. Most patients present with cutaneous lesions followed by systemic dissemination involving lymph nodes, bone marrow, and rarely extranodal sites. An international survey of 398 adult patients with BPDCN indicated the median survival to be 18 months.[25] Cytogenetics was available for 96 patients and an abnormal karyotype was detected in 54%. Major chromosomal abnormalities associated with BPDCN include 5q (70%), 12p (63%), 13q (60%), 6q (52%), and 15q (39%), as well as monosomy 9 (20%). Monosomal and/or complex karyotypes were observed in 36% of patients, and 86% of cases with disseminated disease had this pattern. Most frequent loss of chromosomes include chromosome 13 (38%); and 19% of patients demonstrated loss of chromosomes 5, 7, 8, 9, 15, and 17. Only 15 patients were screened with FISH technology and *ETV* rearrangements were detected in 20%.

HAIRY CELL LEUKEMIA (CHAPTER 77)

Recurrent chromosomal aberrations have not been identified in hairy cell leukemia (HCL) although as shown in Fig. 57.71 some patients may have a complex bone marrow karyotype at diagnosis (Chapter 77). HCL is a mature B-cell malignancy with unique immunophenotypic features. High-density genome-wide SNP genotyping has shown a remarkably balanced genomic profile. In 2011, a *BRAF*-V600E mutation was described for the first time in every one of 47 patients. The *BRAF*-V600E mutation defines HCL, is present in virtually all patients with HCL, and is absent in other B-cell malignancies except a small number of patients with MM. BRAF is a kinase within the RAS-RAF-MEK-ERK pathway. Quantitative sequence analysis revealed a mean *BRAF*-V600E-mutant allele frequency of 4.9% in human hematopoietic stem cells from patients with HCL and functional studies showed that these cells have self-renewal capacity, indicating that this mature B-cell malignancy originates within the hematopoietic stem cell compartment. Detection of the *BRAF*-V600E mutation by PCR is the gold standard for the genetic diagnosis of HCL.

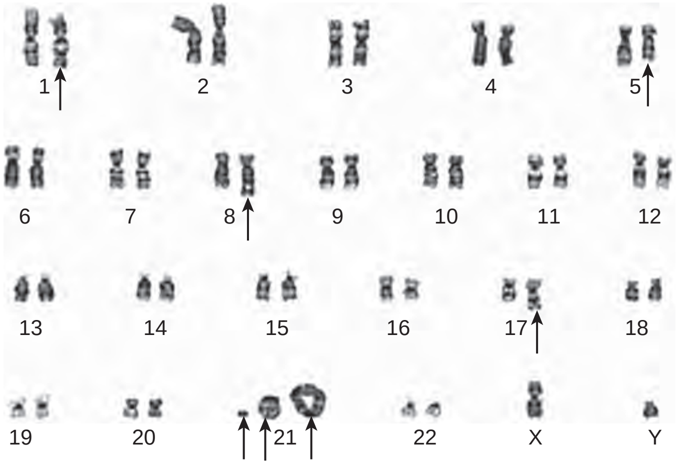


Figure 57.71 A COMPLEX KARYOTYPE FROM A PATIENT WITH HAIRY CELL LEUKEMIA. This complex karyotype with nine complex structural abnormalities, as indicated by *arrows*, 47, XY, del(5)(q15;q31), der(8)t(3;8)(p21p26;q24), der(17)t(1;17) (p36.1;p13)t(17;21)(q25q22.2), −21, +r(21)×2, is an example of profound chromosomal rearrangements in hairy cell leukemia.

T-Cell Lymphoproliferative Neoplasms (Chapter 89)

This is a diverse group of hematologic disorders that includes T-cell ALL and T-cell CLL/PML, as well as several indolent T-cell disorders, large granular lymphocyte leukemia (LGL) (Chapter 33), natural killer leukemia/lymphoma, and anaplastic large cell lymphoma (ALCL) (Chapter 89). Approximately 80% of T-cell ALL harbor oncogenic driver lesions. The WHO and the International Lymphoma Study Group denominated both T-ALL and T-LBL (T-cell lymphoblastic lymphoma) as T lymphoblastic leukemia/lymphoma in an updated Revised European-American Classification of Lymphoid neoplasms and WHO classification but without further specification.

T-cell ALL represents 15% of childhood ALL cases and 25% of adult ALL cases. At diagnosis, approximately 50% of patients have a normal karyotype. Table 57.22 lists recurrent cytogenetic and molecular genomic changes associated with T-cell ALL and Table 57.23 lists the frequency of recurrent chromosomal abnormalities in T cell-ALL. Immunophenotypic and gene expression analyses are consistent with genetic heterogeneity in T-cell ALL, reflecting, to some degree, distinct stages of T-cell maturation arrest.

One of the common themes in T-lymphoid malignancies is the juxtaposition of the *TCR* gene enhancer element adjacent to a variety of transcription factors located at or near breakpoints on the partner chromosome. The chromosomal bands most frequently involved are 14q11, where *TCRA* and *TCRD* are located (Fig. 57.72); 7q35, the site of *TCRB*; and 7p15, the site of the *TCRG*. *TCR* translocations are found in approximately 35% of T-cell ALL cases (see Table 57.22). The rearrangements of *TCRB* and *TCRG* are relatively rare, whereas 14q11 rearrangements involving both *TCRA* and *TCRD* are frequent in T-cell neoplasms (see Table 57.22).

In children, the overall frequency of T-cell ALL (Chapters 66 and 67) translocations is 40% to 50%, and several molecular/cytogenetic abnormalities have prognostic implications (see Table 57.23). *TAL1* rearrangements include t(1;14)(p32;q11), t(1;7)(p32;q35), rare t(1;3)(p32;p21), t(1;5)(p32;q31), and t(14;19)(q11.2;q13.1) (see Fig. 57.72). In general, *TAL1* rearrangements are submicroscopic and best identified with FISH. Disruption of *TAL1* is frequently associated with a submicroscopic interstitial deletion (90 kb) between the 5′ untranslated region of the *TAL1* (1p32) and the *SIL* (*STIL*) genes (1p32) in 9% to 26% of cases depending on the different studies. The *SIL-TAL1* fusion product gives rise to inappropriate expression of TAL1 protein. Among 382 children with T-cell ALL 16% possessed *SIL-TAL1*, which was more frequently observed in older children. These 1p32 rearrangements are less frequent in adult T-cell ALL. *TAL1*-positive ALL is characterized by an arrest in differentiation at the CD4+CD8+ stage of thymocyte development, when the *TAL1* gene is normally silent. The *TAL1* gene encodes a transcription factor and several of the core components of the transcription complex include *GATA3*, LIM domain only, and *RUNX1*.

TAL2, residing at 9q32, is detected in the t(7;9)(q34;q32) rearrangement as a result of juxtaposition of *TAL2* to *TCRB*. *LYL1*, originally described as a fusion partner for *TCRB* in(7;19)(q34;p13), is associated with a poor prognosis. Rearrangements of *HOX11L2*, which include cryptic t(5;14)(q35;q32), t(5;7)(q35;q21), and other variants, occur in 24% of children with T-cell ALL and represents

TABLE 57.22 **Frequency of T-Cell Receptor Rearrangements Using Conventional Cytogenetics Versus Fluorescence In Situ Hybridization**

		FISH (%)	
Locus	Conventional Cytogenetics (%)	Total Karyotype	Abnormal Karyotype
TCRαδ	9.5	17.4	24.7
TCRβ	3.1	19	26.9
TCRγ	0	0	0

FISH, Fluorescence in situ hybridization; *TCR*, T-cell receptor.

TABLE 57.23 Genetic Rearrangements in T-ALL

Genetic Lesion	Gene	Frequency %	Outcome
Transcription Factor Oncogenes			
t(1;14)(p32;q11)	*TAL1*	3	Poor/none
t(1;7)(p32;q34)	*TAL1*	<1	Poor/none
1p32 deletion	*TAL1*	25	Poor/none
5′ super-enhancer generating mutations	*TAL1*	5	Unknown
t(7;9)(q34;q32)	*TAL2*	1	NA
t(19)(q34;p13)	*LYL1*	1	NA
t(14;21)(q11.2;q22)	*BHLHB1*	1	NA
t(11;14)(p15;q11)	*LMO1*	1	NA
t(7;11)(q34;p15)	LMO1		NA
t(11;14)(p13;q11)	*LMO2*	6	No impact
t(7;11)(q34;p13)	*LMO2*	<1	No impact
11p13 deletion	*LMO2*	3	No impact
t(10;14)(q24;q11)	*TLX1*	5–10 Pediatric T-ALL	No impact
t(7;10(q35;q24)	*TLX1*	30 Adult T-ALL	No impact
del(10(q24-q26)	*TLX1*		No impact
t(5;14)(q35;q32)	*TLX3*	20–25 Pediatric ALL and 5 Adult T-ALL	Unknown
inv(7)(p15q34)	*HOXA*	3	
t(7;7)(p15q34)	*HOXA*	3	
t(10;11)(p13;q14)	*PICALM-MLL10*	5–10	Poor
t(11;19)(q23;p13)	*KMT2A-MLL1*	5	Poor
9q34 deletion	*SET-NUP214*	3	Unknown
inv(14)(q11.2q13)	*NKX2-1*	5 Pediatric T-ALL	Unknown
inv(14)((q13q32.33)	*NKX2-1*		
t(7;14)(q34;q13)	*NKX2-1*		
t(14;20(q11;p11)	*NKX2-2*	1	Unknown
t(2;14)(q22;q32)	*ZEB2*	<1	NA
t(6;7)(q23;q34)	*MYB*	3	NA
t(8;14)(q24;q11)	*MYC*	1	NA
NOTH1 Pathway			
t(7;9)(q34;q34.3)	*NOTH1*	<1	NA
Activating mutations	*NOTH1*	>60	Good
Activating mutations	*FBXW7*	8–30	Poor/none
Cell Cycle			
9p21 deletion	*CDKN2A*	>70	Good
t(7;12)(q34;p13)	*CCND2*	3	NA
13q14.2 deletion	*RB1*	12 Pediatric T-ALL	No impact
12p13.2 deletion	*CDKN1B*	12	NA
Transcription Factor Tumor Suppressors			
Inactivating mutation or deletion	*WT1*	10	No impact
Inactivating mutation or deletion	*LEF1*	10–15	NA
Inactivating mutation or deletion	*ETV6*	13	No impact
Inactivating mutation or deletion	*BCL11B*	10	No impact
Inactivating mutation or deletion	*RUNX1*	10–20	No impact
Inactivating mutation or deletion	*GATA3*	5	Poor
Signal Transduction			
10q23 deletion	*PTEN*	10–15	Poor
Inactivating mutation	*PTEN*	10–15	No impact
Episomal 9q34 amplification	*NUP214-ABL1*	5	Poor
t(9;14)(q34;q32	*EML1-ABL1*	<1	NA
Activating mutation	*NRAS*	5	No impact
Activating mutation	*KRAS*	2	NA
Activating mutation	*JAK1*	4–18	No impact
t(9;12)(p24;p13)	*ETV6-JAK2*	<1	No impact
Activating mutation	*FLT3*	5–10	No impact
t(X;7)((q22;q34)	*IRS4*	<1	NA
t(X;14)(q22;q11.2)	*IRS4*		
Inactivating mutation	*DNM2*	15	NA
t(9;22)(q34;q11.2)	*BCR-ABL1*	1	Poor

NA, Not available.
Modified from Belver L, Ferrando A. The genetics and mechanisms of T cell acute lymphoblastic leukaemia. *Nature.* 2016;16(8):494.

a predictor of poor outcomes. Similarly, the presence of t(10;11)(p13;q14), which results in the *CALM-MLL10* fusion gene and occurs in 2% to 5% of children, may be associated with poor outcome. Abnormalities of *HOX11* are more common in adults than in children with T-cell ALL (31% versus 7%) and are associated with t(10;14)(q34;q24) and t(7;10)(q34;q24). t(10;14) is the most frequent chromosomal translocation in patients with T-cell ALL. It is associated with excellent outcome in both children and adults with t(10;14) and in these patients the homeobox gene *HOX11* is fused with *TCRD*. The coding regions of *HOX11* are not disturbed by the translocation. In the variant translocation t(7;10)(q35;q24), *HOX11* is juxtaposed to *TCRB*, which results in overexpression of normal *HOX11* by bringing *HOX11* under the influence of *TCR* promoter sequences.

KMT2A rearrangements are present in 8% of T-cell ALL cases (see Fig. 57.40). The most frequent *KMT2A* translocation partners in T-cell ALL include *ENL*, which results from t(11;19)(q23;p13.3) and is associated with a more favorable prognosis than T-cell ALL with other fusion partners. GEP has characterized T-cell ALL with *KMT2A* rearrangements as a distinct molecular subtype. Homeobox genes, regulators of embryonic development, are known targets of KMT2A and are overexpressed in patients with T-cell ALL and KMT2A rearrangements.

The most predominant oncogenic event in adult T-cell ALL is the aberrant activation of *NOTCH1* signaling through *NOTCH1* activating mutations, rearrangements, or loss of its negative regulator (Chapter 66). Dysregulation of tyrosine kinase genes (*ABL1, JAK2*)

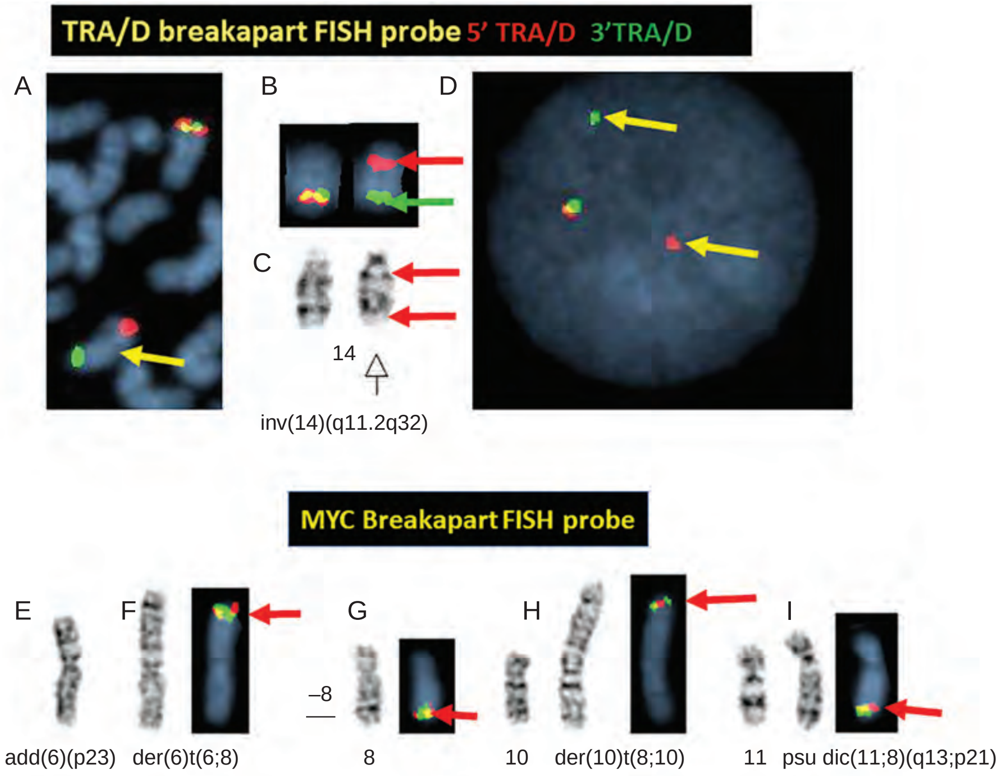


Figure 57.72 REARRANGEMENTS OF T-CELL RECEPTOR (TCR) GENES (TRA/D) AND MYC IN T-CELL MALIGNANCIES. (A) A partial metaphase cell after FISH hybridization with breakapart dual color probe *TRA/D*. Yellow arrow indicates a separation of the 3′ probe red and the 5′ probe *(green)* as a result of inv(14). (B) Isolated a pair of chromosome 14, left shows a normal chromosome 14 with a colocalization of 3′ *(red)* and 5′ *(green)* at 14q32. The right chromosome 14 shows a paracentric inversion with separation of the 3′ and the 5′. (C) An isolated chromosome 14 showing a paracentric inversion (14)(q11.2q32) *(red arrows)*, a characteristic cytogenetic abnormality in T-cell lymphoma. (D) Interphase nuclei after FISH with TRA/D demonstrating a separation of the *TRAD/A* probe *(yellow arrow)* confirming *TCR* rearrangement in a non-diving cell. (E) In addition to the *TCR* rearrangements T-cell lymphoma are characterized by the complex chromosomal rearrangements. This patient, as shown in (F), (G), (H), and (I), had a gain of four copies of 8q as a result of chromosomal abnormalities: der(6)t(6;8)(p25;q13) (F)I, der(10)t(8;10)(q13;p15) (H) and psu dic(11;8)(q13;p21)(I). In each of the 8q rearrangements the *MYC* gene was not rearranged and was present in 4 copies, suggesting a dosage effect. *FISH*, Fluorescence in situ hybridization.

and LIM domain genes (*LMO1, LMO2*) is also common in T-cell ALL. *NOTCH1* is a fusion partner of *TCRB* in t(7;9)(q34;q34.3). These mutations are present in 56% of T-cell ALL cases. The *NOTCH1* gene is important in lymphoid lineage specification, and resequencing of functional domains of *NOTCH1* have revealed that activating *NOTCH1* mutations are present in most primary pediatric and adolescent molecular cases of T-ALL as well as in adult ALL. Mutations in the heterodimer domain as well as ITD result in constitutive ligand-independent activation of *NOTCH1*.

The *LIM* family of genes is found at the breakpoint of rare but consistent chromosomal translocations in T-cell ALL. *LMO1*, located at 11p15, is involved in t(11;14)(p15;q11). *LMO2*, located at 11p13, is involved in t(11;14)(p13;q11) and t(7;11)(q35;p13). As an oncogenic transcription regulator, *LMO2* overexpression in erythroid and T cells leads to an arrest in differentiation, which is a prerequisite for development of T-cell malignancies.

Second in prevalence in T-cell ALL is 9p21 deletions or altered methylation status of *CDKN2A/B* usually accompanied by mutations in *PTEN, NRAS, and P13KCA* genes. Chromosomal rearrangements affecting 9q34 occur in approximately 10% of T-LBL patients compared to 1% to 3% of T-cell-ALL patients and frequently involve t(9;17)((q34;q22–23) rearrangement. The locus at 9q34 includes various oncogenes such as *SET*, *ABL1*, *NUP214*, and *NOTHC1*. Also various T-LBL (Chapter 89) patients have been described to have t(7;14)(p15;q32) aberration in which the TCR gamma chain locus is coupled to the *TCL1A* oncogene.

The *MYB* gene, which is localized on chromosome 6, band q23.3, is rearranged in a rare T-cell ALL subtype observed in young patients (median 2.2 years). Two types of recurrent *MYB* genomic alterations are found in T-cell ALL: (a) a reciprocal t(6;7)(q23;q34) that results in juxtaposition of *MYB* near *TCRB* regulatory sequences on chromosome 7 and (b) a short genomic tandem duplication, identified using genome-wide copy-number analysis. Both rearrangements are cytogenetically cryptic. The breakpoints in t(6;7) are subtelomeric and usually missed. The translocation was discovered using a locus-specific FISH probe for *MYB*. The tandem *MYB* duplication is cryptic using both conventional cytogenetics and locus-specific FISH. LOH 6q is detected in ~13% of T-cell-ALL and 19% of T-LBL. In T-LBL 6qLOH is associated with poor outcome but this was not found to be true for T-ALL. Deletion 6q chromosomal band differs between the two clinical entities, affecting chromosomal band 6q14-15 in T-ALL and 6q16 in T-LBL. 6q deletions are almost exclusively found in patients denoted as TALLMO subtype.

Although only 1% of Ph+ ALL have a T-cell phenotype, the *ABL1 gene* is also involved in fusion with *NUP214* (9q34.11), creating the *NUP214-ABL1* chimeric gene/t(9;22)(q34.11–q22.1), which is identified in up to 6% of T-cell ALLs. This fusion gene is found amplified only in T-cell ALL. When identified with FISH using the *ABL1* probe, it appears as an amplified episome between the chromosomes in metaphase cells, usually showing 5 to 50 copies/cell and is very rarely identified with conventional cytogenetics (Fig. 57.73). The most common fusion gene involves the breakpoint in exon 2 of *ABL1* and the breakpoint in exon 31 of *NUP214*, although variant breakpoints in both genes have been reported. *NUP214-ABL1* is a constitutively activated tyrosine kinase activating similar pathways as *BCR-ABL1* and is sensitive to inhibition with TKIs (nilotinib and dasatinib). These patients usually have homozygous or heterozygous deletions of *CDKN2A* at 9p21 chromosomal site

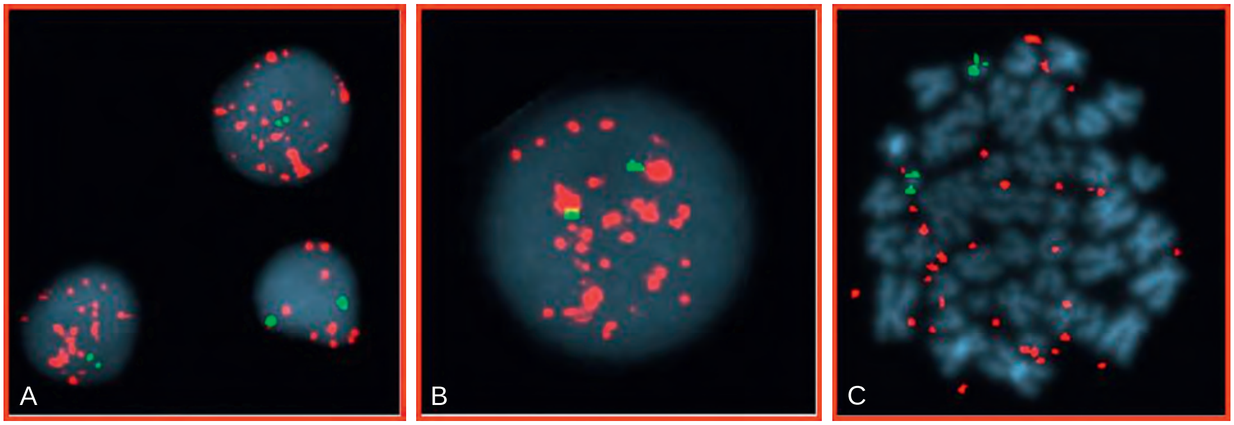


Figure 57.73 *ABL1* AMPLIFICATION IN T-CELL NEOPLASMS. *ABL1* amplification as a result of *NUP214-ABL1* chimeric gene resulting from t(9;22)(q34.11;q22.1) chromosomal translocation in a patient with Ph-positive T-cell acute lymphoblastic leukemia. (A) Three interphase cells showing 2 *BCR* loci *(green)* and numerous copies of *ABL1 (red)*. (B) More than 40 copies of *ABL1 (red)* were identified in this bone marrow nucleus. (C) Approximately 30 copies of *ABL1 (red)* were shown as an amplified episome between the chromosomes in this bone marrow metaphase cell.

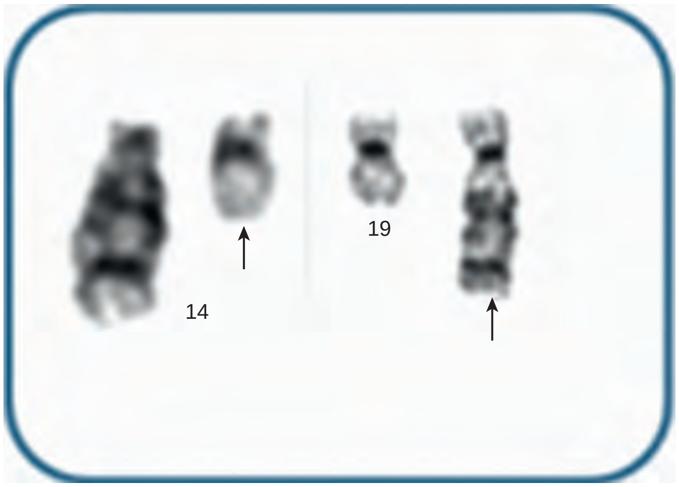


Figure 57.74 A PARTIAL KARYOTYPE FROM A PATIENT WITH T-CELL LEUKEMIA AND t(14;19)(q11.2;q13.1) KARYOTYPE.

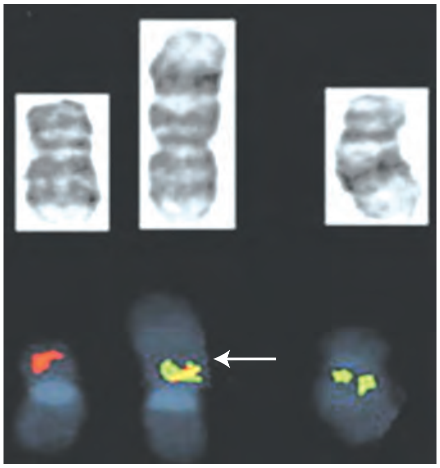


Figure 57.76 JAK-TEL FUSION. Partial karyotype of t(9;12)(p24;q23) *(top)* and after FISH study *(bottom)* with JAK2 *(red)* and TEL/ETV6 *(green)*, showing *JAK2-TEL* fusion *(yellow)* at 9p24.

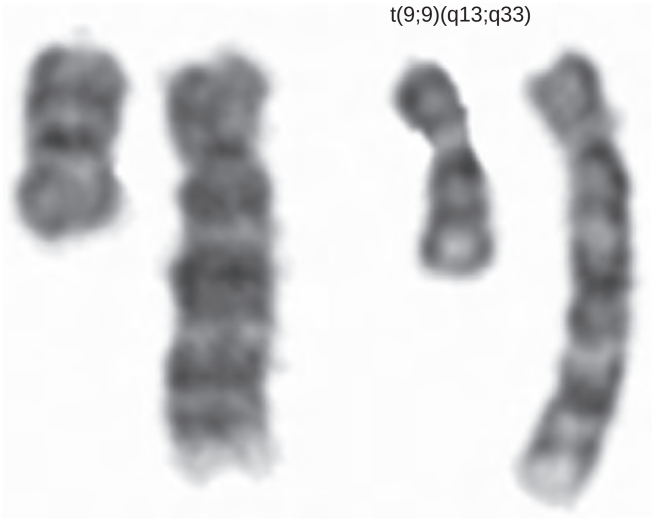


Figure 57.75 A PARTIAL BONE MARROW CHROMOSOME 9 KARYOTYPE FROM TWO METAPHASE CELLS FROM A PATIENT WITH T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA. The rare variant t(9;9)(q22;q34) also results in *SET-NUP214* fusion.

as well as trisomy 8, t(7;10)(q35;q24), or t(10;14)(q24;q11) or (14;19)(q11;q31), indicating that *NUP214-ABL1* is not a primary genetic event (Fig. 57.74). Patients with *NUP214-ABL1* usually present with high-risk T-cell ALL and their outcome is poor. del(9)(q34.11–q34.13) or rare t(9;9)(q34;q34) results in the formation of a *SET-NUP214* fusion gene frequently observed in T-cell ALL (Fig. 57.75). In rare cases of T-cell ALL, *JAK2* has been identified as a partner with *ETV6* in t(9;12)(p24;q13), with *PCM1* in t(8;9) and in multiple amplified copies (Figs. 57.76–57.78), two of the three *JAK2* fusion transcripts seen in other hematologic malignancies (Chapter 72).

Adult T-cell ALL is characterized by an aberrant karyotype in almost 70% of cases and by recurrent acquisition of somatic mutations in 93% (see Table 57.22). The most frequent recurrent molecular lesions observed in adult T-cell ALL include mutations in the following genes: *NOTCH1* (71%), *PHF6* (39.5%), *FBXW7* (18.9%), *DNMT3A* (17.8%), *RUNX1* (15.5%), *PTEN* (10%), *FLT3-IDT* (2.2%), *FLT3-TKD* (1.1%), and *CDKN2A* (3.3%). Combining these data with frequent *CDKN2A/B* deletions and aberrant *CDKN2B* methylation status, 98.9% of adult T-cell ALL have a minimum of one genetic defect. *DNMT3A* and *RUNX1* mutations are preferentially found in early immature T-cell leukemias and are associated with a poor OS.

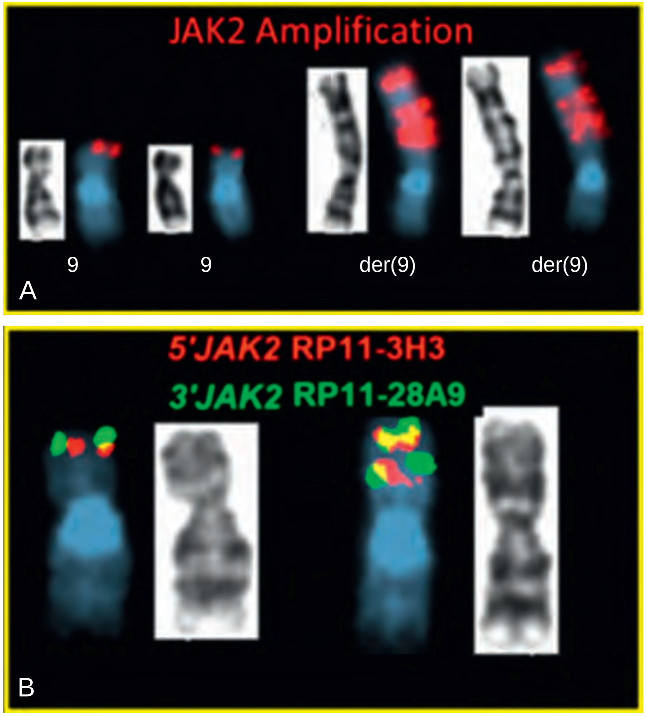


Figure 57.77 GAIN OF *JAK2* IN ADULT T-CELL LEUKEMIA/LYMPHOMA. (A) A partial karyotype of der(9) chromosome from a patient with adult T-cell leukemia/lymphoma showing *JAK2 (red)* amplification. (B) A partial dup(9)(p24) karyotype showing duplication of *JAK2* (*red* is *5′ JAK2*, *green* is *3′ JAK2*).

Early T-cell precursor ALL (ETP) is an aggressive subtype of T-cell ALL that comprises up to 15% of T-cell ALL cases. WGS of 94 T-cell-ALL cases revealed a mutational spectrum similar to AML. ETP ALL is characterized by activating mutations in genes regulating cytokine receptors and RAS signaling (67% of cases: *NRAS*, *KRAS*, *FLT3*, *IL7R*, *JAK3*, *JAK1*, *SH2B3*, and *BRAF*), inactivating lesions disrupting hematopoietic development (58% of cases: *GATA3*, *ETV6*, *RUNX1*, *IKZ1*, and *EP300*), and histone modifying genes (48% of cases: *EZH2*, *EED*, *SUZI2*, *SETD2*, and *EP300*).

MYC translocations identify a genetic subtype of T-cell ALL, which occurs in about 6% of cases, including both children and adults. Two types of translocations have been observed: those involving the *TCR* loci and those with other partners. Specifically, *MYC* translocations were significantly associated with TAL/LMO subtype of T-cell ALL and trisomies for chromosomes 6 and 7. GEP has revealed that *MYC* translocations occur as secondary abnormalities present either in subclones at diagnosis or during disease progression.

In contrast to childhood B-cell ALL, the majority of childhood T-cell ALL cases develop after birth. In utero origin of this form of leukemia is rare.

T-cell CLL and prolymphocytic leukemia is characterized by T-cell leukemia 1 (*TCL1*) gene rearrangements. These include inv(14)(q11q32.1), t(14;14)(q11;q32.1), t(X;14)(q28;q11), and as mentioned above, t(7;14)(q35;q32.1). Approximately 90% of T-CLL and T-PLL have chromosome 14 genomic changes. In these patients *TCL1* is found to be dysregulated. The second most recurrent abnormalities in T-PLL affects chromosome 8 and include idic(8)(p11) t(8;8) and trisomy 8q. Other reported recurrent abnormalities include loss of 11q23 along with additional losses of 6q, 9p, 12p, 13q, 17p, and 22q as well as gains of 22q and 6p. Up to 50% of patients with T-PLL are haplodeficient for *CDKN1B* as a result of 12p13.1 deletion which implicates this gene in the pathogenesis of T-PLL.

Adult T-cell leukemia/lymphoma is associated with human T-cell lymphotropic virus type 1. The most frequent genetic lesions include altered expression of *CDKN2* (cyclin-dependent kinase inhibitor) gene on 9p21 (15% to 20%) and loss of heterozygosity at 6q15 to q21. The most frequent chromosomal abnormalities are gain of 3p, 7q, and 14q and loss of 6q and 13q. Translocations involving 14q32 or 14q11 are frequently observed.

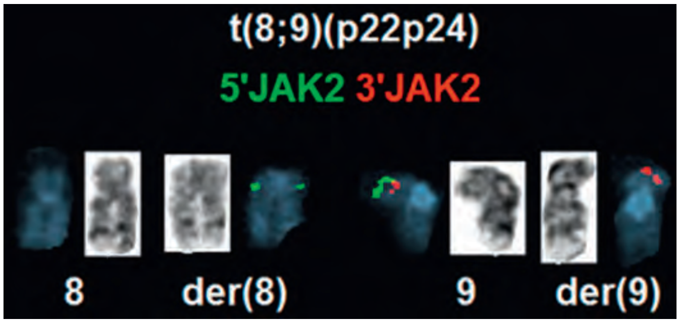


Figure 57.78 t(8;9)/PCM1-JAK2 IN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA. (T-ALL) A partial karyotype from a patient with T-ALL showing t(8;9)(p22;p24) resulting in *PCM1-JAK2* fusion also observed in patients with Ph-negative myeloproliferative neoplasms. In this translocation 5′ JAK2 *(green)* is translocated from 9p24 to 8p22 using *JAK2* two-color breakapart FISH probe. *FISH*, Fluorescence in situ hybridization.

Natural killer lymphoma/leukemia is a group of highly aggressive lymphoid malignancies of natural killer cell origin. They exhibit chromosomal rearrangements in greater than 80% of cases by conventional karyotyping. The most frequent abnormalities include del(6)(q21–23) and gain of the X chromosome. FISH, CGH, and spectral karyotyping have confirmed the presence of del(6) in $CD3^{-}CD56$ tumor cells. Other less common but recurrent karyotypic changes include isochromosome 1q, 6p, and 17q, as well as del(11q), 13q, and 17p, and trisomy 8.

Angioimmunoblastic T-cell lymphoma and unspecified peripheral T-cell lymphoma (Chapter 89) are the most frequent nodal T-cell lymphomas. Losses of 5q/10q/12q, identified by CGH, characterize a subtype of peripheral T-cell lymphoma associated with a more favorable prognosis. Gain of 11q13 may represent a primary change in angioimmunoblastic T-cell lymphoma. In general, chromosomal imbalances are more common in unspecified peripheral T-cell lymphoma than in angioimmunoblastic T-cell lymphoma. Both disorders may share similar genomic imbalances.

The molecular basis of ALCL is the result of t(2;5)(p23;q35), which fuses part of the *NPM* gene on 5q35 with part of the *ALK* receptor tyrosine kinase gene on 2p23 to produce a chimeric *NPM-ALK* gene (Chapter 85). WHO 2017 recognizes both *ALK*-positive (*ALK*+) and *ALK*-negative (*ALK*−) entities. ALK+, chimeric proteins have activated kinase function. *ALK* is thought to play a direct role in the malignant transformation of lymphoid cells, probably by aberrant phosphorylation of intracytoplasmic substrates. ALCL is a distinct clinical entity, with t(2;5) detected by cytogenetics in 60% to 85% of patients. The *NPM-ALK* fusion can be identified by FISH (Fig. 57.79) and by RT-PCR assay in 88% of pediatric patients and 60% of adult patients. The other cases of ALCL have variant translocations with at least 25 different fusion genes and include rearrangements with *TPM3* (1q24), *EML4* (2p24), *RANBP2* (2q13), *ARIC* (2q35), *TCG* (3q21), *SEC31A* (4q21), *CARS* (11p15), *CLTC* (17q23), *ALO17* (17q25), *TPM4* (19p13), *CLTC1* (22q11), *MYH* (22q11.2), and *MSN* (Xq11). Detection of *ALK* rearrangements by FISH corresponds to a cytoplasmic staining pattern associated with translocations other than t(2;5) in 100% of cases. Recently two copies of *NPM-ALK* and *TPM3-ALK* fusions have been reported in children with an aggressive form of ALCL.

The prognosis of patients with t(2;5) or variant translocations is excellent, with 5-year survival rates of 70% to 90%. By contrast, *ALK*− ALCL is associated with a 5-year survival rate of 40% to 60% and is predominantly a disease of adults (median age 55 to 60 years). The true nature of *ALK*− ALCL remains obscure. Most cases have

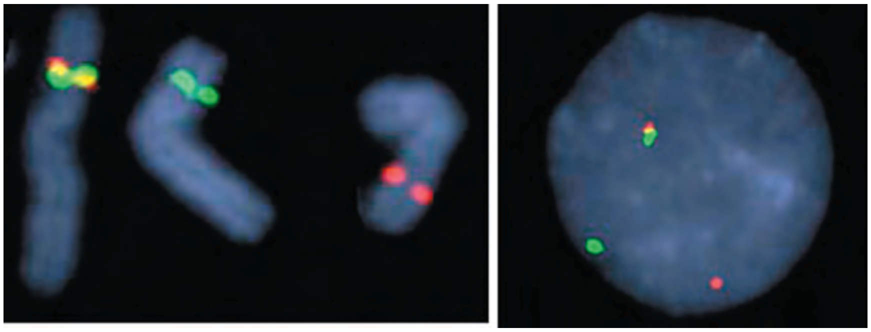


Figure 57.79 FISH STUDY WITH BREAKAPART ALK GENE AT 2P23 IN A PATIENT WITH ANAPLASTIC LARGE CELL LYMPHOMA. Partial karyotype shows a normal chromosome 2 *(left)* with yellow signal, as the 3′ end and the 5′ end of *ALK* gene are in close proximity on chromosome 2. The other chromosome 2 homologue *(middle)* has only a single green signal (5′ end) as a result of t(2;5)(p23;q35), the most frequent translocation in anaplastic large-cell lymphoma. The 3′ end *(red)* of the ALK gene is translocated to 5q35. Abnormal *ALK* gene is also shown in interphase cell *(right)*. *FISH*, Fluorescence in situ hybridization.

clonally rearranged *TCR* genes. Recurrent rearrangements of the *DUSP22-IRF4* locus on 6p25.3 has been identified in about 30% of *ALK*− ALCLs resulting in t(6;7)(p25.3q32.3). Other recurrent rearrangement in *ALK*− cases involves inv(3)(q26q26) resulting in *TBL1XR-TP63* fusion. CGH has shown that *ALK*− ALCL have more complex copy number abnormalities than *ALK*+ ACLC, specifically gains of 1q41-ter, 5q, 6p, 8q, 12q, and 17q and losses of 6q21 and 13q21–22. The interferon regulatory factor 4 (*IRF4*), localized at 6p25.3, is highly expressed in both *ALK*+ and GEP of *ALK*− ALCLs has revealed that *MYC* is the primary target of *IRF4* and essential for ALCL cell survival. Collectively these observations indicate that ALCL is dependent on *IRF4* and *MYC* signaling. Another recurrent alteration, detected in 8% of cases, is rearrangement of the *TP53* homolog, *TP63* (3q23), which is associated with a very poor prognosis. NGS has identified recurrent gene mutations in 18% of ALK− ALCL and they include mutations in *JAK1* and *STAT3* resulting in constitutively activation of the JAK-STAT signaling pathway.

Even though the *ALK* fusion is a key oncogenic event in the pathogenesis of ALK+ ALCL, additional genetic imbalances have been described. By CGH array, 58% of the ALK+ ALCL have chromosomal imbalances including loss of 4q13-q28, 6q13-q22, 11q14-q23, and 13q and gains of 7p11-pter and chromosome 17. By SNP array analysis, ALK+ ALCL displayed losses of 17p13.3-p12 and 6q21 in 35% of the case, and gains of 1q, 7q32.3, and 7p22.3-p21.3 in 15% of samples. The significance of these additional alterations in the pathogenesis of the disease and prognosis is unclear and further studies are required using integrating next generation sequencing approach in order to better define the genomic landscape of ALK+ ALCL.

A variety of genomic rearrangements are seen in the T lymphocytes of patients with mycosis fungoides (MF) and Sézary syndrome (Sz) (Chapter 89). Using aCGH+SNP recurrent copy number abnormalities in Sz include gain of loci on chromosome 17q24 and 8q24 and loss of regions on 17p13 and 10q25, occurring in up to 85% of patients.

Additional evaluation of candidate oncogenes and tumor suppressor genes residing in loci with chromosomal alteration point to dysregulation of the *MYC* oncogene, several of its regulators, and IL-2 receptor signaling pathway components in Sz.

Numerical chromosomal alterations most frequently observed in MF include gain of 7q21–36 and 1p36.2 and loss of 5q13 and 9p21. Amplification of the locus containing the *MYC* gene on 8q24 have been observed in 75% of patients with Sz, but its association with an increased expression of this oncogenic transcription factor is found only in a minority of patients with MF. Interestingly, the most frequently observed CNA, gain of an *MCR* on chromosome 7q36 affecting 59% of MF patients, was associated with an increased expression of the *FASTK* gene. Finally, patients with MF who demonstrated loss of the *MCR* on 9p21, gain 8q24.3, or gain of 1q21–1q22 appear to have significantly poorer survival rates than patients with tumor cells not affected by these CNAs. The integration of high-resolution copy number and gene-expression data has afforded novel insights into molecular genetic alterations underlying MF. Overexpression of *FASTK*, *MCL1*, and *SKAP1* associated with chromosomal gains and reduced expression of *CDKN2A*, *RB1*, and *DLEU1* related to loss are important candidate oncogenic events in MF. In malignant lymphocytes from patients with Sz/MF, *STAT6* (localized at 12q14.1) is constitutively activated. *STAT6* regulates several common pathways in Sz/MF malignant lymphocytes that are associated with control of cell cycle progression and genomic stability.

Post-transplant lymphoproliferative disorder (PTLD) (Chapter 87) is a morphologically diverse group of lymphoid disorders that occurs in immunosuppressed organ transplant recipients. Chromosomal abnormalities are detected in 51% to 72% of B-cell variants and in almost all T-cell variants, as measured by CGH, FISH, and conventional karyotyping. In general, three groups of nonrandom abnormalities are of importance. The pathogenetic roles of abnormalities known to be involved in lymphomas, such as *MYC*, *TP53*, and 18q21 associated with *BCL2* and *MALT1*, and those associated with large genomic imbalances, such as 2p24–25, 9q22–34, 12q22–24, and 14q32, await elucidation. Trisomy of chromosomes 9 and 11 appears to be associated with EBV PTLD and prolonged survival. In contrast, patients with PTLD showing *MYC* rearrangements (about 50%) are associated with BL, an aggressive clinical course. T-cell PTLD develops late after transplantation. These disorders are EBV-negative and are associated with a poor outcome and short survival.

ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

Molecular and cytogenetic analyses can be used to characterize the origin of engrafted cells and the development and evolution of recurrent malignancies after allogeneic HSCT. Hematopoietic cells that emerge after allogeneic HSCT may be of host origin, donor origin, or both. Genetic studies of post-transplant hematopoiesis are termed *chimerism analysis*. Chimerism should be distinguished from mosaicism, which is characterized by two or more different cell populations originating from one zygote. Monitoring chimerism in recipients of allogeneic HSCT is essential to identify early engraftment, monitor residual disease, predict relapse, and optimize post-transplantation therapy in case of graft failure.

Historically, karyotype analyses were used to evaluate engraftment after sex-mismatched allogeneic HCT. Polymorphism of chromosomes 1, 9, and 16, as well as satellite polymorphism of chromosomes 13, 14, 15, 21, and 22, have been used to differentiate donor from recipient cells in sex-matched allogeneic HCT. Karyotype analyses can identify not only chimerism but also recurrence of the hematologic malignancy. However, this is a time-consuming process and has low sensitivity (5%). In the past two decades, many additional methods for detection of chimerism have been developed. All follow the basic principle of using the differences in polymorphic genetic markers to distinguish donor from recipient hematopoiesis. These methods include restriction fragment length polymorphism, red cell phenotyping, and interphase FISH. The most widely used technique is PCR for a variable number of tandem repeats/short tandem repeats (VNTR/STR-PCR). This technique has a sensitivity of 3% to 5%, but the quantitation of donor and recipient cells may be cumbersome. The method that allows study of chimerism in all patients involves fluorescence labeling of the primers and resolution of PCR products with capillary electrophoresis. It provides a high degree of quantitative accuracy with 1% to 5% sensitivity. Real-time PCR or RQ-PCR for analysis of the *SRY* gene on the Y chromosome allows identification of male cells in the background of 100,000 female cells, providing high sensitivity for mixed chimerism. However, this approach is limited to the 50% of patients who receive sex-mismatched transplants. Nevertheless, it remains the most sensitive and the fastest method of chimerism analysis, providing reliable quantitative results within 2 hours.

Detection of SNPs by chimerism analysis (SNP-PCR) is a highly sensitive approach. In one study using 11 different SNP loci, SNP-PCR analysis identified independent predictors of relapse after HSCT. The two most commonly used methods for detection of chimerism after HSCT are fluorescence-based PCR amplification of short tandem repeats (STR-PCR) and interphase FISH. Both methods are accurate and reproducible. The sensitivity of both methods approaches 1%; however, STR-PCR is sex-chromosome independent and can be applied to all patients. FISH analysis, on the other hand, permits simultaneous evaluation of chimerism and residual disease in the same cell when high sensitivity is not a requirement. FISH analysis for diagnostic genomic abnormalities in conjunction with conventional cytogenetics remains useful and reliable in determining the presence of MRD (Fig. 57.80).

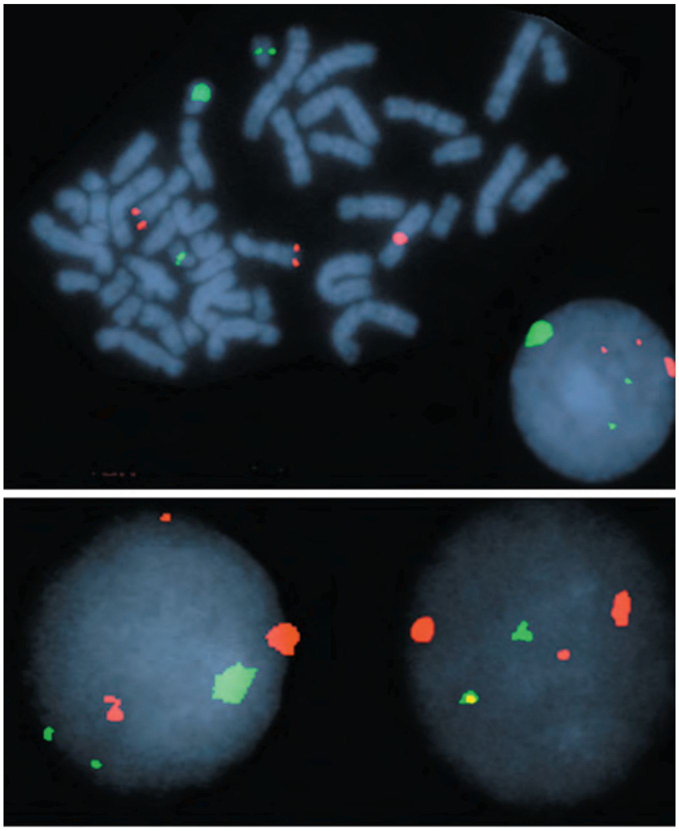


Figure 57.80 DETECTION OF ENGRAFTMENT AND RESIDUAL DISEASE WITH FISH IN SEX-MISMATCHED HEMATOPOIETIC CELL TRANSPLANTATION. Metaphase and non-dividing cell *(blue)* after DAPI counterstaining, hybridized with X *(large red)* and Y *(large green)* for detection of engraftment and with ABL *(small red)* and BCR *(small green)* for detection of residual chronic myelogenous leukemia *(top panel)*. Left nucleus *(bottom panel)* shows a donor male (XY) cell origin and lack of BCR-ABL fusion. In contrast, a host female (XX), BCR-ABL fusion *(yellow)*–positive cell is shown on the *right*. Combination of XY FISH probes with diagnostic genomic markers is a powerful and fast FISH method for simultaneous detection of chimerism and minimal residual disease. *FISH*, fluorescence in situ hybridization.

Donor cell leukemia (DCL) after HSCT is a rare complication occurring in 0.12% to 5% of cases. Careful genetic analysis of relapse cells is essential. VNTR, restriction fragment length polymorphism, STR analysis, or FISH XY analysis alone may not definitively assign the origin of the leukemic clone because genomic deletions or amplifications of chromosomal segments may occur during transplantation or disease progression. The increased use of unrelated cord blood as a source of stem cells for HSCT raises the concern that HSCs containing preleukemic clonal molecular rearrangements may be inadvertently transplanted. Systematic screening of unselected cord blood samples has revealed putative preleukemic rearrangements such as *ETV6-RUNX1* and *RUNX1-RUNXT1*. Cord blood grafts have been reported to be at the highest risk of leading to DCL. The median duration between the occurrence of DCL following cord blood and bone marrow transplantation has been reported to be 14.5 and 36 months, respectively ($P < .0001$). Among 30 patients who developed DCL between 2005 and 2018, an abnormal karyotype involving chromosome 7 were observed in 52.4% of cord blood recipients and 17.3% of marrow recipients ($P < .003$). Patients with DCL generally have a poor prognosis. Proposed mechanisms underlying DCL include: (a) sustained host-origin antigenic stimulation, (b) impaired hematopoietic microenvironment and a defective microenvironmental support system, (c) immune surveillance escape secondary to posttransplant immunosuppressive therapy, (d) similar genetic susceptibility in cases of related donors, (e) viral driven pathogenesis (cytomegalovirus, EBV), (f) delayed effects of conditioning regimens, and (g) transfection of host cell oncogene into donor cells. Most likely, the underlying cause is a combination of these mechanisms operating in individual cases. A very compelling hypothesis underlying DCL is the "2-hit" hypothesis. A donor HSC that has an inherent susceptibility to malignant transformation (hit 1) is placed within a defective microenvironment that elaborates repeated stress signals (hit 2), inducing additional genetic or even epigenetic mutations promoting the appearance of DCL. An examination of every graft prior to transplantation is suggested to determine whether the graft contain abnormal clones (Fig. 57.81).

FUTURE DIRECTIONS

Molecular diversity is the genetic hallmark of many leukemias, lymphomas, and even within a single disease entity such as AML or T-cell ALL. We are at the threshold of understanding additional genetic events, common genetic pathways, and intratumor heterogeneity. Such knowledge is crucial to designing molecular interventions for abnormalities involving specific genetic pathways underlying each hematologic malignancy. It is clear that the genetic revolution has already changed the practice of clinical hematology. Cytogenetics, FISH, aCGH+SNP, and NGS have enhanced the understanding of hematologic malignancies pathogenesis and the origin and evolution of mutations and their temporal sequence, increasing the power for subclassifying these malignancies and allowing earlier diagnosis and more appropriate treatment options. Perhaps most dramatically, the complementary application of these genetic methods has demonstrated intratumor heterogeneity. Nonetheless, modern genomic studies provide a snapshot portrait of the total DNA within the tumor along with a mixture of normal and abnormal cells. Systematic interrogation of subclonal genetic complexity and clonal architecture or phylogeny currently poses a technical challenge because three categories of mutational change—fusion genes, CNVs, and SNPs—need to be identified in a single cell. Currently, we are at the threshold of revolutionary impact of novel single cell technologies which in the near future will provide evidence of single cell tracking, spatial and temporal transcription atlas, as well as the ability to probe the epigenetic landscape at the single cell level. The challenge remains to translate this large body of data into easily interpretable results accessible to the hematologist for the purpose of more accurate and effective therapies.

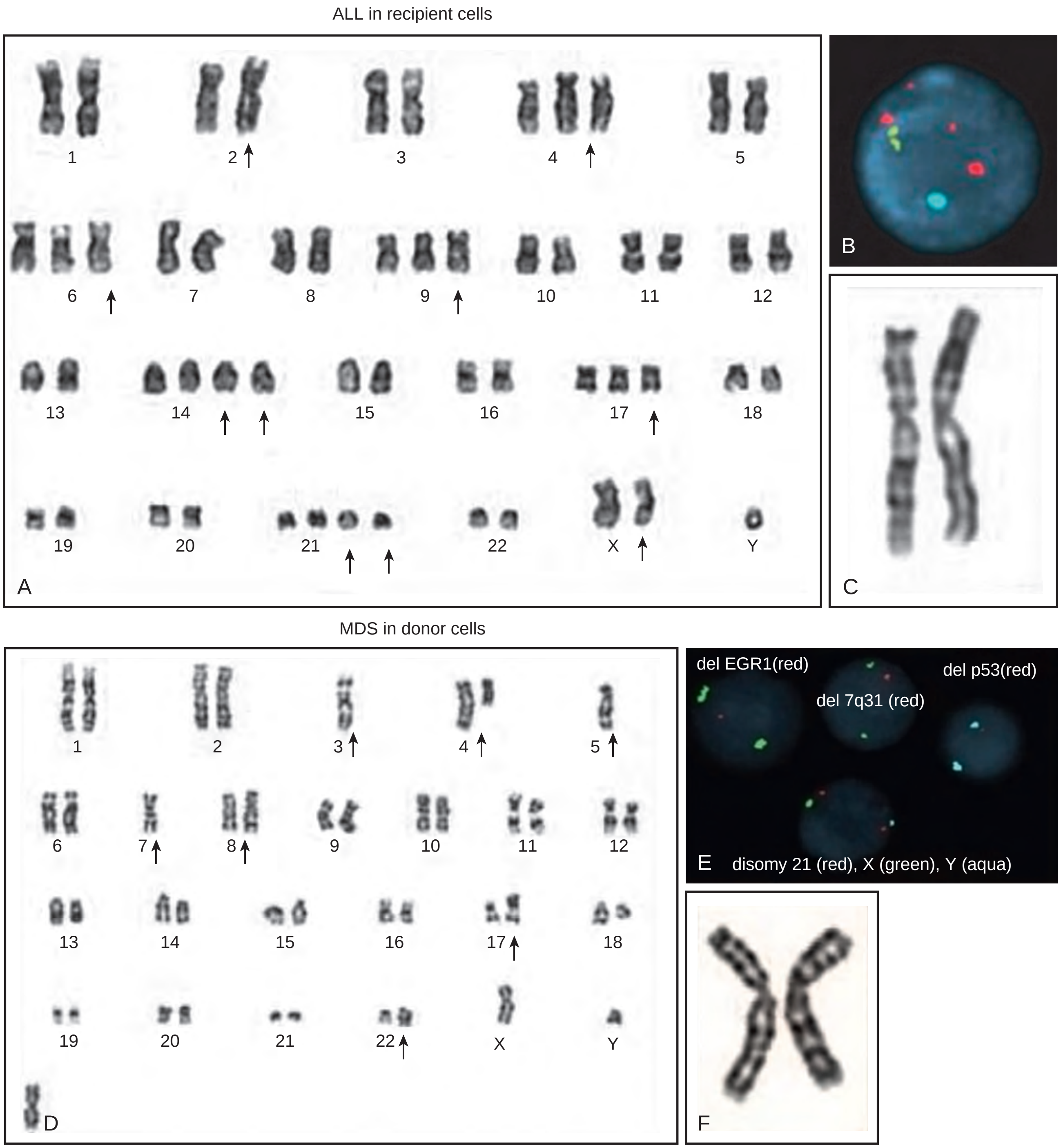


Figure 57.81 DONOR-DERIVED MYELODYSPLASTIC SYNDROME FOLLOWING CORD BLOOD TRANSPLANTATION IN ACUTE LYMPHOBLASTIC LEUKEMIA. (A) At diagnosis of acute lymphoblastic leukemia, in January 2006, cytogenetic analysis of bone marrow revealed a 56, XY, +X, inv(2)(p11.2q13), +4, +6, +9, +14, +14, +17, +18, +21, +21 hyperdiploid karyotype (*arrows* indicate abnormal chromosomes). (B) A bone marrow nucleus from a specimen obtained in January 2007 after FISH showed 5% of cells with tetrasomy 21 *(red signals)*, two copies of X chromosome *(green signals)*, and one copy of Y chromosome *(aqua)*. (C) A partial karyotype of chromosome 2 from the peripheral blood specimen, PHA-stimulated for 72 hours, obtained in May 2006, showing a constitutional inversion (2)(p11.2q13). (D) At the time of the diagnosis of MDS in May 2011, bone marrow cytogenetic analysis revealed 65% of cells with a 44, XY, −3,del(4)(q23q33), der(5;17)(p10;q10), −7,t(8;22)(p21;q13), +mar karyotype (arrows indicate abnormal chromosomes). (E) Five bone marrow nuclei after FISH studies from the June 2011 specimen showing 75% cells with deletion of *EGR1* at 5q31 chromosomal location *(red)* and deletion of P53 at 17p13.1 chromosomal localization *(red)* as a result of der(5;17); 71% showing a loss of 7q31 locus as a result of monosomy 7, as well as disomy 21 *(red)*, one X *(green)*, and one Y *(aqua)* chromosome. (F) A partial bone marrow karyotype of chromosome 2 from May 2011 specimen showing a normal chromosome 2 from the donor cells and absence of inv(2) observed in the bone marrow and PHA-stimulated PB at the time of diagnosis . *FISH*, Fluorescence in situ hybridization. *(Reproduced with permission from Cotter R, Najfeld V, Isola L, et al. An unusual case of donor-derived myelodysplastic syndrome following double-unit umbilical cord blood transplantation in acute lymphoblastic leukemia. Am J Hematol. 2012;87:931.)*

REFERENCES

1. Duncavage EJ, Schroeder MC, O'Loughlin M, et al. Genome sequencing as an alternative to cytogenetic analysis in myeloid cancers. *N Engl J Med*. 2021;384:924.
2. Raskind WH, Steinmann L, Najfeld V. Clonal development of myeloproliferative disorders: clues to hematopoietic differentiation and multistep pathogenesis of cancer. *Leukemia*. 1998;12:108–116.
3. Greaves M. Darwin and evolutionary tales in leukemia. *Hematology Am Soc Hematol Educ Program*. 2009:3–12.
4. Hein D, Borkhardt A, Fischer U. Insights into the prenatal origin of childhood acute lymphoblastic leukemia. *Cancer Metastasis Rev*. 2020;39:161.
5. Guermouche H, Ravalet N, Hirsch P, et al. High prevalence of clonal hematopoiesis in the blood and bone marrow of healthy volunteers. *Blood Adv*. 2020;11:3550.
6. Ouseph MM, Hasserjian RP, Dal Cin P, et al. Genomic alterations in patients with somatic loss of the Y chromosome as the sole cytogenetic finding in bone marrow cells. *Haematologica*. 2021;106:555.
7. Ibáñez M, Such E, Onecha E, et al. Analysis of SNP array abnormalities in patients with de novo acute myeloid leukemia with normal karyotype. *Sci Rep*. 2020;10:5904.
8. Komorowski L, Fidyt K, Patkowska E, Firczuk M. Philadelphia chromosome-positive leukemia in the lymphoid lineage-similarities and differences with the myeloid lineage and specific vulnerabilities. *Int J Mol Sci*. 2020;12:5776.
9. Bernard E, Nannya Y, Hasserjian RP, et al. Implications of *TP53* allelic state for genome stability, clinical presentation and outcomes in myelodysplastic syndromes. *Nat Med*. 2020;26:1549.
10. Döhner K, Thiede C, Jahn N, et al. Impact of NPM1/FLT3-ITD genotypes defined by the 2017 European LeukemiaNet in patients with acute myeloid leukemia. *Blood*. 2020;135:371.
11. Busque L, Buscarlet M, Mollica L, Levine RL. Age-related clonal hematopoiesis: stem cells tempting the devil. *Stem Cells*. 2018;36:1287.
12. Sanz MA, Fenaux P, Tallman MS, et al. Management of acute promyelocytic leukemia: updated recommendations from an expert panel of the European LeukemiaNet. *Blood*. 2019;133:1630.
13. Meyer C, Hoffman J, Burmeister T, et al. The MLL Recombinome of acute leukemias in 2013. *Leukemia*. 2013;27:2165.
14. Bolouri H, Farrar JE, Triche Jr T, et al. The molecular landscape of pediatric acute myeloid leukemia reveals recurrent structural alterations and age-specific mutational interactions. *Nat Med*. 2018;24:103.
15. Arindrarto W, Borràs DM, deGroen RAL, et al. Comprehensive diagnostics of acute myeloid leukemia by whole transcriptome RNA sequencing. *Leukemia*. 2021;35:47.
16. Robert KG, Mullighan CG. Genomics in acute lymphoblastic leukaemia: insights and treatment implications. *Nat Rev Clin Oncol*. 2015;12:344.
17. Yang M, Safavi S, Woodward EL, et al. 13q12.2 deletions in acute lymphoblastic leukemia lead to upregulation of FLT3 through enhancer hijacking. *Blood*. 2020;136:946.
18. Hallek M, Cheson BD, Catovsky D, et al. iwCLL guidelines for diagnosis, indications for treatment, response assessment, and supportive management of CLL. *Blood*. 2018;131(25):2745.
19. Baliakas P, Jeromin S, Iskas M, et al. Cytogenetic complexity in chronic lymphocytic leukemia: definitions, associations, and clinical impact. *Blood*. 2019;14:133 1205.
20. Abdallah N, Greipp P, Kapoor P, et al. Clinical characteristics and treatment outcomes of newly diagnosed multiple myeloma with chromosome 1q abnormalities. *Blood Adv*. 2020;4:3509.
21. Bhalla S, Melekoff DT, Keats J, et al. Patient similarity network of multiple myeloma identifies patient sub-groups with distinct genetic and clinical features. *BioRxiv*. 2020;6:04.
22. Grand BM, Gerhard DS, Jiang A, et al. Genome-wide discovery of somatic coding and noncoding mutations in pediatric endemic and sporadic Burkitt lymphoma. *Blood*. 2019;133:1313.
23. Crombie JL, Phillipe A. Diffuse large B cell lymphoma's new genomics: the bride and the chasm. *J Clin Oncol*. 2020;38:3565.
24. Jain P, Zhang S, Kanagal-Shamanna R, et al. Genomic profiles and clinical outcomes of de novo blastoid/pleomorphic MCL are distinct from those of transformed MCL. *Blood Adv*. 2020;4:1038.
25. Laribi K, Baugier de Materre A, Sobh M, et al. Blastic plasmacytoid dendritic cell neoplasms: results of an international survey on 398 adult patients. *Blood Adv*. 2020;4:4838.

CHAPTER 58

PHARMACOLOGY AND MOLECULAR MECHANISMS OF ANTINEOPLASTIC AGENTS FOR HEMATOLOGIC MALIGNANCIES

Stanton L. Gerson, Paolo F. Caimi, Ehsan Malek, and Benjamin Tomlinson

The treatment of patients with hematologic malignancies has been revolutionized over the past decades as new therapeutic targets continue to be identified through cellular and molecular studies of these conditions. These investigations have spawned the discovery, clinical evaluation, and US Food and Drug Administration (FDA) approval of new mechanistic-based therapeutic agents. A surprising number of these agents have progressed rapidly from the discovery phase to validation, animal modeling, and successful clinical testing. The results have led to an explosion in the therapeutic armamentarium and an increase in the spectrum of drugs, including small molecules, monoclonal antibodies, radiolabeled antibodies, drug immunoconjugates, immunotoxins, and complex delivery systems. This chapter provides information on new and existing therapeutic agents available for the treatment of patients with hematologic malignancies. The chapter reviews the "classic" agents as well as the newly developed, target-based agents. Both cytotoxic and growth-inhibitory agents are covered; however, the use of therapeutic antibodies and antibody conjugates is reviewed within the chapters dealing with specific diseases.

TUMOR CELL HETEROGENEITY OF HEMATOLOGIC MALIGNANCIES

Whereas hematologic malignancies are of clonal origin (i.e., they are derived from a single transformed cell), individual neoplastic cells from a patient's malignancy exhibit a great deal of phenotypic diversity and acquire secondary mutations that affect proliferation, drug sensitivity, and resistance. This diversity likely arises from the progeny of clonal populations and subsets of stem cells. In animal models, it has been shown that the clones themselves can give rise to progeny that can transmit the clonal malignancy after transplantation into secondary recipients, suggesting that stem cells are not required to transmit the malignant phenotype.

New evidence indicates that leukemia stem cells are more quiescent, have higher levels of protective proteins such as efflux pumps for drugs, and have higher levels of DNA repair proteins or antiapoptotic proteins than the more abundant cell making up the circulating population of cells. Tumor cell heterogeneity arises as a consequence of spontaneous mutational events, changes in gene promoter methylation, abnormal expression of transcription factors, lymphoid reactivity, and cytokine responsiveness. For example, a mutation or change in expression that renders a hematopoietic cell clone autonomous or growth factor–independent would be expected to render such cells less susceptible to adverse environmental conditions (e.g., growth factor withdrawal). Similarly, one would also predict that a genetic change facilitating cell cycle entry or disruption of cellular maturation would ultimately lead to overgrowth of affected clones. For obvious reasons, mutations that interfere with drug metabolism or the cell death pathway itself would provide a net survival advantage, particularly under the selection pressure of cytotoxic drug treatment.

Malignant myeloid and lymphoid cells have many reasons to have increased mutational rates. Genomic instability can arise from dysregulation of the cell cycle machinery because of a number of events, including perturbations of cyclins leading to MYC overexpression; AKT (protein kinase B) activation; disruption of replication sequences; loss of DNA repair enzymes such as mismatch repair (MMR) enzymes; loss of proper homologous recombination from a defect in the BRCA (BReast CAncer gene 2)–Fanconi pathways; and loss of ATM (ataxia telangiectasia mutated)/ATR (ataxia telangiectasia and Rad3 related) kinases, which can give rise to chromosomal recombination, loss, and microsatellite instability, and loss of checkpoint regulation. These events can give rise to intraclonal emergent point mutations, translocations, and intragenic losses that might not only result in malignant transformation, but also lead to disruption of genomic stability and selection in favor of proliferative and apoptosis-resistant subclones. Leukemic clonal evolution favors drug resistance.

Common mechanisms may be involved in events associated with malignant transformation and the development of mutations that result in tumor heterogeneity. For example, the cell cycle checkpoint and tumor suppressor gene, *TP53*, is induced during DNA damage, leading to G_1 arrest and, if the damage is too severe to repair, cell death by apoptosis occurs. The presumed goal of this process is to eliminate cells that develop deleterious mutations as a result of damage to the genome. Loss of *TP53* may not only increase cellular survival by inhibiting the cell death process, but may also promote the transmission of mutations that would otherwise be deleted. In this manner, a defect of the cell death pathway can have multiple consequences, including (1) selection of cells exhibiting a growth advantage over their normal counterparts, (2) development of drug resistance, and (3) promotion of mutations that result in either (1) or (2), as well as neoplastic cell heterogeneity. Age-dependent changes in these processes may explain the more favorable behavior of leukemias and lymphomas in response to chemotherapy in young patients than older patients.

A model of the relationship between tumor growth rate, the occurrence of spontaneous mutations, and the development of drug resistance was first described by Goldie and Coldman and is referred to as the Goldie and Coldman hypothesis. In this model, the size of a tumor depends on a complex interaction between tumor growth rate and cell loss, the latter stemming from the status of the cell death process, exhaustion of available nutrients, and outstripping of the blood supply. As tumors increase in size, the cell death rate tends to increase. The heterogeneous nature of additional mutations makes it likely that multiple mechanisms of resistance will develop as well. From an operational standpoint, this model has clear implications for the rational design of therapeutic strategies and provides a basis for early and intensive combination drug therapy. The successful implementation of this strategy is exemplified by the administration of dose-intensive multidrug regimens (i.e., the BEACOPP [bleomycin, etoposide, Adriamycin, cyclophosphamide, vincristine (Oncovin) procarbazine, and prednisone] regimen in Hodgkin lymphoma, CODOX-M-IVAC [cyclophosphamide, vincristine, doxorubicin, high-dose methotrexate alternating with ifosfamide, etoposide and high-dose cytarabine] in Burkitt lymphoma [non-Hodgkin lymphoma (NHL)]) and combinations of cytotoxic agents with monoclonal antibodies, such as CHOP (cyclophosphamide, hydroxydaunorubicin, vincristine [Oncovin], and prednisone)–rituximab, which are potentially curative when given early in the course of the disease. Other examples include combined use of multitargeted agents such as lenalidomide and bortezomib for myeloma, fludarabine, cyclophosphamide and rituximab for chronic lymphocytic leukemia (CLL), or maneuvers to overcome resistance of pretreated disease outside of cell-based therapies. However, as predicted by the model, administration of these or other intensive regimens in patients with relapsed or late-stage disease generally fails because of a generalized resistance of tumor cells to all classes of chemotherapeutic agents.

DEVELOPMENT OF CHEMOTHERAPEUTIC AGENTS

The quest for anticancer agents for hematologic malignancies began with the nitrogen mustard class of compounds developed from the chemical warfare agent sulfur mustard gas used in World War I. Since that time, the National Cancer Institute (NCI) and the pharmaceutical industry have developed complex approaches to drug development, screening, and evaluation. Initial screening consisted of toxicity assessment against murine tumor cell lines. Currently, screening is directed toward numerous cell targets, including receptor and downstream signaling kinases; inducers of cell death pathways, including those at the cell surface; mitochondrial enzymes; nuclear DNA and DNA replication, processing and repair proteins, including base excision repair (BER), poly ADP ribose polymerase (PARP), checkpoint kinase 1 (CHK1), topoisomerases, and telomerase; and inhibitors of histone deacetylase (HDAC) and histone methylation, cell cycle proteins, proteasomes, and the mitosis and spindle machinery. Therapeutic agents targeting microenvironment, angiogenesis, and immune checkpoints round out the spectrum of therapeutic approaches. Whereas killing cells had been the backbone of chemotherapeutic approaches to human malignancies, the field of antineoplastic agent development, which in the first decade of the 21st century was at a tipping point, has now embraced novel targeted and highly effective compounds that block the function of specific kinases with surprising efficacy, even at late stages of disease. Ultimately a balanced approach is likely, with much more precision in drug selection, use of mechanism-based combinations, and attention toward and anticipation of tumor heterogeneity, complex subclonality, and emergence of predictable resistance patterns that dictate proactive drug selection and utilization.

Examples include Bruton tyrosine kinase (Btk) and phosphatidylinositol 3-kinase (PI3K) inhibitors in lymphoid malignancies as well as Janus kinase (JAK) inhibitors for treatment of myelofibrosis and polycythemia vera. A number of differentiating agents (including methyltransferase inhibitors altering DNA methylation and gene expression), kinase inhibitors, and immunomodulatory and cytostatic agents complete the antineoplastic armamentarium. The field of drug development also has to contend with much more complex assessments of toxicities due to both on- and off-target effects, as well as normal tissue effects. In the sections that follow these toxicities will be noted because they restrict the utility of many drugs and toxicities need to be monitored when using new drug combinations.

Screening for Antitumor Activity Among Chemotherapeutic Agents

Identification of critical targets for therapy in cancer cells and the microenvironment starts with the recognition of a known or novel target that appears critical for cancer development, dormancy, growth, or metastasis. The NCI Division of Cancer Treatment has a well molecularly characterized cell bank of malignancies that are available for new drug screening. A more contemporary cell line resource is the Cancer Cell Line Encyclopedia (CCLE), developed to assist in drug analysis. It is a compilation of gene expression, chromosomal copy number, DNA sequence, and RNA data from 947 human cancer cell lines collected across databases and repositories. Many cell lines have drug sensitivity profiles from more than 20 anticancer drugs. Both academia and pharma utilize high-throughput gene knockdown (shRNA and siRNA) library approaches, drug screening using large compound libraries, and in silico screening based on protein and drug structures for docking analysis. Repurposed drugs that are FDA approved or for which there are extensive data provide a rich resource for lead compound identification. Medicinal chemistry can then be applied to improve potency and specificity, solubility, kinetic profile, and tissue penetration and residence. For instance, to screen for a kinase inhibitor, cells overexpressing an activated kinase may be used. After an initial in vitro screen, human tumor cell activity is evaluated using a series of athymic mouse xenograft studies targeting tumors from tissues that show promise in in vitro assays. More informative data is generated from banks of patient-derived tumor cell lines, often using Rho-associated kinase (ROCK) inhibition, and using patient-specific tumor xenografts for drug screening. These in vivo models consist of primary malignancies grown in immunodeficient mice, typically NSG (NOD [non-obese diabnetic] scid gamma) mice. Some genetically engineer mice to express needed human cytokines that promote leukemic cell growth, or reconstitute mice with a human immune system to evaluate immunomodulatory drugs and cell-based immune responses. Another model system mimics the human disease by establishing mice with specific chromosomal translocations and oncogene mutations found in human cancers. Drug efficacy endpoints are complex, since target effects may produce cell death, senescence, apoptosis, differentiation, polyploidy, inhibition of metastasis, and loss of cancer stem cell populations. Drugs with promising efficacy and novel mechanisms of action then go on to formulation and toxicology testing, and are ultimately developed for phase I clinical testing through academic centers, industry, or the NCI Cancer Therapy Evaluation Program. Effective drug screening includes evidence of target effect specificity and potency, consideration of effects across tumor types, recognition of predictors of sensitivity and resistance that depend on the mechanism of action and known pathways of resistance, characterization of genetic changes associated with resistance and acquired resistance, and pharmacokinetic preclinical analysis. In all instances, animal studies often fail to provide accurate prediction of therapeutic efficacy, pharmacokinetics, emergence of resistance, or even toxicities.

Variants of patient-derived and specific tumor xenograft (PDX) models, which allow for direct passage of human tumors in mice, include orthotopic models that assess the tissue microenvironment for tumor growth and drug efficacy, metastatic models that allow for removal of primary tumor and metastasis to the brain, lungs, and bone, and direct implantation and metastasis, for instance into colon, spleen, or marrow sites.

Phase I Clinical Trial Design

New anticancer agents are assessed through a series of clinical trials termed *phase I*, *phase II*, and *phase III* (Table 58.1). The purpose of phase I clinical trials is to establish the safe and optimal biochemically active dose of the compound in question with acceptable toxicity that can be used in disease-targeted phase II testing. During phase I development, pharmacokinetics and pharmacodynamic measures are studied in detail so that appreciable information can be forthcoming from the very first set of patients targeted for treatment and to allow confirmation of these observations in larger phase II disease-focused trials. Dose schedule and route of administration are key considerations in early phase I development. Numerous considerations have guided dose-escalation strategies that accompany phase I trial development. The starting dose is typically 10% of the lethal dose in animals adjusted for species dose equivalency. In classic phase I development, a modified Fibonacci dose schedule is used. Groups of three patients are treated at each of the following doses until the maximum tolerated dose is observed: 1N (N, the starting dose), 2N, 5N, 7N, 9N, 12N, and 16N. Typically, the maximum tolerated dose is defined as the maximum dose not causing irreversible toxicity of any type and causing less than grade 4 toxicity in any organ. Newer agents often have off-target toxicities, and these complicate drug evaluation because the dose-dependent toxicities are replaced by rashes, cardiac effects from prolonged QTc intervals, activation or inhibition of other pathways, and unusual side effect profiles such as pleural and pericardial polyserositis. Typically, if one dose-limiting toxicity is observed, the patient cohort is expanded to six patients, and if two patients develop dose-limiting toxicity, typically defined as grade 4 toxicity except as

TABLE 58.1 Clinical Trial Design

Phase	Patient number	Objective	Patient population	Dosing	Comments
0	Limited. Usually up to 20.	• Feasibility • Pharmacokinetic analyses	Eligible subjects may have different diseases	Limited dosing Usually under therapeutic threshold	Phase 0 trials in oncology are infrequent
I	20 to 200	• Dose finding (maximum tolerated dose) • Recommended phase II dose (RP2D) • Safety profile of drug or drug combination • Pharmacokinetic and pharmacodynamic analyses • Very early efficacy signals	Eligible subjects from one of several cancers vs. studies restricted to a group of disease or a single disease	Escalating doses Range from therapeutic to supra-therapeutic dosing	Classic and adaptive designs available
II	50 to >100	Early efficacy signals. Disease response, survival and progression Extended safety profile Administration schedule evaluation	Eligible subjects restricted to one or few disease targets	Most often fixed dose at therapeutic range May have one or more dosing schedules	Classic and adaptive designs available Randomized phase II trials may be done
III	100 to >1000	• Definitive efficacy signals • Comparison with an accepted standard of care	Eligible subjects restricted to one or few disease targets	Most often fixed dose and schedule Done at therapeutic range	Most frequently are randomized, may be placebo controlled and blinded
IV	100 to >1000	• Post regulatory approval evaluation • Long-term efficacy and safety	Eligible subjects restricted to one or few disease targets	Most often dosing at approved range	Usually observational No randomization

previously noted, then further entry at this dose is not pursued, and the next lower dose level is used to establish the maximum tolerated dose with a total of six patients accrued at that dose level.[1]

Alternative strategies of drug escalation have included the use of toxicity grades to enhance dose escalation in early drug development, allowing that if no toxicity is observed, fewer patients might be accrued to each dose. Using the modified Fibonacci scheme, one patient is entered at each dose level until grade 2 toxicity is observed, at which point cohorts of three patients are entered at each level. Early in drug development, level skipping may take place if no toxicity is observed. The overall impact of this is to reduce the number of patients treated at suboptimal doses of therapy and to increase the number of patients evaluated at biologically active doses. Other dose escalation strategies include Bayesian optimized interval design studies, which permit variable cohort size and incorporate methods to dose escalation of two drugs in combination.[2]

More importantly, biomarker-driven studies can be used to optimize inhibition of the target rather than treating to toxicity. This can then be extended in phase II to determine whether the drug effects on its biochemical target correspond to efficacy and tolerance.

Phase II Drug Development

Phase II drug development uses the dose established in phase I to evaluate therapeutic efficacy. Early phase II studies are conducted typically in a two-stage design, whereby a certain number of responses need to be observed in the first stage to proceed to full trial accrual.[3,4] Modifications include seamless phase Ib/II designs in specific diseases with dose escalations and efficacy assessments in a disease-specific manner. Phase II combination therapies optimize therapeutic efficacy, often with dose escalation of one or both agents. For instance, topotecan was found in phase I testing to have efficacy against refractory leukemias, leading to combinations of topotecan with ara-C. When a new agent is combined with an established agent, the new agent undergoes dose escalation. In the past, strategies for combination chemotherapeutic agents have included the use of non–cross-resistant agents with nonoverlapping toxicities. Mechanism-based therapeutic combinations have been more successful. In rare diseases, and now with genomic-driven studies, randomized phase II trials in a specific disease comparing standard to novel therapy can be used as evidence for drug approval, a “registration” trial.

Phase III Clinical Trials

Most phase III trials randomize patients between an established standard therapy and a new therapy that has appeared promising in the phase II setting. These studies are usually multi-institutional, and many involve large national (e.g., National Clinical Trials Network) and international cooperative groups. The endpoint of these studies is disease response, time to progression (TTP), survival, and patient tolerance. Phase III trials are often registration trials designed with the FDA and intended to guide drug approval for a disease indication. All trials must have rigorous objective data collection, safety and quality review, and reporting. All clinical trials should be registered at clinicaltrials.gov with public reporting of results.

TRADITIONAL CYTOTOXIC ANTINEOPLASTIC AGENTS TARGETING THE CELL CYCLE AND DNA

Targeting Tumor Cell Growth Kinetics

Malignant hematopoietic cells proliferate more and differentiate less than their normal counterparts. The cell cycle consists of a series of stages through which normal and neoplastic cells proceed during the course of cellular replication (shown schematically in Fig. 58.1). The cell cycle is divided into G_1 (pre-DNA synthetic phase), S phase (in which DNA replication takes place), G_2 (post-DNA synthetic phase), and mitosis (M), during which chromosomal division and segregation occur. In addition, nonproliferating, resting cells reside in G_0, a phase that may theoretically last for an indefinite period. Such cells remain in G_0 until they are induced to cycle (at G_1) by specific triggers (e.g., hematopoietic growth factors). The growth fraction of a tumor represents the percentage of cycling cells relative to the total cell population. The generation time represents the time required for a cell to proceed through a single cell cycle (generally 24 to 36 hours for hematopoietic tissues). Surprisingly, in the case of acute myeloid leukemia (AML), the generation time of leukemic blasts is not shorter than that of normal hematopoietic progenitors and may be longer. The proliferative advantage of malignant hematopoietic cells (and of many nonhematopoietic tumors) stems, at least in part, from the fact that a higher percentage of cells are in cycle at any one point

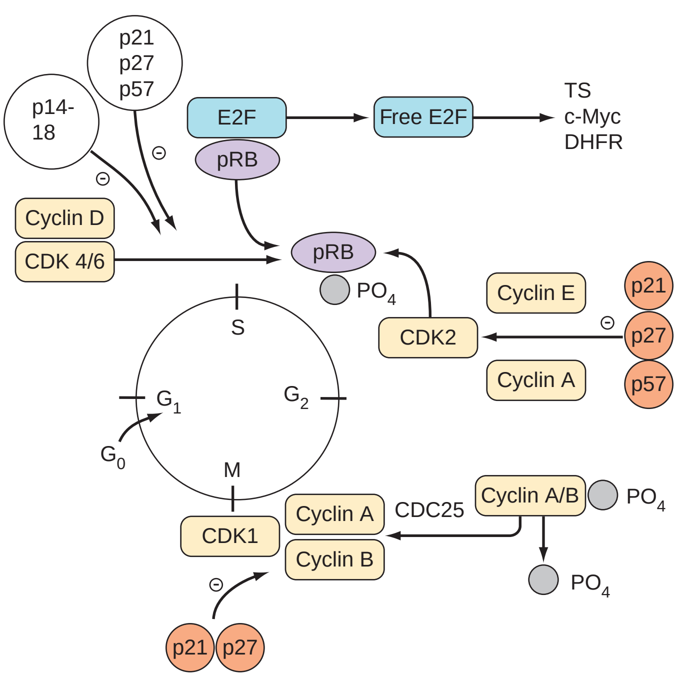


Figure 58.1 Progression through the cell cycle is controlled through complex interactions among cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors. Progression across the G_1S interface and through S phase involves the E2F transcription factor, which activates numerous enzymes (e.g., thymidylate synthase, dihydrofolate reductase) required for DNA replication. The pRb in its dephosphorylated state binds to and inactivates E2F in conjunction with DP proteins, thereby inhibiting S-phase progression. Conversely, phosphorylation of pRb antagonizes binding to E2F allowing S-phase events to proceed. Phosphorylation of pRb results from activation of (1) CDK2:cyclin A/E and (2) CDK4/6:cyclin D complexes. The former complexes are inhibited by the CDK1s p21, p27 (and p57), and the latter are inhibited by the low-molecular-weight CDK inhibitors (p14–18) but also by p21 and p27. The complex formed by CDK1 ($p34^{cdc2}$) and cyclins A and B regulates G_2M progression and is inhibited by the "universal" CDK inhibitor, p21. Moreover, its phosphorylation status, which plays a major role in determining activity, is regulated by the phosphatase cdc25. Proteins such as pRb, E2F, p21, and p27 can influence the response of cells to chemotherapeutic agents by controlling cell cycle progression and possibly via cell cycle–unrelated actions. *CDK*, Cyclin-dependent kinase; *CDK2*, cyclin-dependent kinase-2; *DHFR*, dihydrofolate reductase; *pRb*, retinoblastoma protein; *TS*, Thymidine synthase.

in time (i.e., the growth fraction is higher). The doubling time represents the period required for a tumor to double in mass and is, in general, inversely related to the tumor's growth fraction. Tumor-doubling times range from longer than 120 days in the case of some solid tumors (e.g., lung and colon) to less than 2 weeks (in some leukemias and lymphomas). Tumors with high growth fractions and short doubling times tend to be more sensitive to chemotherapy than slowly growing neoplasms with low growth fractions and long doubling times.

Cell cycle progression is governed by a complex network of proteins consisting of cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors. Progression through S phase is regulated primarily by CDK2 in association with cyclins A and E; progression through G_2M is regulated by CDK1 (p34cdc2) and cyclins A and B; and progression through G_1 involves CDKs 4 to 6 in conjunction with cyclin D. CDK inhibitors fall into two major categories: the low-molecular-weight inhibitors (pINK14, -15, -16, -17, and -18), which primarily inhibit cyclin D (and to some extent, CDK2) complexes, and the higher molecular-weight inhibitors, p21, p27, and p57, which are more universal in their actions and inhibit most or all CDKs. Signals for the progression of cells through G_1S are essential for maintenance of the neoplastic phenotype. In the commonly accepted model of G_1S progression, inactivation of the retinoblastoma protein (pRb) is required. In quiescent cells, pRb is in an active dephosphorylated state and bound to the transcription factor E2F. Phosphorylation of pRb by CDK4, CDK6, and CDK2 leads to release of E2F, which is then free to activate diverse genes essential for S-phase progression, such as MYC (also known as c-Myc), TS (thymidylate synthetase), and DHFR (dihydrofolate reductase). Conversely, induction of CDK inhibitors (e.g., by transforming growth factor-β [TGF-β] or differentiation-inducing agents) results in inactivation of CDK4, CDK6, and CDK2, dephosphorylation of pRb, inactivation of E2F, and inhibition of the progression through S phase. Aberrant expression of cyclins and CDK inhibitors is commonly encountered in hematopoietic malignancies.

In addition to growth control, cell cycle proteins are intimately involved in the regulation of programmed cell death (apoptosis) and checkpoint control mechanisms. Consequently, cell cycle regulatory proteins can exert a major influence on the response of neoplastic cells to cytotoxic agents. For example, when cells undergo DNA damage, they may arrest in G_2M or G_1, during which repair occurs, or if the damage is too severe, the cells undergo apoptosis. In particular, the tumor suppressor gene TP53 and its downstream inducible target p21 have been implicated in the G_1 arrest process after genotoxic insult. Dysregulation of various cell cycle regulatory proteins can have a major impact on the sensitivity of neoplastic cells to chemotherapeutic agents. Loss of the TP53 gene renders cells resistant to diverse chemotherapeutic agents, presumably by preventing cells from undergoing repair in G_1 and thereby inhibiting the cell death processes and allowing DNA damage to accumulate, culminating in cellular transformation. Conversely, transfection of P53-negative cells with wild-type P53 restores responsiveness to most drugs. Dysregulation of the CDK inhibitors p21 (a downstream target of P53) and p27 increases the sensitivity of neoplastic cells to various cytotoxic agents, possibly by uncoupling S-phase progression and mitosis. After DNA damage, checkpoints block the cell cycle, but loss of the CDK inhibitors p21 or p27 prevents cells from arresting in G_1 and cells die during G_2M. Mutations in the E2F protein have been shown to lengthen S phase and increase the sensitivity of malignant cells to S-phase–specific agents. Furthermore, cells lacking functional pRb have been shown to be significantly less sensitive to the actions of antimetabolites, including methotrexate.

In vivo the growth of tumors is limited by various factors such as vascular supply, nutritional requirements, and possibly physical restraints. Consequently, the rate of tumor growth declines as the number of cells increases. To the extent that tumor-doubling times are inversely correlated with drug responsiveness, large, late-stage tumors are less susceptible to cytotoxic drugs than early-stage tumors, with higher growth fractions. Most chemotherapeutic drugs kill by first-order kinetics. The implication of this phenomenon is that it requires the same drug dose to reduce the number of tumor cells from 10^4 to 10^1 cells as it does to reduce the tumor burden from 10^{10} to 10^7 cells.

Hematopoietic malignancy-initiating or stem cells, such as leukemia stem cells, appear to explain resistance and treatment failure. These cells express high levels of hematopoietic stem cell proteins and markers and are resistant to cell cycle–specific agents because of an increase in quiescent cell populations. They overlap with normal hematopoietic stem cells (HSCs) and there appears to be a set of HSCs that contain preleukemic-promoting mutations that both predispose and may be sufficient for conversion to leukemic stem cells. The proteins expressed by these cells have become targets of therapy, including alterations in DNA damage, quiescent cell cycle factors such as the thrombopoietin receptor, MPL, stem cell proliferation signals such as NOTCH and WNT protein families, and niche occupancy proteins such as KIT.

Cytotoxic agents may be divided into several categories with respect to their effects on the cell cycle or the cell cycle specificity of their actions, or both.

1. *Noncycle-active drugs* kill both cycling and noncycling cells in all phases of the cell cycle. Examples include steroids and antitumor antibiotics (except bleomycin).

2. *Cycle-active, nonphase-specific drugs* are more active against cycling cells and can kill cells in each phase of the cell cycle. However, such drugs may preferentially kill cells in a particular phase of the cell cycle. Examples include alkylating agents, cisplatin, and 5-fluorouracil (5-FU).
3. *Cycle-active, phase-specific drugs* primarily kill cells in a specific phase of the cell cycle. Examples include most antimetabolites, which are active against cells engaged in DNA synthesis (S-phase cells), and microtubule-active drugs (e.g., vinca alkaloids, taxanes), which kill cells in G_2M.

An example of a cytokinetically rational approach to chemotherapy involves the combination of a noncycle-active agent (e.g., daunorubicin) with a cycle- and a phase-specific agent (e.g., ara-C, fludarabine, decitabine, gemcitabine, clofarabine, and nelarabine). From a theoretical standpoint, administration of a noncycle-active agent may reduce tumor mass, leading in turn to an increase in the growth fraction caused by recruitment of cells into cycle. Such cells would then be more susceptible to a cycle- and phase-specific agent, particularly one administered over a prolonged interval. In the case of hematopoietic malignancies, attempts have been made to recruit neoplastic cells into the more susceptible S phase of the cell cycle through the use of hematopoietic growth factors. The success of such a strategy has been limited because of several factors, including the inability of growth factors to increase the S-phase fraction significantly, the lack of selectivity of this strategy, and the theoretical possibility that growth factors may protect neoplastic cells from apoptosis.

Unfortunately, cytokinetic differences between normal and neoplastic tissues have been difficult to exploit. Both normal hematopoietic stem cells and hematologic malignant stem cells have a low proportion of cells in G_1. However, prolonged dosage schedules can provoke these malignant cells into cell cycle and may explain their efficacy. Consequently, rapidly dividing normal tissues such as gastrointestinal epithelium and normal hematopoietic progenitors tend to be very sensitive to most chemotherapeutic agents. As a result, mucositis and myelosuppression represent frequent dose-limiting toxicities for many cytotoxic drugs.

Newer agents that target cell cycle proteins are often first utilized in patients with hematologic malignancies. As noted in the section on CDK and CHK1 inhibitors, these agents can have potent cytotoxic effects on dividing cells.

PHARMACOLOGY OF TRADITIONAL CHEMOTHERAPEUTIC AGENTS

Traditional agents are classified by their site of action, and as such all have a targeted mechanism, albeit not that of newer kinase targeted agents. They are divided into alkylating agents, antimicrotubule agents, antimetabolites, topoisomerase I or II inhibitors, platinum analogs, and miscellaneous agents. The pharmacology and cellular mechanisms of action of these agents are schematically presented in Fig. 58.2.

Alkylating Agents

Drug treatment for cancer began with the use of the mustard class of alkylating agents, initially mechlorethamine (nitrogen mustard), which entered into clinical use in the mid-1940s. Alkylating agents are used in many regimens but are rapidly being supplanted by newer classes of agents.

All alkylating agents undergo molecular rearrangements to form covalent bonds to DNA bases. Some form monoadducts, while others form cross-links either with intrastrand or interstrand bases, or with adjacent proteins. All alkylating agents are cytotoxic to tumor cells through interruption of DNA replication, induction of DNA damage repair and stress response, or through checkpoints in the cell cycle that result in apoptosis, senescence, or necrosis. All alkylating agents induce DNA strand breaks directly or through the damage response. Alkylating agents can also cause DNA mutations that can induce a cytotoxic response or can alter proteins, leading to immune responses to novel protein sequences.

Mechanisms of Resistance

Efficient removal of the DNA adducts reduces the lesion burden, while loss of DNA damage recognition can invoke damage tolerance, as in the case of loss of MMR and temozolomide tolerance. Detoxifying enzymes (glucuronidating enzymes and metabolizing enzymes residing in the liver) can serve as acceptor molecules for alkylation, alter the agent prior to DNA attack, or metabolize the parent compound. Loss of TP53 results in loss of cell cycle checkpoint induction of DNA repair signals. Increased AKT signaling promotes cell proliferation to compensate for the DNA damage response-associated toxicity.

Nitrogen Mustard

The nitrogen mustard class includes mechlorethamine, cyclophosphamide, 4-hydroperoxy cyclophosphamide, ifosfamide, chlorambucil, and melphalan. These drugs all share a common bischloroethyl group attached to nitrogen and a substituted “R” group that provides drug specificity (Fig. 58.3). All nitrogen mustards react with DNA in an SN2 reaction, a bimolecular nucleophilic displacement reaction also called a second-order reaction. Although numerous sites are targeted for alkylation, nucleophiles in DNA, including nitrogen (N), oxygen (O), and phosphate (P), attract the chloroethyl moiety attached to the R–N backbone, and the chlorine is displaced by the nucleophilic atom to form an aziridinium moiety. The remaining chloroethyl group is then attracted to a second nucleophilic atom, forming a second aziridinium intermediate, leading to a second alkylation, forming a cross-link. Both intrastrand and interstrand cross-links are formed. The N7 guanine position is the most critical for cytotoxic cross-link formation. Clinical use of mechlorethamine is limited because the MOPP regimen (mechlorethamine, vincristine [Oncovin], procarbazine, prednisone) has been replaced by ABVD (doxorubicin [Adriamycin], bleomycin, vinblastine, dacarbazine [DTIC]) in Hodgkin lymphoma (see Chapters 78 and 80). Local use of mechlorethamine occurs in a dermatologic suspension for the treatment of cutaneous T-cell lymphomas (CTCLs; see Chapter 89).

Cyclophosphamide is a nitrogen mustard with a ringed structure off the end-chloroethyl backbone that decreases spontaneous decomposition (see Fig. 58.3). Enzymatic activation is required through multifunction P450 enzymes in the liver. Phenobarbital and corticosteroids may alter activation. The bioavailability of cyclophosphamide orally and intravenously is quite similar, although most use of the drug is by bolus IV injection. Cyclophosphamide is detoxified through oxidation to 4-keto-cyclophosphamide and carboxyphosphamide by aldehyde dehydrogenase. Cyclophosphamide is used in doses as small as 50 to 100 mg/day orally and in bolus doses of 400 to 700 mg/m^2 for solid tumors and 750 mg/m^2 in combination with doxorubicin and vincristine and prednisone as part of the CHOP regimen for NHLs, or alone or with bortezomib for patients with myeloma. It is also used at doses of up to 60 mg/kg/day for 4 days in autologous and allogeneic bone marrow (BM) transplantation protocols. Cyclophosphamide is used in numerous treatment protocols for NHLs and high-dose therapy regimens designed to eradicate tumor and BM in patients with lymphomas and leukemias and those undergoing BM transplantation. It is commonly used with granulocyte colony-stimulating factor (G-CSF) for mobilizing hematopoietic stem cells to be collected before autologous transplantation.

Cyclophosphamide is metabolically activated by cytochrome P450 mixed function oxidases in the liver to 4-hydroxycyclophosphamide. 4-Hydroxycyclophosphamide is further converted to aldophosphamide and then to phosphoramide mustard, the alkylated species, and acrolein. High levels of aldehyde dehydrogenase detoxify cyclophosphamide in hematopoietic stem cells and, thus, high doses are

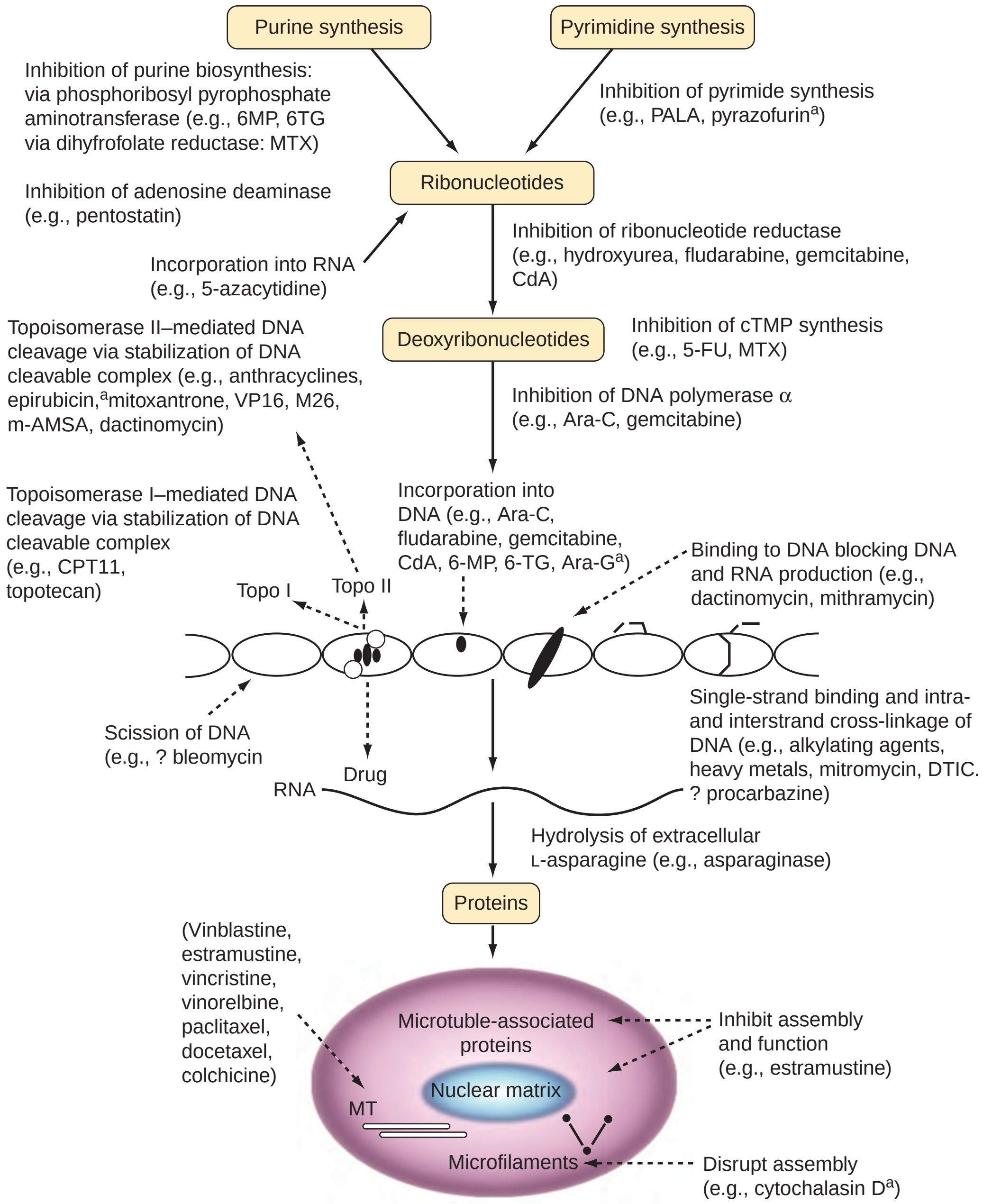

Purine synthesis
Pyrimidine synthesis
Inhibition of purine biosynthesis:
via phosphoribosyl pyrophosphate
aminotransferase (e.g., 6MP, 6TG
via dihyfrofolate reductase: MTX)
Inhibition of pyrimide synthesis
(e.g., PALA, pyrazofurin[a])
Ribonucleotides
Inhibition of adenosine deaminase
(e.g., pentostatin)
Incorporation into RNA
(e.g., 5-azacytidine)
Inhibition of ribonucleotide reductase
(e.g., hydroxyurea, fludarabine, gemcitabine,
CdA)
Topoisomerase II–mediated DNA
cleavage via stabilization of DNA
cleavable complex (e.g., anthracyclines,
epirubicin,[a]mitoxantrone, VP16, M26,
m-AMSA, dactinomycin)
Deoxyribonucleotides
Inhibition of cTMP synthesis
(e.g., 5-FU, MTX)
Inhibition of DNA polymerase α
(e.g., Ara-C, gemcitabine)
Topoisomerase I–mediated DNA
cleavage via stabilization of DNA
cleavable complex
(e.g., CPT11,
topotecan)
Incorporation into
DNA (e.g., Ara-C,
fludarabine, gemcitabine,
CdA, 6-MP, 6-TG, Ara-G[a])
Binding to DNA blocking DNA
and RNA production (e.g.,
dactinomycin, mithramycin)
Topo I
Topo II
Scission of DNA
(e.g., ? bleomycin
Drug
RNA
Single-strand binding and intra-
and interstrand cross-linkage of
DNA (e.g., alkylating agents,
heavy metals, mitromycin, DTIC.
? procarbazine)
Hydrolysis of extracellular
L-asparagine (e.g., asparaginase)
Proteins
(Vinblastine,
estramustine,
vincristine,
vinorelbine,
paclitaxel,
docetaxel,
colchicine)
Microtuble-associated
proteins
Inhibit assembly
and function
(e.g., estramustine)
Nuclear matrix
MT
Microfilaments
Disrupt assembly
(e.g., cytochalasin D[a])

Figure 58.2 OVERVIEW OF SITES AND MECHANISMS OF ACTION OF THE MOST USEFUL CHEMOTHERAPEUTIC AGENTS. *5-FU*, 5-Fluorouracil; *6-MP*, 6-mercaptopurine; *ara-C*, cytarabine; *ara-G*, 9′-β-D-arabinofuranosylguanine; *DTIC*, dacarbazine; *Topo*, topoisomerase.

not marrow ablative. Acrolein is a highly reactive aldehyde and the cause of hemorrhagic cystitis. Mercaptoethane sulfonate (Mesna) is used to provide prophylaxis against hemorrhagic cystitis caused by cyclophosphamide and ifosfamide, and is now standard for doses of cyclophosphamide and ifosfamide above 1000 mg/m^2. Mesna is given in divided doses every 4 hours or as a continuous infusion for 18 to 24 hours in a dose equivalent to either cyclophosphamide or ifosfamide. Other than hemorrhagic cystitis, BM suppression is dose limiting and can be rescued by reinfusion of autologous or allogeneic hematopoietic progenitor cells. Other toxicities include alopecia and cardiac toxicity, which is unusual and most often seen after high-dose therapy.

4-Hydroperoxycyclophosphamide, a chemically stable form of the reactive intermediate of cyclophosphamide, 4-hydroxycyclophosphamide, is more toxic to committed hematopoietic progenitors such as colony-forming unit–granulocyte/macrophages (CFU-GM), burst-forming unit–erythroids (BFU-E), and colony-forming unit–erythroids (CFU-E).

Melphalan, or phenylalanine mustard, has an amino acid side chain that alters its cellular uptake and stabilizes its structure, allowing PO administration (see Fig. 58.3). It is available in both PO and IV forms, and has a similar effect on DNA cross-linking as cyclophosphamide and the other nitrogen mustards. Melphalan uptake by cells is by means of a neutral amino acid transporter. Its rate of cross-link formation is much slower than that of mechlorethamine, presumably because of delayed metabolism. Oral melphalan is used predominantly for the standard treatment of multiple myeloma (MM) and IV in high-dose regimens in preparation for stem cell transplantation (see Chapter 91) for MM patients.

Chlorambucil has been used for more than 40 years for the treatment of CLL. Chlorambucil is the phenylbutyric acid derivative of nitrogen mustard and is very stable, entering the cell by diffusion rather than by a specific uptake mechanism. It is typically administered orally on a daily basis or intermittently. It appears to have greater bioavailability than melphalan and a more consistent half-life of approximately 2 hours.

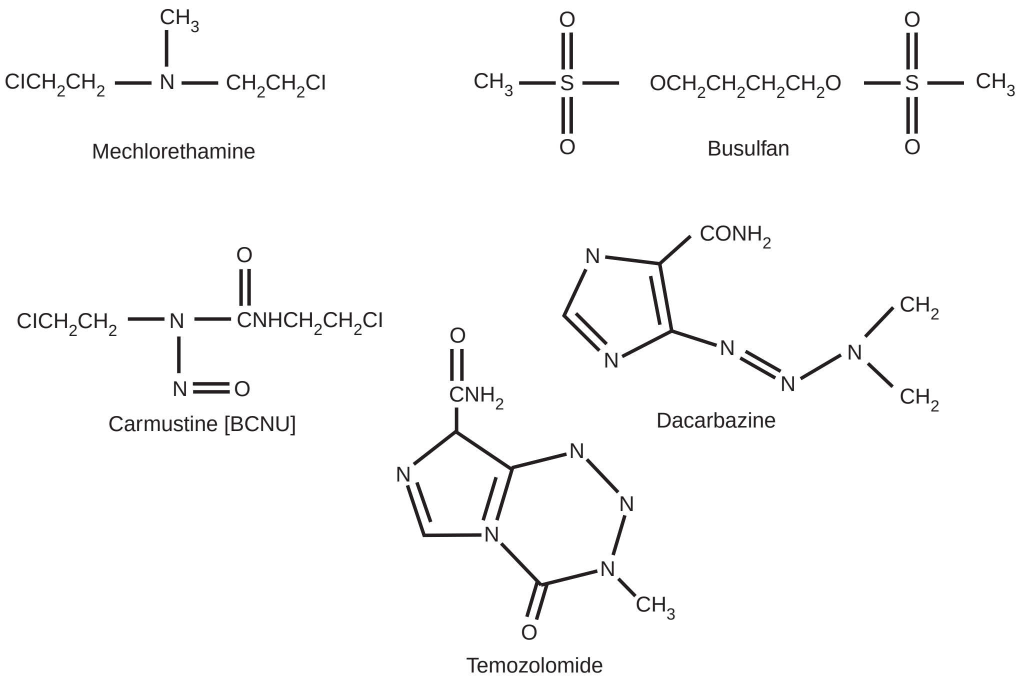


Figure 58.3 STRUCTURE OF COMMON ALKYLATING AGENTS.

Busulfan is an alkylsulfonate unique among alkylating agents because of two sulfur groups and lack of a chloroethyl moiety (see Fig. 58.3). Busulfan, similar to the nitrogen mustards, reacts predominantly at the N^7 position of guanine and produces an N^7–N^7 biguanyl DNA cross-link, although the precise nature of this cross-link appears different than that of the nitrogen mustards. The pharmacokinetics of busulfan is important for its use in high-dose therapy for ablation of the BM in patients undergoing autologous transplantation for acute leukemia or allogeneic stem cell transplantation (see Chapters 104–107). Because the incidence of veno-occlusive disease is lower in patients receiving high-dose busulfan with predosing pharmacokinetics performed, this is now recommended in high-dose regimens, the target being an area under the curve (AUC) of 1125 μ mol/L × min (range 900 to 1350) with every 6-hour dosing. Busulfan is a potent stem cell toxin, killing both early and late hematopoietic progenitor cells and damaging the BM stroma. Other toxicities of busulfan include nausea and vomiting, and pulmonary interstitial and intra-alveolar edema leading to fibrosis. The pulmonary fibrosis is distinct from the interstitial pneumonitis, which accompanies allogeneic stem cell transplantation and is not related to cytomegalovirus (CMV) or other viral infections.

Nitrosoureas

Four chloroethyl nitrosoureas and one methyl nitrosourea are in clinical use. These agents are different from the nitrogen mustards in that they alkylate through an SN^1 reaction, forming a highly reactive intermediate in the presence of N, O, and P nucleophiles in DNA. The commonly used clinical agent is (2-chloroethyl)-*N*-nitrosourea (BCNU). *N*-([4-amino-2-methyl-5-pyrimidinyl] methyl)-*N*-(2-chloroethyl)-*N*-nitrosourea (ACNU) is commonly used in Japan. A third agent, *N*-(2-chloroethyl)-*N*-cyclohexyl-*N*-nitrosourea (CCNU), is used predominantly as an PO nitrosourea in children with brain tumors. All of these compounds have high hydrophobicity, actively penetrating the blood–brain barrier.

The DNA alkylation sites include N^7 and O^6 of guanine. Chloroethylation at the O^6 position of guanine appears critical to cytotoxicity. DNA cross-linking by chloroethyl nitrosoureas include at 1-(3-cytosinyl), 2-(1-guanyl) ethane, and 1-2-bis(7-guanyl) ethane. The former is responsible for much of the cytotoxicity observed with the chloroethyl nitrosoureas. It is formed after alkylation at the O^6 position of guanine. This adduct undergoes intramolecular rearrangement to a circular intermediate, N^1. O^6 ethanoguanine is formed, which can then rearrange by attack at the opposite hydrogen-bonded base N^3 of cystine, forming the interstrand cross-link. This is a unique DNA cross-link and is poorly recognized by DNA repair processes, leading to marked cytotoxic potency of this cross-link.

The pharmacokinetics of the chloroethyl nitrosoureas reveal a very short half-life. BCNU is predominantly used for high-dose treatment of recurrent lymphomas. Regimens including BCNU induce sustained complete remission (CR) rates of approximately 40% to 60%. Doses of between 300 and 600 mg/m^2 have been safely administered. The chloroethyl nitrosoureas cause profound and cumulative BM suppression at conventional doses of 120 to 150 mg/m^2, limiting treatment to three to five cycles at 6-week intervals.

Complications of high-dose BCNU therapy include pulmonary toxicity and renal toxicity at doses higher than 600 mg/m^2. Pulmonary toxicity, as evidenced by a decrease in the DLCO (diffusing capacity of the lung for carbon monoxide), occurs in up to 40% of patients. It can be managed with high-dose oral steroids during the inflammatory phase. Interstitial nephritis with glomerulosclerosis, interstitial fibrosis, and dropout of tubules has been reported with BCNU or CCNU.

Methylating Agents

Four methylating alkylating agents are in clinical use. These include procarbazine, DTIC, streptozotocin, and temozolomide. Procarbazine and DTIC are triazines. Streptozotocin is a monofunctional methyl nitrosourea derivative with an attached sugar moiety, and temozolomide is an imidazotetrazine. All react with DNA by undergoing SN^1 reactions forming a methyldiazonium ion, resulting in methylation of N^7 guanine (67%), O^6 guanine (9%), O^4 and O^2 thymine (2%), and N^3 adenine (3%). None form DNA cross-links. However, all induce high levels of DNA methylation, and their recognition and repair results in both single- and double-strand breaks. N^7 methylguanine is removed through the BER system. Recognition by the *N*-methylpurine glycosylase results in removal of the adducted base with formation of an abasic site that is recognized by the apurinic (AP) endonuclease, which then cleaves the backbone at the AP site. Subsequently, the free 5′ sugar is released by DNA lyase, with repair initiated by β-polymerase and DNA ligase. BER effectively removes N^7 methylguanine and N^3 methyladenine, and restores DNA to normal. Inhibition of BER by the investigational agent methoxyamine (TRC102) blocks this pathway and increases toxicity.

O^6 methylguanine mispairs with thymine during DNA synthesis, resulting in a lesion recognized by the MMR system. Mispair recognition proteins are MSH6, MSH3, and MSH2. Recognition of the mispair recruits additional proteins to the complex, including MLH1 and PMS1/PMS2. These proteins initiate exonuclease cleavage of a long patch in the newly synthesized strand of DNA. This is then repaired by polymerases-δ and -ε. If unrepaired, a thymine is repeatedly inserted opposite the O^6 methylguanine, resulting in multiple single-strand breaks. Cells expressing high levels of the DNA repair protein for O^6 methylguanine, O^6 methylguanine-DNA methyltransferase (MGMT), are approximately 10-fold more resistant to methylating agents than MGMT-negative cells. Cells lacking MMR are very resistant to methylating agents. Acquisition of MMR defects is associated with acquired resistance to methylating agents and cisplatin, which is also recognized by this protein complex.

Procarbazine was synthesized as a monoamine oxidase inhibitor and has been used since the 1950s for the treatment of Hodgkin lymphoma and NHL, as well as a component of combination therapies for gliomas. DTIC is metabolically activated by cytochrome P450 microsomal oxidoreductases, ultimately leading to formation of the methyldiazonium ion and DNA methylation. DTIC is used in combination with ABVD for treating Hodgkin lymphoma (see Chapters 79 and 80) and is also used for patients with metastatic malignant melanoma in combination with BCNU, cisplatin, and tamoxifen. Activation of DTIC requires hydroxylation of one terminal methyl group caused by demethylation forming 5-[3-methyl-triazen-1-yl]-imidazole-4-carboxamide (MTIC), with spontaneous decomposition to the methyldiazonium ion, which alkylates the DNA, as noted earlier. Maximum tolerated doses of DTIC are approximately 1000 mg/m^2, with myelosuppression and gastrointestinal toxicity (including severe watery diarrhea) being the most common side effects.

Temozolomide represents an imidazotetrazinone. It differs from DTIC in that it is chemically degraded to the monomethyl triazine, MTIC, at neutral pH and does not require P450 enzymatic demethylation. Compared with DTIC, temozolomide has much more consistent pharmacokinetic parameters, including peak serum concentrations, volume of distribution and clearance, and conversion to MTIC. Clinical studies documented considerable activity in acute leukemias. The dose-limiting toxicity was thrombocytopenia and, less frequently, neutropenia, with maximum tolerated doses of 1000 mg/m^2 given over 5 days on a daily or twice-daily regimen. Nausea and vomiting were the other common side effects, easily controlled with antiemetics.

Bendamustine

Bendamustine is comprised of a 2-chloroethylamine nitrogen mustard alkylating group, a benzimidazole ring, and a butyric acid side chain. Its mechanism of action is unknown but appears to be different than other alkylating agents, causing DNA damage that is repaired predominantly by the base-excision repair system and has activity against lymphoid cell lines resistant to alkylating agents. Myelosuppression with leukopenia and thrombocytopenia is the most frequent side effect, with mild nonhematologic side effects including nausea, fatigue, constipation, and diarrhea. Phase III studies comparing standard treatment with bendamustine and rituximab in front-line treatment of CLL and indolent lymphomas (see Chapter 76) have established this combination as a first-line treatment. Bendamustine is also active against MM, and combinations with steroids and bortezomib or the immunomodulatory agents thalidomide and lenalidomide have been reported to result in high response rates in patients with relapsed or refractory disease.

Alkylating Agent–Induced Leukemias

Alkylating agents induce dose-limiting myelosuppression and cause sublethal DNA damage to hematopoietic progenitors, causing mutational events that lead to malignant transformation to preleukemic and leukemic states. A concern exists about the use of hematopoietic growth factors after exposure to alkylating agents (see Chapter 59). There is evidence of increased cytotoxicity to hematopoietic progenitors during simultaneous exposure to these agents and growth factors. Treatment-related AML (t-AML) accounts for approximately 15% of all adult AML. Approximately 50% of t-AML patients have a preleukemic phase compared with only 10% of patients with de novo AML. CRs are achieved in 15% to 30% of patients with t-AML and a mean remission duration of 2 months. Chromosomal abnormalities and gene mutations characteristic of t-AML establish this as a distinct disease requiring novel therapeutic approaches. Loss or deletion of all or part of the long arm [q] of chromosomes 5 or 7 is common, as are trisomy 8 and deletions of the short arm of chromosomes 12, 17, and 21.

Historically, patients with Hodgkin lymphoma treated with mechlorethamine and procarbazine in the MOPP regimen or with CCNU were at the highest risk if exposed to radiation as well as an alkylating agent combination. Patients with polycythemia vera treated with chlorambucil were at much higher risk than patients treated with phlebotomy alone, which can contribute to a shift in treatment strategy. Patients with myeloma and ovarian cancer have developed t-AML, especially after prolonged exposure to alkylating agents. Patients treated with alkylating agents for benign diseases such as nephritis, lupus, psoriasis, rheumatoid arthritis, and Wegener granulomatosis also have an increased risk of t-AML. The mean latency between exposure and t-AML from alkylating agents is 4 to 5 years, in contrast to t-AML from etoposide, which has a latency period as short as 1 year. The cumulative risk of developing t-AML is between 10% and 17% at 4 to 6 years for myeloma patients treated with melphalan and between 2% and 10% at 7 to 10 years in patients with Hodgkin lymphoma. Alkylating agent-associated t-AML has also been recognized in patients with breast and colon cancer.

Antimicrotubule Agents

The antimicrotubule drugs include the vinca alkaloids (e.g., vincristine, vinblastine, vinorelbine and vindesine), taxanes (e.g., paclitaxel and docetaxel), and epothilones (ixabepilone).

Molecular Targets

The vinca alkaloids are naturally occurring (vincristine and vinblastine) or semisynthetic (vinorelbine) nitrogenous bases derived from the pink periwinkle plant, *Catharanthus roseus*. Paclitaxel was originally isolated from the bark of the Pacific yew, *Taxus brevifolia*. Paclitaxel can also be isolated from other members of the *Taxus* genus and from a fungal endophyte that grows on the Pacific yew. Docetaxel is derived semisynthetically from 10-deacetyl-baccatin III, which is obtained from the needles of the European yew, *Taxus baccata*.

The vinca alkaloids bind to the protein tubulin at a site distinct from that of the taxanes and, at low concentrations, inhibit microtubule dynamics. At higher concentrations, these vinca alkaloids disrupt microtubules and mitotic spindle, resulting in cell cycle mitotic arrest and apoptosis of cells (see Fig. 58.3). In contrast, after binding to α -tubulin, taxanes kinetically stabilize microtubule dynamics at their plus ends and shift the equilibrium toward tubulin polymerization into microtubule bundles. This also causes mitotic arrest and apoptosis of cells. The mitotic arrest caused by antimicrotubule drugs is associated with phosphorylation of the B-cell lymphoma (BCL2) protein and increased intracellular levels of the BCL2-associated X protein (Bax), which promote apoptosis.

As natural products, overexpression of the efflux pump multidrug resistance gene-1 (MDR-1) and the ABCB-1 transporter mediate resistance. Intracellular resistance to mitotic spindle and microtubule formation is mediated by numerous pathways and proliferative signals including MYC, nuclear factor kappa-B (NFκB), and AKT.

Antimicrotubule agents, particularly the vinca alkaloids, are used in the management of lymphomas and leukemias, and continue to be used in the mainstay of clinical chemotherapeutic regimens. Because of their mechanism of action, they are best used in multiagent combinations, in which potentiation of efficacy with other classes of agents, such as antimetabolites and DNA-damaging agents, provide better therapeutic responses and well-tolerated treatments.

Inhibitors of Nucleotide Synthesis

Within this class are two important agents used for hematologic malignancies, hydroxyurea and methotrexate. They both serve to disrupt nucleotide synthesis, and slow or stop DNA and RNA synthesis.

Hydroxyurea

Hydroxyurea inhibits ribonucleotide diphosphate reductase, blocking de novo synthesis of purines and pyrimidines. S-phase arrest is commonly observed. Given this exquisite cell cycle specificity, resting and quiescent cells are rarely affected and there is virtually no hematopoietic stem cell toxicity. As a consequence of disruption of nucleotide synthesis and direct binding to telomere binding factors, telomere synthesis and function are compromised in leukemic cells. In cells arrested in S phase, apoptosis and senescence are observed.

Hydroxyurea is used in the treatment of myeloproliferative neoplasms including essential thrombocythemia, polycythemia vera, and myelofibrosis (see Chapters 69–72). It may also be used for acute cytoreduction prior to induction therapy of AML and for management of chronic myelomonocytic leukemia. It is commonly used for extended periods of time to induce fetal hemoglobin and reduce sickle crisis in patients with sickle cell anemia (see Chapters 42 and 43).

Folic Acid Analogs (e.g., Methotrexate)

Folic acid analogs block the formation of thymidine by inhibition of thymidylate synthase, resulting in accumulation of uridine-5'-monophosphate (UMP), leading to high levels of uridine-5'-triphosphate (UTP) which is incorporated into DNA and causes purine nucleotide pool imbalance, slowing DNA synthesis. These agents bind thymidylate synthase and DHFR and disrupt cell cycle progression and cell division. Methotrexate polyglutamation results in higher affinity binding to DHFR and improved inhibition. Aminopterin, the first antifolate, was used by Sidney Farber (reported in 1948) and became one the first chemotherapeutic agents to be administered with success to children with acute lymphocytic leukemia (ALL). Methotrexate is the agent currently used in hematologic diseases, while the family of agents includes pemetrexed and trimetrexate. Inhibition of folate-dependent methyl transfer enzymes disrupts purine synthesis pathways. Toxicity of methotrexate is reversed by N^5-formyl FH_4, or leucovorin, which serves as a direct folate coenzyme. Since it has no tumor selectivity, normal tissues in active cell cycle are affected, including mucosa, BM, and hair follicles, resulting in mucositis, pancytopenia, and alopecia. Prolonged use is associated with pulmonary and liver fibrosis. High-dose therapy and excessive periods of high blood levels result in interstitial nephritis, sometimes requiring dialysis.

The most common resistance is due to amplification of DHFR expression by upregulation of translation and gene amplification, including the establishment of minichromosomes with the DHFR gene. A second mechanism is decreased affinity of DHFR to methotrexate. A third mechanism is decreased thymidylate synthase, and the fourth mechanism of resistance is impaired methotrexate polyglutamate formation.

While use has diminished in recent years, methotrexate remains part of the regimen for maintenance in children and adults with ALL (see Chapter 66). It is also used to depress T-lymphocyte proliferation after allogeneic stem cell transplantation to prevent acute graft-versus-host disease (GVHD). High-dose methotrexate, with careful blood level monitoring to prevent acute renal toxicity and mucositis, and leucovorin rescue, is effective in the management of CNS leukemias, primary CNS lymphoma, and highly proliferative fraction (c-myc-positive and high KI67) intermediate- and high-grade lymphomas, including Burkitt.

Nucleoside Analogs

The nucleoside analogs exhibit structural similarities to naturally occurring nucleosides and are incorporated into either DNA or RNA with lethal consequences. Alternatively, they block key enzymes in de novo purine or pyrimidine biosynthesis. There are two broad categories:

1. Pyrimidine analogs (e.g., ara-C, 5-azacytidine, gemcitabine)
2. Purine analogs (e.g., 6-thioguanine [6-TG], 6-mercaptopurine, fludarabine, chlorodeoxyadenosine, deoxycoformycin, clofarabine, nelarabine)

These categories are not mutually exclusive; for example, some nucleoside analogs (e.g., ara-C and 6-TG) also inhibit enzymes involved in DNA or deoxyribonucleotide biosynthesis. These agents are predominantly cycle-active agents and in most cases are phase-specific, being primarily active against cells in S phase. Because the growth fraction of hematologic malignancies tends to be higher than that of nonhematologic malignancies, nucleoside analogs are particularly useful in the former disorders. In contrast to alkylating agents, nucleoside analogs have limited carcinogenic and leukemogenic potential. The fluorinated pyrimidines (e.g., 5-FU) are generally not used in treating hematologic disorders and are not discussed further.

Ara-C has been used in the treatment of acute leukemias—particularly acute nonlymphocytic leukemias, aggressive lymphomas including Burkitt, and CNS lymphomas for over 30 years. It is incorporated into DNA during replication and inhibits polymerase-α, is cell cycle specific, and penetrates the blood–brain barrier. In rapidly replicating cells, it stalls polymerase function, leading to replication fork collapse and strand breaks at the replication fork, signaling apoptosis and differentiation.

Mechanisms of Resistance

Although ara-C remains the backbone of therapy for aggressive hematologic malignancies, resistance has been observed that is both specific to the drug and nonspecific. Specific resistance could be due to nucleotide transport down regulation, uncommon in dividing cells. Inside the cell, ara-C is phosphorylated by deoxycytidine kinase to the active 5′-triphosphate derivative ara-cytidine triphosphate (CTP), whereas catabolism of ara-C by cytidine deaminase (CDD) to the nontoxic metabolite arabinoside uridine is a major pathway of detoxification that can be overcome by high-dose therapy. Low penetration into the cerebrospinal fluid is overcome by bolus administration of very high doses: 1 to 2 g/m^2 over 1 hour. Other resistance mechanisms appear nonspecific and result in leukemia tolerance to cell cycle arrest and induction of apoptosis, tolerance to DNA strand breaks, and rapid cell division after therapy.

Clinical Use

Standard induction therapy for AML includes a 5- to 7-day continuous infusion of 100 to 200 mg/m^2 or high-dose ara-C at doses of 1 to 3 g/m^2 over 1 hour every 12 hours. Many modifications to these schedules have improved tolerance without sacrificing efficacy. For CNS leukemia, a depo form of ara-C has been developed with improved tolerance and sustained cerebrospinal fluid levels.

Subcutaneous (SC) administration has provided responses in older individuals with AML or myelodysplastic syndromes (MDS) who do not respond well to more intensive induction therapy.

5-Azacytidine and Decitabine

These agents were initially developed as antimetabolites but are more effective at low doses that inhibit the function of histone demethylation and are described below.

Gemcitabine

Developed as an agent for the treatment of pancreatic cancer, gemcitabine has gained therapeutic attention for use in patients with Hodgkin lymphoma and NHL, particularly in the relapsed state.

Like other nucleotide analogs, gemcitabine is a prodrug that is phosphorylated by deoxycytidine kinase to gemcitabine diphosphate (FdCDP) and gemcitabine triphosphate (dFdCTP), which, when incorporated into DNA, stalls the polymerase-α, which adds one more deoxynucleotide to the elongating strand. The polymerase replication complex then falls off the DNA, causing replication fork collapse and chain termination. In addition, FdCDP is a potent inhibitor of ribonucleoside reductase, causing depletion of deoxyribonucleotide pools and further encouraging dFdCTP incorporation while disrupting DNA synthesis.

Specific resistance is caused by upregulation of deoxycytidine deaminase, which metabolizes gemcitabine to 2,2′-difluorodeoxyuridine. Nonspecific resistance emerges due to upregulation of membrane transporters, although their role in clinical resistance is less clear.

Numerous studies have identified the utility of adding gemcitabine to cisplatin and other agents for the treatment of both relapsed Hodgkin lymphoma and NHL, and CTCLs. The combination of oxaliplatinum or cisplatin and gemcitabine is effective and well tolerated, and the majority of patients remain eligible for autologous stem cell collection and transplantation.

Fludarabine

Fludarabine was introduced 25 years ago as an agent with potent activity against lymphoid malignancies and remains an active agent in CLL and other low-grade lymphomas. It has since emerged as an agent in combination that is effective in leukemias, and in induction therapy for nonmyeloablative conditioning prior to allogeneic stem cell transplantation. It is recognized for its immunosuppressive function and inducing tolerance to allografts transplantation. Fludarabine phosphate is the 2-fluoro, 5′-monophosphate derivative of vidarabine (9′-β-D-arabinofuranosyladenine [ara-A]) and is converted to the di- and triphosphate by intracellular kinases, as are gemcitabine and ara-C. It has greater potency because it confers resistance to deamination by adenosine deaminase (ADA) and improved solubility. It is incorporated into DNA as a nucleotide and causes chain termination. It is a potent inhibitor of cytosolic 5′-nucleotidase II. It is also a substrate for uracil glycosylase, causing abasic sites as the first stem in BER and has recently been used in combination with TRC102, which binds to these sites and prevents their repair. Like other nucleoside analogs, it causes replication fork collapse, double-strand breaks, and induction of P53, leading to apoptotic signaling. Its efficacy against normal lymphoid T and B cells appears linked to both cytotoxicity against proliferating cells and resting cells; the latter effect is mediated by interference with the normal activity of the nucleotide excision repair pathway.

Fludarabine is a complex agent. Resistance is multifactorial including active transport, decreased cytosolic 5′-nucleotidase II and deoxycytokine kinase, and Ku80 binding to telomerase. Other nonspecific mechanisms include mutations in *p53* or loss due to chromosome deletion, which is important in many cases of CLL, and other proliferation-associated genes such as *NOTCH1*, *SF3B1*, and *BIRC3*. Low-level miR-34a is also associated with fludarabine resistance.

Fludarabine, at a standard dose of 25 mg/m^2 for 5 days and rituximab with or without cyclophosphamide were the mainstay of primary treatment for CLL (see Chapter 76). Fludarabine has also been used in refractory leukemias, marginal cell and other low-grade lymphomas. Recognition of the appearance of lymphopenia after treatment led to studies establishing fludarabine as part of the nonmyelosuppressive preparative regimens for allogeneic transplantation. This is most often at a dose of 30 to 35 mg/m^2 for 5 days and used in combination with radiation, melphalan, or cyclophosphamide. In addition to lymphopenia, multiple cycles are associated with prolonged myelosuppression and a chronic peripheral neuropathy.

Clofarabine

Clofarabine (2-chloro-2′-arabino-flouro-2′-deoxyadenosine) is a purine analog with activity in patients with relapsed acute leukemia. Its activation requires cellular uptake and conversion to the triphosphate nucleotide. It then decreases ribonucleotide reductase; alters nucleotide precursors; inhibits and reduces the function of antiapoptotic proteins such as Bcl-X(L), Mcl-1, and Bax with dephosphorylation of akt; and inhibits DNA synthesis.

It appears to be more active against B-cell than T-cell, especially in AML and MDS. It is well tolerated. Recent studies show its significant efficacy in ara-C–refractory AML. Reversible liver toxicity and myelosuppression can be dose-limiting (see Chapters 60–62).

Nelarabine

Nelarabine (9-β-D-arabinofuranosylguanine) is another FDA-approved purine analog for the treatment of refractory T-cell leukemias and lymphomas (see Chapters 66–68).

As a nucleotide analog, it preferentially accumulates in T cells and is incorporated into DNA, causing chain termination and inhibiting DNA synthesis. The FDA approved this drug after analyzing the results of two phase II clinical trials, one in pediatric T-cell ALL and the other in adults with T-cell lymphoblastic lymphoma. In both cases, patients had relapsed after at least two induction regimens. Because CRs were seen in 13% of the 39% of pediatric patients and in 18% of the 28 adult patients, the FDA granted approval. Neurologic toxicity is dose limiting. Good response rates, including CRs, have been seen in patients with refractory T-cell leukemias.

Reduced ara-G incorporation into DNA is likely due to altered nucleotide transport, increased nucleotidase activity, reduced nucleoside kinase activity, and nonspecific resistance mechanisms as described earlier for this class of agent.

Inhibitors of DNA Topoisomerase I and II

Inhibitors of DNA topoisomerases I and II include such drugs as doxorubicin, daunorubicin, mitoxantrone, etoposide, and topotecan. Before describing the specific inhibitors, a brief review of the drug targets (topoisomerase enzymes) will be presented (Table 58.2).

DNA Topoisomerase I

Topoisomerase I is a ubiquitous enzyme whose function in vivo is to relieve the torsional strain in DNA, specifically to remove positive supercoils generated in front of the replication fork and to relieve negative supercoils occurring downstream of RNA polymerase during transcription. Topoisomerase I is catalytically active as a 100-kDa monomer and is concentrated in nucleoli, although smaller amounts are found in a diffuse nuclear distribution. The gene for this enzyme is located on human chromosome 20q12–13.2. Topoisomerase I does

TABLE 58.2 **Characteristics of Mammalian DNA Topoisomerases**

	Topoisomerase I	Topoisomerase IIα and Topoisomerase IIβ	
Size of monomer (kDa)	100	170	180
mRNA (kb)	4.2	6.2	6.5
Chromosome	20q12–13.2	17q21–22	3p24
DNA cleavage	Single-strand breaks	Double-strand breaks	Double strand
Covalent intermediate	$3'PO_4$-Tyr^{723}	$5'PO_4$-Tyr^{804}	$5'PO_4$-Tyr^{821}
ATP requirement	No	Yes	Yes
Nuclear location	Nucleoli, diffuse	Nuclear matrix and scaffold, nucleoli	Nucleoli Nuclear matrix
Cell cycle dependence	None	Yes, maximum in G_2/M	None
Nuclear localization signal	NH_2-end	COOH-end	COOH-end
Phosphorylation	By CK II and PKC (increases activity)	By CK II, PKC, p34^{odc2}, MAPK	Increases mass to 190 kDa
Role	In replication, transcription, and recombination	In replication, transcription, chromosome condensation/segregation, and recombination	rRNA transcription
Inhibitors	Camptothecins	(see Table 58.3)	

MAPK, Mitogen-activated protein kinase; *PKC*, protein kinase C.

not require adenosine triphosphatase (ATP) for catalytic activity. It binds double-strand DNA over 15 to 25 bp (with a preference for supercoiled or bent DNA) followed by cleavage of one DNA strand and forming a transient covalent phosphotyrosyl bond at the 3′-end of DNA. DNA torsional strain is then relieved by a "controlled rotation" mechanism (see Fig. 58.1), subsequent to which the cleaved DNA is religated. The three-dimensional crystal structure of human topoisomerase I, both in covalent and noncovalent complexes with DNA, has defined the structural elements of the enzyme that contacts DNA. The association between topoisomerase I and the 3′-end of cleaved DNA has been termed the *cleavable complex*, which is stabilized by topoisomerase I inhibitors.

DNA Topoisomerase II

Two isoforms of human topoisomerase II (α and β) exist. They act as homodimers to cleave double-stranded DNA and require ATP for full activity. Their role in vivo is to relieve torsional strain in DNA, and their cellular distribution is determined by nuclear localization signals contained in the C-terminal domain. These isoforms are distinct in that they have different-size monomers (see Table 58.2), their genes are located on separate chromosomes, their nuclear distribution is different, and only the α-isoform shows cell cycle variations in amount and activity (with maximal activity being in G_2/M). The mechanism of action of topoisomerase II involves several steps (Fig. 58.4): DNA recognition and binding (curved and supercoiled DNA, as well as DNA crossovers, are preferred), the sequential cleavage of the two strands of DNA with covalent attachment of a monomer to each 5′-end of the cleaved DNA, passage of another DNA duplex through the break site (e.g., to relieve DNA torsional strain or decatenate daughter chromosomes at the end of replication), religation of the cleaved DNA, and ATP hydrolysis-dependent enzyme turnover. The binding of ATP by topoisomerase II is required for the strand passage reaction. Again, the association between topoisomerase II monomers and the 5′-end of the cleaved DNA has been termed the *cleavable complex*, the stabilization of which generally correlates with the cytotoxic activity of specific topoisomerase II inhibitors.

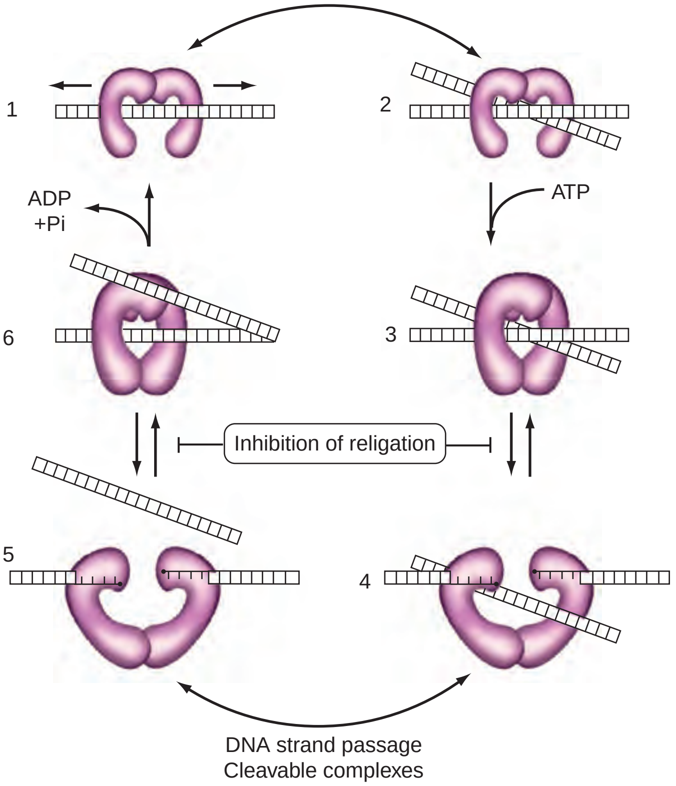


Figure 58.4 DNA TOPOISOMERASE II CATALYTIC CYCLE. (1) Noncovalent binding of DNA by the topoisomerase II homodimer. (2) DNA recognition and preferential binding to crossovers by topoisomerase II. (3) Binding of ATP promotes the formation of a topologic complex. (4) DNA cleavage with covalent linkage of each topoisomerase II monomer to the 5′-DNA terminus of the break. (5) Poststrand passage cleavable complex. (6) Religation of the cleaved DNA is followed by ATP hydrolysis and enzyme turnover. DNA topoisomerase II inhibitors generally increase cleavable complexes by inhibiting the religation activity.

Because topoisomerase I and II inhibitors convert their respective enzymes into DNA-damaging agents, it is usually true that the more enzyme target a cell contains (provided it is in the nucleus), the more cytotoxic is the specific inhibitor. An exception to this generalization is CLL cells, which have abundant topoisomerase I but are not very sensitive to topoisomerase I inhibitors because topoisomerase I inhibitors are S-phase-specific and CLL cells have very few cells in S phase. Finally, in addition to topoisomerase I and II, a mammalian DNA topoisomerase III has been described and found to be essential for early embryogenesis in the mouse. In addition to the presumed lethality of a homozygous deletion of the topoisomerase II gene, topoisomerases I and III appear to be essential for cell growth and division in mammals. The specific role of topoisomerase III in humans is unknown at present.

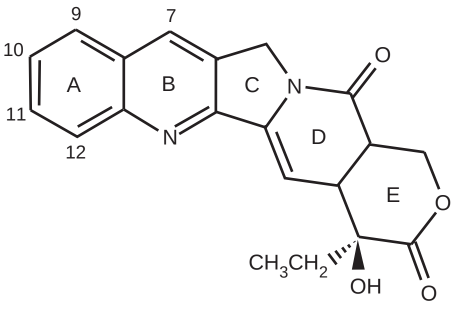
DNA Topoisomerase I Inhibitors

Camptothecin (CPT-11 or irinotecan) is a plant alkaloid first identified in 1966 from the tree *Camptotheca acuminata*. Early clinical studies with camptothecin were stopped primarily because of hemorrhagic cystitis resulting from conversion of the sodium salt form to the active lactone form owing to its acidic pH in the bladder. Renewed interest in camptothecin occurred in 1985 when topoisomerase I was identified as the target of this drug and as new more water-soluble analogs became available. At present, two topoisomerase I inhibitors have been approved by the FDA as second-line agents for the treatment of ovarian carcinoma and colorectal cancer; these are topotecan and irinotecan (CPT-11; Fig. 58.5). Topotecan has been shown to be active in the treatment of MDS and inactive in the treatment of CLL. Responses to topotecan have also been seen in refractory MM, refractory large-cell lymphoma, and refractory acute leukemia.

The lactone forms of topotecan and SN-38 (the active form of CPT-11 generated in vivo by the action of a carboxylesterase) are as much as 1000-fold more active inhibitors of DNA topoisomerase I than are their carboxylate forms. The lactone form predominates at an acidic pH. Topoisomerase I inhibitors stabilize the DNA-enzyme cleavable complex and thus inhibit DNA religation, but the production of DNA double-strand breaks results from a collision of the DNA replication fork with the ternary drug-enzyme-DNA complex, which is the lethal event (see Fig. 58.4). Topoisomerase I inhibitors are considered S-phase-specific agents because they require ongoing DNA synthesis to exert their cytotoxic effect.

DNA Topoisomerase II Inhibitors

Inhibitors of DNA topoisomerase II are commonly used for the treatment of hematologic malignancies. Three general types of topoisomerase II inhibitors exist (Table 58.3). The first are the topoisomerase II poisons, typified by etoposide, which results in the stabilization of cleavable complexes. The second group are the catalytic inhibitors, represented by aclarubicin, merbarone, and the bis-2,6-dioxopiperazine derivatives (ICRF-193, ICRF-159, ICRF-187); these are drugs that, except for aclarubicin, do not bind DNA and do not stabilize cleavable complexes, but rather interfere with some aspect of topoisomerase II catalytic activity (e.g., ICRF-187 inhibits topoisomerase II ATPase activity). The final class includes drugs that can inhibit both DNA topoisomerases I and II, and are represented by intoplicine and saintopin.



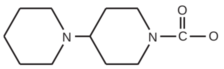
	C-10	C-9	C-7
Camptothecin	H	H	H
Topotecan	OH	$(CH_3)_2NCH_2$	H
9-Aminocamptothecin	H	NH_2	H
SN-38	OH	H	CH_3CH_2
CPT-11		H	CH_3CH_2

Figure 58.5 STRUCTURE OF CAMPTOTHECIN ANALOGS.

DNA topoisomerase II poisons (see Fig. 58.5 for structures) are most likely cytotoxic because they trap DNA topoisomerase II complexes on nascent DNA in the nuclear matrix. The topoisomerase II poison-stabilized enzyme–DNA complex likely acts as a replication fork barrier and leads to the generation of irreversible DNA damage and cell death in proliferating cells. Whereas experiments in yeast show that although DNA synthesis is a major determinant for cell killing by topoisomerase I inhibitors, topoisomerase II poisons are also cytotoxic during other phases of the cell cycle.

Drug Resistance to Topoisomerase Inhibitors

Essentially all of the topoisomerase II poisons (see Tables 58.2 and 58.3) are substrates for the drug efflux pump P-glycoprotein (PGP), and many are substrates for multidrug-resistance protein (MRP) and lung resistance-related protein. In addition, several point mutations and gene deletions have been defined in the gene for topoisomerase II, resulting in the production of an enzyme with altered catalytic or cleavage activity. The third mechanism of resistance is a decrease in expression of the enzyme such that there is less target for the inhibitor to "convert" to a DNA-damaging agent. This can result from a proliferation-dependent or cell cycle–dependent decrease in topoisomerase II, from a specific attenuation of topoisomerase II, or from an intrinsic absence of topoisomerase II (identified in some acute and chronic leukemias). The fourth resistance mechanism involves alterations in the subcellular distribution of the enzyme. Truncation of the COOH-end of topoisomerase II has resulted in the cytoplasmic distribution of enzyme caused by a loss of nuclear localization signals, so that the enzyme cannot interact with DNA in the presence of an inhibitor and the cell is resistant. However, mutations in the gene for topoisomerase II do not appear to be common, because only a single patient with AML has been found to have a point mutation. By contrast, CLL cells are resistant to topoisomerase II inhibitors because they express very low levels of the protein.

Platinum Analogs

Mechanism of Action

During a study of the effects of electric current on growing bacteria, the antibacterial and, later, the antitumor activities of the platinum compounds were fortuitously discovered. The antitumor agent cisplatin, its *cis*-carboxylester analog, carboplatin, and the diaminocyclohexane-containing oxaliplatin, are heavy-metal platinum complexes. They are activated when one of their ligands (cisplatin; chloride and carboplatin; carboxylester) is displaced by water, leading to the formation of positively charged aquated platinum complexes, allowing platinum to stably bind DNA, RNA, proteins, or other critical biomacromolecules (see Fig. 58.2). With DNA, platinum complexes form covalent links to the N^7 position of guanine and adenine. The N^7 adducts at d(GpG) or d(ApG) result in intrastrand or interstrand DNA cross-links that bend the DNA helix and inhibit DNA synthesis. The cytotoxicity of platinum analogs correlates with the total platinum binding to DNA, as well as with the intrastrand or interstrand cross-links. This results in DNA damage, which triggers apoptosis of sensitive cells.

Clinical Activity

Cisplatin, carboplatin, and oxaliplatin are used in the treatment of refractory lymphomas in a variety of combinations, and as part of high-dose and intensification therapy for lymphomas as definitive treatment including prior to autologous stem cell transplantation.

TABLE 58.3 **Mechanism(s) of Action of Antineoplastic Agents That Are Primarily Topoisomerase II Inhibitors**

Drug	Topoisomerase II Inhibition Poison	DNA Suppressor	Free Radical Intercalation	Formation
Epipodophyllotoxins	+++	−	−	+
VP-16				
VM-26				
Anthracyclines				
Doxorubicin				
Daunorubicin	++	++	++	+
Idarubicin				
Epirubicin				
Anthracenedione				
Mitoxantrone	++	++++	++	+
Acridine				
m-AMSA	+++	+	+	−
Catalytic inhibitors				
Aclarubicin	−	+++	+	−
Others (merbarone, fostriecin, bis-2,6-dioxopiperazines)	−	+++	−	−

DNA, Deoxyribonucleic acid; *m-AMSA*, amsacrine.

Miscellaneous Agents

Among the agents included in this category, only plicamycin, bleomycin, L-asparaginase, gallium nitrate, and glucocorticoids are of current interest to hematologists.

PHARMACOLOGY OF TARGETED ANTINEOPLASTIC AGENTS

Since the year 2000, the therapeutic arsenal available for treatment of hematologic malignancies has expanded to include a group of drugs that have collectively been termed as “targeted agents.” Traditional chemotherapeutic drugs affect specific targets, many times identified after the cytotoxic and clinical activity of these agents have been demonstrated. Dobbelstein and Moll describe three “waves” or “epochs” in anticancer drug development.[5] The first wave includes traditional chemotherapeutic agents, comprised mostly of drugs that affect DNA replication, repair and cell division; these drugs are nonspecific and can affect normal cells, but take advantage of the increased proliferation of cancer cells to exert their cytotoxic activity. Second-wave drugs target cellular signals, including those mediated by surface receptors and protein kinases. Monoclonal antibodies are included in this second wave. The targets of signaling inhibitors are diverse, and include the products of oncogenes, essential for the development and survival of neoplasms (“oncogene addition”). The prototype of this class of agents is imatinib, targeting the chimeric product of BCR-ABL in chronic myelogenous leukemia (CML).

Other cellular signaling proteins are not the product of oncogenes but have an essential role in intracellular processes of malignant cells. This state is known as “nononcogene addiction” and has expanded the number of targets that can be used for cancer treatment with signaling inhibitors. Examples of the latter agent class include inhibitors of mammalian target of rapamycin (mTOR) and BTK. The third wave of anticancer drugs target cellular mechanisms and effector systems distinct from those targeted by drugs of the first two waves, but that are still essential for the survival of cancer cells. The cellular processes targeted by drugs of the third wave are those that contain several types of constitutive stress experienced by cancer cells (separate from replicative stress), including proteotoxic stress leading to abnormal protein folding and increased protein degradation (targeted by heat-shock protein inhibitors and proteasome inhibitors), DNA damage (targeted by PARP inhibitors), DNA modifications (targeted by hypomethylating agents and HDACs), prosurvival balance in organelles governing cell survival (targeted by BCL2 inhibitors), increased need for protein transport (inhibited by nuclear transport inhibitors), as well as transcriptional, ribosomal, metabolic, and oxidative stress, targeted by several other drugs in development. Other agents not easily included in these three waves include immunomodulatory drugs (thalidomide, lenalidomide, and pomalidomide), as well as agents promoting cancer cell differentiation (all-*trans* retinoic acid).

Signaling Inhibitors (Table 58.4 and Fig. 58.6)

Imatinib Mesylate and Other BCR-ABL Kinase Inhibitors

Imatinib Mesylate

Imatinib mesylate is a phenylaminopyrimidine developed as an inhibitor of the constitutively active tyrosine kinase BCR-ABL, expressed in the leukemic cells of most patients with CML. The recognition that the constitutively active BCR-ABL played a central role in the pathogenesis of CML led to the search for potential inhibitors. The introduction of the tyrosine kinase inhibitor (TKI) imatinib for treatment of CML is a major milestone in the development of targeted therapy for hematologic and neoplastic disorders. The role of the BCR-ABL kinase in the pathogenesis of CML and ALL and the use of BCR-ABL inhibitors in the treatment of these disorders is discussed in Chapters 67–69.

Imatinib mesylate is the founding agent in the class of TKIs. It causes direct inhibition of the BCR-ABL kinase[6] but is not absolutely specific for this molecule, as it also inhibits other kinases, including KIT, platelet-derived growth factor (PDGF), stem cell factor (SCF), and TEL-ARG.[7]

TABLE 58.4 Targets and Approvals for Signaling Inhibitors

Agent	Target	Approved indication
Acalabrutinib	BTK	CLL, MCL
Alectinib	ALK, RET	Alk positive metastatic NSCLC
Asciminib	BCR-ABL	
Azacitidine	DNA methyltransferase	MDS, CML, and AML
Belinostat	Histone deacetylase	PTCL
Bortezomib	Proteasome	Multiple myeloma, MCL AL amyloidosis
Bosutinib	BCR-ABL, KIT, Src, HCK, Lyn, MAPK1-2	CML
Carfilzomib	Proteasome	Multiple myeloma
Ceritinib	ALK	NSCLC
Copanlisib	PI3K-alpha and PI3K-delta	Follicular lymphoma
Crenolanib	FLT3, PDGFRα, PDGFRβ, c-KIT	
Crizotinib	ALK, HGFR	ALK-positive non-small–cell lung cancer
Dabrafenib	BRAF	Melanoma with BRAF V600E mutation
Decitabine	DNA methyltransferase	AML
Enasidenib	IDH2	AML
Everolimus	mTOR	Renal cell carcinoma
Fedratinib	JAK2, FLT3, RET	Intermediate - 2 or high-risk myelofibrosis
Gilteritinib	FLT3	AML, MDS
Glasdegib	Hedgehog pathway	AML
Ibrutinib	BTK	CLL, MCL, Waldenström's macroglobulinemia, Marginal zone lymphoma, GVHD
Idelalisib	PI3Kδ	Relapsed CLL, SLL, and follicular lymphoma
Ipatasertib	AKT	
Ivosidenib	IDH1	AML, cholangiocarcinoma
Ixazomib	Proteasome	Multiple myeloma
Lenalidomide	IMiD	Multiple myeloma, MCL, 5q- syndrome, DLBCL (in combination with tafasitamab)
Midostaurin	FLT3, c-KIT	AML
Nilotinib	BCR-ABL, Kit, Lck, Ephrins, PDGFRβ, MAPK11, ZAK	CML
Omacetaxine	Ribosomal protein translation	CML with resistance or intolerance to tyrosine kinase inhibitors
Pacritinib	JAK2, FLT3	
Panobinostat	Histone deacetylase	Multiple myeloma, myelofibrosis
Ponatinib	BCR-ABL, Kit, PDGFRα, VEGFR, Src, Lyn, Lck, FGFR1–4, FLT3, TEK	CML with T315I mutation
Quizartinib	FLT3, CRS1R, SCFR, PDGFR	
Romidepsin	Histone deacetylase	CTCL, PTCL
Ruxolitinib	JAK1, JAK2,	Intermediate or high-risk myelofibrosis, GVHD
Sonidegib	Hedgehog pathway	Basal cell carcinoma
Sorafenib	BRAF, VEGFR, FLT3, PDGFRβ, Kit, FGFR1, Ret	Hepatocellular carcinoma
Tazemetostat	EZH2	Follicular lymphoma, epithelioid sarcoma
Temsirolimus	mTOR	Renal cell carcinoma
Thalidomide	IMiD	Multiple myeloma
Umbralisib	PI3K-delta and CK1-epsilon	
Vemurafenib	BRAF	Melanoma with BRAF V600E mutation
Venetoclax	BCL2	CLL, AML
Vismodegib	Hedgehog pathway	Basal cell carcinoma
Vorinostat	Histone deacetylase	CTCL, PTCL
Zanubrutinib	BTK	MCL

ALK, Anaplastic lymphoma kinase; *ALL*, acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *BTK*, Bruton's tyrosine kinase; *CLL*, chronic lymphocytic leukemia; *CML*, chronic myelogenous leukemia; *CTCL*, cutaneous T cell cymphoma; *JAK*, Janus kinase; *FLT3*, fms-like tyrosine kinase 3; *HGFR*, hepatocyte growth factor receptor; *IDH*, isocitrate dehydrogenase; *IMiD*, immunomodulatory imide drugs; *MCL*, mantle cell lymphoma; *MDS*, myelodysplastic syndrome; *mTOR*, mechanistic target of rapamycin; *NSCLC*, non-small cell lung cancer; *PDGFR*, platelet-derived growth factor receptor; *PI3K*, phosphatidylinositol 3-kinase pathway; *PTCL*, peripheral T cell lymphoma; *SCF*, stem cell factor; *SLL*, small lymphocytic lymphoma; *STAT5B*, signal transducer and activator of transcription 5B; *VEGFR*, vascular endothelial growth factor receptor.

Radiographic crystallography studies show that imatinib mesylate competitively binds to the ATP-binding site of the ABL kinase and stabilizes it in an inactive conformation. The 50% inhibitory concentration (IC_{50}) of imatinib mesylate for BCR-ABL is in the submicromolar range, and is substantially lower than the supramicromolar levels achievable in the plasma of patients receiving the drug by the oral route.

Despite the success with imatinib mesylate in the treatment of chronic-phase CML and, to a lesser extent, accelerated or blast-phase CML, the preexistence or development of resistance represents a major therapeutic challenge that can occur at any stage of the disease. Primary or secondary resistance can develop through a variety of mechanisms. BCR-ABL–dependent mechanisms correspond to the emergence of mutations in ABL kinase domain, which can be located in the imatinib binding site, the P loop, the catalytic domain, or the activation loop. Many of these mutations are present at the time of diagnosis and resistant clones emerge after exposure to imatinib. Resistance mechanisms independent of BCR-ABL include development of multidrug resistance mechanisms (e.g., PGP related); decreased levels of human organic cation transporter; acquisition of additional genetic abnormalities (clonal evolution) including acquisition of an additional Philadelphia-positive chromosome (Ph+), trisomy 8 and isochromosome 17q, increased expression of the BCR-ABL protein, and Src kinase overexpression.

Imatinib also has activity in diseases dependent on other kinases, such as PDGF receptor PDGFRα, PDGFRβ, or KIT, and is active against myeloproliferative disorders associated with eosinophilia and FIP1L1/PDGFRα or PDGFRβ fusion genes, as well as against systemic mastocytosis without KIT^{D816V} mutation (see Chapters 71 and 72).

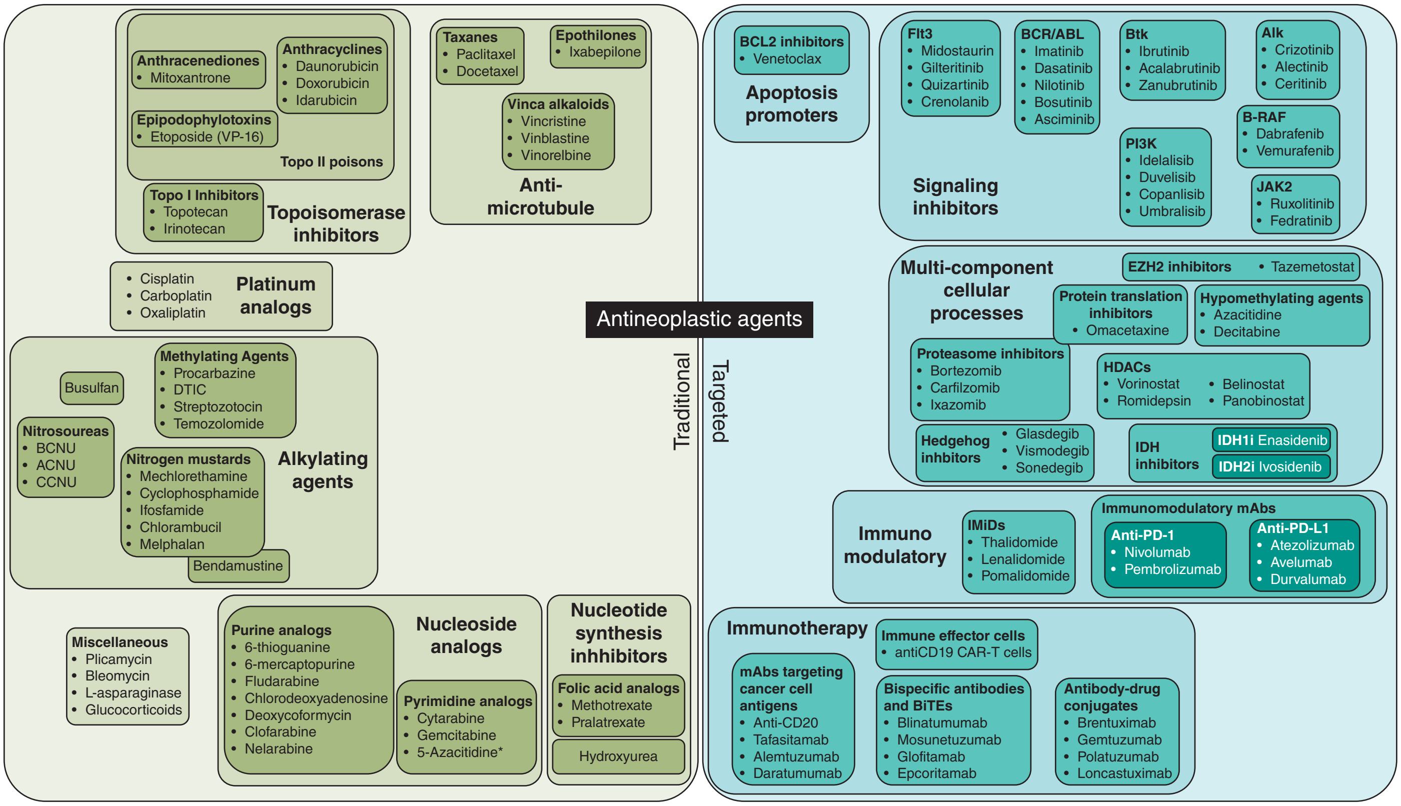

Anthracenediones
• Mitoxantrone
Anthracyclines
• Daunorubicin
• Doxorubicin
• Idarubicin
Epipodophylotoxins
• Etoposide (VP-16)
Topo II poisons
Topo I Inhibitors
• Topotecan
• Irinotecan
Topoisomerase inhibitors
Taxanes
• Paclitaxel
• Docetaxel
Epothilones
• Ixabepilone
Vinca alkaloids
• Vincristine
• Vinblastine
• Vinorelbine
Anti-microtubule
• Cisplatin
• Carboplatin
• Oxaliplatin
Platinum analogs
Busulfan
Methylating Agents
• Procarbazine
• DTIC
• Streptozotocin
• Temozolomide
Nitrosoureas
• BCNU
• ACNU
• CCNU
Nitrogen mustards
• Mechlorethamine
• Cyclophosphamide
• Ifosfamide
• Chlorambucil
• Melphalan
Bendamustine
Alkylating agents
Miscellaneous
• Plicamycin
• Bleomycin
• L-asparaginase
• Glucocorticoids
Purine analogs
• 6-thioguanine
• 6-mercaptopurine
• Fludarabine
• Chlorodeoxyadenosine
• Deoxycoformycin
• Clofarabine
• Nelarabine
Nucleoside analogs
Pyrimidine analogs
• Cytarabine
• Gemcitabine
• 5-Azacitidine*
Nucleotide synthesis inhhibitors
Folic acid analogs
• Methotrexate
• Pralatrexate
Hydroxyurea
Antineoplastic agents
Traditional
Targeted
BCL2 inhibitors
• Venetoclax
Apoptosis promoters
Flt3
• Midostaurin
• Gilteritinib
• Quizartinib
• Crenolanib
BCR/ABL
• Imatinib
• Dasatinib
• Nilotinib
• Bosutinib
• Asciminib
Btk
• Ibrutinib
• Acalabrutinib
• Zanubrutinib
Alk
• Crizotinib
• Alectinib
• Ceritinib
B-RAF
• Dabrafenib
• Vemurafenib
PI3K
• Idelalisib
• Duvelisib
• Copanlisib
• Umbralisib
JAK2
• Ruxolitinib
• Fedratinib
Signaling inhibitors
Multi-component cellular processes
EZH2 inhibitors • Tazemetostat
Protein translation inhibitors
• Omacetaxine
Hypomethylating agents
• Azacitidine
• Decitabine
Proteasome inhibitors
• Bortezomib
• Carfilzomib
• Ixazomib
HDACs
• Vorinostat
• Romidepsin
• Belinostat
• Panobinostat
Hedgehog inhbitors
• Glasdegib
• Vismodegib
• Sonedegib
IDH inhibitors
IDH1i Enasidenib
IDH2i Ivosidenib
Immuno modulatory
IMiDs
• Thalidomide
• Lenalidomide
• Pomalidomide
Immunomodulatory mAbs
Anti-PD-1
• Nivolumab
• Pembrolizumab
Anti-PD-L1
• Atezolizumab
• Avelumab
• Durvalumab
Immunotherapy
Immune effector cells
• antiCD19 CAR-T cells
mAbs targeting cancer cell antigens
• Anti-CD20
• Tafasitamab
• Alemtuzumab
• Daratumumab
Bispecific antibodies and BiTEs
• Blinatumumab
• Mosunetuzumab
• Glofitamab
• Epcoritamab
Antibody-drug conjugates
• Brentuximab
• Gemtuzumab
• Polatuzumab
• Loncastuximab

Figure 58.6 SCHEMATIC REPRESENTATION OF THE THERAPEUTIC ARMAMENTARIUM IN MALIGNANT HEMATOLOGY.

Common adverse events due to imatinib include fluid overload and edema, and development of heart failure, fatigue, rash, and myelosuppression. Gastrointestinal side effects are common, and nausea and vomiting are frequent reasons for poor compliance.

Dasatinib

Dasatinib is a second-generation ABL kinase inhibitor approved for the treatment of CML. Dasatinib is an oral multikinase inhibitor, affecting BCR-ABL, KIT, PDGFR, and Src kinases. It binds the ATP binding site of ABL in an opposite direction to imatinib, can inhibit active and inactive forms of the kinase, and requires fewer points of contact. This reduced structural stringency in kinase inhibition allows for inhibition of all kinase mutations, with the exception of the *T315I* mutation.

Compared with imatinib, dasatinib is several hundred times more potent as an inhibitor of ABL. Initial studies demonstrated dasatinib-induced responses in patients who had developed resistance to imatinib treated in the chronic, accelerated, and blast phase of CML. There is a high response rate in patients with wild-type sequences of *BCR-ABL* and also in patients with mutations in the ABL protein conferring resistance to imatinib, including *E255V* and *Y253H*. The notable exception is *T315* mutations.[8]

As a multikinase inhibitor, dasatinib has a role in myeloid-lymphoid neoplasms with eosinophilia and recurrent genomic translocations. Dasatinib has a broad toxicity profile, with pulmonary edema, pleural effusions, and thrombocytopenia being the more frequent adverse effects. Recently, higher rates of pulmonary hypertension have been observed in patients receiving dasatinib.

Nilotinib

Nilotinib is another second-generation ABL kinase inhibitor approved for treatment of CML. Nilotinib is structurally related to imatinib, but has structural modifications that increase affinity for the ABL kinase site, binding with an improved topological fit to the kinase site in its inactive form. Nilotinib is 10 to 30 times more potent that imatinib.

In contrast with imatinib and dasatinib, nilotinib is a selective inhibitor of BCR-ABL. Nilotinib is active in chronic- and accelerated-phase CML patients who have developed resistance to imatinib. As with dasatinib, nilotinib was compared with imatinib in a phase III randomized trial as initial therapy for chronic-phase CML patients, with nilotinib achieving higher rates of complete cytogenetic and major molecular responses, and with fewer cases of CML phase progression or clonal evolution in the nilotinib-treated cohorts.[9] Nilotinib is active against all *BCR-ABL* mutations that confer resistance to imatinib, with the exceptions of *T315I*.

Common adverse events with nilotinib include rash, gastrointestinal disturbances (nausea, vomiting, diarrhea), as well as neutropenia and thrombocytopenia. Pleural effusion and peripheral edema are less common than with dasatinib. In addition, QTc prolongation and risk of pancreatitis are serious side effects of nilotinib. Post-approval studies and cross-sectional studies have found nilotinib to also be associated with a risk of peripheral arterial disease.

Bosutinib

Bosutinib is a dual kinase inhibitor of Src and ABL kinases. Although originally described as a Src-selective TK inhibitor, cell line screening assays allowed discovery of its ABL inhibitory activity. ABL kinase assay studies showed the IC_{50} was 1.4 nM. Cell line and animal studies supported the potential therapeutic activity of bosutinib against CML. Bosutinib has does not inhibit wild-type c-KIT of PDGFR, although recent studies have shown inhibitory activity of other kinases, including C-terminal Src-kinase (Csk), erythropoietin-producing hepatoma (Eph) receptors, Trk, Tek, and Axl kinases, as well as some epidermal growth factor receptor (EGFR) and KIT mutants.

Bosutinib docks inside the intermediate conformation of the ATP binding site of ABL (in contrast to imatinib, which binds to the inactive conformation of the site). Binding to the intermediate conformation overcomes the resistance to imatinib conferred by mutations

Y253F, *E255K*, and *D276G*, but does not confer activity against cells expressing *T315I*. In addition, Bosutinib appears to be a poor substrate of multidrug resistance (MDR) transporters, overcoming this additional mechanism of resistance. Bosutinib, like dasatinib and nilotinib before it, was approved for frontline use in chronic-phase CML patients with a phase III trial demonstrating statistically significantly higher rates of cytogenetic and molecular responses.[10]

Common adverse events observed with bosutinib include gastrointestinal upset (diarrhea, vomiting, and abdominal pain), whereas the more common serious adverse events include diarrhea, myelosuppression, and elevated serum lipase and transaminases.

Ponatinib

Ponatinib is a third-generation, dual Src-ABL inhibitor. It was developed using computational and structure-based design to have activity against BCR-ABL forms with mutations that convey resistance to other TKIs, in particular *T315I* mutation. Ponatinib binds ABL in its inactive form as imatinib. Differences in the chemical structure allow ponatinib to maintain interaction with ABL even in the presence of isoleucine in position 315, in cases with a *T315I* mutation. Initial cell- and murine-based studies showed that ponatinib inhibits cells expressing mutant or native *BCR-ABL* with IC_{50} values ranging from 0.37-2 nM. Subsequent human studies demonstrated that ponatinib had activity in CML, including those with *T315I* mutation. Toxicities observed in early clinical trials included pancreatitis and elevation in pancreatic enzymes, fatigue, rash, and elevated aminotransferase levels. Ponatinib received FDA-accelerated approval in 2012 for the treatment of resistant or intolerant CML and Ph+ ALL. However, subsequent trials showed increased arterial thrombosis as well as hypertension events in patients randomized to ponatinib arms, leading to limitations in the indications for ponatinib and the requirement for thrombosis prevention strategies in subjects treated with this drug.[11]

Given the structural similarity to imatinib, as expected ponatinib has activity as an inhibitor of several other targets, including FLT3, c-KIT, vascular endothelial growth factor receptor (VEGFR), PDGFR, and c-SRC. However, ponatinib is yet to be used clinically outside of BCR-ABL–containing hematologic malignancies.

Asciminib

Asciminib, also known as ABL001, is an allosteric inhibitor of BCR-ABL. Unlike imatinib, dasatinib, nilotinib, bosutinib, and ponatinib which bind to the ATP-binding pocket, asciminib binds to the myristoyl pocket of the N-terminus of ABL1 kinase. This pocket normally downregulates the kinase activity, but the function is lost with the formation of the fusion protein. Asciminib was designed to change the conformation and restore the regulatory function of the region of the protein. In BCR-ABL positive cell lines, asciminib inhibits growth at concentrations of 1-10 nM while having little effect on the growth of wildtype cells.

As a result of the disparate binding site and mechanism of action, it has been shown to be clinically active regardless of the mutations around the ATP binding pocket, including in patients with *T315* mutations. In the phase I study,[12] examples of resistance did emerge with a *G463S* mutation located at the myristoyl pocket.

The toxicity profile of asciminib had some overlap with other BCR-ABL1 signal blocking TKIs. Elevation of amylase and lipase were relatively common, but reversible. Clinically evident pancreatitis also occurred, and also appeared to be reversible with cessation of drug. Other adverse events described in the phase I study included fatigue, rash, headache, arthralgia, and hypertension with a majority of reported events grade 1 or 2.

Bruton Tyrosine Kinase Inhibitors

Bruton tyrosine kinase (BTK) is a member of the Tec family nonreceptor kinase. BTK plays a central role in the B-cell receptor activation pathway, fundamental in B-lymphocyte development, differentiation, and signaling.[13] This pathway is also critical for the emergence and progression of several B-cell lymphoid malignancies, including CLL, mantle cell lymphoma (MCL), and others. [14] Activation of BTK is followed by 1-phosphatidylinositol-4,5-biphosphate phosphodiesterase gamma-2 (PLCγ2) activation, leading to release of intracellular calcium, diacylglycerol (DAG), and inositol triphosphate (IP3), with activation of nuclear factor kappa B (NF-κ B) and inhibiting the apoptosis of the normal and malignant B cells. [15] BTK inhibitors have demonstrated activity in preclinical models of B-cell malignancies and may also have activity in treatment of autoimmune disorders.[16]

Ibrutinib is the first BTK inhibitor to receive approval, inactivating BTK through an irreversible covalent bond with a cysteine residue (Cys-481) in the kinase domain, blocking also BTK autophosphorylation at Tyr-223. Acalabrutinib and zanubrutinib also form a covalent bond with Cys-481, with higher selectivity for BTK over other kinases. The latter agents have been denominated second-generation BTK inhibitors by some authors, although mechanistically, they do not differ from ibrutinib. Noncovalent inhibitors of BTK do not require interaction with Cys-481 for BTK inhibition, thus overcoming resistance conferred by C481S mutations, a common mechanism of resistance to covalent BTK inhibitors. Noncovalent BTK inhibitors under development have promising activity in lymphoid malignancies but have not yet received regulatory approval.

Class toxicities of BTK inhibitors include an increased risk of opportunistic infections, including Pneumocystis jirovecii and fungal infections[17]; the mechanisms of increased infection are not fully explained, but do not appear to be limited to B-cell function impairment.[18] Other common complications observed across this class of agents include diarrhea and increased bruising, with increased risk of hemorrhagic complications, the latter resulting from inhibition of collagen-induced platelet activation and aggregation. Cardiovascular toxicities are well recognized and have been observed with all three approved agents, including arterial hypertension and atrial fibrillation in up to 15% of patients. There are no sufficient data to indicate the rates of complications are higher with any one agent.

Ibrutinib, acalabrutinib, and zanubrutinib are substrates of CYP3A4. Relevant interactions with CYP3A4 inhibitors azole antifungal agents as well as antiretroviral agents and grapefruit juice. BTK dose should be reduced if concomitant administration with these agents is necessary. Use with CYP3A4 agents is not recommended. There are no dosing modifications for impaired renal function, and doses should be reduced for mild hepatic impairment and use should be avoided with advanced liver failure.

Efficacy of BTK inhibitors

BTK inhibitors have established efficacy in several B lymphoid malignancies. In chronic lymphocytic lymphoma, BTK inhibitors achieve overall response rates (ORR) in 68% to 94% of cases (with or without deletion of 17p).[19–21] In randomized trials, ibrutinib resulted in higher rates of overall survival than ofatumumab in relapsed CLL patients. In the RESONATE-2 trial, continuous treatment with ibrutinib resulted in higher rates of overall and progression free survival.[19,21] Acalabrutinib, alone or in combination with Obinutuzumab had superior outcomes than chlorambucil combined with Obinutuzumab for previously untreated CLL patients. In mantle cell, initial reports showed ibrutinib achieved response rates of 68%, with 21% complete responses,[22] and a subsequent phase III trial showed similar response rates.[23] While studies with acalabrutinib and zanubrutinib have been reported to achieve response rates between 80 and 90%,[24] complete responses still occur in a fraction of subjects. BTK inhibitors have remarkable activity in Waldenström macroglobulinemia, with responses for ibrutinib of 91%, 73% of which were major responses.[25] Initial studies suggest that this class of agents has activity in activated B-cell center diffuse large B-cell lymphoma, but this was not confirmed in a recent phase III trial, with future emphasis on more specific selection of DLBCL patients for whom this class of agents will be effective. BTK inhibitors have limited activity against follicular lymphoma.

PI3K/AKT/mTOR Inhibitors

The PI3K/AKT/mTOR pathway is involved in the regulation of diverse cellular functions, including cell growth, protein synthesis, cell cycle regulation, glucose metabolism, and motility. PI3K represents a family of enzymes with multiple subunits. These subunits cooperate to transduce upstream signals (from RTKs, G-protein–coupled receptors, and other intracellular stimuli) into the enzymatic conversion of phosphatidylinositol diphosphate (PIP$_2$), to phosphatidylinositol triphosphate (PIP$_3$). The dual-specific phosphatase and tensin homolog (PTEN) opposes the actions of PI3K. PTEN is one of the most frequently mutated tumor suppressor genes in human malignancies, with inactivating mutations, genetic losses, and epigenetic silencing leading to PTEN loss. Phosphatidylinositol triphosphate, through phosphoinositide-dependent kinase-1 (PDK1), leads to the phosphorylation and activation of the serine/threonine kinase AKT.

AKT signals to multiple downstream targets, in particular mTORC1, to promote cell survival and growth. Additional targets of AKT include molecules involved in cell survival and proliferation (e.g., Bad, Bim, procaspase 9, CREB, forkhead transcription factors [FHKR], IB), cell cycle regulators (p21^{CIP1}, p27^{KIP1}, cyclin D$_1$), glycogen synthesis (GSK3), and protein synthesis (FRAP1, p70^{S6K}).

The mechanistic target of rapamycin (also known as mTOR) operates through two complexes: mTORC1 (mTOR-Raptor) and mTORC2 (mTOR-Rictor). mTORC1 is activated by AKT and mediates its downstream proliferation, growth, and survival signaling, specifically through phosphorylation and inactivation of the repressor of mRNA translation initiation factor 4E-binding protein 1 (4E-BP) and ribosomal protein S6 kinase 1 (S6K1; see Fig. 58.7), resulting in enhanced translation of transcripts relevant to lymphoma pathogenesis, including CCND1, MYC, and MCL1. The mTORC1 complex is activated by AKT whereas mTORC2 is capable of activating AKT. The majority of the downstream canonical functions are conducted by mTORC1.

Because of the frequency of PTEN losses or mutations (less common in hematologic malignancies) in transformed cells and the dependence of numerous cancers on an intact PI3K/AKT/mTOR pathway for survival, this cascade has become an attractive therapeutic target.

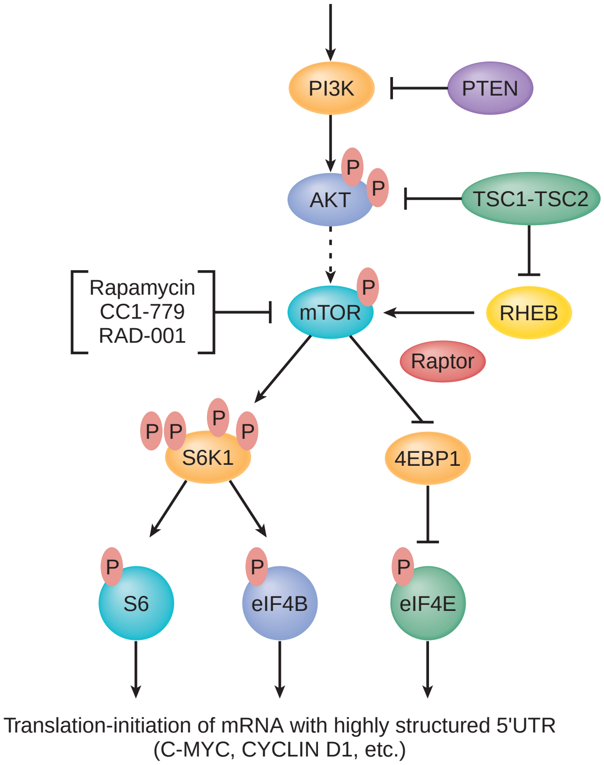


Figure 58.7 RAPAMYCIN OR ITS ANALOGS INHIBIT MAMMALIAN TARGET OF RAPAMYCIN AND THE DOWNSTREAM PHOSPHORYLATION OF S6K1 AND 4EBP1, THEREBY ATTENUATING THE TRANSLATION INITIATION OF mRNAS WITH HIGHLY STRUCTURED 5′-UTR. Activation of the receptor *(FLT-3)* or cytosolic tyrosine kinase (e.g., BCR-ABL) can lead to increased activity of PI3K/AKT. Although it can directly phosphorylate mTOR, AKT activity inhibits the TSC1–TSC2 complex, thereby derepressing RHEB and activating mTOR. The phosphorylation and activation of S6K1, and phosphorylation and inactivation of 4E-BP through Raptor, results in the phosphorylation of S6, eIF4B, and eIF4E, which are involved in the cap-dependent translation of mRNAs with highly structured 5′-UTR. *eIF*, Eukaryotic translation initiation factor; *mTOR*, mammalian target of rapamycin; *PI3K*, phosphatidylinositol 3-kinas; *PTEN*, phosphate and tensin homolog; *RHEB*, Ras homolog enriched in brain; *TSC*, tuberous sclerosis; *UTR*, untranslated region.

PI3K Inhibitors

Phosphatidyl inositol 3-kinases (PI3K) are a family of lipid kinases with highly conserved ATP binding sites that phosphorylate phosphatidylinositides at the 3′ position if the inositol ring.[26] They are classified according to their substrate, and class I PI3Ks perform the enzymatic conversion of phosphatidylinositol diphosphate (PIP2), to phosphatidylinositol triphosphate (PIP3) as a result of upstream signals (from receptor tyrosine kinases [RTKs], G-protein–coupled receptors, and other intracellular stimuli). Class I PI3Ks are heterodimers, with a regulatory subunit p85 and a catalytic subunit, p110. Upstream signals result in activation of the regulatory subunit, which results in release of the catalytic subunit, which then localizes to the cell membrane to convert PIP2 to PIP3. Phosphatidylinositol triphosphate, through phosphoinositoside-dependent kinase-1 (PDK1), leads to the phosphorylation and activation of the serine/threonine kinase AKT, which in turn signals to multiple downstream targets, including mTORC1, BCL2 family members, FOXO, and others, promoting cell survival and growth.

There are four class I PI3K isoforms (PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ), defined by the p110 catalytic isotype. Each member of class I has a well-defined physiologic role, with variable degree of functional overlap among class members.[27] PI3Kα is ubiquitously expressed and plays a role in insulin signaling and glucose homeostasis, whereas PI3Kβ enhances the actions of integrin $\alpha_{IIb}\beta_3$ on platelet adhesion and signaling. PI3Kγ and PI3Kδ have more restricted expression to the hematopoietic system and are involved in lymphocyte activation, mast cell degranulation, and chemotaxis.[28]

The role of PI3Ks and the PI3K/AKT/mTOR pathway in cancer is well recognized. Mutations and abnormal expression of PI3K members are well recognized in solid tumors and in a minority of hematologic malignancies.[27] In addition, the dual-specific PTEN, which opposes the actions of PI3K, is one of the most frequently mutated tumor suppressor genes in human malignancies, with inactivating mutations, genetic losses, and epigenetic silencing leading to PTEN loss. Of note, the relevance of PI3K in hematologic malignancies is not secondary to genetic activations of the PI3K pathway, but rather the central role that PI3Kδ and PI3Kγ play in lymphocyte signaling (i.e., B-cell and T-cell receptor signaling pathways) and microenvironmental responses to tumors.

There are several PI3K inhibitors approved for treatment of hematologic malignancies, and several others are in development. Their mechanism of action is associated with the specific p110 isoform inhibited. Idelalisib (CAL-101, GS 1101) is a highly selective inhibitor of the p110d isoform of PI3Kδ, whereas duvelisib (IPI-145) inhibits the isoforms of PI3Kδ and PI3Kγγ, and copanlisib (BAY-80-6946) is a pan PI3K inhibitor with predominant activity against PI3Kα and PI3Kδ (Table 58.5). The difference between pan-PI3K inhibitor and isoform-specific inhibitors appears to be related to the conformation adopted by the drugs in the adenine-binding pecked of the catalytic p110 subunit. Pan-PI3K inhibitors (i.e., copanlisib) adopt a flat conformation, whereas isotype-specific inhibitors (i.e., idelalisib, duvelisib) adopt a "propeller shaped" conformation.[29]

TABLE 58.5 **Isoform Selectivity Profile of PI3K Inhibitors**

	IC_{50} (nM)			
	Alpha	**Beta**	**Delta**	**Gamma**
Idelalisib	820	855	2.5	89
Duvelisib	1602	85	2.5	27
Umbralisib	>10,000	>10,000	6.2	1400
Copanlisib	0.5	3.7	0.7	6.4
Buparlisib	52	166	116	262
Pictilisib	3.0	33	3.0	75
AZD8835	6.2	431	5.7	90
ME-101	22,867	30	1	>20,000

The adverse event profile of PI3K inhibitors is closely related to their isoform-specific actions. Studies done with idelalisib have predominantly reported autoimmune and infectious adverse events. Autoimmune events have included pneumonitis, rash, hepatitis (manifested by increase in transaminase concentrations) as well as non-infectious colitis. Most events respond to discontinuation of the drug and use of corticosteroids for severe instances. In cases of isolated transaminase elevation, re-exposure to lower doses of idelalisib may be considered. The incidence of autoimmune adverse events appears to have been higher in patients treated earlier in the course of their disease[30] or when the drug is combined with other immunomodulatory agents.[31] An additional gastrointestinal adverse event includes the risk of intestinal perforation, which may occur in the absence of significant diarrhea. Subsequent research has shown that PI3Kdelta is relevant to the function of regulatory T cells (Tregs).[32]

Opportunistic infections have also been reported, specifically *Pneumocystis jirovecii* pneumonia (PJP), CMV reactivation as well as fungal infections. Autoimmune toxicities and increased risk of infection have been reported with other PI3K delta inhibitors duvelisib and umbralisib although reports from clinical trials with the latter appear to show decreased risk of autoimmune events, possibly related to the additional inhibition of casein kinase 1 epsilon (CK1ε).[33,34]

Copanlisib, because of its actions PIK3 alpha, has a distinct pattern of adverse events. Hyperglycemia is an on-target effect related to PIK3alpha inhibition on insulin receptor signaling, and has been observed in studies of other pan-PI3K inhibitors (buparlisib, pictilisib).[29] In addition to hyperglycemia, subjects treated with copanlisib experienced infusion-related hypertension in approximately 40% of cases.[35] Both hyperglycemia and hypertension were infusion-related, reversible, and could be managed with medical interventions. Other adverse events observed with copanlisib likely are secondary to its actions on PIK3 delta, including diarrhea in 37% of cases (grade 3 in less than 5% of cases), as well as neutropenia in approximately 25% of cases. Other inflammatory autoimmune events such as pneumonitis are rare.

All three currently FDA-approved PI3K inhibitors (idelalisib, duvelisib, and copanlisib) are metabolized by CYP3A. Interactions with strong inhibitors and inducers as well as substrates of this system need to be considered when prescribing these agents. Dose modification guidelines are available. Umbralisib is not metabolized through CYP3A and reportedly has limited medication interactions.

Idelalisib is currently approved as a single agent for relapsed follicular lymphoma and in combination with rituximab for CLL patients. The approved dosing is 150 mg PO twice daily. In the randomized trial in relapsed CLL patients, comparing rituximab plus idelalisib vs. rituximab plus placebo, the addition of idelalisib resulted in longer progression-free survival (median 19.4 months vs. 6.5 months), with response rate of 83.6% vs. 15.5%.[36] All responses were partial. As single agent in relapsed follicular lymphoma, idelalisib was evaluated in a single-arm phase II study and resulted in a response rate of 57% (6% complete responses), with median progression-free survival of 11 months.[37]

Duvelisib is currently approved as single agent for treatment of relapsed CLL, SLL, or FL after two prior lines of therapy. Duvelisib was compared in a randomized trial with ofatumumab including relapsed CLL/SLL subjects with two or more prior lines of therapy but excluding prior BTK or PI3K inhibitor therapy. Overall response was 74% (all partial response [PR]) versus 45% with ofatumumab (all PR), while median progression free survival was 13.3 months vs. 9.9 months (HR 0.52, $P<.0001$).[38] A non-randomized phase II trial of duvelisib was conducted in patients with refractory indolent lymphoma treated with at least two previous treatment regimens, but excluding previous treatment with BTK or PI3K inhibitors. In the follicular lymphoma subgroup ($n = 83$), duvelisib resulted in an ORR of 42% (1.2% complete response), and median progression free survival for the whole cohort ($n = 129$) of patients with follicular, marginal, and small lymphocytic lymphoma was 9.5 months.[39] Copanlisib received accelerated approval for patients with follicular lymphoma treated with two prior systemic therapies. The CHRONOS-1 trial was a single-arm phase II trial that included 141 subjects with relapsed/refractory indolent NHL. The response rate among follicular lymphoma ($n = 104$) was 58.7% (14.4% complete response), with median duration of response in follicular lymphoma of 12.2 months. Progression free survival for the complete trial cohort including other indolent lymphomas was 11.2 months.[40]

AKT Inhibitors

The frequent presence of AKT abnormalities in human neoplasms makes this enzyme an attractive target for potential novel treatment strategies. Several drugs have been developed using dephosphorylation of the active enzyme, allosteric enzyme inhibition, and ATP competition as strategies for targeting AKT. Allosteric inhibitors bind the lipid-binding domain of AKT, resulting in a change in conformation that prevents localization to the plasma membrane and subsequent activation. The allosteric inhibitor perifostine had modest clinical activity as a single agent in patients with Waldenström macroglobulinemia and in patients with myeloma alone or in combination with bortezomib. Phase III studies failed to demonstrate clinical benefit. MK2206 is another oral allosteric inhibitor with minimal single-agent activity against solid tumors and hematologic malignancies.

Early ATP-competitive AKT inhibitors had significant off-target effects. More recently, ipatasertib (GDC-0068), an oral pan AKT inhibitor, has been observed to have significant selectivity for AKT. Clinical development has focused on solid tumors. Afuresertib (GSK 2110183) is a reversible ATP-competitive AKT inhibitor with in vitro activity against ALL, CLL, lymphoma, and myeloma. A phase I study of single-agent afuresertib showed activity in MM, with a maximum tolerated dose of 125 mg PO once daily.[41] Subsequent studies have demonstrated activity of afuresertib when used in combination with bortezomib and dexamethasone for the treatment of myeloma, and with ofatumumab for the treatment of CLL. Frequent adverse events include gastrointestinal complaints (diarrhea, nausea, dyspepsia, gastrointestinal reflux, and anorexia) and fatigue. Severe adverse events include rash, fatigue, elevations in liver function tests, and thrombocytopenia.

Inhibitors of the Mammalian Target of Rapamycin

The first mTOR inhibitor discovered was the naturally occurring macrolide rapamycin (sirolimus), widely used as an immunosuppressant (see Chapter 108). The importance of the mTOR pathway in cancer suggested this agent would have antineoplastic activity. Improvements in solubility and pharmacokinetic properties led to development of rapamycin analogs (rapalogs), of which everolimus and temsirolimus are available as antineoplastic agents.[42] The mechanism of rapamycin and its analogs is similar: after complexing with the small protein FKBP12, they bind irreversibly to the FKBP12-rapamycin binding site on the mTOR protein. Acute rapamycin treatment only affects mTORC1 because the FKBP12-rapamycin is occluded in mTORC2. Prolonged rapalog exposure may result in decreased mTOR availability and therefore indirectly decreased mTORC2 activity through limited mTORC2 complex formation. It is well recognized that growth factor signaling initiated through the RTKs or caused by the activity of cytosolic tyrosine kinases (TKs), such as BCR-ABL, results in activation of PI3K/AKT signaling. AKT has been shown to phosphorylate and inactivate tuberous sclerosis (TSC)2 (also known as tuberin), thereby disrupting its interaction with TSC1 (or hamartin; see Fig. 58.7). Inhibition of the TSC1–TSC2 complex derepresses RHEB, which is a small G protein that activates mTOR. When RHEB is in an active GTP-bound state, its localization to the membrane stimulates mTOR-mediated phosphorylation of the downstream eukaryotic initiation 4E-BP and ribosomal protein S6K1 (see Fig. 58.7). However, the association of mTOR with the 150-kDa Raptor (regulatory-associated protein of mTOR) is necessary for the phosphorylation of 4E-BP and S6K1. mTOR-mediated phosphorylation and activation of S6K1 results in the phosphorylation of the ribosomal S6 protein and eIF4B (see Fig. 58.7). AKT has also been shown to directly activate S6K1. S6K1 activity is involved in regulating the translation of a group of mRNAs that have a highly structured 5′ -UTR (untranslated region), including mRNAs that have a 5′-TOP (terminal oligopyrimidines; a stretch of 4 to 14 pyrimidines).[43]

The rapalogs temsirolimus and everolimus have been tested in lymphoid malignancies, and as single agents they have demonstrated activity, although modest, against a variety of lymphomas (see Chapter 82). In the initial phase II study in patients with MCL, temsirolimus was administered at a dose of 250 mg based on a phase II study done in renal cell carcinoma patients. A modest response rate was noted (ORR: 38%), but the majority of patients required a dose reduction because of myelosuppression, particularly thrombocytopenia. A second trial in relapsed refractory MCL patients was conducted to determine if a lower dose of temsirolimus could be used. In a follow-up phase II study, temsirolimus was given at a dose of 25 mg IV weekly and was found to be as effective as the 250-mg IV dose, with a modest improvement in the toxicity profile and dose reductions. This dose and schedule were also noted to be efficacious in nonmantle cell NHL, with thrombocytopenia being the main dose-limiting toxicity. A recent phase III study in relapsed refractory MCL patients evaluated two different dosing regimens of temsirolimus; 175 mg weekly for 3 weeks followed by 75 mg weekly versus 175 mg weekly for 3 weeks followed by 25 mg weekly versus the investigator's choice.[44] At the conclusion of this study, it was determined that the temsirolimus regimen with 175/75 mg dosing had a significant improvement in progression-free survival (PFS) and objective response rate compared with the investigator's choice. Everolimus, given orally at a daily dose of 10 mg has single-agent activity against Hodgkin lymphoma (PMI, Waldenström macroglobulinemia, diffuse large B-cell lymphoma [DLBCL], CLL, and other relapsed lymphomas).

The most common side effect of rapalogs is myelosuppression; other common side effects include fatigue, oral ulcers, and dermatologic abnormalities. Metabolic abnormalities are common, including hyperglycemia, hypercholesterolemia, and hypertriglyceridemia. An uncommon pulmonary toxicity manifested as interstitial lung disease has also been observed with rapalogs.

Anaplastic Lymphoma Kinase Inhibitors

Anaplastic lymphoma kinase (ALK) encodes for a 210 kDa receptor tyrosine kinase, member of the insulin receptor superfamily, closely related to leukocyte tyrosine kinase. While ALK has a normal role in nervous system development, translocations involving ALK have been described in a variety of malignancies, including anaplastic large cell lymphoma (NPM-ALK, TFG-ALK, ATIC-ALK and CLTC-ALK translocations) [45] diffuse large B-cell lymphoma (NPM-ALK, CLTC-ALK, SQSTM1-ALK, SEC31A-ALK), as well as non-small cell lung cancer (NSLCL) (EML4-ALK translocation and others)[46] and inflammatory myofibroblastic tumors in children.[47] These translocations result in ALK chimeras with fusion of the intracytoplasmic domain of ALK with partner proteins that provide a dimerization domain that results in ALK-mediated autophosphorylation and constitutive activation. Subsequent signaling promotes cellular proliferation, survival, and growth via phospholipase Cγ, RAS/MAPK, PI3K, and c-Src. ALK inhibitors are currently approved for treatment of non-small cell lung cancer (NSCLC), including crizotinib, alectinib (CH5424802), and ceritinib.

Crizotinib is a dual ALK/c-MET inhibitor with additional inhibitory activity against the kinase ROS1. It inhibits kinase activation through binding to the kinase domain and displacing the kinase activation loop, interfering with ATP and substrate binding. Crizotinib was shown to be 20-fold more selective for ALK and MET than other kinases, and inhibited cell proliferation and induced apoptosis in NPL-ALK-dependent cell lines. Alectinib is a dual ALK/RET inhibitor that binds to the ATP site of ALK, but can maintain the interaction with the ATP binding site in presence of the common gatekeeper mutation L1196M, overcoming this mechanism of resistance.[48]

The most common adverse events of crizotinib include gastrointestinal symptoms (nausea, vomiting, diarrhea), visual impairment, asthenia, cough, and myelosuppression (neutropenia, lymphopenia) as well as elevations of hepatic function tests. Alectinib has been primarily tested in NSCLC subjects, and its adverse events include fatigue, constipation, edema, and myalgia. Less commonly, alectinib is associated with hepatotoxicity, pneumonitis or interstitial lung disease, and bradycardia as well as increases in creatine phosphokinase.

Crizotinib is metabolized by CYP3A system, with known interactions with strong inhibitors or strong inducers of this system. Alectinib is also metabolized by CYP3A4 into its metabolite M4, but no interactions were seen when coadministered with strong inducers or inhibitors of CYP3A.

The initial clinical studies done in patients with NSCLC showed the maximum tolerated dose was 250 mg orally twice daily. Although initially tested as a c-MET inhibitor in these patients, ALK translocations were reported to be associated with marked responses in patients treated with crizotinib. Subsequent studies in ALK-rearranged NSCLC confirmed the activity of crizotinib in NSCLC with ALK translocations. In 2011, the FDA granted approval for crizotinib in this patient population. Subsequent studies conducted in relapsed and refractory ALK-positive anaplastic large-cell lymphoma showed high rates of response to single-agent crizotinib. Additional trials testing crizotinib for ALK positive lymphomas are ongoing. Studies with alectinib in treatment of ALCL are ongoing in countries with high prevalence of these diseases,[49] and published experience in this disease is limited to case reports and series.[50]

Inhibitors of the RAF1/MEK/ERK Pathway

The MAPK (mitogen-activated protein kinase) pathways consist of three parallel serine-threonine kinase modules that are intimately involved in the control of cell survival, proliferation, and differentiation: JNK (c-Jun N-terminal kinase), p38 MAPK, and ERK (extracellular signal-regulated kinase). Although exceptions exist, JNK and p38 MAPK primarily exert proapoptotic functions, but ERK activation is generally associated with cell survival. The only well-defined activator of ERK is the serine-threonine kinase mitogen-activated protein kinase 1/2 (MEK1/2), but numerous ERK targets have been identified, including ELK-1, CREB, BCL2, Bad, FRAP1 (also known as mTOR), and caspase 9, among numerous others. The activating effects of MEK1/2 on ERK are opposed by phosphatases that dephosphorylate and inactivate the enzyme (e.g., MAP kinase phosphatase 1/2).[51]

The major activator of MEK1/2 is the serine-threonine kinase RAF, of which three forms exist: RAF1, B-RAF, and A-RAF. RAF can be activated by Ras (discussed earlier), as well as through various RAS-independent pathways, including PKC (protein kinase C), KSR (kinase suppressor of Ras), as well as the Src and JAK family of kinases, among others. The activation of RAF involves several processes, including recruitment to the plasma membrane, phosphorylation on serine and threonine residues, and dimerization. Interference with any of these processes can lead to inhibition of RAF activation as well as downstream targets.[52]

Dysregulation of the RAF/MEK/ERK pathway has been observed in most hematopoietic malignancies, including acute leukemia, CLL, MM, and lymphomas. Consequently, there has been considerable interest in the development of pharmacologic inhibitors of the RAF/MEK/ERK pathway in these disorders. In addition to their potential intrinsic activity against malignant hematopoietic cells, evidence indicates that such agents might also enhance the activity of conventional cytotoxic drugs. Nevertheless, translation of RAF/MEK/ERK inhibitors into the treatment of hematologic malignancies has significantly impacted the treatment of certain malignancies.

Vemurafenib and Dabrafenib

Vemurafenib is a selective inhibitor of mutant B-RAF, and was developed with a scaffold-based drug design approach and was able to achieve serum concentrations in excess of the IC50.[53] Although originally approved for metastatic melanoma harboring the B-RAF V600E mutation, it was the first MAPK pathway targeting medication to be approved for the treatment of hematologic malignancy, specifically for hairy cell leukemia (HCL) and Erdheim-Chester disease (ECD). B-RAF V600E was identified as a key genetic lesion in HCL in the early 2000s, and nearly universally mutated in this condition and inhibition with vemurafenib induces responses in over 95% of relapsed and refractory cases (Chapter 77).[54,55] Although ECD was formerly felt to be an inflammatory disorder, the identification of recurrent activating mutations in the RAF/MEK/ERK pathway led to its reclassification as a hematologic malignancy. Similarly, another histiocytic disorder, Langerhans cell histiocytosis (LCH), was identified as a clonal disease through genetic analyses. Both ECD and LCH were found to have a high prevalence (38% to 57%) of mutations at the V600 residue of B-RAF.[56,57] The efficacy of vemurafenib in these diseases was evaluated as part of the histology independent VE-BASKET study, and found an ORR over 60% for these diseases. [58] Vemurafenib was approved by the FDA for the treatment of B-RAF V600E mutant ECD in 2017.

Dabrafenib is a competitive ATP binding site inhibitor for B-RAF, with lower IC^{50} concentrations for mutant B-RAF than wildtype. Notably, dabrafenib has reported activity in CNS lesions for patients with V600E mutant malignancies. Early reports have suggested that it may play a role in the treatment of ECD, with reported high response rates, including in patients with vemurafenib resistance or progression of lesions in the CNS.[59]

Inhibitors of *fms*-like Tyrosine Kinase 3 (FLT3)

The FLT3 is a surface receptor within class III receptor tyrosine kinase family and is also known as CD135.[60] It is transmembrane protein and expressed on normal CD34+ hematopoietic stem cells and immature progenitors of both B-lymphoid and myeloid progenitors. When bound to FLT3 ligand in a dimeric form, it phosphorylates downstream targets, particularly simultaneous activation of non-overlapping pathways of the PI3K/AKT pathway and the RAS/RAF/MEK pathway. Signal transduction leads to transcription of genes important for proliferation and anti-apoptotic pathway. FLT3 is highly implicated in hematologic malignancies, particularly AML. Wild-type FLT3 is usually expressed at a high level in AML. Most crucial has been the identification of activating mutations in over 30% of AML patients. The most common mutations are internal tandem duplications of the transmembrane domain, which have been consistently associated with more adverse outcomes. Point mutations of the tyrosine kinase binding domain (TKD), particularly at the D835 residue, can also be activating and potentially sensitive to inhibition. TKD mutations have not consistently been associated with more adverse outcomes.

Inhibitors of FLT3 may be classified as type I or type II. Type I inhibitors bind to the gatekeeper domain near the ATP binding pocket in both the active and inactive conformations of the protein. Type II inhibitors bind to a hydrophobic region directly adjacent to the ATP binding site when the protein is in the inactive conformation. Type II inhibitors do not inhibit FLT3 with TKD mutations, as these mutations favor the active conformation of the protein.

Sorafenib

Although sorafenib has not found a role as a MAPK pathway inhibitor in solid tumors, it has found a niche as a FLT3 inhibitor in AML. Sorafenib was original developed as an inhibitor of RAF, but early on was found to have broad activity across numerous kinase families including VEGFR, PDGFRβ, c-KIT, and FLT3.[61,62] Early evaluation of sorafenib in AML noted modest single-agent activity, and it was subsequently combined with multiagent chemotherapy in the upfront setting.[63] Response rates and disease-free survival in phase II settings were encouraging, and evaluation of FLT-ITD mutant subsets suggested that the inclusion of sorafenib with initial chemotherapy and subsequent maintenance may have improved the outcomes of this adverse mutation. As a type II inhibitor, correspondingly, analyses were not suggested of improvements in patients with TKD mutations.[64] Studies with sorafenib paved the way for other FLT3 and *multi-kinase inhibitors in AML.*

Midostaurin

Midostaurin is a multikinase inhibitor that is a derivative of the naturally occurring staurosporine, and was originally developed as an inhibitor of protein kinase C. In vitro, it has broad activity, and further characterization of the compound found it capable of inhibiting numerous serine and tyrosine kinases.[65] Its broad activity includes PDGFR, VEGFR, Syk, Src, and c-kit. Midostaurin undergoes metabolism in the cytochrome p450 pathway into at least 2-major metabolites, which also appear to have kinase inhibitory activity. Midostaurin's potential as a FLT3 inhibitor was identified through broad screening of compounds to induce apoptosis in FLT3-ITD expressing cells. Further characterization of midostaurin demonstrated that it is a type I inhibitor of FLT3 and active in both FLT3-ITD and FLT3-TKD, but has a clear affinity for mutant protein, with a ten-fold lower inhibitory concentration of wildtype FLT3.[66] Although only modest activity of midostaurin was identified in single agent studies, it was moved into treatment in combination with front line therapy, and the phase III RATIFY study demonstrated a clear overall survival advantage in FLT3 mutant AML when combined with traditional 7+3 therapy, and was approved for combination therapy in 2017.[67]

The c-KIT inhibitor effects of midostaurin are also clinically important. Midostaurin inhibits c-KIT D816V mutation, which is found in 80% of patients with systemic mastocytosis and confers upfront resistance to inhibition with imatinib. Systemic mastocytosis patients have high response rates to midostaurin, with 50% to 71% of patients with aggressive forms of the disease responding to single-agent therapy. However, inhibition of KIT may lead to myelosuppression that has been observed secondary to midostaurin.[66] Common side effects of midostaurin include elevated liver enzymes, nausea, myelosuppression, edema, and prolonged QTc (requiring monitoring) (see Chapter 60).

Gilteritinib

Gilteritinib is a pyrazine carboxamide derivative and was specifically synthesized to be a potent inhibitor of FLT3. It does target other kinases, such as AXL, but has a very high affinity for mutant FLT3 with an IC_{50} of 1 nM. This compares favorably to the wildtype form, of 5 nM as well as to other kinases, such as AXL with an IC_{50} of 41 nM. Clinical studies with gilteritinib have demonstrated a lower rate of myelosuppression compared to other FLT3 inhibitors. This is often attributed to the fact that gilteritinib does not inhibit c-kit, unlike many of its related compounds. Clinically, single-agent gilteritinib had activity even at low levels in early studies, and in relapsed and refractory disease, 24% of patient achieved a CR with incomplete recovery of counts. The phase III ADMIRAL study compared gilteritinib to other standard forms of salvage therapy in AML patients after failure of frontline intensive therapy, and demonstrated a median OS benefit of 9.3 months versus 5.6 months, as well as an advantage of patients proceeding to allogeneic stem cell transplant. Results of the ADMIRAL study resulted in FDA approval of gilteritinib in 2018.[68] Common side effects of gilteritinib include arthralgias, myalgias, fatigue, and edema (see Chapter 60).

Quizartinib

Quizartinib is a small molecule inhibitor developed specifically as a FLT3 inhibitor, with kinase inhibitor active of c-kit and PDGFR as well. It is a type II inhibitor and does not affect TKD mutations. Nevertheless, preclinical and early clinical data suggested potent antileukemia effects in FLT3-ITD models and patients. IC_{50} values in vitro were 1.1 nM for inhibition of FLT3 autophosphorylation and 0.56 nM for inhibition of FLT-ITD cell models. Early phase trials found in relapsed/refractory AML, high rates of blast clearance including reported remissions over 30% in multiple early trials.[69,70] The phase III QuANTUM-R trial evaluated the efficacy of quizartinib versus high-intensity salvage chemotherapy in adult patients with relapsed FLT3-ITD with less than six months remission, and demonstrated a median OS benefit of 6.2 versus 4.7 months.[71] Based on these outcomes, quizartinib has been approved for relapsed/refractory AML in Japan. In the United States, approval was withheld due to concerns about the quality of remission and persistence of severe cytopenias is a major toxicity. Most FLT3 inhibitors have some concerns of either being an inhibitor of potassium channels, or having a high level of interaction with other medications that inhibitor these channels and associated prolongation of the QTc interval. In the QuANTUM-R study. Common side effects of quizartinib include neutropenia, anemia, thrombocytopenia, and nausea (see Chapter 60).

Crenolanib

Crenolanib, a benzamidine quinolone analog originally developed as an inhibitor of PDGFR, was found to be a type I inhibitor of FLT3 potent effects against both FLT3-TKD and ITD mutations with IC_{50} as low as 1.2 nM. Similar to gilteritinib, it has selective activity against c-kit and appears in early studies to have less myelosuppression. Phase II trials with crenolanib have suggested rates of CR and CRi of 15% to 37% in relapsed/refractory FLT3 mutated AML patients.[72] Crenolanib development is ongoing.

Janus Kinase 2 Inhibitors

JAK2 is a non-RTK that plays a central role in the transduction of differentiation and proliferation signals in hematopoietic progenitor cells. Ligand binding to surface receptors for hematopoietic growth factors leads to phosphorylation of JAK2 with subsequent activation of transcription factors in the JAK/signal transducer and activator of transcription (STAT) pathway, including STAT3 and STAT5 (see Chapters 68 to 70). Identification of the JAKV617F mutation in patients with myeloproliferative neoplasms, including polycythemia vera, essential thrombocythemia, and primary myelofibrosis, represented a fundamental step in understanding the pathophysiology of these disorders. The genetic abnormality corresponds to a point mutation in nucleotide 1849 of the molecule, where guanine replaces thiamine, resulting in substitution of a valine for phenylalanine in the JH2 pseudokinase autoinhibitory domain of the molecule. The result is either hypersensitivity to cytokine signals or constitutive activation of the kinase. Development of JAK2 inhibitors followed as a rational, targeted therapeutic strategy for these neoplasms (see Chapters 70 to 72).

Ruxolitinib

Ruxolitinib (INCB018424, Jakafi) is a small-molecule inhibitor of JAK1 and JAK2, and the first-in-class JAK inhibitor to receive FDA approval for treatment of intermediate- and high-risk myelofibrosis (see Chapter 70).[73,74] It exerts its inhibitory activity through competitive inhibition of the kinase's ATP-binding catalytic site. In preclinical studies, JAK1/2 inhibition with ruxolitinib decreased STAT3/5 signaling both in wild-type cells and those carrying *JAKV617F*. Two large phase III studies of ruxolitinib have evaluated the efficacy of ruxolitinib compared to best alternative care in intermediate-2 and high-risk myelofibrosis, where the primary endpoint was reduction in splenomegaly: COMFORT-I and COMFORT-II. Responses were observed in patients both with the *JAKV617F* mutation as well as those with wild-type *JAK2*, and clearly favored ruxolitinib. Importantly, the majority of patients experienced a decrease in constitutional symptoms and improved exercise tolerance and performance status, as well as weight gain. Additionally, long-term follow-up suggests an overall survival benefit, and ruxolitinib is a standard of care for this population. Nevertheless, the medication was not effective in reversing histologic, cytogenetic, or molecular abnormalities in peripheral blood or BM, suggesting that ruxolitinib is not curative.[75] More recent clinical trials suggest that ruxolitinib in combination therapies, such as hypomethylating agents, may play a role in reversing fibrosis. Ruxolitinib has also been evaluated in polycythemia vera. The phase III RESPONSE study enrolled patients refractory or intolerant to hydroxyurea and demonstrated improvement in spleen size and hematologic response. Rapid redevelopment of splenomegaly and symptom exacerbation can occur after abrupt interruption or discontinuation of ruxolitinib. Common side effects of ruxolitinib include myelosuppression, primarily anemia, and thrombocytopenia requiring dose modifications as expected with inhibition of the JAK:STAT pathway; increased risk of infections and herpes zoster; gastrointestinal symptoms (abdominal pain, diarrhea), fatigue, and headache.

Fedratinib

Fedratinib is a pyrimidine-based JAK2 inhibitor specifically developed to be selective for JAK2 without cross-inhibition of other kinases in the same class, including JAK1, JAK3, and TYK2. In vitro IC_{50} for JAK2 is 3 nM, but over 100 nM for the related kinases, whereas ruxolitinib inhibits JAK1 with an IC_{50} 3.3 nM. Large phase III studies evaluated fedratinib in JAK inhibitor naïve and previously treated patients (JAKARATA and JAKARTA-2) and identified significant improvement in splenic volume and quality of life. Development was halted when multiple cases of an ill-defined encephalopathy were reported, and there was suspicion that fedratinib may cause Wernicke encephalopathy. However, review of the data strongly suggested that all afflicted patients had severe cachexia and developed Wernicke encephalopathy and these events were probably not directly related to fedratinib, but instead due to nutritional deficiencies exacerbated by gastrointestinal toxicities. Fedratinib was subsequently approved with warning to supplement thiamine and immediately cease dosing in the event of encephalopathy[76] (see Chapter 72).

Other JAK2 Inhibitors

Several other inhibitors with activity against JAK2 remain under investigation. Pacritinib is a JAK2 inhibitor with activity against the wild-type kinase as well as the JAKV617F-mutated kinase, and has activity against FLT3. It achieves high rates of reduction in spleen volume and in early phase studies did not appear to be associated with high rates of thrombocytopenia. The phase III PERSIST-1 trial evaluated patients with higher-risk primary myelofibrosis without regard for anemia or thrombocytopenia. Primary endpoint of spleen volume reduction by 35% or more was achieved by 19% of patients.[77] Further studies are examining the efficacy specifically in myelofibrosis patients with severe thrombocytopenia. The main side effects are gastrointestinal, predominantly diarrhea, nausea, vomiting, and abdominal pain, while hematologic adverse events are modest.

Momelotinib (CYT387) is an oral multikinase inhibitor affecting JAK1/2, TYK2, TBK1, PRKD1, ROCK2, PRKCN, MAPK8, and CDK2/cyclin A. Early trials in myelofibrosis patients resulted in decreased splenomegaly and improvement in symptoms in the majority of patients treated and appeared to be effective in patients refractory to ruxolitinib. The phase III SIMPLIFY-1 trial compared momelotinib to ruxolitinib with a noninferiority design. Although there was demonstrable activity, it did not meet criteria for non-inferiority compared to ruxolitinib, which remains a standard of care. Nevertheless, development of momelotinib is continuing to evaluate efficacy in the second-line setting. The most common side effects included myelosuppression, primarily thrombocytopenia. Nonhematologic side effects included headache, QTc prolongation, neuropathy, and abnormal liver test results. The maximum tolerated dose has been established at 300 mg/day given continuously in 28-day cycles.

Cyclin-Dependent Kinase Inhibitors

Orderly progression through the cell cycle is regulated by the coordinated expression of a variety of genes and proteins, and effected by the interactions between cyclins, CDKs 1 to 9 and endogenous CDK inhibitors (e.g., $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$; see Fig. 58.1). CDK1, in association with cyclins A and B, is involved in G_2M progression; CDK2, CDK4, and CDK6, in association with cyclins A, D, and E, are involved in G_1S progression. Activation of CDKs results in phosphorylation of the pRb, which leads to its dissociation from the E2F transcription factor. Once freed, E2F triggers the transcription of diverse genes involved in cell cycle progression (thymidylate synthase and DHFR, among numerous others). Endogenous small-molecule CDK inhibitors such as $P21^{CIP1}$ and $P27^{KIP1}$ bind stoichiometrically to CDKs and inhibit their activity. CDK activity can also be regulated through inhibitory or activating phosphorylation. Interference with the activation of CDKs results in dephosphorylation of pRb, leading in turn to binding and inactivation of E2F and inhibition of cell cycle progression.

Cell cycle dysregulation is a cardinal characteristic of cancer. A classic example of this phenomenon is the association of increased expression of cyclin D_1 in MCL. Regulation of the cell cycle has been found to be closely related to apoptosis, and disruption of normal cell cycle transit has been shown to be a potent cell death stimulus. A corollary of this observation is that agents that interfere with the cell cycle, in addition to blocking cell cycle progression, can be potent inducers of programmed cell death. Cell cycle inhibitors can be subdivided into several categories. For example, they can act directly, as in the case of CDK inhibitors, or indirectly, as in the case of histone deacetylase inhibitors (HDIs), compounds that block cell cycle progression by inducing endogenous cell cycle inhibitors such as $P21^{CIP1}$. The latter agents are discussed later in this chapter. CDK inhibitors can also be classified as specific (i.e., directed against a particular CDK, such as CDK2) or those that nonspecifically inhibit most CDKs (e.g., flavopiridol).

Flavopiridol

Flavopiridol (L86-8275) is a semisynthetic flavonoid derived from the Indian plant *rohitukine*. It was the first CDK inhibitor to enter clinical trials in humans. Flavopiridol binds to the ATP-binding site of CDKs, resulting in reversible, competitive enzyme inhibition at concentrations of less than 100 nM. As noted earlier, flavopiridol is a relatively nonspecific CDK inhibitor and inhibits all CDKs, although it is less effective against CDK7. Flavopiridol induces G_1S or G_2M arrest, presumably a consequence of inhibition of CDK1 and CDK2. Flavopiridol may also act to block cell cycle progression by downregulating cyclin D_1 levels, inhibiting the CDK-activating complex (CDK7), or both.

In addition to blocking cell cycle progression, flavopiridol has been shown to be a potent inducer of apoptosis in malignant hematopoietic cells (e.g., acute and chronic leukemia) at low concentrations (e.g., <100 nM). Moreover, flavopiridol has shown activity against MM cells in vitro. The proapoptotic actions of flavopiridol have been attributed to its capacity to inhibit the positive transcription elongation factor-β (PTEF-β), cyclin T/CDK9 complex by inhibiting phosphorylation of the C-terminal domain of RNA polymerase II. This leads to downregulation of several antiapoptotic proteins, including BIRC4, P_{21}^{CIP1}, and, in the case of MM cells, Mcl-1. Flavopiridol has also recently been shown to block the antiapoptotic actions of the IAP family member survivin.

In clinical studies, flavopiridol was initially administered as a 72-hour continuous infusion every 2 weeks, with a maximally tolerated dose of 40 mg/m^2. Steady-state plasma levels in excess of those necessary to inhibit CDKs and induce apoptosis in leukemia cells (e.g., 350 nM) were achieved. Dose-limiting toxicities were fatigue, diarrhea, nausea, and myelosuppression. However, the occurrence of thromboembolic phenomena and the general lack of single-agent activity have limited enthusiasm for administering flavopiridol by this schedule. Flavopiridol has also been administered as a daily IV bolus for 1, 3, or 5 days every 3 weeks with manageable toxicity, and other schedules are being examined, including a hybrid schedule with half the dose administered as an IV bolus and the other half as a more prolonged infusion. When administered as a daily bolus infusion for 3 days every 3 weeks, flavopiridol exhibited modest activity in patients with MCL. A novel pharmacologically directed schedule of flavopiridol has been developed in which half of the dose (e.g., 30 mg/m^2) is administered as a 30-minute bolus-loading infusion and 30 mg/m^2 as a 4-hour infusion. In a phase I study, objective response rates of 45% were obtained with this schedule in patients with progressive CLL, including some with high-risk disease.[78] Because of the rapidity of response, particularly in patients with high white blood cell counts, aggressive measures designed to avoid tumor lysis syndrome (TLS) (e.g., hydration, alkalinization of the urine, administration of rasburicase) are advisable. Efforts are now underway to use this novel flavopiridol schedule in combination with other agents and in other hematologic malignancies.

Because of limited single-agent activity, combination regimens involving flavopiridol are being explored in hematologic malignancies. On the basis of preclinical evidence of synergism with the antimetabolite ara-C, a regimen combining flavopiridol on a daily IV bolus schedule followed by high-dose ara-C has been initiated in patients with AML and has shown some activity. In a phase II study, flavopiridol was administered as a 1-hour bolus infusion of 50 mg/m^2 daily for 3 days before administration of high-dose ara-C (day 6) and mitoxantrone (day 9). CR rates of 67% were obtained in patients with high-risk AML and more than half were durable. More recently, evidence of synergism between flavopiridol and other signal transduction modulators has become the focus of considerable attention. For example, the observation that flavopiridol interacts synergistically with imatinib mesylate against CML cells, including some that are imatinib mesylate resistant, has prompted the initiation of a phase I trial of flavopiridol and imatinib mesylate in patients with progressive BCR-ABL$^+$ hematologic malignancies. Four out of 21 patients responded. Evidence that flavopiridol interacts synergistically with HDAC and proteasome inhibitors in human leukemia cells has appeared, and clinical trials combining flavopiridol with the HDI vorinostat or the proteasome inhibitor bortezomib in patients with refractory AML/MDS and MM/indolent NHL are currently underway.

Targeting Apoptosis Signaling in Hematologic Malignancies

The processes of cell division and cell death are tightly coupled so that a net increase in cell numbers does not occur. Alterations in the expression or function of the genes controlling cell division and cell death can upset this delicate balance and are hallmarks of cancer. Although conventional anticancer drugs cause cell cycle perturbation or DNA damage, they do not directly interact with the intracellular machinery involved in apoptosis. Tumor selectivity of conventional agents is largely caused by the increased sensitivity to apoptosis of tumor cells after DNA damage or cell cycle perturbation. Novel therapeutic agents or strategies that target critical regulators or effectors of apoptosis are under development and clinical testing, and these agents or strategies have the potential to exert selective cytotoxicity against cancer cells.

Caspases are the “executioners” for apoptosis. They are proteases that exist as inactive zymogens and are activated by proteolytic cleavage of their proforms in response to a variety of death stimuli. This processing occurs at conserved aspartic acid residues, thus generating the enzymatically active caspases. Caspase activation is organized as a cascade, with an upstream initiator and downstream effector caspase. Upstream initiator caspases contain large prodomains that interact with specific proteins involved in triggering the cascade. The downstream caspases, which function as the ultimate effectors of apoptosis, possess small prodomains and are activated predominantly by proteolytic cleavage by upstream caspases. The irreversible cleavage of specific protein death substrates by the downstream effector caspases directly or indirectly accounts for the biochemical and morphologic changes that are recognized as apoptosis.

At least three pathways of caspase activation leading to apoptosis have been identified (Fig. 58.8): (1) the receptor-initiated apoptosis pathway, where the TNF family of cytokine receptors activate initiator caspases such as caspase 8; (2) the mitochondria-initiated apoptosis pathway, where cytochrome c and other prodeath effectors are released from mitochondria into the cytosol that results in activation of caspase 9; and (3) a pathway of caspase activation, which involves a serine protease, granzyme B, that directly cleaves and activates several caspases, including procaspase 3.

Proapoptotic Targets of Anticancer Agents

Most cancer cells have active antiapoptotic pathways that prevent cell death in response to growth, checkpoint, DNA damage, and metabolic stimuli. A number of current anticancer agents in development target these pathways and are designed to be effective alone or in combination with other agents that disrupt the cell cycle, DNA synthesis, invoke DNA damage, and so on.

Death Receptor–Initiated Apoptotic Signaling

Several TNF family receptors are known to transduce signals that result in apoptosis. These include TNFRSF1A (also known as CD120a and previously designated tumor necrosis factor receptor-1 [TNFR1]), TNFRSF6 (also known as CD95, APO-1, or FAS), TNFRSF25 (also designated TRAMP and known as DR3 or APO3), TNFRSF10A (also known as DR4 or TRAIL-R1), and TNFRSF10B (also known as DR5 or TRAIL-R2). These receptors, also called *death receptors*, are characterized by the presence of a death domain within their cytoplasmic region, and have been shown to trigger apoptosis upon binding to their cognate ligands or specific agonist antibody. The activating ligands for these death receptors are structurally related molecules that also belong to the TNF gene superfamily such as TNFSF6 (also known as Fas ligand), TNF, and TRAIL.

Ligation of death receptors produces receptor trimerization and formation of a death-inducing signaling complex (DISC). This is composed of TNFRSF6, Fas (TNFRSF6)-associated via death domain (FADD), and procaspase 8, an apical signaling complex that mediates receptor-induced apoptosis. FADD binds directly to FasR, TNFRSF10A, or TNFRSF10B and indirectly to TNFRSF1A via TNFRSF1A-associated via death domain protein (TRADD). FADD is essential for cell death signaling from all three receptors. FADD interacts through its C-terminal death domain to cross-link TNFRSF6, TNFRSF10A, or TNFRSF10B receptors and recruits procaspase 8 and procaspase 10, or TRADD, through its N-terminal death effector domain (DED) to the DISC (see Fig. 58.8). Oligomerization of caspase 8 within the DISC results in a high local concentration of the zymogen. The induced proximity under these crowded conditions generates low levels of intrinsic proteolytic activity of caspase 8, enough to allow the various proenzymatic molecules to mutually cleave each other. Processing of caspase 8 removes the DED-containing prodomain, thus releasing the activated protease into the cytosol, where it can cleave and activate other downstream procaspases. In certain types of cells (type I), enough caspase 8 is activated by the activation of the death receptor to cause apoptosis. In other cell types, such as hepatocytes (type II), a sufficient level of caspase 8 activation is not achieved and the apoptotic signal is amplified by the mitochondrial pathway. The active caspase 8 cleaves the cytosolic p22 Bid into a BH3-only domain-containing, proapoptotic,

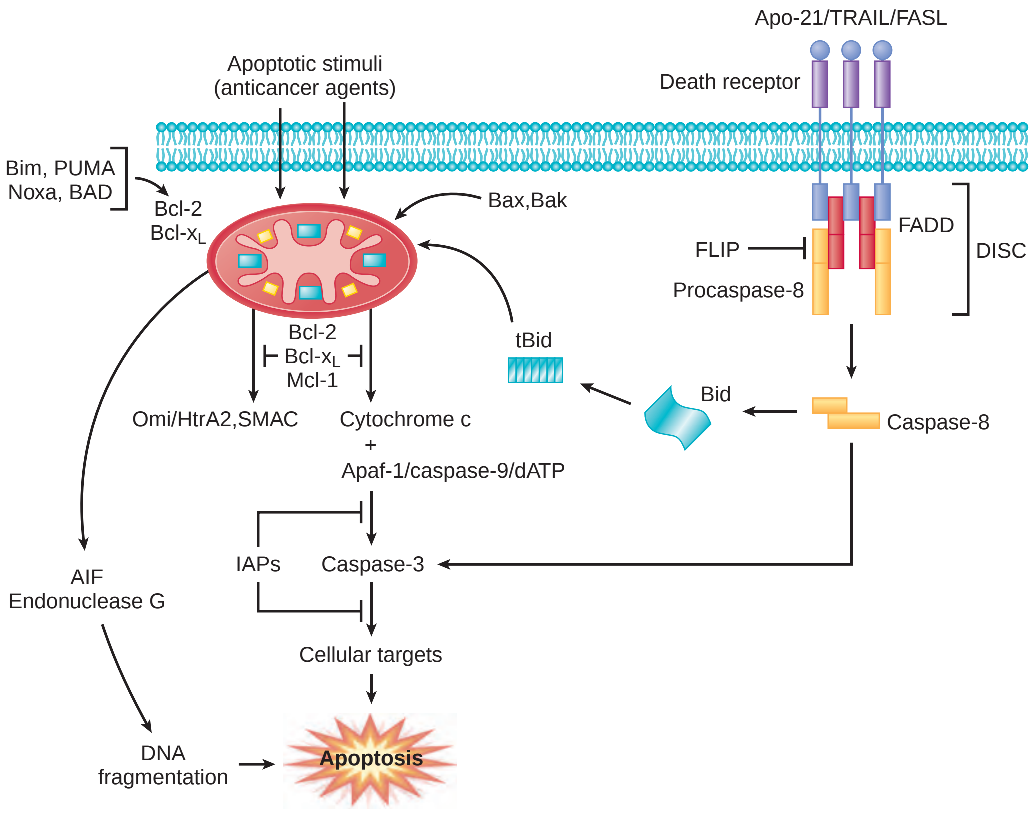


Figure 58.8 SCHEMATIC REPRESENTATION OF APOPTOTIC PATHWAYS.

truncated p15 tBid fragment, which translocates to the mitochondria and triggers the release of cyt c into the cytosol. Cleavage by caspase 8 has been shown to cause exposure of a glycine residue on p15 Bid that is myristoylated, thereby targeting p15 Bid to the mitochondria. Thus, N-myristoylation acts as an activating switch that enhances tBid-induced release of cyt c and apoptosis. The ability to cleave Bid may not be limited to caspase 8. Other caspases, such as caspase 3, as well other proteases, such as granzyme B and lysosomal proteases, have been shown to activate Bid. This indicates that Bid probably serves to amplify the caspase cascade rather than to initiate it (see Fig. 58.8).

Fas ligation has also been shown to initiate an alternative pathway leading to apoptosis, involving activation of JNK/SAPK. After ligation, Fas recruits an adaptor protein called Daxx that interacts with the apoptosis signal-regulating kinase 1 (ASK1), activating the transcription factors AP-1 and ATF-2. After activation, ASK-1 launches a phosphorylation cascade that culminates in the activation of JNK. Activated JNK phosphorylates substrates such as c-Jun, p53, and a pro-death Bcl-2 member Bim. This, in turn, has been shown to trigger mitochondrial or death receptor-initiated apoptotic signaling.

Mitochondria-Initiated Apoptotic Signaling

Mitochondria sequester a potent cocktail of proapoptotic proteins. These proteins promote apoptosis by activating caspases (e.g., by cyt c), by inducing DNA fragmentation (e.g., by endonuclease G or apoptosis-inducing factor [AIF]), or by neutralizing cytosolic inhibitors of apoptosis (IAPs; e.g., by SMAC or Omi/HtrA2). On induction of apoptosis, unlike other proapoptotic proteins, mitochondrial AIF translocates to the nucleus to induce DNA fragmentation and apoptosis. cytochrome c is a well-known component of the mitochondrial electron transfer chain. cytochrome c is released from the mitochondria during apoptosis. After release into the cytosol, cytochrome c binds to apoptosis-activating factor (Apaf-1), a cytosolic adapter protein that contains a caspase recruitment domain (CARD), a nucleotide-binding domain, and multiple WD-40 repeats. Binding of cytochrome c to Apaf-1 increases its affinity for dATP or ATP by approximately 10-fold. It also triggers the oligomerization of Apaf-1 into a multimeric Apaf-1– cytochrome c complex, also called the *apoptosome*. This exposes the CARD domain of Apaf-1, which recruits several molecules of procaspase 9, inducing their autoactivation. Only the caspase 9 bound to the apoptosome is able to efficiently cleave and activate a downstream executioner caspase, caspase 3.

SMAC/DIABLO is a 25-kDa mitochondrial protein that is released by mitochondria into the cytosol during apoptosis. SMAC contains a mitochondria-targeting sequence at its N-terminus. This sequence is removed on import into the mitochondria, generating the mature SMAC protein. In the mitochondria-initiated and common effector pathways of apoptosis, the processing and proteolytic activity of caspase 9 followed by caspases 3 and 7 are inhibited by the IAP family of proteins. IAP family members include XIAP, cIAP1, cIAP2, and survivin. All IAPs contain at least one baculovirus repeat (BIR) domain, although some contain three. Another region, the RING domain, has ubiquitin ligase activity and promotes the self-degradation of IAPs through proteasomes in response to some apoptotic stimuli. Furthermore, during Fas death receptor-mediated apoptosis, XIAP is cleaved by activated caspase 3 into the N-terminal BIR1 and 2 and BIR3-RING finger fragments. The BIR3 domain binds and sequesters the monomeric and inactive caspase 9, thereby inhibiting its activation. Overexpression of XIAP inhibits anticancer drug (including ara-C)–induced caspase activity and apoptosis. In contrast, downregulation of XIAP sensitizes cancer cells to apoptosis induced by chemotherapeutic drugs. The antiapoptotic activity of NFκB has also been shown to be mediated by the induction of IAPs. The first four amino acids of the mature SMAC, Ala-Val-Pro-Ile (AVPI), bind to the BIR domain of IAP proteins. The four amino acids (AVPI) of SMAC that bind to the BIR3 domain of XIAP are similar to the XIAP-binding sequence of active caspase 9. In addition, SMAC has been shown to form a stable complex with the BIR2 domain of XIAP. SMAC binds to the linker-BIR2 domain and presumably disrupts its inhibition of the active caspase 3 and caspase 7 by steric hindrance. On induction of apoptosis, another mitochondrial protein, Omi/HtrA2 (a serine protease), is released from mitochondria and can induce caspase-independent cell death. Additionally, like SMAC, the mature Omi protein contains a conserved IAP-binding motif (AVPS) at its N-terminus. Omi complexes with XIAP at high stoichiometry and promotes apoptosis by neutralizing the caspase-inhibitory effect of XIAP. Recently, Omi has also been shown to process XIAP and promote its proteasomal degradation.

The Bcl-2 family of proteins is divided into three subfamilies. Members of the subfamily that include Bcl-2, Bcl-x_L, and Mcl-1 inhibit apoptosis (see Fig. 58.8). The Bax subfamily members that promote apoptosis, including Bax and Bak, share with Bcl-2 three of the four Bcl-2 homology domains, BH1–BH3. A C-terminus hydrophobic tail is responsible for the localization of these proteins to the outer membranes of the mitochondria and ER. The third BH3-only subfamily of proteins that includes Bid, Bim, and Bad also promotes apoptosis by binding and inactivating prosurvival Bcl-2 family members. The prosurvival Bcl-2 family members, to a variable degree, are bound to the membranes of the mitochondria, the ER, and the nuclear envelope. In the event of an apoptotic stimulus, BH3-only proteins require Bax and Bak to trigger mitochondrial apoptotic signaling. For example, the exposed BH3 domain of tBid oligomerizes with Bak and Bax, causing their mitochondrial membrane insertion and cytochrome c release. In response to apoptotic stimuli, Bax and Bak undergo conformational change and form membrane-associated homo-oligomers, disrupting the outer membrane of the mitochondria and ER and releasing the pro-death molecules into the cytosol. Besides tBid, Humanin peptide has been shown to activate Bax. Bcl-2 can block these events involving Bax activation by titrating tBid or Bim and/or heterodimerization with Bax and preventing mitochondrial permeabilization.

Induction of $P53^{wt}$ in cells triggers apoptosis by transcriptional activation of prodeath effectors, including Bax, Noxa, Puma, Apaf-1, and DR5, and by transcriptional repression of Bcl-2 and IAPs. p53-dependent apoptosis has also been shown to occur in the absence of any gene transcription or translation. In response to apoptotic stimulus such as irradiation, p53 translocates to the mitochondria, where it directly induces permeabilization of the outer membrane by forming complexes with the protective Bcl-2, resulting in the release of cytochrome c into the cytosol. The E2F transcription factor, normally restrained by the Rb tumor suppressor to inhibit cell proliferation, has been shown to induce apoptosis though p53-dependent and p53-independent mechanisms. These mechanisms include transcriptional activation of ARF (the alternate reading frame product of the INK4a/ARF tumor suppressor locus) or p73 (a member of the p53 family), repression of Mcl-1, and inducing the levels of caspase proenzymes.

Selective Antiapoptotic Agents or Strategies

BCL2 Family of Proteins as Targets for Anticancer Drug Design

The antiapoptotic protein BCL2 was originally identified during the process of the discovery of the t(14;18) in human follicular lymphoma. Overexpression of BCL2 has been reported in both hematologic and solid malignancies and to mediate resistance to traditional chemotherapeutic agents, radiation, and other antitumor treatments.

BH3 Peptide/Mimetics or Bcl-2/Bcl-XL Small-Molecule Antagonists

Another strategy to create Bcl-2 inhibitors has focused on developing small molecules that mimic the action of the endogenous Bcl-2-binding death agonists. Compounds that mimic the BH3-only class of death agonists such as Bad inhibit the survival proteins Bcl-2 and

Bcl-x_L but do not appear to have independent proapoptotic activity. Two classes of novel small-molecule cell-permeable inhibitors of the Bcl-x_L-BH3 domain (BH3I) have been identified. Studies have demonstrated that BH3Is induce apoptosis by preventing BH3 domain-mediated interaction between proapoptotic and antiapoptotic members of the Bcl-2 family. Two natural products have been suggested to antagonize the antiapoptotic function of Bcl-2 or Bcl-x_L. Tetrocarcin A was reported to inhibit mitochondrial functions of Bcl-2 and suppress its antiapoptotic activity. In another report, Antimycin-A was shown to mimic activity of BH3 peptides and selectively induce apoptosis in cell lines overexpressing Bcl-x_L. Certain green tea catechins and black tea theaflavins were identified as potent inhibitors (K_i in the nanomolar range) of the antiapoptotic Bcl-2 family of proteins. On the basis of the high-resolution three-dimensional structure of the target receptor, small organic molecules that bind to this interface have been designed. ABT-737 is a BH3 mimetic that binds to Bcl-2, Bcl-xL, and Bcl-w with high affinity (K_i <1 nM) but not to Mcl-1. Preclinical data demonstrate cytotoxic activity in B lymphoid tumor cell lines, human follicular lymphoma, myeloma, and CLL cells, as well as synergism with other chemotherapeutic agents. However, the clinical utility of ABT-737 is limited, as it undergoes rapid metabolism and is not orally bioavailable. Targeted modifications of ABT-737 led to synthesis of navitoclax (ABT-263), an orally bioavailable BH3 mimetic. In the initial phase I trial[79] more than half of patients had a decrease in their lymphocytosis, with 35% achieving partial remission. The main toxicity was dose-dependent thrombocytopenia, which occurred early in the treatment cycle (days 2 to 5 of a 21-day cycle). Neutropenia occurred at higher dose levels. Other adverse events included gastrointestinal side effects and fatigue. The remarkable activity of this agent in early trials validated Bcl-2 as a target for the treatment of CLL. The clinical development of navitoclax was suspended because of thrombocytopenia, which is an off-target effect due to inhibition of Bcl-XL.

Venetoclax (Abt-199) was subsequently developed to replace navitoclax as a highly selective, orally available small-molecule Bcl-2 family protein inhibitor that binds with high affinity (K_i <0.10 nM) to Bcl-2 and with lower affinity to other Bcl-2 family proteins Bcl-XL and Bcl-w (>480-fold and >2000-fold lower affinity than to Bcl-2, respectively). Thrombocytopenia is less frequent with venetoclax due to its lower affinity to Bcl-XL. Early clinical experience with venetoclax is very promising, with remarkable efficacy in a patient population with relapsed or refractory CLL with high ORR (75% to 79%) and impressive CR rates (22% to 29%) and clearance of measurable residual disease (MRD) even in the high-risk subsets of patients with the adverse-risk del(17p) chromosomal abnormality associated with p53 mutation/dysfunction, an unmutated immunoglobulin heavy chain gene (IGHV), and those with disease refractory to fludarabine in a phase I trial. The early clinical trials of venetoclax were suspended initially because patients presented TLS, which led to two deaths. That led to modification of the clinical protocol with a gradual dose escalation of venetoclax over 4 weeks from 20 to 400 mg daily with stringent monitoring and aggressive prophylaxis and management of TLS. These preventive measures have decreased the incidence of TLS with venetoclax. In the MURANO phase III trial, the combination of venetoclax and rituximab was compared with bendamustine and rituximab for treatment of relapsed CLL, demonstrating the venetoclax combination achieved superior PFS and OS,[80] with the potential for treatment discontinuation in patients who achieved MRD-negative disease. Frontline treatment in a sequence—combination with obinutuzumab was done in the CLL14 trial, which included untreated CLL patients with significant comorbidities,[81] with chlorambucil and obinutuzumab used to treat patients in the control arm. Pretreatment with obinutuzumab for three weeks significantly decreased the incidence of TLS, and the venetoclax combination resulted in significant PFS benefit (3-year PFS of 81.9% vs. 49.5%). Based on the studies, venetoclax is currently approved by regulatory agencies for previously untreated CLL patients and for patients with relapsed disease.

BCL-2 dependence is not limited to lymphoid malignancies or overexpression of the target protein. In vitro profiling of mitochondrial out membrane permeabilization by exposure to BH3 peptides in AML cell lines has found that many are dependent on the anti-apoptotic signaling of BCL-2 for survival. These same cell lines are exquisitely sensitive to BCL-2 inhibition by many BH3 mimetics, including navitoclax and venetoclax.[82] The potential for BH3 profiling as a potential biomarker for AML sensitivity to venetoclax was explored in a phase II clinical trial. Single agent venetoclax was given at a dose of 800 mg per day in a population of heavily pretreated, high-risk AML patients. Nineteen percent of patients had an objective response, including 6% of patients achieving a CR or CRi. Patients with BH3 profiles predictive of venetoclax sensitivity were more likely to respond and remain on study. Two landmark phase I studies evaluated both the safety and preliminary efficacy of venetoclax in combination with low-dose cytarabine and hypomethylating agents respectively. Complete response rates of 54% and 61% were reported, both much higher than historical experiences.[83,84] DiNardo et al. further evaluated the pharmacokinetics of venetoclax in combination with CYP34A substrates, and thus established appropriate dose adjustments with antifungal prophylaxis. Due to the outcomes, the FDA granted venetoclax accelerated approval in November of 2018. A phase III study of azacytidine and venetoclax versus azacytidine and placebo reported an overall survival advantage for venetoclax of 14.7 versus 9.6 months and established this combination as a current standard of care for AML patients unfit for more intensive therapies. The CR rate was 36.7% versus 17.9%. Additionally, the response rate for AML patients with IDH1 and IDH2 mutated AML was also confirmed to be significantly higher in the venetoclax arm with 75.4% versus 10.7% entering a CR. Numerous clinical trials evaluating venetoclax in other combinations are ongoing as well as upfront and in relapse settings.

Agents Targeting Complex Multicomponent Cellular Processes

Proteasome Inhibitors

In eukaryotic cells, the lysosomal pathway degrades extracellular proteins imported into the cell through endocytosis or pinocytosis and the ubiquitin proteasome pathway controls the degradation of intracellular proteins. Numerous studies have demonstrated that the ubiquitin–proteasome system controls basic cellular functions such as cell cycle progression, signal transduction, programmed cell death, DNA repair, and antigen presentation.[85] Ubiquitin is a highly conserved 76-amino–acid polypeptide that is expressed in all eukaryotic cells. Under the sequential action of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase), ubiquitin is activated and covalently conjugated to potential proteasome substrates via an isopeptide bond between the C-terminal glycine residue of ubiquitin and the ε-amino group of internal lysine residues in target proteins. The same set of enzymes also catalyzes the formation of the isopeptide bond between G76 and the lysine residue (K48) of previously conjugated ubiquitin, leading to formation of a polyubiquitin chain. Polyubiquitinated substrates are usually targeted for proteasomal degradation.

The 26S proteasome is a large (2000-kDa) threonine protease present in the nucleus and cytoplasm of all eukaryotic cells. This ATP-dependent, multicatalytic protease eliminates damaged or misfolded proteins and regulates cyclins and CDK inhibitor cell cycle regulatory proteins as well as other proteins that govern the transcription factor activation, apoptosis, and cell trafficking. Its structure consists of two parts: the 20S core and the 19S cap regulatory particle (Fig. 58.9). The 19S cap is involved in the recognition, binding, and unfolding of ubiquitinated proteins and in the regulation of the opening of the 20S core. The 20S core is a cylinder composed of four stacked heptameric rings, each containing seven different α or β subunits (α7β7β7α7; Figs. 58.9 and 58.10). Three different active sites are located inside the cylindrical core within the β-subunit rings. Proteasome inhibitors target the 20S subunit of the proteasome. At least three distinct proteolytic sites present in the 20s subunit are named after enzymes with similar proteolytic activity[85]: chymotrypsin-like (β5), trypsin-like

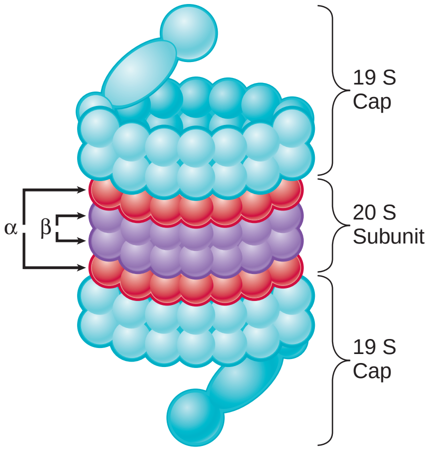


Figure 58.9 26 S PROTEASOME.

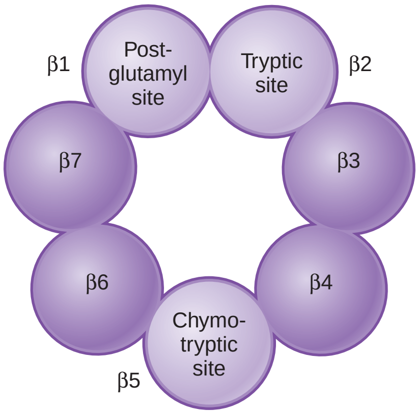


Figure 58.10 CROSS-SECTION OF THE BETA RING OF THE 20 S CORE OF THE 26 S PROTEASOME.

(β2), and caspase-like (post-glutamyl peptide hydrolyzing-like)(β1). After release from the substrate, the polyubiquitin chain is hydrolyzed into single ubiquitin moieties, and tagged proteins are degraded to small peptides. Both the assembly of the 26S proteasome and the degradation of protein substrates are ATP dependent.

Abnormal function of UPP can contribute to oncogenesis through increased or decreased degradation of proteins,[86] including down regulation of tumor suppressor proteins p53 and p27 or decreased degradation of NF-κB. The mechanisms leading to cell death after proteasome inhibition are not fully understood, but it is hypothesized that accumulation of incompatible regulatory proteins within the cell, accumulation of mis-folded proteins from the endoplasmic reticulum (ER) ("ER stress") causing suspension of protein synthesis, and interference with degradation of the NF-κB inhibitor IκB, preventing NF-κB action by blocking its nuclear translocation.

First-Generation Proteasome Inhibitors

Bortezomib. Bortezomib (pyrazylcarbonyl-Phe-Leu-boronate), the first in this class of agents to enter clinical trials, is a dipeptidyl boronic acid that is a specific and selective inhibitor of the 26S proteasome. The boron atom interacts reversibly with the catalytic threonine residue of the proteasome, primarily inhibiting its chymotrypsin-like activity. The inhibition of the ubiquitin–proteasome pathway with bortezomib was demonstrated to arrest the growth of malignant cells (breast, colon, prostate tumor cell lines, Burkitt lymphoma, adult T-cell leukemia, Lewis lung carcinoma, CLL, and myeloma cell lines) and sensitize them to chemotherapeutic agents (5-FU, cisplatin, Taxol, doxorubicin, CPT-11, and gemcitabine). Bortezomib mediates these effects through multiple mechanisms by regulating the expression of proteins involved in cell cycle progression ($P21^{cip1}$, $P27^{Kip1}$), oncogenesis (P53, IκB), apoptosis (BCL2, BIRC2, BIRC3, BIRC4, Bax), and, more recently, DNA repair (DNA-PKcs, ATM). Loss of IκB destabilizes NFκB, reducing expression of a critical plasma cell cytokine stimulatory molecule, interleukin (IL)-6 (see Chapters 86 and p1). The process of cell death appears to be p53-independent and to result in mitotic catastrophe, although the classical caspase 8-dependent apoptosis pathway has also been implicated. A recent study found a strong correlation between immunoglobulin production and apoptotic sensitivity to bortezomib, suggesting that the active requirement for protein folding in the ER provides a direct target and explains sensitivity to proteasome inhibition.[87,88]

Bortezomib is primarily metabolized through cytochrome P450 (CYP) and not via phase II pathways (e.g., glucuronidation and sulfation). In vitro studies indicated that the primary metabolic pathway was deboronation mediated by CYP3A4. Bortezomib was also metabolized by CYP2D6, but the rate of metabolism was slower than that observed with CYP3A4. The deboronated metabolites have been shown to be inactive in the 20S proteasome assay. Bortezomib rapidly exits the plasma compartment, with more than 90% cleared within 15 minutes of IV administration. In whole-body autoradiography of [^{14}C] bortezomib-treated rats, the CNS, testes, and eyes appeared to be protected from bortezomib. Bortezomib specifically and selectively inhibits proteasome function by binding tightly (dissociation constant [K_i] >0.6 nM) and reversibly to the enzyme's chymotrypsin-like site. In ex vivo 20S proteasome activity bioassays, the proteasome was inhibited within 1 hour of bortezomib administration, and baseline proteasome activity was restored within 48 to 72 hours. An intermittent but high level of inhibition (>70%) of proteasome activity was better tolerated than sustained inhibition. Thus, a twice-weekly clinical dosing regimen is better tolerated. Nonetheless, this dose schedule is often associated with significant myelosuppression and the onset of peripheral neuropathy. Reducing the dose schedule to a weekly regimen ameliorates these toxicities and appears to increase patient tolerance without jeopardizing clinical efficacy.

Bortezomib showed significant single-agent clinical activity in phase I and phase II clinical trials in relapsed/refractory MM. In the SUMMIT study of 202 patients with relapsed MM, 27% of patients achieved a PR or complete response (CR), and median TTP was 7 months.[89] The FDA approved the drug for use in relapsed/refractory MM in 2003 on the basis of this study. In 2008, the VISTA trial presented evidence demonstrating the superiority of bortezomib plus melphalan and prednisone (VMP) compared to MP alone for patients with newly diagnosed MM, which led to FDA approval for the use of bortezomib combinations as front-line therapy. In the VISTA trial, the median TTP was 24 months for VMP versus 16.6 months with MP. After initial treatment, many patients remain sensitive to bortezomib and can be retreated upon relapse; 63% of patients who responded to initial treatment with bortezomib respond to retreatment, with a median TTP of 9.3 months.

Second-Generation Proteasome Inhibitors

Carfilzomib. Carfilzomib is a second-generation proteasome inhibitor. It is an epoxy-ketone that selectively inhibits the chymotrypsin-like activity of the proteasome through irreversible binding of N-terminal threonines, resulting in more prolonged proteasome inhibition.[90]

Carfilzomib is active in patients with bortezomib resistant MM. Carfilzomib was approved in 2012 for the treatment of patients with myeloma who have received at least two prior therapies. As a monotherapy in phase II studies, carfilzomib induced an ORR of 20% in patients refractory to bortezomib. In a large, randomized phase III trial, comparison of carfilzomib + dexamethasone (KD) vs. bortezomib + dexamethasone (VD) in relapsed MM, KD was found to be superior to VD (PFS 18.7 months vs. 9.4 months, $P<.0001$). Of note, studies of initial therapy have not shown superiority of carfilzomib- vs. bortezomib-containing regimens.

Ixazomib. Like bortezomib, ixazomib is a boronic acid-containing peptide with chymotrypsin- and caspase-like proteasome inhibitory activity, formulated for oral administration.

Toxicities of Proteasome Inhibitors

Given their broad application, prolonged exposure, and use in combination with other agents with similar toxicities (especially vinca alkaloids and IMiDs), there has been a considerable effort to characterize and mitigate the side effects of proteasome inhibitors. One of the major motivations for the development of the second-generation agents has been to reduce the occurrence of neuropathy. Unlike IMiDs, proteasome inhibitors, as a class, do not appear to induce chromosomal abnormalities and therefore are not associated with an increased risk for secondary malignancies. Proteasome inhibitors may therefore be safer for long-term use/maintenance therapy than other agents.[91]

Peripheral Neuropathy. Bortezomib and ixazomib are more frequently associated with peripheral neuropathy than carfilzomib. The peripheral neuropathy associated with proteasome inhibitors is typically a sensory neuropathy affecting the hands and feet in a "stocking and glove" distribution and is frequently painful. Grade ≥ 3 PN occurs in 5% to 15% of patients but is reversible in most cases (in the phase III VISTA trial, 60% of instances of neuropathy showed complete resolution within a median of 5.7 months). The mechanism of peripheral neuropathy is unknown but is hypothesized to be due to aggresome formation and cytoskeletal collapse in dorsal root ganglion sensory neuron axons, alterations in mitochondrial function, or other off-target effects. It is possible that the boron moiety is implicated in the peripheral neuropathy since carfilzomib (which does not contain a boron atom) is much less likely to cause neuropathy than bortezomib. The incidence of peripheral neuropathy with bortezomib is reduced by SC administration and weekly dosing. The lower incidence of neuropathy of SC vs. IV administration of bortezomib has been attributed to IV dosing achieving peak serum levels of bortezomib that exceed the threshold that causes peripheral neuropathy, whereas the slower pharmacokinetics of SC administration deliver an effective antimyeloma dose without exceeding the neuropathy threshold.

Hematologic Toxicity. Hematologic adverse events appear to be a class effect associated with proteasome inhibitors; all agents tested so far are associated with thrombocytopenia, neutropenia, anemia, and lymphopenia. Differences in the tendency of the different agents to cause hematologic toxicity remain to be established as newer agents undergo further testing in clinical trials (there is hope that second-generation drugs may have lower rates of hematologic toxicity). Bortezomib, carfilzomib, and ixazomib cause transient, cyclical thrombocytopenia, with platelet counts dropping and then returning to baseline prior to the next cycle of treatment. The exact mechanism of bortezomib-induced thrombocytopenia remains to be fully elucidated. Bortezomib does not appear to adversely affect stem cell function. One hypothesis is that proteasome inhibition with bortezomib prevents the activation of NFκB, which leads to impairment of platelet budding from megakaryocytes.

Herpes Zoster Reactivation. Bortezomib has been associated with a significantly increased rate of herpes zoster reactivation. In the phase III study of bortezomib VMP versus MP alone, zoster reactivation was observed in 13% of patients in the VMP group versus 4% in the MP group. In the subgroup of patients in the VMP group who were receiving antiviral prophylaxis, the rate of zoster reactivation was reduced to 3%. The increased susceptibility to herpes zoster reactivation in patients treated with bortezomib may be due to the effect of bortezomib treatment on the number and function of specific lymphocyte subsets.

Other Toxicities. Infusion reactions (chills, fever, and dyspnea) have been observed with carfilzomib. Therefore, it is recommended that dexamethasone (4 mg PO or IV) be administered prior to each dose during cycle 1 and prior to the first of the higher doses during cycle 2. Carfilzomib has been associated with pulmonary complications, renal toxicity, and cardiac events (including congestive heart failure and cardiac arrest) in up to 7% of treated patients. Chest pain and acute congestive heart failure may have been related to prehydration with normal saline. Ixazomib may cause transient rash.

Hypomethylating Agents

Promoter methylation within CpG islands regulates gene expression in all cells. Disruption of normal gene expression profiles accompanies malignant transformation, giving rise to complex patterns of gene expression. As a consequence, promoters of many genes have an altered pattern of methylation, resulting in either gene activation or gene repression. The link between these concepts and the interest in agents that alter promoter methylation began with the realization that azacitidine, an agent used sparingly for treating myeloid leukemias, had efficacy when given at low doses either IV or SC for extended periods of time, and that in these cases, altered gene expression accompanied responses. The primary mechanism of action for both 5-azacytidine (azacitidine) and 5-aza-2-deoxycytidine (decitabine) act by irreversible inhibition of the DNA methyltransferase-1 (DNMT1) responsible for methylation of the cytidine in CpG islands. Inhibition of DNMT1 requires azacitidine and decitabine to be converted into 5-aza-2-deoxycytidine triphosphate and subsequently incorporated into DNA. Thus, both agents are S-phase-dependent requiring active DNA synthesis for activity. Wijermans et al.[92] first described the efficacy of decitabine when given as a continuous infusion to elderly patients with high-risk MDS, with a 54% ORR. The mechanism of action included both a direct change in promoter methylation and independent changes, with up to 70% genome-wide demethylation, suggesting either that genes with altered expression either up- or downregulated a second set of genes through altered pathways such as induction of WAFp21, p15, and p16, or in a more direct fashion through altered transcription factor expression. Additional studies have identified activity of these agents in other myeloid malignancies, particularly AML. Most often, PRs or short-lived CRs are seen in AML, with CRs more common in MDS. Recent studies also describe a number of extended therapy regimens. Kantarjian et al.[93] reported a comparison of 20 mg/m^2 decitabine IV for 5 days with 20 mg/m^2 SC daily for 5 days and 10 mg/m^2 IV for 10 days. The 5-day IV dose schedule was judged superior on the basis of clinical outcome with 39% CR, a higher proportion of reactivation of p15, and achievement of a hypomethylation state as well as clinical tolerance. For MDS, a course of 7 days of azacitidine at 75 mg/m^2 has been established as a standard of care for high-risk MDS with an overall survival benefit compared to supportive care in a phase III study.[94]

Both azacitidine and decitabine have also been given for AML in remission as "maintenance therapies," and have been associated with both leukemia free and disease-free survival. Both decitabine and azacitidine have demonstrated disease free survival benefits in large randomized controlled trials for AML in first complete remission. An oral formulation of azacitidine administered at a high dose has demonstrated overall survival benefit as maintenance therapy for AML patients in first remission who are not candidates for allogeneic stem cell transplant and was approved by the FDA for this population.[95] Even after stem cell transplant, safety and signals of efficacy of maintenance hypomethylating agents has been demonstrated, though lower doses are required to limit toxicity. De Lima et al.[96] established the MTD of azacitidine after transplant as 32 mg/m^2 for 5 days. Posttransplant azacitidine may also augment graft-versus-leukemia effect and attenuate GVHD after transplant through expansion of T-regulatory cells.

One goal of drug development has been oral formulations of both available hypomethylating agents, as this would ease the burden of drug delivery and potentially further permit extended drug exposure during S-phase of cancer cells. A major barrier to oral azacitidine has been high first pass metabolism by cytidine deaminase (CDA), which is highly expressed in gastrointestinal tissues. The high CDA expression also contributes to a different toxicity profile, as oral azacitidine also has a higher rate of gastrointestinal toxicities than IV or SC administration. Co-administration with CDA inhibitors may permit a higher absorption of the active drug. Tetrahydropyridine in combination with azacitidine and decitabine has been showed to improve the pharmacokinetics of oral administration. Cedazuridine is a novel CDA inhibitor designed to have increased stability compared to tetrahydropyridine and was approved by the FDA in combination with an oral formation of decitabine after phase II and III pharmacokinetic studies demonstrated equivalent area-under-the-curve and hypomethylation patterns to intravenously administered decitabine.[97]

Pretreatment with hypomethylating agents has also been postulated to improve malignant cell sensitivity to standard agents by re-expression of tumor suppressor genes. Several early phase studies have suggested that this “epigenetic priming” can restore chemosensitivity. In DLBCL cells in vitro and in clinical samples from patients azacitidine “priming” decreased SMAD1 methylation, and increased SMAD1 mRNA, in DLBCL cells obtained from patients. SMAD1 is the major downstream effector of TGF-β. TGF-β exerts an antiproliferative effect in most NHLs, and loss of antiproliferative response to TGF-β has been demonstrated in the majority of NHLs. SMAD1 hypomethylation has been associated with an abundance of SMAD1 mRNA and restoration of chemosensitivity in DLBCL cells after azacitidine “priming.” In leukemia, priming can demonstrably result in increased hypomethylation in constitutively hypermethylated regions of the genome (HIST1H2AA and LINE1 repetitive elements).[98] Unfortunately, examination of priming in larger-scale studies has yet to suggest a clear role for azacitidine and decitabine prior to standard chemotherapy. Epigenetic priming is still being explored as a method to increase the efficacy of immune checkpoint inhibition. High rates of CR have been observed in relapsed and refractory Hodgkin lymphoma patients with prior exposure to hypomethylating agents.[99]

Histone Deacetylase Inhibitors

Histone acetyl transferases (HATs) and HDAC are two classes of enzymes that mediate the acetylation and deacetylation of histones, respectively, at evolutionarily conserved N-terminal lysine residues. Acetylated histones are negatively charged and do not bind as tightly to negatively charged DNA, thereby facilitating gene transcription. In contrast, deacetylated histones bind closely to DNA, preventing transcription. The acetylation status of chromatin and hence gene transcription is dictated by the balance of the actions of HATs and HDACs. Acetylation of core histone bases has also been implicated in chromatin assembly, DNA repair, and replication timing of specific genomic regions. Crosstalk also exists between acetylation and ubiquitination. Thus, HDACs can decrease the half-lives of substrates by exposing the lysine residue for ubiquitination.

Poorly regulated balance between HATs and HDACs can lead to perturbed gene expression, facilitating the development of malignant cell phenotypes. Eckschlager and colleagues enumerated the possible effects of changes in HAT/HDAC activity[100]: (1) modified gene expression profile resulting in changes in intracellular signaling pathways; (2) Changes in proteasomal degradation; (3) Changes in DNA methylation status; and (4) Modify protein kinase C activity.

Classes of Histone Deacetylase Inhibitors

HDAC inhibitors (HDIs) may act on a specific HDAC subtype or may affect all types (pan-HDIs). Several structurally diverse classes of naturally occurring and synthetic compounds have been investigated for their ability to inhibit HDAC activity (Table 58.6). These include hydroxamic acid derivatives (e.g., vorinostat, Panobinostat, short-chain fatty acids (e.g., valproic acid [VPA]), synthetic benzamides (e.g., entinostat), cyclic tetrapeptides (e.g., romidepsin), and sirtuin inhibitors. HDAC inhibitors of all classes have undergone clinical evaluation.

TABLE 58.6 **Histone Deacetylase Inhibitors[a]**

Name	Type of Compound	Cell Culture (Activity)	Animal Tumor Models	Clinical Trial
Butyrates	Short-chain fatty acids	Yes (μM)	Yes	Phase I/II
Valproic acid	Short-chain fatty acid	Yes	Yes	Phase I/II
Trichostatin A	Hydroxamic acid	Yes (nM)	Yes	–
Pyroxamide	Hydroxamic acid derivative	Yes (nM)	Yes	Phase I
Oxamflatin	Hydroxamic acid derivative	Yes (μM)	Yes	–
SAHA	Hydroxamic acid derivative	Yes (nM)	Yes	Phase I/II
TPX-HA analog (CHAP)	Hydroxamic acid derivative	Yes (nM)	Yes	–
LAQ824	Hydroxamic acid derivative	Yes (nM)	Yes	Phase I
MS-275	Benzamide derivative	Yes (μM)	Yes	–
CI-994 (*N*-acetyl dinaline)	Benzamide derivative	Yes	Yes	Phase I
Depsipeptide (FR901228, FK-228)	Cyclic tetrapeptides	Yes (nM)	Yes	Phase I/II
Trapoxin	Cyclic tetrapeptides	Yes (nM)	–	–
Apicidin	Cyclic tetrapeptides	Yes (nM)	Yes	–

SAHA, Suberoylanilide hydroxamic acid.

[a] Activity reported as “Yes” indicates that the compound has been shown to inhibit histone deacetylase (HDAC) activity, that is, growth of transformed cells in culture and in vivo tumor growth in animal studies. (–) indicates no data reported. CI-994 is reported to inhibit histone deacetylation but does not directly inhibit HDAC.

Vorinostat and Other Hydroxamic Acid Derivative Histone Deacetylase Inhibitors. Members of this class are some of the most potent HDIs. They contain a functional group that interacts with the critical zinc atom at the base of the catalytic pocket of the class I and II HDACs. These HDIs also possess a hydrophobic cap and an aliphatic side chain that interacts with the edge and fits into the hydrophobic catalytic pocket of HDAC, respectively, of the HDACs. Members of this class inhibit both class I and II HDACs. Vorinostat (suberoylanilide hydroxamic acid [SAHA], Zolinza) is a second-generation polar–planar compound that induces in vitro growth arrest, differentiation, or apoptosis of a variety of cancer, leukemia, and MM cells by restoring function of aberrantly silenced genes, among other effects. Most clinically relevant responses have been observed in patients with cutaneous T-cell lymphoma, where an overall response of approximately 30% resulted in regulatory approval.[101] Belinostat is another hydroxamic acid HDI that was recently FDA approved for the treatment of relapsed and refractory peripheral T-cell lymphoma (PTCL) based on a multicenter, single-arm BELIEF trial of 120 evaluable patients with PTCL that was refractory or had relapsed after prior treatment. Among patients with histologically confirmed PTCL ($n = 120$), the ORR was 25.8%. Similar to other

FDA-approved HDIs, belinostat was also tested in phase I and II clinical trials for both solid and hematological cancers. Panobinostat is a second-generation hydroxamic acid-based hybrid polar HDI tested both in in vitro and in vivo tumor models, and in clinical trials. Panobinostat induces apoptosis in a dose-dependent manner on human CML blast crisis K562 cells and acute leukemia MV4-11 cells with the activating length mutation of *FLT-3*. Exposure to panobinostat is associated with hyperacetylation of H3, H4, and Hsp90; increase in p21; and induction of cell cycle G_1 phase accumulation. Panobinostat influences the ubiquitin-proteasome system through hyperacetylation of α-tubulin and uncoupling of HDAC6 and dynein (i.e., involved with lysosomal degradation of misfolded proteins or autophagy) leading to higher levels of ubiquitinated protein and apoptosis.[102,103] This explains the synergistic effect of panobinostat with bortezomib.[102] HDAC6 inhibition augments the anti-myeloma immunity.[104]

Short-Chain Fatty Acid Histone Deacetylase Inhibitors. Sodium butyrate, a well-studied member of this class of compounds, induces in vitro growth arrest and differentiation of human leukemia cells at millimolar concentrations. Its clinical development has been hampered by its short half-life and difficulty in achieving millimolar levels in vivo. Phenylbutyrate, another derivative of butyric acid, induces in vitro growth arrest and differentiation of leukemia cells at clinically achievable submillimolar concentrations. Importantly, at these levels, phenylbutyrate synergizes with retinoids in inducing cell cycle arrest, differentiation, and apoptosis of myeloid leukemia cells, and with ara-C in myeloid leukemias. VPA, a well-tolerated antiepileptic, was shown to be as effective as an HDI at levels ranging between 0.5 and 2.5 mM.

Two phase I trials have examined the therapeutic effects of phenylbutyrate in patients with AML and MDS. No responses were noted in either study, with neurotoxicity being the dose-limiting toxicity. Several studies have used VPA as monotherapy or in combination with other agents in hematologic malignancies. As monotherapy in MDS, response rates have been as high as 16% using the IWG criteria. Neurotoxicity has been the major side effect. Other side effects of VPA include thrombocytopenia, weight gain, asthenia, and rarely hepatic failure and pancreatitis. Given the low response rate, emphasis has shifted to newer HDIs.

Synthetic Benzamide Derivative Histone Deacetylase Inhibitors. Benzamide-containing HDIs are another class of compounds that showed both in vitro and in vivo anticancer activities. Among them, and entinostat is an HDAC Class I selective inhibitor currently approved for treatment of solid tumors. A phase I trial tested entinostat in patients with refractory solid tumors and lymphomas. Prolonged disease stabilization was seen in some patients, and the drug was well tolerated and demonstrated antitumor activity.

Cyclic Tetrapeptide Histone Deacetylase Inhibitors. The principal members of this class of agents are depsipeptide (romidepsin). Romidepsin has been approved by the FDA as an agent for the treatment of patients with relapsed or refractory CTCL. It is a potent HDI that exerts in vitro antitumor effects at nanomolar levels against several cancer cell types. It also induces apoptosis of human acute leukemia and CLL cells. Romidepsin is a natural product derived from *Chromobacterium violaceum*, a bacterium isolated from Japanese soil samples. Additionally, romidepsin was shown to exert antiangiogenic effects by modulating the expression of genes involved in angiogenesis. FDA approval for romidepsin in relapsed/refractory CTCL is based on two large phase II studies: a multi-institutional study based at the NCI in the United States (71 patients), and an international study (96 patients). The treatment schedule was identical across both studies and the ORR was 34% in both studies.[105,106] Romidepsin also induced complete and durable responses in patients with relapsed or refractory PTCL across all major PTCL subtypes, regardless of the number or types of prior therapies, with an objective response rate of 25%, which led to the approval of single-agent romidepsin for the treatment of relapsed or refractory PTCL in the United States.

Toxicity of Histone Deacetylase Inhibitors in Clinical Trials

The most common grade 3 and 4 adverse events observed with the use of HDIs were thrombocytopenia, neutropenia, anemia, fatigue, and diarrhea. In some cases, HDI-induced thrombocytopenia can be rapidly reversible upon withdrawal of the drug. Nausea, vomiting, anorexia, constipation, and dehydration were also seen in patients receiving HDIs. Deaths have been reported in clinical studies involving HDIs. For example, when mocetinostat was tested in patients with relapsed Hodgkin lymphoma four patients died, of which two were treatment-related deaths. Similarly, deaths were also reported in clinical trials involving vorinostat, givinostat, and many other HDIs.

Combinations of Histone Deacetylase Inhibitors With Other Agents

With an increased understanding of the function of HDIs, rational combinations of chemotherapeutic agents and HDI inhibitors have been investigated. The acceptable toxicity profile associated with HDI treatment permits a broad integration into currently approved chemotherapy regimens. A variety of HDIs synergistically enhance the growth inhibition and apoptosis of DNA-damaging agents and irradiation. This occurs, in part, through an HDI-mediated increase in chromatin accessibility and downregulation of DNA repair. A small phase I study examined the MTD of vorinostat with standard-dose CHOP chemotherapy in 14 patients with untreated PTCL. The MTD of vorinostat was 300 mg daily. The combination was tolerated, with diarrhea being the most common toxicity observed, and with a 2-year PFS and OS of 79/70% and 81/75% in the two dosing schedules tested, respectively. Synergy between HDI and proteasome inhibitors has been demonstrated in preclinical and clinical studies in myeloma. The most well-characterized model of synergy between proteasome inhibitors and HDIs are the dual inhibition of the proteasome and aggresomal pathways. Targeting both the proteasome with bortezomib and the aggresome with HDAC6 inhibitors in tumor cells induces greater accumulation of polyubiquitinated proteins, resulting in increased cellular stress and apoptosis. More specifically, proteasome inhibition drives the formation of aggresomes, which are dependent on the interaction of HDAC6 with tubulin and dynein complex. Moreover, the proteasome inhibitor (bortezomib) and HDAC6 inhibitors (tubacin or panobinostat) lead to increased hyperacetylation of tubulin and generation of polyubiquitinated proteins, thus increasing cellular stress response (i.e., c-Jun N-terminal protein kinase activation) and leading to apoptosis, which is, in part, dependent on caspase activity. The addition of panobinostat to bortezomib improved PFS by about 3 months in a recent randomized phase III trial of 768 patients with relapsed/refractory myeloma that led to FDA approval.[107] Clinical trials are ongoing to test the combinations of HDI with hypomethylating agents, mTOR inhibitors, and other proteasome inhibitors.

EZH2 Inhibitors

Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of the Polycomb repressive complex 2 (PRC2). The polycomb group proteins have well characterized roles in cancer, acting as epigenetic regulators.[108] The PRC2 is a multimer that includes one of the catalytic subunits EZH1 or EZH2 and includes other core components, including SUZ12, RBAP46 or RBAP48, JARID2, and AEBP2. This complex catalyzes mono-, di-, and trimethylation of lysine 27 of histone H3(H2K27), with the physiologic role of PRC2 thought to be maintenance of transcriptional silencing rather than its initiation.[108]

The ubiquitous role of PRC2 in epigenetic regulation has led to consideration of the role of EZH2 as both an oncogene and a tumor suppressor gene. Hyperactivating mutations of EZH2 occur in several subtypes of NHLs of germinal center origin,[109] including DLBCL and follicular lymphoma, where EZH2 may occur in up to 27% of cases and represent an early event in the pathogenesis of disease.[110] Several gain of function mutations have been identified in lymphomas,[111] including point mutation at tyrosine 641 (Y641X) within the EZH2 catalytic SET domain, affecting the substrate specificity of the enzyme and enhancing histone trymethylation in the presence of residual wild-type EXH2, which is more efficient at mono and di-methylation.[112] Other activating point mutations within the SET domain of EZH2 have been identified in follicular and diffuse large B-cell lymphoma. In contrast, in myeloid malignancies, EZH2 appears to act as a tumor suppressor gene, and abnormalities are characterized by loss of function mutations.

Tazemetostat (EPZ-6438) is the first EZH2 inhibitor to receive regulatory approval. Tazemetostat is a competitive inhibitor of the s-adenosyl-methionine pocket of the SET domain of EZH2. In preclinical models, tazemetostat inhibited methylation of H3K27 in both EZH2 mutated and wild-type lymphoma cells, with increased cell death in those with EZH2 catalytic point mutations.[113] Animal model studies with lymphoma-bearing mice showed that tazemetostat treatment decreases histone methylation and was capable of tumor control in EZH2-mutant lymphoma xenografts.

The initial phase I trial conducted by Italiano and colleagues[114] included 64 patients with lymphomas and solid tumors. The most frequent all-grade adverse events included asthenia (53%), muscle spasms (22%), gastrointestinal complaints (nausea [20%], vomiting [19%], constipation [17%], abdominal pain [16%]), hematologic toxicities (anemia[17%], thrombocytopenia [11%]), and, although rare, neutropenia (3%). Among grade 3 to 4 adverse events, asthenia (33%) and nausea (20%) were the most common. A subsequent phase II study included EZH2 mutated and wild-type follicular lymphoma ($n = 99$), treated with tazemetostat 800 mg orally twice a day. Fatigue was the most common all-grade adverse event (36%), followed by gastrointestinal complaints (nausea [24%], abdominal pain [20%], diarrhea [18%], vomiting [12%]). Treatment-related grade 3 or worse adverse events included thrombocytopenia (3%), neutropenia (3%), and anemia (2%).

Pharmacokinetic studies done in phase I showed tazemetostat is rapidly absorbed after oral administration, with median t_{max} of 1 to 2 hours, and half- life of 3 to 4 hours. Repeated administration of tazemetostat resulted in decreased AUC, indicating autoinduction of CYP3A4 metabolism. Drug interactions are a result of tazemetostat metabolism by CYP3A4, and co-administration with strong or moderate inhibitors should be avoided or require decreased tazemetostat dosing. Strong CYP3A4 inducers can decrease tazemetostat plasma concentrations and should be avoided. Given that tazemetostat is an inducer of CYP3A, it can decrease the concentrations of its substrates.

In phase I, ORR for relapsed/refractory B-cell lymphoma was 38%, with a median time to response of 3.5 months, and a median duration of response of 12.4 months. Follicular lymphoma patients enrolled in a multi-arm phase II trial presented different response rates according to their EZH2 mutation status, with EZH2-mutated patients presenting an overall response of 74% ((95% CI 57.9% to 87%) versus 34% in EZH2 wild-type follicular lymphoma. Complete response rates were 64% and 28%, respectively. Median duration of response was 40 weeks for EZH2-mutated patients and 56 weeks for those with wild-type EZH2.[115] Based on these results, tazemetostat has received approval as a third-line agent for follicular lymphoma patients with mutations of EZH2 as well as for patients without EZH2 mutations who do not have available effective therapies. Tazemetostat appears to have efficacy in DLBCL, with 17% ORR both in patients with mutated and wild-type EZH2.

Protein Translation Inhibitors

The increased proliferation and cell survival of cancer cells imposes a requirement for increased levels of short-lived proteins involved in cell division and survival. As a result, protein translation is markedly upregulated in cancer cells, making ribosomal function and protein synthesis a potential therapeutic target.

Protein translation is divided into three phases: initiation, elongation, and termination. As discussed in earlier sections of this chapter, mTOR inhibitors disrupt the initiation phase by preventing assembly of the eIF4F complex, responsible for ribosomal recruitment. Other agents aimed at impeding translation by directly targeting the function of eIF4F have not yet had successful clinical applications.

Omacetaxine

Omacetaxine mepesuccinate (homoharringtonine, HHT, Synribo), is a semisynthetic, highly purified compound derived from a plant alkaloid discovered more than 40 years ago. Omacetaxine prevents peptide elongation by binding the A site in the peptidyl cleft of the large ribosomal subunit, thus blocking correct positioning of the amino-acid side chains of tRNAs. The clinical activity of omacetaxine has been correlated with its capacity to decrease translation of short-lived proteins such as c-Myc, MCL-1, and Cyclin D1. The initial clinical studies of omacetaxine in CML were done with IV administration, before the introduction of BCR-ABL inhibitors, and were focused on patients who had failed IFN-α. Interest decreased after approval of imatinib for CML, but subsequent studies focused on the use of SC omacetaxine in patients who developed resistance to TK inhibitors and those carrying the T315I mutation. The maximum tolerated dose was 1.25 mg/m^2 subcutaneously twice daily. Subsequent studies demonstrated omacetaxine was capable of achieving major cytogenetic response in 23% of chronic-phase CML patients carrying the T315I mutation. Omacetaxine dosing was 1.25 mg/m^2 twice daily for 14 days every 28 days until achievement of a hematologic response, after which maintenance was given at the same dose for 7 days every 28 days. Hematologic toxicity was frequent (neutropenia 44%, anemia 39%, and thrombocytopenia 76%), particularly in the initial stages of treatment, and was managed by reducing the number of days of drug administration.[116] The most common nonhematologic toxicities were infections, diarrhea, nausea, and fever. Severe nonhematologic toxicities included infection (10%), fatigue (5%), and increased alanine aminotransferase (3%). Omacetaxine received FDA approval in 2012 for the treatment of CML patients who have failed two or more TKI treatments.

Isocitrate Dehydrogenase Inhibitors

Isocitrate dehydrogenases (IDH) are key enzymes in the citric acid cycle, converting isocitrate to α-ketoglutarate (αKG) and vice versa. IDH1 is typically restricted to the cytoplasm and IDH2 and IDH3 found intramitochondrial space. Somatic mutations at the active site of the enzymes results in aberrant conversion of isocitrate into 2-hydroglutarate (2HG). 2HG is considered an oncometabolite, as high concentrations inhibit histone and DNA demethylases which are usually dependent on αKG. As such, these tumors generally exhibit a high level of DNA hypermethylation and a clear block in terminal differentiation.[117]

IDH1 and IDH2 mutations conferring this altered activity are found in up to 20% of AML and 5% of MDS.[118,119] These activating mutations are well defined with mutations occurring at residues at R132 in IDH1, and at R140 and R172 in IDH2. Inhibition of these mutated enzymes *in vitro* can demonstrably reduce 2HG levels and subsequently change DNA and histone methylation patterns. Moreover, human-derived AML blasts will express surface markers indicative of terminal differentiation.[120]

Two IDH inhibitors have been granted acerated approval by the FDA based on early evidence of clinical efficacy. Enasidenib, an IDH2 inhibitor, was approved for relapsed and refractory IDH2 mutant AML based on outcomes of a phase I/II study of 239 patients with relapsed or refractory AML and a documented R140 or R172 IDH mutation. 23% of patients achieved a CR or CRh and remained in remission for a median of 8 months. Toxicities overall were modest, but included nausea, vomiting, and elevated bilirubin. Hyperbilirubinemia was predominantly indirect bilirubin, and reduced activity of UGT1A1, similar to Gilbert syndrome, was suspected, and little direct liver toxicity was noted.[121] Ivosidenib has been approved both as a single agent for relapsed and refractory IDH1 mutant AML, and in combination with azacitidine for newly diagnosed IDH1 mutant AML. A phase I/II study of 258 relapsed and refractory patients found a CR rate of 21.6% and an ORR of 41.6% with a median response time exceeding 8 months. In patients with de novo AML unfit for more intensive therapy, azacitidine and ivosidenib was studied in 23 patients and demonstrated a CR rate of 56.5% and an ORR of 78.6%. Treatment emergent toxicity that appeared related to ivosidenib in both studies included nausea, diarrhea, and prolonged QTc requiring monitoring.[122]

A unique toxicity of IDH inhibition appears to be a modest, but real risk of differentiation syndrome. Previously well described as a potential adverse event with all-trans retinoic acid in acute promyelocytic leukemia, differentiation syndrome is characterized by fever, weight gain, edema, pulmonary edema, and effusions. Management of differentiation syndrome requires urgent recognition and the start of high-dose steroids. Though correlation with response has yet to be clearly observed, this is felt to be a natural effect of the *in vivo* differentiation of the malignant cells.[123]

Hedgehog Pathway Inhibitors

The Hedgehog (Hh) signaling pathway was originally described as a critical intracellular system in embryonic development, controlling maintenance and differentiation. In the canonical pathway, hedgehog ligands interact with two cell surface receptors, a negative regulator named Patched (PTCH1) and a positive regulator named Smoothened (SMO). When ligands bind to PTCH, SMO is depressed. In the active form, SMO promotes transduction of GLI proteins, which in turn lead to expression of numerous genes, including c-MYC and BCL-2. In some myeloid malignancies including AML and CML, aberrant activation of the Hh pathway appears to result in selective survival of the malignant stem cell. In CML, inhibition of SMO can reduce the cell renewal capacity that the BCR-ABL fusion protein induces.[124] AML cell lines with overexpression of Hh pathway proteins appear to have increased drug resistance in addition to uncontrolled self-renewal. Inhibition of SMO appears to restore sensitivity to traditional cytotoxic medications.[125]

To date, clinically relevant pharmacologic agents targeting the Hh pathway have all been inhibitors of SMO. Indeed, several agents are approved for basal cell cancer and nevoid basal cell cancer syndrome, which are uniquely dependent on the Hh pathway. In vitro, both vismodegib and sonidegib have single agent activity against AML, and clinical trials are ongoing. Despite these agents being available, a different SMO inhibitor, glasdegib, has translated into clinical practice for the treatment of AML. Early phase studies have suggested a favorable toxicity profile with dysgeusia and muscle spasms as common events, and little additional toxicity when added to low-dose citarabine.[126] A phase II study of low-dose cytarabine with glasdegib versus placebo demonstrated an improved OS benefit, and this led to a breakthrough designation and approval for glasdegib by the FDA.[127]

Immunomodulatory Agents and Cereblon E3 Ligase Modulation Drugs

Immunomodulatory drugs (IMiDs), thalidomide, lenalidomide, and pomalidomide, are oral agents derived from glutarimide with high anti-tumor activity against plasma cell dyscrasias and lymphoid malignancies. Despite the established use in the last 15 years, particularly in management of plasma cell malignancies, only recently the target of IMiDs has been described. These agents bind to Cereblon, a critical E3 ligase enzyme part of the ubiquitination system, resulting in enhanced degradation of tumor promoting agents. Of note, Cereblon is the primary target of thalidomide-induced teratogenicity.[128] This finding led to advent of a new generation of compounds, called Cereblon E3 ligase modulation drugs (CELMoDs), which have been tested in later-stage clinical trials.[129]

IMiD-based combination induction therapy is considered as standard-of-care modality for newly diagnosed MM regardless of transplant eligibility as well as single-agent post-transplant maintenance therapy. IMiDs also have noticeable activity when combined with monoclonal anti-CD38, such as (daratumumab),[130] or anti-SLAMF7 (elotuzumab) [131] antibodies, most likely due to enhanced antibody dependent cell-mediated cytotoxicity and NK cell activity with these combinations.

IMiDs have mostly similar chemical structures, however, it is clear that subtle differences in their structures can lead to significant difference in the anti-tumor activity.

Lenalidomide

Lenalidomide was identified as an analog of thalidomide with more potent immunomodulatory functions but less neuropathy. The immunomodulatory effects include modifications in T-cell function which can enhance their function,[132] including enhanced ADCC (antibody-dependent cellular cytotoxicity). Lenalidomide also has antiangiogenic properties and increases the anti-inflammatory cytokine IL-10 while decreasing pro-inflammatory IL-6, IL-1b, IL-2, and TNF-alpha production. Decreases in NFκB and IκB levels are also observed in cells. In addition, lenalidomide induces caspase 8-mediated apoptosis and mitochondrial-mediated cell death. T-cell activation and increased natural killer (NK) activity are observed, thus increasing the anticancer immune response.[133]

Lenalidomide can cause significant myelosuppression, which can be dose limiting. The risk of thrombosis is also increased and the administration of lenalidomide should be accompanied by antithrombotic prophylaxis. Hypersensitivity rash occurs in up to 10% of patients. In trials of CLL and lymphoma, initial treatment with lenalidomide can result in tumor flare, characterized by fever, rash, and painful lymphadenopathy; lower initial doses should be considered in patients with high tumor burden and CLL.

A variety of dosing schedules provides a flexible therapeutic approach and a high response rate in front-line and relapsed myeloma patients, including maintenance therapy after autologous stem cell transplant.[134] In 5q-MDS patients, response in over 40% of patients was observed, indicating more based on the achievement of transfusion independence (see Chapter 60). The activity of lenalidomide has been observed in several lymphoid malignancies, including relapsed mantle cell lymphoma, follicular lymphoma, and diffuse large B-cell lymphoma, all in combination with monoclonal antibodies targeting CD20 or CD19.[133]

Pomalidomide

Pomalidomide is a third-generation IMiDs class. In addition to its immunomodulatory and antiangiogenic activity, pomalidomide has direct activity against myeloma cells, affecting gene expression, and promoting apoptosis and cell cycle arrest. Pomalidomide upregulates expression of p21WAF and downregulates interferon (IFN) regulatory factor 4. p21WAFinhibits CDK2, which in turn results in phosphorylation of the retinoblastoma (Rb) protein and cell cycle arrest at G_1. Apoptosis is also induced via activation of caspase 8. In vitro studies showed that pomalidomide was active in cell lines resistant to thalidomide and lenalidomide.[135]

The most common severe adverse events are myelosuppression (anemia, neutropenia, and thrombocytopenia), infections, and fatigue. Venous thromboembolic events were observed in early-phase studies and subsequent trials have required thromboprophylaxis, under which the rate of VTE with pomalidomide is less than 5%.

Phase I studies established that the maximum tolerated dose of pomalidomide was 4 mg given PO on days 1 to 21 of 28-day cycles, which corresponds to the currently approved starting dose. Results of a phase II study combining pomalidomide with dexamethasone in patients with relapsed myeloma showed an encouraging ORR of 63% (PR or better), including 60% of patients who were refractory to bortezomib and 40% of patients who were refractory to lenalidomide.

CELMoDs

CELMoD CC-885, CC-122, and C-220 are emerging as potent antimyeloma agents. CC-885 has a potent antitumor activity through binding to Cereblon and mediating the ubiquitination and degradation of the translation termination factor, GSPT1.[136] CC-122 is another CELMoDs that includes the glutarimide moiety that is present in IMiDs and is currently being evaluated in diffuse large B-cell lymphoma and a number of other malignancies.[137] CC-220 has a higher affinity than either lenalidomide or pomalidomide for CRBN and more potently degrades IKZF1 and IKZF3. Importantly, core structure of this compound is similar to the IMiDs with additional phenyl and morpholino moieties that seem to increase its binding capacity to Cereblon.[138]

Denileukin Diftitox

Denileukin diftitox (DD) or DAB389IL-2 (Ontak) is a fusion protein in which the receptor binding domain of diphtheria toxin has been exchanged for that of the IL-2 molecule. Because of the specificity of the IL-2 domain, the DT-mediated cytotoxic activity will predominately affect cells that express the IL-2 receptor (IL-2R). The IL-2R is selectively expressed on activated T-lymphocytes, B cells, and NK cells. It has been shown that IL-2 fusion toxin is cytotoxic against in vitro T-cell lines. It has been estimated that approximately 50% of CTCL cases express the IL-2R (CD25), as demonstrated by immunohistochemical staining. DD was approved by the FDA for the treatment of relapsed or persistent $CD25^+$ CTCL based on demonstrated efficacy in a placebo-controlled multinational dose-ranging study that enrolled 144 patients with $CD25^+$ stages Ia–III CTCL. Patients who had three or fewer prior therapies were randomly assigned to receive an IV infusion of either DD at 0.018 mg/kg or 0.009 mg/kg on days 1 to 5 of a 21-day cycle (maximum eight cycles) or saline placebo. The ORR for patients receiving the 0.018 mg/kg dose was 46%, with a median response duration of 220 days. The ORR for patients receiving the 0.009 mg/kg dose was 37%, with a median response duration of 277 days. Patients receiving the saline placebo had an ORR of 16% and median response duration of 81 days. Significant improvements of PFS at both doses of DD was noted (hazard ratio: 0.27 comparing 0.018 mg/kg vs. placebo, $P = .0002$; hazard ratio: 0.42 comparing the 0.009 mg/kg vs. placebo, $P = .02$). Treatment-emergent adverse events that occurred in at least 20% of patients in the 0.018 mg/kg group, and more frequently than in the placebo arm, were fever, nausea, rigors, fatigue, vomiting, headache, peripheral edema, diarrhea, anorexia, rash, and myalgia. Serious adverse events in patients receiving DD included infusion reactions, capillary leak syndrome, and loss of visual acuity, including loss of color vision. Laboratory abnormalities reported included hypoalbuminemia and hepatic transaminitis. Both bexarotene and alitretinoin were shown to increase the expression of CD25 in the CTCL cell line. Cells that were subsequently exposed to DD showed a 50% to 70% decrease in protein synthesis. This observation suggests that increases in expression of components of the IL-2R can be achieved by retinoids and rexinoids, which may enhance the cytotoxicity of DD. A phase I trial investigating the use of bexarotene in conjunction with DD in patients with relapsed or refractory CTCL was undertaken, and eight out of 12 treated patients showed a greater than 50% increase in CD25 expression after treatment with bexarotene. A phase II trial of DD demonstrated an ORR of 48% in relapsed/refractory PTCL (excluding CTCL) with median PFS of 6 months. Higher responses (61%) were seen in $CD25^+$ tumors. In a phase II trial in patients with relapsed/refractory NHL, the ORR was 25%, with similar responses in $CD25^+$ and $CD25^-$ tumors. DD has also demonstrated promising activity in 30 patients with steroid-refractory acute GVHD, with 71% of patients responding with either complete (50%) or partial resolution (21%) of GVHD. Hepatic transaminase elevation was the dose-limiting toxicity.

DRUG RESISTANCE TO CHEMOTHERAPEUTIC AGENTS OR MULTIDRUG RESISTANCE

Leukemia Stem Cells

Clonal Evolution

Although many hematologic malignancies develop resistance to a specific class of chemotherapeutic agents, or to targeted therapeutics. The basis for resistance includes overexpression of anti-apoptotic proteins, preexisting and acquired drug-specific resistance mechanisms often at the active site of the target protein, emerging mutations as part of clonal evolution, DNA repair gene expression-mediated resistance, and overexpression of the ATP-binding cassette (ABC) transporters such as MDR-1 for drug efflux. In general, genomic instability and clonal evolution of hematologic malignancies, the existence and selective regrowth of sub-clone populations, especially leukemic stem cells, and more specific error prone DNA repair provide a background for a high mutational frequency that under selective survival pressure due to both cytotoxic and targeted therapies give rise to an exceptional environment for rapid evolution of resistance phenotypes.

P-Glycoprotein (ABC-B1 Transporter)

Structure and Function

The ABC superfamily of membrane transporters mediates the cross-membrane flux of xenobiotics, naturally occurring toxic compounds, drugs, peptides, and ions. The ABC family has a profound impact on homeostasis and is critically important to proliferation and differentiation signals in normal progenitor cells. It also mediates drug sensitivity. This phosphorylated glycoprotein has a molecular mass of approximately 170 kDa and is localized to the plasma membrane, where it functions as a drug efflux pump (Table 58.7). The family of drug-transporting proteins has been more carefully characterized, leading to the use of the term *ATP-binding cassette transporters*. The observation of double-minute chromosomes and homogeneously staining regions in several MDR cell lines suggested that gene amplification is involved in PGP-mediated MDR. These double minute amplifications[139] contain not only drug resistance genes but also oncogenic drivers on episomes that replicate with the tumor cell. The human *MDR1* gene, which codes for PGP and is involved in antitumor drug resistance, and the human *MDR2* gene (the product of which is expressed by hepatocytes) are located very near each other on human chromosome 7q21.1. PGP is a member of the ABC superfamily, which includes, among more than 100 others, the MRP; the *pfmdr* pump in *Plasmodium falciparum*, which results in chloroquine resistance; STE6, the transporter of the "a" peptide mating factor in yeast; the cystic fibrosis transmembrane conductance regulator (CFTR); and the TAP-1 and TAP-2 proteins that transport antigenic peptides for association with class I molecules and surface antigen presentation.

PGP has a broad specificity for hydrophobic compounds and can both reduce the influx of drugs into the cytosol and increase efflux from the cytosol. To accomplish the former, this "hydrophobic vacuum cleaner" must detect drugs and expel them while they are still in the plasma membrane. Drugs are thought to be effluxed from the cytosol through a single barrel of the PGP transporter, although an exact mechanism has been lacking. PGP is monomeric, with the shape of a cylinder 10 nm in diameter with a maximum height (in the plane of the membrane) of 8 nm (Fig. 58.11). Approximately half of the PGP molecule is within the membrane because the lipid bilayer is approximately 4 nm in depth. When viewed from the extracellular surface of the membrane, PGP is pteroidal with a large central pore 5 nm in diameter. Thus, this large pore has a "gate" on the cytoplasmic side of the membrane that can regulate the transport of different-sized substrates.

TABLE 58.7 **Characteristics of Three Mechanisms of Multidrug Resistance That Result From Overexpression of P-glycoprotein, Multidrug Resistance-Associated Protein, or Lung Resistance-Related Protein**

	PGP	MRP	LRP
Gene on chromosome	7q21.1	16p13.1	16p11.2
Protein			
Molecular mass	170 kDa	190 kDa	110 kDa
Cellular location	Plasma membrane	Plasma membrane	Cytoplasm ≫ nuclear membrane
Function	Efflux pump, chloride channel	Drug transporter	Major vault protein (nucleocytoplasmic transport?)
Energy source	ATP	ATP	
Posttranslational modifications	*N*-glycosylation, phosphorylation	*N*-glycosylation, phosphorylation	No *N*- or *O*-glycosylation
Analogs	Member ABC superfamily	Member ABC superfamily; GS-X pump, MOAT, LTC$_4$ transport	
Drug-Resistance Phenotype			
Antitumor agents	Act-D, *m*-AMSA, dauno, dox, epi, ida, mito-C	Act-D, chlor, CDDP-GSH, dauno, dox, epi, mel, tax (low), vbl (low), vcr, VM-26, VP-16	Carbo, CDDP, dox, mel, vcr, VP-16
	(Low), mtz, nav, tax, txtr, tpt (low), vbl, vcr, VM-26, VP-16	As, Cd, colch (low), GSH	
Other drugs	Colch, rhod	Conjugates, GSSG, LT$_4$, Sb	
Reversing agents	CSA, FK506, nifed, PSC833, quin, rap, verap	CSA, gnstn, indo, nicard, prbn, PSC833, verap, VX-710	
Normal hematopoietic tissues with increased expression	NK (CD56$^+$) T cells, suppressor T cells (CD8$^+$), B cells, CD34$^+$ stem cells	PBMNs (especially T cells), red blood cell membranes; liver and spleen low level	Macrophages
Prognostic significance	AML, MM, NHL	AML (inv 16)	AML, ALL

ABC, ATP-binding cassette; *act-D*, actinomycin D; *ALL*, acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *As*, arsenicals; *ATP*, adenosine triphosphate; *carbo*, carboplatin; *Cd*, cadmium; *CDDP-GSH*; cisplatin glutathione conjugate; *chlor*, chlorambucil; *colch*, colchicine; *CSA*, cyclosporin A; *dauno*, daunomycin; *dox*, doxorubicin; *epi*, epirubicin; *gnstn*, genistein; *GS-X*, glutathione conjugate; *GSSG*, oxidized glutathione; *ida*, idarubicin; *indo*, indomethacin; *LRP*, lung resistance-related protein; *LTC$_4$*, cysteinyl leukotriene; *m*-AMSA, amsacrine; mel, melphalan; *mito-C*, mitomycin C; *MM*, multiple myeloma; *MRP*, multidrug resistance-associated protein; *MOAT*, multispecific organic anion transporter; *mtz*, mitoxantrone; *nav*, navelbine; *NHL*, non-Hodgkin lymphoma; *nicard*, nicardipine; *NK*, natural killer; *nifed*, nifedipine; *PGP*, P-glycoprotein; *prbn*, probenecid; *quin*, quinidine; *rap*, rapamycin; *rhod*, rhodamine; *Sb*, antimonials; *tax*, taxol; *tpt*, topotecan; *txtr*, Taxotere; *vbl*, vinblastine; *vcr*, vincristine; *verap*, verapamil; *VM-26*, teniposide; *VP-16*, etoposide.

Substrates of PGP include (see Table 58.7) anthracyclines (doxorubicin, daunorubicin, epirubicin, and idarubicin), anthracenediones (mitoxantrone), aminoacridines (amsacrine), taxanes (Taxol and Taxotere), epipodophyllotoxins (VP-16 and VM-26), vinca alkaloids (vincristine, vinblastine, and vinorelbine), bortezomib, and actinomycin D. Mitomycin C and one of the topoisomerase I inhibitors (topotecan) are both weak substrates for PGP. Several drugs reverse the resistance mediated by PGP overexpression and sensitize cells to the cytotoxic effects of antineoplastic agents. These drugs compete with antitumor agents for efflux from the cell, effectively increasing the intracellular concentration of the cytotoxic drug, and include immunosuppressants (cyclosporin A [CSA], FK 506, rapamycin, PSC 833), calcium channel blockers (verapamil, nifedipine), antiarrhythmics (quinidine), and other miscellaneous agents. Several of these MDR-modulating agents have been used in clinical trials in an effort to sensitize resistant tumor cells (see later).

P-Glycoprotein Expression in Human Malignancies

Increased expression of PGP has been observed in several human tumors, especially those malignancies that arise in tissues that normally have high levels of PGP expression. An analysis of 61 human tumor cell lines (from leukemia, CNS tumors, melanoma, breast cancer, ovarian cancer, colon cancer, lung cancer, and kidney cancer) that were not selected for resistance to antitumor agents demonstrated co-expression of two or three of the MDR proteins (PGP, LRP, or MRP) in 64% of the cell lines. PGP and LRP were overexpressed in 3% of the tumors; MRP and LRP in 43%; and PGP, LRP, and MRP in 18%. The cell lines with the highest levels of drug resistance were found to overexpress all three proteins. Whether this is true in primary human tumors awaits further investigations.

Acute Myeloid Leukemia

An analysis of 96 untreated patients with AML showed that PGP expression predicted induction failure ($P < .0001$) and decreased OS ($P < .001$), as did unfavorable cytogenetics. PGP expression was not detected in patients with favorable cytogenetic abnormalities [t(15;17), inv(16), t(8;21)], was found in 29% of those samples with a normal karyotype, and was expressed in 62% of patients with an unfavorable cytogenetic abnormality. PGP was also detected in 63% of those with secondary AML compared with 25% of those with de novo disease. PGP analysis with the MRK16 antibody in 211 elderly patients (older than 55 years) with untreated AML again showed that PGP expression is significantly associated with a decreased CR rate and resistant disease. Patients in this report with de novo PGP-negative AML with favorable cytogenetics have a CR rate of 81% compared with 12% for those with secondary AML, which is PGP positive and has unfavorable cytogenetics.

Clinical Studies With Modulators of P-Glycoprotein

The clinical trials that have used various modulators of PGP have been reviewed by several investigators. An early study in VAD (vincristine-Adriamycin-dexamethasone)-refractory MM resulted in short-lived PRs to VAD plus racemic verapamil in five out of 22 patients. Four of the five responders overexpressed PGP; however, cardiac side effects

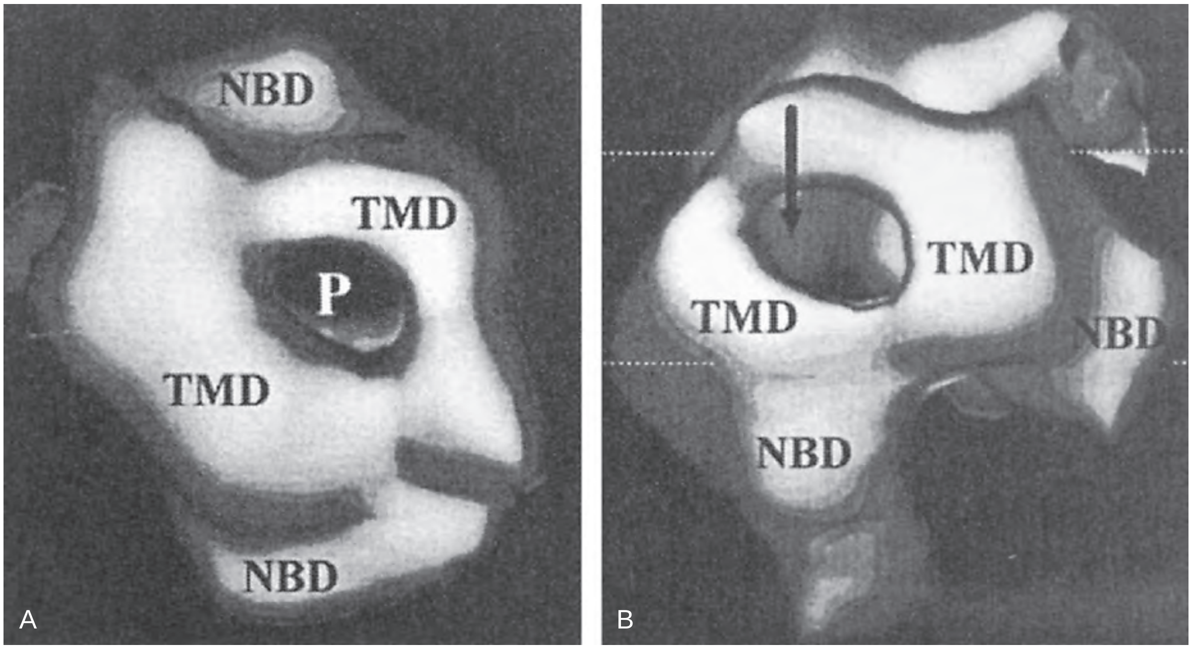


Figure 58.11 STRUCTURE OF P-GLYCOPROTEIN DETERMINED BY ELECTRON MICROSCOPY. A computer graphic representation of the three-dimensional reconstruction is shown as a shaded surface representation of the structure. The *straight arrow* shows the putative adenosine triphosphate binding domains. *P* represents the aqueous pore open at the extracellular face of the membrane. *TMD*, two thumbs, each of which probably corresponds to one of the two transmembrane domains. *NBD*, 3-nm lobes projecting from the structure at the cytoplasmic face of the membrane, probably corresponding to the two nucleotide-binding domains. (A) View perpendicular to the extracellular surface of the lipid bilayer; (B) side view of P-glycoprotein in which the approximate position of the lipid bilayer is indicated by the two horizontal *dashed lines*. *Arrow* indicates asymmetric opening providing access from the lipid phase to the aqueous core of the protein. *(Reproduced with permission from Rosenberg MF, Callaghan R, Ford RC, et al. Structure of the multidrug resistance P-glycoprotein to 2.5 nm resolution determined by electron microscopy and image analysis. J Biol Chem. 1997;272[16]:10685.)*

precluded further dose escalation of IV *R*,*S*-verapamil. Continuous IV infusion of CSA with VAD in VAD-resistant myeloma patients resulted in seven out of 15 responses, which were more common in those who overexpressed PGP. A randomized SWOG phase III study of VAD and PO verapamil in 120 patients with refractory myeloma demonstrated a 41% and 36% response in the VAD and VAD/verapamil arms, respectively, with median survival times of 10 and 13 months, respectively. Continuous-infusion CSA has been dose escalated in combination with daunorubicin and ara-C. A CR was seen in 26 out of 42 patients; however, the MDR phenotype was not found to influence the response. A study of PSC833 in patients with acute leukemia treated with cytarabine, daunorubicin, and etoposide showed a modest benefit in patients younger than 45 years of age. The future role of modulation of PGP in leukemia management remains ill defined.

Multidrug Resistance-Associated Protein (ABC G2 Transporter)

Structure and Function

The MRP was first described in 1992 in the doxorubicin-selected small-cell lung cancer cell line, and its biochemical characteristics and biologic properties have been reviewed.[140] It has now been classified within the larger context of ABC transporters, termed *ABC G2 transporters* (Fig. 58.12).

The overexpression of MRP has been shown in vitro to result in different levels of drug resistance to several classes of antineoplastic agents, represented by actinomycin D, chlorambucil, melphalan, cisplatin (CDDP), daunomycin, doxorubicin, epirubicin, teniposide (VM-26), etoposide (VP-16), and vincristine. Compounds reported to modulate MRP-mediated drug resistance in vitro include the calcium channel blocker verapamil nicardipine; the protein kinase C inhibitor GF109203X; the cyclosporin analog PSC833; the TKI

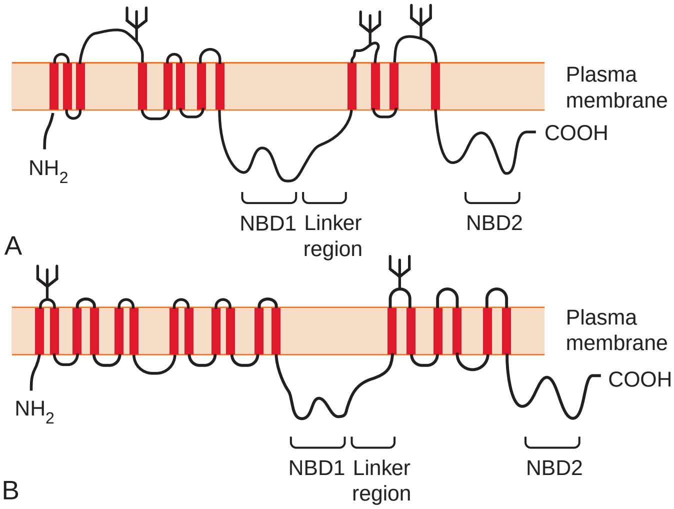


Figure 58.12 MODELS OF MULTIDRUG-RESISTANCE PROTEIN MEMBRANE TOPOLOGY. Multidrug-resistance protein (MRP) possesses features common to all members of the adenosine triphosphate binding cassette (ABC) cassette transporter superfamily in that each half of the protein is predicted to consist of several transmembrane domains followed by a cytosolic nucleotide-binding domain (MBD). The first model (A) is based on computer-assisted hydropathy analyses of the human MRP amino acid sequence and predicts that MRP is composed of 12 transmembrane domains *(solid bars)*, eight of which are within the NH_2-proximal half of the protein. The second model (B), based on a comparison of human and murine MRP with other ABC transporters, suggests that there are up to four additional transmembrane domains in the NH_2-proximal half of the protein. *(Adapted from Loe DW, Deley RG, Cole SPC. Biology of the multidrug resistance-associated protein, MRP. Eur J Cancer. 1996;32A[6]:945.)*

genistein; the gyrase-inhibiting antibiotic difloxacin; and amiodarone VX-710. Amiodarone VX-710, a nonmacrocyclic ligand of the FK506-binding protein FKBP12 and a potent modulator of PGP-mediated MDR, has been found to restore sensitivity of MRP-expressing HL60/ADR cells to the cytotoxic action of doxorubicin, VP-16, and vincristine.

Multidrug-Resistance Protein Expression in Hematologic Malignancies

The expression of MRP mRNA or the level of MRP protein has been assessed in 148 patients with hematopoietic malignancies. MRP mRNA expression was found to be significantly increased in 84% of patients with CLL and in 30% of those with AML. Most patients with ALL, CML, MM, HCL, and NHL were found to have low levels of MRP mRNA expression. MRP protein was assayed using the monoclonal antibody MRPr1, and the results were generally similar with increased expression in most patients with CLL. A study of 40 patients with refractory lymphoma and 16 with newly diagnosed lymphoma suggests a limited role for MRP mRNA expression in drug resistance in NHL because 15 paired samples in the refractory group showed no difference in MRP expression pre- and post-EPOCH treatment. In addition, the untreated patients had MRP mRNA levels that were no different from the pre- or post-EPOCH patient levels. A study of 49 patients with AML and 29 with ALL demonstrated significantly higher expression of MRP in ALL ($P < .007$) and in secondary AML ($P < .016$), but not in de novo AML. Combining overexpression of ABC G2 and FLT3-ITD, an Italian group identified that in adult AML patients, overexpression of ABC G2 was detected in 83 (50%) and FLT3-ITD in 47 (28%) patients. Although the response rate was not affected, the duration of remission was much shorter when these proteins were overexpressed.

The larger family of transporters, the ABC group that includes ABC G2 and ABC B1, is overexpressed in leukemic cells and in many malignant stem cells. In fact, in addition to CD44, high levels of ABC transporters assist in the characterization and isolation of the transplantable subpopulation of malignant stem cells. Brendel and coworkers found that both imatinib and nilotinib used to block the ABL kinase in CML were effectively blocking the ABC transporter in CML cells.[141] This suggested both that these agents might function in part by blocking expression of an important leukemic stem cell protein, and that they would sensitize CML cells to other agents transported by the ABC system.

DNA Repair Pathway Mechanisms of Drug Resistance

O^6-methylguanine-DNA methyltransferase

The nitrosoureas and methylating agents are cytotoxic largely because of formation of DNA adducts at the O^6 position of guanine. The most efficient means of protection from the cytotoxicity of adducts at the O^6 position of guanine is rapid repair by the O^6-MGMT. This protein serves as the stoichiometric acceptor protein for O^6-methylguanine DNA monoadducts, transferring the alkyl group from DNA to the active site of the protein, inactivating the protein, and restoring DNA to normal. However, the $N^1G–N^3C$ DNA cross-link that follows chloroethylation is not a substrate for MGMT. There is a striking correlation between drug resistance and methyltransferase activity. Of interest, high levels of MGMT are found in many leukemias, but low MGMT is observed in normal human CD34 cells, perhaps explaining why nitrosoureas are not used in leukemia management and why nitrosoureas are effective myeloablative agents used in high-dose chemotherapy regimens. A novel inhibitor of MGMT, O^6-benzylguanine (BG) has been used to sensitize human tumors to BCNU. Studies indicate activity of the combination of BG and BCNU in myeloma and in cutaneous lymphomas. Temozolomide has some therapeutic efficacy in leukemia in a manner inversely related to MGGT levels in the tumor cells. A study in five pediatric patients pointed out that response to temozolomide was greater in those with MGMT promoter methylation and no evidence of MMR. This has been tested in a prospective phase II trial of older patients with leukemia. Temozolomide was given in a dosing schedule dependent on MGMT promoter methylation status, with a shorter course of therapy in those with low MGMT expression in the leukemic cells. The ORR in elderly leukemia patients who would otherwise not be candidates for treatment was about 40%, with a median duration of about 29 to 35 weeks.

Mismatch Repair

The spectrum of drug resistance and sensitivity to methylating agents does not end with MGMT. Evidence suggests that methylating agent-induced cell death involves an aborted effort at MMR. Karran and others,[142] in mammalian systems, have shown that the replicative DNA polymerase and the repair polymerase pauses at O^6-mG and preferentially inserts a thymine (T) at the site. The O^6-mG:T base pair is recognized by the MMR system, which initiates (see later). In human cells, the MMR complex consists of at least six proteins involved in the recognition and repair of mismatch lesions: HMLH1, hMSH2, hMSH3, hPMS1, hPMS2, and GTBP (GT-binding protein, also called *MSH6*), all of which appear to be homologs of MMR proteins found in *Escherichia coli* and in yeast. After binding recognition, an endonuclease removes a patch of approximately 100 to 1000 bp containing the T mismatch, DNA polymerase-δ or -γ fills in the patch, with reinsertion of a T opposite the O^6-mG, and a DNA ligase closes the strand break. Because the O^6-mG:T is reformed, cytotoxicity ensues as a result of repetitive efforts at DNA repair and induction of chromosomal breakage, rearrangements, energy depletion, and apoptosis. Drug resistance based on mutation or loss of expression of an MMR protein, owing to mutation within one of the gene coding regions, or promoter methylation leading to loss of gene expression, has been noted in solid tumors and in leukemias and lymphomas. The mutator phenotype was originally described in cells with acquired resistance to methylating agents such as *N*-methyl-*N*-nitroso-*N*-nitrosoguanidine, which were tolerant to G→A point mutations according to the inability to repair O^6-mG and are cross-resistant to 6-TG used in childhood leukemia maintenance regimens, which form the 6-TG:T mismatch.

Major Molecular Response Mutations and Methylating Agent Resistance

MMR defects in humans were initially described in hereditary nonpolyposis colon cancer, which comprises approximately 15% of all colon cancer, lymphomas, and relapsing acute leukemias. The genetic defect results in a high rate of spontaneous mutations within microsatellite DNA, resulting in the RER phenotype arising as the expansion or contraction of mono-, di-, or tri-nucleotide repeats within the microsatellites. In normal hematopoietic stem cells, loss of MMR through promoter methylation of MLH1 in these cells gives rise to microsatellite instability that is age dependent,[143] and may evolve into MDS or acute leukemia.[144] Tumor cells defective in MMR are remarkably resistant to temozolomide regardless of AGT activity or its inhibition by BG, confirming the importance of MMR in sensitivity to methylating agents. Of interest, MMR mutant cells are also two- to threefold resistant to cisplatin, perhaps because the cisplatin DNA adduct is bound by the MMR complex, slowing its recognition and repair by the nucleotide repair pathway and increasing its cytotoxicity. In NHL, loss of MMR results in shorter responses and drug resistance.[145] Such MMR-deficient cells also exhibit microsatellite instability, a measure of genomic instability and the propensity to develop further mutations during therapy, leading to subclones of resistant cells. Loss of PMS2 has been identified in a family of childhood lymphomas, while somatic loss is associated with promoter methylation in acute leukemia.[146] Microsatellite instability is seen in acute leukemias and in T-cell leukemias, suggesting both that these malignancies have lost MMR function and that they are more prone to drug resistance and acquisition of additional mutations that give rise to further drug resistance. Evidence of microsatellite instability and loss of MMR is present in some leukemias but is much more common in treatment-related leukemias, again providing a mechanism of drug resistance.

Base Excision Repair

Methylating agents such as procarbazine and temozolomide form large numbers of N^3-A and N^7-G adducts in addition to O^6-mG (with TMZ, the relative amounts are 72 N^7mG:8 O^6mG:5 N^3mA). Thus, under normal circumstances, cells process many more N^7mG and N^3mA lesions than O^6mG lesions, even though the latter appear much more cytotoxic except in MMR-defective cells. Repair of N^3-A and N^7-G adducts through BER is efficient and normally leads to cell survival rather than cell death. Adducts are recognized by the methylpurine glycosylase with removal of the base, generating an abasic (or AP) site. The AP site is then cleaved by the class II hydrolytic endonuclease (or AP endonuclease) generating a single-strand break with a 5′ PO_4, which becomes the substrate for DNA polymerase-β, and to a lesser extent, polymerases-δ and -γ, followed by DNA ligase.[147] Other compounds induce nucleotide pool imbalance, leading to misincorporation of bases that become substrates for BER. These compounds include folate antagonists such as methotrexate, 5-FdU (floxuridine), and, to a lesser extent, nucleoside analogs such as fludarabine. Misincorporation of uracil alone (after methotrexate) or with 5FU after floxuridine inhibition of thymidylate synthase also leads to BER. Compounds to disrupt BER, such as TRC102, are now being developed and may lead to combination therapy for hematologic malignancies. An initial phase I trial with TRC102 has been completed with fludarabine in CLL[148] and pemetrexed in solid tumors[149]; a trial with temozolomide continues. A third trial using the combination of fludarabine and TRC102 in patients with CLL has found remarkable efficacy in the first cohorts after relapsing from fludarabine (unpublished).

Drug Resistance to Antimetabolites

In addition to their DNA damaging effects and associated resistance patterns, mechanisms of resistance to antimetabolites fall into several broad categories. For example, many antimetabolites are prodrugs in that they must be converted intracellularly into active nucleotide forms to exert their cytotoxic actions. Consequently, events that interfere with cellular accumulation of drug or nucleotide formation will reduce activity. Examples include decreased transport of methotrexate or decreased nucleotide formation of ara-C and 6-TG by reductions in activity of deoxycytidine kinase or hypoxanthine–guanine phosphoribosyltransferase, respectively. Alternatively, enhanced drug catabolism reduces cytotoxicity. Examples include the deamination of ara-C (to inactive ara-U) by cytidine deaminase or catabolism of 6-TG by thiopurine methyltransferase. A third mechanism of resistance stems from the presence of increased intracellular levels of a competing metabolite (e.g., dCTP in the case of ara-C, or hypoxanthine or guanine in the case of 6-TG). Fourth, alterations in the level of activity of a target enzyme or the presence of a mutant form that is a poor target of inhibition will also confer resistance. Examples include increased DHFR activity through gene amplification, most commonly on oncogene-containing episomes termed double minutes DHFR,[150,151] or a mutant form of DHFR (in the case of methotrexate),[152] an altered FLT3-ITD (in the case of ara-C).[153] Finally, cytokinetic factors represent a common theme in the case of most (but not all) antimetabolites, in that a reduction in the S-phase fraction generally leads to reduced drug sensitivity.

Mechanisms of Resistance to Signal Transduction Inhibitors

The introduction of signaling inhibitors to the therapeutic armamentarium represents a significant advance in the treatment of hematologic malignancies. As discussed in the previous sections, these agents can inhibit, with relative selectivity, cellular signaling pathways essential for the survival of neoplastic cells. However, development of resistance has been observed in vitro and in clinical practice.

Genetic Modifications Leading to Resistance to Signal Transduction Inhibitors

Ibrutinib Resistance

Point mutations are the most common mechanism of resistance to TK inhibitors. Emerging ibrutinib resistance due to BTK due to Cys481 mutations occurs in CLL and mantle cell lymphoma, including those with MYD88 mutations.[154] Most often, these BTK^{Cys481} mutations are subclonal and expand over time.[155] While the exact etiology of these mutations is unclear, it is possible that point mutations mediated by BER induced by activation-induced cytidine deamination which is active in B-CLL may be responsible.[156,157]

BCR-ABL Inhibitor Resistance

In CML, the development of multi-generation BCR-ABL inhibitors followed by Src kinase inhibition has provided a plethora of therapeutic options. Resistance to imatinib appears during clonal evolution in a sub population of patients and has the characteristics of a preexisting clone of cells. The T315I BCR-ABL mutation in CML patients is responsible for up to 20% of all clinically observed resistance due to reduced binding to the active site, but may be overcome by addition of interferon.[158] In other patients, a splice variant of BCR-ABL1(35INS) is identified with altered binding to imatinib.[159] While other BCR-ABL inhibitors can be effective for long durations, many other therapeutics to overcome resistance have been identified (see Chapter 69).

Venetoclax Resistance

While very effective, late venetoclax resistance has developed in CLL patients, as noted in those on clinical trials. The novel Gly101Val mutation in BCL2 was identified at progression in 7 of 15 patients with emerging resistance detected after 19 to 42 months of therapy. These mutations were not found at study entry. The point mutation reduces venetoclax affinity of BCL2.[160]

Idelalisib Resistance

The phosphatidylinositol 3-kinase delta (PI3K-δ) inhibitor idelalisib does not have mutational evidence of resistance in CLL but does occur with upregulation of expression of insulin-like growth factor 1 receptor (IGF1R), resulting in enhanced MAPK signaling.[161]

Lenalidomide Resistance

Cereblon, the target for lenalidomide, is often depleted by chromosomal loss or point mutation and results in resistance in myeloma patients. In experimental systems, bromodomain inhibitors can overcome this resistance.

Bortezomib Resistance

Alterations in the proteasome can provide fundamental resistance to proteasome inhibitors in myeloma. High levels of the deubiquitinase USP7, mutations in the proteasome beta-5 subunits, and overexpression of macrophage activating factor (MAF) have been observed.[162–164]

FUTURE DIRECTIONS

Hematologic malignancy research continues to uncover new pathways of pathogenesis, acquired mutations, clonal evolution, drug-induced resistance mutations, and acquired gene activation leading to drug resistance. Artificial intelligence and machine learning are transforming personalized decision making for specific genotypic disorders. Age dependent phenotypes and response rates are emerging as critical in treatment decision making and cell-based therapies and other immune-based therapeutics complement the outlook for patients treated with small molecules.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Abe Y, Ishida T. Immunomodulatory drugs in the treatment of multiple myeloma. *Jpn J Clin Oncol*. 2019;49:695–702.

Blombery P, Anderson MA, Gong J, et al. Acquisition of the recurrent Gly101Val mutation in BCL2 confers resistance to venetoclax in patients with progressive chronic lymphocytic leukemia. *Cancer Discov*. 2019 Mar;9(3):342–353.

Burger JA, Tedeschi A, Barr PM, et al. Ibrutinib as initial therapy for patients with chronic lymphocytic leukemia. N Engl. *J Medicine*. 2015;373: 2425–2437.

Burris HA, Flinn IW, Patel MR, et al. Umbralisib, a novel PI3Kδ and casein kinase-1ε inhibitor, in relapsed or refractory chronic lymphocytic leukaemia and lymphoma: an open-label, phase 1, dose-escalation, first-in-human study. *Lancet Oncol*. 2018;19:486–496.

Comet I, Riising EM, Leblanc B, Helin K. Maintaining cell identity: PRC2-mediated regulation of transcription and cancer. *Nat Rev Cancer*. 2016;16:803–810.

Cortes JE, Heidel FH, Hellmann A, et al. Randomized comparison of low dose cytarabine with or without glasdegib in patients with newly diagnosed acute myeloid leukemia or high-risk myelodysplastic syndrome. *Leukemia*. 2019;33:379–389.

Cortes JE, Khaled S, Martinelli G, et al. Quizartinib versus salvage chemotherapy in relapsed or refractory FLT3-ITD acute myeloid leukaemia (QuANTUM-R): a multicentre, randomised, controlled, open-label, phase 3 trial. *Lancet Oncol*. 2019;20:984–997.

Dimopoulos MA, Oriol A, Nahi H, et al. Daratumumab, lenalidomide, and dexamethasone for multiple myeloma. *N Engl J Medicine*. 2016;375: 1319–1331.

DiNardo CD, Pratz KW, Letai A, et al. Safety and preliminary efficacy of venetoclax with decitabine or azacitidine in elderly patients with previously untreated acute myeloid leukaemia: a non-randomised, open-label, phase 1b study. *Lancet Oncol*. 2018;19:216–228.

Dobbelstein M, Moll U. Targeting tumour-supportive cellular machineries in anticancer drug development. *Nat Rev Drug Discov*. 2014;13:179–196.

Eckschlager T, Plch J, Stiborova M, Hrabeta J. Histone deacetylase inhibitors as anticancer drugs. *Int J Mol Sci*. 2017;18:1414.

Fischer K, Al-Sawaf O, Bahlo J, et al. Venetoclax and obinutuzumab in patients with CLL and coexisting conditions. *N Engl J Med*. 2019;380:2225–2236.

Gallogly MM, Lazarus HM, Cooper BW. Midostaurin: a novel therapeutic agent for patients with FLT3-mutated acute myeloid leukemia and systemic mastocytosis. *Ther Adv Hematology*. 2017;8:245–261.

Hideshima T, Mitsiades C, Akiyama M, et al. Molecular mechanisms mediating antimyeloma activity of proteasome inhibitor PS-341. *Blood*. 2003;101:1530–1534.

Holstein SA, Hillengass J, McCarthy PL. Next-generation drugs targeting the cereblon ubiquitin ligase. *J Clin Oncol*. 2018;36:2101–2104.

Italiano A, Soria J-C, Toulmonde M, et al. Tazemetostat, an EZH2 inhibitor, in relapsed or refractory B-cell non-Hodgkin lymphoma and advanced solid tumours: a first-in-human, open-label, phase 1 study. *Lancet Oncol*. 2018;19:649–659.

Kantarjian HM, Hochhaus A, Saglio G, et al. Nilotinib versus imatinib for the treatment of patients with newly diagnosed chronic phase, Philadelphia chromosome-positive, chronic myeloid leukaemia: 24-month minimum follow-up of the phase 3 randomised ENESTnd trial. *Lancet Oncol*. 2011;12:841–851.

Kantarjian H, Oki Y, Garcia-Manero G, et al. Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia. *Blood*. 2006;109:52–57.

Laubach JP, Schjesvold F, Mariz M, et al. Efficacy and safety of oral panobinostat plus subcutaneous bortezomib and oral dexamethasone in patients with relapsed or relapsed and refractory multiple myeloma (PANORAMA 3): an open-label, randomised, phase 2 study. *Lancet Oncol*. 2021;22:142–154.

Lonial S, Dimopoulos M, Palumbo A, et al. Elotuzumab therapy for relapsed or refractory multiple myeloma. *N Engl J Medicine*. 2015;373:621–631.

LoRusso PM, Boerner SA, Seymour L. An overview of the optimal planning, design, and conduct of phase I studies of new therapeutics. *Clin Cancer Res*. 2010;16:1710–1718.

Lue JK, Amengual JE. Emerging EZH2 inhibitors and their application in lymphoma. *Curr Hematol Malig R*. 2018;13:369–382.

Manasanch EE, Orlowski RZ. Proteasome inhibitors in cancer therapy. *Nat Rev Clin Oncol*. 2017;14:417–433.

Mesa RA, Vannucchi AM, Mead A, et al. Pacritinib versus best available therapy for the treatment of myelofibrosis irrespective of baseline cytopenias (PERSIST-1): an international, randomised, phase 3 trial. *Lancet Haematol*. 2017;4:e225–e236.

Park J, Cho J, Song EJ. Ubiquitin–proteasome system (UPS) as a target for anticancer treatment. *Arch Pharm Res*. 2020;43:1144–1161.

Perl AE, Martinelli G, Cortes JE, et al. Gilteritinib or chemotherapy for relapsed or refractory FLT3-mutated AML. *N Engl J Med*. 2019;381:1728–1740.

Sancar A, Lindsey-Boltz LA, Ünsal-Kaçmaz K, Linn S. Molecular mechanisms of mammalian dna repair and the dna damage checkpoints. *Annu Rev Biochem*. 2004;73:39–85.

Seymour L, Ivy SP, Sargent D, et al. The design of phase II clinical trials testing cancer therapeutics: consensus recommendations from the clinical trial design task force of the national cancer institute investigational drug steering committee. *Clin Cancer Res*. 2010;16:1764–1769.

Simon R. Optimal two-stage designs for phase II clinical trials. *Control Clin Trials*. 1989;10:1–10.

Stone RM, Mandrekar SJ, Sanford BL, et al. Midostaurin plus chemotherapy for acute myeloid leukemia with a FLT3 mutation. *N Engl J Medicine*. 2017;377:454–464.

Stone RM, Manley PW, Larson RA, Capdeville R. Midostaurin: its odyssey from discovery to approval for treating acute myeloid leukemia and advanced systemic mastocytosis. *Blood Adv*. 2018;2:444–453.

Thorpe LM, Yuzugullu H, Zhao JJ. PI3K in cancer: divergent roles of isoforms, modes of activation and therapeutic targeting. *Nat Rev Cancer*. 2015;15:7–24.

Venugopal S, Mascarenhas J. Novel therapeutics in myeloproliferative neoplasms. *J Hematol Oncol*. 2020;13:162.

Wang ML, Rule S, Martin P, et al. Targeting BTK with ibrutinib in relapsed or refractory mantle-cell lymphoma. *N Engl J Medicine*. 2013;369:507–516.

Wang F, Travins J, DeLaBarre B, et al. Targeted inhibition of mutant IDH2 in leukemia cells induces cellular differentiation. *Science*. 2013;340:622–626.

Yuan Y, Hess KR, Hilsenbeck SG, Gilbert MR. Bayesian optimal interval design: a simple and well-performing design for phase I oncology trials. *Clin Cancer Res*. 2016;22:4291–4301.

Zhou J, Chng W-J. Novel mechanism of drug resistance to proteasome inhibitors in multiple myeloma. *World J Clin Oncol*. 2019;10:303–306.

REFERENCE

1. LoRusso PM, Boerner SA, Seymour L. An overview of the optimal planning, design, and conduct of phase I studies of new therapeutics. *Clin Cancer Res*. 2010;16:1710–1718.
2. Yuan Y, Hess KR, Hilsenbeck SG, Gilbert MR. Bayesian optimal interval design: a simple and well-performing design for phase I oncology trials. *Clin Cancer Res*. 2016;22:4291–4301.
3. Seymour L, Ivy SP, Sargent D, et al. The design of phase II clinical trials testing cancer therapeutics: consensus recommendations from the clinical trial design task force of the national cancer institute investigational drug steering committee. *Clin Cancer Res*. 2010;16:1764–1769.
4. Simon R. Optimal two-stage designs for phase II clinical trials. *Control Clin Trials*. 1989;10:1–10.
5. Dobbelstein M, Moll U. Targeting tumour-supportive cellular machineries in anticancer drug development. *Nat Rev Drug Discov*. 2014;13:179–196.
6. Druker BJ, Tamura S, Buchdunger E, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr–Abl positive cells. *Nat Med*. 1996;2:561–566.
7. Hantschel O, Rix U, Superti-Furga G. Target spectrum of the BCR-ABL inhibitors imatinib, nilotinib and dasatinib. *Leukemia Lymphoma*. 2009;49:615–619.
8. Kantarjian H, Shah NP, Hochhaus A, et al. Dasatinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukemia. *New Engl J Medicine*. 2010;362:2260–2270.
9. Kantarjian HM, Hochhaus A, Saglio G, et al. Nilotinib versus imatinib for the treatment of patients with newly diagnosed chronic phase, Philadelphia chromosome-positive, chronic myeloid leukaemia: 24-month minimum follow-up of the phase 3 randomised ENESTnd trial. *Lancet Oncol*. 2011;12:841–851.
10. Cortes JE, Gambacorti-Passerini C, Deininger MW, et al. Bosutinib versus imatinib for newly diagnosed chronic myeloid leukemia: results from the randomized BFORE Trial. *J Clin Oncol*. 2018;363:231–237. JCO.2017.74.716.
11. Boddu P, Jain P, Borthakur G, et al. Outcomes of patients with chronic phase chronic myeloid leukemia (CML-CP) after discontinuation of frontline ponatinib therapy. *Leukemia Lymphoma*. 2019;60:1–9.
12. Hughes TP, Mauro MJ, Cortes JE, et al. Asciminib in chronic myeloid leukemia after ABL kinase inhibitor failure. *N Engl J Med*. 2019;381:2315–2326.
13. Küppers R. Mechanisms of B-cell lymphoma pathogenesis. *Nat Rev Cancer*. 2005;5:251–262.
14. Byrd JC, Furman RR, Coutre SE, et al. Ibrutinib treatment for first-line and relapsed/refractory chronic lymphocytic leukemia: final analysis of the pivotal phase Ib/II PCYC-1102 Study. *Clin Cancer Res*. 2020;26: 3918–3927.
15. Young RM, Phelan JD, Wilson WH, Staudt LM. Pathogenic B-cell receptor signaling in lymphoid malignancies: New insights to improve treatment. *Immunol Rev*. 2019;291:190–213.
16. Honigberg LA, Smith AM, Sirisawad M, et al. The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. *Proc National Acad Sci U S A*. 2010;107:13075–13080.
17. Awan FT, Al-Sawaf O, Fischer K, Woyach JA. Current perspectives on therapy for chronic lymphocytic leukemia. *Am Soc Clin Oncol Educ Book*. 2020;40:320–329.
18. Bercusson A, Colley T, Shah A, Warris A, Armstrong-James D. Ibrutinib blocks Btk-dependent NF-κB and NFAT responses in human macrophages during Aspergillus fumigatus phagocytosis. *Blood*. 2018;132:1985–1988.
19. Byrd JC, Brown JR, O'Brien S, et al. Ibrutinib versus ofatumumab in previously treated chronic lymphoid leukemia. *N Engl J Med*. 2014;371:213–223.
20. Byrd JC, Harrington B, O'Brien S, et al. Acalabrutinib (ACP-196) in relapsed chronic lymphocytic leukemia. *N Engl J Med*. 2016;374:323–332.
21. Burger JA, Tedeschi A, Barr PM, et al. Ibrutinib as initial therapy for patients with chronic lymphocytic leukemia. *N Engl J Med*. 2015;373:2425–2437.
22. Wang ML, Rule S, Martin P, et al. Targeting BTK with ibrutinib in relapsed or refractory mantle-cell lymphoma. *N Engl J Med*. 2013;369:507–516.
23. Dreyling M, Jurczak W, Jerkeman M, et al. Ibrutinib versus temsirolimus in patients with relapsed or refractory mantle-cell lymphoma: an international, randomised, open-label, phase 3 study. *Lancet*. 2016;387:770–778.
24. Wang M, Rule S, Zinzani PL, et al. Acalabrutinib in relapsed or refractory mantle cell lymphoma (ACE-LY-004): a single-arm, multicentre, phase 2 trial. *Lancet*. 2018;391:659–667.
25. Treon SP, Tripsas CK, Meid K, et al. Ibrutinib in previously treated Waldenström's macroglobulinemia. *N Engl J Med*. 2015;372:1430–1440.
26. Miller MS, Thompson PE, Gabelli SB. Structural determinants of isoform selectivity in PI3K inhibitors. *Biomol*. 2019;9:82.
27. Thorpe LM, Yuzugullu H, Zhao JJ. PI3K in cancer: divergent roles of isoforms, modes of activation and therapeutic targeting. *Nat Rev Cancer*. 2015;15:7–24.
28. Castillo J, Iyengar M, Kuritzky B, Bishop K. Isotype-specific inhibition of the phosphatidylinositol-3-kinase pathway in hematologic malignancies. *Oncotargets Ther*. 2014;7:333–342.
29. Krause G, Hassenrück F, Hallek M. Copanlisib for treatment of B-cell malignancies: the development of a PI3K inhibitor with considerable differences to idelalisib. *Drug Des Dev Ther*. 2018;12:2577–2590.
30. O'Brien SM, Lamanna N, Kipps TJ, et al. A phase 2 study of idelalisib plus rituximab in treatment-naïve older patients with chronic lymphocytic leukemia. *Blood*. 2015;126:2686–2694.
31. Barr PM, Saylors GB, Spurgeon SE, et al. Phase 2 study of idelalisib and entospletinib: pneumonitis limits combination therapy in relapsed refractory CLL and NHL. *Blood*. 2016;127:2411–2415.
32. Patton DT, Garden OA, Pearce WP, et al. Cutting edge: the phosphoinositide 3-kinase p110δ is critical for the function of CD4+CD25+Foxp3+ regulatory T cells. *J Immunol*. 2006;177: 6598–6602.
33. Maharaj K, Powers JJ, Achille A, et al. The dual PI3Kδ/CK1ε inhibitor umbralisib exhibits unique immunomodulatory effects on CLL T cells. *Blood Adv*. 2020;4:3072–3084.
34. Burris HA, Flinn IW, Patel MR, et al. Umbralisib, a novel PI3Kδ and casein kinase-1ε inhibitor, in relapsed or refractory chronic lymphocytic leukaemia and lymphoma: an open-label, phase 1, dose-escalation, first-in-human study. *Lancet Oncol*. 2018;19:486–496.
35. Zinzani PL, Santoro A, Mollica L, et al. Copanlisib, a PI3K inhibitor, demonstrates a favorable long-term safety profile in a pooled analysis of patients with hematologic malignancies. *Blood*. 2019;134 4009–4009.
36. Sharman JP, Coutre SE, Furman RR, et al. Final results of a randomized, phase iii study of rituximab with or without idelalisib followed by open-label idelalisib in patients with relapsed chronic lymphocytic leukemia. *J Clin Oncol*. 2019;37 JCO.18.01460.
37. Gopal AK, Kahl BS. Vos Sde, et al. PI3Kδ inhibition by idelalisib in patients with relapsed indolent lymphoma. *N Engl J Med*. 2014;370: 1008–1018.
38. Flinn IW, Hillmen P, Montillo M, et al. The phase 3 DUO trial: duvelisib vs ofatumumab in relapsed and refractory CLL/SLL. *Blood*. 2018;132: 2446–2455.
39. Flinn IW, Miller CB, Ardeshna KM, et al. DYNAMO: A Phase II Study of Duvelisib (IPI-145) in patients with refractory indolent non-hodgkin lymphoma. *J Clin Oncol*. 2019;37 JCO.18.00915.
40. Dreyling M, Santoro A, Mollica L, et al. Phosphatidylinositol 3-kinase inhibition by copanlisib in relapsed or refractory indolent lymphoma. *J Clin Oncol*. 2017;35 JCO.2017.75.464.
41. Uko NE, Güner OF, Matesic DF, Bowen JP. Akt pathway inhibitors. *Curr Top Med Chem*. 2019;20:883–900.
42. Zaytseva YY, Valentino JD, Gulhati P, Evers BM. mTOR inhibitors in cancer therapy. *Cancer Lett*. 2012;319:1–7.
43. Liu Q, Thoreen C, Wang J, Sabatini D, Gray NS. mTOR mediated anti-cancer drug discovery. *Drug Discov Today Ther Strategies*. 2009;6:47–55.
44. Hess G, Herbrecht R, Romaguera J, et al. Phase III study to evaluate temsirolimus compared with investigator's choice therapy for the treatment of relapsed or refractory mantle cell lymphoma. *J Clin Oncol*. 2009;27:3822–3829.
45. Falini B, Martelli MP. Anaplastic large cell lymphoma: changes in the World Health Organization classification and perspectives for targeted therapy. *Haematologica*. 2009;94:897–900.
46. Iragavarapu C, Mustafa M, Akinleye A, et al. Novel ALK inhibitors in clinical use and development. *J Hematol Oncol*. 2015;8:17.
47. Cook JR, Dehner LP, Collins MH, et al. Anaplastic lymphoma kinase (ALK) expression in the inflammatory myofibroblastic tumor. *Am J Surg Pathol*. 2001;25:1364–1371.
48. Sakamoto H, Tsukaguchi T, Hiroshima S, et al. CH5424802, a selective ALK inhibitor capable of blocking the resistant gatekeeper mutant. *Cancer Cell*. 2011;19:679–690.
49. Nagai H, Fukano R, Sekimizu M, et al. Phase II trial of CH5424802 (alectinib hydrochloride) for recurrent or refractory ALK-positive anaplastic large cell lymphoma : study protocol for a non-randomized non-controlled trial. *Nagoya J Med Sci*. 2017;79:407–413.
50. Nakai R, Fukuhara S, Maeshima AM, et al. Alectinib, an anaplastic lymphoma kinase (ALK) inhibitor, as a bridge to allogeneic stem cell transplantation in a patient with ALK-positive anaplastic large-cell lymphoma refractory to chemotherapy and brentuximab vedotin. *Clin Case Rep*. 2019;7:2500–2504.

51. Morrison DK. MAP kinase pathways. *Cold Spring Harb Perspect Biol*. 2012;4:a011254.
52. Braicu Buse Busuioc A comprehensive review on MAPK: a promising therapeutic target in cancer. *Cancers*. 1618;2019:11.
53. Bollag G, Hirth P, Tsai J, et al. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature*. 2010;467:596–599.
54. Tiacci E, Park JH, Carolis LD, et al. Targeting mutant BRAF in relapsed or refractory hairy-cell leukemia. *N Engl J Med*. 2015;373:1733–1747.
55. Tiacci E, Trifonov V, Schiavoni G, et al. BRAF mutations in hairy-cell leukemia. *N Engl J Med*. 2011;364:2305–2315.
56. Goyal G, Heaney ML, Collin M, et al. Erdheim-Chester disease: consensus recommendations for evaluation, diagnosis, and treatment in the molecular era. *Blood*. 2020;135:1929–1945.
57. Badalian-Very G, Vergilio J-A, Degar BA, et al. Recurrent BRAF mutations in Langerhans cell histiocytosis. *Blood*. 2010;116:1919–1923.
58. Oneal PA, Kwitkowski V, Luo L, et al. FDA approval summary: vemurafenib for the treatment of patients with Erdheim-Chester disease with the BRAFV600 mutation. *Oncol*. 2018;23:1520–1524.
59. Bhatia A, Ulaner G, Rampal R, et al. Single-agent dabrafenib for BRAFV600E-mutated histiocytosis. *Haematologica*. 2018 103 haematol.2017.185298.
60. Grafone T, Palmisano M, Nicci C, Storti S. An overview on the role of FLT3-tyrosine kinase receptor in acute myeloid leukemia: biology and treatment. *Oncol Rev*. 2012;6:8.
61. Wilhelm SM, Carter C, Tang L, et al. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res*. 2004;64:7099–7109.
62. Wilhelm S, Carter C, Lynch M, et al. Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. *Nat Rev Drug Discov*. 2006;5:835–844.
63. Pratz KW, Cho E, Levis MJ, et al. A pharmacodynamic study of sorafenib in patients with relapsed and refractory acute leukemias. *Leukemia*. 2010;24:1437–1444.
64. Röllig C, Serve H, Hüttmann A, et al. Addition of sorafenib versus placebo to standard therapy in patients aged 60 years or younger with newly diagnosed acute myeloid leukaemia (SORAML): a multicentre, phase 2, randomised controlled trial. *Lancet Oncol*. 2015;16:1691–1699.
65. Stone RM, Manley PW, Larson RA, Capdeville R. Midostaurin: its odyssey from discovery to approval for treating acute myeloid leukemia and advanced systemic mastocytosis. *Blood Adv*. 2018;2:444–453.
66. Gallogly MM, Lazarus HM, Cooper BW. Midostaurin: a novel therapeutic agent for patients with FLT3-mutated acute myeloid leukemia and systemic mastocytosis. *Ther Adv Hematol*. 2017;8:245–261.
67. Stone RM, Mandrekar SJ, Sanford BL, et al. Midostaurin plus chemotherapy for acute myeloid leukemia with a FLT3 mutation. *N Engl J Med*. 2017;377:454–464.
68. Perl AE, Martinelli G, Cortes JE, et al. Gilteritinib or chemotherapy for relapsed or refractory FLT3-mutated AML. *N Engl J Med*. 2019;381:1728–1740.
69. Zarrinkar PP, Gunawardane RN, Cramer MD, et al. AC220 is a uniquely potent and selective inhibitor of FLT3 for the treatment of acute myeloid leukemia (AML). *Blood*. 2009;114:2984–2992.
70. Fletcher L, Joshi SK, Traer E. Profile of quizartinib for the treatment of adult patients with relapsed/refractory FLT3-ITD-positive acute myeloid leukemia: evidence to date. *Cancer Manag Res*. 2020;12:151–163.
71. Cortes JE, Khaled S, Martinelli G, et al. Quizartinib versus salvage chemotherapy in relapsed or refractory FLT3-ITD acute myeloid leukaemia (QuANTUM-R): a multicentre, randomised, controlled, open-label, phase 3 trial. *Lancet Oncol*. 2019;20:984–997.
72. Galanis A, Ma H, Rajkhowa T, et al. Crenolanib is a potent inhibitor of FLT3 with activity against resistance-conferring point mutants. *Blood*. 2014;123:94–100.
73. Mascarenhas J, Hoffman R. Ruxolitinib: The first FDA approved therapy for the treatment of myelofibrosis. *Clin Cancer Res*. 2012;18:3008–3014.
74. Harrison C, Kiladjian J-J, Al-Ali HK, et al. JAK Inhibition with ruxolitinib versus best available therapy for myelofibrosis. *N Engl J Med*. 2012;366:787–798.
75. Plosker GL. Ruxolitinib: a review of its use in patients with myelofibrosis. *Drugs*. 2015;75:297–308.
76. Venugopal S, Mascarenhas J. Novel therapeutics in myeloproliferative neoplasms. *J Hematol Oncol*. 2020;13:162.
77. Mesa RA, Vannucchi AM, Mead A, et al. Pacritinib versus best available therapy for the treatment of myelofibrosis irrespective of baseline cytopenias (PERSIST-1): an international, randomised, phase 3 trial. *Lancet Haematol*. 2017;4:e225–e236.
78. Tong W-G, Chen R, Plunkett W, et al. Phase I and pharmacologic study of SNS-032, a potent and selective Cdk2, 7, and 9 inhibitor, in patients with advanced chronic lymphocytic leukemia and multiple myeloma. *J Clin Oncol*. 2010;28:3015–3022.
79. Roberts AW, Seymour JF, Brown JR, et al. Substantial susceptibility of chronic lymphocytic leukemia to BCL2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease. *J Clin Oncol*. 2011;30:488–496.
80. Seymour JF, Kipps TJ, Eichhorst B, et al. Venetoclax–rituximab in relapsed or refractory chronic lymphocytic leukemia. *N Engl J Med*. 2018;378:1107–1120.
81. Fischer K, Al-Sawaf O, Bahlo J, et al. Venetoclax and obinutuzumab in patients with CLL and coexisting conditions. *N Engl J Med*. 2019;380:2225–2236.
82. Pan R, Hogdal LJ, Benito JM, et al. Selective BCL-2 inhibition by ABT-199 causes on-target cell death in acute myeloid leukemia. *Cancer Discov*. 2014;4:362–375.
83. Wei AH, Strickland Jr SA, Hou J-Z, et al. Venetoclax combined with low-dose cytarabine for previously untreated patients with acute myeloid leukemia: results from a phase Ib/II study. *J Clin Oncol*. 2019;37(15):1277–1284.
84. DiNardo CD, Pratz KW, Letai A, et al. Safety and preliminary efficacy of venetoclax with decitabine or azacitidine in elderly patients with previously untreated acute myeloid leukaemia: a non-randomised, open-label, phase 1b study. *Lancet Oncol*. 2018;19:216–228.
85. Manasanch EE, Orlowski RZ. Proteasome inhibitors in cancer therapy. *Nat Rev Clin Oncol*. 2017;14:417–433.
86. Park J, Cho J, Song EJ. Ubiquitin–proteasome system (UPS) as a target for anticancer treatment. *Arch Pharm Res*. 2020;43:1144–1161.
87. Hideshima T, Richardson P, Chauhan D, et al. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Res*. 2001;61:3071–3076.
88. Hideshima T, Mitsiades C, Akiyama M, et al. Molecular mechanisms mediating antimyeloma activity of proteasome inhibitor PS-341. *Blood*. 2003;101:1530–1534.
89. Richardson PG, Barlogie B, Berenson J, et al. A phase 2 study of bortezomib in relapsed, refractory myeloma. *N Engl J Med*. 2003;348:2609–2617.
90. Kuhn DJ, Chen Q, Voorhees PM, et al. Potent activity of carfilzomib, a novel, irreversible inhibitor of the ubiquitin-proteasome pathway, against preclinical models of multiple myeloma. *Blood*. 2007;110:3281–3290.
91. Lee JH, Kim S-H. Treatment of relapsed and refractory multiple myeloma. *Blood Res*. 2020;55:S43–S53.
92. Wijermans P, Krulder J, Huijgens P, Neve P. Continuous infusion of low-dose 5-Aza-2′-deoxycytidine in elderly patients with high-risk myelodysplastic syndrome. *Leukemia*. 1997;11:1–5.
93. Kantarjian H, Oki Y, Garcia-Manero G, et al. Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia. *Blood*. 2006;109:52–57.
94. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, et al. Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. *Lancet Oncol*. 2009;10:223–232.
95. Roboz GJ, Montesinos P, Selleslag D, et al. Design of the randomized, Phase III, QUAZAR AML maintenance trial of CC-486 (oral azacitidine) maintenance therapy in acute myeloid leukemia. *Future Oncol*. 2016;12:293–302.
96. Lima Mde, Giralt S, Thall PF, et al. Maintenance therapy with low-dose azacitidine after allogeneic hematopoietic stem cell transplantation for recurrent acute myelogenous leukemia or myelodysplastic syndrome. *Cancer*. 2010;116:5420–5431.
97. Garcia-Manero G, Griffiths EA, Steensma DP, et al. Oral cedazuridine/decitabine for MDS and CMML: a phase 2 pharmacokinetic/pharmacodynamic randomized crossover study. *Blood*. 2020;136:674–683.
98. Scandura JM, Roboz GJ, Moh M, et al. Phase 1 study of epigenetic priming with decitabine prior to standard induction chemotherapy for patients with AML. *Blood*. 2011;118:1472–1480.
99. Falchi L, Sawas A, Deng C, et al. High rate of complete responses to immune checkpoint inhibitors in patients with relapsed or refractory Hodgkin lymphoma previously exposed to epigenetic therapy. *J Hematol Oncol*. 2016;9:132.
100. Eckschlager T, Plch J, Stiborova M, Hrabeta J. Histone deacetylase inhibitors as anticancer drugs. *Int J Mol Sci*. 2017;18:1414.
101. Duvic M, Talpur R, Ni X, et al. Phase 2 trial of oral vorinostat (suberoylanilide hydroxamic acid, SAHA) for refractory cutaneous T-cell lymphoma (CTCL). *Blood*. 2006;109:31–39.
102. Catley L, Weisberg E, Kiziltepe T, et al. Aggresome induction by proteasome inhibitor bortezomib and α-tubulin hyperacetylation by tubulin deacetylase

(TDAC) inhibitor LBH589 are synergistic in myeloma cells. *Blood*. 2006;108:3441–3449.

103. Hideshima T, Richardson PG, Anderson KC. Mechanism of action of proteasome inhibitors and deacetylase inhibitors and the biological basis of synergy in multiple myeloma. *Mol Cancer Ther*. 2011;10:2034–2042.
104. García-Guerrero E, Götz R, Doose S, et al. Upregulation of CD38 expression on multiple myeloma cells by novel HDAC6 inhibitors is a class effect and augments the efficacy of daratumumab. *Leukemia*. 2021;35:201–214.
105. Coiffier B, Pro B, Prince HM, et al. Results from a pivotal, open-label, phase ii study of romidepsin in relapsed or refractory peripheral T-cell lymphoma after prior systemic therapy. *J Clin Oncol*. 2012;30:631–636.
106. Foss F, Pro B, Prince HM, et al. Responses to romidepsin by line of therapy in patients with relapsed or refractory peripheral T-cell lymphoma. *Cancer Med-us*. 2017;6:36–44.
107. Laubach JP, Schjesvold F, Mariz M, et al. Efficacy and safety of oral panobinostat plus subcutaneous bortezomib and oral dexamethasone in patients with relapsed or relapsed and refractory multiple myeloma (PANORAMA 3): an open-label, randomised, phase 2 study. *Lancet Oncol*. 2021;22:142–154.
108. Comet I, Riising EM, Leblanc B, Helin K. Maintaining cell identity: PRC2-mediated regulation of transcription and cancer. *Nat Rev Cancer*. 2016;16:803–810.
109. Morin RD, Johnson NA, Severson TM, et al. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. *Nat Genet*. 2010;42:181–185.
110. Bödör C, Grossmann V, Popov N, et al. EZH2 mutations are frequent and represent an early event in follicular lymphoma. *Blood*. 2013;122: 3165–3168.
111. Lue JK, Amengual JE. Emerging EZH2 inhibitors and their application in lymphoma. *Curr Hematol Malig Rep*. 2018;13:369–382.
112. Sneeringer CJ, Scott MP, Kuntz KW, et al. Coordinated activities of wild-type plus mutant EZH2 drive tumor-associated hypertrimethylation of lysine 27 on histone H3 (H3K27) in human B-cell lymphomas. *Proc Nat Acad Sci U S A*. 2010;107:20980–20985.
113. Knutson SK, Kawano S, Minoshima Y, et al. Selective inhibition of EZH2 by EPZ-6438 leads to potent antitumor activity in EZH2-mutant non-Hodgkin lymphoma. *Mol Cancer Ther*. 2014;13:842–854.
114. Italiano A, Soria J-C, Toulmonde M, et al. Tazemetostat, an EZH2 inhibitor, in relapsed or refractory B-cell non-Hodgkin lymphoma and advanced solid tumours: a first-in-human, open-label, phase 1 study. *Lancet Oncol*. 2018;19:649–659.
115. Morschhauser F, Tilly H, Chaidos A, et al. Tazemetostat for patients with relapsed or refractory follicular lymphoma: an open-label, single-arm, multicentre, phase 2 trial. *Lancet Oncol*. 2020;21:1433–1442.
116. Nicolini FE, Khoury HJ, Akard L, et al. Omacetaxine mepesuccinate for patients with accelerated phase chronic myeloid leukemia with resistance or intolerance to two or more tyrosine kinase inhibitors. *Haematologica*. 2013;98:e78–e79.
117. Wang F, Travins J, DeLaBarre B, et al. Targeted inhibition of mutant IDH2 in leukemia cells induces cellular differentiation. *Science*. 2013;340: 622–626.
118. DiNardo CD, Jabbour E, Ravandi F, et al. IDH1 and IDH2 mutations in myelodysplastic syndromes and role in disease progression. *Leukemia*. 2016;30:980–984.
119. DiNardo CD, Ravandi F, Agresta S, et al. Characteristics, clinical outcome, and prognostic significance of IDH mutations in AML. *Am J Hematol*. 2015;90:732–736.
120. Yen K, Travins J, Wang F, et al. AG-221, a first-in-class therapy targeting acute myeloid leukemia harboring oncogenic IDH2 mutations. *Cancer Discov*. 2017;7:478–493.
121. Pollyea DA, Tallman MS. Botton Sde, et al. Enasidenib, an inhibitor of mutant IDH2 proteins, induces durable remissions in older patients with newly diagnosed acute myeloid leukemia. *Leukemia*. 2019;33:2575–2584.
122. DiNardo CD, Stein EM. Botton Sde, et al. Durable remissions with ivosidenib in IDH1-mutated relapsed or refractory AML. *N Engl J Med*. 2018;378:2386–2398.
123. Golub D, Iyengar N, Dogra S, et al. Mutant isocitrate dehydrogenase inhibitors as targeted cancer therapeutics. *Frontiers Oncol*. 2019;9:417.
124. Irvine DA, Zhang B, Kinstrie R, et al. Deregulated hedgehog pathway signaling is inhibited by the smoothened antagonist LDE225 (Sonidegib) in chronic phase chronic myeloid leukaemia. *Sci Rep*. 2016;6:25476.
125. Kobune M, Takimoto R, Murase K, et al. Drug resistance is dramatically restored by hedgehog inhibitors in CD34+ leukemic cells. *Cancer Sci*. 2009;100:948–955.
126. Savona MR, Pollyea DA, Stock W, et al. Phase Ib study of glasdegib, a hedgehog pathway inhibitor, in combination with standard chemotherapy in patients with AML or high-risk MDS. *Clin Cancer Res*. 2018;24(10):2294–2303.
127. Cortes JE, Heidel FH, Hellmann A, et al. Randomized comparison of low dose cytarabine with or without glasdegib in patients with newly diagnosed acute myeloid leukemia or high-risk myelodysplastic syndrome. *Leukemia*. 2019;33:379–389.
128. Ito T, Ando H, Suzuki T, et al. Identification of a primary target of thalidomide teratogenicity. *Science*. 2010;327:1345–1350.
129. Holstein SA, Hillengass J, McCarthy PL. Next-generation drugs targeting the cereblon ubiquitin ligase. *J Clin Oncol*. 2018;36:2101–2104.
130. Dimopoulos MA, Oriol A, Nahi H, et al. Daratumumab, lenalidomide, and dexamethasone for multiple myeloma. *N Engl J Med*. 2016;375: 1319–1331.
131. Lonial S, Dimopoulos M, Palumbo A, et al. Elotuzumab therapy for relapsed or refractory multiple myeloma. *N Engl J Med*. 2015;373:621–631.
132. Corral LG, Haslett PA, Muller GW, et al. Differential cytokine modulation and T cell activation by two distinct classes of thalidomide analogues that are potent inhibitors of TNF-alpha. *J Immunol*. 1950 1999;163:380–386.
133. Yamshon S, Ruan J. IMiDs new and old. *Curr Hematol Malig R*. 2019;14:414–425.
134. Abe Y, Ishida T. Immunomodulatory drugs in the treatment of multiple myeloma. *Jpn J Clin Oncol*. 2019;49:695–702.
135. Hoy SM. Pomalidomide: a review in relapsed and refractory multiple myeloma. *Drugs*. 2017;77:1897–1908.
136. Matyskiela ME, Lu G, Ito T, et al. A novel cereblon modulator recruits GSPT1 to the CRL4CRBN ubiquitin ligase. *Nature*. 2016;535:252–257.
137. Krönke J, Fink EC, Hollenbach PW, et al. Lenalidomide induces ubiquitination and degradation of CK1α in del(5q) MDS. *Nature*. 2015;523:183–188.
138. Matyskiela ME, Zhang W, Man H-W, et al. A cereblon modulator (CC-220) with improved degradation of ikaros and aiolos. *J Med Chem*. 2018;61:535–542.
139. Shimizu N, Kapoor R, Naniwa S, et al. Generation and maintenance of acentric stable double minutes from chromosome arms in inter-species hybrid cells. *BMC Mol Cell Biology*. 2019;20:2.
140. Mo W, Zhang JT. Human ABCG2: structure, function, and its role in multidrug resistance. Int J Biochem Mol. *Biology*. 2012;3:1–27.
141. Tiwari AK, Sodani K, Wang S-R, et al. Nilotinib (AMN107, Tasigna®) reverses multidrug resistance by inhibiting the activity of the ABCB1/Pgp and ABCG2/BCRP/MXR transporters. *Biochem Pharmacol*. 2009;78: 153–161.
142. Karran P, Macpherson P, Ceccotti S, Dogliotti E, Griffin S, Bignami M. O6-methylguanine residues elicit DNA repair synthesis by human cell extracts. *J Biol Chem*. 1993;268:15878–15886.
143. Kenyon J, Nickel-Meester G, Qing Y, et al. Epigenetic loss of MLH1 expression in normal human hematopoietic stem cell clones is defined by the promoter CpG methylation pattern observed by high-throughput methylation specific sequencing. *Int J Stem Cell Res Ther*. 2016;3(2):031.
144. Qing Y, Gerson SL. Mismatch repair deficient hematopoietic stem cells are preleukemic stem cells. *Plos One*. 2017;12:e0182175.
145. Miyashita K, Fujii K, Yamada Y, et al. Frequent microsatellite instability in non-Hodgkin lymphomas irresponsive to chemotherapy. *Leuk Res*. 2008;32:1183–1195.
146. Mo HY, Choi EJ, Yoo NJ, Lee SH. Promoter mutation analysis of PMS2 gene in solid tumors and acute leukemias. *Pathol Res Pract*. 2019;215:152583.
147. Sancar A, Lindsey-Boltz LA, Ünsal-Kaçmaz K, Linn S. Molecular mechanisms of mammalian DNA repair and the dna damage checkpoints. *Annu Rev Biochem*. 2004;73:39–85.
148. Caimi PF, Cooper BW, William BM, et al. Phase I clinical trial of the base excision repair inhibitor methoxyamine in combination with fludarabine for patients with advanced hematologic malignancies. *Oncotarget*. 2017; 8:79864–79875.
149. Gordon MS, Rosen LS, Mendelson D, et al. A phase 1 study of TRC102, an inhibitor of base excision repair, and pemetrexed in patients with advanced solid tumors. *Invest New Drug*. 2013;31:714–723.
150. Matheson EC, Hogarth LA, Case MC, Irving JAE, Hall AG. DHFR and MSH3 co-amplification in childhood acute lymphoblastic leukaemia, in vitro and in vivo. *Carcinogenesis*. 2007;28:1341–1346.
151. Göker E, Waltham M, Kheradpour A, et al. Amplification of the dihydrofolate reductase gene is a mechanism of acquired resistance to methotrexate in patients with acute lymphoblastic leukemia and is correlated with p53 gene mutations. *Blood*. 1995;86:677–684.
152. Takebe N, Nakahara S, Zhao S-C, et al. Comparison of methotrexate resistance conferred by a mutated dihydrofolate reductase (DHFR) cDNA in two different retroviral vectors. *Cancer Gene Ther*. 2000;7: 910–919.

153. Damdinsuren A, Matsushita H, Ito M, et al. FLT3-ITD drives Ara-C resistance in leukemic cells via the induction of RUNX3. *Leuk Res*. 2015;39:1405–1413.
154. Johnson AR, Kohli PB, Katewa A, et al. Battling BTK mutants with noncovalent inhibitors that overcome Cys481 and Thr474 mutations. *ACS Chem Biol*. 2016;11:2897–2907.
155. Chen JG, Liu X, Munshi M, et al. BTKCys481Ser drives ibrutinib resistance via ERK1/2 and protects BTKwild-type MYD88-mutated cells by a paracrine mechanism. *Blood*. 2018;131:2047–2059.
156. Yuan C, Chu CC, Yan X-J, Bagnara D, Chiorazzi N, MacCarthy T. The number of overlapping AID hotspots in germline IGHV genes is inversely correlated with mutation frequency in chronic lymphocytic leukemia. *Plos One*. 2017;12:e0167602.
157. Whang Y, Silberklang M, Morgan A, et al. Expression of the Epstein-Barr virus gp350/220 gene in rodent and primate cells. *J Virol*. 1987;61: 1796–1807.
158. Itonaga H, Tsushima H, Hata T, et al. Successful treatment of a chronic-phase T-315I-mutated chronic myelogenous leukemia patient with a combination of imatinib and interferon-alfa. *Int J Hematol*. 2012;95: 209–213.
159. Chiarella P, Summa V, Santis SD, et al. BCR/ABL1 fusion transcripts generated from alternative splicing: implications for future targeted therapies in Ph+ leukaemias. *Curr Mol Med*. 2012;12:547–565.
160. Blombery P, Anderson MA, Gong J, et al. Acquisition of the recurrent Gly101Val mutation in BCL2 confers resistance to venetoclax in patients with progressive chronic lymphocytic leukemia. *Cancer Discov*. 2019;9(3):342–353.
161. Scheffold A, Jebaraj BMC, Tausch E, et al. IGF1R as druggable target mediating PI3K-δ inhibitor resistance in a murine model of chronic lymphocytic leukemia. *Blood*. 2019;134:534–547.
162. Yao Y, Zhang Y, Shi M, et al. Blockade of deubiquitinase USP7 overcomes bortezomib resistance by suppressing NF-κB signaling pathway in multiple myeloma. *J Leukocy Biol*. 2018;104:1105–1115.
163. Zhou J, Chng W-J. Novel mechanism of drug resistance to proteasome inhibitors in multiple myeloma. *World J Clin Oncol*. 2019;10:303–306.
164. Dehazya P, Martin GS. pp60src-Dependent protein phosphorylation in membranes from Rous sarcoma virus-transformed chicken embryo fibroblasts. *Virology*. 1985;143:407–421.

CHAPTER 59

PATHOBIOLOGY OF ACUTE MYELOID LEUKEMIA

Andrew M. Brunner and Timothy A. Graubert

Acute myeloid leukemia (AML) is a cancer of hematopoietic stem/progenitor cells, characterized by recurrent genetic and epigenetic alterations. Historically, human leukemias were distinguished according to clinical and histological features and subsequently by morphology. Analysis of the AML genome at increasing resolution, from the level of whole chromosomal changes to individual base pairs, together with an appreciation of epigenetic changes and interactions within the bone marrow microenvironment, has furthered the understanding of the biology and clinical behavior of this disease. Characterizing the patient-specific biology of AML is increasingly important both for understanding the origins of disease and also to guide therapeutic decision-making.

PHENOTYPE OF ACUTE MYELOID LEUKEMIA

Normal hematopoiesis is characterized by self-renewal and differentiation of long-term hematopoietic stem cells (HSCs) to short-term HSCs, multipotent progenitors, and common lymphoid and myeloid progenitors (see Chapter 9). These lineage-committed progenitors further differentiate to mature lymphoid or myeloid cells, including erythrocytes, granulocytes, macrophages, and platelets. This process is regulated by lineage-specific transcription factors at key points during normal hematopoiesis. Functional analysis of recurrent chromosomal, molecular, and epigenetic alterations in AML has revealed that many of these lesions corrupt hematopoietic differentiation programs, impacting proliferation, survival, and maturation of myeloid progenitor cells. Consequently, a hallmark of the AML phenotype is an accumulation of immature myeloid precursors. A myeloblast count of 20% or greater distinguishes AML from other myeloid malignancies.

Historically, leukemias were classified according to morphologic criteria using the French-American-British (FAB) classification, first proposed in 1976. This system classifies AML by the extent of maturation and lineage specificity, ranging from M0 (undifferentiated) to M3 (promyelocytic), M4eo (myelomonocytic with eosinophilia), M6 (erythroid), or M7 (megakaryocytic). Certain FAB subtypes were subsequently found to correlate with underlying cytogenetic abnormalities, notably M3 with t(15;17), M4eo with inv(16), and M2 with t(8;21). As greater understanding of recurrent cytogenetic and now molecular and epigenetic aberrations has developed, it has become clear that morphology alone is inadequate to fully describe the disease spectrum of AML.

ETIOLOGY OF ACUTE MYELOID LEUKEMIA

The majority of patients who develop AML lack any recognized antecedent condition, and for these patients their disease is classified as sporadic or de novo. In these cases, AML appears to result from the accumulation of spontaneously acquired somatic mutations in self-renewing hematopoietic cells. In contrast, a diagnosis of secondary AML (sAML) is preceded by a known predisposing condition, including environmental exposure, antecedent hematologic malignancy, or inherited factors.

Toxins and Exposures

A number of environmental, occupational, and iatrogenic exposures have been identified that contribute to sAML via genotoxic damage to hematopoietic cells. Exposure to benzene, an organic component of many commonly used chemicals including plastics, dyes, pesticides, solvents, and petroleum products, has been linked to the subsequent development of AML. This relationship was identified in the 19th century, when bone marrow aplasia and myeloid leukemia were noted among workers exposed to benzene-containing chemicals. Individuals with occupational benzene exposure have an approximately threefold increased relative risk of developing AML. Workplace benzene exposures have decreased significantly since this discovery, but other sources of benzene exposure remain a concern (e.g., through cigarette smoking). Although cytopenias can occur within months of benzene exposure, there is a latency of several years between benzene exposure and the development of leukemia.

Cases of AML arising after chemotherapy or radiation have been historically designated as therapy-related AML (tAML). Two classes of chemotherapy drugs in particular are associated with an increased risk of tAML (Table 59.1); the first class of drugs with clear links to tAML are the topoisomerase II inhibitors. The most commonly used topoisomerase II inhibitors are anthracyclines, such as doxorubicin, idarubicin, and daunorubicin, and the epipodophyllotoxin etoposide, which are critical components of many treatment regimens for both solid tumors and hematologic malignancies. Topoisomerase II is an adenosine triphosphate (ATP)-dependent enzyme that religates deoxyribonucleic acid (DNA) at sites of double-strand breaks to manage supercoils; inhibition of this enzyme increases the number of double-strand breaks. Resolution of these double-strand breaks may occur via error-prone nonhomologous end joining, resulting in accumulation of DNA damage or apoptotic cell death. tAML arising after exposure to topoisomerase II inhibitors typically occurs with a latency of 1 to 3 years and is often characterized by balanced chromosomal translocations, with the majority involving the *KMT2A* (mixed-lineage leukemia [*MLL1*]) locus on chromosome 11q23. Typical lesions are reciprocal translocations such as t(9;11)(p21;q23) and t(11;19)(q23;p13). Other rearrangements that occur in de novo AML also occur in tAML after topoisomerase II inhibitors, including t(15;17), t(8;21), and inv(16). The risk of tAML varies based on the chemotherapy dosing schedule, cumulative dose received, additional cytotoxic agents, and underlying disease characteristics but generally does not exceed 5% of patients treated with topoisomerase II inhibitors.

Alkylating agents are the second class of chemotherapy drugs with a clear role in the pathogenesis of tAML. The first leukemogenic agents identified in this category were nitrogen mustards. Frequently implicated drugs in contemporary clinical practice include cyclophosphamide, ifosfamide, and melphalan; weaker associations have been described with other alkylating agents such as busulfan, thiotepa, and cisplatin. Alkylating agents create adducts in DNA bases, which are variably mutagenic or cytotoxic. Cytogenetic lesions in alkylator-associated tAML are typically unbalanced, including loss of the long arms of chromosomes 5 or 7 [del(5q), del(7q)], or complete loss of these chromosomes (−5, −7). The risk of tAML following alkylator exposure is up to 1% per year but typically has a longer latency (5 to 7 years) compared with topoisomerase II-associated tAML. The risk increases with age and cumulative exposure to these agents. In

TABLE 59.1 Characteristic Features of Therapy-Related Acute Myeloid Leukemia

Class	Examples	Latency	Associated Phenotypes
Topoisomerase II inhibitors	Doxorubicin, idarubicin, daunorubicin, etoposide, mitoxantrone	1–3 years after chemotherapy	Balanced translocations, often with *KMT2A* (*MLL1*), or with t(15;17), inv(16), or t(8;21)
Alkylating agents	Cyclophosphamide, ifosfamide, chlorambucil, busulfan, carmustine, dacarbazine	~5–7 years after chemotherapy	Monosomal karyotype (–7, del5q, –5, etc.), *TP53* mutations are common, underlying dysplastic features
Irradiation	Therapeutic radiation typically involving bone marrow, nuclear accidents, radioiodine	~5–7 years	Often similar to alkylating agents
Immunosuppressive agents	Azathioprine	~8 years	Abnormalities in chromosomes 5 and 7, possibly related to diminished immune surveillance
PARP inhibitors	Olaparib, rucaparib, niraparib, talozoparib	Poorly defined	Limited to date. Possible increased risk in patients with germline *BRCA1/2* mutations

MLL, Mixed-lineage leukemia; *PARP*, poly (ADP-ribose) polymerase.

some cases of tAML, small clonal populations harboring *TP53* mutations antedate chemotherapy exposure. *TP53* deficiency may confer enhanced fitness on these clones, allowing them to expand under the selective pressure of therapy.

Several other therapies have also been implicated as risk factors for tAML, including some immunosuppressive therapies such as azathioprine, while other new associations continue to be investigated—for instance, possible tAML risk associated with poly (ADP-ribose) polymerase (PARP) inhibitors, particularly in patients with germline *BRCA1/2* mutations.

Exposure to ionizing radiation also has been identified as a causative mechanism for tAML. This relationship was identified in the context of occupational exposures during the development of radiography and subsequently in the setting of mass exposures such as the atomic bomb detonations or nuclear power plant disasters, where a time-limited spike in leukemia incidence occurred following the event. Outside of these events, therapeutic radiation therapy represents the most common setting for significant radiation exposure, which is associated with a small but significant increase in tAML risk and likely varies depending on the site and dose of directed radiation therapy. Radiation-associated tAML is characterized by an increased frequency of mutations otherwise implicated in de novo AML pathogenesis—for instance, mutations in *RUNX1*, as well as balanced translocations such as *RUNX1-RUNX1T1* and *DEK-NUP214*—suggesting some selectivity in the patterns of DNA damage.

Prior Hematologic Malignancy

Other myeloid malignancies, including myelodysplastic syndromes (MDSs) and myeloproliferative neoplasms (MPNs), carry a risk of disease evolution to sAML. The risk varies depending upon the underlying disease and may be facilitated by certain exposures, including genotoxic chemotherapy.

Patients with MPNs have an approximately 10% risk of evolution to AML (MPN in blast phase [MPN-BP]) over 10 years, which varies according to the underlying disease. The risk is lowest in essential thrombocythemia and may be as high as 20% for myelofibrosis. There is a clear association between chemotherapies used in treating MPNs, specifically alkylating agents and radioactive phosphorus, and leukemic evolution; treatment with these agents results in a threefold to fourfold increase in progression to MPN-BP. Another mechanism that may contribute to clonal evolution and disease progression may be a chronic inflammatory state related to the underlying MPN. Sequencing of sAML cases developing in the background of an MPN has identified recurrent mutations in *TET2*, *JAK2*, *IDH*, *IKZF1*, and *ASXL1*. Moreover, a number of patients with a *JAK2*-mutated MPN may progress to MPN-BP that is *JAK2* wild-type, thought to arise either from a common pre-*JAK2* founding clone, or due to parallel expansion of a distinct hematopoietic clone. MPN-BP with mutated *JAK2* typically proceeds through an accelerated myelofibrosis phase, whereas MPN-BP that no longer harbors a *JAK2* mutation tends to arise from chronic phase disease and may be associated with the use of cytotoxic therapies (see Chapter 72).

Prior to the introduction of tyrosine kinase inhibitors (TKIs) for chronic myeloid leukemia (CML), patients with CML typically progressed from chronic phase to blast phase within 5 years, at a rate of more than 20% per year (see Chapter 69). Most cases of blast phase CML have a myeloid phenotype, whereas approximately 30% of patients have a lymphoid phenotype. Additional mutations may occur during transformation of CML, and approximately 80% of patients have additional cytogenetic abnormalities, such as duplication of the Philadelphia chromosome, and other trisomies that are recurrent in de novo AML. Up to one third of patients with CML in myeloid blast phase harbor mutations in the tumor suppressor genes *P16* or *TP53*. In addition, *BCR-ABL* signaling upregulates transcription factors implicated in AML pathogenesis, including *HOXA9* and *EVI1*, which may contribute to leukemic transformation. The rate of transformation to blast phase CML in the TKI era has decreased markedly to approximately 1% per year, which underscores the continued requirement for *BCR-ABL1* signaling in CML evolution.

Approximately one-third of patients with MDS progress to sAML, although this varies significantly according to the underlying MDS subtype and disease characteristics, including the percentage of bone marrow blasts, presence of characteristic cytogenetic abnormalities, and cytopenias (see Chapter 61). Progression to leukemia is associated with acquisition of additional somatic mutations as well as epigenetic alterations within the MDS clone. Mutations in transcription factors and cytokine signaling genes, including *RUNX1*, *NRAS*, and *ETV6*, are more common at progression to sAML, compared with the frequency of these mutations at MDS diagnosis. Epigenetic modifications of the MDS genome appear to also play a significant role in AML progression, particularly through DNA methylation–mediated silencing of tumor suppressor genes. These alterations are also enriched in the subtype of AML with myelodysplasia-related changes, even where a prior MDS diagnosis was not known.

Congenital Bone Marrow Failure Syndromes

A number of inherited bone marrow failure syndromes are associated with an increased risk of developing advanced myeloid malignancies (see Chapter 30). This may be due to the proliferative stress imposed by chronic cytopenias or defects in DNA repair that are hallmarks of several of these syndromes. These syndromes are reviewed in detail in Chapter 30.

Fanconi anemia (FA) is the most common inherited bone marrow failure disorder and is caused by germline mutations in factors involved in DNA repair. These disorders have an autosomal recessive inheritance pattern except for *FANCB*, which is X-linked. To date, more than 20 genes have been identified as a part of the *FANC* gene family, and together their protein products are responsible for identifying DNA damage and targeting these sites for repair. The cumulative risk of AML or MDS among FA patients is approximately 10% to 15%, with peak incidence during the teenage years.

Dyskeratosis congenita (DKC) is a bone marrow failure syndrome characterized by inherited mutations in the telomere maintenance pathway. DKC can be inherited in an autosomal dominant (Online Mendelian Inheritance in Man [OMIM] 127550), autosomal recessive (OMIM 224230), or X-linked recessive pattern (OMIM 305000). Mutations in *TERT*, *DKC1*, *TERC*, or *TINF2* account for most cases. These patients will typically develop bone marrow failure by 20 to 30 years of age, although variable penetrance of short telomere syndromes may result in later onset of leukemia—particularly in those with a telomere mutation but without the DKC "triad" phenotype of skin hyperpigmentation, nail dystrophy, and oral leukoplakia. Transformation to AML occurs in approximately 10% of patients and is thought to occur via genomic instability related to shortened telomeres and associated DNA damage, resulting in dysplasia and an increased risk of hematopoietic malignancy.

Shwachman-Diamond syndrome (OMIM 260400) is an autosomal recessive disorder caused by mutations in *SBDS*. AML or MDS occurs in up to a third of patients by 30 years of age and is thought to relate to chromosomal instability and accelerated rates of apoptosis, which may be due to the role of *SBDS* in stabilizing the mitotic spindle during mitosis.

Severe congenital neutropenia or Kostmann syndrome is associated with neutropenia at birth and has been associated with a variety of genetic mutations. The pattern of inheritance can be autosomal dominant (*ELANE* or *GFI1*), autosomal recessive (*HAX1*, *G6PC3*, *VPS45*, or *JAGN1*), or X-linked (*WAS*). Nearly a third of patients develop AML or MDS during adolescence. Transformation into AML is frequently characterized by the acquisition of somatic mutations in *CSF3R*, which encodes the granulocyte colony-stimulating factor (G-CSF) receptor. The causal relationship to chronic G-CSF therapy remains controversial.

Diamond-Blackfan anemia (DBA) (OMIM 105650) is characterized by red cell aplasia and typically spares the leukocyte and platelet lineages. DBA is typically inherited in an autosomal dominant fashion and is associated with mutations in a number of ribosomal proteins. AML can occur in up to 20% of patients and typically occurs after 40 years of age.

Congenital amegakaryocytic thrombocytopenia (CAMT) (OMIM 604498) and thrombocytopenia with absent radii (TAR) (OMIM 274000) syndrome are both characterized by hypoplastic thrombocytopenia. CAMT is inherited in an autosomal recessive manner via mutations in the *MPL* gene, which encodes the receptor for thrombopoietin (TPO). CAMT is associated with an increased incidence of AML, typically in the second decade of life. TAR syndrome has been associated with mutations in *RBM8A*, which is involved in messenger RNA (mRNA) splicing. The thrombocytopenia in TAR syndrome often improves over time; both acute lymphoblastic leukemia and AML have been reported among patients with this rare disorder.

Down syndrome, caused by trisomy 21, is associated with an approximately 10- to 20-fold elevated relative risk of AML and MDS compared with the general population and in particular an increased risk for acute megakaryocytic leukemia, FAB M7 (see Chapters 29 and 30). Infants with Down syndrome may experience transient abnormal myelopoiesis (TAM), where circulating peripheral blood blasts are seen and may be accompanied by hepatic dysfunction, effusions, and rash; this occurs in approximately 10% of these patients. The majority of TAM cases harbor somatic mutations in *GATA1*, resulting in altered function of this transcription factor that plays an important role in hematopoietic cell maturation, particularly in the megakaryocyte lineage. Indeed, up to 30% of persons with TAM will progress to AML, commonly acute megakaryocytic leukemia. The development of AML in patients with Down syndrome likely relates both to acquired somatic mutations, such as *GATA1*, and also the presence of additional copies of genes on chromosome 21 that facilitate leukemogenesis, such as the oncogenes *RUNX1*, *ERG*, and *ETS2*.

Mendelian Acute Myeloid Leukemia Predisposition Syndromes

A number of genes that are targets of recurrent somatic mutation in AML are also mutated in the germline in families with predisposition to myeloid malignancy without a prodrome of bone marrow failure. These include mutations in *RUNX1*, *CEBPA*, *DDX41*, and *GATA2*. Predisposition to AML is also associated with germline variants in *ANKRD26*, *SRP72*, *ETV6*, and *SAMD9*/*SAMD9L*. Because gene panel testing (see box on Molecular Diagnostics in Acute Myeloid Leukemia) of unpaired tumor samples cannot reliably discriminate germline from somatic mutations, detection of a known pathogenic mutation in one of these genes in a bone marrow or peripheral blood sample should prompt referral for genetic counseling and germline testing. Although these familial syndromes are rare, they are important to recognize because affected individuals and asymptomatic carriers require specific clinical management. In particular, asymptomatic carriers require counseling about risks to themselves and their offspring, and they should be deferred as graft donors for allogeneic stem cell transplantation. The prevalence and risk of MDS/AML development has not been defined for most of these syndromes due to ascertainment bias in most of the reported series (Table 59.2).

TABLE 59.2 **Familial Acute Myeloid Leukemia Syndromes**

Gene	Chromosome	Inheritance	Risk of MDS/AML	Associated Phenotypes
RUNX1	21q22.12	AD	~40%	Thrombocytopenia, platelet dysfunction
ANKRD26	10p12.1	AD	Not defined	Thrombocytopenia, platelet dysfunction
ETV6	12p13.2	AD	Not defined	Thrombocytopenia, platelet dysfunction
CEBPA	19q13.1	AD	~100%	None
DDX41	5q35.3	AD	Not defined	None
GATA2	3q21.3	AD	~80%	Emberger, MonoMAC
SAMD9	7q21.2	AD	Not defined	MIRAGE (myelodysplasia, infection, restriction of growth, adrenal insufficiency, genital phenotypes, enteropathy)
SAMD9L	7q21.2	AD	Not defined	Ataxia, pancytopenia
SRP72	4q12	AD	Not defined	Cytopenias, sensorineural hearing loss

AD, Autosomal dominant; *AML*, acute myeloid leukemia; *MDS*, myelodysplastic syndrome.

MOLECULAR DIAGNOSTICS IN ACUTE MYELOID LEUKEMIA

The various mutations and dysregulated pathways integral to the pathobiology of acute myeloid leukemia (AML) also act as specific therapeutic targets and offer clear prognostic implications for patients. As such, molecular testing at the time of AML diagnosis has become the standard of care, to assist with subclassification of disease, risk stratification, selection of an induction regimen, and consolidation preferences. Diagnosis of AML requires analysis of a specimen demonstrating excess myeloblasts. For patients with high levels of circulating disease, some studies may be performed with the use of peripheral blood samples; however, assessment of a bone marrow biopsy and aspirate is essential.

Standard Evaluation

The cornerstone of the diagnosis of AML remains morphologic assessment. In the current World Health Organization (WHO) guidelines, at least 20% of the bone marrow cellularity or peripheral blood must be composed of myeloblasts, except in the presence of the t(8;21), t(16;16)/inv(16), or t(15;17) rearrangements, which are sufficient for an AML diagnosis regardless of blast count. In addition, promonocytes in acute monocytic leukemia, megakaryoblasts in acute megakaryocytic leukemia, and abnormal promyelocytes in acute promyelocytic leukemia are added to the blast percentage. Only in pure erythroleukemia are erythroblasts included in the blast count.

Flow cytometry uses multiparametric analysis of single cells to assess cellular granularity and size, cell surface, intracellular antigen expression, and other features. The coexpression of certain cell surface markers may help to confirm myeloid cell origin, identify immature blasts, typically CD34$^+$ and CD117$^+$, and also distinguish an aberrant phenotype of a leukemic blast population. Flow cytometry can enumerate small populations of leukemic cells, below the limit of detection by morphology. For this reason, flow cytometric analysis has been developed as a platform to monitor minimal residual disease (MRD).

A critical element in the initial laboratory assessment of AML is cytogenetic analysis of a bone marrow aspirate specimen. This provides important prognostic data for risk stratification and informs therapeutic strategies. Cells from the aspirate are cultured, mitosis is interrupted, and the paired chromosomes are arranged to identify missing, translocated, or duplicated segments. Fluorescence in situ hybridization uses fluorescently labeled DNA probes and can identify gains and losses of chromosomal material, as well as rearrangements that may be cryptic using conventional banding techniques, and may complement, but not replace, standard karyotyping.

Detection of somatic mutations that are known drivers of AML biology can aid in the initial risk classification of patients with AML, particularly those with intermediate-risk cytogenetics. Current National Comprehensive Cancer Network guidelines recommend testing for recurrent gene mutations (*KIT*, *FLT3, NPM1*, *IDH1, IDH2, TP53*, and *CEBPA*) at diagnosis because their prognostic and therapeutic significance has been validated in large cohorts (level 2 A evidence). Testing for mutations in *RUNX1* and *ASXL1* is recommended for WHO classification. In addition, use of targeted therapies is increasingly dependent on detection of a specific tumor genotype. Multigene panels that test for recurrently mutated genes in AML and other myeloid malignancies are increasingly used for these purposes (later).

Investigational Testing

Given the rapidly expanding number of genes that are recognized targets of recurrent somatic mutation in AML, more comprehensive mutational profiling is increasingly part of routine clinical practice. With increasing numbers of genes to query, next-generation sequencing approaches offer advantages in sensitivity, cost, and efficiency over traditional testing methods (e.g., polymerase chain reaction, Sanger sequencing). Large panels of genes can be tested simultaneously by preenriching for the targets of interest (by automated amplicon generation or hybridization capture). These platforms have also been adapted to monitor MRD for specific known alterations, including *NPM1* and *FLT3*, among others. With further improvements in analytical workflow and cost reduction, whole-genome and transcriptome sequencing could displace some existing diagnostic tools because these platforms can provide simultaneous detection of mutations, gene expression, copy number alteration, and structural variation.

Familial platelet disorder with predisposition to acute myelogenous leukemia (OMIM 601399) is associated with autosomal dominant inheritance of germline mutations in *RUNX1*. Mutation carriers frequently present with easy bruising/bleeding due to quantitative or qualitative platelet dysfunction and have an approximately 40% lifetime risk of developing AML, typically in the third or fourth decade. The most frequently reported mutant alleles in *RUNX1* are loss-of-function nonsense, frameshift, or missense mutations in the DNA-binding domain. The second copy of *RUNX1* is frequently mutated in cases that evolve to MDS/AML.

Germline mutations in *CEBPA* are a rare cause of autosomal dominant familial predisposition to AML (OMIM 116897). The germline mutations are typically truncating at the N-terminus of the protein, whereas somatic acquisition of mutations affecting the C-terminus is a nearly invariant event in the development of AML among these patients. These cases have a relatively favorable prognosis, similar to de novo AML with somatically acquired biallelic *CEBPA* mutations.

Germline mutations in *GATA2* cause a spectrum of disorders with overlapping features, including Emberger syndrome (OMIM 614038) and immunodeficiency 21 (IMD21) (OMIM 614172), also described as monocytopenia with susceptibility to mycobacterial, fungal, and papillomavirus infection and myelodysplasia (MonoMAC syndrome) or dendritic cell, monocyte, B-lymphocyte, and natural killer lymphocyte deficiency (DCML). Patients with IMD21 have decreased monocyte counts and natural killer and B-cell deficiency and are at an increased risk of developing viral and nontuberculous mycobacterial infections. Emberger syndrome patients have a similar presentation but also have deafness and lymphedema and often develop pancytopenia. More than 100 germline *GATA2* mutations have been described, most commonly deletions, nonsense, or missense substitution affecting the C-terminal zinc finger of *GATA2*, in addition to intronic mutations and substitutions at a 5′ enhancer. AML or MDS arises in approximately 70% of carriers and is associated with cooperating genetic events, such as mutations in *ASXL1*, *SETBP1*, *STAG2*, or hemizygous deletions involving chromosome 7.

SAMD9 and *SAMD9L* are paralogous genes on chromosome 7q. Inherited missense mutations in both genes appear to confer gain-of-function growth suppressive activity in hematopoietic cells. A variety of compensatory somatic events have been described that allow cells to escape from this antiproliferative pressure, including acquisition of loss-of-function mutations in cis that abrogate the effects of the germline allele, gene correction by homologous recombination, and loss of the chromosome arm harboring the mutant allele—a phenomenon termed “adaptation by aneuploidy.” The latter is associated with increased risk of MDS/AML development, whereas the former mechanisms are protective. Affected individuals may present with MDS/AML without prior sequelae or may have preceding stigmata of ataxia-pancytopenia (*SAMD9L*; OMIM 159550) or the MIRAGE syndrome (*SAMD9*; OMIM 617053).

Germline mutations in *DDX41* on chromosome 5q are associated with autosomal dominant predisposition to MDS/AML with a long latency (OMIM 616871). Affected individuals may present with cytopenias prior to diagnosis of MDS/AML, but often a family history of hematologic malignancies is the only feature that raises suspicion of an inherited syndrome. Pathogenic germline *DDX41* alleles include deletions, frameshift, nonsense, or missense mutations. Somatic mutations in the remaining *DDX41* allele are acquired in nearly half of individuals who progress to MDS/AML.

Clonal Hematopoiesis

HSCs acquire somatic mutations with age (see Chapter 19). Mutations that confer a fitness advantage create a state of clonal hematopoiesis (CH) in which a clonally derived population of cells can be detected in peripheral blood or bone marrow. CH is nearly ubiquitous in the adult population when very sensitive error-corrected sequencing technologies are used. Using more stringent thresholds typically employed in routine clinical sequencing (i.e., variant allele fraction of 2%), CH is detectable in nearly 10% of apparently healthy individuals older than 65 years of age. This is termed clonal hematopoiesis of indeterminate potential (CHIP) if the affected individual has normal blood counts and no antecedent hematologic malignancy. In contrast, CH in patients with idiopathic cytopenias but without a morphologically apparent hematologic malignancy is referred to as clonal cytopenias of undetermined significance (CCUS). The genes most frequently mutated in CHIP and CCUS encode epigenetic regulators (e.g., *DNMT3A*, *TET2*, *ASXL1*) which are also targets of recurrent mutations in MDS/AML.

Patients with CHIP carry a small but significantly increased risk of progression to overt hematologic malignancy, approximately 0.5% to 1% per year, most often to MDS or AML. The risk of progression to a hematologic malignancy is higher in individuals with CCUS, a large clone size, more than one driver mutation, and mutations in specific genes (particularly, spliceosome components). Patients with CH also have a significantly increased risk of cardiovascular events, typically atherosclerosis-related vascular events, possibly related to altered proinflammatory signaling in clonally derived monocytes, among other mechanisms. Additional epidemiologic study will be required to determine how often CH is a precursor to MDS/AML and whether surveillance and/or early intervention are warranted.

GENETIC AND EPIGENETIC ALTERATIONS IN ACUTE MYELOID LEUKEMIA

AML is characterized by a variety of recurrent cytogenetic, molecular, and epigenetic modifications that illustrate the pathophysiologic role of acquired somatic alterations affecting specific gene products. These mutations have been incorporated into strategies for prognostic stratification and risk-adapted treatment, in addition to providing a framework for targeted therapy. The World Health Organization (WHO) classification now recognizes a number of recurrent genetic abnormalities, including balanced translocations and inversions, as defining features of AML.

Early understanding of the pathogenesis of AML suggested a model in which AML occurred in the setting of acquired mutations in two pathways: mutations that block normal mechanisms of differentiation and mutations that activate cell proliferation and survival pathways. This oversimplified model gained early support from genetically engineered mouse models in which at least two genetic "hits" were required to generate leukemia, such as a mutation activating cytokine signaling and a mutation inactivating a hematopoietic transcription factor. However, this model does not account for the wide spectrum of more recently described somatic alterations, nor do all AML cases carry mutations in these two classes.

Chromosomal Abnormalities

The central role of acquired mutations in AML was first recognized through the identification of recurrent nonrandom cytogenetic alterations in the mid-20th century (see Chapter 57). Recurrent karyotypic lesions are frequent events in AML, present in approximately 50% to 60% of patients at diagnosis, and have distinct prognostic significance that are central to treatment decisions, including the identification of patients for whom HSC transplantation should be considered in first remission, as well as patients likely to achieve a favorable outcome with chemotherapy alone (Fig. 59.1).

Common chromosomal abnormalities include chromosome translocations or inversions, chromosome deletions, and monosomies or trisomies. Patients can be stratified according to karyotype into favorable-, intermediate-, and poor-risk categories. Typically, favorable risk includes patients with t(15;17), t(8;21), or inv(16)/t(16;16), and adverse risk includes inv(3q)/t(3;3), t(6;9), monosomy 7, monosomy 5, loss of 5q, 7q, or 17p, and complex (three or more) chromosomal abnormalities, as well as most translocations involving the *KMT2A* (*MLL1*) locus on chromosome 11q23. In lieu of additional molecular studies, all other karyotypic lesions are generally classified as intermediate risk. This includes patients with a normal karyotype, which comprise approximately 45% of all AML cases.

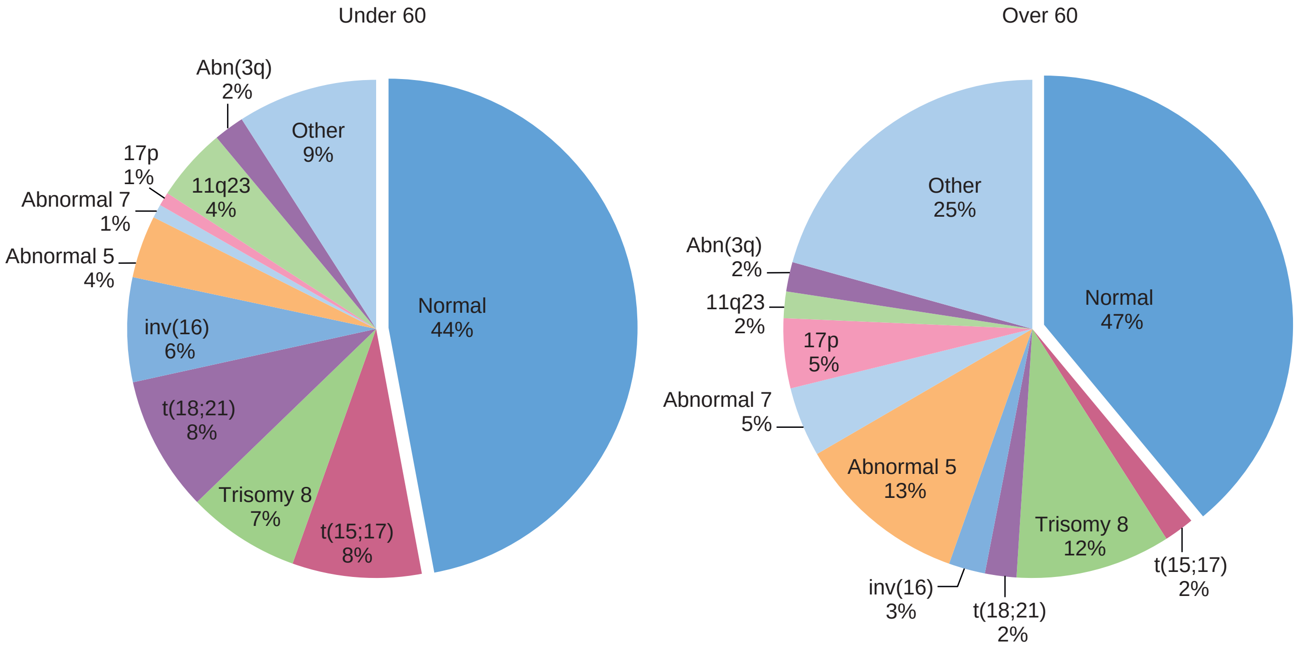


Figure 59.1 FREQUENCY OF CHARACTERISTIC CYTOGENETIC ABNORMALITIES IN ADULT PATIENTS WITH ACUTE MYELOID LEUKEMIA. The proportion of recurrent cytogenetic abnormalities in patients 15 to 60 years old and patients greater than 60 years old at diagnosis is shown. *Abn*, Abnormal; *Inv*, inversion. *(Data derived from previously reported cytogenetic studies by CALGB, MRC, SWOG, ECOG, EBMT, and AMLSG.)*

Retinoic Acid Receptor Rearrangements

A distinct subset of AML, acute promyelocytic leukemia (APL) or FAB M3, comprises approximately 10% of adult AML cases and is defined by the presence of a translocation involving the retinoic acid receptor-α (*RARA*) on chromosome 17q21 (see Chapters 57 and 60). This results in a distinct phenotype characterized by a maturation arrest at the promyelocyte stage. RARs heterodimerize with retinoid X receptors (RXRs) to bind DNA at retinoic acid response elements, where they control gene expression during development and differentiation. In the majority of cases, *RARA* is fused with the *PML* gene on chromosome 15q22 as a consequence of t(15;17)(q22;q21). Other rare fusion partners include PML-like zinc finger (*PLZF*), nucleophosmin (*NPM1*), nuclear mitotic apparatus protein (*NUMA1*), and signal transducer and activator of transcription 5B (*STAT5B*). Each of these fusion partners has a self-association domain, similar to RXRs, and the fusion product interferes with expression of retinoic acid transcriptional targets.

In mouse models, transgenic expression of *PML-RARA* results in APL after a long myeloproliferative phase; the long latency period prior to leukemia development implies that cooperating mutations are typically required for full transformation. Introduction of the *FLT3-ITD* hastens APL onset in mice. Consistent with this finding, more than 35% of human patients with APL have *FLT3-ITD* mutations and nearly 20% have *FLT3-TKD* mutations. *PML-RARA* appears to have a dominant negative effect on RARα transcriptional function, as well as a role in DNA and chromatin modification, resulting in impaired myeloid differentiation. In addition, the fusion protein disrupts the organization of PML nuclear bodies, which are subcellular structures that are involved in a number of cell cycle, metabolic, and apoptotic regulatory pathways.

Core Binding Factor Rearrangements

Rearrangements involving genes encoding components of the core binding factor (CBF) are present in approximately 15% of AML cases (generally, in younger patients). CBF is a heterodimeric transcription factor that is composed of an alpha subunit (encoded by *RUNX1*) that binds to a consensus DNA sequence, and a beta subunit (encoded by *CBFB*) that increases the affinity of the complex for DNA. The t(8;21) is found in up to 10% of AML cases and creates an in-frame fusion of RUNX family transcription factor 1 (*RUNX1*) with RUNX1 partner transcriptional co-repressor 1 (*RUNX1T1*) (encoding CBFA2T1, or ETO). This rearrangement is enriched in the FAB M2 subgroup and composes up to 40% of this patient subgroup. This fusion protein alters the CBF transcription factor complex, allowing it to oligomerize and interact with a nuclear corepressor complex that includes nuclear co-repressor 1 (NCOR1), histone deacetylas 1 (HDAC1), and SIN3 transcription regulator family member a (SIN3A)/HDAC, resulting in reduced RUNX1 target gene expression. The RUNX1-RUNX1T1 fusion protein functions as a dominant negative inhibitor of wild-type CBF and consequently impairs hematopoietic differentiation. The fusion protein also leads to activation of TP53 response genes, which may in part explain the relative chemosensitivity of t(8;21) AML. In addition to t(8;21), *RUNX1* is involved in more than 50 other translocations, including t(16;21)(q24;q22) with myeloid translocation gene on chromosome 16 (*MGT16*), and t(3;21)(q26;q22), with the *MECOM* ("MDS1 and EVI1 complex") locus. RAS pathway mutations (affecting *KIT*, *FLT3*, *N/KRAS, CBL*) are present in more than half of t(8;21) cases and are associated with a higher risk of disease relapse. Frequent co-occurring mutations also include components of the cohesin complex (*SMC1A, SMC3, RAD21*), chromatin modifiers (*ASXL1, ASXL2, EZH2*), and epigenetic regulators (*TET2, IDH1/2*).

Chromosomal alterations involving *CBFB* include inv(16) and t(16;16)(p13;q22), which create an in-frame fusion between *CBFB* and *MYH11*, the gene encoding smooth muscle myosin heavy chain 11. AML with inv(16) or t(16;16) is often associated with the FAB M4eo phenotype. The CBFB-MYH11 gene product exerts a dominant negative effect on RUNX1 and can recruit nuclear corepressors to inhibit transcription of CBF gene targets. Mouse models with a *CBF-MYH11* knock-in mutation require additional mutations to progress to AML. Similar to t(8;21) AML, co-occurring RAS pathway mutations are common in patients with inv(16) AML, but mutations in chromatin modifiers and the cohesin complex are underrepresented in inv(16) AML. Given the clear link between CBF rearrangements, as well as the *PML-RARA* translocation, and the development of AML, the presence of any of these is sufficient for a diagnosis of AML, regardless of the percentage of myeloblasts in the bone marrow or peripheral blood.

Mixed-Lineage Leukemia Gene Rearrangements

Translocations in the *MLL1* gene (also known as *KMT2A*) are seen in up to 10% of patients with AML. *MLL1* rearrangements are common in tAML following exposure to topoisomerase II inhibitors and are enriched in cases with the FAB M5a phenotype. Wild-type MLL1 binds DNA and methylates histone H3K4 via its C-terminal SET domain and regulates expression of target genes, including homeobox (*HOX*) genes. Members of the homeobox A cluster, including *HOXA7* and *HOXA9*, play critical roles in the regulation of hematopoiesis and are normally expressed in early hematopoietic cells.

Rearranged MLL1 retains its N-terminal DNA-binding domain, whereas the C-terminal portion is replaced by a fusion partner. More than 70 unique fusion partners have been identified, including AF4, AF9, AF10, ELL, and ENL; these can interact with and recruit the histone methyltransferase enzyme "disruption of telomeric silencing 1-like" (DOT1L), which methylates H3K79 and results in the expression of *HOXA* genes implicated in leukemic transformation. Expression of the *MLL1-AF9* fusion in mice generates AML with high penetrance and short latency. The oncogenic function of these fusion proteins requires interaction with Menin, a protein encoded by *MEN1* that binds to the N-terminus of MLL1. Inhibition of the Menin/MLL interaction represents a promising new avenue for drug development in AML.

Another mechanism by which *MLL1* plays a role in leukemia is through an in-frame partial tandem duplication (*MLL1-PTD*). Mouse knock-in models with *MLL1-PTD* develop acute leukemias characterized by overexpression of *Hox* genes and an increase in H3/H4 acetylation with associated H3K4 methylation.

Rare Translocations

Less common cytogenetic abnormalities implicated in the pathogenesis of AML include the t(6;9), which fuses the DEK oncogene, encoding a DNA-binding protein involved in transcription regulation and introduction of supercoils, with Nucleoporin 214 (*NUP214* or *CAN*), which encodes a nuclear envelope pore protein that regulates nuclear/cytoplasmic transport. This rearrangement is found in approximately 1% of patients with AML and is associated with a poor prognosis. It typically occurs as a sole chromosomal rearrangement; however, there is a high frequency of concurrent mutations in *FLT3-ITD*. The fusion retains most of the open reading frame from both proteins, but the molecular consequences of the fusion protein are not well understood. Retroviral transduction of long-term HSCs with the *DEK-CAN* fusion generates leukemias after transplantation into mice.

t(3;3)(9q21;q26.2) and inv(3)(q21q26.2) are included in the "acute myeloid leukemia with recurrent cytogenetic abnormalities" category in the WHO classification. Collectively, the inv(3)/t(3;3) rearrangements are present in less than 5% of AML cases and are associated with poor survival. These rearrangements "hijack" an enhancer that normally drives *GATA2* expression, resulting in aberrant activation of *EVI1* and reduced expression of *GATA2*. EVI1 interacts directly with DNA methyltransferase 3 A (DNMT3A) and DNMT3B, which may account for the distinct DNA hypermethylation signature associated with dysregulated EVI1 expression. Patients with this translocation may have a prior MDS, and often the bone marrow morphology shows multilineage dysplasia with atypical megakaryocytes.

t(8;16)(p11;p13) is a rare translocation that occurs in de novo AML and topoisomerase II–associated tAML. The disease typically has an FAB M4 or M5 phenotype, and patients often present with extramedullary disease, coagulopathy, and hemophagocytosis. This translocation fuses two histone acetyltransferases: KAT6A (also known as MOZ or MYST3) and CREB-binding protein. The fusion protein binds to DNA, and the colocalized proteins result in upregulation of the *HOX* genes *HOXA9* and *HOXA10*, as well as their cofactor, *MEIS1*.

The t(1;22) involving one twenty two (*OTT*; or RNA-binding motif protein 15 [*RBM15*]) and megakaryocytic acute leukemia (*MAL* or *MKL1*) is a rare translocation found in infant acute megakaryocytic leukemia. MAL functions as a transcriptional coactivator of DNA-bound serum responsive factors (SRFs) and triggers histone modifications, including acetylation of H3K9. The translocation generates the *OTT-MAL* fusion protein, thus altering MAL function, resulting in SRF-directed expression of *MYL9* and *MMP-9*, which play a role in megakaryocyte development and migration, and may contribute to the phenotype of t(1;22) megakaryocytic leukemia. Mice engineered to express *OTT-MAL* have altered megakaryocyte development and dysregulated NOTCH signaling. With concurrent activating mutations in *MPL*, these mice develop acute megakaryocytic leukemia.

Amplifications and Deletions

Recurrent cytogenetic abnormalities in AML also include somatically acquired chromosome copy or segment gains, chromosomal monosomies, as well as the accumulation of karyotypic abnormalities, classified as complex karyotype, which typically has at least three or more distinct chromosomal abnormalities (see Chapter 57). Common abnormalities include those involving chromosomes 5 and 7, particularly in AML arising out of the background of MDSs, and each of which are seen in approximately 5% to 10% of patients with AML. Enhancer of zeste 2 polycomb repressive complex 2 (PRC2) subunit (EZH2) is a histone methyltransferase that may play a role in leukemogenesis via haploinsufficiency in the setting of loss of material from the long arm of chromosome 7.

Interstitial deletions on the long arm of chromosome 5 at 5q33.1 are associated with the 5q minus syndrome, a subtype of MDS, which has a low risk of progression to sAML but may occur in association with acquired mutations in *TP53* (see Chapter 61). RPS14 and miR145/146a are 5q33.1 genes that have been implicated in the pathogenesis of this syndrome. In contrast, deletions involving a more proximal region at 5q31.2 are associated with higher risk of sAML transformation. Most chromosome 5 deletions in high-risk MDS and AML are large, including most of the long arm or the entire chromosome. Many genes have been implicated in myeloid malignancies associated with these larger deletions, including *APC*, *CTNNA1*, *HSPA9*, *EGR1*, and *NPM1*.

Loss of chromosome 17p, including the *TP53* locus at 17p13, is associated with complex cytogenetics as well as abnormalities in chromosome 5 and 7. Indeed, there appears to be cooperativity between 17p alterations and deletions at chromosome 5q13 at the site of *SSBP4*, another tumor suppressor gene, which may influence the progression to leukemia. Alterations in 17p are enriched in patients with alkylator-associated tAML and sAML arising from an underlying MPN or MDS. In contrast to AML with balanced translocations, leukemias that develop in the context of 17p alterations are characterized by greater genomic instability.

Common examples of trisomy in AML include somatic acquisition of trisomy 8 and trisomy 21, seen in approximately 10% and 3% of patients, respectively. Trisomy 8, the most common chromosomal gain seen in AML, may contribute to leukemogenesis via amplification of *MYC*, which is located at chromosome 8p24 and is implicated in a number of malignancies including AML. Acquired trisomy 21, similar to AML in Down syndrome, appears to progress to AML in association with acquisition of mutations in *RUNX1* or *GATA1*. Unlike Down syndrome, acquired trisomy 21 often occurs in conjunction with other cytogenetic aberrations, in particular with complex cytogenetic rearrangements.

Recurrently Mutated Genes

A recurrent chromosomal abnormality can be detected in a majority of AML patients by either cytogenetic analysis or fluorescence in situ hybridization, using probes for common rearrangements and copy number alterations. However, approximately 45% of AML cases have normal cytogenetics, and this group has historically been categorized as having intermediate prognosis with standard chemotherapy. Molecular testing of this group, as well as patients in other cytogenetic risk groups, has identified a number of recurrent genetic alterations that play critical roles in AML pathogenesis, prognosis, and response to therapy.

On average, AML genomes contain fewer somatic mutations compared with other adult cancers (fewer than 20 mutations in protein-coding genes per case). Despite this relatively low mutation burden, more than 50 genes are recurrently mutated in AML. The large combinatorial matrix of potential co-mutation patterns results in substantial genetic diversity across the AML population (interpatient heterogeneity). This is compounded by clonal diversity (intrapatient heterogeneity), discussed in a later section. AML mutations can be categorized according to the biological pathway involved and the functional impact of the mutation (Fig. 59.2).

Cytokine Signaling

A number of mutations have been identified that result in altered signal transduction, enhancing leukemic cell proliferation and survival. These include mutations in the fms-related tyrosine kinase 3 (*FLT3*) gene, *RAS* genes, and *KIT*, among others.

Mutations in *FLT3* can occur either as an in-frame internal tandem duplication within the juxtamembrane domain of the receptor (*FLT3-ITD*), found in up to 30% of AML patients, or as a mutation in the tyrosine kinase domain (*FLT3-TKD*), the most common being a point mutation resulting in an aspartate to tyrosine substitution at codon 835, detectable in 5% to 10% of AML cases. Both mutations result in constitutive activation of the receptor. *FLT3* signaling activates the PI3K/AKT and RAS/mitogen-activated protein kinase (MAPK) signaling pathways, and mutated *FLT3-ITD* can also activate the STAT5 pathway, which induces the serine-threonine Pim kinases, and results in enhanced cell growth and survival. Murine models that combine *FLT3-ITD* with other mutations, such as *PML-RARA*, *MLL1-ENL*, or *NUP98-HOXD13*, have demonstrated cooperativity between these mutations with respect to latency and penetrance of leukemia, whereas either mutation on its own has low efficiency for disease initiation. The *FLT3-ITD* alteration in particular appears to be associated with increased rates of relapse and worsened patient outcomes; it is less clear whether *FLT3-TKD* mutations have the same impact on outcomes. The *FLT3-ITD* alteration may be present at the time of diagnosis and can be acquired or lost at relapse. Moreover, the length of the duplication, position of the mutation, the allelic fraction, and number of distinct ITDs can vary within a given sample and may have varying prognostic impact. Together, this suggests that the *FLT3-ITD* alteration may be a later event in the development of AML and illustrates the subclonal heterogeneity of AML.

CD117, encoded by *KIT*, is another example of a proto-oncogenic receptor tyrosine kinase implicated in a number of malignancies. KIT is the receptor for stem cell factor and is upregulated in most cases of AML. *KIT* mutations are most frequently seen at codons D816 or N822 in the tyrosine kinase domain or in the extracellular domain; rare ITDs have also been identified in AML. Mutations at D816 or N822 occur in the activating loop of the kinase domain, interrupt autoinhibitory feedback and result in constitutive activation. Mutations in *KIT* occur in less than 10% of patients and are enriched in patients with AML harboring CBF alterations. In mouse models, *KIT* mutations cooperate with *RUNX1-RUNX1T1* and *CBFB-MYH11* to induce AML. *KIT* mutations in patients with CBF AML, particularly with *RUNX1-RUNX1T1*, are associated with increased rates of relapse.

Mutations in the *RAS* proto-oncogenes, most commonly *NRAS* and *KRAS*, are also recurrent events in AML, identified in approximately 10% and 5% of patients, respectively. *RAS* family members are small membrane-associated guanosine triphosphatase signaling proteins that activate the MAPK/extracellular signal-related kinase (ERK) and PI3K

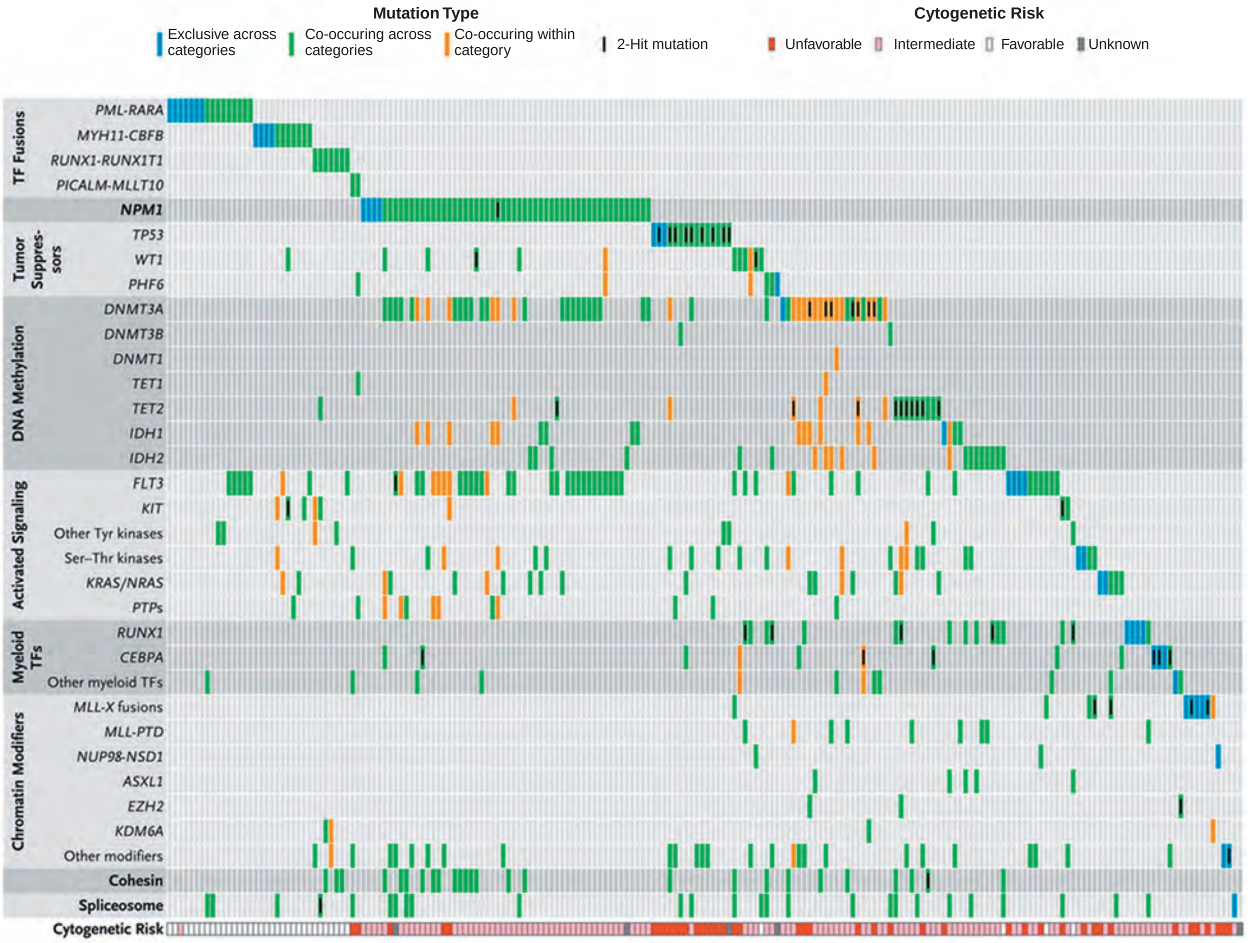


Figure 59.2 CO-OCCURENCE AND MUTUAL EXCLUSIVITY OF SOMATIC MUTATIONS AMONG PATIENTS WITH ACUTE MYELOID LEUKEMIA. A combination of exome, genome, and transcriptome sequencing in 200 patients with de novo acute myeloid leukemia identified a number of mutations which co-occur or are mutually exclusive according to nine categories of biological function: transcription factor fusions, cytokine signaling, transcription factor mutations, tumor suppressor genes, regulators of DNA methylation, polycomb complex, spliceosome, cohesin complex, and nucleophosmin. *TF*, Transcription factor. *(From Ley TJ, Miller C, Ding L, et al. The cancer genome atlas research network. genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. N Engl J Med. 2013;368:2059. Copyright © 2013 Massachusetts Medical Society. Reprinted with permission.)*

pathways, leading to cell growth and survival. The most common mutations in *NRAS* occur at codons 12 and 13 and in *KRAS* at codon 12. These mutations result in a guanosine triphosphate–bound state of the RAS protein, leading to constitutive activation. In murine models, expression of mutant *NRAS* or *KRAS* results in a spectrum of myeloid disease, with variable features of acute leukemia, myeloproliferation, or MDS/MPN overlap. Consistent with this finding, *RAS* is frequently mutated in patients with MPNs and chronic myelomonocytic leukemia (CMML), as well as MDS transforming to AML.

Transcription Factors

In addition to their frequent involvement in chromosomal rearrangements, transcription factors are also recurrently mutated in AML.

RUNX1 mutations occur in approximately 5% to 15% of all patients with AML but are slightly more common in patients with intermediate-risk AML, particularly in association with trisomy 8 or 13. Approximately 80% of *RUNX1* mutations are located in a DNA-binding domain that is homologous to the *Drosophila* runt protein. C-terminal domains of RUNX1 are involved in nuclear matrix localization and recruitment of transcriptional transactivating and repressing factors. Overexpression of mutant *RUNX1* in mice causes AML with dysplastic changes and, in combination with *EVI1* mutations, results in a more rapid AML phenotype.

CEBPA is mutated in almost 10% of AML cases; these mutations are enriched in younger patients and patients with otherwise normal cytogenetics. *CEBPA* encodes the CCAAT/enhancer binding protein-α, a basic leucine zipper transcription factor that is essential for neutrophil development. Mutations may affect the N-terminus (usually frameshift, resulting in preferential expression of a shorter isoform with dominant negative activity) or C-terminus (usually in-frame but also resulting in loss of function). In the majority of CEBPA-mutated AML cases, both alleles are mutated (biallelic, or CEBPA-double mutant). Favorable prognosis is restricted to cases of AML with a normal karyotype and with biallelic *CEBPA* mutations, which is associated with a distinct gene expression signature. When biallelic mutations were engineered in a mouse model, hematopoietic differentiation was impaired and AML developed after a long latency, implying that cooperating mutations were necessary.

Tumor Suppressor Genes

Mutations in tumor suppressor genes facilitate the development of AML. Common examples include the canonical tumor suppressors *TP53* and Wilms tumor 1 (*WT1*).

Mutations in *TP53* are found in fewer than 10% of AML cases overall but are enriched in patients with tAML and patients with a complex karyotype, of whom two-thirds harbor a mutation in *TP53*.

TP53 is a transcription factor that regulates multiple signaling pathways in response to cellular stress, with an output that may culminate in cell cycle arrest, senescence, or apoptosis. Mutations occur throughout the *TP53* gene, usually resulting in loss of function; more than half of cases have concurrent loss of heterozygosity at 17p.

Mutations in *WT1* occur in fewer than 10% of patients with AML, but wild-type *WT1* is frequently overexpressed—such that it has been explored as a way to monitor disease clearance after therapy. WT1 is a zinc finger transcription factor that is required for normal development. Mutations may occur throughout the gene and generally predict loss of function. One mechanism by which loss of WT1 may influence tumorigenesis appears to relate to DNA methylation; mutations in *WT1* result in a DNA methylation pattern similar to that in *TET2* mutated AML, apparently due to a lost interaction between WT1 and wild-type TET2. In addition to loss of function, *WT1* may also have oncogenic effects. In a *RUNX1-RUNX1T1* mouse model of AML, forced overexpression of *WT1* resulted in more rapid progression to AML.

Regulators of Deoxyribonucleic Acid Methylation

Methylation of cytosine residues is an important epigenetic mark that contributes to regulation of gene expression. Genes encoding factors directly or indirectly involved in DNA methylation or demethylation, including *TET2*, *DNMT3A*, and isocitrate dehydrogenase-1 (*IDH1*) or *IDH2*, are recurrently mutated in AML.

DNMT3A encodes a de novo methyltransferase that catalyzes cytosine methylation at CpG dinucleotides. *DNMT3A* mutations are seen in approximately 20% of patients with de novo AML, but nearly 30% of patients with a normal karyotype. Nearly half of all *DNMT3A* mutations are heterozygous missense substitutions at codon R882 (most frequently R882H); the remainder are deletions, frameshifts, and missense substitutions throughout the open reading frame. The unifying mechanism of action appears to be loss of function, through haploinsufficiency or dominant negative activity, resulting in reduced methyltransferase activity. The prognostic significance of *DNMT3A* mutations remains controversial, although most studies have demonstrated an adverse impact on overall and leukemia-free survival.

Mutations in ten-eleven-translocation 2 (*TET2*) have been identified in 10% to 30% of patients with AML and are enriched in patients with prior MDS or MPN. There are three members of the TET gene family, but *TET2* is the most frequently mutated in AML; *TET1* is also rearranged in t(10;11)(p12;q23). TET2 converts 5-methylcytosine to 5-hydroxymethylcytosine, an initial step in the reversion to unmethylated cytosine. Hydroxylation of methylated CpG-rich regions by TET2 activates gene programs important for cellular differentiation, including the homeobox A cluster. By contrast, *TET2* loss-of-function mutations are associated with impaired differentiation. Research in this field has not yet reconciled the opposing biochemical effects of mutant DNMT3A and TET2 (loss and gain of cytosine methylation, respectively) with their shared role in promoting myeloid malignancies. Mice harboring a *TET2*-null allele display enhanced stem cell self-renewal and develop myeloid malignancies with underlying features consistent with CMML or MDS. *TET2* mutations do not have a consistent impact on AML prognosis in multivariate analysis.

Although IDH1 and IDH2 do not directly modify cytosine residues, they impact these epigenetic marks indirectly via their metabolic activity. These enzymes normally decarboxylate isocitrate to form α-ketoglutarate via the reduced form of NADPH, a key reaction in the Krebs cycle. IDH1 is primarily cytosolic, whereas IDH2 localizes to mitochondria. *IDH1/2* mutations are found in approximately 5% to 10% and 15% to 20% of patients with AML, respectively. Mutational hotspots include codon R132 in *IDH1* and codons R140 or R172 in *IDH2*. The mutations are enriched in cases with a normal karyotype and frequently co-occur with *NPM1* mutations. Mutant IDH1/2 catalyzes the conversion of α-ketoglutarate to the oncometabolite 2-hydroxyglutarate (2-HG), which suppresses TET2 activity via competitive inhibition at the α-ketoglutarate binding site. Through this mechanism, excess 2-HG results in a DNA hypermethylation pattern similar to that observed in *TET2*-mutated AML. This interpretation is consistent with the observation that *TET2* mutations are mutually exclusive with *IDH1/2* mutations in AML. Importantly, 2-HG also interferes with other α-ketoglutarate–dependent enzymes, including members of the Jumonji-C domain–containing histone demethylases. Mouse models with *IDH1* mutations in hematopoietic cells develop a disease phenotypically similar to human MDS.

Polycomb-Associated Proteins

The polycomb group proteins play a major role in transcriptional repression of target genes important during development and for maintenance of cell identity in adult organisms. There are two major PRCs in humans: PRC1 and PRC2. EZH2 is the catalytic component of PRC2 and is a target of recurrent loss-of-function mutations in AML.

The additional sex combs-like family includes three members in humans: ASXL1, ASXL2, and ASXL3. These proteins are highly homologous, but only *ASXL1* and *ASXL2* are recurrently mutated in AML. ASXL1 collaborates with the PRC2 complex to repress target genes, including the posterior *HOXA* cluster. ASXL1 also interacts with BAP1 via an N-terminal domain to form a complex that antagonizes PRC1 function. *ASXL1* mutations truncate the protein just upstream of a C-terminal plant homeodomain (PHD) domain that binds methylated H3K4. These mutations were initially interpreted as loss of function, but more recent studies have shown that a truncated protein can be detected by quantitative mass spectrometry and appears to confer gain-of-function activity in transgenic mice. *ASXL1* mutations are more common in older patients with AML and sAML derived from antecedent MDS and are associated with adverse prognosis.

ASXL2 mutations are detectable in more than 20% of patients with t(8;21) AML but are rare in other subtypes of AML. Conversely, *ASXL1* is mutated at a lower frequency in t(8;21) AML and is mutually exclusive with *ASXL2*, implying that these mutations have redundant function or are not tolerated when present in the same cell.

The nuclear receptor binding SET domain protein 1 (*NSD1*) gene encodes a histone methyltransferase, which similarly has a role in normal development; germline mutations result in Sotos syndrome, which is associated with a number of childhood cancers. NSD1 methylates H3K36 and is associated with transcriptional activation. *NSD1* is involved as a fusion partner in the recurrent translocation t(5;11)(q35;p15.5) with *NUP98*, seen in approximately 15% of pediatric AML, but less than 5% of adult AML, and is associated with a poor response to therapy. This fusion protein binds regulatory elements of the polycomb complex and results in *HOX* gene activation.

Spliceosome Complex

The spliceosome is a mega-dalton complex consisting of five small nuclear ribonucleoproteins (snRNPs) and more than 100 auxiliary proteins. The spliceosome functions in a dynamic fashion to define 5′ and 3′ splice junctions, remove intervening introns, and religate exons, resulting in a diversity of mRNA isoforms from each coding gene. Recurrent mutations have been identified in hematologic malignancies in a number of the core splicing components, most often affecting *SF3B1*, *U2AF1*, *SRSF2*, or *ZRSR2*. These mutations are typically heterozygous and tend not to co-occur within patients, suggesting that a second mutation in the pathway confers no additional selective advantage or is not tolerated by hematopoietic cells. Alternative splicing was noted in AML samples prior to the discovery of spliceosome mutations, but the cause and biological consequences were not understood. RNA-seq analysis has demonstrated that *ZRSR2* (X-linked) mutations impair minor (U12) intron excision whereas *SRSF2*, *U2AF1*, and *SF3B1* mutations are "change of function," leading to distinct changes in splice-site preference. Nevertheless, a direct causal connection between alternative splicing and AML pathogenesis has not been established.

Collectively, splicing factor mutations are detectable in approximately 15% of patients with AML. These mutations are frequently

CONTROVERSIES AT THE BOUNDARY BETWEEN MYELODYSPLASTIC SYNDROMES AND ACUTE MYELOID LEUKEMIA

Patients with myelodysplastic syndromes (MDSs) can have a bone marrow myeloblast count up to 19%, but a myeloblast count of 20% or higher defines acute myeloid leukemia (AML). This threshold is useful clinically but does not have a strong biological basis. Based on results from whole-genome sequencing, the majority (>80%) of bone marrow cells are clonally derived in MDSs, even in patients with a normal myeloblast count; this proportion of clonal cells does not significantly increase when patients progress to secondary AML (sAML). This disconnect between morphology and genetics has motivated the search for molecular features that might provide a more robust classifier to define subsets of myeloid malignancies with shared biological and clinical features, particularly at the boundary between MDSs and AML.

Splicing factor mutations have unique biological properties and are common in both MDS (>50% of patients) and AML (~15% of patients). An analysis of a large cohort of MDS and AML cases found that the presence of splicing factor mutations defined a subset of patients with similar molecular (gene expression and DNA methylation) and clinical features. The myeloblast count in splicing factor mutated cases varied widely around the 20% threshold but was lower on average (~30%) compared with AML cases that lacked splicing factor mutations. In another study, the presence of splicing factor mutations (and 4 other MDS-associated mutations) in an otherwise unselected group of AML patients identified a subset with clinical features that closely resembled an independent cohort of patients with rigorously defined MDS-derived sAML. Collectively, these associations suggest that the presence of splicing factor mutations may define a unique clinicopathologic subgroup of MDS/AML cases, independent of myeloblast count.

Erythroleukemia is another diagnostically challenging entity that straddles the boundary between MDS and AML. The original FAB erythroleukemia subtype (M6) was later divided into a group with both elevated erythroid precursors and myeloblasts (M6a) and a group with only increased proerythroblasts (M6b). The World Health Organization (WHO) essentially retained this classification through the 2008 revision, with minor differences in the way blasts were enumerated. This system left open the possibility of classifying cases either as refractory anemia with excess blasts (MDS) or AML (erythroid/myeloid type) based on subtle subjective differences in the percentage of erythroid precursors. The 2016 WHO classification retained only the pure acute erythroleukemia subtype of AML (AEL), requiring at least 80% erythroid precursors of which 30% must be erythroblasts with no excess myeloblasts. The remaining cases (the erythroid/myeloid subtype in the prior classification) were reclassified as MDS (generally, MDS-EB). This led to reclassification of nearly 50% of these cases from AML to MDS.

A comprehensive genomic analysis of adult AEL cases annotated by both the 2008 and 2016 WHO classifications identified four distinct biological groups within this disease subgroup: TP53 mutated, NPM1 mutated, DDX41 mutated, and KMT2A mutated/rearranged. These four groups had distinct clinical outcomes but were randomly distributed across WHO 2016 categories of AML. These data again suggest that classification systems based on molecular features rather than morphology alone may provide more robust prognostic information, particularly in this subset of malignancies at the interface between MDS and AML.

associated with specific subtypes of MDS and MPNs; consequently, AML with splicing factor mutations often have a history of these antecedent disorders (see box on Controversies at the Boundary between MDS and AML). *SF3B1* is the most common mutation in the splicing complex; mutations in this gene are tightly associated with the MDS subtype of single lineage dysplasia with ring sideroblasts. *SRSF2* mutations are common in CMML and are retained when these patients progress to sAML.

Cohesin Complex

The cohesin complex is essential for proper alignment of sister chromatids throughout replication and for organization of topological domains of the genome that are required for coordinated gene expression. The cohesin core complex is comprised of the structural maintenance of chromosomes (SMC) proteins SMC1, SMC3, RAD21 (SCC1), and stromalin antigens STAG1 or STAG2. These core proteins form a ring structure, with a hinge formed by SMC1 and SMC3, and a closed loop formed through the binding of RAD21 with STAG1 or STAG2. Recurrent mutations in the genes for this core complex have been identified in approximately 15% of AML cases, with *STAG2* the most frequently affected. STAG2 loss leads to a reduction in chromatin accessibility, associated with aberrant gene expression and differentiation programs that are not rescued by STAG1. Perturbation of this mechanism may be more relevant to the pathogenesis of AML than sister chromatid cohesion because cohesin mutations have not been associated with increased risk of aneuploidy in AML.

Nucleophosmin

NPM1 is a molecular chaperone with multiple putative functions, including ribosomal protein assembly, the prevention of nucleolar protein aggregation, and regulation of the tumor suppressors TP53 and CDKN2A. *NPM1* mutations are seen in approximately one third of patients with AML and in more than half of patients with normal karyotypes. They occur as insertions in exon 12, most frequently of the four base pairs TCTG (type A), causing a frameshift mutation with an added nuclear export signal motif at the carboxy-terminus and resulting in abnormal cytoplasmic localization (NPM1c). Less common mutations with similar effect include alternate insertions of CATG (type B) or CCTG (type D), among others; there does not appear to be a clinical impact according to the type of mutation. NPM1c mutations without FLT3-ITD constitute a relatively favorable genotype in normal karyotype AML.

The molecular mechanism by which NPM1c contributes to AML remains controversial, but cytoplasmic localization appears essential for maintenance of aberrant *HOXA/B* and *MEIS1* expression and persistence of human AML in xenograft models. NPM1-mutant AML may also be dependent on the MLL1-Menin axis for its survival. Genetically engineered mice harboring the *NPM1* type A mutation have increased megakaryocytes but do not develop AML spontaneously. Consistent with this, mutations in *NPM1* are thought to represent early events in the development of AML and are frequently associated with cooperating mutations in *DNMT3A* and/or *FLT3*. Rarely, *NPM1* is involved in chromosomal translocations, including t(2;5)(p23;q35) with *ALK*, and t(5;17)(q35;q21) with *RARA*, the latter resulting in an uncommon variant of APL.

Epigenetic Alterations in Acute Myeloid Leukemia

AML is characterized by aberrant transcription. As discussed earlier, this is driven in part by somatic mutations in key regulators of DNA methylation, chromatin function, and transcription. In addition, mutations in noncoding regions of the genome can have major effects on gene expression programs that are important for AML pathogenesis.

Methylation of cytosine on DNA at CpG sites, which are enriched at gene promoter regions, is a key regulator of gene expression. The AML genome has increased methylation when compared with normal tissues, and certain genetic subtypes of AML, such as AML with *RUNX1-RUNX1T1* or *PML-RARA* rearrangements, have distinct gene methylation patterns. As discussed earlier, recurrent missense mutations in the de novo DNA methyltransferase, DNMT3A, through haploinsufficiency or dominant negative loss of function lead to focal regions of reduced DNA methylation. TET2 inhibition, either through an acquired loss-of-function mutation or via 2-HG produced preferentially in the setting of *IDH1/2* mutations, results in aberrant hydroxymethylation, particularly at key *HOX* sites, which leads to myeloid expansion and a dysplastic phenotype in mouse models. Causal links between mutations in these regulatory enzymes, cytosine modifications, altered gene expression, and leukemogenesis remain elusive, but it is unlikely that global changes in methylation are sufficient to explain the leukemic phenotype.

DNA methylation and histone modification are often coordinately regulated. For example, EVI1 has DNA methylation activity but also associates with histone deacetylases and methyltransferases. As discussed earlier, PRC2 catalyzes the methylation of histone H3K27, resulting in gene silencing. PRC2 is composed of a noncatalytic subunit, as well as a SET domain with methyltransferase activity via EZH1 or EZH2. Mutations in *EZH2* found in myeloid malignancies are predicted to be loss of function. In mouse models, EZH2 appears to have either tumor suppressor or oncogene characteristics, depending on the timing of the mutation in relation to the stage of disease, and has a role in inhibiting hematopoietic cell differentiation. Similarly, ASXL1 interacts with PRC2, resulting in trimethylation at H3K27; loss-of-function mutations may promote leukemia due to relative activation of PRC2-specific genes. Finally, MLL1 rearrangements bring a diversity of fusion partners to targets normally regulated by MLL1; this interaction depends on the interaction between MLL1 and Menin. Many of these fusion partners interact with DOT1L, a histone H3K79 methyltransferase, which then methylates critical MLL1-dependent regulatory genes, such as *HOXA9*.

In addition to mutations in these *trans*-acting factors, mutations in regulatory regions of the genome can have *cis*-acting effects on gene expression. One example is a super-enhancer downstream of Myc that is required for maintenance of MLL1-AF9 leukemia in mice and is contained within a region of recurrent amplification in human AML. Another example is the *GATA2* enhancer "hijacking" in the case of inv(3) and t(3;3) that was previously discussed.

BIOLOGY OF ACUTE MYELOID LEUKEMIA

The Leukemic Niche

AML arises in hematopoietic cells residing in a bone marrow microenvironment known as the *niche*, where interactions with nonmalignant cells play an important role in leukemia initiation, progression, and response to therapy. Niche cells include endothelial cells, fibroblasts, adipocytes, osteoblasts, osteocytes, mesenchymal stem cells, neurons, and other cell types. There appears to be a reciprocal relationship whereby niche cells can provide signals that promote the development of hematologic malignancies and, conversely, emerging malignant clones can remodel and reprogram niche cells to favor disease progression. Many of the mechanisms that have been implicated derive from observations in animal models and have not been validated in patients. The role of the niche in normal and malignant hematopoiesis is discussed in detail elsewhere (see Chapter 14).

A hallmark of the AML niche is the production of an "inflamed" or proinflammatory microenvironment. This is characterized by aberrant expression of cytokines (e.g., interleukin [IL]-1b, tumor necrosis factor [TNF], IL-6), chemokines (CCL3, CXCL12), and alarmins. These signals are generally growth suppressive for normal hematopoiesis. Leukemic cells are able to evade this hostile microenvironment through poorly understood mechanisms to gain a relative growth advantage. Interactions with niche cells induce prosurvival factors in AML cells (e.g., BCL2, BCLXL, MCL1) through direct cell contact and soluble mediators (e.g., growth arrest specific 6 [GAS6]). Finally, the niche elaborates factors that create an immune-suppressive microenvironment (e.g., indoleamine 2,3-dioxygenase [IDO], arginase) that may facilitate immune evasion by AML cells.

Although it is clear that interactions between leukemic precursors and the bone marrow niche are necessary for leukemogenesis, it is less clear which interaction is most amenable to therapeutic intervention. Early candidates include targeting the interactions between leukemic blasts and existing immune-mediators in the marrow such as T-cell or NK-cell populations, which may be amenable to immunotherapy strategies. Similarly, drug delivery to the niche may concentrate an antileukemic effect; liposomal packaging of chemotherapeutics is based, in part, on this concept. Other strategies have sought to evaluate whether leukemic cells may be more sensitive to chemotherapy if mobilized out of the niche, including inhibiting CXCR4 or mobilizing leukemic blasts with growth factors in combination with chemotherapy.

Clonal Hierarchy of Acute Myeloid Leukemia

The recognition that some mutations may be lost or acquired at relapse (e.g., *FLT3-ITD*), whereas others are present at diagnosis and remain stable (e.g., *NPM1*, *PML-RARA*, and *CBF* rearrangements) suggests that distinct clonal populations can coexist in an individual case of AML. Sequencing of serially obtained samples using bulk and single cell approaches has revealed a clonal hierarchy in AML, beginning with a founding clone that represents the initial population that becomes dominant in the bone marrow. The founding clone spawns subclones that retain founding clone mutations but gain additional mutations that confer a fitness advantage. Initial studies using bulk sequencing approaches suggested that AML evolved largely along a linear trajectory. More recent studies using single cell approaches suggest that more complex branching hierarchies are possible.

The clonal architecture of AML evolves over time as a feature of the natural history of the disease or in response to selective pressure imposed by therapy. Analysis of AML cases that entered morphologic remission after cytotoxic chemotherapy and later relapse revealed that the dominant clone at relapse retains founding clone mutations. These observations raise the hypothesis that therapies directed at eradicating drivers of the founding clone may be more effective than therapies targeting subclones.

Leukemia Stem Cells

Normal human HSCs are characterized by their capacity for self-renewal and multilineage differentiation, properties that can only be assessed in vivo using functional assays such as xenotransplantation into immune-deficient mouse models. The pioneering studies of John Dick and colleagues demonstrated that a similar cellular hierarchy exists in AML, in which rare cells with an immunophenotype similar to normal stem/progenitor cells are enriched for the capacity to initiate leukemia in xenotransplantation models. The stem cell model of AML and other hematologic malignancies is discussed in detail elsewhere (see Chapter 13).

Work from many laboratories has led to the identification of several cell surface antigens that are preferentially expressed on leukemia stem cells (LSCs) compared with normal HSCs, including CD123, CD99, TIM3, and others. No single marker identified to date is sufficient to perfectly discriminate HSCs from LSCs. Combinatorial approaches may improve the ability to identify and selectively target LSCs. LSCs have several biological properties that cause them to resist chemotherapy and create an important reservoir for disease relapse, including expression of drug efflux pumps, resistance to apoptosis, and cell cycle quiescence.

More recently, a "pre-LSC" population has been described. These are cells with an HSC immunophenotype that can be recovered from the bone marrow of patients with AML and are capable of engraftment and multilineage differentiation in vivo in immunodeficient mice. Single cell genotyping has revealed that pre-LSCs often harbor the same class of mutations detected in individuals with CH (e.g., *DNMT3A*, *TET2*) but lack the full complement of mutations found in the bulk AML sample, suggesting that they are ancestral. The contribution of these preleukemic stem cells to chemotherapy resistance and relapse remains to be determined.

Extramedullary Acute Myeloid Leukemia

Typically, AML is characterized by involvement of the bone marrow and peripheral blood, but it may also infiltrate into other tissues, termed extramedullary AML. This may be concurrent with marrow or blood involvement or may be isolated, such as in the case of myeloid sarcoma or chloroma. The pathobiology that results in extramedullary manifestations of AML remains poorly understood. Common sites of extramedullary disease include in the skin, termed leukemia cutis, as well as in soft tissues or the central nervous system (CNS). Certain clinicomorphologic features are associated with extramedullary AML, including monocytic differentiation (notable for mucosal infiltration) and hyperleukocytosis.

Comprehensive or reliable molecular and cytogenetic characterization of extramedullary AML is limited in scope and generally retrospective in nature; however, prior reports suggest an increased frequency of extramedullary disease in CBF-AML—particularly t(8;21)—as well as APL. Other cytogenetic and molecular alterations that appear to be enriched in myeloid sarcoma include trisomy 8, and mutations in *NPM1* or *FLT3-ITD*. There may also be interactions with immune surveillance that lend to extramedullary disease involvement, particularly in disease relapse, including following allogeneic hematopoietic cell transplantation. Some studies have identified T-cell exhaustion as a possible mechanism of extramedullary relapse, with upregulated immune checkpoint expression on biopsy of leukemic infiltrates.

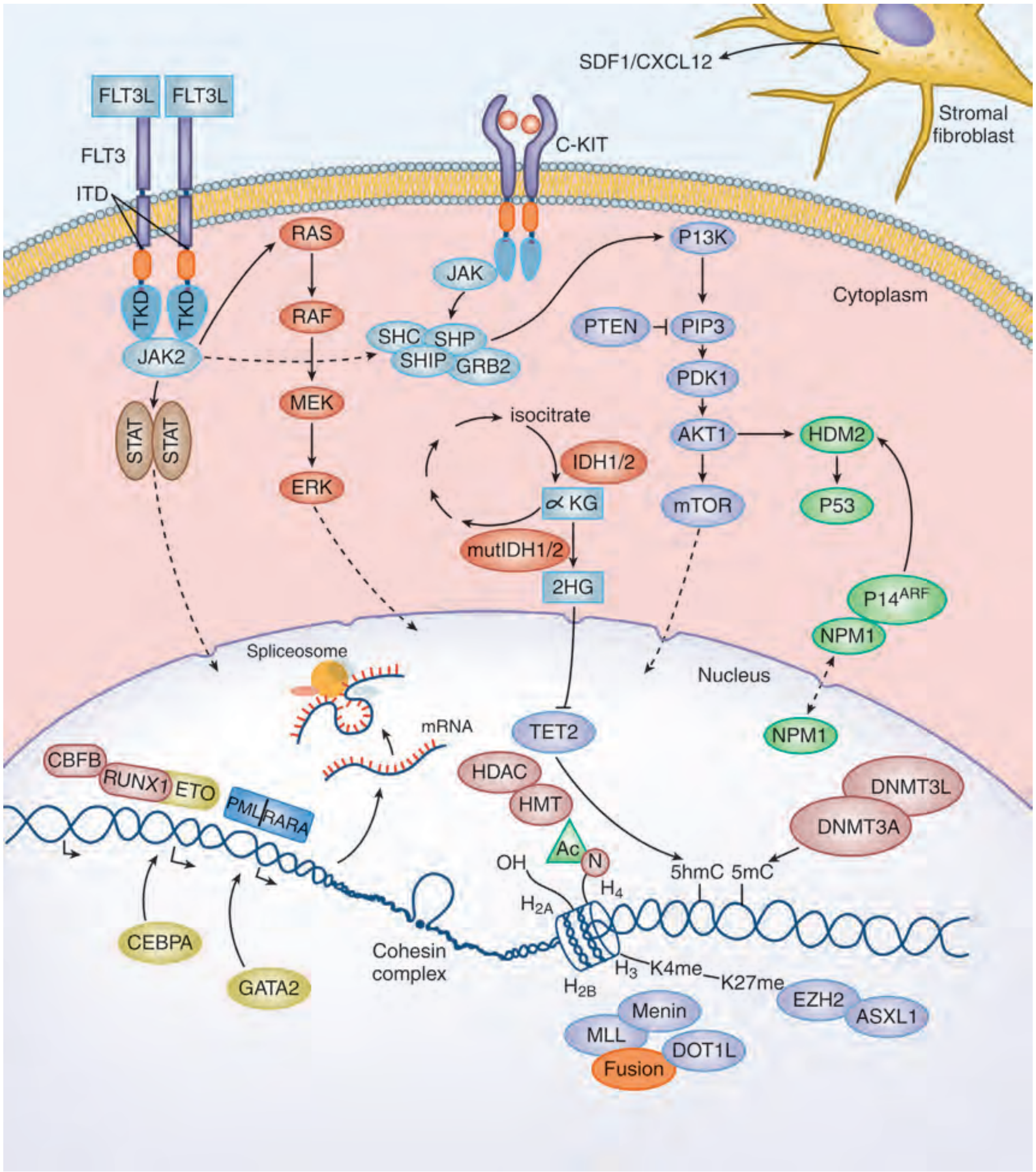

SDF1/CXCL12
Stromal fibroblast
FLT3L
FLT3L
FLT3
ITD
C-KIT
TKD
TKD
JAK2
RAS
RAF
MEK
ERK
JAK
SHC
SHP
SHIP
GRB2
P13K
PTEN
PIP3
PDK1
AKT1
HDM2
P53
mTOR
Cytoplasm
STAT
STAT
isocitrate
IDH1/2
∝KG
mutIDH1/2
2HG
P14ARF
NPM1
Spliceosome
Nucleus
NPM1
mRNA
TET2
CBFB
RUNX1
ETO
PML
RARA
HDAC
HMT
DNMT3L
DNMT3A
Ac
OH
N
H4
5hmC 5mC
H2A
CEBPA
Cohesin complex
GATA2
H3
K4me
K27me
H2B
Menin
MLL
DOT1L
Fusion
EZH2
ASXL1

Figure 59.3 COMMONLY ALTERED CELLULAR PATHWAYS IN ADULT ACUTE MYELOID LEUKEMIA. Mutations in acute myeloid leukemia commonly affect pathways related to cell signaling, differentiation, secondary modifications of deoxyribonucleic acid (DNA), and tumor suppressor genes. Collectively, these lesions interfere with self-renewal, differentiation, and survival pathways. *Ac*, Acetyl; *ARF*, ADP-ribosylation factor; *AKT1*, AKT serine/threonine kinase 1; *ASXL1*, additional sex Combs-like 1; *CBFB*, core binding factor b; *CEBPA*, CCAAT/enhancer binding protein-alpha; *CH*$_3$, methyl; *CXCL12*, CXC-chemokine ligand 12; *DNMT3A*, DNA methyltransferase 3 A; *DNMT3L*, DNA methyltransferase 3 like; *DOTIL*, disruption of telomeric silencing 1-like; *ERK*, extracellular signal-related kinase; *ETO*, eight twenty one or RUNX1T1; *EZH2*, enhancer of zeste homolog 2; *FLT3L*, FMS-like tyrosine kinase-3 ligand; *GATA1*, GATA binding protein 1; *GRB2*, growth factor receptor–bound protein 2; *H*, histone; *HDAC*, histone deacetylase; *HDM2*, human homolog of double minute 2, P53-binding protein; *HMT*, histone methyltransferase; *IDH1/2*, isocitrate dehydrogenase-1/2; *ITD*, internal tandem duplication: *JAK*, Janus-activated kinase; *MEK*, mitogen-activated protein kinase kinase; *MLL*, mixed lineage leukemia; *mTOR*, mammalian target of rapamycin; *N*, nitrogen; *NPMI*, nucleophosmin; *OH*, hydroxyl; *P14*^{ARF}, cyclin-dependent kinase inhibitor 2 A; *p53*, tumor protein p53; *RARA*, retinoic acid receptor alpha; *PDK1*, phosphoinositide-dependent kinase-1; *PI3K*, phosphatidylinositol 3-kinase; *PIP3*, phosphoinositide-3,4,5-trisphosphate; *PML*, promyelocytic leukemia; *PTEN*, phosphatase and tensin homolog; *RAF*, RAF kinase family; *RAS*, RAS family kinase; *RUNX1*, runt-related transcription factor 1; *SDF-1*, stromal cell–derived factor 1; *SHC*, Src Homology 2 Domain-Containing; *SHIP*, SH$_2$-containing inositol phosphatase; *SHP*, small heterodimer partner; *STAT*, signal transducer and activator of transcription; *TET2*, TET oncogene family member 2; *TKD*, tyrosine kinase domain; *α-KG*, α-ketoglutarate.

Biology of Relapsed/Refractory Acute Myeloid Leukemia

Treatment failure is the most important cause of mortality in AML. This includes cases that relapse after achieving remission and cases that are refractory to initial therapy. Chemotherapy, targeted therapy, and immunotherapy impose different therapeutic bottlenecks and are associated with different mechanisms of resistance.

Allogeneic stem cell transplantation exerts its principal antileukemic effect by providing cell-mediated immunity against AML cells ("graft-versus-leukemia"). Therefore it is not surprising that the major mechanisms that have been identified in AML patients who relapse after transplant involve adaptations that allow AML cells to evade immune attack. These include loss of mismatched HLA alleles by somatic recombination after haploidentical transplant and downregulation of class II antigens after matched allogeneic transplant.

Resistance to conventional cytotoxic chemotherapy has been associated with mutations in sensors and effectors of the DNA damage response (e.g., TP53) and a metabolic state that is not sufficiently "primed" towards apoptosis in response to DNA damage (e.g. BCL-2 dependence). Using peptides to probe the status of the mitochondrial apoptotic pathway, retrospective studies have demonstrated that cells from AML patients who were refractory to remission induction had a lower level of priming for apoptosis. Finally, cytotoxic chemotherapy is often successful in removing the bulk AML population but leaves behind a residual population capable of seeding relapse (see Leukemia Stem Cells, earlier) with intrinsic properties (including quiescence and expression of drug efflux pumps) that render cells less sensitive to chemotherapy.

Small molecules that target key driver mutations (e.g., FLT3, IDH1/2) have emerged as important therapeutic tools in AML. Predictably, escape from the selective pressure induced by these agents is frequently associated with mutations in the drug target or pathway. With IDH inhibitors, resistance may be conferred by a second site mutation in the drug target (in *cis* or in *trans*) or by emergence of a subclone harboring a mutation in the other isoform (e.g., IDH1 mutations emerging after treatment of IDH2-mutant AML with an IDH2 inhibitor). Resistance to FLT3 inhibitors may be caused by mutations in the ATP-binding pocket that affects drug binding or by nonmutational processes affecting parallel survival pathways. Finally, acquisition of RAS pathway mutations appears to be a relatively common nonspecific escape mechanism that has been associated with resistance to multiple targeted agents.

FUTURE DIRECTIONS

AML comprises a heterogeneous spectrum of disease, which shares a common myeloid phenotype and is characterized by accumulation of abnormal leukemic myeloblasts. The development of AML occurs through the acquisition of somatic mutations, resulting in clonal expansion and genetic evolution of this disease. As our understanding of the genetic underpinnings has grown, it is clear that there are various pathways to the development of AML (Fig. 59.3). Moreover, some mutations appear to be earlier, founding events, whereas other mutations are typically acquired during disease progression. The further identification of the sequence of mutation acquisition, as well as co-occurring and mutually exclusive genetic pathways, will help to better distinguish different subsets of this disease.

A broader understanding of the mutational landscape of AML has provided a basis for development and testing of novel targeted therapies and monitoring of disease. The role of residual disease monitoring may occur through cytogenetic, flow cytometric, and now also molecular assessment. The incorporation of these residual disease markers into clinical practice remains under intense investigation; similar to patients with chronic myeloid leukemia, it may eventually inform treatment duration and maintenance therapies.

SUGGESTED READINGS

Abelson S, Collord G, Ng SWK, et al. Prediction of acute myeloid leukaemia risk in healthy individuals. *Nature*. 2018;559:400–404.

Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med*. 1997;3:730–737.

Cole C, Russler-Germain DA, Ketkar S, et al. Haploinsufficiency for DNA methyltransferase 3A predisposes hematopoietic cells to myeloid malignancies. *J Clin Inv*. 2017;127:3657–3674.

Corral J, Lavenir I, Impey H, et al. An Mll–AF9 fusion gene made by homologous recombination causes acute leukemia in chimeric mice: a method to create fusion oncogenes. *Cell*. 1996;85:853–861.

Ding L, Ley TJ, Larson DE, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature*. 2012;481:506–510.

Döhner H, Estey EH, Grimwade D, et al. Diagnosis and management of acute myeloid leukemia in adults: 2017 ELN recommendations from an international expert panel. *Blood*. 2017;129(4):424–447.

Erickson P, Gao J, Chang KS, et al. Identification of breakpoints in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, *AML1/ETO*, with similarity to *Drosophila* segmentation gene, *runt*. *Blood*. 1992;80:1825–1831.

Falini B, Mecucci C, Tiacci E, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med*. 2005;352:254–266.

Figueroa ME, Abdel-Wahab O, Lu C, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell*. 2010;18:553–567.

Figueroa ME, Lughart S, Li Y, et al. DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. *Cancer Cell*. 2010;17:13–27.

Furutani E, Shimamura A. Germline genetic predisposition to hematologic malignancy. *J Clin Onc*. 2017;35:1018–1028.

Kakizuka A, Miller Jr WH, Umesono K, et al. Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML. *Cell*. 1991;66:663–674.

Krause D, Fulzele K, Catic A, et al. Differential regulation of myeloid leukemias by the bone marrow microenvironment. *Nat Med*. 2013;19:1513–1517.

Ley TJ, Ding L, Walter MJ, et al. DNMT3A mutations in acute myeloid leukemia. *N Engl J Med*. 2010;363:2424–2433.

Ley TJ, Miller C, Ding L, et al. The cancer genome atlas research network. genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med*. 2013;368:2059–2074.

Lindsley RC, Mar BG, Mazzola E, et al. Acute myeloid leukemia ontogeny is defined by distinct somatic mutations. *Blood*. 2015;125:1367–1376.

Liu P, Tarlé SA, Hajra A, et al. Fusion between transcription factor CBF beta/PEBP2 beta and a myosin heavy chain in acute myeloid leukemia. *Science*. 1993;261:1041–1044.

Micol JB, Duployez N, Boissel N, et al. Frequent ASXL2 mutations in acute myeloid leukemia patients with t(8;21)/RUNX1-RUNX1T1 chromosomal translocations. *Blood*. 2014;124:1445–1449.

Pabst T, Mueller BU, Zhang P, et al. Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. *Nat Genet*. 2001;27:263–270.

Pappaemmanuil E, Gerstung M, Bullinger L, et al. Genomic classification and prognosis in acute myeloid leukemia. *N Engl J Med*. 2016;374:2209–2221.

Paschka P, Marcucci G, Ruppert AS, et al. Adverse prognostic significance of KIT mutations in adult acute myeloid leukemia with inv(16) and t(8;21): a cancer and leukemia group B study. *J Clin Oncol*. 2006;24:3904–3911.

Polpraset C, Schulze I, Sekeres M, et al. Inherited and somatic defects in DDX41 in myeloid neoplasms. *Cancer Cell*. 2015;27:658–670.

Shlush LI, Zandi S, Mitchell A, et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature*. 2014;506:328–333.

Shlush LI, Mitchell A, Heisler L, et al. Tracing the origins of relapse in acute myeloid leukaemia to stem cells. *Nature*. 2017;547:104–108.

Smith CC, Wang Q, Chin CS, et al. Validation of ITD mutations in FLT3 as a therapeutic target in human acute myeloid leukemia. *Nature*. 2012;485:260–263. 2012.

Viny AD, Bowman RL, Liu Y, et al. Cohesin members Stag1 and Stag2 display distinct roles in chromatin accessibility and topological control of HSC self-renewal and differentiation. *Cell Stem Cell*. 2019;25:682–696.

Walter MJ, Shen D, Ding L, et al. Clonal architecture of secondary acute myeloid leukemia. *N Engl J Med*. 2012;366:1090–1098.

Welch JS, Ley TJ, Link DC, et al. The origin and evolution of mutations in acute myeloid leukemia. *Cell*. 2012;150:264–278.

Wong TN, Ramsingh G, Young AL, et al. Role of TP53 mutations in the origin and evolution of therapy-related acute myeloid leukaemia. *Nature*. 2015;518:552–555.

CHAPTER 60

CLINICAL MANIFESTATIONS AND TREATMENT OF ACUTE MYELOID LEUKEMIA

Harry P. Erba

Acute myeloid leukemia (AML) is a clonal neoplasm of hematopoietic stem or myeloid progenitor cells characterized by block in differentiation and unregulated proliferation of hematopoietic cells in the marrow, blood, and extramedullary sites. The major consequence of this malignant transformation is bone marrow failure with mortality predominantly due to infectious and hemorrhagic complications of the disease. Knowledge of the pathobiology of AML as it relates to origin of blast cells, their biologic behavior, sensitivity to therapeutic interventions, and their interactions with and interdependence on the microenvironment continues to increase (Chapters 13 and 59). Extensive application of whole-genome sequencing has identified numerous gene mutations and molecular footprints in AML, highlighting the heterogeneity of AML, devising better tools for prognosis, and identifying abnormal cellular and signaling pathways as central to the development of the leukemic phenotype against which novel targeted therapies are increasingly being developed (Chapter 59).

Recent progress in basic and clinical research has altered the decades-old management paradigm. Incorporation of novel drugs into existing chemotherapy backbones, separate approaches to core-binding factor (CBF), *FLT3*-mutated and *IDH*- mutated AML, successful treatment of most patients with acute promyelocytic leukemias (APL) without cytotoxic chemotherapy, and more effective therapy for older, frail patients with AML are highlights of the current treatment algorithms. New drugs allow old concepts such as maintenance therapy to be revisited. Measurements of minimal residual disease (MRD) have emerged as powerful prognostic markers and may be useful to guide therapeutic options. A major challenge for the future lies in the ability to effectively incorporate and utilize the vast amount of generated data in the most effective way to extend to patients with AML the benefit of this basic and translational research.

EPIDEMIOLOGY

In 2019, 21,450 estimated new cases of patients with AML were diagnosed in the United States and an estimated 10,920 patients with AML died from it. The age-adjusted incidence rate of AML is 3.6/100,000 population. However, with a median age at diagnosis of 68 years, incidence rates are as high as greater than 15/100,000 in the older age group, and about 70% of all diagnoses of AML are in patients over 55 years of age. The 5-year survival rate declines with advancing age for many reasons, including declining overall health, higher incidence of poor risk cytogenetic and mutational features, adverse socioeconomic factors such as absence of adequate caregiver support, and inability to tolerate treatments associated with lower risk of relapse, such as high-dose cytarabine consolidation therapy and allogeneic hematopoietic stem cell transplantation (allo HSCT). AML is slightly more frequent in men than women (lifetime risk of acquiring AML: 1 in 227 [male] to 1 in 278 [female]). Based on Surveillance, Epidemiology, and End Results (SEER) data from 2005 to 2009, small differences in frequency also exist by race; AML is more common in whites than other ethnic groups. African Americans have a worse overall survival (OS) than Whites in National Cancer Institute-sponsored cooperative group clinical trials. According to one study of 27,525 patients with AML, based on the SEER database from 1999 to 2008, African Americans and Hispanics had a 12% and 6% increased hazard of death, respectively. This unfavorable hazard rate pertained despite the higher rate in these two ethnic groups of AML with translocations t(8;21) and t(15;17), both associated with a better outcome.

AML has been associated with exposure to benzene, cigarette smoke, chemotherapy, ionizing radiation, and immunosuppressive agents. AML may develop following immunosuppressive therapy for aplastic anemia (Chapters 31 and 32) or evolve from an antecedent myeloid neoplasm (myelodysplastic syndrome [MDS]; Chapter 73), myeloproliferative neoplasms, and myelodysplastic/myeloproliferative neoplasms) (Chapters 70–73). AML may also be due to an inherited germline mutation and indicate a familial cancer syndrome (Chapter 30). AML patients with two or more first- or second-degree relatives with hematologic neoplasms, prolonged cytopenias, or bleeding disorders should be screened for germline mutations which predispose to myeloid neoplasms. AML patients may have physical stigmata associated with known clinical syndromes. For example, mucosal leukoplakia, reticular hyperpigmentation, nail dystrophy, and interstitial lung disease are all suggestive of short telomere syndromes, such as dyskeratosis congenita (Chapter 30). The European Leukemia Network (ELN) 2017 guidelines suggests testing for germline mutations of *RUNX1*, *CEBPA*, *DDX41*, *TERC*, *TERT*, *TP53*, and others, when appropriate. Some of these variants will be detected by NGS panels at the time of AML diagnosis. The patient should be referred for genetic counseling, especially if present at 50% variant allelic frequency at the time of remission. Identification of other affected family members is an important consideration for selection of family members as hematopoietic stem cell donors.

CLINICAL AND LABORATORY MANIFESTATIONS

The initial presentation of patients with AML may vary from the discovery of relatively asymptomatic cytopenias at the time of routine health maintenance exams to life-threatening complications, requiring urgent intervention. Signs and symptoms of AML mostly reflect the effect of cytopenias. Patients typically present with a short history (few weeks to 3 months) of constitutional symptoms (fatigue, lack of energy, malaise, fever, profuse sweats), infections, and/or manifestations of bleeding (e.g., gingival bleeding, bruising, epistaxis, menorrhagia). Fevers should always be presumed to be secondary to infections even in the absence of an identifiable focus and lead to rapid institution of antibiotic therapy. “Tumor fever” due to AML does occur but remains a diagnosis of exclusion. Extramedullary leukemic infiltrates in the gingiva, skin, lymph nodes, or other organs occur occasionally, and are more often associated with monocytic differentiation. Signs and symptoms referable to central nervous system (CNS) involvement (cranial neuropathies and other focal neurologic abnormalities, encephalopathy, seizures) are rare, except for AML with monocytic/monoblastic differentiation or in any AML with considerable leukocytosis (>100,000/μL). The life-threatening complications requiring emergent intervention include clinical leukostasis, spontaneous tumor lysis syndrome (TLS) with renal failure, and DIC with hemorrhagic or occasionally thrombotic complications (see Initial Therapy).

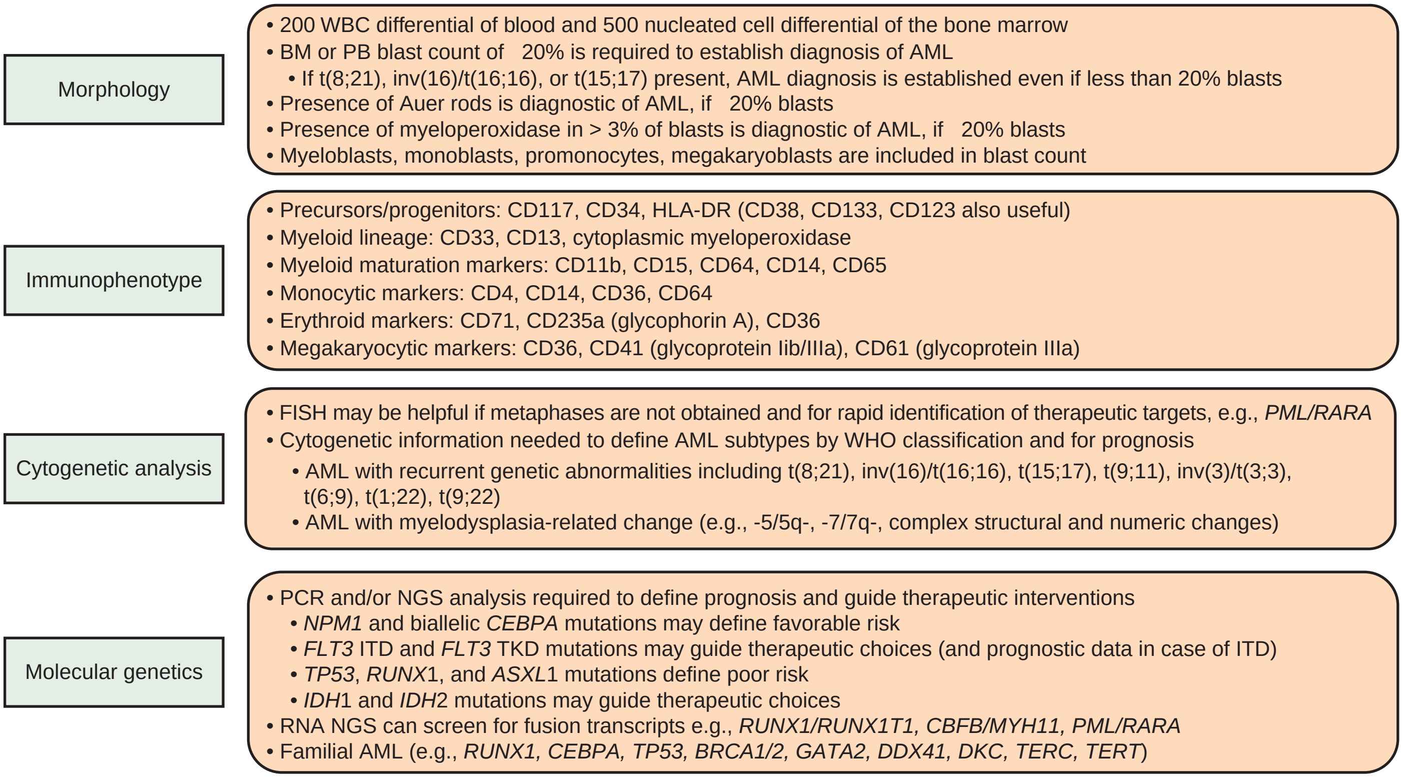
Findings after physical examination are often nonspecific. Patients may demonstrate pallor, tachycardia, oral purpura, ecchymoses, and petechiae due to anemia and thrombocytopenia. There may be signs of extramedullary leukemia such as gingival hypertrophy, enlargement of lymph nodes, cutaneous nodules (leukemia cutis), or hepatosplenomegaly. Examination of the lungs may reveal signs of an infectious process. Many patients with AML have no abnormal findings on physical examination at presentation.

The laboratory evaluation should include blood counts with evaluation of the blood smear, a standard chemistry panel (electrolytes, urea nitrogen, creatinine, total bilirubin, transaminases, uric acid, lactate dehydrogenase [LDH]), and coagulation studies, including prothrombin time (PT), partial thromboplastin time (PTT), and fibrinogen levels. Anemia and thrombocytopenia are almost always present. The white blood cell (WBC) count can vary from low to extremely high (>100,000/μL). Spurious hypoxia and hypoglycemia may occur in the setting of hyperleukocytosis due to continued metabolism by the leukemic blasts prior to processing of a blood sample for analysis; spurious hyperkalemia can occur in the setting of hyperleukocytosis due to cell lysis during clot formation in the preparation of serum. On the other hand, hypokalemia is not infrequently observed with monoblastic AML. DIC may be present at diagnosis and is often seen in patients with monocytic AML and APL (Chapter 137). Subclinical DIC, evident by elevated D-dimers, is common in many forms of AML and can worsen with the institution of therapy. Abnormalities of renal and hepatic values may represent infiltration of these organs; renal dysfunction is commonly observed at presentation due to spontaneous TLS or hypoperfusion due to capillary leak syndrome.

Imaging studies are of little help in diagnosis but allow assessment of complications (pneumonia, cerebral hemorrhage). If patients present with any neurologic deficit, the threshold for computed tomography (CT) scan (non-contrast if bleeding is of concern) or any other imaging modality of the brain should be low. Further evaluations should be based on the clinical assessment of the patients.

DIAGNOSIS AND CLASSIFICATION

The diagnosis of AML relies on morphologic evaluation (cytochemical stains), immunophenotyping by flow cytometry, and assessment of karyotype and molecular studies (Fig. 60.1). Blast percentage is best determined by a 500-cell differential of the marrow aspirate. The presence of ≥20% myeloid blasts in the peripheral blood or bone marrow is required for the diagnosis of AML, except in AML with t(8;21), inv(16), or t(15;17), in which case, these specific cytogenetic changes/gene fusions are sufficient for the diagnosis of AML regardless of the blast percentage. Three broad types of myeloblasts are described based on the granular content and nuclear features of the blasts (type 1: agranular basophilic cytoplasm, nucleus with


Morphology

- 200 WBC differential of blood and 500 nucleated cell differential of the bone marrow
- BM or PB blast count of 20% is required to establish diagnosis of AML
  - If t(8;21), inv(16)/t(16;16), or t(15;17) present, AML diagnosis is established even if less than 20% blasts
- Presence of Auer rods is diagnostic of AML, if 20% blasts
- Presence of myeloperoxidase in > 3% of blasts is diagnostic of AML, if 20% blasts
- Myeloblasts, monoblasts, promonocytes, megakaryoblasts are included in blast count

Immunophenotype

- Precursors/progenitors: CD117, CD34, HLA-DR (CD38, CD133, CD123 also useful)
- Myeloid lineage: CD33, CD13, cytoplasmic myeloperoxidase
- Myeloid maturation markers: CD11b, CD15, CD64, CD14, CD65
- Monocytic markers: CD4, CD14, CD36, CD64
- Erythroid markers: CD71, CD235a (glycophorin A), CD36
- Megakaryocytic markers: CD36, CD41 (glycoprotein Iib/IIIa), CD61 (glycoprotein IIIa)

Cytogenetic analysis

- FISH may be helpful if metaphases are not obtained and for rapid identification of therapeutic targets, e.g., *PML/RARA*
- Cytogenetic information needed to define AML subtypes by WHO classification and for prognosis
  - AML with recurrent genetic abnormalities including t(8;21), inv(16)/t(16;16), t(15;17), t(9;11), inv(3)/t(3;3), t(6;9), t(1;22), t(9;22)
  - AML with myelodysplasia-related change (e.g., -5/5q-, -7/7q-, complex structural and numeric changes)

Molecular genetics

- PCR and/or NGS analysis required to define prognosis and guide therapeutic interventions
  - *NPM1* and biallelic *CEBPA* mutations may define favorable risk
  - *FLT3* ITD and *FLT3* TKD mutations may guide therapeutic choices (and prognostic data in case of ITD)
  - *TP53*, *RUNX*1, and *ASXL*1 mutations define poor risk
  - *IDH*1 and *IDH*2 mutations may guide therapeutic choices
- RNA NGS can screen for fusion transcripts e.g., *RUNX1/RUNX1T1, CBFB/MYH11, PML/RARA*
- Familial AML (e.g., *RUNX1, CEBPA, TP53, BRCA1/2, GATA2, DDX41, DKC, TERC, TERT*)

Figure 60.1 WORKUP OF ACUTE MYELOID LEUKEMIA. The diagnostic workup consists of a morphologic assessment, immunophenotyping by flow cytometry, assessment of the karyotype, and a panel of gene mutations. Whereas morphologic assessment by itself is often not sufficient to render a diagnosis, flow cytometry will confirm the lineage assignment (myeloid vs. lymphoid) and stage of differentiation in more than 95% of cases. In the remainder, either no lineage-specific antigens are expressed (acute undifferentiated leukemia) or antigens of more than one lineage are present (mixed-phenotype acute leukemia). In the latter scenario, antigens of several lineages can be found on one (bi-phenotypic) or separate populations of blasts (bilineal). Karyotyping and gene mutation analysis may add diagnostic information in morphologically ambiguous situations but is otherwise of more interest in determining prognosis. Additional information (exposure to previous chemotherapy and/or radiation therapy, history of an antecedent hematologic disorder, dysplasia) forms the basis for the 2016 revision of the WHO classification of AML. *WHO*, World Health Organization. *(Adapted from Dohner H, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood. 2017;129(4):424–447.)*

fine chromatin, and two to four distinct nucleoli; type 2: basophilic cytoplasm with 20 or fewer azurophilic granules and similar nuclear features as type 1 blasts; type 3: basophilic cytoplasm with more than 20 azurophilic granules), although the morphologic variety of blasts exceeds the defined categories (Fig. 60.2). Auer rods are linear aggregates of primary granules composed predominantly of myeloperoxidase (MPO) that are found in 30% to 50% of newly diagnosed patients with AML and, if present, are one of the hallmark morphologic features to establish a diagnosis of AML (see Fig. 60.2C). Malignant promyelocytes have moderately basophilic cytoplasm with numerous azurophilic granules, frequent Auer rods including cells with multiple Auer rods ("faggot cells"), and bilobed or sliding-plate nuclei. Monoblasts and promonocytes usually exhibit folded or convoluted nuclei and may contain prominent vacuoles. Promyelocytes, promonocytes, and atypical pronormoblasts are all considered blast equivalents. Micromegakaryocytes and pronormoblasts are not considered blasts (see Fig. 60.2K and L).

AML marrows are typically hypercellular with decreased or absent megakaryocytes. Exceptions are marrows of older patients or those with t-AML, which may be hypocellular with dysplastic changes of one or more hematopoietic lineages. Prominent dysplasia may suggest a previous diagnosis of MDS but can also be found in patients with de novo AML, where the prognostic significance of dysplastic changes is less clear. For example, multilineage dysplasia in natural killer (NK) AML with *NPM1* or biallelic *CEBPA* mutations does not appear to affect the otherwise more favorable outcome of AML patients with these mutations. Cases with extensive fibrosis may be due to a preceding myeloproliferative neoplasm or acute megakaryocytic leukemia.

Several cytochemical reactions further highlight morphologic characteristics (MPO, periodic acid–Schiff, Sudan black B, naphthol AS-D chloroacetate esterase [specific esterase], α-naphthyl acetate/butyrate esterase [nonspecific esterase], acid phosphatase; Fig. 60.3). MPO is the most specific granulocytic marker, and MPO positivity in at least 3% of

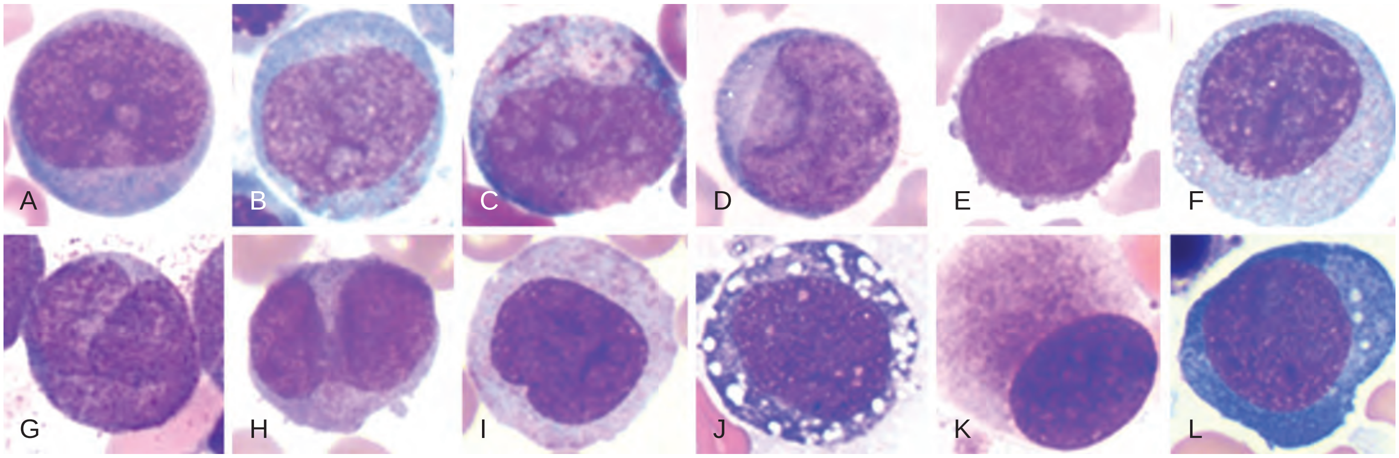


Figure 60.2 SPECTRUM OF BLASTS, BLAST EQUIVALENTS, AND OTHER CELLS. Blast cells in acute myeloid leukemia (AML) exhibit a wide spectrum of morphologic features. The French–American–British classification described three types of blasts depending on the granule content (A–C). However, blasts with nuclear invagination (frequently associated with *NPM1* and/or *FLT3* mutations) (D); blasts with pseudopods (frequently shown to be megakaryoblasts) (E); and monoblasts (F) are also quite distinctive. Blast equivalents include granular or hypogranular promyelocytes (G and H) for acute promyelocytic leukemia, promonocytes (I) for AML with a monocytic component, and atypical pronormoblasts (frequently with cytoplasmic vacuoles) (J) for acute erythroleukemia of the pure erythroid type. Micromegakaryocytes (K) and pronormoblasts (L) are not considered blasts.

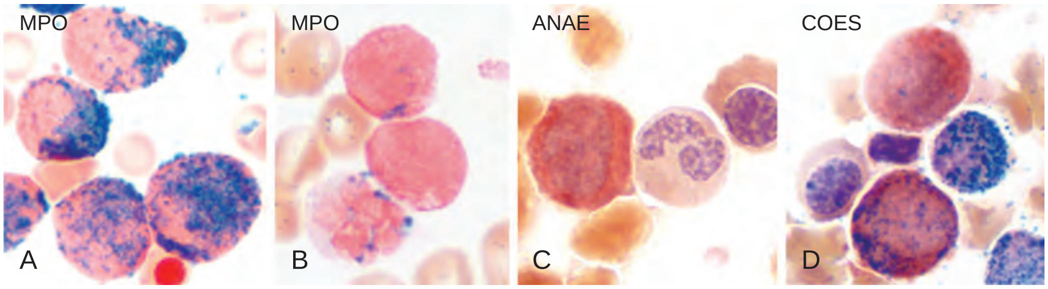


Figure 60.3 CYTOCHEMISTRY: MYELOPEROXIDASE, α-NAPHTHYL ACETATE ESTERASE, AND COMBINED ESTERASE REACTIONS. The MPO reaction is easily performed, can be done in less than a few minutes, and provides important initial information about the lineage of the blasts, particularly in cases in which morphologic assessment is difficult. In many laboratories it is routinely performed for all new acute leukemias. The MPO reaction should be interpreted in the blast population and expressed as a percentage of blasts that are positive. (A) The positive MPO reaction is strong. (B) The reaction is weak and seen in only some blasts. A counterstain would have obscured the weak reaction product. A weak MPO reaction is not uncommon in cases of acute myeloid leukemia (AML) associated with myelodysplasia (as in [B]). The neutrophils in such cases are also only weakly positive (*bottom cell* [B]). The ANAE reaction is a nonspecific esterase reaction positive in most monocytic cells ([C] *orange-brown* cell on *left* compared to negative neutrophil, and erythroid cell, *middle* and *right*). The ANAE reaction is interpreted as positive in cells as a percentage of nonerythroid elements. A significant monocytic component is usually defined as 20% or greater of the nonerythroid elements and is usually required for making a diagnosis of acute myelomonocytic leukemia. It is notable that in some cases of AML with inv(16) (i.e., AML with abnormal eosinophils), the monocytes are ANAE negative. A COES reaction uses another nonspecific esterase reaction for monocytes, α-naphthyl butyrate esterase, together with the specific esterase, chloroacetate esterase, for granulocytes. The combination allows simultaneous evaluation of granulocytes (*blue* reaction product) and monocytes (*orange-brown* reaction product). (D) Acute myelomonocytic leukemia. A monocyte *(top)*, a granulocyte *(right)*, and a myelomonocytic hybrid cell that exhibits both the orange-brown and blue reaction products *(bottom)*. *ANAE*, α-Naphthyl acetate esterase; *COES*, combined esterase; *MPO*, myeloperoxidase.

the blasts is consistent with a diagnosis of AML. On the other hand, lack of MPO staining does not rule out AML because it is often not present in AML with minimal differentiation, acute monoblastic leukemia, and acute megakaryocytic leukemia. Monoblastic leukemias are stained by nonspecific esterase. The diagnostic utility of cytochemical reactions is limited by the more rapid availability of immunophenotypic data by flow cytometry. Nonetheless, cytochemical stains of a touch imprint of the core biopsy can be very useful in the event of a "dry tap." Likewise, immunohistochemical staining for CD34 and CD117 can be used to estimate the blast percentage in a bone marrow core biopsy.

The first systematic attempt to classify AML was made by the French–American–British (FAB) Cooperative Group in 1976 and updated in 1985, and was based solely on morphology (blast percentage, degree of differentiation, lineage involvement). Because of its limited scope, the FAB system is now considered inadequate. Rapidly growing insights from genetic mutation analyses, their association with prognosis, and, in some cases, prediction of response to therapy triggered a revision of the old system and led to the changes in the 2008 edition of the World Health Organization (WHO) classification of AML and updated in 2016. The focus has shifted to identification of recurrent cytogenetic and molecular abnormalities, information regarding exposure to prior leukemogenic therapy, and morphologic features related to dysplasia-related changes as well as morphologic remnants of the FAB system (Table 60.1). Several categories have been defined:

1. AML with recurrent genetic abnormalities. This includes AML with relatively common cytogenetic changes: AML with t(8;21)(q22;q22), *RUNX1-RUNX1T1* (Fig. 60.4); AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22), *CBFB-MYH11* (Fig. 60.5); AML with t(15;17)(q22;q12); *PML-RARA* (Fig. 60.6); and AML with t(9;11)(p22;q23), *MLLT3-KMT2A* (Fig. 60.7). Less common subtypes are AML with t(6;9)(p23;q34), *DEK-NUP214*; AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2), *GATA1*, *MECOM*; and AML with t(1;22) (p13;q13), *RBM15-MKL1* (Fig. 60.8). Normal karyotype AML with nucleophosmin 1 mutation (*NPM1*; see Fig. 60.8G and H), and biallelic *CEBPA* mutations (and in the absence of additional mutations) were initially considered as provisional, but are now incorporated in this subtype, with other molecular subsets expected to be added in subsequent editions.
2. AML with myelodysplasia-related changes (AML-MRC) is defined in one of three ways: (a) AML in patients with a preceding diagnosis of MDS or MDS/MPN; (b) AML with multilineage dysplasia recognized morphologically by the presence of dysplastic features in 50% or more of cells in two separate lineages (Fig. 60.9); and (c) AML associated with a myelodysplasia-related cytogenetic abnormality. Patients with AML-MRC appear to have a worse outcome compared with AML-not otherwise specified (AML-NOS). However, the OS of patients treated with intensive chemotherapy for AML-MRC as defined solely by multilineage dysplasia is similar to that of patients with AML-NOS.
3. The category of t-AML was retained from the 2001 WHO classification, although the distinctions between AML associated with alkylating agents or radiation and those with topoisomerase II inhibitors were abandoned. Many patients receive complex therapeutic regimens, making this distinction difficult and impractical. Nonetheless, AML following exposure to topoisomerase II inhibitors typically have a shorter latency (less than 2 years) and frequently have monoblastic morphology with fusions of the *KMT2A* (previously *MLL*) gene. CBF AML and APL have also been associated with topoisomerase II inhibitors exposure. AML following alkylating agents typically have a longer latency, are preceded by MDS, and have complex cytogenetic changes including monosomy and deletions of chromosomes 5 and/or 7 as well as *TP53* mutations (Chapter 59). Immunosuppressive therapies are now considered potentially leukemogenic.
4. NOS subtypes are AML cases that do not fit the categories already described and for the most part are classified using the FAB scheme (AML with minimal differentiation, AML without maturation, AML with maturation, acute myelomonocytic leukemia, acute monocytic or monoblastic leukemia, acute erythroleukemia,

TABLE 60.1 WHO 2016 Classification of Acute Myeloid Leukemia (AML) and Related Neoplasms

Myeloid Neoplasms With Germline Predisposition
AML and Related Neoplasms
AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1; q22); *CBFB-MYH11*
Acute promyelocytic leukemia with *PML-RARA*
AML with t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*
AML with t(6,9)(p23;q34.1); *DEK-NUP214*
AML with inv(3)(q21.3q26.2) or t(3;23)(p21.3;q26.2); *GATA2,MECOM(EVI1)*
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); *RBM15-MKL1*
Provisional entity: AML with *BCR-ABL1*
AML with mutated *NPMI*
AML with biallelic mutations of *CEBPA*
Provisional entity: AML with mutated *RUNX1*
AML with myelodysplasia-related changes‖
Therapy-related myeloid neoplasms
AML, NOS
AML with minimal differentiation
AML without maturation
AML with maturation
AML and Related Neoplasms (cont'd)
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Pure erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis
Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm
Acute leukemias of ambiguous lineage
Acute undifferentiated leukemia
MPAL with t(9;22)(q34.1;q11.2); *BCR-ABL1*
MPAL with t(v;11q23.3); *KMT2A rearranged*
MPAL, B/myeloid, NOS
MPAL, T/myeloid, NOS

From Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127(20):2391-2405.

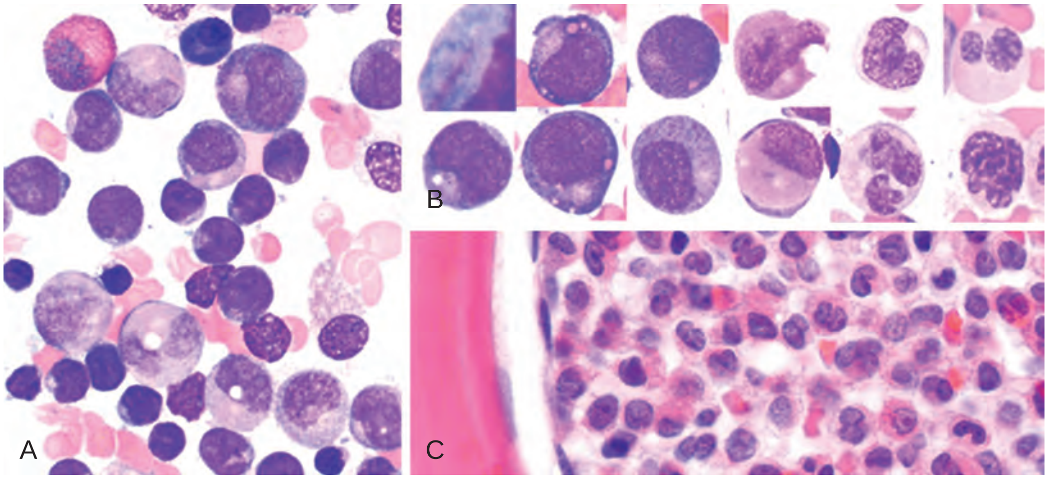


Figure 60.4 ACUTE MYELOID LEUKEMIA WITH T(8;21)(Q22;Q22), (*RUNX1-RUNX1T1*). (A) Low-power, Wright-stained bone marrow aspirate smear showing increased blasts associated with differentiating myeloid cells. (B) Details illustrating some of the features associated with this leukemia. They include blasts with long thin Auer rods *(top left)*, immature cells with abnormal eosinophilic globules *(top and bottom, second from left)*, abnormal salmon-colored granulation in the maturing cells, sometimes associated with a basophilic periphery *(top and bottom, fourth from left)*, and slightly abnormal features in the mature neutrophils *(far right)*. Pseudo–Chediak-Higashi granules were not seen in this case. (C) Biopsy specimen illustrates the significant degree of maturation that can sometimes be seen. In fact, in some cases the blast count can be less than 20%, but the diagnosis of acute myeloid leukemia is still made with the cytogenetic finding of t(8;21).

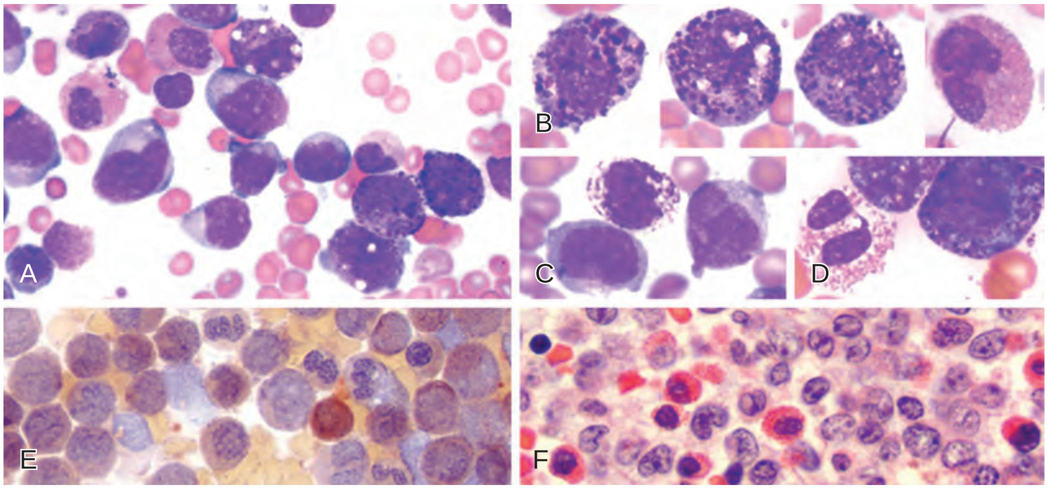


Figure 60.5 ACUTE MYELOID LEUKEMIA WITH ABNORMAL BONE MARROW EOSINOPHILS AND INV(16)(P13.1;Q22) OR T(16;16)(P13.1;Q22), (*CBFB-MYH11*). (A) Low-power, Wright-stained bone marrow aspirate showing blasts, monocytic cells, granulocytic cells, and abnormal eosinophils. (B) Features of the abnormal eosinophils in three abnormal eosinophils *(left three cells)*. Note the abnormal basophilic granules in the eosinophilic myelocytes. These granules are large, tend to cluster or coalesce, and are interspersed among the large eosinophilic granules, which are more difficult to see. As the eosinophils mature, the abnormal basophilic granules are less prominent and sometimes disappear *(far right)*. A common misconception is that the basophilic granules are the granules of basophils and that the abnormal eosinophils are "hybrid cells." (C) A basophil in the same case *(top cell)* for comparison. (D) Cells from a case of reactive eosinophilia, also for comparison. Immature eosinophils *(cell to the right)* do have primary blue granules. However, they are usually less prominent and less atypical than the basophilic granules of the abnormal eosinophils. It is notable that the monocytes in cases of AML with inv(16) or t(16;16) are sometimes α-naphthyl acetate esterase (ANAE) reaction negative. (E) Negative ANAE reaction. (F) Abnormal eosinophils cannot be recognized in hematoxylin and eosin-stained sections of the biopsy.

acute megakaryoblastic leukemia, acute basophilic leukemia). The AML-like disease of acute panmyelosis with myelofibrosis is included here (Fig. 60.10).

5. Lastly, the classification now includes myeloid sarcoma (Fig. 60.11), myeloid proliferations associated with Down syndrome, and the rather rare entity of blastic plasmacytoid dendritic cell neoplasm (BPDCN) (Fig. 60.12) (Chapter 64).

PROGNOSIS

Prognosis in AML is dependent on patient characteristics and disease-related factors. The former determines the likelihood of surviving initial induction chemotherapy as well as eligibility for potentially curative post-remission therapy. Age and performance status are key determinants of early mortality as well as long-term outcome (Chapter 157). Comorbidity scores such as the Charlson comorbidity index and the hematopoietic cell transplantation comorbidity index have been incorporated into predictive models of early mortality with intensive therapy (Chapter 157). Comorbidity indices (ideally as part of a more global geriatric assessment strategy) are particularly useful in older patients where the question of intensive chemotherapy versus lower-intensity interventions assumes more significance. AML prognostic models often include both factors that determine early treatment-related mortality as well as OS, since the impact of these features on early death and long-term outcome are difficult to separate. More recently, the approval of drugs for

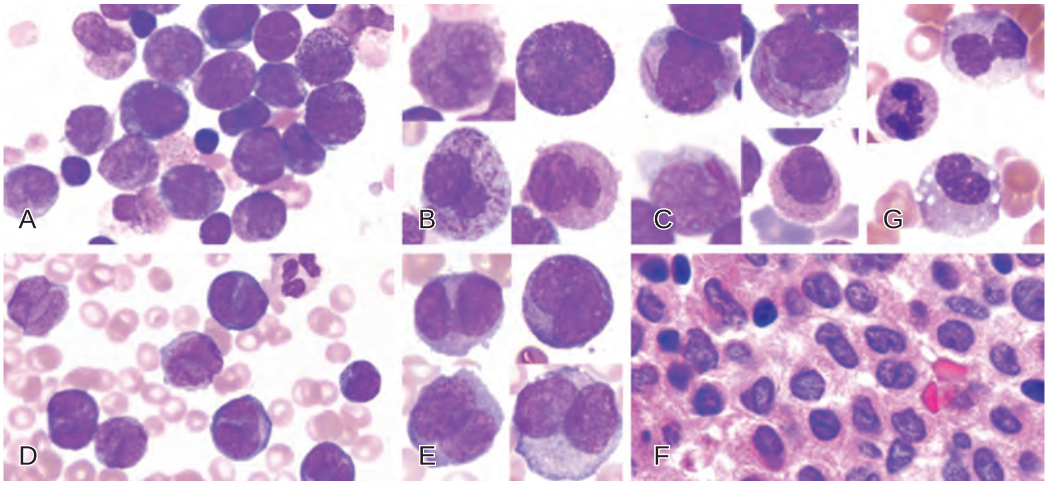


Figure 60.6 ACUTE PROMYELOCYTIC LEUKEMIA, ACUTE MYELOID LEUKEMIA WITH T(15;17)(Q22;Q12), (*PML-RARA*). (A–C) Acute promyelocytic leukemia (APL). (D and E) Hypogranular or microgranular subtype. (F) Bone marrow biopsy. (G) APL cells maturing after all-trans retinoic acid (ATRA) therapy. In the typical granular type of APL (A), the abnormal promyelocytes can exhibit variable morphologic features. Even within the same case, the granules can range from coarse, dark, and dense to fine and dust-like (B). The nuclei in the abnormal promyelocytes frequently exhibit a bilobed, dumbbell, or reniform shape. This is a diagnostically important feature, which can sometimes be difficult to recognize beneath the granules (B). Auer rods can be single, multiple, coalesced into Auer bodies, and even present in maturing cells (C). The microgranular type usually presents with an elevated white blood cell count (D). Although granules cannot be readily appreciated at the light microscope (E), granules can be demonstrated by electron microscopy. The abnormal nuclear shapes (bilobed, dumbbell, and reniform) can be easily appreciated (E). Bone core biopsy sample typically shows sheets of cells with abundant granular cytoplasm (F). After ATRA therapy the abnormal promyelocytes mature to abnormal neutrophils ([G] *top and bottom right*, compared with normal neutrophil, *left*). These can be seen for weeks after therapy and do not signify a failed response to the differentiating agent.

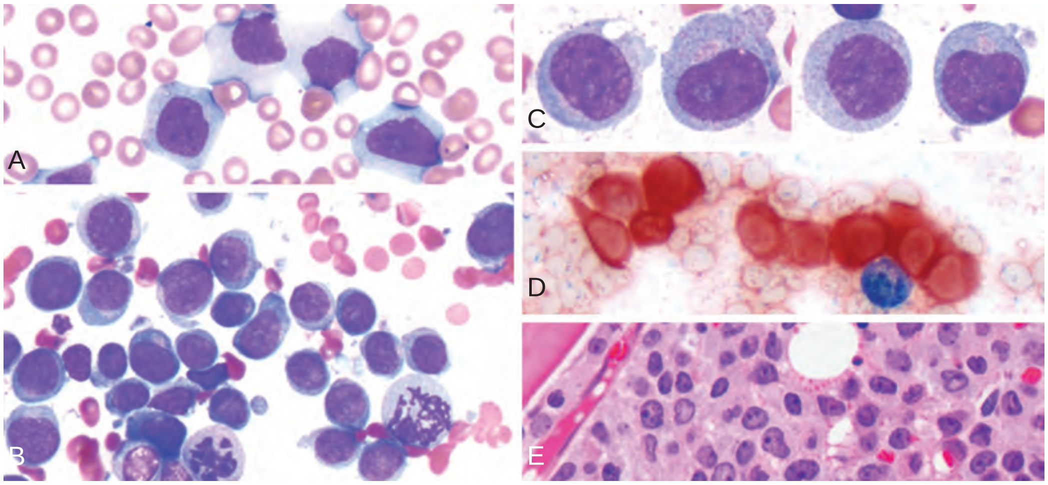


Figure 60.7 ACUTE MONOBLASTIC LEUKEMIA WITH T(9;11)(P22;Q23), (*KMT2A-MLLT3*). Acute monoblastic leukemia with t(9;11). (A) Such cases typically present with high counts because of circulating monoblasts. (B and C) Bone marrow aspirate is packed with monoblasts and shows few granulocytic elements. (D) Absence of a granulocytic component can be illustrated with the combined esterase reaction, in which most of the cells show the α-naphthyl butyrate reaction product *(orange-brown)*, with only rare granulocytes with the blue reaction product from the chloroacetate esterase reaction. (E) The biopsy sample is usually packed with sheets of monoblasts with fine nuclear chromatin and abundant pink cytoplasm.

AML patients unfit for intensive chemotherapy have used some of the criteria identified by Ferrara and colleagues to define this population, including age 75 years or older, performance status greater than 2, left ventricular ejection fraction ≤50%, congestive heart failure, pulmonary DLCO or FEV1 less than 65% predicted, and hepatic disease.

Disease-related factors determine resistance to therapy. Secondary AML, herein defined as AML following an antecedent hematologic neoplasm (MDS, MPN, MDS/MPN) or prior exposure to leukemogenic chemotherapy or ionizing radiation, has been associated with a worse prognosis. The response rates following intensive therapy are lower in patients with an antecedent hematologic neoplasm. Patients with therapy-related AML (t-AML) are typically older and have reduced survival due to other cancers or comorbid illnesses. The cumulative toxicity of prior therapy likely contributes to the higher rate of treatment-related mortality in t-AML patients. Regardless of age, patients with t-AML have lower remission rates, higher relapse rates, and increased early therapy-related mortality compared with de novo AML patients. The multi-drug resistance (MDR) phenotype due to expression of transmembrane drug efflux proteins negatively impacts outcomes of AML patients. Anthracyclines, etoposide, and calicheamicin (a component of Gemtuzumab Ozogamicin [GO]) are substrates for these drug efflux proteins. The MDR phenotype is commonly observed in secondary AML.

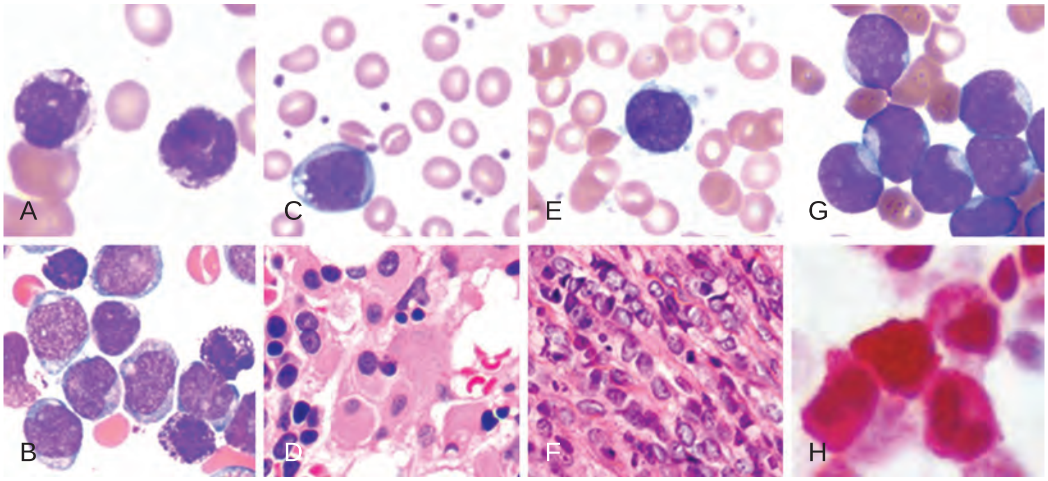


Figure 60.8 ACUTE MYELOID LEUKEMIA WITH LESS COMMON CYTOGENETIC AND GENETIC CHANGES. (A and B) Acute myeloid leukemia (AML) with t(6;9)(p23;q34). These cases are characterized by marrow dysplasia and basophilia. However, the morphologic characteristics can be quite varied among cases. AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2) is characterized by normal or high platelet counts (C), and by a bone marrow with numerous micromegakaryocytes (D). AML with t(1;22)(p13;q13) is uncommon and occurs in infants. The blasts are megakaryoblasts (E) but in the bone marrow can have a sarcomatous appearance (F). AML with *NPM1* or *NPM1* and *FLT3* mutations frequently presents with high peripheral blast counts, and the blasts sometimes have nuclear invaginations (G). *NPM1* mutation is associated with the abnormal cytoplasmic localization of *NPM1* rather than the normal nuclear staining (H).

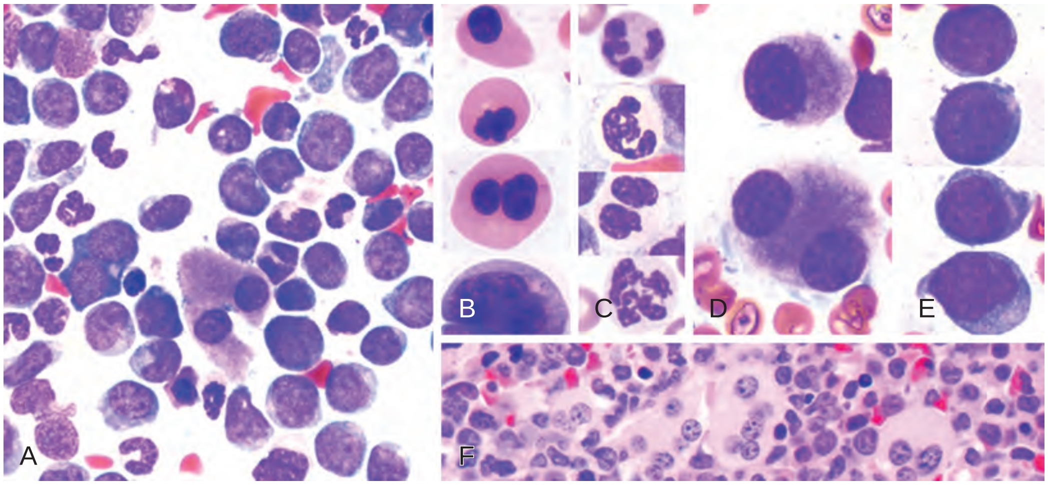


Figure 60.9 ACUTE MYELOID LEUKEMIA WITH MULTILINEAGE DYSPLASIA. (A) Bone marrow aspirate shows increased blasts and maturing cells with dysplastic features. (B–D) Details of the dysplasia. (B) Maturing erythroid elements exhibit megaloblastoid change and bizarre nuclear abnormalities. (C) Dysplastic granulocytes *(bottom three)* are compared with a rare normal granulocyte *(top)*. The dysplastic forms have pale cytoplasm and abnormal nuclear shapes with hypolobation, hypersegmentation, and prominent nuclear excrescences. (D) Dysplastic micromegakaryocytes have single or double small nuclei but mature cytoplasm with platelet material within. (E) Increased blasts. (F) Dysplasia is difficult to appreciate on a biopsied section. This is true except for the megakaryocytes. Dysplastic megakaryocytes can be recognized on the biopsy specimen by their abnormally small nuclei, which are sometimes multiple and widely spaced.

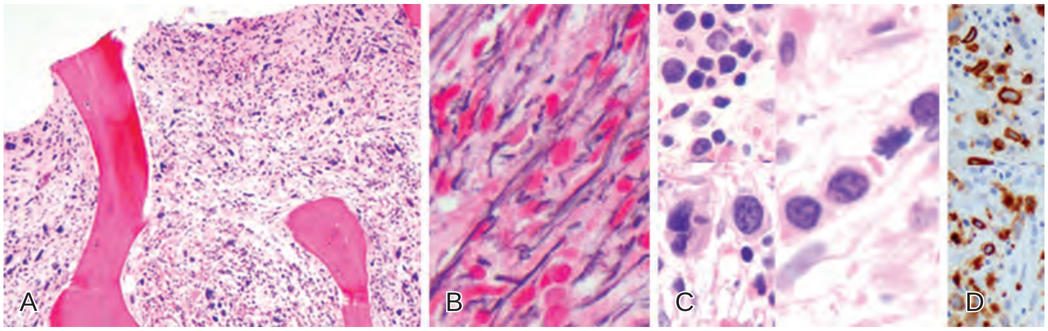


Figure 60.10 ACUTE PANMYELOSIS WITH MYELOFIBROSIS. (A) Bone marrow biopsy specimen shows a loosely packed marrow with a swirling appearance to the cellular elements due to underlying fibrosis. (B) The latter is illustrated on the reticulin stain. (C) Proliferations of erythroid cells *(top left)*, megakaryocytes *(bottom left)*, and immature cells within the fibrotic areas *(right)*. (D) Immunohistochemical staining shows increased megakaryocytes (*top*, CD61) and increased blasts (*bottom*, CD34).

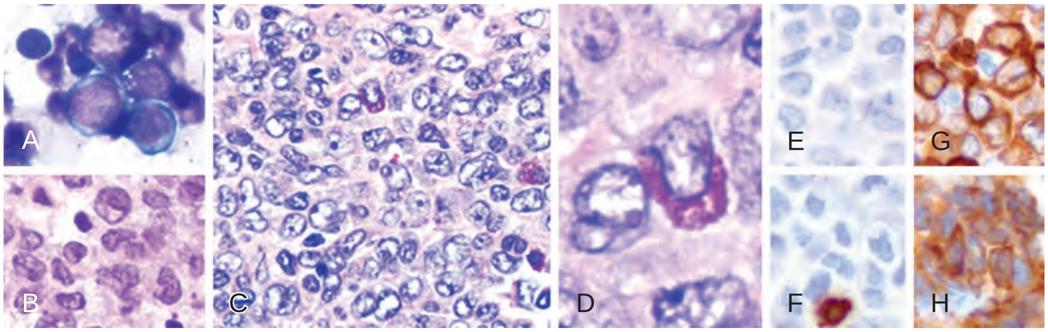


Figure 60.11 MYELOID SARCOMA. Myeloid sarcomas can sometimes present a diagnostic challenge. In this case the touch imprints (A) and frozen section preparation (B) from a mass lesion in the cecal area from a 44-year-old patient were thought to represent a high-grade lymphoma. However, initial immunohistochemical stains did not support the diagnosis, and on closer inspection of the tumor and with subsequent immunomarkers, a diagnosis could be reached. The tumor (C) is composed of sheets of non-cohesive cells. A diagnostic clue to the origin was the presence of eosinophilic myelocytes (D), which indicate that some tumor cells have the capacity to differentiate into eosinophils. The granules of neutrophilic myelocytes cannot be recognized on tissue section. The immunohistochemical stains showed the cell to be CD45$^+$ (not shown), (B) and T marker negative (E and F), but myeloid marker (myeloperoxidase and CD33) positive (G and H). Cytogenetic analysis showed the case was inv(16)(p13.1;q22).

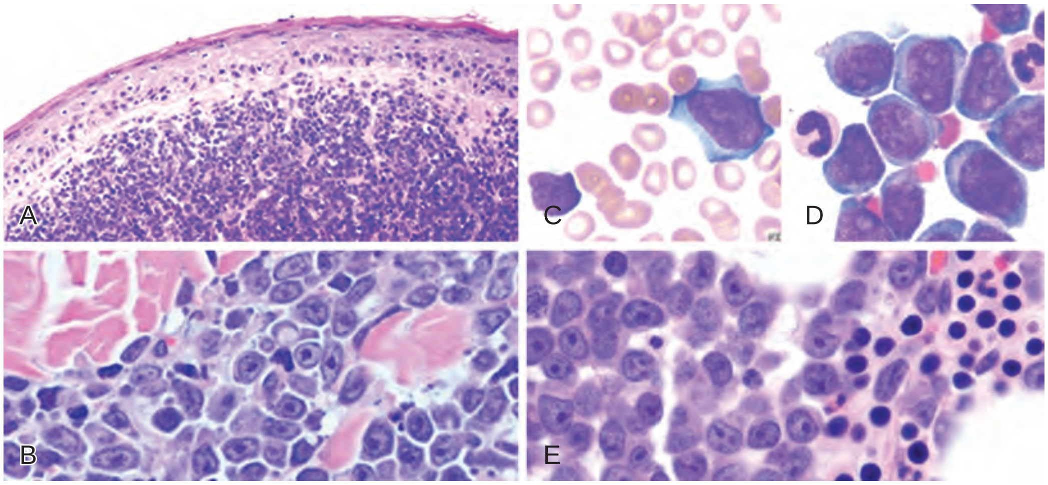


Figure 60.12 BLASTIC PLASMACYTOID DENDRITIC CELL NEOPLASM. The blastic plasmacytoid dendritic cell tumor is an unusually aggressive malignancy that was previously called *hematodermic* malignancy. It is now classified in the acute myeloid leukemia category of myeloid neoplasms. It frequently presents in the skin with a blastic proliferation of cells in the dermis (A and B), which inevitably spreads to the blood (C) and bone marrow (D and E).

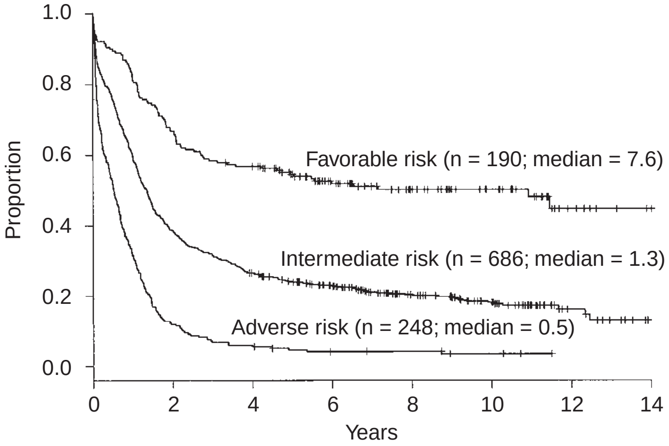


Figure 60.13 DISEASE-FREE SURVIVAL BY CYTOGENETIC RISK GROUP. *(Data from Byrd JC, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: Results from Cancer and Leukemia Group B [CALGB 8461],* Blood *100:4325, 2002.)*

Among the disease-related factors, karyotype (Chapter 57) has historically been considered as the most important prognostic factor dividing patients into those with favorable (CBF leukemias associated with t[8;21] and inv[16] or t[16;16]; APL with t[15;17]), unfavorable (complex abnormalities and monosomies, among others), and intermediate prognosis (mainly diploid; Fig. 60.13). Fluorescence in situ hybridization (FISH) can identify some of the more common AML gene deletions (deletions of chromosomes 5 and/or 7) and AML gene rearrangements in AML, including *RUNX1/RUNX1T1*, *CBFB/MYH11*, *PML/RARA*, and *KMT2A* fusions. FISH analysis can provide more rapid identification of these common changes and can help guide initial therapeutic choices. DNA based assays may replace classical cytogenetic and FISH analyses in the future.

Karyotype is supplemented by analyses of commonly mutated genes (Chapter 59). Whereas prognosis is hardly or not at all augmented by gene mutation testing in cytogenetically unfavorable groups, the situation is different in the intermediate cytogenetic risk group. Identification of *NPM1* mutation or biallelic *CEBPA* mutations in the absence of a *FLT3* ITD mutation has been associated with a more favorable outcome than other patients with intermediate risk karyotype. Normal diploid karyotype, associated with intermediate risk, is the largest subset of both younger and older AML patients.

Even in older, normal karyotype AML patients treated with intensive therapy, the presence of *NPM1* or *CEBPA* mutations, and absence of the *FLT3* ITD mutation, along with age, performance status, and WBC count, determine prognosis. On the other hand, even in the absence of an adverse risk karyotype, identification of the *FLT3* ITD (especially high allelic burden), *ASXL1, RUNX1,* and *TP53* variants are considered poor risk.

Core binding factor (CBF) leukemias are characterized by translocation t(8;21) and inv(16)/t(16;16) and, at the molecular level, by a disruption of the genes for the CBFα and CBFβ subunits, respectively. The CBF transcription factor plays a crucial role in hematopoietic differentiation. CBF AML comprises about 15% to 20% of patients with AML, but only 5% in AML patients over age 60 to 65 years. Although generally considered favorable risk, the prognosis of AML patients with CBF translocations is adversely affected by other clinical, cytogenetic, and mutational features. Poor prognostic factors in t(8;21) AML include high WBC count at diagnosis, presentation with granulocytic sarcoma, and expression of CD56. In general, cytogenetic abnormalities in addition to CBF translocations do not appear to influence outcome; these more complex karyotypes with CBF translocations are still considered favorable risk by ELN 2017 and NCCN risk stratification. However, del(9q) and loss of Y chromosome in t(8;21) AML have been associated with an inferior OS. The presence of a *KIT* tyrosine kinase activating mutation has been considered to have a negative impact on OS in CBF AML due to a higher rate of relapse. More recently, the prognostic impact of *KIT* has been shown to vary according to the variant allelic frequency as well as the concomitant mutations in t(8;21). Mutations in genes for chromatin modifying enzymes and cohesin complex proteins increase the risk of relapse in patients with t(8;21) and *KIT* mutations. The impact of *KIT* mutation is much less clear in inv(16) compared with t(8;21).

The first cancer genome to be completely sequenced was a cytogenetically normal AML. Whole-genome sequencing identifies a finite number of genes that are recurrently mutated in AML samples (Chapter 3,59). These repetitively mutated genes encode proteins/enzymes that generally fall into just a few classes including DNA methylation, epigenetic/chromatin modifiers, transcription factors, RNA splicing, activating signals, and the cohesin complex. These mutations are considered in risk assessment, but often with incongruous results (e.g., *NPM1*, *FLT3*, *CEBPA*, *ASXL1*, *RUNX1*, *TP53*, *KIT*, *IDH1*, *IDH2*, *TET2*, *DNMT3A*). Nonetheless, a comprehensive cytogenetic and mutational analysis of samples from 1540 AML patients treated with intensive chemotherapy identified 5234 driver mutations across 76 genes. At least one pathogenic mutation was found in 96% of the samples. Eleven prognostic groups were identified, allowing classification of 80% of patients into mutually exclusive prognostic subsets (Fig. 60.14). The ELN currently recommends testing for mutations of *NPM1*, *CEBPA*, *FLT3*, *RUNX1*, *ASXL1*, *TP53* in all patients with a new presentation of AML. Evaluation for specific germline mutations associated with familial AML (e.g., *RUNX1*, *CEBPA*, *TP53*, *BRCA1/2*, *GATA2*, *DDX41*, *DKC*, *TERC*, *TERT*) should be performed in those with a family history of AML or patients with physical stigmata of congenital marrow failure syndromes; this information may guide genetic counseling and donor selection for allo HSCT (Chapter 30). The ELN has published a revised risk model based on integration of cytogenetic and gene mutation information (Table 60.2). Mutations of the chromatin modifying proteins (*ASXL1*, *EZH2*, *BCOR*), spliceosome enzymes (*SRSF2*, *U2AF1*, *SF3B1*, *ZRSR2*), and the cohesin complex protein *STAG2* are more commonly identified in AML evolving from MDS compared with de novo AML. In fact, patients with apparently de novo AML with one of these mutations have inferior response to and survival following intensive therapy compared with those who do not have this genotype.

Assessment of post-therapy response provides additional valuable information. Achievement of morphologic complete remission (CR) after one or two courses of induction therapy is associated with improvement in survival. The most powerful post-treatment prognostic factor is the degree of minimal (or measurable) residual disease (MRD). The deeper the response, the better the long-term outcome. MRD is defined as disease that is detected below the arbitrary definition of persistent AML, 5% blasts by morphology and/or immunohistochemical assays. It is estimated that patients in morphologic CR still harbor up to 10^9 leukemic cells, which constitute an important reservoir for later recurrences. Assessing MRD has several advantages: (1) better determination of the quality of response, (2) more timely diagnosis of an impending relapse and possibly improvement of outcome by early intervention, and (3) potential indication to intensify post-remission therapy (allo HSCT) or not.

Persistence of karyotypic abnormalities by cytogenetic analysis at the time of morphologic CR is associated with increased relapse risk and inferior OS. However, the cytogenetic analysis only evaluates 20 metaphases. FISH analysis can detect lower levels of persistent karyotypic abnormalities. MRD, as detected by reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) or multiparameter flow cytometry (MFC), is even more sensitive. RT-PCR requires a suitable molecular target such as fusion transcripts (e.g., *PML-RARA*, *RUNX1-RUNX1T1*, *CBFB-MYH11*, *DEK-CAN*, *BCR-ABL*), *NPM1* insertion mutations, or overexpressed genes (e.g., *WT1*). Persistence of these transcripts following intensive chemotherapy has been associated with higher risk of relapse and inferior survival. For example, detection of the clone-specific NPM1 mutant transcript in the peripheral blood by RT-PCR following 2 cycles of intensive chemotherapy was associated with increased risk of relapse at 3 years (82% if MRD positive vs. 30% if MRD negative) and inferior survival at 3 years (24% if MRD positive vs. 75% if MRD negative). An expert

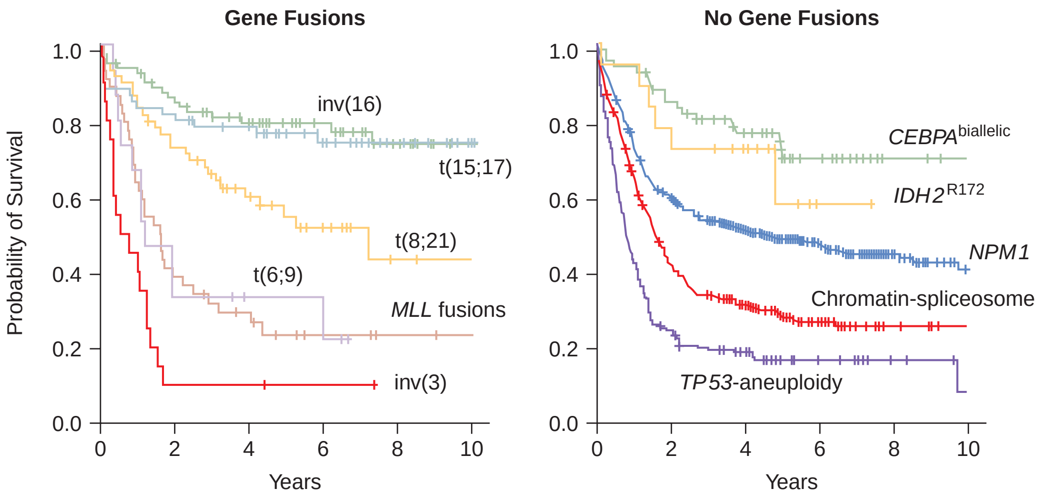


Figure 60.14 MOLECULAR SUBCLASSIFICATION OF AML AND OVERALL SURVIVAL. *(From Papaemmanuil E, et al: N Engl J Med 2016; 374[23]: 2209.)*

TABLE 60.2 **European Leukemia Network 2017 Acute Myeloid Leukemia Risk Stratification**

Risk Category	Genetic Abnormality
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD
	Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD
	Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD (without adverse-risk genetic lesions)
	t(9:11)(p21.3;q23.3); *MLLT3-KMT2A*
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9) (p23;q34.1); *DEK-NUP214*
	t(v;11q23.3); *KMT2A* rearranged
	t(9:22)(q34.1;q11.2); *BCR-ABL1*
	inv(3)(q21.3q26.2) or t(3;3)(q21,3;q26.2); *GATA2, MECOM(EVI1)*
	-5 or del(5q); -7; -17/abn(17p)
	Complex karyotype, monosomal karyotypeII
	Wild-type *NPM1* and *FLT3*-ITD
	Mutated *RUNX1*
	Mutated *ASXL1*
	Mutated *TP53*

From Dohner H, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel *Blood.* 2017;129(4):424–447.

panel has recommended the use of RT-PCR for MRD assessment at informative clinical timepoints only in AML patients with *PML-RARA*, *RUNX1-RUNX1T1*, *CBFB-MYH11*, and *NPM1* insertion mutations.

Since only the minority of AML patients harbor a leukemia-specific marker that can be identified by RT-PCR, MFC is more widely applicable to the assessment of MRD in AML patients. MRD is based on the identification of a leukemia-associated immunophenotype, which can be detected in the majority of patients with AML in various ways: (a) "different-from normal" antigen expression in leukemic blasts; (b) detection of lineage-foreign markers; (c) asynchronous expression of markers; (d) altered density of surface antigens. Commonly, a level of 0.1% has been determined to distinguish patients with MRD from those without. MRD levels following induction or early in consolidation have been associated with relapse and survival in all age groups regardless of cytogenetic and mutational status. Even though MRD levels tend to be higher in patients with unfavorable pretreatment characteristics, the prognostic impact of MRD extends to all risk groups and has shown to improve standard risk classification independently of cytogenetic–molecular markers.

Despite the clear prognostic significance of persistent MRD even in the setting of CR, there are many barriers to fully adopting MRD assessments into the routine care of AML patients. The list of these obstacles includes lack of assay standardization, absence of a universal definition of most appropriate cut-point for reporting positive MRD, choice of peripheral blood versus bone marrow for MRD analysis, quality of the bone marrow aspirate sample, timing of the assay following therapy, and variation in the kinetics of MRD clearance. The persistence of specific mutations by NGS may not actually affect prognosis. For example, persistence of mutations in *DNMT3A*, *TET2*, and *ASXL1* following intensive chemotherapy has not been shown to affect prognosis. Intensification of therapy by means of allo HSCT is associated with the lowest risk of relapse in AML patients with MRD persistence after initial therapy. However, even with a myeloablative preparative regimen, risk of relapse and OS are negatively impacted by persistence of disease by MFC at the time of the allo. Reduced intensity preparative regimens seem to provide little protection from relapse in patients with genomic evidence of persistent AML using highly sensitive duplex DNA sequencing. Effective strategies to eradicate MRD following intensive chemotherapy are not currently available, but novel agents are being assessed for this purpose (e.g., antibody-drug conjugates, Bispecific T-cell engagers [BiTE], targeted therapies, and cellular therapy).

THERAPEUTIC CONSIDERATIONS AT INITIAL PRESENTATION

Leukocytosis with 100,000 WBC/μL or higher is considered an emergency and requires immediate efforts to reduce the disease burden. Hyperleukocytosis may result in leukostasis. The clinical features of leukostasis are due to the rheologic effects of the large blasts in the microvasculature as well as the presence of endothelial adhesion molecules on the leukemic blasts. Leukostasis is a multisystem syndrome due to compromise of blood flow in the lungs (dyspnea, hypoxemia, diffuse alveolar hemorrhage), CNS (encephalopathy, coma, tinnitus, dizziness, visual disturbance, retinal hemorrhages), and heart (myocardial ischemia). Patients with leukostasis require urgent cytoreduction with chemotherapy (immediate start of intensive induction therapy, high-dose hydroxyurea, single dose of cytarabine) and/or leukapheresis. Retrospective studies have not confirmed any survival benefit with leukapheresis compared with chemotherapy alone. Furthermore, leukapheresis is contraindicated in the setting of significant DIC and hypotension; extracorporeal leukapheresis can be associated with respiratory failure due to cytokine release and exacerbation of pulmonary capillary leak. Leukapheresis also requires placement of large-bore central venous catheters which may result in traumatic hemorrhage.

Other complications of AML requiring urgent intervention are TLS and DIC. Either may be present at the time of diagnosis, and both may worsen as therapy is started. Therefore, frequent laboratory assessments (2 to 4 times daily) may be needed, especially as therapy is initiated in patients with high tumor burden, e.g., hyperleukocytosis. DIC is managed with aggressive transfusion of cryoprecipitate, plasma, and platelet transfusion based on close monitoring of the fibrinogen, PT/PTT, and platelet count, respectively (Chapter 137). The use of heparin and/or anti-fibrinolytic agents have not been shown to decrease the risk of serious hemorrhagic complications associated with DIC. However, prospective evaluation of these interventions versus transfusion support alone is not available. TLS should be anticipated, and prophylactic therapy with xanthine oxidase inhibitors (allopurinol, febuxostat) and intravenous hydration are recommended with initial therapy. Urine alkalinization to increase urinary excretion of uric acid is not recommended, since alkalinization may exacerbate metastatic calcinosis due to hyperphosphatemia. Instead, hyperuricemia, especially if accompanied by poor renal function, can be corrected rapidly with intravenous rasburicase. Rasburicase metabolized uric acid to the more soluble allantoin. However, patients with G6PD deficiency are at risk of fulminant oxidative hemolysis with rasburicase due to the concomitant production of hydrogen peroxide. Phosphate binders are given for the hyperphosphatemia. Insulin with glucose, sodium bicarbonate, and sodium polystyrene sulfonate in sorbitol (a potassium exchange resin) are used to control life-threatening hyperkalemia. Patiromer may also be used but has not been evaluated for hyperkalemia associated with TLS. Finally, urgent renal replacement therapy (dialysis) should be considered for management of acute renal failure due to TLS that is not adequately controlled by the management outlined above.

Initial Therapy: Intensive Versus Less Intensive Treatment Algorithm

Until 2017, the general therapeutic algorithm following the diagnosis of AML had not changed substantially in the preceding four decades. The physician first assessed the patient's ability to tolerate intensive chemotherapy approaches, which may ultimately include allo HSCT. Therapeutic options for "unfit" AML patients were quite limited and included DNA methyltransferase inhibitors (also known as DNA hypomethylating agents [HMA]) such as azacitidine and decitabine, Low-Dose Cytarabine (LoDAC), and Best Supportive Care (BSC) alone. Cytarabine 20 mg by subcutaneous injection twice daily for 10 days every 4 weeks (LoDAC) was compared with hydroxyurea and BSC (with or without all trans-retinoic acid) in older, unfit AML patients. Both the median survival and CR rate (18% vs. 1%; $P < .001$) were superior with LoDAC, except for those patients with adverse cytogenetics, who did poorly on either treatment arm. The survival benefit was only observed in those achieving remission.

Azacitidine 75 mg/m^2/day for 7 days improved the survival of AML patients with 20% to 30% marrow blasts compared with conventional care regimens (BSC, LoDAC, and intensive chemotherapy) in a phase III study enrolling high-risk, high-grade MDS patients (median OS 24.5 vs. 16 months, $P = .005$). Subsequently, azacitidine was compared with conventional care regimens (predominantly, LoDAC) in AML patients ages 60 years and older. There was a trend for improvement in the median survival with azacitidine (10.4 vs. 6.5 months, $P = .1009$); however, subsequent therapies confounded the assessment of any survival benefit. The rate of CR was similar with both azacitidine and LoDAC (19.5% vs. 21.9%). Decitabine 20 mg/m^2 IV daily for 5 days every 4 weeks was evaluated in a phase II study of treatment-naïve older AML patients and subsequently compared to either LoDAC (in most subjects) or BSC alone in a large randomized multicenter study. The decitabine arm achieved a higher CR rate (17.8% vs. 7.8%; $P = .001$). In a post hoc sensitivity analysis of the mature data a survival advantage was observed with decitabine at fixed time points over 2 years, and median survival improved from 5.0 to 7.7 months. A phase II study suggested that an extended schedule of decitabine 20 mg/m^2/day for 10 days may result in better response (CR 47%) and survival (median OS 55 weeks). However, a randomized phase II study of 5 versus 10 days did not detect any benefit in terms of CR/CRi rate (43% vs. 40%), median relapse-free survival (RFS) (5.7 vs. 4.6 months), median OS (5.5 vs. 6.0 months), or 1-year survival (25% both arms). With both azacitidine and decitabine, several monthly cycles may be required to achieve the response. Although some analyses suggested a higher response rate in MDS patients with *TET2* mutations, there are no clear predictors of response to HMA therapy in AML patients.

In AML patients over the age of 65 years, there has even been conflicting data regarding the benefit of intensive versus less intensive chemotherapy from randomized trials conducted in the 1980s. A randomized trial conducted by the European Organization for Research and Treatment of Cancer (EORTC) compared immediate induction therapy with daunorubicin, cytarabine, and vincristine with supportive care until disease progression (leukocytosis, thrombocytopenia, pain) and then therapy with hydroxyurea 3 g on days 1 and 4 with cytarabine 100 mg/m^2 twice daily on day 2 to 3 and 5 to 6. The remission rate was higher (58% vs. 0%), median survival longer (21 vs. 1 weeks), and survival at 2.5 years higher (17% vs. 0%) with the immediate induction therapy; early mortality was higher with the more palliative approach. On the other hand, another randomized trial comparing an anthracycline and cytarabine induction therapy with LoDAC demonstrated higher CR rate with intensive therapy, but lower early mortality with LoDAC, resulting in no significant difference in the OS. In a retrospective analysis of AML patients older than 65 years treated at the M.D. Anderson Cancer Center from 2000 to 2010, OS was similar regardless of initial therapy, either intensive chemotherapy or epigenetic-directed therapy. Given the low response rate with less intensive therapy, greater toxicity with intensive therapy, and minimal (if any) survival benefit, it is not surprising that the majority of AML patients age ≥65 years chose not to receive any form of chemotherapy for AML. In an analysis of SEER and Medicare data between 2000 and 2007, only 38.6% of U.S. Medicare recipients with AML received any form of chemotherapy. Increasing age, higher Charlson Comorbidity Index, and prior MDS diagnosis were associated with a lower rate of treatment. However, those who were selected for therapy had a longer median survival than those who were not (6 vs. 2 months).

With the recent availability of more effective, less intensive treatment regimens (see section Less Intensive Therapy), more older AML patients are now eligible for therapy. On the other hand, younger, fit AML patients may potentially benefit from less intensive therapies that have demonstrated efficacy in AML subtypes typically associated with chemotherapy resistance. Conversely, AML patients may become candidates for allo HSCT in first remission even if initially treated with less intensive therapy. The current AML treatment algorithm is summarized in Fig. 60.15.

When selecting initial therapy now, the clinician needs to consider a more global assessment of fitness (performance status, comorbidities, geriatric assessment scores), disease biology, as well as the goal of therapy (see box on Selection of Initial Therapy for the Patient With Acute Myeloid Leukemia). Age alone is considered

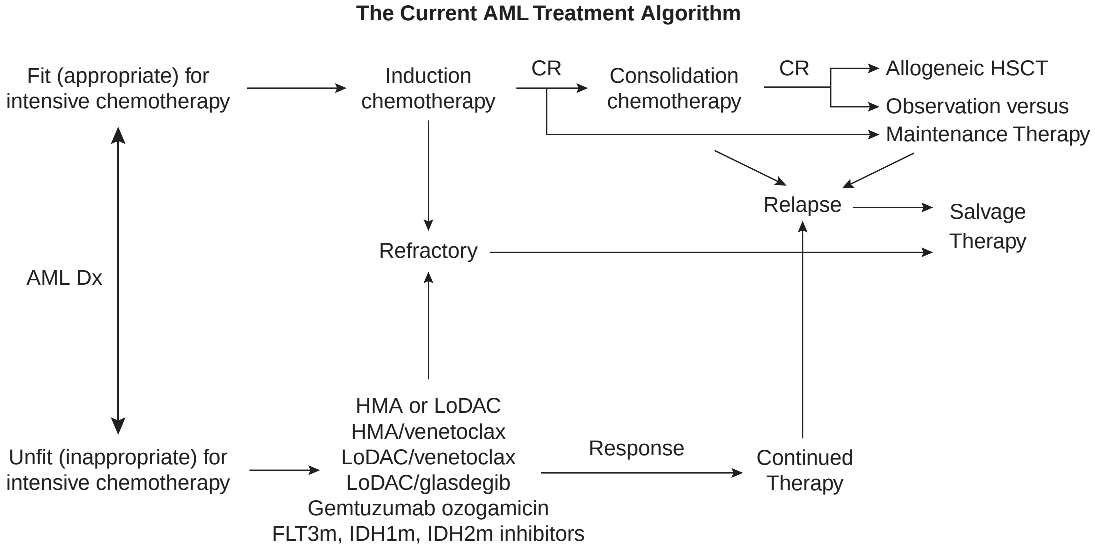

The Current AML Treatment Algorithm
Fit (appropriate) for intensive chemotherapy
Induction chemotherapy
CR
Consolidation chemotherapy
CR
Allogeneic HSCT
Observation versus Maintenance Therapy
Relapse
Salvage Therapy
Refractory
AML Dx
HMA or LoDAC
HMA/venetoclax
LoDAC/venetoclax
LoDAC/glasdegib
Gemtuzumab ozogamicin
FLT3m, IDH1m, IDH2m inhibitors
Response
Continued Therapy
Unfit (inappropriate) for intensive chemotherapy

Figure 60.15 GENERAL APPROACH TO ACUTE MYELOID LEUKEMIA THERAPY.

inadequate for determination of initial and subsequent therapy. On the other hand, comorbidities are frequent in older patients, who are more likely to have a poor performance status. Hence, tolerance to the myelosuppressive and immunosuppressive consequences of intensive chemotherapy is diminished and there is greater risk of early therapy-related mortality. There are also differences intrinsic to the blast biology between older and younger patients. Patients over 60 years of age are more likely to have secondary AML, demonstrate unfavorable cytogenetics, and more often express multidrug-resistant phenotypes. Nevertheless, a select group of patients may still benefit from more intensive interventions. Older patients are not a homogeneous group and several models have been devised to identify variables that predict which patients may do well with intensive therapy versus those who will not. These models are comprised of patient characteristics, easily accessible clinical variables, and tumor characteristics. Efforts are therefore underway to capture multiple patient characteristics (physical and cognitive function, nutritional status, comorbidity, psychological state, and social support) as part of a comprehensive geriatric assessment and to better distinguish patients into fit, vulnerable, and frail (indicating a significantly increased risk of treatment complications). Despite thorough discussions with their oncologist regarding the goals of therapy, patients often misunderstand terms, e.g., equating remission with cure; patients may overestimate the benefit of therapy in terms of long-term survival and cure.

The initial goal of either intensive or less intensive therapy is the prolongation of survival through the achievement of a CR. Following CR, further post-remission therapy is required to maintain remission by eliminating or reducing residual disease. CR is defined as achievement of less than 5% marrow blasts (even if these are known to be leukemic blasts by immunophenotypic, cytogenetic, or mutational analyses), absence of Auer rods, and clearance of the peripheral blood and all extramedullary deposits of leukemic blasts. CR also requires restitution of relatively normal marrow function, indicated by an absolute neutrophil count (ANC) greater than 1000/µL, a platelet count greater than 100,000/µL, and independence of transfusion support. CR defines a landmark point in time because patients who achieve CR after beginning therapy have longer survival than patients who are resistant to therapy. Lesser response criteria (CR with incomplete platelet recovery [CRp], CR with incomplete recovery of neutrophils or platelets [CRi], CR with partial hematologic recovery [CRh], partial remission [PR], and morphologic leukemia-free state [MLFS]) may serve as useful end points in the context of early phase clinical trials, but they do not necessarily carry the same significance for survival as does CR. Each of these (except CRh) are defined by International Working Group and ELN criteria. The definition of CRh is identical to that of CR, except that the ANC must only be greater than 500/µL and the platelet count only greater than 50,000/µL.

After achievement of an initial response, the continued therapeutic path is different for patients treated with intensive versus less intensive therapy. Early clinical trials demonstrated the need to provide some post-remission therapy following achievement of CR with intensive chemotherapy. For example, in an ECOG trial AML patients $\leq$ age 65 years in first CR were assigned to allo HSCT (if family member donor available and age $\leq$40 years), or randomly assigned to observation, 2 years of oral mercaptopurine (MP) maintenance, or 1 cycle of high-dose cytarabine. The observation arm was closed early after 28 subjects assigned to this arm were noted to have inferior remission duration compared with continuing maintenance therapy. Post-remission therapy following intensive remission induction therapy consists of chemotherapy (typically 1 to 4 cycles), and/or HSCT, and/or maintenance therapy. Patients may receive one of these post-remission treatments or all of them, depending on initial risk stratification, eligibility for allo HSCT over time, as well as MRD assessments. For patients receiving less intensive regimens, the same therapy is continued in the absence of disease progression, disease relapse, or unacceptable toxicity (discussed later). However, even patients achieving CR after less intensive therapy may become candidates for potentially curative allo HSCT.

SELECTION OF INITIAL THERAPY FOR THE PATIENT WITH ACUTE MYELOID LEUKEMIA

The selection of either an intensive or less intensive chemotherapeutic regimen for AML patients remains a matter of significant debate and part of the art of medicine. The choice has been made more difficult by the recent approval of less intensive regimens with high initial response rates, even in patients with adverse AML biology. Fitness for intensive chemotherapy has never been adequately defined. I weigh multiple factors that will determine tolerance to chemotherapy including age, functional or performance status, comorbid illnesses, organ dysfunction, tobacco use, and nutritional status. However, I no longer consider only fitness; I also consider whether intensive chemotherapy is appropriate. The disease biology is critical to decision making. AML with adverse features based on cytogenetic and mutational analysis has a low response rate with intensive chemotherapy, and the only potentially curative option will ultimately be allogeneic hematopoietic stem cell transplantation once a remission is achieved.

For patients appropriate for intensive therapy, I base my choice of initial therapy on the following features: documented history of antecedent myeloid neoplasm, prior leukemogenic therapy for another disease, presence of a *FLT3* ITD or TKD mutation, presence of *RUNX1/RUNX1T1* or *CBFB/MYH11* fusion, and presence of the *PML/RARA* fusion. I have partnered with our pathology colleagues to expedite PCR for the *FLT3* ITD and TKD mutations and FISH analysis for *PML/RARA*, *RUNX1/RUNX1T1*, *CBFB/MYH11*, as well as deletions of chromosomes 5q, 7q, and 17p. These results are available within 72 hours. If there are 20% circulating myeloblasts, the diagnosis of AML is confirmed, and these assays can be done on a peripheral blood sample without waiting for a bone marrow biopsy to be performed.

If there is any suggestion of APL at initial presentation, I start ATRA immediately, before the diagnosis is confirmed. A myeloid immunophenotype lacking the stem cell markers CD34 and HLA-DR, blasts with bilobed nuclei without cytoplasmic granules, or even leukopenia with DIC, may suggest the diagnosis of APL. The ATRA can be discontinued, after the diagnosis is excluded. If APL is confirmed, I treat with ATRA and ATO. I add GO 9 mg/m^2, if the WBC count is over 10,000/µL. If the patient has CBF AML, I treat with cytarabine and daunorubicin (7+3) with GO 3 mg/m^2. If the *FLT3* ITD or TKD is present, I treat with daunorubicin 60 mg/m^2/day for 3 days and cytarabine 200 mg/m^2/day for 7 days with midostaurin 50 mg twice daily on days 8–21. Finally, if the patient has t-AML or AML following MDS or MDS/MPN, I treat with liposomal daunorubicin/cytarabine (CPX-351). If the patient apparently has a de novo AML but with multiple abnormalities on the FISH panel including del(5q) and del(7q), I will also treat with CPX-351. However, I will also consider a HMA and venetoclax (VEN) for these patients, especially if there is del(17p), and the patient had not been previously treated with an HMA. If none of these markers are present, I often select 7+3 with idarubicin 12 mg/m^2/day for 3 days, to avoid the controversy surrounding the optimal dose of daunorubicin (90 vs. 60 mg/m^2).

For patients who are truly unfit for intensive chemotherapy, or decline intensive chemotherapy, I treat with azacitidine 75 mg/m^2/day for 7 days plus VEN once daily for up to 28 days. I do not wait for any cytogenetic or mutational data since this information would not change the decision to use AZA/VEN in this approved population. Although certain subsets of patients did not have a survival benefit with AZA/VEN compared with AZA/placebo (e.g., *FLT3* ITD- or *TP53*-mutated), the initial response rate is over 50%. The one possible exception are those patients with *IDH1*- or *IDH2*-mutated AML. If these patients had previously received an HMA for MDS, or if they decline any form of parenteral therapy, ivosidenib and enasidenib would be acceptable alternatives for *IDH1*- and *IDH2*-mutated AML, respectively. Therefore, our pathologists also provide the *IDH* mutation status within 72 hours as well.

I will treat patients with HMA/VEN as an outpatient if they live close to our center, have a caregiver at home, have normal renal function, do not have leukocytosis, and do not already have complications from active AML such as infection. Otherwise, I will admit patients for the first week of therapy. I generally give posaconazole to all patients during initial induction due to the high rates of invasive fungal infection seen in our population. I give venetoclax 100 mg once daily in combination with posaconazole. If there is clearance of the peripheral blood blasts by day 21, I repeat a bone marrow biopsy at that time. If morphologic leukemia-free state (MLFS) or marrow aplasia has been achieved, I hold venetoclax at that time and allow for neutrophil recovery. In that case, I only prescribe 21 days of venetoclax during cycle #2. If the patient has not achieved MLFS, I proceed with cycle #2 as planned at day 29. Generally, remission is achieved with the first 2 cycles, but I will continue AZA/VEN if there appears to be clinical benefit even if CR or CRi has not been achieved.

Of course, I consider every patient for a clinical trial if available and appropriate.

Intensive Therapy: Induction

Since first published by Yates and colleagues in 1973, intensive AML therapy is still built on the same two drugs as 50 years ago: cytarabine (also known as cytosine arabinoside or Ara-C) and an anthracycline. Cytarabine, an analogue of a physiologic pyrimidine nucleoside, is a cell cycle-specific antimetabolite that requires intracellular conversion to its triphosphate compound, ara-CTP, and incorporation into DNA to become active (Chapter 58). When given by itself at standard doses of 100 to 200 mg/m^2 intravenously (IV) daily for 5 to 7 days, it produces CR rates of around 40%. The anthracyclines, daunorubicin and idarubicin, and the anthraquinone, mitoxantrone, have been the most extensively evaluated agents in combination with cytarabine. The anthracycline doxorubicin was associated with more mucositis and gastrointestinal toxicity compared with daunorubicin and is not used in AML therapy. These agents intercalate into DNA and stabilize the complex between DNA and the enzyme topoisomerase II, thereby leading to apoptotic cell death (Chapter 58). Daunorubicin achieves similar CR rates as does cytarabine alone. The combination of cytarabine 100 to 200 mg/m^2 as a continuous IV infusion (its serum half-life is only 15 minutes) daily on days 1 to 7 and daunorubicin 45 to 90 mg/m^2 IV daily on days 1 to 3 has become known as the "7+3" regimen and, for more than 40 years, has been the standard induction combination for AML patients selected for intensive therapy. Remission rates range from 50% to 80% (depending on patient age and cytogenetic risk), and long-term disease-free survival (DFS) is 35% to 40%.

Early clearance of the peripheral blood blasts (e.g., less than 3 days by flow cytometry) is associated with superior long-term survival. In clinical practice, patients undergo a repeat bone marrow examination between 14 and 21 days from the start of treatment (Fig. 60.16). If the marrow continues to show blasts and is cellular, a reinduction is usually given. However, there is little consensus on what degree of persistent disease at day 14 to 21 can be tolerated. It is not uncommon for a patient to be too ill 2 weeks after intensive therapy to receive re-induction therapy for persistent disease, and yet ultimately achieve remission without further therapy. Clinical trials often recommend re-induction, if there are more than 10% blasts, and the marrow cellularity is ≥15%. The reinduction may be an attenuated repetition of the induction ("5+2") or an intensification with intermediate-dose cytarabine (IDAC) or high-dose cytarabine (HiDAC) or fludarabine, HiDAC and granulocyte colony stimulating factor (G-CSF) (FLAG). There has been debate regarding the prognostic significance of requiring 2 versus 1 cycle of induction therapy to achieve first CR. Earlier studies reported a negative impact on RFS whereas others showed no impact on outcome. However, younger AML patients treated on SWOG S0106 and S1203 studies with 7+3 containing daunorubicin 60 and 90 mg/m^2, respectively, the requirement of 2 induction cycles to achieve remission had a significant negative impact on RFS and OS.

On the other hand, if the day 14 or 21 marrow is hypoplastic and appropriately ablated, supportive care continues until bone marrow recovery. If blood counts are not recovering, the bone marrow studies are repeated every 2 weeks to determine if there is recurrent AML causing the pancytopenia or delayed marrow recovery. If in CR, post-remission therapy starts shortly thereafter, once the patient has recovered from the toxicities of induction therapy. The degree of neutrophil and platelet recovery at the time of remission has prognostic significance. Higher neutrophil and platelet count at the time of remission are predictive of better RFS. In some cases, a regenerating marrow may have an increased number of blasts, which may look like persistent leukemia. Immunophenotyping by flow cytometry often helps to make that distinction. If due to regeneration, then follow-up marrow studies will show reduction in blasts concomitant with

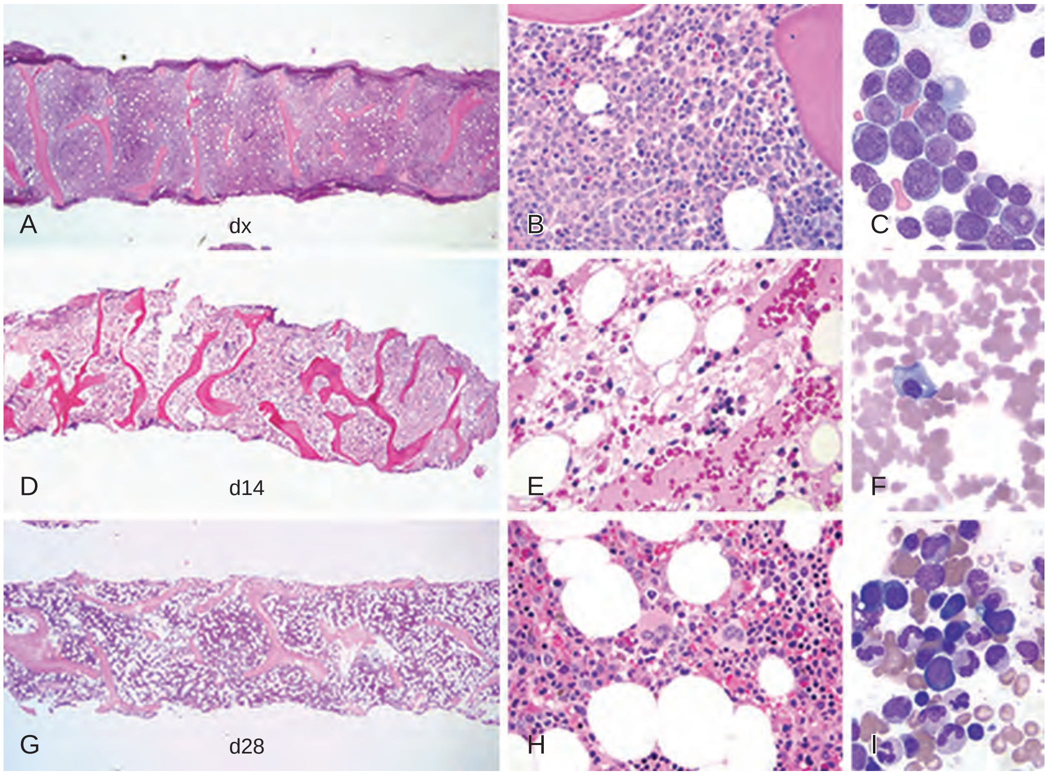


Figure 60.16 ACUTE MYELOID LEUKEMIA AT DIAGNOSIS AND AT DAY 14 AND DAY 28 FOLLOWING THE START OF STANDARD INDUCTION CHEMOTHERAPY. A 43-year-old man presented with fatigue and was found to have a white blood cell count of 70,300/µL composed of mostly blasts. A bone marrow study (A–C) showed a 90% cellular marrow packed with blasts (80%). The blasts were myeloperoxidase positive and had the following phenotype: CD34$^+$, HLA-DR$^+$, CD117$^+$, CD13$^+$, CD33$^+$ with partial CD15 and CD11b. Cytogenetic analysis demonstrated a normal male karyotype, and molecular studies showed wild-type *FLT3* and *NPM1*. The patient was treated with standard induction chemotherapy, and a day-14 bone marrow study (D–F) showed chemoablation effects with an empty marrow, stromal injury, dilated sinuses, and only scattered stromal cells and plasma cells with no obvious blasts. A bone marrow study performed at day 28 (G–I) showed regenerative changes of trilineage hematopoiesis.

a rise of neutrophils and platelets. In case of no response to one or two courses of induction therapy ("primary refractory"), treatment is often changed.

Many modifications to the 7+3 regimen have been evaluated over the years. Until recently, modifications of 7+3 have not improved outcomes for AML patients (see below). Two different standard cytarabine dose regimens were compared: 100 versus 200 mg/m^2 continuous IV infusion once daily days 1 to 7 with daunorubicin. The rates of remission, median DFS, median OS, and 5-year survival were not statistically different. However, there was a higher mortality rate related to chemotherapy with 200 mg/m^2 (21% vs. 13%, $P = .05$). Likewise, 10 days instead of 7 days of cytarabine (10+3) did not improve outcomes. Other cytotoxic agents (e.g., vincristine, etoposide, thioguanine, topotecan, fludarabine) have been added to 7+3 without proven benefit, but often with increased toxicity. Inhibitors of multi-drug efflux protein MDR1 (also known as P-glycoprotein or P-gp) could potentiate the cytotoxicity of P-gp substrates, such as the anthracyclines and etoposide, by allowing intracellular accumulation of these drugs. However, P-gp inhibitors also affect clearance of these drugs. Therefore, the doses of anthracyclines and etoposide needed to be reduced due to increased toxicity in the presence of the P-gp inhibitor. Neither the response rates nor OS of AML patients was improved by the addition of the P-gp inhibitors, valspodar or zosuquidar, to induction chemotherapy in phase III randomized trials. The B-cell lymphoma/leukemia-2 (BCL2) anti-sense oligonucleotide, oblimersen, did not improve OS when administered with 7+3 induction therapy. Since cytarabine is a cell-cycle specific cytotoxic agent, priming with hematopoietic growth factors (G-CSF and granulocyte-macrophage colony stimulating factor [GM-CSF]) has been performed during the week of chemotherapy to force leukemic blasts into S phase. However, priming has not been shown to improve OS. The greatest risk of intensive chemotherapy is morbidity and mortality due to neutropenic infection. The addition of either G-CSF or GM-CSF following chemotherapy shortens the duration of absolute neutropenia by a few days, but did not improve CR rate, early mortality, or OS.

The intracellular concentration of ara-CTP is increased by culturing leukemic cell lines in the presence of both a purine analog and cytarabine, due to increased expression of cytidine kinase. Based on this pharmacokinetic interaction and other effects of the purine analogs, many studies have evaluated the addition of purine analogs to cytarabine-based chemotherapy. The Polish Adult Leukemia Group published the outcome of an open-label, randomized study where addition of cladribine 5 mg/m^2 once daily days 1 to 5, but not fludarabine, to a daunorubicin 60 mg/m^2 for three days and cytarabine 200 mg/m^2 for 7 days resulted in significantly increased rates for remission (68% vs. 56%; P=.01) and 3-year OS (OS; 45% vs. 33%; P=.02). Interestingly, leukemia-free survival was not improved by the addition of cladribine. The survival benefit was less significant when subjects were censored at the time of allo HSCT. The addition of the second-generation purine analog, clofarabine at 20 mg/m^2/day for five days, to idarubicin 10 mg/m^2/day on days 1 to 3 and cytarabine 1 g/m^2/day on days 1 to 5 (IA) led to significantly better event-free survival (EFS) and OS in the three-drug combination when compared with a historical IA-treated group, especially in patients younger than 40 years of age. The effect of ludarabinee on cytarabine accumulation in leukemic blasts was also demonstrated in patients with relapsed/refractory AML receiving high-dose cytarabine salvage therapy with or without fludarabine; however, there was no apparent clinical benefit in this phase III study. The use of fludarabine with idarubicin and high-dose cytarabine in the United Kingdom Medical Research Council (UK MRC) trials is discussed later.

The dose of daunorubicin has been the subject of debate. A study by the Eastern Cooperative Oncology Group (ECOG 1900) randomized patients between the ages of 17 and 60 years with untreated AML to a 7+3 combination with either standard-dose daunorubicin (45 mg/m^2 daily for 3 days) or high-dose daunorubicin (90 mg/m^2 daily for 3 days). The higher dose of daunorubicin achieved higher CR rates (70.6% vs. 57.3%, $P < .001$) and improved the median OS (23.7 vs. 15.7 months, $P = .003$). This improvement was limited to patients younger than 50 years and with intermediate-risk cytogenetics. The daunorubicin dose of 90 mg/m^2/day did not lead to a higher incidence of adverse events (particularly cardiomyopathy and infectious complications). For patients with *FLT3* ITD mutations, CR rates (70% vs. 48%), median survival (15.2 vs. 10.1 months), and 4-year survival estimates (28% vs. 17%) were also superior with 90 versus 45 mg/m^2. The study can be criticized on the basis that the dose of the comparator arm, daunorubicin 45 mg/m^2/dose, is no longer considered the standard of care for younger AML patients, and instead 60 mg/m^2/dose should be considered standard. In older AML patients ages 60 to 83 years (median 67 years), daunorubicin 90 mg/m^2/day for 3 days was compared with the standard dose for this population, 45 mg/m^2/day for 3 days both in combination with cytarabine 200 mg/m^2/day for 7 days. The CR rate was greater with the high-dose daunorubicin (64% vs. 54%, $P = .002$) without an increase in induction mortality. There was no difference in EFS or OS for the entire population. In the patients ages 60 to 65 years, 2-year survival was superior with high-dose daunorubicin (38% vs. 23%). The UK MRC AML 17 trial compared daunorubicin 60 versus 90 mg/m^2 during induction and showed equivalent CR and relapse rates as well as survival. However, in the patients with *FLT3* ITD mutation, the 90 mg/m^2 daunorubicin resulted in higher RFS and OS. The UK MRC AML 17 trial did not settle the debate, because patients received a second cycle of daunorubicin-containing chemotherapy. The cumulative dose of daunorubicin was higher in both arms of the UK MRC AML17 trial compared with the two arms of the ECOG 1900 study.

Idarubicin is a 4-demethoxy anthracycline analogue of daunorubicin, which results in increased lipophilicity and better cellular uptake compared with daunorubicin. Idarubicin, and its metabolite idarubicinol, are not substrates for the multidrug resistance efflux pump. Idarubicin 12 to 13 mg/m^2 for 3 days appeared to be superior to daunorubicin 45 to 50 mg/m^2 for 3 days in several phase III studies. However, the doses did not appear to be biologically equivalent, since idarubicin was associated with greater myelosuppression in those studies continuing the comparison into the post-remission setting. In younger adults with treatment naïve AML receiving cytarabine 200 mg/m^2 for 7 days with either daunorubicin 90 mg/m^2/day (AD) for 3 days or idarubicin 12 mg/m^2/day for 3 days (AI), there was no difference in CR rates (AD 74.5% vs. AI 80.5%), cumulative incidence of relapse (AD 25.1% vs. AI 35.2%), and 4-year survival (AD 54.7% vs. AI 51.1%). However, EFS and OS were significantly better with IA in patients with *FLT3* ITD positive AML.

A meta-analysis of 29 randomized controlled trials compared the efficacy of different anthracyclines and dosing schedules during induction therapy for AML patients less than age 60 years. Idarubicin compared with daunorubicin improved remission rates, although this effect was limited to studies with a daunorubicin/idarubicin ratio of less than 5. Likewise, higher-dose compared with lower-dose daunorubicin improved remission rates. Survival estimates suggest that both high-dose daunorubicin (90 mg/m^2/dose × 3 days or 50 mg/m^2/dose × 5 days) and idarubicin (12 mg/m^2/dose × 3) can achieve 5-year survival rates of between 40% and 50%. On the other hand, there was no difference in CR rates or OS in AML patients older than age 55 years treated with cytarabine 100 mg/m^2/day for 7 days with either idarubicin 12 mg/m^2/day, daunorubicin 45 mg/m^2/day, or mitoxantrone 12 mg/m^2/day for 3 days. However, the induction mortality was greater in the idarubicin treatment arm.

A meta-analysis of 3 randomized clinical trials using HiDAC during induction encompassed 1691 patients and arrived at the following conclusions: there were no differences between HiDAC and standard-dose cytarabine (SDAC) with respect to percent CR and rates of persisting leukemia and early death. On the other hand, 4-year OS and RFS were significantly better with HiDAC but at the cost of more toxicities (infections, nausea/vomiting, CNS). HiDAC benefited mostly patients who already were more likely to achieve CR (i.e., patients with intermediate and favorable karyotype). The major drawback of HiDAC during induction is that it may make further consolidation therapy difficult. It is also not clear if HiDAC should be used during initial induction or is best used as a post-remission therapy after marrow function has been restored. The UK MRC AML 15 trial randomized 1268 treatment-naïve younger AML patients to receive fludarabine, HiDAC, G-CSF, and idarubicin (FLAG-Ida; fludarabine 30 mg/m^2 IV on days 2 to 6, cytarabine 2 g/m^2 IV on days 2 to 6, idarubicin 10 mg/m^2 IV on days 4 to 6, and G-CSF on days 1 to 7) and 1983 to receive cytarabine (ara-C), daunorubicin, and etoposide (ADE; daunorubicin 50 mg/m^2 IV on days 1, 3, 5; cytarabine 100 mg/m^2 IV on days 1 to 10; and etoposide 100 mg/m^2 IV on days 1 to 5), the latter representing the SDAC arm. FLAG-Ida resulted in higher response rates following the first induction and fewer patients on FLAG-Ida relapsed. However, more myelosuppression and deaths in CR offset the survival benefit except in the FLAG-Ida treated patients who were able to receive four courses (FLAG-Ida × 2, HiDAC × 2) where 8-year survival was 63% versus 47% with ADE with intermediate-risk patients and 95% for those with favorable risk.

The EORTC and the Italian Cooperative Group for Hematologic Diseases in Adults (GIMEMA) AML-12 trial randomized 1942 newly diagnosed AML patients between ages 15 and 60 years to an induction with SDAC or high-dose cytarabine (3 g/m^2 every 12 hours on days 1, 3, 5, and 7). The study demonstrated significantly higher CR rates in all patients and significantly improved EFS (43.6% vs. 35.1%; *P* =.003) and OS (51.9% vs. 43.3%; *P* =.009) for patients between 15 and 45 years of age. The impact on survival was particularly evident in younger patients with secondary AML, high-risk cytogenetics, and *FLT3* mutations, emphasizing that HiDAC may overcome poor-risk features during induction that SDAC cannot.

Increasing the efficacy of induction therapy has been attempted with the use of additional cytotoxic chemotherapy shortly after completion of the first course and prior to recovery of normal marrow function. This approach is known as timed-sequential chemotherapy and is based on the observation that leukemic blasts will enter cell cycle shortly after completion of chemotherapy, theoretically increasing the sensitivity to cytarabine-based chemotherapy. OS was improved in pediatric patients treated with multi-agent chemotherapy (dexamethasone, cytarabine, thioguanine, etoposide, daunorubicin, and intrathecal cytarabine) followed by the same regimen at day 10 to 14 regardless of initial response compared to standard sequencing of these two courses based on initial response. On the other hand, the German AML Cooperative Group did not detect any difference in CR rate or OS with a dose dense regimen of high-dose cytarabine and mitoxantrone on days 1 to 4 and 8 to 11 compared with their standard double induction regimen with thioguanine, cytarabine, and daunorubicin (TAD) followed by high dose cytarabine and mitoxantrone (HAM) 21 days later. The duration of neutropenia was shorter with the dose dense sequential HiDAC and mitoxantrone.

Intensive Therapy: Post-Remission

In CR, as many as 10^9 leukemia cells survive below the threshold of detection by morphologic assessment. Further therapy is therefore necessary to reduce this number and minimize the chances of relapse. In a series of clinical studies in which patients did not receive post-remission therapy, relapse was universal, and the median remission duration was about 4 months. Post-remission therapy is mainly predicated by pretreatment prognostic factors and can take the form of continued chemotherapy (termed consolidation) and/or high-dose chemotherapy followed by HSCT (see box on Post-Remission Therapy: Allogeneic Hematopoietic Stem Cell Transplantation Versus Consolidation Alone in First Remission). As for the former, debate continues regarding the optimal cytarabine dose, incorporation of anthracyclines, and number of cycles. Three doses of the anti-CD33 antibody-drug conjugate, GO (see below), was compared with no further therapy for older AML patients in first remission following 2 induction cycles; there was no clinical benefit of post-remission GO (5-year DFS 17% vs. 16%). DFS of younger AML patients in first CR was also not improved with three doses of GO following induction and consolidation chemotherapy (SWOG 0106).

POST-REMISSION THERAPY: ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION VERSUS CONSOLIDATION ALONE IN FIRST REMISSION

Allo HSCT is associated with the lowest risk of relapse of any post-remission intervention. Unfortunately, allo HSCT is also associated with the greatest risk of treatment-related morbidity and mortality. Therefore, I attempt to determine which patients are more likely to be cured with chemotherapy alone, and I will recommend allo HSCT for all others. Regardless of my opinion, I generally refer all my patients to a SCT specialist for consultation regarding risks and benefits of allo HSCT in CR1. Almost all patients will now have an acceptable donor given the expansion of the alternative donor pool (haploidentical donors and umbilical cord blood). Nonetheless, there are significant obstacles to allo HSCT even if the patient would be best served by this post-remission intervention. Some of the barriers to allo HSCT in CR1 include poor performance status, serious infection, or organ dysfunction after initial AML therapy, early relapse, and lack of caregiver. I will consider allo HSCT for AML patients in CR1 regardless of the initial induction therapy: intensive versus less intensive regimens.

I consider three factors in my recommendation for allo HSCT in CR1: initial risk stratification, MRD status following initial therapy, and ability to achieve a second CR if the patient does not undergo allo HSCT in CR1. For patients with adverse risk karyotype, I strongly recommend allo HSCT in CR1. If these patients do not undergo allo HSCT in CR1, I am not convinced that high-dose cytarabine would be beneficial. Instead, I would recommend oral azacitidine maintenance therapy to delay relapse. If the patient has CBF AML regardless of other mutations or biallelic *CEBPA* mutations, I recommend high-dose cytarabine alone (with GO for CBF AML). The situation is more complicated for those with *NPM1* mutation and depends on the presence of other mutations as well as the mutational burden. It is in this population that the *NPM1* mutant transcript level by peripheral blood RT-qPCR after 2 cycles of chemotherapy may be most helpful. If still positive, I recommend allo HSCT. I also generally recommend allo HSCT for those patients without favorable risk features (intermediate risk). If the intermediate-risk AML patient does not proceed with allo HSCT in CR1, I generally prescribe up to 3 cycles of high-dose cytarabine consolidation followed by oral azacitidine maintenance therapy to delay relapse. I make it clear to patients that oral azacitidine is not an equally effective alternative to allo HSCT.

I generally prescribe cytarabine 1.5 g/m^2 for six doses as consolidation therapy: 3 cycles for younger patients and two for older. However, for younger patients with CBF AML in CR1 I still prescribe cytarabine 3 g/m^2 during consolidation. If the patient received CPX-351 during induction, I proceed with 1 or 2 cycles of CPX-351 consolidation prior to allo HSCT. I do not use high dose cytarabine consolidation if the first remission was achieved with CPX-351.

Relapse is also common for AML patients with MRD following allo HSCT. However, if such patient has already received induction and consolidation therapy, I strongly recommend allo HSCT despite the presence of MRD, since further attempts to achieve an MRD-negative remission are likely to be unsuccessful and/or lead to toxicities that will preclude allo HSCT in first remission.

In a landmark study by the Cancer and Leukemia Group B (CALGB), patients with newly diagnosed AML in CR following a 7+3 induction were randomized to SDAC (100 mg/m^2 continuous IV infusion daily × 7), IDAC (400 mg/m^2 continuous IV infusion daily × 5), or HiDAC (3 g/m^2 IV over 3 hours every 12 hours on days 1, 3, and 5) for a total of 4 cycles. This was followed by an additional 4 cycles of 5+2, a consolidation-maintenance omitted from all subsequent CALGB studies. The probability of remaining in continuous CR after 4 years was 24% for SDAC, 29% for IDAC, and 44% for HiDAC (*P* =.002). It is important to recognize an important safety signal identified in this study. In the 31 subjects ages 60 years and older who received HiDAC, 32% developed severe neurologic toxicity (seizures, cerebellar dysfunction), which was permanent in 40% of those affected. In fact, age greater than 40 years, creatinine greater than 1.2 mg/dL, and threefold elevation of alkaline phosphatase were all associated with a higher risk of neurologic toxicity. The study was amended to exclude further enrollment of AML patients over age

60 years based on this observation. Patients with CBF translocations and normal diploid karyotypes benefited from HiDAC consolidation. The survival of patients with any other karyotype with SDAC, IDAC, and HiDAC consolidation were similar and very poor. Over the ensuing years, studies have shown that there is no benefit by extending consolidation therapy by a fifth cycle and that even 3 cycles may be equivalent.

CBF AML is very sensitive to cytarabine-based therapy, which is reflected in high remission rates (80% to 90%) and more patients achieving long-term OS (about 60% at 5 years). Post-remission therapy with HiDAC (e.g., 3 g/m^2 IV every 12 hours on days 1, 3, and 5) for 3 to 4 cycles is commonly used. Questions remain regarding the optimal number of cycles, intensification, and addition of further drugs. One cycle (with a cumulative dose of up to 18 g/m^2 of cytarabine) is worse than 3 or 4 cycles (cumulative dose 54 to 72 g/m^2) for patients with t(8;21) translocation. Repetitive cycles of HiDAC consolidation also benefit patients with inv(16) and t(16;16). DFS and OS were similar for younger AML patients in first CR with either 4 cycles of multiagent chemotherapy with SDAC or 3 cycles of HiDAC consolidation. However, in the subset of patients with CBF AML DFS was significantly better with HiDAC, and there was a trend for improvement in OS as well. Given the excellent long-term outcome with HiDAC consolidation, and the higher risk for treatment-related mortality and morbidity with allo HSCT, allo HSCT is generally not recommended for CBF AML patients in first remission. However, allo HSCT may be considered by some for CBF AML patients with *KIT* mutation in first remission. Less frequently in the United States compared with other regions, autologous HSCT is sometimes performed following 1 or 2 cycles of cytarabine-based consolidation, albeit with comparable results to chemotherapy-only regimens.

Patients in the intermediate-risk group comprise the largest and most heterogeneous AML population (about 60% of AML). The majority demonstrate a diploid karyotype. It is in this group of patients where there is an ongoing debate about the role of consolidation chemotherapy versus allo HSCT for post-remission therapy. It is also in this group where the prognostic impact of gene mutation analysis is most helpful to facilitate this decision. Nevertheless, the reported data can be conflicting. For example, the German AML Cooperative Study Group did not observe any benefit of allo HSCT in terms of RFS for younger AML patients with *NPM1* mutation and normal karyotype based on a donor versus no-donor analysis. On the other hand, the Study Alliance Leukemia (SAL) cooperative group demonstrated superior 3-year RFS of younger AML patients with normal karyotype and *NPM1* mutation (without *FLT3* ITD) in those with a sibling stem cell donor compared with those without (83% vs. 53%, $P = .004$). However, there was no difference in OS (81% vs. 75%, $P = .300$), likely related to the ability to achieve a second remission followed by an alternative donor allo HSCT. The outcome of patients in first CR or second CR at the time of allo HSCT is similar, but second CR must be achieved prior to allo HSCT. Some patients will choose to reserve allo HSCT in the event of relapse. Patients need to be informed that remission rates are typically lower in relapsed AML than in previously untreated AML patients (see Relapsed AML). Monitoring for MRD following intensive therapy may help to identify those patients with *NPM1* mutated AML most likely to benefit from allo HSCT in first CR.

Outcome following standard chemotherapy remains poor for patients with adverse-risk cytogenetics and unfavorable gene mutations. In general, allo HSCT is strongly recommended for this group of patients in first remission. There is an ongoing discussion about the significance of allele burden with respect to *FLT3* ITD mutations. In a German AML Study Group report, the CR rate and OS were adversely affected by increasing burden of *FLT3* ITD mutated AML, as measured by the allelic ratio (AR, defined as ratio of mutated-to-wild type *FLT3* by PCR assay). In fact, in this retrospective analysis, there was no apparent benefit of allo HSCT compared with chemotherapy/auto HSCT for AML patients with *FLT3* ITD AR ≤ 0.5 in first CR. However, there are technical barriers to adopting use of allelic ratio by PCR, and NGS can underestimate the allelic frequency, especially with longer insertions. In the United States, allo HSCT is generally recommended for eligible patients in first CR with unfavorable risk AML including *FLT3* ITD mutated (regardless of allelic ratio); however, risk of relapse following allo HSCT remains high.

The value of intensive consolidation therapy is a matter of debate for older patients with AML. Older patients appear to tolerate intermediate doses of cytarabine, e.g., 6 to 12 doses of 1.5 g/m^2. There is little consensus on the number of post-remission cycles and the required intensity of post-remission therapy. Older AML patients in first CR had similar relapse rate (77% vs. 82%), DFS (11 vs. 10 months), and OS with 4 cycles of cytarabine 100 mg/m^2/day for 5 days versus 2 cycles of cytarabine 0.5 g/m^2 with mitoxantrone 5 mg/m^2 every 12 hours for 3 days. There was a trend for improved DFS and OS for AML patients age 65 years and older in first CR treated with 6 cycles of cytarabine 60 mg/m^2 subcutaneous injection every 12 hours for 5 days and a single dose of idarubicin or daunorubicin administered in the ambulatory setting compared with 1 cycle of intensive chemotherapy similar to induction (idarubicin 9 mg/m^2/day or daunorubicin 45 mg/m^2/day for 4 days with cytarabine 200 mg/m^2 for 7 days) as an inpatient. The patients receiving the ambulatory post-remission therapy spent fewer days in the hospital and required fewer red blood cell and platelet transfusions. It is generally accepted that intensive post-remission consolidation therapy is most likely to benefit older patients with favorable risk AML (CBF AML, *NPM1* mutation without *FLT3*-ITD mutation, and biallelic *CEBPA* mutation). The benefit of intensive consolidation chemotherapy for unfavorable risk AML is doubtful.

Allo HSCT is typically preferred over the use of autologous HSCT (Chapters 105, 106, 107). The benefit of allo HSCT is derived at least in part from the graft-versus-leukemia effect of the donor immune system. Allo HSCT also avoids the potential re-infusion of leukemic cells if autologous stem cells are used as a hematopoietic rescue after high-dose chemotherapy. The allogeneic donor may be HLA-identical, partially HLA-mismatched, or haploidentical. The source of allogeneic stem cells may be a family member (typically first-degree relative), unrelated donor from a registry, or umbilical cord blood. The preparative regimen prior to infusion of the allogeneic stem cells is both immunosuppressive and myelosuppressive. The intensity of the myelosuppressive regimen varies. The choice of a myeloablative versus reduced intensity preparative regimen depends on a number of factors, including patient age, organ function, and disease status (e.g., presence of MRD). Patients with CBF AML do not appear to benefit from allo HSCT in first CR. On the other hand, patients with poor risk cytogenetic or mutational features have better outcomes with allo HSCT in first remission. The debate as to which patients should receive chemotherapy consolidation alone versus allo HSCT is most divisive for patients with intermediate-risk disease. In this population, characterization of the genotype as well as MRD status are helpful in guiding that decision.

Maintenance Therapy

Maintenance therapy (i.e., additional therapy following consolidation) aims to improve the quality of remission, eliminate residual disease, delay relapse, and ideally extend survival. Initial studies focused on stimulation by IL-2 of immune effector cells such as tumor-specific cytotoxic lymphocytes and NK cells. Although IL-2 has demonstrated antileukemic activity in preclinical models, not one study applying low-dose IL-2 (high doses of IL-2 were not feasible because of drug-related toxicities) showed any improvement of DFS or OS. However, the combination of IL-2 plus histamine dihydrochloride did improve leukemia-free survival at 3 years when compared with a randomized observation cohort (40% vs. 26%; $P = .01$). The benefit was restricted to patients in first CR; OS was not extended.

HMA have been studied as maintenance therapy following remission induction, following consolidation therapy, and following allo HSCT. Azacitidine 50 mg/m^2/day for 5 days for up to 12 cycles improved the DFS of older AML patients following 2 cycles of induction therapy compared with observation (12-month DFS

64% vs. 42%); however, there was no improvement in OS with or without censoring at the time of subsequent allo HSCT. The ECOG 2906 study demonstrated improved OS ($P = .06$) with up to 1 year of decitabine maintenance therapy for older patients in first CR. However, the study was terminated before the accrual to the maintenance randomization could be completed. The HMA have also been evaluated as maintenance therapy following allo HSCT; however, the benefit has not yet been established.

CC-486 is an oral formulation of azacitidine with a distinct pharmacokinetic profile from parenteral azacitidine. The QUAZAR AML-001 study was an international, randomized trial of oral azacitidine for 14 consecutive days every 28 days versus placebo as maintenance therapy. Maintenance therapy was continued until relapse or unacceptable toxicity. Eligible patients were age 55 years or older and had achieved first CR with cytarabine- and anthracycline-based induction therapy. Eighty percent of patients had received between 1 and 3 cycles of consolidation. Subjects could not be candidates for allo HSCT in first remission. The median OS was increased from 14.8 months with placebo to 24.7 months with oral azacitidine. The benefit was due to a delay in relapse; the survival at 5 years was similar in the two treatment arms. The survival benefit was observed regardless of MRD status at study registration and number of consolidation cycles. The major toxicities of oral azacitidine included gastrointestinal adverse events (nausea, emesis, and/or diarrhea in 50% or more of subjects, improving over time) and myelosuppression (49% grade 3/4 neutropenia and 23% grade 3/4 thrombocytopenia). CC-486 is being evaluated as a maintenance therapy following allo HSCT.

Recent Advances in Intensive Therapy

Subsets of AML patients have been shown to benefit from specific modifications of intensive therapy. Currently, these subsets and modifications are defined by mutations (midostaurin for *FLT3* ITD or TKD mutations), clinical features (CPX-351 for AML-MRC and t-AML), and cytogenetic features (GO for CBF translocations). Clinical trials are evaluating the addition of investigational agents to intensive therapy in other subgroups, e.g., Menin inhibitors in *KMT2A* rearranged and *NPM1* mutated AML, and KIT inhibitors in CBF AML. Enasidenib and ivosidenib have been approved for the treatment of *IDH*-mutated AML. Identification of these patients requires knowledge of cytogenetic and molecular features at the time of AML diagnosis. However, cytogenetic and mutational assays currently require more time to complete than the assays for confirming the AML diagnosis (morphology and flow cytometry). There has been concern that this could result in a delay from diagnosis to therapy that may adversely affect survival. Two retrospective analyses have not shown any effect of the time from diagnosis to treatment on early mortality, remission rate, or OS. It is expected that advances in whole genome sequencing assays will shorten the turnaround time even further; the cost of NGS assays will likely continue to decrease over time as well.

Midostaurin for *FLT3*-mutated AML

Midostaurin (previously known as PKC412) is an inhibitor with a broad spectrum of activity against many protein kinases. Midostaurin is a type I inhibitor of FLT3 with activity against both ITD and TKD mutations as well as unmutated FLT3. As a single agent, midostaurin has minimal clinical activity in *FLT3*-mutated AML. Pre-clinical studies suggested synergistic activity of midostaurin in combination with chemotherapy. A phase IB study defined the optimal dosing schedule of midostaurin with 7+3, demonstrated an acceptable safety profile of the combination, and showed encouraging activity of midostaurin added to 7+3 in patients with *FLT3*-mutated AML.

The RATIFY trial, an international, double-blind, placebo-controlled, randomized phase III trial, established the benefit of adding midostaurin to induction and consolidation chemotherapy for *FLT3*-mutated AML patients ages 18 to 59 years. At preregistration, 3277 potential subjects were screened for *FLT3* ITD and tyrosine kinase domain (TKD) mutations by a PCR-based assay, and 717 *FLT3*-mutated AML patients (allelic ratio ≥0.05) were randomly assigned 1:1 to 7+3 induction and up to 4 cycles of HiDAC consolidation with either midostaurin or placebo. Midostaurin 50 mg was administered orally every 12 hours on days 8 to 21 of each cycle of induction and consolidation; placebo was given in the same dosing schedule as midostaurin. If in remission, patients received maintenance therapy with daily midostaurin or placebo for 1 year according to their initial treatment assignment. The primary endpoint of the study was reached. OS was significantly longer with the addition of midostaurin; the hazard ratio for death was 0.78, indicating a 22% reduction in the risk of death ($P = .009$). EFS was also significantly longer despite no apparent difference in the CR/Cri rate. At 4 years, 51% of patients were still alive in the midostaurin group compared with 44% with placebo. The survival benefit was observed in *FLT3* ITD high allelic ratio, low allelic ratio, and *FLT3* TKD subsets. The toxicities were similar except for higher rate of anemia and rash with midostaurin. Allo HSCT was performed in first remission in one quarter of study patients (similar number in both arms). The median OS had not been reached in either group, but there was a trend for improved survival with allo HSCT in the midostaurin compared with placebo group.

On April 28, 2017, the US FDA approved midostaurin in combination with daunorubicin and cytarabine induction and cytarabine consolidation therapy for newly diagnosed adult AML patients with *FLT3* ITD or TKD mutations. Based on the trial design, the independent benefit of midostaurin maintenance could not be established. Midostaurin became the first of nine drugs to be approved by the U.S. FDA between 2017 and 2021 for AML patients. Currently, more selective, second-generation FLT3 inhibitors (quizartinib, gilteritinib, and crenolanib) are being evaluated in phase III studies in combination with initial induction and consolidation therapy.

CPX-351 for t-AML and AML-MRC

CPX-351 is a liposomal formulation of cytarabine and daunorubicin at a fixed 5:1 molar ratio within 100-nm bilamellar liposomes. This formulation allows preferential delivery to the bone marrow and leukemic blasts of a maximally synergistic drug ratio that may enhance treatment efficacy. In a phase II randomized study of CPX-351 versus 7+3 in older patients with untreated AML, CPX-351 achieved significantly higher remission rates than standard 7+3; however, the EFS and OS were not better. However, in the subgroup with secondary AML, both EFS and OS were superior with CPX-351. This observation led to a pivotal, prospective, randomized phase III study of induction and consolidation with CPX-351 versus 7+3 followed by 5+2 in treatment-naïve AML patients ages 60 to 75 years and fit for intensive chemotherapy. The patients had either an antecedent MDS or CMML, prior leukemogenic therapy, or de novo AML with poor risk karyotype. The CR+Cri rate was higher with CPX-351 (47.4% vs. 33.3%, $P = .016$) without an increase in induction mortality. The median OS improved from 5.95 years with 7+3 to 9.56 years with CPX-351 (hazard ratio 0.69, $P = .003$). The survival benefit was observed even in patients ages 70 to 75 years. More subjects were able to proceed with allo HSCT following initial therapy with CPX-351 compared with 7+3. A survival analysis from the time allo HSCT favored CPX-351. CPX-351 was associated with delay in both neutrophil and platelet recovery in remission patients compared with those treated with 7+3. The 5-year survival rate was superior for patients treated with CPX-351 versus 7+3 (18% vs. 8%) as well as those patients undergoing allo HSCT (52% vs. 23%). Remarkably, the clinical benefit of CPX-351 over 7+3 chemotherapy was observed even though the total cumulative doses of both daunorubicin and cytarabine were lower with CPX-351, supporting the hypothesis that the fixed molar ratio as well as liposomal formulation targeting the marrow may be critical features.

On August 3, 2017, the US FDA approved liposomal daunorubicin/cytarabine for adults with newly diagnosed t-AML or AML-MRC. On March 30, 2021, the FDA expanded the approved population to patients with t-AML and AML-MRC ages 1 year and older based on safety data gathered from two studies including pediatric patients.

Addition of Gemtuzumab Ozogamicin to Intensive Therapy

CD33 is a cell surface antigen that is present in more than 80% of patients with AML but is absent from pluripotent hematopoietic stem cells. GO is a humanized anti-CD33 monoclonal antibody covalently linked to the DNA-binding cytotoxin, calicheamicin. As a single agent in relapsed/refractory AML patients, nearly 30% achieved either CR or CRp, resulting in accelerated approval by the U.S. Food and Drug Administration (FDA) in 2000. The major toxicities of GO are infusion reactions, myelosuppression, and hepatic toxicity, especially hepatic veno-occlusive disease / sinusoidal obstructive syndrome (VOD/SOS). The risk of VOD/SOS was shown to be related to dose (initially approved at 9 mg/m^2), schedule, and temporal proximity to allo HSCT especially with a myeloablative preparative regimen. In the randomized trial SWOG S0106, the addition of GO 6 mg/m^2 to 7+3 induction therapy failed to improve the survival of previously untreated younger AML patients. Induction mortality was also higher in the GO treatment arm. The results of the SWOG S0106 study led the FDA to revoke the approval in 2010, and GO was voluntarily withdrawn from the US market.

The ALFA 0701 study randomly assigned AML patients ages 50 to 70 years to receive or not a fractionated schedule of GO (3 mg/m^2 on days 1, 4, and 7) with 7+3 induction. The GO dose was capped at 5 mg (a single vial, which is now felt to deliver only 4.5 mg). Patients in remission received 2 cycles of consolidation with daunorubicin and cytarabine 1000 mg/m^2 every 12 hours on days 1 to 4. If randomly assigned to the GO arm, the patient also received a single dose of GO 3 mg/m^2 with each cycle. Hematologic toxicity (especially thrombocytopenia) was increased with GO but without increase in early mortality. There were three cases of hepatic VOD/SOS among the 139 patients in the GO arm. There was no difference in CR rate. The 2-year RFS was superior with the addition of GO to intensive induction and consolidation therapy (50.3% vs. 22.7%, $P = .0003$). At 5 years, there remained a significant improvement in EFS with GO; there was a trend for improved OS with GO.

A meta-analysis of 3325 patients treated in five randomized trials evaluating GO in combination with intensive chemotherapy (including SWOG S0106 and ALFA 0701) confirmed the potential benefit of GO in AML therapy. GO did not increase the remission rate. The 5-year OS is significantly improved by GO (34.6% vs. 30.7%, $P = .01$) due to reduction in the relapse rate. The survival benefit is most obvious in patients with favorable-risk CBF karyotypes (76.3% vs. 55.2%, $P = .0005$). There was also a benefit in the intermediate cytogenetic risk group (39.4% vs. 34.1%, $P = .007$). There was no benefit in the adverse cytogenetic risk group. Doses of 3 mg/m^2 are as effective as higher doses and were associated with fewer early deaths.

A single dose of GO 3 mg/m^2 was added to 1 cycle of induction and 1 cycle of intensification (consolidation) therapy for children, adolescents, and young adults with AML in the Children's Oncology Group AAML0531 study. GO improved the EFS and DFS due to a reduction in the risk of relapse; OS was not improved. A controversial correlate of this study was the demonstration that a CD33 gene splice site polymorphism, resulting in loss of the epitope for GO binding, negated the benefit of GO, even if the polymorphism was heterozygous.

Since CBF AML patients do not typically undergo allo HSCT in first CR, the risk of hepatic VOD/SOS is of lesser concern. The situation is more controversial in *NPM1* mutated patients. In the prospective, randomized AMLSG 09-09 trial, GO 3 mg/m^2 was added on day 1 of induction therapy with idarubicin, cytarabine, etoposide, and ATRA and day 1 of the first of 3 planned cycles of high-dose cytarabine and ATRA consolidation. Nearly 90% of the patients had de novo AML with normal karyotype; 75% were in the favorable ELN risk group. There was no benefit in the primary study endpoint, EFS. However, the cumulative incidence of relapse was significantly lower in the CR patients who had received GO. The benefit was offset by the higher early mortality (10.3% with GO vs. 5.7% without GO, $P = .05$), predominantly due to infection.

Based on the results of the meta-analysis as well as a trial of GO in older AML patients unfit for intensive chemotherapy (see Less Intensive Therapy), on September 1, 2017, the US FDA granted approval of GO in combination with daunorubicin and cytarabine for adults with newly diagnosed CD33 positive AML or for patients ages 2 years and older with relapsed/refractory AML. NCCN guidelines recommend the addition of GO to chemotherapy for CBF AML. Although the dose of GO with cytotoxic chemotherapy is capped at 3 mg/m^2 (4.5 mg maximum), the optimal GO schedule (one dose vs. three doses during induction therapy, number of doses over course of initial therapy) remains uncertain.

Less Intensive Therapy

The OS of AML patients is improved with therapy based on retrospective registry studies. LoDAC has improved the survival of older, unfit AML patients compared with BSC alone. The phase III EORTC-GIMEMA AML 19 trial demonstrated the survival benefit associated with single agent GO compared with BSC alone. Treatment-naïve AML patients ages 61 years and older were randomly assigned to receive GO 6 mg/m^2 on day 1 and 3 mg/m^2 on day 8 versus BSC alone. Patients achieving CR could receive up to 8 monthly doses of GO 2 mg/m^2. The median OS was significantly improved from 3.6 months with BSC to 4.9 months with GO. The benefit was restricted to patients without an unfavorable karyotype and those with higher CD33 expression. However, median survival remained very short (5 to 10 months) for older, unfit AML patients treated with LoDAC, GO, or HMA.

The cytotoxic agents, laromustine (an alkylating agent) and clofarabine, were evaluated in phase II studies of older AML patients felt to be unfit for intensive therapy. Clofarabine is a purine analog with activity in both AML and ALL patients; clofarabine is approved by the U.S. FDA for pediatric patients with relapsed/refractory ALL. The phase II study of clofarabine enrolled 112 treatment naïve AML patients age 60 and over with at least one of the following poor risk features: age ≥70 years, performance status 2, antecedent hematologic disorder, and intermediate or unfavorable risk. The 30-day mortality was 9.8%, overall response rate 46%, and median survival 41 weeks. The potential benefit of these two novel cytotoxic agents in these phase II studies was difficult to discern by comparison with historical controls; many of the subjects ultimately received intensive chemotherapy, further blurring the definition of the population. Clofarabine doubled the overall response rate compared with LoDAC (38% vs. 19%) in a randomized phase III study of 406 older AML (and high-risk MDS) patients unfit for intensive chemotherapy, but there was no improvement in OS. On the other hand, the survival of older patients, fit for intensive therapy, treated with clofarabine induction and consolidation was worse than those patients treated with 7+3 followed by HiDAC consolidation.

The outlook for older AML patients, unfit for intensive therapy, became brighter with the accelerated approval of the B-cell lymphoma-leukemia-2 (BCL2) inhibitor, venetoclax, in combination with azacitidine, decitabine, or LoDAC by the US FDA on November 21, 2018. BCL2 is an anti-apoptotic protein that is over-expressed in cells from patients with hematologic malignancies including AML. Earlier clinical trials investigating other BCL2 inhibitors failed to demonstrate any clinical benefit. Even venetoclax has limited single agent activity in AML patients. Thirty-two AML patients (30 relapsed/refractory, 2 treatment-naïve) were treated with venetoclax 800 mg orally once daily. The overall response rate was 19% (2 patients with CR, 4 with Cri). Among the 12 patients with *IDH1* or *IDH2* mutation, 4 (33%) achieved CR+Cri. The activity of venetoclax in *IDH*-mutated AML may be due to suppression of cytochrome c oxidase activity, which may lower the threshold for induction of apoptosis by BCL2 inhibition.

The concomitant inhibition of DNA methyltransferase and BCL2 synergistically induces apoptosis of AML cell lines. Based on this observation, as well as the need to improve outcomes for older AML patients being treated with HMA alone, a phase Ib study of either azacitidine or decitabine (physician choice) with venetoclax in 145 treatment-naïve, older, unfit AML patients was performed. The standard doses and schedules of azacitidine or decitabine were combined with venetoclax orally once daily on days 1 to 28 of a 28-day cycle. There was a short ramp-up of the venetoclax dose during cycle 1 to mitigate the risk of TLS. There was no laboratory or clinical evidence of TLS; however, patients were required to have a WBC count less than 25,000/μL (hydroxyurea could be used) at study entry and received TLS prophylaxis with allopurinol and IV fluids as an inpatient for the first cycle. The gastrointestinal toxicity was generally mild and did not result in discontinuation from study. The most common adverse events were cytopenias and infections, as expected in this population. The most common cause of dose interruption was neutropenia. The 30- and 60-day mortality were 5% and 11%, respectively. Based on hematologic and gastrointestinal toxicities with higher doses, the recommended venetoclax dose in combination with HMA was 400 mg once daily.

The overall response rate including CR, Cri, PR, and MLFS was 83%, and the median OS 17.5 months for the entire study population. The median time to first response was only 1.2 months. The CR+Cri rate was 73% in those patients treated at venetoclax 400 mg daily with HMA; response rates were similar with azacitidine and decitabine. The median OS for azacitidine with venetoclax 400 mg daily had not been reached; the median OS for decitabine with venetoclax 400 mg daily was 14.2 months. The CR+Cri rate was 67% in both the de novo and secondary AML subsets; however, it should be noted that patients with an antecedent myeloid neoplasm could not have received an HMA prior to study entry. Even in the *TP53*-mutated subset, the CR+Cri rate was 47%, although the median duration of response was only 5.6 months and median OS only 7.2 months. On the other hand, more than 72% of patients with *FLT3* mutation responded. The *NPM1* mutation predicted superior outcomes in multivariate analysis; the CR+Cri rate was 92% in *NPM1* mutated AML, and median OS had not been reached.

The results from this multicenter phase Ib study compared favorably with the prior reports of HMA alone in this population. An international, randomized, placebo-controlled, double-blind phase III study was launched comparing azacitidine 75 mg/m^2/day for 7 days with either venetoclax 400 mg orally once daily on days 1 to 28 with dose ramp-up during the first 3 days of cycle 1 of a 28-day cycle or placebo in the same schedule. Subjects were randomly assigned 2:1 to azacitidine/venetoclax (AZA/VEN, n = 286) or azacitidine/placebo (AZA/PLA, n = 145). The dose of venetoclax was increased from 100 mg on day 1, to 200 mg on day 2, to the final dose of 400 mg daily during cycle 1. Eligible patients had to be either 75 years of age or older or have a significant comorbidity precluding the use of intensive chemotherapy (congestive heart failure, left ventricular ejection fraction 50% or less, unstable angina, diffusing capacity of the lung for carbon monoxide 65% or less, forced expiratory volume in 1 second 65% or less, or ECOG performance status 2 or 3). The WBC count had to be less than 25,000/μL at study entry. Patients with a favorable risk karyotype, and those receiving prior HMA or other chemotherapy for MDS were excluded. The primary endpoint of the study was reached. The median OS was 14.7 months with AZA/VEN and 9.6 months with AZA/PLA; the hazard ratio for death was 0.66. Key secondary endpoints for AZA/VEN versus AZA/PLA were CR+Cri rate 66.4% versus 28.3%, median time to first response 1.3 versus 2.8 months, median duration of CR+CRi 17.5 versus 13.4 months, and CR+CRh 64.7% versus 22.8%, respectively. Remarkably, the rate of CR+Cri and the median OS in the control arm were very similar to these outcomes with azacitidine alone in the AML-001 study a decade. Thirty-day mortality was similar in the two groups (7% AZA/VEN vs. 6% AZA/PLA). Three patients (less than 1%) treated with AZA/VEN had laboratory evidence of TLS. There were no new safety signals with hematologic and mild gastrointestinal adverse events being the most common. Importantly, more patients required dose interruptions due to neutropenia in the AZA/VEN arm.

Venetoclax was also evaluated in combination with LoDAC in 82 treatment-naïve AML patients unfit for intensive therapy. In this phase I/II study, venetoclax 600 mg once daily on days 1 to 28 with dose ramp-up in the first 4 days was determined to be the optimal dose in combination with cytarabine 20 mg/m^2 by subcutaneous injection once daily on days 1 to 10 (LoDAC/VEN). Treatment was continued every 28 days until disease progression or unacceptable toxicity. TLS precautions were identical to those of the HMA/VEN study. Patients previously treated with HMA for MDS were eligible (29% had prior HMA); otherwise, the inclusion criteria were similar to those of the HMA/VEN phase I/II study. The median age was 74 years (range, 57 to 90 years old). The CR/CRi rate was 54% for the entire population, and 62% for those without prior HMA exposure. The median time to first CR or CRi was only 1.4 months. The median OS was 10.1 months. The toxicities were similar to those of the HMA/VEN combination.

Treatment-naïve AML adult patients unfit for intensive chemotherapy were treated with LoDAC with either venetoclax 600 mg or placebo once daily on day 1 to 28 in an international, randomized, double-blind, phase III study. There was a 25% reduction in the risk of death with LoDAC/VEN (P = .11). With further follow up, the improvement in survival became statistically significant (median OS, 8.4 vs. 4.1 months, P = .04). The response rate was higher with LoDAC/VEN (CR/Cri, 48% vs. 13%) in the entire population, as well as across cytogenetic and mutational subsets. Patients were more likely to become transfusion independent with LoDAC/VEN. The 30-day mortality was similar in both treatment arms (13% LoDAC/VEN, 16% LoDAC/PLA).

The combinations of azacitidine, decitabine, or LoDAC with venetoclax are the current standard of care for older AML patients unfit for intensive therapy. There are several important management issues to consider, including drug-drug interactions, TLS management, and management of the expected myelosuppression, that are beyond the scope of this chapter. Practical advice regarding management of AML patients receiving HMA or LoDAC with venetoclax has been published by investigators with extensive experience with these regimens. Some have argued that the HMA/VEN combination should become the new standard of care for all older adults with AML regardless of fitness. For example, the outcomes of patients treated with either decitabine (10 days during induction, 5 days with subsequent cycles) plus venetoclax (DEC/VEN) or intensive chemotherapy including cytarabine ≥1 g/m^2/dose (IC) were compared. The patients were matched by a treatment-related mortality score and other prognostic features. The median age was 72 years in the DEC/VEN cohort (range 69 to 78 years) and 73 years (67 to 76 years) in the IC group. The DEC/VEN combination was associated with higher rate of CR+CRi (81% vs. 52%), lower relapse rate (34% vs. 56%), lower 30-day mortality (1% vs. 24%), and longer median OS (12.4 vs. 4.5 months). The superior outcomes with DEC/VEN compared with IC were also seen in those patients with low treatment-related mortality scores. Nonetheless, there are older AML patients who may be cured of their disease with a time-limited IC approach.

The Hedgehog pathway is important in normal embryonic development. The Hedgehog pathway proteins have been shown to be overexpressed in AML, and activity of the Hedgehog pathway appears to enhance the survival of leukemic stem cells. Therefore, the Hedgehog pathway has been identified as a potential therapeutic target in AML. Glasdegib is an orally bioavailable antagonist of Smoothened, a cell surface protein in the Hedgehog pathway. Glasdegib demonstrated an acceptable safety profile and preliminary evidence of efficacy as a single agent or in combination with chemotherapy in patients with myeloid neoplasms.

The combination of LoDAC with glasdegib 100 mg orally once daily continuously was evaluated in patients with AML (and high-risk MDS) age 55 years and older, and not candidates for intensive therapy based on age 75 years or older or specified organ dysfunction. Subjects were randomly assigned 2:1 to the combination versus LoDAC alone. The assignment was not blinded, and there was no placebo. The median survival was improved from 4.9 months with LoDAC alone to 8.8 months with LoDAC/glasdegib. The survival benefit was only observed in those with intermediate risk karyotype, not adverse risk karyotype. The CR rate was 17.0% with LoDAC/glasdegib versus 2.3 % with LoDAC alone. Subjects receiving glasdegib were more likely to report alopecia, muscle cramps, dysgeusia, anorexia, nausea, emesis, and constipation; they were also more likely to report grade 3/4 fatigue.

Based on the results of this randomized phase II study, on November 21, 2018, the US FDA approved glasdegib in combination with low-dose cytarabine for treatment-naïve AML patients age 75 years or older or with comorbidities precluding use of intensive chemotherapy.

The IDH1 inhibitor ivosidenib and IDH2 inhibitor enasidenib have been approved for relapsed / refractory, *IDH*-mutated AML (see section of Salvage Therapy for Relapsed/Refractory AML). However, both were evaluated as initial therapy for patients unfit for intensive therapy in the phase I study that resulted in initial FDA approval. Thirty-four patients with *IDH1*-mutated AML were treated in a cohort of the phase I study at the maximally tolerated dose, 500 mg orally once daily. The median age of this patient cohort was 76.5 years, 76% had secondary AML, and 47% had received a prior HMA for an antecedent hematologic disorder. The CR+CRh rate was 42.4%, and median OS 12.6 months. Ivosidenib 500 mg daily continuously was evaluated in combination with azacitidine 75 mg/m^2/day for 7 days every 28 days in 23 adults with newly diagnosed, *IDH1* mutated AML ineligible for intensive therapy (median age 76, range 61 to 88 years). The overall response rate was 78.3%, and CR rate 60.9%. The 12-month survival was 82%.

Thirty-nine patients with *IDH2*-mutated AML were treated in a cohort of the phase I study at the maximally tolerated dose, 100 mg of enasidenib orally once daily. The median age was 77 years, and 59% had an antecedent hematologic disorder. The overall response rate was 30.8%, CR rate 18%, and median survival 11.3 months. Older, treatment naïve, *IDH2*-mutated AML patients ($n = 60$) were treated with single agent enasidenib in a sub-study of the BEAT AML program. The CR+Cri was 47% (37% CR), and median survival was 24.4 months. Azacitidine was added to the enasidenib in 17 patients with inadequate response following 5 cycles of enasidenib monotherapy; 41% achieved CR or Cri. The combination of azacitidine and enasidenib (AZA/ENA) was compared with azacitidine alone in a randomized, open label study of newly diagnosed *IDH2*-mutated AML patients ineligible for intensive chemotherapy (median age 75, range 57 to 85 years). The overall response rate (CR+Cri+PR+MLFS) was significantly higher with AZA/ENA compared with AZA alone (71% vs. 42%). The CR rate was also higher (53% vs. 12%), and the median duration of response was longer (24 vs. 12 months) with AZA/ENA versus AZA alone.

The toxicities of ivosidenib and enasidenib (including IDH Differentiation Syndrome [DS]) in newly diagnosed older AML patients were similar to those observed in relapsed/refractory AML. On May 2, 2019, the US FDA approved ivosidenib for newly diagnosed, *IDH1*-mutated AML patients age 75 years or older or those with comorbidities that preclude intensive chemotherapy. The NCCN AML Practice Guidelines list ivosidenib and enasidenib as therapeutic options for newly diagnosed, *IDH1*- and *IDH2*-mutated AML patients who are not candidates for intensive therapy.

Salvage Therapy for Relapsed/Refractory Acute Myeloid Leukemia

Most patients with AML relapse and do so within the first year following achievement of remission. In an analysis of 1069 consecutive AML patients in first CR who were treated and followed at the MD Anderson Cancer Center, the risk for treatment failure during the first year was 69%, 38% in the second year, 17% in the third year, and less than 10% in both the fourth and fifth years. The risk of AML relapse also declines over time following allo HSCT. In an analysis of 1265 patients with relapsed AML following allo HSCT, the median time to relapse was 6.1 months, and less than one per 100 patient-years relapsed after 3 years. The survival following AML relapse post allo HSCT was 19% at 2 years. It is reasonable to consider patients cured if in first CR for at least 3 years.

The prognosis for AML patients following relapse remains poor, but outcomes are variable. An important predictor for prognosis is the duration of first remission. In a study of 243 patients with AML in first relapse, the likelihood of CR was 60% if the duration of first remission exceeded 2 years and 19% if it was shorter than 1 year. In a multivariate analysis of 667 adult patients less than age 60 years in first relapse, a prognostic score was derived from four clinical parameters (duration of first remission, karyotype at diagnosis, age at relapse, and previous HSCT or not). The model identified three risk groups: (a) favorable group: CR 85% and 5-year OS 46%; (b) intermediate group: CR 60% and 5-year survival 34%; (c) poor risk group: CR 18% and 5-year survival 4%. The largest group of patients had poor risk disease (455 of the 667 patients). In another prognostic model for relapsed/refractory AML, first remission less than 12 months, *FLT3* ITD mutation, and high-risk cytogenetics were independent adverse prognostic factors for OS and EFS. Three subgroups with different outcomes at 2 years were identified: (a) favorable (no adverse features): OS 58%, EFS 45%; (b) intermediate (1 adverse factor): OS 37%, EFS 31%; (c) poor (≥2 adverse factors): OS 12%, EFS 12%. Prognosis is worse for patients after more than one salvage attempt; 1-year survival probabilities vary between 2% and 24%. Patients with primary refractory disease following at least 1 cycle of HiDAC-based induction therapy do equally poorly, with a subsequent CR rate of 22%, median CR duration of around 9 months, and survival of 3.8 months. The outcome of patients with relapse following HMA plus venetoclax is also very poor. The median survival of patients with either relapse following or refractory to first-line HMA plus venetoclax was only 2.4 months.

The only potentially curative therapeutic approach for patients with rel/ref AML is to recapture a remission with sufficient reduction of disease burden to then proceed with allo HSCT. For example, 66% of 1535 AML patients treated with intensive chemotherapy alone relapsed. A second CR could be achieved in half, and 3-year OS was 30%. Achievement of second CR, salvage allo HSCT, and first remission duration ≥1 year were independent prognostic factors. Allo HSCT in second CR was associated with superior 3-year survival compared with no HSCT (44% vs. 14%, $P = .001$). The Center for International Blood and Marrow Transplant Research (CIBMTR) evaluated the outcome of 1673 AML patients following allo HSCT for primary induction failure and first relapse. The 3-year OS was 19%. Five adverse risk factors were identified: first CR duration less than 6 months, peripheral blood blasts, donor other than HLA identical sibling, Karnofsky score less than 90%, and adverse risk karyotype. The 3-year OS was 42% if the score was 0, but only 6% for score ≥3. Those with primary refractory AML had a 3-year survival of 16%. Nonetheless, outcomes remain superior to non-transplant therapies across all scenarios.

AML refractory to initial intensive chemotherapy is not uncommon. In an analysis of 3324 adult AML patients treated with idarubicin, cytarabine, and etoposide induction therapy between 1993 and 2009, 31% had primary refractory disease; 845 had disease refractory to the first cycle, and another 180 relapsed after the second induction cycle. The CR/CRi rate was highest in those proceeding directly to allo HSCT compared with intensive chemotherapy. However, the 5-year survival was best for those undergoing allo HSCT in remission (48%) compared with those who proceeded to HSCT directly (36%) or with persistent AML after salvage chemotherapy (25%).

There is no one standard salvage chemotherapy regimen. An intensive chemotherapy regimen is most likely to benefit patients with favorable prognostic features at relapse. Commonly used intensive regimens include combinations of high-dose cytarabine, etoposide, idarubicin, and mitoxantrone. The response rates observed in the early reports of HiDAC-based salvage regimens such as mitoxantrone, etoposide, and cytarabine (MEC) were encouraging, but likely affected by the absence of high-dose cytarabine in the initial therapy of AML patients prior to 1990. The purine analogs (fludarabine and cladribine) administered prior to high-dose cytarabine will increase the intracellular accumulation of ara-CTP. This pharmacokinetic interaction has formed the basis of regimens such as cladribine, cytarabine, G-CSF, and mitoxantrone (CLAG-M) and fludarabine, cytarabine, G-CSF, and idarubicin (FLAG-Ida). Intensive regimens are associated with significant mucositis and treatment-related mortality. The goal of intensive chemotherapy must be carefully considered.

Although randomized clinical trials have been completed comparing different salvage chemotherapy regimens, none of these studies have resulted in the adoption of a clearly superior salvage regimen as standard of care. The outcomes reported in these clinical trials are more likely related to the patient characteristics (e.g., first CR duration, number of prior relapses, age, prior or subsequent allo HSCT, cytogenetics) than to any substantial differences in the biologic activity of the chemotherapy regimens. One international, multicenter trial randomized 326 patients ≥55 years of age with relapsed and refractory AML to cytarabine 1 g/m^2 daily for 5 days with or without clofarabine 40 mg/m^2/day for 5 days. CR rate and EFS were superior in the combination arm, but survival was not improved. In another multicenter study 711 adult patients with first relapse or refractory AML were randomly assigned to cytarabine 1 g/m^2 daily for 5 days with or without vosaroxin, a first-in-class anticancer quinolone derivative. There was no increase in early mortality, and the combination arm achieved significantly higher CR rates (30.1% vs. 16.3%, $P = .00001$). There was a trend for a survival benefit that appeared significant for those subjects age ≥ 60 years and those with early relapse. The apparent response benefit with the addition of either clofarabine or vosaroxin was questioned, since many leukemia experts felt the control arm was a suboptimal salvage regimen.

Although currently only approved for treatment-naïve AML patients unfit for intensive therapy, HMA with the BCL2 inhibitor venetoclax has been evaluated as a salvage regimen for patients with relapsed/refractory AML. Fifty-five patients with relapsed/refractory AML were treated with decitabine 20 mg/m^2 daily for 10 days (the duration was decreased to 5 days with subsequent cycles) with venetoclax 400 mg once daily for 28 days. The median age was 62 years, and 35% were age ≥70 years. The median number of prior therapies was 2 (range, 1 to 3). Seventy-six percent had received prior intensive chemotherapy, 22% had received both intensive chemotherapy and HMA, and 33% had prior allo HSCT. The rate of CR+CRi was 42%; another 18% achieved MLFS. Fourteen of 36 patients with adequate marrow samples were negative for MRD by flow cytometry. The median survival was 7.8 months.

CD33 is a cell surface antigen present on AML blasts in more than 80% of patients but is absent from pluripotent hematopoietic stem cells. GO is a humanized, murine anti-CD33 monoclonal antibody covalently linked to the DNA-binding cytotoxin, calicheamicin. The antibody-drug conjugate is internalized upon binding to CD33, transported to the lysosome, where the linker is hydrolyzed, releasing calicheamicin. Calicheamicin then binds to double-strand DNA, causing double-strand DNA breakage and apoptosis. Patients with AML in first relapse ($n = 277$) were treated with GO 9 mg/m^2 on three separate phase II studies. The rate of CR+CRp was 26%. Sixteen percent died within 30 days of GO. Infusion reactions, myelosuppression, and hepatic toxicity were among the most frequently observed toxicities. Hyperbilirubinemia was observed in 29%, and 5% of patients developed hepatic SOS. However, only 0.9% developed hepatic SOS, if they did not previously or subsequently undergo allo HSCT. Hepatic SOS developed in 8 of 46 patients who underwent allo HSCT in second CR after GO; 5 of 27 patients with relapsed AML following prior HSCT developed SOS with salvage GO. GO has been safely combined at lower doses (3 mg/m^2) with salvage chemotherapy. However, it is not clear how to best incorporate GO into the treatment of patients with relapsed/refractory AML.

The first-generation FLT3 inhibitors have minimal efficacy in *FLT3*-mutated AML as single agents. The first-generation, type I FLT3 inhibitor lestaurtinib was evaluated in combination with intensive salvage chemotherapy (MEC or HiDAC) in a randomized trial of patients with *FLT3*-mutated AML in first relapse. There was no benefit of lestaurtinib in terms of response or OS. However, it is instructive to note the low response rates in either arm (CR+CRp: 21% with chemo alone versus 26% with chemo plus lestaurtinib). Increased production of the FLT3 ligand as well as variability in the lestaurtinib plasma concentration (as measured by a pharmacodynamic plasma inhibitory assay) may account for the lack of observed benefit.

Higher single-agent activity (including objective remissions) has been observed with the more potent and selective second-generation FLT3 inhibitors, quizartinib, gilteritinib, and crenolanib. For example, 194 patients with relapsed/refractory *FLT3*-mutated AML received single agent gilteritinib on a phase I/II study. Gilteritinib demonstrated potent inhibition of the FLT3 tyrosine kinase at doses ≥80 mg once daily. The rate of CR+Cri+CRp in patients receiving greater than 80 mg daily was 41%. Gilteritinib was well tolerated; the adverse event profile was as expected in patients with active AML. Four percent developed QTc prolongation greater than 500 ms. There have been reports of DS with gilteritinib. Based on the efficacy, toxicity, and pharmacodynamic data, the recommended phase II dose was 120 mg once daily.

Patients with first relapse or primary refractory *FLT3*-mutated AML were randomly assigned to single agent gilteritinib 120 mg orally once daily ($n = 247$) versus salvage chemotherapy ($n = 124$). The investigator could treat the patient with intensive chemotherapy (FLAG-IDA, MEC) or less intensive therapy (azacitidine or LoDAC). The primary endpoint of the study was achieved; the median survival was longer with gilteritinib (9.3 vs. 5.6 months, hazard ratio for death = 0.64, $P = .001$). More patients achieved a remission with gilteritinib: CR 21.1% versus 10.5%; CR+CRh 34.0% versus 15.3%; CR+CRi+CRp 54.3% versus 24.8. More patients were able to undergo allo HSCT following gilteritinib (25.5% vs. 15.3%). Those patients who restarted gilteritinib following allo HSCT had a better survival than those who did not. Based on the results of the ADMIRAL trial, on November 28, 2018 the U.S. FDA approved gilteritinib for patients with relapsed or refractory *FLT3*-mutated AML.

Quizartinib is a second-generation, type II FLT3 inhibitor. Quizartinib only inhibits FLT3 with an ITD mutation, not TKD-mutated *FLT3*. A series of phase I and II studies established an effective and safe dose of quizartinib. Quizartinib was compared with salvage chemotherapy for patients with first relapse or primary refractory *FLT3* ITD mutated AML. The median OS was longer with quizartinib: 6.2 versus 4.7 months ($P = .02$). Although not approved in the United States, quizartinib has been approved in Japan. Crenolanib, a second-generation, type I FLT3 inhibitor, is clinically effective for *FLT3* ITD- and D835-mutated and is being evaluated in both treatment-naïve and relapsed/refractory *FLT3*-mutated AML.

FLT3 mutations may be acquired at the time of disease relapse. Presentation with leukocytosis at the time of relapse, especially if not present at initial diagnosis or previous relapse, may indicate the acquisition of a *FLT3* mutation by the leukemic progenitor cells. Therefore, it is strongly recommended to evaluate for *FLT3* mutation at the time of AML relapse or for chemotherapy-refractory AML. Resistance to FLT3 inhibitors is not uncommon. *FLT3* ITD-mutated AML patients treated with a type II FLT3 inhibitor frequently have *FLT3* TKD mutations at the time of resistance. On the other hand, mutations in the *RAS/MAPK* pathway are associated with resistance to the type I FLT3 inhibitors.

Somatic mutations in the genes for isocitrate dehydrogenase isoforms 1 and 2 (IDH1 and IDH2) are gain of function mutations that result in accumulation of the oncometabolite R-2-hydroxyglutarate. The mutations are identified in 20% of AML patients and are typically mutually exclusive; *IDH2* mutations are more common. R-2-hydroxyglutarate (2-HG) is a competitive inhibitor of several histone and DNA demethylating enzymes. High levels of 2-HG lead to epigenetic changes and impaired cellular differentiation. Ivosidenib is a selective, potent, and orally bioavailable inhibitor of IDH1 with an R132 missense mutation. Enasidenib is a selective, potent, and orally bioavailable inhibitor of mutant IDH2 with either R140 or R172 missense mutation. Ivosidenib and enasidenib have minimal activity against the unmutated IDH1 and IDH2, respectively.

Enasidenib was evaluated in a phase I/II study of 176 relapsed/refractory, *IDH2*-mutated AML. The overall response rate (CR + Cri + PR = MLFS) was 40.3%, and the CR rate was 19.3%. The median time to first response was 1.9 months; 87% achieved the first response by cycle 5. The median response duration was 5.8 months, median OS was 9.3 months, and the 12-month OS was 39%. The most notable toxicities were DS (10%), leukocytosis, and indirect hyperbilirubinemia. The approved enasidenib dose is 100 mg orally once daily. Ivosidenib was evaluated in a separate phase I/II study of 179 relapsed/refractory, *IDH1*-mutated AML. Among the 125 patients receiving the recommended phase II dose (500 mg orally once daily), CR or CRh was achieved in 30.4% with CR in 21.6%. Clearance of the *IDH1* R132 variant by PCR analysis was achieved in 7 of the 34 CR/CRh patients. The median duration of CR or CRh was 8.2 months. The most notable toxicities were DS, leukocytosis, and QT prolongation.

The symptoms and signs of IDH-differentiation syndrome (IDH-DS) include dyspnea, hypoxia, fever, weight gain, edema, unexplained hypotension, bone pain, and acute renal failure. Chest radiographic imaging often demonstrates pulmonary infiltrates, pleural effusions, and/or pericardial effusion. IDH-DS is frequently associated with leukocytosis. The incidence of IDH-DS in patients with relapsed/refractory AML treated with ivosidenib and enasidenib ranges from 11% to 14% of patients. When suspected, IDH-DS is treated with dexamethasone 10 mg IV twice daily and patients are observed closely until resolution of the symptoms and signs. Approximately half of patients with IDH-DS received corticosteroid therapy and continue therapy without interruption. If associated with severe pulmonary symptoms and/or renal dysfunction lasting more than 48 hours after corticosteroid treatment is initiated, the IDH inhibitor is withheld until symptoms improve. Symptoms may not resolve immediately. Due to the prolonged half-life of enasidenib and ivosidenib, drug treatment interruption is not an alternative to dexamethasone therapy.

IDH-DS remains a clinical diagnosis; there is no laboratory test that will confirm the diagnosis. However, the signs and symptoms can overlap significantly with those of disease progression, leukostasis, and pulmonary infection. Since CR is only achieved in the minority of *IDH*-mutated AML patients treated with IDH inhibitors, and since IDH-DS does not necessarily predict for subsequent achievement of CR, the distinction between IDH-DS and disease progression with leukostasis, pulmonary infiltration, or pneumonia can be challenging. In practice, when IDH-DS is suspected, the patient will be treated with dexamethasone, antibiotics, and hydroxyurea for leukocytosis. Rapid clinical improvement with steroids and/or the presence of granulocytic precursors in the peripheral blood are consistent with the IDH-DS and are helpful clues when present.

Serial bone marrow samples indicate that the responses are achieved by differentiation; marrow aplasia is not observed. The 2-HG levels decline with therapy but do not predict response. The *IDH1* or *IDH2* variant allelic frequency does not predict response. However, the presence of a greater mutational burden is associated with lower response rates, as is the presence of *FLT3* mutations, *RAS* mutations, and other *MAPK* pathway mutations.

Enasidenib was approved for *IDH2*-mutated, relapsed/refractory AML by the U.S. FDA on August 1, 2017. Ivosidenib was approved for *IDH1*-mutated, relapsed/refractory AML by the US FDA on July 20, 2018. Since these inhibitors were approved based on the results of single arm, open label clinical trials, it remains uncertain which relapsed/refractory, *IDH*-mutated AML patients should be treated with an IDH inhibitor, and which should receive salvage chemotherapy. As indicated, responses with enasidenib and ivosidenib often do not occur quickly, and response rates are low in the presence of *RAS*/*MAPK* pathway and *FLT3* mutations. Enasidenib was compared to salvage chemotherapy (LoDAC, azacitidine, intermediate dose cytarabine) for *IDH2* mutated AML following two or three prior lines of therapy. The response rates were higher with enasidenib (CR + Cri, 29.7% vs. 6.2%), but EFS and OS were similar. Nonetheless, the IDH inhibitors provide an effective oral therapeutic option, especially for those who are not necessarily candidates for curative therapy (i.e., allo HSCT). Both ivosidenib and enasidenib may result in transfusion independence, even in patients who did not experience a morphologic leukemia free state.

ACUTE PROMYELOCYTIC LEUKEMIA

APL is a rare subtype (approximately 10% of all AML diagnoses) with unique pathophysiologic, clinical, and therapeutic features. The median age at diagnosis (40 years) is younger than for other types of AML; APL occurs more frequently in Hispanics and the obese. APL was first recognized as a separate clinical subtype of AML based on the promyelocytic morphology and the rapidly fatal course due to hemorrhage associated with fibrinolysis. Now, if diagnosed promptly and managed appropriately, APL has the most favorable prognosis of all types of AML. Nevertheless, catastrophic bleeding events, due to coagulopathy and thrombocytopenia, may still result in high early mortality (in some reports 20% to 30%, typically in those with leukocytosis at presentation). In 80% of the cases, APL cells are hypergranular with Auer rods (see Fig. 60.6 D and E). Blasts with multiple Auer rods, also known as faggot cells, are commonly observed in this subtype. A microgranular variant (M3v) is often associated with leukocytosis (see Fig. 60.6E). The nuclei of APL blasts have characteristic features including two lobes separated by an isthmus, two overlapping nuclei (sliding plates), or butterfly shape. The APL cells are strongly positive for MPO and CD33, and lack expression of the stem cell markers, CD34 and HLA-DR. The *FLT3* ITD mutation is present in 25% of APL patients and is often associated with leukocytosis at presentation and M3v morphology. However, unlike other AML subtypes, the presence of the *FLT3* ITD is not an independent predictor of increased risk of relapse in APL with current therapy. However, the induction mortality may be higher in this subset given the association of *FLT3* mutation with leukocytosis and risk of hemorrhagic death. FLT3 inhibitors are not included in APL treatment.

APL is almost always the result of the translocation t(15;17)(q24.1;21.1) (Chapter 57), which fuses the retinoic acid receptor alpha (*RARA*) gene on the long arm of chromosome 17 with the promyelocytic leukemia (*PML*) gene on the long arm of chromosome 15. In rare cases (<5%) translocation partners other than *PML* result in alternative fusion genes (e.g., *PLZF-RARA*, *NPM1-RARA*, *NuMA-RARA*, *STAT5b-RARA*, *BCOR-RARA*), which often convey resistance to all trans-retinoic acid (ATRA). The *PLZF-RARA* fusion is clearly resistant to ATRA therapy; the others are very uncommon. PML-RARA is an abnormal retinoic acid (RA) receptor that represses transcription of RA-activated target genes by recruiting large protein complexes through the coiled-coil domain of PML, which in turn act as potent transcriptional corepressors. Pharmacologic doses of RA release the corepressors and allow recruitment of coactivators, resulting in the transcription of previously silenced genes. This leads to a sudden arrest in proliferation and a differentiation response that can be seen in vitro and in vivo within a few days following administration of ATRA (Fig. 60.17). APL is the most dramatic example of the success of differentiating therapy.

Prior to the use of ATRA, the anthracyclines were seen as the most active agents in APL and were used as single agents or in combination with cytarabine. The treatment of APL began to diverge from that of other AML subtypes following the initial report of the clinical activity of ATRA in a study from China. Twenty-three of 24 APL patients, eight of whom had disease resistant to prior chemotherapy, achieved either PR or CR with oral ATRA alone given over 30 to 90 days. The single agent efficacy of ATRA was confirmed by groups in North America and Europe. ATRA has a dramatic effect on the coagulopathy, substantially reducing the risk of serious bleeding events, and should be instituted immediately upon suspicion of a diagnosis of APL, even before confirmatory assay results are available. However, continuation of single agent ATRA does not result in long-term DFS due to inevitable relapse. ATRA resistance may be due to increased metabolism of the drug over time, mutations in the *PML-RARA* ligand-binding domain, acquisition of *FLT3* ITD, and/or additional chromosomal abnormalities.

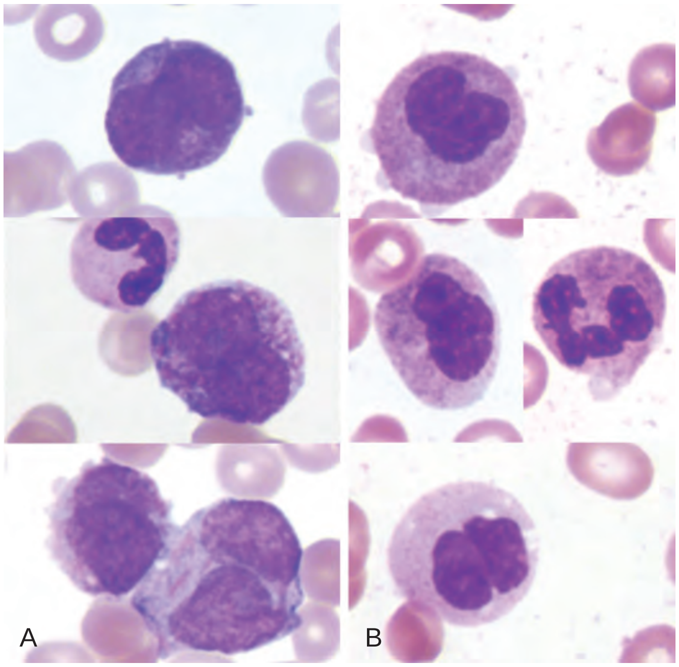


Figure 60.17 EFFECT OF ALL-TRANS RETINOIC ACID THERAPY ON ACUTE PROMYELOCYTIC LEUKEMIA CELLS. All-trans retinoic acid (ATRA) therapy in acute promyelocytic leukemia (APL) causes the malignant promyelocytes (A) to differentiate, and the effects of differentiation can be seen within days of treatment (B). The differentiated APL cells are still somewhat atypical, frequently with bilobed nuclei (compare with normal neutrophil [B, *inset center right*]).

ATRA induces differentiation of APL blasts in patients. ATRA therapy has been associated with the APL DS. APL DS was initially called "retinoic acid syndrome" until it was realized that the syndrome also occurs with the use of arsenic trioxide (ATO) (see below). DS was described in 10% to 25% of patients treated with single agent ATRA. DS can occur within a few days of the start of therapy and has features of a capillary leak syndrome. DS is a clinical diagnosis based on the occurrence of fever, dyspnea, hypoxemia, pulmonary infiltrates, pleural / pericardial effusions, weight gain, peripheral edema, hypotension, and renal and/or hepatic dysfunction. DS carries a high mortality unless recognized and managed promptly. Therapy consists of immediate administration of steroids (dexamethasone 10 mg intravenously twice daily until resolution of signs and symptoms). This intervention will rapidly reverse the manifestations of DS and has substantially reduced mortality to below 5%. If there is no response, or in the case of life-threatening complications, APL treatment should be interrupted. The potential benefit of prophylactic steroids with any APL differentiating therapy is unclear, and the use of prophylactic prednisone, methylprednisolone, or dexamethasone remains controversial. APL DS will not occur during post-remission therapy.

Women of childbearing potential must be warned of the teratogenic effects of ATRA. Other toxicities associated with ATRA include headache, xerosis, mucosal dryness, rash, hypertriglyceridemia (and risk of acute pancreatitis), and hepatic toxicity. Pseudotumor cerebri may occur. Persistent, severe headache with signs of increased intracranial pressure, such as nausea and emesis, should prompt a dilated fundoscopic examination for papilledema. Lumbar puncture can be diagnostic and therapeutic. Acetazolamide is typically prescribed for pseudotumor cerebri, while ATRA is held.

Subsequent clinical studies confirmed significantly better DFS with the combination of ATRA plus chemotherapy. The North American Intergroup study demonstrated superior DFS with ATRA induction, daunorubicin/cytarabine consolidation, followed by ATRA maintenance compared with standard AML induction and consolidation chemotherapy alone (5-year DFS 74% vs. 16%). In the French APL 93 trial, concurrent administration of ATRA with chemotherapy in APL patients with WBC count less than 5000/μL and age ≤ 65 years achieved CR rates of 94%. The relapse rate at 2 years was lower with concurrent (6%) compared with sequential administration (16%, $P = .04$). As standard induction therapy for patients with APL evolved, the combination of ATRA with an anthracycline (daunorubicin or idarubicin) became the standard of care. The role of non-anthracycline agents in ATRA-based therapy was called into question by a comparison of two large trials from Italy (GIMEMA) and Spain (PETHEMA). Both study groups used the same induction [ATRA and idarubicin (AIDA)] and maintenance regimens. However, the GIMEMA consolidations included cytarabine, etoposide, and thioguanine with an anthracycline, whereas the PETHEMA trial only used idarubicin and mitoxantrone during consolidation. The 3-year RFS was not significantly different (90% vs. 86%). A risk-stratification model was proposed based on WBC and platelet count. High risk was defined as a WBC greater than 10,000/μL at diagnosis with significant differences in RFS between patients with high- and those with intermediate- or low-risk disease.

Nonetheless, the role of cytarabine in APL therapy remained controversial. The European APL Group evaluated ATRA/daunorubicin +/−cytarabine during induction followed by daunorubicin +/−cytarabine consolidation (ATRA was omitted from consolidatio n in both arms) in younger, low-/intermediate-risk APL. The CR rates were comparable, but the risk for relapse was increased when cytarabine was omitted from both induction and consolidation (2-year risk of relapse 4.7% vs. 15.9%). On the other hand, in the MRC15 trial daunorubicin, cytarabine, etoposide, and ATRA was compared with the AIDA induction regimen. There was no difference in the risk of relapse or 5-year OS (84% vs. 83%); however, the cytarabine-treated patients experienced greater degree of myelosuppression and higher use of medical resources. In a joint analysis of the European results with those of the PETHEMA group, the benefit of cytarabine was restricted to patients with higher-risk disease.

The APL treatment algorithm continued to evolve with the recognition of arsenic trioxide (ATO as the single most active agent in APL. The precise mechanism of action is not well defined. ATO appears to induce apoptosis of APL cells at higher concentrations and differentiation at lower concentrations. ATO targets the PML/RARA fusion protein for proteasomal degradation. Single-agent ATO was evaluated in patients with relapsed/refractory APL based on the initial reports of activity, again from China. Following a small, single institution pilot study, a larger phase II study of ATO 0.15 mg/kg IV daily until CR was performed in 40 patients with relapsed/refractory APL. Patients received induction followed by 1 cycle of consolidation. Maintenance ATO was administered on a separate clinical trial; 11 patients underwent subsequent allo or auto HSCT. The CR rate was 85%; the rate of complete molecular remission by RT-qPCR at the end of 1 cycle of consolidation was 86%. The 18-month RFS and OS were 56% and 66%, respectively. The most notable toxicities were QT prolongation and risk of torsade de pointes, differentiation syndrome, and leukocytosis. ATO can also cause vasomotor reactions (flushing, hypotension, headache), peripheral neuropathy, and hepatic toxicity. The vasomotor reactions can be ameliorated by slowing the infusion rate.

In two studies, single-agent ATO achieved 85% CR rates in previously untreated APL patients. The estimated 3-year DFS and OS rates were 87% and 86%, respectively, in the report by Mathews. The use of single agent may be particularly attractive where medical resources are scarce and access to cheaper therapies is an economic necessity. In the United States, there is no patient assistance programs for ATRA; some patients face unacceptably high out-of-pocket expenses for ATRA forcing the use of single agent ATO.

The North American Intergroup trial examined the addition of 2 cycles of ATO consolidation to daunorubicin, cytarabine and ATRA induction therapy, daunorubicin plus ATRA consolidation, and ATRA, MP, and methotrexate (MTX) maintenance therapy. The addition of ATO to this backbone resulted in superior 3-year EFS (80% vs. 63%, $P < .0001$), DFS (90% vs. 70%), and OS (86% vs. 81%, $P = .059$). However, the concurrent use of both ATRA and

ATO appeared to result in deeper remissions by RT-qPCR compared with either agent alone. The MD Anderson Cancer Center evaluated the efficacy and safety of ATRA plus ATO for previously untreated APL patients. A single dose of GO 9 mg/m^2 on day 1 was added to therapy for patients with high-risk disease; otherwise, all patients received ATRA 45 mg/m^2 in two divided doses daily with ATO 0.15 mg/kg IV daily. The CR+CRp rate was 92% with an estimated 3-year OS of 85% for the entire study population. The 3-year OS for lower-risk APL patients was 90%. Importantly, therapy with ATRA and ATO was only continued for 28 weeks after achievement of CR; maintenance therapy was not given.

The MD Anderson experience led to the phase III, Italian/German, multicenter APL0406 trial comparing ATRA plus ATO with ATRA plus chemotherapy in low- and intermediate-risk APL patients. ATRA 45 mg/m^2 in two divided doses and ATO 0.15 mg/kg/day were administered concurrently until CR. Patients in CR received 4 cycles of ATO 0.15 mg/kg/day for 5 days/week (20 doses) for 4 consecutive weeks every 8 weeks and 7 cycles of ATRA for 14 days every 4 weeks. CR rates in the ATRA/ATO and the ATRA/chemotherapy group were similar (100% vs. 95%, $P = .12$). Although designed as a noninferiority trial, EFS and OS at 2 years were superior in the ATRA/ATO-treated patients. The ATRA/ATO arm was also associated with less hematologic toxicity and fewer infections. In fact, significant myelosuppression is extremely unlikely during ATRA/ATO consolidation. ATRA/ATO was associated with higher rates of hepatic toxicity and peripheral neuropathy. Moderate to severe DS occurred in 17% of ATRA/ATO-treated patients despite the use of prednisone 0.5 mg/kg/day during induction for DS prophylaxis. The results of the APL0406 trial have been updated. The 50-month OS was 99.2% with ATRA/ATO and 92.6% with ATRA/chemo ($P = .0073$). Two of 132 patients developed therapy-related myeloid neoplasms following ATRA/chemo. There were 2 relapses and one death in CR among the 127 patients treated with ATRA/ATO compared with 15 relapses and 5 deaths in CR among the 132 ATRA/chemo patients. The combination of ATRA and ATO has emerged as the new standard of care for low- and intermediate-risk APL patients. ATO was initially approved in 2000 by the US FDA for relapsed/refractory APL. On January 16, 2018, the FDA expanded the approval of ATO in combination with ATRA for newly diagnosed patients with low-risk APL.

The optimal therapy for high-risk APL is not as clearly defined. Cytoreduction at the beginning of ATRA/ATO induction has been accomplished with either GO or idarubicin. In the MD Anderson study, 26 high-risk patients received ATRA/ATO until CR with a single dose of GO 9 mg/m^2 on day 1. Five patients died during induction; all others achieved remission (81% CR). Patients in CR then received four 8-week cycles of ATO (20 doses per cycle) and seven 14-day cycles of ATRA completed in 28 weeks; maintenance was not prescribed. Three of the 21 high-risk patients in first CR relapsed. In the United Kingdom AML17 trial, 28 of 30 high-risk APL patients received ATRA/ATO induction and consolidation with a single dose of GO 6 mg/m^2 during the first 4 days of induction. The 4-year OS of these 28 high-risk patients was 89%. Maintenance therapy was not administered in the AML17 trial. SWOG S0535 evaluated 70 high-risk APL patients. The treatment algorithm included ATRA/ATO with GO 9 mg/m^2 on day 1 during induction, 2 cycles of ATO consolidation, 2 cycles of ATRA/daunorubicin consolidation, two doses of GO 9 mg/m^2, and 1 year of ATRA/MP/MTX maintenance. The CR rate was 86% and 6-week mortality 11%. The 3-year EFS and OS were 78% and 86%, respectively. In the Australasian APML4 trial, 124 APL patients received induction therapy with ATRA, ATO, and four doses of idarubicin (age-adjusted dose), 2 cycles of ATRA/ATO consolidation, and 2 years of ATRA/MP/MTX maintenance therapy. The 2-year OS of the entire population was 93%. Twenty of the 24 high-risk patients achieved CR; only one of these 20 patients relapsed.

Primary resistance to first-line ATRA/ATO therapy has not been observed, with 100% rate of complete hematologic remission in patients who survive the initial induction therapy. However, at the end of induction and by the time a morphologic remission is achieved, *PML-RARA* transcript is frequently still detectable by RT-qPCR and should not be interpreted as a sign of treatment resistance. RT-qPCR testing is essential in the follow-up of patients with APL. In no other leukemia, apart from chronic myeloid leukemia, does molecular testing influence clinical decision-making processes. Patients who do not achieve a molecular response by the end of the consolidation have a worse prognosis with a higher likelihood of relapse. Positive PCR test results should be confirmed by a second PCR test within 1 to 4 weeks. Single agent GO resulted in a complete molecular remission in 14 of 16 patients with molecular relapse of APL. RT-qPCR monitoring should take place every 3 months for 2 years, especially for those patients with high-risk disease, those older than 60 years, and patients for whom treatment delays occurred during consolidation.

Prior to the addition of ATO to ATRA/chemotherapy-based therapy, maintenance therapy was incorporated into every regimen. For patients who were treated with an ATRA/chemotherapy combination, common practice was to give another two or three anthracycline-based cycles of consolidation. Following consolidation, maintenance therapy for 1 to 2 years with ATRA ± MP/MTX was recommended. The benefit of maintenance appeared to be greatest in those with high-risk disease. However, the addition of ATO consolidation to an ATRA plus chemotherapy regimen eliminated the need for any maintenance therapy in low-/intermediate-risk APL patients. Maintenance was not used in non-high-risk APL patients receiving ATRA/ATO induction and consolidation with excellent long-term outcomes. There remains uncertainty and debate as to the benefit of maintenance in high-risk APL with ATRA/ATO-based therapy, but many leukemia experts do not include maintenance therapy even for these patients.

The choice of therapy for relapsed APL will be determined by the therapeutic agents used for initial therapy, duration of first response, and presence or absence of CNS disease at relapse. The incidence of CNS involvement at first relapse 5 years after initial ATRA and anthracycline therapy without CNS prophylaxis was 1.7%. Two independent risk factors for CNS disease at first relapse in this study were WBC greater than 10,000/μL at diagnosis and occurrence of CNS hemorrhage during induction; the 5-year cumulative incidence of CNS relapse was 5.5% (8/149) and 18.8% (3/18) in patients with these risk factors, respectively. Some experts recommend intrathecal CNS prophylaxis in APL patients with these high-risk features at initial diagnosis and also at relapse. ATO, ATRA, anthracyclines, high-dose cytarabine, and GO (alone or in various combinations) can be considered for relapsed APL patients. HSCT should follow achievement of a second remission. Auto HSCT is recommended for relapsed APL patients who have again achieved a complete molecular remission. For those whose PCR results remain positive, allo HSCT is favored.

The outcome of APL patients has dramatically improved from the initial description by Hillestad in 1957. Primary resistance to ATRA/ATO therapy is virtually unheard of, and relapse is uncommon. HSCT is not considered for patients achieving first CR with negative RT-qPCR for *PML/RARA* fusion transcripts (i.e., complete molecular remission). Current clinical studies focus on improving the quality of life of APL patients undergoing therapy, by replacing intravenous ATO with oral arsenic formulations. However, early mortality due to hemorrhage remains the greatest risk facing patients with APL worldwide. International efforts aim to expedite administration of ATRA to newly diagnosed APL patients.

BLASTIC PLASMACYTOID DENDRITIC CELL NEOPLASM (CHAPTER 64)

BPDCN is a rare hematopoietic neoplasm involving bone marrow, skin, and lymph nodes (Chapter 64). CNS disease may occur with disease progression, especially in patients not receiving CNS-directed therapy. The disease was previously known as agranular CD4+ NK cell leukemia, blastic NK-cell lymphoma, and CD4+, CD56+ hematodermic neoplasm. BPDCN is now classified by the WHO as a myeloid neoplasm, being derived from type II (plasmacytoid) dendritic cells. BPDCN cells express CD4, CD56, and CD123 (the alpha chain of the low-affinity interleukin-3 receptor). The median age at diagnosis is 65 to 70 years but can occur at any age, affecting all races and geographies. Patients may present with a single cutaneous nodule, multiple nodules, hyperpigmented red-brown macules, and/or purpuric macules.

Until recently, there was no standard of care for patients with BPDCN. Although localized radiation therapy is effective for local control, relapse rapidly follows, and systemic therapy is preferred from the time of initial diagnosis. Intensive chemotherapy regimens used for acute lymphoblastic leukemia or AML may lead to remissions with typically short duration. CNS prophylaxis appears to improve outcomes. For eligible patients, allo HSCT is recommended. For patients ineligible for intensive therapy, several regimens have been reported such as bendamustine, asparaginase with MTX, gemcitabine, and HMA with venetoclax.

More recently, CD123 directed therapy has shown promise. Tagraxofusp is a cytotoxic agent composed of interleukin-3 (IL-3) fused to a truncated diphtheria toxin, a protein synthesis inhibitor. IL-3 binds to CD123, leading to internalization of the conjugate and cellular apoptosis. A phase I/II study in newly diagnosed and relapsed/refractory BPDCN patients established the maximally tolerated dose of tagraxofusp. In an expansion of this study, 29 newly diagnosed BPDCN patients were treated with tagraxofusp 12 mcg/kg IV once daily on days 1 to 5 of a 21-day cycle. Treatment is continued until progression or unacceptable toxicity. The CR and clinical CR rate was 72% and 2-year survival 52%. The response rate was 65% in a cohort of 15 relapsed/refractory BPDCN patients. The most serious adverse event is capillary leak syndrome; hepatic transaminase elevations and hypoalbuminemia were also observed. On December 21, 2018, the US FDA approved tagraxofusp for newly diagnosed and relapsed/refractory BPDCN patients ages 2 and older. Other CD123-directed therapies (antibody-drug conjugates, BiTE, and chimeric antigen receptor T cells [CAR-T]) are in development.

EXTRAMEDULLARY ACUTE MYELOID LEUKEMIA

AML blasts may infiltrate any tissue. A tumor composed of myeloid blasts is known as a myeloid sarcoma (or granulocytic sarcoma or chloroma). The term chloroma derives from the green hue of the tumor due to the presence of copper in MPO. Myeloid sarcomas can occur in any tissue but most commonly present in the skin (leukemia cutis), lymph nodes, gastrointestinal tract, testes, ovaries, CNS, soft tissue, and bones. They can easily be confused with lymphomas and soft-tissue sarcomas; immunohistochemical stains are often required to establish the correct diagnosis. Myeloid sarcomas can be isolated or multiple. Myeloid sarcomas may occur at the same time as blood and marrow involvement, may precede the diagnosis of AML in the marrow (primary extramedullary AML), or may occur at the time of relapse without marrow involvement. Isolated extramedullary relapse is particularly common following allo HSCT. Immune checkpoint inhibitors, such as the CTLA-4 inhibitor ipilimumab, appear to be particularly active in extramedullary relapse following allo HSCT. In the context of chronic myeloid malignancies (e.g., MDS, myeloproliferative neoplasms, chronic myeloid leukemia, chronic myelomonocytic leukemia), their appearance represents progression to a blast phase.

Myeloid sarcoma, even if primary extramedullary myeloid sarcoma, should be treated with systemic chemotherapy. In the absence of systemic therapy, subsequent progression to systemic AML is very likely in patients with primary extramedullary AML. Radiation therapy may be of help to optimize local control (especially if residual disease present after systemic therapy) and provide palliation. There is controversy over whether patients with myeloid sarcoma do better or worse than those with AML. It is likely that the prognosis depends more on the biologic features of the disease (e.g., presence of a CBF gene translocation) than on the extramedullary presentation.

CENTRAL NERVOUS SYSTEM DISEASE

CNS involvement occurs in less than 5% of patients with AML. The risk for CNS involvement is higher in AML with monoblastic differentiation and those cases with a high WBC (>100,000/μL) at presentation, and perhaps in FLT3-mutated AML. HiDAC penetrates the blood–brain barrier and appears to have contributed to a lower rate of CNS involvement over the years. For example, prior to the use of HiDAC, inv(16) AML was associated with a 30% incidence of CNS leukemia, particularly intracerebral masses. This problem has been virtually eliminated with the use of HiDAC. Nonetheless, once a patient has achieved a CR, some experts recommend intrathecal therapy for prophylaxis. This is a more common practice after allo HSCT in patients with monoblastic AML or those with CNS disease at presentation.

In the presence of symptoms suggestive of CNS disease (headache, persistent nausea/emesis, cranial nerve dysfunction), further evaluation should be pursued. Symptoms are due to raised intracranial pressure, mass effect, or infiltration of cranial nerves. Although CT scan is adequate to evaluate for large masses and intracerebral hemorrhage, a magnetic resonance image with contrast of the brain and/or spinal cord should be obtained for suspected leukemic meningitis. Lumbar puncture is a crucial component of the evaluation but may need to be repeated several times to establish the diagnosis. Flow cytometry of the CSF sample can help to establish the diagnosis. However, the results of the CSF flow cytometry must be evaluated cautiously and only with knowledge of the CSF cell counts, since a positive result may be due to peripheral blood contamination of the CSF specimen. Treatment consists of intrathecal therapy with cytarabine and/or MTX via lumbar route or an intraventricular catheter (Ommaya reservoir). A typical schedule includes twice-weekly treatments until the cerebrospinal fluid is free of leukemic blasts. The frequency is then changed to weekly for 2 months, then every other week for 2 months, followed by monthly injections for up to 1 year. However, various schedules are used in practice. Radiation therapy can be a useful alternative, especially in the case of localized lesions such as isolated cranial nerve findings as well as for palliation.

PREGNANCY

AML is rarely diagnosed during pregnancy. The response to treatment is not affected by pregnancy. AML therapy during the first trimester is problematic due to the toxic and teratogenic effects of chemotherapy on the fetus; termination of pregnancy before institution of induction therapy should be strongly considered in the first trimester. Pregnancy outcome during the second and third trimester is more favorable, and successful treatment of the AML and delivery of normal infants have been reported. Daunorubicin is preferable over idarubicin because the latter is more lipophilic and has higher DNA affinity, raising the possibility of higher maternal–fetal transfer and toxicity to the fetus. In general, it is not advisable to delay therapy once the diagnosis of AML is made given the high risk for maternal death. However, in situations where the leukemia behaves indolently and women are close to delivery, a conservative approach can be chosen with supportive care and induction of labor as soon as possible. These decisions are based on clinical judgment and discussion with the patient.

FUTURE DIRECTIONS

The survival of younger AML patients has improved over the last 40 years. The use of intensive post-remission therapy (HiDAC) and allo HSCT has contributed to this improvement. Patients are more likely to have stem cell donors for allo HSCT, since the unrelated donor international registries have increased in size, and alternative donors such as haploidentical donors and cord blood are now routinely used. There have been improvements in supportive care. The use of broad-spectrum triazole antibiotics has decreased the incidence and mortality associated with invasive fungal infections following AML induction therapy. The prevention of fungal and viral (CMV) infections, as well as advances in the prevention and treatment of graft-versus-host disease, following allo HSCT have decreased the transplant-related mortality. On the other hand, the survival of older AML patients has not improved. However, with the availability of effective, better tolerated regimens, especially HMA/venetoclax, we expect that the survival of older AML patients will also improve.

Nonetheless, the risk of relapse remains high with currently available therapies, and OS remains poor. With few exceptions, attempts to improve outcomes with intensive chemotherapy have been largely disappointing. Nevertheless, it remains possible that the efficacy of intensive chemotherapy may be improved with the addition of novel agents. For example, the BCL2 inhibitor venetoclax is being evaluated with 7+3, HiDAC, FLAG-Ida, and liposomal daunorubicin/cytarabine. E-selectin inhibitors are hypothesized to increase chemotherapy sensitivity of leukemic stem cells by displacing these cells from a protective niche in the bone marrow; these agents are being evaluated in combination with intensive chemotherapy regimens. Agents that target specific mutations such as *FLT3*, *IDH1*, and *IDH2* are commercially available. However, these targeted inhibitors result in remissions for a minority of patients with these mutations, and the responses are not durable. This should not be surprising, since the pathogenesis of AML (with the possible except of APL) is due to multiple genetic events. Nonetheless, inhibitors of other potential targets are being evaluated such as Menin, which is central to AML pathogenesis due to *KMT2A* rearrangements and *NPM1* mutation. KIT inhibitors are being evaluated in AML with CBF fusions.

Given the genetic heterogeneity of AML, it should not be surprising that targeted therapies do not result in high response rates or long DFS. The lowest risk of relapse occurs following allo HSCT, at least partially due to a graft-versus-leukemia effect. This observation suggests that manipulation of the immune system may improve the outcome of AML patients. Checkpoint inhibitors against PD1 and PD-L1 have been evaluated in AML, but the results of these studies have been disappointing to date. However, it remains possible that we just have not been able to identify a specific immune signature that will predict for response. Other components of the immune regulation are being targeted in clinical trials. CD47, integrin-associated protein, is present on the cell surface of nearly all cells. CD47 binds to SIRP alpha on macrophage, providing a "don't eat me" signal and preventing phagocytosis. Anti-CD47 monoclonal antibodies are in clinical development. Magrolimab, a first generation anti-CD47 monoclonal antibody, has demonstrated very encouraging efficacy in combination with azacitidine for patients with MDS and AML. Durable CRs, including MRD negative responses, have been seen even in *TP53*-mutated MDS and AML. TIM3 is present on leukemic stem cells but is also a T-cell immune checkpoint. Anti-TIM3 monoclonal antibodies are in clinical development. BiTE and CART targeting cell surface antigens present on AML blasts are in development as well. These approaches are complicated by the presence of the target on normal myeloid cells, which may contribute to significant myelosuppression. The cytokine release syndrome and encephalopathy observed with currently available BiTE and CAR-T therapies have also complicated the clinical development of these agents in AML patients.

We need to develop a better understanding of the cellular perturbations that lead to uncontrolled proliferation, block of normal differentiation, and avoidance of apoptosis by neoplastic hematopoietic stem cells. We also need to have a deeper knowledge of the immune dysregulation associated with active disease. Advances in DNA sequencing technology and proteomics will help us identify targets in the individual AML patient more rapidly. We will also develop these technologies to evaluate not just the prognostic significance of measurable residual disease, but hopefully identify targets for earlier intervention to prevent relapse. This knowledge will continue to drive clinical research and highlights the importance of clinical trial participation as an integral component of AML therapy.

SUGGESTED READINGS

Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127:2391–2405.

Burnett A, Russell N, Hills R, et al. A randomized comparison of daunorubicin 90 mg/m2 vs 60 mg/m2 in AML induction: results from the UK NCRI AML17 trial in 1206 patients. *Blood*. 2015;125(25):3878–3885.

Cortes J, Heidel F, Hellmann H, et al. Randomized comparison of low dose cytarabine with or without glasdegib in patients with newly diagnosed acute myeloid leukemia or high-risk myelodysplastic syndrome. *Leukemia*. 2019;33(2):379–389.

Cortes JE, Khaled S, Martinelli G, et al. Quizartinib versus salvage chemotherapy in relapsed or refractory FLT3-ITD acute myeloid leukaemia (QuANTUM-R): a multicentre, randomised, controlled, open-label, phase 3 trial. *Lancet Oncol*. 2019;20(7):984–997.

DiNardo CD, Jonas BA, Pullarkat V, et al. Azacitidine and venetoclax in previously untreated acute myeloid leukemia. *N Engl J Med*. 2020;383:617–629.

DiNardo CD, Maiti A, Rausch CR, et al. 10-day decitabine with venetoclax for newly diagnosed intensive chemotherapy ineligible, and relapsed or refractory acute myeloid leukaemia: a single-centre, phase 2 trial. *Lancet Haematol*. 2020;7(10):e724–e736.

DiNardo CD, Pratz K, Pullarkat V, et al. Venetoclax combined with decitabine or azacitidine in treatment-naive, elderly patients with acute myeloid leukemia. *Blood*. 2019;133(1):7–17.

DiNardo CD, Stein EM, De Botton S, et al. Durable remissions with ivosidenib in IDH1-mutated relapsed or refractory AML. *N Engl J Med*. 2018;378(25):2386–2398.

Dohner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*. 2017;129(4):424–447.

Duncavage EJ, Schroeder MC, O'Laughlin M, et al. Genome sequencing as an alternative cytogenetic analysis in myeloid cancers. *N Engl J Med*. 2021;384(10):924–935.

Erba HP. Has there been progress in the treatment of older patients with acute myeloid leukemia? *Best Pract Res Clin Haematol*. 2010 Dec;23(4):495–501.

Fenaux P, Chastang C, Chevret S, et al. A randomized comparison of all transretinoic acid (ATRA) followed by chemotherapy and ATRA plus chemotherapy and the role of maintenance therapy in newly diagnosed acute promyelocytic leukemia. The European APL Group. *Blood*. 1999;94(4):1192–1200.

Hasserjian RP, Steensma DP, Graubert TA, Ebert BL. Clonal hematopoiesis and measurable residual disease assessment in acute myeloid leukemia. *Blood*. 2020;135(20):1729–1738.

Hills R, Castaigne S, Appelbaum F, et al. Addition of gemtuzumab ozogamicin to induction chemotherapy in adult patients with acute myeloid leukaemia: a meta-analysis of individual patient data from randomised controlled trials. *Lancet Oncol*. 2014;15:986.

Huang M, Ye Y, Chen S, et al. Use of all-transretinoic acid in the treatment of acute promyelocytic leukemia. *Blood*. 1988;72:567–572.

Lancet JE, Uy GL, Newell LF, et al. CPX-351 versus 7+3 cytarabine and daunorubicin chemotherapy in older adults with newly diagnosed high-risk or secondary acute myeloid leukaemia: 5-year results of a randomised, open-label, multicentre, phase 3 trial. *Lancet Haematol*. 2021;8(7):E481–E491.

Larson RA, Sievers EL, Stadtmauer EA, et al. Final report of the efficacy and safety of gemtuzumab ozogamicin (Mylotarg) in patients with CD33-positive acute myeloid leukemia in first recurrence. *Cancer*. 2005;104(7):1442–1452.

LeBlanc TW, Erba HP. Shifting paradigms in the treatment of older adults with AML. *Semin Hematol*. 2019 Apr;56(2):110–117.

Ley TJ, Mardis ER, Ding L, et al. DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. *Nature*. 2008;456(7218):66–72.

Nair R, Salinas-Illarena A, Baldauf HM. New strategies to treat AML: novel insights into AML survival pathways and combination therapies. *Leukemia*. 2021;35:299–311.

Papaemmanuil E, Gerstung M, Bullinger L, et al. Genomic classification and prognosis in acute myeloid leukemia. *N Engl J Med*. 2016;374(23):2209–2221.

Pemmaraju N, Lane AA, Sweet KL, et al. Tagraxofusp in blastic plasmacytoid dendritic-cell neoplasm. *N Engl J Med*. 2019;380(17):1628–1637.

Perl AE, Martinelli G, Cortes JE, et al. Gilteritinib or chemotherapy for relapsed or refractory FLT3-mutated AML. *N Engl J Med*. 2019;381(18):1728–1740.

Platzbecker U, Avvisati G, Cicconi L, et al. Improved outcomes with retinoic acid and arsenic trioxide compared with retinoic acid and chemotherapy in non-high-risk acute promyelocytic leukemia: final results of the randomized Italian-German APL0406 trial. *J Clin Oncol*. 2017;35 (6):605–612.

Pollyea DA, Tallman MS, De Botton S, et al. Enasidenib, an inhibitor of mutant IDH2 proteins, induces durable remissions in older patients with newly diagnosed acute myeloid leukemia. *Leukemia*. 2019;33(11):2575–2584.

Powell BL, Moser B, Stock W, et al. Arsenic trioxide improves event-free and overall survival for adults with acute promyelocytic leukemia: North American Leukemia Intergroup Study C9710. *Blood*. 2010;116(19):3751–3757.

Ravandi F, Estey E, Jones D, et al. Effective treatment of acute promyelocytic leukemia with all-trans-retinoic acid, arsenic trioxide, and gemtuzumab ozogamicin. *J Clin Oncol*. 2009;27(4):504–510.

Roboz GJ, DiNardo CD, Stein EM, et al. Ivosidenib induces deep durable remissions in patients with newly diagnosed IDH1-mutant acute myeloid leukemia. *Blood*. 2020;135(7):463–471.

Sanz MA, Lo Coco F, Martin G, et al. Definition of relapse risk and role of nonanthracycline drugs for consolidation in patients with acute promyelocytic leukemia: a joint study of the PETHEMA and GIMEMA cooperative groups. *Blood*. 2000;96(4):1247–1253.

Schuurhuis G, Heuser M, Freeman S, et al. Minimal/measurable residual disease in AML: a consensus document from the European LeukemiaNet MRD Working Party. *Blood*. 2018;131(12):1275–1291.

Sekeres MA, Guyatt G, Abel G, et al. American Society of Hematology 2020 guidelines for treating newly diagnosed acute myeloid leukemia in older adults. *Blood Adv*. 2020;4(15):3528–3549.

Soignet SL, Frankel SR, Douer D, et al. United States multicenter study of arsenic trioxide in relapsed acute promyelocytic leukemia. *J Clin Oncol*. 2001;19(18):3852–3860.

Sorror M, Storer B, Fathi A, et al. Development and validation of a novel acute myeloid leukemia-composite model to estimate risks of mortality. *JAMA Oncol*. 2017;3(12):1675–1682.

Stein EM, DiNardo CD, Pollyea DA, et al. Enasidenib in mutant IDH2 relapsed or refractory acute myeloid leukemia. *Blood*. 2017;130(6):722–731.

Stone RM, Mandredkar SJ, Sanford BL, et al. Midostaurin plus chemotherapy for acute myeloid leukemia with a FLT3 mutation. *N Engl J Med*. 2017;377 (5):454–464.

Tallman MS, Andersen JW, Schiffer CA, et al. All-trans retinoic acid in acute promyelocytic leukemia: long-term outcome and prognostic factor analysis from the North American Intergroup protocol. *Blood*. 2002;100(13):4298 –4302.

Uy GL, Aldoss I, Foster MC, et al. Flotetuzumab as salvage immunotherapy for refractory acute myeloid leukemia. *Blood*. 2021;137(6):751–762.

Wei A, Montesinos P, Ivanov V, et al. Venetoclax plus LDAC for newly diagnosed AML ineligible for intensive chemotherapy: a phase 3 randomized placebo-controlled trial. *Blood*. 2020;135(24):2137–2145.

Wei A, Strickland S, Hou J-Z, et al. Venetoclax combined with low-dose cytarabine for previously untreated patients with acute myeloid leukemia: results from a phase Ib/II study. *J Clin Oncol*. 2019;37(15):1277–1285.

Wei AH, Dohner H, Pocock C, et al. Oral azacitidine maintenance therapy for acute myeloid leukemia in first remission. *N Engl J Med*. 2020;383(26):2526–2537.

CHAPTER 61

MYELODYSPLASTIC SYNDROMES

Christopher J. Gibson and David P. Steensma

The myelodysplastic syndromes (MDS) include a group of clonal hematopoietic disorders characterized by ineffective hematopoiesis and blood cytopenias, abnormal blood and bone marrow cell morphology (Fig. 61.1), and a risk of clonal evolution including progression to acute myeloid leukemia (AML).[1] The syndromes have a highly variable clinical course, manifesting in some patients as indolent asymptomatic cytopenias or only necessitating occasional transfusions over a course of many years, and in others as aggressive diseases that rapidly evolve into treatment-refractory acute leukemias and cause death from infection or other complications within a few months.[2]

Despite this phenotypic variability, individual cases of MDS share many pathophysiologic mechanisms at the molecular and cellular levels, and our collective understanding of these mechanisms has evolved substantially in the last decade. At the same time, although MDS also shares features with AML and other myeloid malignancies, mounting evidence shows that it is appropriately conceived as a distinct class of diseases, and that the older conceptualization of MDS as "pre-leukemia" is overly simplistic.

While our collective understanding of MDS pathophysiology is improving, the condition remains difficult to treat, and outcomes for many patients with MDS are poor. This chapter describes our current understanding of the classification, pathobiology, clinical features, and treatment approaches for this heterogeneous group of disorders.

Central to an understanding of how MDS is diagnosed and classified is the concept of morphologic dysplasia. Although specific criteria for diagnosis of MDS as a distinct clinical syndrome exist and are discussed below, the term "myelodysplasia" refers more generally to an abnormal appearance of hematopoietic precursors during pathologic examination of bone marrow, which may result from many different causes (e.g., drug toxicity, nutritional deficiency (see chapter on nutritional deficiencies), viral infection).[3] The process by which our understanding of MDS has evolved from that of a morphologic oddity associated with cytopenias to that of a distinct clonal disease process has been a century in the making, propelled forward at several points by paradigm shifts in pathologic and molecular characterization of hematologic diseases.[4]

HISTORY

In describing the history of MDS as a distinctly categorized disease entity, it is perhaps most useful to separately describe the history of its three major conceptual components.[4] The earliest recognized of these, not surprisingly, was that of cellular dysplasia in association with cytopenias. Although studies of blood morphology in anemic patients were reported in the 19th century not long after the quantitative hemocytometer debuted,[5] the first detailed characterization of dysplasia was probably made by Giovanni di Guglielmo in Pavia in 1923, when he described abnormal erythroid forms in the marrows of patients with various types of cytopenias.[6]

A second major concept, that of ineffective hematopoiesis, developed in the 1930s with the description of "refractory anemia" in patients unresponsive to iron pills or liver extract (the precursor to B_{12} supplementation), and later to folic acid supplementation.[7] In the 1950s, others expanded this to include similarly treatment-refractory leukopenia and thrombocytopenia.[8] Importantly, however, ineffective hematopoiesis and marrow dysplasia were initially incompletely linked, and the ineffective hematopoiesis of many early "refractory anemia" patients was probably rooted in other disorders, such as anemia of inflammation due to advanced rheumatologic disease or non-myeloid neoplasia.

The third major component of MDS, and possibly the most prominent, is its conceptualization as a pre-leukemic state. In 1942, a group from France led by Paul Chevallier described a case of anemia evolving into leukemia as "odo-leukemia" ("odo" from the Greek for edge or threshold), which they attributed to benzene exposure.[9] A similar disorder was termed "preleukaemic anemia" by a British hematologist, J.L. Hamilton-Paterson, in 1949.[10] This term was then adopted and expanded by an American group at the University of Chicago including Leon Jacobson in the early 1950s,[11] providing one of the clearest descriptions of a disorder clinically defined by both cytopenias and a risk of leukemia. Other groups published similar descriptions in the 1950s and 1960s under different terminologies.[12]

In 1970, Bernard Dreyfus in Paris and colleagues coined the term refractory anemia with excess blasts (RAEB, described in French as "les anémies réfractaire avec excès de myeloblasts")[13] and later attempted to further characterize components of RAEB based on morphology and clinical characteristics.[14] The term "hematopoietic dysplasia" was used in the early 1970s to describe a heterogeneous group of disorders distinct from AML, and the term was later simplified to "myelodysplasia."

One of the key events in the history of myeloid disease classification was the formation of the French-American-British (FAB) Cooperative Group in 1976, which in that year issued a comprehensive and influential categorization of AML[15] based on the 1975 classification of David Galton and John Dacie in London.[16] The FAB classification persisted as the dominant descriptive system for AML until the early 2000s. The original FAB AML formulation from 1976 included two types of "dysmyelopoietic syndromes" that the group warned should not be confused with AML: RAEB and chronic myelomonocytic leukemia (CMML), which had first been described in the late 1960s.[17] However, in 1982 the FAB recognized MDS as a separate group of diseases and accorded a distinct classification system, described in more detail below.[18]

CLASSIFICATION

The currently accepted classification scheme for MDS was initially published by the World Health Organization (WHO) in 2001, revised in 2008,[19] and revised again in 2016 (Table 61.1).[20] Like the classifications of the FAB group, the WHO classification is principally a morphology-based hematopathology system with limited inclusion of cytogenetic and molecular markers, and classifies disease chiefly based on the number of dysplastic lineages and the percentage of marrow blasts.

The WHO classification was intended to replace the FAB classification system, which included 5 subtypes of MDS. Those 5 FAB subtypes were refractory anemia (RA, <5% blasts and any cytopenia pattern); refractory anemia with ring sideroblasts (RARS, defined by <5% myeloblasts and ring sideroblasts—formerly called "ringed"—representing $\geq$15% of erythroid precursors); RAEB (5% to 19% blasts in the marrow); RAEB in transformation to AML (RAEB-t, defined as 20% to 29% blasts in the marrow); and CMML (defined by $>1 \times 10^9$ peripheral monocytes).[18]

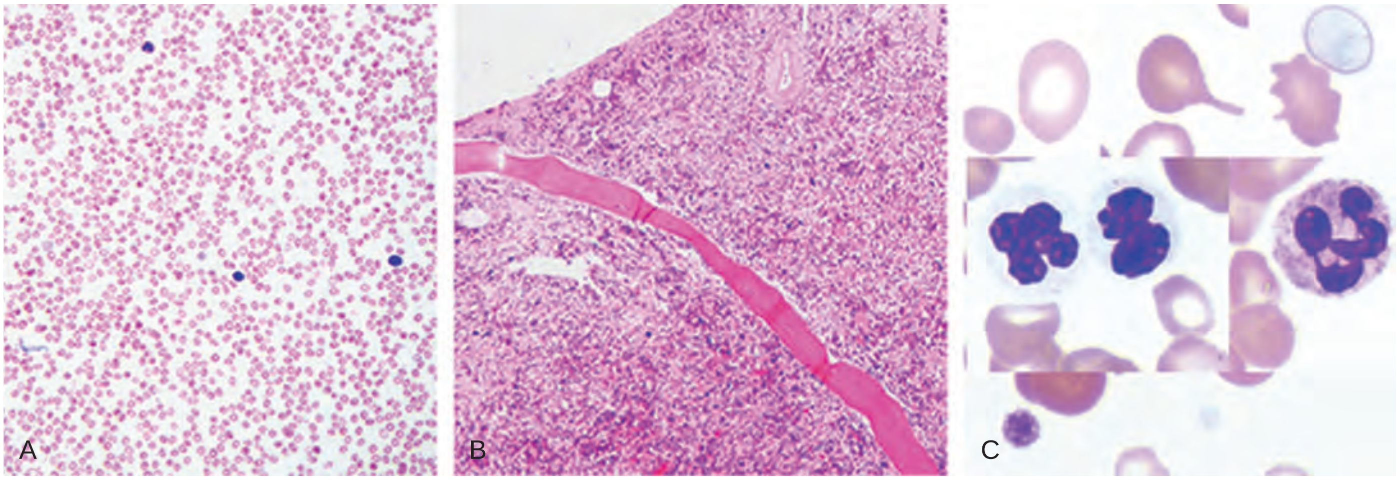


Figure 61.1 ELEMENTS OF MYELODYSPLASTIC SYNDROME. Myelodysplastic syndromes are generally characterized by cytopenias (A) caused by ineffective hematopoiesis (B), which is related to multilineage dysplasia (C). (A) This patient presented with a white blood cell count of 1500/μL, hemoglobin 8.9 g/dL, and platelet count 47,000/μL. (B) The bone marrow was hypercellular, indicating ineffective hematopoiesis. (C) Evidence of trilineage dysplasia was apparent on the peripheral smear. Anisocytosis with macroovalocytes and poikilocytosis is seen in the red blood cells (C, *top*). The latter included the somewhat uncommon finding of Cabot ring forms *(right)*. A large proportion of the granulocytes were severely hypogranular (C, *middle left*) compared to some normal forms still in the circulation *(right)*. Platelets (C, *bottom*) were decreased in number, and many were severely hypogranular (*middle*, barely visible) compared with residual normal platelets *(left)*.

The WHO systems bear some similarities to the FAB scheme, but there are several notable changes. First, the WHO system recognizes that MDS may present with isolated leukopenia or thrombocytopenia, albeit uncommonly, hence the change from RA to "MDS with single lineage dysplasia" (MDS-SLD), and the addition of a second distinct class for patients with more than one affected cell line/ multilineage dysplasia (MDS-MLD). Second, a mounting body of evidence suggested that MDS with concomitant myeloproliferative features such as CMML is clinically and biologically distinct from MDS without such features, especially when the white count is elevated, and thus warranted separate designation.[21] Third, the unique clinical syndrome associated with low-risk MDS with isolated deletion of chromosome 5q, in combination with its particular responsiveness to treatment with lenalidomide, warranted its designation as a specific clinicopathologic entity.[22] Fourth, in the 2016 edition, the WHO removed the obsolete "refractory anemia" terminology from the 1930s.[23] Finally, the blast threshold for diagnosis of AML instead of MDS was changed with the 2001 classification from 30% to 20%, rendering the FAB designation of RAEB-t obsolete, and RAEB was divided into two categories reflecting different prognoses for patients with 5% to 9% versus 10% to 19% blasts.[24]

The WHO revision improved on certain aspects of the FAB system, including refining prognostic categories.[25] However, artificial boundaries such as the 10% cell threshold in a lineage to define dysplasia, 15% ring sideroblasts to define MDS-RS (unless *SF3B1* mutation is present, in which case the threshold is lowered to 5% ring sideroblasts), and 5%/10%/20% blast cutoffs often prove themselves to be of variable relevance, both biologically and clinically. These thresholds fail to capture a number of variables important in MDS, including patient age and comorbidities, kinetics of disease, and cytogenetic and molecular genetic data. Newer classifications are likely to evolve as molecular characterization of MDS improves; for instance, in 2020, an International Working Group (IWG) proposed definition of *SF3B1* mutant MDS as a distinct entity.[26]

While the FAB and WHO subgroups carry some prognostic information (for instance, patients with FAB RAEB or WHO RAEB-2 are at higher risk of progression to AML than subgroups without excess blasts[27]), other systems specifically dedicated to estimating disease risk, such as the International Prognostic Scoring System and its Revised version (IPSS and IPSS-R; see below), are better suited to the task of risk stratification. Future iterations of classification systems will incorporate elements of genetic or clinical data that better group MDS patients based on disease biology, prognosis and natural history, or anticipated responsiveness to treatment modalities.[28] For now, however, the WHO classification remains a key component of several descriptive tools applied to patients with MDS.

EPIDEMIOLOGY

Most available data suggest that MDS is one of the most common hematologic malignancies, although this claim has been difficult to validate until recently due to confusing terminology and incomplete reporting of cases to registries.[29,30] Cases of MDS were not reported to the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) until 2001, and reporting was inconsistent for several years thereafter. Initial reports to the registry suggested an incidence of only about 10,000 new cases per year.[31] Subsequent comparison of these data with Medicare and insurance claims for MDS suggested that a substantial proportion of MDS cases went unreported to SEER, with an estimated age-adjusted incidence of >5.3 per 100,000, compared to the SEER estimate of 3.3.[32]

The reporting difference was especially stark in patients age 65 and older, where it was estimated that the actual incidence of MDS might actually be close to fourfold higher than what was captured in the database (i.e., 75 vs. 20 per 100,000). Some of the underreporting was likely related to specific criteria for entry into the database: for instance, myeloid malignancies could only be counted once, such that patients presenting with a new diagnosis of secondary AML were usually coded as AML, without mention of the antecedent MDS. Much of the underreporting, however, was likely due to the complexity of diagnosing and classifying MDS, as described above. The rate of reporting may be improving with time, with annual incidence in the SEER database in more recent years estimated at around 20,500 and an age-adjusted incidence of 4.9 per 100,000.[33]

Even the most accurate database would probably underestimate the total global burden of MDS, which is likely present in a substantial percentage of older patients with idiopathic cytopenias who never undergo bone marrow analysis.[34] Although some proportion of this uncaptured population likely has biologically indolent disease that would never require therapy, more thorough cross-sectional studies, particularly in older patients, would help clarify the distinction between this end of the MDS spectrum and more aggressive biology that brings patients to clinical attention.

TABLE 61.1 2016 World Health Organization Classification of the Adult Myelodysplastic Syndromes

MDS with Single Lineage Dysplasia (MDS-SLD)	
Dysplasia	≥10% of cells from a single lineage
Blasts	<5% in marrow; <1% in peripheral blood; no Auer rods
MDS With Single Lineage Dysplasia and With Ring Sideroblasts (MDS-SLD-RS)	
Dysplasia	Isolated erythroid dysplasia
Blasts	<5% in marrow; <1% in peripheral blood; no Auer rods
Notes	≥15% of erythroid precursors are ring sideroblasts, or if
	SF3B1 mutation present, ≥5% of erythroid precursors are ring sideroblasts
MDS With Isolated Del(5q)	
Dysplasia	Erythroid dysplasia prominent; granulocytic series variable; normal or increased numbers of megakaryocytes with hypolobated nuclei
Blasts	<20% (though usually much less)
Notes	Must include deletion of chromosome 5q inclusive of band 5q31 as sole chromosomal abnormality or with one other abnormality
MDS With Multilineage Dysplasia (MDS-MLD)	
Dysplasia	≥10% of cells from two or more myeloid lineages
Blasts	<5% in marrow; <1% in peripheral blood; no Auer rods
Notes	Peripheral monocyte count must be $<1 \times 10^9$/L; ring sideroblasts may be present, as above for MDS-SLD-RS, in which case it is classified asMDS-MLD-RS
MDS With Excess Blasts (MDS-EB)	
Dysplasia	No specific requirement
Blasts	MDS-EB1: 5%–9% in marrow, <5% in peripheral blood, AND no Auer rods
	MDS-EB-2: 10%–19% in marrow, 5%–19% in peripheral blood, OR Auer rods
Notes	≥20% blasts is now considered AML
Unclassifiable MDS (MDS–U)	
Dysplasia	May be minimal, or not meeting criteria for another subtype
Blasts	<5% in marrow; no Auer rods
Notes	In presence of clonal cytogenetic finding considered diagnostic of MDS

[a] Note: excludes refractory cytopenias of childhood (RCC). MDS/myeloproliferative neoplasms such as chronic myelomonocytic leukemia (CMML), and therapy-related MDS/acute myeloid leukemia (AML).

As referenced above, MDS is by and large a disease of older adults and reflects the inevitable acquisition of genetic mutations by aging hematopoietic progenitor cells. In the United States, the median age at diagnosis is approximately 71 years,[35] and in the absence of a congenital disorder/germline predisposition or exposure to radiation or cytotoxic chemotherapy for another disease, diagnosis prior to the age of 50 is rare.[36] Recent data suggest that a substantial proportion of older adults harbor hematopoietic clones defined by the presence of mutations recurrently found in MDS and AML at a variant allele frequency (VAF) of at least 2%, and that this state of "clonal hematopoiesis of indeterminate potential" (CHIP) progresses to MDS at a rate of 0.5% to 1% per year (see Chapter 19).[37–41] Smaller clones with a VAF < 1% are ubiquitous by middle age.[42,43] CHIP most commonly is driven by mutations in *DNMT3A, TET2*, or *ASXL1*, with high allelic heterogeneity, and pre-disposes to cardiovascular events due to clonal monocytes causing NLRP3 inflammasome-driven inflammation in the endothelium, which results in accelerated atherogenesis.[44,45]

MDS occurs in children much more rarely, at an estimated annual rate of 1 per 1 million.[46] Most cases have excess blasts and evolve to AML over time; other subtypes such as lower-risk MDS including 5q minus syndrome are even less common.[47] Several genetic syndromes confer an increased risk of MDS (see Chapter 30); these include Down syndrome,[48] Fanconi anemia,[49] dyskeratosis congenita and other telomeropathies,[50] Shwachman-Bodian-Diamond syndrome,[51] and germline mutations in *GATA2*,[52] *RUNX1*,[53] *ETV6*,[54] and *DDX41*[55] among others.[39] In patients with Fanconi anemia, GATA2 mutations, or telomeropathies, MDS arising during young adulthood may be the initial presenting sign of inherited conditions that in other patients affect multiple organ systems and result in diagnosis in early childhood.

Most subtypes of MDS appear to be more common in men than women, with SEER data suggesting respective age-adjusted incidences of 6.7 versus 3.9 per 100,000.[56] One exception to this is MDS with isolated del(5q), which most series show to be slightly more common in women.[57] The reason for these sex differences is unclear; some have postulated a protective factor encoded on the X chromosome, or a similar protective factor on the Y chromosome, which is clonally lost in some cases of MDS, as well as in many older healthy men. Others have suggested that the increased incidence in men could be due to differences in occupational exposures, but this has never been clearly demonstrated. In fact, the only toxic chemical exposure definitively proven to cause MDS is benzene,[58] which is now significantly less prevalent in industrial workplaces than it had been in the past. Other environmental exposures, including cigarette smoking, have been suggested to predispose to MDS.[59] Agent Orange, a defoliant used by the US Army in the Vietnam War and frequently diluted with hydrocarbons to facilitate spraying, has been anecdotally linked to MDS risk, but given the lack of ongoing exposure in the population, a definitive causal connection is difficult to prove.[60]

MDS in the United States and Western Europe are epidemiologically similar, but there are differences between these geographical regions and other parts of the world. In Asia[61] and Eastern Europe,[62] for instance, the average age at MDS diagnosis is younger, and the frequency of both severe cytopenias and risk of progression to leukemia may differ.[63] Some of these differences are likely due to variance in environmental exposures. In Japan, for example, broad population-wide exposure to ionizing radiation from the atomic bombings of Hiroshima and Nagasaki in the 1940s continued to influence MDS incidence well into the 1990s.[64] However, the cause of differences in MDS subtypes, such as the low incidence of MDS with ring sideroblasts in Japan compared with the West, remain unclear.

The two exposures most consistently associated with subsequent development of MDS are ionizing radiation and cytotoxic chemotherapy, and MDS arising in these settings, known as therapy-related MDS or therapy-related (treatment-related) MDS (t-MDS), is frequently characterized by TP53 mutations,[65] multiple chromosomal abnormalities including complex karyotypes (most commonly defined as ≥3 clonal chromosomal anomalies),[66] and frequent transformation to treatment-refractory AML.[67] In the United States, radiation is most frequently encountered as treatment for other cancers, and radiation fields that include the hips or pelvis, the most active sites of hematopoiesis in adults, probably pose the greatest risk.[68] Less commonly, exposure to radiation can occur as the result of occupational exposures (e.g., workers at nuclear reactors) or industrial accidents. Among chemotherapeutic agents, there is a substantial difference in the risk of subsequent MDS. In particular, alkylating agents (e.g., cyclophosphamide and melphalan),[69] platinum-based agents,[70] and fludarabine[71] appear to carry the greatest risk, while the risk with other drugs is lower. PolyADP ribose polymerase (PARP) inhibitors also appear to increase the risk of therapy-related MDS, although many people who receive PARP inhibitors have also received other therapies such that the independent contribution of the PARP inhibitor may be unclear.[72] The rapidly progressive AML seen in association with exposure to topoisomerase inhibitors (e.g., doxorubicin, etoposide) is not typically preceded by MDS.[73]

PATHOBIOLOGY

Our understanding of the complex pathobiology underlying MDS has evolved substantially since their first description. MDS is now recognized to arise from the acquisition of sequential mutations in hematopoietic stem cells (HSCs) that either confer clonal advantage, impair normal leukocyte function, or both. In some cases, this cell-autonomous expansion of a malignant clone is complemented by non-cell autonomous alterations in the marrow microenvironment and immune surveillance that allow the clone's expansion. This section details our current understanding of these concepts.

Myelodysplastic Syndromes Cell of Origin

One of the central challenges in understanding the pathogenesis of MDS has been isolating the cell of origin and understanding that cell's mechanisms of self-renewal and propagation, both of which are necessary for the establishment of a malignant clone.[74] The capacity for self-renewal on its face implies that the origin cell is either a hematopoietic stem cell, and thus possesses intrinsic self-renewal capabilities, or is a more differentiated myeloid progenitor that acquired the ability to self-renew. Additional alterations may confer other characteristics associated with MDS, such as enhanced proliferation, resistance to apoptosis, specific types of morphologic dysplasia, or ineffective hematopoiesis and cytopenias, at which point MDS also becomes clinically evident.[75]

The clonal nature of MDS was first established in the 1980s by studies that showed skewed inactivation of glucose-6-phosphate dehydrogenase (G6PD), an X-linked gene, in the hematopoietic cells of female MDS patients heterozygous for G6PD deficiency.[76] More recent studies have used deep sequencing techniques to track the clonal evolution from MDS into AML and have confirmed that in these cases, the pre-existing MDS is as highly clonal as the resulting secondary AML.[77]

Whether MDS is a disorder of stem or early progenitor cells has been more difficult to prove.[78] Xenotransplant experiments using immunophenotypically defined HSPCs (classically CD34$^+$ CD38$^-$ Lin−) from MDS patients have not shown a striking proliferative or self-renewal advantage for the MDS cells compared to normal controls,[79,80] and the degree to which these experiments accurately depict the clonal dynamics of MDS in humans is unclear. More recent studies combining immunophenotypic analysis with deep sequencing of clonal mutations in MDS cells have shown that the mutations appear to originate exclusively in the most primitive, stem-cell-like compartment,[81] and others have shown that differential expansion of specific progenitor compartments may vary between different phenotypes and risk profiles of MDS.[82] These studies have led to the conclusion that MDS is, in fact, a disorder of transformed HSCs.[83] The conceptualization of MDS as a stem cell disorder partly explains why it is so refractory to most attempts at conventional therapy.

Genetic Alterations

Like other cancers, the core hypothesis underlying MDS pathogenesis is that the originating clone of MDS increasingly diverges from normal HSCs, and ultimately undergoes frank malignant transformation, through the sequential accumulation of acquired genetic abnormalities.[84] Improvement in our understanding of how these abnormalities are acquired, how they interact with each other, and their impact on pathways affecting proliferation, self-renewal, and differentiation, has been one of the major advancements in the study of MDS over the last decade.

Several types of genetic abnormalities are found in MDS. The first to be recognized (beginning in the 1970s) were cytogenetic abnormalities on standard karyotyping,[85] which are present in about 50% of patients (see Chapter 57).[86] A second category of cryptic chromosomal aberrations, including microdeletions and copy number-neutral loss of heterozygosity (CN-LOH), are too small to be detected by karyotype but may be found with fluorescence in-situ hybridization (FISH)[87] or single nucleotide polymorphism (SNP) arrays.[88] The most common type of MDS-associated genetic abnormality, mutations in single genes, has been increasingly well-characterized, and clinical tests for recurrent mutations are becoming increasingly available—though not all mutations have equal clinical relevance.[84,89] Finally, epigenomic alterations—global aberrations in histone and chromatin modification—are common in MDS, and are often, though not always, associated with mutations in genes involved in epigenetic regulation.[90]

GERMLINE PREDISPOSITION

Although MDS is largely a disease of older adults, several inherited genetic disorders predispose to its development as well.[91] While most of these disorders are associated with development of bone marrow failure or MDS in childhood, some (including Shwachman-Diamond syndrome,[51] Familial Platelet Disorder-AML due to germline *RUNX1* mutations,[92] and *SAMD9* mutations[93]) can present in young adulthood, and a few (some telomeropathies[50] and germline *DDX41* mutations[55]) most commonly present in middle age or later (see Chapter 30). Similarly, although most are associated with other characteristic non-hematologic features, a few (such as some *GATA2* mutation disorders,[94] *DDX41* mutations, and familial platelet disorder with propensity for AML [FPD-AML]) have only hematologic abnormalities. A partial summary of the most common germline predisposition disorders is given in Table 61.2.

Somatic Mutations

Of the types of genetic abnormalities found in MDS, acquired mutations in individual genes are the most recently recognized (Table 61.3); they are also the most frequent, currently estimated to be present in at least 80% of MDS patients.[84,95] Most mutations are acquired randomly, either spontaneously (e.g., deamination of methylated cytosine to thymine), during DNA replication prior to cell division, or during DNA repair; mutations accumulate with age.[96,97]

One of the central challenges of understanding the genetics of MDS has been determining which mutations contribute to the pathogenesis of the disease and which do not. While HSCs accumulate many mutations over their lifetimes, the majority are of no pathogenic consequence and are thus termed "passenger" mutations. Meanwhile, HSCs acquire nonsynonymous exonic mutations with translational consequences ("coding" mutations) at a rate of about one mutation per decade,[98] though most of these will not affect clonal dynamics or cellular function. Since the incidence of MDS increases with age, HSCs from an average MDS patient might typically contain between 5 and 10 coding mutations, though the actual number varies widely between patients, against a background of hundreds of non-coding or synonymous single nucleotide variations (SNVs). Only a minority of coding mutations, the "drivers," actually contribute to the development of the MDS.[99]

Although the recurrently mutated genes in MDS can be characterized in several ways,[100–104] one of the simplest strategies categorizes them based on their typical position in MDS ontogeny. In this model, mutations that occur early in MDS pathogenesis, such as those in splicing factors, methylation enzymes, and certain epigenetic modifiers, are capable of driving clonal expansion, but their other phenotypic consequences are variable. Mutations that occur later in MDS development may not be capable of driving clonal expansion on their own, but their effects may complement or amplify the originating mutations, and they may be associated with clinical changes, such as worsening of cytopenias. Finally, the acquisition of activating mutations, especially within the RAS pathway, drives transformation to AML. This section is a brief summary of our current understanding of some of the most important genes and pathways that are recurrently deranged in MDS.

TABLE 61.2 Selected Germline Myelodysplastic Syndromes Predisposition Syndromes

Disorder	Cellular Defect	Gene	Inheritance	Features
Fanconi anemia	DNA repair	*FANC* genes (many)	Variable	Other cancers, facial and skeletal dysmorphologies, short stature, other systemic disorders
Diamond-Blackfan anemia	Ribosomal function	Multiple (*RPL* and *RPS* genes, others)	Mostly AD	Short stature, facial dysmorphologies, cardiac/renal abnormalities
Shwachman-Diamond syndrome	Ribosomal function	*SBDS* (most common), *DNAJC21, EFL1*	AR	Short stature, exocrine pancreatic deficiency, immunodeficiency
Telomeropathies	Telomere maintenance	*DKC1, TERT, TERC*, other telomerase complex components	Variable	Dyskeratosis congenita: nail dystrophy, skin pigmentation abnormalities Other: pulmonary fibrosis, cirrhosis, premature graying
FPD-AML	Abnormal RUNX1 function	*RUNX1*	AD	Thrombocytopenia, platelet dysfunction
MonoMAC, Emberger syndrome	Abnormal GATA2 function	*GATA2*	AD	MonoMAC: monocytopenia, hearing loss, warts, atypical mycobacterial infections; Emberger: lymphedema
SAMD9 mutation	Abnormal SAMD9 function	*SAMD9*	AD	Short stature, adrenal hypoplasia, infections
Li-Fraumeni syndrome	*TP53* defects	*TP53*	AD	Other cancers (breast, osteosarcoma, soft tissue sarcoma, CNS, adrenocortical carcinoma, etc.)
DDX41 mutation	Abnormal DDX41 function	*DDX41*	AD	Other hematologic cancers (AML, CML, lymphoma); presents in middle/older age

AD, Autosomal dominant; *AR*, autosomal recessive; *FPD-AML*, familial platelet disorder with propensity for AML.

Originating Mutations

The unifying feature of these mutations is that they occur early in MDS pathogenesis, which can be either inferred by high VAF indicating presence within the majority of cells in an MDS clone, or from analysis of sequential samples from single individuals. The same mutations are also commonly found in clonal hematopoiesis,[39,40,105] an asymptomatic condition in which driver mutations of MDS and AML are found in the blood of individuals with normal blood counts and without known hematologic malignancies, which shows that they are capable of driving clonal expansion as sole lesions.

Some of the common originating MDS mutations, including *TET2, DNMT3A*, and *ASXL1*, do not have significant phenotypic consequences beyond conferring a selective advantage to mutant HSCs. Splicing factor mutations (e.g., *SF3B1, SRSF2, U2AF1*), in contrast, are frequently associated with development of cytopenias and morphologic dysplasia on bone marrow examination. Somatic mutations in *TP53*, which is responsible for integrating the cellular response to DNA damage, constitute a unique category of high-risk MDS that frequently, though not always, develops after exposure to cytotoxic therapy[71,106,107] and is rarely associated with other cooperating mutations.[104,108]

TET2

TET2, which encodes a member of the Ten-Eleven Translocation gene family, is mutated in 20% to 30% of all MDS cases[109] and in 40% to 50% of CMML.[110] The *TET2* protein is a methylcytosine oxygenase responsible for converting 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) using iron and α-ketoglutarate (α-KG, produced by IDH1 and IDH2), and for further oxidizing 5hmC to 5-formyl- and 5-carboxycytosine.[111] These reactions contribute to demethylation through base excision repair back to unmodified cytosine.[112] Mutations in *TET2* tend to be inactivating frameshift or nonsense mutations or specific missense substitutions predicted to lead to abrogation of protein function, and many patients are either compound heterozygotes or have UPD at chromosome 4q, leading to effective abrogation of TET2 function.[113] Indeed, patients with *TET2* mutations have been shown to have globally altered methylation profiles.[114]

DNMT3A

The *DNMT3A* gene consists of 29 exons and encodes a 908-amino acid protein that, along with DNMT3B, is one of the two enzymes responsible for de novo CpG methylation independent of replication, whereas a third methyltransferase, DNMT1, is responsible for maintenance of baseline hemi-methylation during active replication.[115] Only *DNMT3A* mutations, however, have been found to occur recurrently in myeloid malignancies, perhaps suggesting differential expression in hematopoietic cells. *DNMT3A* mutations in patients with hematologic malignancies, which include truncating mutations and SNVs in key functional domains, are associated with reduced enzymatic function. For unclear reasons, *DNMT3A* mutations are relatively less common in MDS (5% to 15%) than they are in AML (30% to 50%)[116] or clonal hematopoiesis (60% to 80%).[39,40]

ASXL1

ASXL1 codes for a polycomb chromatin-binding protein and is involved in epigenetic regulation of gene expression.[117] It acts as a coactivator of the retinoic acid receptor and directly interacts with chemical modifiers of histones (e.g., NCOA1, a histone acetyltransferase, and LSD1, a histone demethylase). *ASXL1* mutations occur in about 10% to 29% of total MDS and myeloproliferative neoplasm (MPN) patients[100] and 40% of CMML patients.[118] Pathogenic *ASXL1* mutations are exclusively truncating variants in the C-terminal portion of the protein.[119] In studies of MDS cohorts, investigators have observed that *ASXL1* mutations are rarely associated with *DNTM3A* and *JAK2* mutations[120] but frequently co-occur with *TET2*, *SRSF2*, and *RUNX1* abnormalities.[100,121]

TABLE 61.3 **Genes Somatically Mutated in MDS**

	Gene	Frequency (%)	Notes
Originating	*TET2*	20–30 (MDS) 40–50 (CMML)	Enriched in CMML Mutually exclusive with IDH Often seen in older people without MDS (clonal hematopoiesis)
	DNMT3A	8–13	Often seen in older people without MDS (clonal hematopoiesis)
	ASXL1	10–20 (MDS) 30–40 (CMML)	Enriched in CMML Often seen in older people without MDS (clonal hematopoiesis)
	SF3B1	20–30	Strong association with RS
	SRSF2	10–15 (MDS) 40 (CMML)	Enriched in CMML
	U2AF1	5–12	Association with del(20q)
	TP53	10–12	Association with complex karyotype, therapy-related disease, poor prognosis
	PPM1D	<5	Association with therapy-related disease Not clearly a true driver
Intermediate	*RUNX1*	10–15	Can be somatic or germline
	BCOR	5–10	Frequently associated with RAS pathway mutations
	STAG2	5–10	Cohesin class mutations enriched in high-risk MDS and secondary AML
	EZH2	5–10 (MDS) 20–30 (CMML)	May be functionally involved in some cases of 7q−
	IDH1/2	<5	More frequent in AML
	ATRX	Rare	Associated with acquired thalassemia
Transforming mutations	*NRAS*	5–10	Seen in progression to AML
	KRAS	5–10	Seen in progression to AML
	NF1	<5	Can occasionally be germline
	PTPN11	<5	More common in JMML
	BRAF	Rare	Also seen in hairy cell leukemia

AML, Acute myeloid leukemia; *CMML*, chronic myelomonocytic leukemia; *GCPR*, G-coupled protein receptor; *IDH*, isocitrate dehydrogenase; *JMML*, juvenile myelomonocytic leukemia; *MDS*, myelodysplastic syndromes; *MPN*, myeloproliferative neoplasm; *RS*, ring sideroblasts.

SF3B1

SF3B1 encodes the component of the U2 small nuclear riboprotein complex (snRNP) responsible for 3′ branch site recognition and is the most frequently mutated splicing factor gene.[100] *SF3B1* mutations can be found in about 20% to 30% of all MDS patients. They have a particularly strong association with ring sideroblast morphology, with mutations found in >60% to 85% of patients with MDS-MLD-RS, MDS-SLD-RS, or MDS/MPN-RS-T, as well as a substantial proportion of other myeloid neoplasms in which ring sideroblasts can be found.[122,123] The most common mutations are heterozygous missense substitutions that change lysine to glutamate at codon 700 (K700E), with other less common hotspots in the same vicinity such as K666. *SF3B1* mutations tend not to be associated with complex karyotypes or poor-prognosis mutations.[124] They are clinically associated with isolated, transfusion-dependent anemia, preserved WBC and platelet counts, and a lower risk of progression to AML.[125] In cross-sectional studies of MDS patients, *SF3B1* mutations appear to confer a relatively good prognosis,[102] and they are relatively less common in high-risk MDS cohorts, such as those undergoing allogeneic hematopoietic stem cell transplant.[104]

SRSF2

SRSF2 encodes a member of the serine/arginine (SR)-rich family of pre-mRNA splicing factors that interacts with the U2 and U1 components of the spliceosome. After *SF3B1*, it is the second-most commonly mutated splicing factor, with mutations present in 10% to 15% of MDS[126] and 40% of CMML patients.[127] Almost all mutations are heterozygous missense substitutions for proline at codon 95, while a small minority of in-frame insertions or deletions affects the same region. *SRSF2* mutations co-occur with several other mutations, many of which are also frequently found in CMML, including *TET2*, *ASXL1*, *RUNX1*, and *STAG2*.[100] In contradistinction to patients with *SF3B1* mutations, patients with *SRSF2* mutations tend to have more dysplasia in the granulocytic lineage and less in the erythroid lineage, and patients consequently tend to have a less prominent transfusion requirement, more enrichment in the RAEB subtypes, and, at least in some studies, a greater risk of progression to AML.[128]

U2AF1

U2AF1 encodes an auxiliary factor in the U2 spliceosome that is responsible for recognizing the AG splice acceptor dinucleotide at the 3′ end of introns. *U2AF1* mutations occur in about 10% to 15% of patients with MDS.[129] Similar to other commonly mutated splicing factor genes, the two most common mutations are both heterozygous missense substitutions, again implying a dominant negative effect, and both appear to alter sequence specificity of pre-mRNA binding and splicing.[130] The two mutations are in separate zinc finger DNA binding domains, one at codon 34 toward the N terminal of the protein, and one at codon 157 toward the C terminal.

TP53

TP53 mutations, which result in inactivation of the DNA damage response (DDR) pathway,[131] define a unique evolutionary pathway in MDS. *TP53* mutations occur in about 10% to 15% of MDS patients and are universally associated with a poor prognosis.[104,108] *TP53* mutations are often not accompanied by other cooperating point mutations, suggesting that they may be able to drive MDS development as sole lesions. However, they have been shown in cell culture and animal models to be disadvantageous relative to wildtype cells in the absence of chemotherapy or radiation.[132] Consistent with these findings, they are enriched in cases of therapy-related MDS[104,106] and are often associated with complex and monosomal karyotypes that would likely not be tolerated in cells with functioning DDR pathways. They also frequently co-occur with del(5q) and may represent a progression pathway for patients with 5q− syndrome, including those treated with lenalidomide.[133]

PPM1D

PPM1D encodes a phosphatase that negatively regulates multiple components of the DDR pathway, including p53, ATM, ATR, CHK1, and CHK2,[134–136] as well as the MAP kinase pathway[137] and the NFKB pathway.[138] Under normal conditions, its role is to restore homeostasis after periods of cellular stress. Pathogenic *PPM1D* mutations truncate the protein near the C-terminus and confer a gain of phosphatase function,[139–142] thus downregulating the DDR and partially phenocopying *TP53* mutations. *PPM1D* mutations are frequently found in the blood of adults who have previously received cytotoxic therapy.[71,107,143] They have also been found in MDS patients, where they are strongly associated with prior therapy.[144] In MDS, *PPM1D* is frequently co-mutated with *TP53* and does not on its own appear to have a strong correlation either with prognosis or with other phenotypic features, such as complex cytogenetics.[144] It is therefore not clear whether *PPM1D* mutations actually drive MDS development or whether they mark bystander HSC clones that expand in the context of cytotoxic therapy.

Intermediate Mutations

The common feature of these mutations is that, while they are frequent in MDS, they almost never appear as sole lesions, are almost never found in clonal hematopoiesis, and usually appear to have subclonal relationships to founding mutations based on allele fraction. It is thus likely that most of these mutations are incapable of driving clonal expansion on their own but play important cooperative roles in MDS pathogenesis.

EZH2

EZH2 encodes the catalytic subunit of protein repressive complex 2 (PRC2), which promotes the di- and tri-methylation of lysine 27 on histone 3 (H3K27). Locally, methylated H3K27 results in closed chromatin and transcriptional repression, and global H3K27 trimethylation in particular is associated with reduced pluripotency and cellular senescence, suggesting a role for *EZH2* in regulation of cell fate.[145] *EZH2* resides on the long arm of chromosome 7, and its loss has been hypothesized to be at least part of the reason 7q− is a deleterious cytogenetic occurrence in MDS,[146] though not all 7q deletions affect the *EZH2* locus. Mutations in *EZH2* itself are found in 5% to 10% of patients with MDS[102] and 20% to 30% of patients with CMML.[147] Consistent with the model of *EZH2* as a negative regulator of pluripotency and survival, and in contrast to the gain of function mutations in lymphoproliferative neoplasms, *EZH2* mutations in MDS tend either to be inactivating frameshift or nonsense mutations, or missense mutations concentrated in the gene's SET domain, which is critical for DNA binding.[148,149]

RUNX1

RUNX1 (formerly known as *AML1*) is a transcription factor gene commonly mutated in MDS, and its biology is complex. It encodes the alpha subunit of the core binding transcription factor (CBF) and is involved in determining the lineage fate of hematopoietic stem cells.[150] *RUNX1* was initially identified as one of the genes involved in two different common pathogenic translocations: t(8;21), found in AML, and t(12;21), found in ALL.[151,152] Subsequently, germline point mutations in *RUNX1* were identified in autosomal dominant FPD-AML,[53] and later as somatic events in both sporadic AML and MDS.[153] Reflecting the complexity of *RUNX1* biology, mutations occur throughout the gene, can be either monoallelic or biallelic, and can be frameshift insertions or deletions or nonsense or missense substitutions. However, most mutations appear to have an inactivating effect on *RUNX1* function, either by affecting the DNA-binding RUNT domain or by disrupting the C-terminal protein interaction domain.[154] Many of the remaining mutations outside these regions appear to affect RUNX1 interactions with epigenetic regulators like MLL, thereby affecting histone methylation.[155]

BCOR

This gene encodes a corepressor of BCL-6, a transcription factor required for germinal B-cell development. However, it also has a noncanonical role as a component of the PRC1.1 complex, which monoubiquitinates the H2A histone component and leads to epigenetic silencing.[156] Truncating *BCOR* mutations are found in 5% to 10% of MDS cases, likely due to disruption of PRC1.1 function.[100,157,158] They frequently co-occur with TET2 mutations, a combination that in animal models has been shown to induce a rapidly fatal MDS due to derepression of key myeloid and HOXA genes, leading to myeloid skewing and clonal advantage, as well as simultaneous induction of p53 target genes in erythroblasts, which causes dyserythropoiesis and anemia.[159]

ETV6

ETV6 encodes an ets-like transcription factor with mostly repressive activity.[160] It is situated on the short arm of chromosome 12, and its role in hematologic malignancy has been best characterized by its involvement in recurrent translocations, including t(3;12)(q26;p13)[161] and deletions of 12p.[162] In MDS, however, both missense and inactivating frameshift point mutations have been described as well.[163] *ETV6* mutations are relatively rare events in MDS, occurring in at most 5% of cases[100]; recently, familial cases of MDS and AML due to inherited *ETV6* mutations have also been described.[54]

Cohesin Complex Genes

Genes encoding members of the cohesin complex family, including *STAG2*, *SMC3*, *RAD21*, and *SMC1A*, are each mutated in a small minority of MDS cases, but collectively, they can be found in about 10% of MDS.[164] They have multiple physiologic roles, including the promotion of chromosomal stability during mitosis, as well as the generation of chromatin looping structures that enable promoter interactions with distal enhancer elements.[165] Their role in MDS pathogenesis is largely driven by disruption of these epigenetic functions. *STAG2* mutations, which are the most common of this group, appear to have specific interactions with other myeloid transcription factors, including *RUNX1*.[121]

Transforming Mutations

Mutations in tyrosine kinase and growth factor receptor genes are typically associated with pro-proliferative signals and occur in a wide range of myeloid malignancies, including AML (*FLT3*[166]), myeloproliferative neoplasms (*JAK2*[167] and *MPL*[168]), and mast cell disorders (*KIT*[169]). While they do occur in MDS, they are usually late, subclonal events that often mark progression to secondary AML.[170] Activating mutations in *NRAS* are the most frequent tyrosine kinase mutations found in MDS,[171] but still only occur in about 5% of cases and as above tend to begin as subclonal, pro-proliferative events that frequently drive the transition to AML.[172]

Karyotypic Abnormalities

Chromosomal abnormalities, larger-scale genetic aberrations that can be detected on either karyotype or FISH, are also common events in

MDS (see Chapter 70).[173] The most common types of abnormalities in MDS are deletions or duplications of very large chromosomal regions; as opposed to some other hematologic cancers, translocations and inversions are less common. Several of the most common karyotypic abnormalities have been shown to have prognostic value, and one—del(5q)—has enough unique biologic features to be its own subclassification within the WHO criteria.

Del(5q)

Interstitial deletion of the long arm of chromosome 5 (del(5q)) is the most common chromosomal abnormality in MDS,[174] and del(5q) is the only karyotypically-defined subtype recognized by the WHO.[20] Although the specific region affected varies between patients, there are two commonly deleted regions (CDRs), one on 5q31.1 and the other at 5q32–33.3, with most patients having a deletion that includes both CDRs.[175,176] MDS patients in whom del(5q) is the sole karyotypic abnormality often display the "5q-minus syndrome," which is clinically characterized by anemia, normal or elevated platelet count, female predominance,[177] lower risk of transformation to AML, and a striking response to lenalidomide.[178]

The clinical and molecular physiology of 5q− syndrome is complex, and multiple genes lost in the CDRs are responsible for different aspects of the 5q− phenotype. First, haploinsufficiency of RPS14, a ribosomal subunit gene located at 5q31.2, is responsible for the dyserythropoiesis seen in the syndrome,[179] due to p53 expression[180] that is mediated by induction of innate immune signaling.[181] Second, deletion of a key microRNA, miR-145, is responsible for the megakaryocytic component of the phenotype.[182] Separately, haploinsufficiency of CSNK1A1, which is located at 5q32 and encodes casein kinase 1-alpha, is responsible for the sensitivity to lenalidomide, which accelerates the ubiquitination and degradation of remaining casein kinase 1-alpha through a cereblon-dependent process.[183] Importantly, the coordinate loss of RPS14, miR-145, and CSNK1A1 has a greater impact on the del(5q) impact than single loss of any one gene.[184] *TP53* mutations and 17p loss frequently develop in the setting of del(5q),[184] often acting as a resistance mechanism following lenalidomide treatment.

Chromosome 7 Abnormalities

Deletion of one entire copy of chromosome 7 (i.e., monosomy 7) is also characteristic of MDS and portends a poor prognosis.[185] The pathogenesis of chromosome 7 abnormalities is incompletely understood. Several genes recurrently mutated in MDS, including EZH2, *MLL3*,[186] and *CUX1*,[187] lie on 7q, and it has been hypothesized that loss of some or all of chromosome 7 contributes to MDS pathogenesis via a haploinsufficiency mechanism similar to that of del(5q). The evidence for such a mechanism, however, is weak: unlike del(5q), there is no common deleted region on chromosome 7 in which to focus efforts at driver gene discovery.[176,188] It is important to note that in the IPSS-R, del(7q) is considered to be an intermediate-prognosis abnormality distinct from monosomy 7, which is classified as poor prognosis.[189]

Trisomy 8

Trisomy 8 is present in about 5% of MDS patients and can be found in a wide range of other myeloid disorders, including AML, myeloproliferative neoplasms, and aplastic anemia. In MDS, it can often be seen as a late, subclonal event.[190] Although its contribution to pathogenesis is incompletely understood, MDS patients with trisomy 8 appear to upregulate *WT1*, an oncogene that can be mutated in AML (but very rarely in MDS), which appears to behave as a neoantigen that stimulates the expansion of oligoclonal $CD4^+$ and $CD8^+$ T cells.[191] Some evidence suggests that these populations may contribute to impairment of hematopoiesis in MDS patients with trisomy 8,[192] which may explain why some patients with isolated trisomy 8 can have substantial responses to immune suppression with anti-thymocyte globulin (ATG).[191] Trisomy 8 has also been associated with the development of paraneoplastic autoimmune phenomena, such as Behçet disease,[193] again reflective of the immune dysregulation characteristic of this cytogenetic abnormality.

Del(20q)

Del(20q) is an infrequent chromosomal aberration in MDS, occurring in about 2% of patients.[194] Patients with del(20q) frequently have prominent thrombocytopenia, may have concomitant mutations of *U2AF1*, and appear to have an intermediate prognosis,[195] although 20q loss can also be a late event that indicates clonal progression of disease.[196] Although *ASXL1* resides on 20q, it sits outside the CDR.[197]

17p Deletions

Chromosome 17 abnormalities occur most often in MDS in association with complex karyotypes, which is most likely related to the fact that *TP53* resides within the common deleted region on 17p.[198] In MDS, many patients with loss of 17p have an inactivating mutation of their remaining copy of *TP53*, implying that haploinsufficiency is not in and of itself enough to drive pathogenesis,[199] although some common *TP53* hotspot mutations may function as dominant negatives and not require loss of the second allele to drive malignant transformation.[132] As with *TP53* point mutations, loss of 17p is a universally poor prognostic factor in MDS and is frequently seen in cases of therapy-related disease.[69]

Complex and Monosomal Karyotypes

Complex karyotypes, defined by three or more cytogenetic abnormalities within the same clone, are common abnormalities in MDS.[173] About half are associated with *TP53* mutations; conversely, many patients with *TP53* mutations have complex karyotypes.[198] As opposed to 17p abnormalities, which are reflective of the *TP53* loss itself, complex karyotypes may instead be effects of p53 loss and large-scale structural DNA damage that would have triggered p53-mediated apoptosis in normal cells. Monosomal karyotypes, defined by loss of at least two entire chromosomes or one monosomy plus one other abnormality, are also common and also confer a poor prognosis, particularly when associated with monosomy 7 or monosomy 5.[200] When monosomal and complex karyotypes occur together, however, the monosomy does not appear to confer an additional poor prognosis beyond that of the complex karyotype.[201]

Other Karyotypic Abnormalities

A number of other cytogenetic abnormalities have been repeatedly described in MDS, including -Y,[202] del(13q),[70] del(11q), del(12p) or t(12p),[203] del(9q),[194] idic(X)(q13),[194] t(11;16)(q23;p13.3),[204] t(3;21)(q26.2;q22.1),[205] t(1;3)(p36.3;q21),[206] t(2;11)(p21;q23),[204] inv(3)(q21q26.2),[204] and t(6;9)(p23;q34).[207] Most of these do not have specific phenotypes associated with them, but some do confer prognostic information and are included in the IPSS-R. In particular, -Y and del(11q) are designated "very good" prognosis; del(12p), del(20q), isolated del(5q), and normal karyotypes are considered "good"; trisomy 8, del(7q), isochromosome 17(q), trisomy 19, and trisomy 21 are considered "intermediate;" monosomy 7, double deleted (7q), derivative (3q), and complex karyotype with exactly three abnormalities are considered "poor"; and complex karyotypes with more than three abnormalities are considered "very poor."[20]

The Marrow Microenvironment in Myelodysplastic Syndromes

MDS develops not in isolation but within the microenvironment of the bone marrow stroma, also called the "hematopoietic niche" (see Chapter 14).[208] Under normal circumstances, hematopoiesis is in part regulated by paracrine signals released from non-hematopoietic mesenchymal cells,[209] and some of these processes are dysregulated in MDS. For example, some patients with MDS have abnormal circulating levels of cytokines known to affect hematopoiesis, including monocyte colony-stimulating factor (M-CSF), interleukin 1a, and granulocyte–monocyte colony-stimulating factor (GM-CSF),[210] and hematopoietic cells from MDS patients respond abnormally to these cytokines compared to cells from healthy controls.[211] Additionally, hematopoietic progenitor cells transplanted into older mice display a narrower range of clonal expansion than identical cells transplanted into younger mice, suggesting that an aging niche might exert selective pressure on dominant HPC clones.[212] Conditional deletion of the gene encoding dicer1, a microRNA processing enzyme, in osteoprogenitor cells can induce myelodysplasia and secondary AML in mice,[213] and mesenchymal cells of MDS patients display altered expression of DICER and DROSHA, another microRNA-processing enzyme.[214] Finally, introduction of an activating B-catenin mutation in mouse osteoblasts can induce the development of AML through induction of the Notch ligand jagged1, which leads to Notch activation in HSCs.[215]

While these studies show that non-cell autonomous features of the marrow microenvironment can induce myelodysplasia in model systems, it is not clear whether this is a dominant mechanism of MDS initiation in humans. The majority of MDS cases have at least one detectable genetic or karyotypic abnormality,[100,102,144] suggesting that cell-intrinsic dysregulation is usually necessary for disease propagation. Moreover, many of the mutations common in MDS, including those in *TET2*,[45,216] *DNMT3A*,[217] and many of the splicing factor genes,[218] lead to altered inflammatory signaling in terminally differentiated leukocytes. It is therefore likely that at least some microenvironmental dysfunction is a consequence of MDS clones, not a cause.

Immune Dysregulation

In normal human tissues, both the innate and adaptive immune systems play important roles in identifying, isolating, and destroying early clones with malignant potential before they are able to transform into clinically evident cancers. Perturbation of this immune response presumably allows the persistence and propagation of abnormal clones in MDS, but the extent to which this is pathogenically important is not clear.[219]

Several aspects of innate immunity can be abnormal in MDS patients.[220] Some toll-like receptors, including TLR4, are overexpressed on MDS progenitor cells,[221] and increased TLR signaling has been associated with higher degrees of apoptosis and the development of cytopenias. Myeloid-derived suppressor cells are significantly expanded in some patients with MDS, and increased signaling in these cells via interaction with S100A9, an important inflammatory mediator, stimulates inappropriate apoptosis in hematopoietic precursor cells.[222] In fact, activation of the NLRP3 inflammasome may be a convergent mechanism of clonal expansion and pyroptotic cell death that is activated by many of the common drive mutations found in MDS.[218] Coincident with these findings, population-level data suggest that patients with some chronic inflammatory stimulation are at increased risk of developing MDS and AML.[223]

Abnormalities in the adaptive immune system also exist in MDS. These have been particularly well described in hypoplastic variants, which have similarities to aplastic anemia at both the cellular and genetic level, and both can respond to immune suppression.[224] Oligoclonal T-cell receptor gene rearrangements can be found in MDS patients of all subtypes,[225] and clinically detectable abnormalities of NK-cell, B-cell, and T-cell number and function can be detected in a subset of patients as well.[226–228]

Abnormal Apoptosis

Although MDS is typically characterized by normal or increased marrow cellularity, abnormalities of apoptosis are frequently described, particularly in low-risk variants, where increased apoptosis has been hypothesized to contribute to the development of ineffective hematopoiesis.[229] There are two major mammalian apoptosis pathways. The death receptor pathway, also known as the external pathway, is triggered by ligation of tumor necrosis factor (TNF) family members, which leads to the recruitment and activation of caspases at the cell surface.[230] There is ample evidence of abnormalities in this pathway in MDS; for instance, TNF-α levels have been shown to be increased in all MDS subtypes,[231] and other proapoptotic cytokines, including IL-8, transforming growth factor β, interferon-γ, and Fas ligand, have been shown to be elevated as well.[232]

The other major apoptotic pathway, known as the BCL-2 or intrinsic pathway, involves a balance between pro-apoptotic (BAX, BAK, BAD, PUMA, and others) and anti-apoptotic molecules (BCL-2, BCL-XL, MCL-1, and others),[233] and can be abnormal in MDS. For example, ratios of pro-apoptotic (Bax/Bad) to anti-apoptotic (Bcl-2/Bcl-XL) molecules are elevated in patients with lower-risk subtypes, but the ratio reverses in MDS with excess blasts and secondary AML, primarily driven by increased BCL-2 expression.[234] Consistent with this observation, but somewhat paradoxically, inhibition of apoptosis by BCL2 can prevent leukemic transformation in murine models of MDS.[235] Preclinical studies further suggest that inhibition of BCL-2 and other anti-apoptotic molecules may delay progression of disease and may sensitize MDS cells to hypomethylating agents such as azacytidine[236]; clinical trials of BCL-2 inhibitors such as venetoclax in MDS are currently ongoing.

Transformation to Acute Leukemia

Some patients with MDS go undiagnosed until they present with AML. An antecedent unrecognized MDS phase may be suspected on the basis of dysplastic-appearing morphology or long-standing incompletely evaluated cytopenias,[20] but can also be inferred by the presence of MDS-associated mutations, including *SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*, *ASXL1*, *EZH2*, *BCOR*, or *STAG2*.[237] Absence of these mutations does not imply the converse conclusion that the AML arose de novo; while *NPM1* mutations appear to be restricted to de novo AML, mutations in *DNMT3A*, *TET2*, and *IDH1/2* are not ontogeny-specific. *TP53*-mutated AML comprises its own category and is associated with prior chemotherapy and complex karyotypes.[106]

In most cases, progression of MDS to AML involves the acquisition of pro-proliferative mutations, which tend to be late, subclonal events.[238] As discussed above, patients who develop secondary AML from an antecedent MDS tend to have a poorer prognosis, with both a lower rate of complete remission and a poorer overall survival, independent of remission, than patients with de novo AML.[237,239] In unselected populations, some of this difference in outcome can be explained by demographic characteristics associated with secondary AML, including older age and increased number of comorbidities, mirroring the characteristics of the MDS population. However, patients with secondary AML have inferior outcomes even compared to age-matched controls with de novo AML,[237] reflective of the refractory nature of the antecedent MDS. Overcoming the intrinsic resistance to therapy common to most patients with MDS remains one of the central challenges of treating the disease.

CLINICAL MANIFESTATIONS

Compared to other hematologic neoplasms, the clinical features of MDS are often underemphasized relative to laboratory aspects of the disease. This is due in part to the fact that the major common physiologic defect, ineffective hematopoiesis, usually leads to nonspecific signs or symptoms, such as fatigue or exercise intolerance.

Nevertheless, it is important to understand these features within the context of the disease's pathogenesis and management.

Some patients with MDS have distinct clinical phenotypes that are largely related to genetics or specific pathologic features. MDS can also coexist with other hematologic malignancies, including multiple myeloma,[240] hairy cell leukemia,[241] chronic lymphocytic leukemia (CLL),[242] non-Hodgkin lymphoma,[243] and large granular lymphocyte leukemia.[244]

Signs and Symptoms

Although some patients with MDS profess to being asymptomatic, fatigue is extremely common, as is a sense of general malaise.[245] Rarely, patients may complain of diffuse arthralgias or bone pain. Smaller proportions of patients may present with severe or recurrent infections as a result of immune defects, most commonly neutropenia or granulocyte dysfunction.[246] Bleeding or easy bruising as a result of thrombocytopenia or qualitative platelet defects also bring some patients with MDS to medical attention.[247] When present, fever is most commonly a sign of infection, though a small number of patients may have low-grade fevers in the absence of a discernible infection. Splenomegaly or hepatomegaly are rare in MDS and should prompt consideration of either an alternate diagnosis or an MDS/MPN overlap syndrome, such as CMML, which is defined by peripheral monocytosis and exclusion of other neoplasms or reactive conditions that can cause monocytosis.

Dermatologic Manifestations

Although dermatologic manifestations of MDS are uncommon,[248] a few deserve specific mention. Neutrophilic dermatosis (Sweet syndrome; Fig. 61.2) is characterized by painful plaques on the face, neck, trunk or extremities, often in association with fever and diffuse arthralgias.[249] While neutrophilic dermatosis can be seen in any subclass of MDS, it is classically associated with an impending transformation to AML. It frequently responds to steroids or dapsone therapy, but may recur as steroids are tapered.[250]

Pyoderma gangrenosum (PG; Fig. 61.3) is an ulcerative, necrotic lesion that most frequently develops on the extremities, and is occasionally associated with MDS.[251] Histologically, it is characterized by neutrophilic infiltrates; clinically, it may be associated with pathergy where minor trauma such as intravenous catheter insertion leads to the development of skin lesions or ulcers that may be resistant to healing. A high index of suspicion is necessary to distinguish it from necrotizing infections since treatment of PG involves systemic steroids and surgical debridement is contraindicated.

Other dermatologic manifestations of MDS include monocytic infiltrates, classically of the gingiva and other mucosal surfaces, a process most frequently seen in CMML[252]; the development of a chloroma or granulocytic sarcoma, which by definition indicates progression to AML; and petechial lesions, which most frequently develop on the lower extremities and are associated with severe thrombocytopenia.

Autoimmune Manifestations

Some patients with MDS may have rheumatologic features to their disease, which may in part arise from immune dysregulation that occurs during disease pathogenesis.[253] In a few patients, these may be the presenting complaint. Such manifestations can include episodes of seronegative oligoarthritis or polyarthritis,[254] cutaneous vasculitis,[255] inflammatory myositis,[256] lupus-like syndromes, or autoimmune peripheral neuropathies. Other autoimmune phenomena have also been reported, including mucocutaneous ulcerations similar to Behçet disease, iritis, inflammatory bowel disease, and pure red cell aplasia. Many of these conditions respond to the initiation of immunosuppressive agents such as corticosteroids or T-cell directed therapies.[257]

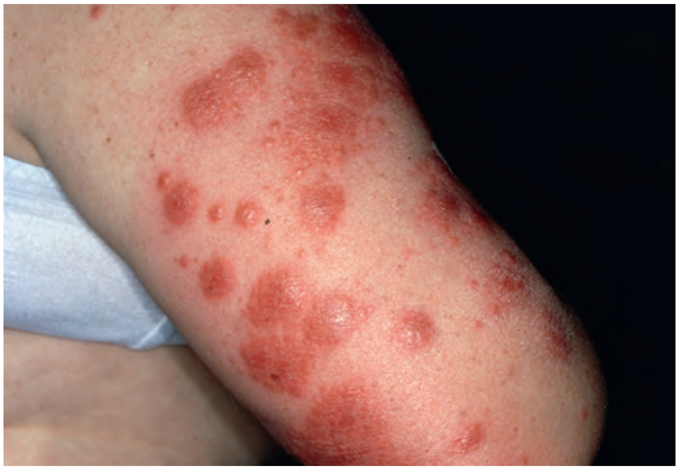


Figure 61.2 SWEET SYNDROME WITH ACUTE MYELOGENOUS LEUKEMIA. *(From James WD, Elston DM, McMahon PJ, et al., eds. Andrews' Diseases of the Skin Clinical Atlas. Amsterdam: Elsevier; 2017.)*

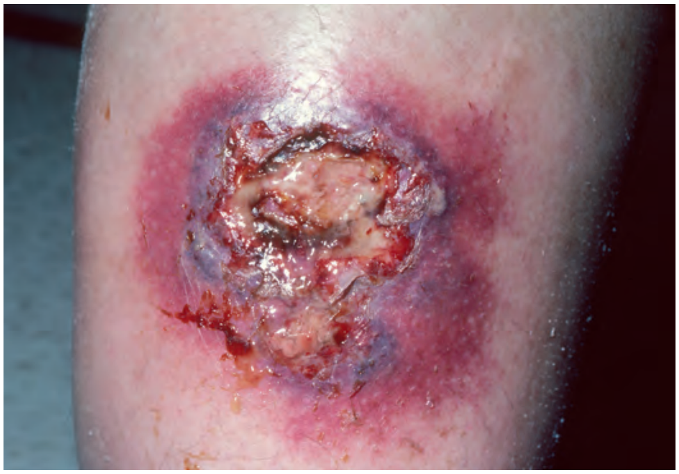


Figure 61.3 PYODERMA GANGRENOSUM. *(From James WD, Elston DM, McMahon PJ, et al., eds. Andrews' Diseases of the Skin Clinical Atlas. Amsterdam: Elsevier; 2017. Courtesy of Steven Binnick, MD.)*

LABORATORY MANIFESTATIONS

Erythroid Lineage

Most patients with MDS present with some degree of anemia, which contributes to fatigue, the most common presenting complaint.[258] The anemia is often macrocytic, and the peripheral blood smears of MDS patients frequently show a variety of abnormal forms, including oval macrocytes, and less commonly dacrocytes, elliptocytes, or acanthocytes.

Anemia in MDS is usually due to ineffective hematopoiesis. Consequently, the serum erythropoietin (EPO) level is typically elevated, though in many elderly patients it is still lower than would be expected for the degree of anemia, in some cases because of subclinical renal insufficiency.[259] Ineffective hematopoiesis causes abnormal iron utilization in a substantial number of patients, many of whom have evidence of iron overload based on ferritin or transferrin saturation even in the absence of transfusions.[260] Studies have shown other abnormalities of erythrocytes as well, including increased levels of fetal hemoglobin,[261] aberrant surface antigens,[262] increased osmotic fragility, and low levels of pyruvate kinase,[263] although none of these are routinely assessed in clinical practice. Hemolysis is rarely observed.

Morphologic changes in the erythroid lineage on bone marrow examination can vary widely from patient to patient. Many patients have megaloblastoid erythroid precursors that contain multiple nuclei or asynchronous maturation of the nucleus and cytoplasm (Fig. 61.4). It is this asynchrony, with continued membrane synthesis in the absence of normal nuclear maturation, which is thought to give rise to the macrocytosis displayed by most patients.

Ring sideroblasts (see Fig. 61.4), erythroid precursors containing iron-laden mitochondria, are another relatively common finding.[264] These are defined by the presence of at least five granules that are positive with the Prussian Blue reaction and surround at least one third of the circumference of the cell nucleus. Ring sideroblasts are not in and of themselves diagnostic of MDS, and can be seen in a number of other disorders including congenital sideroblastic anemias, anti-tuberculosis drugs, and nutritional deficiencies, but their presence in association with other dysplastic changes is highly specific for MDS and, as described above, is tightly linked to acquired *SF3B1* mutations.[122] Ring sideroblasts may be seen in all subtypes of MDS; the specific designation of MDS-SLD-RS or MDS-MLD-RS is made when ring sideroblasts comprise at least 15% of the erythroid precursors and blasts are not increased, or when ring sideroblasts are at least 5% of red cell precursors and an *SF3B1* mutation is present.

In a normal bone marrow, myeloid precursors typically outnumber erythroid precursors by a ratio of 2 to 4 to 1. A special case arises in cases of MDS in which this normal ratio is skewed toward the erythroid lineage, that is, 50% or more of the total cellularity is erythroid. If in this case 30% or more of the remaining cellularity is comprised of myeloblasts, the case may be diagnosed as erythroleukemia (WHO AML, not otherwise specified, subtype acute erythroid leukemia, erythroid/myeloid type; formerly FAB AML-M6A).[20]

Acquired hemoglobin H disease is a rare but well-described development in MDS that is associated with acquired mutations of *ATRX*,[265] a gene encoding a chromatin-remodeling protein. *ATRX* functional loss results in downregulation of globin synthesis in the alpha cluster (the genes themselves remain intact) but not the beta globin cluster. Patients with acquired *ATRX* mutations have clinical features resembling alpha thalassemia, including microcytosis, anisocytosis, poikilocytosis, target cells, schistocytes, dacrocytes, and beta-chain tetramers on crystal violet staining.[266] As opposed to the macrocytosis seen in most MDS patients, those with acquired *ATRX* mutations are typically profoundly microcytic, again similar to thalassemia.

Myeloid Lineage

About one-half of patients with MDS are neutropenic at the time of diagnosis, and more will become neutropenic with time.[267] Many also have defective neutrophil function irrespective of the absolute neutrophil count (ANC), and patients with MDS have been shown to have inadequate inflammatory responses to infections. Many have diminished production of hematopoietic growth factors like G-CSF and GM-CSF, and their neutrophils often have reduced phagocytic, chemotactic, or bactericidal capabilities.[268–270]

The granulocytes of MDS patients also display frequent morphologic abnormalities (Fig. 61.5). In the marrow, either myeloid hyperplasia or hypoplasia can occur, and there is often a prominent left shift toward immature forms. Myeloid precursors often display asynchronous maturation of the nucleus and cytoplasm. In promyelocytes this can manifest as an early hypergranulation coupled with reticulated nuclei and a prominent Golgi apparatus, but more mature myeloid forms are usually hypogranulated and hypolobated.

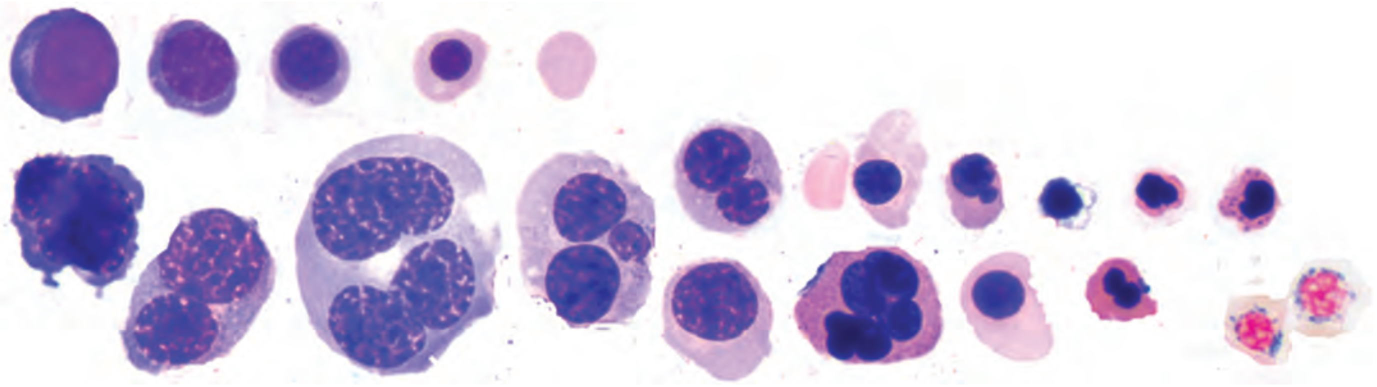


Figure 61.4 ERYTHROID DYSPLASIA. Examples of dysplastic erythroid precursors *(bottom)* compared to those with normal morphology in the sequence of erythroid maturation *(top)*. The dysplastic forms include (*left* to *right*) abnormal immature forms with multinucleation; maturing forms with multinucleation and nuclear-to-cytoplasmic dyssynchrony; and more mature forms with megaloblastoid change, nuclear budding, cloverleaf forms, cytoplasmic vacuolization, and cytoplasmic stippling. Ringed sideroblasts *(far right)* also are evidence of erythroid dysplasia. Photomicrographs are from patients with refractory cytopenia with multilineage dysplasia and ringed sideroblasts.

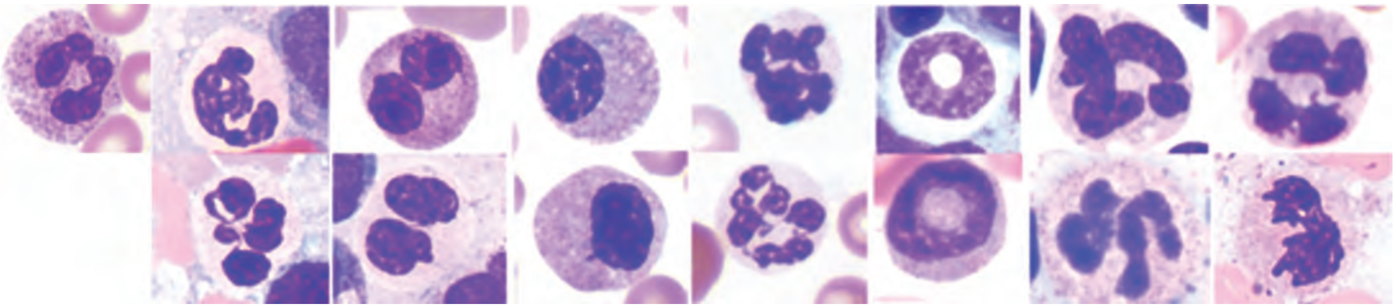


Figure 61.5 GRANULOCYTIC DYSPLASIA. Granulocytic dysplasia is most evident in mature neutrophils and can be contrasted to features of normal forms *(far left, top)*, which are usually still present as a subpopulation of the total cells in most cases. Granulocytic dysplasia is characterized by *(left to right, starting at second column)* reduced cytoplasmic granulation, nuclear hypolobation (resulting in the binuclear or single-lobed pseudo–Pelger-Huët forms), hypersegmentation, ringed forms ("rodent cells"), cells with nuclear twinning, and cells with excessive nuclear excrescences. Photomicrographs are from a number of cases of refractory cytopenia with multilineage dysplasia and refractory anemia with excess blasts.

The myeloid series may also be abnormally localized within the marrow: rather than differentiating in an organized fashion inward from the endosteum, immature cells often cluster centrally in a morphologic process known as abnormal localization of immature precursors (ALIP). ALIP has been shown to occur in most cases of MDS-EB and in about one-third of cases in which the blast percentage is less than 5%.[271] In these latter cases, ALIP may be an independent risk factor for subsequent development of AML.[272]

Morphologic characterization of the myeloid series is particularly important with regard to the blast count, since ≥20% myeloblasts is characterized as AML using the WHO classification, and ≥5% qualifies as MDS-EB. Even patients with lower blast proportions, however, still have an increased risk of developing AML, and some have proposed designating any MDS patient with more than 2% marrow blasts as having "oligoblastic leukemia."[273]

In the blood, morphologic abnormalities include pseudo Pelger-Huët cells, which have condensed chromatin and bilobed nuclei resembling an old-fashioned pince-nez (true Pelger-Huët cells are a benign congenital abnormality). Granulocytes may display other nuclear abnormalities as well, including hypersegmentation reminiscent of vitamin B_{12} deficiency, or aberrant ring shapes. The hypogranulation visible in the marrow typically persists in the peripheral blood, and the left shift typical of MDS marrows is often, though not always, present to some degree in the blood as well.

Despite these quantitative and qualitative defects in leukocytes, only a minority of MDS patients have problems with recurrent infections.[274] When infections do occur, they tend to be bacterial and often arise from the lower respiratory tract, skin, and mucous membranes. Patients without absolute neutropenia may still develop recurrent infections as a consequence of abnormal neutrophil function. Even though only some patients have recurrent infections, an infection is the most common cause of MDS-associated death.

Megakaryocytic Lineage

Thrombocytopenia is present in 25% to 50% of MDS patients at the time of presentation,[267] and some patients also have functional platelet defects, even if the platelet count is normal.[275] Laboratory abnormalities include prolonged bleeding times, defective granulation, and abnormal platelet aggregation indices mediated by hypofunctional platelet glycoprotein IIb/IIIa, leading to a defect similar to Glanzmann syndrome.[276,277] These defects can occasionally manifest as spontaneous bleeding, or can be unmasked after trauma or surgery.

Thrombocytosis, by contrast, is relatively unusual in patients with MDS, except in those with MDS/MPN overlap syndromes or 5q− syndrome.[278] *JAK2* mutations, in particular, are associated with thrombocytosis, and are enriched in MDS/MPN overlap cases.

In the marrow, megakaryocytes are most commonly present in normal or increased numbers. A number of morphologic abnormalities can be observed, including abnormally small forms (micromegakaryocytes), hypersegmentation, and nuclear hypolobation (Fig. 61.6).[279,280] Megakaryocytes may also be abnormally distributed in clusters scattered throughout the marrow in MDS, rather than their normal parasinusoidal positioning.

Lymphoid Lineage

Abnormalities in the lymphoid lineage have been described in subsets of patients with MDS. Some patients have been shown to have global decreases in NK cells,[226] deficient receptor localization on B cells,[228] or abnormal T-cell responses to mitogenic stimuli.[227] Certain types of immune functions, however, appear to be preserved, such as antibody-dependent cellular cytotoxicity.[281]

The mechanism by which these abnormalities develop in MDS is unclear. One hypothesis proposes that they could be indirectly reflective of shared somatic defects in a multipotent lymphoid-myeloid progenitor, which is possible given that most driver mutations in MDS patients are present in the LT-HSC compartment and can therefore be passed both to myeloid and lymphoid cells.[81] Alternatively, a number of immune functions, including both B-cell and T-cell diversity, diminish as a function of normal aging,[282,283] and in this context, deficiencies of immune function could develop as a consequence of lymphoid cells arising from and interacting with an abnormally aged hematopoietic niche.

Other Objective Findings

Patients with MDS may have a variety of other laboratory abnormalities as a consequence of their disease. Ineffective erythropoiesis may lead to inappropriate iron deposition, resulting in abnormally elevated ferritin and transferrin saturation, which may be exacerbated by congenital variants in iron processing.[284] Nonspecific markers of cellular turnover, such as elevations in lactate dehydrogenase,[285] uric acid, and phosphate, may also be seen. Reflective of underlying immune dysregulation, some patients may have abnormalities of immunoglobulins, including hypogammaglobulinemia, polyclonal hypergammaglobulinemia, or monoclonal gammopathies.[286,287] Whether this latter phenomenon is directly related to MDS or to an unrelated, early plasma cell dyscrasia (given that most patients with MDS are elderly, and the risk of monoclonal gammopathy increases with age) has not been definitively established.

Specific Clinical Syndromes

Although MDS is a heterogeneous disorder, it includes a number of specific entities, either defined by cytogenetics, pathologic findings, or clinical features, which possess distinctive clinical features or biologic behaviors. Some of these are discussed in more detail below.

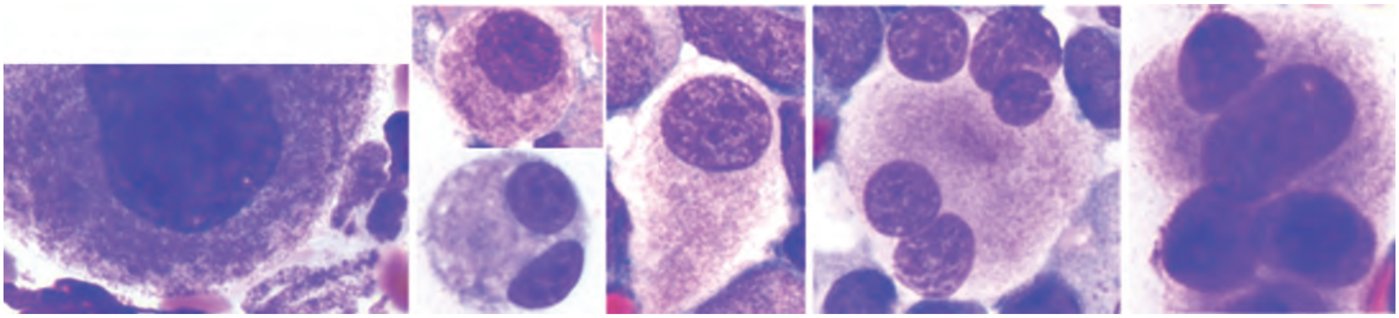


Figure 61.6 MEGAKARYOCYTIC DYSPLASIA. Dysmegakaryopoiesis is most obvious with the presence of micromegakaryocytes and abnormal larger forms *(panels 2–5)*. These are compared with a normal megakaryocyte at same magnification *(panel 1, far left)*. Micromegakaryocytes have single, two, or four small nuclei, which indicate a low-ploidy level. Normal low-ploidy megakaryocytes can be seen in the bone marrow, but these are immature forms and do not have mature granular cytoplasm with platelet material, as do the micromegakaryocytes. Larger dysplastic megakaryocytes have multiple, small, widely spaced nuclei. Photomicrographs are from a number of cases of refractory anemia with excess blasts and refractory cytopenia with multilineage dysplasia.

The 5q– Syndrome

Patients with isolated loss of the long arm of chromosome 5 (so-called del(5q) or 5q minus syndrome) are a unique group with a pathobiology that is described in more detail above. Clinically, 5q– syndrome is characterized by a predominant anemia with normal or elevated platelet counts, and a relatively indolent course with progression to AML in fewer than 25% of cases.[288,289] For unclear reasons, this syndrome is more common in women, who comprise 60% to 70% of cases in most series. The anemia in lower-risk del5q MDS is usually responsive to lenalidomide,[178] which is discussed in more detail in the Treatment section below. Those patients who become refractory to lenalidomide typically are transfusion dependent, and management of iron overload then becomes increasingly important. Many such patients also acquire abnormalities in *TP53*.

The designation of 5q– syndrome applies only to patients with the characteristic morphology and in whom loss of 5q is the only cytogenetic abnormality (Fig. 61.7A–C). Patients in whom 5q– is only one of several cytogenetic aberrations tend to have a poorer prognosis, with a more rapid progression to AML. As referenced above, loss of 5q often occurs in tandem with *TP53* mutations (or loss of 17p), another poor-prognosis combination.[133]

Hypocellular Myelodysplastic Syndromes

Most patients with MDS have hypercellular or normocellular marrows, adjusted for age. Hypocellular marrows, in contrast, are found in fewer than 15% of patients (Fig. 61.8A).[224] These patients have substantial overlap, both morphologically and clinically, with other hypoplastic syndromes, including aplastic anemia,[290] PNH,[291] and T-cell large granular lymphocyte leukemia.[244,292] While extensive dysplasia favors MDS (see Fig. 61.8B), distinction between these entities can sometimes be difficult, as patients with MDS may occasionally have PNH or LGL clones detectable by flow cytometry, though these are usually small and transient. Some of the resemblance may in fact reflect a shared pathogenesis; for instance, studies have shown that a sizable minority of aplastic anemia cases harbor clonal somatic mutations in genes recurrently mutated in MDS, including *ASXL1* and *DNMT3A*,[293] and that these patients have an inferior prognosis and lower likelihood of response to immunosuppressive therapy compared to patients with mutations in *PIGA* and *BCOR*.[294] It is therefore possible that these studies are defining distinct disease processes within a homogeneous-appearing morphology, with *ASXL1* and *DNMT3A* mutations defining disease more similar to MDS, and *BCOR* and *PIGA* mutations defining disease more akin to true, immune-mediated aplasia. Finding somatic mutations characteristic of the alternative diagnoses (for instance, telomerase complex mutations in aplastic anemia,[50] or *STAT3* mutations in LGL[295]) may clarify the picture as well.

Myelodysplastic Syndromes/Myeloproliferative Neoplasm Overlap Syndromes and Myelodysplastic Syndromes With Fibrosis

Although previously included as a subtype of MDS, disorders with overlapping features of both MDS and MPN are no longer included

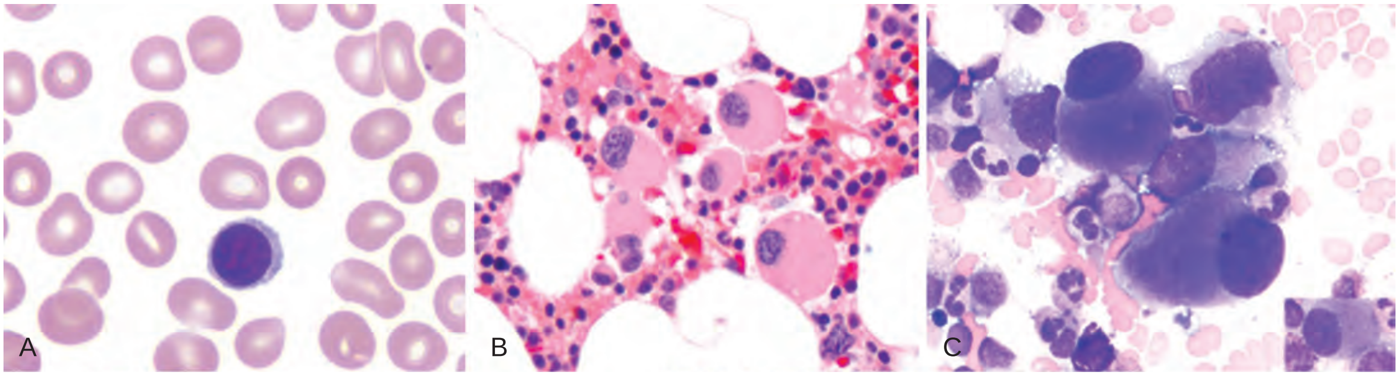


Figure 61.7 THE 5Q– SYNDROME. The 5q– syndrome has specific morphologic correlates. There is a macrocytic anemia (A) and a cellular bone marrow characterized by increased small monolobated megakaryocytes (B) and (C). The megakaryocyte nuclei have little segmentation and are fairly round. Although some true micromegakaryocytes may be present (C, *inset*, same magnification), the typical monolobated forms are not as tiny as the micromegakaryocytes.

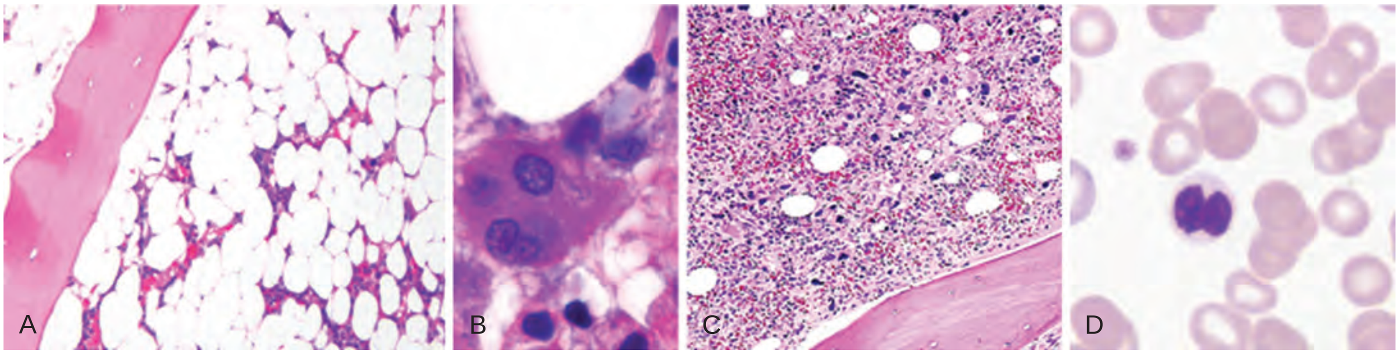


Figure 61.8 HYPOCELLULAR MYELODYSPLASTIC SYNDROME AND MYELODYSPLASTIC SYNDROME WITH FIBROSIS. Hypocellular myelodysplastic syndrome (A and B) can present a diagnostic problem and can be difficult to differentiate from aplastic anemia. Dysplasia may be difficult to evaluate if the smears are paucicellular. The finding of dysplastic megakaryocytes (note small widely separated nuclei, C) on the biopsy sample can be helpful. Myelodysplastic syndrome with fibrosis (D) can be difficult to differentiate from myeloproliferative neoplasms (MPNs). However, the lack of large megakaryocytes, which typically are see in the MPNs along with presence of dysplasia in the circulating neutrophils are useful clues to the correct diagnosis.

in the WHO classification system and are now considered a distinct category. MDS/MPN overlap entities, which include CMML, juvenile myelomonocytic leukemia, atypical chronic myeloid leukemia, MDS/MPN with ring sideroblasts and thrombocytosis (in which *JAK2* or *MPL* and *SF3B1* mutations usually co-exist), and unclassifiable cases, are discussed in more detail elsewhere (see Chapter 70 and 71). CMML can present as MDS with cytopenias but with absolute monocytosis, or with leukocytosis and WBC > 13×10^9/L ("proliferative CMML") driven by aberrant GM-CSF signaling. The monocytes in CMML with have a classical immunophenotype ($CD14^{++}/CD16^{-}$) and precursor states exist akin to CHIP.[296] The mutation profile in CMML is enriched for somatic mutations in *SRSF2, CBL, ASXL1, SETBP1*, and Ras pathway members, compared to MDS without proliferative features.[297,298]

Mildly increased marrow reticulin is common in MDS but marked fibrosis (see Fig. 61.8C) rarely occurs in cases of MDS without myeloproliferative features. When it does occur, it can be difficult to distinguish these cases from MPN.[299] Extensive dysplasia (see Fig. 61.8D) favors MDS.

Therapy-Related Myelodysplastic Syndromes

T-MDS is a well-recognized consequence of cytotoxic chemotherapy or radiotherapy for other cancers, but the overall incidence varies depending on the type of cancer and type of therapy.[300,301] Therapy-related MDS has been best characterized in patients with a prior history of breast cancer,[68,302] lymphoma,[303,304] or myeloma,[305] where the overall incidence is usually reported at around 1%. On the other hand, it is rare following treatment for other types of tumors, such as gastrointestinal and genitourinary cancers. This difference is largely attributed to differences in the types and intensities of chemotherapeutic agents used to treat different tumor types. In particular, high doses of alkylating agents, such as cyclophosphamide, melphalan, chlorambucil, or busulfan, have been recurrently associated with classical t-MDS,[306] as has extensive radiation therapy.[307]

T-MDS was initially thought to be driven by the acquisition of large-scale chromosomal damage induced by the cytotoxic therapy, given both the mechanism of alkylators (which induce double-stranded DNA breaks) and the fact that many patients with t-MDS have complex karyotypes. This model of DNA damage as the initiating event, however, was undercut by analysis showing that cytogenetic abnormalities present in the bone marrow of patients who developed therapy-related MDS after autologous transplant for lymphoma could be found in specimens banked at the time of the transplant, years before the development of t-MDS.[308] Subsequent analysis of a small group of patients with therapy-related AML harboring *TP53* mutations showed that clones harboring the mutations preceded the development of leukemia by years, and in some cases could be detected prior to the original initiation of chemotherapy.[106] Additional studies have confirmed that patients with detectable clonal hematopoiesis at the time of chemotherapy treatment are at increased risk of developing t-MDS due to selection of chemoresistant clones, especially those harboring *TP53* mutations, by cytotoxic therapy.[71,107,309,310] These findings suggest that most cases of t-MDS are driven by the expansion of pre-existing mutant HSC clones that gain a selective advantage upon exposure to cytotoxic therapy, rather than accumulation of DNA damage caused by the therapy itself.

Therapy-related MDS has relatively distinct clinical behavior. It is characterized by a latency of years and recurrent large-scale chromosomal abnormalities, especially of chromosomes 5 and 7 or 11q23. Complex karyotypes are also common.[311] These karyotypic abnormalities, which occur in only about 10% to 15% of patients with de novo MDS, are present in more than half of patients with t-MDS. They have a more aggressive clinical course than patients with de novo disease, with more frequent progression to acute leukemia. They also respond poorly to all classes of treatment, and relapse is common even after allogeneic stem cell transplant.[144,237,312] These clinical associations are driven by the unique biology of t-MDS, including the enrichment of *TP53* mutations and adverse karyotypes, and not by the prior cytotoxic therapy on its own. Patients with clinically defined therapy-related disease who lack these abnormalities have a prognosis more similar to de novo MDS.[144,237]

DIFFERENTIAL DIAGNOSIS

In the absence of clonal markers or excess blasts, MDS is a diagnosis of exclusion (Table 61.4), requiring the exclusion of other potential contributors to cytopenias and dysplasia.[3] Since cytopenias are the most common presenting clinical feature and cytopenias have many potential causes, the differential diagnosis is broad and includes numerous other entities covered in more detail elsewhere in this text. All patients suspected of having MDS should undergo a bone marrow aspirate and biopsy, since the diagnostic criteria are almost entirely pathologic and marrow findings are critical for risk stratification and treatment planning.

TABLE 61.4 **Diagnostic Criteria for Myelodysplastic Syndromes**

A. Presence of at Least One Unexplained Cytopenia:		
Hemoglobin <11 g/dL, *or*		
Absolute neutrophil count <1.5 × 10^9/L, *or*		
Platelet count <100 × 10^9/L		
plus **B. Presence of One or More MDS-Qualifying Criteria:**		
>10% dysplasia in one or more hematopoietic lineage without another explanation, *or*		
5%–19% blasts in bone marrow, *or*		
An MDS-associated cytogenetic abnormality, such as:		
t(1;3)(p36.3;q21.1)	t(2;11)(p21;q23)	inv(3)(q21;q26.2)
t(3;21)(q26.2;q22.1)	−5 or del(5q)	t(6;9)(p23;q34)
−7 or del(7q)		del(9q)
del(11q)	t(11;16)(q23;p13.3)	del(12p) or t(12p)
	i(17q) or del(17p)	idic(X)(q13)
plus **C. Exclusion of Alternative Diagnoses**		
AML (i.e., <20% blasts, and no t(8;21), inv(16), t(16;16), t(15;17), or erythroleukemia) or *ALL*		
Other hematologic diseases (aplastic anemia, PNH, LGL leukemia, lymphoma, myelofibrosis and other MPN)		
Viral infections (HIV, EBV, parvovirus)		
Nutritional deficiencies (iron, copper, B_{12}, folate)		
Medications (methotrexate, azathioprine, isoniazid, cytotoxic chemotherapy)		
Alcohol or other toxins		
Autoimmune diseases (SLE, Felty syndrome, ITP, autoimmune hemolytic anemia)		
Congenital disorders (Diamond-Blackfan anemia, Shwachman-Diamond syndrome, Fanconi anemia, and others)		

[a] Diagnosis can be made earlier than 6 months if no other cause is apparent for cytopenias, or there are excess blasts or an MDS-defining cytogenetic abnormality.

ALL, Acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *EBV*, Epstein-Barr virus; *HIV*, human immunodeficiency virus; *ITP*, immune thrombocytopenic purpura; *LGL*, large granular lymphocyte leukemia; *MDS*, myelodysplastic syndromes; *MPN*, myeloproliferative neoplasm; *PNH*, paroxysmal nocturnal hemoglobinuria; *SLE*, systemic lupus erythematosus.

Several nutritional deficiencies can cause cytopenias and morphology similar to MDS (see chapter on nutritional deficiencies). Folate deficiency can cause macrocytosis and, in extreme cases, megaloblastic morphology in the marrow.[313] Vitamin B_{12} (cobalamin) deficiency is a more classic cause of megaloblastic changes, and patients with severe B_{12} deficiency can have bizarre morphology with an abundance of early forms that can occasionally be confused with evolving AML, especially erythroleukemia.[314] Since the hematologic changes of B_{12} deficiency can occur in the absence of classic neurologic symptoms, B_{12} and folate levels should be checked on all patients in whom a potential diagnosis of MDS is being entertained, and methylmalonic acid and homocysteine levels may help clarify whether deficiency is truly present in cases with borderline levels.[315]

Copper deficiency is less common than B_{12}/folate deficiency but can also be mistaken for MDS. It classically arises in patients who have had gastrectomies or certain forms of gastric bypass surgery, particularly biliopancreatic diversion,[316] but is uncommon in newer forms of gastric bypass, including gastric sleeve procedures and Roux-en-Y procedures. It can also develop in patients with malabsorption syndromes such as uncontrolled celiac disease or those taking high doses of zinc, which competes with copper for absorption in the small bowel.[317] Clinically, patients with copper deficiency develop an anemia that can be normocytic or macrocytic and can occasionally be profound. Mild neutropenia can also be seen, while clinically significant thrombocytopenia is rare.[318] Such patients can also develop neurologic symptoms similar to those seen in B_{12} deficiency, including peripheral neuropathy and in advanced cases myelopathy, demyelination, and rarely optic neuritis.[319] In the marrow, copper deficiency is classically associated with ring sideroblasts and erythroid and neutrophil vacuolization.[320]

Alcohol is a marrow toxin and a common cause of hematologic abnormalities, especially macrocytic anemia.[321] Bone marrow examination often reveals ring sideroblasts, and sometimes megaloblastic morphology, but frank dysplasia that would suggest MDS is uncommon.[322] Patients with borderline marrow findings who consume alcohol heavily should be advised to completely abstain from drinking (such advice is sometimes heeded), with a repeat marrow examination in 2 to 3 months if they are abstinent and cytopenias persist.

Other drugs and toxins have also been associated with dysplastic changes in the marrow. Well-described offenders include valproic acid,[323] mycophenolate mofetil,[324] ganciclovir,[325] alemtuzumab,[326] and the antimetabolites mercaptopurine and methotrexate.[327] These tend to cause macrocytic anemias and can also cause neutropenia and thrombocytopenia. On the other hand, isoniazid[328] and chloramphenicol,[329] and to a lesser extent cycloserine[330] and pyrazinamide,[331] have been associated with modest sideroblastic anemia. The anemia associated with isoniazid, in particular, can often be reversed with high doses of vitamin B_6 (pyridoxine).[332] Chloramphenicol can also cause an idiosyncratic pancytopenia distinguished by prominent vacuolization of erythroid precursors.[333]

HIV infection has specifically been associated with trilineage dysplasia that is most predominant in the erythroid line.[334] The erythroid hematopoiesis almost always is megaloblastic, and reticulin fibrosis can be seen. The marrows also often have polyclonal plasma cell expansion and lymphoid aggregates, and sometimes granulomas. The differential diagnosis of erythrodysplasia in patients with HIV infection includes the influence of medications, opportunistic infections, or a direct effect of HIV on the hematopoietic progenitor cells.[335]

Other primary hematologic disorders are also on the differential diagnosis with MDS. Hypoplastic MDS, for example, can be difficult to distinguish from aplastic anemia, as described above (see Chapter 31).[336] Similarly, some patients with MDS may have a significant degree of co-existing fibrosis, which can complicate the distinction from MPN (see Chapter 71).[337]

Some patients have cytopenias for which no cause is apparent despite extensive evaluation, including marrow aspiration and biopsy.[338] These patients with "idiopathic cytopenias of undetermined significance" (ICUS) can progress to MDS; the cytopenias may also be due to a non-clonal cause (e.g., immunologically mediated) and may be non-progressive, giving ICUS a variable natural history.[25,339] Detection of a somatic mutation in these patients—so-called "clonal cytopenias of undetermined significance" (CCUS)—is associated with a higher likelihood of progression to overt WHO-defined MDS or AML, especially if the multiple mutations are present at a high VAF and there are red cell abnormalities such as broad RDW.[340,341]

PROGNOSIS

As discussed throughout this chapter, MDS is heterogeneous in both morphology and clinical course. Patients with MDS typically have at least one persistent otherwise unexplained cytopenia (hemoglobin less than 11 g/dL, ANC < 1500/mm^3, or platelet count less than 100,000/mm^3), plus one of the following: (1) ≥5% blasts in the bone marrow, (2) any of several chromosomal abnormalities that are found recurrently in MDS; (3) a meaningful degree of dysplasia, usually 10% or more, detectable in at least one cell lineage on bone marrow examination, or (4) other evidence of clonal hematopoiesis.[20] In the past this latter category has usually comprised either karyotype or FISH results demonstrating chromosomal abnormalities missed by conventional karyotype, but evidence of clonal hematopoiesis based on detection of recurrent somatic mutations may need to be considered as well. However, given that at least 10% of the population aged 70 or older harbor point mutations in genes associated with myeloid malignancies,[39] detection of such a mutation in the absence of other diagnostic criteria cannot be considered equivalent to MDS. Patients with unexplained cytopenias who have clonal mutations have a considerably higher likelihood of developing MDS meeting WHO criteria or another myeloid neoplasm compared to those who lack clonal mutations, especially if multiple mutations or splicing mutations are present, or the clonal burden is high.[340]

As described above, the WHO criteria divide adult MDS into several general categories. Practitioners should note that these criteria have several limitations, the most obvious being the complexity of the criteria themselves, which can make them cumbersome to use. Second, as described above, there are a number of conditions that can mimic aspects of MDS, and the diagnostic criteria assume these diagnostic possibilities have been eliminated. Third, morphologic criteria are still based around arbitrary percentages of dysplasia and blasts, which can be interpreted differently by different pathologists or may be vulnerable to sampling variations.[342,343] Finally, although WHO criteria incorporate cytogenetics to a limited extent, the criteria have not yet been updated to include types of newer genetic results that have been shown to have diagnostic value, and it is likely that incorporating some of these features will improve diagnostic clarity.

IPSS and IPSS-R

Although the WHO classification system (and the FAB system before it) is the accepted standard for diagnosing MDS, WHO criteria confer limited prognostic information, so other systems have been developed expressly for this purpose. The most widely used has been the International Prognostic Scoring System (IPSS, Table 61.5)[344] and its newer revised iteration, the IPSS-R (Table 61.6).[345] The original IPSS was first published in 1997 and was derived by analyzing baseline characteristics in over 800 newly diagnosed MDS patients from all diagnostic categories who received supportive care. Patients were scored based on three characteristics: the percentage of blasts in the marrow, the presence of specific cytogenetic abnormalities, and the number of cytopenias in the blood. Patients were then stratified based on total score into four risk groups (low, intermediate-1, intermediate-2, and high) that were shown to have prognostic value, both in estimating the chance of progressing to AML and in estimating the duration of overall survival. The original IPSS excluded patients with therapy-related MDS, patients with proliferative CMML, and patients who underwent AHCT.

The original IPSS had several limitations that ultimately necessitated its revision. First, it was formulated four years prior to the adoption of the first iteration of WHO classification criteria, and some of the changes in those criteria (for instance, changing the cutoff for AML from 30% blasts to 20% blasts) invalidated certain aspects

TABLE 61.5 1997 International Prognostic Scoring System for Myelodysplastic Syndromes

Variable	Score			
	0	0.5	1	1.5
Marrow blasts (%)	<5	5–10	—	11–20
Karyotype	Good	Intermediate	Poor	—
Cytopenias	0–1	2–3	—	—

Survival Based on IPSS (percent alive at milestone timepoint without disease-modifying therapy)

IPSS Risk Group	No. Patients	2 Years	5 Years	10 Years	15 Years
Low	267 (33%)	85	55	28	20
Intermediate-1	314 (38%	70	35	17	12
Intermediate-2	179 (22%)	30	8	<1%	<1%
High	56 (7%)	5	<1%	<1%	<1%

IPSS, International Prognostic Scoring System.

TABLE 61.6 2012 Revised International Prognostic Scoring System for Myelodysplastic Syndromes

Cytogenetic Risk	Included Karyotypic Abnormalities
Very good	del(11q), −Y
Good	Normal, del(20q), del(5q) alone or +1 other abnormality, del(12p)
Intermediate	+8, del(7q), i(17q), +19, +21 Any other single or double abnormality Two or more independent clones
Poor	der(3q), −7, double with del(7q), complex with exactly 3 abnormalities
Very poor	Complex with >3 abnormalities

IPSS-R Scoring Table

Parameter	Category/Score				
Cytogenetic risk	Very good 0 points	Good 1	Intermediate 2	Poor 3	Very poor 4
Marrow blasts (%)	≤2 0 points	3–4 1	5–10 2	10–19 3	—
Hemoglobin (g/dL)	≥10.0 0 points	8.0–9.9 1	<8.0 1.5	—	—
Platelet count ($\times 10^9$/L)	≥100 0 points	50–99 0.5	<50 1	—	—
Neutrophil Count ($\times 10^9$/L)	≥0.8 0 points	<0.8 0.5	-	-	-

IPSS-R Risk Groups and Predicted Outcomes

IPSS-R Risk Group	Total Score	% of Patients	Median Survival (Years)	25% With AML (Years)
Very low	≤1.5	19	8.8	NR
Low	2–3	38	5.3	10.8
Intermediate	3.5–4.5	20	3	3.2
High	5–6	13	1.6	1.4
Very high	>6	10	0.8	0.73

AML, Acute myeloid leukemia; *IPSS-R*, Revised International Prognostic Scoring System.

of the IPSS. Furthermore, broad application of the criteria to larger populations of MDS patients began to reveal that the original IPSS gave insufficient weight to some important clinical features, particularly the presence of severe cytopenias and complex karyotype. The IPSS was only validated in de novo disease, not t-MDS, and only in patients treated with supportive care alone. In response to some of these evident limitations, several alternative prognostic systems were proposed, but none were widely adopted.[346–348]

The IPSS-R, introduced in 2012, revised the MDS blast cutoff to 20% and assigned points for severity of cytopenias. Furthermore, it incorporated a broader range of cytogenetic abnormalities than the original IPSS, rebalanced karyotype importance versus blast proportion, and stratified patients into five risk categories instead of four. The resulting system thus incorporates more informative biology and offers better refinement of prognosis than its predecessor.[349,350]

The median survival times estimated by the IPSS and IPSS-R are largely reflective of natural history since they were originally derived in patients who did not receive any therapy for their MDS. However, IPSS-R stratifications have been shown to retain relevance when applied to treated populations, including risk stratification of patients receiving hypomethylating agents[351] and those who undergo allogeneic transplant.[352]

Nevertheless, the IPSS-R still has limitations. It does not incorporate some laboratory findings, such as the LDH and the serum ferritin, that have been shown to have independent prognostic value in dedicated studies.[353–355] It does not capture the kinetics of disease or comorbid conditions.[356] Additionally, similar to the WHO classification criteria, IPSS-R does not include any mutational data other than cytogenetics.

A molecular classification system, IPSS-RM, has been in development for more than 5 years. A European consortium evaluated more than 2,000 cases and identified 8 distinct mutation clusters with variant prognosis.[357] Currently there is a high degree of variability in how clinicians use molecular profiling in evaluating patients with suspected MDS,[358] and the availability of sequencing in different practice settings. The complexity of developing a molecular classification system appropriate for clinical use is underscored by the heterogeneity of co-mutation patterns, clonal architecture, and clone size associated with even a single allele.[359]

Other Risk Stratification Systems

The limitations of the IPSS prompted attempts at more refined risk stratification using alternative models. Few of these have gained much traction in clinical practice but retain value for use in clinical trials and retrospective studies. For instance, a need for better stratification of lower-risk patients (i.e., IPSS-low or Int-1) led to the creation of a Lower-Risk Prognostic Scoring System (LR-PSS) by the MD Anderson group.[346] The LR-PSS uses criteria similar to the IPSS but sets lower thresholds for point assignment (e.g., 2 points for a platelet count <50 × 10^9/L and 1 point for bone marrow blasts ≥4%). Subsequent studies have confirmed the LR-PSS's ability to risk stratify patients, with those in the highest risk group having the greatest risk of developing AML, and the poorest overall survival.[360]

Other attempts to improve prognostic systems have included attempts to incorporate formal measures of comorbidity into the IPSS,[361] and an attempt to combine the WHO classification system with the IPSS that has been dubbed the WPSS.[362] This latter system was formulated prior to the introduction of the IPSS-R.[363]

TREATMENT OF PATIENTS WITH MYELODYSPLASTIC SYNDROMES

Treating patients with MDS presents a number of challenges. First, MDS patients are often elderly and frequently have serious comorbid conditions, poor performance status, or frailty. Second, the heterogeneous nature of MDS means that treatments appropriate for some patients may be unhelpful for others. Third, a number of biologic factors, including the disease's origin in a quiescent stem cell, make MDS highly refractory to most conventional treatments like intensive cytotoxic chemotherapy, which many MDS patients are not healthy enough to receive anyway. Hematopoietic cell transplantation (HCT) remains the only potentially curative therapy for MDS but is unavailable to many due to age and comorbidities, and even for those who undergo it, relapse is common.

Despite these challenges, most patients with MDS should be offered some form of therapy. For elderly patients or those with serious comorbid conditions who are too unwell to undergo active treatment, this may consist primarily of supportive care and possibly hospice referral. Other patients with lower-risk disease may benefit from a period of observation, or judicious use of transfusion and growth factor support, balanced in carefully selected cases with chelation therapy to prevent or ameliorate iron overload. Patients with higher-risk disease may derive benefit from treatment with hypomethylating agents or other conventional therapies, and those patients with higher-risk disease who meet other selection criteria should be referred for consideration of HCT. Finally, patients should whenever possible be encouraged to participate in clinical trials to the extent that this remains consistent with their personal goals of care.

Once the decision to pursue active treatment has been made, there are at least three major points to consider. One is the choice of initial treatment modality, which is guided in large part by risk stratification (Fig. 61.9). This should typically start by computation of the IPSS-R, but IPSS-R score should not be the sole consideration in choosing therapy; for instance, a very young patient with IPSS-R lower-risk MDS that has evolved quickly and has higher-risk molecular genetic features may still warrant consideration of HCT (see Chapter 104), while an elderly patient with higher-risk disease may nevertheless opt not to pursue treatment with a hypomethylating agent after discussion of the risks and potential benefits. Second, there are a number of special cases for which there is no clear consensus about the single best treatment strategy; these include patients with lower-risk disease who have a dominant cytopenia other than anemia and patients with high-risk disease who have progressed on a hypomethylating agent but are not candidates for transplant.

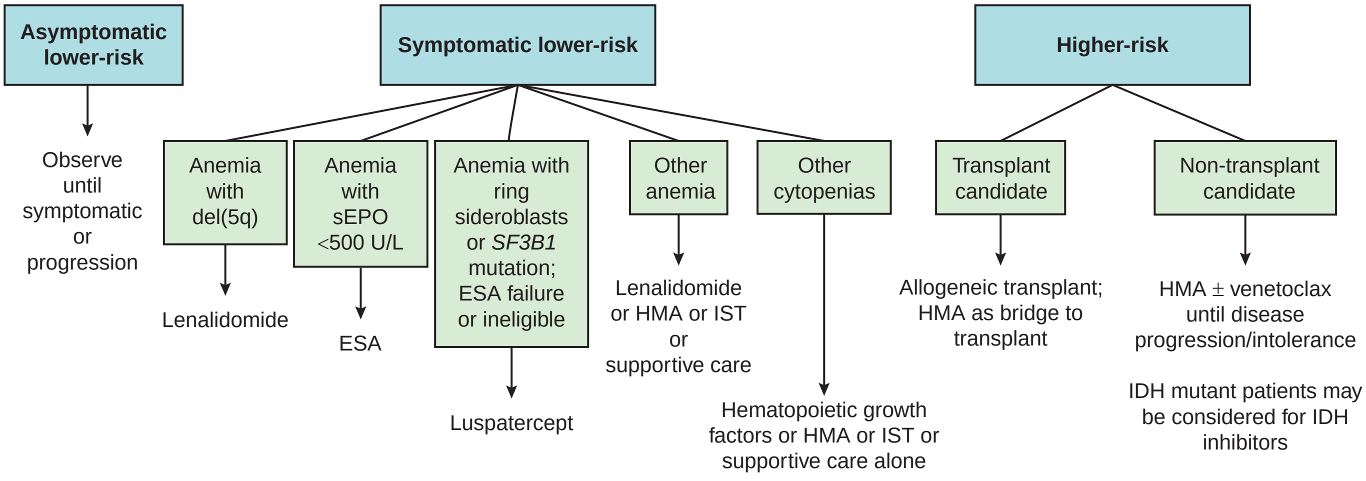
Finally, since therapies other than HCT are rarely if ever curative, defining what constitutes a meaningful response to therapy can be a nontrivial endeavor. Objective criteria for response were proposed by an IWG in 2000 and revised in 2006 and again in 2018,[364] and include improvements in cytopenias, reduction of the number of metaphases with abnormal karyotypes, and reduction in the percentage of the marrow involved by blasts.[365] However, improvements in these objective variables may not always coincide with the ways patients perceive changes in their quality of life[366,367] or MDS-related symptoms,[3] and these perceptions may be more likely to impact patients' decisions to continue or discontinue active therapy for their disease.

Lower-Risk Disease

Many patients with lower-risk MDS do not require active treatment directed at the underlying disease process, but this does not mean they require no care at all. In fact, providing appropriate supportive care is an important undertaking that can at times be complex and time-consuming, and requires a detailed understanding of the evidence behind the available supportive measures.[368]

Management of Anemia

Because most MDS patients become anemic at some point during their disease, appropriate management of anemia is important.[369] Most patients with MDS will require packed RBC transfusions during


Asymptomatic lower-risk
Symptomatic lower-risk
Higher-risk
Observe until symptomatic or progression
Anemia with del(5q)
Anemia with sEPO <500 U/L
Anemia with ring sideroblasts or *SF3B1* mutation; ESA failure or ineligible
Other anemia
Other cytopenias
Transplant candidate
Non-transplant candidate
Lenalidomide
ESA
Luspatercept
Lenalidomide or HMA or IST or supportive care
Hematopoietic growth factors or HMA or IST or supportive care alone
Allogeneic transplant; HMA as bridge to transplant
HMA ± venetoclax until disease progression/intolerance
IDH mutant patients may be considered for IDH inhibitors

*Consider **clinical trial enrollment** for all patients*
***Supportive care** (e.g., transfusions and antimicrobials as needed) for all patients*
***Chelation therapy** for selected lower-risk RBC transfusion-dependent patients*
***Risk stratification** using IPSS-R supplemented by molecular testing*

Figure 61.9 TREATMENT FOR MYELODYSPLASTIC SYNDROMES IS INDIVIDUALIZED TO THE PATIENT'S RISK STATUS, SYMPTOMS, AGE, AND MEDICAL HISTORY. Asymptomatic patients can be observed since there is no clear benefit from early therapy. For higher-risk patients, the key decision is whether to proceed with transplantation; for non-transplant candidates, DNA hypomethylating agents are the treatment of choice. Increasingly, other agents are being added to DNA hypomethylating agents. Lower-risk patients with anemia may be candidates for lenalidomide (if del5q), erythropoiesis stimulating agents (if serum erythropoietin <500 U/L), or luspatercept (if ring sideroblasts or *SF3B1* mutation present). There is no standard therapy for other cytopenia patterns. More detail is given in the text. *EPO*, Erythropoietin; *ESA*, erythropoiesis stimulating agents; *HMA*, hypomethylating agent; *IPSS*, International Prognostic Scoring System; *IPSS-R*, Revised IPSS; *IST*, immunosuppressive therapy; *MDS*, myelodysplastic syndromes.

the course of their disease. To minimize the risks associated with repetitive transfusion, it is usually appropriate to reserve transfusion until the hemoglobin is below 8 to 9 mg/dL or the patient has symptoms referable to anemia. More restrictive transfusion strategies, such as those suggested by studies in inpatient or critical care settings,[370,371] may be appropriate in younger, healthy patients, while some older patients, particularly those with significant comorbid cardiac disease, may benefit from more liberal strategies targeting a hemoglobin of 9 to 10 mg/dL.[372,373] If the patient is an HCT candidate, irradiated blood is essential.[374] Since many patients with MDS will receive hundreds of units of PRBCs over the course of their disease, prevention and appropriate management of iron overload can be important.[375,376]

Transfusion dependency is a negative predictor of outcome in MDS.[377] While transfusion requirement may to some extent be a marker of more severe hematopoietic insufficiency and worse disease not captured by other prognostic markers, the poor outcomes may also be a reflection of the adverse effects of repetitive transfusion, including accumulation of toxic iron species, immune modulation, and a risk of infection.[378] Given these facts, the administration of erythropoiesis-stimulating agents (ESAs) has been heavily investigated in MDS in an attempt to spare patients from unnecessary transfusions.

Dozens of studies of ESAs have been conducted in MDS, and 20% to 50% of patients experience meaningful, sustained improvements in their hemoglobin level in response to ESA supplementation.[379–384] Predictors of response include lower pre-treatment serum EPO levels (<500 U/L, but especially <100 U/L),[385] lower IPSS scores,[386] normal blast counts, normal or low-risk cytogenetics,[387] and lower serum and marrow levels of inflammatory cytokines.[388] The serum EPO level should be evaluated in the context of the degree of anemia; a trial of an ESA is usually reasonable in patients with EPO levels up to 200 to 300 U/L, which, while elevated compared to normal, is still lower than would be predicted for most MDS patients' degree of anemia. Patients with EPO levels over 500 U/L, on the other hand, are unlikely to respond to any dose or duration of ESA.

There are two major formulations of ESA available in the United States. Epoetin alfa, the shorter-acting of the two, can either be given at a dose of 150 to 300 U/kg three times weekly, or in a fixed dose of 40,000 to 60,000 units once weekly.[382] Higher doses above 60,000 U/weekly have not proven to be more effective.[389] Darbepoetin alfa is longer acting due to an altered glycosylation pattern, and is typically given in doses of 200 to 300 μg every 2 weeks or 500 μg once every 3 weeks.[390] Retrospective comparisons of the two agents have not shown a significant difference in efficacy.[391] Other ESA formulations are available outside the US, such as epoetin zeta. Bioequivalent forms of ESAs are also available in many countries.

Although there is some increase in the proportion of responding patients with longer duration of ESA therapy,[392] in practical terms, a trial of 8 to 12 weeks is reasonable. Prior to starting therapy, other causes of anemia should be ruled out. In particular, response can be suboptimal if patients are absolutely or even relatively iron deficient, and oral or parenteral iron supplementation may be reasonable in patients with ferritin levels at the lower end of the normal range.

One limitation of the numerous studies of ESAs in MDS patients is the lack of a consistent definition of response since most studies predated uniform IWG criteria. Some studies define this based on a hemoglobin increase (usually improvement by 1 to 2 g/dL),[393,394] while others have defined response by transfusion reduction or quality of life metrics.[395] In practice, any of these improvements could be a reasonable criterion for continuation of therapy. Supplementation with ESA has not been shown to alter the rate of progression to acute leukemia, and probably has a minimal influence on survival.[396] Additionally, several studies have shown an improvement in response in patients supplemented with a combination of ESA and G-CSF, especially in MDS-SLD-RS, likely due to pleiotropic effects of both growth factors on hematopoietic progenitors.[397]

ESAs are no longer routinely used for management of anemia in solid tumor patients, where studies have shown them to be associated with an increased risk of poor outcomes.[398] Similar findings have also been noted in patients without cancer receiving ESAs for anemia associated with renal failure.[399] Such a risk has not consistently been shown in MDS, although most studies published to date have not had long follow-up periods. The risk of thromboembolism has largely been correlated with the degree of improvement in hemoglobin, with patients targeted to hemoglobin over 12 mg/dL at greatest risk. Given this, the consensus recommendation in MDS is to initiate an ESA at hemoglobin <10 g/dL and target a level between 11 and 12 g/dL.[400]

Luspatercept, a fusion antibody that is an activin receptor IIB ligand trap, binds to members of the transforming growth factor β family that inhibit erythropoiesis in MDS and can release developing erythroid precursor cells from a differentiation block. Luspatercept improves anemia in patients with MDS and ring sideroblasts who are either not ESA treatment candidates due to high serum EPO level or who have been failed by an ESA, and was approved by the US FDA in April 2020.[401] In a randomized, placebo-controlled trial, about 40% of patients treated with subcutaneous luspatercept at a starting dose of 1.0 mg/kg every 21 days (and escalating to 1.75 mg/kg if needed) had an 8-week transfusion free period compared to 15.8% in the placebo arm.[402] The most common adverse events include fatigue. The effectiveness of luspatercept in other subtypes of MDS besides those with ring sideroblasts is an ongoing area of investigation.

A small proportion of anemic patients will benefit from treatment with androgens such as danazol or testosterone.[403] A history of prostate cancer is usually a contraindication, and hepatotoxicity should be monitored.

Management of Neutropenia and Infections

As referenced above, patients with MDS often become neutropenic but also appear to have other qualitative immune defects that are not accurately represented by the ANC,[404,405] and infection is the leading cause of death in MDS.[246] Despite this, supplementation with either G-CSF (filgrastim) or GM-CSF (sargramostim) and their bioequivalents has not been shown to improve outcomes in MDS patients.[406–408] Nevertheless, it is reasonable to consider a trial of growth factor (usually G-CSF, due to less frequent adverse effects) in lower-risk IPSS patients with severe neutropenia, typically defined as an ANC < 200 to 500/mm^3. Supplementation in higher-risk patients, particularly those with any degree of excess blasts, is not usually recommended in the absence of chemotherapy, due to lingering concerns about promoting expansion of leukemic clones.[409]

Appropriate antimicrobial use is an important component in the care of MDS patients. Patients should be counseled to seek medical attention for any fever greater than 100.4°F (38.4°C), and those who are neutropenic should be started on broad-spectrum parenteral antibiotics while an appropriate search for infection is undertaken. A thorough discussion of treating febrile neutropenia can be found elsewhere in the text, but should begin with an antibiotic that covers a broad spectrum of gram-negative organisms including *Pseudomonas* species.[410] Although there is no clear benefit of prophylactic antibiotics, they may be appropriate for selected patients who demonstrate a pattern of recurrent infections. There is less consensus about prophylactic antivirals[411] or antifungal medications,[412] and the efficacy of the latter depends on local microbiological isolates and epidemiological patterns.

Management of Thrombocytopenia and Bleeding

Modest thrombocytopenia is common across many subtypes of MDS, while severe depression of platelet counts is more frequently seen in patients with higher-risk IPSS scores or those undergoing active treatment with hypomethylating agents or chemotherapy. Overall, bleeding is the second most common cause of non-leukemic death in MDS patients.[413] Conventional wisdom and observational studies indicate that the risk of bleeding with trauma begins to increase at a platelet count near 50×10^9 cells/L and that of spontaneous hemorrhage does not increase significantly until the count is below 10 to 20×10^9 cells/L. However, these cutoffs may underestimate the bleeding risk in MDS patients, many of whom have qualitative defects in platelet function that are not captured by the platelet count. A number of retrospective studies have evaluated the significance of thrombocytopenia in MDS, all of which have found that thrombocytopenia confers a poor prognosis due both to the risk of bleeding and, in some cases, an elevated risk of transformation to AML.[414,415]

As with red blood cells, management of thrombocytopenia can either involve transfusions or use of growth factors. Many institutions use a standard transfusion threshold of 10 × 10^9 cells/L,[416] but as discussed above, a higher goal may be appropriate in selected patients who show a predilection for bleeding. In general, patients should be transfused prophylactically rather than waiting for bleeding to occur. Although there are few large studies comparing transfusion strategies specifically for MDS patients, a study of patients with other hematologic malignancies showed that for most patients (including those with AML), a prophylactic strategy using a trigger of 10 × 10^9 cells/L led to fewer significant hemorrhages than a therapeutic strategy in which patients were transfused only when bleeding.[417]

Thrombopoietin analogs, which are widely used for refractory immune thrombocytopenia purpura (ITP),[418] are not approved by any regulatory agencies for use in MDS. Formulations approved in ITP include romiplostim, a "peptibody" consisting of 14 amino acid peptides that bind the extracytoplasmic domain of the thrombopoietin receptor fused to an IgG1 heavy chain, and eltrombopag, a small molecule without TPO homology that nevertheless binds and activates the TPO receptor.[419] Romiplostim is administered as intermittent subcutaneous injections, whereas eltrombopag is an oral formulation that is taken daily.

Both thrombopoietin analogs have shown efficacy in terms of reducing platelet transfusions and clinically significant bleeding events and increasing platelet counts in early-phase trials in MDS.[420–422] In patients with IPSS low or intermediate-1 risk MDS who had platelet counts less than 50 × 10^9/L, romiplostim consistently led to significant improvements in platelet count, which were durable in about half of patients.[423] Modest improvements were also seen in a trial of eltrombopag.[424]

However, concerns have been raised about the safety of TPO agonists in patients with MDS. First, a number of studies in ITP have shown an increase in marrow reticulin fibrosis in some patients.[425] While the absolute risk of fibrosis has not been determined, and fibrosis has been moderate in most patients, it is possible that the abnormal marrow microenvironment of MDS patients might encourage reticulin deposition.[426] Second, there is concern about increased blast proliferation in the presence of TPO agonists, since some blasts have functional TPO receptors. A randomized controlled trial of romiplostim versus placebo was stopped early by the study's safety monitoring committee due to interim analysis suggesting a higher rate of progression to AML in the romiplostim arm.[427] At the time the study was stopped, 10 of 167 patients in the romiplostim group had progressed to AML, compared to 2 of 83 in the placebo group. Since the study accrual was incomplete at the time the study was stopped, the risk of disease progression could not be definitively assessed and the AML progression rate eventually nearly equalized; nevertheless, the manufacturer added a warning of risk of MDS progression to romiplostim's label. In a controlled trial of eltrombopag, there was also a modest increase in disease progression with active therapy.[428] Therefore romiplostim or eltrombopag cannot be recommended for routine treatment of MDS-associated thrombocytopenia outside a clinical trial, although patients with refractory bleeding who fail to respond to platelet transfusions could consider trying one of these agents palliatively.

Other agents approved for thrombocytopenia such avatrombopag, lusutrombopag, or the Syk inhibitor fostamatinib have not been studied in MDS patients. The use of antifibrinolytic agents (tranexamic acid or aminocaproic acid) may be helpful in patients with refractory bleeding, especially those who have platelet alloimmunization.[429]

Management of Iron Overload (Transfusional Hemosiderosis)

Although it is clear that MDS patients who undergo repeated blood transfusions have an increased risk of developing iron overload,[377] the importance of this relative to other disease-related risks, and how to assess and respond to it, remains a matter of debate.[378] MDS is distinct from pediatric illnesses associated with iron overload, in which the benefit of chelation has been well established,[430] in that many MDS patients will not live long enough for the clinical impact of end-organ iron deposition to be realized, and there are competing risks in MDS patients including neutropenic sepsis and leukemia progression that are not present in thalassemia or sickle cell anemia. Registry data suggest that patients with higher transfusion requirements have a greater risk of complications,[431,432] but registry data cannot prove the direction of the relationship—that is, whether the transfusions lead to complications or whether the need for transfusion is simply a marker of more severe disease or underlying comorbidities. Other retrospective studies have provided circumstantial evidence for deleterious effects of iron overload. For instance, one study has shown a higher rate of death from heart failure among MDS patients,[433] and another has shown increased rates of heart and liver failure among MDS patients with high lifetime numbers of transfusions[434]—but these observations again cannot prove causality.

There are two major formulations of chelating agent available in the United States, deferoxamine and deferasirox.[435] (A third agent, deferiprone/L1, is approved for thalassemia patients via a restricted distribution program, and is not often used in MDS because of idiosyncratic neutropenia.) Deferoxamine is administered as a continuous subcutaneous or intravenous infusion, a cumbersome mechanism that has limited its applicability in widespread practice. Deferasirox, an oral formulation, is more convenient so is much more widely used, but is quite costly.

Deferasirox is typically started at 20 mg/kg once daily and escalated to 30 to 40 mg/kg/day using the original formulation and started at 14 mg/kg once daily for a revised formulation with a different lactose-free binder. Although deferasirox has been shown to rapidly mobilize stored iron and reduce labile plasma iron species, it is frequently discontinued either as a result of disease progression or due to side effects, which include GI distress, renal impairment, and rash.

There are data from retrospective studies showing better outcomes for patients who undergo iron chelation, but all of these are subject to patient selection bias. In one study, a Canadian review of 178 patients, only 18 received chelation therapy, suggesting that these patients were carefully selected and destined to have a better outcome anyway.[436] In another, a French study of 97 patients in whom 46% received chelation, the regimen and duration of chelation therapy varied considerably, and no baseline assessment of iron stores was performed.[437] A recent meta-analysis of iron chelation studies in MDS found that use of chelation therapy was associated with an increase in length of survival (overall 61.2 months longer for patients receiving chelation),[438] but since the analysis included only retrospective studies, it is possible that these numbers are again largely reflective of selection bias, with healthier or younger patients more likely to receive chelation therapy than those who were older or sicker.

In a single-arm cohort study of 1744 patients treated with deferasirox, including a prespecified MDS subgroup of 341 patients, median ferritin levels decreased significantly compared to baseline regardless of whether patients were chelation-naïve or not, as did the median alanine aminotransferase (ALT), which was tracked as a marker of hepatic toxicity from iron overload.[439] On the other hand, 48.7% of the MDS patients discontinued treatment within one year of initiation due to side effects. Since there was no control arm and the study period was short, no conclusions could be drawn about the impact of chelation on more meaningful clinical outcomes. Other studies have shown improvements in hematopoiesis in patients started on chelation therapy,[430,431] but these observations were not tied to longer-term outcomes either.

A phase III study, TELESTO, randomized patients with lower-risk MDS who had received 15 to 75 RBC units and had elevated ferritin but preserved organ function to receive either deferasirox or placebo.[440,441] The original accrual goal was 630 patients, but because of narrow entry criteria and clinicians' lack of perceived equipoise about the benefit and risks of chelation therapy, only 210 patients were enrolled, which precluded an assessment of overall survival.

The trial did show benefit with chelation in a complex composite endpoint including elevation of liver tests, infection, congestive heart failure exacerbation, and death, but elevated creatinine was observed in many patients.

Despite the limitations of the available data, there are several published consensus opinion guidelines regarding the use of chelation therapy in MDS.[260,442] Although they differ with respect to their specific recommendations, most operate on similar principles: the patients best suited to iron chelation therapy are either those with lower-risk disease who face a long period of transfusion dependency, or those who could potentially be eligible for HCT, since in the transplant setting numerous studies have shown that a high serum ferritin level is associated with inferior outcomes.[443] A third category of patients comprises those with suspected end-organ damage as a result of iron overload. Some of the guidelines recommend waiting to start chelation therapy until the ferritin is greater than 1000 µg/L or the patient has had more than 20 to 30 lifetime transfusions, but they also note that neither of these criteria is supported by high-level evidence. Serum ferritin is problematic since it is subject to variability based on inflammatory state and is an imperfect marker of total body iron burden. For quantitative assessment of hepatic iron overload, T2*-weighted MRI of the liver or other organs has supplanted biopsy as the preferred method of validation.[444]

Other Therapies for Lower-Risk Disease

The literature is replete with attempts to treat patients with lower-risk MDS with a variety of different drugs, especially so-called "differentiating agents" such as retinoic acid or arsenic trioxide (ATO). With the exception of lenalidomide for 5q− syndrome, most of these have been inconsistently effective at best.

Lenalidomide

As described above, lenalidomide is a derivative of thalidomide that is uniquely effective in reversing the severe anemia associated with isolated del(5q). This was previously thought to be related to poorly characterized immunomodulatory effects (as implied by "Imid," the name given to the class of thalidomide derivatives).[445] However, more recent work has shown that lenalidomide actually exerts its effect in 5q− syndrome by mediating the binding of the E3 ubiquitin ligase component cereblon, which is responsible for modulating substrate specificity, to casein kinase 1α, thus triggering casein kinase's proteasomal degradation.[183] Casein kinase 1α is an essential kinase whose gene, *CSNK1A1*, lies within the minimally deleted region on 5q.[175] In normal cells, treatment with lenalidomide is insufficient to deplete casein kinase 1α to lethal levels, but the haploinsufficiency of *CSNK1A1* induced by loss of 5q renders 5q− cells sensitive to the drug through a p53-dependent mechanism and allows for repopulation of the marrow by wildtype hematopoietic progenitors. This activity appears to be unique to lenalidomide and is not shared, for instance, by thalidomide. Resistance eventually develops in most cases, often through inactivating mutations in *TP53*.[446]

Lenalidomide's efficacy was first observed in MDS-001, a phase I study of 32 patients with symptomatic anemia who had not responded to ESAs.[447] Twenty of the 32 patients became independent of transfusion for some period of time, including 10 of 12 patents with del(5q). Of the responders, those with del(5q) had a significantly longer duration of response. This prompted further study of lenalidomide specifically in del(5q) patients in MDS-003, a phase II study in which 76% of 148 enrolled patients had an improvement in anemia and 67% achieved transfusion independence, with a median improvement in hemoglobin near 5 mg/dL.[178] A second phase II trial, MDS-002, showed that lenalidomide was also effective in a substantially smaller subset of patients without del(5q), with an overall response rate of 43% and a transfusion independence rate of 26%.[448] The factors that determine lenalidomide responsiveness in non-del(5q) MDS have not been determined.

The most conclusive evidence of lenalidomide's efficacy in del(5q) comes from the phase III MDS-004 trial, a randomized, double-blinded study in which patients were randomized 1:1:1 to receive lenalidomide 10 mg/day on days 1 to 21 of a 28-day-cycle, lenalidomide 5 mg/day on days 1 to 28, or placebo on days 1 to 28.[449] Those without an erythroid response at 16 weeks were unblinded and became eligible for open-label treatment. At the conclusion of the study, significantly more patients in both lenalidomide groups had achieved transfusion independence for at least 26 weeks than those in the placebo group (56% in the 10-mg group vs. 43% in the 5-mg group vs. 5.9% in the placebo group), with those in the 10-mg group having a longer median duration of response (82.9 weeks) than those in the 5-mg group (41.3 weeks). Treatment with lenalidomide also appeared to lower the risk of transformation to AML; at a median follow-up of around 3 years, 36% of patients who had received placebo only and 30% who had started with placebo and crossed over to lenalidomide had developed AML, compared to 23% in the 5-mg group and 22% in the 10-mg group, though the crossover model prevented an assessment of statistical significance.

Given recent progress in understanding of lenalidomide's mechanism of action, it is perhaps unsurprising that it demonstrates considerably lower efficacy in MDS patients who do not have del(5q). In these patients, studies including the MDS-005 randomized trial have consistently shown an overall response rate of about 25%, which on average lasts less than a year.[450] It is not known whether the patients with non-del5q MDS who respond have abnormalities of casein kinase 1-α, or whether their sensitivity to lenalidomide occurs via a different mechanism. ESA increases the response rate to lenalidomide.

Lenalidomide is generally well tolerated, but the most common side effects are neutropenia and thrombocytopenia, which are most common in the first month of treatment and which have some correlation to treatment response.[451] Rash is another recurrent adverse event. Pretreatment thrombocytopenia, a feature atypical of 5q− syndrome, appears to negatively correlate with response and perhaps serves as a phenotypic proxy for cooperating genetic events that blunt the molecular sensitivity to the drug.[133] While thromboembolism has been observed in studies of lenalidomide in patients with myeloma, this effect largely seems to be confined to co-administration of lenalidomide and dexamethasone.[452]

Low-Dose Cytarabine and DNA Hypomethylating Agents

Cytarabine (cytosine arabinoside or Ara-C) is a nucleoside analog that is widely used in the treatment of a number of hematologic malignancies. In MDS, its use in both lower- and higher-risk patients dates to the 1980s.[453,454] The typical dosing schedule is 5 to 20 mg/m^2/day via subcutaneous injection (daily or twice daily) or continuous IV infusion. Although 10% to 20% of patients with MDS have some degree of hematologic or blast cytoreductive response during Ara-C therapy, these tend to last only a few months, and treatment has not been shown to improve survival or reduce the rate of transformation to AML.

One of the drawbacks to treatment with cytarabine or any other conventional chemotherapeutic agent is that they cause cytopenias as a primary side effect, and since cytopenias are a central problem in most MDS patients, the agents rapidly become limited in utility.[455] The worsening of cytopenias is likely one of the primary reasons for the lack of survival benefit for cytarabine in MDS.

Reduced schedules of 5-azacytidine and decitabine have been used in lower-risk MDS patients, with complete response rates exceeding 30%.[456] These agents are described further below in the discussion of treatment of higher-risk patients, where they see much greater use.

Anti-Tumor Necrosis Factor Therapy

The observation that MDS patients often have elevated levels of inflammatory cytokines and increased rates of apoptosis led to the postulate that blockade of these pathways might ameliorate the ineffective hematopoiesis found in many patients with MDS. Etanercept, a soluble TNFα inhibitor, has been analyzed in two small pilot studies of MDS patients, with mixed results: cytopenias improved in one trial,[457] whereas no effect was seen in the other.[458] The drug has also been combined with both ATG[459] and 5-azacytidine,[460] but in neither case was it clear that the addition made a difference. A separate case report described sustained erythroid responses in two patients treated with infliximab, a monoclonal anti-TNF antibody.[461] TNFα and other inflammatory mediators may play a greater role in some subsets of MDS than others.[462]

Immunosuppressive Therapy

Corticosteroid therapy was once a mainstay of treatment for MDS, but despite reported response rates in the range of 10% to 20%,[463] the complications of long-term steroid use in this population were found to outweigh the benefits. Nevertheless, immunosuppressive therapy may be reasonable for certain subsets of MDS patients in whom autoreactive T-lymphocytes contribute to the inhibition of effective hematopoiesis. Patients with hypoplastic MDS, in particular, have been thought to be good candidates for treatment with ATG or other parallel approaches, but studies have not consistently shown that marrow cellularity predicts response to anti-T-cell therapy in MDS.[464] Other studies have shown that patients with underlying PNH clones, which can be assessed by flow cytometry for PIG-anchored glycoproteins CD55 and CD59, may also respond to ATG.[465]

Retrospective analysis of data from the International Myelodysplasia Risk Assessment Workshop (IMRAW) showed that treatment with immunosuppression was associated with an improvement in overall survival (8.1 vs. 5.2 years, $P < .001$) and a lower risk of leukemic transformation, but these results could have been subject to selection bias since fewer patients with higher-risk disease are treated up front with immunosuppression. On the other hand, a randomized study comparing ATG plus cyclosporine to best supportive care suggested a slight improvement in hematologic response in the ATG + CsA group, but there was no difference in disease-free or overall survival.[466] Subgroup analysis suggested that patients with hypocellular marrows were more likely to respond to immunosuppression, again hinting at biologic similarities to aplastic anemia. In studies making careful distinction between MDS and aplastic anemia, however, mortality with ATG treatment was higher in MDS than in AA.[290] In the largest multicenter retrospective review, marrow hypocellularity (<20% cellular) was associated with better response to IST therapy, and the use of horse ATG was associated with better outcomes than with rabbit ATG or cyclosporine.[467]

Alemtuzumab, a monoclonal antibody against anti-CD52, has activity in highly selected subsets of MDS patients. In particular, one small phase I/II study of patients yielded a 77% overall response rate among patients with IPSS intermediate-1 disease.[468] Some patients with cytogenetic abnormalities normalized their karyotype, and the rate of complete response appeared to increase with time in those patients who were not lost to follow-up. However, alemtuzumab is severely immunosuppressive and difficult to obtain, and the study was hampered by a high rate of attrition. The biologic basis for the high response rate in this single study remains unclear.

Manipulating the Microenvironment: Vascular Endothelial Growth Factor and Other Targets

Observations of increased microvessel density in the bone marrow of MDS patients[469] led to interest in targeting vascular growth factors and other molecules associated with the marrow microenvironment, but results have been disappointing. For instance, a phase II trial of bevacizumab, a monoclonal antibody directed against vascular endothelial growth factor (VEGF), showed a decrease in VEGF levels and a significant reduction in microvascular density, but only one of 21 patients had any type of hematologic response.[470] Similarly, a phase II study of vatalanib, an oral VEGF receptor inhibitor, showed hematologic responses in only 5% of patients, and many patients had to withdraw from the study due to side effects.[471]

Other therapies directed at the microenvironment have similarly shown responses in only a minority of patients. For instance, the combination of pentoxifylline, a phosphodiesterase inhibitor, with dexamethasone and ciprofloxacin (PCD) has been shown to induce hematologic responses in some patients, but most of these were transient and probably represented neutrophil demargination from the corticosteroid.[231] A study combining PCD with amifostine, a chemoprotectant agent, showed a hematologic response rate of 66%, but many of these responses were dexamethasone-induced increments in neutrophil count and long-term results were not reported.[472] Lenalidomide and thalidomide were both previously thought to act via effects on the microenvironment,[473] but with the discovery of lenalidomide's role as a catalyst for cereblon-mediated ubiquitination, as described above, the extent to which it also affects the microenvironment is unclear.

Signal Transduction Inhibitors

Activating mutations in receptor tyrosine kinases like *NRAS* and *FLT3* are relatively uncommon in MDS because they usually act as catalysts for progression to secondary AML.[238] The considerations for use of FLT3 inhibitors in MDS is similar to those in AML, and data are limited in MDS. A more thorough discussion of these agents may be found in the chapter on AML (see Chapter 60).

Isocitrate dehydrogenase (IDH) is occasionally observed in MDS patients, albeit at a lower frequency than in AML. Ivosidenib, an IDH1 inhibitor, and enasidenib, an IDH2 inhibitor, are approved for AML, and have some activity in MDS with IDH mutations.[474] Other IDH inhibitors are in development.

Other Agents

A number of other agents have been used in MDS and reported either in anecdotes or small, nonrandomized series of patients. Historically, many patients with MDS with ring sideroblasts were started on vitamin B_6 because of its efficacy in familial sideroblastic anemia, but it has shown little efficacy in adults.[475] Other dietary supplements, including retinoids (vitamin A analogues), vitamin D,[476] and vitamin K_2,[477] have all been tried due to favorable effects on differentiation in vitro, but have not been effective in humans.

A number of other drugs have also been trialed in humans based on similar observations of pro-differentiation effects in preclinical experiments, all without comparable efficacy in human patients. These include amifostine as a single agent,[478] the solvent hexamethyl bisacetamide,[479] and alpha and gamma interferon.[480]

Both all-trans retinoic acid (ATRA) and ATO have been trialed in small numbers of MDS patients in an attempt to induce differentiation similar to that seen in acute promyelocytic leukemia. ATO has shown responses in up to 20% of MDS patients in several small series,[481–484] but the molecular basis for this action and how to predict who will respond are unclear and adverse effects including cytopenias and cardiac conduction defects are common. ATRA by itself has largely been ineffective.[485,486]

Higher-Risk Disease

Hypomethylating Agents

MDS genomes frequently display aberrant methylation patterns relative to normal hematopoietic cells. This observation was initially made in the 1990s, when investigators noted hypermethylation in the promoters of numerous tumor suppressor genes, including *CDKN2A, CDKN2B*,[487] *FHIT*,[488] *SOCS1*,[489] *CTNNA*1,[490] and others. Later, with the advent of genome-wide methylation profiling, it was further observed that this aberrant methylation is often a global phenomenon in MDS and that global hypermethylation, in particular, appears to confer a poor prognosis.[491]

This characterization of aberrant methylation in MDS soon led to the identification of hypomethylating agents as a candidate class of MDS drugs. The prototypical hypomethylator, 5-azacytidine (azacitidine), was synthesized in the 1960s and its hypomethylating activity first clearly characterized in 1980.[492] It is a 5-azo-substituted pyrimidine analog that incorporates into RNA and is from there converted to a deoxynucleotide and incorporated into DNA, where it binds and irreversibly inhibits DNA methyltransferase 1. The in vitro result is a global decrease in cytosine methylation, which leads to differentiation in some leukemia cell lines. Azacitidine also appears to have non-epigenetic mechanisms of action, including some degree of direct cytotoxicity and immune stimulation.[493] Both azacitidine and the related compound decitabine (5-aza-2′-deoxycytidine) are approved for and frequently administered in both MDS and AML.

Standard dosing of azacitidine is 75 mg/m^2 administered subcutaneously once per day for seven consecutive days each month.[494] This was the dosing schedule used in the first major study of hypomethylating agents in MDS, CALGB 9221, in which azacitidine was found to be superior to best supportive care both in terms of quality of life and risk of progression to AML.[495] In the CALGB study, about 60% of patients had some degree of response to azacitidine, although consensus response criteria were not used. Most of these responses were minor hematologic improvement, but about 15% of patients had a pathologic complete response. Most of those who responded did so within 6 months. There was a nonsignificant trend toward an improvement in overall survival with azacitidine, and side effects tended to be mild, including nausea and vomiting and transient myelosuppression.

Based on these data and a large increase in requests for compassionate use of the drug after CALGB 9221 was first presented in 2002, the FDA granted approval to azacitidine for MDS in 2004. A subsequent study, AZA-001, compared azacitidine 1:1 to the patient and doctor choice of either standard induction, low-dose cytarabine, and best supportive care in 358 patients with higher-risk MDS or CMML, and showed a median nine-month improvement in overall survival for those treated with azacitidine (24 vs. 15 months).[496] This represents the only major study to date in which a survival benefit has been shown for a drug in MDS, and has not been replicated. Other studies have shown that a more convenient 5-day azacitidine dosing schedule appears to be effective, but the 7-day schedule remains the standard. An oral formulation of azacitidine, CC-486, showed a survival advantage in a maintenance trial in AML and has been tested in MDS with clinical activity but wide variation in bioavailability and cytopenias that resulted in early cessation of a randomized placebo-controlled trial.[497]

Although structurally similar to azacitidine, decitabine has a deoxyribose backbone that permits it to be directly incorporated into DNA. At higher doses it introduces DNA cross-linking and cell-cycle arrest, a mechanism more akin to a cytotoxic agent, but at lower doses it appears to primarily act as a hypomethylating agent.[498] Similar to azacitidine, decitabine has been shown to improve outcomes for patients with MDS, including a reduced transfusion requirement and slower transformation to AML. It may induce responses more quickly than azacitidine; for instance, in the multicenter ADOPT study, 90% of patients responded after four cycles of decitabine therapy, rather than the six cycles seen in the largest azacitidine.[499] In contrast to azacitidine, however, decitabine has never been shown to confer an overall survival benefit. Azacitidine and decitabine have never been compared head to head in a large prospective trial, which is a major failure of the MDS clinical trials community.

Some of the difference in outcomes between decitabine and azacitidine may be explained by difficulties establishing the optimal dose and schedule for decitabine; in the earliest trials it was administered at a dose of 15 mg/m^2 given intravenously every eight hours for three days,[500] but subsequent studies showed better hypomethylation and an improved overall response rate when it was given at a dose of 20 mg/m^2 once daily for five consecutive days,[501] the dosing strategy used in ADOPT. Some recent small studies have suggested a different dosing strategy, using frequent administration of low doses of decitabine (0.1 to 0.2 mg/kg/day) in an attempt to minimize the cytotoxic effects of the drug while catching more cells in S phase, when the demethylating activity of the drug would be most effective.[502] On the other hand, a 10-day schedule has also been explored, and while active (including in TP53 mutant disease) is more myelosuppressive.[503,504] An oral formulation of decitabine in combination with cedazuridine, which inhibits breakdown of oral decitabine by cytidine deaminase in the gut, was approved for MDS by the US FDA in July 2020.[505]

Despite the widespread use of hypomethylating agents in MDS, a number of key questions persist. Although studies have shown that each can demethylate the promoters of specific tumor suppressors (e.g., *P15INK4B*[506] and *PLCB1*[507]), it is not clear that this is the dominant in vivo mechanism. Some studies have shown increased demethylation in patients who respond to HMAs,[508] but this has not been a universal observation, and neither pretreatment methylation levels (either global or at specific promoters) nor the net change in methylation with treatment have been consistently predictive of treatment responsiveness.[509]

Several studies have interrogated the influence of somatic mutations on hypomethylating agent sensitivity. *TET2* mutations may increase likelihood of response and *ASXL1* mutations decrease response,[510] but the effect is modest such that results of mutation studies are not clinically actionable. Patients with *TP53* mutations have a high early response rate to hypomethylating agents,[504] but unfortunately the efficacy is not durable.[511]

Once the patient has been failed by HMA therapy, their prognosis is grim. The median survival for higher-risk patients is less than 6 months, and for those in whom the drug was used for lower-risk disease the median survival is only about 15 months.[512] No second-line therapy for higher-risk MDS is FDA approved.

Venetoclax

The oral BCL-2 inhibitor venetoclax in combination with low-dose cytarabine, azacitidine, or decitabine was approved by the FDA for treatment of AML in 2018 on the basis of phase 1/2 for use in AML; subsequent phase 3 studies in AML showed a survival advantage with venetoclax combinations compared to monotherapy with hypomethylating agents.[513] In early studies, venetoclax plus azacitidine appears to have a higher response rate than would be expected with azacitidine alone, especially when used as initial therapy rather than in patients who are already refractory to azacitidine or decitabine.[514,515] Tumor lysis syndrome is rare in myeloid neoplasms but control of leukocytosis before venetoclax initiation is important. Cytopenias are problematic and antimicrobial therapy is essential, and a shorter schedule (e.g., 14 days per 28 days rather than continuous dosing) may reduce the risk of septic death. Randomized data are awaited but not yet available.

APR-246

The TP53 refolding agent APR-246 (eprenetapopt), an analogue of PRIMA1, showed a promising complete response rate when used in combination with hypomethylating agents in patients with MDS associated with TP53 point mutations.[516,517] The TP53 mutant clone is cytoreduced in responding patients. A unique pattern of neurotoxicity that can be partially ameliorated with prochlorperazine is observed, but the drug is otherwise well tolerated. A randomized trial completed accrual in 2020 and unfortunately did not meet the primary endpoint of improved complete response rate with combination therapy compared to HMA monotherapy.

“Histone” Deacetylase Inhibitors

The observation that MDS frequently involves epigenomic abnormalities has provoked interest in other modalities beyond the hypomethylating agents. To date, the next most-studied class of agent is the histone deacetylase inhibitors, which as their name implies block the deacetylation of histone residues as well as deacetylation of other cellular protein. In vitro, application of these drugs leads to large-scale epigenetic modifications, but in vivo, the drugs proved ineffective, with multiple negative randomized trials. These include the ECOG E1905 study of azacitidine with or without entinostat[518]; the US/Canadian Intergroup S1117, a large three-arm trial comparing azacitidine alone to azacitidine plus vorinostat to azacitidine plus lenalidomide[519]; as well as a study of azacitidine with or without pracinostat.[520]

Induction Chemotherapy

AML-like induction regimens (e.g., cytarabine plus an anthracycline) are not effective for most patients with MDS, with available studies suggesting a remission rate of less than 20%. Hematologic recovery is often incomplete, and in many cases the chemotherapy mainly serves to select for the malignant clone. In fact, a randomized trial in higher-risk MDS compared azacitidine to physician and patient choice of alternative therapy that included the possibility of daunorubicin plus cytarabine, and patients treated with azacitidine had better outcomes, although only small numbers of patients were treated with induction.[496]

Induction chemotherapy, however, may be reasonable in selected younger patients, especially as a bridge to HCT.[521] Adding other agents, such as liposomal doxorubicin,[522] topotecan,[522] and thalidomide, does not seem to improve the rates of remission. Approval of CPX-351, a stoichiometrically defined liposomal encapsulation of daunorubicin and cytarabine, in secondary AML and AML with MDS-related changes has prompted interest in studying reduced doses of this agent in higher-risk MDS.[523]

Allogeneic Hematopoietic Cell Transplantation

HCT is the only known curative therapy for MDS.[524] It leverages both the cytoreductive effect of the conditioning regimen and the anti-tumor effect of the newly transplanted stem cells to eliminate abnormal cells. The reality for most MDS patients is significantly more complicated, although outcomes have steadily improved in recent years as centers have gained more experience in transplanting older patients using unrelated and even unmatched donors.[525]

An exhaustive review of stem cell transplantation is beyond the scope of this chapter, but a few points specific to MDS bear mention. Identification of patients for whom transplant might be a reasonable option is an important first step. This group typically includes patients younger than 75 years who are in otherwise good health and have adequate performance.[526] The stage of disease is also important: transplant is usually reserved for those with excess blasts or other forms of higher-risk disease, based on mathematical models,[527,528] but it is important to proceed to transplant before the disease has evolved into acute leukemia. Some centers occasionally offer transplantation to younger patients with lower-risk categories of disease who have higher-risk molecular markers.

Once a decision has been made to proceed to transplant, other decisions must follow. The first involves the source of the stem cells. If a healthy, HLA-matched sibling is available, this is often preferable[529]; however, reflective of the average age of MDS patients, most siblings are older and often have comorbidities, such as cancer or other illnesses, that preclude them from consideration as donors. Clonal hematopoiesis is common in elderly donors, and even when younger donors are used, donor clonal populations can durably engraft in the recipient, but thus far universal screening of prospective donors for clonal mutations cannot be recommended.[530] If a related donor is unavailable, the next best option is a search of the donor registries for an HLA-matched unrelated donor.[531] Compared to related donors, transplant from unrelated donors carries a higher rate of graft-versus-host disease as a consequence of minor antigen mismatch. If a matched unrelated donor cannot be found, options then include an unrelated donor transplant mismatched at one (or occasionally more) HLA loci, or an "alternative donor source," which includes umbilical cord blood[532] and haploidentical donors.[533] In the case of MDS patients, this latter type of transplant is often obtained from one of the patient's children.

The specific choice of conditioning regimen is likely less important in MDS than in other hematologic diseases given the disease's intrinsic chemoresistance, as described above. Options include fludarabine plus busulfan, fludarabine plus melphalan, busulfan plus cyclophosphamide, treosulfan, or cyclophosphamide plus irradiation. Many older patients with MDS are unlikely to tolerate myeloablative doses of conditioning agents and are instead given reduced doses of conditioning agents. These "reduced intensity conditioning" transplants rely more heavily on the graft-versus-tumor effect of the transplant to eliminate the malignant clone.[527,534] Given the intrinsic chemoresistance of MDS stem cells, however, this is usually a reasonable tradeoff, although a randomized trial in AML showed better disease control with a myeloablative approach.[535] Following transplant, patients must remain on immunosuppressive medications for a period of time to prevent the emergence of graft-versus-host disease. A full discussion of post-transplant considerations is outside the scope of this chapter, but most aspects of this area are less specific to MDS than those discussed above.

As mentioned, despite being potentially curative therapy, transplant in MDS is associated with significant morbidity and mortality. One study has suggested that 50% to 60% of lower-risk patients and 20% to 40% with higher-risk disease can expect long-term disease-free survival following transplant,[536] but the alternative view of these data is that a substantial proportion of patients either relapse or die of non-relapse complications of transplant.

Numerous studies have attempted to dissect patient and disease attributes that may predict outcomes following transplant. Poor-risk cytogenetics, including monosomy 7 and complex karyotypes,[537] have been shown to predict a high rate of relapse, and data from a large genomic analysis show that *TP53* mutations have a universally poor prognosis and that *JAK2* and RAS pathway mutations have a poor prognosis specifically among patients over 40.[144] In this study, RAS pathway mutations also suggested a benefit to myeloablative over reduced intensity conditioning, likely reflective of incipient transformation to AML. Short telomeres in the recipient predict for higher non-relapse mortality. Donor clonal hematopoiesis, especially when defined by *DNMT3A* mutations, predicts a higher likelihood of chronic graft versus host disease and may be associated with a reduced risk of relapse, though available studies published thus far are inconclusive.[538] So far, specific cytogenetic or genetic profiles have not been clearly associated with a favorable response to HCT. Pre-existing neutropenia has been shown to predict an increased risk of serious infections in the post-transplant setting.[539] On the other hand, a low blast count (fewer than 5%) is associated with better outcomes, and other data show that patients who achieved a remission or significant response to pre-transplant chemotherapy have better outcomes as well.[540]

Other Therapies for Higher-Risk Disease

Though rarely used in current practice, infusion of autologous hematopoietic cells has been attempted in the past, primarily for patients with oligoblastic disease.[541,542] There are a number of reasons why this approach is suboptimal for MDS patients. The most obvious drawback is that MDS is by definition a disorder of stem cells, such that any autologous infusion will ultimately lead to repopulation of the marrow with the original malignant clone, but without the graft-versus-leukemia effect imparted by an HCT. Moreover, autologous stem cell infusion is really meant to serve as a rescue after high-dose chemotherapy and is thus most successful when given for a chemotherapy-sensitive disease, which MDS is not. Finally, given the ineffective hematopoiesis many patients experience prior to treatment, hematopoietic recovery following high-dose chemotherapy is often poor. Since most patients who would be candidates for autologous stem cell rescue would also be eligible for other, more effective modalities of therapy, there are usually few reasons to pursue this avenue.

Other Drugs

Most drugs known to have activity in AML have been tried for MDS as well, usually with suboptimal results. These include etoposide,[543] irinotecan, gemcitabine,[544] low-dose melphalan,[545] idarubicin,[521] and clofarabine.[546] Both hydroxyurea and low-dose oral etoposide can be used to control proliferation in patients with excess blasts, but they do not target the underlying disease. Clofarabine is sometimes effective as a bridge to transplant therapy in patients with excess blasts despite HMA therapy, but renal and hepatic toxicity and severe skin rash are limiting.[547]

Immune checkpoint inhibitors targeting PD-1 or PD-L1 have had limited efficacy and some immunotoxicity when used as single agents in MDS and AML, even though PD-1, PD-L1, and CTLA-4 are expressed in MDS and expression of immune checkpoints is augmented by hypomethylating agents.[548] Studies using these approaches in combination are ongoing. Randomized trials of anti-CD47 macrophage immune checkpoint inhibition with magrolimab[549] and anti-TIM3 inhibition with sabatolimab are ongoing. Agents that modulate other immune checkpoints such as LAG3, NKG2D/MICA, and CD70 are also being investigated.[550]

FUTURE DIRECTIONS

While the last ten years have witnessed substantial improvements in our understanding of the molecular pathophysiology underlying MDS, similar strides have not been made in its treatment: only 4 drugs—azacitidine in 2004, lenalidomide in 2005, decitabine in 2006 (supplemented by an oral formulation of decitabine and cedazuridine combination in 2020), and luspatercept in 2020—have achieved FDA approval for MDS-specific indications as of this writing. There are few therapies in clinical trials that offer hope of broad or deep efficacy, and much clinical effort has been directed at incremental changes to current practice, such as adjusting dosing schedules of currently approved therapies or using modestly effective drugs in combination. The lack of progress is to a great extent due to unfortunate facts of MDS biology, including its origin in quiescent stem cells, a paucity of targetable activating mutations, and enrichment for poorly targetable mutations with much more subtle effects on growth and differentiation. Efforts are further complicated by the heterogeneity of biology both between and within individual patients, as well as the advanced age and poor overall health of many MDS patients, which are barriers to clinical trial enrollment.[551]

Despite these significant challenges, there is nevertheless great opportunity for improvements in care. Advances in stem cell transplant techniques, including the availability of alternative donor sources, refinements of post-transplant immunosuppression, and augmentation of the graft-versus-tumor effect, should continue to improve the outcomes for MDS patients able to undergo transplant. An equally difficult challenge lies in treatment of those patients ineligible for transplant, and it is in these patients that effective unification of our improving biological understanding of MDS with clinical care is most essential.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Abel GA, Klaassen R, Lee SJ, et al. Patient-reported outcomes for the myelodysplastic syndromes: a new MDS-specific measure of quality of life. *Blood*. 2014;123:451–452.

Angelucci E, Li J, Greenberg P, et al. Iron chelation in transfusion-dependent patients with low- to intermediate-1-risk myelodysplastic syndromes: a randomized trial. *Ann Intern Med*. 2020;172(8):513–522.

Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127:2391–2405.

Cazzola M. Myelodysplastic syndromes. *New Engl J Med*. 2020;383(14): 1358–1374.

Cogle CR, Craig BM, Rollison DE, List AF. Incidence of the myelodysplastic syndromes using a novel claims-based algorithm: high number of uncaptured cases by cancer registries. *Blood*. 2011;117:7121–7125.

Fenaux P, Mufti GJ, Hellstrom-Lindberg E, et al. Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. *Lancet Oncol*. 2009;10:223–232.

Fenaux P, Platzbecker U, Mufti GJ, et al. Luspatercept in patients with lower-risk myelodysplastic syndromes. *N Engl J Med*. 2020;382:140–151.

Garcia-Manero G, Griffiths EA, Steensma DP, et al. Oral cedazuridine/decitabine for MDS and CMML: a phase 2 pharmacokinetic/pharmacodynamic randomized crossover study. *Blood*. 2020;136:674–683.

Greenberg PL, Tuechler H, Schanz J, et al. Revised International Prognostic Scoring System (IPSS-R) for myelodysplastic syndromes. *Blood*. 2012;120:2454–2465.

Hellstrom-Lindberg E, Gulbrandsen N, Lindberg G, et al. A validated decision model for treating the anaemia of myelodysplastic syndromes with erythropoietin + granulocyte colony-stimulating factor: significant effects on quality of life. *Br J Haematol*. 2003;120:1037–1046.

Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med*. 2014;371:2488–2498.

Kantarjian H, Issa J-PJ, Rosenfeld CS, et al. Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. *Cancer*. 2006;106:1794–1803.

Lindsley RC, Saber W, Mar BG, et al. Prognostic mutations in myelodysplastic syndrome after stem-cell transplantation. *N Engl J Med*. 2017;376:536–547.

List A, Dewald G, Bennett J, et al. Lenalidomide in the myelodysplastic syndrome with chromosome 5q deletion. *N Engl J Med*. 2006;355: 1456–1465.

Platzbecker U, Fenaux P, Ades L, et al. Proposals for revised IWG 2018 hematological response criteria in patients with MDS included in clinical trials. *Blood*. 2019;133:1020–1030.

Silverman LR, Demakos EP, Peterson BL, et al. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. *J Clin Oncol*. 2002;20:2429–2440.

Stahl M, DeVeaux M, de Witte T, et al. The use of immunosuppressive therapy in MDS: clinical outcomes and their predictors in a large international patient cohort. *Blood Adv*. 2018;2:1765–1772.

Steensma DP, Bejar R, Jaiswal S, et al. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood*. 2015;126:9–16.

Walter MJ, Shen D, Ding L, et al. Clonal architecture of secondary acute myeloid leukemia. *N Engl J Med*. 2012;366:1090–1098.

Yoshida K, Sanada M, Shiraishi Y, et al. Frequent pathway mutations of splicing machinery in myelodysplasia. *Nature*. 2011;478:64–69.

REFERENCES

1. Cazzola M. Myelodysplastic syndromes. *N Engl J Med*. 2020;383(14):1358–1374.
2. Greenberg PL, Tuechler H, Schanz J, et al. Revised international prognostic scoring system for myelodysplastic syndromes. *Blood*. 2012;120:2454–2465.
3. Steensma DP. Dysplasia has a differential diagnosis: distinguishing genuine myelodysplastic syndromes (MDS) from mimics, imitators, copycats and impostors. *Curr Hematol Malig Rep*. 2012;7:310–320.
4. Steensma DP. Historical perspectives on myelodysplastic syndromes. *Leuk Res*. 2012;36:1441–1452.
5. Luzzatto AM. Sull' anemia grave megaloblastica senza reporto ematologico corrispondente (anemia pseudoaplastica). *Riv veneta di sc med Venezia*. 1907;47:193–212.
6. di Guglielmo G. Eritremie acute. *Bollettino—Societa Medico Chirurgica*. 1926;1:665–673.
7. Rhoads CP, Barker WH. Refractory anemia: analysis of 100 cases. *J Am Med Assoc*. 1938;110:794–796.
8. Rosati S, Mick R, Xu F, et al. Refractory cytopenia with multilineage dysplasia: further characterization of an 'unclassifiable' myelodysplastic syndrome. *Leukemia*. 1996;10:20–26.
9. Chevallier P. Sur la terminologie des leucoses et les affections-frontieres: les odoleucoses. *Sangre (Barc)*. 1942–1943;15:587–593.
10. Hamilton-Paterson JL. Pre-leukaemic anaemia. *Acta Haematol (Basel)*. 1949;2:309–316.
11. Block M, Jacobson LO, Bethard WF. Preleukemic acute human leukemia. *JAMA*. 1953;152:1018–1028.
12. Saarni MI, Linman JW. Preleukemia. The hematologic syndrome preceding acute leukemia. *Am J Med*. 1973;55:38–48.
13. Dreyfus B. [Refractory anemia or chronic acquired dyserythropoiesis. Definition. Classification. General characteristics]. *Ann Med Interne (Paris)*. 1974;125:621–626.
14. Dreyfus B. Preleukemic states. I. Definition and classification. II. Refractory anemia with an excess of myeloblasts in the bone marrow (smoldering acute leukemia). *Nouv Rev Fr Hematol Blood Cells*. 1976;17:33–55.
15. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol*. 1976;33:451–458.
16. Galton DJ. Classification of the acute leukaemias. *Blood Cells*. 1975;1:17–24.
17. Linman JW. Myelomonocytic leukemia and its preleukemic phase. *J Chronic Dis*. 1970;22:713–716.
18. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of the myelodysplastic syndromes. *Br J Haematol*. 1982;51:189–199.
19. Swerdlow SH, Campo E, Harris NL, eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. 4th ed. Lyon: IARC Press; 2008.
20. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127:2391–2405.
21. Cabello AI, Collado R, Ruiz MA, et al. A retrospective analysis of myelodysplastic syndromes with thrombocytosis: reclassification of the cases by WHO proposals. *Leuk Res*. 2005;29:365–370.
22. Giagounidis AA, Germing U, Wainscoat JS, Boultwood J, Aul C. The 5q− syndrome. *Hematology*. 2004;9:271–277.
23. Steensma DP. Good riddance to the term "refractory anemia" in myelodysplastic syndromes. *Leuk Res*. 2016;51:22–26.
24. Hasserjian RP, Campigotto F, Klepeis V, et al. De novo acute myeloid leukemia with 20–29% blasts is less aggressive than acute myeloid leukemia with ≥30% blasts in older adults: a Bone Marrow Pathology Group study. *Am J Hematol*. 2014;89:E193–E199.
25. Valent P, Bain BJ, Bennett JM, et al. Idiopathic cytopenia of undetermined significance (ICUS) and idiopathic dysplasia of uncertain significance (IDUS), and their distinction from low risk MDS. *Leuk Res*. 2012;36:1–5.
26. Malcovati L, Stevenson K, Papaemmanuil E, et al. SF3B1-mutant MDS as a distinct disease subtype: a proposal from the International Working Group for the Prognosis of MDS. *Blood*. 2020;136:157–170.
27. Riccardi A, Giordano M, Girino M, et al. Refractory cytopenias: clinical course according to bone marrow cytology and cellularity. *Blut*. 1987;54:153–163.
28. Della Porta MG, Travaglino E, Boveri E, et al. Minimal morphological criteria for defining bone marrow dysplasia: a basis for clinical implementation of WHO classification of myelodysplastic syndromes. *Leukemia*. 2015;29:66–75.
29. Sekeres MA. The epidemiology of myelodysplastic syndromes. *Hematol Oncol Clin North Am*. 2010;24:287–294.
30. Craig BM, Rollison DE, List AF, Cogle CR. Underreporting of myeloid malignancies by United States cancer registries. *Cancer Epidemiol Biomarkers Prev*. 2012;21:474–481.
31. Ma X, Does M, Raza A, Mayne ST. Myelodysplastic syndromes: incidence and survival in the United States. *Cancer*. 2007;109:1536–1542.
32. Cogle CR, Iannacone MR, Yu D, et al. High rate of uncaptured myelodysplastic syndrome cases and an improved method of case ascertainment. *Leuk Res*. 2014;38:71–75.
33. Cogle CR, Craig BM, Rollison DE, List AF. Incidence of the myelodysplastic syndromes using a novel claims-based algorithm: high number of uncaptured cases by cancer registries. *Blood*. 2011;117:7121–7125.
34. Pavlu J, Emmerson J, Marks AJ, Bain BJ. Idiopathic cytopenia of undetermined significance and the minimal criteria for a diagnosis of myelodysplastic syndrome. *Leuk Lymphoma*. 2011;52:515–516.
35. Sekeres MA, Schoonen WM, Kantarjian H, et al. Characteristics of US patients with myelodysplastic syndromes: results of six cross-sectional physician surveys. *J Natl Cancer. Inst*. 2008;100:1542–1551.
36. Marisavljevic D, Savic A, Zeremski V, Stanisavljevic N, Jelic S. Myelodysplastic syndromes in adults aged less than 50 years: incidence and clinicopathological data. *J BUON*. 2014;19:999–1005.
37. Jaiswal S, Ebert BL. Clonal hematopoiesis in human aging and disease. *Science*. 2019:366.
38. Shlush LI. Age-related clonal hematopoiesis. *Blood*. 2018;131:496–504.
39. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med*. 2014;371:2488–2498.
40. Genovese G, Kähler AK, Handsaker RE, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med*. 2014;371:2477–2487.
41. Steensma DP, Bejar R, Jaiswal S, et al. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood*. 2015;126:9–16.
42. Young AL, Challen GA, Birmann BM, Druley TE. Clonal haematopoiesis harbouring AML-associated mutations is ubiquitous in healthy adults. *Nat Commun*. 2016;7:12484.
43. Young AL, Wong TN, Hughes AEO, et al. Quantifying ultra-rare pre-leukemic clones via targeted error-corrected sequencing. *Leukemia*. 2015;29:1608–1611.
44. Libby P, Sidlow R, Lin AE, et al. Clonal hematopoiesis: crossroads of aging, cardiovascular disease, and cancer: JACC review topic of the week. *J Am Coll Cardiol*. 2019;74:567–577.
45. Jaiswal S, Natarajan P, Silver AJ, et al. Clonal hematopoiesis and risk of atherosclerotic cardiovascular disease. *N Engl J Med*. 2017;377:111–121.
46. Starý J, Baumann I, Creutzig U, Harbott J, Michalova K, Niemeyer C. Getting the numbers straight in pediatric MDS: distribution of subtypes after exclusion of down syndrome. *Pediatr Blood Cancer*. 2008;50:435–436.
47. Germing U, Aul C, Niemeyer CM, Haas R, Bennett JM. Epidemiology, classification and prognosis of adults and children with myelodysplastic syndromes. *Ann Hematol*. 2008;87:691–699.
48. Lange BJ, Kobrinsky N, Barnard DR, et al. Distinctive demography, biology, and outcome of acute myeloid leukemia and myelodysplastic syndrome in children with Down syndrome: Children's Cancer Group studies 2861 and 2891. *Blood*. 1998;91:608–615.
49. Butturini A, Gale RP, Verlander PC, Adler-Brecher B, Gillio AP, Auerbach AD. Hematologic abnormalities in Fanconi anemia: an International Fanconi Anemia Registry study. *Blood*. 1994;84:1650–1655.
50. Yamaguchi H, Baerlocher GM, Lansdorp PM, et al. Mutations of the human telomerase RNA gene (TERC) in aplastic anemia and myelodysplastic syndrome. *Blood*. 2003;102:916–918.
51. Shimamura A. Shwachman-Diamond syndrome. *Semin Hematol*. 2006;43:178–188.
52. Hahn CN, Chong CE, Carmichael CL, et al. Heritable GATA2 mutations associated with familial myelodysplastic syndrome and acute myeloid leukemia. *Nat Genet*. 2011;43:1012–1017.
53. Owen CJ, Toze CL, Koochin A, et al. Five new pedigrees with inherited RUNX1 mutations causing familial platelet disorder with propensity to myeloid malignancy. *Blood*. 2008;112:4639–4645.
54. Zhang MY, Churpek JE, Keel SB, et al. Germline ETV6 mutations in familial thrombocytopenia and hematologic malignancy. *Nat Genet*. 2015;47:180–185.
55. Polprasert C, Schulze I, Sekeres MA, et al. Inherited and somatic defects in DDX41 in myeloid neoplasms. *Cancer Cell*. 2015;27:658–670.
56. Surveillance, Epidemiology, and End Results (SEER) Program. www.seer.cancer.gov. National Cancer Institute Surveillance Research Program, released April 2021, based on the November 2020 submission.
57. Komrokji RS, Padron E, Ebert BL, List AF. Deletion 5q MDS: molecular and therapeutic implications. *Best Pract Res Clin Haematol*. 2013;26:365–375.
58. Yin SN, Hayes RB, Linet MS, et al. A cohort study of cancer among benzene-exposed workers in China: overall results. *Am J Ind Med*. 1996;29:227–235.

59. Tong H, Hu C, Yin X, Yu M, Yang J, Jin J. A meta-analysis of the relationship between cigarette smoking and incidence of myelodysplastic syndromes. *PLoS One*. 2013;8:e67537.
60. Sperling AS, Leventhal M, Gibson CJ, Ebert BL, Steensma DP. Myelodysplastic syndromes (MDS) occurring in Agent Orange exposed individuals carry a mutational spectrum similar to that of de novo MDS. *Leuk Lymphoma*. 2020;61:728–731.
61. Chen B, Zhao W-L, Jin J, et al. Clinical and cytogenetic features of 508 Chinese patients with myelodysplastic syndrome and comparison with those in Western countries. *Leukemia*. 2005;19:767–775.
62. Gologan R, Georgescu D, Tatic A, Radulescu I, Vasilache D. Epidemiological data from the registry of patients with myelodysplastic syndrome in a single hospital center of Romania. *Leuk Res*. 2009;33:1556–1561.
63. Matsuda A, Germing U, Jinnai I, et al. Difference in clinical features between Japanese and German patients with refractory anemia in myelodysplastic syndromes. *Blood*. 2005;106:2633–2640.
64. Iwanaga M, Hsu W-L, Soda M, et al. Risk of myelodysplastic syndromes in people exposed to ionizing radiation: a retrospective cohort study of Nagasaki atomic bomb survivors. *J Clin Oncol*. 2011;29:428–434.
65. Ben-Yehuda D, Krichevsky S, Caspi O, et al. Microsatellite instability and p53 mutations in therapy-related leukemia suggest mutator phenotype. *Blood*. 1996;88:4296–4303.
66. Pedersen-Bjergaard J, Philip P, Larsen SO, Jensen G, Byrsting K. Chromosome aberrations and prognostic factors in therapy-related myelodysplasia and acute nonlymphocytic leukemia. *Blood*. 1990;76:1083–1091.
67. Karp JE, Sarkodee-Adoo CB. Therapy-related acute leukemia. *Clin Lab Med*. 2000;20:71–81. ix.
68. Curtis RE, Boice Jr. JD, Stovall M, et al. Risk of leukemia after chemotherapy and radiation treatment for breast cancer. *N Engl J Med*. 1992;326:1745–1751.
69. Christiansen DH, Andersen MK, Pedersen-Bjergaard J. Mutations with loss of heterozygosity of p53 are common in therapy-related myelodysplasia and acute myeloid leukemia after exposure to alkylating agents and significantly associated with deletion or loss of 5q, a complex karyotype, and a poor prognosis. *J Clin Oncol*. 2001;19:1405–1413.
70. Pedersen-Bjergaard J, Pedersen M, Roulston D, Philip P. Different genetic pathways in leukemogenesis for patients presenting with therapy-related myelodysplasia and therapy-related acute myeloid leukemia. *Blood*. 1995;86:3542–3552.
71. Gibson CJ, Lindsley RC, Tchekmedyian V, et al. Clonal hematopoiesis associated with adverse outcomes after autologous stem-cell transplantation for lymphoma. *J Clin Oncol*. 2017;35:1598–1605.
72. Moore KN, Mirza MR, Matulonis UA. The poly (ADP ribose) polymerase inhibitor niraparib: management of toxicities. *Gynecol Oncol*. 2018;149:214–220.
73. Super HJ, McCabe NR, Thirman MJ, et al. Rearrangements of the MLL gene in therapy-related acute myeloid leukemia in patients previously treated with agents targeting DNA-topoisomerase II. *Blood*. 1993;82: 3705–3711.
74. Nimer SD. MDS: a stem cell disorder—but what exactly is wrong with the primitive hematopoietic cells in this disease? *Hematology Am Soc Hematol Educ Program*. 2008;2008:43–51.
75. Pang WW, Pluvinage JV, Price EA, et al. Hematopoietic stem cell and progenitor cell mechanisms in myelodysplastic syndromes. *Proc Natl Acad Sci U S A*. 2013;110:3011–3016.
76. Janssen JW, Buschle M, Layton M, et al. Clonal analysis of myelodysplastic syndromes: evidence of multipotent stem cell origin. *Blood*. 1989;73: 248–254.
77. Walter MJ, Shen D, Ding L, et al. Clonal architecture of secondary acute myeloid leukemia. *N Engl J Med*. 2012;366:1090–1098.
78. Magee JA, Piskounova E, Morrison SJ. Cancer stem cells: impact, heterogeneity, and uncertainty. *Cancer Cell*. 2012;21:283–296.
79. Clevers H. The cancer stem cell: premises, promises and challenges. *Nat Med*. 2011:313–319.
80. Thanopoulou E. Engraftment of NOD/SCID-beta2 microglobulin null mice with multilineage neoplastic cells from patients with myelodysplastic syndrome. *Blood*. 2004;103:4285–4293.
81. Woll PS, Kjällquist U, Chowdhury O, et al. Myelodysplastic syndromes are propagated by rare and distinct human cancer stem cells in vivo. *Cancer Cell*. 2014;25:794–808.
82. Will B, Zhou L, Vogler TO, et al. Stem and progenitor cells in myelodysplastic syndromes show aberrant stage-specific expansion and harbor genetic and epigenetic alterations. *Blood*. 2012;120:2076–2086.
83. Jaiswal S, Ebert BL. MDS is a stem cell disorder after all. *Cancer Cell*. 2014;25:713–714.
84. Sperling AS, Gibson CJ, Ebert BL. The genetics of myelodysplastic syndrome: from clonal haematopoiesis to secondary leukaemia. *Nat Rev Cancer*. 2017;17:5–19.
85. Jacobs RH, Cornbleet MA, Vardiman JW, Larson RA, Le Beau MM, Rowley JD. Prognostic implications of morphology and karyotype in primary myelodysplastic syndromes. *Blood*. 1986;67:1765–1772.
86. Kelly L, Clark J, Gilliland DG. Comprehensive genotypic analysis of leukemia: clinical and therapeutic implications. *Curr Opin Oncol*. 2002;14:10–18.
87. Anastasi J, Feng J, Le Beau MM, Larson RA, Rowley JD, Vardiman JW. Cytogenetic clonality in myelodysplastic syndromes studied with fluorescence in situ hybridization: lineage, response to growth factor therapy, and clone expansion. *Blood*. 1993;81:1580–1585.
88. Gondek LP, Tiu R, O'Keefe CL, Sekeres MA, Theil KS, Maciejewski JP. Chromosomal lesions and uniparental disomy detected by SNP arrays in MDS, MDS/MPD, and MDS-derived AML. *Blood*. 2008;111:1534–1542.
89. Damm F, Fontenay M, Bernard OA. Point mutations in myelodysplastic syndromes. *N Engl J Med*. 2011;365:1154–1155. author reply 155.
90. Bravo GM, Lee E, Merchan B, Kantarjian HM, García-Manero G. Integrating genetics and epigenetics in myelodysplastic syndromes: advances in pathogenesis and disease evolution. *Br J Haematol*. 2014;166:646–659.
91. Kennedy AL, Shimamura A. Genetic predisposition to MDS: clinical features and clonal evolution. *Blood*. 2019;133:1071–1085.
92. Preudhomme C, Renneville A, Bourdon V, et al. High frequency of RUNX1 biallelic alteration in acute myeloid leukemia secondary to familial platelet disorder. *Blood*. 2009;113:5583–5587.
93. Nagamachi A, Matsui H, Asou H, et al. Haploinsufficiency of SAMD9L, an endosome fusion facilitator, causes myeloid malignancies in mice mimicking human diseases with monosomy 7. *Cancer Cell*. 2013;24:305–317.
94. Hyde RK, Liu PP. GATA2 mutations lead to MDS and AML. *Nat Genet*. 2011;43:926–927.
95. Lindsley RC, Ebert BL. The biology and clinical impact of genetic lesions in myeloid malignancies. *Blood*. 2013;122:3741–3748.
96. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell*. 2013;153:1194–1217.
97. Behjati S, Huch M, van Boxtel R, et al. Genome sequencing of normal cells reveals developmental lineages and mutational processes. *Nature*. 2014;513:422–425.
98. Welch JS, Ley TJ, Link DC, et al. The origin and evolution of mutations in acute myeloid leukemia. *Cell*. 2012;150:264–278.
99. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz Jr. LA, Kinzler KW. Cancer genome landscapes. *Science*. 2013;339:1546–1558.
100. Papaemmanuil E, Gerstung M, Malcovati L, et al. Clinical and biological implications of driver mutations in myelodysplastic syndromes. *Blood*. 2013;122:3616–3627. quiz 99.
101. Bejar R, Stevenson K, Abdel-Wahab O, et al. Clinical effect of point mutations in myelodysplastic syndromes. *N Engl J Med*. 2011;364:2496–2506.
102. Haferlach T, Nagata Y, Grossmann V, et al. Landscape of genetic lesions in 944 patients with myelodysplastic syndromes. *Leukemia*. 2014;28:241–247.
103. Malcovati L, Papaemmanuil E, Ambaglio I, et al. Driver somatic mutations identify distinct disease entities within myeloid neoplasms with myelodysplasia. *Blood*. 2014;124:1513–1521.
104. Lindsley RC, Saber W, Mar BG, et al. Prognostic mutations in myelodysplastic syndrome after stem-cell transplantation. *N Engl J Med*. 2017;376:536–547.
105. Xie M, Lu C, Wang J, et al. Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nat Med*. 2014;20:1472–1478.
106. Wong TN, Ramsingh G, Young AL, et al. Role of TP53 mutations in the origin and evolution of therapy-related acute myeloid leukaemia. *Nature*. 2015;518:552–555.
107. Coombs CC, Zehir A, Devlin SM, et al. Therapy-related clonal hematopoiesis in patients with non-hematologic cancers is common and associated with adverse clinical outcomes. *Cell Stem Cell*. 2017;21:374–382. e4.
108. Bejar R, Stevenson K, Abdel-Wahab O, et al. Clinical effect of point mutations in myelodysplastic syndromes. *N Engl J Med*. 2011;364:2496–2506.
109. Langemeijer SM, Kuiper RP, Berends M, et al. Acquired mutations in TET2 are common in myelodysplastic syndromes. *Nat Genet*. 2009;41:838–842.
110. Kosmider O, Gelsi-Boyer V, Ciudad M, et al. TET2 gene mutation is a frequent and adverse event in chronic myelomonocytic leukemia. *Haematologica*. 2009;94:1676–1681.
111. Nakajima H, Kunimoto H. TET2 as an epigenetic master regulator for normal and malignant hematopoiesis. *Cancer Sci*. 2014;105:1093–1099.
112. Wu H, Zhang Y. Mechanisms and functions of Tet protein-mediated 5-methylcytosine oxidation. *Genes Dev*. 2011;25:2436–2452.
113. Jankowska AM, Szpurka H, Tiu RV, et al. Loss of heterozygosity 4q24 and TET2 mutations associated with myelodysplastic/myeloproliferative neoplasms. *Blood*. 2009;113:6403–6410.
114. Yamazaki J, Taby R, Vasanthakumar A, et al. Effects of TET2 mutations on DNA methylation in chronic myelomonocytic leukemia. *Epigenetics*. 2012;7:201–207.
115. Yang L, Rau R, Goodell MA. DNMT3A in haematological malignancies. *Nat Rev Cancer*. 2015;15:152–165.

116. Ley TJ, Ding L, Walter MJ, et al. DNMT3A mutations in acute myeloid leukemia. *N Engl J Med*. 2010;363:2424–2433.
117. Abdel-Wahab O, Adli M, LaFave LM, et al. ASXL1 mutations promote myeloid transformation through loss of PRC2-mediated gene repression. *Cancer Cell*. 2012;22:180–193.
118. Gelsi-Boyer V, Trouplin V, Adélaïde J, et al. Mutations of polycomb-associated gene ASXL1 in myelodysplastic syndromes and chronic myelomonocytic leukaemia. *Br J Haematol*. 2009;145:788–800.
119. Boultwood J, Perry J, Pellagatti A, et al. Frequent mutation of the polycomb-associated gene ASXL1 in the myelodysplastic syndromes and in acute myeloid leukemia. *Leukemia*. 2010;24:1062–1065.
120. Carbuccia N, Murati A, Trouplin V, et al. Mutations of ASXL1 gene in myeloproliferative neoplasms. *Leukemia*. 2009;23:2183–2186.
121. Ochi Y, Kon A, Sakata T, et al. Combined cohesin-RUNX1 deficiency synergistically perturbs chromatin looping and causes myelodysplastic syndromes. *Cancer Discov*. 2020;10:836–853.
122. Papaemmanuil E, Cazzola M, Boultwood J, et al. Somatic SF3B1 mutation in myelodysplasia with ring sideroblasts. *N Engl J Med*. 2011;365:1384–1395.
123. Yoshida K, Sanada M, Shiraishi Y, et al. Frequent pathway mutations of splicing machinery in myelodysplasia. *Nature*. 2011;478:64–69.
124. Damm F, Thol F, Kosmider O, et al. SF3B1 mutations in myelodysplastic syndromes: clinical associations and prognostic implications. *Leukemia*. 2012;26:1137–1140.
125. Malcovati L, Papaemmanuil E, Bowen DT, et al. Clinical significance of SF3B1 mutations in myelodysplastic syndromes and myelodysplastic/myeloproliferative neoplasms. *Blood*. 2011;118:6239–6246.
126. Thol F, Kade S, Schlarmann C, et al. Frequency and prognostic impact of mutations in SRSF2, U2AF1, and ZRSR2 in patients with myelodysplastic syndromes. *Blood*. 2012;119:3578–3584.
127. Meggendorfer M, Roller A, Haferlach T, et al. SRSF2 mutations in 275 cases with chronic myelomonocytic leukemia (CMML). *Blood*. 2012;120:3080–3088.
128. Wu S-J, Kuo Y-Y, Hou H-A, et al. The clinical implication of SRSF2 mutation in patients with myelodysplastic syndrome and its stability during disease evolution. *Blood*. 2012;120:3106–3111.
129. Graubert TA, Shen D, Ding L. Recurrent mutations in the U2AF1 splicing factor in myelodysplastic syndromes. *Nat Genet*. 2012;44:53–U77.
130. Okeyo-Owuor T, White BS, Chatrikhi R, et al. U2AF1 mutations alter sequence specificity of pre-mRNA binding and splicing. *Leukemia*. 2014
131. Kastenhuber ER, Lowe SW. Putting p53 in context. *Cell*. 2017;170:1062–1078.
132. Boettcher S, Miller PG, Sharma R, et al. A dominant-negative effect drives selection of TP53 missense mutations in myeloid malignancies. *Science*. 2019;365:599–604.
133. Jädersten M, Saft L, Smith A. TP53 mutations in low-risk myelodysplastic syndromes with del(5q) predict disease progression. *J Clin Oncol*. 2011;29:1971–1979.
134. Fiscella M, Zhang H, Fan S, et al. Wip1, a novel human protein phosphatase that is induced in response to ionizing radiation in a p53-dependent manner. *Proc Natl Acad Sci U S A*. 1997;94:6048–6053.
135. Lu X, Nannenga B, Donehower LA. PPM1D dephosphorylates Chk1 and p53 and abrogates cell cycle checkpoints. *Genes Dev*. 2005;19:1162–1174.
136. Lu X, Nguyen T-A, Donehower LA. Reversal of the ATM/ATR-mediated DNA damage response by the oncogenic phosphatase PPM1D. *Cell Cycle (Georgetown, Tex)*. 2005;4:1060–1064.
137. Takekawa M, Adachi M, Nakahata A, et al. p53-inducible wip1 phosphatase mediates a negative feedback regulation of p38 MAPK-p53 signaling in response to UV radiation. *EMBO J*. 2000;19:6517–6526.
138. Chew J, Biswas S, Shreeram S, et al. WIP1 phosphatase is a negative regulator of NF-kappaB signalling. *Nat Cell Biol*. 2009;11:659–666.
139. Ruark E, Snape K, Humburg P, et al. Mosaic PPM1D mutations are associated with predisposition to breast and ovarian cancer. *Nature*. 2012;493:406–410.
140. Swisher EM, Harrell MI, Norquist BM, et al. Somatic mosaic mutations in PPM1D and TP53 in the blood of women with ovarian carcinoma. *JAMA Oncol*. 2016;2:370–372.
141. Kahn JD, Miller PG, Silver AJ, et al. PPM1D-truncating mutations confer resistance to chemotherapy and sensitivity to PPM1D inhibition in hematopoietic cells. *Blood*. 2018;132:1095–1105.
142. Wong TN, Miller CA, Jotte MRM, et al. Cellular stressors contribute to the expansion of hematopoietic clones of varying leukemic potential. *Nat Commun*. 2018;9:455.
143. Bolton KL, Ptashkin RN, Gao T, et al. Oncologic therapy shapes the fitness landscape of clonal hematopoiesis. *bioRxiv*. 2019:848739.
144. Lindsley RC, Saber W, Mar BG, et al. Prognostic mutations in myelodysplastic syndrome after stem-cell transplantation. *N Engl J Med*. 2017;376:536–547.
145. Ezhkova E, Pasolli HA, Parker JS, et al. EZH2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. *Cell*. 2009;136:1122–1135.
146. Wang X, Dai H, Wang Q, et al. EZH2 mutations are related to low blast percentage in bone marrow and -7/del(7q) in de novo acute myeloid leukemia. *PLoS One*. 2013;8:e61341.
147. Jankowska AM, Makishima H, Tiu RV, et al. Mutational spectrum analysis of chronic myelomonocytic leukemia includes genes associated with epigenetic regulation: UTX, EZH2, and DNMT3A. *Blood*. 2011;118:3932–3941.
148. Ernst T, Chase AJ, Score J, et al. Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. *Nat Genet*. 2010;42:722–726.
149. Nikoloski G, Langemeijer SM, Kuiper RP, et al. Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. *Nat Genet*. 2010;42:665–667.
150. Ito Y, Bae S-C, Chuang LSH. The RUNX family: developmental regulators in cancer. *Nat Rev Cancer*. 2015;15:81–95.
151. Miyoshi H, Shimizu K, Kozu T, Maseki N, Kaneko Y, Ohki M. t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. *Proc Natl Acad Sci U S A*. 1991;88:10431–10434.
152. Golub TR, Barker GF, Bohlander SK, et al. Fusion of the TEL gene on 12p13 to the AML1 gene on 21q22 in acute lymphoblastic leukemia. *Proc Natl Acad Sci U S A*. 1995;92:4917–4921.
153. Harada Y, Harada H. Molecular pathways mediating MDS/AML with focus on AML1/RUNX1 point mutations. *J Cell Physiol*. 2009;220:16–20.
154. Matsuura S, Komeno Y, Stevenson KE, et al. Expression of the runt homology domain of RUNX1 disrupts homeostasis of hematopoietic stem cells and induces progression to myelodysplastic syndrome. *Blood*. 2012;120:4028–4037.
155. Huang G, Zhao X, Wang L, et al. The ability of MLL to bind RUNX1 and methylate H3K4 at PU.1 regulatory regions is impaired by MDS/AML-associated RUNX1/AML1 mutations. *Blood*. 2011;118:6544–6552.
156. Gearhart MD, Corcoran CM, Wamstad JA, Bardwell VJ. Polycomb group and SCF ubiquitin ligases are found in a novel BCOR complex that is recruited to BCL6 targets. *Mol Cell Biol*. 2006;26:6880–6889.
157. Haferlach T, Nagata Y, Grossmann V, et al. Landscape of genetic lesions in 944 patients with myelodysplastic syndromes. *Leukemia*. 2014;28:241–247.
158. Damm F, Chesnais V, Nagata Y, et al. BCOR and BCORL1 mutations in myelodysplastic syndromes and related disorders. *Blood*. 2013;122:3169–3177.
159. Tara S, Isshiki Y, Nakajima-Takagi Y, et al. Bcor insufficiency promotes initiation and progression of myelodysplastic syndrome. *Blood*. 2018;132:2470–2483.
160. Fears S, Gavin M, Zhang DE, et al. Functional characterization of ETV6 and ETV6/CBFA2 in the regulation of the MCSFR proximal promoter. *Proc Natl Acad Sci U S A*. 1997;94:1949–1954.
161. Peeters P, Wlodarska I, Baens M, et al. Fusion of ETV6 to MDS1/EVI1 as a result of t(3;12)(q26;p13) in myeloproliferative disorders. *Cancer Res*. 1997;57:564–569.
162. Wlodarska I, Selleri L, La Starza R, et al. Molecular cytogenetics localizes two new breakpoints on 11q23.3 and 21q11.2 in myelodysplastic syndrome with t(11;21) translocation. *Genes Chromosomes Cancer*. 1999;24:199–206.
163. Padron E, Yoder S, Kunigal S, et al. ETV6 and signaling gene mutations are associated with secondary transformation of myelodysplastic syndromes to chronic myelomonocytic leukemia. *Blood*. 2014;123:3675–3677.
164. Kon A, Shih L-Y, Minamino M, et al. Recurrent mutations in multiple components of the cohesin complex in myeloid neoplasms. *Nat Genet*. 2013;45:1232–1237.
165. Waldman T. Emerging themes in cohesin cancer biology. *Nat Rev Cancer*. 2020;20(9):504–515.
166. Kottaridis PD. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. *Blood*. 2001;98:1752–1759.
167. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of Jak2 in myeloproliferative disorders. *N Engl J Med*. 2005;352:1779–1790.
168. Pardanani AD, Levine RL, Lasho T, et al. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. *Blood*. 2006;108:3472–3476.
169. Furitsu T, Tsujimura T, Tono T, et al. Identification of mutations in the coding sequence of the proto-oncogene c-kit in a human mast cell leukemia cell line causing ligand-independent activation of c-kit product. *J Clin Invest*. 1993;92:1736–1744.
170. Bains A, Luthra R, Medeiros LJ, Zuo Z. FLT3 and NPM1 mutations in myelodysplastic syndromes: Frequency and potential value for predicting progression to acute myeloid leukemia. *Am J Clin Pathol*. 2011;135:62–69.

171. Gallagher A, Darley R, Padua RA. RAS and the myelodysplastic syndromes. *Pathol Biol*. 1997;45:561–568.
172. Side LE, Curtiss NP, Teel K, et al. RAS, FLT3, and TP53 mutations in therapy-related myeloid malignancies with abnormalities of chromosomes 5 and 7. *Genes Chromosomes Cancer*. 2004;39:217–223.
173. Kawankar N, Vundinti BR. Cytogenetic abnormalities in myelodysplastic syndrome: an overview. *Hematology*. 2011;16:131–138.
174. List A, Ebert BL, Fenaux P. A decade of progress in myelodysplastic syndrome with chromosome 5q deletion. *Leukemia*. 2018;32:1493–1499.
175. Ebert BL. Molecular dissection of the 5q deletion in myelodysplastic syndrome. *Semin Oncol*. 2011;38:621–626.
176. Le Beau MM, Espinosa 3rd R, Neuman WL, et al. Cytogenetic and molecular delineation of the smallest commonly deleted region of chromosome 5 in malignant myeloid diseases. *Proc Natl Acad Sci U S A*. 1993;90:5484–5488.
177. Pedersen B. Anatomy of the 5q− deletion: different sex ratios and deleted 5q bands in MDS and AML. *Leukemia*. 1996;10:1883–1890.
178. List A, Dewald G, Bennett J, et al. Lenalidomide in the myelodysplastic syndrome with chromosome 5q deletion. *N Engl J Med*. 2006;355:1456–1465.
179. Ebert BL, Pretz J, Bosco J, et al. Identification of RPS14 as a 5q- syndrome gene by RNA interference screen. *Nature*. 2008;451:335–339.
180. Dutt S, Narla A, Lin K, et al. Haploinsufficiency for ribosomal protein genes causes selective activation of p53 in human erythroid progenitor cells. *Blood*. 2011;117:2567–2576.
181. Schneider RK, Schenone M, Ferreira MV, et al. Rps14 haploinsufficiency causes a block in erythroid differentiation mediated by S100A8 and S100A9. *Nat Med*. 2016;22:288–297.
182. Kumar MS, Narla A, Nonami A, et al. Coordinate loss of a microRNA and protein-coding gene cooperate in the pathogenesis of 5q− syndrome. *Blood*. 2011;118:4666–4673.
183. Krönke J, Fink EC, Hollenbach PW, et al. Lenalidomide induces ubiquitination and degradation of CK1α in del(5q) MDS. *Nature*. 2015;523:183–188.
184. Ribezzo F, Snoeren IAM, Ziegler S, et al. Rps14, Csnk1a1 and miRNA145/miRNA146a deficiency cooperate in the clinical phenotype and activation of the innate immune system in the 5q− syndrome. *Leukemia*. 2019;33:1759–1772.
185. Kere J, Ruutu T, de la Chapelle A. Monosomy 7 in granulocytes and monocytes in myelodysplastic syndrome. *N Engl J Med*. 1987;316:499–503.
186. Chen C, Liu Y, Rappaport AR, et al. MLL3 is a haplo insufficient 7q tumor suppressor in acute myeloid leukemia. *Cancer Cell*. 2014;25:652–665.
187. Wong CC, Martincorena I, Rust AG, et al. Inactivating CUX1 mutations promote tumorigenesis. *Nat Genet*. 2014;46:33–38.
188. Beau MM, Espinosa 3rd R, Davis EM, Eisenbart JD, Larson RA, Green ED. Cytogenetic and molecular delineation of a region of chromosome 7 commonly deleted in malignant myeloid diseases. *Blood*. 1996;88(6):1930–1935.
189. Cordoba I, González-Porras JR, Nomdedeu B, et al. Better prognosis for patients with del(7q) than for patients with monosomy 7 in myelodysplastic syndrome. *Cancer*. 2012;118:127–133.
190. Saumell S, Florensa L, Luño E, et al. Prognostic value of trisomy 8 as a single anomaly and the influence of additional cytogenetic aberrations in primary myelodysplastic syndromes. *Br J Haematol*. 2012;159:311–321.
191. Sloand EM, Mainwaring L, Fuhrer M, et al. Preferential suppression of trisomy 8 compared with normal hematopoietic cell growth by autologous lymphocytes in patients with trisomy 8 myelodysplastic syndrome. *Blood*. 2005;106:841–851.
192. Nilsson L, Astrand-Grundström I, Anderson K, et al. Involvement and functional impairment of the CD34(+)CD38(−)Thy-1(+) hematopoietic stem cell pool in myelodysplastic syndromes with trisomy 8. *Blood*. 2002;100:259–267.
193. Toyonaga T, Nakase H, Matsuura M, et al. Refractoriness of intestinal Behçet's disease with myelodysplastic syndrome involving trisomy 8 to medical therapies—our case experience and review of the literature. *Digestion*. 2013;88:217–221.
194. Haase D, Germing U, Schanz J, et al. New insights into the prognostic impact of the karyotype in MDS and correlation with subtypes: evidence from a core dataset of 2124 patients. *Blood*. 2007;110:4385–4395.
195. Bacher U, Haferlach T, Schnittger S, et al. Investigation of 305 patients with myelodysplastic syndromes and 20q deletion for associated cytogenetic and molecular genetic lesions and their prognostic impact. *Br J Haematol*. 2014;164:822–833.
196. Braun T, de Botton S, Taksin A-L, et al. Characteristics and outcome of myelodysplastic syndromes (MDS) with isolated 20q deletion: a report on 62 cases. *Leuk Res*. 2011;35:863–867.
197. Wang PW, Eisenbart JD, Espinosa 3rd R, Davis EM, Larson RA, Le Beau MM. Refinement of the smallest commonly deleted segment of chromosome 20 in malignant myeloid diseases and development of a PAC-based physical and transcription map. *Genomics*. 2000;67:28–39.
198. Kaneko H, Misawa S, Horiike S, Nakai H, Kashima K. TP53 mutations emerge at early phase of myelodysplastic syndrome and are associated with complex chromosomal abnormalities. *Blood*. 1995;85:2189–2193.
199. Soenen V, Preudhomme C, Roumier C, Daudignon A, Laï JL, Fenaux P. 17p Deletion in acute myeloid leukemia and myelodysplastic syndrome. Analysis of breakpoints and deleted segments by fluorescence in situ. *Blood*. 1998;91:1008–1015.
200. Patnaik MM, Hanson CA, Hodnefield JM, Knudson R, Van Dyke DL, Tefferi A. Monosomal karyotype in myelodysplastic syndromes, with or without monosomy 7 or 5, is prognostically worse than an otherwise complex karyotype. *Leukemia*. 2011;25:266–270.
201. Valcárcel D, Ademà V, Solé F, et al. Complex, not monosomal, karyotype is the cytogenetic marker of poorest prognosis in patients with primary myelodysplastic syndrome. *J Clin Oncol*. 2013;31:916–922.
202. Wong AK, Fang B, Zhang L, Guo X, Lee S, Schreck R. Loss of the Y chromosome: an age-related or clonal phenomenon in acute myelogenous leukemia/myelodysplastic syndrome? *Arch Pathol Lab Med*. 2008;132:1329–1332.
203. Wlodarska I, Marynen P, La Starza R, Mecucci C, Van den Berghe H. The ETV6, CDKN1B and D12S178 loci are involved in a segment commonly deleted in various 12p aberration in different hematological malignancies. *Cytogenet Cell Genet*. 1996;72:229–235.
204. Pedersen-Bjergaard J, Philip P. Balanced translocations involving chromosome bands 11q23 and 21q22 are highly characteristic of myelodysplasia and leukemia following therapy with cytostatic agents targeting at DNA-topoisomerase II. *Blood*. 1991;78:1147–1148.
205. Rubin CM, Larson RA, Anastasi J, et al. t(3;21)(q26;q22): a recurring chromosomal abnormality in therapy-related myelodysplastic syndrome and acute myeloid leukemia. *Blood*. 1990;76:2594–2598.
206. Testoni N, Borsaru G, Martinelli G, et al. 3q21 and 3q26 cytogenetic abnormalities in acute myeloblastic leukemia: biological and clinical features. *Haematologica*. 1999;84:690–694.
207. Gupta M, Ashok Kumar J, Sitaram U, et al. The t(6;9)(p22;q34) in myeloid neoplasms: a retrospective study of 16 cases. *Cancer Genet Cytogenet*. 2010;203:297–302.
208. Boulais PE, Frenette PS. Making sense of hematopoietic stem cell niches. *Blood*. 2015;125(17):2621–2629.
209. Kfoury Y, Scadden DT. Mesenchymal cell contributions to the stem cell niche. *Cell. Stem Cell*. 2015;16:239–253.
210. Verhoef GE, De Schouwer P, Ceuppens JL, Van Damme J, Goossens W, Boogaerts MA. Measurement of serum cytokine levels in patients with myelodysplastic syndromes. *Leukemia*. 1992;6:1268–1272.
211. Schipperus MR, Sonneveld P, Lindemans J, et al. The combined effects of Il-3, GM-CSF and G-CSF on the in vitro growth of myelodysplastic myeloid progenitor cells. *Leuk Res*. 1990;14:1019–1025.
212. Vas V, Senger K, Dörr K, Niebel A, Geiger H. Aging of the microenvironment influences clonality in hematopoiesis. *PLoS One*. 2012;7:e42080.
213. Raaijmakers MH, Mukherjee S, Guo S, et al. Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature*. 2010;464:852–857.
214. Santamaría C, Muntión S, Rosón B, et al. Impaired expression of DICER, DROSHA, SBDS and some microRNAs in mesenchymal stromal cells from myelodysplastic syndrome patients. *Haematologica*. 2012;97:1218–1224.
215. Kode A, Manavalan JS, Mosialou I, et al. Leukaemogenesis induced by an activating β-catenin mutation in osteoblasts. *Nature*. 2014;506:240–244.
216. Fuster JJ, MacLauchlan S, Zuriaga MA, et al. Clonal hematopoiesis associated with TET2 deficiency accelerates atherosclerosis development in mice. *Science*. 2017;355:842–847.
217. Buscarlet M, Bourgoin V, Busque L. Gene-specific lineage involvement of age-related clonal hematopoiesis. American Society of Hematology Annual Meeting and Exposition; 2017.
218. Basiorka AA, McGraw KL, Eksioglu EA, et al. The NLRP3 inflammasome functions as a driver of the myelodysplastic syndrome phenotype. *Blood*. 2016;128:2960–2975.
219. Warlick ED, Miller JS. Myelodysplastic syndromes: the role of the immune system in pathogenesis. *Leuk Lymphoma*. 2011;52:2045–2049.
220. Wei Y, Dimicoli S, Bueso-Ramos C, et al. Toll-like receptor alterations in myelodysplastic syndrome. *Leukemia*. 2013;27:1832–1840.
221. Maratheftis CI, Andreakos E, Moutsopoulos HM, Voulgarelis M. Toll-like receptor-4 is up-regulated in hematopoietic progenitor cells and contributes to increased apoptosis in myelodysplastic syndromes. *Clin Cancer Res*. 2007;13:1154–1160.
222. Chen X, Eksioglu EA, Zhou J, et al. Induction of myelodysplasia by myeloid-derived suppressor cells. *J Clin Invest*. 2013;123:4595–4611.
223. Kristinsson SY, Björkholm M, Hultcrantz M, Derolf ÅR, Landgren O, Goldin LR. Chronic immune stimulation might act as a trigger for the

development of acute myeloid leukemia or myelodysplastic syndromes. *J Clin Oncol*. 2011;29:2897–2903.

224. Nand S, Godwin JE. Hypoplastic myelodysplastic syndrome. *Cancer*. 1988;62:958–964.
225. Epperson DE, Nakamura R, Saunthararajah Y, Melenhorst J, Barrett AJ. Oligoclonal T cell expansion in myelodysplastic syndrome: evidence for an autoimmune process. *Leuk Res*. 2001;25:1075–1083.
226. Anderson RW, Volsky DJ, Greenberg B, et al. Lymphocyte abnormalities in preleukemia—I. Decreased NK activity, anomalous immunoregulatory cell subsets and deficient EBV receptors. *Leuk Res*. 1983;7:389–395.
227. Knox SJ, Greenberg BR, Anderson RW, Rosenblatt LS. Studies of T-lymphocytes in preleukemic disorders and acute nonlymphocytic leukemia: in vitro radiosensitivity, mitogenic responsiveness, colony formation, and enumeration of lymphocytic subpopulations. *Blood*. 1983;61:449–455.
228. Volsky DJ, Anderson RW. Deficiency in Epstein-Barr virus receptors on B-lymphocytes of preleukemia patients. *Cancer Res*. 1983;43:3923–3926.
229. Parker JE, Fishlock KL, Mijovic AJ, et al. "Low-risk" myelodysplastic syndrome is associated with excessive apoptosis and an increased ratio of pro-versus anti-apoptotic bcl-2-related proteins. *Br J Haematol*. 1998;103:1075.
230. Letai A. Restoring cancer's death sentence. *Cancer Cell*. 2006;10:343–345.
231. Reza S, Shetty V, Dar S, et al. Tumor necrosis factor-alpha levels decrease with anticytokine therapy in patients with myelodysplastic syndromes. *J Interferon Cytokine Res*. 1998;18:871–877.
232. Shetty V, Mundle S, Alvi S, et al. Measurement of apoptosis, proliferation and three cytokines in 46 patients with myelodysplastic syndromes. *Leuk Res*. 1996;20:891–900.
233. Letai AG. Diagnosing and exploiting cancer's addiction to blocks in apoptosis. *Nat Rev Cancer*. 2008;8:121–132.
234. Parker JE, Mufti GJ, Rasool F, et al. The role of apoptosis, proliferation, and the Bcl-2-related proteins in the myelodysplastic syndromes and acute myeloid leukemia secondary to MDS. *Blood*. 2000;96:3932–3938.
235. Slape CI, Saw J, Jowett JBM, et al. Inhibition of apoptosis by BCL2 prevents leukemic transformation of a murine myelodysplastic syndrome. *Blood*. 2012;120:2475–2483.
236. Bogenberger JM, Kornblau SM, Pierceall WE, et al. BCL-2 family proteins as 5-Azacytidine-sensitizing targets and determinants of response in myeloid malignancies. *Leukemia*. 2014;28:1657–1665.
237. Lindsley RC, Mar BG, Mazzola E, et al. Acute myeloid leukemia ontogeny is defined by distinct somatic mutations. *Blood*. 2015;125:1367–1376.
238. Takahashi K, Jabbour E, Wang X, et al. Dynamic acquisition of FLT3 or RAS alterations drive a subset of patients with lower risk MDS to secondary AML. *Leukemia*. 2013;27:2081–2083.
239. Pagano L, Pulsoni A, Vignetti M, et al. Secondary acute myeloid leukaemia: results of conventional treatments. Experience of GIMEMA trials. *Ann Oncol*. 2005;16:228–233.
240. Kobayashi Y, Nakayama M, Uemura N, et al. Analysis of myelodysplastic syndrome clones arising after multiple myeloma: a case study by correlative interphase cytogenetic analysis. *Jpn J Clin Oncol*. 1999;29:374–377.
241. Lorand-Metze I, Lima CS, Cardinalli IA, Vassallo J. Association of a myelodysplastic syndrome with hairy cell leukaemia. *Eur J Haematol*. 1995;55:341–343.
242. Mitterbauer G, Schwarzmeier J, Mitterbauer M, Jaeger U, Fritsch G, Schwarzinger I. Myelodysplastic syndrome/acute myeloid leukemia supervening previously untreated chronic B-lymphocytic leukemia: demonstration of the concomitant presence of two different malignant clones by immunologic and molecular analysis. *Ann Hematol*. 1997;74:193–197.
243. Wells DA, Hall MC, Shulman HM, Loken MR. Occult B cell malignancies can be detected by three-color flow cytometry in patients with cytopenias. *Leukemia*. 1998;12:2015–2023.
244. Saunthararajah Y, Molldrem JL, Rivera M, et al. Coincident myelodysplastic syndrome and T-cell large granular lymphocytic disease: clinical and pathophysiological features. *Br J Haematol*. 2001;112:195–200.
245. Steensma DP, Heptinstall KV, Johnson VM, et al. Common troublesome symptoms and their impact on quality of life in patients with myelodysplastic syndromes (MDS): results of a large internet-based survey. *Leuk Res*. 2008;32:691–698.
246. Pomeroy C, Oken MM, Rydell RE, Filice GA. Infection in the myelodysplastic syndromes. *Am J Med*. 1991;90:338–344.
247. Raman BK, Van Slyck EJ, Riddle J, Sawdyk MA, Abraham JP, Saeed SM. Platelet function and structure in myeloproliferative disease, myelodysplastic syndrome, and secondary thrombocytosis. *Am J Clin Pathol*. 1989;91:647–655.
248. Avivi I, Rosenbaum H, Levy Y, Rowe J. Myelodysplastic syndrome and associated skin lesions: a review of the literature. *Leuk Res*. 1999;23: 323–330.
249. Rochet NM, Chavan RN, Cappel MA, Wada DA, Gibson LE. Sweet syndrome: clinical presentation, associations, and response to treatment in 77 patients. *J Am Acad Dermatol*. 2013;69:557–564.
250. Ten Oever J, Kuijper PHM, Kuijpers ALA, Dercksen MW, Vreugdenhil G. Complete remission of MDS RAEB following immunosuppressive treatment in a patient with Sweet's syndrome. *Neth J Med*. 2009;67:347–350.
251. Srivastava M, Rencic A, Nousari HC. A rapidly expanding ulcer. Myelodysplastic syndrome-associated (paraneoplastic) pyoderma gangrenosum. *Arch Dermatol*. 2003;139:531–536.
252. Duguid JK, Mackie MJ, McVerry BA. Skin infiltration associated with chronic myelomonocytic leukaemia. *Br J Haematol*. 1983;53:257–264.
253. Saif MW, Hopkins JL, Gore SD. Autoimmune phenomena in patients with myelodysplastic syndromes and chronic myelomonocytic leukemia. *Leuk Lymphoma*. 2002;43:2083–2092.
254. George SW, Newman ED. Seronegative inflammatory arthritis in the myelodysplastic syndromes. *Semin Arthritis Rheum*. 1992;21:345–354.
255. Green AR, Shuttleworth D, Bowen DT, Bentley DP. Cutaneous vasculitis in patients with myelodysplasia. *Br J Haematol*. 1990;74:364–365.
256. Tsuji G, Maekawa S, Saigo K, et al. Dermatomyositis and myelodysplastic syndrome with myelofibrosis responding to methotrexate therapy. *Am J Hematol*. 2003;74:175–178.
257. Enright H, Jacob HS, Vercellotti G, Howe R, Belzer M, Miller W. Paraneoplastic autoimmune phenomena in patients with myelodysplastic syndromes: response to immunosuppressive therapy. *Br J Haematol*. 1995;91:403–408.
258. Noël P, Solberg Jr. LA. Myelodysplastic syndromes. Pathogenesis, diagnosis, and treatment. *Crit Rev Oncol Hematol*. 1992;12:193–215.
259. Bowen DT, Jacobs A, Cotes PM, Lewis TC. Serum erythropoietin and erythropoiesis in patients with myelodysplastic syndromes. *Eur J Haematol*. 1990;44:30–32.
260. Kushner JP, Porter JP, Olivieri NF. Secondary iron overload. *Hematology Am Soc Hematol Educ Program*. 2001:47.
261. Choi JW, Kim Y, Fujino M, Ito M. Significance of fetal hemoglobin-containing erythroblasts (F blasts) and the F blast/F cell ratio in myelodysplastic syndromes. *Leukemia*. 2002;16:1478–1483.
262. Lopez M, Bonnet-Gajdos M, Reviron M, Janvier D, Huet M, Salmon C. An acute leukaemia augured before clinical signs by blood group antigen abnormalities and low levels of A and H blood group transferase activities in erythrocytes. *Br J Haematol*. 1986;63:535–539.
263. Kornberg A, Goldfarb A. Preleukemia manifested by hemolytic anemia with pyruvate-kinase deficiency. *Arch Intern Med*. 1986;146:785–786.
264. Hast R. Sideroblasts in myelodysplasia: their nature and clinical significance. *Scand J Haematol Suppl*. 1986;45:53–55.
265. Steensma DP, Higgs DR, Fisher CA, Gibbons RJ. Acquired somatic ATRX mutations in myelodysplastic syndrome associated with alpha thalassemia (ATMDS) convey a more severe hematologic phenotype than germline ATRX mutations. *Blood*. 2004;103:2019–2026.
266. Annino L, Di Giovanni S, Tentorì Jr. L, et al. Acquired hemoglobin H disease in a case of refractory anemia with excess of blasts (RAEB) evolving into acute nonlymphoid leukemia. *Acta Haematol*. 1984;72:41–44.
267. French registry of acute leukemia and myelodysplastic syndromes Age distribution and hemogram analysis of the 4496 cases recorded during 1982–1983 and classified according to FAB criteria Groupe Francais de Morphologie Hematologique. *Cancer*. 1987;60:1982–1983. 1385.
268. Cech P, Markert M, Perrin LH. Partial myeloperoxidase deficiency in preleukemia. *Blut*. 1983;47:21–30.
269. Prodan M, Tulissi P, Perticarari S, et al. Flow cytometric assay for the evaluation of phagocytosis and oxidative burst of polymorphonuclear leukocytes and monocytes in myelodysplastic disorders. *Haematologica*. 1995;80:212–218.
270. Ruutu P. Granulocyte function in myelodysplastic syndromes. *Scand J Haematol Suppl*. 1986;45:66–70.
271. Tricot G, De Wolf-Peeters C, Vlietinck R, Verwilghen RL. Bone marrow histology in myelodysplastic syndromes. II. Prognostic value of abnormal localization of immature precursors in MDS. *Br J Haematol*. 1984;58: 217–225.
272. Della Porta MG, Malcovati L, Boveri E, et al. Clinical relevance of bone marrow fibrosis and CD34-positive cell clusters in primary myelodysplastic syndromes. *J Clin Oncol*. 2009;27:754–762.
273. Schanz J, Tüchler H, Solé F, et al. New comprehensive cytogenetic scoring system for primary myelodysplastic syndromes (MDS) and oligoblastic acute myeloid leukemia after MDS derived from an international database merge. *J Clin Oncol*. 2012;30:820–829.
274. Toma A, Fenaux P, Dreyfus F, Cordonnier C. Infections in myelodysplastic syndromes. *Haematologica*. 2012;97:1459–1470.
275. Rasi V, Lintula R. Platelet function in the myelodysplastic syndromes. *Scand J Haematol Suppl*. 1986;45:71–73.

276. Girtovitis FI, Ntaios G, Papadopoulos A, Ioannidis G, Makris PE. Defective platelet aggregation in myelodysplastic syndromes. *Acta Haematol*. 2007;118:117–122.
277. Zeidman A, Sokolover N, Fradin Z, Cohen A, Redlich O, Mittelman M. Platelet function and its clinical significance in the myelodysplastic syndromes. *Hematol J*. 2004;5:234–238.
278. Szpurka H, Tiu R, Murugesan G, et al. Refractory anemia with ringed sideroblasts associated with marked thrombocytosis (RARS-T), another myeloproliferative condition characterized by JAK2 V617F mutation. *Blood*. 2006;108:2173–2181.
279. Kuriyama K, Tomonaga M, Matsuo T, Ginnai I, Ichimaru M. Diagnostic significance of detecting pseudo-Pelger-Huet anomalies and micro-megakaryocytes in myelodysplastic syndrome. *Br J Haematol*. 1986;63:665–669.
280. Queisser W, Queisser U, Ansmann M, Brunner G, Hoelzer D, Heimpel H. Megakaryocyte polyploidization in acute leukaemia and preleukaemia. *Br J Haematol*. 1974;28:261–270.
281. Kerndrup G, Meyer K, Ellegaard J, Hokland P. Natural killer (NK)-cell activity and antibody-dependent cellular cytotoxicity (ADCC) in primary preleukemic syndrome. *Leuk Res*. 1984;8:239–247.
282. Britanova OV, Putintseva EV, Shugay M, et al. Age-related decrease in TCR repertoire diversity measured with deep and normalized sequence profiling. *J Immunol (Baltimore, Md: 1950)*. 2014;192:2689–2698.
283. Gibson KL, Wu Y-C, Barnett Y, et al. B-cell diversity decreases in old age and is correlated with poor health status. *Aging Cell*. 2009;8:18–25.
284. Wells RA, Leber B, Buckstein R, et al. Iron overload in myelodysplastic syndromes: a Canadian consensus guideline. *Leuk Res*. 2008;32:1338–1353.
285. Wimazal F, Sperr WR, Kundi M, et al. Prognostic value of lactate dehydrogenase activity in myelodysplastic syndromes. *Leuk Res*. 2001;25:287–294.
286. Economopoulos T, Economidou J, Giannopoulos G, et al. Immune abnormalities in myelodysplastic syndromes. *J Clin Pathol*. 1985;38:908–911.
287. Mufti GJ, Figes A, Hamblin TJ, Oscier DG, Copplestone JA. Immunological abnormalities in myelodysplastic syndromes. I. Serum immunoglobulins and autoantibodies. *Br J Haematol*. 1986;63:143–147.
288. Van den Berghe H, Cassiman JJ, David G, Fryns JP, Michaux JL, Sokal G. Distinct haematological disorder with deletion of long arm of no. 5 chromosome. *Nature*. 1974;251:437–438.
289. Sokal G, Michaux JL, Van Den Berghe H, et al. A new hematologic syndrome with a distinct karyotype: the 5q– chromosome. *Blood*. 1975;46:519–533.
290. Kulasekararaj AG, Jiang J, Smith AE, et al. Somatic mutations identify a subgroup of aplastic anemia patients who progress to myelodysplastic syndrome. *Blood*. 2014;124:2698–2704.
291. Iwanaga M, Furukawa K, Amenomori T, et al. Paroxysmal nocturnal haemoglobinuria clones in patients with myelodysplastic syndromes. *Br J Haematol*. 1998;102:465–474.
292. Sokol L, Loughran Jr. TP. Large granular lymphocyte leukemia. *Oncologist*. 2006;11:263–273.
293. Lane AA, Odejide O, Kopp N, et al. Low frequency clonal mutations recoverable by deep sequencing in patients with aplastic anemia. *Leukemia*. 2013;27:968–971.
294. Yoshizato T, Dumitriu B, Hosokawa K, et al. Somatic mutations and clonal hematopoiesis in aplastic anemia. *N Engl J Med*. 2015;373:35–47.
295. Koskela HL, Eldfors S, Ellonen P, et al. Somatic STAT3 mutations in large granular lymphocytic leukemia. *N Engl J Med*. 2012;366:1905–1913.
296. Selimoglu-Buet D, Badaoui B, Benayoun E, et al. Accumulation of classical monocytes defines a subgroup of MDS that frequently evolves into CMML. *Blood*. 2017;130:832–835.
297. Zoi K, Cross NC. Molecular pathogenesis of atypical CML, CMML and MDS/MPN-unclassifiable. *Int J Hematol*. 2014;101(3):229–242.
298. Padron E, Steensma DP. Cutting the cord from myelodysplastic syndromes: chronic myelomonocytic leukemia-specific biology and management strategies. *Curr Opin Hematol*. 2015;22:163–170.
299. Ohyashiki K, Sasao I, Ohyashiki JH, et al. Clinical and cytogenetic characteristics of myelodysplastic syndromes developing myelofibrosis. *Cancer*. 1991;68:178–183.
300. Kantarjian HM, Keating MJ, Walters RS, et al. Therapy-related leukemia and myelodysplastic syndrome: clinical, cytogenetic, and prognostic features. *J Clin Oncol*. 1986;4:1748–1757.
301. Leone G, Mele L, Pulsoni A, Equitani F, Pagano L. The incidence of secondary leukemias. *Haematologica*. 1999;84:937–945.
302. Tallman MS, Gray R, Bennett JM, et al. Leukemogenic potential of adjuvant chemotherapy for early-stage breast cancer: the Eastern Cooperative Oncology Group experience. *J Clin Oncol*. 1995;13:1557–1563.
303. Darrington DL, Vose JM, Anderson JR, et al. Incidence and characterization of secondary myelodysplastic syndrome and acute myelogenous leukemia following high-dose chemoradiotherapy and autologous stem-cell transplantation for lymphoid malignancies. *J Clin Oncol*. 1994;12:2527–2534.
304. Friedberg JW, Neuberg D, Stone RM, et al. Outcome in patients with myelodysplastic syndrome after autologous bone marrow transplantation for non-Hodgkin's lymphoma. *J Clin Oncol*. 1999;17:3128–3135.
305. Palumbo A, Bringhen S, Kumar SK, et al. Second primary malignancies with lenalidomide therapy for newly diagnosed myeloma: a meta-analysis of individual patient data. *Lancet Oncol*. 2014;15:333–342.
306. Andersen MK, Christiansen DH, Pedersen-Bjergaard J. Centromeric breakage and highly rearranged chromosome derivatives associated with mutations of TP53 are common in therapy-related MDS and AML after therapy with alkylating agents: an M-FISH study. *Genes Chromosomes Cancer*. 2005;42:358–371.
307. Pedersen-Bjergaard J. Radiotherapy- and chemotherapy-induced myelodysplasia and acute myeloid leukemia. A review. *Leuk Res*. 1992;16:61–65.
308. Abruzzese E, Radford JE, Miller JS, et al. Detection of abnormal pretransplant clones in progenitor cells of patients who developed myelodysplasia after autologous transplantation. *Blood*. 1999;94:1814–1819.
309. Gillis NK, Ball M, Zhang Q, et al. Clonal haemopoiesis and therapy-related myeloid malignancies in elderly patients: a proof-of-concept, case-control study. *Lancet Oncol*. 2017;18:112–121.
310. Takahashi K, Kantarjian H, Doss D, et al. Pre-leukemic clonal hematopoiesis and the risk of therapy-related myeloid neoplasms: a case-control study. *Lancet Oncol*. 2016;18(1):100–111.
311. Smith SM. Clinical-cytogenetic associations in 306 patients with therapy-related myelodysplasia and myeloid leukemia: the University of Chicago series. *Blood*. 2003;102:43–52.
312. Bejar R, Stevenson KE, Caughey B, et al. Somatic mutations predict poor outcome in patients with myelodysplastic syndrome after hematopoietic stem-cell transplantation. *J Clin Oncol*. 2014;32:2691–2698.
313. Chanarin I. Megaloblastic anaemia, cobalamin, and folate. *J Clin Pathol*. 1987;40:978–984.
314. Aitelli C, Wasson L, Page R. Pernicious anemia: presentations mimicking acute leukemia. *South Med J*. 2004;97:295–297.
315. Stabler SP. Clinical practice. Vitamin B12 deficiency. *N Engl J Med*. 2013;368:149–160.
316. Balsa JA, Botella-Carretero JI, Gómez-Martín JM, et al. Copper and zinc serum levels after derivative bariatric surgery: differences between Roux-en-Y gastric bypass and biliopancreatic diversion. *Obes Surg*. 2011;21:744–750.
317. Fiske DN, McCoy 3rd HE, Kitchens CS. Zinc-induced sideroblastic anemia: report of a case, review of the literature, and description of the hematologic syndrome. *Am J Hematol*. 1994;46:147–150.
318. Halfdanarson TR, Kumar N, Li C-Y, Phyliky RL, Hogan WJ. Hematological manifestations of copper deficiency: a retrospective review. *Eur J Haematol*. 2008;80:523–531.
319. Imataki O, Ohnishi H, Kitanaka A, Kubota Y, Ishida T, Tanaka T. Pancytopenia complicated with peripheral neuropathy due to copper deficiency: clinical diagnostic review. *Intern Med (Tokyo, Japan)*. 2008;47:2063–2065.
320. Huff JD, Keung Y-K, Thakuri M, et al. Copper deficiency causes reversible myelodysplasia. *Am J Hematol*. 2007;82:625–630.
321. Chanarin I. Alcohol and haemopoiesis. *Br J Haematol*. 1969;17:515–516.
322. Scott JM, Weir DG. Drug-induced megaloblastic change. *Clin Haematol*. 1980;9:587–606.
323. Hongeng S, May W, Crist WM. Transient myeloid dysplasia due to valproic acid. *Clin Pediatr (Phila)*. 1997;36:361–364.
324. Kennedy GA, Kay TD, Johnson DW, et al. Neutrophil dysplasia characterised by a pseudo-Pelger-Huet anomaly occurring with the use of mycophenolate mofetil and ganciclovir following renal transplantation: a report of five cases. *Pathology*. 2002;34:263–266.
325. Taegtmeyer AB, Halil O, Bell AD, Carby M, Cummins D, Banner NR. Neutrophil dysplasia (acquired pseudo-Pelger anomaly) caused by ganciclovir. *Transplantation*. 2005;80:127–130.
326. Gibbs SDJ, Westerman DA, McCormack C, Seymour JF, Miles Prince H. Severe and prolonged myeloid haematopoietic toxicity with myelodysplastic features following alemtuzumab therapy in patients with peripheral T-cell lymphoproliferative disorders. *Br J Haematol*. 2005;130:87–91.
327. Buchbinder R, Hall S, Ryan PF, Littlejohn GO, Harkness AJ. Severe bone marrow failure due to low dose oral methotrexate. *J Rheumatol*. 1988;15:1586–1588.
328. Piso RJ, Kriz K, Desax M-C. Severe isoniazid related sideroblastic anemia. *Hematol Rep*. 2011;3:e2.
329. Beck EA, Ziegler G, Schmid R, Lüdin H. Reversible sideroblastic anemia caused by chloramphenicol. *Acta Haematol*. 1967;38:1–10.

330. Verwilghen R, Reybrouck G, Callens L, Cosemans J. Antituberculous drugs and sideroblastic anaemia. *Br J Haematol*. 1965;11:92–98.
331. Colucci G, Silzle T, Solenthaler M. Pyrazinamide-induced sideroblastic anemia. *Am J Hematol*. 2012;87:305.
332. Haden HT. Pyridoxine-responsive sideroblastic anemia due to antituberculous drugs. *Arch Intern Med*. 1967;120:602–606.
333. Scott JL, Finegold SM, Belkin GA, Lawrence JS. A controlled double-blind study of the hematologic toxicity of chloramphenicol. *N Eng J Med*. 1965;272:1137–1142.
334. Karcher DS, Frost AR. The bone marrow in human immunodeficiency virus (HIV)-related disease. Morphology and clinical correlation. *Am J Clin Pathol*. 1991;95:63–71.
335. Scadden DT, Zon LI, Groopman JE. Pathophysiology and management of HIV-associated hematologic disorders. *Blood*. 1989;74:1455–1463.
336. Kasahara S, Hara T, Itoh H, et al. Hypoplastic myelodysplastic syndromes can be distinguished from acquired aplastic anaemia by bone marrow stem cell expression of the tumour necrosis factor receptor. *Br J Haematol*. 2002;118:181–188.
337. Pagliuca A, Layton DM, Manoharan A, Gordon S, Green PJ, Mufti GJ. Myelofibrosis in primary myelodysplastic syndromes: a clinico-morphological study of 10 cases. *Br J Haematol*. 1989;71:499–504.
338. Steensma DP. The clinical challenge of idiopathic cytopenias of undetermined significance (ICUS) and clonal cytopenias of undetermined significance (CCUS). *Curr Hematol Malig Rep*. 2019;14:536–542.
339. Malcovati L, Cazzola M. The shadowlands of MDS: idiopathic cytopenias of undetermined significance (ICUS) and clonal hematopoiesis of indeterminate potential (CHIP). *Hematology Am Soc Hematol Educ Program*. 2015;2015:299–307.
340. Malcovati L, Gallì A, Travaglino E, et al. Clinical significance of somatic mutation in unexplained blood cytopenia. *Blood*. 2017;129:3371–3378.
341. Abelson S, Collord G, Ng SWK, et al. Prediction of acute myeloid leukaemia risk in healthy individuals. *Nature*. 2018;559:400–404.
342. Font P, Loscertales J, Benavente C, et al. Inter-observer variance with the diagnosis of myelodysplastic syndromes (MDS) following the 2008 WHO classification. *Ann Hematol*. 2013;92:19–24.
343. Senent L, Arenillas L, Luño E, Ruiz JC, Sanz G, Florensa L. Reproducibility of the World Health Organization 2008 criteria for myelodysplastic syndromes. *Haematologica*. 2013;98:568–575.
344. Greenberg P, Cox C, LeBeau MM, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. *Blood*. 1997;89:2079–2088.
345. Greenberg PL, Tuechler H, Schanz J, et al. Revised international prognostic scoring system (IPSS-R) for myelodysplastic syndromes. *Blood*. 2012;120:2454–2465.
346. Garcia-Manero G, Shan J, Faderl S, et al. A prognostic score for patients with lower risk myelodysplastic syndrome. *Leukemia*. 2008;22:538–543.
347. Kantarjian H, O'Brien S, Ravandi F, et al. Proposal for a new risk model in myelodysplastic syndrome that accounts for events not considered in the original International Prognostic Scoring System. *Cancer*. 2008;113:1351–1361.
348. Malcovati L, Della Porta MG, Strupp C, et al. Impact of the degree of anemia on the outcome of patients with myelodysplastic syndrome and its integration into the WHO classification-based Prognostic Scoring System (WPSS). *Haematologica*. 2011;96:1433–1440.
349. Voso MT, Fenu S, Latagliata R, et al. Revised International Prognostic Scoring System (IPSS) predicts survival and leukemic evolution of myelodysplastic syndromes significantly better than IPSS and WHO Prognostic Scoring System: validation by the Gruppo Romano Mielodisplasie Italian Regional Database. *J Clin Oncol*. 2013;31:2671–2677.
350. Neukirchen J, Lauseker M, Blum S, et al. Validation of the revised international prognostic scoring system (IPSS-R) in patients with myelodysplastic syndrome: a multicenter study. *Leuk Res*. 2014;38:57–64.
351. Zeidan AM, Lee J-W, Prebet T, et al. Comparison of the prognostic utility of the revised International Prognostic Scoring System and the French Prognostic Scoring System in azacitidine-treated patients with myelodysplastic syndromes. *Br J Haematol*. 2014;166:352–359.
352. Della Porta MG, Alessandrino EP, Bacigalupo A, et al. Predictive factors for the outcome of allogeneic transplantation in patients with MDS stratified according to the revised IPSS-R. *Blood*. 2014;123:2333–2342.
353. Germing U, Hildebrandt B, Pfeilstöcker M, et al. Refinement of the international prognostic scoring system (IPSS) by including LDH as an additional prognostic variable to improve risk assessment in patients with primary myelodysplastic syndromes (MDS). *Leukemia*. 2005;19:2223–2231.
354. Kikuchi S, Kobune M, Iyama S, et al. Prognostic significance of serum ferritin level at diagnosis in myelodysplastic syndrome. *Int J Hematol*. 2012;95:527–534.
355. Buckstein R, Wells RA, Zhu N, et al. Patient-related factors independently impact overall survival in patients with myelodysplastic syndromes: an MDS-CAN prospective study. *Br J Haematol*. 2016;174:88–101.
356. Naqvi K, Garcia-Manero G, Sardesai S, et al. Association of comorbidities with overall survival in myelodysplastic syndrome: development of a prognostic model. *J Clin Oncol*. 2011;29:2240–2246.
357. Bersanelli M, Travaglino E, Meggendorfer M, et al. Classification and personalized prognostic assessment on the basis of clinical and genomic features in myelodysplastic syndromes. *J Clin Oncol*. 2021;39:1223–1233.
358. Pine AB, Chokr N, Stahl M, et al. Wide variation in use and interpretation of gene mutation profiling panels among health care providers of patients with myelodysplastic syndromes: results of a large web-based survey. *Leuk Lymphoma*. 2020;61:1455–1464.
359. Todisco G, Creignou M, Gallì A, et al. Co-mutation pattern, clonal hierarchy, and clone size concur to determine disease phenotype of $SRSF_2$(P95)-mutated neoplasms. *Leukemia*. 2020
360. Komrokji RS, Corrales-Yepez M, Al Ali N, et al. Validation of the MD Anderson Prognostic Risk Model for patients with myelodysplastic syndrome. *Cancer*. 2012;118:2659–2664.
361. van Spronsen MF, Ossenkoppele GJ, Holman R, van de Loosdrecht AA. Improved risk stratification by the integration of the revised international prognostic scoring system with the myelodysplastic syndromes comorbidity index. *Eur J Cancer (Oxford, England: 1990)*. 5020143198–3205.
362. Malcovati L, Germing U, Kuendgen A, et al. Time-dependent prognostic scoring system for predicting survival and leukemic evolution in myelodysplastic syndromes. *J Clin Oncol*. 2007;25:3503–3510.
363. Della Porta MG, Tuechler H, Malcovati L, et al. Validation of WHO classification-based prognostic scoring system (WPSS) for myelodysplastic syndromes and comparison with the revised international prognostic scoring system (IPSS-R). A study of the International Working Group for Prognosis in Myelodysplasia (IWG-PM). *Leukemia*. 2015;29:1502–1513.
364. Platzbecker U, Fenaux P, Adès L, et al. Proposals for revised IWG 2018 hematological response criteria in patients with MDS included in clinical trials. *Blood*. 2019;133:1020–1030.
365. Cheson BD, Bennett JM, Kantarjian H, et al. Report of an international working group to standardize response criteria for myelodysplastic syndromes. *Blood*. 2000;96:3671–3674.
366. Jansen AJ, Essink-Bot ML, Beckers EA, Hop WC, Schipperus MR, Van Rhenen DJ. Quality of life measurement in patients with transfusion-dependent myelodysplastic syndromes. *Br J Haematol*. 2003;121:270–274.
367. Abel GA, Klaassen R, Lee SJ, et al. Patient-reported outcomes for the myelodysplastic syndromes: a new MDS-specific measure of quality of life. *Blood*. 2014;123:451–452.
368. Hellström-Lindberg E, Malcovati L. Supportive care and use of hematopoietic growth factors in myelodysplastic syndromes. *Semin Hematol*. 2008;45:14–22.
369. Cazzola M, Malcovati L. Myelodysplastic syndromes—coping with ineffective hematopoiesis. *N Engl J Med*. 2005;352:536–538.
370. Hébert PC, Wells G, Blajchman MA, et al. A multicenter, randomized, controlled clinical trial of transfusion requirements in critical care. Transfusion Requirements in Critical Care Investigators, Canadian Critical Care Trials Group. *N Engl J Med*. 1999;340:409–417.
371. Hajjar LA, Vincent JL, Galas FR, et al. Transfusion requirements after cardiac surgery: the TRACS randomized controlled trial. *JAMA*. 2010;304:1559–1567.
372. Fakhry SM, Fata P. How low is too low? Cardiac risks with anemia. *Crit Care*. 2004;8(Suppl 2):S11–S14.
373. Oliva EN, Schey C, Hutchings AS. A review of anemia as a cardiovascular risk factor in patients with myelodysplastic syndromes. *Am J Blood Res*. 2011;1:160–166.
374. Anderson KC, Weinstein HJ. Transfusion-associated graft-versus-host disease. *N Engl J Med*. 1990;323:315–321.
375. Chacko J, Pennell DJ, Tanner MA, et al. Myocardial iron loading by magnetic resonance imaging T2* in good prognostic myelodysplastic syndrome patients on long-term blood transfusions. *Br J Haematol*. 2007;138:587–593.
376. Pascal L, Beyne-Rauzy O, Brechignac S, et al. Cardiac iron overload assessed by T2* magnetic resonance imaging and cardiac function in regularly transfused myelodysplastic syndrome patients. *Br J Haematol*. 2013;162:413–415.
377. Malcovati L. Impact of transfusion dependency and secondary iron overload on the survival of patients with myelodysplastic syndromes. *Leuk Res*. 2007;31(Suppl 3):S2–S6.
378. Chee CE, Steensma DP, Wu W, Hanson CA, Tefferi A. Neither serum ferritin nor the number of red blood cell transfusions affect overall survival in refractory anemia with ringed sideroblasts. *Am J Hematol*. 2008;83:611–613.

379. Rose EH, Abels RI, Nelson RA, McCullough DM, Lessin L. The use of r-HuEpo in the treatment of anaemia related to myelodysplasia (MDS). *Br J Haematol*. 1995;89:831–837.
380. Bessho M, Jinnai I, Matsuda A, Saito M, Hirashima K. Improvement of anemia by recombinant erythropoietin in patients with myelodysplastic syndromes and aplastic anemia. *Int J Cell Cloning*. 1990;8:445–458.
381. Stebler C, Tichelli A, Dazzi H, Gratwohl A, Nissen C, Speck B. High-dose recombinant human erythropoietin for treatment of anemia in myelodysplastic syndromes and paroxysmal nocturnal hemoglobinuria: a pilot study. *Exp Hematol*. 1990;18:1204–1208.
382. Bowen D, Culligan D, Jacobs A. The treatment of anaemia in the myelodysplastic syndromes with recombinant human erythropoietin. *Br J Haematol*. 1991;77:419–423.
383. Hellström E, Birgegård G, Lockner D, Helmers C, Ost A, Wide L. Treatment of myelodysplastic syndromes with recombinant human erythropoietin. *Eur J Haematol*. 1991;47:355–360.
384. Schouten HC, Vellenga E, van Rhenen DJ, de Wolf JT, Coppens PJ, Blijham GH. Recombinant human erythropoietin in patients with myelodysplastic syndromes. *Leukemia*. 1991;5:432–436.
385. Hellström-Lindberg E, Gulbrandsen N, Lindberg G, et al. A validated decision model for treating the anaemia of myelodysplastic syndromes with erythropoietin + granulocyte colony-stimulating factor: significant effects on quality of life. *Br J Haematol*. 2003;120:1037–1046.
386. Park S, Grabar S, Kelaidi C, et al. Predictive factors of response and survival in myelodysplastic syndrome treated with erythropoietin and G-CSF: the GFM experience. *Blood*. 2008;111:574–582.
387. Rigolin GM, Porta MD, Ciccone M, et al. In patients with myelodysplastic syndromes response to rHuEPO and G-CSF treatment is related to an increase of cytogenetically normal CD34 cells. *Br J Haematol*. 2004;126:501–507.
388. Musto P, Matera R, Minervini MM, et al. Low serum levels of tumor necrosis factor and interleukin-1 beta in myelodysplastic syndromes responsive to recombinant erythropoietin. *Haematologica*. 1994;79:265–268.
389. Goy A, Belanger C, Casadevall N, et al. High doses of intravenous recombinant erythropoietin for the treatment of anaemia in myelodysplastic syndrome. *Br J Haematol*. 1993;84:232–237.
390. Stasi R, Abruzzese E, Lanzetta G, Terzoli E, Amadori S. Darbepoetin alfa for the treatment of anemic patients with low- and intermediate-1-risk myelodysplastic syndromes. *Ann Oncol*. 2005;16:1921–1927.
391. Moyo V, Lefebvre P, Duh MS, et al. Erythropoiesis-stimulating agents in the treatment of anemia in myelodysplastic syndromes: a meta-analysis. *Ann Hematol*. 2008;87:527–536.
392. Terpos E, Mougiou A, Kouraklis A, et al. Prolonged administration of erythropoietin increases erythroid response rate in myelodysplastic syndromes: a phase II trial in 281 patients. *Br J Haematol*. 2002;118:174–180.
393. Verhoef GE, Zachée P, Ferrant A, et al. Recombinant human erythropoietin for the treatment of anemia in the myelodysplastic syndromes: a clinical and erythrokinetic assessment. *Ann Hematol*. 1992;64:16–21.
394. Kurzrock R, Talpaz M, Estey E, et al. Erythropoietin treatment in patients with myelodysplastic syndrome and anemia. *Leukemia*. 1991;5:985–990.
395. Spiriti MA, Latagliata R, Niscola P, et al. Impact of a new dosing regimen of epoetin alfa on quality of life and anemia in patients with low-risk myelodysplastic syndrome. *Ann Hematol*. 2005;84:167–176.
396. Jädersten M, Malcovati L, Dybedal I, et al. Erythropoietin and granulocyte-colony stimulating factor treatment associated with improved survival in myelodysplastic syndrome. *Oncol*. 2008;26:3607–3613.
397. Hellström-Lindberg E, Negrin R, Stein R, et al. Erythroid response to treatment with G-CSF plus erythropoietin for the anaemia of patients with myelodysplastic syndromes: proposal for a predictive model. *Br J Haematol*. 1997;99:344–351.
398. Rizzo JD, Brouwers M, Hurley P, et al. American Society of Hematology/American Society of Clinical Oncology clinical practice guideline update on the use of epoetin and darbepoetin in adult patients with cancer. *Blood*. 2010;116:4045–4059.
399. Phrommintikul A, Haas SJ, Elsik M, Krum H. Mortality and target haemoglobin concentrations in anaemic patients with chronic kidney disease treated with erythropoietin: a meta-analysis. *Lancet*. 2007;369:381–388.
400. National Comprehensive Cancer Network. Practice guidelines for the myelodysplastic syndromes. *Oncology (Williston Park)*. 1998;12:53–80.
401. Platzbecker U, Germing U, Götze KS, et al. Luspatercept for the treatment of anaemia in patients with lower-risk myelodysplastic syndromes (PACE-MDS): a multicentre, open-label phase 2 dose-finding study with long-term extension study. *Lancet Oncol*. 2017;18:1338–1347.
402. Fenaux P, Platzbecker U, Mufti GJ, et al. Luspatercept in patients with lower-risk myelodysplastic syndromes. *N Engl J Med*. 2020;382:140–151.
403. Ahn YS. Efficacy of danazol in hematologic disorders. *Acta Haematol*. 1990;84:122–129.
404. Boogaerts MA, Nelissen V, Roelant C, Goossens W. Blood neutrophil function in primary myelodysplastic syndromes. *Br J Haematol*. 1983;55:217–227.
405. Davey FR, Erber WN, Gatter KC, Mason DY. Abnormal neutrophils in acute myeloid leukemia and myelodysplastic syndrome. *Hum Pathol*. 1988;19:454–459.
406. Vadhan-Raj S, Keating M, LeMaistre A, et al. Effects of recombinant human granulocyte-macrophage colony-stimulating factor in patients with myelodysplastic syndromes. *N Engl J Med*. 1987;317:1545–1552.
407. Antin JH, Smith BR, Holmes W, Rosenthal DS. Phase I/II study of recombinant human granulocyte-macrophage colony-stimulating factor in aplastic anemia and myelodysplastic syndrome. *Blood*. 1988;72:705–713.
408. Negrin RS, Haeuber DH, Nagler A, et al. Treatment of myelodysplastic syndromes with recombinant human granulocyte colony-stimulating factor. A phase I-II trial. *Ann Intern Med*. 1989;110:976–984.
409. Hellström-Lindberg E, Ahlgren T, Beguin Y, et al. Treatment of anemia in myelodysplastic syndromes with granulocyte colony-stimulating factor plus erythropoietin: results from a randomized phase II study and long-term follow-up of 71 patients. *Blood*. 1998;92:68–75.
410. Freifeld AG, Bow EJ, Sepkowitz KA, et al. Clinical practice guideline for the use of antimicrobial agents in neutropenic patients with cancer: 2010 Update by the Infectious Diseases Society of America. *Clin Infect Dis*. 2011;52:427–431.
411. Bergmann OJ, Mogensen SC, Ellermann-Eriksen S, Ellegaard J. Acyclovir prophylaxis and fever during remission-induction therapy of patients with acute myeloid leukemia: a randomized, double-blind, placebo-controlled trial. *J Clin Oncol*. 1997;15:2269–2274.
412. Cornely OA, Maertens J, Winston DJ, et al. Posaconazole vs. fluconazole or itraconazole prophylaxis in patients with neutropenia. *N Engl J Med*. 2007;356:348–359.
413. Kantarjian H, Giles F, List A, et al. The incidence and impact of thrombocytopenia in myelodysplastic syndromes. *Cancer*. 2007;109:1705–1714.
414. Neukirchen J, Blum S, Kuendgen A, et al. Platelet counts and haemorrhagic diathesis in patients with myelodysplastic syndromes. *Eur J Haematol*. 2009;83:477–482.
415. Gonzalez-Porras JR, Cordoba I, Such E, et al. Prognostic impact of severe thrombocytopenia in low-risk myelodysplastic syndrome. *Cancer*. 2011;117:5529–5537.
416. Rebulla P, Finazzi G, Marangoni F, et al. The threshold for prophylactic platelet transfusions in adults with acute myeloid leukemia. Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto. *N Engl J Med*. 1997;337:1870–1875.
417. Wandt H, Schaefer-Eckart K, Wendelin K, et al. Therapeutic platelet transfusion versus routine prophylactic transfusion in patients with haematological malignancies: an open-label, multicentre, randomised study. *Lancet*. 2012;380:1309–1316.
418. Bussel JB, Cheng G, Saleh MN, et al. Eltrombopag for the treatment of chronic idiopathic thrombocytopenic purpura. *N Engl J Med*. 2007;357:2237–2247.
419. Kuter DJ, Begley CG. Recombinant human thrombopoietin: basic biology and evaluation of clinical studies. *Blood*. 2002;100:3457–3469.
420. Kantarjian H, Fenaux P, Sekeres MA, et al. Safety and efficacy of romiplostim in patients with lower-risk myelodysplastic syndrome and thrombocytopenia. *J Clin Oncol*. 2010;28:437–444.
421. Kantarjian HM, Giles FJ, Greenberg PL, et al. Phase 2 study of romiplostim in patients with low- or intermediate-risk myelodysplastic syndrome receiving azacitidine therapy. *Blood*. 2010;116:3163–3170.
422. Sekeres MA, Kantarjian H, Fenaux P, et al. Subcutaneous or intravenous administration of romiplostim in thrombocytopenic patients with lower risk myelodysplastic syndromes. *Cancer*. 2011;117:992–1000.
423. Greenberg PL, Garcia-Manero G, Moore M, et al. A randomized controlled trial of romiplostim in patients with low- or intermediate-risk myelodysplastic syndrome receiving decitabine. *Leuk Lymphoma*. 2013;54:321–328.
424. Svensson T, Chowdhury O, Garelius H, et al. A pilot phase I dose finding safety study of the thrombopoietin-receptor agonist, eltrombopag, in patients with myelodysplastic syndrome treated with azacitidine. *Eur J Haematol*. 2014
425. Kuter DJ, Mufti GJ, Bain BJ, et al. Evaluation of bone marrow reticulin formation in chronic immune thrombocytopenia patients treated with romiplostim. *Blood*. 2009;114:3748–3756.
426. Brierley CK, Steensma DP. Thrombopoiesis-stimulating agents and myelodysplastic syndromes. *Br J Haematol*. 2015;169:309–323.
427. Giagounidis A, Mufti GJ, Fenaux P, et al. Results of a randomized, double-blind study of romiplostim versus placebo in patients with low/

intermediate-1-risk myelodysplastic syndrome and thrombocytopenia. *Cancer*. 2014;120(12):1838–1846.
428. Oliva EN, Alati C, Santini V, et al. Eltrombopag versus placebo for low-risk myelodysplastic syndromes with thrombocytopenia (EQoL-MDS): phase 1 results of a single-blind, randomised, controlled, phase 2 superiority trial. *Lancet Haematol*. 2017;4:e127–e136.
429. Salacz ME, Lankiewicz MW, Weissman DE. Management of thrombocytopenia in bone marrow failure: a review. *J Palliat Med*. 2007;10:236–244.
430. Giardina PJ, Grady RW. Chelation therapy in beta-thalassemia: an optimistic update. *Semin Hematol*. 2001;38:360–366.
431. Lyons RM, Marek BJ, Paley C, et al. Comparison of 24-month outcomes in chelated and non-chelated lower-risk patients with myelodysplastic syndromes in a prospective registry. *Leuk Res*. 2014;38:149–154.
432. Neukirchen J, Fox F, Kündgen A, et al. Improved survival in MDS patients receiving iron chelation therapy—a matched pair analysis of 188 patients from the Dusseldorf MDS registry. *Leuk Res*. 2012;36:1067–1070.
433. Malcovati L, Porta MGD, Pascutto C, et al. Prognostic factors and life expectancy in myelodysplastic syndromes classified according to WHO criteria: a basis for clinical decision making. *J Clin Oncol*. 2005;23:7594–7603.
434. Takatoku M, Uchiyama T, Okamoto S, et al. Retrospective nationwide survey of Japanese patients with transfusion-dependent MDS and aplastic anemia highlights the negative impact of iron overload on morbidity/mortality. *Eur J Haematol*. 2007;78:487–494.
435. Jensen PD, Heickendorff L, Pedersen B, et al. The effect of iron chelation on haemopoiesis in MDS patients with transfusional iron overload. *Br J Haematol*. 1996;94:288–299.
436. Leitch HA, Chan C, Leger CS, et al. Improved survival with iron chelation therapy for red blood cell transfusion dependent lower IPSS risk MDS may be more significant in patients with a non-RARS diagnosis. *Leuk Res*. 2012;36:1380–1386.
437. Rose C, Brechignac S, Vassilief D, et al. Does iron chelation therapy improve survival in regularly transfused lower risk MDS patients? A multicenter study by the GFM (Groupe Francophone des Myelodysplasies). *Leuk Res*. 2010;34:864–870.
438. Mainous 3rd AG, Tanner RJ, Hulihan MM, et al. The impact of chelation therapy on survival in transfusional iron overload: a meta-analysis of myelodysplastic syndrome. *Br J Haematol*. 2014;167:720–723.
439. Cappellini MD, Porter J, El-Beshlawy A, et al. Tailoring iron chelation by iron intake and serum ferritin: the prospective EPIC study of deferasirox in 1744 patients with transfusion-dependent anemias. *Haematologica*. 2010;95:557–566.
440. Gattermann N. Do recent randomized trial results influence which patients with myelodysplastic syndromes receive iron chelation? *Hematol Oncol Clin North Am*. 2020;34:465–473.
441. Angelucci E, Li J, Greenberg P, et al. Iron chelation in transfusion-dependent patients with low- to intermediate-1-risk myelodysplastic syndromes: a randomized trial. *Ann Intern Med*. 2020;172(8):513–522.
442. Bennett JM. Consensus statement on iron overload in myelodysplastic syndromes. *Am J Hematol*. 2008;83:858–861.
443. Armand P, Kim HT, Cutler CS, et al. Prognostic impact of elevated pretransplantation serum ferritin in patients undergoing myeloablative stem cell transplantation. *Blood*. 2007;109:4586–4588.
444. Di Tucci AA, Matta G, Deplano S, et al. Myocardial iron overload assessment by T2* magnetic resonance imaging in adult transfusion dependent patients with acquired anemias. *Haematologica*. 2008;93:1385–1388.
445. List AF. New approaches to the treatment of myelodysplasia. *The Oncologist*. 2002;7(Suppl 1):39–49.
446. Jädersten M, Saft L, Smith A, et al. TP53 mutations in low-risk myelodysplastic syndromes with del(5q) predict disease progression. *J Clin Oncol*. 2011;29:1971–1979.
447. List A, Kurtin S, Roe DJ, et al. Efficacy of lenalidomide in myelodysplastic syndromes. *N Engl J Med*. 2005;352:549–557.
448. Raza A, Reeves JA, Feldman EJ, et al. Phase 2 study of lenalidomide in transfusion-dependent, low-risk, and intermediate-1 risk myelodysplastic syndromes with karyotypes other than deletion 5q. *Blood*. 2008;111: 86–93.
449. Fenaux P, Giagounidis A, Selleslag D, et al. A randomized phase 3 study of lenalidomide versus placebo in RBC transfusion-dependent patients with low-/intermediate-1-risk myelodysplastic syndromes with del5q. *Blood*. 2011;118:3765–3776.
450. Santini V, Almeida A, Giagounidis A, et al. The effect of lenalidomide on health-related quality of life in patients with lower-risk non-del(5q) myelodysplastic syndromes: results from the MDS-005 study. *Clin Lymphoma Myeloma Leuk*. 2018;18:136–144. e7.
451. Sekeres MA, Maciejewski JP, Giagounidis AAN, et al. Relationship of treatment-related cytopenias and response to lenalidomide in patients with lower-risk myelodysplastic syndromes. *J Clin Oncol*. 2008;26:5943–5949.
452. Richardson P, Jagannath S, Hussein M, et al. Safety and efficacy of single-agent lenalidomide in patients with relapsed and refractory multiple myeloma. *Blood*. 2009;114:772–778.
453. Wisch JS, Griffin JD, Kufe DW. Response of preleukemic syndromes to continuous infusion of low-dose cytarabine. *N Engl J Med*. 1983;309:1599–1602.
454. Mufti GJ, Oscier DG, Hamblin TJ, Bell AJ. Low doses of cytarabine in the treatment of myelodysplastic syndrome and acute myeloid leukemia. *N Engl J Med*. 1983;309:1653–1654.
455. Manoharan A. Low-dose cytarabine therapy in hypoplastic acute leukemia. *N Engl J Med*. 1983;309:1652–1653.
456. Jabbour E, Short NJ, Montalban-Bravo G, et al. A randomized phase 2 study of low-dose decitabine vs low-dose azacitidine in lower-risk MDS and MDS/MPN. *Blood*. 2017;130(13):1514–1522.
457. Deeg HJ, Gotlib J, Beckham C, et al. Soluble TNF receptor fusion protein (etanercept) for the treatment of myelodysplastic syndrome: a pilot study. *Leukemia*. 2002;16:162–164.
458. Rosenfeld C, Bedell C. Pilot study of recombinant human soluble tumor necrosis factor receptor (TNFR:Fc) in patients with low risk myelodysplastic syndrome. *Leuk Res*. 2002;26:721–724.
459. Scott BL, Ramakrishnan A, Fosdal M, et al. Anti-thymocyte globulin plus etanercept as therapy for myelodysplastic syndromes (MDS): a phase II study. *Br J Haematol*. 2010;149:706–710.
460. Scott BL, Ramakrishnan A, Storer B, et al. Prolonged responses in patients with MDS and CMML treated with azacitidine and etanercept. *Br J Haematol*. 2010;148:944–947.
461. Stasi R, Amadori S. Infliximab chimaeric anti-tumour necrosis factor alpha monoclonal antibody treatment for patients with myelodysplastic syndromes. *Br J Haematol*. 2002;116:334–337.
462. Braun T, Fenaux P. Myelodysplastic syndromes (MDS) and autoimmune disorders (AD): cause or consequence? *Best Pract Res Clin Haematol*. 2013;26:327–336.
463. Biesma DH, van den Tweel JG, Verdonck LF. Immunosuppressive therapy for hypoplastic myelodysplastic syndrome. *Cancer*. 1997;79:1548–1551.
464. Molldrem JJ, Leifer E, Bahceci E, et al. Antithymocyte globulin for treatment of the bone marrow failure associated with myelodysplastic syndromes. *Ann Intern Med*. 2002;137:156–163.
465. Saunthararajah Y, Nakamura R, Nam J-M, et al. HLA-DR15 (DR2) is overrepresented in myelodysplastic syndrome and aplastic anemia and predicts a response to immunosuppression in myelodysplastic syndrome. *Blood*. 2002;100:1570–1574.
466. Passweg JR, Giagounidis AAN, Simcock M, et al. Immunosuppressive therapy for patients with myelodysplastic syndrome: a prospective randomized multicenter phase III trial comparing antithymocyte globulin plus cyclosporine with best supportive care—SAKK 33/99. *J Clin Oncol*. 2011;29:303–309.
467. Stahl M, DeVeaux M, de Witte T, et al. The use of immunosuppressive therapy in MDS: clinical outcomes and their predictors in a large international patient cohort. *Blood Adv*. 2018;2:1765–1772.
468. Sloand EM, Olnes MJ, Shenoy A, et al. Alemtuzumab treatment of intermediate-1 myelodysplasia patients is associated with sustained improvement in blood counts and cytogenetic remissions. *J Clin Oncol*. 2010;28:5166–5173.
469. Keyhani A, Jendiroba DB, Freireich EJ. Angiogenesis and leukemia. *Leuk Res*. 2001;25:639–645.
470. Legros L, Slama B, Karsenti J-M, et al. Treatment of myelodysplastic syndromes with excess of blasts by bevacizumab is well tolerated and is associated with a decrease of VEGF plasma level. *Ann Hematol*. 2012;91:39–46.
471. Gupta P, Mulkey F, Hasserjian RP, et al. A phase II study of the oral VEGF receptor tyrosine kinase inhibitor vatalanib (PTK787/ZK222584) in myelodysplastic syndrome: Cancer and Leukemia Group B study 10105 (Alliance). *Invest New Drugs*. 2013;31:1311–1320.
472. Erikci AA, Ozturk A, Karagoz B, et al. Results of combination therapy with amifostine, pentoxifylline, ciprofloxacin and dexamethasone in patients with myelodysplastic syndrome and acute myeloid leukemia. *Hematology*. 2008;13:289–292.
473. List AF. Emerging data on IMiDs in the treatment of myelodysplastic syndromes (MDS). *Semin Oncol*. 2005;32:S31–S35.
474. Dang L, Yen K, Attar EC. IDH mutations in cancer and progress toward development of targeted therapeutics. *Ann Oncol*. 2016;27:599–608.
475. Vuylsteke J, Verloop MC, Drogendijk AC. Favourable effect of pyridoxine and ascorbic acid in a patient with refractory sideroblastic anaemia and haemochromatosis. *Acta Med Scand*. 1961;169:113–123.

476. Nagler A, Rikilis I, Tatarsky I, Fabian I. Effect of 1,25-dihydroxyvitamin D3 and 13-cis-retinoic acid on in vitro hematopoiesis in the myelodysplastic syndromes. *J Lab Clin Med*. 1987;110:237–244.
477. Takami A, Nakao S, Ontachi Y, Yamauchi H, Matsuda T. Successful therapy of myelodysplastic syndrome with menatetrenone, a vitamin K2 analog. *Int J Hematol*. 1999;69:24–26.
478. Tefferi A, Elliott MA, Steensma DP, et al. Amifostine alone and in combination with erythropoietin for the treatment of favorable myelodysplastic syndrome. *Leuk Res*. 2001;25:183–185.
479. Andreeff M, Stone R, Michaeli J, et al. Hexamethylene bisacetamide in myelodysplastic syndrome and acute myelogenous leukemia: a phase II clinical trial with a differentiation-inducing agent. *Blood*. 1992;80:2604–2609.
480. Essers MAG, Offner S, Blanco-Bose WE, et al. IFNalpha activates dormant haematopoietic stem cells in vivo. *Nature*. 2009;458:904–908.
481. List A, Beran M, DiPersio J, et al. Opportunities for Trisenox (arsenic trioxide) in the treatment of myelodysplastic syndromes. *Leukemia*. 2003;17:1499–1507.
482. Schiller GJ, Slack J, Hainsworth JD, et al. Phase II multicenter study of arsenic trioxide in patients with myelodysplastic syndromes. *J Clin Oncol*. 2006;24:2456–2464.
483. Bejanyan N, Tiu RV, Raza A, et al. A phase 2 trial of combination therapy with thalidomide, arsenic trioxide, dexamethasone, and ascorbic acid (TADA) in patients with overlap myelodysplastic/myeloproliferative neoplasms (MDS/MPN) or primary myelofibrosis (PMF). *Cancer*. 2011;118(16):3968.
484. Sekeres MA, Maciejewski JP, Erba HP, et al. A Phase 2 study of combination therapy with arsenic trioxide and gemtuzumab ozogamicin in patients with myelodysplastic syndromes or secondary acute myeloid leukemia. *Cancer*. 2011;117:1253–1261.
485. Hast R, Axdorph S, Laurén L, Reizenstein P. Absent clinical effects of retinoic acid and isoretinoin treatment in the myelodysplastic syndrome. *Hematol Oncol*. 1989;7:297–301.
486. Ohno R, Naoe T, Hirano M, et al. Treatment of myelodysplastic syndromes with all-trans retinoic acid. Leukemia Study Group of the Ministry of Health and Welfare. *Blood*. 1993;81:1152–1154.
487. Quesnel B, Guillerm G, Vereecque R, et al. Methylation of the p15(INK4b) gene in myelodysplastic syndromes is frequent and acquired during disease progression. *Blood*. 1998;91:2985–2990.
488. Lin J, Yao D-m, Qian J, et al. Methylation status of fragile histidine triad (FHIT) gene and its clinical impact on prognosis of patients with myelodysplastic syndrome. *Leuk Res*. 2008;32:1541–1545.
489. Brakensiek K, Länger F, Schlegelberger B, et al. Hypermethylation of the suppressor of cytokine signalling-1 (SOCS-1) in myelodysplastic syndrome. *Br J Haematol*. 2005;130:209–217.
490. Liu TX, Becker MW, Jelinek J, et al. Chromosome 5q deletion and epigenetic suppression of the gene encoding alpha-catenin (CTNNA1) in myeloid cell transformation. *Nat Med*. 2007;13:78–83.
491. Shen L, Kantarjian H, Guo Y, et al. DNA methylation predicts survival and response to therapy in patients with myelodysplastic syndromes. *J Clin Oncol*. 2010;28:605–613.
492. Jones PA, Taylor SM. Cellular differentiation, cytidine analogs and DNA methylation. *Cell*. 1980;20:85–93.
493. de Vos D. Epigenetic drugs: a longstanding story. *Semin Oncol*. 2005;32:437–442.
494. Garcia-Manero G. Demethylating agents in myeloid malignancies. *Curr Opin Oncol*. 2008;20:705–710.
495. Silverman LR, Demakos EP, Peterson BL, et al. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. *J Clin Oncol*. 2002;20:2429–2440.
496. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, et al. Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. *Lancet Oncol*. 2009;10:223–232.
497. Garcia-Manero G, Santini V, Almeida A, et al. A phase III placebo-controlled trial of CC-486 in patients with red blood cell transfusion-dependent (RBC-TD) anemia and thrombocytopenia due to IPSS lower-risk myelodysplastic syndromes (LR-MDS). Presented at the European Hematology Association (EHA25 Virtual), 2020.
498. Qin T, Castoro R, El Ahdab S, et al. Mechanisms of resistance to decitabine in the myelodysplastic syndrome. *PLoS One*. 2011;6:e23372.
499. Steensma DP, Baer MR, Slack JL, et al. Multicenter study of decitabine administered daily for 5 days every 4 weeks to adults with myelodysplastic syndromes: the alternative dosing for outpatient treatment (ADOPT) trial. *J Clin Oncol*. 2009;27:3842–3848.
500. Kantarjian H, Issa J-PJ, Rosenfeld CS, et al. Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. *Cancer*. 2006;106:1794–1803.
501. Kantarjian H, Oki Y, Garcia-Manero G, et al. Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia. *Blood*. 2007;109:52–57.
502. Saunthararajah Y, Sekeres M, Advani A, et al. Evaluation of noncytotoxic DNMT1-depleting therapy in patients with myelodysplastic syndromes. *J Clin Invest*. 2015;125(3):1043.
503. Roboz GJ, Mandrekar SJ, Desai P, et al. Randomized trial of 10 days of decitabine ± bortezomib in untreated older patients with AML: CALGB 11002 (Alliance). *Blood Adv*. 2018;2:3608–3617.
504. Welch JS, Petti AA, Miller CA, et al. TP53 and decitabine in acute myeloid leukemia and myelodysplastic syndromes. *N Engl J Med*. 2016;375:2023–2036.
505. Garcia-Manero G, Griffiths EA, Steensma DP, et al. Oral cedazuridine/decitabine for MDS and CMML: a phase 2 pharmacokinetic/pharmacodynamic randomized crossover study. *Blood*. 2020;136:674–683.
506. Quesnel B, Fenaux P. P15INK4b gene methylation and myelodysplastic syndromes. *Leuk Lymphoma*. 1999;35:437–443.
507. Follo MY, Russo D, Finelli C, et al. Epigenetic regulation of nuclear PI-PLCbeta1 signaling pathway in low-risk MDS patients during azacitidine treatment. *Leukemia*. 2012;26:943–950.
508. Figueroa ME, Skrabanek L, Li Y, et al. MDS and secondary AML display unique patterns and abundance of aberrant DNA methylation. *Blood*. 2009;114(16):3448.
509. Fandy TE, Herman JG, Kerns P, et al. Early epigenetic changes and DNA damage do not predict clinical response in an overlapping schedule of 5-azacytidine and entinostat in patients with myeloid malignancies. *Blood*. 2009;114:2764–2773.
510. Bejar R, Lord A, Stevenson K, et al. TET2 mutations predict response to hypomethylating agents in myelodysplastic syndrome patients. *Blood*. 2014;124:2705–2712.
511. Uy GL, Duncavage EJ, Chang GS, et al. Dynamic changes in the clonal structure of MDS and AML in response to epigenetic therapy. *Leukemia*. 2017;31:872–881.
512. Jabbour EJ, Garcia-Manero G, Strati P, et al. Outcome of patients with low-risk and intermediate-1-risk myelodysplastic syndrome after hypomethylating agent failure: a report on behalf of the MDS Clinical Research Consortium. *Cancer*. 2015;121:876–882.
513. DiNardo CD, Pratz KW, Letai A, et al. Safety and preliminary efficacy of venetoclax with decitabine or azacitidine in elderly patients with previously untreated acute myeloid leukaemia: a non-randomised, open-label, phase 1b study. *Lancet Oncol*. 2018;19:216–228.
514. Wei AH, Garcia JS, Borate U, et al. A phase 1b study evaluating the safety and efficacy of venetoclax in combination with azacitidine in treatment-naïve patients with higher-risk myelodysplastic syndrome. *Blood*. 2019;134:568.
515. Zeidan AM, Pollyea DA, Garcia JS, et al. A phase 1b study evaluating the safety and efficacy of venetoclax as monotherapy or in combination with azacitidine for the treatment of relapsed/refractory myelodysplastic syndrome. *Blood*. 2019;134:565.
516. Sallman DA, DeZern AE, Garcia-Manero G, et al. Phase 2 results of APR-246 and azacitidine (AZA) in patients with TP53 mutant myelodysplastic syndromes (MDS) and oligoblastic acute myeloid leukemia (AML). *Blood*. 2019;134:676.
517. Cluzeau T, Sebert M, Rahmé R, et al. APR-246 combined with azacitidine (AZA) in TP53 mutated myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). A phase 2 study by the Groupe Francophone des Myélodysplasies (GFM). *Blood*. 2019;134:677.
518. Prebet T, Sun Z, Figueroa ME, et al. Prolonged administration of azacitidine with or without entinostat for myelodysplastic syndrome and acute myeloid leukemia with myelodysplasia-related changes: results of the US Leukemia Intergroup trial E1905. *J Clin Oncol*. 2014;32:1242–1248.
519. Sekeres MA, Othus M, List AF, et al. Randomized phase II study of azacitidine alone or in combination with lenalidomide or with vorinostat in higher-risk myelodysplastic syndromes and chronic myelomonocytic leukemia: North American Intergroup Study SWOG S1117. *J Clin Oncol*. 2017;35:2745–2753.
520. Garcia-Manero G, Montalban-Bravo G, Berdeja JG, et al. Phase 2, randomized, double-blind study of pracinostat in combination with azacitidine in patients with untreated, higher-risk myelodysplastic syndromes. *Cancer*. 2017;123:994–1002.
521. Beran M, Shen Y, Kantarjian H, et al. High-dose chemotherapy in high-risk myelodysplastic syndrome: covariate-adjusted comparison of five regimens. *Cancer*. 2001;92:1999–2015.
522. Cortes J, Kantarjian H, Albitar M, et al. A randomized trial of liposomal daunorubicin and cytarabine versus liposomal daunorubicin and topotecan with or without thalidomide as initial therapy for patients with poor prognosis acute myelogenous leukemia or myelodysplastic syndrome. *Cancer*. 2003;97:1234–1241.

523. Lancet JE, Uy GL, Cortes JE, et al. CPX-351 (cytarabine and daunorubicin) liposome for injection versus conventional cytarabine plus daunorubicin in older patients with newly diagnosed secondary acute myeloid leukemia. *J Clin Oncol*. 2018;36:2684–2692.
524. Oliansky DM, Antin JH, Bennett JM, et al. The role of cytotoxic therapy with hematopoietic stem cell transplantation in the therapy of myelodysplastic syndromes: an evidence-based review. *Biol Blood Marrow Transplant*. 2009;15:137–172.
525. Hahn T, McCarthy Jr. PL, Hassebroek A, et al. Significant improvement in survival after allogeneic hematopoietic cell transplantation during a period of significantly increased use, older recipient age, and use of unrelated donors. *J Clin Oncol*. 2013;31:2437–2449.
526. Brunner AM, Kim HT, Coughlin E, et al. Outcomes in patients age 70 or older undergoing allogeneic hematopoietic stem cell transplantation for hematologic malignancies. *Biol Blood Marrow Transplant*. 2013;19: 1374–1380.
527. Koreth J, Pidala J, Perez WS, et al. Role of reduced-intensity conditioning allogeneic hematopoietic stem-cell transplantation in older patients with de novo myelodysplastic syndromes: an international collaborative decision analysis. *J Clin Oncol*. 2013;31:2662–2670.
528. Cutler CS, Lee SJ, Greenberg P, et al. A decision analysis of allogeneic bone marrow transplantation for the myelodysplastic syndromes: delayed transplantation for low-risk myelodysplasia is associated with improved outcome. *Blood*. 2004;104:579–585.
529. Sierra J, Pérez WS, Rozman C, et al. Bone marrow transplantation from HLA-identical siblings as treatment for myelodysplasia. *Blood*. 2002;100:1997–2004.
530. Gibson CJ, Lindsley RC. Stem cell donors should not be screened for clonal hematopoiesis. *Blood Adv*. 2020;4:789–792.
531. Castro-Malaspina H, Harris RE, Gajewski J, et al. Unrelated donor marrow transplantation for myelodysplastic syndromes: outcome analysis in 510 transplants facilitated by the National Marrow Donor Program. *Blood*. 2002;99:1943–1951.
532. Majhail NS, Brunstein CG, Shanley R, et al. Reduced-intensity hematopoietic cell transplantation in older patients with AML/MDS: umbilical cord blood is a feasible option for patients without HLA-matched sibling donors. *Bone Marrow Transplant*. 2012;47:494–498.
533. Di Stasi A, Milton DR, Poon LM, et al. Similar transplantation outcomes for acute myeloid leukemia and myelodysplastic syndrome patients with haploidentical versus 10/10 human leukocyte antigen-matched unrelated and related donors. *Biol Blood Marrow Transplant*. 2014;20:1975–1981.
534. Alyea EP, Kim HT, Ho V, et al. Impact of conditioning regimen intensity on outcome of allogeneic hematopoietic cell transplantation for advanced acute myelogenous leukemia and myelodysplastic syndrome. *Biol Blood Marrow Transplant*. 2006;12:1047–1055.
535. Scott BL, Pasquini MC, Logan BR, et al. Myeloablative versus reduced-intensity hematopoietic cell transplantation for acute myeloid leukemia and myelodysplastic syndromes. *J Clin Oncol*. 2017;35:1154–1161.
536. Appelbaum FR, Anderson J. Allogeneic bone marrow transplantation for myelodysplastic syndrome: outcomes analysis according to IPSS score. *Leukemia*. 1998;12(Suppl 1):S25–S29.
537. Armand P, Kim HT, DeAngelo DJ, et al. Impact of cytogenetics on outcome of de novo and therapy-related AML and MDS after allogeneic transplantation. *Biol Blood Marrow Transplant*. 2007;13:655–664.
538. Frick M, Chan W, Arends CM, et al. Role of donor clonal hematopoiesis in allogeneic hematopoietic stem-cell transplantation. *J Clin Oncol*. 2019;37:375–385.
539. Scott BL, Park JY, Deeg HJ, et al. Pretransplant neutropenia is associated with poor-risk cytogenetic features and increased infection-related mortality in patients with myelodysplastic syndromes. *Biol Blood Marrow Transplant*. 2008;14:799–806.
540. Gerds AT, Gooley TA, Estey EH, Appelbaum FR, Deeg HJ, Scott BL. Pretransplantation therapy with azacitidine vs induction chemotherapy and posttransplantation outcome in patients with MDS. *Biol Blood Marrow Transplant*. 2012;18:1211–1218.
541. De Witte T, Van Biezen A, Hermans J, et al. Autologous bone marrow transplantation for patients with myelodysplastic syndrome (MDS) or acute myeloid leukemia following MDS. Chronic and Acute Leukemia Working Parties of the European Group for Blood and Marrow Transplantation. *Blood*. 1997;90:3853–3857.
542. de Witte T, Suciu S, Brand R, et al. Autologous stem cell transplantation in myelodysplastic syndromes. *Semin Hematol*. 2007;44:274–277.
543. Ogata K, Nomura T. Application of low-dose etoposide therapy for myelodysplastic syndromes. *Leuk Lymphoma*. 1993;12:35–39.
544. Mario AD, Pagano L, Mele L, et al. Use of gemcitabine (GEM) in advanced myelodysplastic syndromes. *Ann Oncol*. 2001;12:1494.
545. Denzlinger C, Bowen D, Benz D, et al. Low-dose melphalan induces favourable responses in elderly patients with high-risk myelodysplastic syndromes or secondary acute myeloid leukaemia. *Br J Haematol*. 2000;108:93–95.
546. Bryan J, Kantarjian H, Prescott H, Jabbour E. Clofarabine in the treatment of myelodysplastic syndromes. *Expert Opin Investig Drugs*. 2014;23:255–263.
547. Roberts DA, Wadleigh M, McDonnell AM, et al. Low efficacy and high mortality associated with clofarabine treatment of relapsed/refractory acute myeloid leukemia and myelodysplastic syndromes. *Leuk Res*. 2015;39: 204–210.
548. Yang H, Bueso-Ramos C, Dinardo C, et al. Expression of PD-L1, PD-L2, PD-1 and CTLA4 in myelodysplastic syndromes is enhanced by treatment with hypomethylating agents. *Leukemia*. 2013;28(6):1280.
549. Swoboda DM, Sallman DA. The promise of macrophage directed checkpoint inhibitors in myeloid malignancies. *Best Pract Res Clin Haematol*. 2020;33:101221.
550. Bewersdorf JP, Zeidan AM. Management of patients with higher-risk myelodysplastic syndromes after failure of hypomethylating agents: what is on the horizon? *Best Pract Res Clin Haematol*. 2021;34:101245.
551. Steensma DP, Brunner AM, DeZern AE, et al. Low clinical trial accrual of patients with myelodysplastic syndromes: causes and potential solutions. *Cancer*. 2018;124:4601–4609.

CHAPTER 62

ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION FOR ACUTE MYELOID LEUKEMIA AND MYELODYSPLASTIC SYNDROME IN ADULTS

John Koreth, Joseph H. Antin, and Corey Cutler

Recent advances in molecular diagnostics continue to shed light on genetic underpinnings of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). However, with notable exceptions, both diseases remain therapeutic challenges. Fortunately, our ability to provide more precise prognostic information has corresponded with advances in transplantation technology. We are increasingly able to apply transplantation to older people and those with comorbidities and to use alternative donors in patients without matched family members. By applying molecular prognostic criteria, we can provide transplantation to patients with poor outcomes on conventional therapy, and we can avoid transplant-related toxicity in those patients who are likely to do well with supportive care or nontransplant therapy.

Allogeneic hematopoietic stem cell transplantation (HSCT) is curative in MDS and AML due to a combination of the cytotoxicity of the preparative conditioning regimen and the donor-mediated immunologic graft-versus-leukemia (GVL) effect. Regimen intensity ranges from high-dose myeloablative conditioning (MAC) that induces profound and prolonged pancytopenia to reduced-intensity conditioning (RIC) that induces milder cytopenias and is more appropriate for older patients and those with comorbidities in whom MAC may be intolerable. The risk-benefit ratio of conditioning regimen intensity varies with factors like patient age, comorbidities, and relapse risk. In general, more intense regimens cause more treatment-related morbidity and mortality but are associated with a lower relapse rate. Conversely, there are more relapses in RIC HSCT, but the procedure is more tolerable.[1–3]

HEMATOPOIETIC STEM CELL TRANSPLANTATION FOR ACUTE MYELOID LEUKEMIA

AML remains the leading indication for allogeneic HSCT in the USA per the Center for International Blood and Marrow Transplant Research (CIBMTR), accounting for almost 40% of the 9028 allogeneic transplants performed in 2018.[4] AML displays considerable heterogeneity with markedly variable survival, traditionally defined by prognostic factors of patient age, comorbidities, white blood cell count, prior MDS or cytotoxic therapy, morphologic remission status, and karyotype, but further refined by its molecular taxonomy and assessment of minimal residual disease. In addition to new prognostic indicators, the availability of novel AML therapeutic agents constitutes an alternative or addition to cytotoxic chemotherapy, and they are also potentially intercalatable with HSCT.

Moreover, on the transplant side, the use of molecular tissue typing in conjunction with novel transplant modalities and better control of treatment-related complications has increased the pool of available donors. As shown in a recent analysis from the European Society for Blood and Marrow Transplantation (EBMT), the ability to use donors that are not fully histocompatible increases the pool of patients in whom HSCT can be an effective therapy.[5,6] Transplants performed using alternative donors are increasing.[4]

In the subsequent sections, we review allogeneic HSCT indications for patients with AML in first complete remissions (CR1) and the challenges for the treatment of relapsed/refractory and secondary AML, highlighting recent changes, including the inclusion of molecular disease markers and assessment of measurable (or minimal) residual disease (MRD). Additionally, we delineate approaches to transplantation for older patients and the role of alternative donor transplantation in AML. Finally, we note the poor prognosis of patients with morphologic AML relapse after HSCT and highlight trials of novel leukemia prophylaxis and therapeutic strategies for such patients.

Hematopoietic Stem Cell Transplantation for Acute Myeloid Leukemia in First Complete Remission

Adult AML patients can be stratified into good-risk, intermediate-risk, and poor-risk groups based on the disease characteristics including numeric and/or structural chromosomal abnormalities and the presence of specific mutations that can also direct choice of curative post-remission therapy, especially for patients less than 60 years of age (see Chapter 60). For instance, current European LeukemiaNet (ELN) consensus incorporates molecular mutations to derive AML prognostic risk groups (Table 62.1).[7] In current thinking, allogeneic HSCT is generally recommended when the relapse incidence with non-transplant approaches is expected to be above 35% to 40%.

Numerous groups have evaluated prospectively the relative benefits of allogeneic HSCT versus non-allogeneic therapies (consolidation chemotherapy or autologous HSCT) in adult AML patients 18 to 60 years of age in CR1. Treatment allocation was by biologic assignment as a surrogate for true randomization, with allogeneic HSCT for patients with available human leukocyte antigen (HLA)–matched sibling donors (donor group) and non-allogeneic consolidation for those lacking matched sibling donors (no-donor group). Some studies further stratified post-remission outcomes by cytogenetic risk. Individual studies, when analyzed on an intent-to-treat (ITT) donor versus no-donor basis, typically demonstrated that allogeneic HSCT was effective at improving disease-free survival, but overall survival benefit was hard to document because of graft-versus-host disease (GVHD) and other treatment-related mortality. Moreover, transplantation in CR2 salvages some of the patients who relapsed, thus making overall survival similar with the two approaches.

A meta-analysis of over 6000 patients in 24 biologic treatment assignment trials, however, confirmed that overall survival was in fact better in allogeneic matched related donor transplantation in AML-CR1, with a hazard ratio (HR) of death at 0.90 (95% confidence interval [CI], 0.82 to 0.97).[7] Importantly, when stratified by cytogenetic risk, there was an overall survival benefit of allogeneic transplantation (Table 62.2) for both intermediate-risk (HR, 0.83; 95% CI, 0.74 to 0.93) and poor-risk (HR, 0.73; 95% CI, 0.59 to 0.90) cytogenetic groups, but not for good-risk AML-CR1 (HR, 1.07; 95% CI, 0.83 to 1.38) (Fig. 62.1). These findings were particularly relevant for patients with normal cytogenetic AML, who

TABLE 62.1 **European LeukemiaNet Acute Myeloid Leukemia Risk Classification 2017**

Risk Category	Genetic Abnormality
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD$^{\text{low}}$[a]
	Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD$^{\text{high}}$[a]
	Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD$^{\text{low}}$[a] (without adverse-risk genetic lesions)
	t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*[b]
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214*
	t(v;11q23.3); *KMT2A* rearranged
	t(9;22)(q34.1;q11.2); *BCR-ABL1*
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)*
	−5 or del(5q); −7; −17/abn(17p)
	Complex karyotype,[c] monosomal karyotype[d]
	Wild-type *NPM1* and *FLT3*-ITD$^{\text{high}}$[a]
	Mutated *RUNX1*[e]
	Mutated *ASXL1*[e]
	Mutated *TP53*[f]

[a] Low, low allelic ratio (<0.5); high, high allelic ratio ($\geq$0.5); semiquantitative assessment of *FLT3*- internal tandem duplication (ITD) allelic ratio (using DNA fragment analysis) is determined as the ratio of the area under the curve "*FLT3*-ITD" divided by area under the curve "*FLT3*-wild type"; recent studies indicate that acute myeloid leukemia (AML) with *NPM1* mutation and *FLT3*-ITD low allelic ratio may also have a more favorable prognosis and patients should not routinely be assigned to allogeneic hematopoietic cell transplantation (HCT).

[b] The presence of t(9;11)(p21.3;q23.3) takes precedence over rare, concurrent adverse-risk gene mutations.

[c] Three or more unrelated chromosome abnormalities in the absence of 1 of the WHO-designated recurring translocations or inversions, that is, t(8;21), inv(16) or t(16;16), t(9;11), t(v;11)(v;q23.3), t(6;9), inv(3), or t(3;3); AML with *BCR-ABL1*.

[d] Defined by the presence of one single monosomy (excluding loss of X or Y) in association with at least one additional monosomy or structural chromosome abnormality (excluding core-binding factor AML).

[e] These markers should not be used as an adverse prognostic marker if they co-occur with favorable-risk AML subtypes.

[f] *TP53* mutations are significantly associated with AML with complex and monosomal karyotype.

From Dohner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*. 2017;129:424–447.

constitute the largest subgroup and for whom no consensus regarding optimal post-remission treatment was previously available. These studies however did not have the benefit of prognostic assignment based on FLT3, NPM1, or other molecular markers.

Prognostic Factors for Acute Myeloid Leukemia in First Complete Remission

Good-Risk Acute Myeloid Leukemia

In general, the 15% to 20% of AML patients with core binding factor (CBF) leukemia—t(8;21) (q22;q22) and inv(16)(p13.q22)—are considered to have good-risk disease, with a long-term disease-free survival of approximately 50% to 60% after consolidation chemotherapy, and allogeneic HSCT is not routinely recommended for patients achieving CR1. However, retrospective studies have identified activating mutations in C-KIT (mKIT)—a member of the type III receptor tyrosine kinase family—at exon 17 (mKIT 17) or exon 8 (mKIT 8) in approximately 30% of CBF AMLs, in particular AML with t(8;21), that are associated with increased relapse incidence and likely poorer survival, especially if higher mutant *KIT* levels are present.[8–11] Consensus opinions differ on the implications: ELN guidelines suggest that occurrence of a *KIT* mutation should not assign a patient to a different risk category; rather, patients should be monitored for MRD, whose absence can abrogate the effect of *KIT*.[9] 2019 guidelines of the US National Comprehensive Cancer Network (NCCN) in contrast highlight considering them for clinical trials targeting the molecular abnormality, or for referral to allogeneic transplantation.[12]

Intermediate-Risk Acute Myeloid Leukemia

The intermediate-risk cytogenetic group is heterogeneous and includes cytogenetically normal disease as well as those with karyotypic abnormalities not meeting criteria for good- or poor-risk AML. It previously constituted the largest risk category, accounting for approximately 40% to 50% of adult AML patients less than 60 years of age, but more recently with the refinement of molecular risk predictors, this category has shrunk. Long-term disease-free survival of approximately 40% to 45% is anticipated after consolidation chemotherapy in intermediate-risk AML. However, identification of mutations with prognostic importance such as mutant FLT3 internal tandem duplication (FLT3-ITD), NPM1, and CEBPA (CCAAT/enhancer-binding protein-α) have further individualized treatment decision-making, including allogeneic transplantation.

Over the past decade, "normal" karyotype AML (NK-AML, the largest category within intermediate-risk AML) has been documented to encompass molecular abnormalities with divergent risk. The presence of an isolated NPM1 or biallelic CEBPA mutation improves disease prognosis to slightly below that of AML with CBF translocations, placing them in the favorable-risk category, and most investigators do not offer HSCT in first remission.[13] The favorable impact of NPM1 mutations appears to persist albeit to a lesser extent in older patients, and allogeneic HSCT may be a consideration in older AML.[14–16] In contrast, patients with an isolated FLT3-ITD mutation and NK-AML have outcomes similar to those with poor-risk karyotype.[17] In a report evaluating the 2010 ELN risk classification in a large cohort of patients, for those in the "intermediate I" risk group (includes all patients with NK-AML with FLT3 abnormalities and those lacking both FLT3 and NPM1 mutations), relapse-free survival (RFS) was improved with allogeneic HSCT (94 vs. 7.9 months without allogeneic HSCT).[18]

Although the better-prognosis subgroup within NK-AML may not benefit from early allogeneic transplantation, HSCT appears to be the preferred post-remission therapy for other intermediate-risk AML patients at higher risk for relapse, including those lacking favorable gene mutations like NPM1 without FLT3-ITD, or double mutant CEBPA. However, the dissection of disease complexity requires detailed analysis to identify optimal therapy. For instance, Dohner et al. validated the 2017 NPM1/FLT3-ITD ELN AML risk classification, documenting that allogeneic HSCT benefit is restricted to patients in the "adverse-risk" group (NPM1$^{\text{wt}}$/FLT3-ITD$^{\text{high}}$; NPM1$^{\text{mut}}$/FLT3-ITD$^{\text{high}}$ with high-risk molecular mutations *(RUNX1*, *ASXL1*, *TP53)*; and NPM1$^{\text{wt}}$/FLT3-ITD$^{\text{low}}$ AML with high-risk molecular markers and/or adverse-karyotype). For the "intermediate-risk" group (NPM1$^{\text{mut}}$/FLT3-ITD$^{\text{high}}$; and NPM1$^{\text{wt}}$/FLT3-ITD$^{\text{low}}$, both without adverse-risk karyotype), allogeneic HSCT benefit was restricted to patients who did not receive midostaurin-based treatment on the CALGB 10603/RATIFY trial.[13] Of note, this finding is relevant to AML patients aged 18 to 60 years and requires validation in independent datasets prior to its routine incorporation in clinical decision making. Further, extrapolation to patients aged above 60 years is not warranted, given their overall worse disease outcomes.

TABLE 62.2 **Allogeneic Transplantation Guidelines for Adult Acute Myeloid Leukemia Based on Commonly Assessed Cytogenetic and Molecular Markers**

AML Category	Prognostic Impact	Allogeneic Transplantation	Notes
AML-CR1: Younger Adults			
Good-risk disease			
APL	Favorable	No	APL is treatable by chemotherapy.
CBF-AML *without mKIT*	Favorable	No	t(8;21) AML with high WBC count at diagnosis may have worse prognosis.
CBF-AML *with mKIT*	Intermediate	Possible: MRD, MUD Uncertain: MMUD, UCB, haplo	
Intermediate-Risk Disease			
CN-AML *with CEBPA*	Favorable	No	Benefit likely restricted to *DM-CEBPA*.
CN-AML *with mutant NPM1 but not FLT-3-ITD*	Favorable	Possible: MRD	Emerging data suggests allogeneic hematopoietic stem cell transplantation benefit for this category, with reduced relapse and improved DFS in patients >40 years.
CN-AML *with FLT-3-ITD*	Unfavorable[a]	Yes: MRD, MUD Possible[b]: MMUD, UCB, haplo	Unfavorable risk may be restricted to AML *with FLT-3-ITD allelic ratio >0.51*.
Other intermediate-risk disease	Intermediate or Unfavorable	Yes: MRD Likely acceptable[a]: MUD Possible[b]: MMUD, UCB, haplo	Likely considerable underlying clinical heterogeneity. Molecular risk profiling may further delineate risk in this category.
Poor-Risk Disease			
Monosomal karyotype *absent*	Unfavorable	Yes: MRD, MUD Likely acceptable[b]: MMUD, UCB, haplo	
Monosomal karyotype *present*	Very unfavorable	Yes: MRD, MUD Acceptable[b]: MMUD, UCB, haplo	
Abnormal 17(p)	Very unfavorable	Yes: MRD, MUD Acceptable[b]: MMUD, UCB, haplo	
AML-CR1: older adults	Unfavorable	Yes: MRD, MUD Likely acceptable[b]: MMUD, UCB, haplo	
AML-CR1: t-AML, AML/MDS	Unfavorable	Yes: MRD, MUD Acceptable[b]: MMUD, UCB, haplo	Molecular risk profiling may supersede clinical classification of secondary AML, especially in older patients.
AML-CR2	Very unfavorable	Yes: MRD, MUD Acceptable[b]: MMUD, UCB, haplo	
AML not in remission	Very unfavorable	Yes: MRD, MUD Uncertain: MMUD, UCB, haplo	For selected patients: good performance status, little comorbidity, lower leukemic burden; CIBMTR risk score may be useful.

[a] If no sibling donor available.
[b] If no timely matched donor available.
AML, Acute myeloid leukemia; *APL*, acute promyelocytic leukemia; *CBF*, core binding factor; *CIBMTR*, Center for International Blood and Marrow Transplant Research; *CN*, cytogenetically normal; *CR1*, first complete remission; *CR2*, second complete remission; *DFS*, disease-free survival; *haplo*, haploidentical; *MDS*, myelodysplastic syndrome; *MMUD*, mismatched unrelated donor; *MRD*, matched related donor; *MUD*, matched unrelated donor; *t-AML*, therapy-related AML; *UCB*, umbilical cord blood; *WBC*, white blood cell.

Poor-Risk Acute Myeloid Leukemia

In poor-risk AML, relapse rates are high and survival rates are anticipated to be 15% or lower with conventional therapy. Allogeneic HSCT with a matched sibling or unrelated donor results in long-term survival of 30% to 40% and is considered the treatment of choice for adults less than 60 years of age with poor-risk AML. There remains, however, a subgroup of poor-risk AML that may have particularly adverse prognosis, for whom additional novel therapeutic strategies may be necessary.

The monosomal karyotype (MK) is defined by the presence of a single autosomal monosomy, in association with at least one additional monosomy or non-good-risk structural chromosomal abnormality (i.e., excluding CBF mutation AML).[16] In the original report, MK-positive AML patients had a long-term survival of only 3% to 4%, and these generally dismal chemotherapeutic outcomes have been confirmed by other investigators.[19] Allogeneic transplantation appears to only partly ameliorate the impact of MK-positive karyotype, which remained an adverse prognostic factor after HSCT, and was associated with a relapse risk of 62% and poor survival of 15% at 4 years.[20]

Importantly, some if not all of the negative impact of MK and complex karyotype (CK) AML is its association with deletions of chromosome 5 (−5/5q−) and especially with abnormal (abnl)17p (site of TP53 gene). In a retrospective analysis of 236 high-risk AML-CR1 patients of whom nearly half (49%) received reduced-intensity allogeneic HSCT, patients with abnl(17p) had a 2-year EFS of 11%, those with −5/5q− but no abnl(17p) had a 2-year EFS of 29%, and those with high-risk AML (including MK or CK) without either abnl(17p) or −5/5q− had a 2-year EFS of 49%.[21] Further, in

Source	No. of patients: Donor group	No. of patients: No-donor group	No. of trials	HR (95% CI)
Shiller et al,[1] 1992	28	54		1.91 (0.95-3.84)
Archimbaud et al,[2] 1994	27	31		1.04 (0.52-2.11)
Hewlett et al,[3] 1995	53	110		0.98 (0.65-1.49)
Sierra et al,[4] 1996	47	68		1.60 (0.96-2.65)
Keating et al,[5] 1998	295	377		0.88 (0.72-1.09)
Slovak et al,[6] 2000	89	174		0.82 (0.57-1.18)
Suciu et al,[7] 2003	293	441		0.85 (0.67-1.08)
Jourdan et al,[8] 2005	182	290		0.81 (0.63-1.05)
Burnett et al,[9] 2006	428	877		0.87 (0.74-1.03)
Cornelissan et al,[10] 2007	326	599		0.89 (0.74-1.08)
Overall survival benefit (fixed effects)	**1768**	**3021**	**15**	**0.90 (0.82-0.97)**
Sensitivity analyses for overall survival benefit for AML in first remission				
Random effects meta-analysis	1768	3021	15	0.90 (0.82-1.00)
Adjusted HR	1768	3021	15	0.89 (0.81-0.97)
Including cytogenetic risk-stratified trials (fixed effects)	2057	3549	21	0.87 (0.80-0.94)
Including cytogenetic risk-stratified trials (random effects)	2057	3549	21	0.87 (0.78-0.98)
Including cytogenetic risk-stratified trials (adjusted HR)	2057	3549	21	0.86 (0.79-0.93)
Overall survival benefit by cytogenetic risk				
Good-risk AML	188	359	10	1.07 (0.83-1.38)
Intermediate-risk AML	864	1635	14	0.83 (0.74-0.93)
Poor-risk AML	226	366	14	0.73 (0.59-0.90)

Test for heterogeneity: $\chi^2_2 = 5.29$; $P = .07$; $J^2 = 62.2\%$

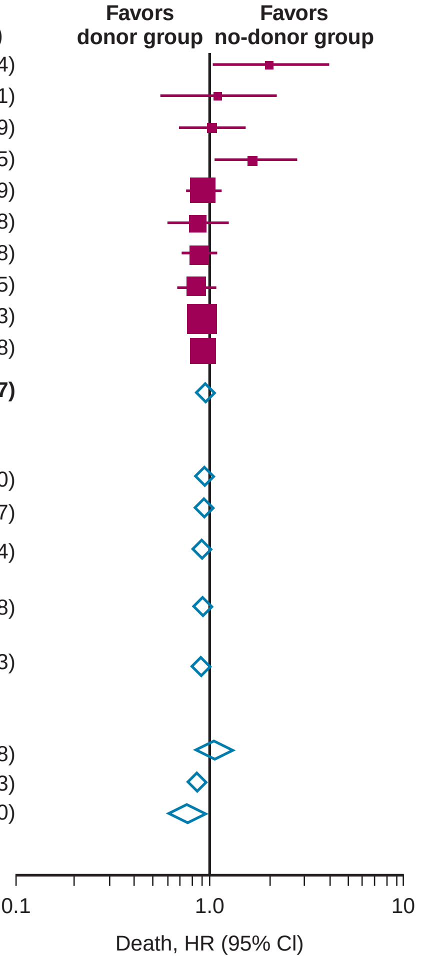


Figure 62.1 META-ANALYSIS OF OVERALL SURVIVAL BENEFIT IN FIRST COMPLETE REMISSION OF ACUTE MYELOID LEUKEMIA FOR YOUNGER ADULTS. *AML*, Acute myeloid leukemia; *CI*, confidence interval; *HR*, hazard ratio. *(Redrawn from Koreth J, Schlenk R, Kopecky KJ, et al. Allogeneic stem cell transplantation for acute myeloid leukemia in first complete remission: systematic review and meta-analysis of prospective clinical trials. JAMA. 2009;301:2349.)* Sources cited within Fig. 62.1 can be found following the reference list online at Elsevier eBooks for Practicing Clinicians.

updated analyses, the negative impact of abnl(17p) appears minimally improved after allogeneic HSCT.[22,23] Improving HSCT outcomes in TP53 mutant and other extremely poor-risk AML remains a high priority for the future, and clinical trials of novel conditioning (e.g., Venetoclax-based) and post-transplant maintenance (e.g., APR-246) approaches are strongly encouraged for such patients.

Measurable Residual Disease Assessment in Acute Myeloid Leukemia

Sequential monitoring of MRD via sensitive phenotyping (e.g., multicolor flow cytometry [MFC]) or molecular methods (e.g., quantitative PCR [qPCR], digital droplet PCR [ddPCR], deep error-corrected next-generation sequencing [NGS]) to detect AML-associated immunophenotypes or mutations respectively, at a sensitivity of at least 1×10^{-3}, is applicable to the vast majority of AML and provides a useful guide to post-remission AML management. Indeed, MRD negativity is emerging as an alternative therapeutic endpoint that supplements (and may replace) morphologic AML remission criteria.

As reviewed by Dohner et al., mutational assessment at early post-remission time points can distinguish patients at a differing probability of relapse.[24] As they highlight, MRD status has been found to be a better predictor of relapse risk than the presence of cooperating mutations involving *KIT* and *FLT3*-ITD in CBF-AML, or *FLT3-ITD*, *DNMT3A*, and *WT1* in *NPM1*-mutated AML. Sequential MRD-monitoring studies have shown that persistent high-level PCR positivity, or a rising level of leukemic transcripts after an initial molecular response, invariably predict relapse. Validated AML-associated mutations for MRD monitoring include *RUNX1-RUNX1T1*, *CBFB-MYH11*, and *PML-RARA* translocations (*KMT2A* and *DEK-NUP214* translocations are also AML-associated).[25] Other likely prognostic MRD mutations include *NPM1*, RAS pathway mutations (*FLT3-ITD* and *-TKD*), *N*- and *K-RAS*, *PTPN11* or *KIT*, whose persistence in CR indicates a likely higher risk of relapse. However, in the non-transplant context, the persistence of AML-associated mutations needs to be distinguished from the persistence of underlying clonal-hematopoiesis (CH) or MDS-associated mutations (e.g., DTA: DNMT3A, TET2, ASXL1). Future studies will help further distinguish mutations that are reliable indicators of leukemic clones associated with AML relapse versus mutations associated with preleukemic clones that are poorly predictive of relapse, despite persistence at high levels after chemotherapy and during remission. Whether MRD-based early interventions can prevent overt relapse remains under investigation. Preemptive therapy may be particularly relevant with allogeneic HSCT where MRD status may inform conditioning strategy, or post-hematopoietic cell transplantation (HCT) measures aiming to avoid frank relapse. These data support routine inclusion of MRD assessment to help inform transplant decision making in AML-CR1.

Patients with persistent MRD or with early MRD reoccurrence can receive salvage therapy as a bridge to allogeneic HSCT before hematologic relapse or proceed directly to transplant depending on donor availability and the likelihood of success with salvage therapy. The GIMEMA AML1310 study undertook risk-adapted treatment assignment of young adults with newly diagnosed intermediate-risk AML to allogeneic HSCT based on MFC-based MRD assessments, reporting promising 2-year disease-free survival and overall survival of 67% and 70% respectively for MRD$^+$ intermediate-risk AML patients assigned allogeneic HSCT.[26] Importantly, their finding of similar survival of MRD$^+$ intermediate-risk AML patients (assigned allogeneic HSCT) versus those who were MRD$^-$ (assigned autologous HSCT) suggests that allotransplantation may ameliorate the negative impact of persistent MRD, although other studies suggest it does not fully abrogate the negative effect of unfavorable pre-transplant MRD. For instance, in *NPM1*-mutant AML, high-level MRD for *NPM1*, or low-level MRD for *NPM1* plus presence of *FLT3-ITD*, had impaired post-transplant survival.[27] More intensive conditioning may offer therapeutic benefit for patients with persistent MRD pre-transplant. Deep error-corrected 13-gene NGS-based reanalysis of the BMTCTN 0901 study randomizing older AML patients to MAC versus RIC documented relapse protection and survival benefit for pre-transplant MRD$^+$ patients assigned MAC HSCT.[28] Importantly, the benefit was restricted to those with persistent AML-associated mutations, but not for those with CH-associated (*DNMT3A*, *TET2*, *ASXL1*) mutations pre-transplant. These findings need to be validated prior to incorporation in routine clinical practice.

In the post-transplant context, persistent or recurrent recipient MRD positivity is also associated with an increased risk of AML relapse.[29] However, many uncertainties remain, for instance with regards to MRD assessment modalities, thresholds, assessment frequency and timepoints, and the prognostic implications of recurrence or persistence of specific mutations and their recrudescence patterns. Importantly, for post-transplantation testing, detection of *either* AML- or CH-associated mutations would be concerning for recrudescent donor hematopoiesis and disease. However, therapeutic interventions based on the post-transplant assessment of MRD, ranging from immune suppression (IS) taper, donor lymphocyte infusion (DLI) and modified DLI (mDLI), hypomethylating agents (HMAs), and AML mutation-specific targeted therapies (e.g., *FLT3* inhibitors) may offer utility and are currently undergoing prospective evaluation.[29]

Transplantation Regimen Intensity in Acute Myeloid Leukemia

Myeloablative HSCT remains the standard of care for younger adults with AML. However, treatment-related mortality after MAC remains appreciable. Lower-intensity regimens offering less treatment-related toxicity are gaining in popularity. One report retrospectively compared RIC transplantation with chemotherapy in high-risk AML on a donor versus no-donor basis. They identified a leukemia-free survival benefit in the donor group (54% vs. 30%, $P = .01$).[30] This indicates that both MAC and RIC HSCT offer antileukemic benefit compared to non-transplant therapies.

Several groups compared MAC versus RIC transplantation, documenting similar overall and leukemia-free survival, with some reporting lower treatment-related mortality offset by increased relapse risk, especially for patients not in complete remission at the time of HSCT. A CIBMTR study compared 3731 MAC, 1041 RIC, and 407 nonmyeloablative (NMA) transplantations, reporting adjusted 5-year overall survival (OS) of 34%, 33%, and 26%, respectively.[31] The authors concluded that, although NMA transplantation increased relapse and impaired survival, RIC and MAC regimens had similar overall and leukemia-free survival. While a small randomized trial documented similar outcomes of MAC versus RIC transplantation in AML-CR1,[1] a larger prospective randomized trial of MAC versus RIC transplantation in AML or MDS patients closed after the accrual of 272 of planned 356 patients due to an increased relapse and impaired long-term survival in the RIC arm.[2] Additionally, as discussed previously, post-hoc analysis of this study documented that ablative conditioning can offer therapeutic benefit for patients with detectable pre-transplant MRD, offering relapse protection and survival benefit compared to RIC HSCT.[28]

RIC transplantation does, however, offer the option of curative allogeneic HSCT in older or sicker AML-CR patients for whom the toxicity of MAC transplantation might be prejudicial, especially if combined with novel approaches to augment the GVL effect in the peri- and post-transplant setting. Conversely, patients with good performance status but high AML relapse risk could be preferentially selected for MAC.

Alternative Donor Transplantation in Acute Myeloid Leukemia

HSCT from an HLA-matched sibling donor has been the standard of care for prospective biologic treatment assignment studies evaluating post-remission therapies. Patients lacking sibling donors could be considered for alternative donors: unrelated adult donors, umbilical cord blood (UCB), or haploidentical donor transplantation.

Unrelated Adult Donors

Given the very poor outcomes after non-allogeneic therapy in poor-risk AML, and the documented benefit of HLA-matched sibling donor HSCT, clinical practice has been to undertake HLA-matched unrelated donor transplantation for younger adults with poor-risk AML in CR1 who lack sibling donors. A retrospective analysis of HLA-matched siblings compared with unrelated donor HSCT in poor-risk AML-CR1 documented comparable outcomes with HLA-matched siblings and HLA well-matched unrelated donors with 3-year OS of 45% compared with 53% ($P = .63$).[32] Outcomes were not as good for partially HLA-mismatched unrelated donors (one-locus HLA mismatch), with 3-year OS of 31% ($P = .026$). Smaller prospective studies have also shown equivalent results, confirming the suitability of well-matched unrelated donor HSCT in poor-risk AML.[33,34]

There is limited data comparing HLA-matched sibling with unrelated donors in intermediate-risk AML, although at least one study documented similar outcomes with either donor type.[35] Current practice is to offer HLA-matched unrelated donor HSCT to intermediate-risk AML patients in first remission, especially if there are adverse molecular lesions.

Umbilical Cord Blood Donors

Another important issue is the use of UCB transplantation for AML in CR1. These data are harder to interpret because most studies reported outcomes for “acute leukemia,” rather than AML-CR1. A European registry study documented similar outcomes of UCB versus unrelated donor bone marrow (BM) transplantation in adults with acute leukemia, whereas a CIBMTR study showed better outcomes with HLA-matched unrelated donor BM transplantation but similar outcomes of UCB and HLA-mismatched unrelated donor BM transplantation.[36,37] A large analysis of adult acute leukemia (including 880 AML patients) compared the outcomes of single-unit 4–6/6 HLA-matched (HLA-A, -B, -DRB1) UCB with 8/8 or 7/8 HLA-matched (HLA-A, -B, -C, DRB1) BM or peripheral blood stem cell (PBSC) transplantation and documented similar leukemia-free survival regardless of stem cell source or degree of HLA match. There was higher treatment-related mortality after UCB transplantation.[38] Transplantation in CR1 resulted in similar 2-year leukemia-free survivals of 44%, 50%, 52%, 39%, and 41% with UCB, 8/8 PBSC, 8/8 BM, 7/8 PBSC, and 7/8 BM transplantation, respectively. In contrast, a Japanese study evaluated outcomes of 484 AML patients receiving 4 to 6/6 UCB compared with 8/8 unrelated donor BM transplantation and documented a similar relapse risk with UCB transplantation, but higher treatment-related mortality and worse overall and leukemia-free survival.[39]

Double unit UCB (DUCB) transplantation is commonly used for larger patients. Indeed, since 2005, the number of adults receiving DUCB has exceeded those receiving single-unit UCB products. Limited data suggest similar outcomes of myeloablative DUCB, HLA-matched unrelated, and sibling donor HSCT in hematologic malignancies.[40] However, only a subset of patients had AML. The role of DUCB in AML remains to be better delineated. Overall, from 2003 to 2011, there was a steady increase in the numbers of transplants using UCB, after which, however, UCB transplant activity declined, in part owing to issues around UCB treatment-related mortality, including delayed engraftment, infections, and NRM. In general, current clinical practice envisages the use of UCB transplantation in younger adults with high-risk AML in CR1 who lack suitable HLA-matched donors. However, the increasing utilization of haploidentical transplant (discussed below) is associated with and may be the cause of a concurrent decline in UCB transplantation, in part due to cost and convenience advantages, with comparable outcomes to HLA-matched transplantation.

Reducing treatment toxicity will be important to advance UCB transplantation. A trial of 79 adult AML cord recipients (60% DUCB), identified a low 2-year NRM incidence of 20%, suggestive of progress in this metric.[41] Importantly, a retrospective report highlighting potential curative benefit in acute leukemia and MDS patients with detectable pre-transplant MRD with UCB compared to URD HSCT, if validated, would be a reason to reconsider the trend against UCB HSCT.[42] Additionally, recent results of the international, multicenter, randomized Phase III study treating 125 patients aged 12 to 65 years with high-risk hematologic malignancies (including AML) undergoing transplant, documenting significant shortening of the median time to neutrophil engraftment for patients who received omidubicel UCB compared to the comparator group receiving usual UCB (12 days; 95% CI: 10 to 15 days vs. 22 days; 95% CI: 19 to 25 days),[43] offer a means to alleviate some of the clinical concerns regarding UCB HSCT to better harness its antileukemic potential.

Haploidentical Donors

Per CIBMTR data, transplants performed using alternative donors are increasing. From 2012 onward, there has been an increase in the numbers of transplants from "Other Relatives," likely due to the use of haploidentical (haplo) donors. This trend has continued to rise with these transplants representing 21% of transplants and nearing numbers of HLA-identical sibling transplants that represent 25% of allogeneic transplants in the United States in 2018. Haplo-transplantation (haplo-HSCT) is particularly interesting given the potential enhancement of GVL activity by natural killer cells and allogeneic T cells responding to the HLA disparity.[44] An advantage of this approach is the rapidity and near-universal availability of donors.

Of current haplo-HSCT regimens, those incorporating post-transplantation cyclophosphamide (PTCy) for the prevention of GVHD have gained increasing prominence, with low rates of treatment-related mortality and GVHD.[45,46] For AML/MDS, a retrospective analysis found no significant differences between outcomes of matched unrelated ($n = 108$) or haploidentical ($n = 32$) donor HSCT with PTCy.[47] A large CIBMTR analysis documented similar 3-year OS for AML after MUD versus haplo-HSCT,[48] while another documented equivalent post-HSCT outcomes, with less chronic GVHD in PTCy-based haplo-HSCT.[49] In a systemic review and meta-analysis comparing MRD versus PTCy-based haplo-HSCT, haplo-HSCT was associated with ~50% lower risk of chronic GVHD (HR, 0.55; 95% CI, 0.41–0.74), but higher risk of NRM (HR, 1.36; 95% CI, 1.12–1.66).[50] Relapse, survival, acute GVHD, and GVHD-free relapse-free survival (GRFS) were not significantly different between the groups. Indeed, in a recent large CIBMTR registry analysis, haplo-HSCT had the best GRFS and OS outcomes compared to UCB and MMUD donors.[51]

The reduction in chronic GVHD risk is likely PTCy-related and independent of donor source, as documented by Phase II randomized comparison in HLA-matched and mismatched unrelated donor transplantation, wherein a PTCy-based GVHD regimen offered significant improvement in GVHD and GRFS endpoints.[52] The recent Phase III randomized HOVON-96 study confirms the benefit of PTCy-based versus cyclosporine/mycophenolate mofetil GVHD prophylaxis for HLA-matched HSCT.[53] Overall, however, these results highlight the increasing utilization and acceptability of PTCy-based haplo-HSCT, though issues with regards haplo-donor selection (parent vs. haplo-sibling vs. child), donor-specific antibodies, cytokine release syndrome (especially after infusion of haplo PBSC grafts, as is increasingly popular), post-PTCy immunologic dysfunction and reconstitution, and unique aspects of AML relapse in the haplo-HSCT context are areas of ongoing study.

Hematopoietic Stem Cell Transplantation for Relapsed, Refractory, and Induction-Failure Acute Myeloid Leukemia

Outcomes are typically poor for AML patients not in morphologic remission at the time of allogeneic HSCT, although the literature in this regard is limited, heterogeneous, and likely subject to patient selection and publication bias. A large analysis of MAC HSCT for acute leukemia in relapse or in primary induction failure (including 1673 AML patients) documented a 3-year OS of 19% in AML.[54] On multivariable analysis, risk factors at time of HSCT of poor-risk cytogenetic AML, CR1 duration of less than 6 months, circulating AML blasts, performance score of less than 90%, and lack of an HLA-matched sibling donor predicted for 3-year survivals ranging between 42% (zero risk factors) and 6% (three or more risk factors). The major cause of death remains AML relapse, and innovative strategies to reduce leukemic burden before transplantation and enhance GVL effect after transplantation are likely required to improve survival of this extremely high-risk patient population.

In one instance for this patient population, the use of novel apoptosis-inducing agents (e.g., venetoclax) or agents targeting specific AML molecular pathways (e.g., FLT3, IDH 1/2, mTOR) with limited off-target toxicity as a bridge to allogeneic HSCT could offer important clinical benefit. In another instance, the use of novel adoptive cell therapies (e.g., cytokine-induced memory-like NK cells[55]) or graft-engineering approaches (e.g., αβ T cell-[56] or regulatory T cell- [Treg] engineered grafts[57]) to enhance GVL while controlling GVHD are important innovations under evaluation for high-risk leukemia. Finally, the ability to rapidly proceed to allogeneic transplantation may also be important given the dismal outcome of chemotherapy alone, suggesting a role for expeditious transplantation in this setting, especially for patients temporarily achieving a remission.

Hematopoietic Stem Cell Transplantation for Therapy-Related and Secondary Acute Myeloid Leukemia

Therapy-related AML (t-AML) currently accounts for ~10% to 20% of newly diagnosed AML, and its incidence is likely to rise in the future, given rising cancer survivorship. Post-remission therapy using conventional chemotherapy is usually not curative in secondary AML (s-AML: t-AML and AML arising from antecedent MDS or MPD) with the rare exception of CBF-AML, specifically inv(16) and t(15;17). Allogeneic HSCT is less effective in s-AML. The poorer survival appears multifactorial due to poor-risk cytogenetics and tumor-associated mutations (e.g., TP53, PPM1D), lower likelihood of entering remission, older patient age, and impaired organ function from prior cancer therapy; although some reports documented similar post-transplantation survival compared to de novo AML when adjusted for risk factors like disease status and cytogenetics.[32,58] An observational analysis of 545 patients with t-AML identified risk factors influencing HSCT outcomes: age greater than 35 years, poor-risk cytogenetics, AML not in remission, and lack of well-matched donors. A prognostic score could stratify outcomes from 5-year OS of 50% (zero risk factors) to 4% (four risk factors).[59]

Molecular genotyping offers insights into the clinical heterogeneity of s-AML. In a seminal analysis, Lindsley et al identified eight genes specifically mutated in s-AML (SRSF2, SF3B1, U2AF1, ZRSR2, SXL1, EZH2, BCOR, STAG2) that also identify worse prognosis "de novo" elderly AML, likely indicating undiagnosed antecedent MDS. They also identified a molecular signature of "de novo" type AML. Finally, they confirmed that TP53, PPM1D mutations comprised a distinct set of AML with the worst outcomes. Molecular risk stratification offers the potential to redefine the clinical diagnosis of s-AML, refine AML risk prognostication, and potentially better select remission-inducing and post-remission therapy.[60]

Hematopoietic Stem Cell Transplantation for Acute Myeloid Leukemia in Older Adults

AML incidence rises with age, with a median age at diagnosis of 67 years.[61] For AML patients older than 60 years, consolidation chemotherapy is unlikely to be curative (Fig. 62.2). Even after adjusting for increased incidence of poor prognosis features like adverse cytogenetic and molecular markers, secondary AML, comorbidities, and poorer performance status, older age remains an independent predictor of poor survival with conventional chemotherapy. A priori, age-related CH may be a relevant variable for impaired survival, though this remains to be confirmed.[62,63] Although allogeneic HSCT is a curative therapy in the older age-group, there is considerable reluctance to refer these patients for transplantation, in contrast to younger adults with AML, in part due to outdated assumptions regarding the tolerability of allogeneic transplantation but also due to lack of comparative data regarding outcomes.

Several reports have documented the feasibility of RIC transplantation with acceptable toxicity and reasonable survival in patients 60 years of age and above, including those with AML. Indeed, chronologic age alone does not appear to be a prognostic factor in RIC allogeneic transplantation for AML.[64,65] In a case-control study comparing RIC transplantation with chemotherapy for AML patients 60 to 70 years of age, allogeneic HSCT was associated with lower relapse risk (32% vs. 81%, $P < .001$), greater treatment-related mortality (36% vs. 4%; $P < .001$), and longer 3-year leukemia-free survival (32% vs. 15%, $P = .001$), with a borderline 3-year OS benefit (37% vs. 25%, $P = .08$).[66]

Overall, for physically fit older AML patients, especially those between 60 and 70 years of age, a donor search should be undertaken promptly at diagnosis, disease control with intensive induction chemotherapy is beneficial, and they should be considered for potentially curative allogeneic transplantation upon entering complete remission. Broadly speaking, molecular and karytotypic AML risk prognostication also applies to older patients being considered for RIC HSCT, with several caveats:

First, favorable-risk (e.g., CBF-AML) disease retains a relatively favorable impact in older patients, though data indicating equivalent outcomes of non-transplantation and allogeneic HSCT treatment cannot be extrapolated to older patients. Allogeneic HSCT likely offers additional benefit. For instance, in the UK National Cancer Research Institute (NCRI) AML16 trial of patients with median age of above 65 years, NPM1 mutant FLT3-ITD negative AML treated with intensive chemotherapy had a 3-year OS of only 26%, with a relapse incidence of 73%.[67] Additionally, in a combined SWOG/NCRI cohort, patients with NPM1mutant FLT3-ITD negative AML who were aged 55 to 65 years had a significantly improved 2-year OS of 70% versus 27% for those aged above 65 years ($P < .001$). The survival benefit of NPM1 mutant FLT3-ITD negative AML was absent in patients aged above 65 years.[68] Importantly, in a separate donor versus no-donor analysis, NPM1 mutant AML patients with an allogeneic donor had significantly lower 3-year relapse rates (14% vs. 46%) and improved RFS (71% vs. 47%), indicating a beneficial antileukemic effect of allogeneic HSCT in this AML subgroup.[69]

Second, certain AML cohorts (e.g., MK and TP53 mutant AML) have an extremely poor prognosis with high risk of disease relapse,[22,23,70] and the adequacy of "usual" RIC HSCT is questionable in this AML subgroup. Such patients should be appropriately counseled prior to proceeding with allogeneic HSCT, with consideration to conditioning intensity escalation if possible. Novel clinical trials focused on enhancing GVL are a priority for this ultra-high-risk AML subgroup.

Acute Myeloid Leukemia Relapse After Transplantation

AML relapse after transplant is common, associated with dismal outcomes, and remains a major obstacle to HSCT success.[71] Up to 40% of patients with AML/MDS relapse after undergoing HLA-matched HSCT.[72] Powerful donor-derived immune responses underlie the GVL effect, and immune escape (i.e., tumor evasion of the donor immune system) contributes to relapse after HSCT.

In HLA-matched HSCT, presentation of host hematopoietic and/or tumor epitopes within the concordant HLA context can invoke T cell receptor (TCR)-mediated recognition and elimination by donor T effector (Teff) cells to induce curative GVT responses that may be antagonized by loss of Class I HLA or suppression of Class II HLA on tumor cells, by suppression of co-stimulatory immune pathways, by immune suppressive T regulatory (Treg) cells, or by activation of inhibitory immune checkpoint pathways.[73–78] While the loss of tumor Class I HLA (typically mismatched allele) can occur during relapse after HLA-mismatched and haploidentical HSCT, it is uncommon in HLA-matched and absent in UCB HSCT,[77–79] highlighting the importance of individualized tumor versus donor immune interactions in each clinical context.

Inhibitory immune checkpoint pathways play an important role in relapse after HSCT.[75] The engagement of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed death 1 (PD-1)

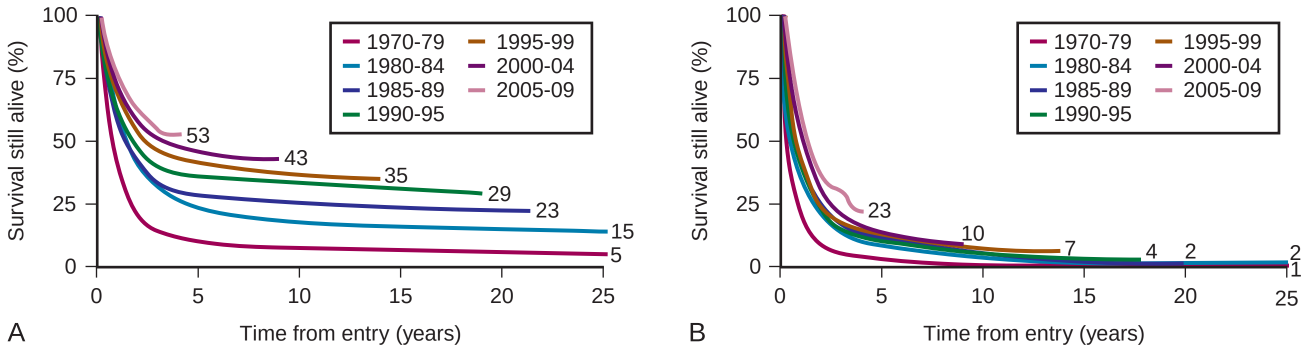


Figure 62.2 CHANGE IN ACUTE MYELOID LEUKEMIA OVERALL SURVIVAL WITH TIME. (A) Age 15 to 59 years. (B) Age 60 years or greater. *(Data from Burnett A, Wetzler M, Löwenberg B. Therapeutic advances in acute myeloid leukemia. J Clin Oncol. 2011;29:487.)*

receptors by their ligands B7-1/2 and PD-L1/2, respectively, inhibits effector T cell function. Tumor cells often express these inhibitory ligands, thereby selectively blocking antitumor immunity. In murine models, PD-1 blockade led to an increase in GVHD, whereas selective CTLA-4 blockade with Ipilimumab (Ipi) to treat late HSCT relapses augmented GVT without accelerating GVHD.[80,81] Outside of HSCT, Ipi monotherapy has limited efficacy in relapsed AML/MDS.[82]

For patients with targetable mutations, or for therapies broadly augmenting GVL and controlling disease relapse, maintenance strategies to prevent AML relapse in the post-transplant context are worth evaluating. For instance, early-phase trials of maintenance therapy with HMAs for MDS/AML (e.g., azacytidine), and targeting of FLT3-ITD AML (e.g., sorafenib) reported preliminary safety and efficacy.[83–85] In the randomized phase II SORMAIN study, 83 FLT3-ITD AML patients who had undergone HSCT and were stably engrafted without grade II or higher acute GVHD were randomly assigned to sorafenib maintenance for up to 24 months versus placebo. At 30 months, OS favored the sorafenib arm, with a HR of 0.447 ($P = .03$).[86] Preliminary results of the randomized phase II RADIUS trial of post-transplant midostaurin likely support this conclusion, with a 43% reduction in the risk of FLT3-ITD AML relapse.[87] Interestingly, recent data show that sorafenib also promotes GVL activity in mice and humans through IL-15 production in FLT3-ITD-mutant leukemia cells.[88]

While falling donor chimerism after HSCT can identify cohorts at higher relapse risk,[89,90] more specific and sensitive MRD assessments in the post-HSCT context can better predict relapse. Importantly, early identification of patients at high risk of relapse after allogeneic HSCT offers the opportunity for targeted early intervention prior to morphologic relapse. Prospective prevention trials focused on the high-risk setting offer an informative population for evaluation of intervention efficacy and are a high priority. The ongoing randomized phase III trial of gilteritinib (BMTCTN 1506, NCT02997202) will help definitively address the issue of FLT3-ITD maintenance post-transplant, especially in those at high risk, as a highly sensitive and specific MRD assay will be used throughout the trial to provide information about which patients may or may not benefit from maintenance therapy. Similar trials for the prevention of AML relapse after transplant in patients with other targetable mutations (e.g., IDH1/2) are eagerly anticipated.

The treatment options for patients with morphologic disease relapse after HSCT include withdrawal of IS, DLI, chemotherapy (including novel agents), second allogeneic transplantation, or supportive care.[91] Taper of IS and DLI are used especially in patients without GVHD to enhance antileukemic GVL effect. DLI has limited efficacy, in the range of 15% to 30% for patients with relapsed AML, and many responses are incomplete or temporary. Of note, recommended DLI dose is ~1-log lower in haplo-HSCT; and it is not expected to have efficacy in relapse associated with mismatched HLA loss on tumor cells but still carries a risk of GVHD and should be avoided in that context. DLI outcomes are better in patients who relapsed beyond 6 months after HSCT, those with lower leukemic burden (less than 35% BM blasts), patients with good-risk cytogenetics, and those who achieved remission before DLI.[92]

Novel options to enhance curative GVL effect include infusion of selected donor lymphocyte subpopulations via mDLI (e.g., CD25 Treg-depleted DLI[93]), other immune effectors (e.g., cytokine-induced memory-like [CIML] NK cells[55]), and use of immune checkpoint inhibitors (e.g., CTLA-4 blockade[94]) A second allogeneic HSCT has demonstrable but limited efficacy, and the duration of remission of more than a year after initial HSCT is the major predictor of survival.[95]

TRANSPLANTATION FOR MYELODYSPLASTIC SYNDROME

MDS comprises a heterogeneous group of clonal hematologic stem cell disorders characterized by varying degrees of cytopenias and risk for transformation into acute leukemia (see Chapter 61).[96] The incidence and prevalence of MDS has increased significantly over the last 20 years as a result of increasing longevity of the population and increasing physician awareness.[97–99] New therapeutic options such as HMAs, immunomodulatory drugs, and differentiation induction agents may improve hematologic parameters and reduce transfusion requirements,[100–102] and even prolong survival,[102] but allogeneic HSCT is the only known curative therapeutic option.

MDS is currently the third most common indication for allogeneic HSCT as reported to the CIBMTR.[103] Outcomes following transplantation for MDS have steadily improved over time, and now 40% to 65% of patients undergoing transplants are long-term survivors. MDS is a disease predominantly of older individuals, and with the increased use and acceptance of RIC into the eighth decade of life, it is anticipated that transplantation volume for MDS will continue to increase in the coming years.

Transplantation Trials in Myelodysplastic Syndrome—Comparison With Non-Transplantation Therapies

It is now clear that allogeneic transplantation offers a survival advantage over conventional therapies in MDS, and prospective trials have confirmed this assertion. In the first prospective trial that assigned subjects in a donor versus no donor fashion, a survival advantage in the donor arm ($n = 112$) over those without a donor ($n = 50$) was noted. At 4 years from the time of registration, OS was 37% in the donor arm in comparison with 15% in the no donor arm ($P < .02$) and importantly, there did not appear to be an initial decrement in early survival in the donor arm, suggesting that early TRM after transplantation is comparable to mortality associated with non-transplant therapies in a high-risk subject cohort. In this trial, however, subjects with CMML, secondary MDS, and AML were included, as were subjects with lower-risk MDS.[104] In a prospective randomized biologic assignment study, the German MDS group treated all enrolled subjects with 4 cycles of azacytidine, followed by a biologic assignment to further azacytidine or transplantation. The authors reported superior outcomes in the group that proceeded to transplantation ($n = 83$) in comparison to the continued azacytidine group ($n = 26$), with 49% survival in the transplant arm in comparison with 22% in the continued azacytidine arm at 3 years ($P = .027$).[105]

At the Dana-Farber Cancer Institute, we followed the outcomes of two distinct groups of patients who were referred for transplantation. The first group comprised patients with higher-risk MDS, defined as either IPSS Intermediate-2 or High-Risk disease, the presence of high-risk cytogenetics, or those with secondary MDS ($n = 175$). The second group comprised patients whose transplant indication was refractory cytopenias alone ($n = 115$). In the high-risk group, subjects who chose transplantation had improved outcomes in comparison with those who did not (multivariable HR for death 0.57, $P = .01$) while no advantage was noted in the group with cytopenias alone (multivariable HR 1.33, $P = .36$). Moreover, the benefits of transplantation were associated with "early" transplantation, defined by the median time to transplant in the study.[106]

The most definitive prospective trial comparing transplantation and non-transplantation approaches was recently published by the Blood and Marrow Transplantation Clinical Trials Network. In this biologic assignment trial, 384 subjects (260 donor, 124 no donor) between the ages of 50 to 75 with higher-risk MDS were followed prospectively after assignment. At 3 years, there was a 21.3% improvement in OS (47.9% vs. 26.6%, $P = .0001$) and a 15.2% improvement in leukemia-free survival (35.9% vs. 21.8%, $P = .003$) in an ITT analysis.[107] Based on this trial results, we concluded that all patients who are potential candidates for HSCT should be referred to a transplantation center early in their disease course so that a discussion regarding the appropriateness of transplantation can occur and a donor search can be initiated, where appropriate.

Contemporary Results in Ablative and Reduced-Intensity Transplantation for Myelodysplastic Syndrome

The role of conditioning intensity in transplantation has been debated since the introduction of reduced-intensity regimens (RIC), and several comparative studies have now been published. The premise behind all of these comparisons is that RIC should be associated with a reduction in early treatment-related mortality, whereas myeloablative (MAC) approaches should be associated with a reduction in disease relapse. The net effect of these two opposing risks is anticipated to be no difference in outcomes. In an analysis by the EBMT of over 800 patients with MDS, the 3-year relapse rate was significantly higher after RIC (HR, 1.64; $P = .001$), but with less treatment-related mortality (HR, 0.61; $P = .015$), leading to similar 3-year OS (45% vs. 41%, $P = 0.8$). When limited to individuals above 50 years of age, no difference in outcome stratified by conditioning intensity (32% vs. 30%, $P = .73$) was noted (Fig. 62.3).[108] Two recent CIBMTR analyses additionally address this controversy. In the first study, 951 subjects with MDS were stratified by conditioning regimen. In this study, myeloablative regimens (including BuCy, FluBu4, and FluMel) were associated with the best overall outcomes, using RFS as the main study endpoint.[109] In the other CIBMTR second study, where only subjects aged 40 to 65 were included, myeloablation resulted in superior outcomes only in subjects with low or intermediate disease risk index (DRI) scores, with no advantage for dose intensity when high DRI was noted.[110] It is to be noted that the latter two studies included subjects with AML as well as MDS.

As retrospective studies are generally not considered definitive, prospective trials to answer this important question have now been performed. In a multicenter prospective study by the Blood and Marrow Transplant Clinical Trials Network, patients aged 18 to 65 were randomized to allogeneic transplant using reduced intensity or MAC. This trial was halted early due to an observed survival advantage in the AML cohort with MAC but too few MDS patients were enrolled to make definitive conclusions in these patients.[2] A second prospective study by the EBMT randomized patients up to the age of 65 to reduced intensity or MAC similarly showed no statistical difference in nonrelapse mortality, relapse, or disease-free survival.[111] Based on these studies, consensus favors MAC for those under 65 years old with an acceptable HCT comorbidity index score and RIC for all patients 65 years and older or those with higher comorbidity scores.

Using Prognostic Models to Define the Timing of Transplantation

While the prospective comparative trials have defined the superiority of transplantation over non-transplant therapies for select groups of patients,

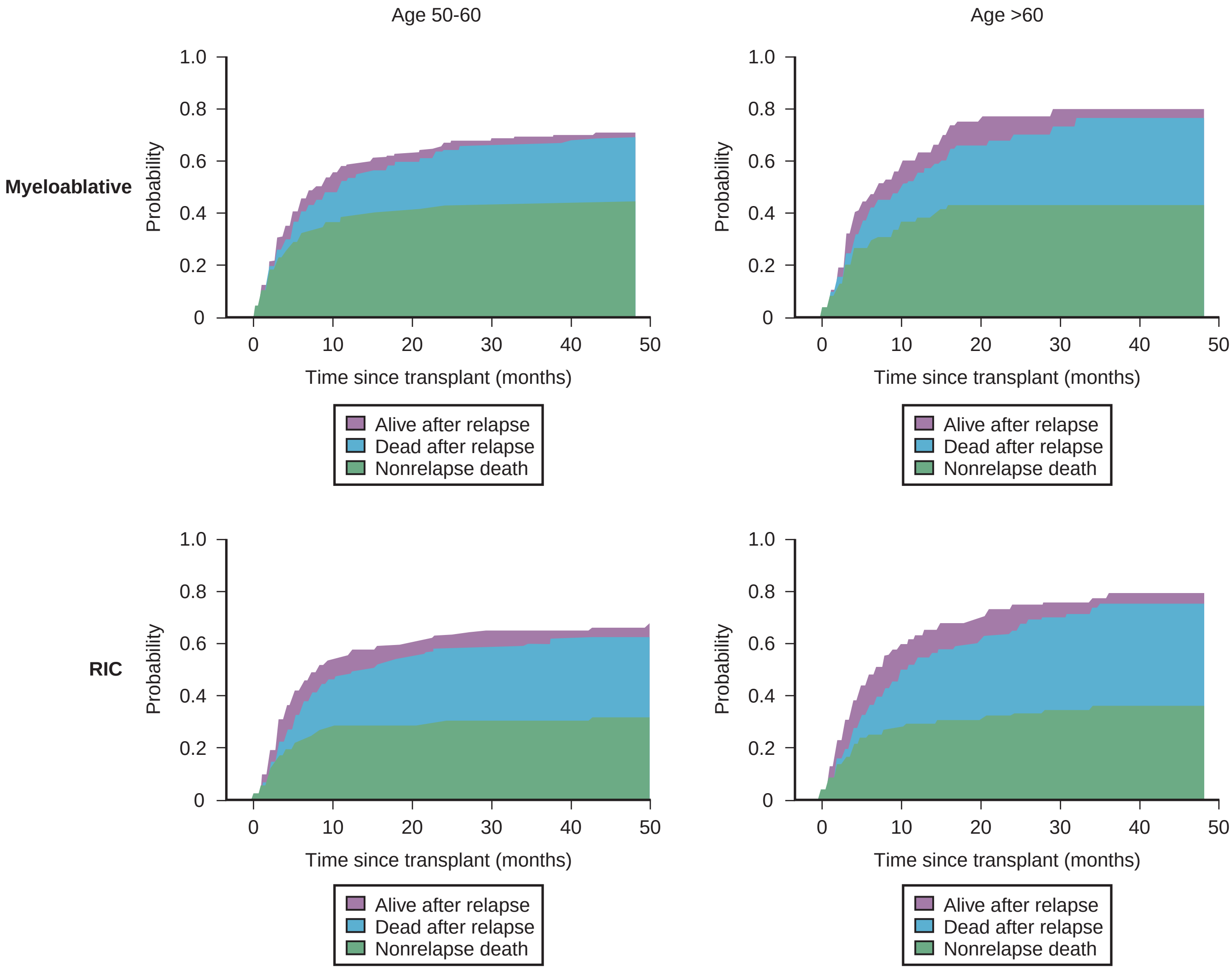


Figure 62.3 COMPETING RISK CUMULATIVE INCIDENCES FOR RELAPSE AND NONRELAPSE MORTALITY AMONG INDIVIDUALS ABOVE AGE 50 YEARS. *RIC*, Reduced-intensity conditioning. *(Data from Lim Z, Brand R, Martino R, et al. Allogeneic hematopoietic stem-cell transplantation for patients 50 years or older with myelodysplastic syndromes or secondary acute myeloid leukemia. J Clin Oncol. 2010;28:405.)*

the optimal timing for transplant informing the prospective trials was approached using Markov modeling and scoring systems that use clinical, pathological, and cytogenetic risk assignment. The most widely used clinical prognostic system is the IPSS, which can predict both non-transplantation outcomes as well as myeloablative transplantation outcomes.[112–114] The IPSS-R has recently supplanted the IPSS as the dominant prognostic scoring system.[115] Several groups have attempted to validate this newer prognostic construct with transplant outcomes. A cohort of 519 MDS patients was reported by the GITMO, and their outcomes were stratified by IPSS-R. In addition to the IPSS-R, MK and the HCT comorbidity index independently predicted outcomes. The 5-year survival outcomes were 71%, 58%, 39%, and 23% in the low, intermediate, high, and very high-risk patients when classified by the IPSS-R.[116]

Markov decision models have suggested that the optimal timing of transplantation is at the time of diagnosis of Intermediate-2 or high-risk disease,[114,117] although one analysis suggested that even patients with Intermediate-1 risk disease should be offered transplantation.[118] The first model utilizing the IPSS-R has been presented, suggesting optimal outcomes were obtained with immediate transplantation for patients with Intermediate risk or higher disease and delayed transplantation for low and very low risk disease.[119] A shortcoming of all of these analyses is the inability to provide treatment decision guidance at clinically relevant times for patients not undergoing immediate HSCT because other important clinical events, such as a new transfusion requirement, recurrent infection, or recurrent bleeding episodes certainly could be considered triggers to move on to transplantation. Thus, it is useful to subdivide patients with lower-risk MDS into more-refined prognostic groups that may benefit from HSCT.[120]

Transfusion frequency and resultant iron overload have both been demonstrated to be important prognostic factors in HSCT outcomes. Platzbecker et al. compared the effects of transfusion dependency at the time of HSCT showing that transfusion-dependent patients have more low-risk features but still have inferior overall survival, with a 3-year OS of 49% compared with 60% in a transfusion-independent group ($P = .1$).[121] The Pavia group examined a cohort of patients stratified by IPSS to determine the relevance of the newer World Health Organization (WHO) histologic classification scheme and to determine if transfusion requirement influenced outcomes among different IPSS groups. For patients with low- and intermediate-1 risk IPSS scores, the WHO histologic classification scheme significantly differentiated survival among the different histologic subtypes within each IPSS range.[122] More importantly, the authors demonstrated a significant effect on survival based on the requirement for transfusion support. As would be expected, outcomes in patients with higher-grade myelodysplasia were affected less by transfusion requirement than were patients with less-advanced WHO histologic classifications. As a result, the impact of the transfusion requirement was then incorporated into the WHO Prognostic Scoring System (WPSS).[123] In this system, the effect of regular transfusion requirement (defined as requiring at least one transfusion every 8 weeks in a 4-month period) was given the same regression weight as progressing between cytogenetic risk groups. In contrast to the initial IPSS publication, which carries prognostic information only at the time of initial MDS diagnosis, this model was time-dependent, such that patients could be continually reevaluated by the scoring model and updated prognostic information could be generated at any time in the patient's treatment course. As such, the development of new cytogenetic changes or the development of a transfusion need adds useful information that helps guide treatment decisions, including the decision to proceed to HSCT as well as estimating transplantation outcomes. A decision analysis incorporating WPSS stage to determine the optimal timing of transplantation demonstrated a survival advantage for transplantation performed with intermediate WPSS stage,[118] although this prognostic scoring system is infrequently used.

Molecular Prognostic Factors

Perhaps even more than in AML, mutation analysis undoubtedly led to a shift from rudimentary clinical prognostic models to molecularly defined models for prognosis and to guide therapy in MDS. As many as 75% of patients can be found to have one or more mutations.[124] Numerous individual genes and whole-genome expression profiling have demonstrated significant associations with MDS outcome, and now, some of these mutational profiles have been associated with the transplantation outcome.

Examining a cohort of 87 patients who underwent allogeneic transplantation at Dana-Farber Cancer Institute, Bejar et al. were able to demonstrate the adverse prognostic impact of three specific mutations on outcome after transplantation.[125] In this cohort, while 92% of subjects had at least one identifiable mutation, those with TET2 and DNMT3A had worse prognoses than those without these mutations, while those with mutations in TP53 had the worst outcomes, with no subjects surviving beyond 2 years from transplantation. Mutations in TP53 were extremely powerful predictors of relapse and were better prognosticators than cytogenetic status; subjects with CKs in the absence of TP53 mutations had similar outcomes to subjects without CKs, possibly heralding the decline of the cytogenetic signature as the most important prognostic marker in transplantation. More recent studies have confirmed the adverse impact of TP53 mutations along with mutations in RAS pathway genes and CK.[126] The impact of molecular mutations was further confirmed in an analysis of the prospective randomized trial of reduced intensity or myeloablative transplantation, where MDS subjects with mutations in one or more genes of a 10-gene panel were associated with poorer outcomes among subjects randomized to reduced-intensity transplantation when compared with MAC.[127]

The most comprehensive analysis of genomic alterations and transplantation outcome was performed in a cohort of 1514 patients in the CIBMTR registry.[128] Importantly, nearly 1200 patients undergoing transplantation had one or more mutations noted (79%). The most important prognostic factor that emerged from this analysis was the importance of TP53 mutations, which were associated with far inferior outcomes related to excess relapse. RAS pathway mutations (including mutations in *NRAS, KRAS, PTPN11, CBL, NF1, RIT1, FLT3,* and *KIT*) were similarly associated with excess risk of relapse, which could be overcome partially by increasing conditioning intensity. Mutations in JAK2, however, were associated with excess treatment-related mortality.

When examining characteristics of TP53 mutations in the transplant cohort, subjects with missense TP53 mutations were noted to have better outcomes when compared with those with truncated TP53 mutations.[128] In the non-transplant setting, patients with monoallelic TP53 mutations had presentations similar to patients with bi-allelic mutations and fare better.[129] At least one group has demonstrated reasonable outcomes for patients transplanted with known TP53 mutations.[130] Patients with TP53 and other adverse-risk prognostic mutations should be encouraged to participate in novel clinical trials to reduce the rate of relapse after transplantation.

The Role of Pretransplantation Therapy in Myelodysplastic Syndrome

The role of cytoreductive chemotherapy before HSCT for MDS remains controversial. Consensus guidelines[131] recommend debulking therapy in patients with greater than 10% blasts in the marrow, although this recommendation is not based on prospective evidence. Many analyses have demonstrated that outcomes are improved with lower disease burden at the time of HSCT,[132] but previously, only conventional chemotherapy was offered. The analyses that compare chemotherapy to no chemotherapy before HSCT are inherently biased because patients who do poorly or expire with chemotherapy are not included in HSCT outcome data and might have had favorable outcomes if not exposed to cytotoxic agents. On the other hand, responsiveness to chemotherapy may indicate more favorable disease biology, and this has been correlated with improved outcomes after HSCT.[133] With the advent of DNA hypomethylating therapy, the use of conventional chemotherapy has markedly diminished prior to transplantation in MDS. The effect of DNA hypomethylating therapy on

HSCT outcomes has recently been studied by several groups.[134–136] Compared retrospectively, it does not appear that pretransplantation hypomethylating therapy offers a survival advantage, but these agents continue to be used widely to prevent disease progression and to reduce transfusion needs, while donor selection is performed in patients destined to undergo transplantation. Older analyses have compared the use of traditional chemotherapy with hypomethylating therapy prior to transplantation. In the smaller of the older analyses, there was no advantage to hypomethylating therapy over conventional chemotherapy,[136] while in the larger of the two, the use of both chemotherapy and hypomethylating therapy prior to transplantation was associated with adverse outcomes, presumably on the basis of identifying subjects with more aggressive disease biology.[134] More recent analyses continue to demonstrate similar outcomes when transplant without debulking is compared to pretransplant therapy,[137] and an EBMT analysis failed to demonstrate differences in survival measured from the time of transplantation when pre-transplant hypomethylating therapy was compared with traditional chemotherapy[138]; however, only a prospective randomized trial with survival measured from the time of randomization to pre-transplant therapy can rationally answer the question as to the most appropriate pre-transplant therapy.

Theoretical advantages associated with the use of HMAs prior to transplant include upregulation of the expression of cancer-testis antigens,[139,140] killer immunoglobulin-like receptor (KIR) ligands,[141] HLA molecules,[142] and other minor antigens on tumor cells,[143] all of which may enhance graft-versus-MDS effects. HMAs have been used after transplantation to reduce the risk for relapse,[85] but randomized trials in myeloid malignancies have not yet demonstrated efficacy.[144]

Transplantation in Older Patients and in Patients With Comorbidity

Older adults represent the fastest-growing fraction of transplantation activity, and there has been a dramatic increase in transplantation for MDS for individuals in the eighth decade of life.[145] As such, specific accommodations need to be considered for these patients. However, it is critical to realize that with proper patient selection, transplantation outcomes in older individuals are very similar to those of younger patients. In a landmark study conducted by the CIBMTR, the outcomes of 688 patients aged 65 or older were compared with 592 subjects aged 55 to 64 at the time of transplantation. 3-year OS was 37% versus 42% in the older and younger groups respectively, with no differences noted in multivariable analyses (HR for mortality 1.09, $P = .23$).[146] This analysis, as well as an older study,[64] therefore demonstrates that age alone cannot be used as a discriminator for transplantation appropriateness. However, with increasing age comes increasing comorbidity, and the CIBMTR analysis clearly demonstrated that a comorbidity index score of 4 or greater was associated with inferior transplantation outcomes.[146] The Hematopoietic Cell Transplantation Comorbidity Index, developed by Sorror and colleagues,[147,148] has been shown to carry the greatest prognostic value, even in patients with MDS not undergoing transplantation.[149] More recently, an enhanced index that incorporates both age and clinical comorbidity was developed and demonstrated to have even more prognostic power than either of the components alone. In this scoring system, those with the highest age-adjusted comorbidity scores have nonrelapse mortality that might exceed 50% at 5 years, with concomitant survival that is less than 20%.[150] The consideration of comorbidity before HSCT is even more relevant now that RIC has been shown to be equally effective in the treatment of MDS.[2]

An elevated pre-HSCT serum ferritin level was strongly associated with lower overall and disease-free survival in patients with AML and MDS after transplantation, and this was largely attributable to increased treatment-related mortality.[151] Similarly, a prospective single-institution study of 190 adult patients undergoing MAC HSCT demonstrated that elevated pretransplantation serum ferritin level was associated with increased risk for 100-day mortality, acute GVHD, and bloodstream infections or death as a composite endpoint.[152] It has now been recognized that the elevated ferritin level can predict inferior outcomes even after RIC transplantation.[153] As a laboratory prognostic factor, ferritin should be considered a marker of comorbidity, as ferritin is a marker of inflammation as well as iron overload. As such, ferritin levels could influence the decision to pursue high-intensity or reduced-intensity HSCT, and iron chelation therapy should be considered prior to transplantation when feasible.

There are several approaches to the transplantation of older patients with MDS that can improve outcomes. In older subjects, careful attention to frailty with geriatric assessment should be performed prior to transplantation. A comprehensive intervention strategy to preserve function has been demonstrated to improve nonrelapse mortality in a group of older subjects, not limited to MDS patients.[154] Selection of the most appropriate conditioning regimen has been demonstrated to have an impact in transplantation for MDS, and one CIBMTR study suggested that a more intensive Fludarabine-Melphalan regimen, while associated with slightly higher rates of treatment-related mortality, was associated with improved outcomes in comparison with a lower intensity Fludarabine-Busulfan regimen.[155] However, excess treatment-related mortality is noted in subjects undergoing transplantation with more intensive regimens who have unrecognized shorter telomere lengths, and in these analyses, routine assessment of telomeropathy should be considered in all older subjects planning to undergo melphalan-based conditioning and transplantation.[156]

Alternative Donor Transplantation in Myelodysplastic Syndrome

Early results using alternative donors in MDS were not encouraging, with treatment-related mortality approaching 30% and long-term survival of only 30% using mismatched unrelated, haploidentical, and UCB.[157] More contemporary registry and comparative analyses suggest that outcomes have improved marginally, now demonstrating survival in approximately 40% of patients, with only minor differences between haploidentical or mismatched unrelated donors, but with UCB faring the worst.[158]

The largest series reporting on outcomes in UCB transplantation reported on 176 subjects with a median age of 56 years at the time of transplantation (2004 to 2013). Despite this relatively young age, OS was only 30%, with high rates of treatment-related mortality.[159] An older comparative analysis demonstrated no advantage of mismatched unrelated donors over UCB,[160] but the more recent analysis suggesting a disadvantage in UCB.[158]

There has been more interest recently in the use of haploidentical donors given the ease of identifying donors and lower costs associated with graft procurement. In one comparative analysis that included both patients with AML and MDS, no major differences in outcomes were noted when matched related, unrelated or haploidentical donors were used.[161] However, a larger CIBMTR analysis restricted to RIC in subjects with MDS demonstrated higher relapse and lower disease-free survival in recipients of haploidentical transplantation when compared with matched unrelated donors.[162]

Treatment and Prevention of Relapse Following Transplantation

The treatment of relapse following allogeneic HSCT for MDS is similar to the treatment of AML; manipulation of immunosuppression with or without DLI is of modest utility[163] and generally requires re-institution of pre-transplant chemotherapy for success. As there are no targeted therapy agents available for MDS at this time, DNA hypomethylating therapy is most commonly used. DNA hypomethylating therapy has been attempted as post-transplant maintenance therapy in an attempt to reduce the risk of relapse. In the initial phase 1 trial, escalating doses of azacytidine were given to 45 subjects with AML or MDS. The maximum-tolerated dose was 32 mg/m^2 given for 5 days of a 30-day cycle.[85] When tested in a prospective, randomized trial of 187 subjects, however, there was no benefit noted in either relapse-free or overall survival.[144] As maintenance therapy has become the standard in AML with a molecular target (i.e., Flt3), the search for a maintenance strategy that improves outcomes is actively being pursued. The addition of the bcl-2 inhibitor, venetoclax, might hold promise in this regard.[164]

FUTURE DIRECTIONS

Despite the progress that has been made in conventional therapy for both AML and MDS, allogeneic HSCT has the best track record for curing the disease. Although HSCT has limitations, reductions in toxicity, improvements in donor availability, and new strategies to enhance GVL while limiting GVHD promise to further improve survival. Advances in mutation analysis provide insights into the pathophysiology of MDS and AML and suggest routes for targeted therapy. However, in many circumstances targeted therapy may not be feasible; thus the utility of molecular prognostic indicators is to identify patients who will benefit from early transplantation.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Atallah E, Logan BR, Chen M, et al. Comparison of patient age groups in transplantation for myelodysplastic syndrome: the Medicare Coverage with Evidence Development Study. *JAMA Oncol*. 2020;6:486–493.

Bejanyan N, Zhang M, Bo-Subait K, et al. Myeloablative conditioning for allogeneic transplantation results in superior disease-free survival for acute myelogenous leukemia and myelodysplastic syndromes with low/intermediate but not high disease risk index: a center for international blood and marrow transplant research study. *Biol Blood Marrow Transplant*. 2021;27:68.e1–68.e9.

Bejar R, Stevenson KE, Caughey B, et al. Somatic mutations predict poor outcome in patients with myelodysplastic syndrome after hematopoietic stem-cell transplantation. *J Clin Oncol*. 2014;32:2691–2698.

Bornhäuser M, Kienast J, Trenschel R, et al. Reduced-intensity conditioning versus standard conditioning before allogeneic haemopoietic cell transplantation in patients with acute myeloid leukaemia in first complete remission: a prospective, open-label randomised phase 3 trial. *Lancet Oncol*. 2012;13:1035–1044.

Christopher MJ, Petti AA, Rettig MP, et al. Immune escape of relapsed AML cells after allogeneic transplantation. *N Engl J Med*. 2018;379:2330–2341.

Ciurea SO, Zhang M-J, Bacigalupo AA, et al. Haploidentical transplant with posttransplant cyclophosphamide vs matched unrelated donor transplant for acute myeloid leukemia. *Blood*. 2015;126:1033–1040.

Cutler CS, Lee SJ, Greenberg P, et al. A decision analysis of allogeneic bone marrow transplantation for the myelodysplastic syndromes: delayed transplantation for low-risk myelodysplasia is associated with improved outcome. *Blood*. 2004;104:579–585.

De Jong CN, Meijer E, Bakunina K, et al. Post-transplantation cyclophosphamide after allogeneic hematopoietic stem cell transplantation: results of the prospective randomized HOVON-96 trial in recipients of matched related and unrelated donors. *Blood*. 2019;134:1.

Di Stasi A, Milton DR, Poon LM, et al. Similar transplantation outcomes for acute myeloid leukemia and myelodysplastic syndrome patients with haploidentical versus 10/10 human leukocyte antigen-matched unrelated and related donors. *Biol Blood Marrow Transplant*. 2014;20:1975–1981.

Dillon LW, Gui G, Logan BR, et al. Impact of conditioning intensity and genomics on relapse after allogeneic transplantation for patients with myelodysplastic syndrome. *JCO Precision Oncology*. 2021;5:265–274.

Döhner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*. 2017;129:424–447.

Eapen M, Brazauskas R, Hemmer M, et al. Hematopoietic cell transplant for acute myeloid leukemia and myelodysplastic syndrome: conditioning regimen intensity. *Blood Adv*. 2018;2:2095–2103.

Fasslrinner F, Schetelig J, Burchert A, et al. Long-term efficacy of reduced-intensity versus myeloablative conditioning before allogeneic haemopoietic cell transplantation in patients with acute myeloid leukaemia in first complete remission: retrospective follow-up of an open-label, randomised phase 3 trial. *Lancet Haematol*. 2018;5:e161–e169.

Horwitz ME, Cutler C, Stiff P, et al. Improved clinical outcomes with omidubicel versus standard myeloablative umbilical cord blood transplantation: results of a phase III randomized, multicenter study. *Transplant Cell Ther*. 2021;27:S33–S34.

Hourigan CS, Dillon LW, Gui G, et al. Impact of conditioning intensity of allogeneic transplantation for acute myeloid leukemia with genomic evidence of residual disease. *J Clin Oncol*. 2020;38:1273–1283.

Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med*. 2014;371:2488–2498.

Koreth J, Schlenk R, Kopecky KJ, et al. Allogeneic stem cell transplantation for acute myeloid leukemia in first complete remission: systematic review and meta-analysis of prospective clinical trials. *JAMA*. 2009;301:2349–2361.

Kröger N, Iacobelli S, Franke G-N, et al. Dose-reduced versus standard conditioning followed by allogeneic stem-cell transplantation for patients with myelodysplastic syndrome: a prospective randomized phase III study of the EBMT (RICMAC trial). *J Clin Oncol*. 2017;35:2157–2164.

Lim Z, Brand R, Martino R, et al. Allogeneic hematopoietic stem-cell transplantation for patients 50 years or older with myelodysplastic syndromes or secondary acute myeloid leukemia. *J Clin Oncol*. 2010;28:405–411.

Lindsley RC, Saber W, Mar BG, et al. Prognostic mutations in myelodysplastic syndrome after stem-cell transplantation. *N Engl J Med*. 2017;376:536–547.

Mehta RS, Holtan SG, Wang T, et al. Composite GRFS and CRFS outcomes after adult alternative donor HCT. *J Clin Oncol*. 2020:JCO1900396.

Meybodi MA, Cao W, Luznik L, et al. HLA-haploidentical vs matched-sibling hematopoietic cell transplantation: a systematic review and meta-analysis. *Blood Adv*. 2019;3:2581–2585.

Milano F, Appelbaum FR, Delaney C. Cord-blood transplantation in patients with minimal residual disease. *N Engl J Med*. 2016;375:2204–2205.

Myllymäki M, Redd R, Reilly CR, et al. Short telomere length predicts nonrelapse mortality after stem cell transplantation for myelodysplastic syndrome. *Blood*. 2020;136:3070–3081.

Nakamura R, Saber W, Martens MJ, et al. Biologic assignment trial of reduced-intensity hematopoietic cell transplantation based on donor availability in patients 50–75 years of age with advanced myelodysplastic syndrome. *J Clin Oncol*. 2021;39(30):3328–3339.

Oran B, de Lima M, Garcia-Manero G, et al. A phase 3 randomized study of 5-azacitidine maintenance vs observation after transplant in high-risk AML and MDS patients. *Blood Adv*. 2020;4:5580–5588.

Oran B, Woo Ahn K, Fretham C, et al. Fludarabine and melphalan compared with reduced doses of busulfan and flurabine improves transplant outcomes in older MDS patients. *Blood*. 2019;134:253.

Rashidi A, Hamadani M, Zhang MJ, et al. Outcomes of haploidentical vs matched sibling transplantation for acute myeloid leukemia in first complete remission. *Blood Adv*. 2019;3:1826–1836.

Scott BL, Pasquini MC, Logan BR, et al. Myeloablative versus reduced-intensity hematopoietic cell transplantation for acute myeloid leukemia and myelodysplastic syndromes. *J Clin Oncol*. 2017;35:1154–1161.

Toffalori C, Zito L, Gambacorta V, et al. Immune signature drives leukemia escape and relapse after hematopoietic cell transplantation. *Nat Med*. 2019;25:603–611.

Yoshizato T, Nannya Y, Atsuta Y, et al. Genetic abnormalities in myelodysplasia and secondary acute myeloid leukemia: impact on outcome of stem cell transplantation. *Blood*. 2017;129:2347–2358.

REFERENCES

1. Bornhäuser M, Kienast J, Trenschel R, et al. Reduced-intensity conditioning versus standard conditioning before allogeneic haemopoietic cell transplantation in patients with acute myeloid leukaemia in first complete remission: a prospective, open-label randomised phase 3 trial. *Lancet Oncol*. 2012;13:1035–1044.
2. Scott BL, Pasquini MC, Logan BR, et al. Myeloablative versus reduced-intensity hematopoietic cell transplantation for acute myeloid leukemia and myelodysplastic syndromes. *J Clin Oncol*. 2017;35:1154–1161.
3. Fasslrinner F, Schetelig J, Burchert A, et al. Long-term efficacy of reduced-intensity versus myeloablative conditioning before allogeneic haemopoietic cell transplantation in patients with acute myeloid leukaemia in first complete remission: retrospective follow-up of an open-label, randomised phase 3 trial. *Lancet Haematol*. 2018;5:e161–e169.
4. Current Use and Outcome of Hematopoietic Stem Cell Transplantation: CIBMTR Summary Slides. 2019. http://www.cibmtr.org.
5. Brunstein CG, Fuchs EJ, Carter SL, et al. Alternative donor transplantation after reduced intensity conditioning: results of parallel phase 2 trials using partially HLA-mismatched related bone marrow or unrelated double umbilical cord blood grafts. *Blood*. 2011;118:282–288.
6. Passweg JR, Baldomero H, Bader P, et al. Hematopoietic SCT in Europe 2013: recent trends in the use of alternative donors showing more haploidentical donors but fewer cord blood transplants. *Bone Marrow Transplant*. 2015;50:476–482.
7. Koreth J, Schlenk R, Kopecky KJ, et al. Allogeneic stem cell transplantation for acute myeloid leukemia in first complete remission: systematic review and meta-analysis of prospective clinical trials. *JAMA*. 2009;301:2349–2361.
8. Paschka P, Du J, Schlenk RF, et al. Secondary genetic lesions in acute myeloid leukemia with inv(16) or t(16;16): a study of the German–Austrian AML study group (AMLSG). *Blood*. 2013;121:170–177.
9. Jourdan E, Boissel N, Chevret S, et al. Prospective evaluation of gene mutations and minimal residual disease in patients with core binding factor acute myeloid leukemia. *Blood*. 2013;121:2213–2223.
10. Allen C, Hills RK, Lamb K, et al. The importance of relative mutant level for evaluating impact on outcome of KIT, FLT3 and CBL mutations in core-binding factor acute myeloid leukemia. *Leukemia*. 2013;27:1891–1901.
11. Duployez N, Marceau-Renaut A, Boissel N, et al. Comprehensive mutational profiling of core binding factor acute myeloid leukemia. *Blood*. 2016;127:2451–2459.
12. Tallman MS, Wang ES, Altman JK, et al. Acute myeloid leukemia, version 3.2019, NCCN clinical practice guidelines in oncology. *J Natl Compr Canc Netw*. 2019;17:721–749.
13. Döhner K, Schlenk RF, Habdank M, et al. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. *Blood*. 2005;106:3740–3746.
14. Becker H, Marcucci G, Maharry K, et al. Favorable prognostic impact of NPM1 mutations in older patients with cytogenetically normal de novo acute myeloid leukemia and associated gene- and microRNA-expression signatures: a Cancer and Leukemia Group B study. *J Clin Oncol*. 2010;28:596–604.
15. Rockova V, Abbas S, Wouters BJ, et al. Risk stratification of intermediate-risk acute myeloid leukemia: integrative analysis of a multitude of gene mutation and gene expression markers. *Blood*. 2011;118:1069–1076.
16. Breems DA, Van Putten WLJ, De Greef GE, et al. Monosomal karyotype in acute myeloid leukemia: a better indicator of poor prognosis than a complex karyotype. *J Clin Oncol*. 2008;26:4791–4797.
17. Schnittger S, Kohl TM, Haferlach T, et al. KIT-D816 mutations in AML1-ETO-positive AML are associated with impaired event-free and overall survival. *Blood*. 2006;107:1791–1799.
18. Röllig C, Bornhauser M, Thiede C, et al. Long-term prognosis of acute myeloid leukemia according to the new genetic risk classification of the European LeukemiaNet recommendations: evaluation of the proposed reporting system. *J Clin Oncol*. 2011;29:2758–2765.
19. Breems DA, Lowenberg B. Acute myeloid leukemia with monosomal karyotype at the far end of the unfavorable prognostic spectrum. *Haematologica*. 2011;96:491–493.
20. Oran B, Dolan M, Cao Q, Brunstein C, Warlick E, Weisdorf D. Monosomal karyotype provides better prognostic prediction after allogeneic stem cell transplantation in patients with acute myelogenous leukemia. *Biol Blood Marrow Transplant*. 2011;17:356–364.
21. Middeke JM, Beelen D, Stadler M, et al. Outcome of high-risk acute myeloid leukemia after allogeneic hematopoietic cell transplantation: negative impact of abnl(17p) and −5/5q−. *Blood*. 2012;120:2521–2528.
22. Mohr B, Schetelig J, Schafer-Eckart K, et al. Impact of allogeneic haematopoietic stem cell transplantation in patients with abnl(17p) acute myeloid leukaemia. *Br J Haematol*. 2013;161:237–244.
23. Middeke JM, Fang M, Cornelissen JJ, et al. Outcome of patients with abnl(17p) acute myeloid leukemia after allogeneic hematopoietic stem cell transplantation. *Blood*. 2014;123:2960–2967.
24. Dohner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*. 2017;129:424–447.
25. Hasserjian RP, Steensma DP, Graubert TA, Ebert BL. Clonal hematopoiesis and measurable residual disease assessment in acute myeloid leukemia. *Blood*. 2020;135:1729–1738.
26. Venditti A, Piciocchi A, Candoni A, et al. GIMEMA AML1310 trial of risk-adapted, MRD-directed therapy for young adults with newly diagnosed acute myeloid leukemia. *Blood*. 2019;134:935–945.
27. Dillon R, Hills R, Freeman S, et al. Molecular MRD status and outcome after transplantation in NPM1-mutated AML. *Blood*. 2020;135:680–688.
28. Hourigan CS, Dillon LW, Gui G, et al. Impact of conditioning intensity of allogeneic transplantation for acute myeloid leukemia with genomic evidence of residual disease. *J Clin Oncol*. 2020;38:1273–1283.
29. Spyridonidis A. How I treat measurable (minimal) residual disease in acute leukemia after allogeneic hematopoietic cell transplantation. *Blood*. 2020;135:1639–1649.
30. Mohty M, de Lavallade H, Ladaique P, et al. The role of reduced intensity conditioning allogeneic stem cell transplantation in patients with acute myeloid leukemia: a donor vs no donor comparison. *Leukemia*. 2005;19:916–920.
31. Luger SM, Ringdén O, Zhang M-J, et al. Similar outcomes using myeloablative vs reduced-intensity allogeneic transplant preparative regimens for AML or MDS. *Bone Marrow Transplant*. 2012;47(2):203–211.
32. Gupta V, Tallman MS, He W, et al. Comparable survival after HLA-well-matched unrelated or matched sibling donor transplantation for acute myeloid leukemia in first remission with unfavorable cytogenetics at diagnosis. *Blood*. 2010;116:1839–1848.
33. Basara N, Schulze A, Wedding U, et al. Early related or unrelated haematopoietic cell transplantation results in higher overall survival and leukaemia-free survival compared with conventional chemotherapy in high-risk acute myeloid leukaemia patients in first complete remission. *Leukemia*. 2009;23:635–640.
34. Schlenk RF, Dohner K, Mack S, et al. Prospective evaluation of allogeneic hematopoietic stem-cell transplantation from matched related and matched unrelated donors in younger adults with high-risk acute myeloid leukemia: German–Austrian trial AMLHD98A. *J Clin Oncol*. 2010;28:4642–4648.
35. Walter RB, Pagel JM, Gooley TA, et al. Comparison of matched unrelated and matched related donor myeloablative hematopoietic cell transplantation for adults with acute myeloid leukemia in first remission. *Leukemia*. 2010;24:1276–1282.
36. Laughlin MJ, Eapen M, Rubinstein P, et al. Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. *N Engl J Med*. 2004;351:2265–2275.
37. Rocha V, Labopin M, Sanz G, et al. Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. *N Engl J Med*. 2004;351:2276–2285.
38. Eapen M, Rocha V, Sanz G, et al. Effect of graft source on unrelated donor haemopoietic stem-cell transplantation in adults with acute leukaemia: a retrospective analysis. *Lancet Oncol*. 2010;11:653–660.
39. Atsuta Y, Suzuki R, Nagamura-Inoue T, et al. Disease-specific analyses of unrelated cord blood transplantation compared with unrelated bone marrow transplantation in adult patients with acute leukemia. *Blood*. 2009;113:1631–1638.
40. Brunstein CG, Gutman JA, Weisdorf DJ, et al. Allogeneic hematopoietic cell transplantation for hematologic malignancy: relative risks and benefits of double umbilical cord blood. *Blood*. 2010;116:4693–4699.
41. Rio B, Chevret S, Vigouroux S, et al. Decreased nonrelapse mortality after unrelated cord blood transplantation for acute myeloid leukemia using reduced-intensity conditioning: a prospective phase II multicenter trial. *Biol Blood Marrow Transplant*. 2015;21:445–453.
42. Milano F, Appelbaum FR, Delaney C. Cord-blood transplantation in patients with minimal residual disease. *N Engl J Med*. 2016;375:2204–2205.
43. Horwitz ME, Cutler C, Stiff P, et al. 34—Improved clinical outcomes with omidubicel versus standard myeloablative umbilical cord blood transplantation: results of a phase III randomized, multicenter study. *Transplant Cell Ther*. 2021;27:S33–S34.
44. Aversa F. Haploidentical haematopoietic stem cell transplantation for acute leukaemia in adults: experience in Europe and the United States. *Bone Marrow Transplant*. 2008;41:473–481.
45. O'Donnell PV, Luznik L, Jones RJ, et al. Nonmyeloablative bone marrow transplantation from partially HLA-mismatched related donors using posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant*. 2002;8:377–386.

46. Luznik L, O'Donnell PV, Symons HJ, et al. HLA-haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose, posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant*. 2008;14:641–650.
47. Di Stasi A, Milton DR, Poon LM, et al. Similar transplantation outcomes for acute myeloid leukemia and myelodysplastic syndrome patients with haploidentical versus 10/10 human leukocyte antigen-matched unrelated and related donors. *Biol Blood Marrow Transplant*. 2014;20:1975–1981.
48. Ciurea SO, Zhang M-J, Bacigalupo AA, et al. Haploidentical transplant with posttransplant cyclophosphamide vs matched unrelated donor transplant for acute myeloid leukemia. *Blood*. 2015;126:1033–1040.
49. Rashidi A, Hamadani M, Zhang MJ, et al. Outcomes of haploidentical vs matched sibling transplantation for acute myeloid leukemia in first complete remission. *Blood Adv*. 2019;3:1826–1836.
50. Meybodi MA, Cao W, Luznik L, et al. HLA-haploidentical vs matched-sibling hematopoietic cell transplantation: a systematic review and meta-analysis. *Blood Adv*. 2019;3:2581–2585.
51. Mehta RS, Holtan SG, Wang T, et al. Composite GRFS and CRFS outcomes after adult alternative donor HCT. *J Clin Oncol*. 2020:JCO1900396.
52. Bolanos-Meade J, Reshef R, Fraser R, et al. Three prophylaxis regimens (tacrolimus, mycophenolate mofetil, and cyclophosphamide; tacrolimus, methotrexate, and bortezomib; or tacrolimus, methotrexate, and maraviroc) versus tacrolimus and methotrexate for prevention of graft-versus-host disease with haemopoietic cell transplantation with reduced-intensity conditioning: a randomised phase 2 trial with a non-randomised contemporaneous control group (BMT CTN 1203). *Lancet Haematol*. 2019;6:e132–e143.
53. De Jong CN, Meijer E, Bakunina K, et al. Post-Transplantation cyclophosphamide after allogeneic hematopoietic stem cell transplantation: results of the prospective randomized HOVON-96 trial in recipients of matched related and unrelated donors. *Blood*. 2019;134:1.
54. Duval M, Klein JP, He W, et al. Hematopoietic stem-cell transplantation for acute leukemia in relapse or primary induction failure. *J Clin Oncol*. 2010;28:3730–3738.
55. Romee R, Rosario M, Berrien-Elliott MM, et al. Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. *Sci Transl Med*. 2016;8:357ra123.
56. Locatelli F, Merli P, Pagliara D, et al. Outcome of children with acute leukemia given HLA-haploidentical HSCT after alphabeta T-cell and B-cell depletion. *Blood*. 2017;130:677–685.
57. Pierini A, Ruggeri L, Mancusi A, et al. T cell depletion and no post transplant immune suppression allow separation of graft versus leukemia from graft versus host disease. *Bone Marrow Transplant*. 2019;54:775–779.
58. Armand P, Kim HT, Cutler CS, et al. A prognostic score for patients with acute leukemia or myelodysplastic syndromes undergoing allogeneic stem cell transplantation. *Biol Blood Marrow Transplant*. 2008;14:28–35.
59. Litzow MR, Tarima S, Perez WS, et al. Allogeneic transplantation for therapy-related myelodysplastic syndrome and acute myeloid leukemia. *Blood*. 2010;115:1850–1857.
60. Lindsley RC, Mar BG, Mazzola E, et al. Acute myeloid leukemia ontogeny is defined by distinct somatic mutations. *Blood*. 2015;125:1367–1376.
61. National Cancer Institute: Surveillance, Epidemiology and End Results Program. 2021. Accessed March 31, 2021. www.seer.cancer.gov.
62. Genovese G, Kahler AK, Handsaker RE, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med*. 2014;371:2477–2487.
63. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med*. 2014;371:2488–2498.
64. McClune BL, Weisdorf DJ, Pedersen TL, et al. Effect of age on outcome of reduced-intensity hematopoietic cell transplantation for older patients with acute myeloid leukemia in first complete remission or with myelodysplastic syndrome. *J Clin Oncol*. 2010;28:1878–1887.
65. Koreth J, Aldridge J, Kim HT, et al. Reduced-intensity conditioning hematopoietic stem cell transplantation in patients over 60 years: hematologic malignancy outcomes are not impaired in advanced age. *Biol Blood Marrow Transplant*. 2010;16:792–800.
66. Farag SS, Maharry K, Zhang M-J, et al. Comparison of reduced-intensity hematopoietic cell transplantation with chemotherapy in patients age 60–70 years with acute myelogenous leukemia in first remission. *Biol Blood Marrow Transplant*. 2011;17:1796–1803.
67. Lazenby M, Gilkes AF, Marrin C, Evans A, Hills RK, Burnett AK. The prognostic relevance of flt3 and npm1 mutations on older patients treated intensively or non-intensively: a study of 1312 patients in the UK NCRI AML16 trial. *Leukemia*. 2014;28:1953–1959.
68. Ostronoff F, Othus M, Lazenby M, et al. Prognostic significance of NPM1 mutations in the absence of FLT3-internal tandem duplication in older patients with acute myeloid leukemia: a SWOG and UK National Cancer Research Institute/Medical Research Council report. *J Clin Oncol*. 2015;33:1157–1164.
69. Röllig C, Bornhäuser M, Kramer M, et al. Allogeneic stem-cell transplantation in patients with NPM1-mutated acute myeloid leukemia: results from a prospective donor versus no-donor analysis of patients after upfront HLA typing within the SAL-AML 2003 trial. *J Clin Oncol*. 2015;33:403–410.
70. Kayser S, Zucknick M, Döhner K, et al. Monosomal karyotype in adult acute myeloid leukemia: prognostic impact and outcome after different treatment strategies. *Blood*. 2012;119:551–558.
71. Kekre N, Koreth J. Novel strategies to prevent relapse after allogeneic haematopoietic stem cell transplantation for acute myeloid leukaemia and myelodysplastic syndromes. *Curr Opin Hematol*. 2015;22:116–122.
72. Saber W, Opie S, Rizzo JD, et al. Outcomes after matched unrelated donor versus identical sibling hematopoietic cell transplantation in adults with acute myelogenous leukemia. *Blood*. 2012;119:3908–3916.
73. Nishiyama Y, Saikawa Y, Nishiyama N. Interaction between the immune system and acute myeloid leukemia: a model incorporating promotion of regulatory T cell expansion by leukemic cells. *Biosystems*. 2018;165:99–105.
74. van den Brink M, Uhrberg M, Jahn L, et al. Selected biological issues affecting relapse after stem cell transplantation: role of T-cell impairment, NK cells and intrinsic tumor resistance. *Bone Marrow Transplant*. 2018;53:949–959.
75. Armand P. Immune checkpoint blockade in hematologic malignancies. *Blood*. 2015;125:3393–3400.
76. Christopher MJ, Petti AA, Rettig MP, et al. Immune escape of relapsed AML cells after allogeneic transplantation. *N Engl J Med*. 2018;379:2330–2341.
77. Jan M, Leventhal MJ, Morgan EA, et al. Recurrent genetic HLA loss in AML relapsed after matched unrelated allogeneic hematopoietic cell transplantation. *Blood Adv*. 2019;3:2199–2204.
78. Toffalori C, Zito L, Gambacorta V, et al. Immune signature drives leukemia escape and relapse after hematopoietic cell transplantation. *Nat Med*. 2019;25:603–611.
79. Vago L, Perna SK, Zanussi M, et al. Loss of mismatched HLA in leukemia after stem-cell transplantation. *N Engl J Med*. 2009;361:478–488.
80. Blazar BR, Carreno BM, Panoskaltsis-Mortari A, et al. Blockade of programmed death-1 engagement accelerates graft-versus-host disease lethality by an IFN-gamma-dependent mechanism. *J Immunol*. 2003;171:1272–1277.
81. Blazar BR, Taylor PA, Panoskaltsis-Mortari A, et al. Opposing roles of CD28:B7 and CTLA-4:B7 pathways in regulating in vivo alloresponses in murine recipients of MHC disparate T cells. *J Immunol*. 1999;162:6368–6377.
82. Boddu P, Kantarjian H, Garcia-Manero G, et al. The emerging role of immune checkpoint based approaches in AML and MDS. *Leuk Lymphoma*. 2018;59:790–802.
83. Brunner AM, Li S, Fathi AT, et al. Haematopoietic cell transplantation with and without sorafenib maintenance for patients with FLT3-ITD acute myeloid leukaemia in first complete remission. *Br J Haematol*. 2016;175:496–504.
84. Chen Y-B, Li S, Lane AA, et al. Phase I trial of maintenance sorafenib after allogeneic hematopoietic stem cell transplantation for fms-like tyrosine kinase 3 internal tandem duplication acute myeloid leukemia. *Biol Blood Marrow Transplant*. 2014;20:2042–2048.
85. de Lima M, Giralt S, Thall PF, et al. Maintenance therapy with low-dose azacitidine after allogeneic hematopoietic stem cell transplantation for recurrent acute myelogenous leukemia or myelodysplastic syndrome: a dose and schedule finding study. *Cancer*. 2010;116:5420–5431.
86. Burchert A, Bug G, Finke G, et al. Sorafenib as maintenance therapy post allogeneic stem-cell transplantation for FLT3-ITD–positive AML: results from the randomized, double-blind, placebo-controlled multicentre SORMAIN trial. *Blood*. 2018;132:661.
87. Maziarz RT, Patnaik MM, Scott BL, et al. A phase 2 randomized trial investigating standard of care ± midostaurin after allogeneic stem cell-transplant in FLT3-ITD–mutated AML. *Blood*. 2018;132:662.
88. Mathew NR, Baumgartner F, Braun L, et al. Sorafenib promotes graft-versus-leukemia activity in mice and humans through IL-15 production in FLT3-ITD-mutant leukemia cells. *Nat Med*. 2018;24:282–291.
89. Koreth J, Kim HT, Nikiforow S, et al. Donor chimerism early after reduced-intensity conditioning hematopoietic stem cell transplantation predicts relapse and survival. *Biol Blood Marrow Transplant*. 2014;20:1516–1521.
90. Reshef R, Hexner EO, Loren AW, et al. Early donor chimerism levels predict relapse and survival after allogeneic stem cell transplantation with reduced-intensity conditioning. *Biol Blood Marrow Transplant*. 2014;20:1758–1766.

91. Porter DL, Alyea EP, Antin JH, et al. NCI first international workshop on the biology, prevention, and treatment of relapse after allogeneic hematopoietic stem cell transplantation: report from the committee on treatment of relapse after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2010;16:1467–1503.
92. Schmid C, Labopin M, Nagler A, et al. Donor lymphocyte infusion in the treatment of first hematological relapse after allogeneic stem-cell transplantation in adults with acute myeloid leukemia: a retrospective risk factors analysis and comparison with other strategies by the EBMT acute leukemia working party. *J Clin Oncol*. 2007;25:4938–4945.
93. Nikiforow S, Kim HT, Daley H, et al. A phase I study of CD25/regulatory T-cell-depleted donor lymphocyte infusion for relapse after allogeneic stem cell transplantation. *Haematologica*. 2016;101:1251–1259.
94. Davids MS, Kim HT, Bachireddy P, et al. Ipilimumab for patients with relapse after allogeneic transplantation. *N Engl J Med*. 2016;375:143–153.
95. Eapen M, Giralt SA, Horowitz MM, et al. Second transplant for acute and chronic leukemia relapsing after first HLA-identical sibling transplant. *Bone Marrow Transplant*. 2004;34:721–727.
96. Cazzola M. Myelodysplastic syndromes. *N Engl J Med*. 2020;383:1358–1374.
97. Aul C, Germing U, Gattermann N, Minning H. Increasing incidence of myelodysplastic syndromes: real or fictitious? *Leuk Res*. 1998;22:93–100.
98. Goldberg SL, Chen E, Corral M, et al. Incidence and clinical complications of myelodysplastic syndromes among United States Medicare beneficiaries. *J Clin Oncol*. 2010;28:2847–2852.
99. Ma X, Does M, Raza A, Mayne ST. Myelodysplastic syndromes: incidence and survival in the United States. *Cancer*. 2007;109:1536–1542.
100. Fenaux P, Platzbecker U, Mufti GJ, et al. Luspatercept in patients with lower-risk myelodysplastic syndromes. *N Engl J Med*. 2020;382:140–151.
101. Garcia JS. Prospects for venetoclax in myelodysplastic syndromes. *Hematol Oncol Clin North Am*. 2020;34:441–448.
102. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, et al. Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. *Lancet Oncol*. 2009;10:223–232.
103. Phelan RA, Chen M. Current use and outcome of hematopoietic stem cell transplantation: CIBMTR US summary slides. 2020. https://www.cibmtr.org/ReferenceCenter/SlidesReports/SummarySlides/pages/index.aspx.
104. Robin M, Porcher R, Adès L, et al. HLA-matched allogeneic stem cell transplantation improves outcome of higher risk myelodysplastic syndrome A prospective study on behalf of SFGM-TC and GFM. *Leukemia*. 2015;29:1496–1501.
105. Kroeger N, Sockel K, Wolschke C, et al. Prospective multicenter phase 3 study comparing 5-azacytidine (5-Aza) induction followed by allogeneic stem cell transplantation versus continuous 5-aza according to donor availability in elderly MDS patients (55–70 years) (vidazaallo study). *Blood*. 2018;132:208.
106. Abel GA, Kim HT, Hantel A, et al. Fit older adults with advanced myelodysplastic syndromes: who is most likely to benefit from transplant? *Leukemia*. 2021;35(4):1223.
107. Nakamura R, Saber W, Martens MJ, et al. Biologic assignment trial of reduced-intensity hematopoietic cell transplantation based on donor availability in patients 50–75 years of age with advanced myelodysplastic syndrome. *J Clin Oncol*. 2021;39(30):3328–3339.
108. Lim Z, Brand R, Martino R, et al. Allogeneic hematopoietic stem-cell transplantation for patients 50 years or older with myelodysplastic syndromes or secondary acute myeloid leukemia. *J Clin Oncol*. 2010;28:405–411.
109. Eapen M, Brazauskas R, Hemmer M, et al. Hematopoietic cell transplant for acute myeloid leukemia and myelodysplastic syndrome: conditioning regimen intensity. *Blood Adv*. 2018;2:2095–2103.
110. Bejanyan N, Zhang M, Bo-Subait K, et al. Myeloablative conditioning for allogeneic transplantation results in superior disease-free survival for acute myelogenous leukemia and myelodysplastic syndromes with low/intermediate but not high disease risk index: a Center for International Blood and Marrow Transplant Research study. *Transplant Cell Ther*. 2021;27(1):68.e1-68.e9.
111. Kroger N, Iacobelli S, Franke GN, et al. Dose-reduced versus standard conditioning followed by allogeneic stem-cell transplantation for patients with myelodysplastic syndrome: a prospective randomized phase III study of the EBMT (RICMAC Trial). *J Clin Oncol*. 2017;35:2157–2164.
112. Greenberg P, Cox C, LeBeau MM, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. *Blood*. 1997;89:2079–2088.
113. Lee J-H, Lee J-H, Lim S-N, et al. Allogeneic hematopoietic cell transplantation for myelodysplastic syndrome: prognostic significance of pre-transplant IPSS score and comorbidity. *Bone Marrow Transplant*. 2010;45:450–457.
114. Cutler CS, Lee SJ, Greenberg P, et al. A decision analysis of allogeneic bone marrow transplantation for the myelodysplastic syndromes: delayed transplantation for low-risk myelodysplasia is associated with improved outcome. *Blood*. 2004;104:579–585.
115. Greenberg PL, Tuechler H, Schanz J, et al. Revised international prognostic scoring system for myelodysplastic syndromes. *Blood*. 2012;120:2454–2465.
116. Della Porta MG, Alessandrino EP, Bacigalupo A, et al. Predictive factors for the outcome of allogeneic transplantation in patients with MDS stratified according to the revised IPSS-R. *Blood*. 2014;123:2333–2342.
117. Koreth J, Pidala J, Perez WS, et al. A decision analysis of reduced-intensity conditioning allogeneic hematopoietic stem cell transplantation for older patients with de-novo myelodysplastic syndrome (MDS): early transplantation offers survival benefit in higher-risk MDS. *ASH Annual Meeting Abstracts*. 2011;118:115.
118. Alessandrino EP, Della Porta MG, Malcovati L, et al. Optimal timing of allogeneic hematopoietic stem cell transplantation in patients with myelodysplastic syndrome. *Am J Hematol*. 2013;88:581–588.
119. Della Porta MG, Jackson CH, Alessandrino EP, et al. Decision analysis of allogeneic hematopoietic stem cell transplantation for patients with myelodysplastic syndrome stratified according to the revised international prognostic scoring system. *Leukemia*. 2017;31:2449–2457.
120. Garcia-Manero G, Shan J, Faderl S, et al. A prognostic score for patients with lower risk myelodysplastic syndrome. *Leukemia*. 2008;22:538–543.
121. Platzbecker U, Bornhäuser M, Germing U, et al. Red blood cell transfusion dependence and outcome after allogeneic peripheral blood stem cell transplantation in patients with de novo myelodysplastic syndrome (MDS). *Biol Blood Marrow Transplant*. 2008;14:1217–1225.
122. Alessandrino EP, Della Porta MG, Bacigalupo A, et al. WHO classification and WPSS predict posttransplantation outcome in patients with myelodysplastic syndrome: a study from the Gruppo Italiano Trapianto di Midollo Osseo (GITMO). *Blood*. 2008;112:895–902.
123. Malcovati L, Della Porta MG, Pascutto C, et al. Prognostic factors and life expectancy in myelodysplastic syndromes classified according to WHO criteria: a basis for clinical decision making. *J Clin Oncol*. 2005;23:7594–7603.
124. Bejar R, Stevenson K, Abdel-Wahab O, et al. Clinical effect of point mutations in myelodysplastic syndromes. *N Engl J Med*. 2011;364:2496–2506.
125. Bejar R, Stevenson KE, Caughey B, et al. Somatic mutations predict poor outcome in patients with myelodysplastic syndrome after hematopoietic stem-cell transplantation. *J Clin Oncol*. 2014;32:2691–2698.
126. Yoshizato T, Nannya Y, Atsuta Y, et al. Genetic abnormalities in myelodysplasia and secondary acute myeloid leukemia: impact on outcome of stem cell transplantation. *Blood*. 2017;129:2347–2358.
127. Dillon L, Gui G, Logan BR, et al. Impact of conditioning intensity and genomics on relapse after allogeneic transplantation for patients with myelodysplastic syndrome. *JCO Precis Oncol*. 2021;5:265–274.
128. Lindsley RC, Saber W, Mar BG, et al. Prognostic mutations in myelodysplastic syndrome after stem-cell transplantation. *N Engl J Med*. 2017;376:536–547.
129. Bernard E, Nannya Y, Hasserjian RP, et al. Implications of TP53 allelic state for genome stability, clinical presentation and outcomes in myelodysplastic syndromes. *Nat Med*. 2020;26:1549–1556.
130. Aldoss I, Pham A, Li SM, et al. Favorable impact of allogeneic stem cell transplantation in patients with therapy-related myelodysplasia regardless of TP53 mutational status. *Haematologica*. 2017;102:2030–2038.
131. de Witte T, Bowen D, Robin M, et al. Allogeneic hematopoietic stem cell transplantation for MDS and CMML: recommendations from an international expert panel. *Blood*. 2017;129:1753–1762.
132. Warlick ED, Cioc A, DeFor T, et al. Allogeneic stem cell transplantation for adults with myelodysplastic syndromes: importance of pretransplant disease burden. *Biol Blood Marrow Transplant*. 2009;15:30–38.
133. Nakai K, Kanda Y, Fukuhara S, et al. Value of chemotherapy before allogeneic hematopoietic stem cell transplantation from an HLA-identical sibling donor for myelodysplastic syndrome. *Leukemia*. 2005;19:396–401.
134. Damaj G, Duhamel A, Robin M, et al. Impact of azacitidine before allogeneic stem-cell transplantation for myelodysplastic syndromes: a study by the Societe Francaise de Greffe de Moelle et de Therapie-Cellulaire and the Groupe-Francophone des Myelodysplasies. *J Clin Oncol*. 2012;30:4533–4540.
135. Field T, Perkins J, Huang Y, et al. 5-Azacitidine for myelodysplasia before allogeneic hematopoietic cell transplantation. *Bone Marrow Transplant*. 2010;45:255–260.
136. Gerds AT, Gooley TA, Estey EH, et al. Pretransplantation therapy with azacitidine vs induction chemotherapy and posttransplantation outcome in patients with MDS. *Biol Blood Marrow Transplant*. 2012;18:1211–1218.
137. Schroeder T, Wegener N, Lauseker M, et al. Comparison between upfront transplantation and different pretransplant cytoreductive treatment approaches in patients with high-risk myelodysplastic syndrome and

secondary acute myelogenous leukemia. *Biol Blood Marrow Transplant*. 2019;25:1550–1559.
138. Potter VT, Iacobelli S, van Biezen A, et al. Comparison of intensive chemotherapy and hypomethylating agents before allogeneic stem cell transplantation for advanced myelodysplastic syndromes: a study of the myelodysplastic syndrome subcommittee of the Chronic Malignancies Working Party of the European Society for Blood and Marrow Transplant Research. *Biol Blood Marrow Transplant*. 2016;22:1615–1620.
139. Roman-Gomez J, Jimenez-Velasco A, Agirre X, et al. Epigenetic regulation of human cancer/testis antigen gene, HAGE, in chronic myeloid leukemia. *Haematologica*. 2007;92:153–162.
140. Almstedt M, Blagitko-Dorfs N, Duque-Afonso J, et al. The DNA demethylating agent 5-aza-2'-deoxycytidine induces expression of NY-ESO-1 and other cancer/testis antigens in myeloid leukemia cells. *Leukemia Res*. 2010;34:899–905.
141. Liu Y, Kuick R, Hanash S, Richardson B. DNA methylation inhibition increases T cell KIR expression through effects on both promoter methylation and transcription factors. *Clin Immunol*. 2009;130:213–224.
142. Campoli M, Ferrone S. HLA antigen changes in malignant cells: epigenetic mechanisms and biologic significance. *Oncogene*. 2008;27:5869–5885.
143. Hambach L, Ling K-W, Pool J, et al. Hypomethylating drugs convert HA-1-negative solid tumors into targets for stem cell-based immunotherapy. *Blood*. 2009;113:2715–2722.
144. Oran B, de Lima M, Garcia-Manero G, et al. A phase 3 randomized study of 5-azacitidine maintenance vs observation after transplant in high-risk AML and MDS patients. *Blood Adv*. 2020;4:5580–5588.
145. Muffly L, Pasquini MC, Martens M, et al. Increasing use of allogeneic hematopoietic cell transplantation in patients aged 70 years and older in the United States. *Blood*. 2017;130:1156–1164.
146. Atallah E, Logan B, Chen M, et al. Comparison of patient age groups in transplantation for myelodysplastic syndrome: the Medicare Coverage with Evidence Development Study. *JAMA Oncol*. 2020;6:486–493.
147. Sorror ML, Sandmaier BM, Storer BE, et al. Comorbidity and disease status based risk stratification of outcomes among patients with acute myeloid leukemia or myelodysplasia receiving allogeneic hematopoietic cell transplantation. *J Clin Oncol*. 2007;25:4246–4254.
148. Sorror ML, Maris MB, Storb R, et al. Hematopoietic cell transplantation (HCT)-specific comorbidity index: a new tool for risk assessment before allogeneic HCT. *Blood*. 2005;106:2912–2919.
149. Zipperer E, Pelz D, Nachtkamp K, et al. The hematopoietic stem cell transplantation comorbidity index is of prognostic relevance for patients with myelodysplastic syndrome. *Haematologica*. 2009;94:729–732.
150. Sorror ML, Storb RF, Sandmaier BM, et al. Comorbidity-age index: a clinical measure of biologic age before allogeneic hematopoietic cell transplantation. *J Clin Oncol*. 2014;32:3249–3256.
151. Armand P, Kim HT, Cutler CS, et al. Prognostic impact of elevated pretransplantation serum ferritin in patients undergoing myeloablative stem cell transplantation. *Blood*. 2007;109:4586–4588.
152. Pullarkat V, Blanchard S, Tegtmeier B, et al. Iron overload adversely affects outcome of allogeneic hematopoietic cell transplantation. *Bone Marrow Transplant*. 2008;42:799–805.
153. Lim ZY, Fiaccadori V, Gandhi S, et al. Impact of pre-transplant serum ferritin on outcomes of patients with myelodysplastic syndromes or secondary acute myeloid leukaemia receiving reduced intensity conditioning allogeneic haematopoietic stem cell transplantation. *Leukemia Res*. 2010;34:723–727.
154. Popat UR, Saliba RM, Szewczyk N, et al. Enhanced recovery program in older patients undergoing allogeneic stem cell transplantation (ER-SCT): first year outcomes. *Biol Blood Marrow Transplant*. 2020;26:S49–S50.
155. Oran B, Woo Ahn K, Fretham C, et al. Fludarabine and melphalan compared with reduced doses of busulfan and flurabine improves transplant outcomes in older MDS patients. *Blood*. 2019;134:253.
156. Myllymäki M, Redd R, Reilly CR, et al. Short telomere length predicts nonrelapse mortality after stem cell transplantation for myelodysplastic syndrome. *Blood*. 2020;136:3070–3081.
157. Saber W, Cutler CS, Nakamura R, et al. Impact of donor source on hematopoietic cell transplantation outcomes for patients with myelodysplastic syndromes (MDS). *Blood*. 2013;122:1974–1982.
158. Robin M, Porcher R, Ruggeri A, et al. HLA-mismatched donors in patients with myelodysplastic syndrome: an EBMT registry analysis. *Biol Blood Marrow Transplant*. 2019;25:114–120.
159. Gerds AT, Woo Ahn K, Hu ZH, et al. Outcomes after umbilical cord blood transplantation for myelodysplastic syndromes. *Biol Blood Marrow Transplant*. 2017;23:971–979.
160. Robin M, Ruggeri A, Labopin M, et al. Comparison of unrelated cord blood and peripheral blood stem cell transplantation in adults with myelodysplastic syndrome after reduced-intensity conditioning regimen: a collaborative study from Eurocord (Cord blood Committee of Cellular Therapy & Immunobiology Working Party of EBMT) and chronic malignancies working party. *Biol Blood Marrow Transplant*. 2015;21:489–495.
161. Bashey A, Zhang X, Sizemore CA, et al. T-cell-replete HLA-haploidentical hematopoietic transplantation for hematologic malignancies using post-transplantation cyclophosphamide results in outcomes equivalent to those of contemporaneous HLA-matched related and unrelated donor transplantation. *J Clin Oncol*. 2013;31:1310–1316.
162. Bashey ZA, Zhang X, Brown S, et al. Comparison of outcomes following transplantation with T-replete HLA-haploidentical donors using post-transplant cyclophosphamide to matched related and unrelated donors for patients with AML and MDS aged 60 years or older. *Bone Marrow Transplant*. 2018;53:756–763.
163. Schroeder T, Rautenberg C, Krüger W, et al. Treatment of relapsed AML and MDS after allogeneic stem cell transplantation with decitabine and DLI-a retrospective multicenter analysis on behalf of the German Cooperative Transplant Study Group. *Ann Hematol*. 2018;97:335–342.
164. Garcia JS, Kim HT, Brock J, et al. A phase 1 dose-escalation study of adding venetoclax to a reduced intensity conditioning (RIC) regimen prior to allogeneic hematopoietic cell transplantation for patients with high risk myeloid malignancies. *Blood*. 2019;134:258.

CHAPTER 63

ACUTE MYELOID LEUKEMIA IN CHILDREN

C. Michel Zwaan, Olaf Heidenreich, and E. Anders Kolb

Acute myeloid leukemia (AML) is a complex and heterogeneous group of malignancies in which genetic and epigenetic alterations lead to differentiation arrest and/or uncontrolled expansion of myeloid cell precursors. Pediatric AML is characterized by a wide array of genetic aberrations that are often not seen or differ in frequency when compared to AML diagnosed in adults and mainly comprise different fusion genes.[1,2] Though the mutational landscape of childhood AML is now well-described,[2] and despite the recent approvals of various targeted compounds for use in adults with AML, there are no new targeted therapies approved for use in children.[3] This is due to several factors, such as delays to implement clinical studies in children, relevance of some targets in children, and/or class-related toxicity concerns (e.g., Hedgehog pathway inhibitors that may induce skeletal toxicity).

Improvements in survival have been largely achieved through the successful intensification of broadly active chemotherapy (cytarabine and anthracyclines), the effective use of hematopoietic stem cell transplantation (HSCT), improved methods for risk stratification including early response assessments, advanced diagnostics to detect genetic aberrations, improvements in supportive care, and increasing retrieval rates at relapse.[4,5]

Overall survival (OS) rates for children with AML who are treated on contemporary clinical trials are now greater than 70% overall, and for some genotypes, OS rates are above 80%.[5] However event-free survival (EFS) rates still fall behind, ranging from 50% to 65% (summarized in Table 63.1),[1] and the OS for other genotypes are unacceptably low despite maximal intensification of standard chemotherapy. Genomic and biologic insights into the mechanisms of leukemogenesis have both helped to identify patients a priori who will have a poor prognosis, and may potentially benefit from an HSCT as well, and identified potential targets for new therapies. Survivors of childhood AML are at significant risk for serious long-term side effects including cardiomyopathy, infertility, and second malignancies.

Future advances in childhood AML must include a better understanding of whether targeted therapies approved in older adults can be effective in children with AML by determining the dependency of childhood leukemia cells on targetable oncogene products and understanding the compensatory mechanism of resistance to conventional and targeted therapies. This should lead to higher cure rates with less long-term toxicity.

EPIDEMIOLOGY

AML accounts for approximately 20% of cases of acute leukemia in children and adolescents younger than 20 years of age. The incidence rates have remained constant over the past 40 years, are similar between boys and girls, and are highest during the first 2 years of life before the incidence rises again in adolescents/young adults. However, the age distribution may vary between subtypes. For example, acute promyelocytic leukemia (APL) and core-binding factor (CBF) leukemia are rare in children younger than 3 years of age, whereas the incidence of acute megakaryoblastic leukemia (AMKL) and "myeloid leukemia of Down syndrome" (ML DS) is highest in infants and young children, yet rare among teenagers. The distribution of AML subtypes may also vary among ethnic groups, with some studies suggesting a higher incidence of APL among Hispanic/Mediterranean populations.

Constitutional syndromes that are associated with an increased predisposition to AML include Down syndrome (DS), Fanconi anemia, Bloom syndrome, neurofibromatosis, Noonan syndrome, congenital neutropenia, and germline haploinsufficiency of the specific oncogenes (e.g., the *Runt-related transcription factor-1* or *RUNX1* gene),[6] though these constitute only a small subset of AML diagnosed in children. The World Health Organization (WHO) 2016 classification also provides a listing of myeloid malignancies that can develop in patients with germline predispositions (Table 63.2).[7] Exposure to ionizing radiation, alkylating agents, topoisomerase II inhibitors, and benzene are among the few environmental factors proven to increase the risk of AML. In the majority of cases of childhood AML, neither a genetic nor environmental cause can be identified.

PATHOBIOLOGY

The French–American–British (FAB) classification system uses morphologic and histochemical features of the leukemic cells to sub-classify AML, though with limited prognostic and therapeutic significance.[8] The WHO 2016 classification system (see Table 63.2) uses specific cytogenetic alterations and molecular genetic lesions to classify AML.[7] Recent genomic approaches have validated the distinct genomic heterogeneity within AML.[2] In addition, these approaches have identified new genetic subtypes as well as lesions that are enriched within leukemias that have normal karyotypes, that is, genetic changes not identifiably by standard karyotype analysis or miscellaneous chromosomal alterations (Fig. 63.1). This chapter discusses many of the known molecular subtypes and their associated prognosis.

Recent DNA and RNA sequencing efforts have significantly expanded the list of molecular lesions that underlie AML.[2,9–11] Pediatric AML is characterized by very few somatic mutations per cell, though there are many distinct mutations described. Data support models for childhood AML where single and catastrophic translocation events, that result in the generation of an entirely novel gene product such as *RUNX1/RUNX1T1*, initiate leukemogenesis in early hematopoietic stem and progenitor cells. Additional acquired leukemogenic mutational "hits" ultimately lead to a malignant transformation. Common initiating mutations described for adults with AML include *isocitrate dehydrogenase1/2* (*IDH1/2*) and *DNA methyltransferase 3 alpha* (*DNMT3A*) that alter gene function. In contrast to chromosomal rearrangements, mutations in these genes are exceedingly rare in children. There may also be a developmental context that mediates the potency of these chromosomal alterations and their requirement for secondary mutations.[12] Hematopoietic progenitor cells in children are fundamentally different than those of older adults. Because of their much higher proliferative and self-renewal capacity, the fetal, infant and pediatric hematopoietic stem and progenitor cell compartment provides a much more supportive environment for initiating genetic lesions than the adult one. It should be no surprise that stem cells within different developmental phases will require different oncogenic events needed for a malignant transformation.

It is useful to conceptualize mutations in AML as falling into two broad classes: class I mutations confer a proliferative or survival advantage; and class II mutations block differentiation and promote self-renewal.[13] In general, class I and class II mutations frequently cooperate in leukemogenesis. Interestingly, many of the class II mutations arise from translocation events that lead to chimeric transcription factors with oncogenic properties. Moreover, the relationship

TABLE 63.1 Results of Recent Clinical Trials for Pediatric Acute Myeloid Leukemia

Type of AML	Study	Years of Enrollment	Eligible Age (Years)	Number of Patients	CR (%)	EFS (%) and (Median Follow-Up)	OS (%) and (Median Follow-Up)	Reference
AML	AIEOP AML 2002/01	2002–2011	≤18	482	87	55 (8-year)	68 (8-year)	64
	AML-BFM 2004	2004–2010	<18	611 (521 randomized)	89	All: 55 (5-year); L-DNR: 59; Ida: 53	All: 74 (5-year); L-DNR: 76; Ida: 75	65
	AML-BFM 2012 registry	2012–2017	<18	324	90	62 (5-year)	80 (5-year)	47
	COG AAML0531	2006–2010	<30	1022	87	No GO 46.9%; GO 53.1% (3-year)	No GO 65.4%; GO 69.4% (3-year)	46
	COG AAML 1031	2011–2016	<30	1097	90	No Bort: 45; Bort: 47 (3-year)	No Bort: 64; Bort 67 (3-year)	66
	JPLSG AML05	2006–2010	≤18	443		54 (3-year)	73 (3-year)	67–69
	JACLS	2003–2006	<18	146	96	67 (5-year)	78 (5-year)	70
	MRC AML12	1995–2002	<16	529	92	54 (10-year)	63 (10-year)	71
	NOPHO AML 2004	2004–2009	≤18	151	92	57 (3-years)	69 (3-years)	72,73
	SJCRH AML08	2008–2017	≤21	285	92	Clo-AraC 53 & HD-ADE 52 (3-year)	Clo-AraC 75 & HD-ADE 65 (3-year)	74
	DB AML01	2010–2014	<17	112	94	53 (3-year)	74 (3-year)	75
	ELAM02	2005–2011	<18	438	89	57 (4-years)	73 (4-years)	76
ML DS	AML D05 study	2008–2010	<18	72	96	83 (3-year)	88 (3-year)	77
	ML DS 2006	2007–2015	<4	170		87 (5-year)	89 (5-year)	35
	COG AAML 0431	2007–2011	<4	204		90 (5-year)	93 (5-year)	26
TMD	COG A2971	1999–2004	neonates	135		57 (3-year)	77 (3-year)	78
	TMD prevention 2007 study	2007–2015	neonates	102		72 (5-year)	91 (5-year)	79
APL	AML P05 study (ATRA/chemo)	2006–2011	<18	43	86	84 (3-year)	91 (3-year)	80
	AML-BFM 93/-98/-2004 (ATRA/chemo)	1993–2007	<18	81	93	73 (5-year)	89 (5-year)	81
	AML-BFM Standard-Risk APL (ATRA/ATO)	2013–2016	<18	11	100	100 (2.4-year)	100 (2.4-year)	82
	ICC-APL-01 (ATRA/chemo)	2008–2015	<21	258	97	80 (5-year)	95 (5-year)	20
	GIMEMA/AIEOP AIDA 2000 ATRA/chemo)	2000–2008	<18	127	96	85 (10-year)	94 (10-year)	20
	CALGB 9710 (ATRA/chemo/ATO)	1999–2005	<18	83	83	54 (5-year)	82 (5-year)	83
	COG AAML 0631 (ATRA/chemo/ATO)	2009–2012	<22	101		91 (3-year)	94 (3-year)	21

AML, Acute myeloid leukemia; *APL*, acute promyelocytic leukemia; *AIEOP*, Associazione Italiana di Ematologia e Oncologia Pediatrica; *ATO*, arsenic trioxide; *ATRA*, all-trans-retinoic acid; *BFM*, Berlin–Frankfurt–Münster Study Group; *Bort*, bortezomib arm; *CALGB*, Cancer and Leukemia Group B; *COG*, Children's Oncology Group; *CR*, complete remission; *DB*, Dutch Belgian; *EFS*, event-free survival; *ELAM*, Enfants Leucémie Aiguë Myéloïde; *ICC-APL*, International Consortium for Childhood APL; *Ida*, idarubicin arm; *JACLS*, Japan Association of Childhood Leukemia Study; *JPLSG*, Japanese Pediatric Leukemia/Lymphoma Study Group; *L-DNR*, liposomal daunorubicin arm; *ML DS*, myeloid leukemia of Down syndrome; *MRC*, Medical Research Council; *NOPHO*, Nordic Society of Paediatric Haematology and Oncology; *OS*, overall survival; *SJCRH*, St Jude Children's Research Hospital; *TMD*, transient myeloproliferative disease.

TABLE 63.2 **The 2016 World Health Organization Classification of Acute Myeloid Leukemia and Related Neoplasms**

WHO 2016 Classification	Classification of Myeloid Neoplasms With Germline Predisposition
Acute myeloid leukemia with recurrent genetic abnormalities AML with t(8;21)(q22;q22); RUNX1-RUNX1T1 AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11 APL with t(15;17)(q22;q12); PML-RARA AML with t(9;11)(p22;q23); KMT2A-KMT2AT3 AML with t(6;9)(p23;q34); DEK-NUP214 AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1 AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1 AML with mutated NPM1 AML with mutated CEBPA *Provisional entity*: AML with BCR-ABL1 *Provisional entity*: AML with mutated RUNX1 Acute myeloid leukemia with myelodysplasia-related changes Therapy-related myeloid neoplasms Acute myeloid leukemia, not otherwise specified AML with minimal differentiation AML without maturation AML with maturation Acute myelomonocytic leukemia Acute monoblastic/monocytic leukemia Pure erythroid leukemia Acute megakaryoblastic leukemia Acute basophilic leukemia Acute panmyelosis with myelofibrosis Myeloid sarcoma Myeloid proliferations related to Down syndrome Transient abnormal myelopoiesis Myeloid leukemia associated with Down syndrome	Myeloid neoplasms with germline predisposition without a pre-existing disorder or organ dysfunction AML with germline *CEBPA* mutation Myeloid neoplasms with germline *DDX41* mutation Myeloid neoplasms with germline predisposition and pre-existing platelet disorders Myeloid neoplasms with germline RUNX1 mutation Myeloid neoplasms with germline ANKRD26 mutation Myeloid neoplasms with germline ETV6 mutation Myeloid neoplasms with germline predisposition and other organ dysfunction Myeloid neoplasms with germline GATA2 mutation Myeloid neoplasms associated with bone marrow failure syndromes Myeloid neoplasms associated with telomere biology disorders Myeloid neoplasms associated with Down syndrome

Modified from Arber DA. The 2016 WHO classification of acute myeloid leukemia: what the practicing clinician needs to know. *Semin Hematol.* 2019;56:90–95.
AML, Acute myeloid leukemia; *APL*, acute promyelocytic leukemia; *NMP1*, nucleophosmin 1; *WHO*, World Health Organization.

between these class I and class II mutations is non-random, for example, *KIT* (class I) mutations are mostly found in CBF AML (class II), and *fms-like tyrosine kinase 3* (*FLT3*) mutations (class I) are mostly found in AML with *nucleoporin 98* (*NUP98*) fusions (class II) or *nucleophosmin* (*NPM1*) mutations (class II). However, although more amenable to conventional drug design than class II mutations, class I mutations are generally secondary events and, thus, frequently subclonal.

In this chapter, we discuss the major subtypes of pediatric AML, our current understanding of the biologic processes that mediate disease, and how these major subtypes may impact survival following conventional therapy. The following section highlights some of the major genetic driver events in leukemogenesis in children. Chapter 59 provides a more detailed overview of the pathobiology of AML with a focus on genetics in adult AML.

Fusion Genes Resulting From Chromosomal Rearrangements

About 60% of all pediatric AML cases are associated with six genetic loci targeting five different protein complexes: the transcription factor CBF with 21q22 (*RUNX1*) and 16q22 (*CBFB*) rearrangements, the epigenetic modifier KMT2A with 11q23 (*KMT2A*, previously known as *MLL*) translocations, the transcription factor retinoic acid receptor alpha with 17q21 (*RARA*) translocations, and the nuclear pore component nucleoporin 98 and 96 precursor with 11p15 (*NUP98*).

Chromosomal Rearrangements Affecting Core-Binding Factor

The CBF transcription complex is a heterodimeric complex composed of a DNA-binding RUNX member (either RUNX1, 2, or 3) and CBFβ. The RUNX1:CBFβ complex functions as a master regulator of the definitive hematopoiesis. Mutations in the genes encoding the RUNX1/CBFβ transcription factor complex are one of the most common lesions seen in de novo AML, occurring in approximately 25% of cases.[2]

The translocation t(8;21)(q22;q22) is the most common translocation found in pediatric AML and fuses the DNA-binding domain of RUNX1 to almost the entire coding region of RUNX1T1. The closely related translocation t(16;21) (q24;q22) fuses *RUNX1* to *CBF2A3*, a paralog of *RUNX1T1*. Following the discovery of RUNX1, the inv(16)(p13;q22) and the less common t(16;16)(p13;q22) result in a fusion between CBFβ and MYH11, frequently seen in acute monoblastic leukemias with eosinophilia (FAB-M4Eo). The CBFβ/MYH11 fusion protein retains the RUNX1 binding domain and therefore its ability to interact with wild-type RUNX1. Both fusion proteins interfere with normal RUNX1 functions but exert also RUNX1-independent functions. In particular, RUNX1/RUNX1T1 can initiate leukemogenesis but requires secondary mutations for establishing AML. Consistent with this, *RUNX1/RUNX1T1* expressing progenitors can persist in the bone marrow for years without expanding into overt leukemia.[14] Nevertheless, AMLs are addicted to these fusion proteins and their transcriptional programs.[15] Cooperating mutations include both classic class I mutations including *FLT3*, *KIT*, and *RAS* members and epigenetic regulators such as *ASXL1* and 2 or *EZH2*.

Patients with CBF mutations tend to have an improved survival with conventional therapy and even improved survival following relapse, although this is more apparent for inv(16) than for t(8;21) rearranged cases.[16] As a result, in some collaborative group protocols, patients with CBF AML receive fewer courses of intensive chemotherapy.

KMT2A Gene Rearrangements

The *KMT2A* gene, located on chromosome 11 band q23, encodes the MLL histone methylase. Upon proteolytic cleavage, it is part of a multiprotein complex involved in chromatin remodeling. Knockout of *KMT2A* inhibits myeloid and lymphoid differentiation and rapidly leads to bone marrow failure in mice. These results suggest that the MLL protein is not only required for definitive hematopoiesis but is also required for maintenance of HSCs in postnatal hematopoiesis.

Chromosomal translocations involving the *KMT2A* gene are associated with both AML and ALL leukemias. There are more

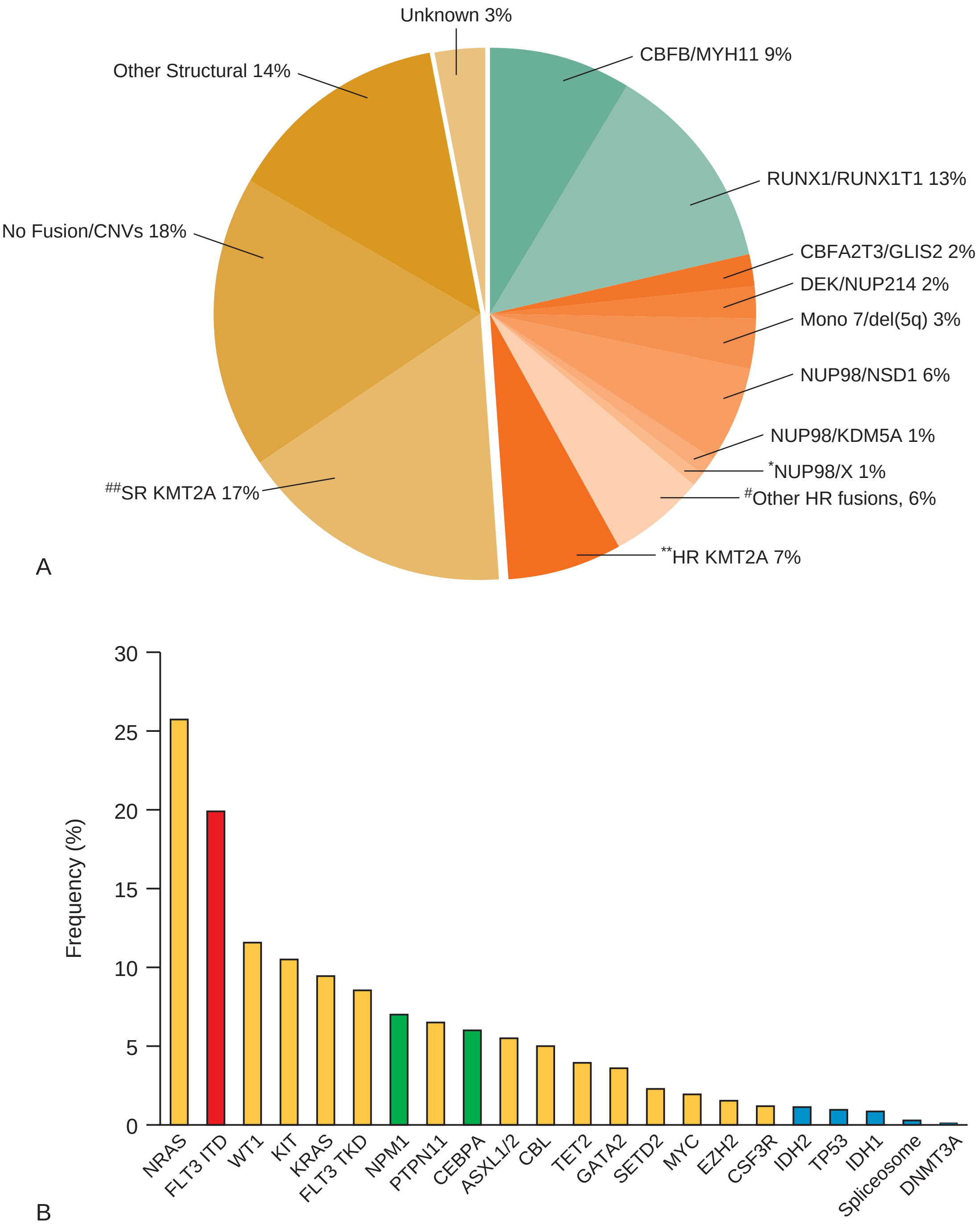


Figure 63.1 FREQUENCIES OF RECURRENT GENETIC LESIONS IN CHILDHOOD ACUTE MYELOID LEUKEMIA (AML). DNA and RNA sequencing results on 2273 children with AML treated on Children's Oncology Group Phase 3 trials from 2006 to 2018 reveal the distribution of gene fusion events (A) and small insertions and deletions (B). *Continued.*

than 100 different fusion partners identified, although nine specific genes account for more than 90% of the fusion events.[17] *KMT2A* rearrangements are found in 60% to 70% of infants with leukemia (predominantly *KMT2A/AFF1*), regardless of immunophenotype, but are less common in older children and adults. The distribution of translocation partners varies depending on the age of the patient and the immunophenotype of leukemia, with *KMT2A/MLLT10* being more frequent in infant and pediatric AML. The majority of active KMT2A fusion proteins contain the N-terminus of KMT2A and the C-terminus of the fusion partner gene, while the function of reciprocal fusion proteins harboring the KMT2A C-terminus are not well understood. The prognostic significance of *KMT2A* mutations is often dictated by the fusion partner and can range from favorable to highly unfavorable.[18] Next-generation sequencing of *KMT2A*-rearranged leukemias demonstrated a paucity of cooperating mutations, although approximately 50% carry an activating tyrosine kinase mutation.[19] However, those mutations are often absent at relapse, confirming they may represent secondary events in irrelevant subclones. *KMT2A* fusion genes alone are sufficient for leukemogenesis, and the targeting of cooperating mutations may not result in a therapeutic benefit.

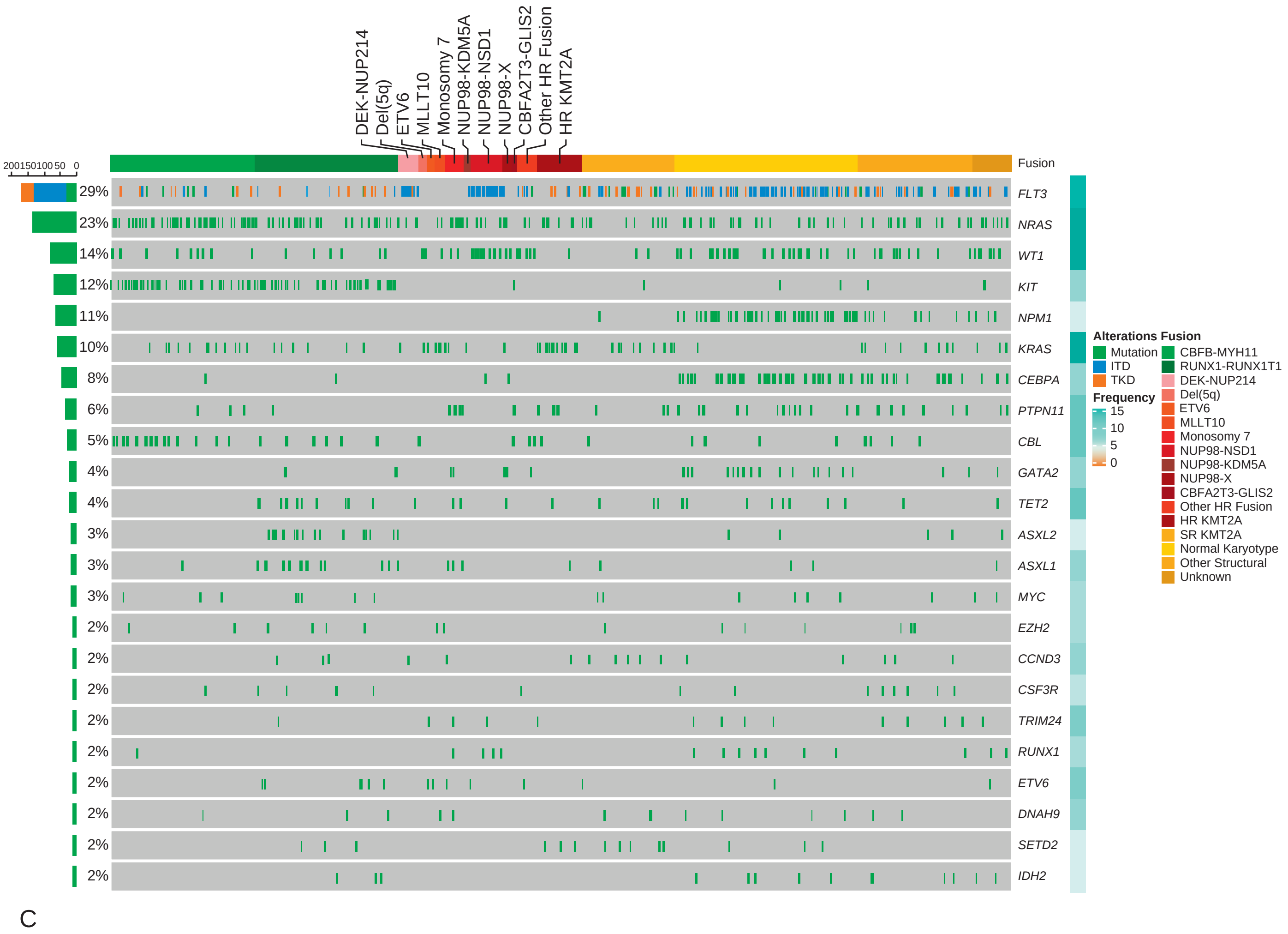


Figure 63.1 cont'd Fusions and small insertions and deletions are often co-occurring (C), though the pattern of co-occurring mutations varies by fusion type. In (C) each hash mark represents an individual patient.

RARA Gene Rearrangements

The hallmark of APL is chromosomal translocations involving the *RARA* gene at 17q22 coding for the retinoic acid receptor alpha. The binding of retinoid acid to a heterodimer of RARA and retinoid X receptor (RXR) leads to a conformational change and converts a transcriptional repressor to an activator that drives granulopoiesis. About 98% of all APL cases harbor the translocation t(15;17)(q24;q21) fusing the *PML* gene to *RARA*. The remaining 2% consist of eight additional translocations with the fusion proteins ZBTB16/RARA (also known as PLZF/RARA) and STAT5B/RARA, being resistant to retinoid acids including all-trans-retinoic acid (ATRA). The most frequently observed secondary mutations with 25% to 60% of all cases are internal tandem duplications of *FLT3*.

PML/RARA represses RARA-RXR-driven gene expression and also alters the localization of wild-type PML away from nuclear bodies, thereby interfering with processes such as DNA damage response and telomere maintenance ultimately inhibiting senescence. Importantly, PML/RARA-expressing APL is highly sensitive to ATRA, inducing a conformational change that relieves the transcriptional repression and drives terminal granulocytic differentiation associated with apoptosis. At higher doses, ATRA also induces degradation of the fusion protein. ATRA alone achieves remission very efficiently but has a high relapse rate, whereas combinations of ATRA with combination chemotherapy yield cure rates of more than 90%. Arsenic trioxide (ATO) has also been shown to induce remissions in patients who had failed ATRA and conventional chemotherapy. Similar to ATRA, ATO triggers degradation of PML/RARA and induces apoptosis of APL, while low doses promote maturation and differentiation. The combination of ATRA with ATO achieves similar cure rates as ATRA with standard chemotherapy but avoids the side effects associated with anthracycline-based combination chemotherapy.[20,21] This regimen may convert APL into the first leukemia that can be cured by targeted therapy alone.

NUP98 Rearrangements

The *NUP98* gene at chromosome 11p15 encodes a 186 kDa precursor protein that by autoproteolytic cleavage matures in two nucleoporins of 98 kDa 96 kDa. Both proteins are components of the nuclear pore complex. In addition, NUP98 binds to promoter regions and activates gene expression by recruiting histone methylases that methylate lysine 4 of histone 3 (H3K4). Initially shown to be a fusion partner with *HOXA9* in the t(7;11)(p15;p15) translocation, it is similar to *KMT2A* in that multiply other fusion partners have been subsequently identified. Most *NUP98* fusions are the result of inversions or balanced translocations. Due to their cryptic nature—NUP98 rearrangements are not visible by standard karyotype analysis—their frequency has initially been underestimated. Recent next-generation sequencing analyses established that 5% to 9% of infant and pediatric AMLs harbor *NUP98* rearrangements, an incidence significantly higher than in adult AML.[2,9,22] Some standard fluorescent in situ hybridization (FISH) panels now include probes capable of identifying *NUP98* fusions.

The most common *NUP98* translocation in childhood AML is t(5;11)(q35;p15) fusing *NUP98* to *NSD1*, encoding a histone methylase with a preference for methylating lysine residues 36 and 20 in histones 3 and 4 (H3K36 and H4K20), respectively, thereby modulating gene expression. The NUP98/NSD1 contains the NSD1 SET domain, harboring the methylase activity. Thus, NUP98/NSD1 activates and maintains gene expression by methylating H3K36 of its target genes. Another prominent *NUP98* fusion is t(11;12)(p15;p13), *NUP98/KDM5A*, that occurs mainly in AMKL, where it constitutes approximately 8% of pediatric non–DS-AMKL cases.[9]

AMLs with *NUP98* rearrangements are characterized by high expression of both the *HOXA* and the *HOXB* cluster, thereby driving leukemic self-renewal. H3K4 methylation by MLL has been shown to direct NUP98/JARID1 binding to and activation of *HOXA* expression. Similarly, NSD1 binds to methylated H3K4 and H3K9 by its PHD domain, which is also kept in the NUP98/NSD1 fusion protein. Furthermore, NUP98/KDM5A has also been demonstrated to bind to H3K4me3 nucleosomes via a PHD finger. This dependency of NUP98 fusion proteins on a functional MLL complex suggests that this group of AMLs may also benefit from drugs developed for *KMT2A* mutated AML.

Interestingly, the majority of NUP98/NSD1-positive AMLs also harbor the *FLT3-ITD* mutation and, to a lesser extent, *Wilms tumor 1 (WT1)* mutations. Such patients have a dismal outcome with current chemotherapy regimens.[22] In this context, FLT3 inhibitors may offer another targeted option for this hard-to-treat AML cohort.

Chromosomal Rearrangements Involving E26 Transformation Specific Family Members

Several members of the E26 transformation specific (ETS) transcription factor family including ERG, FLI1, and SPI1 (also known as PU.1) are crucial regulators of hematopoiesis. They support both normal and malignant hematopoiesis by cooperating with CBF and its fusion proteins RUNX1/RUNX1T1 and CBFB/MYH11. Moreover, the expression of *ERG* is directly regulated by these leukemic fusion proteins.

In particular, *ERG* (21q22) and *FLI1* (11q24) are targets of chromosomal rearrangements leading to a fusion with *FUS* (16p11) or its paralog *EWSR1* (22q12). These translocations are prominent in Ewing sarcoma but are also observed mainly in older children and young adults with AML.

The FUS protein orchestrates both transcription and splicing by binding to RNA polymerase II with its N-terminus while interacting with RNA and the U1 snRNP via C-terminal domains. The translocations fuse the N-terminal regions of FUS and EWSR1 to the C-terminal ETS DNA-binding domains of ERG or FLI1. The resultant fusion protein dysregulates gene expression both by aberrantly activating gene expression and by interfering with RNA splicing. Interestingly, FUS/ERG co-occupies genomic binding sites with the RXR:RARA nuclear receptor complex. In line with this observation, treatment of a FUS/ERG-positive AML cell line with ATRA has been shown to block proliferation and induce differentiation. However, in particular, *FUS/ERG*-positive AML has a very poor prognosis with current treatment regimens.

Another member of the ETS genes is ETV6, whose fusion to RUNX1 is found in 25% of all pediatric ALL cases. The translocation t(7;12)(q36;p13) fuses *ETV6* to the homeobox gene *MNX1*. This translocation generates a very heterogeneous fusion; the corresponding fusion transcript is only detected in 50% of all cases and it has remained unclear if the ETV6 moiety is expressed at all, suggesting that high expression of MNX alone may be the leukemic driver. The translocation is with 30% incidence one of the most frequent rearrangements in infant AML.[23] AMLs with this translocation have an unfavorable prognosis.

DEK/NUP214

The translocation t(6;9)(p23;q34), identified in less than 2% of all pediatric AML, is a rare location that is associated with a poor clinical outcome. Similar to NUP98, NUP214 is part of the nuclear pore complex; by interaction with transporter proteins such as CRM1, XPO1, and NXF1, it is involved in protein and mRNA transport across the nuclear membrane. DEK recruits histone deacetylases to DNA and is an epigenetic regulator of transcription and splicing. Its transcriptional signature includes the activation of HOXA and HOXB expression. Another similarity with *NUP98* fusions is the more than 50% incidence of the FLT3-ITD mutation. *DEK/NUP214* predicts a poor clinical outcome with lower rates of complete remission (CR) and OS.[24]

KAT6A Fusions

The *KAT6A* gene at chromosome 8p11 encodes the histone acetyl transferase (HAT) MOZ (also known as MYST) that is a coactivator for transcription factors including RUNX1 and is required for maintaining the hematopoietic stem cell pool. The two main translocations involve with *CREBBP* and *EP300* at 16p13 and 22q13, respectively, another two closely related HATs. Interestingly, each fusion protein contains both HAT domains from the fusion partners and leads to increased HOXA expression. Both fusions are with less than 1% of all AML cases rare and are mainly associated with monocytic AML of FAB subtype 4 and 5 in infants and young children. Although occasional spontaneous regressions have been reported for *KAT6A/CREBBP*, especially in young children, both translocations predict an unfavorable clinical outcome.[25]

RBM15/MRTFA

The *RBM15* gene at chromosome 1p13 encodes an RNA-binding protein that controls the methylation of RNA at the N6 of adenosines and the splicing of genes relevant of megakaryopoiesis including *MPL*. *MPL* is the receptor for thrombopoietin and an inducer of HOX-gene expression. About 10% of all pediatric AMKL cases harbor the translocation t(1;22)(p13;q13), which fuses RBM15 to MRTFA (also known as MKL). MRTFA is a regulator of myogenesis and cell migration. By interacting with serum response factor, it integrates RAC, RHO, and CDC42 GTPase signaling by binding to G-acting and activates the expression of cytoskeletal genes. Notably, RBM15/MRTFA retains all functional domains of each partner protein. This translocation is associated with an intermediate clinical outcome.[9]

CBF2A3/GLIS2

Until recently, with the exception of the *RBM15/MRTFA* fusion, the genetic etiology of non-DS-AMKL had remained elusive. Transcriptome sequencing of a small cohort identified a cryptic inversion on chromosome 16 [inv(16)(p13.3q24.3)] in half of the patients that resulted in the joining of *CBFA2T3*, a member of the ETO family of nuclear corepressors also involved in *RUNX1* translocations, to *GLIS2*, a member of the GLI family of transcription factors. This translocation is almost exclusively found in children younger than 4 years. *CBFA2T3/GLIS2* AMKL has a distinct gene expression signature: the transcription factor GATA1 required for megakaryopoiesis, and which is mutated in DS-associated AMKL, is downregulated, while ERG as a promoter of self-renewal is upregulated. Patients harboring this mutation have an extremely poor prognosis.[11,26]

Cytogenetically Normal Acute Myeloid Leukemia

Approximately 10% of all infant AML and 25% of pediatric AML cases lack chromosomal aberrations detectable by traditional karyotyping. Different from adult AML single-gene mutations are still uncommon in this group, RNA sequencing has identified cryptic gene fusions are most common in this normal karyotype population.

Small Insertion and Deletions

While fusion genes found in pediatric AML affect transcription factor or epigenetic modulator complexes, smaller insertions and deletions (Indels) may impact the function of key members of signal-transduction pathways such as the RAS-MAPK, JAK-STAT, and PI3K-AKT pathways. Some of them such as *CEBPA* and *nucleophosmin 1 (NPM1)* mutations are leukemia-initiating events and clonal, while others and in particular mutations of signal transducers are secondary events and tend to be subclonal. It is currently unclear if and to what extent subclonal populations may affect the pathobiology of the whole AML by cell-cell contact or secretion of growth-promoting factors.

CCAAT/Enhancer-Binding Protein Alpha (C/EBPα)

C/EBPα is a transcription factor that contains two N-terminal transcriptional activation domains and a C-terminal DNA-binding basic region followed by a leucine zipper domain that mediates homo- and hetero-dimerization with other CEBP family members. In the hematopoietic system, the *CEBPA* gene is expressed in adult HSCs, myeloid progenitors, and granulocytes but not macrophages. C/EBPα is a key regulator of granulopoiesis by regulating the expression of many myeloid genes. Conditional knockout of *CEBPA* in adult mice induces proliferation of HSCs and blocks the transition from common myeloid progenitors to the more differentiated granulocyte monocyte progenitor, leading to an accumulation of myeloid blasts. Mutations that reduce the transcriptional activity of C/EBPα occur in between 5% and 14% of AML patients, primarily in FAB-M1/2 AMLs with a normal karyotype.[27] Two classes of *CEBPA* mutations have been defined, and often occur together: mutations that occur within the first 300 bp of the CEBPA gene that eliminate expression of the p42 isoform but have no effect on the translation of p30; and mutations in the 3′ end of the gene that affect dimerization and DNA-binding. The most frequent pattern seen in patients is a combination of the two types of mutations, one on each allele. Patients with "double mutations" usually have good clinical outcome.[28] Interestingly, CEBPA is repressed by RUNX1/RUNX1T1 and *CEBPA* mutations, and *RUNX1/RUNX1T1* are mutually exclusive.

Wilms' Tumor 1

The *WT1* gene is located on chromosome 11p13 and is a zinc-finger protein binding to nucleic acids. WT1 is significantly expressed only in a few adult tissues including HSCs. Therefore, it is currently being studied as a target for T-cell receptor (TCR) gene therapy of AML.[29]

Similar to CEBPA, it regulates quiescence in early progenitors and promotes myeloid differentiation. Subject to alternative splicing itself, isoforms act as transcription factors or modulators of alternative splicing. *WT1* mutations are found in 10% of pediatric AMLs, are mostly in-frame, and seem to affect its DNA-binding. Among children with *WT1* mutations, 35% are cytogenetically normal, 27% have otherwise favorable cytogenetics, and 36% have the *FLT3/ITD* mutation.[30] However, *WT1* mutations also frequently co-occur with *NUP98/NSD1* fusions, a group with very poor outcome. Event-free and OS in children with *WT1* mutations tends to be lower than those with wild-type WT1, especially in combination with *FLT3-ITD*.[31]

Nucleophosmin 1

NPM1 is often mutated in cytogenetically normal AML and is unique in that it has both oncogenic and tumor suppressor functions. The protein shuttles between the nucleus and cytoplasm, taking part in many cellular processes including regulating ribosomal RNA transcription/processing, transport of pre-ribosomal particles to the cytoplasm, DNA-histone and nucleosome assembly, DNA repair as well as regulating the activity and stability of tumor suppressors such as p53 and its regulator ARF. Alterations of *NPM1* in AML include rare chromosomal translocations such as t(3;5)(q25;q34)[*NPM1-MLF1*] in AML and t(5;17)(q35;q21)[*NPM1-RARA*] in variant APL; the vast majority of changes comprise point mutations that alter its C-terminus, resulting in the creation of a new nuclear export signal. Each of these genetic alterations alters the normal shuttling of NPM between the cytoplasm and nucleus, resulting in constitutive cytoplasmic localization. AMLs with *NPM1* mutations drive *HOX*-gene expression and respond to treatment with a menin inhibitor, suggesting a crucial involvement of the MLL-menin complex in the transcriptional program of mutated NPM. Mutations in *NPM1* are found in 35% of cytogenetically normal adult AMLs, but only in 12% of pediatric AMLs, mostly with normal cytogenetics. *NPM1* mutations are associated with a favorable prognosis even in the context of FLT3-ITD co-occurrence.[32]

GATA1 Mutations

ML DS is associated with a hematologic disorder in infancy, termed transient myeloproliferative disease (TMD; also referred to as "transient abnormal myelopoiesis" or TAM). Approximately 20% of TMD cases patients will later develop AMKL, typically between 1 and 4 years of age. AMKL is the most frequent type of AML in children with DS, and the incidence in these patients is 500-fold higher than in the general population.

The family of GATA transcription factors comprises three members that are expressed in hematopoietic cells (*GATA1*, *GATA2*, and *GATA3*). GATA1 is required for the development of erythrocytes, megakaryocytes, eosinophils, and mast cells. Mutations detected in DS patients with TMD or AMKL consist of short deletions, insertions, and point mutations, typically in exon 2, that eliminate the exon 2 transcription start site. Transcription may still occur, utilizing an exon 3 start site and resulting in a shorter mutant protein (GATA1s) that retains the ability to bind DNA and interact with its cofactor but lacks the transcriptional activation domain and hence has reduced transactivation potential.

GATA1 mutations can be already found at birth in TMD patients and are maintained in patients progressing to DS-AMKL. However, AMKL blasts carry a higher burden of mutations, with additional lesions in epigenetic and kinase-signaling genes leading to the progression of the disease. Collectively, these findings support a model whereby TMD blasts arise secondary to *GATA1* mutations, acquiring this so-called *first hit*, and migrate to and persist in the bone marrow. The occurrence of additional mutations provides the cooperating events that are necessary for full-blown leukemia to develop.[33]

FMS-Like Tyrosine Kinase 3

FLT3 is a class III receptor tyrosine kinase that is normally expressed in early hematopoietic progenitors that are CD34+/c-KIT+. When bound by its ligand (FL), the receptor dimerizes, leading to activation of the receptor's intrinsic tyrosine kinase activity. The activated kinase signals through a variety of pathways including the phosphatidylinositol 3-kinase (PI3K) and RAS signal-transduction cascades through phosphorylation of cytoplasmic substrates.

FLT3 mutations with an incidence of more than 20% of all cases frequent cooperating lesions in pediatric AML.[2] It results from a fragment of the juxtamembrane-domain coding sequence that is duplicated and inserted, the length of which varies from 3 to 400 bp. The juxtamembrane domain is a negative regulator of the kinase activity and the ITD leads to a disruption of this autoinhibitory activity. As a result of the disruption of this domain, the mutant *FLT3*-ITD undergoes ligand-independent dimerization and tyrosine autophosphorylation, and this constitutively active tyrosine kinase activates the downstream targets that are normally regulated by the native receptor. Transplantation of bone marrow cells transduced with *FLT3*-ITD into mice leads to a myeloproliferative disorder but not leukemia, underscoring the importance of cooperative mutations in *FLT3*-ITD–positive malignancy.

FLT3-TKD mutations occur in the activation loop of the kinase domain, leading to constitutive activation. In a wild-type setting, ligand-induced activation of FLT3 causes an active configuration to form, allowing kinase activity. The TKD mutations interfere with the inhibitory loop and thus are similar to ITD in disrupting the regulation of signaling but differ in that they do not require dimerization for constitutive activation. While both mutations lead to constitutive activation of AKT and extracellular signal-related kinase (ERK)1/2 from the PI3K and RAS signaling cascades, respectively, data suggest that strong signal transducer and activator of transcription (STAT5) activation is only observed in *FLT3*-ITD cells.[34] The negative prognostic implications of *FLT3*-ITD are more pronounced than of TKD mutations. Outcome is also affected by co-occurring mutations: *NPM1* mutations yielded better outcomes, while *WT1* mutations and the *NUP98/NSD1* fusion gene further diminished survival when co-occurring with *FLT3-ITD*. Several small-molecule inhibitors targeting FLT3 are currently under clinical evaluation and are discussed elsewhere in this chapter.

RAS Mutations

RAS proteins couple receptor activation with downstream effector pathways, altering proliferation, differentiation, and apoptosis. In AML, *NRAS* mutations predominate, followed in frequency by *KRAS*, while *HRAS* mutations have not been reported. RAS proteins cycle between an inactive GDP-bound state and an active GTP-bound state. Somatic mutations occur at amino acid residues, which impair their intrinsic GTPase activity and also confer resistance to GTPase-activating proteins leading to persistence of a GTP-bound (and therefore active) state. Less common mutations affecting RAS proteins, occurring in the *NF1, CBL*, and *PTPN11* genes, can also promote the active GTP-bound state. Activation of the RAS pathway mediates effects through cell cycle progression, promotes survival, and stimulates actin reorganization and vesicle trafficking, all of which have been shown in various model systems to contribute to RAS-induced tumorigenesis. *RAS* mutations are frequently co-occurring with chromosomal rearrangements affecting *KMT2A* or CBF. Though among the most common mutations observed in children with AML, paired diagnostic and relapse specimens show that RAS pathway mutations may appear, disappear, or change allele frequency from diagnosis to relapse, suggesting that the mutations are mainly subclonal and may not influence outcome.[2,31]

Specific Acute Myeloid Leukemia Subtypes

Acute Promyelocytic Leukemia

APL is morphologically identified as AML-M3. In the late 1970s, it was discovered that leukemia cells could be forced to differentiate in vitro when exposed to retinoic acid. Based on this observation, a 5-year-old girl with refractory APL was treated with ATRA and remained disease-free. Subsequently, in 24 patients the CR rate improved to 96% with ATRA alone. In addition to high CR rates, ATRA has improved long-term outcomes. Of relevance, ATRA diminishes the initial bleeding tendency the disease is associated with and reduces early mortality.[20]

ATRA is not the only targeted agent for APL. Ai-ling 1, a Chinese remedy historically used to treat a variety of illnesses, was tested in the 1970s in more than 1000 patients with different cancers and was found to induce remissions in approximately two-thirds of patients with APL. The effective component of this remedy was arsenic (III) trioxide (ATO). Importantly, ATO was able to induce remissions in patients who had failed ATRA and conventional chemotherapy. At high concentrations, ATO induces apoptosis of APL, while low doses promote maturation and differentiation.

Approaches to the diagnosis, supportive care measures, and treatment of childhood APL are similar to those in adults with APL, including the immediate initiation of ATRA when APL is suspected, followed by the continued use of ATRA and ATO, with or without the addition of conventional chemotherapy.[20,21] Follow-up of patients after the end of treatment using Q-polymerase chain reaction (PCR) is essential, as treating molecular relapse yields better results than salvaging overt relapse. An overview of recent pediatric APL studies is given in Table 63.1.

Transient Myeloproliferative Disease and Myeloid Leukemia of Down Syndrome

ML DS is associated with a hematologic disorder in infancy, termed TMD. In this disorder, a clonal population of megakaryoblasts accumulates in the peripheral blood, originating from the fetal liver where hematopoiesis takes place prenatally. In the majority of cases, remission is spontaneous within 3 months in the absence of treatment. In certain cases, treatment with cytarabine may be indicated, for example, to avoid liver failure in case of severe hyperbilirubinemia or in case of massive organomegaly. Both Children's Oncology Group (COG) and BFM have described the natural history of children with TMD. The BFM group tried unsuccessfully to treat children with persistent TMD with low-dose cytarabine to prevent later ML DS. Results of the two studies are detailed in Table 63.1.

In approximately 20% of TMD cases, patients will later develop AMKL, typically between 1 and 4 years of age. AMKL is the most frequent type of AML in children with DS, and the incidence in these patients is 500-fold higher than in the general population.

Children with ML DS have a favorable prognosis, with OS rates greater than 90%, and should be treated in cooperative group trials that are designed to minimize toxicity while maintaining high cure rates by reducing cumulative chemotherapy exposure, as first demonstrated by Ravindranath and colleagues in the AML8498 study.[35,36] An overview of more recent trials in ML DS is given in Table 63.1. The excellent outcome has been at least partly attributed to increased drug sensitivity of the leukemic blasts to chemotherapeutic agents, allowing for dose-reducing chemotherapy. This nicely makes up for the greater risk of toxicity (especially mucositis and infections) with chemotherapy in these children, especially for anthracyclines. Unfortunately, children with ML DS suffering from relapse are almost incurable, which is a poorly understood phenomenon.

Clinical and Laboratory Manifestations and Diagnosis of Acute Myeloid Leukemia in Children

Most children with AML present with symptoms of bone marrow failure, including pallor, fatigue, bleeding (spontaneous nose or gum bleeding), bruising (petechiae), fever, and/or infection. Hepatosplenomegaly, lymphadenopathy, and bone pain are common but usually less prominent than in children with ALL. Because of the acute nature of the process, weight loss and other signs of chronic disease are rare. In some cases, the predominant clinical signs result not from bone marrow disease, but from extramedullary myeloid tumors, referred to as *chloromas* or *granulocytic sarcomas*, which most often arise in the orbital or spinal regions. Other clinical manifestations include gingival hypertrophy, commonly seen in AML cases with a monocytic component, and skin or subcutaneous nodules. Cutaneous lesions may be single or multiple violaceous papules or nodules, are most common in infants, and may be mistaken for the blueberry muffin lesions that are associated with congenital infection. Skin nodules sometimes develop before bone marrow involvement and may regress spontaneously. Patients with highly elevated leukocyte counts (hyperleukocytosis) may also present with central nervous system (CNS) symptoms (bleeding, seizure, or stroke) or pulmonary symptoms related to hyperviscosity. Testicular infiltration may occur in a minority of patients.

Among children with AML, the median leukocyte count at diagnosis is approximately 20 × 10^9/L, with a median hemoglobin concentration of approximately 9 g/dL and a median platelet count of 60 × 10^9/L. Although a minority of children with AML have no morphologically detectable circulating blasts, careful examination of the peripheral blood smear and/or flow cytometry will reveal leukemic cells in most cases. Sometimes a diagnosis will only become evident with a bone marrow aspirate/trephine or biopsy of a chloroma. Prolonged prothrombin, thrombin, and partial thromboplastin times, as well as decreased fibrinogen levels, are seen in the majority of patients with APL and about 5% of other AML cases. Clinically, these patients present with significant bleeding tendency typical for clotting disorders such as large bruises rather than just petechiae.

Abnormalities of serum chemistries may include hyperuricemia, although this is less common and less severe than hyperuricemia associated with ALL. The presence of hypokalemia, a rare finding in patients with ALL, suggests a diagnosis of monoblastic leukemia. Tumor lysis may also be evident from low calcium and high phosphate levels as well as increased creatinine levels. High LDH levels may be indicative of tumor burden and rates of cellular turn-over.

Among patients who present with circulating blasts, the differential diagnosis is generally limited to various types of leukemia, including AML, ALL, and juvenile myelomonocytic leukemia (JMML). For patients who do not have blasts in their blood at the time of presentation, the differential diagnosis is determined by signs and symptoms and may include aplastic anemia, autoimmune or inflammatory disease, infection, and solid malignancies.

In most cases, the final diagnosis of AML is made by bone marrow examination. A bone marrow aspiration should be performed for morphologic examination, immunophenotyping, genetic, and molecular studies, whereas a bone marrow biopsy is used to assess cellularity and dysplastic features, or when an aspirate fails.[5] A diagnosis of AML is confirmed when 20% or more of nucleated bone marrow cells are blasts of myeloid origin, or when the blasts contain AML-specific genetic lesions, regardless of blast percentage. In children, the blast percentage may not be the most reliable marker to differentiate pediatric AML from myelodysplastic syndrome (MDS), and other clinical factors may need to be taken into account.[37] Nowadays, a morphological diagnosis of AML is confirmed using multiparameter flow cytometry, including markers that diagnose precursor stage such as CD34, CD117, CD133, and HLA-DR, as well as lineage-specific markers, for example, CD41, CD42, and CD61 for megakaryocytic differentiation.[5] The mandatory minimal panel required to fulfill WHO and EGIL criteria for AML includes CD34, CD117, CD11b, CD11c, CD13, CD14, CD15, CD33, CD64, CD65, iMPO, i-lysozyme, CD41, and CD61; and for MPAL: CD19, iCD79a, iCD22, CD10, and iCD3. Aberrant markers may be present and may help to define patient-specific leukemia-associated immune-phenotype (LAIP) that can be used for flow cytometric minimal residual disease (MRD) detection during treatment, including for example, CD2, CD7, CD19, or CD56.[38,39] In case a LAIP cannot be established, MRD can still be followed using the "different from normal" approach, which uses aberrant differentiation/maturation profiles compared to normal, as described in the European Leukemia Net (ELN) MRD Working party guidelines.[40] Moreover, flow cytometry may help to assess response after the first and second course of chemotherapy, as a reliable blast percentage after chemotherapy in the context of regenerating bone marrow may be difficult to assess. In difficult cases, FISH or Q-PCR may also be helpful in response determination (see box on Diagnosis of Acute Myeloid Leukemia).

When 20% to 30% of blasts are present and advanced MDS is considered in the differential diagnosis, repeated bone-marrow punctures may be needed to assess disease progression over time, as well as clinical signs such as organomegaly or CNS involvement that favor a diagnosis of AML over advanced MDS.[37] Identifying underlying MDS is relevant as children with MDS need to be transplanted as definitive therapy.

When a diagnosis of AML is confirmed, most investigators classify each case according to the WHO 2016 criteria (see Table 63.2). This requires at least some genetic studies, and groups make use of various techniques including G-banding (conventional karyotyping), which can detect structural and numerical cytogenetic abnormalities in 70% to 80% of children with AML[41]; FISH to confirm karyotype findings and identify cryptic rearrangements undetectable by G-banding; and molecular genetic approaches such as PCR, target-panel sequencing, genome-wide RNA sequencing, exome sequencing, or whole-genome sequencing.[2] Diagnostic testing may be dependent on resources and the requirements to use these markers for risk-group differentiation. Quantitative PCR of certain markers such as fusion genes may also be used for tracking residual disease during therapy.

Since AML may involve the CNS, examination of the cerebrospinal fluid (CSF) should also be performed as part of the initial workup of all patients. Although CNS leukemia is traditionally defined as the presence of at ≥5 leukocytes/μL of CSF with leukemic blast cells present, the significance of lower levels of CNS involvement differs between studies. The diagnosis of CNS involvement may also be based on the presence of cranial nerve palsies or radiologic evidence of leukemic infiltration. Some groups defer examination of the CSF until day 8 of therapy, especially in cases with hyperleukocytosis.

DIAGNOSIS OF ACUTE MYELOID LEUKEMIA

With a suspected acute leukemia or a suspected acute myeloid leukemia, immunophenotyping of the bone marrow is required to confirm the diagnosis. Histologic and immunohistochemical evaluation of a bone marrow biopsy is helpful, but immunophenotyping by flow cytometry is necessary for both diagnostic and monitoring purposes. When a bone marrow aspiration is not feasible, a diagnosis can be made on peripheral blood. Omission of a bone marrow aspiration should be reserved for exceptional circumstances.

Karyotyping by G-banding and FISH is necessary for classification according to WHO guidelines. It is important to ensure that the FISH probes used account for the unique molecular subtypes of pediatric AML. Confirmation of e.g., KMT2A fusion partners, NUP98 fusions, loss of ETV6, all have a profound impact on risk stratification, treatment decisions (e.g., HSCT), and prognosis.

Molecular diagnostics to identify FLT3-ITD, NPM1, and CEBPA mutations are a minimum requirement for correct WHO classification of AML. Additional DNA/RNA sequencing panels for confirmation of fusion partners, detection of cryptic fusions, and identifying of other disease-modifying mutations should be strongly considered if available.

Prognostic Factors

In the past, FAB-type and WBC were considered prognostic factors, but they have been replaced by more robust predictors of outcome, such as genetic features, which are strongly associated with outcome (see risk-group stratification in Table 63.3).

Response to therapy reflects features specific to leukemia (genetic alterations and inherent sensitivity to chemotherapy), characteristics of the host (pharmacokinetics and genomics and drug metabolism), as well as the intensity and components of therapy, and is therefore a key predictor of outcome. However, morphologic examination of the bone marrow, especially during periods of brisk hematopoietic recovery after intensive chemotherapy, is subjective and lacks the sensitivity and specificity required to accurately assess response. Methods that rely on leukemia-specific features that distinguish residual leukemia cells from normal hematopoietic precursors can provide more precise estimates of MRD. Techniques applicable to AML include RNA-based PCR analysis of leukemia-specific gene fusions, quantitative analysis of *WT1*-expression, deep sequencing to detect leukemia-specific mutations, and flow cytometric detection of aberrant immunophenotypes. Although PCR detection of fusion transcripts is sensitive to a level of 0.01% to 0.001%, it can be applied to only about 50% of cases. In addition, the significance of persistence of fusion transcript expression is not consistent across subtypes of AML. For example, persistent expression of *PML-RARA* is associated with a high risk of relapse among patients with APL, whereas the *RUNX1-RUNX1T1* and *CBFβ-MYH11* transcripts can be detected in patients with CBF leukemia who are in long-term remission, unless there are rising MRD-levels using Q-PCR. In contrast, the sensitivity of flow-based MRD assays is only 0.1% to 0.01%, but this technique can be applied to more than 90% of cases.[40,42] However, even a deep initial

TABLE 63.3 Risk-Group Classification of Pediatric Acute Myeloid Leukemia Used by the Various Collaborative Groups

		Collaborative Groups/Studies						
Risk-Group Stratification	**Mutation, Gene**	**AIEOP**	**BFM**	**COG**	**JPLSG**	**NOPHO-DB-SHIP**	**MyeChild[a]**	**SJCRH**
Low/standard risk	(8;21)(q21.3;q22), *RUNX1/RUNX1T1*	✓	✓	✓	✓		✓	✓
	inv(16)/ t(16;16)(p13.1q22.1), *CBFB/MYH11*	✓	✓	✓	✓	✓	✓	✓
	NPM1 mutation positive	✓	✓	✓			✓	✓
	CEBPA bZIP mutation positive			✓			✓[b]	✓
	Requires patient to be MRD negative at EOI1	✓		✓				✓
	Requires patient to be MRD negative at EOI2						✓	
	Requires patient to be in CR at EOI2					✓		
	Includes Intermediate risk cytogenetics if EOI1 MRD negative						✓	
Intermediate risk	Absence of group specific low/standard-risk or high-risk criteria	✓		✓	✓	✓		✓
	Low/standard risk with poor response at EOI1		✓					
High risk	inv(3)(q21.3q26.2)/t(3;3)(q21.3q26.2), *RPN1/MECOM*			✓			✓	-
	t(3;21)(26.2;q22), *RUNX1/MECOM*			✓			✓	
	t(3;5)(q25;q34), *NPM1/MLF1*			✓				
	t(6;9)(p22.3;q34.1), *DEK/NUP214*	✓	✓	✓			✓	
	t(8;16)(p11.2;p13.3), *KAT6A/CREBBP*	✓		✓[c]				✓
	t(16;21)(p11.2;q22.2), *FUS/ERG*			✓	✓			
	inv(16)(p13.3q24.3), *CBFA2T3/GLIS2*			✓			✓	✓
	t(5;11)(q35;p15.5), *NUP98/NSD1*	✓	✓	✓	✓		✓	✓
	t(4;11)(q21;q23.3), *KMT2A/AFF1 (MLL/AF4)*	✓	✓	✓			✓	
	t(6;11)(q27;q23.3), *KMT2A/AFDN* (*MLL/MLLT4*)	✓	✓	✓	✓		✓	✓
	t(9;22)(q34;q11), *BCR/ABL*	✓	✓		✓		✓	
	t(10;11)(p12.3;q23.3), *KMT2A/MLLT10*	✓	✓	✓			✓	✓
	t(10;11)(p12.1;q23.3), *KMT2A/ABI1*	✓	✓	✓			✓	✓
	t(11;19)(q23.3;p13.3), *KMT2A/MLLT1*(*MLL-ENL*)			✓				
	11p15 rearrangement, *NUP98-any partner gene*			✓			✓	✓
	12p13.2 rearrangement, *ETV6 any partner gene*		✓	✓			✓	✓
	Deletion 12p to include 12p13.2, Loss of *ETV6*	✓		✓			✓	
	Monosomy5/Del(5q) to include 5q31, Loss of *EGR1*	✓		✓	✓		✓	✓
	Monosomy 7	✓	✓	✓	✓		✓	✓
	10p12.3 rearrangement			✓				
	FLT3-ITD (allelic ratios cut-offs may vary)	✓	✓[d]	✓[e]	✓	✓	✓[e]	✓[d]
	Complex karyotype	✓	✓					
	FAB M0	✓						✓
	FAB M6	✓						✓
	Infants excluding FAB M7 with t(1;22)	✓						
	Non-LR/SR and MRD+ at EOI1			✓				✓
	LR/SR cytogenetics an MRD+ at end of consolidation 3						✓	
	Failure to achieve CR at EOI1	✓	✓ (day 42)		✓	✓[f]		
	Day 22 blasts >15%					✓		

AIEOP MRD negative requires <0.1% blasts. COG—MRD negative requires <0.05% blasts. MyeChild—MRD negative requires <0.1% blasts. SJCRH—MRD negative requires <0.1% blasts.

COG, Children's Oncology Group; *CR*, Complete remission; *FAB*, French-American-British; *MRD*, minimal residual disease; *NMP1, nucleophosmin 1*.

[a] MyeChild—United Kingdom and France.

[b] Only *CEBPA* bZIP mutation positive patients without a FLT3-ITD are low risk in MyeChild.

[c] Only if 90 days or older at diagnosis.

[d] FLT3-ITD with a WT1 mutation (BFM) and/or *NUP98* fusion (SJCRH).

[e] FLT3-ITD without NPM1 or *CEBPA* bZIP mutations.

[f] NOPHO-DB-SHIP defines a poor response as: EOi2 and ≥15% leukemic cells in the bone marrow at day 22 or any other time-point prior to course 2, or Flow MRD ≥0.1%–4.9% in the bone marrow before start of consolidation, or FLT3-ITD/NPM1 wild type.

response, which suggests that the bulk of cells is killed by chemotherapy, is no guarantee that the leukemia will not relapse from remaining clonal cells. Currently, not yet enough information is available on the prognostic value of MRD response per genetic subcategory. It is clear, however, regardless of the method used, that we need a better understanding of why some MRD negative patients relapse and some MRD positive patients do not.

Risk-Group Stratification

Most collaborative groups apply a combination of genetic aberrations and early response to therapy as criteria for risk stratification, as shown in Table 63.3.

Investigators from almost all study groups consider children with CBF leukemias to have low or standard-risk AML. Approximately a third of children with CBF mutations will have co-occurring *KIT* mutations.[16] Although *KIT* mutations confer an inferior prognosis in adults with CBF leukemia, their prognostic significance in children is variable and may be treatment-dependent.

Mutations of the *NPM1* gene are seen primarily in AML cases with normal karyotypes, with or without internal tandem duplications of the *FLT3* gene (*FLT3*-ITD). Children whose blasts contain *NPM1* mutations, normal karyotypes, and wild-type *FLT3* appear to have a more favorable prognosis, although the data to support their classification as low-risk patients are not as strong as that for children with CBF leukemia. Similarly, biallelic mutations of *CEBPA* are associated with normal karyotypes and a favorable outcome in adults with AML but occur in less than 5% of childhood AML cases. Nevertheless, they are likely to be associated with a favorable outcome and are included as a low-risk feature in many treatment protocols. Although *IDH* mutations are rare in children, they have been reported as having relatively good clinical outcome, perhaps because of their association with *NPM1* mutations.

Genetic abnormalities for which there is strong evidence of an association with a high risk of relapse include *WT1* mutations and *FLT3*-ITD, especially when occurring together in the leukemic clone. The outcome of patients with *FLT3*-ITD is especially poor in cases with high allelic ratios of *FLT3*-ITD to wild-type *FLT3* but may also be dependent on the associated fusion gene when present. For example, the prognosis of *FLT3*-ITD in the context of APL differs vastly from a combination with *NUP98-NSD1*. Translocations that create chimeric fusion genes and likely confer a poor prognosis are summarized in Table 63.3. The prognostic impact of other lesions, such as mutations of *RUNX1*, *TET2*, or *DNMT3A*, is not well documented.

Therapy

CR and OS rates are now greater than 90% and 70% on collaborative group study protocols for children with AML, respectively (see Table 63.1, Fig. 63.2).

In general, treatment consists of four to five blocks of intensive chemotherapy followed by HSCT in CR1 in selected high-risk patients. There is not a lot of evidence to support maintenance therapy after intensive chemotherapy.

Most induction regimens are based on the combination of cytarabine and daunorubicin, first developed in the 1970s. Attempts to improve remission induction rates have included the addition of etoposide or other nucleoside analogs (cladribine or fludarabine), dose-intensification of cytarabine, the replacement of daunorubicin with idarubicin, mitoxantrone, or liposomal daunorubicin, and the addition of gemtuzumab ozogamicin (GO). Although all these interventions were generally found to be safe, randomized clinical trials mostly demonstrated similar remission rates regardless of the intervention. Most protocols assess CR rates after 1 to 2 blocks of treatment using both morphologies (using Cheson or ELN criteria)[43,44] combined with flow cytometry.

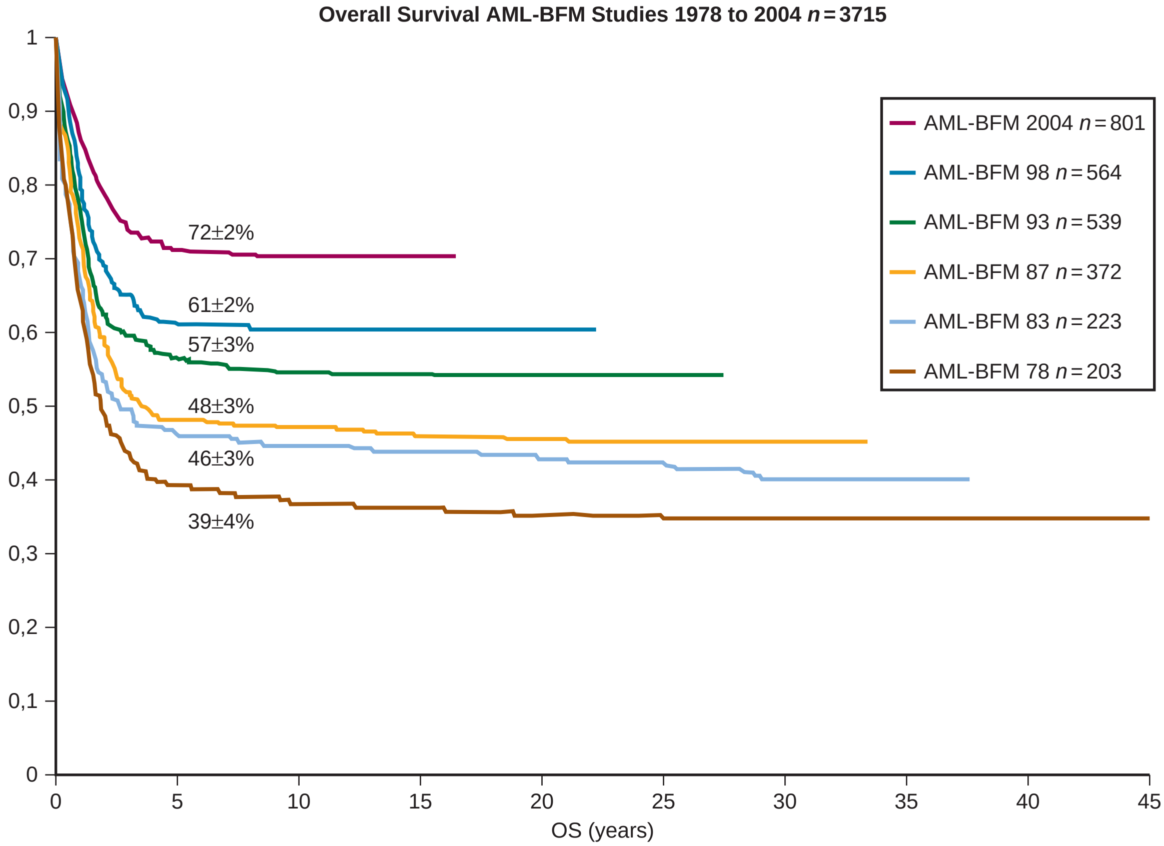


Figure 63.2 Improvement in overall survival for children with acute myeloid leukemia (AML) treated on successive AML-BFM Study Group protocols. *(Data kindly provided by Prof Dr. D Reinhardt, AML-BFM Study Group, Essen, Germany, June 2020.)*

Infants are in general treated on AML protocols that are also used for older children with specific—however, often empiric—dose modification guidelines. There is no indication—different from infants in ALL—that they have a significantly worse outcome than older children with AML.[45]

Clinical trials conducted during the 1980s and 1990s demonstrated that intensive post-remission therapy, administered as intensive chemotherapy and/or HSCT, significantly improved outcome and hence is an essential component of therapy for all children with AML. By contrast, low-dose maintenance therapy may actually lower the survival rates due to poor salvage rates at relapse. The role of autologous HSCT has been studied but without clear benefit.

Trials performed during the past 20 years (see Table 63.1) have sought to determine the optimal duration of post-remission therapy, the benefit of new agents, the value of MRD monitoring, and the role of HSCT. However, like the interventions used to improve remission induction rates, many of the post-remission interventions have had only modest effects on survival. For example, in the COG AAML0531 trial,[46] 1022 patients were randomly assigned to receive standard chemotherapy with or without the addition of three doses of GO. Although the incorporation of GO was associated with a better EFS (53% vs. 47%), there was no difference in OS between arms (69% vs. 65%). In this trial, GO had the greatest impact among patients with favorable karyotypes, *KMT2A* fusions, and *FLT3*/ITD, suggesting that the efficacy of even broadly targeted therapies may be defined by the molecularly defined subtypes of AML.

In the non-randomized 2012 AML-BFM registry ($n = 324$), the impact of a revised risk-group stratification without changes in the chemotherapy regimen but resulting in a higher frequency of HSCT in CR1 was studied using a historical comparator (AML-BFM 04). During the registry time-period, a slightly higher number of patients were allocated to the very high-risk group based on the identification of newly identified high-risk molecular lesions. Moreover, the indication for HSCT was broadened (using molecular subtype and induction response) which led to a higher percentage of patients with HSCT in CR1 (34% compared to 15% in study AML-BFM 04). As a result of these changes and general improvements in supportive care, EFS improved from 52% in AML-BFM 04 to 62%, and OS improved from the historical 71% to 80%.[47]

Improvements in HSCT, such as more comprehensive HLA typing, improved donor availability, have led to fewer short- and long-term side effects. In recent trials, there is no significant difference in survival regardless of whether the donor was related or unrelated. There is clear evidence of anti-leukemic efficacy of HSCT for some children with AML. A well-balanced approach considering procedural morbidity and mortality is important.[48,49]

All children with AML receive intrathecal chemotherapy for prevention of CNS-relapse although the exact number of required intrathecals is not known. Prophylactic cranial irradiation is abandoned by all collaborative groups. Some collaborative groups mainly use cytarabine, others use triple therapy for intrathecal administration. Children with initial CNS involvement are considered candidates for more intensive intrathecal therapy and/or cranial irradiation. High-dose cytarabine may also have a protective effect on CNS-relapse given expected CNS-penetration.

Despite all efforts in finding better ways of cure, intensive cytarabine/anthracycline-based chemotherapy is still the standard of care in pediatric AML treatment. An increase in OS over time is clear despite modest improvements in EFS. OS improvements are most likely due to improvements in risk-group stratification, post-relapse therapy, supportive care (transfusions, infectious complications, nutritional support), and HSCT.[4]

Relapses occur in 25% to 30% of AML patients, and time to relapse is associated with genetic features and with outcome.[50] Especially early relapse after HSCT in CR1 has a dismal prognosis. Late relapse, however, is curable in many patients with the use of 1 to 2 blocks of intensive chemotherapy plus HSCT in all patients. In North America, first relapse patients are often recruited in studies of novel agents (summarized in Table 63.4). Given the concern for long-term cardiac toxicity with high cumulative dosages of anthracyclines, in Europe, liposomal daunorubicin was used off-label as the anthracycline of choice in these patients. However, this compound is no longer available. Recent results with CPX-351 (Vyxeos), a liposomal cytarabine/daunorubicin combination in a fixed ratio, generated in the COG, may set the new standard for relapsed AML if results hold-up in larger studies (see box on Treatment of Relapsed Acute Myeloid Leukemia).[51]

TREATMENT OF RELAPSED ACUTE MYELOID LEUKEMIA

The survival for pediatric patients with AML who have suffered relapse of their disease is at best 40%–50%. At the same time outcome in AML overall has improved in part by better retrieval of patients at relapse compared to decades ago. The largest study to date is the AML 2002/01 study in which 394 patients were enrolled showing approximately 40% survival. There was a clear distinction in outcome between early first and late first relapse, with the latter doing much better. For patients in second or later relapse, the outcome is even worse. This indicates that especially early first relapse and second relapse patients are candidates for experimental approaches. Such approaches should include the use of novel tyrosine kinase inhibitors, targeted agents, and antibody-drug conjugates or immunotherapy. Despite the fact that in recent years many drugs were approved for use in adult AML, this has not happened for children. Therefore, recently, a meeting with all stakeholders was organized to speed up drug development for children with AML.[63] It was concluded that patients with newly diagnosed or relapsed *FLT3*-mutated AML should be enrolled in clinical trials that incorporate second- or third-generation *FLT3* inhibitors, and that T-cell engaging antibodies and antibody-drug conjugates should be prioritized for development, focusing on targets such as CD123 or CD33. As a result of this workshop trans-Atlantic collaboration was initiated to test these agents in children presenting with relapse. Such collaboration is needed to have sufficient numbers to generate data in a reasonable time window. Because it is imperative to study new agents in scientifically rational and clinically safe trials, we believe that efforts should be made to enroll all patients with newly diagnosed and relapsed AML on research protocols.

Supportive Care

Supportive care is an essential component of the management of children with AML. For example, infectious complications are a major cause of morbidity and mortality in children with AML, with a cumulative incidence of documented infection greater than 60% in patients who do not receive prophylactic antibiotics. Randomized controlled trials conducted in adults with AML demonstrated that prophylactic antibiotics, such as oral levofloxacin, are effective at reducing the incidence of bacterial infection. A randomized study in North America in children with leukemia receiving intensive therapy showed that receipt of levofloxacin prophylaxis compared with no prophylaxis resulted in a significant reduction in rates of bacteremia.[52]

Apart from gram-negative infections, viridans group streptococcal infections are also a potential risk, especially after using high-dose cytarabine. Studies are needed to show how this can be prevented, given the limited efficacy of pencilline prohylaxis. Moreover, many children receive prophylaxis against *Pneumocystis jirovecci* pneumonia during intensive chemotherapy treatment.

Disseminated fungal infections, most commonly caused by *Candida* and *Aspergillus* species, are also frequently seen in children with AML. Because several randomized, controlled trials demonstrated the benefits of prophylactic antifungal therapy in adults with cancer, many pediatric oncologists recommend antifungal prophylaxis for children with AML.[53] Nowadays, many agents are available for prophylaxis or treatment of invasive fungal infections. The choice of agent should depend on species and the resistance pattern, as well as on, for example, CNS involvement of the infection. Apart from improvements in treatment, there are also major improvements in the early detection of fungal infections, such as serum galactomannan testing and early CT-scans of the lungs to detect nodules that are suggestive of fungal infection. However, better detection methods are needed.

TABLE 63.4 Results of Recent Clinical Trials for Relapsed/Refractory Pediatric Acute Myeloid Leukemia

Study	Years of Enrollment	Study Question	Relapse Status	Number of Patients	CR (%)	EFS (%) and (median follow-up)	OS (%) and (median follow-up)	Reference
I-BFM AML 2001/01	2001–2009	FLAG vs FLAG-DNX	First relapse	394	64 69 FLAG-DNX, 59 FLAG		38 (4-year)	84
NOPHO relapse	1993–2012	Various regimens	First relapse	208	70		39 (5-year)	85
Japanese Childhood AML Cooperative Study	2000–2005	Various regimens	First relapse after AML99 protocol	71	50		37 (5-year)	86
BFM	1987–2007	Various regimens	First relapse after AML-BFM-87/-93/-98	379	54		23 (5-year)	87
TACL	1995–2004	Retrospective cohort review	First and second relapse	99	First relapse 56%, 2nd relapse 25%	First relapse 24 (5-year)	First relapse 29 (5-year)	88
COG AAML1421, Phase 1–2	2016–?	CPX351, followed by FLAG	Relapsed/refractory	38	ORR 81			51
ITCC 020 Phase 1B study	2010–2014	Clofarabine with FLA-DNX	Relapsed/refractory	34	ORR 68	35 (1-year)	50 (1 year)	89
I-BFM AML 2001/02, Phase 2 study	2001–2009	GO single-agent	Relapsed/refractory	30	ORR 37		27% (3 year, responders only)	59
COG AAML 0532, Phase 1–2 study	2007–2012	Clofarabine with Cytarabine	Relapsed/refractory	49	ORR 48		46% (3 year, responders only)	90
Phase 1 venetoclax	2017–2019	Venetoclax with Cytarabine	Relapsed/refractory	38	ORR 69			91
Phase 2 bortezomib		Bortezimib with Ida/Cytarabine or Cytarabine/Etoposide	Relapsed/refractory	46	ORR 57 (Ida) and 48 (Etoposide)		39 (2-year)	92
BFM compassionate use series	1995–2014	GO/Cytarabine	Relapsed/refractory (mostly 2nd R/R, 2rd line)	76			18 for all patients, 27 for transplanted pts. (4-years)	93

AML, Acute myeloid leukemia; *Ara-C*, cytarabine; *BFM*, Berlin–Frankfurt–Münster Study Group; *COG*, Children's Oncology Group; *CR*, complete remission; *DNX*, liposomal daunorubicin; *EFS*, event-free survival; *FLAG*, fludarabine, cytarabine, GCSF; *GO*, gemtuzumab ozogamicin; *I-BFM*, International BFM study group; *Ida*, idarubicin; *ITCC*, Innovative Therapies for Children with Cancer Consortium; *NOPHO*, Nordic Society of Paediatric Haematology and Oncology; *ORR*, overall response rate (usually comprising CR plus CRp, CRi); *OS*, overall survival.

Antiviral prophylaxis is often added using chemotherapy that induces severe lymphopenia such as clofarabine or fludarabine or post-HSCT to avoid viral reactivations, for example, using prophylactic dosing of valaciclovir.

Initial leukocyte counts greater than 100,000/μL are associated with risks of intracranial hemorrhage and respiratory insufficiency secondary to leukostasis. The potential benefit of leucapheresis and exchange transfusions over direct initiation of chemotherapy remains a matter of debate, but aggressive management to reduce tumor burden is critical to the prevention of morbidity and mortality.

Other relevant supportive care measurements include transfusion support for anemia and thrombocytopenia, and nutritional support using either tube feeding or total parenteral nutrition. Measures for cardio-protection are discussed in the following section.

Late Effects

AML treatment may be associated with serious long-term toxicity, largely dependent on the cumulative dose of chemotherapy agents, side effects of treatment, and the need for irradiation and/or HSCT.

A well-known risk is that of long-term cardiac toxicity, especially using mitoxantrone, although most collaborative groups have tried to reduce anthracycline exposure and/or use cardioprotective agents or liposomal anthracycline formulations.[54] In a recent study, 12% of children suffered from cardiac toxicity during 5-year follow-up, with 70% occurring during protocol treatment, also compromising EFS and OS rates.[55] In the Dutch Cancer Survivor study, 6165 childhood cancer survivors (CCSs) diagnosed between 1963 and 2002 were included, and cardiac follow-up was available in 95% of patients. The cumulative incidence of developing heart failure 40 years after childhood cancer diagnosis was 4.4% (3.4% to 5.5%) among all CCS.[54] CCS treated with mitoxantrone, cyclophosphamide, anthracyclines, or radiotherapy involving the heart were at high risk. To prevent cardiac toxicity, in Europe, liposomal daunorubicin (Daunoxome) has been used off-label as preferred anthracycline at relapse,[4] based on the anticipation that the liposomal encapsulation avoids penetration in the heart muscle. Unfortunately, this drug is no longer available, and in fact, the real evidence for a cardioprotective effect was scant. Whether the new liposomal formulation of daunorubicin and cytarabine (Vyxeos) can prevent such damage is currently unknown. Another drug that may be used to prevent cardiac toxicity is dexrazoxane, which can be given together with anthracylines, but there have been concerns about potentially reducing anticancer efficacy. This was not confirmed in a meta-analysis, nor was there evidence for a higher rate of second malignancies.[56]

High-dose cytarabine may be associated with peripheral or central neurological toxicity such as, for example, cerebellar toxicity. Children who have received cranial irradiation may suffer from neuropsychological long-term sequelae. HSCT may be associated with long-term toxicity depending on the agents used in the preparative regimen, as well as the occurrence of acute and chronic graft versus host disease. Testicular irradiation may lead to infertility and/or hormonal dysfunction. Children receiving long-term mechanical ventilation due to for example serious infectious complications may have reduced pulmonary function. Cerebral bleeding or infarction due to hyperleukocytosis may affect neurological functioning.

Despite all these potential side effects, many children survive without noticeable long-term effects, but this summary clearly demonstrates that children treated for AML should be followed in specialized long-term follow-up clinics. Moreover, new treatment modalities are needed to increase outcome but also ensure a better quality of life for those that survive.

Future Directions

Tyrosine Kinase Inhibitors

AML cells commonly possess aberrant receptor tyrosine kinase activity as a result of genetic alterations, such as *FLT3*-ITD. These alterations are seen in approximately 10% to 15% of pediatric and 30% of adult AML cases, and are associated with a poor outcome, and are druggable targets.

Recent studies have demonstrated that sorafenib, sunitinib, and other FLT3 inhibitors are active in patients with *FLT3* mutations but that prolonged use of these agents is associated with the development of resistance, most commonly caused by the acquisition of secondary point mutations in the kinase domain.[57,58] In adults, two FLT3 inhibitors have been authorized for use by the Food and Drug Administration (FDA), including midostaurin in newly diagnosed patients in combination with chemotherapy and gilteritinib as monotherapy for relapsed AML. In children studies with various FLT3 inhibitors are planned or ongoing, including the two approved drugs, as well as quizartinib and the recently completed COG study of sorafenib. These studies are urgently needed to determine the optimal dose and schedule required to improve the long-term outcome of patients with *FLT3* mutations.

Antibody-Based Therapies

Most antibody-directed therapies for AML have focused on CD33, which is expressed on the surface of leukemia blasts in greater than 90% of cases. GO, a humanized anti-CD33 antibody conjugated to calicheamicin, was approved by the United States FDA in 2000 but was later withdrawn from the market because of concerns of toxicity. However, the results of randomized trials that were completed after the withdrawal of GO suggest that the addition of GO to conventional chemotherapy reduces the risk of relapse and improves event-free survival in newly diagnosed patients.[46] Meta-analyses demonstrate that the benefit of GO was greatest among low-risk patients, with only modest benefits seen in intermediate-risk patients and no benefits in patients with high-risk disease. In pediatrics, GO is only labeled for use in children 15 years of age and older. Despite the availability of early-phase studies and a randomized phase 3 study by COG, none of these studies used the fractionated administration used in the adult studies.[46,59] The MyeChild trial, a collaboration between the French and UK AML groups, currently investigates this in newly diagnosed pediatric AML. New antibody-drug conjugates (ADCs) are currently under development, including CD123 directed agents. It is anticipated that CD123 is better expressed on leukemic stem cells and that therefore its anti-leukemic potential might be more pronounced.

An alternative approach to enhancing the efficacy of CD33/CD123-directed therapy is through the development of CD33/CD123-CD3-directed bispecific T-cell engaging antibodies. By bridging CD33/CD123 with TCRs, these antibodies can direct T-cell effector functions to AML cells. In preclinical models, the CD33/CD3 compound AMG 330 was able to recruit T-cells, resulting in potent CD33-dependent cytotoxicity. Other compounds targeting CD123 are currently in the early stages of clinical development, including a pediatric flotetuzumab study that has just recruited its first subjects (NCT04158739).

Epigenetic Agents

Abnormal regulation of epigenetic modification, such as histone acetylation and DNA methylation, is likely a key factor in the pathogenesis of AML. However, studies using HDAC inhibitors and hypomethylating agents (HMAs) in pediatric AML have not significantly changed the treatment landscape to date. Drug development in elderly patients with AML often uses an HMA backbone to which a new agent is added; however, in children, more aggressive backbone therapy is often needed due to the blastic nature of the disease.

Chimeric Antigen Receptor-Modified T Cells

T cells can be redirected to target cells of interest through the use of chimeric antigen receptors (CARs), which link a target cell ligand-recognition domain to signaling regions from the TCR. First-generation CARs typically incorporated the TCRζ cytoplasmic domain, but it was later demonstrated that tandem signaling domains incorporating the TCR along with costimulatory or co-receptor signals provided more robust stimulation and enhanced the survival, expansion, and activity of CAR-modified T cells. The clinical effectiveness of CAR-modified T cells was first established in B-cell malignancies and led to the market authorization of tisagenlecleucel.

A major challenge to extending the success achieved with CD19-specific CARs (tisagenlecleucel) to AML is identifying the proper recognition domain because effective therapy will require eradication of the leukemic initiating cells as well as their more mature descendants.[60] The widespread expression of CD33 on leukemic cells and the absence of down-modulation after GO treatment indicate that it is a robust target. Preclinical studies performed in NOD-SCID mouse models suggest that CD33 CAR-modified T cells should be investigated. However, the potential depletion of normal hematopoietic precursors or committed myeloid progenitor cells may cause unacceptable long-term neutropenia and require rescue with an allogeneic HSCT. In addition, the absence of CD33 expression on leukemic stem cells in some cases may limit the efficacy of such therapy. Future clinical trials and additional modifications of CD33 and/or CD123 CARs will address these important issues. Other targets for CAR-T-cell therapy may include CD70, CLL-1, or FLT3. All studies are very early in development.

Regulatory Landscape and Initiatives

In Europe, companies are obliged to perform pediatric studies when they develop new agents for a disease that also exists in children. The development plan needs to be laid down in a Pediatric Investigational Plan (PIP) that needs to be approved prior to obtaining market authorization in adults.[61,62] In North America, FDA Regulatory requirements and incentives have led to clinical evaluation of few agents for children with AML. With the Research to Accelerate Cures and Equity (RACE) for Children Act, there is hope that regulatory requirements for target-driven testing in children will improve drug development in pediatric AML. In preparation for the RACE for Children Act, the FDA developed a pediatric molecular target list that may trigger pediatric development or may result in a waiver when a target is on the “automatic waiver” list (https://www.fda.gov/media/120331/download). Some of the targets are high priority for pediatric AML. However, as the number of available relapsed/refractory patients is limited, prioritization needs to take place in selection agents for clinical trials in children with relapsed/refractory AML.

The Accelerate group (https://www.accelerate-platform.org/) organized a meeting in 2019 with all stake holders involved in the treatment of pediatric AML to discuss how to speed up pediatric drug development for childhood AML.[63] This Pediatric Strategy Forum prioritized the development of FLT3 inhibitors and CD123-directed compounds, which are discussed elsewhere in this chapter. This meeting helped to create an international environment to develop drugs for children with AML, which is still a disease with high medical need, and at the same time may fulfill the needs of the companies and regulators.

CONCLUSION

The prognosis of pediatric AML has increased stepwise over the last decades but is still heavily based on intensive chemotherapy and/or HSCT which has remained relatively unchanged over the last decade. Recent studies of the genetics and biology of AML have identified new therapeutics targets and have led to the development of novel therapies, many of which are now in early-phase clinical trials. We hope that these efforts will lead to the development of more effective and less toxic treatments in the near future. However, the evaluation of these treatment strategies will require collaboration between cooperative groups to ensure that adequate numbers of patients to which each therapy is directed can be studied.

SUGGESTED READING

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Aplenc R, Meshinchi S, Sung L, et al. Bortezomib with standard chemotherapy for children with acute myeloid leukemia does not improve treatment outcomes: a report from the Children’s Oncology Group. *Haematologica*. 2020;105(7):1879–1886.

Arber DA. The 2016 WHO classification of acute myeloid leukemia: what the practicing clinician needs to know. *Semin Hematol*. 2019;56:90–95.

Balgobind BV, Raimondi SC, Harbott J, et al. Novel prognostic subgroups in childhood 11q23/MLL-rearranged acute myeloid leukemia: results of an international retrospective study. *Blood*. 2009;114:2489–2496.

Bolouri H, Farrar JE, Triche Jr. T, et al. The molecular landscape of pediatric acute myeloid leukemia reveals recurrent structural alterations and age-specific mutational interactions. *Nat Med*. 2018;24:103–112.

Creutzig U, Dworzak MN, Bochennek K, et al. First experience of the AML-Berlin–Frankfurt–Münster group in pediatric patients with standard-risk acute promyelocytic leukemia treated with arsenic trioxide and all-trans retinoid acid. *Pediatr Blood Cancer*. 2017;64(8).

Creutzig U, van den Heuvel-Eibrink MM, Gibson B, et al. Diagnosis and management of acute myeloid leukemia in children and adolescents: recommendations from an international expert panel. *Blood*. 2012;120:3187–3205.

de Rooij JDE, Branstetter C, Ma J, et al. Pediatric non-Down syndrome acute megakaryoblastic leukemia is characterized by distinct genomic subsets with varying outcomes. *Nat Genet*. 2017;49:451–456.

Faber ZJ, Chen X, Gedman AL, et al. The genomic landscape of core-binding factor acute myeloid leukemias. *Nat Genet*. 2016;48:1551–1556.

Flasinski M, Scheibke K, Zimmermann M, et al. Low-dose cytarabine to prevent myeloid leukemia in children with Down syndrome: TMD Prevention 2007 study. *Blood Adv*. 2018;2:1532–1540.

Gamis AS, Alonzo TA, Gerbing RB, et al. Natural history of transient myeloproliferative disorder clinically diagnosed in Down syndrome neonates: a report from the Children’s Oncology Group Study A2971. *Blood*. 2011;118:6752–6759. quiz 6996.

Gamis AS, Alonzo TA, Meshinchi S, et al. Gemtuzumab ozogamicin in children and adolescents with de novo acute myeloid leukemia improves event-free survival by reducing relapse risk: results from the randomized phase III Children’s Oncology Group trial AAML0531. *J Clin Oncol*. 2014;32:3021–3032.

Getz KD, Sung L, Ky B, et al. Occurrence of treatment-related cardiotoxicity and its impact on outcomes among children treated in the AAML0531 clinical trial: a report from the Children’s Oncology Group. *J Clin Oncol*. 2019;37:12–21.

Heidenreich O, Krauter J, Riehle H, et al. AML1/MTG8 oncogene suppression by small interfering RNAs supports myeloid differentiation of t(8;21)-positive leukemic cells. *Blood*. 2003;101:3157–3163.

Hollink IHIM, van den Heuvel-Eibrink MM, Arentsen-Peters STCJM, et al. NUP98/NSD1 characterizes a novel poor prognostic group in acute myeloid leukemia with a distinct HOX gene expression pattern. *Blood*. 2011;118:3645–3656.

Kaspers GJL, Zimmermann M, Reinhardt D, et al. Addition of liposomal daunorubicin (DaunoXome®) to FLAG significantly improves treatment response in pediatric relapsed AML: final results from the International Randomised Phase III Study Relapsed AML 2001/01. *Blood*. 2009;114:13–14.

Kutny MA, Alonzo TA, Gerbing RB, et al. Arsenic trioxide consolidation allows anthracycline dose reduction for pediatric patients with acute promyelocytic leukemia: report from the Children’s Oncology Group Phase III Historically Controlled Trial AAML0631. *J Clin Oncol*. 2017;35:3021–3029.

Labuhn M, Perkins K, Matzk S, et al. Mechanisms of progression of myeloid preleukemia to transformed myeloid leukemia in children with Down syndrome. *Cancer Cell*. 2019;36:340.

Loken MR, Alonzo TA, Pardo L, et al. Residual disease detected by multidimensional flow cytometry signifies high relapse risk in patients with de novo acute myeloid leukemia: a report from Children’s Oncology Group. *Blood*. 2012;120:1581–1588.

Meyer C, Burmeister T, Gröger D, et al. The MLL recombinome of acute leukemias in 2017. *Leukemia*. 2018;32:273–284.

Miyamoto T, Weissman IL, Akashi K. AML1/ETO-expressing nonleukemic stem cells in acute myelogenous leukemia with 8;21 chromosomal translocation. *Proc Natl Acad Sci USA*. 2000;97:7521–7526.

Moreno L, Pearson ADJ, Paoletti X, et al. Early phase clinical trials of anticancer agents in children and adolescents—an ITCC perspective. *Nat Rev Clin Oncol*. 2017;14:497–507.

Pearson ADJ, Zwaan CM, Kolb EA, et al. Paediatric strategy forum for medicinal product development for acute myeloid leukaemia in children and adolescents: ACCELERATE in collaboration with the European Medicines Agency with participation of the Food and Drug Administration. *Eur J Cancer*. 2020;136:116–129.

Rasche M, Zimmermann M, Borschel L, et al. Successes and challenges in the treatment of pediatric acute myeloid leukemia: a retrospective analysis of the AML-BFM trials from 1987 to 2012. *Leukemia*. 2018;32:2167–2177.

Sander A, Zimmermann M, Dworzak M, et al. Consequent and intensified relapse therapy improved survival in pediatric AML: results of relapse treatment in 379 patients of three consecutive AML-BFM trials. *Leukemia*. 2010;24:1422–1428.

Taub JW, Berman JN, Hitzler JK, et al. Improved outcomes for myeloid leukemia of Down syndrome: a report from the Children's Oncology Group AAML0431 trial. *Blood*. 2017;129:3304–3313.

Testi AM, Pession A, Diverio D, et al. Risk-adapted treatment of acute promyelocytic leukemia: results from the International Consortium for Childhood APL. *Blood*. 2018;132:405–412.

Tierens A, Bjørklund E, Siitonen S, et al. Residual disease detected by flow cytometry is an independent predictor of survival in childhood acute myeloid leukaemia; results of the NOPHO-AML 2004 study. *Br J Haematol*. 2016;174:600–609.

Uffmann M, Rasche M, Zimmermann M, et al. Therapy reduction in patients with Down syndrome and myeloid leukemia: the international ML-DS 2006 trial. *Blood*. 2017;129:3314–3321.

Zwaan CM, Kolb EA, Reinhardt D, et al. Collaborative efforts driving progress in pediatric acute myeloid leukemia. *J Clin Oncol*. 2015;33:2949–2962.

REFERENCES

1. Zwaan CM, Kolb EA, Reinhardt D, et al. Collaborative efforts driving progress in pediatric acute myeloid leukemia. *J Clin Oncol*. 2015;33: 2949–2962.
2. Bolouri H, Farrar JE, Triche Jr. T, et al. The molecular landscape of pediatric acute myeloid leukemia reveals recurrent structural alterations and age-specific mutational interactions. *Nat Med*. 2018;24:103–112.
3. Lai C, Doucette K, Norsworthy K. Recent drug approvals for acute myeloid leukemia. *Hemato Oncol*. 2019;12(1):100.
4. Rasche M, Zimmermann M, Borschel L, et al. Successes and challenges in the treatment of pediatric acute myeloid leukemia: a retrospective analysis of the AML-BFM trials from 1987 to 2012. *Leukemia*. 2018;32:2167–2177.
5. Creutzig U, van den Heuvel-Eibrink MM, Gibson B, et al. Diagnosis and management of acute myeloid leukemia in children and adolescents: recommendations from an international expert panel. *Blood*. 2012;120:3187–3205.
6. Zhang J, Walsh MF, Wu G, et al. Germline mutations in predisposition genes in pediatric cancer. *N Engl J Med*. 2015;373:2336–2346.
7. Arber DA:. The 2016 WHO classification of acute myeloid leukemia: What the practicing clinician needs to know. *Semin Hematol*. 2019;56: 90–95.
8. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol*. 1976;33:451–458.
9. de Rooij JDE, Branstetter C, Ma J, et al. Pediatric non-Down syndrome acute megakaryoblastic leukemia is characterized by distinct genomic subsets with varying outcomes. *Nat Genet*. 2017;49:451–456.
10. Faber ZJ, Chen X, Gedman AL, et al. The genomic landscape of core-binding factor acute myeloid leukemias. *Nat Genet*. 2016;48:1551–1556.
11. Gruber TA, Gedman AL, Zhang J, et al. An Inv(16)(p13.3q24.3)-encoded CBFA2T3-GLIS2 fusion protein defines an aggressive subtype of pediatric acute megakaryoblastic leukemia. *Cancer Cell*. 2012;22:683–697.
12. Porter SN, Cluster AS, Yang W, et al. Fetal and neonatal hematopoietic progenitors are functionally and transcriptionally resistant to Flt3-ITD mutations. *Elife*. 2016;5:e18882.
13. Deguchi K, Gilliland DG:. Cooperativity between mutations in tyrosine kinases and in hematopoietic transcription factors in AML. *Leukemia*. 2002;16:740–744.
14. Miyamoto T, Weissman IL, Akashi K. AML1/ETO-expressing nonleukemic stem cells in acute myelogenous leukemia with 8;21 chromosomal translocation. *Proc Natl Acad Sci U S A*. 2000;97:7521–7526.
15. Heidenreich O, Krauter J, Riehle H, et al. AML1/MTG8 oncogene suppression by small interfering RNAs supports myeloid differentiation of t(8;21)-positive leukemic cells. *Blood*. 2003;101:3157–3163.
16. Klein K, Kaspers G, Harrison CJ, et al. Clinical impact of additional cytogenetic aberrations, cKIT and RAS mutations, and treatment elements in pediatric t(8;21)-AML: results from an International Retrospective Study by the International Berlin-Frankfurt-Münster Study Group. *J Clin Oncol*. 2015;33:4247–4258.
17. Meyer C, Burmeister T, Gröger D, et al. The MLL recombinome of acute leukemias in 2017. *Leukemia*. 2018;32:273–284.
18. Balgobind BV, Raimondi SC, Harbott J, et al. Novel prognostic subgroups in childhood 11q23/MLL-rearranged acute myeloid leukemia: results of an international retrospective study. *Blood*. 2009;114:2489–2496.
19. Hyrenius-Wittsten A, Pilheden M, Sturesson H, et al. De novo activating mutations drive clonal evolution and enhance clonal fitness in KMT2A-rearranged leukemia. *Nat Commun*. 2018;9:1770.
20. Testi AM, Pession A, Diverio D, et al. Risk-adapted treatment of acute promyelocytic leukemia: results from the International Consortium for Childhood APL. *Blood*. 2018;132:405–412.
21. Kutny MA, Alonzo TA, Gerbing RB, et al. Arsenic trioxide consolidation allows anthracycline dose reduction for pediatric patients with acute promyelocytic leukemia: report from the Children's Oncology Group Phase III Historically Controlled trial AAML0631. *J Clin Oncol*. 2017;35: 3021–3029.
22. Hollink IHIM, van den Heuvel-Eibrink MM, Arentsen-Peters STCJM, et al. NUP98/NSD1 characterizes a novel poor prognostic group in acute myeloid leukemia with a distinct HOX gene expression pattern. *Blood*. 2011;118:3645–3656.
23. von Bergh ARM, van Drunen E, van Wering ER, et al. High incidence of t(7;12)(q36;p13) in infant AML but not in infant ALL, with a dismal outcome and ectopic expression of HLXB9. *Genes Chromosomes Cancer*. 2006;45:731–739.
24. Sandahl JD, Coenen EA, Forestier E, et al. t(6;9)(p22;q34)/DEK-NUP214-rearranged pediatric myeloid leukemia: an international study of 62 patients. *Haematologica*. 2014;99:865–872.
25. Coenen EA, Zwaan CM, Reinhardt D, et al. Pediatric acute myeloid leukemia with t(8;16)(p11;p13), a distinct clinical and biological entity: a collaborative study by the International-Berlin-Frankfurt-Münster AML-study group. *Blood*. 2013;122:2704–2713.
26. Thirant C, Ignacimouttou C, Lopez CK, et al. ETO2-GLIS2 hijacks transcriptional complexes to drive cellular identity and self-renewal in pediatric acute megakaryoblastic leukemia. *Cancer Cell*. 2017;31:452–465.
27. Pabst T, Mueller BU, Zhang P, et al. Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. *Nat Genet*. 2001;27:263–270.
28. Hollink IHIM, van den Heuvel-Eibrink MM, Arentsen-Peters STCJM, et al. Characterization of CEBPA mutations and promoter hypermethylation in pediatric acute myeloid leukemia. *Haematologica*. 2011;96:384–392.
29. Chapuis AG, Egan DN, Bar M, et al. T cell receptor gene therapy targeting WT1 prevents acute myeloid leukemia relapse post-transplant. *Nat Med*. 2019;25:1064–1072.
30. Hollink IHIM, van den Heuvel-Eibrink MM, Zimmermann M, et al. Clinical relevance of Wilms tumor 1 gene mutations in childhood acute myeloid leukemia. *Blood*. 2009;113:5951–5960.
31. Balgobind BV, Hollink IHIM, Arentsen-Peters STCJM, et al. Integrative analysis of type-I and type-II aberrations underscores the genetic heterogeneity of pediatric acute myeloid leukemia. *Haematologica*. 2011;96:1478–1487.
32. Hollink IHIM, Zwaan CM, Zimmermann M, et al. Favorable prognostic impact of NPM1 gene mutations in childhood acute myeloid leukemia, with emphasis on cytogenetically normal AML. *Leukemia*. 2009;23:262–270.
33. Labuhn M, Perkins K, Matzk S, et al. Mechanisms of progression of myeloid preleukemia to transformed myeloid leukemia in children with Down syndrome. *Cancer Cell*. 2019;36:340.
34. Mizuki M, Fenski R, Halfter H, et al. Flt3 mutations from patients with acute myeloid leukemia induce transformation of 32D cells mediated by the Ras and STAT5 pathways. *Blood*. 2000;96:3907–3914.
35. Uffmann M, Rasche M, Zimmermann M, et al. Therapy reduction in patients with Down syndrome and myeloid leukemia: the international ML-DS 2006 trial. *Blood*. 2017;129:3314–3321.
36. Taub JW, Berman JN, Hitzler JK, et al. Improved outcomes for myeloid leukemia of Down syndrome: a report from the Children's Oncology Group AAML0431 trial. *Blood*. 2017;129:3304–3313.
37. Hasle H, Niemeyer CM, Chessells JM, et al. A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative diseases. *Leukemia*. 2003;17:277–282.
38. Rubnitz JE, Inaba H, Dahl G, et al. Minimal residual disease-directed therapy for childhood acute myeloid leukaemia: results of the AML02 multicentre trial. *Lancet Oncol*. 2010;11:543–552.
39. Tierens A, Bjørklund E, Siitonen S, et al. Residual disease detected by flow cytometry is an independent predictor of survival in childhood acute myeloid leukaemia; results of the NOPHO-AML 2004 study. *Br J Haematol*. 2016;174:600–609.
40. Schuurhuis GJ, Heuser M, Freeman S, et al. Minimal/measurable residual disease in AML: a consensus document from the European LeukemiaNet MRD Working Party. *Blood*. 2018;131:1275–1291.
41. Harrison CJ, Hills RK, Moorman AV, et al. Cytogenetics of childhood acute myeloid leukemia: United Kingdom Medical Research Council Treatment trials AML 10 and 12. *J Clin Oncol*. 2010;28:2674–2681.
42. Loken MR, Alonzo TA, Pardo L, et al. Residual disease detected by multidimensional flow cytometry signifies high relapse risk in patients with de novo acute myeloid leukemia: a report from Children's Oncology Group. *Blood*. 2012;120:1581–1588.
43. Döhner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*. 2017;129:424–447.
44. Cheson BD, Cassileth PA, Head DR, et al. Report of the National Cancer Institute-sponsored workshop on definitions of diagnosis and response in acute myeloid leukemia. *J Clin Oncol*. 1990;8:813–819.
45. Blais S, Boutroux H, Pasquet M, et al. Is acute myeloblastic leukemia in children under 2 years of age a specific entity? A report from the FRENCH ELAM02 Study Group. *Hemasphere*. 2019;3:e316.
46. Gamis AS, Alonzo TA, Meshinchi S, et al. Gemtuzumab ozogamicin in children and adolescents with de novo acute myeloid leukemia improves event-free survival by reducing relapse risk: results from the randomized phase III Children's Oncology Group trial AAML0531. *J Clin Oncol*. 2014;32:3021–3032.

47. Rasche M, Steidel E, Kondryn D, et al. Impact of a risk-adapted treatment approach in pediatric AML: a report of the AML-BFM registry 2012. *Blood*. 2019;134.
48. Sauer MG, Lang PJ, Albert MH, et al. Hematopoietic stem cell transplantation for children with acute myeloid leukemia-results of the AML SCT-BFM 2007 trial. *Leukemia*. 2020;34:613–624.
49. Niewerth D, Creutzig U, Bierings MB, et al. A review on allogeneic stem cell transplantation for newly diagnosed pediatric acute myeloid leukemia. *Blood*. 2010;116:2205–2214.
50. Kaspers GJL, Zimmermann M, Reinhardt D, et al. Addition of liposomal daunorubicin (DaunoXome®) to FLAG significantly improves treatment response in pediatric relapsed AML: final results from the international randomised phase III study relapsed AML 2001/01. *Blood*. 2009;114:13–14.
51. Cooper TM, Absalon M, Alonzo TA, et al. AAML 1421, a phase I/II study of CPX-351 followed by fludarabine, cytarabine, and G-CSF (FLAG) for children with relapsed acute myeloid leukemia (AML): a report from the Children's Oncology Group. *J Clin Oncol*. 2019;38:2170–2177.
52. Alexander S, Fisher BT, Gaur AH, et al. Effect of levofloxacin prophylaxis on bacteremia in children with acute leukemia or undergoing hematopoietic stem cell transplantation: a randomized clinical trial. *JAMA*. 2018;320:995–1004.
53. Lehrnbecher T, Groll AH:. Pre-emptive versus empirical antifungal therapy in immunocompromised children. *Lancet Child Adolesc Health*. 2019;3:518–520.
54. Feijen EAM, Font-Gonzalez A, Van der Pal HJH, et al. Risk and temporal changes of heart failure among 5-year childhood cancer survivors: a DCOG-LATER study. *J Am Heart Assoc*. 2019;8:e009122.
55. Getz KD, Sung L, Ky B, et al. Occurrence of treatment-related cardiotoxicity and its impact on outcomes among children treated in the AAML0531 clinical trial: a report from the Children's Oncology Group. *J Clin Oncol*. 2019;37:12–21.
56. van Dalen EC, van den Berg H, Raphäel MF, et al. Should anthracyclines and dexrazoxane be used for children with cancer? *Lancet Oncol*. 2011;12:12–13.
57. Zwaan CM, Söderhäll S, Brethon B, et al. A phase 1/2, open-label, dose-escalation study of midostaurin in children with relapsed or refractory acute leukaemia. *Br J Haematol*. 2019;185:623–627.
58. Inaba H, Rubnitz JE, Coustan-Smith E, et al. Phase I pharmacokinetic and pharmacodynamic study of the multikinase inhibitor sorafenib in combination with clofarabine and cytarabine in pediatric relapsed/refractory leukemia. *J Clin Oncol*. 2011;29:3293–3300.
59. Zwaan CM, Reinhardt D, Zimmerman M, et al. Salvage treatment for children with refractory first or second relapse of acute myeloid leukaemia with gemtuzumab ozogamicin: results of a phase II study. *Br J Haematol*. 2010;148:768–776.
60. Maude SL, Laetsch TW, Buechner J, et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *N Engl J Med*. 2018;378:439–448.
61. Pearson ADJ, Pfister SM, Baruchel A, et al. From class waivers to precision medicine in paediatric oncology. *Lancet Oncol*. 2017;18:e394–e404.
62. Moreno L, Pearson ADJ, Paoletti X, et al. Early phase clinical trials of anticancer agents in children and adolescents—an ITCC perspective. *Nat Rev Clin Oncol*. 2017;14:497–507.
63. Pearson ADJ, Zwaan CM, Kolb EA, et al. Paediatric strategy forum for medicinal product development for acute myeloid leukaemia in children and adolescents: ACCELERATE in collaboration with the European Medicines Agency with participation of the Food and Drug Administration. *Eur J Cancer*. 2020;136:116–129.
64. Pession A, Masetti R, Rizzari C, et al. Results of the AIEOP AML 2002/01 multicenter prospective trial for the treatment of children with acute myeloid leukemia. *Blood*. 2013;122:170–178.
65. Creutzig U, Zimmermann M, Bourquin J-P, et al. Randomized trial comparing liposomal daunorubicin with idarubicin as induction for pediatric acute myeloid leukemia: results from Study AML-BFM 2004. *Blood*. 2013;122:37–43.
66. Aplenc R, Meshinchi S, Sung L, et al. Bortezomib with standard chemotherapy for children with acute myeloid leukemia does not improve treatment outcomes: a report from the Children's Oncology Group. *Haematologica*. 2020;105(7):1879–1886.
67. Tomizawa D, Tawa A, Watanabe T, et al. Appropriate dose reduction in induction therapy is essential for the treatment of infants with acute myeloid leukemia: a report from the Japanese Pediatric Leukemia/Lymphoma Study Group. *Int J Hematol*. 2013;98:578–588.
68. Tomizawa D, Tawa A, Watanabe T, et al. Excess treatment reduction including anthracyclines results in higher incidence of relapse in core binding factor acute myeloid leukemia in children. *Leukemia*. 2013;27:2413–2416.
69. Taga T, Tomizawa D, Takahashi H, et al. Acute myeloid leukemia in children: current status and future directions. *Pediatr Int*. 2016;58:71–80.
70. Imamura T, Iwamoto S, Kanai R, et al. Outcome in 146 patients with paediatric acute myeloid leukaemia treated according to the AML99 protocol in the period 2003–06 from the Japan Association of Childhood Leukaemia Study. *Br J Haematol*. 2012;159:204–210.
71. Gibson BE, Webb DK, Howman AJ, et al. Results of a randomized trial in children with Acute Myeloid Leukaemia: medical research council AML12 trial. *Br J Haematol*. 2011;155:366–376.
72. Abrahamsson J, Forestier E, Heldrup J, et al. Response-guided induction therapy in pediatric acute myeloid leukemia with excellent remission rate. *J Clin Oncol*. 2011;29:310–315.
73. Hasle H, Abrahamsson J, Forestier E, et al. Gemtuzumab ozogamicin as postconsolidation therapy does not prevent relapse in children with AML: results from NOPHO-AML 2004. *Blood*. 2012;120:978–984.
74. Rubnitz JE, Lacayo NJ, Inaba H, et al. Clofarabine can replace anthracyclines and etoposide in remission induction therapy for childhood acute myeloid leukemia: the AML08 multicenter, randomized phase III trial. *J Clin Oncol*. 2019;37:2072–2081.
75. De Moerloose B, Reedijk A, de Bock GH, et al. Response-guided chemotherapy for pediatric acute myeloid leukemia without hematopoietic stem cell transplantation in first complete remission: results from protocol DB AML-01. *Pediatr Blood Cancer*. 2019;66:e27605.
76. Petit A, Ducassou S, Leblanc T, et al. Maintenance therapy with interleukin-2 for childhood AML: results of ELAM02 phase III randomized trial. *Hemasphere*. 2018;2:e159.
77. Taga T, Watanabe T, Tomizawa D, et al. Preserved high probability of overall survival with significant reduction of chemotherapy for myeloid leukemia in Down syndrome: a nationwide prospective study in Japan. *Pediatr Blood Cancer*. 2016;63:248–254.
78. Gamis AS, Alonzo TA, Gerbing RB, et al. Natural history of transient myeloproliferative disorder clinically diagnosed in Down syndrome neonates: a report from the Children's Oncology Group Study A2971. *Blood*. 2011;118:6752–6759. quiz 6996.
79. Flasinski M, Scheibke K, Zimmermann M, et al. Low-dose cytarabine to prevent myeloid leukemia in children with Down syndrome: TMD Prevention 2007 study. *Blood Adv*. 2018;2:1532–1540.
80. Takahashi H, Watanabe T, Kinoshita A, et al. High event-free survival rate with minimum-dose-anthracycline treatment in childhood acute promyelocytic leukaemia: a nationwide prospective study by the Japanese Paediatric Leukaemia/Lymphoma Study Group. *Br J Haematol*. 2016;174:437–443.
81. Creutzig U, Zimmermann M, Dworzak M, et al. Favourable outcome of patients with childhood acute promyelocytic leukaemia after treatment with reduced cumulative anthracycline doses. *Br J Haematol*. 2010;149:399–409.
82. Creutzig U, Dworzak MN, Bochennek K, et al. First experience of the AML-Berlin-Frankfurt-Münster group in pediatric patients with standard-risk acute promyelocytic leukemia treated with arsenic trioxide and all-trans retinoid acid. *Pediatr Blood Cancer*. 2017;64.
83. Kutny MA, Geyer S, Laumann KM, et al. Outcome for pediatric acute promyelocytic leukemia patients at Children's Oncology Group sites on the Leukemia Intergroup Study CALGB 9710 (Alliance). *Pediatr Blood Cancer*. 2019;66:e27542.
84. Kaspers GJL, Zimmermann M, Reinhardt D, et al. Improved outcome in pediatric relapsed acute myeloid leukemia: results of a randomized trial on liposomal daunorubicin by the International BFM Study Group. *J Clin Oncol*. 2013;31:599–607.
85. Karlsson L, Forestier E, Hasle H, et al. Outcome after intensive reinduction therapy and allogeneic stem cell transplant in paediatric relapsed acute myeloid leukaemia. *Br J Haematol*. 2017;178:592–602.
86. Nakayama H, Tabuchi K, Tawa A, et al. Outcome of children with relapsed acute myeloid leukemia following initial therapy under the AML99 protocol. *Int J Hematol*. 2014;100:171–179.
87. Sander A, Zimmermann M, Dworzak M, et al. Consequent and intensified relapse therapy improved survival in pediatric AML: results of relapse treatment in 379 patients of three consecutive AML-BFM trials. *Leukemia*. 2010;24:1422–1428.
88. Gorman MF, Ji L, Ko RH, et al. Outcome for children treated for relapsed or refractory acute myelogenous leukemia (rAML): a Therapeutic Advances in Childhood Leukemia (TACL) Consortium study. *Pediatr Blood Cancer*. 2010;55:421–429.
89. van Eijkelenburg NKA, Rasche M, Ghazaly E, et al. Clofarabine, high-dose cytarabine and liposomal daunorubicin in pediatric relapsed/refractory acute myeloid leukemia: a phase IB study. *Haematologica*. 2018;103:1484–1492.

90. Cooper TM, Alonzo TA, Gerbing RB, et al. AAML0523: a report from the Children's Oncology Group on the efficacy of clofarabine in combination with cytarabine in pediatric patients with recurrent acute myeloid leukemia. *Cancer*. 2014;120:2482–2489.
91. Karol SE, Alexander TB, Budhraja A, et al. Venetoclax in combination with cytarabine with or without idarubicin in children with relapsed or refractory acute myeloid leukaemia: a phase 1, dose-escalation study. *Lancet Oncol*. 2020;21:551–560.
92. Horton TM, Perentesis JP, Gamis AS, et al. A phase 2 study of bortezomib combined with either idarubicin/cytarabine or cytarabine/etoposide in children with relapsed, refractory or secondary acute myeloid leukemia: a report from the Children's Oncology Group. *Pediatr Blood Cancer*. 2014;61:1754–1760.
93. Niktoreh N, Lerius B, Zimmermann M, et al. Gemtuzumab ozogamicin in children with relapsed or refractory acute myeloid leukemia: a report by Berlin-Frankfurt-Münster study group. *Haematologica*. 2019;104:120–127.

CHAPTER 64

BLASTIC PLASMACYTOID DENDRITIC CELL NEOPLASM

Andrew A. Lane

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a hematopoietic-derived malignancy thought to occur by transformation of plasmacytoid dendritic cells (pDCs) or pDC progenitors. Normal pDCs are immune cells that recognize microbes via Toll-like receptors and are the principal producers of type 1 interferons in the setting of infection. The disease was formally named BPDCN in 2008, based on recognition that the malignant cells shared features with pDCs, such as cell surface markers, gene expression, and ability to stimulate T-cell function. Previous names for the disease included blastic natural killer (NK) cell lymphoma, CD4+CD56+ hematodermic neoplasm, and acute agranular CD4-positive NK cell leukemia. In 2016, BPDCN was added as a distinct malignancy in the World Health Organization (WHO) pathological classification, after previously having been categorized as a subtype of acute myeloid leukemia (AML).[1]

EPIDEMIOLOGY

It remains challenging to accurately quantify the incidence of BPDCN due to the variety of names the disease had previously and because it was only recently given a code in the International Classification of Diseases (ICD). BPDCN incidence has been estimated by various sources as approximately 0.5% of all hematologic malignancies, 0.45/1,000,000 population, or 200 to 500 cases per year in the United States.[2] The true incidence of BPDCN may be underestimated due to unfamiliarity with the disease among clinicians and pathologists, and because at least 20% of patients with BPDCN have a pre-existing myeloid neoplasm, such as chronic myelomonocytic leukemia (CMML), myelodysplastic syndrome (MDS), or AML. Careful clinical and pathological examination is necessary to distinguish between BPDCN and AML, particularly in patients with a known myeloid disorder (discussed in more detail later). In particular, BPDCN should be considered when patients with a myeloid neoplasm develop skin lesions. As recognition of BPDCN as a distinct entity improves and because there is an approved BPDCN-specific targeted therapy available and others in development, biopsy-confirmed incidence of the disease may continue to rise.

The median age of diagnosis in adult patients is approximately 60 to 65 years old. The disease also occurs in children and there is a bimodal incidence pattern, similar to what is observed in B-cell acute lymphoblastic leukemia, with a separate pediatric peak of BPDCN incidence at age 8 to 10 years. There is a striking male predominance in adults with BPDCN, with men affected 3 to 4 times more frequently than women. The disease is not nearly as gender-biased in children, where the male incidence is only 1.2 times higher than in females.[3] There is no exposure history clearly linked to BPDCN, such as prior chemotherapy, radiation, or environmental toxins. Different from conventional dendritic cells (cDCs), which present processed antigens to lymphocytes, pDCs' primary role is to sense pathogens and activate the immune system by producing interferon α and β. Thus, there has been interest in determining if BPDCN has an infectious etiology. However, there has been no infection linked to BPDCN, neither in empirical assessment of specific viruses, such as Epstein-Barr virus, nor in unbiased microbial metagenomics using next-generation sequencing.[4]

PATHOBIOLOGY

pDCs develop from a common myeloid-dendritic progenitor that is found in the bone marrow. After maturation, they circulate as 0.5% to 1% of peripheral blood mononuclear cells and are also found in secondary lymphoid organs such as lymph nodes, tonsil, and spleen. They are not frequently detected in the skin under normal circumstances. It is not clear if a specific stage of hematopoietic stem cells, pDC progenitors, or mature pDCs is the target for transformation to BPDCN. Similarly, we do not know if the initial transformation event takes place in bone marrow, peripheral blood, lymphoid organs, or skin. Investigation of these questions regarding the developmental ontogeny of BPDCN is an active area of ongoing research.[5]

At least 50% of BPDCNs have an abnormal karyotype by cytogenetic analysis.[6] However, there is no consistent translocation, mutation, deletion, or amplification event that defines all cases of BPDCN. Recurrent copy loss of specific chromosomes is a common feature, including minimal deleted regions that include known tumor suppressor genes, many of which are cell cycle regulators: 7p12 (*IKZF1*), 9p21 (*CDKN2A/CDKN2B*), 12p13 (*CDKN1B*), 13q11 (*LATS2*), 13q13 (*RB1*), and 17p (*TP53*). Rearrangements of 8q24 involving the *MYC* gene were reported in 40% of cases in a Japanese BPDCN population, with at least half only detectable using fluorescence in situ hybridization (FISH).[7] In that series, *MYC* rearrangement was associated with poorer overall survival (OS) in univariate analysis. In a subset, the translocation t(6;8)(p21;q24) positions an enhancer region of the dendritic lineage-specific gene *RUNX2* on 6p21 upstream of the *MYC* gene, driving its high expression. Despite these findings, in our center's BPDCN population, we have not seen these high rates of *MYC* rearrangement, possibly related to differences in North American and Japanese populations. Therefore, we do not routinely perform FISH for *MYC* abnormalities. Pediatric BPDCN may also be distinct cytogenetically as it is enriched compared to adults for translocations involving the *MYB* locus and does not have other adult-type BPDCN-associated gene mutations.[8]

Somatic mutations acquired in BPDCN cells overlap with the genes mutated in myeloid malignancies, supporting a similar cell of origin.[9] The affected genes are particularly overlapping with those observed in patients with MDS and CMML, and include *TET2*, *ASXL1*, RNA splicing factors (e.g., *SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*), *TP53*, and others mutated in AML, including *IDH1*, *IDH2*, *KRAS*, and *NRAS*. Some series identified mutations in transcription factors that may be involved in pDC lineage-related transformation, including *IKZF1*, *IKZF2*, *IKZF3*, and *ZEB2*. Unlike AML, mutations in *DNMT3A*, *FLT3*, and *NPM1* are not common.

Gene expression profiling, including by single cell RNA sequencing, support that the closest normal counterpart of BPDCN is the pDC or its immediate precursors.[10] BPDCNs also express high levels of the antiapoptotic gene *BCL2*[11] and have upregulated expression of nuclear factor kappa B (NF-κB) gene signatures,[12] which may suggest targets for therapy.

CLINICAL MANIFESTATIONS AND RECOMMENDED DIAGNOSTIC EVALUATION

The most striking and unique feature of BPDCN is its involvement of the skin. At least 80% of patients have skin involvement at the time of initial diagnosis. The skin lesions can be single, or widespread and numerous; they can be nodular and indurated or can be patchy erythema that mimics inflammatory or allergic conditions. However, the most common skin presentation is one or more violaceous, bruise-like plaques or tumors that can be found in any region of the body (Fig. 64.1). The disease usually begins with a single lesion, which often then spreads to contiguous areas or more distantly in the skin. Interestingly, up to 50% of patients will not have concurrent bone marrow involvement (so-called "skin-only" disease). In those with bone marrow disease, only a minority will have circulating peripheral blood BPDCN cells. Anemia or thrombocytopenia are signs that the bone marrow may be involved. Lymph node and/or splenic involvement is present in approximately 40%. BPDCN has an increased propensity to involve other visceral sites such as the lung, breast, oral cavity, and other mucosa such as the cervix. In our experience, extramedullary involvement by BPDCN is more frequent than in AML, including compared to AMLs with monocytic differentiation (FAB M4 and M5), which can involve mucosal surfaces, gingiva, and other organs including the central nervous system.

We recommend that the initial evaluation for a patient with suspected BPDCN includes a full body skin exam, preferably by a dermatologist or oncologist with experience in skin malignancies, with skin biopsy of suspicious lesions; a bone marrow aspiration and biopsy; and full body imaging with either contrast-enhanced CT scan or positron emission tomography (PET)-CT scan.

Central nervous system (CNS) involvement by BPDCN is more frequent than in AML or other myeloid neoplasms such as MDS and CMML. Occult CNS involvement at diagnosis may be as high as 30%, and symptomatic CNS disease is often a feature of relapsed or refractory disease. Therefore, we recommend lumbar puncture for cytology and flow cytometry, plus prophylactic intrathecal chemotherapy for all patients at diagnosis, and for all patients with neurologic symptoms or signs at any time in their disease course. For patients with no CNS involvement at diagnosis, we recommend a full course of prophylactic intrathecal chemotherapy, concurrent with systemic therapy, following a regimen similar to that used for high-risk acute lymphoblastic leukemia.

HISTOPATHOLOGY

Skin biopsy reveals a dense monomorphous infiltrate in the dermis and subcutis of blastoid cells (Fig. 64.2). The epidermis is typically spared and there is usually a clear "grenz zone," an uninvolved strip of papillary dermis just below the epidermis. The individual tumor cells are medium to large sized with scant cytoplasm and have similar appearance in skin, marrow, lymph nodes, blood, and other involved sites. Some have a plasmacytoid appearance with eccentric nuclei, cytoplasmic vacuolation, and/or extended cytoplasmic projections similar to "hand-mirror" cells that are seen in acute lymphoblastic leukemia. Mitotic figures are often present.

The classic immunophenotype of BPDCN cells is positivity for CD123 (IL-3 receptor α chain), CD4, and CD56, on CD45-positive cells indicating hematopoietic origin. The cells fall in the CD45 dim, side-scatter low "blast gate" on flow cytometry. Staining for the proto-oncogene T-cell leukemia/lymphoma protein 1A (TCL-1) is often positive, although this marker is not specific for BPDCN and can also be expressed in several lymphoid malignancies. Terminal deoxynucleotidyl transferase (TdT) is positive in approximately one-third of cases. The pDC antigen CD303 (BDCA-2) is lost in some BPDCNs, but when positive, its expression supports the diagnosis. CD4 or CD56 may be negative in a minority (approximately 8%) of cases. One diagnostic schema suggests that BPDCN is likely if the tumor cells express at least 4 of 5 markers among CD123, CD4, CD56, TCL-1, and CD303.[13] CD34 is usually negative. Myeloperoxidase and lysozyme are negative in BPDCN and when either is positive makes AML more likely.

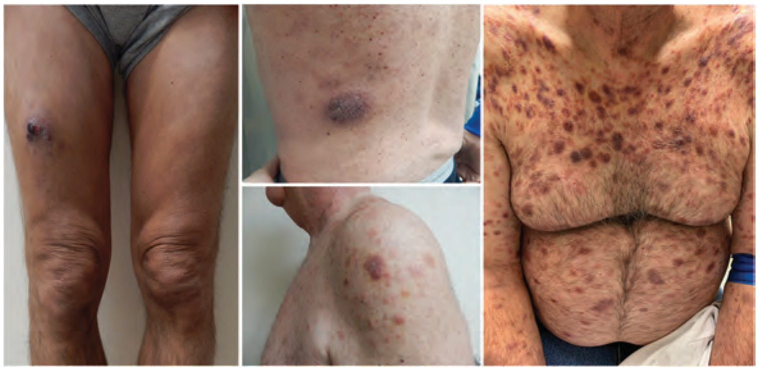


Figure 64.1 BLASTIC PLASMACYTOID DENDRITIC CELL NEOPLASM (BPDCN) SKIN LESION PHOTOGRAPHS FROM FOUR INDIVIDUALS SHOWING DIFFERENT PRESENTATIONS OF CUTANEOUS DISEASE.

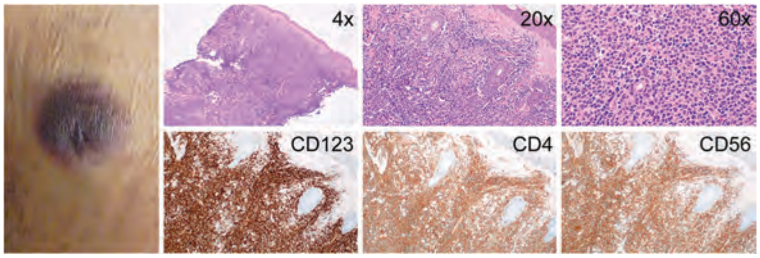


Figure 64.2 BLASTIC PLASMACYTOID DENDRITIC CELL NEOPLASM (BPDCN) SKIN LESION, SKIN BIOPSY PHOTOMICROGRAPHS, AND IMMUNOHISTOCHEMISTRY (AT 20× MAGNIFICATION) FOR CD123, CD4, AND CD56.

DIFFERENTIAL DIAGNOSIS

Acute Myeloid Leukemia and Other CD56+ Neoplasms With Skin Involvement

Given the association with preexisting hematopoietic neoplasms, all patients with myeloid disorders, including MDS, CMML, AML, myeloproliferative neoplasms, and clonal hematopoiesis who develop skin lesion(s) should undergo a biopsy and evaluation for BPDCN (Box 64.1). Collaboration with a dermatologist and hematopathologist is recommended. The principal differential diagnoses include hematologic malignancies that are CD56 positive and/or present with skin lesions (Table 64.1). AML can be CD123, CD4, and CD56 positive, and some are CD34 negative. In these cases, myeloperoxidase or lysozyme positivity, or presence of other monocytic/myelomonocytic markers such as CD13, CD15, or CD117 favor a diagnosis of AML over BPDCN. Negativity for CD123 or TCL-1 also favors AML over BPDCN. The most challenging situation is when there are no skin lesions; in those cases, immunophenotyping on bone marrow biopsy immunohistochemistry and flow cytometry must be used to distinguish between AML and BPDCN. Extranodal NK/T-cell lymphoma may involve the skin and express CD56 but is associated with Epstein-Barr virus positivity and is CD123 negative. Cutaneous T-cell lymphoma (CTCL) can also be CD56 positive, but expresses mature T-cell markers (CD2, CD3, CD5, CD7, CD8) and is negative for CD123.

Mature Plasmacytoid Dendritic Cell Proliferation

Mature plasmacytoid dendritic cell proliferation (MPDCP) is associated with myeloid neoplasms, particularly CMML, MDS, and AML, but can be seen in any myeloid neoplasm and especially those with monocytic differentiation.[14] MPDCP is usually detected as clonally expanded aggregates of pDCs distinguishable from BPDCN by their clustered localization, mature morphology, and CD56 negativity. MPDCPs are characterized by a low Ki-67 proliferation index (<10%) and they lack TdT. In contrast, in BPDCN, TdT is positive in about one-third of cases, with expression in 10% to 80% of the cells and the Ki-67 proliferation index is 20% to 80%. There is no clear evidence that patients with MPDCP are more likely to develop subsequent BPDCN, but this has not been studied in great depth.

BOX 64.1 Diagnostic Dilemma: Skin Lesions in a Patient With Myelodysplastic Syndrome

A 71-year-old man has a 9-month history of myelodysplastic syndrome (MDS), classified as MDS-EB2 (with 12% bone marrow blasts at the time of diagnosis). The MDS blasts were CD45 positive, CD34 positive, myeloperoxidase positive, CD123 negative, and HLA-DR negative. After 4 cycles of decitabine monotherapy he has stable bone marrow blast percentage and no significant change in his pre-treatment anemia and thrombocytopenia. He presents to clinic with a new purple, painless, 3 cm diameter raised tumor on his forehead. Skin biopsy reveals a dense dermal infiltrate of medium to large-sized cells with blastoid appearance that are CD45 positive and concerning for myeloid sarcoma on histopathology. However, upon further staining, the skin tumor cells are CD34 negative, myeloperoxidase and lysozyme negative, CD123 bright, and HLA-DR positive, raising the possibility of BPDCN. Additional immunohistochemistry revealed that the skin tumor cells were also CD4, CD56, and TCL1 positive, confirming the diagnosis of BPDCN.

PROGNOSIS

The median survival of adults with BPDCN is between 12 and 24 months, irrespective of initial pattern of disease.[15,16] Children have better outcomes, likely owing to higher rates of complete remission after initial chemotherapy and lower likelihood of relapse after entering remission.[3] Positivity for TdT, negativity for CD303, and high Ki-67 proliferation index are associated with improved survival, whereas age over 60 and abnormal karyotype portend worse survival.[16] Biallelic loss of 9p21.3 and haploinsufficiency of 5q31 are associated with poor prognosis, as may be mutation of *TET2*, *IDH1*, *IDH2*, or *DNMT3A*.[17,18] Independent association of specific clinical or molecular features with prognosis remains difficult given the relatively small sample size of most studies.

THERAPY

Hematopoietic cell transplantation (HCT) is the only known curative therapy for BPDCN, with most data suggesting that allogeneic HCT (allo-HCT) is associated with better outcomes than autologous hematopoietic cell transplantation (auto-HCT). Therefore, our overall management strategy is to induce a complete remission and then proceed to allo-HCT (Fig. 64.3). The only drug approved specifically for BPDCN is tagraxofusp-erzs (previously called SL-401), a recombinant protein consisting of interleukin-3 (IL-3) fused to a truncated diphtheria toxin (DT) payload. Tagraxofusp delivers DT to cells that express CD123, which is the α chain of the IL-3 receptor and is highly expressed on all BPDCNs. Our practice is to treat adult patients with tagraxofusp as first-line induction therapy. If ineligible for tagraxofusp, due to specific safety parameters such as low baseline serum albumin (<3.2 mg/dL) or if tagraxofusp is unavailable, we recommend intensive induction with acute leukemia chemotherapy, preferably an acute lymphoid leukemia (ALL) regimen (e.g., hyper-CVAD, parts A and B). There are no data comparing tagraxofusp to intensive induction chemotherapy for initial treatment of BPDCN. We recommend using tagraxofusp first, followed by salvage intensive chemotherapy if a CR is not achieved, rather than the reverse order, because tagraxofusp is less likely to cause lasting toxicities that preclude subsequent intensive induction

TABLE 64.1 Hematologic Malignancies That Involve the Skin and are Often CD56+

	CD123	CD4	CD56	MPO and/or Lysozyme	EBV Markers	Mature T-Cell Markers (CD2, CD3, CD5, CD7, CD8)
BPDCN	++	+	+	–	–	–
AML or myeloid sarcoma	– to ++	– to +	– to +	+	–	–
NK/T-cell lymphoma	–	–	+	–	+	+
CTCL	–	–	+	–	–	+

These are the most common immunophenotypes. Aberrant expression of any one of these markers can occur in some cases, so no single marker should be used to make a diagnosis. *AML*, Acute myeloid leukemia; *BPDCN*, plastic plasmacytoid dendritic cell neoplasm; *CTCL*, cutaneous T-cell lymphoma; *EBV*, Epstein Barr virus; *MPO*, myeloperoxidase; *NK*, natural killer.

Adapted from Venugopal S, Zhou S, El Jamal SM, Lane AA, Mascarenhas J. Blastic plasmacytoid dendritic cell neoplasm—current insights. *Clin Lymphoma Myeloma Leuk*. 2019;19:545–54.

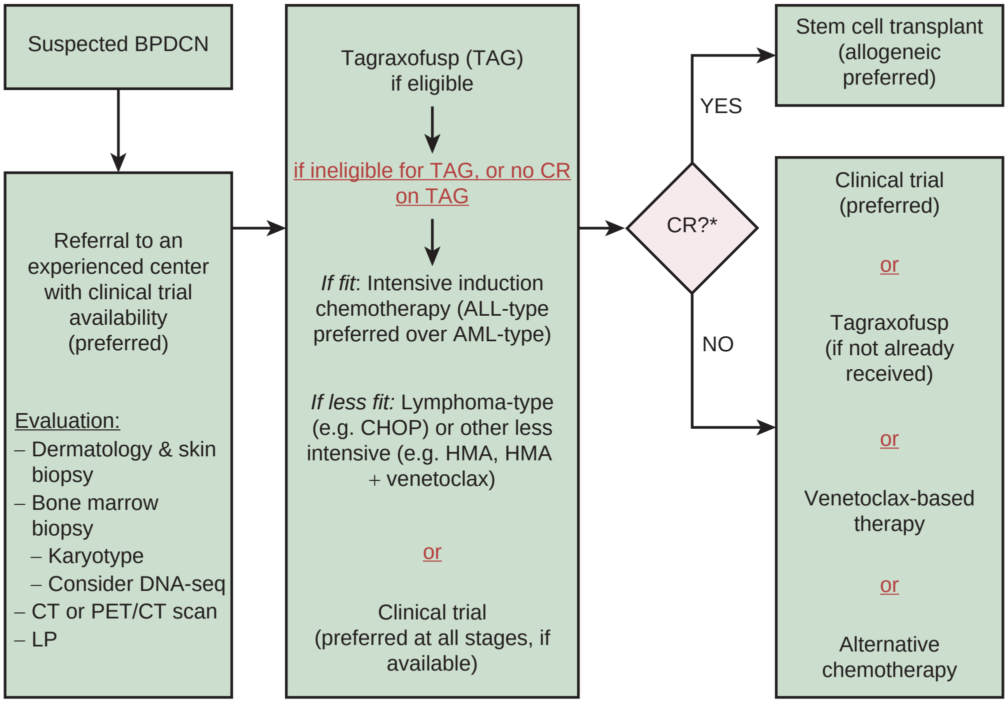

Suspected BPDCN
Referral to an experienced center with clinical trial availability (preferred)
Evaluation:
– Dermatology & skin biopsy
– Bone marrow biopsy
– Karyotype
– Consider DNA-seq
– CT or PET/CT scan
– LP
Tagraxofusp (TAG) if eligible
if ineligible for TAG, or no CR on TAG
If fit: Intensive induction chemotherapy (ALL-type preferred over AML-type)
If less fit: Lymphoma-type (e.g. CHOP) or other less intensive (e.g. HMA, HMA + venetoclax)
or
Clinical trial (preferred at all stages, if available)
CR?*
YES
NO
Stem cell transplant (allogeneic preferred)
Clinical trial (preferred)
or
Tagraxofusp (if not already received)
or
Venetoclax-based therapy
or
Alternative chemotherapy

Figure 64.3 EVALUATION AND TREATMENT ALGORITHM FOR ADULTS WITH BLASTIC PLASMACYTOID DENDRITIC CELL NEOPLASM (BPDCN). *CT*, Computed tomography; *PET*, 18-fluoro-deoxyglucose positron emission tomography; *LP*, lumbar puncture; *ALL*, acute lymphoid leukemia; *AML*, acute myeloid leukemia; *CHOP*, cyclophosphamide, doxorubicin, vincristine, prednisone; *HMA*, hypomethylating agent (e.g., azacitidine or decitabine); *CR*, complete remission (*including CRc and CRi, for definitions see text).

chemotherapy. In contrast, patients who have received intensive induction chemotherapy may have infectious complications after myelosuppression or hypoalbuminemia following prolonged hospitalization that could make them ineligible for tagraxofusp. Patients who achieve complete remission with induction therapy, either with tagraxofusp or chemotherapy, are then referred for allo-HCT. However, given the relatively small sample size of studies to date and lack of head-to-head comparative data, clinical trial participation is preferred at all stages of the disease.

Tagraxofusp

Tagraxofusp was approved by the US Food and Drug Administration in 2018 based on the results of a phase 1/2 multicenter, prospective, open-label clinical trial for patients with either treatment-naïve or relapsed/refractory BPDCN.[19] The median age was 70 (range 22 to 84) years old. Among the 29 subjects treated at the FDA-approved dose of 12 μg/kg daily for 5 days as first-line therapy, the rate of complete response (CR) or clinical complete response (CRc, defined as complete response in the marrow and lymph nodes/extramedullary sites with only microscopic disease remaining on skin biopsy) was 72% (95% confidence interval [CI], 53 to 87). The median time until response was 43 days (range, 14 to 131). The rate of complete, clinical complete, and partial responses (CR + CRc + PR) among the 29 therapy-naïve patients was approximately 90% ($n = 26$ of 29). About 45% of the treatment-naïve patients went to HCT (10 allogeneic, 3 autologous) after receiving tagraxofusp, all of whom were in a CR or CRc. The median OS for first-line treated patients was not reached (25 months follow-up), nor was the median duration of response (19 months follow-up). Two patients remained in CR on continued tagraxofusp without transplant for more than 1 year (>15 and >30 months), but with these small numbers of patients the long-term efficacy of tagraxofusp alone is not clear. In those with relapsed or refractory disease, treatment with tagraxofusp resulted in CR/CRc/CRi (complete response with incomplete count recovery) in 5 of 15 patients and an overall response rate (PR or better) of 67% (95% CI, 33 to 88). One patient with relapsed BPDCN achieved a CRc with tagraxofusp and went on to allo-HCT.

Tagraxofusp Side Effects and Management of Capillary Leak Syndrome

Grade 3 or higher thrombocytopenia and liver enzyme elevations (alanine/aspartate aminotransferases [ALT/AST]) were seen in about 40% of patients on the tagraxofusp trial, but these did not lead to clinical complications and resolved within 4 weeks of the first dose in nearly all patients. Approximately half of patients developed hypoalbuminemia or peripheral edema and 20% developed frank capillary leak syndrome (CLS). CLS is related to endothelial damage and manifests as one or more of: weight gain, hypoalbuminemia, edema, pleural or pericardial effusions, hypotension, and fever. CLS is a known side effect of diphtheria and pseudomonas toxin conjugate drugs and therefore, tagraxofusp should not be given to patients with baseline hypoalbuminemia or reduced cardiac function. Our practice is to follow the eligibility criteria from the registration trial and only give tagraxofusp to patients with baseline albumin of ≥ 3.2 g/dL (in the absence of exogenous supplementation with intravenous albumin) and a normal left ventricular ejection fraction.

The FDA approved dose of tagraxofusp is 12 μg/kg daily over 15 minutes for 5 days with cycles repeated every 3 weeks. However, dosing should be interrupted or halted in a given cycle if there are signs or symptoms of clinically important CLS, such as falling albumin concentration, weight gain, pulmonary edema, or hypotension. Specifically, if any of the following parameters occur, then no further tagraxofusp should be given during a cycle: albumin below 3.0 g/dL or ALT/AST greater than 5 times the upper limit of normal. Other clinical and lab abnormalities suggestive of CLS warrant holding tagraxofusp during a given cycle until the findings resolve: weight gain of more than 1.5 kg in 24 hours; albumin below 3.5 g/dL if baseline was 4.0 g/dL or higher, or any drop of more than 0.5 g/dL in 24 hours. In these situations, we recommend giving intravenous albumin, 25 g every 6 to 12 hours until albumin is greater or equal to 3.5 g/dL AND within 0.5 g/dL of baseline. Diuretics are recommended as tolerated for volume overload. For patients with CLS and signs of a systemic inflammatory response (fever, hypotension) consider giving corticosteroids, such as solumedrol 1 mg/kg daily, until resolution (Box 64.2).

Chemotherapy

An alternative to tagraxofusp for patients who are otherwise "fit" is intensive acute leukemia-type induction chemotherapy. Similar to tagraxofusp, if the patient is eligible for allo-HCT the goal of chemotherapy is to induce a complete remission and proceed to transplant. Given that there are no head-to-head comparisons of induction regimens, we recommend tagraxofusp as our first choice, as discussed previously. If unable to use tagraxofusp, we recommend induction chemotherapy. In retrospective studies and in our personal experience, less-intensive therapies (e.g., CHOP) appear to be inferior to conventional intensive acute leukemia-type regimens.[16] Among intensive induction regimens, ALL-type may be more effective than AML-type, although they have not been compared in randomized trials. Intensive induction therapies such as hyper-CVAD (cyclophosphamide, vincristine, doxorubicin (Adriamycin), and dexamethasone) have substantial response rates in BPDCN but the duration of response is often short with relapsed disease being difficult to treat and control. Another practical challenge is the advanced median age of patients with BPDCN who are, in most cases, unable to tolerate intensive induction chemotherapy, which is why tagraxofusp may be particularly useful in achieving remission. When tagraxofusp is not an option, our recommendation is for ALL-type intensive induction (e.g., hyper-CVAD or an L-asparaginase-containing induction regimen) for younger, "fit" patients. For older or "less fit" patients, we recommend age-adjusted ALL induction chemotherapy regimens or anthracycline-based lymphoma chemotherapy such as CHOP. Additional less intensive regimens with activity are discussed later.

Disease Response Assessment in Blastic Plasmacytoid Dendritic Cell Neoplasm

There are no formal standardized disease assessment criteria specifically for BPDCN, but most clinical trials are using the schema defined in the tagraxofusp registration trial. This has become the de facto standard in our clinical practice. Each disease compartment is assessed using criteria adopted from other hematologic malignancies: AML criteria for bone marrow biopsy and peripheral blood; lymphoma criteria for lymph nodes, spleen, and other extramedullary sites by CT or PET-CT scan; and the modified Severity Weighted Assessment Tool (mSWAT) for quantitative skin malignancy assessment originally designed for cutaneous T-cell lymphoma trials.[19] To be considered in a CR, a patient must have a CR in all three compartments. For patients who receive tagraxofusp, we usually assess for response after 2 cycles and then every 2 to 4 cycles based on the clinical situation. There are no data on the minimum number of cycles of tagraxofusp necessary prior to allo-HCT. Our practice is to proceed with allo-HCT once CR is achieved (here, we consider CR, CRc, or CRi acceptable to undergo allo-HCT), continuing tagraxofusp every 3 weeks with cessation of therapy immediately prior to the procedure. For patients receiving chemotherapy instead of tagraxofusp, we use these same response criteria and similar approach toward allo-HCT, with no minimum required number of chemotherapy cycles.

BOX 64.2 Management of Capillary Leak Syndrome During Tagraxofusp Therapy

A 65-year-old man with newly diagnosed blastic plasmacytoid dendritic cell neoplasm (BPDCN) involving skin and bone marrow is being treated with tagraxofusp. He is admitted to the inpatient hospital for cycle 1, days 1–6 (until at least 1 day after the last dose of tagraxofusp), as we recommend for all patients in the first cycle to provide close monitoring for tagraxofusp-associated capillary leak syndrome (CLS). On the fourth day of planned 5 days of therapy, he is found to have gained 2 kg from the prior day, his blood oxygen saturation is 91% on room air (down from 98%), he has new peripheral edema, and his albumin has dropped to 2.9 g/dL from 3.6 g/dL at baseline. The fourth and fifth doses of tagraxofusp in cycle 1 are cancelled. He is treated with intravenous albumin, 25 g twice daily, and intravenous furosemide for diuresis. Over the next 2 days, his weight returns to baseline and serum albumin recovers to 3.5 g/dL. He is discharged from the hospital and has no further signs or symptoms of CLS. Tagraxofusp is restarted in cycle 2 at the standard dose, with inpatient monitoring given prior CLS. He has no recurrence of CLS and future tagraxofusp cycles are administered in the outpatient setting.

Alternative Regimens for First-Line Therapy and Treatment of Relapsed/Refractory Disease

Clinical trials are preferred (Table 64.2). For those treatment-naïve patients unlikely to tolerate intensive induction, there are many regimens that have reported activity in BPDCN. None have been compared in randomized studies. These include lymphoma regimens such as CHOP, hypomethylating agent-based regimens with azacitidine or decitabine, pralatrexate, gemcitabine +/− docetaxel, bendamustine, and bortezomib-based therapy. In addition, BPDCN is dependent on the antiapoptotic protein BCL2 for survival and is sensitive to the BCL2 inhibitor, venetoclax.[11] Disease responses have been reported with single-agent venetoclax and in combination with hypomethylating agents or the hyper-CVAD regimen.[16,20,21] Mutations in *IDH1* and *IDH2* are present in 10% to 15% of BPDCNs and there are data demonstrating activity of enasidenib, an IDH2 inhibitor, in BPDCN with an *IDH2* mutation.[16] *FLT3* mutations are not common in BPDCN but are occasionally observed. Therefore, given that there are approved IDH1, IDH2, and FLT3 inhibitors for AML, it is reasonable to perform DNA sequencing to evaluate for these mutations and consider using them per AML treatment guidelines in patients with relapsed or refractory BPDCN.[22]

Radiation therapy is active against BPDCN cells and can be useful for emergent treatment of lesions with mass effect. Radiation has been used to treat skin-only disease, but most patients eventually progress with systemic disease and radiation is therefore not commonly used as part of curative-intent therapy.[23] CNS involvement is more frequent in the setting of relapsed/refractory disease and should be evaluated in any patients with symptoms. Lumbar puncture may be beneficial in all patients, including at initial diagnosis, to evaluate for asymptomatic disease. Active CNS BPDCN is associated with very poor outcomes and is commonly managed by intrathecal chemotherapy (methotrexate and/or cytarabine) or radiation, although there are very little data to guide treatment. Systemic corticosteroids can be useful as short-term palliative treatment for relapsed BPDCN or if needed acutely to control lesions with mass effect or symptomatic CNS BPDCN.

There are several additional therapies undergoing clinical evaluation in BPDCN, including other agents targeting CD123 and small molecule targeted therapies (Fig. 64.4).[22] Analysis of patient samples before and after tagraxofusp suggest that loss of CD123 is not a common mechanism of resistance, but instead, resistance is related to downregulation of the diphthamide synthesis pathway that is necessary for DT sensitivity.[24] Thus, clinical evaluation of sequential CD123-targeting agents is justified. Novel agents targeting CD123 include anti-CD123 antibody-drug conjugates, CD3 × CD123 bispecific T-cell engagers, and CD123-directed chimeric antigen receptor T cells (CD123 CAR-T).

Hematopoietic Cell Transplantation

Allo-HCT is recommended for all eligible adult patients with BPDCN in first complete remission (CR1). Long-term overall and disease-free survival are higher in patients with BPDCN who receive transplant compared to chemotherapy alone, although the data are

TABLE 64.2 **Selected Clinical Trials for Patients With Blastic Plasmacytoid Dendritic Cell Neoplasm**

Agent	Phase	Key Eligibility	Status	Clinicaltrials.Gov ID#
Venetoclax +/– azacitidine	1	Relapsed disease, or treatment-naïve but ineligible for standard therapy	Recruiting	NCT03485547
IMGN632 (anti-CD123 ADC)	1	Treatment-naïve or relapsed disease	Recruiting	NCT03386513
XmAb14045 (CD123 x CD3 bispecific T-cell engager)	1	Relapsed disease	Recruiting	NCT02730312
CD123 CAR-T cell (autologous)	1	Relapsed disease	Recruiting	NCT02159495
Combination chemotherapy (idarubicin, methotrexate, L-asparaginase, dexamethasone) followed by hematopoietic cell transplantation (auto- or allo- transplant)	2	Treatment-naïve	Recruiting	NCT03599960
Hyper-CVAD/mini-CVD + venetoclax + tagraxofusp	2	Treatment-naïve or relapsed disease	Recruiting	NCT04216524
Flotetuzumab (CD123 × CD3 bispecific T-cell engager)	2	Relapsed disease	Anticipated	NCT03739606

ADC, Antibody-drug conjugate; *CAR*, chimeric antigen receptor.
Clinicaltrials.gov accessed June 10, 2020.

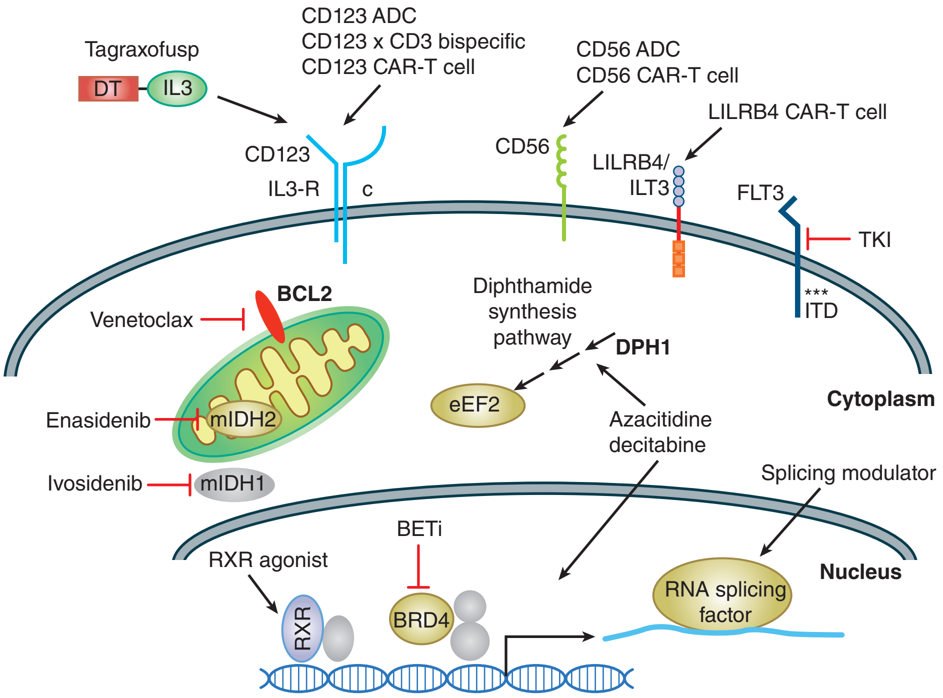


Figure 64.4 NOVEL TARGETS AND AGENTS UNDER INVESTIGATION IN BLASTIC PLASMACYTOID DENDRITIC CELL NEOPLASM (BPDCN). Tagraxofusp is approved for treatment of BPDCN in the United States. Tagraxofusp is being combined with chemotherapy, including azacitidine, and/or venetoclax in clinical trials. Several additional CD123-targeting agents are in clinical trials. The other drugs listed are described as having activity in clinical case reports or supportive evidence via mutation profiling or pre-clinical models of BPDCN. *ADC*, Antibody-drug conjugate; *BETi*, bromodomain and extraterminal motif inhibitor; *CAR*, chimeric antigen receptor; *DT*, diphtheria toxin; *eEF2*, eukaryotic elongation factor 2; *IL3*, interleukin 3; *mIDH1* and *mIDH2*, mutant isocitrate dehydrogenase 1 and 2; *RXR*, retinoid X receptor; *TKI*, tyrosine kinase inhibitor; ****ITD*, internal tandem duplication mutation in *FLT3*. *(Image is from Figure 1 [page 590] of Lane AA. Novel therapies for blastic plasmacytoid dendritic cell neoplasm. Hematol Oncol Clin North Am. 2020;34:589–600.)*

retrospective and subject to inclusion and eligibility biases. Transplant in the setting of any residual active disease is associated with dismal prognosis. A meta-analysis of 128 adult patients (from four studies) who received allogeneic transplant for BPDCN reported approximately 50% (95% CI, 41 to 59) OS at 4 years. OS was 67% (95% CI, 52 to 80) in those who were transplanted in CR1 versus 7% (95% CI, 0 to 32) in those transplanted in any state less than CR or after first disease relapse.[25] The analysis also confirmed decreased relapse rates using myeloablative compared to reduced intensity conditioning (RIC; 18% vs. 40%, respectively). These results translated into higher OS of 55% for myeloablative compared to 44% for RIC. Based on these and results from a European Blood and Marrow Transplantation (EBMT) group study,[26] our practice is to offer allo-HCT, preferably with myeloablative conditioning (MAC), to all eligible adult patients in CR (including CRc and CRi).

In our practice we have observed complete regression of disease following rapid immunosuppressive taper in patients with relapsed disease after allo-HCT suggestive of a graft-versus-BPDCN immune effect. This observation supports the utility of RIC transplants in older and frailer patients who cannot tolerate MAC. In contrast, autologous transplantation relies on the high-dose chemotherapy effect. One study in a Japanese population reported 4-year disease-free survival of over 80% in 11 patients after auto-HCT.[27] However, a retrospective analysis in a North American population found that auto-HCT was associated with only 11% 1-year OS.[28] In our practice, we do not recommend autologous transplantation for BPDCN except in limited situations. It is reasonable to consider consolidation with auto-HCT for patients with chemosensitive disease in CR who are ineligible or decline allo-HCT.

Treatment of Blastic Plasmacytoid Dendritic Cell Neoplasm in Children (<18 Years of Age)

Response to therapy and disease-free survival are better in children than in adults with BPDCN.[3] A high-risk ALL-type induction chemotherapy regimen with screening lumbar puncture and prophylactic CNS-directed therapy per standard lymphoid leukemia practice is recommended. Given the relatively higher disease-free survival after achieving remission in children, allogeneic transplant is usually not recommended in CR1. Pediatric patients should continue on consolidation and maintenance chemotherapy regimens as used for high-risk ALL in CR1. Allo-HCT is recommended for children who experience relapse and achieve CR2. Published experience with tagraxofusp in children is limited to the relapsed/refractory setting. Tagraxofusp was approved by the FDA for ages 2 and up based on one report of safety and short-term efficacy in three patients with relapsed BPDCN.[29] However, we do not recommend tagraxofusp as first-line therapy in pediatric BPDCN (<18 years of age) given the absence of efficacy and survival data in treatment-naïve disease and because of the relatively favorable outcomes with conventional ALL-type chemotherapy.[30] Whether young adults benefit most from a pediatric or adult approach is not known. In our practice, we have treated patients 18 years of age and older with the adult algorithm outlined previously.

FUTURE DIRECTIONS

Progress in BPDCN research requires improved scientific understanding of the unique disease pathobiology and performance of disease-specific clinical trials. We recommend enrolling patients on clinical studies whenever possible and encourage investigators to design BPDCN-specific trials that evaluate response in all sites of disease involvement. Important questions that are unanswered include comparison of tagraxofusp with intensive induction chemotherapy, particularly in the first-line setting for younger patients. We must determine if combination therapy is superior to currently available treatments, including testing tagraxofusp with intensive or nonintensive chemotherapy, and adding venetoclax to tagraxofusp or to chemotherapy. We also need to determine if maintenance therapy in the setting of CR after chemotherapy or HCT (autologous or allogeneic) has clinical benefit and which agent(s) are most effective in the maintenance setting. Finally, a better understanding of how to assess measurable residual disease (MRD) in BPDCN may improve comparison of active therapies and should be incorporated into clinical trial endpoints, particularly in studies of maintenance therapy and after transplant.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

DiNardo CD, Rausch CR, Benton C, et al. Clinical experience with the BCL2-inhibitor venetoclax in combination therapy for relapsed and refractory acute myeloid leukemia and related myeloid malignancies. *Am J Hematol*. 2018;93:401–407.

Kharfan-Dabaja MA, Reljic T, Murthy HS, et al. Allogeneic hematopoietic cell transplantation is an effective treatment for blastic plasmacytoid dendritic cell neoplasm in first complete remission: systematic review and meta-analysis. *Clin Lymphoma Myeloma Leuk*. 2018;18:703–709 e1.

Kim MJ, Nasr A, Kabir B, et al. Pediatric blastic plasmacytoid dendritic cell neoplasm: a systematic literature review. *J Pediatr Hematol Oncol*. 2017;39:528–537.

Lane AA. Novel therapies for blastic plasmacytoid dendritic cell neoplasm. *Hematol Oncol Clin North Am*. 2020;34:589–600.

Montero J, Stephansky J, Cai T, et al. Blastic plasmacytoid dendritic cell neoplasm is dependent on BCL2 and sensitive to venetoclax. *Cancer Discov*. 2017;7:156–164.

Pemmaraju N, Lane AA, Sweet KL, et al. Tagraxofusp in blastic plasmacytoid dendritic-cell neoplasm. *N Engl J Med*. 2019;380:1628–1637.

Sun W, Liu H, Kim Y, et al. First pediatric experience of SL-401, a CD123-targeted therapy, in patients with blastic plasmacytoid dendritic cell neoplasm: report of three cases. *J Hematol Oncol*. 2018;11:61.

Taylor J, Haddadin M, Upadhyay VA, et al. Multicenter analysis of outcomes in blastic plasmacytoid dendritic cell neoplasm offers a pretargeted therapy benchmark. *Blood*. 2019;134:678–687.

Togami K, Pastika T, Stephansky J, et al. DNA methyltransferase inhibition overcomes diphthamide pathway deficiencies underlying CD123-targeted treatment resistance. *J Clin Invest*. 2019;129:5005–5019.

Venugopal S, Zhou S, El Jamal SM, et al. Blastic plasmacytoid dendritic cell neoplasm-current insights. *Clin Lymphoma Myeloma Leuk*. 2019;19:545–554.

REFERENCES

1. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127:2391–2405.
2. Pagano L, Valentini CG, Grammatico S, Pulsoni A. Blastic plasmacytoid dendritic cell neoplasm: diagnostic criteria and therapeutical approaches. *Br J Haematol*. 2016;174:188–202.
3. Kim MJ, Nasr A, Kabir B, et al. Pediatric blastic plasmacytoid dendritic cell neoplasm: a systematic literature review. *J Pediatr Hematol Oncol*. 2017;39:528–537.
4. Nomburg J, Bullman S, Chung SS, et al. Comprehensive metagenomic analysis of blastic plasmacytoid dendritic cell neoplasm. *Blood Adv*. 2020;4:1006–1011.
5. Griffin GK, Togami K, Morgan EA, Lane AA. Developmental ontogeny of blastic plasmacytoid dendritic cell neoplasm (BPDCN) revealed by recurrent high burden clonal hematopoiesis, including in “Skin-Only“ disease. *Blood*. 2018;132:2755.
6. Sakamoto K, Takeuchi K. Cytogenetics of blastic plasmacytoid dendritic cell neoplasm: chromosomal rearrangements and DNA copy-number alterations. *Hematol Oncol Clin North Am*. 2020;34:523–538.
7. Sakamoto K, Katayama R, Asaka R, et al. Recurrent 8q24 rearrangement in blastic plasmacytoid dendritic cell neoplasm: association with immunoblastoid cytomorphology, MYC expression, and drug response. *Leukemia*. 2018;32:2590–2603.
8. Suzuki K, Suzuki Y, Hama A, et al. Recurrent MYB rearrangement in blastic plasmacytoid dendritic cell neoplasm. *Leukemia*. 2017;31:1629–1633.
9. Sapienza MR, Abate F, Melle F, et al. Blastic plasmacytoid dendritic cell neoplasm: genomics mark epigenetic dysregulation as a primary therapeutic target. *Haematologica*. 2019;104:729–737.
10. Villani AC, Satija R, Reynolds G, et al. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science*. 2017:356.
11. Montero J, Stephansky J, Cai T, et al. Blastic plasmacytoid dendritic cell neoplasm is dependent on BCL2 and sensitive to venetoclax. *Cancer Discov*. 2017;7:156–164.
12. Sapienza MR, Fuligni F, Agostinelli C, et al. Molecular profiling of blastic plasmacytoid dendritic cell neoplasm reveals a unique pattern and suggests selective sensitivity to NF-κB pathway inhibition. *Leukemia*. 2014;28: 1606–1616.
13. Julia F, Dalle S, Duru G, et al. Blastic plasmacytoid dendritic cell neoplasms: clinico-immunohistochemical correlations in a series of 91 patients. *Am J Surg Pathol*. 2014;38:673–680.
14. Tzankov A, Hebeda K, Kremer M, et al. Plasmacytoid dendritic cell proliferations and neoplasms involving the bone marrow: summary of the workshop cases submitted to the 18th Meeting of the European Association for Haematopathology (EAHP) organized by the European Bone Marrow Working Group, Basel 2016. *Ann Hematol*. 2017;96:765–777.
15. Pagano L, Valentini CG, Pulsoni A, et al. Blastic plasmacytoid dendritic cell neoplasm with leukemic presentation: an Italian multicenter study. *Haematologica*. 2013;98:239–246.
16. Taylor J, Haddadin M, Upadhyay VA, et al. Multicenter analysis of outcomes in blastic plasmacytoid dendritic cell neoplasm offers a pretargeted therapy benchmark. *Blood*. 2019;134:678–687.
17. Lucioni M, Novara F, Fiandrino G, et al. Twenty-one cases of blastic plasmacytoid dendritic cell neoplasm: focus on biallelic locus 9p21.3 deletion. *Blood*. 2011;118:4591–4594.
18. Menezes J, Acquadro F, Wiseman M, et al. Exome sequencing reveals novel and recurrent mutations with clinical impact in blastic plasmacytoid dendritic cell neoplasm. *Leukemia*. 2014;28:823–829.
19. Pemmaraju N, Lane AA, Sweet KL, et al. Tagraxofusp in blastic plasmacytoid dendritic-cell neoplasm. *N Engl J Med*. 2019;380:1628–1637.
20. DiNardo CD, Rausch CR, Benton C, et al. Clinical experience with the BCL2-inhibitor venetoclax in combination therapy for relapsed and refractory acute myeloid leukemia and related myeloid malignancies. *Am J Hematol*. 2018;93:401–407.
21. Pemmaraju N, Konopleva M, Lane AA. More on blastic plasmacytoid dendritic-cell neoplasms. *N Engl J Med*. 2019;380:695–696.
22. Lane AA. Novel therapies for blastic plasmacytoid dendritic cell neoplasm. *Hematol Oncol Clin North Am*. 2020;34:589–600.
23. Ishibashi N, Maebayashi T, Aizawa T, et al. Radiation therapy for cutaneous blastic plasmacytoid dendritic cell neoplasm: a case report and review of the literature. *Int J Clin Exp Med*. 2015;8:8204–8209.
24. Togami K, Pastika T, Stephansky J, et al. DNA methyltransferase inhibition overcomes diphthamide pathway deficiencies underlying CD123-targeted treatment resistance. *J Clin Invest*. 2019;129:5005–5019.
25. Kharfan-Dabaja MA, Reljic T, Murthy HS, et al. Allogeneic hematopoietic cell transplantation is an effective treatment for blastic plasmacytoid dendritic cell neoplasm in first complete remission: systematic review and meta-analysis. *Clin Lymphoma Myeloma Leuk*. 2018;18:703–709 e1.
26. Roos-Weil D, Dietrich S, Boumendil A, et al. Stem cell transplantation can provide durable disease control in blastic plasmacytoid dendritic cell neoplasm: a retrospective study from the European Group for Blood and Marrow Transplantation. *Blood*. 2013;121:440–446.
27. Aoki T, Suzuki R, Kuwatsuka Y, et al. Long-term survival following autologous and allogeneic stem cell transplantation for blastic plasmacytoid dendritic cell neoplasm. *Blood*. 2015;125:3559–3562.
28. Kharfan-Dabaja MA, Al Malki MM, Deotare U, et al. Haematopoietic cell transplantation for blastic plasmacytoid dendritic cell neoplasm: a North American multicentre collaborative study. *Br J Haematol*. 2017;179: 781–789.
29. Sun W, Liu H, Kim Y, et al. First pediatric experience of SL-401, a CD123-targeted therapy, in patients with blastic plasmacytoid dendritic cell neoplasm: report of three cases. *J Hematol Oncol*. 2018;11:61.
30. Venugopal S, Zhou S, El Jamal SM, et al. Blastic plasmacytoid dendritic cell neoplasm-current insights. *Clin Lymphoma Myeloma Leuk*. 2019;19:545–554.

CHAPTER 65

MYELODYSPLASTIC SYNDROMES AND MYELOPROLIFERATIVE NEOPLASMS IN CHILDREN

Elliot Stieglitz, Christopher C. Dvorak, and Benjamin S. Braun

Myelodysplastic syndromes (MDS) and myeloproliferative neoplasms (MPN) are a heterogeneous group of clonal stem cell disorders that result in ineffective hematopoiesis and an increased risk of developing acute myeloid leukemia (AML). Whereas in MDS ineffective hematopoiesis results in progressive cytopenias, in MPN ineffective hematopoiesis leads to excessive proliferation frequently characterized by increased peripheral blood counts. MDS and MPN are rare in children, and the only malignancy with overlapping features of both MDS and MPN in childhood is referred to as juvenile myelomonocytic leukemia (JMML). Chronic myeloid leukemia (CML), characterized by the Philadelphia chromosome (*BCR/ABL*-positive), is more common in adults and seen infrequently in children. However, other forms of MPN including polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis, chronic neutrophilic leukemia, chronic eosinophilic leukemia, chronic basophilic leukemia, chronic myelomonocytic leukemia, systemic mastocytosis (SM), and stem cell leukemia–lymphoma syndrome) are exceedingly rare in children. Additional information regarding disorders that are predominantly found in adults are referred to in Chapters 70–75. In contrast, two MPNs are uniquely pediatric: JMML and Down syndrome–associated transient abnormal myelopoiesis (TAM) will be the main focus of this chapter.

MYELODYSPLASTIC SYNDROMES

The MDS are a heterogeneous group of clonal disorders characterized by ineffective hematopoiesis, impaired maturation of hematopoietic cells, progressive cytopenias, and dysplastic changes in the bone marrow (BM).

Epidemiology

MDS is a common malignancy of adults, with an incidence of 50 cases per million in people older than the age of 60 years.[1] In contrast, MDS accounts for only 3% to 7% of all hematologic malignancies in children, with an unknown true incidence, owing in part to the inclusion of children with Down syndrome in some estimates. Several population-based studies have been performed with reported incidences of 4.0 cases per million in Denmark,[2] 3.1 per million in British Columbia (Canada),[3] and 1.35 per million in the United Kingdom.[4] The median age of presentation is 6.8 years with an equal sex distribution.[4–6] However, in a study of children with advanced or high-risk MDS, the median age of presentation was older, at 10.7 years with a 2:1 male-to-female ratio.[7]

Pathobiology

As in adults, MDS in children can be considered as primary (de novo) or secondary. In adults, two predominant patterns of primary, de novo MDS have been observed. In the first of these patterns, the disease is indolent in nature and is characterized by prolonged survival, little accumulated genetic damage, and a low probability of progression to AML. This group of diseases is best exemplified by the 5q− syndrome, an entity not seen in children. Far more common in adults is a disease characterized by the accumulation of genetic changes, progression to BM failure, and a high probability of developing AML. This form of the disease is characterized as a mutator phenotype.[8] The primary MDS seen in children appears to share this mutator phenotype.

As in adults, secondary MDS in children can also arise as sequelae from exposure to chemotherapy and radiation, and from genetic conditions including Down syndrome, paroxysmal nocturnal hemoglobinuria (PNH), neurofibromatosis type 1 (NF1), Bloom syndrome, and Li-Fraumeni syndrome. However, MDS in children may often result from inherited constitutional BM failure syndromes including Fanconi anemia (FA), severe congenital neutropenia, Shwachman-Diamond syndrome, congenital amegakaryocytic thrombocytopenia, dyskeratosis congenital, Diamond-Blackfan anemia, and MonoMAC syndrome.

Ongoing studies are now defining the molecular pathogenesis and interrelationships among MDS, MPN, and AML.[8–10] Accumulating data suggest that aberrant signal transduction resulting from acquired somatic mutations encoding proteins, leading to hyperactivation of the Ras pathway, may stimulate proliferation without concomitant differentiation.[9] This has been clearly demonstrated in CML (*BCR-ABL*).[11,12] A number of other putative pathogenetic mutations have been identified in other myeloproliferative disorders, including *JAK2* V617F and *CALR* mutations in PV, ET, and primary myelofibrosis[13]; *KIT* D816V mutations in SM[14]; *FIPL1-PDGFRA* in chronic eosinophilic leukemia-SM[15]; *ZNF198-FG4FR1* mutations in stem cell leukemia–lymphoma syndrome[16]; *RAS/NF1/PTPN11* mutations in JMML (Fig. 65.1)[17–19]; and *GATA1* mutations in TAM.[20]

AML is the result of cooperating mutations in genes that confer a proliferative and survival advantage (e.g., activating mutations in receptor tyrosine kinases [FLT3, c-kit]), and genes that impair differentiation and apoptosis (e.g., loss-of-function mutations in transcription factors [CBF, AML/ETO]) (Fig. 65.2). This multistep model for the pathogenesis of AML is supported by murine models,[21,22] the analysis of leukemia in twins,[23–26] and the analysis of patients with familial platelet disorder with a propensity to develop AML (FDP/AML syndrome).[27]

The patterns of genetic and karyotypic abnormalities in children with MDS are typically distinct from that of adults. Although a few karyotypic abnormalities are shared (e.g., 7/7q−), many that are common in adults are only rarely found in children (e.g., 5q−). Importantly, mutations in mRNA spicing genes are only rarely found in children with de novo and secondary MDS.[28–31] Mutations in epigenetic genes that control DNA methylation and histone function are also only rarely identified in children, making their role in MDS uncertain. For example, although mutations in *TET2* are identified in 20% to 25% of adults with MDS, only 1 out of 19 children with refractory cytopenia of childhood (RCC) had a mutation in *TET2*.[32] Instead, the genetic abnormalities in children with MDS are most often those associated with inherited BM failure syndromes and other genetic disorders (*FANC* member genes, *DKC*, *TERT*, *TREC*, *WAS*, *GATA-2*, *SBDS*, etc.).

Finally, three exceedingly rare familial forms of MDS/AML are associated with mutations in *RUNX1/AML1* (familial platelet disorder with a predisposition to AML), *CEBPα* (familial AML), and *GATA-2*.[31,33–35] Most recently, germline mutations in the genes *SAMD9* and *SAMD9L* have been shown to lead to Mirage and ataxia–pancytopenia syndrome, respectively. Patients with these disorders are at a higher risk of developing MDS compared to the general population and are frequently present with monosomy 7.

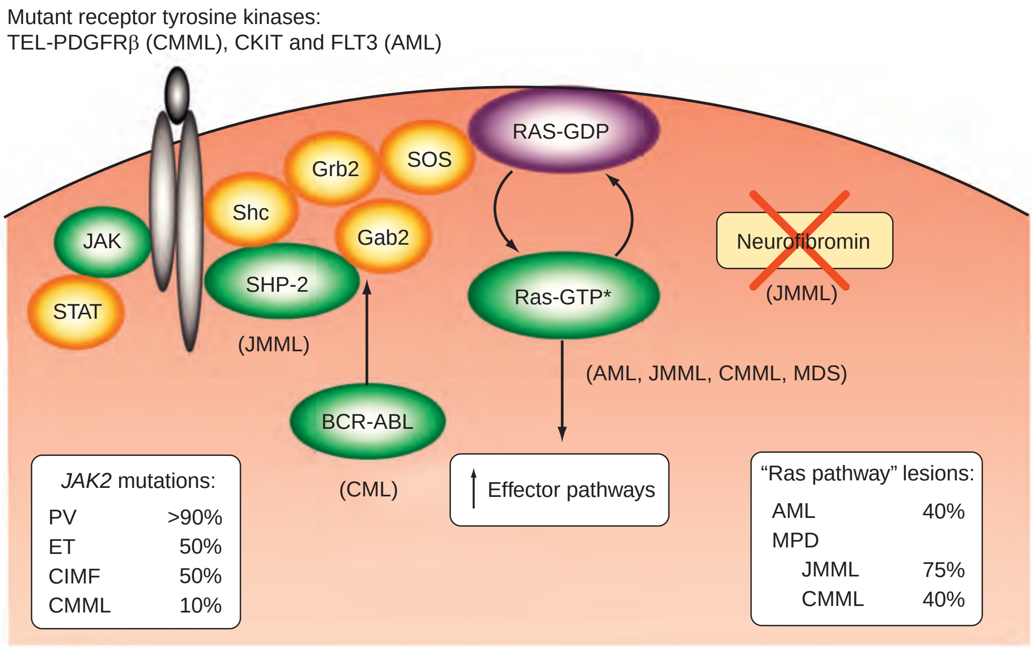

Mutant receptor tyrosine kinases:
TEL-PDGFRβ (CMML), CKIT and FLT3 (AML)
RAS-GDP
Grb2
SOS
Shc
JAK
Gab2
Neurofibromin
SHP-2
STAT
Ras-GTP*
(JMML)
(JMML)
(AML, JMML, CMML, MDS)
BCR-ABL
JAK2 mutations:
PV >90%
ET 50%
CIMF 50%
CMML 10%
(CML)
Effector pathways
"Ras pathway" lesions:
AML 40%
MPD
JMML 75%
CMML 40%

Figure 65.1 OVERVIEW OF RAS SIGNALING WITH MOLECULES HARBORING MUTATIONS IN PATIENTS WITH MYELOID MALIGNANCIES. *AML*, Acute myeloid leukemia; *CML*, chronic myeloid leukemia; *CMML*, chronic myelomonocytic leukemia; *ET*, essential thrombocythemia; *GDP*, guanosine diphosphate; *GTP*, guanosine triphosphate; *JAK*, Janus-activated kinase; *JMML*, juvenile myelomonocytic leukemia; *MDS*, myelodysplastic syndromes; *MPD*, myeloproliferative disorder; *PV*, polycythemia vera.

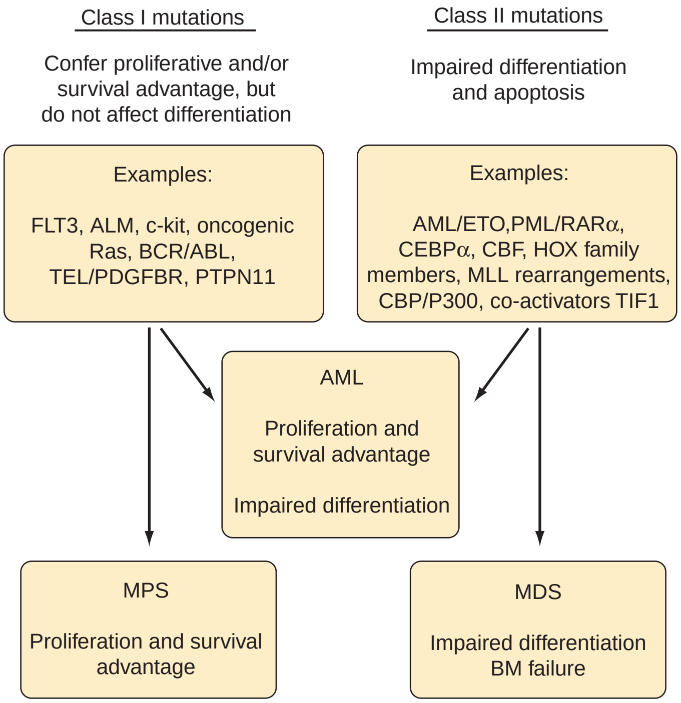

Class I mutations
Confer proliferative and/or survival advantage, but do not affect differentiation
Class II mutations
Impaired differentiation and apoptosis
Examples:
FLT3, ALM, c-kit, oncogenic Ras, BCR/ABL, TEL/PDGFBR, PTPN11
Examples:
AML/ETO,PML/RARα, CEBPα, CBF, HOX family members, MLL rearrangements, CBP/P300, co-activators TIF1
AML
Proliferation and survival advantage
Impaired differentiation
MPS
Proliferation and survival advantage
MDS
Impaired differentiation
BM failure

Figure 65.2 COOPERATING MUTATIONS IN ACUTE MYELOID LEUKEMIA, MYELOPROLIFERATIVE SYNDROMES, AND MYELODYSPLASTIC SYNDROMES. *AML*, acute myeloid leukemia; *BM*, Bone marrow; *MDS*, myelodysplastic syndromes; *MPS*, myeloproliferative syndromes.

Classification

Until recently, MDS in children was poorly defined, characterized, classified, and reported. In fact, MDS was not included in the International Classification of Childhood Cancer until 2005.[36] Also contributing to this lack of information was the use of classification and prognostic systems designed for adults that have had limited applicability to children. A number of classification systems for children and adults have now been proposed. Taken together, it may be useful to think about childhood MDS as primary or secondary in nature, with secondary MDS arising either from a known inherited BM failure syndrome, prior acquired aplastic anemia, or as a complication from prior chemotherapy or radiation therapy. A diagnosis of primary MDS would then apply to all other cases.

Historically, one of the most commonly used classification systems was the French-American-British (FAB) system, originally proposed in 1982.[37] This classification system recognized five forms of MDS in adults: refractory anemia (RA), RA with ringed sideroblasts (RARS), RA with excess of blasts (RAEB), RAEB in transformation (RAEB-T), and CMML. While RAEB and RAEB-T were commonly reported in children, RA and RARS were thought to be rare in children. However, a population-based study in the United Kingdom showed 25% of childhood MDS cases to be RA or RARS, suggesting inaccurate diagnosis or reporting of these subtypes in other pediatric studies. CMML has only rarely been reported in children.

Additional subtypes of MDS are now recognized that do not fit well into the FAB system, including hypoplastic MDS, therapy-related MDS, refractory cytopenias with trilineage dysplasia, MDS associated with myelofibrosis, and MDS associated with inherited disorders (congenital neutropenias, Shwachman-Diamond syndrome, FA), Down syndrome, NF1, and mitochondrial cytopathies.[38,39] Therefore, the World Health Organization (WHO) proposed changes to the FAB criteria to account for many of these subtypes.[40,41] Importantly, whereas adults commonly present with RA without cytopenias in the myeloid or platelet lineages, this is very rare in children since they more often have cytopenias in more than one cell line. Therefore, children with low-grade MDS are classified as having refractory cytopenia as opposed to RA. Thus in 2008 the WHO classification of pediatric MDS included the provisional category of RCC based on persistent cytopenia with <5% blasts in the BM and <2% blasts in the peripheral blood.[42] Under this classification, it is recommended that children with refractory cytopenia with multilineage dysplasia (RCMD) be classified as RCC until it is clarified whether the number of lineages involved is an important prognostic discriminator in childhood MDS. Under the 2016 revision of the WHO classification of myeloid and neoplasms and acute leukemia, RCC remains a provisional entry.[43]

Clinical Manifestations

Signs and symptoms of MDS are nonspecific and are usually attributable to pancytopenia (fever, infections, pallor, fatigue, bruising, and petechiae). Lymphadenopathy, hepatomegaly, and splenomegaly are uncommon presenting signs in children with MDS.

Laboratory Manifestations

Commonly accepted minimal diagnostic criteria for pediatric MDS include the absence of common de novo AML karyotypic abnormalities and at least two of the following: (1) sustained, unexplained anemia; neutropenia or thrombocytopenia; dysplastic morphology in the erythroid; granulocytic or megakaryocytic lineages (at least bilineage), and (2) an acquired, sustained clonal cytogenetic abnormality with 5% or more blasts in the BM.[8,44] Almost half of all children with MDS in one series presented with refractory cytopenia, most notably neutropenia and thrombocytopenia.[45]

Morphologically, the BM may be hypocellular, normocellular, or hypercellular. A diagnosis of MDS is made based on the presence of dysplastic changes in at least two cell lineages. The dysplastic changes in the granulocytes (hypogranulation, nuclear hyposegmentation, megaloblastoid maturation, and a left shift with an increased number of myeloblasts), megakaryocytes (micromegakaryocytes, abnormal megakaryocyte nuclei), monocytes (increase in BM monocytes, abnormal granulation with persistence of azurophilic granules, hemophagocytosis, abnormal nuclei, and giant forms), or erythroid lineages (megaloblastoid maturation, nuclear budding and multinucleated forms, and ringed sideroblasts) can be multiple and varied. Similar dysplastic changes can occur in the peripheral blood for each of these lineages. Although dysplastic changes in the BM are a common feature of MDS, it is important to remember that dysplasia, unto itself, is not diagnostic of MDS because dysplastic features are associated with other conditions, and can be found in normal BM donors.[46]

Flow cytometric analysis can serve as a useful addition to histopathology and to quantitate the number of blasts based on aberrant cell surface antigen expression and to exclude AML. It is also helpful in detecting populations of PNH-like cells.[47,48] However, while beneficial, flow cytometric findings are not generally diagnostic of MDS.

Cytogenetic abnormalities are seen in approximately half of children diagnosed with de novo MDS. Karyotypic abnormalities most commonly seen are −7, 7q−, and +8. Abnormalities in chromosomes 6, 9, 11, 12, and 13 are rare in children. Deletions of chromosome 20q can be seen in pediatric patients with SDS. Specific abnormalities seen in adults, including −5, 5q−, and −Y, are very rarely seen in children.

Differential Diagnosis

Although the history, physical examination, evaluation of the BM and peripheral blood, and cytogenetic analysis often make the diagnosis of MDS, other diseases should be considered. Congenital disorders such as Down syndrome, FA, Shwachman-Diamond syndrome, Diamond-Blackfan anemia, congenital dyserythropoietic anemias, and hereditary sideroblastic anemia should be considered. The differential should also include AML with a low blast count, mitochondrial cytopathies such as Pearson syndrome, rheumatic diseases including juvenile idiopathic arthritis, and myeloproliferative disorders. Specifically, PNH, although rare in children, should be considered. Deficiencies of vitamin B_{12} and folate can cause megaloblastic changes that resemble the dysplastic changes seen in MDS. Other nutritional deficiencies, including copper, iron, thiamine, riboflavin, and pyridoxine, should be considered. Infections caused by human immunodeficiency virus, parvovirus, Epstein-Barr virus, cytomegalovirus, and human herpes virus 6 can cause changes that resemble MDS. Finally, the differential diagnosis should include toxins (insecticides, chemotherapy agents, and arsenic), as well as cytokine exposure and radiation.

Hypoplastic MDS (RCC) can be difficult to distinguish from severe aplastic anemia and inherited BM failure (BMF) syndromes, especially when no chromosomal aberrations are detected. One interesting area of research that may help in differentiating between these diagnoses is the use of cytokine-based programs. In one preliminary study, thrombopoietin and IL-17 levels were useful in differentiating hypoplastic MDS from aplastic anemia.[49] When the diagnosis is unclear, prospective monitoring and serial BM examinations may serve as useful aids in making an accurate diagnosis.

Therapy

Although MDS is a heterogeneous, clonal disease of hematopoietic stem cells (HSCs) that can manifest different clinical courses, it is not readily curable by conventional chemotherapy, and requires allogeneic hematopoietic cell transplantation (HCT) to be cured in most cases. Some children with RCC and RCMD, who do not have life-threatening neutropenia nor require transfusions, may only require close observation (see box on Treatment Overview for Children With MDS). Although children with this disease may eventually develop progressive disease requiring HCT, they may have long periods when minimal treatment is required.[45] The use of AML-like chemotherapy for patients with RCMD with excess blasts (RCMD-EB) is controversial, but may serve to “debulk” patients with a high percentage of blasts before HCT.[6,50,51] However, this potential benefit may be offset by toxicities associated with AML-like chemotherapy. Finally, patients with advanced MDS (RAEB-T or t-MDS) should be treated like those with AML.

Prognosis

Although a number of methods have been developed to predict the outcome of adults with MDS, the systems now most commonly used in adults are the International Prognostic Scoring System[88], that uses percentage BM blasts, karyotype, and number of cytopenias to assign a score that is then used to predict outcome, and the WHO classification-based prognostic scoring system (WPSS) that includes more recently identified prognostic factors (e.g., transfusion dependency and multilineage dysplasia).[89] A recent study demonstrated that both systems “well represent” the prognostic risk for patients whose MDS is defined by the WHO classification criteria.[90] Although these are effective tools for adults, their value for children is very limited.[91]

Secondary Myelodysplastic Syndrome

Secondary MDS can develop in both children and adults after exposure to chemotherapy and radiation. Alkylating agents used to treat Hodgkin disease, non-Hodgkin lymphoma, and Ewing sarcoma are particularly concerning in children.[92–104] Interestingly, there is also evidence to suggest that the cardioprotectant dexrazoxane, a topoisomerase II inhibitor with a mechanism of action that is different from etoposide and doxorubicin, may have increased the incidence of secondary MDS and AML in children treated for Hodgkin disease.[105]

Treatment options for children with secondary MDS are limited. Although AML-like chemotherapy can induce a period of remission and reduction in BM blasts, it is not curative. As noted above, allogeneic HCT has curative potential, but outcomes remain poor, with only 20% to 30% of children surviving in the reported

TREATMENT OVERVIEW FOR CHILDREN WITH MYELODYSPLASTIC SYNDROMES

A multitude of agents have been studied for the treatment of myelodysplastic syndromes (MDS) in adults, but only rarely in children. These include low-dose chemotherapy (cytosine arabinoside, melphalan, hydroxyurea, etoposide, topotecan, 6-mercaptopurine, and busulfan), hormones (glucocorticoids and androgens), differentiating agents (13-*cis*-retinoic acid, all-*trans* retinoic acid), hematopoietic growth factors (granulocyte-macrophage colony-forming factor, granulocyte colony-forming factor, and erythropoietin), demethylating agents (decitabine, 5-azacytidine), proteasome inhibitors, antiangiogenic agents, and arsenic.[52–67] This has resulted in three drugs (lenalidomide, azacitidine, and decitabine) that are now approved by the US Food and Drug Administration for the treatment of MDS in adults. However, as there are currently no safety or efficacy data to support the use of these agents in children with MDS, these drugs are not approved for MDS in children.

In addition, a large number of agents are being tested in clinical trials in the broad categories of kinase inhibitors, deacetylase inhibitors and DNA methyltransferase inhibitors, altered cell metabolism, cytotoxics, cell cycle inhibitors, immunomodulators and immunosuppressive agents, apoptosis modulators, and others.[68]

The difficulty in assessing the safety and efficacy of new agents in children with MDS is illustrated by the Children's Oncology Group's recent prospective study of amifostine.[69] This prospective Phase II cooperative group study was unable to be completed because of lack of accrual. As a result, the safety and efficacy of amifostine in children with MDS remains uncertain. Despite a lack of successful prospective clinical trials in children with MDS, there are limited retrospective data on the use of the hypomethylating agent azacytidine in children with newly diagnosed MDS, and in the palliative setting, children with relapsed MDS. These children had refractory cytopenia of childhood (RCC), advanced and secondary MDS,[70] and prior to allogeneic hematopoietic cell transplantation (HCT).[71] These retrospective reviews suggest that azacytidine is tolerable and responses are possible, although the safety and efficacy in these various clinical settings have not been demonstrated in prospective clinical trials in children.

The utility of chemotherapy prior to allogeneic HCT for childhood MDS is controversial. One retrospective report suggested that use of pre-HCT chemotherapy was associated with poorer post-HCT survival; however, interpretation was limited by the lack of randomization, such that patients with more aggressive disease may have been the ones treated with chemotherapy.[72] Another report suggested less post-HCT relapse after pre-HCT chemotherapy in more advanced cases.[7] Rather than using traditional cytotoxic chemotherapy, a single-center report of non-random use of azacitidine pre-HCT decreased blast counts and improved survival post-HCT.[71] This approach has also been demonstrated to result in better outcomes after HCT for adults with MDS.[73,74]

Most children with MDS require allogeneic HCT for curative therapy. Although children have been included in published HCT studies for MDS that are largely focused on adult patients, several studies have focused specifically on children.[7,75–80] These studies suggested a probability of disease-free survival in about 50% of patients undergoing human leukocyte antigen (HLA)–matched related donor HCT. However, HCT outcomes have improved in recent years. A single-center report compared OS rates between the time-period of 1997–2007 (50%) and 2008–2017 (87%; $P = .006$).[81] The authors attributed much of this improvement to advances in haploidentical donor HCT.

Given the rarity of this disease in children, the best approach for HCT is not clear. A small single center report suggested that reduced intensity conditioning (RIC) was at least equivalent to fully myeloablative conditioning (MAC) for patients with RCC.[82] Another small single-center report also suggested high rates of EFS after RIC for patients with RCC.[75] Treosulfan, if available, may be an especially attractive agent to use in conditioning for patients with MDS, given low toxicity and relapse rates.[83,84] Conversely, patients with more advanced forms of childhood MDS, including RCC associated with monosomy 7, 7q−, or multiple genetic abnormalities, continue to have high rates of post-HCT relapse and probably should be treated with MAC.[7] Algorithms have been proposed to help guide this decision.[85] There are also single-arm adult trials which have attempted to prevent post-HCT relapse with the using of hypomethylating agents,[86,87] though these have been fraught with high rates of toxicity, and pediatric data is lacking.

series.[79,103,106,107] HCT has also been reported in children and young adults with secondary MDS, arising from hereditary BMF syndromes, as well as MDS secondary to chemotherapy exposure.[108–113] In the setting of hereditary BMF syndromes, careful evaluation of potential familial donors needs to be factored into donor choice.[113,114] HCT outcomes for MDS secondary to chemotherapy exposure have been poor, with both high rates of relapse and transplant-related mortality.[110,111] Current areas of investigation include the utility of monitoring hematopoietic chimerism after transplantation, and intervening with preemptive immunotherapy to try to prevent relapse.[115,116]

MYELOPROLIFERATIVE NEOPLASMS

Juvenile Myelomonocytic Leukemia

JMML is classified by the WHO as an overlap of MDS and MPN. It is an aggressive myeloid malignancy of young children with poor outcomes to conventional therapies. The diagnostic criteria are complex (Table 65.1), but recent advances in elucidating the molecular genetics of the disorder demonstrate that approximately 95% of children will harbor an alteration in one of several genes in the Ras pathway. Thus, there is now international agreement on the diagnostic criteria (see Table 65.1),[117] with an emphasis on incorporating these molecular criteria, as well as a recent definition of common response criteria.[118]

Epidemiology

The incidence of JMML in the United States has been estimated as 0.69 to 1.2 per million.[4,119] There is a male predominance with a median age of diagnosis of 1.8 years. The apparent incidence and demographic distribution may change in the coming years because of the refinement of diagnostic techniques such as molecular testing. In particular, mutation analysis may identify patients with less aggressive disease or at a younger age.

Pathogenesis

Early clonality studies suggested that JMML arose at the level of at least an immature myeloid precursor cell.[117,120–124] More recent data suggest that JMML may arise in a pluripotent stem cell with involvement of the myeloid, erythroid, and megakaryocyte lineages, as well as B lymphocytes and T lymphocytes.[117,125–128] This is supported by finding pathogenic mutations in immature hematopoietic cells identified by flow cytometry.[129]

Somatic mutations that augment signaling through the Ras pathway occur in approximately 90% to 95% of JMML patients. These include biallelic inactivation of *NF1* (15%),[18] activating mutations in *NRAS* or *KRAS* (25%),[130–132] mutations in the *PTPN11* gene (see Fig. 65.1) (35%), and mutations in *CBL* (10% to 15%).[133–136] Less frequent but more recently reported mutations occur in the genes *RRAS*, *RRAS2*, *RAC2* and *SH2B3*. This synopsis reflects decades of research, beginning with the seminal observation that JMML is associated with NF1. A rough comparison of incidence rates suggests that NF1 carries a 500-fold increased risk of acquiring JMML. At a molecular level, these children inherit a defective allele of the *NF1* gene, which encodes a negative regulator of Ras named *neurofibromin*. In JMML, the normal allele is lost as a somatic event in the initiating cell, most often by loss of heterozygosity in which the overall copy number remains unchanged (uniparental isodisomy). This completely ablates neurofibromin and thereby increases Ras activity.

Such interplay of inherited and somatic mutations is also relevant to the other JMML genes. Germline mutations in *PTPN11* cause Noonan syndrome (NS), a common genetic condition that overlaps

TABLE 65.1 **Diagnostic Criteria for Juvenile Myelomonocytic Leukemia**

Category 1 All of the Following:	Category 2 At Least 1 of the Following:	Category 3 At Least 2 of the Following:
Splenomegaly[a]	Somatic mutation in *RAS* or *PTPN11*	Circulating myeloid precursors
AMC >1000/μL	Clinical diagnosis of NF1 or *NF1* gene mutation	WBC >10,000/μL
Blasts in PB/BM <20%	Homozygous mutation in *CBL*	Increased Hgb F for age
Absence of the t(9;22) *BCR/ABL* fusion gene	Monosomy 7	Clonal cytogenetic abnormality excluding monosomy 7
		GM-CSF hypersensitivity

The diagnosis of juvenile myelomonocytic leukemia is made if a patient meets all of the Category 1 criteria and one of the Category 2 criteria without needing to meet the Category 3 criteria. If there are no Category 2 criteria met, then the Category 3 criteria must be met.

[a] For the 7%–10% of patients without splenomegaly, the diagnostic criteria must include all other features in Category 1 AND one of the parameters in Category 2 OR no features in Category 2 but two features in Category 3.

AMC, Absolute monocyte count; *BM*, bone marrow; *GM-CSF*, granulocyte-macrophage colony-stimulating factor; *Hgb F*, fetal hemoglobin; *NF1*, neurofibromatosis type 1; *PB*, peripheral blood; *WBC*, white blood cell.

From Chan RJ, Cooper T, Kratz CP, et al. Juvenile myelomonocytic leukemia: a report from the 2nd International JMML Symposium. *Leuk Res.* 2009;33:355.

phenotypically with NF1 and sometimes includes a mild JMML-like myeloproliferative syndrome. This finding led directly to the discovery of somatic *PTPN11* mutations in sporadic JMML, a lesion now recognized as the most common pathogenic mutation in this disease. Furthermore, rare germline mutations in *KRAS* have also been associated with related genetic syndromes including NS and cardiofaciocutaneous syndrome.[137–142] The *PTPN11* and *KRAS* mutations present in the germline generally have less severe biochemical consequences than those in sporadic cases. This suggests that strong signal activation is not tolerated during development. Accordingly, the most common NS-associated *PTPN11* alleles have relatively weak effects on signal transduction, and the myeloproliferative syndrome in these patients is usually transient.[143–145] However, a more recent and comprehensive analysis has suggested that a cohort of NS patients may inherit more deleterious mutations and develop fatal neonatal JMML.[146] Thus, cytoreductive therapy or HCT may be considered for select NS patients with severe disease.

Similar to *NF1*, mutations in *CBL* are most often heterozygous in the germline, with subsequent reduction to homozygosity in hematopoietic cells. However, recent reports demonstrate that JMML patients can harbor somatic *CBL* mutations, similar to adults with MPNs.[135] The more typical heterozygous germline mutations appear to cause a genetic syndrome with features that can overlap with NS, termed *CBL syndrome* or *NS-like disorder with or without JMML*. The phenotypic manifestations of NSLL are variable and incompletely penetrant, and they include cryptorchidism, hearing loss, and skeletal abnormalities. Importantly, major vascular abnormalities have been described in patients with *CBL*-mutant JMML, and this could also reflect an NSLL phenotype. As expected for a heterozygous mutation, transmission is autosomal dominant, although 50% of cases arise spontaneously.[134,136,147,148] In contrast to the loss of function mutations in *NF1*, the mutations in *CBL* selectively inactivate the ubiquitin ligase activity of the Cbl protein while leaving the molecule otherwise intact. When homozygous, this alteration eliminates the negative regulatory activity of Cbl while sparing positive signaling functions.[135]

These so-called *Ras pathway* or *driver* mutations are largely mutually exclusive, supporting the idea that each provides a similar function in JMML. However, patients with two such lesions (approximately 10% of patients have co-occurring Ras pathway mutations) or duplication of the mutant allele have been reported, typically co-occurring in the same cells.[149,150] This likely reflects clonal evolution and positive selection for increased Ras signaling even beyond that provided by the initiating mutation. Growing evidence demonstrates that negative feedback prevents the Ras pathway from being fully activated by a single lesion, leading to a dosage effect of mutant alleles on signaling outputs.[151] Consistent with predictions from model systems, cases with multiple Ras pathway mutations have a more aggressive clinical course.[150]

Genomic studies have also begun to address the question of whether mutations outside the Ras pathway contribute to the pathogenesis of JMML.[129,149,150,152] A wide variety of so-called secondary mutations have now been recognized. They are primarily associated with relapse of JMML after HCT, or with transformation to AML. However, they often can be detected at a low level in the blood or BM when JMML is first diagnosed. This suggests that clonal evolution depends on chemotherapy resistance and eventually leukemic transformation. This is supported by the striking observation that secondary mutations of any kind have a major prognostic impact, even when they are present in a very small proportion of hematopoietic cells at diagnosis.

In the few JMML cases in which clonal history has been inferred, mutations appear to be acquired sequentially, beginning with a Ras pathway lesion. This linear evolutionary path contrasts with the highly branched patterns typical of high-grade neoplasms. Thus, despite the discovery of a variety of secondary mutations in JMML, it remains a disease with much less genetic complexity than most cancers. This may have important implications for therapy, as any individual patient is likely to harbor a relatively small number of distinct subclones that need to be eradicated to achieve cure.

The cellular function of cooperating mutations in JMML is under active investigation. Most can be categorized into three groups. Some amplify signaling beyond the level provided by the initial Ras pathway mutation. As described earlier, some second mutations directly alter core Ras pathway genes. Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT) signaling is enhanced by activating mutations in *JAK3* or loss-of-function mutations in *SH2B3*, which encodes the negative regulatory adaptor protein LNK. Mutations in *RRAS*, *RRAS2*, and *RAC2* represent a novel signaling complex in JMML that is implicated in regulating the PI3K/Akt pathway.[150] Dominant point mutations in *SETBP1* remain somewhat unexplored, but may augment signal transduction by inhibiting the phosphatase PP2A, or regulate gene transcription and strengthen self-renewal programs. Other secondary lesions have broad effects on gene expression, through inactivation of the PRC2 polycomb repressor complex or alteration of spliceosome components. Many of these genes and cellular functions are also implicated in the pathogenesis of other myeloid neoplasms such as CMML or AML.

Several genome-wide studies have now been published documenting the role of altered DNA methylation as a predictor of the outcome in JMML.[153–155]

Patients with a hypermethylated profile are typically present at an older age, have higher levels of fetal hemoglobin, and more often have more than one somatic alteration in their JMML cells.

In multivariable analyses, DNA methylation has been the most significant predictor of outcome with hypermethylated patients experiencing the highest rates of relapse post-HSCT. In contrast, patients with a hypomethylated profile tend to be younger at presentation, have normal levels of fetal hemoglobin and invariably have only one somatic mutation. These patients are more likely to be cured with HSCT but of interest may also be the most likely to experience spontaneous resolution in the absence of transplant. International efforts are now underway to establish consensus definitions of altered DNA methylation and develop techniques to bring DNA methylation testing into the clinic in order to prospectively test the predictive nature of DNA methylation. The exact mechanism that altered DNA methylation plays in this disease has yet to be elucidated and is the focus of ongoing investigations.

Model Systems

Genetically engineered mouse models of JMML have been based on directed mutation of *Nf1*, *Kras*, *Nras*, *Ptptn11*, and *c-Cbl*. These are reviewed in detail elsewhere.[156,157] In general terms, these mice reproduce many key features of JMML, but penetrance and severity of disease vary across these models. In an alternative approach, induced pluripotent stem cells have recently been generated from germline and neoplastic cells from JMML patients.[158] These can be differentiated into myeloid progenitors that also recapitulate signal transduction and cell biology phenotypes characteristic of JMML. Most recently, several groups have reported the feasibility of generating patient derived xenograft models using a variety of immunocompromised mouse strains. Together, these systems provide a robust set of tools for investigating the biology of JMML and also enable preclinical testing of novel therapeutic strategies.

Clinical Manifestations

Children with JMML typically present with signs and symptoms attributable to a heavy burden of organ-infiltrating cells that results in hepatosplenomegaly, lymphadenopathy, and skin rash. As a result of the association with neurofibromatosis, patients may also have café-au-lait spots or juvenile xanthogranulomas. Death is usually the result of organ dysfunction caused by infiltrating cells, infection, or bleeding. Approximately 10% to 20% of children progress to a blast-like phase consistent with AML.

Laboratory Manifestations

Laboratory abnormalities may include an elevated white blood cell count with absolute monocytosis, anemia, and thrombocytopenia (Figs. 65.3 and 65.4). Monocytes, either circulating in the peripheral blood or in the BM, frequently appear dysplastic. The peripheral smear shows leukoerythroblastic changes, and there are often circulating nucleated red blood cells. Fifty percent of patients may also present with elevated fetal hemoglobin levels and hypergammaglobulinemia, which is of interest given that there are patients recently described with autoimmune lymphoproliferative syndromes who also harbor *RAS* mutations (see later). International criteria mandate that the BM have fewer than 20% blast cells at diagnosis. Other findings typical in the BM may include micromegakaryocytes.

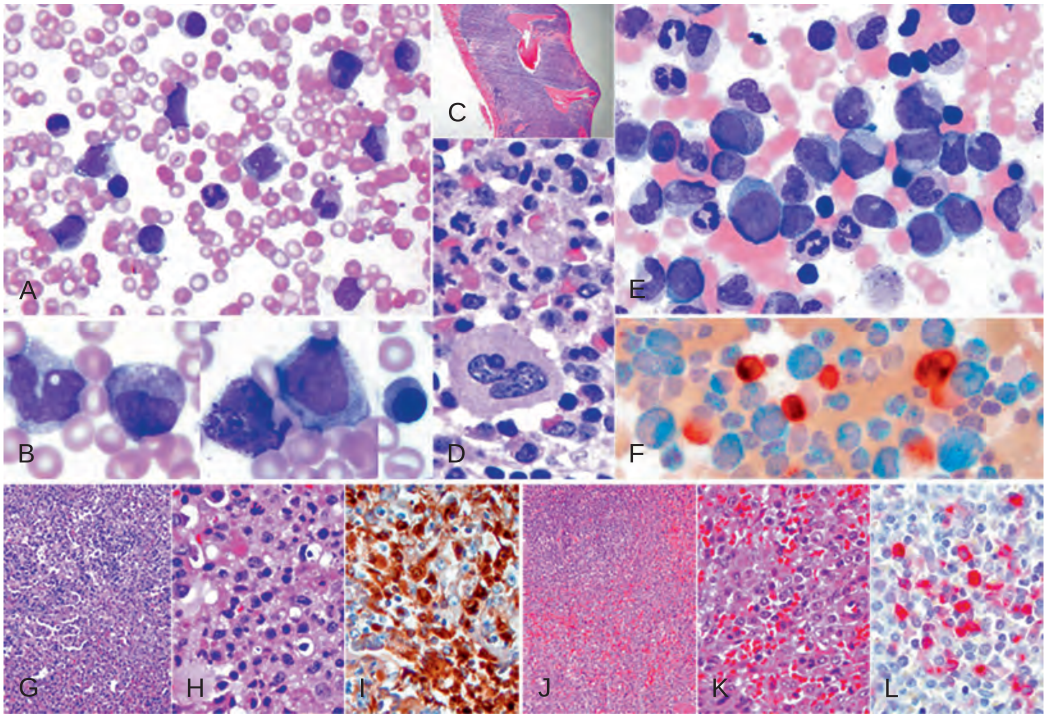


Figure 65.3 JUVENILE MYELOMONOCYTIC LEUKEMIA: BLOOD, BONE MARROW, LUNG, AND SPLEEN. The illustrations are from the case of a 3-year-old boy who was diagnosed with neurofibromatosis at birth. At 1 year of age, he presented with leukocytosis (58 K/μL). The peripheral blood (A and B) showed left-shifted granulocytes and increased monocytes (16%). A bone marrow biopsy (C and D) was hypercellular as a result of increased granulocytic and monocytic cells that could also be appreciated on the aspirate (E). Blasts accounted for only 4% of the bone marrow elements. A combined esterase reaction (F) illustrated the increased monocytes (α-naphthol butyrate esterase reaction positive; *orange/brown*) in the background of granulocytes (chloroacetate esterase reaction positive; *blue*). Cytogenetic analysis revealed monosomy 7. At age 2 years, the patient presented with respiratory distress, and a lung biopsy (G and H) demonstrated a monocyte infiltrate (lysozyme stain, I) consistent with involvement by juvenile myelomonocytic leukemia. This is not uncommon in such patients. At age 3 years, his blast count began to rise, and he underwent a splenectomy (which showed a marked infiltrate of immature and mature monocytes and granulocytic cells [J and K; lysozyme stain, L]). After the splenectomy the patient underwent a successful stem cell transplant.

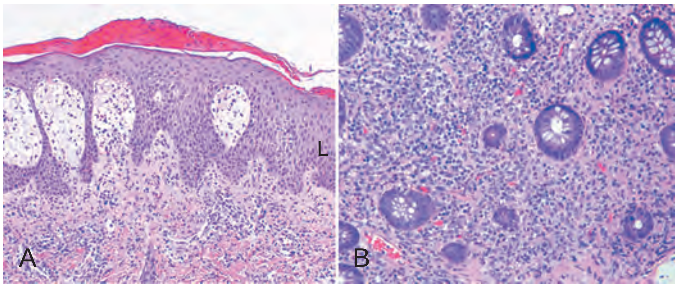


Figure 65.4 JUVENILE MYELOMONOCYTIC LEUKEMIA: SKIN AND GASTROINTESTINAL TRACT. Patients with juvenile myelomonocytic leukemia sometimes present with or develop skin nodules, which on biopsy show a myelomonocytic infiltrate in the upper and lower dermis (A). Involvement can also be seen in the gastrointestinal tract (B). *(The case was kindly provided by Dr. Elizabeth Hyjek, University of Chicago.)*

Differential Diagnosis

Traditionally, establishing a diagnosis of JMML was challenging because its clinical and laboratory presenting features can also be associated with other disorders, including Wiskott-Aldrich syndrome,[159] infantile osteopetrosis,[160] infections (Epstein-Barr virus, cytomegalovirus, human herpesvirus 6, histoplasmosis, mycobacterium, and toxoplasmosis), class I Langerhans cell histiocytosis, hemophagocytic lymphohistiocytosis, FA, Kostmann syndrome, Shwachman syndrome, and Down syndrome. However, with a confirmed molecular mutation, patients who exhibit the category 1 features are now more easily diagnosed.

Recently, the term "Ras-associated lymphoproliferative disorder (RALD)" has been coined to describe patients with autoimmunity and lymphoid proliferation with activating *RAS* mutations in the blood.[161] These mutations were initially thought to be in the germline, but later were established to be somatically acquired in the BM. These patients also exhibit splenomegaly and monocytosis, and they tend to meet clinical diagnostic criteria for JMML. At this time, there is no accepted method for distinguishing JMML from RALD, except to note that patients diagnosed with RALD have less fulminant myeloid disease and relatively more prominent immune dysregulation. Further study is needed to determine whether these diagnoses represent distinct clinicopathologic entities, or rather represent the spectrum of hematologic phenotypes specified by Ras pathway mutations. Along with reports of rare JMML patients who survive without chemotherapy despite persistent Ras pathway mutations, this idea has raised the controversial question as to whether or not select patients can be monitored without definitive therapy. However, given the difficulty of prospectively diagnosing RALD, its extremely low incidence, and the strong tendency of JMML to become more aggressive over time, most patients meeting JMML criteria should be treated as such.

Therapy

Children with JMML can have a variable course, with rare patients having a spontaneous remission and long-term survival without treatment, and others having a rapidly fatal course despite aggressive treatment.[162,163] The patients likely to do well without HCT are those with somatic *NRAS* or *KRAS* mutations, or germline mutations in *CBL*.[135,164,165] Older age, lower platelet count, and elevated hemoglobin F levels have been used to predict a more aggressive clinical course for patients.[4,166–169] Currently, the most adverse prognostic factors for outcome are the presence of >20% blasts, an AML-like gene expression profile,[166,168] or a hypermethylated DNA signature, which have survival rates of 10% to 20% even with allogeneic HCT.

A wide variety of agents have been used to treat children with JMML, including AML-like chemotherapy,[51,170–175] low-dose chemotherapy,[176,177] interferon-α (IFN-α),[178] and 13-*cis*-retinoic acid. Tipifarnib, a farnesyl transferase inhibitor, was tested in a Phase II window by the Children's Oncology Group (COG) where it produced some responses, but did not appear to impact long-term survival.[179] Azacitidine, a DNA hypomethylating agent has now been tested in a prospective clinical trial in Europe with promising results. Trametinib, an oral, MEK1/2 inhibitor is currently being tested through the COG for JMML patients with relapsed or refractory disease (NCT03190915).

Perhaps because the evaluation of these treatment regimens has been complicated by small patient numbers, a prior lack of consistent response criteria, and until recently, lack of assays to measure residual disease, the utility of chemotherapy prior to HCT is unclear. One report showed no difference in univariate post-HCT outcomes in patients who did or did not receive AML-like chemotherapy, but this was limited by the inclusion of patients with AML and the lack of multivariate analysis.[166] A report in patients undergoing umbilical cord blood transplant shows a trend towards better post-HCT EFS after receipt of AML-like chemotherapy.[167] Another report demonstrated trends to better EFS and lower relapse in patients who had normalization of WBC count and organomegaly prior to HCT.[179] With the advent of uniform response criteria[118] and the ability to follow molecular disease burden in patients with mutations in *PTPN11*, *NRAS*, and *KRAS*,[180] the role of pre-HCT chemotherapy has evolved. A single center report demonstrated that patients who achieved low levels of molecular disease burden pre-HCT had excellent post-HCT outcomes,[181] though this needs to be confirmed in larger datasets.

For most children with JMML, allogeneic HCT offers the only known potential for cure, with about half of transplanted children surviving disease-free.[166,167,182] Rapid withdrawal of immunosuppression appears to be important in this disease, as mounting evidence supports a graft-versus-leukemia effect.[181,183,184] A number of issues related to HCT for JMML remain uncertain. Among these are the value of pre-transplant splenectomy, and the optimal conditioning regimen and graft-versus-host disease prophylaxis. Splenectomy demonstrated a trend towards improved outcomes in patients undergoing umbilical cord blood transplant,[167] but no benefit in those getting transplants from other donors,[166,179] and can be associated with increased risk of overwhelming sepsis from encapsulated organisms. Triple alkylator-based conditioning appears to be associated with lower rates of relapse for patients with high disease burdens, either through direct anti-leukemic effects or via tissue-injury mediated induction of GVHD and GVL.[185] However, these very intensive regimens might not be needed for patients who enter HCT in molecular remission.[181] Radiation-containing preparative regimens, which have significant long-term risks in small children, have not shown benefit when compared with other published reports,[179] although they did decrease the risk of rejection in patients undergoing cord blood transplant.[167] JMML appears to be highly sensitive to a GVL effect.[181] As such, preemptive withdrawal of immunosuppression may be beneficial in the setting of low disease burden and donor leukocyte infusions may have some response.[184,186] While the most common cause of death after HCT is recurrent disease, a second HCT can be lifesaving for some children.[166,187,188]

Down Syndrome–Associated Transient Abnormal Myelopoiesis

Epidemiology

Down syndrome is one of the most common congenital disorders, affecting approximately one in every 800 to 1000 live births. A population-based study estimated that children with Down syndrome have a 10- to 20-fold overall increased risk of developing leukemia,[189] a 150-fold increased risk of developing AML, and a 500-fold increased risk of acute megakaryocytic leukemia. The median age of diagnosis of myeloid leukemia of Down syndrome (ML-DS), as defined by the WHO classification system, in children with Down syndrome is 2 years, and it is rarely diagnosed after age 4.[190] TAM, also known as *transient myeloproliferative disorder (TMD)* and *transient leukemia*, is thought to occur in at least 10% of children with trisomy 21 Down syndrome, although 7% to 16% of TAM cases have mosaic trisomy 21. It has also been suggested that when cases of TAM that develop and resolve in utero or result in death before delivery are taken into account, the incidence may be as high as 20%.[191] Currently, it is estimated that approximately 10% to 20% of children with TAM subsequently develop ML-DS, usually by 3 years of age.[192–194]

Pathobiology

The molecular pathogenesis of TAM and ML-DS in children with Down syndrome has provided valuable insights into myeloid leukemogenesis.[195] Current research suggests that the development of TAM and subsequent progression to ML-DS result from a three-step process: perturbation of fetal hematopoiesis by trisomy 21; an acquired *GATA1* mutation; and the acquisition of additional oncogenic mutations.[195,196]

It is increasingly clear that trisomy 21 results in genome-wide changes in gene expression that affect, directly or indirectly, most chromosomes.[197,198] This results in perturbed hematopoiesis with increased numbers and clonality of HSCs, increased frequency of megakaryocyte and erythroid progenitors, and reduced numbers of granulocyte- and macrophage-committed progenitor cells.[199,200]

Recent studies have shown that virtually all patients with TAM, and most patients with ML-DS, harbor mutations in exon 2 and, occasionally, in exon 3 of the hematopoietic transcription factor *GATA1*.[201–205] *GATA1* is a double–zinc finger DNA-binding transcription factor expressed primarily in hematopoietic cells. It is required for the development of red blood cells, megakaryocytes, mast cells, and eosinophils. A number of different mutations in *GATA1* have been identified, including insertions, deletions, missense mutations, nonsense mutations, and splice site mutations. All of these mutations lead to a block in the expression of the full-length 50-kd isoform of *GATA1* but allow for the expression of a smaller, 40-kd isoform (*GATA1s*).[206] This smaller isoform lacks the N-terminal transactivation domain, but retains both zinc fingers involved in DNA binding as well as interactions with its cofactor, friend of *GATA1* (*FOG1*).[201] Recent studies have shown that mutations that alter *GATA1-FOG1* binding in the N-terminal zinc finger or result in the expression of the *GATA1s* isoform uncouple megakaryocyte growth and differentiation.[207–209] Similar studies in cell lines derived from children with ML-DS have demonstrated that expression of *GATA1* led to erythroid differentiation whereas expression of *GATA1s* did not alter the characteristics of the cell line.[205] Taken together, current data suggests that the loss of *GATA1* and expression of *GATA1s* directly contribute to leukemogenesis. Importantly, TAM and ML-DS reflect an interaction between *GATA1s* and trisomy 21. This likely reflects the combined effects of multiple genetic interactions, with studies implicating various genes on chromosome 21 such as *ERG*, *ETS2*, *RUNX1*, *CHAF1B*, and mir-125b2.[195]

Although *GATA*1 mutations in the context of trisomy 21 may be sufficient to cause TAM, these mutations are not sufficient for the development of ML-DS, as evidenced by the latency period between resolution of TAM and the development of ML-DS, as well as the observation that not all children with TAM and *GATA1* mutations will ultimately develop ML-DS. Therefore, a multistep pathogenesis model is proposed in patients with Down syndrome in whom ML-DS develops in clones with *GATA1* mutations and additional cooperating mutations. This has been corroborated by genomic analyses of TAM and ML-DS cases.[198,210] These studies have revealed common molecular functions in genes mutated upon progression to ML-DS. One such class increases cytokine signaling, with typical lesions affecting genes in receptors (*MPL*. *CSF2RB*, *KIT*), associated kinases (*JAK2*, *JAK3*), and downstream signal transduction molecules (*NRAS*, *KRAS*, *NF1*, *SH2B3*). Another class of mutations disrupts the cohesin complex (*STAG2*, *RAD21*, *CTCF*, *SMC1A*). This multifunctional complex regulates sister chromosome pairing during mitosis and also participates in epigenetic regulation of gene expression in a broad fashion. Epigenetic regulation is also implicated by a third large class of mutations (*EZH2*, *SUZ12*, *KANSL1*, *DNMT3A*), often reducing function of the PRC2 complex and/or DNA methylation. Importantly, some of these same genes can be mutated in TAM, but without the same deleterious biochemical or functional consequences; thus, mutations of unclear functional significance must be interpreted with caution in TAM.[198]

Clinical Manifestations

Patients with Down syndrome typically present with TAM within 3 months after birth, although TAM can be manifest at birth. Some of these children present with hydrops fetalis, secondary to anemia and cardiac dysfunction. Although some patients may be asymptomatic, others can have myeloblast infiltration of the heart, liver, and spleen that can result in hepatosplenomegaly; hepatic fibrosis; pleural, pericardial, and peritoneal effusions; and disseminated intravascular coagulopathy. In some cases, organ dysfunction can be severe, with failure of the liver, heart, kidneys, and lungs. In the COGs prospective study, death occurred in 21% of patients, although only 10% died as a result of TAM-associated problems.[192]

Laboratory Manifestations

The complete blood count in infants with TAM typically demonstrates an elevated white blood cell count with myeloblasts present (Fig. 65.5). One difficulty that arises when evaluating a baby for TAM is that circulating blasts are present to some degree in almost newborns with Down syndrome. However, in a landmark study that included molecular analysis, *GATA1* mutations were found only in patients with peripheral blasts present at levels >10%.[211] Thus, some groups require *GATA1* mutation for diagnosing TAM (with a detection threshold of >5%); however, this may not be universally available, and the clinical significance of rare *GATA1* mutant clones (e.g., <1%) remains unclear. In the same study, deep sequencing revealed that 20% of babies lacking clinical or hematologic features of TAM had such small *GATA1* mutant clones. While these authors coined the term "silent TAM" for these patients and recommended close observation, a widely accepted standard of care has yet to be defined. Rarely, a neonate without stigmata of Down syndrome will present with similar features—in these cases, workup for either trisomy 21 mosaicism or a *PTPN11* mutation should be pursued.

Myeloblasts from TAM and ML-DS patients have an immunophenotype that is distinct from other types of AML in children.[212,213] Specifically, all TAM and ML-DS blasts express CD45, CD38, and CD33, but the majority of cases express CD36 and CD34. CD41 and CD61 also are expressed, consistent with megakaryocyte differentiation. CD14 and CD64 are usually negative. Most cases have

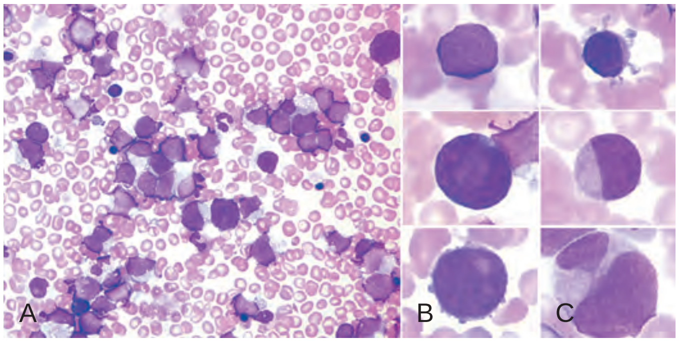


Figure 65.5 TRANSIENT ABNORMAL MYELOPOIESIS AND TRANSIENT LEUKEMIA OF DOWN SYNDROME. The patient was a premature newborn girl with trisomy 21 and a white blood cell count of 195,000/μL with 67% blasts (A). The blasts were CD34+, CD33+, and CD117+, and a portion exhibited CD41, a megakaryocyte marker. Morphologically, some of the blasts appeared to be megakaryoblasts (B), sometimes with slight differentiation toward megakaryocytes (C).

aberrant expression of CD7, a T-lineage antigen. Finally, regenerating BM in children with Down syndrome often demonstrates a population of nonmalignant CD34$^+$/CD56$^+$ precursors.[214]

Therapy and Prognosis

Supportive care is sufficient management for most babies with TAM.[192,215,216] In the COGs prospective study, peripheral blood blasts and TAM-associated symptoms resolved in 36 and 49 days, respectively, for observation patients.[192] However, approximately 25% of patients with clinically significant TAM exhibit life-threatening organ dysfunction, and these patients warrant urgent medical intervention. Risk factors for mortality have been defined by several studies (summarized in Box 65.1). Importantly, hepatopathy can be fatal after a protracted, indolent course that eventually leads to irreversible hepatic fibrosis. This represents one of the most common causes of death associated with TAM. Thus, liver dysfunction is an urgent indication for chemotherapy even when it appears to be physiologically well tolerated. By contrast, mild hepatomegaly with adequate function is not itself an indication for treatment unless it compromises the function of other vital organs. Guidelines for management of TAM that incorporate international studies have recently been published by the British Society for Haematology.[217] Though not part of these guidelines, prematurity (gestation <37 weeks) has also been noted as a recurrent risk factor for early death.[192,215,216]

A consensus has emerged that a short course of low-dose cytarabine (e.g., 1 to 1.5 mg/kg/day for 5 to 7 days) improves outcomes for babies with TAM at risk for mortality. In a non-randomized trial, the BFM group reported a substantial benefit of low dose cytarabine among infants with TAM and high risk features.[215] The overall survival was 76% in the treated patients versus 28% in the untreated patients. Similarly, the Japanese TAM-10 study demonstrated that patients with leukocytosis (>100 × 10^8/l) had survival rates of 78% if treated with cytarabine versus 39% if not.[216] While neutropenia is a common adverse effect of cytarabine, the benefits appear to greatly outweigh the risks for high risk TAM patients. Therapy should be initiated promptly for patients with these risk factors; supportive care is insufficient in patients with life-threatening symptoms.

Most patients with TAM lacking high risk features do well with supportive care. Approximately 10% to 15% of these patients die in early infancy.[192,216] However, the COG study reported that almost all of these deaths were attributable to causes unrelated to TAM. These patients must be closely monitored for resolution of TAM and possible development of life-threatening symptoms such as hepatopathy, or emergence of ML-DS. Failure to resolve hematologic abnormalities by 4 months of age is atypical and should raise consideration of potential transformation to ML-DS or MDS.

Therapy for ML-DS has been adapted from conventional AML protocols, after early studies noted that these patients had higher cure rates but also more severe toxicities. These observations have been attributed partly to an increase in cytarabine sensitivity in cells with trisomy 21. Subsequent studies have attempted to improve the risk-benefit ratio of chemotherapy by reducing intensity. AAAML0431 adapted standard AML therapy for ML-DS by (a) moving high-dose cytarabine earlier in the treatment regimen (from cycle 5 to cycle 2), and (b) reducing anthracycline exposure by 25%. This successfully preserved the excellent outcomes in ML-DS patients, with >90% having long term event-free survival.[218] Similarly, a European study, ML-DS 2006, was able to reduce etoposide exposure by ~50%.[219] The most recent North American study, AAML1531, attempted to reduce therapy further in low-risk ML-DS patients by eliminating a course of high dose cytarabine and asparaginase. However, relapse (and salvage) rates were unacceptably high in these patients. Thus, prolonged and intensive myelosuppressive chemotherapy is still considered essential for curing ML-DS. Interestingly, high dose chemotherapy is effective for ML-DS when it presents as MDS without excessive blasts in the marrow. This is distinct from sporadic MDS in the general population, for whom the benefit of chemotherapy is less clear.

BOX 65.1 Life-Threatening TAM Symptoms Indicating Need for Intervention[192,217]

Multi-organ failure
White blood cell count >100 × 10^8/L or leukostasis
Hepatopathy (conjugated bilirubin >83 μmol/L or >5 mg/dL, ascites or massive hepatomegaly)
Hepatosplenomegaly (beyond umbilicus or causing respiratory or feeding compromise)
Hydrops fetalis
Pleural or pericardial effusions
Renal failure
Disseminated intravascular coagulation/coagulopathy with bleeding

Chronic Myeloid Leukemia

Epidemiology

Nearly 1 in 500 adults will be diagnosed with CML in their lifetime. CML in children on the other hand is rare with an annual incidence of between one and two cases for every million children under the age of 18 in the United States.[220] Both children and adults can present in one of three phases: (1) nearly 90% present in the chronic phase which is the most common and indolent, (2) accelerated phase is more aggressive and (3) blast phase, upon transformation to an acute leukemia, either ALL or AML.[221] In general, children appear to have higher risk disease with more frequent presentation in accelerated or blast phase compared to adults.[222]

Pathogenesis

CML is an MPN that occurs in HSCs. In children, it always presents with the Philadelphia chromosome t(9;22)(q34;q11), leading to the fusion gene, *BCR-ABL1*.[223] This encodes for an oncoprotein, leading to a constitutively active tyrosine kinase as a result of oligomerization of the coiled coil region of BCR, and deletion of the inhibitory SH3 domain of ABL. Several downstream pathways are then activated including JAK2, RAS, and STAT1/5. Nearly 90% of patients present in the chronic phase of the disease, but the events leading to the more advanced accelerated phase and blast crisis are incompletely understood. Typically, cytogenetic changes involving the acquisition of additional oncogenes or loss of tumor suppressors is noted. The single most common alteration involves deletion of 17q, where p53 resides, but non-random changes involving chromosomes 7, 8, 9, and 22 are frequent. Patients who transform to acute lymphoblastic leukemia frequently present with loss of CDKN2A/B. In general, it is thought that constitutive activation of BCR/ABL1 leads to an unstable genome within the mutated HSC, which results in the increased likelihood of non-random changes involving genes with critical functions that are more likely to be tolerated due to impaired apoptosis.[224]

Clinical Manifestations

Children with CML frequently present with elevated white blood cell and platelet counts.[222] These can be detected in the asymptomatic patient but more frequently due to fatigue, malaise or weight loss. Patients can also present with concomitant infections due to nonfunctional neutrophils. Enlargement of the spleen is nearly universal at diagnosis and can cause abdominal discomfort. Bleeding due to thrombocytopenia, coagulation defects, or even thrombocytosis with an acquired von Willebrand's disease can be presenting symptoms.

Laboratory Manifestations

CML patients present with a median elevated white blood count of 250 × 10^8/l per an international registry study of nearly 200 pediatric patients with a median age of 12 years.[225] Owing to the myeloproliferative nature of the disease, nearly all types of myeloid cells will be visible on a peripheral blood smear, with basophilia and eosinophilia being nearly universal. Blasts are typically <2%, although it can be higher depending on the phase of presentation. A CBC and FISH, or RT-PCR for BCR/ABL, can be performed although a BM examination that rules out the more common diagnosis of AML is required. Cytogenetic culture is typically sent from the BM. Next-generation sequencing is more commonly sent at diagnosis to determine the presence of tyrosine kinase inhibitor (TKI) resistance mutations although the therapeutic utility of this approach has not been definitively documented.[226]

Therapy

As a poster child for precision medicine, the advent of TKIs demonstrated remarkable efficacy in CML, first in adults and later in children.[227] A paradigm shift occurred in adults, wherein CML became a chronic manageable condition in the setting of TKIs, of which several generations of increasingly potent drugs are now available. In pediatrics, whereas HSCT was indicated for nearly all patients in the pre-TKI era, it is now reserved for patients who present in an accelerated or blast crisis phase.[228] For the approximately 90% of pediatric patients who present in the chronic phase of the disease, monotherapy with a TKI is the standard of care. The choice between the several generations of TKIs including, but not limited to, imatinib (first generation), dasatinib or nilotinib (second generation), ponatinib or bosutinib (third generation) to name just a few, is mainly stylistic or resource-dependent, as there is no clear winner from the few randomized studies in pediatrics.[222] The goal with all treatment is to induce rapid molecular remission and prevent the development of mutations that cause resistance to TKIs. Studies have demonstrated that adult patients who achieve early and/or major molecular remissions can safely stop TKI therapy and maintain deep remissions. TKIs in children have been shown to affect growth among other side effects and, in pediatrics, there is great interest in safely discontinuing TKIs. Prospective studies testing this strategy in pediatrics are currently ongoing. In summary, TKIs have transformed CML, but the optimal TKI, duration, and the exact role of HSCT are still not defined in pediatrics.

Prognosis

There are several prognostic scoring schemas that accurately predict the outcome in adults in the context of a variety of therapies including TKIs. Prognostic include age, sex, spleen size, white count, basophil, eosinophil and blast count.[229] However, none of these prognostic models have ever been prospectively tested in pediatric CML. In general, early cytogenetic and molecular responses are associated with favorable outcomes in both adult and pediatric studies.

Other Myeloproliferative Neoplasms

Essential Thrombocythemia

ET has an estimated incidence of 1 to 1.25 cases per million in adults, but it is even rarer in children, with an estimated incidence of 0.09 cases per million.[230–234] ET is a genetically heterogeneous disease. Mutations in *JAK2* have been reported in adults with *BCR-ABL*–negative MPNs, including ET, PV, and idiopathic myelofibrosis (IM). It has been proposed that ET consists of several subtypes, with some children and adults having monoclonal disease but others having polyclonal disease. Further investigation into the mutational status of *JAK2* V617F also demonstrates mutation-positive (roughly 55%) and mutation-negative patients.[231,235] Another cause of ET was recently demonstrated to be mutations in CALR (calreticulin).[236] Studies suggest that a similar proportion of children with sporadic ET display polycythemia rubra vera-1 (PRV-1) RNA overexpression, *JAK2* V617F mutations, and monoclonal disease, as compared to adults with sporadic ET.[237] Rare cases of familial ET have been shown to be caused by dominant activating mutations in both *c-MPL*[237] and in *JAK2*.[238]

Patients with ET have thrombocythemia with an increased number of megakaryocytes in the BM. Platelet clumps on peripheral blood smears are common. Some patients have a concomitant increase in the number of peripheral blood granulocytes. The differential diagnosis includes familial ET, other forms of MPS, and increased platelets as a reactive process.

Although the clinical course can be uncomplicated, patients can develop thromboembolic complications, including deep venous thrombosis, and arterial thrombosis such as transient cerebral ischemia and peripheral vascular ischemia. The clinical course for children is typically less aggressive than in adults, with fewer thrombotic episodes.[237,239] Adult patients have a median survival of at least 10 years, with most deaths caused by thrombosis.[240,241] Progression to AML has been reported in adult patients, usually those with a prior exposure to chemotherapy agents.

Asymptomatic children do not require treatment. There is no clear association with platelet count and thrombotic events. As such, there is not a clear role for treatment with aspirin, which can increase the risk of paradoxical bleeding episodes.[242] Risk factors for thrombosis include leukocytosis and the presence of a *JAK2* V617F mutation.[243] A number of treatment approaches aimed at controlling thrombocytosis have been attempted for symptomatic patients, including hydroxyurea, IFN-α, and anagrelide.[244–246] The JAK inhibitor ruxolitinib may benefit some patients with ET, especially those with *JAK2* V617F mutations, although pediatric-specific data with this agent are lacking. The only potentially curative therapy for ET is allogeneic HCT.[247] Because ET is primarily a disease of older individuals, the data on its efficacy is mainly limited to adult patients with ET that had transformed to myelofibrosis or AML.[248–250] However, younger patients, and those with matched related donors, did especially well, suggesting that allogeneic transplant should be considered in the rare pediatric patient with symptomatic ET.

Idiopathic Myelofibrosis

Idiopathic MF is very rarely diagnosed in children[251] and should be differentiated from acute megakaryocytic leukemia; metastatic neoplasms; and connective tissue, metabolic, and bone diseases. It is characterized by BM fibrosis, megakaryocytes with bizarre morphology, splenomegaly, anemia, and extramedullary hematopoiesis. Idiopathic MF is a clonal disorder, with approximately half of adult patients having mutations in *JAK2* V617F.[252] Recent studies have suggested that angiogenic cytokines produced by megakaryocytes and monocytes and their receptors (platelet-derived growth factor receptors, vascular endothelial growth factor receptor-2, and fibroblast growth factor receptor) may be involved in the pathogenesis of the myelofibrosis.[253]

Treatment algorithms for adult MF have been proposed (Fig. 65.6).[254] Numerous treatment approaches have been attempted with variable degrees of success. These include splenectomy, splenic radiation, transfusions, androgens, corticosteroids, hydroxyurea, and IFN-α.[255–257] More recently, based on preliminary studies about the potential role of angiogenic cytokines and their receptors, newer approaches have been tested, including the tyrosine kinase inhibitor imatinib[258,259] and the antiangiogenic agent thalidomide.[260,261] Selective JAK inhibitors have been successfully used in patients with IM with a dramatic improvement in the quality of life, but without generalized reduction of the *JAK2* V617F burden allele.[262] In 2011, the United States Food and Drug Administration granted approval to the first of these agents, ruxolitinib, for adults with IM. However, the safety and efficacy of ruxolitinib in children is still unknown. Further trials are investigating the combination of JAK inhibition in combination with other therapeutic modalities, such as PI3K inhibition, mammalian target of rapamycin inhibition, histone deacetylase inhibition, and antifibrotic agents.[263]

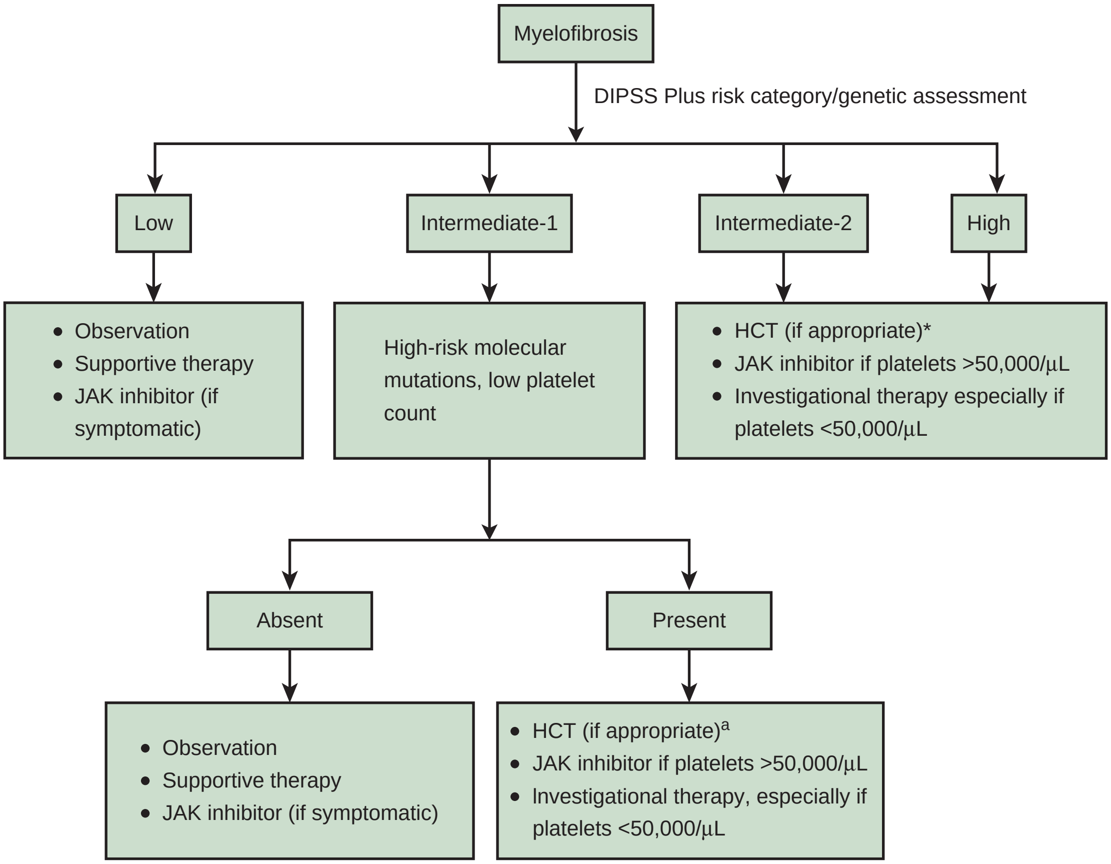

Myelofibrosis
DIPSS Plus risk category/genetic assessment
Low
Intermediate-1
Intermediate-2
High
Observation
Supportive therapy
JAK inhibitor (if symptomatic)
High-risk molecular mutations, low platelet count
HCT (if appropriate)*
JAK inhibitor if platelets >50,000/µL
Investigational therapy especially if platelets <50,000/µL
Absent
Present
Observation
Supportive therapy
JAK inhibitor (if symptomatic)
HCT (if appropriate)a
JAK inhibitor if platelets >50,000/µL
Investigational therapy, especially if platelets <50,000/µL

Figure 65.6 TREATMENT ALGORITHM FOR ADULT MYELOFIBROSIS. [a]Directly to hematopoietic cell transplantation or after bridging therapy to decrease marrow blasts. *(From Jain T, Mesa RA, Palmer JM. Allogeneic stem cell transplantation in myelofibrosis. Biol Blood Marrow Transplant. 2017;23(9):1429–1436.)*

Retrospective adult studies suggest that MF patients who respond to JAK2 inhibitors have significantly improved post-HCT OS,[264,265] though a prospective trial was complicated by signs of ruxolitinib withdrawal, including cardiogenic shock and tumor lysis.[266] When used, it appears important to continue JAK2 inhibition until close to the start time of conditioning chemotherapy.

Allogeneic HCT is the only potentially curative option for MF, with resolution of BM fibrosis in donor-engrafted patients, but high rates of transplant-related complications, even with reduced intensity conditioning.[249,267–271] Risk-prediction models (assessing age, performance status, WBC and platelet parameters, mutation status, and donor type) have been built for adults undergoing HCT for MF, which identifies groups with 5-year survival rates as low as 22% and as high as 83%,[272] though whether this data is applicable to children is unknown. Algorithms to help determine whether adult patients should undergo HCT have been proposed (Fig. 65.7).[273]

Polycythemia Vera

PV has an incidence of three cases per 100,000 in adults but is very rarely seen in children, with fewer than 0.1% of PV patients diagnosed before 20 years of age.[270] PV in children is far more often the result of congenital or acquired causes of an increased red blood cell mass. These include primary familial polycythemia (characterized by specific mutations in the erythropoietic receptor gene)[274] and secondary polycythemias caused by abnormal hemoglobins, cardiac and pulmonary disease, excessive erythropoietin, and a relative polycythemia that is the result of decreased plasma volume.

PV in children and adults is associated with clonal hematopoiesis,[275] *JAK2* V617F mutations (in 95% of patients; with mutations in *JAK2* exon 12 representing 2% to 4%),[237,276–281] overexpression of PVR-1 RNA,[282,283] and endogenous erythroid colony growth.[284,285] In contrast to adults, children with PV may be less likely to have *JAK2* V617F mutations and endogenous erythroid colony growth,[237,286] such that the proposed revision to WHOs diagnostic criteria,[287] may be less helpful in the diagnosis of PV in children, although other reports suggest that the proposed WHO criteria are equally applicable to children.[288] The proposed criteria include: (1) a hemoglobin >16.5 g/dL in men or >16.0 g/dL in women; (2) a BM biopsy showing trilineage myeloproliferation and pleomorphic megakaryocytes; and (3) either a *JAK2* mutation or a serum erythropoietin level below the normal reference range.[289] Although the pathogenesis is currently unclear, patients also have abnormalities in tissue factor, endogenous anticoagulants mechanisms, hyperhomocysteinemia, and acquired von Willebrand syndrome.

Children with PV may present with signs and symptoms associated with an increased red blood cell mass such as headache, dizziness, fatigue, pruritus, night sweats, and a ruddy complexion. Mild splenomegaly may be present, but massive hepatosplenomegaly is rare. As a result of hyperviscosity of the blood, elevated platelet counts, and coagulation abnormalities, patients can develop thromboses and CNS ischemia, although the incidence of thrombotic events may be lower than that reported in adults.[237,286]

Many different agents have been used to treat patients with PV, including chlorambucil,[290] melphalan,[291] 6-thioguanine,[292] uracil mustard,[293] busulfan,[294] carboquone,[295] anagrelide,[296] imatinib,[297] hydroxyurea,[298] radioactive phosphorous,[299] and IFN.[300] Despite these efforts, the best approach to patients with PV remains unclear. Therefore, the British Committee for Standards in Haematology formulated recommendations for the initial management of PV.[301] Recommendations include phlebotomy to maintain a hematocrit level <45%;[302] low-dose aspirin unless contraindicated; and cytoreduction if there is poor tolerance to phlebotomy, symptomatic or progressive splenomegaly, evidence of disease progression, or thrombocytosis. For patients younger than 40 years of age, pegylated IFN was the recommendation for first-line cytoreduction, with hydroxyurea as the

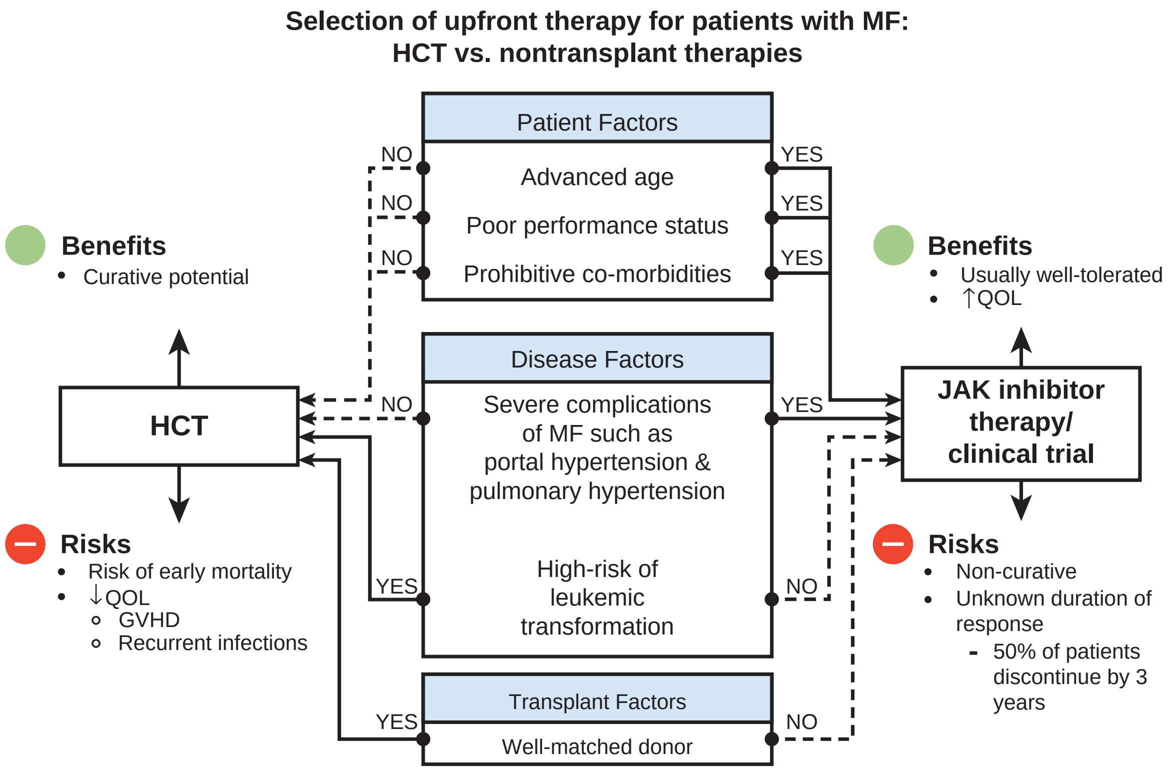

Selection of upfront therapy for patients with MF:
HCT vs. nontransplant therapies
Patient Factors
Advanced age
Poor performance status
Prohibitive co-morbidities
NO
NO
NO
YES
YES
YES
Benefits
Curative potential
HCT
Risks
Risk of early mortality
↓QOL
GVHD
Recurrent infections
Disease Factors
Severe complications of MF such as portal hypertension & pulmonary hypertension
High-risk of leukemic transformation
NO
YES
YES
NO
Transplant Factors
Well-matched donor
YES
NO
Benefits
Usually well-tolerated
↑QOL
JAK inhibitor therapy/ clinical trial
Risks
Non-curative
Unknown duration of response
50% of patients discontinue by 3 years

Figure 65.7 SELECTION OF UPFRONT THERAPY FOR PATIENTS WITH MYELOFIBROSIS: HEMATOPOIETIC CELL TRANSPLANTATION (HCT) VERSUS NONTRANSPLANT THERAPIES. *(Adapted from Devlin R, Gupta V. Myelofibrosis: to transplant or not to transplant? Hematology. 2016;2016[1]:543–551.)*

second choice. However, the JAK inhibitor ruxolitinib was recently found to have excellent results in controlling the hematocrit and splenomegaly with minimal side-effects in an open-label phase III trial.[303] This may eventually supplant IFN and hydroxyurea as front-line therapy once pediatric-specific dosing is available. Finally, allogeneic transplantation has been successfully used to treat some adult patients with PV,[247–250] especially those with progression to myelofibrosis or AML. Younger patients, and those with matched related donors, did especially well, suggesting that allogeneic transplant should be considered in pediatric patients with PV, though data is currently limited to case reports.[288,304]

FUTURE DIRECTIONS

Although a clearer understanding of the biologic mechanisms underlying MDS in children is slow in coming, dramatic progress has been made in our understanding of the genetic and biologic mechanisms contributing to TMD, JMML, PV, ET, and IM. There exists a potential for targeted therapeutics for many of these disorders, with the first targeted agents now receiving approval for use in adults. However, the use of these approved agents in children remains investigational.

Progress for both MDS and MPN in children will require international cooperation. The rarity of these disorders and limitations in resources for pediatric cooperative cancer groups make clinical trials within each individual cooperative group increasingly difficult.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Caye A, Strullu M, Guidez F, et al. Juvenile myelomonocytic leukemia displays mutations in components of the RAS pathway and the PRC2 network. *Nat Genet*. 2015;47(11):1334–1340.

Chaudhury S, HiJiya N, Hu ZH, et al. Outcomes of allogeneic hematopoietic stem cell transplantation in children and young adults with chronic myeloid leukemia: a CIBMTR Cohort analysis. *Blood*. 2014;124(21).

Devlin R, Gupta V. Myelofibrosis: to transplant or not to transplant? *Hematology*. 2016;2016(1):543–551.

Gamis AS, Alonzo TA, Gerbing RB, et al. Natural history of transient myeloproliferative disorder clinically diagnosed in Down syndrome neonates: a report from the Children's Oncology Group Study A2971. *Blood*. 2011;118(26):6752–6759.

Hasford J, Baccarani M, Hoffmann V, et al. Predicting complete cytogenetic response and subsequent progression-free survival in 2060 patients with CML on imatinib treatment: the EUTOS score. *Blood*. 2011;118(3):686–692.

Hecht A, Meyer J, Chehab FF, et al. Molecular assessment of pretransplant chemotherapy in the treatment of juvenile myelomonocytic leukemia. *Pediatr Blood Cancer*. 2019;66(11). e27948-e27948.

Jain T, Mesa RA, Palmer JM. Allogeneic stem cell transplantation in myelofibrosis. *Biol Blood Marrow Transplant*. 2017;23(9):1429–1436.

Klusmann J-H, Creutzig U, Zimmermann M, et al. Treatment and prognostic impact of transient leukemia in neonates with Down syndrome. *Blood*. 2008;111(6):2991–2998.

Labuhn M, Perkins K, Matzk S, et al. Mechanisms of progression of myeloid preleukemia to transformed myeloid leukemia in children with Down syndrome. *Cancer Cell*. 2019;36(2):123–138. e110.

Lipka DB, Witte T, Toth R, et al. RAS-pathway mutation patterns define epigenetic subclasses in juvenile myelomonocytic leukemia. *Nat Commun*. 2017;8(1):2126.

Locatelli F, Nöllke P, Zecca M, et al. Hematopoietic stem cell transplantation (HSCT) in children with juvenile myelomonocytic leukemia (JMML): results of the EWOG-MDS/EBMT trial. *Blood*. 2005;105(1):410–419.

Locatelli F, Strahm B. How I treat myelodysplastic syndromes of childhood. *Blood*. 2018;131(13):1406–1414.

Lock R, Cichowski K. Loss of negative regulators amplifies RAS signaling. *Nat Genet*. 2015;47(5):426–427.

McNulty M, Crispino JD. Acute megakaryocytic leukemia. *Cold Spring Harb Perspect Med*. 2020;10(2).

Millot Fdr, Suttorp M, Guilhot J, et al. The International Registry for Chronic Myeloid Leukemia (CML) in children and adolescents (I-CML-Ped-Study): objectives and preliminary results. *Blood*. 2012;120(21). 3741-3741.

Muramatsu H, Watanabe T, Hasegawa D, et al. Prospective study of 168 infants with transient abnormal myelopoiesis with Down syndrome: Japan Pediatric Leukemia/Lymphoma Study Group, TAM-10 study. *Blood*. 2015;126(23). 1311–1311.

Nižetić D, Groet J. Tumorigenesis in Down's syndrome: big lessons from a small chromosome. *Nat Rev Cancer*. 2012;12(10):721–732.

Roberts I, Alford K, Hall G, et al. GATA1-mutant clones are frequent and often unsuspected in babies with Down syndrome: identification of a population at risk of leukemia. *Blood*. 2013;122(24):3908–3917.

Sakaguchi H, Okuno Y, Muramatsu H, et al. Exome sequencing identifies secondary mutations of SETBP1 and JAK3 in juvenile myelomonocytic leukemia. *Nat Genet*. 2013;45(8):937–941.

Schönung M, Meyer J, Nöllke P, et al. International consensus definition of DNA methylation subgroups in Juvenile myelomonocytic leukemia. *Clin Cancer Res*. 2021;27(1):158–168.

Stieglitz E, Mazor T, Olshen AB, et al. Genome-wide DNA methylation is predictive of outcome in juvenile myelomonocytic leukemia. *Nat Commun*. 2017;8(1):2127.

Stieglitz E, Taylor-Weiner AN, Chang TY, et al. Corrigendum: the genomic landscape of juvenile myelomonocytic leukemia. *Nat Genet*. 2016;48(1):101.

Taub JW, Berman JN, Hitzler JK, et al. Improved outcomes for myeloid leukemia of Down syndrome: a report from the Children's Oncology Group AAML0431 trial. *Blood*. 2017;129(25):3304–3313.

Tunstall O, Bhatnagar N, James B, et al. Guidelines for the investigation and management of transient leukaemia of Down syndrome. *Br J Haematol*. 2018;182(2):200–211.

Uffmann M, Rasche M, Zimmermann M, et al. Therapy reduction in patients with Down syndrome and myeloid leukemia: the International ML-DS 2006 Trial. *Blood*. 2017;129(25):3314–3321.

Waespe N, Van Den Akker M, Klaassen RJ, et al. Response to treatment with azacitidine in children with advanced myelodysplastic syndrome prior to hematopoietic stem cell transplantation. *Haematologica*. 2016;101(12):1508–1515.

Yoo JW, Im HJ, Kim H, et al. Improved outcomes of allogeneic hematopoietic stem cell transplantation including haploidentical transplantation for childhood myelodysplastic syndrome. *Bone Marrow Transplant*. 2020;55(8):1595–1603.

Yoshida K, Toki T, Okuno Y, et al. The landscape of somatic mutations in Down syndrome-related myeloid disorders. *Nat Genet*. 2013;45(11):1293–1299.

REFERENCES

1. Look AT. Molecular pathogenesis of MDS. *Hematol Am Soc Hematol Educ Program*. 2005:156–160.
2. Hasle H, Kerndrup G, Jacobsen BB. Childhood myelodysplastic syndrome in Denmark: incidence and predisposing conditions. *Leukemia*. 1995;9(9):1569–1572.
3. Hasle H, Wadsworth LD, Massing BG, McBride M, Schultz KR. A population-based study of childhood myelodysplastic syndrome in British Columbia, Canada. *Br J Haematol*. 1999;106(4):1027–1032.
4. Passmore SJ, Chessells JM, Kempski H, Hann IM, Brownbill PA, Stiller CA. Paediatric myelodysplastic syndromes and juvenile myelomonocytic leukaemia in the UK: a population-based study of incidence and survival. *Br J Haematol*. 2003;121(5):758–767.
5. Luna-Fineman S, Shannon KM, Atwater SK, et al. Myelodysplastic and myeloproliferative disorders of childhood: a study of 167 patients. *Blood*. 1999;93(2):459–466.
6. Sasaki H, Manabe A, Kojima S, et al. Myelodysplastic syndrome in childhood: a retrospective study of 189 patients in Japan. *Leukemia*. 2001;15(11):1713–1720.
7. Strahm B, Nöllke P, Zecca M, et al. Hematopoietic stem cell transplantation for advanced myelodysplastic syndrome in children: results of the EWOG-MDS 98 study. *Leukemia*. 2011;25(3):455–462.
8. Gilliland DG. Molecular genetics of human leukemia. *Leukemia*. 1998;12(Suppl 1):S7–S12.
9. Tefferi A, Gilliland DG. Oncogenes in myeloproliferative disorders. *Cell Cycle*. 2007;6(5):550–566.
10. Tefferi A, Gilliland G. Classification of chronic myeloid disorders: from Dameshek towards a semi-molecular system. *Best Pract Res Clin Haematol*. 2006;19(3):365–385.
11. Daley GQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. *Science*. 1990;247(4944):824–830.
12. Kelliher MA, McLaughlin J, Witte ON, Rosenberg N. Induction of a chronic myelogenous leukemia-like syndrome in mice with v-abl and BCR/ABL. *Proc Natl Acad Sci U S A*. 1990;87(17):6649–6653.
13. Tefferi A, Gilliland DG. JAK2 in myeloproliferative disorders is not just another kinase. *Cell Cycle*. 2005;4(8):1053–1056.
14. Garcia-Montero AC, Jara-Acevedo M, Teodosio C, et al. KIT mutation in mast cells and other bone marrow hematopoietic cell lineages in systemic mast cell disorders: a prospective study of the Spanish Network on Mastocytosis (REMA) in a series of 113 patients. *Blood*. 2006;108(7):2366–2372.
15. Pardanani A, Brockman SR, Paternoster SF, et al. FIP1L1-PDGFRA fusion: prevalence and clinicopathologic correlates in 89 consecutive patients with moderate to severe eosinophilia. *Blood*. 2004;104(10):3038–3045.
16. Xiao S, Nalabolu SR, Aster JC, et al. FGFR1 is fused with a novel zinc-finger gene, ZNF198, in the t(8;13) leukaemia/lymphoma syndrome. *Nat Genet*. 1998;18(1):84–87.
17. Loh ML, Vattikuti S, Schubbert S, et al. Mutations in PTPN11 implicate the SHP-2 phosphatase in leukemogenesis. *Blood*. 2004;103(6):2325–2331.
18. Shannon KM, O'Connell P, Martin GA, et al. Loss of the normal NF1 allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeloid disorders. *N Engl J Med*. 1994;330(9):597–601.
19. Lauchle JO, Braun BS, Loh ML, Shannon K. Inherited predispositions and hyperactive Ras in myeloid leukemogenesis. *Pediatr Blood Cancer*. 2006;46(5):579–585.
20. Crispino JD. GATA1 mutations in Down syndrome: implications for biology and diagnosis of children with transient myeloproliferative disorder and acute megakaryoblastic leukemia. *Pediatr Blood Cancer*. 2005;44(1):40–44.
21. Castilla LH, Garrett L, Adya N, et al. The fusion gene Cbfb-MYH11 blocks myeloid differentiation and predisposes mice to acute myelomonocytic leukaemia. *Nat Genet*. 1999;23(2):144–146.
22. Higuchi M, O'Brien D, Kumaravelu P, Lenny N, Yeoh E-J, Downing JR. Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia. *Cancer Cell*. 2002;1(1):63–74.
23. Ford AM, Ridge SA, Cabrera ME, et al. In utero rearrangements in the trithorax-related oncogene in infant leukaemias. *Nature*. 1993;363(6427):358–360.
24. Gill Super HJ, Rothberg PG, Kobayashi H, Freeman AI, Diaz MO, Rowley JD. Clonal, nonconstitutional rearrangements of the MLL gene in infant twins with acute lymphoblastic leukemia: in utero chromosome rearrangement of 11q23. *Blood*. 1994;83(3):641–644.
25. Megonigal MD, Rappaport EF, Jones DH, et al. t(11;22)(q23;q11.2) In acute myeloid leukemia of infant twins fuses MLL with hCDCrel, a cell division cycle gene in the genomic region of deletion in DiGeorge and velocardiofacial syndromes. *Proc Natl Acad Sci U S A*. 1998;95(11):6413–6418.
26. Wiemels JL, Ford AM, Van Wering ER, Postma A, Greaves M. Protracted and variable latency of acute lymphoblastic leukemia after TEL-AML1 gene fusion in utero. *Blood*. 1999;94(3):1057–1062.
27. Song WJ, Sullivan MG, Legare RD, et al. Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. *Nat Genet*. 1999;23(2):166–175.
28. Jekic B, Novakovic I, Lukovic L, et al. Lack of TP53 and FMS gene mutations in children with myelodysplastic syndrome. *Cancer Genet Cytogenet*. 2006;166(2):163–165.
29. Jekic B, Novakovic I, Lukovic L, et al. Low frequency of NRAS and KRAS2 gene mutations in childhood myelodysplastic syndromes. *Cancer Genet Cytogenet*. 2004;154(2):180–182.
30. Hirabayashi S, Flotho C, Moetter J, et al. Spliceosomal gene aberrations are rare, coexist with oncogenic mutations, and are unlikely to exert a driver effect in childhood MDS and JMML. *Blood*. 2012;119(11):e96–e99.
31. Glaubach T, Robinson LJ, Corey SJ. Pediatric myelodysplastic syndromes: they do exist! *J Pediatr Hematol Oncol*. 2014;36(1):1–7.
32. Coutinho DF, Monte-Mór BCR, Vianna DT, et al. TET2 expression level and 5-hydroxymethylcytosine are decreased in refractory cytopenia of childhood. *Leuk Res*. 2015;39(10):1103–1108.
33. Owen C, Barnett M, Fitzgibbon J. Familial myelodysplasia and acute myeloid leukaemia—a review. *Br J Haematol*. 2008;140(2):123–132.
34. Liew E, Owen C. Familial myelodysplastic syndromes: a review of the literature. *Haematologica*. 2011;96(10):1536–1542.
35. Hahn CN, Chong CE, Carmichael CL, et al. Heritable GATA2 mutations associated with familial myelodysplastic syndrome and acute myeloid leukemia. *Nat Genet*. 2011;43(10):1012–1017.
36. Steliarova-Foucher E, Stiller C, Lacour B, Kaatsch P. International classification of childhood cancer, third edition. *Cancer*. 2005;103(7):1457–1467.
37. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of the myelodysplastic syndromes. *Br J Haematol*. 1982;51(2):189–199.
38. Gattermann N, Aul C, Schneider W. Is acquired idiopathic sideroblastic anemia (AISA) a disorder of mitochondrial DNA? *Leukemia*. 1993;7(12):2069–2076.
39. Bader-Meunier B, Rötig A, Mielot F, et al. Refractory anaemia and mitochondrial cytopathy in childhood. *Br J Haematol*. 1994;87(2):381–385.
40. Harris NL, Jaffe ES, Diebold J, et al. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. *J Clin Oncol*. 1999;17(12):3835–3849.
41. Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*. 2009;114(5):937–951.
42. Baumann I, Niemeyer C, Bennett J. Childhood myelodysplastic syndrome. In: Swerdlow S, Campo E, Harris N, eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon: IARC Press; 2008:104.
43. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127(20):2391–2405.
44. Hasle H, Niemeyer CM, Chessells JM, et al. A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative diseases. *Leukemia*. 2003;17(2):277–282.
45. Kardos G, Baumann I, Passmore SJ, et al. Refractory anemia in childhood: a retrospective analysis of 67 patients with particular reference to monosomy 7. *Blood*. 2003;102(6):1997–2003.
46. Ramos F, Fernández-Ferrero S, Suárez D, et al. Myelodysplastic syndrome: a search for minimal diagnostic criteria. *Leuk Res*. 1999;23(3):283–290.
47. Dunn DE, Tanawattanacharoen P, Boccuni P, et al. Paroxysmal nocturnal hemoglobinuria cells in patients with bone marrow failure syndromes. *Ann Intern Med*. 1999;131(6):401–408.
48. Aalbers AM, van den Heuvel-Eibrink MM, Baumann I, et al. Bone marrow immunophenotyping by flow cytometry in refractory cytopenia of childhood. *Haematologica*. 2015;100(3):315–323.
49. Elmahdi S, Hama A, Manabe A, et al. A cytokine-based diagnostic program in pediatric aplastic anemia and hypocellular refractory cytopenia of childhood. *Pediatr Blood Cancer*. 2016;63(4):652–658.
50. Webb DKH, Passmore SJ, Hann IM, Harrison G, Wheatley K, Chessells JM. Results of treatment of children with refractory anaemia with excess blasts (RAEB) and RAEB in transformation (RAEBt) in Great Britain 1990–99. *Br J Haematol*. 2002;117(1):33–39.
51. Woods WG, Barnard DR, Alonzo TA, et al. Prospective study of 90 children requiring treatment for juvenile myelomonocytic leukemia or myelodysplastic syndrome: a report from the Children's Cancer Group. *J Clin Oncol*. 2002;20(2):434–440.

52. Bagby Jr. GC, Gabourel JD, Linman JW. Glucocorticoid therapy in the preleukemic syndrome (hemopoietic dysplasia): identification of responsive patients using in-vitro techniques. *Ann Intern Med*. 1980;92(1):55–58.
53. Hurtado R, Sosa R, Majluf A, Labardini JR. Refractory anaemia (RA) type I FAB treated with oxymetholone (OXY): long-term results. *Br J Haematol*. 1993;85(1):235–236.
54. Najean Y, Pecking A. Refractory anemia with excess of blast cells: prognostic factors and effect of treatment with androgens or cytosine arabinoside. Results of a prospective trial in 58 patients. Cooperative Group for the Study of Aplastic and Refractory Anemias. *Cancer*. 1979;44(6):1976–1982.
55. Cines DB, Cassileth PA, Kiss JE. Danazol therapy in myelodysplasia. *Ann Intern Med*. 1985;103(1):58–60.
56. Letendre L, Levitt R, Pierre RV, et al. Myelodysplastic syndrome treatment with danazol and cis-retinoic acid. *Am J Hematol*. 1995;48(4):233–236.
57. Koeffler HP, Heitjan D, Mertelsmann R, et al. Randomized study of 13-cis retinoic acid v placebo in the myelodysplastic disorders. *Blood*. 1988;71(3):703–708.
58. Clark RE, Ismail SA, Jacobs A, Payne H, Smith SA. A randomized trial of 13-cis retinoic acid with or without cytosine arabinoside in patients with the myelodysplastic syndrome. *Br J Haematol*. 1987;66(1):77–83.
59. Kurzrock R, Estey E, Talpaz M. All-trans retinoic acid: tolerance and biologic effects in myelodysplastic syndrome. *J Clin Oncol*. 1993;11(8):1489–1495.
60. Ohno R, Naoe T, Hirano M, et al. Treatment of myelodysplastic syndromes with all-trans retinoic acid. Leukemia Study Group of the Ministry of Health and Welfare. *Blood*. 1993;81(5):1152–1154.
61. Schuster MW. Granulocyte-macrophage colony-stimulating factor (GM-CSF) for myelodysplastic syndrome: results of a multi-center randomized controlled trial. *Blood*. 1990;76:318a.
62. Greenberg P. Phase III randomized multicenter trial of G-CSF vs observation for MDS [abstract]. *Blood*. 1993;82(suppl 1):196a.
63. Hellström-Lindberg E. Efficacy of erythropoietin in the myelodysplastic syndromes: a meta-analysis of 205 patients from 17 studies. *Br J Haematol*. 1995;89(1):67–71.
64. Negrin RS, Stein R, Vardiman J, et al. Treatment of the anemia of myelodysplastic syndromes using recombinant human granulocyte colony-stimulating factor in combination with erythropoietin. *Blood*. 1993;82(3):737–743.
65. Negrin RS, Stein R, Doherty K, et al. Maintenance treatment of the anemia of myelodysplastic syndromes with recombinant human granulocyte colony-stimulating factor and erythropoietin: evidence for in vivo synergy. *Blood*. 1996;87(10):4076–4081.
66. Sandor V, Bakke S, Robey RW, et al. Phase I trial of the histone deacetylase inhibitor, depsipeptide (FR901228, NSC 630176), in patients with refractory neoplasms. *Clin Cancer Res*. 2002;8(3):718–728.
67. Marshall JL, Rizvi N, Kauh J, et al. A phase I trial of depsipeptide (FR901228) in patients with advanced cancer. *J Exp Ther Oncol*. 2002;2(6):325–332.
68. Bejar R, Steensma DP. Recent developments in myelodysplastic syndromes. *Blood*. 2014;124(18):2793–2803.
69. Mathew P, Gerbing R, Alonzo TA, et al. A phase II study of amifostine in children with myelodysplastic syndrome: a report from the Children's Oncology Group study (AAML0121). *Pediatr Blood Cancer*. 2011;57(7):1230–1232.
70. Cseh AM, Niemeyer CM, Yoshimi A, et al. Therapy with low-dose azacitidine for MDS in children and young adults: a retrospective analysis of the EWOG-MDS study group. *Br J Haematol*. 2016;172(6):930–936.
71. Waespe N, Van Den Akker M, Klaassen RJ, et al. Response to treatment with azacitidine in children with advanced myelodysplastic syndrome prior to hematopoietic stem cell transplantation. *Haematologica*. 2016;101(12):1508–1515.
72. Smith AR, Christiansen EC, Wagner JE, et al. Early hematopoietic stem cell transplant is associated with favorable outcomes in children with MDS. *Pediatr Blood Cancer*. 2013;60(4):705–710.
73. Yahng S-A, Kim M, Kim T-M, et al. Better transplant outcome with pre-transplant marrow response after hypomethylating treatment in higher-risk MDS with excess blasts. *Oncotarget*. 2017;8(7):12342–12354.
74. Voso MT, Leone G, Piciocchi A, et al. Feasibility of allogeneic stem-cell transplantation after azacitidine bridge in higher-risk myelodysplastic syndromes and low blast count acute myeloid leukemia: results of the BMT-AZA prospective study. *Ann Oncol*. 2017;28(7):1547–1553.
75. Strahm B, Locatelli F, Bader P, et al. Reduced intensity conditioning in unrelated donor transplantation for refractory cytopenia in childhood. *Bone Marrow Transpl*. 2007;40(4):329–333.
76. Yusuf U, Frangoul HA, Gooley TA, et al. Allogeneic bone marrow transplantation in children with myelodysplastic syndrome or juvenile myelomonocytic leukemia: the Seattle experience. *Bone Marrow Transpl*. 2004;33(8):805–814.
77. Trobaugh-Lotrario AD, Kletzel M, Quinones RR, et al. Monosomy 7 associated with pediatric acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS): successful management by allogeneic hematopoietic stem cell transplant (HSCT). *Bone Marrow Transpl*. 2005;35(2):143–149.
78. Kalwak K, Wójcik D, Gorczyńska E, et al. Allogeneic hematopoietic cell transplantation from alternative donors in children with myelodysplastic syndrome: is that an alternative? *Transpl Proc*. 2004;36(5):1574–1577.
79. Woodard P, Carpenter PA, Davies SM, et al. Unrelated donor bone marrow transplantation for myelodysplastic syndrome in children. *Biol Blood Marrow Transpl*. 2011;17(5):723–728.
80. Madureira ABM, Eapen M, Locatelli F, et al. Analysis of risk factors influencing outcome in children with myelodysplastic syndrome after unrelated cord blood transplantation. *Leukemia*. 2011;25(3):449–454.
81. Yoo JW, Im HJ, Kim H, et al. Improved outcomes of allogeneic hematopoietic stem cell transplantation including haploidentical transplantation for childhood myelodysplastic syndrome. *Bone Marrow Transpl*. 55(8):1595–1603. doi: 10.1038/s41409-020-0814-8.
82. Inagaki J, Fukano R, Kurauchi K, Noguchi M, Tanioka S, Okamura J. Hematopoietic stem cell transplantation in children with refractory cytopenia of childhood: single-center experience using high-dose cytarabine containing myeloablative and aplastic anemia oriented reduced-intensity conditioning regimens. *Biol Blood Marrow Transpl*. 2015;21(3):565–569.
83. Nemecek ER, Hilger RA, Adams A, et al. Treosulfan, fludarabine, and low-dose total body irradiation for children and young adults with acute myeloid leukemia or myelodysplastic syndrome undergoing allogeneic hematopoietic cell transplantation: prospective phase II trial of the pediatric blood and marrow transplant consortium. *Biol Blood Marrow Transpl*. 2018;24(8):1651–1656.
84. Sakellari I, Mallouri D, Gavriilaki E, et al. Survival advantage and comparable toxicity in reduced-toxicity treosulfan-based versus reduced-intensity busulfan-based conditioning regimen in myelodysplastic syndrome and acute myeloid leukemia patients after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transpl*. 2017;23(3):445–451.
85. Locatelli F, Strahm B. How I treat myelodysplastic syndromes of childhood. *Blood*. 2018;131(13):1406–1414.
86. de Lima M, Giralt S, Thall PF, et al. Maintenance therapy with low-dose azacitidine after allogeneic hematopoietic stem cell transplantation for recurrent acute myelogenous leukemia or myelodysplastic syndrome: a dose and schedule finding study. *Cancer*. 2010;116(23):5420–5431.
87. Pusic I, Choi J, Fiala MA, et al. Maintenance therapy with decitabine after allogeneic stem cell transplantation for acute myelogenous leukemia and myelodysplastic syndrome. *Biol Blood Marrow Transpl*. 2015;21(10):1761–1769.
88. Greenberg P, Cox C, LeBeau MM, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. *Blood*. 1997;89(6):2079–2088.
89. Malcovati L, Germing U, Kuendgen A, et al. Time-dependent prognostic scoring system for predicting survival and leukemic evolution in myelodysplastic syndromes. *J Clin Oncol*. 2007;25(23):3503–3510.
90. Della Porta MG, Tuechler H, Malcovati L, et al. Validation of WHO classification-based prognostic scoring system (WPSS) for myelodysplastic syndromes and comparison with the revised international prognostic scoring system (IPSS-R). A study of the International Working Group for Prognosis in Myelodysplasia (IWG-PM). *Leukemia*. 2015;29(7): 1502–1513.
91. Hasle H, Baumann I, Bergsträsser E, et al. The international prognostic scoring system (IPSS) for childhood myelodysplastic syndrome (MDS) and juvenile myelomonocytic leukemia (JMML). *Leukemia*. 2004;18(12):2008–2014.
92. Harrison CN, Vaughan G, Devereux S, Linch DC. Outcome of secondary myeloid malignancy in Hodgkin's disease: the BNLI experience. *Eur J Haematol*. 1998;61(2):109–112.
93. Harrison CN, Gregory W, Hudson GV, et al. High-dose BEAM chemotherapy with autologous haemopoietic stem cell transplantation for Hodgkin's disease is unlikely to be associated with a major increased risk of secondary MDS/AML. *Br J Cancer*. 1999;81(3):476–483.
94. Milligan DW, Ruiz De Elvira MC, Kolb HJ, et al. Secondary leukaemia and myelodysplasia after autografting for lymphoma: results from the EBMT. EBMT lymphoma and late effects working parties. European Group for Blood and Marrow Transplantation. *Br J Haematol*. 1999;106(4):1020–1026.
95. Laver JH, Yusuf U, Cantu ES, Barredo JC, Holt LB, Abboud MR. Transient therapy-related myelodysplastic syndrome associated with monosomy 7 and 11q23 translocation. *Leukemia*. 1997;11(3):448–450.
96. Thirman MJ, Larson RA. Therapy-related myeloid leukemia. *Hematol Oncol Clin North Am*. 1996;10(2):293–320.

97. Kushner BH, Heller G, Cheung NK, et al. High risk of leukemia after short-term dose-intensive chemotherapy in young patients with solid tumors. *J Clin Oncol*. 1998;16(9):3016–3020.
98. Schneider DT, Hilgenfeld E, Schwabe D, et al. Acute myelogenous leukemia after treatment for malignant germ cell tumors in children. *J Clin Oncol*. 1999;17(10):3226–3233.
99. Travis LB, Curtis RE, Storm H, et al. Risk of second malignant neoplasms among long-term survivors of testicular cancer. *J Natl Cancer Inst*. 1997;89(19):1429–1439.
100. Dunst J, Ahrens S, Paulussen M, et al. Second malignancies after treatment for Ewing's sarcoma: a report of the CESS-studies. *Int J Radiat Oncol Biol Phys*. 1998;42(2):379–384.
101. Burdach S, van Kaick B, Laws HJ, et al. Allogeneic and autologous stem-cell transplantation in advanced Ewing tumors. An update after long-term follow-up from two centers of the European Intergroup study EICESS. Stem-Cell Transplant Programs at Düsseldorf University Medical Center, Germany and St. Anna Kinderspital, Vienna, Austria. *Ann Oncol*. 2000;11(11):1451–1462.
102. Rodriguez-Galindo C, Poquette CA, Marina NM, et al. Hematologic abnormalities and acute myeloid leukemia in children and adolescents administered intensified chemotherapy for the Ewing sarcoma family of tumors. *J Pediatr Hematol Oncol*. 2000;22(4):321–329.
103. Tsurusawa M, Manabe A, Hayashi Y, et al. Therapy-related myelodysplastic syndrome in childhood: a retrospective study of 36 patients in Japan. *Leuk Res*. 2005;29(6):625–632.
104. Bhatia S, Krailo MD, Chen Z, et al. Therapy-related myelodysplasia and acute myeloid leukemia after Ewing sarcoma and primitive neuroectodermal tumor of bone: a report from the Children's Oncology Group. *Blood*. 2007;109(1):46–51.
105. Tebbi CK, London WB, Friedman D, et al. Dexrazoxane-associated risk for acute myeloid leukemia/myelodysplastic syndrome and other secondary malignancies in pediatric Hodgkin's disease. *J Clin Oncol*. 2007;25(5):493–500.
106. Yakoub-Agha I, de La Salmonière P, Ribaud P, et al. Allogeneic bone marrow transplantation for therapy-related myelodysplastic syndrome and acute myeloid leukemia: a long-term study of 70 patients-report of the French society of bone marrow transplantation. *J Clin Oncol*. 2000;18(5):963–971.
107. Barnard DR, Lange B, Alonzo TA, et al. Acute myeloid leukemia and myelodysplastic syndrome in children treated for cancer: comparison with primary presentation. *Blood*. 2002;100(2):427–434.
108. Sabloff M, Sobecks RM, Ahn KW, et al. Does total body irradiation conditioning improve outcomes of myeloablative human leukocyte antigen-identical sibling transplantations for chronic lymphocytic leukemia? *Biol Blood Marrow Transpl*. 2014;20(3):421–424.
109. Grossman J, Cuellar-Rodriguez J, Gea-Banacloche J, et al. Nonmyeloablative allogeneic hematopoietic stem cell transplantation for GATA2 deficiency. *Biol Blood Marrow Transpl*. 2014;20(12):1940–1948.
110. Woodard P, Barfield R, Hale G, et al. Outcome of hematopoietic stem cell transplantation for pediatric patients with therapy-related acute myeloid leukemia or myelodysplastic syndrome. *Pediatr Blood Cancer*. 2006;47(7):931–935.
111. Kobos R, Steinherz PG, Kernan NA, et al. Allogeneic hematopoietic stem cell transplantation for pediatric patients with treatment-related myelodysplastic syndrome or acute myelogenous leukemia. *Biol Blood Marrow Transpl*. 2012;18(3):473–480.
112. Ahmed IA, Farooqi MS, Vander Lugt MT, et al. Outcomes of hematopoietic cell transplantation in patients with germline SAMD9/SAMD9L mutations. *Biol Blood Marrow Transpl*. 2019;25(11):2186–2196.
113. Simonis A, Fux M, Nair G, et al. Allogeneic hematopoietic cell transplantation in patients with GATA2 deficiency—a case report and comprehensive review of the literature. *Ann Hematol*. 2018;97(10):1961–1973.
114. Galera P, Hsu AP, Wang W, et al. Donor-derived MDS/AML in families with germline GATA2 mutation. *Blood*. 2018;132(18):1994–1998.
115. Willasch AM, Kreyenberg H, Shayegi N, et al. Monitoring of hematopoietic chimerism after transplantation for pediatric myelodysplastic syndrome: real-time or conventional short tandem repeat PCR in peripheral blood or bone marrow? *Biol Blood Marrow Transpl*. 2014;20(12):1918–1925.
116. Bader P, Niemeyer C, Willasch A, et al. Children with myelodysplastic syndrome (MDS) and increasing mixed chimaerism after allogeneic stem cell transplantation have a poor outcome which can be improved by pre-emptive immunotherapy. *Br J Haematol*. 2005;128:649–658.
117. Emanuel PD. Myelodysplasia and myeloproliferative disorders in childhood: an update. *Br J Haematol*. 1999;105(4):852–863.
118. Niemeyer CM, Loh ML, Cseh A, et al. Criteria for evaluating response and outcome in clinical trials for children with juvenile myelomonocytic leukemia. *Haematologica*. 2015;100(1):17–22.
119. Hasle H. Myelodysplastic and myeloproliferative disorders in children. *Curr Opin Pediatr*. 2007;19(1):1–8.
120. Hays T, Humbert JR, Peakman DC, et al. Missing Y chromosome in juvenile chronic myelogenous leukemia. *Humangenetik*. 1975;29(3):259–264.
121. Inoue S, Ravindranath Y, Thompson RI, Zuelzer WW, Ottenbreit MJ. Cytogenetics of juvenile type chronic granulocytic leukemia. *Cancer*. 1977;39(5):2017–2024.
122. Inoue S, Shibata T, Ravindranath Y, Gohle N. Clonal origin of erythroid cells in juvenile chronic myelogenous leukemia. *Blood*. 1987;69(3):975–976.
123. Brodeur GM, Dow LW, Williams DL. Cytogenetic features of juvenile chronic myelogenous leukemia. *Blood*. 1979;53(5):812–819.
124. Amenomori T, Tomonaga M, Yoshida Y, et al. Cytogenetic evidence for partially committed myeloid progenitor cell origin of chronic myelomonocytic leukaemia and juvenile chronic myeloid leukaemia: both granulocyte-macrophage precursors and erythroid precursors carry identical marker chromosome. *Br J Haematol*. 1986;64(3):539–546.
125. Busque L, Gilliland DG, Prchal JT, et al. Clonality in juvenile chronic myelogenous leukemia. *Blood*. 1995;85(1):21–30.
126. Emanuel PD, Snyder RC, Wiley T, Gopurala B, Castleberry RP. Inhibition of juvenile myelomonocytic leukemia cell growth in vitro by farnesyltransferase inhibitors. *Blood*. 2000;95(2):639–645.
127. Cooper LJ, Shannon KM, Loken MR, Weaver M, Stephens K, Sievers EL. Evidence that juvenile myelomonocytic leukemia can arise from a pluripotential stem cell. *Blood*. 2000;96(6):2310–2313.
128. Lapidot T, Grunberger T, Vormoor J, et al. Identification of human juvenile chronic myelogenous leukemia stem cells capable of initiating the disease in primary and secondary SCID mice. *Blood*. 1996;88(7):2655–2664.
129. Stieglitz E, Troup CB, Gelston LC, et al. Subclonal mutations in SETBP1 confer a poor prognosis in juvenile myelomonocytic leukemia. *Blood*. 2015;125(3):516–524.
130. Miyauchi J, Asada M, Sasaki M, Tsunematsu Y, Kojima S, Mizutani S. Mutations of the N-ras gene in juvenile chronic myelogenous leukemia. *Blood*. 1994;83(8):2248–2254.
131. Largaespada DA, Brannan CI, Jenkins NA, Copeland NG. Nf1 deficiency causes Ras-mediated granulocyte/macrophage colony stimulating factor hypersensitivity and chronic myeloid leukaemia. *Nat Genet*. 1996;12(2):137–143.
132. Bollag G, Clapp DW, Shih S, et al. Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in haematopoietic cells. *Nat Genet*. 1996;12(2):144–148.
133. Tartaglia M, Niemeyer CM, Fragale A, et al. Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. *Nat Genet*. 2003;34(2):148–150.
134. Loh ML, Sakai DS, Flotho C, et al. Mutations in CBL occur frequently in juvenile myelomonocytic leukemia. *Blood*. 2009;114(9):1859–1863.
135. Niemeyer CM, Kang MW, Shin DH, et al. Germline CBL mutations cause developmental abnormalities and predispose to juvenile myelomonocytic leukemia. *Nat Genet*. 2010;42(9):794–800.
136. Muramatsu H, Makishima H, Jankowska AM, et al. Mutations of an E3 ubiquitin ligase c-Cbl but not TET2 mutations are pathogenic in juvenile myelomonocytic leukemia. *Blood*. 2010;115(10):1969–1975.
137. Niihori T, Aoki Y, Narumi Y, et al. Germline KRAS and BRAF mutations in cardio-facio-cutaneous syndrome. *Nat Genet*. 2006;38(3):294–296.
138. Schubbert S, Zenker M, Rowe SL, et al. Germline KRAS mutations cause Noonan syndrome. *Nat Genet*. 2006;38(3):331–336.
139. Carta C, Pantaleoni F, Bocchinfuso G, et al. Germline missense mutations affecting KRAS Isoform B are associated with a severe Noonan syndrome phenotype. *Am J Hum Genet*. 2006;79(1):129–135.
140. Zenker M, Lehmann K, Schulz AL, et al. Expansion of the genotypic and phenotypic spectrum in patients with KRAS germline mutations. *J Med Genet*. 2007;44(2):131–135.
141. Bertola DR, Pereira AC, Brasil AS, Albano LMJ, Kim CA, Krieger JE. Further evidence of genetic heterogeneity in Costello syndrome: involvement of the KRAS gene. *J Hum Genet*. 2007;52(6):521–526.
142. Schulz AL, Albrecht B, Arici C, et al. Mutation and phenotypic spectrum in patients with cardio-facio-cutaneous and Costello syndrome. *Clin Genet*. 2008;73(1):62–70.
143. Keilhack H, David FS, McGregor M, Cantley LC, Neel BG. Diverse biochemical properties of Shp2 mutants. Implications for disease phenotypes. *J Biol Chem*. 2005;280(35):30984–30993.
144. Kratz CP, Niemeyer CM, Castleberry RP, et al. The mutational spectrum of PTPN11 in juvenile myelomonocytic leukemia and Noonan syndrome/myeloproliferative disease. *Blood*. 2005;106(6):2183–2185.
145. Tartaglia M, Martinelli S, Stella L, et al. Diversity and functional consequences of germline and somatic PTPN11 mutations in human disease. *Am J Hum Genet*. 2006;78(2):279–290.
146. Strullu M, Caye A, Lachenaud J, et al. Juvenile myelomonocytic leukaemia and Noonan syndrome. *J Med Genet*. 2014;51(10):689–697.

147. Pérez B, Mechinaud F, Galambrun C, et al. Germline mutations of the CBL gene define a new genetic syndrome with predisposition to juvenile myelomonocytic leukaemia. *J Med Genet*. 2010;47(10):686–691.
148. Matsuda K, Taira C, Sakashita K, et al. Long-term survival after nonintensive chemotherapy in some juvenile myelomonocytic leukemia patients with CBL mutations, and the possible presence of healthy persons with the mutations. *Blood*. 2010;115:5429–5431.
149. Stieglitz E, Taylor-Weiner AN, Chang TY, et al. Corrigendum: the genomic landscape of juvenile myelomonocytic leukemia. *Nat Genet*. 2016;48(1):101.
150. Caye A, Strullu M, Guidez F, et al. Juvenile myelomonocytic leukemia displays mutations in components of the RAS pathway and the PRC2 network. *Nat Genet*. 2015;47(11):1334–1340.
151. Lock R, Cichowski K. Loss of negative regulators amplifies RAS signaling. *Nat Genet*. 2015;47(5):426–427.
152. Sakaguchi H, Okuno Y, Muramatsu H, et al. Exome sequencing identifies secondary mutations of SETBP1 and JAK3 in juvenile myelomonocytic leukemia. *Nat Genet*. 2013;45(8):937–941.
153. Stieglitz E, Mazor T, Olshen AB, et al. Genome-wide DNA methylation is predictive of outcome in juvenile myelomonocytic leukemia. *Nat Commun*. 2017;8(1):2127.
154. Lipka DB, Witte T, Toth R, et al. RAS-pathway mutation patterns define epigenetic subclasses in juvenile myelomonocytic leukemia. *Nat Commun*. 2017;8(1):2126.
155. Schönung M, Meyer J, Nöllke P, et al. International consensus definition of DNA methylation subgroups in juvenile myelomonocytic leukemia. *Clin Cancer Res*. 2021;27(1):158–168.
156. Ward AF, Braun BS, Shannon KM. Targeting oncogenic Ras signaling in hematologic malignancies. *Blood*. 2012;120(17):3397–3406.
157. Chang TY, Dvorak CC, Loh ML. Bedside to bench in juvenile myelomonocytic leukemia: insights into leukemogenesis from a rare pediatric leukemia. *Blood*. 2014;124(16):2487–2497.
158. Gandre-Babbe S, Paluru P, Aribeana C, et al. Patient-derived induced pluripotent stem cells recapitulate hematopoietic abnormalities of juvenile myelomonocytic leukemia. *Blood*. 2013;121(24):4925–4929.
159. Yoshimi A, Kamachi Y, Imai K, et al. Wiskott-Aldrich syndrome presenting with a clinical picture mimicking juvenile myelomonocytic leukaemia. *Pediatr Blood Cancer*. 2013;60(5):836–841.
160. Hoyoux C, Dresse MF, Forget P, et al. Osteopetrosis mimicking juvenile myelomonocytic leukemia. *Pediatr Int*. 2014;56(5):779–782.
161. Calvo KR, Price S, Braylan RC, et al. JMML and RALD (Ras-associated autoimmune leukoproliferative disorder): common genetic etiology yet clinically distinct entities. *Blood*. 2015;125(18):2753–2758.
162. Aricò M, Biondi A, Pui CH. Juvenile myelomonocytic leukemia. *Blood*. 1997;90(2):479–488.
163. Castro-Malaspina H, Schaison G, Passe S, et al. Subacute and chronic myelomonocytic leukemia in children (juvenile CML). Clinical and hematologic observations, and identification of prognostic factors. *Cancer*. 1984;54(4):675–686.
164. Matsuda K, Shimada A, Yoshida N, et al. Spontaneous improvement of hematologic abnormalities in patients having juvenile myelomonocytic leukemia with specific RAS mutations. *Blood*. 2007;109(12):5477–5480.
165. Imamura M, Imai C, Takachi T, Nemoto T, Tanaka A, Uchiyama M. Juvenile myelomonocytic leukemia with less aggressive clinical course and KRAS mutation. *Pediatr Blood Cancer*. 2008;51(4):569.
166. Locatelli F, Nöllke P, Zecca M, et al. Hematopoietic stem cell transplantation (HSCT) in children with juvenile myelomonocytic leukemia (JMML): results of the EWOG-MDS/EBMT trial. *Blood*. 2005;105(1):410–419.
167. Locatelli F, Crotta A, Ruggeri A, et al. Analysis of risk factors influencing outcomes after cord blood transplantation in children with juvenile myelomonocytic leukemia: a EUROCORD, EBMT, EWOG-MDS, CIBMTR study. *Blood*. 2013;122(12):2135–2141.
168. Bresolin S, Zecca M, Flotho C, et al. Gene expression-based classification as an independent predictor of clinical outcome in juvenile myelomonocytic leukemia. *J Clin Oncol*. 2010;28(11):1919–1927.
169. Yoshida N, Yagasaki H, Xu Y, et al. Correlation of clinical features with the mutational status of GM-CSF signaling pathway-related genes in juvenile myelomonocytic leukemia. *Pediatr Res*. 2009;65(3):334–340.
170. Niemeyer CM, Arico M, Basso G, et al. Chronic myelomonocytic leukemia in childhood: a retrospective analysis of 110 cases. European Working Group on Myelodysplastic Syndromes in Childhood (EWOG-MDS). *Blood*. 1997;89(10):3534–3543.
171. Chan HS, Estrov Z, Weitzman SS, Freedman MH. The value of intensive combination chemotherapy for juvenile chronic myelogenous leukemia. *J Clin Oncol*. 1987;5(12):1960–1967.
172. Chan GC, Wang WC, Raimondi SC, et al. Myelodysplastic syndrome in children: differentiation from acute myeloid leukemia with a low blast count. *Leukemia*. 1997;11(2):206–211.
173. Hasle H, Kerndrup G, Yssing M, et al. Intensive chemotherapy in childhood myelodysplastic syndrome. A comparison with results in acute myeloid leukemia. *Leukemia*. 1996;10(8):1269–1273.
174. Estrov Z, Dubé ID, Chan HS, Freedman MH. Residual juvenile chronic myelogenous leukemia cells detected in peripheral blood during clinical remission. *Blood*. 1987;70(5):1466–1469.
175. Festa RS, Shende A, Lanzkowsky P. Juvenile chronic myelocytic leukemia: experience with intensive combination chemotherapy. *Med Pediatr Oncol*. 1990;18(4):311–316.
176. Lilleyman JS, Harrison JF, Black JA. Treatment of juvenile chronic myeloid leukemia with sequential subcutaneous cytarabine and oral mercaptopurine. *Blood*. 1977;49(4):559–562.
177. Wasserman LR. The management of polycythaemia vera. *Br J Haematol*. 1971;21(4):371–376.
178. Ohta H, Kawai M, Sawada A, et al. Juvenile myelomonocytic leukemia relapsing after allogeneic bone marrow transplantation successfully treated with interferon-alpha. *Bone Marrow Transpl*. 2000;26(6):681–683.
179. Stieglitz E, Ward AF, Gerbing RB, et al. Phase II/III trial of a pre-transplant farnesyl transferase inhibitor in juvenile myelomonocytic leukemia: a report from the Children's Oncology Group. *Pediatr Blood Cancer*. 2015;62(4):629–636.
180. Archambeault S, Flores NJ, Yoshimi A, et al. Development of an allele-specific minimal residual disease assay for patients with juvenile myelomonocytic leukemia. *Blood*. 2008;111(3):1124–1127.
181. Hecht A, Meyer J, Chehab FF, et al. Molecular assessment of pretransplant chemotherapy in the treatment of juvenile myelomonocytic leukemia. *Pediatr Blood Cancer*. 2019;66(11). e27948-e27948.
182. Manabe A, Okamura J, Yumura-Yagi K, et al. Allogeneic hematopoietic stem cell transplantation for 27 children with juvenile myelomonocytic leukemia diagnosed based on the criteria of the International JMML Working Group. *Leukemia*. 2002;16(4):645–649.
183. Yoshimi A, Niemeyer CM, Bohmer V, et al. Chimaerism analyses and subsequent immunological intervention after stem cell transplantation in patients with juvenile myelomonocytic leukaemia. *Br J Haematol*. 2005;129(4):542–549.
184. Inagaki J, Fukano R, Nishikawa T, et al. Outcomes of immunological interventions for mixed chimerism following allogeneic stem cell transplantation in children with juvenile myelomonocytic leukemia. *Pediatr Blood Cancer*. 2013;60(1):116–120.
185. Dvorak CC, Satwani P, Stieglitz E, et al. Disease burden and conditioning regimens in ASCT1221, a randomized phase II trial in children with juvenile myelomonocytic leukemia: A Children's Oncology Group study. *Pediatr Blood Cancer*. 2018;65(7). e27034-e27034.
186. Yoshimi A, Bader P, Matthes-Martin S, et al. Donor leukocyte infusion after hematopoietic stem cell transplantation in patients with juvenile myelomonocytic leukemia. *Leukemia*. 2005;19(6):971–977.
187. Faraci M, Micalizzi C, Lanino E, et al. Three consecutive related bone marrow transplants for juvenile myelomonocytic leukaemia. *Pediatr Transpl*. 2005;9(6):797–800.
188. Smith FO, King R, Nelson G, et al. Unrelated donor bone marrow transplantation for children with juvenile myelomonocytic leukaemia. *Br J Haematol*. 2002;116(3):716–724.
189. Patja K, Pukkala E, Sund R, Iivanainen M, Kaski M. Cancer incidence of persons with Down syndrome in Finland: a population-based study. *Int J Cancer*. 2006;118(7):1769–1772.
190. Lange B. The management of neoplastic disorders of haematopoiesis in children with Down's syndrome. *Br J Haematol*. 2000;110(3):512–524.
191. Zipursky A. Transient leukaemia—a benign form of leukaemia in newborn infants with trisomy 21. *Br J Haematol*. 2003;120(6):930–938.
192. Gamis AS, Alonzo TA, Gerbing RB, et al. Natural history of transient myeloproliferative disorder clinically diagnosed in Down syndrome neonates: a report from the Children's Oncology Group Study A2971. *Blood*. 2011;118(26):6752–6759.
193. Khan I, Malinge S, Crispino J. Myeloid leukemia in Down syndrome. *Crit Rev Oncog*. 2011;16(1-2):25–36.
194. Maloney KW, Taub JW, Ravindranath Y, Roberts I, Vyas P. Down syndrome preleukemia and leukemia. *Pediatr Clin North Am*. 2015;62(1):121–137.
195. McNulty M, Crispino JD. Acute Megakaryocytic Leukemia. *Cold Spring Harb Perspect Med*. 2020;10(2):a034884.
196. Nižetić D, Groet J. Tumorigenesis in Down's syndrome: big lessons from a small chromosome. *Nat Rev Cancer*. 2012;12(10):721–732.
197. Liu B, Filippi S, Roy A, Roberts I. Stem and progenitor cell dysfunction in human trisomies. *EMBO Rep*. 2015;16(1):44–62.

198. Labuhn M, Perkins K, Matzk S, et al. Mechanisms of progression of myeloid preleukemia to transformed myeloid leukemia in children with Down syndrome. *Cancer Cell*. 2019;36(2):123–138. e110.
199. Tunstall-Pedoe O, Roy A, Karadimitris A, et al. Abnormalities in the myeloid progenitor compartment in Down syndrome fetal liver precede acquisition of GATA1 mutations. *Blood*. 2008;112(12):4507–4511.
200. Roy A, Cowan G, Mead AJ, et al. Perturbation of fetal liver hematopoietic stem and progenitor cell development by trisomy 21. *Proc Natl Acad Sci U S A*. 2012;109(43):17579–17584.
201. Wechsler J, Greene M, McDevitt MA, et al. Acquired mutations in GATA1 in the megakaryoblastic leukemia of Down syndrome. *Nat Genet*. 2002;32(1):148–152.
202. Groet J, McElwaine S, Spinelli M, et al. Acquired mutations in GATA1 in neonates with Down's syndrome with transient myeloid disorder. *Lancet*. 2003;361(9369):1617–1620.
203. Hitzler JK, Cheung J, Li Y, Scherer SW, Zipursky A. GATA1 mutations in transient leukemia and acute megakaryoblastic leukemia of Down syndrome. *Blood*. 2003;101(11):4301–4304.
204. Mundschau G, Gurbuxani S, Gamis AS, Greene ME, Arceci RJ, Crispino JD. Mutagenesis of GATA1 is an initiating event in Down syndrome leukemogenesis. *Blood*. 2003;101(11):4298–4300.
205. Xu G, Nagano M, Kanezaki R, et al. Frequent mutations in the GATA-1 gene in the transient myeloproliferative disorder of Down syndrome. *Blood*. 2003;102(8):2960–2968.
206. Calligaris R, Bottardi S, Cogoi S, Apezteguia I, Santoro C. Alternative translation initiation site usage results in two functionally distinct forms of the GATA-1 transcription factor. *Proc Natl Acad Sci U S A*. 1995;92(25):11598–11602.
207. Kuhl C, Atzberger A, Iborra F, Nieswandt B, Porcher C, Vyas P. GATA1-mediated megakaryocyte differentiation and growth control can be uncoupled and mapped to different domains in GATA1. *Mol Cell Biol*. 2005;25(19):8592–8606.
208. Li Z, Godinho FJ, Klusmann J-H, Garriga-Canut M, Yu C, Orkin SH. Developmental stage-selective effect of somatically mutated leukemogenic transcription factor GATA1. *Nat Genet*. 2005;37(6):613–619.
209. Muntean AG, Crispino JD. Differential requirements for the activation domain and FOG-interaction surface of GATA-1 in megakaryocyte gene expression and development. *Blood*. 2005;106(4):1223–1231.
210. Yoshida K, Toki T, Okuno Y, et al. The landscape of somatic mutations in Down syndrome-related myeloid disorders. *Nat Genet*. 2013;45(11):1293–1299.
211. Roberts I, Alford K, Hall G, et al. GATA1-mutant clones are frequent and often unsuspected in babies with Down syndrome: identification of a population at risk of leukemia. *Blood*. 2013;122(24):3908–3917.
212. Karandikar NJ, Aquino DB, McKenna RW, Kroft SH. Transient myeloproliferative disorder and acute myeloid leukemia in Down syndrome. An immunophenotypic analysis. *Am J Clin Pathol*. 2001;116(2):204–210.
213. Langebrake C, Creutzig U, Reinhardt D. Immunophenotype of Down syndrome acute myeloid leukemia and transient myeloproliferative disease differs significantly from other diseases with morphologically identical or similar blasts. *Klin Padiatr*. 2005;217(3):126–134.
214. Wilson ER, Brodersen LE, Zehentner BK, et al. Down syndrome AML is unique in phenotype both at diagnosis and in post chemotherapy regeneration. *Blood*. 2016;128(22). 1687-1687.
215. Klusmann J-H, Creutzig U, Zimmermann M, et al. Treatment and prognostic impact of transient leukemia in neonates with Down syndrome. *Blood*. 2008;111(6):2991–2998.
216. Muramatsu H, Watanabe T, Hasegawa D, et al. Prospective study of 168 infants with transient abnormal myelopoiesis with Down syndrome: Japan Pediatric Leukemia/Lymphoma Study Group, TAM-10 study. *Blood*. 2015;126(23). 1311-1311.
217. Tunstall O, Bhatnagar N, James B, et al. Guidelines for the investigation and management of transient leukaemia of Down syndrome. *Br J Haematol*. 2018;182(2):200–211.
218. Taub JW, Berman JN, Hitzler JK, et al. Improved outcomes for myeloid leukemia of Down syndrome: a report from the Children's Oncology Group AAML0431 trial. *Blood*. 2017;129(25):3304–3313.
219. Uffmann M, Rasche M, Zimmermann M, et al. Therapy reduction in patients with Down syndrome and myeloid leukemia: the international ML-DS 2006 trial. *Blood*. 2017;129(25):3314–3321.
220. Linabery AM, Ross JA. Childhood and adolescent cancer survival in the US by race and ethnicity for the diagnostic period 1975–1999. *Cancer*. 2008;113(9):2575–2596.
221. Hijiya N, Schultz KR, Metzler M, Millot F, Suttorp M. Pediatric chronic myeloid leukemia is a unique disease that requires a different approach. *Blood*. 2016;127(4):392–399.
222. Kalmanti L, Saussele S, Lauseker M, et al. Younger patients with chronic myeloid leukemia do well in spite of poor prognostic indicators: results from the randomized CML study IV. *Ann Hematol*. 2014;93(1):71–80.
223. Suryanarayan K, Hunger SP, Kohler S, et al. Consistent involvement of the BCR gene by 9;22 breakpoints in pediatric acute leukemias. *Blood*. 1991;77(2):324–330.
224. Bhatia R. Novel approaches to therapy in CML. *Hematol Am Soc Hematol Educ Program*. 2017;2017(1):115–120.
225. Millot F, Suttorp M, Guilhot J, et al. The International Registry for Chronic Myeloid Leukemia (CML) in children and adolescents (i-CML-Ped-Study): objectives and preliminary results. *Blood*. 2012;120(21). 3741-3741.
226. Radich JP, Deininger M, Abboud CN, et al. Chronic myeloid leukemia, version 1.2019, NCCN clinical practice guidelines in oncology. *J Natl Compr Canc Netw*. 2018;16(9):1108–1135.
227. Druker BJ, Sawyers CL, Kantarjian H, et al. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *N Engl J Med*. 2001;344(14):1038–1042.
228. Chaudhury S, HiJiya N, Hu ZH, et al. Outcomes of allogeneic hematopoietic stem cell transplantation in children and young adults with chronic myeloid leukemia: a CIBMTR cohort analysis. *Blood*. 2014;124(21).
229. Hasford J, Baccarani M, Hoffmann V, et al. Predicting complete cytogenetic response and subsequent progression-free survival in 2060 patients with CML on imatinib treatment: the EUTOS score. *Blood*. 2011;118(3):686–692.
230. Sceats DJ, Baitlon D. Primary thrombocythemia in a child. *Clin Pediatr (Phila)*. 1980;19(4):298–300.
231. Randi ML, Putti MC, Scapin M, et al. Pediatric patients with essential thrombocythemia are mostly polyclonal and V617FJAK2 negative. *Blood*. 2006;108(10):3600–3602.
232. El-Moneim AA, Kratz CP, Böll S, Rister M, Pahl HL, Niemeyer CM. Essential versus reactive thrombocythemia in children: retrospective analyses of 12 cases. *Pediatr Blood Cancer*. 2007;49(1):52–55.
233. Hasle H. Incidence of essential thrombocythaemia in children. *Br J Haematol*. 2000;110(3):751.
234. Dame C, Sutor AH. Primary and secondary thrombocytosis in childhood. *Br J Haematol*. 2005;129(2):165–177.
235. Campbell PJ, Scott LM, Buck G, et al. Definition of subtypes of essential thrombocythaemia and relation to polycythaemia vera based on JAK2 V617F mutation status: a prospective study. *Lancet*. 2005;366(9501):1945–1953.
236. Klampfl T, Gisslinger H, Harutyunyan AS, et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med*. 2013;369(25):2379–2390.
237. Teofili L, Giona F, Martini M, et al. Markers of myeloproliferative diseases in childhood polycythemia vera and essential thrombocythemia. *J Clin Oncol*. 2007;25(9):1048–1053.
238. Marty C, Saint-Martin C, Pecquet C, et al. Germ-line JAK2 mutations in the kinase domain are responsible for hereditary thrombocytosis and are resistant to JAK2 and HSP90 inhibitors. *Blood*. 2014;123(9):1372–1383.
239. Hoagland HC, Silverstein MN. Primary thrombocythemia in the young patient. *Mayo Clin Proc*. 1978;53(9):578–580.
240. Tartaglia AP, Goldberg JD, Berk PD, Wasserman LR. Adverse effects of antiaggregating platelet therapy in the treatment of polycythemia vera. *Semin Hematol*. 1986;23(3):172–176.
241. Rozman C, Giralt M, Feliu E, Rubio D, Cortés MT. Life expectancy of patients with chronic nonleukemic myeloproliferative disorders. *Cancer*. 1991;67(10):2658–2663.
242. Finazzi G, Carobbio A, Thiele J, et al. Incidence and risk factors for bleeding in 1104 patients with essential thrombocythemia or prefibrotic myelofibrosis diagnosed according to the 2008 WHO criteria. *Leukemia*. 2012;26(4):716–719.
243. Carobbio A, Thiele J, Passamonti F, et al. Risk factors for arterial and venous thrombosis in WHO-defined essential thrombocythemia: an international study of 891 patients. *Blood*. 2011;117(22):5857–5859.
244. Gilbert HS. Historical perspective on the treatment of essential thrombocythemia and polycythemia vera. *Semin Hematol*. 1999;36(1 Suppl 2):19–22.
245. Barbui T, Barosi G, Grossi A, et al. Practice guidelines for the therapy of essential thrombocythemia. A statement from the Italian Society of Hematology, the Italian Society of Experimental Hematology and the Italian Group for Bone Marrow Transplantation. *Haematologica*. 2004;89(2):215–232.
246. Giles FJ, Singer CR, Gray AG, et al. Alpha-interferon therapy for essential thrombocythaemia. *Lancet*. 1988;2(8602):70–72.

247. Ballen KK, Woolfrey AE, Zhu X, et al. Allogeneic hematopoietic cell transplantation for advanced polycythemia vera and essential thrombocythemia. *Biol Blood Marrow Transpl*. 2012;18(9):1446–1454.
248. Anderson JE, Sale G, Appelbaum FR, Chauncey TR, Storb R. Allogeneic marrow transplantation for primary myelofibrosis and myelofibrosis secondary to polycythaemia vera or essential thrombocytosis. *Br J Haematol*. 1997;98(4):1010–1016.
249. Kerbauy DM, Gooley TA, Sale GE, et al. Hematopoietic cell transplantation as curative therapy for idiopathic myelofibrosis, advanced polycythemia vera, and essential thrombocythemia. *Biol Blood Marrow Transpl*. 2007;13(3):355–365.
250. Lussana F, Rambaldi A, Finazzi MC, et al. Allogeneic hematopoietic stem cell transplantation in patients with polycythemia vera or essential thrombocythemia transformed to myelofibrosis or acute myeloid leukemia: a report from the MPN Subcommittee of the Chronic Malignancies Working Party of the European Group for Blood and Marrow Transplantation. *Haematologica*. 2014;99(5):916–921.
251. Sekhar M, Prentice HG, Popat U, et al. Idiopathic myelofibrosis in children. *Br J Haematol*. 1996;93(2):394–397.
252. Reilly JT. Idiopathic myelofibrosis: pathogenesis to treatment. *Hematol Oncol*. 2006;24(2):56–63.
253. Chou JM, Li C-Y, Tefferi A. Bone marrow immunohistochemical studies of angiogenic cytokines and their receptors in myelofibrosis with myeloid metaplasia. *Leuk Res*. 2003;27(6):499–504.
254. Jain T, Mesa RA, Palmer JM. Allogeneic stem cell transplantation in myelofibrosis. *Biol Blood Marrow Transpl*. 2017;23(9):1429–1436.
255. Tefferi A, Silverstein MN, Noël P. Agnogenic myeloid metaplasia. *Semin Oncol*. 1995;22(4):327–333.
256. Barosi G, Ambrosetti A, Buratti A, et al. Splenectomy for patients with myelofibrosis with myeloid metaplasia: pretreatment variables and outcome prediction. *Leukemia*. 1993;7(2):200–206.
257. Silverstein MN, ReMine WH. Splenectomy in myeloid metaplasia. *Blood*. 1979;53(3):515–518.
258. Tefferi A, Mesa RA, Gray LA, et al. Phase 2 trial of imatinib mesylate in myelofibrosis with myeloid metaplasia. *Blood*. 2002;99(10):3854–3856.
259. Cortes J, Giles F, O'Brien S, et al. Results of imatinib mesylate therapy in patients with refractory or recurrent acute myeloid leukemia, high-risk myelodysplastic syndrome, and myeloproliferative disorders. *Cancer*. 2003;97(11):2760–2766.
260. Elliott MA, Mesa RA, Li C-Y, et al. Thalidomide treatment in myelofibrosis with myeloid metaplasia. *Br J Haematol*. 2002;117(2):288–296.
261. Marchetti M, Barosi G, Balestri F, et al. Low-dose thalidomide ameliorates cytopenias and splenomegaly in myelofibrosis with myeloid metaplasia: a phase II trial. *J Clin Oncol*. 2004;22(3):424–431.
262. Verstovsek S, Kantarjian H, Mesa RA, et al. Safety and efficacy of INCB018424, a JAK1 and JAK2 inhibitor, in myelofibrosis. *N Engl J Med*. 2010;363(12):1117–1127.
263. Geyer HL, Mesa RA. Therapy for myeloproliferative neoplasms: when, which agent, and how? *Blood*. 2014;124(24):3529–3537.
264. Stübig T, Alchalby H, Ditschkowski M, et al. JAK inhibition with ruxolitinib as pretreatment for allogeneic stem cell transplantation in primary or post-ET/PV myelofibrosis. *Leukemia*. 2014;28(8):1736–1738.
265. Shanavas M, Popat U, Michaelis LC, et al. Outcomes of allogeneic hematopoietic cell transplantation in patients with myelofibrosis with prior exposure to Janus Kinase 1/2 inhibitors. *Biol Blood Marrow Transpl*. 2016;22(3):432–440.
266. Robin M, Francois S, Huynh A, et al. Ruxolitinib before allogeneic hematopoietic stem cell transplantation (HSCT) in patients with myelofibrosis: a preliminary descriptive report of the JAK ALLO study, a phase II trial sponsored by Goelams-FIM in collaboration with the SFGMTC. *Blood*. 2013;122(21). 306-306.
267. Guardiola P, Esperou H, Cazals-Hatem D, et al. Allogeneic bone marrow transplantation for agnogenic myeloid metaplasia. French Society of Bone Marrow Transplantation. *Br J Haematol*. 1997;98(4):1004–1009.
268. Shanavas M, Messner HA, Atenafu EG, et al. Allogeneic hematopoietic cell transplantation for myelofibrosis using fludarabine-, intravenous busulfan- and low-dose TBI-based conditioning. *Bone Marrow Transpl*. 2014;49(9):1162–1169.
269. Claudiani S, Marktel S, Piemontese S, et al. Treosulfan based reduced toxicity conditioning followed by allogeneic stem cell transplantation in patients with myelofibrosis. *Hematol Oncol*. 2016;34(3):154–160.
270. Osgood EE. Polycythemia vera: age relationships and survival. *Blood*. 1965;26:243–256.
271. Gupta V, Malone AK, Hari PN, et al. Reduced-intensity hematopoietic cell transplantation for patients with primary myelofibrosis: a cohort analysis from the center for international blood and marrow transplant research. *Biol Blood Marrow Transpl*. 2014;20(1):89–97.
272. Gagelmann N, Ditschkowski M, Bogdanov R, et al. Comprehensive clinical-molecular transplant scoring system for myelofibrosis undergoing stem cell transplantation. *Blood*. 2019;133(20):2233–2242.
273. Devlin R, Gupta V. Myelofibrosis: to transplant or not to transplant? *Hematology*. 2016;2016(1):543–551.
274. Bento C, Percy MJ, Gardie B, et al. Genetic basis of congenital erythrocytosis: mutation update and online databases. *Hum Mutat*. 2014;35(1):15–26.
275. Spivak JL. The chronic myeloproliferative disorders: clonality and clinical heterogeneity. *Semin Hematol*. 2004;41(2 Suppl 3):1–5.
276. Zhao R, Xing S, Li Z, et al. Identification of an acquired JAK2 mutation in polycythemia vera. *J Biol Chem*. 2005;280(24):22788–22792.
277. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet*. 2005;365(9464):1054–1061.
278. James C, Ugo V, Le Couédic JP, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*. 2005.
279. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med*. 2005;352(17):1779–1790.
280. Levine RL, Wadleigh M, Cools J, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell*. 2005;7(4):387–397.
281. Passamonti F, Elena C, Schnittger S, et al. Molecular and clinical features of the myeloproliferative neoplasm associated with JAK2 exon 12 mutations. *Blood*. 2011;117(10):2813–2816.
282. Temerinac S, Klippel S, Strunck E, et al. Cloning of PRV-1, a novel member of the uPAR receptor superfamily, which is overexpressed in polycythemia rubra vera. *Blood*. 2000;95(8):2569–2576.
283. Teofili L, Pierconti F, Di Febo A, et al. The expression pattern of c-mpl in megakaryocytes correlates with thrombotic risk in essential thrombocythemia. *Blood*. 2002;100(2):714–717.
284. Prchal JF, Axelrad AA. Letter: bone-marrow responses in polycythemia vera. *N Engl J Med*. 1974;290(24):1382.
285. Michiels JJ, Juvonen E. Proposal for revised diagnostic criteria of essential thrombocythemia and polycythemia vera by the Thrombocythemia Vera Study Group. *Semin Thromb Hemost*. 1997;23(4):339–347.
286. Giona F, Teofili L, Moleti ML, et al. Thrombocythemia and polycythemia in patients younger than 20 years at diagnosis: clinical and biologic features, treatment, and long-term outcome. *Blood*. 2012;119(10):2219–2227.
287. Tefferi A, Thiele J, Orazi A, et al. Proposals and rationale for revision of the World Health Organization diagnostic criteria for polycythemia vera, essential thrombocythemia, and primary myelofibrosis: recommendations from an ad hoc international expert panel. *Blood*. 2007;110(4):1092–1097.
288. Cario H, McMullin MF, Pahl HL. Clinical and hematological presentation of children and adolescents with polycythemia vera. *Ann Hematol*. 2009;88(8):713–719.
289. Tefferi A, Thiele J, Vannucchi AM, Barbui T. An overview on CALR and CSF3R mutations and a proposal for revision of WHO diagnostic criteria for myeloproliferative neoplasms. *Leukemia*. 2014;28(7):1407–1413.
290. Berlin NI, Wasserman LR. Polycythemia vera: a retrospective and reprise. *J Lab Clin Med*. 1997;130(4):365–373.
291. Logue GL, Gutterman JU, McGinn TG, Laszlo J, Rundles RW. Melphalan therapy of polycythemia vera. *Blood*. 1970;36(1):70–86.
292. Milligan DW, Thein SL, Roberts BE. Secondary treatment of polycythemia rubra vera with 6-thioguanine. *Cancer*. 1982;50(5):836–839.
293. Toh BT, Gregory SA, Knospe WH. Acute leukemia following treatment of polycythemia vera and essential thrombocythemia with uracil mustard. *Am J Hematol*. 1988;28(1):58–60.
294. Treatment of polycythaemia vera by radiophosphorus or busulphan: a randomized trial. "Leukemia and Hematosarcoma" Cooperative Group, European Organization for Research on Treatment of Cancer (E.O.R.T.C.). *Br J Cancer*. 1981;44(1):75–80.
295. Higuchi T, Okada S, Mori H, Niikura H, Omine M, Terada H. Leukemic transformation of polycythemia vera and essential thrombocythemia possibly associated with an alkylating agent. *Cancer*. 1995;75(2):471–477.
296. Petitt RM, Silverstein MN, Petrone ME. Anagrelide for control of thrombocythemia in polycythemia and other myeloproliferative disorders. *Semin Hematol*. 1997;34(1):51–54.
297. Silver RT. Imatinib mesylate (Gleevec(TM)) reduces phlebotomy requirements in polycythemia vera. *Leukemia*. 2003;17(6):1186–1187.
298. Donovan PB, Kaplan ME, Goldberg JD, et al. Treatment of polycythemia vera with hydroxyurea. *Am J Hematol*. 1984;17(4):329–334.

299. Harman JB, Ledlie EM. Survival of polycythaemia vera patients treated with radioactive phosphorus. *Br Med J*. 1967;2(5545):146–148.
300. Castello G, Lerza R, Cerruti A, Cavallini D, Bogliolo G, Pannacciulli I. The in vitro and in vivo effect of recombinant interferon alpha-2a on circulating haemopoietic progenitors in polycythaemia vera. *Br J Haematol*. 1994;87(3):621–623.
301. McMullin MF, Bareford D, Campbell P, et al. Guidelines for the diagnosis, investigation and management of polycythaemia/erythrocytosis. *Br J Haematol*. 2005;130(2):174–195.
302. Marchioli R, Finazzi G, Specchia G, et al. Cardiovascular events and intensity of treatment in polycythemia vera. *N Engl J Med*. 2013;368(1):22–33.
303. Vannucchi AM, Kiladjian JJ, Griesshammer M, et al. Ruxolitinib versus standard therapy for the treatment of polycythemia vera. *N Engl J Med*. 2015;372(5):426–435.
304. Reinhard H, Klingebiel T, Lang P, Bader P, Niethammer D, Graf N. Stem cell transplantation for polycythemia vera. *Pediatr Blood Cancer*. 2008;50(1):124–126.

CHAPTER 66

PATHOBIOLOGY OF ACUTE LYMPHOBLASTIC LEUKEMIA

Melissa A. Burns, Alejandro Gutierrez, and Lewis B. Silverman

INTRODUCTION

Normal lymphoid precursors undergo somatic recombination at their immunoglobulin (Ig) or T-cell receptor (TCR) gene loci, and the successful completion of V(D)J recombination, with the resultant formation of a functional Ig or TCR, is required for the survival of lymphocyte precursors. Positive and negative selection steps ensure that only lymphocytes with Ig or TCRs that function appropriately within the context of an individual's immune microenvironment are allowed to proceed through the proliferation and differentiation steps required for the development of mature lymphocytes. This developmental process generates a repertoire of mature lymphocytes with unique variations in the antigen-recognition portions of the Ig or TCR genes; together, these form the foundation of the adaptive immune system that can recognize a countless variety of foreign antigens. The acquisition of mutations of oncogenes or tumor suppressors during this developmental process, which can be mediated by the off-target activity of the V(D)J recombination machinery, can generate lymphocyte clones with uncontrolled proliferation and differentiation arrest, features characteristic of acute lymphoblastic leukemia (ALL). Many of these genetic alterations have prognostic significance and are used in contemporary ALL treatment protocols to adjust the intensity of therapy. Although the incidence of specific genetic alterations in ALL varies according to patient age and immunophenotype (Fig. 66.1), evidence suggests that the pathogenesis underlying malignant transformation in molecularly defined subsets of ALL is similar across age groups.

LINEAGE-SPECIFIC FEATURES OF LEUKEMIC LYMPHOBLASTS

Malignant lymphoblasts share many of the features of normal lymphoid progenitors. Thus, ALL cells rearrange their Ig and TCR genes and express components of antigen receptor molecules and other differentiation-linked cell-surface glycoproteins in ways that correspond to features of developing normal B and T lymphocytes. In some cases, leukemic cells appear to represent the clonal expansion of a lymphoid progenitor that has arrested its development at an early stage of B- or T-cell differentiation, while in others, the blast cell phenotypes differ from those of normal lymphocyte progenitors, which is likely a result of aberrant regulation of gene expression. Still, the general concept that leukemic cells should be classified according to their "normal" developmental stage remains an important one, providing a basis for the study of immunophenotype-specific genetic changes.

Mature B-Cell Acute Lymphoblastic Leukemia

The diagnosis of mature B-cell ALL, also termed Burkitt leukemia, is based on the detection of surface Ig on leukemic blasts. This rare phenotype accounts for only 2% to 3% of ALL cases, and the lymphoblasts often have distinctive morphology, with deeply basophilic cytoplasm containing prominent vacuoles; this morphologic pattern was designated L3 in the French-American-British (FAB) system. Mature B-cell ALL is a disseminated form of Burkitt lymphoma, as these conditions share common cytogenetic, molecular, phenotypic, and clinical features. Mature B-cell ALL does not respond well to chemotherapy traditionally used for childhood ALL. However, favorable outcomes have been obtained with treatments designed for Burkitt lymphoma, which involves therapy of greater intensity and shorter duration. Thus, mature B-cell leukemia was the first subtype of ALL to be recognized as a distinct clinical entity based on immunophenotypic and cytogenetic features and the first to be treated by separate protocols designed specifically for the leukemia's unique features.

B-Cell Acute Lymphoblastic Leukemia

Approximately 80% of ALL patients have lymphoblasts with phenotypes corresponding to those of B-cell progenitors. These cases can be identified on the basis of cell-surface expression of CD19 and at least one other recognized B lineage–associated antigen: CD20, CD24, CD22, CD21, or CD79. The most common subtype of B-cell ALL, previously termed common precursor B-cell ALL, also expresses CD10. These lymphoblasts may also express nuclear terminal deoxynucleotidyl transferase (TdT) or CD34. About one-fourth of B-cell ALL cases express cytoplasmic Ig μ heavy-chain proteins and were historically designated pre-B-ALL.

DNA rearrangement of Ig genes occurs before heavy-chain gene expression in B-cell development, providing a genetic marker of B-lymphocyte ontogeny. Korsmeyer and colleagues pioneered the use of heavy- and light-chain gene rearrangements to support an early B-lineage origin of most ALL blasts. However, Ig heavy-chain gene rearrangements are observed in about 15% of T-cell ALL cases and in a similar percentage of AML cases. Thus, caution must be exercised when assigning cell lineage on the basis of studies of Ig gene rearrangement.

The identification of specific immunophenotypic, genetic, and clinical features that predict response to therapy in patients with B-lineage ALL and the use of these predictors to stratify intensity of therapy are now common features of contemporary ALL treatment protocols. This ability to predict outcome has been closely tied to the remarkable improvements in therapy for children with this disease, which 60 years ago was universally fatal. However, many subgroups of pediatric and adult patients continue to face a poor prognosis, and much progress remains to be made.

T-Cell Acute Lymphoblastic Leukemia

Lymphoblasts with a T-cell phenotype, defined by the WHO as cell-surface expression of TdT with variable expression of CD1a, CD2, CD3, CD4, CD5, CD7, and CD8, comprise approximately 15% of cases of childhood ALL and a higher proportion of ALL in adults. These cases can be further classified according to the sequence of expression of T-cell-associated surface antigens during normal thymocyte ontogeny. Numerous investigators, using a battery of monoclonal antibodies specific for T-cell surface glycoproteins, have confirmed the close relationship between the recognizable patterns of surface antigen expression on leukemic T cells and the normal stages of thymocyte development. T-cell ALL is often associated with distinctive clinical features that include high circulating leukocyte counts, male predominance, central nervous system involvement, and a radiographically evident thymic mass in many

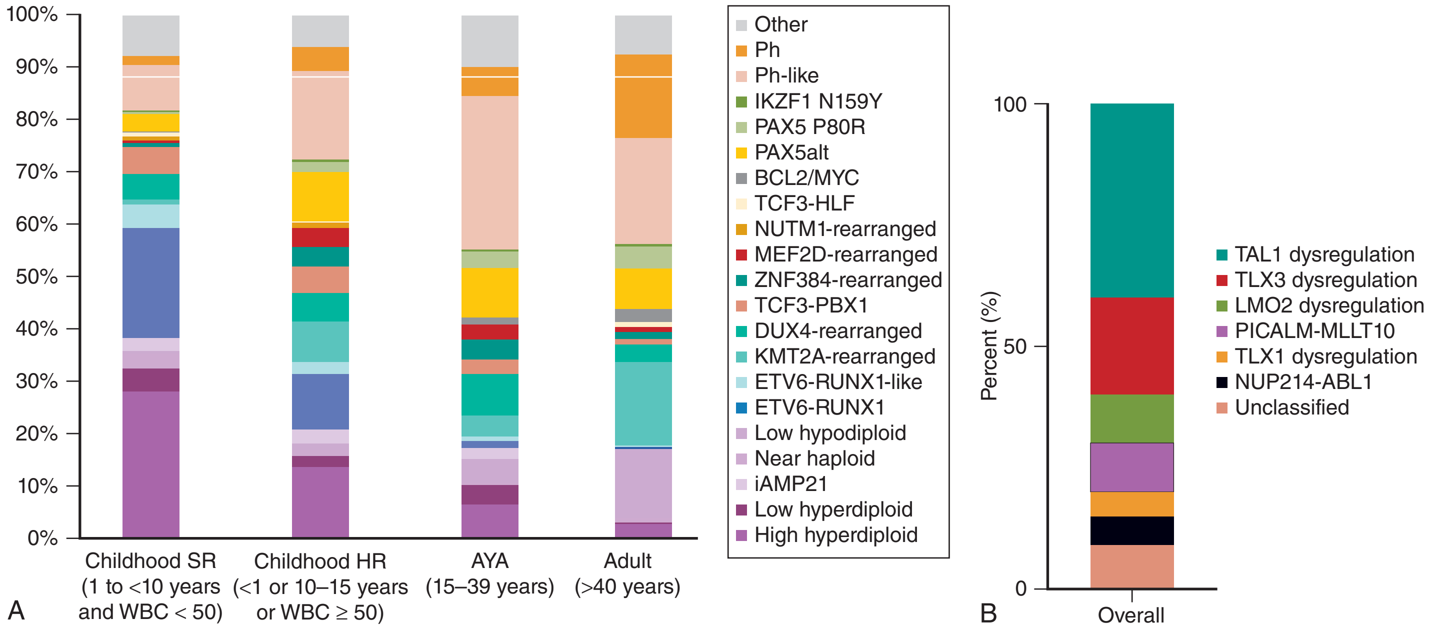


Figure 66.1 FREQUENCY OF THE MAJOR MOLECULAR CLASSIFICATIONS IN (A) PEDIATRIC AND ADULT B-CELL AND (B) PEDIATRIC T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (ALL). *(A, Adapted from Inaba H, Mullighan CG. Pediatric acute lymphoblastic leukemia. Haematologica. 2020;105:11 [Fig. 2], with permission; B, From Williams LA, Yang JJ, Hirsch BA. Is there etiologic heterogeneity between subtypes of childhood acute lymphoblastic leukemia? A review of variation in risk by subtype. Cancer Epidemiol Biomarkers Prev. 2019;28:846–856 [Fig. 1].)*

cases at presentation. Historically, patients with T-cell ALL had an adverse prognosis compared with patients with B-cell ALL; however, with contemporary therapy, outcomes are similar. In marked contrast to B-cell ALL, in which the availability of a wide range of clinical and genetic prognostic markers has allowed the development of treatment protocols tailored to an individual patient's risk of relapse, robust pretreatment prognostic markers are lacking in T-cell ALL. Recent discovery of biomarkers of differentiation arrest at the earliest stages of T-cell development has identified potential markers of treatment resistance.

A distinctive subset of T-cell ALL has been identified that is associated with an increased risk of slow early response and/or early treatment failure, the so-called early T-cell precursor (ETP) subset of T-cell ALL. ETP ALL comprises 8% to 15% of pediatric T-ALL cases but accounts for a much higher fraction of T-cell ALL in adults and is characterized by differentiation arrest at the earliest identifiable stages of T-cell development. These cases are most commonly identified by a specific immunophenotype, which can be defined by the lack of expression of the T-lineage cell-surface markers CD1a and CD8, weak or absent expression of CD5, and aberrant expression of one or more myeloid or stem cell markers. ETP cases also express a distinctive gene expression profile and often have an absence of biallelic TCRγ deletion (ABD), indicating differentiation arrest prior to the completion of TCR gene rearrangement. In addition to harboring oncogenic lesions typical of T-cell ALL, ETP cases also harbor gene mutations that are characteristic of acute myeloid leukemia, including mutations of Polycomb repressive complex 2 (*PRC2*) genes (*EZH2, EED,* or *SUZ12*) and *WT1* mutations, suggesting that the cell of origin of ETP leukemia may be a multipotent hematopoietic stem or progenitor cell rather than a committed T-cell progenitor. Evidence also indicates that a subset of AML cases have features characteristic of T-cell differentiation, suggesting that current WHO classification schemes may have resulted in the separation of a biologically unique entity with features of both T-lymphoblastic and myeloid differentiation across the T-cell ALL, AML, and mixed-phenotype acute leukemia (MPAL) diagnostic categories. The term acute myeloid/T-lymphoblastic leukemia has been proposed for this entity, although defining the optimal therapeutic approach for these cases requires additional investigation.

Mixed-Phenotype Acute Leukemia

MPAL defines a heterogenous class of leukemias in which blast cells coexpress antigens of both lymphoid and myeloid lineage. Historically, two distinct forms of these leukemias have been recognized: biphenotypic leukemia, which accounts for the majority of MPAL and can be the result of cells coexpressing B-cell or T-cell antigens together with myeloid-associated antigen (B/myeloid MPAL and T/myeloid MPAL, respectively), or more rarely cells that coexpress B-cell and T-cell antigens (B/T MPAL) or B-cell, T-cell, and myeloid antigens (B/T/myeloid MPAL), and bilineal leukemia, in which there are two distinct populations of cells (generally one lymphoid and one myeloid). Despite recent work investing the complex genomic and epigenomic landscape of MPAL, the origin of this subtype remains unknown. One possibility is the malignant transformation of pluripotent hematopoietic stem or progenitor cells that retain the ability to differentiate in both the myeloid and lymphoid lineages; another is the immortalization of rare progenitor cells that normally coexpress features of both lineages; and a third is aberrant gene expression caused by specific genetic alterations. Despite initial controversy, co-expression of myeloid antigens in ALL, or of lymphoid markers in acute myeloid leukemia, does not appear to have prognostic significance in the setting of contemporary treatment regimens.

GENETIC BASIS OF ACUTE LYMPHOBLASTIC LEUKEMIA

Genome-wide profiling studies, including whole-genome sequencing, transcriptome sequencing, and microarray analyses, have led to major advances in understanding the genetics of ALL through the identification of recurrently mutated cellular pathways, much of which has been shown to have direct clinical relevance. While an in-depth analysis of the genetics of ALL is beyond the scope of this chapter, we present here an overview of select genetic lesions that have provided key insights into mechanisms of leukemogenesis. Mutations are classified based on their predominant cellular consequence, which is an imperfect and simplified classification scheme because some mutations can induce more than one type of cellular aberration. Nevertheless, we believe this provides a useful conceptual framework for understanding the molecular pathogenesis of ALL.

Aberrant Activation of Oncogenic Transcriptional Programs

Dysregulated activation of oncogenic transcriptional programs is a central theme in the pathogenesis of ALL. Many of the proto-oncogenes recurrently activated by genetic lesions are transcription factors that regulate proliferation and differentiation during the development of various embryonic tissues. Thus, co-option of normal developmental pathways plays a central role in human leukemogenesis. Transcription factors are commonly activated by chromosomal translocations in ALL, but the consequences of translocations tend to vary according to the ALL subtype. In mature B-cell ALL and in T-cell ALL, these genetic lesions often lead to the aberrant expression of structurally intact genes. By contrast, the chromosomal translocations that often occur in B-cell ALL generally lead to the expression of chimeric fusion proteins.

Activation of Structurally Intact Transcription Factors

MYC in Mature B-Cell Acute Lymphoblastic Leukemia

The vast majority of cases of mature B-cell ALL (Burkitt leukemia) are characterized by a translocation that places one allele of MYC from chromosome 8 under the control of the regulatory elements of an *Ig* gene, either the heavy-chain gene on chromosome 14q32 or the κ or λ light-chain genes on chromosomes 2 and 22, respectively. These translocations are oncogenic because they result in lineage-specific overexpression of MYC, a prototypical basic helix–loop–helix oncogenic transcription factor. The mechanisms through which MYC exerts its potent oncogenic effects have been the subject of intense investigation. MYC has been estimated to regulate the expression of 15% of the genome and leads to the transcriptional activation of a large number of genes involved in cell division, growth, metabolism, adhesion, and motility. MYC also exerts post-transcriptional effects on gene expression by regulating microRNA expression and ribosome biogenesis. MYC exerts its transcriptional activity via the formation of heterodimers with its DNA-binding partner protein MAX. MYC–MAX heterodimers bind to canonical hexameric E-box DNA sequences (5′-CACGTG-3′) where they activate transcription. MAX can also heterodimerize with other bHLH proteins, including MAD, MXI-1 (MAD2), and MNT. Whereas transcriptional activation by MYC–MAX complexes promotes proliferation, binding by MAD–MAX and other MAX heterodimers produces opposite effects. For example, MAD inhibits MYC function both by competing with MYC for binding to MAX and by directly inhibiting transcription.

Recent work has revealed that a major consequence of MYC overexpression is the global amplification of gene expression. Investigation of transcriptional regulation mechanisms has revealed two distinct steps in the regulation of gene expression by transcription factors. First, RNA polymerase II and its associated transcriptional apparatus are loaded onto a gene promoter by one set of transcription factors, but RNA polymerase is often initially “paused” near the proximal promoter. Subsequent release from transcriptional pause is a distinct and highly regulated step in the control of gene expression. Unexpectedly, recent studies have revealed that a major consequence of MYC overexpression is the release of transcriptional pause at genes that were already loaded with RNA polymerase, rather than the recruitment of RNA polymerase to new target genes. These findings support a model in which MYC overexpression functions to amplify the expression of genes that are already being transcribed, thus “locking in” a cell’s existing transcriptional program. The acquisition of an MYC-activating lesion in a cell with a highly proliferative gene expression program that is normally transient, such as an immature B-cell progenitor, can lock this cell in this highly proliferative state, thus providing one mechanism to explain MYC-driven oncogenesis.

TAL1 and LMO Genes in T-Cell Acute Lymphoblastic Leukemia

In leukemia with a T-cell phenotype, the breakpoints of recurrent chromosomal translocations consistently juxtapose *TCR* gene-regulatory elements, which are highly active in committed T-cell progenitors, to the protein-coding sequence of oncogenic transcription factors, which then become aberrantly overexpressed as a result of these translocations. The best characterized of the oncogenic transcription factors involved is TAL1 (also known as SCL). TAL1 is overexpressed as a result of the recurrent t(1;14) translocation or intra-chromosomal deletions in approximately one-fourth of childhood T-cell ALL cases. However, TAL1 is aberrantly expressed in the leukemic cells of 60% of children and 45% of adults with T-cell ALL, implicating additional pathogenic mechanisms leading to TAL1 overexpression. One such recently described mechanism is an activating mutation of a noncoding gene-regulatory element, which is described later in this chapter. TAL1 acts as a master regulatory protein during early hematopoietic development and is required for the generation of all blood cell lineages. However, it does not seem to be required for the generation and function of hematopoietic stem cells (HSCs) during adult hematopoiesis. *TAL1* is a bona fide T-cell ALL oncogene, as its aberrant expression in murine T-cell progenitors induces T-cell ALL.

TAL1 binds DNA in complex with other transcription factors including TCF3 (also known as E2A), HEB, LMO1/2, GATA3, RUNX1, and MYB. TAL1 binding can activate or repress the expression of its target genes, and a number of these targets have been implicated in T-cell transformation. In murine T-cell progenitors, TAL1 expression inhibits TCF3 transcriptional activity, and loss of TCF3 function induces T-cell leukemias in mice, supporting a role for TAL1-mediated inhibition of TCF3 function in leukemogenesis. The TAL1 complex binds and upregulates the expression of several of its own core components, including TAL1, GATA3, RUNX1, and MYB, thus forming a positive feedback loop that reinforces the activity of this oncogenic transcriptional complex. TAL1 also upregulates the expression of *TRIB2*, a gene whose overexpression in mouse bone marrow cells induces myeloid leukemia and whose expression is required for the survival of *TAL1*-overexpressing T-cell ALL. Furthermore, TAL1 also upregulates the expression of the microRNA mir223, which promotes leukemic cell survival by downregulating the expression of the *FBXW7* tumor suppressor.

The LIM-only domain genes, *LMO1* and *LMO2*, are also involved in recurrent chromosomal translocations in T-cell ALL. These genes encode transcription factors that interact with TAL1 in erythroid cells and in T-cell leukemias. Homozygous disruption of *LMO2* in mice phenocopies the hematopoietic defect of *TAL1* knock-outs, suggesting that these proteins function together during hematopoietic development. In addition, overexpression of *LMO1* and *LMO2* in murine thymocytes leads to T-cell transformation and accelerates the onset of leukemias in *TAL1* transgenic mice.

Homeobox Genes

The homeobox gene *TLX1* (also known as *HOX11*) is the founding member of a family of homeobox genes that includes *TLX2 (HOX11L1)* and *TLX3 (HOX11L2)*, each of which plays key roles in embryonic development. *TLX1* was originally isolated from the recurrent t(10;14) translocation in T-cell ALL and is aberrantly expressed in 5% of pediatric and approximately 25% of adult T-cell ALL cases. *TLX3* is also involved in a recurrent t(5;14)(q35;q32) translocation and is overexpressed in approximately 25% of pediatric but in only 5% of adult T-cell ALL. *TLX1* and *TLX3* encode very similar proteins, suggesting they have similar oncogenic mechanisms. Overexpression of *TLX1* or *TLX3* induces differentiation arrest at a cortical stage of T-cell development, an effect that is mediated by TLX-induced transcriptional repression of the pre-TCR alpha, whose expression is required for progression beyond this stage in normal T-cell development. *TLX1* expression in murine T-cell progenitors induces T-cell ALL, and these tumor cells have a defective mitotic checkpoint due to transcriptional repression of the checkpoint kinase *CHEK1*. These findings, together with the previous observation that TLX1 binds the catalytic subunits of the phosphatases PP2A and PP1 and disrupts the G2/M checkpoint, thus provide a mechanistic explanation for the association of *TLX1*/*TLX3* overexpression with aneuploidy, which is otherwise rare in human T-cell ALL. RUNX1, which is directly bound and repressed by TLX1 and TLX3, has been implicated as a downstream mediator of the oncogenic function of these transcription factors in T-cell ALL.

The cluster of *HOXA* genes on chromosome 7 is affected by a recurrent chromosomal inversion that places it in the vicinity of *TCR* beta gene-regulatory elements, leading to aberrant expression of the entire *HOXA* cluster in approximately 5% of cases of T-cell ALL. Many of these cases also carry cooperating oncogenic lesions consisting of *NOTCH1* gene mutations and deletions of 9p21. This translocation provides additional evidence for the role of aberrant *HOXA* activation in leukemogenesis and in the pathogenesis of *KMT2A*- and *CALM-AF10*-rearranged leukemias, as reviewed in more detail in the following sections.

DUX4 Gene Rearrangements in B-Cell Acute Lymphoblastic Leukemia

The double homeobox 4 (*DUX4*) gene encodes a homeobox-containing protein and is located on chromosome 4q within a sub-telomeric D4Z4 repeat region. In normal lymphoid cells, *DUX4* undergoes epigenetic silencing. Recent genomic studies, however, have identified recurrent gene rearrangements in approximately four percent of B-cell ALL cases that fuse *DUX4* to either *IGH* or, less commonly, *ERG*, a member of the erythroblast transformation-specific (ETS) family of transcription factors and an important regulator of hematopoietic cells. The juxtaposition of *DUX4* to the regulatory region of *IGH* or *ERG* results in *DUX4* overexpression in lymphoblasts. This overexpression leads to deregulation of *ERG* through binding of DUX4 to an alternative transcription initiation site within intron 6 of ERG, leading to truncated form of *ERG* that lacks the N-terminal pointed domain and central regulatory domains, but retains the DNA-binding ETS and transactivation domains, called ERGalt. Mice transplanted with ERGalt develop lymphoid malignancies, suggesting that expression of ERGalt directly induces lymphoid leukemogenesis. *DUX4* gene rearrangements commonly co-occur with *ERG* deletions in B-cell ALL, suggesting that *ERG* deletions are secondary cooperating events in this subtype of B-cell ALL. Interestingly, *IKZF1* deletions are commonly found in *DUX4*/*ERG* mutated B-cell ALL; however, unlike other ALL subtypes, *IKZF1* deletions are not associated with a poor outcome in this group of patients.

Chimeric Transcription Factor Oncogenes

Chromosomal translocations resulting in the formation of chimeric proteins represent a second mechanism for aberrant transcription factor activation, which is more prevalent in B-cell ALL. These translocations juxtapose exons that encode the DNA-binding and protein-binding domains of different genes, resulting in the expression of a chimeric fusion protein. The generation of such fusions is facilitated by the modular structure of transcription factor genes, in which discrete exons encode particular functional domains. This feature of gene structure facilitates organismal evolution but is commonly co-opted during oncogenesis.

ETV6-RUNX1 Fusions in B-Cell Acute Lymphoblastic Leukemia

Although most t(12;21) translocations are not detectable by standard cytogenetic analysis, this translocation is detectable by molecular techniques in approximately 25% of childhood B-cell ALL, making this the most common translocation in pediatric ALL (see Fig. 66.1). The *ETV6–RUNX1* translocation often arises prenatally and is likely to be the initiating mutation in at least a subset of ALL, as evidenced by the identification of identical *ETV6–RUNX1* translocations in identical twins with concordant ALL, and in retrospectively analyzed neonatal blood specimens of children who were diagnosed with ALL many years later. However, *ETV6–RUNX1* alone is not sufficient for leukemogenesis because the incidence of detectable *ETV6–RUNX1* fusions in the blood of normal newborns is about 100-fold greater than the incidence of leukemia.

The molecular mechanisms mediating *ETV6–RUNX1* induced leukemogenesis remain poorly understood. This fusion gene encodes a chimeric protein that contains the helix–loop–helix (HLH) domain of *ETV6* fused to nearly all of *RUNX1* (also known as *AML1* or *CBFA2*), including both the transactivation domain and the DNA- and protein-binding Runt homology domain. Both of these genes are found in other leukemia-related translocations, and both are essential for normal hematopoiesis. *ETV6* was first identified in the t(5;12) in chronic myelomonocytic leukemia, where it is fused to the platelet-derived growth factor receptor gene (*PDGFRB*), and is also fused to *ABL, MN1,* and *EVI1* in AML and to *JAK2* in T-cell ALL. *ETV6* is required for fetal hematopoiesis in the mouse. Interestingly, the inactivation of *ETV6* in adult mice leads to the selective loss of HSCs from adult bone marrow, but hematopoiesis is sustained by committed precursors. Many *ETV6–RUNX1* leukemias show the loss of the normal *ETV6* allele, suggesting that the leukemogenic effect of *ETV6-RUNX1* may be mediated in part by the loss of wildtype (WT) *ETV6* function. Interestingly, germline loss-of-function *ETV6* mutations have been identified in patients with familial thrombocytopenia and a predisposition to hematologic malignancies including ALL (as described in further detail later in this chapter), further implicating WT *ETV6* as an ALL tumor suppressor.

RUNX1 is the DNA-binding component of the *RUNX1–CBFß* transcription factor complex disrupted by the t(8;21), t(3;21), and inv(16) in AML. RUNX1 is a transcription factor that is required for the expression of several hematopoietic genes involved in myeloid and lymphoid development, including *PU.1* and *IL-3*, although it can also act as a transcriptional repressor in some settings. Homozygous disruption of the murine *RUNX1* or *CBFB* genes results in the lack of definitive hematopoiesis, indicating that genes regulated by *RUNX1* are essential for normal hematopoietic development. Additionally, rare familial mutations in the *RUNX1* DNA-binding domain lead to the familial platelet disorder and predisposition to both myeloid and lymphoid malignancies.

The presence of the *ETV6–RUNX1* translocation is associated with a favorable prognosis, with long-term event-free survival rates of approximately 90% in a number of studies.

TCF3-PBX1 Fusion Genes in B-Cell Acute Lymphoblastic Leukemia

Another example of an oncogenic chimeric transcription factor is the TCF3-PBX1 (previously known as E2A-PBX1) rearrangement, which results from the t(1;19)(q23;p13) chromosomal translocation present in about 5% of all B-lineage ALLs and in 25% of cases with a pre-B (cytoplasmic Ig-positive) phenotype. This translocation fuses the two N-terminal transactivation domains of the *TCF3* transcription factor on chromosome 19 to the DNA-binding domain of the homeobox gene *PBX1*, leading to the expression of hybrid TCF3-*PBX1* oncoproteins. The transforming potential of TCF3–PBX1 was first demonstrated by the rapid induction of AML in lethally irradiated mice repopulated with hematopoietic progenitors transduced with *TCF3–PBX1* genes. This fusion has also been shown to transform NIH-3T3 fibroblasts and induce T-cell lymphomas in transgenic mice. Additional studies have shown that deletion of one of the *TCF3* activation domains diminishes its transforming activity, but deletion of the *PBX1* homeodomain has no effect. Historically, the presence of the *TCF3–PBX1* translocation was associated with an inferior prognosis, but this no longer is the case with contemporary protocols for childhood ALL.

TCF3–HLF Fusion Genes in B-Cell Acute Lymphoblastic Leukemia

The t(17;19) is a rare recurrent chromosomal translocation that fuses the amino-terminal transactivation domains of *TCF3* to the C-terminal DNA binding and dimerization domains of *HLF*, a basic leucine zipper domain transcription factor. Although TCF3–HLF can bind DNA either as a homodimer or as a heterodimer with HLF and related proteins, no other PAR proteins are expressed in hematopoietic cells, and the TCF3–HLF fusion binds DNA as a homodimer in cells harboring the t(17;19). Similar to TCF3–PBX1, TCF3–HLF can transform NIH–3T3 fibroblasts, a process that requires the *HLF* leucine zipper domain and the *TCF3* transactivation domains, and can induce lymphoid tumors in transgenic mice. Recent transcriptomic analysis has revealed that the *TCF3–HLF* fusion leads to a gene expression signature enriched for stem cell and myeloid markers, suggesting that this fusion leads to cellular reprogramming of a lymphoid progenitor to a more primitive state. Additionally, another major consequence of TCF3–HLF expression in lymphoid precursors is the inhibition of apoptotic cell death. In normal pro-B lymphocytes, the expression of TCF3–HLF blocks apoptosis induction by either IL-3 or p53. In TCF3-HLF expressing human ALL cells, the inhibition of TCF3–HLF function by expression of a dominant-negative form of TCF3–HLF results in apoptosis induction. HLF is the mammalian homolog of the worm protein ces-2, a transcription factor that is necessary for the death of two specific nerve cells during *Caenorhabditis elegans* development. This pathway, which is evolutionarily conserved, is inhibited by the TCF3-HLF fusion. Thus, in contrast to the proapoptotic role of the WT HLF homolog (ces-2) in worms, TCF3–HLF blocks apoptosis by inducing the expression of SLUG, a transcription factor that blocks DNA damage-induced apoptosis in hematopoietic cells. The t(17;19) occurs in less than 1% of ALL cases, is associated with characteristic clinical features including adolescent age, disseminated intravascular coagulation and hypercalcemia at diagnosis, as well as a high risk of treatment failure leading to a dismal prognosis.

MEF2D Gene Fusions in B-Cell Acute Lymphoblastic Leukemia

Cryptic fusions involving *MEF2D* at chromosome 1q21–22 have recently been identified through RNA sequencing studies. *MEF2D*, located at chromosome 1q21–22, encodes myocyte enhancer factor 2D and is a member of the myocyte enhancer factor family of transcription factors that are expressed throughout normal B-cell development. Previous studies have shown that the inactivation of *MEF2D* Results in maturation arrest at the pre-B-cell stage of lymphoid development. In these cases, the amino terminus of *MEF2D* fuses in frame with the carboxy terminus of the fusion partner. Several fusion partners of *MEF2D* have been identified, including most commonly *BCL9*, which is a component of the *WNT*/β-catenin signaling cascade. The prognostic implication of *MEF2D* gene fusions has not yet been established.

ZNF384 Gene Fusions in B-Cell Acute Lymphoblastic Leukemia

Fusions involving the zinc-finger protein 384 (*ZNF384*) gene, which encodes a transcription factor that regulates promoters of the extracellular matrix, have recently been identified through RNA sequencing and transcriptomic analyses in B-cell ALL. To date, eight fusion partners have been identified, including *BMP2K, TCF3, TAF15, EWSR, EP300, CREBBP, SYNRG,* and *ARID1B. ZNF384*-related fusion genes have demonstrated the transformation of NIH3T3 fibroblasts in vitro; however, their exact cellular consequences and role in leukemogenesis remain unknown. B-cell ALL cases that harbor *ZNF384*-related fusions are characterized by a unique immunophenotype that is weak or negative for CD10 and positive for the myeloid markers, CD13 and CD33, but are variable in clinical presentation. *ZNF384* gene fusions have also been observed in up to 50% of B-cell/myeloid cases of MPAL.

CALM–AF10 Fusion Gene in T-Cell Acute Lymphoblastic Leukemia

The t(10;11)(p13;q14) is detected in approximately 3% to 10% of T-cell ALL cases and in occasional AML cases. This translocation results in the fusion of *CALM* (also known as *PICALM*), encoding a protein with high homology to the murine clathrin assembly protein ap3, with *AF10*, a gene identified as a *KMT2A* partner in the *KMT2A–AF10* fusion resulting from the t(10;11)(p13;q23). The expression of the *CALM-AF10* fusion transcript has been associated with early arrest in T-cell development and with differentiation into the gamma-delta lineage in T-cell ALL. Additionally, aberrant upregulation of *HOX* gene expression appears to be involved in *CALM–AF10*-mediated leukemogenesis, at least in acute myeloid leukemia cells that carry this translocation. Interestingly, analysis of a mouse model of *CALM–AF10*-induced acute myeloid leukemia suggests that the leukemic stem cell in this model has lymphoid characteristics, and cells from human patients with AML can be identified that have similar characteristics to the disease-propagating cell in this animal model.

SPI1 Fusion Genes in T-Cell Acute Lymphoblastic Leukemia

Sequencing studies pf pediatric T-cell ALL have recently identified recurrent *SPI1* fusions (*STMN1-SPI1* and *TCF7-SPI1*) in approximately 4% of cases and are associated with poor outcome. *SPI1* is a member of the ETS family of transcription factors and is expressed in hematopoietic cells, where its tight regulation is critical for normal HSC development. During normal T-cell development, *SPI1* is expressed in prethymic and early T-cell progenitors and turned off during later stages of T-cell development. The fusion places SPI1 under the control of *TCF7* or *STMN1* promoter and results in the overexpression of *SPI1*, regardless of fusion partner. In vitro studies demonstrate that expression of *SPI1* fusion genes results in cell proliferation and differentiation arrest during the double-negative (DN) stage of T-cell development, thereby likely contributing to T-cell leukemogenesis.

Activating Point Mutations of Oncogenic Transcription Factors

NOTCH1 in T-Cell Acute Lymphoblastic Leukemia

The *NOTCH1* gene was originally discovered as a partner gene in an exceedingly rare t(7;9) chromosomal translocation in T-cell ALL, in which *NOTCH1* is truncated and placed under the control of the *TCRβ* locus. Despite the rarity of *NOTCH1* translocations, a search for point mutations within the *NOTCH1* gene revealed activating mutations in more than 50% of cases of T-cell ALL. *NOTCH1* plays several critical roles during T-cell development, and its overexpression in murine hematopoietic cells potently drives T-cell ALL. NOTCH1 is a transmembrane protein that is proteolytically processed during its transit to the cell surface, where it exists as a heterodimer consisting of extracellular and transmembrane subunits. Upon ligand binding, the transmembrane subunit undergoes additional proteolytic cleavage within the plasma membrane, leading to the release of its intracellular domain, known as ICN1 (intracellular domain of NOTCH1), into the cytosol. ICN1 subsequently translocates into the nucleus, where it is active as a transcription factor. Activating *NOTCH1* mutations in T-cell ALL can occur as either missense mutations in the heterodimerization domain, allowing constitutive proteolytic activation of the ICN1 domain, or as frameshift or stop codon mutations that lead to truncation of the PEST domain. The PEST domain regulates proteasomal degradation of the protein, and these PEST-inactivating mutations result in aberrant stabilization of ICN1 protein. Mutations in both regions are often found on the same allele in cases of T-cell ALL and are synergistic in increasing NOTCH1 transcriptional output. Additionally, the NOTCH1 oncoprotein can also be stabilized by inactivating mutations of tumor suppressor genes involved in its proteasomal degradation, including *FBXW7* and Cyclin C, as discussed later in this chapter. *MYC* is an important transcriptional target of NOTCH1, and it mediates many of the leukemogenic properties of NOTCH1 in human T-cell ALL cells. However, NOTCH1 also has MYC-independent oncogenic activity, as evidenced by its ability to induce T-cell ALL in zebrafish, where NOTCH1 does not upregulate *MYC* expression. Additional NOTCH1 targets implicated in T-cell ALL pathogenesis include the *IL7R* interleukin receptor and the long noncoding RNA *LUNAR1*, which is upregulated by NOTCH1 and functions to upregulate expression of the growth factor receptor *IGF1R*.

Small-molecule inhibitors of γ-secretase impair the proteolytic activation of NOTCH, but early clinical trial results with these agents in relapsed/refractory T-cell ALL have been disappointing. Investigation of mechanisms of resistance to NOTCH1 inhibition has revealed several potential strategies for therapeutic intervention. Activation of the phosphoinositide-3-kinase (PI3K)-AKT pathway, which commonly results from loss of its negative regulator *PTEN* in T-cell ALL, provides one mechanism for resistance to NOTCH1 inhibition. The PTEN-PI3K-AKT pathway also mediates resistance to MYC inhibition in this disease. However, work in a murine model of Kras-induced T-ALL, where leukemias commonly acquire activating *NOTCH1* mutations and are sensitive to PI3K inhibitors, revealed that the development of resistance to PI3K inhibition was unexpectedly associated with the loss of oncogenic NOTCH1 signaling. This finding thus raises the possibility that dual PI3K-NOTCH1 inhibition may actually promote the emergence of drug resistance. Additional studies are needed to define the optimal strategy for clinical application of NOTCH1 and PI3K inhibition in patients with T-cell ALL.

Recent work has also revealed that some T-cell ALL cases in which the bulk cell population is sensitive to NOTCH1 inhibition harbor minor populations of so-called “persister” cells that are resistant to NOTCH1 inhibitors. This “persister” state is not driven by genetic mutations but is instead a reversible cellular state characterized by chromatin compaction. These NOTCH1 inhibitor-resistant cells maintain the expression of *MYC*, a crucial downstream target of *NOTCH1*, despite effective inhibition of NOTCH1 activity. However, the survival of these cells is specifically dependent on BRD4, a transcriptional regulator whose ability to bind acetylated chromatin can be specifically inhibited using small molecules. Combination therapy with inhibitors of both BRD4 and NOTCH1 can block the emergence of this resistance mechanism, and this approach has promising in vivo activity in preclinical models.

MYC Mutations in Mature B-Cell Lymphoblastic Leukemia

As reviewed previously in this chapter, mature B-cell ALL is characterized by chromosomal translocations that place the *MYC* coding sequence under the control of *Ig* gene-regulatory elements. Although the *MYC* coding region is not structurally altered by these translocations in most cases, point mutations of *MYC* commonly arise in these tumors at codons 58 or 62. These codons encode phosphorylation sites that regulate the activity and proteasomal degradation of MYC. These mutations lead not only to the aberrant stabilization of MYC protein but also inhibit the ability of MYC to activate apoptosis, while its ability to stimulate proliferation remains intact.

Mutations of Histone-Modifying Enzymes

DNA exists in cells in complex with histone proteins and other molecules in a complex known as chromatin. Post-translational modifications of histone proteins play prominent roles in the regulation of chromatin structure. Chromatin structure at individual loci can be broadly categorized as euchromatin, or “open” chromatin where transcription factor binding sites in DNA are readily accessible to the transcriptional machinery, or heterochromatin, where chromatin is compacted and DNA is generally not accessible to the transcriptional machinery. Alternations in chromatin structure can have profound effects on the gene expression program activated by transcription factors. Several enzymes that catalyze covalent histone modifications are recurrently mutated in ALL, highlighting a central role for dysregulation of chromatin structure in human leukemogenesis.

KMT2A Fusion Genes

Translocations involving the *KMT2A* gene (previously known as *MLL*) on chromosome 11q23 occur in approximately 80% of infant ALL cases, 5% of AML cases, and 85% of secondary AML cases that occur in patients treated with topoisomerase II inhibitors. *KMT2A* is the human ortholog of the *Drosophila* trithorax gene. Trithorax proteins are positive regulators of homeobox gene expression and act antagonistically to polycomb proteins. WT KMT2A positively regulates *HOX* gene expression and is required for both primitive and definitive hematopoiesis. WT KMT2A is a member of a large transcriptional regulatory complex, together with histone deacetylases and members of the SWI/SNF chromatin-remodeling complex.

The KMT2A protein undergoes proteolytic processing by Taspase1, a specialized protease that cleaves the KMT2A protein into N-terminal ($KMT2A^N$) and C-terminal ($KMT2A^C$) fragments that remain associated through intramolecular protein–protein interaction domains. $KMT2A^N$ contains several DNA-binding domains, including AT-hook domains that nonspecifically bind the minor groove of DNA, a methyltransferase homology region (CxxC domain) that specifically binds unmethylated DNA, four plant homeodomain zinc fingers with an embedded bromodomain, and a transcriptional repression domain. The C-terminal fragment $KMT2A^C$ contains a transcriptional activation domain that recruits the histone acetyltransferase cAMP response element-binding protein (CBP) and a SET domain that is responsible for its histone 3 lysine 4 (H3K4) methyltransferase activity.

WT KMT2A encodes an H3K4 lysine methyltransferase, and H3K4 methylation is associated with transcriptional activation. *KMT2A* fusion oncogenes result from translocations whose breakpoints cluster between exons 5 and 11 of *KMT2A*, and the resultant fusion proteins retain the N-terminal region of KMT2A, including the AT-hook and CxxC domains that bind DNA in a sequence-nonspecific manner. By contrast, the C-terminal domains that mediate the association of WT KMT2A with its endogenous chromatin-remodeling complex and its H3K4 methyltransferase activity are invariably lost from oncogenic KMT2A fusion proteins. Instead, the C-terminus of KMT2A fusion proteins is provided by one of more than 60 different translocation partners, with common translocations such as the t(4;11), t(9;11), and t(11;19)(q23;p13.3) resulting in the in-frame fusion of KMT2A to AF4, AF9, and ENL, respectively. The unrelated t(11;19)(q23;p13.1) translocation results in the fusion of KMT2A to ELL, the RNA polymerase II elongation factor.

Formal proof that *KMT2A* fusions play a critical role in the development of leukemias has come from the generation of murine models of *KMT2A*-induced leukemias. Chimeric mice harboring a *KMT2A–AF9* fusion gene generated by homologous recombination developed leukemias with a latency of 4 to 12 months. Retroviral transduction of *KMT2A–ENL, KMT2A–ELL,* and *KMT2A–CBP* fusion genes in hematopoietic precursors induces transformation upon transplantation into recipient mice. Similar results were obtained with a model in which chromosomal translocations involving the *KMT2A* locus are induced by directed interchromosomal recombination in mice, a strategy that reproduces experimentally the initiating events in the pathogenesis of *KMT2A*-rearranged leukemias. Interestingly, the introduction of *KMT2A–AF9* into committed granulocyte-macrophage progenitors in the mouse leads to the reactivation of a subset of genes normally expressed only in HSCs and transforms these committed precursors into AML leukemic stem cells by imparting the properties of self-renewal, suggesting that the leukemogenic lesion in *KMT2A*-rearranged leukemia might occur in a committed progenitor rather than in a pluripotent HSC.

KMT2A-rearranged B-lineage leukemias have a characteristic gene expression signature that includes the upregulation of several *HOX* genes and the expression of numerous myeloid markers. Both early B- and T-cell ALLs with *KMT2A* rearrangements showed a characteristic upregulation of specific *HOX* genes, including *HOXA9, HOXA10,* and *HOXC6,* and the *HOX* gene regulator *MEIS1*. These results, together with the demonstration that *HOXA9* plays important roles in the transformation of hematopoietic precursors by *KMT2A* fusion oncogenes in murine leukemia models, emphasize the central role of *HOX* gene dysregulation in the pathogenesis of *KMT2A*-rearranged leukemias. Additionally, as discussed later in this chapter, overexpression or activating mutations of the *FLT3* receptor tyrosine kinase (RTK) are frequent in *KMT2A*-rearranged leukemias.

Until recently, the precise mechanisms mediating the oncogenic activity of KMT2A fusion proteins were unclear because *KMT2A* translocation partners have no sequence similarity. However, recent work has shown that several distinct *KMT2A* translocation partners are functionally linked through their association in protein complexes that regulate transcriptional elongation. Oncogenic KMT2A-fusion proteins have been implicated in the DOT1L, SEC, and PAFc transcriptional complexes. The DOT1L complex consists of DOT1L, a histone H3 lysine 79 (H3K79) methyltransferase, and multiple KMT2A fusion partners, including AF9, ENL, and AF10. The SEC (also known as p-TEFb or AEP) complex consists of a CDK9 and cyclin T heterodimer (known as p-TEFb) that phosphorylates RNA polymerase II, interacts with several KMT2A fusion partners, including ENL, ELL, AF4, and AF5, and co-purifies with several KMT2A fusion proteins. PAFc, the polymerase-associated factor complex, regulates RNA polymerase II and associates with the amino terminus of both WT KMT2A and KMT2A fusions.

The observation that *DOT1L* interacts with multiple KMT2A translocation partners suggests that KMT2A fusion proteins may drive ectopic gene expression through recruiting excessive *DOT1L* activity to their target loci. *DOT1L* encodes a histone H3K79 methyltransferase, and many of the target genes directly upregulated by oncogenic KMT2A fusions are characterized by aberrant H3 lysine 27 (H3K27) methylation, including the 5′ *HOXA* cluster genes and *MEIS1*. Interestingly, the DOT1L methyltransferase is required for the transformation of murine bone marrow progenitors by KMT2A fusion oncoproteins, but it plays a less prominent role in normal hematopoiesis.

The oncogenic activity of KMT2A fusions also requires their physical interaction with Menin, the tumor suppressor protein encoded by the multiple endocrine neoplasia 1 (*MEN1*) gene. Menin binds an N-terminal domain of KMT2A that is conserved in all KMT2A fusion proteins. Moreover, abrogating the interaction of Menin with KMT2A, either by deleting Menin or by mutating the Menin-binding domain of KMT2A, impairs both transcriptional and oncogenic functions of KMT2A fusions. These findings spurred the development of small-molecule inhibitors of the KMT2A-menin interaction. Studies of small molecular inhibitors have provided critical insight into the mechanism by which KMT2A-Menin inhibition acts through the displacement of KMT2A chromatin occupancy at select target genes (Fig. 66.2) and have demonstrated promising activity in preclinical models, both in vitro and in vivo, including complete eradication of leukemia in a patient-derived xenograft model of *KMT2A*-rearranged acute leukemia, providing the rationale for early phase clinical trials of Menin inhibitors in relapsed/refractory *KMT2A*-rearranged acute leukemias.

The presence of *KMT2A* rearrangements is associated with inferior outcomes despite aggressive chemotherapy, especially in infants with ALL, 80% of whom present with *KMT2A*-rearrangements.

PRC2 Mutations in T-Cell Acute Lymphoblastic Leukemia

The PRC2 is a transcriptional repressor complex best known as a "writer" of histone H3K27 methylation, a chromatin mark associated with transcriptional repression. Up to 25% of T-cell ALL cases harbor inactivating mutations of core components of the PRC2, including *EZH2, SUZ12,* and *EED*. EZH2 deficiency is sufficient to initiate T-cell leukemogenesis in murine models, implicating PRC2 as a tumor suppressor in this disease. Genome-wide analysis of NOTCH1 occupancy and histone methylation patterns have revealed that NOTCH1 binding is associated with loss of the repressive histone mark placed by the PRC2 complex, H3K27 trimethylation. Recent work has revealed that the H3K27 "eraser" demethylase JMJD3 (also known as KDM6B) is a component of the NOTCH1 transcriptional complex. These data thus support a model in which the NOTCH1 recruits the H3K27 "eraser" demethylase JMJD3 to its target genes, where it antagonizes the activity of the H3K27 "writer" PRC2. Thus, one pathogenic consequence of PRC2 inactivation in T-cell ALL may be the release of inhibition of oncogenic NOTCH1 signaling. Interestingly, a small-molecule inhibitor whose effects include inhibition of JMJD3 function has therapeutic activity in T-cell ALL, thus suggesting that JMJD3 inhibition may be a potential therapeutic strategy for patients with PRC2-mutant T-cell ALL. Additionally, recent work has demonstrated that PRC2 plays a role in chemotherapy resistance via inhibition of mitochondrial apoptosis. In T-ALL cells, knockdown of *EZH2*, a core component of the PRC2 complex, resulted in resistance to multiple chemotherapy agents that are commonly used in T-cell ALL therapy. RNA sequencing and in vitro functional studies revealed downregulation of CRIP2 and the mitochondrial chaperone protein TRAP1 in PRC2 deficient T-cell ALL cells, suggesting that these play a key role in apoptosis resistance and treatment response and may provide a novel therapeutic strategy in PRC2-deficient T-cell ALL.

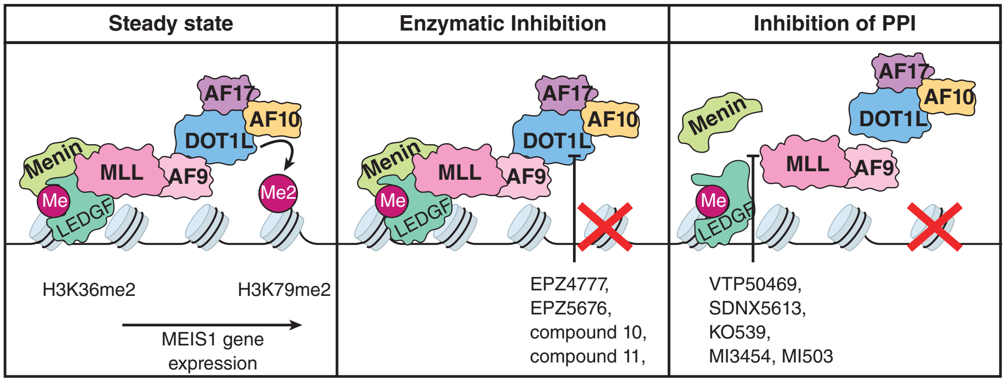


Figure 66.2 MODEL FOR THE CONSEQUENCES OF DOT1L VERSUS MENIN INHIBITION FOR THE INTEGRITY OF THE MLL-AF9 COMPLEX ON CHROMATIN. At steady state, the intact MLL-AF9 complex stably binds to its target genes via Menin and recruits the DOT1L complex to drive histone H3 lysine 79 methylation (H3K79me2) and gene expression of canonical target genes (e.g., *MEIS1*) (*left*). DOT1L inhibition removes H3K79me2 by blocking the enzymatic activity of the methyltransferase while leaving the complex largely intact (*middle*). Inhibition of the Menin-MLL interaction disrupts the integrity of the MLL-AF9 complex, leading to a more rapid and profound response (*right*). *(Adapted from Perner F, Armstrong SA. Targeting chromatin complexes in myeloid malignancies and beyond: from basic mechanisms to clinical innovation. Cells. 2020;9:2721, with permission).*

Focal Activating Mutations of Noncoding Gene-Regulatory Elements

Activating mutations of cis-acting gene-regulatory elements are common events in ALL pathobiology. This mechanism was first revealed through the investigation of recurrent translations that juxtapose the protein-coding sequences of *MYC* with *Ig* enhancer elements in mature B-cell ALL (Burkitt leukemia), as discussed earlier in this chapter. However, mono-allelic oncogene overexpression often occurs in the absence of identifiable chromosomal translocations, suggesting the existence of unrecognized mutations of cis-acting gene-regulatory elements. Our ability to identify pathogenic mutations of non-protein-coding elements has been hampered by the difficulty of distinguishing pathogenic "driver" mutations from nonspecific "passenger" events that represent nonspecific consequences of genetic instability, which are much more common in cancer genomes. Two recent studies provide experimental paradigms for the identification of such pathogenic mutations of gene-regulatory elements based on co-localization of genetic lesions with markers of active gene-regulatory elements.

TAL1 Super-Enhancer Mutations in T-Cell Acute Lymphoblastic Leukemia

Advances in the ability to map transcription factor occupancy and chromatin modifications genome-wide have revealed the presence of unusually dense clusters of enhancer elements at select genomic locations. These elements have been termed super-enhancers (also stretch enhancers or locus control regions) and are characterized by dense and broad occupancy by core components of the transcriptional machinery, such as Mediator, as well as histone marks associated with active enhancer elements, including histone H3K27 acetylation. Super-enhancers have been implicated in the expression of genes responsible for cellular identity in normal and malignant cells.

Recent work has revealed the presence of focal mutations affecting a specific hotspot in a noncoding locus 23 kb upstream of the *TAL1* oncogene in 5% of T-cell ALL cases. These mutations uniformly introduce a binding motif for the MYB transcription factor, and this occurs at a locus that already harbors binding motifs for core components of the TAL1 complex, including TAL1 itself, RUNX1, and GATA3. These mutations lead to the discovery that the *MYB* oncogene is a core component of the TAL1 complex. Indeed, the introduction of this novel MYB binding site is sufficient to recruit the entire TAL1 transcriptional complex to this locus (Fig. 66.3), leading to the generation of an aberrant super-enhancer that drives aberrant overexpression of TAL1 specifically from the mutated allele.

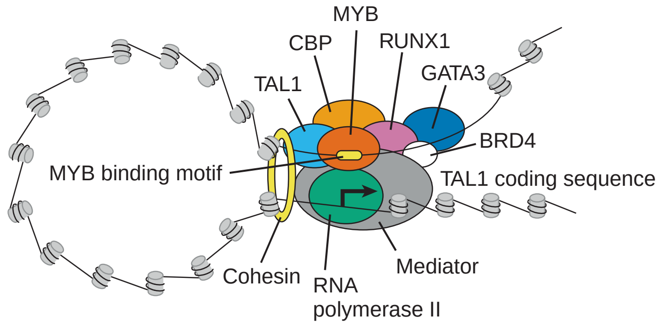


Figure 66.3 ONCOGENIC SUPER-ENHANCER IN T-CELL ALL. Noncoding insertion mutations upstream of TAL1 create binding sites for the MYB transcription factor, leading to the recruitment of core components of a major leukemogenic transcriptional complex containing RUNX1, GATA-3, and TAL1, and to the formation of a de novo super-enhancer that drives expression of the *TAL1* oncogene in a subset of T-cell ALL. *(Adapted from Hnisz D, Abraham BJ, Lee TI. Super-enhancers in the control of cell identity and disease. Cell. 2013;155(4):934–947).*

Duplications of the NOTCH-Driven Enhancer of MYC in T-Cell Acute Lymphoblastic Leukemia

Aberrant NOTCH1 activation is pathogenic, at least in part, due to NOTCH1-driven overexpression of *MYC*, as reviewed earlier in this chapter. Recent work has revealed an enhancer element downstream of *MYC* that is highly bound by NOTCH1 and is affected by recurrent somatic duplications in 3% of T-cell ALL. This enhancer forms a looping interaction with the *MYC* proximal promoter, and it drives NOTCH1-dependent overexpression of *MYC*. Mice in which this enhancer element has been deleted have a defect in T-cell development that specifically mimics that seen in mice with T-cell specific *MYC* inactivation. Moreover, murine bone marrow cells lacking this enhancer element are completely resistant to leukemic transformation by NOTCH1. Finally, duplication of this enhancer element drives increased gene expression in a reporter assay, suggesting that this enhancer duplication is pathogenic because it potentiates signaling through the oncogenic NOTCH1-MYC axis.

Aberrant Growth Factor Signaling

BCR-ABL1 in Acute Lymphoblastic Leukemia

The Philadelphia (Ph) chromosome, which arises from the t(9;22)(q34;q11), was originally identified in patients with chronic myelogenous leukemia (CML); however, it is also found in about 2% to 4% of childhood cases and 20% of adult cases of ALL, which are almost always of the B-cell ALL subtype. The t(9;22) generates a *BCR–ABL1* fusion gene consisting of 5′ (upstream) sequences from *BCR* and 3′ (downstream) sequences of *ABL1*. The t(9;22) breakpoints on the distal tip of the long arm of chromosome 9 are scattered over a distance of nearly 200 kb within the first intron of the *ABL* proto-oncogene, upstream of the tyrosine kinase domain. The breakpoints in the *BCR* gene on chromosome 22 cluster in two separate regions of that gene, known as the major breakpoint cluster region (M-bcr) or minor breakpoint cluster region (m-bcr). In two-thirds of cases of Ph-positive ALL, the breakpoint in the *BCR* gene occurs in the m-bcr, but in all cases of CML and about one-third of cases of ALL, the breaks occur in the M-bcr. The fusion transcript more commonly present in ALL (m-bcr) encodes a 190-kd protein (p190), whereas the transcript found in CML and in some cases of ALL (M-bcr breakpoint) encodes a 210-kd hybrid protein (p210). Both types of fusions generate chimeric oncoproteins that are activated as a tyrosine-specific protein kinase, similar to the v-abl protein.

The ABL1 tyrosine kinase is localized both in the nucleus and in the cytoplasm of proliferating cells. It is normally activated by DNA damage downstream of ATM and appears to promote p53-mediated growth arrest. Mice deficient in ABL develop a wasting syndrome and die soon after birth. In contrast to the nuclear and cytoplasmic distribution of normal ABL, the BCR–ABL1 fusion oncoprotein has a cytoplasmic location and shows increased tyrosine kinase activity. When expressed in murine hematopoietic precursors, both p190 and p210 transform hematopoietic cells in vitro and induce a syndrome similar to CML in mice. Transformation by the BCR–ABL1 oncoprotein involves activation of the RAS–MAPK (mitogen-activated protein kinase) pathway, PI-3 and JUN kinases, c-CBL and CRKL, JAK–STAT, NFκB (nuclear factor kappa-B), Src, and cyclin D1. The BCR–ABL1 oncoprotein affects multiple aspects of cell homeostasis, including apoptosis, differentiation, and cell adhesion. An important cellular effect of BCR–ABL1 is the induction of cellular resistance to DNA damage agents such as cytostatic drugs and irradiation. After DNA damage, BCR–ABL1 extends the duration of the G2/M cell cycle checkpoint and facilitates DNA repair. It also upregulates the antiapoptotic *BCL-XL* gene, contributing to the suppression of apoptotic cell death.

The presence of the Ph chromosome historically was associated with an extremely poor prognosis in ALL patients despite treatment with intensified chemotherapeutic regimens. Better outcomes were achieved with allogeneic stem cell transplantation in first complete remission, but overall prognosis remained inferior to other ALL subtypes. The development of imatinib mesylate, a pharmacologic tyrosine kinase inhibitor targeting the BCR–ABL1 oncoprotein, has transformed the management of Ph-positive ALL. The utility of imatinib as a single agent for Ph-positive ALL is limited by the rapid development of drug resistance. However, the combination of imatinib and other BCR-ABL1 kinase inhibitors, such as dasatinib, with cytotoxic chemotherapy has led to significant improvements in outcome for patients with Ph-positive ALL over the last two decades.

Resistance to tyrosine kinase inhibitors can emerge in Ph-positive lymphoblasts, most commonly because of point mutations in the kinase domain of *BCR–ABL1*. As a result, a series of novel BCR–ABL1 kinase inhibitors have been developed that retain activity against many of these mutant oncoproteins. Although not all *BCR–ABL1* mutations that confer resistance can be overcome with newer agents, the potential of these newer BCR–ABL inhibitors with broader specificity to further improve outcomes is actively being investigated. Additionally, a novel pathway mediating resistance to BCR–ABL1 inhibition has been uncovered in Ph-positive ALL. Upon BCR–ABL1 inhibition, these cells markedly upregulate expression of the *BCL6* transcription factor, a well-known oncogene that is often translocated in diffuse large B-cell lymphomas. *BCL6* then blocks activation of the p53 pathway via transcriptional repression of *ARF* and thus blocks the therapeutic efficacy of monotherapy with imatinib in Ph-positive ALL cells. Importantly, inhibition of BCL6 activity genetically or using a peptide inhibitor of BCL6 has demonstrated marked activity in patient-derived BCR–ABL xenografts grown in immunodeficient mice, highlighting the therapeutic relevance of these findings.

BCR-ABL1-Like Acute Lymphoblastic Leukemia

Analysis of gene expression signatures of patients with ALL has led to the identification of a subtype of B-cell ALL termed BCR-ABL1-like or Ph chromosome-like (Ph-like) ALL. These cases were identified because they lack BCR-ABL1 translocations yet are characterized by a gene expression signature nearly identical to that of BCR-ABL1 ALL. Ph-like ALL occurs in approximately 15% of B-ALL, increasing in frequency with age, and has been associated with an inferior prognosis compared to other subtypes of B-cell ALL, similar to BCR-ABL1 ALL. One genetic feature common to both BCR-ABL1 and BCR-ABL1-like ALL is the deletion of the *IKZF1* tumor suppressor. Approximately half of the cases of Ph-like ALL contain *CRLF2* genomic alterations and half of those cases contain concomitant *JAK* mutations. The remaining cases of Ph-like ALL have been characterized by rearrangements involving various kinases, including *ABL1, ABL2, CSF1R, JAK2,* and *PDGFRB* (Fig. 66.4). In a small subset of cases, rearrangements leading to overexpression of the erythropoietin receptor *EPOR* have been observed. These findings have important clinical implications, as many of these mutations are potentially targetable with clinically relevant inhibitors

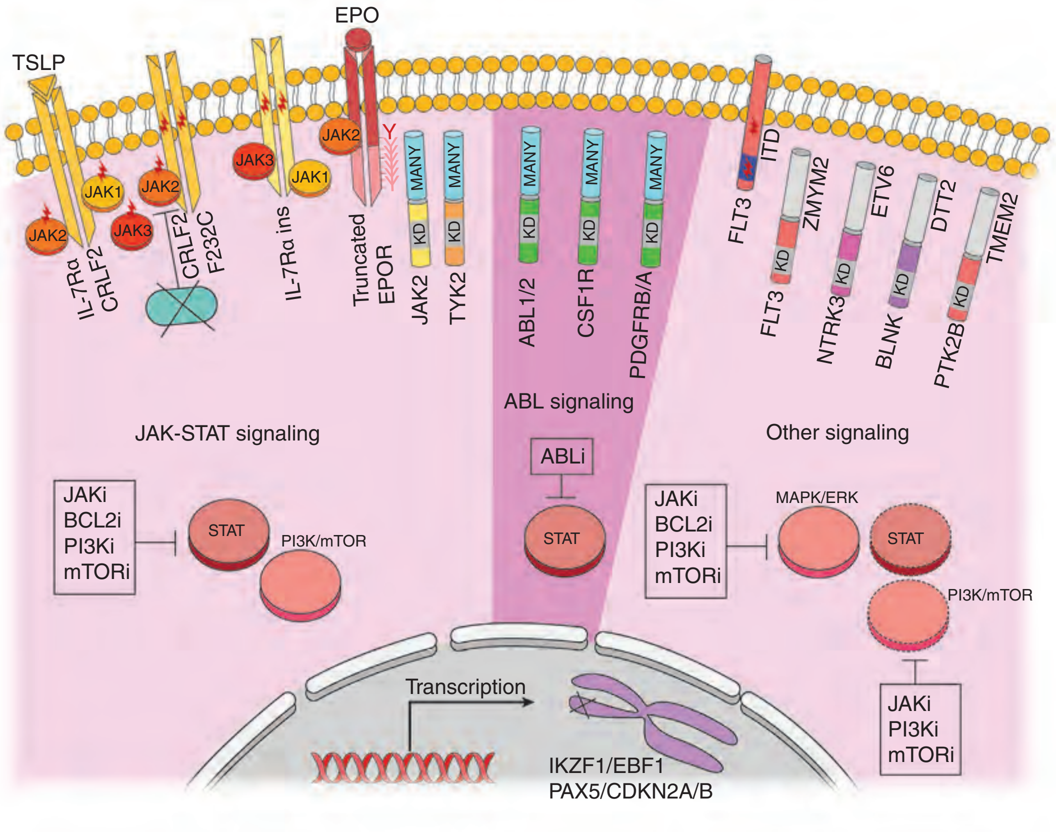


Figure 66.4 ABERRANT ACTIVATION OF SIGNAL TRANSDUCTION PATHWAYS IN BCR-ABL1-LIKE ALL. Deregulation of JAK2, ABL, and other signaling pathways in BCR-ABL1-like ALL is caused by activating mutations (*lightning bolts*), fusion genes, or genomic deletions (*X*) leading to overexpression of cytokine receptors (e.g., CRLF2, IL-7, EPOR), expression of constitutively active receptors (e.g., EPOR), mislocalized and constitutively active kinases. Some downstream signaling pathways are shown, as well as potential targets for therapeutic intervention with small-molecule inhibitors (i). *(Adapted from Iacobucci I, Mullighan CG. Genetic basis of acute lymphoblastic leukemia. J Clin Oncol. 2017;35:975, with permission.)*

NUP214–ABL1 in T-Cell Acute Lymphoblastic Leukemia

Although the BCR–ABL1 translocation is rare in T-cell ALL, amplified episomes containing *NUP214–ABL1* fusion genes have been described in approximately 6% of children and adults with T-cell ALL. These episomes appear to arise via a mechanism in which the genomic region of chromosome 9q34, which contains both the *NUP214* and *ABL1* genes, is circularized in a manner that leads to the fusion of these two genes. The breakpoint in the *ABL1* gene in all of these cases occurs in intron 1, which is the same breakpoint observed in Ph-positive CML and B-cell ALL, but the *NUP214* breakpoints are variable. The WT NUP214 protein is a component of the nuclear pore complex and may contribute oligomerization motifs to the *NUP214–ABL1* fusion oncogene. The NUP214-ABL1 fusion protein has constitutively activated ABL1 tyrosine kinase activity, which is inhibited by the BCR–ABL kinase inhibitor imatinib, suggesting the potential therapeutic role of imatinib or other tyrosine kinase inhibitors in these patients.

B-Cell Receptor Signaling in Acute Lymphoblastic Leukemia

Despite its importance in the molecular pathogenesis of mature B-cell lymphomas, the role of B-cell receptor signaling in ALL was poorly understood. However, in one study, 10% to 15% of cases of B-cell ALL were characterized by active signaling through the B-cell receptor. Such B-cell receptor-positive cases were identifiable by expression of the Ig μ heavy chain or of BCL6 protein on flow cytometry and were strongly associated with the presence of the *TCF3-PBX1* rearrangement or 6q21 deletion, but they are rare in other ALL subsets. Interestingly, the oncogenic *TCF3-PBX1* translocation itself directly binds and upregulates genes encoding key components of the B-cell receptor in these cases. Active B-cell receptor signaling leads to downstream activation of SRC, SYK, and PI3K oncogenic signaling pathways. Importantly, these cases are sensitive to small-molecule inhibitors of kinases that mediate B-cell receptor signaling in preclinical models, thus providing a novel potential therapeutic strategy for these patients.

RAS Gene Mutations

Pioneering studies into the molecular etiology of cancer led to the identification of activated homologs of either *HRAS* or *KRAS* in human tumor DNA. These proto-oncogenes were originally identified on the basis of their homology with viral oncogenes. Gene transfer methods identified an additional member of the *RAS* gene family, called *NRAS*, that had not been observed as a component of a transforming retrovirus. Proto-oncogenes of the *RAS* family (*HRAS, KRAS,* and *NRAS*) encode 21-kDa proteins that are associated with the inner surface of the cytoplasmic membrane and are involved in growth factor receptor signaling. *RAS* proteins are guanosine nucleotide-binding proteins (G proteins) that are GTP-bound in their active form, and GDP-bound in their inactive form. The *RAS* proto-oncogenes are converted to the status of transforming oncogenes by somatic mutations that most commonly alter the amino acids specified by codons 12, 13, or 61. Mutated *RAS* genes lose their intrinsic GTPase activity, thus accumulating in their active, GTP-bound conformation, even in the absence of growth factor binding to surface receptors. Aberrant RAS-mediated signaling contributes to transformation through activation of the PI3K and MAPK pathways.

In ALL, mutations of codons 12, 13, or 61 of *NRAS* have been found in approximately 10% of patients, while *KRAS* mutations have been identified in 5% to 10% of patients. *RAS* mutations are considerably more common in specific molecular subsets of ALL. Up to 50% of cases of *KMT2A*-rearranged ALL harbor mutations of *NRAS* or *KRAS*, although these mutations can be subclonal and lost at relapse. *RAS* mutations have also been identified in approximately 30% of Down syndrome-associated B-cell ALL, and appear to be mutually exclusive of *JAK2* mutations in this setting. *RAS* mutations appear to be more frequently found in the setting of relapsed ALL and are associated with a poor prognosis in this setting. The development of an effective pharmacologic approach for direct inhibition of *RAS* has been elusive. However, recent studies in preclinical models of *RAS*-driven ALL have revealed the therapeutic potential of inhibitors of key downstream effectors of *RAS* signaling in ALL, including MEK and PI3K, thus providing a rationale for clinical trials testing such an approach for *RAS*-mutant ALL.

PTEN–PI3K–AKT Mutations in T-Cell Acute Lymphoblastic Leukemia

The *PI3K–AKT* signal transduction pathway, which is negatively regulated by the *PTEN* tumor suppressor, induces cellular growth and proliferation while inhibiting apoptosis and is aberrantly activated in a range of human cancers. In T-cell ALL, recent work has identified a very high frequency of mutational activation of oncogenic signaling through this pathway, most often via deletions or truncating mutations of *I*, but activating mutations of *PI3K* and *AKT* genes also occur. Moreover, deletions of *PTEN* have been found to predict treatment resistance in clinical specimens, and mutations within the *PI3K* pathway are associated with inferior clinical outcomes. Studies in zebrafish and patient-derived xenograft mouse models have identified that acquisition of *PI3K-AKT* pathway during clonal evolution of leukemic blasts leads to primary glucocorticoid resistance, an effect mediated by direct phosphorylation of the glucocorticoid receptor by *AKT*. In addition, both *MYC* and the *PI3K-AKT* pathway have been identified as key suppressors of the proapoptotic factor, *BIM*, which is required for mitochondrial apoptosis in normal T-cell development. Taken together, these findings thus suggest the potential clinical utility of *PI3K-AKT* pathway inhibitors in high-risk T-cell ALL.

FLT3 Mutations and Overexpression in Acute Lymphoblastic Leukemia

FLT3 encodes an RTK that is highly expressed in early hematopoietic precursors, where it plays important functional roles. Multiple studies have shown that activating mutations of *FLT3*, which lead to constitutive RTK activity even in the absence of ligand, are common in leukemic myeloblasts in patients with AML but are rare in adults with ALL. However, gene expression studies demonstrated high expression of *FLT3* in pediatric ALL cases that involve *KMT2A* gene rearrangements or high hyperdiploidy. Additionally, activating mutations were identified in 18% of infants with *KMT2A*-rearranged ALL, in 21% to 24% of hyperdiploid ALL cases, and in all three cases of the prothymic CD117/KIT$^+$ subtype of T-cell ALL in adults examined.

In the absence of the FLT3 ligand, WT FLT3 receptors are inactive because of autoinhibition mediated by the juxtamembrane domain of the receptor. Upon binding of FLT3 ligand, normal FLT3 receptors homodimerize, become activated by phosphorylation, and lead to the activation of signal transduction pathways that promote proliferation and cell survival. Activating mutations of FLT3 found in leukemias occur in two separate regions of the gene. In-frame tandem duplications in the juxtamembrane domain lead to loss of the autoinhibition mediated by this domain, with subsequent dimerization and receptor activation in the absence of FLT3 ligand. Alternatively, point mutations or insertions in the second tyrosine kinase domain of the FLT3 receptor lead to autophosphorylation and activation of downstream signaling in the absence of the FLT3 ligand. Small-molecule inhibitors of the FLT3 kinase lead to in vitro apoptosis in AML cell lines and *KMT2A*-rearranged ALL cell lines and are used clinically in *FLT3*-mutated AML, but a beneficial impact of these agents in ALL with *FLT3* mutations and/or overexpression has not been demonstrated.

CRLF2 and *JAK2* Mutations in B-Cell Acute Lymphoblastic Leukemia

The CRLF2 cytokine receptor binds its ligand, thymic stromal lymphopoietin (TSLP), as a heterodimeric complex with the IL-7 receptor subunit (IL-7R). TSLP–CRLF signaling plays physiologic roles during normal B-cell development and in inflammation. Mutations and rearrangements involving *CRLF2* have been observed in 5% to 10% of cases of pediatric B-ALL but are much more frequent in the Ph-like subtype and in Down syndrome patients (approximately 50% of each). The abnormalities that commonly lead to *CRLF2* overexpression include translocations of the *IGH* locus (chromosome 14) to *CRLF2* and interstitial deletions in pseudoautosomal regions of Xp22.3/Yp11.3, resulting in a *P2RY8-CRLF2* fusion. Aberrant *CRLF2* expression very frequently co-occurs with *JAK2* activating mutations, and these two genetic lesions collaborate to induce ligand-independent activation of downstream signal transduction pathways, suggesting that CRLF2 may act as a scaffold that is required for activation of oncogenic JAK–STAT signaling by *JAK2* mutations in B-cell ALL. In addition to co-occurring mutations in the JAK/STAT pathway, *CRFL2*-rearrangements frequently co-occurs with *IKZF1* gene deletions as well as mutations with the *RAS* pathway, leading to activation of *MAPK, PI3K*, and *mTOR* pathways. The exact mechanism by which these mutations cooperate with *CRLF2* rearrangements in B-cell ALL remains unknown. When first identified, *CRLF2* rearrangements were thought to portend a poor prognosis in pediatric B-cell ALL; however, more recent analyses suggest that this observation was due to their high frequency in Ph-like ALL and co-occurrence with *IKZF1* deletions but that CRLF2 rearrangements themselves are not independent predictors of adverse outcome.

Interleukin-7 Receptor Mutations in Acute Lymphoblastic Leukemia

The interleukin 7 receptor alpha (*IL7R*) is required for normal lymphoid development, with loss-of-function *IL7R* mutations leading to severe combined immunodeficiency. The IL-7R protein can heterodimerize with either IL2Rγ, resulting in the receptor for IL-7, or with CRLF2 to form the receptor for TSLP. Gain of function mutations characterized by either a serine-to-cysteine substitution at amino acid 185 within the extracellular domain or in-frame insertions and deletions in the transmembrane domain have been described in both B-cell and T-cell ALL. In the case of the point mutations, this permits the formation of a neomorphic disulfide bond between mutant IL-7R homodimers and leads to ligand-independent receptor dimerization and activation of downstream oncogenic signal transduction pathways, including JAK–STAT and PI3K–AKT. A potential therapeutic strategy to target oncogenic activation of mutant IL-7R proteins is the reduction of the neomorphic disulfide bond. Indeed, N-acetylcysteine, a reducing agent developed clinically for the treatment of acetaminophen overdose, has been shown to reduce the extracellular cysteine bond and inhibit signaling by mutant IL-7R proteins. Downstream activation of the JAK–STAT pathway in gain of function IL7R mutated ALL would suggest that inhibition of JAK2 with ruxolitinib may be an effective therapeutic approach. Preclinical data has confirmed that ruxolitinib treatment has robust activity in murine xenograft models of Ph-like ALL, including those with *IL7R* mutations. This treatment approach is being studied in a Phase 2 study of de novo JAK pathway mutant Ph-like childhood ALL through the Children's Oncology Group.

Tumor Suppressor Gene Inactivation

Much attention has been focused on tumor suppressors, whose loss of function via deletion or mutational inactivation leads to malignant transformation. Knudson first proposed that inactivation of both alleles of a single locus is needed to initiate the development of retinoblastoma, basing his hypothesis on the observed frequencies of hereditary and sporadic forms of this disease. Allelic loss of defined regions of many different chromosomes has been linked to specific types of human tumors. By analogy with the findings in retinoblastoma, a reasonable hypothesis is that each of these regions harbors a tumor suppressor gene whose product is uniquely involved in the inhibition of cell cycle progression and promotion of terminal differentiation of the normal cells that give rise to these different types of tumors.

p53 and *CDKN2A* Mutations

p53 (also known as *TP53*) is a classic tumor suppressor gene whose activation induces apoptosis, cell cycle arrest, or senescence in response to distinct stimuli, including DNA damage or aberrant oncogene activation. p53 is mutated or deleted in a wide variety of human tumors and occurs as a heritable cause of cancer in families with Li-Fraumeni syndrome. p53 is inactivated in a variety of hematopoietic malignancies, including mature B-cell ALL, but is mutated or deleted in fewer than 3% of pediatric B-cell or T-cell ALL cases at diagnosis. Recently, large-scale genomic profiling of childhood B-cell ALL has revealed germline p53 mutations in about 2% of cases sequenced (which is discussed further in the following sections), thus adding B-cell ALL to the constellation of malignant neoplasms that patients with Li-Fraumeni syndrome are at risk for developing. While mutations in p53 are overall uncommon in B-cell ALL, they are characteristic of low hypodiploid ALL, occurring in more than 90% of cases with this specific subtype. About half of the p53 mutations observed in hypodiploid ALL are germline and the rest are somatic. Additionally, p53 mutations are seen in approximately 25% of relapsed T-cell ALL cases, as well as in association with early treatment failure, suggesting a role for p53 inactivation in the development of resistant disease.

Despite the rarity of p53 mutations in ALL specimens at the time of diagnosis, the p53 pathway is commonly inactivated by loss-of-function mutations of the *CDKN2A* locus in ALL. *CDKN2A* encodes two distinct tumor suppressor proteins, ARF (also known as p14ARF in humans and p19ARF in mice) and p16INK4a. ARF is induced by oncogene stress, such as acute *MYC* overexpression, and it binds and sequesters MDM2, leading to p53 activation. p16INK4a is a cyclin-dependent kinase inhibitor, a family of proteins that also include p15INK4B, p18INK4C, p19INK4D, p21, p27, and p57. These constitute a family of tumor suppressors that negatively regulate the cell cycle by inhibiting CDK phosphorylation of pRB. In particular, human leukemia and lymphoma show a high frequency of 9p21 deletions involving both the p16INK4A/p14ARF and the p15INK4B loci. Epigenetic silencing of these tumor suppressor genes through hypermethylation of their promoter sequences represents an alternative mechanism of gene inactivation. Although p16INK4A/p14ARF and p15INK4B are homozygously deleted in 20% to 30% of B-cell ALL cases and in 70% to 80% of T-cell ALL cases, epigenetic silencing of the p15INK4B promoter has been observed in 44% of primary B-lineage ALLs.

FBXW7 and Cyclin C in T-Cell Acute Lymphoblastic Leukemia

FBXW7 (also known as *FBW7*) is an E3 ubiquitin ligase that targets the transcriptionally active intracellular form of NOTCH1 (ICN), MYC, and cyclin E for degradation, and this gene is inactivated by mutation or deletion in approximately 10% of T-cell ALL cases. In T-cell ALL cell lines, *FBXW7* mutation or homozygous deletion leads to resistance of NOTCH1 pathway inhibition by γ-secretase inhibitor therapy, presumably because intracellular NOTCH protein levels remain high in the absence of FBXW7-mediated degradation despite inhibition of γ-secretase activity. Additionally, tumor-derived *FBXW7* mutations maintain their ability to bind MYC but do not lead to its degradation and may act as dominant-negative mutants that protect MYC from degradation. *FBXW7* also targets the antiapoptotic protein MCL1 for proteasomal degradation in T-cell ALL, providing an additional oncogenic consequence resulting from the inactivation of the *FBXW7* tumor suppressor that may provide a novel therapeutic strategy using MCL1 inhibitors.

Recent work has implicated cyclin C as a key component of the *FBXW7*-dependent pathway that mediates the degradation of NOTCH1. Cyclin C, which was originally identified as a growth-promoting cyclin, is involved in recurrent heterozygous deletions in human T-cell ALL. In a murine model, loss of one cyclin C allele accelerates the onset of NOTCH1-induced T-cell ALL, implicating this gene as a tumor suppressor. Mechanistically, cyclin C binds and activates the cyclin-dependent kinases CDK18, CDK8, and CDK3 to phosphorylate the intracellular domain of NOTCH1 and to promote its FBXW7-mediated proteasomal degradation. Thus, cyclin C deficiency and FBXW7 are both key components of a degradation pathway that controls physiologic NOTCH1 activity. The model that emerges from these findings is that impaired function of either of these tumor suppressors promotes T-cell oncogenesis by inducing aberrant nuclear accumulation of transcriptionally active NOTCH1.

PAX5 and Other B-Cell Developmental Gene Alterations in B-Cell Acute Lymphoblastic Leukemia

A high-resolution genome-wide analysis of B-cell ALL cases using single nucleotide polymorphism arrays identified copy number alterations in a number of genes that play important roles in B-cell development. Genes involved in B-cell development were found to be altered by deletion, amplification, mutation, or rearrangement in 40% of cases of B-cell ALL. The most common abnormalities identified were deletions of *PAX5*, and upon further analysis in other cases, other mechanisms that led to the inactivation of *PAX5* were identified. These included a number of translocations that led to fusion proteins that maintained the ability to bind to *PAX5* transcriptional targets but lost regulatory ability, thus having dominant-negative activity, and inactivating point mutations that altered the transcriptional activity of *PAX5*. As discussed later in this chapter, a recurrent germline *PAX5* mutation affecting Gly183 in the octapeptide domain of the gene has been identified in two unrelated kindreds, thus implicating *PAX5* in an autosomal dominant leukemia predisposition syndrome. Deletions were also detected in the *TCF3, EBF1, LEF1, IKZF1,* and *IKZF3* genes, all of which play important roles in B-cell development. More recently, genomic analyses of pediatric and adult B-cell ALL have identified two specific *PAX5* subtypes. The first, *PAX5*alt, was found in 7% of cases and is characterized by diverse genetic alterations, including rearrangements, intragenic amplifications, and mutations. The second is characterized by a specific *PAX5* SNV that results in a nonsynonymous amino acid substitution of Arginine for Proline at position 80 (p.P80R). This particular residue is essential for the binding of PAX5 to its targets and results in a nonfunctional protein and B-cell maturation arrest. Interestingly cases with PAX5 p.P80R almost uniformly lost the wildtype allele. In total, PAX5alt and PAX5 p.P80R account for about 9% of the B-other ALL subtype and are associated with intermediate-poor outcomes in both children and adults with B-cell ALL.

IKZF1 Deletions in B-Cell Acute Lymphoblastic Leukemia

IKZF1 (Ikaros) is a DNA-binding transcription factor that is required for the development of all lymphoid lineages, and expression of a dominant-negative isoform of *IKFZ1* in mice was shown to lead to T-cell lymphomas. However, the role of *IKZF1* in human leukemias was not appreciated until genomic analyses of B-cell ALL patient samples revealed *IKZF1* deletions in 16% to 29% of pediatric samples. *IKZF1* deletions are strongly associated with BCR–ABL1-positive ALL and Ph-like ALL and are also acquired at the transformation of CML to ALL (lymphoid blast crisis). *IKZF1* deletions are associated with an inferior 5-year EFS and an increased 5-year cumulative incidence of relapse, even in children with low end-induction minimal residual disease (MRD), and on multivariable analyses have been shown to be an independent predictor of adverse outcome. Interestingly, recent work has identified co-occurring mutations, which have a significant impact on the prognostic significance of *IKZF1* mutations. B-cell ALL cases harboring deletions of *IKZF1* that occur in combination with deletions of *CDKN2A, CDKN2B, PAX5,* or *PAR1* and in the absence of an *ERG* deletion, termed *IKZF1*$^{\text{plus}}$, had significantly inferior outcome than in B-cell ALL cases that harbor an *IKZF1* deletion but do not meet criteria for *IKZF1*$^{\text{plus}}$.

Inactivation of *LEF1* in T-Cell Acute Lymphoblastic Leukemia

The LEF1 transcription factor, a member of the LEF/TCF (lymphoid enhancer-binding factor/T-cell-specific transcription factor) family of DNA-binding transcription factors, is best known for its role as a positive mediator of β-catenin transcriptional activity, a well-established oncogene. Genomic analyses have identified mono- and biallelic deletions and truncating mutations of *LEF1* in 18% of primary T-cell ALL patient samples, implicating *LEF1* as a T-cell ALL tumor suppressor. *LEF1* inactivation is associated with a very young age at diagnosis, T-cell differentiation arrest at an early cortical stage of T-cell development, and activating *NOTCH1* mutations. Moreover, T-cell ALL cases with *LEF1* inactivation are characterized by very high expression of MYC mRNA, a surprising finding given that LEF/TCF transcription factors transactivate MYC expression when bound to β-catenin. In addition to their well-established functions as transcriptional activators, LEF/TCF transcription factors can also act as transcriptional repressors when bound to Groucho/TLE family members in the absence of β-catenin activation. Although the precise mechanisms responsible for the pathogenic effect of *LEF1* inactivation in T-cell ALL have not yet been established, one potential explanation is that *LEF1* may actively repress *MYC* in T-cell ALL cells lacking β-catenin activation, and that *LEF1* inactivation in this context may promote maximal *MYC* overexpression downstream of other oncogenic lesions that drive *MYC* overexpression, such as *NOTCH1* mutations.

BCL11B Inactivation in T-Cell Acute Lymphoblastic Leukemia

The *BCL11B* transcription factor is required for normal T-cell development. In murine T-cell progenitors, inactivation of *BCL11B* leads to developmental arrest at DN stages, acquisition of NK-like features, and aberrant self-renewal activity. By contrast, knockdown of *BCL11B* in human cells revealed a species-specific transcriptional program, but the overall effect demonstrated conservation of the negative regulatory effect of *BCL11B* on stem, myeloid, and NK genes. Monoallelic *BCL11B* deletions or point mutations have been identified in 9% to 16% of primary T-cell ALL patient samples. The point mutations identified typically occur within the DNA-binding domains of *BCL11B* and are predicted to disrupt their ability to bind DNA. Moreover, previous work in murine models has shown that *BCL11B* suppresses T-lymphoblastic malignancies induced by p53 haploinsufficiency, radiation, or the *BCR–ABL1* oncogene, and *BCL11B* inactivation is a particularly common cooperating lesion in murine T-cell ALL induced by the *TLX1* oncogene or by *ATM* deficiency. In human T-cell ALL, recurrent cryptic t(5;14)(q35;q32) translocations juxtaposing *BCL11B* and *TLX3* have been described, resulting in *BCL11B* gene-regulatory elements driving overexpression of *TLX3*. These translocations were long thought to be pathogenic because of the resultant overexpression of the *TLX3* oncogene. However, these recent findings indicate that both *BCL11B* inactivation and *TLX* oncogene overexpression are important pathogenic consequences of this translocation, thus representing two oncogenic events from a single genomic lesion. BCL11B has been implicated as a core component of the SWI/SNF chromatin-remodeling complex, a key tumor suppressor in diverse human malignancies, thus SWI/SNF complex inactivation provides one potential mechanism to explain the pathogenic consequences of BCL11B in T-cell ALL.

PHF6 Mutations in T-Cell Acute Lymphoblastic Leukemia

T-cell ALL has a male predominance, suggesting the potential involvement of tumor suppressors on the X chromosome. Targeted mutational analysis of the X chromosome revealed one such candidate tumor suppressor, PHF6, which is nucleolus, ribosomal RNA (rRNA) promoter-associated protein that directly interacts with upstream binding factor (UBF) through its PHD1 domain and suppresses rRNA. Germline mutations of *PHF6* were first described in association with Borjeson-Forssman-Lehmann syndrome (OMIM 301900), which is associated with severe mental retardation and facial dysmorphisms but until recently had not been associated with T-cell ALL. Ferrando and colleagues identified somatic nonsense and frameshift mutations leading to loss of *PHF6* expression in 16% of pediatric and in 38% of adult T-cell ALL patient samples and which were associated with overexpression of the *TLX1* or *TLX3* oncogenic transcription factors. Recently, studies of murine models have demonstrated that the loss of *PHF6* is an early event in T-cell leukemogenesis and can accelerate the onset of Notch1-induced T-cell ALL, implicating a leukemia-initiating tumor suppressor role for *PHF6*. While the pathogenic consequences of *PHF6* inactivation in T-cell ALL remain an area of ongoing investigation, recent comprehensive sequencing studies by Ferrando and colleagues have demonstrated that inactivation of *PHF6* induces a transcriptional program that increases the self-renewal capacity of HSCs. Additionally, the loss of *PHF6* has been shown to lead to increased chromatin accessibility of *MYC* target genes implicated in cell cycle control. Other work has also demonstrated a role for *PHF6* in cell proliferation with loss of *PHF6* causing arrest at the G_2/M checkpoint and increased DNA damage at the ribosomal locus. Thus, it is postulated that *PHF6* acts as a tumor suppressor with a regulatory role in rRNA synthesis and genome maintenance.

Ribosomal Protein Gene Mutations in T-Cell Acute Lymphoblastic Leukemia

Mutations in ribosomal proteins that lead to impaired ribosome biogenesis and function are well characterized in disorders of normal hematopoiesis, such as Diamond-Blackfan anemia, and in 5q− myelodysplastic syndrome. Using whole-exome sequencing, recurrent mutations in *RPL5* and *RPL10* have also been identified in pediatric T-cell ALL. Moreover, gene expression analysis of pediatric T-cell ALL samples has identified monoallelic focal deletions of *RPL22*, a ribosomal gene that is required for the development of normal T-cell progenitors. While functional studies investigating ribosomal proteins in T-cell ALL have revealed a role in transformation and cell proliferation, the exact mechanism through which this may provide an advantage to the lymphoblasts remains unknown.

Hedgehog Pathway Activation in T-Cell Acute Lymphoblastic Leukemia

Mutations within the Hedgehog pathway, most commonly within *PTCH1*, have been identified in 16% of primary T-ALL cases and have been associated with resistance to induction chemotherapy. The Hedgehog signal transduction pathway stimulates growth and proliferation in multiple cell types during embryonic development, and loss-of-function mutations of *PTCH1*, a negative regulator of Hedgehog signaling, are known to result in aberrant Hedgehog pathway activation and are well-described driver oncogenic mutations initially described in association with Gorlin syndrome (OMIM #109400), a rare inherited disorder characterized by early-onset basal cell carcinoma and increase the risk of medulloblastoma. The Hedgehog pathway may play a role in normal and malignant T-cell development. Studies in human T-cell ALL cell lines have shown that the Hedgehog pathway mutations lead to aberrant ligand-independent activation of Hedgehog signaling and that Hedgehog pathway mutation are required for survival signaling in *PTCH1*-mutant T-ALL cells. Moreover, the disruption of *ptch1* accelerated the onset and increased the penetrance of *notch1*-induced T-cell ALL in a zebrafish model. While the mechanism by which Hedgehog activation leads to T-cell leukemogenesis and chemotherapy resistance is not well understood, human and murine studies have demonstrated a potential role for Hedgehog pathway inhibitors in *PTCH1*-mutant T-cell ALL.

Fanconi-*BRCA* Mutations in T-Cell Acute Lymphoblastic Leukemia

The Fanconi pathway plays a critical role in DNA repair, and mutations in Fanconi genes have been linked to the development of both sporadic and inherited cancers. Additionally, studies have demonstrated the development of T-cell lymphomas in BRCA2-deficient mice. Recent genomic analysis of childhood T-cell ALL has identified frequent loss of function mutations within the Fanconi-*BRCA* pathway. Interestingly, these cases retained expression of the wild-type allele, suggesting that partial inactivation of the Fanconi-*BRCA* pathway may cooperate with other genetic lesions in T-cell ALL transformation. BRCA2 haploinsufficiency revealed a gene expression profile consistent with activation of the DNA damage repair pathway and being hypersensitive to UV irradiation. Together, this suggests a potential role for targeted therapy with ATR inhibitors, which are in clinical testing in a wide spectrum of tumors.

Abnormalities of Leukemia Cell Ploidy

Abnormalities of chromosome number, which generally occur in the absence of specific chromosomal translocations, have important prognostic and biologic implications in childhood ALL.

Hyperdiploidy

Found in 25% to 30% of childhood ALL cases, high hyperdiploidy, defined as the presence of 51 to 65 chromosomes in the leukemic clone, is the most frequent in childhood ALL and, in general, associated with a favorable prognosis. High hyperdiploid ALL is characterized by nonrandom gain of chromosomes X, 4, 6, 10, 14, 17, 18, and 21; trisomies of chromosomes 4, 10, and 17 impart a particularly good prognosis. Genomic analyses have revealed that chromosomal gains are commonly early events, corroborating with other evidence that high hyperdiploidy is a genetic driver event during B-cell leukemogenesis and have demonstrated frequent involvement of the RTK-RAS-ERK pathway and histone modifiers in this subtype of B-cell ALL. High hyperdiploidy occurs more frequently in younger children than in adolescents and adults and is more common in B-ALL than T-ALL. The mechanisms accounting for the favorable outcome of patients with high hyperdiploid ALL remain unknown but may reflect increased sensitivity to antimetabolite therapy and a greater propensity to undergo apoptosis. Mutations in genes of the RTK/*RAS* pathway are frequently observed in high hyperdiploid ALL, and *FLT3* mutations/overexpression have also been observed in these patients.

Near triploid (68 to 80 chromosomes) and near tetraploid ALL (>80 chromosomes) are much less common and appear to be biologically distinct from high hyperdiploidy (51 to 65 chromosomes). The prognostic significance of these subtypes is less well characterized. Near tetraploidy is frequently associated with *ETV6-RUNX1* fusions.

Hypodiploidy

In marked contrast to the favorable outcomes associated with high hyperdiploidy, the occurrence of hypodiploidy (<45 chromosomes), which is much less common, carries a poor prognosis, especially in cases with less than 40 chromosomes. A large-scale genomic study has revealed that hypodiploid ALL comprises two molecularly distinct biologic entities. Near-haploid ALL (24 to 31 chromosomes) commonly harbors activating mutations in the *RAS* pathway, with activating mutations of *RAS* genes or loss-of-function mutations of its negative regulator *NF1* being particularly common. By contrast, low hypodiploid ALL (32 to 39 chromosomes) has an exceptionally high frequency of p53 mutations, which are otherwise very rare in ALL. Strikingly, nearly half of the p53 mutations observed in low hypodiploid ALL appear to be germline. A subgroup of patients has also recently been identified with so-called "masked hypodiploidy," in which the hypodiploid clone doubles, thus appearing by karyotype to have 50 to 78 chromosomes. Careful evaluation of the DNA index fluorescence in situ hybridization (FISH), and karyotype, as well as the evaluation of co-occurring mutations can be useful in identifying cases of masked hypodiploidy, which is essential for risk stratification. Despite the differences in underlying genetic alterations, both near-haploid and low-hypodiploid ALL are associated with activation of *RAS* and *PI3K* signaling pathways, and preclinical data support the potential therapeutic utility of PI3K pathway inhibitors for these patients. Moreover, preclinical studies of hypodiploid ALL have demonstrated a dependence on *BCL-2*, suggesting that the selective BH3 mimetic, venetoclax, may have therapeutic utility in hypodiploid B-cell ALL. In vivo studies of venetoclax have demonstrated increased survival in patient-derived xenograft models of hypodiploid B-cell ALL.

Polysomy 21

Gains of additional copies of chromosome 21 are the most common somatic aneuploidy in B-cell ALL, and patients with germline trisomy 21 (Down syndrome) are at increased risk of this disease. Additionally, chromosome 21 is never lost in ALL cases with hypodiploidy, highlighting the role of additional copies of chromosome 21 in ALL pathobiology. Until recently, the molecular mechanisms linking trisomy 21 to B-cell leukemogenesis were unknown. Recent work with a murine model harboring triplication of 31 genes that are orthologous to the segment of chromosome 21q22 revealed that chromosome 21q22 triplication leads to aberrant self-renewal and differentiation arrest of B-cell progenitors. Moreover, chromosome 21q22 triplication accelerates the onset of B-ALL induced by either *BCR-ABL1* or the combination of *CRLF2* and activated *JAK2*. Polysomy 21 was found to induce loss of the repressive histone mark H3K27 trimethylation, indicating suppression of the activity of the PRC2. This effect was mediated, at least in part, by overexpression of *HMGN1*, a gene that encodes a histone remodeling protein and is located on the triplicated region of chromosome 21q22. These findings thus formally prove that polysomy 21 is oncogenic in the B-cell lineage and provide a molecular mechanism linking this chromosomal lesion to B-cell pathobiology.

In addition to whole-chromosome 21 gains, 1% to 2% of B-cell ALL cases are characterized by amplification of material from an approximately 5 Mbp region of chromosome 21 that includes *RUNX1*, the so-called intra-chromosomal amplification of chromosome 21 (iAMP21). Interestingly, iAMP21 is 2,700 fold more common in individuals with a germline Robertsonian translocation involving chromosomes 15 and 21. These translocations result in the fusion of the long arms of chromosomes 15 and 21 into a single chromosome. Both of these chromosomes are acrocentric with little genetic material on their short arms; thus, little genetic material is lost and heterozygous carriers are phenotypically normal. Whole-genome sequencing analysis of ALL cases with iAMP21 has revealed highly complex structural and copy number rearrangements within the amplified region. Interestingly, reconstruction of the evolution of this lesion strongly suggests that the initiating event for this lesion is chromothripsis, or the shattering of a chromosome in a single catastrophic event, followed by additional amplification via breakage-fusion-bridge cycles. These data strongly suggest that the abnormal structure of the Robertsonian chromosome predisposes it to focal genomic instability involving chromosome 21, possibly because it is prone to mitotic segregation errors, thus providing a potential mechanism linking germline chromosome 21 Robertsonian translocations and iAMP21. Children with i*Amp*21 ALL are often older at presentation with a mean age of 9 years and have a low white blood cell count. Several studies have demonstrated an increased risk of relapse with this chromosomal aberration; however, more recent studies have shown improved outcomes with the intensification of therapy for patients with *iAmp21*.

GERMLINE PREDISPOSITION IN ACUTE LYMPHOBLASTIC LEUKEMIA

While somatically acquired genomic alterations have provided significant insight into the pathobiology of ALL, the importance of inherited genetic variation, termed germline variants, on ALL susceptibility has more recently been uncovered. ALL most commonly develops between the ages of 2 and 5 years of age, and acquired genomic alterations have been identified at birth. Together, this suggests there may be a strong association with inheritable risk factors. Recently, both rare variants associated with a high risk of development of ALL and common variants identified in genome-wide association studies (GWAS) that are associated with a more modest risk of ALL have been identified.

Susceptibility Loci in Acute Lymphoblastic Leukemia

GWAS in childhood B-cell ALL have identified several susceptibility loci, including *ARID5B*, *IKZF1*, *GATA3*, *PIP4K2A*, *CEBPE*, *BMI1*, *CDKN2A, BAK1*, and *IGF2BP1*. The population frequency of these common variants differs significantly by ancestry and may account for racial differences in the development of ALL. Interestingly, many of the risk loci identified by GWAS undergo frequent somatic mutation in ALL, such as deletions of *IKZF1* and loss of *CDKN2A*, both described elsewhere in this chapter. There does not appear to be cosegregation, however, suggesting that inherited common risk alleles and somatically acquired mutations function independently. Furthermore, while there is speculation that these risk alleles may alter normal hematopoiesis (*ARID5B*, *IKZF1*, *GATA3*, *CEBPE*, *BMI1*, *BAK1*) and/or cell cycle regulatory control (*CDKN2A* and *IGF2BP1*) based on the function of somatic mutations within these genes, the molecular mechanisms by which these common variants increase the risk for the development of ALL remain largely unknown, highlighting the need for functional studies to further elucidate the inherited basis of B-ALL.

Recently, a large GWAS performed in childhood T-cell ALL revealed two susceptibility loci: *CDKN2A* and the novel risk locus, *USP7*. The variants identified in *CDKN2A* overlap with those identified in B-cell ALL. As was seen in B-cell ALL, somatic mutations of both *CDKN2A* and *USP7* frequently occur in T-cell ALL. Further analysis of the functional consequence of the identified *USP7* variants suggests that they impair USP7 function, similar albeit to a lesser extent than somatic mutations. Further studies are needed, however, to better understand the exact mechanism by which common variation of *USP7* influences T-cell leukemogenesis.

Rare Variation in Familial Acute Lymphoblastic Leukemia

Through kindred studies, inherited, rare variants have been identified over the past decade in association with familial predisposition to ALL.

Inherited *ETV6* Loss of Function Mutations in B-Cell Acute Lymphoblastic Leukemia

ETV6 is a transcription factor important in the regulation of normal hematopoiesis, and alterations in *ETV6*, including single nucleotide variants, copy number loss, and chromosomal rearrangements, are among the most common genetic lesions in B-cell ALL. Recently, *ETV6* has been identified in association with familial thrombocytopenia and increased risk for development of leukemia, particularly B-cell ALL, constituting a novel leukemia predisposition syndrome that accounts for approximately 1% of ALL cases. Functional analysis of identified germline variants identified two classes of mutations: damaging mutations, which were predominately restricted to the ETS domain, and WT-like mutations that did not show a preference for the ETS domain. The damaging germline variants resulted in reduced DNA binding and impaired transcriptional repressor activity. Importantly, these variants retained their ability to dimerize and function in a dominant-negative manner by sequestering wildtype ETV6 protein to the cytoplasm. In contrast, the WT-like variants retained normal protein function. Genomic and transcriptomic analysis demonstrated that the germline *ETV6*-mutated cases clustered with hyperdiploid ALL, characterized by secondary somatic mutations in the RAS pathway, or diploid ALL (clustering with ETV6-RUNX1 cases) with recurrent somatic *PAX5* alterations and copy number loss of *ETV6*. Similar to other genes that predispose to leukemia, the location of the germline variant and secondary somatic mutations appear to determine both the penetrance and molecular classification of disease in *ETV6* leukemia predisposition syndrome.

Inherited *PAX5* Mutations in B-Cell Acute Lymphoblastic Leukemia

Somatic mutations in the lymphoid transcription factor, *PAX5*, have long been a hallmark of B-cell ALL development (as described earlier). More recently, however, a recurrent germline variant of PAX5, p.Gly183Ser, has been implicated in ALL development. This missense variant, identified in three unrelated kindreds, appears to cosegregate with deletion of *CDKN2A* on chromosome 9p and loss of the wildtype *PAX5* allele, suggesting that this mutation leads to loss of normal protein function. In contrast to other ALL predisposition syndromes, all affected individuals within the identified kindreds develop B-cell ALL, suggesting an autosomal dominant inheritance pattern. Further comprehensive sequencing studies of *PAX5* in B-cell ALL should be pursued to define the role that *PAX5* may play in ALL susceptibility.

Inherited Alterations of *IKZF1* in B-Cell Acute Lymphoblastic Leukemia

IKZF1 is a master transcriptional regulator critical for lymphoid development, and somatic alterations, particularly deletions, of *IKZF1* are well described in B-cell ALL. More recently, rare germline alterations of the N-terminal DNA binding domain of *IKZF1* have been described in association with inherited immunodeficiencies and risk for development of B-cell ALL. Further genomic analysis of sporadic B-cell ALL identified germline alterations in *IKZF1* in 0.9% of B-cell ALL cases sequenced. These unique alterations included missense, nonsense, and frameshift mutations and in contrast to those identified in association with immunodeficiencies did not preferentially localize to the DNA binding domain and had minimal effect on transcriptional repressor activity. In fact, functional assays demonstrated that these mutations had variable effects on IKZF1 dimerization, localization, cell adhesion, and drug sensitivity.

Germline *TP53* Mutations in Hypodiploid B-Cell Acute Lymphoblastic Leukemia

Germline variation in *TP53*, the genetic basis for Li-Fraumeni syndrome, has been reported in association with childhood hypodiploid ALL and is thought to confer an inferior prognosis. A study of 3801 children with newly diagnosed ALL identified rare loss of function variants largely restricted to a critical DNA binding domain, consistent with what has been described with somatic *TP53* mutations in ALL. Functional in vitro assays identified pathogenic variants resulting in loss of transcriptional activation. The molecular basis for cosegregation of *TP53* germline variants and hypodiploid ALL remains unknown. There is speculation that impaired p53-mediated DNA damage repair may lead to global genomic instability, resulting in aneuploidy; however, aneuploidy is not enriched in other Li-Fraumeni–associated cancers. Alternatively, this aneuploidy may be a function of the interaction between *TP53* germline mutations and other somatic alterations in hypodiploid ALL. The overall inferior clinical outcome associated with *TP53*-mutated hypodiploid ALL is likely related to chemotherapy resistance, in part mediated by impaired DNA damage response. Additionally, patients with *TP53* germline mutations have a high rate of treatment-related secondary cancers, which further impacts clinical outcome in this group of patients.

While rare germline variants account for only a very small proportion of ALL cases, the recognition of inherited susceptibility to ALL has become increasingly important, as it can have a profound clinical impact on screening, disease surveillance, and treatment. Moreover, further studies and advancements in the field of ALL genetic predisposition will no doubt provide critical insights into the molecular basis of ALL.

CHEMOTHERAPY RESISTANCE MECHANISMS AND NOVEL THERAPEUTIC TARGETS

Apoptosis Resistance and Mitochondrial Apoptosis

Despite the clinical application of conventional cytotoxic chemotherapy for more than 50 years, the molecular determinants of chemotherapy response have received relatively little attention. Recent work has implicated a major role in resistance to cell death through the mitochondrial pathway. The mitochondrial pathway of apoptosis is regulated by the relative activity of pro- and antiapoptotic BCL2 family members, which converge at the mitochondrial outer membrane. When survival signals predominate, the BAX and BAX effector proteins are in an inactive conformation. However, induction of mitochondrial apoptosis culminates in the activation of BAX and BAK, which oligomerize to form pores in the mitochondrial outer membrane. This step triggers mitochondrial depolarization, cytochrome c release, and subsequent caspase activation and is generally considered the point of irreversible commitment to cell death. Recent work using an assay to measure the ability of a fixed proapoptotic stimulus to trigger mitochondrial depolarization has revealed that a tumor cell's sensitivity to mitochondrial apoptosis is a major determinant of chemotherapy response. Indeed, resistance to mitochondrial apoptosis can predict the clinical outcome for patients with a wide range of tumors, including ALL. Investigation of the dependence of ALL cells on specific antiapoptotic factors revealed that most cases of B-cell ALL and of ETP ALL are dependent on the antiapoptotic BCL2 protein, whereas "typical" T-cell ALL cases are most often dependent on a distinct antiapoptotic protein, BCL-XL. Small-molecule inhibitors of both of these proteins have been developed and demonstrate promising activity in preclinical models.

NT5C2 Nucleosidase Gene Mutations in Acute Lymphoblastic Leukemia

Genomic analyses of relapsed ALL clinical specimens have revealed activating point mutations in the cytosolic 5′-nucleotidase II gene (*NT5C2*), occurring in approximately 20% of relapsed T-cell ALL cases and in 3% of relapsed B-cell ALL. *NT5C2* encodes a 5′-nucleotidase enzyme that metabolizes and inactivates nucleoside analog chemotherapeutics, including 6-mercaptopurine and 6-thioguanine. Point mutations within *NT5C2* lead to increased enzyme activity in vitro, and their expression is sufficient to induce resistance to 6-mercaptopurine and 6-thioguanine in ALL cells. Patients with *NT5C2* mutations presented with early relapse (occurring within 36 months of achieving remission) and had a uniformly poor prognosis.

Pharmacologic PP2A Activation in T-Cell Acute Lymphoblastic Leukemia

A small-molecule screen in a zebrafish model of MYC-induced T-cell ALL revealed that perphenazine has activity against zebrafish and human T-cell ALL. Perphenazine is an FDA-approved antipsychotic best known as an inhibitor of dopaminergic signaling, but its anti-leukemic activity could not be explained by any of its known molecular targets. Using a mass spectrometry-based approach, PP2A was identified as a novel target of perphenazine. Perphenazine was found to bind and activate the phosphatase activity of PP2A, leading to dephosphorylation and inactivation of several oncoproteins with well-known roles in leukemogenesis, including MYC and AKT. Despite its key role as a tumor suppressor, PP2A inactivating mutations have not been described as a recurrent event in T-cell pathobiology. This is likely because each subunit of PP2A is encoded by multiple redundant genes, making it easier for a tumor cell to inactivate PP2A function by overexpression of a negative regulator, such as the *TLX1* oncogene. Although the dopaminergic neurotoxicity of perphenazine is an obstacle to immediate clinical translation of these findings, the development of more potent and specific PP2A activators could have important therapeutic applications for patients with T-cell ALL and other cancers driven by hyper-phosphorylated PP2A substrates.

FUTURE DIRECTIONS

The immediate applications of the emerging molecular information include a refinement of risk classification schemes to emphasize the roles of somatically acquired genetic abnormalities that carry a defined prognosis and likelihood of therapeutic failure. Currently, patients are assigned to treatment according to their initial clinical features and, increasingly, the genetic and biologic properties of their leukemic cells. We are now in a position to view ALL as a group of heterogeneous diseases defined by discrete molecular lesions. As these lesions have been systematically analyzed in larger numbers of patients, it has been possible to devise new classification schemes for ALL that reflect prognosis with precision. The development of new drugs based on the molecular biology of ALL, whose promise is highlighted by the early success of imatinib in BCR–ABL1–positive ALL, is clearly a priority for the future and will likely take the form of compounds developed to specifically interfere with oncoprotein function and other pro-survival mechanisms specific to each patient's leukemic blasts. Additionally, the discovery that enzymes such as kinases and histone methyltransferases play important roles in ALL pathogenesis has provided new opportunities for targeted drug development. With these advances in the understanding of the pathobiology of various ALL subsets, there is potential to improve outcomes through clinical trials coordinated on a nationwide or even worldwide scale that focus on patients with ALL whose lymphoblasts harbor specific genetic abnormalities.

SUGGESTED READINGS

Comprehensive Reviews of Acute Lymphoblastic Leukemia

Iacobucci I, Mullighan CG. Genetic basis of acute lymphoblastic leukemia. *J Clin Oncol*. 2017;35(9):975–983.

Raetz EA, Teachey DT. T-cell acute lymphoblastic leukemia. *Hematology Am Soc Hematol Educ Program*. 2016;2016(1):580–588.

Somatic Genetic Lesions in Acute Lymphoblastic Leukemia

Gu Z, Churchman ML, Roberts KG, et al. PAX5-driven subtypes of B-progenitor acute lymphoblastic leukemia. *Nat Genet*. 2019;51(2):296–307.

Gutierrez A, Sanda T, Grebliunaite R, et al. High frequency of PTEN, PI3K, and AKT abnormalities in T-cell acute lymphoblastic leukemia. *Blood*. 2009;114(3):647–650.

Holmfeldt L, Wei L, Diaz-Flores E, et al. The genomic landscape of hypodiploid acute lymphoblastic leukemia. *Nat Genet*. 2013;45(3):242–252.

Inaba T, Roberts WM, Shapiro LH, et al. Fusion of the leucine zipper gene HLF to the E2A gene in human acute B-lineage leukemia. *Science*. 1992;257(5069):531–534.

Lane AA, Chapuy B, Lin CY, et al. Triplication of a 21q22 region contributes to B cell transformation through HMGN1 overexpression and loss of histone H3 Lys27 trimethylation. *Nat Genet*. 2014;46(6):618–623.

Li Y, Buijs-Gladdines JGCAM, Canté-Barrett K, et al. IL-7 receptor mutations and steroid resistance in pediatric T cell acute lymphoblastic leukemia: a genome sequencing study. *PLoS Med*. 2016;13(12):e1002200.

Liu Y, Easton J, Shao Y, et al. The genomic landscape of pediatric and young adult T-lineage acute lymphoblastic leukemia. *Nat Genet*. 2017;49(8):1211–1218.

Mansour MR, Abraham BJ, Anders L, et al. Oncogene regulation. An oncogenic super-enhancer formed through somatic mutation of a noncoding intergenic element. *Science*. 2014;346(6215):1373–1377.

Mullighan CG, Goorha S, Radtke I, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature*. 2007;446(7137):758–764.

Paulsson K, Lilljebjorn H, Biloglav A, et al. The genomic landscape of high hyperdiploid childhood acute lymphoblastic leukemia. *Nat Genet*. 2015;47(6):672–676.

Seki M, Kimura S, Isobe T, et al. Recurrent SPI1 (PU.1) fusions in high-risk pediatric T cell acute lymphoblastic leukemia. *Nat Genet*. 2017;49(8):1274–1281.

Ueno H, Yoshida K, Shiozawa Y, et al. Landscape of driver mutations and their clinical impacts in pediatric B-cell precursor acute lymphoblastic leukemia. *Blood Adv*. 2020;4(20):5165–5173.

Zhang J, McCastlain K, Yoshihara H, et al. Deregulation of DUX4 and ERG in acute lymphoblastic leukemia. *Nat Genet*. 2016;48(12):1481–1489.

Germline Predisposition to Acute Lymphoblastic Leukemia

Brown AL, de Smith AJ, Gant VU, et al. Inherited genetic susceptibility to acute lymphoblastic leukemia in Down syndrome. *Blood*. 2019;134(15):1227–1237.

Churchman ML, Qian M, Te Kronnie G, et al. Germline genetic IKZF1 variation and predisposition to childhood acute lymphoblastic leukemia. *Cancer Cell*. 2018;33(5):937–948. e8.

Perez-Andreu V, Roberts KG, Harvey RC, et al. Inherited GATA3 variants are associated with Ph-like childhood acute lymphoblastic leukemia and risk of relapse. *Nat Genet*. 2013;45(12):1494–1498.

Qian M, Cao X, Devidas M, et al. TP53 germline variations influence the predisposition and prognosis of B-cell acute lymphoblastic leukemia in children. *J Clin Oncol*. 2018;36(6):591–599.

Clinical Implications and Novel Therapeutic Strategies in Acute Lymphoblastic Leukemia

Bernt KM, Zhu N, Sinha AU, et al. MLL-rearranged leukemia is dependent on aberrant H3K79 methylation by DOT1L. *Cancer Cell*. 2011;1:66–78.

Borkin D, He S, Miao H, et al. Pharmacologic inhibition of the Menin-MLL interaction blocks progression of MLL leukemia in vivo. *Cancer Cell*. 2015;27(4):589–602.

Burns MA, Place AE, Stevenson KE, et al. Identification of prognostic factors in childhood T-cell acute lymphoblastic leukemia: results from DFCI ALL consortium protocols 05-001 and 11-001. *Pediatr Blood Cancer*. 2021;68(1):e28719.

Chonghaile TN, Roderick JE, Glenfield C, et al. Maturation stage of T-cell acute lymphoblastic leukemia determines BCL-2 versus BCL-XL dependence and sensitivity to ABT-199. *Cancer Discov*. 2014;4(9):1074–1087.

Coustan-Smith E, Mullighan CG, Onciu M, et al. Early T-cell precursor leukaemia: A subtype of very high-risk acute lymphoblastic leukaemia. *Lancet Oncol*. 2009;10(2):147–156.

Fischer U, Forster M, Rinaldi A, et al. Genomics and drug profiling of fatal TCF3-HLF-positive acute lymphoblastic leukemia identifies recurrent mutation patterns and therapeutic options. *Nat Genet*. 2015;47(9):1020–1029.

Hinze L, Pfirrmann M, Karim S, et al. Synthetic lethality of Wnt pathway activation and asparaginase in drug-resistant acute leukemias. *Cancer Cell*. 2019;35(4):664–676. e7.

Krivtsov AV, Evans K, Gadrey JY, et al. A menin-MLL inhibitor induces specific chromatin changes and eradicates disease in models of MLL-rearranged leukemia. *Cancer Cell*. 2019;36(6):660–673. e11.

Schultz KR, Bowman WP, Aledo A, et al. Improved early event-free survival with imatinib in Philadelphia chromosome-positive acute lymphoblastic leukemia: a children's oncology group study. *J Clin Oncol*. 2009;27(31):5175–5181.

Stanulla M, Dagdan E, Zaliova M, et al. IKZF1^{plus} defines a new minimal residual disease-dependent very-poor prognostic profile in pediatric B-cell precursor acute lymphoblastic leukemia. *J Clin Oncol*. 2018;36(12):1240–1249.

Tzoneva G, Perez-Garcia A, Carpenter Z, et al. Activating mutations in the NT5C2 nucleotidase gene drive chemotherapy resistance in relapsed ALL. *Nat Med*. 2013;19(3):368–371.

CHAPTER 67

CLINICAL MANIFESTATIONS AND TREATMENT OF CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

Rayne H. Rouce and Rachel E. Rau

Serial risk-directed clinical trials have optimized the combination of chemotherapeutic agents and, along with advances in supportive care, have led to current cure rates of childhood acute lymphoblastic leukemia (ALL) exceeding 85% compared with those of less than 10% in the 1960s.[1] However, because ALL is the most common cancer in children, relapsed ALL remains a leading cause of death from disease in this age group. Over the last two decades, minimal residual disease (MRD) has become the most important determinant in risk stratification. Prophylactic cranial irradiation has been successfully omitted from virtually all patients in several frontline protocols, and the Abelson (ABL) tyrosine kinase inhibitors (TKIs) imatinib and dasatinib have revolutionized the treatment of patients with Philadelphia chromosome (Ph)-positive ALL. Significant advances in biotechnology, such as next-generation sequencing (NGS), have led to a deeper understanding of the molecular pathways involved in ALL, which in turn helped identify new ALL subtypes and their driver mutations.[1] Precision medicine ALL strategies based on inherited and leukemia-specific genomic features, host pharmacogenomics, and targeted molecular and immunologic treatment approaches are increasingly implemented to improve cure rates and reduce toxicity. The remarkable success of bispecific T-cell engagers and chimeric antigen receptor modified T cells (CAR-T cells) targeting CD19, once considered only as treatment of relapsed or refractory B-ALL, has led to their testing in upfront clinical trials, while changing the way practitioners assess allogeneic hematopoietic stem cell transplant (allo-HSCT).

EPIDEMIOLOGY

ALL accounts for approximately 75% of all cases of childhood leukemia, and is the most common pediatric cancer, representing 23% of cancer diagnoses among children younger than 15 years of age. About 3600 children and adolescents are diagnosed with ALL each year in the United States, with an annual rate of 30 to 40 per million. The peak incidence of ALL occurs around 3 to 5 years of age. This young age peak historically has been associated with major periods of industrialization in many countries, suggesting a causative role for environmental changes. ALL, especially T-cell subtype, occurs more frequently in boys than in girls.[1]

Certain inherited genetic and acquired factors are associated with the development of ALL, but most patients have no recognized risk factors. Children with constitutional trisomy 21 (Down syndrome) are up to 15 times more likely to develop leukemia than children without Down syndrome. Genetic instability and DNA repair disorders (e.g., Bloom syndrome, ataxia-telangiectasia, Fanconi anemia) are also associated with an increased risk of developing ALL.[2] Among identical twins, if one is diagnosed with ALL during the first year of life, the risk that the other twin will develop ALL is over 70%, approaching 100% in twins with a single monochorionic placenta. The extraordinarily high concordance rate in monozygotic infant twins compared with dizygotic infant twins or non-twinned siblings results from the metastasis of leukemic cells from one twin to the other through shared placental circulation. Highest in infancy, this risk diminishes with increasing age. If the first twin develops ALL by 5 to 7 years of age, the risk to the second twin is approximately twice that in the general population, regardless of zygosity.[3] After the age of 7 years, the risk to the unaffected twin is similar to that for persons in the general population. The majority of infants with ALL have a chromosome translocation that results in the fusion of the *KMT2A* (formerly *MLL*) gene at 11q23 with a variety of partner genes, the most common of which in B-ALL is *AF4*. Identical twin infant pairs with concordant ALL share the same acquired *KMT2A* gene rearrangements.

Several genetic, dietary, and environmental factors have been proposed to modify the risk of leukemia initiation. Children with various congenital immunodeficiency diseases, including Wiskott-Aldrich syndrome, congenital hypogammaglobulinemia, and ataxia-telangiectasia, have an increased risk of developing lymphoid malignancies, as do patients undergoing chronic treatment with immunosuppressive drugs. The loss of cellular immune surveillance capability for tumor antigens and the inability to self-regulate lymphoproliferative processes may contribute to malignant transformation in these patients. Absence of exposure to common infections in the first year of life is associated with a higher risk of developing *ETV6-RUNX1*–positive or hyperdiploid ALL (>50 chromosomes) in older children. A possible explanation is that in the absence of infection-driven modulation of the naive immune network in infants, subsequent infectious exposures could result in a highly dysregulated response to infections in older children contributing to leukemogenesis. Exposure to ionizing radiation and certain toxic chemicals could also facilitate the development of acute leukemia. Other evidence linking most environmental exposures to risk of childhood ALL has largely been inconsistent. Causation pathways are likely to be multifactorial and it is probable that the risk of ALL from environmental exposure is influenced by germline genetic variations through the co-inheritance of multiple low-risk variants.

PATHOBIOLOGY

Acute leukemias comprise a group of clonal disorders of maturation at an early phase of hematopoietic differentiation. ALL subtypes are a heterogeneous group of malignancies with distinctive immunophenotype and molecular pathogenesis that result in varying clinical characteristics and response to therapy. Accurate pathobiologic diagnosis is not only important for prognostic stratification, but can also help define patient-specific therapeutic approaches.[1]

Lymphoblastic leukemias can arise from either B- or T-cell mutant hematopoietic progenitor cells capable of indefinite self-renewal. T-cell ALL can be classified into several distinct genetic subgroups that correspond to specific T-cell development stages and is frequently associated with translocations of T-cell receptor genes on chromosome 14q11 or 7q34 with other gene partners. Early T-cell precursor (ETP) ALL comprises 12% to 15% of T-ALL and is characterized by immature genetic and immunophenotypic features (CD1a-negative, CD8-negative, and CD5-weak and the co-expression of stem-cell or myeloid markers), and gene expression reminiscent of double-negative 1 thymocyte that retains the ability to differentiate into both T-cell and myeloid, but not B-cell lineages.[4] The discovery of its mutational spectrum recapitulated that of acute myeloid leukemia (AML) by whole genome sequencing (see discussion in Genomics of ALL) and global transcription profile similar to that of normal hematopoietic stem cells, and granulocyte macrophage precursors suggest that this subtype of leukemia is a stem-cell leukemia.[4] Most B-lineage leukemias are early precursor B cells, expressing CD19 and CD10 (or cALLa, the common acute leukemia antigen) but lacking surface or cytoplasmic immunoglobulin. The mature B-cell ALL, or Burkitt cell leukemia, is stratified separately and not included in this chapter.

GENOMICS OF ACUTE LYMPHOBLASTIC LEUKEMIA

Most cases of ALL harbor driver genetic aberrations. In B-ALL in particular, a number of recurrent structural chromosomal anomalies have been identified that are of pathogenic and prognostic importance. Recent advances in global genome analysis have enabled the identification of recurring alterations in genes and pathways with key roles in cell growth and tumorigenesis. The identification of such lesions may help further refine risk stratification and could represent targetable genetic vulnerabilities.

B-Acute Lymphoblastic Leukemia Genomics

Chromosomal aneuploidy is common in B-ALL. Hyperdiploidy (modal chromosome number 51 to 65 or DNA index of >1.16) is present in 20% to 25% of pediatric patients with B-ALL, most commonly in younger children with decreasing frequency with age. Hyperdiploidy is associated with a more favorable outcome and is incorporated into the risk stratification algorithm of several childhood cancer consortia worldwide. Studies from the Pediatric Oncology Group (POG) and subsequently the Children's Oncology Group (COG) have found that patients with trisomies of chromosomes 4 and 10 (double trisomies, DT) in particular (with or without meeting the definition of hyperdiploidy) have excellent outcomes with standard therapy, thus the COG incorporates DT as a favorable cytogenetic lesion in its risk stratification.[5]

Hypodiploidy (modal chromosome number <44 or DNA index of <0.81) is a relatively rare (1% to 2%) cytogenetic lesion associated with a particularly poor outcome in B-ALL. While data based on small numbers suggests patients with hypodiploidy who clear bone marrow (BM) MRD by the end of induction therapy have reasonably good outcomes with chemotherapy alone, analysis of a larger COG cohort revealed the outcomes were overall dismal and not improved by HSCT in first complete response (CR).[6] Thus, there is a dire need for novel therapeutic options for patients with hypodiploidy B-ALL.

Chromosomal translocations are also prevalent in B-ALL, a number of which have prognostic importance. Fusion of the *ETV6* and *RUNX1* genes by translocation t(12;21)(p12;q22) occurs in 20% to 25% of childhood B-ALL, and is associated with favorable outcome. Conversely, rearrangements of *KMT2A* on chromosome 11q23 are associated with poor prognosis. *KMT2A* rearrangements drive approximately 80% of infant B-ALL, and *KMT2A*r infant ALL is a particularly poor prognosis disease. *KMT2A* rearrangements are also found in 3% to 5% of older children with B-ALL, and while associated with increased MRD and inferior outcomes, the risk of relapse can largely be mitigated with intensification of chemotherapy.

Another recurrent structural chromosomal lesion conferring an increased relapse risk is intrachromosomal amplification of chromosome 21 (iAMP21) which is present in 1% to 3% of pediatric B-ALL cases. Similar to *KMT2A* rearrangements, outcomes are greatly improved with appropriate intensification of chemotherapy.[7] The *TCF3-HLF* fusion gene results from another, rare (<1%) translocation in B-ALL, t(17;19). B-ALL patients with *TCF3-HLF* fusions characteristically present with coagulopathy and hypercalcemia and have a dismal prognosis, thus warranting novel therapies and consideration of HSCT in first CR.

The t(9;22) Philadelphia chromosome (Ph+) is another recurrent and well-characterized genomic driver of B-ALL. This translocation results in the *BCR-ABL1* fusion gene. A number of potent and specific TKIs targeting *BCR-ABL1* such as imatinib and dasatinib have been developed, and their continuous administration in combination with standard ALL chemotherapy has dramatically improved the outcome of Ph+ B-ALL.[8]

In more recent years, gene expression studies of large B-ALL cohorts identified a subset of Ph-negative patients characterized by a gene expression profile highly similar to Ph+ B-ALL, subsequently designated Ph-like B-ALL (or *BCR-ABL1*-like) B-ALL. Whereas Ph+ B-ALL steadily increases with age, the incidence of Ph-like disease peaks in young adulthood. In B-ALL ~10% of children under 10 years, ~20% of adolescents, and greater than 30% of young adults have Ph-like disease with rates then decreasing with increasing age. Ph-like B-ALL is associated with poor prognosis, particularly in the AYA (adolescent and young adult) population. B-ALL patients of Hispanic ethnicity and Native American genetic ancestry have significantly higher rates of Ph-like disease and an association has been identified between the risk of Ph-like B-ALL and specific germline polymorphisms of *GATA3*.[9] While Ph-like B-ALL lacks the BCR-ABL1 kinase fusions, a number of lesions driving Ph-like B ALL have been identified via seminal genomic studies. The identified causative lesions are characterized by the activation of a number of different kinase signaling pathways.[10] Many of the activated kinase pathways can be selectively inhibited by targeted small molecule TKIs, thus providing a possible approach to improve the historically inferior outcomes of affected patients.

The most common subsets of Ph-like ALL are those with aberrantly increased expression of the gene, *cytokine receptor-like factor 2* (*CRLF2*) secondary to fusion with either *IGH* or *P2RY8*. Thymic stromal lymphopoietin receptor (TSLPR) is the cell surface receptor protein encoded by *CRLF2*. As a cell surface protein, TSLPR is readily detected by flow cytometry. Thus, cell surface expression of TSLPR on diagnostic flow cytometry is suggestive of Ph-like disease with a driving *CRLF2* fusion, though confirmatory testing by fluorescence in situ hybridization (FISH) is required to confirm the diagnosis. The signaling pathway responsible for the Ph-like gene expression signature in *CRLF2*-rearranged B-ALL is the pro-proliferative, pro-survival, anti-apoptotic Janus kinase/signal transducers and activators of transcription (JAK)/STAT) pathway. TSLPR forms a heterodimeric cell surface receptor with IL7 receptor α (IL7Rα) which activates the JAK/STAT signaling pathway when bound by ligand. Overexpression of *CRLF2* in Ph-like B-ALL results in constitutive JAK/STAT signaling hyperactivation. *CRLF2* rearranged B-ALL is also characterized by frequent activating *JAK1/2* mutations, highlighting the critical role of JAK signaling in disease pathogenesis. *CRLF2* alterations are enriched in B-ALL patients of Hispanic ethnicity and occur in approximately 50% of the B-ALL cases in children with Down syndrome.

Another, less frequent subset of Ph-like B-ALL with aberrant JAK/STAT activation are those with rearrangements of the erythropoietin receptor gene (*EPOR*) that produce a truncated protein lacking the negative regulatory domain. Other, rare, JAK/STAT activating lesions are recurrently identified in Ph-like B-ALL, including *IL7R* insertion or deletions, fusions of *JAK2*, and SH2B3 mutations/deletions.[10] As JAK/STAT activation drives a large subset of Ph-like B-ALL, ongoing trials are testing if the JAK1/2 inhibitor, ruxolitinib, in combination with standard chemotherapy will be tolerable and efficacious for the treatment of JAK-activated Ph-like B-ALL (NCT02723994, NCT03117751, NCT02420717).

Another 3% to 5% of pediatric B-ALL cases have kinase fusions resulting in constitutive activation of ABL-class kinases including, *ABL1/2*, *PDGFRA/B*, *CSF1R*, and *LYN*. ABL-class Ph-like disease may be effectively targeted by ABL kinase TKIs such as imatinib and dasatinib. The combination of ABL TKIs and chemotherapy is being explored for the treatment of ABL-class Ph-like disease (NCT02883049, NCT03117751, and NCT02420717). Rarely, lesions of other targetable activating signaling kinases such as *FLT3, FGFR1*, and *NTRK3* drive Ph-like A-ALL, suggesting the possibility to improve outcome with kinase inhibition.

The era of genome sequencing has essentially defined the landscape of genetic lesions driving B-ALL.[11] Recent work has uncovered recurrent genomic lesions in pediatric B-ALL characterized by distinct gene expression programs and clinical characteristics, of potential prognostic and therapeutic relevance. These include cases with combined deregulation of *DUX4*, a homeobox transcription factor, and *ERG*, a member of the erythroblast transforming-specific (ETS) transcription factor family (~7%); rearrangement of the zinc-finger protein 384 gene (*ZNF384*) (~4% to 5%); and monocyte enhancer factor D2 (*MEF2D*) rearrangements (~3% to 4%). *MEF2D*-rearrangements are more common in older children, whereas *ZNF384* rearrangements are enriched in younger B-ALL cases and tend to have weak CD10 expression and variable expression of myeloid and stem markers on immunophenotyping and gene expression analyses. *DUX4-ERG* dysregulated B-ALL has been associated with a favorable prognosis, and small studies suggest *MEF2D*-rearranged B-ALL may be associated with inferior outcomes. In B-ALL, *MEF2D* has multiple fusion partners, but all MEF2D fusions all are characterized by a gene expression profile with overexpression of *HDAC9* which is a known MEF2D target. Thus, inhibition of HDACs may represent a viable therapeutic adjuvant for the treatment of affected individuals. Although no significant difference in outcome was found in patients with *ZNF384* rearranged B-ALL as a whole, ZNF384 has multiple potential fusion partners (*TCF3, EWS1, CREBBP*, and *EP300*) and outcomes may vary depending on the specific fusion. Patients with the *TCF3-ZNF384* fusions are relatively resistant to corticosteroids and carry an increased risk of relapse. Prospective studies of larger, uniformly treated patients harboring these novel fusions will be necessary to precisely define their prognostic impact with modern era therapy.

Recent genomic studies of large cohorts of B-ALL patients have identified a number of collaborating genetic lesions with biologic and potentially prognostic and therapeutic significance. A single nucleotide polymorphism (SNP) array demonstrated substantial differences in the frequency of copy number abnormality (CNA) among various ALL subtypes. *KMT2A*-rearranged cases had less than one CNA per case, suggesting that *KMT2A* fusions are potent oncogenes that requires very few cooperating lesions to induce leukemia transformation, especially in infants, while other subtypes such as *ETV6-RUNX1* and *BCR-ABL1* leukemias have over eight lesions per case. Such SNP array studies have identified recurrent deletion of several transcription factors critical to normal B-cell development including *PAX5*, *EBF1*, and *IKZF1*. While deletion of each of these genes likely contributes to the development of B-ALL, only *IKZF1* deletion appears to impact prognosis, with *IKZF1* deletions and mutations being associated with a poor prognosis.[12] Deletions or sequence mutation of the *IKZF1* are present in 10% to 20% of pediatric B-ALL patients and are enriched in high-risk subsets including over 80% of Ph-positive (*BCR-ABL1*–positive) B-ALL cases and is common in Ph-like ALL. *IKZF1* deletions are also common in DUX4/ERG dysregulated B-ALL, but in this context are not associated with a poor prognosis. Cooperative groups have attempted to incorporate *IKZF1* status into risk stratification. The Associazione Italiana Ematologia ed Oncologia Pediatrica-Berlin-Frankfurt-Muenster (AIEOP-BFM) ALL 2000 trial demonstrated that patients with *IKZF1* deletion with concomitant deletion of *CDKN2*A, *CKN2*B, *PAR1*, or *PAX5* without deletion of *ERG* (termed IKZF1^{plus}) had a significantly worse 5-year event-free survival (EFS) than those with *IKZF1* deletions without the IKZF1^{plus} status, and those without *IKZF1* deletions (53 ± 6% vs 79 ± 5% vs 87 ± 1%, respectively).[13]

Recent genome-wide association studies have identified germline polymorphic variants in several genes (including *ARID5B*, *CEBPE*, *GATA3*, *CDKN2*A, *BMI1-PIP4K2A*, and *IKZF1*) that are associated with an increased risk of ALL or specific ALL subtypes. Rare germline mutations in *PAX5* and *ETV6* are linked to familial ALL.[9] In addition to identifying common, low-penetrance susceptibility alleles, these data provide insights into disease causation by identifying risk variants annotating genes involved in transcriptional regulation and differentiation of B-cell progenitors.

T-Acute Lymphoblastic Leukemia Genomics

Unlike B-ALL, genomic classifiers are not widely used to risk stratify patients with T-ALL. However, some consortia are exploring possible integration of clinical features, MRD, and recurrent genomic lesions for refinement of risk-adapted therapy for T-ALL.[14]

T-ALL is characterized by a number of structural chromosomal lesions and genetic mutations. Translocation involving transcription factors and epigenetic regulators (e.g., *TAL1*, *TLX1*, *TLX*3, *KMT2A*, *MLLT10*) are common. Deletions of *CDKN2A/B* and *PTEN* and mutations of signaling pathway and epigenetic regulators are also recurrent in T-ALL.[11]

Additionally, over 70% of T-ALL patients have activating mutations of *NOTCH1*, which encodes a transmembrane receptor and critical regulator of normal T-cell development and another 20% have loss-of-function mutations of *FBXW7*, a ubiquitin ligase that negatively regulates NOTCH1. Given the frequency of NOTCH1 activating lesions in T-ALL, inhibition of NOTCH1 has been explored. Most notably, gamma secretase inhibitors (GSIs) which block the cleavage and release of the active intracellular component of NOTCH1 have been clinically tested, but demonstrated limited efficacy and excess rates of intolerable gastrointestinal toxicity.[15]

Early T-Cell Precursor Acute Lymphoblastic Leukemia Genomics

Early T-cell precursor or early thymic precursor (ETP) ALL is a distinct subtype of T-ALL characterized by a stem-progenitor cell gene expression signature and an immature immunophenotype. While the first descriptions of ETP ALL found and associated with poor outcome, more recent data indicates that with appropriate risk-adapted therapy based on MRD outcomes of patients with ETP ALL are the same as those with non-ETP T-ALL.[16]

Importantly, genomic profiling of ETP-ALL samples has identified several potentially targetable lesions. Most prominently, ETP-ALL is characterized by mutations of activated signaling including activating Ras-pathway and *FLT3* mutations as well as epigenetic regulator mutations.[4]

CLINICAL MANIFESTATIONS

Children with ALL present with nonspecific symptoms and signs reflecting the degree of disruption in BM function and the extent of extramedullary infiltration. The most common presenting symptoms are fever, fatigue, pallor, petechiae, bruising, bleeding from mucosal surfaces, and pain. Patients, especially young children, may present with bone pain, arthralgia, or refusal to walk due to leukemic infiltration of the bone or joint, or to expansion of the marrow cavity by leukemic cells (Table 67.1). The evolution of symptoms may proceed over a few days, weeks, or months. Less common presenting symptoms include headache, visual complaints, vomiting, respiratory distress, oliguria, and anuria. Occasionally, patients present with life-threatening infection or bleeding.

On physical examination, fever, pallor, petechiae, and ecchymoses may be present. The lymphoproliferative nature of the disease may be manifested as lymphadenopathy, splenomegaly, or hepatomegaly. Overt central nervous system (CNS) involvement is uncommon at presentation, but leukemic cells can be detected by screening lumbar puncture (Fig. 67.1A,B) in as many as 20% of children with ALL, especially those with high-risk disease who are asymptomatic at the time of the puncture. Papilledema, retinal hemorrhages, and cranial nerve palsies may be present. CNS involvement usually is restricted to leptomeninges, and parenchymal mass lesions are uncommon. Epidural spinal cord compression is a rare but serious presenting finding and requires immediate chemotherapy including high-dose glucocorticoid therapy. Laminectomy or radiotherapy is generally not necessary because leukemias are very sensitive to chemotherapy at diagnosis. Overt testicular involvement occurs in only 2% of boys and usually presents as painless, asymmetric enlargement that can be distinguished from hydrocele by ultrasonography (Fig. 67.1C,D). Less common presenting features include ocular involvement, subcutaneous nodules (leukemia cutis) (Fig. 67.1E,F), and enlarged salivary glands (Mikulicz syndrome). Approximately 55% of T-cell cases present with an anterior mediastinal mass. A bulky mediastinal mass can compress the great vessels and trachea, resulting in superior vena cava syndrome and respiratory distress. Patients with large mediastinal mass generally present with cough, dyspnea, orthopnea, dysphagia, stridor, cyanosis, facial edema, increased intracranial pressure, and sometimes syncope. When significant tracheal compression is present, general anesthesia should be avoided and procedures should be performed under local anesthesia. Immediate diagnosis and initiation of steroids and chemotherapy is essential to prevent respiratory failure. Lumbar puncture and intrathecal therapy can be delayed for a few days, allowing the relief of airway compression and decrease in circulating blasts, without compromising ultimate clinical outcome.

Clinical laboratory data often reveal a broad spectrum of abnormal findings. Various degrees of anemia and thrombocytopenia are usually present at diagnosis. The presenting leukocyte counts range widely from 0.1 to 1500 × 10^9/L. Leukemic blasts may not be appreciated morphologically in the peripheral blood smear in 10% of the patients. Approximately 45% of children have leukocyte counts less than 10 × 10^9/L, and 15% present with hyperleukocytosis (>100 × 10^9/L). Patients with hyperleukocytosis are at increased risk of CNS disease, tumor lysis syndrome, and leukostasis. Leukostasis may manifest as dyspnea, chest pain, alteration in mental status, cranial nerve palsies, or priapism. The majority of childhood ALL cases are B cell in derivation with approximately 12% to 15% of children with ALL having a T-cell immunophenotype. T-cell ALL usually occurs in patients older than 9 years of age with elevated leukocyte count, and is associated with CNS involvement. Coagulopathy, usually mild, can occur in T-cell ALL and is only rarely associated with severe bleeding. Elevated serum uric acid and lactate dehydrogenase levels are common in patients with a large leukemic cell burden. Patients with massive

TABLE 67.1 **Clinical Presentation of Acute Lymphoblastic Leukemia**

Symptoms/ Signs	Etiology	Management
Fever	Disease or infection	Always conduct fever work up and provide broad antimicrobial coverage until infectious etiology is ruled out
Fatigue, pallor	Anemia (ALL infiltrating bone marrow)	Packed red blood cell transfusion (slow if anemia is severe, avoid in hyperleukocytosis)
Petechiae, bruising, bleeding	Thrombocytopenia (ALL infiltrating bone marrow)	Transfuse with platelets
Pain	Leukemia infiltrating bones/joints, or expanding marrow cavity	Establish diagnosis and start chemotherapy
Respiratory distress/ superior vena cava syndrome	Mediastinal mass	Avoid sedation in presence of tracheal compression. Establish diagnosis as soon as possible and start chemotherapy

ALL, Acute lymphoblastic leukemia.

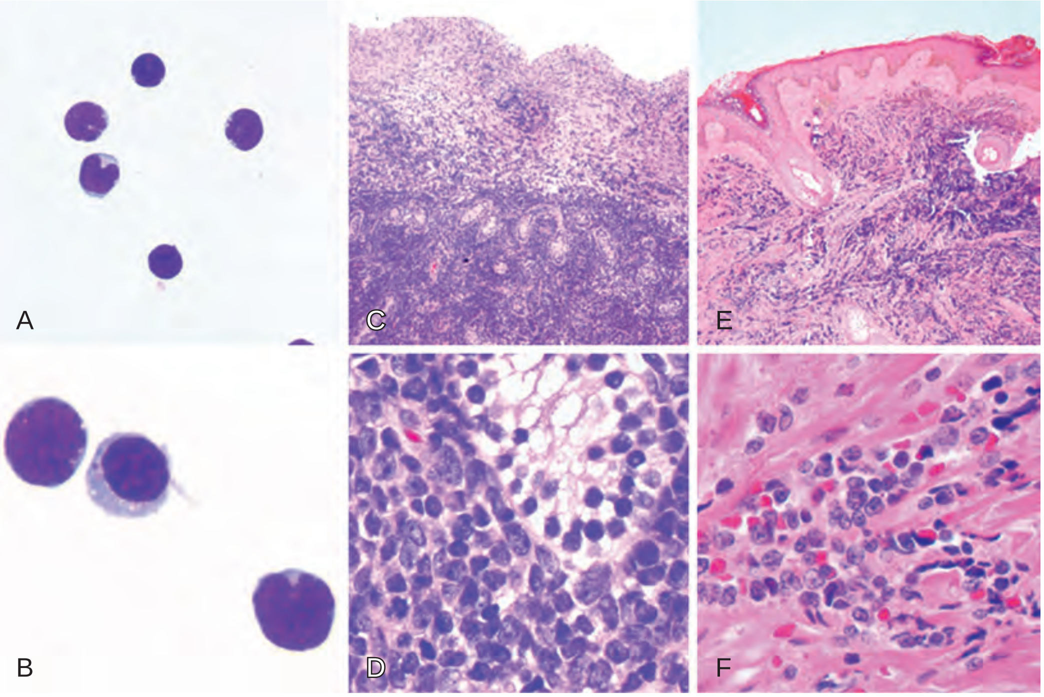


Figure 67.1 CENTRAL NERVOUS SYSTEM (CNS), TESTICULAR, AND SUBCUTANEOUS INVOLVEMENT IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA (ALL). CNS disease identified in the cerebrospinal fluid (CSF) by screening lumbar puncture at the time of diagnosis in a 12-year-old boy with high-risk precursor B-cell ALL. The total count of the CSF specimen was 6131/μL with 6076 white blood cells/μL and 98% blasts. (A, B) The cytospin preparation shows mostly blasts, slightly altered morphologically by the preparation. In (B), there is a small lymphocyte *(middle)* for comparison with the blasts. (C, D) Testicular disease noted at relapse in a 13-year-old boy with precursor B-cell ALL. Note the infiltrate of blasts in the parenchyma of the testes, surrounding the seminiferous tubules. Immunostaining (not shown) demonstrated that the blasts were $CD19^+$, $CD10^+$, and TdT^+. (E, F) Cutaneous disease at diagnosis. The patient was an 8-year-old boy with a scalp lesion for 2 months that was initially treated with antibiotics. (F) On biopsy there was much crush artifact, but deep in the specimen there was an infiltrate of blasts separating fibers shown to be B-cell lineage. Interestingly, the patient had a normal complete blood count, but bone marrow was packed with blasts that had a precursor B-cell phenotype and a hyperdiploid karyotype.

renal involvement can have increased levels of creatinine, urea nitrogen, uric acid, and phosphorus; intrathecal methotrexate should be delayed for a few days to avoid undue delay in methotrexate clearance resulting in systemic toxicity in these patients. Approximately 0.5% of patients have hypercalcemia at diagnosis, attributable to the release of parathyroid hormone–like protein from lymphoblasts and leukemic infiltration of bone; these patients tend to be in the older age group and present with low blast cell count, and this complication generally resolves rapidly with hydration and chemotherapy. As described above, the chromosomal rearrangement t(17;19), observed in less than 1% of precursor B-ALL, has been associated with hypercalcemia and coagulopathy. Liver dysfunction due to leukemic infiltration occurs in 10% to 20% of patients, is usually mild, and has no prognostic consequences. Because vincristine and daunorubicin are metabolized primarily through biliary excretion, modifications of the dosage of these agents are recommended if direct bilirubin level is elevated.

Abnormalities of the bone, such as metaphyseal banding, periosteal reactions, osteolysis, osteosclerosis, or osteopenia, can be demonstrated by radiographic studies in one-half of the patients, especially those with low presenting leukocyte counts. Routine bone surveillance is not necessary because these findings have no clinical or prognostic implication. Vertebral compression fracture that can be found in 2% of the cases in routine chest X-ray to detect mediastinal mass, is usually associated with low leukocyte count and hyperdiploidy (>50 chromosomes), and is not associated with adverse prognosis.

DIFFERENTIAL DIAGNOSIS

Children with ALL present with a variety of nonspecific symptoms that may mimic other conditions. Pancytopenia and fever are also presenting symptoms for aplastic anemia. Failure of a single cell line, as in transient erythroblastic anemia, idiopathic thrombocytopenic purpura, and congenital or acquired neutropenia, sometimes produces a clinical picture that is difficult to distinguish from ALL. Routine BM aspiration is not necessary for patients with severe thrombocytopenia and no other hematologic or physical evidence of leukemia. However, BM aspiration should be performed to exclude leukemia in patients who require glucocorticoid treatment. Children with infectious mononucleosis or other acute viral illnesses may present with fever, adenopathy, splenomegaly, lymphocytosis, or pancytopenia. Fever, arthralgia, or a limp may frequently be confused with juvenile rheumatoid arthritis, which can also be associated with anemia, leukocytosis, and mild splenomegaly. Children with prominent bone pain frequently have nearly normal blood counts, a finding that can contribute to a delay in diagnosis. Immunostains and molecular studies help differentiate ALL from AML and other small blue cell malignancies that invade the BM including neuroblastoma, rhabdomyosarcoma, Ewing sarcoma, and retinoblastoma. Infants may present with subcutaneous nodules (leukemia cutis) that look clinically like Langerhans cell histiocytosis.

PROGNOSIS

Contemporary regimens have abolished the prognostic impact of many clinical and biologic features, demonstrating that the single most important prognostic factor in childhood ALL is appropriate risk-directed therapy (Table 67.2). Accurate assessment of relapse hazard is an integral part of ALL therapy, so that only high-risk patients are treated aggressively, with less toxic therapy reserved for cases at lower risk of failure.

To facilitate comparison of treatment results among different clinical trials, participants in a 1993 workshop sponsored by the United States National Cancer Institute adopted a uniform risk classification based on age and presenting leukocyte count. Two-thirds of the patients who were 1 to 9 years old with precursor B-cell ALL and a leukocyte count less than 50×10^9/L were considered to be at standard risk of relapse, while the other third were classified as high risk. This prognostic value of the United States National Cancer Institute (NCI) risk classification is limited to B-ALL, and cannot be applied to T-cell ALL. Importantly, the prognostic impact of age and, to a lesser extent, leukocyte count may largely be due to their association with specific genetic abnormalities. For example, the overall poor prognosis of infants less than 12 months of age can be explained by the very high frequency of *MLL* rearrangements (70% to 80%) in this age group, and the overall favorable outcome of patients aged 1 to 9 years is related to the preponderance of cases (70%) with hyperdiploidy (>50 chromosomes) or *ETV6-RUNX1* fusion, both favorable genetic features. Thus, the most appropriate strategy incorporates clinical features including NCI risk status and presence or absence of extramedullary disease as well as genetic characteristics, and perhaps most importantly, early response to remission induction treatment, as determined by MRD level.

B-ALL, lacking immunoglobulin synthesis, is the most common form of acute leukemia in children, and can be further divided into several subtypes based on the stage of differentiation. The high-risk features previously ascribed to pre-B-ALL (presence of cytoplasmic IgM) are closely associated with the presence of the t(1;19) translocation with *E2A* (*TCF3*)*-PBX1* fusion. Prognostic distinctions among ALL immunophenotypes, including the negative prognostic impact once associated with T-cell ALL and pre-B-ALL with *E2A-PBX1* fusion, have been abolished by recent improvements in risk-directed treatment. Aberrant expression of myeloid-associated antigens has been observed with certain genetic subtypes. CD15, CD33, and CD65 are expressed in ALL cases with a rearranged *MLL* gene, and CD13 and CD33 are expressed in cases with the *ETV6-RUNX1* fusion. Once associated with a poor outcome in some studies, myeloid-associated antigen expression has no prognostic impact in contemporary risk-directed treatment programs.

As described in detail in the genomics of ALL sections above, B-ALL can be risk stratified on the basis of recurrent genetic lesions, with hyperdiploidy (modal chromosome number >50/cell) and *ETV6-RUNX1* fusions associated with favorable response to therapy and in general excellent prognosis. Conversely, hypodiploidy (modal chromosome number <44/cell), iAMP21, *KMT2A* rearrangement, and t(17;19) are consistently associated with poor response to therapy and increased risk of treatment failure and/or relapse. For *KMT2A*r in older children and iAMP21, this relapse risk can largely be overcome by appropriate intensification of therapy, whereas means to improve outcomes for those with hypodiploidy and t(17;19) have yet to be established. Additionally, Ph+ and Ph-like B-ALL is associated with poor outcome, but in Ph+ disease this has been greatly improved by incorporation of ABL-targeting TKIs and the hope is for similar improvement in outcome for Ph-like patients with targetable kinase fusions. While not universally incorporated into risk stratification, *IKZF1* status is used by some ALL groups. In T-ALL while tumor genetics are not yet routinely incorporated into risk stratification schemas, clinical evidence suggests *KMT2A*r may lead to worse outcomes in T-ALL.

Genetic features do not entirely account for treatment outcome and their prognostic impact also depends on the treatment efficacy. Interindividual variability in the pharmacokinetics and pharmacodynamics of many antileukemic agents also contributes to the heterogeneity in treatment response among patients with specific genetic abnormalities.

The degree of reduction of the leukemic cell clone early during remission induction therapy is determined by leukemic cell genetics, microenvironment, and host pharmacogenetics and pharmacodynamics, and has shown greater prognostic strength than any other individual biologic or host-related feature.[17,18] Measurements of MRD, by flow-cytometric detection of aberrant immunophenotypes or by polymerase chain reaction (PCR) of clonal antigen-receptor gene rearrangements and more recently by next generation deep sequencing, provides a level of sensitivity and specificity that cannot be attained by traditional morphologic assessment of early treatment response. Patients who achieve an immunologic or molecular remission, traditionally defined as leukemic involvement of less than 10^{-4} nucleated BM cells on completion of remission induction, have a much more favorable prognosis than do those who do not

TABLE 67.2 Prognostic Factors in Acute Lymphoblastic Leukemia

Factor	Prognosis	Clinical Application
Age		
<1 year	*KMT2A*r (70%–80% infants) poor outcome; *KMT2A*^{WT} same outcome as older children	*KMT2A*^{WT} do well on standard ALL therapy Potential role for proteasome inhibitors, histone deacetylase inhibitors, hypomethylating agents, BCL2 targeted agents, immunotherapy for *KMT2A*r
1–9 years	Lower (standard) risk	ALL biology may change risk
>9 years	Higher risk	ALL biology may change risk
WBC		
$<50 \times 10^9$/L	Lower (standard) risk	ALL biology may change risk
$\geq 50 \times 10^9$/L	Higher risk	ALL biology may change risk
CNS		
CNS3	Higher risk of CNS and bone marrow relapse	Therapy intensification, +/– cXRT
CNS2 Traumatic lumbar puncture with blasts	Higher risk of CNS relapse	CNS directed therapy intensification
Testicular	Higher risk	Therapy intensification, radiation for persistent testicular leukemia
Immunophenotype		
T cell	Higher risk	Poor outcome abolished with current therapy; nelarabine an active agent
pre-B (cIgM+)	Standard risk	Poor outcome abolished with current therapy
Early pre-B	Standard risk	Genetics may change risk
Early T-cell precursor	Adverse prognosis	Poor outcome abolished with current therapy
Ploidy		
>50 (DI >1.16) (or trisomies of chromosomes 4 and 10)	Low risk	Good response to antimetabolites, may have delayed MRD clearance, but do well provided MRD <0.1% at end induction
<44	Higher risk	Therapy intensification, high prevalence of germline *TP53* mutations
Genetic Alterations		
t(9;22)/*BCR-ABL1*	Higher risk	ABL TKI
t(4;11)/*KMT2A-AF4*	Higher risk	Potential role for proteasome inhibitors, histone deacetylase inhibitors, hypomethylating agents, BCL2 targeted agents, immunotherapy
t(1;19)/*E2A-PBX1*	Higher risk of CNS relapse	Improved outcome with current therapy
t(12;21)/*ETV6-RUNX1*	Low risk	
IKZF1	Poor prognosis Present in 80% Ph+ and also in Ph-like ALL	Potential role for tyrosine kinase, JAK inhibitors
ABL-class fusions	High risk	Potential benefits from ABL-targeting TKI
CRLF2 rearrangements	In half of Ph-like cases, associated with Hispanic/Latino, poor outcome	Potential role for JAK inhibitors
Other JAK activating lesions *(JAK1/2* fusions and activating mutations, *EPOR* rearrangements, *IL7R* lesions, *SH2B3* lesions*)*	Poor prognosis	Potential role for JAK inhibitors
DUX4 rearranged	Favorable prognosis Commonly accompanied by ERG dysregulation Frequent IKZF1 deletions but these not associated with poor prognosis	
MEF2D fusions	Possible poor prognosis, older age	Potential role for HDAC inhibition
ZNF384 fusions	Younger age, immunophenotype with weak CD10 and expression of myeloid markers; stem cell like gene expression signature; *TCF3-ZNF384* fusions associated with poor prognosis	
NT5C3 mutations	Associated with 6MP/6TG resistance and relapse	Possible role for NT5C3 inhibitors, inosine-5'-monophosphate dehydrogenase inhibition
CREBBP	Associated with drug resistance and relapse	Potential benefit from histone deacetylase inhibitors

Continued

TABLE 67.2 Prognostic Factors in Acute Lymphoblastic Leukemia—cont'd

Factor	Prognosis	Clinical Application
MRD		
Day 15 <0.01%	Excellent outcome	No benefit from 2nd delayed intensification
Day 8 PB <1%	Associated with excellent outcome in NCI SR patients with favorable cytogenetics, end induction MRD <0.01%	Do well with relatively low-intensity therapy
Day 8 PB ≥1%	Associated with higher risk for NCI SR patients with neutral cytogenetics even if end induction BM MRD <0.01%	Benefit from intensified post-induction therapy
Slow early responders	Higher MRD=higher risk of relapse Prognostic MRD threshold may be genotype specific (0.01% for most, but 0.1% for some favorable cytogenetic subsets)	Benefit from intensified post-induction therapy
End of consolidation (or after 4 months of therapy) >0.01%	Dismal outcome	Transplantation in first CR; CD19 targeting CART being explored

ALL, Acute lymphoblastic leukemia; *BM*, bone marrow; *CART*, chimeric antigen receptor T-cell therapy; *CNS*, central nervous system; *CR*, complete remission; *DI*, DNA index; *HDAC*, histone deacetylase; *JAK*, Janus kinase; *KMT2Ar*, KMT2A-rearranged; *KMT2A*^{WT}, *KMT2A* wild-type; *MRD*, minimal residual disease; *NCI SR*, National Cancer Institute standard risk; TKI, tyrosine kinase inhibitor; WBC, white blood cell.

achieve this status. Patients who are in morphologic remission but have a postinduction MRD level of 1% or more fare as poorly as those who do not achieve clinical remission by conventional criteria (≥5% blasts). Even earlier disease assessment can further refine risk stratification. The COG incorporates day 8 peripheral blood MRD into its risk stratification schema and about half of all patients show a disease reduction in the BM to 10^{-4} or lower after only 2 weeks of remission induction, and they appear to have an exceptionally good treatment outcome.[18] The persistence of MRD (≥0.01%) beyond 4 months from diagnosis was associated with an estimated 70% cumulative risk of relapse. Patients with 0.1% MRD or more at 4 months had an especially dismal outcome. COG data suggests that NCI HR patients with persistent MRD (≥0.01%) after consolidation therapy have a dismal outcome, warranting consideration of HSCT in first CR.[19] Most contemporary clinical trials have incorporated MRD detection into the risk-classification system. Although MRD positivity is strongly associated with known presenting risk features, it has independent prognostic strength, and is increasingly used in risk stratification of ALL in contemporary regimens. (See box on Minimal Residual Disease.)

MINIMAL RESIDUAL DISEASE

The rapidity of response to induction therapy is perhaps the most powerful independent predictor of outcome. There is strong concordance between the assessment of minimal residual disease (MRD) by flow cytometry and by polymerase chain reaction (PCR) methods. Most North American institutions routinely monitor MRD using the flow cytometry method because it is simple and rapid, and can be used in the vast majority of acute lymphoblastic leukemia (ALL) cases. Most European groups on the other hand more commonly use PCR of the immunoglobulin heavy chain. When compared these methodologies show strong concordance, and both readily achieve detection of residual disease to 10^{-4}. Persistence of MRD of 10^{-4} at end of consolidation or more at 4 months from diagnosis is associated with an especially dismal outcome. The adverse prognosis of high-risk slow early responders can be improved with intensification of induction and consolidation. Low-risk cases may be spared the increased risk of early morbidity and mortality from intensive induction provided that they receive postinduction intensification therapy. Novel approaches at measuring MRD at levels down to and even below 10^{-6} using next-generation sequencing of clonotypic B- and T-cell receptors are being evaluated.

Currently, pediatric patients with ALL are typically classified into risk categories based on age, leukocyte count at diagnosis, blast cell immunophenotype and genotype, as well as early treatment response. More recently, genome-wide analyses with SNP array and especially NGS have discovered many nonrandom genetic abnormalities, several of which have already been found to have prognostic and therapeutic implications.

THERAPY, INCLUDING STEM CELL TRANSPLANTATION

In contrast to Burkitt leukemia, which is treated with short-term intensive chemotherapy, therapy for B-ALL or T-ALL is administered over 2 to 3 years. Treatment starts with a 4- to 6-week remission-induction phase aiming at eradicating the initial leukemic cell burden and restoring normal hematopoiesis. The induction phase typically includes the administration of a glucocorticoid (prednisone or dexamethasone), vincristine, and asparaginase. A three-drug induction regimen appears sufficient for most low-risk cases, provided they receive appropriate post-remission therapy risk-stratified on the basis of tumor genetics and response to induction therapy. The benefit in long-term survival of addition of an anthracycline during induction is widely accepted in higher-risk patients but less clear in lower-risk patients. With this approach, 98% to 99% of patients can attain remission, as defined by less than 5% blasts in the marrow and a return of neutrophil and platelet counts to near normal levels. Intrathecal chemotherapy is usually initiated at the start of treatment but may be delayed in selected patients, as mentioned earlier.

Following remission induction, 6 to 8 months of intensive chemotherapy (generally referred to as consolidation) is given to eradicate drug-resistant residual leukemic cells and prevent CNS leukemia development. Consolidation therapy is tailored to the leukemia subtype and risk group and varies by pediatric ALL consortia. Most patients benefit from a delayed intensification (or delayed re-induction) based on the Berlin-Frankfurt-Munster protocol I, consisting of drugs similar to those used in remission induction therapy after a 3- to 4-month period of a less intensive, interim maintenance chemotherapy. Some pediatric ALL consortia utilize a double-delayed intensification with a second re-induction at week 32 of treatment as on some chemotherapy backbones this has been found to improve outcome in patients with intermediate-risk leukemia but does not benefit patients with rapid early response. (See box on Consolidation Therapy.)

CONSOLIDATION THERAPY

The importance of a consolidation phase following remission induction is undisputed, but the treatment regimen and duration varies in the different childhood acute lymphoblastic leukemia (ALL) studies. Commonly used strategies include high-dose methotrexate plus mercaptopurine, frequent pulses of vincristine and corticosteroid plus high-dose asparaginase for 20–30 weeks, and re-induction treatment with the same agents given during initial remission induction. Re-induction treatment has become an integral component of contemporary protocols. In one randomized study, double re-induction further improved treatment outcome in patients with intermediate-risk ALL, while additional pulses of vincristine and prednisone after a single re-induction course was not beneficial, suggesting that the increased dose intensity of other drugs, such as asparaginase, was responsible for the observed improvement. An "augmented" regimen including additional doses of vincristine and asparaginase during periods of myelosuppression improved outcomes of patients with a slow early response to therapy.

After completion of induction and consolidation, patients receive a 2- to 2.5-year maintenance (or continuation) phase consisting of low-intensity metronomic chemotherapy designed to eradicate any residual leukemic cell burden. Weekly low-dose methotrexate (mostly given orally) and daily oral mercaptopurine form the backbone of most maintenance regimens. Many groups add regular pulses of vincristine and corticosteroids to this regimen, although the benefit of these pulses and their optimal duration and frequency of administration in the context of contemporary therapy have not been established. Adjusting chemotherapy doses to maintain white cell count between 2 and 3 × 10^9/L and neutrophil counts between 0.5 and 1.5 × 10^9/L has been associated with a better clinical outcome. Overzealous use of mercaptopurine, to the extent that neutropenia necessitates chemotherapy interruption, reduces overall dose intensity and is counterproductive. The optimal duration of therapy remains unknown. Attempts to shorten therapy duration from 24 months to 12 or 18 months have resulted in a significant increase in relapses. Several studies showed no advantage to prolonging treatment beyond 3 years. Some consortia have historically given boys a longer duration of maintenance therapy, though the benefit of this extended maintenance phase has not been definitively established in the context of modern-era therapy.[20] Thus, most consortia have eliminated this sex-based difference in length of therapy. A small number of patients with particularly poor prognostic features may undergo allogeneic hematopoietic cell transplantation during first remission.

Radiation therapy was the first modality successfully used to prevent CNS relapse. The effectiveness of cranial radiation as preventive therapy was offset by substantial late effects in long-term survivors, including learning disabilities, multiple endocrinopathies, and an increased risk of second malignancies. Subsequent trials demonstrated that, in the context of optimal systemic and intrathecal therapy, cranial irradiation can be omitted in the vast majority of patients but reserved for a few patients with significant CNS disease at diagnosis and those who developed subsequent CNS relapse.[21] Some consortia have eliminated CNS radiation for all upfront ALL patients regardless of presenting CNS status on the basis of a collaborative group study showing that prophylactic cranial irradiation failed to improve outcome of any high-risk subgroups and in fact was associated with poor survival in several subgroups. Patients with high-risk genetic features, T-lineage ALL, and leukemic cells in the cerebrospinal fluid (CSF) even from iatrogenic introduction from a traumatic lumbar puncture at diagnosis, require more intensive CNS-directed therapy to reduce CNS relapse.[21] Studies have successfully used early intensified intrathecal methotrexate alone or triple intrathecal therapy with methotrexate, hydrocortisone, and cytarabine. Systematically administered agents including high-dose methotrexate, dexamethasone, and asparaginase also contribute to prevention of extramedullary relapses including CNS.

Based on reports of more potent in vitro antileukemic activity and better CNS penetration, dexamethasone has replaced prednisone in many induction and continuation regimens. Prednisone remains the preferred glucocorticoid for older B-ALL patients during induction because of the relative increased toxicity associated with dexamethasone use in individuals above 10 years. Polyethylene glycol–conjugated (PEG) *Escherichia coli* asparaginase is now the widely accepted first-line asparaginase preparation. Asparaginase derived from *Erwinia chrysanthemi* has a short half-life and its use is currently limited to patients who develop antibody mediated hypersensitivity to or silent inactivation of *E. coli* formulations given limited to no cross-reactivity.[22] The dose schedule for asparaginase should take into account the variability in the pharmacokinetic profile and potency among the different preparations. Intensifying asparaginase therapy during the early phase of treatment benefits high-risk patients, particularly those with T-cell disease. Significant improvement was also reported in the outcome of patients receiving early intensification consisting of intermediate- or high-dose antimetabolite therapy.[23] The optimal dose of methotrexate depends on the leukemic cell genotype and phenotype, as well as host pharmacogenetic and pharmacokinetic parameters. Escalating low-dose methotrexate or intermediate-dose methotrexate of up to 2.5 g/m^2 is adequate for most patients with standard-risk B-ALL, but a higher dose (5 g/m^2) may benefit those with high-risk B-ALL. While previously high-dose methotrexate was favored for the treatment of T-ALL patients, a study conducted by the COG suggest excellent outcomes with escalating low-dose IV methotrexate plus asparaginase.[24] Leucovorin, a folinic acid that rescues normal cells from the toxicity of methotrexate, is necessary after treatment with high-dose methotrexate. While the intensive asparaginase and high-dose methotrexate treatment has significantly improved the outcome for patients with T-cell ALL, the emergence of specific therapy like the purine nucleoside analog nelarabine will likely increase the tendency to assign patients with T-cell ALL to a specific treatment protocol or stratum.

About 10% of the population inherit one wild-type gene encoding thiopurine methyltransferase (TPMT) and one nonfunctional variant allele, resulting in intermediate enzyme activity, while 1 in 300 people inherit two nonfunctional variant alleles and are completely deficient of this enzyme that catalyzes the *S*-methylation of mercaptopurine to its inactive metabolite. Patients with heterozygous and especially homozygous deficiency of TPMT are at high risk of severe myelosuppression. Patients with poor tolerance to antimetabolite treatment should be tested for TPMT genotype or activity for selective dose reduction of mercaptopurine. Recently, *NUDT15* polymorphism was found to account for the poor tolerance of Asian and to a lesser degree Hispanic populations to mercaptopurine. Substituting thioguanine for mercaptopurine during continuation therapy was associated with a high incidence of profound thrombocytopenia and hepatic sinusoidal obstructive syndrome (formerly veno-occlusive disease). Thioguanine use has therefore been limited to short pulses administered during consolidation therapy in some trials.

As optimizing the administration of existing therapies is reaching its limit, further improvements in outcome will require the development of therapeutic approaches directed against rational therapeutic targets. The expanded understanding of the biologic, immunologic, and genetic heterogeneity of ALL has enabled development of several novel therapeutic strategies. Various monoclonal antibodies, bispecific T-cell engager antibody (blinatumomab), and autologous CD19 chimeric antigen receptor (CAR) T cells have shown promise in clinical trials leading to commercialization for treatment of B-ALL that is refractory or in second or greater relapse. More recent frontline B-ALL trials are evaluating the incorporation of blinatumomab, CD19 CAR T cells, and inotuzumab into upfront ALL regimens in standard and high-risk patients with persistent residual disease, respectively.

Dose Schedule

The biologically equivalent doses among the different formulations of corticosteroids, thiopurines, and asparaginase are not clear. Trials comparing such agents should be cautiously interpreted taking into account the dose schedule and route of administration, and the effect of variability in the pharmacokinetic profile and potency among the agents involved. Simple modification of dose, schedule, or route of administration may result in significant differences in efficacy and toxicity. Also, when comparing regimens containing high-dose intravenous methotrexate, the dose schedule of leucovorin rescue should not be ignored as it plays a crucial role in modulating the activity and toxicity of methotrexate.

Autologous transplantation has failed to improve the outcome in ALL, and thus is not incorporated into the treatment of pediatric ALL. Comparisons between allogeneic hematopoietic cell transplantation and intensive chemotherapy for high-risk patients have yielded inconsistent results due to the small number of patients studied and differences in case selection criteria. It is generally accepted that allogeneic transplantation is a treatment modality for patients with ALL who are predicted to respond poorly to intensive chemotherapy including those with persistence of MRD after post-induction phases of therapy. While continuing to evolve, at present, many would consider HSCT in first CR for patients with refractory leukemia (failure to enter morphologic remission after 4 to 6 weeks of induction therapy), high level of MRD (>1%) after remission induction, persistent MRD after consolidation treatment, and early hematologic relapse. It is crucial to reduce residual disease to, or close to, undetectable levels as the outcome is superior if MRD is undetectable prior to transplantation, and worsens with increasing MRD levels at time of transplant. Whether CAR-T-cell therapy can replace transplantation in selected patients is being tested in prospective clinical trials, which remain in their early stages (NCT03876769).

Therapeutic Innovations Being Incorporated Into Contemporary Personalized Acute Lymphoblastic Leukemia Therapy

1. Targeting genetic alterations that drive high-risk Ph-like ALL with ABL TKIs and JAK inhibitors
2. Immunotherapeutic approaches with bispecific T-cell engager antibodies, antibody-drug conjugates, and CAR-T cells
3. Proteasome inhibitors
4. Epigenetically targeted agents
5. Inhibitors of *BCL2* family members

Treatment approaches for adolescents and young adults with ALL have evolved considerably with the widespread adoption of pediatric-based protocols that appears to have significantly improved survival and decreased the need for transplantation in this age group. The benefit of allogeneic transplantation in infants with t(4;11) ALL remains controversial, and should be evaluated in the context of emerging therapies such as epigenetically targeted agents, immunotherapy, and other novel agents. As detailed above, patients with Ph-like ALL may benefit from molecularly targeted therapy that will improve their outcome without relying on transplantation. Matched unrelated-donor, haploidentical, or cord blood transplantation has yielded outcomes comparable to those obtained with matched related-donor transplantation, and should be considered reasonable alternatives if a matched donor is not available. Many advances have been made in stem cell transplantation, such as prevention of graft-versus-host disease, expansion of the pool of suitable unrelated or related donors, donor selection and tissue typing, acceleration of engraftment, enhancement of the graft-versus-leukemia effect, and supportive care. Because improvements in transplantation tend to parallel those in chemotherapy, the indications for transplantation in newly diagnosed and relapsed patients should be re-evaluated periodically. For example, the presence of the Philadelphia chromosome is no longer a clear indication for transplantation with the incorporation of ABL TKIs in treatment regimens

ACUTE LYMPHOBLASTIC LEUKEMIA RELAPSE

Most relapses occur during treatment or within the first 2 years after its completion, although relapses have been reported as late as 10 years after initial ALL diagnosis. The most common site of relapse is the BM. Relapse in extramedullary sites, such as the CNS and testes, has decreased to less than 3% and 1%, respectively. Leukemia relapse occasionally occurs at other sites, including the eye, ovary, uterus, bone, muscle, tonsil, kidney, mediastinum, pleura, and paranasal sinus. Extramedullary relapse in children with ALL frequently presents as an isolated clinical finding. However, in studies that included the MRD assay, many extramedullary recurrences were associated with MRD in the BM. A small fraction of patients experience a recurrence of acute leukemia with an immunophenotype different from that determined at diagnosis. Some of the cases represent relapse of original leukemic clones with a shift in immunophenotype, but others are secondary malignancies caused by the mutagenic effects of leukemia treatment, especially from epipodophyllotoxin. Patients with isolated BM relapse generally fare worse than those with isolated extramedullary relapse or combined BM and extramedullary relapse. Factors indicating an especially poor prognosis are short initial remission and T-cell immunophenotype. The presence of MRD at the end of second remission induction is also a strong adverse prognostic indicator. While chemotherapy may secure a prolonged second remission in children with ALL who experience late relapse (defined as more than 6 months after cessation of therapy by some consortia and BM relapse ≥36 months from diagnosis or isolated extramedullary relapse ≥18 months from diagnosis), allogeneic transplantation is the treatment of choice for patients with early relapse of B-ALL, or late B-ALL relapse with residual MRD at end re-induction I and all T-ALL relapse. A subset of B-ALL patients treated with CD19 CAR T cells as definitive therapy following relapse have experienced sustained remissions without undergoing allogeneic HSCT.[25] Patients with late-onset isolated CNS relapse who had not received cranial irradiation as initial CNS-directed therapy have a very high remission retrieval rate, with long-term prognosis approaching that of newly diagnosed patients.

Genome-wide studies using paired diagnosis and relapse samples from the same patients are exploring the genetic basis of relapse. Although 90% of the cases exhibit differential gaining or losing genetic lesions from diagnosis to relapse, most relapse samples are clonally related to diagnosis samples. Relapse clones could be present as minor populations at diagnosis and selected during treatment to emerge in the predominant clone at relapse, displaying alterations of genes that have been implicated in treatment resistance. Gain of function mutations of the gene *NT5C3*, a 5’nucleotidase, are also common in relapsed ALL. These relapse-specific *NT5C3* mutations inactivate the active metabolite of 6-mercaptopurine, rendering the mutated leukemic blasts resistant to this important component of therapy, particularly during maintenance. These paired diagnosis/relapse studies have also revealed a key role for epigenetic dysregulation in chemotherapy resistance and relapse in ALL. Almost 20% of relapsed cases have sequence or deletion mutations of *CREBBP*, which impair histone acetylation and transcriptional regulation of CREBBP targets. Other epigenetic regulators with mutations enriched at relapse include *EP300*, *KMT2D*, *NSD2*, and *SETD2*.[11] The striking enrichment of epigenetic regulator mutations at relapse suggest they are key drivers of therapy resistance, and thus epigenetically targeted therapy may be a viable means to overcome chemoresistance in ALL. The next generation of deep sequencing technologies promises to unravel many more, if not the full repertoire of genetic alterations in leukemia.

IMMUNOTHERAPEUTIC OPTIONS FOR ACUTE LYMPHOBLASTIC LEUKEMIA

The homogenous and stable expression of CD19 on greater than 95% of B-ALL, coupled with the accessibility of leukemic blasts within marrow and peripheral blood, have contributed to the considerable success of targeted immunotherapies for relapsed or refractory B-ALL. The two immunotherapeutic modalities that have had the most success to date are the bispecific T-cell engager (BiTE) blinatumomab and CAR-T-cells, both targeting CD19.

Blinatumomab (Amgen Inc., Thousand Oaks, CA, USA) is a BiTE antibody construct with dual specificity for CD19 and CD3 which allows it to simultaneously bind patients' endogenous $CD3^+$ cytotoxic T cells and $CD19^+$ leukemic blasts, resulting in T-cell-mediated cytotoxicity of malignant B cells. The short half-life of blinatumomab requires administration as a continuous infusion, typically administered in 28-day cycles. The success of blinatumomab as a single agent in relapsed, refractory adult B-ALL patients led to early phase studies in children with refractory B-ALL and more recently incorporation of cycles into upfront standard-risk clinical trials for B-ALL (NCT NCT03914625).[26,27]

Chimeric Antigen Receptor-Modified T Cells Targeting Acute Lymphoblastic Leukemia

CAR-T cells are artificial molecules manufactured in the laboratory to combine the antigen recognition capabilities of a monoclonal antibody (derived from a single chain variable fragment [scFv]) with the signaling and killing capacity of the T cell. In addition to the antigen recognition region and T-cell zeta chain, CAR-T cells contain an endodomain (responsible for providing the T-cell activation signal) and an additional costimulatory domain responsible for enhanced *in vivo* persistence.[28] Second-generation CAR-T cells containing 4-1BB or CD28 costimulatory domains can be manufactured from the peripheral blood of patients with r/r B-ALL following a leukapheresis procedure (less commonly venipuncture). CAR molecules are introduced into patients' T cells typically using a retroviral or lentiviral vector, then expanded in the presence of cytokines in the laboratory, typically over a period of 2 to 3 weeks. While artificial CAR receptors have been studied in a variety of malignancies, the CD19 CAR has been the most successful to date, and effectively altered the manner in which practitioners approach relapsed or refractory pre-B-ALL. Lymphodepleting chemotherapy (designed to deplete inherent lymphocytes and create a homeostatic cytokine milieu) followed by a single infusion of autologous CD19 CAR-T cells has produced remarkable initial responses (upwards of 70% MRD negative CRs across studies of various CD19 CAR products) in heavily pretreated B-ALL patients with limited treatment options.[29] This groundbreaking therapy led to FDA approval of the first cellular gene therapy in 2017, a CD19 CAR-T cell by the company Novartis (Kymriah [tisagenlecleucel]), approved for the treatment of pediatric B-ALL that is refractory or in second or greater relapse.[25,28] While a subset of patients experience sustained remissions, with ~60% of patients with an initial CR at 4 weeks remaining in MRD negative remission at one-year post CAR-T-cell infusion, the majority of patients receive CAR-T cells as a bridge to allogeneic HSCT. Major focuses of current research efforts include enhancing the persistence of autologous CD19 CAR-T cells (which is directly related to ongoing clinical response), reducing antigen-escape relapse variants (by targeting multiple tumor antigens including CD22 and CD20), identifying factors predictive of response, and exploring HSCT donor-derived and "off-the-shelf" immediately available sources.

It is worth mentioning that both blinatumomab and CD19-directed CAR-T cells are unable to distinguish malignant from normal B cells, thus successful treatment typically results in eradication of normal CD19-expressing B cells, necessitating long-term replacement with intravenous immune globulin in patients treated with CD19 CAR-T cells. Expanded use of these immunotherapeutic options has also required development of algorithms to manage commonly encountered adverse events such as cytokine release syndrome and neurotoxicity (see Chapter 96).

While immunotherapeutic targeting of ALL has primarily centered around the CD19 antigen, monoclonal antibodies and antibody drug conjugates targeting CD22 (inotuzumab ozogamicin [InO], BESPONSA) approved for use in relapsed or refractory adult B-ALL[30] have shown promising results and a favorable safety profile as a single agent in Phase 1 and 2 trials in pediatric patients. Thus, the current COG high-risk B-ALL study incorporates InO into frontline chemotherapy in patients who achieve End of Consolidation MRD negative status and are thus not anticipated to require allogeneic SCT, which is especially relevant given the risk of sinusoidal obstructive syndrome of the liver particularly in patients who undergo SCT and have previously received InO (NCT NCT03959085).

Promising targeted immunotherapy agents for T-ALL are also gaining traction, with daratumumab, a CD38 monoclonal antibody approved for treatment of multiple myeloma showing promising results in relapsed or refractory T-ALL (which commonly expresses CD38) in combination with standard reinduction chemotherapy (NCT03384654).

Ongoing immunotherapy research efforts are focused on increasing persistence of ALL-targeting CAR-T cells, reducing the risk of antigen loss relapse (by targeting multiple leukemia antigens on one CAR-T cell), identification of additional targets sensitive to CAR-T-cell or antibody-mediated cytotoxicity, and identifying off-the-shelf platforms for CAR-T cells including alternative immune effectors such as natural killer cells.

SUPPORTIVE CARE

Stringent supportive care significantly contributes to a favorable ALL outcome, and should be initiated at diagnosis as remission induction is associated with an increased risk from cardiovascular, metabolic, and infectious complications. All febrile patients with or without documented infection should be given broad-spectrum intravenous antibiotics until an infectious disease can be excluded. Rapid turnover of leukemia cells before and immediately after the initiation of chemotherapy leads to metabolic disturbances including hyperkalemia, hyperuricemia, hyperphosphatemia, and secondary hypocalcemia. Patients with high levels of uric acid are at risk for the development of acute kidney injury secondary to uric acid deposition in the kidneys. All patients require intravenous hydration to prevent or treat hyperuricemia and hyperphosphatemia. Allopurinol, a xanthine oxidase inhibitor, can prevent uric acid formation. Rasburicase, a recombinant urate oxidase that breaks down uric acid to allantoin (a readily excretable metabolite with 5- to 10-fold higher solubility than uric acid), is more effective than allopurinol but is associated with methemoglobinemia or hemolytic anemia in patients with glucose-6-phosphate dehydrogenase deficiency because hydrogen peroxide is a byproduct of the uric acid breakdown. Phosphate binders should also be used to prevent or treat hyperphosphatemia. Transfusions should be administered slowly in patients with severe anemia to prevent congestive heart failure. In patients with extreme hyperleukocytosis, prompt diagnosis can be established on peripheral blood, and chemotherapy should be urgently initiated (usually steroids with gradual introduction of other agents to achieve adequate response while avoiding massive tumor lysis). Packed red blood cell transfusion should be delayed until after the leukocyte count is decreased to prevent complications of leukostasis, but platelet transfusion should be administered to prevent bleeding. All blood products should be irradiated in patients who are receiving immunosuppressive therapy to prevent graft-versus-host disease. Adolescents, obese individuals, and patients with Down syndrome are at increased risk of hyperglycemia and other complications. Patients with Down syndrome are at very high risk for infectious mortality during induction and other intense phases of therapy, thus monitoring in the hospital through count recovery is recommended by many. Prophylactic use of trimethoprim-sulfamethoxazole (or pentamidine or atovaquone in patients with poor tolerance to trimethoprim-sulfamethoxazole) successfully prevents *Pneumocystis jirovecii* pneumonia. Dental evaluation at diagnosis and meticulous oral hygiene during chemotherapy minimizes the oral complications of leukemia and its treatment. It is important to distinguish between herpes simplex viral infection and chemotherapy-induced oral mucositis. Occasionally, patients have nausea and substantial pain on swallowing because of esophageal herpes simplex viral infection, candidiasis, or both. Oral candidiasis occurs frequently, especially in young children. Azole compounds (e.g., fluconazole, itraconazole, ketoconazole) are frequently used to treat fungal infections. It should be recognized that they can inhibit cytochrome P450 enzymes and increase the toxicities of various antileukemic agents, especially vincristine. On the other hand, concomitant administration of anticonvulsants that induce cytochrome P450 enzymes (e.g., phenytoin, phenobarbital, carbamazepine) increases the systemic clearance of several antileukemic agents and may adversely affect treatment outcome. Anticonvulsants that are less likely to induce the activity of cytochrome P450 enzymes (e.g., levetiracetam [Keppra]) are recommended in patients receiving chemotherapy. Photosensitive skin rash can occur during antimetabolite therapy. The rashes are erythematous, maculopapular, similar to atopic eczema, and most prominent on the face. Topical administration of simple emollients or a weak steroid preparation, and avoidance of external exposure to sunlight, should improve the skin condition. Patients with Down syndrome tolerate methotrexate poorly, and appropriate dose adjustment is indicated in them. During each clinic visit, a thorough review of all drugs should be undertaken because of potential adverse interactions among them. In fact, chemotherapy can also interact with various food (e.g., grapefruit) and supplements (e.g., St. John's wort, folic acid).

DRUG INTERACTIONS

We have not yet reached a full understanding of the contribution of genetic polymorphisms to interindividual differences in drug effects that allows us to translate this new knowledge into clinical practice. However, by simply avoiding drug interactions, one can prevent increased toxicity or reduced efficacy of chemotherapy. Phenytoin and phenobarbital induce the activity of cytochrome P450 enzymes, significantly increasing the systemic clearance of several antileukemic agents that may adversely affect treatment outcome. We substitute these anticonvulsants with gabapentin or levetiracetam in patients receiving antileukemic therapy. On the other hand, azole compounds (e.g., fluconazole, itraconazole, ketoconazole, posaconazole) can inhibit cytochrome P450 enzymes and increase the toxicities of various antileukemic agents, especially vincristine.

LATE EFFECTS OF TREATMENT

The most problematic late effects of contemporary ALL therapy include neuropsychologic impairments, bone morbidity, and obesity.[31] While neuropsychologic deficits are well-recognized side effects of cranial irradiation, intrathecal and systemic chemotherapy (especially methotrexate) can also cause brain atrophy and spinal cord dysfunction and contribute to the development of neurocognitive toxicities. Severe CNS toxicity has been attributed to cranial irradiation at doses of 2400 cGy or higher, but lower doses have also been associated with long-term neuropsychologic impairments, especially in younger children. Obesity, most prevalent among female survivors of childhood ALL, may be related to metabolic syndrome caused by treatment with cranial radiation and corticosteroids. Osteopenia, fractures, and osteonecrosis have been observed in up to 30% of survivors of childhood ALL. Osteonecrosis, which can lead to significant pain, loss of function, and total joint replacement, has been reported in approximately 8% of children with ALL, with the highest frequency observed in adolescence and young adult females. Testicular function (in boys treated with less than 6 g/m^2 cumulative dose of cyclophosphamide) and especially ovarian function are relatively unaffected by antileukemic therapy. Offspring of patients successfully treated for childhood ALL are generally expected to be as normal as the general population. However, recent studies showed that about 5% of patients with ALL inherit major cancer predisposing genes, some of which (such as TP53) have important clinical implication to the immediate family members.[9] Second malignant neoplasms, including malignant gliomas, meningiomas, and myeloid malignancies, occur with increased frequency in patients treated with regimens that included irradiation, epipodophyllotoxins, or alkylating agents.

FUTURE DIRECTIONS

As the cure rate approaches 90%, treatment response assessed by MRD measurements of submicroscopic leukemia has emerged as a powerful and independent prognostic indicator for gauging the intensity of ALL therapy. Children at high risk of relapse may now benefit from early intensification of therapy. The next goal is to reduce the intensity of therapy in children at very low risk of relapse, hence avoiding undue toxicity. The successful elimination of preventive cranial irradiation indicates that treatment reduction is feasible if done with caution and appropriate substitution with less toxic alternatives. Global genome analysis, in addition to refining leukemia classification, is helping identify potential molecular targets for therapy. Expanding the application of pharmacogenomics, a science that aims to define the genetic determinants of drug effects, while continuing to expand immunotherapeutic strategies will allow further personalized therapy in the future.

REFERENCES

1. Hunger SP, Mullighan CG. Acute lymphoblastic leukemia in children. *N Engl J Med*. 2015;373(16):1541–1552.
2. Bienemann K, Burkhardt B, Modlich S, et al. Promising therapy results for lymphoid malignancies in children with chromosomal breakage syndromes (Ataxia teleangiectasia or Nijmegen-breakage syndrome): a retrospective survey. *Br J Haematol*. 2011;155(4):468–476.
3. Schmiegelow K, Lausten Thomsen U, Baruchel A, et al. High concordance of subtypes of childhood acute lymphoblastic leukemia within families: lessons from sibships with multiple cases of leukemia. *Leukemia*. 2012;26(4):675–681.
4. Zhang J, Ding L, Holmfeldt L, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. *Nature*. 2012;481(7380): 157–163.
5. Borowitz MJ, Pullen DJ, Shuster JJ, et al. Minimal residual disease detection in childhood precursor-B-cell acute lymphoblastic leukemia: relation to other risk factors. A Children's Oncology Group study. *Leukemia*. 2003;17(8):1566–1572.
6. McNeer JL, Devidas M, Dai Y, et al. Hematopoietic stem-cell transplantation does not improve the poor outcome of children with hypodiploid acute lymphoblastic leukemia: a report from children's oncology group. *J Clin Oncol*. 2019;37(10):780–789.
7. Loh ML, Raetz E, Devidas M, et al. Outcomes of children, adolescents, and young adults with acute lymphoblastic leukemia based on blast genotype at diagnosis: a report from the children's oncology group. *Blood*. 2016;128(22):451.
8. Biondi A, Schrappe M, De Lorenzo P, et al. Imatinib after induction for treatment of children and adolescents with Philadelphia-chromosome-positive acute lymphoblastic leukaemia (EsPhALL): a randomised, open-label, intergroup study. *Lancet Oncol*. 2012;13(9):936–945.
9. Zhang J, Nichols KE, Downing JR. Germline mutations in predisposition genes in pediatric cancer. *N Engl J Med*. 2016;374(14):1391.
10. Roberts KG, Li Y, Payne-Turner D, et al. Targetable kinase-activating lesions in Ph-like acute lymphoblastic leukemia. *N Engl J Med*. 2014;371(11):1005–1015.
11. Rau RE, Loh ML. Using genomics to define pediatric blood cancers and inform practice. *Hematol Am Soc Hematol Educ Program*. 2018;2018(1):286–300.
12. Mullighan CG, Su X, Zhang J, et al. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. *N Engl J Med*. 2009;360(5):470–480.
13. Stanulla M, Dagdan E, Zaliova M, et al. IKZF1(plus) Defines a new minimal residual disease-dependent very-poor prognostic profile in pediatric B-cell precursor acute lymphoblastic leukemia. *J Clin Oncol*. 2018;36(12):1240–1249.
14. Petit A, Trinquand A, Chevret S, et al. Oncogenetic mutations combined with MRD improve outcome prediction in pediatric T-cell acute lymphoblastic leukemia. *Blood*. 2018;131(3):289–300.
15. Sanchez-Martin M, Ferrando A. The NOTCH1-MYC highway toward T-cell acute lymphoblastic leukemia. *Blood*. 2017;129(9):1124–1133.
16. Conter V, Valsecchi MG, Buldini B, et al. Early T-cell precursor acute lymphoblastic leukaemia in children treated in AIEOP centres with AIEOP-BFM protocols: a retrospective analysis. *Lancet Haematol*. 2016;3(2):e80–e86.
17. Borowitz MJ, Devidas M, Hunger SP, et al. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia and

its relationship to other prognostic factors: a Children's Oncology Group Study. *Blood*. 2008;111(12):5477–5485.

18. Pui CH, Pei D, Coustan-Smith E, et al. Clinical utility of sequential minimal residual disease measurements in the context of risk-based therapy in childhood acute lymphoblastic leukaemia: a prospective study. *Lancet Oncol*. 2015;16(4):465–474.
19. Borowitz MJ, Wood BL, Devidas M, et al. Prognostic significance of minimal residual disease in high risk B-ALL: a report from Children's Oncology Group Study AALL0232. *Blood*. 2015;126(8):964–971.
20. Teachey DT, Hunger SP, Loh ML. Optimizing therapy in the modern age: differences in length of maintenance therapy in acute lymphoblastic leukemia. *Blood*. 2020
21. Pui CH, Campana D, Pei D, et al. Treating childhood acute lymphoblastic leukemia without cranial irradiation. *N Engl J Med*. 2009;360(26): 2730–2741.
22. van der Sluis IM, Vrooman LM, Pieters R, et al. Consensus expert recommendations for identification and management of asparaginase hypersensitivity and silent inactivation. *Haematologica*. 2016;101(3): 279–285.
23. Matloub Y, Bostrom BC, Hunger SP, et al. Escalating intravenous methotrexate improves event-free survival in children with standard-risk acute lymphoblastic leukemia: a report from the Children's Oncology Group. *Blood*. 2011;118(2):243–251.
24. Winter SS, Dunsmore KP, Devidas M, et al. Improved survival for children and young adults with T-lineage acute lymphoblastic leukemia: results from the children's oncology group AALL0434 methotrexate randomization. *J Clin Oncol*. 2018;36(29):2926–2934.
25. Maude SL, Laetsch TW, Buechner J, et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *N Engl J Med*. 2018;378(5):439–448.
26. Gore L, Locatelli F, Zugmaier G, et al. Survival after blinatumomab treatment in pediatric patients with relapsed/refractory B-cell precursor acute lymphoblastic leukemia. *Blood Cancer J*. 2018;8(9):80.
27. Topp MS, Kufer P, Gokbuget N, et al. Targeted therapy with the T-cell-engaging antibody blinatumomab of chemotherapy-refractory minimal residual disease in B-lineage acute lymphoblastic leukemia patients results in high response rate and prolonged leukemia-free survival. *J Clin Oncol*. 2011;29(18):2493–2498.
28. Rouce RH, Sharma S, Huynh M, Heslop HE. Recent advances in T-cell immunotherapy for haematological malignancies. *Br J Haematol*. 2017;176(5):688–704.
29. Maude SL, Teachey DT, Porter DL, Grupp SA. CD19-targeted chimeric antigen receptor T-cell therapy for acute lymphoblastic leukemia. *Blood*. 2015;125(26):4017–4023.
30. Kantarjian HM, DeAngelo DJ, Stelljes M, et al. Inotuzumab ozogamicin versus standard therapy for acute lymphoblastic leukemia. *N Engl J Med*. 2016;375:740–753.
31. Essig S, Li Q, Chen Y, et al. Risk of late effects of treatment in children newly diagnosed with standard-risk acute lymphoblastic leukaemia: a report from the Childhood Cancer Survivor Study cohort. *Lancet Oncol*. 2014;15(8):841–851.

CHAPTER 68

ACUTE LYMPHOBLASTIC LEUKEMIA IN ADULTS

Shira Dinner, Sandeep Gurbuxani, Alexandra E. Rojek, Nitin Jain, and Wendy Stock

Acute lymphoblastic leukemia (ALL) is a heterogeneous group of diseases characterized by clonal proliferation of lymphoid progenitors (lymphoblasts). Improved diagnostic tools permit accurate and prompt diagnosis and aid in evaluation of minimal residual disease (MRD). There have been significant advances in the past decade toward understanding disease pathogenesis, refinement of prognostic groups, and the development of exciting new therapies directed toward specific disease subsets. These molecular targeted and immunotherapeutic approaches are transforming the treatment strategies for adults with ALL and are beginning to result in significant improvements in survival.

EPIDEMIOLOGY

It is estimated that in the year 2019, approximately 5930 new cases of ALL were diagnosed and 1500 deaths occurred due to the disease in the United States. ALL is primarily a cancer of childhood; the peak incidence (7.7 in 100,000) occurs between the ages of 1 and 4 years, and approximately 60% of the patients are diagnosed before the age of 20 years.[1] The incidence of ALL begins to decline with increasing age after the first decade of life. A second upward trend starts to emerge in the sixth decade of life, and a much smaller peak is seen in patients older than 85 years of age (1.8 in 100,000). Men have a slightly higher incidence of ALL than do women (male-to-female ratio, 1.4:1). The overall age-adjusted rate of ALL has increased from 0.93 in 100,000 in the year 1975 to 1.38 in 100,000 in the year 2015.[2] Similar increases in the incidence of ALL have also been reported in Scandinavia, the United Kingdom, and Italy. Although most investigators agree that this increase in the incidence is beyond what might be expected from better reporting, the actual cause(s) of this increased incidence remains largely speculative.

ETIOLOGY

A small minority of ALL cases (<5%) are associated with predisposing inherited syndromes such as Down syndrome, Bloom syndrome, ataxia telangiectasia, and Nijmegen breakage syndrome. However, the underlying etiology is not known in most cases. Although alcohol use, exposure to pesticides or solvents, and cigarette smoking have all been implicated, only ionizing radiation has been significantly linked to an increased risk of developing ALL. The vast majority of attempts to identify causative agents for ALL have been at best correlative; most of it borders on pure speculation and conjecture. Only recently has it been appreciated that the interaction between genetic predisposition and environmental factors involved in leukemogenesis is complex and may vary for different subtypes of ALL. Epidemiologic studies designed to identify environmental agents involved in leukemogenesis will therefore have to take into account the biologic subsets defined by morphology, immunophenotype, karyotype, and the molecular abnormalities and consider the possibility that for each of these subtypes the causative agent may well be different. To adequately investigate these issues, future studies will require collaborative efforts studying large patient populations.

Despite the limitations described, it is relevant to review some of the recent advances in our understanding of the etiology of ALL. Greaves and colleagues have used DNA obtained from neonatal blood spots to demonstrate that ALL-associated chromosomal translocations were acquired in utero (and hence a preleukemic clone) because they could be demonstrated in neonatal blood spots.[1] Furthermore, these ALL-associated chromosomal translo cations were present at a 100-fold higher rate than the incidence of leukemia. These investigators hypothesized that overt leukemia evolves as a consequence of an abnormal lymphoid proliferation that occurs in response to exposure to an as yet unidentified infectious agent(s). Supporting this hypothesis are data that suggest that day care attendance associated with early exposure to common infectious agents is associated with a lower incidence of ALL. Similarly, industrialization associated with improved socioeconomic status and exposure to common infectious agents later in life have been postulated to result in abnormal and excessive lymphoid proliferation and leukemic transformation. Finally, an infectious etiology can also be evoked to explain leukemic clusters that occur when a previously unexposed community is exposed to infectious agents brought into the community by a large influx of residents, as happens during urbanization of rural communities. However, it should be reiterated that all of these studies are at best correlative and not supported by direct experimental evidence. Furthermore, the infectious or environmental agent(s) responsible for this abnormal lymphoid proliferation remains elusive.

More recently genome-wide association studies (GWASs) demonstrated that susceptibility to ALL is polygenic. Several GWASs identified single nucleotide polymorphisms (SNPs) in eight loci influencing ALL risk at 7p12.2 *(IKZF1)*, 9p21.3 *(CDKN2A)*, 10p12.2 *(PIP4K2A)*, 10q26.13 *(LHPP)*, 12q23.1 *(ELK3)*, 10p14 *(GATA3)*, 10q21.2 *(ARID5B)*, and 14q11.2 *(CEBPE)*. ALL is biologically heterogeneous, and subtype associations have been identified for 10q21.2 *(ARID5B)* associated with high-hyperdiploid B-acute lymphoblastic leukemia (B-ALL) (i.e., >50 chromosomes) and 10p14 *(GATA3)* associated with B-cell receptor (BCR) *ABL*-like B-ALL. Vijayakrishnan and colleagues also applied GWASs to identify risk loci for B-ALL at 8q24.21 and for *ETV6-RUNX1* fusion-positive B-ALL at 2q22. Although these SNPs have been identified as risk factors for B-ALL, they do not appear to correlate with survival outcomes based on these early data.[3]

Retrospective analyses have also reported cases of therapy-related ALL (t-ALL) in patients who received chemotherapy and/or radiation for a prior malignancy. Several institutions in the United States jointly reported a higher frequency of poor-risk cytogenetic features, including KMT2A rearrangements and myelodysplastic syndrome–like abnormalities (e.g., monosomal karyotype) in t-ALL. In addition, they observed a variety of mutations among t-ALL patients, with the majority of patients exhibiting mutations that were more common in myeloid malignancies (e.g., DNMT3A, RUNX1, ASXL1), whereas others had ALL-type mutations (e.g., CDKN2A, IKZF1). Median overall survival (OS) was significantly shorter in the t-ALL cohort compared with patients with de novo ALL. Patients who were eligible for allogeneic stem cell transplant had improved long-term survival.[4] Washington University reported similar findings in a retrospective analysis at their institution, and a meta-analysis showed t-ALL had a higher risk of relapse and death than de novo ALL but outcomes were better in those who underwent transplant.[5]

Thus predisposing genetic alterations and prior chemotherapy and/or radiation exposure are emerging as some of the few known causes of ALL.

CLINICAL MANIFESTATIONS

The clinical presentation of ALL encompasses a wide spectrum of symptoms that correlate with the degree of bone marrow (BM) involvement and the resultant cytopenias, as well as the leukemic cell burden. Typical symptoms include fatigue, anorexia, night sweats, pallor, shortness of breath, bone pain, fever, and bleeding diathesis. Involvement of extramedullary sites may present with lymphadenopathy, hepatomegaly, or splenomegaly. Less commonly, ALL can involve the central nervous system (CNS), leading to headache, vomiting, lethargy, and cranial nerve palsies. Other extramedullary sites of involvement include the testis, tonsils, adenoids, breast, and gastrointestinal (GI) tract. Precursor T-cell ALL often presents with a large mediastinal mass with associated respiratory distress or possible signs of superior vena cava syndrome. Burkitt leukemia/lymphoma is frequently associated with CNS involvement and bulky adenopathy.

CLINICAL AND LABORATORY EVALUATION

The initial work-up for patients with suspected ALL is detailed in Table 68.1. All patients should undergo a detailed history and physical examination. Family history should be ascertained. Laboratory evaluation should include a complete blood count (CBC) with differential; and comprehensive metabolic panel, including liver function tests, lactate dehydrogenase (LDH), and uric acid. A coagulation profile should also be obtained, although coagulation parameters are frequently normal at diagnosis. Human leukocyte antigen (HLA) typing should be performed for patients who are potential candidates for autologous stem cell transplantation (ASCT).

All patients should undergo BM aspiration and biopsy for confirmation of diagnosis and for cytogenetic and molecular genetic evaluation. It is particularly important to send an aspirate (or peripheral blood if lymphoblasts are present) to the molecular oncology diagnostic laboratory to evaluate for the presence of the *BCR-ABL* fusion transcript using reverse-transcriptase polymerase chain reaction (PCR) because these patients will receive frontline therapy that includes a tyrosine kinase inhibitor (TKI). Skin biopsy for fibroblast culture for germline testing can also be obtained at the time of diagnostic BM biopsy if there is concern for a familial leukemia predisposition syndrome. Increasingly, chromosomal microarrays, gene expression profiling, RNA sequencing (RNAseq), and whole-genome sequencing are being incorporated into the work-up of ALL patients.

TABLE 68.1 Initial Evaluation of a Patient With Acute Lymphoblastic Leukemia

- Complete history (including family history)
- Physical examination
- CBC with differential
- Comprehensive metabolic profile, including LFTs
- LDH, uric acid
- Coagulation profile
- BM aspiration and biopsy (morphology, immunohistochemistry, flow cytometry, molecular and cytogenetic analysis)
- HLA typing of the patient (if a potential aSCT candidate)
- Lumbar puncture
- Chest radiography or CT imaging of the chest

aSCT, Allogeneic stem cell transplantation; *BM*, bone marrow; *CBC*, complete blood count; *CT*, computed tomography; *HLA*, human leukocyte antigen; *LDH*, lactate dehydrogenase; *LFT*, liver function test.

A lumbar puncture should be performed at diagnosis to determine CNS involvement. In the event of increased risk of bleeding caused by severe thrombocytopenia or risk of cerebrospinal fluid contamination caused by high peripheral blood blasts, the lumbar puncture should be performed by an experienced operator. It is prudent to administer intrathecal chemotherapy at the time of the diagnostic lumbar puncture after obtaining the necessary samples. A white blood cell (WBC) count of greater than 5/μL with morphologically identifiable blasts is considered as evidence of cerebrospinal fluid involvement by leukemia[1].

APPROACH TO DIAGNOSIS

Although evaluation of morphology and immunophenotype are sufficient for making the diagnosis, current risk stratification relies on additional cytogenetic and molecular genetic information. Data from these tests are therefore an important adjunct to the initial diagnostic work-up.

Initial laboratory evaluation starts with a CBC and morphologic evaluation of a Giemsa-stained peripheral blood smear. An abnormality of at least one of the CBC parameters is detected in more than 90% of ALL patients at the time of diagnosis. Anemia and thrombocytopenia are common. The anemia is usually a normochromic, normocytic anemia accompanied by reticulocytopenia. The hemoglobin levels range from 30 to 174 g/L, and almost 50% of the patients have hemoglobin levels below 100 g/L. The median platelet count at presentation is approximately 55 to 60 × 10^9/L, and almost 60% to 70% of patients have platelet counts below 100 × 10^9/L. Although the total WBC count may be low, normal, or elevated, neutropenia is commonly present. In a Cancer and Leukemia Group B (CALGB) study, the median WBC count at presentation was 19.3 × 10^9/L. Almost one-third of the patients are likely to present with a WBC count greater than 30 × 10^9/L. Blasts account for a variable proportion of the circulating WBCs, and the percent blast population can range from 0% to 100%. A leukoerythroblastic picture can sometimes be seen. In an extreme form, immature myeloid precursors and myeloblasts constitute the vast majority of cells in the peripheral blood. This should be kept in mind when attempting to make a diagnosis exclusively from peripheral blood. Eosinophilia as a presentation of ALL is extremely uncommon and is seen in association with specific chromosomal abnormalities, including t(5;14)(q31;q32) or, even less frequently, with 8p11-associated ALL (see Chapter 57). Eosinophilia associated with t(5;14) is reactive and due to overexpression of interleukin-3 on chromosome 5 driven by the immunoglobulin H (IgH) promoter on chromosome 14. The eosinophilia can be extremely pronounced and mask the blast population in this subset of patients.

Several metabolic abnormalities are present at the time of diagnosis and frequently reflect tumor burden. For example, LDH levels are frequently elevated, and almost 50% of the patients have levels between 300 and 1000 U/L. Elevated serum levels of calcium, potassium, and phosphorous have been noted. More importantly, elevated serum uric acid levels are frequently present and reflect tumor burden. Hyperuricemia needs to be carefully monitored and aggressively corrected to avoid renal failure, especially at the time of starting induction therapy.

MORPHOLOGY

Romanowsky-based stains such as Wright-Giemsa and Giemsa provide the greatest cytoplasmic detail for evaluation of cytomorphology of the cells in the peripheral blood smear, BM aspirate smear, and touch imprints (Fig. 68.1). Most frequently, lymphoblasts are small to intermediate in size and have scant, agranular cytoplasm. B lymphoblasts are morphologically indistinguishable from T lymphoblasts, and this distinction relies on immunophenotyping. The nuclei are usually round, with uniformly dispersed “smudgy”

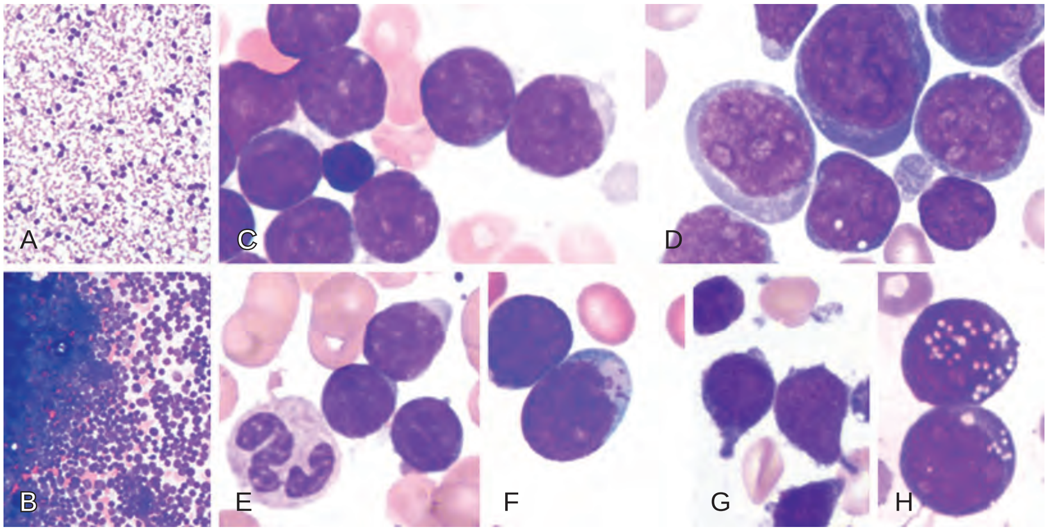


Figure 68.1 MORPHOLOGIC FEATURES OF ACUTE LYMPHOBLASTIC LEUKEMIA IN THE BLOOD AND BONE MARROW ASPIRATE. (A) In acute lymphoblastic leukemia (ALL), the peripheral blood count can be low, normal, or high, although frequently it is high and composed of mostly blasts. (B) A similar range in cellularity is true regarding the bone marrow. Typically, lymphoblasts are small to intermediate in size and have round nuclei with dispersed chromatin and indistinct nucleoli. (C) They have scant pale blue cytoplasm. A small lymphocyte (C, *middle*, *left*) is useful for comparison. (D) In many cases, the lymphoblasts are monotonous, but in some cases, the lymphoblast morphology is varied with some large cells, some cells with abundant cytoplasm, and other cells with prominent nucleoli. In the older French-American-British classification, these two patterns were referred to as ALL-L1 and ALL-L2, respectively, although they have been shown not to have any clinical significance. Other cytologic variants of lymphoblasts are shown in (E–H). These include (E) lymphocyte-like blasts, (F) blasts with azurophilic granules, (G) blasts with "hand-mirror" morphology, and (H) blasts with vacuoles. The small lymphocyte-like blasts can be difficult to distinguish from normal mature lymphocytes or chronic lymphocytic leukemia cells in the blood, making flow immunophenotyping important in the distinction. Lymphoblasts with granules can be misleading because they can be mistaken for myeloblast or monoblasts. The hand-mirror cells appear to be an artifact because they can be seen only focally on a smear. Vacuoles in the blasts can make the blasts difficult to distinguish from Burkitt cells (see also Fig. 68.7). However, flow cytometry can easily distinguish the immature ALL blasts from the mature B cells seen in a leukemic presentation of Burkitt lymphoma.

chromatin and inconspicuous nucleoli (see Fig. 68.1C). However, variations in morphology are common, and larger cells with abundant bluish gray cytoplasm, larger, somewhat irregular nuclei, and variably prominent nucleoli can be frequently seen (see Fig. 68.1D). Even though the nuclear chromatin of these cells can be fine, it is never as finely dispersed as in a myeloblast. At the other end of the morphologic spectrum are smaller cells with uniformly condensed, mature lymphocyte-like chromatin (see Fig. 68.1E), and distinction from mature B-cell malignancies relies on immunophenotyping. Coarse azurophilic granules (see Fig. 68.1F) can be seen in a subset of blasts in 5% to 8% of childhood ALLs and even more frequently in adult ALL patients. These have been reported in association with Philadelphia chromosome-positive (Ph$^+$) ALL and in ALL in Down syndrome patients. The granules are coarser than the granules seen in myeloblasts and are invariably myeloperoxidase negative (see later discussion of cytochemistry). Morphologic distinction between the L1 and L2 category recommended in the French-American-British (FAB) classification has proven to be poorly reproducible and of little prognostic value. It has been abandoned in the current World Health Organization (WHO) classification.

Cytoplasmic vacuolation (see Fig. 68.1H) can be seen in as many as 28% of childhood ALL patients. These lymphoblasts can be distinguished from leukemic presentation of Burkitt lymphoma (BL) (see later discussion) based on other morphologic features such as a smaller cell size, lack of deep blue cytoplasm, and less coarse chromatin. However, when morphology is confounding, the distinction relies on immunophenotyping of the malignant cells. In contrast to BL cells, ALL blasts are precursor B cells that express terminal deoxynucleotidyl transferase (TdT) and lack surface immunoglobulin (Ig) expression (see later discussion).

The trephine biopsy sections show hypercellular BM (Fig. 68.2). The sections are evaluated after staining with hematoxylin and eosin. The BM is usually packed with a relatively uniform population of small round blasts with round to oval nuclei. Less frequently, the blasts can be more pleomorphic, with indented, convoluted, and variably sized nuclei. The chromatin is described as being finely dispersed or stippled, and the nucleoli are usually not conspicuous. Brisk mitotic activity is almost always present. The effacement of the BM space is almost complete and uniform at the time of initial presentation. Minimal residual hematopoiesis is present; in most instances this is represented by a few megakaryocytes and some erythropoiesis. Normocellular or even hypocellular BMs at presentation have been described but are uncommon. Rarely, the initial presentation of ALL can be with an aplastic or markedly hypocellular BM. Making the diagnosis in this hypocellular context can be particularly challenging because of a paucity of material available for supporting studies such as cytogenetics and immunophenotyping.

The BM biopsy can reveal partial or complete necrosis (see Fig. 68.2C). When extensive necrosis is present, making a diagnosis can be challenging or almost impossible. A repeat BM biopsy should be attempted and will usually provide diagnostic material. Some increase in reticulin fibrosis is present in 60% to 70% of ALL patients. When fibrosis is extensive, an aspirate cannot be obtained, limiting material available for ancillary studies such as flow cytometry and cytogenetics. For these patients, the immunophenotyping can be performed by immunoperoxidase immunohistochemistry on the bone core biopsy. A second core can be obtained and submitted without fixation for cytogenetic analyses.

Organs other than the BM can be frequently involved. Extramedullary or lymphomatous presentation is more common with T-acute lymphoblastic leukemia (T-ALL) than B-ALL. The cytomorphology of the malignant cells in extramedullary disease is similar to that described in the bone core biopsy. Lymph node involvement is usually diffuse but can be partial with sparing of the follicles.

CYTOCHEMISTRY

The use of cytochemistry to assign lineage has been largely replaced by flow cytometry evaluation of the leukemic blast immunophenotype. However, when available, the myeloperoxidase reaction

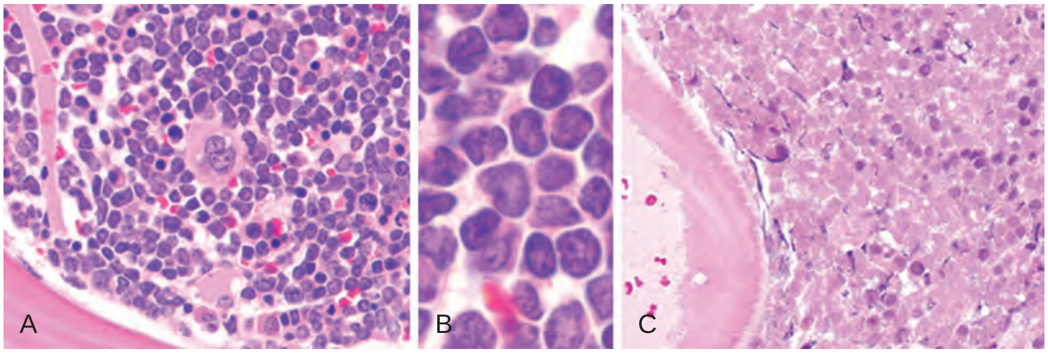


Figure 68.2 MORPHOLOGIC FEATURES OF ACUTE LYMPHOBLASTIC LEUKEMIA IN THE BONE MARROW BIOPSY. (A) The findings in the bone marrow (BM) biopsy in acute lymphoblastic leukemia are varied, but frequently the biopsy is 100% cellular and packed with blasts. Sometimes there is some residual hematopoiesis with sparing of megakaryocytes as shown. (B) A high power of the lymphoblasts is shown, illustrating the fine, dispersed, and "blastic" chromatin pattern of the cells. Sometimes the BM can be hypocellular (not shown); however, the majority of the cells in such cases are blasts. (C) Sometimes there is necrosis. The necrotic cells are referred to as "ghost" cells. They may retain some of their antigen expression as identified by immunostains; however, if the entire biopsy shows necrosis, a diagnosis should be made from the findings in the blood or a repeat BM may be necessary. Other tumors can present with BM necrosis.

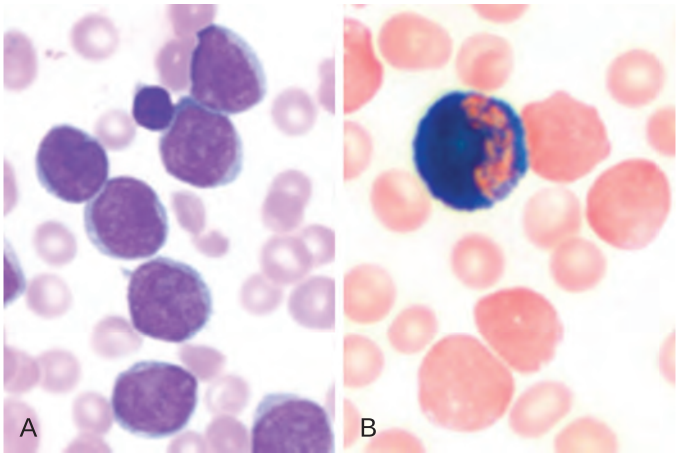


Figure 68.3 CYTOCHEMISTRY IN ACUTE LYMPHOBLASTIC LEUKEMIA. (A) The blasts in acute lymphoblastic leukemia are always myeloperoxidase reaction negative. (B) Compare the blasts with a single positively reactive granulocyte with a black-blue reaction product. Evaluating for nonspecific esterase reactivity (α-naphthyl acetate esterase, or α-naphthyl butyrate esterase) might also be performed when the blasts are difficult to distinguish from monoblasts.

(Fig. 68.3A and B) permits a rapid distinction from acute myeloid leukemia (AML). The reaction detects the myeloperoxidase enzyme in the primary granules of myeloblasts and is specific for the myeloid lineage. An acute leukemia in which 3% or more of the cells are myeloperoxidase positive is considered myeloid. There is excellent concordance between the myeloperoxidase reaction detected by cytochemistry and the myeloperoxidase molecule detected by flow cytometry.

IMMUNOPHENOTYPE

Based on large cooperative group studies in Europe and the United States, B-ALL comprises 75% to 80% and T-ALL from 15% to 25% of cases of ALL. B lymphoblasts cannot be distinguished from T lymphoblasts by morphology. Extensive immunophenotypic characterization is therefore required for the appropriate classification of ALL and indeed distinction from certain subtypes of AML. When adequate material is available, immunophenotyping should be performed using multicolor flow cytometry so that multiple antigens can be detected simultaneously on the lymphoblasts (Fig. 68.4). When interpreting the immunophenotypic data, it is important to remember that no single antigen is specific for any given lineage and multiple antigens need to be evaluated to establish the correct diagnosis. The panel of antibodies used for flow cytometry of a new leukemia and the pattern of expression seen in B-ALL and T-ALL are shown in Table 68.2. In addition, the combination of markers expressed on the B or T lymphoblasts can be reflective of the stage of development at which the transformation happened (Table 68.3). Of note, expression of myeloid antigens is seen frequently in B-ALL and T-ALL, as is the expression of T-cell antigens in B-ALL and B-cell antigens in T-ALL. Expression of individual myeloid antigens should not be a deterrent to making the diagnosis of ALL. Of particular significance is the subgroup of T-ALL that lack CD1a, CD8, and CD5 but show expression of one or more myeloid or stem cell markers and have been recently designated as *early T-cell precursor ALL*. Although these leukemias can have a gene expression profile and spectrum of genetic mutations that overlap significantly with biphenotypic leukemia, most studies suggest improved outcomes using T-ALL therapy. It is therefore best to recognize this type of ALL as a type of T-ALL rather than mixed lineage leukemia. The criteria for diagnosis of acute leukemia of ambiguous lineage, which would include mixed phenotype acute leukemia, have been extensively revised in the current WHO classification.

CYTOGENETICS AND MOLECULAR GENETICS

Chromosomal abnormalities can be detected in almost 80% of B-ALLs and 70% of T-ALLs (see Chapter 57). Cytogenetic classification remains the single most important prognostic factor in both pediatric and adult ALL. Numerical abnormalities, as well as structural abnormalities that disrupt the function of transcription factors involved in hematopoietic development and differentiation, are common. These genetic abnormalities define the biology of the disease and have an impact on treatment outcome. In addition, specific cytogenetic or molecular abnormalities are associated with unique phenotypic characteristics and are amenable to targeted therapy. *BCR-ABL1*$^+$ (referred to as Ph$^+$) ALL was the genetically defined subtype of B-ALL incorporating molecularly targeted TKI therapy in the frontline treatment. Studies over the past decade have identified the existence of a subset of B-ALL with a gene expression profile similar to that of *BCR-ABL1*$^+$ patients but without the *BCR-ABL1* fusion. This group of patients is referred to as *BCR-ABL1*–like (or Ph-like) and is also likely to benefit from targeted TKI therapy (see discussion on Philadelphia chromosome–like ALL [Ph-like ALL] later). The 2016 revision of WHO classification added the category of B-lymphoblastic leukemia/lymphoma BCR-ABL1–like to the previously recognized genetically defined entities (Table 68.4).[6]

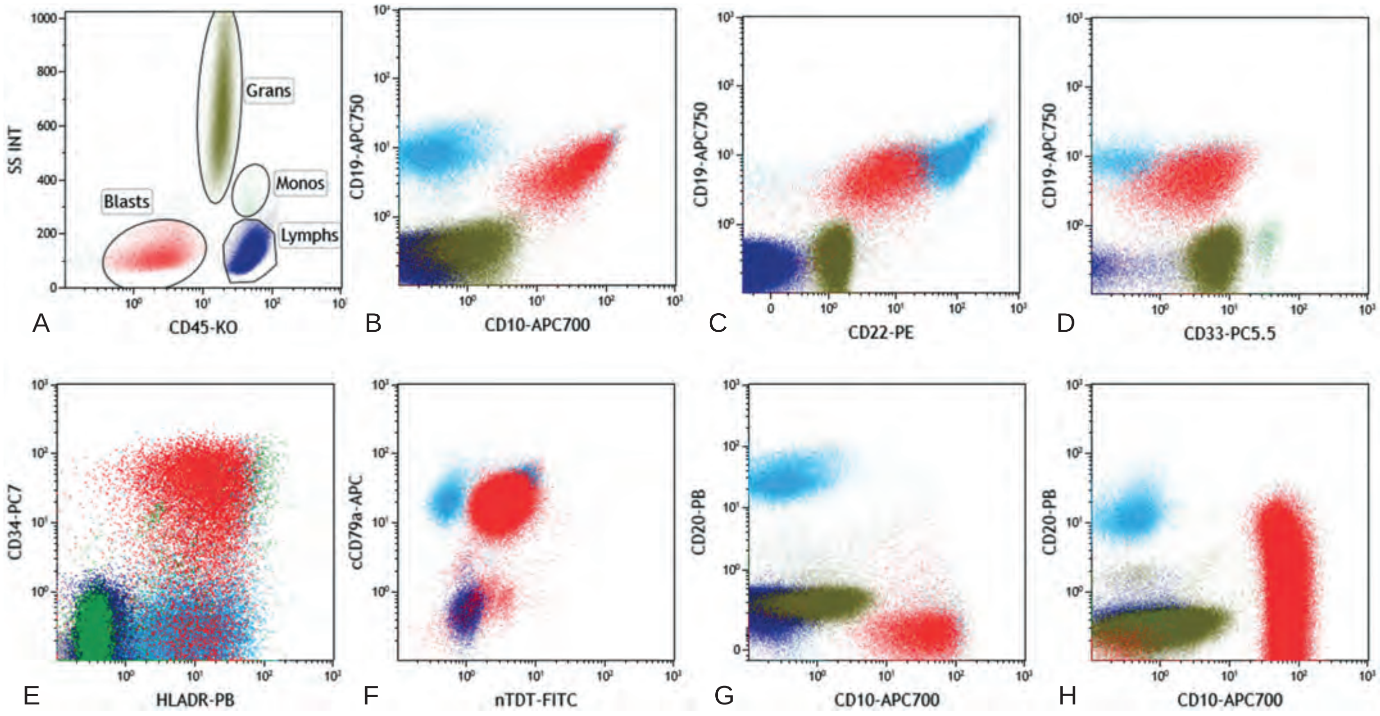


Figure 68.4 AN EXAMPLE OF FLOW CYTOMETRIC EVALUATION IN A CASE OF B-ACUTE LYMPHOBLASTIC LEUKEMIA. Selected histograms from a panel of markers used in the evaluation of acute lymphoblastic leukemia by flow cytometry are illustrated. The blasts are first identified by weak CD45 expression and low side scatter *(Blasts, red)* (A). The residual normal populations within the specimen serve as internal controls used as a reference to determine the presence or absence of an antigen as well as intensity of expression. Because no single antigen is specific for a lineage, multiple antigens are evaluated using multicolor flow cytometry. In this case, the blasts express CD19, CD10 (B), CD22 (C), myeloid antigen CD33 (intensity of expression comparable to normal granulocyte present in the specimen, D), CD34, HLADR (E), nuclear terminal deoxynucleotidyl transferase (nTDT), cytoplasmic CD79a (F), and CD10 (G). A different example shows aberrant variable expression of CD20 on leukemic blasts that express bright CD10 (H).

Hyperdiploidy, defined by the presence of more than 50 chromosomes, is seen in almost 25% of pediatric patients and 4% to 5% of adult ALL patients. In addition to routine karyotyping or fluorescence in situ hybridization (FISH) analysis, hyperdiploid DNA content can be determined by flow cytometry using DNA-binding fluorescent dyes and corresponds to a DNA content between 1.16 and 1.6. However, some studies have demonstrated that hyperdiploidy resulting from duplication of specific chromosomes (4, 10, and 17) is a better indicator of a favorable prognosis than the actual ploidy or the DNA content. Careful analysis of the specific pattern of chromosomal gains and losses or flow cytometry peaks for DNA content is required to distinguish true hyperdiploid cases from near-haploid cases that have undergone endoreplication (vide infra). In contrast to hyperdiploidy, a hypodiploid karyotype is associated with an adverse prognosis. Hypodiploid ALL with near-diploid chromosome numbers (44 to 45) is associated with somewhat distinct biology (loss of sex chromosome, dicentric chromosomes, presence of recurrent cytogenetic abnormalities such as ETV6-RUNX1) and is associated with a better outcome. In contrast, hypodiploidy with chromosome numbers less than 44 is invariably associated with poor outcome. The most common chromosome complements within the hypodiploid group are near haploid (24 to 31 chromosomes) and low hypodiploid (32 to 39 chromosomes) and are both associated with a poor outcome. Of note, low-hypodiploid ALL is frequently associated with germline *TP53* mutations and at least in a subset of cases appears to be a manifestation of Li-Fraumeni syndrome (see Chapter 66). Both near-haploid and low-hypodiploid ALL have activation of phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) and MEK-ERK signaling that can potentially be targeted by PI3K inhibitors.

A structural abnormality seen commonly in the pediatric age group but extremely rarely in the adult age group is a cryptic translocation, the t(12;21)(p13;q22) (*ETV6-RUNX1*). When present, it is associated with a favorable prognosis. Leukemias that harbor rearrangements of the mixed-lineage leukemia (*MLL*; now referred to as *KMT2A*) gene at chromosome 11q23, most notably t(4;11)(q21;q23), present with high WBC counts and frequent CNS involvement and are associated with poor clinical outcomes. Ph$^+$ ALL associated with t(9;22)(q34;q11.2) is seen in 25% of adults diagnosed with ALL. The translocation results in *BCR-ABL1* fusion, and the resulting leukemia has a poor outcome without the inclusion of TKIs into frontline treatment. Intrachromosomal amplification of chromosome 21 (iAMP21) is defined as a gain of at least three copies of regions of chromosome 21 that include *RUNX1*. The abnormality seen in 2% of B-ALL patients is associated with an older age at diagnosis and poor outcome with standard-risk therapy. iAMP21 is usually the sole cytogenetic abnormality but can be seen in patients with Ph-like ALL (see later). In addition, use of technology to assess copy number variation has identified submicroscopic genomic alterations (see Chapter 57) that have significant impact on the biology of B-ALL. These involve transcription factors such as *IKZF1* (Ikaros), *EBF*, and *PAX5*. The role of these transcription factors in B-cell development and the biology of B-ALL are discussed in greater detail in Chapter 66.

One of the most critical and clinically relevant discoveries with respect to ALL has been the description of high-risk ALL with a gene expression profile similar to that of Ph$^+$ ALL but without the *BCR-ABL1* fusion gene. This subtype of leukemia is referred to as *BCR-ABL1*–like or *Ph-like ALL*, and the kinase-activating alterations are a result of a diverse group of genetic lesions that result in dysregulated cytokine receptor and tyrosine kinase signaling. The lesions can be grouped into a surprisingly small number of broad categories. The most frequent category involves *CRLF2* rearrangements that account for almost 50% of Ph-like ALLs. The rearrangements involve either translocation of *IGH2* at locus on chromosome 14q32 to *CRLF2* on Xp22.3/Yp11.3 (pseudoautosomal region 1) or a 320-kb interstitial deletion centromeric of *CRLF2* resulting in *P2RY8-CRLF2* fusion. *CRLF2* overexpression is frequently associated with activating mutations of *JAK1*/*JAK2*, *IL7R*, or deletion of the JAK-STAT negative regulator *SH2B3*, resulting in activation of JAK-STAT signaling. JAK-STAT signaling activation also

ı a n (x p s y
u s e o n l y .

TABLE 68.2 Antigens Used for Immunophenotyping of Acute Lymphoblastic Leukemia[a]

	Commonly Positive	Variable Expression
B-ALL	CD19[b]	CD20
	cCD22[b]	CD34
	cCD79a[b]	CD45
	Pax5[c]	CD13[d]
	CD10	CD33[d]
	sCD22	sIgM[e]
	CD24	CD58[d]
	TdT	CD38[d]
T-ALL	TdT	CD1a
	cCD3[f]	CD2
	CD7	sCD3
		CD4[g]
		CD5
		CD8[g]
		CD10
		CD34
		CD99
		CD19
		CD33[h]
		CD79a
		CD117[h]
		CD56
		CD13[h]

[a] Antigens are listed approximately in order of frequency.
[b] Almost always positive.
[c] Most specific for B lineage but can be positive in t(8;21) acute myeloid leukemia.
[d] Altered expression provides leukemia associated phenotype crucial for detection of minimal residual disease.
[e] Rarely present.
[f] Only marker considered lineage-specific.
[g] Frequently coexpressed.
[h] Along with CD5^{lo}, CD1a^{-}, CD8^{-}, the expression of these antigens is helpful in identifying early T-cell precursor acute lymphoblastic leukemia. For prognostic significance, please refer to discussion in text.
B-ALL, B-acute lymphoblastic leukemia; *c*, cytoplasmic; *IgM*, immunoglobulin M; *s*, surface; *T-ALL*, T-acute lymphoblastic leukemia.

occurs in a second category of Ph-like ALL with *JAK2/EPOR* rearrangements. The third broad category of Ph-like ALL involves *ABL1*, *ABL2*, *CSF1R*, and *PDGRB*, described together as *ABL1* class rearrangements. In addition, other kinases such as *NTRK3*, *PTK2B*, and *TYK2* have also been shown to be involved in rearrangements in sporadic cases. As preclinical studies and case reports continue to document dramatic responses to targeted therapy for this group of patients, it is imperative that these lesions be identified prospectively. Because a wide variety of lesions result in a Ph-like phenotype, the laboratory diagnosis of Ph-like ALL remains a challenge. One approach to work-up of Ph-like ALL is highlighted in Fig. 68.5.

In addition to identification of Ph-like B-ALL, recent studies that use multiple techniques including chromosomal microarrays, gene expression profiling, RNAseq, and whole-genome sequencing have identified novel genetic subtypes that had were not detectable by karyotype and FISH analyses. These genetic subtypes show alteration in PAX5, MEF2D, ZNF384, and DUX4 genes (reviewed in references 7 and 8). The incidence and impact on outcome for these abnormalities remain an area of active investigation.

Similar to B-ALL, translocations involving transcription factors are common in T-ALL. The most commonly involved genes include *HOX11* and *HOX11L2*. Other genes that are involved in T-ALL are *MYC*, *TAL1*, *LMO2*, and *LYL1*. Similar to t(12;21), translocations involving the *TAL1* gene are cryptic and require detection by molecular techniques (see Chapter 57). Activating mutations of the *NOTCH1* gene are detected in almost 50% of ALL patients (see Chapter 66). Although activating mutations of *NOTCH1* appear to be associated with disease pathogenesis, they do not appear to be associated with an adverse prognosis; in fact, some pediatric studies suggest that NOTCH1-mutated T-ALL may have a relatively favorable response to current treatment regimens. Deletions of the *CDKN2A* gene on chromosome 9p are also particularly frequent as are mutations in the *FBXW7* gene, but neither has clear prognostic significance as single abnormalities. The significance of these mutations remains an area of active research.

DIFFERENTIAL DIAGNOSIS

ALL blasts can be easily distinguished from the reactive lymphocytes which accompany viral infections, because of the precursor phenotype of these cells. Low to weak expression of CD45 and expression of one or more precursor antigens such as TdT or CD34 is useful. In addition, precursor T-ALL cells express only cytoplasmic CD3 and no surface CD3, and B-ALLs frequently lack expression of CD20 while being CD19 positive. As mentioned previously, ALL cells can have some cytoplasmic vacuoles and need to be distinguished from leukemic presentation of BL. Unlike ALL, BL cells are mature B cells with bright surface Ig expression, with very strong CD20 expression, and that lack expression of CD34. Diagnosis of BL is confirmed by the presence of an *MYC* translocation using FISH or karyotypic analyses. Other entities that require distinction from ALL depend on the age of presentation. In the pediatric age group, ALL blasts need to be distinguished from hematogones. Hematogones are normal B-cell precursors present within the BM. These are more abundant in childhood and decrease with increasing age. Hematogones may also be increased during hematopoietic regeneration, particularly after chemotherapy or BM engraftment after stem cell transplant. Hematogones possess a distinct pattern of antigen expression that recapitulates progressive B-cell maturation. This is reflected in progressive loss of antigens such as CD34, TdT, and CD10 and acquisition of CD20 and surface Ig expression (Fig. 68.6). Other diseases that need to be morphologically distinguished from ALL include small blue cell tumors, including Ewing sarcoma, neuroblastoma, and medulloblastoma. Ancillary studies, including immunophenotyping and cytogenetics, are helpful in making the distinction. In older adults, entities that can morphologically mimic ALL include blastoid mantle cell lymphoma, chronic lymphocytic leukemia, and prolymphocytic leukemia (see Chapters 76 and 86). These latter are all mature B-cell malignancies that can be distinguished from ALL based on the mature B-cell phenotype, including consistent expression of CD20 and surface Igs.

PROGNOSIS

Prognostication based on clinical and biologic risk factors has been useful in making informed decisions about postremission treatment options. Established risk factors for a poor prognosis with current chemotherapeutic approaches include age older than 60 years, elevated WBC count at diagnosis (>30,000/µL for B-cell ALL; >100,000/µL for T-cell ALL), pro–B-cell or early T-cell immunophenotype, and cytogenetics (t[4;11][q21;q23] and other *MLL* rearrangements, hypodiploidy, or a complex karyotype). The presence of the Philadelphia chromosome, t(9;22)(q34;q11.2) resulting in the *BCR-ABL* fusion gene, was previously associated with very poor treatment outcomes. Recent addition of ABL kinase inhibitors (molecularly targeted therapy), discussed in detail later, has improved the prognosis for these patients. Time to achievement of complete remission (CR) longer than 4 weeks has also been associated with a poor clinical outcome (Table 68.5).

Although different study groups have used slightly different variations of these risk factors, the presence of any one of the

TABLE 68.3 Immunophenotypes of B- and T-Lymphocyte Progenitors

B Lineage	CD10	CD19	CD22	CD79a	TdT	CyIgμ	
Early precursor (pro-B)	–	+	+	+	+	–	
Intermediate (common)	+	+	+	+	+	–	
Pre-B	±	+	+	+	+	+	
T Lineage	**CD1a**	**CD2**	**CD3**	**CD4**	**CD7**	**CD8**	**CD34**
ETP[a]	–	±	**C**	±	±	–	+
Pro-T	–	–	C	–	+	–	±
Pre-T	–	+	C	–	+	–	±
Cortical T	+	+	C	+	+	+	–
Medullary T	–	+	C, S	[b]	+	[b]	–

[a] ETP ALL CD5-/lo, CD1a$^-$, CD8$^-$ with stem cell myeloid markers, notably CD34, CD117, CD33, and CD13.
[b] Medullary T lymphocytes are positive for either CD4 or CD8, but not both.
C, Cytoplasmic; *CyIgμ*, cytoplasmic μ heavy chain; *ETP*, early T-cell precursor; *S*, surface; *TdT*, terminal deoxynucleotidyl transferase.

TABLE 68.4 Significant Features of B-Acute Lymphoblastic Leukemia With Recurrent Cytogenetic Abnormalities in the 2008 World Health Organization Classification

Cytogenetic Abnormality	Phenotype	Clinical Correlates	Incidence
t(9;22)(q33;q11.2)	CD19$^+$, CD10$^+$, CD25$^+$; frequent expression of myeloid antigens	Seen more frequently in adults; traditionally associated with extremely poor outcome; improved early event-free survival with targeted therapy	19% of all adult ALL patients; incidence increases with age
t(v;11q23) Common fusion partners include AF4 (4q21) and ENL (19p13)	CD19$^+$, CD10$^-$; aberrant expression of myeloid antigen CD15	Frequent presentation with high WBC count, CNS involvement	9% of Ph$^-$ adult ALL
t(12;21)(p13;q22) Cryptic translocation; requires FISH	CD19$^+$, CD10$^+$; aberrant expression of myeloid antigen CD13	Sensitive disease with favorable outcome on standard therapy	2% to 3% of Ph$^-$ adult ALL
Hyperdiploidy chromosome number >50, <66 extra copies of nonrandom chromosomes, most frequently 21,X,14, and 4	CD19$^+$, CD10$^+$; no distinctive phenotype	Sensitive disease with favorable outcome on standard therapy	10% of Ph$^-$ adult ALL
Hypodiploidy chromosome number <46	CD19$^+$, CD10$^+$; no distinctive phenotype	Poor prognosis	4% of Ph$^-$ adult ALL
t(5;14)(q31;q32)	CD19$^+$, CD10$^+$; no distinctive phenotype	Reactive eosinophilia driven by IL-3 overexpression driven by the translocation; blasts may be <20% in the BM and undetectable in peripheral blood	Rare in adults
t(1;19)(q23;p13.3)	CD19$^+$, CD10$^+$; cytoplasmic μ$^+$	No significant association with response to therapy on current protocols	3% of Ph$^-$ adult ALL

ALL, Acute lymphoblastic leukemia; *BM*, bone marrow; *CNS*, central nervous system; *FISH*, fluorescence in situ hybridization; *IL-3*, interleukin-3; *Ph*, Philadelphia chromosome; *WBC*, white blood cell.
Data from Moorman AV, Harrison CJ, Buck GAN, et al. Karyotype is an independent prognostic factor in adult acute lymphoblastic leukemia (ALL): analysis of cytogenetic data from patients treated on the Medical Research Council (MRC) UKALLXII/Eastern Cooperative Oncology Group (ECOG) 2993 trial. *Blood*. 2007;109:3189; and Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. *N Engl J Med*. 2004;350:1535.

following is generally accepted as high risk (HR): high WBC count at diagnosis (>30,000/μL in B-cell ALL or >100,000/μL in T-cell ALL); cytogenetic abnormalities [hypodiploidy, t(4;11), t(9;22)]; age older than 60 years; pro–B-cell phenotype; and time to remission longer than 4 weeks. All other patients are considered standard risk (SR).

Nevertheless, despite being labeled as SR, up to 40% to 50% of these adults eventually relapse. Thus there is a need for refinement of prognostic markers for ALL patients. Recently, an early T-cell phenotype, the expression of CD20$^+$, and several recently described genetic mutations (*IKAROS*, *CRLF2*, and *JAK2*; reviewed earlier in this chapter) have been identified as being associated with adverse outcomes from retrospective analyses.

MINIMAL RESIDUAL DISEASE

The identification and quantification of measurable or MRD (below the level of morphologic disease detection) are now considered the key prognostic marker of disease-free survival (DFS). A variety of methods are used to detect MRD, including multiparameter flow cytometry (MFC), quantitative PCR, and next-generation sequencing (NGS); highest sensitivity is yielded from BM samples as compared with peripheral blood. While flow cytometry–based methods have issues with lack of standardization, they can detect aberrant patterns of cell surface antigen expression unique to the leukemic clone. PCR methods detect particular fusion transcripts such as *BCR-ABL* translocation, where its use has been most widespread and very well

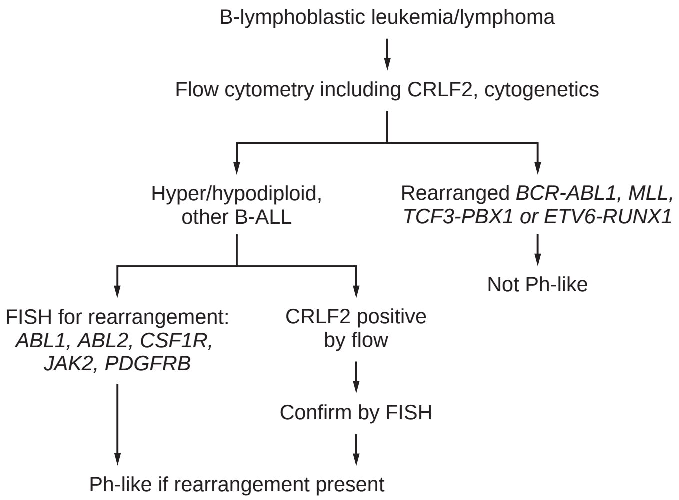

B-lymphoblastic leukemia/lymphoma
Flow cytometry including CRLF2, cytogenetics
Hyper/hypodiploid, other B-ALL
Rearranged *BCR-ABL1, MLL, TCF3-PBX1 or ETV6-RUNX1*
Not Ph-like
FISH for rearrangement: *ABL1, ABL2, CSF1R, JAK2, PDGFRB*
CRLF2 positive by flow
Confirm by FISH
Ph-like if rearrangement present

Figure 68.5 ALGORITHM TO EVALUATE FOR PHILADELPHIA CHROMOSOME–LIKE ACUTE LYMPHOBLASTIC LEUKEMIA. Initial evaluation includes standard karyotype analysis to exclude cases with *BCR-ABL1*, *MLL*, *TCF3-PBX1*, and *ETV6-RUNX1* rearrangements. Flow cytometry for *CRLF2* can identify as many as 50% of Philadelphia chromosome–like (Ph-like) acute lymphoblastic leukemia. In the remaining cases FISH analysis can be used to identify recurrent rearrangements that contribute to a Ph-like gene expression profile. However, some cases will require additional analysis by next-generation sequencing. *B-ALL*, B-acute lymphoblastic leukemia; *FISH*, fluorescence in situ hybridization. *(Modified from Roberts KG, Li Y, Payne-Turner D, et al. Targetable kinase-activating lesions in Ph-like acute lymphoblastic leukemia. N Engl J Med. 2014;371:1005.)*

standardized as an outstanding marker for MRD detection, or clone-specific rearrangements as quantitative PCR (clone-specific IgH, or T-cell receptor [TCR] rearrangements) which are well standardized in European centers. NGS methods identify similar clone-specific gene rearrangements with a higher threshold of sensitivity at 10^{-6}. Regardless of method, many prospective studies have been performed and demonstrate the utility of MRD monitoring for prognosis and treatment stratification. The NGS methodology perhaps holds the most promise for future MRD testing given its ability to standardize methods, its higher limit of sensitivity, specificity for residual disease, and ability to track minor subclones of evolving disease. Consensus opinion has recommended MRD testing at the end of induction, in early consolidation, and approximately for every 3 months thereafter for at least 3 years, as well as immediately prior to stem cell transplantation (SCT) and serially thereafter.[8]

A recent meta-analysis of MRD in pediatric and adult ALL demonstrated that MRD negativity is associated with improved event-free survival (EFS) and OS at 10 years, when analyzing studies from 2000 to 2014.[9] Among studies of pediatric ALL, MRD negativity was associated with an EFS of 77% at 10 years and those with detectable MRD had an EFS of 32%. Among studies of ALL in adults, MRD negativity was associated with an EFS of 64% at 10 years, compared with 21% among those with detectable MRD. These findings, importantly, held true across modalities of MRD detection method, timing of MRD testing, karyotype of underlying disease, and phenotype of ALL—with none of these factors holding significance on the effect of MRD on hazard ratios of EFS or OS.

Individual studies of MRD among specific phenotypes of ALL have revealed new insights on prognosis and potential future study and/or treatment. Adult patients with Ph-like ALL, *MLL* gene rearrangements, and early T-cell precursor ALL have had relatively poor outcomes with standard aggressive chemotherapy regimens and are more likely to have persistent MRD positivity after intensive therapy,[10] guiding many physicians to intervene with allogeneic stem cell transplant during first CR for these subsets.[11] By contrast, with the addition of novel TKIs and targeted immune therapies, the absence of MRD during intensive frontline treatment of Ph$^+$ ALL may be changing the general recommendation away from allogeneic stem cell transplant in first remission for all eligible patients. Among Ph$^+$ ALL patients receiving a *BCR-ABL1* TKI who achieved a complete molecular remission (CMR), defined as absence of detectable MRD, after intensive chemotherapy and a TKI, who additionally did not undergo a stem cell transplant, a 4-year OS rate of 66% was achieved. Of note, the median OS for those achieving a CMR at 3 months was a statistically significant 127 months versus 38 months for those who did not achieve CMR by real-time-PCR detection of *BCR-ABL1* transcripts.[12] Similarly, Ph$^+$ ALL patients who received lower-intensity regimens after achieving CMR have been shown in a GIMEMA (Gruppo Italiano Malattie Ematologiche) study to have superior survival outcomes on TKI maintenance therapy post induction, despite not continuing on to higher-intensity chemotherapy and/or allogeneic stem cell transplant.[13]

In addition to its prognostic relevance, MRD is becoming more widely accepted as an acceptable clinical end point in clinical trials and drug approval. Blinatumomab, the bispecific monoclonal T-cell engager antibody binding CD3 and CD19, has been approved for treatment for MRD-positive disease. The use of MRD as an end point or prognostic marker may facilitate more rapid evaluation of new, emerging therapies, which might allow one to assign highest priority for early-phase trials which demonstrate low rates of MRD for continued development and approval in phase III studies to evaluate survival.[14]

TREATMENT OF ACUTE LYMPHOBLASTIC LEUKEMIA

Historical Perspective

Combination chemotherapy is the cornerstone of ALL management. In the 1960s, investigators at the National Institutes of Health designed multidrug chemotherapy regimens given over many different courses for pediatric ALL patients. A similar approach was then introduced for adults with ALL. The Berlin-Frankfurt-Muenster (BFM) group in the 1980s conducted pioneering studies and showed that an intensive multidrug induction and consolidation chemotherapy followed by delayed intensification led to improvement in survival in the majority of children. These regimens combine drugs with varying mechanisms of action at different doses, often in complex schedules, which have largely evolved empirically. Only a few of these drugs have been tested individually in randomized clinical trials in adults with ALL; therefore the relative contribution of each of the drugs in a multidrug regimen to the overall outcome is difficult to assess. Because there is an increased propensity for CNS involvement in ALL patients, all treatment regimens must also include CNS prophylaxis with intrathecal chemotherapy with or without cranial radiation. Adult ALL regimens have typically evolved from the BFM regimen using similar but often less-intensive treatment.[15]

The therapy for ALL is typically divided into three phases: (1) the remission induction phase, (2) remission consolidation or intensification, and (3) the maintenance (or continuation) phase. The remission induction and consolidation phases typically involve blocks of monthly treatment for 6 to 8 months followed by long-term maintenance, which is given for up to 3 years; thus the treatment of ALL is long and challenging and requires tremendous attention to detail, compliance, and support. Because of these challenges and the relative rarity and biologic heterogeneity of the disease, it is recommended that patients are referred to larger academic centers for evaluation and treatment to ensure the best possible survival rates. In the ensuing sections, the various components of treatment are reviewed. Special attention to the treatment of certain disease subsets is addressed because treatment of ALL is becoming increasingly based on its heterogeneity and the ability to adapt therapy to each of these subsets.

Effective chemotherapy regimens must be supplemented with adequate supportive care measures to obtain the best patient outcomes. Given the highly immunosuppressive nature of ALL treatment regimens, prophylaxis for *Pneumocystis carinii* with trimethoprim-sulfamethoxazole should be initiated early during treatment and

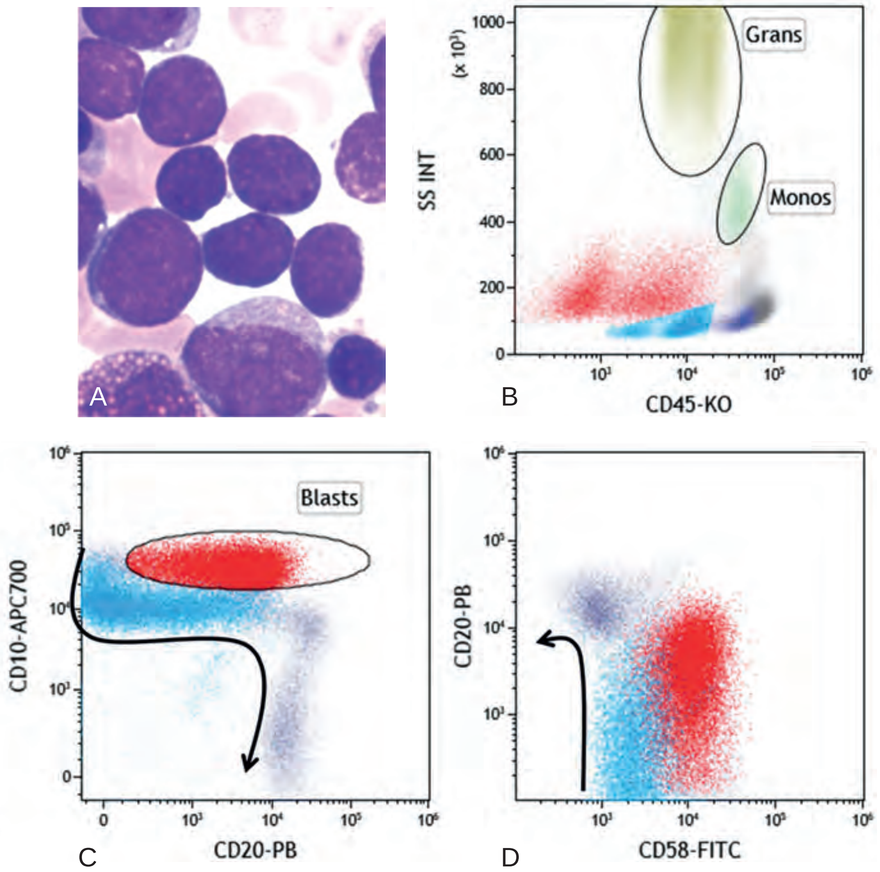


Figure 68.6 HEMATOGONES AND FLOW CYTOMETRIC EVALUATION OF MINIMAL RESIDUAL DISEASE. Hematogones are nonmalignant immature precursor B cells that are present in the bone marrow (BM). They are more commonly seen in pediatric patients but can be seen in adults during BM regeneration or associated with other conditions. (A) They can be difficult to distinguish morphologically from malignant lymphoblasts because the cytologic features overlap significantly. (B–D) Unlike malignant lymphoblasts, hematogones exhibit a spectrum of maturation that can allow distinction from leukemic blasts. The example shown here is for a bone marrow with residual B-acute lymphoblastic leukemia (B-ALL) along with hematogones. Blasts are shown in red, hematogones in aqua, and mature B cells in dark blue. Hematogones have a distinct side scatter/CD45 profile when compared with hematogones (B). Like leukemic blasts, hematogones are CD10$^+$, but as CD20 is expressed, CD10 is diminished. Lymphoblasts, on the other hand, frequently exhibit maturation arrest and expression of antigens with abnormal intensity. In the example shown (C), hematogones show progressive maturation while the blasts show maturation arrest with aberrantly bright expression of CD10. Similarly, image D shows aberrantly bright expression of CD58 when compared with hematogones. These patterns can be used to identify minimal residual disease *(right histogram)* and distinguish regenerative hematogones from residual or recurrent blasts *(red)* after therapy. Multiple markers and parameters are usually used to study posttherapy specimens in this way.

continued throughout the consolidation and maintenance phases of chemotherapy. It is also reasonable to consider antifungal and antiviral prophylaxis during periods of neutropenia. Consideration may be given to antibacterial prophylaxis during periods of neutropenia. Fluoroquinolones such as levofloxacin are the preferred agent, although the optimal antibiotic choice should depend on local bacterial resistance patterns. Neutropenic fever in this group of patients must be treated as a medical emergency with immediate institution of intravenous (IV) antibiotics after obtaining the necessary cultures.

In an attempt to decrease the duration of neutropenia and improve remission rates, several groups have studied the role of granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF) during induction therapy. In the CALGB 9111 trial, 198 adults with untreated ALL were randomized to receive G-CSF (beginning 4 days after the start of induction until neutrophil recovery) versus placebo.[16] The median time to neutrophil recovery (≥1000/μL for 2 days) was significantly shortened to 16 days in the G-CSF arm compared with 22 days in the placebo arm. The duration of hospitalization was also significantly shorter in the G-CSF arm. There was a trend toward increased CR rates in the G-CSF arm. However, the infectious complications, DFS, and OS were similar in the two arms. Thomas and colleagues randomized 236 ALL patients treated on the France-Belgium Group for Lymphoblastic Leukemia in Adults (LALA)-94 trial to receive G-CSF, GM-CSF, or no CSF. Use of G-CSF was associated with a significant decrease in time to neutrophil recovery, duration of hospitalization, and infections that were grade 3 or higher.[17] Thus use of G-CSF can allow for faster hematopoietic recovery, especially in older adult patients, and may decrease infectious complications; however, the prophylactic use of G-CSF has not been uniformly adopted, and many studies simply follow standard guidelines for use of growth factors in the setting of neutropenic fever.

All patients should be monitored carefully for tumor lysis syndromes, especially those with risk factors such as elevated WBC counts, renal dysfunction, and elevated LDH at presentation. Patients must be provided adequate hydration and prophylaxis or treatment for hyperuricemia with allopurinol for the first week of induction. Rasburicase, a urate oxidase, can be considered for the treatment of hyperuricemia in cases with high proliferation rates.

REMISSION INDUCTION

The aim of induction therapy is to achieve maximal reduction of the leukemic burden to result in morphologic remission (<5% blasts with trilineage hematopoiesis) and normalization of the blood counts, with

TABLE 68.5 Markers for Poor Prognosis in Adult Acute Lymphoblastic Leukemia

Established Risk Factors	
Age	>60 years
Presenting WBC count	>30,000/µL (B-cell ALL); >100,000/µL (T-cell ALL)
Immunophenotype	Pro-B cell; early T cell[a]
Cytogenetics	t(4;11)(q21;q23) and other *MLL* rearrangements
	t(9;22)(q34;q11.2)—Philadelphia chromosome
	Hypodiploidy (<44 chromosomes)
	Complex (>5 abnormalities)
Therapy response	Time to complete remission >4 weeks
MRD	≥0.01% at 3–6 months after initiation of therapy[b]
Emerging Risk Factors	
Immunophenotype	CD20
Molecular	BAALC
	FUS
	ERG
	IKZF1[c]
	Ph-like ALL

[a] Initial report characterizing ETP ALL showed a poor outcome. However, subsequent studies have shown variable association with response to therapy.
[b] Different studies have used different time points for MRD assessment.
[c] Focal deletions in IKZF1 are present in up to 70% of Ph-like ALL. However, IKZF1 deletions are associated with adverse outcome irrespective of association with Ph-like phenotype.
ALL, Acute lymphoblastic leukemia; *ETP*, early T-cell precursor; *MRD*, minimal residual disease; *Ph*, Philadelphia chromosome; *WBC*, white blood cell.

as little toxicity as possible. This is typically achieved using combination chemotherapy consisting of four or five systemically administered drugs and intrathecal chemotherapy over a period of approximately 3 to 4 weeks. Most current ALL induction regimens in adults are modeled after pediatric regimens, and include a prolonged course of oral corticosteroid (prednisone or dexamethasone), weekly vincristine, and either weekly or pulsed anthracycline (doxorubicin, daunorubicin, or mitoxantrone). Many groups (CALGB,[15] French Group for Research in Adult Acute Lymphoblastic Leukemia [GRAALL] 2003,[18] Japanese Acute Leukemia Study Group [JALSG][19]) have also used twice-weekly L-asparaginase as part of induction therapy, and some have added cyclophosphamide (GRAALL 2003,[18] German Multicenter Acute Lymphoblastic Leukemic protocol [GMALL] 07/2003,[20] CALGB 8811[15]). With the current treatment regimens, very high CR rates in adults with ALL have been achieved in the range of 84% to 94% (Table 68.6). There have been few randomized comparisons between these different induction regimens; therefore the choice of the regimen should depend on the treating physician's experience with a particular regimen. Most ALL regimens are designed to optimally deliver a variety of chemotherapeutic agents; thus an entire treatment program should be selected at the time of diagnosis based on the patient's performance status and biologic risk factors. Whenever possible, these patients should be referred to centers that offer expertise in the treatment of ALL and where enrollment into clinical trials is available. Once a specific regimen has been selected, the goal (and challenge) is for both the medical team and the patient to adhere to the recommended treatments, which are arduous and lengthy in duration.

Traditionally, prednisone was the corticosteroid of choice during induction therapy for ALL trials. Studies in pediatric patients have shown lower relapse rates but higher toxicity (more sepsis, higher incidence of osteonecrosis) with dexamethasone. Dexamethasone penetrates the blood-brain barrier effectively and therefore may offer more direct CNS protection than prednisone. Randomized studies of prednisone versus dexamethasone during induction have not been performed in adults; however, both have been used during induction and postremission therapy. Anthracyclines form an important part of induction therapy. In an early trial from the CALGB,[26] patients were randomized to receive vincristine, prednisone, and L-asparaginase with or without daunorubicin. Significant improvements in CR rates and remission duration were noted with the addition of daunorubicin; anthracyclines have since become standard components of induction chemotherapy in adult and pediatric ALL. Different anthracyclines (doxorubicin, daunorubicin, and mitoxantrone) are thought to be clinically equivalent, and, to date, dose intensification of anthracycline during induction has been tested in a variety of prospective clinical trials but has not been shown to definitely improve already high CR rates or to result in significant benefits in DFS.[22,27] Addition of pulsed cyclophosphamide to induction chemotherapy has been studied with conflicting results. In the CALGB 8811 trial, addition of cyclophosphamide to the induction regimen was shown to lead to improved responses compared with historical control participants.[15] However, in a prospective, randomized Italian GIMEMA 0288 study, the addition of cyclophosphamide did not influence CR rate or survival.[23]

L-asparaginase (asparaginase) is also an important component of ALL therapy and has been incorporated into most ALL trials beginning with induction. ALL cells are unable to produce asparagine and are dependent on plasma levels of this amino acid for protein synthesis. Depletion of asparagine results in inhibition of protein synthesis and subsequent apoptotic leukemic cell death. Traditionally, native enzyme derived from *Escherichia coli* has been used. However, this preparation can be immunogenic, leading to hypersensitivity reactions and development of cross-reacting antibodies; this preparation, although still used elsewhere, is no longer available for administration in the United States. Polyethylene glycosylated (PEG)-asparaginase, formed by covalently attaching PEG to the native *E. coli* enzyme, has been developed and offers lower immunogenicity and a longer half-life. Douer and colleagues treated 25 newly diagnosed adult ALL patients (median age: 27 years; range: 17 to 55 years) with the adult ALL BFM protocol in which 14 injections of *E. coli* asparaginase were replaced by a single dose of pegaspargase (2000 IU/m^2 IV) on day 16 of induction therapy. After the single dose, asparagine deamination was complete in all patients after 2 hours and in 100%, 81%, and 44% on days 14, 21, and 28, respectively. No allergic reactions or pancreatitis was observed, and a CR was achieved in 24 of the 25 patients.[28] The CALGB 9511 trial used pegaspargase (2000 IU/m^2 subcutaneously, two doses during induction and two during first intensification) as part of the five-drug induction regimen. Of the 85 evaluable patients, those with effective asparagine depletion (defined by enzyme levels >0.03 U/mL plasma for 14 consecutive days after pegaspargase administration) had improved DFS and OS compared with those without effective asparagine deletion.[29] Thus use of pegaspargase is generally safe and effective in adults and has replaced native L-asparaginase as the agent for asparagine depletion. In 2011 the US Food and Drug Administration (FDA) approved asparaginase derived from *Erwinia chrysanthemi* to treat ALL patients who develop a hypersensitivity reaction to *E. coli*–derived asparaginase and pegaspargase; 25,000u/m^2 of Erwinia asparaginase given every 48 hours for six doses (for every one dose of pegasparaginase) has demonstrated safety and results in effective asparagine depletion in pediatric and adult ALL. Patients receiving *Erwinia* asparaginase are still at risk for known toxicities of asparaginase, including bleeding, clotting, transaminitis, and pancreatitis. In 2020, due to supply issues, a new *Erwinia* asparaginase has been approved.[30] To avoid hypersensitivity reactions, a novel preparation of L-asparaginase in which L-asparaginase is encapsulated within red blood cells (GRASPA [erythrocytes encapsulating L-asparaginase]) has been in clinical development in Europe but has not to date received approval from the regulatory agencies.

One of the regimens that is widely used in the United States and that also results in high CR rates of approximately 90% is the hyper-CVAD (cyclophosphamide, vincristine, adriamycin, and dexamethasone) regimen developed at the MD Anderson Cancer Center. The hyper-CVAD regimen differs from the pulsed weekly therapy developed by the pediatric groups and consists of four cycles of intensive

TABLE 68.6 Clinical Trials With Various Chemotherapy Regimens for Adult Acute Lymphoblastic Leukemia

Trial	Patients *(n)*	Age in Years, Median (Range)	CR Rate (%)	Disease-Free Survival	Overall Survival	Comments
CALGB 8811[15]	197	32 (16–80)	85	46% at 3 years	36 months (median)	Five-drug induction regimen based on pediatric trials with earlier and more intensive L-asparaginase
CALGB 9111[16]	198	35 (16–83)	85	40% at 3 years	23 months (median)	Patients randomized to G-CSF vs. placebo; G-CSF significantly decreased time to neutrophil recovery and CR rate with no effect on DFS, OS, or toxicities
MDACC hyper-CVAD[21]	288	40 (15–92)	92	38% at 5 years	32 months (median)	Long-term follow-up results of hyper-CVAD regimen; regimen does not include asparaginase
GMALL 07/2003[20]	713	34 (15–55)	89		54% at 5 years	Risk-adapted SCT for HR and very HR groups
LALA 94[17]	922	33 (18–79)	84	30% at 5 years	23 months (median)	CR rate similar between IDA and DNR arms; significantly higher TRM with IDA; improved DFS for the IDA arm for patients receiving only chemotherapy; for SR patients, intensive consolidation did not affect outcomes
JALSG ALL-93[19]	263	31 (15–59)	78	30% at 6 years	33% at 6 years	Doxorubicin dose intensity did not improve outcomes; during maintenance phase, early sequential intensification compared with intermittent intensification did not affect DFS
JALSG ALL-97[22]	404	38 (15–64)	74	33% at 5 years	32% at 5 years	Induction and maintenance based on CALGB 8811; dose-intensive doxorubicin during consolidation did not improve outcomes
GIMEMA 0288[23]	778	28 (12–60)	82	33% at 9 years	29% at 9 years	Addition of cyclophosphamide to induction did not influence CR rate or survival; responders to prednisone pretreatment had favorable outcomes
MRC UKALLXII/ ECOG E2993[24]	1646 (Ph$^-$)	(15–64)	90		43% at 5 years	Largest ALL trial to date; in a donor vs. no-donor analysis, those with a donor had improved OS and lower relapse rate; ASCT cannot replace consolidation or maintenance chemotherapy in any risk group
PETHEMA ALL-93[25]	222 high-risk ALL	27 (15–50)	82	35% at 5 years	34% at 5 years	Did not show beneficial effect of aSCT compared with ASCT or chemotherapy
GRAALL-2003[18]	225 Ph$^-$ ALL	31 (15–60)	94	59% at 3.5 years	60% at 3.5 years	Pediatric-inspired treatment regimen; in a historical comparison, significant increases in CR, EFS, and OS rates compared with LALA-94 trial; OS was improved for patients younger than 45 years only

ALL, Acute lymphoblastic leukemia; *aSCT*, allogeneic stem cell transplantation; *ASCT*, autologous stem cell transplantation; *CALGB*, Cancer And Leukemia Group B; *CR*, complete remission; *CVAD*, cyclophosphamide, vincristine, adriamycin, and dexamethasone; *DFS*, disease-free survival; *DNR*, daunorubicin; *EFS*, event-free survival; *G-CSF*, granulocyte colony-stimulating factor; *HR*, high risk; *IDA*, idarubicin; *OS*, overall survival; *Ph*, Philadelphia chromosome; *SCT*, stem cell transplantation; *SR*, standard risk; *TRM*, transplant-related mortality.

infusional cyclophosphamide, vincristine, doxorubicin, and dexamethasone alternating with four cycles of high-dose methotrexate and high-dose cytarabine for a total of eight intensive treatment cycles.[21]

Because CD20 is expressed on 20% to 40% of pre-B lymphoblasts and has been noted to be associated with adverse prognosis, the investigators at MD Anderson subsequently added rituximab to a modified hyper-CVAD regimen for patients with CD20 expression greater than or equal to 20%. In younger patients (≤60 years of age), rituximab improved 3-year OS to 75% compared with 47% in the historical control participants; however, no difference was seen for older adults.[31] The GRAALL 2005 study in ALL patients 18 to 59 years old included a randomization for the addition of rituximab to chemotherapy in CD20$^+$ patients. Rituximab plus chemotherapy resulted in an improved EFS of 65% compared with 52% in the chemotherapy only arm.[32] Based on these and other studies, rituximab is now generally recommended for all B-ALL patients with CD20 expression.

Some groups have also routinely instituted a 5- to 7-day steroid prophase before starting the induction therapy. This results in gentle cytoreduction and reduces the risk of tumor lysis. The achievement of rapid cytoreduction during this steroid prophase has also been shown to be of prognostic value in several studies.[33]

In summary, traditional induction regimens in adults with ALL result in very high CR rates of 80% to 90%. Until recently, OS rates have hovered at 40% to 45% (see Table 68.6) due to emergence of resistant disease. Advances that are described later are resulting in significant improvements in survival for both younger and older adults with ALL.

POSTREMISSION THERAPY

As mentioned earlier, 80% to 90% of adults with previously untreated ALL achieve CR with induction chemotherapy. However, all patients relapse if no further chemotherapy is given. This underscores the need for effective post-remission management strategies for these patients in first CR (CR1). As described earlier, adult patients with ALL have traditionally been stratified into SR or HR.

The type of recommended postremission strategy is to be based on the risk (risk-adapted approach), which implies more aggressive therapeutic approaches for HR groups compared with the SR group. There have been three tested approaches for postremission therapies: (1) postremission chemotherapy modules followed by long-term maintenance chemotherapy as per pediatric regimens; (2) allogeneic stem cell transplant; and least frequently, (3) ASCT. Studies using these postremission strategies are reviewed later.

The optimal postremission strategy for SR adult patients is not clear. Postremission chemotherapy typically follows pediatric regimen and consists of a variety of non–cross-resistant chemotherapeutic agents administered in treatment "modules" for a total of 6 to 8 months after achievement of remission. The postremission modules are typically 4 to 6 weeks in length and are modeled after successful pediatric regimens and often consist of consolidation module(s), interim maintenance modules that often focus on CNS prophylaxis (described in more detail below), and a late intensification module(s), which is often quite similar to the induction chemotherapy course. The specific drugs and the sequence or combinations in which they used are different for each protocol but typically include cyclophosphamide, L-asparaginase (or pegasparaginase), methotrexate, cytarabine, 6-mercaptopurine, vincristine, and doxorubicin. Many of these protocols have evolved empirically with few randomized trials evaluating the impact of any of the modifications that have been developed (see Table 68.6).

A different approach to postremission therapy has been taken by the MD Anderson Cancer Center, which has reported long-term results of the hyper-CVAD regimen (also listed in Table 68.6). As described earlier in the induction section, patients received alternating cycles of hyperfractionated cyclophosphamide, vincristine, adriamycin, and dexamethasone (hyper-CVAD) (courses 1, 3, 5, and 7) alternating with high-dose methotrexate and cytarabine (courses 2, 4, 6, and 8) followed by maintenance therapy for 2 years with 6-mercaptopurine, methotrexate, vincristine, and prednisone (POMP). They identified the following factors to be independent poor prognostic factors for survival: older age, Ph^+ disease, leukocytosis, thrombocytopenia, poor performance status, and hepatomegaly. The 5-year OS for the good-risk (risk score: 0 to 1), intermediate-risk (risk score: 2 to 3), and poor-risk groups (risk score: ≥ 4) were 62%, 34%, and 5%, respectively.[21]

Adults older than the age of 60 years have uniformly fared poorly with traditional chemotherapy approaches, with an OS below 10%. The most significant adverse risk factor is the presence of MRD after induction or consolidation chemotherapy. Blinatumomab, a bispecific CD19-directed CD3 T-cell engager monoclonal antibody was investigated in a phase II, single-arm study for adults with B-cell precursor ALL in hematologic CR with MRD (N^{-3}). Patients received blinatumomab 15 μg/m^2/day by continuous IV infusion for up to four cycles. Eighty-eight (78%) of 113 evaluable patients achieved a complete MRD response. Complete MRD responders had longer relapse-free survival (RFS) (23.6 vs. 5.7 months) and OS (38.9 vs. 12.5 months) compared with MRD nonresponders.[14] Based on these findings blinatumomab was granted accelerated FDA approval for the treatment of adult and pediatric patients with B-cell precursor ALL in first or second CR with MRD greater than or equal to 0.1%. These data suggest that chemotherapy alone was not effectively managing MRD and that the addition of immunotherapies such as blinatumomab to chemotherapy may improve outcomes. The Eastern Cooperative Oncology Group (ECOG) 1910 clinical trial in adults 30 to 70 years old is currently addressing the use of blinatumomab in frontline B-ALL treatment during consolidation. The study randomized all MRD-negative patients to receive blinatumomab in addition to chemotherapy versus chemotherapy alone. The primary objective of the study is to determine if the addition of blinatumomab to chemotherapy in newly diagnosed B-ALL improves OS. The study has completed accrual and is pending analysis for publication.

Table 68.6 summarizes the major treatment questions, therapeutic approach, and results of several large prospective cooperative group trials performed in the United States and Europe.

CENTRAL NERVOUS SYSTEM DISEASE: PROPHYLAXIS AND TREATMENT

Involvement of the CNS (Fig. 68.7) is uncommon at the time of diagnosis, with most series reporting it at less than 10%. However, because many patients with CNS involvement may not be referred for clinical trial enrollment, this may be an underestimate of the true rate. In the MRC United Kingdom Acute Lymphoblastic Leukemia (UKALL) XII/ECOG E2993 trial, of the 1508 eligible patients, 77 (5%) had CNS involvement at diagnosis.[24] Similarly, the French group reported a 7% (104 out of 1493 patients) incidence of CNS disease at diagnosis. Risk factors for CNS disease at diagnosis have varied in different studies. In the French trial (LALA-94), risk factors included mediastinal mass associated with precursor T-cell immunophenotype, lymphadenopathy, higher hemoglobin level, and absence of the Ph chromosome.[17] In the MRC trial, the risk factors included a higher WBC count, precursor T-cell immunophenotype, and mediastinal mass. The presence of CNS disease at diagnosis has been shown not to affect the CR rate. The French group also reported no significant difference in the OS in patients with and without CNS disease at diagnosis. However, in the MRC trial, the OS at 5 years was lower in the group with CNS disease (29% vs. 38%; $P = .03$), and they also reported that patients with CNS disease at diagnosis were twice as likely to relapse in the CNS. In the French study, younger age (<30 years), male gender, absence of the Ph chromosome, achievement of CR with one course, and use of transplant as postremission therapy had favorable impact on OS for patients presenting with CNS disease at diagnosis.

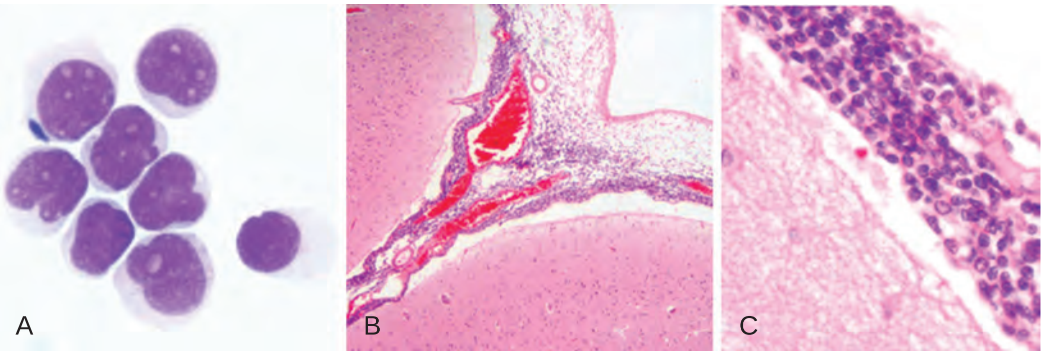


Figure 68.7 CEREBROSPINAL FLUID AND CENTRAL NERVOUS SYSTEM DISEASE. Blasts in the cerebrospinal fluid can be identified on a cytospin preparation. (A) They have similar cytologic features as the blasts in the blood or bone marrow aspirate. Histologic section of the brain is illustrated from a patient with acute lymphoblastic leukemia and leukemic meningitis. (B) The sections show blasts within the leptomeninges (higher power, C).

Induction therapy for ALL usually includes lumbar punctures with introduction of intrathecal chemotherapy during the first weeks of therapy. For those presenting with CNS disease at diagnosis or in whom CNS disease develops later in the course of the treatment, immediate intensified CNS treatment is imperative. These patients should receive intrathecal chemotherapy (intrathecal methotrexate alone or together with cytarabine and steroid [triple intrathecal therapy]) twice weekly until clearing of cerebrospinal fluid along with initiation of systemic chemotherapy. Cranial irradiation is often added for patients who present with active CNS involvement and may be delivered during postremission therapy.[35]

CNS prophylaxis is an essential component of therapy for all patients given the high incidence (30% to 40%) of CNS relapse if the CNS prophylaxis is omitted. Now with the universal adoption of CNS prophylaxis in the treatment regimens, the rates of CNS relapse have decreased substantially. CNS prophylaxis is typically administered by multiple rounds of intrathecal chemotherapy that are staggered over the entire treatment duration. In addition, the use of high-dose methotrexate and cytarabine also provides for CNS prophylaxis because both agents penetrate the blood-brain barrier. The intensive use of high-dose systemic methotrexate and frequent intrathecal therapy has often replaced the more traditional combination of CNS irradiation and intrathecal therapy that were the hallmarks of the earlier ALL regimens. This non-radiation-containing approach has resulted in effective CNS protection, with rates of CNS relapse now routinely reported as occurring in fewer than 5% to 10% of cases. Adolescents and young adults (AYAs) treated with pediatric regimens with more intensive CNS prophylaxis have reported CNS relapse rates as low as 1%. The addition of nelarabine to frontline therapy in children and young adults with T-ALL has improved EFS, largely as a result of decreasing CNS relapses.[35] The omission of cranial irradiation may prevent neurocognitive damage in adults. Although this has not been demonstrated in adult studies, definitive evidence from pediatric studies indicates that avoidance of cranial irradiation may prevent declines in cognitive function.[36]

MAINTENANCE THERAPY

The goal of maintenance treatment is to prevent disease relapse by elimination of leukemia clones by long-term exposure to cytotoxic drugs. One of the commonly used regimens uses daily 6-mercaptopurine and weekly oral methotrexate with monthly vincristine and prednisone (POMP regimen). Some groups replaced prednisone with dexamethasone. The duration of maintenance treatment is generally 2 to 3 years (2 years for women and 3 years for men). Similar to the other components of adult ALL regimens, the adoption of long-term maintenance therapy is based upon the benefit of maintenance therapy that has been demonstrated in pediatric trials.

Although the benefit of maintenance therapy in adult trials has not been demonstrated in randomized trials, it has been shown that omission of maintenance treatment leads to worse clinical outcomes. In an early CALGB study, 164 newly diagnosed ALL patients were randomized to receive daunorubicin or mitoxantrone during induction followed by four cycles of consolidation. No maintenance therapy was planned. There were significantly more relapses noted in this study, with median remission duration of only 10 to 12 months. The study was stopped earlier than planned because remission duration was shorter than in historical control participants who had received maintenance therapy. Similar results were obtained by the Dutch-Belgian Haemato-Oncology Cooperative Group (HOVON), which treated 130 ALL patients with induction followed by three cycles of consolidation. No maintenance therapy was given. The estimated 5-year OS and DFS were only 22% and 28%, respectively. Pediatric regimens routinely incorporate CNS prophylaxis during the maintenance phase. Pediatric studies have also reported favorable impact on DFS of achieving adequate myelosuppression with the chemotherapy drugs during the maintenance phase. Thus it appears that careful adjustment of the doses of the oral agents used during maintenance therapy to achieve optimal yet safe myelosuppression improves treatment outcomes, although the optimal duration of maintenance therapy varies from 2 to 3 years.[37]

ALLOGENEIC STEM CELL TRANSPLANT IN FIRST COMPLETE REMISSION

Despite attempts to improve DFS in adult ALL, OS with the standard chemotherapy regimens described earlier has been “fixed” at approximately 35% to 45% for the past decade. Over the years, the question of whether to recommend allogeneic transplant in first remission. A meta-analysis of seven studies (with 1274 patients) that prospectively assessed OS using genetic randomization based on donor availability reported significantly better OS in the donor group versus the no-donor groups (hazard ratio: 1.29; $P = .037$), which was more pronounced in patients with HR features.[38]

As treatment approaches and outcomes for adults with ALL have improved in the past 15 years, the role of allogeneic stem cell transplant is evolving. An intensive pediatric regimen administered to adult up to 40 years old was compared with registry data from the Center for International Blood and Marrow Transplant Research (CIBMTR) and demonstrated superior survival for chemotherapy versus transplant (3-year OS 77% vs. 53%, $P < .0001$).[39]

Importantly, the role of MRD has improved the ability to risk-stratify patients who might be most likely to benefit from allogeneic transplant in CR1. A number of more recent trials have demonstrated that patients with detectable MRD during postremission therapy have improved survival if they receive allogeneic transplant as postremission intensification.[11,20] In the GMALL study, MRD-positive patients who underwent allogeneic stem cell transplant had better OS than for those without allogeneic stem cell transplant (54% vs. 33%).[20] Thus detection of MRD in postremission therapy identifies patients who are at HR for relapse and may benefit from allogeneic transplant in CR1. Blinatumomab or other MRD “erasers” are now being used to eradicate MRD as a bridge to transplant and result in better outcomes following allogeneic transplant.[14]

Stem Cell Sources and Preparative Regimens for Allogeneic Stem Cell Transplantation

Matched sibling donors (MSDs) remain the preferred modality for the stem cell source undergoing allogeneic stem cell transplant; however, unrelated donor (URD) transplant outcomes have improved. For patients lacking an MSD, a matched URD (MUD) or an alternative donor source (e.g., umbilical cord blood [UCB]) should be pursued. In a CIBMTR registry study, outcomes of 1458 patients with ALL in CR1 who underwent URD transplants between 2000 and 2011 were analyzed. HLA 8/8 allele-matched URD recipients had similar transplant-related mortality (TRM) and all-cause mortality in comparison with related donor (RD) recipients. Seven of eight MUD recipients had a greater risk of TRM and all-cause mortality in comparison with RD recipients. The risk of TRM and all-cause mortality was also greater for 7/8 MUD recipients versus 8/8 MUD recipients. Compared with RD recipients, both 8/8 and 7/8 allogeneic stem cell transplant URD recipients had a lower risk of relapse. Both 8/8 and 7/8 MUD recipients had a greater risk of acute graft-versus-host disease (GVHD) and chronic GVHD in comparison with RD recipients.[40] The Minnesota group reported outcomes of myeloablative allogeneic stem cell transplant for ALL patients transplanted at their center and compared retrospectively the outcomes with respect to different donor sources. In an analysis restricted to 91 adult patients who received allogeneic stem cell transplant in CR1 or CR2 from 1990 to 2005, a difference between the allogeneic donor sources (matched related donor vs. MUD vs. UCB donor) was not observed for OS, DFS, or TRM. Similarly, in the ALL-SCT-BFM 2003 study of children and adolescents, a difference was not observed between matched related donor sources and at

TABLE 68.7 **Clinical Trials Evaluating Role of Myeloablative Allogeneic Stem Cell Transplant in Acute Lymphoblastic Leukemia**

Trial	Patients (*n*)	Age in Years, Median (Range)	Conditioning Regimen/Donor	TRM	DFS/OS	Comments
GOELALMO02[41]	41 (HR)	34 (15–52)	Etoposide/cyclophosphamide/TBI (12 Gy in six fractions) MSD	15%	DFS: 75% at 6 years; OS: 75% at 6 years	Showed improved survival with aSCT compared with ASCT
LALA 94[17]	100 (HR ALL excluding Ph$^+$ ALL)	33 (15–55) for the entire study cohort of 922 patients	Cyclophosphamide/TBI (10 Gy as a single dose or 12 Gy in six fractions) MRD	18% at 3 years	3-year DFS: 47%	For HR ALL, aSCT was superior to an ASCT
PETHEMA-93[25]	222 HR patient underwent MSD (if available); if not, randomized to ASCT vs. chemotherapy	27 (15–50)	Cyclophosphamide/TBI (12 Gy in six fractions) MSD		5-year OS: 35%	No benefit of an MSD transplant for HR patients (compared with ASCT or chemotherapy only approach)
MRC UKALLXII/ ECOG E2993[24]	Patients assigned to aSCT if an MSD available (donor group); those without donors randomized to ASCT vs. chemotherapy	15–64	Etoposide/TBI (13.2 Gy in six fractions) MSD	NRM HR (36% donor; 14% no-donor); SR (20% donor; 7% no donor)	5-year OS: HR (41% donor, 35% no donor) SR (62% donor vs. 52% donor)	Beneficial effect of all-SCT limited to SR group only; relapse rate decreased with aSCT in both HR and SR groups
HOVON[42]	ALL in CR1 according to sibling donor vs. no-donor comparison HR: 46 donor; 73 no donor SR: 50 donor; 88 no donor	Donor 31 (16–55)	Cyclophosphamide/TBI (12 Gy in six fractions) MRD	HR: NRM at 5 years (15% donor vs. 4% no-donor) SR: NRM at 5 years (16% donor vs. 2% no-donor, $P = .01$)	HR: OS at 5 years (53% donor vs. 41% no donor) SR: OS at 5 years (69% donor vs. 49% no donor, $P = .05$)	Similar to the MRC UKALLXII/ECOG E2993 trial, benefit of aSCT seems limited to SR patients

ALL, Acute lymphoblastic leukemia; *aSCT*, allogeneic stem cell transplantation; *ASCT*, autologous stem cell transplantation; *CR1*, first complete remission; *DFS*, disease-free survival; *HR*, high risk; *MRD*, minimal residual disease; *MSD*, matched sibling donor; *NRM*, nonrelapse mortality; *OS*, overall survival; *Ph*, Philadelphia chromosome; *SCT*, stem cell transplantation; *SR*, standard risk; *TBI*, total-body irradiation; *TRM*, transplant-related mortality.

least 9 out of 10 matched MUD sources. Summary of these studies is presented in Table 68.7.

Because the majority of older adults are not candidates for a myeloablative allogeneic stem cell transplant, reduced-intensity conditioning (RIC) approaches have been explored in this patient population (Table 68.8). The Seattle group reported outcomes of allogeneic stem cell transplant using nonmyeloablative conditioning in 51 patients with ALL (median age: 56 years), half of whom were Ph positive. For the Ph-negative group, the 3-year OS for those transplanted in CR1 was 52%. However, the incidence of chronic GVHD was high, at 44%.[43] A registry study examined the efficacy of RIC in 93 ALL patients and compared it with 1428 ALL patients receiving myeloablative conditioning for allogeneic stem cell transplant using either a sibling or URD in CR1/CR2. Interestingly, the TRM at 3 years was similar between the RIC and myeloablative regimens (32% vs. 33%, respectively). The relapse rate at 3 years was slightly higher in the RIC arm, although this was not statistically significant (35% vs. 26%; $P = .08$). The OS at 3 years was similar between the two groups (38% for RIC arm vs. 43% for myeloablative arm, $P = .39$). This was a retrospective registry study; however, it provides evidence that RIC regimens can lead to similar survival in adults with ALL as an allogeneic stem cell transplant with a more intensive, myeloablative conditioning regimen. This is particularly compelling because the median age was significantly older for those receiving RIC (median age in RIC arm: 45 years vs. 28 years for myeloablative arm). The group from Minnesota has also reported encouraging results with UCB donor transplantation using a reduced-intensity conditioning (RIC) regimen. Eighteen adults (median age: 49 years; range: 24 to 68 years) with high-risk ALL (majority Ph ALL) received a UCB transplant using a RIC regimen and reported 3-year OS, TRM, and relapse rates of 49%, 28%, and 33%, respectively. Notably, patients in CR1 had a TRM of only 8% with no events after 6 months. These results with UCB donor transplantation are promising and compare favorably with conventional allogeneic stem cell transplant with relatively low TRM in this high-risk population.[44] Thus RIC-based allogeneic stem cell transplant regimens with alternative donor sources should be considered for older adults participating in prospective clinical trials.

In summary, allogeneic stem cell transplant is a curative option for patients with ALL and can be considered the standard recommendation for patients with a good performance status but with adverse disease risk factors in CR1. There have been significant improvements in transplantation techniques (better supportive care, refinement of preparative regimens, and uses of alternative donors as a source of stem cells) that are resulting in greater availability and improved transplant outcomes. At the same time, the TRM and mortality are significant, and allogeneic stem cell transplant has largely been offered to patients only younger than 60 years of age because of the poor tolerance of older adults to standard myeloablative transplant preparative regimens. Prospective incorporation of MRD evaluation may refine the ability to risk-stratify patients who are at HR of relapse and might benefit from allogeneic stem cell transplant in CR1. However, the benefit of an allogeneic stem cell transplant for MRD-positive

TABLE 68.8 Clinical Trials Evaluating Role of Reduced-Intensity Conditioning Allogeneic Stem Cell Transplant in Acute Lymphoblastic Leukemia

Trial	Number of Patients	Age in Years, Median (Range)	Conditioning Regimen/Donor	Mortality	DFS/OS	Comments
Arnold[45]	22 (11 Ph^{+})	38 (21–58)	Fludarabine/busulfan ± ATG MSD or MUD	TRM: 45%	Median survival: 354 days	High TRM of the group; all patients with aGVHD had CR indicating toward GVL effect
Martino[46]	27 (11 Ph^{+})	50 (18–63)	Fludarabine/melphalan (most cases) MSD or MUD	2-year TRM: 23%	2-year OS: 31%	100-day acute grade II–IV GVHD: 48%; cGVHD 72% (extensive in 39%)
Hamaki[47]	33 (14 Ph^{+})	55 (17–68)	Different RIC regimens MSD or MUD	TRM: 27%	1-year OS: 39.6%	Grade II–IV aGVHD: 45%; cGVHD: 64%
Mohty[48]	97 (37 Ph^{+})	38 (17–65)	Different RIC regimens MSD or MUD	NRM: 28% (for those transplanted in CR1: 18%)	2-year OS: 31% (for those transplanted in CR1: 52%)	Three factors associated with improved OS:CR1 at time of transplant, chronic GVHD, and woman donor
Stein[49]	24 (10 Ph^{+})	47.5 (23–68)	Fludarabine/melphalan MSD or MUD	NRM at 2 years: 21.5%	OS at 2-years: 61.5%	75% aGVHD (62% grade II–IV aGVHD) 86% cGVHD (62% extensive)
Bachanova[44]	22 (14 Ph^{+})	49 (24–68)	Fludarabine/cyclophosphamide/TBI 200 cGy MRD ($n = 4$) or umbilical cord blood donor graft ($n = 18$)	TRM at 3 years: 27%	OS at 3 years: 50% (for CR1: 81%)	3-year OS for those in CR1 81% vs. 15% for those in CR2 or more ($P < .01$). For CR1: 8% TRM
Ram[43]	51 (25 Ph^{+})	56 (8–69)	Fludarabine/TBI 200 cGy MSD or MUD	NRM at 3 years: 28%	OS at 3-years: 34%	Grade II–IV aGVHD: 53% Chronic extensive GVHD: 42%

aGVHD, Acute graft-versus-host disease; *cGVHD*, chronic graft-versus-host disease; *ATG*, antithymocyte globulin; *CR1*, first complete remission; *CR2*, second complete remission; *DFS*, disease-free survival; *GVHD*, graft-versus-host disease; *GVL*, graft-versus-leukemia; *MSD*, matched sibling donor; *MUD*, matched unrelated donor; *NRM*, nonrelapse mortality; *OS*, overall survival; *Ph^{+}*, Philadelphia chromosome positive; *RIC*, reduced-intensity conditioning; *TBI*, total-body irradiation; *TRM*, transplant-related mortality.

patients remains to be proven and has been studied only prospectively in a single study to date. Conversely, patients who were MRD negative in CR1 may best be treated by chemotherapy alone. Overall, the role of allogeneic stem cell transplant in CR1 merits further study, and it is important for these patients to participate in clinical trials exploring such approaches.

Autologous Stem Cell Transplantation

The role of autologous transplant for patients with ALL is controversial. ASCT in CR1 has also been studied prospectively in several clinical trials. Dhedin and colleagues reviewed the data from the French LALA 85, 87, and 94 trials and compared the outcomes of patients in CR1 who were randomized to chemotherapy versus ASCT. There was no improvement in DFS or OS with the use of ASCT compared with postremission chemotherapy, but there was a lower incidence of relapse at 10 years (66% vs. 78%; $P = .05$).[50] In the MRC UKALLXII/ECOG E2993 trial, 456 patients were randomized to chemotherapy versus ASCT in CR1. Patients randomized to chemotherapy had significantly better 5-year EFS (41% vs. 32%; $P = .02$) and OS (46% vs. 37%; $P = .03$).[24] Thus outside of a clinical trial, an ASCT cannot be recommended for ALL patients.

THERAPY FOR SPECIFIC DISEASE SUBSETS

Philadelphia Chromosome–Positive Acute Lymphoblastic Leukemia

The Ph chromosome [t(9;22)(q34;q11)] is the most common cytogenetic abnormality in adult ALL, and its incidence increases with age. Among patients older than 60 years, 40% to 50% have the Ph chromosome. Before the introduction of molecularly targeted therapy, Ph^{+} ALL was associated with lower remission rates and a very poor prognosis, with a median survival time of only approximately 9 months.

With the introduction of therapy using TKIs targeted to the aberrant BCR-ABL protein, the outcome of Ph^{+} ALL has improved substantially. Imatinib, the first targeted TKI developed for use in Ph leukemia, has been combined with multiagent chemotherapy by various groups, and this has substantially improved both CR rates and DFS (Table 68.9).

The MD Anderson group was the first to report on the successful combination of a targeted TKI, imatinib, to frontline chemotherapy (hyper-CVAD). The 3-year CR duration and OS rates were significantly superior in the hyper-CVAD–imatinib arm compared with historical control participants treated with hyper-CVAD alone (68% vs. 24% and 54% vs. 15%, respectively; $P < .001$).[51,52] The Japanese also reported impressive improvements compared with their control participants. In the JALSG ALL202 trial, imatinib, 600 mg/day, was added to standard induction followed by alternating cycles of high-dose methotrexate and high-dose cytarabine and single agent imatinib 600 mg/day. Later maintenance therapy with imatinib 600 mg/day with monthly vincristine and prednisone was given. After longer follow-up, they reported results on 103 patients with Ph^{+} ALL with a 97% CR rate and 3-year OS of 57%, both of which were significantly better than in historical control participants.[53,54]

Older adults with Ph^{+} ALL have also demonstrated improved survival with the addition of imatinib to reduced-intensity chemotherapy regimens. Ottmann and colleagues reported for the German study group in which older adults (>55 years of age) Ph^{+} ALL patients were randomized to receive induction cycle with imatinib alone ($n = 28$) versus combination chemotherapy ($n = 27$)[58]. The CR rate was 96% in the imatinib arm, which was significantly better than the CR rate of 50% in the chemotherapy alone arm. This was the first prospective randomized controlled trial comparing imatinib with chemotherapy and clearly showed the superiority of the imatinib arm.[58] The Italian GIMEMA group treated 30 Ph^{+} ALL patients (median age: 69 years; range: 61 to 83 years)

TABLE 68.9 Clinical Trials With Tyrosine Kinase Inhibitor Plus Chemotherapy for Adult Philadelphia Chromosome–Positive Acute Lymphoblastic Leukemia

Trial	Patients (*n*)	Age in Years, Median (Range)	CR Rate	Disease-Free Survival	Overall Survival	Comments
MDACC Hyper-CVAD Imatinib[51,52]	54	51 (17–84)	93%	68% at 3 years	In de novo patients 40 years of age or younger: 3-year OS was 90% with aSCT ($n = 10$) vs. 33% without aSCT ($n = 6$), $P = .05$	First report of the combination of chemotherapy with a TKI Imatinib 400 mg/day on days 1–14 of induction; 600 mg continuously during courses 2–8; 600 mg during 2 years of maintenance therapy with monthly vincristine–prednisone; then imatinib indefinitely; aSCT in CR1 as feasible
JALSG ALL202[53,54]	103	45 (15–64)	97%	For those younger than age 55 years, 54 of 74 patients in CR1 who had aSCT, only 13% relapsed; among the 20/74 patients in CR1 who did not undergo SCT, 90% relapsed	57% at 3 years For patients younger than age 55 years, OS at 3 years was 75.0% for the transplanted group vs. 36.4% for the nontransplanted group	Standard induction plus imatinib 600 mg/day followed by alternating cycles of high-dose methotrexate and high-dose cytarabine and imatinib 600 mg/day; imatinib 600 mg/day with monthly vincristine–prednisone for maintenance
GRAAPH-2003[55,56]	45	45 (16–59)	96%	43% at 4 years	52% at 4 years The 4-year OS in the aSCT, ASCT, and no SCT groups were 55%, 80%, and 25%, respectively	Imatinib was started with consolidation in good early responders (corticosensitive and chemosensitive ALL) or during the induction course in poor early responders
GMALL[57]	47 (alternating imatinib and chemotherapy)	46 (21–65)	NA because only CR patients were eligible	52% at 2 years	36% at 2 years	Coadministration of imatinib with induction cycle 2 led to CR rate of 95% and molecular CR rate of 52% of patients compared with 19% (significantly worse) in patients in the alternating treatment cohort
	45 (concurrent imatinib and chemotherapy starting after induction I)	41 (19–63)	95%	61% at 2 years	43% at 2 years	
GMALL[58]	28 (imatinib arm)	66 (54–79)	96%	30% at 1.5 years	57% at 1.5 years	Patients older than age 55 years with de novo ALL and not eligible for aSCT; randomized to single-agent imatinib induction vs. multiagent chemotherapy induction; subsequent consolidation or maintenance with imatinib plus chemotherapy for all patients
	27 (chemotherapy arm)	68 (58–78)	50% (significantly inferior to imatinib arm)	35% at 1.5 years	41% at 1.5 years	
GIMEMA[59]	29	69 (61–83)	100%	48% at 1 year	74% at 1 year	7-day steroid pretreatment followed by induction treatment with imatinib (800 mg/day) plus steroids
NILG protocol 09/00[60]	59	45 (20–66)	92%	39% at 5 years	38% at 5 years	Imatinib (600 mg/day) was added to each chemotherapy course for 7 consecutive days, starting from day 15 of chemotherapy course 1 and from 3 days before chemotherapy during courses 2 to 8

Continued

TABLE 68.9 **Clinical Trials With Tyrosine Kinase Inhibitor Plus Chemotherapy for Adult Philadelphia Chromosome–Positive Acute Lymphoblastic Leukemia—cont'd**

Trial	Patients (*n*)	Age in Years, Median (Range)	CR Rate	Disease-Free Survival	Overall Survival	Comments
UKALLXII/ ECOG2993[61]	175	Not reported	92%	54% at 3 years	42% at 3 years	Outcomes (CR, DFS, and OS) were significantly better compared with the historical preimatinib cohort; for the imatinib group, 3-year OS for patients who received aSCT was 59% vs. 28% for those who did not receive aSCT
PACE[62]	32 (relapsed/TKI intolerant)	62	41%	7% at 12 months	40% at 12 months	Ponatinib is active in Ph$^+$ ALL with T135I mutation
GIMEMA LAL1811[63]	44	68 (27–85)	95%		87.5% at 1 year	Mostly elderly patients. Only 1 death attributed to ponatinib
Ponatinib HyperCVAD[64]	86	46 (21–80)	100%	84% at 3 years	73% at 5 years	18 (21%) underwent aSCT in CR1. Trend for better OS in patients who did not undergo HSCT in first remission
Dasatinib Blinatumomab[65]	63	54 (24–82)		88% at 18 months	95% at 18 months	Complete molecular response increased from 29% with dasatinib to 60% after two cycles blinatumomab
EWALL-PH-02[66]	36	66	97%	89% at median follow-up of 7 months		
CALGB 10001[67]	58	45	8 patients converted from MMR to CMR after transplant	Median DFS 5.9 months after auto-HSCT	Median OS 4.8 years. 47% OS with allo-HSCT vs. 42% OS with auto-HSCT	TRM associated with ASCT occurred in one (5%) of 19 patients, while TRM associated with aSCT occurred in 3 (20%) of 15 patients

ALL, Acute lymphoblastic leukemia; *aSCT*, allogeneic stem cell transplantation; *ASCT*, autologous stem cell transplantation; *CMR*, complete molecular response; *CR*, complete remission; *CR1*, first complete remission; *DFS*, disease-free survival; *HSCT*, hematopoietic stem cell transplantation; *MMR*, major molecular response; *NA*, not applicable; *OS*, overall survival; *Ph$^+$*, Philadelphia chromosome positive; *SCT*, stem cell transplantation; *TKI*, tyrosine kinase inhibitor; *TRM*, treatment-related mortality.

with 7-day steroid pretreatment followed by induction treatment with only imatinib (800 mg/day) plus steroids. Impressively, all 29 evaluable patients achieved CR with 1-year DFS and OS of 48% and 74%, respectively.[59] These early studies demonstrated that TKI therapy is crucial and that the TKI should be started early during induction chemotherapy and continued throughout postremission and maintenance therapy and, potentially, indefinitely.

Despite these significant advances in the treatment of Ph$^+$ ALL, relapse remains a major challenge. Resistance to imatinib develops and most frequently results from the emergence of a resistant clone with ABL kinase domain mutations, most commonly the T315I mutation. These kinase domain mutations cause conformational changes in the ABL protein that prevent effective binding. Other potential mechanisms of resistance that have been described include reduced intracellular availability of imatinib and activation of alternative signaling pathways such as the Src-kinase pathways. Second-generation TKIs, dasatinib and nilotinib, are more potent kinase inhibitors and are also effective against many imatinib-resistant mutations (although not the T315I mutation). In addition, dasatinib has been shown to penetrate the CNS and thus may be particularly effective in Ph$^+$ ALL.

Dasatinib has been tested in several prospective trials for previously untreated Ph$^+$ ALL. MD Anderson Cancer Center investigators evaluated hyper-CVAD plus dasatinib in newly diagnosed Ph$^+$ ALL patients. The median age of the 35 patients studied was 53 years (range: 21 to 79 years); 94% achieved CR. Of the patients who achieved CR, 61% of patients achieved CMR, meaning that there was no detectable MRD using quantitative PCR for *BCR-ABL*. Later, the same group reported that 24% of patients underwent allogeneic stem cell transplant (16% in CR1; 8% CR2), and 3-year DFS and OS for the entire cohort were 49% and 62%, respectively.[68]

In the GIMEMA LAL1205 study, dasatinib monotherapy (70 mg twice daily) with steroids was investigated for frontline treatment of 53 adult Ph$^+$ ALL patients (median age: 54 years). All patients achieved a complete hematologic response, and there were no induction deaths. Allogeneic stem cell transplant in CR1 was performed in 18 (34%) patients. At 20 months, the OS was 69% and DFS was 51%. BCR-ABL levels of less than 0.001% at day 85 correlated with DFS. These results with dasatinib without any cytotoxic chemotherapy are encouraging but demonstrated that single-agent dasatinib is not sufficient to cure this disease[69]. In a subsequent trial, the GIMEMA LAL1509, if patients did not achieve complete molecular response after a prolonged 84-day induction with dasatinib and steroids, they received more intensive combination chemotherapy and/or allogeneic stem cell transplant. At day 85, 58 out of 60 (97%) patients achieved a complete hematologic remission; however, only 11 (18.6%) patients achieved a CMR with TKI plus steroid induction, suggesting that dasatinib and steroids are insufficient to achieve a molecular remission in most patients and that additional postremission intensification is needed. Using this stratified treatment approach, the DFS was 62% with 18 months of follow-up and OS was 69%.[70]

Nilotinib is also a second-generation TKI that has been evaluated as frontline therapy. In a prospective, single arm phase II trial, induction treatment consisted of vincristine, daunorubicin, oral prednisolone, and nilotinib (400 mg orally twice daily). Patients in CR received either five courses of consolidation followed by 2-year maintenance therapy or an allogeneic stem cell transplant, depending on donor availability. More than 70% of patients received an allogeneic stem cell transplant. Estimated DFS and OS at 2 years were 71% and 66%, respectively, similar to rates observed with other TKIs.[66] Although these results are similar to other studies with dasatinib, most of the current trials in Ph$^+$ ALL have incorporated dasatinib in the frontline therapy because of its potential advantage over nilotinib with respect to CNS penetration.

Ponatinib is a third-generation ABL kinase inhibitor that maintains ABL kinase inhibitory effects even in the presence of the T315I mutant kinase, which has been the most commonly occurring mutation at time of relapse in Ph ALL, and is approved for relapsed disease based on data from the PACE trial.[62] In the chronic myeloid leukemia blast phase/Ph$^+$ ALL cohort, 37% (11 out of 30) of patients who were resistant or intolerant to second-generation TKIs and 27% (6 out of 22) of patients with T315I mutations achieved a major hematologic remission.

Ponatinib has also been studied for frontline treatment of Ph$^+$ ALL, both in combination with intensive chemotherapy and in combination with low dose chemotherapy. In a study investigating ponatinib combined with hyper-CVAD, 45 mg was given daily for the first 14 days of cycle 1 and then continuously for the subsequent cycles.[64] The CMR rate was 84%. The estimated 5-year continuous CR, EFS, and OS rates were 84%, 68%, and 73%, respectively. Two patients experienced ponatinib-related deaths from myocardial infarction. Therefore, due to concern for vascular toxicity associated with long-term use of high-dose ponatinib, the protocol was amended to reduce ponatinib to 30 mg daily starting at cycle 2, with further reduction to 15 mg once a CMR was achieved. In a propensity score analysis, ponatinib hyper-CVAD therapy was superior to the historical control dasatinib and hyper-CVAD. The 3-year EFS rates for ponatinib hyper-CVAD and dasatinib hyper-CVAD were 69% and 46%, respectively, and the 3-year OS rates were 83% and 56%, respectively.[71] Using ponatinib with minimal chemotherapy, the GIMEMA LAL1811 investigated ponatinib with steroids (without allogeneic transplant) in untreated older adults with Ph$^+$ ALL patients. Steroids were administered from day −14 to day 29 during course 1. Despite this being an older population that may have cardiovascular comorbidities, patients received ponatinib 45 mg daily for eight consecutive courses of 6 weeks. Only one death on study was attributed to ponatinib. Ninety-five percent of patients achieved CR after course 1. Sixty percent of evaluable patients were in CMR at 24 weeks (end of course 4). OS was 87.5% at 1 year follow-up. Although the rate of CMR in this study clearly exceeds those of prior dasatinib and steroids studies, longer-term follow-up is necessary to determine if patients can safely receive high-dose ponatinib for 48 weeks and if this results in meaningful improvement in OS.[63] Further definition of the incidence of serious vascular events with ponatinib in the frontline treatment setting of Ph ALL is required with longer-term follow-up of these studies. However, these studies have demonstrated high rates of CMR compared with other TKIs, and ponatinib is being used increasingly in the frontline treatment of Ph$^+$ ALL.

Recently immunotherapies have been added to TKIs in Ph$^+$ ALL as another approach to minimize toxicity and enhance response rates. In the first effort to investigate this approach, blinatumomab (a CD19 bispecific T-cell engager [BITE]) was added following dasatinib and prednisone induction. High CMR rates were achieved, and with a median follow-up of 18 months, OS was 95% and DFS was 88%. The regimen was well tolerated, with fever, neutropenia, and cytomegalovirus (CMV) reactivation being most common adverse events.[65] Based on these exciting results with a second-generation TKI, studies are underway that evaluate ponatinib and blinatumomab with low-intensity chemotherapy in newly diagnosed Ph ALL.

Role of Transplantation for Philadelphia Chromosome–Positive ALL in the Era of Tyrosine Kinase Inhibitor–Based Therapy

Allogeneic stem cell transplant in CR1 was the only curative treatment option for Ph$^+$ ALL in the pre-TKI era. Although the addition of TKI therapy has significantly improved the outcome of Ph$^+$ ALL, many recent studies have also highlighted the continued role of allogeneic stem cell transplant for Ph$^+$ ALL for eligible patients, at least when a first or second generation TKI is used.[67,72] In a landmark analysis, in patients under 60 years of age who received allogeneic transplant following intensive chemotherapy with dasatinib, allogeneic transplant showed statistically significant improvements in RFS and OS compared with those who were not transplanted in CR1 with 12 month OS of 87% in the transplanted group.[73] Potentially the use of a third-generation TKI and/or of frontline immunotherapy with TKI will obviate the recommendation for allogeneic transplant. In the hyper-CVAD with ponatinib study mentioned previously, the 3-year OS rate was 66% for patients who underwent allogeneic stem cell transplant and 90% for patients who did not undergo allogeneic stem cell transplant; however, this was not statistically significant.[64]

The role of autologous transplantation for Ph$^+$ ALL has also been explored and remains uncertain as the field advances with new TKIs and immunotherapies.[67,72] In a study of 64 adults with a median age of 60 years, dasatinib with dexamethasone induction followed by allogeneic, autologous, or continued chemotherapy with TKI yielded similar 3-year survival approaches; however, patients with the P210 isoform had poor outcome.[72] Finally, the role and duration of TKI therapy after intensive chemotherapy and transplant is currently an area of active investigation. Currently, it appears that continuing TKI therapy indefinitely for those patients receiving chemotherapy alone and for a minimum of 1 to 2 years after transplant is an appropriate strategy.[74] Studies evaluating MRD may provide guidance with the question of duration of posttransplant TKI therapy.

In summary, during the past two decades the addition of TKIs to combination chemotherapy has resulted in significant improvement in DFS and OS for both younger and older adults with Ph$^+$ ALL. High CMR rates can now be achieved and have been demonstrated to be associated with improved outcomes. While allogeneic stem cell transplant remains the current treatment of choice for eligible patients, the next generation of studies in Ph$^+$ ALL may help to define the role of transplantation when CMRs are achieved with combination TKI and chemotherapy alone.

Burkitt Lymphoma/Leukemia

Burkitt lymphoma (BL) is a mature B-cell lymphoma with an extremely short doubling time that often presents in extranodal sites or as acute leukemia. Thus a brief description of the therapy is warranted in this chapter. Morphologically (Fig. 68.8), the malignant cells are intermediate in size, have round nuclei with small nucleoli, are intensely basophilic, and frequently have vacuolated cytoplasm. In a BM biopsy and in tissue sections, the tumor is classically described as having a “starry sky” appearance because of the presence of multiple tingible body macrophages with phagocytized cellular debris. Immunophenotypically, the tumor cells express moderate to strong levels of surface IgM with light chain restriction, indicating origin of the tumor from a mature B cell. The tumor cells also universally express the B-cell antigens CD19 and CD20 and germinal center–associated markers such as CD10 and BCL6. Unlike B-ALL, BL cells do not express CD34 or TdT. The genetic basis of the disease is the underlying MYC translocation at band 8q24 to either the Ig heavy chain region on chromosome 14 or less commonly at the lambda (22q11) or kappa (2p12) loci.

Prompt diagnosis and recognition of this entity are essential because this is now a highly curable leukemia (in the range of 65% to 80% in recent trials); however, failure to institute appropriate therapy at diagnosis for BL results in emergence of early resistance and

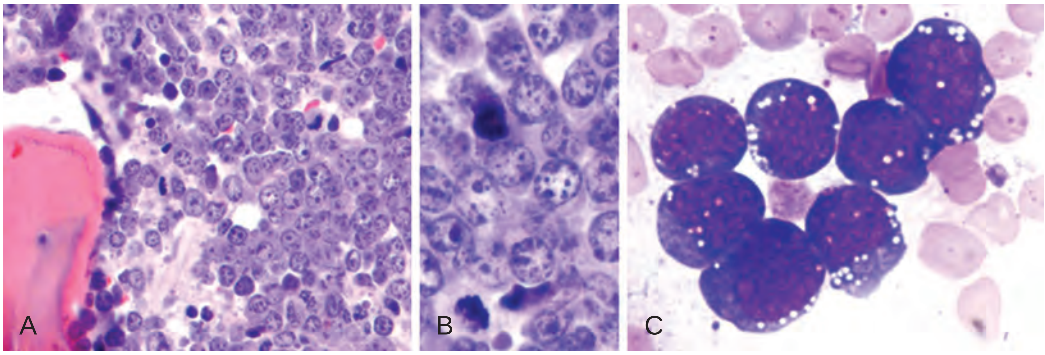


Figure 68.8 BURKITT LEUKEMIA/LYMPHOMA (A–C). Bone marrow biopsy and aspirate features of Burkitt leukemia/lymphoma are illustrated. The bone marrow (or lymph node) will show sheets of highly proliferating intermediate-sized neoplastic cells with a syncytial appearance. The cells are monotonous but have a stippled intermediate chromatin pattern with multiple small nucleoli. (C) On the aspirate, the Burkitt cells have an intermediate size, a denser chromatin than lymphoblasts, and deeply blue cytoplasm with prominent vacuoles.

dismal outcomes. Traditional CHOP (cyclophosphamide, hydroxydaunomycin, vincristine [Oncovin], and prednisone) chemotherapy is inadequate and should not be used. Treatment should be initiated quickly and consists of aggressive combination chemotherapy with CNS prophylaxis. These patients are at HR for the development of tumor lysis syndrome; therefore aggressive hydration and administration of allopurinol and/or rasburicase is important. All current regimens rely on short intensive courses of chemotherapy that incorporate fractionated doses of alkylating agents, high doses of methotrexate and cytarabine, and intensive intrathecal prophylaxis. In the United States, commonly used regimens include CODOX-M/IVAC (cyclophosphamide, vincristine, doxorubicin, high-dose methotrexate/ifosfamide, etoposide, and high-dose cytarabine) CALGB 9251, and hyper-CVAD. With these regimens, approximately 80% achieve CR, and 2-year survival is approximately 60% to 70%. In the CALGB 9251 trial, 52% of the patients were alive and in continuous CR at a median follow-up of 5.1 years. Because BL has a predisposition for CNS involvement, aggressive CNS-directed therapy is essential and typically consists of intrathecal administration of methotrexate, cytarabine, and hydrocortisone. Recurrence after the first 2 years rarely occurs; therefore maintenance therapy has not been shown to be beneficial and is not recommended.

Because of the strong expression of CD20 in mature B-cell ALL, several groups have incorporated rituximab into the frontline chemotherapy regimen, with impressive further improvements in survival. The hyper-CVAD–rituximab regimen was reported in 31 patients with newly diagnosed BL, with a CR rate of 86% and a 3-year survival rate of 89%. Similarly, the addition of rituximab to the treatment in both the GMALL and CALGB regimens has proven feasible with OS of 91% (3 years) and 79% (2 years), respectively. Based on these exciting improvements in survival, the addition of anti-CD20 targeting to frontline therapies for BL is now considered the standard of care.

ADOLESCENTS AND YOUNG ADULTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA: THE INTERSECTION BETWEEN PEDIATRIC AND ADULT CARE

Evaluation of the outcomes of young adult patients, here defined as patients between the ages of 15 and 39 years, presents specific challenges. Because of community referral patterns, these patients may be treated by either pediatric or adult oncologists. As such, the treating physician may view a patient in this age group either as an older child or as a younger adult. The oncologist will choose a regimen most appropriate for the population usually seen by that particular physician. A number of retrospective comparisons of the clinical outcome of adolescents enrolled on adult and pediatric clinical trials have resulted in interesting observations about what that appropriate treatment regimen should be and have guided the design of a number of prospective clinical trials designed specifically for AYAs with ALL and typically include patients from 15 to 39 years of age. The majority of these retrospective comparison studies demonstrated a significant survival advantage for AYA patients treated by the pediatric versus the adult cooperative group.[75] Although the specific treatment approaches differ among countries, several treatment themes have emerged as being potentially important. Pediatric studies throughout the world use considerably more treatment with nonmyelosuppressive drugs, including glucocorticoids (both dexamethasone and prednisone), vincristine, and L-asparaginase. CNS prophylaxis is typically administered earlier, with a greater frequency, and for a more prolonged period during pediatric group trials. Finally, long-term maintenance therapy was also continued for a longer period in pediatric cooperative group trials. Based on additional retrospective data from Finland and the MD Anderson Cancer Center, it appears that treating patients in a uniform fashion by an experienced group of physicians and nurses is also a very important component in the successful treatment of young adults with ALL.

To address the many unanswered questions that have been raised by the retrospective comparison trials and to determine whether AYA patients treated by adult hematologists and oncologists can achieve similarly improved outcomes to the pediatricians for this age group, prospective cooperative group trials have been conducted in North America and in Europe and validate the results of retrospective studies—AYAs fare better with intensive pediatric regimens, and several of these trials[76–78] are summarized in Table 68.10 and additionally reviewed in reference 75. A few important points about these prospective studies can be made. (1) Use of a pediatric regimen is feasible and results in low treatment related mortality of less than 5%; however, morbidity and mortality rates increase over age 45 to 50 years, limiting the exact use of these regimens in older adults. (2) EFS and OS are significantly improved compared with historical controls, with survival rates routinely now greater than 70% for both B- and T-cell ALL. (3) Outcomes were equivalent for both B- and T-cell ALL. (4) Achievement of MRD negativity in early treatment is the most important prognosticator of DFS and may inform design of future clinical trials. The design and the results of the Nordic Society of Pediatric Hematology and Oncology (NOPHO) ALL2008 highlight some of these important issues.[78] This was the first study of newly diagnosed Ph-negative B- and T-ALL patients that treated all patients ages 1 to 45 years on a single risk-adapted protocol. They reported a 4-year EFS of 84% in those aged 16 to 39 years, which is superior to both the Dana Farber Cancer Institute and CALGB results. Their protocol was less intensive during induction and therapy for those with SR but had more intensive postremission therapy for those with higher risk, largely defined by MRD status at the end of induction. This illustrates a remarkable effort to successfully perform an innovative protocol that aimed to coordinate care of children, AYAs using rigorous monitoring and strong centralized support to facilitate treatment adherence.

TABLE 68.10 Outcomes of Adolescent and Young Adult Patients Treated With Pediatric Intensive Regimens: Several Examples of Recent Trials

	ALL-96 (PETHEMA)[79]	DFCI Adult ALL 01–175 [76]	CALGB 10403 (NCTN)[77]
Patient Population			
Patients (*n*)	81	92	318
Median age (range)	20 years (15–30)	28 years (18–50)	24 years (17–39)
Gender	Male: 62%	Male: 61%	Male: 61%
Immunophenotype	Precursor B- and T-ALL	Precursor B- and T-ALL	Precursor B- and T-ALL
Outcomes			
EFS	61% (6 year)	58% (4 year)	66% (2 year)
OS	69% (6 year)	67% (4 year)	78% (2 year)

ALL, Acute lymphoblastic leukemia; *EFS*, event-free survival; *OS*, overall survival.

While these prospective trials demonstrate that an intensive pediatric regimen results in improved survival rates for AYAs, many challenges remain to ensure access to care, to minimize treatment toxicity, and to develop and follow specific survivorship monitoring plans. As stated previously, the successful outcome of a patient with ALL is, largely, based on the ability of both the medical team and the patient to adhere to the rigorous and lengthy regimens that currently remain the gold standard of care. The next generation of studies, linked to important biologic, psychosocial, and pharmacologic correlates, will result in further insights into optimizing the comprehensive approach to treatment and follow-up for this significant group of patients with ALL and may be the next step to achieving the high cure rate and successful transition back to "normal" life now routinely achieved in children with ALL. In particular, due to significant pharmacogenomic variability and age-related changes in drug metabolism, further evaluation and potential dose modifications of some of the agents, including glucocorticoids, vincristine, and asparaginase, crucial to the successful outcomes of children, will need to be addressed in future studies of AYA and older adults with ALL, to optimize these regimens for these populations. Enrollment in clinical trials, which has led to the survival rates now approaching more than 90% in pediatric ALL, will be the key to making progress in survival rates for both younger and older adults with ALL.

Differences in Treatment for B- Versus T-Cell Acute Lymphoblastic Leukemia

To date, different phenotypes of ALL (Ph-negative B-ALL and T-ALL) have been treated with the same management approach, as described earlier. Aforementioned studies have also elucidated whether there are outcome differences based on immunophenotype, and what implications this may have on future therapy development as our understanding of the underlying biology evolves.

The Children's Oncology Group (COG) AALL0434 study enrolled exclusively T-ALL patients aged 1 to 31 and showed improved DFS when nelarabine was added to an augmented BFM regimen with a 4-year DFS of 88.9% as compared with 83.3% without nelarabine,[80] largely as a result of reduction in the number of CNS relapses. This has not yet been prospectively tested in adults older than the age of 30 years, but given these encouraging results, it is possible that future treatment of T-ALL will incorporate nelarabine into frontline therapy, similar to what was done in the COG study.

Emerging data on differences in the molecular genetic underpinnings of T- versus B-ALL and the heterogeneity within each of these subsets will undoubtedly lead to more individualized "targeted" approaches (see Novel Therapies later). As these new approaches are incorporated into novel frontline treatment trials, it may be possible to eradicate resistant subclones that give rise to relapse and result in higher survival rates.

Comparison of Pediatric-Inspired Regimens With Allogeneic Transplant for Adolescents and Young Adults

Given these very encouraging improvements in outcome for AYAs with ALL using intensified pediatric regimens, the role of allogeneic stem cell transplant in CR1 has been reexamined in two retrospective comparison studies[81,82]. These investigators reported significantly better outcomes for patients receiving the intensive pediatric approaches compared with transplant. Overall, treatment using a pediatric-inspired regimen for young adults with ALL appears to result in significantly improved survival compared with allogeneic stem cell transplant in CR1. Similarly, data from the GRAALL pediatric-inspired protocols showed that there was no difference in relapse, nonrelapse mortality, or RFS overall in patients who went onto allogeneic stem cell transplant compared with those who did not undergo transplant.[18] However, allogeneic stem cell transplant did result in a longer RFS in patients who were MRD positive after induction chemotherapy, suggesting there is a select HR group of patients who still benefit from transplant.

OLDER ADULTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA

Older adults (generally defined as older than 60 years old) constitute a challenging subset of ALL patients. They have a worse prognosis as compared with their younger counterparts. Most studies demonstrate that adults older than 60 years old have survival rates below 15%. Various factors contribute to the poor outcome of these patients, both patient related (e.g., the presence of comorbidities, less likely to be eligible for allogeneic stem cell transplant, poor tolerance of intensive chemotherapies) and disease related (more likely to have unfavorable disease characteristics such as the presence of the Ph chromosome, complex karyotype, and genetic alterations such as TP53 and IKZF1 mutations). It is important to note that many large clinical trials have excluded patients older than 60 years of age; therefore clinical outcome data for this group are sparse. In historical trials, although CR rates for older adults range from 60% to75% with traditional intensive combination therapies, due to treatment-related complications and disease resistance, OS at 3 to 5 years has been poor, with fewer than 15% to 20% achieving long-term survival (for review and analysis, see Reference 83).

One of the major problems with application of traditional BFM therapies in older adults has been the unacceptable toxicity from the key agents—asparaginase, prednisone/dexamethasone, and vincristine. The GMALL successfully introduced dose-reduced PEG asparaginase 500 IU/m^2 in postinduction phases and has reported on its safety and encouraging results in patients 75 years old or younger.[84] A modified protocol based on the DFCI ALL regimen was applied to older patients with ALL (older than 50 years old). The study met the primary end point, with a 1-year OS of 63%. However, the administration of the standard dose PEG asparaginase 2000 IU/m^2 resulted in a high rate of hyperbilirubinemia, and this led to amending the PEG asparaginase dose twice during the study course, down to only 500 IU/m^2. In a smaller study of a modified pediatric regimen in adults up to the age 70 years (median = 46 years), therapeutic drug monitoring of asparaginase levels was used to optimize dosing of asparaginase, a dose of 1000u/m^2 resulted in therapeutic levels in all patients and 3-year EFS of 54%.[85,86] These studies suggest that it may be possible to modify intensive pediatric regimens with some reduction in toxicity; however, new approaches may be changing the treatment paradigm for older adults.

Given the poor prognosis of older adults with ALL and difficulty tolerating intensive chemotherapy regimens, other studies are also investigating the use of the immunotherapies blinatumomab or inotuzumab ozogamicin, which are approved for relapsed disease, in the frontline setting with or without reduced intensity chemotherapy. These new trials result in high CR rates and improved EFS in early studies with limited long-term follow-up. The MD Anderson group used inotuzumab ozogamicin in frontline therapy to allow combination with reduced-intensity chemotherapy in frontline treatment of adults 60 years old or older with ALL. Patients received mini-hyper-CVD (cyclophosphamide and dexamethasone at 50% dose reduction, no anthracycline) for up to eight cycles. Median age was 68 years, and notably 41% of patients had a p53 mutation. Ninety-eight percent of patients achieved a CR, CRp (complete remission with incomplete platelet recovery), or CRi (complete remission with incomplete hematologic recovery), and 80% of these patients were MRD negative after the first cycle. No early deaths were reported. The 4-year continuous remission and OS rates were 78% and 50%, respectively. However, 4-year OS was only 34% in patients 70 years old or older, which was attributed to higher rates of infection and therapy-related MDS or AML. To decrease treatment-related mortality, the protocol has been amended to eliminate chemotherapy for patients 70 years of age or older. In addition, 4-year OS was only 22% in patients with HR cytogenetic abnormalities; additional studies are warranted.[87] The Southwest Oncology Group (SWOG) 1318 clinical trial studied blinatumomab as a single agent in newly diagnosed Ph$^-$ ALL, as well as in combination with dasatinib in Ph$^+$ ALL, in patients older than 65 years old. Patients received up to five cycles of blinatumomab followed by 18 months of POMP maintenance chemotherapy.[88] Among the Ph$^-$ patients, the median age was 75 years old and no patients died during the first 28 days of induction. Sixty-six percent of patients achieved a CR or CRi with single agent blinatumomab induction. At 1 year the DFS was 56% and OS was 65%.[77] The regimen was well tolerated and resulted in a 3-year OS of 34%. Additional ongoing studies both in Europe and in the United States are taking this approach one step further to evaluate the use of the new antibodies to eliminate entirely standard chemotherapy in older adults with ALL; the results of these trials are eagerly awaited. Consideration should be given to enrollment of older adults into these new frontline clinical trials, given the lack of progress with dose intensification of traditional agents and interest in reduced chemotherapy and addition of immunotherapies. Management of older adult patients with Ph$^+$ ALL is discussed in the Ph$^+$ ALL section; the incorporation of TKIs into frontline therapy has already made significant improvements in DFS for these high-risk patients.

RELAPSED ACUTE LYMPHOBLASTIC LEUKEMIA

As described earlier, with standard therapies for ALL, long-term DFS is only approximately 30% to 40%. Thus the majority of patients with ALL relapse despite the administration of postremission treatment. Until recently postrelapse therapies have resulted in CR2 in 30% to 40% of patients with a 5-year OS of only approximately 10%.[89–91] However, new immune-targeting approaches have revolutionized the approach to relapsed B-ALL and are resulting in high CR rates of 60% to 80% in patients with relapsed disease. An allogeneic stem cell transplant is still considered the standard potentially curative approach for all eligible patients in CR2 and beyond.

Vincristine is an important component of ALL treatment. In preclinical models, encapsulation of vincristine into sphingomyelin liposomes or “sphingosomes” has been shown to lead to increased efficacy without increased neurotoxicity compared with conventional vincristine. Sphingosomal vincristine administered every 2 weeks in relapsed or refractory ALL reported an overall response rate of 14%. A weekly schedule resulted in a higher CR rate of 19% overall and 29% in first salvage treatment.[92] Based on an improved toxicity profile and comparable efficacy in relapsed/refractory (RR) ALL, particularly as a single agent, in 2012 the FDA approved vincristine sulfate liposome injection (VSLI) for the treatment of adult patients with Ph-negative ALL in second or greater relapse or whose disease has progressed following two or more antileukemia therapies. However, with the advent of the targeted approaches described later, remission rates are now much higher for relapsed B-ALL.

Blinatumomab is a novel BITE antibody targeted against CD3 on T cells and CD19 on leukemia B cells. When blinatumomab concurrently binds CD3 and CD19, the T cells are activated and directed to CD19-expressing target B cells, resulting in a cytotoxic T-cell response. A pivotal phase III study of blinatumomab compared with chemotherapy that randomized patients with relapsed CD19$^+$ ALL in a 2:1 fashion. Blinatumomab demonstrated higher rates of CR, CRp, or CRi (44%) compared with chemotherapy (25%).[93] The study met its primary end point and confirmed median OS was significantly improved to 7.7 months in the blinatumomab group compared with 4.0 months in the chemotherapy group. Treatment with blinatumomab also resulted in a higher EFS than that with chemotherapy (6-month estimates, 31% vs. 12%). The unique side effects of blinatumomab that require special attention are cytokine release syndrome (CRS) and neurologic toxicity. Grade 3 or higher CRS and neurologic events occurred in only 5% and 9%, respectively, of patients. Eighty-eight percent of all CR/CRh responders achieved MRD negativity.[93,94] The FDA approval for blinatumomab also includes Ph$^+$ ALL. Another targeted approach is inotuzumab ozogamicin, which is a humanized anti-CD22 monoclonal antibody conjugated to calicheamicin, a cytotoxic antibiotic agent. Upon binding CD22 on the B-ALL blast surface, inotuzumab ozogamicin is rapidly internalized, and the link between the antibody and calicheamicin is cleaved, releasing calicheamicin into the cell. Calicheamicin binds to the minor groove of DNA and thus induces double-strand cleavage and subsequent apoptosis. A phase III trial randomized patients with Ph$^+$ and Ph$^-$ ALL in first or second relapse to receive inotuzumab ozogamicin or standard salvage chemotherapy.[95] The CR rate was significantly higher with inotuzumab ozogamicin with chemotherapy (81% vs. 29%). For those who achieved CR, 78% achieved MRD-negative response with inotuzumab ozogamicin, compared with only 28% with chemotherapy. Although PFS and OS with inotuzumab ozogamicin did improve upon chemotherapy, they were still unfortunately limited (5 months vs. 1.8 months, and 7.7 months vs. 6.7 months, respectively). The most serious nonhematologic adverse events with inotuzumab ozogamicin were liver-related. Venoocclusive liver disease of any grade occurred in 15 patients (11%) who received inotuzumab ozogamicin and in 1 patient (1%) who received standard therapy. Based on these results, inotuzumab ozogamicin was FDA approved for Ph$^+$ and Ph$^-$ B-ALL in 2017. Thus, although both blinatumomab and inotuzumab can result in high CR rates, remission duration is short and additional therapy such as allogeneic transplant still must be considered once remissions are achieved.

The newest approach to relapsed B-ALL is the approval of chimeric antigen receptor (CAR)-T cells which are genetically modified T cells that express antigen-binding moieties and T-cell activation domains. The basic structure of modern CARs includes an extracellular antigen binding domain, an intracellular T-cell signaling domain, and one or two intracellular T-cell costimulatory domains. The costimulatory domains play an important role in CAR-T-cell persistence. Two products for CD19$^+$ ALL have now been approved: one for children and young adults up to age 25 and one for adults with relapsed B-ALL. Tisagenlecleucel (formerly CTL019) is an anti-CD19 CAR-T-cell therapy that was investigated in a phase II study in children and young adults up to the age of 25 years with RR B-ALL.[96] Sixty-one percent of patients had relapsed after prior allogeneic stem cell transplant. The overall remission rate within 3 months was 81%, with all patients who had a response to treatment found to be negative for MRD. The rates of EFS and OS were 50% and 76%, respectively, at 12 months. The median duration of remission was not reached. Persistence of tisagenlecleucel in the blood was observed for as long as 20 months. In adults, KTE-X19 (brexucabtagene autoleucel) was approved in 2021 based on the results of a phase II trial of 71 patients with relapsed CD10$^+$ ALL and resulted in a 71% CR/CRi rate with a median relapse-free survival of 11.6 months and OS of 18.2 months (not reached among responders); 97% of responding patients achieved MRD-negative status.[97] Although CAR-T cells are highly effective in disease eradication, they are also associated with complicated toxicities, including serious CRS and neurologic toxicities, that require oversight in specialized medical centers. Severe neurologic events occurred more frequently in patients with higher-grade CRS. The requirement for allogeneic transplant for long-term survival following CAR-T-cell therapy–induced remissions remains an open question. New approaches to CAR-T therapy are evolving rapidly and are discussed briefly in the novel therapy section later.

The targeted antibody approaches have not yet been developed for T-ALL, although efforts are ongoing. To date, the only approved agent in adults with relapsed T-ALL is nelarabine, a prodrug that is demethylated by adenosine deaminase to the deoxyguanosine analog (ara-G). T lymphoblasts are sensitive to the cytotoxic effects of nelarabine. In the study that resulted in approval of nelarabine, 26 patients with relapsed T-ALL (age range: 16 to 66 years) were treated. The CR rate was 31%, with a 1-year OS of 28%. Neurotoxicity was seen in many patients.[98] Nelarabine is currently approved in the United States for both pediatric and adult patients with T-cell ALL and T-cell lymphoblastic lymphoma who have failed at least two chemotherapeutic regimens.

Despite the approval of these new agents, patients with relapsed ALL continue to be a challenging subgroup of patients, in particular because the duration of response is limited to months. Importantly, these agents approved for RR ALL are now entering trials in the frontline setting, with the hope that they will decrease relapse rates, obviate the need for rescue therapy, and improve OS in ALL.

NOVEL THERAPIES

Immunotherapy

Novel Chimeric Antigen Receptor T-Cell Therapies

Potentially one of the most exciting breakthroughs in ALL therapy is the development and results from trials evaluating CAR-T cells (Table 68.11). Two CD19 CAR-T-cell products are now FDA approved for relapsed disease as described earlier. One of the challenges of multiple CD19-directed immunotherapies such as blinatumomab, tisagenlecleucel, and brexucabtagene autoleucel is the occurrence of relapse with CD19-negative disease, or antigen escape by the ALL. Several research groups are developing bispecific CAR-T cells to concurrently target CD19 and CD22, or other antigens such as CD20, to mitigate this risk. A CD19 and CD22 bivalent CAR with a 41BB costimulatory domain in a phase I trial in pediatric and adult RR ALL and DLCBL has been published, reporting good tolerability and high MRD-negative CR rates (88% of the 100% of CR patients) for patients with B-ALL[99]; however, CD19-negative relapses still occurred that were not “salvaged” by the CD22 target. This and other early-phase dual targeting CAR-T-cell trials highlight the ongoing challenges of engineering multispecific CAR-T cells with equivalent potency across targets.

Another focus of CAR-T-cell research is allogeneic products that may help to shorten the time to treatment that is required for apheresis and manufacturing of autologous products. UCART22 is an example of a genetically modified T-cell product manufactured from non-HLA-matched healthy donor cells. Donor-derived T-cells are transduced using a lentiviral vector to express anti-CD22 CAR (anti-CD22 scFv-41BB-CD3ζ) and are further modified to disrupt the T-cell receptor alpha constant (TRAC) and CD52 genes. These modifications minimize the risk of GVHD and allow the use of anti-CD52–directed drugs following a lymphodepleting chemotherapy regimen.[100] Another area of intense interest is development of CAR-T-cell therapy directed to T-ALL, which poses a particular challenge due to the very real risk of host-engineered T-cells killing themselves, known as “fratricide.” Significant engineering has taken place using CRISPR (clustered regularly interspaced short palindromic repeats) and other techniques to avoid these issues, and the early CD7 CAR-T-cell trials are now being reported with promising early results in patients with highly refractory disease T-ALL.[101]

Daratumumab

As discussed earlier, therapy for relapsed T-ALL remains an unmet need. CD38 is a type II transmembrane glycoprotein expressed on immature T and B lymphocytes, natural killer (NK) cells, activated T cells, plasma cells, monocytes, and red blood cells. CD38 is strongly expressed by B- and T-ALL blasts. Daratumumab is a human IgG1 κ monoclonal antibody that binds CD38 and has been demonstrated to be safe and effective in patients with multiple myeloma. Daratumumab demonstrated efficacy in vitro and in vivo in T-ALL xenograft models.[102] Furthermore, daratumumab cleared MRD in T-ALL xenograft models as a single agent and when used in combination with chemotherapy. The degree of CD38 expression on cells did not correlate with daratumumab response. Finally, recent case reports demonstrated daratumumab efficacy in several T-ALL patients with RR MRD after allogeneic stem cell transplant.[103] Daratumumab is currently under investigation in combination with chemotherapy in

TABLE 68.11 Immunotherapies in Relapsed/Refractory Acute Lymphoblastic Leukemia

Trial	Cell Surface Antigen Target and Agent	Class	Disease Status	*N*	CR (%)	MRD Negativity[a] (%)	Survival Outcomes
	CD19						
Kantarjian[93]	Blinatumomab	BITE	RR	271	119 (44%)	90 (33)	Median OS 7.7 months
Maude[96]	Tisagenlecleucel	CAR-T cell	Pediatric RR	75	61 (81)	61 (81)	OS 76% at 12 months
Shah[97]	Brexucabtagene autoleucel	CAR-T cell	RR	71	39(71)	38(97%)	Median OS 18.2 months
	CD22						
Kantarjian[95]	Inotuzumab ozogamicin	Antibody-drug conjugate	adult RR	49	28 (57)		Median OS 7.9 months

[a] MRD in ALL refers to the presence of leukemic cells less than the threshold of detection by conventional morphologic methods. Patients who achieved a CR by morphologic assessment alone can potentially harbor a large number of leukemic cells in the bone marrow. Current multicolor flow cytometry or PCR methods can detect leukemic cells at a sensitivity threshold of $<1 \times 10^{-4}$ ($<0.01\%$) bone marrow mononuclear cells.

ALL, Acute lymphoblastic leukemia; *BITE*, bispecific T-cell engager; *CAR-T cell*, chimeric antigen receptor–T cell; *CR*, complete remission; *DOR*, duration of response; *EFS*, event-free survival; *MRD*, minimal residual disease; *OS*, overall survival; *PCR*, polymerase chain reaction; *RFS*, relapse-free survival; *RR*, relapsed/refractory ALL; *SCT*, stem cell transplantation.

RR ALL patients 30 years old or younger and may be an exciting agent for treatment of MRD states.

BH3 Mimetics

Venetoclax is a selective BH3 mimetic inhibitor of BCL2, an antiapoptotic protein conferring enhanced survival in leukemia cells, including chronic lymphocytic leukemia, AML, and ALL. Venetoclax is safe and effective in the treatment of CLL and AML as both monotherapy and combined with other agents. Preclinical studies have shown that venetoclax is also active against ALL cells, which are dependent on BCL2 and other antiapoptosis family members, including BCL-XL, for survival. Venetoclax demonstrated synergy with chemotherapy in ALL cell lines and appears to be safe and effective in early phase clinical trials.[104] Preliminary data from an ongoing trial demonstrates high efficacy in the frontline setting with 9/10 patients (90%) achieving CR, but for RR patients the response was limited to 3/8 patients (37.5%).[105] Based on these safety and efficacy data, phase II studies are underway.

In addition to BCL2, preclinical studies have shown BCL-XL may be an important target, particularly for precursor T-ALL. In a recent phase I trial, venetoclax was combined with the BCL-XL inhibitor navitoclax in children and adults with RR B- and T-ALL. Patients could receive chemotherapy at investigator discretion, consisting of PEG-asparaginase, vincristine, and dexamethasone. The most common adverse events were febrile neutropenia and neutropenia. Best response of CR/CRi/CRp was achieved by 25 (54%) patients, with 15 (33%) achieving MRD negativity. Median OS was 9.7 months for B-ALL patients and 6.6 months for T-ALL patients. Eleven (24%) patients went on to allogeneic stem cell transplant or CAR-T cells.[106] These agents are now being considered for combination therapy in frontline T-ALL trials.

Kinase Inhibitors for ALL

Asciminib (ABL001) is a potent, specific, orally bioavailable BCR-ABL1 inhibitor that was approved in 2021 for treatment of TKI-resistant chronic myeloid leukemia (CML) (see Chapter 69) including those with the T315I mutation, which as previously mentioned, is a common mechanism of treatment resistance in patients with Ph $^+$ ALL. Although studies using this agent have focused on CML,[107] there are early-phase data supporting the use of asciminib for patients with advanced T315I-mutant Ph$^+$ ALL and a phase I trial of asciminib with dasatinib and prednisone in newly diagnosed Ph$^+$ ALL unfit for intensive therapy or with relapsed disease has been initiated.

Other TKIs are being explored for patients with Ph-like ALL. Multiple anecdotal reports (reviewed in reference 108) demonstrate efficacy of dasatinib for Ph-like ALL with ABL class fusions. While targeting CRLF2/JAK activation in PH-like ALL has been a greater challenge, an ongoing COG study suggests feasibility and several ongoing studies are evaluating both dasatinib or ruxolitinib added to frontline therapy for patients with Ph-like ALL.[109]

Targeting Intracellular Signaling Pathways

The *NOTCH1* gene encodes a transmembrane receptor protein that drives stem cell commitment to T-cell differentiation. *NOTCH1* activation occurs in more than 50% of patients with precursor T-cell ALL and has been used as a therapeutic target. γ-Secretase is required for *NOTCH1* proteolytic activation. The γ-secretase inhibitor MK-052 caused significant GI toxicity and failed to induce responses in RR T-ALL. To date, due to the off-target effects of γ -secretase inhibition,[110] particularly on the GI tract, the impact of γ-secretase inhibition in T-ALL has been limited and no recent trials have been initiated.

The PI3K/AKT/mTOR pathway is also thought to be active in Ph$^+$ and Ph$^-$ B-ALL. As a single agent, the rapalog sirolimus resulted in only stable disease in RR pediatric ALL. Temsirolimus, everolimus, and sirolimus are each under investigation in combination with chemotherapy in RR ALL. BEZ-235 is a dual inhibitor of PI3K and mTORC1/mTORC2, which in vitro, caused cell cycle arrest and apoptosis in T- and B-ALL, including Ph$^+$ cells, and acted synergistically with chemotherapy.[111] In a phase I trial of BEZ-235 in RR acute leukemia, one out of 10 ALL patients achieved a CR and two additional patients had improvement in blood counts. Similar to rapalogs, this agent may be more effective in combination with chemotherapy.

Epigenetic Modulation

MLL gene rearrangements at position 11q23 occur in approximately 10% of ALL, AML, or MLL and are associated with an aggressive disease course. MLL gene mutations result in oncogenic fusion proteins that associate with disruption of telomeric silencing 1-like (DOT1L), leading to hypermethylation and activation of MLL target genes that drive leukemogenesis. The DOT1L inhibitor EPZ5676 was evaluated in a phase I trial in RR acute leukemias with modest impact.[112] Another exciting approach to targeting *MLL(KMT2A)* fusions, is under active investigation. MLL fusion proteins bind to menin, and this interaction causes leukemia by inducing an aberrant stem cell gene expression program mediated by the homeobox (HOX) genes. Compounds that disrupt binding of KMT2A to menin have shown to have promising antileukemic activity by inhibiting aberrant gene expression programs that result downstream of the MLL fusion. Ongoing early-phase clinical trials demonstrate tolerability and efficacy of this exciting new therapeutic approach to these highly resistant leukemias.[113]

SURVIVORSHIP

Although specific data on long-term complications of young adult survivors of ALL is lacking, the long-term complications of successful treatment of children with ALL have been well described and include neurocognitive and neurologic dysfunction, endocrine and metabolic abnormalities (including obesity), bone toxicity (osteonecrosis), cardiac toxicity, and secondary malignancies. To guide the frequency and focus of medical visits and the ordering of appropriate surveillance tests, comprehensive guidelines have been published in many countries, including North America, where guidelines created by the COG, titled “Long-Term Follow-up Guidelines for Survivors of Childhood, Adolescent and Young Adult Cancers” are available at http://www.survivorshipguidelines.org and should be adopted by the oncology community treating such patients.

FUTURE DIRECTIONS

ALL is a heterogeneous group of diseases with varied clinical outcomes, depending on molecular, cytogenetic, and clinical characterization. Historically, all ALL subtypes behave aggressively and were associated with poor survival outcomes, particularly in adults. The use of intensive pediatric regimens for younger adults with ALL has become standard of care and has resulted in survival rates approaching 70% to 75%. For patients with Ph$^+$ ALL, survival rates have also improved with implementation of next-generation TKIs and reduction in the proportion of patients requiring allogeneic transplant for prolonged survival. In the past several years, immunotherapies such as blinatumomab, inotuzumab ozogamicin, and CAR-T cells remarkably increased our abilities to treat RR ALL and allow more patients to received potentially curative transplants—future generations of these agents/studies may allow for long-term survival without the need for transplant. To that end, studies are now investigating the use of immunotherapies earlier in treatment, including frontline, to improve the depth of response and survival. This may be most relevant for older adults with this disease, where less-intensive combination therapies are already demonstrating significant benefits with less toxicity and improved survival, and it is possible that "chemotherapy"-free regimens will prove to be highly effective for patients with ALL. We have also made significant advances in our understanding of the molecular pathogenesis of this disease, which are translating into effective new targeted therapies as described in the novel agents section. Given the rarity of this disease, it is strongly recommended that patients are referred to centers of expertise, and it is crucial that patients be enrolled in clinical trials designed to evaluate subset-specific therapies to improve survival.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Epidemiology, Classification, and Prognosis

Borowitz MJ, Chan JKC, Downing J, et al. Precursor lymphoid neoplasms. In: Swerdlow SH, Campo E, Harris NL, eds. *WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues (Revised 4th Edition)*. Lyon: International Agency for Research on Cancer; 2017.
Fielding AK, Richards SM, Chopra R, et al. Outcome of 609 adults after relapse of acute lymphoblastic leukemia (ALL); an MRC UKALL12/ECOG 2993 study. *Blood*. 2007;109:944.
Gale KB, Ford AM, Repp R, et al. Backtracking leukemia to birth: identification of clonotypic gene fusion sequences in neonatal blood spots. *Proc Natl Acad Sci USA*. 1997;94:13950.
Moorman AV, Chilton L, Wilkinson J, et al. A population-based cytogenetic study of adults with acute lymphoblastic leukemia. *Blood*. 2010;115:206.
Mullighan CG, Su X, Zhang J, et al. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. *N Engl J Med*. 2009;360:470.
Roberts KG, Li Y, Payne-Turner D, et al. Targetable kinase-activating lesions in Ph-like acute lymphoblastic leukemia. *N Engl J Med*. 2014;371:1005.
Wetzler M, Dodge RK, Mrózek K, et al. Prospective karyotype analysis in adult acute lymphoblastic leukemia: the cancer and leukemia Group B experience. *Blood*. 1999;93:3983.

Clinical Significance of Minimal Residual Disease

Bassan R, Spinelli O, Oldani E, et al. Improved risk classification for risk-specific therapy based on the molecular study of minimal residual disease (MRD) in adult acute lymphoblastic leukemia (ALL). *Blood*. 2009;113:4153.
Berry DA, Zhou S, Higley H, et al. Association of minimal residual disease with clinical outcome in pediatric and adult acute lymphoblastic leukemia: a meta-analysis. *JAMA Oncol*. 2017 Jul 13;3(7):e170580.
Campana D. Minimal residual disease in acute lymphoblastic leukemia. *Hematology Am Soc Hematol Educ Program*. 2010;2010:7.

Hematopoietic Stem Cell Transplantation

Bachanova V, Verneris MR, DeFor T, et al. Prolonged survival in adults with acute lymphoblastic leukemia after reduced-intensity conditioning with cord blood or sibling donor transplantation. *Blood*. 2009;113:2902.
Cornelissen JJ, van der Holt B, Verhoef GE, et al. Myeloablative allogeneic versus autologous stem cell transplantation in adult patients with acute lymphoblastic leukemia in first remission: a prospective sibling donor versus no-donor comparison. *Blood*. 2009;113:1375.
Dhédin N, Huynh A, Maury S, et al. Role of allogeneic stem cell transplantation in adult patients with Ph-negative acute lymphoblastic leukemia. *Blood*. 2015;125(16):2486–2496. quiz 2586.
Goldstone AH, Richards SM, Lazarus HM, et al. In adults with standard-risk acute lymphoblastic leukemia, the greatest benefit is achieved from a matched sibling allogeneic transplantation in first complete remission, and an autologous transplantation is less effective than conventional consolidation/maintenance chemotherapy in all patients: final results of the International ALL Trial (MRC UKALL XII/ECOG E2993). *Blood*. 2008;111:1827.
Marks DI, Wang T, Pérez WS, et al. The outcome of full-intensity and reduced-intensity conditioning matched sibling or unrelated donor transplantation in adults with Philadelphia chromosome-negative acute lymphoblastic leukemia in first and second complete remission. *Blood*. 2010;116:366.
Tomblyn MB, Arora M, Baker KS, et al. Myeloablative hematopoietic cell transplantation for acute lymphoblastic leukemia: analysis of graft sources and long-term outcome. *J Clin Oncol*. 2009;27:3634.

Standard Treatment Regimens

Kantarjian HM, O'Brien S, Smith TL, et al. Results of treatment with hyper-CVAD, a dose-intensive regimen, in adult acute lymphocytic leukemia. *J Clin Oncol*. 2000;18:547.
Larson RA, Dodge RK, Burns CP, et al. A five-drug remission induction regimen with intensive consolidation for adults with acute lymphoblastic leukemia: cancer and leukemia group B study 8811. *Blood*. 1995;85:2025.
Lazarus HM, Richards SM, Chopra R, et al. Central nervous system involvement in adult acute lymphoblastic leukemia at diagnosis: results from the international ALL trial MRC UKALL XII/ECOG E2993. *Blood*. 2006;108:465.
Maury S, Chevret S, Thomas X, et al. Rituximab in B-lineage adult acute lymphoblastic leukemia. *N Engl J Med*. 2016;375:1044.
Rowe JM, Buck G, Burnett AK, et al. Induction therapy for adults with acute lymphoblastic leukemia: results of more than 1500 patients from the international ALL trial: MRC UKALL XII/ECOG E2993. *Blood*. 2005;106:3760.
Salzer WL, Asselin B, Supko JG, et al. Erwinia asparaginase achieves therapeutic activity after pegaspargase allergy: a report from the Children's Oncology Group. *Blood*. 2013;122:507.
Vignetti M, Fazi P, Cimino G, et al. Imatinib plus steroids induces complete remissions and prolonged survival in elderly Philadelphia chromosome-positive patients with acute lymphoblastic leukemia without additional chemotherapy: results of the Gruppo Italiano Malattie Ematologiche dell'Adulto (GIMEMA) LAL0201-B protocol. *Blood*. 2007;109:3676.

Pediatric Intensive Regimens for Adolescents and Young Adults

DeAngelo DJ, Stevenson KE, Dahlberg SE, et al. Long-term outcome of a pediatric-inspired regimen used for adults aged 18–50 years with newly diagnosed acute lymphoblastic leukemia. *Leukemia*. 2015 Mar;29(3): 526–534.
Stock W, Luger SM, Advani AS, et al. A pediatric regimen for older adolescents and young adults with acute lymphoblastic leukemia: results of CALGB 10403. *Blood*. 2019;133(14):1548–1559.
Testi AM, Canichella M, Vitale A, et al. Adolescent and young adult acute lymphoblastic leukemia. Final results of the phase II pediatric-like GIMEMA LAL-1308 trial. *Am J Hematol*. 2021;96(3):292–301.
Toft N, Birgens H, Abrahamsson J, et al. Results of NOPHO ALL2008 treatment for patients aged 1–45 years with acute lymphoblastic leukemia. *Leukemia*. 2018;32(3):606–615.
Wieduwilt MJ, Stock W, Advani A, et al. Superior survival with pediatric-style chemotherapy compared to myeloablative allogeneic hematopoietic cell transplantation in older adolescents and young adults with Ph-negative acute lymphoblastic leukemia in first complete remission: analysis from CALGB 10403 and the CIBMTR. *Leukemia*. 2021;35(7):2076–2085.

Ph+ ALL

Chalandon Y, Thomas X, Hayette S, et al. Randomized study of reduced-intensity chemotherapy combined with imatinib in adults with Ph-positive acute lymphoblastic leukemia. *Blood*. 2015;125(24):3711–37119.
Foà R, Bassan R, Vitale A, et al. Dasatinib–Blinatumomab for Ph-positive acute lymphoblastic leukemia in adults. *N Engl J Med*. 2020;383:1613–1623.
Foà R, Vitale A, Vignetti M, et al. Dasatinib as first-line treatment for adult patients with Philadelphia chromosome-positive acute lymphoblastic leukemia. *Blood*. 2011;118:6521.
Jabbour E, Short NJ, Ravandi F, et al. Combination of hyper-CVAD with ponatinib as first-line therapy for patients with Philadelphia chromosome-positive acute lymphoblastic leukaemia: long-term follow-up of a single-centre, phase 2 study. *Lancet Haematol*. 2018 Dec;5(12):e618–e627.
Ravandi F, Othus M, O'Brien SM, et al. US intergroup study of chemotherapy plus dasatinib and allogeneic stem cell transplant in Philadelphia chromosome positive ALL. *Blood Adv*. 2016;1(3):250–259.

T-ALL

Dunsmore KP, Winter SS, Devidas M, et al. Children's Oncology Group AALL0434: a phase III randomized clinical trial testing nelarabine in newly diagnosed T-cell acute lymphoblastic leukemia. *J Clin Oncol*. 2020 Oct 1; 38(28):3282–3293.

Quist-Paulsen P, Toft N, Heyman M, et al. T-cell acute lymphoblastic leukemia in patients 1–45 years treated with the pediatric NOPHO ALL2008 protocol. *Leukemia*. 2020;34:347–357.

Relapsed Disease and Novel Therapies

Hughes TP, Mauro MJ, Cortes JE, et al. Asciminib in chronic myeloid leukemia after ABL Kinase inhibitor failure. *N Engl J Med*. 2019;381:2315–2326.

Kantarjian H, Stein A, Gökbuget N, et al. Blinatumomab versus chemotherapy for advanced acute lymphoblastic leukemia. *N Engl J Med*. 2017;376: 836–847.

Kantarjian HM, DeAngelo DJ, Stelljes M, et al. Inotuzumab ozogamicin versus standard therapy for acute lymphoblastic leukemia. *N Engl J Med*. 2016;375:740–753.

Maude SL, Laetsch TW, Buechner J, et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *N Engl J Med*. 2018;378: 439–448.

Ofran Y, Ringelstein-Harlev S, Slouzkey I, et al. Daratumumab for eradication of minimal residual disease in high-risk advanced relapse of T-cell/CD19/ CD22-negative acute lymphoblastic leukemia. *Leukemia*. 2020;34(1): 293–295.

Pullarkat VA, Lacayo NJ, Jabbour E, et al. Venetoclax and navitoclax in combination with chemotherapy in patients with relapsed or refractory acute lymphoblastic leukemia and lymphoblastic lymphoma. *Cancer Discov*. 2021;11(6):1440–1453.

Shah BD, Bishop MR, Oluwole OO, et al. KTE-X19 anti-CD19 CAR T-cell therapy in adult relapsed/refractory acute lymphoblastic leukemia: ZUMA-3 phase 1 results. *Blood*. 2021;8;138(1):11–22.

REFERENCES

1. Greaves MF. A causal mechanism for childhood acute lymphoblastic leukaemia. *Nat Rev Cancer*. 2018;18:471–484.
2. Surveillance, Epidemiology, and End Results (SEER) Program Populations (1969–2019). National Cancer Institute, DCCPS, Surveillance Research Program. Released February 2021. www.seer.cancer.gov/popdata.
3. Vijayakrishnan J, Studd J, Broderick P, et al. Genome-wide association study identifies susceptibility loci for B-cell childhood acute lymphoblastic leukemia. *Nature Commun*. 2018;9:1340.
4. Saygin C, Kishtagari A, Cassaday RD, et al. Therapy-related acute lymphoblastic leukemia is a distinct entity with adverse genetic features and clinical outcomes. *Blood Adv*. 2019;3(24):4228–4237.
5. Ferraro F, Gao F, Stockerl-Goldstein K, et al. Secondary acute lymphoblastic leukemia, a retrospective analysis from Washington University and meta-analysis of published data. *Leuk Res*. 2018;72:86–91.
6. Borowitz MJ, Chan JKC, Downing J, Le Beau MM, Arber DA. Precursor lymphoid neoplasms. In: Swerdlow SH, Campo E, Harris NL, eds. *WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues (Revised 4th Edition)*. Lyon: International Agency for Research on Cancer; 2017.
7. Schwab C, Harrison CJ. Advances in B-cell precursor acute lymphoblastic leukemia genomics. *Hemasphere*. 2018;2(4):e53.
8. Brüggemann M, Raff T, Kneba M. Has MRD monitoring superseded other prognostic factors in adult ALL? *Blood*. 2012;120(23):4470–4481. https://doi.org/10.1182/blood-2012-06-379040.
9. Berry DA, Zhou S, Higley H, et al. Association of minimal residual disease with clinical outcome in pediatric and adult acute lymphoblastic leukemia: a meta-analysis. *JAMA Oncol*. 2017;3(7):e170580. https://doi.org/10.1001/jamaoncol.2017.0580.
10. Beldjord K, Chevret S, Asnafi V, Group for Research on Adult Acute Lymphoblastic Leukemia (GRAALL) Oncogenetics and minimal residual disease are independent outcome predictors in adult patients with acute lymphoblastic leukemia. *Blood*. 2014;123(24):3739–3749. https://doi.org/10.1182/blood-2014-01-547695.
11. Dhédin N, Huynh A, Maury S, GRAALL Group. Role of allogeneic stem cell transplantation in adult patients with Ph-negative acute lymphoblastic leukemia. *Blood*. 2015;125(16):2486–2496. https://doi.org/10.1182/blood-2014-09-599894. quiz 2586.
12. Sasaki K, Kantarjian HM, Short NJ, et al. Prognostic factors for progression in patients with Philadelphia chromosome-positive acute lymphoblastic leukemia in complete molecular response within 3 months of therapy with tyrosine kinase inhibitors. *Cancer*. 2021;127(15):2648–2656. https://doi.org/10.1002/cncr.33529.
13. Chiaretti S, Ansuinelli M, Vitale A, et al. A multicenter total therapy strategy for *de novo* adult Philadelphia chromosome positive acute lymphoblastic leukemia patients: final results of the GIMEMA LAL1509 protocol. *Haematologica*. 2021;106(7):1828–1838. https://doi.org/10.3324/haematol.2020.260935.
14. Gökbuget N, Dombret H, Bonifacio M, et al. Blinatumomab for minimal residual disease in adults with B-cell precursor acute lymphoblastic leukemia. *Blood*. 2018;131(14):1522–1531. https://doi.org/10.1182/blood-2017-08-798322.
15. Larson RA, Dodge RK, Burns CP, et al. A five-drug remission induction regimen with intensive consolidation for adults with acute lymphoblastic leukemia: cancer and leukemia group B study 8811. *Blood*. 1995;85:2025.
16. Larson RA, Dodge RK, Linker CA, et al. A randomized controlled trial of filgrastim during remission induction and consolidation chemotherapy for adults with acute lymphoblastic leukemia: CALGB study 9111. *Blood*. 1998;92:1556.
17. Thomas X, Boiron JM, Huguet F, et al. Outcome of treatment in adults with acute lymphoblastic leukemia: analysis of the LALA-94 trial. *J Clin Oncol*. 2004;22:4075.
18. Huguet F, Leguay T, Raffoux E, et al. Pediatric-inspired therapy in adults with Philadelphia chromosome-negative acute lymphoblastic leukemia: the GRAALL-2003 study. *J Clin Oncol*. 2009;27:911.
19. Takeuchi J, Kyo T, Naito K, et al. Induction therapy by frequent administration of doxorubicin with four other drugs, followed by intensive consolidation and maintenance therapy for adult acute lymphoblastic leukemia: the JALSG-ALL93 study. *Leukemia*. 2002;16:1259.
20. Gökbuget N, Arnold R, Böhme A, et al. Improved outcome in high risk and very high risk ALL by risk adapted SCT and in standard risk ALL by intensive chemotherapy in 713 adult ALL patients treated according to the prospective GMALL study 07/2003. *Blood*. 2007;110:12.
21. Kantarjian H, Thomas D, O'Brien S, et al. Long-term follow-up results of hyperfractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone (Hyper-CVAD), a dose-intensive regimen, in adult acute lymphocytic leukemia. *Cancer*. 2004;101:2788.
22. Jinnai I, Sakura T, Tsuzuki M, et al. Intensified consolidation therapy with dose-escalated doxorubicin did not improve the prognosis of adults with acute lymphoblastic leukemia: the JALSG-ALL97 study. *Int J Hematol*. 2010;92:490.
23. Annino L, Vegna ML, Camera A, et al. Treatment of adult acute lymphoblastic leukemia (ALL): long-term follow-up of the GIMEMA ALL 0288 randomized study. *Blood*. 2002;99:863.
24. Goldstone AH, Richards SM, Lazarus HM, et al. In adults with standard-risk acute lymphoblastic leukemia, the greatest benefit is achieved from a matched sibling allogeneic transplantation in first complete remission, and an autologous transplantation is less effective than conventional consolidation/maintenance chemotherapy in all patients: final results of the International ALL Trial (MRC UKALL XII/ECOG E2993). *Blood*. 2008;111:1827.
25. Ribera JM, Oriol A, Bethencourt C, et al. Comparison of intensive chemotherapy, allogeneic or autologous stem cell transplantation as post-remission treatment for adult patients with high-risk acute lymphoblastic leukemia. Results of the PETHEMA ALL-93 trial. *Haematologica*. 2005; 90:1346.
26. Larson RA, Stone RM, Mayer RJ, Schiffer CA. Fifty years of clinical research by the leukemia committee of the cancer and leukemia group B. *Clin Cancer Res*. 2006 Jun 1;12(11 Pt 2). https://doi.org/10.1158/1078-0432.CCR-06-9001. 3556s-63s.
27. Stock W, Johnson JL, Stone RM, et al. Dose intensification of daunorubicin and cytarabine during treatment of adult acute lymphoblastic leukemia: results of Cancer and Leukemia Group B Study 19802. Cancer. 2013;119(1):90-8. doi:10.1002/cncr.27617. (Erratum in: Cancer. 2014;120(14):2222. Dosage error in article text. Erratum in: Cancer. 2014;120(14):2222.)
28. Douer D, Yampolsky H, Cohen LJ, et al. Pharmacodynamics and safety of intravenous pegaspargase during remission induction in adults aged 55 years or younger with newly diagnosed acute lymphoblastic leukemia. *Blood*. 2007;109(7):2744–2750. https://doi.org/10.1182/blood-2006-07-035006.
29. Wetzler M, Sanford BL, Kurtzberg J, et al. Effective asparagine depletion with pegylated asparaginase results in improved outcomes in adult acute lymphoblastic leukemia: Cancer and Leukemia Group B Study 9511. *Blood*. 2007;109(10):4164–4167. https://doi.org/10.1182/blood-2006-09-045351.
30. Douer D, Gökbuget N, Stock W, Boissel N. Optimizing use of L-asparaginase-based treatment of adults with acute lymphoblastic leukemia. *Blood Rev*. 2021:100908. https://doi.org/10.1016/j.blre.2021.100908.
31. Thomas DA, O'Brien S, Faderl S, et al. Chemoimmunotherapy with a modified hyper-CVAD and rituximab regimen improves outcome in de novo Philadelphia chromosome-negative precursor B-lineage acute lymphoblastic leukemia. *J Clin Oncol*. 2010;28(24):3880–3889.
32. Maury S, Chevret S, Thomas X, et al. Rituximab in B-lineage adult acute lymphoblastic leukemia. *N Engl J Med*. 2016;375:1044.
33. Gökbuget N, Arnold R. Buechner Th, et al: Intensification of induction and consolidation improves only subgroups of adult ALL: analysis of 1200 patients in GMALL study 05/93. *Blood*. 2001;98:802.
34. Brown PA, Shah B, Advani A, et al. Acute lymphoblastic leukemia, version 2.2021, NCCN clinical practice guidelines in oncology. *J Natl Compr Canc Netw*. 2021;19(9):1079–1109. https://doi.org/10.6004/jnccn.2021.0042.
35. Dunsmore KP, Winter SS, Devidas M, et al. Children's Oncology Group AALL0434: a phase III randomized clinical trial testing nelarabine in newly diagnosed T-cell acute lymphoblastic leukemia. *J Clin Oncol*. 2020;38(28): 3282–3293. https://doi.org/10.1200/JCO.20.00256.
36. Pui CH, Campana D, Pei D, Bowman WP, Sandlund JT, Kaste SC, et al. Treating childhood acute lymphoblastic leukemia without cranial irradiation. *N Engl J Med*. 2009;360(26):2730–2741. https://doi.org/10.1056/NEJMoa0900386.
37. Teachey DT, Hunger SP, Loh ML. Optimizing therapy in the modern age: differences in length of maintenance therapy in acute lymphoblastic leukemia. *Blood*. 2021;137(2):168–177. https://doi.org/10.1182/blood.2020007702.
38. Yanada M, Matsuo K, Suzuki T, Naoe T. Allogeneic hematopoietic stem cell transplantation as part of postremission therapy improves survival for adult patients with high-risk acute lymphoblastic leukemia: a metaanalysis. *Cancer*. 2006;106(12):2657–2663.
39. Wieduwilt MJ, Stock W, Advani A, et al. Superior survival with pediatric-style chemotherapy compared to myeloablative allogeneic hematopoietic cell transplantation in older adolescents and young adults with Ph-negative acute lymphoblastic leukemia in first complete remission: analysis from CALGB 10403 and the CIBMTR. *Leukemia*. 2021;35(7):2076–2085. https://doi.org/10.1038/s41375-021-01213-5. (Erratum in: *Leukemia*. Jun 4, 2021.).
40. Segal E, Martens M, Wang H, et al. Comparing outcomes of matched related donor and matched unrelated donor hematopoietic cell transplants in adults with B-cell acute lymphoblastic leukemia. *Cancer*. 2017;123(17): 3346–3355.
41. Hunault M, Harousseau JL, Delain M, et al. Better outcome of adult acute lymphoblastic leukemia after early genoidentical allogeneic bone marrow transplantation (BMT) than after late high-dose therapy and autologous BMT: a GOELAMS trial. *Blood*. 2004;104:3028.

42. Cornelissen JJ, van der Holt B, Verhoef GE, et al. Myeloablative allogeneic versus autologous stem cell transplantation in adult patients with acute lymphoblastic leukemia in first remission: a prospective sibling donor versus no-donor comparison. *Blood*. 2009;113:1375.
43. Ram R, Storb R, Sandmaier BM, Maloney DG, Woolfrey A, Flowers ME, et al. Non-myeloablative conditioning with allogeneic hematopoietic cell transplantation for the treatment of high-risk acute lymphoblastic leukemia. *Haematologica*. 2011;96(8):1113–1120. https://doi.org/10.3324/haematol.2011.040261.
44. Bachanova V, Verneris MR, DeFor T, et al. Prolonged survival in adults with acute lymphoblastic leukemia after reduced-intensity conditioning with cord blood or sibling donor transplantation. *Blood*. 2009;113:2902.
45. Arnold R, Massenkeil G, Bornhäuser M, et al. Nonmyeloablative stem cell transplantation in adults with high-risk ALL may be effective in early but not in advanced disease. *Leukemia*. 2002;16:2423.
46. Martino R, Giralt S, Caballero MD, et al. Allogeneic hematopoietic stem cell transplantation with reduced-intensity conditioning in acute lymphoblastic leukemia: a feasibility study. *Haematologica*. 2003;88:555.
47. Hamaki T, Kami M, Kanda Y, et al. Reduced-intensity stem-cell transplantation for adult acute lymphoblastic leukemia: a retrospective study of 33 patients. *Bone Marrow Transplant*. 2005;35:549.
48. Mohty M, Labopin M, Tabrizzi R, et al. Reduced intensity conditioning allogeneic stem cell transplantation for adult patients with acute lymphoblastic leukemia: a retrospective study from the European Group for Blood and Marrow Transplantation. *Haematologica*. 2008;93:303.
49. Stein AS, Palmer JM, O'Donnell MR, et al. Reduced-intensity conditioning followed by peripheral blood stem cell transplantation for adult patients with high-risk acute lymphoblastic leukemia. *Biol Blood Marrow Transplant*. 2009;15:1407.
50. Dhedin N, Dombret H, Thomas X, et al. Autologous stem cell transplantation in adults with acute lymphoblastic leukemia in first complete remission: analysis of the LALA-85, -87 and -94 trials. *Leukemia*. 2006;20:336–344.
51. Thomas DA, Faderl S, Cortes J, et al. Treatment of Philadelphia chromosome-positive acute lymphocytic leukemia with hyper-CVAD and imatinib mesylate. *Blood*. 2004;103:4396.
52. Thomas DA, O'Brien SM, et al. Long-term outcome after hyper-CVAD and imatinib (IM) for de novo or minimally treated Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph-ALL). *J Clin Oncol*. 2010;28(15s).
53. Towatari M, Yanada M, Usui N, et al. Combination of intensive chemotherapy and imatinib can rapidly induce high-quality complete remission for a majority of patients with newly diagnosed BCR-ABL-positive acute lymphoblastic leukemia. *Blood*. 2004;104:3507.
54. Yanada M, Takeuchi J, Sugiura I, et al. High complete remission rate and promising outcome by combination of imatinib and chemotherapy for newly diagnosed BCR-ABL-positive acute lymphoblastic leukemia: a phase II study by the Japan Adult Leukemia Study Group. *J Clin Oncol*. 2006;24:460.
55. de Labarthe A, Rousselot P, Huguet-Rigal F, et al. Imatinib combined with induction or consolidation chemotherapy in patients with de novo Philadelphia chromosome-positive acute lymphoblastic leukemia: results of the GRAAPH-2003 study. *Blood*. 2007;109:1408.
56. Tanguy-Schmidt A, de Labarthe A, Chalandon Y, et al. Long-term results of the Imatinib GRAAPH-2003 study in newly-diagnosed patients with de novo Philadelphia chromosome-positive acute lymphoblastic leukemia: a GRAALL study. *Biol Blood Marrow Transplant*. 2013;19:150.
57. Wassmann B, Pfeifer H, Goekbuget N, et al. Alternating versus concurrent schedules of imatinib and chemotherapy as front-line therapy for Philadelphia-positive acute lymphoblastic leukemia (Ph+ ALL). *Blood*. 2006;108:1469.
58. Ottmann OG, Wassmann B, Pfeifer H, et al. Imatinib compared with chemotherapy as front-line treatment of elderly patients with Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ALL). *Cancer*. 2007;109:2068.
59. Vignetti M, Fazi P, Cimino G, et al. Imatinib plus steroids induces complete remissions and prolonged survival in elderly Philadelphia chromosome-positive patients with acute lymphoblastic leukemia without additional chemotherapy: results of the Gruppo Italiano Malattie Ematologiche dell 'Adulto (GIMEMA) LAL0201-B protocol. *Blood*. 2007;109:3676.
60. Bassan R, Rossi G, Pogliani EM, et al. Chemotherapy-phased imatinib pulses improve long-term outcome of adult patients with Philadelphia chromosome-positive acute lymphoblastic leukemia: Northern Italy Leukemia Group protocol 09/00. *J Clin Oncol*. 2010;28:3644.
61. Fielding AK, Buck G, Lazarus HM, et al. Imatinib significantly enhances long-term outcomes in Philadelphia positive acute lymphoblastic leukaemia; final results of the UKALLXII/ECOG2993 trial. *Blood*. 2010;116(21):169.
62. Cortes JE, Kim DW, Pinilla-Ibarz J, et al. A phase 2 trial of ponatinib in Philadelphia chromosome positive leukemias. *N Engl J Med*. 2013;369:1783.
63. Martinelli G, Piciocchi A, Papayannidis C, et al. First report of the Gimema LAL1811 phase II prospective study of the combination of steroids with ponatinib as frontline therapy of elderly or unfit patients with Philadelphia chromosome-positive acute lymphoblastic leukemia. *Blood*. 2017;130 (suppl 1):99.
64. Jabbour E, Short NJ, Ravandi F, et al. Combination of hyper-CVAD with ponatinib as first-line therapy for patients with Philadelphia chromosome-positive acute lymphoblastic leukaemia: long-term follow-up of a single-centre, phase 2 study. *Lancet Haematol*. 2018;5(12):e618–e627. https://doi.org/10.1016/S2352-3026(18)30176-5.
65. Foà R, Bassan R, Vitale A, et al. Dasatinib–blinatumomab for Ph-positive acute lymphoblastic leukemia in adults. *N Engl J Med*. 2020;383:1613–1623.
66. Ottmann OG, Pfeifer H, Cayuela J-M, et al. Nilotinib (Tasigna®) and chemotherapy for first-line treatment in elderly patients with de novo Philadelphia chromosome/BCR-ABL1 positive acute lymphoblastic leukemia (ALL): a trial of the European Working Group for Adult ALL (EWALL-PH-02). *Blood*. 2018;132(suppl 1):31.
67. Wetzler M, Watson D, Stock W, et al. Autologous transplantation for Philadelphia chromosome positive acute lymphoblastic leukemia achieves outcomes similar to allogeneic stem cell transplantation: results of CALGB Study 10001 (Alliance). *Haematologica*. 2014;99:111.
68. Benjamini O, Dumloa T, Kantarjian H, et al. Phase II trial of HyperCVAD and dasatinib in patients with relapsed Philadelphia chromosome positive acute lymphoblastic leukemia or blast phase chronic myeloid leukemia. *Am J Hematol*. 2014;89(3):282–287.
69. Foà R, Vitale A, Vignetti M, et al. Dasatinib as first-line treatment for adult patients with Philadelphia chromosome-positive acute lymphoblastic leukemia. *Blood*. 2011;118(25):6521–6528.
70. Chiaretti S, Vitale A, Elia L, et al. Multicenter total therapy GIMEMA LAL 1509 protocol for de novo adult Ph+ acute lymphoblastic leukemia (ALL) patients. Updated results and refined genetic-based prognostic stratification. *Blood*. 2015;126(23):81.
71. Sasaki K, Jabbour E, Ravandi F, et al. Hyper-CVAD + ponatinib vs. hyper-CVAD + dasatinib as frontline therapy for Ph-positive ALL: a propensity score analysis. *Cancer*. 2016;122(23):3650–3656.
72. Wieduwilt MJ, Yin J, Wetzler M, et al. Dasatinib and dexamethasone followed by hematopoietic cell transplantation for adults with Ph-positive ALL. *Blood Adv*. 2021;5(22):4691–4700. https://doi.org/10.1182/bloodadvances.2021004813.
73. Ravandi F, Othus M, O'Brien SM, et al. US intergroup study of chemotherapy plus dasatinib and allogeneic stem cell transplant in Philadelphia chromosome positive ALL. *Blood Adv*. 2016;1(3):250–259. https://doi.org/10.1182/bloodadvances.2016001495.
74. Giebel S, Czyz A, Ottmann O, et al. Use of tyrosine kinase inhibitors to prevent relapse after allogeneic hematopoietic stem cell transplantation for patients with Philadelphia chromosome-positive acute lymphoblastic leukemia: a position statement of the Acute Leukemia Working Party of the European Society for Blood and Marrow Transplantation. *Cancer*. 2016;122(19):2941–2951. https://doi.org/10.1002/cncr.30130.
75. Siegel SE, Stock W, Johnson RH, et al. Pediatric-inspired treatment regimens for adolescents and young adults with Philadelphia chromosome-negative acute lymphoblastic leukemia: a review. *JAMA Oncol*. 2018;4(5):725–734. https://doi.org/10.1001/jamaoncol.2017.5305.
76. DeAngelo DJ, Stevenson KE, Dahlberg SE, et al. Long-term outcome of a pediatric-inspired regimen used for adults aged 18–50 years with newly diagnosed acute lymphoblastic leukemia. *Leukemia*. 2015;29(3):526–534. https://doi.org/10.1038/leu.2014.229.
77. Stock W, Luger SM, Advani AS, et al. A pediatric regimen for older adolescents and young adults with acute lymphoblastic leukemia: results of CALGB 10403. *Blood*. 2019;133(14):1548–1559. https://doi.org/10.1182/blood-2018-10-881961. (Erratum in: *Blood*. 2019;134(13):1111.).
78. Toft N, Birgens H, Abrahamsson J, et al. Results of NOPHO ALL2008 treatment for patients aged 1–45 years with acute lymphoblastic leukemia. *Leukemia*. 2018;32(3):606–615. https://doi.org/10.1038/leu.2017.265.
79. Ribera JM, Oriol A, Sanz MA, et al. Comparison of the results of the treatment of adolescents and young adults with standard-risk acute lymphoblastic leukemia with the Programa Espanol de Tratamiento en Hematologia pediatric-based protocol ALL-96. *J Clin Oncol*. 2008;26:1843.
80. Dunsmore KP, Winter SS, Devidas M, et al. A phase III randomized clinical trial testing nelarabine in newly diagnosed T-cell acute lymphoblastic leukemia. *J Clin Oncol*. 2020;38(28):3282–3293. https://doi.org/10.1200/JCO.20.00256.
81. Seftel MD, Neuberg D, Zhang MJ, et al. Pediatric-inspired therapy compared to allografting for Philadelphia chromosome-negative adult ALL in first complete remission. *Am J Hematol*. 2016;91(3):322–329. https://doi.org/10.1002/ajh.24285.

82. Wieduwilt MJ, Stock W, Advani A, et al. Superior survival with pediatric-style chemotherapy compared to myeloablative allogeneic hematopoietic cell transplantation in older adolescents and young adults with Ph-negative acute lymphoblastic leukemia in first complete remission: analysis from CALGB 10403 and the CIBMTR. *Leukemia*. 2021;35(7):2076–2085. https://doi.org/10.1038/s41375-021-01213-5. (Erratum in: *Leukemia*. Jun 4, 2021.)
83. Gökbuget N. How I treat older patients with ALL. *Blood*. 2013;122(8):1366–1375. https://doi.org/10.1182/blood-2012-07-379016.
84. Gökbuget N, Beck J, Brüggemann M, et al. Moderate intensive chemotherapy including CNS-prophylaxis with liposomal cytarabine is feasible and effective in older patients with Ph-negative acute lymphoblastic leukemia (ALL): results of a prospective trial from the German Multicenter Study Group for Adult ALL (GMALL). *Blood*. 2012:120 abstr 1493a.
85. Derman BA, Streck M, Wynne J, et al. Efficacy and toxicity of reduced vs. standard dose pegylated asparaginase in adults with Philadelphia chromosome-negative acute lymphoblastic leukemia. *Leuk Lymphoma*. 2020;61(3):614–622. https://doi.org/10.1080/10428194.2019.1680839.
86. Patel AA, Heng J, Dworkin E, et al. Efficacy and tolerability of a modified pediatric-inspired intensive regimen for acute lymphoblastic leukemia in older adults. *eJHaem*. 2021;2(3):413–420.
87. Kantarjian H, Ravandi F, Short NJ, et al. Inotuzumab ozogamicin in combination with low-intensity chemotherapy for older patients with Philadelphia chromosome-negative acute lymphoblastic leukaemia: a single-arm, phase 2 study. *Lancet Oncol*. 2018;19(2):240–248. https://doi.org/10.1016/S1470-2045(18)30011-1.
88. Advani AS, Moseley A, O'Dwyer KM, et al. SWOG 1318: A phase II trial of blinatumomab followed by POMP maintenance in older patients with newly diagnosed Philadelphia chromosome–negative B-cell acute lymphoblastic leukemia. *J Clin Oncol*. 2022;40(14):1574–1582.
89. Fielding AK, Richards SM, Chopra R, et al. Outcome of 609 adults after relapse of acute lymphoblastic leukemia (ALL); an MRC UKALL12/ECOG 2993 study. *Blood*. 2007;109:944.
90. Tavernier E, Boiron J-M, Huguet F, et al. Outcome of treatment after first relapse in adults with acute lymphoblastic leukemia initially treated by the LALA-94 trial. *Leukemia*. 2007;21(9):1907–1914.
91. Oriol A, Vives S, Hernández-Rivas J-M, et al. Outcome after relapse of acute lymphoblastic leukemia in adult patients included in four consecutive risk-adapted trials by the PETHEMA Study Group. *Haematologica*. 2010;95(4):589–596.
92. O'Brien S, Schiller G, Lister J, et al. High-dose vincristine sulfate liposome injection for advanced, relapsed, and refractory adult Philadelphia chromosome-negative acute lymphoblastic leukemia. *J Clin Oncol*. 2013;31(6):676–683.
93. Kantarjian H, Stein A, Gökbuget N, et al. Blinatumomab versus chemotherapy for advanced acute lymphoblastic leukemia. *N Engl J Med*. 2017;376:836–847.
94. Rambaldi A, Ribera J-M, Kantarjian HM, et al. Blinatumomab compared with standard of care for the treatment of adult patients with relapsed/refractory Philadelphia chromosome–positive B-precursor acute lymphoblastic leukemia. *Cancer*. 2020;126(2):304–310.
95. Kantarjian HM, DeAngelo DJ, Stelljes M, et al. Inotuzumab ozogamicin versus standard therapy for acute lymphoblastic leukemia. *N Engl J Med*. 2016;375:740–753.
96. Maude SL, Laetsch TW, Buechner J, et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *N Engl J Med*. 2018;378:439–448.
97. Shah BD, Ghobadi A, Oluwole OO, et al. KTE-X19 for relapsed or refractory adult B-cell acute lymphoblastic leukaemia: phase 2 results of the single-arm, open-label, multicentre ZUMA-3 study. *Lancet*. 2021;398(10299):491–502. https://doi.org/10.1016/S0140-6736(21)01222-8.
98. Deangelo D, Yu D, Johnson J, et al. Nelarabine induces complete remissions in adults with relapsed or refractory T-lineage acute lymphoblastic leukemia or lymphoblastic lymphoma: Cancer and Leukemia Group B study 19801. *Blood*. 2007;109(12):5136–5142.
99. Spiegel JY, Patel S, Muffly L, et al. CAR T cells with dual targeting of CD19 and CD22 in adult patients with recurrent or refractory B cell malignancies: a phase 1 trial. *Nat Med*. 2021;27(8):1419–1431. https://doi.org/10.1038/s41591-021-01436-0.
100. Jain N, Roboz G, Konopleva M, et al. Preliminary results of balli-01: a phase I study of UCART22 (allogeneic engineered T-cells expressing anti-CD22 chimeric antigen receptor) in adult patients with relapsed or refractory (R/R) CD22+ B-cell acute lymphoblastic leukemia. *Blood*. 2020;136:7–8. abstract 163.
101. Yang J, Zhang X, Liu Y, et al. High effectiveness and safety of anti-CD7 CAR T-cell therapy in treating relapsed or refractory (R/R) T-cell acute lymphoblastic leukemia (T-ALL). *Blood*. 2021;138(suppl 1):473. https://doi.org/10.1182/blood-2021-147667.
102. Ofran Y, Ringelstein-Harlev S, Slouzkey I, et al. Daratumumab for eradication of minimal residual disease in high-risk advanced relapse of T-cell/CD19/CD22-negative acute lymphoblastic leukemia. *Leukemia*. 2020;34(1):293–295.
103. Richard-Carpentier G, Jabbour E, Short NJ, et al. Clinical experience with venetoclax combined with chemotherapy for relapsed or refractory T-cell acute lymphoblastic leukemia. *Clin Lymphoma Myeloma Leuk*. 2020;20(4):212–218.
104. Jain N, Stevenson KE, Winer ES, et al. A multicenter phase I study combining venetoclax with mini-hyper-CVD in older adults with untreated and relapsed/refractory acute lymphoblastic leukemia. *Blood*. 2019;134(suppl_1):3867.
105. Pullarkat VA, Lacayo NJ, Jabbour E, et al. Venetoclax and navitoclax in combination with chemotherapy in patients with relapsed or refractory acute lymphoblastic leukemia and lymphoblastic lymphoma. *Cancer Discov*. 2021;11(6):1440–1453. https://doi.org/10.1158/2159-8290.CD-20-1465.
106. Hughes TP, Mauro MJ, Cortes JE, et al. Asciminib in chronic myeloid leukemia after ABL Kinase inhibitor failure. *N Engl J Med*. 2019;381:2315–2326.
107. Tasian SK, Loh ML, Hunger SP. Philadelphia chromosome-like acute lymphoblastic leukemia. *Blood*. 2017;130(19):2064–2072. https://doi.org/10.1182/blood-2017-06-743252.
108. Tasian SK, Assad A, Hunte DA. A phase 2 study of ruxolitinib with chemotherapy in children with Philadelphia chromosome-like acute lymphoblastic leukemia (INCB18424-269/AALL1521): dose-finding results from the part 1 safety phase. *Blood*. 2018;132(suppl 1):555. https://doi.org/10.1182/blood-2018-99-110221.
109. Papayannidis C, DeAngelo DJ, Stock W, et al. A phase 1 study of the novel gamma-secretase inhibitor PF-03084014 in patients with T-cell acute lymphoblastic leukemia and T-cell lymphoblastic lymphoma. *Blood Cancer J*. 2015;5(9):e350. https://doi.org/10.1038/bcj.2015.80.
110. Lang F, Wunderle L, Badura S, et al. A phase I study of a dual PI3-kinase/mTOR inhibitor BEZ235 in adult patients with relapsed or refractory acute leukemia. *BMC Pharmacol Toxicol*. 2020;21(1):70. https://doi.org/10.1186/s40360-020-00446-x.
111. Stein EM, Garcia-Manero G, Rizzieri DA, et al. The DOT1L inhibitor pinometostat reduces H3K79 methylation and has modest clinical activity in adult acute leukemia. *Blood*. 2018;131(24):2661–2669. https://doi.org/10.1182/blood-2017-12-818948.
112. Stein EM, Aldoss I, DiPersio JF, et al. Safety and efficacy of menin inhibition in patients (Pts) with MLL-rearranged and NPM1 mutant acute leukemia: a phase (Ph) 1, first-in-Human Study of SNDX-5613 (AUGMENT 101). *Blood*. 2021;138(suppl 1):699. https://doi.org/10.1182/blood-2021-146944.

CHAPTER 69

CHRONIC MYELOID LEUKEMIA

Michael W. Deininger

INTRODUCTION AND HISTORICAL PERSPECTIVE

Chronic myeloid leukemia (CML), a rare condition, has had a profound impact on the development of hematology/oncology and modern medicine as a whole (Fig. 69.1A and B). The story started in the 1830s with Alfred Francois Donné of Paris, a remarkable innovator who pioneered the use of microscopy in medicine and was the first to describe the different types of blood cells. When asked to examine the blood specimen of a 44-year-old woman suffering from a painless left-abdominal mass, he noticed the excess of white cells and speculated that the patient's blood may contain pus.[1,2] Donné also organized a *Cours de Microscopie*, which was attended by Edinburgh pathologist John Hughes Bennet who in 1845 published a "Case of Hypertrophy of the Spleen and Liver in which Death Took Place from Suppuration of the Blood."[3] Only a few weeks later Rudolf Virchow of Berlin reported on a very similar patient in an article titled "Weisses Blut" (white blood).[4] Although we do not know for sure that these patients indeed suffered from CML, they highlight CML's most salient features: leukocytosis and splenomegaly. There was disagreement about the cause of the new disease. While Bennett believed it was an infection, Virchow recognized its neoplastic nature and coined the term "leukemia," from the Greek words "λενκον αιμα" meaning "white blood." The ensuing academic dispute was settled cordially: Virchow acknowledged Bennett's priority of discovery and Bennett that leukemia was a cancer of the blood. Perhaps it was the ability of these pioneers to put collaboration first and ego second that turned leukemia research into an engine of medical progress. Initial steps however were tedious. In 1872 Ernst Neumann established the bone marrow as the origin of leukemia and of blood cells in general, but then not much progress was made for almost a century.[5] In 1951 William Dameshek, a visionary with a rare ability of seeing an organizing concept in the unstructured, posited that CML belonged to a larger group of related disorders characterized by overproduction of blood cells in the bone marrow. He called these disorders myeloproliferative disorders (MPDs), a name that stuck.[6]

Just as better microscopes had enabled the first description of leukemia, improvements in chromosome banding led to a breakthrough by cytogeneticists Peter Nowell and David Hungerford from the city of Philadelphia. In 1960 they described an abnormally small G-group chromosome in metaphase spreads from several patients with CML.[7] Ironically, in a now mostly forgotten manuscript, a team from Edinburgh led by Ishbel Tough reported similar findings just a few months later.[8] The discovery of a consistent karyotypic abnormality associated with a cancer settled the debate as to whether DNA or proteins were responsible for transmitting the neoplastic phenotype to the next generation of cells. Anticipating that this was just the first in a long list of cancer chromosomes, the scientists called the new chromosome Philadelphia^1 (Ph^1, now usually referred to as Ph) after the place of its discovery. One lesion in all CML cells suggested that the leukemia traced its origins back to a single ancestral cell. Formal proof for this idea was provided by Philip Fialkow who showed that the neutrophils from a female CML patient expressed only one isoform of the X chromosomal gene glucose-6-phosphate dehydrogenase (G6PD). This was consistent with inactivation of either a maternal or paternal X chromosome in all cells rather than the random inactivation characteristic of polyclonal tissues.[9]

In 1973, Janet Rowley recognized that Ph was the product of a t(9;22)(q34;q11.1) reciprocal translocation rather than only a shortened chromosome 22.[10] Over the following 25 years, the genetic anatomy and biochemical consequences of t(9;22)(q34;q11.1) were revealed with increasing resolution. Groups led by Eli Cannani, John Groffen/Nora Heisterkamp, and Owen Witte contributed to the identification of the genes juxtaposed by t(9;22).[11–13] The chromosome 9 fusion partner was found to be *ABL1* (formerly *ABL*), the human homolog of v-abl, the oncogene of the Abelson murine leukemia virus (A-MuLV).[14,15] On the derivative chromosome 22, *ABL1* sequences consistently translocated to a genetic region that became known as the "breakpoint cluster region" (*BCR*), a name that was subsequently used for the new gene fused upstream of *ABL1*. The next critical step was the discovery that BCR-ABL1, similar to v-Abl, was a constitutively active tyrosine kinase and that kinase activity was required for cellular transformation.[16] Lastly, mice transplanted with bone marrow engineered to express BCR-ABL1 were shown to develop an MPD resembling CML, another milestone in the history of cancer research.[17]

CML therapy developed slowly. Arsenicals, in use for cancer treatment since ancient times, were the only treatment available in the 19th century, usually in the form of Fowler's solution, which contained potassium arsenite. The German physician Heinrich Lissauer is credited with the first publication on the remarkable efficacy of Fowler's solution in a patient with leukemia.[18] We may suspect that the reviewers of his paper gave him a pass on novelty, since the use of arsenic for cancer therapy had been described by the poet Valmiki in the Indian Ramayana in approximately 500 BC. As a curiosity, in an 1882 *Lancet* paper, Conan Doyle, better known for his Sherlock Holmes detective stories, published on a patient with the clinical presentation of CML who achieved a partial response to arsenic.[19] In the 1920s, radiotherapy was introduced for symptomatic relief of tender splenomegaly, and remained the mainstay of CML therapy for the first half of the 20th century. Busulfan (1959), an alkylating agent discovered at the Chester Beatty Laboratories in London, was the first drug that reliably controlled white blood cell counts in CML and has retained a place in conditioning regimens for allogeneic hematopoietic cell transplantation (HCT). Ten years later, hydroxyurea was introduced as the first intervention that prolonged survival, although by only a small margin.[20,21] A breakthrough was achieved in the late 1970s when the Seattle group reported the disappearance of Ph in CML patients who underwent HCT, the first cures of CML.[22] Soon after that, interferon-α (IFNα) was found to induce durable complete cytogenetic responses (CCyRs) and long-term survival in 10% to 20% of patients.[23] In 1992, Alexander Levitzki proposed the use of ABL inhibitors to treat leukemias driven by ABL oncogenes, but unfortunately, none of his so-called tyrphostins were developed for clinical use.[24] At about the same time, scientists at Ciba-Geigy had synthesized a potent inhibitor of ABL1 termed GCP57148B that is now known as imatinib.[25,26] Of note, GCP57148B's anti-ABL activity was the serendipitous byproduct of a drug development effort to identify inhibitors of platelet-derived growth factor receptor (PDGFR) for cardiovascular indications. Clinical trials initiated by Brian Druker, against the skepticism of the manufacturer, rapidly established the compound's activity in patients with CML and revolutionized CML therapy.[26–28] Solving the crystal structure of ABL1 in complex with imatinib provided the foundation for subsequent studies into the auto-regulation of ABL1 kinase activity and its disruption in BCR-ABL1.[29–31] These studies were of fundamental importance for our understanding of kinases and their regulation in physiological

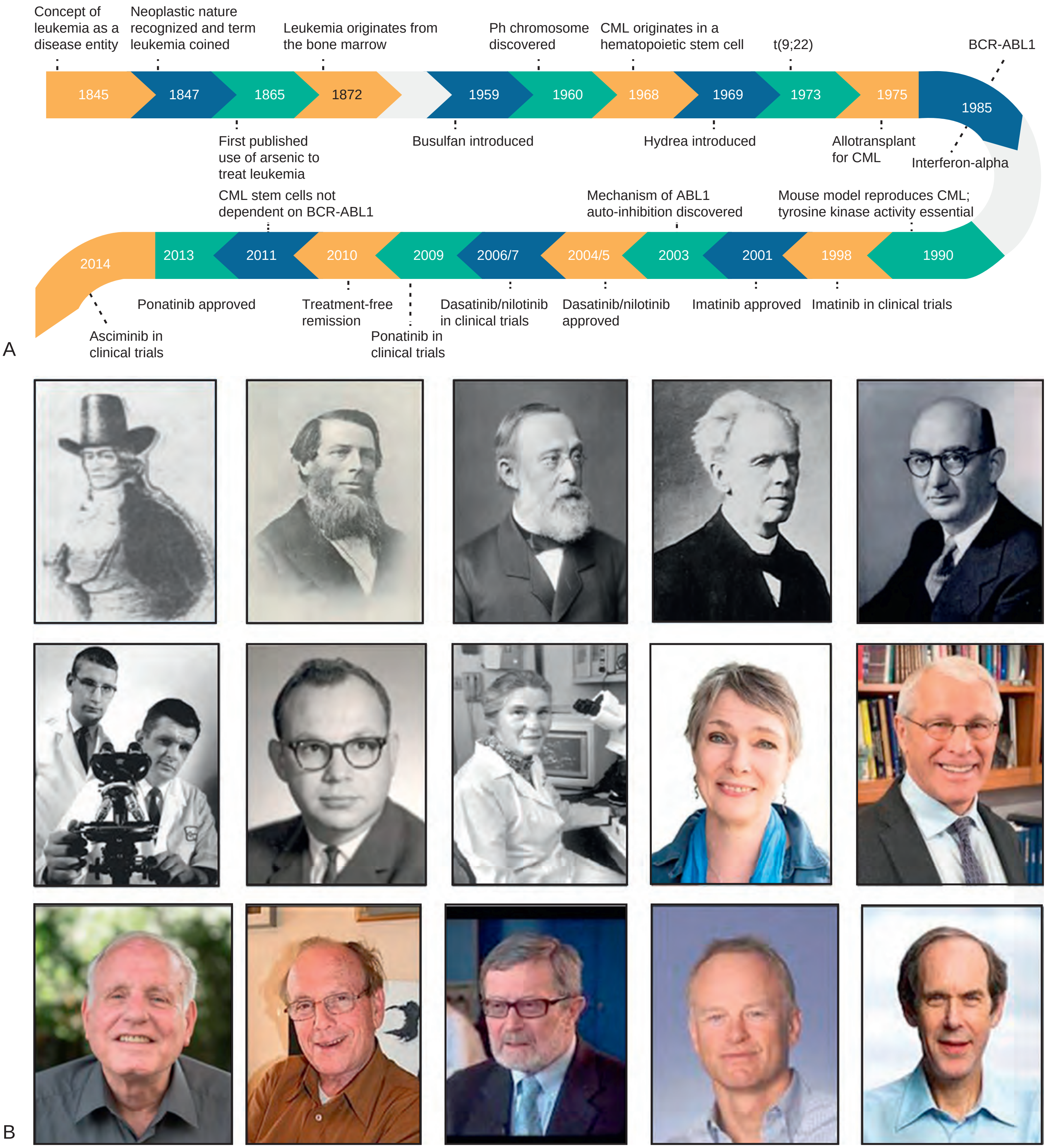

Concept of leukemia as a disease entity
Neoplastic nature recognized and term leukemia coined
Leukemia originates from the bone marrow
Ph chromosome discovered
CML originates in a hematopoietic stem cell
t(9;22)
BCR-ABL1
1845
1847
1865
1872
1959
1960
1968
1969
1973
1975
1985
First published use of arsenic to treat leukemia
Busulfan introduced
Hydrea introduced
Allotransplant for CML
Interferon-alpha
CML stem cells not dependent on BCR-ABL1
Mechanism of ABL1 auto-inhibition discovered
Mouse model reproduces CML; tyrosine kinase activity essential
2014
2013
2011
2010
2009
2006/7
2004/5
2003
2001
1998
1990
Ponatinib approved
Treatment-free remission
Dasatinib/nilotinib in clinical trials
Dasatinib/nilotinib approved
Imatinib approved
Imatinib in clinical trials
Asciminib in clinical trials
Ponatinib in clinical trials
A
B

Figure 69.1 THE HISTORY OF CHRONIC MYELOID LEUKEMIA. (A) Timeline of important developments in leukemia/CML. (B) Major contributors to CML biology and treatment. From left upper to right lower corner: Alfred Donné, Hughes Bennet, Rudolf Virchov, Ernst Neumann, William Dameshek, Peter Nowell and David Hungerford, Phillip Fialkow, Janet Rowley, Nora Heisterkamp, Owen Witte, Eli Canaani, Alexander Levitzki, Alois Matter, Nick Lydon, Brian Druker. Note that many others have made significant and lasting contributions, but for the sake of space could not be included here. *CML*, Chronic myeloid leukemia.

and aberrant signal transduction. Despite imatinib's general efficacy, subsets of patients developed resistance, frequently due to point mutations in BCR-ABL1, stimulating the development of subsequent generations of tyrosine kinase inhibitors (TKIs).[32–34] The latest development is asciminib, an allosteric ABL inhibitor that mimics the physiological autoinhibition of ABL1.[35,36]

There is compelling evidence that the most primitive CML cells are not dependent on BCR-ABL1 kinase activity,[37,38] explaining why residual leukemia remains detectable in many CML patients treated with TKIs. Surprisingly a plethora of studies has demonstrated that 40% to 60% of patients with deep molecular responses (DMRs) maintain remission after discontinuation of TKIs, a state termed treatment-free remission (TFR).[39] While survival remains the universal goal for all CML patients, TFR has gained traction as a new target for selected patient populations, particularly young individuals.[40]

The unprecedented success of TKIs to treat CML established a paradigm for molecularly targeted therapy that informs approaches in other types of cancer. However, many scientific, clinical, and societal questions remain. CML mortality is not zero, and the prognosis for patients who progress to blast phase CML (BP-CML) remains very poor. Despite improved understanding of blastic transformation at the molecular level, no fundamentally new treatments have emerged beyond chemotherapy followed by HCT. At the other extreme, increasing the pool of TRF eligible patients and improving the success rate will be crucial to minimize long-term TKI toxicity. However, as the biological underpinnings of TFR have remained largely elusive, rational approaches to improve over the current situation are lacking. Last but not least, the exorbitant costs of TKIs, particularly in the United Stated, raise ethical and societal questions that cannot be answered on medical grounds alone.[41] Again, CML has prepared the ground for fundamental discussions far beyond what one might expect from an orphan disease.

EPIDEMIOLOGY

CML occurs with an annual incidence of 1.0 to 1.5 per 100,000, without significant racial or geographic differences, but a slight male predominance of approximately 1.5. The median age at CML diagnosis is above 60 years in the developed world, but lower in developing countries, reflecting differences in access to medical care or—much less likely—disease biology. CML is generally rare in children, but cases have been reported even in infants. Familial occurrence has been observed, but there is no evidence for a general familial predisposition. The only well-documented risk factor is ionizing radiation. In the survivors of the atomic bombings in Japan, CML incidence increased, peaking 8 to 10 years post exposure.[42] Elevated rates of CML were also seen in patients treated with radiotherapy for ankylosing spondylitis and those exposed to thorotrast, an alpha-emitting contrast medium widely used in the 1930s and 1940s.[43,44] In contrast, there is no association between benzene exposure and CML.[45] As a result of the vastly improved survival afforded by TKIs, CML prevalence in the United States is predicted to rise from 70,000 in 2010 to 180,000 in 2050.[46] At that point CML will become the most prevalent myeloid neoplasm, with considerable implications for health care costs.

MOLECULAR BIOLOGY

BCR-ABL1 Translocation and Fusion Gene (Chapter 57)

Ph is the result of a reciprocal translocation between the long arms of chromosomes 9 and 22 [t(9;22)(q34;q11.2)] that fuses sequences from the *ABL1* gene on 9q34 downstream of the *BCR* gene on 22q11 (Fig. 69.2A). The chromosome 22 breaks localize almost exclusively to one of three *BCRs* and determine which parts of *BCR* are retained in the *BCR-ABL1* fusion mRNA and protein. In contrast, the chromosome 9 breaks can occur over a large genetic region, 5′ of *ABL1* exon Ib, 3′ of *ABL1* exon Ia, or most commonly between the two alternative first *ABL1* exons (Fig. 69.3B). Despite the different breakpoints, splicing almost invariably leads to fusion mRNAs that encompass *ABL1* exons 2 to 11.[47] Breakpoints in the minor *BCR* (m-*BCR)* generate an e1a2 fusion mRNA and p190$^{BCR-ABL1}$, which is found in two-thirds of Ph$^+$ acute lymphoblastic leukemia (ALL) cases, but very rarely in CML, where it is associated with monocytosis and an aggressive clinical course.[48,49] The origin and biology of p190$^{BCR-ABL1}$-positive CML is mysterious. Either m-*BCR* rearrangements are much less likely to occur in hematopoietic stem cells (HSCs) than the major *BCR* (M-BCR) rearrangements typical of CML, or the cell of origin is different, or an as yet unknown concomitant somatic mutation creates a state conducive to transformation by p190$^{BCR-ABL1}$. In the vast majority of CML patients, the break localizes to the M-*BCR*, generating e13a2 or e14a2 (formerly referred to as b2a2 and b3a2) fusion mRNAs and a p210$^{BCR-ABL1}$ fusion protein. p230$^{BCR-ABL1}$, the largest of the fusion proteins, is derived from a break in the micro *BCR* (μ-*BCR*) that generates e19a2 mRNA. p230$^{BCR-ABL1}$ is associated with neutrophilic predominance and a more benign disease.[50] There are a number of rare *BCR-ABL1* variants, some of which may be associated with a more aggressive clinical course.[51] Additionally variants can give rise to misleading reverse transcription polymerase chain reaction (RT-PCR) results and cannot be followed using standard quantitative PCR (qPCR) assays.[51–53] Approximately two-thirds of CML cases also express the reciprocal *ABL1-BCR* mRNA, but there is no definitive evidence that this influences disease biology or prognosis.[54,55] However, absence of *ABL1-BCR* mRNA is an indirect indication for deletions flanking the breakpoints in *BCR*, *ABL1*, or both. For unknown reasons, these deletions are associated with significantly reduced survival in patients treated with IFN-α, but their adverse impact is reduced or abolished by TKI therapy.[56,57]

BCR and ABL1 Proteins and Their Contribution to Cellular Transformation

BCR is a ubiquitously expressed 160kD cytoplasmic protein encompassing several functional domains, including an N-terminal coiled-coil motif, followed by a serine/threonine kinase motif whose only known substrate is the 14-3-3 family protein Bap-1,[58] a *dbl*-like (DH) domain and a pleckstrin-homology (PH) domain (see Fig. 69.3A). DH had been thought to stimulate exchange of guanidine triphosphate (GTP) for guanidine diphosphate (GDP) on Rho guanidine exchange factors,[59] but recent structural and biochemical studies failed to confirm this.[60,61] The PH domain binds to various phosphatidylinositol (PI)-phosphates, impacts subcellular protein localization, and is required for interactions with selected proteins.[60,61] The BCR C-terminus contains a putative calcium-dependent lipid binding (CaLB) site and a GTPase activating function (RAC-GAP). The latter constrains activity of Ras-related C3 botulinum toxin substrate (RAC), a small GTPase that regulates actin polymerization and an nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase in phagocytic cells.[62,63] *Bcr*$^{-/-}$ mice are viable and fertile, the only known defect being an increased oxidative burst in neutrophils.[64] BCR can be phosphorylated on several tyrosine residues, most importantly tyrosine 177, which binds growth factor receptor bound protein 2 (GRB2), an adapter molecule involved in rat sarcoma virus (RAS) activation in CML cells.[65–67] The question why p190$^{BCR-ABL1}$ and p210$^{BCR-ABL1}$ are associated with ALL and CML, respectively, has long been a matter of debate.[59,68,69] Recent work has shown that the protein networks entertained by p190$^{BCR-ABL1}$ and p210$^{BCR-ABL1}$ are profoundly different, and the differences have been attributed to the retention of the PH domain in p210$^{BCR-ABL1}$.[60] It remains unclear how this impacts lineage commitment and why HSCs are susceptible to transformation by p210$^{BCR-ABL}$, while p190$^{BCR-ABL1}$ targets pre-B cells.[70]

ABL1 is the human homologue of the v-*abl* oncogene of the A-MuLV.[71] The 145-kDa ABL1 protein is ubiquitously expressed and encodes a non-receptor tyrosine kinase.[72] Two isoforms arise from alternative splicing of the first exon. ABL1a lacks the first 19 amino acids, which are retained in ABL1b and include a glycine in position 2 that can be myristoylated.[73] The N-terminal region contains three SRC homology domains (SH1 to SH3). SH1 carries the tyrosine kinase function, while the SH2 and SH3 domains engage in protein-protein interactions and participate in regulation of kinase activity (see Fig. 69.3B).[74] The center of ABL1 features proline rich sequences that interact with SH3 domains of other proteins such as Crk.[75,76] The C-terminus contains nuclear localization, DNA binding,[77] and actin-binding motifs.[78] Several functions have been attributed to ABL1, including inhibition of cell cycle progression and proliferation,[79,80] integrin signaling,[81] and DNA repair.[82–86] Mice with germline deletion of *Abl1* exhibit high perinatal mortality, runting, and skeletal and immune system defects.[87,88] ABL1 kinase activity is tightly controlled in physiological conditions through allosteric mechanisms that involve a myristate-binding pocket at the base of the SH1 domain. Mice null for *Abl1* and the *Abl*-related gene (formerly

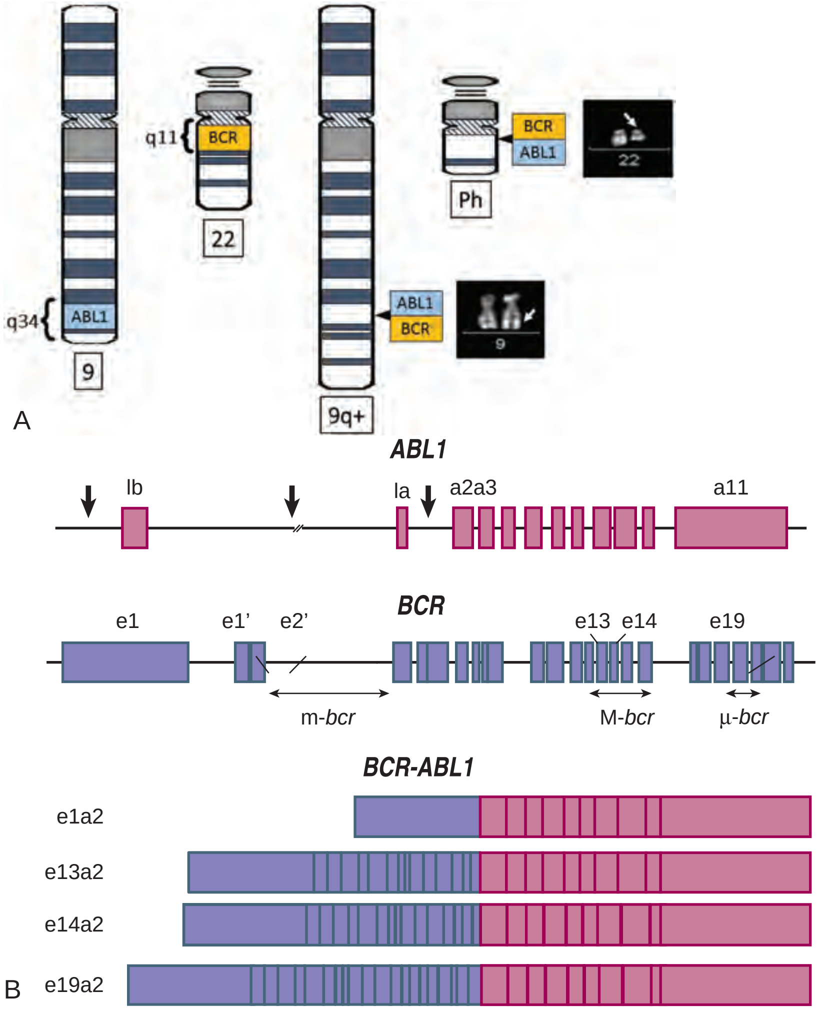


Figure 69.2 GENETIC ANATOMY OF THE *BCR-ABL1* TRANSLOCATION. (A) Schematic of the t(9;22)(q34;q11.1) and R-banding images of normal chromosome 9, derivative chromosome 9 *(white arrow)*, normal chromosome 22, and Ph *(white arrows)*. (B) *(upper panels)* Genomic loci of *ABL1* and *BCR* with exon numbering. The three breakpoint cluster regions in *BCR* are also indicated. *(lower panel)* Fusion mRNAs derived from the chimeric genes. Note that the e13a2 and e14a2 fusion mRNAs were previously referred to as b2a2 and b3a2, respectively. *BCR*, Breakpoint cluster region

Arg, now *Abl2*) are embryonically lethal due to absence of neurulation.[89] Gain of function in the germline is also detrimental, as shown by a recent report of mutations in the myristate binding pocket that confer constitutive kinase activation and are associated with cardiac and skeletal defects.[90] The emerging complex picture suggests that ABL1 may be best characterized as a cellular module that integrates extra- and intracellular signals to influence decisions with regard to cell cycle and apoptosis. The latter observation raised considerable concerns about the potential side effects of clinical ABL inhibitors that fortunately were not confirmed.

Constitutive Kinase Activation in the BCR-ABL1 Fusion Protein

In contrast to ABL1, BCR-ABL1 is constitutively active and excluded from the nucleus. Revealing the mechanism by which replacing the ABL1 N-terminus with BCR sequences leads to kinase activation has led to deep insights into the regulation of ABL1.[91] The coiled-coil domain of BCR (see Fig. 69.3A) promotes dimerization, which allows for an initial transphosphorylation event, followed by autophosphorylation of additional tyrosine residues that fully activate the kinase.[92] Other proteins with the ability to form dimers can substitute for BCR, such as ETV6 and NUP214 that form ABL1 fusion genes associated with a myeloproliferative neoplasm (MPN) or ALL.[93,94] The N-terminal “cap” region of ABL1, when myristoylated, binds to a hydrophobic pocket at the base of the kinase domain, resulting in a conformation that resembles a latch that holds the kinase in an inactive state.[30,31,95] However, small molecules binding to the myristate pocket inhibit kinase activity, indicating that the concept of a “mechanical” latch is too simplistic. Rather than that, an allosteric mechanism controls kinase activity that does not require the ABL1 N-terminal sequences and hence is preserved and amenable to therapeutic targeting in BCR-ABL1.[35,96] How precisely information is communicated from the myristate pocket to the catalytic site is unknown. Recent data suggest that reverse signaling is also possible, as mutations in the ATP site can influence binding of small molecules to the myristate pocket.[97] Additional levels of regulation exist: certain residues within the SH2 domain participate in the regulation of kinase activity,[98] and trans-acting binding partners such as the ABL1 interacting proteins have been implicated as physiological inhibitors.[99,100]

Several domains or amino acid residues in BCR-ABL1 are critical for cellular transformation. In the ABL1 portion they include the kinase domain, SH2, and actin-binding domains (see Fig. 69.3C).[101] Additionally the SH3 domain contributes to aberrant regulation of homing, adhesion, and migration.[102] Transformation-relevant domains in the BCR portion include a coiled–coil motif contained in amino acids 1 to 63, the tyrosine at position 177, and phosphoserine–threonine-rich sequences between amino acids 192 to 242 and 298 to 413.[103] Whether or not a particular feature is essential may depend on the cellular context. For example, SH2 deletion mutants of BCR-ABL1 are defective for fibroblast transformation, but they retain the capacity to transform cell lines to factor independence and are leukemogenic in mice.[103]

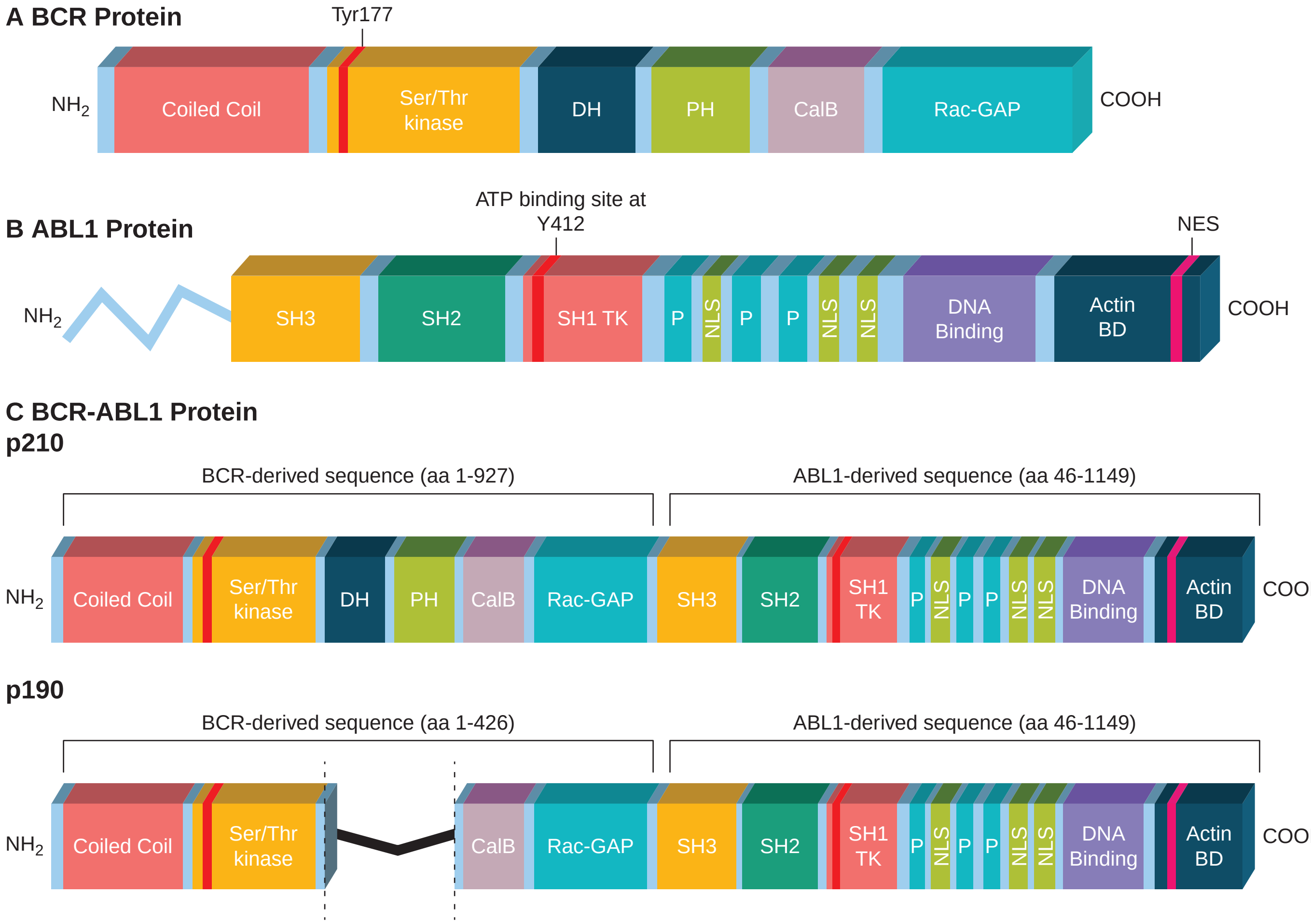

A BCR Protein
Tyr177
NH2
Coiled Coil
Ser/Thr kinase
DH
PH
CalB
Rac-GAP
COOH
B ABL1 Protein
ATP binding site at Y412
NES
NH2
SH3
SH2
SH1 TK
P
NLS
P
P
NLS
NLS
DNA Binding
Actin BD
COOH
C BCR-ABL1 Protein
p210
BCR-derived sequence (aa 1-927)
ABL1-derived sequence (aa 46-1149)
NH2
Coiled Coil
Ser/Thr kinase
DH
PH
CalB
Rac-GAP
SH3
SH2
SH1 TK
P
NLS
P
P
NLS
NLS
DNA Binding
Actin BD
COOH
p190
BCR-derived sequence (aa 1-426)
ABL1-derived sequence (aa 46-1149)
NH2
Coiled Coil
Ser/Thr kinase
CalB
Rac-GAP
SH3
SH2
SH1 TK
P
NLS
P
P
NLS
NLS
DNA Binding
Actin BD
COOH

Figure 69.3 STRUCTURAL DOMAINS AND SIGNALING MOTIFS IN BCR, ABL1, AND BCR-ABL1 PROTEINS. (A) BCR protein. Shown are the N-terminal coiled coil (oligomerization), serine/threonine kinase, Dbl-like domain (DH), pleckstrin homology (PH) domain, calcium binding domain (CalB), and Rac-GAP domain. (B) ABL1 protein. Shown are SRC homology domains 1–3 (SH1–3), proline-rich motifs (P), nuclear localization signals (NLS), DNA binding domain, and actin binding domain. (C) p210BCR-ABL1 and p190BCR-ABL1 proteins. Note that p210BCR-ABL1 retains the DH and PH domains, which are missing from p190BCR-ABL1. *BCR*, Breakpoint cluster region.

Aberrant Signaling in BCR-ABL1 Transformed Cells

BCR-ABL1 signaling is one of the most intensely studied areas in biomedical research. Three decades of research have identified numerous BCR-ABL1 substrates, binding partners, and downstream signaling molecules involved in cellular transformation, and the number is still growing (Fig. 69.4). Efforts have been directed at linking these pathways to specific characteristics of CML cells, such as increased proliferation, reduced apoptosis, abnormal adhesion and migration, or genetic instability. The accumulated data are overwhelming, and a comprehensive review of the multiple pathways implicated in BCR-ABL1 transformation is beyond the scope of this chapter. As a note of caution, despite evidence from CML cell lines and primary CML cells, mouse models using homozygous deletions of signaling proteins have identified very few pathway components with a truly essential role for BCR-ABL1–induced leukemia. This speaks to the extensive redundancy of the BCR-ABL1 transformation network and/or to the limitations of the disease models.

Phosphatidylinositol-3 Kinase

BCR-ABL1 activates phosphatidylinositol-3 kinase (PI3K) through at least two separate mechanisms. The first is initiated by autophosphorylation of BCR-ABL1 tyrosine 177 (Y177), which generates a docking site for the GRB2 adapter protein. GRB2 recruits GAB2, another adapter, into a complex that activates PI3K.[104,105] Alternatively, PI3K can be activated by complex formation between the PI3K p85 regulatory subunit, and the BCR-ABL1 substrates casistas B-lineage lymphoma (CBL) and CRK like (CRKL), which bind to the SH2 and proline-rich domains of BCR-ABL1.[106] The main downstream target of PI3K is the serine-threonine kinase AKT, a major conduit for oncogenic signals in many types of cancer. In BCR-ABL1$^+$ cells, AKT enhances survival by phosphorylating forkhead O transcription factor 3a (FOXO3a) and pro-apoptotic proteins like BCL2 associated agonist of cell death (BAD).[107–109] Phosphorylated FOXO3 is excluded from the nucleus, leading to downregulation of important transcriptional targets such as p27. Phosphorylated BAD is no longer able to neutralize anti-apoptotic BCL2 family members such as BCL2 and BCLX$_L$, promoting survival. Furthermore, AKT activates mammalian target of rapamycin (mTOR), which phosphorylates ribosomal proteins p70S6 kinase (S6K) and 4E-BP1. S6K, a serine/threonine kinase, phosphorylates multiple substrates that collectively promote cell proliferation. Phosphorylated mTOR has been shown to inactivate 4EBP-1, releasing the translation initiation factor eIF4E from inhibition to enhance protein synthesis.[110,111] In addition to activating AKT, PI3K promotes proteasomal degradation of p27 through upregulation of SKP2, the F-Box recognition protein of the SCF^{SKP2} E3 ubiquitin ligase. Accordingly, absence of SKP2 from leukemia cells prolongs survival in a murine CML model.[112]

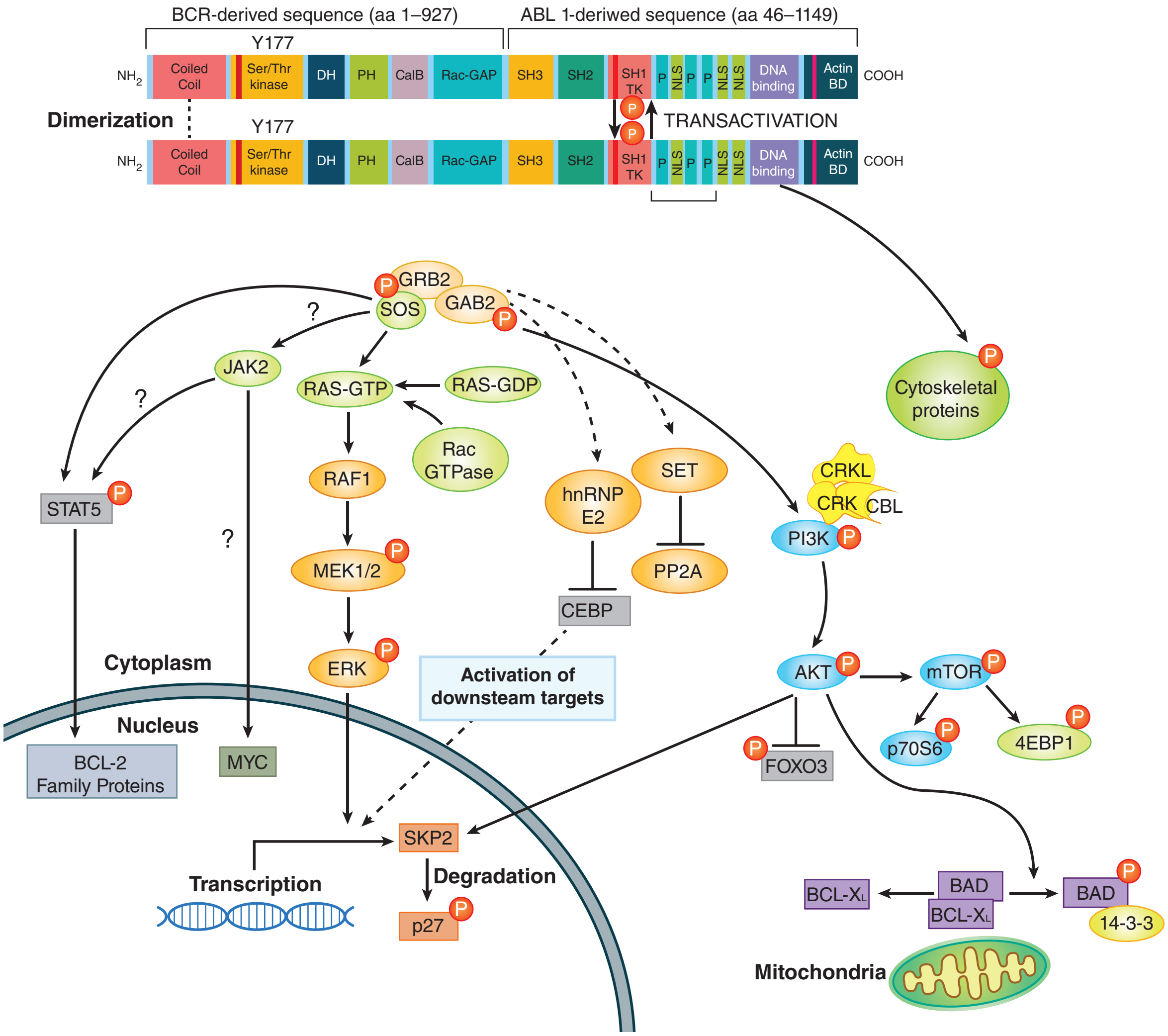

BCR-derived sequence (aa 1–927)
ABL 1-deriwed sequence (aa 46–1149)
Y177
NH_2
Coiled Coil
Ser/Thr kinase
DH
PH
CalB
Rac-GAP
SH3
SH2
SH1 TK
P
NLS
DNA binding
Actin BD
COOH
Dimerization
TRANSACTIVATION
GRB2
SOS
GAB2
JAK2
RAS-GTP
RAS-GDP
Rac GTPase
RAF1
MEK1/2
ERK
hnRNP E2
SET
PP2A
CEBP
Activation of downsteam targets
STAT5
?
Cytoplasm
Nucleus
BCL-2 Family Proteins
MYC
SKP2
Transcription
Degradation
p27
Cytoskeletal proteins
CRKL
CRK
CBL
PI3K
AKT
mTOR
p70S6
4EBP1
FOXO3
BCL-XL
BAD
14-3-3
Mitochondria

Figure 69.4 GROSSLY SIMPLIFIED REPRESENTATION OF SIGNALING PATHWAYS ACTIVATED IN CML CELLS. Autophosphorylation generates docking sites on BCR-ABL1 that promote binding of intermediary proteins, some of which are also BCR-ABL1 substrates such as CRKL and CBL, forming a multimeric complex. The BCR-ABL1 signaling complex activates multiple downstream pathways that inhibit apoptosis, enhance proliferation, perturb adhesion and migration, and impart genomic instability. Only few of these downstream mediators are essential to BCR-ABL's ability to transform cells, indicating that the signaling network is highly redundant. For details see text. *BCR*, Breakpoint cluster region.

RAS/Mitogen-Activated Protein Kinase Pathways

A long-known pathway of RAS activation is phosphorylation of tyrosine 177 with recruitment of GRB2 and Son of Sevenless (SOS) into a complex that promotes exchange of GTP for GDP on RAS.[104,113] GTP-bound RAS activates the serine-threonine kinase RAF-1,[114,115] which in turn activates mitogen-activated protein kinase (MAPK). Recently, a genetic screen identified inactivation of LZTR1 as a promoter of RAS activation in TKI-treated CML cells.[116] Mechanistically LZTR1 controls KRAS expression by enhancing interaction with the CUL3 E3 ligase, promoting proteasomal degradation. Other small GTPases like RAC1/2 have also been implicated in BCR-ABL1 signaling[117] and genetic absence of RAC1/2 has been shown to attenuate *BCR-ABL1* leukemia in a murine model.[118]

Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) Pathway

The STAT5 transcription factor is constitutively tyrosine-phosphorylated in CML cells. Initial experiments in mice failed to demonstrate a critical role for STAT5.[119] However, it was subsequently discovered that the presumed *Stat5* knockout mice used in this study were not null for *Stat5*, but expressed a partially functional N-terminally deleted protein. While STAT5 is not required for the initiation of CML, there is convincing evidence that it plays a rate-limiting role in leukemogenesis.[120,121] Absence of only STAT5A or overexpression of a dominant-negative STAT5 mutant in BCR-ABL1 expressing bone marrow cells attenuates CML.[122] Conditional deletion of STAT5 prevents the establishment of active CML in mice, but does not eliminate the BCR-ABL1 expressing cells.[120] The consequence of constitutive STAT5 activation is inhibition of apoptosis by enhancing transcription of anti-apoptotic proteins like MCL-1 and BCL-X_L.[123] BCR-ABL1 may activate STAT5 through direct phosphorylation or indirectly by promoting phosphorylation by HCK or JAK2.[124,125] The role of JAK2 itself is controversial. Previous studies had suggested that JAK2 plays an important if not essential role,[126] while a recent report using conditional knockout technology showed that murine CML does not require JAK2.[127] In contrast, JAK2 may have an important role in promoting survival of CML stem cells (from here onward referred to as LSCs).[128,129]

Cytoskeletal Proteins

Several BCR-ABL1 substrates are involved in adhesion and migration, including focal adhesion kinase (FAK), CRKL, paxillin, p130CAS, and HEF1.[130–135] It is thought that this aberrant phosphorylation is responsible for the defect in integrin-mediated adhesion of CML progenitors to bone marrow stroma and extracellular matrix. Alternatively, integrin function may be compromised through RAS activation or BCR-ABL1 binding to F-actin.[104,136] Lastly one report found that a kinase inactive BCR-ABL1 variant causes an adhesion defect, consistent with a process that is independent of BCR-ABL kinase activity.[137] Given that adhesion of integrins inhibits proliferation of hematopoietic progenitors, the defect in integrin function may contribute to the premature circulation as well as the abnormal proliferation of Ph^+ progenitor cells.[137]

DNA Damage Surveillance and Repair Pathways

Without effective therapy, chronic phase of CML (CP-CML) invariably progresses to BP-CML, testimony to the profound genetic instability of CML cells. CML $CD34^+$ cells contain more reactive oxygen species (ROS) compared to normal controls, and this is particularly pronounced in CML-BP cells.[138–140] ROS cause DNA damage by generating oxidized bases and double-strand breaks (DSB), which are up to eightfold more frequent in CML than in normal cells.[141] While ROS generation in CML cell lines and progenitor cells is controlled by BCR-ABL1 through activation of PI3K, studies in mice have shown that LSCs continue to accumulate ROS-induced DNA damage despite TKI inhibition of BCR-ABL1.[142,143] This suggests that genetic evolution may continue in patients on TKIs as long as they harbor residual leukemia. Fortunately, this concern was not borne out in the clinic, at least in patients with a CCyR or major molecular response (MMR), whose progression rate is low. Additional mutagenic mechanisms operational in lymphoid blast phase cells are activation of activation-induced cytidine deaminase (AID), which promotes point mutations, and RAG-mediated recombination, which promotes structural recombination events.[144,145] The consequences of increased DNA damage are aggravated by the impairment of DNA damage surveillance and repair. Multiple mechanisms have been implicated, including BCR-ABL1 kinase-independent mechanisms. For instance, BCR-ABL1 impairs the intra-S-phase cell cycle checkpoint through suppression of checkpoint kinase 1 (CHK1), either by inhibiting the nuclear protein kinase ATR[146] or by downregulating BRCA1, a substrate of ataxia telangiectasia mutated (ATM).[147] There is also aberrant regulation of DSB repair pathways. In addition to BRCA1, the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) is downregulated in $CD34^+$ CML progenitors, leading to error-prone non-homologous end-joining (NHEJ), rather than more faithful D-NHEJ,[148] thereby enhancing the effects of BCR-ABL1–induced ROS. While these deficiencies result in genomic instability and increase the risk of progression, they also offer therapeutic opportunities. Cycling cells have access to a range of DNA repair mechanisms, including homologous recombination (HR), single strand annealing (SSA), transcription-associated homologous recombination (TA-HR), and NHEJ. In contrast, quiescent cells have limited options, using mostly NHEJ (Fig. 69.5A). There are differences in the usage of the various DNA repair pathways between LSCs and HSCs that in the future may provide individualized therapeutic opportunities, based on the principle of synthetic lethality. For instance BRCA1/2 is frequently downregulated in CML. Additionally, some cases have downregulated one of the components of D-NHEJ, rendering them vulnerable to a combination of PARP inhibitors such as olaparib and inhibitors of RAD51, while cycling HSCs can resort to BRCA1/2-based HR, and quiescent HSCs to D-NHEJ (see Fig. 69.5B, C).[149,150] There is little evidence that TKIs themselves cause DNA damage, although imatinib was reported to induce centrosome abnormalities in vitro.[151] Conflicting data have been reported regarding the risk of second (non-hematologic) malignancies in CML patients on TKIs but there is consensus that any excess incidence is related to CML rather than TKI therapy.[152,153] Similarly there is no evidence that the clonal cytogenetic abnormalities in Ph-negative cells (CCA/Ph^-) detected in 5% to 10% of CML patients with a cytogenetic response to TKI therapy are induced by TKIs.[154]

A wealth of data on BCR-ABL1 signaling has been amassed, yet a complete picture is still elusive. As conventional approaches have typically focused on single pathways, efforts are now underway to characterize more comprehensively BCR-ABL1 signaling by quantitative genomics.[60,155] These data suggest that cellular processes in CML rely on integrated networks rather than single pathways, which cooperate to fully realize the leukemogenic potential of BCR-ABL1.

CELLULAR BIOLOGY

Cell of Origin

Current thinking holds that *BCR-ABL1* is both necessary and sufficient to produce the CP-CML. Unlike some fusion genes associated with acute myeloid leukemia (AML), such as *MOZ-TIF2*, *BCR-ABL1* does not confer HSC self-renewal, implying that the initial translocation event must occur in a multipotent hematopoietic HSC already endowed with this property.[156] Evidence in support of this comes from mouse models and clinical observations. Mice transplanted with bone marrow cells infected with a *BCR-ABL1* retrovirus develop an oligoclonal CML-like MPN, suggesting that a second event is not required.[13] Similarly mice expressing a doxycycline-repressible BCR-ABL1 allele develop CML upon withdrawal of doxycycline, which regresses upon re-exposure to doxycycline.[25] TKIs restore polyclonal hematopoiesis as assessed by X chromosome inactivation.[154] However the clinical variability of CP-CML, particularly the highly variable time to transformation to AP/BP-CML, indicates the presence of major disease modifying factors. Between 5% and 10% of CP-CML cases have additional clonal cytogenetic abnormalities in Ph^+ cells (CCA/Ph^+) at diagnosis, and this is associated with a more aggressive clinical course.[157] In almost all cases, CCA/Ph^+ disappear upon achievement of a cytogenetic response, suggesting they reflect evolution of the Ph^+ cell clone (Fig. 69.6). Next generation sequencing (NGS) has revealed mutations in epigenetic modifier genes such as *TET2, DNMT3A*, and *ASXL1* in ~30% of CP-CML patients at diagnosis.[158–160] In most but not all cases, these mutations became undetectable at the time of cytogenetic or molecular response, indicating that CML may develop either truly de novo or on the background of clonal hematopoiesis (CH) (see Fig. 69.6). This model is consistent with the observation that CML can occur at any age, while complex myeloid neoplasms such as chronic myelomonocytic leukemia (CMML) almost invariably develop from preceding CH and as such are restricted to older individuals.[161,162] The impression is that patients with additional somatic mutations at diagnosis have a worse prognosis, but this is less well established compared to CCA/Ph^+, and more data is needed.[159,163] That somatic mutations in addition to Ph may happen at any point during the evolution of CML speaks to the remarkable capacity of BCR-ABL1 to drive the CML phenotype, either alone or on a genetically modified background.

Chronic Myeloid Leukemia Hematopoiesis

Unlike in acute leukemia, the hierarchical organization of hematopoiesis is initially maintained in CML, although there is lineage imbalance in favor of the myeloid series and a left-shift of granulopoiesis. Although maturation is delayed, cellular function is mostly normal, evidenced by the fact that CML patients in the chronic phase are not at increased risk of infection or bleeding. *BCR-ABL1* is detected in cells belonging to all hematopoietic lineages, including B cells and T cells.[164] Consistent with the clinical presentation, the thrust of *BCR-ABL1*–induced cellular expansion targets the myeloid progenitor cell compartment, which is almost exclusively Ph^+ (see Fig. 69.6). In contrast, in most newly diagnosed patients the majority of the most primitive cells (defined as long-term culture initiating cells, LTC-ICs, or quiescent $CD34^+$ cells) are partially or even predominantly Ph^-.[165] These normal HSCs are the basis for cytogenetic and molecular

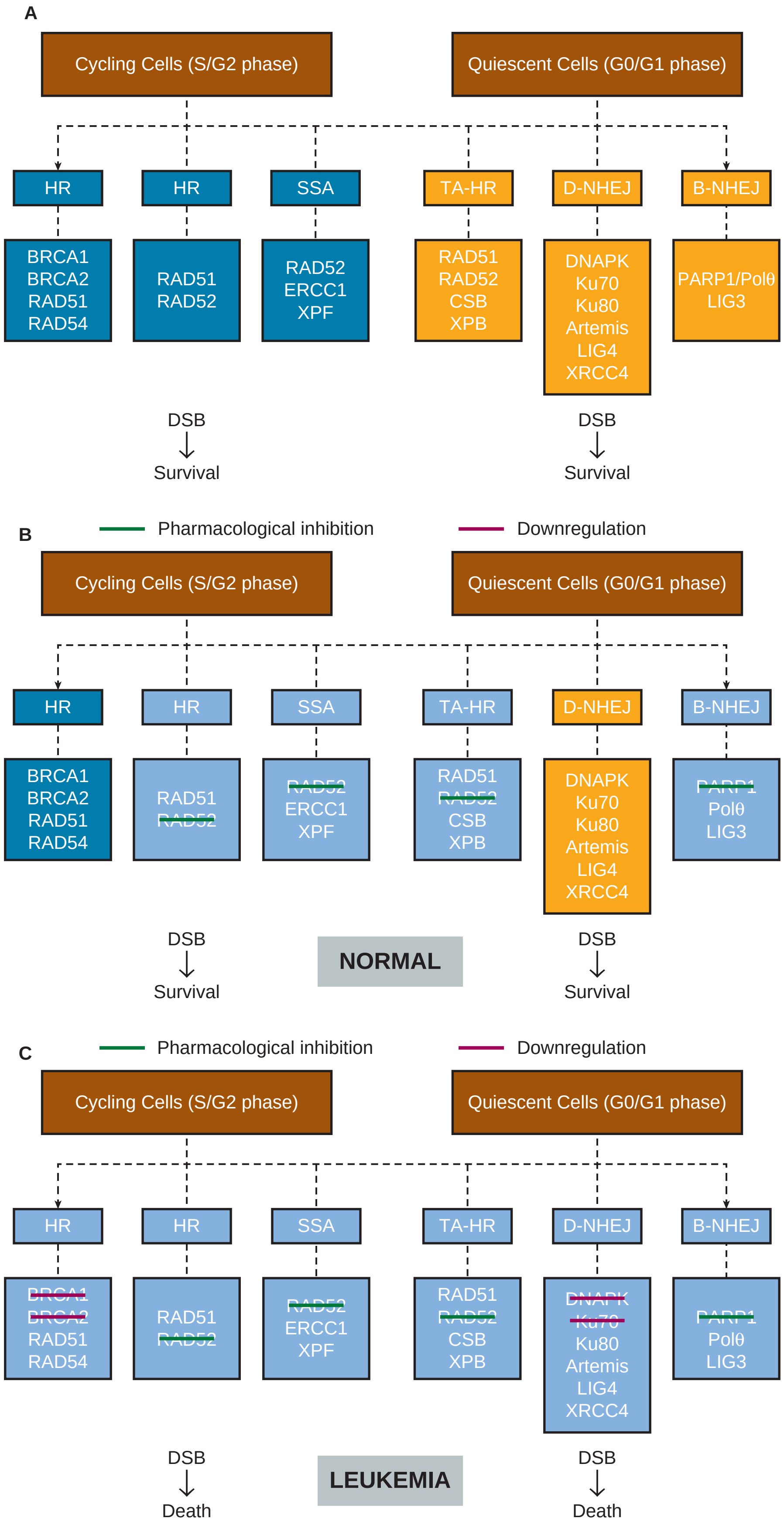

A
Cycling Cells (S/G2 phase)
Quiescent Cells (G0/G1 phase)
HR
HR
SSA
TA-HR
D-NHEJ
B-NHEJ
BRCA1 BRCA2 RAD51 RAD54
RAD51 RAD52
RAD52 ERCC1 XPF
RAD51 RAD52 CSB XPB
DNAPK Ku70 Ku80 Artemis LIG4 XRCC4
PARP1/Polθ LIG3
DSB
Survival
DSB
Survival
B
Pharmacological inhibition
Downregulation
Cycling Cells (S/G2 phase)
Quiescent Cells (G0/G1 phase)
HR
HR
SSA
TA-HR
D-NHEJ
B-NHEJ
BRCA1 BRCA2 RAD51 RAD54
RAD51 ~~RAD52~~
~~RAD52~~ ERCC1 XPF
RAD51 ~~RAD52~~ CSB XPB
DNAPK Ku70 Ku80 Artemis LIG4 XRCC4
~~PARP1~~ Polθ LIG3
DSB
Survival
NORMAL
DSB
Survival
C
Pharmacological inhibition
Downregulation
Cycling Cells (S/G2 phase)
Quiescent Cells (G0/G1 phase)
HR
HR
SSA
TA-HR
D-NHEJ
B-NHEJ
~~BRCA1~~ ~~BRCA2~~ RAD51 RAD54
RAD51 ~~RAD52~~
~~RAD52~~ ERCC1 XPF
RAD51 ~~RAD52~~ CSB XPB
~~DNAPK~~ ~~Ku70~~ Ku80 Artemis LIG4 XRCC4
~~PARP1~~ Polθ LIG3
DSB
Death
LEUKEMIA
DSB
Death

Figure 69.5 DNA REPAIR PATHWAYS IN CYCLING AND QUIESCENT HSCS AND LSCS. (A) Fully active DNA repair. (B) Cycling HSCs maintain BRCA1/2-dependent homologous recombination (HR) upon inhibition of RAD52-dependent HR, single strand annealing and transcription-dependent homologous recombination, and DNA-PK dependent non-homologous end joining (D-NHEJ) upon PARP inhibition of B-NHEJ with drugs such as olaparib. (C) Cycling and HSCs from some patients downregulate BRCA1/2 and/or components of the D-NHEJ (DNA-PK, Ku70, Ku80, LIG4), respectively, and become susceptible to synthetically lethal combinations of RAD52 and PARP inhibitors. *DSB*, Double-strand break; *SSA*, single-strand annealing; *TA-HR*, transcription-associated homologous recombination.

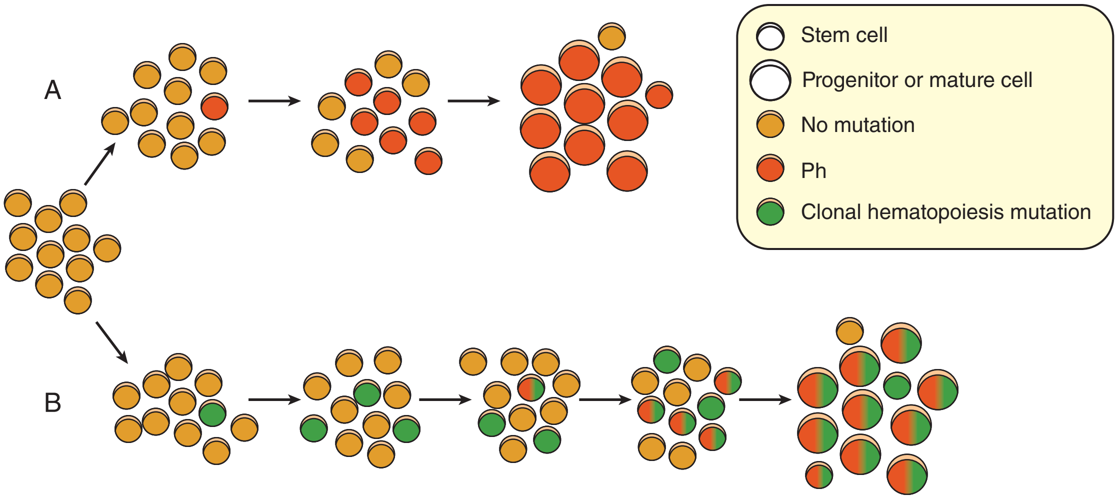


Figure 69.6 ORIGIN AND DEVELOPMENT OF THE CHRONIC PHASE OF CHRONIC MYELOID LEUKEMIA. (A) Origin through acquisition of Ph in a diploid hematopoietic stem cell (HSC) without prior clonal hematopoiesis mutation. (B) Origin from a somatically mutated HSC.

responses to therapy. The cause of the myeloid bias is incompletely understood. One possible explanation is that BCR-ABL1 alone is insufficient to overcome the powerful metabolic growth restraint placed on lymphoid precursor cells, which is thought to minimize the risk of malignant transformation during V(D)J rearrangement or T-cell receptor diversification.[166] As such BCR-ABL1-positive chronic lymphocytic leukemia does not exist. However, when disruption of orderly B-cell (and rarely T-cell) differentiation abrogates the growth restraint, BCR-ABL1-positive ALL ensues. In the B-cell lineage this is accomplished by inactivation of lineage-specific transcription factors such as IKZF1 or PAX5.[166]

Leukemia stem cells (LSCs) and what distinguishes them from HSCs have been the topic of intense research. It is important to remember that the ultimate diagnostic test for stemness is functional—the ability of a cell to generate progeny of all lineages in vivo, while phenotypic markers are surrogates by definition. As such, the terms HSC and LSC frequently denote populations of cells that are enriched for functionally defined "true" stem cells, but not pure populations. For instance, in many studies stem cells are conveniently defined as Lineage$^-$CD34$^+$CD38$^-$, but less than 10% of these cells are functionally stem cells. As of 2020, no universal immunophenotypic marker had been identified that separates LSCs from HSCs, but strategies using multiple markers have achieved considerable enrichment for BCR-ABL$^+$ cells. The issue is complicated by the fact that some but not all markers are regulated by BCR-ABL1 kinase activity, and that TKIs predominantly eliminate proliferating LSCs with a "late" myeloid signature, while relatively sparing the most primitive LSCs.[167] As a consequence, the immunophenotype of LSCs in untreated CML patients is different from that in patients with residual disease on TKIs (Table 69.1). Some clinical features of CML can be linked to biological aberrancies of LSCs. For instance LSCs exhibit reduced integrin-mediated adhesion to bone marrow stroma and abnormal migration to CXCL12 (SDF1), which may account for leukocytosis and extramedullary hematopoiesis in spleen and liver.[168,169] Transcriptomic profiling of CD34$^+$38$^-$ cells showed that quiescent LSCs are primed to proliferate compared to HSCs, explaining their hypersensitivity to cytokines such as IL-3 and G-SCF.[149,170]

TABLE 69.1 **Immunophenotypic Markers of Primitive Chronic Myeloid Leukemia Cells in Chronic Phase at Diagnosis and on Tyrosine Kinase Inhibitor Therapy**

	HSC	LSC (Diagnosis)	LSC (on TKI)
Lineage	–	–	–
CD34	+++	+++	+
CD38	Low/–	Low/–	Low/–
CD11c	+	+	–
CD25	–	++	–
CD26	Low/–	+	+
CD32 (FcγRII)	+	++	++
CD45RA	Low/–	Low/–	–
CD90	+++	++	++
CD117	+++	+	–
CD276	–	–	–
TIM3	–	–	–
IL1RAP	–	++	–
ITGB7	–	–	–

HSC, Hematopoietic stem cell; *TKI*, tyrosine kinase inhibitor.
Adapted from Warfvinge R, Geironson L, Sommarin MNE, et al. Single-cell molecular analysis defines therapy response and immunophenotype of stem cell subpopulations in CML. *Blood*. 2017;129(17):2384–2394.

A question of considerable clinical importance is whether CML stem cells are dependent ("addicted") to BCR-ABL1 or not. The fact that residual BCR-ABL1$^+$ cells remain detectable by PCR in most patients on TKIs has been interpreted as evidence for persistence of LSCs despite continued suppression of BCR-ABL1 kinase activity. Consistent with this, primitive CML cells survive TKI treatment ex vivo.[37,38] However, recent data have revealed that long-lived B cells and less frequently T cells account for most of the positive PCR results in patients in TFR, while granulocytes are consistently BCR-ABL1-negative. These data do not prove that multipotent CML stem cells are absent, but they explain the puzzling observation of positive PCR results in patients with stable TFR for years. Conceptually the central question is whether an LSC with kinase-disabled BCR-ABL1 is equal to a normal HSC or is still "rewired." This could happen through at least three mechanisms. First there is compelling data that some BCR-ABL1 functions are kinase-independent.[137,150] Whether these functions alone are sufficient to support long-term survival of LSCs is unknown. Second epigenetic reprogramming as a result of exposure to unopposed BCR-ABL1 kinase activity could persist even when BCR-ABL1 is no longer active.[171] Third the presence of a large multi-domain protein like BCR-ABL1 may interfere with physiological signaling, for instance through sequestration of adaptor proteins. Indeed considerable evidence has accumulated to suggest that LSCs exhibit specific vulnerabilities that may be exploitable therapeutically, either alone or in combination with TKIs (Table 69.2). Although several pathways such as β-catenin, PP2A, and JAK2 are supported by multiple independent studies, translation of these data into hypothesis-driven clinical trials has been excruciatingly slow. As a result, conclusive data regarding clinical efficacy are mostly missing or inconclusive, and thus far no new clinical approaches have emerged that could be considered standard of care.

TRANSFORMATION TO ACCELERATED AND BLAST PHASE

As the CP-CML is compatible with life, one could argue that the main objective in CML therapy is the prevention of transformation to BP-CML, an acute leukemia with myeloid (M-BP) or pre-B-cell phenotype (L-BP). A host of genetic alterations has been associated with transformation to accelerated phase (AP)/BP (Fig. 69.7). CCA/Ph$^+$ is present in 70% to 80% of cases and includes a number of nonrandom abnormalities. Classically, +8, +Ph, i(17q), and +19 were considered as "major route" abnormalities, although this is not a universally accepted definition.[172] Several other recurrent abnormalities are also associated with a poor prognosis, including −7 and 3q26 abnormalities.[157] Many other cytogenetic changes have been described in smaller subsets of patients, and their prognostic impact is less well established. However, the acquisition of any CCA/Ph$^+$ in a patient on TKI therapy is consistent with a diagnosis of AP-CML irrespective of other disease features.[40] NGS has revealed point mutations, indels, and fusion genes in addition to BCR-ABL1 (see Fig. 69.7). For example, inactivating mutations of *RUNX1* occur in ~25% of M-BP cases, respectively, while ~55% of L-BP cases have inactivating mutations in *IKZF1*.[159,173,174]

BCR-ABL1 expression tends to increase with progression to BP. In some patients, rising *BCR-ABL1* mRNA levels are associated with duplication of Ph, but in the majority this seems to result from transcriptional upregulation due to as yet unknown mechanisms.[175] The increase in BCR-ABL1 expression promotes expression of SET, which inhibits the tumor suppressor phosphatase PP2A by forming a complex with the PP2A catalytic subunit (PP2Ac). PP2A activates SHP-1 to de-phosphorylate BCR-ABL1 and other important substrates.[176] Reduced PP2A activity increases BCR-ABL1 activity, further enhancing the signal in a positive feedback loop. Reduced SHP-1 activity has been associated with resistance to TKIs, emphasizing the importance of this pathway.[177] In addition to SET, cancerous inhibitor of PP2A (CIP2A) was implicated in PP2A inhibition in CML and high CIP2A activity predicts a high risk of transformation to BP.[178]

The most salient feature of transformation from the chronic to the blast phase is the loss of terminal differentiation capacity. Rare patients acquire AML-type translocations such as *CBFβ-MYH11* at the time of transformation.[179] Mutant RUNX1 functions in a dominant-negative fashion to block neutrophil differentiation.[180] In the majority of M-BC patients, myeloid differentiation is impaired due to suppression of CAAT enhancer binding protein α (C/EBPα), which is the result of enhanced expression of the RNA-binding protein heterogeneous nuclear ribonucleoprotein E2 (hnRNP-E2), which inhibits C/EBPα translation. Two mechanisms underlie the increased expression and activity of hnRNP-E2. First the increase in BCR-ABL1 activity promotes expression of hnRNP-E2.[181] Second, miR-328, which binds to hnRNP-E2 to block its activity, is downregulated.[182] Disruption of other myeloid transcription factors such as C/EBPβ and over-expression of MECOM (EVI-1) have also been implicated in blastic transformation.[183–185] With the loss of differentiation capacity, the organization of CML hematopoiesis is disrupted, as granulocyte-macrophage progenitor cells acquire self-renewal capacity, possibly by activation of nuclear β-catenin, undermining the hierarchical structure of chronic phase hematopoiesis.[186] Various mechanisms have been implicated in β-catenin activation, including stabilization by BCR-ABL1 tyrosine phosphorylation, inactivation of glycogen synthase kinase 3β by mis-splicing, and activation of MNK family kinases.[187–189] Transformation to L-BC, which has almost invariably a pre-B-cell phenotype, is caused by inactivation of transcription factors that are critical for orderly B-cell development, such as IKZF1 or PAX-5. It is thought that physiologically these transcription factors impose a metabolic restraint on developing B cells that limits the risk of malignant transformation during antigen receptor diversification.[166] Interestingly the types of structural rearrangements in L-BC suggest that they originate from aberrant RAG-mediated recombination.[145]

The variability of somatic mutations associated with blastic transformation is consistent with conversion of multiple aberrancies into a relatively uniform phenotype. Recent work using multiple genome-wide "omic" platforms has implicated epigenetic dysregulation as the overarching mechanism. Specifically downregulation of polycomb repressive complex 2 (PRC2) and upregulation of PRC1 activity may explain phenotypic conversion despite heterogeneous upstream mutations.[174] In this framework, histone and DNA methylation work hand in hand to achieve profound epigenetic reprogramming, consistent with the frequent mutation of epigenetic regulators observed in multiple studies.[159,160] PRC2 directs BP DNA hypermethylation, which in turn silences key genes involved in myeloid differentiation and tumor suppressor function through epigenetic switching, while PRC1 represses an overlapping and distinct set of genes, including BP tumor suppressors. This may provide a rationale for therapeutic opportunities that are broadly applicable, for instance the combination of EZH2 (PRC2) and BMI1 (PRC1) inhibitors.

MOLECULAR BASIS OF TYROSINE KINASE INHIBITOR RESISTANCE

TKI resistance is classified as primary resistance (failure to achieve a desired response) and acquired resistance (loss of response). Mechanistically TKI resistance broadly bifurcates into BCR-ABL1–dependent resistance (reactivation of BCR-ABL1 kinase activity) and BCR-ABL1–independent resistance (resistance despite continued suppression of BCR-ABL1 kinase activity). Interestingly, for unknown reasons, a given CML case tends to re-use the same strategy to escape the next line of therapy, i.e. to acquire yet another BCR-ABL1 mutation if it has used this strategy before.[190] From a clinical standpoint the distinction between BCR-ABL1–dependent and –independent resistance is critical for rationalizing salvage strategy. (See box on Resistance, Relapse, Recurrence, Intolerance.)

RESISTANCE, RELAPSE, RECURRENCE, INTOLERANCE

Resistance implies the failure to achieve a desired level of response (primary resistance) or the loss of response while on therapy (secondary or acquired resistance). Relapse is used synonymously with acquired resistance, while the term recurrence is reserved (and should be used) to denote increasing leukemia burden in the context of treatment-free remission (i.e., off TKI therapy). Keeping the semantics clear is important, given that the biology and prognostic impact of relapse and recurrent are vastly different. In practice it can be challenging to distinguish between true (biological) resistance and resistance due to insufficient dose intensity because of intolerance.

BCR-ABL1–Dependent Tyrosine Kinase Inhibitor Resistance

The most common mechanism of BCR-ABL1 reactivation is mutations in the kinase domain of BCR-ABL1 that impair drug binding through steric hindrance or by stabilizing a kinase conformation from which a given inhibitor is excluded.[32,191,192] The spectrum of mutations conferring clinically relevant resistance is broadest for imatinib, reflecting its relatively low potency. Hot spots include the ATP binding loop (p-loop), activation loop, and threonine 315, which controls access to a hydrophobic pocket in the catalytic site and is frequently referred to as the gatekeeper residue (Fig. 69.8).[193] Compared to

TABLE 69.2 **Pathways Implicated in the Survival of CML Stem Cells**

Pathway	Publications	Clinical Trial	Status	Final Report
WNT/β-Catenin	Zhao et al., Cancer Cell 2007[1]; McWeeney et al., Blood 2010[2]; Heidel et al., Cell Stem Cell 2012[3]; Schürch et al., JCI 2012[4]; Lim et al., PNAS 2013[5]; Zhang et al., Blood 2013[6]; Eiring et al., Leukemia 2015[7]; Agarwal et al., Blood 2017[8]	No	NA	NA
HDAC	Zhang et al., Cancer Cell 2010[9,10]	Panobinostat	Lack of efficacy	No
Hedgehog	Dierks et al., Cancer Cell 2008; Zhao et al., Nature 2009[11]	LDE225+NIL	Terminated	No
		BMS-833923+DAS	Terminated	No
5-Lipoxygenase	Chen et al., Nat Genet. 2009[12]	Zileuton+IM	Terminated	No
BCL6	Hurtz et al., J Exp Med. 2011[13]	No	NA	NA
MYC	Ravie et al., Cancer Cell 2013[14] Abraham et al., Nature 2016[15]	No	NA	NA
PP2A	Neviani et al., J Clin Invest 2013[16]; Lai et al., Sci Trans Med[17]	No	NA	NA
SIRT1	Bhatia et al., Cancer Cell 2012[18]	No	NA	NA
PRMT5	Jin et al., JCI 2016[19]	No	NA	NA
Rad52	Cramer-Morales et al., Blood 2013[20]	No	NA	NA
PIM2	Ma et al., PNAS 2019[21]	No	NA	NA
BCL2	Goff et al., Cancer Stem Cell 2013[22]	Venetoclax+DAS	Recruiting	NA
PPRγ	Prost et al., Nature 2015[23]	Pioglitazone	Potentially active	Rousselot et al., Cancer 2017[24]
Autophagy	Bellodi et al., JCI 2009[25]; Baqero et al., Blood 2019[26]	Chloroquine	Modest activity	Horne et al., Leukemia 2020[27]
PML	Ito et al., Nature 2008[28]	Arsenic trioxide	Terminated or not reported	NA
JAK2/STAT3/ STAT5	Ye et al., Blood 2006[29]; Traer et al., Leukemia 2012[30]; Neviani et al., JCI 2013[16]; Gallipoli et al., Blood 2014[31]; Eiring et al., Leukemia 2015[32]	Ruxolitinib+TKI	Potentially active	Sweet et al., Leuk Res 2018[33]
Mitochondrial protein translation	Kuntz et al., Nature Medi 2017[34]	No	No	NA
TGFβ	Naka et al., Nature 2010[35]	No	No	NA
Musashi2	Ito et al., Nature 2010[36]	No	No	NA
ADAR1	Jiang et al., PNAS 2013[37]	No	No	NA
EZH2	Scott et al., Cancer Discov 2016[38]; Xie et al., Cancer Discov 2016[39]			
PIM2	Ma et al., PNAS 2019[21]			

DAS, Dasatinib; *IM*, imatinib; *NA*, not applicable; *NIL*, nilotinib.

[1] Zhao C, Blum J, Chen A, et al. Loss of beta-catenin impairs the renewal of normal and CML stem cells in vivo. *Cancer Cell.* 2007;12:528–541.

[2] McWeeney SK, Pemberton LC, Loriaux MM, et al. A gene expression signature of CD34+ cells to predict major cytogenetic response in chronic-phase chronic myeloid leukemia patients treated with imatinib. *Blood.* 2010;115:315–325. doi:10.1182/blood-2009-03-210732.

[3] Heidel FH, Bullinger L, Feng Z, et al. Genetic and pharmacologic inhibition of beta-catenin targets imatinib-resistant leukemia stem cells in CML. *Cell Stem Cell.* 2012;10:412–424. doi:10.1016/j.stem.2012.02.017.

[4] Schurch C, Riether C, Matter MS, Tzankov A, Ochsenbein AF. CD27 signaling on chronic myelogenous leukemia stem cells activates Wnt target genes and promotes disease progression. *J Clin Invest.* 2012;122:624-638. doi:10.1172/JCI45977.

[5] Lim S. Saw TY, Chang, M, et al. Targeting of the MNK-eIF4E axis in blast crisis chronic myeloid leukemia inhibits leukemia stem cell function. *Proc Natl Acad Sci U S A.* 2013;110:E2298–E2307, doi:10.1073/pnas.1301838110.

[6] Zhang B, Li M, McDonald T, et al. Microenvironmental protection of CML stem and progenitor cells from tyrosine kinase inhibitors through N-cadherin and Wnt-beta-catenin signaling. *Blood.* 2013;121: 1824–1838. doi:10.1182/blood-2012-02-412890.

[7] Eiring AM, Khorashad JS, Anderson DJ, et al. β-Catenin is required for intrinsic but not extrinsic BCR-ABL1 kinase-independent resistance to tyrosine kinase inhibitors in chronic myeloid leukemia. *Leukemia.* 2015;29:2328–2337. doi:10.1038/leu.2015.196.

[8] Agarwal P, Zhang B, Ho Y, et al. Enhanced targeting of CML stem and progenitor cells by inhibition of porcupine acyltransferase in combination with TKI. *Blood.* 2017;129:1008–1020. doi:10.1182/blood-2016-05-714089.

[9] Zhang J, Adrián FJ, Jahnke W, et al. Targeting Bcr-Abl by combining allosteric with ATP-binding-site inhibitors. *Nature.* 2010;463:501–506. doi:10.1038/nature08675.

[10] Dierks C, Beigi R, Guo G-R, et al. Expansion of Bcr-Abl-positive leukemic stem cells is dependent on Hedgehog pathway activation. *Cancer Cell.* 2008;14:238–249.

[11] Zhao C, Chen A, Jamieson CH, et al. Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. *Nature.* 2009;458:776–779

[12] Chen Y, Hu Y, Zhang H, Peng C, Li S. Loss of the Alox5 gene impairs leukemia stem cells and prevents chronic myeloid leukemia. *Nat Genet.* 2009;41:783–792. doi:10.1038/ng.389.

[13] Hurtz C, Hatzi K, Cerchietti L, et al. BCL6-mediated repression of p53 is critical for leukemia stem cell survival in chronic myeloid leukemia. *J Exp Med.* 2011;208:2163–2174. doi:10.1084/jem.20110304.

[14] Reavie L, Buckley SM, Loizou, E, et al. Regulation of c-Myc ubiquitination controls chronic myelogenous leukemia initiation and progression. *Cancer Cell.* 2013;23:362–375. doi:10.1016/j.ccr.2013.01.025.

Continued

TABLE 69.2 **Pathways Implicated in the Survival of CML Stem Cells—cont'd**

[15] Abraham SA, Hopcroft LEM, Carrick E, et al. Dual targeting of p53 and c-MYC selectively eliminates leukaemic stem cells. *Nature*. 2016;534:341–346. doi:10.1038/nature18288.

[16] Neviani P, HarbJG, Oaks JJ, et al. PP2A-activating drugs selectively eradicate TKI-resistant chronic myeloid leukemic stem cells. *J Clin Invest*. 2013;123:4144–4157. doi:10.1172/JCI68951.

[17] Lai D, Chen M, Su J, et al. PP2A inhibition sensitizes cancer stem cells to ABL tyrosine kinase inhibitors in BCR-ABL(+) human leukemia. *Sci Transl Med*. 2018;10:eaan8735. doi:10.1126/scitranslmed.aan8735.

[18] Li L, Wang L, Li L, et al. Activation of p53 by SIRT1 inhibition enhances elimination of CML leukemia stem cells in combination with imatinib. *Cancer Cell*. 2012;21:266–281. doi:10.1016/j.ccr.2011.12.020.

[19] Jin Y, Zhou J, Xu F, et al. Targeting methyltransferase PRMT5 eliminates leukemia stem cells in chronic myelogenous leukemia. *J Clin Invest* 2016;126:3961–3980. doi:10.1172/jci85239.

[20] Cramer-Morales K, Nieborowska-Skorska M, Scheibner K, et al. Personalized synthetic lethality induced by targeting RAD52 in leukemias identified by gene mutation and expression profile. *Blood*. 2013;122:1293–1304. doi:10.1182/blood-2013-05-501072.

[21] Ma L, Pak ML,Ou J, et al. Prosurvival kinase PIM2 is a therapeutic target for eradication of chronic myeloid leukemia stem cells. *Proc Natl Acad Sci U S A*. 2019;116:10482–10487. doi:10.1073/pnas.1903550116.

[22] Goff DJ, Recart AC, Sadarangani A, et al. A Pan-BCL2 inhibitor renders bone-marrow-resident human leukemia stem cells sensitive to tyrosine kinase inhibition. *Cell Stem Cell*. 2013;12:316–328. doi:10.1016/j.stem.2012.12.011.

[23] Prost S, Relouzat F, Spentchian M, et al. Erosion of the chronic myeloid leukaemia stem cell pool by PPARγ agonists. *Nature*. 2015;525:380–383. doi:10.1038/nature15248.

[24] Rousselot P, Prost S, Guilhot J, et al. Pioglitazone together with imatinib in chronic myeloid leukemia: a proof of concept study. *Cancer*. 2017;123:1791–1799. doi:10.1002/cncr.30490.

[25] Bellodi C, Lidonnici MR, Hamilton A, et al. Targeting autophagy potentiates tyrosine kinase inhibitor-induced cell death in Philadelphia chromosome-positive cells, including primary CML stem cells. *J Clin Invest*. 2009;119:1109–1123. doi:10.1172/jci35660.

[26] Baquero P, Dawson A, Mukhopadhyay A, et al. Targeting quiescent leukemic stem cells using second generation autophagy inhibitors. *Leukemia*. 2019;33:981–994. doi:10.1038/s41375-018-0252-4.

[27] Horne GA, Stobo J, Kelly C, et al. A randomised phase II trial of hydroxychloroquine and imatinib versus imatinib alone for patients with chronic myeloid leukaemia in major cytogenetic response with residual disease. *Leukemia*. 2020; 34:1775-1786. doi:10.1038/s41375-019-0700-9.

[28] Ito K, Bernardi R, Morotti A, et al. PML targeting eradicates quiescent leukaemia-initiating cells. *Nature*. 2008;453:1072–1078.

[29] Ye D, Wolff N, Li L, Zhang S, Ilaria RL Jr. STAT5 signaling is required for the efficient induction and maintenance of CML in mice. *Blood*. 2006;107:4917–4925.

[30] Traer E, MacKenzie R, Snead, J, et al. Blockade of JAK2-mediated extrinsic survival signals restores sensitivity of CML cells to ABL inhibitors. *Leukemia*. 2012;26:1140–1143. doi:10.1038/leu.2011.325.

[31] Gallipoli P, Cook A, Rhodes S, et al. JAK2/STAT5 inhibition by nilotinib with ruxolitinib contributes to the elimination of CML CD34+ cells in vitro and in vivo. *Blood*. 2014;124:1492–1501. doi:10.1182/blood-2013-12-545640.

[32] Eiring AM, Kraft IL, Page BD, et al. STAT3 as a mediator of BCR-ABL1-independent resistance in chronic myeloid leukemia. *Leuk Suppl*. 2014;3:S5–S6. doi:10.1038/leusup.2014.3.

[33] Sweet K, Hazlehurst L, Sahakian E, et al. A phase I clinical trial of ruxolitinib in combination with nilotinib in chronic myeloid leukemia patients with molecular evidence of disease. *Leuk Res*. 2018;74:89–96. doi:10.1016/j.leukres.2018.10.002.

[34] Kuntz EM, Baquero P, Michie AM, et al. Targeting mitochondrial oxidative phosphorylation eradicates therapy-resistant chronic myeloid leukemia stem cells. *Nat Med*. 2017;23:1234–1240. doi:10.1038/nm.4399.

[35] Naka K, Hoshii T, Muraguchi T, et al. TGF-beta-FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. *Nature*. 2010;463:676–680. doi:10.1038/nature08734.

[36] Ito T, Kwon HY, Zimdahl B, et al. Regulation of myeloid leukaemia by the cell-fate determinant Musashi. *Nature*. 2010;466:765–768. doi:10.1038/nature09171.

[37] Jiang Q, Crews LA, Barrett CL, et al. ADAR1 promotes malignant progenitor reprogramming in chronic myeloid leukemia. *Proc Natl Acad Sci U S A*. 2013;110:1041–1046. doi:10.1073/pnas.1213021110.

[38] Scott MT, Korfi K, Saffrey P, et al. Epigenetic reprogramming sensitizes CML stem cells to combined EZH2 and tyrosine kinase inhibition. *Cancer Discov*. 2016;6:1248–1257. doi:10.1158/2159-8290.cd-16-0263.

[39] Xie H, Peng C, Huang J, et al. Chronic myelogenous leukemia- initiating cells require polycomb group protein EZH2. *Cancer Discov*. 2016;6:1237–1247. doi:10.1158/2159-8290.cd-15-1439.

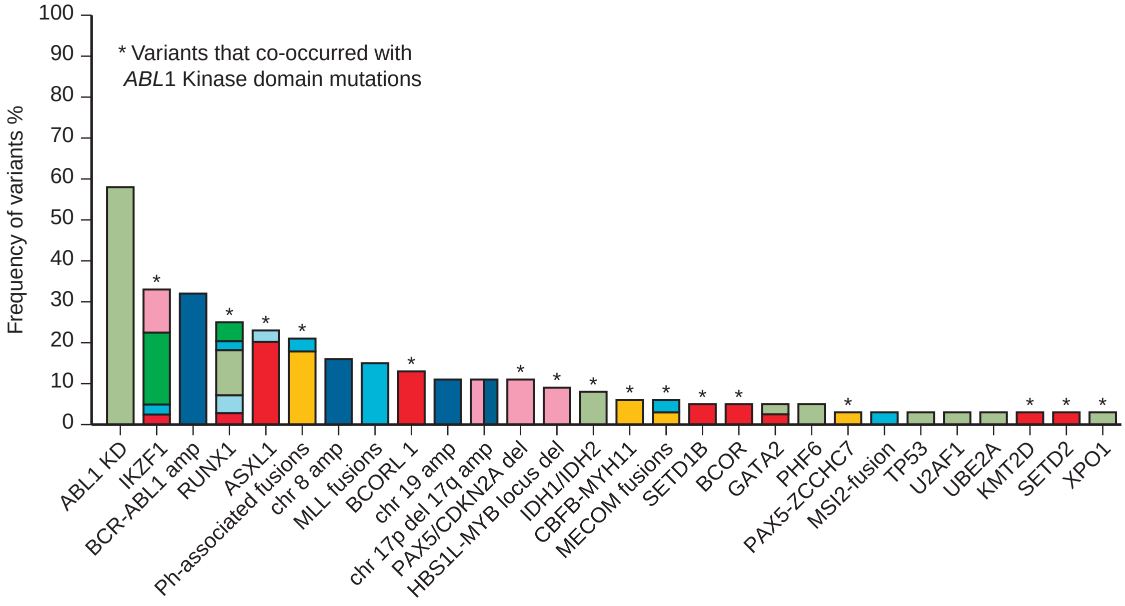


Figure 69.7 SOMATIC MUTATIONS ASSOCIATED WITH BLAST PHASE CML. *(Reproduced with permission from Branford S, Wang P, Yeung D, et al. Integrative genomic analysis reveals cancer-associated mutations at diagnosis of CML in patients with high-risk disease. Blood. 2018;132[9]:948–961.)*

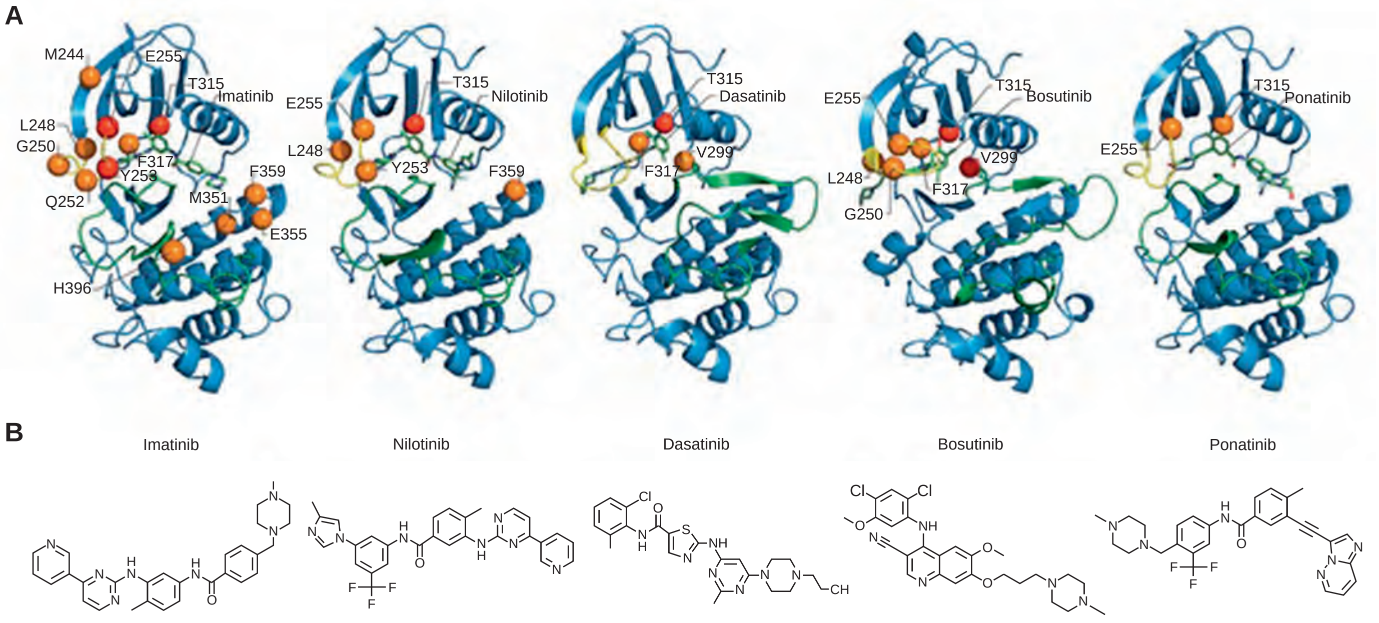


Figure 69.8 TYROSINE KINASE INHIBITORS (TKIS) APPROVED FOR THE TREATMENT OF CHRONIC MYELOID LEUKEMIA AND CLINICAL RESISTANCE MUTATIONS. (A) Crystal structure of the ABL1 kinase domain in complex with the indicated TKI. Highlighted residues indicate mutations that confer resistance to the indicated TKI in vitro and in vivo. *Orange* (moderate) and *red* (severe) spheres indicate the level of TKI resistance. (B) Chemical structures of the TKIs. *(Adapted with permission from Eiring AM, Deininger MW. Individualizing kinase-targeted cancer therapy: the paradigm of chronic myeloid leukemia. Genome Biol. 2014;15[9]:461.)*

imatinib, second-generation TKIs exhibit a much more contracted spectrum of resistance mutations, and in aggregate cover all clinical mutations except T315I. This is due to the fact that all first- and second-generation TKIs make a hydrogen bond with T315 and require access to the hydrophobic pocket. In contrast, ponatinib skirts T315I through a rigid triple carbon bond.[194] Kinase domain mutations are more common in acquired resistance than in primary resistance and in AP/BP-CML than in CP-CML.[195] BCR-ABL1 genotyping is part of a workup for clinical resistance, as detection of a mutation allows rationalizing TKI selection. One should bear in mind that the value of genotyping is mostly to avoid selecting an inactive TKI, while the presence of a "sensitive" mutation does not guarantee a clinical response, indicating that other mechanisms, including BCR-ABL1 independent pathways, contribute to clinical resistance.[192] Studies in cell lines have implicated overexpression of BCR-ABL1 by gene amplification or transcriptional upregulation, but the importance of these findings for clinical resistance is less clear.[196] All TKIs are substrates for various efflux pumps, including ABCB1 (MDR1), and several polymorphisms have been correlated with the depth of response.[197] One study showed that overexpression of MDR1 may be sufficient to protect CML stem and progenitor cells, until they acquire a BCR-ABL1 kinase domain mutation that is able to drive overt resistance.[198]

BCR-ABL1–Independent Resistance

Extrinsic or intrinsic activation of growth and survival mechanisms can substitute for BCR-ABL1 kinase activity. Multiple different pathways have been implicated, including several that are also downstream effectors of BCR-ABL1, such as PI3K and MAPK.[192] This is consistent with the observation that TKI resistant CML is phenotypically still CML. The mechanistic heterogeneity of BCR-ABL1–independent resistance implies that no obvious universal therapeutic vulnerabilities exist, presenting a challenge for the development of rational therapeutic strategies. There is extensive overlap in the pathways involved in transformation to AP/BP and BCR-ABL1–independent resistance, both of which employ sweeping epigenetic reprogramming, e.g., through imbalanced activity of PRC1 and PRC2.[174] Such common denominator may in the future inform rational strategies to overcome BCR-ABL1–independent resistance. In reality, many cases of advanced TKI-resistant CML combine BCR-ABL1–dependent and –independent mechanisms, which explains why deep responses in these patients are difficult to achieve.

DIAGNOSIS AND INITIAL WORKUP

Clinical Evaluation

Signs and Symptoms

A complete history and physical exam are mandatory. CML may present with a plethora of constitutional symptoms such as fatigue, weight loss, fever, night sweats, and musculoskeletal aches. Severe constitutional symptoms raise concerns about advanced disease. Splenomegaly may cause abdominal pain and fullness; excruciating, sharp pain should raise suspicion of a splenic infarct or rupture, rare but potentially life-threatening CML complications.[199] Excessively high white blood cell counts can lead to leukostasis, especially in case of a high proportion of blasts in the differential. Clinically leukostasis may manifest as priapism, chest pain, respiratory compromise, neurological deficits, or visual impairment. Physical exam may reveal splenomegaly and sometimes hepatomegaly, while involvement of other extramedullary sites is uncommon. Spleen size (in cm below the left costal margin in the mid-clavicular line) must be documented. Despite its inaccuracy, this simple clinical test has remained a component of most CML risk scores.[200–202] Extramedullary disease other than hepatosplenomegaly is uncommon at presentation, and if suspected must be verified histologically. Some reactive lymphadenopathy may occur, but significant lymph node enlargement requires a biopsy to rule out blastic transformation. An immature cellular infiltrate (chloroma) indicates blast phase, but if there is differentiation it can be difficult to distinguish between true extramedullary disease from an inflammatory process that recruits CML cells. There are no signs or symptoms that are pathognomonic for CML. In developed countries, many patients are diagnosed incidentally, when an abnormal complete count blood obtained for an unrelated reason leads to a diagnostic workup.

Laboratory Manifestations

Complete Blood Count (CBC)

Standard hematologic parameters typically show leukocytosis, frequently thrombocytosis, and commonly mild anemia. The diagnosis of CML is usually suspected based on the morphology of a Wright- or Giemsa-stained blood smear. Granulopoiesis is left-shifted and may encompass the full spectrum of granulocytic precursors including blasts and promyelocytes. Basophilia is typical and a useful hint to distinguish CML from other MPNs. Significant eosinophilia is rare and raises the question of an alternative diagnosis. A manual differential is good clinical practice, as some automated cell counters do not provide the percentage of promyelocytes and/or basophils, which are required to determine disease phase. As all risk scores to stratify CML-CP are based on pre-therapeutic data, it is important to document the counts before any therapy is initiated. Flow cytometry is indicated only if AP/BP is suspected. Difficulties can arise in cases of discrepancies between morphological and immunophenotypic blast counts based on CD34 expression. In ambiguous cases, morphological blast counts should take precedence over flow cytometry.

Bone Marrow Aspirate and Biopsy

The bone marrow is hypercellular with myeloid predominance and left shift and frequently basophilia. Abnormally small, monolobated megakaryocytes (micromegakaryocytes) are typical and there may be pseudo-Gaucher cells (Chapter 54) (large macrophages with foamy cytoplasm and a lobated eccentric nucleus) and harlequin cells (dysplastic eosinophils with metachromatic granules). It is essential to obtain bone marrow morphology at diagnosis, as failure to do so may miss AP/BP-CML and lead to under-treatment. Whether all patients should have a biopsy is debatable. We routinely perform a biopsy at diagnosis to identify the occasional patient with nests of blasts missed by cytology. This is particularly important in patients with an aggressive presentation and those with inadequate aspirates or dry taps. As with peripheral blood, flow cytometry is not routinely indicated.

Bone Marrow Karyotyping

Metaphase karyotyping of bone marrow cells using G- or R-banding is mandatory to confirm the diagnosis of CML (Chapter 57). As a rule, a minimum of 20 metaphase spreads is analyzed. More than 95% of CML patients are Ph$^+$, including ~5% of patients in whom Ph is part of complex translocations involving additional chromosomes. These complex translocations do not affect the outcome of patients on TKI therapy, and they must not be confused with clonal cytogenetic abnormalities in Ph$^+$ cells (CCA/Ph$^+$, also referred to as clonal cytogenetic evolution).[157] Rare (<5%) of patients have a silent Ph that is not identified by karyotyping, and molecular testing is required to establish the diagnosis of CML. A critical piece of information from karyotyping is CCA/Ph$^+$, since these patients have inferior progression-free and overall survival on TKI therapy, even in the absence of other features of AP/BP.[157,203,204]

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) uses fluorescent DNA probes that hybridize to *BCR* and *ABL*1 genetic regions adjacent to the translocation breakpoints—typically red fluorescence to label *ABL1* and green fluorescence to label *BCR*. Most FISH assays in current use identify both *BCR-ABL1* (on Ph) and *ABL1-BCR* fusion signals (on the derivative chromosome 9) and have low false-positive and -negative rates (Chapter 57).[205] In contrast to karyotyping, FISH is usually performed on interphase nuclei, which increases sensitivity as 200 to 500 nuclei can be analyzed routinely. FISH is an excellent test to establish the diagnosis of CML in cases of silent Ph, but it should not routinely replace karyotyping, as CCA/Ph$^+$ will be missed. Modern FISH probes also identify deletions in *ABL1* adjacent to the translocation breakpoint, which confer an adverse prognosis to patients treated with IFN and second-line imatinib, but not to patients on frontline TKI therapy.[56,57,206] Altogether, FISH at diagnosis does not add actionable information in addition to standard karyotyping and its routine use in addition to conventional karyotyping is not justified. However, as a negative result by FISH has a very high positive predictive value for a CCyR by karyotyping, FISH is an extremely useful test to monitor patients with atypical BCR-ABL1 transcripts that are not detected by standard qPCR assays.[207]

Reverse Transcription Polymerase Chain Reaction

Qualitative RT-PCR is used to identify a *BCR-ABL1* fusion mRNA in patients with suspected CML who are Ph$^-$ by metaphase karyotyping, essentially providing the same information as FISH. It is important to make sure that the assay detects all types of BCR-ABL1 transcripts, including rare atypical transcripts.[208] As most *quantitative* PCR assays are designed to detect the typical e13a2 (b2a2) and e14a2 (b3a2) transcripts, but will miss e1a2, e19a2, and atypical transcripts, qPCR is not suitable for ruling out CML. A more detailed discussion of qPCR for *BCR-ABL1* is provided in the section on monitoring response.

Myeloid Mutation Screening

NGS assays to screen for mutations in genes involved in myeloid malignancies have become part of the routine diagnostic work-up of patients with MPNs. Approximately one-third of patients with CP-CML have mutations in genes such as *TET2* and *ASXL1* at diagnosis, but the prognostic impact of these mutations has not been established.[159] NGS is currently not mandatory, as results do not typically inform clinical decisions, but this may change as more data become available. A reasonable approach in view of this uncertainty may be to consider NGS in high-risk CP-CML and AP/BP-CML patients.

Imaging

An abdominal ultrasound or computerized tomography (CT) scan is frequently performed at diagnosis and may reveal splenomegaly and/or hepatomegaly. Imaging studies are indicated only in patients where palpation of spleen and liver is not possible due anatomical barriers or in whom extramedullary involvement other than spleen and liver is suspected. Importantly, there is no easy conversion of spleen size measured by ultrasound or CT scan to the clinical measurement in centimeters under the rib cage, which is required for calculating risk scores. Altogether imaging as part of the routine CML workup is discouraged.

RISK STRATIFICATION

Disease Phases

CML progresses in stages termed chronic phase (CP-CML), accelerated phase (AP-CML), and blast phase (BP-CML). Various definitions of AP-CML and BP-CML are in use that are based on clinical, morphological, and cytogenetic features. Unfortunately, criteria are still mostly based on outcomes obtained in the pre-TKI era, and some lack any association with sufficiently powered studies. A major inconsistency is the proportion of blasts required to establish a diagnosis of blast phase. While ≥20% blasts are used by the WHO classification of hematologic neoplasm, the European Leukemia Net (ELN), National Comprehensive Cancer Network (NCCN), and Center for

International Blood and Marrow Transplant Research (CIBMTR) use a cutoff of ≥30%. A pragmatic approach is to use the definitions employed by major prospective TKI trials, which form the basis for current treatment algorithms (Table 69.3).[209] There is controversy regarding clonal cytogenetic evolution, i.e. the presence of additional cytogenetic abnormalities in the Ph$^+$ cell clone (CCA/Ph$^+$). There is agreement that acquisition of CCA/Ph$^+$ on treatment is diagnostic of AP-CML,[210] but this is not universally accepted for newly diagnosed patients, although recent studies showed that these patients have inferior outcomes even in the absence of any other features of AP-CML, raising the question of whether the criteria should be changed.[157,204] Failure of one or more TKIs is an ominous sign indicative of advanced CML, even in patients with CP-CML, but has not yet been integrated into the phase definitions.[40] Lastly molecular data support the existence of two, but not three distinct disease phases.[211,212] The hope is that more consistent criteria will be developed in the future that include relevant clinical and molecular findings, such as somatic mutations detected by NGS.[213]

TABLE 69.3 Definition of Accelerated and Blast Phase

Accelerated Phase[a]
15%–29% blasts in blood or marrow
Greater than 30% blasts plus promyelocytes in blood or marrow (provided <30% blasts are present)
≥20% peripheral basophils in the blood or marrow
Platelet counts <100 × 10^9/L, unrelated to therapy
Additional cytogenetic abnormalities in Ph$^+$ cells while on TKI therapy
Blast Phase[a]
≥30% blasts in blood or marrow
Extramedullary blast infiltrates

[a]At least one criterion must be satisfied.
TKI, Tyrosine kinase inhibitor.

The CP-CML blood smear is characterized by left-shifted granulocytosis with maintenance of terminal differentiation, frequently accompanied by basophilia and sometimes eosinophilia. Significant dysplasia raises the question of alternative diagnoses, such as atypical CML. Bone marrow aspirate and biopsy are hypercellular for age, but maturation is complete (Fig. 69.9A, B). Micromegakaryocytes are typical and an important diagnostic hint. Grade 1 to 2 reticulin fibrosis may be present, but severe fibrosis is uncommon and may be associated with poorer outcomes.[214] In the absence of effective therapy, CP-CML inexorably progresses to BP-CML within 2 to 3 years. BP-CML can exhibit a pre B-lymphoid (25%), myeloid (70%), or indeterminate (5%) phenotype[215] and develop suddenly or over a period of time through the intermediary stage of AP-CML (see Fig. 69.9C, D). In addition to a blast count of ≥30% in the blood or bone marrow, extramedullary myeloblasts (chloroma) in tissues other than liver or spleen define BP-CML, and any proportion of pre-B lymphoblasts strongly suggests lymphoid blast phase.

Risk Scores in Chronic Phase of Chronic Myeloid Leukemia

There are several prognostication systems designed to subclassify CP-CML (Table 69.4). The oldest system, reported by Joseph Sokal more than 35 years ago, uses a sophisticated formula based on age, blast count in the blood, platelet count, and spleen size (in cm below

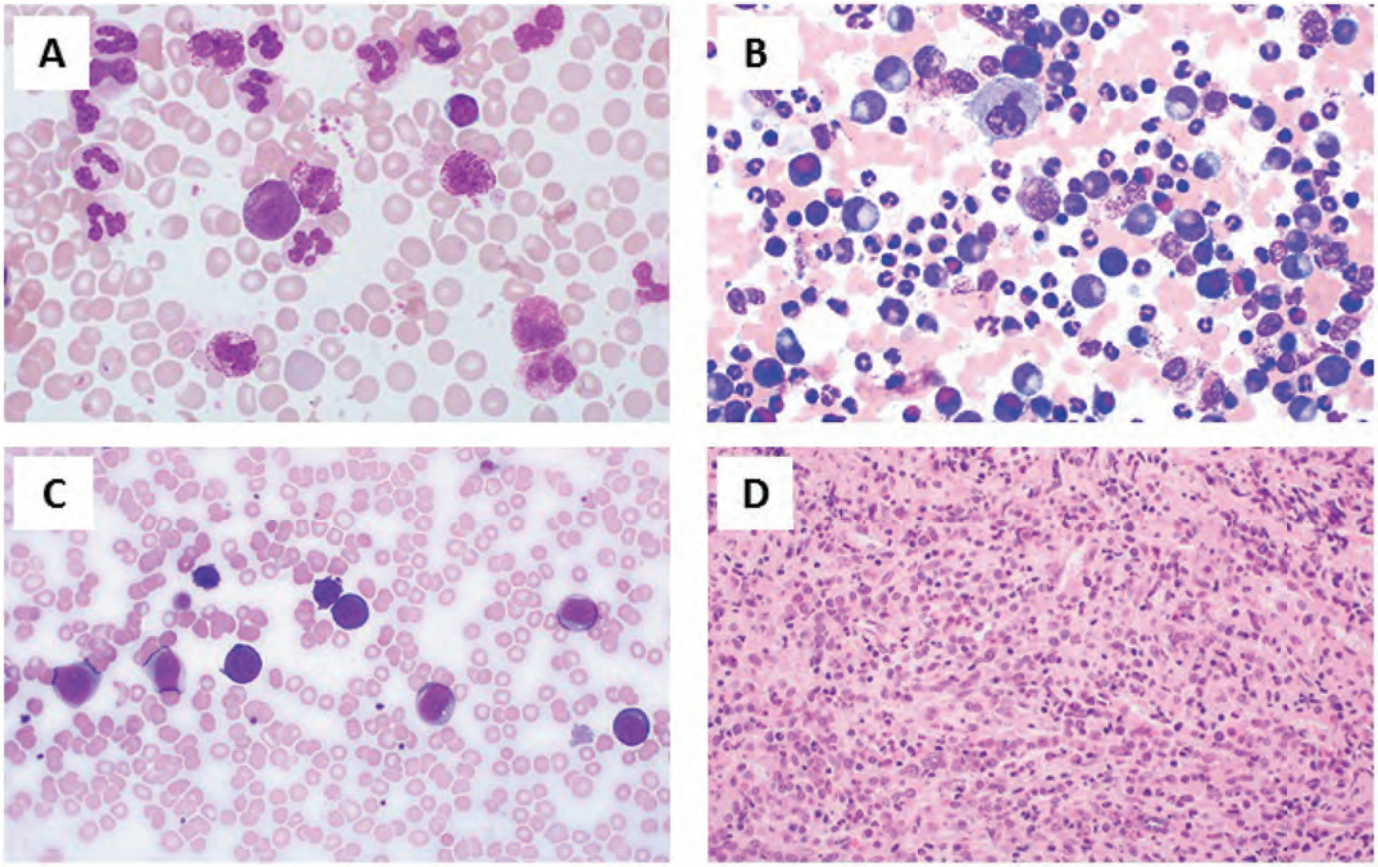


Figure 69.9 CHRONIC MYELOID LEUKEMIA MORPHOLOGY. (A) Blood smear, chronic phase. (B) Bone marrow aspirate smear, chronic phase. (C) Blood smear, blast phase. (D) Bone marrow biopsy, blast phase.

TABLE 69.4 Prognostic Scores in Newly Diagnosed Chronic Phase of Chronic Myeloid Leukemia

Score	Formula	
Sokal Sokal et al. 1984	Exp 0.0116 × (age − 43.4) + 0.0345 × (spleen − 7.51) + 0.188 × [(platelet count ÷ 700)^2 − 0.563] + 0.0887 × (myeloblasts − 2.10)	Low risk: <0.8 Intermediate risk: 0.8–1.2 High risk: >1.2
ELTS Pfirrmann et al. 2016	0.0025 × (age in completed years/10)^3 + 0.0615 × spleen size below costal margin + 0.1052 × blasts in peripheral blood + 0.4104 × (platelet count/1000)$^{-0.5}$	Low risk: ≤1.5680 Intermediate risk: 1.5680–2.2185 High risk: >2.2185

the left costal margin). Scores of less than 0.8, 0.8 to 1.2, and greater than 1.2 define low, intermediate, and high risk.[200] Although the Sokal score was built on patients treated with busulfan-based chemotherapy, it proved resilient to changes in the treatment paradigm and has been applied successfully to risk-stratify patients treated with IFN or TKIs.[216,217] Subsequently, Hasford developed a score optimized for patients on IFN.[201] Recently, a third prognostication system (EUTOS score) optimized for patients on imatinib was reported.[202] The EUTOS score is simple, relying on basophils and spleen size only, but since an independent study failed to validate the score, its use is not recommended.[218] An important advance is the EUTOS long-term survival score (ELTS), which takes into account the fact that most CML patients with access to TKIs and appropriate monitoring die from non-CML causes.[219] Although reliant on the same parameters as the Sokal score, the ELTS was superior to all other scores in predicting CML-related death in CP-CML patients treated with imatinib, and its use is recommended in this population. Data on second-generation TKIs are not yet available. As with the definitions of disease phases, molecular markers may eventually replace clinical risk scores.[213]

DIFFERENTIAL DIAGNOSIS

Other MPNs, including atypical CML, CMML, chronic neutrophilic leukemia, MPN associated with rearrangements within the platelet *PDGFRs*, or fibroblast growth factor receptor 3 (*FGFR3*) can mimic CML clinically and morphologically. Conversely, CML patients may present with features indistinguishable from essential thrombocythemia (ET) or primary myelofibrosis. It is therefore mandatory to ascertain the presence or absence of *BCR-ABL1* in every patient with MPN to prevent misdiagnosis with potentially catastrophic consequences. Demonstration of Ph or *BCR-ABL1* establishes the diagnosis of CML in a patient with MPN, irrespective of other clinical, morphological, or genetic features. Tests like vitamin B12 levels (high in CML) or the leukocyte alkaline phosphatase (LAP) score (low in CML, high in leukemoid reactions) are of historical interest only. CML may present in BP and these cases can be extremely difficult to distinguish from de novo Ph$^+$ ALL or Ph$^+$ AML, a rare and sometimes disputed entity.[220] In the pre-TKI era, such patients were treated with multi-agent chemotherapy, and upon count recovery the underlying CML often became evident by typical morphology and the continued presence of Ph. With all BCR-ABL1-positive patients receiving TKIs up front, this accidental diagnostic test is no more valid. Preceding CP-CML may be suspected if there are residual left-shifted but maturing granulocytic precursors, basophilia, and splenomegaly. Given that the therapeutic approaches are almost identical, the distinction is mainly of academic interest. A *BCR* breakpoint in the m-*BCR* with e1a2 *BCR-ABL1* transcript establishes a diagnosis of Ph$^+$ ALL for all practical purposes.

MONITORING RESPONSE TO THERAPY

Response to therapy is assessed by clinical, hematological, cytogenetic, and molecular parameters that reflect the decrease of leukemia burden (Fig. 69.10A). Response to TKI therapy is highly predictive of subsequent outcome, and milestones of response have been identified by NCCN and ELN consensus panels (see Fig. 69.10B).

Hematologic Response (CHR)

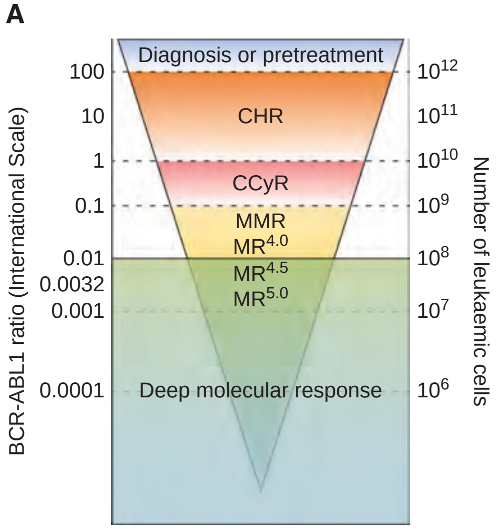
Complete hematologic response (CHR) is defined as the resolution of all CML-related signs and symptoms (including palpable splenomegaly) and normalization of white blood cell count, white blood cell differential, and platelet count. Normalization of hemoglobin is not part of the CHR definition. Of note, bulky splenomegaly is often slow to resolve and the significance of persistent minimal splenomegaly in patients who meet all other CHR criteria is unknown. The term "major hematologic response" (MHR) denotes a situation where blasts are absent from the blood and less than 5% in the bone marrow, but reconstitution of peripheral blood counts has not occurred. Although MHR was used as an endpoint in some clinical trials, its prognostic significance is unknown.

Cytogenetic Response

Bone marrow karyotyping was the first method used to assess restoration of normal (polyclonal) hematopoiesis. Since karyotyping is laborious and time consuming, a minimum of 20 metaphases was set as the standard to provide reasonable response definitions despite a limited sample. The 95% confidence intervals of correctly identifying 50% Ph$^+$ metaphases are 35% and 65%. Therefore a partial cytogenetic response (PCyR) was defined as 35% or less Ph$^+$ metaphases, reflecting 50% or less Ph$^+$ metaphases with 95% confidence. CCyR denotes the absence of Ph$^+$ metaphases, and CCyR and PCyR combined are referred to as major cytogenetic response (MCyR). Some studies also report minor (36% to 65% Ph$^+$) and minimal (66% to 95% Ph$^+$) cytogenetic responses, but these response levels have minimal clinical significance, since correlations with outcome are weak or absent. Given the strong correlation between CCyR and overall survival, MCyR and PCyR are of mostly historical interest and should be avoided as an efficacy endpoint in clinical trials.

Molecular Response

qRT-PCR for *BCR-ABL1* has emerged as the assay of choice for monitoring patients on TKI therapy or after allogeneic stem cell transplantation (SCT). In the past, the use of numerous technical platforms by commercial vendors and academic labs had created a confusing landscape, where results could not be compared between labs and with data from clinical studies. Following an initial consensus meeting, a multi-center collaboration led to the introduction of the international scale (IS) as a universal metric to express *BCR-ABL1* transcript levels.[221] In principle results from any qRT-PCR assay with linear performance characteristics over a sufficiently large range can be converted to IS units by using an assay-specific conversion factor. An exhaustive technical discussion of qRT-PCR monitoring is beyond the scope of this chapter and the reader is referred to comprehensive reviews of the subject,[221] but a few critical points deserve mentioning. First, compared to metaphase karyotyping and FISH, qPCR has a vastly greater dynamic range, which can span up to 5 logs. However, this sensitivity is reached only if the sample is of optimal quality. Thus, laboratories must report the sensitivity reached for a given sample to allow for correct interpretation of results. Second, not all housekeeping genes are equally suitable as control genes for normalization of RNA input; acceptable choices include *ABL1*, *BCR*, *GUS*, and *G6PD*.[221] β-actin is not acceptable due to high-level expression that may conceal degradation of the sample. Third, in clinical practice it is



B

BCR-ABL1 (IS)	3 months	6 months	12 months
>10%	YELLOW	RED	
>1%–10%	GREEN		YELLOW
>0.1%–1%	GREEN		LIGHT GREEN
≤0.1%	GREEN		

Figure 69.10 (A) Response to treatment and residual leukemia in chronic myeloid leukemia. *CHR*, Complete hematologic response; *CCyR*, complete cytogenetic response; *MMR*, major molecular response; *DMR*, deep molecular response. *(Adapted from O'Hare T, Zabriskie MS, Eiring AM, et al. Pushing the limits of targeted therapy in chronic myeloid leukaemia*. Nat Rev Cancer. *2012;12[8]:513–526.)* (B) Milestones of response to first line TKI therapy. Green is considered optimal, and no change to therapy is required. Yellow indicates a suboptimal response or 'warning,' which may trigger more intense monitoring or efforts to increase compliance, but not necessarily a change of treatment. Red denotes treatment failure, warranting a diagnostic workup and change of therapy.

often necessary to decide whether a rise of *BCR-ABL1* mRNA is real and should trigger a more extensive workup. The most important factor is the quality of the test, which determines the extent of inter-test variation and, as such, the optimal compromise between sensitivity and specificity.[222] Current recommendation is that a 5- to 10-fold rise in transcript levels be regarded as significant.[221] In excellent laboratories this may decrease to twofold,[223–226] while in poor laboratories even 10-fold rises may not be dependable. For the clinician, it is important to know the performance of the laboratory and to adjust standard operating procedures accordingly. It is good clinical practice to repeat the test in case of rising *BCR-ABL1* mRNA levels before rushing to far-reaching conclusions, particularly if the changes occur at a low level, where the coefficient of variation is largest. Fourth, key anchoring points of the IS are 100%, which is equivalent to the average *BCR-ABL1* mRNA expression of 30 patients treated on the International Randomized Study of Interferon and STI571 (IRIS) study[227] and 0.1%, corresponding to a 3-log reduction compared to this baseline and referred to as MMR. Achievement of MMR on TKI therapy is an important milestone that portends a good prognosis. Further reductions of *BCR-ABL1* mRNA by 4 or 4.5 logs are referred to as MR^4 or $MR^{4.5}$, respectively. $MR^{4.0}$ is frequently referred to as DMR and is the minimum depth of response required to consider a trial of TFR. It is important to be aware that the fold reductions of *BCR-ABL1* expressed on IS refer to a standardized baseline rather than the patient's individual baseline, thus representing an absolute measurement of leukemia burden. If results expressed on some obscure percentage scale are mistaken as IS results, response may be overestimated, giving false reassurance. Fifth, in the past a complete molecular response (CMR) was defined as negativity for *BCR-ABL1* mRNA. However, in contrast to MMR, which is an absolute value due to its anchoring to a standardized baseline, a universal definition of CMR is impossible, as it depends on the sensitivity of the assay and the quality of the sample. As such the term CMR should be abandoned altogether.

Despite universal agreement that standardized testing is superior, and years of education, some commercial labs in the US still do not express results on the IS. These assays are out of date, and should not be covered by insurance carriers. New technologies have refined the quantification of BCR-ABL1 on several levels. Digital PCR is based on amplification in single wells and Poisson statistics to estimate transcript numbers. Sensitivity is increased by 0.5 to 1 log compared to conventional assays. DNA-based assays require individualized primer design due to the variation in genomic breakpoints, but achieve 1-log higher sensitivity.[228] Other improvements include automated point of care assays that do not require sample processing in a laboratory, avoiding the need for shipment. Amplification systems that function without temperature cycling will further reduce infrastructure requirements. Technological advances of this kind will hopefully lead to greater access to PCR-based diagnosis and monitoring in resource-deprived countries.

BCR-ABL1 Mutation Testing

BCR-ABL1 mutations are the most extensively characterized mechanism of TKI resistance. The current NCCN guideline is to test for mutations in patients with failure to achieve therapeutic milestones or with relapse or progression to AP/BP-CML on therapy. Mutation analysis at baseline in patients with AP/BP-CML is not indicated due to the low yield and the absence of prospective studies supporting therapy modification based on the results. Similarly, routine mutation surveillance in high-risk patients is not indicated. For a long time Sanger sequencing of *BCR-ABL1* amplicons had been the gold standard of mutation screening, although it has limited sensitivity (20% to 30% mutant allele).[221] Several technologies such as high performance liquid chromatography, Sequenom MassARRAY, and allele-specific PCR were optimized to detect specific *BCR-ABL1* mutations with higher sensitivity. Since most of these studies were retrospective, it was difficult to assess the clinical significance of low-level mutations, and it remained controversial whether a positive test result should trigger a change of therapy.[185,186,229] Recently NGS has emerged as a technology that reliably increases sensitivity to between 1% and 5%, depending on sequencing depth. A recent Italian multi-institutional study demonstrated that NGS testing for BCR-ABL1 mutations is feasible and cost-effective in a routine setting. Confirming prior results, the study showed that mutant clones are invariably selected if the patient is treated with a TKI that is inactive against the mutant in question.[230] Even in the absence of a survival endpoint, it seems common sense to avoid exposing any patient to an ineffective therapy. In the future, error-corrected NGS may allow for ultra-sensitive mutation detection.[231] As with all other biomarkers, the clinical significance of such very small clones must be validated. Compound mutations, that is, the presence of two or more mutations in the same BCR-ABL1 molecule, add another level of complexity. If the variant allele frequencies add up to more than 100%, the presence of a compound mutation can be inferred. If the mutations are in close proximity, they may be covered in the same NGS amplicon. However, in many cases, sequencing of individual PCR clones is required to confirm the presence of a compound mutation. Clinically, many compound mutations, particularly those including the *BCR-ABL1*^{T315I} "gatekeeper" mutation, confer high level resistance to most or all currently approved TKIs and are associated with poor prognosis.[97,232]

THERAPY

Cytotoxic Agents

The alkylating agent busulfan was the first drug that effectively controlled white blood cell and platelet counts, but is rarely used today except as part of conditioning regimens for HCT.[20] Busulfan is highly toxic to HSCs, and must be dosed with great caution due to delayed myelosuppression. Chronic busulfan exposure is associated with pulmonary fibrosis, and proper dosing can be challenging due to prolonged cytopenias. Hydroxyurea is a ribonucleotide reductase inhibitor currently used as an effective method for lowering excessive white blood cell counts until the diagnosis of CML is confirmed. Although at conventional doses hydroxyurea acts predominantly on more mature progenitor cells, and myelosuppression is usually short-lived and reversible, overzealous WBC cosmetics is counterproductive. Prolonged cytopenias can result, particularly if aggressive hydroxyurea therapy is followed by a TKI, which not only puts the patient at risk of bleeding and infection, but may impair response to TKIs due to prolonged dose interruptions. In general practice, the only solid indication to rapidly lower the WBC is clinical signs of leukostasis, such as retinal ischemia, hypoxia, or priapism.

Interferon-α

IFN was introduced as a CML therapy in the early 1980s. Initial reports of CHR were followed by the surprising observation of CCyR in some cases, although only in a small minority of patients. The biological basis for IFN-induced responses has been the subject of much research, but to date the mechanism remains elusive, with evidence supporting cytotoxic, differentiating, and immunomodulatory effects. Subsequent studies combined IFN with cytarabine and found the combination superior to IFN monotherapy. Prior to the introduction of TKIs, IFN/Ara-C was the gold standard for drug therapy of CML.[233,234] Pegylated IFN formulations were a major improvement, due to better tolerability and the convenience of weekly injections. Today IFN remains an option for the rare patients who are intolerant of all TKIs. Moreover, IFN is a safe and frequently effective strategy to maintain responses in women who wish to become pregnant.[235] Lastly, combining TKIs with IFN improves the depth of molecular responses,[236] and IFN maintenance may increase the stability of TFR.[237]

Omacetaxine

Omacetaxine (previously homoharringtonine) is a subcutaneously administered protein synthesis inhibitor approved for the treatment of CP-CML and AP-CML with resistance to at least two TKIs. In CP-CML, the rate of MCyR was 18.4%, with a median duration of 12.5 months.[238] Due to the inconvenience of administration and significant myelosuppression, omacetaxine is used infrequently, but is an option as a palliative strategy for patients with intolerance of or resistance to all TKIs.

Tyrosine Kinase Inhibitors

TKIs are the standard of care for newly diagnosed CML patients in all disease phases. Six TKIs are currently approved—imatinib, dasatinib, nilotinib, bosutinib, ponatinib, and radotinib (only in South Korea). The first in class inhibitor imatinib is referred to as a first-generation (1 G) TKI; dasatinib, nilotinib, bosutinib, and radotinib are second-generation (2 G) TKIs; and ponatinib is a third-generation TKI. The TKIs are distinct based on their potency, activity against *BCR-ABL1* mutants, spectrum of activity against kinases other than *BCR-ABL1*, and side effect profiles. Today' treatment algorithms are based on a series of phase 3 trials (Table 69.5).

Imatinib

A phase 1 trial of imatinib initiated in 1998 included CML patients in all disease phases who had failed conventional therapy. Activity was seen across the spectrum, including CCyR, although mainly in CP-CML.[27,28] No maximum tolerated dose (MTD) was identified, and a phase 2 dose of 400 mg daily was selected based on the cytogenetic responses. These trials in CP-, AP-, and BP-CML confirmed or exceeded the phase 1 results and led to rapid approval of imatinib for patients who had failed prior IFN therapy due to resistance or intolerance.[239–242] Responses in CP-CML were mostly durable, but there was a high rate of resistance in advanced CML, which prompted an increase of the starting dose to 600 mg imatinib daily. The IRIS study compared imatinib 400 mg daily versus a combination of IFN and cytarabine, and found imatinib to be vastly superior in all major endpoints, but most importantly in progression-free survival.[242] Crossover was permitted for resistance or intolerance and occurred almost exclusively in patients failing IFN, and the fact that many of these patients were effectively salvaged by imatinib may explain why no difference in overall survival between the two experimental arms has been observed.[244] At the last follow-up at 10 years, the estimated overall survival of patients started on first-line imatinib was 83.3% (95% CI, 80.1 to 86.6).[245] Considering that overall survival of CML patients in the pre-TKI era was approximately 5 years, it is evident that imatinib revolutionized CML therapy, turning a lethal condition into a manageable ailment for the majority of patients. Subsequent phase 3 studies of imatinib in newly diagnosed CP-CML patients mainly addressed dose optimization and the role of combining imatinib with IFN or cytarabine. Single-arm/single-center studies in comparison with historical controls had suggested higher rates of CCyR and MMR for 800 mg imatinib daily, commonly referred to as high-dose imatinib.[246,247] Two prospective randomized studies compared 400 mg vs. 800 mg imatinib in patients with newly diagnosed CP-CML. The Tyrosine Kinase Inhibitor Optimization and Selectivity Study (TOPS) included patients from all Sokal risk groups, while an Italian trial focused on high Sokal risk patients. Both studies failed in their primary endpoints, MMR or CCyR at 12 months, respectively.[248,249] The German CML IV study reported superior rates of MMR at 12 months for 800 mg compared to 400 mg imatinib daily (59% vs. 44%, $P < .001$), but there was no difference in progression-free survival.[250,251] Interestingly, in this study, imatinib dosing was flexible and was maximized based on the individual patient's tolerability, resulting in a median dose of 627 mg imatinib daily. Similarly, the French SPIRIT study reported higher MMR rates at 12 months (49 vs. 38%) for patients treated with 600 versus 400 mg imatinib daily.[236] Thus, the optimal dose of imatinib may be approximately 600 mg daily, equal to the recommended dose for AP/BC.[252]

Dasatinib

A phase 1 study tested dasatinib in CML patients who had failed prior imatinib. Activity was seen in all disease phases.[33] Subsequent phase 2 studies tested different doses and dosing schedules.[253–257] Contrary to expectations based on dasatinib's short plasma half-life of 3 to 5 hours, once daily dosing proved to be equally effective compared to twice daily dosing, but was better tolerated, and 100 mg dasatinib once daily for CML-CP, and 140 mg once daily for AP/BP-CML emerged

TABLE 69.5 Results of Major Frontline Studies in Patients With Newly Diagnosed Chronic Phase of Chronic Myeloid Leukemia

Trial	Treatment	N =	CCγR	MMR	$MR^{4.5}$	TFS	OS
IRIS (18 months)	IM 400 mg/day	553	74% (est. at 18 months)	57% (est. at 12 months)[a]	NA/NR	96.7% (at 18 months)	97.2% (est. at 18 months)
IRIS (18 months)	IM 400 mg/day	553	74% (est. at 18 months)	57% (est. at 12 months)[a]	NA/NR	96.7% (at 18 months)	97.2% (est. at 18 months)
IRIS (60 months)	IM 400 mg/day	553	87% (est. at 60 months)	NA/NR	NA/NR	93% (at 60 months)	89% (est. at 60 months)
IRIS (10.9 years)	IM 400 mg/day	553	83% (at 10.9 years)	NA/NR	NA/NR	92% (at 10.9 years)	83.3% (at 10.9 years)
TOPS (42 months)	IM 400 mg/day	157	80.3% (by 42 months)	51.6% (at 42 months)	NA/NR	94.4% (est. at 48 months)	94.0% (est. at 48 months)
	IM 800 mg/day	319	81.5% (by 42 months)	50.2% (at 42 months)	NA/NR	95.8% (est. at 48 months)	93.4% (est. at 48 months)
ENESTnd (60 months)	NIL 600 mg/day	282	NA/NR	77.0% (by 60 months)	53.5% (by 60 months)	96.3% (est. by 60 months)	93.7% (est. by 60 months)
	NIL 800 mg/day	281	NA/NR	77.2% (by 60 months)	52.3% (by 60 months)	97.8% (est. by 60 months)	96.2% (est. by 60 months)
	IM 400 mg/day	283	NA/NR	60.4% (by 60 months)	31.4% (by 60 months)	92.6% (est. by 60 months)	91.7% (est. by 60 months)
DASISION (60 months)	DAS 100 mg/day	259	NA/NR	76% (by 60 months)	33% (by 60 months)	95.4% (by 60 months)	91% (est. by 60 months)
	IM 400 mg/day	260	NA/NR	64% (by 60 months)	42% (by 60 months)	92.7% (by 60 months)	90% (est. by 60 months)
BFORE (12 months)	BOS 400 mg/day	268	77% (by 12 months)	47% (at 12 months)	8.1% (at 12 months)	98.6% (by 12 months)[b]	99.6% (est. by 12 months)
	IM 400 mg/day	268	66% (by 12 months)	37% (at 12 months)	3.3% (at 12 months)	97.5% (by 12 months)[b]	97.9% (est. by 12 months)

[a] Only patients with CCyR had qPCR monitoring.
[b] 3/4 (BOS) and 2/6 patients (IM) met criteria solely on the basis of basophil count within 2 weeks of randomization. All continued study drug; 4/5 achieved MMR
BOS, Bosutinib; *CCyR*, complete cytogenetic response; *est.*, estimated; *MMR*, major molecular response; *DA*, dasatinib; *IM*, imatinib; *MR4.5*, ≥4.5-log reduction of BCR-ABL1 transcripts; *NA/NR*, not available/ or nor reported; *NIL*, nilotinib; *OS*, overall survival; *TFS*, transformation (to accelerated or blast phase)-free survival.

as the recommended doses. In laboratory studies, 4 hours of dasatinib exposure was sufficient to reduce colony formation by primary CML and irreversibly commit CML cell lines to apoptosis.[258] Collaborative group (SWOG S0325) and industry-sponsored (DASatinib versus Imatinib Study In treatment-Naive CML patients, DASISION) phase 3 studies compared dasatinib 100 mg daily with imatinib 400 mg daily in newly diagnosed CML-CP patients. Dasatinib proved superior in the primary endpoint, CCyR at 12 months.[257,259] Progression to AP/BP-CML was reduced, but the difference did not reach statistical significance, and no differences in overall survival were observed. Based on the results of DASISION, dasatinib was approved for use in newly diagnosed CML patients.

Nilotinib

A phase 1 study of nilotinib in a cohort of CML and Ph$^+$ ALL patients who failed prior imatinib therapy revealed considerable activity in CP-CML and AP-CML, while results in BP were less impressive.[34] Based on the confirmatory results in phase 2 studies, nilotinib 400 mg twice daily was approved for second-line therapy of CP-CML and AP-CML.[260,261] Although the phase 2 results for BP-CML are comparable to those of dasatinib, nilotinib is not currently approved for this indication.[262] The Evaluating Nilotinib Efficacy and Safety in Clinical Trials–Newly Diagnosed (ENESTnd) study evaluated nilotinib (300 or 400 mg twice daily) in comparison with imatinib 400 mg daily in first-line therapy of newly diagnosed CP-CML. Both experimental arms were superior to the standard arm in the major endpoint, MMR at 12 months.[263] Unlike any other second-generation TKI, nilotinib significantly improved progression-free survival. Based on the ENESTnd results, nilotinib (300 mg twice daily) received approval for frontline therapy of CML-CP. The 5-year follow-up of the study confirmed the superior rates of molecular responses. Progression free survival was improved for both experimental arms, and overall survival for the nilotinib 400 mg twice daily arm.[264]

Bosutinib

Formerly known as SKI-606, bosutinib was initially approved at a dose of 500 mg daily for third-line therapy (after failure of imatinib and one second-generation TKI) of CML in all phases.[265,266] A phase 3 trial (Bosutinib Efficacy and Safety in Newly Diagnosed Chronic Myeloid Leukemia, BELA) comparing bosutinib 500 mg daily vs. imatinib 400 mg daily as frontline therapy for CP-CML failed to meet its primary endpoint, CCyR at 12 months. Regulatory approval was denied despite superior rates of MMR.[267,268] It was speculated that inadequate management of adverse events led to a high rate of discontinuation in the experimental arm, including patients who discontinued bosutinib before their first response assessment. A second phase 3 study using a reduced dose of bosutinib at 400 mg daily (Bosutinib Trial in First-Line Chronic Myelogenous Leukemia Treatment BFORE) demonstrated superiority for MMR at 12 months (47.2 [40.9 to 53.4] vs. 36.9 [30.8 to 43.0]%). Overall and progression free survival were not different.[269] Based on the BFORE study, bosutinib became the fourth TKI approved for the first-line therapy of CP-CML.

Ponatinib

In aggregate, first- and second-generation TKIs cover all single BCR-ABL1 mutants, with the exception of BCR-ABL1^{T315I}.[270] Ponatinib was designed to address this specific need. Unlike other approved TKIs, ponatinib skirts isoleucine 315 through a carbon–carbon triple bond and exhibits potent activity against native BCR-ABL1 and all single mutants.[194] Based on its long half-life and prolonged target occupancy time, ponatinib is considered the most potent BCR-ABL1 TKI currently approved. However, this potency comes at the expense of reduced selectivity, and multiple additional tyrosine kinases are inhibited at clinically relevant plasma concentrations.[271] Ponatinib at an initial dose of 45 mg daily was effective in CML patients in all phases and Ph$^+$ ALL patients who had failed at least two prior TKIs or had the BCR-ABL1^{T315I} mutation.[272,273] Responses were generally durable in CP-CML, less stable in AP-CML, and transient in BP-CML and Ph$^+$ ALL. A strong cardiovascular safety signal emerged with longer follow-up of the Ponatinib efficacy and safety in Philadelphia chromosome-positive leukemia (PACE) study, leading to premature termination of an ongoing phase 3 trial [Ponatinib versus Imatinib in Chronic Myeloid Leukemia (EPIC)].[274] It was initially thought that ponatinib's vascular toxicity was caused by inhibition of vascular system kinases such as VEGF, PDGFR, and TIE2. Recent work in mice revealed that ponatinib-induced vascular toxicity involves VWF-mediated platelet adhesion and a secondary microvascular angiopathy that produces ischemic cardiac motion abnormalities with angiographically normal coronary arteries. Interventions known to reduce VWF multimer size mitigated toxicity.[275] There is evidence that ponatinib toxicity is dose-dependent, leading to the recommendation of dose reduction as soon as a response has been achieved. A dose-finding study compared starting doses of 45, 30, and 15 mg ponatinib daily, with mandatory dose reduction to 15 mg daily upon achieving BCR-ABL1 <1%. Responses were best for the 45 mg daily starting dose, with mostly comparable toxicity between arms. Cardiovascular events were lower compared to the PACE study.[276]

Drug Combinations

Imatinib has been tested in combination with multiple cytotoxic agents and signal transduction inhibitors, mostly in the setting of TKI resistance or AP/BP-CML. In the frontline setting, data are limited to combinations with cytarabine or IFN. Imatinib at 400 mg daily was compared with 600 mg daily as well as 400 mg daily combined with pegylated IFN or cytarabine, with MMR at 12 months as the major endpoint.[236] Patients in the IFN/imatinib arm had significantly higher rates of MMR than all other arms of the study (57% vs. 38% at 12 months for the comparison with single agent imatinib). Importantly, these data were confirmed by a smaller Scandinavian study.[277] Thus far, no differences in overall and progression-free survival have been seen, and IFN/imatinib combinations should be considered experimental. Imatinib and other TKIs were combined with AML-type multiagent chemotherapy in patients with myeloid BP, and with ALL-type chemotherapy in patients with lymphoid BP, with acceptable toxicity. Imatinib also demonstrated activity when combined with hypomethylating agents in TKI-resistant CML.[278] Unfortunately all these studies were single-armed, and the evidence supporting the addition of chemotherapy to TKIs is limited.

Allogeneic Stem Cell Transplantation

Prior to the introduction of imatinib, HCT was recommended to all eligible patients, and even today it is still regarded by some as the only therapy with curative potential. CML is more susceptible to immunotherapy in an HCT setting than most hematologic cancers and HCT was the first treatment modality that restored Ph$^-$ hematopoiesis and induced durable responses in CML.[22] Donor leukocyte infusions (DLIs) were first used in CML patients, demonstrating their effectiveness and proving the existence of a graft-versus-leukemia effect.[279] Unfortunately the transplant field is plagued by a paucity of controlled trials and only one single study prospectively compared HCT vs. drug therapy for CML. In this study, newly diagnosed patients with CML-CP were biologically randomized to a matched sibling transplant vs. IFN-based drug therapy, the best drug therapy available when the trial was initiated.[280] Initially patients managed with IFN had superior survival, with the biggest difference observed in patients with low Sokal risk. However survival curves crossed after approximately 8 years, suggesting that transplant would be superior in the long run if IFN was the alternative. In view of the efficacy of TKIs in the frontline setting, and the fact that early transplant-related mortality is higher than the risk of early disease progression, allotransplant is no longer justified in newly diagnosed patients with CML-CP.[210]

The European Group for Blood and Marrow Transplantation (EBMT) developed risk scores for allografting in CML and identified disease duration of greater than 12 months, advanced disease, higher age, unrelated donor type, and the combination of a male recipient with a female donor as adverse prognostic factors, with advanced disease having the greatest negative impact.[281] While this analysis dates back to the pre-imatinib era, it still provides some guidance. Little data is available for haploidentical transplants, but one retrospective study found them to be equally effective as matched sibling transplants.[282] There is consensus that HCT should be offered to all patients with progression to AP/BP.[40,283] There is no evidence that TKIs prior to allografting negatively impact the outcome. On the contrary, for unknown reasons, prior imatinib seems to reduce the risk of chronic graft-versus-host disease (GVHD) and possibly relapse.[284–286] A clinical challenge is not to miss the optimal timing for transplant, as HCT in first chronic phase yields far superior results compared to second chronic phase or in AP/BP. For instance, in the CML study IV 3-year overall survival was 91% for patients transplanted in CP after failing imatinib vs. 59% for patients transplanted in AP/BP.[287] The exceptional sensitivity of CML to the graftVersus-leukemia effect provides opportunities to select the transplant regimen based on the aggressiveness of the disease. There is consensus that bone marrow is preferred over peripheral blood stem cells (PBSCs) in patients with CML-CP, where GVHD is of greater concern than disease control.[288] In contrast patients with transformation to AP/BP, even if they have achieved a second chronic phase, may benefit from PBSCs, accepting a higher risk of GVHD. High relapse risk patients should receive a TKI post-transplant. In the absence of clear data it is our practice to treat patients with a TKI for at least one year post HCT, even if they are PCR-undetectable. This approach must be balanced against the toxicity of TKIs in the post-transplant setting, particularly myelosuppression. *BCR-ABL1* mutation analysis at the time of resistance may aid in TKI selection. Given the high median age at the time of diagnosis, many patients will be eligible only for reduced-intensity conditioning regimens. An in-depth discussion of the various conditioning regimens, selection of bone marrow vs. PBSCs, and post-transplant immunosuppression is beyond the scope of this chapter.

APPROACH TO THE NEWLY DIAGNOSED CHRONIC PHASE PATIENT

After completion of staging and risk assessment, the question is how to choose among the four approved TKIs. Several considerations factor into drug selection, not all of which are strictly medical. Thus far (2021), the only trial showing improved overall survival for a second generation TKI compared to imatinib 400 mg daily is the high-dose nilotinib arm (400 mg twice daily) of the ENESTnd study.[263,289,290] However, this dose is not approved as frontline therapy. At the next level, progression free survival was lower for all approved second-generation TKIs compared to standard dose imatinib, although this difference reached significance only for nilotinib. In contrast, all three second-generation TKIs are clearly superior in terms of MMR and DMR, which are achieved sooner and in a larger proportion of patients (see Table 69.5). Across all studies, differences in progression free survival, MMR, and DMR tend to be greatest in Sokal high-risk patients, somewhat less in Sokal intermediate-risk, and small in Sokal low-risk. Unfortunately, the available data is patchy due to the use of the Euro Score in the DASISION trial and inconsistent analyses. Nonetheless, both the ELN and the NCCN guidelines recommend a second-generation TKI for Sokal high- and intermediate-risk patients, noting that imatinib is an acceptable choice.[40,283] An increasingly important consideration is DMR, which is the basis for a trial of TFR. Since the success rate of TFR is very consistent across different populations of CP-CML patients, higher rates of DMR are expected to translate into higher rates of TFR (see also section on "TFR"). An equally important factor is the patient's past medical history and risk factors. Some adverse effects are shared across all or the majority of TKIs approved for first-line therapy, while others are specific to a particular drug (Table 69.6). For instance nilotinib is associated with arterial occlusive events, impaired glucose tolerance, and dyslipidemia. It is contraindicated in patients with a history of diabetes, hypercholesterolemia, or cardiovascular disease. Dasatinib frequently causes pleural effusions, and sometimes pulmonary hypertension, and is contraindicated in patients with pre-existing pulmonary disease. Bosutinib is associated with liver toxicity and renal impairment. Overall second-generation TKIs demonstrated improved tolerability compared to imatinib in phase 3 studies. While imatinib is associated with many low-grade toxicities that can significantly impair quality of life, it has proven to be extremely safe over more than 20 years of clinical use. In contrast,

TABLE 69.6 **Tyrosine Kinase Inhibitors Approved for the First-Line Treatment of Chronic Myeloid Leukemia**

Inhibitor	Imatinib	Nilotinib	Dasatinib
Indication	First line CML-CP, AP, BP	First line and salvage CML-CP, AP	First line and salvage CML-CP, AP, BP
Binding mode	Inactive conformation (type II)	Inactive conformation (type II)	Active conformation (type I)
IC_{50} native BCR-ABL1 expressing cells [nM]	260	13	0.8
IC_{50} ABL1 substrate phosphorylation [nM]	280	15	0.6
Additional targets	KIT, PDGFRA/B, CSF1R, LCK	KIT, PDGFRA/B	SRC family and multiple other kinases
Dose	400 mg once daily (CML-CP) 600 mg once daily (CML-AP, CML-BP)	300 mg twice daily (CML-CP, first line) 400 mg twice daily (CML-CP, CML-AP, salvage)	100 mg once daily (CML-CP) 140 mg once daily (CML-AP, CML-BP)
Adverse events in newly diagnosed patients irrespective of relation to drug (only grade 3&4)	Fluid retention 62 (3) • Superficial edema 60 (2) • Other fluid retention reactions 7 (1) Nausea 50 (1) Muscle cramps 49 (2) Musculoskeletal pain 47 (5) Diarrhea 45 (3) Rash 40 (3) Fatigue 39 (2) Headache 37 (<1) Joint pain 31 (3) Abdominal pain 37 (4) Nasopharyngitis 31 (0) Hemorrhage 29 (2) • GI hemorrhage 2 (<1) • CNS hemorrhage 0.2 (0) Myalgia 24 (2) Vomiting 23(2) Dyspepsia 19 (0) Cough 20 (<1) Pharyngolaryngeal pain 18 (<1) Upper respiratory tract infection 21 (<1) Dizziness 19 (<1) Pyrexia 18 (<1) Weight gain 16 (2) Insomnia 15 (0) Depression 15 (<1) Influenza 14 (<1) Bone pain 11 (2) Constipation 11 (<1)	Rash 33 (2) Pruritus 29 (1) Nausea 31 (1) Diarrhea 22 (3) Constipation 21 (<1) Vomiting 21 (<1) Abdominal pain 11 (1) Headache 31 (3) Fatigue 28 (1) Pyrexia 14 (1) Asthenia 14 (0) Edema, peripheral 11 (0) Arthralgia 18 (2) Myalgia 14 (2) Pain in extremity 13 (1) Bone pain 11 (<1) Muscle spasms 11 (<1) Back pain 10 (<1) Cough 17 (<1) Dyspnea 11 (1) Nasopharyngitis 16 (<1)	Fluid retention 23 (1) Pleural effusion 12 (<1) Superficial localized edema 10 (0) Generalized edema 3 (0) Congestive heart failure/cardiac dysfunction 2 (<1) Pericardial effusion 2 (<1) Pulmonary hypertension 1 (0) Pulmonary edema <1(0) Diarrhea 18 (<1) Headache 12 (0) Musculoskeletal pain 12 (0) Rash 11 (0) Nausea 9 (0) Fatigue 8 (<1) Myalgia 6 (0) Hemorrhage 6 (1) Gastrointestinal bleeding 2 (1) Other bleeding 5 (0) Vomiting 5 (0) Muscle inflammation 4 (0)

some toxicities of second-generation TKIs are potentially fatal, which may be one reason why improved progression free survival has not translated into improved overall survival, but studies directly comparing the different second-generation TKIs are unavailable. Additional considerations include co-medications, convenience (once vs. twice daily dosing; food interactions), and costs (financial toxicity). Second-generation TKIs are consistently more expensive than imatinib, and the introduction of generic imatinib has led to an enormous difference in countries such as Canada. In many cases, there are no firm contraindications against any one of the four TKIs, and it can be a matter of trial and error to identify the drug with the optimal tolerability in a given patient. In this complicated space of abundant and yet incomplete data, it is important to define the goals of therapy in an informed discussion with the patient that aims at shared decision making. In young patients, TFR should receive strong consideration as the treatment goal. Women who wish to start a family may want to maximize their chances of achieving DMR and subsequently TFR to avoid the need to interrupt TKI therapy during pregnancy. In older patients with cardiovascular risk, overall survival is the key consideration, and imatinib with its low (or absent) cardiovascular toxicity is the drug of choice. In this context, recent data for dasatinib suggest that a starting dose of 50 mg daily, half the current label dose, produces excellent results.[291] Using this approach, drug interruptions due to toxicity were minimized, which may have compensated for the reduction in daily dose. Similar observations were made for imatinib, where a flexible dosing strategy aimed to minimize protocol-driven dose interruptions seemed to improve efficacy.[291] More research into this strategy is clearly warranted. (See boxes on Cardiovascular Tyrosine Kinase Inhibitor Toxicity, Finding the Best Tyrosine Kinase Inhibitor Dose, and Rapid Tyrosine Kinase Inhibitor Cycling.)

APPROACH TO THE PATIENT WITH TYROSINE KINASE INHIBITOR RESISTANCE

Chronic Phase of Chronic Myeloid Leukemia

Failure to achieve therapeutic milestones or loss of response suggests TKI resistance. As TKI resistance is a consequential diagnosis associated with less favorable outcomes, it is mandatory to exclude other causes. The first call of order is a thorough history to assess drug compliance. Drug level testing, while available in some specialized labs, is not routine practice, and patients have been found to make up for skipped doses during the last few days prior to the

FINDING THE BEST TYROSINE KINASE INHIBITOR DOSE

Most CML patients are on long-term TKI therapy, and managing toxicity is critically important to maintain compliance. Recommended TKI dosing as per package insert is based on early efficacy endpoints that do not reflect the reality of chronic therapy. Dose reduction strategies have received relatively little attention, although a recent study suggests that it is safe and may improve the rate of TFR.[292] This suggests that lower doses of dasatinib, nilotinib, or bosutinib can be considered in patients with good disease control (MMR or better) who experience severe toxicity, but are not candidates for or not comfortable with a trial of TFR. We have found that dasatinib at 20 mg daily can be an effective and very well tolerated maintenance, especially in older patients, and the same holds true for lower doses of bosutinib or nilotinib. It is important to discuss with the patient that relatively little data is available, and that regular PCR monitoring is essential.

CARDIOVASCULAR TYROSINE KINASE INHIBITOR TOXICITY

Arterial occlusive events and other types of cardiovascular (CV) toxicity are of significant concern in patients on TKI therapy, with the notable exception of imatinib. Although incidence is highest with ponatinib and nilotinib, CV risk is also increased with dasatinib and to a lesser extent bosutinib.[293] It is likely that different mechanisms are operational with the different TKIs, although it remains possible that inhibition of ABL1 itself contributes to toxicity. Regardless, there is no uniform preventive strategy other than minimizing CV risk and selecting the most appropriate TKI for a given patient. In our practice we initially determine CV risk using the Framingham score. While patients with low risk (<5%) are eligible in principle for all TKIs, we avoid nilotinib in patients with higher CV risk. Imatinib is strongly considered for patients with high CV risk, and we have a low threshold in obtaining a cardiology consult, particularly if a high-risk agent such as ponatinib is under consideration based on the patient's CML. All patients are followed using a set of practice guidelines tailored to the specific TKI.

Provisional Recommendation for Cardiovascular Monitoring of Patients on Tyrosine Kinase Inhibitors

		Imatinib	Nilotinib	Dasatinib	Bosutinib	Ponatinib
Base line	Clinical cardiovascular assessment, including blood pressure	Follow good clinical practice	REC	REC	REC	REC
	Fasting glucose		REC	ACI	ACI	REC
	Fasting lipid panel		REC	ACI	ACI	REC
	Echocardiogram		ACI	ACI	ACI	ACI
	Electrocardiogram		REC	REC	ACI	ACI
	Ankle-brachial index		REC	ACI	ACI	REC
1-month follow-up	Clinical cardiovascular assessment		REC	REC	ACI	REC
	Blood pressure check		ACI	ACI	ACI	REC
3–6 monthly follow-up	Clinical cardiovascular assessment		REC	REC	REC	REC
	Blood pressure check		REC	ACI	ACI	REC
	Fasting glucose		REC	ACI	ACI	ACI
	Fasting lipid panel		REC	ACI	ACI	REC
	Echocardiogram		ACI	ACI	ACI	ACI
	Electrocardiogram		ACI	ACI	ACI	ACI
	Ankle-brachial Index		REC	ACI	ACI	REC

ACI, As clinically indicated; *REC*, recommended.
Adapted from Moslehi JJ, Deininger M. Tyrosine kinase inhibitor-associated cardiovascular toxicity in chronic myeloid leukemia. *J Clin Oncol*. 2015;33(35):4210–4218.

RAPID TYROSINE KINASE INHIBITOR CYCLING

With the availability of multiple TKIs, it is tempting to switch therapy rather than first making an attempt to manage toxicity to fully exploit the potential of a given TKI. As a result, some patients rapidly cycle through all approved TKIs and are then labelled as pan-TKI intolerant. While pan-TKI intolerance clearly exists, it is rare, and the diagnosis should me made with great caution, given that alternative therapies (IFN-α and SCT) are typically more toxic than TKIs. Perhaps the most important strategy to avoid this scenario is to be realistic and properly manage expectations up front. Every TKI has side effects, and may impair quality of life. On the positive side, for CP-CML patients there is an almost normal life expectancy, an incredible achievement compared to the pre-TKI era. In selected cases of severe or unusual toxicity, drug level testing can provide useful information to inform the use of unusually low doses.[294] It can be challenging to distinguish between TKI toxicity and the many other causes of headaches, fatigue, and other hard to objectively evaluate complaints. Exploring other causes such as stress at the workplace or in the family can reveal surprising results. As everywhere in medicine, taking the time to listen and pick up clues is essential. Lastly non-drug strategies, such as exercise and mediation, should be part of the management to minimize poly-pharmacy. Sometimes it is helpful to emphasize that not every day is perfect, even for perfectly healthy individuals. In other words, that life itself has side effects.

office visit.[295] Possible drug interactions should be reviewed, ideally involving a clinical pharmacologist, especially in patients on multiple co-medications. It is very important to include herbal preparations, supplements, and over-the-counter remedies—for example, St. John's Wort, which can drastically lower imatinib plasma concentrations.[296] It is nearly impossible to accurately predict drug interactions in patients on polypharmacotherapy, and the situation is even more complicated in cases of impaired renal or hepatic function. If non-adherence or drug interactions seem unlikely, a complete resistance workup is indicated, including physical exam, CBC, bone marrow aspirate and biopsy, bone marrow metaphase karyotyping, and *BCR-ABL1* qPCR and mutation screen. Key data gained from this workup are disease phase, *BCR-ABL1* mutation status, and karyotype, including CCA/Ph$^+$.

Patients with resistance to imatinib can be managed with a second-generation TKI as long as they are in chronic phase and do not have the *BCR-ABL1*^{T315I} mutation. The selection of dasatinib, nilotinib, or bosutinib is based on past medical history to minimize side effects and the type of *BCR-ABL1* mutation if present (Table 69.7). Ponatinib is the only option for patients with *BCR-ABL1*^{T315I}. It is good clinical practice to have the patient evaluated or re-evaluated by a transplant center. Analogous to newly diagnosed patients, patients on second-line TKIs must be assessed at regular intervals to determine whether their response meets expectations. One study showed that only 7% of patients who failed to achieve a minor cytogenetic response ($\leq$65% Ph$^+$ metaphases) to dasatinib or nilotinib at 3 months were in MCyR at 12 months, while MCyR at 12 months predicted progression-free and overall survival.[297] Although data are limited, we generally follow the consensus response milestones used for first-line TKI therapy. Patients who fail frontline or second-line second-generation TKI are at high risk, even if still in CP, and have limited drug treatment options. Switching from one second-generation TKI to another produces CCyR rates of only ~20%, and responses are generally not durable.[298] Except in patients where a specific *BCR-ABL1* mutation suggests that another second-generation will be effective, ponatinib is the TKI of choice, with an estimated 5-year overall survival of 73%.[299] Evidently, ponatinib's cardiovascular risk must be taken into account. Additionally, experimental agents and/or allogeneic stem cell transplant must be considered.

Accelerated Phase/Blast Phase Chronic Myeloid Leukemia

Patients who progress to AP/BP on TKI therapy are candidates for HCT, and the purpose of salvage drug therapy is to establish a second chronic phase. Patients with AP-CML are managed with an appropriate TKI, following the same strategy as described for CP-CML. A clinical trial should be strongly considered and is the best option for patients who have failed ponatinib and are ineligible for SCT. Patients with progression to BP-CML may be managed with a combination of TKI and chemotherapy. In patients who still have a TKI option, it is important to weigh the risk of chemotherapy against the potential gain in efficacy, since severe chemotherapy-induced toxicity may delay SCT and increase transplant-related toxicity. Additionally the superiority of TKI/chemotherapy combinations versus TKI alone in AP/BP-CML has never been established in a controlled trial setting. Reduced intensity approaches such as combinations of TKIs with hypomethylating agents in M-BP and vincristine/prednisone in patients with L-BP should be considered, particularly in older patients and those with a reduced performance status.

TREATMENT-FREE REMISSION

The Stop Imatinib Trial (STIM-1) was the first prospective study that systematically evaluated whether TKI discontinuation is feasible.[39] Patients with $MR^{5.0}$ on imatinib for at least 2 years were eligible. Molecular recurrence was defined as any two consecutive positive PCR results or loss of MMR in a single test. At 12 months of follow-up, the projected recurrence free survival was 41%, with the overwhelming majority of recurrences occurring within 6 months of discontinuation, and no recurrence after 21 months. At 67 months, 39% of patients maintained TFR.[300] Patients who experienced recurrence recaptured MMR upon resumption of TKI therapy. Longer TKI exposure and longer duration of DMR were associated with a higher probability of successful TFR, while patients with high Sokal scores were at a higher risk of recurrence. Multiple subsequent TFR studies used modifications to the eligibility and recurrence criteria, but yielded remarkably similar results. TFR rates varied mostly between 40% and 60%, with higher rates observed when loss of MMR was used as a more lenient definition of failure.[301] Several independent studies have identified associations between cellular immune parameters and TFR, including higher NK cells, lower T regulatory cells, and lower numbers of CD86$^+$ plasmacytoid dendritic cells.[302–304] Studies to mechanistically link these immunologic phenomena with TFR have yet to be conducted. In practice, longer duration of TKI exposure and DMR are the most consistent clinical parameters predicting successful TFR.

After the initial TFR studies following imatinib therapy, the same approach was tested in patients with DMR on a second-generation TKI, again with very similar results, although follow-up was shorter compared to imatinib.[305–310] Given the higher rates of DMR in CP-CML patients treated up front with a second-generation TKI, this should translate into higher overall rates of TFR, which argues for starting patients on a second-generation TKI. The risk/benefit assessment of this situation is complex, as any TFR gain with second-generation TKIs must be balanced against the increased risk of serious AEs, such as cardiovascular events on nilotinib.[311,312]

About one-quarter of patients who discontinue TKIs develop a "TKI withdrawal syndrome," consisting primarily of musculoskeletal pain, sometimes accompanied by flushing.[313] Patients with preexisting arthritis appear to be especially susceptible. Over-the-counter anti-inflammatory agents or a short course of steroids are usually effective, but some patients may require opiates or even resumption of TKI therapy. The etiology of the TKI withdrawal syndrome remains enigmatic, but cytokine release, regrowth of mast cells, and reactivation of an underlying, TKI-suppressed inflammatory condition are under consideration. Patients experiencing TKI withdrawal syndrome appear to have a higher likelihood of successful TFR.

The sobering news is that only a minority of newly diagnosed CML patients are likely to enter successful TFR. This is based on the following calculations, which apply to developed countries only. Approximately 10% of patients present with AP/BP and are not considered TFR candidates. Of the remaining 90%, at best 60% will achieve sustained DMR, even if started on a 2G TKI. Of these, at

TABLE 69.7 Sensitivity of BCR-ABL1 Kinase Domain Mutants to Approved Tyrosine Kinase Inhibitors

		$1C_{50}$-fold increase (WT = 1)				
location of Mutation	**Mutation**	**Imatinib**	**Bosutinib**	**Dasatinib**	**Nilotinib**	**Ponatinib**
	Parental	10.8	38.3	568.3	38.4	570.0
	WT	1	1	1	1	1
P-loop	M244V	0.9	0.9	2.0	1.2	3.2
	L248R	**14.6**	**22.9**	**12.5**	**30.2**	*6.2*
	L248V	*3.5*	*3.5*	*5.1*	*2.8*	*3.4*
	G250E	*6.9*	*4.3*	*4.4*	*4.6*	*6.0*
	Q252H	1.4	0.8	*3.1*	*2.6*	*6.1*
	Y253F	*3.6*	1.0	1.6	*3.2*	*3.7*
	Y253H	*8.7*	0.6	*2.6*	**36.8**	*2.6*
	E255K	*6.0*	*9.5*	**5.6**	*6.7*	*8.4*
	E255V	**17.0**	*5.5*	**3.4**	**10.3**	**12.9**
C-helix	D276G	*2.2*	0.6	1.4	2.0	*2.1*
	E279K	*3.6*	1.0	1.6	2.0	*3.0*
	E292L	0.7	1.1	1.3	1.8	2.0
ATP binding region	V299L	1.5	**26.1**	*8.7*	1.3	0.6
	T315A	1.7	*6.0*	**58.9**	*2.7*	0.4
	T315I	**17.5**	**45.4**	**75.0**	**39.4**	*3.0*
	T315V	**12.2**	**29.3**	**738.8**	**57.0**	*2.1*
	F317L	*2.6*	*2.4*	*4.5*	*2.2*	0.7
	F317R	*2.3*	**33.5**	**114.8**	*2.3*	*4.9*
	F317V	0.4	**11.5**	**21.3**	0.5	*2.3*
SH2-contact	M343T	1.2	1.1	0.9	0.8	0.9
	M351T	1.8	0.7	0.9	0.4	1.2
Substrate binding region	F359I	*6.0*	*2.9*	*3.0*	**16.3**	*2.9*
	F359V	*2.9*	0.9	1.5	*5.2*	*4.4*
A-loop	L384M	1.3	0.5	*2.2*	*2.3*	*2.2*
	H396P	*2.4*	0.4	1.1	*2.4*	1.4
	H396R	*3.9*	0.8	1.6	*3.1*	*5.9*
C-terminal lobe	F486S	*8.1*	*2.3*	*3.0*	1.9	*2.1*
	L248R 1 F359I	**11.7**	**39.3**	**13.7**	**96.2**	**17.7**
Sensitive	≤-2					
Moderately resistant	*2.1–10*					
Highly resistant	**>10**					

From Redaelli S, Mologni L, Rostagno R, et al. Three novel patient-derived BCR/ABL mutants show different sensitivity to second and third generation tyrosine kinase inhibitors. *Am J Hematol.* 2012;87: E125-E128. 10.1002/ajh.23338.

best 60% (or 32.4% of all newly diagnosed patients) will enter TFR if recurrence is defined as loss of MMR. For the remaining majority, lifelong TKI therapy is required. (See box on Selecting Patients for Treatment-Free Remission.)

FUTURE DIRECTIONS

CP-CML patients treated with TKIs have a life expectancy close to aged-matched controls.[152] In fact the survival of these patients is driven much more by comorbidities than by CML.[315] In practice this means that hematologists treating CML patients need to pay attention to concomitant medical conditions or carefully coordinate care with other specialists, such as cardiologists. The fact that the decades-old goal of CML therapy, a normal life span, has been attained, raises the question whether treatment goals need to change from overall survival to TFR. If TFR is the accepted goal, then using a second-generation TKI up front is justified. Unfortunately second-generation TKIs are sometimes associated with irreversible toxicity, and only one-third of patients will achieve TFR, creating a difficult competing risk scenario. Contrary to expectations, starting patients on a second-generation TKI is not cost-effective, even if the higher TFR rates are taken into account and assuming a relatively high price for generic imatinib.[316] As most CML patients will require lifelong therapy to avoid disease recurrence, developing strategies to increase rates of DMR and successful TFR is paramount. Unfortunately, implementing the clinical trials needed to test the many rational drug combinations

SELECTING PATIENTS FOR TREATMENT-FREE REMISSION

TFR has fairly rapidly moved from anecdote through clinical trials to mainstream practice, including the NCCN guidelines and ELN recommendations.[40,283] Although multiple clinical studies have shown that TFR is safe, observational data from the community are sparse. One observation deserves particular attention. While most tightly controlled and/or industry-sponsored trials showed that most recurrences occur relatively early, with a stable plateau thereafter, there is a slow but continuous attrition of the recurrence free survival curve in the international multi-institutional EuroSKI trial, the largest study conducted thus far.[314] The reasons for this discrepancy are unknown. One possibility is that patients with less deep responses were included. As the EuroSKI trial is a better reflection of the "real world," this suggests that monitoring must continue indefinitely. In a fragmented health care system like the one on the US, with frequent changes of insurance, this can be challenging. On the other hand, given that long-term TKI toxicity remains a concern, a TFR attempt is more than just a consideration, but close to a mandate in well selected patients. By analogy, would one continue antibiotics if there was a 50% chance that the infection had been cleared and re-treatment was practically always effective? In our practice we recommend a TRF trial to every patient who qualifies based on the NCCN consensus guidelines, which include

- Age ≥18 years
- History of good compliance
- CP-CML, no prior AP/BP
- TKI therapy for ≥3 years
- Quantifiable BCR-ABL1 transcript
- Stable DMR for ≥2years
- Access to reliable qPCR testing, with results expressed on the IS
- Monthly testing for month 1–6, 2-monthly for month 7–12, and every 3 months there after
- Prompt resumption of TKI therapy upon loss of MMR

identified in preclinical models has been challenging, and the few studies that performed thus far failed to yield a conclusively positive result (see Table 69.2). Establishing pre-clinical models that more faithfully reflect human CML and simplifying clinical trials will be critical for progress. Another question is whether we have exploited the entire potential of inhibiting BCR-ABL1 in the first place. The abandoned EPIC study of ponatinib 45 mg daily in newly diagnosed patients with CP-CML may provide some insights. Although only 73 patients were evaluable for response at 6 months, the DMR rate was 32% compared to ~5% with nilotinib 300 mg twice daily in the ENESTnd study. Although one cannot exclude that off-target effects of ponatinib have contributed to this high efficacy, these data may suggest that a very potent yet well-tolerated TKI is the first step to maximizing TFR rates.[274] In this context, asciminib, the first clinical allosteric ABL1 TKI, is of considerable interest. Asciminib targets the myristate binding pocket of BCR-ABL1, enforcing an inactive kinase conformation. The drug inhibits BCR-ABL1 at sub-nanomolar concentration, on par with ponatinib and dasatinib.[35,317] A phase 1 study showed considerable clinical activity in patients who failed multiple ATP site TKIs. Toxicity was low, reflecting extremely high selectivity and suggesting that high doses may be tolerated, achieving deep suppression of BCR-ABL1 kinase activity.[318] A subsequent phase 3 study compared asciminib 80 mg daily with bosutinib 500 mg daily in patients with failure to two or more prior TKIs, but without the BCR-ABL1T315I mutation.[319] Asciminib was superior in the primary endpoint, MMR at 6 months (25.5 vs. 13.2%, a 12.2% (95% CI, 2.19–22.30) after adjusting for MCyR status at baseline, leading to FDA approval. Asciminib has further enriched the treatment options for CML. How exactly asciminib will fit into the treatment paradigm in the salvage and ultimately frontline setting will require additional studies.

TKI therapy of CML established the paradigm of molecularly targeted therapy, and lessons continue to be learned from the ongoing history of CML, some of which have reached the broader societal context. CML TKIs are extremely expensive, particularly in the United States, which does not have the political will to put mechanisms in place to prevent excessive health care system exploitation. As a result, the price of imatinib increased in leaps throughout a patent's lifetime, without apparent reason. Even today generic imatinib continues to be many times more expensive than in neighboring countries. While patients may eventually gain access to the TKI most suitable for them through assistance programs, precious time and resources are wasted on bureaucratic processes that are of no societal value. It is my impression that most patients who die from CML in 2020 are the victims of a society that spends more on health care than any other and yet fails to provide equal and easy access to care for its most vulnerable members. For this to improve, more is needed than another TKI.

ACKNOWLEDGMENTS

This work was supported in part by Leukemia & Lymphoma Society (LLS) Translational Research Program Award (6086-12) (M.W.D.), an LLS Specialized Center of Research Program Award (GCNCR0314A-UTAH) (M.W.D.), National Institutes of Health (NIH) National Cancer Institute grant R01CA178397 (M.W.D. and T.O.) and NIH grant R01CA257602 (M.W.D).

I thank Claire Davis and Kristin Dahlin, Huntsman Cancer Institute, for assistance with illustrations, and my wife, Jutta Deininger, MD, for her unwavering support and ever-present inspiration.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Bigenzahn JW, et al. LZTR1 is a regulator of RAS ubiquitination and signaling. *Science*. 2018;362:1171–1177.

Branford S, et al. Integrative genomic analysis reveals cancer-associated mutations at diagnosis of CML in patients with high-risk disease. *Blood*. 2018;132:948–961.

Bumm T, et al. Emergence of clonal cytogenetic abnormalities in Ph-cells in some CML patients in cytogenetic remission to imatinib but restoration of polyclonal hematopoiesis in the majority. *Blood*. 2003;101:1941–1949.

Corbin AS, et al. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J Clin Invest*. 2011;121:396–409.

Cortes JE, et al. A phase 2 trial of ponatinib in Philadelphia chromosome-positive leukemias. *N Engl J Med*. 2013;369:1783–1796.

Cortes JE, et al. Bosutinib versus imatinib for newly diagnosed chronic myeloid leukemia: results from the randomized BFORE Trial. *J Clin Oncol*. 2018;36:231–237.

Druker BJ, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat. Med*. 1996;2:561–566.

Druker BJ, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med*. 2006;355:2408–2417.

Eide CA, et al. Combining the allosteric inhibitor asciminib with ponatinib suppresses emergence of and restores efficacy against highly resistant BCR-ABL1 mutants. *Cancer Cell*. 2019;36:431–443. e435.

Etienne G, et al. Long-term follow-up of the french stop imatinib (STIM1) study in patients with chronic myeloid leukemia. *J Clin Oncol*. 2017;35:298–305.

Gorre ME, et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science*. 2001;293:876–880.

Hehlmann R, et al. Assessment of imatinib as first-line treatment of chronic myeloid leukemia: 10-year survival results of the randomized CML study IV and impact of non-CML determinants. *Leukemia*. 2017;31:2398–2406.

Hehlmann R, et al. Tolerability-adapted imatinib 800 mg/d Versus 400 mg/d versus 400 mg/d plus interferon-α in newly diagnosed chronic myeloid leukemia. *J Clin Oncol*. 2011;29:1634–1642.

Hochhaus A, et al. Long-term outcomes of imatinib treatment for chronic myeloid leukemia. *N Engl J Med*. 2017;376:917–927.

Hughes TP, et al. Asciminib in Chronic Myeloid Leukemia after ABL Kinase Inhibitor Failure. *N Engl J Med*. 2019;381:2315–2326.

Huntly BJ, et al. MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. *Cancer Cell*. 2004;6:587–596.

Jamieson CHM, et al. Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N Engl J Med*. 2004;351:657–667.

Kantarjian H, et al. Dasatinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukemia. *N Engl J Med*. 2010;362:2260–2270.

Lipton JH, et al. Ponatinib versus imatinib for newly diagnosed chronic myeloid leukaemia: an international, randomised, open-label, phase 3 trial. *The Lancet Oncol*. 2016;17:612–621.

Mahon FX, et al. Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. *Lancet Oncol*. 2010;11:1029–1035.

Marin D, et al. Adherence is the critical factor for achieving molecular responses in patients with chronic myeloid leukemia who achieve complete cytogenetic responses on imatinib. *J Clin Oncol*. 2010;28:2381–2388.

Mauro MJ, Hughes TP. Asciminib in relapsed chronic myeloid leukemia. Reply. *N Engl J Med*. 2020;382:1379.

Moslehi JJ, Deininger M. Tyrosine kinase inhibitor-associated cardiovascular toxicity in chronic myeloid leukemia. *J Clin Oncol*. 2015;33:4210–4218.

Neviani P, et al. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. *Cancer Cell*. 2005;8:355–368.

O'Hare T, et al. AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. *Cancer Cell*. 2009;16:401–412.

Pfirrmann M, et al. Prognosis of long-term survival considering disease-specific death in patients with chronic myeloid leukemia. *Leukemia*. 2016;30:48–56.

Prost S, et al. Erosion of the chronic myeloid leukaemia stem cell pool by PPARγ agonists. *Nature*. 2015;525:380–383.

Schindler T, et al. Structural mechanism for STI-571 inhibition of abelson tyrosine kinase. *Science*. 2000;289:1938–1942.

Warfvinge R, et al. Single-cell molecular analysis defines therapy response and immunophenotype of stem cell subpopulations in CML. *Blood*. 2017;129:2384–2394.

Wylie AA, et al. The allosteric inhibitor ABL001 enables dual targeting of BCR-ABL1. *Nature*. 2017;543:733–737.

REFERENCES

1. Thorburn AL. Alfred François Donné, 1801–1878, discoverer of Trichomonas vaginalis and of leukaemia. *Br J Vener Dis*. 1974;50:377–380. https://doi.org/10.1136/sti.50.5.377.
2. Donné, A. *Cours de Microscopie*. (Paris: Baillière; 1844.).
3. Bennett JH. Case of hypertrophy of the spleen and liver in which death took place from suppuration of the blood. *Edinb Med and Surgi J*. 1845;64:413–423.
4. Virchow R. Weisses Blut. *Frorieps Notizen*. 1845;36:151–156.
5. Neumann E. Ein Fall von Leukamie mit Erkrankung des Knochenmarks. *Archive Heilkunde*. 1870
6. Dameshek W. Some speculations on the myeloproliferative syndromes. *Blood*. 1951;6:372–375.
7. Nowell P, Hungerford D. A minute chromosome in human chronic granulocytic leukemia. *Science*. 1960;132:1497.
8. Baikie AG, et al. A possible specific chromosome abnormality in human chronic myeloid leukaemia. *Nature*. 1960;188:1165–1166. https://doi.org/10.1038/1881165a0.
9. Fialkow PJ, Gartler SM, Yoshida A. Clonal origin of chronic myelocytic leukemia in man. *Proc Natl Acad Sci USA*. 1967;58:1468–1471.
10. Rowley JD. A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature*. 1973;243:290–293.
11. Groffen J, et al. Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. *Cell*. 1984;36:93–99.
12. Bartram CR, et al. Translocation of c-abl oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. *Nature*. 1983;306:277–280.
13. Shtivelman E, Lifshitz B, Gale RP, Canaani E. Fused transcript of abl and bcr genes in chronic myelogenous leukaemia. *Nature*. 1985;315:550–554. https://doi.org/10.1038/315550a0.
14. Abelson HT, Rabstein LS. Lymphosarcoma: virus-induced thymic-independent disease in mice. *Cancer Res*. 1970;30:2213–2222.
15. Heisterkamp N, et al. Chromosomal localization of human cellular homologues of two viral oncogenes. *Nature*. 1982;299:747–749.
16. Lugo TG, Pendergast AM, Muller AJ, Witte ON. Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. *Science*. 1990; 247:1079–1082.
17. Daley GQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. *Science*. 1990;247:824–830.
18. Lissauer H. Zwei Faelle von Leucaemie. *Berl. Klin. Wochenschr*. 1865;2: 403–405.
19. Cowan Doyle A. Notes on a case of leukocythaemia. *The Lancet*. 1882;119:490.
20. Hehlmann R, et al. Randomized comparison of interferon-alpha with busulfan and hydroxyurea in chronic myelogenous leukemia. The German CML Study Group. *Blood*. 1994;84:4064–4077.
21. Djaldetti M, Padeh B, Pinkhas J, De Vries A. Prolonged remission in chronic myeloid leukemia after one course of busulfan. *Blood*. 1966;27:103–109.
22. Fefer A, et al. Disappearance of Ph1-positive cells in four patients with chronic granulocytic leukemia after chemotherapy, irradiation and marrow transplantation from an identical twin. *N Engl J Med*. 1979;300:333–337. https://doi.org/10.1056/NEJM197902153000702.
23. Talpaz M, Kantarjian H, Kurzrock R, Trujillo JM, Gutterman JU. Interferon-alpha produces sustained cytogenetic responses in chronic myelogenous leukemia. Philadelphia chromosome-positive patients. *Ann Intern Med*. 1991;114:532–538.
24. Anafi M, Gazit A, Gilon C, Ben-Neriah Y, Levitzki A. Selective interactions of transforming and normal abl proteins with ATP, tyrosine-copolymer substrates, and tyrphostins. *J Biol Chem*. 1992;267:4518–4523.
25. Buchdunger E, et al. Selective inhibition of the platelet-derived growth factor signal transduction pathway by a protein-tyrosine kinase inhibitor of the 2-phenylaminopyrimidine class. *Proc Natl Acad Sci USA*. 1995;92:2558–2562.
26. Druker BJ, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat Med*. 1996;2:561–566.
27. Druker BJ, et al. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *N Engl J Med*. 2001;344:1038–1042.
28. Druker BJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med*. 2001;344:1031–1037.
29. Schindler T, et al. Structural mechanism for STI-571 inhibition of abelson tyrosine kinase. *Science*. 2000;289:1938–1942.
30. Nagar B, et al. Structural basis for the autoinhibition of c-Abl tyrosine kinase. *Cell*. 2003;112:859–871.
31. Hantschel O, et al. A myristoyl/phosphotyrosine switch regulates c-Abl. *Cell*. 2003;112:845–857.
32. Gorre ME, et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science*. 2001;293:876–880.
33. Talpaz M, et al. Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. *N Engl J Med*. 2006;354:2531–2541.
34. Kantarjian H, et al. Nilotinib in imatinib-resistant CML and Philadelphia chromosome-positive ALL. *N Engl J Med*. 2006;354:2542–2551.
35. Wylie AA, et al. The allosteric inhibitor ABL001 enables dual targeting of BCR-ABL1. *Nature*. 2017;543:733–737. https://doi.org/10.1038/nature21702.
36. Hughes TP, et al. Asciminib in chronic myeloid leukemia after ABL kinase inhibitor failure. *N Engl J Med*. 2019;381:2315–2326. https://doi.org/10.1056/NEJMoa1902328.
37. Corbin AS, et al. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J Clin Invest*. 2011;121:396–409. https://doi.org/10.1172/JCI35721.
38. Hamilton A, et al. Chronic myeloid leukemia stem cells are not dependent on Bcr-Abl kinase activity for their survival. *Blood*. 2012;119:1501–1510. https://doi.org/10.1182/blood-2010-12-326843.
39. Mahon FX, et al. Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. *Lancet Oncol*. 2010;11:1029–1035.
40. Hochhaus A, et al. European LeukemiaNet 2020 recommendations for treating chronic myeloid leukemia. *Leukemia*. 2020;34:966–984. https://doi.org/10.1038/s41375-020-0776-2.
41. Experts in Chronic Myeloid Leukemia The price of drugs for chronic myeloid leukemia (CML) is a reflection of the unsustainable prices of cancer drugs: from the perspective of a large group of CML experts. *Blood*. 2013;121:4439–4442. https://doi.org/10.1182/blood-2013-03-490003.
42. Ichimaru M, Tomonaga M, Amenomori T, Matsuo T. Atomic bomb and leukemia. *J Radiat Res*. 1991;32(Suppl_1):162–167. https://doi.org/10.1269/jrr.32.supplement_162.
43. Little MP, et al. Risks of leukemia in Japanese atomic bomb survivors, in women treated for cervical cancer, and in patients treated for ankylosing spondylitis. *Radiat Res*. 1999;152:280–292.
44. Van Kaick G, Wesch H, Luhrs H, Liebermann D, Kaul A. Neoplastic diseases induced by chronic alpha-irradiation—epidemiological, biophysical and clinical results of the German Thorotrast Study. *J Radiat Res. Tokyo*. 1991;32(Suppl 2):20–33.
45. Glass DC, Schnatter AR, Tang G, Irons RD, Rushton L. Risk of myeloproliferative disease and chronic myeloid leukaemia following exposure to low-level benzene in a nested case-control study of petroleum workers. *Occup Environ Med*. 2014;71:266–274. https://doi.org/10.1136/oemed-2013-101664.
46. Huang X, Cortes J, Kantarjian H. Estimations of the increasing prevalence and plateau prevalence of chronic myeloid leukemia in the era of tyrosine kinase inhibitor therapy. *Cancer*. 2012;118:3123–3127. https://doi.org/10.1002/cncr.26679.
47. Melo JV. The diversity of BCR-ABL fusion proteins and their relationship to leukemia phenotype. *Blood*. 1996;88:2375–2384.
48. Verma D, et al. Chronic myeloid leukemia (CML) with P190 BCR-ABL: analysis of characteristics, outcomes, and prognostic significance. *Blood*. 2009;114:2232–2235. https://doi.org/10.1182/blood-2009-02-204693.
49. Melo JV, Myint H, Galton DA, Goldman JM. P190BCR-ABL chronic myeloid leukaemia: the missing link with chronic myelomonocytic leukaemia? *Leukemia*. 1994;8:208–211.
50. Pane F, et al. Neutrophilic chronic myeloid leukemia: a distinct disease with a specific molecular marker (BCR/ABL with C3/A2 junction). *Blood*. 1996;88:2410–2414.
51. Hochhaus A, et al. A novel BCR-ABL fusion gene (e6a2) in a patient with Philadelphia chromosome-negative chronic myelogenous leukemia. *Blood*. 1996;88:2236–2240.
52. Demehri S, et al. e8a2 BCR-ABL: more frequent than other atypical BCR-ABL variants? *Leukemia*. 2005;19:681–684.
53. Al Ali HK, et al. CML with an e1a3 BCR-ABL fusion: rare, benign, and a potential diagnostic pitfall. *Blood*. 2002;100:1092–1093.
54. Melo JV, Gordon DE, Cross NC, Goldman JM. The ABL-BCR fusion gene is expressed in chronic myeloid leukemia. *Blood*. 1993;81:158–165.
55. Huntly BJ, et al. Derivative chromosome 9 deletions in chronic myeloid leukemia: poor prognosis is not associated with loss of ABL-BCR expression, elevated BCR-ABL levels, or karyotypic instability. *Blood*. 2002;99:4547–4553.
56. Huntly BJ, et al. Imatinib improves but may not fully reverse the poor prognosis of patients with CML with derivative chromosome 9 deletions. *Blood*. 2003;102:2205–2212.

57. Quintas-Cardama A, et al. Imatinib mesylate therapy may overcome the poor prognostic significance of deletions of derivative chromosome 9 in patients with chronic myelogenous leukemia. *Blood*. 2005;105:2281–2286.
58. Maru Y, Witte ON. The BCR gene encodes a novel serine/threonine kinase activity within a single exon. *Cell*. 1991;67:459–468.
59. Harnois T, et al. Differential interaction and activation of Rho family GTPases by p210bcr-abl and p190bcr-abl. *Oncogene*. 2003;22:6445–6454.
60. Reckel S, et al. Differential signaling networks of Bcr-Abl p210 and p190 kinases in leukemia cells defined by functional proteomics. *Leukemia*. 2017 https://doi.org/10.1038/leu.2017.36.
61. Reckel S, et al. Structural and functional dissection of the DH and PH domains of oncogenic Bcr-Abl tyrosine kinase. *Nature Commun*. 2017;8:2101. https://doi.org/10.1038/s41467-017-02313-6.
62. Diekmann D, et al. Bcr encodes a GTPase-activating protein for p21rac. *Nature*. 1991;351:400–402.
63. Diekmann D, Nobes CD, Burbelo PD, Abo A, Hall A. Rac GTPase interacts with GAPs and target proteins through multiple effector sites. *EMBO J*. 1995;14:5297–5305.
64. Voncken JW, et al. Increased neutrophil respiratory burst in bcr-null mutants. *Cell*. 1995;80:719–728.
65. Liu J, et al. BCR-ABL tyrosine kinase is autophosphorylated or transphosphorylates P160 BCR on tyrosine predominantly within the first BCR exon. *Oncogene*. 1993;8:101–109.
66. Liu J, et al. Inhibition of Bcr serine kinase by tyrosine phosphorylation. *Mol Cell Bio*. 1996;16:998–1005.
67. Ma G, Lu D, Wu Y, Liu J, Arlinghaus RB. Bcr phosphorylated on tyrosine 177 binds Grb2. *Oncogene*. 1997;14:2367–2372.
68. Kin Y, Li G, Shibuya M, Maru Y. The Dbl homology domain of BCR is not a simple spacer in P210BCR-ABL of the Philadelphia chromosome. *J Biol Chem*. 2001;276:39462–39468.
69. Demehri S, et al. The function of the pleckstrin homology domain in BCR-ABL-mediated leukemogenesis. *Leukemia*. 2010;24:226–229. https://doi.org/10.1038/leu.2009.196.
70. Castor A, et al. Distinct patterns of hematopoietic stem cell involvement in acute lymphoblastic leukemia. *Nat Med*. 2005;11:630–637.
71. Rabstein LS, Gazdar AF, Chopra HC, Abelson HT. Early morphological changes associated with infection by a murine nonthymic lymphatic tumor virus. *J Natl Cancer Inst*. 1971;46:481–491.
72. Konopka JB, Watanabe SM, Witte ON. An alteration of the human c-abl protein in K562 leukemia cells unmasks associated tyrosine kinase activity. *Cell*. 1984;37:1035–1042.
73. Bernards A, Rubin CM, Westbrook CA, Paskind M, Baltimore D. The first intron in the human c-abl gene is at least 200 kilobases long and is a target for translocations in chronic myelogenous leukemia. *Mol Cell Bio*. 1987;7:3231–3236.
74. Laneuville P. Abl tyrosine protein kinase. *Semin. Immunol*. 1995;7:255–266.
75. Feller SM, Ren R, Hanafusa H, Baltimore D. SH2 and SH3 domains as molecular adhesives: the interactions of Crk and Abl. *Trends Biochem Sci*. 1994;19:453–458.
76. Feller SM, Knudsen B, Hanafusa H. c-Abl kinase regulates the protein binding activity of c-Crk. *EMBO J*. 1994;13:2341–2351.
77. Kipreos ET, Wang JY. Cell cycle-regulated binding of c-Abl tyrosine kinase to DNA. *Science*. 1992;256:382–385.
78. McWhirter JR, Wang JY. An actin-binding function contributes to transformation by the Bcr-Abl oncoprotein of Philadelphia chromosome-positive human leukemias. *EMBO J*. 1993;12:1533–1546.
79. Kipreos ET, Wang JY. Differential phosphorylation of c-Abl in cell cycle determined by cdc2 kinase and phosphatase activity. *Science*. 1990;248:217–220.
80. Sawyers CL, McLaughlin J, Goga A, Havlik M, Witte O. The nuclear tyrosine kinase c-Abl negatively regulates cell growth. *Cell*. 1994;77:121–131.
81. Lewis JM, Baskaran R, Taagepera S, Schwartz MA, Wang JY. Integrin regulation of c-Abl tyrosine kinase activity and cytoplasmic-nuclear transport. *Proc Natl Acad Sci USA*. 1996;93:15174–15179.
82. Baskaran R, et al. Ataxia telangiectasia mutant protein activates c-Abl tyrosine kinase in response to ionizing radiation. *Nature*. 1997;387:516–519.
83. Yuan ZM, et al. Inhibition of phosphatidylinositol 3-kinase by c-Abl in the genotoxic stress response. *J Biol Chem*. 1997;272:23485–23488.
84. Kharbanda S, et al. Functional interaction between DNA-PK and c-Abl in response to DNA damage. *Nature*. 1997;386:732–735.
85. Yuan ZM, et al. Regulation of Rad51 function by c-Abl in response to DNA damage. *J Biol Chem*. 1998;273:3799–3802.
86. Yuan ZM, et al. Activation of protein kinase C delta by the c-Abl tyrosine kinase in response to ionizing radiation. *Oncogene*. 1998;16:1643–1648.
87. Schwartzberg PL, et al. Mice homozygous for the ablm1 mutation show poor viability and depletion of selected B and T cell populations. *Cell*. 1991;65:1165–1175.
88. Tybulewicz VL, Crawford CE, Jackson PK, Bronson RT, Mulligan RC. Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. *Cell*. 1991;65:1153–1163.
89. Koleske AJ, et al. Essential roles for the Abl and Arg tyrosine kinases in neurulation. *Neuron*. 1998;21:1259–1272.
90. Wang X, et al. Germline mutations in ABL1 cause an autosomal dominant syndrome characterized by congenital heart defects and skeletal malformations. *Nature Genet*. 2017;49:613–617. https://doi.org/10.1038/ng.3815.
91. Zhao X, Ghaffari S, Lodish H, Malashkevich VN, Kim PS. Structure of the Bcr-Abl oncoprotein oligomerization domain. *Nat Struct. Biol*. 2002;9:117–120.
92. Smith KM, Yacobi R, Van Etten RA. Autoinhibition of Bcr-Abl through its SH3 domain. *Mol Cell*. 2003;12:27–37.
93. Golub TR, et al. Oligomerization of the ABL tyrosine kinase by the Ets protein TEL in human leukemia. *Mol Cell Biol*. 1996;16:4107–4116. https://doi.org/10.1128/mcb.16.8.4107.
94. Graux C, et al. Fusion of NUP214 to ABL1 on amplified episomes in T-cell acute lymphoblastic leukemia. *Nat Genet*. 2004;36:1084–1089. https://doi.org/10.1038/ng1425.
95. Pluk H, Dorey K, Superti-Furga G. Autoinhibition of c-Abl. *Cell*. 2002;108:247–259.
96. Adrian FJ, et al. Allosteric inhibitors of Bcr-abl-dependent cell proliferation. *Nat Chem Biol*. 2006;2:95–102. https://doi.org/10.1038/nchembio760.
97. Eide CA, et al. Combining the allosteric inhibitor asciminib with ponatinib suppresses emergence of and restores efficacy against highly resistant BCR-ABL1 mutants. *Cancer Cell*. 2019;36:431–443. https://doi.org/10.1016/j.ccell.2019.08.004. e435.
98. Grebien F, et al. Targeting the SH2-kinase interface in Bcr-Abl inhibits leukemogenesis. *Cell*. 2011;147:306–319. https://doi.org/10.1016/j.cell.2011.08.046.
99. Dai Z, Pendergast AM. Abi-2, a novel SH3-containing protein interacts with the c-Abl tyrosine kinase and modulates c-Abl transforming activity. *Genes Dev*. 1995;9:2569–2582.
100. Dai Z, et al. Oncogenic Abl and Src tyrosine kinases elicit the ubiquitin-dependent degradation of target proteins through a Ras- independent pathway. *Genes Dev*. 1998;12:1415–1424.
101. McWhirter JR, Galasso DL, Wang JY. A coiled-coil oligomerization domain of Bcr is essential for the transforming function of Bcr-Abl oncoproteins. *Mol Cell Biol*. 1993;13:7587–7595.
102. Skorski T, et al. The SH3 domain contributes to BCR/ABL-dependent leukemogenesis in vivo: role in adhesion, invasion, and homing. *Blood*. 1998;91:406–418.
103. Pendergast AM, Muller AJ, Havlik MH, Maru Y, Witte ON. BCR sequences essential for transformation by the BCR-ABL oncogene bind to the ABL SH2 regulatory domain in a non-phosphotyrosine-dependent manner. *Cell*. 1991;66:161–171. https://doi.org/10.1016/0092-8674(91)90148-r.
104. Chu S, Li L, Singh H, Bhatia R. BCR-tyrosine 177 plays an essential role in Ras and Akt activation and in human hematopoietic progenitor transformation in chronic myelogenous leukemia. *Cancer Res*. 2007;67:7045–7053.
105. Sattler M, et al. Critical role for Gab2 in transformation by BCR/ABL. *Cancer Cell*. 2002;1:479–492.
106. Gaston I, et al. Coexistence of phosphotyrosine-dependent and -independent interactions between Cbl and Bcr-Abl. *Exp Hematol*. 2004;32:113–121.
107. Komatsu N, et al. A member of Forkhead transcription factor FKHRL1 is a downstream effector of STI571-induced cell cycle arrest in BCR-ABL-expressing cells. *J Biol Chem*. 2003;278:6411–6419.
108. Atfi A, Abecassis L, Bourgeade MF. Bcr-Abl activates the AKT/Fox O_3 signalling pathway to restrict transforming growth factor-beta-mediated cytostatic signals. *EMBO Reports*. 2005;6:985–991. https://doi.org/10.1038/sj.embor.7400501.
109. Neshat MS, Raitano AB, Wang HG, Reed JC, Sawyers CL. The survival function of the Bcr-Abl oncogene is mediated by Bad- dependent and -independent pathways: roles for phosphatidylinositol 3- kinase and Raf. *Mol Cell Biol*. 2000;20:1179–1186.
110. Markova, B. et al. Novel pathway in Bcr-Abl signal transduction involves Akt-independent, PLC-gamma1-driven activation of mTOR/p70S6-kinase pathway. *Oncogene* 29, 739-751.
111. Ly C, Arechiga AF, Melo JV, Walsh CM, Ong ST. Bcr-Abl kinase modulates the translation regulators ribosomal protein S6 and 4E-BP1 in chronic myelogenous leukemia cells via the mammalian target of rapamycin. *Cancer Res*. 2003;63:5716–5722.
112. Agarwal A, et al. Absence of SKP2 expression attenuates BCR-ABL-induced myeloproliferative disease. *Blood*. 2008;112:1960–1970.

113. Kardinal C, et al. Chronic myelogenous leukemia blast cell proliferation is inhibited by peptides that disrupt Grb2-SoS complexes. *Blood*. 2001;98:1773–1781.
114. Marais R, Light Y, Paterson HF, Marshall CJ. Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *EMBO J*. 1995;14:3136–3145.
115. Salomoni P, et al. Expression of constitutively active Raf-1 in the mitochondria restores antiapoptotic and leukemogenic potential of a transformation-deficient BCR/ABL mutant. *J Exp Med*. 1998;187:1995–2007.
116. Bigenzahn JW, et al. LZTR1 is a regulator of RAS ubiquitination and signaling. *Science*. 2018;362:1171–1177. https://doi.org/10.1126/science.aap8210.
117. Thomas EK, Cancelas JA, Zheng Y, Williams DA. Rac GTPases as key regulators of p210-BCR-ABL-dependent leukemogenesis. *Leukemia*. 2008;22:898–904.
118. Thomas EK, et al. Rac guanosine triphosphatases represent integrating molecular therapeutic targets for BCR-ABL-induced myeloproliferative disease. *Cancer Cell*. 2007;12:467–478.
119. Sexl V, et al. Stat5a/b contribute to interleukin 7-induced B-cell precursor expansion, but abl- and bcr/abl-induced transformation are independent of stat5. *Blood*. 2000;96:2277–2283.
120. Walz C, et al. Essential role for Stat5a/b in myeloproliferative neoplasms induced by BCR-ABL1 and JAK2(V617F) in mice. *Blood*. 2012;119:3550–3560. https://doi.org/10.1182/blood-2011-12-397554.
121. Hoelbl, A. et al. Stat5 is indispensable for the maintenance of bcr/abl-positive leukaemia. *EMBO Mol Med*. 2, 98–110.
122. Ye D, Wolff N, Li L, Zhang S, Ilaria Jr. RL. STAT5 signaling is required for the efficient induction and maintenance of CML in mice. *Blood*. 2006;107:4917–4925.
123. Klejman A, et al. The Src family kinase Hck couples BCR/ABL to STAT5 activation in myeloid leukemia cells. *EMBO J*. 2002;21:5766–5774.
124. Frank DA, Varticovski L. BCR/abl leads to the constitutive activation of Stat proteins, and shares an epitope with tyrosine phosphorylated Stats. *Leukemia*. 1996;10:1724–1730.
125. Ilaria Jr. RL, Van Etten RA. P210 and P190(BCR/ABL) induce the tyrosine phosphorylation and DNA binding activity of multiple specific STAT family members. *J Biol Chem*. 1996;271:31704–31710.
126. Samanta AK, Lin H, Sun T, Kantarjian H, Arlinghaus RB. Janus kinase 2: a critical target in chronic myelogenous leukemia. *Cancer Res*. 2006;66:6468–6472.
127. Hantschel O, et al. BCR-ABL uncouples canonical JAK2-STAT5 signaling in chronic myeloid leukemia. *Nat Chem Biol*. 2012;8:285–293. https://doi.org/10.1038/nchembio.775.
128. Lai D, et al. PP2A inhibition sensitizes cancer stem cells to ABL tyrosine kinase inhibitors in BCR-ABL(+) human leukemia. *Sci Transl Med*. 2018;10 https://doi.org/10.1126/scitranslmed.aan8735. eaan8735.
129. Prost S, et al. Erosion of the chronic myeloid leukaemia stem cell pool by PPARγ agonists. *Nature*. 2015;525:380–383. https://doi.org/10.1038/nature15248.
130. Gotoh A, et al. Tyrosine phosphorylation and activation of focal adhesion kinase (p125FAK) by BCR-ABL oncoprotein. *Exp Hematol*. 1995;23:1153–1159.
131. Salgia R, et al. Molecular cloning of human paxillin, a focal adhesion protein phosphorylated by P210BCR/ABL. *J Biol Chem*. 1995;270:5039–5047.
132. Salgia R, et al. p130CAS forms a signaling complex with the adapter protein CRKL in hematopoietic cells transformed by the BCR/ABL oncogene. *J Biol Chem*. 1996;271:25198–25203.
133. Sattler M, et al. Differential signaling after beta1 integrin ligation is mediated through binding of CRKL to p120(CBL) and p110(HEF1). *J Biol Chem*. 1997;272:14320–14326.
134. Uemura N, Griffin JD. The adapter protein Crkl links Cbl to C3G after integrin ligation and enhances cell migration. *J Biol Chem*. 1999;274:37525–37532.
135. Oda T, et al. Crkl is the major tyrosine-phosphorylated protein in neutrophils from patients with chronic myelogenous leukemia. *J Biol Chem*. 1994;269:22925–22928.
136. Verfaillie CM, et al. Pathophysiology of CML: do defects in integrin function contribute to the premature circulation and massive expansion of the BCR/ABL positive clone? *J Lab Clin Med*. 1997;129:584–591.
137. Ramaraj P, et al. Effect of mutational inactivation of tyrosine kinase activity on BCR/ABL-induced abnormalities in cell growth and adhesion in human hematopoietic progenitors. *Cancer Res*. 2004;64:5322–5331.
138. Cramer K, et al. BCR/ABL and other kinases from chronic myeloproliferative disorders stimulate single-strand annealing, an unfaithful DNA double-strand break repair. *Cancer Res*. 2008;68:6884–6888. https://doi.org/10.1158/0008-5472.CAN-08-1101.
139. Nowicki MO, et al. BCR/ABL oncogenic kinase promotes unfaithful repair of the reactive oxygen species-dependent DNA double-strand breaks. *Blood*. 2004;104:3746–3753.
140. Koptyra M, et al. BCR/ABL kinase induces self-mutagenesis via reactive oxygen species to encode imatinib resistance. *Blood*. 2006;108:319–327.
141. Perrotti D, Jamieson C, Goldman J, Skorski T. Chronic myeloid leukemia: mechanisms of blastic transformation. *J Clin Invest*. 2010;120:2254–2264. https://doi.org/10.1172/JCI41246.
142. Sattler M, et al. The BCR/ABL tyrosine kinase induces production of reactive oxygen species in hematopoietic cells. *J Biol Chem*. 2000;275:24273–24278.
143. Bolton-Gillespie E, et al. Genomic instability may originate from imatinib-refractory chronic myeloid leukemia stem cells. *Blood*. 2013;121:4175–4183. https://doi.org/10.1182/blood-2012-11-466938.
144. Feldhahn N, et al. Activation-induced cytidine deaminase acts as a mutator in BCR-ABL1-transformed acute lymphoblastic leukemia cells. *J Exp Med*. 2007;204:1157–1166. https://doi.org/10.1084/jem.20062662.
145. Thomson DW, et al. Aberrant RAG-mediated recombination contributes to multiple structural rearrangements in lymphoid blast crisis of chronic myeloid leukemia. *Leukemia*. 2020;34:2051–2063. https://doi.org/10.1038/s41375-020-0751-y.
146. Melo JV, Barnes DJ. Chronic myeloid leukaemia as a model of disease evolution in human cancer. *Nature Rev*. 2007;7:441–453.
147. Risch HA, et al. Population BRCA1 and BRCA2 mutation frequencies and cancer penetrances: a kin-cohort study in Ontario, Canada. *J Natl Cancer Inst*. 2006;98:1694–1706.
148. Deutsch E, et al. BCR-ABL down-regulates the DNA repair protein DNA-PKcs. *Blood*. 2001;97:2084–2090.
149. Graham SM, Vass JK, Holyoake TL, Graham GJ. Transcriptional analysis of quiescent and proliferating CD34+ human hemopoietic cells from normal and chronic myeloid leukemia sources. *Stem Cells*. 2007;25:3111–3120. https://doi.org/10.1634/stemcells.2007-0250.
150. Agarwal A, et al. BCR-ABL1 promotes leukemia by converting p27 into a cytoplasmic oncoprotein. *Blood*. 2014;124:3260–3273. https://doi.org/10.1182/blood-2013-04-497040.
151. Fabarius A, et al. Induction of centrosome and chromosome aberrations by imatinib in vitro. *Leukemia*. 2005;19:1573–1578.
152. Gambacorti-Passerini C, et al. Multicenter independent assessment of outcomes in chronic myeloid leukemia patients treated with imatinib. *J Natl Cancer Inst*. 2011;103:553–561. https://doi.org/10.1093/jnci/djr060.
153. Gunnarsson N, et al. Second malignancies following treatment of chronic myeloid leukaemia in the tyrosine kinase inhibitor era. *Br J Haematol*. 2015;169:683–688. https://doi.org/10.1111/bjh.13346.
154. Bumm T, et al. Emergence of clonal cytogenetic abnormalities in Ph-cells in some CML patients in cytogenetic remission to imatinib but restoration of polyclonal hematopoiesis in the majority. *Blood*. 2003;101:1941–1949.
155. Brehme M, et al. Charting the molecular network of the drug target Bcr-Abl. *Proc Natl Acad Sci U S A*. 2009;106:7414–7419.
156. Huntly BJ, et al. MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. *Cancer Cell*. 2004;6:587–596.
157. Fabarius A, et al. Impact of additional cytogenetic aberrations at diagnosis on prognosis of CML: long-term observation of 1151 patients from the randomized CML Study IV. *Blood*. 2011;118:6760–6768. https://doi.org/10.1182/blood-2011-08-373902.
158. Schmidt M, et al. Molecular-defined clonal evolution in patients with chronic myeloid leukemia independent of the BCR-ABL status. *Leukemia*. 2014;28:2292–2299. https://doi.org/10.1038/leu.2014.272.
159. Branford S, et al. Integrative genomic analysis reveals cancer-associated mutations at diagnosis of CML in patients with high-risk disease. *Blood*. 2018;132:948–961. https://doi.org/10.1182/blood-2018-02-832253.
160. Nteliopoulos G, et al. Somatic variants in epigenetic modifiers can predict failure of response to imatinib but not to second-generation tyrosine kinase inhibitors. *Haematologica*. 2019;104:2400–2409. https://doi.org/10.3324/haematol.2018.200220.
161. Deininger MWN, Tyner JW, Solary E. Turning the tide in myelodysplastic/myeloproliferative neoplasms. *Nature Rev*. 2017;17:425–440. https://doi.org/10.1038/nrc.2017.40.
162. Srour SA, et al. Incidence and patient survival of myeloproliferative neoplasms and myelodysplastic/myeloproliferative neoplasms in the United States, 2001–12. *Br J Haematol*. 2016;174:382–396. https://doi.org/10.1111/bjh.14061.
163. Hehlmann R, et al. High-risk additional chromosomal abnormalities at low blast counts herald death by CML. *Leukemia*. 2020;34:2074–2086. https://doi.org/10.1038/s41375-020-0826-9.
164. Takahashi N, Miura I, Saitoh K, Miura AB. Lineage involvement of stem cells bearing the Philadelphia chromosome in chronic myeloid leukemia in

the chronic phase as shown by a combination of fluorescence-activated cell sorting and fluorescence in situ hybridization. *Blood*. 1998;92:4758–4763.

165. Petzer AL, et al. Characterization of primitive subpopulation of normal and leukemic cells present in the blood of patients with newly diagnosed as well as established chronic myeloid leukemia. *Blood*. 1996;88:2162–2171.
166. Chan LN, et al. Metabolic gatekeeper function of B-lymphoid transcription factors. *Nature*. 2017;542:479–483. https://doi.org/10.1038/nature21076.
167. Warfvinge R, et al. Single-cell molecular analysis defines therapy response and immunophenotype of stem cell subpopulations in CML. *Blood*. 2017;129:2384–2394. https://doi.org/10.1182/blood-2016-07-728873.
168. Jiang Y, Zhao RC, Verfaillie CM. Abnormal integrin-mediated regulation of chronic myelogenous leukemia CD34+ cell proliferation: BCR/ABL up-regulates the cyclin-dependent kinase inhibitor, p27Kip, which is relocated to the cell cytoplasm and incapable of regulating cdk2 activity. *Proc Natl Acad Sci U S A*. 2000;97:10538–10543. https://doi.org/10.1073/pnas.190104497.
169. Peled A, et al. Immature leukemic CD34+CXCR4+ cells from CML patients have lower integrin-dependent migration and adhesion in response to the chemokine SDF-1. *Stem Cells*. 2002;20:259–266. https://doi.org/10.1634/stemcells.20-3-259.
170. Schemionek M, et al. BCR-ABL enhances differentiation of long-term repopulating hematopoietic stem cells. *Blood*. 2010;115:3185–3195. https://doi.org/10.1182/blood-2009-04-215376.
171. Pellicano F, et al. BMS-214662 induces mitochondrial apoptosis in chronic myeloid leukemia (CML) stem/progenitor cells, including CD34+38− cells, through activation of protein kinase Cbeta. *Blood*. 2009;114:4186–4196. https://doi.org/10.1182/blood-2009-05-219550.
172. Johansson B, Fioretos T, Mitelman F. Cytogenetic and molecular genetic evolution of chronic myeloid leukemia. *Acta Haematol*. 2002;107:76–94.
173. Mullighan CG, Williams RT, Downing JR, Sherr CJ. Failure of CDKN2A/B (INK4A/B-ARF)-mediated tumor suppression and resistance to targeted therapy in acute lymphoblastic leukemia induced by BCR-ABL. *Genes Dev*. 2008;22:1411–1415. https://doi.org/10.1101/gad.1673908.
174. Ko TK, et al. An integrative model of pathway convergence in genetically heterogeneous blast crisis chronic myeloid leukemia. *Blood*. 2020;135:2337–2353. https://doi.org/10.1182/blood.2020004834.
175. Elmaagacli AH, Beelen DW, Opalka B, Seeber S, Schaefer UW. The amount of BCR-ABL fusion transcripts detected by the real-time quantitative polymerase chain reaction method in patients with Philadelphia chromosome positive chronic myeloid leukemia correlates with the disease stage. *Ann Hematol*. 2000;79:424–431.
176. Neviani P, et al. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. *Cancer Cell*. 2005;8:355–368. https://doi.org/10.1016/j.ccr.2005.10.015.
177. Esposito N, et al. SHP-1 expression accounts for resistance to imatinib treatment in Philadelphia chromosome-positive cells derived from patients with chronic myeloid leukemia. *Blood*. 2011;118:3634–3644. https://doi.org/10.1182/blood-2011-03-341073.
178. Lucas CM, et al. Cancerous inhibitor of PP2A (CIP2A) at diagnosis of chronic myeloid leukemia is a critical determinant of disease progression. *Blood*. 2011;117:6660–6668. https://doi.org/10.1182/blood-2010-08-304477.
179. Wu Y, Slovak ML, Snyder DS, Arber DA. Coexistence of inversion 16 and the Philadelphia chromosome in acute and chronic myeloid leukemias: report of six cases and review of literature. *Am J Clin Pathol*. 2006;125:260–266.
180. Gerritsen M, et al. RUNX1 mutations enhance self-renewal and block granulocytic differentiation in human in vitro models and primary AMLs. *Blood Adv*. 2019;3:320–332. https://doi.org/10.1182/bloodadvances.2018024422.
181. Perrotti D, et al. BCR-ABL suppresses C/EBPalpha expression through inhibitory action of hnRNP E2. *Nat Genet*. 2002;30:48–58.
182. Eiring AM, et al. miR-328 functions as an RNA decoy to modulate hnRNP E2 regulation of mRNA translation in leukemic blasts. *Cell*. 2010;140:652–665. https://doi.org/10.1016/j.cell.2010.01.007.
183. Guerzoni C, et al. Inducible activation of CEBPB, a gene negatively regulated by BCR/ABL, inhibits proliferation and promotes differentiation of BCR/ABL-expressing cells. *Blood*. 2006;107:4080–4089.
184. Cuenco GM, Ren R. Both AML1 and EVI1 oncogenic components are required for the cooperation of AML1/MDS1/EVI1 with BCR/ABL in the induction of acute myelogenous leukemia in mice. *Oncogene*. 2004;23:569–579.
185. De Weer A, et al. EVI1 activation in blast crisis CML due to juxtaposition to the rare 17q22 partner region as part of a 4-way variant translocation t(9;22). *BMC Cancer*. 2008;8:193. https://doi.org/10.1186/1471-2407-8-193.
186. Jamieson CHM, et al. Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N Engl J Med*. 2004;351:657–667.
187. Coluccia AM, et al. Bcr-Abl stabilizes beta-catenin in chronic myeloid leukemia through its tyrosine phosphorylation. *EMBO J*. 2007;26:1456–1466.
188. Abrahamsson AE, et al. Glycogen synthase kinase 3beta missplicing contributes to leukemia stem cell generation. *Proc Natl Acad Sci U S A*. 2009;106:3925–3929.
189. Lim S, et al. Targeting of the MNK-eIF4E axis in blast crisis chronic myeloid leukemia inhibits leukemia stem cell function. *Proc Natl Acad Sci U S A*. 2013;110:E2298–E2307. https://doi.org/10.1073/pnas.1301838110.
190. Parker WT, et al. BCR-ABL1 kinase domain mutations may persist at very low levels for many years and lead to subsequent TKI resistance. *Br J Cancer*. 2013;109:1593–1598. https://doi.org/10.1038/bjc.2013.318.
191. Shah NP, et al. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer Cell*. 2002;2:117–125. https://doi.org/10.1016/s1535-6108(02)00096-x.
192. O'Hare T, Zabriskie MS, Eiring AM, Deininger MW. Pushing the limits of targeted therapy in chronic myeloid leukaemia. *Nature Rev*. 2012;12:513–526. https://doi.org/10.1038/nrc3317.
193. Eiring AM, Deininger MW. Individualizing kinase-targeted cancer therapy: the paradigm of chronic myeloid leukemia. *Genome Biol*. 2014;15:461. https://doi.org/10.1186/s13059-014-0461-8.
194. O'Hare T, et al. AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. *Cancer Cell*. 2009;16:401–412. https://doi.org/10.1016/j.ccr.2009.09.028.
195. Soverini S, et al. Contribution of ABL kinase domain mutations to imatinib resistance in different subsets of Philadelphia-positive patients: by the GIMEMA Working Party on Chronic Myeloid Leukemia. *Clin Cancer Res*. 2006;12:7374–7379. https://doi.org/10.1158/1078-0432.ccr-06-1516.
196. Mahon FX, et al. Selection and characterization of BCR-ABL positive cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanisms of resistance. *Blood*. 2000;96:1070–1079.
197. Dulucq S, et al. Multidrug resistance gene (MDR1) polymorphisms are associated with major molecular responses to standard-dose imatinib in chronic myeloid leukemia. *Blood*. 2008;112:2024–2027. https://doi.org/10.1182/blood-2008-03-147744.
198. Eadie LN, Hughes TP, White DL. ABCB1 overexpression is a key initiator of resistance to tyrosine kinase inhibitors in CML cell lines. *PloS One*. 2016;11:e0161470. https://doi.org/10.1371/journal.pone.0161470.
199. Sawyers CL. Chronic myeloid leukemia. *N Engl J Med*. 1999;340:1330–1340.
200. Sokal JE, et al. Prognostic discrimination in "good-risk" chronic granulocytic leukemia. *Blood*. 1984;63:789–799.
201. Hasford J, et al. A new prognostic score for survival of patients with chronic myeloid leukemia treated with interferon alfa. Writing Committee for the Collaborative CML Prognostic Factors Project Group. *J Natl Cancer Inst*. 1998;90:850–858.
202. Hasford J, et al. Predicting complete cytogenetic response and subsequent progression-free survival in 2060 patients with CML on imatinib treatment: the EUTOS score. *Blood*. 2011;118:686–692. https://doi.org/10.1182/blood-2010-12-319038.
203. Luatti S, et al. Additional chromosomal abnormalities in Philadelphia-positive clone: adverse prognostic influence on frontline imatinib therapy: a GIMEMA Working Party on CML analysis. *Blood*. 2012;120:761–767. https://doi.org/10.1182/blood-2011-10-384651.
204. Alhuraiji A, et al. Prognostic significance of additional chromosomal abnormalities at the time of diagnosis in patients with chronic myeloid leukemia treated with frontline tyrosine kinase inhibitors. *Am J Hematol*. 2018;93:84–90. https://doi.org/10.1002/ajh.24943.
205. Sinclair PB, Green AR, Grace C, Nacheva EP. Improved sensitivity of BCR-ABL detection: a triple-probe three-color fluorescence in situ hybridization system. *Blood*. 1997;90:1395–1402.
206. Sinclair PB, et al. Large deletions at the t(9;22) breakpoint are common and may identify a poor-prognosis subgroup of patients with chronic myeloid leukemia. *Blood*. 2000;95:738–743.
207. Testoni N, et al. Chronic myeloid leukemia: a prospective comparison of interphase fluorescence in situ hybridization and chromosome banding analysis for the definition of complete cytogenetic response: a study of the GIMEMA CML WP. *Blood*. 2009;114:4939–4943. https://doi.org/10.1182/blood-2009-07-229864.
208. Cross NC, Melo JV, Feng L, Goldman JM. An optimized multiplex polymerase chain reaction (PCR) for detection of BCR-ABL fusion mRNAs in haematological disorders. *Leukemia*. 1994;8:186–189.
209. Kantarjian HM, Talpaz M. Definition of the accelerated phase of chronic myelogenous leukemia. *J Clin Oncol*. 1988;6:180–182.

210. Baccarani M, et al. Chronic myeloid leukemia: an update of concepts and management recommendations of European LeukemiaNet. *J Clin Oncol*. 2009;27:6041–6051.
211. Radich JP, et al. Gene expression changes associated with progression and response in chronic myeloid leukemia. *Proc Natl Acad Sci U S A*. 2006;103:2794–2799.
212. Lauseker M, et al. Prognosis of patients with chronic myeloid leukemia presenting in advanced phase is defined mainly by blast count, but also by age, chromosomal aberrations and hemoglobin. *Am J Hematol*. 2019;94:1236–1243. https://doi.org/10.1002/ajh.25628.
213. Mitchell B, Deininger M. Techniques for risk stratification of newly diagnosed patients with chronic myeloid leukemia. *Leuk Lymphoma*. 2011;52(Suppl 1):4–11. https://doi.org/10.3109/10428194.2010.546916.
214. Thiele J, et al. Bone marrow features and clinical findings in chronic myeloid leukemia—a comparative, multicenter, immunohistological and morphometric study on 614 patients. *Leuk Lymphoma*. 2000;36:295–308.
215. Hehlmann R. How I treat CML blast crisis. *Blood*. 2012;120:737–747. https://doi.org/10.1182/blood-2012-03-380147.
216. Bonifazi F, et al. Chronic myeloid leukemia and interferon-alpha: a study of complete cytogenetic responders. *Blood*. 2001;98:3074–3081.
217. Druker BJ, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med*. 2006;355:2408–2417.
218. Jabbour E, et al. EUTOS score is not predictive for survival and outcome in patients with early chronic phase chronic myeloid leukemia treated with tyrosine kinase inhibitors: a single institution experience. *Blood*. 2012;119:4524–4526. https://doi.org/10.1182/blood-2011-10-388967.
219. Pfirrmann M, et al. Prognosis of long-term survival considering disease-specific death in patients with chronic myeloid leukemia. *Leukemia*. 2016;30:48–56. https://doi.org/10.1038/leu.2015.261.
220. Arber DA, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127:2391–2405. https://doi.org/10.1182/blood-2016-03-643544.
221. Hughes T, et al. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. *Blood*. 2006;108:28–37.
222. Press RD, Willis SG, Laudadio J, Mauro MJ, Deininger MW. Determining the rise in BCR-ABL RNA that optimally predicts a kinase domain mutation in patients with chronic myeloid leukemia on imatinib. *Blood*. 2009;114:2598–2605. https://doi.org/10.1182/blood-2008-08-173674.
223. Branford S, et al. Real-time quantitative PCR analysis can be used as a primary screen to identify patients with CML treated with imatinib who have BCR-ABL kinase domain mutations. *Blood*. 2004;104:2926–2932.
224. Müller MC, et al. An international study to standardize the detection and quantitation of BCR-ABL transcripts from stabilized peripheral blood preparations by quantitative RT-PCR. *Haematologica*. 2007;92:970–973.
225. White HE, et al. Establishment of the first World Health Organization International Genetic Reference Panel for quantitation of BCR-ABL mRNA. *Blood*. 2010;116:e111–e117.
226. Branford S, et al. Desirable performance characteristics for BCR-ABL measurement on an international reporting scale to allow consistent interpretation of individual patient response and comparison of response rates between clinical trials. *Blood*. 2008;112:3330–3338.
227. Hughes TP, et al. Frequency of major molecular responses to imatinib or interferon alfa plus cytarabine in newly diagnosed chronic myeloid leukemia. *N Engl J Med*. 2003;349:1423–1432.
228. Ross DM, et al. Patients with chronic myeloid leukemia who maintain a complete molecular response after stopping imatinib treatment have evidence of persistent leukemia by DNA PCR. *Leukemia*. 2010;24:1719–1724. https://doi.org/10.1038/leu.2010.185.
229. Willis SG, et al. High-sensitivity detection of BCR-ABL kinase domain mutations in imatinib-naive patients: correlation with clonal cytogenetic evolution but not response to therapy. *Blood*. 2005;106:2128–2137.
230. Soverini S, et al. Prospective assessment of NGS-detectable mutations in CML patients with nonoptimal response: the NEXT-in-CML study. *Blood*. 2020;135:534–541. https://doi.org/10.1182/blood.2019002969.
231. Schmitt MW, et al. Detection of ultra-rare mutations by next-generation sequencing. *Proc Natl Acad Sci U S A*. 2012;109:14508–14513. https://doi.org/10.1073/pnas.1208715109.
232. Zabriskie MS, et al. BCR-ABL1 compound mutations combining key kinase domain positions confer clinical resistance to ponatinib in Ph chromosome-positive leukemia. *Cancer Cell*. 2014;26:428–442. https://doi.org/10.1016/j.ccr.2014.07.006.
233. Guilhot F, et al. Interferon alfa-2b combined with cytarabine versus interferon alone in chronic myelogenous leukemia. French Chronic Myeloid Leukemia Study Group. *N Engl J Med*. 1997;337:223–229.
234. Baccarani M, et al. A randomized study of interferon-alpha versus interferon-alpha and low-dose arabinosyl cytosine in chronic myeloid leukemia. *Blood*. 2002;99:1527–1535.
235. Lasica M, et al. The effect of tyrosine kinase inhibitor interruption and interferon use on pregnancy outcomes and long-term disease control in chronic myeloid leukemia. *Leuk Lymphoma*. 2019;60:1796–1802. https://doi.org/10.1080/10428194.2018.1551533.
236. Preudhomme C, et al. Imatinib plus peginterferon alfa-2a in chronic myeloid leukemia. *N Engl J Med*. 2010;363:2511–2521.
237. Burchert A, et al. Interferon alpha 2 maintenance therapy may enable high rates of treatment discontinuation in chronic myeloid leukemia. *Leukemia*. 2015;29:1331–1335. https://doi.org/10.1038/leu.2015.45.
238. Cortes JE, et al. Final analysis of the efficacy and safety of omacetaxine mepesuccinate in patients with chronic- or accelerated-phase chronic myeloid leukemia: Results with 24 months of follow-up. *Cancer*. 2015;121:1637–1644. https://doi.org/10.1002/cncr.29240.
239. Kantarjian H, et al. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. *N Engl J Med*. 2002;346:645–652.
240. Talpaz M, et al. Imatinib induces durable hematologic and cytogenetic responses in patients with accelerated phase chronic myeloid leukemia: results of a phase 2 study. *Blood*. 2002;99:1928–1937.
241. Sawyers CL, et al. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood*. 2002;99:3530–3539.
242. Ottmann OG, et al. A phase 2 study of imatinib in patients with relapsed or refractory Philadelphia chromosome-positive acute lymphoid leukemias. *Blood*. 2002;100:1965–1971.
243. O'Brien SG, Deininger MW. Imatinib in patients with newly diagnosed chronic-phase chronic myeloid leukemia. *Semin Hematol*. 2003;40:26–30.
244. Guilhot F, et al. High rates of durable response are achieved with imatinib after treatment with interferon alpha plus cytarabine: results from the International Randomized Study of Interferon and STI571 (IRIS) trial. *Haematologica*. 2009;94:1669–1675. https://doi.org/10.3324/haematol.2009.010629.
245. Hochhaus A, et al. Long-term outcomes of imatinib treatment for chronic myeloid leukemia. *N Engl J Med*. 2017;376:917–927. https://doi.org/10.1056/NEJMoa1609324.
246. Kantarjian H, et al. High-dose imatinib mesylate therapy in newly diagnosed Philadelphia chromosome-positive chronic phase chronic myeloid leukemia. *Blood*. 2004;103:2873–2878.
247. Cortes J, et al. Result of high-dose imatinib mesylate in patients with Philadelphia chromosome—positive chronic myeloid leukemia after failure of interferon-alpha. *Blood*. 2003;102:83–86.
248. Cortes JE, et al. Phase III, randomized, open-label study of daily imatinib mesylate 400 mg versus 800 mg in patients with newly diagnosed, previously untreated chronic myeloid leukemia in chronic phase using molecular end points: tyrosine kinase inhibitor optimization and selectivity study. *J Clin Oncol*. 2010;28:424–430. https://doi.org/10.1200/JCO.2009.25.3724.
249. Castagnetti F, et al. Results of high-dose imatinib mesylate in intermediate Sokal risk chronic myeloid leukemia patients in early chronic phase: a phase 2 trial of the GIMEMA CML Working Party. *Blood*. 2009;113:3428–3434. https://doi.org/10.1182/blood-2007-08-103499.
250. Hehlmann R, et al. Tolerability-adapted imatinib 800 mg/d versus 400 mg/d versus 400 mg/d plus interferon- α in newly diagnosed chronic myeloid leukemia. *J Clin Oncol*. 2011;29:1634–1642.
251. Hehlmann R, et al. Assessment of imatinib as first-line treatment of chronic myeloid leukemia: 10-year survival results of the randomized CML study IV and impact of non-CML determinants. *Leukemia*. 2017;31:2398–2406. https://doi.org/10.1038/leu.2017.253.
252. Hoffmann VS, et al. Systematic review and meta-analysis of standard-dose imatinib vs. high-dose imatinib and second generation tyrosine kinase inhibitors for chronic myeloid leukemia. *J Cancer Res Clin Oncol*. 2017;143:1311–1318. https://doi.org/10.1007/s00432-017-2385-7.
253. Hochhaus A, et al. Dasatinib induces notable hematologic and cytogenetic responses in chronic-phase chronic myeloid leukemia after failure of imatinib therapy. *Blood*. 2007;109:2303–2309. https://doi.org/10.1182/blood-2006-09-047266.
254. Cortes J, et al. Dasatinib induces complete hematologic and cytogenetic responses in patients with imatinib-resistant or -intolerant chronic myeloid leukemia in blast crisis. *Blood*. 2007;109:3207–3213. https://doi.org/10.1182/blood-2006-09-046888.
255. Guilhot F, et al. Dasatinib induces significant hematologic and cytogenetic responses in patients with imatinib-resistant or -intolerant chronic myeloid leukemia in accelerated phase. *Blood*. 2007;109:4143–4150.

256. Shah NP, et al. Intermittent target inhibition with dasatinib 100 mg once daily preserves efficacy and improves tolerability in imatinib-resistant and -intolerant chronic-phase chronic myeloid leukemia. *J Clin Oncol*. 2008;26:3204–3212.
257. Kantarjian H, et al. Phase 3 study of dasatinib 140 mg once daily versus 70 mg twice daily in patients with chronic myeloid leukemia in accelerated phase resistant or intolerant to imatinib: 15-month median follow-up. *Blood*. 2009;113:6322–6329. https://doi.org/10.1182/blood-2008-11-186817.
258. Snead JL, et al. Acute dasatinib exposure commits Bcr-Abl-dependent cells to apoptosis. *Blood*. 2009;114:3459–3463. https://doi.org/10.1182/blood-2007-10-113969.
259. Deininger MW, et al. Imatinib 800 mg daily induces deeper molecular responses than imatinib 400 mg daily: results of SWOG S0325, an intergroup randomized PHASE II trial in newly diagnosed chronic phase chronic myeloid leukaemia. *Br J Haematol*. 2014;164:223–232. https://doi.org/10.1111/bjh.12618.
260. Le CP, et al. Nilotinib (formerly AMN107), a highly selective BCR-ABL tyrosine kinase inhibitor, is active in patients with imatinib-resistant or -intolerant accelerated-phase chronic myelogenous leukemia. *Blood*. 2008;111:1834–1839.
261. Kantarjian HM, et al. Nilotinib (formerly AMN107), a highly selective BCR-ABL tyrosine kinase inhibitor, is effective in patients with Philadelphia chromosome-positive chronic myelogenous leukemia in chronic phase following imatinib resistance and intolerance. *Blood*. 2007;110:3540–3546.
262. Giles FJ, et al. Nilotinib is effective in imatinib-resistant or -intolerant patients with chronic myeloid leukemia in blastic phase. *Leukemia*. 2012;26:959–962. https://doi.org/10.1038/leu.2011.355.
263. Saglio G, et al. Nilotinib versus imatinib for newly diagnosed chronic myeloid leukemia. *N Engl J*. 2010;362:2251–2259.
264. Hochhaus A, et al. Long-term benefits and risks of frontline nilotinib vs imatinib for chronic myeloid leukemia in chronic phase: 5-year update of the randomized ENESTnd trial. *Leukemia*. 2016;30:1044–1054. https://doi.org/10.1038/leu.2016.5.
265. Khoury HJ, et al. Bosutinib is active in chronic phase chronic myeloid leukemia after imatinib and dasatinib and/or nilotinib therapy failure. *Blood*. 2012;119:3403–3412. https://doi.org/10.1182/blood-2011-11-390120.
266. Cortes JE, et al. Long-term bosutinib for chronic phase chronic myeloid leukemia after failure of imatinib plus dasatinib and/or nilotinib. *Am J Hematol*. 2016;91:1206–1214. https://doi.org/10.1002/ajh.24536.
267. Brummendorf TH, et al. Bosutinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukaemia: results from the 24-month follow-up of the BELA trial. *Br J Haematol*. 2015;168:69–81. https://doi.org/10.1111/bjh.13108.
268. Cortes JE, et al. Bosutinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukemia: results from the BELA trial. *J Clin Oncol*. 2012;30:3486–3492. https://doi.org/10.1200/JCO.2011.38.7522.
269. Cortes JE, et al. Bosutinib versus imatinib for newly diagnosed chronic myeloid leukemia: results from the randomized BFORE trial. *J Clin Oncol*. 2018;36:231–237. https://doi.org/10.1200/JCO.2017.74.7162.
270. O'Hare T, Eide CA, Deininger MW. Bcr-Abl kinase domain mutations, drug resistance, and the road to a cure for chronic myeloid leukemia. *Blood*. 2007;110:2242–2249.
271. Moslehi JJ, Deininger M. Tyrosine kinase inhibitor-associated cardiovascular toxicity in chronic myeloid leukemia. *J Clin Oncol*. 2015;33:4210–4218. https://doi.org/10.1200/JCO.2015.62.4718.
272. Cortes J, et al. A phase 1 trial of oral ponatinib (AP24534) in patients with refractory chronic myelogenous leukemia (CML) and other hematologic malignancies: emerging safety and clinical response findings. *ASH Annual Meeting Abstracts*. 2010;116:210.
273. Cortes JE, et al. A phase 2 trial of ponatinib in Philadelphia chromosome-positive leukemias. *N Engl J Med*. 2013;369:1783–1796. https://doi.org/10.1056/NEJMoa1306494.
274. Lipton JH, et al. Ponatinib versus imatinib for newly diagnosed chronic myeloid leukaemia: an international, randomised, open-label, phase 3 trial. *Lancet Oncol*. 2016;17:612–621. https://doi.org/10.1016/S1470-2045(16)00080-2.
275. Latifi Y, et al. Thrombotic microangiopathy as a cause of cardiovascular toxicity from the BCR-ABL1 tyrosine kinase inhibitor ponatinib. *Blood*. 2019;133:1597–1606. https://doi.org/10.1182/blood-2018-10-881557.
276. Cortes J, et al. Ponatinib dose-ranging study in chronic-phase chronic myeloid leukemia: a randomized, open-label phase 2 clinical trial. *Blood*. 2021;138(21):2042–2050.
277. Simonsson B, et al. Combination of pegylated IFN-alpha2b with imatinib increases molecular response rates in patients with low- or intermediate-risk chronic myeloid leukemia. *Blood*. 2011;118:3228–3235. https://doi.org/10.1182/blood-2011-02-336685.
278. Ruggiu M, et al. Azacytidine in combination with tyrosine kinase inhibitors induced durable responses in patients with advanced phase chronic myelogenous leukemia. *Leuk Lymphoma*. 2018;59:1659–1665. https://doi.org/10.1080/10428194.2017.1397666.
279. Kolb HJ, et al. Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. *Blood*. 1990;76:2462–2465.
280. Hehlmann R, et al. Drug treatment is superior to allografting as first-line therapy in chronic myeloid leukemia. *Blood*. 2007;109:4686–4692. https://doi.org/10.1182/blood-2006-11-055186.
281. Gratwohl A, et al. Risk assessment for patients with chronic myeloid leukaemia before allogeneic blood or marrow transplantation. Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation. *Lancet*. 1998;352:1087–1092.
282. Ma YR, et al. Transplantation from haploidentical donor is not inferior to that from identical sibling donor for patients with chronic myeloid leukemia in blast crisis or chronic phase from blast crisis. *Clin Transplant*. 2016;30:994–1001. https://doi.org/10.1111/ctr.12779.
283. Radich JP, et al. Chronic Myeloid Leukemia, Version 1.2019, NCCN Clinical Practice Guidelines in Oncology. *J Natl Compr Canc Netw*. 2018;16:1108–1135. https://doi.org/10.6004/jnccn.2018.0071.
284. Deininger M, et al. The effect of prior exposure to imatinib on transplant-related mortality. *Haematologica*. 2006;91:452–459.
285. Oehler VG, et al. The effects of imatinib mesylate treatment before allogeneic transplantation for chronic myeloid leukemia. *Blood*. 2007;109:1782–1789. https://doi.org/10.1182/blood-2006-06-031682.
286. Lee SJ, et al. Impact of prior imatinib mesylate on the outcome of hematopoietic cell transplantation for chronic myeloid leukemia. *Blood*. 2008;112:3500–3507. https://doi.org/10.1182/blood-2008-02-141689.
287. Saussele S, et al. Allogeneic hematopoietic stem cell transplantation (allo SCT) for chronic myeloid leukemia in the imatinib era: evaluation of its impact within a subgroup of the randomized German CML Study IV. *Blood*. 2010;115:1880–1885. https://doi.org/10.1182/blood-2009-08-237115.
288. Zhang H, Chen J, Que W. Allogeneic peripheral blood stem cell and bone marrow transplantation for hematologic malignancies: meta-analysis of randomized controlled trials. *Leuk Res*. 2012;36:431–437. https://doi.org/10.1016/j.leukres.2011.10.016.
289. Kantarjian H, et al. Dasatinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukemia. *N Engl J Med*. 2010;362:2260–2270. https://doi.org/10.1056/NEJMoa1002315.
290. Cortes JE, et al. Bosutinib versus imatinib for newly diagnosed chronic myeloid leukemia: results from the Randomized BFORE Trial. *J Clin Oncol*. 2018;36:231–237. https://doi.org/10.1200/jco.2017.74.7162.
291. Naqvi K, et al. Long-term follow-up of lower dose dasatinib (50 mg daily) as frontline therapy in newly diagnosed chronic-phase chronic myeloid leukemia. *Cancer*. 2020;126:67–75. https://doi.org/10.1002/cncr.32504.
292. Clark RE, et al. De-escalation of tyrosine kinase inhibitor therapy before complete treatment discontinuation in patients with chronic myeloid leukaemia (DESTINY): a non-randomised, phase 2 trial. *Lancet Haematol*. 2019;6:e375–e383. https://doi.org/10.1016/s2352-3026(19)30094-8.
293. Douxfils J, et al. Association between BCR-ABL tyrosine kinase inhibitors for chronic myeloid leukemia and cardiovascular events, major molecular response, and overall survival: a systematic review and meta-analysis. *JAMA Oncol*. 2016;2:625–632. https://doi.org/10.1001/jamaoncol.2015.5932.
294. Blasdel C, Egorin MJ, Lagattuta TF, Druker BJ, Deininger MW. Therapeutic drug monitoring in CML patients on imatinib. *Blood*. 2007;110:1699–1701. https://doi.org/10.1182/blood-2007-03-079871. author reply 1701.
295. Marin D, et al. Adherence is the critical factor for achieving molecular responses in patients with chronic myeloid leukemia who achieve complete cytogenetic responses on imatinib. *J Clin Oncol*. 2010;28:2381–2388.
296. Frye RF, Fitzgerald SM, Lagattuta TF, Hruska MW, Egorin MJ. Effect of St John's wort on imatinib mesylate pharmacokinetics. *Clin Pharmacol Ther*. 2004;76:323–329. https://doi.org/10.1016/j.clpt.2004.06.007.
297. Tam CS, et al. Failure to achieve a major cytogenetic response by 12 months defines inadequate response in patients receiving nilotinib or dasatinib as second or subsequent line therapy for chronic myeloid leukemia. *Blood*. 2008;112:516–518.
298. Garg RJ, et al. The use of nilotinib or dasatinib after failure to 2 prior tyrosine kinase inhibitors: long-term follow-up. *Blood*. 2009;114:4361–4368. https://doi.org/10.1182/blood-2009-05-221531.
299. Cortes JE, et al. Ponatinib efficacy and safety in Philadelphia chromosome-positive leukemia: final 5-year results of the phase 2 PACE trial. *Blood*. 2018;132:393–404. https://doi.org/10.1182/blood-2016-09-739086.
300. Etienne G, et al. Long-term follow-up of the French stop imatinib (STIM1) study in patients with chronic myeloid leukemia. *J Clin Oncol*. 2017;35:298–305. https://doi.org/10.1200/jco.2016.68.2914.

301. Rousselot P, et al. Loss of major molecular response as a trigger for restarting tyrosine kinase inhibitor therapy in patients with chronic-phase chronic myelogenous leukemia who have stopped imatinib after durable undetectable disease. *J Clin Oncol*. 2014;32:424–430. https://doi.org/10.1200/JCO.2012.48.5797.
302. Ilander M, et al. Increased proportion of mature NK cells is associated with successful imatinib discontinuation in chronic myeloid leukemia. *Leukemia*. 2017;31:1108–1116. https://doi.org/10.1038/leu.2016.360.
303. Schütz C, et al. Expression of the CTLA-4 ligand CD86 on plasmacytoid dendritic cells (pDC) predicts risk of disease recurrence after treatment discontinuation in CML. *Leukemia*. 2017;31:829–836. https://doi.org/10.1038/leu.2017.9.
304. Irani YD, et al. Successful treatment-free remission in chronic myeloid leukaemia and its association with reduced immune suppressors and increased natural killer cells. *Br J Haematol*. 2020 https://doi.org/10.1111/bjh.16718.
305. Kimura S, et al. Treatment-free remission after first-line dasatinib discontinuation in patients with chronic myeloid leukaemia (first-line DADI trial): a single-arm, multicentre, phase 2 trial. *Lancet Haematol*. 2020;7:e218–e225. https://doi.org/10.1016/s2352-3026(19)30235-2.
306. Okada M, et al. Final 3-year results of the dasatinib discontinuation trial in patients with chronic myeloid leukemia who received dasatinib as a second-line treatment. *Clin Lymphoma Myeloma Leuk*. 2018;18:353–360. https://doi.org/10.1016/j.clml.2018.03.004. e351.
307. Imagawa J, et al. Discontinuation of dasatinib in patients with chronic myeloid leukaemia who have maintained deep molecular response for longer than 1 year (DADI trial): a multicentre phase 2 trial. *Lancet Haematol*. 2015;2:e528–e535. https://doi.org/10.1016/S2352-3026(15)00196-9.
308. Rea D, et al. Discontinuation of dasatinib or nilotinib in chronic myeloid leukemia: interim analysis of the STOP 2G-TKI study. *Blood*. 2017;129:846–854. https://doi.org/10.1182/blood-2016-09-742205.
309. Shah NP, et al. Dasatinib discontinuation in patients with chronic-phase chronic myeloid leukemia and stable deep molecular response: the DASFREE study. *Leuk Lymphoma*. 2020;61:650–659. https://doi.org/10.1080/10428194.2019.1675879.
310. Ross DM, et al. Durable treatment-free remission in patients with chronic myeloid leukemia in chronic phase following frontline nilotinib: 96-week update of the ENESTfreedom study. *J Cancer Res Clin Oncol*. 2018;144:945–954. https://doi.org/10.1007/s00432-018-2604-x.
311. Aichberger KJ, et al. Progressive peripheral arterial occlusive disease and other vascular events during nilotinib therapy in CML. *Am J Hematol*. 2011;86:533–539. https://doi.org/10.1002/ajh.22037.
312. Kim TD, et al. Peripheral artery occlusive disease in chronic phase chronic myeloid leukemia patients treated with nilotinib or imatinib. *Leukemia*. 2013;27:1316–1321. https://doi.org/10.1038/leu.2013.70.
313. Richter J, et al. Musculoskeletal pain in patients with chronic myeloid leukemia after discontinuation of imatinib: a tyrosine kinase inhibitor withdrawal syndrome? *J Clin Oncol*. 2014;32:2821–2823. https://doi.org/10.1200/jco.2014.55.6910.
314. Saussele S, et al. Discontinuation of tyrosine kinase inhibitor therapy in chronic myeloid leukaemia (EURO-SKI): a prespecified interim analysis of a prospective, multicentre, non-randomised, trial. *Lancet Oncol*. 2018;19:747–757. https://doi.org/10.1016/s1470-2045(18)30192-x.
315. Saussele S, et al. Impact of comorbidities on overall survival in patients with chronic myeloid leukemia: results of the randomized CML study IV. *Blood*. 2015;126:42–49. https://doi.org/10.1182/blood-2015-01-617993.
316. Yamamoto C, et al. Analysis of the cost-effectiveness of treatment strategies for CML with incorporation of treatment discontinuation. *Blood Adv*. 2019;3:3266–3277. https://doi.org/10.1182/bloodadvances.2019000745.
317. Schoepfer J, et al. Discovery of Asciminib (ABL001), an Allosteric Inhibitor of the Tyrosine Kinase Activity of BCR-ABL1. *J Med Chem*. 2018;61:8120–8135. https://doi.org/10.1021/acs.jmedchem.8b01040.
318. Mauro MJ, Hughes TP. Asciminib in relapsed chronic myeloid leukemia. Reply. *N Engl J Med*. 2020;382:1379. https://doi.org/10.1056/NEJMc2000116.
319. Réa D, et al. A phase 3, open-label, randomized study of asciminib, a STAMP inhibitor, vs bosutinib in CML after 2 or more prior TKIs. *Blood*. 2021;138(21):2031–20041.

CHAPTER 70

THE POLYCYTHEMIAS

Marina Kremyanskaya, Vesna Najfeld, John Mascarenhas, and Ronald Hoffman

Under normal conditions, the red blood cell (RBC) mass in humans is tightly controlled and remains relatively constant in a given individual. The numbers of senescent RBCs lost daily are replaced by newly formed ones by a carefully controlled network of growth factors, progenitor and precursor cells. Erythropoiesis can be augmented by a variety of stimuli that increase the delivery of oxygen to tissues. This delicate balance can be disturbed by various pathologic conditions and can result in either reduced numbers of RBCs (anemia) or excessive numbers of RBCs (polycythemia). Hematocrit values over 49% in males and 48% in females are abnormal and require further evaluation to determine if the patient has an absolute increase in their RBC mass and if investigation of its cause should be pursued. The RBC mass is increased if it is greater than 125% above that expected for sex and body mass. The measurement of the RBC mass is a diagnostic study that is now available at a dwindling number of tertiary care centers, making other diagnostic studies pivotal in evaluating patients with elevated hematocrit levels. Polycythemic states can be due to a variety of disorders that can be attributed to several pathophysiologic mechanisms. Determination of the etiology of an individual's polycythemia is a critical step in defining the patient's appropriate prognosis and treatment plan. Primary polycythemias are the result of innate abnormalities involving hematopoietic progenitors and stem cells that lead to constitutive overproduction of RBCs, accompanied by low erythropoietin (EPO) levels. By contrast, secondary polycythemias are the consequence of a number of conditions that lead to increased EPO production, which acts on normal progenitors to overproduce RBCs. In a small number of patients, the cause of erythrocytosis cannot be determined; these patients are classified as having idiopathic erythrocytosis.

ERYTHROPOIESIS

RBC production can be influenced by numerous factors, including nutrients, growth factors, numbers and function of bone marrow (BM) progenitor and precursor cells, and cellular receptors and transcription factors (Chapter 27). EPO is considered to be the physiologic regulator of the terminal phases of erythropoiesis. Alterations in its production are followed by adjustments in the rate of formation of RBCs. In humans, EPO production is controlled by the relative supply of oxygen to the kidneys, the major site of EPO production. In states of severe hypoxia, EPO production can be increased up to 1000-fold. In a healthy person after phlebotomy, EPO production increases, and an inverse logarithmic relationship between hematocrit and EPO production rates exists. Patients with secondary erythrocytosis caused by chronic hypoxia have either normal or increased basal EPO values, but they also have increased values after reduction of the hematocrit to normal levels by phlebotomy. By contrast, EPO excretion is invariably subnormal in patients with polycythemia vera (PV), which demonstrates that this disorder is not a result of excessive EPO production.

ERYTHROPOIETIN, OXYGEN SENSING, AND HYPOXIA-INDUCIBLE FACTOR

Under normal conditions, EPO production is mediated by a reduced oxygen content, termed *hypoxemia*, which leads to decreased oxygen delivery to tissues (Chapter 27). Regulation of oxygen homeostasis is critical to survival. In humans, oxygen sensing occurs at many levels, leading to both acute and chronic adaptation. The acute reduction of the availability of oxygen leads to the initiation of a cascade of adaptive events that sets in place compensatory events to correct the lack of oxygen supply.[1] Low oxygen levels, or hypoxia (60 mm Hg), in humans causes oxygen-sensing chemosensory cells to undergo rapid membrane depolarization within seconds, leading to the production of action potentials, influx of calcium ions, and release of the neurotransmitters that result in stimulation of the brain stem that controls the respiratory and cardiovascular systems. These chemosensory cells are found within the glomus cells of the carotid body located at the bifurcation of the internal and external carotid arteries. The released neurotransmitters activate the nerve endings of the carotid body sensory nerve to convey to the CNS signals that command ventilation to fight hypoxia, resulting in an increase of the ventilation rate and restoration of normal oxygen tension to vital organs. In addition, there are changes in blood pressure and heart rate to maximize oxygen delivery. The carotid body is the organ with the greatest blood flow within the body. Activation of the carotid body results in the sensation of breathlessness experienced by individuals at high altitudes. During chronic hypoxia when the carotid body is permanently active, there is marked enlargement of the carotid body because of an increase in capillaries and a marked reduction in the mean distance from the capillaries to the edge of the chemoreceptor cells.

In response to chronic hypoxia, multiple compensatory mechanisms come into play over several days within the kidneys, the major site of EPO production. Lack of oxygen activates the cellular signaling pathway of the hypoxia-inducible transcription factors (HIF) that has three isoforms, hypoxia-inducible factor (HIF)-1, HIF-2, HIF-3, regulating expression of several thousand genes. Within the kidney, specialized interstitial cells are the source of EPO. These cells employ the HIF pathway, a pathway that is otherwise present in essentially all cells of the body, to regulate EPO production in an oxygen-sensitive manner. Hypoxic stimulation results in production of HIF-1/HIF-2, the major factors responsible for transcriptional activation of the *EPO* gene.[2] The HIF transcriptional system is a master regulator of the hypoxic response controlling a large number of genes in multiple cell types. HIF-1 is a heterodimeric protein consisting of HIF-1/HIF-2α and HIF-1β, which is required for normal development of the heart, blood vessels, and blood cells. As the key mediator of cellular oxygen maintenance, HIF-1 facilitates body oxygen delivery and responses to oxygen deprivation by regulating the expression of gene products that are involved in cellular energy metabolism and glucose transport, angiogenesis, erythropoiesis and iron metabolism, pH regulation, apoptosis, cell proliferation, and cell–cell and cell–matrix interactions. Classic HIF target genes include phosphoglycerate kinase, glucose transporter-1, vascular endothelial growth factor (VEGF), and EPO. The HIF proteins are members of the Per–ARNT–Sim family of heterodimeric basic helix–loop–helix transcription factors (Fig. 70.1).

In contrast to the constitutively expressed, HIF-1β subunits, HIF-1α is an oxygen-labile protein that becomes stabilized in response to hypoxia. HIF-1α mRNA and protein levels are induced by hypoxia, and HIF-1α protein levels decay rapidly with return to normoxia. The posttranslational regulation of HIF-1α protein accounts for the majority of the regulation of this gene. Normoxia-induced, ubiquitin-mediated degradation of the HIF-1α protein is the major regulator of HIF-1α levels, thereby reducing the stimulus for additional EPO production. The targeting and subsequent polyubiquitination of HIF-1α require the von Hippel-Lindau (VHL) protein, oxygen, and three different iron-requiring proline hydroxylase (PHD) enzymes (see Fig. 70.1). The PHD proteins exist in three isoforms, PHD1, PHD2, and PHD3. HIF-1α is hydroxylated by all three isoforms but primarily by PHD2. The prolyl hydroxylation of HIF-1α is necessary for the binding of HIF-1α to VHL, which is the substrate-recognition subunit of an E3 ubiquitin-protein ligase. Different parts of the HIF-1α chains have different functions. The N-terminus part of HIF-1α is involved in DNA binding and dimerization, and the C-terminus portion has regulatory functions. One domain at the C-terminus influences transcriptional activity without affecting HIF-1α protein levels, and the other region, termed the oxygen-dependent degradation domain (ODD), affects protein abundance. The VHL protein physically interacts with the ODD of HIF-1α, targeting it for ubiquitination and destruction by the proteasome. Iron-chelating drugs can also block the interaction of HIF-1α with the VHL protein, suggesting a role for iron in the degradation of HIF-1α.

Under normoxic conditions, hydroxylation of HIF-1α/HIF-2α is essential for HIF proteolytic degradation by promoting interaction with the VHL tumor-suppressor protein through hydrogen bonding to the hydroxy proline-binding pocket in the VHL-β domain. As oxygen levels decrease, hydroxylation of HIF decreases, and HIF-1α/HIF-2α then no longer binds VHL. As a result, HIF-1α/HIF-2α become stabilized, dimerize with HIF-1β, and activate transcription of target genes. The activity of PHDs depends on the availability of molecular oxygen, which qualifies these enzymes as oxygen sensors. In addition, these dioxygenases require 2-oxyglutarate which acts as a cosubstrate with vitamin C to keep nonheme iron in the ferrous state. Although PHD-2 appears to be the hydroxylase that is essential for HIF-1α/HIF-2α degradation under normoxic conditions, PHD-3 is important for hydroxylation of HIF-1α/ HIF-2α during reoxygenation. Different effects of individual PHDs on HIF-1α and HIF-2α hydroxylation indicate that the stability of individual HIF-1α subunits and their target gene expression might be affected by tissue- and cell-type differences in PHD expression and activity levels. The activity of PHDs can be modulated by mitochondrial reactive oxygen species, implicating mitochondria in oxygen sensing. HIF-2α under hypoxic conditions dimerizes with HIF-1β and activates the transcription of a set of target genes that overlap with the target genes regulated by HIF-1α/HIF-1β heterodimers. HIF-1α is expressed by all nucleated cells, but HIF-2α is expressed by specific cell types, including vascular endothelial cells, renal interstitial cells, hepatocytes, cardiomyocytes, and astrocytes. HIF-2α appears to play a critical role in regulating EPO production in adult mammals[2] and HIF-1α is important during yolk sac erythropoiesis. HIF-1 also controls the absorption and delivery of iron to the BM by repressing of hepcidin and activation of genes encoding transferrin and the transferrin receptor.

HIF3α is another HIF protein which is an oxygen-dependent transcription factor which activates a distinct transcriptional response to hypoxia. Mammalian *HIF-3* genes use different promoters, different transcription initiation sites, and alternative splicing to transcribe a large number of mRNA variants. While some short HIF-3α variants act as dominant-negative regulators of HIF-1/2 actions, other HIF-3 variants can inhibit HIF-1/2 actions by competing for the common HIF-β. It is thought that HIF3 acts as a negative regulator of HIF-1 and HIF-2. Similar to HIF1 and HIF2, *PHD1*, *PHD2*, *PHD3*, and *VHL* also cause HIF-3 protein degradation. Further studies of HIF-3α variants will provide new and important insights into HIF biology.[3]

VHL syndrome is a hereditary cancer syndrome that is associated with exaggerated responses to hypoxia caused by posttranslational abnormalities in HIF. VHL syndrome is characterized by a propensity for developing clear-cell renal carcinomas, retinal

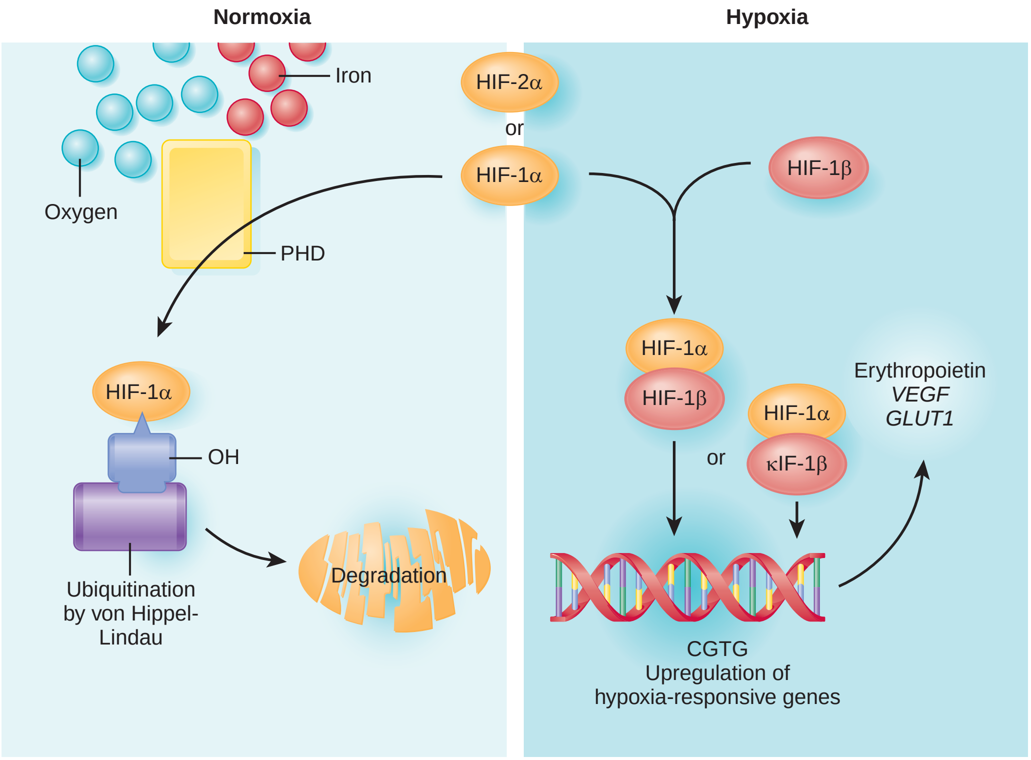


Figure 70.1 SCHEMATIC REPRESENTATION OF THE RELATIONSHIP BETWEEN HYPOXIA SENSING AND ERYTHROPOIETIN PRODUCTION. *GLUT*, Glucose transporter 1; *HIF*, hypoxia-inducible factor; *PHD*, proline hydroxylase; *VEGF*, vascular endothelial growth factor.

hemangioblastomas, cerebellar and spinal hemangiomas, pancreatic and renal cysts, islet cell tumors of the pancreas, and pheochromocytomas.[4] The tumors result from somatic mutations that cause a loss of heterozygosity (LOH) of the *VHL* gene. VHL inactivation leads to aberrant stabilization and accumulation of HIF-2α, which drives tumor cell growth. VHL disease affects approximately 1 in 35,000 individuals and is transmitted in an autosomal dominant manner. Individuals with VHL disease carry one wild-type (WT) *VHL* allele and one inactivated *VHL* allele. This inactivation can occur by somatic mutation or hypermethylation. Tumor or cyst development is linked to somatic inactivation or loss of the remaining WT *VHL* allele. Approximately 20% to 37% of VHL patients have large or partial germ-line deletions, 23% to 27% have nonsense or frame-shift mutations, and 30% to 35% have missense mutations. More than 150 different *VHL* mutations linked to VHL disease have been reported. The tumors linked to *VHL* inactivation are often highly vascular and can produce angiogenic factors such as VEGF. In addition, renal cell carcinoma, cerebellar hemangioblastomas, and pheochromocytomas have been associated with paraneoplastic erythrocytosis caused by overproduction of EPO. Overproduction of HIF-inducible mRNAs is the hallmark of VHL protein defective cells. Genotype–phenotype correlates in VHL disease suggest that VHL has functions independent of HIF regulation that might play a role in tumor formation. VHL protein has other binding partners, including atypical protein kinase C and a family of deubiquitinating enzymes called VHL-interacting deubiquitinating enzymes 1 and 2. In addition, VHL has been involved in numerous cellular processes, including regulation of extracellular matrix, cytoskeleton stability, cell cycle control, and differentiation. VHL disease is not associated with erythrocytosis.

This growing understanding of intracellular responses to hypoxia has led to the development of new therapeutic strategies for treating solid tumors which was recognized in 2019 by the Nobel Prize in Physiology and Medicine being awarded to William G. Kaelin, Jr., Sir Peter J. Ratcliffe, and Gregg L. Semenza. Many cancers contain areas of intra-tumoral hypoxia, and primary tumors with low oxygen levels are associated with an increased risk of metastasis and patient mortality. Increased HIF-1 levels are associated with increased risk of mortality in patients with many human cancers, including those involving the bladder, brain, breast, colon, esophagus, head/neck/oropharynx, liver, lung, pancreas, skin, stomach, and uterus, as well as in acute lymphocytic and myeloid leukemia. By contrast, in colon carcinoma and clear cell renal cell carcinoma HIF-2 overexpression is associated with disease progression and mortality.[5]

The possibility that HIF family member inhibitor therapy might be used to treat solid tumors was hypothesized by many and recently confirmed by the FDA approving belzutifan. Belzutifan is an oral HIF-2α inhibitor used to treat adults with VHL syndrome who require therapy for associated renal cell carcinoma, central nervous system tumors, hemangioblastomas, or pancreatic neuroendocrine tumors not requiring immediate surgery. In a phase I study of patients with clear cell renal cell carcinoma reduced EPO levels were observed at all doses administered. The confirmed objective response rate was 25% (all partial responses), and the median progression-free survival was 14.5 months. The most common grade $\geq$ 3 adverse events were anemia (27%) and hypoxia (16%). Belzutifan was well tolerated and demonstrated preliminary antitumor activity in heavily pre-treated patients, suggesting that HIF-2 α inhibition might offer an effective treatment for clear cell renal cell carcinoma.[6]

THE ERYTHROPOIETIN RECEPTOR

Interaction of EPO with the EPO receptor (EPOR) present on the erythroid progenitor and precursor cells leads to its homodimerization, resulting in (1) stimulation of cell division, (2) differentiation by induction of erythroid-specific gene expression, and (3) prevention of erythroid progenitor and precursor cell apoptosis. EPOR is a member of the type I cytokine receptor superfamily. Signal transduction through the receptor is initiated by ligand binding, which induces dimerization of EPOR monomers. The predominant signaling cascade activated by EPOR and other cytokine receptors is the Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. JAK tyrosine kinases are constitutively associated with the membrane proximal regions of cytokine receptor cytoplasmic domains and are activated by receptor dimerization. The EPOR associates predominantly with JAK2. JAK2 binds to the EPOR in the endoplasmic reticulum and acts as a chaperone protein transporting the EPOR to the plasma membrane. Immediately after EPO binding, JAK2 phosphorylates itself and the EPOR on multiple tyrosine residues in its cytoplasmic domain, thus creating a platform for the recruitment and activation of multiple key signaling regulators. One such protein is STAT5. JAK2–STAT5 signaling plays an essential role in EPO/EPOR-mediated regulation of erythropoiesis. Consistent with their essential roles in erythropoiesis, EPO-, EPOR-, and JAK2-deficient mice die embryonically from severe anemia.

The C-terminal cytoplasmic portion of the EPOR also possesses a negative regulatory domain. Hematopoietic cell phosphatase (HCP; also known as *SHP-1* or *PTP N6*) interacts with this portion of the EPOR and downregulates signal transduction by promoting dephosphorylation (Fig. 70.2). Inactivation of the HCP-binding site leads to prolonged phosphorylation of JAK2–STAT5. Another negative regulator of erythropoiesis, suppressor of cytokine signaling-3 (SOCS-3), binds to the cytoplasmic portion of the EPOR and suppresses EPO-dependent JAK2–STAT5 signaling. Thus, deletion of the distal C-terminal cytoplasmic portion of the EPOR abolishes negative regulatory elements and results in increased proliferation of erythroid progenitor cells. Mutations in the *EPOR* gene have been observed in some patients with primary familial and congenital polycythemia (PFCP) and are occasionally found in erythroleukemia (see Fig. 70.2). Such secondary growth factors as insulin-like growth factor-1 (IGF-1) and the components of the renin–angiotensin system (RAS) may also influence the production of RBCs.

THE RENIN–ANGIOTENSIN SYSTEM AND HEMATOPOIESIS

The RAS regulates fluid and electrolyte homeostasis and blood pressure, and has been hypothesized to also play a role in the regulation of erythropoiesis. The primary function of angiotensin during development is the regulation of tissue growth and differentiation. Angiotensin II (Ang II) is a ligand for two distinct receptors, type 1 and type 2 (AT1 and AT2). AT1 appears to play a major role in the regulation of cell proliferation.[7]

The RAS was first postulated to influence erythropoiesis in the 1980s after the use of angiotensin-converting enzyme (ACE) inhibitors to treat hypertension was shown to result in anemia. In animals, increased blood levels of renin (a major regulator of Ang II synthesis) were found to result in elevated serum EPO levels and erythrocytosis. In humans, the infusion of Ang II in healthy volunteers increased serum EPO levels by 35% or higher via activation of the Ang II type I receptor, and ACE inhibitors significantly decrease plasma EPO levels by as much as 20% to 30%. The pathway underlying Ang II-driven EPO secretion is unknown. However, some investigators have suggested that Ang II modulates renal EPO production through changes in renal perfusion and sodium reabsorption. This hypothesis is based on the presumption that reduced oxygen pressure in the kidneys triggers HIF-1α/HIF-2α to induce release of EPO. Ang II also directly stimulates proliferation of hematopoietic progenitors in vitro, and inhibition of this effect with ACE inhibitors induces apoptosis of erythroid progenitors in renal transplantation patients. ACE-1 knockout mice develop a normocytic anemia that can be reversed by infusion

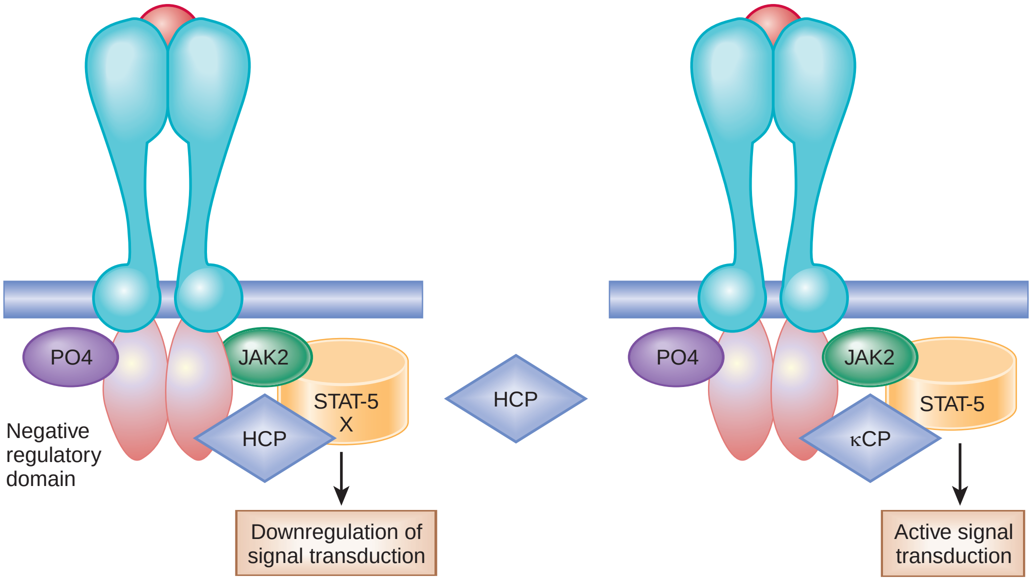


Figure 70.2 SCHEMATIC REPRESENTATION OF THE ERYTHROPOIETIN RECEPTOR AND THE DEFECT IN THE RECEPTOR UNDERLYING PRIMARY FAMILIAL AND CONGENITAL POLYCYTHEMIA. *HCP*, Hematopoietic cell phosphatase; *JAK*, Janus kinase; *STAT*, signal transducer and activator of transcription.

of Ang II. ACE-related anemia is most pronounced in patients with renal insufficiency or end-stage renal disease and in patients who have received a renal allograft. The pathogenesis of this anemia is not clear, but reduced levels of circulating EPO are not solely responsible, suggesting that there might be other contributing factors. The AT1 receptor is present on erythroid progenitors, and its ligand, Ang II, augments EPO stimulation of erythropoiesis. The involvement of JAK2 kinase in Ang II signaling suggests that this signal transduction pathway mediated by EPO and Ang II might overlap. Postrenal transplant erythrocytosis likely can be accounted for by activation of the RAS.

DEFINITION AND CLASSIFICATION OF POLYCYTHEMIA

The term *polycythemia* is a literal translation from Greek, meaning "too many cells in the blood," and refers to an increase in the RBC mass; it is frequently used interchangeably with the term *erythrocytosis*. Polycythemia may be due to a myriad of causes (Table 70.1). The polycythemias can be classified as relative and absolute. Relative polycythemia is a disorder in which the patient characteristically has a modest elevation of the hematocrit level without an elevated RBC mass but rather because of contraction of the plasma volume. The absolute polycythemias are accompanied by an actual increase in the circulating RBC mass. Polycythemias can also be classified according to the responsiveness of their erythroid progenitor cells to growth factors or the circulating levels of such growth factors. Primary polycythemias are characterized by increased sensitivity of the erythroid progenitors to regulatory growth factors as a result of acquired somatic or inherited germ-line mutations expressed by hematopoietic progenitor cells (HPCs). In contrast, secondary polycythemias are characterized by an increase in regulatory growth factors, primarily EPO, and normal responsiveness of their erythroid progenitors to these growth factors. These conditions can usually be distinguished by in vitro assays of erythroid progenitor cells, quantitation of serum EPO levels, and detection of somatic *JAK2* mutations. In a small number of patients, the cause of erythrocytosis cannot be determined; these patients are classified as having idiopathic erythrocytosis.

RELATIVE POLYCYTHEMIA

Individuals with a modestly increased venous hematocrit level that is not accompanied by an increased RBC mass are frequently thought to be polycythemic by imprecise yet widely accepted medical practice. Frequently, these individuals are thought to be polycythemic owing to the lack of appreciation by a clinician of what constitutes the upper limit of normal values for a hematocrit (49% in males and 48% in females). Such individuals frequently prove not to have an absolute polycythemia as defined by an actual increase in the measured RBC mass. *Relative* or *spurious polycythemia* is a term used to describe an elevation of the hematocrit level either caused by an acute transient state of hemoconcentration associated with intravascular fluid depletion or a chronic sustained relative polycythemia caused by contraction of the plasma volume (see Table 70.1).

Transient polycythemias may be a result of acute depletion of the plasma volume from a variety of disorders, including protracted vomiting or diarrhea, plasma loss from external burns, sudden cold exposure or protracted exercise, insensible fluid loss from fever, sepsis, diabetic ketoacidosis, or acute ethanol intoxication. These elevations of hematocrit can be easily corrected by appropriate replacement of intravascular fluids.

Gaisböck syndrome, first described in 1905, is a condition observed mainly in obese, hypertensive, middle-aged, and male smokers. Alcohol, diuretics, obesity, hypoxia, psychologic stress, and excess catecholamine secretion have been identified as possible causes of relative polycythemia. Such individuals can have a chronic modest-to-moderate elevation of the hematocrit level associated with a normal RBC mass and low plasma volume, which has been attributed to reduced venous compliance, or they can have a high normal RBC mass with either a normal or slightly decreased plasma volume. The primary significance of the identification of a patient with relative polycythemia is the recognition of the increased risk of developing thrombotic vascular events likely caused by excessive smoking, hypertension, and obesity associated with this disease. Treatment is generally directed at correction of the patient's underlying cardiovascular risk factors.

It is also important to emphasize that overfilling of blood collection vacuum tubes can result in pseudopolycythemia, pseudothrombocytopenia, and pseudoleukopenia as a result of inadequate sample mixing. Careful attention to such a seemingly trivial detail can help avoid expensive, unnecessary diagnostic workups.

TABLE 70.1 Differential Diagnosis of the Polycythemias

Relative or Spurious Polycythemia

1. Decreased plasma volume—reduced fluid intake, marked loss of body fluids (diaphoresis, vomiting, diarrhea, “third spacing”)
2. Gaisböck syndrome
3. Overfilling of blood in collection vacuum tubes

Absolute Polycythemia

1. Secondary polycythemia
    - A. Acquired
        - Hypoxia
            - Pulmonary disease
            - Cyanotic congenital heart disease
            - Hypoventilation syndromes: sleep apnea, Pickwickian syndrome
            - High altitude
            - Smokers’ polycythemia, hookah polycythemia, carbon monoxide intoxication caused by industrial exposure
        - Postrenal transplantation erythrocytosis
        - Aberrant erythropoietin production
            - Tumors: renal cell carcinoma, Wilms tumor, hepatic carcinoma, uterine leiomyomata, virilizing ovarian tumors, vascular cerebellar tumors
            - Miscellaneous renal and hepatic disorders: solitary renal cysts, polycystic kidney disease, renal artery stenosis hydronephrosis, viral hepatitis
        - Endocrine disorders: Cushing syndrome, primary aldosteronism
        - Androgen use
        - Erythropoietin use
    - B. Congenital polycythemias
        - Abnormal high-affinity hemoglobin variants
        - Bisphosphoglycerate deficiency
        - Congenital methemoglobinemia
        - Chuvash polycythemia (von Hippel-Lindau mutations)
        - Prolyl hydroxylase mutations
        - Hypoxia-inducible factor gene mutations
2. Primary polycythemias
    - Primary congenital and familial polycythemia
    - Polycythemia vera

ABSOLUTE POLYCYTHEMIAS

Primary Familial and Congenital Polycythemia

This is an autosomal dominant disorder. Although PFCP is uncommon, it is more prevalent than polycythemia caused by high-oxygen–affinity hemoglobin mutants or a 2,3-biphosphoglycerate (2,3-BPG) deficiency. Unlike patients with PV, patients with PFCP lack splenomegaly and do not progress to acute leukemia. It is not unusual for these patients to present with headaches, dizziness, epistaxis, and exertional dyspnea that resolve with normalization of the hematocrit level.[8] An increased incidence of cardiovascular events and premature morbidity and mortality has been reported in some affected members, but many appear to have a benign clinical course. Although clinical symptoms are relieved by phlebotomy, the increased risk of cardiovascular morbidity is not corrected by maintaining a normal hematocrit. Characteristic laboratory findings are (1) an increased hematocrit and RBC mass without an increased leukocyte or platelet count, (2) an absence of an activating mutation of *JAK2*, (3) a normal hemoglobin–oxygen dissociation curve, (4) low serum EPO levels, and (5) in vitro hypersensitivity of erythroid progenitors to EPO. Even though PFCP is present at birth, many affected patients are incidentally diagnosed later in life after the performance of routine blood counts or when evaluated in the context of multiple family members having polycythemia. It is of interest that one individual so affected was an accomplished cross-country skier who had won medals at the Olympic Games. Numerous mutations of the EPOR associated with PFCP have been described, leading to a loss in the negative regulatory domain of the EPOR.

The physiologic basis for EPO-mediated activation of erythropoiesis is as follows: EPO activates its receptor by conformational changes of its dimers, leading to initiation of an erythroid-specific cascade of events. The first signal is initiated by the binding of a tyrosine kinase to the EPOR and its phosphorylation and activation of a transcription factor, STAT5, which regulates erythroid-specific genes. This “on” signal is negated by dephosphorylation of the EPOR by HCP, that is, the “off” signal. Truncation of the EPOR leads to a loss in the negative regulatory domain of the EPOR, a binding site for HCP, leading to a gain-of-function mutation of the EPOR (see Fig. 70.2). In addition, the negative regulation of erythropoiesis by SOCS-3, cytokine-inducible SH2 domain containing protein (CIS), and Src homology region 2 domain-containing phosphatase-1 (SHP-1) is presumed to contribute to the underlying cause of PFCP.

Alternative explanations for the increased sensitivity of erythroid progenitors to EPO of patients with PFCP have been proposed. EPOR downregulation provides another mechanism by which EPO desensitization can occur. EPOR downregulation is a complex process that involves EPOR-induced internalization or ubiquitination and degradation by proteasomes. EPO-induced receptor internalization is an efficient means of rapidly reducing EPO responsiveness. This process is mediated by binding of the EPOR to the p85 subunit of phosphatidylinositol 3-kinase (PI3K) but does not involve its kinase activity as the PI3K inhibitor wortmannin does not impair EPOR internalization. All of the truncated mutants associated with PFCP are associated with failure to internalize the EPOR, contributing to prolonged signaling through the EPOR. The EPOR degradation process removes all of the phosphorylated tyrosine residues in the intracellular domain of the receptor, thereby preventing further signal transduction. The remaining part of the EPO–EPOR complex is then internalized and degraded by lysosomes. The E3 ligase B-transducin repeat containing protein-1 (B-Trcp-1) is responsible for EPOR ubiquitination and degradation. Mutations in *B-Trcp-1* abolish EPOR ubiquitination and degradation, making cells expressing the EPORs hypersensitive to EPO. Each of the *PFCP* mutations involving the EPOR results in loss of the binding site for B-Trcp-1. These findings suggest that the EPO hypersensitivity in PFCP might not be attributable to a failure to recruit negative regulators such as phosphatases to inactivate JAK2, or that these mutant receptors are defective in EPO-induced receptor downregulation.

The effect of a truncated EPOR is not always predictable. Some patients who inherit an EPOR mutation are not polycythemic. This observation suggests that undefined environmental or genetic factors may mask the development of polycythemia. Also, the heterogeneity of the polycythemic phenotype observed in a PFCP animal model appears to be strain dependent. This indicates that gene modifiers or epigenetic factors may mask the development of the full PFCP phenotype. Four different rearrangements of the EPOR have been observed in Philadelphia chromosome-like (Ph-like) acute lymphoblastic leukemia (ALL) B cells (Chapters 66 and 68). Normal B cells do not express the EPOR, but the EPOR has been described in ETV6-RUNX1–positive ALL blast cells. These rearrangements are different from those observed in PFCP but result in truncation of the cytoplasmic tail of the EPOR at residues similar to those mutated in PFCP, with preservation of the proximal tyrosine essential for receptor activation and loss of distal regulatory residues. This leads to dysregulated EPOR expression, hypersensitivity to EPO stimulation, and heightened JAK–STAT activation. Expression of truncated EPOR in murine B-cell progenitors leads to the development of ALL in vivo (see Chapters 66 and 68). Several observations suggest that the EPOR mutations in human leukemic cells in Ph-like ALL are driver mutations that are acquired during early stages of leukemogenesis and therefore JAK inhibitor therapy can be used in combination with current ALL treatment strategies to treat patients with this type of leukemia.

SECONDARY POLYCYTHEMIAS

Secondary polycythemias can be either congenital or acquired (see Table 70.1). Conditions leading to hypoxia, such as high altitude, cyanotic heart disease, obstructive sleep apnea or chronic lung disease, may result in physiologic polycythemia mediated by increased levels of EPO. There are marked variations in EPO levels and subsequent erythroid responses in the face of chronic hypoxia, suggesting that some of these factors may be genetically determined. The same degree of renal tissue hypoxia may induce substantially different levels of EPO production in response to high altitude. It is likely that these individual variations are a function of genetic differences in hypoxia sensing and the hypoxia response pathways. For purposes of simplicity and clinical diagnostic usefulness, the secondary polycythemic disorders are divided into those that are acquired and those that are congenital. It should be kept in mind that this division, although useful for purposes of differential diagnosis, is artificial. Patients with inherited germ-line mutations, for instance, can develop an EPO-secreting pheochromocytoma or renal cell cancer, and a patient with PV can smoke and have chronic obstructive pulmonary disease (COPD). In other instances, polycythemia caused by a germ-line mutation can be masked by an acquired environmental factor or another gene-modifying mutation.

Acquired Secondary Polycythemias

Polycythemias of Cyanotic Heart Disease and Pulmonary Disease

Patients with cyanotic heart disease and pulmonary disease frequently have arterial hypoxemia, leading to increased production of EPO and polycythemia. Excessive EPO production occurs when the PaO_2 is sustained below 67 mmHg as a result of severely impaired pulmonary mechanics. Because patients with severe pulmonary disease and secondary erythrocytosis frequently have elevated plasma volumes, the degree of elevation of the hematocrit level may be modest. Hematocrit levels as high as 65% or rarely 75% have, however, been reported. Moderate elevations of hematocrit have been estimated to occur in 20% of patients with COPD. Polycythemia in this setting can contribute to pulmonary hypertension, pulmonary endothelial cell dysfunction, reduced cerebral blood flow, hyperuricemia, gout, and an increased risk of venous thromboembolic disease.

Why some patients with pulmonary disease and congenital heart disease develop polycythemia but others do not remains unclear. Increased oxygen-carrying capacity may improve oxygen delivery; however, it is not obvious at what hematocrit level the resultant elevation in blood viscosity impairs blood flow to the tissues, leading to a reduction in oxygen uptake. In addition, oxygen uptake to the tissues is markedly influenced by whole blood volume. Thus, whereas the optimal hematocrit level for oxygen delivery is about 45% in normovolemic subjects, it rises to over 60% in hypervolemic states, likely as a result of engorgement of the vascular bed and a decrease in peripheral resistance. Furthermore, chronic exposure to hypoxia leads to respiratory alkalosis that in turn promotes the synthesis of 2,3-BPG, facilitating increased oxygen delivery to tissues.

The practical relevance of an elevated hematocrit level in this clinical situation is whether and at what level it is harmful or beneficial. An extremely elevated hematocrit level may be detrimental to optimal oxygen delivery. Extreme but not moderate polycythemia caused by chronic hypoxia may affect systemic vascular function by altering blood viscosity, vessel wall shear stress, reduced endothelial cell–derived nitric oxide release, and increasing the secretion of endothelin. Although it is widely accepted that polycythemic pediatric patients with cyanotic heart disease are at an increased risk for developing cerebrovascular accidents, the literature provides conflicting data as relates to the prevalence of such events among adults. A 10% to 13.6% prevalence of stroke and transient ischemic attacks (TIAs) has been reported in a cohort of adult patients with cyanotic heart disease, but others have claimed that such events are rare. Iron deficiency occurs in more than 30% because of the total depletion of iron stores to support erythropoiesis. Microcytic RBCs are, however, rarely found, and despite the iron deficiency, these patients frequently have normal mean corpuscular volumes and high mean corpuscular hemoglobin concentrations, which might maximize the amount of hemoglobin within an individual RBC, thereby maximizing oxygen delivery. In the past, compensatory erythrocytosis was thought to lead to an increased plasma viscosity, leading to a compromised microcirculation, resulting in symptoms such as headache, sluggish mentation, dizziness, blurry vision, muscle weakness, or paresthesias. In reality, such symptoms are rare in patients with chronic compensated secondary erythrocytosis, and the secondary erythrocytosis is viewed as a physiologically desirable response to chronic hypoxia. The symptoms delineated above are likely attributable to decreased tissue oxygen delivery rather than hyperviscosity.

The treatment of hyperviscosity secondary to erythrocytosis in cyanotic heart disease with prophylactic phlebotomy is rarely used. In fact, phlebotomy has been reported to have harmful rather than beneficial effects in adults with cyanotic congenital heart disease. Because almost one-third of these patients are iron deficient even though their RBC indices do not reflect this, routine assessment of the patient's iron status is suggested with gradual supplementation with sufficient iron to attain appropriate compensatory levels of erythropoiesis but avoiding excessive sudden increases in the degree of erythrocytosis. The present evidence indicates that prophylactic phlebotomy promotes the development of iron deficiency, decreases exercise tolerance, and increases the number of cerebrovascular events. Currently, experts in this field recommend that phlebotomy should be restricted to individuals with symptoms with extreme erythrocytosis (hematocrit >65%) and preoperatively to improve hemostasis. Clinical data to justify these recommendations are lacking. Phlebotomy should be followed by the infusion of an equal volume of fluids to maintain intravascular volume and blood flow, as well as to provide a dilutional effect to reduce the hematocrit level. Hydroxyurea (HU) therapy has been used occasionally to reduce erythropoiesis in this situation to reduce the need for phlebotomy, but little evidence exists for this approach. The superiority of HU therapy versus phlebotomy therapy has not been documented. HU might act not only by suppressing RBC production but also by promoting macrocytic RBC formation, thereby increasing RBC deformability and decreasing RBC adhesiveness.

Chronic oxygen therapy in patients with severe COPD has resulted in relief of hypoxia and a modest reduction in hematocrit levels. Pharmacologic interventions, including theophylline, inhaled nitric oxide, sildenafil, or antagonism of the renin–angiotensin pathway with losartan, may also reduce the degree of pulmonary hypertension or secondary erythrocytosis. In a retrospective study, hematocrit levels were found to be significantly lower in 50 COPD patients with erythrocytosis receiving theophylline as compared to levels in the 61 patients not receiving theophylline. Since the levels of oxygen saturation were similar in both groups, the normal hematocrit levels in the theophylline treated group could not be explained by improved oxygen availability. Serum EPO levels, however, declined in 70% of theophylline treated patients. Theophylline has previously been shown to reduce the production of EPO in both normal subjects and patients with erythrocytosis after renal transplantation and is likely acting in a similar fashion to reduce the degree of erythrocytosis in COPD patients.[9]

Obstructive Sleep Apnea–Induced Polycythemia

Obstructive sleep apnea syndrome is characterized by repetitive episodes of partial or complete obstruction of airflow during sleep. Common symptoms include loud snoring and breathing pauses observed by a bed partner, feelings of nonrefreshing sleep, and excess daytime sleeping. Although the evidence is largely anecdotal and somewhat controversial, secondary polycythemia is a recognized complication of long-standing sleep apnea. The factor that seems to have the strongest prediction for polycythemia is nocturnal mean oxygen saturation.[10] On the other hand, most studies demonstrate that the apnea-hypopnea index does not reliably associate with polycythemia except in severe cases. The mechanism by which sleep apnea can cause polycythemia is unclear. Differences in EPO levels between normoxic and hypoxemic patients referred for suspected sleep apnea have not been documented. Obstructive sleep apnea is also associated with an increased risk of developing cardiovascular diseases, including systemic hypertension, pulmonary hypertension, cardiac arrhythmias, atherosclerosis, ischemic heart disease, and stroke. Intermittent hypoxia is thought to be a major cause of cardiovascular complications. These patients undergo repeated episodes of hypoxia and normoxia. The hypoxia leads to ischemia, and the reoxygenation causes a sudden increase of oxygen. This reoxygenation phase results in the production of reactive oxygen species and the promotion of oxidative stress, leading to an inflammatory response and the development of vascular complications. Nguyen and Holly analyzed 1604 consecutive veterans undergoing sleep evaluations for suspected obstructive sleep apnea to identify the incidence and causes of erythrocytosis. They reported four important findings: (1) Clinically significant erythrocytosis (HCT >48%) was relatively uncommon in those with suspected or confirmed OSA occurring in only 8.0% of cases with only 1.6% having clinical erythrocytosis as defined as Hct ≥51% in males, Hct ≥48% in females. (2) The severity of obstructive sleep apnea as measured by Apnea Hypopnea Index was not associated with higher hematocrit levels or absolute erythrocytosis. (3) Both nocturnal hypoxemia and hypoxemia occurring while individuals were awake were independent predictors of erythrocytosis. (4) The severity of obstructive sleep apnea was predictive of nocturnal hypoxemia. These authors concluded that factors other than obstructive sleep apnea should initially be evaluated for unexplained secondary erythrocytosis, although nocturnal oximetry might be of diagnostic use. They concluded that in patients with unexplained nocturnal hypoxemia and erythrocytosis, an obstructive sleep apnea evaluation using a formal sleep study was warranted.[11] Conversely, PV may induce central sleep apnea by decreasing cerebral blood flow to diencephalic respiratory centers, and patients so affected can have complete resolution of their sleep disorder with normalization of their blood counts.

Pickwickian Syndrome and Polycythemia

Pickwickian syndrome or obesity–hypoventilation syndrome, seen in morbidly obese individuals, is characterized by chronic hypoxemia and hypercapnia caused by alveolar hypoventilation, with a resultant increase in EPO production, polycythemia, and cor pulmonale. The three principal causes are the high cost of the work of respiration in morbidly obese individuals, dysfunction of the respiratory centers, and repeated episodes of nocturnal obstructive apnea. Effective treatments include surgically induced weight loss, nasal continuous positive airway pressure ventilation, and the respiratory stimulant medroxyprogesterone acetate.

Polycythemia Caused by High Altitude

Polycythemia caused by the hypoxic conditions encountered by high-altitude dwellers would appear at first glance to represent a universal adaptive process to altitude. High altitude results in hyperventilation, alkalosis, and shifting of the O_2 dissociation curve to the left, leading to the impaired release of O_2 from hemoglobin and ultimately tissue hypoxia. This tissue hypoxia results in markedly increased EPO production, leading to increased plasma iron turnover, reticulocytosis, and a rising hematocrit level. Residents of the Andes Mountains who live 4200 m above sea level frequently have 30% higher hematocrit levels than individuals living at sea level.

People native to high altitudes (highlanders) live in a hypobaric hypoxic environment characterized by a low ambient partial pressure of oxygen. In response to this environment, they develop alveolar hypoxia, hypoxemia, and polycythemia. Healthy highlanders develop pulmonary hypertension, right ventricular hypertrophy, and an increased amount of smooth muscle cells in the distal pulmonary arterial branches, which leads to increased pulmonary vascular resistance and pulmonary artery pressure compared with individuals living at sea level. The importance of these structural changes in the pulmonary vasculature in highlanders is confirmed by the slow decline of pulmonary artery pressure, which is normalized after living for 2 years at sea level. Despite these adaptive changes, healthy highlanders are able to perform physical activities similar to or often even more strenuous than those living at sea level. In fact, there are differences in ventilation rates between athletes performing at sea level and those at high altitudes. Ventilation rates of athletes increase normally during exercise at sea level, but relative hypoventilation occurs in highlanders. This relative hypoventilation is characteristic of Andean natives and has been attributed to desensitization of the carotid bodies to the hypoxic stimulus. The erythrocytosis observed in individuals who reside at high altitudes for relatively short periods of time (days) can also be attributed in part to excessive water loss and contraction of the plasma volume. Total acclimatization of an individual who moves from sea level to a high altitude may actually require years. Individuals who reside at sea level and are acutely exposed to high altitudes are at increased risks of developing a deep venous thrombosis, pulmonary infarction, retinal hemorrhage, or ischemic digits because of increased blood viscosity. High-altitude climbers frequently combat these problems by intravenous administration of isotonic saline, with considerable success.

The chronic responses of various ethnic and racial groups to high altitudes are quite variable. Andean natives, known as the Quechua and Aymara Indians, experience a gradual increase in their hemoglobin levels with age. In addition, hemoglobin values are almost 10% higher in those living at 5500 m above sea level than in those living at 4355 m above sea level. Curiously, their Tibetan and Ethiopian counterparts living at similar altitudes do not respond to the resultant chronic hypoxia by increasing their hematocrits. It has been suggested that high levels of nitrous oxide in the exhaled breath of Tibetans may improve oxygen delivery by inducing vasodilatation and increasing blood flow to tissues, thus making the compensatory increased RBC volume unnecessary. Interestingly, Tibetans and Ethiopians have lived much longer as mountain dwellers than the Quechua or Aymara Indians, suggesting that extreme elevation of the RBC mass is a maladaptation that Tibetans and Ethiopians have avoided by adopting more physiologic compensatory mechanisms. Many residents of the Tibetan plateau reside at elevations exceeding 4000 m and experience oxygen concentrations that are about 40% lower than experienced at sea level. Human adaptation to a high-altitude environment is believed to be the result of advantageous genetic mutations and selective pressure. These genetic adaptations are shared by common ancestors within East Asian but not Central and South Asian populations and confer characteristics including adaptation to hypoxia, the absence of chronic mountain sickness (CMS), and high offspring survival rates. Polymorphisms in the *EPAS1* gene that encodes HIF-2α, and the *EGLN1* gene, which encodes PHD2, have been positively selected and have been shown to be associated with the key adaptive features in Tibetans. The putative advantageous haplotypes of *EGLN1* and *EPAS1* have revealed negative correlations with hemoglobin levels in Tibetans compared with lowlander Han Chinese. A variant in EGLN1, c.[12 C>G; 380 G>C], contributes functionally to the Tibetan high-altitude phenotype. PHD2 triggers the degradation of HIFs. The *PHD2* p.[Asp4Glu; Cys127Ser] variant exhibits a lower Km value for oxygen, suggesting that it promotes increased HIF degradation under hypoxic conditions. Whereas hypoxia stimulates the proliferation of WT erythroid progenitors, the proliferation of progenitors with the c.[12 C>G; 380 G>C] mutation in *EGLN1* is significantly impaired under hypoxic culture conditions. The c.[12 C>G; 380 G>C] mutation abrogates hypoxia-induced and HIF-mediated augmentation of erythropoiesis, which provides a molecular mechanism for the observed protection of Tibetans from polycythemia at high altitude.

This individual variability of elevation of serum EPO levels in high-altitude dwellers and the resultant increase in RBC mass appears widespread. For example, acclimatization to moderately high altitudes when combined with low-altitude training (so-called *living high*, *training low*) improves sea-level performance in endurance athletes, in part because of the erythropoietic effects of altitude exposure. This substantial individual variability in response to all forms of altitude training correlates with improved athletic performance and with elevation of EPO levels. A large component of this individual variability appears to be related to differences in the peak and rate of decay of the increase in EPO in response to altitude exposure. These observations suggest that genetically determined variables account for individual responses to hypoxia.

Chronic CMS is a pathological loss of adaptation to altitude. CMS is a clinical syndrome that occurs in a fraction of natives or lifelong residents living above 2500 m. Prevalence of CMS has considerable variability in different high-altitude parts of the world. The prevalence of CMS increases with age and varies among the mountainous regions of the world. CMS is an adult disease, and its prevalence is the lowest in Ethiopians (<1%) and Tibetans (~1%), higher in South Asian Indians (6.2%) and Han Chinese relocated to high altitudes (6%), and highest among South American Andeans (15%). This variability is mostly attributed to differences in ethnicity. Various ethnic groups have had different periods of adaptation to chronic hypoxia. Those living at high altitudes for the longest time are more likely to be better adapted. It appears that native Tibetans and Ethiopians are the most adapted ethnic groups as compared to Andeans and Han Chinese immigrants. CMS is characterized by excessive erythrocytosis (females, Hb >19 g/dL; males, Hb >21 g/dL); severe hypoxemia; and in some cases, moderate or severe pulmonary hypertension that may lead to the development of cor pulmonale and congestive heart failure. The clinical picture of CMS gradually disappears after descending to lower altitudes and reappears after returning to high altitudes. The prevalence of CMS is higher in men than women and increases with altitude, aging, associated lung disease, history of smoking, and air pollution. CMS is a public health problem in those living in mountainous regions of the world above 2500 m. In China alone, 80 million people live above that altitude, while in South America, 35 million people live above 2500 m. The CMS Andean phenotype has been associated with a single-nucleotide polymorphism (SNP) in the Sentrin-specific Protease 1 (*SENP1*) gene. The *SENP1* gene encodes for a protease that regulates the function of hypoxia-relevant transcription factors such as HIF and GATA1 and thus regulates erythropoiesis in individuals with CMS by modulating expression of EPO and the EPOR. The critical role of GATA1 in erythropoiesis is attributed to its ability to drive expression of many erythroid specific genes including enzymes involved in heme biosynthesis, hemoglobin, and the EpoR (see Chapter 27). *SENP1* has been shown to regulate EPO production by regulating the stability of HIF1 α during hypoxia, and indeed *SENP1*$^{-/-}$ mice die of anemia during early life. *SENP1* also mediates a positive-feedback loop under hypoxic conditions that is responsible for VEGF production and angiogenesis. SENP1-mediated regulation of EpoR expression is likely to occur through GATA-1. Compared with normoxic controls, hypoxic conditions induce a significant downregulation of SENP1 expression in non-CMS cells and an upregulation in CMS cells. Therefore, it is likely that hypoxia in patients with CMS induces a higher expression of SENP1 which favors exaggerated erythropoiesis. Thus Andeans, but not Tibetans and Ethiopians, possess a proerythropoietic SNP rather than SNPs that protect individuals from developing erythrocytosis. The presence of *SENP1* C/G or C/C SNPs are significantly associated with more severe forms of CMS. SENP-dependent stabilization of HIF2α would be anticipated to lead to increased Epo expression. Surprisingly, elevated Epo values are uncommon in CMS patients, yet a subgroup of CMS patients do have significantly higher Epo levels which is associated with slightly higher hematocrits. These CMS patients with high and normal EPO levels likely reflects genetic differences between these two populations. The development of CMS is associated with relative alveolar hypoventilation. Healthy highlanders characteristically hyperventilate. A gradual decline in the rate of alveolar ventilation in these individuals leads to progressive loss of adaptation to chronic hypoxia and the development of CMS. The main components of this syndrome include (1) alveolar hypoventilation leading to relative hypercapnia and increasing hypoxemia; (2) excessive erythrocytosis leading to increased blood viscosity and expansion of the total lung blood volume; (3) pulmonary hypertension and right ventricular hypertrophy that may evolve to hypoxic cor pulmonale and heart failure; (4) neuropsychiatric symptoms, including sleep disorders, headache, dizziness, and mental fatigue; (5) proteinuria in spite of a normal glomerular filtration rate; and (6) a contracted plasma volume which might potentially lead to more extreme polycythemia and clinical symptoms.[12]

Physical examination reveals cyanosis of the nail beds, ears, and lips in contrast to the ruddy complexion that is characteristic of a healthy highlander. In some cases, the face is almost black, and the mucosa and conjunctiva are dark red. The fingers are frequently clubbed, and auscultation of the heart reveals an increased pulmonary second sound. The patients are frequently hypertensive and have evidence of heart failure. Chest radiographic and electrocardiographic findings are characteristic of right atrial and right ventricular hypertrophy. Criteria for the diagnosis of CMS have been published and are useful in identifying CMS patients as well as monitoring their response to treatment.

The definitive treatment for CMS is descent to lower altitudes or sea level. The degree of polycythemia decreases after a few weeks or months, and eventually the hematocrit level returns to sea-level values. Pulmonary hypertension and right ventricular hypertrophy gradually resolve and disappear after 1 to 2 years of living at sea level. Phlebotomy or isovolemic hemodilution can reduce the excessive erythrocytosis and hyperviscosity, improve oxygenation, and lead to relief from symptoms. Due to its transient effects phlebotomy is not a long-term treatment for CMS since at high altitude hematocrit reaches or exceeds pretreatment values within a few weeks. A variety of drugs has also been evaluated for the treatment of patients with CMS. Ten weeks of the respiratory stimulant medroxyprogesterone acetate at doses of 60 mg/day was reported to lead to a reduction of hematocrit levels from 60% to 52% and an increase in arterial oxygen saturation from 84% to 90% in 17 highlanders with CMS. Medroxyprogesterone use, however, was associated with a loss of libido in men and therefore is infrequently used in this population. Therapy with almitrine, a respiratory stimulant, or enalapril, an ACE inhibitor (10 mg/day for 30 days), has resulted in even more modest reductions of hematocrit levels. Therapy with acetazolamide is the most useful treatment for CMS. Acetazolamide therapy has been evaluated in two double-blind, placebo-controlled, randomized clinical trials of patients with CMS. Acetazolamide is an inhibitor of carbonic anhydrase and stimulates ventilation by promoting the development of metabolic acidosis. Furthermore, acetazolamide reduces renal EPO production. Patients with CMS were randomized to receive placebo or acetazolamide therapy at a dose of 250 or 500 mg/day for 21 days. Drug therapy at both doses of acetazolamide resulted in reduction of hematocrit levels by 7% and serum EPO levels by 50% to 67%, with an increase in nocturnal oxygen saturation levels of 5%. Presently, 250 mg of acetazolamide daily appears to be an inexpensive, nontoxic, and effective therapy for CMS.

Smokers' Polycythemia or Carbon Monoxide-Induced Polycythemia

Smoking is the most common cause of secondary polycythemia. Those affected have a carboxyhemoglobin-induced increase in RBC mass or decrease in plasma volume, either of which is reversible with smoking cessation. Excessive carbon monoxide exposure can also be attributed to exposure to industrial emissions and automobile exhaust. Carbon monoxide binds to hemoglobin with a more than 200-times greater affinity than oxygen, resulting in not only occupation of one of the heme groups of hemoglobin but also an increase in the O_2 affinity by the remaining heme group. Individuals smoking even one pack of cigarettes a day frequently have elevated hematocrit levels. These patients characteristically have normal blood gases and elevation of carboxyhemoglobin levels, resulting in a reduction in $P_{50}O_2$. The elevation of the hematocrit level is reversed with interruption of the smoking behavior. Increased hematocrit levels have been observed in 3% to 5% of heavy smokers. Although these patients are not immune to thrombotic complications, the number of thromboembolic events is lower than in patients with PV. Polycythemia has also been reported to be associated with hookah use. A hookah is an oriental pipe containing tobacco often mixed with molasses and fruit flavors connected by a long flexible tube that draws the smoke to the bowl of water. Hookah use exposes the user to generous amounts of carbon monoxide, resulting in erythrocytosis.

Postrenal Transplantation Erythrocytosis

Postrenal transplantation erythrocytosis (PTE) is a potentially dangerous condition found in approximately 10% to 15% of renal allograft recipients. PTE usually develops within 8 to 24 months after a successful renal transplantation and resolves spontaneously within 2 years in about 25% of patients. There is no clear definition of PTE, but general consensus is hematocrit greater than 53% in men and greater than 51% in women that persists over a period of 3 to 6 months without other explanation for erythrocytosis. PTE is more common in males and may recur in the same patient after a second successful renal transplantation. Factors that increase the likelihood of its development are a lack of EPO therapy before transplantation, a history of smoking, diabetes mellitus, transplant-related renal artery stenosis, retention of the native kidney, low serum ferritin levels, and normal or high EPO levels prior to transplantation. PTE is also more frequent in patients who do not experience rejection. There is some evidence that the use of cyclosporine as an immunosuppressive agent is associated with increased risk of PTE. At higher hematocrit levels (usually >60%), thrombotic events may complicate the clinical course of patients with PTE.

Approximately 60% of patients with PTE experience malaise, headache, plethora, lethargy, and dizziness. In addition, from 10% to 20% develop thromboembolic complications involving either arteries or veins. Retention of the native kidney is essential for the development of PTE in most cases. Although the transplanted kidney produces EPO under normal regulatory mechanisms, the native kidney overproduces EPO despite the development of erythrocytosis. Frequently, the PTE resolves with removal of the native kidney. Plasma EPO levels can be normal or high in patients with PTE.

The molecular basis of PTE remains unclear; Ang II is believed, however, to play an important role in its pathogenesis by sustaining the secretion of EPO. Growing evidence indicates that increased AT1R expression makes erythroid progenitor cells hypersensitive to Ang II. Furthermore, Ang II can modulate release of EPO and IGF-1. Androgens are thought to play a role in the development of the syndrome. Androgens can directly affect erythroid progenitor cells or stimulate EPO production or actually the RAS. Treatment of patients with PTE includes intermittent phlebotomy or administration of an ACE inhibitor which suppresses the renin–angiotensin pathway and virtually eliminates the need for therapeutic phlebotomy in these patients. Maximal reduction of hemoglobin levels is evident 6 months after starting therapy with either an ACE inhibitor or angiotensin receptor blockers. Some patients are exquisitely sensitive to these medications and may become severely anemic. Therapy is usually begun at hematocrit levels above 55% with the hope of maintaining hematocrit levels below 50% to reduce the risk of thrombosis. ACE inhibitor fosinopril in an open-label crossover trial with theophylline was shown to produce a dramatic reduction in hematocrits in patients with PTE (51.3% to 43.7%; $P < .005$). Low doses of ramipril, another ACE inhibitor, normalized the hematocrit level in 26 out of 27 patients with PTE after a mean of 127 days of therapy.

Polycythemia Accompanying Kidney and Liver Diseases and Neoplastic Disorders

Polycythemia has been reported in association with kidney diseases such as renal cell carcinoma, renal artery stenosis, hydronephrosis, Wilms tumor, polycystic kidney disease, paragangliomas, and pituitary adenomas. Renal tumors account for approximately one-third of cases of tumor-associated polycythemias and secondary erythrocytosis can be seen in 1% to 5% of patients with renal cell carcinomas. The tumor tissue has been demonstrated to produce excessive amounts of EPO, and the erythrocytosis resolves with surgical resections of the tumor. The mechanisms underlying the activation of the *EPO* gene have been related to somatic mutations of the *VHL* gene in clear-cell renal carcinomas causing increased expression of EPO mRNA, HIF-1α, and HIF-2α. These abnormalities are related to a point mutation in the *VHL* gene that impaired the bindings of HIF-1α to VHL, leading to its accumulation and increased production of EPO. Polycythemia is also a well-described paraneoplastic manifestation of hepatocellular carcinoma in 2.5% to 10% of patients and is again caused by the production of EPO by the tumor. Polycythemia in hepatoma patients is strongly related to tumor burden and elevated α-fetoprotein levels. Polycythemia has also been associated with cerebellar hemangioblastomas and very large uterine fibromas. In tumor-associated erythrocytosis, EPO production has been shown to be autonomous of hypoxic stimuli. Paragangliomas are tumors arising from extraadrenal paragangliar neural crest cells. Most paragangliomas arise retroperitoneally or within the peritoneal cavity and are associated with high EPO levels and erythrocytosis. After tumor resection, reduction of the EPO levels and erythrocytosis occurs. Germ-line mutations involving two Krebs cycle enzymes, fumarate hydratase and succinate dehydrogenase, have been implicated in the hereditary paraganglioma–pheochromocytoma syndrome. In tumors deficient in these enzymes, fumarate and succinate accumulate, which inhibits PHD, leading to accumulation of HIF-1/HIF-2 and eventually resulting in erythrocytosis. In another patient with a paraganglioma who did not have the inherited syndrome but had extreme erythrocytosis, a germ-line mutation in *PHD2* was demonstrated. In this patient's tumor, tumor analysis showed LOH with the mutant allele predominating, which was associated with a loss of function. The degradation of both HIF-1α and HIF-2α was decreased. These data indicate that PHD2 can act as a tumor suppressor gene. The tumor suppressor activity of PHD2 has also been reported in sporadic endometrial, breast, and pancreatic cancers, with inactivating mutations ultimately leading to the increased production of growth factors that contribute to tumor growth.

Two plasma cell dyscrasias, **P**olyneuropathy, **O**rganomegaly, **E**ndocrinopathy, **M**onoclonal protein, **S**kin changes (POEMS) syndrome and **T**elangiectasias, **E**rythrocytosis with elevated erythropoietin levels, **M**onoclonal gammopathy of unknown significance [MGUS], **P**erinephric fluid collections, and **I**ntrapulmonary shunting syndrome (TEMPI) have recently been reported to be associated with erythrocytosis. Treatment is directed at the underlying disease. In TEMPI syndrome (22 patients reported in 11 countries worldwide as of December 2019), the most effective treatment has been plasma cell dyscrasia-directed therapies such as bortezomib, lenalidomide, and daratumumab, suggesting that TEMPI syndrome is a consequence of the abnormal plasma cell clone.[13]

Polycythemia in Endocrine Disorders

Polycythemia is also associated with Cushing syndrome, acromegaly, and primary aldosteronism. Secondary polycythemia can also be seen in men receiving long-term androgen-replacement therapy. A meta-analysis of randomized trials of testosterone therapy in hypogonadal men showed that participants in testosterone arms had statistically significant higher frequency of erythrocytosis compared to placebo.[14] The endocrine society guidelines suggest stopping therapy if hematocrit levels increase to greater than 54%. When hematocrit normalizes, a lower dose of testosterone can be attempted. Secondary polycythemia can also be seen in a significant number of competitive athletes taking anabolic steroids. For instance, in a small study of professional bodybuilders, 67% of those examined had hematocrit levels above 50%. Recombinant human EPO has also been abused by athletes competing in endurance sports and can be detected by analyzing the individual's hematocrit level, reticulocyte count, percentage of macrocytic RBCs, serum EPO level, serum transferrin receptor level, and electrophoretic mobility of the EPO molecule (recombinant EPO is less negatively charged than endogenous EPO).

Congenital Secondary Polycythemias

High-oxygen-affinity hemoglobins and bisphosphoglycerate deficiency. More than 100 mutations of hemoglobin lead to increased oxygen affinity and thus decreased oxygen delivery and compensatory polycythemia (see Chapter 44). These mutations involve either the α- or β-chain globin chains. Such polycythemias are usually well tolerated in young patients but may lead to thrombotic complications in older patients. High-oxygen–affinity hemoglobin variants are transmitted as autosomal dominants. Phlebotomy therapy of such patients has not been reported to be of any beneficial value and has been shown to decrease exercise tolerance. The best test to detect high-oxygen–affinity hemoglobin relies on the determination of hemoglobin dissociation kinetics and P_{50} (partial pressure of O_2 at which hemoglobin is 50% oxygenated). If cooximetry is not available, the P_{50} can be mathematically estimated from a venous blood gas measurement by using a computer algorithm, which is available online.

Whereas a $P_{50}O_2$ below 17 mmHg is indicative of a mutant hemoglobin with high oxygen affinity, a level above 35 mmHg is strongly suggestive of a mutant hemoglobin with a low oxygen affinity. Hemoglobin electrophoresis is not a reliable screen to rule out such a hemoglobin mutation because only about half of these mutants are electrophoretically distinguishable. Hemoglobin electrophoresis will reveal the presence of an abnormal hemoglobin only if the mutation leads to a change in electrical charge. If such a charge differential is not present, high-performance liquid chromatography or mass spectrometry followed by routine DNA sequencing of both α- and β-globin genes is required to establish the diagnosis.

A rare cause of congenital polycythemia is 2,3-BPG (previously known as 2,3-DPG) deficiency (see Chapter 44). 2,3-BPG is synthesized in RBCs and binds to hemoglobin, thereby reducing its affinity for oxygen. An absence of 2,3-BPG therefore leads to increased affinity of hemoglobin for oxygen, resulting in a lifelong hypoxic stimulus and erythrocytosis.

Congenital methemoglobinemias, whether caused by cytochrome b5 reductase mutations or globin mutations, may be associated with mild polycythemia (see Chapter 45). When hemoglobin is oxidized to methemoglobin, all of the four subunits of the tetramer may be affected, eliminating the oxygen transport capacity of hemoglobin.

Hypoxia-Inducible Factor Pathway Mutations Leading to Erythrocytosis

Alterations of a number of proteins in the HIF pathway have been shown to lead to increased EPO production and erythrocytosis (see Fig. 70.1). Chuvash polycythemia (CP) is an autosomal recessive disorder associated with germ-line mutations of *VHL* and erythrocytosis. CP was first described in the mid-1970s and is endemic in the Chuvash population of the Russian republic. The serum EPO concentration in affected individuals is elevated compared with healthy first-degree family members, although some patients may have normal serum EPO levels. They also have elevated levels of VEGF and plasminogen activator inhibitor-1. The erythroid progenitors of patients with CP are also hypersensitive to EPO; thus, CP has characteristics of both primary and secondary polycythemias. A homozygous missense mutation in the *VHL* gene, VHL598C→T mutation, was identified in CP patients. The disorder is characterized by high hemoglobin levels (usually >20 g/L), increased plasma EPO levels, varicose veins, vertebral hemangiomas, low blood pressure, and an elevated concentration of VEGF. The defective *VHL* gene product is not capable of promoting the ubiquitin-mediated degradation of HIF-1α and HIF-2α, thereby leading to increased levels of both of these proteins and increased elaboration of EPO. The CP-VHL mutants have been shown to affect EPO signaling in erythroid progenitor cells of patients with CP. JAK2 phosphorylation of STAT5 triggers not only erythroid progenitor development but also a negative feedback mechanism by transactivating the expression of SOCS family members, which bind and inhibit activated JAKs by promoting their ubiquitination and protosomal degradation. The CP mutation causes conformational changes, leading to a tight CP–VHL–SOCS-1 association, thereby slowing phosphorylated JAK2 degradation. These events lead to hyperactivation of the JAK2–STAT pathway in erythroid progenitor cells, causing their hypersensitivity to EPO, which likely contributes to the excessive erythrocytosis characteristic of CP. The frequency of the allele has been estimated to be at 0.057 in the Chuvash population. Several other similar types of mutations in VHL have been observed in a variety of other ethnic groups. A number of heterozygotes with erythrocytosis have been reported, which can likely be accounted for by an as yet undiscovered additional defect. CP is associated with a high mortality rate from thrombotic and hemorrhagic vascular complications. Most patients complain of chronic headache, fatigue, and/or lower extremity pain, and are noted to have lower blood pressures. Cerebrovascular events are especially common causes of death. The median age of death from cerebrovascular events is 42 years. Estimated survival to age 65 years is 29% for individuals with CP and 64% for age-matched community members. There is a perfect genotype–phenotypic correlation, with all patients with CP being homozygotes for the mutation. Interestingly, heterozygous carriers do not develop erythrocytosis but do have lower blood pressures and do not seem to be at increased risk for tumors, which contrasts with patients with VHL syndrome. Patients with CP also have larger livers, spleens, and kidneys than control patients, due to enhanced cellular proliferation caused by HIF-2α suppression of p21Cip1.

Germ-line mutations of *VHL* alleles have been reported in several patients with apparent congenital polycythemia that had no evidence of developing tumors; some of these subjects had germline mutations of both *VHL* alleles. Other subjects with congenital polycythemia of various ethnicities, including Pakistanis, Punjabis, African Americans, and Whites, harboring homozygosity for the CP *VHL* mutation or double heterozygosity for CP and other *VHL* mutations, have been found. The VHL598C→T mutation has been shown to originate in a single haplotype in the Chuvash patients, as well as in Whites, South Asian Indians, and one African American individual. Homozygosity of this mutation appears to be the most common genetic defect leading to congenital polycythemia. The mutation originated from a single founder 12,000 to 51,000 years ago. This suggests that such wide dissemination from the original founder may be associated with some survival advantage for heterozygotes carrying this mutation. Such an advantage might be related to a subtle improvement of iron metabolism, erythropoiesis, embryonic development, energy metabolism, or some other yet unknown effect.

VHL mutations are likely the most common cause of congenital polycythemias, greatly exceeding the number of patients with hemoglobin chain mutations. In point of fact, in one study of more than 200 unrelated subjects with apparently congenital polycythemia, none had a 2,3-BPG deficiency, two had a globin mutation (Hgb Vanderbilt and Hemoglobin San Diego), and 12 had *EPOR* mutations to account for their polycythemia. By contrast, when 50 patients were examined for the *VHL* mutation, 22 had *VHL* mutations, most of them being the CP *VHL* mutation, either being homozygous or in combination with another *VHL* mutation. The failure of patients with CP to develop VHL syndrome tumors is consistent with the concept that dysregulation of H1F-1α and VEGF are not sufficient to cause tumors. A cluster of patients with clinical features identical to patients with CP has been documented on the island of Ischia in the Bay of Naples, Italy. All of these patients also had the VHL598C →T mutation. Twelve of the 14 patients were homozygotes, and two were heterozygotes. The homozygotes had symptoms identical to patients in Chuvashia. Unlike heterozygotes in Chuvashia, the heterozygotes from Ischia developed erythrocytosis associated with high EPO levels, which raises the possibility that genetic alterations of additional components of the oxygen-sensing pathway other than VHL may contribute to the development of erythrocytosis in the Italian patients. The disorder in Ischia has a gene frequency even higher than that in Chuvashia, with 14% of the population estimated to be heterozygotes, which again has led to the suggestion that heterozygotes have a survival advantage, perhaps conferring protection from developing anemia. Recently a new VHL cryptic exon (termed E1') was identified deep in intron 1, which is naturally expressed in many tissues. Mutations in E1' were identified in seven families with erythrocytosis and in one family with typical VHL disease but without any alterations in other VHL exons. These mutations induced dysregulation of VHL splicing with excessive retention of E1' and downregulation of VHL protein expression.[15]

The Chuvash mutation has been shown to have a profound effect on the cardiopulmonary system. Patients with CP have significant abnormalities of cardiopulmonary physiology, including elevated basal ventilation rates and increased pulmonary vascular tone, with extremely high ventilatory rates and heightened pulmonary vasoconstriction and heart rates in response to acute hypoxia. These observations indicate that the VHL–HIF pathway might also play a central role in calibrating the pulmonary system to hypoxic challenges. Such undesirable exaggerated hypoxic responses and the resulting elevated pulmonary vascular hypertension may contribute to the morbidity and mortality associated with CP. Increased expression of endothelin-1 has been documented in patients with CP. Endothelins are vasoconstricting peptides produced primarily in the endothelium which play a pivotal role in vascular constriction. Hypoxia stimulates endothelin expression via HIF-1, while endothelins stabilize HIF-1 leading to expression of VEGF. Endothelin-1 has been associated with the development of hypoxia-related pulmonary hypertension. Endothelin-1 receptor inhibitors might therefore be useful in reversing the associated pulmonary hypertension in these patients. Treatment strategies for patients with CP have included phlebotomy, aspirin, and occasionally chemotherapy. Many patients are treated empirically with phlebotomy therapy, but this treatment is controversial and some experts believe that repeated phlebotomies can actually increase the risk of thrombotic events. The use of the JAK1/2 inhibitor, ruxolitinib, has been shown to be effective in reducing phlebotomy requirements in three patients with CP. More extensive evaluation of this treatment strategy is clearly warranted. Recently, administration of the allosteric HIF-2α inhibitor, belzutifan, to animal models of CP has been shown to decrease EPO production and reverse polycythemia. Drug treatment also decreased right ventricular pressure and pulmonary hypertension in these mice to near normal levels and normalized the movement of the cardiac interventricular septum. These data indicate that oral intake of belzutifan might be a potential effective treatment of CP patients with polycythemia, pulmonary hypertension, pulmonary fibrosis, and complications caused by elevated expression of HIF-2α.[16]

Additional genetic abnormalities of oxygen sensing have been reported that lead to familial polycythemia. A heterozygous C-to-G change at base 950 of the coding sequence of *PHD2* (*P317R* mutation) was first detected in three affected family members with this syndrome. Hydroxylation of HIF-1 by PHD2 facilitates its interaction with VHL, thereby favoring its ubiquitination and degradation by the proteosome. The failure of *PHD2 P317R* to bind HIF-1 and HIF-2α and promote HIF hydroxylase activity ultimately leads to increased HIF-1/HIF-2 and EPO levels, resulting in the development of erythrocytosis. The *PHD2 P317R* mutation is inherited as an autosomal dominant trait in contrast to the CP defect, which is an autosomal recessive disorder. Polycythemic patients with *PHD2 P317R* do not have any evidence of tumors characteristic of the VHL syndrome. Additional mutations in *PHD2* have been reported that lead to erythrocytosis. Over 20 erythrocytosis-associated PHD2 heterozygous missense, nonsense, and frameshift mutations have been reported. A series of HIF-2 gain-of-function mutations have also been reported to lead to familial erythrocytosis in an autosomal dominant fashion. These mutations lead to weakened bonds between PHD2 and HIF-2, resulting in less hydroxylation of HIF-2 and subsequent recognition of HIF-2 by VHL. Many of these patients present in their twenties, although patients have also been diagnosed in their fifties with erythrocytosis associated with high EPO levels. Interestingly some HIF-2α mutations are also associated with neuroendocrine tumors.

Neonatal Polycythemia

Because of the high oxygen affinity of fetal hemoglobin, many neonates have markedly elevated hematocrit levels. Babies with hematocrit levels over 65% have neurologic and functional impairments, and are more likely to be born to diabetic mothers. Although phlebotomy has been recommended, there is little evidence that it is beneficial for these babies.

Drug-induced Erythrocytosis

The administration of EPO, corticosteroids, or androgens to an excessive degree has each been associated with reversible erythrocytosis. Androgens have been shown to stimulate EPO production and to directly affect erythroid progenitor cells. Approximately 5% of patients receiving long-term testosterone therapy for testosterone-deficiency syndrome develop erythrocytosis. A higher proportion of erythrocytosis has been reported in transmasculine individuals receiving intramuscular testosterone enantate (23.3%) as compared to transdermal testosterone (0%).[17] Androgens activate HIF-1 and HIF-1-regulated gene expression, leading to increased expression of VEGF and presumably EPO. Rarely, erythrocytosis can be the presenting manifestation of Cushing syndrome. Blood doping refers to the use of EPO to increase the RBC mass and enhance oxygen delivery with the hope of increasing endurance performance in athletes. A monitoring of an athlete's hematologic profile longitudinally provides some insight into the likelihood of blood doping playing a role in the development of erythrocytosis.

The development of paradoxical polycythemia in three patients with VHL syndrome and CNS or retinal hemangioblastomas treated with a VEGF receptor inhibitor has been reported. The cause of these phenomena remains unknown. In addition, reversible paradoxical erythrocytosis has been reported with the use of VEGF tyrosine kinase inhibitors, including sunitinib, sorafenib, axitinib, pazopanib, and the anti-VEGF monoclonal antibody bevacizumab, primarily in patients with renal cell carcinomas. These patients become symptomatic due to fatigue, pruritis, myalgias, headaches, and malignant hypertension. The cause of the erythrocytosis associated with these drugs has been attributed to either elevation of EPO levels or sensitization of erythroid cells to EPO. The erythrocytosis in this situation can be controlled with phlebotomy therapy with relief of symptoms.

POLYCYTHEMIA VERA

PV is a clonal, chronic, progressive myeloproliferative neoplasm (MPN) often of insidious onset, characterized by an absolute increase in RBC mass and often by leukocytosis, thrombocytosis, splenomegaly, and driver mutations affecting JAK2. PV leads to excessive proliferation of erythroid, myeloid, and megakaryocytic elements within the BM. Vaquez first described this clinical entity in 1892, noting the characteristic physical findings. At the turn of this century, Cabot and Osler independently associated the name PV with this clinical disorder.

PV differs from many other hematologic malignancies in that prolonged survival is enjoyed by most patients if the excessive production of RBCs and platelets can be controlled. This prolonged survival, however, can be punctuated by the development of other syndromes, such as myelofibrosis (MF), termed *post-PV MF*, and a form of acute leukemia termed MPN-blast phase (MPN-PB) (Fig. 70.3). Frequently, patients present at the time of a thrombotic event or when asymptomatic to a physician only to find that they have splenomegaly, isolated erythrocytosis, or thrombocytosis. Left untreated, these patients will become progressively symptomatic owing to the excessive production of RBCs, platelets, and white blood cells, leading to arterial or venous thromboses, aquagenic pruritus, and systemic symptoms caused by increasing splenomegaly. After a number of years, the erythrocytotic phase of the disease can become inactive, and the patient may no longer have the sequelae of excessive RBC production. Subsequently, these patients can develop post-PV MF, which is frequently indistinguishable from another MPN, primary MF (PMF) (see Chapter 72). Finally, a significant proportion of these patients will go on to develop MPN-BP. Most patients undergo this orderly transition, but some patients transition from the polycythemic phase directly into an MPN-BP or a myelodysplastic disorder. The outcome of patients with PV is largely influenced by the increased risk of thrombosis, evolution to PV-related MF, and MPN-BP. Patients who are at the greatest risk to progress to post PV-MF or MPN-BP frequently have additional mutations in myeloid malignancy genes including TP53 and genes encoding chromatin and spliceosome regulators.[18]

The transition from one phase of MPN to another is not necessarily unidirectional. About 10% to 15% of patients with *JAK2V617F*-positive essential thrombocythemia (ET) eventually develop erythrocytosis and are reclassified as having PV (see Chapter 71). A number of cases of presumed PMF either spontaneously or after chemotherapy have been described where the patient develops erythrocytosis and a syndrome that is virtually indistinguishable from de novo PV. The constantly changing clinical picture of this malignant hematologic disorder requires careful observation and treatment to deal with the numerous problems that can be encountered.

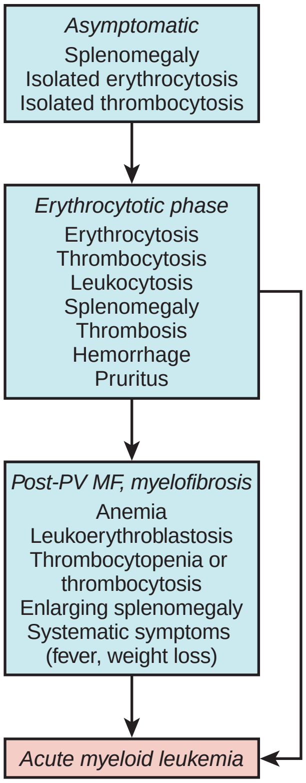


Figure 70.3 EVOLUTION OF POLYCYTHEMIA VERA. *MF*, Myelofibrosis; *PV*, polycythemia vera.

Epidemiology

PV is the most common primary polycythemia. Incidence rates (IRs) have been reported to be around 2.8 per 100,000 persons per year. It is important to emphasize that no or very few population-based estimates of the prevalence of this disorder are presently available. Actual determination of its prevalence has been a difficult process because of the need in the past to pursue an extensive diagnostic evaluation to differentiate this disorder from other causes of spurious or absolute erythrocytosis. The diagnosis of PV has been simplified by the identification of PV-associated mutations (*JAK2V617F* and *JAK2* exon 12 mutations), permitting for the first time molecular epidemiologic studies. The prevalence of the *JAK2V617F* mutation in a normal population in Denmark was recently determined to be 0.2%, with 63% of these individuals not having a previously detected hematological malignancy. The presence of the mutation was associated with increasing age, male sex, and a lower cumulative survival. Recently available 2001 to 2012 data from the Surveillance, Epidemiology and End Results (SEER) Program from the US National Cancer Institute provided new insight into the IR of the MPNs. This study utilized the World Health Organization (WHO) Diagnostic Criteria and during the last 5 years of the study *JAK2V617F* testing was included. The age-adjusted IR of PV and ET were 10.9 and 9.6 per 1 million per year, respectively. The IR for PMF was 3.1. In order to grasp the magnitude of the IR of these various MPNs, the IR of CML was 3.3, demonstrating that the IR of these Ph^- MPNs were sevenfold higher than CML. Both PV and PMF were more frequent in males while the IR for ET was greater in females. ET, PV, and PMF were associated with lower IRs among Hispanic whites than non-Hispanic whites.[19] Several groups have reported that 50% of patients with a splanchnic vein thrombosis without an overt MPN are *JAK2V617F* positive and that more than 50% of these individuals subsequently develop an MPN. However, the incidence of the mutation with unprovoked thromboembolism in the more usual sites (deep venous thrombosis involving the leg veins or pulmonary embolism) ranges between 0.2 and 1.0%. Such data have led to the conclusion that systematic screening for *JAK2V617* in such a patient population is not warranted.

The prevalence of PV has been reported by several investigators to be higher among American Jews and lower among African Americans and Hispanics. The reported lower incidence in African Americans might reflect a referral bias characteristic of centers with research interest in MPN because the authors, when practicing in several large urban areas, have observed a considerable number of African Americans with MPNs, including PV. The incidence of the disorder is greater among Ashkenazi Jews, who originate from eastern and central Europe, than among Arabs and Sephardic Jews. Interestingly, extremely low IRs have been reported from Japan. These findings suggest that important genetic factors might be involved in the biogenesis of this disorder. The importance of genetic factors in the origin of this disease is further emphasized by reports of multiple cases of *JAK2V617F* or *JAK2* exon 12 mutation-positive and -negative MPN, including PV, within multiple generations of a number of families. These forms of familial PV must be distinguished from PFCP, CP, and polycythemia associated with mutations in the HIF pathway. The reports of families in which multiple members have PV first raised the possibility that a genetic predisposition to acquire such mutations exists in these families that is inherited in an autosomal dominant pattern with decreased penetrance. Clinical analyses of affected family members confirmed that they have clonal hematopoiesis (CH) and that their clinical manifestations are identical to patients with sporadic PV. In a large population study from Sweden, the relatives of MPN patients were shown to have a significantly increased risk of developing a Ph^- MPN and possibly chronic myeloid leukemia. It was estimated that first-degree relatives of MPN patients have a five- to sevenfold greater risk of developing an MPN, again supporting the hypothesis that common strong susceptibility genes predispose one to develop PV, essential thrombocytosis (ET), PMF, and possibly CML.

One notable exception to the low prevalence of PV in Japan has been the higher incidence observed among populations exposed to atomic bomb explosions. The possibility that radiation exposure is an etiologic factor in the generation of PV was also raised by the observation in the United States of four cases of PV 10 to 20 years after the Smokey nuclear weapons test in which 3000 military observers were exposed. An epidemiologic investigation that focused on occupational exposure among petroleum refinery and chemical plant workers has revealed an increased incidence of PV relative to the general population. In this study, the increased incidence of PV was linked to similar increases in the frequency of multiple myeloma and non-Hodgkin lymphoma, suggesting involvement of a putative environmental toxin that may have broad hematopoietic toxicity. The possibility of environmental factors contributing to the development of PV was raised by the identification of a cluster of patients with *JAK2V617F*-positive PV in an area of Eastern Pennsylvania that contained numerous sources of hazardous materials, including waste coal power plants and United States Environmental Protection Agency Superfund sites.

In several large studies, 5% of patients with PV were younger than 40 years of age, 1% were younger than 25 years at diagnosis, and 0.1% were younger than 20 years. Small numbers of patients with PV have been reported who presented during childhood. Interestingly, females comprise the majority of PV patients diagnosed before the age of 40 years, suggesting a unique risk factor for developing the disease at early age. These young female patients tend to present with unique complications such as splanchnic vein thromboses (SVT). However their risk of transformation to PV-MF or MPN-BP has been thought to be similar to males. Additional risk factors for female PV patients developing SVTs is their use of oral contraceptives and estrogen replacement therapy. Karantos and coworkers has reported provocative data which indicates that male sex serves as a poor prognostic parameter for PV patients. Men had higher JAK2 V617F allele burdens in their $CD34^+$ cells, acquired more somatic mutations apart from the MPN driver mutations, and had an increased frequency of acquiring high-risk mutations. By contrast, disease progression in females was reported to be more dependent on higher JAK2V617F variant allele burdens (VAFs) rather than on acquisition of other somatic mutations (Fig. 70.4).[20]

The *JAK2V617F* mutation has a prevalence of 0.1% to 0.2% in the general population. These individuals usually have a VAF of less than 10%. Some of these patients have an undiagnosed MPN or represent an early molecular stage of a MPN, which will evolve over time. The normal individuals with a low JAK2V617F VAFs are thought to have CH of indeterminate potential (CHIP) (see Chapter 19). Although these individuals with JAK2V617F+ CHIP do not have an overt MPN they have significantly higher platelet counts, white blood cell counts, and hematocrit levels and are at higher risk of developing arterial and venous thrombotic events than normal individual with wild type JAK2. Most of these CHIP patients never develop an MPN but some eventually develop a full-blown MPN.[21]

Pathobiology

Considerable speculation has centered on the pathobiology of the erythrocytosis that characterizes PV. The expanded RBC mass in PV patients is due to a two- to three-fold increase in the production of RBCs by a hyperplastic BM and is not attributable to prolongation of the RBC lifespan. Granulocyte and platelet production are also increased in this disorder. This overly exuberant production of all cellular elements of the blood suggests that the basic defect resides at the level of the pluripotent hematopoietic stem cell (HSC).

Serum EPO levels have been shown to be subnormal in patients with PV, elevated in many but not all cases of secondary erythrocytosis, and normal in patients with relative polycythemia, indicating that PV cannot be an abnormality of EPO production.

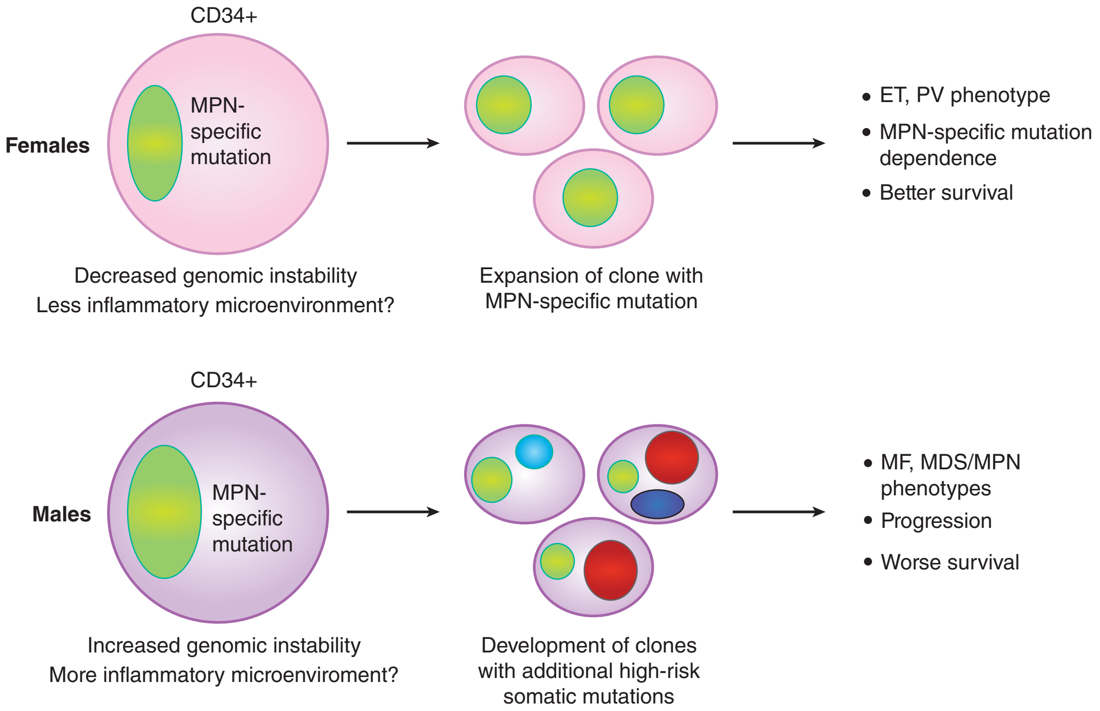

CD34+
Females
MPN-specific mutation
Decreased genomic instability
Less inflammatory microenvironment?
Expansion of clone with MPN-specific mutation
• ET, PV phenotype
• MPN-specific mutation dependence
• Better survival
CD34+
Males
MPN-specific mutation
Increased genomic instability
More inflammatory microenviroment?
Development of clones with additional high-risk somatic mutations
• MF, MDS/MPN phenotypes
• Progression
• Worse survival

Figure 70.4 HYPOTHETICAL MODEL OF THE DIFFERENT BIOLOGY OF MPN DEVELOPMENT AND PROGRESSION BETWEEN WOMEN AND MEN. *ET*, Essential thrombocythemia; *MDS*, myelodysplastic syndromes; *MF*, myelofibrosis; *MPN*, myeloproliferative neoplasm. *(From Karantanos T, Chaturvedi S, Braunstein EM, et al. Sex determines the presentation and outcomes in MPN and is related to sex-specific differences in the mutational burden. Blood Adv. 2020;4[12]:2567–2576.)*

In 1951, Dameshek postulated that chronic myeloid leukemia, PV, essential thrombocythemia, and PMF were related disorders, which he called *myeloproliferative disorders*, but are now termed *MPNs* because of the evidence that these disorders are hematologic malignancies.

Since the mid-1970s, data have accumulated that conclusively demonstrate PV to be the result of a neoplastic proliferation of hematopoietic cells. The cellular origin of the disorder was first established by the analysis of glucose-6-phosphate dehydrogenase (G6PD) isoenzymes in women who were heterozygous for this X-linked gene. This approach was based on the random irreversible inactivation of one X chromosome in each female somatic cell during embryogenesis. Inactivation of the same X chromosome occurs in the progeny of these cells. A normal female heterozygous for G6PD will therefore have approximately equal populations of BM cells with a different G6PD isoenzyme. The G6PD isoenzymes can be readily distinguished by electrophoretic methods.

This approach was exploited in a seminal study by Adamson and coworkers in an effort to determine the cellular origin of PV. They presumed that cells comprising a tumor that arises from a single cell in a G6PD heterozygote would express a single isoenzyme type, but a neoplasm originating from multiple cells would express both isoenzyme types. These investigators found that circulating RBCs, granulocytes, and platelets obtained from female patients who were G6PD heterozygotes express the same isoenzyme, but skin and cultured BM fibroblasts obtained from these same patients demonstrate both isoenzymes. They concluded that PV represented a clonal proliferation of neoplastic HSCs and was not multicellular in origin or the consequence of excessive proliferation of normal HSCs.

The clonality of blood cell production in PV has subsequently been confirmed using restriction fragment length polymorphisms of the active X chromosome. A monoclonal pattern of X chromosome inactivation has been defined in RBCs, granulocytes, monocytes, and platelets in female patients with PV.

On the basis of the knowledge that RBC production in PV is not associated with excessive EPO production, numerous investigators hypothesized that the erythroid progenitor cell in this disorder was no longer subject to physiologic regulators. PV BM can form substantial numbers of erythroid colonies in vitro in the absence of exogenous EPO, but normal human BM is incapable of forming such colonies without the addition of EPO. These erythroid colonies were termed *endogenous colonies*. When erythroid PV and normal BM were subsequently assayed in the presence of EPO, PV BM was characterized by a higher cloning efficiency. Additional observations suggested that the altered response to cytokines was not restricted to EPO but included a variety of other cytokines and that this cytokine hypersensitivity was characteristic of progenitor cells committed to a variety of other lineages.

In addition, BM cells cloned from G6PD heterozygous females with PV in the presence and absence of exogenous EPO revealed that the erythroid colonies that formed in the absence of exogenous EPO contained the same G6PD isoenzyme type as that expressed by peripheral blood elements. Thus, the so-called *endogenous erythroid colonies* arose from the abnormal clone that was responsible for supplying RBCs, granulocytes, and platelets to the peripheral blood. When exogenous EPO was added, increasing numbers of colonies were formed containing cellular elements expressing the other G6PD isoenzymes; presumably, these colonies originated from cells not involved in the malignant process. Similarly, small numbers of granulocyte–macrophage colonies not originating from the PV clone were also observed in these assays. These data collectively indicate the existence of malignant and nonmalignant populations of HPCs in PV BM. The relative frequency of the neoplastic clone in relation to normal progenitor cells was further examined by Adamson and coworkers, who, by monitoring the proportion of neoplastic erythroid clones and their numeric relationship to normal clones over a period of several years, showed disease progression was associated with a significant decline in the frequency of normal progenitor cells and increasing proportion of the neoplastic clone. Erythroid progenitor cells from PV patients are, in fact, abnormally sensitive to the actions of this EPO. This increased responsiveness allows these cells to form colonies in the presence of small amounts of EPO. Most PV patients possess two distinct populations of erythroid progenitor cells: a normally EPO-responsive population and a population of cells similar in proliferative and maturational behavior in vitro but requiring little or no EPO. These investigators suggested that the proliferation of the normal progenitor cells in vivo was at a disadvantage. A number of investigators have demonstrated that the increased responsiveness of these BM progenitor populations extends to their responses to other cytokines, including SCF, IL-3, GM-CSF, IGF-1, G-CSF, and thrombopoietin (TPO).

Identification of *JAK2V617F* Mutation in Polycythemia Vera

For more than two decades a number of research groups searched for the genetic defect underlying PV. Most groups predicted that this defect involved the signaling pathways downstream of the EPOR. These pathways include the tyrosine kinase JAK2 and the transcriptional signal transducers and activators of transcription, STAT3 and STAT5. The initial discovery of a mutation was predicated on a somewhat simplistic yet revealing set of experiments performed by Vainchenker and colleagues in France.[22] They observed that the inhibition of JAK2 by a small molecule (AG490) or by small interfering RNA (siRNA) reduced EPO-independent colony formation by PV BM mononuclear cells. This observation prompted them to directly sequence JAK2 in the hematopoietic cells of PV patients and to discover a single recurrent point mutation. A guanine-to-thymine mutation was observed that resulted in a substitution of valine to phenylalanine at codon 617 within the pseudokinase domain (JH2) of *JAK2* (*JAK2V617F)* (Fig. 70.5). These findings were quickly confirmed by several different groups. Kralovics et al. had previously identified a region of LOH on chromosome 9p in PV and identified a 6.2-Mbp region common to all PV patients screened.[23] Because this region contained *JAK2*, with its known role in erythropoiesis, it was screened for mutations and the same *JAK2V617F* mutation identified. Three other groups targeted *JAK2* as part of a global sequencing screen of tyrosine kinases and phosphatases in MPNs. *JAK2V617F* is an acquired somatic mutation present exclusively in hematopoietic cells. All JAK2V616F+ patients with PV have a population of erythroid progenitor cells that are homozygous for the mutation. Using quantitative PCR, patients can be divided into those with a low allele burden of *JAK2V617F* in granulocytes (<50%) and patients with a higher burden of *JAK2V617F* (>50%). A subset of patients with PV are homozygous for *JAK2V617F*, which is the result of mitotic recombination and duplication of the mutant allele (Fig. 70.6). The occurrence of mitotic recombination has been observed during the clinical course of individual patients, leading to *JAK2V617F* heterozygous patients becoming homozygous over time. The concept of the conversion of *JAK2V617F* low burden to high burden is further supported by the observation that the median duration of disease at the time of evaluation was 48 months in high-burden PV patients compared with 23 months in low-burden PV. These observations are consistent with a multistep pathogenesis of PV. The first step consists of the acquisition of *JAK2V617F*, which results in a low allele burden of *JAK2V617F*, followed by a second step, homologous recombination, that leads to *JAK2V617F* homozygous progenitor cells and eventually granulocytes with a high burden of *JAK2V617F*.

Several groups have recently reported that *JAK2V617F* can be acquired several decades before the appearance of an overt MPN. In fact in some individuals it has been suggested using mathematical modeling that the mutation in *JAK2* is acquired in utero. It remains unknown at present if a lesion occurring prior to acquisition of the *JAK2V617F* mutation predisposes an individual to acquire *JAK2V617F*.

JAK2V617F is present in greater than 95% of PV cases. Approximately 50% to 60% of patients with ET and PMF are also *JAK2V617F* positive. ET is distinguished from PV by being associated with a low allele burden. In the overwhelming majority of cases,

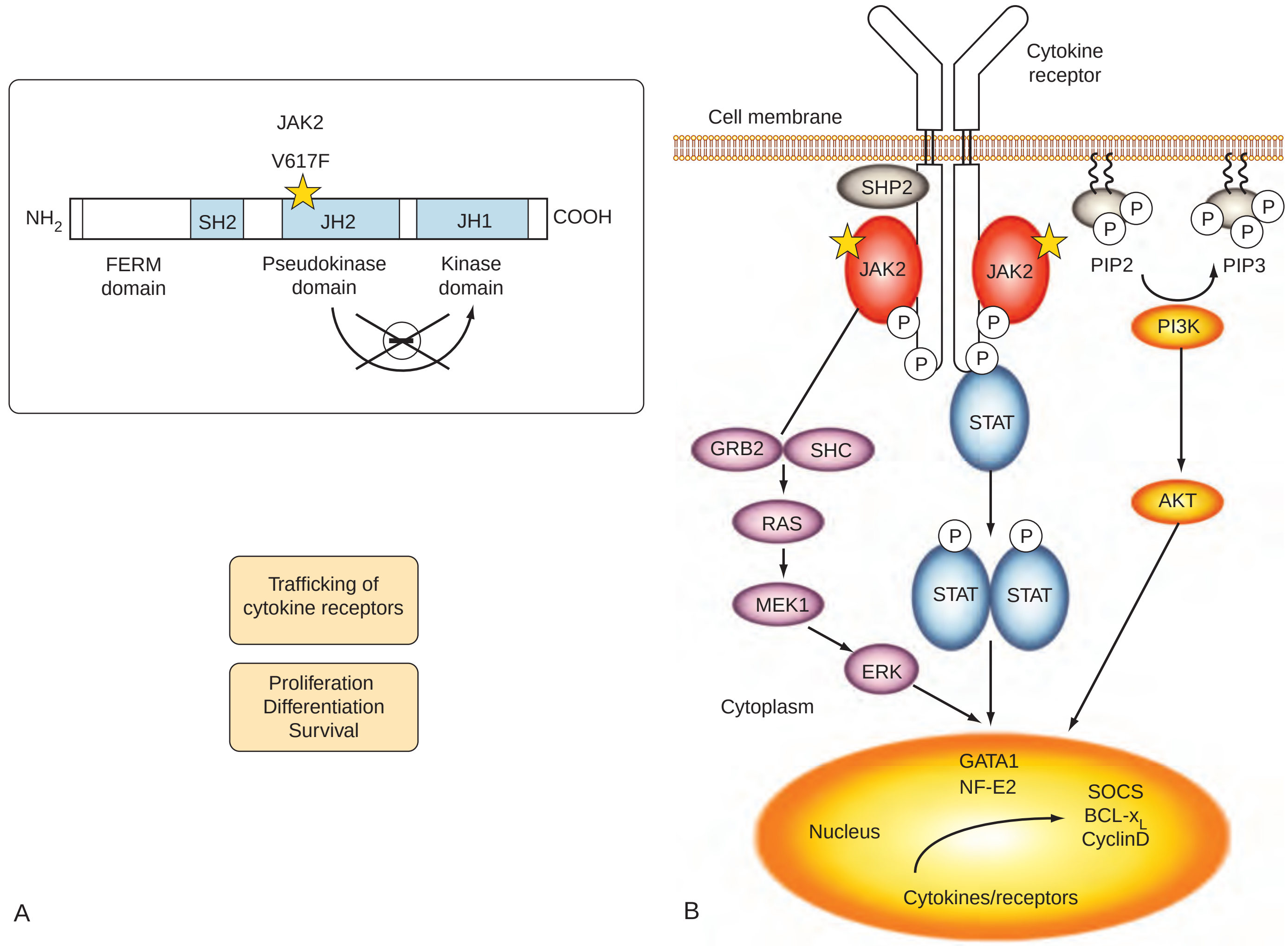


Figure 70.5 *JAK2V617F* SIGNALING IN MYELOPROLIFERATIVE DISORDERS. (A) Structure of *JAK2V617F*: the mutation is located in pseudokinase JAK JH2 and disrupts the autoinhibition of this regulatory domain. Consequently, the tyrosine kinase corresponding to the JH1 domain is constitutively activated. (B) In the presence of a homodimeric cytokine receptor (e.g., erythropoietin receptor), the two *JAK2V617F* proteins bound to the intracellular domain of the receptor transphosphorylate its tyrosine residues. In turn, STAT5, PI3K, and RAS signaling pathways are activated, leading to the downstream modulation of transcription and protein levels for cell cycle, proliferation, and apoptosis-related factors. *Bcl-X*$_L$, B-cell lymphoma-extra large; *ERK*, extracellular signal-related kinase; *GATA*, GATA-binding factor; *GRB*, growth factor receptor-bound protein; *JAK*, Janus kinase; *JH2*, JAK homology domain 2; *MEK1*, dual specificity mitogen-activated protein kinase kinase 1; *NF*, nuclear factor; *P*, Phosphate; *PI3K*, phosphatidylinositol 3-kinase; *PIP2* and *PIP3*, phosphatidylinositol bi- and triphosphate; *RAS*, renin–angiotensin system; *SH2*, src homology 2; *SOCS*, suppressor of cytokine signaling; *STAT*, signal transducer and activator of transcription. *(From Delhommeau F, Pisani DF, James C, et al. Oncogenic mechanisms in myeloproliferative disorders. Cell Mol Life Sci. 2006;6363:2939.)*

PV is characterized by a population of *JAK2V617F* homozygous colonies with some heterozygous and WT colonies. By contrast, in ET, there are few homozygous colonies, with the majority being heterozygous or WT. The majority of ET and PMF patients who are negative for *JAK2V617F* have CH, which indicates that these *JAK2V617F* negative diseases likely are the consequences of other genetic events including mutations in the TPO receptor, MPL, or calreticulin, which are discussed in Chapters 71 and 72. The *JAK2V617F* allele has also been observed in a limited number of patients with chronic myelomonocytic leukemia, myelodysplastic syndromes (MDS), refractory anemia with ring sideroblasts and thrombocytosis (see Chapter 73), and acute myeloid leukemia (AML), although most *JAK2V617F* mutations in AML occur in patients with a preceding diagnosis of PV, ET, or PMF. *JAK2V617F* is an acquired somatic mutation, does not appear in nonhematopoietic cells, and has not been detected in patients with secondary erythrocytosis. Moreover, *JAK2V617F* has not been observed in lymphoid malignancies, although other mutations in *JAK2* have been identified in 10% of patients with pediatric high-risk ALL (see Chapter 66).

Other *JAK2* Mutations in Polycythemia Vera

Other mutations of *JAK2* associated with erythrocytosis, however, can also constitutively activate JAK2 kinase activity. Several gain-of-function mutations affecting *JAK2* exon 12 within an area immediately adjacent to the pseudokinase domain of *JAK2V617F*-negative patients have been identified in 2.5% to 3.4% of PV patients and approximately 30% of *JAK2V617F*-negative PV cases. To date, greater than 40 different such mutations have been identified. Two-thirds of patients with a *JAK2* exon 12 mutation presented with an isolated erythrocytosis and distinctive BM morphology, and had reduced serum EPO levels, but the remainder of patients have erythrocytosis plus leukocytosis, thrombocytosis, or both. These exon 12 mutations perturb the autoinhibitory domain of *JAK2*. *JAK2* exon 12 mutations have not been reported in patients with ET or PMF, but have been occasionally observed in patients with refractory anemia and ring sideroblasts associated with thrombocytosis. Erythroid colonies cloned from their blood samples in the absence of exogenous EPO were most frequently heterozygous for the mutation, with homozygous colonies only rarely

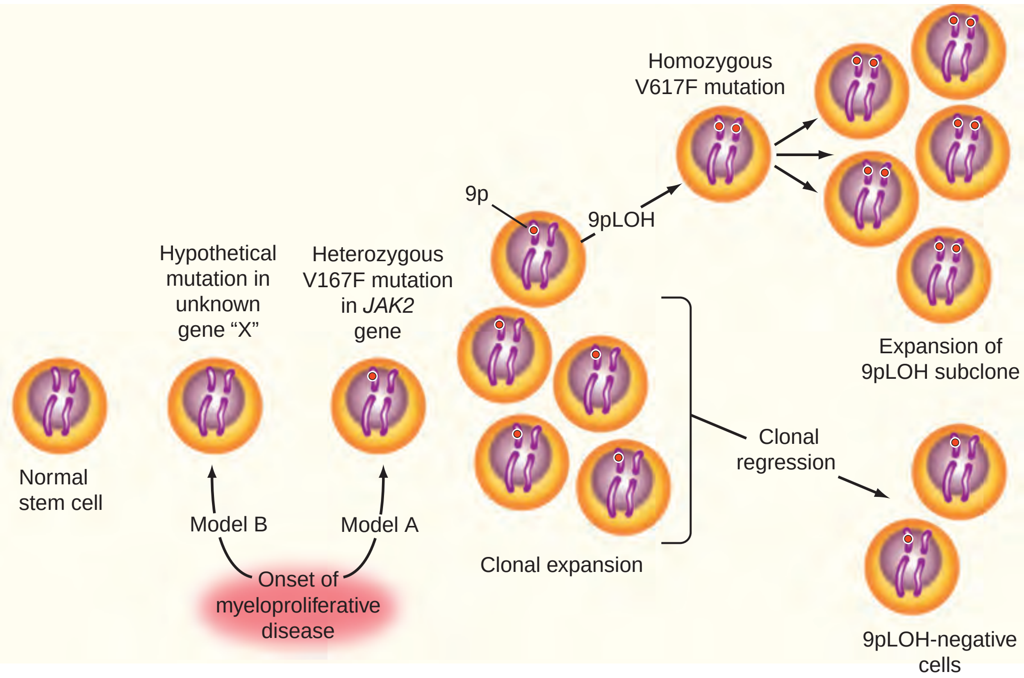


Figure 70.6 POSSIBLE ROLE OF *JAK2V617F* IN THE BIOLOGY OF MYELOPROLIFERATIVE DISORDERS. The chromosome 9 with the wild-type *JAK2* sequence (G) is depicted in *white*, and the chromosome 9 with the G–T transversion (T) is shown in *red*. *Circles* symbolize the nuclei of the cells. Deletion of the telomeric part of wild-type chromosome 9p as a potential mechanism for 9pLOH is shown on the *left*. Alternatively, mitotic recombination could also result in 9pLOH, shown on the *right*. The events during mitosis and the resulting cell progeny after mitotic recombination of chromosome 9p are also shown. *(Adapted from Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med. 2005;352:1779.)*

occurring, but colonies homozygous for the mutation occur in most PV patients with *JAK2V617F*, suggesting that a *JAK2* exon 12 mutation results in a stronger activation of the JAK2-mediated intracellular signaling pathways. Exon 12 mutations tend to occur in younger patients compared to *JAK2V617F* positive PV patients. After taking the younger age of exon 12 *JAK2* mutated PV patients into account, the clinical outcomes of patients with these two different *JAK2* mutations have been shown to be similar. Patients with exon 12 mutations can develop thrombotic episodes and can evolve into PV-related MF or MPN-BP. In several series, approximately 2.7% of patients with a clinical syndrome that resembles PV have been observed who have a WT *JAK2*. The existence of such patients may be the result of several factors, including limited sensitivity of the assay used for genotyping, or lack of efforts to exclude inherited genetic disorders associated with erythrocytosis that have been described in this chapter. Alternatively, additional acquired genetic lesions that have yet to be described may be responsible for the disease phenotype.

JAK2V617F Is Likely Not the Only Event That Determines if Polycythemia Vera Develops

Although data generated using a variety of mouse models suggests that *JAK2V617F* might be sufficient for the development of PV, increasing evidence shows that PV is not solely dependent on acquisition of *JAK2V617F*. The fact that *JAK2V617F* has been identified in patients with three phenotypically related but clinically distinct MPNs suggests that additional genetic or epigenetic events likely contribute to the phenotypic divergence of PV, ET, and PMF. These differing disease phenotypes have been hypothesized to be caused by striking differences in the degree to which the mutation activates the JAK-STAT pathway. Mutant *JAK2* activates multiple cytokine receptor-associated pathways, including STAT1 and STAT5, which can have competing consequences. STAT1 appears to be activated in association with *JAK2V617F* ET but not PV. Inhibition of STAT1 in ET progenitor cells enhances erythropoiesis, indicating that in ET, the phospo-STAT1 response to *JAK2V617F* constrains erythropoiesis and promotes megakaryocytic differentiation, but in PV, the reduced pSTAT1 response removes the break on erythropoiesis, allowing enhanced erythropoiesis to occur.

The role of *JAK2V617F* in the underlying pathogenesis of PV has been extensively explored using either restricted fragment length polymorphism analysis or the presence of marker cytogenetic abnormalities in patients with MPNs. The percentage of granulocytes and platelets that are *JAK2V617F* positive is often lower than the percentage of granulocytes belonging to the malignant clone. In addition, these marker cytogenetic abnormalities may occur before or after the acquisition of *JAK2V617F*. Furthermore *JAK2V617F*-negative erythroid colonies have been cloned in vitro in the absence of the addition of EPO, a hallmark of PV, indicating the presence of an undefined molecular lesion that precedes the *JAK2V617* mutation.

Several investigators have reported families in which multiple members have MPNs, including PV, ET, PMF, and CML, and have analyzed the *JAK2V617F*, *MPL*, and *CALR* status of family members. In affected patients, the *JAK2V617F* mutation was the most commonly acquired followed by *CALR* exon 9 mutations, with no *MPL W515L/K* mutations being detected. Interestingly, in some families, both *JAK2V617F*-positive and -negative members with MPN were observed. A small number of relatives who were *JAK2V617F* negative and did not have a diagnosis of PV, ET, or PMF had hematopoietic cells that formed endogenous erythroid colonies in vitro. Disease evolution can be highly variable within families presenting with the same type of MPN. These results suggest that an as yet unidentified genetic event, either germ-line or somatic, might contribute to the pathogenesis of PV, ET, and PMF, regardless of *JAK2* mutational status, and that there may be "initiating events" that precede the acquisition of *JAK2V617F* in these disorders.

Evidence has accumulated from epidemiological and familial studies that indicate that common low-penetrance factors present in the general population contribute to the risk of developing an MPN and possibly to the phenotype of the particular MPN. There is a 5- to 7-fold increased risk of MPN acquisition in first-degree relatives of individuals with the disease, amounting to a familial risk greater than that observed in breast, prostate, and colorectal cancer. Genetic studies have identified a limited set of loci associated with MPN predisposition. The germ-line constitutive *JAK2* haplotype, called GGCC or 46/1, has been shown to be a susceptibility factor for the development of *JAK2V617F*-positive PV. The *JAK2* 46/1 haplotype is also weakly associated with exon 12 *JAK2* PV, *MPL W515* MPNs, as well as MPNs that lack *MPL* mutations. How this *JAK2* SNP promotes the development of *JAK2V617F* MPNs remains the subject of great speculation, but two hypotheses have been proposed: (1) hypermutability of the chromosome region facilitates the acquisition of somatic mutation; or (2) the *JAK2* SNP confers a selective proliferative advantage, the so-called *fertile ground hypothesis*. Germ-line polymorphisms in the *TERT* gene have also been reported to predispose to *JAK2V617F*-positive and -negative sporadic and familial MPNs. The predisposition was far stronger in familial than sporadic MPNs, suggesting that low-penetrance variants might be responsible for the familial clustering of MPNs. Two additional SNPs involving TERT and HBS1L/MYB have been shown to have a stronger association with MPN populations with *CALR* or *MPL* mutations that lacked *JAK2V617F*. Reduced expression of MYB has been linked to ET-like disease in several animal models. In *JAK2V617F*-positive individuals, the reduced MYB associated with the MYB SNP favored the development of an ET phenotype. In addition, polymorphisms of the glucocorticoid receptor have been associated with PV and PMF but not ET. Bao and coworkers have extensively examined germline MPN predisposition SNPs by using an integrative approach to map risk variants to target genes. Remarkably, 11 of the 15 genes identified affect HSC self-renewal and function, including *ZNF521*, *GATA2*, *MECOM*, *RUNX1*, *HMGA1*, *ATM*, *FOXO1*, *TET2*, *JAK2*, *SH2B3*. They have hypothesized that MPN risk alleles expand the baseline pool of HSCs, which increases the risk of any one HSC acquiring an MPN-specific driver mutation.[24]

In the MPNs, acquisition of mutations in either *TET2* or *JAK2* may occur first, but those patients that acquire *JAK2V617F* first are more likely to develop PV. The concept that the MPN phenotype is the consequence of the order of mutational acquisition was first proposed by Ortmann and coworkers. *JAK2* and *TET2* mutations each occurred first in 50% of patients. *JAK2V617F* homozygosity was not required for acquisition of a *TET2* mutation to occur. *JAK2V617F* first patients were more likely to present with PV, were younger, more sensitive to ruxolitinib treatment, and were more likely to suffer from a thrombotic event than those individuals who acquired a *TET2* mutation initially. These data indicate that the order of acquisition of mutations might influence the clinical phenotype of the resultant MPN. *TET2* mutations have been shown to result in expansion of the malignant clone in elderly persons with normal blood counts, and in murine studies, a double-mutant *TET2–JAK2* clone was out competed by its *TET2* single-mutant ancestor. In mice, expression of *JAK2 V617F* but not TET2 resulted in increased erythropoiesis. A *TET2*-inactivating mutation but not *JAK2V617F* leads to HSC expansion. The report by Ortmann and coworkers suggests that in patients who acquire TET2 first, TET2 single-mutant hematopoietic stem and progenitor cells expand but do not give rise to excess differentiated megakaryocytic and erythroid cells until a *JAK2* mutation is acquired. By contrast, in patients who acquire JAK2 first, *JAK2* single-mutant hematopoietic stem and progenitor cells generate increased numbers of megakaryocytic and erythroid cells, and stem cell numbers only expand after acquisition of a *TET2* mutation. This model is consistent with the earlier clinical presentation of patients who first acquire a *JAK2* mutation since they more rapidly generate excess megakaryocytic and erythroid cells. The initial *TET2* mutation may modify the epigenetic program of HSCs and progenitor cells, and thus alter the consequences of the second mutation. A prior mutation of *TET2* alters the transcriptional consequences of *JAK2 V617F* in a cell-intrinsic manner and prevents *JAK2 V617F* from upregulating stem and progenitor cell proliferation.

The *JAK2V617F* Mutation Is Present in Hematopoietic Stem Cells in Polycythemia Vera

Previous studies had demonstrated that the majority of patients with PV had clonal involvement of multiple lineages, including myeloid, erythroid, and lymphoid cells. These results suggested that PV originates in HSCs with the ability to differentiate into multiple lineages. In addition, LOH at 9p24, known to correspond to homozygous *JAK2V617F* mutations, can be identified in both myeloid and lymphoid cells in some patients with PV, further suggesting that the underlying mutations occur in progenitor cells with the ability to differentiate into multiple hematopoietic lineages.

The *JAK2V617F* mutation has been detected in hematopoietic colony-forming cells and more mature progeny, such as neutrophils and platelets. Populations of cells enriched for HSCs, common myeloid progenitors (CMPs), granulocyte–macrophage progenitors (GMPs), and megakaryocytic–erythroid progenitors (MEPs) from patients with PV have been analyzed for the presence of the *JAK2V617F* mutation. *JAK2V617F* was detected in HSCs, CMPs, GMPs, and MEPs from patients with PV, supporting that PV is a disorder that arises in HSCs and involves the myeloid, erythroid, and megakaryocytic lineages. These data indicate that *JAK2V617F* in PV originates in lymphomyeloid progenitor cells. Analyses of hematopoietic cells from PV patients has suggested that *JAK2V617F* amplifies the terminal stages of hematopoiesis but not the more primitive hematopoietic stem/progenitor cells. In mouse models, however, *JAK2V617F* has been shown to lead to the amplification of both stem and progenitor cells, providing them with a competitive advantage compared with their normal counterpart through increased cell cycling and a reduced rate of apoptosis resulting in the emergence of the MPN phenotype. *JAK2V617F* may give only a subtle advantage, which will require several years for mutated stem and progenitor cells to predominate in humans over their normal counterparts. In man, other mutations such as *ASXL1*, *DNMT3A*, and *TET2* may further alter the biology of the *JAK2V617F*-mutated clone in a manner that affects phenotype as well as disease evolution.

Structural and Functional Aspects of *JAK2V617F*-Mediated Transformation

The *JAK2V617F* mutation occurs within the JH2 domain of JAK2, which has significant homology to the kinase domain of JAK2 (JH1) but lacks catalytic activity (see Fig. 70.5). The JH2 domain exerts an inhibitory effect on JAK2 kinase activity, and the *V617F* mutation is predicted to disrupt this inhibition. In vitro kinase assays with *JAK2V617F* and WT *JAK2* have revealed that *JAK2V617F* has greatly increased kinase activity, as assessed by autophosphorylation and by substrate phosphorylation. Ectopic expression of *JAK2V617F* in either epithelial or hematopoietic cell lines results in autophosphorylation of mutant *JAK2*, but not the WT *JAK2*, and activation of downstream signaling events. BAF3 or FDCP cell lines expressing the EPOR and engineered to stably express *JAK2V617F* are largely independent of the addition of exogenous growth factors and are hypersensitive to EPO. Coexpression of *JAK2V617F* and a homodimeric type 1 cytokine receptor (EPOR, TPOR, or G-CSFR) facilitates the transformation of cells to growth factor independence, suggesting that the mutant *JAK2* requires a receptor scaffold to be active. This contrasts with the effects of the *TEL-JAK2* fusion gene, which can readily transform cells on its own, presumably because of the strong homodimerization effects of the TEL moiety. Ectopic expression of *JAK2V617F* can also sensitize cells to the effects of IGF1, a characteristic feature of PV progenitors.

Expression of *JAK2V617F* in hematopoietic cells activates intracellular signaling pathways downstream of the EPOR, including STAT5, STAT3, the MAP kinase pathway, and the PI3K–Akt pathway. STAT5 is normally phosphorylated by the cytokine receptor–JAK2 complex, and phosphorylated STAT5 then translocates to the nucleus and activates the transcription of target genes. The target genes of STAT5 include Bcl-X_L, an important antiapoptotic protein known to be expressed in increased levels in PV proerythroblasts. The possibility that STAT5-mediated activation of Bcl-X_L is important in the pathogenesis of PV was suggested by observations that expression of either constitutively active STAT-5 or Bcl-X_L resulted in spontaneous erythroid colony formation. Furthermore, the degree of apoptosis can also be altered by TP53 levels. *JAK2V617F* appears to functionally inactivate TP53 by upregulating MDM2, an E3 ubiquitin ligase, thereby decreasing the degree of apoptosis. Preclinical studies have shown that MDM2 is overexpressed in PV stem/progenitor cells. In addition, cells expressing *JAK2V617F* display constitutive activation of the MAP kinase pathway (as assessed by phosphorylation of ERK), and of the PI3K pathway (as assessed by phosphorylation of AKT).

Furthermore, JAK2 has been shown to play a role in cellular MPL trafficking. Decreased expression of MPL on the cell surface of platelets and megakaryocytes is an established feature of PV and MF which leads to increased TPO levels. Lower expression of MPL on the cell surface of platelets and Mks in MPNs has been reported to be due to both a reduction in the recycling and maturation of the receptor as well as an increase in MPL proteasomal degradation mediated by *JAK2V617F* protein. Spivak and Moliterno and their group have performed seminal studies that indicate that the MPL/TPO axis plays a pivotal role in the biogenesis of PV. They have shown that MPL expression by HSCs is also reduced due to abnormal post-translational glycosylation. They have hypothesized that MPL processing plays a pivotal role in the biology of MPNs since MPL is the only hematopoietic growth factor receptor expressed by HSCs. To prove this they genetically manipulated MPL expression and maturation in a transgenic mouse model of PV. Elimination of MPL expression completely eliminated the development of PV phenotype, which was partially restored by expression of one MPL allele. Most importantly, elimination of TPO expression also eliminated the PV phenotype in this mouse model, and the PV phenotype could be restored with expression of a single TPO allele. These data indicate that PV is in part a TPO-dependent malignancy and that therapeutic strategies targeting the MPL-TPO axis should be evaluated in the future for the treatment of PV patients.[25]

Many PV patients have a low burden of *JAK2V617F*, as assessed by DNA sequencing, suggesting that there is either a subpopulation of cells that are homozygous for *JAK2V617F* mixed with WT cells or a clonal population of cells with one WT copy of *JAK2* and one mutant copy of *JAK2*. In PV, data from clonality or quantitative *JAK2V617F* assessment and from colony assays suggest that most PV patients have a subpopulation of cells homozygous for *JAK2V617F*, but in ET, clonal progenitor cells are heterozygous for *JAK2V617F*. The *JAK2V617VF* mutation occurs in a homozygous state in 25% to 30% of PV patients.[26] PV patients who are heterozygous for *JAK2V617F* have more mutated, committed progenitors than other PV patients, suggesting a selective amplification of mutated cells during the early phases of hematopoiesis. Dupont and coworkers have also demonstrated that mutated erythroid progenitors were more sensitive to EPO than normal progenitors, and that most *JAK2V617F* homozygous erythroid progenitors are actually EPO independent. The presence of *JAK2V617F* heterozygosity and homozygosity influences the clinical phenotype of PV patients. Vannucchi and coworkers have shown after a retrospective clinical analysis of a large number of PV patients that *JAK2V617F* homozygous patients were older, displayed a higher leukocyte count and hematocrit levels at diagnosis, and had larger spleens. Aquagenic pruritus was also more common in *JAK2V617F* homozygous PV patients. Furthermore, *JAK2V617VF* homozygosity was associated with more frequent evolution into PV related MF.[27]

Additional Mutations Associated With Polycythemia Vera

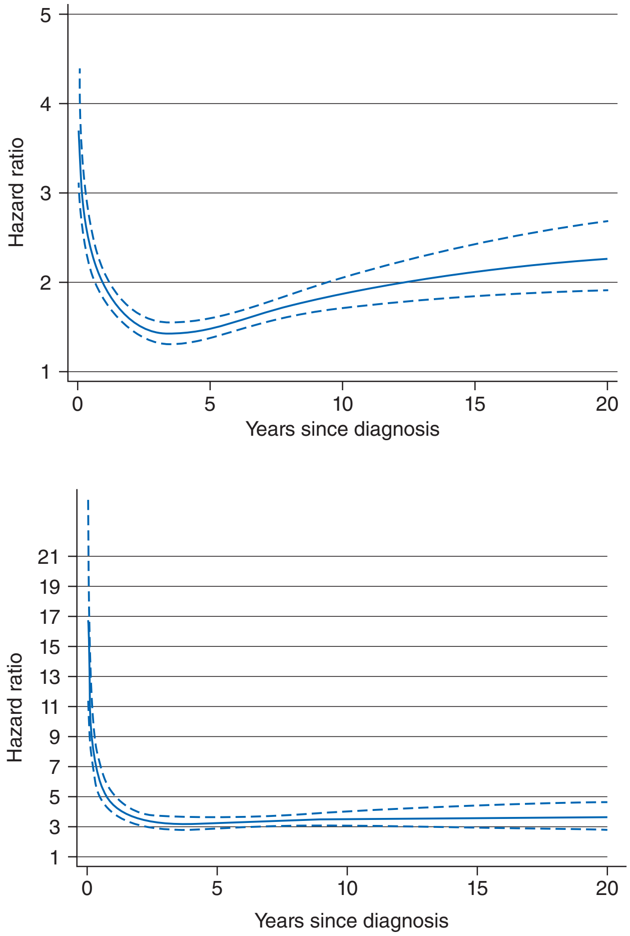
After the application of whole-genome assays (comparative genomic hybridization and SNPs) as well as whole-genome sequencing, an increasing number of mutations have been observed in patients with Philadelphia chromosome-negative MPNs. These mutations are not unique to any of the MPNs and are associated with AML and MDS. Four of these genes—*EZH2*, *ASXL*, *DNMT3a*, and *TET2*—participate in the epigenetic control of transcription and are discussed in greater detail in Chapter 59. *EZH2* mutations occur in 3% of PV patients, 7% to 16% of PV patients have *TET2* mutations, and 5% to 7% have mutations of *DNMT3a*; *ASXL* mutations are rare in PV (<7%). Each of these mutations may precede *JAK2V617F*, but the converse may also occur. Each of these mutations are far less frequent in PV and ET than PMF, supporting that these events may combine to generate a more accelerated phase of the classic MPN that phenotypically presents as MF. In addition to epigenetic regulators, mutations in genes that encode proteins that participate in the process of splicing immature mRNA have also been described in MPNs, specifically SF3B1, SRSF2, and U2AF1. *SF3B1* mutations are mostly found in PMF where they cluster within exons 12 to 16. Alterations in SRSF2 are mostly found in PMF and MPN evolved to AML. *U2AF1* mutations are detected in up to 15% of PMF cases, but are not present in ET or PV patients. *IDH1/2*, *IKZF* deletions, *NRAS/KRAS*, *p53* mutations, and *RUNX1* mutations rarely occur in PV but are all associated with transformation of an underlying MPN such as PV to MPN-BP.

The Hypercoagulable State That Characterizes Polycythemia Vera

Thrombosis is a major cause of morbidity and mortality in PV patients. These thrombotic events are most frequently microcirculatory and arterial, but venous thromboses are also of important clinical significance. In a population-based study from Sweden, Hultcrantz reported that the rate of arterial and venous thrombosis was significantly higher in patients with MPNs than in the general Swedish population. They demonstrated that the rate of thrombosis was significantly elevated in patients with MPNs across all age groups The absolute number of first arterial events was greater than the number of venous thrombotic events. The highest rate ratios for arterial and venous thrombosis were observed shortly after MPN diagnosis (3.0 and 9.7 respectively). The risk decreased during follow-up but continued to be elevated (Fig. 70.7).[28] The increased risk for thrombosis can be attributed to abnormalities in the vessel wall, blood cell components, a chronic inflammatory state, and the dynamics of blood flow. A number of risk factors for thrombosis have been investigated, but most of them are not firmly established.

In the European Collaboration on Low-Dose Aspirin in Polycythemia Vera (ECLAP) study, the incidence of cardiovascular complications was higher in patients older than 65 years (5.0% of patients per year; hazard ratio [HR]: 2.0; 95% confidence interval [CI]: 1.22 to 3.29; $P < .006$) or with a history of thrombosis (4.93% of patients per year; HR: 1.96; 95% CI: 1.29 to 2.97; $P = .0017$). Patients both with a history of thrombosis and older than 65 years had the highest risk of developing additional cardiovascular events (10.9% of patients per year; HR: 4.35; 95% CI: 2.95 to 6.41; $P < .0001$).[29] These data confirm previous findings that increasing age and history of thrombosis are the two most important prognostic factors for the development of vascular complications.

This information does not negate the increased incidence of thrombotic incidents also observed in younger patients with this disorder. In a series of 58 PV patients younger than 40 years of age, a disturbingly high incidence of life-threatening thrombotic events was observed. In fact, seven of the 10 patients in this series who died during the period of observation died from thrombotic events—four from Budd–Chiari syndrome (BCS), one from a pulmonary embolism, and two from cerebral thrombosis. Therefore, although a significant factor, preexisting atherosclerotic disease is not the sole etiologic factor in the genesis of thrombosis in PV. Analysis of the ECLAP study identified smoking in addition to older age as an important risk factor for major thrombosis. A retrospective study of 450 patients with PV demonstrated that hypertension and tobacco use were associated



Note: The beginning of the curve has been cropped to better visualize the hazard ratio during follow-up

Figure 70.7 Arterial (*top*) and venous (*bottom*) thrombosis during follow-up in patients with myeloproliferative neoplasms versus matched control participants. *(From Hultcrantz M, Björkholm M, Dickman PW, et al. Risk for arterial and venous thrombosis in patients with myeloproliferative neoplasms: a population-based cohort study. Ann Intern Med. 2018;168[5]:317–325.)*

with arterial thrombosis ($P < .02$) and diabetes mellitus was associated with venous thrombosis (P=.002).

The principal hemorheologic abnormality in PV is an elevated whole blood viscosity. The blood viscosity in PV is higher than that of normal control participants at all shear rates. In a retrospective analysis of PV patients with histories of vascular thrombosis, a strong correlation in univariate analysis between hematocrit level and the development of thrombotic episodes, including many cerebrovascular occlusions, was demonstrated. Cerebral blood flow is reduced in patients with PV in whom the hematocrit level is 53% to 62%. These abnormalities were observed even in patients with hematocrits at the lower levels, that is, 46% to 52%. Reductions in cerebral blood flow are correctable with phlebotomy. Reduction of the hematocrit by relatively small amounts frequently led to substantial improvements in whole blood viscosity and cerebral blood flow. Some PV patients apparently still maintain a higher-than-normal whole blood viscosity despite the normalization of the hematocrit, suggesting that an increase in hematocrit may not be the only factor responsible for increased blood viscosity. There appear to be important gender differences as related to the location of thromboses in PV patients. Women appear to have a higher incidence of thromboses within the abdominal cavity involving the portal, mesenteric, or hepatic vessels but a comparable rate of other vascular complications. Several groups have reported that patients with SVT frequently have endothelial cells that are affected by *JAK2V617F*, suggesting that in such individuals, their MPN might originate not at the level of the HSC but rather at the level of a hemangioblast, from which myeloid and endothelial cells originate. Such JAK2V61F-positive endothelial cells have been shown to exhibit a high efficiency of adhering to normal mononuclear cells, thereby likely leading to an increased risk of thrombosis.

A number of possible explanations have been suggested for the observed relationship between hematocrit level and the development of thrombotic events in PV patients. Platelet adhesion and thrombus formation on the vascular subendothelium are determined in part by the rate at which platelets are transported to the vascular surface. In a polycythemic condition in which increased numbers of RBCs are present, a greater number of intercellular collisions between RBCs and platelets occurs. These collisions could lead to increased platelet movement in a direction perpendicular to blood flow. This facilitation of platelet transport to the vessel wall may be an important factor in the development of thrombosis. An alternative explanation for the association between hematocrit level and the risk of thrombosis is based on the knowledge that blood viscosity is particularly sensitive to hematocrit levels. Increased hematocrits lead to increased blood viscosity, in turn leading to increased peripheral vascular resistance and an actual reduction in blood flow to a variety of organs, predisposing them to the development of thrombosis. The issue of hematocrits and thrombosis in PV has been prospectively investigated in an analysis of 1638 patients enrolled in the ECLAP study. In this prospective study, despite recommendations of maintaining hematocrit levels lower than 45%, only 50% of patients achieved this target during the follow-up, and 10% had hematocrit levels above 50%. These different hematocrit values were not associated with different thrombotic outcomes as assessed using univariate and multivariable analysis. However, in a recent prospective randomized clinical trial of 365 patients with PV, it was shown that patients with hematocrit maintained at less than 45% had a lower incidence of cardiovascular events compared with patients whose hematocrits were maintained at 45% to 50% (4.4% vs. 10.9%; $P = .02$). The group with lower hematocrit also had lower death rates from cardiovascular events or major thrombosis.[30] This study confirmed the need for strict hematocrit control in PV patients.

Additional factors have been implicated in the development of thrombosis in PV patients. Almost all patients with PV are iron-deficient. RBC size is a major determinant of platelet adherence, with larger RBCs leading to increased platelet adherence and smaller RBCs to decreased platelet adherence. Whether the increased membrane stiffness associated with iron deficiency is counterbalanced by the decreased platelet adherence associated with smaller RBCs is yet to be determined.

Inflammation may be another predictor of thrombosis in patients with PV.[21] In a retrospective study patients with increased inflammation as evidenced by an increased C-reactive protein (CRP) had significantly greater risk for thrombosis based on a multivariant analysis that adjusted for age, sex, ET, or PV diagnosis, cardiovascular risk factors, and *JAK2V617F* mutation status. Elevated circulating levels of pro-inflammatory cytokines generated by MPN cells and stromal cells in their microenvironment create a chronic systemic pro-inflammatory state potentially creating a pro-thrombotic environment. Conversely, inflammatory states may predispose to acquiring JAK2V617F and other MPN driver mutation and their clonal evolution.[31] CH and systemic inflammation are both linked to aging and risk of thrombosis. CHIP is a presence of a mutation known to occur in hematologic malignancies at relatively low allele frequency but without any clinically evident hematologic neoplasm. Patients with CHIP are at high risk of developing a true hematologic neoplasm such as MPN over time. CHIP has been shown to be associated with increased risk of coronary artery disease (CAD) and stroke. In an elderly population with known CAD, higher levels of high-sensitivity CRP were found to be present in those patients with CH again supporting the relationship between CH and inflammation (Fig. 70.8).

Thrombocytosis and qualitative platelet abnormalities occur frequently and are likely to be important contributory factors to the development of thrombosis. Some investigators have implicated uncontrolled thrombocytosis as a cause of thrombosis in these patients, but this relationship has not been confirmed by others. Increased plasma and urinary thromboxane production has been linked to increased platelet activation in these patients. A low-dose aspirin regimen selective for inhibition of platelet cyclooxygenase has been found to suppress increased thromboxane production in vivo and to clinically benefit patients with PV. Furthermore, elevated levels of serum VEGF and plasma TPO have been observed in patients with PV. These growth factors have been shown to lead to platelet activation.

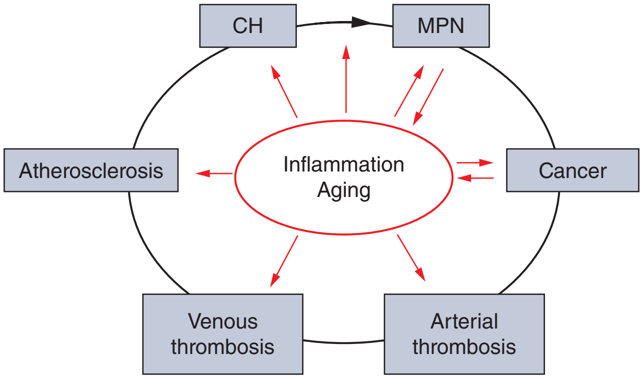


Figure 70.8 The relationship between inflammation and aging as risk factors (*red arrow*) for the development of CH, MPN, cancer, thrombosis, and atherosclerosis. *CH*, Clonal hematopoiesis; *MPN*, myeloproliferative neoplasm. *(From Moliterno AR, Ginzburg YZ, Hoffman R. Clinical insights into the origins of thrombosis in myeloproliferative neoplasms. Blood. 2021;137[9]:1145–1153.)*

Despite conflicting data, no clear clinical relationship between platelet number or function and the incidence of hemorrhage or thrombosis in PV patients has been delineated. An argument in favor of a role of elevated platelet numbers in the genesis of thrombosis in PV and ET is the observation in patients with ET that a reduction of excessive platelet numbers with the use of HU is associated with a reduction in the risk of developing thrombotic events. However, this should not be taken as evidence that the reduction in developing additional vascular events was caused by platelet count normalization alone; very likely, it may be related to the suppression by HU of each of three myeloid lineages. In line with this interpretation are the results of a randomized study performed by the Medical Research Council in the United Kingdom (PT-01) in which patients with ET were randomized to receive either HU or anagrelide therapy. This study showed that HU therapy rather than anagrelide, a selective platelet number-reducing drug, was associated with a reduction in the number of arterial thrombotic events, especially in *JAK2V617F* patients. In this study the rate of thrombosis in ET patients during follow-up did not vary according to different platelet numbers. Select patients with PV have, however, been afforded prompt resolution of vascular complications such as erythromelalgia or TIAs after institution of platelet antiaggregating agents or cytoreduction. It is important to emphasize that erythromelalgia does not resolve in PV patients with phlebotomy alone or with anticoagulation, but requires the use of platelet antiaggregating agents or reduction of platelet numbers. What distinguishes the clinical courses of these patients from those of others is unknown. These reports, coupled with the knowledge of abnormal thromboxane metabolism of platelets in PV, provide substance to the belief that platelets contribute to the generation of the thrombotic and hemorrhagic tendencies observed in PV. Although a number of clinical assessments of platelet function have been used to identify patients who are potentially at a high risk of developing a life-threatening hemorrhagic or thrombotic event, the results of these studies to date have been very disappointing. It appears that the etiology of thrombosis and hemorrhage in PV is multifactorial, and that the available tools are inadequate to identify patients at highest risk.

An elevation of WBC number occurs in 50% to 60% of PV patients, which may also have a detrimental effect on the rheology of the microcirculation in PV. An analysis of the ECLAP database showed that a baseline WBC count above $15{,}000 \times 10^6\,L^{-1}$ was associated with the development of major arterial events, mainly myocardial infarction. A retrospective study of 459 patients with PV showed a significant association between baseline leukocyte count greater than $15 \times 10^9/L$ and venous thrombotic events during a median follow-up of 64 months. Similar results were shown in two additional studies in patients with ET. On the other hand, a recent retrospective study of 520 PV patients seen at 10 academic institutions across the United States used group-based trajectory modeling and demonstrated that persistently elevated leukocyte trajectories were not associated with the hazard of a thrombotic event, but were actually significantly associated with increased hazard of disease evolution.[32]

Activated leukocytes may release proteases and oxygen radicals that alter endothelial cells and platelets so as to favor the development of a prothrombotic state. A series of markers of leukocyte activation, including expression of membrane CD11b and leukocyte alkaline phosphatase antigen, cellular elastase content, plasma elastase levels, and myeloperoxidase levels, are elevated in patients with PV. Limited and conflicting data are available as to whether or not there is a correlation between the presence of platelet–leukocyte interactions and thrombotic events. Increased expression of leukocyte adhesion molecules increases the adhesion of leukocytes to platelets and the endothelium. These cell-to-cell interactions stimulate the activation of endothelial cells and platelets, and induce the release from activated leukocytes reactive oxygen species as well as proteases that are capable of impairing a number of hemostatic processes. Platelet–leukocyte aggregates are increased in number in PV and are associated with an increased propensity to thrombose. In patients with PV and ET, increased levels of soluble plasma selectins were observed as compared to controls. In addition, platelets and vascular endothelial cells produce pro-coagulant microparticles. In a study of MPN patients microparticles were found to be significantly increased compared to controls. Furthermore, the increase was higher in patients with MPNs who had thrombosis. A recent study performed unbiased RNA sequencing in granulocytes and platelets of PV patients and found that expression of several thrombotic, inflammatory, and HIF-regulated genes is increased in these cells of PV patients.[33]

JAK2V617F allele burden has also been implicated as a potential risk factor for thrombosis. In a prospective study of 173 patients with PV that were followed for a median of 24 months, patients with a *JAK2V617F* allele burden of greater than 75% had a sevenfold increased risk of thrombosis compared with patients with an allele burden of less than 25%. However, another prospective study comparing patients with an allele burden of less than 50% versus less than 50% did not confirm the association between *JAK2V617F* allele burden and thrombotic risk. In a retrospective study of 186 patients with MPN, individuals harboring the *JAK2V617F* mutation were at highest risk for VTE. *JAK2* allele burdens higher than 20% identified patients with a sevenfold increased risk of VTE but not arterial thrombosis. In a recent retrospective study of 1537 MPN patients in China, of which 931 patients had PV, a multivariate analysis indicated that JAK2V617F allele burden greater than 50% was one of the risk factors for thrombotic events.[34] More studies are needed to clearly establish higher *JAK2V617F* allele burden as a risk factor for thrombosis.

Clearly, the thrombotic tendencies in PV are multifactorial and in the future it is likely that the thrombotic risk will be assessed on an individual basis, taking into account many of the factors mentioned previously.

Polycythemia Vera and the Risk of Hemorrhage

Patients with PV are also at an increased risk of developing minor as well as life-threatening hemorrhagic complications. The median incidence of all bleeding events and major bleeding events was reported in PV patients after a meta-analysis by Nicol et al. to be 6.5 and 1.0% patients/year respectively. Bleeding events occurred less frequently than thrombotic events and occurred at a similar incidence throughout the course of the disease. These bleeding events involved most commonly the gastrointestinal tract, skin, and occasionally the central nervous system. Bleeds within the central nervous system were the causes of death due to bleeding. Bleeding after surgery or trauma can also lead to significant morbidity and mortality. Abnormalities in platelet function and number have been implicated as the cause of this hemorrhagic tendency. Qualitative platelet abnormalities frequently found in these patients include platelet hypofunction, as demonstrated by defective in vitro platelet aggregation, acquired storage pool disease, and platelet membrane defects, and increased platelet reactivity, as demonstrated by enhanced platelet aggregation, increased plasma β-thromboglobulin levels, and shortened platelet survival. With platelet counts greater than 1000 to 1500 × 10^9 L^{-1}, the development of acquired von Willebrand syndrome is common (incidence of 49%) and can be associated with life-threatening hemorrhagic episodes. The use of anticoagulants or platelet function antagonists frequently contribute to the development of bleeding events especially in patients with acquired von Willebrand syndrome.[35]

Post-Polycythemia Vera Myelofibrosis and MPN-BP

A major cause of morbidity and mortality in PV results from the transition from the polycythemic phase of the disease to post-PV MF and/or to MPN-BP. These events usually occur a decade or more after the initial diagnosis of PV is made. Among a large cohort of 267 patients with PV, 12.5% to 21.0% of PV progressed to PV-related MF while MPN-PB has been estimated to occur in 6.8% of PV patients.[36] The PV patients who are at the highest risk of developing post PV MF and MPN-BP, in addition to their driver mutations frequently have additional malignancy mutations including TP53 and genes encoding chromatin (*IDH1/2, ASXL1, EZH2*) and spliceosome regulators (*SRSF2,U2AF1, SF3B1* and *ZRSR2*), transcriptional regulators (RUNX1 and NRAS) as well as homozygosity for *JAK2V617F*. Although *TET2* gene mutations are the most frequent non-driver mutations encountered in PV patients, they are not predictive of disease evolution. Also those patients with multiple complex cytogenetic abnormalities including loss of 17p which results in loss of TP53, multiple copies of 1q, and deletion of chromosome 7 also are at risk of having their PV progress to post PV MF or MPN-BP. The hematopoietic cells from patients destined to develop post PV MF and/or MPN-BP produce a number of cytokines that create a pro-inflammatory microenvironment within the BM and spleen which favors disease progression. In PVs, the fibroblastic component of the BM has been shown not to be directly involved in the malignant process but to react to the neoplastic clone by creating a tumor-promoting microenvironment. Several investigators have suggested that the release of growth factors, particularly VEGF, fibroblast growth factor, transforming growth factor-β, lipocalin, interleukin-6, interleukin-8, and tumor necrosis factor-α from myeloid cells are responsible for the marrow fibroblastic proliferation, increased marrow microvessel density, osteosclerosis, as well as the systemic symptoms observed in patients with PV-related MF. Whether the use of any particular therapeutic agents for treatment of PV accelerates the development of PV-related MF remains hotly debated, although it is well established that the use of alkylating agents such as pipobroman or chlorambucil increases the risk of developing acute leukemia. It remains a source of debate if treatment with HU increases the risk of developing MF or AML. The bulk of evidence does not support a clear leukemogenic role for this drug that is the standard of care for high-risk PV patients. Nevertheless, as a cautionary principle, it is wise to consider carefully the use of this agent in very young patients, in those carrying cytogenetic abnormalities, and in patients previously exposed to alkylating agents. In a report in which patients were treated exclusively with HU with an average follow-up of 16.3 years, the cumulative incidence of MPN-BP/MDS was 7.3%, 10.7%, and 16.6% at 10, 15, and 20 years, respectively, and was significantly lower than in patients treated with the alkylating agent pipobroman. However, patients treated with HU had a greater chance of developing MF (at 20 years, 31.6% vs. 21.3%). Whether HU really is leukemogenic is difficult to say from this study. The most likely possibility is that these rates of evolution reported with HU use merely reflect the natural history of the disease.

Post-PV MF is characterized by cytopenias, marrow fibrosis, an increasing burden of systemic symptoms, and extramedullary hematopoiesis. MPN BP can be preceded by an accelerated phase which is characterized by the appearance of 10% to 19% blasts in the peripheral blood which evolves to full blown BP when the blast cell count exceeds 20%. The MPN-BP patients frequently experience increasing consequences of their progressively enlarging spleen and/or liver, thrombocytopenia, leukocytosis, increasing numbers of blast cells in the peripheral blood and/or marrow, and increasing systemic symptoms. In some patients the increasing blast cell numbers observed in the peripheral blood are surprisingly not accompanied by increasing blast cells in the marrow, indicating that the cellular origin of the MPN-BP likely occurs in an extramedullary site, most frequently the spleen. Genetic studies of paired samples before and after leukemic transformation have suggested there are at least two distinct routes for leukemic transformation that are operational. Some patients who present with a *JAK2V617F/MPL* mutation-positive MPN progress to *JAK2V617F/MPL* mutation-positive MPN-BP that is associated with the acquisition of additional genetic alterations. A second, more complex, route to MPN-BP from the original MPN has been described in which a *JAK2V617F/MPL* mutation-positive MPN is followed by *JAK2V617F/MPL* mutation-negative MPN-BP. Clonality studies using X-chromosome inactivation in females demonstrated that *JAK2V617F/MPL* mutation-positive MPN and *JAK2V617F/MPL* mutation-negative MPN-BP are clonally related, consistent with transformation of an antecedent, pre-*JAK2V617F/MPL* mutation mutant clone that can progress to MPN-BP. Patients with *JAK2V617F*-positive MPN who developed *JAK2V617F*-negative MPN-BP had a shorter time between the diagnosis of the original MPN and the development of MPN-BP (3 + 2 vs. 10 + 7 years, respectively) than patients with *JAK2V617F*-positive leukemias. SNP array analysis has shown that genomic alterations occur at an increased frequency during the period of blastic transformation and that no single gene or molecular pathway is sufficient to cause transformation. A surprising correlation has been observed between the phenotype of the preceding MPN and the *JAK2* mutational status of the leukemic blasts after transformation. In contrast to *JAK2* WT MPN-BP, evolution to *JAK2V617F*-positive leukemia is invariably preceded by a myelofibrotic transformation of ET/PV or *JAK2V617F*-positive PMF. Because of these observations, myelofibrotic transformation of ET or PV is thought by many to represent an accelerated phase of the initial MPN preceded by genetic changes that result in evolution to MF and eventually MPN-BP. *JAK2* WT MPN-BP, by contrast, usually arises in patients with chronic-phase PV or ET that do not undergo evolution to MF. Some have suggested that these leukemias are therapy related. The reversion from *JAK2V617F* to WT *JAK2* in these leukemias has been shown not to be caused by homologous recombination. Two models have been proposed to account for the clonal relationship between *JAK2* WT MPN-BP and its preceding MPN: (1) both the chronic MPN and the MPN-BP arise from a shared pre-*JAK2V617F* founder clone; and (2) the chronic MPN and the MPN-BP arise from two independent stem cells. It remains possible that each model is viable and operates in different individual patients. Mutations in *CALR*, *JAK2*, *TP53*, *IDH2*, and *ASXL1* are frequent events in MPN BP, while mutations in *NPM1*, cohesin complex members, *FLT3*, and *CEBPA*, which are common events in de novo AML, are rarely, if ever, observed in MPN-BP. These data provide genetic evidence that MPN-BP is a distinct disease from classical AML with a unique mutational profile and molecular pathogenesis and that lack of response to AML chemotherapy regimens reflects the divergent biology of MPN-BP. Important differences in the mutational spectrum of *JAK2V617F* and *JAK2*-WT in post-MPN AML have been observed. *TP53* mutations are frequent, co-occurring events in patients with *JAK2V617F* mutations but not in patients with *CALR* mutations. In patients with co-occurring *JAK2V617F* and *TP53* mutations, the *JAK2*/*TP53* mutant clone dominates, consistent with potent cooperativity in vivo and with a dominant role in inducing transformation from the chronic *JAK2V617F*-positive MPN to AML. By contrast, mutations in members of the RAS pathway, *SRSF2*, and in *ASXL1* were more common in *JAK2* WT MPN-BP, and these mutations often were present as subclonal disease alleles at the time of transformation, suggesting they are not rate limiting in the transformation from the chronic to MPN-BP.

CLINICAL MANIFESTATIONS

The initial clinical manifestations of PV can largely be attributed to the excessive production of cells belonging to each of the myeloid lineages affected by the malignant process, including RBCs, platelets, and WBCs. With the implementation of laboratory tests during annual physical examinations, increasing numbers of people are being diagnosed with PV before the symptoms related to this neoplastic process become apparent. Symptomatic patients with PV may present to a physician with a myriad of nonspecific complaints, including headaches, weakness, pruritus, dizziness, excessive sweating, visual disturbances, paresthesias, joint symptoms, abdominal distress, a thrombotic or hemorrhagic episode, and weight loss. Thrombosis is a frequent presenting event. Two-thirds of such thrombotic events occur either at presentation or before diagnosis and the remainder most often during the first 10 years of follow-up. At diagnosis, one-third of patients have already lost 10% of their body weight, presumably secondary to the hypermetabolism associated with this disorder, and complaints of fatigue are common. Arthropathies are frequently observed and are largely caused by the clinical manifestations of gout. The hyperproliferative BM state characteristic of PV and the increased nucleoprotein degradation are contributory factors in the development of hyperuricemia.

The principal findings on physical examination of a patient with PV include ruddy cyanosis, conjunctival plethora, hepatomegaly, splenomegaly, and hypertension. Untreated patients are at particularly high risk for thrombotic and hemorrhagic events. In several large series of patients with PV, thrombosis was the cause of death in 30% to 40% of patients. Arterial thrombotic events account for two-thirds of such events, with venous thrombotic events representing the remainder. Ischemic stroke, myocardial infarction, and TIAs are the most common arterial thrombotic events. Patients may also present with deep venous thrombosis in the lower extremities, pulmonary embolism, or peripheral vascular occlusions. The cumulative rate of thrombosis ranges from 2.5% to 5% per patient per year. The prevalence of thrombosis at diagnosis ranges from 34% to 39%. It is not unusual for patients with PV to develop thromboses at unusual anatomic sites; in particular, thromboses are relatively frequent in the splanchnic veins, including the splenic, hepatic, portal, and mesenteric vessels or cerebral sinus veins, thrombosis of the vena cava, and intraventricular thrombosis. A particularly serious thrombotic event associated with PV is BCS, which results from hepatic venous or inferior vena caval thrombosis and obstruction. These events lead to hepatic venous outflow obstruction, increased hepatic sinusoidal pressure, and portal hypertension. Portal venous perfusion of the liver is frequently reduced, leading to portal venous thrombosis and hypoxic damage of liver parenchymal cells. This cascade of events results in centrilobular hepatic necrosis, centrilobular fibrosis, and nodular regenerative fibrosis, which culminates in the development of cirrhosis of the liver. The cause of BCS can be identified in 75% of cases, including hereditary and acquired prothrombotic disorders, trauma, and infection. PV account for 10% to 40% of all cases of BCS. Paroxysmal nocturnal hemoglobinuria is also a frequent cause of BCS (see Chapter 32). Because BM cells from 87% of patients with idiopathic BCS form erythroid colonies in the absence of EPO, such patients were in the past believed to have a forme fruste of an MPN. The identification of these latent MPNs, without elevated blood counts, has been facilitated by screening for *JAK2V617F*. These patients tend to be younger and female, and to have normal blood counts caused by hemodilution and hypersplenism, which mask an elevated RBC mass. Many of these patients (37%) also have another predisposing factor for thrombosis such as exposure to oral contraceptives, antiphospholipid antibodies, factor V Leiden, or protein C deficiency. The *JAK2V617F* mutation has been reported in 32.7% of 831 patients with an idiopathic splanchnic vein thrombosis. In another study, the mutation was present in 49% of patients at diagnosis of the idiopathic form of splanchnic vein thrombosis (SVT), of which 60% were diagnosed as having a coexisting MPN; more than 52% of the remaining patients went on to develop an MPN after a median of 49 months. *JAK2V617F* was detected in 45% of patients with BCS, and at least in one series, BCS is occasionally observed in PV patients with exon 12 *JAK2* mutations.[37]

Furthermore, additional patients (6.9%) who have idiopathic SVT have a BM histopathologic picture diagnostic of an MPN but are *JAK2V617F* negative. The diagnostic value of low serum EPO level in BCS has been questioned because elevated levels have been attributed in *JAK2V617F*-positive patients with necrotic liver tissue.

There are fulminant, acute, subacute, and chronic forms of BCS. These clinical manifestations depend on the extent and rapidity of hepatic vein occlusion and the development of venous collaterals to decompress the venous sinusoids. One should always consider a diagnosis of BCS in any patient with an MPN with ascites, upper abdominal pain, and liver function abnormalities. This syndrome is characterized by hepatosplenomegaly, ascites, edema of the peripheral extremities, and distension of superficial abdominal veins caused by resultant portal hypertension. Routine biochemical determinations of hepatocellular function and injury are frequently of little diagnostic value in patients with suspected BCS. Doppler ultrasonography is the best tool for screening patients for BCS. This test has a sensitivity and specificity of 85%. Characteristic findings include an absence of flow in the hepatic veins or nonvisualization of the hepatic vein. Contrast-enhanced computed tomography scanning and magnetic resonance imaging (MRI) are useful in better defining the hepatic venous anatomy. Hepatic venous and inferior vena caval catheterization is a key diagnostic procedure indicating the sites of venous obstruction. The diagnosis can be definitively made by a spider web pattern on hepatic venography. Transjugular liver biopsy specimens usually reveal intense congestion and cellular atrophy.

It has been emphasized that patients with PV can present with portal or hepatic vein thrombosis with normal hemoglobin or hematocrit values. Such patients may have leukocytosis, thrombocytosis, or splenomegaly. Screening for the *JAK2* mutation should be systematically carried out in such situations. Gastrointestinal bleeding or an increase in plasma volume that is a consequence of splenomegaly often accounts for the normal blood counts in such patients with BCS. The factors operational in the PV patient that lead to the development of hepatic vein thrombosis are believed to be multiple. Whereas splenomegaly causes increased portal blood flow, extramedullary hematopoiesis within the hepatic sinusoids frequently obstructs hepatic blood flow, and *JAK2V617F*-positive endothelial cells might affect adhesion to monocytes, leading to aggregation of blood elements. These processes are surely important contributory factors in addition to the other previously discussed risk factors that lead to the development of thrombosis in this patient population.

Neurologic abnormalities occur in almost 60% to 80% of untreated or poorly controlled PV patients and include TIAs, cerebral infarction, cerebral hemorrhage, fluctuating dementia, confusional states, and choreic syndromes. PV-associated chorea[59] (Video 70.1) has been reported as a presenting complaint, primarily in older females, which typically involves the orolingual or appendicular musculature and often resolves with phlebotomy or cytoreductive therapy. In addition, complaints of dizziness, paresthesias, visual disturbances, tinnitus, and headaches have been attributed to the increased blood viscosity and reduced cerebral blood flow caused by erythrocytosis. The transient neurologic symptoms can also be the consequence of small infarcts in the region of the basal ganglia, which can be detected by computed tomography. These small infarcts are known as *lacunae* and result from the occlusion of small penetrating arteries, which are particularly susceptible to thrombosis. Cerebrovascular thrombosis occurs more often in PV patients than in the general population. Symptoms caused by intermittent carotid or vertebral basilar artery insufficiency (or both) occur so frequently in PV that it is suggested that every patient with focal cerebrovascular insufficiency should at least have a complete blood count to exclude the diagnosis of an underlying MPN.

Thrombosis of the dural sinus or cerebral veins (CVT) is an uncommon form of stroke, usually affecting young individuals. CVT represents about 0.5% to 1% of all strokes. While 3.8% of CVT patients are diagnosed with MPNs, 0.4% of MPN patients have a CVT during their clinical course. Headache, generally indicative of an increase in intracranial pressure, is the most common symptom in CVT. Clinical manifestations of CVT may also depend on the location of the thrombosis. The superior sagittal sinus is most commonly involved, which may lead to headache, increased intracranial pressure, and papilledema. A motor deficit, sometimes with seizures, can also occur. Scalp edema and dilated scalp veins may be seen on examination. For lateral sinus thromboses, symptoms related to an underlying condition (middle-ear infection) may be noted, including constitutional symptoms, fever, and ear discharge. Pain in the ear or mastoid region and headache are typical. Hemianopia, contralateral weakness, and aphasia may sometimes be seen owing to cortical involvement. Approximately 16% of patients with CVT have thrombosis of the deep cerebral venous system (internal cerebral vein, vein of Galen, and straight sinus), which can lead to thalamic or basal ganglial infarction. Cavernous sinus thrombosis is usually associated with a primary infectious etiology involving a focus in the face, throat, mouth, ear, or sinuses. Aseptic cavernous sinus thrombosis is an extremely rare phenomenon that has been reported in patients with PV. These patients present with monocular blindness and the characteristic features of ipsilateral cavernous sinus thrombosis, and only retrospectively is the diagnosis of PV made. Therefore, patients found to have this symptom complex who have no known infectious predisposing causes should be carefully evaluated to rule out a diagnosis of PV. The most sensitive diagnostic technique is MRI in combination with magnetic resonance venography.

Thrombosis of large-caliber arteries is a relatively rare event in PV patients, but there have been case reports of thromboses within the chambers of the heart, leading to refractory congestive heart failure and acute aortic occlusion. Such catastrophic thrombotic events in the heart or large vessels would suggest that cardiac catheterization be performed with some caution.

PV frequently manifests with symptoms caused by peripheral vascular disease. In these cases, patients may first be seen by surgeons or dermatologists. Intense redness or cyanosis of the digits with or without burning, classic erythromelalgia, digital ischemia with palpable pulses, or thrombophlebitis without another known cause may be the presenting symptoms.

Erythromelalgia is characterized by burning pain in the digits, an objective sensation of increased temperature, and relief by cooling. PV is the most common cause of erythromelalgia and is one of the few disorders in which digital ischemia with or without ulceration may exist in the presence of palpable pulses. Other disorders that can lead to this abnormality include embolism, trauma, cutaneous infarction, neuritis, infection, and various types of arteritis. Painful and ulcerating toes and fingers have been observed to be presenting symptoms in patients with PV. The likelihood that arterial insufficiency is the cause of such ulceration is quite small in patients who have a palpable dorsalis pedis and posterior tibialis pulses; in this situation, the possibility of an underlying hematologic disorder such as PV should be entertained. Foot pain at rest is a distressing but not widely recognized symptom of PV. In patients with this complaint, peripheral pulses are of normal character, and cutaneous circulation appears to be adequate. The pain is most severe at night, is dull in nature, and occurs primarily in the feet or legs. These symptoms have been shown to be the results of platelet activation and aggregation in vivo, which preferentially occur in arterioles. If untreated, erythromelalgia can progress to ischemic acrocyanosis or gangrene. Phlebotomy alone in PV does not improve erythromelalgia. These symptoms can be abolished by reducing the platelet counts to normal levels and can be rapidly reversed after the institution of antiplatelet aggregation therapy but not anticoagulation. Therefore, the cause of erythromelalgia appears to be closely linked to abnormal arachidonic acid metabolism that occurs within PV platelets.

Retrospective reviews have revealed a higher-than-expected number of patients with PV and pulmonary artery hypertension. PV is associated with nearly a threefold higher risk for developing pulmonary artery hypertension.[38] This diagnosis usually requires transthoracic echocardiography followed by a right heart catheterization. Proposed etiologies include direct obstruction of pulmonary arteries by circulating megakaryocytes, extramedullary hematopoiesis in the pulmonary parenchyma, smooth muscle hyperplasia induced by release of PDGF from activated platelets, chronic disseminated intravascular coagulation, and unrecognized recurrent pulmonary emboli. The diagnosis of pulmonary hypertension is made, on average, 9.5 years after the diagnosis of the underlying MPN and is associated with a poor prognosis, with death usually occurring as a result of congestive heart failure or pneumonia. Although anecdotal reports have claimed improvements in patients' pulmonary artery pressure with control of the underlying disease, others have not found any change in serial measurements of 11 patients' pulmonary arterial pressures despite good disease control. Ruxolitinib therapy has been reported to result in improved outcomes, pulmonary and echocardiographic findings, as well as correction of cytokine dysregulation in MPN patients with pulmonary hypertension. Ironically, several case reports have linked ruxolitinib exposure to the development of pulmonary hypertension. The use of ruxolitinib in treating pulmonary hypertension remains uncertain and patients should be monitored carefully if it is used for these purposes

As many as 30% to 40% of patients with PV experience some sort of hemorrhagic event, which can be relatively trivial, such as epistaxis or gingival hemorrhage, or can be life-threatening, such as gastrointestinal hemorrhage or hematomas involving vital organs. The gastrointestinal tract is a frequent site of hemorrhagic complications because patients with PV are predisposed to portal vein thrombosis and resultant variceal bleeding and peptic ulcer disease. Gastroduodenal erosions and ulcers, and *Helicobacter pylori* infection are all significantly more common in PV patients than in control patients with dyspepsia. This may be partly attributable to altered mucosal blood flow as a result of increased plasma viscosity or increased histamine release caused by peripheral blood basophilia. Cerebral hemorrhage is a common cause of morbidity and mortality. Bleeding events frequently occur with the use of aspirin or other nonsteroidal antiinflammatory agents; an association between the hemorrhage and the use of high doses of these platelet-paralyzing drugs has been made in almost one-third of such instances. Low-dose aspirin therapy has, however, been reported not to lead to an increased incidence of life-threatening hemorrhagic events. Spontaneous bleeding in patients with PV is relatively rare, although spontaneous retropharyngeal hematomas leading to acute upper airway obstruction or hematomas in the groin or retroperitoneum have been reported.

Patients with PV who undergo surgical procedures are at a very high risk of developing postoperative complications. In one series of 62 major operations on 54 patients with PV, postoperative complications occurred in 49% of patients; 52% of complications were from hemorrhage, 18% from thrombosis, and 14% from hemorrhage and thrombosis. The postoperative mortality rate in this patient population was 18%. In another series of 15 patients, five had serious complications secondary to thrombosis and hemorrhage. A study by an Italian group retrospectively evaluated 311 surgical interventions in 105 patients with PV and 150 with ET: 24 arterial or venous thromboses (7.7%), 23 major hemorrhages (7.3%), and five surgery-related deaths (1.6%) were observed within 3 months of the procedure. PV patients with uncontrolled erythrocytosis before surgery have been shown to have the highest complication rate. Patients with inadequately controlled disease had a 79% incidence of complications, but in those with adequate hematologic control before surgery, the rate of perioperative and postoperative complications was reduced to 28%. In addition, the duration of disease control was an important factor in decreasing surgical risk; a prolonged period of effective disease control before surgery reduced the complication rate to 5%. Complication rates after surgery can therefore be dramatically reduced by appropriate therapeutic interventions with normalization of blood counts. The chief deterrent to such an approach has been the failure by physicians to recognize the risk associated with PV in the surgical setting.

Another situation that puts patients with PV and ET at high risk for thrombosis is pregnancy. Maternal and fetal complications have been reported. Specifically, the rate of fetal loss is higher than that of the general population.

Generalized pruritus occurs in approximately 40% of cases of PV. Water contact, such as during showers or bathing, induces attacks of intolerable pruritus. There appears to be no clear relationship between the degree of the pruritus and severity of the disease, and 20% of patients continue to experience itching despite reduction of their hematocrits to normal levels. Aquagenic pruritus is significantly more common among *JAK2V617F* homozygous patients than heterozygotes. The degree of pruritus is so severe in some patients that they are unable to tolerate bathing at all and find it necessary to substitute gentle skin swabbing or to simply not bathe. The etiology of the pruritus in PV remains uncertain. Several groups have attempted to implicate elevated blood and urine histamine levels in its pathobiology. A strong correlation between skin mast cell numbers and the severity of itching has been demonstrated, and mast cells in PV have been shown to be *JAK2V617F* positive. However, the failure of the pruritus to respond to antihistamine therapy in many patients suggests that abnormally high histamine levels probably do not constitute the sole factor in its development.

Iron deficiency has also been implicated as a factor contributing to pruritus in PV patients who are almost invariably iron-deficient. Iron-substitution therapy has resulted in symptomatic improvement, but this approach is less than optimal because it frequently results in uncontrollable erythrocytosis. One theoretical approach to combat this therapeutic challenge is to use hepcidin to correct iron deficiency in PV patients while restricting the iron available for erythropoiesis at the same time. A phase II study of hepcidin mimetic PTG 300 (rusfertide) is currently testing this hypothesis. Ruxolitinib therapy has been reported to be effective in alleviating the patient's aquagenic pruritis in 80% of patients.

The risk of development post-PV MF is increased with prolonged duration of disease and is directly related to the duration of long-term follow-up, but the influence of the modality used to treat the initial PV phase of the disease on the rate of transformation to this more accelerated form of the disease remains uncertain. Post-PV MF should be considered the natural evolution of PV. Criteria for the diagnosis of post-PV MF have been established (Table 70.2) and can be used as a guide for documenting this transition in disease phenotype, which in reality represents a point in the continuum of PV. For patients with disease duration greater than 10 years, the HR was 15.24 (95% CI: 4.22 to 55.06; $P < .0001$). The median interval between the diagnosis of PV and the development of post-PV MF is 13 years. Post-PV MF is characterized by (1) increasing splenomegaly; (2) teardrop RBC morphology; (3) extensive BM fibrosis; (4) a leukoerythroblastic blood picture, systemic symptoms including fatigue, weight loss, night sweats, bone pain, and fevers; and (5) anemia and or thrombocytopenia. The patients may be entirely asymptomatic but often complain of fatigue, dizziness, weight loss, and anorexia. Splenomegaly can lead to abdominal pain caused by repeated splenic infarcts and to early satiety caused by mechanical obstruction of the upper gastrointestinal tract. Patients with post-PV MF are virtually all *JAK2V617F* positive and characteristically have high *JAK2V617F* allele burdens.

The anemia that characterizes post-PV MF is primarily a result of splenic pooling, ineffective erythropoiesis, hemolysis, and extramedullary production of RBCs with a shortened RBC survival. Patients positive for *JAK2V617F* are less likely to require blood transfusions but have a poorer survival. Occasionally, the anemia is exacerbated by folate or iron deficiency. Before assuming that a patient has entered

TABLE 70.2 **International Working Group for Myelofibrosis Research and Recommended Treatment Criteria for Post-Polycythemia Vera Myelofibrosis**

Required Criteria

1. Documentation of a previous diagnosis of polycythemia vera as defined by the WHO criteria
2. Bone marrow fibrosis grade 2–3 (on 0–3 scale) or grade 3–4 (on 0–4 scale)

Additional Criteria (Two Are Required)

1. Anemia or sustained loss of requirement of either phlebotomy (in the absence of cytoreductive therapy) or cytoreductive treatment for erythrocytosis
2. A leukoerythroblastic peripheral blood picture
3. Increasing splenomegaly defined as either an increase in palpable splenomegaly of ≥5 cm (distance of the tip of the spleen from the left costal margin) or the appearance of a newly palpable splenomegaly
4. Development of ≥ one of three constitutional symptoms: >10% weight loss in 6 months, night sweats, unexplained fever (>37.5°C)

Adapted from Barosi G, Mesa RA, Thiele J, et al. Proposed criteria for the diagnosis of post-polycythemia vera and post-essential thrombocythemia myelofibrosis: a consensus statement from the international working group for myelofibrosis research and treatment. *Leukemia*. 2008;22:437.
WHO, World Health Organization.

post-PV MF, it is prudent to assess BM iron stores. Replacement therapy with iron may lead to the resurgence of erythropoiesis and prevent the faulty categorization of disease progression.

Bleeding abnormalities caused by thrombocytopenia or qualitative platelet abnormalities are especially common during this phase of the disease. Frequent instances of epistaxis or ecchymoses occur, and gastrointestinal hemorrhage caused by esophageal varices arising from portal hypertension is a recurrent problem. The majority of hemorrhagic events are minor in nature. Frequently, patients have generalized wasting characterized by progressive asthenia and weight loss. Severe hyperuricemia, leading to secondary gout or uric acid nephropathy, may also complicate the clinical course.

The median survival for patients with post-PV MF is 5.7 years. Patients with post-PV MF are at a high risk for the development of MPN-BP. Of patients who develop post-PV MF, approximately 18% will undergo leukemic transformation after 3 years and likely a greater number with longer follow-up.

The leukemic transformation of PV has been extensively described. The possibility that a relationship exists between the therapeutic modality used during the erythrocytotic phase and the frequency of development of MPN-BP has been a point of heated discussion. Some of the controversy surrounding this question was formerly caused by a lack of understanding of the basic origins of PV. Clinical hematologists in the 1950s and 1960s frequently thought of PV as a benign hematologic abnormality and believed that therapeutic interventions either with alkylating agents or radiotherapy were solely responsible for the development of MPN-BP. That concept has proved erroneous, and PV, similar to the other MPNs, has been shown to be a clonal malignant hematologic disorder. The evolution to MPN-BP can therefore be thought of as a natural consequence of this malignant disorder, which can be accentuated by the therapeutic interventions already discussed. In fact, 25% of patients who develop MPN-BP have never been exposed to any form of cytotoxic therapy.

Further insight into the relationship between MPN-BP and PV has been best provided by the results of the PV Study Group (PVSG), which described a randomized trial comparing the use of phlebotomy, chlorambucil, and ^{32}P for the treatment of this disorder. The incidence of MPN-BP was approximately 1.5% in patients treated with phlebotomy alone, 17.5% in patients treated with chlorambucil, and 10.9% in patients treated with ^{32}P after over 15 years of follow-up. The incidence of MPN-BP in the patients treated with phlebotomy alone is therefore much higher than that expected in a normal age-matched control group, again indicating that leukemia is a natural evolutionary event in the clinical course of an individual with PV. The incidence of MPN-BP can be increased, however, by the institution of therapy with either alkylating agents or ^{32}P. The time course for the development of MPN-BP appears to be dependent on the treatment used to control the polycythemia. The development of MPN-BP in patients treated with phlebotomy in the PVSG trial was limited to the first 5 years of treatment, suggesting that the development of MPN-BP is not solely attributable to the prolongation of survival. In contrast, analysis of the hazard function was virtually flat for patients treated with chlorambucil from years 2 to 7 after randomization; however, the risk for MPN-BP became alarmingly high after 10 years of study, suggesting that the risk of MPN-BP increases with time even after the drug has been stopped. One half of the cases of MPN-BP in the chlorambucil arm occurred during the first 5 years, with the remainder equally split between the second and third 5-year periods. In contrast, 60% of the cases of MPN-BP in the group treated with radioactive phosphorus occurred 6 to 10 years after randomization. Of particular concern is the high incidence of MPN-BP reported in patients who were initially treated with radioactive phosphorous or busulphan and then switched to maintenance therapy with HU, previously believed to be a nonleukemogenic agent. Some light was shed on the genetic basis of the early and delayed onset MPN-BP following PV by Paz and co-workers. They identified groups of PV associated with a distinct time to leukemic transformation. Transformation to MPN-BP after short-term follow-up was characterized by a complex molecular landscape with mutations in *IDH1/2*, *RUNX1*, and *U2AF1* genes occurring, while evolution to MPN-BP over longer periods of time was associated with mutations in *TP53*, *NRAS*, and *BCORL1* genes.[39] The association between *TP53* mutations and long-term transformation to MPN-BP is usually related to the necessary inactivation of the second allele of *TP53* for leukemic transformation either by a mutation in the other allele, deletion of 17p where the gene for TP53 is located or the acquisition over time of multiple copies of 1q in which MDMD4, a degrader of P53, is encoded. Also transcripts for MDM2, as well as the protease PPM1D, which also degrade P53 activity, are progressively upregulated in PV over time leading to further loss of P53 activity. Furthermore activating mutation of PPM1D can also contribute to this process. This progressive loss of P53 activity during the course of PV due to multiple genetic events has been proposed by Marcellino and coworkers to play a pivotal role in the development of MPN-BP (Fig. 70.9).[40] These mutations were frequently already present during the PV phase, either with a high VAF (short-term transformation) or with a low VAF (especially *TP53* mutations). These data suggest that the evolution of PV to MPN-BP may be driven by distinct time-dependent molecular and cytogenetic events. The use of a combination of an alkylating agent, busulphan, pipobroman, or melphalan, may particularly increase the risk of leukemia. Approximately 30% to 50% of patients with PV who develop MPN-BP have previously entered the post-PV MF phase. In contrast,

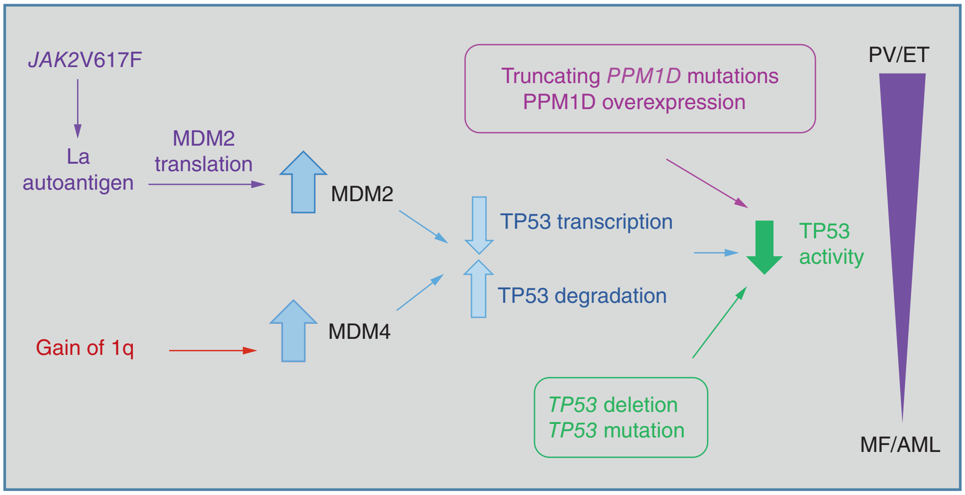


Figure 70.9 MULTIPLE MECHANISMS TO DOWNREGULATE TP53 ACTIVITY IN PV. *ET*, Essential thrombocythemia; *MF*, myelofibrosis; *PV*, polycythemia vera.

approximately 50% of patients progress directly from the erythrocytotic phase to MPN-BP. The phenotype of the leukemia cells that characterize the MPN-BP is overwhelmingly myeloid, although rare cases of lymphoblastic and biphenotypic leukemias have been reported. Patients with *JAK2V617F*-positive MPN are also at a higher risk of developing both additional hematologic malignancies and solid tumors. The SNPs for TERT, which is a susceptibility factor for familial and sporadic MPNS, have also been shown to be associated with a variety of solid tumors. The presence of this SNP predisposes to the development of MPNs and the co-occurrence of solid tumors, especially in patients receiving cytoreductive therapy. Some investigators have suggested that prolonged cytoreductive therapy be avoided in patients with the TERT polymorphism. In addition, low-grade lymphoproliferative disorders such as chronic lymphocytic leukemia and MGUS have been shown to coexist in patients with a variety of MPNs, including PV, and not to have an adverse effect on prognosis.

In some instances, a preleukemic phase characterized by refractory anemia with excess blasts has been described. In fact, half of such cases of MPN-BP in one series were preceded by a myelodysplastic disorder.

PV can be associated with unanticipated consequences. Johansson reported that in 10,752 patients diagnosed with an MPN in Sweden from 1995 to 2015 there was a significant increased risk of both hip and vertebral fracture for the total group of MPN-patients compared with the total Swedish population and this effect was especially noticeable in PV patients. The risk for hip fracture was increased in PV patients by 37% and the risk for vertebral fracture was increased by 109% in patients with PV. Studies of PV mouse models have provided some insights into this increased risk of bone fractures. PV mouse models are associated with decreased osteoblast activity in mice, and polycythemia due to chronically elevated EPO levels in mice also results in bone loss, and implications on patients treated with rEPO should be evaluated.[41]

LABORATORY MANIFESTATIONS

Laboratory evaluation of patients with erythrocytosis involves the careful use of a broad range of diagnostic studies. Because PV is a panmyelosis, the overwhelming number of patients has elevated hematocrits, WBC counts, and platelet counts. The diagnosis of PV has been greatly simplified by the discovery of the *JAK2V617F* mutation, which is present in more than 95% of PV patients. Hematocrit values greater than 49% in males and greater than 48% in females are abnormal and require further evaluation. Documentation of the absolute increase in RBC mass is rarely required and is a test that is available at few institutions. A hematocrit value greater than 60% in men or greater than 55% in women is almost always associated with an absolute erythrocytosis. Occasionally, an elevated RBC mass can actually be present in the face of a normal hematocrit value. In cases of splenomegaly caused by portal hypertension, an expanded plasma volume may mask an elevated RBC mass. In addition, iron deficiency can also lead to a normalization of the hematocrit in PV, making the diagnosis difficult. PV is associated with a 20% to 30% frequency of peptic ulcers and gastritis, which can be associated with blood loss. In this situation, thrombocytosis may be exacerbated as a consequence of the iron deficiency. Iron supplementation is not necessary to make a diagnosis of an MPN because of the availability of molecular diagnostic studies

Leukocytosis is present in approximately two-thirds of cases and seems to be proportional to the burden of *JAK2V617F*. Thrombocytosis is observed in 50% of cases. Abnormalities of RBC, WBC, and platelet morphology are frequently observed. The morphologic RBC changes observed during the erythrocytotic phases are characteristic of iron deficiency and include microcytosis, hypochromia, and frequently polychromatophilia. Some anisocytosis and poikilocytosis can be seen as well. Fetal hemoglobin levels and the number of RBCs containing fetal hemoglobin, known as *F cells*, may be increased. The WBCs are characterized by normal morphology, although the numbers of basophils, eosinophils, and immature myeloid forms can be increased. Platelet morphology is also quite striking in PV. Frequently, megathrombocytes (platelets the sizes of RBCs) are seen on the peripheral blood smear. Patients typically have platelet counts of less than $1 \times 10^6\,\text{mm}^{-3}$, but it is not unusual to observe a patient with a platelet count higher than this value. PV-related MF is characterized by a leukoerythroblastic blood picture, with the appearance in the peripheral blood of dacryocytes or teardrop RBCs, myelocytes, metamyelocytes, and (rarely) blasts and promyelocytes in addition to nucleated RBCs in the peripheral blood.

Platelet aggregation studies do not correlate frequently with the risk of bleeding episodes. The most common abnormalities are decreased primary and secondary aggregation to either or both epinephrine and adenosine diphosphate, and decreased response to collagen with generally a normal response to arachidonic acid. An abnormal platelet storage pool disease is a characteristic feature and is caused by abnormal platelet activation. Others have employed rotational thromboelastometry to screen for PV patients who are at an increased risk of developing bleeding or thrombotic events. The utility of this approach is yet to be determined. Prothrombin times (PT) and partial thromboplastin times (aPTT), as well as fibrinogen levels, are usually normal. Profound abnormalities of the PT and aPTT, however, are frequently reported. This is largely a laboratory artifact caused by the extreme erythrocytosis, which results in a relatively smaller volume of plasma being present in the whole blood sample. Coagulation assays are performed on blood anticoagulated with sodium citrate, and the citrate concentration in the anticoagulant is calibrated to chelate the plasma calcium and inhibit coagulation reactions. All coagulation assays include the addition of calcium chloride to neutralize the excess citrate and provide free calcium to mediate coagulation reactions. In patients with extreme erythrocytosis, the ratio of citrate in the collection tube to the volume of plasma is too high; therefore, excess citrate is present, and the standard amount of calcium chloride added during the performance of the PT and aPTT is insufficient to neutralize the excessive citrate, and the coagulation assays are frequently and factually prolonged. To avoid this problem, the clinician should calculate the relative amount of plasma compared with the normal amount and remove the corresponding volume of sodium citrate from the blood collection tube. Normal values can then be confidently anticipated in patients with erythrocytosis. A shortened fibrinogen half-life has been detected in some patients with PV and a significantly increased fractional catabolic rate of the plasma fibrinogen pool per day. In addition, elevated platelet β-thromboglobulin and plasma β-thromboglobulin levels are observed. The constellation of findings is indicative of increased platelet turnover. Prothrombin fragments F1 and 2, thrombin–antithrombin complex, and D-dimer levels are frequently elevated in PV patients. These are enzyme–inhibitor complexes or byproducts of active thrombosis that serve as a biochemical signature of the hypercoagulable state that characterizes PV.

Acquired von Willebrand syndrome occurs frequently in patients with PV and ET with over 1×10^6/L platelet numbers (see Chapter 133). This syndrome is characterized by a normal or prolonged bleeding time, normal factor VIII level, and normal von Willebrand factor (vWF) antigen level, but abnormal vWF ristocetin cofactor actively associated with a decrease or absence of large vWF multimers. This acquired defect resembles type II vWF disease. Because the molecular size of vWF is a major determinant of its adhesive function and the larger multimers are most active in achieving hemostasis, the deficiency of large vWF multimers is associated with a bleeding tendency. The decrease in the frequency of large vWF multimers occurs in patients with platelet counts over $1000 \times 10^9\,\text{L}^{-1}$. This abnormality has been reported not only with patients with severe thrombocytosis caused by MPN, but also in patients with reactive thrombocytosis. An inverse correlation between the proportion of large vWF multimers and platelet numbers has been observed. In addition, normalization of the platelet count is accompanied by restoration of a normal vWF multimer pattern. These findings suggest that thrombocytosis of any etiology may favor the adsorption of larger forms of vWF multimers onto platelet membranes, resulting in their removal from the circulation and subsequent degradation by platelet-associated proteases. Although patients with MPN frequently have bleeding tendencies, this is not the case in secondary thrombocytosis, possibly because of the limited periods of extreme thrombocytosis observed in such patients. Patients with PV with clinical courses punctuated by hemorrhagic events, extreme thrombocytosis, and acquired von Willebrand syndrome should avoid aspirin therapy if they are having a thrombotic episode but should be phlebotomized, and platelet-reduction therapy should be initiated. Patients with PV and acquired von Willebrand syndrome have recurrent bleeding from mucous membranes and the digestive tract, and easy bruising. These symptoms frequently resolve with normalization of the platelet count.

Deficiencies of one or more natural anticoagulants as well as the antiphospholipid antibody syndrome have been observed in patients with PV and thrombosis. These studies indicate that either familial or acquired antithrombin III deficiency, protein C or S deficiency, factor V Leiden mutation, or the prothrombin gene mutation may contribute to the hypercoagulable state observed in PV (see Chapter 138). Of note, although hyperhomocysteinemia caused by deficiency of cobalamin or folate can be found in 32% to 56% of PV patients, there is no agreement as to its role in the genesis of thrombotic episodes. Such inherited disorders associated with the erythrocytosis of PV provide a scenario that frequently favors thrombosis in affected individuals. In attempting to determine if a patient with PV and an active thrombosis has such an inherited predisposition, it is important to be aware that proteins C and S as well as antithrombin III levels can be low in patients with an ongoing acute thrombosis or liver cirrhosis. Normal prothrombin and factor VII levels in such patients eliminate liver cirrhosis as a cause of the reduction of these circulating anticoagulants. Individuals with inherited prothrombotic conditions frequently have levels of specific proteins below 10% to 20% of normal during periods of active thrombosis.

Whereas serum vitamin B_{12} concentrations have been found to be elevated in 40% of patients, serum vitamin B_{12}-binding proteins are elevated in 70% of patients. Hyperuricemia occurs in an overwhelming number of patients, and elevated histamine levels are also frequently observed. BM aspirates and biopsies obtained at the time of diagnosis of patients with PV are hypercellular and display characteristic erythroid, granulocytic, and megakaryocytic hyperplasia. The cellular elements (Fig. 70.10) are frequently morphologically normal. Iron stores are almost uniformly absent in pretreatment biopsy specimens. Significant increases in BM reticulin may be present in biopsies obtained early in the course but also may develop during the erythrocytotic phase and may be present for long periods before the onset of the post-PV MF. It is important to emphasize that individuals may have considerable BM fibrosis, which occurs as a consequence of the underlying MPN. The presence of minimal BM fibrosis should not be considered a harbinger of the development of post-PV MF. In patients with post-PV MF, a moderate-to-marked increase in reticulin fiber is observed, either simultaneously with or within 1 year of this clinical transformation.

Several investigators have attempted to use BM biopsy morphology as a differential diagnostic tool to differentiate between PV and secondary forms of erythrocytosis. The marked hypercellularity, erythroid and megakaryocytic hyperplasia with pleiomorphic enlarged megakaryocytes that are the hallmarks of MPNs are useful parameters for identifying such individuals (see Fig. 70.10). It is imperative to use the BM biopsy rather than the aspirate specimens for this purpose. It has been suggested that those PV patients with minor BM fibrosis are at an increased risk to develop a thrombotic episode or to evolve into post-PV MF but not acute leukemia during the course of their disease.

The pathologic appearance of the spleen in PV depends on the stage of the disease at which the organ is examined. Spleens from patients in the erythrocytotic phase of the disease are characterized by striking congestion with mature erythrocytes. Small numbers of hematopoietic precursor cells are frequently present. By contrast, spleens examined during the PV-related MF phase are characterized by prominent numbers of foci of extramedullary hematopoiesis, with representation of all BM precursor elements.

Allele-specific PCR methods or next generation sequencing can be used to detect the *JAK2V617F* or a *JAK2* exon 12 mutation in approximately 95% of patients with PV. The very high frequency of *JAK2V617F* in PV has dramatically improved our ability to diagnose this disease. Patients with isolated erythrocytosis associated with normal neutrophil and platelet counts but low serum EPO levels and the formation of erythroid colonies by peripheral blood mononuclear cells in the absence of EPO in vitro are the characteristic phenotype of patients with the exon 12 mutation. BM biopsies from these patients were slightly hypercellular with isolated erythroid hyperplasia. Megakaryocytes were morphologically normal and not clustered. The exon 12 mutations were frequently present at low levels in granulocyte DNA but were readily identifiable in endogenous erythroid colonies generated in vitro. Because granulocyte involvement with *JAK2* exon 12 mutations is low, it is important to sequence DNA from BM cells or from endogenous erythroid colonies generated in vitro to make this diagnosis (if available). At diagnosis, serum EPO levels in PV are either reduced or at the lower limits of normal. Even after normalization of the hematocrit, the serum EPO level in PV remains low in two-thirds of patients.

Arterial blood gas measurements are frequently performed to rule out hypoxia as a cause of erythrocytosis. In PV with extreme thrombocytosis, such routine measurements can prove to be misleading. Spurious hypoxemia can frequently be attributed to either significant leukocytosis or thrombocytosis caused by in vitro consumption of oxygen, the so-called *platelet and leukocyte larceny*. In this situation, pulse oximetry can be a useful tool for establishing the patient's true oxygenation status.

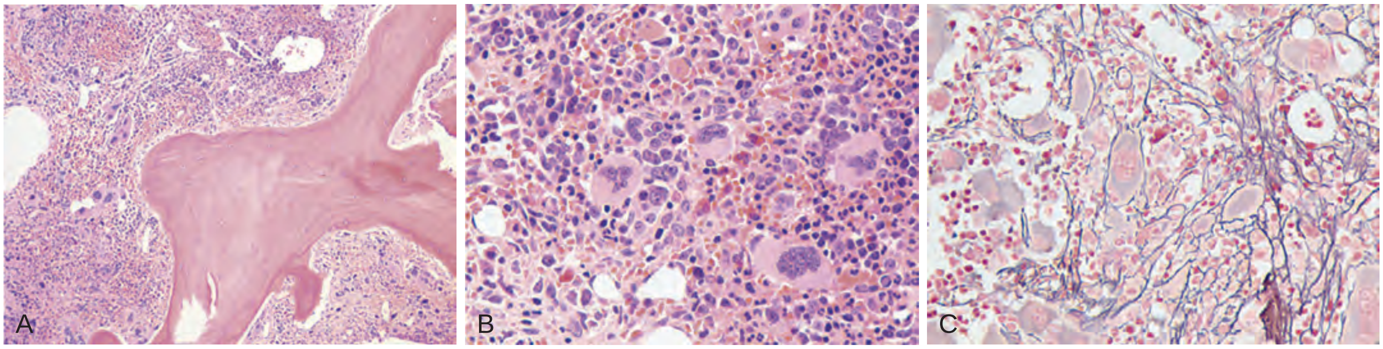


Figure 70.10 POST-POLYCYTHEMIC MYELOFIBROSIS. (A, B) Hyperplastic bone marrow with increases in all lineages, and megakaryocytic pleomorphism, with clustering. There are changes consistent with fibrosis, including streaming of hematopoietic cells and dilated sinusoids. (C) Reticulin fibers are increased, with prominent coarse fibers (grade MF2). (A, Hematoxylin-eosin [H&E] stain, ×100; B, H&E stain, ×400; C, reticulin stain, ×400.) *(From Kremyanskaya M, et al. Myeloproliferative neoplasms. In Salama M, et al. (eds). Atlas of Diagnostic Hematology. Elsevier, 2021.)*

CYTOGENOMIC ABNORMALITIES

The occurrence of nonrandom cytogenetic abnormalities in PV is anticipated because this is a feature of most hematologic malignancies. Such abnormalities have been observed without a single characteristic abnormality defined; the most frequent abnormalities are the gain of chromosome 9 as well as 9p, deletion of the long arm of chromosome 20, gain of the long arms of chromosome 1, trisomy 8 alone or co-occurrence of +8, +9, and deletion of chromosome 13 (Fig. 70.11). Balanced translocations are rarely but recurrently observed in PV although recently translocations involving JAK2, such as t(8;9)(p22;p24.1)/PCM1-JAK2), t(3;9)(q21;p24)/RPN1-JAK2, t(1;9)(p36;p24.1)/(PEX14-JAK2), t(9;22)(p24.1;q11.2)/BCR-JAK2 were described. At diagnosis, approximately 28% of patients have a recurrent clonal chromosome marker. Interphase fluorescence in situ hybridization (FISH) testing with 12 probes for loci most frequently associated with PV and other Ph^- MPNs occasionally identifies cryptic +9p, del(20q) or del(13q) abnormalities and thus may increase the frequency of detecting a chromosomal rearrangement at diagnosis to 29% to 30%.[42] However, interphase FISH (I-FISH) does not appear to significantly increase the detection rate in untreated patients. Among 452 patients with PV cytogenetically studied at the authors' institution between 1984 and April 2011, 28% had cytogenetic abnormalities at diagnosis. I-FISH studies alone (without cytogenetic examination) using an MPN panel of 12 probes revealed that 29% of patients had genomic changes (Fig. 70.12). The frequency of detection of cytogenetic abnormalities in PV increases over time, with 35% to 55% of patients having clonal cytogenetic abnormalities after extended follow-up and more than 80% of those patients in whom acute leukemia eventually develops. Progression from a normal to an abnormal karyotype is an important adverse prognostic parameter. Most of the PV-related chromosomal abnormalities are occurring in JAK2V617F mutated cells. However, clonal single cells studies have documented that abnormalities such as del(20q) and +8,+9 can occasionally precede acquisition of JAK2V671F mutation in single CD34 HPC, suggesting that clonality can occasionally precede development of JAK2V617F mutation.

Trisomy of the long arms of chromosome 1 is a recurrent abnormality present in about 4% to 6% of patients with PV with an abnormal karyotype. This abnormality has also been described in PMF and other myeloid malignancies. Specifically, 70% of patients with post-PV MF develop trisomy 1q as a result of unbalanced translocations. Trisomy 1q rarely occurs alone and is most frequently found translocated to another chromosome, creating an unbalanced +1q translocation. The recipient chromosome most frequently involved is chromosome 6 followed by chromosome 9. Jumping translocations are a rare recurrent cytogenetic phenomenon whereby a part of one chromosome is translocated to several recipient chromosomes, creating multiple related clones within a single patient. Jumping 1q in MPN occurs in 4.2% of cytogenetically abnormal MPN; 86% of such patients progress to AML after an average of 8 months, suggesting that 1q jumping translocations are associated with both disease progression and poor prognosis. Gain of 1q is most prevalent in patients with a history of PV and *JAK2V617F+* mutation (~50%) and is accompanied by an increased transcript level of *MDM4* (residing at 1q32.1). Since these patients have co-occurrence of either del(17p) or TP53 mutation, the current hypothesis is that gain of 1q leads to further dysregulation of the TP53 pathway and contributes to the progression of disease.[40]

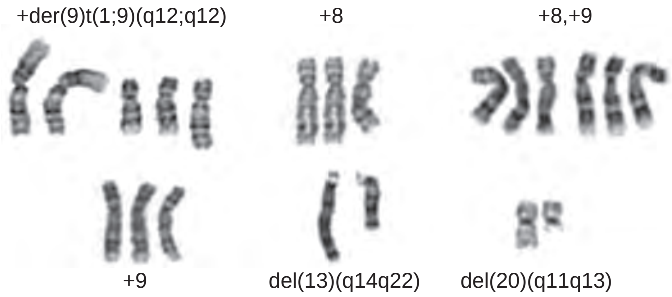


Figure 70.11 Most frequent chromosomal abnormalities associated with polycythemia vera at diagnosis include *(top row)* a gain of derivative(9)t(1;9), resulting in three copies of the long arms of chromosome 1 and three copies of the long arms of chromosome 9, including Janus kinase 2; gain of chromosome 8 and simultaneous gain of both chromosomes 8 and 9 *(bottom row)*; gain of chromosome 9 alone; interstitial deletion of chromosome 13; and interstitial deletion of chromosome 20.

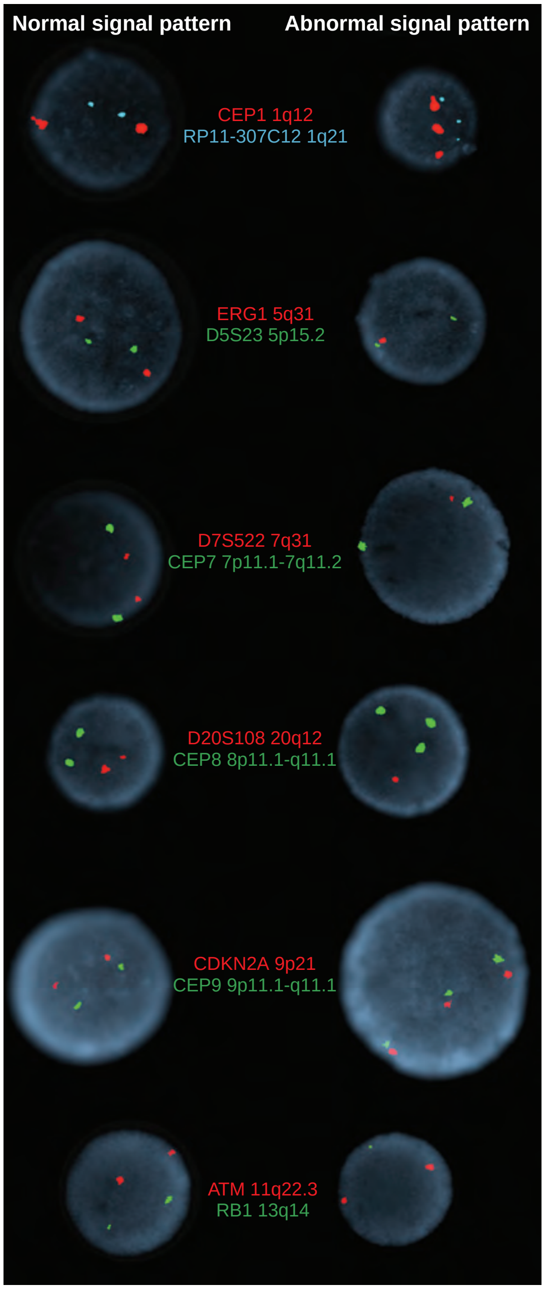


Figure 70.12 Myeloproliferative neoplasm fluorescence in situ hybridization (FISH) panel includes 12 loci on eight different chromosomes for detection of the most frequent chromosomal rearrangements when cytogenetics are either not available or in conjunction with conventional cytogenetics for detection of cryptic abnormalities. Discrepancies in frequency of abnormality detected by interphase FISH and conventional cytogenetics are present and often suggest a proliferative advantage of the abnormal clone.

Gain of chromosome 8 is a recurrent abnormality not only in Ph$^-$ MPN, but it is also one of the three most frequent abnormalities in MDS and is present in 10% of patients with malignant hematopoietic disorders of both myeloid and lymphoid lineages. The prognostic significance in PV is unknown. Some patients with PV with trisomy 8 do not acquire other abnormalities after 20 years. The simultaneous presence of both +8 and +9 is PV specific, and is observed in 3% to 4% of PV cases; it is rarely seen in other hematologic malignancies. In PV, trisomy 8 has been demonstrated in myeloid cells but not in lymphoid cells.

Trisomy 9 and gain of the short arms of chromosome 9 is most frequently and almost exclusively observed in PV. There are three types of 9p abnormalities in PV: (1) about 30% of patients have uniparental disomy of the 9p region; (2) numerical gain, such as different chromosomal rearrangements that contribute to trisomy, tetrasomy, or amplification of 9p; and (3) unbalanced translocations, of which the most frequent, +der(9)t(1;9) t(1;9)(q12, results in a trisomy of both 9p and 1q, and appears to be a relatively specific abnormality in PV patients (Fig. 70.13). This rearrangement provides an extra copy of mutated *JAK2*. Trisomy 9p and three copies of mutated *JAK2* are rare recurrent abnormalities resulting from unbalanced chromosome 9 translocations such as der(1)t(1;9)((q12;q13, der(1)t(1;9)p13;p13) der(18)t(9;18)(p13;p11)/der(9;18)(p10;q10) or isochromosome 9(p10). I-FISH has been used to detect cryptic chromosome 9 rearrangements. FISH has uncovered chromosome 9 rearrangements in 53% of patients with abnormal FISH patterns, indicating that a gain of 9p is the most frequent genomic alteration in PV. The association between the trisomy of 9p and PV was one of the keys to the identification of the *JAK2V617F* mutation. Cytogenetic studies of 9p and LOH indicate that the LOH is attributable to mitotic recombination that enables genetic variants to progress from a heterozygous to a homozygous state without change in DNA copy number.

Interstitial deletions of the long arms of chromosome 13 are not specific for PV and are more frequently observed in PMF than in PV. About 1% to 13% of cytogenetically abnormal patients with PV have del(13q). Among patients with del(13q), about 91% have breakpoints in 13q12–14 to q21–22 regions. Fine FISH mapping has defined the commonly deleted region (CDR) to 13q13.3-q14.3 encompassing Rb1 and two microsatellite loci, D13S319 and D13S25. Cryptic del(13q) occurs and is easily identified with the FISH method. Deletions are heterogeneous and may involve one, two, or all three loci, including Rb1. del(20)(q11q13), an interstitial deletion of the long arm of chromosome 20, is the second most common cytogenetic abnormality in PV (Fig. 70.14). Del(20)(q11q13) is not diagnostic of PV because it also occurs in PMF and ET, as well as in MDS, AML, and multiple myeloma. Heterogeneity of the breakpoints is suggested by the observation that two minimally deleted regions (MDRs) characterize different disorders. A 2.7-Mb region spanning D20S10-8 (proximal) and D20S481 (distal) is identified in Ph$^-$ MPN, and a 2.6-Mb region spanning R52161 (proximal) and Wi-12515 (distal) is found in other malignancies. Two CDR, CDR1 spanning 2.4 Mb between bands 20q11.23 and 20q12, and CDR2 encompassing 1.8 Mb within 20q13.12, have been identified. The commonly retained region (CRR)1 spans 1.9 Mb within 20q11.21 and CRR2 encompasses 2.5 Mb within 20q13.33. High-resolution genotyping has not revealed any somatic copy-neutral LOH within these regions. Investigations to date of candidate genes within the deleted segments have failed to identify any mutations within deleted segments. In contrast, haploinsufficiency of the *L3MBTL1* gene, the human homolog of the *Drosophila* L(3)MBT polycomb group tumor suppressor gene located on the 20q12 within a region commonly deleted in several myeloid malignancies, may play a role in erythropoiesis in PV. Downregulation of *L3MBTL1* expression in primary hematopoietic stem progenitor cells (HSPCs) causes enhanced commitment to and acceleration of erythroid differentiation. Moreover, overexpression of *L3MBTL1* in primary hematopoietic CD34$^+$ cells as well as in 20q cell lines limits erythroid differentiation. Therefore, haploinsufficiency of *L3MBTL1* may contribute to erythroid differentiation in PV. It has also been demonstrated that *L3MBTL1* is important for the normal progression of cells through mitosis because both overexpression and loss of activity can affect cell division. There is evidence that 20q deletions may impair the release of granulocytes into the peripheral blood. Some patients with PV and 20q deletion in BM cells have cytogenetically normal peripheral blood granulocytes, which has been attributed to del(20q) cells being preferentially retained or destroyed in the BM. The significance of this abnormality remains unknown because it may be dormant for many years before cells with del(20q) gain proliferative advantage. It is important to emphasize, however, that patients with del(20q) have been observed without further karyotypic instability for more than 10 years. Within ~3 Mb of minimal common deleted region spanning 20q12–20q12.12, a gene *STK4*, encoding Hippo kinase *MST1* is downregulated in patients with del(20q). Functionally deletion of these two genes in normal hematopoietic cells induces thrombocytopenia, enlarged platelets, and myeloid skewing. The level of *STK4* in patients with del(20q) is down regulated below haploinsufficiency. In JAK2V617F mouse model heterozygous Hippo kinase inactivation led to accelerated development of MF. This transformation was associated with cytokine production including IL1 and IL6, suggesting a novel pathway through chronic innate immune activation, and possible novel mechanism of disease progression in patients with del(20q).

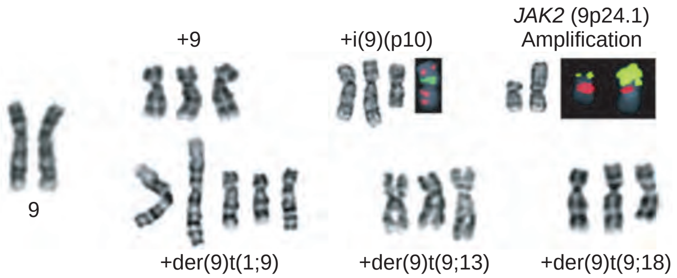


Figure 70.13 NUMEROUS CHROMOSOMAL ABNORMALITIES RESULTING IN TRISOMY, TETRASOMY, OR AMPLIFICATION OF THE SHORT ARMS OF CHROMOSOME 9. *JAK2*, Janus kinase 2.

Other rare recurrent chromosomal abnormalities may occur at the onset of the disease or are associated with disease progression. Both interstitial deletions of the long arms of chromosomes 5 and 7 have been reported at diagnosis and are associated with disease progression. Loss of P53 as a result of del(17p) or other chromosomal rearrangements is a rare finding in PV and appears to be related to disease progression. Del(17p) is not PV specific because it is found in many other myeloid malignancies.

Chromosome abnormalities including +8, +9, and del(20)(q11q13) are related to the biogenesis of the disease rather than occurring as a consequence of the chemotherapy. However, in some patients an abnormal clone (abnormalities of chromosome 5, 7, or 17) appears to develop as a consequence of exposure to chemotherapeutic agents. Some investigators have suggested that patients with cytogenetic abnormalities at diagnosis have a statistically significant poorer survival rate than those in whom a normal karyotype is observed. This influence of cytogenetic abnormalities on prognosis has, however, not been verified.

Among ~70% of PV patients who are cytogenetically normal, SNP array technology, a powerful complement to metaphase cytogenetics, revealed that at diagnosis 66% show copy number abnormalities and this number is extended to 100% of those who progressed to MF. Moreover, this exon-level technology when combined with an integrative approach such as mutational burden and cytogenetics revealed that even those PV or PV-related MF patients with a normal karyotype who are triple negative for MPN driver mutations harbor cells with cryptic recurrent lesions such as 9p21 cn-LOH or MDM4 strongly indicating that very few PV patients, when extensively worked up at diagnosis, are genomically normal.

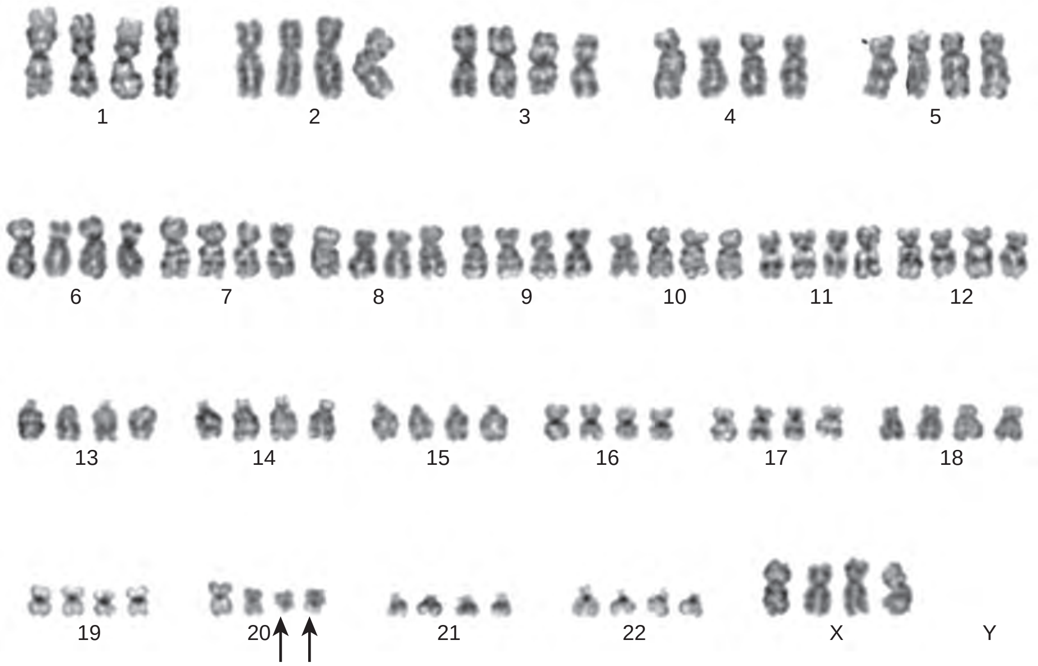


Figure 70.14 A karyotype from a dividing megakaryocyte showing *(arrows)* two copies of deleted 20q in a cell with 92 chromosomes.

DIFFERENTIAL DIAGNOSIS

In most patients, establishing the cause leading to erythrocytosis is not difficult. Initially, it is critical to be certain that one is dealing with a patient with absolute erythrocytosis. Testing for *JAK2V617F* and *JAK2* exon 12 mutations can be extremely useful in diagnosing PV and is now standard practice. The presence of splenomegaly is an important finding on clinical examination, and adjunctive laboratory findings include a normal arterial oxygen saturation, elevated LDH levels as well as biochemical evidence of iron deficiency in an erythrocytotic individual. Splenic sizing by ultrasonography can be useful in documenting splenic enlargement when the spleen is not palpable by physical examination.

It is initially important to differentiate PV from the large number of other causes of secondary erythrocytosis. Characteristically, the patient with PV will present with erythrocytosis, leukocytosis, thrombocytosis, and splenomegaly, and is positive for *JAK2V617F*. The BM biopsy shows hypercellularity with trilineage hyperplasia. In individuals who lack a mutated *JAK2*, it is important to determine the SaO_2 using an arterial blood gas, the carboxyhemoglobin level, and the $P_{50}O_2$ in patients with other family members with erythrocytosis to exclude obvious causes of secondary erythrocytosis. Because smokers' polycythemia is the most frequent cause of erythrocytosis, it is wise to measure carboxyhemoglobin levels early on in the investigation. In addition, a PaO_2 greater than 67 mm Hg or an O_2 saturation greater than 95%, as quantitated on an arterial blood gas, is helpful in ruling out hypoxic conditions that lead to erythrocytosis. In patients with intermittent hypoxia, such as sleep apnea syndrome or alveolar hypoventilation caused by obesity, such blood gas determinations can be normal. A low $P_{50}O_2$ is indicative of a hemoglobin mutant with high O_2 affinity, leading to tissue hypoxia and erythrocytosis. Patients with PV characteristically exhibit serum EPO levels below the 95% CIs for the range observed in normal control participants. It is not unusual to have a normal EPO level in some patients with hypoxic causes of secondary erythrocytosis, unless the hypoxia exists over an extended period of time. A normal EPO level cannot be used to exclude a hypoxic cause of erythrocytosis. EPO measurements do, however, remain an important diagnostic tool. Elevation of EPO levels in the face of erythrocytosis is indicative of a hypoxic cause of secondary erythrocytosis, but extremely low levels of EPO (<4 mIU/mL) are virtually diagnostic of PV.

Incorporating a mutational screen into the algorithm for investigating a case of suspected polycythemia helps to streamline the diagnosis of PV, although the presence of a *JAK2* mutation alone does not distinguish PV from PMF or ET. Clinical criteria for the diagnosis of PV have been defined by the WHO, which serves as a uniform platform with which to diagnose PV. These criteria are the consensus of many experts working in this field. It is important, however, for the clinician to realize that some patients undoubtedly have an MPN disorder resembling PV but do not fulfill all the diagnostic criteria of the PVSG or the WHO (Table 70.3). There will always be unusual cases with clinical characteristics that cannot be pigeonholed into a particular diagnostic category. If these patients have serious symptoms, the individual physician must make treatment decisions on the basis of the risk–benefit ratio for that patient.

A particularly difficult dilemma occurs when evaluating patients with isolated pure erythrocytosis. These patients have elevated RBC masses, normal WBC counts, normal platelet counts, no evidence of splenomegaly, and no evidence of any recognizable cause of secondary erythrocytosis. Clearly, some of these patients have *JAK2V617F*-positive PV or mutations of exon 12 *JAK2*, leading to isolated erythrocytosis. The PFCPs are caused by truncation of the EPOR, characterized by increased sensitivity of erythroid progenitor cells to EPO, and can be easily distinguished from PV. These patients frequently present in childhood, and this disorder is characterized by isolated erythrocytosis that is associated with an increased risk of thrombotic events. There is a strong family history of polycythemia. The BM progenitor cells are hypersensitive to EPO, yet no colonies form in the absence of EPO. The patient with profound erythrocytosis who is negative for *JAK2V617F* as well as exon 12 *JAK2* mutations and does not have erythrocytosis caused by smoking, cyanotic heart disease, an EPO-secreting tumor, sleep apnea, or resides at a high altitude remains a diagnostic dilemma. All patients should have a good family and drug history, an arterial oxygen saturation study and a carboxyhemoglobin measurement, and measurement or calculation of p50, and quantitation of 2,3BPG and serum EPO levels. If these studies are unrewarding, appropriate patients should then be investigated for mutations in the hypoxia-responsive element of the human *EPO* gene, HIF-2α and HIF-1α, VHL, PHD1,2,3, STAT5, and LNK. Some of these tests can be performed by commercial laboratories or by specialized academic reference laboratories (www.mayomedicallaboratories.com/articles/features/erythrocytosis/testing).

PV must also be differentiated from the other MPNs, such as CML, ET, and PMF. Such classification has major prognostic implications and influences important therapeutic decisions, including the use of tyrosine kinase inhibitors. With the distinctive cytogenetic abnormalities and molecular genetic abnormalities that are unique to CML (Philadelphia chromosome, *BCR-ABL* gene fusion), these two disorders should not be difficult to differentiate. Occasional patients with an MPN have been shown to have both *JAK2V617F* as well as *BCR-ABL*; whether this unusual occurrence that takes place in two different clones and thereby represents two independent MPNs or a single clone and is caused by the predisposition of such patients to acquire mutations in their hematopoietic cells is a subject of speculation.

Patients with PMF can present with abnormalities that are virtually indistinguishable from those of patients with PV-related MF. Almost 50% of PMF are *JAK2V617F* positive, but more than 90% of patients with post-PV MF are *JAK2V617F* positive. The survival of patients with the latter disorder is much shorter than that of patients with the former condition. Unusual patients with PMF may actually develop PV after iron supplementation or the institution of chemotherapy. ET with a borderline elevated hematocrit and PV with marked thrombocytosis can easily be confused. When the RBC mass is used as a definitive diagnostic test, a distinction between ET and the erythrocytotic phase of PV is usually readily apparent. This measurement is not widely available and can also be normal or actually low in patients with PV who are iron-deficient because of bleeding or excessive phlebotomy. *JAK2V617F*-positive ET patients were characterized by multiple features that resembled those of patients with PV, including higher hemoglobin levels, higher WBC counts, more prominent BM erythroid and granulocytic hyperplasia, a higher incidence of venous but not arterial thrombosis, low serum EPO levels, and lower serum ferritin levels. Surprisingly, *JAK2V617F*-negative patients who likely had calreticulin mutations had higher platelet counts. Furthermore, *JAK2V617F*-positive ET patients have a higher probability of developing PV with longer follow-up. Acquisition of homozygosity for the *JAK2V617* caused by homologous recombination is likely the critical event in the development of PV in *JAK2V617F* ET patients. Homozygosity for *JAK2V617F* occurs in at least 30% of patients with PV but is extremely rare in ET.

TABLE 70.3 World Health Organization Criteria for Polycythemia Vera

2016 WHO Diagnostic Criteria for PV
Major criteria:
1. Hemoglobin >16.5 g/dL in men, 16 g/dL in women, or hematocrit > 49% (men), >48% (women), or increased (RCM)[a]
2. BM biopsy showing hypercellularity for age with trilineage growth (panmyelosis) including prominent erythroid, granulocytic, and megakaryocytic proliferation with pleomorphic, mature megakaryocytes
3. Presence of *JAK2V617F* or *JAK2* exon 12 mutation
Minor criteria:
1. Serum EPO level less than the reference range for normal
Diagnosis requires meeting either all three major criteria or the first two major criteria and the minor criterion

[a] More than 25% above mean normal predicted value.

BM, Bone marrow; *EPO*, erythropoietin; *PV*, polycythemia vera; *RCM*, red cell mass; *WHO*, World Health Organization.

Data from Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127(20):2391–2405. *Blood*, 2016. 128(3):462–463.

PROGNOSIS

The prognosis of a patient with PV depends on the nature and severity of the complications that occur during the clinical course of that particular patient's disorder. In addition, an individual patient's prognosis depends on the duration of the erythrocytotic phase or the time to transition to post-PV MF or MPN-BP. Survival is also influenced by whether appropriate treatment is instituted during the erythrocytotic phase of the illness. Patients who have uncontrolled erythrocytosis are at an extremely high risk for the development of thromboses. Determination of the optimal management of patients with PV has been a difficult task because the disease, when treated, is associated with a survival period of approximately 17 years. Studies of new potential therapeutic interventions therefore require prospective studies with prolonged follow-up times before meaningful results can be generated determining their ability to improve upon overall survival.

Prognostic models in PV typically assess the risk of thrombotic events and the risk of leukemic transformation. Increasing age and history of vascular events have consistently proven to be independent predictors of developing additional thromboses in patients with PV. In the ECLAP trial, the incidence of cardiovascular complications was higher in patients older than 69 years of age, patients with a history of thrombosis, and patients with WBC counts higher than $15{,}000 \times 10^9\,L^{-1}$. Conventional cardiovascular risk factors, including hypertension, hyperlipidemia, diabetes, and smoking, are assumed to be associated with the same relative risk of developing thrombosis in PV patients as that observed in the general population. Although practicing hematologists are frequently concerned about elevated platelet counts in PV patients, several reports have failed to show any association between platelet count and thrombotic events, suggesting that therapies in PV targeting reduction of platelet numbers are ill conceived.

These data have led to stratification of therapy in patients with PV based on clearly identifiable risk factors (Table 70.4) of developing additional thrombotic events. The effect of *JAK2V617F* allele frequency on the prognosis of PV patients has been examined. More than 95% of patients with PV are *JAK2V617F* positive, and their mutational load can be determined by allele-specific or digital PCR. Approximately 30% of PV patients have a high *JAK2V617F* burden (>50%), and the remainder have a lower burden (<50%), with the small numbers being WT. Some investigators, but not all, have reported that high burdens of *JAK2V617F* in PV patients are associated with larger splenic volumes, more frequent aquagenic pruritus, a higher incidence of thrombotic events, and a higher rate of evolution into MF and MPN-BP.

High leukocyte counts ($>15{,}000 \times 10^9/L$) at the time of diagnosis have been identified in some studies as another independent predictor of developing a major thrombosis in PV patients. In addition, others have suggested that this parameter is predictive of evolution to MF and MPN-BP and an overall inferior survival time. In a retrospective study of 520 PV patients from 10 academic institutions across the United States, group-based trajectory modeling was used to identify

TABLE 70.4 Risk Stratification in Polycythemia Vera Based on Thrombotic Risk

Risk Category	Age >60 Years or History of Thrombosis	Cardiovascular Risk Factors[a]
Low	No	No
Intermediate	No	Yes
High	Yes	

[a] Hypertension, hypercholesterolemia, diabetes, and smoking (see text). Extreme thrombocytosis (platelet count $>1500 \times 10^9\,L^{-1}$) is a risk factor for bleeding. Its role as a risk factor for thrombosis is uncertain. An increasing leukocyte count has been identified as a novel risk factor for thrombosis, but confirmation is required.

Data from Finazzi G, Barbui T. How I treat patients with polycythemia vera. *Blood*. 2007;109:5104.

latent clusters of patients who follow distinct trajectories with regard to their hematologic laboratory data over time. The authors found that persistently elevated leukocyte trajectories were not associated with the hazard of thrombotic event, but were significantly associated with increased hazard of disease evolution. Interestingly, neither hematocrit nor platelet count was significantly associated with the hazard of thrombosis or disease evaluation.[32]

In a study of more than 1500 patients with PV, risk factors for survival included advanced age, leukocytosis, venous thrombosis, and abnormal karyotype. Median survival (14.1 years) was significantly worse than that of the age and sex-matched US population. A prognostic model based on the age, presence of leukocytosis, and venous thrombosis delineated risk groups with median survival of 10.9 to 27.8 years. In another study of 327 patients with PV from France and Sweden, multivariable analysis identified age greater than 70 years, leukocyte count greater than 13×10^9/L, and thrombosis at diagnosis as risk factors for survival. Patients with two or more of these risk factors had a 10-year relative survival of 26%, as opposed to patients with zero or one risk factors, who had a 10-year survival of 84% and 59%, respectively.

Recently, it has become more apparent that young patients with MPN comprise a unique disease population with different survival characteristics and potentially a more indolent disease course. A study at Mayo clinic identified 361 patients with MPN age 40 seen between 1967 and 2017, constituting 12% of all MPN patients seen during that time period. Compared to their older counterparts, younger patients with PV had lower incidence of arterial events and higher incidence of venous thrombotic events and a normal karyotype. Furthermore, PMF patients in this cohort had a lower incidence of high molecular risk myeloid malignancy gene mutations. With a median follow-up of 11.3 years, leukemic transformation was documented in 4% of PV patients. Median survival in young patients was 37 years compared to 22 years for patients 41 to 60 years and 10 years for patients over 60 years.[43] In order to take a look at the youngest MPN population, a recent systemic review evaluated patients with MPN diagnosed before 20 years of age.[44] They identified 75 cases of PV. The median age at diagnosis was 12 years for PV patients. Half of the patients were asymptomatic at diagnosis. Interestingly, 73% of these patients were triple negative for driver mutations suggesting that these individuals might actually have an inherited form of erythrocytosis. The incidence of thrombosis was 9.3% during the follow-up period. Venous events predominated. The risk of disease evolution was low with 2% developing MF and no reports of MPN-BP, however the median follow-up was only 50 months. While this data is not mature enough to make any realistic conclusions, it does point out that the prognosis for younger patients should not be based on risk factors used for an older population.

The choice of therapeutic agents used to treat the erythrocytotic phase of PV clearly influences patient outcomes. The use of alkylating agents is associated with an established risk for leukemic transformation or evolution to MDS. Clearly, this risk is also associated with increased duration of the disease. The effect of HU on leukemic transformation rates is limited, if any. In addition, the influence of long-term IFN therapy on disease outcomes is not at present clearly defined.

As the mutational landscape in myeloid malignancies takes a prominent role and various prognostic scoring systems based on mutational analysis become more standardized for patients with MF, attempts are being made to use mutational analysis to help prognosticate PV and ET patients as well. A study of 906 molecularly-annotated patients at Mayo Clinic and University of Florence, including 502 ET and 404 PV patients, tried to integrate genetic information together with clinical information for predicting survival. Multivariable analysis identified spliceosome mutations, and specifically in PV, SRSF2 to adversely affect MF free and overall survival. They were able to confirm the independent survival effect of adverse mutations, age greater than 67 years, leukocytosis $\geq 15 \times 10^9$/L and thrombosis history in PV patients and thus develop and validate mutation-enhanced international prognostic systems (MIPSS) which they reported was superior to the conventional scoring systems.[45]

THERAPY

The cumulative duration of survival for PV patients treated with modern strategies is between 15 and 17 years. Despite the implementation of standard therapeutic strategies, the mortality rate of PV patients is increased by 1.84 compared with age- and sex-matched populations. The primary goals of treatment are to reduce the risk for thrombosis and ameliorate the PV symptom burden. At present, therapeutic strategies which prevent evolution to MF and/or MPN-BP are not available. The European Leukemia Net (ELN) expert panel defined a complete response as a hematocrit less than 45% without phlebotomy, platelet count less than 400×10^9/L, leukocyte count less than 10×10^9/L, normal spleen size, and no disease-related symptoms.[46] However, in a study of 261 patients with PV who were given HU and followed up for a median of 4.4 years, no association was observed between achieving an ELN or a hematocrit response and better survival or fewer vascular complications. No particular platelet or leukocyte count could be shown to be protective against thrombosis. The ELN response criteria were updated in 2013, but have not been validated to predict survival or progression to MF or AM. Therapy is also stratified based on the presumed risk of an individual patient to develop additional thrombotic episodes, with low-risk patients being treated with phlebotomy therapy alone plus a therapy directed toward paralysis of platelet function, and high-risk patients receiving not only phlebotomy plus antiplatelet therapy but also some form of myelosuppressive therapy (HU, IFN, busulfan, melphalan, ruxolitinib). At present, the therapeutic goals are to reduce the risk of thrombosis by normalizing the hematocrit levels to 45% (some experts advocate reducing the hematocrit to 42% in females) based on a randomized prospective study that demonstrated a decreased risk of cardiovascular morbidity and mortality with a goal hematocrit of less than 45% compared to 45% to 50%.[30] Previous studies have indicated a lack of a relationship between thrombocytosis at diagnosis or during the period of follow-up with thrombotic complications. Whether therapy should be directed at normalization of platelets remains uncertain at present and should not be pursued unless within the context of a clinical trial. Extreme thrombocytosis ($>1 \times 10^9$/L), if associated with spontaneous hemorrhage, is an indication for normalization of platelet numbers. Phlebotomy therapy alone is the standard of care for low-risk PV patients (see box on General Principles of Therapy).

A series of studies, although completed more than 30 years ago, by the PVSG answered several very important questions regarding the efficacy and associated complications of particular therapeutic modalities. These investigations have aided in the identification of optimal therapy for individual patients, which must be selected on the basis of age and comorbid disease status to minimize treatment-related complications.

The first PVSG randomized trial (01 trial) evaluated three treatment strategies: (1) phlebotomy alone to maintain the hematocrit level at less than 45%; (2) intravenous ^{32}P at 2.3 mCi/m^2 repeated every 12 weeks, if needed (maximum, 5 mCi per dose), supplemented by phlebotomy to maintain the hematocrit level at less than 45%; and (3) myelosuppression with chlorambucil 10 mg/day orally for 6 weeks and then daily on alternate months with necessary dose reductions and supplemental phlebotomy. More than 400 patients were randomly assigned to this protocol. The median survival duration from entry into the study until death was 9.1 years for patients treated with chlorambucil, 10.9 years for those treated with ^{32}P, and 12.6 years for the phlebotomy group. Long-term survival was inferior for patients treated with chlorambucil compared with those treated with ^{32}P or phlebotomy. An early finding was the appearance during the first 5 years of a significant excess of deaths from MPN-BPin the chlorambucil arm, which reached 17% after 15 years of follow-up. As a result, the chlorambucil arm was discontinued, and patients were assigned randomly to one of the other two arms. Even though no statistical difference in overall survival between ^{32}P and phlebotomy alone was apparent through the first 10 years, the morbidity and mortality associated with each type of therapy were attributable to distinctly different causes. Thrombosis as a cause of death was much more frequent in the phlebotomy-only group during the first 5 to 7 years

GENERAL PRINCIPLES OF THERAPY

1. The etiology of erythrocytosis must be correctly categorized to be certain the patient has polycythemia vera (PV). This will avoid inappropriate exposure of patients with nonmalignant disorders to the adverse effects of myelosuppressive agents. To this end, a major contribution is played by incorporating molecular marker testing (*JAK2V617F* and exon 12 *JAK2* mutational determinations) in the diagnostic workup, and in this way, early phases of PV can be recognized as well. In individuals with erythrocytosis without a *JAK2* mutation, search for causes or secondary causes of erythrocytosis as well as inherited disorders that lead to erythrocytosis, which includes mutations in *EPOR*, *VHL*, *PHD2*, *HIF-1*, and *HIF-2*.
2. Therapy should be individualized.
3. Initially, the hematocrit should be reduced to 45% as soon as possible. The speed of phlebotomy will depend on patients' general medical conditions (250–500 mL every other day). Elderly patients with compromised cardiovascular or pulmonary systems should be more carefully phlebotomized (twice a week), or smaller volumes of blood should be removed.
4. Hematocrit levels should be consistently maintained at 45%.
5. The use of chemotherapeutic agents should be avoided in low-risk PV patients, and treatment with hydroxyurea, interferon (IFN), or ruxolitinib should be reserved for high-risk patients or patients with severe uncontrolled systemic symptoms. Be on the lookout for toxicities from each of these agents.
6. Hyperuricemia is treated with allopurinol (100–300 mg/day).
7. Pruritus may be improved with PV-directed therapy (phlebotomy, hydroxyurea, or IFN). Empiric therapy with antihistamines can be useful in selected patients. Selective serotonin uptake inhibitors (paroxetine 20 mg/day or fluoxetine 10 mg/day) or phototherapy can also be of use. Dramatic relief, however, can be achieved in almost all cases with the institution of ruxolitinib.
8. Elective surgery or dental procedures should be delayed until hematocrit levels have been normalized for more than 2 months. Aspirin should be held before surgery. If emergency surgery is contemplated, phlebotomy and cytapheresis should be pursued.
9. Women and men who are contemplating having children should be treated by phlebotomy plus low-dose aspirin therapy (81 mg/day) or with IFN-α to avoid teratogenic effects of chemotherapy. Such avoidance will also prevent deleterious effects on fertility. During pregnancy, therapy is frequently not necessary; if it is, phlebotomy plus low-dose aspirin should be exclusively used. If phlebotomy control is inadequate, treatment with IFN-α should be pursued.

of follow-up. Analyses of factors associated with thrombosis revealed that the performance of phlebotomy, the rate of phlebotomy, advancing age, and history of previous thrombosis were statistically significant factors predictive of this outcome. Historical studies have raised concerns about therapy with phlebotomy alone. The need for more than four therapeutic phlebotomies a year in the phlebotomy-alone arm was associated with an increased thrombotic risk. This led to the belief that frequent phlebotomy requirements were detrimental and necessitated cytoreductive therapy. However, overall survival and risk of evolution to MF and MPN-BP were lower in the phlebotomy alone arm. Iron deficiency is often present at diagnosis in PV patients and is also a consequence of repeated therapeutic phlebotomy; but in fact, it should be viewed as a therapeutic goal to further limit erythropoiesis. Iron deficiency however, can be associated with a number of clinical signs and symptoms, including fatigue, glossitis, restless leg syndrome, pruritis, dysphagia, cheilosis, koilonychia, fatigue, global weakness, cognitive deficits, neuromuscular disturbances, and pica syndrome. The neurocognitive effects of iron deficiency have been termed "foggy brain syndrome" and can limit PV patients in their successful performance of complex activities. Fatigue as a general symptom of non-anemic iron deficiency is likely related to the ubiquitous requirement for iron in all cells, particularly for the formation of heme as part of myoglobin, cytochromes, and nitric oxide synthase as well as in multiple enzymes required for the generation of adenosine triphosphate. Myoglobin is the oxygen storage protein in not only erythroid cells but also skeletal and cardiac muscle. In fact, iron repletion in non-anemic iron-deficient patients with congestive heart failure resulted in improved function, exercise tolerance, quality of life, and a significant reduction in recurrent hospitalizations, cardiovascular mortality, and all-cause mortality. Furthermore, iron is critical for energy metabolism due to its contribution to heme-containing enzymes such as cytochrome *c* (in the electron transport chain) and cytochrome *c* oxidase (in the oxidative phosphorylation pathway) as well as (2) non-heme-containing enzymes including NADH dehydrogenase and succinate dehydrogenase (in the respiratory chain) and aconitase (in the Krebs cycle). Lastly, iron is a necessary component of key proteins involved in DNA replication, repair, and cell cycle progression. Hepcidin, the master regulator of iron metabolism, is regulated by circulating iron levels, erythroblast secretion of erythroferrone, and inflammation. Insufficiently suppressed hepcidin given the degree of iron deficiency in PV patients strongly suggests that disordered iron metabolism is an important component of the pathobiology of PV. These findings have led to therapeutic trials aiming to restore iron stores but consistently normalize hematocrit levels with the administration of a hepcidin mimetic. The outcome of this phase II trial is eagerly awaited.[47]

In contrast, the use of ^{32}P led to a lower rate of thrombosis during the first 5 years of the PVSG study, but the incidences of leukemias, lymphomas, and nonhematologic malignancies increased during the next 5 years to nearly 10%. After a 15-year period of observation, the incidences of leukemia and lymphoma in the chlorambucil group had risen to 17%. A statistically significant increase in skin and gastrointestinal cancers occurred in the ^{32}P- and chlorambucil-treated cohorts compared with the group treated with phlebotomy alone (see box on Algorithm for Management of Patients With Polycythemia Vera). Based on these studies, therapy with chlorambucil and ^{32}P are no longer recommended.

Impetus for the use of nonchemotherapeutic agents for the treatment of PV was provided by an extensive natural history study of 1213 patients reported by the Gruppo Italiano Studio Policitemia. They showed that the age- and gender-standardized mortality rate of patients with PV was 1.7-times greater than the mortality rate of control participants in the general Italian population. In addition, four times as many patients who had previously received ^{32}P, alkylating agents, or HU died of cancer compared with patients treated with phlebotomy alone. When this group combined the total number of deaths and the number of nonfatal myocardial infarctions and strokes, they found an unsatisfactory risk-to-benefit profile in patients treated with chemotherapeutic agents and suggested that antithrombotic strategies, such as low-dose aspirin, be carefully evaluated for use in the care of patients with PV.

PV platelets are known to have a generalized abnormality of arachidonate metabolism that is characterized by enhanced synthesis of thromboxane A_2, which likely reflects stimuli to platelet activation. The exact mechanism responsible for enhanced platelet synthesis of thromboxane A_2 in PV remains unknown and requires further investigation. A low-dose aspirin regimen (50 mg/day for 7 to 14 days) has been shown to suppress more than 80% of the excretion of the metabolites of thromboxane A_2. A pilot study was performed in which the toxicity of low-dose aspirin therapy in PV patients was evaluated. A very low-dose aspirin regimen (40 mg/day) was chosen to prevent thrombosis yet minimize the risk of bleeding. After follow-up of the low-dose aspirin treatment group and control group, low-dose therapy was shown not to be associated with an increased incidence of bleeding complications. Aspirin therapy was well tolerated and was associated with complete inhibition of platelet cyclooxygenase activity. A large, randomized, placebo-controlled clinical trial testing the risk-to-benefit ratio of low-dose aspirin therapy in preventing thrombotic episodes in PV has been completed.[29] Treatment with low-dose aspirin (100 mg/day) compared with placebo reduced the risk of the combined end points of nonfatal myocardial infarction, nonfatal stroke, pulmonary embolism, major venous thrombosis, or death from cardiovascular courses. Overall mortality and cardiovascular mortality, however, was not reduced significantly by aspirin therapy. Importantly, the incidence of major bleeding episodes was not significantly increased in the aspirin group. It is important to emphasize that patients require aggressive phlebotomy therapy to

ALGORITHM FOR MANAGEMENT OF PATIENTS WITH POLYCYTHEMIA VERA

Low-risk young patients (age <60 years) and no history of thrombosis, platelet count $<1.5 \times 10^6\,\text{mm}^{-3}$

Phlebotomy + low-dose aspirin (81 mg/day) to maintain hematocrit lower than 45%. Aspirin should be used with caution in patients with histories of a hemorrhagic episode or with extreme thrombocytosis ($>1.5 \times 10^6\,\text{mm}^{-3}$) or acquired von Willebrand syndrome.

↓

Thrombosis or hemorrhage
Systemic symptoms
Severe pruritus refractory to histamine antagonists
Painful splenomegaly

↓

Hydroxyurea 15–20 mg/kg or

↓

Pegylated IFN 45–180 μg/week (alter dose depending on response and toxicity) or ruxolitinib 10 mg twice daily and titrate dose depending on the response. →

In a patient with a prior thrombosis or a history of bleeding due to acquired von Willebrand's syndrome, normalization of platelet numbers is necessary. If platelet control is inadequate or the patient cannot tolerate interferon, one option is the use of anagrelide. In this case, supplemental phlebotomy is required to maintain hematocrit lower than 45%, and the use of hydroxyurea should be considered, especially if the patient continues to have thrombotic episodes.

↓

If the patient has increasing splenomegaly, systemic symptoms, or repeated thromboses despite adequate dose of hydroxyurea (2 g/day) or if unable to tolerate hydroxyurea, start ruxolitinib 10 mg twice daily and titrate up or down based on hematologic parameters. Ruxolitinib therapy should be especially considered in patients with severe aquagenic pruritis which is unresponsive to other myelosuppressive therapies. If patient is infected with COVID-19 virus do not discontinue ruxolitinib.

For patients >80 years of age who cannot tolerate the other therapeutic approaches outlined above or have access issues to medical care, low doses of busulphan should be administered until the blood counts are normalized. Therapy should then be discontinued since patients frequently enjoy drug-free prolonged remissions lasting months. Therapy should only be reinstituted at the time the blood counts begin to be elevated again. Such therapy with alkylating agents should not be used in younger patients. It should be mentioned that the sequential use of hydroxyurea and alkylating agents is associated with an increased risk of leukemia and should be avoided. As a general approach we suggest periodic supplemental phlebotomy to achieve hematocrit control rather than administering increasing doses of myelosuppressive agents and risking drug-related adverse events.

Painful splenomegaly

↓

Patients should be treated with ruxolitinib.
If unable to tolerate or insufficient response
Splenectomy + continued systemic therapy.

↓

High-risk patients (age >60 years), previous thrombosis, platelet count $>1.5 \times 10^6\,\text{mm}^{-3}$

Phlebotomy to hematocrit of 45%

Aspirin (81 mg/day) to be given only in patients with platelet counts $<1.5 \times 10^6\,\text{mm}^{-2}$

Myelosuppressive therapy with hydroxyurea 30 mg/kg orally for 1 week then 15–20 mg/kg

or 45–180 μg/week of pegylated IFN.

↓

If patient continues to have thrombotic episodes and has extreme thrombocytosis or cannot tolerate hydroxyurea or pegylated IFN, initiate therapy with ruxolitinib 10 mg twice daily and titrate the dose to achieve hematocrit control. If the patient is refractory to other agents and is >80 years of age add intermittent busulphan.

If on busulphan, stop when blood counts are normalized or platelet count is lower than $300{,}000\,\text{mm}^{-3}$.

Occasional supplemental phlebotomy if hematocrit is >45%; when these elderly patients relapse (patient is symptomatic) initiate busulphan therapy again at same dose.

Patient age >70 years
Phlebotomy + low-dose aspirin + hydroxyurea

↓

No response or poor compliance
Ruxolitinib or busulfan.

the appropriate target hematocrit levels as well as the appropriate use of myelosuppressive agents as primary therapy, with the addition of aspirin serving as an adjunct to this strategy. The conclusions of this study have been questioned by several investigators. In addition, a retrospective analysis performed by independent investigators of the 1630 patients treated by the ECLAP group has concluded that low-dose aspirin therapy in PV patients was associated with a statistically nonsignificant reduction in the risk of fatal thrombotic episodes without an increased risk of bleeding. This meta-analysis is surely not a ringing endorsement of the widespread use of aspirin therapy, which is a strategy that has been indiscriminately used worldwide to treat such patients. Because it is unlikely that the value of aspirin therapy will be examined in a properly powered randomized trial, it is suggested that since aspirin therapy is a relatively innocuous therapy it should be used in high-risk patients judiciously. However, it should be avoided in patients with extremely high platelet counts who are at risk for hemorrhage.

After the disappointing results experienced with the alkylating agent chlorambucil, the PVSG began a nonrandomized phase II investigation of HU, an S-phase–specific ribonucleotide reductase inhibitor. The hope was that this agent would be nonleukemogenic. Of 53 patients with PV treated with HU who had never received other forms of myelosuppression, after follow-up for a median period of 8.6 years and a maximum follow-up of 795 weeks, 5.4% developed MPN-BP compared with 1.5% of patients treated with phlebotomy alone on the original PVSG randomized study.

In a comparable trial from Israel, 71 patients were treated with HU for a mean duration of 7.3 years. Remarkably, the incidence of thrombosis was only 6%, indicating the potential of HU to lower the incidence of thrombosis in patients with PV, confirming an observation previously made by another group. The incidence of MPN-BP in the Israeli trial was 5.6%. In another retrospective series, the incidence of MPN-BP and myelodysplasia in patients with PV treated with HU alone was 6.9%. In patients treated first with busulphan and then HU, the rate of MPN-BP and myelodysplasia was 13.8%. A recent propensity score analysis of patients enrolled in the ECLAP study examined 1042 patients who during follow-up received only phlebotomy or HU with a goal of hematocrit less than 45%. Over comparable period of follow-up they demonstrated superiority of HU over phlebotomy alone with respect to the incidence of fatal/not-fatal cardiovascular events. In addition, this same population of patients was used to demonstrate a significant advantage of HU over phlebotomy alone with respect to the proportion of non-fatal arterial thrombosis. This advantage was not present at reducing unprovoked venous thromboembolic events. Twenty-two patients developed MPN-BP and MDS in the ECLAP study that enrolled 1638 patients. AML and MDS were diagnosed after a median of 8.4 years from the diagnosis of PV; the variable associated with progression was older age, but overall disease duration (>10 years) failed to reach statistical significance. Exposure to ^{32}P, busulphan, and pipobroman but not HU alone had an independent role in producing an excess risk for progression to MPN-BP and MDS compared with treatment with phlebotomy or IFN.

The leukemia-promoting potential of HU has been readdressed in two additional studies. Kiladjian and coworkers reported the results of a randomized trial of HU versus pipobroman that was initiated in 1980; the overall survival was 20.3 years in the HU-treated group compared with 15.4 years for the patients treated with pipobroman. The incidence of MPN-BP and MDS in the pipobroman group was dramatically higher, which is not unexpected because pipobroman is an alkylating agent. This trial was designed with a cross-over option, but 93 patients only received pipobroman. The incidence of MPN-BP and MDS in the patient group that only received HU was 7.3%, 10.7%, and 16.6% at 10, 15, and 20 years, respectively. Since a control arm treated with phlebotomy alone was not included, one cannot determine if this rate of evolution to MPN-BP/MDS represents the natural history of PV or a possible limited leukemogenic effect of HU. Interestingly, no difference in the incidence of vascular events was seen between the two arms. This same question was again addressed in a nested case–control study performed in Sweden. The most important observation was that 25% of patients with various

MPNs (68% had PV) who evolved to MPN-BP and MDS had not been exposed to any chemotherapeutic agent, indicating that the risk of leukemic evolution was higher than had been previously appreciated. The use of radioactive phosphorous and alkylating agents, but not HU, was associated with a higher rate of MPN-BP and MDS. These reports indicate that nontreatment-related factors play a major role in the development of AML and MDS, and the contribution of HU based on the best available evidence is at best minimal. These data and the ease of administration make HU a very useful chemotherapeutic agent in older, high-risk patients with disease that cannot be controlled with phlebotomy alone. Excessive myelosuppression, macrocytosis, hypersegmentation of polymorphonuclear leukocytes, cutaneous actinic keratosis, squamous cell carcinoma of the skin, hyperpigmentation of the skin and nail beds, stomatitis, painful leg ulcers, creatinine elevations, and jaundice have been attributed to the use of HU. Aphthous and leg ulcers occur in 9% of patients, usually after approximately 10 months of therapy (Fig. 70.15). Rarely, cycling of platelet and leukocyte counts but not RBCs occurs while patients are being treated with HU. In this situation, the authors have maintained patients on a fixed dose of HU rather than chasing cycling platelet counts. In addition, the use of HU requires patient compliance and careful monitoring of blood counts to avoid the sequelae of excessive myelosuppression.

Patients resistant to HU are defined by need for additional phlebotomies, increasing splenomegaly, increasing leukocytosis in the face of the highest tolerable dose. HU resistance develops in 20% to 30% of PV patients after a median time of 4.6 months and is associated with an increased risk of developing a thrombosis, disease progression and a limited survival. The patients have an unfavorable mutational profile involving JAK2V617F homozygosity rather than heterozygosity as well as mutations in genes encoding chromatin modifiers, spliceosome genes, or TP53.[48] Anagrelide, a selective inhibitor of platelet production, is occasionally used to treat uncontrolled thrombocytosis not responsive to HU, interferon, or ruxolitinib in PV patients with thrombotic or hemorrhagic complications. This agent is nonleukemogenic and acts by impairing megakaryocyte maturation. Its use leads to a selective reduction in platelet numbers, and it has been effective in patients refractory to HU and IFN. This drug does not effectively control the erythrocytosis and leukocytosis or systemic symptoms associated with PV, and therefore has limited utility and is best used as a supplement to phlebotomy therapy. The time to complete response generally ranges between 17 and 25 days. The dose of anagrelide required to control thrombocytosis remains constant over time in most patients. When anagrelide is discontinued, platelet counts returned to pretreatment levels within 5 to 7 days. The simultaneous administration of anagrelide and low-dose aspirin has been reported to lead to a significant increase in bleeding manifestations. Clinicians presume that the effects of anagrelide observed in ET patients will be relevant to patients with PV. This assumption has not been tested in a randomized clinical trial. Anagrelide therapy should be reserved for patients with recent thromboses (e.g., stroke, migraines, erythromelalgia) and with thrombocytosis that cannot be controlled with IFN, HU, or ruxolitinib therapy alone. It can also be used in combination with HU to minimize adverse events that accompany the use of each drug alone. Anagrelide should not be used in pregnant patients because it can easily cross the placenta, leading to adverse effects on the platelet count of the fetus.

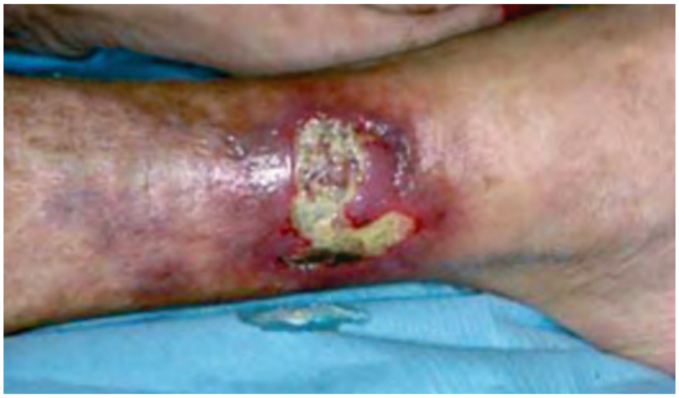


Figure 70.15 EXAMPLE OF A HYDROXYUREA-INDUCED LEG ULCER. *(From Soutou B, Aractingi S. Myeloproliferative disorder therapy: assessment and management of adverse events–a dermatologist's perspective. Hematol Oncol. 2009;27:11.)*

Approximately 15% to 20% of patients treated with anagrelide discontinue the medication because of nonmyelosuppressive side effects. The spectrum of adverse effects involved neurologic (headaches and dizziness), cardiac (vasodilatation, fluid retention, congestive heart failure, palpitations, and tachycardia), and gastrointestinal (nausea) toxicities. These toxicities reflect the novel mechanism of action of anagrelide as a cyclic nucleotide phosphodiesterase inhibitor. Anagrelide should be used with caution in patients with known or suspected cardiac disease because of its ability to promote fluid retention. Because many patients with PV are elderly, careful attention to fluid status should be maintained to avoid slipping into congestive heart failure after the initiation of anagrelide.

IFN-α therapy has been explored for several decades for the treatment of PV patients, but its beneficial effects have been more greatly appreciated during the past 12 to 17 years. Its effects on myeloid malignancies are likely the consequence of its broad range of biologic activities, including direct effects on malignant cells, enhancement of antitumor immune responses, induction of proapoptotic genes, inhibition of angiogenesis, and promotion of the cycling of dormant malignant stem cells. The induction of apoptosis by IFN-α involves the activation of a number of IFN-stimulated genes including TP53 that mediate this response. Gene expression studies have identified more than 15 IFN-stimulated genes with proapoptotic functions. Although these IFN-stimulated genes alone are probably not sufficient to induce apoptosis, their cumulative effects likely result in apoptosis. Type 1 IFNs suppress the ability of normal human HPCs to proliferate in vitro in the presence of cytokine combinations. IFN-α acts directly against HPCs; this inhibitory activity has been documented using CD34$^+$ cell populations. PV CD34$^+$ cells are more sensitive to the inhibitory effects of IFN-α than normal HPCs.

The ability of type 1 IFNs to inhibit HPC proliferation and maturation has been documented to occur independently of the STAT pathway. Type 1 IFNs activate the p38 MAP kinase, a proline-directed serine/threonine kinase that is required for IFN-stimulated gene transcription through IFN-sensitive response elements (IRSE). The pharmacologic blockade of p38 in IFN-treated PV HPCs reverses the inhibitory effects of IFN-α. Activation of p38 MAP kinase results in mitochondrial translocation of the proapoptotic protein Bax, leading to the induction of apoptosis.

IFN has also been shown to be able to affect HSC behavior. High levels of IFN-α induce murine HSCs to exit from a normally quiescent state and to transiently proliferate. Several studies using either primary PV CD34$^+$ cells or murine models of *JAK2V617*-positive PV have provided evidence that IFN-α is able to selectively deplete malignant PV stem cells. IFN has also been shown in murine models to promote the predominance of JAK2V617F positive megakaryocyte-biased stem cells with decreased long-term repopulating potential. Such a skewing of stem cell potential could lead to a reduction in JAK2V617F VAFs.[49]

Whole-blood transcriptional profiling studies demonstrated downregulation of HLA genes in MPNs and it has been postulated that this could be one of the ways that MPN cells evade immune surveillance with deregulation of several immune genes and contributing to the risk of second cancers which has been demonstrated in MPN patients. Treatment with IFN-alpha2 results in upregulation of these downregulated HLA genes.

In the United States, the pioneering independent studies by Richard Silver and Harriet Gilbert first documented the clinical effectiveness of IFN-α in controlling erythrocytosis as well as pruritus and other constitutional symptoms in PV patients, and Austrian and French groups reported during the same period evidence that IFN-α was also effective in reducing thrombocytosis in ET patients. A number of clinical trials have been performed subsequently using several different commercial preparations of IFN. In almost all PV and ET trials, IFN-α therapy rapidly normalized platelet numbers and corrected the degree of leukocytosis and erythrocytosis, allowing reduction in the requirement for phlebotomies within a few months. In both diseases, an objective hematologic response was observed in about 80% of patients, including complete freedom from phlebotomies in PV in 60% of patients. However, toxicity associated with IFN-α therapy was not trivial, leading to the discontinuation of treatment in almost 25% of patients. A pegylated form of IFN has been used with increasing frequency to treat patients with a variety of MPNs with great success. This form of IFN can be administered once weekly, and its use is associated with a more favorable toxicity profile. Several phase II studies using peg-IFNα-2a in PV and ET showed similarly impressive hematologic response rates compared with standard IFN-α, but with less associated toxicity (<10% of patients discontinued therapy during the first year of therapy). In addition, these studies showed for the first time evidence of significant molecular responses, as documented by a clear reduction in the *JAK2V617F* allele burden after IFN-α treatment. Overall, the two clinical trials with peg-IFNα-2a showed a meaningful and progressive reduction in the *JAK2V617F* allele burden in about 70% of PV and 40% of ET patients. Importantly, the *JAK2V617F* mutation became undetectable (with 1% sensitivity PCR assays) in 24% of PV patients in the French PVN-1 study after about 3 years' median follow-up, and in 14% and 6% of PV and ET patients, respectively, in the United States study after about 2 years' median follow-up. Combining both studies, 12 out of 64 (19%) PV patients achieved a molecular complete response (MCR). Of note, many of these MCRs occurred after the 12th month of treatment, but hematologic responses occurred within a few weeks. Prolonged exposure (>12 months) to peg-IFNα-2a seems to be an important factor in achieving MCR. The reduction in the *JAK2V617F* allele burden was not influenced by the cumulative dose of peg-IFNα-2a in the PVN-1 study, but toxicity was clearly dose dependent in the United States study. Taken together, these results indicate that peg-IFNα-2a therapy is best initiated at very low doses that are gradually increased until hematologic response is achieved; this strategy avoids cessation of therapy and allows sufficient exposure to the drug to allow for the achievement of a molecular response. These results are in agreement with studies from Denmark showing major molecular responses in PV after long-term treatment with IFNα-2b that could persist after IFN-α was discontinued. Finally, several who achieved MCR in the PVN-1 study have maintained this response after peg-IFNα-2a was discontinued (≤30 months after discontinuation), with none experiencing hematologic relapse. Such long-term clinical and molecular responses after the discontinuation of treatment have previously been reported after IFN-α therapy in MPN patients, an effect that has not been reported with other currently available therapies. It is important to emphasize that the elimination of *JAK2V617F* in this setting is not necessarily indicative of cure of the MPN, and that clinical implications of these molecular responses remain uncertain and require validation. It has been shown that molecular relapse can rapidly occur after IFN-α discontinuation in some cases. In one patient with a biclonal *JAK2V617F*/*TET2*-mutated PV, peg-IFNα-2a treatment did not affect the *TET2*-mutated cells, but the *JAK2V617F* clone was eradicated. Nevertheless, prolonged periods of time (≤40 months, personal unpublished data) during which the patient remained in complete hematologic remission without any cytoreductive therapy after peg-IFNα-2a withdrawal was observed in several patients of the PVN-1 study even though low levels of the *JAK2V617F* allele (≈5%) were still detected. Such results suggest that despite the disease not being eradicated, long-term exposure to peg-IFNα-2a was sufficient to modify the clinical expression of the MPN for a sustained period of time.

Recently, the results of randomized trials comparing the peg-IFNα-2a to HU in first-line treatment were published. In the MPD-RC-112 phase III trial high-risk untreated ET/PV patients were randomized to either peg-IFNα-2a or HU. The overall response rate was similar with 78% in peg-IFNα-2a group and 70% in HU group at 12 months and 91% and 88%, respectively at 24 months. Molecular responses were achieved in 11% of peg-IFNα-2a patients and 20% of HU patients. Best BM responses occurred in 17% of peg-IFNα-2a patients and 33% of patients treated with HU. More adverse events were reported in peg-IFNα-2a (41.9% >grade 3) arm compared to HU (24.4% >grade 3) arm. In a phase III DALLIAH trial 205 previously untreated patients were randomized to IFN alfa-2a, IFN alpha-2b, or HU. Interim analysis showed 49% overall response rate in MPN patients compared to 75% response rate seen with HU. It has been demonstrated that optimal response rates achieved with IFN can take a long time, so longer follow-up is needed to truly establish final response rates. In a second-line trial of peg-IFNα-2a in PV and ET patients that were resistant or intolerant to HU (MPD-RC 111), the ORR in the PV cohort at 12 months was 60%, and 22% of patients experienced CHR. The median absolute reduction in JAK2V617F allele burden was -6% (range −84% to 47%). Drug discontinuation related to AEs occurred in 13.9% of subjects.[50]

In addition to clinical, hematologic, and MCRs achieved in a significant proportion of PV patients, IFN-α therapy has also been shown recently to reverse BM histopathologic abnormalities in selected cases of both PV and PMF. Thus, IFN-α seems to be a drug able to deplete PV stem cells, and induce complete resolution of all clinical, biologic, and morphologic abnormalities in selected MPN patients, raising the hope that a curative outcome might be possible in a limited subset of such patients. These observations lead one to question the validity of the current therapeutic strategies for PV and ET patients in which myelosuppressive therapy is exclusively used in subpopulations of patients at high risk for developing additional thrombotic events.

IFN is not a leukemogenic drug but it is also not an innocuous agent, necessitating careful follow-up of patients receiving such treatment. Initially, many patients experience flu-like symptoms, which are controllable with acetaminophen or aspirin. Such symptoms usually resolve spontaneously after several months of therapy. More serious side effects, including excessive suppression of blood counts, irritability, high fevers, severe asthenia, reversible lower extremity bilateral neuritis, retinopathy, hypothyroidism, depression, sarcoidosis, and left-sided heart failure have been reported and may require cessation of IFN-α therapy. Exacerbation of depression and other psychiatric manifestations are serious potential side effects of IFN, and it should probably not be used in patients with history of clinically significant psychiatric conditions. Patients and family members should be counseled to watch for subtle evidence of depression, mood swings, and personality changes and to immediately report their concerns to the treating physician. The use of IFN should also be avoided in patients with any history of autoimmune diseases and new development of autoimmune disorders should be high on the differential diagnosis of patients being treated with any formulation of IFN.

Since a major limitation of peg-IFN is its toxicity profile and the need for weekly injections, a novel monopegylated interferon Ropeginterferon alpha-2b (ropeg) (PharmaEssentia) has already gained regulatory approval in the EU. It is administered every 2 weeks and with time even less frequently and its toxicity profile appears to be more favorable. The PROUD-PV study was a phase III registration study done in 48 clinics in Europe where 257 PV patients who were either treatment naive or had less than 3 years of HU treatment were randomly assigned to ropeg or HU. After 1 year of study, patients could opt to enter the extension part of the study, CONTINUATION-PV. At 12 months, Ropeg was shown to be non-inferior to HU at reaching the primary endpoint of complete hematologic response with normal spleen. In CONTINUATION-PV, complete hematologic response was met in 70.5% of patients in the ROPEG arm versus 51.4% of patients in the HU arm at 36 months (P=0.01). This difference was not apparent early on in the study when at 12 months of treatment CHR rates were 43% in the ROPEG arm and 46% in standard therapy arm. Over the 36-month treatment period, the proportion of patients who maintained their response was significantly higher in the ROPEG arm compared to the HU arm in terms of CHR as well as CHR with improved disease burden. Molecular responses in PROUD-PV were not significantly different between the two groups at 12 months. In both groups, a reduction in mean JAK2V617F allele burden compared to baseline was evident at 12 months. In the CONTINUATION-PV study, patients treated with ROPEG showed a steady decrease in the means absolute JAK2V617F allele burden to less than half the baseline level by month 36. However in the HU group the reduction was transient and was lost by month 36. Interestingly, Ropeg was relatively well tolerated with minimal grade 3 to 4 adverse events reported.[51] Jäger et al. performed a study of germline SNPs to identify those patients with the greatest potential to achieve MR with Ropeg therapy. They demonstrated a statistically significant effect at the interferon lambda 4 (*IFNL4*) locus diplotype (haplotype pair, including the protein-coding variants rs368234815/rs117648444) on MR as reflected in differential *JAK2*V617F mutational burden changes according to *IFNL4* diplotype status. These investigators showed that the absence of IFNL4 increased the chance of developing a MR. They proposed that a personalized approach to the use of Ropeg based upon IFN4 genotype/phenotype in patients with PV based on IFNL4 functionality might allow for the identification of patients most likely to benefit from Ropeg therapy.[52]

The reduction of JAK2 mutation VAFs by Ropeg therapy has led several investigators to propose that such therapy was capable of altering the natural history of PV thereby reducing the risk of additional thrombotic events as well as reducing the risk of progression to MF and MPN-BP. Unfortunately the effects of HU and Ropeg on these outcomes in the CONTINUATION-PV study were similar. Perhaps with longer follow-up such effects on alteration of the natural history of high-risk PV will be observed but at present these claims must be considered speculative. Recently this optimistic outlook for Ropeg therapy led to a randomized phase II trial of 100 low-risk PV patients randomized to receive either Ropeg every 2 weeks or phlebotomy alone. Ropeg was shown to be capable of reducing the phlebotomy requirements by only 15% as compared to 3% in the phlebotomy group.[53] Although this study was stopped by the data safety and monitoring committee prematurely due to the meeting of the primary endpoints, the clinical significance of these results are questionable. To answer the fundamental question of whether Ropeg is indeed capable of influencing the natural history of low-risk PV patients, the follow-up of the treated patients would have to be extended for a decade or two in order to accumulate enough events to determine the strength of this claim.

The use of small-molecule inhibitors of JAK1/2 ruxolitinib was first approved in the United States in 2011 for the treatment of advanced forms of MF, and in 2015 was approved for the treatment of patients with PV who were intolerant or resistant to HU. In a phase III study (RESPONSE trial), HU resistant or intolerant patients were randomized to receive either ruxolitinib or standard care. To be eligible for the study entry patients had to have splenomegaly with a spleen volume of at least 450 cm^3 and ongoing phlebotomy requirements, and be resistant to or intolerant of HU according to the standardized criteria. Standard care was selected by the investigator and included HU. Patients receiving standard therapy were able to cross over to ruxolitinib if they failed to achieve the primary end point by 32 weeks. The primary end point was achievement of hematocrit control and reduction in spleen size (at least 35% by MRI). This was achieved in 21% of patients in the ruxolitinib arm versus 1% of patients in the standard therapy arm. Hematocrit control was achieved in 60% of patients receiving ruxolitinib and 20% of patients receiving standard therapy, and 38% of patients in the ruxolitinib arm met the reduction in spleen end point versus 1% of patients in the standard therapy arm. By week 80, almost 60% of patients treated with ruxolitinib had normalization of their platelet counts. Patients on ruxolitinib enjoyed improvement in such PV-related symptoms as pruritus, headaches, fatigue, and night sweats. The mean change in *JAK2V617F* allele burden from baseline to week 32 was 12.2% in the ruxolitinib group and 1.2% in the standard therapy group. Based on an analysis after 80 weeks of follow-up, there was a suggestion that those patients receiving ruxolitinib had a reduced incidence of serious thrombotic events but that ruxolitinib therapy did not affect progression to MF and MPN-BP; these conclusions are not well substantiated since the study was not powered to examine these events. It also did not provide information on correction of the BM histopathological findings and chromosomal abnormalities in patients treated with ruxolitinib. In general, ruxolitinib was well tolerated with 85% of patients remaining on ruxolitinib at 81 weeks. The most common nonhematological adverse event was diarrhea, while the hematological toxicity included anemia, lymphopenia, and thrombocytopenia. In addition, the rates of nonmelanoma skin cancer were higher in the ruxolitinib arm compared with those patients receiving standard therapy. An increase in infectious complications was observed on ruxolitinib treatment, specifically herpes zoster infection. This has also been observed in MF patients treated with ruxolitinib. Cases of tuberculosis activation, toxoplasmosis, and other atypical infections have been reported in ruxolitinib-treated patients. The increased number of infections has been attributed to the inhibition of both T- and NK-cell function by ruxolitinib. Recently, a 5-year follow-up analysis of RESPONSE trial as well the RESPONSE 2 where the participating patients lacked splenomegaly on entry became available.[54] The majority of patients remained on the drug and maintained their clinical response at 5 years. Still conclusions could not be made regarding the effect of ruxolitinib therapy on the risk of progression to MF or MPN-BP or the risk of thrombosis. In the era of COVID-19 the use of ruxolitinib therapy presents some challenges. In a retrospective study of 175 MPN patients infected with the COVID-19 virus from clinical centers throughout Europe, ruxolitinib was used more frequently in patients who died than those who survived. Multivariable analysis, however, did not suggest any effect of ruxolitinib on mortality, but rather demonstrated an increased risk of death in patients who discontinued ruxolitinib treatment. These data indicate that PV patients treated with ruxolitinib should remain on this JAK1/2 inhibitor during the period of infection with the virus.

A recent phase II trial looked at the combination of INF-α2 and ruxolitinib in patients with PV and MF. The rationale for the combination was to target the malignant clone early and prevent clonal evolution and progression. In addition, the investigators raised the possibility that by decreasing inflammation, ruxolitinib might improve IFN signaling. This was a multicenter single arm study done in Denmark. Patients were treated with low-dose IFN and ruxolitinib ranging from 5 to 20 mg twice daily. After 2 years of treatment, of 32 patients with PV, 10 (31%) achieved a remission, of which 3 (9%) achieved complete remission. Overall improvements in total symptom score were also reported. The median *JAK2V617F* allele burden decreased from 47% to 12%, and 41% of patients achieved a molecular response. Total dropout rate was 16%. The majority of patients required a dose reduction of at least one drug. This is an interesting approach and further studies are clearly needed. The use of this combination drug therapy is associated with a high degree of myelosuppression and other overlapping toxicities from these drugs.[55]

Pruritus in PV patients occurs on exposure to sudden body cooling, especially after a warm bath, and is experienced by as many as 40% to 60% of patients treated with phlebotomy. The onset of pruritus may occur prior to the diagnosis of PV or at later time points following the diagnosis. In some studies, basophils and mast cells have been shown to be involved with *JAK2V617F* in patients with aquagenic pruritus and PV, likely leading to the elaboration of mediators that lead to itching. The frequency of pruritus appears to be somewhat lower in patients treated with myelosuppressive agents. This observation is related to the probable relationship between pruritus and degranulation of tissue mast cells and circulating basophils. Aged transgenic mice expressing *JAK2V617F* suffer from pruritis and the massive accumulation of mast cells in the skin. Cultured mast cells from these mice exhibited enhanced proliferative signals, relative resistance to cell death upon growth factor deprivation, and a growth advantage over control cells under suboptimal growth conditions. A JAK inhibitor was shown to reduce mast cell numbers and alleviate pruritus in *JAK2V617F* transgenic mice. Some uncontrolled studies have attributed pruritus to hyperhistaminemia or severe iron deficiency, with relief associated with the use of histamine antagonists or ferrous sulfate. Iron replacement is frequently not possible because it can lead to dangerous elevations of the RBC mass. In a number of instances, however, minimal iron replacement can be possible with disease control with cytoreductive therapy. The association between pruritus and tissue infiltration by mast cells would appear to explain the response of occasional patients to photochemotherapy with psoralens and ultraviolet irradiation. In addition, in one small study, 80% of patients with pruritus have been reported to respond to the selective serotonin uptake inhibitors paroxetine or fluoxetine. Other effective options include anticonvulsant drugs such as pregabalin. Ruxolitinib in both PV and PMF has a remarkable effect on reducing the severity of intractable pruritus. Ruxolitinib is much more effective in treating this particular symptom of PV compared with both HU and IFN and has become the treatment of choice for this particular symptom of PV. In occasional patients this effect, unfortunately, is not sustained.

Budd-Chiari syndrome (BCS) is a catastrophic illness that can lead to significant morbidity and mortality in patients with PV. Patients with MPN are at a high risk of developing this syndrome. Independently, the use of oral contraceptive pills and congenital and acquired thrombophilic factors are involved in its development. A number of cases of hepatic vein thrombosis have been reported in nonpolycythemic women taking oral contraceptives.

The optimal approach to the problem of BCS is obviously preventive and involves maintenance of normal blood values in the patient with PV. When BCS develops, the prognosis without treatment is dismal. The goals of therapy are to prevent further propagation of thrombus, relieve the intense hepatic congestion, and manage the severe ascites that often plague these patients. If untreated, these patients often have a slowly progressive course, with deterioration and death occurring within 3.5 years. Spontaneous resolution of the hepatic vein occlusion rarely occurs. There are no prospective randomized trials of anticoagulation in BCS, but retrospective studies suggest a survival benefit. Anticoagulation should be continued indefinitely unless there is a contraindication such as bleeding esophageal varices. Typically, low-molecular–weight heparin (LMWH) is used as the initial treatment with subsequent switch to warfarin. New direct acting oral anticoagulants (DOACs) that act by direct thrombin or factor Xa inhibition are being increasingly used for various prophylactic and therapeutic indications, and their use for these patients is becoming more widespread due to ease of administration and reversal agents are now available for some of these agents. However, evidence for efficacy and safety of using these agents in this setting is extremely limited. The use of DOACs in MPNs patients is discussed in greater detail in Chapter 71. The use of thrombolytic therapy directly in the thrombosed vein is infrequent due to the lack of data and potentially high risk of bleeding. Angioplasty with or without the use of stents attempts to restore hepatic blood flow and is often used if anticoagulation is not successful, and should be considered if liver function is deteriorating despite therapeutic anticoagulation. The overall success rate is about 95%, with a low periprocedural complication rate. The clinical deterioration of patients with BCS results from damage to the hepatocytes from necrosis associated with marked elevation in sinusoidal pressure coupled with ischemia from reduced hepatic arteriole perfusion. The only rational therapeutic intervention, therefore, involves some sort of portal vein decompression to achieve an effective reduction of sinusoidal pressure. Portosystemic shunting such as transjugular intrahepatic portosystemic shunt (TIPS) has been used extensively to treat refractory portal hypertension. Recent data show good long-term outcomes when using TIPS, and it is now considered standard management if anticoagulation or angioplasty are not successful, or if patients have high-risk disease. It has been shown that about a third of patients with BCS require TIPS placement. One-year survival in a recent large UK single-center study was 97% and 5-year survival 72%. Procedure-related complications are frequent but mortality rates are low.

Liver transplantation is a potential option for treatment of PV BCS patients with continued hepatic decompensation. The indications for liver transplantation are cirrhosis, fulminant hepatic failure, and failure of a portosystemic shunt. The overall actuarial survival at 1 year was 76% and was 68% at 10 years. Because PV is a slowly progressive disease, transplantation should not be withheld from these patients. Pretransplant predictors of mortality based on a multivariable analysis were impaired renal function and a history of a shunt. The hematologic consequences of polycythemia must be aggressively treated in the posttransplantation setting; hepatic vein occlusion may reoccur in the transplanted liver.

Therapy of CVT and sinuses includes anticoagulation with heparin to arrest the thrombotic process and to prevent pulmonary embolism. LMWH should be started as soon as the diagnosis is confirmed even in the presence of hemorrhagic infarcts. After the acute phase, life-long oral anticoagulant therapy is recommended. There is currently no available evidence from randomized clinical trials regarding the efficacy or safety of thrombolytic therapy. Many patients also receive long-term myelosuppressive therapy with HU, ruxolitinib, or pegylated forms of IFN. De Stefano however recently reported that HU does not prevent reoccurrences of splanchnic vein thromboses in MPN patients,[56] while Mascarenhas failed to observe a single rethrombosis in the same patient population with pegylated IFN therapy.[57] Based on this limited data we usually maintain such patients of pegylated IFN in order to normalize blood counts in a sustained manner.

The performance of any surgical procedures on patients with PV is, as previously discussed, accompanied by excessively high morbidity and mortality. Elective surgery should not be contemplated unless the patient's hematologic values have been normalized for several months. The longer the hematologic control has been in effect, the lower the incidence of postoperative complications. If emergency surgery is required, the patient should be phlebotomized rapidly until a normal hematocrit is reached, and platelets should be available in case excessive perioperative or postoperative bleeding occurs. After emergency or elective surgery, the patient should be mobilized as soon as possible, and strong consideration should be given to anticoagulation with LMWH unless the patient has some contraindication. Dental extractions can also result in excessive hemorrhage and should not be performed unless the patient is under strict hematologic control.

Perhaps the most difficult and frustrating period encountered during the clinical course of a patient with PV is the development of post-PV MF. The origins, manifestations, and management options are discussed in detail in Chapter 72.

Limited information is available concerning the treatment of patients who develop MPN-BP after PV. The overwhelming majority of such cases involve myeloid leukemias. The leukemia is frequently preceded by post-PV MF but not always. Patients with post-PV MF and MPN-BP frequently have greater than 20% blasts in the peripheral blood but far fewer blast cells in the BM, which has led to a hypothesis that the leukemia originates from an extramedullary site, particularly the spleen. These cases tend to have a more indolent course, but patients with a greater degree of BM infiltration have a more highly proliferative form of leukemia that is associated with an even more aggressive course. The optimal treatment of such patients is unknown. In elderly adults with significant comorbidities, the choice not to institute chemotherapy is a reasonable option. These patients are frequently elderly, and poor results with standard regimens have been reported. Patients rarely achieve a complete remission but rather return to a clinical condition that resembles their original MPN for limited periods of time. In a retrospective analysis on 23 patients with leukemic transformation of PV, the median patient age was 68 years, and MPN-BP developed a median of 12.8 years from the diagnosis of PV. Twelve of the 14 patients in whom cytogenetic analyses were performed had complex cytogenetic abnormalities associated with high-risk leukemias. Fifteen patients were treated with palliative measures and had a median survival of 2.5 months. Of the eight patients treated with standard induction therapy, one obtained a complete remission, and seven died without obtaining a response. The median survival time of these chemotherapy-treated patients was 5.6 months. The median survival time of the entire cohort of 23 patients was 2.9 months. This poor outcome is likely the result of clinical and biologic features of the acute leukemia, advanced patient age, unfavorable cytogenetics, and patient comorbidities associated with advanced age. Selected patients are likely to tolerate and have a favorable outcome with allogeneic stem cell transplantation and reduced-intensity conditioning. In fact, the only long-term survival with post-PV leukemia has been observed in patients receiving allogeneic stem cell transplantation. For eligible patients, remission induction should be attempted using either standard AML induction therapy or combination of DNA hypomethylating agents (HMA) with or without the bcl 2 inhibitor venetoclax. Once remission is achieved patients should proceed to transplant as soon as possible. Promising results with the use of HMAs such as decitabine or azacitidine alone or in combination with ruxolitinib have been reported, with 50% to 60% of patients achieving clinically significant responses persisting for 6 to 24 months in the majority of cases. More recently the use of bcl-2 inhibitor venetoclax has changed the landscape of AML treatment as it has shown remarkable efficacy in combination with HMAs. The data for the use of venetoclax in AML secondary to MPN is mixed. Gangat and coworkers in a multicenter trial of a HMA plus venetoclax reported a CR rate of 44% versus 4% with a HMA alone. Most importantly, this combination successfully allowed many patients to move forward with allogenic stem cell transplantation.[58] These chemotherapeutic regimens can frequently be administered as an outpatient but require treatment for multiple monthly cycles before clinical responses can be evaluated. Recently we have begun to treat these patients with oral decitabine with impressive outcomes. Although many of these patients do not achieve true complete remissions, their prolonged survival and quality of life, at least based upon single-institutional studies, appears to be superior to that achieved with standard induction chemotherapy.

PV occurs frequently during childbearing years. A discussion of contraception options is imperative because the use of oral contraceptive pills may be associated with an increased risk of deep venous thrombosis as well as splanchnic vein thrombosis. It seems prudent to entertain alternative forms of contraception with such individuals. Discontinuation of HU in both men and women desiring to have a child is recommended. HU is capable of inducing azoospermia in men, frequently limiting their ability to father a child. Pregnancy is itself a prothrombotic condition. PV in a pregnant individual has been reported to lead to an increased incidence of fetal demise, with up to 30% of pregnancies in PV patients terminating in spontaneous abortions. In addition, preeclampsia occurs more frequently in these women. A team approach requiring close communication between an obstetrician skilled in providing care for high-risk pregnancies and the responsible hematologist provides the optimal integration of care to the mother and the child. Pregnancy in PV patients is frequently associated with a gradual normalization of blood values, and it is not unusual for a woman who has required extensive therapy for control of her disease to no longer require phlebotomies during pregnancy. Delivery appears not to be complicated by excessive hemorrhage, but the postpartum period carries an increased risk of venous thrombosis. Although some degree of hematocrit level normalization can be explained by expansion of the plasma volume or by nutritional deficiencies that occur during pregnancy, it is unlikely that these factors can be solely responsible. It seems more reasonable to assume that the high estrogen levels characteristic of pregnancy suppress erythropoiesis. After completion of the pregnancies, the patients' hematologic values slowly drift back to their previously elevated values in parallel with the return to normal estrogen levels. Because pregnancy is usually associated with spontaneous control of the polycythemic state, no specific therapy is required except for careful observation. If needed, therapy should be limited to phlebotomy and low-dose aspirin therapy because of the mutagenic effects of chemotherapeutic agents. Aggressive treatment of hypertension is imperative, as well as attention to the risk of developing preeclampsia and toxemia, which should be treated in a standard fashion. In individuals with a history of repeated spontaneous abortions associated with placental infarction, consideration should be given to low-dose aspirin with LMWH therapy, making sure that these agents are discontinued at the time of delivery to avoid the risk of excessive hemorrhage. If this approach is not successful, IFN therapy is suggested because it is not known to be leukemogenic or teratogenic and does not cross the placenta. In the puerperium, thromboprophylaxis with 6 weeks of LMWH therapy or a DOAC is recommended. Aggressive intervention with hematocrit levels maintained below 45% and aspirin therapy with LMWH is associated with a positive outcome for the pregnancy. Breastfeeding is recommended if the mother is receiving therapy with heparin or Coumadin as long as the baby receives adequate supplementation with vitamin K. Rapid mobilization of the mother after delivery is of great importance.

Because PV is ultimately a stem cell disorder, it should be possible to achieve a cure with stem cell transplantation. Thus far, the majority of patients undergoing allogeneic stem cell transplantation have been transplanted after evolution to MF, MDS, or acute leukemia. Transplantation during the polycythemia phase of the disease is at present not appropriate.

FUTURE DIRECTIONS

The discovery of *JAK2V617F* mutations has led to a more comprehensive understanding of the pathophysiology of the MPNs, including PV. There is no question that the diagnostic tests for *JAK2V617F* and exon 12 mutations of *JAK2*, as well as mutations in *VHL*, *HIF-1*, *HIF-2*, the *EPOR*, and *PHD* have revolutionized the diagnostic approach to patients with erythrocytosis. PV is a stem cell disease that will ultimately require curative therapies at a minimum to deplete or eliminate malignant stem cells if curative small-molecule therapies are to become a reality. Although the *JAK2V617F* VAF has been used as a biomarker for outcomes of novel therapeutic approaches to treat patients with PV, studies are needed to determine the extent of reduction of allele burden that would be predictive of altering the natural history of PV and eliminating complications from thrombotic episodes or evolution to MF or MPN-BP. The use of drugs affecting only JAK2 is unlikely to be curative because multiple genetic and epigenetic events likely contribute to the origins and progression of PV. Randomized clinical trials of combinations of drugs, rather than single drugs, and comparing these outcomes with the standard of care are more likely to lead to significant advances in improving the natural history of PV patients. The use of novel immunotherapeutic approaches as well as strategies to interrupt the TPO/MPL axis should serve as new therapeutic platforms to develop novel therapeutic strategies.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Barbui T, et al. Ropeginterferon alfa-2b versus phlebotomy in low-risk patients with polycythaemia vera (Low-PV study): a multicentre, randomised phase 2 trial. *Lancet Haematol*. 2021;8(3):e175–e184.

Barosi G, et al. Revised response criteria for polycythemia vera and essential thrombocythemia: an ELN and IWG-MRT consensus project. *Blood*. 2013;121(23):4778–4781.

Choueiri TK, et al. Inhibition of hypoxia-inducible factor-2alpha in renal cell carcinoma with belzutifan: a phase 1 trial and biomarker analysis. *Nat Med*. 2021;27(5):802–805.

Gangat N, et al. Venetoclax with azacitidine or decitabine in blast-phase myeloproliferative neoplasm: a multicenter series of 32 consecutive cases. *Am J Hematol*. 2021;96(7):781–789.

Gisslinger H, et al. Ropeginterferon alfa-2b versus standard therapy for polycythaemia vera (PROUD-PV and CONTINUATION-PV): a randomised, non-inferiority, phase 3 trial and its extension study. *Lancet Haematol*. 2020;7(3):e196–e208.

Karantanos T, et al. Sex determines the presentation and outcomes in MPN and is related to sex-specific differences in the mutational burden. *Blood Adv*. 2020;4(12):2567–2576.

Moliterno AR, Ginzburg YZ, Hoffman R. Clinical insights into the origins of thrombosis in myeloproliferative neoplasms. *Blood*. 2021;137(9):1145–1153.

James C, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*. 2005;434(7037):1144–1148.

Kralovics R, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med*. 2005;352(17):1779–1790.

Landolfi R, et al. Efficacy and safety of low-dose aspirin in polycythemia vera. *N Engl J Med*. 2004;350(2):114–124.

Marchioli R, et al. Cardiovascular events and intensity of treatment in polycythemia vera. *N Engl J Med*. 2013;368(1):22–33.

Hasselbalch HC, Elvers M, Schafer AI. The pathobiology of thrombosis, microvascular disease, and hemorrhage in the myeloproliferative neoplasms. *Blood*. 2021;137(16):2152–2160.

Luque Paz D, et al. Leukemic evolution of polycythemia vera and essential thrombocythemia: genomic profiles predict time to transformation. *Blood Adv*. 2020;4(19):4887–4897.

Marcellino BK, et al. Advanced forms of MPNs are accompanied by chromosomal abnormalities that lead to dysregulation of TP53. *Blood Adv*. 2018;2(24):3581–3589.

Ginzburg YZ, et al. Dysregulated iron metabolism in polycythemia vera: etiology and consequences. *Leukemia*. 2018;32(10):2105–2116.

Kiladjian J-J, et al. Long-term efficacy and safety of ruxolitinib versus best available therapy in polycythaemia vera (RESPONSE): 5-year follow-up of a phase 3 study. *Lancet Haematol*. 2020;7(3):e226–e237.

Mascarenhas J, et al. A prospective evaluation of pegylated interferon alfa-2a therapy in patients with polycythemia vera and essential thrombocythemia with a prior splanchnic vein thrombosis. *Leukemia*. 2019;33(12):2974–2978.

Szuber N, et al. Myeloproliferative neoplasms in the young: Mayo Clinic experience with 361 patients age 40 years or younger. *Am J Hematol*. 2018;93(12):1474–1484.

Tefferi A, et al. Mutation-enhanced international prognostic systems for essential thrombocythaemia and polycythaemia vera. *Br J Haematol*. 2020;189(2):291–302.

Yacoub A, et al. Pegylated interferon alfa-2a for polycythemia vera or essential thrombocythemia resistant or intolerant to hydroxyurea. *Blood*. 2019;134(18):1498–1509.

REFERENCES

1. Hirota K. Basic biology of hypoxic responses mediated by the transcription factor HIFs and its implication for medicine. *Biomedicines*. 2020;8(2):32.
2. Lappin TR, Lee FS. Update on mutations in the HIF: EPO pathway and their role in erythrocytosis. *Blood Rev*. 2019;37:100590.
3. Ravenna L, Salvatori L, Russo MA. HIF3α: the little we know. *FEBS J*. 2016;283(6):993–1003.
4. Crespigio J, et al. Von Hippel-Lindau disease: a single gene, several hereditary tumors. *J Endocrinol Invest*. 2018;41(1):21–31.
5. Semenza GL. HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations. *J Clin Invest*. 2013;123(9):3664–3671.
6. Choueiri TK, et al. Inhibition of hypoxia-inducible factor-2alpha in renal cell carcinoma with belzutifan: a phase 1 trial and biomarker analysis. *Nat Med*. 2021;27(5):802–805.
7. Vlahakos DV, Marathias KP, Madias NE. The role of the renin-angiotensin system in the regulation of erythropoiesis. *Am J Kidney Dis*. 2010;56(3):558–565.
8. Pasquier F, et al. New pathogenic mechanisms induced by germline erythropoietin receptor mutations in primary erythrocytosis. *Haematologica*. 2018;103(4):575–586.
9. Bakris GL, et al. Effects of theophylline on erythropoietin production in normal subjects and in patients with erythrocytosis after renal transplantation. *N Engl J Med*. 1990;323(2):86–90.
10. Li N, et al. Nocturnal mean oxygen saturation is associated with secondary polycythemia in young adults with obstructive sleep apnea, especially in men. *Nat Sci Sleep*. 2019;11:377–386.
11. Nguyen CD, Holty JC. Does untreated obstructive sleep apnea cause secondary erythrocytosis? *Respir Med*. 2017;130:27–34.
12. Steele AR, et al. Global REACH 2018: volume regulation in high-altitude Andeans with and without chronic mountain sickness. *Am J Physiol Regul Integr Comp Physiol*. 2021;321(3):R504–r512.
13. Sykes DB, O'Connell C, Schroyens W. The TEMPI syndrome. *Blood*. 2020;135(15):1199–1203.
14. Ponce OJ, et al. The efficacy and adverse events of testosterone replacement therapy in hypogonadal men: a systematic review and meta-analysis of randomized, placebo-controlled trials. *J Clin Endocrinol Metab*. 2018; 103(5):1745–1754.
15. Lenglet M, et al. Identification of a new VHL exon and complex splicing alterations in familial erythrocytosis or von Hippel-Lindau disease. *Blood*. 2018;132(5):469–483.
16. Ghosh MC, et al. Therapeutic inhibition of HIF-2α reverses polycythemia and pulmonary hypertension in murine models of human diseases. *Blood*. 2021;137(18):2509–2519.
17. Nolan BJ, et al. Prevalence of polycythaemia with different formulations of testosterone therapy in transmasculine individuals. *Intern Med J*. 2021; 51(6):873–878.
18. Grinfeld J, et al. Classification and personalized prognosis in myeloproliferative neoplasms. *N Engl J Med*. 2018;379(15):1416–1430.
19. Srour SA, et al. Incidence and patient survival of myeloproliferative neoplasms and myelodysplastic/myeloproliferative neoplasms in the United States, 2001-12. *Br J Haematol*. 2016;174(3):382–396.
20. Karantanos T, et al. Sex determines the presentation and outcomes in MPN and is related to sex-specific differences in the mutational burden. *Blood Adv*. 2020;4(12):2567–2576.
21. Moliterno AR, Ginzburg YZ, Hoffman R. Clinical insights into the origins of thrombosis in myeloproliferative neoplasms. *Blood*. 2021;137(9): 1145–1153.
22. James C, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*. 2005;434(7037):1144–1148.
23. Kralovics R, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med*. 2005;352(17):1779–1790.
24. Bao EL, et al. Inherited myeloproliferative neoplasm risk affects haematopoietic stem cells. *Nature*. 2020;586(7831):769–775.
25. Spivak JL, et al. Thrombopoietin is required for full phenotype expression in a JAK2V617F transgenic mouse model of polycythemia vera. *PLoS One*. 2020;15(6):e0232801.
26. Dupont S, et al. The JAK2 617V>F mutation triggers erythropoietin hypersensitivity and terminal erythroid amplification in primary cells from patients with polycythemia vera. *Blood*. 2007;110(3):1013–1021.
27. Vannucchi AM, et al. Clinical profile of homozygous JAK2 617V>F mutation in patients with polycythemia vera or essential thrombocythemia. *Blood*. 2007;110(3):840–846.
28. Hultcrantz M, et al. Risk for arterial and venous thrombosis in patients with myeloproliferative neoplasms: a population-based cohort study. *Ann Intern Med*. 2018;168(5):317–325.
29. Landolfi R, et al. Efficacy and safety of low-dose aspirin in polycythemia vera. *N Engl J Med*. 2004;350(2):114–124.
30. Marchioli R, et al. Cardiovascular events and intensity of treatment in polycythemia vera. *N Engl J Med*. 2013;368(1):22–33.
31. Hasselbalch HC, Elvers M, Schafer AI. The pathobiology of thrombosis, microvascular disease, and hemorrhage in the myeloproliferative neoplasms. *Blood*. 2021;137(16):2152–2160.
32. Ronner L, et al. Persistent leukocytosis in polycythemia vera is associated with disease evolution but not thrombosis. *Blood*. 2020;135(19): 1696–1703.
33. Gangaraju R, et al. Thrombotic, inflammatory, and HIF-regulated genes and thrombosis risk in polycythemia vera and essential thrombocythemia. *Blood Adv*. 2020;4(6):1115–1130.
34. Zhang Y, et al. Thrombosis among 1537 patients with JAK2V617F - mutated myeloproliferative neoplasms: risk factors and development of a predictive model. *Cancer Med*,. 2020;9(6):2096–2105.
35. Nicol C, et al. Hemorrhage in essential thrombocythemia or polycythemia vera: epidemiology, location, risk factors, and lessons learned from the literature. *Thromb Haemost*. 2021;121(5):553–564.
36. Tefferi A, et al. Long-term survival and blast transformation in molecularly annotated essential thrombocythemia, polycythemia vera, and myelofibrosis. *Blood*. 2014;124(16):2507–2513. quiz 2615.
37. Magaz M, et al. Next-generation sequencing in the diagnosis of non-cirrhotic splanchnic vein thrombosis. *J Hepatol*. 2021;74(1):89–95.
38. Stempel JM, et al. Pulmonary arterial hypertension in hospitalized patients with polycythemia vera (from the National Inpatient Database). *Am J Cardiol*. 2021;143:154–157.
39. Luque Paz D, et al. Leukemic evolution of polycythemia vera and essential thrombocythemia: genomic profiles predict time to transformation. *Blood Adv*. 2020;4(19):4887–4897.
40. Marcellino BK, et al. Advanced forms of MPNs are accompanied by chromosomal abnormalities that lead to dysregulation of TP53. *Blood Adv*. 2018.;2(24):3581–3589.
41. Johansson P, et al. Highly increased risk of fracture in patients with myeloproliferative neoplasm. *Leuk Lymphoma*. 2021;62(1):211–217.
42. Najfeld V, et al. Exploring polycythaemia vera with fluorescence in situ hybridization: additional cryptic 9p is the most frequent abnormality detected. *Br J Haematol*. 2002;119(2):558–566.
43. Szuber N, et al. Myeloproliferative neoplasms in the young: Mayo Clinic experience with 361 patients age 40 years or younger. *Am J Hematol*. 2018;93(12):1474–1484.
44. Ianotto JC, et al. Characteristics and outcomes of patients with essential thrombocythemia or polycythemia vera diagnosed before 20 years of age: a systematic review. *Haematologica*. 2019;104(8):1580–1588.
45. Tefferi A, et al. Mutation-enhanced international prognostic systems for essential thrombocythaemia and polycythaemia vera. *Br J Haematol*. 2020;189(2):291–302.
46. Barosi G, et al. Revised response criteria for polycythemia vera and essential thrombocythemia: an ELN and IWG-MRT consensus project. *Blood*. 2013;121(23):4778–4781.
47. Ginzburg YZ, et al. Dysregulated iron metabolism in polycythemia vera: etiology and consequences. *Leukemia*. 2018;32(10): 2105–2116.
48. Alvarez-Larrán A, et al. Genomic characterization of patients with polycythemia vera developing resistance to hydroxyurea. *Leukemia*. 2021; 35(2):623–627.
49. Rao TN, et al. JAK2-V617F and interferon-α induce megakaryocyte-biased stem cells characterized by decreased long-term functionality. *Blood*. 2021;137(16):2139–2151.
50. Yacoub A, et al. Pegylated interferon alfa-2a for polycythemia vera or essential thrombocythemia resistant or intolerant to hydroxyurea. *Blood*. 2019;134(18):1498–1509.
51. Gisslinger H, et al. Ropeginterferon alfa-2b versus standard therapy for polycythaemia vera (PROUD-PV and CONTINUATION-PV): a randomised, non-inferiority, phase 3 trial and its extension study. *Lancet Haematol*. 2020;7(3):e196–e208.
52. Jäger R, et al. Germline genetic factors influence the outcome of interferon-α therapy in polycythemia vera. *Blood*. 2021;137(3):387–391.
53. Barbui T, et al. Ropeginterferon alfa-2b versus phlebotomy in low-risk patients with polycythaemia vera (Low-PV study): a multicentre, randomised phase 2 trial. *Lancet Haematol*. 2021;8(3):e175–e184.
54. Kiladjian J-J, et al. Long-term efficacy and safety of ruxolitinib versus best available therapy in polycythaemia vera (RESPONSE): 5-year follow-up of a phase 3 study. *Lancet Haematol*. 2020;7(3):e226–e237.
55. Mikkelsen SU, et al. Safety and efficacy of combination therapy of interferon-α2 and ruxolitinib in polycythemia vera and myelofibrosis. *Cancer Med*. 2018;7(8):3571–3581.

56. De Stefano V, et al. Hydroxyurea prevents arterial and late venous thrombotic recurrences in patients with myeloproliferative neoplasms but fails in the splanchnic venous district. Pooled analysis of 1500 cases. *Blood Cancer J*. 2018;8(11):112.
57. Mascarenhas J, et al. A prospective evaluation of pegylated interferon alfa-2a therapy in patients with polycythemia vera and essential thrombocythemia with a prior splanchnic vein thrombosis. *Leukemia*. 2019;33(12): 2974–2978.
58. Gangat N, et al. Venetoclax with azacitidine or decitabine in blast-phase myeloproliferative neoplasm: a multicenter series of 32 consecutive cases. *Am J Hematol*. 2021;96(7):781–789.
59. Lew J, et al. Hemichorea in a patient with JAK2V617F blood cells. *Blood*. 2013;121(7):1239–1240.

CHAPTER 71

ESSENTIAL THROMBOCYTHEMIA

Bridget K. Marcellino, John Mascarenhas, Camelia Iancu-Rubin,
Marina Kremyanskaya, Vesna Najfeld, and Ronald Hoffman

Essential thrombocythemia (ET) is a chronic myeloproliferative neoplasm (MPN) characterized by platelet counts in excess of 450 × 10^9/L, profound bone marrow (BM) megakaryocyte hyperplasia, leukocytosis, splenomegaly, a clinical course punctuated by hemorrhagic and/ or thrombotic episodes, and a possible evolution to myelofibrosis (MF) and MPN blast phase (MPN-BP). ET is a clinically heterogeneous disorder, with more than half of patients meeting the criteria for diagnosis being asymptomatic at presentation. ET is associated with the clonal acquisition of driver mutations (*JAK2*), the thrombopoetin receptor (*MPL*), or calreticulin (*CALR*) that activate the JAK-STAT signaling pathway, allowing hematopoiesis to occur in the absence of exogenous cytokines. The *JAK2*V617F mutation occurs in 50% to 60% of ET patients, while recurrent *CALR* mutations occur in 15% to 24% of patients. Finally, about 3% to 5% have *MPL* mutations. The ET patients who lack such driver mutations but have histomorphologic features diagnostic of ET are referred to as being “triple negative.” ET was first described in 1934 by Epstein and Goedel, reporting on a patient with an elevated platelet count who had repeated hemorrhagic episodes.

EPIDEMIOLOGY

The true incidence of ET is unknown because extensive epidemiologic studies are not available. The incidence of ET has been estimated to be approximately 1.5 to 2.4 patients per 100,000 populations annually. A Swedish study indicated that first-degree relatives of patients with an MPN, including ET, had a five- to sevenfold increased risk of developing an MPN, supporting the concept that there is a strong genetic predilection. ET occurs in individuals with a median age of 67 to 73 years. To gain additional insight into the patterns of occurrence of MPNs by age, sex, race/ethnicity, and susceptible populations and provide a population-based assessment of patient survival, Srour et al.[1] used data from the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute in the United States, to better assess the incidence of MPNs in the United States from 2001 to 2012. Importantly, during a part of this period of time, the use of mutational analyses in making the diagnosis of MPNs became widespread. There were 31,904 MPN cases diagnosed among residents of the 18 SEER registries evaluated. The age-adjusted incidence rates (IRs) were as follows: polycythemia vera (PV; 10.9 per 1 million patient years), ET (9.6), chronic myeloid leukemia (CML; 3.3), and primary myelofibrosis (PMF; 3.1). ET was the only MPN with a significantly lower IR among males than females; the average age at diagnosis was 68 years. The female predominance was most pronounced in individuals less than 60 years of age. Surprisingly, the IR of ET was 18% higher among Blacks than among non-Hispanic Whites; ET was associated with a female predominance among non-Hispanic Whites, White Hispanics, Blacks, and Asian–Pacific islanders, suggesting shared gender-specific risk factor(s) across these racial/ethnic groups. A recent study compared the impact of sex on MPNs and found that male sex was an independent predictor of a poorer overall survival and higher rate of progression to MF and MPN-BP which might be attributable to their having higher *JAK2*V617F variant allele frequencies (VAF) as well as an increased number of acquired non-MPN specific myeloid malignancy gene mutations (*ASXL1*, *EZH2*, *SRSF2*, *U2AF1*, and *IDH1/2*).

The incidence patterns observed by Srour et al.[1] support inherent differences in susceptibility to developing an MPN. This study was of importance since it represented the first step in appreciating the potential for molecular diagnostics to improve our understanding of the epidemiology of the MPNs. Recent studies have confirmed that in addition to the *JAK2*V617F mutation, mutations in *CALR* and *MPL* are also key drivers of this disease. These data suggest that ET is a relatively common hematological malignancy that has an especially significant impact on younger women due to their propensity to develop venous thromboses.

There are shared susceptibility genes that predispose individuals to develop MPNs. The Janus kinase 2 (*JAK2*) mutations (V617F) are not acquired randomly but arise preferentially on a specific *JAK2* haplotype (46/1). The *JAK2* 46/1 haplotype has been shown not only to predispose *JAK2*V617F-positive ET but also to ET harboring *MPL* and *CALR* mutations, again indicating that genetic factors play a role in the susceptibility to developing ET. Tapper et al. also identified additional two single-nucleotide polymorphisms (SNPs), r14339666 within *JAK2* and rs2201862, 153 kb downstream of the DS1 and EVI1 complex locus protein EVI1 (*MECOM*), which were associated with *JAK2*V617F-negative MPNs. Two additional SNPs, rs2736100 in telomerase reverse transcriptase (*TERT*), and rs9376092 between *HBS1L* and *MYB*, were associated with *JAK2*V617F-positive MPNs. The SNP between *HBS1L* and *MYB*, rs9376092, however, had a stronger effect on MPNs associated with *CALR* and/or *MPL* mutations, whereas in *JAK2*V617F-positive cases, rs9376092 was associated with ET rather than PV. These investigators demonstrated that the candidate risk allele at rs9376092, which had a strong association with ET, was associated with reduced *MYB*. Prior functional analyses have shown that mice expressing low levels of MYB develop a transplantable ET-like disease. These findings indicate that multiple germline variants predispose to the development of each of the MPNs and link constitutional differences in *MYB* expression, in particular to ET. Additionally, germline mutations in ET patients have been shown to affect their clinical course. For instance, ET patients with a specific SNP variant in the ERCC2 gene have nearly a fourfold higher risk of progressing to MPN-BP.

ET has rarely been reported in the pediatric age group. The incidence of ET in childhood has been reported to be approximately 1 per 10^7 population, which is 60 times less than that in adults. Approximately 30% of children with this disorder experience thrombotic or hemorrhagic complications at diagnosis or later in their course, and 50% have splenomegaly. Mutations in one of the established MPN driver genes, *JAK2*, *CALR*, or *MPL*, were present in a lower percentage of pediatric cases (34%), compared with adult MPN patients (90%). The subgroup of patients without a detectable driver mutation tended to have higher platelet counts, compared with patients with mutations. One must always exclude inherited disorders that are known to lead to isolated thrombocytosis before being certain that a child who lacks a known driver mutation has ET.

Several families with multiple members having ET have been described. The prevalence of the *JAK2*V617F mutation in familial cases of MPN has been analyzed in 72 families, including 174 patients (68 with ET). The *JAK2* mutation was found in half of patients with ET and a similar proportion as observed in sporadic, nonfamilial cases. Among 46 families with at least two cases of PV, ET, or PMF, the *JAK2* mutation was absent in six families, heterogeneously distributed in 18, and present in all patients with MPN in 22. Thus, the *JAK2* mutation does not seem to be required for the development of ET or other MPNs, and this familial clustering cannot be accounted for by the prevalence of the *JAK2* 46/1 haplotype. In familial MPNs, *CALR* mutations can also be somatically acquired and are associated with an ET or PMF phenotype.

PATHOBIOLOGY

ET is a clonal hematological malignancy originating at the level of the hematopoietic stem cell. The thrombocytosis that characterizes ET is caused by increased platelet production due to increased numbers of megakaryocytes. Effective platelet production is increased as much as 10-fold and is associated with an increase in megakaryocyte clustering, volume, nuclear lobe number, and nuclear ploidy.

Although ET originates at the level of the pluripotent hematopoietic stem cell, a significant proportion of nonclonally derived leukocytes exist, in addition to the clonally derived population of leukocytes in patients with ET. In one study of 42 patients with ET, 31 patients exhibited clonality of at least one hematopoietic lineage, but the remaining 11 patients had a polyclonal origin of all the lineages studied. The biogenesis of polyclonal ET remains a subject of investigation. Interestingly, in some patients, monoclonality of hematopoiesis is restricted to platelets, despite the polyclonal origin of cells belonging to other lineages. Presently it is possible that some patients with polyclonal triple negative thrombocytosis cases might have ET, which might be missed since the *JAK2*V617F VAF in such a case is higher in platelet RNA than in granulocyte DNA, which is routinely used for the performance of mutational analyses. Therefore it is important to directly perform WES or whole-genome sequencing on megakaryocytes or to sequence platelet RNA in order to establish the diagnosis and the presence of clonality in such triple negative ET cases. Alternatively, the isolated thrombocytosis in such cases of presumed polyclonal triple negative ET could be due to diseases other than MPNs, particularly hereditary forms of thrombocytosis associated with germline gain-of-function mutations in *JAK2* and *MPL or reactive causes of thrombocytosis*. In addition, another type of polyclonal ET has also been reported in patients with a *CALR* mutation. Two *CALR* frameshift mutation-positive patients were reported with an oligo-clonal disease: one patient had one clone that was positive for a *CALR* type-1 mutation and a second clone possessed a *CALR* type-2 mutation, while the second patient had separate clones that were positive for either *JAK2*V617F or a *CALR* type-2 mutation.

Increased numbers of megakaryocyte progenitor cells are present in the BM and the peripheral blood of patients with ET. These data support the concept that the principal abnormality is an expansion of the progenitor cell pool. In addition, progenitor cells were noted to either be hypersensitive, or independent of the addition of exogenous cytokines, including interleukin (IL)-3, IL-6, and thrombopoietin. A second subpopulation of colony-forming unit–megakaryocyte (CFU-MK) assayed from patients with ET remained dependent on the addition of exogenous cytokines.

Mutational Spectrum in Essential Thrombocythemia

The hypersensitivity of ET progenitor cells to a variety of cytokines is due to the clonal acquisition of driver mutations (*JAK2*, *MPL*, or *CALR*) that activate the JAK-STAT signaling pathway, allowing hematopoiesis to occur in the absence of exogenous cytokines. The *JAK2*V617F mutation occurs in 50% to 60% of ET patients, while recurrent *CALR* mutations occur in 15% to 24% of patients, and 3% to 5% have *MPL* mutations. The patients with ET who lack such driver mutations are said to be "triple negative." While the driver mutations were originally thought to be mutually exclusive, it has recently been identified that a small subset of ET patients with low VAF *JAK2*V617F can have co-occurring *CALR* or *MPL* mutations.

JAK2 is a cytoplasmic tyrosine kinase that plays a key role in mediating intracellular signaling from a variety of growth factors, including IL-3, erythropoietin, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and thrombopoietin. Co-expression of *JAK2*V617F with a homodimeric type 1 cytokine receptor (including erythropoietin, thrombopoietin, or G-CSF) is necessary for hormone activation of JAK-STAT (signal transducer and activator of transcription) signaling pathways and for hematopoietic cell proliferation to become growth factor–independent. The *JAK2*V617F mutation is present in ET patients with both clonal and polyclonal hematopoiesis. Patients with clonal hematopoiesis have a higher *JAK2*V617F allele burden (26%) than patients with polyclonal hematopoiesis (16%). The relative size of the *JAK2V617F* clone is often small and remains stable over time in patients with both clonal and polyclonal ET. A VAF higher than 50% indicating the presence of granulocytes, homozygous for *JAK2*V617F, has been found in 70% of PV patients, but occurs less frequently in ET. In ET patients, the increase in the *JAK2*V617F VAF from a level well below 50% at presentation, to a level above 50% after years of follow-up could be indicative of the imminent evolution of the patient's ET to PV. All PV patients have assayable erythroid colonies that are homozygous for *JAK2*V617F, even in PV patients with a low burden of *JAK2*V617F. By contrast, hematopoietic colonies cloned from ET patients are only occasionally *JAK2*V617F homozygous colonies. The transition from *JAK2* heterozygous to homozygous progenitors is a consequence of homologous recombination. These studies suggest that such an event is characteristic of PV but rarely occurs in ET. If there is a substantial increase in the numbers of *JAK2*V617F homozygous progenitors in an ET patient, it would likely lead to a transition from an ET to a PV phenotype.

STATs are activated downstream of *JAK2*V617F. STAT3 is a pivotal regulator of megakaryocytopoiesis, which might provide an explanation for its exclusive upregulation in ET. To further examine the differences between hematopoiesis in *JAK2*V617F ET and PV, the gene expression profiles of *JAK2*V617F-heterozygous erythroid colonies have been examined. Erythroblasts from ET patients were characterized by enhanced expression of genes associated with interferon (IFN) signaling and phosho-STAT1, compared with PV erythroblasts. STAT1 is essential for IFN-γ signaling. Increased STAT1 in normal CD34$^+$ cells has been shown to favor megakaryocytic differentiation but reduce erythroid differentiation, creating a differentiation pattern that resembles ET. Furthermore, inhibition of STAT1 signaling in ET hematopoietic progenitor cells led to enhanced erythropoiesis and reduced megakaryocytopoiesis. These studies suggest that in ET, *JAK2*V617F induces the simultaneous activation of STAT5 and STAT1 pathways, but in PV, the relatively reduced levels of phospho-STAT1 reduce the brake on erythropoiesis. Further functional annotation analyses have also demonstrated that clusters of gene ontology terms related to inflammation, immune system, apoptosis, RNA metabolism, and secretory system are also significantly dysregulated terms in ET, PV, and MF. These data support the concept of MPNs being a biological continuum transitioning from ET to PV to PMF.

Thrombopoietin is the primary physiologic regulator of thrombopoiesis, which acts by binding to its cell surface receptor, MPL. Thrombopoietin levels in a normal individual are inversely related to megakaryocyte mass. MPL is expressed by CD34$^+$ hematopoietic stem and progenitor cells, MKs, and platelets. One would anticipate that thrombopoietin levels in ET would be low due to the characteristic megakaryocytic hyperplasia. Normal or slightly elevated thrombopoietin levels have, however, been observed in patients with ET. Furthermore, Wang et al. have reported that thrombopoietin levels were significantly higher in patients with ET than in patients with reactive thrombocytosis who had similar levels as control. This unanticipated elevation of thrombopoietin levels in ET patients might be explained by the observation that the expression of the thrombopoietin receptor and its mRNA is dramatically reduced in the platelets of patients with ET. Thrombopoietin serum levels are controlled by platelet mass through MPL-mediated thrombopoietin uptake and degradation. The reduced platelet MPL expression occurs not only in ET but also in PV and PMF and has been shown to be a downstream event of *JAK2*V617F, which promotes the proteasomal degradation of MPL. The reduced MPL likely results in the decreased capacity of platelets to absorb thrombopoietin, contributing to the increased megakaryocyte mass and thrombocytosis. The mutations in the thrombopoietin receptor, *MPL*W515L and *MPL*515K, are present in approximately 3% to 5% of patients with ET, and additional novel *MPL* mutations have been identified in ET patients in a recent study utilizing deep mutational scanning of MPNs and will require further investigation. About 60% of patients with *MPL* mutations have the W515L mutation and 40% have the W515K mutation. The mutant allele burden is greater than 50% in 50% of W515K patients compared with 17% of W515L patients. The most prevalent *MPL* mutations in ET occur on tryptophan 515, an amino acid that maintains MPL in an inactive form in the absence of cytokines. Rarely, in ET patients, another *MPL* mutation, S505N, serves as a driver mutation. It is located in the exon 10 domain that encodes the transmembrane domain of MPL and induces dimerization of the transmembrane helix in an active confirmation. The *MPL* mutations occurring in ET trigger conformational changes in the receptor, bringing into close proximity two molecules of bound JAK2, for transphosphorylation and activation of the JAK-STAT signaling cascade. The loss of tryptophan, but not the acquisition of a particular residue, induces the constitutive activation of MPL. In ET, both *JAK2*V617F and *MPL* mutations arise preferentially on a specific constitutional *JAK2* 46/1 haplotype. Two hypotheses have been proposed to account for this predilection: 46/1 is inherently genetically more unstable (hypermutability hypothesis) or 46/1 confers a growth advantage that favors the predominance of *JAK2*V617F hematopoiesis (fertile ground hypothesis). The association of *MPL* mutations with the *JAK2* 46/1 haplotype strongly favors the hypermutability hypothesis rather than the fertile ground hypothesis. The presence of *MPL*W515K mutations in ET patients is associated with lower hemoglobin levels and higher platelet counts, as well as preferential expansion of the numbers of megakaryocytes at the expense of erythroid precursors, as observed in BM biopsy specimens.

Of patients with ET or MF who do not have mutations in *JAK2* or *MPL*, the overwhelming majority have a mutation in *CALR*. Mutations in the *CALR* gene occur in 15% to 24% of ET patients and rarely occur together with mutations of *JAK2* or *MPL*. The clinical phenotype of patients with *JAK2*-mutated and *CALR*-mutated ET differs. The presence of the mutated *CALR* is associated with a younger age, male predominance, higher platelet counts, lower hemoglobin levels, lower leukocyte counts, and a lower risk of thrombotic complications. Recent studies have suggested that the lower thrombotic rates in *CALR*-mutated ET may in part be due to reduced leukocyte activation or less reactive platelets, compared with their *JAK2*V617F-positive counterparts. *CALR*-mutated ET patients do not evolve to PV while this transition occurs in 20% to 30% of *JAK2*V617F+ ET patients over a 15-year period. Grinfeld et al. have independently reported that *CALR* mutations are independently associated with an increased risk of developing MF.[2]

The wild-type *CALR* gene encodes for an evolutionarily conserved, multifunctional protein, involved in multiple cellular processes ranging from calcium homeostasis and protein folding in the endoplasmic reticulum, to apoptotic cell death clearance and cellular adhesion. The *CALR* mutations (*CALR*: 19p13.2) identified in MPN mainly consist of deletions (i.e., type I) or insertions (i.e., type II) occurring within exon 9, which create a novel epitope in the C-terminal domain of the protein. Despite the heterogeneity of these mutations, the new C-terminus sequence is identical and results in the loss of the KDEL domain, which is critical for CALR retention in the endoplasmic reticulum and its ability to regulate calcium homeostasis. Original studies describing *CALR* mutations in MPN indicated that such a uniform defect may confer a proliferative advantage to malignant cells via activation of the JAK-STAT pathway.

Mutations of *CALR* are very rare in PV but are found in patients with MF and ET, the two MPN entities in which megakaryocytic hyperplasia is a hallmark of the disease. The most frequent *CALR* mutations, accounting for more than 80% of the total, are the type-1 variant, a 52 bp deletion (p.L367fs*46), and the type-2, a 5 bp TTGTC insertion (p.K385fs*47). The other types of mutations have been observed at much lower frequencies, and many have been detected only in individual patients. Overall, type-1 is more frequent (53%) than type 2 mutations (31.7%), but the incidence of type-2 mutations is higher in ET as compared to MF. Type 1 mutations are more frequent in MF patients. Some investigators have conjectured that type 1 *CALR* mutations are a forme fruste of MF. Most *CALR* mutations are present in a heterozygous state, although rare patients are homozygous for *CALR* mutations, which are associated with uniparental disomy of chromosome 19p. The *CALR* mutations target the hematopoietic stem cell and the mutation can be detected in granulocytes, monocytes, platelets, B, and NK cells. Occasionally in ET patients, they may be seen in T cells. Both types of *CALR* mutations lead to an increase in the number of megakaryocyte progenitors that are hypersensitive to cytokines. A series of subsequent reports have shed light on the mechanisms responsible for the ability of the mutated *CALR* to induce MK hyperplasia and thrombocytosis in MPN. Thus four independent laboratories have demonstrated that the oncogenic activity of mutated *CALR* is mediated by MPL, which is critical for both hematopoietic stem cell and megakaryocytic lineage development. Thus mutant *CALR* expressed by hematopoietic cells in vitro or in vivo in mouse models binds and activates MPL but not other type I hematopoietic cytokine receptors. This, in turn, leads to JAK-STAT pathway activation, resulting in the development of thrombocytosis and an ET-like phenotype. Interestingly, in vivo overexpression of either of the mutation variants (type I, deletion; or type II, insertion) induces thrombocytosis and constitutively activates MPL and JAK-STAT signaling. Yet, while type II mutations favor MK proliferation, type I mutations confer clonal dominance of the hematopoietic stem cell, resulting in splenomegaly, BM hypocellularity, and fibrosis, a phenotype reminiscent of "post-ET" MF. These findings mirror the clinical observations in which type I mutations are more prevalent in MF patients, while type II mutations are more frequent in ET patients. Moreover, CD34$^+$ hematopoietic progenitors from ET patients harboring the type II *CALR* mutation are capable of forming spontaneous CFU-MK, validating the initial suggestions that the mutated *CALR* confers cytokine-independent cell growth.

Elegant molecular and biochemical studies dissecting the interaction between mutant *CALR* and *MPL* and its downstream consequences revealed that the N-glycosylation sites of the MPL extracellular domain are required for its activation by mutated *CALR* and are independent of the thrombopoietin binding site. Furthermore, the mutated *CALR* translocates to the cell surface and acts in an autocrine manner by binding and activating MPL. Recently, it has been postulated that the interaction between mutant CALR and MPL may occur in the endoplasmic reticulum. Although the exact mechanisms by which the mutated CALR binds to the MPL are under investigation, preliminary evidence suggests that the oncogenic properties of the mutated CALR can be attributed to the positive electrostatic charge of the novel peptide, which may be responsible not only for the physical interaction with MPL but also for other cellular functions involving the normally negatively charged portion of the protein

(Fig. 71.1). Mutated *CALR* can also affect the immune system. When CALR is present on the cell surface, it acts as an "eat me" signal that facilitates the recognition of stressed cells by phagocytes that express the CALR receptor, CD91. This type of innate immune response facilitates the transfer of tumor antigens to dendritic cells, thereby facilitating the induction of cancer-specific cytotoxic T cells. The mutated CALR proteins escape from the ER system and can be secreted into the tissue microenvironment. Liu et al.[3] have reported that soluble CALR proteins can, in fact, act as decoy receptors, preventing the uptake of CALR-expressing cells by dendritic cells, thereby leading to profound immunosuppression. *CALR* mutations may therefore play two roles in ET and MF (1) activating cell autonomous oncogenic drivers and (2) blunting the immune response. In fact, immune suppressive mechanisms, including T-cell exhaustion, have been reported in patients harboring mutant *CALR*, perhaps due to its effects on the dendritic cell activation reported above. This immune suppressive effect of CALR-mutated proteins has been proposed as the reason why *CALR*-mutated MPNs arise 10 years earlier than *JAK2*V617F+ MPNS. Mutant *CALR* has recently been proposed as an MPN-specific neoantigen that might serve as a target for immunotherapeutic approaches. CALR-specific T-cell responses have been partially restored in vitro and in vivo by antibodies directed against immune checkpoint inhibitors. The success of such vaccine strategies, however, might require the development of strategies directed towards restoring immune responses in patients by overcoming the effects of secreted mutated CALR.

The SH2B adaptor protein 3 (SH2B3) gene, also known as the *LNK* gene, encodes a negative regulator of cytokine signaling. Occasional cases of ET are associated with loss-of-function *LNK* (SH2B3) mutations. In mouse models, the inhibitory adaptor protein, LNK, has been shown to be associated with the downmodulation of erythropoietin and thrombopoietin signaling. LNK can bind to wild-type *JAK2*, *JAK2*V617F, wild-type *MPL*, and *MPL*W515L. Lnk levels are upregulated and they correlate with an increase in the *JAK2*V617F VAF in MPN patients. In *JAK2*V617F-positive ET, LNK mRNA expression is upregulated and serves to modulate *JAK2*V617F-mediated cell regulation. Thrombopoietin-mediated signaling regulates LNK expression at both the mRNA and protein levels. Furthermore, acquired *LNK* mutations have been observed in less than 1% of ET. The inactivating *LNK* mutations in ET patients result in JAK-STAT activation, leading to high levels of STAT3 and STAT5 activation. Cabagnois and coworkers used whole-exome sequencing and next-generation sequencing, targeting *JAK2* and *MPL*, with the intent of detecting additional mutations in triple negative ET patients. They found several signaling mutations, including *JAK2*V617F with very low VAFs, as well as additional mutations such as: *LNK* mutation, *MPL*-S505N, *MPL*-W515R, and *MPL*-S204P. *MPL*-S204P and *MPL*-Y591N were shown to be weak gain-of-function mutants, increasing MPL signaling and conferring either thrombopoietin hypersensitivity or independence to expressing cells, but with low efficiency. These data demonstrate that some clonal, noncanonical *MPL* gain-of-function mutations are associated with triple negative cases of ET. "Triple negative" ET patients have also been shown to harbor clonal markers involving other myeloid associated genes, including ten–eleven translocation (*TET2*) and *KIT*. In addition, a recent study showed that nearly a quarter of those ET patients diagnosed as "triple negative" actually harbored low-level *JAK2* or *MPL* mutations, detected using deep next-generation sequencing. The study also revealed that "true triple negative" ET patients and ET patients with known driver

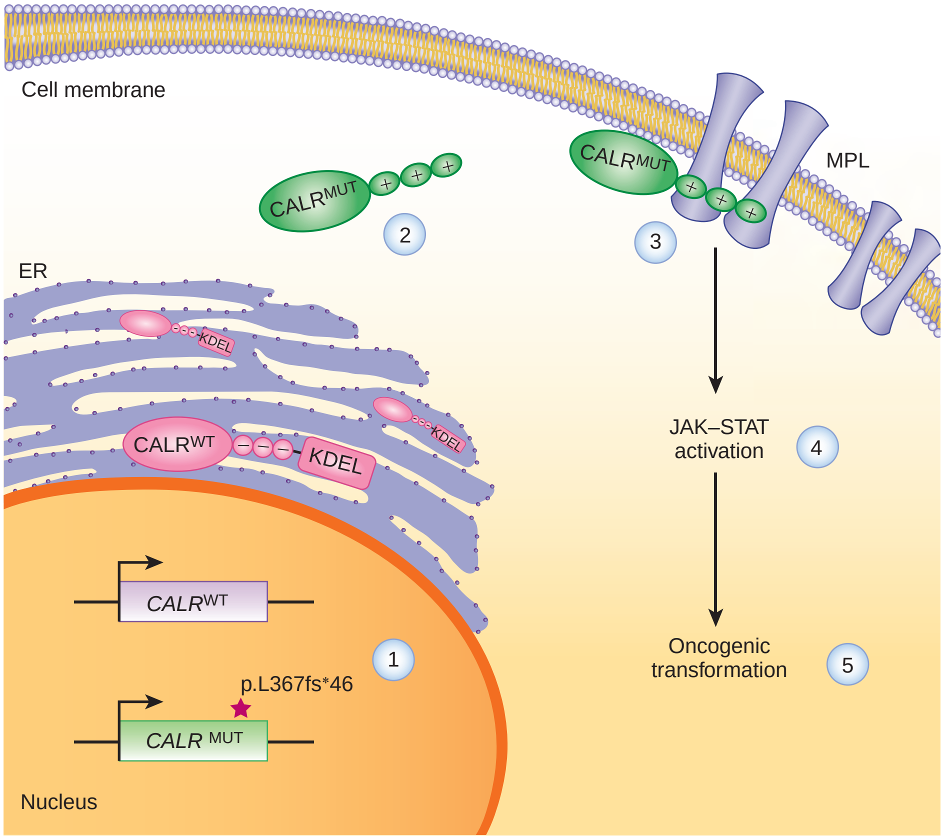


Figure 71.1 SCHEMATIC REPRESENTATION OF THE POTENTIAL MECHANISM OF ACTION OF MUTATED CALRETICULIN. Deletions (type I mutations) or insertions (type II mutations) occurring in the exon 9 of *calreticulin (CALR)* gene (1) result in a novel epitope in the C-terminus of the protein which lacks the endoplasmic reticulum (ER)–retention signal (KDEL) and is positively charged compared with the negatively charged wild-type protein (2). The mutated *CALR* is capable of binding thrombopoietin receptor (MPL) (3), which, in turn, activates the Janus-activated kinase–signal transducer and activator of transcription (JAK-STAT) signaling pathway (4), thus providing proliferative advantage of the malignant clone (5). *(From Stanley RF, Steidl U. Molecular mechanism of mutant CALR-mediated transformation. Cancer Discov. 2016;6(4):344–346.)*

mutations had similar gene expression profiles consistent with activation of the JAK/STAT and nuclear factor kappa B (NFkB) pathways.[4] Ultimately, patients with triple negative ET cannot always be classified as having an MPN and should be evaluated for inherited forms of thrombocytosis.

Mutations in epigenetic regulators (such as *TET2*, *DNMT3A*, *ASXL1*, *EZH2*, and isocitrate dehydrogenase [*IDH*]*1*/*IDH2*) and in spliceosome components (such as *SRSF2*, *U2AF1*, and *SF3B1*) have been shown to be present in MPN patients with *JAK2*/*MPL*/*CALR* mutations. Other mutations were also directly associated with leukemic progression, such as *TP53*, *RUNX1*, *CBL*, and deletion in *IKAROS*. Several of these mutations can occur in the same patient, and most frequently *SRSF2* can occur with *TET2*, *ASXL1*, or *IDH* mutations. In contrast to *MPN* driver mutations, which are rare in other myeloid malignancies, these additional mutations are not specific to MPNs and are found with a higher frequency in patients with myelodysplastic syndrome (MDS) and in MDS/MPN overlap syndromes, such as chronic myelomonocytic leukemia (see Chapter 73) as well as AML. Biologic studies and mouse models have shown that these additional mutations may cooperate with *MPN* driver mutations to favor clonal dominance (*TET2* or *DNMT3A*), to modify disease phenotype, or to promote either progression to MF or leukemic transformation (*ASXL1*, *IDH1/2*, *EZH2*, and *TP53*). Tefferi and coworkers have reported that the percentage of ET patients with 1, 2, or ≥3 sequence variants/mutations was 41%, 8%, and 4%, respectively, with the most frequent sequence variants/mutations in *TET2* and *ASXL1*. In addition, a significant association between the MPN driver mutational status and the number or type of other sequence variants/mutations in the Mayo Clinic series of 183 patients with ET was not observed. Especially important was their stratification of survival by the presence or absence of "adverse" (*SH2B3*, *IDH2*, *SF3B1*, *U2AF1*, *EZH2*, *TP53*) or "other" (*TET2*, *ASXL1*, *PTP11*, *SUZ12*, *ZRSR2*, *CBL*, *CEBPA*, *CSF3R*, *DNMT3A*, *SRSF2*, *FLT3*, *KIT*, *NRAS*, *RUNX1*, *SETBP1*) sequence variants/mutations. Those patients in a cohort of 174 Italian patients with ET, with adverse mutations, had a median survival of 18.4 years as compared to 29 years for those individuals who harbored other vatiants that were not high-risk.[5] A recent study evaluating the genomic profiles of ET and PV patients who undergo leukemic transformation revealed that *DNMT3A*, *U2AF1*, *IDH1*, *IDH2*, and *EZH2* mutations were associated with early transformation (median time to transformation 3 years) and *TP53*, *BCORL1*, and *NRAS* mutations were associated with late transformation (median time to transformation 21 years).[6]

The *TET2* gene encodes a hydroxylase that is able to hydroxylate methylated cytosine. These mutations result in a loss of function, leading to increased DNA methylation and a reduction in hydroxymethylcytosine. *TET2* mutations occur in 11% of ET patients. Mutations in *TET2* can occur either before or after mutations of *JAK2* or *MPL*. Mutations in the gene for IDH occur in 0.9% of ET patients and can functionally lead to similar effects as *TET2* mutations on DNA methylation. Furthermore, disrupting mutations of *ASXL1*, which occur in 36% of PMF patients, are infrequent in ET, as are mutations of *CBL* or *EZH2*.

Furthermore, the transcription factor NFE2 has been shown to be overexpressed in the cells of patients with MPNs independent of the presence or absence of *JAK2*V617F. The NFE2 acts as an epigenetic transcriptional regulator and a chromatin modifier. It is part of a complex regulatory network, including transcription factors such as GATA1 and RUNX1, controlling megakaryocytic and/or erythroid cell function. The knockout mouse model displays only mild erythroid abnormalities, while the major phenotype is a defect in megakaryocyte biogenesis. Indeed, the absence of NFE2 leads to severely impaired platelet production. Genetically engineered mice that overexpress NF-E2 are characterized by extreme thrombocytosis and leukocytosis, normal hemoglobin levels, and BM hypercellularity, which is a clinical picture similar to that observed in ET patients. Frameshift and deletion *NFE2* mutations are found in approximately 2% to 4% of ET patients and have been shown to be preferentially associated with *MPL* (30%) and *CALR* (40%) mutations.[7] While there was no direct correlation between *NF-E2* and *JAK2*V617F VAF in different MPNs, the frequency of *NFE2* mutations resulting in a functionally enhanced truncated form of *NF-E2* was doubled in patients with greater than 50% VAF (9.7% vs. 4.1%). Furthermore, Grinfeld and coworkers reported that the presence of *NF-E2* mutations was correlated with the transition of *JAK2*V617F+ ET patients to a PV phenotype. Mice expressing *NF-E2* mutations develop erythrocytosis and thrombocytosis due to the expansion of their progenitor and stem cell compartments, and in the long term may develop leukemias with concomitant and/or isolated myelosarcoma. Marcault and coworkers reported that MPN patients with *NF-E2* mutations with VAFs greater than 5% were at an increased risk of transformation to MPN-BP.

Thrombosis and Hemorrhage in Essential Thrombocythemia

The cause of the increased risk of developing hemorrhagic and thrombotic events, associated with ET, remains the subject of investigation. The following increase the risk of thrombotic complications in ET: age greater than 60 years, a history of a thrombotic event, the *JAK2*V617F mutation, leukocytosis (>11,0000/L), and the presence of cardiovascular risk factors, including tobacco use, hypertension, or diabetes mellitus. Hemorrhagic events occur almost exclusively in individuals with extremely high platelet counts ($>1000 \times 10^9$/L).[8] Extreme thrombocytosis is most common in young females. Although anxiety-provoking for many practicing hematologists, extreme thrombocytosis in an otherwise low-risk patient is not thought to be associated with a greater risk of developing thrombotic episodes. Thrombosis-free survival has been reported to be similar between patients presenting with or without extreme thrombocytosis and the development of arterial and venous thromboses were similar in patients with and without extreme thrombocytosis.

The incidence of thrombotic events in ET patients ranges from 1.5% to 6.6% per patient-year. Age-related differences in the frequency of thromboses have been attributed to the coexistence of vascular disease in older patients. The thrombotic events in ET are largely arterial, but venous thromboses also occur. Patients with *JAK2*V617F+ ET compared with those with a *CALR* mutation or those who are triple negative have a higher risk of developing thrombotic events, but a dose-dependent correlation between allele burden and the development of clinical symptoms has not been demonstrated. Recent data show that the *CALR*-type 2 mutation, but not the type 1 mutation, is protective against thrombosis when compared to *JAK2*V617F. Recently, Eaton and coworkers have provided data that indicate that JAK2 plays a role in primary hemostasis, affecting thrombus formation by facilitating platelet adhesion and spreading on collagen by altering collagen receptor GPV1 signaling. These data suggest that the enhanced risk of thrombosis in *JAK2*V617F+ ET patients might be a consequence, in part, of this continued enhanced platelet GPV1 signaling.

Many investigators have struggled to identify ET patients who are at the greatest risk of developing thrombotic episodes. A thrombotic scoring system has been developed by investigators on both sides of the Atlantic.[9] In the latest iteration of this risk stratification schema, four categories were delineated: very low-risk (no thrombosis history, age ≤60 years, and *JAK2*-unmutated); low-risk (no thrombosis history, age ≤60 years, and *JAK2*-mutated); intermediate-risk (no thrombosis history, age >60 years, and *JAK2*-unmutated) and high-risk (thrombosis history, age >60 years, and *JAK2* mutation). The use of aspirin in low-risk patients is debated and treatment of *CALR*-mutated ET patients with aspirin did not affect the risk of thrombosis but was associated with a higher incidence of bleeding.[10] How to personalize therapy based upon such a schema remains problematic and to date untested.[11]

Some progress has been made in defining the relationship between platelet numbers and the risks of thrombotic and hemorrhagic events in ET. In a randomized trial of patients at a high-risk of developing a thrombotic event (>60 years of age, a history of a thrombotic episode, or both), the reduction of platelet numbers was highly effective

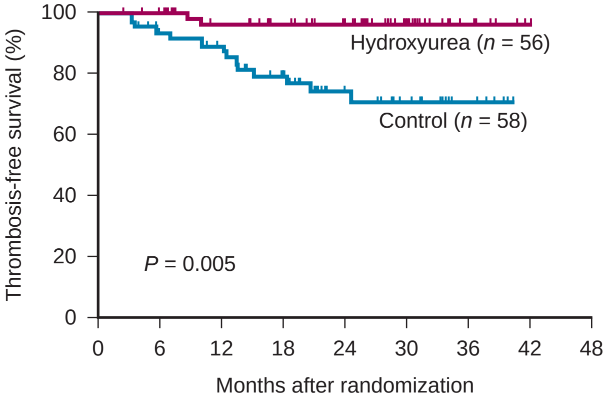


Figure 71.2 PROBABILITY OF THROMBOSIS-FREE SURVIVAL IN 114 PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA TREATED WITH HYDROXYUREA OR LEFT UNTREATED. *(From Cortelazzo S, Finazzi G, Ruggeri M, et al. Hydroxyurea for patients with essential thrombocythemia and a high risk of thrombosis. N Engl J Med. 1995;332:1132.)*

in preventing additional thrombotic events (Fig. 71.2). Furthermore, the incidence of thrombotic events has been shown to be closely correlated with the duration of thrombocytosis in a case–control study. Unfortunately, there has been difficulty in defining what particular target platelet count should serve as an endpoint for myelosuppressive therapies to maximally reduce the degree of thrombotic risk. Many investigators have provided data that indicate that interactions between platelets and activated leukocytes participate in the thrombotic risk of ET patients, suggesting to some that normalization of leukocyte numbers should also be a therapeutic endpoint.

Several groups have confirmed that the degree and duration of bleeding in ET patients is correlated with platelet numbers. The clinical spectrum of bleeding in ET patients closely resembles that observed in von Willebrand disease. Several groups have now shown that high platelet counts ($>1000 \times 10^9$/L) are associated with an acquired form of von Willebrand syndrome, and that reduction of platelet numbers is associated with correction of the von Willebrand syndrome-like abnormalities and cessation of bleeding episodes. The mean platelet count in patients with ET and acquired von Willebrand disease is $2050 \pm 1107 \times 10^9$/L. An increase in the number of circulating platelets appears to favor the adsorption of larger von Willebrand multimers onto platelet membranes, resulting in their removal from the circulation and their subsequent degradation.

Platelets in patients with ET have been known, for a considerable time, to be qualitatively abnormal. Although both increased and decreased platelet reactivity have been described, these findings have not been definitively associated with thrombohemorrhagic complications, with two noteworthy exceptions—erythromelalgia, in which the prompt relief of symptoms by cyclooxygenase inhibitors provides direct evidence that prostaglandins play a role in the development of vascular occlusion, and acquired von Willebrand syndrome, which is a major cause of bleeding in patients with ET.

Abnormal platelet aggregation has been reported in 35% to 100% of patients with ET. The majority of such studies have used conventional platelet aggregation studies performed on platelet-rich plasma. The simultaneous measurement of platelet aggregation and adenosine triphosphate–dense granule release by whole-blood platelet lumiaggregometry has been used to study platelet function in ET patients with the hope of identifying patients at risk of developing thrombosis. A prospective analysis of large cohorts of patients has not been performed to confirm the utility of such assays. Platelets derived from patients with ET and PV have been shown to contain a large proportion of immature platelets that have been recently released from the BM. Such immature platelets have increased hemostatic activity, as demonstrated by a heightened response to thrombin and greater expression of P-selectin. Hydroxyurea has been shown to be capable of reducing the number of immature platelets in ET, which might partly be responsible for the reduced thrombotic risk associated with its use.

Abnormal aggregation studies have not been successful in predicting the incidence of episodes of hemorrhage or thrombosis. In ET, platelet aggregation is classically defective in response to epinephrine, adenosine diphosphate (ADP), and collagen but is usually normal with arachidonic acid and ristocetin. Characteristically, in ET, the first wave of aggregation is diminished, and the second wave of aggregation is absent in response to epinephrine. Interestingly, preincubation of ET platelets with thrombopoietin partially corrects the impaired aggregation in response to epinephrine, ADP, and collagen. An acquired form of platelet storage pool disease occurs frequently in ET. Platelet α-granule content and the release of the content of granules are abnormal, resulting in elevated plasma levels of platelet factor-4 and β-thromboglobulin.

Numerous individual functional platelet abnormalities have been demonstrated. A defect in the metabolism of arachidonic acid by lipoxygenase has been documented, as have decreased numbers of platelet receptors for prostaglandin D_2 and adrenergic receptors for epinephrine. Platelets from patients with thrombotic episodes have been found to be capable of increased generation of thromboxanes and to have an increased affinity for fibrinogen. Elevations in β-thromboglobulin and serum thromboxane levels in ET patients are validated indices of enhanced in vivo platelet activation and possibly thrombin generation. Such abnormalities are not present in patients with secondary thrombocytosis and may provide some explanation for the high incidence of thrombosis associated with ET. The antithrombotic activity of aspirin has been attributed to its ability to permanently and selectively inactivate platelet cyclooxygenase-1 (COX-1) and thereby block thromboxane biosynthesis. This action has served as the rationale for the widespread use of aspirin in ET patients for thrombosis prophylaxis in the absence of valid controlled clinical trials. COX-2 has been shown to be overexpressed by the megakaryocytes and platelets of ET patients; it can also contribute to the enhanced biosynthesis of thromboxanes in ET. Low-dose aspirin can only partially correct this enhanced thromboxane synthesis, likely because of the increased COX-2 activity associated with the increased rate of platelet generation associated with ET, providing the rationale for the use of more aggressive aspirin scheduling or the addition of thromboxane receptor A_2 antagonists to reduce the incidence of thrombotic events. Although laboratory data indicate a rationale for twice-daily dosing of aspirin, prospective clinical data for this approach are lacking.

The platelet activation observed in ET has also been linked to Src, which is a nonreceptor tyrosine kinase particularly abundant in platelets. In thrombin-stimulated platelets, Src kinase is required for platelet aggregation. This preactivation of Src that is characteristic of ET and the related platelet hyperactivity is likely to account for the hypercoagulable state that is emblematic of ET. In vitro studies also implicate *JAK2*V617F-driven heparinase overexpression in ET as a novel thrombotic mechanism. Heparinase has previously been shown to complex and enhance the activity of tissue factor, resulting in activation of the coagulation cascade.

Microvascular thrombosis causing digital or central nervous system ischemia leads to a variety of clinical syndromes closely associated with ET. The survival of platelets in ET patients with erythromelalgia and thrombosis has been shown to be reduced. Thrombosis in this setting is associated with an increased platelet turnover. Treatment of erythromelalgia with aspirin increased mean platelet survival from 4.0 ± 0.3 days to 6.9 ± 0.4 days and was associated with a significant elevation of platelet numbers. These findings suggest that erythromelalgia results from platelet-mediated thrombosis of the arterial microvasculature of the extremities. Complete correction of this ischemic circulatory defect is associated with the use of platelet cyclooxygenase inhibitors, such as aspirin and indomethacin. Agents that do not inhibit platelet cyclooxygenase, such as coumadin, sodium salicylate, dipyridamole, sulfinpyrazone, and ticlopidine, are not active in the treatment of this disorder.

One intriguing explanation for the increased risk of thrombosis in patients with ET has been the observation that the total amount of thrombin generated on the platelet surfaces of patients with ET is markedly greater than that generated on the platelet surfaces of normal control participants or patients with reactive thrombocytosis. The molecular basis of this abnormality has not been defined. Increased numbers of platelet microparticles, as well as increased platelet–neutrophil and platelet–monocyte complexes, have been observed in patients with ET. Platelet microparticles support thrombin generation and leukocyte activation. Increased numbers of platelet microparticles have been associated with the development of vascular thrombosis.

In vivo leukocyte activation has been shown to occur in ET and to be associated with signs of activation of both the coagulation cascade and endothelial cells. Such platelet and leukocyte activation may play a role in the generation of the prethrombotic state that characterizes ET. Interestingly, the presence of the *JAK2* mutation is associated with a greater degree of platelet and leukocyte activation. Activated neutrophils are able to bind platelets, which triggers the expression of tissue factor, as well as endothelial cell activation and damage. From a clinical point of view, several studies have demonstrated that an increased leukocyte count (>11,000 × 10^9) in patients with ET is an independent risk factor for developing arterial thrombosis, especially myocardial infarction, and is associated with an inferior survival rate. Therefore, an important role for leukocytes in the pathogenesis of thrombosis in ET is becoming more evident. Direct involvement of endothelial cells of MPN patients by *JAK2*V617F has been reported by several groups, which might lead to endothelial dysfunction in subsets of MPN patients, further enhancing the thrombotic predisposition to the development of splanchnic vein thromboses.

CLINICAL MANIFESTATIONS

The presenting symptoms of patients with ET are quite variable. Many patients (12% to 67%) reach medical attention fortuitously as a result of the extreme degree of thrombocytosis detected when obtaining a routine blood cell count. Most patients present with symptoms related to small- or large-vessel thrombosis, or minor bleeding. The thrombotic events at diagnosis and during follow-up occurred at rates of 10% to 29% and 8% to 31%, respectively. In general, arterial events predominate over venous events. Presentation with a major bleeding episode is unusual. Neurologic complications are common. Table 71.1 lists representative neurologic complaints, of which headache was the most common, with paresthesias of the extremities a close second. There was an extremely high incidence of transient ischemic attacks involving both the anterior and posterior cerebral circulation. These attacks have a sudden onset, last for a few moments, and are frequently associated with a pulsatile headache. The various symptoms occur sequentially rather than simultaneously and can be preceded or followed by erythromelalgia. Transient neurologic symptoms include unsteadiness, dysarthria, dysphoria, motor hemiparesis, scintillating scotomas, amaurosis fugax, vertigo, dizziness, migraine-like symptoms, syncope, and seizures. The syndrome is caused by platelet-mediated ischemia and thrombosis in end-arterial microvasculature. It is not unusual for these symptoms to eventually progress to definitive cerebral infarcts.

TABLE 71.1 Frequency of Neurologic Complaints Associated With Essential Thrombocythemia

Manifestations	Patients *(n)*
Headache	13
Paresthesias	10
Posterior cerebral circulatory ischemia	9
Anterior cerebral circulatory ischemia	6
Visual disturbances	6
Epileptic seizures	2
Total number of patients	33

Data from Jabaily J, Iland HJ, Laszlo J, et al. Neurologic manifestations of essential thrombocythemia. *Ann Intern Med.* 1983;99(4):513–518.

Microvascular circulatory insufficiency involving the toes and fingers is frequent. Such events can lead to digital pain, enhanced by warmth; distal-extremity gangrene; and classic erythromelalgia. The term *erythromelalgia* refers to a syndrome of redness and burning pain in the extremities. Erythromelalgia, which is characterized by a burning pain and a dusky congestion of swollen extremities, is usually preceded by paresthesias. Cold provides relief from these symptoms, while heat intensifies them. Patients prefer to wear shoes or slippers without socks and elevate their feet. These symptoms may progress in intensity and lead to the peeling of skin in the affected appendages or affected toes or fingers, which then may become cold and ischemic, with a dark purplish tinge. Erythromelalgia symptoms are asymmetric in the majority of cases. Symptoms related to coronary artery disease or transient ischemic attacks may precede or accompany the onset of erythromelalgia. Occasionally, hemorrhagic episodes may occur in patients experiencing erythromelalgia. Platelet counts in patients with erythromelalgia are frequently below 1000 × 10^9/L. The relief of such pain for several days after a single dose of aspirin is diagnostic of erythromelalgia. The specific microvascular syndrome of erythromelalgia is readily explained by platelet-mediated arteriolar inflammation and occlusive thrombosis, leading to acrocyanosis and even gangrene. Skin biopsies from the affected sites reveal arteriolar lesions without involvement of venules, capillaries, or nerves. The arteriolar endothelial cells are swollen and the vessel walls thickened by cellular swelling and deposition of intracellular material. Compared with atherosclerotic circulatory obstruction, arterial pulses in patients with erythromelalgia remain normal. Other patients develop platelet-mediated acral inflammation and arterial thrombosis, which can progress to ischemic acrocyanosis or necrosis of fingers, toes, and, rarely, the tip of the nose (Fig. 71.3).

Although thrombosis of the microvasculature is generally more frequent, thrombosis of large veins and arteries in patients with ET still occurs commonly. Patient symptoms frequently occur related to large-vessel thrombosis, mostly in the arteries of the legs, the coronary arteries, and the renal arteries. Involvement of the carotid, mesenteric, and subclavian arteries is frequent, and patients characteristically have venous thromboses involving the splenic vein, hepatic veins, or veins of the legs and pelvis. Unexplained thrombosis of the hepatic veins leads to Budd-Chiari syndrome, and thrombosis of the renal vein can result in the development of nephrotic syndrome. Splanchnic vein thromboses occur predominantly in young women with ET. Patients with this complication are at a high risk of having poor survival because of hepatic failure or transformation to MF or acute leukemia. It is worth noting that a fraction of patients with idiopathic splanchnic vein thrombosis may present with normal or near-normal blood counts but have an occult MPN based on genetic or

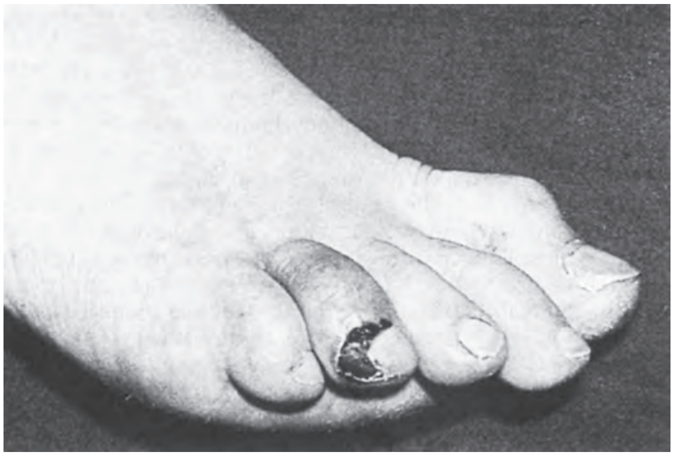


Figure 71.3 GANGRENE OF THE TOE IN A PATIENT WITH ESSENTIAL THROMBOCYTHEMIA.

histopathologic abnormalities. In a review of more than 800 patients with splanchnic vein thrombosis without a diagnosis of an MPN, a mean of 32.7% of patients were *JAK2*V617F-positive. More than 50% of these patients were subsequently diagnosed with an MPN. In addition, 2.6% of patients with cerebral sinus and vein thrombosis were also shown to be *JAK2*V617F-positive. These studies suggest that all cases of splanchnic vein thrombosis, likely cerebral sinus, and vein thrombosis should be tested for *JAK2*V617F to identify patients with an occult MPN. Occasional patients with idiopathic splanchnic vein thromboses with *CALR* mutations have been reported. In those patients who are *JAK2*V617F-negative, it is prudent to also search for *CALR* mutations. Priapism is a rare complication of ET, presumably caused by platelet sludging in the corpus cavernosum. In addition, myocardial ischemia and infarction associated with normal coronary angiograms have been reported in patients with ET, as has a high incidence of anginal symptoms. Acute renal failure has been observed after thrombosis of renal arteries and veins in patients with ET. Pulmonary hypertension, secondary to alveolar capillary plugging by platelets and megakaryocytes, has also been reported in patients with ET, with one meta-analysis reporting approximately 20% of ET patients having pulmonary hypertension.

Hemorrhagic events occur in 3% to 18% of patients with ET; the primary sites of bleeding are the gastrointestinal tract, mouth, nose, and throat. Other sites of bleeding may be the skin, eyes, urinary tract, tooth sockets (after extraction), joints, or brain. Nicol and coworkers reported that major bleeding episodes were relatively rare, ranging from 0.43 to 5.3 per 100 patient events. Central nervous system bleeds were the most frequent cause of fatal bleeds. A high incidence of bleeding episodes during the immediate postoperative period, including dental extractions, is likely caused by postsurgical thrombocytosis and the development of acquired von Willebrand syndrome or the use of antithrombotic prophylaxis therapy. Bleeding is closely correlated with a significant increase in platelet counts in excess of 1500 × 10^9/L and is associated with pseudohyperkalemia. It is important to emphasize that individual patients can experience both thrombotic and hemorrhagic episodes.

Appreciation of the risk of developing thrombohemorrhagic events in asymptomatic patients with ET who are younger than 40 years of age is imprecise at best. Such patients are thought to be at a low risk of developing thrombotic episodes, unless they have experienced a prior thrombotic episode or have associated cardiovascular risk factors. The most common thrombotic complications include migraine headaches in 20% of the patients and erythromelalgia in 5% of the patients. Life-threatening hemorrhagic episodes are rare. The degree of leukocytosis has been suggested by some investigators to be useful in discriminating between young patients with a low or high risk of developing a thrombotic episode.

A meta-analysis has revealed that the *JAK2*V617 mutation is associated with a twofold higher risk of developing either a venous or arterial thrombosis but does not influence the risk of suffering from a hemorrhagic event. Regardless, it is conceivable that the significantly more advanced age and elevated hematocrit and leukocyte levels in mutation-positive patients might contribute to the apparent association between *JAK2*V617F and thrombosis reported in some studies.

Patients with ET who are older than 60 years of age and who have had a prior thrombotic event have a greater risk of developing additional thrombotic events (Table 71.2). By contrast, the incidence of thrombotic and hemorrhagic complications in asymptomatic patients with ET who are younger than 60 years of age and who have platelet counts of less than 1500 × 10^9/L has been shown to be comparable to that of a normal control population. Gender, hypertension, diabetes mellitus, hypercholesterolemia, and smoking have been shown to be independent risk factors for developing arterial thrombotic complications in ET (see Table 71.2). Screening of patients for other acquired and inherited thrombophilic states may identify patients at an even higher risk for both arterial and venous thrombotic events.

Pregnancy is not contraindicated in patients with ET. The outcome of pregnancy in patients with ET has been the subject of intense investigation. The rate of having a successful pregnancy is 61%, compared with an 85% to 90% rate in normal women. The rate of spontaneous abortions ranges from 39% to 44%, compared with the miscarriage rate of 10% to 15% in normal pregnancies. Placental infarction is often responsible for intrauterine fetal growth retardation (5%). Abruptio placenta has been reported in 3.6% of cases, a rate that is higher than that observed in the general population (1%). Major thrombotic episodes occur in 3% of these pregnancies, while major bleeding episodes occur in 2% of cases. These rates are higher than those observed in the overall population of pregnant women. Baseline platelet count is not predictive of pregnancy outcome. ET patients with the *JAK2*V617F mutation have been reported to be at a higher risk of developing complications with pregnancy. In the postpartum period, the platelet counts return to their earlier levels, and rebound thrombocytosis may occur in some patients. This is thought to increase the probability of vascular complications during this period to a level similar to that observed in other conditions of thrombophilia.

TABLE 71.2 **Revised-IPSET Risk Stratification for Thrombotic Risk in ET Patients**

Risk Category	Age >60 Years	History of Thrombosis	*JAK2V617*-Positive
Very low	No	No	No
Low	No	No	Yes
Intermediate	Yes	No	Yes
High	Yes[a]	Yes[a]	Yes

[a]Either age >60 years or history of thrombosis.
ET, Essential thrombocythemia; *IPSET*, international prognostic score for essential thrombocythemia.
Data from Barbui T, Vannucchi AM, Buxhofer-Ausch V, et al. Practice-relevant revision of IPSET-thrombosis based on 1019 patients with WHO-defined essential thrombocythemia. *Blood Cancer J*. 2015;5(11):e369.

In the large majority of cases, the fetal losses in pregnant ET women occur during the first trimester. A previous history of spontaneous abortion may be the greatest risk factor for the development of subsequent spontaneous abortions.

Physical examination findings are relatively unremarkable in patients with ET. Most patients are not severely ill at diagnosis, with a median Karnofsky score of 90% being reported in one series. Splenomegaly is detectable in 40% to 50% of patients, and approximately 20% have hepatomegaly. During the course of the disorder, a further increase in the degree of hepatosplenomegaly may be observed in patients who are developing post-ET MF. Skin manifestations of the MPN have been reported to be relatively common, ranging from paraneoplastic lesions, including vascular, neutrophilic plaques, and unexplained dermatoses. Rarely, vascular changes result in microcirculatory flow abnormalities, leading to a vasculitis resulting in classic purpura that may progress to skin necrosis. This should be distinguished from leg ulcers that occur in patients being treated with hydroxyurea. Occasionally, patients also develop pyoderma gangrenosum or Sweet syndrome.

LABORATORY MANIFESTATIONS

The hallmark of ET is a sustained and unexplained elevation of the platelet count (≥450 × 10^9/L). Accompanying leukocytosis is a common finding. A leukoerythroblastic blood picture, as well as teardrop-shaped RBCs, are not features of ET but are suggestive of an early form of MF. Mild eosinophilia (>400/mm^3) and basophilia (>100/mm^3) have been reported in more than one-third of patients.

The most common morphologic abnormalities are variations in RBC size and shape and the presence of megathrombocytes (Fig. 71.4). The BM is usually normocellular or slightly hypercellular without

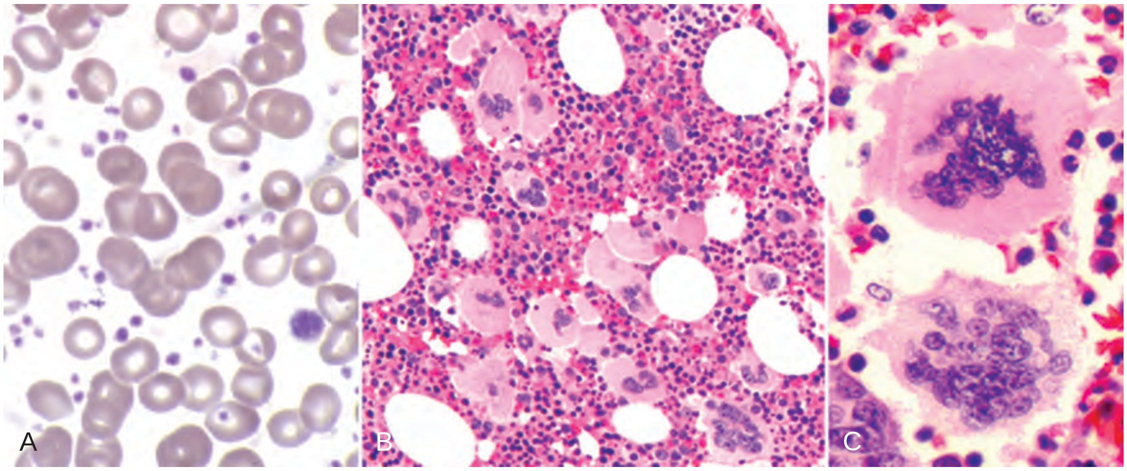


Figure 71.4 ESSENTIAL THROMBOCYTHEMIA: PERIPHERAL BLOOD SMEAR AND BONE MARROW BIOPSY. The peripheral blood smear in essential thrombocythemia (ET) shows a marked thrombocytosis with anisocytosis (varying sizes) of the platelets (A). The bone marrow (B) is hypercellular and exhibits a marked proliferation of large and giant megakaryocytes in loose clusters with other hematopoietic elements in the background. The large megakaryocytes (C) tend to be extensively lobulated.

a significant increase in granulopoiesis or erythropoiesis. Increased numbers of enlarged megakaryocytes with hyperlobulated or deeply folded nuclei that cluster in small groups along sinuses are the hallmarks of ET (see Fig. 71.4). Reticulin fibrosis is not significantly evident. A great deal of controversy currently surrounds distinguishing "true ET" from an early prefibrotic form of MF (pre-PMF), in which the BM is characteristically hypercellular with pronounced proliferation of granulocytes and reduced erythroid precursors. The megakaryocytes are increased in number but are loosely clustered or located along the endosteal bone surface. The megakaryocytes contain hyperchromatic, hypolobulated bulbous, or irregularly folded nuclei with an abnormal nuclear-to-cytoplasmic ratio. The histopathologic criteria for this form of pre-PMF have been combined with clinical criteria (minor criteria) by the World Health Organization (WHO). The 2016 criteria for the diagnosis of ET and pre-MF are particularly useful (Table 71.3). These new criteria are heavily dependent on mutational analyses. The *JAK2*V617F mutation occurs in 50% to 60% of ET patients, while recurrent mutations in *CALR* mutations occur in 25% of patients, and 3% to 5% have *MPL* mutations. Approximately 25% of patients harbor mutated *CALR* as either a type I (52-bp del) or type II (5-bp insertion). Investigators have claimed in a series which requires validation in a larger sample size that each driver mutation was associated with a specific morphological pattern in the BM.[12]

The patients with ET who lack such driver mutations are said to be "triple negative" (see Table 71.3). Based on the 2016 WHO Diagnostic Criteria, the presence of modest BM reticulin fibrosis does not exclude the diagnosis of ET. Although select hematopathologists can reproducibly distinguish ET from pre-PMF, it remains uncertain whether this distinction is broadly applicable. In 70% to 80% of ET patients, iron stores were present in the BM, albeit at reduced levels. Almost all patients have normal serum ferritin levels. The absence of iron stores in 30% of patients may merely be an epiphenomenon of a chronic MPN and not truly reflective of an iron deficiency state. Platelet aggregation study results are frequently abnormal and most often demonstrate an impaired aggregation response to epinephrine, ADP, and collagen but not to arachidonic acid and ristocetin. Spontaneous platelet aggregation has been reported to occur frequently in such patients, but this has not been a universal finding.

Approximately 25% of patients with ET have been reported to have elevated uric acid levels at diagnosis. The average value of the serum potassium at diagnosis is usually within the normal range, although 23% of patients have been reported to have pseudohyperkalemia, caused by the degranulation of platelets when in vitro clotting releases potassium. Both excessive numbers of RBCs and leukocytes can also be associated with these phenomena. Pseudohyperkalemia can be documented by measuring plasma instead of serum potassium levels and the lack of electrocardiographic findings associated with true hyperkalemia. Pseudohypoxemia has also been observed in ET patients with extreme degrees of thrombocytosis. Acquired von Willebrand syndrome is associated almost uniformly with a platelet count greater than 1500×10^9/L, a prolonged bleeding time, normal factor VIII coagulant activity, and a normal von Willebrand antigen level but decreased von Willebrand factor–ristocetin cofactor activity and collagen binding activity, as well as a decrease or absence of large von Willebrand factor multimers, simulating a type II von Willebrand disorder. The serum vitamin B_{12} level can be increased in 25% of cases.

In ET, BM karyotypes are characteristically normal. The absence of the Philadelphia chromosome and the *BCR-ABL* rearrangement excludes the diagnosis of CML. Studies to exclude CML as a cause of isolated thrombocytosis should be uniformly pursued in order to be certain that patients with CML presenting with isolated thrombocytosis are identified and receive appropriate therapy with tyrosine kinase inhibitors. Unfortunately, we have seen several such cases where CML was not excluded, which led to the implementation of inappropriate therapeutic strategies. Rare patients share two molecular markers of these two MPNs (*JAK2*V617F and *BCR-ABL*), but the CML phenotype predominates. Aneuploidy is seen in the minority of cases at diagnosis. In fact, ET is associated with a definite chromosomal abnormality in only 7.8% of cases, but with disease progression, there is an increased risk of aneuploidy. Specific chromosome abnormalities associated with ET have not been described, but abnormalities observed in PV and PMF, as well as in MDS, such as trisomy 1q, deletions of 5q, 13q, and 20q, trisomy 8, as well as monosomal karyotypes, have been reported. Many nonspecific chromosomal rearrangements have also been observed, such as a complex karyotype, the presence of a marker chromosome, and unusual translocations. More sensitive cytogenetic studies (see Chapter 57) in ET have not been performed extensively. In one reported study, the addition of fluorescence in situ hybridization increased detection of chromosomally abnormal clones by 15%. However, in 21 patients studied with oligoarray comparative genomic hybridization, one additional patient with deletion 13q was identified compared with conventional cytogenetics. Leukemic transformation of ET is characterized by the development of an abnormal karyotype in 60% to 100% of patients. Consistent chromosomal abnormalities associated with ET progression in *JAK2*V617F-positive patients are der(1)t(1;9) and der(1;7)(q10;p10). Of the eight reported ET patients with der(1;7), five received previous chemotherapy (chlorambucil, hydroxyurea,

TABLE 71.3 2016 Revised WHO Criteria for the Diagnosis of Essential Thrombocythemia, Pre-Fibrotic Form of PMF, and Overt PMF, and British Committee for Standards in Hematology Criteria for Diagnosis of Essential Thrombocythemia

WHO Essential Thrombocythemia Criteria

Major Criteria

1. Platelet count $\geq 450 \times 10^9$/L
2. BM biopsy showing proliferation mainly of the megakaryocyte lineage with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei. No significant increase or left shift in neutrophil granulopoiesis or erythropoiesis and very rarely minor (grade 1) increase in reticulin fibers
3. Not meeting WHO criteria for *BCR-ABL1*$^+$ CML, PV, PMF, myelodysplastic syndromes, or other myeloid neoplasms
4. Presence of *JAK2*, *CALR*, or *MPL* mutation

Minor Criteria

Presence of a clonal marker or absence of evidence for reactive thrombocytosis

Diagnosis of ET requires meeting all four major criteria, or the first three major criteria and the minor criterion

WHO Criteria for Pre-Fibrotic Form of PMF

Major Criteria

1. Megakaryocytic proliferation and atypia, without reticulin fibrosis >grade 1,[a] accompanied by increased age-adjusted BM cellularity, granulocytic proliferation, and often decreased erythropoiesis
2. Not meeting the WHO criteria for *BCR-ABL1*$^+$ CML, PV, ET, myelodysplastic syndromes, or other myeloid neoplasms
3. Presence of *JAK2*, *CALR*, or *MPL* mutation, or in the absence of these mutations, presence of another clonal marker,[b] or absence of minor reactive BM reticulin fibrosis[c]

Minor Criteria

Presence of at least one of the following, confirmed in two consecutive determinations:

a. Anemia not attributed to a comorbid condition
b. Leukocytosis $\geq 11 \times 10^9$/L.
c. Palpable splenomegaly
d. LDH increased to above upper normal limit of institutional reference range

Diagnosis of pre-PMF requires meeting all three major criteria, and at least one minor criterion

[a] See Table 71.8.

[b] In the absence of any of the three major clonal mutations, the search for the most frequent accompanying mutations (e.g., *ASXL1*, *EZH2*, *TET2*, *IDH1*/*IDH2*, *SRSF2*, *SF3B1*) is of help in determining the clonal nature of the disease.

[c] Minor (grade 1) reticulin fibrosis secondary to infection, autoimmune disorder or other chronic inflammatory conditions, hairy cell leukemia or other lymphoid neoplasm, metastatic malignancy, or toxic (chronic) myelopathies.

WHO Criteria for Overt PMF

Major Criteria

1. Presence of megakaryocytic proliferation and atypia, accompanied by either reticulin and/or collagen fibrosis grades 2 or 3[a]
2. Not meeting WHO criteria for ET, PV, *BCR-ABL1*$^+$ CML, myelodysplastic syndromes, or other myeloid neoplasms
3. Presence of *JAK2*, *CALR*, or *MPL* mutation or in the absence of these mutations, presence of another clonal marker,[b] or absence of reactive myelofibrosis[c]

Minor Criteria

Presence of at least one of the following, confirmed in two consecutive determinations:

a. Anemia not attributed to a comorbid condition
b. Leukocytosis $\geq 11 \times 10^9$/L
c. Palpable splenomegaly
d. LDH increased to above upper normal limit of institutional reference range
e. Leukoerythroblastosis

Diagnosis of overt PMF requires meeting all three major criteria, and at least one minor criterion

[a] See Table 69.8.

[b] In the absence of any of the three major clonal mutations, the search for the most frequent accompanying mutations (e.g., *ASXL1*, *EZH2*, *TET2*, *IDH1*/*IDH2*, *SRSF2*, and *SF3B1*) are of help in determining the clonal nature of the disease.

[c] BM fibrosis secondary to infection, autoimmune disorder, or other chronic inflammatory conditions, hairy cell leukemia or other lymphoid neoplasm, metastatic malignancy, or toxic (chronic) myelopathies.

British Committee for Standards in Hematology Criteria for Diagnosis of Essential Thrombocythemia

Requires A1–A3 or A1 + A3–A5:
A1: Sustained platelet count $>450 \times 10^9$/L
A2: Presence of an acquired pathogenic mutation (e.g., in *JAK2* or MPL)
A3: No other myeloid malignancy, especially PV, PMF, CML, or MDS
A4: No reactive cause for thrombocytosis and normal iron stores
A5: BM aspirate and trephine biopsy showing increased megakaryocyte numbers displaying a spectrum of morphology with predominant large megakaryocytes with hyperlobated nuclei and abundant cytoplasm

BM, Bone marrow; *CML*, chronic myeloid leukemia; *ET*, essential thrombocythemia; *JAK2*, Janus kinase 2; *MDS*, myelodysplastic syndrome; *MPL*, thrombopoietin receptor; *PMF*, primary myelofibrosis; *PV*, polycythemia vera; *WHO*, World Health Organization.

From Barbui T, Thiele J, Gisslinger H, et. al. The 2016 WHO classification and diagnostic criteria for myeloproliferative neoplasms: document summary and in-depth discussion. *Blood Cancer J*. 2018;8:15.

pipobroman, melphalan, cytosine arabinoside, anthracycline), and six transformed into acute leukemia. Although der(1)t(1;9) is a consistent nonrandom rearrangement associated with de novo PV and with PV progression, four ET patients had der(1)t(1;9) with different breakpoints (p13;p13) and (q10;p10). In ET, similar to PV, PMF, and other myeloid disorders, formation of trisomy 1q alone or subsequent formation of jumping +1q translocations should be considered as a clonal marker associated with disease progression or transformation to AML. As has been previously mentioned, approximately 50% of ET patients are *JAK2*V617F-positive, with only 4% having a high allele burden. Approximately 3% to 5% of patients have activating *MPL* mutations. Approximately 25% of patients harbor mutated *CALR* as either a type I (52-bp deletion) or type II (5-bp insertion).

Recent studies have indicated that there is cooperation between MPN driver gene mutations and other acquired somatic mutations within the hematopoietic clone. The effect of this cooperation on cellular phenotype is dependent on the type of nondriver mutation present. For instance, mutations in *TET2* or *DNMT3A* are associated with increasing dominance of the MPN clone and mutations in *ASXL1*, *IDH1/2*, *EZH2* or *TP53* are associated with transformation to MF or MPN-BP. The prevalence of mutations in *TET2* or *DNMT3A* is similar across all forms of MPNs (~12% in ET and 18% in PMF), whereas all other nondriver mutations have a higher prevalence in PMF than ET. For instance, splicing factor and epigenetic modifier mutations are present in more than 80% of PMF patients but less than 25% of ET patients. Approximately half of PMF cases have two or more mutations in nondriver genes. Therefore, the type and number of mutations in nondriver genes can be helpful in discriminating between ET and early forms of MF. It should also be noted that the sequence of acquisition of mutations can differ based on the driver mutation in ET. When the *CALR* mutation is the driver, this is almost always the initial mutation in the clone, whereas when *JAK2*V617F is the driver mutation, it can be the initial mutation or preceded by mutations in *DNMT3A*, *TET2*, and *ASXL1*.

TABLE 71.4 **Conditions Associated With Thrombocytosis**

Primary Thrombocytosis
Malignancies
Essential thrombocythemia
Polycythemia vera
Primary myelofibrosis
Chronic myeloid leukemia
Refractory anemia with ringed sideroblasts and thrombocytosis
Chronic myelomonocytic leukemia
MDS/MPN overlap
Familial thrombocythemia (inherited mutations in thrombopoietin or thrombopoietin receptor)
Secondary (Reactive) Thrombocytosis
Blood loss or iron deficiency
Infection
Inflammation
Disseminated malignancy
Hemolysis
Drug therapy
Hyposplenism
Cytokine administration
Spurious Thrombocytosis
Schistocytes
Cytoplasmic fragmentation of neoplastic cells
Cryoglobulinemia
Bacteria

MDS, Myelodysplastic syndrome; *MPN*, myeloproliferative neoplasm.

DIFFERENTIAL DIAGNOSIS

There are numerous causes of primary, secondary, and spurious forms of thrombocytosis. A listing and description of the conditions that can lead to thrombocytosis are provided in Table 71.4 and Fig. 71.5. Primary thrombocytosis includes both acquired and hereditary forms. In acquired forms of primary thrombocytosis, the genetic abnormalities are present exclusively in hematopoietic cells, but in the hereditary forms, these underlying defects can be detected in both somatic and germline cells and are inherited. The discovery of *JAK2*, *MPL*, and *CALR* mutations has provided definitive diagnostic tools with which to diagnose the MPNs, including ET. These mutations, however, can be found only in approximately 75% of ET patients. Hence, it is reasonable to consider *JAK2*V617F analysis and search for a mutation in *CALR* or *MPL* when evaluating patients with otherwise unexplained thrombocytosis. A positive test result indicates an underlying MPN. However, further investigations, including a BM biopsy (see Table 71.3) as well as cytogenetic analyses, are still required to differentiate ET from the other chronic MPNs, as well as from MDS presenting with thrombocytosis, such as the 5q syndrome and refractory anemia with ring sideroblasts and thrombocytosis (RARS-T; see Table 71.3) and pre-PMF.

For those patients who present with thrombocytosis and who are triple negative for driver mutations, the first step in determining the cause of thrombocytosis is to exclude reactive forms of thrombocytosis. The causes of secondary or reactive forms of thrombocytosis are numerous, but the most common causes are infection, inflammation, hemolysis, severe exercise, malignancy, hyposplenism, and other causes of an acute-phase response (see Table 71.4). In a hospital population, patients with extreme thrombocytosis (>1000 × 10^9/L) are not particularly rare in adult or pediatric patient populations. Examination of the blood smear is important to avoid confusion with so-called *pseudothrombocytosis*. This occurs in a number of conditions in which platelet-sized particles of red or white blood cell fragments (CLL, TTP, hemoglobin H disease), schistocytes, microspherocytosis, and cryoglobulinemia are erroneously enumerated as platelets by automatic particle counters. Confirmation of increased numbers of platelets by examination of the peripheral smear will avoid misdiagnosis and unnecessary clinical evaluation.

Reactive thrombocytosis accounts for more than 88% to 97% of cases, whereas thrombocytosis caused by an MPN accounts for only a minority of cases. Reactive thrombocytosis is more common in all age groups except those in their eighth decade and older. Thrombotic and hemorrhagic events infrequently occur in patients with reactive thrombocytosis. These findings are in contrast to the enhanced risk of these two complications in patients with ET. This relatively high frequency of extreme thrombocytosis in an acute-care hospital emphasizes the need for caution in making a diagnosis of ET (Table 71.5). A number of groups have shown that reactive thrombocytosis may be a consequence of the elaboration of known cytokines in response to the underlying inflammatory or neoplastic disorder and is accompanied by an elevated erythrocyte sedimentation rate or a high C-reactive protein (CRP). Elevated levels of IL-1, IL-6, GM-CSF, G-CSF, and thrombopoietin have been detected in such patient populations and frequently in individuals with thrombocytosis caused by an underlying MPN. Elevation of thrombopoietin levels has not only been found in patients with reactive thrombocytosis but also in patients with ET. IL-6–induced thrombocytosis is mediated in part by secondary thrombopoietin production by the liver in inflammatory disorders and malignant diseases. CRP is an acute-phase reactant, the hepatic synthesis of which is mediated by IL-6. CRP levels are high in patients with high levels of IL-6. In one series, whereas 81% of patients with reactive thrombocytosis had elevated IL-6 or CRP levels, patients

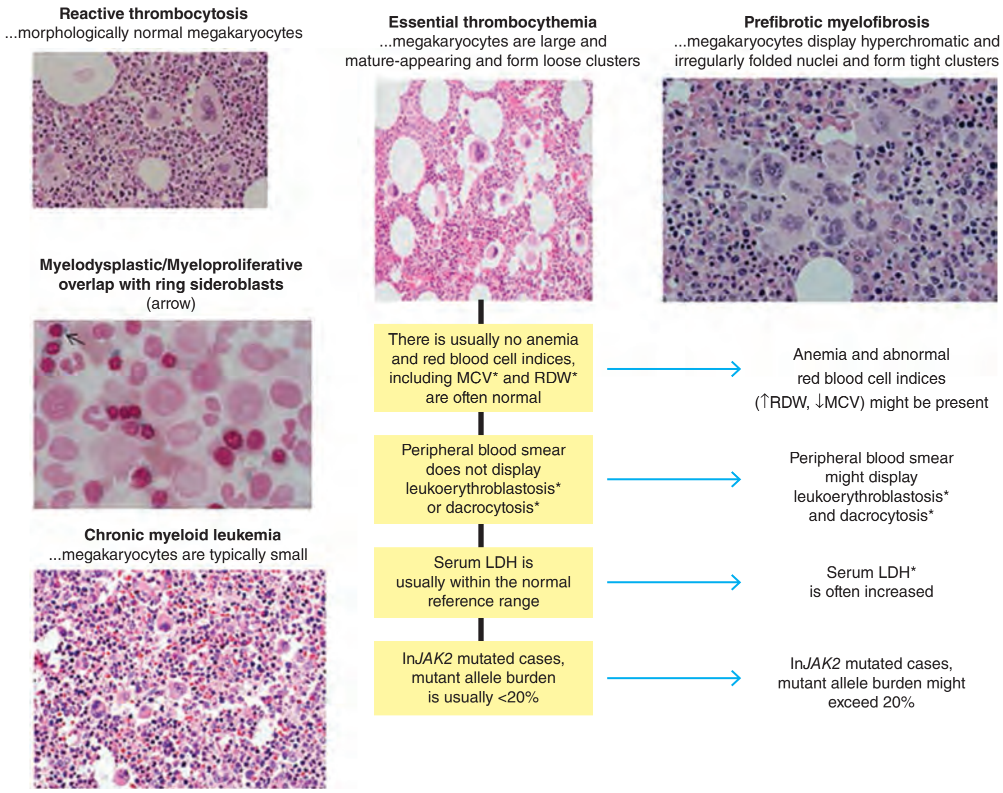

Reactive thrombocytosis
...morphologically normal megakaryocytes
Essential thrombocythemia
...megakaryocytes are large and mature-appearing and form loose clusters
Prefibrotic myelofibrosis
...megakaryocytes display hyperchromatic and irregularly folded nuclei and form tight clusters
Myelodysplastic/Myeloproliferative overlap with ring sideroblasts (arrow)
Chronic myeloid leukemia
...megakaryocytes are typically small
There is usually no anemia and red blood cell indices, including MCV* and RDW* are often normal
Anemia and abnormal red blood cell indices (↑RDW, ↓MCV) might be present
Peripheral blood smear does not display leukoerythroblastosis* or dacrocytosis*
Peripheral blood smear might display leukoerythroblastosis* and dacrocytosis*
Serum LDH is usually within the normal reference range
Serum LDH* is often increased
InJAK2 mutated cases, mutant allele burden is usually <20%
InJAK2 mutated cases, mutant allele burden might exceed 20%

Figure 71.5 FEATURES TO DISTINGUISH BETWEEN DIFFERENT CAUSES OF THROMBOCYTOSIS. Laboratory and bone marrow characteristics of most common cause of thrombocytosis including reactive thrombocytosis, essential thrombocythemia, chronic myeloid leukemia, prefibrotic myelofibrosis and myelodysplastic/myeloproliferative overlap with ringed sideroblasts. **MCV*, mean corpuscular volume; *RDW*, red cell distribution width; *LDH*, lactate dehydrogenase; leukoerythroblastosis, presence of immature myeloid cells and nucleated red cells; dacrocytes, "teardrop" cells. *(From Tefferi A, Barbui T. Polycythemia vera and essential thrombocythemia: 2021 update on diagnosis, risk-stratification and management. Am J Hematol. 2020;95:1599–1613. Adapted from Tefferi A, Pardanani A. Essential thrombocythemia. N Engl J Med. 2019;381(22):2135–2144.)*

TABLE 71.5 Clinical and Laboratory Features Helpful in Distinguishing Essential Thrombocythemia From Reactive Thrombocytosis[a]

Feature	ET	RT
Chronic platelet increase	+	–
Known causes of RT	–	+
Thrombosis or hemorrhage	+	–
Splenomegaly	+	–
BM reticulin fibrosis	+	–
BM megakaryocyte clusters	+	–
Abnormal cytogenetics	+	–
Increased acute phase reactants	–	+
Spontaneous colony formation[b]	+	–
JAK2V617F mutation	+	–

[a] Acute-phase reactants include C-reactive protein and fibrinogen.
[b] Erythroid colonies.
BM, Bone marrow; *ET*, essential thrombocythemia; *RT*, reactive thrombocytosis.
Modified from Tefferi A, Hoagland HC. Issues in the diagnosis and management of primary thrombocythemia. *Mayo Clin Proc.* 1994;69:651.

with uncomplicated thrombocytosis secondary to an MPN had undetectable IL-6 levels. The mechanism by which IL-6 can stimulate thrombopoietin has recently been uncovered. In the liver, the Ashwell-Morell receptor (AMR) binds aging desialylated platelets, enabling their clearance but also activating transcription and translation of the thrombopoietin, facilitating platelet production (see Chapter 29). When IL-6 binds to its hepatic receptor (IL-6R), the signal transducing gp130 subunit is activated and this leads to STAT3 tyrosine phosphorylation and activation by gp130-associated JAK1.[13] There is evidence for cross talk between the AMR and the IL-6R to regulate TPO levels. TPO mRNA expression in hepatocytes is stimulated by IL-6. Loss of IL-6R, in animal models, results in increased expression of AMR, allowing for compensated TPO expression. Furthermore, IL-6R mRNA levels are increased in the liver of AMR knockout mice. After partial hepatectomy in mice, IL-6 played critical roles in hepatocyte proliferation and liver regeneration. Altogether, these studies indicate that both desialylated platelets and IL-6 lead to STAT3-mediated hepatic TPO mRNA expression downstream of the AMR-JAK2 and IL-6R-JAK1 signaling cascades, respectively. Interestingly, JAK1/2 inhibitors inhibit STAT3 activation and TPO mRNA production downstream of AMR binding, contributing potentially to the thrombocytopenia observed with the use of JAK inhibitors.[13] Low levels of both IL-6 and CRP are strongly indicative of the thrombocytosis being the consequence of an underlying MPN.

Both familial forms of thrombocytosis and the thrombocytosis that accompanies hematologic malignancies are examples of primary thrombocytosis. The MPNs are characterized by clonal hematopoiesis, but in the familial forms of thrombocytosis, hematopoiesis is polyclonal. Familial thrombocytosis is frequently inherited as an autosomal dominant disorder, and the affected individuals have elevated megakaryocyte and platelet numbers but normal WBCs and HCT levels. These familial forms of thrombocytosis are due to two types of germline mutations. Familial thrombocytosis, also known as hereditary thrombocytosis, exhibits autosomal dominant Mendelian transmission. It is characterized by the proliferation of megakaryocytes with overproduction of platelets in the absence of elevated hemoglobin and white blood cell count. In contrast to familial erythrocytosis, the OMIM database does not recognize different categories of familial thrombocytosis, considering all germline mutations under a single OMIM reference number (187950). There are two main causes of hereditary thrombocytosis, and they can arise from mutations involving either the thrombopoietin gene (THP) on chromosome 3, or more rarely, the thrombopoietin receptor gene (MPL) on chromosome 1.

Familial thrombocytosis, also known as hereditary thrombocytosis, exhibits autosomal dominant Mendelian transmission. It is characterized by the proliferation of megakaryocytes with overproduction of platelets in the absence of elevated hemoglobin and white blood cell count. The familial forms of thrombocytosis have been attributed in 20% of such families to two types of germline mutations. In the remaining families, the gene responsible for the thrombocytosis has yet to be identified.[14] An abnormality of thrombopoietin production or of the thrombopoietin receptor has been documented to be the basis of these inherited disorders leading to thrombocytosis. Because the median age of diagnosis of patients with these familial forms of thrombocytosis is 17 years, these disorders should be carefully considered in all *JAK2*V617F-, *MPLW*515L-, and *MPL*515K-, *CALR* mutation children with multiple family members with thrombocytosis.

Several different mutations have been reported in families in which excessive thrombocytosis has been attributed to increased thrombopoietin production.[15] A Dutch family with 11 family members and a Japanese family with eight family members were reported with a hereditary form of thrombocytosis that was inherited as an autosomal dominant. The thrombopoietin receptor in a Dutch family was normal, yet there was a G-to-C transversion in the splice donor site of intron 3 of the THP. All of the affected members of the Dutch family were shown to have elevated thrombopoietin levels. In this family, a point mutation in the THP was believed to lead to systemic overproduction of thrombopoietin, leading to a familial form of thrombocytosis. This was the first example of a human disease caused by increased efficiency of mRNA production. Actually, the translation of full-length thrombopoietin is almost completely inhibited by the presence in the 5′ untranslated region of seven AUG codons, which create seven upstream open reading frames (uORFs). These uORFs are potent inhibitors of translation, and mutations in this area cause thrombocytosis by eliminating the normal physiological inhibition of thrombopoietin mRNA translation. Additional families with thrombocytosis caused by a similar genetic mechanism have been identified.

Hereditary mutations of *MPL* can either result in loss of function and thrombocytopenia, or in gain of function and thrombocythemia, and are important models to analyze the mechanism of c-MPL activity. Familial thrombocytosis has been attributed to germline mutations of *MPL* (*MPL*-S505N, *MPL*-K39N, and *MPL*-P106L). *MPL*-S505N was first described in a Japanese pedigree of familial thrombocytosis that is inherited in an autosomal dominant fashion. This disorder has been attributed to a dominant-positive activating mutation of the cellular receptor of MPL. Eight additional Italian families with thrombocytosis and *MPL*-S505N have been identified. In these individuals, hematopoiesis is polyclonal. Etheridge et al.[16] described a novel, autosomal dominant germline mutation that causes familial ET resulting from a single-nucleotide substitution, generating the mutant kinase *JAK2*R564Q. Their data indicate that this mutation leads to isolated thrombocytosis. Mild thrombocytosis was observed as well as polyclonal hematopoiesis. Furthermore, increased activation of JAK2 was confirmed in the platelets of *JAK2*R564Q-positive family members compared with those without the mutation.

A polymorphism of the thrombopoietin receptor (MPL Baltimore, *MPL*-K39N) that is accompanied by thrombocytosis has also been described. This germline polymorphism is caused by a single-nucleotide substitution that results in a lysine-to-asparagine (K39N) substitution in the ligand-binding domain of MPL. The polymorphism occurs exclusively in African Americans and appears to have an autosomal dominant pattern of inheritance with incomplete penetrance because some heterozygotes have normal platelet counts while others have thrombocytosis. Approximately 7% of African Americans are heterozygous for *MPL*K39N. The mutation in the homozygous state is associated with extreme thrombocytosis with a reduced expression of platelet MPL, which has been proposed to affect the receptor's ability to bind thrombopoietin, resulting in its reduced clearance and increased stimulation of megakaryocytopoiesis. *MPL*-P106L is another germline mutation associated with thrombocytosis that has been found in 3.3% of Arabs. It leads to severe thrombocytosis in homozygotes and occasionally to mild thrombocytosis in heterozygotes. In the families described with this germline mutation, extreme thrombocytosis was associated with homozygosity and the mode of inheritance was regarded as being autosomal recessive with possible mild manifestations occurring in heterozygotes.

Subjects with familial forms of thrombocytosis are characteristically diagnosed at earlier ages than patients with ET. These disorders were initially considered to be associated with a benign clinical course, but follow-up of such families for longer periods of time has corrected this misperception. Overall, 23 members of a Dutch and a Polish family with a particular form of thrombocytosis attributed to excessive thrombopoietin production were shown to have similar thrombotic and hemorrhagic complications as individuals with ET. Also, these family members experienced vasomotor symptoms, including erythromelalgia and Raynaud phenomena, which responded to aspirin therapy but not hydroxyurea therapy. Furthermore, many of these patients developed splenomegaly as well as BM histopathologic findings that resemble an MPN, including BM hypercellularity, clustering of megakaryocytes, and a mild increase in BM fibrosis. Surprisingly, more prolonged follow-up of members of the Dutch family due to overproduction of thrombopoietin has revealed progression to symptomatic MF in one individual and progression to acute leukemia in a second family member. The patient with acute leukemia had not received any chemotherapeutic agents, and evaluation of the strength of the relationship between this high thrombopoietin condition and the development of acute leukemia requires further investigation of these families. Similarly, patients with an activating mutation of *MPL*, *MPL*-S505N, also have a high incidence of major thrombotic events, including stroke, myocardial infarction, and Budd-Chiari syndrome. In adult patients, overt BM reticulin and collagen fibrosis associated with mild reductions in hemoglobin levels have been observed, but differences in platelet counts, incidence of thrombotic episodes, or splenomegaly have not been observed when one compares these individuals with ET patients. In women with *MPL-S*505N, hematopoiesis was polyclonal, and the mutation was observed not only in hematopoietic tissues but also in other somatic tissues. Family members who are affected by this mutation appear to have a significantly shorter survival time than nonaffected family members who did not have thrombocytosis, with affected individuals dying most frequently of thrombotic events or complications of MF. By contrast, individuals with either *MPL*-K39N or *MPL*-P106L, which both involve the extracellular domain of MPL, affecting its ability to bind thrombopoietin, were not observed to have an increased risk for thrombosis, splenomegaly, or BM fibrosis.

Table 71.3 outlines the 2016 WHO criteria for the diagnosis of ET that incorporates *JAK2V7*617F, *CALR*, and *MPL* mutational analyses. RBC mass and plasma volume studies, if available, are sometimes helpful in differentiating between *JAK2*V617F-positive ET with borderline elevated hematocrits from patients with PV, but such patients likely represent a continuum of evolution of a *JAK2*V617F-positive hematologic malignancy. BM karyotypic analysis or studies of the *BCR-ABL* fusion gene are imperative in every patient to exclude the diagnosis of CML or to detect another clonal hematologic malignancy. This step is necessary because the natural history of these disorders is very different, and early therapeutic intervention with specific medical therapy for CML, such as a BCR-ABL tyrosine kinase inhibitor, is essential.

Occasionally, ET may be distinguished from refractory anemia with ringed sideroblasts associated with marked thrombocytosis (RARS-T), which is a myelodysplastic/MPN overlap syndrome (see Chapter 73). These patients present with thrombocytosis that is associated with moderate-to-severe anemia and frequently splenomegaly. Their BMs are characterized by the morphologic features of ET and the presence of more than 15% ringed sideroblasts. This entity likely represents a heterogeneous, poorly defined disorder that includes a spectrum of conditions sharing features of an MPN and a myelodysplastic disorder. RARS-T may develop from an *SF3B1*-mutated RARS through the acquisition of a *JAK2*, *MPL*, or *CALR* mutations in a subclone of hematopoietic cells. *SF3B1* is a gene encoding a core component of the RNA splicing machinery. This entity is associated with *JAK2*V617F in 58% of reported patients, *MPL*W515 mutation in 7% of reported patients, and *CALR* mutation in less than 5% of patients. Occasional patients with thrombocytosis and increased ringed sideroblasts but without anemia have also been described. Patients with RARS-T have a similar prognosis as ET patients. About 25% of patients with RARS-T have wild-type *SF3B1* and a subset of these have mutations in other splicing factor genes such as *U2AF1* and *SRSF2*.

Because patients with pre-MF frequently present with thrombocytosis, this form of PMF can frequently be difficult to distinguish from ET, and a recent study showed that 14% of patients diagnosed as ET prior to the revised 2016 WHO criteria defining pre-PMF were actually cases of pre-PMF. This early form of PMF was previously referred to as a *prefibrotic form of PMF*. In pre-PMF, nucleated RBCs, teardrop-shaped RBCs, immature myeloid cells, and megathrombocytes are observed in the peripheral blood. In the BM biopsy, the megakaryocytes are markedly abnormal, a morphologic finding that is helpful in distinguishing this entity from ET (see Fig. 71.5). In pre-PMF, the megakaryocytes often appear in clusters adjacent to the sinusoids; deviations in the nuclear cytoplasmic ratio in the megakaryocytes are observed with abnormal patterns of chromatin clumping; and plump clouds similar to a balloon-shaped lobulation of the nuclei are observed associated with minimal fibrosis or even absent reticulin fibrosis during this stage of PMF. The prevalence of each driver mutation was similar between pre-PMF and MF. However, certain nondriver gene mutations were more prevalent in overt PMF. The discrepancy between the prevalence of mutations in *ASXL1* (33.7% vs. 18%) and *EZH2* (12% vs. 3.6%) in overt versus pre-PMF, respectively, leads to more overt PMF patients meeting criteria to be high molecular risk (44.4% vs. 27.0%). Not all high-risk molecular mutations were discrepant between overt and pre-PMF as seen with *SRSF2* and *IDH1/2* mutations. Overt PMF was more likely to harbor ≥2 HMR mutations (13.6%) than pre-PMF (5.4%).[17] A recent study showed that increased plasma levels of dickkopf-related protein 1 (Dkk-1), an inhibitor of Wnt signaling, could differentiate different MPN subtypes, including discriminating between ET and pre-PMF.[18]

The presence of clonal hematopoiesis, at least in one lineage, quickly establishes the diagnosis of ET. Unfortunately, techniques to study clonality are currently not widely available and are restricted to the evaluation of female patients. Such studies may be particularly useful in young triple negative female patients with thrombocytosis. Probes for a variety of genes on the X chromosome can be informative for clonal analysis of blood cell production in more than 72% of female Americans. In such patients, the analysis of restriction fragment length polymorphisms can be used to establish a pattern of clonal hematopoiesis, which is indicative of a hematologic malignancy and established the diagnosis of ET in a young female patient with thrombocytosis who was lacking a driver mutation. Polyclonal hematopoiesis is found in all cases of reactive and familial thrombocytosis. Polyclonal hematopoiesis, however, does not exclude the diagnosis of ET because in several series, almost one-third of patients who met the clinical criteria for ET had polyclonal hematopoiesis in all studied lineages. The biogenesis of polyclonal ET has been described above.

At times, it is impossible to define the cause of an individual patient's thrombocytosis. In an asymptomatic patient, follow-up to determine whether the degree of thrombocytosis undergoes an increase is warranted. If additional clues to the cause of the thrombocytosis are subsequently revealed, a diagnosis will become apparent. Some reassurance is provided by reports of larger cohorts of patients, each with platelet counts of greater than 1000 × 10^9/L, which have been followed for years. Virtually none of the patients with reactive thrombocytosis demonstrated an increased risk of developing a cerebrovascular accident, thrombophlebitis, myocardial infarction, or a peripheral arterial thrombosis.

PROGNOSIS

The prognosis of ET patients is largely determined initially by their propensity during the early stages after diagnosis to develop thrombotic episodes and later to evolve to PV, MF, or eventually MPN-BP. Thrombotic events are largely the consequences of quantitative and qualitative abnormalities in blood cell numbers, while the evolution to MF and MPN-BP is now appreciated, partly due to the acquisition of additional myeloid gene mutations during the prolonged clinical course of ET patients that play a role in disease progression. Although the thrombotic risk of *JAK2*V617F+ ET patients is now appreciated to be greater than those with *CALR* mutations, Type 1 CALR-mutated patients appear to be at a higher risk of evolving to MF.[19] Significant efforts in the past have been spent trying to predict the outcomes solely based on clinical parameters. The probability that a patient with ET will survive 10 years ranges from 64% to 80%. In a large study from Spain with extensive follow-up, there was no substantial difference between the probability of survival of patients with ET and that of a control population. However, a study of 322 consecutive patients seen at the Mayo Clinic and followed for a median follow-up of 13.6 years showed a different pattern. The survival of patients with ET was similar to that of the control population during the first decade of disease, but it became significantly worse thereafter. Multivariable analysis identified an age at diagnosis of 60 years or older, leukocytosis (>15,000/µL), prior venous thrombosis, tobacco use, and diabetes mellitus as independent predictors of poor survival. The risk of developing leukemia or MF was low in the first 10 years (1.4% and 3.8%, respectively) but increased substantially in the second (8.1% and 19.9%, respectively) and third (24.0% and 28.9%, respectively) decades of the disease. The presence of the *JAK2*V617F mutation did not influence either survival or the rate of leukemic transformation in this analysis. The rate of leukemic transformation was higher in patients with platelet counts above 1000 × 10^9/L and abnormal hemoglobin levels. The development of MF was heralded by the appearance of immature myeloid precursors and dacryocytes in the blood smear and increased serum lactate dehydrogenase levels, followed by a reduction in platelet numbers and progressive splenomegaly. The presence of *CALR*, which occurs in 15% to 25% of patients with ET and is almost uniformly mutually exclusive with mutations in *JAK2* and *MPL*, is associated with younger age, male sex, higher platelet counts, lower hemoglobin levels, and a lower risk of thrombotic complications.

The International Prognostic Score for Essential Thrombocythemia (IPSET) was developed from the retrospective review of the clinical outcomes of 891 patients with ET diagnosed by WHO criteria in which age ≥60 years, leukocyte count ≥11 × 10^9/L, and history of thrombosis were determined to have a prognostic significance for survival, with weighted values of 2, 1, and 1 points, respectively.[9] The IPSET categories of low (no points), intermediate (1 to 2 points), or high (3 to 4 points) had corresponding median survivals of not yet reached, 24.5 years, and 13.8 years, respectively. Analysis of this cohort for thrombotic risk determined age greater than 60 years (1 point), history of thrombosis (2 points), presence of cardiovascular risk factors (1 point; diabetes, hypertension, smoking) and the presence of mutated *JAK2* (2 points) as independent risk factors. Low-risk (0 to 1 points), intermediate-risk (2 points), and high-risk (3+) categories were associated with 1.03%, 2.35%, and 3.56%/year risk of thrombosis, respectively.[20]

The IPSET-thrombosis score was revised by reanalysis of the original IPSET-thrombosis data in 1019 ET patients for whom *JAK2*V617F mutational status was known. This study is of importance since it was the initial attempt to include mutational status into prognostic scoring systems. The aim of this reanalysis was to define the impact of the *JAK2* mutation and cardiovascular risk factors on thrombosis-free survival in ET patients.[11] The presence of *JAK2*V617F clearly impacted the risk of thrombosis. Cardiovascular risk factors did not further worsen the risk of thrombosis in patients who had a history of thrombosis. Results from this analysis led to a four-tier risk stratification based on three adverse features: age greater than 60, thrombosis history, and *JAK2V617F*. Risk categories include very low (no adverse variables), low (presence of *JAK2V617F* alone), intermediate (age >60 years) and high (thrombosis history or age >60 years with *JAK2V617F*). Although this revised prognostic scoring system was validated in 585 ET patients, it will require validation using prognostic studies before it is universally implemented.[19]

Paz and coworkers initially recognized that the prognosis of ET patients is due to two major complications: thrombosis in the time period prior to, or in, the short term following diagnosis; and the evolution to MF and MPN-BP during the long term follow-up of ET patients. Grinfield et al.[2] utilized this concept to develop a genomic classification of MPNs. They performed whole-genome sequencing of 2035 MPN patients, of whom 1321 had ET and had participated in the ET trials conducted by this group. Using this approach, they were able to identify eight genomic subgroups of MPN patients to predict which patients were at the highest risk of progressing to MF or MPN-BP.[2] They concluded that death in the early phases of ET was primarily influenced by age, not genomic factors. However, genomic features were found to be predictive of the progression of ET or preMF to MF or more overt forms of MF. The efforts of Grinfeld and coworkers are important in understanding ET disease progression, but unfortunately, at present, it does not affect treatment options due to the lack of therapeutic options that specifically target these genomic changes.

Recently, work by Tefferi et al. determined that certain non-driver mutations influenced survival in ET patients. Next-generation sequencing results of 502 ET patients were analyzed and splicesosome mutations in *SF3B1* and *SRSF2* predicted inferior overall survival, while mutations in *SF3B1* and *U2AF1* predicted inferior MF-free survival. *TP53* mutations were associated with transformation to MPN-BP. This enabled the development of a mutation-enhanced prognostic model for ET that incorporates the presence of adverse mutations (*SRSF2*, *SF3B1*, *U2AF1*, and *TP53*) (2 points) with high-risk clinical parameters: age greater than 60 years (4 points), male sex (1 point), and leukocyte count $\geq 11 \times 10^9$/L (1 point). Based on data from 451 ET patients merged from the Mayo Clinic and University of Florence, the three-tiered modified MIPSS for ET categories of low-risk (0 to 1 points), intermediate-risk (2 to 5 points) and high-risk ($\geq$ 6 points) corresponded to median survivals of 34.4 years, 14.1 years, and 7.9 years, respectively. Mutational-based prognostic scoring of ET patients will need to be further validated in larger cohorts.

A major determinant of the prognosis of a patient with a presumptive diagnosis of ET depends on the discrimination of whether such patients actually have true ET or an early form of MF, based on histopathologic and clinical criteria adopted by the WHO (see Table 71.3).[14] In a group of 891 patients with a prior diagnosis of ET with BM biopsies evaluated retrospectively, 16% were reclassified as having pre-PMF through histopathology. Thrombosis rates and *JAK2*V617F positivity were similar in the two groups. However, patients with pre-PMF had higher leukocyte counts, lower hemoglobin levels, higher platelet counts, higher lactic dehydrogenase levels, greater numbers of circulating CD34$^+$ cells, and a greater incidence of palpable splenomegaly than patients with true ET. Survival was reported to be significantly inferior for those patients with pre-PMF compared with patients with histopathologically validated ET (Fig. 71.6). In one series with a median follow-up of approximately 7 years, the median survival of patients with ET ranged from 16 to 21 years, and the survival of patients with pre-MF ranged from 10.8 to 14.4 years, with the incidence of thrombosis being similar and the majority of deaths being attributed to progression to overt PMF and acute leukemia. Although the patients with ET enjoyed a more favorable outcome, the 15-year cumulative incidences of overt MF and acute leukemia were still 9.3% and 2.1%, respectively, emphasizing the seriousness of this diagnosis, especially in younger patients.[21]

Death of ET patients predominantly results from thrombotic complications, but transformation to MPN-BP is an important cause of mortality. The phenotype of the blast cell can be myeloid, myelomonocytic, megakaryocytic, of mixed lineage, or even lymphoblastic. According to the data mentioned earlier, the risk of patients with ET transforming into acute MPN-BP is greater than that of normal individuals. This is a phenomenon shared with the other MPNs. The risk of developing MPN-BP after treatment with hydroxyurea alone has been reported to increase only slightly (3% to 4%), but the sequential use of hydroxyurea with other cytotoxic agents, such as busulfan or pipobroman, significantly increases the risk of developing a secondary leukemia.

The development of MPN-BP is associated with a deletion of the short arm of chromosome 17, trisomy of the long arm of chromosome 1, and monosomy 7q. The 17p loss is associated with the loss of TP53. ET patients with such abnormalities of TP53 have been shown by Grinfeld and coworkers to have an inferior outcome due to disease progression.[2] 17p loss has been attributed to hydroxyurea treatment by some but not all investigators. The median survival after the development of myelodysplasia or MPN-BP is 4 months. MPN-BP, which develops following ET, is frequently refractory to standard induction chemotherapy but has been recently reported to be responsive to hypomethylating agents, including 5-azacytidine and decitabine. Because allogeneic stem cell transplantation can be curative, rapid referral to a transplant center is suggested if the patient has an appropriate performance status and an allogeneic stem cell donor is available.

THERAPY

At present, the goal of treating patients with ET is to prevent additional thrombotic and hemorrhagic events[20] without increasing

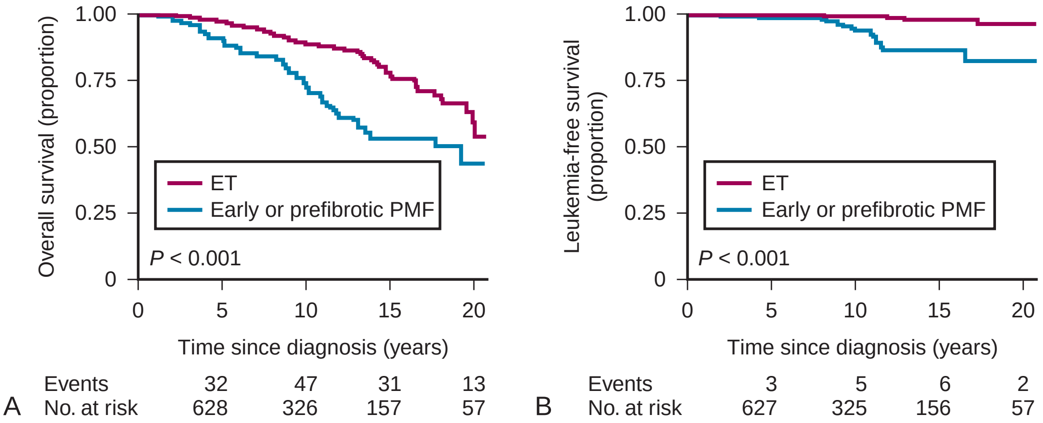


Figure 71.6 Overall (A) and leukemia-free (B) survival of patients with true essential thrombocythemia (ET) versus early or prefibrotic primary myelofibrosis (PMF). *(Data from Barbui T, Thiele J, Passamonti F, et al. Survival and disease progression in essential thrombocythemia are significantly influenced by accurate morphologic diagnosis: an international study. J Clin Oncol. 2011;29:3179.)*

TABLE 71.6 Risk Factors for Thrombosis in 100 Patients With Essential Thrombocythemia

Risk Factor	Incidence of Thrombosis (% Patient-Year)	Relative Risk (95% CI)	*P*
Age (Years)			
<40	1.7	1.0[a]	
40–60	6.3	3.9 (0.7–21.5)	NS
>60	15.1	10.3 (2.1–51.5)	< 0.001
Previous Thrombosis			
No	3.4	1.0[a]	< 0.0005
Yes	31.4	13.0 (4.1–1.5)	

[a] Reference category.

CI, Confidence interval; *NS*, not significant.

Data from Finazzi G, Barbui T. Risk-adapted therapy in essential thrombocythemia and polycythemia vera. *Blood Rev*. 2005;19:243.

the risk of transformation to post-ET MF, MDS, or MPN-BP. Age (>60 years of age), the presence of *JAK2*V617F, and the history of a prior thrombosis are widely accepted predictors of a patient developing additional thrombotic events during follow-up (Table 71.6). Other predictors of cardiovascular morbidity include a history of smoking, hypertension, obesity, diabetes mellitus, and congestive heart failure, and a white blood cell count greater than 11 × 10^9/L. Ironically, patients with platelet counts greater than 1000 × 10^9/L have a lower risk of developing arterial thrombosis and a higher risk of bleeding. These parameters have been widely used by clinicians to stratify ET patients according to their risk of developing additional thrombotic events but are not known to delay disease progression. Such stratification strategies have been used to make treatment decisions to reduce the platelet count. It is important to emphasize that such risk factors have been identified based on retrospective analyses of registries of ET patients that have largely not been created using modern molecular and histopathologic diagnostic tools and are not known to alter the risk of a patient progressing to MF or MPN-BP. Using such patient-stratification strategies, patients have been placed into high-, intermediate-, or low-risk or very low-risk groups based on their predicted risk of developing an additional life-threatening thrombotic event. This strategy is uniformly implemented throughout Europe and North America. The benefit of this strategy is based on a single clinical trial that indicated that the reduction of platelet numbers to less than 600 × 10^9/L with hydroxyurea therapy was associated with a reduction in the development additional thrombotic events compared with a control group (see Fig. 71.2). This study involved a total of 114 patients, and the median follow-up period was only 27 months. Greater degrees of platelet suppression have not been shown to be associated with a further reduction in the possibility of developing a thrombotic episode, which in hydroxyurea-treated high-risk patients has been reported to be 1.66% per year.

Although the implementation of such a strategy is widely accepted, it is important to be aware that this approach is not based on robust data that one associates with modern-day evidence-based medicine. In fact, in several studies, an elevated leukocyte count of above 11 × 10^9/L has been more closely associated with the risk of developing additional thromboses than the degree of elevation of the platelet count, and a recent prospective study of 261 patients for the anagrelide registry confirmed that increased leukocyte count not only increases the risk of major thrombosis but also decreases the time to such an event.[22] The effects of leukocytosis on thrombotic risk remains, however, controversial. Carobbio and coworkers, however, by performing a systematic literature review and meta-analysis of greater than 30,000 patients with ET or PV, found that the greatest effect of leukocytosis on thrombotic risk was in ET, rather than PV, and that this effect was exclusively related to arterial events and not venous events. This study has important limitations since a consensus definition of leukocytosis to optimally predict the risk of developing vascular events could not be identified.[23] These conflicting reports in the literature among experts in this field make it increasingly difficult to be dogmatic about who to treat with platelet-lowering agents and what the target platelet count should be. The risk stratification of patients should not be ignored, and patients with life-threatening thrombosis or disabling symptoms because of microcirculatory problems should receive drugs that lower the platelet count. If a patient of any age has excessive thrombocytosis and a thrombotic or hemorrhagic event, that patient must be treated with platelet lowering agents. However, for asymptomatic ET patients in so-called *high-risk categories based on age alone*, the decision to embark upon a strategy including platelet-lowering agents remains murky and is more based on personal treatment decisions rather than volumes of data. The treatment of asymptomatic low-risk patients with ET remains largely problematic, yet greater insight into the management of such patients has recently been gained.

Management of ET patients with life-threatening hemorrhagic or thrombotic episodes is more straightforward. Life-threatening thrombotic events in patients with extreme thrombocytosis rarely require plateletpheresis, but when implemented, it should be combined with the institution of myelosuppressive therapy. To place this practice in perspective, over the last 14 years at our institution, we have not used this approach on a single patient. Plateletpheresis should not be used in patients undergoing major bleeding events due to its ability to lead to hemodynamic instability or in patients with thrombotic events with modest or moderate thrombocytosis. In an ET patient with an active thrombosis and extreme thrombocytosis, immediate physical removal of large numbers of platelets is an option because chemotherapeutic agents generally require several weeks before platelet counts can be reduced to normal levels. It is recommended to reduce the platelet count to below 600,000/mm^3 by each plateletpheresis and it is suggested that achievement of such a goal requires the passage of two blood volumes over a 3 to 4-hour period. Long-term plateletpheresis is an ineffective means of controlling thrombocytosis, presumably because of the rapid rate of production of platelets. Therefore most clinicians begin by administering a chemotherapeutic agent that has a rapid onset of action, such as hydroxyurea at doses of 2 to 4 g/day, simultaneously with the institution of platelet-pheresis. The dose of hydroxyurea requires close monitoring with an appropriate reduction of dose to avoid excessive myelosuppression. At present, we avoid plateletpheresis in ET patients with acute thrombosis but rather choose to institute hydroxyurea and anticoagulation immediately. Plateletpheresis is not recommended for asymptomatic patients with extreme thrombocytosis such as a platelet count greater than 2000 × 10^9/L in spite of the anxiety that such numbers might create in the treating physician.

In patients found to have ET and a current or past history of a thrombotic event, little controversy exists as to the need for lowering platelet numbers. The large number of thrombotic complications that occur in patients with ET who smoke points to the urgent need for smoking cessation. Most investigators try to normalize the platelet count or reach a platelet count at which the symptoms of the high-risk patient resolve. Although major bleeding episodes requiring hospitalization are rare, patients with extreme thrombocytosis (>1500 × 10^9/L), acquired von Willebrand syndrome, and a history of a hemorrhagic episode are clearly at the risk of developing additional bleeding complications. These patients require reduction of the increased platelet numbers to the normal range with the use of a variety of agents, including hydroxyurea, anagrelide, or IFN-α.

Another situation that requires treatment is discomfort caused by erythromelalgia, or the progression of erythromelalgia, to frank gangrene. Erythromelalgia patients respond within days to low-dose aspirin therapy or platelet-reduction therapy. Patients with frank gangrene require sustained normalization of platelet numbers with myelosuppressive therapy and aspirin with the goal of preserving digits or limbs that remain in danger.

During the 1980s and 1990s, hydroxyurea became the drug of choice for the treatment of ET. The impetus for this practice was based on the knowledge that agents such as ^{32}P and alkylating agents such as melphalan and busulfan were leukemogenic. The popularity of the ribonucleoside reductase inhibitor, hydroxyurea, for the management of ET was due to the belief in the early 1970s that it was nonleukemogenic. Hydroxyurea can be administered at a dose of 15 mg/kg initially, with an adjustment of the dose to maintain a platelet count (at the least) below $600 \times 10^9/L$ without inducing significant neutropenia. Evidence to target lower platelet counts to further reduce the risk of additional thrombotic events is not available. After the agent is started, frequent monitoring of blood counts is mandatory to avoid the development of neutropenia or thrombocytopenia until the maintenance dose is determined. The use of this drug in a high-risk group of patients with a reduction of platelet numbers to less than $600 \times 10^9/L$ has resulted in a reduction of thrombotic events compared with a control population. In addition, hydroxyurea is capable of normalizing the WBC, which is thought by many to be of potential importance since elevated WBCs are thought to contribute to the increased thrombotic risk in ET patients. The reduction of platelet numbers to normal levels reduces but does not entirely eliminate the occurrence of additional thrombotic episodes.

The role of hydroxyurea plus aspirin versus aspirin alone was analyzed in a randomized clinical trial of " intermediate-risk" ET patients who were 40 to 59 years old and lacked high-risk features including a history of thrombosis, ischemia, embolism, hemorrhage, extreme thrombocytosis (platelet count $\geq 1500 \times 10^9/L$), hypertension, or diabetes. These investigators classified ET patients of ages from 40 to 59 years into the intermediate-risk group since they had previously shown that this patient population has a higher thrombotic risk than younger patients. Most investigators in this field would have classified such patients as being low-risk. The addition of hydroxyurea to aspirin therapy did not reduce vascular complications of ET or myelofibrotic/leukemia transformation, confirming that low-risk patients should not receive cytoreductive therapy.[24] This report provides evidence for the lack of benefit of myelosuppressive therapy in low-/intermediate-risk ET patients. This type of therapy should be reserved exclusively for patients with a history of a thrombotic or bleeding event. This report should dissuade clinicians from reflexively treating the platelet count irrespective of the degree of thrombocytosis. Such defensive approaches to treating ET patients are discouraged.

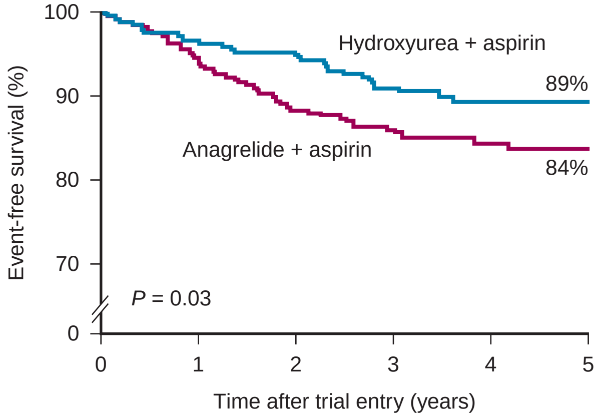
Hydroxyurea use is associated with some toxicity, including dose-related neutropenia, fever, nausea, stomatitis, hair loss, azoospermia, infertility, nail discoloration, and lower extremity and oral ulcerations, as well as an increased risk of developing squamous cell carcinoma of the skin. Many of these problems resolve with the withdrawal of the drug or dose reduction, but leg ulcers can be persistent, sometimes requiring skin grafting. These ulcers typically heal within 1 to 9 months of the cessation of therapy. Such leg ulcers have been reported to occur in 9% of the patients treated with hydroxyurea and are an indication for the immediate discontinuation of therapy and elimination of any rechallenge with the drug. Hydroxyurea is also not universally successful in controlling thrombocytosis; resistance to hydroxyurea has been reported in 11% to 17% of cases. The criteria for defining resistance or intolerance to hydroxyurea have been established by an International Working Group. They include a platelet count greater than $600 \times 10^9/L$ after 3 months of at least 2 g/day of hydroxyurea (2.5 g/day in patients with a body weight >80 kg); a WBC less than $2500/mm^3$ or hemoglobin less than 10 g/dL at any dose of hydroxyurea; presence of leg ulcers or other unacceptable mucocutaneous manifestations at any dose of hydroxyurea; and hydroxyurea-related fever. In such situations, hydroxyurea can be substituted for (or combined with) other platelet-lowering agents. These criteria for hydroxyurea resistance are imperfect because they do not include the development of a thrombotic event while on therapy, which is the central goal of therapy. Whether such patients who develop a new thrombosis would benefit from the continued use of hydroxyurea or use of another therapeutic agent has not been explored.

The risk of evolving to acute leukemia is extremely low in untreated ET patients. More recent prospective studies, both in ET and PV, have confirmed that hydroxyurea therapy is associated with a low incidence of leukemic transformation when used alone (<5%) and with long-term follow-up (≤14 years). However, the leukemic risk increased significantly when the drug was used before or after treatment with alkylating agents, particularly busulfan. One can conclude from these studies that hydroxyurea therapy alone is less leukemogenic than alkylating agents or ^{32}P alone, but a small increased risk of the development of leukemia secondary to its use cannot be completely excluded. Of concern is the observation that a high proportion of AML and MDS occurring in ET patients treated with hydroxyurea alone have morphologic, cytogenetic, and molecular characteristics of the 17p deletion syndrome that is associated with the deletion of TP53. These patients are reported to have a typical form of dysgranulopoiesis characterized by hypolobulated polymorphonuclear leukocytes with small vacuoles in neutrophils and *TP53* mutations. By contrast, Kubesova and coworkers reported that in MPN patients, age was the strongest factor affecting the incidence of low VAF *TP53* mutations and that they could not document a significant age-independent association between the *TP53* mutations and the administration of hydroxyurea. The phobia concerning the use of hydroxyurea in ET patients articulated by some is clearly excessive. A more practical approach would be to avoid its use in younger patients who are in their reproductive years, and due to its ease of administration, to use this agent in older high-risk patients as the standard of care. It should be emphasized that the effects of hydroxyurea are frequently quickly reversible and therapy should be continued as long as the patient has thrombocytosis. Ironically, if ET patients have progressive reductions in platelet numbers into the normal range, allowing hydroxyurea therapy to be discontinued, that scenario should raise the possibility of progression to MF.

When considering the risk-to-benefit ratio, one can conclude that hydroxyurea therapy is the first-line therapy for ET patients with a high-risk of developing an additional thrombosis, including those older than 60 years of age or with a history of a thrombotic episode. As previously suggested, such nonleukemogenic drugs as IFN-α, anagrelide, or pegylated IFN are preferred in patients younger than 40 years of age who have experienced a major thrombotic or bleeding event. An alternative to hydroxyurea is therapy with anagrelide. Data on the use of anagrelide in ET suggests that it is nonleukemogenic. Anagrelide is a member of the imidazo(2,1-b)quinazolin-2-1 series of compounds. When studied in humans, it was noted that anagrelide, in small doses, produced thrombocytopenia. The drug acts primarily by reducing the megakaryocyte size and ploidy. It also decreases megakaryocyte proliferation. Anagrelide therefore appears to lower platelet counts, primarily by interfering with the development of megakaryocytes. Anagrelide suppresses megakaryocytopoiesis by selectively reducing the expression levels of GATA-1, FLI-1, NFE-2, and FOG-1 in cells belonging to the megakaryocytic lineage. The effects of anagrelide do not involve thrombopoietin-mediated signal transduction events. GATA-1 and FOG-1 play critical roles in megakaryocytic differentiation. Anagrelide in low doses is effective in lowering the platelet count in 93% of patients. Most importantly, it is effective despite resistance to prior therapies. Resistance to anagrelide therapy has not been documented, but occasionally patients have been observed to require extraordinarily high doses to control excessive thrombocytosis. The recommended initial dose is 0.5 mg orally, two to four times a day. The dose should be increased by 0.5 mg/week to control thrombocythemia. The dose of anagrelide should not exceed 10 mg/day or a 2-mg/dose. Excessive use will result in predictable thrombocytopenia and increase the likelihood of side effects. The median maintenance dose in patients with ET is 2 mg/day administered in divided doses. Data on more than 3000 patients with a variety of MPNs complicated by extreme thrombocytosis are available. In addition, follow-up of more than 500 patients for more than 5 years has been reported. Anagrelide has been shown to be an effective drug in the treatment of ET, resulting in a median time to response of 2.5 to 4 weeks. An effect on platelet numbers is usually noted within 6 to 10 days. Anagrelide leads to a reduction in hematocrit in 25% to 36% of patients, but it has no effect on white blood cell numbers, systemic symptoms, or the degree of splenomegaly. Buxhofer-Ausch and co-workers using a registry of 309 ET patients with normal WBC numbers have reported the effectiveness of lowering the platelet count with anagrelide below 600×10^9/L on the risk of developing a major thrombotic event in ET patients.

The most common side effects of anagrelide result from its vasodilatory and positive inotropic actions. These effects resulted in complaints of headache, dizziness, fluid retention, palpitations, and high-output cardiac failure. The vasodilatory effect leads to reduced renal blood flow, resulting in fluid retention. In addition, gastrointestinal complications, such as nausea, abdominal pain, and diarrhea, are prominent. These side effects usually develop within 2 weeks of initiation of therapy and frequently diminish in severity or resolve within 2 weeks of continued therapy. Because of its ability to promote fluid retention and the development of tachyarrhythmias, anagrelide therapy should be used with caution in patients with cardiac disease and should be administered carefully to elderly patients. If congestive heart failure or arrhythmias other than tachycardia develop, anagrelide therapy should be discontinued. Prolonged anagrelide therapy may be associated with a potentially irreversible drug-induced cardiomyopathy that is reminiscent of tachycardia-induced cardiomyopathy. Dose reduction can be used to lessen the degree of tachycardia or fluid retention. Before the institution of anagrelide, a basic cardiac evaluation should be performed, including an assessment of cardiac risk factors, a careful cardiac history, and an electrocardiogram, which can be used as a baseline if the patient develops rhythm disturbances. Although most adverse effects are mild or moderate, in one series, therapy was discontinued in 16% of patients because of intolerable side effects, especially headache, nausea, fluid retention, and, rarely, frank congestive heart failure. Anagrelide has no mutagenic activity, but its use is not currently advised during pregnancy. Because of its small molecular weight, it is believed to be capable of crossing the placenta and thus may lead to fetal thrombocytopenia. Anagrelide appears to be a suitable drug for the treatment of young, symptomatic patients with ET and for those who are resistant or refractory to front-line treatment with hydroxyurea.

Hydroxyurea and anagrelide have been compared in a large, randomized trial of 809 “high-risk” patients with ET in combination with low-dose aspirin (75 to 100 mg/day). The dose of each cytoreductive agent was titrated to achieve a platelet count less than 400×10^9/L and long-term control was attained in each treatment arm. Overall, patients randomized to anagrelide (and aspirin) were more likely to reach the composite primary endpoint of major thrombosis (arterial or venous), major hemorrhage, or death from vascular causes ($P = 0.03$; Fig. 71.7). When individual endpoints were assessed, arterial thrombosis, major hemorrhage, and the development of MF were all significantly more frequent in patients treated with anagrelide (7% vs. 2%; P = 0.004, 0.008, and 0.01, respectively). Anagrelide and aspirin seemed to offer at least partial protection from thrombosis because the prevalence of thrombotic events (8% at 2 years) was significantly less than that observed in the control arm of a previous study (28%). Intriguingly, the number of venous thromboses was less frequent in patients treated with anagrelide ($P = 0.006$). In addition, patients with *JAK2*V617F-positive ET were more sensitive to therapy with hydroxyurea but not anagrelide. Recent analyses of anagrelide-treated ET patients indicate responsiveness irrespective of the driver mutation status. In long-term follow-up of 67 patients with ET treated with anagrelide, therapy-related anemia was more frequent in *CALR*-mutated patients without differences in thrombotic rates among the four molecular subtypes. Also, the incidence of discontinuation of anagrelide therapy because of drug-related adverse events was significantly greater than that observed with patients receiving hydroxyurea. Based on this large randomized remarkable study, hydroxyurea is presently considered first-line therapy for patients with high-risk ET (Table 71.7). Although equivalent control of the platelet count was achieved with both agents, hydroxyurea proved superior, perhaps because of its ability to reduce not only platelet numbers but also leukocyte numbers, which have been associated with arterial thrombosis in ET patients. In addition, the combination of anagrelide and low-dose aspirin was associated with a higher incidence of bleeding episodes, suggesting that aspirin therapy might be best avoided in patients being treated with anagrelide.



No. at risk						
Hydroxyurea plus aspirin	404	388	298	204	129	57
Anagrelide plus aspirin	405	379	272	190	119	52

Figure 71.7 EVENT-FREE SURVIVAL IN A HYDROXYUREA-TREATED GROUP COMPARED WITH AN ANAGRELIDE-TREATED GROUP. Primary end points were arterial or venous thrombosis, serious hemorrhage, or death from any of these causes. *(From Harrison CN, Campbell PJ, Buck G, et al. Hydroxyurea compared with anagrelide in high-risk essential thrombocythemia. N Engl J Med. 2005;353:33.)*

TABLE 71.7 **Choice of Drugs for Treatment of Patients With High-Risk Essential Thrombocythemia**

Age (Years)	Treatment of Choice	Second-Line
<50	Interferon	Anagrelide
		Hydroxyurea
50–75	Hydroxyurea	Interferon
		Anagrelide
>75	Hydroxyurea	Anagrelide
		Busulfan

The ANAHYDRET study was a prospective, randomized, noninferiority study comparing hydroxyurea to anagrelide treatment in 259 patients with treatment-naïve, high-risk ET (WHO diagnosis confirmed). Anagrelide was determined to be noninferior to hydroxyurea based on equivalent rates of thrombotic (arterial and venous) and hemorrhagic (major and minor) complications. A criticism of this study was the low rate of aspirin use in this study, which was approximately 28% in both arms, and the lack of data indicating relative rates of transformation to MF.

Analysis of 3649 high-risk ET patients undergoing cytoreductive treatment as participants of a phase IV Evaluation of Anagrelide Efficacy and Long-term Safety revealed fewer thrombotic events in patients treated with anagrelide. However, a greater degree of hemorrhage and transformation to MF in patients treated with anagrelide. Patients who received cytoreductive therapies other than anagrelide had a greater risk of leukemic transformation and the development of other malignancies.

Interestingly, Alvarez-Larrán et al., while reporting the results of myelosuppressive therapy from the Spanish Registry of ET, indicated that *CALR*-mutated ET is less responsive to such a therapeutic approach. About 1104 ET patients were in the study and either received cytoreductive treatment with hydroxyurea ($n = 977$), anagrelide ($n = 113$), or other agents ($n = 14$). Hydroxyurea and anagrelide were similarly effective in *CALR*-mutated ET patients. At 12 months, rates of complete hematological response were 40% in *CALR*-mutated cases and 67% of *JAK2*V617F+ cases. Median time to complete hematological remissions was longest in the *CALR*-mutated cases (705 days) compared to cases with other driver mutations (192 days for *JAK2*V617F, 433 for *MPL*-mutated) and cases that were triple negative (343 days) ($P < 0.0001$). Complete remission duration was shortest in *CALR*-mutated ET. Resistance/intolerance to hydroxyurea occurred more frequently in *CALR*- mutated patients (23%) and *MPL*-mutated patients (27%) than in *JAK2*V617F+ (5%) and triple negative cases (15%) ($P < 0.0001$). This report is of importance since it indicates for the first time that *CALR*-mutated ET is less sensitive to conventional myelosuppressive agents, suggesting that new therapeutics are needed for this genotype. This report has limitations since it did not evaluate the effects of such interventions on the incidence of thrombotic events. Since *CALR*-mutated patients have higher platelet numbers, lower WBCs, and a lower risk for thrombotic events, the application of lessons learned from treating a group of ET patients irrespective of genotype might not be appropriate. The Spanish group also suggested that platelets to be targeted with myelosuppressive therapies might be different for patients with other mutations. Such a personalized approach to myelosuppressive therapy in high-risk ET patients is worthy of further evaluation in a prospective study.

IFN-α has been used to treat MPN-associated thrombocytosis since the 1990s. IFN-α likely acts by promoting cycling of MPN hematopoietic stem cells, activating the TP53 pathway, directly inhibiting megakaryocyte colony formation and secondarily by inhibiting the expression of thrombopoietic-stimulating cytokines, such as GM-CSF, G-CSF, IL-3, and IL-11, and by stimulating the production of negative regulators of megakaryocytopoiesis, such as IL-1ra (receptor agonist) and macrophage inflammatory protein 1 (MIP-1a). IFN-α inhibits thrombopoiesis by suppressing thrombopoietin-induced phosphorylation of the JAK2 substrates, MPL and STAT5. Furthermore, IFN-α also induces the production of suppressor of cytokine signalling-1, which inhibits thrombopoietin-mediated cell proliferation. In a total of 212 patients treated with IFN-α in a total of 11 different clinical trials, a response rate of approximately 90% has been reported. Therapy was administered to outpatients, most frequently at an initial dose of 3 million units daily, and usually produced a rapid decrease in platelets within 2 months. The mean time to complete response with a daily dose of 3 million units was about 3 months. IFN-α was effective in patients who had received other chemotherapeutic agents and in patients resistant to conventional cytotoxic drugs. In the majority of patients, the IFN dose required to maintain a normal platelet count during maintenance therapy was lower than the induction dose. In one study, 61% of patients required 3 million units three times a week, 15% once a week, and 24% daily. In addition, sustained remissions that persisted for 3 to 36 months were achieved with IFN-α therapy in 9% to 16% of patients. IFN-α is reported to be nonmutagenic and does not cross the placenta, making it a useful drug for the treatment of symptomatic pregnant patients with ET. Toxicity, especially in older patients, the need for parenteral administration, and cost limit the usefulness of IFN-α. Side effects include flu-like symptoms during induction therapy, such as fever, bone and muscle pain, fatigue, lethargy, and depression. Symptoms are frequently controlled with acetaminophen. Long-term administration of IFN-α can result in mild weight loss; alopecia; retinal abnormalities; and rarely, a reversible form of sarcoidosis and a reversible form of left-sided heart failure; and the development of autoimmune conditions, including thyroiditis leading to hypothyroidism and autoimmune hemolytic anemia. Patients may develop neutralizing antibodies to recombinant IFN, leading to a concomitant rise in platelet numbers. Approximately 25% of IFN therapy-treated ET patients discontinued therapy due to poor compliance or limiting side effects.

A semisynthetic protein-polymer conjugate of IFN-α2b, pegylated IFN (peg-IFN-α2b), was anticipated to be superior to unmodified IFN as related to its adverse event profile and efficacy when used to treat ET patients. This formulation of IFN provides prolonged activity that permits once-weekly dosing. Normalization of blood counts occurred after a median time of 2 to 3 months; 12% of patients discontinued therapy because of inability to tolerate the drug, and 17% did not achieve normalization of their platelet counts. The majority of side effects were WHO grade 1 or 2, although some encountered grade 3 toxicity, primarily fatigue and flu-like symptoms. More importantly, no thromboembolic or hemorrhagic complications occurred during the period of treatment, although 12 thrombotic events occurred in 42 patients (24%) in the 24 months before the institution of therapy. This form of IFN, however, appears to lead to a similar frequency and severity of side effects during long-term use as experienced with conventional IFN.

Interestingly, the use of another pegylated form of IFN (peg-IFN-α2a) in patients with PV was able to decrease the percentage of mutated *JAK2* allele in 24 out of 27 treated patients, from a mean of 49% to a mean of 27%. The use of this form of IFN appeared to be associated with fewer side effects than standard forms of IFN or peg-IFN-α2b. This form of pegylated IFN has been used to treat approximately 40 patients with ET, with more than 75% of patients achieving a complete hematologic remission. Almost 40% of patients with *JAK2*V617F-positive ET had a reduction in their *JAK2*V617F allele burden, with occasional patients no longer having detectable *JAK2*V617F, leading to their being classified as having achieved a complete molecular remission. Although hematologic remissions were frequently achieved after 3 months of therapy, the effects on *JAK2*V617F were observed after 6 months of treatment. Most patients were successfully treated with 45 to 90 μg/weekly, and 22% of patients ceased therapy because of toxicity. Surprisingly, the hematologic and molecular responses persist for a number of months after discontinuation of therapy, suggesting that intermittent therapy may be an acceptable means of chronically administering this drug. Additionally, molecular responses in a cohort of 31 *CALR*-mutated ET patients treated with peg-IFN-α2b were reported with a baseline allelic frequency of 41% reduced to 26% and two complete responses. Patients with additional subclonal mutations (*TET2*, *ASXL1*, *IDH2*, and *TP53*) were less likely to achieve molecular responses. This is consistent with the finding that patients harboring concurrent mutated *JAK2* and *TET2* can still have evidence of persistent *TET2*, despite complete response of *JAK2*V617F after peg-IFN-α2b therapy. Czech and coworkers noted that the effects of IFN on hematological responses were independent of driver mutational status but that the overall molecular remission rate was 78% for *JAK2*V617F-positive patients and 30% for *CALR*-mutated patients ($P = 0.004$).[25] These data reflect important biological differences between *CALR*-mutated and *JAK2* mutated ET, which might have important therapeutic implications.

Yacoub et al.[26] investigated the outcomes of pegylated-rIFN-α2a therapy in ET patients previously treated with hydroxyurea. The Myeloproliferative Neoplasm Research Consortium (MPN-RC)-111 study was an investigator-initiated, international, multicenter, prospective phase 2 trial which evaluated pegylated-rIFN-α2a therapy in 65 high-risk ET patients that were either refractory or intolerant to hydroxyurea. At 12 months, the overall response rate (complete or partial remission) in ET patients was 69.2%. Complete remission rates were higher in ET patients with *CALR* mutations than without (56.5% vs. 28.0%; $P = 0.01$), however there was no significant effect of pegylated-rIFN-α2 on VAF of the *CALR* mutation. However, pegylated-rIFN-α2a did have an effect on *JAK2*V617F VAF with a median absolute reduction of −6% in patients achieving a CR versus +4% in patients with only a partial response or a nonresponse. There was a significant rate of adverse events with pegylated-rIFN-α2a treatment; most were manageable, with only 13.9% of patients requiring discontinuation of pegylated-rIFN-α2a due to adverse events. There was no major bleeding events, and at 1 year, the cumulative incidence of major vascular events was 2%. The effect of this treatment on disease progression cannot be evaluated due to the limited study period. These investigators concluded that pegylated-rIFN-α2a is an effective therapy for patients with ET who were previously refractory and/or intolerant of hydroxyurea.

To gain a more comprehensive assessment of the clinical usefulness of pegylated-rIFN-α2a in newly diagnosed ET patients, a prospective clinical trial comparing pegylated IFN-α2a with hydroxyurea in patients with high-risk ET and PV was completed at numerous sites in North America and Europe by the MPN-RC. The primary objective of this phase 3 trial was to compare the complete remission rate after 12 months of treatment with either pegylated IFN-α2a or hydroxyurea in newly diagnosed patients with ET, and the secondary objectives were to compare toxicity and tolerability. The overall response rate (complete remissions + partial remissions) at 12 months was 69.8% for hydroxyurea, 78% for pegylated IFN-α2a ($P = 0.22$), and for those patients remaining on trial at 24 months, the overall response rate was 40.7% for hydroxyurea and 59.6% for pegylated IFN-α2a ($P = 0.04$). Pegylated IFN-α2a and hydroxyurea were similar in their effect on spleen size and histopathologic resolution, and both were associated with low incidence of transformation to MF/leukemia and thrombosis. Overall short-term, pegylated IFN-α2a treatment of ET was not found to be superior to hydroxyurea in remission rate or in decreasing symptom burden. However, it was associated with a higher rate of grade (¾) toxicity.

Based on the literature discussed above, we recommend that IFN-α or anagrelide be considered as reasonable alternatives to hydroxyurea in a patient younger than 60 years of age who has had a previous thrombotic episode (see Table 71.7). Which drug should be considered the standard of care in so-called *high-risk patients* remains a topic of debate.

A number of small-molecule inhibitors of *JAK2* have been evaluated for the treatment of ET patients. These agents reproducibly lower platelet counts and have been shown to decrease symptom burden. Harrison et al. evaluated the utility of ruxolitinib in ET patients resistant or intolerant to hydroxyurea in a randomized phase 2 trial of ruxolitinib vs best available therapy in ET. Fifty-eight and 52 patients were randomized to receive ruxolitinib or best available therapy, respectively. There was complete response within 1 year in 27 (46.6%) patients treated with ruxolitinib vs 23 (44.2%) with best available therapy ($P = 0.40$), indicating ruxolitinib did not elicit a better response rate. Similarly, rates of transformation, thrombosis, and hemorrhage were not significantly different at 2 years. There was, however, some improvement in disease-related symptoms in patients treated with ruxolitinib, relative to best available therapy. Patients treated with best available therapy did not experience any grade 3 or 4 anemia or thrombocytopenia, whereas a proportion of ruxolitnib-treated patients did. Based on these findings, it is clear that ruxolitinib is not superior to other second-line treatments for ET. Ruxolitinib is currently not approved for use in ET patients.[27]

The use of platelet antiaggregating agents remains an important area of investigation. Patients with ET have an increased predisposition to hemorrhage, which is likely potentiated by the use of drugs that affect platelet function. Transient ischemic attacks and erythromelalgia associated with ET respond rapidly to aspirin alone. In erythromelalgia, symptoms disappear for 2 to 4 days after the administration of a single dose of aspirin. Although these agents surely have a role in the treatment of these specific complications, their use should be pursued with extreme caution because of the increased risk of hemorrhage. Low-dose aspirin therapy has been uniformly recommended for virtually all patients with ET, independent of their risk of developing a thrombotic event. These recommendations are somewhat surprising because they are based on two clinical trials of the effects of low-dose aspirin therapy in PV patients, and such a trial with aspirin has never been performed with a population of ET patients. An independent analysis of the data from these two trials by the Cochrane Collaboration indicated that the use of low-dose aspirin therapy was associated with a statistically nonsignificant reduction in the risk of fatal thrombotic events and was not associated with an increased risk of bleeding episodes.

After a retrospective study of 198 patients with low-risk ET, Alvarez-Larrán et al.[10] concluded that antiplatelet therapy did not reduce the incidence of thrombotic events and might increase the bleeding risk if platelet counts are greater than 1000×10^9/L or aspirin is used in patients with a bleeding history (Table 71.8). A retrospective study of 433 patients (*CALR* = 271, *JAK2*V617F = 162) with low-risk ET receiving aspirin demonstrated no significant reduction in thrombotic complications but rather an increase in hemorrhagic complications in the *CALR*-mutated cohort (12.9 vs. 1.8 × 1000 patient-years, $P = 0.03$. Interestingly, the presence of *JAK2*V617F and cardiovascular risk factors was associated with a higher risk of thrombosis, which was reduced with administration of aspirin.

Chu et al.[28] also performed a meta-analysis to evaluate the risks and benefits of aspirin therapy in adult ET patients. This systematic review analyzed the benefits and risks of antithrombotic therapy in ET, including 24 observational studies with 6153 patients in total, with most being categorized as high-risk. This analysis highlights the great uncertainty in this area, reporting that the relative risk of thrombosis and bleeding with antiplatelet therapy, compared to without, ranged from 0.26 to 3.48 and 0.48 to 11.04, respectively. They concluded that the presently available evidence for aspirin in adult ET patients was imprecise, inconsistent, and "at best at high-risk of bias." They recommended that randomized studies be pursued in subgroups, perhaps based on their risk for thrombosis, or their driver mutational status. One can only conclude from these studies that the data supporting the universal use of aspirin in ET patients is limited. Most clinicians use aspirin indiscriminately in ET, but this practice requires re-consideration.

Most recently, a randomized double-blind trial assessed the utility of 3 aspirin regimens (aspirin 100 mg one, two, or three times daily for 2 weeks) on platelet inhibition in ET. Dosing of aspirin once daily led to incomplete inhibition in most patients. Twice daily dosing of aspirin improved platelet inhibition, and thrice dosing did not provide any further inhibition).[29] It remains to be determined whether greater inhibition of platelet activity correlates with symptom burden and risk of thrombosis in ET patients.

Aspirin should be used with caution in patients with peptic ulcer disease. Such patients who require aspirin benefit from the concurrent use of a proton pump inhibitor such as omeprazole rather than switching them to clopidogrel. This more critical evaluation of the evidence supporting the indiscriminate use of aspirin therapy in ET patients requires serious reconsideration of this practice. In patients who absolutely require aspirin therapy, one should avoid the simultaneous

TABLE 71.8 **Outcome in Patients With Low-Risk Essential Thrombocythemia Followed With Careful Observation or Treated With Antiplatelet Therapy**

	Observation (848 Person-Years)		Antiplatelet Therapy (802 Person-Years)		*P*
	Events (*n*)	Incidence Rate (95% CI)	Events (*n*)	Incidence Rate (95% CI)	
Thrombosis (arterial and venous)	15	17.7 (107–29.3)	17	21.2 (13.2–34.1)	0.6
Arterial thrombosis	8	9.4 (4.7–18.9)	13	16.2 (9.4–27.9)	0.2
Venous thrombosis	7	8.2 (3.9–17.3)	4	4.9 (1.9–13.3)	0.4
Bleeding	5	6.0 (2.5–14.5)	10	12.6 (6.8–23.4)	0.09

CI, Confidence interval.
From Alvarez-Larrán A, Cervantes F, Pereira A, et al. Observation versus antiplatelet therapy as primary prophylaxis for thrombosis in low-risk essential thrombocythemia. *Blood*. 2010;116(8):1205–1387.

administration of aspirin with a nonsteroidal antiinflammatory drug (NSAID) such as ibuprofen or naproxen because such NSAIDs are known to compete with aspirin for a common binding site on COX-1 that prevents aspirin from gaining access to and acetylating its target serine. Low-dose aspirin therapy must be restricted to patients with platelet counts of less than 1500 × 10^9/L and not be used in patients receiving anagrelide therapy unless they have experienced an arterial thrombotic event. The diagnosis of acquired von Willebrand syndrome should be excluded before aspirin use and considered a contraindication to the use of aspirin.

Whether all patients with low-risk ET should be uniformly treated with aspirin remains speculative because prospective randomized clinical trials including appropriate numbers of patients so as to assure the resolution of this dilemma have not been completed to date.

A continuing clinical controversy revolves around the question of whether any treatment is indicated in patients with ET in whom the platelet count elevation is initially detected fortuitously and who remain largely asymptomatic. In a retrospective study of 99 consecutive low-risk ET patients (age <60 years) who presented with extreme thrombocytosis (platelet count >1000 × 10^9/L) but without a previous history of thrombohemorrhagic complications, the incidence of major thrombosis and hemorrhagic events was shown to be similar during follow-up to those who were treated with prophylactic cytoreductive therapy and those who did not receive such therapy. If the clinician feels compelled to use some therapeutic intervention in young, asymptomatic patients, low-dose aspirin (81 mg/day) appears to be effective in the treatment of microvascular complications, and its use is associated with limited toxicity. Still, it seems reasonable to withhold therapy in younger, asymptomatic patients until the development of a clinically significant thrombotic or hemorrhagic event. Patients older than 60 years of age with other significant risk factors for cardiovascular complications are probably best served by immediate institution of therapy. A sensible strategy for administering aspirin therapy to ET patients was provided by Robinson and Godfrey. Their practice is to provide aspirin therapy to low-risk *JAK2*- and *MPL*-mutated ET patients. However, for low-risk *CALR*-mutated ET patients, they are reluctant to treat them with aspirin due to a lack of evidence available justifying its use given the relatively higher risk of bleeding. They concluded that aspirin is indicated in ET patients with increasing age or with cardiovascular risk factors but should be avoided in patients with platelet counts greater than 1000 × 10^9/L. With a platelet count 1000 to 1500 × 10^9/L, patients are requested to monitor for bleeding or bruising episodes and they should be evaluated for acquired von Willebrand syndrome. If these patients have a major bleeding episode, aspirin should be discontinued immediately and platelet transfusion therapy should be considered if the severity of the bleed merits such an intervention. Aspirin should be withheld in any patient with overt bleeding, including those patients with acquired von Willebrand syndrome; the bleeding in such patients will likely resolve in with normalization of platelet numbers with hydroxyurea. At this time of platelet normalization aspirin therapy can reconsidered.

The management of pregnant patients with ET remains problematic. The major goal of any therapeutic intervention in pregnant patients with ET should be the prevention of the vasoocclusive events that lead to placental infarction; intrauterine fetal growth retardation; and, in some cases, fetal death. Patients with *JAK2*V617F-positive ET are at a higher risk of developing such complications. In one large series, there was no significant relationship between the fetal outcome and the degree of maternal thrombocytosis or the presence of disease complications. In this series, there were no instances of excessive bleeding or other related complications during delivery. This group did not recommend the use of therapeutic plateletpheresis and, in fact, claimed that specific therapy (aspirin, heparin, or plateletpheresis) did not alter the clinical course. Low-dose aspirin (81 mg/day), because of its profound effect on events involving the microcirculation such as erythromelalgia and transient ischemic events, has been used with increasing frequency in pregnant patients during the first and second trimesters. Low-dose aspirin therapy is safe in pregnant women.

It is recommended that aspirin be discontinued at least 1 week before delivery to avoid bleeding complications such as an epidural hematoma during delivery or during the postpartum period. Because of the high-risk of bleeding in patients with platelet counts greater than 1000 × 10^9/L with acquired von Willebrand syndrome, aspirin therapy is contraindicated. There is limited experience with aspirin therapy alone, and although the results are promising, the sample size is too small to confirm a beneficial effect. The observed true birth rate, however, was 75% in those receiving aspirin, compared with 43% in the group in the literature who received no therapy. Aspirin therapy has, however, been found to be ineffective in preventing complications in *JAK2*V617-positive pregnant ET patients. Chemotherapeutic drugs should be avoided during the period of conception and especially during the first trimester. Both hydroxyurea and busulfan are known teratogens in animal models. In addition, busulfan and hydroxyurea reduce fertility in men. Because the greatest risk of thrombosis is postpartum, thrombosis prophylaxis should be initiated in the form of low-molecular-weight heparin and low-dose aspirin after delivery, unless the patient is hemorrhaging. These measures should be continued for approximately 6 weeks. Mothers receiving IFN, anagrelide, or hydroxyurea should refrain from breastfeeding.

IFN-α therapy is not known to be leukemogenic or teratogenic, and because it does not cross the placenta, its use may be considered during pregnancy. The manufacturers of IFN-α still advise that IFN-α not be used during pregnancy because adverse effects on the fetus cannot be ruled out. The effect of IFN-α on male fertility remains uncertain. Schrickel et al.[30] however, have reported a 73.5% live birth rate in 34 high-risk pregnancies in 23 ET patients who had previously experienced a thrombosis, a serious bleeding event, microcirculatory events or extreme thrombocytosis after treatment with IFN. Such promising results should not be dismissed and provide a rationale for IFN therapy in high-risk ET patients during their pregnancy. Anagrelide therapy should be avoided in pregnant patients because of its potential to lead to fetal thrombocytopenia.

Hormone-replacement therapy, including oral contraceptives and estrogen-replacement hormone therapy, remains controversial in patients with ET. Each of these agents in the normal population is associated with an increased incidence of arterial and venous thrombosis. Intuitively, it would seem wise to avoid such agents in patients with ET who are already at an increased risk of developing thrombosis. Gangat et al. retrospectively reviewed the consequences of such hormonal interventions in 305 women. Oral contraceptive therapy was associated with a high incidence of venous thrombosis occurring within the abdominal cavity, but estrogen-replacement hormone therapy in menopausal women did not appear to be associated with an increased incidence of thrombosis. This observation is surprising and might be a consequence of the limited numbers of patients included within this study.

If a patient with ET develops a thrombotic episode, anticoagulation with low-molecular–weight heparin and then transition to an oral anticoagulant is recommended. Vitamin K antagonists such as warfarin have traditionally been the agents of choice in MPN. Treatment of MPN patients with vitamin K antagonists after thrombosis has been shown to result in nearly a threefold decrease in risk of rethrombosis. Recently, the use of direct oral anticoagulants (DOACs) as alternatives to vitamin K antagonists for the prevention of rethrombosis in MPNs has been explored. However, this is limited to small retrospective studies. The DOACs currently in use include Factor Xa inhibitors (apixaban, rivaroxaban, and edoxaban) and the direct thrombin inhibitor dabigatran. Overall, it appears that the use of DOACs prevents rethrombosis to a similar degree as vitamin K antagonists and that risk of bleeding events is not significantly different between these two types of anticoagulants. A study of 442 MPN patients receiving DOACs indicated that dabigtran was associated with a higher risk of bleeding than the other DOACs. It must be noted, however, that these studies are small, heterogenous, and include MPN patients with varying diagnoses. Therefore results are difficult to compare. A randomized trial comparing vitamin K antagonists and DOACs in

MPNs would be necessary to determine the superior anticoagulant class in MPNs, but due to the rarity of the disease and long-term follow-up needed, this is unlikely to be performed. In our practice, we routinely utilize DOACs for prevention of rethrombosis in our MPN patients, finding that adherence to these agents in comparison to warfarin is much more feasible.

A thrombotic episode places a patient into a high-risk group in which myelosuppressive therapy is clearly indicated. Whether patients should have lifelong anticoagulation or should receive only 6 to 12 months of anticoagulation after they are in a complete hematologic remission has not been investigated in a systematic fashion. Because the data necessary to make this decision are not available, this decision can only be made at the discretion of the treating physician. It is our practice and that of many others to continue lifelong myelosuppressive therapy and anticoagulation in those high-risk ET patients with a prior unprovoked thrombotic episode. However in those high-risk ET patients who have had a provoked thrombotic events (surgery, sedentary behavior) it is our practice to discontinue anticoagulation therapy after 12 months but to continue myelosuppressive therapy. Antiplatelet therapy has been shown to reduce the risks of deep venous thrombosis and pulmonary embolism in a variety of high-risk groups. In patients who have a new thrombosis while they are in complete hematologic remission and are optimally anticoagulated, some consideration should be given to adding low-dose aspirin if the risk of hemorrhage is not excessive.

COVID-19 in Essential Thrombocythemia Patients

The incidence of COVID-19 infection in patients with MPNs appears to be relatively low when compared to patients with other neoplasms. However, ET patients infected with COVID-19 appear to be at a particularly heightened risk of complications when compared with PV and MF patients. In a retrospective, multicenter European study of 162 MPN with COVID-19 and all but one patient on low-molecular-weight heparin prophylaxis, ET patients were found to be at a greater risk of developing venous thromboembolism and experiencing mortality from pneumonia than PV and MF patients. Interestingly, COVID-19 infection was associated with a lowering of platelet count in MPN patients, with the greatest reductions being in ET patients. The effectiveness of vaccination in preventing COVID-19 in the MPN population remains to be determined. However, an initial study shows that one dose of the SARS-CoV-2 mRNA vaccine results in an antibody and T-cell response at a high frequency. Further studies are essential to understand the long-term effects of COVID-19 infection in ET patients and the optimal vaccination strategy to prevent infection in these vulnerable patients. At present, we strongly recommend that all ET patients be vaccinated against COVID-19 infection. Although aspirin therapy has been proposed to improve outcomes of normal individuals with COVID-19 infections, a recent meta-analysis indicated that aspirin therapy did not have a protective effect among different groups of patients.

FUTURE DIRECTIONS

ET is a hematologic malignancy with its own distinct clinical manifestations and associated complications. Better means of identifying patients at risk for developing fatal thrombotic or hemorrhagic complications are necessary to provide the basis with which to develop the optimal care of such patients. The ability to reduce the incidence of thrombohemorrhagic episodes with cytoreductive therapy in high-risk patients is well established, yet the optimal agent to employ remains a subject of controversy. The resolution of this dilemma will require a randomized clinical trial that uses clinical events as endpoints and that would require over a decade of follow-up. Whether the resources, both financial and patient numbers, would be available for performing such a trial remains questionable. The use of low-dose aspirin therapy to reduce the number of episodes of erythromelalgia and transient ischemic attacks is widely practiced, but whether aspirin therapy should be indiscriminately used remains a subject of dispute that will only be resolved with the completion of appropriately powered clinical trials. Another pressing question that requires resolution is the degree of reduction of platelet or leukocyte numbers required for optimal management of ET patients. In multiinstitutional trials, pegylated IFN has been shown to be an effective therapy in patients resistant and/or intolerant to hydroxyurea and is also an acceptable alternative to hydroxyurea in high-risk ET patients.

Finally, the discovery of the *JAK2*V617F, *MPL*, and *CALR* mutations has had a major impact on disease classification during routine clinical practice. Diagnostic strategies have now incorporated screening for these driver mutations. This new understanding of the molecular pathogenesis of MPN has led to the development of novel targeted therapies. Now that the clinical phenotype and the risk for disease progression of ET patients are known to be related to not only the ET- related driver mutation but also the burden of additional myeloid malignancy associated mutations, a more personalized approach to treatment options will likely be a goal in the future. This goal will require the discovery of new drugs or therapeutic approaches such as immunotherapeutic strategies.

It is important to note that, at present, a cure for ET is not on the horizon. While current available treatments reduce symptom burden and thrombotic risk, they have not been shown to prevent myelofibrotic or leukemic transformation. Preclinical and clinical evaluation of potential therapies that will target the malignant stem cell are essential for advancement in the care of ET patients.

PERSONAL APPROACH TO THERAPY OF ESSENTIAL THROMBOCYTHEMIA

The optimal therapy for all patients with essential thrombocythemia (ET) remains uncertain. There are currently no therapies that are confirmed to prevent progression of ET to MF or MPN blast phase. The current goal of therapy is to decrease thrombotic risk. Patients with the greatest risk of developing a thrombus have a number of characteristics, including age 60 years or older, history of a thrombotic event, leukocytosis (platelet count ≥11,000 × 10^9/L), and cardiovascular risk factors (hypertension, hypercholesterolemia, diabetes mellitus, obesity). All patients with ET should stop smoking to minimize the risk factors associated with atherosclerotic disease. Indiscriminant use of high doses of nonsteroidal antiinflammatory drugs should be avoided because this practice can lead to an increased risk of hemorrhage. Oral contraceptives should not be used due to their association with a heightened risk of venous thromboembolism. We currently recommend that ET patients receive COVID-19 vaccination.

In high-risk patients, cytoreductive therapy has been shown to lessen the chance of developing additional thrombotic events with the reduction of extreme thrombocytosis to platelet counts below 600,000 × 10^9/L. High-risk patients include patients older than 60 years of age and patients with a history of a previous thrombotic episode, including erythromelalgia, transient ischemic attacks, or large-vessel thrombosis. Even though this treatment philosophy has been considered common practice, the recommendation to treat patients older than the age of 60 years who have not experienced a thrombotic episode with cytoreductive therapy is not based on robust data from multiple randomized trials. Asymptomatic high-risk patients without cardiovascular risk factors may not necessarily benefit from this treatment, and the decision on how to treat them should be based on individual assessment.

Those patients who have venous thromboembolism should be treated with an anticoagulant to prevent rethrombosis. We often initially treat with low-molecular-weight heparin and then switch to an oral anticoagulant, either warfarin or a direct oral anticoagulant. Patients with a provoked thrombosis should receive at least 1 year of anticoagulation, and patients with unprovoked thromboses should receive lifelong myelosuppressive therapy and anticoagulation if the risk-benefit ratio warrants it.

At present, no therapy is indicated in asymptomatic patients younger than 60 years of age. If a patient has a platelet count greater than or equal to 1500 × 10^9/L and acquired von Willebrand syndrome with bleeding symptoms, platelet-reduction therapy is indicated to avoid the high risk of hemorrhage. In totally asymptomatic patients with platelet counts greater than 1500 × 10^9/L, we frequently observe the patients and do not feel compelled to treat them. Patients with acquired von Willebrand syndrome should clearly avoid the use of aspirin.

In patients requiring platelet reduction therapy, the choice among the use of anagrelide, IFN-α, pegylated IFN, or hydroxyurea therapy is based on patient age, ease of administration, and drug-related toxicity. In patients older than 50 years, hydroxyurea or pegylated-IFN therapy is the treatment of choice, but in younger patients, we prefer to initiate therapy with pegylated-IFN. IFN therapy should be avoided in patients with a history of depression, autoimmune disorders, or retinitis. If the patient cannot tolerate IFN or it is not available, we feel comfortable treating symptomatic patients younger than 50 years of age with anagrelide or hydroxyurea. The leukemogenic potential of hydroxyurea appears to be low if not associated with the prior use of an alkylating agent. The development of malleolar ulcers is a frequent complication of hydroxyurea treatment and is a signal for the elimination of hydroxyurea as a therapeutic agent for that particular patient.

Patients who initially receive hydroxyurea and no longer respond to this agent or experience toxicity, thus requiring another agent, should not receive an alkylating agent. This sequence of administration is associated with an extremely high risk of leukemic transformation. Patients who have had a trial of hydroxyurea and require further treatment should receive either IFN-α, pegylated IFN, or anagrelide. Doses of each of these agents required for disease control will, of course, be dependent on the target platelet level that one hopes to achieve. Strict control to a platelet count of lower than 600 × 10^9/L does not appear to be necessary. In these patients, the addition of low-dose aspirin (81 mg/day) should be considered; it is less clear to us if patients who achieve better platelet control with cytoreductive therapy should also be so treated. However, in studies from Europe addressing this question in polycythemia vera, the approach of combining aspirin with cytoreduction therapy appears to minimize thrombotic complications. The use of anagrelide and aspirin in combination should be avoided because of the higher risk of hemorrhage. In patients with thrombotic episodes, especially episodes involving the microcirculation or large vessels, we administer low-dose aspirin (81 mg/day). This dose of aspirin does increase the number of bleeding episodes to a modest degree but is effective in the treatment of thrombotic events. This low-dose aspirin therapy is given in addition to an agent that reduces platelet numbers.

Hydroxyurea can be started at a dose of 1 g/day and then adjusted to achieve the target platelet count (≤600 × 10^9/L) without developing leukopenia. Anagrelide is initiated at 0.5 mg twice daily and increased by 0.5 mg/day every 5 to 7 days if platelet counts do not begin to drop. The usual dose to achieve platelet number control is 2.0 to 2.5 mg/day. Alternatively, combination therapy with anagrelide and hydroxyurea may be considered. Some patients do not tolerate either hydroxyurea or anagrelide. In this patient group, IFN-α therapy with a pegylated form should be given, starting at a dose of 45 mcg/weekly and increasing the dose monthly until a target platelet count is achieved. Patients receiving IFN-α therapy should be closely monitored for adverse events, including yearly ophthalmological examinations as well as mood assessments. Busulfan at 4 mg/day for 2-week courses every time the platelet count rises above the normal range is another therapeutic option. Busulfan therapy is typically reserved for patients older than 70 years of age.

Complications even in young, otherwise healthy patients with platelet counts greater than 2000 × 10^9/L are unusual. However, these marked elevations of platelet numbers can be anxiety-provoking situations for the patient and the clinician.

In certain situations, in young, low-risk patients, treatment should be instituted. Surgery can increase the risk of thrombosis, and the use of anti-inflammatory agents can increase the risk of bleeding postoperatively. Under these circumstances, the platelet count should be lowered to the normal range. In pregnant patients with ET, low-dose aspirin therapy is the first treatment option. If the patient develops symptoms as a result of thrombosis, platelet reduction therapy is necessary, and IFN-α therapy is the treatment of choice.

In a patient with ET and a serious acute hemorrhagic event, the site of bleeding should be determined immediately, and any antiplatelet-aggregating agents should be stopped. Although the platelet count may be high, these platelets should be considered to be qualitatively abnormal, leading to defective hemostasis. The patient may have acquired von Willebrand syndrome. In patients with acquired von Willebrand syndrome, desmopressin (DDAVP) or factor VIII concentrates containing von Willebrand factor can be used immediately at the same time chemotherapy is being administered. If acquired von Willebrand syndrome is not present, the transfusion of normal platelets is suggested. In patients with persistent hemorrhage, immediate reduction of the platelet count can be achieved by plateletpheresis. If this approach fails, some consideration to the use of activated factor VIIa should be given. Hydroxyurea at 2 to 4 g/day for 3 to 5 days should be administered immediately and then reduced to 1 g/day. All patients receiving hydroxyurea should be monitored for the onset of granulocytopenia or thrombocytopenia. Reduction of platelet counts is usually observed within 3 to 5 days of hydroxyurea treatment.

In contrast, patients with acute arterial thrombosis require immediate institution of platelet antiaggregating agents. Aspirin at a dose of 81 mg/day is suggested. Patients with erythromelalgia or transient ischemic attacks will have a rapid cessation of symptoms after the use of low-dose aspirin. In a patient with a life-threatening arterial thrombosis, the platelet count should be lowered with either a combination of apheresis and hydroxyurea or with hydroxyurea alone, depending on the severity of the event. If the arterial thrombosis involves the microcirculation and is not life-threatening (transient ischemic attacks or erythromelalgia), immediate low-dose aspirin therapy is indicated, and platelet-reduction therapy (hydroxyurea, anagrelide, or IFN-α) can be initiated using a standard dose and schedule.

REFERENCES

1. Srour SA, Devesa SS, Morton LM, et al. Incidence and patient survival of myeloproliferative neoplasms and myelodysplastic/myeloproliferative neoplasms in the United States, 2001–12. *Br J Haematol*. 2016;174(3):382–396.
2. Grinfeld J, Nangalia J, Baxter EJ, et al. Classification and personalized prognosis in myeloproliferative neoplasms. *N Engl J Med*. 2018;379(15): 1416–1430.
3. Liu P, Zhao L, Loos F, et al. Immunosuppression by mutated calreticulin released from malignant cells. *Mol Cell*. 2020;77(4):748–760. e749.
4. Alimam S, Villiers W, Dillon R, et al. Patients with triple-negative, JAK2V617F- and CALR-mutated essential thrombocythemia share a unique gene expression signature. *Blood Adv*. 2021;5(4):1059–1068.
5. Tefferi A, Lasho TL, Guglielmelli P, et al. Targeted deep sequencing in polycythemia vera and essential thrombocythemia. *Blood Adv*. 2016;1(1): 21–30.
6. Luque Paz D, Jouanneau-Courville R, Riou J, et al. Leukemic evolution of polycythemia vera and essential thrombocythemia: genomic profiles predict time to transformation. *Blood Adv*. 2020;4(19):4887–4897.
7. Guglielmelli P, Pacilli A, Coltro G, et al. Characteristics and clinical correlates of NFE2 mutations in chronic myeloproliferative neoplasms. *Am J Hematol*. 2020;95(1):E23–E26.
8. Carobbio A, Thiele J, Passamonti F, et al. Risk factors for arterial and venous thrombosis in WHO-defined essential thrombocythemia: an international study of 891 patients. *Blood*. 2011;117(22):5857–5859.
9. Barbui T, Finazzi G, Carobbio A, et al. Development and validation of an international prognostic score of thrombosis in World Health Organization-essential thrombocythemia (IPSET-thrombosis). *Blood*. 2012;120(26):5128–5133. quiz 5252.
10. Alvarez-Larrán A, Pereira A, Guglielmelli P, et al. Antiplatelet therapy versus observation in low-risk essential thrombocythemia with a CALR mutation. *Haematologica*. 2016;101(8):926–931.
11. Barbui T, Vannucchi AM, Buxhofer-Ausch V, et al. Practice-relevant revision of IPSET-thrombosis based on 1019 patients with WHO-defined essential thrombocythemia. Blood. *Cancer J*. 2015;5:e369.
12. Pich A, Riera L, Francia di Celle P, et al. JAK2V617F, CALR, and MPL mutations and bone marrow histology in patients with essential thrombocythaemia. *Acta Haematol*. 2018;140(4):234–239.
13. Grozovsky R, Begonja AJ, Liu K, et al. The Ashwell-Morell receptor regulates hepatic thrombopoietin production via JAK2-STAT3 signaling. *Nat Med*. 2015;21(1):47–54.
14. McMullin MF. Diagnostic workflow for hereditary erythrocytosis and thrombocytosis. *Hematology Am Soc Hematol Educ Program*. 2019; 2019(1):391–396.

15. Karow A, Nienhold R, Lundberg P, et al. Mutational profile of childhood myeloproliferative neoplasms. *Leukemia*. 2015;29(12):2407–2409.
16. Etheridge SL, Cosgrove ME, Sangkhae V, et al. A novel activating, germline JAK2 mutation, JAK2R564Q, causes familial essential thrombocytosis. *Blood*. 2014;123(7):1059–1068.
17. Guglielmelli P, Pacilli A, Rotunno G, et al. Presentation and outcome of patients with 2016 WHO diagnosis of prefibrotic and overt primary myelofibrosis. *Blood*. 2017;129(24):3227–3236.
18. Mambet C, Necula L, Mihai S, et al. Increased Dkk-1 plasma levels may discriminate disease subtypes in myeloproliferative neoplasms. *J Cell Mol Med*. 2018
19. Haider M, Gangat N, Lasho T, et al. Validation of the revised international prognostic score of thrombosis for essential thrombocythemia (IPSET-thrombosis) in 585 Mayo Clinic patients. *Am J Hematol*. 2016;91(4):390–394.
20. Tefferi A, Pardanani A. Essential thrombocythemia. *N Engl J Med*. 2019; 381(22):2135–2144.
21. Barbui T, Thiele J, Passamonti F, et al. Survival and disease progression in essential thrombocythemia are significantly influenced by accurate morphologic diagnosis: an international study. *J Clin Oncol*. 2011;29(23):3179–3184.
22. Buxhofer-Ausch V, Steurer M, Sormann S, et al. Impact of white blood cells on thrombotic risk in patients with optimized platelet count in essential thrombocythemia. *Eur J Haematol*. 2018;101(2):131–135.
23. Carobbio A, Ferrari A, Masciulli A, et al. Leukocytosis and thrombosis in essential thrombocythemia and polycythemia vera: a systematic review and meta-analysis. *Blood Adv*. 2019;3(11):1729–1737.
24. Godfrey AL, Campbell PJ, MacLean C, et al. Hydroxycarbamide plus aspirin versus aspirin alone in patients with essential thrombocythemia age 40 to 59 years without high-risk features. *J Clin Oncol*. 2018;36(34):3361–3369.
25. Czech J, Cordua S, Weinbergerova B, et al. JAK2V617F but not CALR mutations confer increased molecular responses to interferon-α via JAK1/STAT1 activation. *Leukemia*. 2019;33(4):995–1010.
26. Yacoub A, Mascarenhas J, Kosiorek H, et al. Pegylated interferon alfa-2a for polycythemia vera or essential thrombocythemia resistant or intolerant to hydroxyurea. *Blood*. 2019;134(18):1498–1509.
27. Harrison CN, Mead AJ, Panchal A, et al. Ruxolitinib vs best available therapy for ET intolerant or resistant to hydroxycarbamide. *Blood*. 2017; 130(17):1889–1897.
28. Chu DK, Hillis CM, Leong DP, et al. Benefits and risks of antithrombotic therapy in essential thrombocythemia: a systematic review. *Ann Intern Med*. 2017;167(3):170–180.
29. Rocca B, Tosetto A, Betti S, et al. A randomized double-blind trial of 3 aspirin regimens to optimize antiplatelet therapy in essential thrombocythemia. *Blood*. 2020;136(2):171–182.
30. Schrickel L, Heidel FH, Sadjadian P, et al. Interferon alpha for essential thrombocythemia during 34 high-risk pregnancies: outcome and safety. *J Cancer Res Clin Oncol*. 2021;147(5):1481–1491.

CHAPTER 72

PRIMARY MYELOFIBROSIS AND CHRONIC NEUTROPHILIC LEUKEMIA

Sangeetha Venugopal, Vesna Najfeld, Alla Keyzner, Siraj M. El Jamal,
Ronald Hoffman, and John Mascarenhas

Primary myelofibrosis (PMF) is a Philadelphia chromosome-negative, clonal, myeloproliferative neoplasm (MPN) that is often but not always accompanied by the driver mutations *JAK2V617F*, calreticulin exon 9 (*CALR*), and the thrombopoietin receptor, *MPL515L/K*. PMF is clinically characterized by progressive splenomegaly, cytopenias, and cytokine-driven constitutional symptoms with a propensity for leukemic transformation (20%). Pathologically, PMF is associated with megakaryocyte hyperplasia and atypia, reactive bone marrow (BM) fibrosis, angiogenesis, ineffective erythropoiesis, osteosclerosis, and extramedullary hematopoiesis (EMH), resulting in hepatosplenomegaly. Although there has been exceptional progress in unraveling the complex molecular underpinnings and refinement of the prognostic factors for PMF risk stratification, unfortunately, none of the currently available therapies reliably alters the disease trajectory. Since the last edition of this book, another JAK2 inhibitor, fedratinib, has been added to the therapeutic armamentarium of PMF, which previously included only ruxolitinib. Both these JAK2 inhibitors provide important symptom and spleen relief, but allogeneic hematopoietic stem cell transplantation (HSCT) remains the only curative therapy for PMF. This chapter describes the current understanding of the pathobiology, clinical features, and treatment approaches of PMF.

PHYLOGENY OF THE NOMENCLATURE: PRIMARY MYELOFIBROSIS

PMF was first reported in the literature in 1879, when a German surgeon, Gustav Heuck, described two young patients with massive splenomegaly, circulating nucleated red blood cells (RBCs), and abnormal leukocytes, which he referred to as "splenic-medullary leukemia." He noted that this disease differed from chronic myeloid leukemia (CML) due to the presence of bone marrow fibrosis (BMF), osteosclerosis, and EMH. Since then, several other terms have been used *synonymously* to describe PMF, including angiogenic myeloid metaplasia, myeloid metaplasia with myelofibrosis, and chronic idiopathic myelofibrosis. In 2007, the International Working Group for Myelofibrosis Research and Treatment (IWG-MRT) expert consensus proposed the term PMF to standardize the terminology.

In 1951, William Dameshek included PMF among a group of related disorders, which he termed *myeloproliferative disorders* (MPDs), based on clinical observations of patients with polycythemia vera (PV), CML, and essential thrombocythemia (ET) who developed BM fibrosis and a clinical picture resembling PMF. Additionally, several others described that these disorders originated from a common ancestral cell, reflecting its neoplastic nature. Accordingly, in 2008, the World Health Organization (WHO) modified the terminology from MPD to MPN.[1]

EPIDEMIOLOGY

PMF is rare among the MPNs. Epidemiologic studies have reported a variable incidence of PMF across Europe, Australia, and the United States (US), ranging between 0.5 and 1.3 per 100,000 per year. The annual incidence rate of PMF noted in Olmstead County (MN, USA) was 1.5 per 100,000. Recent Surveillance, Epidemiology, and End Results (SEER) database analysis (2000 to 2016) indicates that the incidence of PMF has remained constant. The median age of diagnosis is 69 years, with most diagnosed between 50 and 69 years of age. PMF is rare in the pediatric age-group. Evidence of genetic transmission does exist: a higher incidence has been reported in Ashkenazi Jews than in Arabs, who both live in Northern Israel, and a familial predisposition to MPNs, including PMF, has been identified. Gender distribution of PMF is reported variably, with some studies reporting male predominance while others do not.

PMF in the United States and Europe is epidemiologically similar, but there are differences in other parts of the world, which may be attributed to environmental exposures. These patients may be younger with an accelerated rate of disease progression. In Japan, PMF is considered a rare disorder. However, the incidence of myelofibrosis (MF) among survivors who were close to the hypocenter of the atomic bomb explosion at Hiroshima and Nagasaki was 18 times higher than the general population of Japan, and they became symptomatic approximately 6 years after the bomb blast. Such data indicate a strong link between excessive radiation exposure and development of PMF, which is further substantiated by the high incidence of PMF in patients who received the contrast material thorotrast (which contains ^{232}Th, a radioactive element with a half-life of 1.41×10^{10} years). Thorotrast is retained indefinitely by the reticuloendothelial cells, resulting in lifelong irradiation to the liver, spleen, lymph nodes, and BM. Less commonly, chronic exposure to industrial solvents (benzene and toluene) is associated with the development of PMF. Unlike PV, where clusters of patients identified in Eastern Pennsylvania raise the concern for possible environmental effects of waste-coal and Superfund sites, no epidemiologic studies definitively support environmental exposure in PMF.

PATHOBIOLOGY OF PRIMARY MYELOFIBROSIS

PMF originates from the pluripotent hematopoietic stem cell, and clonality was first established as early as the 1970s by glucose 6-phosphate dehydrogenase (G6PD) isoenzyme analysis at the post-DNA level, followed by several studies in the 1990s, including an X-linked DNA analysis that revealed a monoclonal X chromosome inactivation pattern in peripheral-blood leukocytes, granulocytes, bone marrow mononuclear cells, and, occasionally, T lymphocytes. The central dogma underlying PMF pathogenesis is based on the concept of a sequential accumulation of acquired genetic or epigenetic alterations within the founder clone population that ultimately drive disease progression and evolution to acute myeloid leukemia. Additionally, the bone marrow niche, megakaryocyte hyperplasia, and the sequent cytokine-induced inflammatory response, as well as aging process, further contribute to the pathobiology of PMF.

Akin to related MPNs, PMF mononuclear cells when cloned in semisolid media generate autonomous erythroid and megakaryocyte colony formation in addition to growth factor-dependent hematopoietic colony growth. Endogenous erythroid and megakaryocyte colonies are a hallmark of abnormal growth of hematopoietic progenitors in vitro, indicative of aberrant hyperactive growth factor signaling. The quest for the genetic basis of autonomous hematopoiesis in MPNs propelled the discovery of *JAK2* V671F in more than 90% of patients with PV and approximately 50% of patients with PMF.[2]

DRIVER MUTATIONS

JAK2 V617F is a gain-of-function mutation in the autoinhibitory domain of the JAK2 tyrosine kinase, resulting in constitutive activation of JAK2 that promotes signal transducer and activator of transcription (STAT)-mediated gene transcription and the resultant autonomous hematopoietic cell proliferation. In a murine transplant model, BM cells transduced with Jak2 V617F resulted in a two-stage MPN clinical phenotype, with the initial stage closely resembling PV with erythrocytosis and neutrophilia, followed by an early MF-like phenotype comprising anemia, thrombocytopenia, splenomegaly, and bone marrow fibrosis.

Furthermore, a small proportion of PMF patients harbor mutations in the lymphocyte adaptor protein (LNK or SH2B3) or Casitas B-cell lymphoma (CBL) genes encoding negative regulators of JAK-STAT signaling. Loss-of-function mutations in *LNK*, predictably result in JAK-STAT hyperactivation, and concomitant mutations in *JAK2* V617F and *LNK* are associated with leukemic transformation of PMF. Gain-of-function mutations in *CBL* are associated with acquired uniparental disomy of chromosome 11q, resulting in homozygous deletion of *CBL* and the resultant loss of its tumor suppressor function. *CBL* mutations occur in 6% of PMF patients and are associated with leukemic transformation. The *JAK2* V617F mutation is homozygous only in 13% of patients with PMF as opposed to 30% in PV, which is attributed to homologous recombination. Homozygosity of *JAK2* V617F in PMF patients is associated with frequent occurrence of unfavorable cytogenetic abnormalities (CGA). Although *JAK2* V617F occurs in almost all patients with PV, a sizeable number of patients with ET and PMF are wild-type for *JAK2*, which led to the pursuit of identifying other mutations that drive hyperactive JAK-STAT signaling.

In 2006, Levine et al., through exome sequencing, identified a somatic activating mutation in the transmembrane domain of the thrombopoietin receptor *MPL* (W515L) in 9% (4/45) of patients with *JAK2* V617F wild-type PMF.[3] *MPL* signals the phosphorylation of both MPL and JAK2/TYK2 and subsequently activates STAT3, STAT5, ERK/MAPK, and PI3K/AKT signaling pathways. In contrast to *MPL* wild-type, the W515L variant promotes ligand-independent JAK-STAT signal activation and confers cytokine-independent growth to 32D, Ba/F3, and UT7 cells. In a murine bone marrow transplant assay, isolated expression of *MPL* W515L led to fully penetrant lethal MPN (18 days) characterized by marked thrombocytosis, splenomegaly, and increased reticulin fibrosis but not erythrocytosis. These data suggest that the *MPL* mutation favors the development of thrombocytosis, but *JAK2* V617F favors erythrocytosis. Compared with wild-type, PMF patients with *MPL* 515L/K are older, severely anemic, and more likely to require transfusion support.

The next breakthrough occurred in 2013, when two groups independently reported frameshift mutations in exon 9 of calreticulin (CALR) in patients with ET and PMF. Approximately 25% of patients with ET and PMF harbor insertion/deletion mutations in *CALR*.[4] Furthermore, 80% of *CALR* mutations fall into either of the two categories: type 1 (52-bp deletion) or type 2 (5-bp insertion). Retrospective studies show that *CALR*-mutated PMF patients present with higher platelet counts, low hemoglobin levels, and leukocyte counts, with a lower thrombotic risk and better overall prognosis than patients with *JAK2* V617F. Studies also implicate a worse prognosis for type 2 *CALR*-mutated patients compared to patients with type 1 mutations.

CALR, located in chromosome 19p13.2, encodes a calcium-binding protein in the endoplasmic reticulum (ER), functions as a chaperone protein and maintains calcium homeostasis. The frameshift mutations in *CALR* exon 9 result in a mutant calreticulin protein with a novel C-terminus sequence lacking the ER-targeting KDEL sequence. The fact that c-MPL is an ER client protein and that *CALR* mutations occurred exclusively in patients with ET or PMF alluded to the possibility that mutant *CALR* somehow activates MPL. Consistent with this observation, *CALR* exon 9 mutation was shown to induce constitutive phosphorylation of JAK2 and activation of STAT transcription only in an MPL-dependent manner.

The extracellular domain of MPL, especially its sites for N-linked glycosylation (CALR binding site), is necessary for the mutant *CALR* to drive transformation. While the glycan-binding sites of *CALR* are crucial for activating MPL, the polypeptide-binding regions and chaperone activity of *CALR* are dispensable. Additionally, positively charged C-terminal residues of mutant *CALR* are necessary for activating MPL signaling; mutation of these residues to uncharged glycine residues abrogates its transforming ability. Of note, truncation of exon 9 terminated the transforming potential of mutant *CALR*, further underscoring the functional importance of the novel C-terminus. Analogously, removal of the 36 most distal residues from the new C-terminus prevented mutant *CALR* from transforming Ba/F3-MPL cells. However, this Δ36 mutant was still able to bind to MPL, suggesting that binding and activation of the receptor may be distinct processes. The exact pathobiological link between the *CALR* mutation and the dysregulated JAK-STAT pathway remains an area of ongoing investigation. Collectively, hyperactive JAK-STAT signaling is central to the pathogenesis of PMF.

Approximately 10% of PMF patients are "triple negative," that is, they lack any of the three driver mutations. Milosevic-Feenstra and coworkers, with whole-exome sequencing, identified noncanonical *MPL* mutations in 11.4% ($n = 70$) and *JAK2* mutations in 8.8% ($n = 57$) of patients with triple negative ET and PMF. These newly identified mutations were heterozygous, and the mutations in *MPL* and *JAK2* were mutually exclusive. 50% of triple negative patients lacked evidence for clonality, but the presence of germ-line mutations indicated that some of these individuals likely had a hereditary rather than an acquired MPN-like disorder. Based on these findings, sequencing of all coding exons of *MPL* and *JAK2* is recommended during the diagnostic work-up of triple-negative ET and PMF patients.

THE ROLE OF EPIGENETIC MUTATIONS IN THE MOLECULAR LANDSCAPE OF PRIMARY MYELOFIBROSIS

With the advent of next-generation sequencing (NGS) technologies, the identification of multiple epigenetic mutations has provided further insight into the complex molecular pathogenesis of PMF and offers rationale for the evaluation of novel targeted therapeutics. Table 72.1 lists the common epigenetic mutations recognized in patients with PMF.[5]

Translocation ten-eleven oncogene family member 2 (*TET2*), located on chromosome 4q24, occurs in approximately 15% of patients with myeloid malignancies. TET2 catalyzes the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine (5hmC), which are key intermediates in DNA demethylation. The resultant DNA hypermethylation affects HSC self-renewal and lineage differentiation. Hematopoietic-specific conditional deletion of Tet2 in mice led to global promoter hypermethylation, increased HSC survival, and a propensity towards chronic myelomonocytic leukemia (CMML)-like phenotype. Furthermore, the Tet2-null (Tet2−/−) background in $Jak2^{V617F}$ mice augments splenomegaly and accelerates the MPN phenotype, suggestive of clonal dominance of Jak2V617F/Tet2−/− double mutants. However, Jak2V617F/Tet2−/− mice did not show increased bone marrow fibrosis or leukemic transformation and

TABLE 72.1 **Acquired Genetic Lesions Identified in Patients With Primary Myelofibrosis**

Gene	Location	Mutation	Frequency
Janus kinase 2 (*JAK2*)	9p24 exon 14	*JAK2V617F*	65%
	9p24 exon 12		Infrequent
Calreticulin (*CALR*)	19p13.2 exon 9	Type 1 (52 bp del) and Type 2 (5 bp in)	25%
Ten-eleven translocation 2 (*TET2*)	4q24		17%
Serine/arginine-rich splicing factor 2 (*SRSF2*)	17q25.1		17%
U2 small nuclear RNA auxiliary factor 1 (*U2AF1*)	21q22.3		16%
DNA methyltransferase 3 alpha (*DNMT3a*)	2p23		15%
Enhancer of zeste homolog 2 (*EZH2*)	7q36.1		13%
Myeloproliferative leukemia virus (*MPL*)	1p34 exon 10	*MPL W515L/K*	5%–10%
Casitas B-lineage lymphoma (*CBL*)	11q23.3	Exons 8 and 9	6%
Splicing factor 3B unit 1 (*SF3B1*)	2q33.1		6%
Isocitrate dehydrogenase (*IDH1* and *2*)	2q33.3/15q26.1		4%
Lymphocyte specific adapter protein (*LNK*)	12q24		Infrequent
Ikaros family zinc finger 1 (*IKZF1*)	7p12		Infrequent
Additional sex Combs-like 1 (*ASXL1*)	20q11.1		10%–20%

resembled chronic phase MF in humans in which *TET2* mutations were associated with increasing age and the presence of *JAK2* V617F did not predict disease severity or survival. Clonality studies in MPN patient samples have failed to define a consistent temporal sequence of acquisition of *TET2* and *JAK2* mutations. These two genetic events appear to occur independently of each other, and *TET2* may even be acquired late in the progression of an MPN. Taken together, there is no evidence at present to show that restoration of TET2 activity may have disease-modifying effects in PMF.

Isocitrate dehydrogenase 1 and 2 (*IDH1/IDH2*), located on chromosome 2q33.3 and 15q26.1, respectively, encode $NADP^+$-dependent enzymes that catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate in the Kreb's cycle. The neomorphic mutant IDH preferentially transforms α-ketoglutarate to 2-hydroxyglutarate (oncometabolite), the accumulation of which inhibits *TET2* activity. Approximately 4% (12/301) of patients with PMF harbor *IDH1/IDH2* mutations (7 *IDH2* [5 R140Q, 1 R140W, and 1 R172G] and 5 *IDH1* [3 R132S and 2 R132C]) and they can co-occur with *JAK2*, *MPL*, and *TET2*. In patients with *JAK2*-mutated PMF, the presence of *IDH1/IDH2* mutations portends a poor prognosis in terms of leukemia-free survival (LFS) and overall survival (OS). Furthermore, *Idh+/Jak2 V617F+* double mutant transgenic MPN murine model demonstrated augmented blast cell expansion and differentiation arrest, consequently promoting progression to blast phase disease, thereby highlighting *JAK2-IDH1/2* cooperativity in the leukemic transformation of MPN. Retrospective clinical studies have also confirmed the relatively poor outcome of this double mutant subgroup of patients on both OS and risk of leukemic transformation.

The enhancer of zeste homolog 2 (*EZH2*) gene, located on chromosome 7q36.1, encodes a histone methyltransferase (H3K27) and is a catalytic subunit of polycomb repressor complex 2 (PRC2) required for HSC maintenance. PRC2 is a multiprotein enzyme complex (EZH2, SUz12, EED, and YY1) responsible for the trimethylation of lysine 27 on histone H3 (H3K27me3). Additionally, PRC2 can recruit other polycomb complexes, DNA methyltransferases (DNMTs), and histone deacetylases to the gene site, resulting in chromatin compaction and additional repressive activity. Frameshift mutations in *EZH2* resulting in truncated protein isoforms or missense mutations within the highly conserved methyltransferase domain suggest a tumor suppressor role of EZH2. In contrast to Tet2, conditional murine knockout of Ezh2 (Ezh2−/−) in Jak2V617F background rapidly induced bone marrow fibrosis and splenomegaly. While Jak2V617F knock-in mice exhibited a PV-like disease, Jak2V617F/Ezh2−/− mice displayed decreased erythroid differentiation and expansion of megakaryocytic precursors, correlating with peripheral blood thrombocytosis. Given that *EZH2* encodes an H3K27 methyltransferase, loss of EZH2 function reduced H3K27me3 levels, leading to transcriptional activation of target genes, including two proinflammatory cytokine-like mediators, S100a8 and S100a9, as well as high-mobility AT-hook 2 (HMGA2). Thus, loss of EZH2 transcriptional repression in the background of *JAK2* V617F results in a more severe MPN phenotype than *JAK2* V617F alone and favors progression to MF. Approximately 13% of patients with PMF harbor an *EZH2* mutation, which commonly co-occurs with either *JAK2* V617F or additional sex combs-like 1 (*ASXL1*) mutation. Most of these patients exhibited a high-risk phenotype: leukocytosis, increased blast counts, and massive splenomegaly in addition to reduced LFS and OS independent of the dynamic international prognostic scoring system (DIPSS) risk category and *JAK2* V617F allele burden.

ASXL1 mutation occurs in 10% to 20% of patients with PMF, predominantly in those who are triple negative. *ASXL1*, located on chromosome 20q11.1, encodes an enhancer of the trithorax group (trxG) and PRC. The PRC proteins (repress) and trxG proteins (promote) regulate gene expression of homeotic genes, such as *Hox* genes, via histone methylation. *ASXL1* mutations result in loss of PRC2-mediated H3 lysine (H3K27me3) trimethylation. Although conditional deletion of Asxl1 in murine hematopoietic stem cells resulted in progressive anemia and leukemia with multilineage dysplasia akin to human myelodysplastic syndrome (MDS) without myelofibrosis, it is of prognostic importance in PMF. The presence of *ASXL1* is an independent predictor of poor prognosis, and its presence negates the favorable prognosis of *CALR*-mutated PMF. Additionally, the acquisition of an *ASXL1* mutation (approximately 30%) during ruxolitnib therapy is associated with poor outcome at the time of discontinuation of the drug.

Ikaros is a Kruppel-like zinc finger transcription factor integral to normal hematopoiesis and is encoded by the Ikaros family zinc finger 1 (*IKZF1*) gene located at 7p.12. Compared to chronic phase MPNs (0.2%), abnormalities in *IKZF1* occur at a higher rate (21%) of patients with MPN blast phase (MPN-BP), mostly in association with del 7p. IKZF1 is believed to modulate the expression of lineage-specific genes through chromatin remodeling, which results in effective lymphoid development and tumor suppression. Induced *Ikzf1* haploinsufficiency in primary murine stem cells resulted in increased Stat5 phosphorylation and cytokine-dependent growth, suggesting that reduced expression of *IKZF1* is sufficient to perturb growth regulation and that *IKZF1* loss is an important step in the leukemic transformation in a subset of MPN patients.

RNA spliceosome mutations such as Serine and Arginine Rich Splicing Factor 2 (*SRSF2* P95H) and U2 Small Nuclear RNA Auxiliary Factor 1 (*U2AF1* Q157) occur in 14% and 8% patients with PMF, respectively. *SRSF2* mutations cluster with *IDH* mutations and are independently predictive of poor outcomes in terms of shortened LFS and OS. *U2AF1* Q157 occurs primarily in older patients with PMF and is associated with anemia, thrombocytopenia, and inferior survival. In a large series of patients with myelofibrosis that received HSCT, the presence of *U2AF1* mutation was associated with poor survival outcomes.

EXTRAMEDULLARY HEMATOPOIESIS IN PRIMARY MYELOFIBROSIS

Frequently observed peripheral blood abnormalities in PMF are likely attributed to the EMH inherent to this disease. Abnormal CD34$^+$ cell trafficking, preferential homing to the spleen than the marrow, and continuous proliferation of the malignant clone leads to progressive splenomegaly in PMF. The number of CD34$^+$ hematopoietic progenitor cells constitutively mobilized into the blood is dramatically increased in patients with PMF. The number of circulating CD34$^+$ cells in patients with PMF is 360 times higher than in healthy controls and 18 to 30 times higher than in patients with PV or ET. This striking observation led to the suggestion that the quantitation of CD34$^+$ cells in the peripheral blood might serve as a means of discriminating PMF from other MPNs. A level of 15×10^6 CD34$^+$ cells/L in peripheral blood allows differentiation of PMF from PV and ET. The numbers of circulating CD34$^+$ cells tend to increase as the disease progresses, and there is a close correlation between patients presenting with more than 300×10^6 CD34$^+$ cells/L of peripheral blood and imminent evolution to leukemia. These findings suggest that CD34$^+$ cell trafficking abnormality in PMF is caused by the inability of these cells to be retained within the BM or their premature release into the peripheral blood.

PMF is characterized not only by the constitutive mobilization of CD34$^+$ hematopoietic cells but also endothelial progenitor cells. Endothelial progenitor cell mobilization predominates during the prefibrotic phase of PMF, while hematopoietic stem/progenitor cell mobilization occurs characteristically in advanced PMF. This perturbed stem cell trafficking likely results in the seeding of extramedullary sites with primitive hematopoietic and endothelial cells, leading to EMH within the liver and spleen, as well as other organs.

Several proteolytic pathways play a significant role in cytokine-mediated stem cell mobilization. Proteases released by activated neutrophils cleave vascular adhesion molecule-1 (VCAM-1) expressed by stromal cells, disrupting the crucial adhesive interaction between VCAM-1 and very late antigen-4 (VLA-4) expressed by HSCs and progenitor cells. The interaction between stromal cell–, endothelial cell–, and osteoblast-derived stromal cell–derived factor-1 (SDF-1 or CXCL12) and the CXC chemokine receptor-4 (CXCR-4) expressed by HSCs and progenitor cells determine the stem cell trafficking pattern. Proteases, including neutrophil elastase, soluble matrix metalloproteinase-9 (MMP-9), and cell-bound MMP-9, have been shown to play a role in the constitutive mobilization of CD34$^+$ cells in PMF patients. Levels of soluble VCAM-1, a degradation product of VCAM-1, are increased in the plasma of PMF patients, which correlates with the number of circulating CD34$^+$ cells. These proteases degrade the chemokine CXCL12 that plays a role in the retention of CD34$^+$ cells within the BM because the degradation products lack the ability to attract CD34$^+$ cells, thereby favoring their mobilization. By contrast, the levels of intact CXCL12 within the spleen are increased, likely supporting the homing and localization of HPC/HSC to extramedullary sites such as the spleen. Furthermore, CXCR-4 expression by PMF CD34$^+$ cells is epigenetically downregulated, which may account for altered CXCL12–CXCR-4 interactions, participating in CD34$^+$ cell mobilization. This downregulation of *CXCR4* expression can be reversed in vitro by treatment with chromatin-modifying agents. Reduced CD34/CXCR4 expression was shown to be associated with a shorter interval to development of severe anemia, massive splenomegaly, cytopenias, increased circulating CD34-positive blood cells, leukemic transformation and death.

Furthermore, *CXCR4* expression was significantly lower in patients with a higher burden of *JAK2* V617F (allele frequency: 75%) compared with patients with a low allele burden, suggesting a dependence upon the *JAK2* variant allele frequency. Similar degrees of CD34$^+$ cell mobilization are observed in post-ET and post-PV MF as well. Passamonti and coworkers demonstrated a relationship between the degree of constitutive mobilization of CD34$^+$ and *JAK2* V617F allele burden in patients with PV, suggesting that such mobilization may be a consequence of the transition from *JAK2* V617F heterozygosity to homozygosity accompanied by granulocyte activation. Agents targeting proteases responsible for constitutive CD34$^+$ cell mobilization may be a possible therapeutic strategy to prevent EMH in patients with PMF.

Most recently, Abdelouahab et al. explored the crosstalk between JAK2 and CXCL12/CXCR4 signaling pathways in MPN. They demonstrated that JAK2 cooperates with CXCL12/CXCR4 signaling to increase the chemotactic response of human cell lines and primary CD34$^+$ cells through signal crosstalk with the phosphatidylinositol-3-kinase (PI3K) signaling pathway, and JAK2 inhibition abrogated CXCL12/CXCR4 signaling in primary MPN cells. These results suggest that inhibition of this signal crosstalk may further contribute to the therapeutic effect (reduction in splenomegaly) of JAK2 inhibitors.

BONE MARROW FIBROSIS IN PRIMARY MYELOFIBROSIS

While the cell of origin in PMF is the pluripotent HSC, BM fibrosis is secondary to BM stromal cell reaction. BM fibrosis is characterized by collagen and reticulin fibrosis composed of type III and type I collagen, respectively. While collagen fibrosis appears to be characteristic of MPNs and associated with severe disease, reticulin fibrosis may also occur in nonneoplastic conditions and does not clearly correlate with disease severity. Although the exact pathogenesis of PMF is unknown, it appears to be intimately linked with megakaryocyte proliferation, differentiation, and the elaboration of fibrogenic cytokines from the malignant megakaryocyte, its progeny.

The ability of primitive human hematopoietic stem cells to engraft sublethally irradiated immunodeficient mice is the standard surrogate in vivo assay for human HSCs. Peripheral blood (PB)-derived CD34$^+$ HSCs from patients with PMF are capable of engrafting nonobese nondiabetic/severe combined immune-deficient (NOD/SCID) mice and generating clonal cells of multiple hematopoietic lineages. The differentiation program of PMF PB CD34$^+$ cells in the engrafted NOD/SCID mice differed from that of healthy CD34$^+$ cells as they produced increased numbers of CD33$^+$ myeloid cells and CD41$^+$ megakaryocytes but fewer CD19$^+$ B cells. This predisposition to megakaryocyte hyperplasia was further explored by incubating CD34$^+$ cells from patients with PMF, PV, and healthy human volunteers in the presence of stem cell factor and thrombopoietin (TPO). PMF CD34$^+$ cells predictably induced megakaryocyte hyperplasia in vitro, and these megakaryocytes exhibited a delayed apoptotic pattern secondary to overexpressed antiapoptotic factor B-cell lymphoma-extra-large (Bcl-XL) protein. Therefore the megakaryocyte hyperplasia in PMF is likely attributed to the enhanced ability of CD34$^+$ cells to generate megakaryocytes and the concomitant Bcl-xL overexpression, resulting in delayed apoptosis and megakaryocyte accumulation. The megakaryocyte role in the elaboration of BM fibrosis in PMF is further suggested by megakaryocytic hyperplasia with dysplastic megakaryocytes, increased circulating megakaryocytes, and megakaryocyte progenitors; by the association of BM fibrosis and acute megakaryocytic leukemia; and by the presence of MF in gray platelet syndrome, an inherited disorder of platelet α-granules. It has been hypothesized that megakaryocytes in PMF overproduce cytokines involved in collagen fibrosis, neo-angiogenesis, and osteosclerosis.

In PMF, the levels of platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) were 2.0- to 3.0-fold and 1.5- to 3.0-fold higher, respectively, than in healthy controls. However, fibroblast growth factor (FGF) levels were similar in both, suggesting that PDGF and TGF-β not only promote fibroblastic proliferation but also influence the synthesis, secretion, and degradation of extracellular matrix (ECM) components. TGF-β upregulates the expression of fibronectin and collagen types I, III, and IV involved in ECM synthesis and downregulates ECM-degrading enzymes (collagenase), resulting in ECM accumulation and progressive fibrosis. Additionally, cytokines like lipocalin 2 (LCN2) promote bone marrow fibrosis through osteoblast production. Basic FGF (bFGF) is a potent angiogenic factor and mitogen for human BM stromal cells. It may be released or leaked from dysplastic and necrotic PMF megakaryocytes or platelets. These findings suggest that bFGF may also contribute to progressive fibrosis and pronounced angiogenesis frequently observed in PMF. Investigators have suggested that impaired megakaryocyte emperipolesis might underlie this fibrogenic cytokine release mechanism, which is supported by the correlation of BM fibrosis and the degree of abnormal emperipolesis noted in PMF BM biopsies. *Emperipolesis* is defined as the random entry of hematopoietic cells into the cytoplasm of megakaryocytes. Impaired emperipolesis of neutrophils and eosinophils in PMF and extrusion of myeloperoxidase-positive granules by the engulfed neutrophils has been reported. Abnormal P-selectin distribution in megakaryocytes likely accounts for the selective sequestration of granulocytes by PMF megakaryocytes.[6]

Furthermore, the elevation in TPO levels observed in patients with PMF is not due to enhanced synthesis but rather reduced expression of MPL by platelets and megakaryocytes of PMF patients, leading to decreased TPO clearance. In ET and PMF, platelets and megakaryocytes are characterized by lower MPL protein levels, and most of the receptors are immature. Endo-H–sensitive activated *JAK2* promotes cell surface localization and enhances protein levels of MPL through stabilization of the mature endoglycosidase H-resistant form of the receptor. But *JAK2* V617F may promote receptor ubiquitinylation and proteasomal and lysosomal degradation, resulting in reduced expression of *MPL*, suggesting that proteasome inhibitors or *JAK2* inhibitors may restore platelet MPL levels. By contrast, persistent MPL expression by progenitor or stem cells is linked to myeloproliferation. These observations support a model where a decrease in MPL mass in platelets and megakaryocytes results in increased TPO levels, which act on the primitive stem cells, contributing to myeloproliferation.

Vannucchi and colleagues demonstrated that impaired expression of the transcription factor GATA1 might contribute to the development of myelofibrosis and sought to further define the role of megakaryocytes in the development of BM fibrosis using *Gata1*^{low} mutant mouse model. Mutations in the GATA1 functional pathway in human PMF have not been described. However, at the protein level, a large number of the megakaryocytes in the BM of PMF patients are GATA1-negative, suggesting that whatever the genetic defect leading to PMF is, it involves the pathway that affects the posttranscriptional or posttranslational modification of GATA1 in megakaryocytes.

Most recent studies have demonstrated that MPN driver mutations induce a ribosomopathy that decreases GATA1 levels in megakaryocytes, halting their maturation. *Gata1*^{low} mice showed high numbers of immature megakaryocytes and severe bone marrow fibrosis, as is seen in patients with PMF. Furthermore, treatment with an inhibitor of Aurora kinase A (AURKA), a protein overexpressed in MF megakaryocytes, rescued GATA1 expression in these cells, reducing marrow fibrosis in animal models, suggesting GATA1 as a druggable target. AURKA inhibition induced polyploidization, features of maturation, and subsequent apoptosis of megakaryocytes from primary MPN patient samples. This concept was tested with the oral AURKA inhibitor, alisertib, in a phase I trial of 25 MF patients in which forced maturation of megakaryocytes and resolution of bone marrow fibrosis in treated patients was associated with clinically significant spleen and symptom responses (NCT02530619).

Using the *Gata1*^{low} mouse model, Migliaccio and colleagues demonstrated that increased bioavailability of TGF-β plays a significant role in promoting disease progression in MF. Impaired megakaryocyte maturation coupled with high IL-8 expression, in an autocrine fashion, induces megakaryocytes to increase TGF-β, which in turn is responsible for suppressing hematopoiesis by normal HSCs (leading to bone marrow failure) and promoting an MF-HSC-supporting microenvironment in the spleen, facilitating the transition of pre-MF to overt MF. Although TGF-β inhibition completely rescued MF in *Gata1*^{low} mice, it was difficult to target TGF-β due to the lack of specificity and off-target effects. However, the TGF-β1 specific ligand trap AVID200 has made it possible to evaluate this therapeutic concept in the clinic (NCT03895112).

Intrasinusoidal hematopoiesis in the BM is a conspicuous finding in PMF. The BM vascular architecture in PMF is characterized by increased type IV collagen deposits coupled with increased BM microvessel density, resulting in increased blood flow. Vessels from patients with PMF are markedly abnormal and appear as localized vascular nests consisting of numerous short but highly branched and tortuous vessels. Increased BM microvessel density in PMF is probably mediated by α-granules of megakaryocytes as well as several angiogenic growth factors, including overexpressed bFGF and increased serum levels of vascular endothelial growth factor (VEGF). Immunohistochemical methods have confirmed that this increased BM microvessel density in PMF is correlated with increased spleen size and is an independent risk factor for OS.

Osteosclerosis is prominent in PMF, which often manifests clinically as bone pain. Osteosclerosis is a consequence of cytokines released by the neoplastic BM cells or stroma (conditioned and activated) interaction with PMF cells. PMF-associated osteosclerosis can be reversed after HSCT, restoring normal hematopoiesis. In both animal models and patients with PMF, TGF-β and stromal cell-derived osteoprotegerin, a member of the TNF receptor family, play pivotal roles in the development of osteosclerosis. Osteoprotegerin is a decoy receptor for the receptor activator of the nuclear factor kappa-B ligand (RANKL). RANKL is a transmembrane protein expressed on the cell surface of osteoblasts that can be cleaved into a soluble form by proteases. Both soluble and membrane-bound RANKL attach to RANK, a cell receptor expressed by osteoclast precursors to stimulate osteoclastogenesis. RANKL and osteoprotegerin are positive and negative regulators of osteoclast differentiation, respectively. Osteoprotegerin reduces the production of osteoclasts by inhibiting osteoclast differentiation, leading to the development of osteosclerosis. In patients with PMF, it remains unknown if the degree of osteosclerosis is reversed by normalization of levels of osteoprotegerin.

Megakaryocytes are not the only cells capable of releasing cytokines that promote BM fibrosis. Serum levels of macrophage colony-stimulating factor (M-CSF), a cytokine that regulates macrophage development and proliferation, are also elevated in patients with PMF. Monocytes and macrophages from patients with PMF engender and amplify the inflammatory cytokines TGF-β and interleukin-1 (IL-1) over those seen with healthy controls. TGF-β and IL-1 are fibroblast mitogens that promote ECM accumulation. Monocyte adhesion through CD44 appears to induce these fibrogenic cytokines by facilitating monocyte-ECM interaction. The proinflammatory transcriptional factor nuclear factor kappa-B (NF-kB) plays a pivotal role in the elaboration of IL-1 as well as in promoting PMF monocyte adhesion. Most recently, Kleppe et al. through integrated RNA sequencing and ChIP-seq data, demonstrated that the NF-kB-dependent transcriptional network fuels the inflammatory state in MPNs and this transcriptional network in PMF could be successfully targeted by inhibiting bromodomain and extra terminal (BET) proteins of the chromatin reader family and significant anticlonal synergy with combined inhibition of BET proteins and JAK2, which was notable in reversing the MF phenotype of an *MPL*-driven murine model of MF. This concept is being evaluated in a phase II trial of the oral pan-BET inhibitor CPI-0610 with three cohorts of monotherapy, combination therapy with ruxolitinib in inadequate ruxolitinib responders, and as upfront treatment in JAK inhibitor-naïve patients (NCT02158858). Early signs of clinical activity with spleen and symptom benefit, most pronounced in the combination arm are also coupled with anemia responses and reduction in bone marrow fibrosis in approximately one-third of treated patients.

Abnormal cytokine expression in PMF represents an inflammatory response to the disease phenotype that not only contributes to the development of BM fibrosis, osteosclerosis, and increased BM microvessel density but also PMF-associated constitutional symptoms including weight loss, anorexia, pruritus, bone pain, and night sweats. Increased levels of IL-8 and IL-2R closely correlate with constitutional symptoms, leukocytosis, requirement for RBC transfusions, as well as inferior LFS and OS. These observations evoke the possibility that mutational events leading to the malignant transformation of hematopoietic cells in PMF may also lead to the activation of transcriptional programs that promote hematopoietic cell survival and disease progression. Increased cytokine levels may, therefore, not only be responsible for the numerous epiphenomena that occur as a consequence of these malignant cells but also act on the malignant clone, affecting the proliferation and differentiation in differing microenvironments characteristic of the BM and various extramedullary sites, including the spleen. Ultimately, this may increase the risk of disease progression or leukemic transformation.

CLONAL EVOLUTION OF PRIMARY MYELOFIBROSIS

Skoda et al., through next-generation sequencing of 104 genes from a cohort of 197 MPN patients, provided insight into the clonal evolution of the clinical phenotype. Somatic mutations were observed in 90% of patient samples, in which 40% harbored mutations other than *JAK2* V617F and *CALR*. *JAK2* V617F was the most common mutation (69%), followed by *CALR* (15%), *TET2* (12%), *ASXL1* (5%), and *DNMT3A* (5%). The presence of two or more acquired somatic mutations portended a poor outcome and increased risk of leukemic transformation. By contrast, none of the patients with isolated *JAK2*, *MPL*, or *CALR* mutations evolved to MPN-BP. However, the rate of acquisition of new mutations was low when assayed from serial samples (two mutations were detected during a total follow-up of 133 patient-years). Most mutations were present at a low allelic frequency from the time of diagnosis, and in the case of *TP53*, rapid evolution to blast phase occurred with the loss of the wild-type allele either by chromosomal deletion or uniparental disomy. These data argue against the concepts of hypermutability and genomic instability in MF; however, it supports a complex clonal architecture in MPNs, where some individuals may develop a biclonal disease and others, a linear acquisition pattern.

In 50% of *JAK2* V617F-mutated MPNs, the blast cells that represent the progeny of leukemia-initiating clone are *JAK2* V617F-negative, suggesting that leukemia originates from a clone distinct from the *JAK2* V617F-positive clone. This coexistent *JAK2* V617F-negative clone appears to have a higher propensity for leukemic transformation. *JAK2* V617F, therefore, does not appear to be a prerequisite for the leukemic transformation of MPNs, suggesting that additional genetic and epigenetic events are required for full transformation to occur. Single nucleotide polymorphism (SNP) array analysis has shown that genomic alterations occur increasingly at the time of blastic transformation and that no single gene or molecular pathway is sufficient to cause transformation. Acquisition of somatic mutations in *TET2*, *IDH*, *TP53*, and *ASXL1* have all been implicated as genetic events leading to leukemic transformation in MF patients. In general, mutations in *TET2* and *DNMT3A* appeared earlier in the disease course, whereas mutations in *ASXL1*, *EZH2*, and *IDH1* were often acquired after *JAK2* V617F.

A surprising correlation was observed between the phenotype of preceding MPN and the *JAK2* mutational status of the leukemic blasts after transformation. In contrast to *JAK2* wild-type MPN-BP in this setting, evolution to BPs with *JAK2* V617F-positive blast cells is invariably preceded by the evolution of ET or PV to MF. Because of these observations, the myelofibrotic transformation of ET or PV likely represents an accelerated phase of the initial MPN preceded by genetic changes that result in evolution to MF and eventually BP. *JAK2* wild-type leukemia, by contrast, usually arises in patients with chronic-phase PV or ET who have not evolved to MF. Although not universally accepted, some have suggested that these leukemias are therapy-related and are the consequence of the administration of myelosuppressive agents administered during the chronic phase. The reversion from *JAK2* V617F to *JAK2* wild-type in these leukemias is not due to homologous recombination. Two models have been proposed previously to account for the clonal relationship between *JAK2* wild-type BP and its preceding MPN: (1) both the chronic MPN and the MPN-BP arise from a shared pre-*JAK2* V617F founder clone and (2) the chronic MPN and MPN-BP arise from two independent stem cells. The model proposed by Lundberg and colleagues also reflects similar models, incorporating a newer understanding of the phylogenetic ordering of mutated *JAK2* and *CALR* with respect to additional acquired somatic mutations (Fig. 72.1).[7] It is possible that each of these models is viable and operates in different patients.

Studies have shown that triple negative patients with PMF have a shorter LFS as compared with *CALR*-, *JAK2*-, or *MPL*-mutated status. Conversely, CALR-mutated patients are at a lower risk of leukemic transformation compared with triple negative and *JAK2*-mutated cases but not with *MPL*-mutated cases. Optimally, patients should receive a HSCT before the leukemic transformation, as MPN-BP is notoriously resistant to conventional remission induction strategies employed in de novo AML. The median survival after blast transformation is a meager 2.6 months, owing to the patient's age and aggressive biology of this form of leukemia.

CLINICAL MANIFESTATIONS

Table 72.2 lists the symptoms and physical findings of patients with PMF at presentation.

Approximately 25% are asymptomatic and seek evaluation for incidental splenomegaly or an abnormal blood picture detected on a routine medical exam. Symptomatic patients present with symptoms related to cytokine release or splenomegaly. Cytokine-related symptoms include fatigue, fever, night sweats, bone pain, weight loss, sexual dysfunction, insomnia, difficulties in concentration, and reduced Quality of Life (QoL). Splenomegaly-associated symptoms include a dull, heavy sensation in the left upper quadrant, early satiety, diarrhea secondary to splenic pressure on the intestines or symptoms due to a splenic infarct (left-sided abdominal pain radiating to the left shoulder and fever).

Arterial or venous thromboses occur variably in PMF (10% to 12%). Venous thromboses include cerebral venous sinus thrombosis, splanchnic vein thrombosis, deep venous thrombosis, and pulmonary thromboembolism, while arterial thromboses include stroke, transient ischemic attacks, retinal artery occlusion, myocardial infarction, angina pectoris, and peripheral arterial disease. Established risk factors for thrombosis include the cellular phase of PMF, thrombocytosis (platelet count $>450 \times 10^9$/L), presence of cardiovascular risk factors, Hb greater than 11 g/dL, and *JAK2* V617.[8] The prefibrotic phase of MF (pre-PMF), as defined by WHO 2016 criteria, is associated with a higher thrombotic risk than that associated with ET. In pre-PMF, arterial events occurred at a rate of 1.7% patient-year as compared to venous events of 0.6% patient-year. Although pre-PMF behaves like ET and is managed similarly, retrospective studies have shown that survival of patients with pre-PMF is inferior to that of ET but better than patients with overt PMF. Therefore it is essential to distinguish between ET and pre-PMF in order to inform the patients of their prognosis.

Bleeding diatheses in patients with PMF could be either due to a qualitative or quantitative platelet defect with a heterogeneous clinical presentation, from minor petechiae/ecchymoses to life-threatening bleed, including variceal or intracranial bleed. Thus far, no definitive risk factors have been identified to predict bleeding in patients with PMF except severe thrombocytopenia and excessive thrombocytosis, leading to an acquired form of von Willebrand disease. Patients with pre-PMF are at an increased risk of bleeding when compared to ET and bleeding events occurred at the rate of 1.39 and 0.79 patient-year, respectively. Independent predictors of bleeding in pre-PMF include previous hemorrhage, leukocytosis, an acquired form of von Willebrand disease, and aspirin therapy.

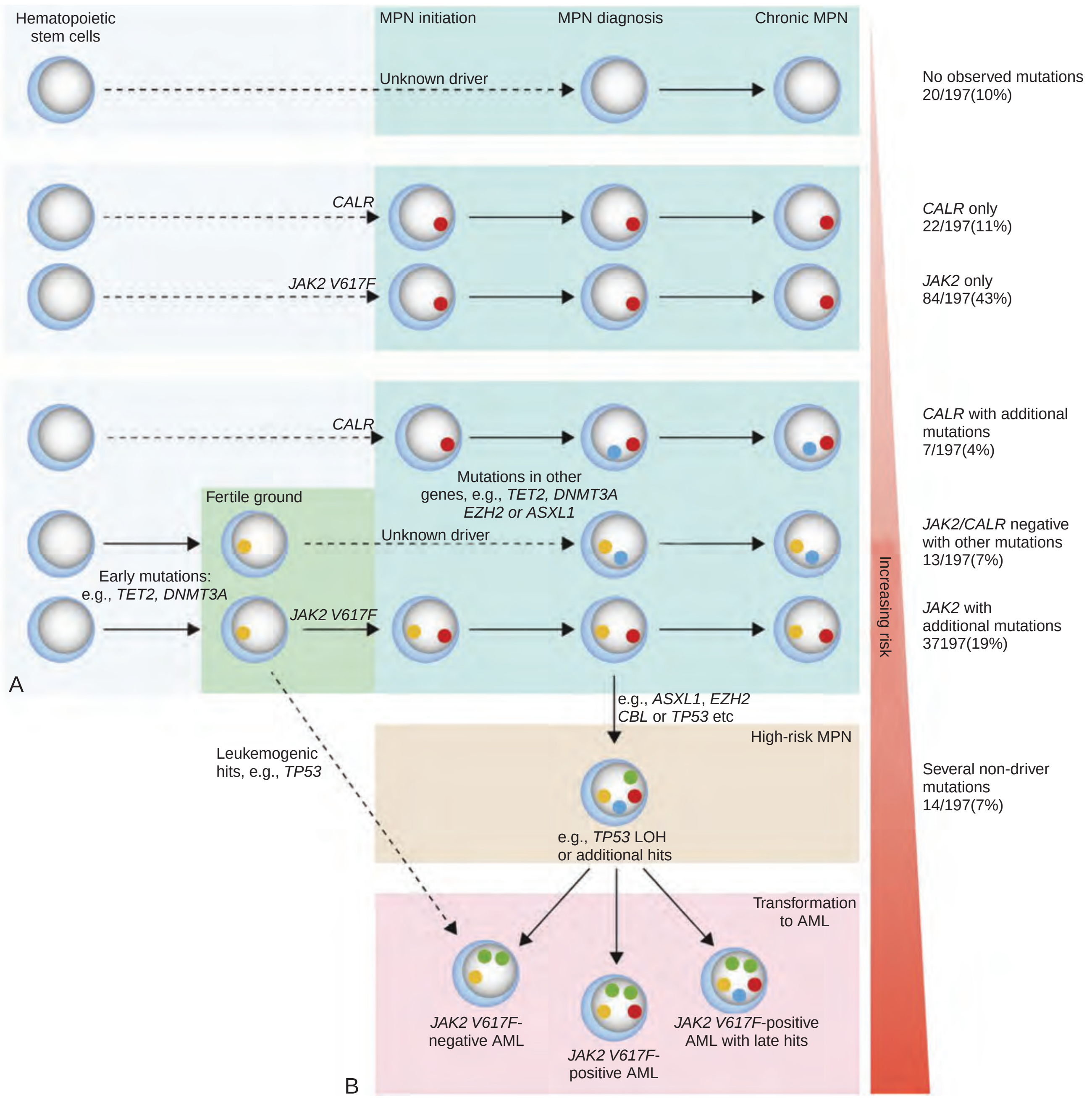

Hematopoietic stem cells
MPN initiation
MPN diagnosis
Chronic MPN
Unknown driver
No observed mutations 20/197(10%)
CALR
CALR only 22/197(11%)
JAK2 V617F
JAK2 only 84/197(43%)
CALR
CALR with additional mutations 7/197(4%)
Mutations in other genes, e.g., TET2, DNMT3A EZH2 or ASXL1
Fertile ground
Unknown driver
JAK2/CALR negative with other mutations 13/197(7%)
Early mutations: e.g., TET2, DNMT3A
JAK2 V617F
JAK2 with additional mutations 37197(19%)
A
Increasing risk
e.g., ASXL1, EZH2 CBL or TP53 etc
High-risk MPN
Leukemogenic hits, e.g., TP53
Several non-driver mutations 14/197(7%)
e.g., TP53 LOH or additional hits
Transformation to AML
JAK2 V617F-negative AML
JAK2 V617F-positive AML
JAK2 V617F-positive AML with late hits
B

Figure 72.1 MODEL OF MYELOPROLIFERATIVE NEOPLASM EVOLUTION AND RISK STRATIFICATION IN CORRELATION TO MUTATIONAL EVENTS. In 10% of myeloproliferative (MPN) patients, a driver mutation (*JAK2*, *MPL*, or *CALR*) is not detected. In approximately 50% to 60% of MPN patients, either *JAK2V617F* or *CALR* is found, and this group collectively has a favorable prognosis with a low risk of evolution to acute myeloid leukemia (AML). In patients with mutated *CALR*, additional molecular events appear to occur after this initiating event, whereas in the majority of patients with mutated *JAK2*, similar somatic mutations appear to occur prior to the acquisition of *JAK2V617F*. The presence of additional somatic mutations correlates with an increased risk of disease evolution, and acquisition of *TP53* mutation is associated with development of *JAK2*-mutated or wild-type AML. In model 1 (A), the two phases of disease are phylogenetically related, having arisen from a shared (pre-*JAK2*) founder clone, but in model 2 (B), the two phases of disease are clonally unrelated, reflecting transformation of independent stem cells. *AML*, Acute myeloid leukemia; HSC, hematopoietic stem cell; *LOH*, loss of heterozygosity. *(From Lundberg P, Karow A, Nienhold R, et al. Clonal evolution and clinical correlates of somatic mutations in myeloproliferative neoplasms. Blood. 2014;123:2220.)*

Massive splenomegaly is a prominent clinical finding that occurs in 25% to 50% of patients with PMF, which may be attributed partially to EMH. Palpable hepatomegaly is observed in 40% to 70% of the patients. Non-hepatosplenic EMH can occur in the pulmonary, central nervous, gastrointestinal, and genitourinary systems. The symptoms vary depending on the location of EMH: cough, headache, or paralysis resulting from “brain tumors or spinal cord tumors,” small-bowel obstruction, or intractable ascites. Ascites may be due to either peritoneal or mesenteric implants of EMH or portal hypertension, and if secondary to peritoneal EMH, the exudative ascitic fluid frequently contains myeloid, erythroid, and megakaryocytic elements.

Portal hypertension may be secondary to increased splanchnic blood flow due to splenomegaly and/or intrahepatic obstruction associated with EMH. Complications include ascites or esophageal varices that occur in 9% to 18% of patients with PMF. Occasionally, cirrhosis or evidence of portal or hepatic vein thrombosis has been reported. Nodular regenerative liver hyperplasia occurred in 14.6% of cases and correlated closely with portal vein lesions.

TABLE 72.2 Summary of Symptoms and Physical Findings of Patients With Primary Myelofibrosis Detected at Diagnosis

Symptom or Finding	Incidence (%)
Asymptomatic	16–30
Fatigue	47–71
Fever	5–15
Weight loss	7–39
Night sweats	6–21
Symptoms due to enlarged spleen	11–48
Bleeding	5–20
Gout or renal stones	6–13
Pallor	60
Petechiae or ecchymoses	15–20
Splenomegaly	89–99
Hepatomegaly	39–70
Peripheral edema	13
Evidence of portal hypertension	2–6
Lymphadenopathy	1–10
Jaundice	0–4

Patients with PMF may sometimes present with documented evidence of pulmonary hypertension (progressive dyspnea, signs of biventricular heart failure, elevated pulmonary artery pressures noted in right-heart catheterization), and acutely worsening hepatosplenomegaly. Many succumb to cardiopulmonary complications within 18 months of their initial diagnosis of pulmonary artery hypertension. Pulmonary artery hypertension in this patient subset is likely secondary to chronic thromboembolic disease, EMH within the lung, and elaboration of fibrogenic cytokines from dysfunctional circulating megakaryocytes, or portal hypertension. Patients with primary pulmonary hypertension may have abnormal blood counts (anemia and thrombocytopenia) and BM fibrosis. They can be distinguished from those with PMF by the lack of high levels of circulating CD34$^+$ cells, leukoerythroblastosis, and absence of driver mutations (*JAK2* V617F, *CALR*, or *MPL*).

Renal EMH is uncommon and may present a challenge for diagnosis. Renal EMH manifests mostly as renal dysfunction and proteinuria/nephrotic syndrome. The proteinuria is caused by concurrent glomerular lesions, which is present in most cases of renal EMH. Such lesions include focal segmental glomerulosclerosis, chronic thrombotic microangiopathy, and fibrillary-like glomerulonephritis. Immune complex deposition with subepithelial electron-dense deposits caused by immune dysfunction of PMF is proposed as a pathogenetic mechanism. Renal EMH may be misdiagnosed as tubulointerstitial nephritis due to the presence of eosinophils and their precursors. EMH should be suspected in cases of kidney masses or enlargement in the setting of renal dysfunction or proteinuria

EMH can rarely involve the skin, manifesting as nontender, pruritic red, pink, or violaceous plaques, papules, or hemangioma-like nodules. These dermal infiltrates are composed of combinations of myeloid, erythroid, and megakaryocytic cells. Occasionally, a Sweet syndrome-like picture may precede an overt PMF diagnosis.

Acute monoarticular inflammation caused by secondary gout related to increased cell turnover is seen in 6% of patients. Other physical findings include pallor, icterus, signs of cachexia, moderate lymphadenopathy, peripheral edema, and bony tenderness.

LABORATORY MANIFESTATIONS

Careful examination of the peripheral blood smear and BM (Fig. 72.2) permits a ready diagnosis of PMF. Leukoerythroblastosis with teardrop RBCs (i.e., dacryocytes) strongly suggests this diagnosis. Leukoerythroblastosis is characterized by the presence of nucleated RBCs and immature myeloid elements in 96% of cases. Megathrombocytes and megakaryocytic fragments are frequent findings. The reduction in the number of dacryocytes, postsplenectomy, or chemotherapy initiation has led some to invoke splenic fibrosis as an etiology.

In approximately 60% of patients, hemoglobin levels drop to less than 10 g/dL. The degree of anemia is frequently challenging to estimate through conventional hemoglobin or hematocrit determinations because of dilutional anemia secondary to plasma volume expansion due to massive splenomegaly. Most patients have normochromic, normocytic, and RBC indices. The anemia is a combination of decreased production due to erythroid hypoplasia or ineffective erythropoiesis and shortened RBC survival. Hemolytic anemia in PMF is multifactorial, including hypersplenism, RBC membrane defects resembling paroxysmal nocturnal hemoglobinuria (see Chapter 32), or antierythrocyte autoantibodies. About 5% of patients with PMF may develop iron deficiency anemia secondary to blood loss, either due to GI bleed (variceal bleed or duodenal ulceration), or intravascular hemolysis. Occasionally, a patient with PMF may develop an occult malignancy or a site of EMH within the gastrointestinal tract, which may serve as a bleeding source. Unexplained microcytosis (mean corpuscular volume <80 fL) has been reported in PMF and has not been shown to have prognostic relevance. Macrocytic anemia may complicate PMF. Although folic acid absorption is normal, folic acid deficiency may result from increased cell turnover.

Leukopenia can occur in 13% to 25% of patients, and leukocytosis is seen in one third. Occasional blast cells and granulocytes with the pseudo–Pelger-Huët anomaly are frequent findings. The leukocyte alkaline phosphatase (LAP) score is high in more than half of patients, but low in about one-third, and therefore does not add diagnostic value.

Platelet counts of less than 100,000/mm^3 are observed in 31% of patients, and platelet counts of more than 800,000/mm^3 have been observed in 12%. In pre-PMF, almost 90% of patients had platelet counts higher than 500,000/mm^3 and may develop acquired von Willebrand disease, often posing a bleeding risk. Defective platelet function is common, and platelets frequently do not respond to collagen or epinephrine stimulation. A variety of qualitative platelet anomalies have been documented as abnormal using automated platelet function analyzers such as the platelet function analyzer PF100 or light transmission platelet aggregometry. In 15% of patients, features of ongoing DIC are found, including decreased platelet counts, factor V and VIII, and increased fibrin split products. DIC in PMF is rare but may occur after an invasive surgical procedure.

Additional laboratory abnormalities are quite common. Hyperuricemia frequently occurs as a result of increased cell turnover. In one series, nonspecific elevated levels of lactic acid (95%), bilirubin (40%), alkaline phosphate, and serum glutamic oxaloacetic transaminase (50%) were observed. Patients with PMF have decreased levels of total cholesterol, likely secondary to inflammation and cachexia. The ratio of high-density lipoprotein cholesterol to low-density lipoprotein cholesterol is low.

A variety of immunologic abnormalities have been reported in PMF, including the presence of ANAs, elevated titers of rheumatoid factor, direct Coombs test positivity, lupus-type circulating anticoagulants, hypocomplementemia, BM lymphoid nodules, and increased circulating immune complexes. In one series of 50 patients with PMF, increased quantities of circulating immune complexes were detected in 39% and found to be associated with increased disease activity as manifested by increased transfusion requirements, bone pain, and fever. Some investigators have suggested that abnormalities of the complement system may be significant in the disease

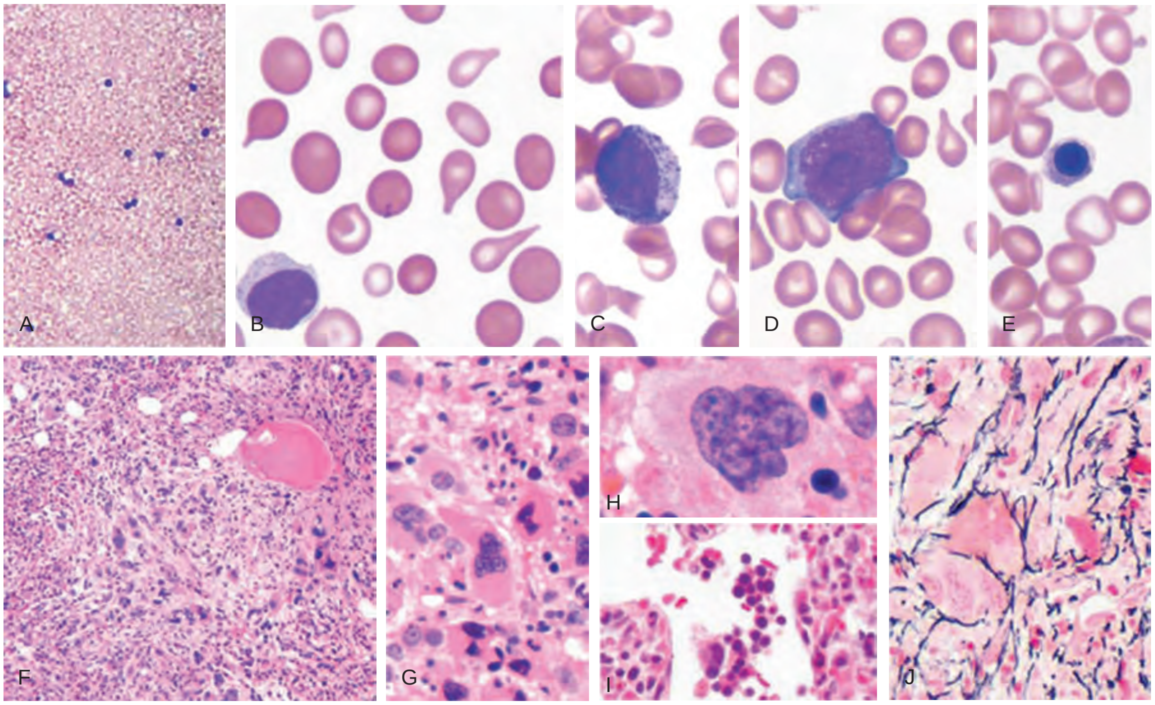


Figure 72.2 PRIMARY MYELOFIBROSIS. Peripheral blood and bone marrow (BM) biopsy. The leukocyte count can vary in primary myelofibrosis from leukopenia to marked leukocytosis. In the case illustrated, the count was normal (A). However, the smear showed numerous dacryocytes, or teardrop forms (B), and a leukoerythroblastic picture (C–E), which is the presence of leukoblasts or immature granulocytic precursors (C), including myeloblasts (D), and circulating nucleated red blood cells or erythroblasts (E). The BM biopsy is frequently hypercellular (F) and comprised of an atypical megakaryocytic and granulocytic proliferation (G) in which some of the megakaryocytes have atypical and pyknotic nuclei. Other megakaryocytes (H) are considered to have nuclei that are "cloud-like." The BM biopsy frequently shows sinusoidal hematopoiesis (I) and significant fibrosis, as illustrated by a reticulin stain (J).

progression of PMF, and others have hypothesized that low levels of C3 may predispose these patients to develop serious bacterial infections.

A remarkably high incidence of monoclonal gammopathies has been reported in PMF, with some series reporting 8% to 10% of benign gammopathies. Several case reports mention concurrent plasma cell or B-cell dyscrasias with PMF.

Successful BM aspiration is unusual, accomplished only in 5% to 10% of cases, with a dry tap in 50% of cases. A BM biopsy is an absolute diagnostic requirement for PMF to determine the presence of atypical megakaryocytes, myeloid hyperplasia, assess the amount of residual hematopoietic cellular tissue, and establish the degree of BM fibrosis. BM biopsies in PMF are typically hypercellular with atypical megakaryocyte morphology and myeloid hyperplasia. The atypical features of the megakaryocytes are paramount in establishing the diagnosis of PMF. Such features include hyperchromasia, high nuclear to cytoplasmic ratio, cloud-like nuclear contour, and tight clustering. Naked megakaryocytic nuclei and open sinuses are fairly common, especially in advanced stages. Although BM fibrosis is expected, prefibrotic PMF lacks this finding (see below). BM fibrosis and osteosclerosis were seen in 67% and 54% of cases, respectively. The characteristic morphologic features include patchiness of hematopoietic cellularity and reticulin fibrosis, with some microscopic fields being cellular and others void of hematopoietic cells. The amount of reticulin may vary from field to field. Additionally, BM biopsies reveal a substantial increase in vascularity. BM microvessels are more tortuous and branched, and the increased microvessel density correlates with increased VEGF expression by megakaryocytes (Fig. 72.3 A and B).

A rare histologic variant, *MF with fatty BM*, is characterized by myeloid hypoplasia associated with fairly complete fat substitution, mimicking the BM of aplastic anemia. These patients frequently have areas of clusters of densely aggregated hematopoietic elements exhibiting the histopathologic characteristics of PMF and large numbers of hematopoietic progenitors circulating in their peripheral blood. This variant of PMF is likely caused by the abnormal trafficking of hematopoietic cells from the BM to extramedullary sites, consistent with the osteosclerosis observed in patients with PMF, increased thickness of some bone units with new lamellae, and focal areas of woven bone. There is a net decrease in osteoclast number and conversion of trabecular pillars into plates.

Currently, pre-PMF is recognized as a distinct type of MPN that is characterized by the absence or presence of minimal BM reticulin fibrosis.[8] Pre-PMF may pose a diagnostic challenge, as clearly differentiating overt PMF from ET may be difficult. However, the myeloid hyperplasia and the tight clustering of megakaryocytes are quite specific for pre-PMF. Progressive fibrosis is frequently observed in such patients as the disease evolves. Based on a careful histomorphometric evaluation of the BM, a progressive fibro-osteosclerotic process paralleled by an increase in numbers of small megakaryocytes with irregular perimeters and megakaryocytes with naked nuclei has been observed during disease evolution. The clinical and morphologic findings of patients with the pre-PMF have been further characterized. Although a steady progression to BM fibrosis has been demonstrated in patients with the prefibrotic phase, fibrosis may remain static or diminish in the more advanced stages of PMF.

Different pathological scoring systems have been used for grading BM cellularity and fibrosis, with the aim of staging and documenting the progression of the disease. The European Consensus Criteria recognized the importance of age-dependent decrease in cellularity (Table 72.3). Grading of MF was simplified using four easily reproducible categories, including differentiation between reticulin and collagen. A consensus was reached that the density of fibers must be assessed in relation to the hematopoietic tissue, which is especially important to avoid a false impression of reduced fiber content in fatty or edematous BM samples after treatment. The progression of BM fibrosis in PMF is accompanied by the expression of subsets of collagenases that is independent of *JAK2* V617F status.

Morphologic examination of the spleen frequently reveals follicular atrophy in the white pulp (Fig. 72.4A) with foci of EMH

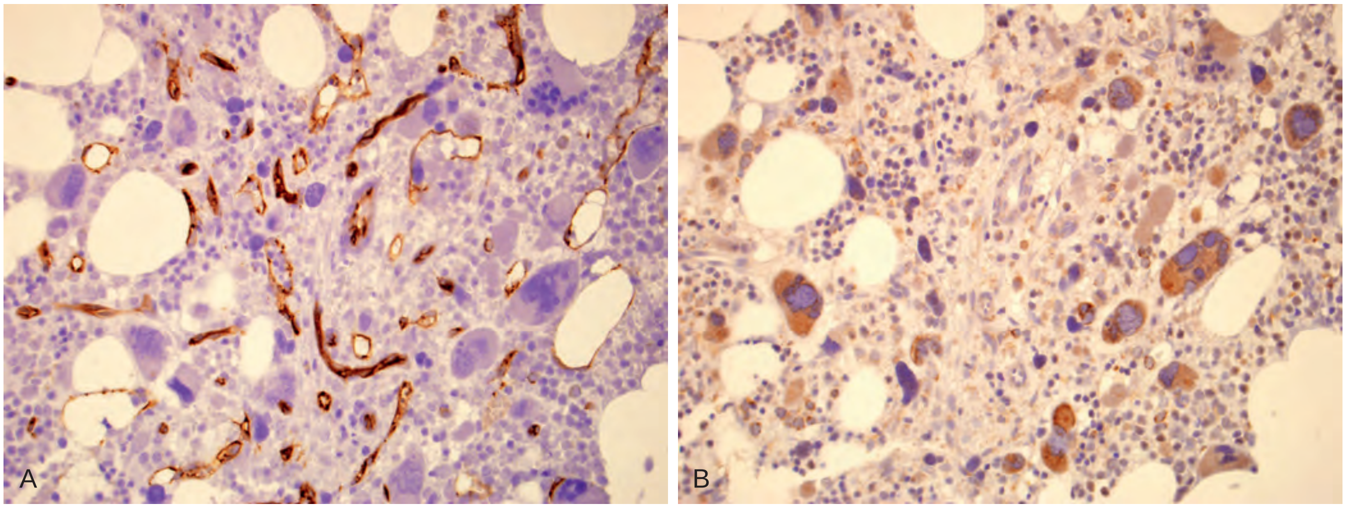


Figure 72.3 IMMUNOHISTOCHEMICAL STAIN OF BONE MARROW BIOPSY FROM A PATIENT WITH PRIMARY MYELOFIBROSIS. The left side shows immunohistochemical stain for CD34 antibody, which highlights the increased microvessel density, as noted with the increased number of vascular structures with the lumen. Please note also the increased number of scattered CD34+ myeloid blasts that are highlighted with granular staining (A). The right side shows immunohistochemical stain for vascular endothelial growth factor (VEGF)C antibody from a matched area of the bone marrow biopsy from the same patient. Note the increased expression of VEGF, as indicated by the brown staining in the cytoplasm of the dysplastic megakaryocytes (B).

TABLE 72.3 **Grading of Myelofibrosis According to the European Consensus Criteria**

Grading	Description[a]
MF-0	Scattered linear reticulin with no intersections (crossovers) corresponding to normal bone marrow
MF-1	Loose network of reticulin with many intersections, especially in perivascular areas
MF-2	Diffuse and dense increase in reticulin with extensive intersections, occasionally with focal bundles of collagen, focal osteosclerosis, or both
MF-3	Diffuse and dense increase in reticulin with extensive intersections and coarse bundles of collagen, often associated with osteosclerosis

[a] The quality of the reticulin stain should be assessed by detection of normal staining in vessel walls as internal control. The degree of myelofibrosis should be assessed by disregarding lymphoid nodules and vessels and by disregarding fibers framing adipocytes. Areas of prominent scleredema or scarring should be included in the overall grading of myelofibrosis. Fiber density should be assessed in hematopoietic areas.

MF, Myelofibrosis.

Data from Thiele J, Kvasnicka HM, Facchetti F, et al. European consensus on grading bone marrow fibrosis and assessment of cellularity. *Haematologica*. 2005;90:1128.

in the sinusoids of the red pulp (see Fig. 72.4B), where megakaryocytes, myeloid elements, and nucleated erythroid elements are seen. The extramedullary hematopoietic cells belonging to each of the myeloid lineages can be distributed in the spleen diffusely or limited to macro nodules. The predominance of immature granulocytic forms is associated with a dismal prognosis. Pathologic examination of the liver reveals hematopoietic cellular elements within the sinusoids. Sinusoidal dilatation is a common finding, as well as prominent intrahepatocyte and Kupffer cell hemosiderin deposition. A marked increase in the hepatic reticulin network has also been observed.

Approximately 50% of patients with PMF harbor karyotypic abnormalities at the time of diagnosis. It is essential to perform appropriate cytogenetic analyses to exclude rare cases of CML with associated BM fibrosis. Because of BM fibrosis, it is often difficult to obtain an optimal number of metaphase cells for cytogenetic analysis. In the past, a substantial percentage of patients with PMF had a “dry tap” or were uninformative, which made the cytogenetic analysis challenging. Although cytogenetic analysis of PMF remains time-consuming and laborious, chromosome studies are now informative in 100% of patients from unstimulated peripheral blood specimens. This success is due to the presence of large numbers of circulating immature mitotic hematopoietic cells (including CD34$^+$ cells) combined with the use of an MPN specific interphase FISH panel and array CGH +SNP that allows for identification of recurrent and novel genomic changes in virtually all patients with MPN (Fig. 72.5). There is a high concordance rate (92%) between conventional cytogenetics and interphase FISH for the 12 most frequent chromosomal abnormalities detected in PMF.

Among the Philadelphia chromosome-negative MPNs, PMF has the highest rate of chromosomal abnormalities at diagnosis. Deletions of the long arms of chromosomes 13 and 20, trisomy 8, and abnormalities of chromosomes 1q, 7q, and 9p constitute more than 80% of all chromosomal abnormalities detected in PMF (see Fig. 72.5), although none is specific to PMF. The deletion of the long arm of chromosome 13 is more frequent in PMF than in PV. Fine FISH mapping has defined the commonly deleted region as 13q13.3–q14.3, encompassing *RB1*, *D13S319*, and *D13S25* loci. As mentioned in Chapter 70, del (20q) and +9/+9p are more frequent in PV than in PMF. Multiple copies of 9p result in trisomy/tetrasomy or amplification of *JAK2*, and each of these abnormalities has been reported in PMF. A 2.7-Mb region on chromosome 20 spanning *D20S108* (proximal) and *D20S481* (distal) is deleted in all Philadelphia chromosome-negative MPNs. A different region is deleted in other myeloid malignancies, but a common 1.6-kb region may constitute the major site responsible for the loss of heterozygosity.

With disease progression from PV/ET to MF, the frequency of CGA is increased up to 100%. Longitudinal studies over a period of 16 years have demonstrated that an increased number of cells with +1q is associated with disease progression. Moreover, loss of 17p13, which leads to a deletion of TP53 in patients with +1q, is associated with perturbations of the TP53 pathway, which contributes to PMF progression. The chromosomal abnormalities observed are similar to those seen at diagnosis of PV/ET or PMF, but through subclonal evolution, they may become very complex. The number of genomic alterations is two or three times greater in the blast phase than it is in the chronic phase. Specific regions on 1q (*MDM4*), 12p (*ETV6*), 17p (*P53*), and 21q (*RUNX1*) are frequently altered and associated with disease progression. The use of comparative genomic hybridization techniques suggests that genomic aberrations are much

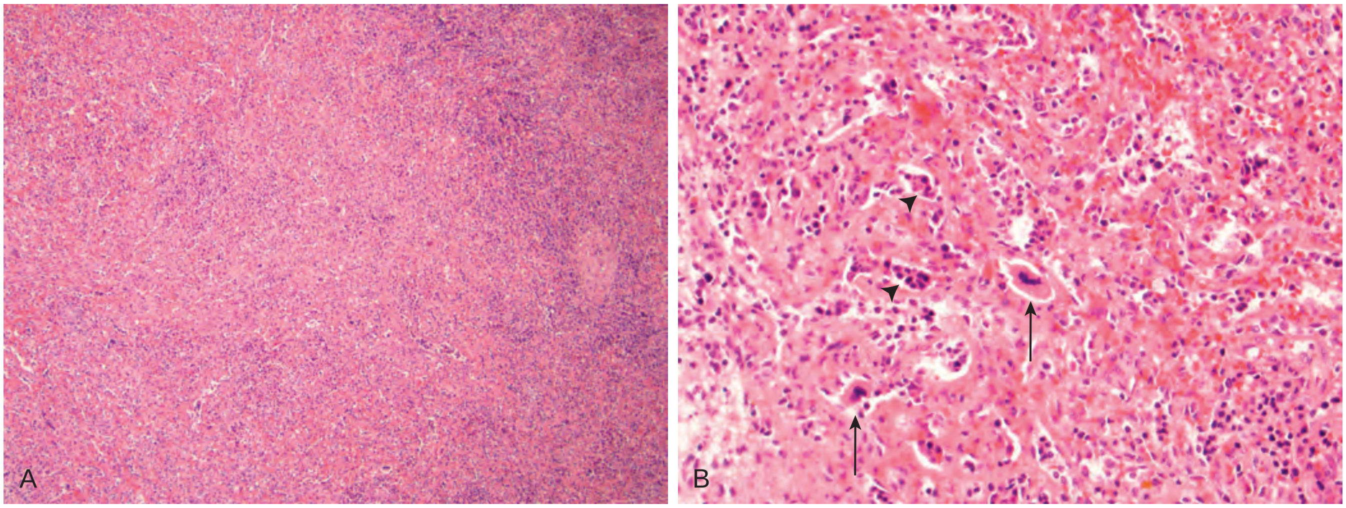


Figure 72.4 SPLEEN MORPHOLOGY IN PRIMARY MYELOFIBROSIS. The left side shows low-power magnification of spleen tissue from a patient with PMF with expanded red pulp and reduced-to-absent white pulp (A). The right side shows higher power magnification with extramedullary hematopoiesis with intrasinusoidal dysplastic megakaryocytes (*long arrows*) and erythroid progenitor clusters (*arrowheads*) along with scattered myeloid cells (B).

more common than what has been previously indicated by conventional cytogenetic analysis and occur in the majority of cases. Even among cytogenetically normal patients with PMF and array CGH+ SNPs, show at least one genomic lesion in 87% of patients. 9p cnLOH occurs in approximately 18% of PMF patients while a gain of 9p and/or cnLOH 9p is present in 88% of cytogenetically normal patients with de novo PMF suggesting that genes located on 9p may play a crucial role in the pathogenesis of PMF. In the majority of patients, CGAs are detected in cells that harbor driver mutations, and single cell analysis has demonstrated that an increased frequency of chromosomally abnormal colonies in *JAK2V617* homozygous progenitor cells may be due to a hyper-recombination phenotype induced by *JAK2V617F*. Others have reported that chromosomal abnormalities such as del(20q), +8, +9 can occur before acquisition of *JAK2V617F* indicating that clonality can also precede acquisition of *JAK2V617*.

Mutational analysis is invaluable in the diagnosis and prognostication of PMF. In PMF, the proportion of patients with *JAK2* V617F in granulocytes has been reported to range from 35% to 95%. The detection rate for *JAK2* V617F is much higher for patients with post-PV MF (91%) than PMF (45%) or post-ET MF (39%). In PV, a high allele burden of the *JAK2* V617F has been associated with an increased risk of evolution to MF. Such differences in allele frequencies are attributed to the variable sensitivity of the diagnostic techniques and differences in the case-mix of the reported series (i.e., the proportion of primary PMF and secondary (SMF) cases). *JAK2* V617F PMF is associated with older age at diagnosis and a history of thrombosis and pruritus. A common *JAK2* germ-line haplotype (46/1) that is identified by the rs12343867 SNP was found to influence susceptibility to developing PMF regardless of *JAK2* mutational status.

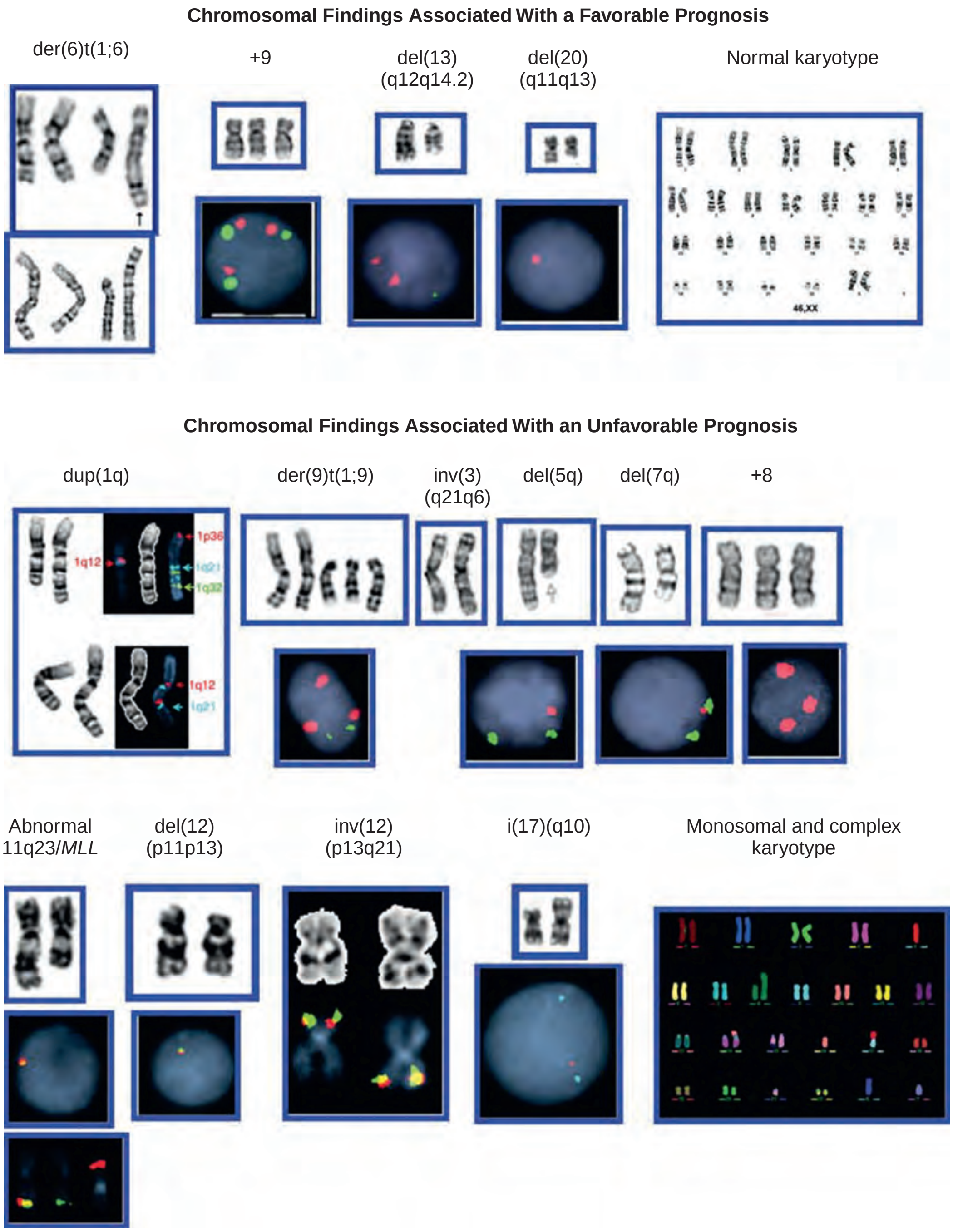
Gain-of-function mutations of MPL, *MPL W515L*, and *MPL W515K* are present in approximately 5% of patients with PMF, 1% with ET, but none with PV. *MPL* mutations may occur concurrently with *JAK2* V617F, suggesting that these alleles may have functional complementation in MPN. In all cases, *MPL W515K/L* VAF is in excess of the *JAK2* V617F VAF. The molecular profile at the time of leukemic transformation of PMF is distinct with a predominance of epigenetic and RNA splicing mutations (*TET2*, *IDH1/IDH2*, *ASXL1*, and *SRSF2*) as opposed to de novo AML in which mutations in the signaling receptor tyrosine kinases (*KIT* and *FLT3)* are common.

The number of circulating cells expressing the CD34 antigen, a phenotypic marker of hematopoietic stem, progenitor cells, and endothelial cells, in patients with PMF is reported to be ≥300-times higher than healthy controls and 18- to 30-times higher than in patients with PV or ET. The clinical utility of the cytofluorimetric measurement of CD34$^+$ cells as a diagnostic marker of PMF is hampered by the observation that a small number of subjects with PMF exhibit a normal number of CD34$^+$ cells in the peripheral blood. Cases with very mild disease or absent or slight reticulin BM fibrosis account for the majority of such patients. High values of CD34$^+$ cells ($>200 \times 10^6$/L) have been proposed as an indicator of an accelerated phase of the disease.

The characteristic radiographic features of PMF include a diffuse increase in bone density and increased prominence of the bony trabeculae. This increased bone density may be patchy and can produce a mottled appearance. Such abnormalities have been reported in 25% to 66% of patients with PMF. Noninvasive imaging of BM is a promising means of evaluating the BM cellularity and distribution in PMF. Magnetic resonance imaging (MRI) can portray the conversion or reconversion of fatty to cellular BM. Fibrotic BM is easily distinguished from cellular BM by its strikingly low signal intensity with all pulse signals. The BM patterns in the proximal femurs of PMF patients are reported to correlate with the clinical severity of the disease. BM MRI has been used to differentiate PMF from ET, where the BM adipose tissue is preserved, but not so in PMF.

DIFFERENTIAL DIAGNOSIS

A patient with hepatosplenomegaly, peripheral cytopenias, teardrop poikilocytosis, leukoerythroblastosis, and BM fibrosis probably has PMF, but other disorders may have a similar clinical picture (Table 72.4 and Fig. 72.6). The WHO diagnostic criteria were revised, incorporating testing for all the driver mutations as well as a greater emphasis on histomorphologic criteria, which allows one to distinguish early phases of PMF and pre-PMF from ET (Table 72.5A). The WHO criteria are based on the recognition of a pre-PMF without reticulin fibrosis and that the primary diagnostic features of PMF are increased megakaryocyte numbers, megakaryocyte morphology, and myeloid hyperplasia (Table 72.5B). SMF frequently occurs in patients with lymphoma or metastatic carcinomas. The clinician should be cautious in making the diagnosis of PMF in a patient who has a history of a primary neoplasm. Demonstration of carcinoma cells in the BM establishes that metastatic carcinoma is the cause of the BM fibrosis (Fig. 72.7A and B). The finding of blastic or lytic bone lesions in patients with MF suggests the presence of an underlying carcinoma. Disseminated tuberculosis and histoplasmosis are associated with the development of SMF corroborated by the presence of caseating or noncaseating granulomas in BM biopsy, which should prompt appropriate microbiological isolation techniques to confirm the diagnosis.


Chromosomal Findings Associated With a Favorable Prognosis

Chromosomal Findings Associated With an Unfavorable Prognosis

Figure 72.5 CYTOGENETIC FINDINGS IN PRIMARY MYELOFIBROSIS. The *top row* shows chromosomal abnormalities and the corresponding interphase fluorescence in situ hybridization (FISH) findings were associated with a favorable prognosis. They include unbalanced translocations between chromosomes 1 and 6 (both the short and long arms of chromosome 6) resulting in a gain of 1q, sole abnormality of chromosomes 9, 13, and 20, as well as the normal karyotype. Abnormalities include a gain of chromosome 9 and interstitial deletions of the long arm of chromosome 13 and 20. Note that gain of 1q and jumping 1q is associated with disease progression to acute myeloid leukemia. The *bottom row* shows chromosomal abnormalities and corresponding interphase nuclei after FISH studies associated with an unfavorable prognosis. They include duplication and trisomy 1q. The most frequent abnormality associated with polycythemia vera-related primary myelofibrosis (PMF) is der (9)t(1;9), resulting in a gain of 1q (*red*) and 9p. Inversion of chromosome 3, -5/del(5q) (*red*), and -7/del(7q) (*red*) are rare in PMF, as are rearrangement of 11q23 and deletion of the short arms of chromosome 12. Sole trisomy 8 is a frequent abnormality associated with PMF, and rearrangements of 12q are almost exclusively identified in PMF. The chromosomal abnormalities associated with the most dismal prognosis are heterozygous 17p loss (*red*) and monosomal or complex karyotype.

Several other primary hematologic disorders can also be accompanied by BM fibrosis (see Table 72.4). MPN–MDS overlap syndromes are frequently seen in clinical practice, and they share clinic-pathological features of both MPN and MDS. These overlap syndromes are characterized by BM hypercellularity, erythroid or myeloid lineage dysplasia, and proliferative features, as well as ineffective hematopoiesis, modest hepatosplenomegaly, and some degree of BM fibrosis. The bone marrow of MDS/MPN overlap syndromes (see Chapter 73) is characterized by dysplastic megakaryocytes rather than the characteristic classic atypical megakaryocytes seen in PMF (hyperchromasia, high n/c ratio, and tight clustering). These patients are occasionally *JAK2* V617F positive and frequently harbor *TET2* mutations. Such patients often present with cytopenias and are at a high risk of developing acute leukemia. These cases indicate the limitations of adhering to strict

TABLE 72.4 Conditions Associated With Myelofibrosis

Nonmalignant Conditions
Infections: tuberculosis, histoplasmosis
Renal osteodystrophy
Vitamin D deficiency
Hypoparathyroidism
Hyperparathyroidism
Gray platelet syndrome
Systemic lupus erythematosus
Scleroderma
Radiation exposure
Osteopetrosis
Paget disease
Benzene exposure
Thorotrast exposure
Gaucher disease
Primary autoimmune myelofibrosis
Malignant Disorders
Primary myelofibrosis
Other chronic myeloproliferative disorders: polycythemia vera, chronic myeloid leukemia, essential thrombocythemia
Acute myelofibrosis
Acute myeloid leukemia
Acute lymphocytic leukemia
Hairy cell leukemia
Hodgkin lymphoma
Myelodysplasia with myelofibrosis
Multiple myeloma
Systemic mastocytosis
Non-Hodgkin lymphoma
Carcinomas: breast, lung, prostate, stomach

disease classifications and underscore the continuum between MPNs and MDS. The peripheral blood and BM findings that allow differentiation of these disorders can be enhanced by *JAK2* V617F, *MPL W515L/K*, and *CALR* mutational analyses. Patients with the variant MDS with MF frequently present with cytopenias and have dysplastic cellular abnormalities indistinguishable from those with myelodysplasia (see Chapter 61). Their BM, however, is characterized by the presence of BM fibrosis and striking megakaryocytic hyperplasia, with a predominance of small hypolobulated forms, in some cases with striking surrounding fibrosis.

Hairy cell leukemia can also be confused with PMF. In one study, five out of 61 patients who had initially been diagnosed as having PMF were shown retrospectively to have had hairy cell leukemia. Hairy cell leukemia can present as pancytopenia with splenomegaly and is associated with a dry BM tap. In one series, BM reticulin content was increased in 26 out of 29 patients with hairy cell leukemia. The presence of hairy mononuclear cells possessing tartrate-resistant acid phosphatase or the appropriate phenotype in the peripheral blood or BM should facilitate differentiation of PMF from hairy cell leukemia (see Chapter 77). This exercise is essential because of the different modalities of treatment that can be successfully used for hairy cell leukemia.

BM fibrosis can occur in patients with other MPNs, especially PV and CML, and less frequently with ET. In CML, progressive BM fibrosis may herald the onset of accelerated disease or blast crisis. MF in CML occurs in two distinct patterns: one in which patients present with CML and associated BM fibrosis; and a second, in which the MF develops late in the course of the CML (see Chapter 69). The MF in the latter group appears at a mean of 36 months after the diagnosis of CML, is associated with a mean survival time of 4.9 months from the detection of MF, and therefore represents an ominous prognostic sign. In 5% to 15% of patients with PV, post-PV MF occurs approximately 10 years after the initial diagnosis of PV (see Chapter 70). PMF may be clinically and pathologically indistinguishable from post-PV MF except for the prior history of erythrocytosis. Among patients with post-PV MF, 25% to 50% develop MPN-BP, and 70% are dead within 3 years of this transition. Post-PV MF represents a transitional MPN with relatively grave prognostic implications. MF has also been reported after ET and the estimated probability of developing post-ET MF is 3% at5 years after ET diagnosis, 8% at 10 years, and 15% at 15 years.

Acute panmyelosis with myelofibrosis (APMF) represents a clinical entity distinct from PMF (see Fig. 72.6). This disorder has also been termed *acute MF*, *acute myelosclerosis*, *acute megakaryocytic MF*, and *acute myelodysplasia with MF*. APMF is exceedingly rare and corresponds to less than 1% of the cases of AML. Patients characteristically present with pancytopenia, fever, absence of clinically significant splenomegaly, minimal or absent teardrop poikilocytosis, and fibrotic BM. The BM is characterized by the appearance of immature myeloid cells and blast cells, which frequently express the megakaryocytic phenotype. Survival ranges from 1 to 9 months after diagnosis. Its distinction from PMF is vital because aggressive chemotherapy and HSCT are the treatments of choice.

Autoimmune myelofibrosis (AIMF) is another cause of BMF that occurs in patients with autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. AIMF occurs predominantly in females with a broad clinical spectrum. Such patients universally have a positive ANA test result or an elevated anti-DNA titer. Because the physical manifestations of an autoimmune disease may not be evident, all patients with MF should have an ANA test to exclude an autoimmune etiology. An important distinguishing feature is that patients with primary AIMF lack MPN driver mutations and clonality inherent to PMF. AIMF likely represents a distinct clinicopathologic syndrome unrelated to other well-defined autoimmune disorders. Vergara-Lluri and colleagues proposed the following diagnostic criteria that favor AIMF rather than PMF, including: rarity or absence of a leukoerythroblastic reaction in the peripheral blood, absence of peripheral eosinophilia or basophilia, mild degree of BMF (usually MF1), absence of osteosclerosis and bone changes, absence of granulocytic hyperplasia that is seen in PMF, and absence of the characteristic atypical features of the PMF megakaryocytes.

PROGNOSIS

PMF is associated with an estimated median OS of approximately 6 to 7 years (Fig. 72.8). The primary causes of death, in descending order, include leukemic transformation, a progression of PMF, thrombosis and cardiovascular causes, bleeding, and portal hypertension. The incidence of leukemic transformation in PMF ranges between 5% and 22% (Fig. 72.9), with an estimated actuarial cumulative risk of death of 2% and 16% at 1 and 5 years from diagnosis, respectively.

The long-term outcomes of PMF depend on the clinical and cytogenomic features. Based on multivariable analyses, several large studies reported that older age, constitutional symptoms, Hb less than 10 g/dL, leukocytosis, thrombocytopenia, circulating blasts greater than 2%, and transfusion dependency is associated with inferior OS.

Although CGAs are common in PMF, they are nonspecific. However, certain CGAs have an independent prognostic impact on PMF. A Mayo Clinic study developed a two-tier cytogenetic risk stratification model. Patients with a favorable karyotype include chromosome del(13q), del(20q), trisomy 9, isolated or with other single abnormalities, two abnormalities excluding unfavorable abnormalities, and a normal karyotype. The 5-year survival rate was 51%, with a median survival of 5.2 years for patients with a favorable karyotype.

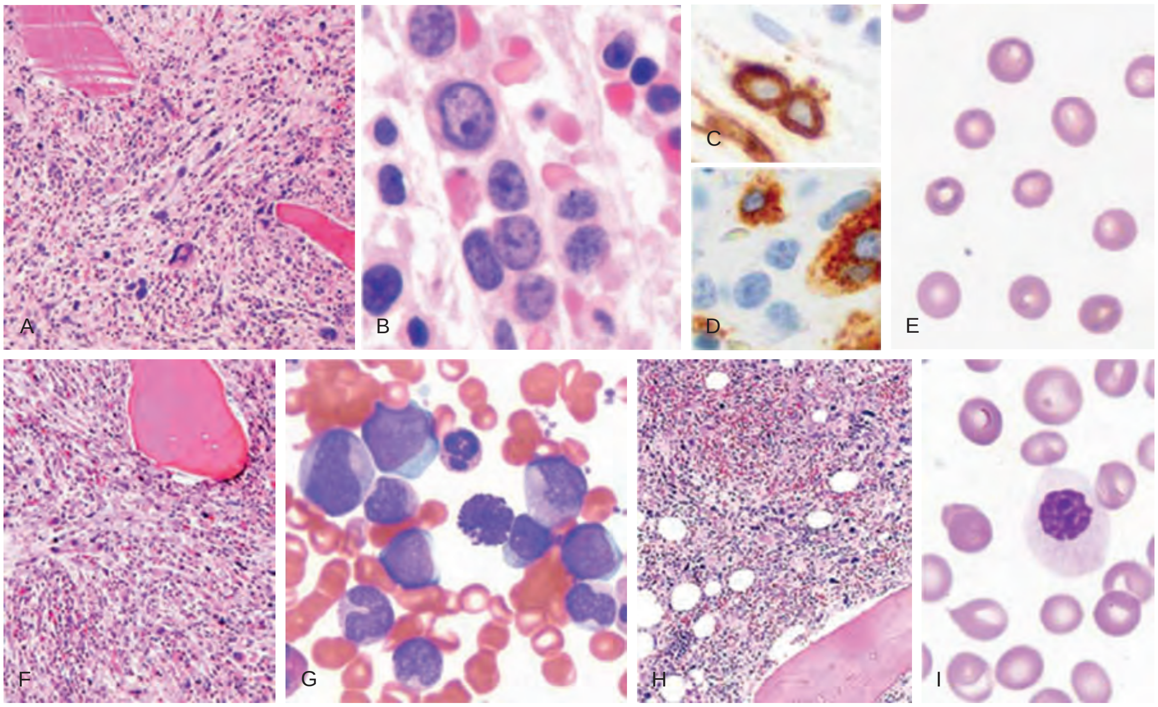


Figure 72.6 DIFFERENTIAL DIAGNOSTIC CONSIDERATIONS IN PRIMARY MYELOFIBROSIS. (A–E) Acute panmyelosis with myelofibrosis (APMF). (F and G) Chronic myelogenous leukemia (CML) in an advance phase with fibrosis. (H and I) Myelodysplastic syndrome (MDS) with fibrosis. The bone marrow (BM) biopsy of APMF, CML in advanced phase with fibrosis, and MDS with fibrosis can all look similar to PMF at low power (A, F, and H). In APMF, the distinction from PMF is made, in part, by seeing increased immature cells on the biopsy (B) interspersed with other hematopoietic precursors. These are usually CD34+ (C), but in contrast to acute megakaryoblastic leukemia are usually CD61– (D). In addition, the peripheral blood usually shows pancytopenia with neither teardrop red blood cells nor leukoerythroblastosis (E). Although the biopsy of CML presenting in an advanced phase can resemble PMF (F), the peripheral blood usually shows classic granulocytosis with left shift and basophilia (G). In the case illustrated, the 30-year-old female patient had fibrotic BM (F) but presented with a white blood cell count of 148,000/L showing a full spectrum of granulocytes, increased blasts (22%), and basophilia. P210 BCR/ABL1 was demonstrated. The bone marrow biopsy in MDS with fibrosis has small megakaryocytes (H), but dysplasia is otherwise difficult to evaluate in the absence of an aspirate, and one must rely on the peripheral blood to identify dysplasia, as in the severely dysplastic neutrophil (F).

A gain of chromosome 1q, which occurs in 22% of PMF patients, is associated with disease progression and jumping 1q translocations with an imminent transformation to AML. In PMF, the region on chromosome 1 that is most frequently duplicated or occurring in trisomy/tetrasomy is between 1q21–32 and 1q32–44 band regions (see Fig. 72.5).

The 5-year survival for patients with an unfavorable karyotype, including abnormalities of chromosomes 5 or 7, inversion of chromosome 3, isochromosome 17q, deletion of 12p or 11q23 rearrangement, as well as a complex karyotype, was 8% with a median survival of 2 years. The 5-year leukemic transformation rates for patients with an unfavorable versus favorable karyotype were 46% and 7%, respectively ($P < 0.0001$). A monosomal karyotype is associated with an unfavorable risk. A monosomal karyotype is defined as having two or more autosomal monosomies or a single autosomal monosomy associated with at least one structural abnormality. This rare CGA is associated with a median survival of 6 months and a 2-year leukemic transformation rate of 29.4%. Evidence suggests that even isolated del(20q), identified in most studies as a marker of favorable prognosis, has been found to promote disease progression through Hippo-kinase *STK4* downregulation as a result of deleted q11 segment on chromosome 20. In patients with *JAK2* 617 F mutation and del(20q) haploinsufficiency of *STK4* gene results in dysregulated inflammatory cytokine production, including IL-1β and IL-6 and disease progression.

The molecular profile of PMF plays a central role in the risk prognostication of PMF and the risk differs according to the presence or absence of driver mutations, in particular *CALR* or high molecular risk mutations (HMR). Triple negative patients displayed significantly worse survival (2.3 years), compared with *CALR-* (15.9 years), *JAK2-* (5.9 years), or *MPL-* (9.9 years) mutated patients. Conversely, survival was significantly better in *CALR*-mutated patients, compared with triple negative (HR, 5.1) and *JAK2-* (HR, 2.5) mutated cases, and no difference in survival rates was observed between *CALR-* and *MPL*-mutated PMF. Guglielmelli and colleagues identified that HMR mutations adversely affect the prognosis of PMF and that outcomes worsen proportionately with the number of HMR mutations. HMR mutations include *ASXL1, SRSF2, U2AF1*Q157*, EZH2*, and *IDH1/2* that occur in descending order of frequency in patients with PMF and the median survivals for those with none, one, or two or more mutations in these genes, were 12.3, 7.0, and 2.6 years, respectively. Additionally, *ASXL1, SRSF2*, and loss of heterozygosity for *TP53* were associated with inferior LFS in patients with PMF.

EVOLUTION OF PROGNOSTIC MODELS IN PRIMARY MYELOFIBROSIS

Prognostication of patients into different risk categories allows us to choose appropriate risk-adapted therapy. In 1996, Dupriez and associates developed the Lille scoring system that stratified patients into three risk categories based on hemoglobin level (<10 g/dL) and leukocyte count (<4000/mm^3 or more than 30,000/mm^3) with a median survival of 93, 26, and 13 months in the low (score-0), intermediate (1), and high (2) categories, respectively. Subsequent studies showed that the Lille score did not discriminate between the intermediate- and high-risk patient groups.

In 2009, the IWG-MRT developed an international prognostic scoring system (IPSS) based on the clinical characteristics of 1054 patients with PMF.[9] Tables 72.6 and 72.7 compare the six validated prognostic scoring systems for patients with PMF. The median OS for this group was 69 months, and the multivariate analysis obtained at a diagnosis-identified age greater than 65 years, presence

TABLE 72. 5A World Health Organization Criteria for Primary Myelofibrosis[a]

Major Criteria

1. Presence of megakaryocyte proliferation and atypia,[b] usually accompanied by either reticulin or collagen fibrosis
2. Not meeting WHO criteria for ET, PV, *BCR-ABL1*⁺ CML, myelodysplastic syndromes, or other myeloid neoplasms
3. Demonstration of *JAK2*, *CALR*, or *MPL* mutation or in the absence of these mutations, presence of another clonal marker[c], or absence of reactive myelofibrosis[d]

Minor Criteria

1. Leukocytosis $\geq 11 \times 10^9$/L
2. Increase in serum lactate dehydrogenase level above upper normal limit of institutional reference range
3. Anemia not related to a comorbid condition
4. Palpable splenomegaly
5. Leukoerythroblastosis

[a] Diagnosis requires meeting all three major criteria and one minor criterion.

[b] Small-to-large megakaryocytes with an aberrant nuclear-to-cytoplasmic ratio and hyperchromatic, bulbous, or irregularly folded nuclei and dense clustering.

[c] In the absence of any of the 3 major clonal mutations, the search for the most frequent accompanying mutations (e.g., *ASXL1*, *EZH2*, *TET2*, *IDH1/IDH2*, *SRSF2*, *SF3B1*) are of help in determining the clonal nature of the disease.

[d] BM fibrosis secondary to infection, autoimmune disorder, or other chronic inflammatory conditions, hairy cell leukemia or other lymphoid neoplasm, metastatic malignancy, or toxic (chronic) myelopathies.

CML, Chronic myeloid leukemia; *MDS*, myelodysplastic syndrome; *PV*, polycythemia vera; *WHO*, World Health Organization.

Modified from Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood.* 2016;127(20):2391–2405.

TABLE 72.5 B World Health Organization Criteria for Prefibrotic or Early-Stage Myelofibrosis (Pre-Primary Myelofibrosis)

Major Criteria

1. Megakaryocytic proliferation and atypia, without reticulin fibrosis > grade 1, accompanied by increased, age-adjusted bone marrow cellularity, granulocytic proliferation, and in many cases decreased erythropoiesis
2. Criteria for BCR-ABL1-positive chronic myeloid leukemia, polycythemia vera, essential thrombocythemia, myelodysplastic syndrome, or other myeloid neoplasm not met
3. JAK2 V617F, CALR, or MPL mutation or presence of another clonal marker or of minor reactive reticulin fibrosis in bone marrow[a]

Minor Criteria

1. Anemia not attributed to a coexisting condition
2. Leukocytosis (≥11,000 cells per cubic millimeter)
3. Palpable splenomegaly
4. Lactate dehydrogenase level above upper limit of normal of institutional reference range

[a] In the absence of any of the three major clonal mutations, a search for other mutations associated with myeloid neoplasms (e.g., ASXL1, EZH2, TET2, IDH1, IDH12, SRSF2, and SF3B1 mutations) may be helpful in determining the clonal nature of the disease. Minor (grade 1) reticulin fibrosis caused by infection is noteworthy, as are autoimmune disorders or other chronic inflammatory conditions, hairy cell leukemia or other lymphoid neoplasms, metastatic cancer, or toxic (i.e., chronic) myelopathies.

Modified from Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood.* 2016;127(20):2391–2405.

of constitutional symptoms, hemoglobin below 10 g/dL, leukocyte count above 25×10^9/L, and 1% or higher peripheral blood blasts as significant risk factors. Based on the presence of 0 (low risk), 1 (intermediate risk-1), 2 (intermediate risk-2) or greater than or equal to 3 (high risk) of these variables, four discrete risk groups with non-overlapping survival curves were delineated with a median survival of 135, 95, 48, and 27 months, respectively. Although the presence of *JAK2* V617F was associated with age older than 65 years, it did not influence survival in this study. The presence of CGA, most notably the gain of 1q, influenced the survival of the intermediate-risk groups only.

Subsequently, a cohort of 525 PMF patients was followed over time to assess the prognostic influence of the five IPSS clinical variables acquired during the course of disease rather than at diagnosis. The IPSS variables were analyzed as time-dependent covariates in a multivariate Cox proportional hazard model. Anemia was found to have the most significant hazard ratio (HR) for survival and hence a greater weight. Comparing IPSS and DIPSS, the time-dependent analysis confers a higher prognostic power to anemia because anemia is acquired most frequently and earlier during follow-up. The advantage of DIPSS is its ability to assess the real-time prognosis of patients with PMF. The DIPSS-plus further refined DIPSS with the incorporation of an unfavorable karyotype, platelet count below 100×10^9/L, and transfusion-dependent anemia. A poorer LFS was predicted by the presence of thrombocytopenia or an unfavorable karyotype with a 10-year risk of 31% (HR: 3.3; 95% confidence interval [CI]: 1.9 to 5.6).

With expanding understanding of the molecular pathogenesis of PMF and identification of HMR mutations as independent prognostic variables, the genetically inspired prognostic scoring system (GIPSS) and mutation-enhanced international prognostic scoring system plus karyotype, version 2.0 (MIPSS70+ v2.0) were developed. The GIPSS incorporates only mutation and karyotype data. The GIPSS confirmed the independent prognostic relevance of very high-risk (VHR) karyotype (single or multiple abnormalities of −7, inv(3), i(17q), del(12p), del(11q), and autosomal trisomy other than +8 or +9), unfavorable karyotype (other than normal karyotype or sole abnormalities of del(20q), del(13q), +9, chromosome 1 translocation/duplication or sex chromosome abnormality, including loss of Y chromosome), and specific mutations including the prognostically favorable type 1/like *CALR* mutation and the unfavorable *ASXL1*, *SRSF2*, and *U2AF1* Q157 mutations. Based on HR-weighted risk points, a four-tiered GIPSS model was devised: low (zero points), intermediate-1 (1 point), intermediate-2 (2 points), and high (≥3 points); the respective median survivals were 26.4, 8.0, 4.2, and 2 years in an international cohort of 641 cytogenetically annotated patients with PMF.

Prognostic models for transplant-age patients (≤70 years) named the MIPSS70 (mutation-enhanced international prognostic scoring system) and MIPSS70+ (karyotype-enhanced MIPSS70) have been developed. MIPSS70 considered six clinical risk variables (hemoglobin < 10 g/dL; leukocytes $>25 \times 10^9$/L; platelets $<100 \times 10^9$/L; circulating blasts ≥2%; bone marrow fibrosis grade ≥2; and constitutional symptoms), five HMR mutations (*ASXL1*, *SRSF2*, *EZH2*, *IDH1*, and *IDH2*), and one favorable mutation (*CALR* type 1/like). MIPSS70+ considered the same list of mutations but only three clinical risk factors (hemoglobin <10 g/dL, circulating blasts ≥2%, and constitutional symptoms), in addition to a two-tiered cytogenetic risk variable (unfavorable *vs* favorable). These clinical and genetic risk factors were used to compose three risk categories for MIPSS70 (low, intermediate, and high) and four risk categories for MIPSS70+ (low, intermediate, high, and very high).

However MIPSS70+ model did not distinguish between VHR and unfavorable karyotype; furthermore, *U2AF1* Q157 mutation was newly deemed HMR and new sex- and severity-adjusted hemoglobin thresholds defined by hemoglobin levels of less than 8 g/dL in women and less than 9 g/dL in men, and moderate anemia, defined by hemoglobin levels of 8 to 9.9 g/dL in women and 9 to 10.9 g/dL in men were identified. Therefore MIPSS70+ was revised to MIPSS70 + version 2.0.c.

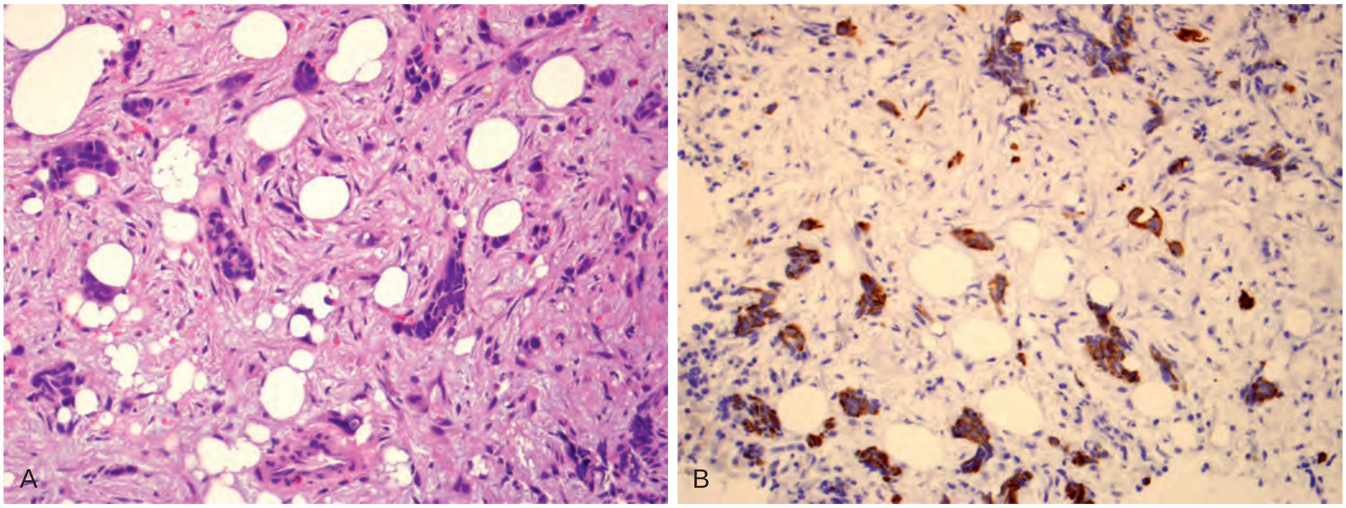


Figure 72.7 Metastatic carcinoma to the bone marrow is often associated with bone marrow fibrosis, as noted in this hematoxylin and eosin-stained section with clusters of carcinoma cells (A) that are highlighted by cytokeratin immunostain (B).

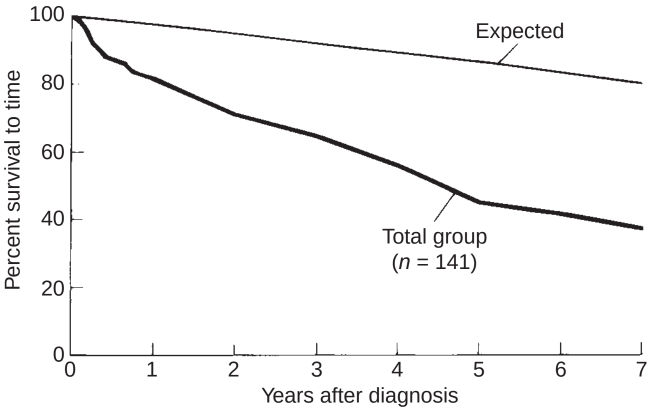


Figure 72.8 OVERALL SURVIVAL FROM THE TIME OF DIAGNOSIS OF 141 PATIENTS WITH PRIMARY MYELOFIBROSIS. *(Data from Silverstein MN. Agnogenic Myeloid Metaplasia. Acton, MA: Publishing Sciences Group; 1975:197.)*

In the multivariable analysis of clinical and molecular characteristics of 406 patients with PMF, HRs were 5.9 and 2.6 for VHR and unfavorable karyotypes, respectively; 2.6 for ≥2 HMR mutations; 1.8 for one HMR mutation; 2.1 for the absence of type 1/like *CALR* mutation; 1.9 for constitutional symptoms; 1.9 and 1.5 for severe anemia and moderate anemia, respectively; and 1.5 for circulating blasts ≥2%. HR-weighted risk points were subsequently allocated to VHR karyotype (4 points), unfavorable karyotype (3 points), ≥2 HMR mutations (3 points), one HMR mutation (2 points), absence of type 1/like *CALR* mutation (2 points), presence of constitutional symptoms (2 points), severe anemia (2 points), moderate anemia (1 point), and ≥ 2% circulating blasts (1 point). This resulted in a new five-tiered MIPSS70+ v2.0 prognostic model: VHR ≥ 9 points; high risk-5 to 8 points; intermediate risk-3 to 4 points; low risk-1 to 2 points; and very low risk-zero points. MIPSS70+ v2.0 further refined low risk to low and very low risk; high to high and VHR. The 10-year median survival was not reached in patients with very low and low risk, but for intermediate-, high-, and very high-risk patients, the median survival was 10.3, 7, 3.5, and 1.8 years, respectively.

Most recently, Nangalia et al., using whole-exome sequencing (WES) of 69 myeloid malignancy genes, developed a personalized genomic prognostic risk model for patients with MPNs.[10] In a large cohort of 2035 patients (ET-1321, PV-356, PMF-309, others—49), 45% harbored *JAK2*, *CALR*, or *MPL* mutations as the sole abnormality. They used Bayesian modeling to identify genomic subgroups of MPNs with maximum within-group similarity and maximum between-group discrimination. *TP53* mutations, often co-occurring with aberrations at chromosome 17p, and deletions at chromosome 5q form the first genomic subgroup; this group had a worse prognosis with a higher risk for leukemic transformation and early death, regardless of the initial driver mutation. The second subgroup was defined by the presence of one or more mutations in 16 myeloid malignancy genes, especially chromatin and spliceosome regulators, loss of heterozygosity at chromosome 4q, and aberrations in chromosomes 7 and 7q. These patients were at increased risk for development of myelofibrosis and shorter event-free survival (EFS) regardless of the MPN subtype and initial driver mutation. The remaining six groups were classified according to their dominant MPN phenotypic driver mutation. These eight genomic subgroups showed distinct clinical phenotypes, including blood counts, risk of leukemic transformation, and EFS. Integrating these clinical and genomic variables, they created multivariate, multistate prognostic models that could generate a personally tailored prognosis. The predicted and observed outcomes correlated in the training as well as in an independent external validation cohort. This model will be most useful for intermediate-risk patients as it allows more informative predictions in a group with otherwise uncertain outcomes.

MANAGEMENT OF PRIMARY MYELOFIBROSIS

The decision to treat an individual patient is based on a comprehensive assessment of the patient’s clinical characteristics, performance status, and prognosis. While “wait and watch policy” is adopted for low-risk patients, most patients with intermediate-/high-risk disease need therapy.

In general, therapy is indicated in the following situations: systemic symptoms (fevers, night sweats, bone pain, and weight loss), splenomegaly-related symptoms including portal hypertension, symptomatic anemia, life-threatening thrombocytopenia, increasing blast cell numbers in the peripheral blood, and hyperuricemia. Hyperuricemia should be aggressively managed with hydration and antihyperuricemic agents, including allopurinol, in all patients with PMF.

Patients with pre-PMF or low-risk MF, particularly if they are young, are especially challenging for most physicians. These patients are mostly asymptomatic but at risk for evolution to symptomatic MF or acute leukemia. Many patients are eager to entertain options that might prevent disease progression, but presently, an approach that will successfully achieve this goal is not available. HSCT is not an option for these patients due to the inherent morbidity and mortality associated with this approach.

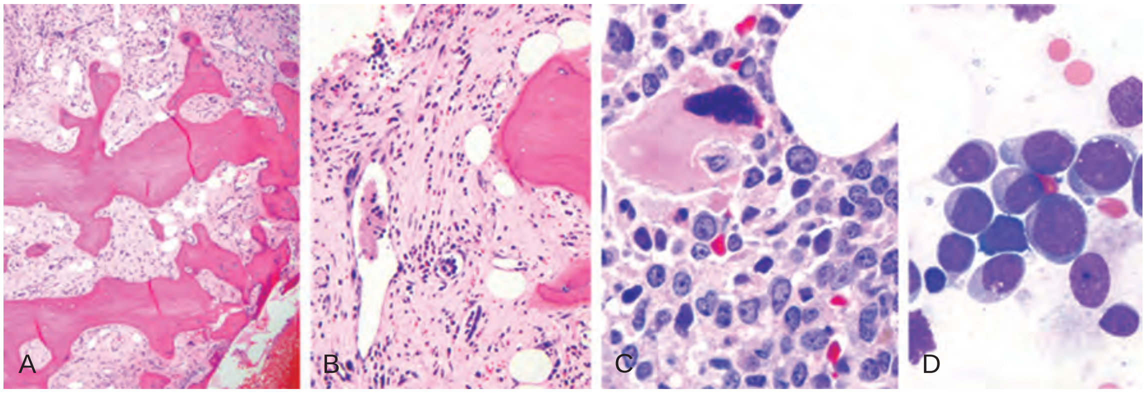


Figure 72.9 DISEASE PROGRESSION IN PRIMARY MYELOFIBROSIS. Marked osteosclerosis (A and B) and acute leukemia (C and D). In some patients, primary myelofibrosis progresses to severe osteosclerosis, in which there is markedly thickened and irregular bone formation (A) and a bone marrow space that is fibrotic and nearly depleted of hematopoietic elements (B). A terminal transformation to acute leukemia (C and D) occurs in 5% to 22% of cases.

TABLE 72.6 **Prognostic Scoring Systems for Primary Myelofibrosis**

Risk Factor	Lille	IPSS	DIPSS	DIPSS Plus	GIPSS	MIPSS70+ v2.0
Anemia (hemoglobin)	X (<10 g/dL)	X (<10 g/dL)	X (<10 g/dL)	X (<10 g/dL)		X (adjusted for sex) Severe (<9 g/dL in males; <8 g/dL in females) Moderate (9–10.9 g/dL in males; 8–9.9 g/dL in females)
Leukocytosis (white blood cell count)	X (<4 or > 30 × 10^9/L)	X (>25 × 10^9/L)	X (>25 × 10^9/L)	X (>25 × 10^9/L)		
Peripheral blood blasts		X (≥1%)	X (≥1%)	X (≥1%)		X (≥2%)
Constitutional symptoms		X	X	X		X
Age (years)		X (>65)	X (adjusted for age)	X		
Karyotype				X (unfavorable[a])	Very high risk[b] Favorable[c] Unfavorable[d]	X
Platelet				X (<100 × 10^9/L)		
Transfusion status				X		
Driver mutations					Absence of type 1-like *CALR*	X
High molecular risk mutations					*ASXL1SRSF2U2AF1*Q157	X

[a] Unfavorable karyotype includes complex or sole or two abnormalities that include +8, −7/7q−, i7/7q−, i(17q), −5/5q−, 12p−, inv (3), or 11q23 rearrangements.

[b] VHR karyotype includes single or multiple abnormalities of −7, i(17q), inv(3)/3q21, 12p−/12p11.2, 11q−/11q23, or other autosomal trisomies not including +8/+9 (eg, +21, +19).

[c] Favorable karyotype includes normal karyotype or sole abnormalities of +9, 13q−, 20q−, chromosome 1 translocation/duplication, or sex chromosome abnormality including −Y.

[d] Unfavorable karyotype includes all other abnormalities.

DIPSS, Dynamic international prognostic scoring system; *IPSS*, international prognostic scoring system; *GIPSS*, genetically inspired prognostic scoring system; *MIPSS 70+ v2.0*, mutation-enhanced international prognostic scoring system plus karyotype, version 2.0.

During the last decade, several investigators have promoted interferon alpha (IFN-α) therapy as an agent that might be capable of delaying disease progression by decreasing the rate of fibrosis if administered chronically during the early course of this disease but not when advanced. In one series, only four of 11 patients were able to complete 1 year of therapy; the other seven patients discontinued the drug because of unacceptable toxicity or severe cytopenias. None of these patients experienced clinically significant improvement (degree of BM fibrosis, osteosclerosis, or angiogenesis). A small phase II pilot study in PMF patients demonstrated responses in patients with pre-PMF treated with low doses of IFN-α-2b over an extended period (median duration: 3 years). Four patients with *JAK2* V617F-positive PMF were followed over time, and although each had clinical responses, only one had a significant reduction in mutant allele burden. The drug was generally well tolerated at doses between 1 and 2 million units weekly, and responses in the BM included a reduction in the degree of reticulin fibrosis and abnormal cellular morphology. Among the eleven PMF patients treated with a pegylated form of IFN-α-2b (PEG-IFN-α-2b; starting doses were between 2 and 3 μg/kg/week), only a single patient responded. Although the pegylated formulation allows for weekly injections, the toxicity profile was similar to the standard formulation and

TABLE 72.7 **Median Survival of Each Risk Group in Six Prognostic Scoring Systems for Primary Myelofibrosis**

Risk Group	Lille (Months)	IPSS (Months)	DIPSS	DIPSS Plus (Months)	GIPSS (Months)	MIPSS70+ v2.0
Low	93	135 s	Not reached	185	367	Very low—not reached
						Low—124 months
Intermediate-1	26	95	170 months	78	95	83 months
Intermediate-2		48	48 months	35	50	
High	13	27	18 months	16	24	42 months
						Very high—21 months

DIPSS, Dynamic international prognostic scoring system; *IPSS*, international prognostic scoring system; *GIPSS*, genetically inspired prognostic scoring system; *MIPSS 70+ v2.0*, mutation-enhanced international prognostic scoring system plus karyotype, version 2.0.

hence limited the duration of therapy in many patients. The most common grade 3/4 toxicity was fatigue and thrombocytopenia. Encouraging results of PEG-IFN-α-2a (Pegasys) in PV patients prompted the evaluation of IFN-α in MF patients. The French Groupe d'Etudes des Myelofibrosis (GEM) and France Intergroupe des Syndromes Myeloproliferatifs conducted a study of 18 patients with PMF and post-ET/PV MF treated with Pegasys. Of the four PMF patients included in this study, one had a complete response, two had a minor response, and one had no response. Responses included improvement in the degree of anemia, leukocytosis, and thrombocytosis but not splenomegaly, and appeared to be irrespective of *JAK2* mutational status. A comprehensive literature review suggests that IFN-α therapy in PMF may be of only moderate benefit in those with advanced phases of the disease. Prospective randomized studies are warranted in patients with pre-MF to determine the effects of IFN-α on disease-free survival, LFS, OS, and QoL. Without systematic evaluation, IFN therapy in any phase of PMF should be considered experimental and not embarked upon unless the patient is participating in a clinical trial.[11]

TREATMENT OF THE ANEMIC PATIENT

Anemia is common in patients with PMF and usually multifactorial in origin; nutrional deficiency (iron, folate), ineffective erythropoiesis, impaired erythroid differentiation, erythroid hypoplasia, increased splenic clearance, and hemolysis. Patients with documented nutritional deficiencies should receive folate, iron supplementation, or both. Packed RBC transfusion is indicated in patients with symptomatic anemia, and hemoglobin levels should be maintained at a level that provides symptomatic relief. Long-term transfusion therapy potentially may lead to iron-overload syndrome. It remains unclear if iron chelation is of meaningful clinical benefit to patients with MF, and data is mostly extrapolated from the MDS literature and anecdotal reports. Low-dose dexamethasone may be useful in patients with transfusion-dependent PMF.

Corticosteroids (e.g., prednisone 1 mg/kg/day taken orally) have been successfully used to treat hemolytic anemia associated with PMF. Other therapeutic options in this setting include intravenous immunoglobulins and recombinant human erythropoietin-α (rEPO). In a retrospective analysis of prednisone monotherapy (0.5 to 1 mg/kg daily, with tapering to the minimum effective dose in responders) in patients with intermediate-2 or high-risk PMF ($n = 30$) and anemia/thrombocytopenia, 12 patients (40%) achieved an anemia response by IWG-MRT criteria. The median response duration was 12.3 months. Patients with constitutional symptoms or greater than 2% circulating blasts had a lower response rate. Median survival from the initiation of prednisone therapy was significantly longer in anemia responders (5.0 years vs. 1.5 years, $P = 0.002$). Additional information supporting the use of corticosteroids for the treatment of MF-related anemia is derived from a prospective clinical trial designed to evaluate the safety and efficacy of the immunomodulatory agent, pomalidomide. This study surprisingly revealed that prednisone alone could induce significant responses in unselected MF patients with severe anemia. In this randomized study, the active control arm that included 21 patients consisted of prednisone (30 mg/day during the first 28-day cycle, 15 mg/day in the second cycle, and 15 mg every other day in the third cycle) plus placebo (up to 12 cycles). Overall, four out of 16 (25%) patients assigned to the control arm who completed three cycles of treatment achieved an anemia response according to the IWG-MRT criteria. Three of the four responses were short-lived, lasting between 2.3 and 5.5 months. This data is similar to our own experience indicating that prednisone therapy can improve PMF-associated anemia and thrombocytopenia even after the failure of standard therapies.

It is important to be aware that hemolytic anemia in patients with PMF may also rarely be a consequence of a coexisting secondary form of paroxysmal nocturnal hemoglobinuria that can be responsive to eculizumab therapy (see Chapter 32). Furthermore, patients with PMF who receive multiple transfusions are at a risk of developing delayed hemolytic transfusion reactions that occasionally may be severe and persistent. The direct antiglobulin test result is usually positive in this setting, and if possible, further transfusions should be limited whenever possible.

Anemia secondary to erythroid hypoplasia or ineffective erythropoiesis in PMF may respond to anabolic steroids. A good response is defined as a decrease, or total avoidance of transfusion therapy, occurring in about 20% to 40% of patients. A course of 3 to 6 months of androgen therapy is indicated to identify responsive patients, but the development of hepatic dysfunction or virilizing side effects may limit long-term androgen administration. Patients with CGAs are less likely to respond to androgen therapy. Danazol, a synthetic attenuated androgen, is useful in reducing RBC transfusion requirements and correcting thrombocytopenia in 20% to 40% of treated patients, in several series. Variables associated with response were a lack of a transfusion requirement and higher hemoglobin levels.

The use of rEPO in PMF was reviewed by Cervantes and colleagues in a total of 51 patients, documenting that 28 (55%) of these patients responded to rEPO treatment, including 16 complete and 12 partial responses. Inappropriately low endogenous serum EPO levels for the degree of anemia are predictive of a favorable response to rEPO. Serum EPO levels below 125 U/L were found to be associated with a favorable response to rEPO therapy. Responses can be associated with a limited enlargement of the spleen. The use of darbepoetin, a hyperglycosylated erythropoiesis-stimulating protein, has been reported in 20 PMF patients. With an initial weekly dose of 150 µg, increased to 300 µg after 4 to 8 weeks if there was a lack of response, eight patients (40%) responded with six complete and two partial responses, and five maintained their response after a median follow-up of 12 months (range: 4 to 22 months). The median time to response was 2 months. Univariate analysis indicated that older age was the only factor associated with a favorable response to treatment ($P < 0.006$). None of the patients with elevated serum EPO levels responded. Treatment was well tolerated, and patients could be successfully switched from rEPO to darbepoetin.

Multiple trials have explored the use of thalidomide in the treatment of anemia in PMF based on the drug's antiangiogenic, immunomodulatory, and antiinflammatory properties. A pooled analysis of five small phase II studies indicated that 29% of patients with moderate to severe anemia experience an increase in hemoglobin or reduction or elimination of blood transfusion requirements with thalidomide therapy at a standard dose of 200 to 800 mg/day. Nevertheless, most of the patients treated with these doses suffered adverse effects that resulted in an attrition rate of greater than 50% after 3 months. Moreover, an increase in white blood cell or platelet counts was frequently associated with serious adverse events such as pericardial effusions secondary to myeloid metaplasia. In a dose-escalation study design, at a low starting dose of thalidomide (50 mg/day), 31% of patients with transfusion-dependent anemia responded to therapy. A combination of low-dose thalidomide (50 mg/day) with prednisone was better-tolerated and equally or more effective than the standard-dose regimen. This regimen also had a significant effect on the degree of thrombocytopenia and resulted in a modest reduction of splenomegaly in almost 10% of the patients. These responses were sustained even after cessation of therapy. Higher doses of thalidomide were not more effective than lower doses. In a prospective phase IIB, randomized, double-blind, multicenter trial that compared 200 to 400 mg of thalidomide with placebo, only 10 out of 26 patients in the thalidomide arm completed 6 months of treatment. No difference was observed between the thalidomide and placebo arms in regard to the improvement of hemoglobin levels or decrease in RBC transfusion requirement. In a two-stage, phase II dose-escalation trial of thalidomide (200 mg dose-escalated to 800 mg as tolerated) in 44 patients with advanced PMF, the median tolerated dose was 400 mg for a median duration of 3 months, and 20% of the patients experienced an improvement in their degree of anemia (21% became transfusion-independent).

A more potent thalidomide analog, lenalidomide, was evaluated in 68 symptomatic patients with PMF at two institutions. Oral lenalidomide was administered at a dose of 10 mg/day if the platelet count was greater than 100,000/mm^3 or at a dose of 5 mg/day if the platelet count was less than 100,000/mm^3 for 3 to 4 months. The overall response rates were 22% for patients with anemia, 33% for patients with splenomegaly, and 50% for thrombocytopenia. Several patients normalized their hemoglobin levels or became RBC transfusion independent. The most common associated toxicities were grade 3 and 4 neutropenia and thrombocytopenia, which occurred in approximately 30% of patients but resolved with therapy cessation. In several patients with del(5)(q31)-associated PMF or post-PV MF, lenalidomide therapy was associated with a cytogenetic response as determined by FISH and a reduction in *JAK2* V617F VAF. Lenalidomide and prednisone combination therapy in PMF was evaluated in a phase II trial within the Eastern Cooperative Oncology Group and appeared to be modestly active but myelosuppressive. 42 MF patients with anemia were treated with lenalidomide at 10 mg/day in combination with a 3-month prednisone taper. Grade 3 or higher myelotoxicity was noted in 88% of patients, with an IWG-MRT response rate of 23% (19% clinical improvement in anemia or 10% clinical improvement in spleen size). Whether either of these immunomodulatory drugs (IMiDs) is superior to the other is a subject of speculation that could only be answered by a large randomized clinical trial.

Because IMiDs as a class have shown activity in MF and their use is often undermined by neurotoxicity or myelosuppression, recent interest in the structurally related but more potent pomalidomide in the treatment of MF-associated anemia has led to the completion of several early-phase studies and a randomized phase III study. The first study to evaluate the response of pomalidomide in MF was a phase II randomized, double-blind, placebo-controlled adaptive design study with four treatment arms. This study reported an overall response in anemia of 24% (10 patients), and 15 of these patients achieved transfusion independence that was durable at 7.5 months. Pomalidomide at 0.5 mg/day with or without a prednisone taper was found to have better anemia responses with less toxicity compared with pomalidomide at 2 mg/day with or without prednisone. Grade 3/4 myelosuppression and neurotoxicity were not frequent adverse events. A single-center phase II study of low-dose pomalidomide was conducted at Mayo Clinic. A cohort of 58 patients was treated with pomalidomide at 0.5 mg/day. Of the 42 transfusion-dependent MF patients, ten had significant anemia responses, with nine patients achieving transfusion independence. Although there were no spleen responses, a 58% response in thrombocytopenia for those with baseline platelets below 100 × 10^9 cells/L was noted. Anemia response was predicted by peripheral blood basophilia within the first month of therapy and the absence of massive splenomegaly and was restricted to *JAK2* V617F-mutated PMF patients. Pomalidomide was also evaluated in phase I/II dose-escalation setting to determine if higher doses of pomalidomide were more effective in reversing the anemia associated with MF. The maximum tolerated dose (MTD) was 3 mg/day given for 21 out of 28 consecutive days, and myelosuppression was the dose-limiting toxicity without any added effects on the number of anemia responses. The placebo-controlled, randomized, phase III RESUME trial compared pomalidomide at 0.5 mg/day with placebo with an opportunity to crossover and enrolled both *JAK2*-mutated and wild-type MF patients with documented anemia. The primary endpoint of anemia response was not met in this large global trial as both arms achieved TI in 16% of treated patients. However, 21% of patients achieved clinical improvement in platelet count with pomalidomide therapy compared to none of the placebo-treated patients. Whether this trial would have had positive results if eligibility had been restricted to MF patients with *JAK2* V617F and limited splenomegaly remains conjecture.

TGF-β facilitates aberrant signaling of stromal cells in the bone marrow microenvironment of MF, resulting in erythroid maturation arrest during erythropoiesis. The activin receptor TGF-β family ligand traps, sotatercept and luspatercept, administered subcutaneously every 3 weeks, prevent ligand binding to activin receptors IIA (sotatercept) and IIB (luspatercept), reduce aberrant SMAD signaling, and promote terminal erythroid differentiation. In a phase II study of sotatercept monotherapy and in combination with ruxolitinib, in 20 evaluable MF patients with anemia, 35% of patients responded to sotatercept monotherapy, of which three patients achieved RBC-transfusion independence (TI); 23% of TI patients experienced an increase in hemoglobin percentage with ruxolitinib combination therapy (NCT01712308). The recently presented study of luspatercept monotherapy and combination therapy with ruxolitinib evaluated TI ($n = 34$) and TD ($n = 40$) patients with MF. Among the TI patients, 10% in the monotherapy arm and 21% in the combination arm achieved a sustained hemoglobin increase ≥1.5 g/dL at ≥12 weeks. Among the TD patients, 10% in the monotherapy arm and 32% in the combination arm achieved RBC-TI for ≥12 consecutive weeks (NCT03194542). Sotatarcept and luspatercept were reasonably well tolerated and shared class-specific treatment-emergent adverse events (TEAEs), that is, hypertension and bone pain. Further evaluation of luspatercept is ongoing, both as a single agent and in combination with ruxolitinib in MF patients with anemia.

TREATMENT OF SPLEEN-RELATED AND SYSTEMIC SYMPTOMS

Historically, pressure symptoms related to splenomegaly have been treated with cytotoxic chemotherapy, namely busulfan, melphalan, and hydroxyurea. Occasionally, a significant reduction in spleen size with symptom alleviation occurs in patients receiving chemotherapy. Unfortunately, these responses are short-lived, lasting a median of only 4.5 months. Only 16% of patients enjoy sustained relief of symptoms on long-term maintenance therapy. Accompanying hematologic toxicity often necessitates curtailing therapy within a short duration. This evident lack of effective treatment options in PMF led to the development of oral small-molecule tyrosine kinase inhibitors targeting the hyperactive JAK-STAT signaling pathway.

Ruxolitinib, a selective JAK1 and JAK2 tyrosine kinase inhibitor, was the first drug to be approved by the United States Food and Drug

Administration (US FDA) in 2011 for the treatment of patients with intermediate- or high-risk MF. The phase I/II study determined 15 mg twice daily as the optimal starting dose, with thrombocytopenia and worsening anemia as dose-limiting toxicities. Dose-dependent suppression of phospho-STAT3 was observed in both *JAK2*-mutated and nonmutated patients treated with ruxolitinib, thus demonstrating ruxolitinib's inhibitory activity regardless of the *JAK2* mutational status. 52% of treated patients achieved a greater than 50% reduction in splenomegaly for more than 12 months, irrespective of their *JAK2* mutational status. The debilitating symptoms associated with MF dramatically improved, which correlated with a decrease in inflammatory cytokines mediated through inhibition of JAK1 signaling. Ruxolitinib was then evaluated in two randomized phase III trials: COMFORT 1 (United States, Canada, and Australia), in which ruxolitinib therapy was compared with placebo, and COMFORT 2 trial (Europe), in which ruxolitinib was compared with the best available therapy.[12,13] The results of each of these trials are remarkably similar, and each trial was adequately powered. Patients with intermediate-2 or high-risk MF as assessed using the IPSS were eligible regardless of *JAK2* mutational status. All patients had platelet counts over 100 × 10^9/L with a palpable spleen at least 5 cm below the left costal margin.

In COMFORT 1, the primary endpoint, 35% or greater splenic volume reduction (SVR) was met at 24 weeks in 41.9% of the ruxolitinib-treated cohort and only 0.7% in the placebo group. This response was maintained for 48 weeks or more in 67% of treated patients. Fig. 72.10 shows the percent change in spleen volume from baseline at week 24 for individual patients on both arms of this trial, which depicts the striking contrast of spleen reduction in ruxolitinib-treated patients compared with the majority of patients in the placebo arm who had an increase in spleen volume. There were 13 deaths in the ruxolitinib arm and 24 deaths in the placebo arm, revealing a modest survival benefit for MF patients treated with ruxolitinib. Although grade 3/4 anemia and thrombocytopenia were frequent in the ruxolitinib arm (45.2% and 12.9%, respectively), discontinuation for these events was extremely rare.

The primary endpoint in the COMFORT-2 trial was met at 48 weeks, with 28% of patients in the ruxolitinib arm and 0% in the BAT arm achieving at least a 35% SVR. The mean length of palpable spleen decreased by 56% at 48 weeks, compared with an increase of 4% in the BAT arm. Sophisticated disease-specific QoL tools were used to evaluate the effects of ruxolitinib therapy on systemic symptoms secondary to the elaboration of inflammatory cytokines modulated by JAK1 signaling. The effects of ruxolitinib were, however, limited in the reversal of histomorphologic abnormalities, including BM fibrosis or osteosclerosis, a significant reduction in the *JAK2* V617F allele burden, and loss of marker CGAs, which indicates that it does not modify the underlying neoplastic potential of PMF. Abrupt cessation of ruxolitinib therapy can lead to "ruxolitinib withdrawal syndrome" characterized by rapid onset splenomegaly, worsening of cytopenias, acute relapse of disease symptoms, and occasional hemodynamic and pulmonary decompensation, which may at times be fatal. Gradual tapering and/or the use of a short steroid taper is recommended to avoid this complication.

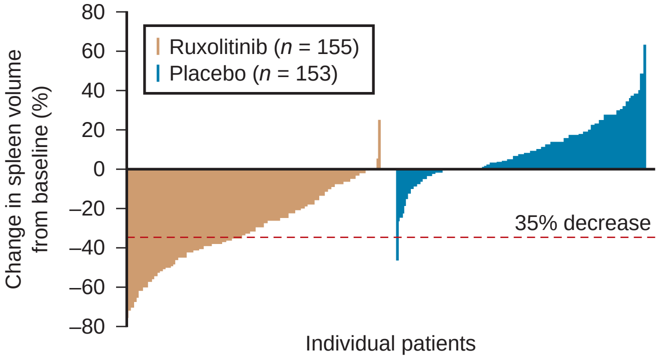


Figure 72.10 REDUCTION IN SPLENIC VOLUME WITH RUXOLITINIB THERAPY IN THE COMFORT I TRIAL. The waterfall plot shows the percent change from baseline in spleen volume at week 24 (in 139 patients in the ruxolitinib group and 106 in the placebo group) or at the last evaluation before week 24 (in 16 patients in the ruxolitinib group and 47 in the placebo group). Data for one patient with a missing baseline value are not included on the graph. Most patients in the ruxolitinib group (150 out of 155) had a reduction in spleen volume, but most patients in the placebo group had either an increase in spleen volume (102 out of 153 patients) or no change (15 out of 153 patients). *(From Verstovsek S, Mesa RA, Gotlib J, et al. A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. N Engl J Med. 2012;366:799.)*

Ruxolitinib is an important agent for the palliation of symptomatic splenomegaly and the debilitating symptoms seen in patients with PMF. The favorable survival data from the COMFORT 1 trial, although modest, are intriguing and suggest that even if a considerable impact on abnormal BM histopathology or molecular or cytogenetic responses is not seen with ruxolitinib, pharmacologic suppression of the JAK-STAT pathway decreases the risk of death, perhaps simply by improving the overall performance status, and reversing the cancer cachexia of an individual. Ruxolitinib has been demonstrated to be safe in patients with a minimum platelet count of 50 × 10^9/L. The need to treat patients who are asymptomatic with limited splenomegaly is not apparent as the treatment does not modify the disease course. The most recent planned analysis of COMFORT-1 at 5 years demonstrated a durable response in spleen reduction and symptom improvement in those subjects who remained on ruxolitinib. No new/unexpected toxicities or late-onset emergent hematologic adverse events were reported. However, it should be noted that a 50% discontinuation rate at 3 years was reported in the arm randomized initially to ruxolitinib, and the potential for leukemic transformation with extramedullary involvement has also been reported.

Patients treated with ruxolitinib are at an increased risk for infectious complications, including reactivation of latent herpes zoster infections leading to shingles, toxoplasmosis, cryptococcal pneumonia, reactivation of latent herpes simplex virus and hepatitis B, and disseminated tuberculosis. Ruxolitinib affects components of both innate and adaptive immunity, including natural killer cells, dendritic cells, and T cells (Th and Tregs). A concern was raised regarding an association between ruxolitinib exposure and the risk of aggressive C-MYC-driven lymphoproliferative neoplasms in these patients. However, several retrospective studies have failed to show an association between the development of lymphoma and ruxolitinib exposure. Importantly, the clinical consequences of ruxolitinib therapy in patients with preexisting B-cell clones warrant further prospective studies.

Following the approval of ruxolitinib in 2011, FDA approval of the selective JAK2 inhibitor fedratinib in August 2019 provided an alternative JAK2 inhibitor for the treatment of patients with intermediate-2 or high-risk PMF with a baseline platelet count ≥50,000/mm^3 irrespective of prior JAK inhibitor treatment. JAKARTA, a randomized, multicenter, double-blind, placebo-controlled phase III trial, evaluated the safety and efficacy of fedratinib in patients with PMF.[14] Patients were evaluated in three arms: fedratinib 400 or 500 mg daily, or placebo for 24 weeks with crossover from placebo after that time. The primary endpoint was ≥35% SVR at 24 weeks. The secondary endpoint was a 50% reduction in symptom burden indicated by the Myelofibrosis Symptom Assessment Form (MFSAF). Among the 289 enrolled patients (96 at 400 mg of fedratinib, 97 at 500 mg of fedratinib, and 96-placebo), 36% and 40% of patients in the fedratinib 400-mg and 500-mg arms, respectively, achieved spleen response as opposed to 1% in the placebo arm ($P < 0.001$). MFSAF score reduction was achieved in 36% and 34%, in those who received 400 mg and 500 mg of fedratinib, respectively, as opposed to 7% in the placebo arm. No appreciable changes in the *JAK2*V617F VAF were observed. The drug was generally well tolerated and anemia was the most common hematological TEAE. Nonhematological TEAE include gastrointestinal toxicities, predominantly low-grade nausea, and diarrhea in the first month that diminished over time. Of note, four patients in the 500 mg fedratinib arm experienced Wernicke encephalopathy, which led to an abrupt FDA-mandated clinical hold and closure of the JAKARTA study in 2013.

JAKARTA-2, conducted in parallel with the JAKARTA study, was an open-label, phase II study that evaluated the safety and efficacy of fedratinib in 97 patients with intermediate-1-high-risk MF who were deemed intolerant or resistant to ruxolitinib by the investigator.[15] A precise definition of ruxolitinib intolerance or resistance was not described at that time. Dose escalation up to 600 mg from 400 mg was permitted if there was less than 50% reduction in palpable spleen size by the end of cycles 2 and 4. This study was also halted early due to the FDA clinical hold. A recent reappraisal of the JAKARTA-2 study data reported that a 31% SVR and 27% symptom response at 24 weeks in the intention to treat cohort was observed. The hematologic and nonhematologic TEAEs were as reported previously. Unfortunately, long-term follow-up data are not available due to the early cessation of the study.

The path to FDA approval of fedratinib was challenging due to the clinical hold placed between 2013 and 2017. An independent central review determined that among the original eight reported potential cases of Wernicke encephalopathy, there was only one definitive confirmed case of Wernicke encephalopathy (clinical and confirmatory MRI). This led to the FDA black box warning of the risk of serious and fatal encephalopathy, including Wernicke encephalopathy. These concerns have resulted in prophylactic oral thiamine administration routinely in patients being treated with fedratinib. The ongoing postmarketing phase III/IIIb FREEDOM studies (NCT03755518, NCT03952039) will add to the overall safety data of fedratinib. Given that both ruxolitinib and fedratinib are only approved for the treatment of patients with MF with a baseline platelet count ≥50,000/mm^3, there is an urgent unmet need for effective treatment options in MF patients with anemia and thrombocytopenia. Momelotinib (JAK1/JAK2 inhibitor) and pacritinib (JAK2/FLT3 inhibitor) are currently being evaluated in phase III registration trials and have the potential to be used as either first-line agents in ruxolitinib-naïve patients with significant baseline cytopenias, or as second-line agents for patients experiencing ruxolitinib-associated cytopenias or loss of clinical response.

Momelotinib, in addition to the JAK1/2 inhibition, was also shown to inhibit bone morphogenic protein receptor kinase activin A receptor type I (ACVR1)–mediated hepatocyte hepcidin expression, which has been shown to stimulate erythropoiesis in murine models of chronic anemia. This suppression of hepcidin was thought to lead to increased mobilization of sequestered iron from cellular stores and subsequent stimulation of erythropoiesis. In the phase III SIMPLIFY-1 study of momelotinib versus ruxolitinib in treatment naïve patients with intermediate- or high-risk MF, momelotinib achieved its prespecified primary endpoint (SVR 35%) of noninferiority to ruxolitinib (momelotinib: 26.5%; ruxolitinib: 29.0%; 95% CI: −11.2% to +5.6%; $P = 0.011$). However, the key secondary endpoint, TSS50, did not meet the noninferiority, therefore formal sequential statistical testing was not undertaken for the three additional anemia secondary endpoints [proportion of patients who are TI, TD, or transfusion rate]. About 66.5% of patients treated with momelotinib achieved TI compared with those treated with ruxolitinib (49.3%; nominal $P < 0.001$). In the phase III SIMPLIFY-2 study of momelotinib versus BAT in patients with intermediate- or high-risk MF with prior JAK inhibitor exposure, momelotinib did not achieve its primary endpoint (only 7% in the momelotinib group and 6% in the BAT group attained SVR 35%; 95% CI –0.09 to 0.10; $P = 0.90$). However, in secondary endpoint analyses, 43% of patients in the momelotinib arm were TI at week 24 compared to 21% patients in the BAT arm ($P = 0.0012$), and 40% of momelotinib-treated patients did not require transfusions over the treatment phase, compared with 27% of patients in the BAT group (nominal $P = 0.10$). The effects of momelotinib on stimulating erythropoiesis, inducing anemia responses, and achieving transfusion independence suggest that this drug may particularly benefit patients with MF-associated anemia. Therefore MOMENTUM, a randomized, double-blind global phase III trial, will evaluate the efficacy of momelotinib compared to danazol in patients with MF and anemia after previous treatment with ruxolitinib (NCT04173494).

Pacritinib is a JAK2/FLT3/IRAK1 inhibitor with substantial early clinical development data indicating efficacy and minimal myelosuppression in MF. The phase III multicenter randomized, controlled, PERSIST-1 trial evaluated pacritinib in a JAK inhibitor-naïve population ($n = 327$), and met its primary endpoint of SVR35% (19% with pacritinib compared to 5% with BAT) at week 24. The phase III PERSIST-2 trial was conducted in thrombocytopenic patients (baseline platelet count $\leq 100 \times 10^9$/L) not excluding prior ruxolitinib exposure and compared two doses of pacritinib (400 mg once daily, the dose evaluated in PERSIST-1, and 200 mg twice daily) to BAT. Although 311 patients were recruited, only 221 (75, 74, and 72 in the three arms) could be included in the ITT efficacy population as trial accrual was affected by the imposition of a "full clinical hold" on pacritinib development by the FDA in February 2016 due to concern over high-grade cardiac and hemorrhagic events leading to increased mortality risk in the PERSIST studies. This ultimately led to the dose-finding PAC 203 trial in MF patients with ruxolitinib failure and a platelet count $<100 \times 10^9$/L (200 mg BID; 100 mg QD; and 100 mg BID). Greatest SVR35% rate (17%) and improvement in symptom scores were observed with 200 mg twice daily of pacritinib. Of note, SVR benefit was seen in subgroups of patients with thrombocytopenia in the PERSIST-1, PERSIST-2, and PAC 203 studies, suggesting that pacritinib may be a reasonable therapeutic option in patients with MF and baseline severe thrombocytopenia. The phase III PACIFICA study will compare pacritinib to Physician's Choice therapy for patients with MF and severe thrombocytopenia (platelet counts <50,000/μL) with less than 3 months of prior ruxolitinib exposure (NCT03165734).

Splenic irradiation was frequently used in the past for treatment of the painful large-spleen syndrome but is now considered a last-resort option with the approval of JAK1/2 inhibitor therapy. Radiation in fractions of 0.15 to 1 Gy administered daily or by an intermittent fractionation schedule (i.e., two or three times a week) to a total dose per treatment course of 2.5 to 6.5 Gy may be effective. Responses were transient, lasting an average of 3.5 months, and hematopoietic toxicity was frequently significant. Splenic irradiation is still useful for the treatment of splenic pain of sudden onset and treatment of ascites caused by implants of hematopoietic tissue. Radiation therapy should be considered as a temporary measure to be used in patients who are too ill to tolerate splenectomy or chemotherapy. Splenic irradiation is limited by myelosuppression and prolonged cytopenias, which is neither predictable nor does it correlate with the radiation dose.

Radiotherapy offers a viable treatment option and sometimes maybe the therapy of choice for patients with symptomatic hepatomegaly, peritoneal and pleural implants or pulmonary infiltration leading to ascites or pleural effusions, and EMH in vital organs leading to organ dysfunction. Given the exquisite sensitivity of myeloid tissue to radiation and profound BM suppression that may occur after irradiation, therapy is usually initiated at low doses (20 to 25 cGy/day) and modified as the clinical situation dictates. An alternative approach using intraperitoneal administration of cytosine arabinoside has been used to treat ascites in PMF. Low-dose, single-fraction, whole-lung radiotherapy has also been useful in treating pulmonary arterial hypertension associated with PMF.

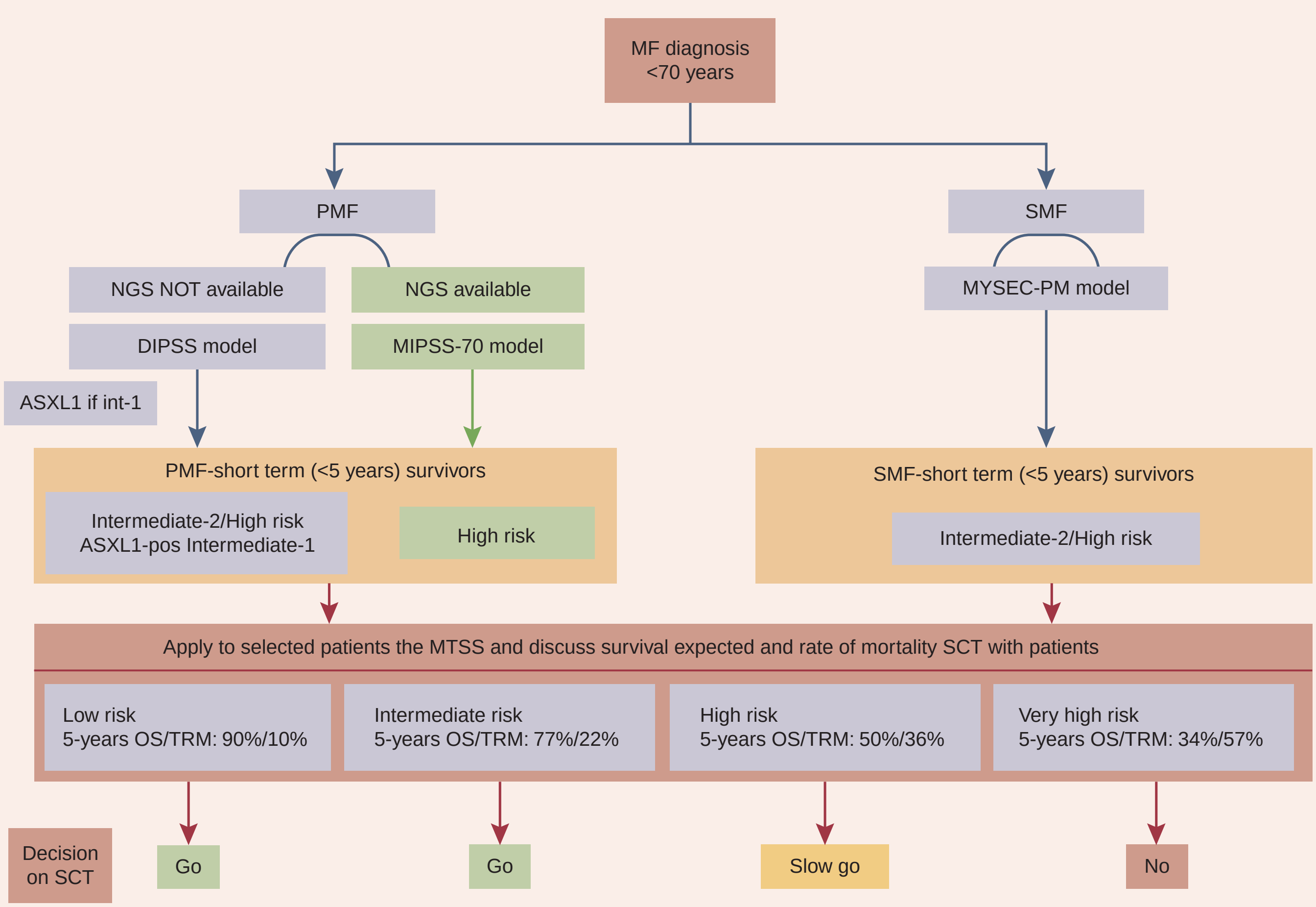
Splenectomy remains a viable option even in the face of recent availability of JAK2 inhibitors in patients with hemolysis, thrombocytopenia, painful splenomegaly, recurrent splenic infarction, and portal hypertension refractory to other therapeutic modalities or in individuals with leukopenia or thrombocytopenia that prevents one from resorting to therapy with chemotherapeutic agents or a JAK2 inhibitor. Splenectomy in patients with PMF is associated with a postoperative morbidity rate of 15% to 30% and a mortality rate of almost 10%. The appropriate implementation of splenectomy can improve QoL in PMF patients who frequently do not have other therapeutic options. Because splenectomy is associated with significant morbidity and mortality, the physician should only resort to this strategy if thrombocytopenia, anemia, or symptomatic splenomegaly is unresponsive to less invasive approaches such as JAK2 inhibitor therapy. Progressive hepatomegaly and marked thrombocytosis occurred in 16% and 22% of patients, respectively, after splenectomy and can often be controlled with JAK inhibitor therapy.

ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

Allogeneic HSCT is a potentially curative therapeutic option in patients with MF and successful transplantation is associated with gradual resolution of BM fibrosis, reduction in splenomegaly, and normalization of hematopoiesis (see box on Algorithm for Selection of Appropriate Patients for Stem Cell Transplantation Stratified According to Risk Status). Historically, allo-HSCT has been recommended for the transplantation of eligible patients with intermediate-2 and high-risk MF. More recently, a 2018 update of European LeukemiaNet (ELN) guidelines also included an intermediate-1 risk score group with refractory transfusion-dependent anemia, having ≥2% blasts, adverse cytogenetics, or high-risk mutations.

Most of our understanding of the role of HSCT in primary and post-PV/ET MF with a conventional myeloablative conditioning (MAC) regimen is derived from two retrospective series of transplants with more than 50 patients performed between 1979 and 2002. The median age of transplanted patients was 42 and 43 years;

ALGORITHM FOR SELECTION OF APPROPRIATE PATIENTS FOR STEM CELL TRANSPLANTATION STRATIFIED ACCORDING TO RISK STATUS


MF diagnosis
<70 years
PMF
SMF
NGS NOT available
NGS available
MYSEC-PM model
DIPSS model
MIPSS-70 model
ASXL1 if int-1
PMF-short term (<5 years) survivors
Intermediate-2/High risk
ASXL1-pos Intermediate-1
High risk
SMF-short term (<5 years) survivors
Intermediate-2/High risk
Apply to selected patients the MTSS and discuss survival expected and rate of mortality SCT with patients
Low risk
5-years OS/TRM: 90%/10%
Intermediate risk
5-years OS/TRM: 77%/22%
High risk
5-years OS/TRM: 50%/36%
Very high risk
5-years OS/TRM: 34%/57%
Decision on SCT
Go
Go
Slow go
No

	Age	Hb, g/dL	PLT, x10^9/L	WBC, x10^9/L	Blast, %	PS	BMF	Driver mutation	Other mut.	Donor
DIPSS	>65 (1)	<10 (2)	-	>25 (1)	≥1% (1)	CS (1)		-	-	–
MIPSS-70	-	<10 (1)	<100 (2)	>25 (2)	≥2 (1)	CS (1)	≥2 (1)	Non-CALR T1 (1)	HMR (1)/>2 (2)	-
MYSECPM	Cont (0.15)	<11 (2)	<150 (1)	–	≥3 (2)	CS (1)		Non-CALR (2)	-	-
MTSS	>57 (1)	-	<150 (1)	>25 (1)	-	K < 90% (1)	-	Non-CALR/MPL (2)	ASXL1 (1)	MMU (2)

Critical information to personalize management of patients with MF. *(Top)* Decision flowchart for each patient with MF suitable for SCT. *(Bottom)* All prognostic models in use with variables and score in parentheses for assessing risk category to each patient. DIPSS categories: low (0); intermediate-1 (1–2), intermediate-2 (3–4), high risk (5–6); MIPSS-70 categories: low (0–1); intermediate (2-4), high risk (≥5); MYSEC-PM (myelofibrosis secondary prognostic model) categories: low (<11); intermediate-1 (11–13), intermediate-2 (14–15), high risk (≥16); MTSS categories: low (0–2); intermediate (3–4), high (5), very high risk (6–9). Cont, continuous; CS, constitutional symptoms; Hb, hemoglobin value; HMR, high molecular risk; K, Karnofsky; MMU, mismatched unrelated; mut., mutation; PLT, platelet count; PS, performance status; WBC, white blood cell count.

From Passamonti F. Stem cell transplant in MF: it's time to personalize. *Blood.* 2019;133(20):2118–2120.

treatment-related mortality (TRM) was 27% and 33%, with a 5-year survival of 47% and 58%, respectively. The 5-year probability of treatment failure caused by non-engraftment, relapse, or persistent disease after transplantation was 36%, and the failure of sustained engraftment was 10.7%, which occurred solely in patients receiving BM transplants from mismatched or unrelated donors. The probability of grade III to IV acute graft-versus-host disease (GVHD) was 33% and 21%, and extensive chronic GVHD was 35% and 50%, respectively. Older age and factors associated with more advanced disease (Hg < 10, increased number of RBC transfusions, presence of osteomyelosclerosis, increased number of clonal abnormalities) adversely affected the outcome. The Seattle group updated their 15-year experience with 95 PMF and post-PV/ET MF patients who received MAC with busulfan and/or TBI-containing regimens. The 7-year actual survival rate was 61%, although 10% of patients died of recurrent or persistent disease. NonTBI–containing regimen of targeted busulfan/cytoxan resulted in an improved probability of survival of 68%. These retrospective reports of MAC allo-HCT in PMF provided evidence that engraftment can be achieved, and that a complete and durable remission of the disease can occur in approximately 50% of patients. However, owing to the poor transplant outcomes in older patients, the use of MAC HSCT represents a major therapeutic dilemma in PMF, where patients are primarily diagnosed in their 60s.

The advent of non-MAC, reduced-intensity conditioning (RIC) regimens for aHCT has resulted in decreased TRM and expanded the use of this treatment modality to older patients. The MPD-Research Consortium (MPD-RC) study by Rondelli and coworkers provided one of the initial prospective reports on the outcome of RIC-based transplant (PBSC, matched related or unrelated donor) in 21 intermediate- to high-risk PMF patients (without leukemic transformation) with a median age of 54 years (range: 27 to 68 years). Predominantly fludarabine-based RIC regimens allowed engraftment in all but one patient with a TRM of 10% and 2-year OS rates of 87%. Durable remission was achieved in 17 out of 18 patients alive at 12 to 122 months (median: 31 months) after transplant. Three patients relapsed, with two of them able to achieve remission following a second transplant and donor lymphocyte infusion. Thirty-three percent of patients developed acute GVHD grades II to IV (9% grade III to IV), and 72% developed chronic GVHD.

A CIBMTR retrospective data of 223 patients who underwent RIC fludarabine-based HCT between 1997 and 2010 showed a 47% probability of 5-year survival. Donor type was the only significant factor influencing survival, with 56% vs. 48% vs. 34% 5-year OS in the related donor, matched unrelated donor, and mismatched unrelated donor, respectively. A trend toward decreased mortality was observed in patients with a low/intermediate-1 DIPSS score versus intermediate-2/high-risk DIPSS.

The use of RIC PBSC transplantation in patients with advanced MF was evaluated prospectively in two phase II trials by the European Group for Blood and Marrow Transplantation (EBMT) and MPD-RC. The EBMT trial included 103 MF patients transplanted from related ($n = 33$) or unrelated donors ($n = 70$), with 21% of donors having at least one allele or antigen mismatch using a fludarabine/busulfan/ATG regimen. Although all but two patients achieved initial engraftment, secondary graft failure (GF) or poor graft function (PGF) occurred in 11% of patients. The estimated 5-year EFS and OS were 51% and 67%, respectively. One-year nonrelapse mortality was 16%—similar among related and unrelated donor transplants but significantly higher in mismatched transplants (38% vs. 12%). One-third of patients developed grade II-IV acute GVHD (11% grade III to IV), with half of the patients affected by chronic GVHD (24% extensive). Relapse rate/treatment failure at 3 and 5 years was 22% and 29%, respectively. As can be seen in Fig. 72.11, age over 55 years, human leukocyte antigen (HLA) mismatching, and advanced disease are associated with decreased PFS and OS. However, even in this high-risk group, the OS was approximately 40%. Remarkably, patients younger than 55 years of age had a 5-year OS rate of 82%. Elimination of *JAK2* V617F after transplant was associated with a reduced incidence of relapse.

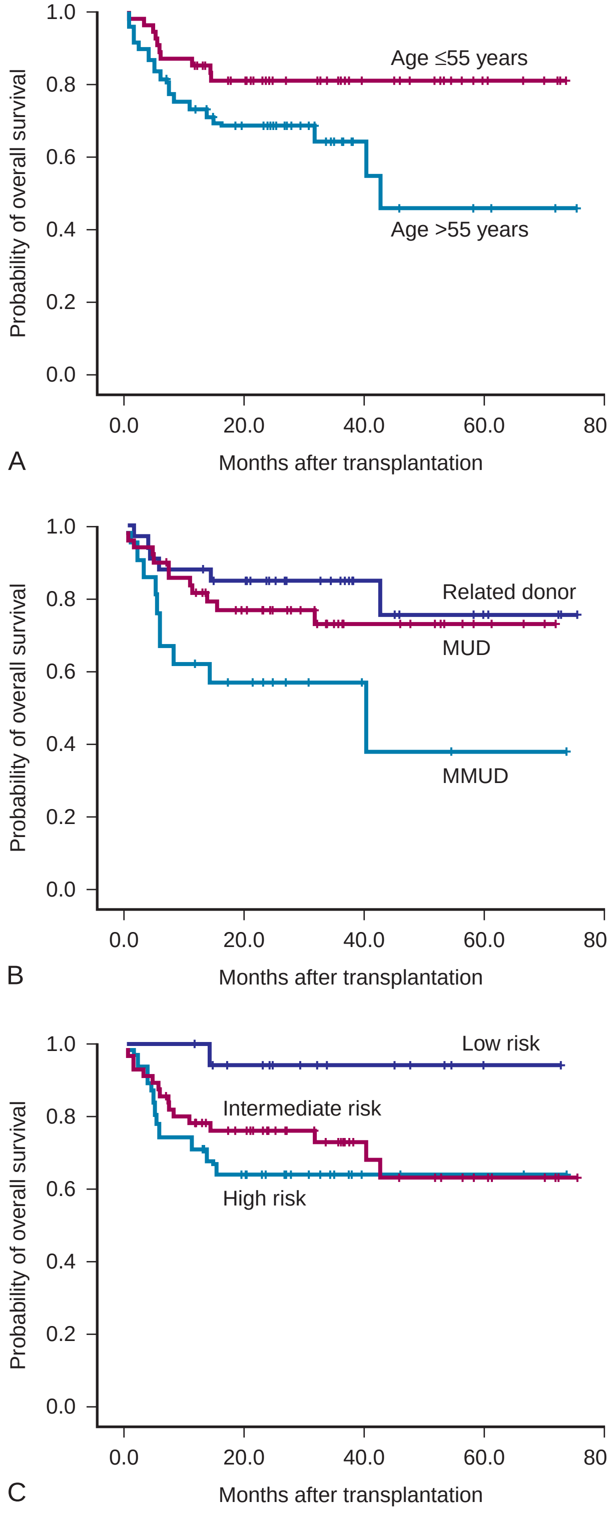


Figure 72.11 Survival of patients with myelofibrosis after reduced-intensity allogeneic stem cell transplantation according to age (A), donor (B), and Lille risk profile (C). *MMUD*, Mismatched unrelated donor; *MUD*, matched unrelated donor.

The prospective, multicenter phase II MPD-RC 101 trial included two parallel cohorts of patients: related donors ($n = 32$, 94% HLA matched) and unrelated donors ($n = 34$, 74% HLA matched). Fludarabine/melphalan was used as a conditioning regimen with rabbit antithymocyte globulin (rATG) added to the unrelated donor group. Patient characteristics were similar in the two groups, with 63 out of 66 patients having intermediate- to high-risk disease based on the Lille score. After a median of 25 months follow-up, OS was 75% (median not reached) in the related donor cohort and 32% (median:

6 months) in the unrelated donor. Nonrelapse mortality at 59% was significantly higher in the unrelated group compared with 22% in the related group. GF occurred in 36% (24% primary) of unrelated transplants and 6% (3% primary) of related. The unrelated cohort had double the rate of severe acute GVHD grade III to IV (21% vs. 12%) but comparable rates of chronic GVHD (38% vs. 36%), including extensive form (20% vs. 25%). Patients who were able to achieve sustained stem cell engraftment experienced an overall response rate of 93% in the related group and 69% in the unrelated group, with only four patients suffering progression of disease. Neither the diagnosis (primary or secondary MF), HLA mismatch, presence of *JAK2* V617F mutation, nor age greater than 57 years showed a statistically significant effect on survival. The only factor that correlated with improved survival was related donor type, as seen in Fig. 72.12. The low/intermediate-1–risk patients in the unrelated group had better survival than the intermediate-2/high-risk patients, whereas there was no difference in survival between these two DIPSS groups in the related group.

The German group analyzed the effect of multiple risk factors related to the patient, disease, and transplant characteristics on survival after RIC HCT and developed a predictive model based on three risk factors: age ≥57 years, *JAK2* WT status, and presence of constitutional symptoms. Five-year OS in the absence of any risk factors was 90%. The presence of one or two risk factors reduced 5-year OS to 74% and 51%, respectively. Patients who had all three risk factors did extremely poorly after RIC HCT, with a 1-year OS of 25%. Despite inferior outcomes in older patients, HCT is feasible, as demonstrated by a retrospective study of HCT of 30 PMF patients between the ages of 60 and 78 (median: 65) years transplanted at four major US academic centers with 3-year OS and PFS rates of 45% and 40%, respectively.

More recently, Gagelmann et al. utilized clinical variables including transplant related (age ≥57 years, Karnofsky performance status <90%, platelet count <150 × 10^9/L, leukocyte count >25 × 10^9/L, HLA-mismatched unrelated donor) and molecular variables (presence of *ASXL1* mutation, and a non-*CALR*/*MPL* genotype) to develop an integrated model (Myelofibrosis Transplant Scoring System [MTSS]) to specifically predict the outcomes of HSCT in MF. In the multivariable analysis of 361 patients with MF (both primary and secondary to PV/ET) who underwent their first HSCT, HR weighted risk points were allotted 2 points each to HLA-mismatch unrelated donor and non-*CALR*/*MPL* genotype; 1 point each to age ≥57 years,

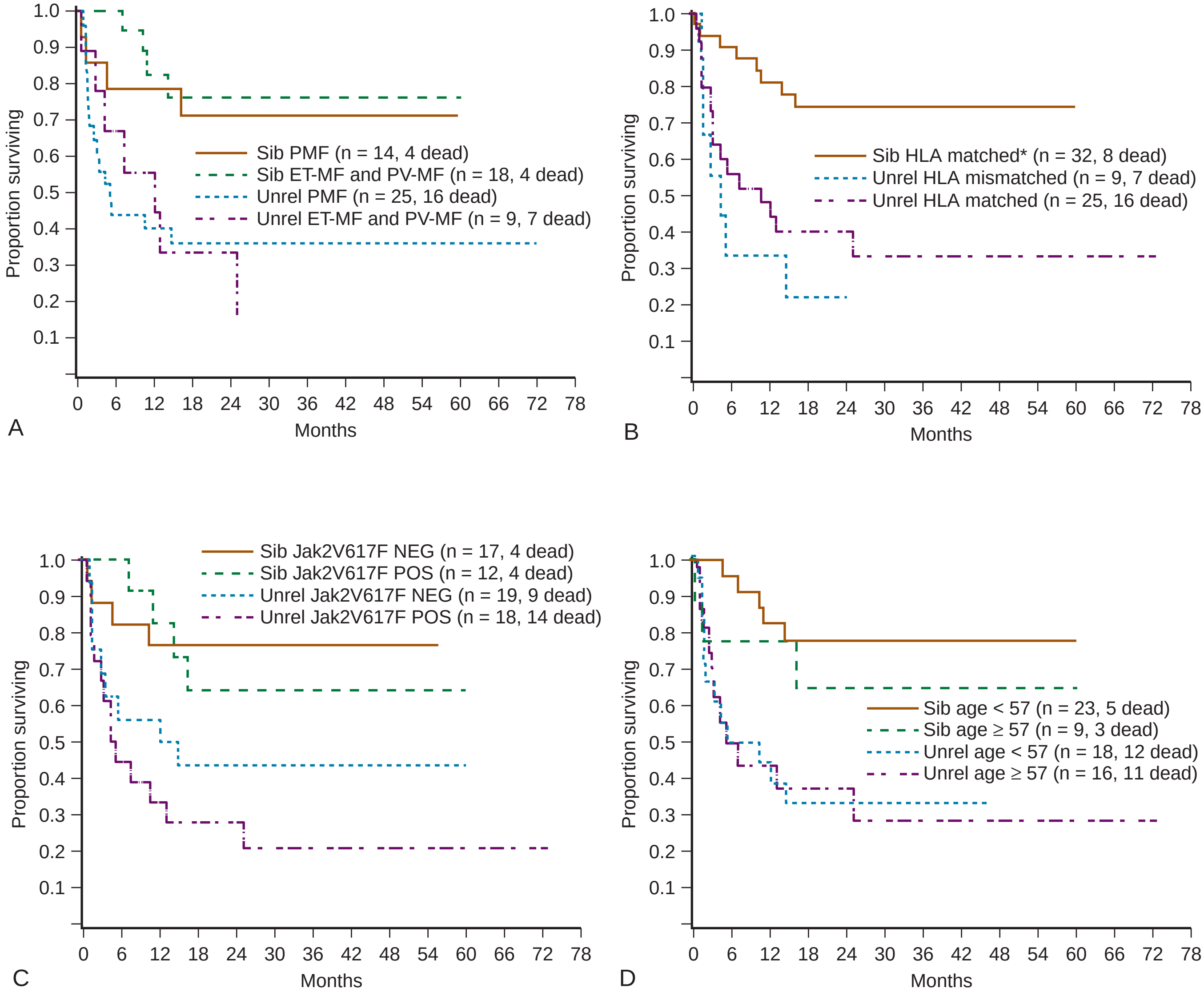


Figure 72.12 Survival for patients with myelofibrosis after receiving reduced-intensity allogeneic stem cell transplantation was worse in the unrelated donor cohort, compared with the related donor cohort irrespective of (A) diagnosis subtype, (B) degree of HLA matching, (C) *JAK2* mutational status, and (D) age. Final results from the multicenter phase II MPD-RC 101 trial. *ET-MF*, Essential thrombocythemia myelofibrosis; *HLA*, human leukocyte antigen; *NEG*, negative; *PMF*, primary myelofibrosis; *POS*, positive; *PV-MF*, polycythemia vera myelofibrosis. *(Data from Rondelli D, Goldberg JD, Isola L, et al. MPD-RC 101 prospective study of reduced-intensity allogeneic hematopoietic stem cell transplantation in patients with myelofibrosis. Blood. 2014;124:1183.)*

Karnofsky performance status less than 90%, platelet count less than 150×10^9/L, leukocyte count greater than 25×10^9/L, and presence of the *ASXL1* mutation. This resulted in a 4-tiered risk predictive model: low (0 to 2 points), intermediate (3 to 4 points), high (5 points), and very high (>5 points) with 5-year OS posttransplant of 83%, 64%, 37%, and 22%, respectively ($P < 0.001$). The MTSS performed exceedingly well compared to similar models (MIPSS70 or the MYSEC-PM) validated in a nontransplant setting.

Wider access to allogeneic HSCT for MF patients has been limited by the availability of HLA-matched donors. Over the past two decades, use of alternative donors such as umbilical cord blood (UCB) and related half-HLA-matched (haploidentical) donors has greatly expanded the donor pool (see Chapters 106, 107, and 108). Takagi and coworkers reported encouraging results of RIC-HCT with UCB grafts in adults (median age, 57.5 years) with various hematologic disorders associated with extensive bone marrow fibrosis. The estimated probability of survival at 4 years was 28.6%, which is modest but considerably superior to that which would be anticipated with currently available therapeutic regimens for such high-risk patients. A retrospective analysis of 35 high-risk MF patients (20% MPN-BP, median age: 54) who underwent UCB transplantation reported by Eurocord showed a 2-year OS and EFS of 44% and 30%, respectively.

An Italian group reported a retrospective analysis of 95 patients demonstrating improvement in HSCT outcomes for patients transplanted between 2011 and 2014 when predominantly using siblings and haploidentical donors (see Chapter 107) with myeloablative thiotepa/busulfan/fludarabine (TBF) conditioning, as compared to patients transplanted between 2000 and 2010 ($n = 58$) using predominantly siblings and unrelated donors and RIC. Bone marrow was the predominant source of stem cells in both groups. Factors leading to improvement in transplant outcomes were attributed to the general improvement in posttransplant care over time (as demonstrated by improved outcomes of the matched-related transplants), TBF conditioning, as well as the use of haploidentical rather than unrelated donors. Retrospective analysis by EBMT society of 56 patients with MF who underwent haploidentical transplant (see Chapter 107) confirmed favorable outcomes with low rates of acute and chronic GVHD, comparable rates of GF, and relapse to matched donors. These studies provide the rationale for the further evaluation of RIC allogeneic transplants for eligible PMF patients lacking an HLA-matched sibling or unrelated donor with the use of alternative sources of grafts such as UCB and haploidentical donors.

Despite the curative nature of HSCT in the treatment of MF, up to 30% of patients experience relapse of their disease. Elimination of *JAK2*V617F after transplant was associated with a reduced risk of relapse, while persistence of known molecular markers (*JAK2, CALR, MPL*) at day + 30 and + 180 posttransplant has been associated with an increased risk of relapse. In patients who lack molecular markers, incomplete or falling donor chimerism can be used to identify patients at risk for relapse. Use of preemptive donor-lymphocyte infusions (DLI) in these patients achieved complete molecular remission. Survival following relapse varies, with patients who relapse in the first 7 months having significantly poorer survival as compared to individuals who relapse later. DLI with or without chemotherapy, as well as a second HSCT, have been used with beneficial effects.

GF and PGF leading to increased risk of morbidity and mortality remain a challenge in patients with MF. GF occurs either due to primary failure to engraft or due to loss of donor cells following successful engraftment. Risk factors for GF include HLA mismatch, T-cell depletion, RIC, major ABO mismatch, and a low stem cell dose. Some of these patients experience autologous stem cell recovery with temporary improvement in disease control. Our group has demonstrated that a second HCT from the same or different donor can allow successful salvage in these patients. PGF, defined as cytopenias involving at least two cell lineages for at least 2 weeks in the setting of full donor chimerism and in the absence of relapse, GVHD, infection, or drug-related myelosuppression, affected 17% of patients in a retrospective study. Pretransplant splenomegaly and persistent splenomegaly after transplantation were identified as significant risk factors. Sequestration of stem cells in the spleen as well as alteration in homing proteins (such as VCAM-1 and CXCL12) are thought to contribute to delayed engraftment and PGF. In a retrospective study, CD34+ selected stem cell boost without prior conditioning resulted in hematological improvement in 81% of patients with hematological malignancies.

The feasibility of utilizing HSCT as the front-line therapy for all intermediate- and high-risk PMF patients is limited by the presence of competing comorbidities or a poor performance status due to the systemic effects of the underlying PMF. Although the JAK2 inhibitors are mostly palliative, they do reduce the degree of splenomegaly and often improve the performance status of such patients, frequently resulting in weight gain and improvement of their candidacy for HSCT. The reported outcomes with JAK2 inhibitor therapy as a component of the conditioning regimen for HSCT have resulted in conflicting results, with some studies showing improved posttransplant outcomes in patients with clinical response to ruxolitinib and others showing increased risk of GVHD, GF, and NRM.

The MPD-RC 114 two-stage, Simon, phase II trial of HSCT with preconditioning with intensive ruxolitinib therapy prior to a RIC regimen in patients with higher risk of MF with a primary endpoint of alive and engrafted was terminated prematurely because of poor accrual and significant GF (16%) and NRM (28%), as well as high rates of acute and chronic GVHD (64% and 76%). Since then, two small prospective studies have shown safety of ruxolitinib administration throughout conditioning and stable engraftment with low levels of aGVHD without increase in GFs. Further rigorous testing is required to determine the effects of JAK2 inhibition on immediate and long-term survival and transplant-related complications.

The fact that allogeneic RIC transplant is curative in appropriate patients is undeniable, and the improvements in the design of conditioning regimens, expansion of the donor pool, and supportive therapy makes this a viable first-line approach for individuals with advanced disease. Whether such an approach should also be implemented in patients with earlier phases of the disease, especially those with adverse cytogenetics or myeloid gene mutations, is a point of contention that requires further careful investigation since some of the adverse mutations have also been shown to have a negative impact on posttransplant outcomes.

Many unresolved issues remain to be clarified concerning the optimal strategy for HSCT in PMF. Understanding of peritransplant role of JAK2 inhibitors requires carefully designed prospective clinical trials. Outcomes of haploidentical donor transplants in management of PMF need to be compared to matched, unrelated donors in randomized prospective trial to determine the optimal donor in cases when both are available. Impact of molecular mutations on transplant outcomes require additional studies.

The need for splenectomy before HCT remains a major issue. Splenectomy before HSCT in PMF results in faster hematopoietic recovery but at the cost of potentially prolonged and complicated postoperative recovery. Although some analyses suggest improved survival with pretransplant splenectomy, it has also been associated with a potential increased risk of relapse. Moreover, even extensive splenomegaly (>30-cm longitudinal size by computed tomography scan) does not appear to prolong hematologic reconstitution after transplant.

The establishment of valid, complete remission criteria for PMF after HSCT remains a significant issue because the conventional criteria for response after HSCT are often influenced by GVHD, infections, or PGF and cannot be used. Conversely, normal blood counts and disappearance of disease-related symptoms do not exclude residual disease. In *JAK2* V617F-positive patients, the mutational allele burden following transplantation may serve as a surrogate marker for complete remission assessment and as a guide for instituting adoptive immunotherapy.

INVESTIGATIONAL THERAPEUTIC OPTIONS

Several targeted therapeutic agents aimed at modifying epigenetics, immunity, cellular survival, metabolic and apoptotic pathways, cell cycle, DNA repair, protein folding, and degradation are in various stages of clinical investigation. Aberrant methylation is commonly encountered in MPNs, which logically led to the evaluation of the DNA methyltransferase inhibitors in PMF. A phase II trial evaluated 5-azacitidine in 34 MF patients and showed an overall response rate of 24% after a median of 5 months of treatment. Grade 3/4 myelosuppression was seen in 29% of patients, requiring dose reduction in 47% of treated patients. Interestingly, there was no difference in global hypomethylation between responders and nonresponders, suggesting an alternative mechanism of action other than induction of DNA promoter site demethylation was responsible for the beneficial effects. Studies designed to evaluate the methylation status of specific genes thought to be silenced by this epigenetic modification (e.g., $p15^{INK4b}$ and $p16^{INK4a}$) need to be done in the future. Ruxolitinib with sequential addition of azacytidine from cycle 4 onwards and a gradual dose titration from 25 to 75 mg/m^2 daily for 5 days of each 28-day cycle showed preliminary activity in a phase II trial enrolling chronic phase MF. Seventy-four percent of treated patients achieved objective responses (OR) by IWG-MRT 2013 criteria, and 60% had greater than 1 grade reduction in BMF. Cytopenias were transient and only four patients discontinued treatment because of cytopenias (NCT01787487).

Histone deacetylase inhibitors (HDACi) downregulate JAK2 signaling by interfering with the chaperone function of heat shock protein 90 (HSP90) through modulation of acetylation. LBH589 (panobinostat) is a potent pan-deacetylase inhibitor that alleviated constitutional symptoms, reduced splenomegaly with improvement in hemoglobin, reduced BM reticulin and collagen fibrosis, and restored normal BM morphology in a small number of patients with PMF. However, a phase II study of panobinostat monotherapy at a dose of 25 mg BID was poorly tolerated and failed to show measurable clinical benefit. A phase I trial (PRIME) reported the safety and efficacy of the combination of ruxolitinib and panobinostat in 15 patients with MF. Forty percent of the treated patients experienced clinical improvement (spleen $n = 5$, anemia $n = 1$), and this combination was found to be safe and tolerable without dose-limiting thrombocytopenia. The MTD of both agents, in combination, was not determined. Further development of this combination has been hampered by modest activity and the need to determine the optimal dosing schedule of panobinostat to avoid myelosuppression and maximize on-target activity.

Modifying the dysregulated transcriptome (e.g., c-Myc, NF-kB overexpression) in MF through inhibition of bromodomain extra terminal (BET) proteins appears to be an exciting therapeutic strategy. Preclinical studies in MF demonstrated synergistic activity between ruxolitinib and the small-molecule BET protein inhibitor JQ1 with a reduction of inflammatory cytokine production, disease burden, and reversal of BMF in an MPL-driven murine model of MF. CPI-0610 is an oral pan-BET protein inhibitor that is being evaluated as monotherapy and in combination with ruxolitinib in patients who have failed and discontinued ruxolitinib, with a suboptimal response, or are naïve to JAK inhibition (NCT02158858). As the preclinical models would predict, activity in terms of SVR35% (29%) and TSS50% (77%) was most notable in the combination arm and particularly impressive in those that were RBC transfusion-dependent at baseline, with 42% of treated patients achieving transfusion independence.

HOW SHOULD PRIMARY MYELOFIBROSIS PATIENTS WITH THROMBOCYTOPENIA BE TREATED?

Platelet Count 50–100 × 10⁹/L

It is important to be certain that vitamin B_{12} or folate deficiencies and/or sustained suppression from prior chemotherapeutic agents are not contributing to the development of a low platelet count. Chemotherapeutic agents such as hydroxyurea can be used in patients with marked splenomegaly, which can result in an improvement in the platelet count as the spleen volume is reduced. Patients can also be treated with thalidomide or lenalidomide in combination with prednisone. These first-generation IMiDs can sometimes improve cytopenias and reduce splenomegaly in PMF patients, and in some cases, this response can persist even after drug discontinuation. Lenalidomide can induce cytopenias to a greater extent than thalidomide, which needs to be taken into consideration before its use in a thrombocytopenic patient. In a randomized phase III trial, pomalidomide failed to meet the primary endpoint of anemia response (transfusion independence: ≥84 days), but the platelet response rate was 22% in the treatment arm. This finding supported the previous phase II findings of a response rate of 58% in increasing the baseline platelet count by greater than 50% in patients with baseline platelets above 50 × 10^9/L. This second-generation IMiD has not been evaluated in patients with platelet counts below 50 × 10^9/L. A course of prednisone therapy should also be considered, since 30% to 40% of patients have been reported to respond to this agent alone.

Ruxolitinib has more recently been shown to be safe and effective in patients with baseline platelet counts as low as 50 × 10^9/L, and the FDA label has been expanded to include such MF patients. Sustained thrombocytopenia with ruxolitinib therapy is an indication for a dose reduction or discontinuation of the drug. Patients can also be enrolled in other JAK2 inhibitor clinical trials in which reduced platelet counts are acceptable (such as the PACIFICA phase III pacritinib trial) or other agents in clinical development. If thrombocytopenia worsens with the current treatment plan, then proceed with the strategy outlined below.

Platelet Count 20–50 × 10⁹/L

Thalidomide or lenalidomide, in combination with prednisone, can be used to correct anemia in these PMF patients but rarely improves the platelet counts in a clinically meaningful way. Most clinical trials involving a variety of therapeutic agents, including JAK2 inhibitors, will require baseline platelets above 50 × 10^9/L. In patients who are experiencing life-threatening bleeding, in addition to aggressive, immediate platelet transfusions, splenectomy is a reasonable therapeutic option. Pacritinib, as first-line JAK inhibitor therapy in JAK inhibitor-naïve MF patients with baseline platelet counts <50 × 10^9/L or AVID200 in those previously treated with a JAK inhibitor, would be a clinical trial consideration.

Platelet Count <20 × 10⁹/L

Treatment choices are truly limited for these patients. Intervention is also dependent on the clinical picture, with emphasis on addressing bleeding. Supportive therapy with frequent platelet transfusions is a possibility, but it is likely not sustainable in the long term. It is our practice to transfuse if the platelet count is below 10 × 10^9/L unless there is no evidence of mucosal bleeding or life-threatening hemorrhage. Occasionally, patients may have an improvement in the degree of thrombocytopenia with steroids. About 20%–30% of patients with severe thrombocytopenia will have significant improvement in platelet counts after a splenectomy. Aggressive platelet transfusional support is necessary before, during, and after splenectomy in many cases. However, splenectomy in patients with PMF is associated with a postoperative morbidity rate of 15%–30% and a mortality rate close to 10%. These numbers, however, are highly dependent on the institutional experience and the operating surgeon. Splenectomy can also result in EMH in the liver, causing hepatomegaly, which may require the administration of a judicious amount of chemotherapeutic agents (hydroxyurea, busulfan, and cladribine) or a JAK2 inhibitor. Severe thrombocytopenia is an adverse prognostic feature. Such patients who are eligible for HSCT and have available donors should be considered for immediate transplantation. Thrombopoietin mimetics have been associated with increased bone marrow fibrosis when used for the treatment of immune thrombocytopenic purpura and have not been formally evaluated in the setting of PMF-associated thrombocytopenia. Additionally, patients with extreme thrombocytopenia and signs of leukemic transformation can be considered for therapy with decitabine or azacitidine with aggressive platelet transfusional support. They then, ideally, should proceed to allogeneic HCT if a donor is available and the patient's performance status is appropriate.

WHICH PATIENTS SHOULD BE CONSIDERED FOR ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION?

Allogeneic hematopoietic stem cell transplantation (HSCT) is the only potential curative treatment option for primary myelofibrosis (PMF). Patients with poor-risk features and decreased probability of survival should be considered for HSCT because the risk of transplant-related morbidity and mortality is not insignificant. All patients younger than 70 years of age and their siblings should be typed for human leukocyte antigens at the time of diagnosis to determine if there is a potential match. Patients between the ages of 65 and 70 years old with available related donors should be evaluated based on their performance status and the presence of comorbid conditions. Patients younger than 70 years of age with good performance status and available donors (either related or fully matched unrelated) should be encouraged to undergo evaluation for HSCT soon after the diagnosis and preferably within a clinical trial. Patients with a high- or intermediate-risk disease with available donors should consider transplantation before developing debilitating symptoms or significant worsening in their performance status. Patients with low-risk disease can be closely monitored without intervention but should proceed to transplantation with evidence of disease progression. For patients with available donors but poor performance status because of the symptoms of myelofibrosis, a course of ruxolitinib can be considered with the goal of improvement in performance status associated with the reduction of splenomegaly and constitutional symptoms. These patients may become more viable transplant candidates with this pretransplant treatment approach. Splenectomy before transplant is not essential to ensure adequate engraftment. Currently, HSCT using reduced-intensity conditioning regimens, haploidentical donors, and cord blood grafts is being actively evaluated.

WHICH PRIMARY MYELOFIBROSIS PATIENTS ARE APPROPRIATE FOR JAK2 INHIBITOR THERAPY?

Not all PMF patients require treatment with JAK2 inhibitor therapy. Patients with symptomatic splenomegaly or debilitating MF-related symptoms are potential candidates for treatment with ruxolitinib or fedratinib. The inhibition of JAK1/2 is associated with myelosuppression and could further compromise existing cytopenias. In patients with isolated splenomegaly and adequate blood counts, a course of hydroxyurea could be considered. If the patient fails hydroxyurea or has debilitating symptoms, then ruxolitinib/fedratinib would be the appropriate agent. Both ruxolitinib and fedratinib are currently approved for patients with intermediate- and high-risk MF and have yet to be formally explored in cases of symptomatic patients with low-risk disease. Additionally, the use of these drugs are not advised in MF patients with platelet counts below 50 × 10^9/L. Ruxolitinib/fedratinib should be used in patients with a poor performance status before HSCT as a means to improve their performance status and promote weight gain in an attempt to optimize them for SCT. Although the COMFORT 1 trial reported a modest improvement in survival at a median of 51 weeks of treatment with ruxolitinib, the exact mechanism that underlies this survival benefit remains unknown, and this agent should not be given with the primary goal of disease process modification and promoting increased survival. Patients started on ruxolitinib need to have frequent monitoring of blood counts. If discontinued, the dose should be either tapered or accompanied by a pulse of concurrent steroid therapy to avoid the life-threatening rebound of systemic symptoms and splenomegaly, which can occur at times.

Fedratinib is approved in the United States as a front-line JAK2 inhibitor option as well as the second line in the treatment of MF. A patient can be safely switched from ruxolitinib to fedratinib, without tapering, to address suboptimal spleen/symptom response or loss of initial response. As with ruxolitinib, blood counts have to be monitored frequently. Gastrointestinal side effects are common but mitigated by antiemetics, and these adverse effects decrease over continued fedratinib administration.

HOW DOES CHARACTERIZING THE MUTATIONAL PROFILE OF A PRIMARY MYELOFIBROSIS PATIENT INFLUENCE CARE?

Assessing the status of the driver mutations *JAK2*, *MPL*, and *CALR* in PMF can help refine prognostication and influence therapeutic decision making. Retrospective studies suggest that molecular risk stratification can further refine the individual risk for leukemic transformation and overall survival. Patients with triple negative status (lacking mutations of *JAK2*, *MPL*, and *CALR*) or with an *ASXL1* mutation are at the highest risk for a poor outcome. They should be evaluated for early HSCT or experimental therapy even if they are assessed to have a low-risk disease by conventional risk-stratification tools. Such patients should be sent for transplant at the earliest time of evidence of disease evolution. It is also important to remember that *JAK2* wild-type status, and *MPL*- and *CALR*-mutated patients respond equally well to ruxolitinib therapy. The characterization of nearly 20 recurrent gene mutations in PMF patients implicates a complex and heterogeneous molecular pathophysiology that will likely not allow for a single therapeutic target drug approach. Whether the presence of certain specific gene mutations will ultimately predict a therapeutic response with different agents has yet to be shown. The presence of these mutations can also be used to detect minimal residual disease after HCT. The combination and number of mutations adversely affect the outcomes in a patient with PMF. *TP53* mutations are rare in chronic phase MF (~5%), but more frequent at the time of transformation to AML. Additionally, those with *ASXL1* and non-*CALR/MPL* mutations also suffer from poor outcomes. PMF patients who harbor *TP53, ASXL1*, or non-*CALR/MPL* mutations should undergo HCT evaluation early in their disease course.

Approximately one-third of treated patients also attained at least one grade reduction in bone marrow fibrosis. A cohort of ruxolitnib-naïve patients was treated with combination therapy and initial results are very encouraging with a SVR35% of 70%. Myelosuppression was minimal and Grade 3/4 anemia and thrombocytopenia occurred in 7% and 10% of treated patients, respectively.

Another epigenetic target of interest in MF is lysine-specific demethylase 1 (LSD1). In preclinical studies, bomedemstat (IMG-7289), an irreversible LSD1 inhibitor, selectively abrogated proliferation and induced apoptosis of *JAK2*^{V617F} cells, normalized blood counts, and reduced bone marrow fibrosis. Bomedemstat is currently under clinical investigation in patients with MF who are resistant or intolerant to ruxolitinib. Fifty percent (7/14) of treated patients achieved a modest SVR, and 79% (11/14) had TSS improvement. Bomedemstat was reasonably well tolerated and is undergoing further clinical evaluation (NCT03136185).

JAKs regulate the antiapoptotic protein BCL-XL; the subsequent demonstration of synergism with JAK2 and BCL-2/-XL inhibition in preclinical *JAK2*^{V617F} MPN models has prompted the investigation of antiapoptotic agents in MF. A phase II clinical trial of navitoclax and ruxolitinib in patients with MF showed preliminary efficacy (30% [9/30] achieved SVR35%, 65% [11/17] experienced reduction in TSS, and 25% had >1 grade reduction in BMF), and the evaluation is ongoing (NCT03222609). On target, thrombocytopenia was observed and was manageable. Another agent of clinical interest targeting the apoptotic pathway is the second mitochondrial activator of caspases (SMAC)-mimetic. The SMAC mimetic LCL-161 antagonizes the inhibitor of apoptosis (IAP) proteins, thereby targeting a different arm of the antiapoptotic machinery. LCL-161 is being evaluated in ruxolitinib-pretreated or ruxolitinib-ineligible patients with MF. Thirty percent (9/30) had OR by IWG-MRT 2013 criteria, with 20% attaining improvement in TSS. Weekly dosing schedule was well tolerated. Further evaluation is ongoing (NCT02098161).

A novel and exciting therapeutic approach to the treatment of MF is an infusional telomerase inhibitor, imetelstat, which is a 13-mer lipid-conjugated oligonucleotide competitive inhibitor of telomerase RNA (hTR), resulting in telomere shortening and cell cycle arrest and apoptosis. A pilot study of imetelstat in 33 patients with MF reported complete morphologic and molecular remissions even in those harboring HMR mutations. The occurrence of elevated liver function tests led to an initial study hold, whose release was followed by the large, multicenter randomized phase II IMbark trial (NCT02426086) that evaluated two dose levels (9.4 and 4.7 mg/kg) of imetelstat in patients with MF who were refractory to or had relapsed after treatment with a JAK inhibitor. The low-dose arm was closed due to a lack of clinical efficacy. Imetelstat 9.4 mg/kg administered intravenously every 3 weeks was well tolerated and demonstrated clinical activity (10% SVR35%, 30% TSS50%), but most importantly, it was associated with a median OS that approached 30 months, which is double the reported median OS in patients who are resistant to JAK2 inhibitors (12 to 14 months). This survival advantage was seen across all molecular risk categories, including triple negative patients. Baseline short telomeres increased telomerase activity and increased hTERT RNA expression level predicted response and pharmacodynamic biomarkers of response, such as reduction in telomerase activity, were enriched in clinical responders, which also included reductions in BMF grade. Given the encouraging results, a global phase III clinical trial of imetelstat in MF patients refractory to JAK2 inhibitor therapy will be initiated with an OS primary endpoint. Of note, the imetelstat phase III trial will be the first study in the clinical investigation of MF to have OS as the primary endpoint.

FUTURE DIRECTIONS

More rational therapies for PMF are evolving from an increased understanding of the cellular and molecular biologic abnormalities underlying PMF. Studies defining the relative contributions of acquired genetic mutations, epigenetic events, as well as the influence of the BM and splenic microenvironment on the origins of PMF, disease progression, and events leading to leukemic transformation are ongoing. Because PMF originates at the level of the HSC, the identification of novel drugs that eliminate such malignant stem cells is likely the most direct route to potentially curative pharmacologic strategies. The evaluation of the use of a combination of active agents, including the JAK2 inhibitors, TGF-β inhibitors, IMiDS, HDACi, BETi, antiapoptotic agents, and telomerase inhibitors, is anticipated to lead to significant clinical improvements in the outcomes of PMF patients. The evaluation of these combination therapies will undoubtedly require the use of novel strategies for the design of clinical trials that will evaluate the effects of several active agents administered either simultaneously or sequentially, and meaningful endpoints such as survival and progression-free-survival will need to be considered. Overall, the implementation of any therapeutic approach by the practicing community of hematologists should only occur after evidence is provided by the completion of well-planned and adequately powered phase III trials comparing the experimental approach to the standard of care.

HSCT remains the only potentially curative therapy available for patients under 70 years of age with acceptable performance status and a suitable available donor. Although there is a reluctance to expose PMF patients to the risks of HSCT if their underlying disorder is associated with a sufficiently poor prognosis, this risk appears to be warranted. Because early forms of PMF are associated with survival of well over a decade, many hematologists have been even more reluctant to expose such patients to experimental therapeutic agents or HSCT. Unfortunately, progression to more advanced forms of MF is anticipated in younger patients. Exploration of therapeutic agents that can delay or even prevent disease progression or identification of the appropriate role of HSCT in this low-risk group will require additional study. Such trials will be time-consuming due to the prolonged survival of such patients with low-risk disease. The decision to proceed to transplant requires a detailed discussion of the risks and benefits of such a high-risk but potentially curative procedure.

The recent establishment of multiinstitutional cooperative groups to evaluate such novel therapies and treatment strategies for PMF patients has met the need of ensuring rapid evaluation of candidate therapeutic agents. No single institution has sufficient numbers of patients to perform this ever-growing number of required investigative efforts. Only with the completion of such rigorous evaluations of individual therapeutic strategies will one be able to determine the value of a continuously growing number of potentially active agents used alone or in combination. Each new therapy will likely be evaluated using not only a number of clinical endpoints but also surrogate biomarkers and consideration of the immediate and long-term toxicities associated with their documented use. Independently developed objective criteria by which responses to experimental therapeutic agents can be judged must be implemented for the evaluation of promising agents. Furthermore, incorporation of validated QoL tools into the clinical trials provides patient-reported outcome data, which is invaluable in assessing the efficacy of novel therapeutic agents. Efforts must be made to evaluate these new classes of agents in the preconditioning regimens for HSCT as this approach may contribute to significant improvements in outcomes in PMF patients undergoing HCT.

CHRONIC NEUTROPHILIC LEUKEMIA

Chronic neutrophilic leukemia (CNL) is a rare Philadelphia chromosome-negative MPN characterized by sustained, mature neutrophilic leukocytosis and bone marrow granulocytic hyperplasia, often accompanied by hepatosplenomegaly. The true incidence of CNL remains unknown, with less than 300 reported cases in the literature, thus attesting to its rarity. CNL is predominantly a disease of older age, with a median age of diagnosis at 66.5 years and a slight predilection for males. CNL was first described by Tuohy in 1920 in a female with symptomatic splenomegaly and mature neutrophilic leukocytosis noted on the peripheral smear. Tanzer et al. in 1964 put forth the terminology “Chronic neutrophilic leukemia of mature cell type” when they described a patient with mature neutrophilia and massive splenomegaly in the absence of t (9;22). Although the WHO recognized CNL as a distinct MPN in 2001, it was only after the discovery of mutations involving the colony-stimulating factor 3 receptor (CSF3R) oncogene in 2013, that the diagnostic criteria for CNL were formalized and the disease was seen as distinct diagnosis with a defined biomarker.[16]

MOLECULAR PATHOGENESIS OF CHRONIC NEUTROPHILIC LEUKEMIA

The growth factor, granulocyte colony-stimulating factor (G-CSF), now referred to as CSF3, principally regulates neutrophil production in basal and emergency conditions (in response to infection, and stress). CSF3R belongs to the cytokine receptor type I superfamily and exploits the canonical Janus kinase (JAK)-STAT signaling pathway, among others. Direct evidence for a possible role of CSF3 in propagating leukemic evolution materialized from patients with severe congenital neutropenia (SCN) (see Chapter 31). Approximately 80% of SCN patients were known to harbor frameshift or nonsense mutations of *CSF3R* that truncate the cytoplasmic tail of CSF3R at the time of leukemic transformation. These truncation mutations were thought to hamper normal neutrophil differentiation and instead allow for unchecked neutrophilic proliferation. Occasionally, these truncation mutations are accompanied by an activating point mutation in the proximal membrane domain of *CSF3R* in addition to the truncation mutation. It is postulated that *CSF3R* mutations in SCN provide a clonal advantage to the hematopoietic stem cell through oncogene cooperativity.

Maxson et al. hypothesized that patients with CNL or atypical CML may harbor oncogenes sensitive to small-molecule kinase inhibitors. They used an elegant, integrated approach of deep sequencing coupled with the screening of primary leukemia cells obtained from patients with CNL or atypical CML against panels of tyrosine kinase-specific, small interfering RNAs or small-molecule kinase inhibitors. The deep sequencing screen identified *CSF3R* mutations in 16 of 27 patients with CNL (89%) and atypical CML (40%). Two mutational variants were reported: the more common membrane proximal, point mutations consisting primarily of *CSF3R* T618I and T615A variants, and frameshift or nonsense mutations leading to premature truncation of the cytoplasmic tail of CSF3R (D771fs, S783 fs, Y752X, and W791Z). The truncation mutations, which also occur in SCN, largely coexisted with membrane proximal or transmembrane *CSF3R* mutations as compound mutations.

Next, they sought to determine if *CSF3R* mutations showed in vitro sensitivity to small molecule tyrosine kinase inhibitors or small interfering RNA directed against kinases known to be activated by normal CSF3R. While frameshift or nonsense mutations result in dysregulation of SRC and TNK2 kinases (and thus were sensitive to the multikinase inhibitor dasatinib), membrane-proximal mutations appeared to cause ligand-independent hyperactive JAK-STAT signaling and were susceptible to the JAK inhibitor ruxolitinib.

Mechanistically, truncation mutations lead to loss of negative regulatory motifs, namely the di-leucine internalization motif in the cytoplasmic domain of the CSF3R and the binding site for suppressor of cytokine signaling 3 (SOCS3), resulting in decreased lysosomal trafficking of the receptor. This, in turn, increases the cell-surface expression of CSF3R, thus conferring ligand hypersensitivity and sustained neutrophil proliferation. In contrast, the membrane-proximal mutations prevent *O*-glycosylation of CSF3R, resulting in ligand-independent homodimerization, constitutive activation of JAK-STAT signaling pathway and resultant autonomous cell proliferation. Fleishman et al., through a murine transplant model, established the role of *CSF3R* T618I as an oncogenic driver in CNL. The *CSF3R* T618I murine transplant model developed a myeloproliferative disorder akin to CNL that was uniformly fatal.[17] Treatment with the JAK1/2 inhibitor ruxolitinib decreased the white blood count and reduced spleen weight, suggesting that activating mutations in *CSF3R* are sensitive to pharmacologic JAK inhibition. Taken together, these studies indicate that the type of *CSF3R* mutation influences sensitivity to different mechanism-based therapeutic agents, thus providing a rationale for molecularly directed therapeutic targeting in CNL.

Approximately 33% of patients harbor dual truncation and membrane-proximal mutations on the same allele (compound mutation). They rely on the mitogen-activated protein kinase (MAPK) pathway for oncogenic signaling and leukemogenesis. These compound mutants are characteristically impervious to ruxolitinib or dasatinib, given their reliance on MAPK signaling, and are predictably sensitive to MEK1/2 inhibitor trametinib. These results demonstrate the oncogenic role of MAPK signaling in patients with *CSF3R* compound mutations.[18]

Additional mutations involving the spliceosome complex and epigenetic modification, genes that govern cell proliferation, growth factors, and kinase signaling are also known to occur in CNL. The cooperative role of these mutations in the pathogenesis of CNL and their prognostic significance are yet to be determined (Table 72.8).

CLONAL EVOLUTION

Zhang and colleagues, using WES and RNA sequencing (RNA-seq), characterized the genomic profile, including co-occurrence and the order of mutational acquisition in CNL, atypical CML, and other MPN/MDS overlap syndromes.[19] The median number of mutations observed per patient was 3.6, suggestive of a byzantine genomic landscape in these related disorders. *CSF3R* mutations frequently tend to co-occur with *SETBP1* and ASXL1 in CNL. Upon analysis of the clonal architecture of patients harboring *SRSF2*, *ASXL1*, and signaling gene mutations, they observed that *SRSF2* mutations were present in the dominant clone, whereas signaling genes and *ASXL1* mutations were observed variably in the dominant clone and subclones in some cases. A similar pattern was also observed in patients with *EZH2*, *TET2*, and signaling gene mutations.

TABLE 72.8 **Frequency of Concurrent Mutations Reported in CSF3R-Mutated Chronic Neutrophilic Leukemia**

Mutation	Frequency (%)
SETBP1	41
JAK2/CBL/SH2B3	7.7/5.1/0.0
NRAS/KRAS	10.3/0.0
DNMT3A/TET2/ASXL1	5.1/20.5/76.9
SRSF2/U2AF1	43.6/15.4
EZH2	20.5
TP53/PTPN11	2.6/10.3

Adapted from Zhang H, Wilmot B, Bottomly D, et al. Genomic landscape of neutrophilic leukemias of ambiguous diagnosis. *Blood*. 2019;134(11): 867–879.

In most cases, variant allele frequencies (VAF) of two of the three pathway mutations were greater than 50%, suggesting the co-occurrence of these mutations in the dominant clone. The third mutation, present at a lower VAF, suggests latter acquisition in a subclone harboring both original mutations. This finding supports a linear evolution of a single, dominant clone, unlike AML, where multiple independent subclones are observed with a branching pattern of mutational acquisition. Moreover, 19% of patients harbored ≥2 different signaling pathway mutations: one where *CSF3R* was shown to cooccur with *NRAS*, *CBL*, *PTPN11*, *SH2B3*, *NTRK2*, and *ABL1* mutations; and the second where *NRAS* mutations co-occurred with *CSF3R*, *KRAS*, *JAK2*, *CBL*, *STAT3*, *NTRK2*, *FLT3*, and *GNB1* mutations. VAF analysis demonstrated that the majority of signaling pathway mutations were present in the same clone. Drug sensitivity assays of cells transformed by coexisting *CSF3R* and *NRAS* mutations demonstrated reduced drug sensitivity to either ruxolitinib (*CSF3R*) or a MEK inhibitor, trametinib (*NRAS*), which was salvaged by combination ruxolitinib and trametinib treatment targeting both pathways, suggesting that these signaling mutations represent important therapeutic targets in CNL.

Furthermore, Stoner et al. analyzed the mutational patterns of seven CNL patients with disease progression on ruxolitinib therapy to understand the changes in the clonal architecture of CNL. In three patients, *RUNX1* mutations were detected at the time of AML transformation but not at baseline. Additionally, an earlier anecdotal report mentions *RUNX1* clonal expansion at the time of disease progression (WBC, splenomegaly) without AML transformation in a CNL patient receiving ruxolitinib therapy. Collectively, this data suggests that *RUNX1* may cooperate with *CSF3R* mutation to drive disease progression and may even be an early marker of AML transformation. Moreover, *STAT3* and *CBL* subclonal expansion have been detected in patients who lost their initial response to ruxolitinib. Further research is needed to clarify the disease-causing properties of these subclones and their contribution to ruxolitinib resistance in CNL.

CLINICAL AND LABORATORY FEATURES OF CHRONIC NEUTROPHILIC LEUKEMIA

The clinical features of CNL resemble any other MPN with a constellation of constitutional symptoms: fatigue, bone pain, pruritus, gout, and symptomatic splenomegaly. Patients with CNL are prone to bleeding diathesis due to quantitative or qualitative (acquired von Willebrand disease) dysfunction.

Laboratory findings of CNL are characterized by sustained, chronic, mature neutrophilic leukocytosis in the absence of monocytosis, basophilia, or eosinophilia. In contrast to CML, leukocytosis in CNL consists of mainly mature neutrophils without a left shift. Circulating blasts are not seen in CNL. Mild anemia may be seen. Mild thrombocytopenia may be seen with disease progression and development of splenomegaly. Uric acid, lactate dehydrogenase (LDH) levels, as well as the LAP score are typically elevated. Vitamin B12 levels are also often elevated due to transcobalamin release from granulocytes and their precursors.[20]

A bone marrow biopsy is essential to diagnose CNL (Fig. 72.13). The bone marrow is usually hypercellular for patients with absolute myeloid hyperplasia (myeloid-to-erythroid ratio often exceeding 20:1). There is abundant granulocytic proliferation with increased myelocytes, metamyelocytes, bands, and segmented neutrophils but without significant granulocytic or myeloid dysplasia. Blasts and promyelocytes are not increased. Normoblastic erythroid precursors are seen but reduced. Megakaryocytes are morphologically normal. Reticulin fibrosis is not typical. Association with other myeloid and lymphoid disorders is suggested, most commonly polycythemia vera and plasma cell disorders, where either entity may be present concurrently with CNL. Therefore it is suggested to examine CNL bone marrow carefully for nonclonal plasma cell populations.

Hepatosplenomegaly is caused by neutrophilic infiltration in the tissues. In the spleen, the infiltration is limited to red pulp cords and sinuses, resembling a leukemic pattern, whereas in the liver, the sinuses, or portal areas, or both, may be infiltrated.

Most patients with CNL exhibit a normal karyotype at diagnosis, and CGAs are usually observed during clonal evolution. Most common CGAs include trisomy 8, trisomy 21, deletion 11q, and deletion 20q, which are not specific to CNL but observed in myeloid malignancies. Identification of *CSF3R*-activating mutations through NGS clinches the diagnosis of CNL.

DIAGNOSIS

According to the most recent WHO criteria, CNL is defined by mature neutrophilic leukocytosis (≥25 × 10^9/L) and neutrophil precursors comprising less than 10% of white blood cell count; absent to minimal monocytosis and dysgranulopoiesis; hypercellular bone marrow with less than 5% myeloblasts; presence of CSF3R T618I or other activating CSF3R mutation; and absence of BCR-ABL1, PDGFRA, PDGFRB, or FGFR1 rearrangement or PCM1-JAK2 and classic MPNs (i.e., PV, ET, PMF) (Table 72.9).[21] In patients lacking a CSF3R mutation, CNL is established by sustained neutrophilia (at least 3 months) or by splenomegaly with no appreciable cause of reactive neutrophilia without plasma-cell neoplasm. If neutrophilia occurs in the setting of a plasma-cell dyscrasia, clonality of myeloid cells must be documented. The absence of monocytosis, lack of marrow dysplasia and less than 10% circulating myeloid precursor cells, and presence of a CSF3R-activating mutation differentiate CNL from a CML or CMML.

DIFFERENTIAL DIAGNOSIS

Neutrophilic leukocytosis may either be benign or malignant (Fig. 72.14). CNL diagnosis must be carefully distinguished from reactive neutrophilia/leukemoid reaction, CML, neutrophilic-CML (CML-N), and MPN/myelodysplastic (MDS) overlap disorders such as atypical chronic myeloid leukemia (aCML) and CMML (Table 72.10).

PROGNOSIS

Overall prognosis of CNL is poor, with a median OS of 24 months in untreated patients. Life-threatening complications include intracranial hemorrhage, progressive disease, leukemic transformation,

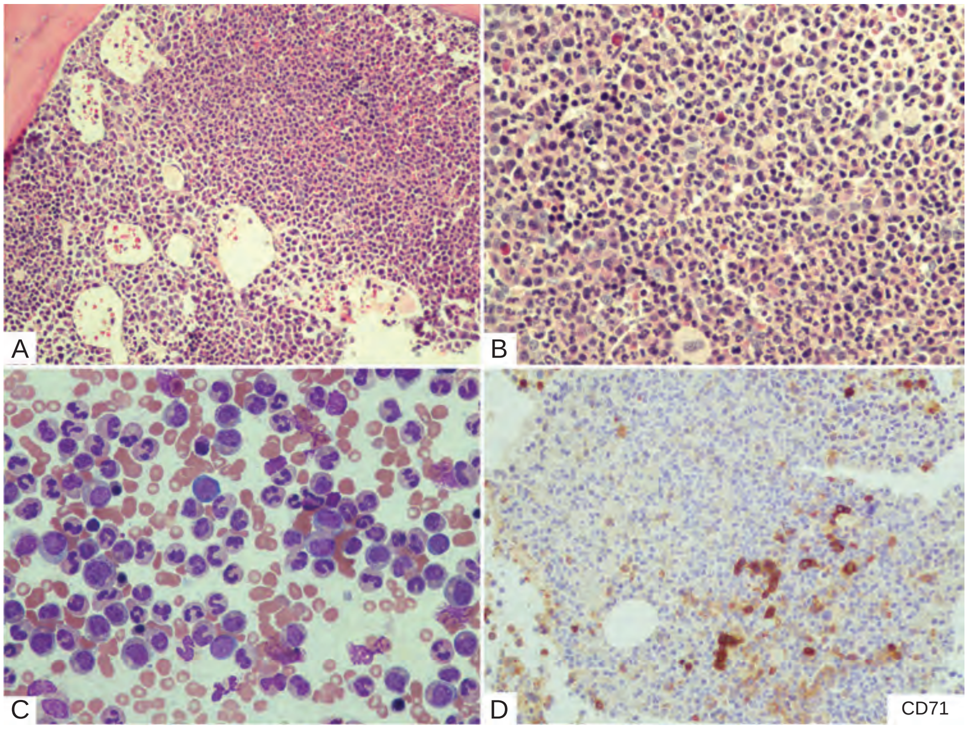


Figure 72.13 BONE MARROW BIOPSY IN CHRONIC NEUTROPHILIC LEUKEMIA. (A and B) Low- and high-power images showing the hypercellular marrow and the myeloid hyperplasia with high numbers of mature neutrophils. (C) Bone marrow smear showing the myeloid hyperplasia with mostly mature forms and no increase in the immature forms. (D) CD71, an erythroid precursors marker, showing decreased and sparse erythroid colonies, indicative of the high myeloid to erythroid ratio.

and treatment-related toxicity following chemotherapy or HCT. Progressive disease is characterized by loss of therapeutic response, progressive refractory neutrophilia, increasing transfusion requirements, acquisition of additional molecular or CGA, and eventually AML. The median time to AML transformation is 21 months (3 to 94 months), and occasionally CNL may transform to PV or CMML.

Thus far, a prognostic scoring system has not been validated for risk stratification in CNL. The Mayo Clinic group developed the first prognostic model predictive of long-term survival in CNL, capturing data from 19 *CSF3R*-mutated, molecularly annotated patients.[22] In this model, platelets less than 160 × 10^9/L (2 adverse points), leukocytes greater than 60 × 10^9/L (1), and presence of an ASXL1 mutation (1) maintained their statistical significance as negative prognostic factors in both univariate and multivariate analyses. When patients were allocated to low-risk (0 to 1 points; $n = 9$) or high-risk (2 to 4 points; $n = 10$) categories, OS times were "not yet reached" and 22.4 months ($P = .0016$), respectively. Zhang et al. further elucidated that *NRAS, ASXL1, GATA2*, and *DNMT3A* mutations were associated with a trend towards shorter OS, while *CBL* mutations predicted favorable survival in CNL.

TABLE 72.9 **WHO Diagnostic Criteria for Chronic Neutrophilic Leukemia**[6]

1. Peripheral blood leukocytosis, ≥25 × 10^9/L
    - Segmented neutrophils plus band forms ≥80% of WBCs
    - Neutrophil precursors (promyelocytes, myelocytes, and metamyelocytes) <10% of WBC
    - Myeloblasts rarely observed
    - Monocyte count <1 × 10^9/L
    - No dysgranulopoiesis
2. Bone marrow biopsy that shows a greater than normal percentage of bone marrow cells
    - Neutrophils increased in percentage and number
    - Neutrophil maturation appears normal
    - Myeloblasts (immature cells) <5%
3. Not meeting WHO criteria for CML *BCR-ABL1*-positive CML, PV, ET or PMF
4. No genetic rearrangement (mutation) of *PDGFRA, PDGFRB*, or *FGRF1*, or *PCM1-JAK2*
5. Presence of CSF3R T618I or other activating CSF3R mutation, or in the absence of a CSF3R mutation:
    - Persistent neutrophilia (at least 3 months)
    - Enlarged spleen (splenomegaly)
    - No identifiable cause of reactive neutrophilia including absence of a plasma cell neoplasm
    - If reactive neutrophilia is present, demonstration of malignant myeloid cells by cytogenetic or molecular studies

MANAGEMENT OF CHRONIC NEUTROPHILIC LEUKEMIA

There is no standard of care for the effective management of CNL. Given the noticeable lack of established treatment guidelines, clinical trial participation is suggested whenever feasible. HSCT remains the definitive curative therapeutic strategy in those patients who are eligible, and available chemotherapeutic agents do not improve OS.

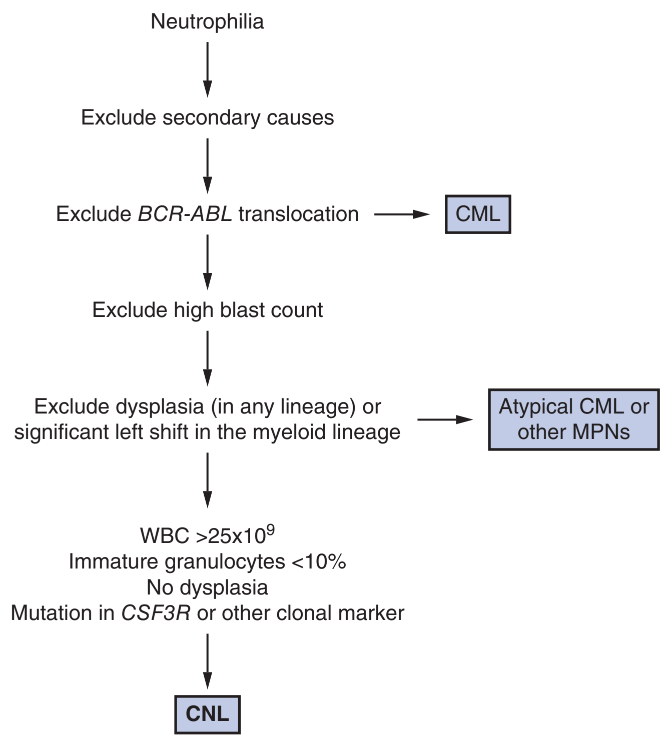

Neutrophilia
Exclude secondary causes
Exclude BCR-ABL translocation
CML
Exclude high blast count
Exclude dysplasia (in any lineage) or significant left shift in the myeloid lineage
Atypical CML or other MPNs
WBC >25x10^9
Immature granulocytes <10%
No dysplasia
Mutation in CSF3R or other clonal marker
CNL

Figure 72.14 WORK-UP FOR NEUTROPHILIC LEUKOCYTOSIS. *CML*, Chronic myeloid leukemia; *CNL*, chronic neutrophilic leukemia; *MPN*, myeloproliferative neoplasm. *(Adapted from Uppal G, Gong J. Chronic neutrophilic leukaemia. J Clin Pathol. 2015;68:680–684.)*

TABLE 72.10 **Differential Diagnosis of Chronic Neutrophilic Leukemia**

Characteristic	CNL	aCML	CMML	CML-N
Philadelphia chromosome	–	–	–	+
Proliferating cells	Mature neutrophils	Neutrophils	Monocytes and neutrophils	Granulocytes and megakaryocytes
Dysplasia	No	Trilineage dysplasia	More than one lineage	Minimal to absent
Blasts in peripheral blood or bone marrow	0%–5%	<20%	<20%	<10%
Genetic abnormality	*CSF3R* mutation	*SETBP1* and/or *ETNK1* in ~ 35% of patients and *CSF3R* in <10%	*SETBP1, TET2, ASXL1, SRSF2*	*BCR-ABL* rearrangement resulting in p230 transcript

CNL, Chronic neutrophilic leukemia; *aCML*, atypical chronic myeloid leukemia; *CMML*, chronic myelomonocytic leukemia; *CML-N*, chronic myelogeneous leukemia:neutrophilic variant

Cytoreductive therapy is recommended in patients deemed unfit for HSCT or clinical trials, or those with highly symptomatic CNL. Historically, splenic irradiation and splenectomy were used as therapeutic options in CNL patients with symptomatic splenomegaly. However, given the risk of postoperative neutrophilic leukocytosis, splenectomy as a therapeutic option is no longer recommended. In a retrospective case series of 40 patients, hydroxyurea was the most common first-line chemotherapeutic agent (80%), and approximately 75% of the patients treated experienced clinical improvement in terms of reduced leukocytosis and/or splenomegaly, with a median response of 12 months (range, 1 to 87 plus months). IFN-α has been shown to be tolerable and provide a durable response lasting 25 months (range, 16 to 41 months) even in the setting of hydroxyurea refractoriness.[23]

Ruxolitinib is currently approved for treatment of patients with intermediate- or high-risk MF, and for PV patients intolerant or refractory to hydroxyurea. Due to inhibition of the oncogenic JAK-STAT signaling pathway, ruxolitinib was hypothesized to provide clinical benefit in CNL patients. Dao et al. evaluated the safety and efficacy of ruxolitinib in patients with CNL and aCML, irrespective of *CSF3R* mutation status.[24] In this phase II study of 44 patients (21 CNL and 23 aCML) that utilized a Simon's two-stage minimax design, the primary endpoint was overall hematologic response rate (ORR includes complete response-CR and partial response-PR) by the end of six continuous 28-day cycles for the first 25 patients enrolled, and the secondary endpoints included grade ≥3 adverse events, spleen volume, symptom assessment, genetic correlates of response, and 2-year survival.

Ruxolitinib was well tolerated and demonstrated an estimated response rate of 32% for the first 25 enrolled patients (8 PR [7 CNL and 1 aCML]). In the larger cohort of 44 patients, 35% had a response (11 PR [9 CNL and 2 aCML] and 4 CR [CNL]). Compared to wild-type, *CSF3R*-mutated CNL patients achieved a greater reduction in white blood cell count and SVR35% with ruxolitinib treatment, suggesting that *CSF3R* mutations in CNL are sensitive to ruxolitinib therapy. Grade ≥3 anemia and thrombocytopenia were observed in 34% and 14% of patients, respectively. No serious adverse events attributed to ruxolitinib were observed. Median OS was 23.1 months vs. 15.6 months for responders and nonresponders, respectively. These results suggest that patients with CNL harboring *CSF3R*-T618I are most likely to respond to ruxolitinib, which is a reasonable therapeutic option to address spleen, symptom and disease burden in transplant ineligible patients with CNL.

Given the rarity of CNL, HSCT experience in CNL is limited. Several anecdotal case reports note long-term remission with HSCT. Although HSCT is done at all phases of the disease, maximal benefit is obtained only if patients are transplanted during the early phase of the disease. A Japanese group retrospectively analyzed the outcomes of HSCT in 14 aCML and five CNL patients transplanted between 2003 and 2014. Most were matched unrelated ($n = 14$) and received a MAC regimen. One-year OS rate was 40% in CNL, emphasizing the lack of prospective data on HSCT outcomes and the natural history of this rare MPN.[25]

FUTURE DIRECTIONS

CNL is a clinically aggressive MPN with no established standard of care treatment. The identification of CSF3R mutations has provided crucial insight into the molecular pathogenesis of CNL and has enabled avenues for therapeutic exploitation. Given the evident lack of effective management strategies in CNL, eligible patients should be considered for HSCT early on in the disease course or for enrollment in clinical trials when available. Furthermore, elucidating critical prognostic clinical and genomic markers to integrate into a validated prognostic model will inform a risk-adapted therapeutic strategy that would significantly improve outcomes in patients with CNL.

REFERENCES

1. Swerdlow SH, Campo E, Harris NL, et al. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. 4th ed. Lyon: International Agency for Research on Cancer; 2008.
2. James C, Ugo V, Le Couédic JP, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*. 2005;434:1144.
3. Pardanani AD, Levine RL, Lasho T, et al. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. *Blood*. 2006;108:3472.
4. Nangalia J, Massie CE, Baxter EJ, et al. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *N Engl J Med*. 2013;369(25):2391.
5. Tefferi A. Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH and IKZF1. *Leukemia*. 2010;24:1128.
6. Schmitt A, Jouault H, Guichard J, et al. Pathologic interaction between megakaryocytes and polymorphonuclear leukocytes in myelofibrosis. *Blood*. 2000;96:1342.
7. Lundberg P, Karow A, Nienhold R, et al. Clonal evolution and clinical correlates of somatic mutations in myeloproliferative neoplasms. *Blood*. 2014;123(14):2220.
8. Thiele J, Kvasnicka HM, Werden C, et al. Idiopathic primary osteo-myelofibrosis: a clinico-pathological study on 208 patients with special emphasis on evolution of disease features, differentiation from essential thrombocythemia and variables of prognostic impact. *Leuk Lymphoma*. 1996;22:303.
9. Cervantes F, Dupriez B, Pereira A, et al. New prognostic scoring system for primary myelofibrosis based on a study of the International Working Group for Myelofibrosis Research and Treatment. *Blood*. 2009;113:2895.
10. Grinfeld J, Nangalia J, Baxter EJ, et al. Classification and personalized prognosis in myeloproliferative neoplasms. *N Engl J Med*. 2018;379(15):1416–1430.
11. Kiladjian JJ, Mesa RA, Hoffman R. The renaissance of interferon therapy for the treatment of myeloid malignancies. *Blood*. 2011;117:4706.
12. Verstovsek S, Mesa RA, Gotlib J, et al. A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. *N Engl J Med*. 2012;366:799.
13. Harrison C, Kiladjian JJ, Al-Ali HK, et al. JAK inhibition with ruxolitinib versus best available therapy for myelofibrosis. *N Engl J Med*. 2012;366:787.
14. Pardanani A, Harrison C, Cortes JE, et al. Safety and efficacy of fedratinib in patients with primary or secondary myelofibrosis: a randomized clinical trial. *JAMA Oncol*. 2015;1(5):643–651.
15. Harrison CN, Schaap N, Vannucchi AM, et al. Janus kinase-2 inhibitor fedratinib in patients with myelofibrosis previously treated with ruxolitinib (JAKARTA-2): a single-arm, open-label, non-randomised, phase 2, multicentre study. *Lancet Haematol*. 2017;4(7):e317–e324.
16. Maxson JE, Gotlib J, Pollyea DA, et al. Oncogenic CSF3R Mutations in Chronic Neutrophilic Leukemia and Atypical CML. *N Engl J Med*. 2013;368(19):1781–1790.
17. Fleischman AG, Maxson JE, Luty SB, et al. The CSF3R T618I mutation causes a lethal neutrophilic neoplasia in mice that is responsive to therapeutic JAK inhibition. *Blood*. 2013;122(22):3628–3631.
18. Rohrabaugh S, Kesarwani M, Kincaid Z, et al. Enhanced MAPK signaling is essential for CSF3R-induced leukemia. *Leukemia*. 2017;31(8):1770–1778.
19. Zhang H, Wilmot B, Bottomly D, et al. Genomic landscape of neutrophilic leukemias of ambiguous diagnosis. *Blood*. 2019;134(11):867–879.
20. Elliott MA, Dewald GW, Tefferi A, et al. Chronic neutrophilic leukemia (CNL): a clinical, pathologic and cytogenetic study. *Leukemia*. 2001;15(1):35–40.
21. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127(20):2391–2405.
22. Szuber N, Finke CM, Lasho TL, et al. CSF3R-mutated chronic neutrophilic leukemia: long-term outcome in 19 consecutive patients and risk model for survival. *Blood Cancer J*. 2018;8(2):21.
23. Elliott MA, Tefferi A. Chronic neutrophilic leukemia 2014: Update on diagnosis, molecular genetics, and management. *Am J Hematol*. 2014;89(6):651–658.
24. Dao KT, Gotlib J, Deininger MMN, et al. Efficacy of ruxolitinib in patients with chronic neutrophilic leukemia and atypical chronic myeloid leukemia. *J Clin Oncol*. 2020;38(10):1006–1018.
25. Itonaga H, Ota S, Ikeda T, et al. Allogeneic hematopoietic stem cell transplantation for the treatment of BCR-ABL1-negative atypical chronic myeloid leukemia and chronic neutrophil leukemia: A retrospective nationwide study in Japan. *Leuk Res*. 2018;75:50–57.

CHAPTER 73

MYELODYSPLASTIC SYNDROME/MYELOPROLIFERATIVE NEOPLASM OVERLAP SYNDROMES

Douglas Tremblay, Jonathan Feld, Nicole Kucine, Noa Rippel, Siraj M. El Jamal, and John Mascarenhas

Myelodysplastic syndrome (MDS)/myeloproliferative neoplasm (MPN) overlap syndromes are a group of rare myeloid malignancies that paradoxically exhibit both dysplastic and proliferative hematopoietic cell characteristics. They share phenotypic features of both MDS, such as dysplasia and cytopenias, and of MPNs, including constitutional symptoms, splenomegaly, and terminally differentiated blood cell expansion. There are four adult MDS/MPN overlap syndromes: chronic myelomonocytic leukemia (CMML), atypical chronic myeloid leukemia (aCML), MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T), and MDS/MPN unclassifiable (MDS/MPN-U). There is also a pediatric syndrome, juvenile myelomonocytic leukemia (JMML). These disorders were historically classified as either an MDS or an MPN, but in 2002, the World Health Organization (WHO) first designated MDS/MPN overlap syndromes as an independent category of tumors of hematopoietic tissues, in recognition of the unique features of these myeloid malignancies.[1] Recurrent mutations shared between the subtypes of MDS/MPNs, and the MPN and MDS highlight the interrelated nature of these diseases.

MDS/MPNs present challenges both from a diagnostic and therapeutic standpoint. As rare diseases, an accurate diagnosis can be challenging and often relies on referral to tertiary care centers with a team of experienced hematopathologists and clinicians. In addition, there is a paucity of clinical research and novel therapeutic approaches for treating these malignancies included within the MDS/MPN overlap syndromes. Treatment approaches are, therefore, largely borrowed from those used to treat MDS or the MPNs. Given advances in the molecular and pathobiologic understanding of MDS/MPNs overlap syndromes, novel therapeutic development is starting to advance, which has the potential to alter the generally poor outcomes seen with such patients.

EPIDEMIOLOGY

The epidemiology of MDS/MPN overlap syndromes is poorly described. CMML is the most common MDS/MPN overlap syndrome, with an incidence of 0.35 cases per 100,000. As expected, the incidence of CMML increases significantly with age based on SEER (Surveillance, Epidemiology, and End Results) data. There is a male predominance, and the disease is more common in those of European descent than Blacks or Asian/Pacific Islanders.

Epidemiologic data are limited for aCML, although one study suggests a slight male predominance with a median age of onset of 69 years. There is no published epidemiologic information concerning MDS/MPN-RS-T or MDS/MPN-U. JMML has an estimated incidence of 1.2 cases per million annually and accounts for approximately 2% of leukemias occurring during childhood. Males are affected more frequently than females, and approximately 50% of cases occur in infants.

DIAGNOSTIC EVALUATION AND CRITERIA

The diagnostic features for the MDS/MPN overlap syndromes, although diverse, essentially encompass a hybrid of both marrow cell dysplasia and myeloproliferation. The dysplastic features are similar to the classic features associated with MDS involving one or more of the hematopoietic lineages. The myeloproliferative component results frequently in monocytosis in CMML, thrombocytosis in MDS/MPN-RS-T, or granulocytosis in aCML. Establishing the diagnosis of a patient with an MPN/MDS overlap syndrome usually requires an integrated approach incorporating clinical presentation, laboratory data, peripheral blood and marrow morphologic evaluation, cytogenetics, and myeloid gene mutational profiling.

In general, special considerations should be taken when making the diagnosis of an MDS/MPN overlap syndrome. The diagnosis should be made prior to the patient receiving any therapy and other genetically-defined myeloid neoplasms should be excluded using cytogenetic analyses and molecular testing. In essence, MDS/MPN overlap syndromes are frequently diagnoses of exclusion.

MDS/MPN overlap syndrome patients present with abnormal blood counts with at least a single lineage cytopenia (the MDS component) or a single lineage cytosis (the MPN component). Of particular interest are the monocyte counts (associated with CMML), eosinophilia (may suggest myeloid neoplasm with *PDGFRα*, *PDGFRβ*, or *FGFR1* abnormalities), and basophilia (may suggest chronic myeloid leukemia [CML] rather than MDS/MPN overlap syndromes). Clinically, patients experience symptoms of MDS including fatigue, frequent infections, and bleeding/bruising. In addition, features of the MPNs such as constitutional symptoms and extramedullary hematopoiesis (often manifested as splenomegaly) can be observed. However, there is a wide spectrum of clinical manifestations in MDS/MPN overlap syndrome patients ranging from the patients being asymptomatic to having debilitating symptoms.

Chronic Myelomonocytic Leukemia

CMML is characterized by sustained monocytosis in the setting of bone marrow (BM) cellular dysplasia. Monocytosis is defined by having an absolute monocyte count greater than 1×10^9/L with greater than 10% monocytes being present in the white blood cell (WBC) differential count that persists for at least 3 months. An examination of the BM is critical to exclude acute myeloid leukemia (AML) from the diagnosis since CMML patients, unlike AML patients, have a blast cell percentage less than 20%. Many other entities can lead to a reactive form of monocytosis, which are discussed in detail in Chapter 49, and must be excluded. Non-neoplastic causes of monocytosis include chronic infections, autoimmune diseases, and drug reactions, among others. Other myeloid neoplasms with associated monocytosis should be excluded as well as CML, primary myelofibrosis, and MDS. The WHO diagnostic criteria for CMML are listed in Table 73.1.[1] CMML can be further subtyped based on the degree of elevation of the peripheral blood WBC count into MPN-CMML (WBC $\geq 13 \times 10^9$/L) and MDS-CMML (WBC $<13 \times 10^9$/L).

The marrow is typically hypercellular with myeloid hyperplasia and monocytosis. The dysplasia is common in the three lineages; however, dysplasia is more common and pronounced in the megakaryocyte lineage, with micromegakaryocytes and small megakaryocytes with

TABLE 73.1 **World Health Organization Diagnostic Criteria for Chronic Myelomonocytic Leukemia**

Persistent PB monocytosis $\geq 1 \times 10^9$/L, with monocytes accounting for $\geq$10% of the WBC count
Not meeting WHO criteria for *BCR-ABL1*$^+$ CML, PMF, PV, or ET[a]
No evidence of *PDGFRA*, *PDGFRB*, or *FGFR1* rearrangement or *PCM1-JAK2* (should be specifically excluded in cases with eosinophilia)
<20% blasts in the blood and BM[b]
Dysplasia in 1 or more myeloid lineages. If myelodysplasia is absent or minimal, the diagnosis of CMML may still be made if the other requirements are met and
An acquired clonal cytogenetic or molecular genetic abnormality is present in hemopoietic cells[c]
or
The monocytosis (as previously defined) has persisted for at least 3 months and
All other causes of monocytosis have been excluded

[a] Cases of MPN can be associated with monocytosis or they can develop it during the course of the disease. These cases may simulate CMML. In these rare instances, a previous documented history of MPN excludes CMML, whereas the presence of MPN features in the BM and/or of MPN-associated mutations (*JAK2, CALR*, or *MPL*) tend to support MPN with monocytosis rather than CMML.

[b] Blasts and blast equivalents include myeloblasts, monoblasts, and promonocytes. Promonocytes are monocytic precursors with abundant light gray or slightly basophilic cytoplasm with a few scattered, fine lilac-colored granules, finely distributed, stippled nuclear chromatin, variably prominent nucleoli, and delicate nuclear folding or creasing. Abnormal monocytes, which can be present both in the PB and BM, are excluded from the blast count.

[c] The presence of mutations in genes often associated with CMML (e.g., *TET2*, *SRSF2*, *ASXL1*, *SETBP1*) in the proper clinical context can be used to support a diagnosis. It should be noted, however, that many of these mutations can be age-related or be present in subclones. Therefore, caution would have to be used in the interpretation of these genetic results.

BM, Bone marrow; *CML*, chronic myeloid leukemia; *CMML*, chronic myelomonocytic leukemia; *ET*, essential thrombocythemia; *MPN*, myeloproliferative neoplasm; *PB*, peripheral blood; *PMF*, primary myelofibrosis; *PV*, polycythemia vera; *WBC*, white blood cell; *WHO*, World Health Organization.

Adapted from Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127(20):2391–2405.

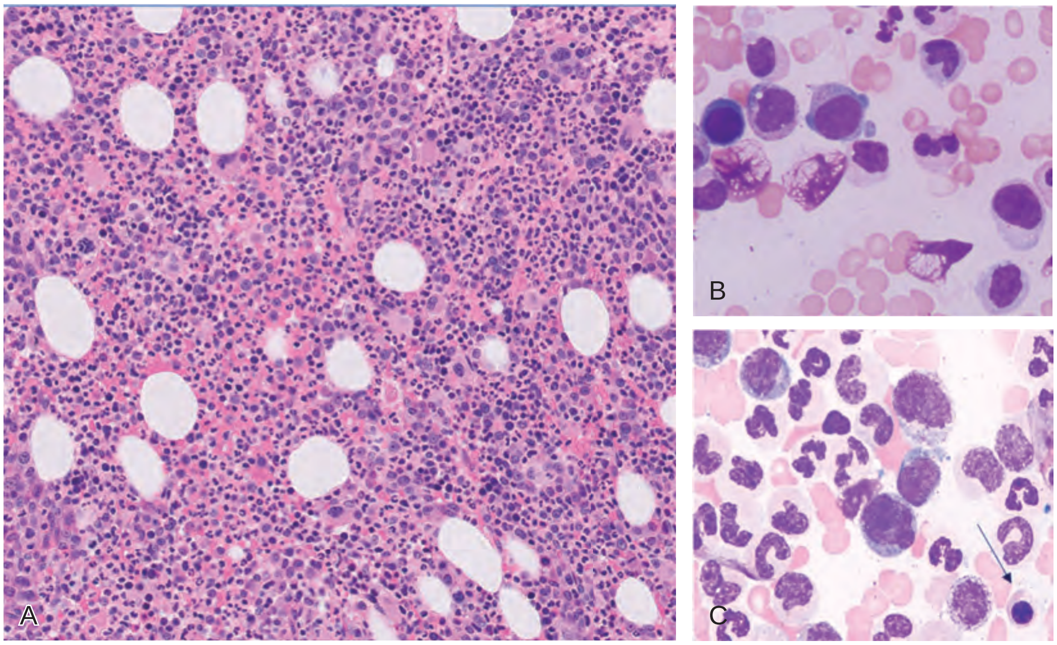


Figure 73.1 CHRONIC MYELOMONOCYTIC LEUKEMIA MORPHOLOGICAL FINDINGS. (A) Bone marrow (BM) biopsy shows hypercellular marrow with myeloid hyperplasia and dysplastic megakaryocytes. (B) Peripheral blood (PB) shows atypical granulocytes and monocytes. (C) BM smears show the myeloid hyperplasia with hypogranular and atypical forms, note the erythroid precursor with megaloblastoid changes *(arrow)*.

hypolobated nuclei being a prominent feature (Fig. 73.1). In rare cases where the dysplasia may not be overt, the diagnosis of CMML can be made if a cytogenetic or myeloid mutational abnormality is detected or if the monocytosis is persistent with no secondary causes identified. Mild BM fibrosis is not uncommon in CMML.

A plasmacytoid dendritic cell (PDC) proliferation (CD123-positive) is a feature of CMML that is observed in 20% of cases but is a non-specific finding and can be seen in other myeloid neoplasms. Of note, CMML is the most common myeloid neoplasm that is associated with systemic mastocytosis (see Chapter 75).

Recently, an entity termed “oligomonocytic chronic myelomonocytic leukemia” has been described. This condition is characterized by blood monocytes $\geq$10% of the WBC without an absolute monocyte count greater than 1×10^9/L. These patients, when compared to CMML, are typically younger and have lower WBCs and absolute neutrophils counts. The molecular signature is similar to CMML with mutations in *ASXL1, TET2*, and *SRSF2*. Progression to overt CMML occurs in approximately 40% of cases over a median time of 12 months. Thus, these cases should not be classified as a type of MDS but rather to represent a forme fruste of CMML.

TABLE 73.2 World Health Organization Diagnostic Criteria for Atypical Chronic Myeloid Leukemia

- Peripheral blood leukocytosis (WBC count ≥13 × 10^9/L) because of increased numbers of neutrophils and their precursors with prominent dysgranulopoiesis
- Neutrophil precursors (promyelocytes, myelocytes, metamyelocytes) ≥10% of leukocytes
- No Ph chromosome or *BCR-ABL1* fusion gene and not meeting criteria for PV, ET, or PMF[a]
- No evidence of *PDGFRA*, *PDGFRB*, *FGFR1* rearrangement, or *PCM1-JAK2*
- Minimal absolute basophilia; basophils usually <2% of leukocytes
- No or minimal absolute monocytosis; monocytes usually <10% of leukocytes
- Hypercellular bone marrow with granulocytic proliferation and granulocytic dysplasia, with or without dysplasia in the erythroid and megakaryocytic lineages
- Less than 20% blasts in the blood and bone marrow

[a] Cases of myeloproliferative neoplasm *(MPN)*, particularly those in accelerated phase and/or in postpolycythemic or postessential thrombocythemic myelofibrosis, if neutrophilic, may simulate aCML. A previous history of MPN, the presence of MPN features in the bone marrow, and/or MPN-associated mutations (in *JAK2*, *CALR*, or *MPL*) tend to exclude a diagnosis of aCML. Conversely, a diagnosis of aCML is supported by the presence of *SETBP1* and/or *ETNK1* mutations. The presence of a *CSF3R* mutation is uncommon in aCML and, if detected, should prompt a careful morphologic review to exclude an alternative diagnosis of chronic neutrophilic leukemia or other myeloid neoplasm.

aCML, Atypical chronic myeloid leukemia; *ET*, essential thrombocythemia; *Ph*, Philadelphia; *PMF*, primary myelofibrosis; *PV*, polycythemia vera; *WBC*, white blood cell; *WHO*, World Health Organization. Adapted from Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127(20):2391–2405.

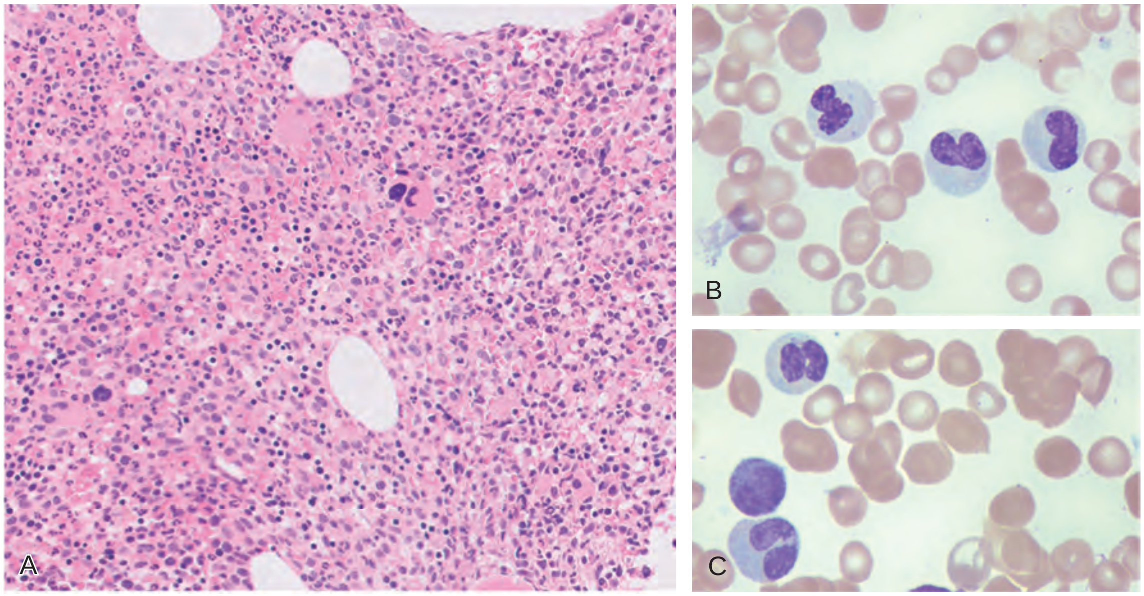


Figure 73.2 ATYPICAL CHRONIC MYELOID LEUKEMIA. (A) Bone marrow (BM) biopsy shows hypercellular marrow with myeloid hyperplasia. The megakaryocytes are morphologically normal. (B and C) The dysplastic features are limited to the granulocytic lineage as seen here.

Atypical Chronic Myeloid Leukemia

aCML is defined by left-shifted neutrophilic leukocytosis with severe dysgranulopoiesis and greater than 10% immature myeloid precursor cells present in the peripheral blood. Anemia and thrombocytopenia are common. These patients lack the *BCR-ABL-1* fusion protein that is diagnostic of CML (see Chapter 69). Basophilia and eosinophilia are not typical features of aCML and should prompt careful consideration of a diagnosis of another myeloid neoplasm. aCML often presents with extramedullary disease, especially in the spleen and liver. The two other diagnoses that resemble aCML and should be ruled out are CMML (which exhibits monocytosis) and CML (which is *BCR-ABL-1*-positive). The WHO diagnostic criteria for aCML are listed in Table 73.2.

The BM in aCML is hypercellular with myeloid hyperplasia and a high myeloid-to-erythroid (M:E) (Fig. 73.2). aCML is characterized by dysplastic hematopoiesis and the presence of immature myeloid precursor cells in the peripheral blood that distinguishes it from chronic neutrophilic leukemia (CNL) (see Chapter 72). In addition to the granulocytic dysplasia, erythroid and megakaryocytic dysplasia are also common. Importantly, aCML is characteristically associated with mutations in *SETBP1* and/or *ETKN1*. Since the *CSF3R* mutation is seen in less than 10% of patients with aCML, the presence of this mutation, in conjunction with the absence of BM dysplasia and immature myeloid precursors, suggests a diagnosis of CNL rather than aCML. The presence of MPN mutations involving *JAK2*, *MPL*, or *CALR* tends to exclude the diagnosis of aCML

Myelodysplastic/Myeloproliferative Syndrome With Ring Sideroblasts and Thrombocytosis

Patients with MDS/MPN-RS-T can present with vasomotor symptoms, such as migraine headaches, erythromelalgia, and paresthesias, or can have fatigue related to anemia. Additionally, some patients initially present incidentally after routine laboratory testing that reveals thrombocytosis and anemia. Table 73.3 lists the WHO diagnostic criteria for MDS/MPN-RS-T,[1] which includes refractory anemia, ≥15% ring sideroblasts in the BM, and thrombocytosis. The presence of the spliceosome mutation *SF3B1* co-mutated with an MPN driver mutation (in particular *JAK2V617F*) is highly suggestive of MDS/MPN-RS-T in the appropriate clinical setting.[2] Distinction between MDS with ring sideroblasts is essential, which in contrast to MDS/MPN-RS-T, lacks thrombocytosis and has hypolobulated, instead of hyperlobulated, atypical megakaryocytes (Fig. 73.3). Other non-clonal disorders can result in the appearance of ring sideroblasts including lead or zinc toxicity, copper or pyridoxine deficiency, the use of particular medications (linezolid, isoniazid), excessive alcohol use, and congenital forms of sideroblastic anemias (see Chapter 39). Reactive causes of thrombocytosis, including infection, rheumatologic conditions, recent trauma/surgery, blood loss, iron deficiency, and hemolysis, should be ruled out (see Chapters 29 and 71).

TABLE 73.3 World Health Organization Diagnostic Criteria for Myelodysplastic Syndrome/Myeloproliferative Neoplasm With Ring Sideroblasts and Thrombocytosis

- Anemia associated with erythroid lineage dysplasia with or without multilineage dysplasia, ≥ 15% ring sideroblasts,[a] <1% blasts in PB, and <5% blasts in the BM
- Persistent thrombocytosis with platelet count ≥450 × 10^9/L
- Presence of an *SF3B1* mutation or, in the absence of *SF3B1* mutation, no history of recent cytotoxic or growth factor therapy that could explain the myelodysplastic/myeloproliferative features[b]
- No *BCR-ABL1* fusion gene, no rearrangement of *PDGFRA*, *PDGFRB*, or *FGFR1*; or *PCM1-JAK2*; no (3;3)(q21;q26), inv(3)(q21q26) or del(5q)[c]
- No preceding history of MPN, MDS (except MDS-RS), or other type of MDS/MPN

[a] At least 15% ring sideroblasts are required even if *SF3B1* mutation is detected.
[b] A diagnosis of MDS/MPN-RS-T is strongly supported by the presence of *SF3B1* mutation together with a mutation in *JAK2* V617F, *CALR*, or MDS-RS, myelodysplastic syndrome with ring sideroblasts; *MPL* genes.
[c] In a case which otherwise fulfills the diagnostic criteria for MDS with isolated del(5q)-no or minimal absolute basophilia; basophils usually less than 2% of leukocytes.

BM, Bone marrow; *MDS*, myelodysplastic syndrome; *MDS-RS*, myelodysplastic syndrome with ring sideroblasts; *MPN*, myeloproliferative neoplasm; *MPN-RS-T*, myeloproliferative neoplasm with ring sideroblasts and thrombocytosis; *PB*, peripheral blood.

Adapted from Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127(20):2391–2405.

GENERAL DIAGNOSTIC APPROACH

MDS/MPN overlap syndromes should be suspected in patients who display phenotypic properties of both bone marrow failure and myeloproliferation. Fig. 73.4 summarizes the diagnostic approach for patients with MDS/MPN overlap syndromes. As noted in the WHO diagnostic criteria (Tables 73.1–73.4), a bone marrow biopsy is essential for the diagnosis of MDS/MPN overlap syndromes in order to distinguish among the diagnoses and from other myeloid malignancies and to determine the type of MDS/MPN overlap syndrome. Histopathology review by an expert hematopathologist is key for proper diagnosis.

In all MDS/MPN overlap syndrome patients, exclusion of chronic myeloid leukemia *(CML)* with polymerase chain reaction (PCR) for *BCR-ABL1* must be performed. CMML, the most common MDS/MPN, can be diagnostically challenging given that monocytosis is seen in other related hematologic disorders or can be reactive due to chronic infections, autoimmune disorders, or other systemic inflammatory processes. Therefore, persistent monocytosis lasting more than 3 months must be documented, unless there is a clonal cytogenetic or molecular marker specific for myeloid malignancies, such *TET2*, *SRSF2*, *ASXL1*, or *SETBP1*. The diagnosis of aCML should be considered in a patient with neutrophilia with greater than 10% immature myeloid cells being the differentiating feature among other MDS/MPN overlap syndromes. Careful consideration of CNL must also be given when considering a diagnosis of aCML given that both have a high frequency of the *CSF3R* mutation. Among the differentiating features, the presence of ≥10% immature granulocytes strongly favors aCML and the presence of less than 10% with greater than 80% neutrophils and bands favors CNL. MDS/MPN-RS-T is characterized by thrombocytosis, concurrent anemia, and ring sideroblasts which differentiates this diagnosis from ET. Cases that harbor features of both MDS and MPN but do not meet WHO diagnostic criteria for any MDS/MPN subtype can be categorized in the “grab-bag” category of MDS/MPN-U.

aCML, Atypical chronic myeloid leukemia; *CMML*, chronic myelomonocytic leukemia; *CNL*, chronic neutrophilic leukemia; *ET*, essential thrombocythemia; *MDS*, myelodysplastic syndrome; *MPN*, myeloproliferative neoplasm; *MPN-RS-T*, myeloproliferative neoplasm with ring sideroblasts and thrombocytosis; *MPN-U*, myeloproliferative neoplasm unclassifiable; *WHO*, World Health Organization.

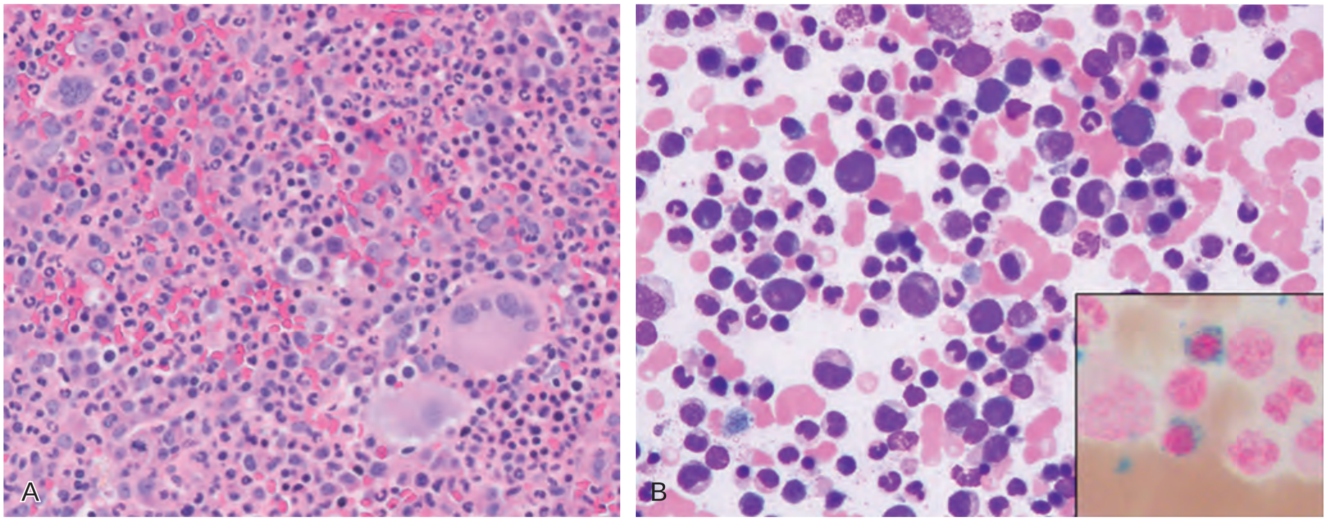


Figure 73.3 MYELODYSPLASTIC SYNDROME/MYELOPROLIFERATIVE NEOPLASM WITH RING SIDEROBLASTS AND THROMBOCYTOSIS. (A) Bone marrow is hypercellular with megakaryocytic hyperplasia. The megakaryocytes are hyperlobulated and atypical with some dysplastic forms. (B) The smears show prominent dyserythropoiesis. The hallmark to make the diagnosis is the presence of greater than 15% ring sideroblasts.

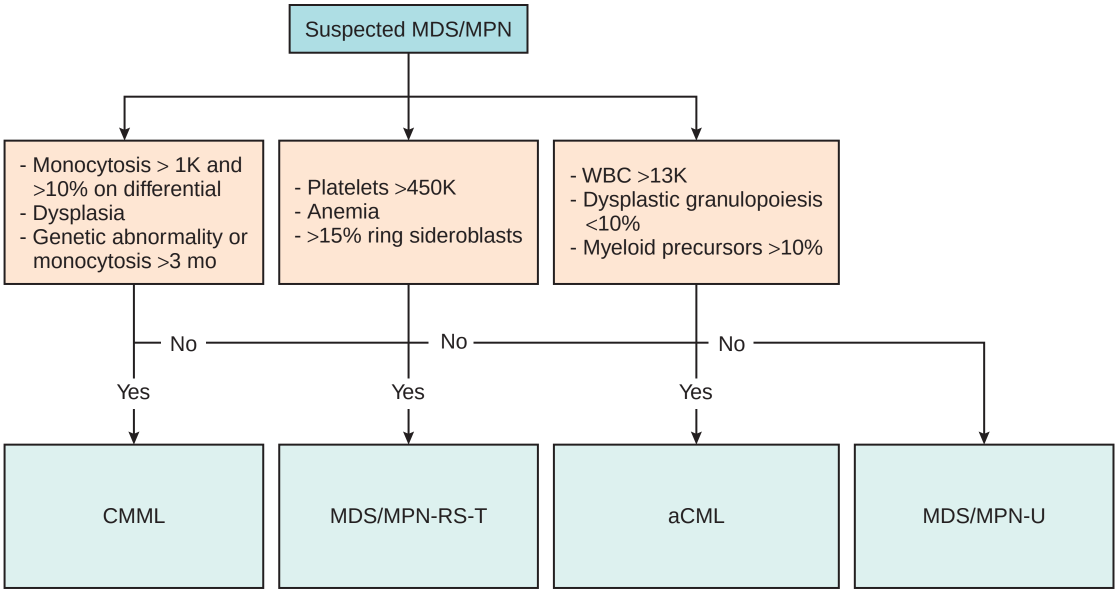

Suspected MDS/MPN
- Monocytosis > 1K and >10% on differential
- Dysplasia
- Genetic abnormality or monocytosis >3 mo
- Platelets >450K
- Anemia
- >15% ring sideroblasts
- WBC >13K
- Dysplastic granulopoiesis <10%
- Myeloid precursors >10%
No
No
No
Yes
Yes
Yes
CMML
MDS/MPN-RS-T
aCML
MDS/MPN-U

Figure 73.4 DIAGNOSTIC ALGORITHM FOR DIAGNOSIS OF PATIENTS WITH AN MYELODYSPLASTIC SYNDROME/MYELOPROLIFERATIVE NEOPLASM OVERLAP SYNDROME. Patients with both dysplasia and myeloproliferative features should be evaluated for the presence of monocytosis, thrombocytosis, and neutrophilia. Patients who do not meet the criteria for specific myelodysplastic syndrome *(MDS)*/myeloproliferative neoplasm *(MPN)* subtypes can be diagnosed with the catch-all diagnosis of MDS/myeloproliferative neoplasm unclassifiable (MPN-U). *aCML*, Atypical chronic myeloid leukemia; *CMML*, chronic myelomonocytic leukemia; *MPN-RS-T*, myeloproliferative neoplasm with ring sideroblasts and thrombocytosis.

Myelodysplastic/Myeloproliferative Syndrome, Unclassifiable

MDS/MPN-U is, by definition, a diagnosis of exclusion, and the clinical presentation varies widely. By definition, MDS/MPN-U is characterized by at least one dysplastic lineage, less than 20% blasts in the marrow and/or peripheral blood, and absence of *BCR-ABL-1* (which defines CML) and the fusions genes involving *PDGFRA, PDGFRB*, and *FGFR1* (which define eosinophilia-associated myeloid/lymphoid neoplasms). In addition, there must be dysplastic and proliferative features that do not meet the diagnostic criteria for CMML, JMML, aCML, or MDS/MPN-RS-T U. This "wastebasket" category of MDS/MPN overlap syndromes can include patients who will eventually evolve to a definitive MPN/MDS overlap syndrome or a rare form of an MPN that is associated with dysplastic changes without documentation of a prior chronic phase.

The mutational landscape of MDS/MPN-U is heterogeneous, with *ASXL1* being the most commonly mutated gene followed by *TET2* and *SRSF2*. *JAK2* mutations can be seen in up to 30% of patients with MDS/MPN-U, while this mutation is rare in MDS. The presence of a *JAK2* mutation in the setting of dysplasia without features of the other MDS/MPN overlap syndromes strongly suggests a diagnosis of MDS/MPN-U. However, cases of MDS with isolated del(5q) may be associated with a *JAK2* mutation and are better classified as MDS with isolated del(5q) and concurrent *JAKV617F* rather than MDS/MPN-U. In comparison to other MDS/MPN overlap syndrome diagnostic categories with strict definitions, the diagnostic criteria for MDS/MPN-U are poorly defined and therefore misclassification is common. Therefore, consultation with an expert hematopathologist is essential for the proper diagnosis of MDS/MPN-U (Fig. 73.4).

Juvenile Myelomonocytic Leukemia

JMML is a rare MPN/MDS overlap syndrome that occurs almost exclusively in infants and young children. It is characterized by an aggressive clinical course, with monocytic and granulocytic proliferation, organ infiltration with significant splenomegaly, and activating mutations in the Ras/Erk pathway (Fig. 73.5). Ras signaling proteins are small guanosine triphosphatase (GTPase) molecules that are ubiquitously expressed. They play diverse cellular roles including regulation of proliferation, survival, and differentiation. Ras pathway activation stimulates a number of signaling pathways including Raf/MEK/ERK and PI3K/AKT/mTOR. Mutations in *PTPN11, NRAS, KRAS, CBL, or NF1* account for approximately 90% of JMML cases. These canonical Ras mutations are associated with five clinically and biologically distinct subtypes of JMML. Germline mutations in Ras family genes, so-called RASopathies, are a clinically distinct group of genetic syndromes that are described below.

PTPN11 is the most common mutation found in JMML, encompassing approximately 40% of all cases. The *PTPN11* gene encodes a cytoplasmic protein tyrosine phosphatase SHP-2, a dual-specificity phosphatase, which contains two tandem SH-2 domains at the N-terminus and a catalytic PTP domain at the C-terminus. All *PTPN11* genes are gain-of-function, missense mutations resulting in constitutive Ras activation. *PTPN11*-associated JMML is associated with an older age at presentation and a more aggressive course. Noonan syndrome is an autosomal-dominant condition associated with a germline *PTPN11* mutation in approximately 50% of patients that causes abnormal development of multiple organs. Most individuals with Noonan syndrome have normal intelligence, but some may have special educational needs or intellectual disability. Noonan syndrome occurs in about 1 in 2500 births and is clinically associated with short stature, facial dysmorphism, neurodevelopmental problems, and lymphedema. Congenital heart disease is present in 80% of patients with Noonan syndrome, most frequently pulmonary stenosis, although atrial septal defects, and hypertrophic cardiomyopathy can also be seen. Cardiac manifestations usually present within the first year of life.[3] Noonan syndrome can be associated with a transient myeloproliferative disorder that is more indolent than JMML but in some cases can progress to JMML (more commonly with certain *PTPN11* or *KRAS* mutations). The *PTPN11*-associated MPN can be present at birth or develop during infancy or childhood.

NRAS and *KRAS* mutations are heterozygous somatic RAS mutations that lock RAS into the active guanosine triphosphate-bound state, thereby diminishing GTPase activity. *NRAS or KRAS* mutations are present in approximately 25% of JMML cases. A small proportion of patients with Noonan syndrome will harbor a mutation in *NRAS* or *KRAS*, with the latter associated with more severe intellectual

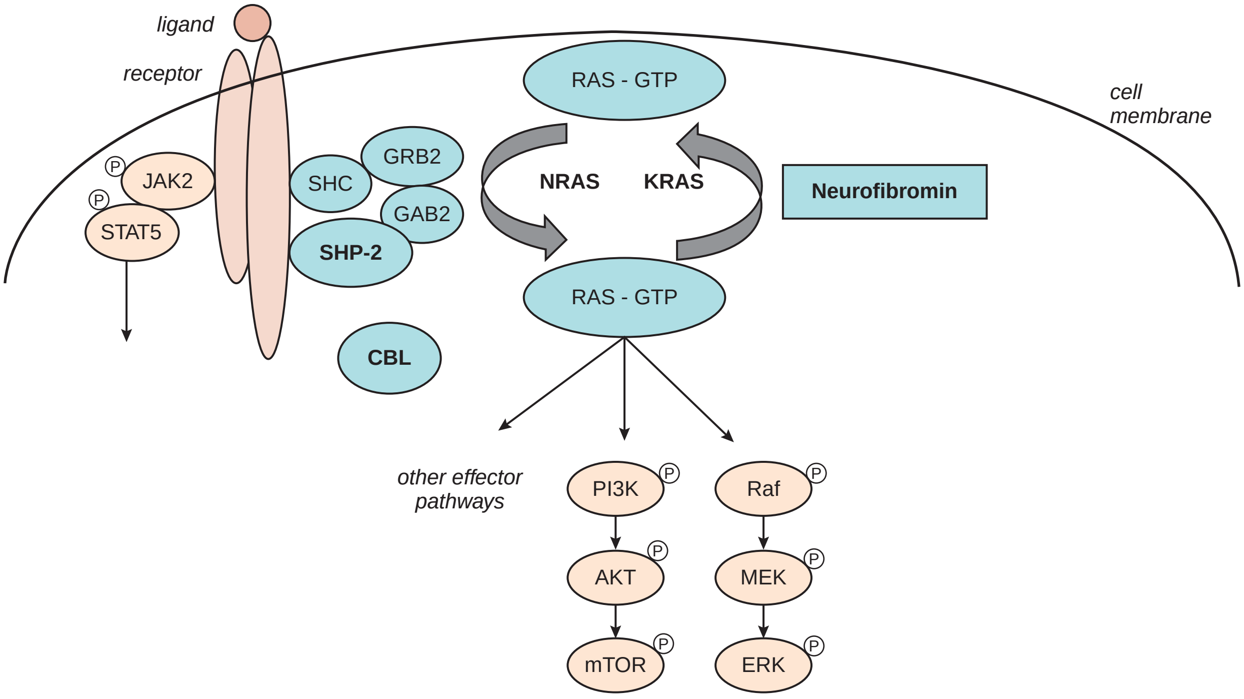


Figure 73.5 RAS PATHWAY WITH MUTATIONS IN JUVENILE MYELOMONOCYTIC LEUKEMIA. Mutations that are recurrently mutated are highlighted in bold. *(Adapted from Niemeyer CM. JMML genomics and decisions. Hematology Am Soc Hematol Educ Prog. 2018;2018[1]:307–312.)*

disability. *NRAS*-mutated JMML has a highly varied clinical presentation and course, with some young infants experiencing an indolent course with spontaneous hematologic improvement being observed during a 2- to 4-year follow-up period. By contrast, *KRAS*-mutated disease is a particularly aggressive form of JMML.

CBL encodes a member of the Cbl family of proteins, which functions as an E3 ubiquitin ligase. CBL syndrome is an autosomal-dominant developmental disorder resulting from germline missense *CBL* mutations, which are characterized by impaired growth, developmental delay, cryptorchidism, vasculitis, and a predisposition to develop JMML. *CBL* mutations account for approximately 15% of cases of JMML. Many individuals with CBL syndrome experience spontaneous regression of their JMML. Importantly, JMML specimens from affected children are characterized by loss of the normal CBL allele through acquired isodisomy.

NF1, located on chromosome 17q11.2, encodes neurofibromin, a GTP-ase activating protein that acts as a negative modulator of RAS function. Loss of a normal *NF1* allele in leukemic cells therefore results in RAS hyperactivity. The syndrome characterized by *NF1* mutations, neurofibromatosis 1 (NF1), is an autosomal-dominant disorder clinically characterized by café-au-lait macules, iris hamartomas (Lisch nodules), axillary and inguinal freckling, and neurofibromas. Germline loss-of-function mutations in NF1 are the molecular cause for neurofibromatosis type 1 is a so-called "cancer predisposition syndrome." Additional tumors, including optic gliomas and soft tissue sarcomas, appear after the first year of life. Although these signs and symptoms are often present, children carrying germline NF1 mutations have a high likelihood of developing JMML, and JMML may, in some cases, actually be the presenting phenotype (without the presence of the aforementioned skin changes) that highlights the importance of *NF1* and hyperactive RAS signaling in normal myeloid cell growth and leukemogenesis.[4,5] *NF-1* mutated JMML encompasses approximately 10% of JMML cases and is clinically characterized by higher platelet counts, higher marrow blast percentages, and older age at diagnosis as compared with other JMML subtypes.

It is important to distinguish germline versus somatic mutations given that germline mutations can be associated with MPNs that are transient and may resolve spontaneously. Searching for the characteristic mutations in non-hematopoietic tissues (such as fibroblasts) can determine the germline versus somatic nature of a RAS pathway mutation.

TABLE 73.4 World Health Organization Diagnostic Criteria for Juvenile Myelomonocytic Leukemia

I Clinical and hematologic features (all 4 features mandatory)
• PB monocyte count ≥1 × 10^9/L
• Blast percentage in PB and BM <20%
• Splenomegaly
• Absence of Philadelphia chromosome (*BCR/ABL1* rearrangement)
II Genetic studies (1 finding sufficient)
• Somatic mutation in *PTPN11*[a] or *KRAS*[a] or *NRAS*[a]
• Clinical diagnosis of NF1 or *NF1* mutation
• Germ line *CBL* mutation and loss of heterozygosity of CBL[b]
III For patients without genetic features, besides the clinical and hematologic features listed under I, the following criteria must be fulfilled:
• Monosomy 7 or any other chromosomal abnormality or at least 2 of the following criteria:
• Hemoglobin F increased for age
• Myeloid or erythroid precursors on PB smear
• GM-CSF hypersensitivity in colony assay
• Hyperphosphorylation of STAT5

[a] Germ line mutations (indicating Noonan syndrome) need to be excluded.
[b] Occasional cases with heterozygous splice site mutations.
BM, Bone marrow; NF1, neurofibromatosis 1; *PB*, peripheral blood.
From Locatelli F, Niemeyer CM. How I treat juvenile myelomonocytic leukemia. *Blood.* 2015;125(7):1083–1090.

The diagnostic criteria for JMML (Table 73.4) include a combination of clinical, hematologic, and genetic features. Patients must have an elevated peripheral monocyte count over 1 × 10^9/L, blast numbers in the BM and peripheral blood below 20%, splenomegaly, and the absence of a *BCR-ABL1* rearrangement. In addition to these features, a genetic mutation is required (a somatic mutation in *PTPN11*, *KRAS*, or *NRAS*, a germline mutation in *CBL* or loss of heterozygosity of *CBL*, or a diagnosis of neurofibromatosis type 1 or a mutation in *NF1*). In patients without somatic mutations, a combination of other features is needed to establish a diagnosis, such as monosomy 7, or other chromosomal abnormalities in addition to an elevated hemoglobin F level for the patient's age, myeloid or erythroid precursors observed on the peripheral smear, *in vitro* hypersensitivity of hematopoietic progenitor cells to granulocyte-macrophage colony-stimulating factor (GM-CSF), or hyperphosphorylation of STAT5.[1]

Children presenting with JMML are often quite ill and present with disease features secondary to marrow and organ infiltration. Common presenting symptoms include fevers, failure to thrive, and splenomegaly. Hepatomegaly may also be present, and lung involvement due to leukemic infiltration manifesting as cough and dyspnea, with abnormal findings on chest X-ray, is possible. Blood tests frequently show leukocytosis with monocytosis, and thrombocytopenia. A small population of blasts may be seen in the peripheral blood.

PATHOGENESIS AND MOLECULAR CHARACTERIZATION

Shared Pathogenic Mechanisms

Despite clinical differences, the MDS/MPN overlap syndromes share a multistep pathogenesis, with alterations initially in genes typically associated with MDS, followed by additional mutations characteristic of other gene classes. Commonly mutated genes in MDS/MPNs include those involved in epigenetic regulation, transcriptional regulation, post-transcriptional modulation (in the form of splicesome mutations), and signaling pathways such as JAK-STAT. Notably, mutations in *TP53*, while common in MDS and AML, are very rare in these patients, although they can be seen in up to 10% of MDS/MPN-U patients. Fig. 73.6 demonstrates the frequency of specific mutations in adult MDS/MPN overlap syndrome subtypes based on a study of 367 patients where 30 genes were recurrently mutated in ≥3% of patients. Among all MDS/MPN overlap syndrome patients, the most common mutated genes were *ASXL1* (51%), *TET2* (45%), *SRSF2* (35%), *SF3B1* (24%), *JAK2* (19%), *EZH2* (17%), *RUNX1* (17%), *SETBP1* (15%), *NRAS* (13%), *CBL* (13%), and *KRAS* (10%). Of note, patients with MDS/MPN-RS-T had a lower mean number of variants per patient compared with other MDS/MPN overlap syndrome subtypes. Certain mutations are highly enriched in different subtypes. For example, *SF3B1* mutations are found in nearly all patients with MDS/MPN-RS-T and *ASXL1* is identified in nearly all patients with aCML.[6]

As described, monocytosis is the hallmark of both CMML and JMML. Interestingly, the two diseases usually reach a similar phenotype via different genetic mechanisms. A pre-disease CMML state is thought to occur when initiating mutations are acquired, most frequently in *TET2*, *SRSF2*, *ASXL1*, or the Ras pathway associated genes. These mutations favor increased monocyte-precursors and monocyte production in the BM. However, the disease only presents itself after an additional second-hit mutation occurs. Mutations in the Ras pathway typify MPN-CMML, a more proliferative subtype of CMML, while additional MDS-associated mutations usually result in dysplasia and MDS-CMML phenotype.

Interestingly, deletion of *TET2* in murine models leads to an increased Lin−Sca-1+c-Kit+ (LSK) cell pool. These cells have an increased hematopoietic repopulating capacity and abnormal cell differentiation pattern that skews heavily toward monocytic/granulocytic lineages. These mice develop a myeloid malignancy resembling CMML, suggesting that *TET2* functions as a tumor suppressor to regulate hematopoietic stem cell (HSC) differentiation.

JMML is characterized by either germline or somatic mutations in the Ras pathway, as previously described. Germline mutations (i.e., RASopathies) in NF1 (*NF1* germline mutation), Noonan syndrome (*PTPN11, NRAS*, or *KRAS* germline mutations), or CBL syndrome (*CBL1* germline mutation) often have different functional effects. The *PTPN11* mutations that are germline have a weaker gain-of-function effect as compared with somatically acquired mutations. Interestingly, some somatically acquired Ras mutations can occur very early in life, even in utero. In a study of patients who developed JMML at ages 2 to 19, sequencing of dried blood from Guthrie cards taken at birth detected acquired mutations in *PTPN11*, *NRAS*, and *KRAS*, suggesting that the JMML clone originated in utero in these children.

Importantly, mutations in the RASopathy genes (*PTPN11, NRAS, KRAS, CBL, and NF1*) are mutually exclusive within a given patient, implying that a mutation in any of these genes is sufficient

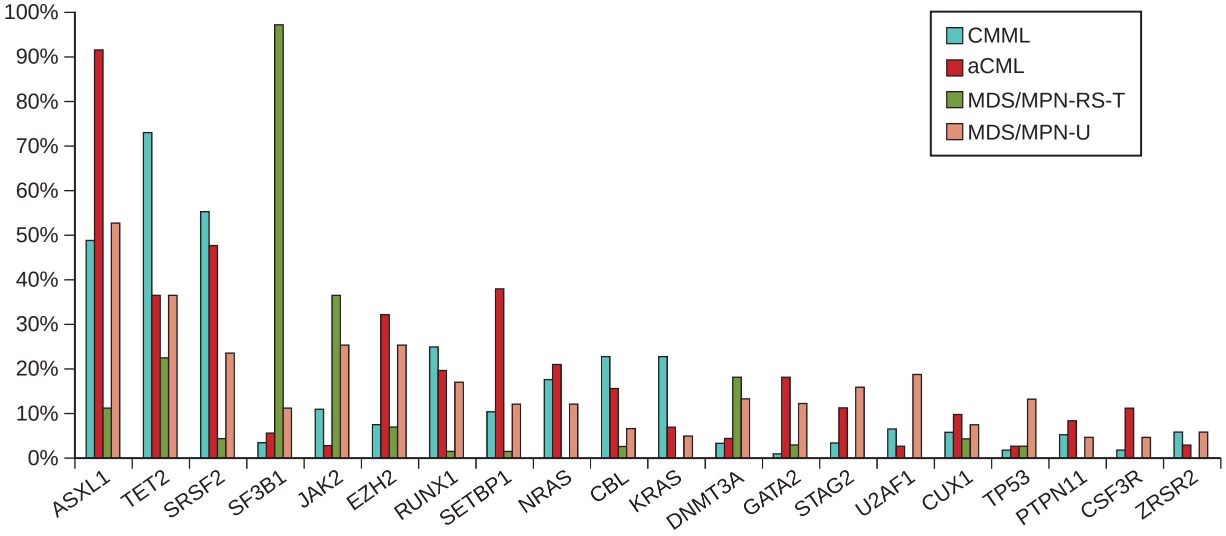


Figure 73.6 DISTRIBUTION OF SOMATIC MUTATIONS IN A SERIES OF 367 MYELODYSPLASTIC SYNDROME/MYELOPROLIFERATIVE NEOPLASM PATIENTS. The distribution that differed among different subtypes, although many genes are recurrent mutated across disease categories. *aCML*, Atypical chronic myeloid leukemia; *CMML*, chronic myelomonocytic leukemia; *MDS*, myelodysplastic syndrome; *MPN-RS-T*, myeloproliferative neoplasm with ring sideroblasts and thrombocytosis; *MPN-U*, myeloproliferative neoplasm unclassifiable. *(Adapted from Palomo L, Meggendorfer M, Hutter S, et al. Molecular landscape and clonal architecture of adult myelodysplastic/myeloproliferative neoplasms. Blood. 2020;136[16]:1851–1862.)*

to drive the JMML disease phenotype. In patients who lack one of these five Ras pathway mutations, there may be abnormal Ras signaling in the BM microenvironment that can result in JMML. For instance, a murine model where *PTPN11* mutation is present in the BM stroma cells, and not hematopoietic cells, results in a phenotype that is indistinguishable from JMML where *PTPN11* is present in hematopoietic cells.

Recently, the origin of the leukemia stem cell (LSC) in JMML has been studied by Louka et al. using primary patient specimens, FACS, and single-cell RNA sequencing revealed that the LSC is markedly heterogeneous in origin. Specifically, the granulocyte monocyte progenitors (GMP, Lin-CD34+CD38-CD90+CD45RA-) and a novel double-positive cell population (Lin-CD34+CD38-CD90+CD45RA+) were aberrantly increased in JMML samples as compared with healthy pediatric controls. Using an in vivo xenotransplantation model, the authors demonstrated that the LSC resided within the JMML HSC compartment, in addition to the GMP and the novel double-positive cell population. Single-cell index sorting of Lin-CD34+ stem cell followed by colony genotyping demonstrated that the JMML HSCs and progenitor cells contain clonally dominant RAS pathway mutations, as well as subclones with additional canonical myeloid mutations such as *ASXL1*, *SETBP1*, and monosomy 7.[7] They proposed model for LSC clonal evolution in JMML that proposes that "first hit "occurs within the RAS pathway, followed later by additional hits leading to a phenotypically heterogeneous HSC pool (Fig. 73.7).[8]

MDS/MPN-RS-T is thought to develop in a similar fashion as MDS-RS, with mutations in *SF3B1* occurring initially, as 85% to 95% of patients with this disease entity harbor this mutation. A second mutation, most commonly *JAK2V617F*, is then required to convert the MDS-RS into the MDS/MPN-RS-T phenotype. aCML and MDS/MPN-U do not appear to closely follow the above pathogenic paradigm, with the later disease especially difficult to define due to its heterogeneity. A study to evaluate the genetic signatures of the different MDS/MPN subsets from a large and diverse cohort of 177 adult MDS/MPN overlap syndrome patients revealed similarities between aCML and CMML, with MDS/MPN-RS-T having a distinct transcriptome, and MDS/MPN-U showing features of both disorders.[9] In another large study of 367 adult patients with MDS/MPN overlap syndrome that utilized genome-wide sequencing, the authors were able to reclassify MDS/MPN-U cases into molecular subtypes that resembled other MDS/MPN overlap syndromes. For instance, a co-mutation in *SRSF2* with either *TET2* or *RUNX1* were termed CMML-like and had similar hematologic parameters as pathologically defined CMML (i.e., monocytosis). Similarly, co-mutation of *ASXL1* and *SETBP1* in MDS/MPN-U patients displayed hematologic features similar to aCML, and the presence of *SF3B1* and *JAK2*-mutations resembled MDS/MPN-RS-T, with a higher percentage of ringed sideroblasts and an excellent prognosis. Overall, 61% of all MDS/MPN-U cases in this study could be genomically reclassified as another MDS/MPN overlap syndrome subtype even though they did not meet the standard pathologic diagnostic criteria. This study highlights that genomic features can be useful in reclassifying pathologically ambiguous cases of MDS/MPN-U.[6]

Epigenetic/Transcription Factor Mutations

Epigenetic regulatory genes are recurrently mutated in the various MDS/MPN overlap syndromes. *DNMT3A*, *ASXL1*, *TET2*, and *EZH2* are the most commonly mutated epigenetic genes in MDS/MPN overlap syndromes, while *RUNX1* is the most frequently mutated transcription factor. *ASXL1* is perhaps the most commonly and widely mutated gene in cases with an MDS/MPN overlap syndrome and can be found in the majority of patients with aCML, CMML, and MDS/MPN-RS-T. *TET2* mutations can be seen in each type of MDS/MPN overlap syndrome but are most common in CMML. *EZH2* mutations are most frequently identified with aCML and MDS/MPN-U. Mutations in *RUNX1* are most common in aCML. *DNMT3A* mutations are rare, though occasionally seen in MDS/MPN-RS-T. The above gene mutations have prognostic implications. *ASXL1* universally portends a poor prognosis across the spectrum of adult MDS/MPN overlap syndromes, while outcomes are worse with *TET2* mutations in aCML, *RUNX1* mutations in CMML and aCML, and *EZH2* mutations in MDS/MPN-RS-T and MDS/MPN-U.[10]

SETBP1 is a DNA-binding protein that forms part of a group of proteins that act together to affect histone methylation, making chromatin more accessible and thereby regulating gene expression. *SETBP1* mutations are observed in each of the MDS/MPN overlap syndromes but most commonly in aCML and least frequently in JMML. *SETBP1* alterations are gain-of-function mutations frequently associated with advanced age and monosomy 7/deletion 7q (−7/del(7q)). This mutation is associated with a poor prognosis and higher risk of leukemic transformation in JMML, CMML, and MDS/MPN-RS-T patients.

In JMML, aberrant DNA methylation is also a prominent pathogenic feature. Specifically, there is frequent hypermethylation of four specific CpG gene islands (*BMP4, CALCA, CDKN2B*, and *RARB*). Interestingly, this methylation status can be classified as high, intermediate, and low. This classification correlates with survival and relapse after allogeneic HSC transplant (alloSCT), with higher methylation status patients having a 42% 5-year survival in contrast to 72% in the low methylation group. After multivariable analysis, DNA methylation status retained independent prognostic significance. Aberrant

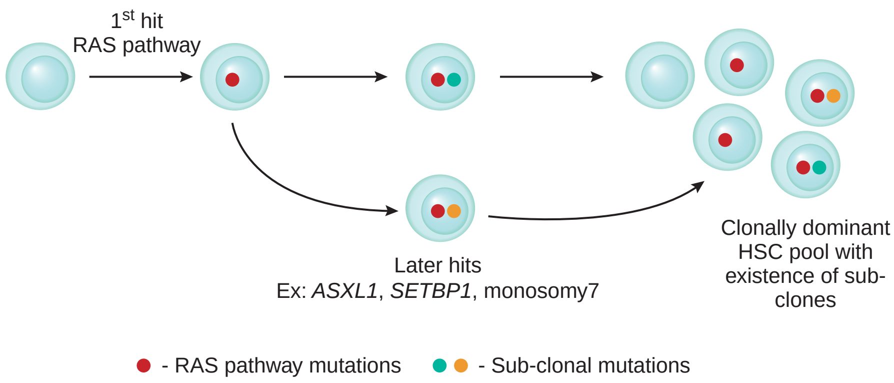

1st hit
RAS pathway
Later hits
Ex: *ASXL1*, *SETBP1*, monosomy7
Clonally dominant HSC pool with existence of sub-clones
● - RAS pathway mutations
●● - Sub-clonal mutations

Figure 73.7 Proposed model of hematopoietic stem cells *(HSC)* clonal evolution in juvenile myelomonocytic leukemia, with clonally dominant RAS pathway mutations occurring first followed by mutations in canonical myeloid mutations, such as *ASXL1, SETPB1*, and monosomy 7, occurring later and resulting in a phenotypically heterogeneous hematopoietic stem cell pool. *(Adapted from Sundaravel S, Steidl U. Stem cell origins of JMML. J Exp Med. 2021;218[2]:e20202152.)*

methylation also appears to be specific for JMML leukemic cell progeny and represents an early pathogenetic event.

Spliceosome Mutations

The spliceosome apparatus is a large ribonucleoprotein (RNP) complex of 100+ proteins and 5 small nuclear proteins that is responsible for the removal of introns from pre-mRNA and preparation of mRNA for protein expression. Thus, mutations in these genes can lead to aberrant protein expression. It is currently hypothesized that less than 20% of spliced proteins are affected by spliceosome mutations, with disease due to gain or loss of functional splicing of targeted oncogenes or tumor suppressor genes, respectively. The most common splicing gene mutations in myeloid malignancies occur in the *SF3B1*, *SRSF2*, *U2AF1*, and *ZRSR2* genes. *SRSF2* is most commonly mutated in CMML, aCML, and MDS/MPN-U; its role in determining patient prognosis in these diseases, especially CMML, is controversial. As previously discussed, *SF3B1* occurs in up to 90% of MDS/MPN-RS-T. *U2AF1* is associated with a worse overall survival (OS), and *ZRSR2* mutations are most common in CMML. Spliceosome mutations are extremely rare in JMML.

Cell Signaling Gene Mutations

Genes mutated in cell signaling pathways typically promote proliferative changes. Recurrently mutated genes in MDS/MPN overlap syndromes include those in the Ras pathway and MPN driver mutations (overwhelmingly *JAK2V617F*). Ras pathway mutations are frequently found in CMML and aCML, although at lower frequencies, and their presence is correlated with an inferior prognosis. *JAK2* mutations are most commonly seen in MDS/MPN-RS-T, more rarely observed in MDS/MPN-U, and to a lesser extent, in CMML.

RISK STRATIFICATION AND PROGNOSIS

Chronic Myelomonocytic Leukemia Risk Stratification

Given the clinical heterogeneity of CMML, tailoring predicted patient outcomes to treatment options has been a fundamental cornerstone in developing treatment strategies. Multiple CMML-specific risk stratification models have been proposed and validated in recent years (Fig. 73.8).[11] Initial models were based on clinicopathologic features, and more recently, CMML-associated genetic mutations and their respective prognostic impact have led to the creation of the following three molecularly integrated models.

In 2013, the CMML-specific prognostic scoring system (CPSS) was conceived based on WHO- and FAB-defined CMML subtypes, CMML-specific cytogenetic abnormalities, and RBC transfusion dependence (Table 73.5). Three years later, the establishment of mutated *ASXL1* as a predictor of aggressive disease and inferior OS in CMML, as well as the concomitant adverse survival implications of *RUNX1*, *NRAS*, and *SETBP1* mutations led to the mutational status of these genes being incorporated into the CPSS-Mol risk stratification system. This score is calculated by first determining the CMML genetic score by assigning 1 point for intermediate risk cytogenetics and 2 points for high-risk cytogenetics, 1 point for an *ASXL1* mutation, 1 point for a *NRAS* mutation, 2 points for a *RUNX1* mutation, and 1 point for a *SETBP1* mutation. The genetic risk is then determined to be low (0 points), intermediate-1 (1 point), intermediate-2 (2 points) or high (3 or more points). The CPSS-Mol score is then calculated

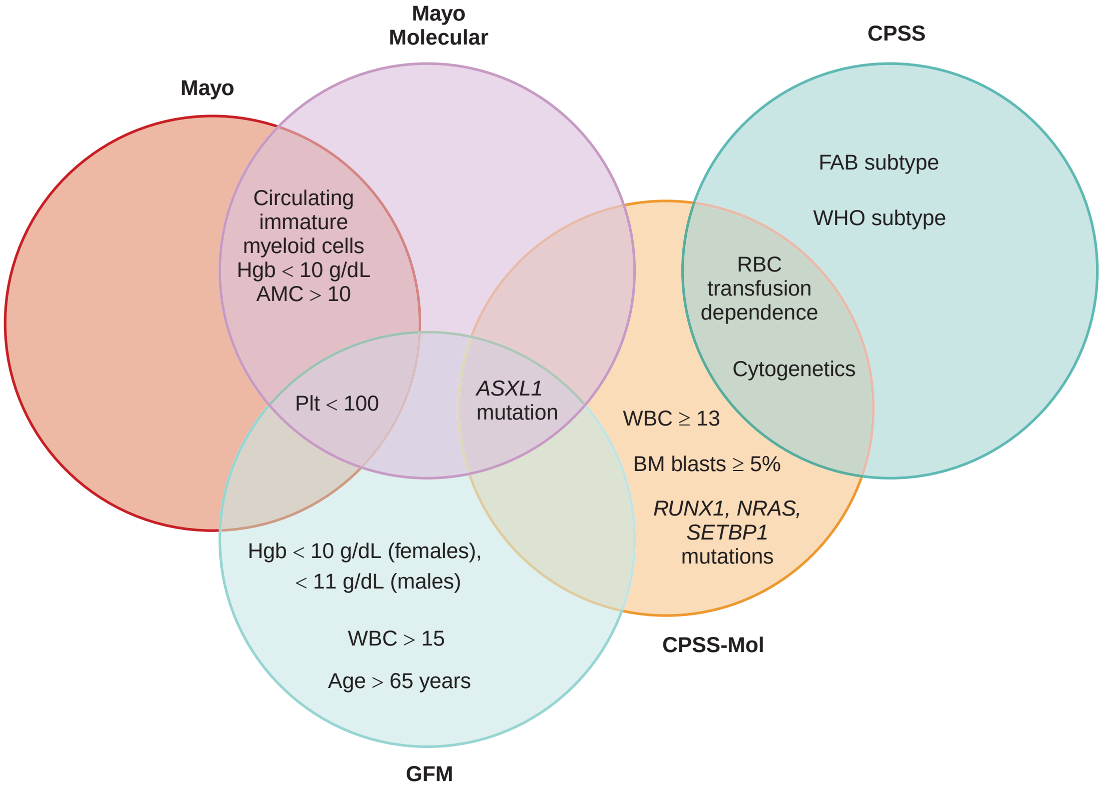


Figure 73.8 COMPARISON OF CHRONIC MYELOMONOCYTIC LEUKEMIA PROGNOSTIC STRATIFICATION SCORING SYSTEM. Five CMML prognostic stratification scoring systems are compared and contrasted via the individual risk factors recognized by each. *AMC*, Absolute monocyte count; *ASXL1*, additional sex combs like-1; *BM*, bone marrow; *CMML*, chronic myelomonocytic leukemia; *CPSS*, CMML-specific prognostic scoring system; *FAB*, French-American-British; *GFM*, Groupe Français des Myélodysplasies; *Hgb*, hemoglobin; *NRAS*, neuroblastoma RAS; *Plt*, platelets; *RBC*, red blood cell; *RUNX1*, Runt-related transcription factor 1; *SETBP1*, SET binding protein 1; *WBC*, white blood cells; *WHO*, World Health Organization. *(Adapted from Tremblay D, Rippel N, Feld J, et al. Contemporary risk stratification and treatment of chronic myelomonocytic leukemia. Oncologist. 2021;26[5]:406–421.)*

TABLE 73.5 **Comparison of Chronic Myelomonocytic Leukemia Prognostic Scoring Systems**

Prognostic Model	No. of Patients	Variables	Points for Each Variable	Median Survival (Months)			
				Low	Intermediate-1	Intermediate-2	High
GFM	312	i. WBC >15×10^9/L	3	*0–4 points*	*5–7 points*		*8 points*
		ii. Platelets <100×10^9/L	2	NR	38.5		14.4
		iii. Hemoglobin <10 g/dL in females, <11 g/dL in males	2				
		iv. Mutation status of *ASXL1*	2				
		v. Age >65 years	2				
Mayo prognostic model	226	i. AMC >10×10^9/L	1	*0 points*	*1 point*		*≥2 points*
		ii. Hemoglobin <10 g/dL	1	32	18.5		10
		iii. Platelets ≤100×10^9/L	1				
		iv. Presence of circulating immature myeloid cells	1				
Mayo molecular model	466	i. AMC >10×10^9/L	1	*0 points*	*1 point*	*2 points*	*≥3 points*
		ii. Hemoglobin <10 g/dL	1	97	59	31	16
		iii. Platelets ≤100×10^9/L	1				
		iv. Presence of circulating immature myeloid cells	1				
		v. Mutation status of *ASXL1* (frameshift or nonsense)	1				
CPSS	558	i. WHO subtype[a]	1	*0 points*	*1 point*	*2 points*	*≥3 points*
		ii. FAB subtype[b]	1	72	31	13	5
		iii. CPSS cytogenetic risk groups[c]	1				
		iv. RBC transfusion dependence[d]	1				
CPSS-Mol	214	i. RBC transfusion dependence[d]	1	*0 points*	*1 point*	*2–3 points*	*≥4 points*
		ii. WBC count ≥13×10^9/L	1	NR	68	30	17
		iii. BM blast proportion ≥5%	1				
		iv. CPSS cytogenetic intermediate risk group*	1				
		v. CPSS cytogenetic high-risk group*	2				
		vi. *RUNX1* mutation	2				
		vii. *ASXL1* mutation	1				
		viii. *NRAS* mutation	1				
		ix. *SETBP1* mutation	1				

[a] WHO subtypes: CMML-1: blasts including promonocytes less than 5% in peripheral blood (PB), less than 10% in bone marrow (BM). CMML-2: blasts including promonocytes 5% to 19% in PB, 10% to 19% in BM, or when Auer rods are present irrespective of blast count.

[b] FAB subtypes—CMML-MD: WBC less than13 × 10^9/L, CMML-MP: WBC ≥13 × 10^9/L.

[c] CPSS cytogenetic risk groups: low risk (diploid karyotype or isolated -Y), intermediate risk (all abnormalities other than those of low-/high-risk groups), high risk (trisomy 8, abnormalities of chromosome 7, or complex karyotype).

[d] At least 1 RBC transfusion every 8 weeks over 4 months.

AMC, Absolute monocyte count; *ASXL1*, additional sex combs like-1; *(BM)*, bone marrow; *CPSS*, CMML-specific prognostic scoring system; *FAB*, French-American-British; *GFM*, Groupe Français des Myélodysplasies; *NRAS*, neuroblastoma RAS; *RBC*, red blood cell; *RUNX1*, Runt-related transcription factor 1; *SETBP1*, SET binding protein 1; *WBC*, white blood cells; *WHO*, World Health Organization.

by giving 1 point each for: BM blasts ≥5%, WBC ≥13 × 10^9/L, and RBC transfusion dependency and adding this to the genetic risk score (0 points for low, 1 point for intermediate-1, 2 points for intermediate-2, and 3 points for high risk). A total score of 0 indicates low risk, 1 indicates intermediate-1 risk, 2 to 3 indicates intermediate-2 risk, and ≥4 indicates high risk. The CPSS-Mol model provides significantly improved prognostic accuracy, subclassifying CMML patients into four distinct prognostic cohorts that are highly variable in terms of both survival and risk of evolution to AML (see Table 73.5).[12]

Similarly, the Mayo prognostic model was devised in 2013 based on studying clinical variables and outcomes of 226 CMML patients in which absolute monocyte count greater than 10 × 10^9/L, hemoglobin less than 10 g/dL, platelets ≤100 × 10^9/L, and the presence of circulating immature myeloid cells were found to have independent prognostic significance after multivariable analysis. Using these risk variables, patients were categorized as low risk (0 risk factors), intermediate risk (1 risk factor), or high risk (2 or more risk factors). This was followed by a 2014 two-center study ($n = 466$) that demonstrated the adverse impact of *ASXL1* mutations on survival outcomes and subsequently led to its integration into an updated Mayo molecular model (MMM) that identified four prognostically discrete risk groups. One point is assigned to each of the four features listed in the Mayo prognostic model, in addition to one point for an *ASXL1* frameshift or nonsense mutation. Low-risk group (0 points) have an estimated OS of 97 months, intermediate-1 risk group (1 point) have an estimated OS of 59 months, intermediate-2 risk group (2 points)

TABLE 73.6 Myelodysplastic Syndrome/Myeloproliferative Neoplasm Overlap Syndromes: Cytogenetic Abnormalities and Overall Outcomes

MDS/MPN Subtype	Overall Frequency Cytogenetic Abnormalities (%)	Most Common Cytogenetic Aberrations and Associated Frequency (%)	Median OS (Months)	Leukemic Transformation Rate
CMML	30%	• Trisomy 8 (23%) • -Y (20%) • Monosomy 7, del7q (14%) • 20q deletion (8%) • Trisomy 21 (8%) • Derivative 3q (8%)	20–40	15%–30%
aCML	30%–40%	• Trisomy 8 (17–27%) • Monosomy 7, del7q (6%) • Trisomy 9 (5%) • Trisomy 21 (5%) • Other: complex karyotype, del(12q)	14–30	40%
MDS/MPN-RS-T	20%	• Trisomy 8 (4%) • -Y (4%) • Other: complex karyotype	60–84	< 5%
MDS/MPN-U	50%	• Trisomy 8 (15%) • Monosomy 7/del(7q) (11%) • Del(20q) (7%) • Complex karyotype (15%)	19–26	16%
JMML	35%	• Monosomy 7 (26%)		Rare

aCML, Atypical chronic myeloid leukemia; *CMML*, chronic myelomonocytic leukemia; *JMML*, juvenile myelomonocytic leukemia; *MDS*, myelodysplastic syndrome; *MPN*, myeloproliferative neoplasm; *MPN-RS-T*, myeloproliferative neoplasm with ring sideroblasts and thrombocytosis; *MPN-U*, myeloproliferative neoplasm unclassifiable.

gave an estimated OS of 31 months, and high-risk patients (3 or more points) have an estimated OS of 16 months.

The third molecularly-integrated risk stratification tool was developed following a retrospective analysis of 312 patients with CMML conducted by the Groupe Français des Myélodysplasies (GFM) and includes five independent prognostic determinants; these include age greater than 65 years (2 points), hemoglobin less than 10 g/dL in females or less than 11 g/dL in males (2 points), platelets less than 100×10^9/L (2 points), WBC greater than 15×10^9/L (3 points), and *ASXL1* mutation status (2 points). The GFM model stratifies patients into three risk groups: low risk (0 to 4 points), intermediate risk (5 to 7 points), and high risk (8 to 12 points) with associated survivals not reached, 38.5 months and 14.4 months, respectively.

While no clear consensus has been established in terms of the optimal CMML prognostic model, mutational data have been repeatedly shown to be invaluable for the prognostication of CMML patients. Accordingly, the European Hematology Association and European LeukemiaNet panel have recommended the use of any of the above three molecularly integrated models for CMML risk stratification, or alternatively the CPSS or MD Anderson Prognostic Scoring System in the absence of mutational data.[12] Until a unified approach to CMML risk stratification is reached with further studies of large, mutationally and cytogenetically characterized CMML databases, the utilization of a molecularly-guided approach, when feasible, is recommended for refined CMML prognostication.

Chromosomal Abnormalities Associated With Myelodysplastic Syndrome/Myeloproliferative Neoplasm Overlap Syndromes

While the MDS/MPN overlap syndromes share many clinicopathologic and molecular features, they retain important cytogenetic heterogeneity that has prognostic implications. There are no specific cytogenetic abnormalities for this group of disorders. Most of the recurrent chromosomal aberrations are also detected in other myeloid disorders. Cytogenetic abnormalities are seen in variable numbers among MDS/MPN overlap syndrome patients, namely in approximately 30%, 30% to 40%, 20%, 50%, and 35% in CMML, aCML, MDS/MPN-RS-T, MDS/MPN-U, and JMML patients, respectively (Table 73.6).[13–15] The presence of genomic abnormalities in MDS/MPN overlap syndrome patients can be used for risk stratification. Overall, these aberrations have been associated with inferior survival outcomes in all overlap syndromes, except for aCML. In a study of 367 MDS/MPN overlap patients, an abnormal karyotype was documented in 29% of patients, while a complex karyotype (defined as ≥3 aberrations) was only observed in 4% of patients. An abnormal karyotype was associated with inferior OS in all subtypes except aCML. Importantly, in MDS/MPN-RS-T, cytogenetic abnormalities are rare, but when present confer a poor prognosis.[6]

In CMML, the most common cytogenetic abnormalities are trisomy 8 (23%), loss of chromosome Y (20%), monosomy 7/7q deletion (14%), trisomy 21 (8%), 20q deletion (8%), and derivative 3q (8%). Two three-tiered systems have guided CMML-specific cytogenetic risk stratification. The Spanish group classified patients into low risk (normal karyotype or -Y), intermediate risk (all chromosomal abnormalities except those in the high and low risk categories), and high risk (trisomy 8, chromosome 7 abnormalities, and complex karyotype) categories, with associated five-year OS rates of 35%, 26%, and 4%, respectively. The Mayo-French system then revised the previously proposed categories by adding derivative 3q to the low-risk group, and including all monosomal karyotypes in the high-risk group, with resultant median OS of 41, 21, and 3 months in the low, intermediate, and high-risk categories, respectively.

Atypical Chronic Myeloid Leukemia Prognosis

aCML is associated with an aggressive disease course, with a median OS of 14 to 30 months and an AML transformation rate of 40% within 11–18 months. Multivariable analyses have implicated several factors as independent adverse prognosticators in terms of overall or progression-free survival in aCML, namely advanced age, progressive anemia, leukocytosis (with suggested peripheral leukocyte count cut-off of >50 × 10^9/L), increased numbers of immature precursors in the peripheral blood, female gender, and presence of *SETBP1* and/or *TET2* mutations.[16]

Multiple studies have investigated the cytogenetics of aCML, with variable frequencies of abnormalities detected; among the most frequent being trisomy 8 (17% to 27%) and chromosome 7 abnormalities (6%).[6,14]

Myelodysplastic Syndrome/Myeloproliferative Neoplasm With Ring Sideroblasts and Thrombocytosis Prognosis

Of the MDS/MPN overlap syndromes, MDS/MPN-RS-T patients have the most favorable outcome, with a median OS of 5 to 7 years and a leukemic transformation rate of <5%. Cytogenetic abnormalities are infrequent in MDS/MPN-RS-T, with a normal karyotype being identified in 80% of patients, and the most frequent chromosomal abnormalities being trisomy 8 (4%) and -Y (4%). Nonetheless, when present, an abnormal karyotype, along with anemia (hemoglobin <10 g/dL), and/or the presence of *SETBP1* and *ASXL1* mutations, have been linked to an inferior survival. In contrast, age less than 80 years, as well as *JAK2* and *SF3B1* mutations, independently predict a more favorable survival.

Myelodysplastic Syndrome/Myeloproliferative Neoplasm Unclassifiable

Among the MDS/MPN overlap syndromes, MDS/MPN-U remains the least characterized. In total, 50% of MDS/MPN-U patients have cytogenetic abnormalities, most commonly trisomy 8 (15%), a complex karyotype (15%), and monosomy 7 (10%).[15] The median OS of MDS/MPN-U is 19 to 26 months, with a 16% leukemic transformation rate. Advanced age (>65 years), adverse cytogenetics (involving chromosome 7 or a complex karyotype, increased BM or peripheral blood blasts (≥5%), and *CBL* and/or *TP53* mutations have all been shown to independently portend an inferior OS.[15,16]

While specific prognostic tools are not present for MDS/MPN-U patients, two studies have suggested the use of the utility of the MDS international prognostic scoring system (MDS-IPSS) for risk stratification.[15,17] Using the MDS-IPSS in MDS/MPN-U patients is limited, however, as the majority of patients are classified as being low-risk. Despite this categorization, low-risk MDS/MPN-U patients have an inferior survival as compared to their similarly-categorized MDS counterparts. The median survivals of MDS/MPN-U categorized by the IPSS-R as very low, low, intermediate, and high risk are 33.4, 25.1, 12.8, and 6.0 months.

Juvenile Myelomonocytic Leukemia Prognosis

Without appropriate treatment, JMML patients have a median survival of 10 to 12 months, with spontaneous complete remissions being reported in rare cases. Even with alloSCT, which is the only curative option for most, 5-year event-free survival (EFS) remains dire at about 50%.

As previously described, in JMML, germline mutations in Ras pathway genes (*NRAS*, *KRAS*, *PTPN11*) are associated with Noonan syndrome and confer a good prognosis and, on occasion, have even been associated with spontaneous remissions. On the contrary, the secondary acquisition of somatic *RAS* mutations and mutations in *SETBP1* and *JAK3* have been implicated in both disease progression to AML and an inferior OS. Other factors associated with adverse prognosis in terms of EFS and OS include older age at presentation (>2 years), increased fetal hemoglobin levels, peripheral and/or BM greater than 20%, increased DNA methylation profile, and a complex cytogenetic pattern. Cytogenetic abnormalities are seen in about 35% of JMML patients, usually in *KRAS*-mutated JMML, with monosomy 7 being the most common (26%).

TREATMENT APPROACHES

General Treatment Considerations

Treatment of patients with MDS/MPN overlap syndromes can be challenging given their clinical heterogeneity and the rarity of these diseases, resulting in many clinicians being unfamiliar with proper management strategies. Therapies in MDS/MPN overlap syndromes are largely borrowed from MDS or MPN treatment paradigms. Few clinical trials have evaluated agents dedicated for treating patients with MDS/MPN overlap syndrome. Among MDS/MPNs, CMML is by far the most thoroughly evaluated; thus, similar treatment strategies have been applied to the other MDS/MPN overlap syndromes given the shared biologic and clinical features. In general, asymptomatic patients without significant cytopenias can often be managed with observation alone. Patients with proliferative symptoms (e.g., leukocytosis) are often treated with hydroxyurea (HU) and occasionally the JAK1/JAK2 inhibitor ruxolitinib, particularly if splenomegaly or constitutional symptoms compromise the patient's quality of life. For patients whose MDS/MPN overlap syndromes with dysplastic features, hypomethylating agents (HMAs) are often employed. AlloSCT is the only curative therapy for all patients with an MDS/MPN overlap syndrome.

GENERAL TREATMENT CONSIDERATIONS

Risk stratification is key for the proper management of MDS/MPN. There are numerous prognostic scoring systems available for CMML, with no clear advantage between the three systems that incorporate mutational information: MMM, CPSS-Mol, and GFM. In practice, we use the CPSS-Mol given its ability to discriminate between risk groups. This scoring system is the only one that incorporates both cytogenetic and molecular data. Other MDS/MPNs overlap syndromes do not have formal risk stratification scoring systems, but certain risk factors portend worse prognosis. Specifically, *SETBP1* and *ASXL1* mutation in MDS/MPN-RS-T, anemia and leukocytosis in aCML, and *CBL* and *TP53* mutations in MDS/MPN-U are associated with inferior survival.

Independent of MDS/MPN subtype, patients who are high risk and eligible should proceed with alloSCT. In high-risk patients, HMA therapy can be employed in patients with cytopenias or elevated blast counts either in preparation for alloSCT or in transplant-ineligible patients. Ruxolitinib may be utilized to treat constitutional symptoms and splenomegaly, particularly in patients with CMML and MDS/MPN-U that are driven by activation of the JAK-STAT signaling pathway. Management of anemia in MDS/MPNs can be challenging, although ESAs can be successfully employed when the serum EPO level is less than 500 IU/L. Lenalidomide may be an option for some patients, particularly those with MDS/MPN-RS-T. Cytoreduction with HU is generally reserved for asymptomatic CMML patients with leukocytosis or splenomegaly and for select MDS/MPN-RS-T patients where anemia is not severe. Intensive chemotherapy should only be employed for rapidly progressive disease with elevated blast counts in preparation for definitive alloSCT.

aCML, Atypical chronic myeloid leukemia; *CMML*, chronic myelomonocytic leukemia; *ESA*, erythropoietin stimulating agent; *EPO*, erythropoietin; GFM, Groupe Français des Myélodysplasies; *HMA*, hypomethylating agent; HU, hydroxyurea; *MDS*, myelodysplastic syndrome; *MMM*, Mayo molecular model; *MPN*, myeloproliferative neoplasm; *MPN-RS-T*, myeloproliferative neoplasm with ring sideroblasts and thrombocytosis; *MPN-U*, myeloproliferative neoplasm unclassifiable

Chronic Myelomonocytic Leukemia Management

The first treatment decision for a patient with CMML is to determine if they are candidates for alloSCT. While advanced age, comorbidities, and lack of available donors remain exclusion criteria for many patients, advances in utilizing reduced intensity conditioning regimens and alternative donor sources are broadening the application of alloSCT. Importantly, to date, there has not been a prospective evaluation of alloSCT in CMML patients. However, many well-conducted retrospective studies provide evidence that alloSCT plays a key role in the treatment of CMML. A European Society for Blood and Marrow Transplantation (EBMT) study of 513 CMML patients undergoing alloSCT estimated a 4-year relapse-free survival and OS of 27% and 33% and a non-relapse mortality of 41%. Grade 2 to 4 acute and chronic graft-versus-host disease (GVHD) occurred in 33% and 24% of patients, respectively. The only statistically significant prognostic factor after multivariable analysis was attaining a complete response (CR) as judged by clinical parameters prior to transplant.[18] AlloSCT was associated with a longer OS as compared with non-transplant approaches (4.3 years vs. 2.0 years) in one retrospective study. However, the benefit for alloSCT extended only to CPSS intermediate-2 or high-risk patients and not to patients with intermediate-1 or low-risk disease.

Risk stratification is the first step for determining the appropriate treatment course for a patient with CMML. As previously discussed, the molecularly enhanced models are preferred but a consensus as to the optimal risk stratification scoring system does not exist. We typically employ the CPSS-Mol score given its ability to categorize patients into four risk groups with disparate clinical outcomes including OS and risk of leukemia transformation; however, each of the molecularly-informed prognostic models is acceptable in clinical practice when mutational information is available. In low-risk asymptomatic patients without anemia, observation may be appropriate. Low-risk patients should be closely monitored for the development of new symptoms, splenomegaly, or changes in their peripheral blood counts. Recent evidence suggests that leukocytosis (WBC above 34.25×10^9/L) in MPN-CMML patients is associated with higher rates of end organ damage, including renal insufficiency, liver dysfunction, pleural effusions, and cardiovascular events. In this study, patients with a WBC above 34.25×10^9/L who were treated with HU had a superior median OS compared to those who were not (24.7 months vs. 12.3 months, $P = .003$). Therefore, consideration of cytoreduction with HU in asymptomatic patients with a WBC greater than 34.5 g/dL is appropriate, although the true benefit has not yet been confirmed in a prospective study.

In low-risk patients with anemia, erythropoietin stimulating agents (ESAs) are frequently used to alleviate symptomatic anemia. In a study of 94 CMML patients who were treated with an ESA, 64% had an erythroid response (defined as an increase in hemoglobin of 1.5 g/dL) and 31% achieved transfusion independence. Similar to MDS, lower erythropoietin (EPO) levels were significantly associated with a response. This study also confirmed that ESAs are best utilized in low-risk patients. Although this study viewed EPO levels as a continuous variable, a cutoff level was not found to be predictive of an optimal response, although prior work in low-risk MDS suggests that patients with an EPO level of $\geq$500 U/L are unlikely to respond to an ESA. Therefore, it is reasonable to consider an ESA in anemic CMML patients who have an EPO level less than 500 U/L.

Lenalidomide has been evaluated in two clinical trials in CMML. In a phase I trial, lenalidomide 5 mg daily administered continuously was identified as the maximally tolerated dose and resulted in one partial response (PR) by MDS response criteria. Combination therapy with lenalidomide and azacitidine has also been explored in the Southwest Oncology Group (SWOG) S1117 study. This phase II/III clinical trial randomized CMML or high-risk MDS patients to azacitidine alone or in combination with lenalidomide or the histone deacetylase inhibitor vorinostat in a 1:1:1 fashion. There was a signal for superior efficacy in the CMML population with azacitidine plus lenalidomide as compared to azacitidine alone (overall response rate [ORR] of 68% vs. 28%). The median duration of response was 19 months. Unfortunately, the type of response (e.g., erythroid hematologic improvement) was not reported. Skin rashes were more common with the combination therapy as compared with azacitidine alone. In clinical practice, lenalidomide is occasionally utilized in CMML patients who have transfusion-dependent anemia based on data extrapolated from MDS where lenalidomide can result in conversion to transfusion independence in approximately a quarter of non-del(5q) MDS patients.

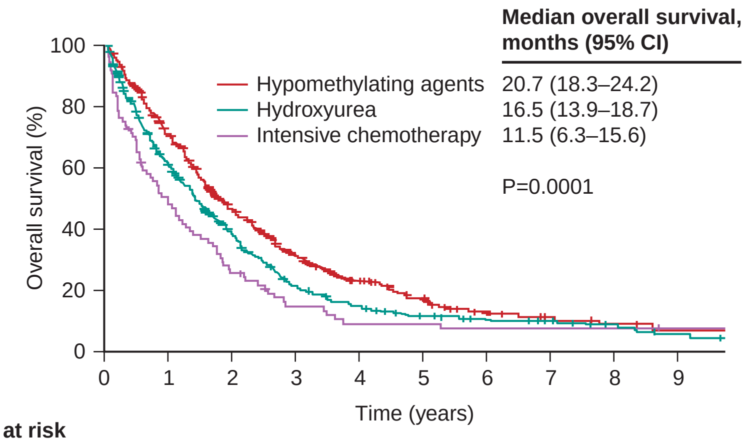
Another therapy that may improve erythropoiesis and ameliorate transfusion dependence in CMML patients is luspatercept, a novel fusion protein that contains an extracellular domain of the activin receptor II B fused to the Fc domain of human IgG1 and acts to trap activin and GDF11, which in turn inhibits Smad2/3 signaling thereby promoting differentiation of cells in the erythroid series. This agent is FDA approved for the treatment of MDS (see Chapter 61) and has been explored in CMML. In the phase I/II PACE-MD trial, which included patients with lower-risk MDS or MDS-CMML, the rate of erythroid improvement was 63% at 12 weeks. Responses were higher in patients who harbored an *SF3B1* mutation or had ring sideroblasts. However, patients with CMML were not included in the phase III MEDALIST trial that led to the approval of luspatercept in MDS patients with ring sideroblasts and MDS/MPN-RS-T. Further studies of CMML patients are needed to better understand the true role of luspatercept therapy.

Cytoreduction is the mainstay of therapy in MPN-CMML. While several cytoreductive agents have been explored, HU has been shown to produce superior response rates, longer OS, and improved tolerability as compared to more intensive therapies, as demonstrated in a trial of 105 CMML patients who were randomized to either HU or etoposide. The study closed after the first interim analysis, and the HU arm resulted in significantly longer survival compared to the etoposide arm (20 months vs. 9 months). In addition, HU treatment resulted in a higher response rate as compared with etoposide (60% vs. 36%).

There is only a limited role for intensive AML type induction chemotherapy in the treatment of CMML. In a retrospective analysis of MDS patients that also included patients with CMML, intensive induction therapy was associated with an inferior OS as compared to treatment with decitabine (12 months vs. 22 months). More recently, a large retrospective study of 949 CMML patients, including 83 who received intensive chemotherapy, demonstrated that intensive chemotherapy was associated with a survival of only 14 months as compared with 20.7 months in patients treated with an HMA (Fig. 73.9).[19] As such, most experts reserve intensive chemotherapy for fit patients who have rapidly increasing blast cell counts as a bridge to definitive therapy with alloSCT.

HMAs are the only FDA-approved agents for the treatment of CMML patients. The approval is based on prospective data of a limited number of CMML patients who were included in studies evaluating these agents in the MDS population. Specifically, the phase III registration studies of decitabine and azacitidine included only 14 patients each with CMML and presumably these patients had MDS-CMML. Despite this limited population of CMML patients, the efficacy of HMA has been subsequently confirmed in a prospective study of decitabine that included 43 CMML patients. In this study, the ORR was 47% and the CR rate was 16%. The median OS was 17 months, with responders having a significantly longer OS as compared with non-responders. Importantly, 9 out of 18 transfusion-dependent patients became transfusion independent.[20] Numerous retrospective studies of HMAs in CMML have confirmed their efficacy and safety, with most studies reporting an ORR of approximately 40% and CR rates of 10% to 20%.

The impact of HMA therapy, however, on OS and quality of life in CMML is less clear. In one matched-pair analysis, there was a trend toward increased two-year OS (62% vs. 41%, $P = .067$) in azacitidine-treated patients compared with best supportive case (BSC).[21] There is also no change in myeloid gene mutational burden following HMA therapy of CMML patients, as demonstrated by a study of mutation-specific discrimination analysis of treated CMML cases over



Number at risk (number censored)										
Hypomethylating agents	412 (0)	267 (26)	159 (44)	92 (61)	50 (81)	27 (91)	14 (98)	9 (102)	5 (104)	3 (105)
Hydroxyurea	391 (0)	218 (23)	123 (37)	64 (41)	42 (43)	29 (48)	22 (52)	15 (58)	11 (60)	5 (62)
Intensive chemotherapy	83 (0)	38 (2)	20 (2)	10 (4)	6 (4)	6 (4)	5 (4)	5 (4)	5 (4)	4 (5)

Figure 73.9 Overall survival of 949 chronic myelomonocytic leukemia from treatment start comparing frontline intensive chemotherapy, hypomethylating agents, and hydroxyurea. Overall survival was significantly longer in patients treated with upfront hypomethylating agents. *(Adapted from Pleyer L, Leisch M, Kourakli A, et al. Outcomes of patients with chronic myelomonocytic leukaemia treated with non-curative therapies: a retrospective cohort study. Lancet Haematol. 2021;8[2]:e135–e148.)*

time. The authors observed amplification of more mutated clones in patients following treatment with HMA. In addition, there was ongoing clonal evolution despite clinical responses seen in patients with CMML treated with HMAs. Thus, while there are clear benefits for HMA therapy, it does not appear to significantly deplete the CMML stem cell clone.

HMAs have been compared to HU both in retrospective studies and in a phase 3 clinical trial. In a previously mentioned multicenter retrospective study including 949 CMML patients, first-line HMA treatment was associated with an improved OS as compared with HU (20.7 months vs. 15.6 months, $P = .0002$) (see Fig. 73.8). However, this survival advantage was not seen in patients with CMML-0, MDS-CMML, or lower-risk patients as defined by the CPSS score, highlighting the importance of patient selection.[19] HU and HMA therapy have also been prospectively compared in MPN-CMML patients in the phase 3 DACOTA study. One-hundred and seventy patients with MPN-CMML and advanced features (meeting at least two criteria: BM blasts ≥5%, abnormal karyotype (except -Y), absolute neutrophil count $\geq 16 \times 10^9$/L, hemoglobin <10 g/dL, platelets $<100 \times 10^9$/L or splenomegaly >5 cm below costal margin) were randomized to either decitabine or HU. After an intention-to-treat analysis, the ORR was 56% and 30% at 3 months for the decitabine and HU arms, respectively, a difference that was statistically significant ($P = .0011$). The duration of response was longer in the decitabine arm but not to a statistically significant degree. While there was no significant difference in EFS, OS, or AML-free survival between the two arms, one-third of patients in the HU arm went on to receive an HMA and more patients in the HMA arm went to alloSCT as compared with the HU arm. This prospective evaluation offers a few important insights. First, this trial demonstrates that randomized clinical trials in CMML are feasible, paving the way for more rigorous therapeutic studies of patients with this rare disease. Second, both HU and HMA are active agents in MPN-CMML. However, there may be an advantage to using HMAs upfront given that one-third of patients in the HU arm eventually went on to receive an HMA and upfront decitabine was associated with higher response rates and more patients proceeding to alloSCT. Long-term follow-up of this trial is eagerly awaited to clarify the upfront treatment for MPN-CMML patients.

Administration of HMAs has recently become more convenient with the approval of oral decitabine/cedazuridine. This agent combines oral decitabine with the cytidine deaminase inhibitor cedazuridine, resulting in bioequivalent doses of decitabine as compared with parenteral decitabine. In a randomized phase II study, 63 patients with MDS and 17 with CMML were randomized patients 1:1 to either oral cedazuridine 100 mg/decitabine 35 mg for 5 days in cycle 1 followed by IV decitabine 20 mg/m^2 in cycle 2 or the reverse (intravenous followed by oral). The ORR was 60%, and the CR rate of 21%. Grade ≥3 or adverse events included 46% with neutropenia, 38% with thrombocytopenia, and 22% with anemia. A phase III trial with a nearly identical design was then performed using a fixed-dose capsule and enrolled 133 patients (117 with MDS and 16 with CMML). The primary endpoint of bioequivalence between oral decitabine/cedazuridine and IV decitabine was achieved.[22] Based on this bioequivalence data, oral decitabine/cedazuridine was approved by the FDA approved for the treatment of MDS and CMML. Of note, the results of these studies do not report the outcomes of the subgroup of CMML patients. Importantly, based on the trial design, oral decitabine/cedazuridine was not compared directly to parenteral decitabine in terms of clinical endpoints and safety, but the response rates, cytopenias, and survival data appear consistent with what is expected for HMA therapy in CMML patients. A phase III trial comparing oral versus intravenous decitabine appears unlikely.

Novel therapies are currently in development for the treatment of CMML. Among the most promising is the JAK1/JAK2 inhibitor, ruxolitinib. In a phase 1 study of 20 patients with CMML, ruxolitinib treatment led to improvement in splenomegaly in 5 of 9 patients and symptomatic improvement in 10 patients out of 11.[23] In an expansion phase 2 trial which included an additional 29 patients, the ORR (by MDS/MDS International Working Groups criteria) was 46% and the OS was significantly prolonged as compared with historical controls. Although not FDA approved for CMML, ruxolitinib can be effective in patients with MPN-CMML who have constitutional symptoms or splenomegaly. Other novel agents in development for CMML include the GM-CSF blocking antibody lenzilumab, the CD123-directed cytotoxin tagraxofusp, and the farnesyltransferase inhibitor tipifarnib. In addition, multiple agents currently in clinical development for MDS may have a role in the future treatment of CMML.

One potential therapy that has recently been evaluated is pevonedistat, a neural precursor cell-expressed, developmentally downregulated 8 (NEDD8) activating enzyme inhibitor that regulates the activity of the cullin-dependent E3 ligases as part of the ubiquitin-proteasome system which leads to the accumulation of certain substrates involved in cell-cycle progression, DNA damage, and stress responses, causing cell death of AML and MDS cells. A phase II randomized trial of azacitidine either alone or in combination with pevonedistat, enrolled 120 patients with higher-risk MDS ($n = 67$), low-blast count AML ($n = 36$), or CMML ($n = 17$) patients has been completed. A significant increase in median OS with azacitidine in combination with pevonedistat versus azacitidine alone (21.8 months vs. 19.0 months, $P = 0.33$) was not observed. Since the number of CMML patients studied was small, further evaluation of this agent or other agents targeting this pathway are merited.

Atypical Chronic Myeloid Leukemia Management

As a rare and heterogeneous disease, there is a paucity of clinical trial data for treating aCML and an established standard of care is not available. Similar to other MDS/MPNs, HMAs, HU, and ESAs have been employed with varied success. The only curative therapy is alloSCT. The largest study of alloSCT in aCML was performed by the EBMT, which retrospectively analyzed the outcomes of 42 patients with aCML who received an alloSCT (64% with matched sibling and 36% with unrelated donor grafts). After a median follow up of 89 months, 87% of patients had achieved a CR with a median OS of 70 months. Acute and chronic GVHD was the major contributor to treatment-related mortality.[24] Therefore, for appropriate aCML patients, alloSCT should be pursued.

HMA therapy can be employed in alloSCT-ineligible patients or to control disease as a bridge to transplant in select patients. Data are largely borrowed from other MDS/MPNs, although several case reports and/or series describe the use of HMAs, largely decitabine, for the treatment of aCML. In the largest series of 19 aCML patients who received an HMA, the ORR was 26%. However, the median duration of response was only 2.7 months. The impact of HMA therapy on cytogenetic and mutational burdens in aCML patients has not been documented. At present, HMAs are likely best utilized as a cytoreductive bridge to alloSCT, although they are frequently administered in transplant-ineligible patients with unclear efficacy.

Additional therapies are being actively evaluated. With the discovery of the *CSF3R T618I* membrane proximal mutation that results in downstream JAK-STAT pathway activation, there is laboratory-based rationale for the utilization of ruxolitinib. There are numerous case reports demonstrating the activity of ruxolitinib in patients with aCML, including a phase II trial of single agent ruxolitinib. In this study, which included 44 patients with aCML or CNL, the ORR was 35%. However, only 2 of the 23 patients with aCML responded (both were a PR). The duration of response was not reported, but the median time of ruxolitinib administration was only 5.5 months in the aCML group (compared to 15.3 months in the CNL arm). Responses were more likely in patients harboring a *CSF3R* mutation and survival between the CNL cohort and aCML were not statistically different. Ruxolitinib therapy led to a greater reduction of median WBC/absolute neutrophil count in patients with CNL (vs. patients with aCML) and in *CSF3R*-mutated patients (vs. *CSF3R*–wild type patients). Spleen volume reduction ≥ 35% occurred more frequently in *CSF3R*-mutant patients (79% vs. 29% in *CSF3R*-wild type patients; but this did reach statistical significance). The majority of patients did not experience CR or PR and eventually succumbed to disease-related complications unless they proceeded to alloSCT.[25] Therefore, ruxolitinib should only be considered for the treatment of aCML in the presence of the *CSF3R T618I* mutation, but this therapy is associated with treatment-emergent myelosuppression and an unclear survival benefit. Given the complexity of aCML, referral to a center with expertise in this area is essential for the management of aCML.

Myelodysplastic Syndrome/Myeloproliferative Neoplasm With Ring Sideroblasts and Thrombocytosis Management

The management of MDS/MPN-RS-T patients is primarily aimed at symptom management, with an emphasis on risk stratification. Treatment strategies are borrowed from both essential thrombocythemia (ET) (see Chapter 71) and MDS-RS (see Chapter 61). However, given the rarity of MDS/MPN-RS-T, the true efficacy and safety of these treatment strategies have not been completely explored.

In patients with low-risk MDS/MPN-RS-T, aspirin may be employed (although it has not been formally evaluated in MDS/MPN-RS-T) to reduce vasomotor symptoms, such as migraine headaches and erythromelalgia. Aspirin may also reduce the thrombotic risk; however, the true benefit of this strategy is debated even in low-risk ET patients (see Chapter 71). Of note, there is no established relationship between the height of the platelet count and the degree of thrombotic risk in ET. Therefore, it is assumed that such a correlation does not exist in MDS/MPN-RS-T. Thus, cytoreductive agents should not be employed to lower the platelet count in the absence of vasomotor symptoms or thrombotic events, especially as these agents will likely exacerbate the underlying anemia. Instead, reduction in symptom burden should be the goal of cytoreductive therapy. In addition, in patients who have severe bleeding events secondary to acquired von Willebrand syndrome due to excessive thrombocytosis, cytoreductive therapy with HU is appropriate. Another option is anagrelide, which selectively reduces platelets but is associated frequently with cardiac and gastrointestinal toxicity and anemia (see Chapter 71). Pegylated interferon-2α can also be used in select patients who are intolerant to HU but will likely lead to greater degrees of anemia. However, none of these therapeutic agents has been thoroughly evaluated specifically in MDS/MPN-RS-T. Ruxolitinib may have a role in appropriate patients with constitutional symptoms who can tolerate treatment-related anemia associated with this agent.

Amelioration of anemia is a key challenge in MDS/MPN-RS-T. Similar to MDS and CMML, ESAs are most appropriately employed in low-risk patients. Given the experience in MDS and CMML with ESAs, patients with lower EPO levels (i.e., <500 mU/mL) are most likely to respond. Lenalidomide has also been explored in MDS/MPN-RS-T patients. Case reports have consistently demonstrated erythroid responses and platelet reductions with occasional molecular responses. However, changes in BM morphology have not been noted. Luspatercept is also likely to have a role in improving anemia in MDS/MPN-RS-T patients, given its efficacy in patients with MDS-RS and in patients that harbor the *SF3B1* mutation. However, luspatercept has not been systematically evaluated in MDS/MPN-RS-T patients. However, in patients with anemia who do not respond to ESAs and/or lenalidomide, luspatercept is a reasonable option. As with other MDS/MPNs, alloSCT should be strongly considered in appropriate high-risk patients.

Myelodysplastic Syndrome/Myeloproliferative Neoplasm Unclassifiable Management

Similar to other MDS/MPNs, there are no dedicated guidelines for the treatment of MDS/MPN-U. Given that this diagnosis is associated with a poor prognosis, with a median OS of approximately 12 to 24 months, appropriate patients should be rapidly referred for alloSCT. In terms of treatment options, HMAs are most commonly employed but their efficacy is not well established. In a series of 135 patients with MDS/MPN-U, 59 patients (44%) were treated with an HMA. In the 27 patients who completed at least 6 cycles of HMA treatment and, therefore were evaluable for response, only 1 patient (4%) attained a CR while 2 patients (7%) attained a partial PR. In addition, 3 patients (11%) had a marrow response and 1 patient (4%) had a complete cytogenetic response, leading to an ORR of 19%. Of note, the duration of response in this series was not reported. Notably, 1 patient with a CR and 1 with a PR eventually transformed to AML while receiving treatment. Therefore, while HMA responses are possible in MDS/MPN-U, the response rate is suboptimal.

As with the other MDS/MPN overlap syndromes, alloSCT is the only curative option. In a Japanese study utilizing a nationwide registry, 86 patients were identified who received an alloSCT. The 3-year OS was 48.5%, with cumulative incidence of relapse of 23.7% and non-relapsed mortality of 26.3%. Age older than 50 years and active disease at time of alloSCT were predictive of an inferior survival.

In terms of novel therapies for MDS/MPN-U, ruxolitinib in combination with azacitidine has been prospectively explored in a phase 2 study of MDS/MPN overlap syndrome patients (CMML, aCML, MDS/MPN-U) that included 14 patients with MDS/MPN-U. The median duration of treatment was 13.8 months. Of the entire cohort of 35 patients, 57% had a response. In the 14 patients with splenomegaly at baseline, 9 (64%) had a spleen response. The median duration of response was 8 months. The median OS of the entire study population was 16.6 months, but the median OS was longest in patients with MDS/MPN-U (26.5 months), as compared with CMML (15.1 months) or aCML (8 months). Therefore, ruxolitinib in combination with an HMA may be employed in MDS/MPN-U patients, particularly if splenomegaly and constitutional symptoms are present.

Juvenile Myelomonocytic Leukemia Management

The majority of children with JMML will require alloSCT. However, as previously discussed, patients with germline *PTPN11, NRAS,* or *KRAS* patients (Noonan syndrome) or those with germline *CBL* mutation may have spontaneous remission of their myeloproliferation and, therefore, a watch and wait approach is appropriate. In patients with somatic *PTPN11* or *KRAS* mutations or germline *NF1*, upfront alloSCT should be offered. In *NRAS* somatically mutated JMML patients who have favorable features, such as HbF levels ≤10% and normal platelet counts, observation may be appropriate.

It is sometimes necessary to provide therapy to bridge a JMML patient to alloSCT, particularly in the case of mild organ dysfunction or symptomatic splenomegaly. Azacitidine, oral 6-mercaptopurine, and low-dose cytarabine have been employed in this setting to leukoreduce patients. In children with blastic transformation or pulmonary infiltration causing respiratory compromise, fludarabine and high-dose cytarabine may be useful. However, there remains controversy whether pre-transplant JMML-directed therapy is appropriate for all patients. To date, standard chemotherapy regimens have not been shown to have an impact on the rate of post-alloSCT relapse. Of note, novel Ras-targeting therapies are in development but have not been clinically evaluated in patients with JMML.

If alloSCT is pursued, total body irradiation should be avoided given the inferior efficacy and late-onset adverse effects that have been reported. A busulfan-based myeloablative conditioning regimen is recommended. In terms of donor source, recent analyses suggest negligible differences between using grafts harvested from unrelated donors and HLA-identical siblings. In the largest study to date, the 5-year OS after alloSCT was 64% with 35% having recurrence of their leukemia. In patients who require timely transplantation or those who do not have a matched donor, umbilical cord blood transplantation (UBCT) has been shown to be efficacious with the 5-year disease free survival of 44%. Patients at an increased risk of death or relapse post-transplant include older children (≥2 years), those with platelet counts ≤40 × 10^9/L, and those with fetal hemoglobin levels ≥10%.

In children who experience post-alloSCT mixed donor/recipient chimerism or reappearance of cytogenetic or molecular alterations after alloSCT, there are several salvage treatment options. In children who are still on immunosuppression for GVHD prophylaxis, withdrawal of the immunosuppressive agents can lead to eradication of the malignant cells. In children off immunosuppressive therapy or with overt leukemic relapse, a second alloSCT should be pursued. Of note, donor lymphocyte infusion has been shown to be ineffective in this setting.

Clinical trials are ongoing looking at alternative therapies for JMML. Leveraging knowledge about the Ras/Erk pathway, the Children's Oncology Group is conducting a study evaluating trametinib (an oral MEK inhibitor) in relapsed or refractory JMML. Given the rare and severe nature of JMML, international and cooperative group studies are needed to identify additional therapies to improve survival and decrease toxicity associated with treatments for these young patients.

FUTURE DIRECTIONS

Since the designation of MDS/MPN overlap syndromes as a distinct diagnostic entity in 2002, advances in molecular and biologic characterization have generated translational science that will likely lead to the next generation of therapeutics. A key theme that has emerged is the genetic similarities between MDS/MPN overlap syndromes. Despite differing morphologic features, whole-exome and RNA sequencing studies have revealed that these overlap syndromes do not cluster and instead exist as a continuum with the majority of patients harboring ≥3 co-occurring mutations involving chromatin modifiers, epigenetic regulators, splicing factors, and signaling pathway genes.[26] Therefore, the future of therapeutic development in MDS/MPN overlap syndromes should involve the creation of shared treatment paradigms targeting key pathway mutations rather than the development of disease-specific therapies. With that in mind, the use of shared response criteria (Table 73.7) has enabled the evaluation of treatment options across the various MDS/MPNs overlap syndromes to facilitate future clinical investigation.[27] To further the development of novel therapeutic in MDS/MPNs, international collaboration will be necessary to combine expertise and optimize enrollment in these rare diseases.

Agents that are currently in development for other myeloid malignancies may also have a role in the treatment of patients with MPN/MDS overlap syndromes. For instance, the BCL-2 inhibitor venetoclax was recently reported to have activity in combination with an HMA for the treatment of CMML and AML secondary to CMML. In addition, targeting agents by mutational subtypes may hold promise in the future. At present, mutations in *IDH1* and *IDH2* are rare in patients with MDS/MPN overlap syndromes and can be targeted with approved small molecule inhibitors. In addition, novel agents in development that target spliceosome mutated myeloid malignancies, such as protein arginine methyltransferase inhibitors (i.e., PRMT5 inhibitor), may be particularly effective in MDS/MPNs given the high prevalence of splicing mutations.

CONCLUSION

MDS/MPN overlap syndromes are rare and heterogeneous entities featuring both proliferative features and BM failure. Morphologic differences in the subtypes are in contrast to recently defined shared pathogenetic features. Risk stratification to assess patient prognosis is key for the appropriate management of MDS/MPN, with alloSCT being the only curative option in fit, high-risk patients. Patients who experience symptomatic splenomegaly or constitutional symptoms may benefit from a JAK2 inhibitor therapy. HMA therapy can also be employed for the treatment of patients with MDS/MPNs overlap syndromes and can produce responses but such agents have an unclear impact on OS and the rate of disease progression. Novel therapies are in development, but multicenter, cooperative trials are needed to expand treatment options for MDS/MPN overlap syndromes, which have traditionally been associated with poor outcomes.

TABLE 73.7 Proposed Criteria for Measurement of Treatment Response in Adult Myelodysplastic Syndrome/Myeloproliferative Neoplasm Overlap Syndromes

CR (presence of all of the following improvements)[a]

- Bone marrow: $\leq$5% myeloblasts (including monocytic blast equivalent in case of CMML) with normal maturation of all cell lines and return to normal cellularity[a]
- Osteomyelofibrosis absent or equal to “mild reticulin fibrosis” ($\leq$ grade 1 fibrosis)[b]
- Peripheral blood[c]
  - WBC $\leq 10 \times 10^9$ cells/L
  - Hgb $\geq$11 g/dL
  - Platelets $\geq 100 \times 10^9$/L; $\leq 450 \times 10^9$/L
  - Neutrophils $\geq 1.0 \times 10^9$/L
  - Blasts 0%
  - Neutrophil precursors reduced to $\leq$2%
  - Monocytes $\leq 1 \times 10^9$/L
- Extramedullary disease: Complete resolution of extramedullary disease present before therapy (e.g., cutaneous disease, disease-related serous effusions), including palpable hepatosplenomegaly

Provisional category of CR with resolution of symptoms:[c] CR as described above, and complete resolution of disease-related symptoms as noted by the MPN-SAF TSS

Persistent low-level dysplasia is permitted given subjectivity of assignment of dysplasia[a]

Complete cytogenetic remission

- Resolution of previously present chromosomal abnormality (known to be associated with myelodysplastic, syndrome myeloproliferative neoplasms, or MDS/MPN), as seen on classic karyotyping with minimal of 20 metaphases or FISH[d]

Partial remission

- Normalization of peripheral counts and hepatosplenomegaly with bone marrow blasts (and blast equivalents) reduced by 50%, but remaining >5% of cellularity except in cases of MDS/MPN with $\leq$5% bone marrow blasts at baseline

Marrow response

- Optimal marrow response: Presence of all marrow criteria necessary for CR without normalization of peripheral blood indices as presented above.
- Partial marrow response: Bone marrow blasts (and blast equivalents) reduced by 50%, but remaining >5% of cellularity, *or* reduction in grading of reticulin fibrosis from baseline on at least 2 bone marrow evaluations spaced at least 2 months apart

Clinical benefit

- Requires 1 of the following in the absence of progression or CR/partial response and independent of marrow response (cord blood response must be verified at $\geq$8 weeks) to be considered a clinical benefit
- Erythroid response
  - Hgb increase by $\geq$2.0 g/dL
  - TI for $\geq$8 weeks for patients requiring at least 4 packed red blood cell transfusions in the previous 8 weeks
  - Only red blood cell transfusions given based on physician’s judgment for a pretreatment Hgb of $\leq$8.5 g/dL will count in the red blood cell TI response evaluatione[e]
- Platelet response
  - Transfusion independence when previously requiring platelet transfusions of at least a rate of 4 platelet transfusions in the previous 8 weeks
  - Pretreatment $\leq 20 \times 10^9$/L: increase from $< 20 \times 10^9$/L to $> 20 \times 10^9$/L and by at least 100%
  - Pretreatment $> 20 \times 10^9$/L but $\leq 100 \times 10^9$/L: absolute increase of $\geq 30 \times 10^9$/L[e]
- Neutrophil response
  - Pretreatment $\leq 0.5 \times 10^9$/L at least 100% increase and an absolute increase $\geq 0.5 \times 10^9$/L
  - Pretreatment, $> 0.5 \times 10^9$/L and $\leq 1.0 \times 10^9$/L At least 50% increase and an absolute increase $\geq 0.5 \times 10^9$/L[e]
- Spleen response
  - Either a minimum 50% reduction in palpable splenomegaly of a spleen that is at least 10 cm at baseline or a spleen that is palpable at more than 5 cm at baseline becomes not palpable
- Symptom response
  - Improvement in symptoms as noted by decrease of $\geq$50% as per the MPN-SAF TSS scoring <20 were not considered eligible for measuring clinical benefit.[f]

---

[a] Presence of dysplastic changes, which may be interpreted within the scope of normal range of dysplastic changes, may still exist in the presence of CR as allowed in MDS International Working Group. Marrow should exhibit age-adjusted normocellularity in CR.

[b] If there is no significant fibrosis present on the initial bone marrow biopsy, a second biopsy is not required to prove resolution of fibrosis. Grading of fibrosis in measurement of treatment response should be according to the European Consensus System.

[c] Given the current lack of a validated tool to assess complete resolution of symptoms in MDS/MPN, “CR with resolution of symptoms” (a complete resolution of disease-related symptoms as noted by the MPN-SAF TSS in presence of CR) will be a provisional category of disease response.

[d] Loss of cytogenetic burden of disease by (via FISH or classic karyotyping) known to adversely affect prognosis is required to reach complete cytogenetic remission. Decrease in the cytogenetic burden of disease must be by $\geq$50% (via FISH or classic karyotyping) to be indicative of a partial cytogenetic response. Given variability of fluorescent probes used in FISH, cytogenetic normalization via FISH will depend on the performance characteristics of the specific probes used.

[e] Resolution of abnormal peripheral blood counts must persist for at least 2 separate analyses over at least 8 weeks. In the case of proliferative MDS/MPN, CR will include resolution of thrombocytosis to a normal platelet count ($150–450 \times 10^9$/L) and resolution of leukocytosis to WBC $\leq 10 \times 10^9$ cells/L but $\geq 1.5 \times 10^9$/L. Hgb should be maintained greater than 11 g/dL and platelets $\geq 100 \times 10^9$/L without the support of transfusions. Clinical benefit may occur when these changes occur in absence of other changes required for CR or marrow response. Platelet and packed red blood cell TI would be considered for clinical benefit, and duration of TI should be monitored. Reduction in myeloid precursors (promyelocytes, myelocytes, metamyelocytes, nucleated red blood cells) to less than appreciable levels ($\leq$2% to 3%) and/or 1×10^9/L monocytosis in the absence of infection, cytokine treatment, or other reactive causes.

[f] MPN-SAF TSS validation among patients with MDS/MPN is currently under way (R.A. Mesa, personal communication, 2014).

CMML, Chronic myelomonocytic leukemia; *CR*, complete response; *FISH*, fluorescence in situ hybridization; *MDS*, myelodysplastic syndrome; *MPN*, myeloproliferative neoplasm; *MPN-SAF TSS*, MPN Symptom Assessment Form total symptom score; *WBC*, white blood cell.

Adapted from Savona MR, Malcovati L, Komrokji R, et al. An international consortium proposal of uniform response criteria for myelodysplastic/myeloproliferative neoplasms (MDS/MPN) in adults. *Blood.* 2015;125(12):1857–1865.

REFERENCES

1. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127(20):2391–2405.
2. Malcovati L, Papaemmanuil E, Bowen DT, et al. Clinical significance of SF3B1 mutations in myelodysplastic syndromes and myelodysplastic/myeloproliferative neoplasms. *Blood*. 2011;118(24):6239–6246.
3. Linglart L, Gelb BD. Congenital heart defects in Noonan syndrome: diagnosis, management, and treatment. *Am J Med Genet C Semin Med Genet*. 2020;184(1):73–80.
4. Largaespada DA, Shaughnessy Jr. JD, Jenkins NA, Copeland NG. Retroviral integration at the Evi-2 locus in BXH-2 myeloid leukemia cell lines disrupts Nf1 expression without changes in steady-state Ras-GTP levels. *J Virol*. 1995;69(8):5095–5102.
5. Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA. Tumour predisposition in mice heterozygous for a targeted mutation in Nf1. *Nature Genet*. 1994;7(3):353–361.
6. Palomo L, Meggendorfer M, Hutter S, et al. Molecular landscape and clonal architecture of adult myelodysplastic/myeloproliferative neoplasms. *Blood*. 2020;136(16):1851–1862.
7. Louka E, Povinelli B, Rodriguez-Meira A, et al. Heterogeneous disease-propagating stem cells in juvenile myelomonocytic leukemia. *J Exp Med*. 2021;218(2).
8. Sundaravel S, Steidl U. Stem cell origins of JMML. *J Exp Med*. 2021;218(2):e20202152.
9. Manja M, Sabine J, Claudia H, Wolfgang K, Torsten H. The mutational landscape of 18 investigated genes clearly separates four subtypes of myelodysplastic/myeloproliferative neoplasms. *Haematologica*. 2018;103(5):e192–e195.
10. Itzykson R, Kosmider O, Renneville A, et al. Prognostic score including gene mutations in chronic myelomonocytic leukemia. *J Clin Oncol*. 2013;31(19):2428–2436.
11. Tremblay D, Rippel N, Feld J, El Jamal SM, Mascarenhas J. Contemporary risk stratification and treatment of chronic myelomonocytic leukemia. *Oncologist*. 2021;26(5):406–421.
12. Elena C, Galli A, Such E, et al. Integrating clinical features and genetic lesions in the risk assessment of patients with chronic myelomonocytic leukemia. *Blood*. 2016;128(10):1408–1417.
13. Tang G, Zhang L, Fu B, et al. Cytogenetic risk stratification of 417 patients with chronic myelomonocytic leukemia from a single institution. *Am J Hematol*. 2014;89(8):813–818.
14. Patnaik MM, Barraco D, Lasho TL, et al. Targeted next generation sequencing and identification of risk factors in World Health Organization defined atypical chronic myeloid leukemia. *Am J Hematol*. 2017;92(6): 542–548.
15. Mangaonkar AA, Swoboda DM, Coltro G, et al. Clinicopathologic characteristics, prognostication and treatment outcomes for myelodysplastic/myeloproliferative neoplasm, unclassifiable (MDS/MPN-U): Mayo Clinic-Moffitt Cancer Center study of 135 consecutive patients. *Leukemia*. 2020;34(2):656–661.
16. Wang SA, Hasserjian RP, Fox PS, et al. Atypical chronic myeloid leukemia is clinically distinct from unclassifiable myelodysplastic/myeloproliferative neoplasms. *Blood*. 2014;123(17):2645–2651.
17. Bose P, Nazha A, Komrokji RS, et al. Mutational landscape of myelodysplastic/myeloproliferative neoplasm-unclassifiable. *Blood*. 2018;132(19):2100–2103.
18. Symeonidis A, van Biezen A, de Wreede L, et al. Achievement of complete remission predicts outcome of allogeneic haematopoietic stem cell transplantation in patients with chronic myelomonocytic leukaemia. A study of the Chronic Malignancies Working Party of the European Group for Blood and Marrow Transplantation. *Br J Haematol*. 2015;171(2):239–246.
19. Pleyer L, Leisch M, Kourakli A, et al. Outcomes of patients with chronic myelomonocytic leukaemia treated with non-curative therapies: a retrospective cohort study. *Lancet Haematol*. 2021;8(2):e135–e148.
20. Santini V, Allione B, Zini G, et al. A phase II, multicentre trial of decitabine in higher-risk chronic myelomonocytic leukemia. *Leukemia*. 2018;32(2):413–418.
21. Pleyer L, Germing U, Sperr WR, et al. Azacitidine in CMML: matched-pair analyses of daily-life patients reveal modest effects on clinical course and survival. *Leukemia Res*. 2014;38(4):475–483.
22. Garcia-Manero G, McCloskey J, Griffiths EA, et al. Pharmacokinetic exposure equivalence and preliminary efficacy and safety from a randomized cross over phase 3 study (ASCERTAIN study) of an oral hypomethylating agent ASTX727 (cedazuridine/decitabine) compared to IV decitabine. *Blood*. 2019;134(suppl 1). 846-846.
23. Padron E, Dezern A, Andrade-Campos M, et al. A multi-institution phase I trial of ruxolitinib in patients with chronic myelomonocytic leukemia (CMML). *Clin Cancer Res*. 2016;22(15):3746–3754.
24. Onida F, de Wreede LC, van Biezen A, et al. Allogeneic stem cell transplantation in patients with atypical chronic myeloid leukaemia: a retrospective study from the Chronic Malignancies Working Party of the European Society for Blood and Marrow Transplantation. *Br J Haematol*. 2017;177(5):759–765.
25. Dao KT, Gotlib J, Deininger MMN, et al. Efficacy of ruxolitinib in patients with chronic neutrophilic leukemia and atypical chronic myeloid leukemia. *J Clin Oncol*. 2020;38(10):1006–1018.
26. Zhang H, Wilmot B, Bottomly D, et al. Genomic landscape of neutrophilic leukemias of ambiguous diagnosis. *Blood*. 2019;134(11):867–879.
27. Savona MR, Malcovati L, Komrokji R, et al. An international consortium proposal of uniform response criteria for myelodysplastic/myeloproliferative neoplasms (MDS/MPN) in adults. *Blood*. 2015;125(12):1857–1865.

CHAPTER 74

EOSINOPHILIA, EOSINOPHILIC NEOPLASMS, AND THE HYPEREOSINOPHILIC SYNDROMES

Peter Valent, Andreas Reiter, and Jason Gotlib

Eosinophils are highly specialized granulocytic effector cells that produce and store numerous biologically active mediators, including cytotoxic proteins, lipid mediators, chemotactic peptides, and cytokines (Table 74.1). Under various pathologic conditions, blood eosinophils transmigrate through the endothelial layer and invade various target organs, where these cells secrete their products into the surrounding tissues, thereby triggering inflammation, toxic damage, and tissue remodeling.[1] Since their initial characterization by Paul Ehrlich in 1879, eosinophils have been implicated in a number of systemic diseases and conditions characterized by blood and/or tissue eosinophilia (Table 74.2). During the past few decades, researchers have also gained a better understanding of the unique features and functions of activated eosinophils and of the specific roles some of their granule proteins and inducible lipid mediators may play in the pathogenesis of allergic, parasitic, neoplastic, and other diseases.[1] In addition, recognition of the eosinophil as a major pro-inflammatory and tissue-remodeling effector cell has fueled a surge of interest in this granulocyte.

Studies of the biochemistry, biologic activities, and tissue localization of distinct enzymatic and non-enzymatic cationic proteins derived from eosinophils have provided convincing evidence for their role in the pathogenesis of inflammation and tissue damage in eosinophil-associated diseases. The five cationic granule proteins that may play a role in eosinophil-related pathologies include two major basic proteins (MBP-1 and MBP-2), eosinophil peroxidase (EPX), and two ribonucleases, namely eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN; see Table 74.1). Eosinophils also have the capacity to express toxic oxidative intermediates and other mediators of inflammation, as well as molecules that may contribute to the development of thrombosis and fibrosis. In addition, eosinophils are a rich source of DNA traps that may facilitate fibrin deposition, as well as the killing of microbial invaders.[2] A clinically important aspect is that the various mediators produced and released by (activated) eosinophils often act together to trigger thrombosis and tissue damage, especially when eosinophil expansion and activation is chronic and "treatment-resistant."

The process of determining the cause of eosinophilia is often frustrating for both the physician and the patient, and, in many instances, the resulting diagnosis is "eosinophilia of unknown etiology." Depending on laboratory standards and local guidelines, eosinophilia is defined as more than 0.4 to 0.5×10^9/L blood, detected by microscopy.

Diurnal variations in eosinophil counts are well documented, with minimum numbers appearing early in the morning and greatest numbers appearing late at night, mirroring circadian rhythms in adrenal corticosteroids. Although these variations are usually limited to a certain extent, basal eosinophil counts should be routinely measured during the daytime. Transient mild eosinophilia is commonly seen in allergic reactions, during and shortly after a bacterial infection, and in many other reactive states. Sometimes, transient eosinophilia may be substantial or even excessive. Such transient (<4 weeks) forms of reactive eosinophilia are considered harmless in most instances but should prompt the physician to search for certain underlying diseases, such as an occult allergy or an unrecognized infection.

Eosinophil disorders and related syndromes are a heterogeneous group of diseases characterized by marked expansion and persistent accumulation of eosinophils in the peripheral blood (PB) and other organ systems. In general, eosinophil disorders can be divided into (A) neoplastic states where eosinophils are monoclonal and (B) reactive states where eosinophil expansion is considered to be "poly-clonal" and triggered by eosinotropic cytokines, such as interleukin-5 (IL-5).[3] In both instances, hypereosinophilia (HE), defined by a persistent eosinophil count of $\geq 1.5 \times 10^9$/L blood, is typically present. Depending on the underlying disease and other factors, the HE state may or may not be accompanied by specific (HE-mediated) organ damage, also referred to as *hypereosinophilic syndrome* (HES). In many instances, HES-related organ damage manifests as overt thromboembolism (thrombosis) or fibrosis.[3] In other patients, organ damage is associated with less-specific findings, and may involve the skin, gastrointestinal (GI) tract, or the central nervous system (CNS). Based on the underlying condition, both HE and HES can be divided into familial, primary (neoplastic), and secondary (reactive) forms (Table 74.3).[3] Apart from these classical forms of HES, more specific syndromes associated with HE and HE-related organopathies have been described. Some of these syndromes are associated with a hereditary (germ-line) defect, whereas others are accompanied by distinct immunological abnormalities (Table 74.4A). Finally, HE may develop in the context of organ-restricted inflammatory conditions such as eosinophilic pneumonia or eosinophilic colitis (Table 74.4B).

A thorough examination of all clinical and laboratory parameters, including radiologic and imaging studies, bone marrow (BM) investigations, karyotype, and molecular studies, are required in order to establish the correct diagnosis (underlying disease and organ damage) in patients with initially unexplained HE.[3] This chapter provides an overview on the epidemiology, pathogenesis, course, prognosis, and clinical features of various eosinophil disorders. We also discuss developments in the field and the impact of molecular markers and targets. In addition, this chapter provides diagnostic algorithms and recommendations for the management and treatment of patients with eosinophil disorders.

EPIDEMIOLOGY

Little is known about the prevalence and incidence of primary eosinophil disorders (eosinophilic neoplasms) and HE-related syndromes, including HES. Based on the available literature, most eosinophil neoplasms and all types of HES are rare, and the same holds true for other HE-related conditions, such as the Gleich syndrome or Churg-Strauss syndrome (CSS), also known as eosinophilic granulomatosis with polyangiitis (EGPA). In an attempt to estimate the incidence of HES, data collected by the Surveillance, Epidemiology and End Results (SEER) database, sponsored by the National Cancer Institute, have been reviewed. A crude incidence of 0.035 cases per 100,000 person-years was found, and the male-to-female ratio was reported to be 1.47 to 1. The average age at diagnosis was 52.5 years, and the peak incidence was recorded in individuals aged 65 to 74 years.

TABLE 74.1 Eosinophil-Derived Molecules and Their Potential Role in Health and Disease

Eosinophil Product/ Molecule	Relevant Function(s)
Eosinophil-Related Enzymes	
Nonspecific esterase	Cytotoxic effects (microbes and tissue cells)
Catalase	Cytotoxic effects (microbes and tissue cells)
Acid phosphatase	Cytotoxic effects (microbes and tissue cells)
Lysophospholipase	Tissue inflammation
Phospholipase D	Migration, adhesion, vesicle transport, secretion
Hexosaminidase	Tissue inflammation and tissue remodeling
Arylsulphatase B	Lysosomal hydrolase
5-Lipoxygenase	Leukotriene production, angiogenesis
Leukotriene C4 synthase	Leukotriene production
Cyclooxygenase	Prostaglandin production
Histaminase	Histamine degradation
Eosinophil Basic Proteins	
Eosinophil peroxidase (EPX)	Cytotoxic effects (parasites, microbes, tissue cells)
Major basic protein-1 (MBP-1)	Cytotoxic effects (parasites, microbes, tissue cells)
Major basic protein-2 (MBP-2)	Cytotoxic effects (parasites, microbes, tissue cells)
Eosinophil cationic protein (ECP)	Cytotoxic effects, mucus secretion, fibrosis
Eosinophil-derived neurotoxin (EDN)	Cytotoxic effects (antiviral, RNase activity)
Chemokines	
Interleukin-8 (IL-8)	Leukocyte recruitment and activation
MIP-1-alpha (CCL3)	Leukocyte recruitment and activation
RANTES (CCL5)	Leukocyte recruitment and activation
Eotaxin (CCL11)	Further eosinophil recruitment
Growth Factors/Cytokines	
Interleukin-1-α (IL-1α)	Endothelial activation, inflammation
Interleukin-2 (IL-2)	Activation of T lymphocytes
Interleukin-3 (IL-3)	Amplification of eosinophil accumulation/ activation and basophil activation
Interleukin-4 (IL-4)	B-cell maturation and mast cell differentiation
Interleukin-5 (IL-5)	Amplification of eosinophil accumulation/ activation
Interleukin-6 (IL-6)	Lymphocyte maturation
Interleukin-13 (IL-13)	Bronchial hyperreactivity, mucus production, B-cell maturation
GM-CSF	Leukocyte/eosinophil accumulation/activation
TGF-α	Fibrosis, growth inhibition
TGF-β	Fibrosis, growth inhibition
TNF-α	Endothelial activation, inflammation, cachexia
Oncostatin-M (OSM)	Fibrosis, angiogenesis, paracrine mobilization of eosinophils via upregulated SDF-1 production in fibroblasts
Lipid Membrane-Derived Substances	
PAF	Bronchoconstriction, edema formation
TXB2	Platelet aggregation
LTC4	Mucus secretion
15-HETE	Diverse effects on blood and tissue cells
PGE1 and PGE2	Diverse effects on platelets, endothelial cells, fibroblasts and other tissue cells
Antifibrinolytic Mediators	
PAI-2	Antifibrinolytic and prothrombotic
Extracellular DNA traps	Fibrin deposition, antibacterial effect

CCL, Chemokine (C-C motif) ligand; *GM-CSF*, granulocyte–macrophage colony-stimulating factor; *LTC4*, leukotriene C4; *MIP-1-alpha*, macrophage inflammatory protein-1-alpha; *PAF*, platelet-activating factor; *PAI-2*, plasminogen activator inhibitor-2; *RANTES*, regulated on activation, normal T-cell expressed and secreted; *SDF*, stromal cell-derived factor; *TGF*, transforming growth factor; *TNF*, tumor necrosis factor; *TXB2*, thromboxane B2.

Childhood cases of HES have been reported but are very rare. Unfortunately, all these data have serious limitations and emphasize the need for more comprehensive epidemiologic studies using currently available diagnostic criteria and robust registries. A complicating issue is that HES may coexist with an underlying primary eosinophil disorder, a lymphoma, but also an allergy or another reactive condition.[3] In eosinophil neoplasms presenting with the *FIP1L1::PDGFRA* fusion gene (*F/P*), a clear male predominance is found. As a consequence, any HES registry, including patients with *F/P*+ eosinophilic leukemia and/or other *F/P*+ myeloproliferative neoplasms (MPNs) with eosinophilia (MPN-eo), will report an overall male predominance of HES, even if other HES types are evenly distributed between males and females. Indeed, no clear gender predominance has been reported in other groups of HE or HES. Familial forms of HE and HES have been described but are very rare. In these cases, a hyper-immunoglobulin (Ig)E syndrome or Omenn syndrome (OS) have to be excluded. No cases of familial *F/P*+ leukemia have been reported to date.

MORPHOLOGY AND PHENOTYPE OF EOSINOPHILS

Eosinophils contain three populations of granules, namely (1) electron-dense, round, primary progranules present mainly at the promyelocyte and myelocyte stages, (2) specific (secondary) eosinophilic granules with an electron-dense crystalloid core, specifically found in mature eosinophils, and (3) small-sized granules, which may contain catalase, acid phosphatase, and arylsulfatase.[1] The large, specific granule is the major source of cytotoxic and pro-inflammatory cationic proteins. Eosinophils also contain lipid bodies that may serve as a source of eosinophil eicosanoide-forming enzymes, including 5-lipoxygenase, leukotriene C4 (LTC4) synthase, and cyclooxygenase (see Table 74.1).

The immunological phenotype of human eosinophils is well established and may assist in eosinophil detection and enumeration by flow cytometry. In common with all leukocytes, eosinophils express leukosialin (CD43), the homing cell adhesion molecule HCAM (CD44), and the pan-leukocyte tyrosine phosphatase C (CD45).[4,5] In addition, eosinophils express several myeloid differentiation antigens, including LFA-1 (CD11a/CD18) and Siglec-3 (CD33).[5] These cell-surface antigens are also expressed on other myeloid cells, including monocytes and basophils. However, a few cell-surface structures are largely restricted to eosinophils and thus serve as cell-specific markers through which eosinophils can be detected and isolated. One of these markers is Siglec-8, an inhibitory receptor that, when crosslinked, mediates eosinophil apoptosis.[6] Apart from eosinophils, basophils also display Siglec-8 (Fig. 74.1).

TABLE 74.2 Conditions Associated With Eosinophilia

Reactive Conditions
Helminth infections (filariasis, toxocariasis, schistosomiasis, trichinosis, onchocerciasis, fascioliasis, strongyloidiasis, ascariasis, hookworm, echinococcus/hydatid disease)
Fungal infections (aspergillosis, coccidioidomycosis, cryptococcosis)
Scabies and other infestations
Bacterial infections (convalescent phase of pneumococcal pneumonia, chlamydial pneumonia, scarlet fever, other bacterial infections)
Viral infections (HIV infection, chronic active hepatitis)
Allergic reactions (asthma, rhinitis, bronchitis, urticaria,
Atopic diseases (atopic asthma, atopic dermatitis)
Drug reactions (allergic, toxic)
Administration of IL-2, IL-3, or GM-CSF
Autoimmune diseases/connective tissue diseases/vasculitis (Sjögren syndrome, rheumatoid arthritis, lupus erythematodes, hypersensitivity vasculitis, bullous pemphigoid, others)
Acute and chronic graft-versus-host disease
Angioblastic lymphoid hyperplasia (Kimura disease)
Lymphocytic/lymphoid variant HES (HES$_L$)
Endocrinologic Conditions
Addison disease
Neoplastic Conditions Involving the Hematopoietic System
Myeloid neoplasms
Mast cell neoplasms
Lymphoid neoplasms
Paraneoplastic Conditions
Solid tumors/malignancy
Lymphoproliferative neoplasms (B- or T-cell lymphoma, Hodgkin disease)
Langerhans cell histiocytosis
Idiopathic Forms
Idiopathic eosinophilia[a]
HE of uncertain (undetermined) significance (HE$_{US}$)
Idiopathic hypereosinophilic syndrome (HES$_I$)
Hereditary HE with unknown etiology or a known genetic disorder

GM-CSF, Granulocyte–macrophage colony-stimulating factor; *HE*, hypereosinophilia; *HES*, hypereosinophilic syndrome; *HES$_I$*, idiopathic HES; *HES$_L$*, lymphoid variant HES; *HE$_{US}$*, hypereosinophilia of undetermined (unknown) clinical significance; *HIV*, human immunodeficiency virus; *IL-2*, interleukin-2; *IL-3*, interleukin-3.

[a] Idiopathic mild eosinophilia (500 to 1500 eosinophils/µL blood = less than HE) of unknown etiology.

TABLE 74.3 Classification of Hypereosinophilia and Hypereosinophilic Syndromes

HE Variant	Abbreviation	Associated HES Variant
Familial/hereditary HE	HE$_{FA}$	Familial HES = HES$_{FA}$
HE of undetermined/ Unknown significance	HE$_{US}$[a]	Idiopathic HES = HES$_I$[a]
Neoplastic/primary HE	HE$_N$	Neoplastic HES = HES$_N$
Reactive/secondary HE	HE$_R$	Reactive HES = HES$_R$
		Lymphocytic variant = HES$_L$[b]

HE, Hypereosinophilia; *HES*, hypereosinophilic syndrome; *HES$_{FA}$*, familial HES; *HES$_I$*, idiopathic HES; *HES$_L$*, lymphocytic variant HES; *HES$_N$*, primary (neoplastic) HES; *HES$_R$*, reactive HES; *HE$_{US}$*, hypereosinophilia of undetermined (unknown) clinical significance.

[a] In patients with HE$_{US}$, the presence of HES is excluded by definition; as soon as HES is diagnosed in a patient with HE$_{US}$, the diagnosis changes to HES$_I$, unless an underlying etiology (condition/disease) is also found.

[b] HES$_L$ is regarded a special variant of HES$_R$.

TABLE 74.4A Rare Syndromes Accompanied by Hypereosinophilia

Syndrome	Characteristic Features and Laboratory Abnormalities
Gleich syndrome	Cyclic recurrent angioedema, fever, weight gain, elevated polyclonal IgM, increased interleukin-5 production, evidence of clonal T cells (CD3$^-$ T cells)[a]
Churg-Strauss syndrome (CSS) = eosinophilic granulomatosis with polyangiitis (EGPA)	Stage I: Asthma and rhinosinusitis (isolated)
	Stage II: Eosinophilic phase (HES-like)
	Stage III: Vasculitic phase defined by necrotizing (small-vessel) vasculitis and HE with pulmonary infiltrates and/or neuropathy (ANCA+ and ANCA– subvariants have been described)
Eosinophilia myalgia syndrome (EMS)	Severe myalgia ± fever (flu-like), edema, fatigue, weight loss, neurologic symptoms, skin abnormalities
	Epidemic cases have been reported to result from exposure to L-tryptophan (toxic oil syndrome).
Eosinophilic fasciitis (EF) = Shulman syndrome	Scleroderma-like condition with painful swelling of the skin and chronic induration (limbs and trunk) as well as laboratory signs of systemic inflammation
Omenn syndrome (OS)	Severe combined immunodeficiency (SCID) with well-populated lymphatic organs (OS paradox)
	Autosomal recessive genetic (pediatric) disease (recurrent mutations in *RAG1* or *RAG2*)
	Clinical findings: erythroderma, hepatosplenomegaly, lymphadenopathy, increased IgE, diarrhea, weight loss (GVHD-like condition with autoreactive T cells)
Hyper-IgE syndrome (HIES)	Hereditary immunodeficiency syndrome with elevated IgE, recurrent severe infections, often with skin eczema and facial anomalies
	Examples of mutations associated with HIES: Autosomal dominant variant: *STAT3* mutations Autosomal recessive variant: *DOCK8* mutations *PGM3* mutations

ANCA, Anti-neutrophil cytoplasmic antibodies; *DOCK8*, dedicator of cytokinesis 8; *GVHD*, graft-versus-host disease; *HE*, hypereosinophilia; *HES*, hypereosinophilic syndrome; *IgE*, immunoglobulin E; *PGM3*, phosphoglucomutase 3; *RAG*, recombination-activating gene; *STAT3*, signal transducer and activator of transcription-3.

[a] EGPA/CSS with clonal T cells is often regarded as special variant of lymphoid variant HES (HES$_L$).

Eosinophils express several cytokine receptors, such as the IL-5 receptor, granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor, and IL-3 receptor. Moreover, eosinophils exhibit various chemokine receptors, including eotaxin receptors and CXC-chemokine receptor 4 (CXCR4), a receptor for stromal cell-derived factor (SDF)-1 (Table 74.5). Finally, eosinophils express diverse complement receptors (CRs), such as CR3 (CD11b/CD18) and C5aR (CD88), adhesion receptors, and Ig (Fc) receptors.[5] Several of these cell-surface markers have been considered as potential targets of therapy. However, aberrantly expressed or disease-specific markers for neoplastic eosinophils or reactive eosinophils have not been identified yet. Several studies have shown that eosinophils in HES patients may express increased amounts of CD11b, CD16, CD25, or/and HLA-DR compared to normal blood

TABLE 74.4B Organ-Restricted (Inflammatory) Conditions Accompanied by Eosinophilia

- Eosinophilic gastrointestinal disorders (EGIDs)
    - Eosinophilic esophagitis
    - Eosinophilic gastritis
    - Eosinophilic gastroenteritis
    - Eosinophilic colitis
- Eosinophilic pancreatitis
- Eosinophilic hepatitis
- Eosinophilic ascites
- Pulmonary eosinophilic syndromes
    - Eosinophilic asthma
    - Eosinophilic bronchitis
    - Eosinophilic pneumonia
    - Eosinophil pleuritis
- Eosinophilic nephritis
- Eosinophilic cystitis
- Eosinophilic endometritis and myometritis
- Eosinophilic mastitis
- Eosinophilic ocular disorders
- Eosinophilic myocarditis
- Eosinophilic synovitis
- Skin diseases/conditions associated with eosinophilia[a]
    - Allergic contact dermatitis
    - Atopic dermatitis
    - Drug reactions
    - Bullous pemphigoid and pemphigoid variants
    - Eosinophilic cellulitis (Wells syndrome)
    - Radiotherapy-related eruptions associated with eosinophilia
    - Eosinophilic pustular folliculitis
    - Infestations (parasitic) and infections (bacterial, viral, fungal) involving the skin
    - Mycosis fungoides
    - Pachydermatous eosinophilic dermatitis
- Kimura disease (skin and lymph nodes)
- Eosinophilic panniculitis

[a] Many other skin abnormalities can be accompanied by eosinophilia, especially when a systemic inflammatory disease process is present and triggers eosinophilia.

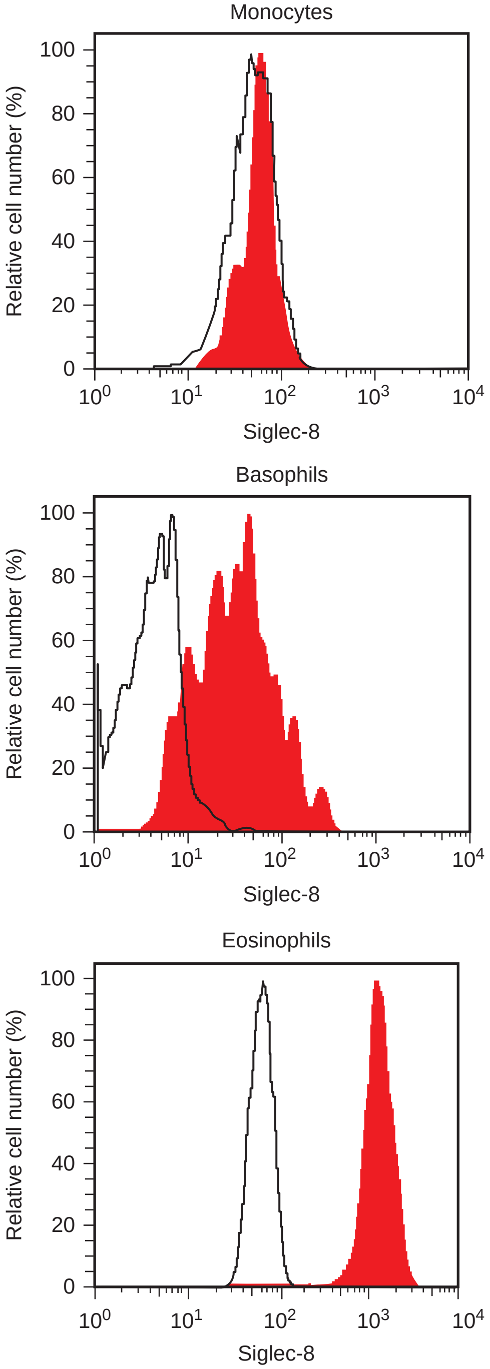


Figure 74.1 EXPRESSION OF SIGLEC-8 ON PERIPHERAL BLOOD EOSINOPHILS. Peripheral blood cells were obtained from a patient suffering from a myeloproliferative neoplasm with eosinophilia (MNP-eo). Cells were stained with an antibody against Siglec-8, as well as antibodies against CD203c (for basophil detection) and CD14 (for monocyte detection), and analyzed by multicolor flow cytometry. Siglec-8 is expressed on eosinophils (detected by their characteristic side-scatter properties and autofluorescence) as well as on blood basophils, whereas monocytes (like most other leukocytes) lack Siglec-8.

eosinophils. However, overexpression of these antigens on eosinophils is not disease-specific.

Apart from cell surface antigens, eosinophils also express more or less specific marker antigens in their cytoplasm by which these cells can be detected in various tissues by immunohistochemistry (IHC). Useful eosinophil IHC stains include ECP, EPX, and MBP-1 (Fig. 74.2A). Depending on the condition and underlying neoplasm, these antibodies may also react with (immature) basophils. Otherwise, however, the staining reaction appears to be largely specific for eosinophil granulocytes. Another important aspect is that some of the eosinophil proteins, like MBP, can be detected in tissue sections as deposit material after complete degranulation of eosinophils and even after their subsequent destruction (disappearance), which is often seen in the context of chronic HE-related tissue damage (HES).[7] Therefore, the definition of tissue HE includes not only the detection of a local eosinophil (cellular) infiltrate but also the presence of eosinophil protein deposits, even in the absence of infiltrating eosinophils.

Eosinophils from the PB and tissues of patients with HE may differ from their normal counterparts by morphologic, biochemical, and functional characteristics. The most important morphologic distinction is the one between immature and mature eosinophils. In most

TABLE 74.5 Receptors and Ligands Regulating Growth and Function of Eosinophils

Receptor (R) on Eosinophils	Ligand	Effects on Eosinophils
IL-2RA/CD25[a]	IL-2	Activation?[a] Migration?[a]
IL-3R/ CD123 + CD131	IL-3	Differentiation, survival, adhesion, migration, activation, priming
IL-4R/CD124	IL-4	Priming for effects of chemotaxins
IL-5R/ CD125 + CD131	IL-5	Differentiation, survival, adhesion, migration, activation, priming
GM-CSFR/ CD116 + CD131	GM-CSF	Differentiation, survival, adhesion, migration, activation, priming
IL-10R	IL-10	Inhibitory (activation, survival)
IL-12R	IL-12	Inhibitory (activation)
IL-13R	IL-13	Unknown
CD4	IL-16 (LCF)	Activation, priming
IL-25R	IL-25	Survival, activation
IL-27R	IL-27	Survival, activation
IL-33R/ST2	IL-33	Activation, survival
VEGFR-1/FLT-1	VEGF	Chemotaxis, activation
Tie-2/TEK	Angiopoietin-1	Chemotaxis, activation?
PDGFRA/B	PDGF	Activation and growth?
FGFR	FGF	Activation?
TGFβ1R	TGFβ1	Inhibitory (differentiation)
TGFβ2R	TGFβ2	Inhibitory (differentiation)
IFN-α-R	IFN-α	Inhibitory (growth)
IFN-γ-R	IFN-γ	Inhibitory (growth, migration)
CCR3 (CD193)	RANTES (CCL5)	Chemotaxis, activation
	MCP-3 (CCL7)	Chemotaxis, activation
	MCP-4 (CCL13)	Chemotaxis, activation
	Eotaxin-1 (CCL11)	Chemotaxis, activation
	Eotaxin-2 (CCL24)	Chemotaxis, activation
	Eotaxin-3 (CCL26)	Chemotaxis, activation
CXCR4 (CD184)	SDF-1 (CXCL12)	Chemotaxis
PAF-R	PAF	Chemotaxis, activation
C5aR (CD88)	C5a	Chemotaxis, activation
TLR1, 4, 7, 9, 10	Toll-like R-ligands	Survival, activation
Corticosteroid R	Corticosteroids	Inhibitory (activation)

CCL, Chemokine ligand; *CCR*, chemokine receptor; *CXCR4*, CXC-chemokine receptor 4; *FGF*, fibroblast growth factor; *GM-CSF*, granulocyte–macrophage colony-stimulating factor; *IFN*, interferon; *IL*, interleukin; *LCF*, lymphocyte chemoattractant factor; *MCP*, monocyte chemotactic protein; *PAF*, platelet-activating factor; *PDGF*, platelet-derived growth factor; *SDF*, stromal cell–derived factor; *TGF*, transforming growth factor; *VEGF*, vascular endothelial growth factor.

[a] Eosinophils derived from patients with hypereosinophilic syndromes (activated eosinophils) may express CD25. However, the role of CD25 in eosinophil function remains unknown.

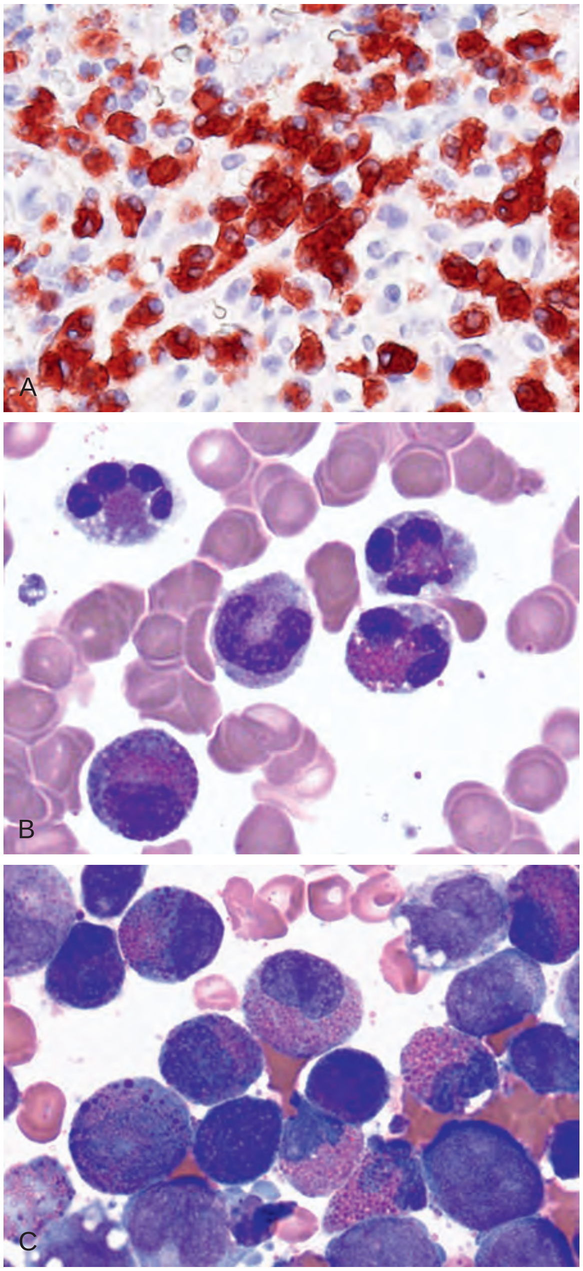


Figure 74.2 IMMUNOHISTOCHEMICAL AND MORPHOLOGIC PROPERTIES OF NEOPLASTIC EOSINOPHILS. (A) Expression of major basic protein (MBP) in neoplastic eosinophils in a patient suffering from a myeloid neoplasm with eosinophilia. Paraffin-embedded bone marrow sections were stained with an antibody against MBP by indirect immunohistochemistry. (B) Wright-Giemsa-stained bone marrow smear in a patient with chronic eosinophilic leukemia. Note the presence of atypical eosinophils, some of which contain multi-lobed nuclei. (C) Wright-Giemsa-stained bone marrow smear in a patient with an acute eosinophilic leukemia. The smear contains a mixture of immature and mature eosinophils. Some of the immature eosinophils exhibit a blast-like morphology. Other immature forms contain "basophilic" dark-blue granules, but these cells still belong to the eosinophil lineage.

instances, this distinction is a straightforward approach, as immature forms exhibit distinct nuclear and cytoplasmic morphologic features (Fig. 74.2B and C). However, sometimes the distinction may be challenging, especially in reactive states with massive inflammation and in certain myeloid neoplasms. In immature myeloid neoplasms, eosinophil precursor cells may exhibit dark basophilic granules that must not be confused with the specific granules of blood basophils. These immature eosinophils may also be misinterpreted as mixed-lineage (eosinophil/basophil) cells.

Immature eosinophils also differ from mature eosinophils in several other aspects, including density, nuclear shape, protein composition, and cell surface antigens. A difficult task is to differentiate normal eosinophils from activated (sometimes immature) eosinophils in reactive states. In previous years, researchers focused on cell density. In particular, a distinction was made between the "normodense" (normal-density) and the "hypodense" (light-density) eosinophil. The blood of normal individuals contains less than 10% eosinophils with densities less than 1.082 g/mL, whereas patients with eosinophilia can have markedly increased numbers of hypodense cells. Hypodense eosinophils often appear to be cytokine-activated cells that are characterized by increased vacuolization, decreased granule size, decreased content of granule cationic proteins, and increased numbers of cytoplasmic lipid bodies. The numbers of surface receptors for a variety of eosinophil agonists, including complement components, Igs, and platelet-activating factor (PAF), are expressed at higher levels by hypodense eosinophils. Functionally, hypodense eosinophils show increased metabolic activity and oxygen consumption, an increased capacity for the synthesis and secretion of LTC4 and certain cytokines, enhanced chemotaxis, and augmented cytotoxicity. So far, no clear relationship between the percentage of hypodense eosinophils and the etiology of eosinophilic disorders has been established. The percentage of these cells increases in most reactive forms of HE and HES, but also in patients with primary HES. Ultrastructurally, HES eosinophils may show a selective loss of secondary granule components (crystalloid MBP-containing core or granular matrix, or both), a decreased number and size of granules, and increased numbers of cytoplasmic lipid bodies and tubulovesicular structures that may be involved in eosinophil secretion during the process of piecemeal degranulation.

ORIGIN, DIFFERENTIATION, RECRUITMENT, AND ACTIVATION OF EOSINOPHILS

Eosinophilopoiesis

Eosinophils originate from pluripotent and granulocyte-committed, $CD34^+$, hematopoietic progenitor cells that are detectable in the BM and in the PB, and under certain conditions (local inflammation) also in various extramedullary organ sites. Eosinophil development from their multipotent and lineage-restricted progenitors is controlled by a network of transcription factors, including GATA-1, GATA-2, C/EBP-A, and C/EBP-E (see Chapter 9). Lineage-specific signaling as well as transcription factor expression is controlled by a network of cytokines and cytokine receptors. In fact, eosinophils develop from their progenitors in response to T-cell–derived growth factors, including IL-3, GM-CSF, and IL-5 (see Chapter 11). The most potent and specific eosinophilopoietic growth factor is IL-5.[1,8,9] Eosinophils are derived from multipotent or bipotent colony-forming progenitor cell units (CFUs). Bipotent CFUs giving rise to eosinophils and basophils (CFU-eo/ba) are frequently detected in the BM and PB in healthy individuals, as well as in various disease states.[8] By contrast, progenitor cells selectively giving rise to eosinophils and mast cells (CFU-eo/mast) are very rare. At an early stage of progenitor cell development, eosinophil-committed progenitors exhibit CD34 and CD38, and coexpress receptors for IL-3, GM-CSF, and IL-5. Later, CD34 expression is diminished, whereas the interleukin receptors continue to be expressed, and several additional, functionally important, cell-surface antigens are acquired by maturing eosinophils.

IL-3, GM-CSF, and IL-5 promote eosinophil differentiation of hematopoietic progenitor cells and eosinophil maturation of lineage-restricted precursors, as well as the recruitment, priming, activation, and survival of mature eosinophils.[9] Whereas IL-3 and GM-CSF also affect other hematopoietic lineages, including basophils, IL-5 acts as a more lineage-restricted (eosinophil-targeting) cytokine. Therefore, IL-5 and IL-5 receptors have been recognized as major targets of therapy in diseases associated with (reactive) HE. The high-affinity receptor for IL-5 is composed of an IL-5–specific alpha chain (CD125) and a beta chain (CD131) that mediates intracellular signaling and is shared also by the high-affinity receptors for GM-CSF (CD116/CD131) and IL-3 (CD123/CD131; see Table 74.5) (see Chapter 11). Expression of high-affinity IL-5 receptors is an important prerequisite for specific cellular responses to "physiologic doses" of IL-5. Overexpression of IL-5 is observed in many reactive conditions and disease states associated with (reactive) HE. Correspondingly, IL-5 transgenic mice develop profound eosinophilia, and IL-5 knockout mice do not develop HE in response to allergic stimuli or a helminth infection. Under various reactive conditions accompanied by HE, IL-5 is produced primarily by activated Th2-type helper cells. However, although activated T-cells are considered a primary and most important source of eosinophil-triggering cytokines, like IL-5, other cell types, such as mast cells, macrophages, natural killer (NK) cells, and stromal cells, also produce these cytokines. In myeloid neoplasms with HE, clonal expansion of eosinophils usually is a growth factor-independent process. Moreover, depending on the underlying disease, neoplastic eosinophils may produce eosinophilopoietic cytokines in an autocrine manner, thereby augmenting tissue and blood HE.

Mobilization and Migration of Eosinophils to Sites of Inflammation

In healthy individuals as well as in reactive states, eosinophils circulate only briefly in the PB, and then transit to extravascular sites.[9] Depending on environmental factors and demand, eosinophils localize preferentially in tissues and organs exposed to external environments, principally the submucosal membranes and loose connective tissues of the skin, GI tract, genital tract, and lungs. Depending on the underlying pathology, eosinophils can migrate into any vascularized organ. During an acute or chronic inflammatory process, larger numbers of eosinophils may be recruited actively into local tissue sites.[9] The mobilization of eosinophils from the vasculature into (vascularized) tissues is a multistep process that involves their rolling on vascular endothelial cells and their subsequent adhesion to endothelium via L-selectin, followed by binding to intercellular adhesion molecule-1 (ICAM-1 = CD54) through a CD18/CD11a/b-dependent mechanism (Chapter 14 and 16).[4] Expression of selectins on endothelial cells is triggered by histamine and other mediators, whereas expression of ICAM-1 on endothelial cells is induced by various pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α). As both histamine and TNF-α are produced and provided by (activated) mast cells, these cells are thought to play a crucial role in eosinophil recruitment in various allergic and other inflammatory reactions. However, the recruitment of eosinophils is a complex process involving many different mechanisms and molecules, such as E-selectin, endothelial-leukocyte adhesion molecule 1 (ELAM-1 = CD62E) or vascular cell-adhesion molecule-1 (VCAM-1 = CD106).[4] Recruitment of eosinophils is also mediated by adhesion to VCAM-1 via the β1 integrin very late activation antigen-4 (VLA-4 = CD49d/CD29), which is expressed by eosinophils but not by blood neutrophils.

Eosinophil migration is regulated by several different chemotactic factors, including various chemokines (SDF-1, eotaxins, IL-8, and others), the complement fragment C5a, PAF, and several eosinotropic cytokines, such as IL-5 or GM-CSF, which also prime eosinophils for enhanced migratory responses to PAF and IL-8. In addition, IL-2, leukotriene B_4, and the lymphocyte chemoattractant factor (LCF = IL-16) can induce eosinophil migration. At least for neoplastic eosinophils, a most potent chemoattractant appears to be SDF-1, a stromal cell-derived chemokine that binds to CXCR4 (CD184) on normal and neoplastic eosinophils.[10] Other potent chemoattractants for (neoplastic) eosinophils are the eotaxins (eotaxin-1, -2, -3) that bind to and signal through the chemokine receptor CCR3

(CD193; see Table 74.5).[9] Depending on the underlying disease, the cell type(s) involved, and the presence of comorbidities (triggering, e.g., local or systemic inflammation) all these chemotactic factors may act together to trigger eosinophil recruitment and accumulation in the affected (often inflamed) tissue sites. Likewise, in several reactive conditions, IL-5 and eotaxin may cooperate locally to promote eosinophil migration and tissue HE.

The primary source of eosinophil chemotactic factors often remains unknown. In many disease states accompanied by HE, eosinophil chemotactic factors may be produced by microenvironmental cells, including fibroblasts, endothelial cells, T-cells, macrophages, and mast cells. Several of these chemokines, like SDF-1, are produced by stromal cells in a paracrine fashion (see Chapter 12). For example, in neoplastic eosinophils, the *F/P* mutant triggers expression and release of oncostatin M (OSM), which in turn induces fibroblast and endothelial cell proliferation but also the expression and release of SDF-1 from these cells, thereby augmenting the accumulation of additional eosinophils.[11] In this regard, it is noteworthy that the SDF-1 receptor CXCR4 is not only expressed on mature eosinophils but also on hematopoietic stem cells and eosinophil-committed precursor cells. Depending on the type of underlying neoplasm, eosinophil chemotactic cytokines may also be produced in an autocrine manner.

Role of Mast Cells in Eosinophil Recruitment and Accumulation

In various inflammatory and allergic reactions, several different interactions between eosinophils and mast cells have been postulated (see Chapter 75). For example, mast cells provide the immediate stimuli upon activation to initiate allergic inflammation, resulting in eosinophil recruitment during the late phase following an allergen encounter. In addition, mast cells and eosinophils are often increased in numbers in allergic reactions, but also in nonallergic inflammatory conditions and even in neoplastic states. Both cell types express a similar profile of chemotactic receptors, such as CCR3, and both respond to eotaxins and RANTES (CCL5), leading to their recruitment into local tissue sites (see Chapter 12). Mast cells and eosinophils also interact with each other via cell–cell contact and through several different soluble mediators. Likewise, mast cell-derived heparin binds and stabilizes various chemokines, including the eotaxins. In addition, mast cells are a source of various cytokines involved in the regulation of eosinophil adhesion, migration, and function. As previously mentioned, mast cells express and release histamine and TNF-α, and thereby facilitate eosinophil recruitment. Mast cell proteases appear to have a dual action on eosinophil functions. Mast cell-derived chymase suppresses eosinophil apoptosis and induces the release of IL-6 and various chemokines (CXCL1, CXCL8, CCL2) from eosinophils. Beta-tryptase, on the other hand, can cleave eotaxin and RANTES, and thereby may limit eosinophil chemotaxis. Apart from these interactions, eosinophils and mast cells may sometimes also be derived from the same stem or progenitor cell compartment, and therefore may express the same driver mutant, which has been documented for cases of *F/P*+ chronic eosinophilic leukemia (CEL) with accompanying mast cell expansion, as well as for patients with *KIT* D816V+ systemic mastocytosis (SM) with concomitant HE (see Chapter 75). Therefore, mast cells and eosinophils may often appear as a dual-target cell population, both in reactive and neoplastic states, which is a critical point when considering the development of specific therapies. For example, simultaneous downregulation of eosinophil and mast cell activation might be achieved by certain inhibitory surface molecules, such as CD300a or Siglec-8.[12] Several other target receptors, such as Siglec-3 (CD33), are expressed on eosinophils, mast cells, and basophils. In patients with *F/P*+ neoplasms, imatinib can induce apoptosis in both eosinophils and mast cells, as both cell types express the mutant platelet-derived growth factor receptor (PDGFR) target in this neoplasm.

MONITORING OF EOSINOPHIL NUMBERS AND ACTIVITY IN HEALTH AND DISEASE

In the PB, eosinophils can easily be measured by microscopy or flow cytometry based on their characteristic side-scatter properties and their autofluorescence. In case of atypical (immature) cells or questionable results, immunophenotyping can be performed to confirm the presence of eosinophils. In tissue sections, the quantification of eosinophils is a more difficult task that makes routine clinical evaluations somewhat impractical. Alternative approaches such as analysis of tissue secretions from affected organs (e.g., bronchoalveolar lavage [BAL]) have been used with success in evaluating local eosinophil numbers and function in asthma. In addition to routine histochemical identification and enumeration of eosinophils and the immunochemical localization of secreted eosinophil granule cationic proteins in tissue biopsies, two other methods have been employed to demonstrate and monitor eosinophil activation and involvement: (1) the identification of activated eosinophils by staining with anti-ECP antibody EG2, recognizing a secreted (deglycosylated) form of the protein, and (2) measurement of eosinophil granule proteins, such as MBP or ECP by radioimmunoassay (RIA) or enzyme-linked immunosorbent assay in various body fluids, including serum, plasma, urine, sputum, nasal lavage, and BAL. Indeed, these antigens may serve as reliable biomarkers of eosinophil involvement and activation in allergic, parasitic, and inflammatory diseases. In addition, treatment responses can be demonstrated and measured with these assays.

ETIOLOGY AND PATHOBIOLOGY OF HYPEREOSINOPHILIA

In patients with documented HE, four etiologies have to be considered: (1) myeloid (and rarely myeloid/lymphoid) neoplasms where eosinophils are usually derived from neoplastic stem and progenitor cells, (2) lymphoid neoplasms or nonhematopoietic (solid) tumors where HE is a paraneoplastic phenomenon, (3) reactive conditions, such as an allergy, infection or an autoimmune disease where HE is a reactive process, and (4) rare syndromes accompanied by HE, including rare and inherited disorders (see Table 74.4A).[3] In patients with clonal (primary) HE, eosinophils and their progenitors often display rearrangements in *PDGFRA*, *PDGFRB*, or *FGFR1* genes.[13] Therefore, the World Health Organization (WHO) has employed these mutations as primary criteria to describe (classify) hematopoietic neoplasms accompanied by HE (Table 74.6).[14] The resulting "fusion proteins" act as prooncogenic drivers and are considered to contribute to cytokine-independent differentiation of eosinophil progenitor cells. The most frequently detected molecular abnormality is the *F/P* fusion gene, a mutant gene created by an 800-kb interstitial deletion on chromosome 4q12. The fusion gene is detectable by polymerase chain reaction (PCR) as well as by fluorescence in situ hybridization (FISH; deleted *CHIC2* gene) but cannot be detected by conventional karyotyping. However, conventional cytogenetics may reveal translocations, deletions, or inversions on chromosome 5q31-33, indicating rearrangements of *PDGFRB*, or abnormalities on chromosome band 8p11-12, indicating a rearrangement of the *FGFR1* gene. From a clinical point of view, it is of great importance to screen for these rearrangements in all patients using the correct techniques, as many of them, including *F/P*, are responsive to imatinib or other tyrosine kinase inhibitors (TKIs). However, clonal HE may also be caused by other mutations, including *BCR::ABL1*, rearranged *JAK2* (e.g., TK fusion partners *PCM1*, *BCR*, and *ETV6*) or *FLT3* (e.g., most common partner *ETV6*), or *KIT* D816V.[14] In a substantial subset of patients with MPN-eo, no mutation is found.

In paraneoplastic and reactive states, HE is usually triggered by eosinophilopoietic cytokines, including IL-5, IL-3, or GM-CSF. These cytokines, especially IL-5, may be detected in the serum of these

TABLE 74.6 **Molecular Classification of Eosinophil-Related Neoplasms Proposed by the WHO and Related Molecular and Cytogenetic Defects**[a]

1. Myeloid/lymphoid neoplasms with eosinophilia and tyrosine kinase gene fusions (MLN-TK):

   MLN-TK with *PDGFRA* rearrangement

   Most common variant: *FIP1L1::PDGFRA*$^{+}$ MLN-TK

   MLN-TK with *PDGFRB* rearrangement

   MLN-TK with *FGFR1* rearrangement

   MLN-TK with rearranged *JAK2*

   FLT3-rearranged MLN-TK

   ETV6::ABL1-rearranged MLN-TK

   MLN-TK with other fusion genes, such as *ETV6*::*FGFR2*, *ETV6*::*LYN*, or *ETV6*::*NTRK3*.
2. Chronic eosinophilic leukemia (CEL):

   BCR::*ABL1* and abnormalities in *PDGFRA*, *PDGFRB*, or *FGFR1* are excluded

   Other MPN-related molecular lesionsand MLN-TK-defining fusion genes are also excluded (CEL is now regarded as a separate form of MPN by the WHO)
3. WHO-defined myeloid neoplasms with HE, not included in "1" or "2":
   - a. Ph^{+} (*BCR::ABL1*$^{+}$) chronic myeloid leukemia (CML-eo)
   - b. *JAK2* V617F^{+} myeloproliferative neoplasms (MPN-eo)
   - c. *KIT* D816V^{+} systemic mastocytosis (SM-eo)
   - d. *CBFβ*-fusion gene-related acute myeloid leukemia (AML-eo/M4-eo)
   - e. Myelodysplastic syndromes with HE (MDS-eo)
   - f. Other WHO-defined myeloid neoplasms with HE

aCML, Atypical chronic myeloid leukemia; *CEL*, chronic eosinophilic leukemia; *CMML*, chronic myelomonocytic leukemia; *FGFR*, fibroblast growth factor receptor; *HE*, hypereosinophilia; *MDS*, myelodysplastic syndrome; *MLN-TK*, myeloid/lymphoid neoplasms with eosinophilia and tyrosine kinase gene fusions; *MPN*, myeloproliferative neoplasm; *PDGFR*, platelet-derived growth factor receptor; *Ph+*, Philadelphia chromosome positive; *SM*, systemic mastocytosis; *WHO*, World Health Organisation.

[a]The table refers to the WHO classification of eosinophil neoplasm.[14]

patients and are considered to be produced by activated T cells or other activated immune cells. In a few patients with HE and HES, activated (clonal) T cells are detectable by phenotyping and PCR, but no underlying lymphoma or other underlying condition is found. In these cases, the lymphoid variant of HES (HES_L) is diagnosed.[15] In some of these patients, an overt T-cell lymphoma or Sézary syndrome may develop in the follow-up. A number of different reactive conditions and disorders, including helminth infections, fungal infections, autoimmune disorders, allergies, or drug reactions, can lead to reactive HE and reactive HES (HES_R). These conditions are listed in Table 74.2.

Pathogenesis of Eosinophil-Associated End-Organ Damage in Patients With Hypereosinophilic Syndrome

Regardless of the underlying etiology (neoplastic or reactive), sustained HE may lead to typical end-organ damage defined as HES. Even in patients with idiopathic HE, such organ damage may occur and is then called *idiopathic HES* (HES_I).[3] However, not all patients presenting with HE develop organ damage even if followed over a longer time period. For example, patients with an underlying allergic disease usually fail to develop the organ damage seen in patients with typical HES. When neither an underlying disease nor HE-related organ damage is found, the diagnosis HE of undetermined significance (HE_US) is established.[3] In general, the risk of HES is lower in reactive forms of HE, especially when the underlying disease can be treated successfully. Correspondingly, IL-5 transgenic mice with massive HE do not develop significant end-organ damage, suggesting that other factors (in addition to IL-5) are likely necessary for the occurrence of an HE-related organ damage. In patients with an underlying MPN or other underlying myeloid neoplasm, the risk of HE-related organ damage (HES) is relatively high. A particularly high risk is found in patients with mutations in *PDGFR* genes. Likewise, most untreated patients with *F/P*+ MPN-eo or CEL may develop HES with cardiac involvement over time. Additional risk factors may also contribute to the development of HES in these patients, including, among others, an additional prothrombophilic state, late detection of the disease, and late initiation of specific (anti-CEL) therapy. There may also be a certain genetic predisposition for early occurrence of tissue HE in patients with *F/P*+ CEL (*F/P*+ MPN-eo). In particular, the severity (extent) of tissue HE seems to correlate with a polymorphic variant (SNP in the 5′-UTR) in the *IL-5RA* gene.[16] However, it remains unclear whether this SNP predisposes to HES development or progression, and thus is relevant clinically. Today, the general recommendation is to initiate therapy with imatinib early in all *F/P*+ patients, regardless of genetic, molecular or other factors, in order to prevent any HES occurrence and to avoid disease progression (to acute leukemia).

A number of different organ systems are affected in patients with HES.[3] The most common manifestations are summarized in Table 74.7. Although multiple organ systems are involved and the manifestation patterns are quite complex with varying courses and outcomes, some common pathogenetic factors have been described. One common feature is the mobilization of the tissue microenvironment by eosinophil-derived mediators and cytokines, which leads to tissue remodeling, fibrosis, and increased angiogenesis.[3,7,17] Another common factor is tissue inflammation, which is typically triggered by eosinophil-derived cytokines and chemokines.[3,17] Finally, HE-related organopathies may often result from direct cytotoxic damage that is induced by the various toxic mediators produced and released by (reactive and neoplastic) eosinophils.[3,17] With regard to thromboembolic events, several different eosinophil-derived mediators have been implicated, including TNF-α, plasminogen activator inhibitors (PAIs), and eosinophil-derived DNA traps (see Table 74.1).[2,17] In addition, ECPs have the capacity to alter thrombomodulin activity. A number of different compounds produced by eosinophils, including cationic proteins and various cytokines, may induce fibrosis. Among these cytokines, OSM may play a particular role as a HES-related profibrogenic cytokine.[11] HE-related neoangiogenesis may be triggered by IL-8, OSM, and vascular endothelial growth factor (VEGF). All these cytokines are produced and secreted by normal/reactive and also by neoplastic eosinophils. However, neoplastic eosinophils produce some of these cytokines in excess over their normal counterparts. For example, *F/P*+ eosinophils express and release huge amounts of OSM, IL-8, and VEGF.[11] The F/P mutant induces the production and secretion of these mediators in neoplastic cells in a STAT5-dependent manner. Concerning the mobilization of the microenvironment in various organs in patients with HES, a most critical *F/P* target gene may be *OSM*. Notably, OSM initiates not only fibrosis and angiogenesis by triggering fibroblast proliferation and tube formation in endothelial cells, but also by inducing the production of multiple cytokines and chemokines in stromal cells. Among these mediators are IL-8, SDF-1, and other chemotactic factors that can recruit additional CXCR4+ precursor cells and eosinophils to local tissue sites.[11]

An unresolved question is why HE-related fibrosis and thrombosis affect certain tissues and organs, including the heart and lungs. One explanation may be that eosinophils invade heart and lung tissues via "physiologic" routes, which may not be the case in other MPNs where HE usually does not occur. An additional explanation may be that eosinophil-derived mediators are toxic to certain (tissue-specific) cell types such as cardiomyocytes. Indeed, as noted earlier, eosinophils express a number of different cationic proteins capable of inducing endothelial and endocardial damage and neurotoxicity. In addition, the eosinophil has the capacity to generate reactive oxidative species that can augment tissue damage.

TABLE 74.7 **End-Organ Damage Typically Observed in Patients With Hypereosinophilic Syndrome**

Cardiac
Intramural thrombi
Valve regurgitation
Constrictive pericarditis
Endomyocardial fibrosis
Myocarditis
Cardiomyopathy
Neurologic
Fatigue
Thromboembolism and stroke
Peripheral neuropathy
Central nervous system dysfunction and dementia
Paresthesias and/or sensory deficits
Epilepsy
Dermatologic
Angioedema
Urticaria
Papular and/or nodular lesions
Mucosal ulcers
Vesicobullous lesions
Microthrombi
Pulmonary
Pulmonary infiltrates
Pulmonary thromboembolism
Pneumonitis
Fibrosis
Pleural effusion
Ocular
Microthrombi
Vasculitis
Retinal arteritis
Connective Tissue
Arthralgias
Effusions
Polyarthritis
Raynaud phenomenon
Digital necrosis
Gastrointestinal
Ascites
Diarrhea
Gastritis
Colitis
Cholangitis

DEFINITION AND CLASSIFICATION OF HYPEREOSINOPHILIA AND HYPEREOSINOPHILIC SYNDROME

The term HES was initially proposed for a syndrome characterized by idiopathic HE and HE-related organ damage, as well as by exclusion of various conditions and diseases that are typically accompanied by HE. More recently, the definition of HES has been refined as any type of HE (not just idiopathic) associated with typical HE-related end-organ damage.[3] According to the proposal of an international working group on eosinophil disorders (ICOG-EO), the term HES should be reserved for clinical syndromes fulfilling HES criteria, but not for underlying (hematologic) malignancies or immunological disorders presenting with HE.[3] In other words, the diagnosis HES must prompt the physician to search for (detect) an underlying (HES-triggering) disease. Accordingly, the final diagnosis is either idiopathic HES or HES based on a recognized underlying disorder, and both need to be documented in the final diagnosis. In previous WHO classifications, the term HES was sometimes used as a synonym of CEL, and sometimes to discriminate CEL from other less well-defined myeloid neoplasms with HE. However, in the 2008 edition of the WHO monograph, the term HES was no longer recommended as a synonym of WHO-defined neoplasms, a distinction that is in agreement with the proposal of the ICON group and the proposal of the ICOG-EO group.[3]

Definition of Hypereosinophilia

HE is currently defined as a persistent, microscopically confirmed increase in PB eosinophils to $\geq 1.5 \times 10^9$/L. In previous definitions, persistent eosinophilia over 6 months was required to establish the diagnosis HE. However, based on improved diagnostics and treatment, and the necessity to introduce such treatment rapidly to avoid organ damage, the proposed definition of HE has been revised to a 4-week observation interval by the ICOG-EO group.[3] The term "tissue HE" has also been proposed by several experts, and it may be useful to apply it in certain conditions, especially in the context of (to demonstrate) HE-related organ damage (HES). However, isolated tissue HE (without blood HE) is rare, and the documentation of tissue HE often requires special immunostains directed against eosinophil granule proteins.[7] Therefore, the presence of tissue HE is often overlooked or is not documented, and PB HE clearly remains the key diagnostic marker in daily practice. In some of these patients, criteria for blood HE are not met, but molecular and clinical signs are strongly indicative of a particular eosinophil disorder with or without accompanying HES. These patients should be followed closely, as they may progress to an overt eosinophil disease (neoplasm) over time. The same holds true for WHO-based neoplasms presenting with *PDGFR* or *FGFR1* fusion genes without overt HE.[14] In fact, many patients with *FGFR1*-rearranged neoplasms do not have eosinophilia at presentation.

Diagnostic Algorithm

When arriving at the provisional pre-diagnostic "checkpoint" HE, two critical questions have to be addressed in order to approach to a final diagnosis: (1) is there any underlying disease or condition triggering HE? *and* (2) are there clinical signs and symptoms or laboratory abnormalities that point to the presence of HE-induced organ damage, so that the diagnosis of HES can be established? For example, the hematologic work-up in a patient with HE revealed CEL, and staging investigations showed endocardial thrombus formation: the final diagnosis in this patient is CEL with concomitant primary HES (HES_N). A diagnostic algorithm is shown in Fig. 74.3. In patients with typical HES and typical clinical manifestation, histopathologic evaluation is generally not warranted to confirm the presence of tissue HE. However, in patients with rare or atypical manifestations, such as renal failure, isolated myocarditis or bloody diarrhea, a tissue biopsy is required to document the presence of tissue HE and to confirm HE-induced organ damage, so that the diagnosis HES can be established. The demonstration of extensive extracellular deposition of eosinophil-derived proteins supports the conclusion the organ damage is "HE-related."[7]

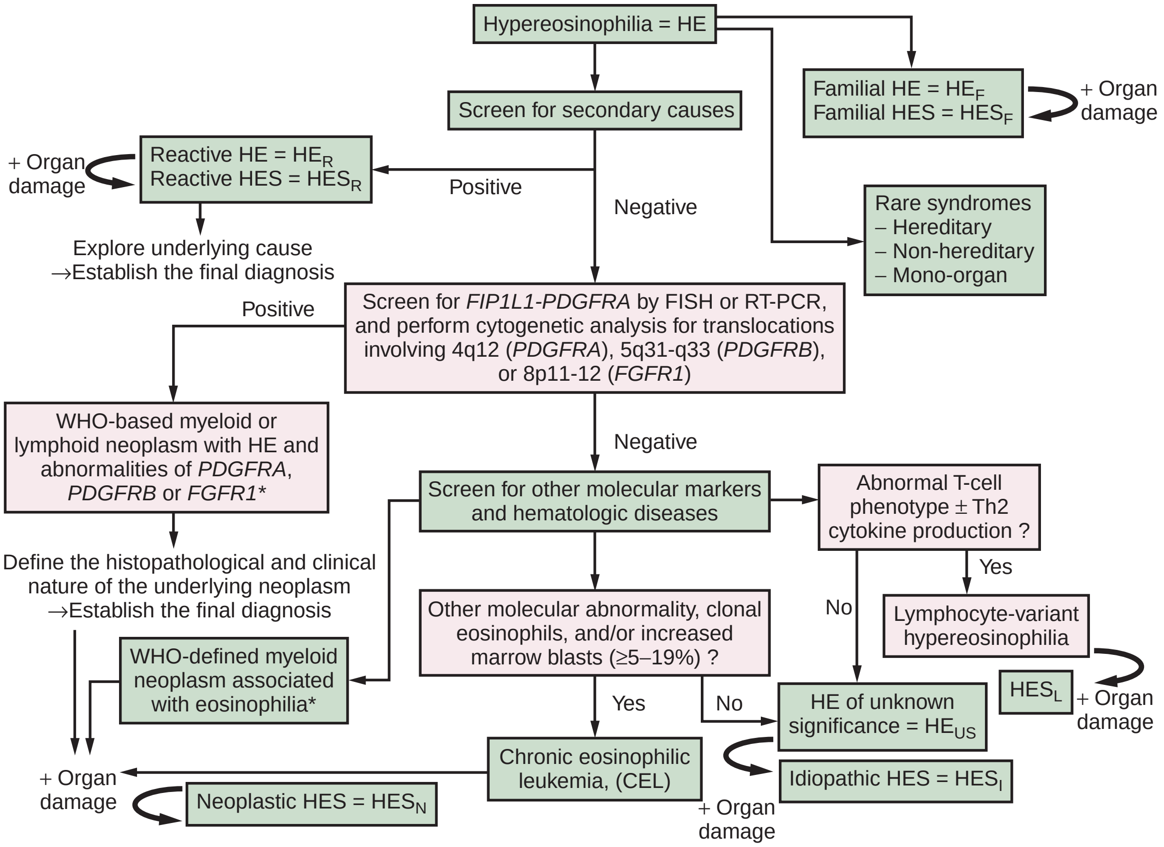

Hypereosinophilia = HE
Screen for secondary causes
Familial HE = HE$_F$
Familial HES = HES$_F$
+ Organ damage
Reactive HE = HE$_R$
Reactive HES = HES$_R$
+ Organ damage
Positive
Negative
Rare syndromes
– Hereditary
– Non-hereditary
– Mono-organ
Explore underlying cause
→Establish the final diagnosis
Positive
Screen for *FIP1L1-PDGFRA* by FISH or RT-PCR, and perform cytogenetic analysis for translocations involving 4q12 (*PDGFRA*), 5q31-q33 (*PDGFRB*), or 8p11-12 (*FGFR1*)
WHO-based myeloid or lymphoid neoplasm with HE and abnormalities of *PDGFRA*, *PDGFRB* or *FGFR1**
Negative
Screen for other molecular markers and hematologic diseases
Abnormal T-cell phenotype ± Th2 cytokine production ?
Yes
No
Define the histopathological and clinical nature of the underlying neoplasm
→Establish the final diagnosis
Other molecular abnormality, clonal eosinophils, and/or increased marrow blasts (≥5–19%) ?
Lymphocyte-variant hypereosinophilia
WHO-defined myeloid neoplasm associated with eosinophilia*
HES$_L$
+ Organ damage
Yes
No
HE of unknown significance = HE$_{US}$
+ Organ damage
Chronic eosinophilic leukemia, (CEL)
Idiopathic HES = HES$_I$
Neoplastic HES = HES$_N$
+ Organ damage

Figure 74.3 DIAGNOSTIC ALGORITHM FOR PATIENTS WITH EOSINOPHIL DISORDERS. The first important step in the algorithm is to confirm the presence of HE, defined by a persistent (>4 weeks) increase in eosinophils above 1500/µL blood. The next important question is whether HE is reactive (HE$_R$) or neoplastic (clonal = HE$_N$) in nature. In patients with HE$_N$, WHO criteria should be applied in order to define the underlying molecular lesion and the WHO category of disease. *In patients with HEN associated with oncogenic fusion genes involving, for example, PDGFRs, the WHO diagnosis is: myeloid/lymphoid neoplasm with eosinophilia and tyrosine kinase gene fusions (MLN-TK). In addition, morphologic and histopathologic criteria are used to arrive at a final diagnosis regarding the underlying disease. In patients with HE$_R$, an underlying disease must also be defined. In both groups of patients, the next important question is whether (or not) HE-related organ damage is present. If such pathological changes (tissue damage) are found, it is appropriate to diagnose a HES. According to the underlying etiology (disease), HES is classified again into HES$_R$ and HES$_N$. In some patients with HES, a clonal T-cell population (but no overt lymphoma) is detected. In these patients, the HES$_L$ variant is diagnosed. If no clonal T cells and no underlying reactive or neoplastic condition is detected in a patient with HES, the (provisional) diagnosis of an HES$_I$ is established. If no underlying disease and no HES are detected, the patient has HE$_{US}$ by definition. Familial cases of HE or HES are very rare, and the same holds true for hereditary syndromes and mono-organ syndromes accompanied by HE. *HE*, Hypereosinophilia; *HES*, hypereosinophilic syndrome; *HES$_F$*, familial HES; *HES$_I$*, idiopathic HES; *HES$_L$*, lymphoid variant of HES; *HES$_N$*, primary (neoplastic) HES; *HES$_R$*, reactive HES; *HE$_{US}$*, HE of undermined (unknown) significance; *WHO*, World Health Organization.

Classification of Hypereosinophilia and Hypereosinophilic Syndrome

The classification of HE and HES is essentially based on the related disease and underlying etiology. Accordingly, HE and HES are both divided into a familial variant (HE$_F$, HES$_F$), a primary/neoplastic form (HE$_N$, HES$_N$), and reactive entities (HE$_R$, HES$_R$; see Table 74.3).[3] In a smaller fraction of patients, the etiology of HE and HES remains uncertain. In other rare cases, two different causative factors are detected. When the etiology of HE remains uncertain after a thorough investigation and appropriate testing and no organ damage is detected, the final diagnosis is HE of uncertain (undetermined) significance (HE$_{US}$); and if HES is diagnosed, but no underlying disease is detected, the final diagnosis is idiopathic HES (HES$_I$).[3]

In general, four major groups of underlying disorders (conditions) have to be considered in HE (HES) patients: (1) myeloid (and rarely myeloid/lymphoid) neoplasms including eosinophilic leukemia (HE$_N$; HES$_N$), (2) lymphoid or nonhematopoietic neoplasms (paraneoplastic HE, a special variant of HE$_R$), (3) common allergic, reactive, or immunologic conditions (HE$_R$ and HES$_R$), and (4) clinically defined, rare syndromes and conditions accompanied by HE, including rare inherited disorders and organ-restricted inflammatory diseases with tissue HE.[3,17] Overall, the classification of HE/HES remains an important step in the diagnostic algorithm (see Fig. 74.3). Using generally accepted criteria and parameters, this approach should lead to a final diagnosis concerning the presence or absence of HES and the presence or absence of an underlying disease.

CLINICAL MANIFESTATIONS OF HYPEREOSINOPHILIC SYNDROME IN VARIOUS ORGAN SYSTEMS AND DIFFERENTIAL DIAGNOSIS

Depending on the type of disease and other factors, a number of different organ systems may be involved in patients with HES. The most commonly involved organs are the skin, lungs, GI tract, heart, and the CNS (see Table 74.7). In some patients, the relationship between HE and the typical clinical signs and symptoms are pathognomonic, whereas in other cases a tissue biopsy may be required to confirm HE-related organopathy.[3,17]

Cardiac Manifestations in Patients With Hypereosinophilic Syndrome

Prior to the advent of improved diagnostics and management, and novel therapeutic approaches, cardiac disease was the leading cause of morbidity and mortality in patients with HES. Endomyocardial thrombosis and fibrosis are often documented in HES_N, particularly in association with the *F/P* fusion gene, but are also seen in other variants of HES, including tropical forms induced by (persistent) parasitic infections and other reactive forms of HES. A thorough cardiac evaluation including an ECG and echocardiogram is mandatory in all patients with HE, and cardiac magnetic resonance imaging (MRI) may be helpful in distinguishing a HE-related cardiac disease from other etiologies.[3,17] Troponin levels and B-type natriuretic peptide levels may also be helpful parameters. An endomyocardial biopsy may be required in some of these patients in order to establish the definitive diagnosis, but biopsies are usually not performed in daily practice.

The cardiac damage seen in HES, usually progresses from early necrotic changes to thrombosis and fibrosis. The presence of eosinophils in the myocardium is always abnormal and highly suggestive of eosinophil-mediated organopathy. Identical forms of cardiac pathologies can develop in patients with HES forms of diverse etiologies, including tropical eosinophilias caused by parasitic infections, drug reactions, eosinophilic leukemias, MPN-eo, solid tumors, CSS and lymphomas. However, some of these patients may never go on to develop cardiac involvement, suggesting that disease manifestation likely involves as yet undefined factors required for HE-related tissue damage. The histopathology of cardiac involvement in HES is well characterized and can evolve through three defined stages in which eosinophils and eosinophil-derived mediators may be involved. These include (1) an initial acute necrotic stage of short duration (a few weeks) involving active endomyocarditis, (2) a later thrombotic stage (several months) with mural thrombus formation over endocardial lesions, and (3) a late fibrotic stage (after approximately 2 years of illness) with development of endomyocardial fibrosis. The early necrotic stage of cardiac disease is usually not recognized clinically. Echocardiography and angiography may fail to detect abnormalities at this early stage because ventricular thickening has not yet occurred and endomyocardial biopsies, generally from the right ventricle, are required to make the correct diagnosis of cardiac involvement. In the second stage, thrombi form over the damaged endocardium in either of the ventricles or the atrium, generally with sparing of the aortic and pulmonary valves. Progressive scarring at sites of mural thrombus formation ultimately leads to the late fibrotic stage, with endomyocardial fibrosis resulting in a restrictive cardiomyopathy and mitral or/and tricuspid valve regurgitation (see Table 74.7). The more common clinical manifestations in the later progressive stages of endomyocardial fibrosis include dyspnea, chest pain, signs of left or right ventricular congestive heart failure, or both, murmurs from mitral valve regurgitation, cardiomegaly, and T-wave inversions. Most patients who progress to this stage of HES-related cardiopathy have echocardiographic abnormalities, with thickening of the left ventricular wall being a common finding. However, electrocardiographic and MRI changes in these patients are not specific to HES. Differential diagnoses include infectious etiologies (endomyocarditis), autoimmune disorders such as rheumatoid arthritis, Marfan syndrome, thyroid disorders, and ischemic heart disease. Although various imaging techniques are helpful in evaluating HES-related cardiopathy, endomyocardial biopsy from the right and left ventricle remains a gold standard in diagnostic evaluation in patients with HE-related cardiopathy.

Pulmonary Manifestations

Approximately 50% of HES patients have pulmonary involvement, with the most common symptom being a chronic and persistent (nonproductive) cough. In order to confirm pulmonary manifestation in HES, a number of studies may be helpful, including pulmonary function tests, chest radiography and/or CT scan, bronchoscopy with BAL studies, and a tissue biopsy if necessary. The physiologic basis of pulmonary involvement in HES remains unknown. As in the heart, several different eosinophil-derived (cytotoxic and stroma-targeting) mediators and cytokines may act together to induce tissue damage and tissue remodeling, as well as fibrosis and thrombosis. Some of the pulmonary features may also reflect changes secondary to congestive heart failure. Although bronchospasm has been noted in some patients, full-blown asthma is rare in HES patients. Transudative pleural effusions are mostly seen in patients with frank congestive heart failure. In contrast to chronic eosinophilic pneumonia, the pulmonary infiltrates seen in HES patients are either diffuse or focal, without any preference for particular regions of the lung. Pulmonary infiltrates in HES often clear with prednisone treatment, but not all patients are responders. Pulmonary fibrosis is rare but can develop in patients with endomyocardial fibrosis. The differential diagnosis of HES_R with pulmonary involvement includes drug allergy, CSS, chronic eosinophilic pneumonia, severe allergic bronchial asthma with eosinophilia, allergic bronchopulmonary aspergillosis, and other eosinophilic lung infections.

Cutaneous Manifestations

The skin is frequently involved in HES, with cutaneous lesions present in over 50% of all cases. In these patients, the cutaneous lesions fall into three categories: (1) angioedematous and urticarial lesions; (2) erythematous, pruritic papules and nodules; and (3) mucosal ulcerations (see Table 74.7).[18] Patients with angioedema and urticaria are more likely to have a benign disease that is responsive to corticosteroids, without the development of cardiac or neurologic symptoms. A subgroup of patients with cyclic angioedema and eosinophilia are considered to have a syndrome (episodic angioedema with eosinophilia = Gleich syndrome) that is distinct from classical HES. These patients have a disorder with recurrent attacks of angioedema and urticaria accompanied by fever and weight gain. In HES patients with papular or nodular lesions, dermal biopsies usually show mixed cellular infiltrates, including eosinophils, without signs of vasculitis. Perivascular eosinophilic infiltrates are also found in these lesions. In a group of HES patients with skin involvement, the erythematous pruritic eruptions and indurated papules and nodules may respond to psoralen and ultraviolet light A. In other patients with HES-associated pruritus, the nodular lesions may respond to dapsone and corticosteroids. Oral sodium cromoglycate (cromolyn sodium), administered before meals, has also been reported to be efficacious, although neither dapsone nor cromolyn is able to reduce eosinophil counts in the blood. Severe and sometimes incapacitating mucocutaneous ulcerations may be a prodrome to HES and indicate a subset of HES patients with a poor prognosis. These lesions can appear at multiple sites, including the mouth, nose, pharynx, penis, esophagus, stomach, and anus. The lesions can flare up independently of other clinical manifestations of HES. Biopsies of the ulcerative lesions usually show mixed cellular infiltrates, without a predominance of eosinophils or any evidence of vasculitis or microthrombi. These ulcers are usually resistant to treatment with corticosteroids, colchicine, and hydroxyurea (HU), but they often respond to interferon-α (IFN-α), with complete and durable remissions in most patients. There are a number of additional diagnoses that have to be excluded in HES patients with suspected skin involvement, including drug reactions, local infections, and atopic eczema.

Neurologic Manifestations

Neurologic involvement is quite common in HES, affecting approximately 50% of all patients. In these cases, three different types of manifestations have been described. The first form of neurologic involvement is caused by thromboemboli, which may originate from intracardiac thrombi in the left ventricle but may also develop locally in cerebral vessels. Some of these thromboembolic episodes may occur before an overt cardiac manifestation has been documented. Patients with thrombotic complications may experience embolic strokes or transient ischemic attacks that may be multiple and recurrent, and these episodes may occur even though the patient is adequately anticoagulated. The second type of neurologic manifestation is primary diffuse CNS involvement. These patients may variably exhibit changes in behavior, confusion, ataxia, and loss of memory. The third neurologic abnormality noted in HES is the development of peripheral neuropathy, which can occur in approximately 50% of HES patients exhibiting neurologic involvement. This includes symmetric or asymmetric sensory polyneuropathies, including sensory deficits, painful paresthesias, or mixed sensory and motor defects (see Table 74.7). These neuropathies may improve with corticosteroid administration or other treatments and may be stable or continue to progress despite therapy, or may improve or even resolve with time. The histopathology of the involved nerves usually shows varying degrees of axonal loss, without evidence of vasculitis or direct or peripheral eosinophil infiltration. Whether EDN and/or ECP, known to cause neurotoxicity in experimental animals, are involved in the development of neuropathies in HES remains unknown. Based on immunohistochemical studies, these proteins are not detectable at the sites of ongoing neuropathology in HES patients. Overall, the etiology of HES-related neuropathy remains poorly defined. Differential diagnoses of HES-related neuropathies are manifold and include, among others, neurodegenerative disorders, multiple sclerosis, and CNS infections.

Gastrointestinal Manifestations

Compared to lung or skin, GI tract involvement is less common in patients with HES. Although any form of HES can present with GI tract manifestation, GI tract symptoms are mostly recorded in patients with HES_R or the idiopathic form of HES. In these patients, a wide variety of symptoms and findings have been described, including abdominal pain, vomiting, diarrhea, or ulcerative disease, but also ascites or an overt colitis (see Table 74.7). These patients may also report bloody diarrhea and weight loss. Histologically, GI tract involvement with HES is characterized by an infiltration of the mucosa and submucosa with eosinophils. Immunohistochemical examination may reveal a huge infiltration with eosinophils as well as extensive deposition of extracellular eosinophil proteins in the gastric mucosa. In most patients with reactive HES, the GI tract disease component responds to treatment with oral corticosteroids. Differential diagnoses of GI tract involvement with HES include localized (organ-restricted) inflammatory disorders, such as eosinophilic esophagitis, eosinophilic gastroenteritis, eosinophilic colitis, autoimmune diseases, chronic inflammatory bowel disease, GI infections, GI tract lymphomas, and GI tract involvement in patients with SM. Especially in patients with SM, the underlying disease is often overlooked, since neoplastic mast cells are outnumbered by the infiltrating eosinophils. In these cases, the use of mast cell-related immunohistochemical markers, including tryptase and KIT (CD117), and CD25, is recommended to diagnose an underlying SM.

Hematologic Manifestations and Bone Marrow Involvement

In HES patients suffering from an underlying myeloid neoplasm (HE_N, HES_N), the BM is almost always involved. In these patients, the BM is often hypercellular and shows a substantial increase in eosinophil numbers. In addition, alterations in the BM microenvironment, including increased microvessel density and BM fibrosis, is usually detected in these patients. Depending on the underlying neoplasm, other hematopoietic lineages may also be affected and may show typical morphological or phenotypic aberrations. Depending on the underlying neoplasm, eosinophils may be mature or (rather) immature cells (see Fig. 74.2B and C). The BM is also affected in patients with reactive or idiopathic HES. However, in these patients, eosinophils are usually mature cells and no major alterations in the BM microenvironment or abnormalities in other hematopoietic lineages are found.

Apart from eosinophilic leukemia, a number of different underlying BM neoplasms may be identified, such as an MPN, myelodysplastic syndrome (MDS), MDS/MPN overlap syndrome, acute myeloid leukemia (AML), or, rarely, a lymphoid malignancy.[17] In these patients, various blood count abnormalities may be present, such as neutrophilia, monocytosis, basophilia, thrombocytosis, a left-shifted white blood count, or an increase in blasts. In some cases, anemia or/and thrombocytopenia with or without increased blast cells or dysplasia is found, consistent with the diagnosis of an overt MDS, an MDS/MPN overlap syndrome, or AML. In rare cases, a stem cell neoplasm with involvement of both the myeloid and lymphoid lineage is detected. In these patients, an *FGFR1*-rearranged neoplasm (historically referred to as 8p11 myeloproliferative syndrome or stem cell leukemia/lymphoma) may be diagnosed.[13] The prognosis is poor in these patients as the *FGFR1*-mutated cells have historically been resistant to AML- or ALL induction-type combination-chemotherapy or nonselective TKIs with anti-FGFR activity. More recently, a number of more potent and rather selective FGFR inhibitors, such as pemigatinib, have demonstrated considerable efficacy with hematologic and cytogenetic remissions in patients with *FGFR1*-rearranged neoplasms.

In some patients with CEL, a massive leukocytosis is found. In these cases, eosinophilia of more than 90% may be seen, and total leukocyte counts may exceed 50 × 10^9/L or even 100 × 10^9/L[3].[3,17] Extremely high leukocyte (eosinophil) counts are more commonly seen in patients with HES_N in the context of CEL.[3] Blood smears from patients with HES_R and HES_I generally show more or less normal, mature eosinophil morphologies, including typical bilobed nuclei and granule-rich cytoplasm. However, hypodense eosinophils and eosinophilic precursor cells may also be observed, although less commonly, and eosinophils may also exhibit morphologic abnormalities, including nuclear hypersegmentation, decreased size and/or numbers of secondary granules, and cytoplasmic vacuolization. BM mast cell numbers, often in an interstitial pattern, may be increased in HES patients. This is often associated with an increased level of serum tryptase, especially in *F/P*+ cases, where these cells usually belong to the neoplastic clone. The presence of myeloblasts and/or dysplastic findings in the PB may suggest the presence of AML or MDS and is thus associated with a more unfavorable prognosis. Splenomegaly is present in a substantial subset of patients with HES, mostly in those with an underlying myeloid neoplasm, but sometimes also in cases with a reactive form of HES or idiopathic HES. In patients with markedly enlarged spleens, hypersplenism may develop and may contribute to thrombocytopenia and anemia. In these patients, splenic pain induced by capsular distention or infarction are well-known complications.

Underlying Hematologic Disorders and Differential Diagnoses

Although various hematologic neoplasms may be accompanied by eosinophilia, only a few of these are associated with persistent HE (HE_N) and HE-related organ damage (= HES_N). For example, mild-to-moderate eosinophilia may be detected in Philadelphia chromosome-positive (Ph^+; BCR::ABL1$^+$) chronic myeloid leukemia (CML) and various Ph^- MPNs, but marked HE is unusual and HES is rarely found in these patients. However, in a few patients with CML or MPNs, excessive HE may develop (see Chapter 69). In these patients, HE is usually responsive to BCR::ABL1 kinase inhibitors in CML, and to conventional cytostatic drugs, such as HU or IFN-α, in a patient with an MPN. In a few myeloid neoplasms substantial or even excessive HE is a typical finding. Based on the proposal of an international consensus group (ICOG-EO) these malignancies include the rare variant of acute eosinophilic leukemia (AEL), the more common variant of CEL that is often associated with a *F/P* fusion gene, and other forms or CEL or MPN-eo with rearrangements involving *PDGFRA*, *PDGFRB*, or *FGFR1*.[3,13] Other patients with CEL may fulfill WHO criteria of CEL, listed as WHO MPN (see Table 74.6).[3,14] Rarely, HE_N is found in patients with MDS. In most cases with dysplastic BM and HE, however, additional signs of myeloproliferation are found, consistent with a diagnosis of a MDS/MPN overlap syndrome or frank MPN (see Chapter 73).[3] Another myeloid neoplasm that is often accompanied by HE is SM (see Chapter 75).[19] In fact, HE_N is frequently seen in patients with advanced SM, including aggressive SM and mast cell leukemia (see Table 74.6). Most of these patients develop HE without HE-related organ damage (HES). In rare cases, lymphoid neoplasms may also present with HE. In these patients, a T-cell lymphoma is most commonly detected as underlying disease during initial staging investigations or during follow-up. In most patients with T-cell lymphomas accompanied by HE, eosinophils are nonclonal cells. By contrast, in patients with fusions involving *FGFR1*, where stem cells are considered to give rise to malignant cells, both eosinophils and lymphocytes are clonally involved. The prognosis in these patients has been historically poor.

Several different proposals to classify eosinophil neoplasms have been presented in the past.[3,14,20] The WHO has classified eosinophil-related disorders according to the presence of certain molecular lesions, including abnormalities in the *PDGFR* or *FGFR* genes or *PCM1::JAK2* (see Table 74.6).[14] In the 2022 update, the WHO has included additional fusion genes in this category, whihc is now termed MLN-TK (see Table 74.6). The advantage of this approach is that it refers to molecular targets of therapy. As a result, therapeutic decisions may be directly based on a WHO-based diagnosis. However, independent of the clinical presentation and markers, the WHO classification has to be complemented by a final histopathologic diagnosis, which in turn is based on thorough morphologic, histopathologic, and immunohistochemical studies of the BM.[3,17] Based on a proposal of the ICOG-EO group,[3] the following underlying neoplasms have to be delineated (Table 74.8A).

Acute Eosinophilic Leukemia

This type of leukemia is defined by HE, more than 19% (≥20%) myeloblasts and greater than 29% (≥30%) eosinophils in BM smears (see Table 74.8A).[3] Other classical types of MPNs and CML must be excluded. AEL is an extremely rare disease. Eosinophils in these patients may be quite immature, hypogranulated cells, or represent a mixture of mature and immature eosinophils. In some of these patients, a prodrome of MDS or MPN without HE occurs. In addition, *F/P*+ patients with CEL or *F/P*+ MPN-eo may progress to AEL during their terminal phase. However, (other) recurrent cytogenetic or molecular marker have not been identified in AEL patients. The clinical course in AEL is unfavorable. Chemotherapy and stem cell transplantation are usually recommended. Major differential diagnoses include AML M4-eo with inv16, CEL with signs of progression, and SM-associated with another hematolological malignancy (AHN)-eo (e.g., SM-AML-eo).

TABLE 74.8A **Histopathologic Classification and Criteria of Eosinophilic Leukemias and Other Myeloid Neoplasms Presenting With Hypereosinophilia**

Neoplasm(s)	Abbreviation	Definition/Criteria
i. Acute eosinophilic leukemia	AEL	HE and eosinophils ≥30% and myeloblasts ≥20%
ii. Chronic eosinophilic leukemia	CEL[a]	HE and eosinophils ≥30% and myeloblasts <20% and no underlying stem cell-, myeloid or lymphoid neoplasm found
iii. Other myeloid neoplasm (MN) or stem cell neoplasm with HE: MPN-eo, MDS-eo, SM-eo,	MN-eo	MN or stem cell neoplasm by WHO or FAB criteria and HE, but eosinophils <30%

The histopathological classification of eosinophil disorders assists the WHO-based delineation of neoplasms presenting with eosinophilia (HE). In a first step, the diagnosis is based on the WHO definition as molecular and cytogenetic abnormalities are examined. In a second step, the final histomorphologically defined diagnosis needs to be established using the criteria of the ICOG-EO depicted in this table.[3] Values for eosinophils and blast cells refer to the bone marrow smear. In rare cases (acute leukemia), eosinophils may be quite immature and may escape conventional morphological identification. In these cases, immunophenotyping or electron microscopy may be required.

AEL, Acute eosinophilic leukemia; *CEL*, chronic eosinophilic leukemia; *FAB*, French–American–British Cooperative Study Group; *HE*, hypereosinophilia; *ICOG-EO*, International Cooperative Study Group on Eosinophil Disorders *MDS*, myelodysplastic syndromes; *MPN*, myeloproliferative neoplasm; *WHO*, World Health Organization.

[a] In order to diagnose CEL, the following cytogenetic and molecular defects have to be excluded as primary reason of HE: *BCR::ABL1*, inv(16), t(16;16), *JAK2* V617F.

Chronic Eosinophilic Leukemia

In patients with CEL, eosinophils comprise ≥30% of all nucleated BM cells and/or blood leukocytes. As per ICOG-EO definition, patients with CEL have overt HE_N and less than 20% myeloblasts in their BM and/or blood smears (see Table 74.8A).[3] Usually, blast counts are below 5%. In most patients, neoplastic cells exhibit rearrangements (mutations) in genes coding for *PDGFRA*, *PDGFRB*, or *FGFR1*. Therefore, most patients also fit into the WHO category of "myeloid/lymphoid neoplasm with eosinophilia and tyrosine kinase gene fusions (MLN-TK)." Other BM neoplasms need to be excluded by BM examination. Major differential diagnoses include AEL, MPN-eo, MPN/MDS-eo, SM-eo, SM-AHN-eo, and reactive forms of HE/HES. Progression from CEL to AEL is an extremely rare event. However, in untreated patients with *PDGFR* mutations and those with *FGFR1* mutations, progression of CEL to AEL or even AML may occur. The prognosis in CEL depends largely on the molecular lesions detected. In patients with CEL exhibiting *PDGFR* mutations, the prognosis is excellent, as in most cases the disease responds to imatinib. By contrast, in patients with *FGFR1* mutations, the diagnosis is dismal, even when treated with chemotherapy and early allogeneic hematopoietic stem cell transplantation (HSCT) should be considered. However, more recently, pemigatinib, a potent and selective small molecule inhibitor of the FGFR, has been reported to be efficacious in patients with *FGFR1*-rearranged myeloid/lymphoid neoplasms. Pemigatinib is a selective and potent inhibitor of FGFR1–3 approved in the United States, EU, and Japan for the treatment of adults with previously treated, unresectable, locally advanced, or metastatic cholangiocarcinoma with a *FGFR2* fusion or other rearrangement.

MPN-eo, MPN/MDS-eo, MDS-eo, and SM-eo

The exact nomenclature of myeloid or mast cell neoplasms with marked eosinophilia (HE) is still under debate.[20] The ICOG-EO group has proposed the use of the appendix "-eo" for patients in whom HE_N is detected but criteria of AEL or CEL are not met.[3] In fact, in these patients an underlying MPN, MPN/MDS, MDS, or SM is diagnosed, and HE is present. However, eosinophils comprise less than 30% of all nucleated BM and PB leukocytes. In patients with MPN-eo, MPN/MDS-eo, or MDS-eo, HE may be detected at diagnosis or may develop during the follow-up period. In a small group of patients, mutant forms of *PDGFR*s or the *FGFR* are found. In other patients, karyotype abnormalities are detected, but *PDGFR* or *FGFR* mutants are not found. The molecular mechanisms underlying HE development in these patients usually remains unknown. Differential diagnoses include Ph^+ CML, CEL, AEL, and SM-AHN-eo (see Chapter 75). The overall prognosis depends on the underlying neoplasm, especially on blast cell counts, karyotypes and the expression of *PDGFR* or *FGFR1* mutants. Therefore it is important to apply all markers and diagnostic tests in order to define the exact diagnosis. In MDS and MPN/MDS, the presence of eosinophilia is an independent prognostic factor and indicative of poor survival, especially when HE is accompanied by blood basophilia.[21] Treatment of patients depends largely on the type of underlying malignancy, but also on the presence of distinct molecular lesions and targets. In advanced stages of the disease, a complex karyotype and complex mutational patterns are often found. These patients are candidates for intensive treatment, including HSCT.

Lymphoid Neoplasms, Lymphomas

A number of different lymphomas may produce eosinophilia, mostly through the generation of eosinophilopoietic cytokines in neoplastic cells. An exception is the 8p11 myeloproliferative syndrome (*FGFR1*-rearranged neoplasm), where eosinophils are derived from the malignant clone even if the primary tumor was classified as a lymphoma.[13] Most lymphoma patients with paracrine HE suffer from a (peripheral) T-cell non-Hodgkin lymphoma (NHL) (see Chapter 89). In these patients, clonal T cells are considered to represent the primary source of eosinophilopoietic cytokines, such as IL-5. A prephase of HES$_{L}$ or HE$_{US}$ may be present. The prognosis of patients with peripheral T-cell NHLs is poor, even when treated with chemotherapy, such as cyclophosphamide, hydroxydaunomycin, vincristine, and prednisone. Therefore allogeneic HSCT is usually recommended for these patients, provided that the patient has an acceptable performance status and a suitable donor is available, and the same holds true for lymphoma patients presenting with an *FGFR1* rearrangement. Relapsing disease after HSCT has an extremely poor outcome. These patients are treated with investigational agents and palliative cytoreduction. A summary of BM neoplasms presenting with HE is provided in Table 74.8B.

TABLE 74.8B Hematopoietic Neoplasms Accompanied by Eosinophilia

Neoplasms in Which Eosinophils Are Likely to Be Clonal Cells
Acute eosinophilic leukemia (AEL)
Chronic eosinophilic leukemia (CEL)
Acute myeloid leukemia with inv(16) (FAB AML M4eo)
Chronic myeloid leukemia (CML, BCR::ABL1+)
Myeloid neoplasms (MLN-TK) with *PDGFR* abnormalities (WHO types)
MLN-TK with *FGFR1* abnormalities (WHO types)
Smoldering systemic mastocytosis
Aggressive systemic mastocytosis (ASM)
Mast cell leukemia (MCL)
SM-AHN (SM-CEL)
Neoplasms in Which Eosinophils May or May Not Be Part of the Malignant Clone
Other myeloproliferative neoplasms (MPN) with eosinophilia
Myelodysplastic syndromes (MDS) with eosinophilia
Other MDS/MPN overlap syndromes with eosinophilia
Indolent systemic mastocytosis
Neoplasms in Which Eosinophils Usually Are Not Part of the Malignant Clone
Hodgkin disease
B- or T-cell non-Hodgkin lymphoma
Acute lymphoblastic leukemia (ALL)
Chronic lymphocytic leukemia (CLL)
Langerhans cell histiocytosis

[a]Other MPN or MPN/MDS: neoplasms where no abnormalities in the *PDGFR* or *FGFR1* genes are detectable.
AHN, Associated hematologic neoplasm; *FAB*, French–American–British Cooperative Study Group; *MLN-TK*, myeloid/lymphoid neoplasms with tyrosine kinase gene fusions; *PDGFR*, platelet-derived growth factor receptor; *SM*, systemic mastocytosis; *WHO*, World Health Organization.

Reactive, Immunologic and Paraneoplastic Conditions Associated With Hypereosinophilia

A number of different nonhematologic malignancies may be accompanied by HE. These include, among others, solid tumors of the GI tract, adenocarcinomas of the lung, carcinomas in other internal organs, skin cancer, urogenital malignancies, and stromal cell-derived tumors. In several of these patients, the cancer cells or the cancer-related microenvironment (e.g., fibroblasts) may produce eosinophilopoietic cytokines. In many cases, PB HE but also tissue eosinophilia (tissue HE) may be recorded. In some of these cancer patients, the tumor cells are tightly surrounded and sometimes even outnumbered by the infiltrating eosinophils. So far, it remains unknown whether tissue HE and eosinophil-derived mediators contribute to tumor formation (through neoangiogenesis or tissue remodeling) or even hinder tumor formation in these patients. As mentioned earlier, lymphomas may also be accompanied by reactive/paracrine HE.

A number of different immunologic and other reactive conditions can cause reactive HE (HE$_{R}$). These include infectious diseases (e.g., helminth infections, HIV, and certain fungal infections), allergic disorders (e.g., asthma, food allergy, atopic dermatitis, and drug reactions), chronic inflammatory reactions, and autoimmune processes (see Table 74.2).[17] Treatment of the underlying disease is usually followed by resolution of HE$_{R}$, thereby confirming the etiology of HE. In most reactive conditions, HE is triggered by cytokines derived from activated immune cells, such as T cells or mast cells. In some of these patients, an underlying disease is not detectable, but clonal T lymphocytes and/or lymphocytes with an aberrant surface immunophenotype are present. In such cases, HE-related organ damage may also be seen, which leads to the diagnosis of the lymphoid variant of HES (HES$_{L}$).[15]

Specific Syndromes and Conditions Associated With Hypereosinophilia

Several specific conditions and syndromes defined by HE and organ damage may fulfill the criteria of a HES, but based on traditional views and their unique etiologies, these syndromes are still regarded as distinct entities that should be discriminated from the classical HES variants. In the future, however, most or all of these syndromes may be regarded as a special form of HES. These syndromes include, among others, episodic angioedema and eosinophilia (Gleich syndrome), CSS, eosinophilia myalgia syndrome (EMS), OS, and hyper-IgE syndrome (see Table 74.4A).

Gleich syndrome is characterized by recurrent angioedema associated with eosinophilia (HE), elevated serum cytokine (IL-5) levels, and increased (polyclonal) IgM production.[22] The episodes of angioedema and eosinophilia typically occur at monthly intervals and often resolve spontaneously without therapy. Patients with Gleich syndrome may suffer from fever and increasing body weight. The clinical course is usually benign without involvement of internal organs, thereby contrasting with the typical HES variants. In a subset of patients, the symptoms and HE persist. These patients usually respond to low-dose corticosteroids. So far, little is known about the pathogenesis of Gleich syndrome. Several lines of evidence suggest that multiple cell types are involved, including neutrophils, mast cells, eosinophils, and T lymphocytes. Whereas most of these cells are considered to be cytokine-mobilized cells, T cells are often found to be clonal in these patients. In fact, in many patients with Gleich syndrome, T-cell phenotyping reveals an abnormal phenotype of CD4^{+} lymphocytes, with decreased or absent CD3 expression (see Table 74.4A).[22] In addition, in several of these patients a clonal T-cell receptor rearrangement has been found. Therefore, Gleich syndrome has also been considered as a special (cycling) subvariant of HES$_{L}$.

CSS, also known as EGPA, is defined by a disseminated necrotizing vasculitis that is accompanied by asthma and (tissue) eosinophilia (HE).[23] EGPA is a small-vessel vasculitis characterized by the formation of extravascular granulomas and the production of antineutrophil cytoplasmic antibodies (ANCA). Both, tissue HE and the ANCAs produced in a subset of EGPA patients are considered to play an active role in disease pathogenesis. However, the exact etiology of EGPA remains unknown. More recent data suggest that EGPA may be a Th2-mediated disease. Clinically, EGPA develops in three distinct phases: the initial (early) phase is characterized by asthma and rhinosinusitis, the eosinophilic phase is defined by HE_R and HE-related organ involvement, and the late ("vasculitic") phase is defined by clinical manifestations originating from the small-vessel vasculitis (see Table 74.4A). EGPA is a systemic disease that may affect a number of different organ systems, including the lungs, skin, kidneys, nerves, and heart. Criteria for EGPA have been proposed by various expert groups. According to the American College of Rheumatology, the following criteria define EGPA: asthma, eosinophilia (>10%), neuropathy (mononeuropathy or polyneuropathy), pulmonary infiltrates, paranasal sinus abnormalities, and extravascular eosinophil infiltrates on tissue biopsies. When four out of these six criteria apply, the diagnosis EGPA can be established. Based on the considerable clinical overlap with classical HES variants, EGPA is also regarded as a special HES variant, defined by an ANCA+ vasculitis. The prognosis of patients with CSS depends on a number of clinical variables, including lung involvement, duration of disease, and response to therapy. Treatment of patients with CSS is based on the individual situation in each case and the presence or absence of prognostic factors. A combination of high-dose corticosteroids and cyclophosphamide remains a standard for treatment. More recently, antibody-based drugs, such as rituximab or mepolizumab, a humanized IL-5 antagonist monoclonal antibody, have been proposed as therapeutic alternatives and should be considered in cases resistant to cyclophosphamide.

EMS is a flu-like condition defined by recurrent episodes of myalgia and persistent eosinophilia. In many patients, neurological symptoms and skin abnormalities are found. In addition, patients may suffer from fever, weight loss, edema, and severe fatigue. The etiology of EMS remains uncertain. A clue to pathogenesis was the detection or a relationship between EMS and exposure to contaminated L-tryptophan or rapeseed oil (toxic oil syndrome). EMS is an incurable disease, but fatal cases are rare.

Eosinophilic fasciitis (EF) is a scleroderma-like syndrome with HE, polyclonal hypergammaglobulinemia, and signs of systemic inflammation. The etiology of EF remains unknown, but most authors regard EF as an immunologic hyper-reactivity disorder manifesting in the skin. Painful swelling of the skin with progressive induration and thickening of the skin and soft tissues of the limbs and trunk are typical clinical findings. Corticosteroids are usually prescribed and have been shown to be efficacious in EF patients. In a subset of patients, spontaneous improvement is found. Again, based on clinical findings and HE, it is difficult to differentiate between EF and skin involvement in HES_R. EF has been associated with the development of aplastic anemia (see Chapter 31).

OS is a rare, autosomal recessive form of severe combined immunodeficiency (SCID) associated with leukocytosis, HE, elevated IgE levels, and a high mortality rate. Recognition in early childhood is required in order to initiate life-saving therapy. OS is caused by hypomorphic missense-mutations in relevant T-cell genes, such as the recombination-activating genes *RAG1* and *RAG2* (see Table 74.4A). Unlike patients with typical SCID, patients with OS have well-populated lymphatic organs and usually suffer from diarrhea, hepatosplenomegaly, lymphadenopathy, and weight loss. In addition, severe erythroderma, increased IgE levels, and eosinophilia are found in most cases. HE and inflammation observed in these patients may result from the activity of clonally expanded T cells, which are predominantly of the Th2 type. Overall, the disease presents very similar to a graft-versus-host reaction, which is due to patients producing T cells with limited recombination ability (mutant *RAG* genes) and a specific affinity for self-antigens, making them autoreactive T lymphocytes. Other SCID-related mutations may also lead to an OS-like disease, including certain mutations in the *IL-7 receptor (IL-7R)* gene. The treatment of choice in OS is allogeneic HSCT. For patients who cannot be transplanted or need a bridging therapy prior to allogeneic HSCT, cyclosporin A (CSA) may be considered.

Hyper-IgE syndromes (HIES) are a group of rare hereditary immunodeficiency syndromes characterized by elevated IgE levels, severe recurrent infections, and HE. In many cases, skin eczema and facial anomalies are found. So far, little is known about the pathogenesis of the HIES. The disease can manifest as an autosomal dominant or autosomal recessive variant. Several different genes may be affected. In the autosomal dominant variant, *STAT3* mutations are most commonly described. Since STAT3 acts as a key transcription factor downstream of multiple cytokine receptors regulating diverse immunologic responses, impaired STAT3 function is likely to cause immunodeficiency. In the autosomal recessive HIES variants, mutations in *DOCK8* and, less commonly, in the phosphoglucomutase 3 (*PGM3*) gene, have been reported (see Table 74.4A). The natural course of HIES patients is variable, but overall the long-term prognosis is poor. Therefore, early allogeneic HSCT should be considered whenever possible.

Organ-Restricted Hypereosinophilic Syndromes

There are other clinical conditions and syndromes accompanied by HE-related organ damage that cannot be classified (diagnosed) as typical HES. A special group of such disorders are organ-restricted inflammatory conditions, such as eosinophilic colitis, eosinophilic pneumonia, or eosinophilic esophagitis (see Table 74.4B). In these patients, cytokine production and disease manifestations are typically restricted to one single-organ system, in contrast to the classical forms of HES and most of the above-described specific syndromes. The etiology of these organ-restricted HES remains largely unknown. In many instances, infectious, toxic agents or specific allergens are felt to play a pathogenetic role. In addition, genetic or epigenetic factors may contribute to disease manifestations. Depending on disease severity, suspected etiology, and presence of comorbidities, a treatment-plan is established. In many cases, corticosteroids are prescribed. In those with suspected allergy, additional drugs, such as antihistamines or leukotriene antagonists, have been recommended. The prognosis is variable and depends largely on the underlying etiology and response to treatment. Most patients respond to corticosteroids. In some patients, an underlying (initially unrecognized) systemic disease, infectious disease, or local tumor is diagnosed during follow-up. In these patients, the prognosis and treatment depend on the type and stage of the underlying disease.

TREATMENT ALGORITHM, TREATMENT OPTIONS, AND PROGNOSIS

When establishing the management plan for a patient with HE, several important questions have to be addressed. First, the final diagnosis concerning the presence or absence of HES and the presence and nature of the underlying disease, if present, have to be firmly established. Second, it is important to decide whether the patient needs symptomatic or/and interventional therapy. In some of these HE patients, a wait-and-watch strategy may be acceptable, whereas in other cases (when HES is diagnosed) specific therapy needs to be introduced quickly. Finally, the optimal treatment plan and most useful drugs need to be defined. The type of therapy varies depending on the underlying condition, targets, and the type of HES.[3,17,24]

Primary Management and Algorithm

In many cases, treatment of HES is a straightforward approach. For example, reactive HES is often managed (and resolved) quite easily by treating the underlying disease, such as a helminth infection or an autoimmune disease. In cases with idiopathic HE or HES and those with advanced or aggressive disease, however, treatment should always be performed in collaboration with a specialized center and in an interdisciplinary approach involving hematologists, immunologists, pathologists, and/or laboratory specialists, as needed.

In patients with HE_{US} and HE_F, it seems appropriate to follow the patient without treatment, provided there are no signs or symptoms of eosinophil-related organ damage despite careful medical monitoring. However, both HE_{US} and HE_F must be regarded as provisional diagnoses, and in both conditions organ damage may develop over time. In addition, a hematologic or other underlying disease may be detected during follow-up of these cases. In the reactive form of HES, it is important to define and to treat the underlying condition. Additional symptomatic therapy, often in the form of corticosteroids, may be required to control HE and eosinophil activation in these patients, especially when eosinophil counts are high or/and cells undergo rapid lysis, which may occur during treatment with cytotoxic drugs.[24]

In the past, most patients with HES received corticosteroids as front-line therapy. However, this strategy has changed with more extensive knowledge about the pathogenesis of HE and HES etiologies, and the advent of novel drugs such as PDGFR-targeting TKIs. Notably, today, front-line therapy is largely dependent on the underlying disease and the molecular targets detectable in neoplastic cells.[24] In patients in whom eosinophils display mutated forms of PDGFRs, imatinib is considered standard front-line therapy. By contrast, in patients with idiopathic HES (HES_I) and those with HES_L, corticosteroids remain standard front-line therapy. However, there are also patients in whom an initial staging and grading and/or molecular studies suggest that neither steroids nor imatinib will work. These cases include, among others, patients with an advanced myeloid neoplasm without a *PDGFR* mutation (e.g., AML-eo, MDS-eo, or SM-eo) and patients with rearranged *FGFR1* genes. In many of these cases, the primary treatment plan includes consideration of a FGFR inhibitor, for example pemigatinib or futibatinib as a bridge to HSCT.

All in all, a number of different therapeutic approaches for the treatment of HES and of the underlying (eosinophil) disorder(s) detected in these patients are available. In the following sections, these treatment approaches are briefly reviewed and discussed.

Corticosteroids

In patients with idiopathic HES (HES_I) and HES_L, corticosteroids are considered standard front-line therapy. By contrast, in patients with HES_R and HES_N, corticosteroids should only be considered as an adjunct to more specific treatment, in order to stop eosinophil activation and eosinophil recruitment (HE) as early as possible. In patients with a (suspected) infection, diabetes mellitus, an ulcerative GI tract disease, or arterial hypertension, corticosteroids should be administered with caution and with recognition of potential side effects and the related risk. In patients with HES_I and HES_L, corticosteroid therapy, usually in form of prednisone at 1 mg/kg/day (initial dose), is usually effective in reducing eosinophil counts to normal levels, with overall response rates of greater than 75% in HES_I. In contrast, most patients with HES_N do not respond to corticosteroid therapy.

The mechanisms of action by which corticosteroids suppress HE in HES_I and HES_L patients are only partly understood. Steroids act through specific corticosteroid receptors that are present in the cytoplasm of eosinophils and eosinophil precursor cells, but also in the cytoplasm of T lymphocytes and mast cells. It has also been described that steroids effectively block the production of various eosinotropic cytokines by T lymphocytes. In addition, corticosteroids effectively counteract T-cell expansion. Based on these effects, corticosteroids may act directly and indirectly to block HE in HES patients, namely by (1) inducing growth inhibition and apoptosis of eosinophils and their precursor cells, by (2) directly blocking eosinophil recruitment and migration, and by (3) inhibiting the production and release of eosinophilopoietic cytokines by T lymphocytes and other immune cells.

As mentioned earlier, long-term treatment with steroids is associated with a risk of (severe) side effects. Therefore, steroids should be tapered down (and discontinued) whenever possible, especially in cases with HES_R and HES_N. However, in many patients with HES_I, maintenance therapy with corticosteroids is required to control disease activity. In these patients, the steroid dose applied should be reduced to a minimum. In fact, even though corticosteroids are "natural" substances and very effective in reducing eosinophil counts in HES_I, chronic use of steroids is associated with the risk of serious adverse events and long-term toxicity. Once reduction of the eosinophil count to below 1.5×10^9/L and symptom control have been achieved, the corticosteroid dose should be tapered. Increases in eosinophil numbers on a prednisone dose greater than 10 mg daily and/or reappearance of symptoms or signs of organ damage are indications for the addition of other agents to the steroid regimen. A number of different drugs may be useful as steroid-sparing agents in patients with HES. These drugs include, among others, conventional cytostatic agents such as HU or cyclophosphamide, IFN-α, and IL-5 and IL-5-receptor-targeting antibodies, including mepolizumab and benralizumab, respectively.[25]

Conventional Cytoreduction and Chemotherapy

Several studies have shown that HU (0.5 to 2 g/day) is efficacious in steroid-nonresponsive patients with HES, including those with HES_I and HES_N. In patients with HES_I, HU is administered as an alternative drug or in conjunction with corticosteroids as a steroid-sparing medication. A typical starting dose is 500 to 1000 mg daily. In combination with corticosteroids, the overall response rate exceeds 50%. Eosinophil counts begin to decrease 7 to 14 days after starting HU treatment. Depending on the dose, HU may induce cytopenias. Otherwise, HU is usually well tolerated unless very high doses are administered and is therefore also used as a palliative drug in HES_N patients resistant to targeted drugs and chemotherapy. However, not all patients with HES may respond to HU. Historically, a number of cytotoxic agents have been used to treat HES refractory to steroids and HU, including vincristine, etoposide (VP-16), and chlorambucil. However, each of these agents can produce cytopenias and other side effects, and their use should be limited to select patients with HES in whom neoplastic cells develop resistance against other agents including HU. In patients with advanced, resistant CEL or AEL, but also in patients with AML-eo, intensive combination chemotherapy with or without subsequent allogeneic HSCT is usually recommended. These chemotherapy regimens are usually the same as applied in patients with refractory leukemias without HE/HES. Chemotherapy before allogeneic HSCT is often a preferable initial approach for two reasons: first, debulking may result in remission, and patients in remission are considered to have, in general, a better overall outcome after allogeneic HSCT. In addition, chemotherapy can provide some indication as to whether a patient can tolerate intensive therapy and are chemo-responsive. On the other hand, multiple cycles of chemotherapy increase the risk of infection, which may be problematic in the context of a planned allogeneic HSCT.

Interferon-Alpha

IFN-α has been used to treat HES for more than 25 years, both as monotherapy and in combination with other agents. A number of studies have shown that this type of therapy can successfully counteract HE and HES in patients in whom treatment with prednisone and HU has failed. Response rates to IFN-α vary depending on the underlying disease and dose but may be approximately 50% when used as monotherapy and 75% in combination with other agents. Usually, therapy is initiated at a dose of 1 million units three times a week and is titrated up to 4 million units three times weekly. In the responding patients, eosinophil counts decrease, and improvement in splenomegaly and hepatomegaly has been reported. Moreover, the risk of cardiac and thromboembolic complications can be reduced by treatment with IFN-α. Finally, mucosal ulcerative lesions and other skin manifestations may substantially improve. The unprocessed and the pegylated form of IFN-α appear to be equally effective and are both recommended for use in clinical practice. However, despite efficacy, the administration of IFN-α is problematic because of side effects. These side effects are often dose dependent and include fever, a flu-like syndrome, fatigue, impotence, depression, suicidal ideation, and psychosis. In addition, IFN-α therapy may lead to exacerbations of autoimmune disorders, such as autoimmune thyroiditis, psoriasis, ulcerative colitis, and others, but also to exacerbation of depression or other psychiatric diseases. Other serious adverse events observed with IFN-α include excessive suppression of blood counts, retinopathy, sarcoidosis, and left-sided heart failure. All these (potential) side effects have to be taken into account, especially when prescribing IFN-α on a long-term basis at relatively high doses. In contrast, IFN-α is nonmutagenic and is considered a drug that can be prescribed even during pregnancy. The mechanism of action of IFN-α is not well understood. Receptors for this cytokine are expressed on multipotent hematopoietic progenitor cells, eosinophil-committed precursors, and mature blood eosinophils. Therefore, in contrast to corticosteroids, IFN-α works in HES patients with diverse etiologies, including HES_L, but also in HES_N and even in HES_I. IFN-α inhibits eosinophil activation and the release of various toxic mediators from eosinophils. In addition, IFN-α may also exert an anti-inflammatory effect on other cell types that may contribute to tissue damage in HES.

Cyclosporin A

CSA, an immunosuppressant that interferes with multiple functions and growth of immunocompetent T lymphocytes, has been reported to be effective in a subset of patients with HES. The goal of CSA treatment in HES is to inhibit production of eosinophilopoietic cytokines, including IL-5, by T cells in these patients. CSA has been used as a single agent or in conjunction with low-dose corticosteroids. In those with steroid-sensitive disease, CSA may act as a steroid-sparing agent. However, good responses may only be seen in distinct variants of HES, namely those HES variants where T-cell activation is a causative etiology. Another important point is that efficacy has so far only been described for CSA in short-term–treated HES patients (10 months), whereas long-term efficacy has not been determined so far. In each case, the side effects and immunosuppressive effects of CSA have to be taken into account. In patients with HES_N, CSA should not be administered.

Targeted Treatment Approaches Using Tyrosine Kinase Inhibitors

Several different oncogenic kinases have been detected in primary eosinophilic disorders (MLN-TK, CEL, and MPN-eo) and serve as targets of therapy.[13] The most relevant and most frequent target kinase detectable in these patients is *F/P*. This fusion gene product is a target of imatinib and also sensitive to other TKIs, such as nilotinib, dasatinib, midostaurin, or ponatinib. A number of other oncoproteins may also serve as targets of imatinib and are detectable in primary eosinophil disorders. Several of these fusion genes involve *PDGFRA* or *PDGFRB*, whereas FGFR1 mutants are resistant against imatinib. The hematologic benefit of imatinib in *F/P*-positive neoplasms and *PDGFRB*-rearranged neoplasms has been confirmed in several clinical trials.[26,27] Therefore imatinib is regarded as standard first-line therapy in these patients. Given the poor prognosis of patients with overt organ damage (HES_N), patients with an *F/P*+ neoplasm or another mutation involving *PDGFRA* or *PDGFRB* genes should be treated with imatinib, even in the absence of end-organ damage, in order to prevent any HES occurrence. Although the optimal dosing strategy for imatinib has not been fully defined, a number of studies have shown that a daily dose of 100 mg appears to be sufficient to induce complete hematologic and molecular remission in most patients.[26] Imatinib is several hundred–fold more potent against *FIP1L1::PDGFRA* compared to BCR-ABL1 as a target. Moreover, 100 mg imatinib/day appears to be a safe approach in all patients, including those who suffer from cardiac involvement. Therefore, 100 mg/day is regarded a standard dose of initial therapy with imatinib in *F/P*+ patients. However, in a few patients with *PDGFRA*- or *PDGFRB*-rearranged neoplasms, higher doses of imatinib (400 mg daily) are required to induce a durable remission. In those who enter a hematologic and molecular remission, imatinib is usually maintained, as discontinuation is often followed by a relapse.[28] However, it remains unknown whether all patients need to continue on their initial dose on a life-long basis. In fact, many of these patients do not relapse when lowering the dose of imatinib or even when imatinib is discontinued. In other patients, however, resistance to imatinib may occur. In relapsing patients, *F/P* point mutations associated with decreased (or loss of) drug-binding capacity may be detected. For these patients, novel TKIs or chemotherapy may be considered. The most commonly detected mutant, T674I, leads to replacement of threonine by isoleucine at residue 674 in the FIP1L1::PDGFRA kinase domain, resulting in resistance to imatinib. This T674I exchange is analogous to the *BCR::ABL1* T315I mutation, which is a pan-resistant mutation to several TKIs except for ponatinib. Although in vitro sensitivity has been demonstrated to a number of different TKIs with this mutation (e.g., midostaurin, nilotinib, sorafenib, and ponatinib), clinical responses are often disappointing. Other secondary mutations in *F/P*+ disease, such as D842V, are very rare. However, once these secondary mutations occur during treatment with multiple TKIs, they usually exhibit pan-resistance. In fact, durable responses to TKIs with either the T674I or D842V mutation are not generally observed, and especially the *F/P* D842V mutant is resistant to almost all available TKIs. It has also been described that ponatinib can kill neoplastic eosinophils exhibiting all types of *F/P* mutations in vitro at clinically achievable drug concentrations. Whether this also holds true in vivo in patients with TKI-resistant CEL or acute leukemias exhibiting this *F/P* mutant, presently remains unknown. Avapritinib, which is now approved for D842V-mutated gastrointestinal stromal tumors, may have activity in *FIP1L1::PDGFRA*-positive disease with the D842V mutant, but this has not yet been reported at the time of this writing. Another approach in patients with TKI-resistant eosinophil neoplasms is to apply cytoreductive agents (IFN-α, HU) or experimental drugs. In patients who transform to an acute leukemia, combination chemotherapy and allogeneic HSCT are usually recommended.

A number of different markers have been proposed for the monitoring of treatment responses in patients with *F/P*+ CEL. Close hematologic follow-up is recommended for the first weeks of therapy, where a rapid decline in eosinophils (and leukocytes) is usually observed and is regarded a good prognostic sign. After a few months, the BM should be examined in order to document and confirm the hematologic response. A complete remission is obtained in most patients, even if the initial BM revealed marked involvement at diagnosis, including fibrosis and/or increased blast cells. In patients with a complete hematologic remission, molecular monitoring and repeated PCR and FISH analysis is recommended in order to define the molecular response. Quantitative measurement of the *F/P* mRNA burden by reverse-transcriptase-PCR is available in many centers and can be employed to define the depth of the remission. In those with an initially elevated serum tryptase, serial determinations of tryptase are also useful to document the response to imatinib or other TKIs. Finally, in those with end-organ damage, including cardiac disease, reinvestigation of all involved organ systems is recommended to demonstrate potential reversion of end-organ damage. In high-risk patients (e.g., cardiac history), echocardiography and troponin as well as B-type natriuretic peptide levels before initiating therapy may be recommended to gauge the potential risk.

Although imatinib and other TKI therapies in HESN patients is generally well tolerated, all these agents can produce severe side effects. Even imatinib may produce severe side effects, especially when applied to HES patients with cardiopathy at 400 mg daily. In fact, in a few cases severe left ventricular dysfunction (presumably due to rapid cytoreduction of eosinophil infiltration of the endomyocardium) has been reported soon after initiation of imatinib therapy; therefore, in patients with known or suspected cardiac involvement, it is recommended that corticosteroids be initiated for the first 7 to 10 days of imatinib therapy and close monitoring is recommended during this period. The second- and third-generation TKIs used to treat CML and CEL may also produce severe side effects, and many of them may affect the cardiovascular system or metabolic parameters, which is relevant clinically in the context of HES.

Targeted Antibodies

A number of targeted antibodies and antibody–toxin conjugates have been considered for patients with HE-related disorders and HES.[25,29] These antibodies are directed either against (neoplastic) eosinophils and their precursor cells or against eosinotropic cytokines, such as IL-5. Anti-eosinophil antibodies are considered useful in neoplastic states and should ideally be directed against both eosinophils and their precursor cells, or even neoplastic stem cells. One such antibody is alemtuzumab. This humanized antibody is directed against CD52 (= Campath-1), a surface receptor expressed on neoplastic stem cells in various leukemias as well as on eosinophils and their precursor cells. Alemtuzumab therapy in patients with drug-resistant eosinophil disorders has been reported in a limited number of studies. In about 80% to 90% of the patients the drug induced remission. However, in over 80% of these patients, relapses occurred after cessation of therapy. In addition, alemtuzumab produced substantial side effects, including severe hematological toxicity with subsequent opportunistic infections. Several other antibodies directed against cell surface receptors expressed by eosinophils are currently in preclinical development. The epidermal growth factor-like module-containing, mucin-like hormone receptor 1 (EMR1) is expressed specifically on human blood eosinophils and may serve as a potential target of therapy. A novel, humanized afucosylated anti-EMR1 IgG1 antibody has recently been shown to augment NK cell-mediated killing of eosinophil granulocytes. Benralizumab is an antibody directed against the IL-5 receptor on eosinophils and their precursor cells. So far, benralizumab has been tested in normal subjects, patients with asthma, and patients with HES.[29,30] BM studies have shown that this antibody induces a remarkable depletion of eosinophils and their precursor cells. It is noteworthy that the IL-5 receptor is also expressed on basophils, albeit at lower levels. The trial showed that benralizumab therapy was effective in a small group of patients of reducing blood and tissue eosinophilia with few or no toxic effects in patients with severe, treatment-refractory HES, despite having markedly higher eosinophil levels. The eosinophil-lowering effect of benralizumab was sustained in the majority of patients allowing the tapering of other therapies that had substantial long-term toxic effects. A larger, well-controlled, multicenter trial of benralizumab is ongoing to confirm these results and further explore the role of clinical disease subtype and other factors in the treatment response in patients with HES. Another interesting target receptor is Siglec-8. This antigen is expressed on eosinophils and mast cells, but not on neoplastic stem cells. Remarkably, Siglec-8 crosslinking induces eosinophil apoptosis and inhibits IgE-mediated mast cell activation, thereby providing a common mechanism to limit inflammation caused by the coexistence and functional interactions of these cells.[6] Whether this concept is clinically effective, remains at present unknown. Since Siglec-8 is not expressed on immature eosinophilic progenitor cells or neoplastic stem cells, the drug may not be useful in eosinophil malignancies. A humanized non-fucosylated IgG1 monoclonal antibody, AK002, has entered clinical trials for the treatment of various eosinophilic diseases including eosinophilic gastritis and/or gastroenteritis, antihistamine-resistant chronic urticaria, and severe allergic conjunctivitis. AK002 was found to reduced GI eosinophils and symptoms in a phase 2 study of symptomatic eosinophilic gastritis and/or duodenitis.

Siglec-3 (CD33), another established drug target, is displayed by normal and neoplastic eosinophils and even by neoplastic stem cells. Gemtuzumab ozogamicin (GO) is a humanized CD33 antibody conjugated with calicheamicin. The drug is highly effective in targeting myeloid cells and their progenitors in AML. The drug can also produce prolonged cytopenia, especially when used with combination chemotherapy. However, when used at limited doses and at reasonable time intervals, the toxicity of such antibodies may be acceptable.

Another approach, for patients with reactive HE and HES (HES_R) as well as HES_L, is to target eosinotropic cytokines using specific antibodies, where overproduction of such cytokines is known to play a pathogenetic role. These agents include antibodies directed against IL-5, against eotaxin, or other cytokine agonists. Mepolizumab is a fully humanized IgG1 antibody that binds IL-5 and prevents its binding to the IL-5 receptor alpha chain.[29] Mepolizumab was tested against placebo in a randomized, multicenter, double-blind, placebo-controlled, phase III trial of mepolizumab. Patients were randomized (1:1) to subcutaneous mepolizumab (300 mg) or placebo every 4 weeks for 32 weeks, plus existing HES therapy. The primary outcome was the proportion of patients with 1 or more flares (worsening of HES-related symptoms necessitating therapy escalation or ≥2 courses of blinded rescue oral corticosteroids). The proportion of patients experiencing one or more flares/withdrawing from the study was 50% lower with mepolizumab versus placebo.[25] Adverse events were tolerable and similar in the treated and in the placebo groups. Based on these data, mepolizumab was approved by the FDA in 2020 for the treatment of patients with HES, and follows prior approvals for severe eosinophilic asthma and EGPA. Reslizumab is a humanized IgG4 anti IL-5 antibody that is currently being tested in clinical trials for eosinophilic esophagitis and in pediatric patients for eosinophilic asthma, but it has not been studied in the context of HES.[29] Initial results suggest that the drug is effective in reducing eosinophil counts and clinical symptoms. Bertilimumab is a human anti-eotaxin-1 antibody that is currently being tested in a phase II clinical trial for ulcerative colitis but has not been tested yet in HES patients. Together, more controlled clinical trials in HES patients are needed to learn which antibodies directed against eosinophilic receptors and eosinotropic cytokines are most effective in patients with primary or reactive eosinophil disorders and HES.

Leukapheresis

Leukapheresis has no defined role in the management of HES. However, leukapheresis may be useful in emergency situations for patients with excessively high eosinophil counts. Since cell counts rebound rapidly to pretreatment levels, such a maneuver should always be combined with cytoreductive therapy in order to keep eosinophil counts under control.

Anticoagulation and Antiplatelet Agents

Thromboembolic complications may be life-threatening in HES patients. Therefore, prophylactic anticoagulation may be considered in patients with HES, especially in those in whom the risk for thromboembolic complications is high, such as in patients with a *F/P*+ disease, cardiac involvement, or neurologic symptoms. In patients with overt HES and associated thromboembolic events, anticoagulation is a standard approach. Commonly used agents are warfarin, antiplatelet agents, direct acting oral anticoagulants and heparin. The efficacy of these drugs has not been formally established in HES, and some of the patients may continue to have thrombotic events, despite adequate anticoagulation. Therefore, it is always important to treat the underlying disease if possible, in order to reduce the thromboembolic risk.

Splenectomy

Splenectomy is usually not required in patients with MPN-eo, AEL, or CEL. However, in rare cases, splenomegaly has been performed to correct hypersplenism-related cytopenias, or to avoid splenic infarction and pain induced by capsular distention.

Allogeneic Hematopoietic Stem Cell Transplantation

Although the experience with allogeneic HSCT in patients with advanced BM neoplasms accompanied by HE (with or without HES) is quite limited, allogeneic HSCT should be considered in certain groups of patients, especially those who (1) are young and fit, (2) have a suitable transplant donor, and (3) have a progressive and treatment-resistant form of an underlying myeloid or lymphoid neoplasm. More or less definitive indications in this regard include *FGFR1*-rearranged neoplasms, drug-resistant forms of AEL or CEL, chemotherapy-resistant peripheral T-cell lymphomas accompanied by HE/HES, and treatment-resistant (progressive) cases of MPN-eo, AML-eo, or MDS-eo. When the burden of neoplastic cells is high, debulking therapy should be introduced prior to conditioning and HSCT. Such debulking may be performed with combination chemotherapy or with experimental drugs. Several but not all transplanted patients may enter a complete remission. Transplant-related mortality rates may not be higher compared to age-matched patients with other advanced hematopoietic neoplasms. As in other BM neoplasms, age, comorbidities, and the disease status are major risk factors concerning survival and transplant-related mortality. In this regard, it should be noted that the presence of HES-related organ damage, such as overt cardiomyopathy or thromboembolism, but also neuropathies or GI disease, may count as relevant comorbidities, and if severe in nature, may represent a contraindication to allogeneic HSCT. In those who are successfully transplanted, the clinical course may be complicated by graft-versus-host disease requiring treatment with corticosteroids, cyclosporin-A, or/and other immunosuppressive drugs. Little is known about long-term outcomes after allogeneic HSCT. In smaller case series and single cases reports, long-term survivors have been described. However, relapses have also been reported in most groups of patients.

Cardiac Surgery

In a subset of patients with HES, surgical intervention of cardiac lesions is required. Interestingly, such intervention does not appear to carry a major risk for disease recurrence at the operative sites. Indications for surgery include a significant compromise of valvular function and the presence of a clinically significant thrombotic mass. Cardiac surgery has the capacity to provide a substantial clinical and quality-of-life benefit in these patients. In fact, successful mitral valve and/or tricuspid valve repair or replacements have been reported in HES patients in several studies. For mitral valve replacements, mechanical valves have proven problematic because of recurrent thrombosis despite adequate anticoagulation, suggesting the use of porcine valves whenever possible. HES patients who have received valve replacements have generally experienced long-term improvement in cardiac function, provided the underlying disease and the related HE can be kept under control.

SUMMARY AND FUTURE PERSPECTIVES

The diagnosis, classification, and management of patients with persistent HE remain a challenge in clinical practice, especially when no underlying disease is identified and/or HE-related organ damage (HES) develops. Because of heterogeneity and complexity of the disease, each case needs to be investigated and managed using the full battery of diagnostic markers and assays, and according to contemporary diagnostic and therapeutic algorithms.[3,14] Depending on the overall situation, initial investigations include BM studies (histology, cytology, and cytogenetics with FISH), molecular analyses, immunologic parameters, and a complete staging of all potentially involved end-organ systems. In benign disease variants, the course can sometimes be monitored without specific treatment. However, in patients with (1) advanced hematopoietic neoplasms, (2) overt HE-related end-organ damage (HES), (3) an active underlying (systemic) immunologic, infectious, or allergic disease, (4) evidence of a specific clinical syndrome with systemic organ involvement, or (5) organ-restricted marked inflammation associated with eosinophilia, such as eosinophilic colitis or eosinophilic pneumonia, specific treatment needs to be initiated. In reactive conditions, symptomatic treatment and corticosteroids are usually administered. In addition, it is of utmost importance to treat and eradicate (or control) the underlying disease, such as an NHL, helminth infection or IgE-dependent allergy, in these patients. Such treatment is often followed by complete resolution of reactive HE and of the related HES. In treatment-resistant patients in whom reactive eosinophilia is likely to be triggered by eosinophilotropic cytokines such as IL-5, anti-cytokine therapy, such as mepolizumab, may be considered.[29,30] This drug has shown promising results in patients with steroid-resistant reactive HES and other HE-related conditions, such as eosinophilic esophagitis. In advanced hematopoietic neoplasms, treatment with targeted drugs or less specific cytoreductive agents is recommended.[31,32] In *F/P*+ patients and those with related *PDGFR* fusion genes, imatinib is an undisputable standard of therapy. For patients with imatinib-resistant *F/P*+ CEL or MPN-eo, MPN-eo without a *PDGFR* mutant, or HE-related malignancies expressing *FGFR1* or *JAK2* fusion genes, novel TKIs are available.[31,32] In patients in whom secondary mutations in *F/P* are detectable, novel PDGFR-targeting TKIs such as ponatinib or avapritinib may work. In cases with *FGFR1*-rearranged neoplasms, novel FGFR inhibitors such as pemigatinib may overcome resistance, and in patients with *JAK2*-mutated MPN-eo, ruxolitinib may elicit clinically meaningful responses. In rapidly progressive and/or drug-resistant cases, high-dose chemotherapy and allogeneic HSCT need to be considered. In patients who are TKI resistant and not eligible for allogeneic HSCT, HU, other cytostatic agents, or IFN-α serve as palliative drugs. Current research aims at exploring the evolution of HES and at defining mechanisms regulating the development of HE-mediated end-organ damage, which should lead to more selective and improved therapies in the future. Clinically relevant targets for these efforts are likely to include (1) neoplastic stem cells producing eosinophil neoplasms such as CEL or MPN-eo, (2) molecular targets created specifically by neoplastic cells, such as mutant forms of *PDGFRs* or *FGFRs*, (3) eosinophilotropic cytokines and their receptors, (4) underlying T-cell clones triggering eosinophil expansion and/or activation through production of eosinopoietic cytokines, (5) adhesion receptors and other molecules mediating eosinophil homing and redistribution, such as VLA-4, and (6) signaling molecules responsible for cytokine-dependent or -independent eosinophil proliferation, migration, adhesion, or/and activation. Another aim for the future is to better understand cellular interactions and related molecular mechanisms underlying HE-induced organ damage (HES) in various organ systems. There is hope that an improved knowledge of mechanisms regulating end-organ damage will lead to the development of new treatment concepts and better therapies for patients with HES.

REFERENCES

1. Kita H. Eosinophils: multifaceted biological properties and roles in health and disease. *Immunol Rev*. 2011;242:161.
2. Yousefi S, Simon D, Simon HU. Eosinophil extracellular DNA traps: molecular mechanisms and potential roles in disease. *Curr Opin Immunol*. 2012;24:736.
3. Valent P, Klion AD, Horny H-P, et al. Contemporary consensus proposal on criteria and classification of eosinophilic disorders and related syndromes. *J Allergy Clin Immunol*. 2012;130:607.
4. Bochner BS, Schleimer RP. Mast cells, basophils, and eosinophils: distinct but overlapping pathways for recruitment. *Immunol Rev*. 2001;179:5.
5. Valent P. The phenotype of human eosinophils, basophils, and mast cells. *J Allergy Clin Immunol*. 1994;94:1177.
6. Bochner BS. Siglec-8 on human eosinophils and mast cells, and Siglec-F on murine eosinophils, are functionally related inhibitory receptors. *Clin Exp Allergy*. 2009;39:317.
7. Tai PC, Ackerman SJ, Spry CJ, et al. Deposits of eosinophil granule proteins in cardiac tissues of patients with eosinophilic endomyocardial disease. *Lancet*. 1987;1:643.
8. Gauvreau GM, Ellis AK, Denburg JA. Haemopoietic processes in allergic disease: eosinophil/basophil development. *Clin Exp Allergy*. 2009;39:1297.
9. Lampinen M, Carlson M, Håkansson LD, et al. Cytokine-regulated accumulation of eosinophils in inflammatory disease. *Allergy*. 2004;59:793.
10. Sadovnik I, Lierman E, Peter B, et al. Identification of Ponatinib as a potent inhibitor of growth, migration, and activation of neoplastic eosinophils carrying FIP1L1-PDGFRA. *Exp Hematol*. 2014;42:282.
11. Hoermann G, Cerny-Reiterer S, Sadovnik I, et al. Oncostatin M is a FIP1L1/PDGFRA-dependent mediator of cytokine production in chronic eosinophilic leukemia. *Allergy*. 2013;68:713.

12. Youngblood BA, Leung J, Falahati R, et al. Discovery, function, and therapeutic targeting of Siglec-8. *Cells*. 2020;10:19.
13. Cross NC, Reiter A. Fibroblast growth factor receptor and platelet-derived growth factor receptor abnormalities in eosinophilic myeloproliferative disorders. *Acta Haematol*. 2008;119:199.
14. Shomali W, Gotlib J. World Health Organization-defined eosinophilic disorders: 2019 update on diagnosis, risk stratification, and management. *Am J Hematol*. 2019;94:1149.
15. Roufosse F, Garaud S, de Leval L. Lymphoproliferative disorders associated with hypereosinophilia. *Semin Hematol*. 2012;49:138.
16. Burgstaller S, Kreil S, Waghorn K, et al. The severity of FIP1L1-PDGFRA-positive chronic eosinophilic leukaemia is associated with polymorphic variation at the IL5RA locus. *Leukemia*. 2007;21:2428.
17. Valent P, Gleich GJ, Reiter A, et al. Pathogenesis and classification of eosinophil disorders: a review of recent developments in the field. *Expert Rev Hematol*. 2012;5:157.
18. Plötz SG, Hüttig B, Aigner B, et al. Clinical overview of cutaneous features in hypereosinophilic syndrome. *Curr Allergy Asthma Rep*. 2012;12:85.
19. Gotlib J, Akin C. Mast cells and eosinophils in mastocytosis, chronic eosinophilic leukemia, and non-clonal disorders. *Semin Hematol*. 2012;49:128.
20. Valent P, Horny H-P, Bochner BS, et al. Controversies and open questions in the definitions and classification of the hypereosinophilic syndromes and eosinophilic leukemias. *Semin Hematol*. 2012;49:171.
21. Wimazal F, Germing U, Kundi M, et al. Evaluation of the prognostic significance of eosinophilia and basophilia in a larger cohort of patients with myelodysplastic syndromes. *Cancer*. 2010;116:2372.
22. Gleich GJ, Schroeter AL, Marcoux JP, et al. Episodic angioedema associated with eosinophilia. *N Engl J Med*. 1984;310:1621.
23. Vaglio A, Moosig F, Zwerina J. Churg-Strauss syndrome: update on pathophysiology and treatment. *Curr Opin Rheumatol*. 2012;24:24.
24. Schwartz LB, Sheikh J, Singh A. Current strategies in the management of hypereosinophilic syndrome, including mepolizumab. *Curr Med Res Opin*. 2010;26:1933.
25. Roufosse F, Kahn JE, Rothenberg ME, et al. Efficacy and safety of mepolizumab in hypereosinophilic syndrome: a phase III, randomized, placebo-controlled trial. *J Allergy Clin Immunol*. 2020;146:1397.
26. Jovanovic JV, Score J, Waghorn K, et al. Low-dose imatinib mesylate leads to rapid induction of major molecular responses and achievement of complete molecular remission in FIP1L1-PDGFRA-positive chronic eosinophilic leukemia. *Blood*. 2007;109:4635.
27. Apperley JF, Gardembas M, Melo J, et al. Response to imatinib mesylate in patients with chronic myeloproliferative diseases with rearrangements of the platelet-derived growth factor receptor beta. *N Engl J Med*. 2002;347:481.
28. Klion AD, Robyn J, Maric I, et al. Relapse following discontinuation of imatinib mesylate therapy for FIP1L1/PDGFRA-positive chronic eosinophilic leukemia: implications for optimal dosing. *Blood*. 2007;110:3552.
29. Radonjic-Hoesli S, Valent P, Klion AD, et al. Novel targeted therapies for eosinophil-associated diseases and allergy. *Annu Rev Pharmacol Toxicol*. 2015;55:633.
30. Harish A, Schwartz SA. Targeted anti-IL-5 therapies and future therapeutics for hypereosinophilic syndrome and rare eosinophilic conditions. *Clin Rev Allergy Immunol*. 2020;59:231.
31. Reiter A, Gotlib J. Myeloid neoplasms with eosinophilia. *Blood*. 2017;129:704.
32. Gerds AT, Gotlib J, Bose P, et al. Myeloid/lymphoid neoplasms with eosinophilia and TK fusion genes, version 3.2021, NCCN Clinical Practice Guidelines in Oncology. *J Natl Compr Canc Netw*. 2020;18:1248.

CHAPTER 75

MAST CELLS AND MASTOCYTOSIS

Jason Gotlib, Hans-Peter Horny, and Peter Valent

Paul Ehrlich used his 1878 doctoral thesis to characterize a new cell type—the mast cell (MC)—based on its reactivity to aniline dyes and the metachromatic appearance of its cytoplasmic granules.[1] He referred to MCs as *Mastzellen* and he speculated that their intracellular granules contained phagocytosed materials or nutrients. Ehrlich also described a close relationship in tissues between MCs and blood vessels, nerves, gland excretory ducts, as well as their proximity within the environment of tumors and chronic inflammation. In 1894, the pathobiologic association of MCs with lesions of urticaria pigmentosa was reported,[2] and from the 1930s forward, basic incremental discoveries about normal MC functions were made. Their capacity to undergo degranulation in response to various stimuli, their source of histamine and heparin, and their important role in anaphylaxis was examined.[3–10]

Perturbations of MC growth and function may lead to mastocytosis, a condition defined by pathologic expansion and accumulation of MCs in diverse tissues such as the skin, bone marrow (BM), spleen, liver, lymph nodes, and gastrointestinal (GI) tract. In 1949, the first case report of systemic mastocytosis (SM) was published. Later, between 1950 and 1980, several different subtypes of SM were described, including a leukemic variant termed *mast cell leukemia* (MCL). The first classification of mastocytosis was created by the Kiel group of pathologists, and in 1991 the first consensus classification was published. Criteria for SM and other variants of the disease were established between 1990 and 2000. In 2000, these criteria were discussed by a consensus group and were employed to establish a robust consensus classification of mastocytosis,[11] that was adopted by the World Health Organization (WHO) in 2001 and also in 2008.[12] In consecutive years, the consensus group published treatment response criteria and established a widely accepted update of the classification of mastocytosis in 2007.[13] In this updated classification, the smoldering state is defined as a separate category of SM which was confirmed by the WHO in 2016. In 2002, the European Competence Network on Mastocytosis (ECNM) was founded. Since then, the ECNM has facilitated basic science and translational research dealing with mastocytosis and related MC disorders. In 2019, the American Initiative in Mast Cell Diseases (AIM) was formed with similar and complementary goals to the ECNM, including fostering collaborative translational and clinical research projects in MC disorders. This chapter provides an overview of normal MC physiology and highlights contemporary issues related to the classification, diagnosis, and treatment of SM.

ORIGIN AND DEVELOPMENT OF MAST CELLS

The marrow origin of MCs was first demonstrated by engrafting BM cells into irradiated mice. Human MCs can be generated from CD34$^+$ hematopoietic stem and progenitor cells in response to stimulation with stem cell factor (SCF). In vitro MC differentiation models showed that circulating MC progenitors express CD13, CD34, and KIT, but lack CD14 and CD17.[14,15] MC progenitors are released from the BM into the circulation in a primitive state and undergo terminal maturation and differentiation after their migration to tissues. Although shared histomorphologic and biologic features of MCs and basophils include cytoplasmic basophilic granules, expression of high-affinity immunoglobulin (Ig)E receptors (FcεRI), and release of histamine upon stimulation, basophils exhibit several distinctive characteristics. Basophils circulate as mature cells, which are incapable of proliferation, and subsequently undergo apoptosis after their recruitment and activation in the tissues. Differential expression of the transcription factor CCAAT-enhancer-binding protein (CEBP)α may be a primary determinant of whether development is skewed toward basophil progenitors (CEBPα present) or MC progenitors (CEBPα absent). Although eosinophils and basophils have been found to share a common bipotential progenitor in humans, definitive data for a similar progenitor giving rise to both basophils and MCs are lacking. Rather, based on in vitro colony assays, MCs are directly derived from multilineage and lineage-restricted progenitors, but not from such a bi-potent precursor cell.[6] In line with this observation, evidence against a bilineage basophil/MC progenitor also came from tracking lineage involvement of *KIT* D816V in patients with SM, where *KIT* D816V was only found in basophils in a small subset of patients, namely those with multilineage involvement of the mutation. In addition, when highly enriched by cell sorting, neither mature blood basophils nor mature blood monocytes give rise to MCs in vitro.[16]

Collectively, these data suggest that normal human MCs are derived from CD34$^+$ stem and progenitor cells but not from a more mature myeloid cell and that MCs form a unique cell lineage in the hematopoietic cell system. Correspondingly, in 2019, Eisenwort and colleagues described that the leukemia-initiating stem cell in MCL resides within the CD34$^+$/CD38$^-$ fraction, but not in CD34$^+$/CD38$^+$ progenitors or bulk KIT$^+$/CD34$^-$ MCs.[17]

KIT and Stem Cell Factor (KIT Ligand)

KIT

Kit is the cellular homolog of the *v-Kit* oncogene of the Hardy-Zuckerman 4 feline sarcoma virus. The human *KIT* gene on chromosome 4q11–12 was found to be allelic to the white spotting locus (*W*) in mice, in which over 30 mutations have been identified. The common theme of mutant alleles at the *W* locus is decreased kit kinase activity either through missense mutations that generate kinase-defective kit (e.g., W^{42} [*kit* D790N]; W^{37} [*kit* E582K]; W^{v} [*kit* T660M]; and W^{41} [*kit* V831M]) or decreased expression of kit on the cell surface (e.g., the *W* allele, caused by a 78-amino acid deletion that involves the transmembrane (TM) region of the kit protein).

Double mutants at the murine *W* locus not only result in markedly decreased MC numbers, but also in pleiotropic phenotypes, including white coat color/spotting, sterility, and anemia, that respectively relate to the failure of melanocytes, germ cells, and hematopoietic progenitors to migrate and/or proliferate effectively during development (see Chapter 9). Heterozygosity or homozygosity for the different alleles at the *W* locus results in variable phenotypic effects on hematopoiesis, pigmentation, and fertility. Mutations at the *W* locus can also result in abnormalities of the interstitial pacemaker cells of Cajal, leading to functional gut abnormalities, such as megacolon. Intriguingly, some of these clinical correlates can also be seen in patients undergoing long-term treatment with a strong KIT inhibitor, like imatinib: these individuals can develop MC deficiency, depigmented skin areas, cytopenias, and fertility problems.

KIT is a member of the type III receptor tyrosine kinases (TKs) that also include platelet-derived growth factor receptor (PDGFR)-α and -β, FMS-like tyrosine kinase-3 (FLT3), vascular endothelial growth factor receptor 2 (VEGFR2), and the receptor for macrophage colony-stimulating factor (FMS).[18,19] These TKs share common structural motifs including an extracellular domain containing five Ig-like motifs that bind their specific ligands, a short TM domain that anchors KIT to the cell membrane, a cytoplasmic TK domain that is split by an insert sequence into ATP-binding and phosphotransferase regions, and a juxtamembrane (JM) domain that lies between the TM and TK domains. KIT is expressed on hematopoietic stem/progenitor cells and is also essential for gametogenesis and melanogenesis. KIT expression is lost when hematopoietic cells undergo differentiation. However, MCs do retain persistent and high-level expression of KIT (CD117). This can be exploited for the purposes of MC identification by immunohistochemistry and/or flow immunophenotyping (in conjunction with other surface and cytoplasmic markers).

The two major splice variants of KIT differ by the presence or absence of four amino acids (GNNK) at the extracellular JM region. In NIH3T3 cells, the GNNK$^-$ isoform induced loss of contact inhibition, anchorage-independent growth, and tumorigenicity in mice, whereas the GNNK$^+$ isoform did not exhibit most of these characteristics. Also, despite similar binding of SCF to KIT, the GNNK$^-$ isoform displayed more rapid and extensive tyrosine autophosphorylation and faster internalization. Preferential expression of the GNNK isoform has been noted in small cohorts of patients with SM and germ cell tumors, but larger series are needed to determine the relevance of these KIT isoforms to disease pathogenesis.

Stem Cell Factor

SCF (KIT ligand) was identified by three groups in 1990 as the principal growth and differentiation factor for human MCs.[20–22] In fact, SCF was found to induce MC differentiation from CD34$^+$ cells or blood/BM mononuclear cells.[23,24] SCF is produced by fibroblasts and endothelial cells, and promotes the proliferation, differentiation, survival, and migration of hematopoietic progenitors, melanocytes, MCs, and germ cells.

Loss-of-function mutations at the *Sl* (steel) locus on chromosome 10 in mice were found to phenocopy mutations at the *W* locus (see Chapter 9). Ultimately, the gene product of the *Sl* locus was found to be the ligand for kit, SCF. In the case of mutations affecting the *W* (kit) locus, transplantation of BM cells from congenic +/+ mice into W/W^v mice corrected MC deficiency and anemia, consistent with an intrinsic progenitor/stem cell defect. In contrast, skin and BM engraftment experiments demonstrated that mutations at the *Sl* locus were cell extrinsic (e.g., microenvironmental) in nature. When skin from either W/W^v or Sl/Sl^d mice was transplanted onto the backs of congenic +/+ mice, MCs were capable of engrafting the skin from W/W^v mice but not the skin from Sl/Sl^d mice. In addition, hematologic abnormalities in Sl/Sl^d mice failed to correct with transplantation of BM cells from congenic +/+ animals. These experiments formally established that SCF is a physiologically relevant ligand of kit and that development is critically dependent on this ligand-receptor interaction. Therefore, SCF has also been termed *MC growth factor*.

Alternative splicing results in two isoforms of membrane-bound SCF with different susceptibility to proteolytic cleavage: a longer isoform that retains exon 6 and contains a cleavage site that results in soluble SCF, and a shorter isoform lacking the cleavage site, which remains membrane-bound and serves as a stem cell homing receptor in the BM niche. Both the membrane-bound and soluble form of SCF (which exists in a homodimeric confirmation) are capable of binding to and inducing homodimerization of KIT, which, in turn, leads to autophosphorylation of tyrosine residues located in intracellular portions of the molecule. A cascade of signaling events ensues via downstream effector molecules, including Src kinases, c-Jun N-terminal kinase (Jnk), mitogen-activated protein (MAP) kinases, and the Janus-activated kinase (JAK)-signal transducer and activator of transcription and phosphatidylinositol 3-kinase (PI3K)/AKT pathways.[25–27] In addition to promoting MC differentiation and survival, SCF can regulate MC adhesion to extracellular matrix proteins as well as mediator release via IgE-dependent and IgE-independent mechanisms. Likewise, when applied at relatively high concentrations for 90 minutes, SCF is capable of directly inducing mediator secretion from human MCs. Whereas in mice several different cytokines are involved in the regulation of growth and activation of MCs, SCF seems to be a rather specific cytokine for human MCs. Other studies have shown that certain cytokines or hormonal factors exert inhibitory effects on MCs (e.g., interferon [IFN]-γ), and some cytokine pairs counterbalance each other's effects (e.g., T-helper 2 [Th2] cytokine interleukin [IL]-4 vs. regulatory T-cell cytokine TGF-β1) on MC homeostasis. Expression of the death receptor tumor necrosis factor–related apoptosis-inducing ligand and inhibitor receptors CD300a and Siglec-8 may also contribute to downregulation of MC activation, survival, and IgE-mediated responses.[28,29]

MAST CELL ACTIVATION AND FUNCTION

MCs commonly reside at the interface between the host and environment, usually in the skin, or in the mucosa lining the lungs or GI tract. Migration and homing of MCs to various sites is regulated by the interaction between surface expression of numerous types of chemokine receptors (e.g., CXC chemokine receptor 2) and integrins (e.g., α4β7, α4β1) on MCs to tissue-specific endothelial binding sites such as mucosal vascular address in cell adhesion molecule-1 (MAdCAM-1) and vascular cell adhesion molecule-1 (VCAM-1). In addition to playing a primary role in mediating allergic responses and inflammation, MCs act as one arm of the adaptive immune system by playing a role in host defense against pathogens such as bacteria, fungi, and viruses.

MCs serve as a rich source of a variety of biologically active molecules (Table 75.1).[10] These include preformed mediators such as vasoactive amines (histamine), anionic proteoglycans (heparin, chondroitin sulfate), and proteases (tryptase, chymase, and carboxypeptidase). Activated MCs also contribute to de novo synthesis of lipid-derived mediators that constitute the slow-reacting substance of anaphylaxis (SRS-A). These mediators include lipoxygenase-derived metabolites of arachidonic acid—the cysteinyl leukotrienes LTC4, LTD4, and LTE4, and cyclooxygenase-derived prostaglandin (PG)D2. These substances are involved in mediating vasodilation and vasopermeability, smooth muscle constriction, mucus secretion, and other proinflammatory sequelae. IgE- and non-IgE–dependent mechanisms (e.g., IgG, complement [C3a, C5a], neuropeptides, narcotics, physical stimuli, and activation of Toll-like receptors by bacterial products) can induce MCs to release a variety of cytokines (e.g., TNF-α, macrophage inflammatory protein [MIP]-1α and -1β, monocyte chemoattractant protein [MCP]-1, IFN-α, -β, and -γ, and various ILs) and growth factors (see Table 75.1) that are implicated in the aforementioned physiologic roles of inflammation, allergic responses, and host defense.[10,30] MCs mediate early-phase (e.g., anaphylaxis, acute asthma) and late-phase allergic responses, as well as non–type I hypersensitivity reactions through their proinflammatory mediators. Furthermore, MCs mediate upregulation of Th2 responses and allergen-specific IgE biosynthesis, which contribute to host defense against parasitic infections. MCs have also been implicated in the pathogenesis of various nonallergic conditions such as infectious or autoimmune diseases (e.g., multiple sclerosis, psoriasis, rheumatoid arthritis, inflammatory bowel disease), and also in repair after tissue injury, such as wound healing, thrombolysis, or tissue remodeling.

Coupling of MCs and eosinophils often occurs in the same tissue niches in allergic/inflammatory and neoplastic disorders such as eosinophilic esophagitis, chronic gastritis, GI neoplasms, parasitic infections, and inflammatory bowel disease. MCs can initiate the allergic inflammatory cascade, resulting in recruitment of eosinophils

TABLE 75.1 Human Mast Cell Cell–Derived Mediators

Class	Mediators	Physiologic Effects
Preformed mediators	Histamine, serotonin, heparin, neutral proteases (tryptase and chymase, carboxypeptidase, cathepsin G), major basic protein, acid hydrolases, peroxidase, phospholipases	Vasodilation, vasoconstriction, angiogenesis, mitogenesis, pain, protein processing/degradation, lipid/proteoglycan hydrolysis, arachidonic acid generation, tissue damage and repair, inflammation
Lipid mediators	LTB4, LTC4, PGE2, PGD2, PAF	Leukocyte chemotaxis, vasoconstriction, bronchoconstriction, platelet activation, vasodilation
Cytokines	TNF-α, TGF-β, IFN-α, IFN-β, IL-1α, IL-1β, IL-5, IL-6, IL-13 IL-16, IL-18	Inflammation, leukocyte migration/proliferation
Chemokines	IL-8 (CXCL8), I-309 (CCL-1), MCP-1 (CCL2), MIP-1αS (CCL3), MIP1β (CCL4), MCP-3 (CCL7), RANTES (CCL5), eotaxin (CCL11), MCAF (MCP-1)	Chemoattraction and tissue infiltration of leukocytes
Growth factors	SCF, M-CSF, GM-CSF, bFGF, VEGF, NGF, PDGF	Growth of various cell types, vasodilation, neovascularization, angiogenesis

The mediators in the table are examples only. In addition, many mediators are identified in mouse mast cells, human mast cell lines, or primary cultures of human mast cells and may not be produced in vivo.
IFN, Interferon; *IL*, Interleukin; *MCP*, monocyte chemoattractant protein; *MIP*, macrophage inflammatory protein; *SCF*, stem cell factor.
From Metcalfe DD. Mast cells and mastocytosis. *Blood*. 2008;112:946.

during the later phases of an allergen encounter. Both MCs and eosinophils express CCR3 and respond to eotaxins and RANTES (CCL5), leading to recruitment of both cell types into inflamed tissue (see Chapters 14 and 15).[31] Moreover, MC-derived heparin can bind and stabilize eotaxins. In addition, MCs and eosinophils can interact with each other through soluble mediators or via direct cell-to-cell contact. At least in the murine system, MCs can also be induced to produce IL-3 and IL-5, which can potentiate eosinophil recruitment in tissues.[32] The two principal MC-derived proteases exert dual actions on eosinophils. For example, MC-derived chymase suppresses eosinophil apoptosis and induces release of IL-6, CXCL8, CCL2, and CXCL1 from eosinophils. In contrast, β-tryptase can cleave eotaxin and RANTES, and can limit eosinophil chemotaxis. Eosinophils in turn produce SCF, which can attract more tissue MCs and protect them from apoptosis.

IgE-mediated hypersensitivity reactions (e.g., allergic rhinitis or asthma, anaphylaxis) are mediated by binding of allergen-specific IgE to the high-affinity FcεRI receptor, which is constitutively expressed at a high level on MCs. Engagement of FcεRI leads to its inclusion in lipid rafts, and phosphorylation of its β- and γ-chain immunoreceptor tyrosine-based activation motifs (ITAMs) by Lyn kinase and subsequent activation of Syk kinase through its binding to the ITAMs.[27] These proximal signaling events also activate Fyn kinase, which is important for phosphorylation of the adaptor protein Gab2 and activation of the PI3K pathway. Lyn kinase also regulates phosphorylation of the protein scaffolds LAT and NTAL, which coordinate multiple signaling pathways including Ras/MAP kinase, TEC family kinases, and PLCγ activation.[33] PLCγ, in turn, is a critical mediator of MC cytokine production and degranulation.

Experimental data indicate that the functions of KIT and FcεRI in MCs are interdependent. It has been observed that SCF enhances FcεRI-mediated MC degranulation, and that phosphorylation of the membrane adaptor molecule NTAL is a crucial link between the signaling cascades following KIT activation and FcεRI aggregation. In addition, BTK is a mediator of FcεRI-mediated MC degranulation and plays a critical role in the amplification of FcεRI activation by KIT.[34]

TOOLS TO STUDY HUMAN MAST CELLS

The challenges of studying MCs relate to heterogeneity in their morphology and function between different species, and also within different tissues of the same organism. Although several techniques, including flow cytometry-based cell sorting, exist for ex vivo isolation of BM MCs, yields are typically low, reflecting the low numbers of MCs in the BM and the difficulties in isolating them from other tissues, such as the lung. Ex vivo generation of human cord blood–derived or peripheral blood–derived MCs from their CD34$^+$ or CD133$^+$ progenitor cells using SCF and IL-6 can generate large numbers of functionally mature MCs that can be interrogated for various types of biologic investigations. Many studies of MCs and/or mastocytosis have relied on MC lines such as the SCF-dependent cell lines LAD1/LAD2, derived from patients with MC sarcoma (MCS) with *KIT* D816V (this cell line actually has normal *KIT* despite its origin, and is SCF-dependent); the LUVA cell line (derived from CD34$^+$ cells, growth factor–independent, normal *KIT*, and tendency to lose FcεRI expression with long-term culture); and the SCF-independent human MC line HMC-1 derived from a patient with MCL. Two HMC-1 subclones are available—HMC-1.1, which contains the *KIT* V560G mutation, and HMC-1.2, which expresses both *KIT* V560G and D816V. Both clones have been useful for in vitro evaluation of the differential sensitivity of different small-molecule inhibitors of KIT. None of these human MC lines, however, express both a functional IgE receptor and *KIT* D816V. However, a human cord blood–derived cell line that is SCF-dependent and FcεRI$^+$ (ROSA$^{\text{KIT WT}}$) has been developed which converts to SCF independence and produces tumors with transfection by *KIT* D816V (ROSA$^{\text{KIT D816V}}$), and is widely used as a tool for studying the biology and pharmacologic aspects of mastocytosis.[35] Other human MC lines that have been studied in the context of mastocytosis are LAD2, LADR, and MCPV-1.

Mouse Models to Study Human Mastocytosis

A number of murine models of mastocytosis are available, including several transgenic models and xenotransplantation models. First, all the cell lines described earlier have been applied successfully in xenotransplantation models. In most instances, NSG mice were employed. In addition, several transgenic models have been established. In one model, transgenic mice harboring a fusion transgene consisting of the 571-bp primate chymase gene (Δ571-bchm) promoter fragment and the human Kit proto-oncogene cDNA with the codon 816 Asp→Val substitution were generated. These mice were found to slowly develop a MC disease-like condition with focal accumulations of MCs in the spleen and other organs, resembling indolent SM (ISM). In another model, a transgenic mouse exhibiting murine KIT *D814V* was established. These mice developed GI MC accumulations. Both models may not reflect all aspects of the human disease, but may be helpful for future investigations.

FIP1L1::PDGFRA- and *NPM::ALK*-Based Murine Models

Expression of the *FIP1L1::PDGFRA* fusion alone in murine BM cells was not sufficient to cause eosinophilia, but only a general myeloproliferative syndrome.[36] However, overexpression of IL-5 together with *FIP1L1::PDGFRA* produced typical features closely resembling HES, including tissue infiltration by eosinophils.[37] However, a phenotype of mastocytosis in addition to eosinophilia developed in another murine model by introducing *FIP1L1::PDGFRA* into hematopoietic stem cells and progenitors with T-cell overexpression of IL-5.[38] Transplantation of *NPM::ALK* (nucleophosmin-anaplastic lymphoma kinase)–transduced progenitors into normal mice or IL-9–transgenic mice without *NPM::ALK* each result in MC hyperplasia; however, both "single-hit" models are insufficient to generate a histopathologic picture of SM.[39] SM is only observed when *NPM::ALK* is transduced into mouse BM progenitors of lethally irradiated IL-9 transgenic mice. Similar to the model of *FIP1L1::PDGFRA* and IL-5, this model of the *NPM::ALK* fusion and IL-9 highlights the biologic interdependence of a fusion oncogene and cytokine-related pathways in promoting the full expression of neoplastic MC growth.

Germline Susceptibility to Mast Cell Disorders and Familial Mastocytosis

In general, mastocytosis behaves as an acquired, somatic disease. However, several familial cases (more frequently presenting as cutaneous rather than systemic disease, and sometimes associated with GI stromal tumors [GIST]) have been reported. In most of these families, mutations in various codons of the *KIT* gene (e.g., K509I, A533D, W557R,V559A, V560del, D579del, K622E/T, D820Y, N822I, M835K, S849I, or deletion of amino acids 419 or 559–560) have been detected in the germline.[40–42] However, in a few families, the *KIT* mutation was only expressed in hematopoietic cells, but not in the germline of the affected individuals, suggesting that predisposing germline factors may contribute to disease development. However, so far, little is known about what gene polymorphisms serve as susceptibility alleles. In one study, polymorphisms within certain cytokine genes have been identified as exerting a potential predisposition to development of SM. In particular, a polymorphism in the promoter of the *IL-13* gene, −1112 C/T, was significantly more frequent in SM patients versus both cutaneous mastocytosis (CM) patients and healthy controls.[43] In addition −1112 C/T was associated with increased serum tryptase levels and adult-onset disease. MCs express IL-13 receptors and IL-13–containing medium provides enhanced support for the growth of MCs. However, it remains unclear how −1112 C/T relates to these specific biologic findings. In contradistinction to the IL-13 −1112 C/T germline allele, the *IL-4α* chain receptor Q576 polymorphism was associated with more limited forms of mastocytosis. IL-4 modulates the growth and differentiation of MCs, and induces IgE receptor expression in MCs.[44] Polymorphisms in the IL-4 receptor have been implicated in allergic and inflammatory conditions. In a study of patients with cutaneous and systemic forms of mastocytosis, this polymorphic allele was significantly more associated with cutaneous disease, childhood-onset disease, and lower levels of serum tryptase and soluble CD117. The Q576 polymorphism in the *IL-4α* chain receptor therefore appears to protect against MC hyperplasia and more aggressive forms of the disease. The Asp358Ala polymorphism in the *IL-6* receptor was associated with a 2.5-fold lower risk for mastocytosis compared with those with the AC or CC genotypes.[45] However, no association was found between the *IL-6* 174 G/C polymorphism and increased susceptibility to SM.

EPIDEMIOLOGY AND CLASSIFICATION OF MASTOCYTOSIS

In 2008, SM was included in the WHO category of myeloproliferative neoplasms (MPNs).[12] However, based on the complex clinical picture, biology, and pathology, SM was again defined by the WHO as a separate unique myeloid neoplasm in 2016. SM is an orphan disease and sparse data exist regarding its incidence or other epidemiologic features. A slight male predominance has been observed, and in one study, the median age at time of SM diagnosis was 55 years. A retrospective study from Denmark that canvassed the years from 1997 to 2010 estimated the incidence of SM at 0.89 per 100,000 persons per year, with an estimated prevalence of 9.59 per 100,000 individuals.[46] A Dutch analysis estimated the prevalence of ISM to be 13 cases per 100,000 inhabitants.[47]

The first diagnostic checkpoint is to distinguish SM from CM, which is characterized by predominant skin involvement. Criteria to diagnose SM are not fulfilled in these patients. However, in rare cases with CM, a discrete involvement of the BM by clonal cells may be detected, and serum tryptase levels may be slightly elevated. SM primarily occurs in adults, whereas CM is more common in children and its natural history is typically defined by spontaneous resolution of skin lesions usually at the time of puberty.[48] CM variants include urticaria pigmentosa/maculopapular CM, diffuse CM, and solitary mastocytoma of the skin.[12,49] In the overwhelming majority of adults with cutaneous involvement, systemic disease (SM) is diagnosed. However, absence of skin lesions does not exclude the presence of SM. Skin involvement occurs in over 80% of all patients with adult SM. However, sometimes no BM examination is performed for some time, especially when the patient has not been referred to a hematologist. In such cases, cutaneous lesions are referred to as *mastocytosis in the skin* (MIS).

Diagnostic criteria for SM were initially formulated by a working group of MC disease experts at a consensus conference in Vienna in 2000,[11] and adopted by the WHO in both 2001[50] and 2008.[12] In 2022, the WHO[51a] and International Consensus Classification (ICC)[51b] generated some minor modifications to the classification of SM and its variants. Minimal diagnostic criteria for SM requires at least one major plus one minor criterion, or at least three minor criteria (Table 75.2). The major criterion is the presence of multifocal dense aggregates of MCs (>15 MCs in aggregates) in sections of BM or other extracutaneous organ(s); minor criteria include: (1) >25% of the MCs in the infiltrate or in the BM smear are of spindle shape or of otherwise atypical morphology; (2) activating mutation at codon 816 in the *KIT* gene (or other activating *KIT* mutation) in an extracutaneous organ; (3) expression of CD25 +/− CD2 or CD30 in/on MCs (2022 WHO and ICC modifications); and (4) serum tryptase level >20 ng/mL, unless there is an associated myeloid disorder that makes this parameter not valid.[51]

SM is further divided into several subtypes that reflect the presence or absence of certain clinical or laboratory findings (Tables 75.3 and 75.4). The most common form of SM in adults is ISM, characterized by a slightly to markedly increased burden of neoplastic MCs in the BM and/or other extracutaneous organ(s). By definition, ISM patients do not exhibit MC-related organ damage or an associated

TABLE 75.2 **2022 World Health Organization and International Consensus Classification (ICC) Diagnostic Criteria for Systemic Mastocytosis**[a]

Major criterion	Multifocal dense infiltrates of mast cells (>15 mast cells in aggregates) detected in sections of bone marrow and/or other extracutaneous organ(s)
Minor criteria	a. In biopsy sections of bone marrow or other extracutaneous organs, >25% of the mast cells in the infiltrate are spindle shaped or have atypical morphology or, of all mast cells in bone marrow aspirate smears, >25% are immature or atypical b. Detection of an activating point mutation at codon 816 in *KIT* (or other activating *KIT* mutation in bone marrow, blood, or another extracutaneous organ) c. Mast cells in bone marrow, blood, or other extracutaneous organs express CD25 with or without CD2 or CD30 in addition to normal mast cell markers d. Serum total tryptase persistently exceeds 20 ng/mL (unless there is an associated clonal myeloid disorder, in which case this parameter is not valid)

[a] Requires at least 1 major + 1 minor criterion or 3 minor criteria.

TABLE 75.3 2022 World Health Organization Variants of Mastocytosis

Cutaneous mastocytosis (CM)

a. Maculopapular CM
b. Diffuse CM
c. Mastocytoma of skin

Systemic mastocytosis (SM)

a. Indolent systemic mastocytosis (ISM)
b. Bone marrow mastocytosis (BMM)[a]
c. Smoldering systemic mastocytosis (SSM)
d. Aggressive systemic mastocytosis (ASM)
e. Systemic mastocytosis with an associated hematologic neoplasm (SM-AHN)[b]
    SM-MDS
    SM-MPN (e.g., PV, ET, MF, CML)
    SM-MDS/MPN (e.g., CMML, MDS/MPN-unclassifiable)
    SM-CEL
    SM-AML
f. Mast cell leukemia (MCL)
    Aleukemic vs. leukemic MCL
    Chronic vs. acute MCL
    Primary vs. secondary MCL
    MCL with or without an AHN

Mast cell sarcoma (MCS)

[a] In the 2022 WHO Classification and 2020 Working Conference consensus proposal, BMM is a separate category from ISM; in the International Consensus Classification (ICC), it remains a subtype of ISM.[57a] BMM is a separate category from ISM. It is defined the same as ISM but without skin lesions and is typically characterized by lower tryptase levels and absence of both B and C findings.

[b] The table shows typical examples of SM-AHN. Most AHN are myeloid neoplasms.

AML, Acute myeloid leukemia; *CEL, NOS*, chronic eosinophilic leukemia, not otherwise specified; *CLL*, chronic lymphocytic leukemia; *CML*, chronic myeloid leukemia; *CMML*, chronic myelomonocytic leukemia; *ET*, essential thrombocythemia; *MDS*, myelodysplastic syndrome; *MF*, myelofibrosis; *MPN*, myeloproliferative neoplasm; *NHL*, non–Hodgkin lymphoma; *PV*, polycythemia vera.

TABLE 75.4 2022 World Health Organization Diagnostic Criteria for Variants of (Systemic) Mastocytosis

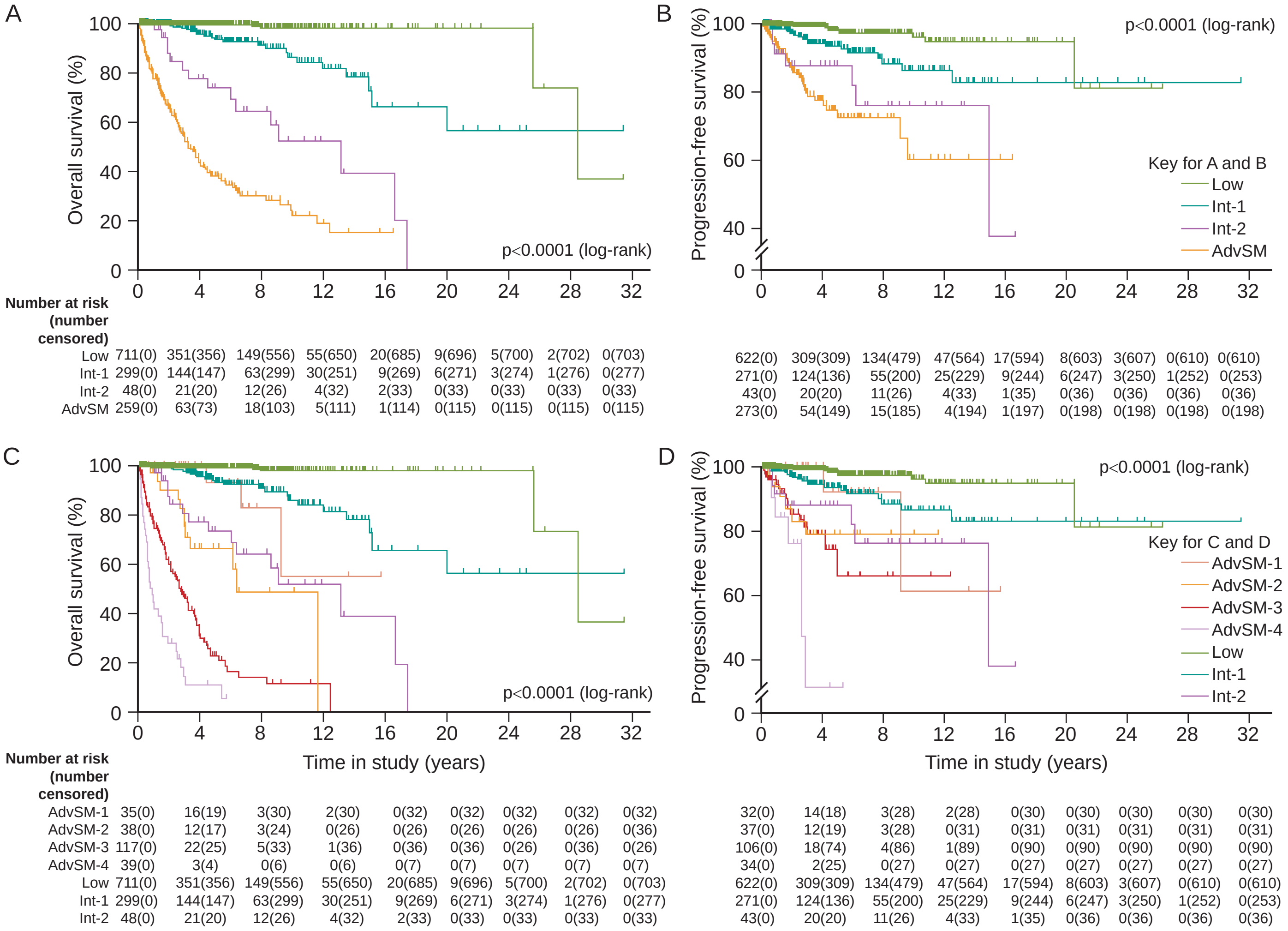
1. **Indolent systemic mastocytosis (ISM):** Meets criteria for SM. No "B" or "C" findings. No evidence of associated clonal hematologic malignancy/disorder. In this variant, the mast cell burden is usually low; skin lesions are almost invariably present.
2. **Bone marrow mastocytosis:** As above, but no skin lesions and a serum tryptase level <125 ng/mL with bone marrow involvement, but no skin lesions.
3. **Smoldering systemic mastocytosis (SSM):** Meets criteria for SM. Two or more "B" findings and no "C" findings.
4. **Systemic mastocytosis with an associated hematologic neoplasm (SM-AHN):** Meets criteria for SM and criteria for an associated hematologic neoplasm (MDS, MPN, MDS/MPN, CEL, AML, lymphoma, or other hematologic neoplasm that meets the criteria for a distinct entity in the WHO classification).
5. **Aggressive systemic mastocytosis (ASM):** Meets criteria for SM. One or more "C" findings. No associated clonal hematologic neoplasm. No evidence of mast cell leukemia.
6. **Mast cell leukemia (MCL):** Meets criteria for SM. Bone marrow biopsy shows diffuse infiltration, usually interstitial pattern, by atypical, immature mast cells. Bone marrow aspirate smears show 20% or more mast cells. Cases in which <10% of circulating WBCs are mast cells are referred to as "aleukemic mast cell leukemia."
7. **Mast cell sarcoma (MCS):** Unifocal mast cell tumor. No evidence of SM. No skin lesions. Destructive growth pattern. High-grade cytology.

AML, Acute myeloid leukemia; *CEL*, chronic eosinophilic leukemia; *MDS*, myelodysplastic syndrome; *MPN*, myeloproliferative neoplasm; *SM*, systemic mastocytosis; *WBC*, white blood cell; *WHO*, World Health Organization.

hematologic disorder, and life expectancy in this variant has been shown to be similar to that of an age-matched healthy population,[52] with an analysis showing a 10-year overall survival (OS) of 93.5% (Fig. 75.1).[53] However, ISM patients often experience mediator symptoms related to MC degranulation (e.g., flushing, pruritis, diarrhea, abdominal cramping, neuropsychiatric complaints). Although triggers may not always be identifiable, known provokers of MC activation and/or anaphylaxis include non-IgE and IgE-mediated causes: physical or emotional stress, exercise, hot or cold temperature stimuli, medications such as aspirin, NSAIDs, opioid analgesics, or antibiotics, alcohol, radiocontrast dye, and IgE-mediated allergies. One critical comorbidity is Hymenoptera (e.g., wasps, bees, and ants) venom allergy. In particular, these patients are at high risk of developing severe or even fatal anaphylaxis upon exposure to the venom. Cutaneous involvement usually develops in conjunction with the ISM subtype, both in younger individuals as well as in older patients. However, those with advanced disease may also develop MIS. Interestingly, skin disease usually portends a decreased risk of anaphylaxis, especially due to Hymenoptera stings.[54,55] Bony disease (e.g., localized bone pain, diffuse osteopenia, osteoporosis with or without pathologic fractures, osteosclerosis) is also well described in ISM, but can cause morbid complications across the spectrum of SM variants.[56,57] Bone marrow mastocytosis (BMM) is defined similarly as ISM but without skin lesions and typically exhibits lower tryptase levels, e.g. <125 ng/mL. It is listed as a subtype of ISM the 2016 WHO diagnostic criteria, but as a separate category in the 2020 Working Conference consensus proposal of mastocytosis.[57a]

In the 2008 WHO classification, smoldering SM (SSM) was designated as a subvariant of ISM. However, based on the current classification proposed by the consensus group, SSM was included in the revised 2016 WHO classification as a separate variant of SM, which is meaningful because these patients exhibit a higher chance of progression to more advanced disease.[12,51,52,58–60] SSM is defined by two or more "B" findings (Table 75.5), for example, (1) hepatomegaly without impairment of liver function, and/or palpable splenomegaly without hypersplenism, and/or lymphadenopathy on palpation or imaging; (2) BM MC burden >30% and serum tryptase level >200 ng/mL; and (3) signs of dysplasia or myeloproliferation, in non-MC lineage(s), but insufficient criteria for definitive diagnosis of an additional hematopoietic neoplasm, with normal or only slightly abnormal blood counts.[12,51,52] Compared with ISM, SSM patients often exhibit clonal multilineage involvement with the *KIT* D816V mutation, which is also a prognostically relevant variable.[61,62] In the 2022 WHO classification, a KIT D816V variant allele frequency ≥10% qualifies as a "B" finding.

Advanced SM (AdvSM) is an umbrella term for three variants that are characterized by decreased life expectancy: SM with an associated hematologic neoplasm (SM-AHN), aggressive SM (ASM), and MCL. SM-AHN comprises 30% of SM variants,[63] and approximately two-thirds of advSM cases, although this figure may vary depending on referral patterns. In the revised 2016 WHO classification, the term "SM-AHN" can be used interchangeably with or in lieu of the prior term "SM-AHNMD" (SM with an associated hematologic non-MC lineage disease).[51] The vast majority of AHNs are myeloid neoplasms: myelodysplastic syndrome (MDS), MPNs, MDS/MPN overlap disorders such as chronic myelomonocytic leukemia (CMML) or MDS/MPN-unclassifiable, chronic eosinophilic leukemia, not otherwise specified (CEL, NOS), and acute myeloid leukemia (AML).[64,65] Rarely, lymphoid neoplasms such as chronic lymphocytic leukemia, myeloma, or lymphomas have been found in association with



Number at risk (number censored)

	0	4	8	12	16	20	24	28	32
Low	711(0)	351(356)	149(556)	55(650)	20(685)	9(696)	5(700)	2(702)	0(703)
Int-1	299(0)	144(147)	63(299)	30(251)	9(269)	6(271)	3(274)	1(276)	0(277)
Int-2	48(0)	21(20)	12(26)	4(32)	2(33)	0(33)	0(33)	0(33)	0(33)
AdvSM	259(0)	63(73)	18(103)	5(111)	1(114)	0(115)	0(115)	0(115)	0(115)

0	4	8	12	16	20	24	28	32
622(0)	309(309)	134(479)	47(564)	17(594)	8(603)	3(607)	0(610)	0(610)
271(0)	124(136)	55(200)	25(229)	9(244)	6(247)	3(250)	1(252)	0(253)
43(0)	20(20)	11(26)	4(33)	1(35)	0(36)	0(36)	0(36)	0(36)
273(0)	54(149)	15(185)	4(194)	1(197)	0(198)	0(198)	0(198)	0(198)

Number at risk (number censored)

	0	4	8	12	16	20	24	28	32
AdvSM-1	35(0)	16(19)	3(30)	2(30)	0(32)	0(32)	0(32)	0(32)	0(32)
AdvSM-2	38(0)	12(17)	3(24)	0(26)	0(26)	0(26)	0(26)	0(26)	0(36)
AdvSM-3	117(0)	22(25)	5(33)	1(36)	0(36)	0(36)	0(26)	0(36)	0(26)
AdvSM-4	39(0)	3(4)	0(6)	0(6)	0(7)	0(7)	0(7)	0(7)	0(7)
Low	711(0)	351(356)	149(556)	55(650)	20(685)	9(696)	5(700)	2(702)	0(703)
Int-1	299(0)	144(147)	63(299)	30(251)	9(269)	6(271)	3(274)	1(276)	0(277)
Int-2	48(0)	21(20)	12(26)	4(32)	2(33)	0(33)	0(33)	0(33)	0(33)

0	4	8	12	16	20	24	28	32
32(0)	14(18)	3(28)	2(28)	0(30)	0(30)	0(30)	0(30)	0(30)
37(0)	12(19)	3(28)	0(31)	0(31)	0(31)	0(31)	0(31)	0(31)
106(0)	18(74)	4(86)	1(89)	0(90)	0(90)	0(90)	0(90)	0(90)
34(0)	2(25)	0(27)	0(27)	0(27)	0(27)	0(27)	0(27)	0(27)
622(0)	309(309)	134(479)	47(564)	17(594)	8(603)	3(607)	0(610)	0(610)
271(0)	124(136)	55(200)	25(229)	9(244)	6(247)	3(250)	1(252)	0(253)
43(0)	20(20)	11(26)	4(33)	1(35)	0(36)	0(36)	0(36)	0(36)

Figure 75.1 OVERALL SURVIVAL AND PROGRESSION-FREE SURVIVAL OF PATIENTS WITH MASTOCYTOSIS ACCORDING TO THE INTERNATIONAL PROGNOSTIC SCORING SYSTEM FOR MASTOCYTOSIS (IPSM). Overall survival was determined in all patients in whom the score could be calculated. Mast cell leukemia, the most aggressive mast cell disorder (cannot progress into a more aggressive variant) and mastocytosis in the skin, a provisional diagnosis for patients with proven skin involvement but unknown/unavailable bone marrow results, were excluded from analysis of progression-free survival. Overall survival (A) and progression-free survival (B) were estimated according to the method of Kaplan and Meier. In these analyses, patients with Low, Intermediate (Int)-1, and Int-2 risk groups differed significantly in their overall survival and progression-free survival and had a favorable outcome compared to patients with advanced systemic mastocytosis (AdvSM) according to log rank test ($P < .0001$). Estimated overall survival (C) and progression-free survival (D) according to the IPSM for patients with AdvSM. overall survival and progression-free survival differed significantly between the cohort without risk factors (AdvSM-1), patients with one risk factor (AdvSM-2), those with two to three risk factors (AdvSM-3), and those with four to five risk factors (AdvSM-4) ($P < .0001$). The overall survival of patients in the risk groups AdvSM-1 and AdvSM-2 were similar to that of non-advanced mastocytosis patients in the int-1 and int-2 risk group, respectively. *AdvSM*, Advanced systemic mastocytosis.

TABLE 75.5 **"B" Findings: Indication of High Mast Cell Burden**[a]

1. Bone marrow mast cell burden >30% by histology and serum tryptase level >200 ng/mL
2. Hypercellular marrow with loss of fat cells, discrete signs of dysmyelopoiesis without substantial cytopenias, and without WHO criteria for an MDS or MPN
3. Organomegaly: palpable hepatomegaly, splenomegaly, or lymphadenopathy (on CT or ultrasound) >2 cm without impaired organ function

[a] In the 2022 WHO 5th edition. a KIT d816V variant allele frequency ≥10% qualifies as a "B" finding.

MDS, Myelodysplastic syndrome; *MPN*, myeloproliferative neoplasm; *WHO*, World Health Organization.

SM.[66,67] Identifying a concomitant myeloid neoplasm may depend on several factors, including the expertise of the evaluating pathologist, and whether one disease is masked by the presence of another. For example, in cases of SM-AML, MC aggregates may only be unmasked after induction chemotherapy with achievement of BM hypoplasia, because neoplastic MCs may persist after such therapy.[68,69] Distinguishing nonhematologic or hematologic organ damage due to the SM component versus the associated myeloid disease can be very difficult, if not impossible, in some patients. Even when a biopsy of the involved extramedullary organ is analyzed to elucidate the burden of neoplastic MCs versus associated myeloid neoplasm, it is sometimes impossible to define the relative impact of the SM versus AHN component on organ dysfunction, especially when both disease components are of an aggressive type.

Although the prognosis of SM-AHN frequently relates to the AHN component, the burden of SM as well as the type and stage of the associated myeloid neoplasm need to be considered on an individual basis. Treatment plans are tailored to the histopathologic and molecular findings and the clinical sequelae that are felt to be attributable to each disease component. A commonly cited therapeutic approach has been to treat the SM component as if the myeloid neoplasm were not present, and to treat the myeloid neoplasm as if

TABLE 75.6 **"C" Findings: Indication of Organ Damage Attributable to Neoplastic Mast Cell Infiltration**

1. Cytopenia(s): Absolute neutrophil count <1 x10^9/L or hemoglobin <10 g/dL or platelets <100 x10^9/L
2. Hepatomegaly with ascites and/or impaired liver function
3. Palpable splenomegaly with hypersplenism
4. Malabsorption with hypoalbuminemia and weight loss
5. Skeletal lesions: large-sized osteolyses (≥2 cm) and/or pathologic fractures
6. Life-threatening organopathy in other organ systems that is definitively caused by an infiltration of the tissue by neoplastic mast cells

SM were not present.[70–72] Because the *KIT* D816V mutation may be present in the cells belonging to both disease compartments,[73] small molecule inhibitors of dysregulated KIT may provide benefit for both the SM and AHN in selected cases.

ASM comprises 5% to 10% of SM variants and is defined by one or more "C findings" (Table 75.6) reflecting organ dysfunction because of neoplastic MC infiltrates.[12,13,51,70–72] Examples of C findings include marked cytopenias because of extensive BM involvement (defined by an absolute neutrophil count [ANC] $<1 \times 10^9$/L, hemoglobin <10 g/dL, and/or platelet count $<100 \times 10^9$/L) and/or hypersplenism; hepatomegaly with liver dysfunction, and/or portal hypertension or ascites; hypoalbuminemia, which may relate to liver dysfunction and/or gut infiltration by neoplastic MCs; weight loss; and severe bone disease manifested by large osteolyses (≥ 2 cm) and/or pathologic fractures. In a majority of patients with ASM, the percentage of MCs in the BM smear is below 5%, which is a favorable prognostic sign. Notably, in the less frequent ASM patients in whom MCs comprise ≥5% of all nucleated BM cells on a Giemsa-stained BM smear, the prognosis is poor, as many of these patients progress and transform to MCL or an ASM-AHN. Therefore these patients have recently been described as ASM in transformation (ASM-t; see later also under diagnostic evaluation of mastocytosis).[74] As soon as the percentage of MCs in these patients increases to ≥20% in the BM smear, the diagnosis changes to MCL.

MCL is a very rare form of SM (~1% of SM variants) and is defined by MCs comprising ≥20% of nucleated cells on BM aspirate smears.[12,51,74] On the core biopsy, MCs form a diffuse, compact infiltrate with usually low levels of fibrosis. High-grade cytologic features of MCs can be observed in MCL, including multilobed or clefted nuclei, clumping of metachromatic granules, metachromatic blasts (rare, more typical for myelomastocytic leukemia), and, rarely, mitotic figures. In MCL, circulating MCs (≥10% of nucleated cells) may be found; however, the aleukemic MCL variant (<10% MCs in the peripheral blood) is more common.[12,51] MCL may arise de novo or progress from less advanced forms of SM, such as ASM with elevated MC counts on the BM aspirate. MCL typically has a dismal prognosis with a life expectancy of 6 months or less in most patients,[75] but survival can be heterogeneous, extending to 1 to 2 years or longer, and can be adversely impacted by high-risk mutations involving *SRSF2*, *ASXL1*, and/or *RUNX1*.[76] A chronic form of MCL has also been described, wherein patients meet histopathologic criteria for the disease, but without C findings (see later). In these cases, MCs tend to show a more mature morphology, and may exhibit imatinib-sensitive JM *KIT* mutations.[74,77–80] However, even those with the initially more indolent form of (chronic) MCL are expected to show progression over time with development of organ damage (transformation to acute MCL) and limited survival. In addition to the distinction of MCL into acute and chronic forms, and the historical convention of dividing MCL into leukemic and aleukemic variants, other useful subclassifications include MCL with or without an AHN, and primary (de novo) versus secondary MCL,[76] the latter transformed from less advanced disease or MCS.

MCS is a rare MC tumor that can invade local tissues and has a high potential to develop advanced systemic disease with a fulminant course.[81] In particular, most if not all patients with MCS progress to MCL within a relatively short time period. Vice versa, MCS-like tumors ("secondary MCS") may develop during the course of SM, usually the advanced forms. Extracutaneous mastocytoma is another rare SM variant that typically follows a benign course.[82] However, because of its rarity, this MC disease entity was eliminated from the last update of the WHO classification.[51]

Well-differentiated SM (WDSM) is a more recently described rare variant of SM that is not yet formally recognized by the WHO (see later for histopathologic characteristics). The major reason for this is that the well-differentiated (WD) morphology of MCs can be found in all WHO subvariants of SM, including ISM, ASM, and MCL.[77–80] Therefore the WD phenotype should be used as a descriptive term to complement the WHO diagnosis rather than as a tool to formulate a new entity.

Mast Cell Activation Syndromes

Diagnostic criteria and a classification have been formulated for MC activation syndromes (MCAS) by the consensus group.[83–85] MCAS are clinical conditions defined by MC activation but are not regarded as subtypes of SM (Table 75.7).[83–85] Patients with MCAS exhibit symptoms (e.g., anaphylaxis) and/or biochemical evidence of massive MC degranulation, with an event-related increase in serum tryptase. Other mediators produced by MCs may also increase during anaphylaxis. These mediators include plasma histamine, and spot or 24-hour urine *N*-methylhistamine, PGD2 or metabolite 11-beta PGF2. Several different variants of MCAS have been described (Table 75.8). In patients with reactive MCAS, an underlying allergy is most commonly detected, whereas no signs of MC clonality are found. By contrast, in patients with primary (clonal) MCAS, MC monoclonality can be demonstrated. In these patients, MCs in the BM may be increased, often with atypical morphology, and evidence for their clonality can be established by identification of *KIT* D816V or cell surface expression of CD25.[86,87] In patients with primary MCAS, there are two subsets of patients. One group of patients fulfills the criteria of an underlying MC disease, usually in form of SM (criteria to diagnose SM are fulfilled). In the second group of patients, the burden of MCs is very low, and despite the documented presence of clonal MCs, neither SM nor CM can be

TABLE 75.7 **Criteria for the Diagnosis of Mast Cell Activation Syndrome**

A. Typical symptoms (see below) that are related to two organ systems
B. Increase in serum total tryptase by at least 20% above baseline plus 2 ng/mL during or within 4 h after a symptomatic period
C. Response of clinical symptoms to histamine receptor blockers or MC-targeting agents, e.g., cromolyn

Typical Symptoms

Flushing
Pruritis
Urticaria
Angioedema
Nasal congestion
Nasal pruritis
Wheezing
Throat swelling
Headache
Hypotension
Diarrhea

Modified from Valent P, Akin C, Arock M, et al. Definitions, criteria and global classification of mast cell disorders with special reference to mast cell activation syndromes: a consensus proposal. *Int Arch Allergy Immunol.* 2012;157:215.
MC, Mast cell.

TABLE 75.8 Classification of Mast Cell Activation Syndromes

Category and Variants	Proposed Criteria
Primary MCAS = (mono)clonal MCAS	MCA criteria fulfilled and MC (mono) clonality proven ($CD25^+$ MC and/or *KIT* D816V)[a]
• Mastocytosis • Primary MCAS without evidence of mastocytosis	
Secondary MCAS	MCA criteria fulfilled and criteria for the diagnosis of allergy or other diseases that can produce MCA fulfilled as well
• Allergy • Other underlying disorder[b]	
Idiopathic MCAS[c]	MCA criteria fulfilled, but no disease that could lead to MCA diagnosed

[a] $CD25^+$ MCs plus *KIT* D816V detectable, or *KIT* D816V detectable, but MCs cannot be demonstrated to express CD25.
[b] Disorders associated with MCA include autoimmune diseases, certain bacterial infections, and some adverse drug reactions.
[c] Idiopathic MCAS is a final diagnosis but needs an extensive workup in order to exclude all potential underlying conditions. Idiopathic and secondary MCA episodes may occur at different time points in the same patient.
MC, Mast cell; *MCA*, mast cell activation; *MCAS*, mast cell activation syndrome.
From Valent P, Akin C, Arock M, et al. Definitions, criteria and global classification of mast cell disorders with special reference to mast cell activation syndromes: a consensus proposal. *Int Arch Allergy Immunol.* 2012;157:215.

diagnosed. In these patients, one or two minor SM criteria are typically present. However, at the time of diagnosis, a diagnosis of an underlying MC disease cannot be established.[84] In both instances, the diagnosis is primary (clonal) MCAS. Because of the very low numbers of MCs, techniques to enrich for MCs and/or use of sensitive techniques to detect *KIT* D816V (e.g., allele-specific PCR) may be necessary to detect neoplastic MCs in these patients. Currently, there is no evidence that these two entities are "prodromal" SM conditions with a defined rate of progression to systemic MC disease. If neither an underlying allergy or another underlying reactive process nor a clonal population of MCs can be detected in an MCAS patient, the final diagnosis is idiopathic MCAS.[84] Criteria for MCAS include an event-related increase in serum tryptase, typical symptoms related to at least two organ systems, and response of these symptoms to drugs targeting MC activation, MC-derived mediators, or their specific receptors.[84] All three MCAS criteria must be fulfilled to characterize the condition as MCAS. The minimal increase in tryptase is defined by the following formula: 20% of baseline plus absolute 2 ng/mL.[88] Example: if in an SM patient with 100 ng/mL baseline tryptase, an anaphylactic response to a bee sting is followed by an increase to 150 ng/mL, the reaction is called MCA ($100 + 20 + 2 = 122$; this means any value above 122 qualifies as a MCAS criterion). It is important to know that after such an anaphylactic reaction, it takes at least 24 to 48 hours (after complete resolution of symptoms) until the baseline of tryptase is again reached.

In patients with IgE-mediated hymenoptera venom anaphylaxis, particularly individuals with an elevated baseline tryptase level (separate from the acute rise after a sting), the suspicion for an underlying clonal MC disorder is increased.[89–92] The differential diagnosis for MCAS includes allergic disorders resulting in secondary MC activation such as chronic urticaria/angioedema, exposures to certain foods, drugs, or environmental allergens, physical or temperature stimuli (exercise-induced anaphylaxis; cold or cholinergic or delayed-pressure urticaria), asthma, and idiopathic anaphylaxis.[93]

An autosomal dominant genetic condition called hereditary alpha-tryptasemia (HαT) has also been described.[94] In these patients, extra copies of the human tryptase alpha gene, *TPSAB1*, are found. The genetic trait is characterized by slightly elevated basal serum tryptase levels, cutaneous flushing and pruritus, connective tissue abnormalities, symptoms suggestive of autonomic dysfunction, vibration-induced reactions, and a certain risk for severe immediate hypersensitivity reactions, especially when an allergy and/or a MC disease is also present. Although the mechanisms are not completely understood, HαT should be regarded as a potential risk factor for severe mediator-related symptoms in patients with MC disorders.[95,96]

Diagnostic Evaluation of Mastocytosis

Tissue biopsy in conjunction with clinical and pathology expertise are critical in establishing the diagnosis of SM. BM MC burden is best quantified by morphologic analysis and immunohistochemical stains such as tryptase, CD117 (KIT), and CD25 on the core biopsy.[12,13,51,97] Multiparameter flow cytometric analysis of BM aspirates can also be used to quantify the percentage of MCs, and generally correlates with SM burden defined by morphologic/immunohistochemical evaluation.[98,99] Morphologic evaluation of the BM aspirate is additionally important for evaluating dysplasia and excess myeloblasts, and most relevant to MCL, the number of atypical MCs. Based on this evaluation, the presence of MCL can be excluded or can be confirmed during the diagnostic work-up.

The most important morphologic feature of mastocytosis, in particular its systemic variants, is the presence of compact infiltrates consisting of densely packed atypical MCs.[12,51] Compact MC infiltrates are almost never detected in reactive states, even in those with marked MC hyperplasia. Accordingly, compact MC infiltrates can be regarded as the only major criterion for diagnosis of mastocytosis, which also underlines the important role of the hematopathologist in making these diagnoses. Investigation of an adequate BM biopsy trephine specimen is crucial to assess or exclude a diagnosis of SM. In most patients a few compact infiltrates can be identified even by using conventional stains like Giemsa-Wright or toluidine blue, which both facilitate detection of the pathognomonic metachromatic granules.[12,51] The MCs are round or spindle shaped, and often exhibit at least some hypogranulation. The nuclei are round or elongated depending on the shape of the cell. Prominent nucleoli are not seen. Almost always such compact infiltrates also contain mature eosinophils, histiocytes, and sometimes large follicle-like aggregates of

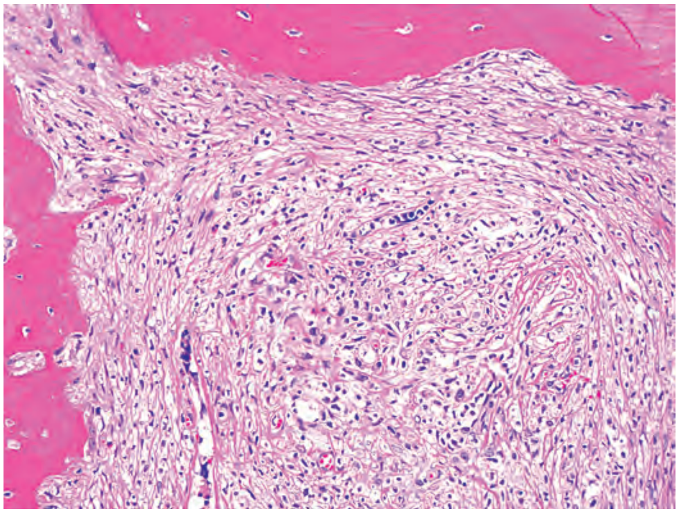


Figure 75.2 AGGRESSIVE SYSTEMIC MASTOCYTOSIS (SM). Core biopsy, hematoxylin, and eosin stain. Extremely hypercellular bone marrow with subtotal depletion of fat cells and normal blood cell precursors. Packed infiltration by atypical hypogranulated spindle-shaped mast cells that are embedded in a dense fibrotic stroma is the typical finding in aggressive systemic mastocytosis (ASM). However, only the presence of C-finding(s) qualifies such cases to be subtyped as ASM. Similar pictures can be seen in smoldering systemic mastocytosis with "B" findings. Note that the morphologic features of nonspecific fibrosis or granulation tissue may be very similar and appropriate immunostaining is necessary to confirm the diagnosis of SM.

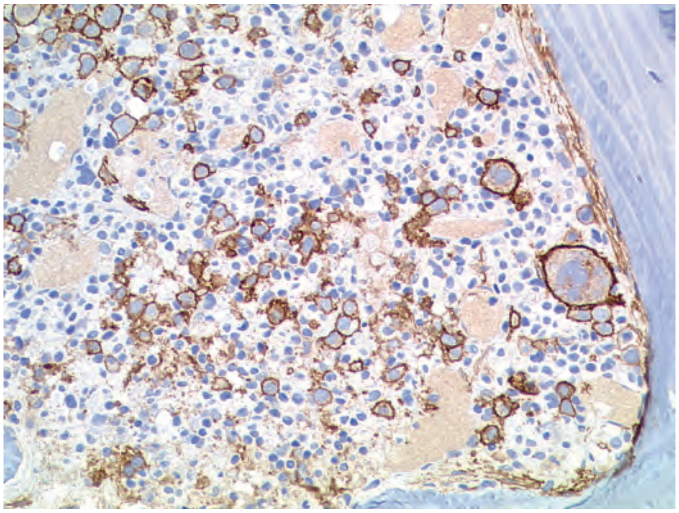


Figure 75.3 ACUTE MAST CELL LEUKEMIA WITH CD30 EXPRESSION. Core biopsy with positive staining of mast cells for surface CD30. Bone marrow section shows extreme hypercellularity with diffuse-packed infiltration by atypical round mast cells coexpressing tryptase and CD117 (not depicted). A significant number of the mast cells stain positive by an antibody against CD30, among them a few multinucleated giant cells that resemble Reed-Sternberg cells. A diagnosis of Hodgkin lymphoma should not be established in such cases.

lymphocytes that are polyclonal in nature. Reticulin fiber density is always increased with the exception of a few very small but diagnostic infiltrates. Larger compact and mixed infiltrates often show patchy collagen fibrosis. The degree of reticulin fibrosis in most cases of MCL is low, which can be used as a criterion that allows one to distinguish MCL from ASM, which usually exhibits marked reticulin or even collagen fibrosis (Fig. 75.2).[12,51] Immunohistochemistry should be performed in all cases in order to enumerate the numbers of loosely scattered MCs and to detect small but diagnostic compact infiltrates. The degree of MC infiltration should always be documented by the hematopathologist: it is recommended to refer to the section area and to the overall cellularity in this respect. This enables monitoring of the disease during and after therapy. MCs almost always coexpress tryptase and CD117; in most SM cases, MCs also aberrantly express CD25, whereas aberrant expression of other antigens such as CD2 is more rarely encountered and therefore of minor diagnostic importance (Fig. 75.3). Interestingly, it could be shown that MCs may lose immunohistochemically detectable CD25 or tryptase expression during or after therapy with TK inhibitors such as midostaurin,[100] and MCs in cases of intestinal involvement by SM may lack detectable tryptase expression at diagnosis (e.g., incomplete immunophenotype). However, it can be stated definitively that a cell lacking expression of CD117 (KIT) cannot be a MC. Although ISM and isolated mastocytosis of the BM (BMM) usually show a minor degree of BM involvement with multifocal compact infiltrates (<10% of the section area), smoldering and advSM but also MCL usually exhibit a more diffuse or packed infiltration occupying more than 30% and up to 100% of the section area. In a significant proportion of cases compact MC infiltrates are missing and the degree of tissue involvement may be very low (<1% regarding cellularity and section area). Accordingly, such cases could be termed "occult SM," and are difficult to detect for the pathologists. Moreover, SM is a great mimicker with regard to morphologic and immunophenotypical features of MCs, and a broad spectrum of differential diagnoses should be considered. The following list only contains the main entities and is therefore incomplete:

1. monocytic leukemias (clear cell feature + CD14)
2. histiocytoses (clear cell feature + CD68)
3. clear-cell carcinomas (clear cell feature + epithelial antigens like keratin)
4. basophilic leukemias (metachromatic granules + tryptase but missing CD117)
5. myelomastocytic leukemia (metachromatic blasts + tryptase + CD117)
6. Hodgkin lymphoma (CD30)
7. hairy cell leukemia (clear cell feature + CD25)
8. granulation tissue and scars

Evaluation of blood and BM smears is crucial for both identification and subtyping of MCL and separation of MCL from a recently described subtype of ASM, namely ASM-t (see earlier). In almost all patients with ISM and SSM, but also in most cases with ASM, the MC numbers in BM smears are lower than 5%, in ISM and SSM usually even below 1%. Crushed particles with higher numbers of MCs must not be considered in this respect. In a few patients with ASM the number of MCs is unusually high, exceeding 5% but not 19% of all nucleated cells. It is this subgroup of patients that has a worse prognosis but do not fulfill criteria for MCL and have therefore been termed ASM-t.[74]

Cytomorphologic findings in SM include atypically shaped MCs with spindled appearance (type I) on the one hand and round immature MCs with bilobated or monocytoid nuclei on the other (type II).[12,51] Atypical type I MCs are usually encountered in ISM, SSM, and often also in ASM, but also in isolated BMM, whereas atypical type II MCs dominate the picture in most cases of MCL, and are often found also in patients with ASM and ASM-t. Exceedingly rare cases of MCL with predominance of spindle-shaped cells of type I are associated with a relatively good prognosis and the disease accordingly has been termed *chronic MCL* (see earlier) (Fig. 75.4).[74,77] There is one peculiar phenotypic variant of SM termed *WDSM* (see earlier) that consists exclusively of round hypergranular-appearing MCs that form the typical cohesive clusters in BM smears.[78,79] Such MC clusters are not seen in all of the subvariants of SM. The feature of WDSM has been encountered in almost all defined subvariants of SM including MCL. WDSM usually lacks aberrant expression of CD25 and does also not show the typical activating point mutations at codon 816 of *KIT*, but instead the aforementioned JM *KIT* mutations which often exhibit sensitivity to imatinib (Fig. 75.5).[78,80] In addition, MCs in WDSM usually express CD30 which can be employed as additional disease indication (criterion) in these patients.

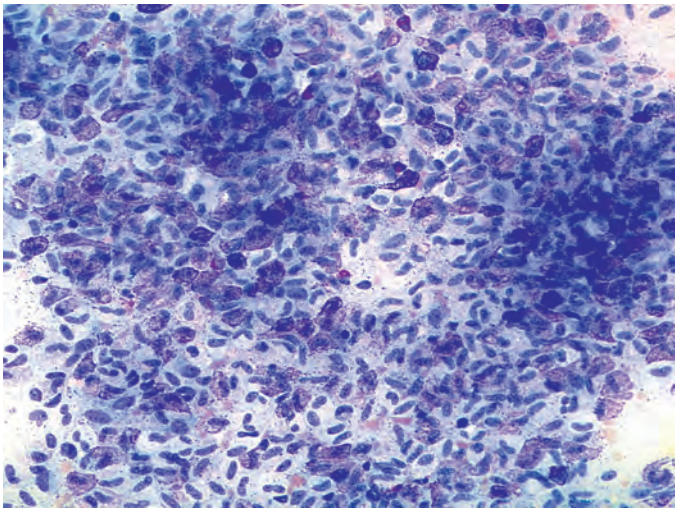


Figure 75.4 CHRONIC MAST CELL LEUKEMIA (BY CLINICAL BEHAVIOR). Bone marrow aspirate (Pappenheim stain) shows a very unusual picture with an extreme increase of spindle-shaped and often slightly hypogranulated mast cells. Note their elongated nuclei. Nucleoli are inconspicuous. Mast cell number is almost 100% and a diagnosis of mast cell leukemia can easily be established. The prolonged survival of this patient and the unique cytomorphologic aspect qualifies this case to be subtyped as chronic mast cell leukemia.

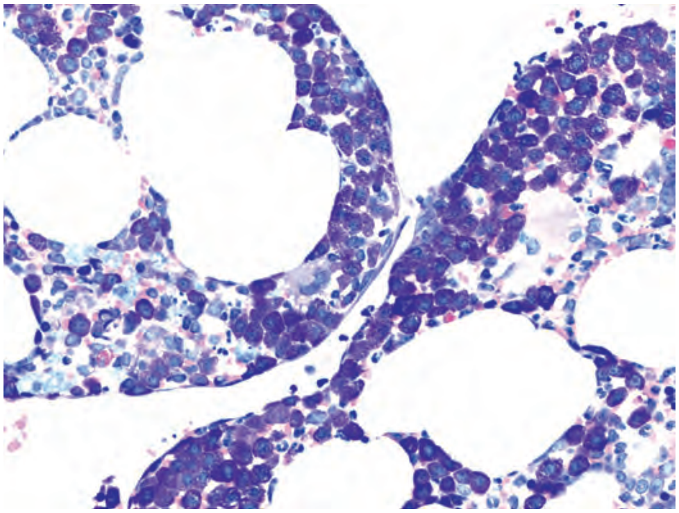


Figure 75.5 WELL-DIFFERENTIATED SYSTEMIC MASTOCYTOSIS. Core biopsy, Giemsa stain. Bone marrow shows aggregates of exclusively round mast cells with an abundance of metachromatic granules. This is the typical phenotypical appearance of a well-differentiated systemic mastocytosis. The disease was otherwise subtyped as indolent systemic mastocytosis with skin involvement but missing *KIT* D816V mutation and also lack of CD25 expression by the mast cells (not depicted).

CD30 (Ki-1) is a cytoplasmic and membrane-bound antigen expressed by neoplastic cells in Hodgkin lymphoma and anaplastic large-cell lymphoma. However, CD30 has also been shown to be expressed by neoplastic MCs in SM.[101,102] Although the antigen was originally reported as an immunohistochemical marker that is primarily expressed in the cytoplasm of MCs in more advanced forms of SM,[102] other studies have detected CD30 by immunohistochemistry in less advanced forms of MC disease, including CM, as well as ISM and SSM.[103–106] CD30 is not only expressed in the cytoplasm of neoplastic MCs but also on the cell surface, which has clinical implications as a CD30 antibody-toxin conjugate is available and was tested in a phase II trial of patients with CD30$^+$ advSM.[107] More recently, it was found that neoplastic MCs express higher levels of CD44 compared to normal MCs and that the levels of measurable CD44 on MC and in the plasma of patients with SM correlates with aggressiveness of the disease.[108]

Altogether, a complete diagnosis of SM reported by the hematopathologist should read like this:

SM-AHN; SM shows a marked multifocal BM infiltration (>50% section area) and is best subtyped as ASM since there is a reported C-finding and MC numbers in BM smears are low; AHN is a myeloid neoplasm that cannot be subtyped with certainty but morphological features are consistent with a disorder of the MDS/MPN category (MDS/MPN-unclassifiable).

Serum Tryptase Level

Tryptase is considered to be a most reliable and robust initial parameter with which to screen patients with suspected SM.[109–111] Tryptase is a serine protease primarily produced by MCs, and to a lesser extent by mature basophils and myeloid progenitors.[112–116] Commercially available ELISA-based assays measure total serum tryptase levels which consist of both mature tryptase (β-tryptase) and protryptases (e.g., α-tryptase).[117] The basal serum tryptase level reflects the MC burden in healthy individuals as well as in patients with SM, whereas the event-related (further) increase in tryptase is a marker of MC activation. β-tryptase is stored in MCs and released together with all other granular substances upon degranulation/anaphylaxis (tryptase levels peak 1 to 2 hours after degranulation event and return to baseline after 24 to 48 hours); the α-tryptase form is constitutively produced by MCs and thus more reflects the total burden of MCs.[115,118,119] For many centers, the upper normal reference range for the serum tryptase level is 10 or 11.4 ng/mL. However, healthy individuals may exhibit levels in the range of 5 to 15 ng/mL or higher, which may be confounded by tryptase haplotype, sex, and by the association between advancing age and increasing serum tryptase levels.[120,121] Although a serum tryptase level >20 ng/mL serves as a minor diagnostic criterion for SM, a minority of patients with histopathologically proven SM can exhibit values lower than this threshold.[12,51,109,122,123] In adult patients with MIS or hymenoptera allergy, a serum tryptase level >20 ng/mL is suggestive of the presence of SM.[54,122,124–126] Conversely, patients with CM usually exhibit a serum tryptase level <20 ng/mL, or it may be within normal range.[122,124,127] Other medical conditions besides SM and anaphylaxis that can produce an elevated tryptase level include renal failure, chronic helminth infections (MC hyperplasia), SCF treatment, and myeloid neoplasms, including MDS, MPN, AML, and CEL—notably patients with the *FIP1L1::PDGFRA* fusion gene.[128–135]

With the diagnosis of HαT, germline duplications and triplications in the *TPSAB1* gene encoding α-tryptase segregate with the inherited increase in basal serum tryptase and aforementioned systemic signs and symptoms.[94] As basal serum tryptase levels are elevated in 4% to 6% of the general population, and the clinical presentation of HαT can overlap with MCAS or systemic mastocytosis, it should be considered in the differential diagnosis of individuals presenting with an elevated tryptase level.[95,96]

Although there is a close correlation between high tryptase levels and advSM,[12,51,52,65,127] serum tryptase levels do not necessarily reflect the aggressiveness of the disease. Likewise, SSM is partially defined by a very high serum tryptase level exceeding 200 ng/mL, and in many of these cases, progression does not occur for many years.[12,51,52,58–60,127,130] Contrasting the smoldering state, most patients with typical ISM have lower serum tryptase levels, although enzyme levels can vary widely among patients according to the MC burden.[12,51,111,127,130] Patients with ASM and MCL may exhibit serum tryptase levels in the several-hundred range to over 1000 ng/mL.[76,127,130] In many of these patients, serum basal tryptase levels increase rapidly over time (in contrast to ISM and SSM). Although discordances can exist between the serum tryptase level and the degree of MC infiltration of the marrow and other organs, it is the most useful and widely available biomarker that can be used to assess changes in the MC burden in response to cytoreductive therapy.[100,111]

Clinical Manifestations

Organomegaly

Splenomegaly and hepatomegaly, without associated organ damage, have traditionally been referred to as "B" findings and are one of the defining features of SSM.[12,51,58–60] Similarly, SM-related substantial (palpable, >2 cm) lymphadenopathy has been included in the category of "B" findings. Organomegaly by itself does not indicate inexorable progression to advSM or organ damage. However, even in the absence of organ damage, symptomatic splenomegaly may warrant therapeutic intervention similar to patients with myelofibrosis or other hematologic neoplasms.

Nonhematologic Organ Damage

The challenge of defining response parameters related to hepatic function is several-fold. First, for patients with SM with associated myeloid neoplasm, it is unclear whether liver dysfunction is related to the SM or the accompanying myeloid disease.[136] Concomitant diseases such as MPN or MDS/MPN such as CMML, CEL, or AML may (also) involve the liver and result in a hepatitis-like syndrome or obstructive liver disease. It is unknown what level of liver involvement by MC consistently results in liver dysfunction. Elevation of the alkaline phosphatase is the most common laboratory marker of clinically relevant liver involvement by SM.[137] Liver biopsy is sometimes performed and may help to assess the grade and extent of liver involvement by SM. Although liver biopsy may be informative, the potential information gleaned from the procedure must be weighed against potential complications such as bleeding, especially in the setting of severe thrombocytopenia and/or coagulopathy.

Hypoalbuminemia is an adverse prognostic factor for OS in SM[52]; it can reflect worsening synthetic function of the liver, and/or worsening nutritional status or even malabsorption because of GI infiltration by neoplastic MCs. The development of ascites in SM usually reflects aggressive liver disease and may be accompanied not only by hepatomegaly but also by abnormal liver function tests (e.g., elevated alkaline phosphatase as above) and/or portal hypertension.[12,51] More uncommonly, ascites may develop without evidence of liver disease, or in conjunction with massive abdominal lymphadenopathy. Similar to ascites, pleural effusions may develop in advSM, although it is an uncommon event. Every attempt should be made to establish that the ascites or pleural effusion is related to SM and/or the associated myeloid neoplasm, including evaluation of MC content.[136] Other causes of ascites or effusion should be excluded including infection, heart failure, and drugs such as dasatinib or avapritinib, that are used to treat advSM.[138,139]

Hematologic Organ Involvement

The etiology of cytopenias in advSM includes compromise of normal marrow reserve by infiltration with neoplastic MCs (often seen in ASM and MCL), ineffective hematopoiesis in the context of SM with an associated myeloid neoplasm, hypersplenism, and other causes such as recent myelosuppressive therapy or GI bleeding.[136] The coexistence of another myeloid neoplasm (AHN) often makes it difficult to ascertain the specific cause of the low blood counts. This distinction is not relevant to ISM, where the relatively lower MC burden is not expected to contribute to the development of cytopenias.

Molecular Features of Systemic Mastocytosis

Following the discovery of the somatic *KIT* D816V mutation in MC neoplasms in the mid-1990s,[140] it has become evident that this molecular abnormality is found in approximately 80% to greater than 90% of SM patients depending on the sensitivity of the assay and the patient population examined. *KIT* D816V has been detected throughout the hematopoietic hierarchy of SM patients, from hematopoietic stem cells to more lineage-restricted, MC-committed progenitors.[141] *KIT* D816V serves as a minor diagnostic criterion for the disease, and is considered a major driver of SM pathogenesis.[12,40,51] However, although promoting differentiation of progenitor cells into MCs, *KIT* D816V is neither a proliferation-promoting nor an oncogenesis-driving oncoprotein, and therefore, other additional mutations are required to fully transform a premalignant neoplastic state such as ISM to advSM. Rare codon 816 variants such as D816A/F/H/I/N/T/Y have also been described and are considered functionally equivalent to D816V.[40,100,142] Mutations affecting codon 816 are located in exon 17 and affect the second TK domain (TK2) that contains the phosphotransferase site and activation loop of KIT. Other mutations that have been identified in exon 17, for example in cases of ASM and MC sarcoma, include D820G and N822K.[143,144] *KIT* mutations in exons 8 to 9 of the extracellular region (del419, S451C, ITD 502–503) and the TM region (exon 10; e.g., F522C), and JM domain (exon 11; e.g., V560G/I) comprise some of the additional rare mutations in *KIT* that have been observed (Fig. 75.6).[40] With a few exceptions (e.g., V559I), mutations affecting these domains are more likely to exhibit sensitivity to imatinib, whereas codon 816 variants are imatinib-resistant.[145,146] Contrary to other SM subtypes, the frequency of *KIT* D816V is more variable in MCL; *KIT* D816V was found in 13 of 28 (46%) evaluable patients in one study, but was found in 24/28 (86%) MCL patients in another series.[75,76] MCL patients may exhibit other variants within *KIT*, or sequencing of the entire *KIT* gene may reveal no mutations. Increasingly sensitive assays

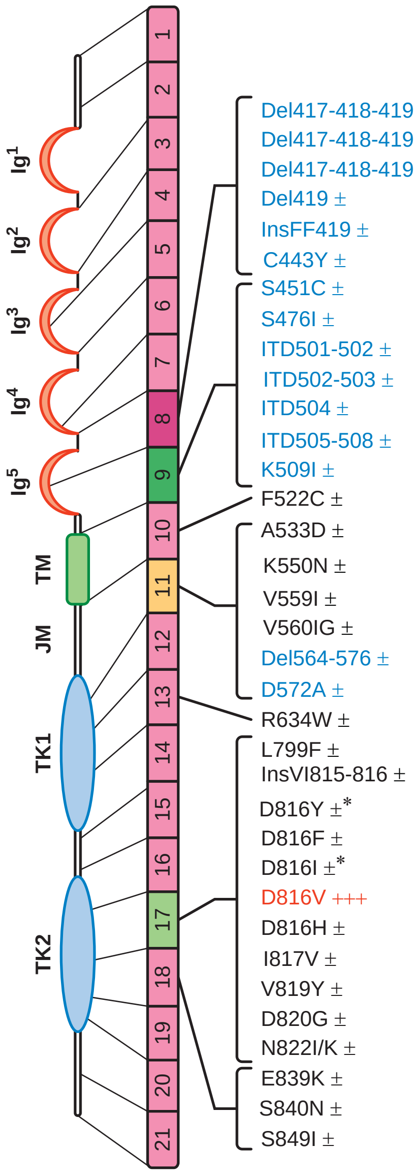


Figure 75.6 STRUCTURE OF THE *KIT* GENE AND MUTATIONS IN MASTOCYTOSIS. Representation of the structure of *KIT*, illustrating the localization of the more frequently observed mutations in the *KIT* sequence in pediatric and adult patients with mastocytosis. The receptor is presented under its monomeric form, whereas its WT counterpart dimerizes upon ligation with SCF before being activated in normal cells. In children, the *KIT* D816V PTD mutant *(in red)* is found in nearly 30% of the patients, whereas the ECD mutants *(in blue)* are found in nearly 40% of the affected children, the most frequent being the deletion 419. In adults, depending on the category of mastocytosis, the *KIT* D816V mutant *(in red)* is found in at least 80% of all patients. The complete list of *KIT* mutants retrieved in the literature for mastocytosis is depicted here. In children, the structure of *KIT* is found WT in ~25% of the patients analyzed, whereas in adults, *KIT* is found WT in <20% of all patients analyzed so far. Some of the mutations are found only in a very few number of patients. The symbols indicate the following: ±, mutation found in <10% of the pediatric or adult patients; +, mutations found in 1% to 5% of pediatric patients; ++, mutation found in 5% to 20% of pediatric patients; +++, mutation found in ~30% of pediatric patients and in >80% of all adult patients. *Mutation also found in children at low frequency. *Del*, Deletion; *ECD*, extracellular domain; *Ig*, immunoglobulin; *Ins*, insertion; *ITD*, internal tandem duplication; *JM*, juxtamembrane domain; *KI*, kinase insert; *PTD*, phosphotransferase domain; *TK*, tyrosine kinase; *TM*, transmembrane domain. *(From Arock M, Sotlar K, Akin C, et al. KIT mutation analysis in mast cell neoplasms: recommendations of the European Competence Network on Mastocytosis. Leukemia. 2015;29:1223,; Yarden Y, Kuang WJ, Yang-Feng T, et al. Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J. 1987;6:3341.)*

(e.g., sensitivity of 0.01% to 0.1%), such as droplet digital PCR or allele-specific quantitative PCR for *KIT* D816V, are now available and can detect the mutation in the peripheral blood of almost all adult patients with SM.[147] Where available, it has been recommended that such sensitive assays be used as a valuable screening test for patients with suspected SM.[110,148] However, a low MC burden or the presence of other rare *KIT* or non-*KIT* mutations could lead to negative results; therefore a full workup with BM biopsy is warranted in typical cases where clinical symptoms and findings are highly suggestive of the presence of SM.

In a large cohort of pediatric mastocytosis patients, 36% exhibited *KIT* D816V mutations, and three (6%) patients had other codon 816 mutations (D816Y [$n = 2$] and D816I [$n = 1$]).[149] Another 44% demonstrated *KIT* mutations outside exon 17, with half of these being located in exons 8 and 9 (D417-418, D419Y, C443Y, S476I, ITD502-503, K509I) and exon 11 (D572A).[149] A retrospective systematic review of 1747 pediatric SM patients identified *KIT* D816V in 34% of 215 sequenced cases.[150]

SM-AHN cases commonly harbor molecular abnormalities related to the myeloid disease component in addition to *KIT* D816V. For example, *JAK2* V617F has been found in SM with myelofibrosis[151]; *BCR::ABL1* in rare cases of concurrent SM-CML[152,153]; and in SM-AML, the t(8;21)(q22;q22) *RUNX1::RUNX1T1* rearrangement is a well-known association.[154–156] In cases of *KIT* D816V-positive SM, several reports have detailed the coexistence of additional myeloid neoplasm-related mutations, which are implicated in differentiation, epigenetic regulation, and control of the splicing machinery.[157–163] Molecular profiling of myeloid progenitor cells from SM-AHN patients using logical hierarchy analysis showed that somatic mutations in *TET2*, *SRSF2*, and *ASXL1* often precede *KIT* D816V.[164] The general themes that have emerged from multiple genetic studies are the following: (1) ISM tends to be more a pure *KIT* D816V-driven disease with absence of, or low frequency of, additional (known) molecular abnormalities; (2) *KIT* D816V restricted to the MC lineage is observed in the majority of ISM patients; however, multilineage representation of *KIT* D816V is associated with progressive ISM, SSM, or AdvSM; and (3) the presence of a multi-mutated molecular profile is usually associated with more advanced disease subtypes, particularly SM-AHN.[100,157–165] *TET2*, *SRSF2*, and *ASXL1* are the most common of the myeloid neoplasm-associated mutated genes (~20% to 35% of patients), but mutations in *DNMT3A*, *CBL*, *JAK2*, *K/NRAS*, *UA2F1*, *ZRSR2 EZH2*, *RUNX1*, *ETV6*, and *SETBP1* have also been identified.[157–165] The prognostic impact of some of these mutations is discussed below (see Survival and Prognostic Factors). Mutations in the ethanolamine kinase gene *ETNK1* were found to be enriched in SM patients with eosinophilia, but are commonly present with *KIT* D816V or other myeloid disease-associated mutations.[166] In addition, *ETNK1* mutations have been identified in ~9% of patients with atypical CML and in <5% of patients with CMML.[167]

KIT D816V has been demonstrated in both MCs and the AHN component (e.g., monocytes in CMML) by laser cell microdissection;[73] *TET2* and *KIT* D816V have been found together in flow-sorted MCs;[159] the *SRSF2* P95 hotspot mutation has been uncovered in both MCs and monocytes from a case of SM-AHN;[162] and finally, t(8;21) has been identified by fluorescence in situ hybridization (FISH) in the BM MCs and the leukemic clone of a patient with SM and AML.[154]

In 2003 the *FIP1L1::PDGFRA* fusion oncogene was identified in patients with a diagnosis of idiopathic hypereosinophilic syndrome.[168] This molecular abnormality is not visible by standard chromosome analysis and its detection requires either use of reverse-transcriptase polymerase chain reaction or, more commonly, FISH (see Chapter 74). Because this genetic lesion results from an interstitial 800-kb deletion on chromosome 4q12, which removes a segment of DNA involving the *CHIC2* gene, the diagnostic test is referred to as "FISH for the *CHIC2* deletion."[169] Patients with *FIP1L1::PDGFRA*-associated eosinophilia present with features of a MPN: splenomegaly, a hypercellular BM, and clinicopathologic characteristics that overlap with SM, including increased numbers of abnormal-appearing BM MCs, marrow fibrosis, and elevated serum tryptase levels. Although *FIP1L1::PDGFRA*-positive myeloid neoplasms may exhibit increased numbers of BM MCs, they usually form interstitial or loose clusters,[169] and the WHO does not consider this disease entity a subtype of SM.[12,51] However, in a smaller subset of patients, criteria for SM may be fulfilled even if a *KIT* mutation is not detected. Notably, in almost all cases with *FIP1L1::PDGFRA*-positive myeloid neoplasms, including those who have SM-CEL, MCs do not exhibit *KIT* D816V, even if they express CD25.[170] Although the *FIP1L1:PDGFRA* genetic abnormality was once considered mutually exclusive of *KIT* D816V, rare cases with both mutations have now been reported, including their coexistence in tryptase-positive microdissected MCs.[171]

TABLE 75.9 **Clinicopathologic Features of Eosinophilia-Associated *FIP1L1::PDGFRA*–Rearranged Myeloid Neoplasms Versus *KIT* D816V-Positive Systemic Mastocytosis**

Features	*FIP1L1::PDGFRA*–Rearranged	*KIT* D816V–Positive
Gender	Overwhelmingly male	Less gender skewing
Bone marrow mast cell aggregates	Loose clusters/interstitial	Dense aggregates
AEC/tryptase ratio	>100	≤100
Treatment	Imatinib sensitive	Imatinib-resistant; second-generation TKIs (e.g., midostaurin)
Symptom profile	Cardiac/pulmonary	Gastrointestinal/urticaria pigmentosa/anaphylaxis
Vitamin B_{12} level	Elevated	Often normal

AEC, Absolute eosinophil count; *TKI*, tyrosine kinase inhibitor.
Modified from Maric I, Robyn J, Metcalfe DD, et al. KIT D816V-associated systemic mastocytosis with eosinophilia and FIP1L1/PDGFRA-associated chronic eosinophilic leukemia are distinct entities. *J Allergy Clin Immunol.* 2007;120(3):680–687.

Investigators identified a set of clinical and laboratory features that could reliably distinguish patients with *KIT* D816V-positive SM with eosinophilia from individuals with the *FIP1L1::PDGFRA*-positive myeloid neoplasms with eosinophilia (Table 75.9).[170] These criteria may be particularly useful when molecular testing is not readily available. In the D816V *KIT*-positive group, GI symptoms, urticaria pigmentosa, thrombocytosis, median serum tryptase values, and the presence of dense MC aggregates in the BM were statistically significantly increased or more frequently represented compared with patients with the *FIP1L1::PDGFRA* gene fusion. Conversely, male sex, cardiac and pulmonary symptoms, median peak absolute eosinophil count, the eosinophil to tryptase ratio, and elevated serum B_{12} levels were higher or more common in the group with *FIP1L1::PDGFRA*.[170] A scoring system incorporating these clinical and laboratory parameters could reliably predict whether patients with peripheral eosinophilia and increased marrow MC burden carried the *FIP1L1::PDGFRA* gene fusion or the *KIT* D816V mutation, which is important for guiding targeted therapy options.[170] Treatment of *FIP1L1::PDGFRA*-positive myeloid neoplasms associated with eosinophilia is discussed in Chapter 74.

Additional Diagnostic Studies

In all patients with symptomatic SM, a thorough work-up for allergies, including total and specific IgE levels and HαT (tryptase gene copy number),[94–96] should be performed. Biochemical studies to evaluate for MC activation/mediator release include 24-hour urine evaluation of *N*-methylhistamine, prostaglandin D2, or 11-β-prostaglandin F2.[84] Bleeding diatheses have been reported in patients with MCAS or SM, and increased heparin levels can be found in patients with SM or MC activation.[172] Dual energy X-ray absorptiometry (DEXA) scans should be undertaken to screen for osteoporosis, and plain X-ray films (e.g., metastatic skeletal survey) are used to detect osteolytic lesions and/or pathologic fractures.[56] MC-related GI symptoms such as diarrhea and signs of malabsorption (e.g., hypoalbuminemia and weight loss) can be evaluated with endoscopy/colonoscopy with biopsy using appropriate immunohistochemical stains (e.g., CD117, tryptase, CD25, and CD3 as a control T-cell marker) to highlight abnormal MCs.[173] Ultrasonography, CT, and/or MRI of the abdomen/pelvis (with or without volumetric imaging) are utilized to determine the presence and extent of B and C findings such as hepato/splenomegaly, portal hypertension, ascites, and lymphadenopathy.

Diagnostic Decision Making

In patients presenting with MC activation symptoms or anaphylaxis without signs of cutaneous involvement, and a normal or elevated serum tryptase level, the diagnostic workup is centered on establishing whether WHO diagnostic criteria for SM are met (Fig. 75.7).[12,51] If these criteria are not satisfied, a workup is performed to rule out or diagnose MCAS or HαT, and to document (other) primary and secondary causes of MC expansion or MC activation.[110,148,174]

In adult patients with cutaneous MC lesions, the large majority of such patients will ultimately be found to have SM according to WHO diagnostic criteria. A BM biopsy is generally recommended in these patients to establish a diagnosis of SM (see Fig. 75.7). In patients with modest elevations of the serum tryptase level, mild or no symptomology, nor evidence of blood count abnormalities or other signs of organ damage, diagnostic testing may not elicit short-term changes in management even if SM is found. However, given the differences in OS between adult CM and SM, and the fact that only those with SM develop severe bone disease (osteoporosis) requiring therapy, staging investigations should be extended in SM. BM analysis can be very helpful as an initial “forensic” assessment of these potential disease trajectories.[110,148,174,175] Children with skin lesions rarely have systemic disease. Therefore, a BM biopsy is generally not recommended in children unless the serum tryptase level is unusually high or progressively increasing, or organomegaly and/or blood count abnormalities emerge.

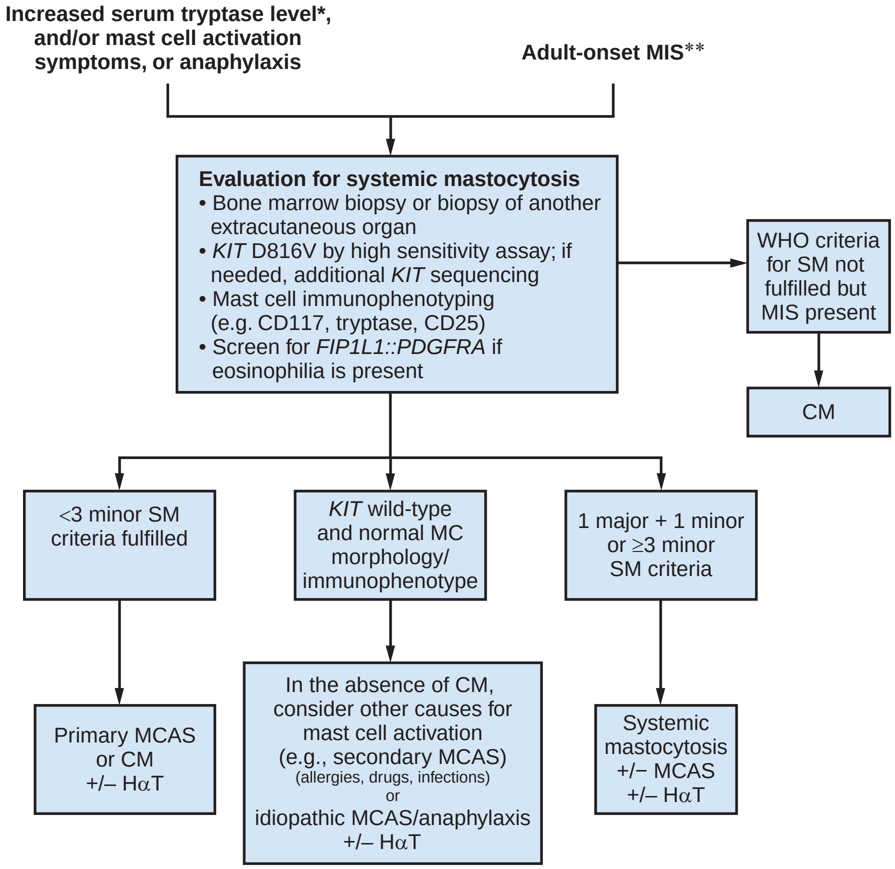

Increased serum tryptase level*, and/or mast cell activation symptoms, or anaphylaxis
Adult-onset MIS**
Evaluation for systemic mastocytosis
• Bone marrow biopsy or biopsy of another extracutaneous organ
• *KIT* D816V by high sensitivity assay; if needed, additional *KIT* sequencing
• Mast cell immunophenotyping (e.g. CD117, tryptase, CD25)
• Screen for *FIP1L1::PDGFRA* if eosinophilia is present
WHO criteria for SM not fulfilled but MIS present
CM
<3 minor SM criteria fulfilled
KIT wild-type and normal MC morphology/ immunophenotype
1 major + 1 minor or ≥3 minor SM criteria
Primary MCAS or CM +/– HαT
In the absence of CM, consider other causes for mast cell activation (e.g., secondary MCAS) (allergies, drugs, infections) or idiopathic MCAS/anaphylaxis +/– HαT
Systemic mastocytosis +/− MCAS +/– HαT

Figure 75.7 DIAGNOSTIC DECISION PATHWAYS FOR PATIENTS WITH MAST CELL ACTIVATION SYMPTOMS (MCAS) OR ADULT-ONSET MASTOCYTOSIS IN THE SKIN (MIS). For patients with mast cell activation symptoms or anaphylaxis (and/or increased serum tryptase level), or adult-onset MIS, screening to assess whether the diagnostic criteria for SM are met should be the first diagnostic checkpoint. For patients not meeting criteria for SM, evaluation for MCAS (primary vs. secondary vs. idiopathic) and idiopathic anaphylaxis is the next phase of evaluation. Patients with MIS for whom no signs of SM can be found may be categorized as cutaneous mastocytosis. Hereditary alpha-tryptasemia is also on the differential diagnosis of individuals with an elevated basal serum tryptase level and/or mast cell activation symptoms/anaphylaxis, and its prevalence is enriched in patients with systemic mastocytosis and severe idiopathic anaphylaxis. *The serum tryptase level may be below the 20 ng/mL threshold, or only transiently elevated. **Although an evaluation for systemic mastocytosis is generally recommended in adults with MIS with no blood count abnormalities or organ dysfunction, and a normal or mildly increased serum tryptase level, the value of performing a bone marrow biopsy should be discussed with the patient. *CM*, Cutaneous mastocytosis; *HαT*, Hereditary alpha-tryptasemia; *MC*, Mast cell; *MCAS*, mast cell activation syndrome; *MIS*, mastocytosis in the skin; *PDGFRA*, platelet-derived growth factor receptor A; *SM*, systemic mastocytosis; *WHO*, World Health Organization. *(Adapted from Pardanani A. How I treat patients with indolent and smoldering mastocytosis (rare conditions but difficult to manage). Blood. 2013;121:3085; Valent P, Akin C, Arock M, et al. Definitions, criteria and global classification of mast cell disorders with special reference to mast cell activation syndromes: a consensus protocol. Int Arch Allergy Immunol. 2012;157:215.)*

Survival and Prognostic Factors

In a Mayo series of 342 patients with SM (46% ISM, 12% ASM, 40% SM-AHN, 1% MCL), life expectancy in ISM was similar to age- and sex-matched normal controls.[52] By contrast, median OS (and leukemia-free survival) was inferior in patients with more advanced forms of SM. For example, the median OS was 41 months and 24 months, respectively for ASM ($n = 41$ patients) and SM-AHN ($n = 138$ patients), and only 2 months for MCL ($n = 4$) patients.[52] In a multivariate analysis, independent adverse prognostic factors included advanced age, weight loss, anemia, thrombocytopenia, hypoalbuminemia, and excess BM blasts.[52] In addition to an increased percentage of MCs on marrow aspirate smears, Spanish investigators found that multilineage involvement of the *KIT* D816V mutation in more than just MCs (e.g., other myeloid lineages, and sometimes lymphoid cells) is a poor prognostic factor and leads to an increased risk of progression to advSM.[61] Many of these patients may suffer from overt SSM. The identification of *KIT* D816V in BM mesenchymal stem cells from ISM patients was associated with more advanced disease features, a greater rate of disease progression, and a shorter progression-free survival (PFS).[176] In patients with SM, eosinophilia was associated with inferior outcomes in two studies of the ECNM registry, including a recent analysis of a large number of SM patients,[177,178] whereas another series found that eosinophilia was prognostically neutral.[65] SM patients with eosinophilia tend to suffer from SSM, ASM, or MCL, but even in ISM, eosinophilia may be detected.

Cytogenetic/Molecular Determinants of Prognosis and the Evolution of Prognostic Scoring Systems

In SM-AHN patients, the SM subtype wherein cytogenetic abnormalities are almost exclusively identified, progression to MCL or secondary AML, as well as shortened OS, were associated with monosomies or a complex karyotype, and were independent of mutational status.[179] However, there are also data to suggest that among SM-AHN patients, karyotype did not carry independent prognostic value in a multivariate analysis, in contrast to adverse mutations, anemia, and thrombocytopenia.[180]

An initial analysis of the molecular profile of SM patients found that the presence of one or more mutations in addition to *KIT* D816V was associated with inferior OS.[160] A separate analysis found that mutation of *ASXL1* had a significant negative impact on OS among a cohort of 62 patients with SM-AHN.[161] Inferior survival has also been observed in SM patients based on the presence of combined *TET2/DNMT3A/ASXL1* mutations independent of *KIT* and sole *TET2* mutations.[158] In a series of 70 multi-mutated advSM patients, OS was adversely influenced by the number of mutated genes in the *SRSF2/ASXL1/RUNX1* (S/A/R) gene panel.[181] In an independent series of 19 advSM patients, non-*KIT* mutations were frequent (79%), particularly in *TET2*, *ASXL1*, and *CBL*.[182] Presence of *ASXL1* and/or *CBL* mutations or occurrence of ≥3 non-*KIT* mutations was independently associated with significantly inferior OS, but not leukemia-free survival. A more recent study found that the presence of ≥1 multilineage mutation in the *S/A/R* gene panel and/or the *EZH2* gene was the sole independent predictor for PFS and OS.[183]

These correlative clinical and genetic studies provided the foundation for the development of clinical-molecular prognostic scoring systems that continue to undergo evolution and further refinement (reviewed in 100). An initial prognostic scoring system employed clinical features (elevated alkaline phosphatase and increased spleen volume) and the presence of additional mutations besides *KIT* D816V.[137] The International Prognostic Scoring System for Mastocytosis (IPSM) uses clinical and laboratory features to further stratify outcomes of patients comprising cohorts of both non-advanced and advSM patients.[53] Other prognostic models are molecularly-anchored; these include the Spanish Network on Mastocytosis (REMA) prognostic scoring system for ISM patients,[184] and their Global Prognostic Score for Mastocytosis (GPSM), which is currently undergoing validation.[185] Additional hybrid clinical-molecular prognostic scoring systems include the Mutation-Adjusted Risk Score (MARS) for advSM patients,[186] as well as the Mayo Alliance Prognostic Scoring System (MAPS), which also includes a separate cohort analysis based on clinical and laboratory information only, excluding data regarding WHO subgroup classification and genetic profile.[187,188] The clinical, laboratory, and molecular variables that comprise these prognostic scoring systems are shown in Table 75.10.

TABLE 75.10 Prognostic Scoring Systems in Systemic Mastocytosis

Parameters		REMA (for ISM)			IPSM			GPSM	
		OS	PFS	MAPS	Non-Adv	Adv	MARS	OS	PFS
	WHO (AdvSM)			+					
	Age ≥60			+	+	+	+		
Anemia	≤10 g/dL						+		
	≤11 g/dL					+		+	
Thrombocytopenia	<100 × 10⁹/L					+	+		+
	<150 × 10⁹/L			+					
Leukocytosis	≥16 × 10⁹/L					+			
Increased serum markers	Baseline serum tryptase					+			+
	β2-Microglobulin		+						+
	Alkaline phosphatase			+	+			+	
Genetics	BM *KIT* D816V VAF ≥1%		+						
	Additional somatic mutations	+ (*A/R/D*)	+ (*A/R/D*)	+ (*A/R/NRAS*)			+ (*S/A/R*)	+ (*S/A/R/D*)	

A, ASXL1; *advSM*, advanced SM; *BM*, bone marrow; *D*, DNMT3A; *GPS*, Global Prognostic Score for Mastocytosis; *IPSM*, International Prognostic Scoring System for Mastocytosis; *ISM*, indolent SM; *MAPS*, Mayo Alliance Prognostic Scoring System; *MARS*, mutation-adjusted risk score; *OS*, overall survival; *PFS*, progression-free survival; *R*, RUNX1; *REMA*, Spanish Network on Mastocytosis; *S*, SRSF2; *VAF*, variant allele frequency; *WHO*, World Health Organization.
Modified from Reiter A, George TI, Gotlib J. New developments in diagnosis, prognostication, and treatment of advanced systemic mastocytosis. *Blood.* 2020;135(16):1365–1376.

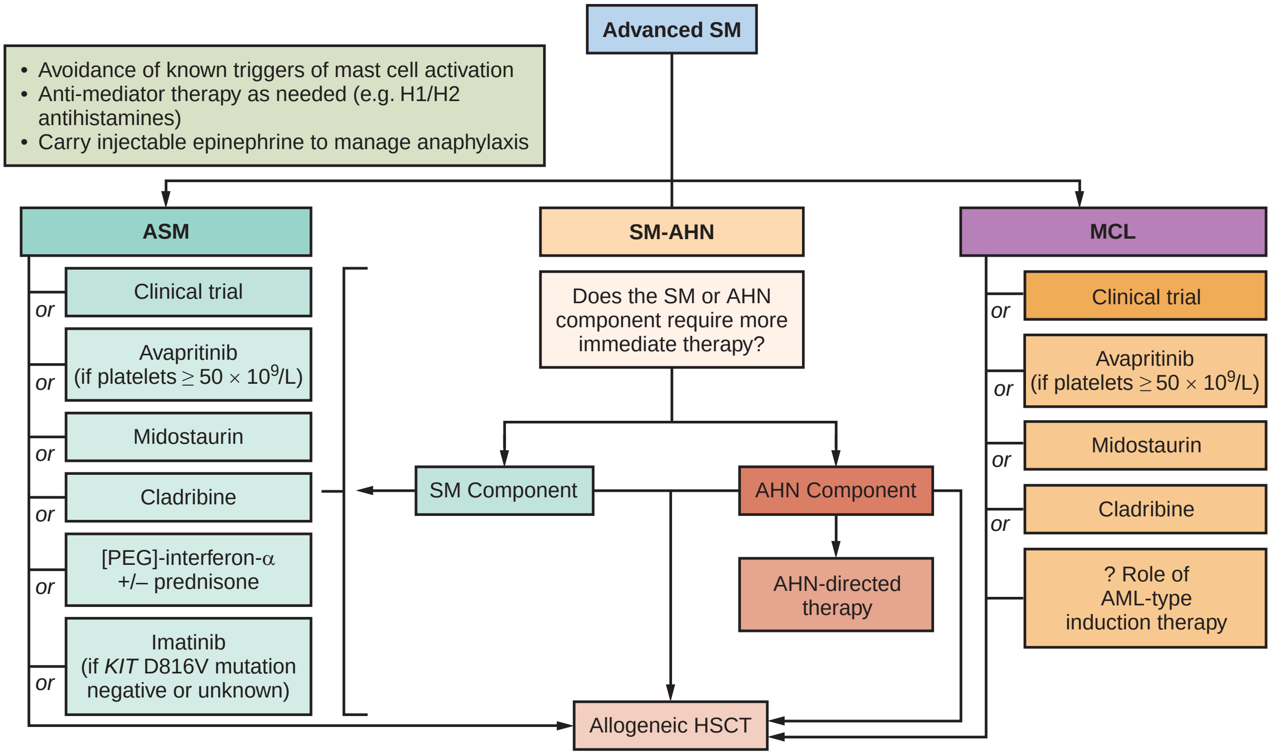

Advanced SM
• Avoidance of known triggers of mast cell activation
• Anti-mediator therapy as needed (e.g. H1/H2 antihistamines)
• Carry injectable epinephrine to manage anaphylaxis
ASM
SM-AHN
MCL
Clinical trial
or
Avapritinib (if platelets ≥ 50 × 10^9/L)
or
Midostaurin
or
Cladribine
or
[PEG]-interferon-α +/– prednisone
or
Imatinib (if *KIT* D816V mutation negative or unknown)
or
Does the SM or AHN component require more immediate therapy?
SM Component
AHN Component
AHN-directed therapy
Allogeneic HSCT
Clinical trial
or
Avapritinib (if platelets ≥ 50 × 10^9/L)
or
Midostaurin
or
Cladribine
or
? Role of AML-type induction therapy

Figure 75.8 TREATMENT OPTIONS FOR ADVANCED SYSTEMIC MASTOCYTOSIS VARIANTS. Treatment options are listed based on the subvariant of advanced SM which comprises aggressive systemic mastocytosis, systemic mastocytosis with an associated hematologic neoplasm, and mast cell leukemia. For SM-AHN, the initial decision is whether treatment is more immediately necessary for the SM or AHN component. *ASM*, Aggressive systemic mastocytosis; *HSCT*, hematopoietic stem cell transplantation; *MCL*, mast cell leukemia; *SM*, systemic mastocytosis; *SM-AHN*, systemic mastocytosis with an associated hematologic neoplasm.

TREATMENT

Therapy of mastocytosis centers on addressing symptoms related to MC activation (in all categories), and using cytoreductive agents to reverse organ damage caused by neoplastic MC infiltrates in advSM. In the United States, the National Comprehensive Cancer Network (NCCN) has produced guidelines which continuously update algorithms for the diagnosis, prognostication, and treatment of the spectrum of SM subtypes.[148] Fig. 75.8 illustrates the treatment options for the advSM variants ASM, SM-AHN, and MCL. For SM-AHN patients, the first major therapeutic consideration is whether the SM or AHN component requires more immediate therapy. The role and timing and allogeneic hematopoietic stem cell transplantation (HSCT) is still being defined, especially in the age of KIT-targeting agents.

Mast Cell Activation Symptoms/Anaphylaxis

All adult mastocytosis patients, regardless of prior anaphylactic events, should carry two doses of an adrenaline autoinjector (and should be instructed in its safe use, e.g., by an allergist) to treat potential future episodes of anaphylaxis. In addition, emergency medicines such as diphenhydramine or oral prednisolone should be considered, although their utility for mitigating acute symptoms is less well-established.[11,13,174] Because anaphylaxis can also be a feature of MCAS, these patients are candidates for similar therapy even if no overt SM was diagnosed.[84–87,92] An additional trigger of severe symptoms may be HαT. Therefore, HαT should also be confirmed or excluded in these patients, regardless of the confirmed presence of an IgE-dependent allergy.[94–96] The treatment of choice for life-threatening Hymenoptera allergy/anaphylaxis is venom immunotherapy that should be undertaken with caution using a multidisciplinary approach led by allergists.[92,174,189–192] Similarly, perioperative management of anesthetics, which can precipitate immediate and delayed MC activation, requires consultation with the surgical, anesthesiology, and allergy teams to minimize the occurrence and severity of anaphylaxis. General guidelines regarding anesthesia and analgesic management in patients with mastocytosis undergoing procedures or surgery, as well as management of pregnancy, have been published.[148,191,193–206]

The treatment of MC activation symptoms follows a stepwise approach using antimediator drugs and other supportive care measures.[11,13,70,174] These medications include H1-histamine blockers for flushing and pruritis, and H2-histamine blockers for SM-related GI symptomatology such as diarrhea, abdominal discomfort/cramping, and peptic ulcer disease. Leukotriene antagonists such as Montelukast are commonly added to H1-histamine blockers for persistent flushing and pruritis. In rare cases, aspirin can be an alternative therapy for refractory flushing, but needs to be used with caution because of its ability to precipitate MC activation, bleeding tendency, and GI tract ulcerative disease. In general, however, aspirin is not recommended for use in SM. Although the MC stabilizer sodium cromoglycate (Gastrocrom) is geared to persistent GI symptoms such as diarrhea, proton pump inhibitors may be specifically useful for patients with refractory GERD/peptic ulcer disease. Alternative treatments that have been used for refractory mediator symptoms include the off-label antihistamine/MC stabilizer ketotifen,[207] and the anti-IgE antibody omalizumab[208–212] has also been used for anaphylaxis, as well as asthma. Although bisphosphonates (alendronate 70 mg q week; risedronate 35 mg q week; pamidronic acid 90 mg intravenously (IV) q 4 weeks; and zoledronic acid 4 mg IV q 4 weeks) are recommended for bony disease, reports of their use in SM primarily consist of single cases or small cases series.[56,70,148,174,213–217] A generally accepted rule is to start with a bisphosphonate when the T-score drops to below −2.[84] IFN-α has been employed with bisphosphonates and/or as single agent therapy, especially for patients with refractory bony pain and/or MC-related osteoporosis and pathologic fractures.[218,219] Anti-RANKL monoclonal antibody therapy (e.g., denosumab) is generally used as second-line therapy for patients with bone pain not responding to bisphosphonates or for patients who are not candidates for bisphosphonates because of renal insufficiency.[148,220] Concerns regarding the cosmetic appearance of cutaneous lesions or skin-related mediator symptoms refractory to the aforementioned therapies can be helped for a limited period of time with phototherapy (UVA$_1$, narrow-band UVB, and UVA plus psoralen). In addition, occlusion dressings embedded with topical steroids with or without phototherapy, and both topical and oral forms of sodium cromoglycate, have been used with some benefit.[221]

Cytoreductive Agents

IFN-α and cladribine (2-chlorodeoxyadenosine) have typically been used for patients with progressive SSM and for cytoreduction in advSM patients (ASM, MCL, and SM-AHN) to reduce or reverse organ damage. In most instances, cladribine is favored over IFN-α to induce debulking of neoplastic MCs in advSM patients.[72,100,148,174] The use of both drugs is recommended to treat patients with less rapidly progressive disease (PD). In addition, cladribine and IFN-α are sometimes used to control mediator symptoms, especially when these symptoms are refractory to conventional anti-mediator-type drugs. In addition, low-dose IFN-α has been suggested for ISM patients with drug-resistant osteoporosis.[218,219] Otherwise, however, these agents are not recommended for use in ISM or SSM. In patients with rapidly progressive ASM or MCL, more intensive therapy is usually recommended, whereas cladribine alone or IFN-α alone are insufficient to control rapid disease expansion or transformation in SM.

Interferon-α

IFN-α has been used as monotherapy or in combination with corticosteroids, mostly in patients with ASM with slow progression or mono-organ involvement. For example, the drug works effectively in patients with ASM with liver involvement and ascites. Prednisone or prednisolone 0.5 to 1 mg/kg is initiated with IFN-α (or as a lead-in dose for several days prior) in order to improve its efficacy and tolerability, and tapered over 2 to 3 months if feasible.[70,72,148] In some patients with severe and persistent mediator symptoms or organ damage that is particularly responsive to corticosteroids, a longer-term maintenance dose (e.g., ≤10 mg daily) may be required. IFN-α dosing has been variable between studies and an optimal dose has not been identified. Starting doses have ranged from 1 million units (MU)/day to 3 MU three times weekly, with median total weekly maintenance doses in the range of 15 to 30 MU.[70,72,148] Because of the (low) risk of anaphylactic reactions, consideration should be given to hospitalization of patients during the first days of treatment, if possible.

In the Mayo experience with IFN-α (with or without prednisone), 21 of 40 evaluable patients responded (53%); response rates by subgroup were: six of 10 ISM, six of 10 ASM, and nine of 20 SM-AHN.[70,222] The 21 responses consisted of one complete remission (CR), six major responses (MRs), and 14 partial responses (PRs). The median duration of response (DOR) was 12 months (range, 1 to 67) with no statistical difference between responders and nonresponders regarding use of prednisone or the median weekly dose of IFN-α. However, the absence of mediator-related symptoms was associated with a significantly lower response rate.

In the French experience with 20 SM patients (four ISM and 16 ASM), IFN-α-2b was initiated at 1 MU daily and progressively increased as tolerated up to 5 MU/m^2 daily.[223] Among the 13 patients treated for at least 6 months (mean daily dose: 3.2 MU daily), all had partial or complete resolution of systemic or cutaneous disease manifestations and/or MC mediator levels, but no significant reduction in BM MC burden. The lack of a substantive effect on tumor load and rapid relapse in four responding patients after drug withdrawal suggests that IFN-α exerts a cytostatic effect on MCs.

Responses to IFN-α primarily include amelioration of MC mediator symptoms and associated laboratory markers of MC activation such as levels of histamine or its metabolites. Single cases and small case series, in addition to the aggregate data from the aforementioned larger trials, indicate variable potential to ameliorate skin lesions, osteoporosis, and C findings such as cytopenias, hepato/splenomegaly with liver dysfunction and/or ascites, and weight loss.[218,219,222–227] Toxicities associated with IFN-α such as flu-like symptoms, myelosuppression, transaminitis, hypothyroidism, depression, and bone pain are not uncommon, and result in a moderate proportion of patients requiring dose-reduction or drug discontinuation.

Cladribine

Cladribine has been used off-label across a spectrum of SM subtypes. Cladribine has received orphan designation (not marketing authorization) for the treatment of SM in Europe. Following an initial report indicating that cladribine could elicit improvement of mediator symptoms, urticaria pigmentosa lesions, and reduction of MC burden and the serum tryptase level,[228] Kluin-Nelemans and colleagues published a series of 10 patients, including 3 with ISM, 1 SSM, 3 SM-AHN, and 3 ASM.[229] Cladribine was administered for 5 days every 4 to 8 weeks at a dose of 0.10 to 0.13 mg/kg over 2 hours IV. Among 9 evaluable patients (1 discontinued because of toxicoderma), the drug elicited improvement in symptoms, and a decrease (often rapid) in urticaria pigmentosa lesions, MC burden, serum tryptase levels, and urine metabolites of MC activation.

In the Mayo Clinic experience, cladribine was administered for 5 days at a dose of 5 mg/m^2/day (or 0.13 to 0.17 mg/kg/day) IV.[70,222] A median of 3 cycles (range, 1 to 9) was administered. The overall response rate was 55% (12/22 patients) consisting of 1 CR, 7 MR, and 4 PR. Responses were observed in 5 of 9 ISM, 1 of 2 ASM, and 6 of 11 SM-AHN patients. Improvement in mediator symptoms, organomegaly, C-findings (e.g., ascites, anemia), and markers of MC burden and/or activation (e.g., serum tryptase level and MC-derived urine metabolites) were observed. The median response duration was 11 months, and inferior outcomes were predicted by leukocytosis, monocytosis, and circulating immature myeloid cells, the latter remaining significant in a multivariate analysis.

The long-term French experience with cladribine in both indolent ($n = 36$) and advanced ($n = 32$) SM patients have been published.[230] Cladribine was administered as an infusion or subcutaneously at a dose of 0.14 mg/kg, for 1 to 5 days, every 4 to 12 weeks. A median of 3.7 courses was administered (range, 1 to 9). The overall response rate was 72%, split between 92% and 50% for indolent and advanced disease, respectively. Among patients with advSM, the respective CR, MR, and PR rates were 0%, 37.5%, and 12.5%. Significant decrease in serum tryptase levels was only observed in ISM patients, and changes in BM MC burden were only evaluated in nine patients, precluding a substantive conclusion regarding this endpoint. Median durations of response were 3.71 (range, 0.1 to 8) and 2.47 (range, 0.5 to 8.6) years for indolent and advSM, respectively. Lymphopenia (82%), neutropenia (47%), and opportunistic infections (13%) were the most common grade 3/4 adverse events. Although cladribine has activity in selected patients with SM, its use in subjects with indolent disease (even those with refractory mediator symptoms) needs to be approached very cautiously because of the potential for substantial high-grade toxicities in these individuals who otherwise exhibit similar survival to age-matched controls.

Tyrosine Kinase Inhibitors

Dasatinib and Nilotinib

Multikinase inhibitors with activity against KIT have been evaluated in patients with advSM. Dasatinib is a dual SRC/ABL kinase inhibitor that is approved for front-line treatment of chronic myeloid leukemia. Despite dasatinib exhibiting nanomolar activity against KIT D816V in vitro,[231,232] a case series and a phase II trial of dasatinib (140 mg total daily dose) revealed limited activity in

SM.[138,233] In the phase II trial of 33 patients (ISM, $n = 18$; ASM, $n = 9$; SM-AHN), 11 (33%) responded.[138] Two complete responses were recorded in patients who were negative for the *KIT* D816V mutation, including a patient with *JAK2* V617F-positive SM-PMF and a patient with SM-CEL. The other 9 responses provided symptomatic benefit only, without clinically significant reductions in either BM MC burden or serum tryptase levels. The drug's short half-life may be one contributory reason for its lack of activity in SM patients. In fact, although dasatinib can block both MC growth and IgE-dependent MC activation in vitro, no reproducible effects are seen in SM patients in vivo.

Nilotinib was evaluated in an open-label trial of 61 patients with SM; among the cohort of 37 advSM patients, 8 (22%) exhibited objective responses with reductions in MC burden and/or serum tryptase level.[234] Taken together, these agents have shown low overall response rates in phase II trials and are no longer being pursued in advSM.

Imatinib

Imatinib is approved for ASM without the *KIT* D816V mutation or with unknown *KIT* mutational status. The clinical benefit of imatinib in SM is therefore limited given the high prevalence of the *KIT* D816V mutation, and the increasing sensitivity and widespread use of assays that can detect this molecular abnormality. Although imatinib lacks activity against KIT D816V in vitro and in vivo,[235–239] it has led to clinical benefit in patients with alternative *KIT* mutations or wild-type *KIT*. For example, imatinib has elicited excellent responses in cases of the WDSM variant with either the F522C TM *KIT* mutation[78] or wild-type *KIT*;[79] in a patient with familial SM carrying the germline *KIT* K509I mutation;[240] with deletion of codon 419 in exon 8 of *KIT* in pediatric CM;[241] and in a case of MCL with mutation in exon 9 (p.A502_Y503dup).[80] It is also noteworthy that long-term treatment with imatinib (400 mg/day) is associated with an almost complete MC deficiency, which may be of clinical relevance in reactive (nonclonal) MCAS and other MC-dependent diseases.

Masitinib

Masitinib (AB1010), is an inhibitor of Lyn, Fyn, PDGFR-α/β, and wild-type KIT.[242] After an initial study II trial in patients with ISM or CM with symptoms unresponsive to prior therapy,[243] masitinib was studied in a phase III, randomized, double-blind trial of 135 patients with ISM or SSM (108 subjects formally satisfied the WHO criteria for SM). Subjects were randomized to oral masitinib (6 mg/kg daily in two daily doses) or placebo.[244] The primary end-point of the study was based on a 75% or more improvement in one or more symptom categories: pruritis, flushing, depression, or fatigue. Of the patients taking masitinib, 18.7% achieved this endpoint compared to 7.4% of patients taking placebo. At week 24, there was an 18% decrease in the serum tryptase level in the masitinib arm versus an increase of 2.2% in the placebo arm ($P < .0001$). Also, urticaria pigmentosa lesions on masitinib therapy decreased by an average body surface area of 12.3% versus an increase of 15.9% for the placebo group. Masitinib-associated clinical benefits were generally sustained during a two-year extension period. Although treatment was generally well tolerated, there was an excess incidence of diarrhea, rash, and asthenia in 9%, 6%, and 4% of patients, respectively, in the masitinib group. In addition, 24% of patients in the masitinib arm discontinued therapy because of an adverse event, compared with 10% in the placebo arm. Masitinib has not received health regulatory approval for ISM or SSM.

Midostaurin

Midostaurin (*N*-benzoylstaurosporine; PKC412) is an inhibitor of multiple TKs including wild-type and D816V-mutated KIT, FLT3, PDGFR-α/β, FGFR1, and VEGFR2. In Ba/F3 cells transformed by *KIT* D816V, the IC_{50} of midostaurin was 30 to 40 nM compared with greater than 1 μM with imatinib.[236,237] A PR with midostaurin in a patient with MCL and an associated MDS/MPN,[237] and encouraging responses in a phase II trial of 26 patients with advSM[245] led to a global, multicenter, open-label trial of midostaurin (100 mg twice daily on 28-day continuous cycles) in patients with ASM, MCL, and SM-AHN.[246]

The trial employed a steering committee and central pathology review to adjudicate eligibility, response, and histopathology.[246] Among 89 evaluable patients, the ORR was 60%, of which 75% were major responders (MRs). Responses in organ damage (e.g., normalization of cytopenias/red blood cell or platelet transfusion dependence) and liver function abnormalities (hypoalbuminemia) were observed regardless of *KIT* D816V status, prior therapy, or the presence of an AHN. The median best reduction in serum tryptase level was −58%. In addition, the median change in BM MC burden was −59%, and 57% of patients had a ≥50% reduction in BM MCs. After a median follow-up of 26 months, the median duration of overall response (DOR) and median OS were 24.1 and 28.7 months, respectively. Median OS in responders was 44.4 months compared with 15.4 months in nonresponders. Of the 16 patients with MCL, 8 responded, including 7 MR (44%); among MCL patients, the median DOR was not reached, with three MRs ongoing at 49, 33, and 19 months at the time of data cut-off. The median OS was 9.4 months among all patients with MCL but was not reached among responding MCL patients. Symptoms and quality of life, measured by the Memorial Symptom Assessment Scale (MSAS) and Short-Form 12 (SF-12) survey, respectively, were significantly improved with midostaurin treatment. Improved symptoms and quality of life may relate to the ability of midostaurin to block IgE-dependent mediator release from MCs in vitro.[247] The drug was generally well tolerated with a manageable toxicity profile consisting mostly of GI side effects, including nausea and vomiting, primarily grade 1 to 2 in nature.

Among a cohort of 38 advSM patients treated with midostaurin, response rate and OS were significantly higher in patients with at least a 25% reduction in *KIT* D816V RNA-expressed allele burden (i.e., *KIT* responders) and in patients without *S/A/R* mutations.[248] In a multivariate analysis, *KIT* responder status was the strongest and only on-treatment variable associated with prolonged OS. Progression of midostaurin-treated patients to MCL or secondary AML was associated with ≥1 *S/A/R* mutations, an increase in the allele burden of preexisting mutations, or on-treatment development of new mutations in genes such as *K/NRAS*, *RUNX1*, *IDH2*, and *NPM1*.

Avapritinib

Avapritinib (BLU-285; Blueprint Medicines) is an oral type 1 multikinase inhibitor with highly selective and potent activity against *PDGFRA* A-loop mutants and *KIT* D816V ($IC_{50} = 0.27$ nM), which is 10-fold more potent than midostaurin.[249] A multicenter phase I trial accrued 69 patients into dose escalation ($n = 37$) and dose expansion ($n = 32$) cohorts, with the resulting recommended go-forward dose of 200 mg daily[139] currently being evaluated in an ongoing phase II study. Among the 53 patients evaluable using modified IWG-MRT-ECNM response criteria in the phase I trial,[139] the overall response rate was 75%; this comprised a 36% CR rate with full or partial hematologic recovery ([CR, 8%] + CR with partial hematologic recovery [CRh, 15%]), 34% PR, and 6% clinical improvement (CI).[139] Response rates by advSM subgroup were 100%, 76%, and 69% in patients with ASM, SM-AHN, and MCL, respectively. A >50% in BM MCs was observed in 92% of patients, and a >50% decrease in serum tryptase level was achieved in 99% of patients. Notably, *KIT* D816V became undetectable in 30% of patients using digital droplet PCR with a sensitivity of 0.17%. The most common adverse events (all grades/grade 3 to 4, %) were periorbital edema (69/2), anemia (55/30), diarrhea (45/1), nausea (44/3), thrombocytopenia (44/23), fatigue (41/9), and peripheral edema (40/0). Intracranial bleeding (ICB) occurred in 13% overall, and in 1% of patients without severe thrombocytopenia. A pre-specified interim analysis of the phase II PATHFINDER study evaluating 32 advSM patients demonstrated an ORR of 75% and similar safety profile with only 1 case of ICB due to implementation of mitigation procedures for thrombocytopenia.[250] Avapritinib was approved for patients with ASM, SM-AHN, and MCL in June of 2021. The recommended starting dose for patients with advSM is 200 mg daily. This drug is not recommended for the treatment of patients with platelet counts less than 50 × 10^9/L due to the higher risk of ICB in these patients.

Avapritinib is also being evaluated in patients with ISM; in stage 1 of a double-blind, placebo-controlled, randomized study, a 25 mg daily dose was chosen as the recommended dose for further evaluation in stage 2, the open-label portion of the trial.[251]

The oral type 2 switch control kinase inhibitor ripretinib (DCC-2618; Deciphera Pharmaceuticals) exhibits activity against multiple KIT and PDGFR-α mutants, with antineoplastic activity in preclinical models using *KIT* D816V-transfected MC lines (IC_{50} = 133 to 256 nM).[252] Enrollment of advSM patients in a phase II trial of ripretinib at a dose of 150 mg twice daily has been completed. Bezuclastinib (CGT9486) is an oral, highly selective TK inhibitor with potent activity against *KIT* D816V which is also commencing trials in both indolent and advSM.

Other Agents

Hydroxyurea has been used to control leukocytosis and splenomegaly in patients with a concurrent AHN such as MPN, CEL/HES, or MDS/MPN.[225] Multiagent, intensive chemotherapy is usually reserved for patients with MCL, especially for patients with kinetically active disease, but the results thus far have generally been disappointing.[253] Anthracycline plus cytarabine-based induction chemotherapy is the standard of care for patients with AML who are suitable for high-intensity therapy, including those in whom SM is also present. In such cases, treatment of the AML almost always takes priority, and the finding of the *KIT* D816V mutation may provide an opportunity to consider the addition of KIT inhibitors such as midostaurin to the induction, consolidation, and/or maintenance phases of treatment. However, the safety and efficacy of this approach has yet to be validated in the context of clinical trials. Several antibodies and antibody-drug conjugates have been considered for use in advSM. For example, the anti-CD30 antibody-drug conjugate brentuximab vedotin (BV) induces growth inhibition and apoptosis of $CD30^+$ MCs. However, a clinical trial evaluating BV did not demonstrate activity in advSM patients with $CD30^+$ MC.[107] Other agents that may be useful for the eradication of neoplastic MCs and possibly also neoplastic stem cells include anti-CD33, CD44, CD52, and CD123 antibody-drug immunoconjugates (or naked monoclonal antibodies).[254–257]

Allogeneic Hematopoietic Stem Cell Transplantation

Until recently, the role of HSCT in advSM remained poorly characterized, and only on a limited number of case reports and series had appeared. In a large, multicenter retrospective analysis, Ustun and colleagues evaluated the outcomes of 57 SM patients (SM-AHN, $n = 38$ [AML = 20]; ASM, $n = 7$; and MCL, $n = 12$) who underwent allogeneic HSCT.[258] Donor types consisted of HLA-matched identical ($n = 34$) and unrelated ($n = 17$) donors, umbilical cord blood ($n = 2$), and haploidentical ($n = 1$), and three unknown. Responses were observed in 70% of patients, including a 16% CR rate. The remaining 30% of responses were split between stable disease (21%) and primary refractory disease (9%). All 38 patients with SM-AHN achieved CR regarding the AHN component, but 10 subsequently relapsed with AHN, and half of these patients died.

The median OS for all patients at 3 years was 57%, consisting of 74% for patients with SM-AHN, and 43% and 17% for ASM and MCL patients, respectively.[258] The strongest risk factor for a poor OS was a diagnosis of MCL. In addition, inferior survival was observed in patients undergoing reduced-intensity versus fully myeloablative conditioning. However, patient age, donor age, donor type (sibling or unrelated donor), graft source (BM or peripheral HSCT, *KIT* mutation status, karyotype), and total-body irradiation used in myeloablative conditioning had no impact on overall or PFS. Treatment-related mortality at 6 months and 1 year was 11% and 20%, respectively, and was highest in MCL patients. Although a prospective trial is needed to better define the role of HSCT in advSM, these data suggest that transplantation can provide extended survival in selected patients, particularly for patients with SM-AHN. Consensus opinions have been developed for the use of HSCT in advSM,[259] but will continually evolve in the context of KIT-targeting drugs and combination therapies to address both the SM and AHN components.

Response Criteria for Advanced Systemic Mastocytosis

Response criteria for advSM were first published in 2003 and reiterated in a consensus conference report in 2007.[84] In this system, evaluation of clinical evidence of organ damage, or “C” findings, was the foundation for distinguishing levels of response. Changes in BM MC burden, serum tryptase level, and hepatosplenomegaly were additionally employed to subcategorize levels of MR. These original criteria or their modified version have been used to adjudicate responses in clinical trials of new agents.

Several shortcomings emerged regarding these SM response criteria. First, achievement of an “MR” is permitted in patients with baseline laboratory values just outside of the normal reference range. Second, responses in C-findings such as ascites, weight loss, and bone lesions are often difficult to quantify. Third, criteria for baseline red blood cell and platelet transfusion dependence were not codified. Lastly, the minimal DOR was not clearly defined, although a minimum response duration of 8 weeks has been incorporated ad hoc into many trials.

In 2013, the International Working Group for Myeloproliferative Neoplasms Research and Treatment (IWG-MRT) and the ECNM established revised response criteria for advSM that better characterize nonhematologic and hematologic organ damage findings, and lend more specificity and quantitation to evaluation of clinical and histopathologic improvement, which require 12-week duration.[136] IWG-MRT-ECNM response criteria, or modifications of these definitions are now being incorporated into clinical trials and are being used by regulatory health agencies such as the Food and Drug Administration and European Medicines Agency to evaluate new agents for approval.

FUTURE DIRECTIONS

Several clinical and translational initiatives are being pursued in MC disease and should reach fruition in the near future. In 2020, an AIM/ECNM (US/EU) Working Conference developed a proposal for revised diagnostic criteria and classification of MC disorders and is expected to achieve broad integration into international guidelines.[260] Efforts to combine data from the ECNM registry,[261] which has enlisted over 5000 patients, with the nascent efforts of AIM, will be a high priority. Updated criteria for MCASs[262,263] and guidelines for vaccination against COVID-19 in patients with MC disorders[264] have provided further clarity for these challenging clinical scenarios.

In conjunction with biopharma, research groups are also validating SM-specific patient-reported outcome tools to qualitatively and quantitatively measure patients’ symptom burden and quality of life in the context of drug development for indolent and advSM. Regulatory health agencies are increasingly focused on these patient-reported outcomes as endpoints in phase II trials as well as phase III placebo-controlled, double-blind, randomized trials.

Clinical trials are under development to investigate targets and pathways relevant to MC pathobiology. These include JAK inhibitors (e.g., ruxolitinib), inhibitors of BCL-2 (e.g., venetoclax), and agonist antibodies against inhibitory receptors such as siglec-8 in order to induce MC apoptosis. Major unmet needs include the development of combination therapies for SM-AHN using AHN-directed therapies plus KIT inhibition, or syncopated schedules of cladribine + KIT inhibitors in advSM patients with refractory disease or suboptimal responses with KIT inhibitor monotherapy.[100] Biologic correlates of therapeutic response such as minimal residual disease analysis using sensitive PCR assays to interrogate *KIT* mutant allele burden,[139] as well as circulating tumor DNA and cytokine profiling are now being incorporated into trials. Multi-omic analyses (e.g., genomic, transcriptomic, and proteomic profiling) of MCs and AHN populations and their progenitors using single cell assays will help further define the biologic heterogeneity of SM-AHN.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Arock M, Sotlar K, Akin C, et al. KIT mutation analysis in mast cell neoplasms: recommendations of the European Competence Network on Mastocytosis. *Leukemia*. 2015;29:1223.

Barete S, Lortholary O, Damaj G, et al. Long-term efficacy and safety of cladribine (2-CdA) in adult patients with mastocytosis. *Blood*. 2015;126:1009.

Bonadonna P, Brockow K, Nedoszytko M, et al. COVID-19 vaccination in mastocytosis: recommendations of the European Competence Network on Mastocytosis (ECNM) and American Initiative in Mast Cell Diseases (AIM). *J Allergy Clin Immunol Pract*. 2021;9:2139.

Eisenwort G, Sadovnik I, Schwaab J, et al. Identification of a leukemia-initiating stem cell in human mast cell leukemia. *Leukemia*. 2019;33:2673.

Escribano L, Diaz-Augustin B, López A, et al. Immunophenotypic analysis of mast cells in mastocytosis: when and how to do it. Proposals of the Spanish Network on Mastocytosis (REMA). *Cytometry B Clin Cytom*. 2004;58:1.

Garcia-Montero AC, Jara-Acevedo M, Teodosio C, et al. KIT mutation in mast cells and other bone marrow haematopoietic cell lineages in systemic mast cell disorders: a prospective study of the Spanish Network on Mastocytosis (REMA) in a series of 113 patients. *Blood*. 2006;108:2366.

Gilfillan AM, Rivera J. The tyrosine kinase network regulating mast cell activation. *Immunol Rev*. 2009;228:149.

Gotlib J, Kluin-Nelemans HC, George TI, et al. Efficacy and safety of midostaurin in advanced systemic mastocytosis. *N Engl J Med*. 2016;374:2530.

Gotlib J, Pardanani A, Akin C, et al. International Working Group-Myeloproliferative Neoplasms Research and Treatment (IWG-MRT) & European Competence Network on Mastocytosis (ECNM) consensus response criteria in advanced systemic mastocytosis. *Blood*. 2013;121:2393.

Gotlib J, Reiter A, Radia DH, et al. Efficacy and safety of avapritinib in advanced systemic mastocytosis: interim analysis of the phase 2 PATHFINDER trial. *Nat Med*. 2021;27(12):2192–2199.

Grootens J, Ungerstedt JS, Ekoff M, et al. Single-cell analysis reveals the KIT D816V mutation in haematopoietic stem and progenitor cells in systemic mastocytosis. *EBioMedicine*. 2019;43:150.

Gülen T, Akin C, Bonadonna P, et al. Selecting the right criteria and proper classification to diagnose mast cell activation syndromes: a critical review. *J Allergy Clin Immunol Pract*. 2021:S2213–2198. 00676-0.

Horny H-P, Akin C, Arber DA, et al. Mastocytosis. In: Swerdlow SH, ed. *World Health Organization (WHO) Classification of Tumours Pathology and Genetics of Tumours of the Haematopoietic and Lymphoid Tissues*. Lyon: International Agency for Research and Cancer (IARC); 2016:61.

Jawhar M, Schwaab J, Meggendorfer M, et al. The clinical and molecular diversity of mast cell leukemia with or without associated hematologic neoplasm. *Haematologica*. 2017;102:1035.

Kluin-Nelemans HC, Reiter A, Illerhaus A, et al. Prognostic impact of eosinophils in mastocytosis: analysis of 2350 patients collected in the ECNM Registry. *Leukemia*. 2020;34:1090.

Lim K-H, Tefferi A, Lasho TL, et al. Systemic mastocytosis in 342 consecutive adults: survival studies and prognostic factors. *Blood*. 2009;113:5727.

Lyons JJ, Yu X, Hughes JD, et al. Elevated basal serum tryptase identifies a multisystem disorder associated with increased TPSAB1 copy number. *Nat Genetics*. 2016;48:1564.

Maric I, Robyn J, Metcalfe DD, et al. KIT D816V-associated systemic mastocytosis with eosinophilia and FIP1L1/PDGFRA-associated chronic eosinophilic leukemia are distinct entities. *J Allergy Clin Immunol*. 2007;120:680.

Metcalfe DD. Mast cells and mastocytosis. *Blood*. 2008;112:946.

Nagata H, Worobec AS, Oh CK, et al. Identification of a point mutation in the catalytic domain of the protooncogene c-kit in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. *Proc Natl Acad Sci USA*. 1995;92:10560.

Pardanani A, Lim K-H, Lasho TL, et al. Prognostically relevant breakdown of 123 patients with systemic mastocytosis associated with other myeloid malignancies. *Blood*. 2009;114:3769.

Reiter A, George TI, Gotlib JR. New developments in diagnosis, prognostication, and treatment of advanced systemic mastocytosis. *Blood*. 2020;135:1365.

Saleh R, Wedeh G, Herrmann H, et al. A new human mast cell line expressing a functional IgE receptor converts to tumorigenic growth by KIT D816V transfection. *Blood*. 2014;124:111.

Schwaab J, Schnittger S, Sotlar K, et al. Comprehensive mutational profiling in advanced systemic mastocytosis. *Blood*. 2013;122:2460.

Schwartz LB, Sakai K, Bradford TR, et al. The alpha form of human tryptase is the predominant type present in blood at baseline in normal subjects and is elevated in those with systemic mastocytosis. *J Clin Invest*. 1995;96:2702.

Sperr WR, Kundi M, Alvarez-Twose I, et al. International prognostic scoring system for mastocytosis (IPSM): a retrospective cohort study. *Lancet Haematol*. 2019;6:e638.

Ustun C, Reiter A, Scott BL, et al. Hematopoietic stem-cell transplantation for advanced systemic mastocytosis. *J Clin Oncol*. 2014;32:3264.

Valent P, Akin C, Arock M, et al. Definitions, criteria and global classification of mast cell disorders with special reference to mast cell activation syndromes: a consensus proposal. *Int Arch Allergy Immunol*. 2012;157:215.

Valent P, Akin C, Escribano L, et al. Standards and standardization in mastocytosis: consensus statements on diagnostics, treatment recommendations and response criteria. *Eur J Clin Invest*. 2007;37:435.

Valent P, Akin C, Hartmann K, et al. Updated diagnostic criteria and classification of mast cell disorders: a consensus proposal. *Hemasphere*. 2021;5(11):e646.

Valent P, Akin C, Nedoszytko M, et al. Diagnosis, classification and management of mast cell activation syndromes (MCAS) in the era of personalized medicine. *Int J Mol Sci*. 2020;21:9030.

Valent P, Escribano L, Broesby-Olsen S, et al. Proposed diagnostic algorithm for patients with suspected mastocytosis: a proposal of the European Competence Network on Mastocytosis. *Allergy*. 2014;69:1267.

Valent P, Horny HP, Escribano L, et al. Diagnostic criteria and classification of mastocytosis: a consensus proposal. *Leuk Res*. 2001;25:603.

Valent P, Sotlar K, Sperr WR, et al. Refined diagnostic criteria and classification of mast cell leukemia (MCL) and myelomastocytic leukemia (MML): a consensus proposal. *Ann Oncol*. 2014;25:1691.

REFERENCES

1. Ehrlich P. *Beitrage Zur Theorie Und Praxis Der Histologischen Färbung [Thesis]*. Leipzig, Germany: University of Leipzig;; 1878.
2. Unna PG. Die spezifische färbung der mastzel-lenkörnung. *Monatsh Prakt Dermatol*. 1894;19:367.
3. Webb RL. Peritoneal reactions in the white rat with especial reference to the mast cells. *Am J Anat*. 1931;49:283.
4. Jorpes JE. The site of formation of heparin. In: Jorpes JE, ed. *Heparin: Its Chemistry, Physiology, and Application in Medicine*. London, United Kingdom: Humphrey Milford (Oxford University Press); 1939:30.
5. Riley JF. Histamine in tissue mast cells. *Science*. 1953;118:332.
6. Uvnäs B, Thon IL. Isolation of "biologically intact" mast cells. *Exp Cell Res*. 1959;18:512.
7. Austen KF, Brocklehurst WE. Anaphylaxis in chopped guinea pig lung. II. Enhancement of the anaphylactic release of histamine and slow reacting substance by certain dibasic aliphatic acids and inhibition by monobasic fatty acids. *J Exp Med*. 1961;113:541.
8. Baker AR, Bloch KJ, Austen KF. In vitro passive sensitization of chopped guinea pig lung by guinea pig 7s antibodies. *J Immunol*. 1964;93:525.
9. Orange RP, Valentine MD, Austen KF. Antigen-induced release of slow reacting substance of anaphylaxis (SRS-A rat) in rats prepared with homologous antibody. *J Exp Med*. 1968;127:767.
10. Metcalfe DD. Mast cells and mastocytosis. *Blood*. 2008;112:946.
11. Valent P, Horny HP, Escribano L, et al. Diagnostic criteria and classification of mastocytosis: a consensus proposal. *Leuk Res*. 2001;25:603.
12. Horny H-P, Akin C, Metcalfe DD, et al. Mastocytosis. In: Swerdlow SH, Campo E, Harris NL, eds. *WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues*. Lyon: International Agency for Research and Cancer (IARC); 2008:54.
13. Valent P, Akin C, Escribano L, et al. Standards and standardization in mastocytosis: consensus statements on diagnostics, treatment recommendations and response criteria. *Eur J Clin Invest*. 2007;37:435.
14. Kirshenbaum AS, Kessler SW, Goff JP, et al. Demonstration of the origin of human mast cells from CD34+ bone marrow progenitor cells. *J Immunol*. 1991;146:1410.
15. Kirshenbaum AS, Goff JP, Semere T, et al. Demonstration that human mast cells arise from a progenitor cell population that is CD34(+), c-kit(+), and expresses aminopeptidase N (CD13). *Blood*. 1999;94:2333.
16. Agis H, Willheim M, Sperr WR, et al. Monocytes do not make mast cells when cultured in the presence of SCF. Characterization of the circulating mast cell progenitor as a c-kit+, CD34+, Ly-, CD14-, CD17-, colony-forming cell. *J Immunol*. 1993;151:4221.
17. Eisenwort G, Sadovnik I, Schwaab J, et al. Identification of a leukemia-initiating stem cell in human mast cell leukemia. *Leukemia*. 2019;33:2673.
18. Yarden Y, Kuang WJ, Yang-Feng T, et al. Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. *EMBO J*. 1987;6:3341.
19. Reilly JT. Class III receptor tyrosine kinases: role in leukaemogenesis. *Br J Haematol*. 2002;116:744.
20. Zsebo KM, Wypych J, McNiece IK, et al. Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver–conditioned medium. *Cell*. 1990;63:195.
21. Huang E, Nocka K, Beier DR, et al. The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. *Cell*. 1990;63:225.
22. Anderson DM, Lyman SD, Baird A, et al. Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. *Cell*. 1990;63:235.
23. Kirshenbaum AS, Goff JP, Kessler SW, et al. Effect of IL-3 and stem cell factor on the appearance of human basophils and mast cells from CD34+ pluripotent progenitor cells. *J Immunol*. 1992;148:772.
24. Valent P, Spanblöchl E, Sperr WR, et al. Induction of differentiation of human mast cells from bone marrow and peripheral blood mononuclear cells by recombinant human stem cell factor/kit-ligand in long-term culture. *Blood*. 1992;80:2237.
25. Linnekin D. Early signaling pathways activated by c-Kit in hematopoietic cells. *Int J Biochem Cell Biol*. 1999;31:1053.
26. Brizzi MF, Dentelli P, Rosso A, et al. STAT protein recruitment and activation in c-Kit deletion mutants. *J Biol Chem*. 1999;274:16965.
27. Gilfillan AM, Rivera J. The tyrosine kinase network regulating mast cell activation. *Immunol Rev*. 2009;228:149.
28. Bochner BS. "Siglec"ting the allergic response for therapeutic targeting. *Glycobiology*. 2016;26:546.
29. Hudson SA, Herrmann H, Du J, et al. Developmental, malignancy-related, and cross-species analysis of eosinophil, mast cell, and basophil siglec-8 expression. *J Clin Immunol*. 2011;31:1045.
30. Brown JM, Wilson TM, Metcalfe DD. The mast cell and allergic diseases: role in pathogenesis and implications for therapy. *Clin Exp Allergy*. 2008;38:4.
31. Gotlib J, Akin C. Mast cells and eosinophils in mastocytosis, chronic eosinophilic leukemia, and non-clonal disorders. *Semin Hematol*. 2012;49:128.
32. Shakoory B, Fitzgerald SM, Lee SA, et al. The role of human mast cell-derived cytokines in eosinophil biology. *J Interferon Cytokine Res*. 2004;24:271.
33. Tkaczyk C, Horejsi V, Iwaki S, et al. NTAL phosphorylation is a pivotal link between the signaling cascades leading to human mast cell degranulation following Kit activation and FcRI aggregation. *Blood*. 2004;104:207.
34. Iwaki S, Tcaczyk C, Satterthwaite AB, et al. Btk plays a crucial role in the amplification of FcRI-mediated mast cell activation by kit. *J Biol Chem*. 2005;280:40261.
35. Saleh R, Wedeh G, Herrmann H, et al. A new human mast cell line expressing a functional IgE receptor converts to tumorigenic growth by KIT D816V transfection. *Blood*. 2014;124:111.
36. Cools J, Stover EH, Boulton CL, et al. PKC412 overcomes resistance to imatinib in a murine model of FIP1L1-PDGFRα-induced myeloproliferative disease. *Cancer Cell*. 2003;3:459.
37. Yamada Y, Rothenberg ME, Lee AW, et al. The FIP1L1-PDGFRA fusion gene cooperates with IL-5 to induce murine hypereosinophilic syndrome (HES)/chronic eosinophilic leukemia (CEL)-like disease. *Blood*. 2006;107:4071.
38. Yamada Y, Sanchez-Aguilera A, Brandt EB, et al. FIP1L1/PDGFRalpha synergizes with SCF to induce systemic mastocytosis in a murine model of chronic eosinophilic leukemia/hypereosinophilic syndrome. *Blood*. 2008;112:2500.
39. Merz H, Kaehler C, Hoefig KP, et al. Interleukin-9 (IL-9) and NPM-ALK each generate mast cell hyperplasia as single "hit" and cooperate in producing a mastocytosis-like disease in mice. *Oncotarget*. 2010;1:104.
40. Arock M, Sotlar K, Akin C, et al. KIT mutation analysis in mast cell neoplasms: recommendations of the European Competence Network on Mastocytosis. *Leukemia*. 2015;29:1223.
41. Halpern AL, Torphy RJ, McCarter MD, et al. A familial germline mutation in KIT associated with achalasia, mastocytosis and gastrointestinal stromal tumors shows response to kinase inhibitors. *Cancer Genet*. 2019;233-234:1.
42. Hartmann K, Wardelmann E, Ma Y, et al. Novel germline mutation of KIT associated with familial gastrointestinal stromal tumors and mastocytosis. *Gastroenterology*. 2015;129:1042.
43. Nedoszytko B, Niedoszytko M, Lange M, et al. Interleukin-13 promoter gene polymorphism -1112C/T is associated with the systemic form of mastocytosis. *Allergy*. 2009;64:287.
44. Daley T, Meetcalfe DD, Akin C. Association of the Q576R polymorphism in the interleukin-4 receptor alpha chain with indolent mastocytosis limited to the skin. *Blood*. 2001;98:880.
45. Rausz E, Szilágyi A, Nedoszytko B, et al. Comparative analysis of IL6 and IL6 receptor gene polymorphisms in mastocytosis. *Br J Haematol*. 2013;160:216.
46. Cohen SS, Skovbo S, Vestergaard H, et al. Epidemiology of systemic mastocytosis in Denmark. *Br J Haematol*. 2014;166:521.
47. van Doormaal JJ, Arends S, Brunekreeft KL, et al. Prevalence of indolent systemic mastocytosis in a Dutch region. *J Allergy Clin Immunol*. 2013;131:1429.
48. Ben-Amitai D, Metzker A, Cohen HA. Pediatric cutaneous mastocytosis: a review of 180 patients. *Isr Med Assoc J*. 2005;7:320.
49. Hartmann K, Escribano L, Grattan C, et al. Cutaneous manifestations in patients with mastocytosis: consensus report of the European Competence Network on Mastocytosis; the American Academy of Allergy, Asthma & Immunology; and the European Academy of Allergology and Clinical Immunology. *J Allergy Clin Immunol*. 2016;137:35.
50. Valent P, Horny H-P, Li CY, et al. Mastocytosis. In: Jaffe ES, Harris NL, Stein H, JW V, eds. *World Health Organization (WHO) Classification of Tumours Pathology and Genetics of Tumours of the Haematopoietic and Lymphoid Tissues*. Lyon: International Agency for Research and Cancer (IARC); 2001:291.
51. Horny H-P, Akin C, Arber DA, et al. Mastocytosis. In: Swerdlow SH, Campo E, Harris NL, eds. *World Health Organization (WHO) Classification of Tumours Pathology and Genetics of Tumours of the Haematopoietic and Lymphoid Tissues*. Lyon: International Agency for Research and Cancer (IARC); 2016:61.

51a. Khoury JD, Solary E, Abla O, et al. The 5th edition of the World Health Organization classification of haematolymphoid tumours: myeloid and histiocytic/dendritic neoplasms. *Leukemia*. 2022;36(7):1703–1719.

51b. Arber DA, Orazi A, Hasserjian RP, et al. International Consensus Classification of myeloid neoplasms and acute leukemia: integrating

morphological, clinical, and genomic data. *Blood*. 2022 June 29. Online ahead of print.
52. Lim K-H, Tefferi A, Lasho TL, et al. Systemic mastocytosis in 342 consecutive adults: survival studies and prognostic factors. *Blood*. 2009;113:5727.
53. Sperr WR, Kundi M, Alvarez-Twose I, et al. International prognostic scoring system for mastocytosis (IPSM): a retrospective cohort study. *Lancet Haematol*. 2019;6:e638.
54. González de Olano D, de la Hoz Caballer B, Núñez López R, et al. Prevalence of allergy and anaphylactic symptoms in 210 adult and pediatric patients with mastocytosis in Spain: a study of the Spanish network on mastocytosis (REMA). *Clin Exp Allergy*. 2007;37:1547.
55. Alvarez-Twose I, Zanotti R, González-de-Olano D, et al. on behalf of the Spanish Network on Mastocytosis (REMA) and the Italian Network on Mastocytosis (RIMA). Nonaggressive systemic mastocytosis (SM) without skin lesions associated with insect-induced anaphylaxis shows unique features versus other indolent SM. *J Allergy Clin Immunol*. 2014;133:520.
56. Barete S, Assous N, de Gennes C, et al. Systemic mastocytosis and bone involvement in a cohort of 75 patients. *Ann Rheum Dis*. 2010;69:1838.
57. Rossini M, Zanotti R, Bonadonna P, et al. Bone mineral density, bone turnover markers and fractures in patients with indolent systemic mastocytosis. *Bone*. 2011;49:880.
57a. Valent P, Akin C, Hartmann K, et al. Updated diagnostic criteria and classification of mast cell disorders: a consensus proposal. *Hemasphere*. 2021;5(11):e646.
58. Akin C, Scott LM, Metcalfe DD. Slowly progressive systemic mastocytosis with high mast-cell burden and no evidence of a non-mast-cell hematologic disorder: an example of a smoldering case? *Leuk Res*. 2001;25:635.
59. Jordan JH, Fritsche-Polanz R, Sperr WR, et al. A case of "smouldering" mastocytosis with high mast cell burden, monoclonal myeloid cells, and C-KIT mutation Asp-816-Val. *Leuk Res*. 2001;25:627.
60. Valent P, Akin C, Sperr WR, et al. Smouldering mastocytosis: a novel subtype of systemic mastocytosis with slow progression. *Int Arch Allergy Immunol*. 2002;127:137.
61. Garcia-Montero AC, Jara-Acevedo M, Teodosio C, et al. KIT mutation in mast cells and other bone marrow hematopoietic cell lineages in systemic mast cell disorders: a prospective study of the Spanish Network on Mastocytosis (REMA) in a series of 113 patients. *Blood*. 2006;108:2366.
62. Escribano L, Alvarez-Twose I, Sánchez-Muñoz L, et al. Prognosis in adult indolent systemic mastocytosis: a long-term study of the Spanish Network on Mastocytosis in a series of 145 patients. *J Allergy Clin Immunol*. 2009;124:514.
63. Travis WD, Li CY, Yam LT, et al. Significance of systemic mast cell disease with associated hematologic disorders. *Cancer*. 1988;62:965.
64. Sperr WR, Horny H-P, Valent P. Spectrum of associated clonal hematologic non-mast cell lineage disorders occurring in patients with systemic mastocytosis. *Int Arch Allergy Immunol*. 2002;127:140.
65. Pardanani A, Lim K-H, Lasho TL, et al. Prognostically relevant breakdown of 123 patients with systemic mastocytosis associated with other myeloid malignancies. *Blood*. 2009;114:3769.
66. Du S, Rashidi HH, Le DT, et al. Systemic mastocytosis in association with chronic lymphocytic leukemia and plasma cell myeloma. *Int J Clin Exp Pathol*. 2010;3:448.
67. Schipper EM, Posthuma W, Snieders I, et al. Mastocytosis and diffuse large B-cell lymphoma, an unlikely combination. *Neth J Med*. 2011;69:132.
68. Bernd HW, Sotlar K, Lorenzen J, et al. Acute myeloid leukaemia with t(8;21) associated with "occult" mastocytosis. Report of an unusual case and review of the literature. *J Clin Pathol*. 2004;57:324.
69. Arrendondo AR, Gotlib J, Scier L, et al. Myelomastocytic leukemia versus mast cell leukemia versus systemic mastocytosis associated with acute myeloid leukemia: a diagnostic challenge. *Am J Hematol*. 2010;85:600.
70. Pardanani A. Systemic mastocytosis in adults: 2019 update on diagnosis, risk stratification and management. *Am J Hematol*. 2019;94:363.
71. Valent P, Akin C, Sperr WR, et al. Aggressive systemic mastocytosis and related mast cell disorders: current treatment options and proposed response criteria. *Leuk Res*. 2003;27:635.
72. Valent P, Sperr WR, Akin C. How I treat patients with advanced systemic mastocytosis. *Blood*. 2010;116:5812.
73. Sotlar K, Fridrich C, Mall A, et al. Detection of c-kit point mutation Asp-816 → Val in microdissected pooled single mast cells and leukemic cells in a patient with systemic mastocytosis and concomitant chronic myelomonocytic leukemia. *Leuk Res*. 2002;26:979.
74. Valent P, Sotlar K, Sperr WR, et al. Refined diagnostic criteria and classification of mast cell leukemia (MCL) and myelomastocytic leukemia (MML): a consensus proposal. *Ann Oncol*. 2014;25:1691.
75. Georgin-Lavialle S, Lhermitte L, Dubreuil P, et al. Mast cell leukemia. *Blood*. 2013;121:1285.
76. Jawhar M, Schwaab J, Meggendorfer M, et al. The clinical and molecular diversity of mast cell leukemia with or without associated hematologic neoplasm. *Haematologica*. 2017;102:1035.
77. Valent P, Sotlar K, Sperr WR, et al. Chronic mast cell leukemia: a novel leukemia-variant with distinct morphological and clinical features. *Leuk Res*. 2015;39:1.
78. Akin C, Fumo G, Yavuz AS, et al. A novel form of mastocytosis associated with a transmembrane c-kit mutation and response to imatinib. *Blood*. 2004;103:3222.
79. Alvarez-Twose I, Gonazález P, Morgado JM, et al. Complete response after imatinib mesylate therapy in a patient with well-differentiated systemic mastocytosis. *J Clin Oncol*. 2012;30:e126.
80. Mital A, Piskorz A, Lewandowski K, et al. A case of mast cell leukaemia with exon 9 KIT mutation and good response to imatinib. *Eur J Haematol*. 2011;86:531.
81. Ryan RJH, Akin C, Castells M, et al. Mast cell sarcoma: a rare and potentially under- recognized diagnostic entity with specific therapeutic implications. *Mod Pathol*. 2013;26:533.
82. Castells MC. Extracutaneous mastocytoma. *J Allergy Clin Immunol*. 2006;117:1513.
83. Akin C, Valent P, Metcalfe DD. Mast cell activation syndrome: proposed diagnostic criteria. *J Allergy Clin Immunol*. 2010;126:1099.
84. Valent P, Akin C, Arock M, et al. Definitions, criteria and global classification of mast cell disorders with special reference to mast cell activation syndromes: a consensus proposal. *Int Arch Allergy Immunol*. 2012;157:215.
85. Hamilton MJ, Hornick JL, Akin C, et al. Mast cell activation syndrome: a newly recognized disorder with systemic clinical manifestations. *J Allergy Clin Immunol*. 2011;128:147.
86. Akin C, Scott LM, Kocabas CN, et al. Demonstration of an aberrant mast-cell population with clonal markers in a subset of patients with "idiopathic" anaphylaxis. *Blood*. 2007;110:2331.
87. Pollyea DA, George TI, Corless C, et al. When yellow jackets attack: recurrent and severe anaphylactic reactions to insect bites and stings. *Am J Hematol*. 2009;84:843.
88. Valent P, Bonadonna P, Hartmann K, et al. Why the 20% + 2 tryptase formula is a diagnostic gold standard for severe systemic mast cell activation and mast cell activation syndrome. *Int Arch Allergy Immunol*. 2019;180:44.
89. Brockow K, Jofer C, Behrendt H, et al. Anaphylaxis in patients with mastocytosis: a study on history, clinical features and risk factors in 120 patients. *Allergy*. 2008;63:226.
90. van Anrooij B, van der Veer E, de Monchy JGR, et al. Higher mast cell load decreases the risk of Hymenoptera venom-induced anaphylaxis in patients with mastocytosis. *J Allergy Clin Immunol*. 2013;132:125.
91. Florian S, Krauth M-T, Simonitsch-Klupp I, et al. Indolent systemic mastocytosis with elevated serum tryptase, absence of skin lesions, and recurrent severe anaphylactoid episodes. *Int Arch Allergy Immunol*. 2005;136:273.
92. Niedoszytko M, Bonadonna P, Oude Elberlink JN, et al. Epidemiology, diagnosis, and treatment of Hymenoptera venom allergy in mastocytosis patients. *Immunol Allergy Clin North Am*. 2014;34:365.
93. Akin C. Mast cell activation disorders. *J Allergy Clin Immunol Pract*. 2014;2:252.
94. Lyons JJ, Yu X, Hughes JD, et al. Elevated basal serum tryptase identifies a multisystem disorder associated with increased TPSAB1 copy number. *Nat Genetics*. 2016;48:1564.
95. Greiner G, Sprinzi B, Górska A, et al. Hereditary alpha tryptasemia is a valid genetic biomarker for severe mediator-related symptoms in mastocytosis. *Blood*. 2021;137(2):238–247.
96. Lyons JJ, Chovanec J, O'Connell MP, et al. Heritable risk for severe anaphylaxis associated with increased α-tryptase-encoding germline copy number at TPSAB1. *J Allergy Clin Immunol*. 2021;147(2):622–632.
97. Horny H-P, Sotlar K, Valent P. Mastocytosis: immunophenotypical features of the transformed mast cells are unique among hematopoietic cells. *Immunol Allergy Clin North Am*. 2014;34:315.
98. Escribano L, Diaz-Augustin B, López A, et al. Immunophenotypic analysis of mast cells in mastocytosis: when and how to do it. Proposals of the Spanish Network on Mastocytosis (REMA). *Cytometry B Clin Cytom*. 2004;58:1.
99. Sánchez-Muñoz L, Teodosio C, Morgado JMT, et al. Flow cytometry in mastocytosis: utility as a diagnostic and prognostic tool. *Immunol Allergy Clin North Am*. 2014;34:297.
100. Reiter A, George TI, Gotlib JR. New developments in diagnosis, prognostication, and treatment of advanced systemic mastocytosis. *Blood*. 2020;135:1365.
101. Sotlar K, Cerny-Reiterer S, Petat-Dutter K, et al. Aberrant expression of CD30 in neoplastic mast cells in high-grade mastocytosis. *Mod Pathol*. 2011;24:585.

102. van Anrooij B, Kluin PM, Oude Elberink JN, et al. CD30 in systemic mastocytosis. *Immunol Allergy Clin North Am*. 2014;34:341.
103. Moonim MT, Kossier T, van Der Walt J, et al. CD30/CD123 expression in systemic mastocytosis does not correlate with aggressive disease. *Blood (ASH Annual Meeting Abstracts)*. 2012;116:1982.
104. Chiu A, Czader MB, Ochs RC, et al. Subtypes of systemic mastocytosis exhibit distinct immunomorphologic features in bone marrow biopsies. *Lab Invest*. 2013;93:324A.
105. Arredondo AR, Jennings CD, Shier L, et al. CD30 expression in mastocytosis. *Lab Invest*. 2011;91:285A.
106. Morgado JM, Perbellini O, Johnson RC, et al. CD30 expression by bone marrow mast cells from different diagnostic variants of systemic mastocytosis. *Histopathology*. 2013;63:780.
107. Gotlib J, Baird JH, George TI, et al. A phase 2 study of brentuximab vedotin in patients with CD30-positive advanced systemic mastocytosis. *Blood Adv*. 2019;3:2264.
108. Mueller N, Wicklein D, Eisenwort G, et al. CD44 is a RAS/STAT5-regulated invasion receptor that triggers disease expansion in advanced mastocytosis. *Blood*. 2018;132:1936.
109. Schwartz LB, Sakai K, Bradford TR, et al. The alpha form of human tryptase is the predominant type present in blood at baseline in normal subjects and is elevated in those with systemic mastocytosis. *J Clin Invest*. 1995;96:2702.
110. Valent P, Escribano L, Broesby-Olsen S, et al. Proposed diagnostic algorithm for patients with suspected mastocytosis: a proposal of the European Competence Network on Mastocytosis. *Allergy*. 2014;69:1267.
111. Valent P, Sperr WR, Sotlar K, et al. The serum tryptase test: an emerging robust biomarker in clinical hematology. *Expert Rev Hematol*. 2014;7:683.
112. Schwartz LB, Lewis RA, Seldin D, et al. Acid hydrolases and tryptase from secretory granules of dispersed human lung mast cells. *J Immunol*. 1981;126:1290.
113. Schwartz LB, Irani AM, Roller K, et al. Quantitation of histamine, tryptase, and chymase in dispersed human T and TC mast cells. *J Immunol*. 1987;138:2611.
114. Castells MC, Irani AM, Schwartz LB. Evaluation of human peripheral blood leukocytes for mast cell tryptase. *J Immunol*. 1987;138:2184.
115. Miller JS, Westin EH, Schwartz LB. Cloning and characterization of complementary DNA for human tryptase. *J Clin Invest*. 1989;84:1188.
116. Jogie-Brahim S, Min H-K, Fukuoka Y, et al. Expression of alpha-tryptase and beta-tryptase by human basophils. *J Allergy Clin Immunol*. 2004;113:1086.
117. Schwartz LB, Bradford TR, Rouse C, et al. Development of a new, more sensitive immunoassay for human tryptase: use in systemic anaphylaxis. *J Clin Immunol*. 1994;190
118. Schwartz LB, Min H-K, Ren S, et al. Tryptase precursors are preferentially and spontaneously released, whereas mature tryptase is retained by HMC-1 cells, Mono-Mac-6 cells, and human skin-derived mast cells. *J Immunol*. 2003;170:5667.
119. Dvorak AM, Schleimer RP, Lichtenstein LM. Human mast cells synthesize new granules during recovery from degranulation. In vitro studies with mast cells purified from human lungs. *Blood*. 1988;71:76.
120. Min H-K, Moxley G, Neale MC, Schwartz LB. Effect of sex and haplotype on plasma tryptase levels in healthy adults. *J Allergy Clin Immunol*. 2004;114:48.
121. Schliemann S, Seyfarth F, Hipler U-C, et al. Impact of age and heterophilic interference on the basal serum tryptase, a risk indication for anaphylaxis, in 1,092 dermatology patients. *Acta Derm Venereol*. 2012;92:484.
122. Schwartz LB. Diagnostic value of tryptase in anaphylaxis and mastocytosis. *Immunol Allergy Clin North Am*. 2006;26:451.
123. Schwartz LB, Metcalfe DD, Miller JS, et al. Tryptase levels as an indicator of mast-cell activation in systemic anaphylaxis and mastocytosis. *N Engl J Med*. 1987;316:1622.
124. Schwartz LB. Clinical utility of tryptase levels in systemic mastocytosis and associated hematologic disorders. *Leuk Res*. 2001;25:553.
125. Valent P, Sperr WR, Schwartz LB, et al. Diagnosis and classification of mast cell proliferative disorders: delineation from immunologic diseases and non-mast cell hematopoietic neoplasms. *J Allergy Clin Immunol*. 2004;114:3.
126. Bonadonna P, Perbellini O, Passalacqua G, et al. Clonal mast cell disorders in patients with systemic reactions to Hymenoptera stings and increased serum tryptase levels. *J Allergy Clin Immunol*. 2009;123:680.
127. Sperr WR, Jordan J-H, Fiegl M, et al. Serum tryptase levels in patients with mastocytosis: correlation with mast cell burden and implication for defining the category of disease. *Int Arch Allergy Immunol*. 2002;128:136.
128. Dugas-Breit S, Schöpf P, Dugas M, et al. Baseline serum levels of mast cell tryptase are raised in hemodialysis patients and associated with severity of pruritus. *J Dtsch Dermatol Ges*. 2005;3:343.
129. Sirvent AE, González C, Enríquez R, et al. Serum tryptase levels and markers of renal dysfunction in a population with chronic kidney disease. *J Nephrol*. 2010;23:282.
130. Sperr WR, El-Samahi A, Kundi M, et al. Elevated tryptase levels selectively cluster in myeloid neoplasms: a novel diagnostic approach and screen marker in clinical haematology. *Eur J Clin Invest*. 2009;39:914.
131. Cairoli R, Ripamonti CB, Beghini A, et al. Total serum tryptase: a predictive marker for KIT mutation in acute myeloid leukemia. *Leuk Res*. 2009;33:1282.
132. Sperr WR, Jordan JH, Baghestanian M, et al. Expression of mast cell tryptase by myeloblasts in a group of patients with acute myeloid leukemia. *Blood*. 2001;98:2200.
133. Sperr WR, Mitterbauer M, Mitterbauer G, et al. Quantitation of minimal residual disease in acute myeloid leukemia by tryptase monitoring identifies a group of patients with a high risk of relapse. *Clin Cancer Res*. 2005;11:6536.
134. Sperr WR, Stehberger B, Wimazal F, et al. Serum tryptase measurements in patients with myelodysplastic syndromes. *Leuk Lymphoma*. 2002;43:1097.
135. Klion AD, Noel P, Akin C, et al. Elevated serum tryptase levels identify a subset of patients with a myeloproliferative variant of idiopathic hypereosinophilic syndrome associated with tissue fibrosis, poor prognosis, and imatinib responsiveness. *Blood*. 2003;101:4660.
136. Gotlib J, Pardanani A, Akin C, et al. International Working Group-Myeloproliferative Neoplasms Research and Treatment (IWG-MRT) & European Competence Network on Mastocytosis (ECNM) consensus response criteria in advanced systemic mastocytosis. *Blood*. 2013; 121:2393.
137. Jawhar M, Schwaab J, Hausmann D, et al. Splenomegaly, elevated alkaline phosphatase and mutations in the SRSF2/ASXL1/RUNX1 gene panel are strong adverse prognostic markers in patients with systemic mastocytosis. *Leukemia*. 2016;30:2342.
138. Verstovsek S, Tefferi A, Cortes J, et al. Phase II study of dasatinib in Philadelphia chromosome-negative acute and chronic myeloid diseases, including systemic mastocytosis. *Clin Cancer Res*. 2008;14:3906.
139. DeAngelo DJ, Radia DH, George TI, et al. Safety and efficacy of avapritinib in advanced systemic mastocytosis: the phase 1 EXPLORER trial. *Nat Med*. 2021;27:2183–2191.
140. Nagata H, Worobec AS, Oh CK, et al. Identification of a point mutation in the catalytic domain of the protooncogene c-kit in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. *Proc Natl Acad Sci USA*. 1995;92:10560.
141. Grootens J, Ungerstedt JS, Ekoff M, et al. Single-cell analysis reveals the KIT D816V mutation in haematopoietic stem and progenitor cells in systemic mastocytosis. *EBioMedicine*. 2019;43:150.
142. Bibi S, Langenfeld F, Jeanningros S, et al. Molecular defects in mastocytosis: KIT and beyond KIT. *Immunol Allergy Clin North Am*. 2014;34:239.
143. Georgin-Lavialle S, Aguilar C, Guieze R, et al. Mast cell sarcoma: a rare and aggressive entity–report of two cases and review of the literature. *J Clin Oncol*. 2013;31:e90.
144. Pignon JM, Giraudier S, Duquesnoy P, et al. A new c-kit mutation in a case of aggressive mast cell disease. *Br J Haematol*. 1997;96:374.
145. Akin C, Brockow K, D'Ambrosio C, et al. Effects of tyrosine kinase inhibitor STI571 on human mast cells bearing wild-type or mutated c-kit. *Exp Hematol*. 2003;31:686.
146. Growney JD, Clark JJ, Adesperger J, et al. Activation mutations of human c-KIT resistant to imatinib mesylate are sensitive to the tyrosine kinase inhibitor PKC412. *Blood*. 2005;106:721.
147. Kristensen T, Vestergaard H, Bindslev-Jensen C, et al. Sensitive KIT D816V mutation analysis of blood as a diagnostic test in mastocytosis. *Am J Hematol*. 2014;89:493.
148. Gotlib J, Gerds AT, Bose P, et al. Systemic mastocytosis, version 2.2019, NCCN clinical practice guidelines in oncology. *J Natl Compr Canc Netw*. 2018;16:1500.
149. Bodemer C, Hermine O, Palmérini F, et al. Pediatric mastocytosis is a clonal disease associated with D816V and other activating c-KIT mutations. *J Invest Dermatol*. 2010;130:804.
150. Méni C, Bruneau J, Georgin-Lavialle S, et al. Paediatric mastocytosis: a systematic review of 1747 cases. *Br J Dermatol*. 2015;172:642.
151. Sotlar K, Bache A, Stellmacher F, et al. Systemic mastocytosis associated with chronic idiopathic myelofibrosis: a distinct subtype of systemic mastocytosis associated with a clonal hematological non-mast cell lineage disorder carrying the activating point mutations KITD816V and JAK2V617F. *J Mol Diagn*. 2008;10:58.
152. Agis H, Sotlar K, Valent P, et al. Ph-chromosome-positive chronic myeloid leukemia with associated bone marrow mastocytosis. *Leuk Res*. 2005;29:1227.

153. Hussein K, Horny HP, Büsche G, et al. Systemic mastocytosis (SM) with associated BCR-ABL-positive myelogenous leukaemia (SM-AHNMD): evidence that mast cells do not belong to the leukaemic clone. *Leukemia*. 2011;25:1050.
154. Pullarkat ST, Pullarkat V, Lagoo A, et al. Characterization of bone marrow mast cells in acute myeloid leukemia with t(8;21) (q22;q22); RUNX1-RUNX1T1. *Leuk Res*. 2013;37:1572.
155. Johnson RC, Savage NM, Chiang T, et al. Hidden mastocytosis in acute myeloid leukemia with t(8;21)(q22;q22). *Am J Clin Pathol*. 2013;140:525.
156. Won D, Chi HS, Shim H, et al. The prognostic impact of c-KIT mutation in systemic mastocytosis associated with acute myeloid leukaemia patients. *Leuk Res*. 2013;37:883.
157. Tefferi A, Levine RL, Lim K-H, et al. Frequent TET2 mutations in systemic mastocytosis: clinical, KITD816V and FIP1L1-PDGFRA correlates. *Leukemia*. 2009;23:900.
158. Traina F, Visconte V, Jankowska AM, et al. Single nucleotide polymorphism array lesions, TET2, DNMT3A, ASXL1 and CBL mutations are present in systemic mastocytosis. *PLoS One*. 2012;7:e43090.
159. Soucie E, Hanssens K, Mercher T, et al. In aggressive forms of mastocytosis, TET2 loss cooperates with c-KITD816V to transform mast cells. *Blood*. 2012;120:4846.
160. Schwaab J, Schnittger S, Sotlar K, et al. Comprehensive mutational profiling in advanced systemic mastocytosis. *Blood*. 2013;122:2460.
161. Damaj G, Joris M, Chandesris O, et al. ASXL1 but not TET2 mutations adversely impact overall survival of patients suffering systemic mastocytosis with associated clonal hematologic non-mast-cell diseases. *PLoS One*. 2014;9:e85362.
162. Hanssens K, Brenet F, Agopian J, et al. SRSF2-p95 hotspot mutation is highly associated with advanced forms of mastocytosis and mutations in epigenetic regulator genes. *Haematologica*. 2014;99:830.
163. Wilson TM, Maric I, Simakova O, et al. Clonal analysis of NRAS activating mutations in KIT-D816V systemic mastocytosis. *Haematologica*. 2011;96:459.
164. Jawhar M, Schwaab J, Schnittger S, et al. Molecular profiling of myeloid progenitor cells in multi-mutated advanced systemic mastocytosis identifies KIT D816V as a distinct and late event. *Leukemia*. 2015;29:1155.
165. Martelli M, Monaldi C, De Santis S, et al. Recent advances in the molecular biology of systemic mastocytosis: implications for diagnosis, prognosis, and therapy. *Int J Mol Sci*. 2020;21:3987.
166. Lasho TL, Finke CM, Zblewski D, et al. Recurrent mutations in ethanolamine kinase 1 (ETNK1) gene in systemic mastocytosis with eosinophilia and chronic myelomonocytic leukemia. *Blood Cancer J*. 2015;5:e275.
167. Gambacorti-Passerini CB, Donadoni C, Parmiani A, et al. Recurrent ETNK1 mutations in atypical chronic myeloid leukemia. *Blood*. 2015;125:499.
168. Cools J, DeAngelo DJ, Gotlib J, et al. A tyrosine kinase created by fusion of the PDGFRA and FIP1L1 genes as a therapeutic target of imatinib in idiopathic hypereosinophilic syndrome. *N Engl J Med*. 2003;348:1201.
169. Pardanani A, Brockman SR, Paternoster SF, et al. FIP1L1-PDGFRA fusion: prevalence and clinicopathologic correlates in 89 consecutive patients with moderate to severe eosinophilia. *Blood*. 2004;104:3038.
170. Maric I, Robyn J, Metcalfe DD, et al. KIT D816V-associated systemic mastocytosis with eosinophilia and FIP1L1/PDGFRA-associated chronic eosinophilic leukemia are distinct entities. *J Allergy Clin Immunol*. 2007;120:680.
171. Schmitt-Graeff AH, Erben P, Schwaab J, et al. The FIP1L1-PDGFRA fusion gene and the KIT D816V mutation are coexisting in a small subset of myeloid/lymphoid neoplasms with eosinophilia. *Blood*. 2014;123:595.
172. Seidel H, Molderings GJ, Oldenburg J, et al. Bleeding diathesis in patients with mast cell activation disease. *Thromb Haemost*. 2011;106:987.
173. Hahn HP, Hornick JL. Immunoreactivity for CD25 in gastrointestinal mucosal mast cells is specific for systemic mastocytosis. *Am J Surg Pathol*. 2007;31:1669.
174. Pardanani A. How I treat patients with indolent and smoldering mastocytosis (rare conditions but difficult to manage). *Blood*. 2013;121:3085.
175. Akin C, Valent P. Diagnostic criteria and classification of mastocytosis in 2014. *Immunol Allergy Clin North Am*. 2014;34:207.
176. Garcia-Montero AC, Jara-Acevedo M, Álvarez-Twose I, et al. KIT D816V-mutated bone marrow mesenchymal stem cells in indolent systemic mastocytosis are associated with disease progression. *Blood*. 2016;127:761.
177. Böhm A, Födinger M, Wimazal F, et al. Eosinophilia in systemic mastocytosis: clinical and molecular correlates and prognostic significance. *J Allergy Clin Immunol*. 2007;120:192.
178. Kluin-Nelemans HC, Reiter A, Illerhaus A, et al. Prognostic impact of eosinophils in mastocytosis: analysis of 2350 patients collected in the ECNM Registry. *Leukemia*. 2020;34:1090.
179. Naumann N, Jawhar M, Schwaab J, et al. Incidence and prognostic impact of cytogenetic aberrations in patients with systemic mastocytosis. *Genes Chromosomes Cancer*. 2018;57:252.
180. Shah S, Pardanani AD, Elala YC, et al. Cytogenetic abnormalities in systemic mastocytosis: WHO subcategory-specific incidence and prognostic impact among 348 informative cases. *Am J Hematol*. 2018;93:1461.
181. Jawhar M, Schwaab J, Schnittger S, et al. Additional mutations in SRSF2, ASXL1 and/or RUNX1 identify a high-risk group of patients with KIT D816V(+) advanced systemic mastocytosis. *Leukemia*. 2016;30:136.
182. Pardanani AD, Lasho TL, Finke C, et al. ASXL1 and CBL mutations are independently predictive of inferior survival in advanced systemic mastocytosis. *Br J Haematol*. 2016;175:534.
183. Muñoz-González JL, Álvarez-Twose I, Jara-Acevedo M, et al. Impact of somatic and germline mutations on the outcome of systemic mastocytosis. *Blood Adv*. 2018;2:2814.
184. Muñoz-González JI, Álvarez-Twose I, Jara-Acevedo M, et al. Frequency and prognostic impact of KIT and other genetic variants in indolent systemic mastocytosis. *Blood*. 2019;134:456.
185. Muñoz-González JI, Orfao A. Validation of the MARS, IPSM, Mayo and REMA prognostic scores for systemic mastocytosis. Presented at the 17th Annual Meeting of the European Competence Network on Mastocytosis. *Salzburg, Austria*. 2019
186. Jawhar M, Schwaab J, Álvarez-Twose I, et al. MARS: mutation-adjusted risk score for advanced systemic mastocytosis. *J Clin Oncol*. 2019;37:2846.
187. Pardanani A, Shah S, Mannelli F, et al. Mayo alliance prognostic system for mastocytosis: clinical and hybrid clinical-molecular models. *Blood Adv*. 2018;2:2964.
188. Pardanani A, Lasho TL, Reichard KK, Hanson CA, Tefferi A. World Health Organization class-independent risk categorization in mastocytosis. *Blood Cancer J*. 2019;9:29.
189. González de Olano D, Alvarez-Twose I, Esteban-López MI, et al. Safety and effectiveness of immunotherapy in patients with indolent systemic mastocytosis presenting with Hymenoptera venom anaphylaxis. *J Allergy Clin Immunol*. 2008;121:519.
190. Bonadonna P, Zanotti R, Müller U. Mastocytosis and insect venom allergy. *Curr Opin Allergy Clin Immunol*. 2010;10:347.
191. Castells MC, Hornick JL, Akin C. Anaphylaxis after hymenoptera sting: is it venom allergy, a clonal disorder, or both? *J Allergy Clin Immunol Pract*. 2015;3:350.
192. Carter MC, Metcalfe DD, Matito A, et al. Adverse reactions to drugs and biologics in patients with clonal mast cell disorders: a Work Group Report of the Mast Cells Disorder Committee, American Academy of Allergy, Asthma & Immunology. *J Allergy Clin Immunol*. 2019;143:880.
193. Chaar CIO, Bell RL, Duffy TP, et al. Guidelines for safe surgery in patients with systemic mastocytosis. *Am Surg*. 2019;75:74.
194. Ahmad N, Evans P, Lloyd-Thomas AR. Anesthesia in children with mastocytosis—a case-based review. *Paediatr Anaesth*. 2009;19:97.
195. Konrad FM, Schroeder TH. Anaesthesia in patients with mastocytosis. *Acta Anaesthesiol Scand*. 2009;53:270.
196. Mertes PM, Malinovsky JM, Jouffroy L, Working Group of the SFAR and SFA Aberer W, Terreehorst I, Brockow K, Demoly P. ENDA; EAACI Interest Group on Drug Allergy. Reducing the risk of anaphylaxis during anesthesia: 2011 updated guidelines for clinical practice. *J Invest Allergol Clin Immunol*. 2011;21:442.
197. Butterfield JH, Kao PC, Klee GC, et al. Aspirin idiosyncrasy in systemic mast cell disease: a new look at mediator release during aspirin desensitization. *Mayo Clin Proc*. 1995;70:481.
198. Brockow K, Bonadonna P. Drug allergy in mast cell disease. *Curr Opin Allergy Clin Immunol*. 2012;12:354.
199. Matito A, Morgado JM, Sánchez-López P, et al. Management of anesthesia in adult and pediatric mastocytosis: a study of the Spanish Network on Mastocytosis (REMA) based on 726 anesthetic procedures. *Int Arch Allergy Immunol*. 2015;167:47.
200. Dewachter P, Castells MC, Hepner DL, Mouton-Faivre C. Perioperative management of patients with mastocytosis. *Anesthesiology*. 2014;120:753.
201. Castells M, Butterfield J. Mast cell activation syndrome and mastocytosis: Initial treatment options and long-term management. *J Allergy Clin Immunol Pract*. 2019;4:1097.
202. Lei D, Akin C, Kovalszki A. Management of mastocytosis in pregnancy: a review. *J Allergy Clin Immunol Pract*. 2017;5:1217.
203. Madendag IC, Madendag Y, Tarhan I, Altinkaya SO, et al. Mastocytosis in pregnancy. *Taiwan J Obstet Gynecol*. 2010;49:192.
204. Woidacki K, Zenclussen AC, Siebenhaar F. Mast cell-mediated and associated disorders in pregnancy: a risky game with an uncertain outcome? *Front Immunol*. 2014;5:231.

205. Ciach K, Niedoszytko M, Abacjew-Chmylko A, et al. Pregnancy and delivery in patients with mastocytosis treated at the Polish Center of the European Competence Network on Mastocytosis (ECNM). *PLoS One*. 2016;11:e0146924.
206. Matito A, Álvarez-Twose I, Morgado JM, et al. Clinical impact of pregnancy in mastocytosis: a study of the Spanish Network on Mastocytosis (REMA) in 45 cases. *Int Arch Allergy Immunol*. 2011;156:104.
207. Kurosawa M, Amano H, Kanbe N, et al. Heterogeneity of mast cells in mastocytosis and inhibitory effect of ketotifen and ranitidine on indolent systemic mastocytosis. *J Allergy Clin Immunol*. 1997;100:S25.
208. Carter MC, Robyn JA, Bressler PB. Omalizumab for the treatment of unprovoked anaphylaxis in patients with systemic mastocytosis. *J Allergy Clin Immunol*. 2007;119:1550.
209. Molderings GJ, Raithel M, Kratz F, et al. Omalizumab treatment of systemic mast cell activation disease: experiences from four cases. *Intern Med*. 2011;50:611.
210. Slapnicar C, Trinkaus M, Hicks L, Vadas P. Efficacy of omalizumab in indolent systemic mastocytosis. *Case Rep Hematol*. 2019;2019:3787586.
211. Distler M, Maul JT, Steiner UC, et al. Efficacy of omalizumab in mastocytosis: Allusive indication obtained from a prospective, double-blind, multicenter study (XOLMA Study). *Dermatology*. 2020;236(6):529–539. https://doi.org/10.1159/000504842.
212. Jendoubi F, Gaudenzio N, Gallini A, et al. Omalizumab in the treatment of adult patients with mastocytosis: a systematic review. *Clin Exp Allergy*. 2020;50:654.
213. Lim AY, Ostor AJ, Love S, et al. Systemic mastocytosis: a rare cause of osteoporosis and its response to bisphosphonate treatment. *Ann Rheum Dis*. 2005;64:965.
214. Brumsen C, Hamdy NA, Papapoulos SE. Osteoporosis and bone marrow mastocytosis: dissociation of skeletal responses and mast cell activity during long-term bisphosphonate therapy. *J Bone Miner Res*. 2002;17:567.
215. Marshall A, Kavanagh RT, Crisp AJ. The effect of pamidronate on lumbar spine bone density and pain in osteoporosis secondary to systemic mastocytosis. *Br J Rheumatol*. 1997;36:393.
216. Laroche M, Bret J, Brouchet A, et al. Clinical and densitometric efficacy of the association of interferon alpha and pamidronate in the treatment of osteoporosis in patients with systemic mastocytosis. *Clin Rheumatol*. 2007;26:242.
217. Laroche M, Livideanu C, Paul C, et al. Interferon alpha and pamidronate in osteoporosis with fracture secondary to mastocytosis. *Am J Med*. 2011;124:776.
218. Weide R, Ehlenz K, Lorenz W, et al. Successful treatment of osteoporosis in systemic mastocytosis with interferon alpha-2b. *Ann Hematol*. 1996;72:41.
219. Lehmann T, Beyeler C, Lämmle B, et al. Severe osteoporosis due to systemic mast cell disease: successful treatment with interferon alpha-2B. *Br J Rheumatol*. 1996;35:898.
220. Orsolini G, Gavioli I, Tripi G, et al. Denosumab for the treatment of mastocytosis-related osteoporosis: a case series. *Calcif Tissue Int*. 2017;100:595.
221. Vieira Dos Santos R, Magerl M, Martus P, et al. Topical sodium cromoglicate relieves allergen- and histamine-induced dermal pruritus. *Br J Dermatol*. 2010;162:674.
222. Lim KH, Pardanani A, Butterfield JH, et al. Cytoreductive therapy in 108 adults with systemic mastocytosis: outcome analysis and response prediction during treatment with interferon-alpha, hydroxyurea, imatinib mesylate or 2-chlorodeoxyadenosine. *Am J Hematol*. 2009;84:790.
223. Casassus P, Caillat-Vigneron N, Martin A, et al. Treatment of adult systemic mastocytosis with interferon-alpha: results of a multicentre phase II trial on 20 patients. *Br J Haematol*. 2002;119:1090.
224. Kluin-Nelemans HC, Jansen JH, Breukelman H, et al. Response to interferon alfa-2b in a patient with systemic mastocytosis. *N Engl J Med*. 1992;326:619.
225. Worobec AS, Kirshenbaum AS, Schwartz LB, et al. Treatment of three patients with systemic mastocytosis with interferon alpha-2b. *Leuk Lymphoma*. 1996;22:501.
226. Butterfield JH. Response of severe systemic mastocytosis to interferon alpha. *Br J Dermatol*. 1998;138:489.
227. Hauswirth AW, Simonitsch-Klupp I, Uffmann M, et al. Response to therapy with interferon alpha-2b and prednisolone in aggressive systemic mastocytosis: report of five cases and review of the literature. *Leuk Res*. 2004;28:249.
228. Tefferi A, Li CY, Butterfield JH, et al. Treatment of systemic mast-cell disease with cladribine. *N Engl J Med*. 2001;344:307.
229. Kluin-Nelemans HC, Oldhoff JM, Van Doormaal JJ, et al. Cladribine therapy for systemic mastocytosis. *Blood*. 2003;102:4270.
230. Barete S, Lortholary O, Damaj G, et al. Long-term efficacy and safety of cladribine (2-CdA) in adult patients with mastocytosis. *Blood*. 2015;126:1009.
231. Schittenhelm MM, Shiraga S, Schroeder A, et al. Dasatinib (BMS-354825), a dual SRC/ABL kinase inhibitor, inhibits the kinase activity of wild-type, juxtamembrane, and activation loop mutant KIT isoforms associated with human malignancies. *Cancer Res*. 2006;66:473.
232. Shah NP, Lee FY, Luo R, et al. Dasatinib (BMS-354825) inhibits KITD816V, an imatinib-resistant activating mutation that triggers neoplastic growth in most patients with systemic mastocytosis. *Blood*. 2006;108:286.
233. Purtill D, Cooney J, Sinniah R, et al. Dasatinib therapy for systemic mastocytosis: four cases. *Eur J Haematol*. 2008;80:456.
234. Hochhaus A, Baccarini M, Giles FJ, et al. Nilotinib in patients with systemic mastocytosis: analysis of the phase 2, open-label, single-arm nilotinib registration study. *J Cancer Res Clin Oncol*. 2015;141:2047.
235. Akin C, Brockow K, D'Ambrosio C, et al. Effects of tyrosine kinase inhibitor STI571 on human mast cells bearing wild-type or mutated c-kit. *Exp Hematol*. 2003;31:686.
236. Growney JD, Clark JJ, Adelsperger J, et al. Activation mutations of human c-KIT resistant to imatinib mesylate are sensitive to the tyrosine kinase inhibitor PKC412. *Blood*. 2005;106:721.
237. Gotlib J, Berubé C, Growney JD, et al. Activity of the tyrosine kinase inhibitor PKC 412 in a patient with mast cell leukemia with the D816V KIT mutation. *Blood*. 2005;106:2865.
238. Droogendijk HJ, Kluin-Nelemans HJ, van Doormaal JJ, et al. Imatinib mesylate in the treatment of systemic mastocytosis: a phase II trial. *Cancer*. 2006;107:345.
239. Vega-Ruiz A, Cortes JE, Sever M, et al. Phase II study of imatinib mesylate as therapy for patients with systemic mastocytosis. *Leuk Res*. 2009;33:1481.
240. Zhang LY, Smith ML, Schultheis B, et al. A novel K509I mutation of KIT identified in familial mastocytosis-in vitro and in vivo responsiveness to imatinib therapy. *Leuk Res*. 2006;30:373.
241. Hoffmann KM, Moser A, Lohse P, et al. Successful treatment of progressive cutaneous mastocytosis with imatinib in a 2-year-old boy carrying a somatic KIT mutation. *Blood*. 2008;112:1655.
242. Dubreuil P, Letard S, Ciufolini M, et al. Masitinib (AB1010), a potent and selective tyrosine kinase inhibitor targeting KIT. *PLoS One*. 2009; 4:e7258.
243. Paul C, Sans B, Suarez F, et al. Masitinib for the treatment of systemic and cutaneous mastocytosis with handicap: a phase 2a study. *Am J Hematol*. 2010;85:921.
244. Lortholary O, Chandesris MO, Livideanu CB, et al. Masitinib for treatment of severely symptomatic indolent systemic mastocytosis: a randomised, placebo-controlled, phase 3 study. *Lancet*. 2017;389:612.
245. DeAngelo DJ, George TI, Linder A, et al. Efficacy and safety of midostaurin in patients with advanced systemic mastocytosis: 10-year median follow-up of a phase II trial. *Leukemia*. 2018;32:470.
246. Gotlib J, Kluin-Nelemans HC, George TI, et al. Efficacy and safety of midostaurin in advanced systemic mastocytosis. *N Engl J Med*. 2016;374:2530.
247. Krauth M-T, Mirkina I, Herrmann H, Baumgartner C, Kneidinger M, Valent P. Midostaurin (PKC412) inhibits immunoglobulin E-dependent activation and mediator release in human blood basophils and mast cells. *Clin Exp Allergy*. 2009;39:1711.
248. Jawhar M, Schwaab J, Naumann N, et al. Response and progression on midostaurin in advanced systemic mastocytosis: KIT D816V and other molecular markers. *Blood*. 2017;130:137.
249. Evans EK, Gardino AK, Kim JL, et al. A precision therapy against cancers driven by KIT/PDGFRA mutations. *Sci Transl Med 9:eaao1690,*. 2017
250. Gotlib J, Reiter A, Radia DH, et al. Efficacy and safety of avapritinib in patients with advanced systemic mastocytosis: interim analysis of the phase 2 PATHFINDER trial. *Nat Med*. 2021;27(12):2192–2199.
251. Akin C, Sabato V, Gotlib J, et al. PIONEER: a randomized, double-blind, placebo-controlled, phase 2 study of avapritinib in patients with indolent or smoldering systemic mastocytosis with symptoms inadequately controlled with standard therapy. *American Academy of Allergy, Asthma, and Immunology Annual Meeting*. 2020.
252. Smith BD, Kaufman MD, Lu W-P, et al. Ripretinib (DCC-2618) is a switch control kinase inhibitor of a broad spectrum of oncogenic and drug-resistant KIT and PDGFRA variants. *Cancer Cell*. 2019;35:738.
253. Valenti CG, Rondoni M, Pogliani EM, et al. Mast cell leukemia: a report of ten cases. *Ann Hematol*. 2008;87:505.
254. Alvarez-Twose I, Martínez-Barranco P, Gotlib J, et al. Complete response to gemtuzumab ozogamicin in a patient with refractory mast cell leukemia. *Leukemia*. 2016;30:1753.
255. Pardanani A, Lasho T, Chen D, et al. Aberrant expression of CD123 (interleukin-3 receptor-α) on neoplastic mast cells. *Leukemia*. 2015;29:1605.

256. Yun HD, Felices M, Vallera DA, et al. Trispecific killer engager CD16xIL15xCD33 potently induces NK cell activation and cytotoxicity against neoplastic mast cells. *Blood Adv*. 2018;2:1580.
257. Gotlib J. Tyrosine kinase inhibitors and therapeutic antibodies in advanced eosinophilic disorders and systemic mastocytosis. *Curr Hematol Malig Rep*. 2015;10:351.
258. Ustun C, Reiter A, Scott BL, et al. Hematopoietic stem-cell transplantation for advanced systemic mastocytosis. *J Clin Oncol*. 2014;32:3264.
259. Ustun C, Gotlib J, Popat U, et al. Consensus opinion on allogeneic hematopoietic cell transplantation in advanced systemic mastocytosis. *Biol Blood Marrow Transplant*. 2016;22:1348.
260. Valent P, Akin C, Hartmann K, et al. Updated diagnostic criteria and classification of mast cell disorders: a consensus proposal. *Hemasphere*. 2021;5(11):e646.
261. Valent P, Oude Elberink JNG, Gorska A, et al. The data registry of the European Competence Network on Mastocytosis (ECNM): set up, projects, and perspectives. *J Allergy Clin Immunol Pract*. 2019;7:81.
262. Valent P, Akin C, Nedoszytko M, et al. Diagnosis, classification and management of mast cell activation syndromes (MCAS) in the era of personalized medicine. *Int J Mol Sci*. 2020;21:9030.
263. Gülen T, Akin C, Bonadonna P, et al. Selecting the right criteria and proper classification to diagnose mast cell activation syndromes: a critical review. *J Allergy Clin Immunol Pract*. 2021;9(11):3918–3928.
264. Bonadonna P, Brockow K, Nedoszytko M, et al. COVID-19 vaccination in mastocytosis: recommendations of the European Competence Network on Mastocytosis (ECNM) and American Initiative in Mast Cell Diseases (AIM). *J Allergy Clin Immunol Pract*. 2021;9:2139.

CHAPTER 76

CHRONIC LYMPHOCYTIC LEUKEMIA

Farrukh T. Awan and John C. Byrd

Over the past several years, major advances have been realized in terms of improved understanding of the pathophysiology and therapeutic options for chronic lymphocytic leukemia (CLL). The plethora of information about CLL has increased dramatically and made management of what was a relatively straightforward disease quite complex but more rewarding. This chapter provides a reference source focused on critical issues in routine clinical management of CLL.

EPIDEMIOLOGY

CLL is one of the most prevalent types of leukemia in the Western Hemisphere. Surveillance, Epidemiology, and End Results (SEER) estimates for 2021 indicate that approximately 21,250 patients (13,040 men and 8210 women) would be diagnosed in the United States and that 4320 patients would die from CLL. The median age at diagnosis for CLL was 70 years in 2020, and incidence increases proportionately by decade of life as shown in Fig. 76.1. This figure illustrates that CLL is a very uncommon diagnosis before 45 years of age and infrequent (33% of total patients diagnosed) in patients before 65 years of age. The age-adjusted incidence rate was 5 per 100,000 men and women per year; 0.6% of men and women born from 2015 to 2017 are expected to be diagnosed with CLL during their lifetime. The estimated time of survival after diagnosis at 5 years is 86.1%, which explains the estimated prevalence of 186,422 in 2017. Similar to other types of leukemia, the risk of dying from disease-specific causes increases proportionately with increasing age. Another analysis of the SEER database, comparing outcome of elderly patients with CLL with age- and sex-matched healthy control participants, demonstrated that CLL has the greatest impact on survival in the most elderly group of patients. However, recent therapeutic advances continue to result in significant improvement in outcomes across all age groups.

CLL is more common in men than women. Women diagnosed with CLL have 5- and 10-year overall survival (OS) rates that exceed those of men. CLL is most common in whites and decreases in frequency in a descending order among blacks, Hispanics, Native Americans and Native Alaskans, and Asians and Pacific Islanders. While adverse outcome based upon race has been reported, this has not been uniformly confirmed in other studies. The rarity of CLL among Asians and Pacific Islanders persists even in immigrants from these areas who have migrated to the Western Hemisphere. This implicates a possible genetic predisposition to the development of CLL. The relationship of environmental factors such as exposure to benzene, radon, and other chemicals to the development of CLL is suggestive but not clearly established. However, CLL is recognized as a service-connected illness among Vietnam War veterans who were exposed to Agent Orange. Occupational or environmental exposure to radiation does not clearly appear to predispose patients to a higher risk of developing CLL. For example, although the frequency of acute myeloid leukemia (AML), chronic myeloid leukemia (CML), and acute lymphoblastic leukemia (ALL) were increased among survivors of the atomic bomb at Hiroshima, an increase in CLL was not appreciated as reported by the Life Span Study (LSS) of survivors of that event. This could be secondary to a baseline low incidence of CLL in patients with Asian heritage. However, recent reports of Chernobyl disaster survivors and the international nuclear workers (INWORKS) study suggest an increased incidence of CLL along with a shorter survival. In addition, the International Lymphoma Epidemiology Consortium non-Hodgkin lymphoma Subtypes Project (InterLymph Study) identified multiple factors that were associated with the presence of CLL, including (1) family history of a first-degree relative with hematologic malignancy including lymphomas, leukemias, and myeloma; (2) a history of working or living on a farm; (3) hairdressers; and (4) a history of hepatitis C infection. Protective factors included a history of allergies, blood transfusions, sun exposure, and smoking.

FAMILIAL CHRONIC LYMPHOCYTIC LEUKEMIA

Up to 10% of CLL patients have a first- or second-degree relative with CLL, making CLL one of the most common types of malignancy with familial predisposition. The higher frequency of monoclonal B-cell lymphocytosis (MBL) reported in these families provides further evidence of inheritance in a subset of patients. However, unlike other types of familial cancers, it is very uncommon for CLL patients to have large pedigrees with many affected distant relatives throughout an extensive family tree. Rather, the more common finding is for most patients to have one or two first- or second-degree relatives with this diagnosis. Large, case-control studies concluded that the risk ratio (RR) for first-degree relatives of CLL probands to also have CLL was higher than that for most other cancers. Although the average RR for all cancers in a U.S. study was approximately 2.1, CLL showed an RR of 5.0, the fourth highest of all cancers. Relatives of patients with CLL also appear to have a higher frequency of other lymphoproliferative disorders and autoimmune diseases. Unlike a variety of other cancers that have known predisposing genes, identification of divergent genes in CLL has been generally unsuccessful. Multiple association studies have identified numerous putative genes, polymorphisms, and genetic factors including death-associated protein kinase (DAPK) and LEU7 (CD57), IRF4, BAK, CD38, CD5, tumor necrosis factor (TNF)-α, and others, but weak evidence for linkage and conclusive studies demonstrating strong mechanistic contribution to pathogenesis are lacking. The role of anticipation in identifying other family members has been reported by several groups; patients with such a family history are generally diagnosed a median of 10 years earlier than other patients. However, other clinical features of CLL at diagnosis do not appear to be different. Thus, patients with familial CLL do not appear to be genetically or clinically different from individuals with sporadic CLL.

PATHOBIOLOGY

The complexity of the biology of CLL has become increasingly apparent, as the knowledge of the basic science of this disease has expanded. Despite these advances, many questions remain unsolved, including (1) the cell of origin from which CLL is derived; (2) the existence of a CLL stem cell as occurs in other leukemias; (3) the biological etiology of the divergent natural histories of immunoglobulin heavy chain variable region (IGHV)-mutated versus unmutated CLL; and (4) the existence and identification of infectious or other naturally occurring

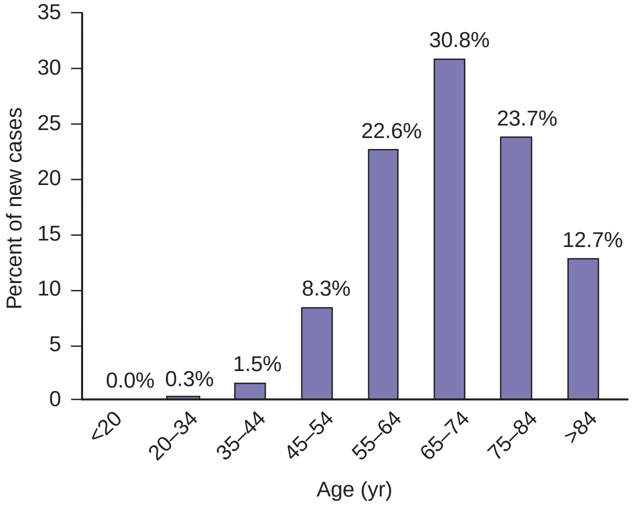


Figure 76.1 Graph demonstrating age distribution of chronic lymphocytic leukemia *(CLL)* as an uncommon diagnosis before the age of 45 years and infrequent (~30% of all patients diagnosed) before the age of 65 years (SEER 21 database, 2014–2018).

antigens that might drive the B-cell clone in this disease. Nonetheless, significant advances in our understanding of the roles of cytogenetics, immunology, and other relevant biological markers in predicting the natural history of disease progression and response to therapy in CLL make an understanding of the basic biology of CLL increasingly relevant and important to clinicians caring for CLL patients.

For many years, CLL was believed to represent a single disease that had a varied natural history. Basic research focused on understanding (1) the normal cell of origin from which CLL is derived; (2) the function of the B-cell receptor (BCR) in CLL; (3) the maturational point in B-cell development at which CLL occurs; (4) the relevance and contribution of a self- or acquired antigen to driving the disease; and (5) the role of the microenvironment in disease development and progression (see Chapter 81). All of these observations are important scientific areas of active, ongoing study, and readers are referred to several recent definitive reviews and Chapter 81 for further information on the basic biology of CLL. The biology and genetics covered within this chapter focus predominately on areas relevant to clinicians who care for patients with CLL.

B-CELL RECEPTOR PATHWAY AND ITS ROLE IN PATHOGENESIS

Modulation of the BCR plays an integral part in the development and maturation of B cells. Its constitutive activation through antigen dependent or independent signaling provides an important survival signal for the propagation of CLL B cells. Signal transduction occurs through a variety of kinases including LYN, PI3K, SYK, and Bruton tyrosine kinase (BTK) with resultant phosphorylation of PLC-γ2 and induction of downstream second messengers that further modulate cell survival regulators. These kinases have been effectively targeted and result in abrogation of the survival signal and B-cell apoptosis. Combinations of these inhibitors with existing and novel therapeutic agents have already changed the natural history of the disease.

X-linked agammaglobulinemia is a disease characterized by a severe immunodeficiency state that results from deficient BTK (see Chapter 52). Characterization of this defect and its resultant impact on causing severe impairment in B-cell development and humoral immunity has been critical to our understanding of CLL disease biology. Activating mutations of BTK have not been identified in CLL or other cancers but CLL B cells tend to have higher levels of BTK that can be induced through the BCR signaling pathway. The enzyme BTK is at an essential node for survival of CLL cells as deletion of this gene prevents development of CLL in mouse models and pharmacologic inhibition results has similar phenotype. Indeed, ibrutinib was the first-in-class irreversible, covalent, BTK inhibitor that efficiently targets BTK resulting in significant abrogation of downstream survival signaling transduced through this pathway and inhibition of cell survival and proliferation. Multiple other BTK inhibitors have also shown similar results. These include both reversible and non-covalent BTK inhibitors. Similarly, both SYK and PI3K can be induced by activation of BCR pathway. The PI3-kinase isoform delta inhibitors, idelalisib and duvelisib, and syk inhibitors have been shown to antagonize these survival signals and result in clinically meaningful disease responses.

IGHV Mutational Status

Two seminal manuscripts in 1999 demonstrated that the IGHV gene had undergone somatic mutation, indicating that CLL arose after this point in B-cell maturation, in 60% of patients at diagnosis. Detailed epigenetic studies examining the methylation changes occurring in normal B cells during development have subsequently suggested that immunoglobulin mutational analysis may indicate simply where in normal B-cell development transformation occurred. In an attempt to identify surrogate genes associated with IGHV-unmutated disease, ZAP-70 overexpression was identified. The majority of IGHV-unmutated CLL cells have ZAP-70 expression and demonstrate evidence of syk activation and other essential BCR downstream activation signals after ligation of surface IgM (sIgM) that is related to overexpression of this protein. In contrast, virtually all IGHV-mutated patients lack significant ZAP-70 expression and do not signal after ligation by sIgM, but can often weakly signal through other alternative BCRs. Thus, although gene expression profiling demonstrates all CLL cells to be most closely related to memory B cells, IGHV-unmutated and -mutated CLL cells differ significantly with respect to their ability to transduce intracellular signals after sIgM ligation. A common repertoire of mutational changes among CLL patients has also been documented that differs significantly from that found in the normal adult B-cell repertoire. These studies have prompted the hypothesis that CLL may represent an antigen-driven disease. IGLV3–21-expressing B cells have recently been described in a subset of patients to acquire a single point mutation (R110) that triggers autonomous BCR signaling and which subsequently identifies a subpopulation of CLL patients with a poor outcome. A multitude of mechanisms of BCR activation are indeed present in CLL. Targeting BCR signaling based on this has already resulted in the development of several therapeutic agents directed against signaling pathways downstream of the BCR.

Defective Apoptosis

Since its initial description and early characterization, CLL has been considered a disease of slow accumulation of tumor cells caused by disrupted or defective apoptosis. Multiple studies have demonstrated that CLL cells overexpress several antiapoptotic proteins, including bcl-2, mcl-1, bak, and X-linked inactivator of apoptosis protein (XIAP), and have diminished expression of compensatory proapoptotic proteins such as bax. Overexpression of bcl-2 and mcl-1 and an increase of the ratio of these proteins to bax have correlated not only with disrupted apoptosis but also shortened OS and poor response to therapy. Studies have demonstrated that CLL cells have constitutive activation of several anti-apoptotic transcription factors, including nuclear factor kappa-B (NFκB), nuclear factor of activated T cells (NFAT), and signal transducer and activator of transcription 3 (STAT3). Each of these transcription factors can influence one or more of the antiapoptotic proteins that promote survival in vivo. The source of activation of these different transcription factors is not completely defined but may be partly attributable to autocrine and paracrine networks involving B-cell activation factor (BAFF), a proliferation inducing ligand (APRIL) vascular endothelial growth factor

(VEGF), interleukin-4 (IL-4), and CD40 among others. CLL cells are also maintained through contact with stromal cells (bone marrow [BM] and dendritic) and nurse like cells through a complex interface of adhesion molecules and stromal survival factors such as stromal cell–derived factor-1 (SDF-1). The importance of the in vivo environment to CLL survival is supported by the increase in apoptosis when CLL cells are cultured in vitro. Moreover, targeting the overexpressed anti-apoptotic protein like bcl-2 with a specific bcl-2 inhibitor venetoclax has already led to excellent clinical efficacy.

Genetic Abnormalities

Detailed study of the genetics of CLL is hindered by the inability to effectively induce proliferation of tumor cells for standard metaphase cytogenetic analysis and the poor response of CLL cells to B-cell mitogens (see Chapter 57). Nonetheless, several cytogenetic studies identified a variety of deletions, including del(11q22.3), del(17p13.1), del(13q14), and del(6q21-q23), as well as trisomy 12, as common abnormalities in CLL. The frequency of these abnormalities has been further refined by using fluorescence in situ hybridization (FISH), which does not require isolation of dividing cells. These studies have demonstrated that del(13q14) is by far the most common cytogenetic abnormality in CLL followed by trisomy 12, del(11q22.3), del(17p13.1), and del(6q22.3). Stimulation studies with CpG oligonucleotides plus IL-4 or CD40 confirmed the prevalence of these abnormalities and identified unbalanced translocations not generally observed with traditional metaphase cytogenetics. The prognostic implications of these unbalanced translocations appear to be significant. Similarly, complexity of karyotype appears to be a driving poor prognostic factor in CLL. Interestingly, balanced translocations, which are more frequently observed in ALL and AML, are generally not observed in CLL.

The presence of recurrent deletions in CLL suggests the possibility of unique tumor suppressor genes in these different regions. This was confirmed by the discovery of miR15 and miR16, two noncoding microRNAs, in the deleted region of 13q14. Noncoding RNAs range in size from 21 to 25 nucleotides and represent a class of gene products whose function is to silence genes through binding to the 3′-untranslated region of specific genes to inhibit translation. It was later shown that miR16 regulates expression of bcl-2, which is overexpressed in CLL and other B-cell lymphoproliferative disorders. Multiple studies have associated specific miR expression with rapid disease progression, fludarabine resistance, poor prognosis, and modulation of BCR pathway and bcl-2 signaling. In addition, miR34a has been directly related to the adverse outcome associated with p53 dysfunction.

Recurring Mutations in Chronic Lymphocytic Leukemia

Until the advent of whole-exon and whole-genomic sequencing, CLL was not typically associated with recurring mutations early in the pathogenesis of the disease. Probably best characterized are p53 mutations, which occur in approximately 10% of patients at diagnosis, often in conjunction with deletion of the alternative allele (at 17p13.1 loci) that is associated with rapid disease progression and poor survival. With treatment and subsequent relapse, the frequency of p53 mutations continues to increase proportionately and is most common in patients with Richter transformation. Thus, p53 mutations are considered by most to be a secondary abnormality in CLL associated with progression. Nonetheless, p53 mutations or deletions have significant impact on consideration of treatment of CLL, and conventional chemoimmunotherapy-based approaches have no role in the treatment of patients with this abnormality. Other recurring mutations in CLL, including SF3B1, NOTCH1, MYD88, XPO1, and ERK1 and more than 100 other mutations have been described with varying physiologic significance. A summary of important genetic abnormalities and their impact is provided in Table 76.1.

TABLE 76.1 Summary of Important Prognostic Markers and Their Role in Disease Biology

Prognostic Markers	Frequency (%)	Putative Role in Disease Biology
Del17p/loss of TP53	~7–10	Loss of p53 protein and decreased levels of miR34a results in cell cycle dysfunction. Predicts for poor response to treatment and worse survival
Del11q	~18–20	Results in ATM gene deletion in the majority of patients and subsequently a lack of repair of double-stranded DNA breaks. Predicts for shorter time to treatment failure and survival. Impact may be overcome with the use of alkylator based chemoimmunotherapy
Trisomy 12	~16–20	Impact may be through a gene dosage effect especially of genes encoding CDK4 and MDM2. Predicts for intermediate risk disease
Del13q	~55–60	Results in the loss of miR-15a and miR16-1 along with DLEU7 that results in loss of tumor suppressor activity. Generally associated with favorable outcomes including response to chemoimmunotherapy and survival
NOTCH1 mutation	~10	Constitutive activation of NOTCH1 results in increased cell survival and resistance to apoptosis and predicts for inferior response to rituximab and shorter treatment-free interval
SF3B1 mutation	~9–18	Mutually exclusive with NOTCH1 mutations. May cause abnormal transcription and splicing events. Associated with rapid disease progression
BIRC3 mutation	~5	Associated with unmutated IGHV, a shorter treatment free interval, and del11q. Mutually exclusive with TP53 mutations
MYD88 mutation	~3–13	Important adapter of the IL-1 and toll-like receptor pathway. Gain-of-function mutations result in increased cytokine production and activation of the NF-κB pathway. Associated with improved survival

MYD88, myeloid differentiation primary response 88; *SF3B1*, NA splicing factor 3B, subunit 1.

Immune and Micro-Environmental Features

The importance of progressive immune suppression with progression of CLL has become appreciated in both human CLL and murine models of this disease. The absolute number of T cells and natural killer (NK) cells in CLL patients at diagnosis has been shown to predict OS. In particular, expansion of suppressive T-regulatory cells has been documented as patients approach the time of requiring therapy. The immunosuppressive effects observed in CLL lie predominately with the CLL clone as demonstrated in human and mouse models of CLL. Similarly, the importance of microenvironmental compartments of CLL such as lymph nodes and BM, where stromal cells provide proliferative signals as well as survival signals to protect from apoptosis, are recognized. Derived from this work are biomarkers of measurable chemokines responsible for T-cell recruitment to the microenvironment. Given the link of these stimuli to BCR signaling, inhibitory molecules directed at kinases involved in this interaction have resulted in significant clinical advances.

CLINICAL MANIFESTATIONS

At diagnosis, CLL most often does not have clinical manifestations associated with the disease. For early-stage CLL patients, the diagnosis is often identified as part of routine blood tests for evaluation of an unrelated problem such as infection, kidney stone, or preoperative assessment in which an elevated leukocyte count is noted with increased numbers of mature lymphocytes observed on the blood smear. For a much smaller subset of patients with CLL, presentation of disease occurs as a consequence of fatigue, weight loss, early satiety (from spleen enlargement), petechiae (from low platelets), or new palpable lymph nodes. Patients with symptomatic CLL at diagnosis represent only 15% of those seen, due to the more indolent nature of this disease at diagnosis. With additional follow-up, the majority of CLL patients will eventually manifest symptoms of the disease that ultimately lead to the need for treatment. The most common symptoms of progression include increasing fatigue (as a consequence of anemia and cytokines from disease), increasing lymph node and spleen size, worsening hematologic parameters (anemia and thrombocytopenia), and rarely infiltration of other organs (kidney, lung, pleural space, skin) that necessitates initiation of treatment to palliate symptoms. Unusual symptoms generally not associated with CLL progression are night sweats, fevers, and weight loss. These are generally suggestive of Richter transformation.

Separate from direct CLL progression, the disease is also immunosuppressive, and with more advanced disease, an increase in infections is generally observed. This represents a major morbidity of CLL and is a leading contribution to mortality associated with this disease. Other manifestations of immune suppression, including higher rate of secondary malignancies and autoimmune complications, are also increased in CLL (see box on Initial Evaluation of Young Patients With Chronic Lymphocytic Leukemia).

DIAGNOSIS AND LABORATORY MANIFESTATIONS

The diagnosis of CLL, as defined by the International Workshop on Chronic Lymphocytic Leukemia (iwCLL) 2018 criteria, requires an absolute malignant B-cell lymphocyte count of greater than 5000/μL that co-express CD5 and CD23 on peripheral blood immunophenotyping. Morphologically, the lymphocytes must appear mature with fewer than 55% prolymphocytes (Fig. 76.2A–E). The BM aspirate smear if done should show greater than 30% of all nucleated cells to be lymphoid, or the BM core biopsy must show lymphoid infiltrates consistent with CLL (see Fig. 76.2F and G). The overall cellularity must be normocellular or hypercellular. Immunophenotyping must reveal a predominant B-cell monoclonal population coexpressing the B-cell markers CD19, CD20, and CD23 and the T-cell antigen CD5 in the absence of other pan-T-cell markers (see later section). In some cases a hypocellular marrow can be present as an artifact of marrow aspiration.

Patients may present with tumor cells immunophenotypically consistent with CLL but have predominately lymph node disease or organomegaly, without a peripheral B-cell lymphocyte count of 5000/μL despite BM involvement. These patients are considered to have small lymphocytic lymphoma (SLL) and both entities share similar immunophenotypic features, genetic findings, natural history, and complications. The clinical management of SLL patients should be similar to CLL with respect to diagnostic testing and treatment.

INITIAL EVALUATION OF YOUNG PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

Only 10% of patients diagnosed with CLL are younger than 50 years of age, and these patients often present a diagnostic and therapeutic dilemma to hematologists initially evaluating them. The great majority of patients diagnosed before the age of 50 years will have early-stage CLL with a slightly higher incidence of a prior first-generation relative with this disease. Additionally, these patients are generally of a higher economic status or have chronic fatigue or medical illnesses for which they have been undergoing routine blood testing, leading to diagnosis of CLL. When the diagnosis of CLL is made, these younger patients have a more challenging time understanding how the disease will impact them. For patients with no symptoms referable to CLL, we generally discuss complications of the disease during the first visit and have a detailed discussion regarding assessment of genetic risk factors predisposing to early disease progression, including select interphase chromosomal abnormalities [del(17p13.1) and del(11q22.3)] and IGHV mutational status (unmutated). During this time, it is important to counsel patients that identification of high-risk genomic features can actually increase anxiety because no treatment intervention is indicated in the absence of symptoms, regardless of genomic profile, outside of a clinical trial. In our experience, the great majority of patients desire this testing. We provide considerable discussion about the excellent outcomes with novel agents and the limited role of chemotherapy for the management of this disease. Despite the potential benefit of allogeneic SCT in younger patients with CLL and possibility of a curative option, we generally avoid mention of this option and do not pursue consultation or tissue typing of patients or siblings. More recent advances with chimeric antigen receptor T-cell (CAR-T) therapy are occasionally mentioned. During the second visit 4 to 6 weeks later, we review the results of these prognostic factors and answer additional questions that have arisen, and reiterate the rationale behind active surveillance and the lack of improvement in OS with early intervention. Since “watchful waiting” is a difficult concept to grasp by a patient who has recently been diagnosed with a malignancy, we use “active surveillance” as a preferred term for our management approach. Ultimately, the majority of patients have low-risk disease, and knowing this allows patients to take partial control of their disease and move on with their lives. Serial assessment of the psychological well-being of patients with CLL during this first year is incredibly important. At no place during the evaluation do we refer to CLL as being a good or favorable leukemia. In our experience, the most common reason for dissatisfaction toward the initial hematology evaluation is lack of explanation of the disease process or the minimization of CLL as a “good leukemia to have.”

For young patients presenting with other chronic medical problems who are asymptomatic from their CLL, we follow the approach outlined above. More commonly, these patients have fatigue, mild anemia, or other symptoms that could be referable to the CLL. Additionally, this group is more commonly overweight or obese. In either setting, it is important to first think like an internist and pursue other causes for symptoms potentially referable to CLL. In particular, an effective weight loss and exercise plan often results in improvement in quality of life and reduction in symptoms of fatigue, along with better control of other medical comorbidities. In some cases methylphenidate can be effective for the management of CLL related fatigue. It is very important to note that younger patients with CLL can often go a decade or more without therapy, and early treatment of this patient group in the absence of symptoms still offers no proven long-term advantage. For this reason, we remain very conservative on starting therapy for young patients with CLL unless they have high-risk disease and are treated on a clinical trial. To date, trials with chemotherapy, chemoimmunotherapy, and BTKi have not demonstrated survival differences with early intervention although follow-up with the later therapies is short.

MONOCLONAL B-CELL LYMPHOCYTOSIS

The increase in diagnostic blood testing for other medical conditions and easy availability of immunophenotyping has led to recognition of a precursor condition to CLL called MBL. Patients with MBL have circulating peripheral blood B cells immunophenotypically consistent with CLL but do not have enlarged lymph nodes, a malignant lymphocyte count greater than 5000/μL, cytopenias, organomegaly, extramedullary involvement, or other features of a lymphoproliferative disorder. Immunophenotyping can further classify this entity into three separate categories: (1) CLL-type MBL; (2) Non-CLL type MBL; (3) Atypical CLL-type MBL. Care must be taken to

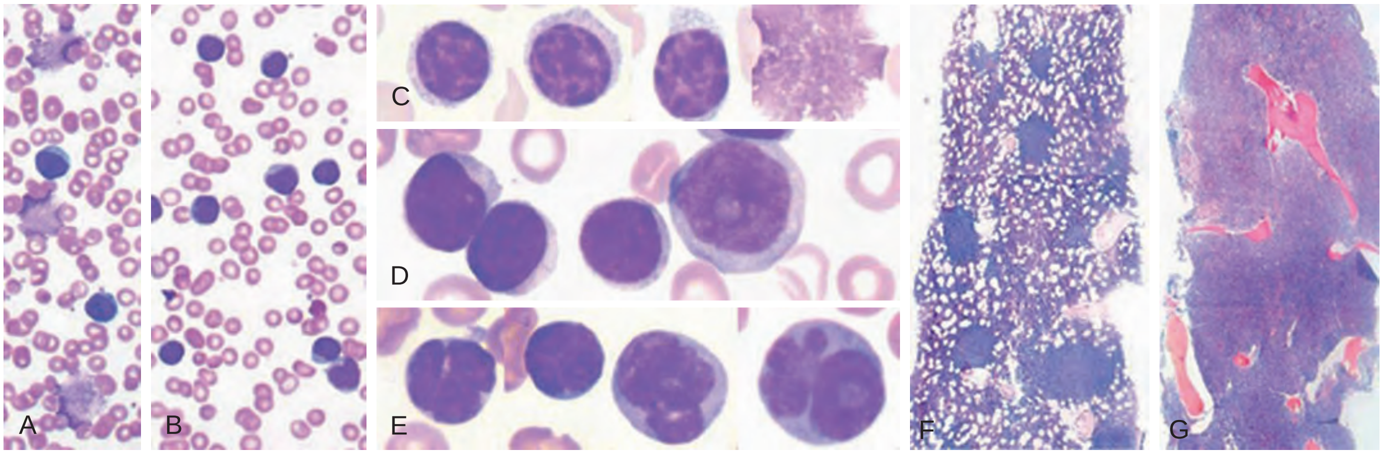


Figure 76.2 CHRONIC LYMPHOCYTIC LEUKEMIA (CLL). The peripheral blood smear (A) typically shows lymphocytosis and increased smudge cells as a result of the fragility of the CLL cells (see also smudge cell in C, *right side*). These can be avoided by making a preparation of blood and bovine serum albumin (22%) at a ratio of 11 drops of blood and 1 drop of albumin before preparing the slide (B). Cytologic features of CLL cells differ. Classic cells have a small nucleus with a "soccer ball" chromatin pattern (C). Some cases have increased large cells, or prolymphocytes, with more open chromatin and prominent "punched-out" nucleoli (D; prolymphocyte, *right side*). Other cases, sometimes referred to as "atypical," have clefted cells and large cells (E). The bone marrow can show nodular infiltrates of CLL cells (F), an interstitial infiltrate, or a diffuse infiltrate (G).

differentiate this entity from circulating disease frequently observed in a variety of non-Hodgkin lymphomas (NHLs).

The frequency of MBL increases with age; 0.3% of patients younger than the age of 40 years have MBL compared with 3.5% to 6.7% of 40- to 60-year-old patients and 5% to 9% of 60- to 90-year-old patients. The frequency of MBL in family members of patients with a first-degree relative with CLL is significantly higher in both young and older patients. Similar to the relationship of monoclonal gammopathy of undetermined significance and multiple myeloma, it appears that only a small proportion of patients with MBL develop overt CLL over time. These are mostly patients with high count MBL, defined as a monoclonal B-cell count of 500 to 5000/μL with a risk of progression to CLL of 1% to 2%/year. However, a similar incidence of 1%/year has also been reported in patients with low count MBL (<500/μL) despite earlier studies suggesting minimal risk of progression. Since the classification of SLL is based on lymphadenopathy evaluated on physical examination, patients with MBL who are found to have lymphadenopathy on CT scans are frequently misclassified as SLL. These patients without palpable lymphadenopathy and only limited adenopathy on imaging have similar risk of progression as patients without lymphadenopathy on imaging with concurrent MBL. We therefore do not recommend the routine use of CT scans in the initial workup or routine follow-up of patients with CLL, SLL, or MBL. Similarly, the degree of BM involvement is not correlated with risk of progression, and while the diagnostic criteria for MBL does not include BM involvement, the presence of BM disease alone does not change the diagnosis from MBL to CLL or SLL.

Patients with MBL are generally evaluated similarly to patients with early-stage CLL with comprehensive prognostic testing and IGHV mutational status and cytogenetic and mutational abnormalities. They also generally have many of the long-term complications of CLL related to immune suppression including a higher frequency of infections and secondary malignancies. We therefore approach the initial counseling of these patients in a similar manner as newly diagnosed CLL patients with respect to interventions (vaccines, dermatologic evaluation, and education) to minimize these complications. We also continue to follow these patients with yearly blood counts and exams.

LABORATORY MANIFESTATIONS

For many years, the diagnosis of CLL was made based on morphologic examination of the peripheral blood smear, which demonstrated mature lymphocytes with an abundance of smudge cells. Despite rigorous morphology, many diseases can mimic CLL in both appearance and clinical presentation, as summarized in Table 76.2, resulting in incorrect diagnoses. With the advent of new, more effective targeted therapies in CLL and other related diseases listed in Table 76.2, determining the correct diagnosis is of great importance. Flow cytometry, or immunophenotyping, is now the standard approach to establish the diagnosis of CLL (Fig. 76.3, for example). The diagnosis of CLL relies on immunophenotypic confirmation, and flow cytometry should therefore be performed on all CLL patients at diagnosis. CLL cells have a relatively consistent immunophenotype, which differentiates CLL from mantle cell lymphoma, hairy cell leukemia, follicular lymphoma, marginal zone lymphoma, and other indolent B-cell malignancies. Specifically, CLL cells express a variety of B-cell markers, including dim sIg, CD19, dim CD20 and CD23, CD200, as well as the pan-T-cell marker CD5. κ or λ restriction is always present, establishing the presence of a clonal B-cell population, although sIg expression may be so dim that light chain restriction may be difficult to determine. In contrast, the presence of CD10, FMC7, or CD79b (all typically absent on CLL cells) or bright expression of CD11c, CD20, or CD25 (all typically dim on CLL cells) suggests an alternative low-grade B-cell lymphoproliferative malignancy. Expression of CD5 without CD23 suggests mantle cell lymphoma (see Chapter 86), and FISH for t(11;14) should be performed to exclude mantle cell lymphoma. Some genetic subsets of CLL are predisposed to variant antigen expression, particularly patients with trisomy 12. Repeating immunophenotyping after the initial diagnosis is not required unless there is a suspicion of transformation to a more aggressive histology or there is a need to assess BM response or antigen expression for an antibody-directed therapeutic agent. Transformation of CLL (Fig. 76.4A–C) to either prolymphocytic leukemia (PLL) or large cell lymphoma (Richter transformation) is often associated with

TABLE 76.2 Diseases That Can Mimic Chronic Lymphocytic Leukemia

- Follicular lymphoma
- Mantle cell lymphoma
- Marginal zone lymphoma
- Hairy cell leukemia
- Acute lymphoblastic leukemia
- T-cell prolymphocytic leukemia
- Large granular natural killer or T-cell leukemia

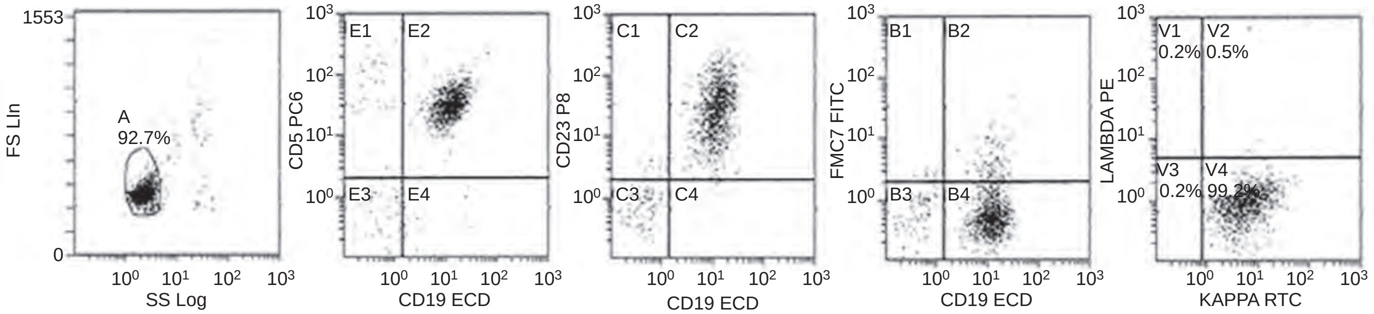


Figure 76.3 FLOW IMMUNOPHENOTYPING IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL). Flow data in a typical case of CLL. The phenotype is CD19$^+$ clonal B cells with κ light chain restriction (weak to moderately intense), with coexpression of CD5 and CD23, but lack of FMC-7. The cells were CD38-negative.

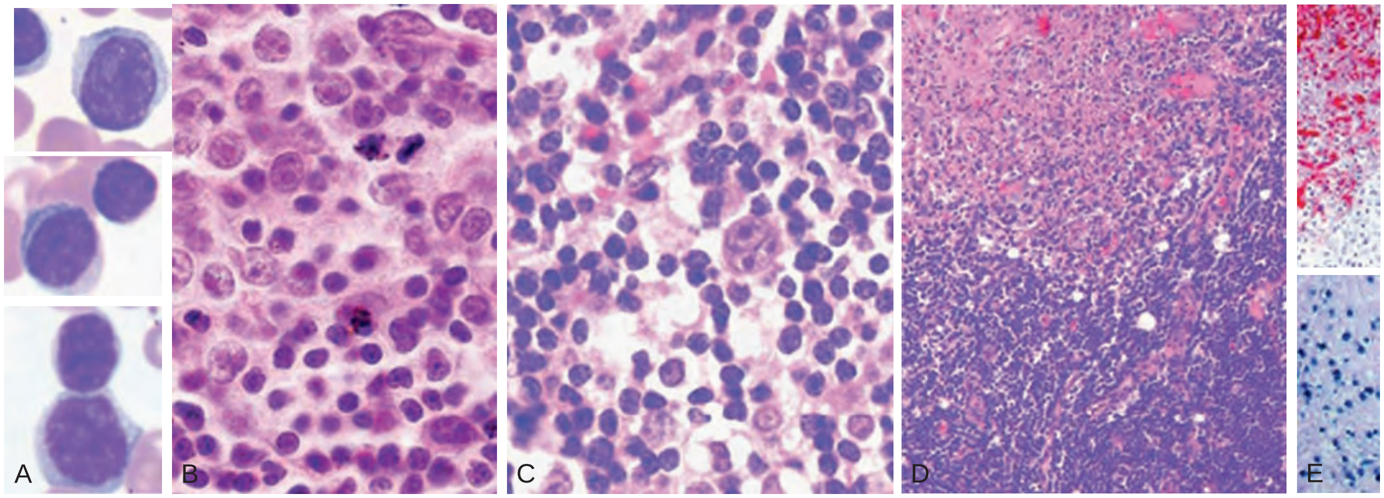


Figure 76.4 TRANSFORMATION IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL). Some patients of CLL develop increasing numbers of prolymphocytes (A) and a "prolymphocytic transformation." A Richter transformation is to a large-cell lymphoma (B; large cells, *upper left*, residual CLL, *lower right*). Occasionally, cases can transform to Hodgkin lymphoma (i.e., the Hodgkin variant of Richter syndrome) (C). With the use of fludarabine and other immunosuppressants, patient can also develop Epstein-Barr virus–related lymphadenopathies or lymphadenopathies with large areas of necrosis caused by herpes simplex virus (HSV). The case illustrated in (D) and (E) had both (E; *upper panel* shows an immunostain of HSV-1/2, and lower is EBER in situ hybridization).

immunophenotypic drift, where CD5 is lost and FMC7 expression is acquired. Additionally, expression of CD20 and surface immunoglobulin typically becomes brighter in PLL or Richter transformation. Although the morphologic appearance of prolymphocytes or large lymphoid cells in blood, BM, or lymph nodes is typically adequate to make the diagnosis of transformation, immunophenotyping may be useful in cases when morphologic findings are less clear.

Patients with CLL often present with no symptoms, with the diagnosis being made as a consequence of asymptomatic enlarged lymph nodes or splenomegaly detected on physical examination or routine blood work done for another cause. Other patients present with symptoms of BM replacement (fatigue, dyspnea, or petechiae secondary to anemia and thrombocytopenia), symptomatic lymphadenopathy or hepatosplenomegaly, autoimmune complications (hemolytic anemia or idiopathic thrombocytopenic purpura), or B symptoms (fevers, night sweats, and weight loss). A small proportion of CLL patients will have pulmonary infiltrates at diagnosis that are representative of CLL involvement in some cases and active infection in others.

In addition to blood and BM lymphocytosis, a few abnormal laboratory findings are commonly observed in CLL. Neutropenia, anemia, and thrombocytopenia can develop as a consequence of BM infiltration or myelosuppressive therapy administered to eliminate the leukemia. A positive direct antibody, or Coombs, test result is observed in approximately 10% to 25% of CLL patients at some time during the course of the disease. Similarly, autoimmune thrombocytopenia or neutropenia may be present, although other causes such as BM replacement or a chemotherapy effect are much more common and should be excluded. Pure red blood cell (RBC) aplasia can sometimes be observed with isolated anemia and absence of RBC precursor cells (see Chapter 33). Hypogammaglobulinemia is common in CLL and becomes more frequent and marked as the disease progresses. A monoclonal protein, most commonly IgM, can be present in a subset of CLL. By contrast, hypercalcemia and a markedly elevated lactate dehydrogenase (LDH) level are not common in CLL and suggest progression to Richter transformation. Clearly, a wide spectrum of presentations exists for patients with CLL.

PROGNOSIS

Historically, patients with CLL were staged using either the Rai or Binet system. Both of these discriminate CLL by the sites of disease and degree of cytopenias induced by BM replacement by the leukemia cells. Patients can be categorized into three groups on the basis of these features. According to the modified Rai criteria, patients in the low-risk group (stage 0) have lymphocytosis without any other abnormality; patients in the intermediate-risk group (stages I and II) have, in addition to their lymphocytosis, enlarged lymph nodes, spleen, or liver; and patients in the high-risk group (stages III and IV) have anemia (hemoglobin <11.0 g/dL) or thrombocytopenia (platelets <100 × 10^9/L).

TABLE 76.3 Rai Staging System

Rai Stage at Diagnosis	Percent of Patients Never Requiring Chronic Lymphocytic Leukemia Therapy	Expected Survival in Months From Initial Diagnosis
0—Lymphocytosis >5 × 10^9/L only	59	150
1—Lymph node enlargement	21	101
2—Spleen or liver enlargement	23	71
3—Anemia with hemoglobin <11 g/dL	5	19
4—Thrombocytopenia <100 × 10^{12}/L	0	19

Adapted from Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. *Blood*. 1975;46(2):219–234.

The historical median survival times for the Rai low-, intermediate-, and high-risk groups are similar to those of Binet stages A, B, and C: 12+, 8, and 2 years (Table 76.3). For early-stage patients with CLL (Rai low and intermediate stage and Binet stages A and B), a significant range of time to developing symptoms of CLL exists. The lack of survival advantage with early treatment, the observation that a subset of CLL patients will never require therapy, and the varied natural history of the disease have resulted in the identification of specific biologic or clinical prognostic factors that predict time to progression. Multiple tools have been developed and validated to address this issue, and the CLL-International Prognostic Index (CLL-IPI) score appears to have the most discriminative ability for predicting disease progression and OS in patients with newly diagnosed and previously untreated CLL. This weighted score incorporates five prognostic factors including age, clinical stage, β2-microglobulin, IGHV mutational status, and del17p or TP53 mutation status, with del17p or TP53 mutation status being the most significant variable. This allows separation of patients in low-risk to very high-risk cohorts with respect to survival outcomes, and also provides management suggestions, e.g. active surveillance in patients in the low-risk group. Given the impact of BTKi and other targeted therapies for CLL, the impact of historical staging systems relative to survival for newly diagnosed patients is limited.

Although lymphadenopathy is a common clinical feature of CLL and is incorporated into both major clinical staging systems, computed tomography (CT) scans are not commonly used to determine staging or to evaluate response to therapy outside of clinical trials. However, several studies have examined whether the incorporation of CT scans in initial staging or response evaluation may affect the ability to predict disease progression at diagnosis and assessment of clinical response to therapy. Although a few studies have suggested that these may help predict disease progression at diagnosis, studies done serially at the time of treatment and for response show that CT scans generally do not impact assessment of response, progression-free survival (PFS), or OS. Additionally, CT scan identification of new nodal areas did not impact decisions for retreatment. Thus, routine CT scans should not be performed for staging and response evaluation in CLL. Similarly, PET scans appear to be sensitive for Richter transformation, with a high negative predictive value, but specificity is poor. We generally use these studies to identify patients who warrant biopsy for suspected Richter transformation and to localize where to biopsy.

With recent advances in the molecular biology of CLL, some prognostic factors such as BM infiltration pattern, which requires an invasive procedure at diagnosis, have not maintained their usefulness in predicting disease progression. Prognostic features outside of the traditional staging systems outlined above relative to daily practice are summarized in Table 76.4.

TABLE 76.4 Evaluation of Chronic Lymphocytic Leukemia Patients at Diagnosis

History

- B-symptom and fatigue assessment
- Infectious history assessment
- Occupational assessment for chemical exposure
- Familial history of CLL and lymphoproliferative disorders
- Preventive interventions for infections and secondary cancers

Physical Examination

Laboratory Assessment

- CBC with differential
- Morphology assessment of lymphocytes
- Chemistry, LFT enzymes, LDH
- Flow cytometry assessment to confirm immunophenotype of CLL
- Serum immunoglobulins
- Serum β_2M levels
- Interphase cytogenetics for del(17p13.1), del(11q22.3), del(13q14), del(6q21), and trisomy 12
- IGHV mutational analysis
- Stimulated metaphase karyotype (if available)

Selected Tests Under Certain Circumstances

- DAT, haptoglobin, reticulocyte count if anemia present
- CT scan if unexplained abdominal pain or enlargement present
- PET scan or biopsy (or both) if large nodal mass present
- BM aspirate and biopsy if cytopenias present
- Familial counseling if first-degree relative with CLL

Teaching

- Varicella zoster identification instruction
- Skin cancer identification
- Disease education (Leukemia and Lymphoma Society)

BM, Bone marrow; *CBC*, complete blood count; *CLL*, chronic lymphocytic leukemia; *CT*, computed tomography; *DAT*, direct antiglobulin test; *LDH*, lactate dehydrogenase; *LFT*, liver function test; *PET*, positron emission tomography.

Thymidine Kinase Activity and β_2-Microglobulin

Thymidine kinase is an enzyme involved in the salvage pathway of DNA synthesis and correlates with proliferative activity. Elevated thymidine kinase activity (TKA) has been observed to be predictive of early progression in a subgroup of untreated patients with early-stage CLL. β_2-Microglobulin (β_2M) is an extracellular protein component of the human leukocyte antigen (HLA) class I complex. β_2M has been shown to have significant prognostic relevance in lymphoma and multiple myeloma and correlates with disease burden in CLL. Both these variables have been validated as independent predictors of shortened PFS, and shorter survival time for both untreated and previously treated patients. Elevated β_2M was also observed in patients with high tumor burden and extensive BM infiltration. In addition to disease progression, both β_2M and TKA have been associated with short duration of remission and inferior survival after treatment with chemoimmunotherapy. In some studies, β_2M also predicts for shorter remission after targeted therapies as well.

IGHV Mutational Status and Surrogates

Although the malignant cell of CLL morphologically resembles a mature lymphocyte, genetic, immunologic, and phenotypic studies suggest that this cell is better designated as either a pregerminal or postgerminal B cell. Somatic mutations in the first and second complementarity-determining regions (CDR1 and CDR2) of the IGHV genes are thought to occur in the germinal centers. Examination of IGHV genes in patient cells suggests that there are two subsets of CLL: leukemias whose cell of origin has successfully traversed the germinal center, resulting in the mutated IGHV phenotype, and leukemias that are derived from naive B cells with the unmutated (germline) IGHV sequence. Whereas approximately 60% of CLL patients have cells with mutated IGHV genes (<98% sequence homology to germline), the remaining patients have cells exhibiting unmutated IGHV (≥98% sequence homology with germline), typical of pregerminal B cells. The prognostic significance of the absence of IGHV gene mutations is substantial, with studies uniformly noting an inferior survival and high predisposition to requiring early treatment in this patient subset. A CLL Research Consortium study examined the impact of IGHV mutation in 307 untreated CLL patients enrolled in a prospective tissue collection study. A total of 53% of these patients exhibited unmutated IGHV genes, and this population had a significantly shorter median time to initial therapy (3.5 years) than those with mutated IGHV (9.2 years; $P < .001$). IGHV 3–21 rearrangements irrespective of low or high concordance with germ line sequence have poor outcome. This can be further refined by the presence or absence of the single point mutation (R110) that triggers autonomous BCR signaling with shorter time to treatment and inferior outcome with chemoimmunotherapy. The impact of this abnormality on targeted therapeutics for CLL is uncertain at this time.

While IGHV testing is widely available, its utilization in routine clinical practice has historically been dismal, with less than 20% of patients being tested for this mutation. Alternative markers that correlate with IGHV mutation status are also frequently utilized and include expression of the cell surface molecule CD38 on CLL cells and ZAP-70 intracellularly, among others.

High CD38 (>30%) expression is associated with both a shorter time from diagnosis to treatment and inferior survival. CD38 interaction with its ligand, CD31, in the presence of IL-2 results in upregulation of the survival receptor CD100 exclusively on proliferating CLL cells. This occurs with concomitant down-modulation of CD72, a negative regulator of immune response. The interaction between CD38 and CD100 on CLL cells and CD31 and plexin B1, respectively, on transfected fibroblasts results in enhanced survival and growth of CLL cells. Furthermore, the presence of nurse-like cells in CLL patients expressing high levels of CD31 and plexin B1 corroborates the interplay of CD38 and CD31 and provides further evidence of activation of circulating CD38-positive CLL cells by the microenvironment. This finding also may explain the aggressive nature of these CLL clones.

ZAP-70 is a T-cell receptor–associated tyrosine kinase that is aberrantly expressed in CLL cells, and ZAP-70 expression is generally found in patients with unmutated IGHV but not in patients with mutated IGHV. Functional studies in ZAP-70 positive cases (>20%) have shown that BCR ligation leads to increased phosphorylation of cytosolic proteins (Syk, BLNK, PLCγ), calcium mobilization, degradation of IκB, and ultimately NFκB target gene activation. Despite clear data showing that ZAP-70 is a prognostic factor, the reproducibility of this assay across laboratories has been problematic. Methylation of select regions in the proximal 5′ region of the ZAP-70 gene has been shown to correlate closely with expression of ZAP-70 and influence treatment outcome and has been utilized as an alternative to ZAP-70 testing. Overall, despite the apparent usefulness of ZAP-70 in research laboratories for discriminating CLL patients at high risk for progression, this test is of limited utility for routine clinical practice.

Other Prognostic Markers

Various other markers are utilized for predicting disease course of CLL. Serum LDH levels are associated with aggressive disease and with Richter syndrome and prolymphocytic lymphoma. Similarly, patients with a lymphocyte doubling time (LDT) of 12 months or less have been shown to have worse overall and treatment free survival. Other markers include soluble factors like CD23, CD44, VCAM-1, CD27 and MMP-9, IL-6, IL-8, miR-15a and miR-16-1, etc. However, none of these are routinely utilized in clinical practice.

Chromosomal Aberrations

Conventional metaphase cytogenetics can identify chromosomal aberrations in only 20% to 50% of CLL cases because of the low in vitro mitotic activity of CLL cells (see Chapter 57). Abnormalities noted in descending frequency of occurrence include trisomy 12; deletions at 13q14; structural aberrations of 14q32; and deletions of 11q, 17p, and 6q. In addition, a complex karyotype (three or more abnormalities) occurs in approximately 15% of patients and predicts for rapid disease progression, Richter transformation, and inferior survival. The use of CD40L or the combination of IL-2 and CpG stimulation revealed translocations in 33 of 96 patients (34%). These translocations were both balanced and unbalanced, occurring in 13q14, 11(q21q25), 14q32, or regions also seen in lymphomas such as 1(p32p36), 1(q21q25), 2(p11p13), 6(p11p12), 6(p21p25), and 18q21. These data define a prognostic subgroup of patients with significantly shorter median time from diagnosis to requiring therapy (24 vs. 106 months) and OS (94 vs. 346 months) compared with those without translocations. The frequency of these translocations in untreated patients is less common, suggesting that these accumulate with disease progression.

Given the limitations of standard or stimulated karyotype analysis, interphase FISH has become the state-of-the-art technique for accurately distinguishing genetic subtypes of CLL. The largest study of interphase FISH resulted in improved sensitivity to detect partial trisomies (12q12, 3q27, 8q24), deletions (13q14, 11q22–23, 6q21, 6q27, 17p13), and translocations (band 14q32) in more than 80% of all cases. In a large study of 325 patients by Dohner and colleagues, a hierarchical model consisting of five genetic subgroups was constructed on the basis of regression analysis of CLL patients with chromosomal aberrations. Patients with a 17p deletion had the shortest median survival time (32 months) and the shortest treatment-free interval (TFI, months); patients with an 11q deletion followed closely with 79 months and 13 months, respectively. Whereas the favorable 13q14 deletion group had a long TFI of 92 months and a median survival of 133 months, the group without detectable chromosomal anomalies and those with trisomy 12 fell into the intermediate group with median survival of 111 and 114 months, respectively, and TFI of 33 and 49 months, respectively. According to this pivotal study, CLL patients are prioritized in a hierarchical order (deletion 17p13 > deletion 11q22-q23 > trisomy 12 > no aberration > deletion 13q14). The hierarchical model of cytogenetic abnormalities in predicting disease progression by FISH has been further confirmed by other studies. Of interest, patients with high-risk interphase cytogenetic abnormalities or other complex abnormalities are almost always found to have IGHV-unmutated CLL. The impact of high-risk interphase cytogenetics relative to disease progression, outside of its association with IGHV-unmutated CLL, in at least one study had less impact on outcome.

Mutational Analysis

Numerous recurrent mutations and or deletions have been described in CLL that are often associated with IGHV mutational status. While the majority of these abnormalities do not impact clinical outcome of CLL, several including p53, ATM, NOTCH1, SF3B1, XPO1, and BIRC3 have been shown to impact time to initiation of treatment and also OS. Outside of p53 mutations, the frequency of these are often low and therefore prevent definitive independent determination of impact on outcome with respect to other features. Several other mutations including ERK1, BRAF, and MYD88 may afford themselves to therapeutic targeting with novel agents that are FDA approved or in clinical trials. Despite routine mutational tests being available, it is not our practice to examine these broadly at diagnosis or at time of treatment. The exception to this is p53 mutations since the presence of both del(17) and p53 mutation predict for a worse clinical outcome as compared to either of these abnormalities alone. We therefore recommend IGHV and p53 mutational testing, along with conventional karyotyping and interphase FISH testing

HOW SHOULD STAGING AND BIOMARKERS BE USED FOR TREATMENT DECISIONS IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS?

Our understanding of the biology of CLL has improved dramatically, and many relevant biomarkers are now available for predicting when CLL will clinically progress. However, no study to date has demonstrated that earlier treatment will alter the natural history of the patient in even the higher-risk groups with high progression rates. Therefore, at the present time, the use of staging and predictive biomarkers should be used only to provide patients with information relative to the expected course of their disease. Outside of a clinical trial, these results should never be used to initiate therapy in patients with asymptomatic disease and are not an indication for treatment. Before performing predictive tests, a detailed discussion of how these tests will be used with the patient should occur and the option of not performing them should be provided. In a subset of patients, significant anxiety can be produced by identifying high-risk features for which observation, without therapeutic intervention, remains the standard of care. Table 76.4 provides an example of the initial evaluation provided by us when seeing a newly diagnosed patient. Because lymphocyte-doubling time is a prognostic feature in the progression of CLL, our approach is to follow patients every 3 months during the first year; if little change in clinical or laboratory parameters occurs at this point, we extend this time period to every 6 months in the absence of new complaints.

for all patients as part of their initial genetic workup for CLL (see box on How Should Staging and Biomarkers Be Used for Treatment Decisions in Chronic Lymphocytic Leukemia Patients?).

TREATMENT

Initiation of Treatment of Newly Diagnosed or Previously Untreated Patients

Many patients are incidentally diagnosed with CLL on routine complete blood count examination and are asymptomatic with a normal hemoglobin and platelet count at time of initial diagnosis. Expectant observation without therapy is the standard practice for such asymptomatic patients, and patients receive treatment only when their disease progresses. This practice is based on several studies that failed to show an improvement in OS when early therapeutic intervention with chlorambucil therapy was administered to asymptomatic patients. A meta-analysis of 2048 patients in six trials demonstrated no difference in death rate between patients who were randomized to early therapy (42.6%) and those with treatment deferred (41.6%). Thus, patients with asymptomatic or early-stage disease derive no therapeutic benefit from early alkylating agent therapy. However, in these studies, chlorambucil was administered as initial therapy, and chlorambucil achieves complete response (CR) in few patients. Similarly, fludarabine when employed early for patients who do not meet the conventional criteria for treatment resulted in higher response rate but failed to demonstrate a survival advantage. A subsequent CLL7 trial of the German CLL study group of high-risk CLL patients did not demonstrate advantage of even more aggressive chemoimmunotherapy with fludarabine, cyclophosphamide, and rituximab (FCR) in improving OS in this group as compared to observation alone. More recently, early results from the German CLL study group CLL12 trial of early intervention with ibrutinib as compared to placebo in patients with intermediate, high, or very high risk of disease progression, demonstrated longer time to next treatment, event free survival, and progression free survival in patients treated with ibrutinib. However, the incidence of adverse events (AEs) was higher in the ibrutinib arm and no difference in OS was observed after limited follow up. While these are promising early results the long-term outcomes of these patients and clonal evolution of the disease with regard to resistance development to BTK inhibitors remain unanswered questions. We therefore recommend ongoing active surveillance of patients with previously untreated CLL including patients with high- and very high-risk CLL-IPI scores until they meet the iwCLL 2018 criteria for treatment. Early intervention strategies should only be considered on a clinical trial at this time.

TABLE 76.5 Modified Indications for Treatment of Chronic Lymphocytic Leukemia

- Grade 2 or greater fatigue limiting life activities
- B symptoms persisting for ≥2 weeks
- Lymph nodes >10 cm or progressively enlarging lymph nodes causing symptoms
- Spleen or liver with progressive enlargement or causing symptoms
- Anemia (hemoglobin <11 g/dL) referable to CLL
- Thrombocytopenia (platelets $<100 \times 10^{12}$/L) referable to CLL or ITP poorly responsive to traditional therapy
- Severe paraneoplastic (e.g., insect hypersensitivity, vasculitis, myositis) process related to CLL not responsive to traditional therapies

AIHA, Autoimmune hemolytic anemia; *CLL*, chronic lymphocytic leukemia; *ITP*, idiopathic thrombocytopenic purpura; *WBC*, white blood cell.

To establish uniform clinical practice standards and ensure reproducible eligibility criteria for entrance into clinical studies, the iwCLL established guidelines for initiation of treatment. Indications to begin therapy include non-autoimmune cytopenias (hemoglobin <10 g/dL, platelets <100,000/μL); bulky (≥10 cm in longest diameter) or symptomatic lymphadenopathy or splenomegaly (≥6 cm below the left costal margin); disease-related B symptoms (unintentional weight loss ≥10% within the previous 6 months, fevers ≥100.5°F or 38°C for 2 or more weeks without evidence of infection, night sweats for ≥1 month without evidence of infection) or fatigue (ECOG PS 2 or worse; cannot work or unable to perform usual activities); rapid lymphocyte-doubling time (<6 months or ≥50% increase over a 2-month period for an initial absolute lymphocyte count >30,000/μL); or LDT of less than 6 months; and autoimmune hemolytic anemia (AIHA) or thrombocytopenia not controlled with steroids. It is imperative to determine if a patient's symptoms are attributable to CLL or a comorbid medical condition because constitutional symptoms such as fatigue are nonspecific and can be caused by many etiologies in elderly patients with comorbid illnesses. In addition to the official iwCLL 2018 criteria for therapy, an increasing frequency of infections and slowly progressive anemia are other indications that can aid the practicing physician in deciding when to initiate therapy in patients with CLL. A summary of the iwCLL 2018 guidelines for treatment is provided in Table 76.5 with some minimal modifications used by us in our practice.

A useful paradigm for clinical practice is to institute treatment in CLL only for cytopenias or directly referable symptoms. When treatment is necessary, a variety of agents have been examined in clinical trials and are currently approved. Previously detailed biomarkers are used in selecting the initial treatment for CLL. Below, we summarize the findings of clinical studies of different treatments for CLL; where such data exist, we integrate genomic biomarkers as well as clinical features, including age and comorbid illnesses. Different therapies are outlined below with attention to details related to their initial use in previously untreated CLL. Because many of these regimens were also developed in relapsed CLL, these data are also reviewed and later referred to in the section Treatment of Patients With Relapsed Chronic Lymphocytic Leukemia.

TARGETED THERAPY

Treatment of CLL has undergone dramatic changes in the last 5 years and chemotherapy with or without the use of CD20 targeting antibodies now has a very limited role in the management of patients with this disease. For historical perspective and for practitioners in resource-limited areas, readers are advised to refer to previous editions of this textbook for detailed discussions on chemoimmunotherapeutic use. The subsequent therapy discussion will be focused on the role of targeted therapies for the management of patients with CLL. Chemoimmunotherapy-related data will be detailed in reference to the comparison to targeted therapies. These authors rarely rely on chemotherapy or chemoimmunotherapy for CLL outside of the rare individual with young IGHV mutated disease where FCR-based therapy may produce cure, albeit with a 3% to 10% risk of treatment-related myeloid malignancy and extended immune suppression that may be associated with development of other secondary malignancies.

BCR signaling and microenvironmental signals are now known to be important in both proliferation and protection of CLL cells in BM and nodal sites. These findings prompted the introduction of several novel, orally available kinase inhibitors. In particular, agents targeting the δ isoform of PI3-kinase and BTK have established clinical activity and, more importantly, durability of remission over time in refractory CLL patients. Early results from these trials led to the approval of ibrutinib, acalabrutinib, and idelalisib for the treatment of patients with relapsed CLL. The results of these studies are summarized below.

BRUTON TYROSINE KINASE INHIBITORS

BTK inhibitors have been a significant advancement for patients with CLL. They have radically transformed the treatment of CLL in the last few years. Ibrutinib was the first-in-class irreversible, covalent BTK inhibitor of BTK that was approved for all indications in patients with CLL. While the initial approval was for patients with high risk and relapsed disease, this was quickly followed by approvals across all disease states including patients with previously untreated disease. The second-in-class BTK inhibitor acalabrutinib was also approved both for patients with untreated and relapsed disease based on similar exciting data. Both agents are potent, irreversible, covalent inhibitors of BTK. BTK is a tyrosine kinase in the Tec family of tyrosine kinases and is an essential element of the BCR signaling pathway. Genetic knock-out or inactivation of BTK in mice produces predominantly a B-cell defect with absent B1 lymphocytes, diminished B cells, and disrupted BCR signaling. Mutations in BTK in humans result in a more profound humoral immune defect with absent B cells, immunoglobulins, and infections. Preclinical work with these inhibitors demonstrated disruption of BCR signaling and in vivo activity in non-human lymphoma mouse models with documented targeted inhibition of BTK. Preclinical studies also established their role in promoting apoptosis of CLL cells, inhibiting activation of PI3-kinase, ERK1, and NFκB by external microenvironment signals, and also prevention of CLL proliferation. While these agents have differing specificities with regard to kinase targeting, their clinical efficacy and utility are now well established.

Ibrutinib in Untreated Chronic Lymphocytic Leukemia

In the pivotal RESONATE-2, phase III, randomized control trial ibrutinib was compared to chlorambucil in 269 patients over 65 years of age or older with previously untreated CLL. Ibrutinib use resulted in significant improvement in progression free and OS at 5 years (PFS estimates at 5 years: 70% vs. 12%; HR [95% CI]: 0.146 [0.098 to 0.218]; OS estimates at 5 years: 83% vs. 68%; HR [95% CI]: 0.450 [0.266 to 0.761). Overall response rate (ORR) was 92% with ibrutinib with 30% complete remissions. Depth of response also improved with prolonged use of ibrutinib. Most frequent AEs observed with ibrutinib were diarrhea, cough, and fatigue and most of these events improved with ongoing use of ibrutinib. With regard to AEs of special interest, ibrutinib increased the severity of hypertension in 26% of patients. Atrial fibrillation was observed in 5% of patients and major bleeding was observed in 6% of patients. Median PFS was 56 months in patients who discontinued ibrutinib in CR or CRi for toxicity-related or other reasons. Importantly, patients who discontinued ibrutinib because of progressive disease had a median OS of 20 months. Early results from this trial resulted in the approval of ibrutinib for patients with previously untreated disease but more importantly, it also demonstrated that in the event that patients have to discontinue ibrutinib for reasons related to toxicity and if they are in a good disease control state, they can maintain disease control for an extended period of time.

Given the exciting results observed with ibrutinib monotherapy, multiple trials were conducted of ibrutinib in various combinations in comparison to existing treatment options. In the ALLIANCE A041202 phase III study of ibrutinib alone or in combination with rituximab, versus bendamustine and rituximab, 447 patients older than 65 years of age were randomly assigned to either ibrutinib, ibrutinib with rituximab, or bendamustine and rituximab arms. PFS at 2 years was 87% with ibrutinib alone, 88% with ibrutinib and rituximab, and 75% with bendamustine and rituximab. While there was no difference between the ibrutinib-containing arms both these arms were significantly better than the bendamustine and rituximab-containing arm. In addition, serious AEs were much higher for bendamustine and rituximab as compared to ibrutinib-containing arms.

Similarly, the combination of ibrutinib and rituximab was also compared to FCR chemotherapy in the Eastern Cooperative Oncology Group (ECOG) E1912 randomized phase III study of patients with previously untreated CLL over 70 years of age or younger. A series of 354 patients were randomized in a 2:1 manner to ibrutinib and rituximab (IR) versus FCR. At a median follow-up of 3 years, the combination of ibrutinib and rituximab significantly improved PFS and more importantly OS as compared to FCR (PFS estimate 89.4% vs. 72.9% at 3 years; HR, 0.35; $P < .001$; OS estimate 98.8% vs. 91.5% at 3 years; HR, 0.17; $P < .001$). While the incidence of serious AEs was similar in both groups, grade ≥3 treatment-related AEs were experienced by 70% of IR-treated and 80% of FCR-treated patients (odds ratio, 0.56; $P = .013$). A total of 23 deaths were reported (IR cohort, 11; FCR cohort, 11) and the use of FCR resulted in a higher incidence of infectious complications. In addition, similar to results seen with RESONATE-2 trial, the median time to progression or death after discontinuing ibrutinib was 23 months.

Subgroup analysis of the ECOG E1912 study by IGHV mutation status demonstrated that IR was superior to FCR for IGHV-unmutated patients (HR, 0.28; 95% CI, 0.17 to 0.48; $P < .0001$), but not for IGHV-mutated patients (HR, 0.42; 95% CI, 0.16 to 1.16; $P = .086$) with current follow-up. These results clearly demonstrate the superiority of ibrutinib as compared to chemoimmunotherapy-based regimens. The benefit of CD20 antibodies for CLL with ibrutinib is of less value, particularly in the elderly population and not yet tested in trials for younger patients.

FCR and BR chemoimmunotherapy have been the most utilized regimens for the treatment of patients with CLL. One of the arguments that is utilized for the use of FCR in the current era is the long-term follow-up that resulted in a plateau in progression free survival curves over the long term in a select subset (~50%) of patients who have mutated IGHV, and achieve minimal residual disease-negative state after FCR treatment. Patients in this select group had a progression free survival of 79.8% at 12.8 years of follow-up as compared to only 8.7% in patients who had unmutated IGHV. These plateaus in progression free survival have not been seen in patients on BR-based regimens. The prolonged and sustained remissions seen in a small subset of patients treated with FCR definitely raises the issue of a potentially curative option for a group of patients with good risk features. While ibrutinib has not demonstrated improvement in outcomes in this particular subgroup, it appears to be equally effective and with prolonged follow-up it might even establish its superiority in this cohort of patients too. Nevertheless, for young, fit patients with mutated IGHV and requiring treatment, and who might be considered candidates for chemoimmunotherapy, we generally discuss this issue in detail and inform them about the increased risk of secondary malignancies and treatment-related myeloid neoplasms associated with the use of FCR chemotherapy. We generally do not recommend the use of chemoimmunotherapy-based regimens in the current era.

Acalabrutinib in Untreated Chronic Lymphocytic Leukemia

Initial results from a phase IB/II clinical trial of acalabrutinib demonstrated promising efficacy and tolerability and resulted in the approval of acalabrutinib for patients with relapsed and refractory disease. In the subsequent ELEVATE TN multicenter, open label, phase III, randomized clinical trial, 675 patients were randomized in a 1:1:1 manner to acalabrutinib, acalabrutinib plus obinutuzumab (a humanized anti-CD20 monoclonal antibody), or obinutuzumab plus chlorambucil. The trial was designed for patients who would not be ordinarily considered candidates for conventional chemotherapy because of an age of 65 years or older, or younger patients with compromised kidney function or a high comorbidity burden. With a median reported follow-up of 28.3 months, PFS was significantly prolonged in the acalabrutinib-containing arms as compared to obinutuzumab plus chlorambucil (estimated 24-month PFS 93% with acalabrutinib-obinutuzumab (95% CI 87% to 96%), 87% with acalabrutinib monotherapy (95% CI 81% to 92%), and 47% with obinutuzumab-chlorambucil (95% CI 39% to 55%)). While there was improved PFS with acalabrutinib in combination with obinutuzumab, the study was not designed to evaluate this comparison. Future studies examining this question are warranted. Treatments were generally well-tolerated with the most common AE being neutropenia and infectious complications in 30% and 21% of patients in the acalabrutinib plus obinutuzumab arm, 9% and 14% in the acalabrutinib alone arm, and 41% and 8% in the obinutuzumab plus chlorambucil arm respectively. With regard to AEs of clinical interest, atrial fibrillation was observed in 3% versus 4% versus 1%; grade 3 hypertension in 3% versus 2% versus 3%; bleeding events of any grade in 43% versus 39% versus 12% in the acalabrutinib versus acalabrutinib plus obinutuzumab versus acalabrutinib plus chlorambucil arms respectively . Based on these results acalabrutinib was approved for treatment of patients with previously untreated CLL either as a single agent or in combination with obinutuzumab. At time of completion of this chapter, the Elevate R/R trial has been completed, comparing the efficacy of ibrutinib versus acalabrutinib in relapsed, del(11q) and del(17p) CLL patients with a non-inferiority design. This trial demonstrated similar progression free survival with use of acalabrutinib but diminished adverse cardiovascular (atrial fibrillation and hypertension), treatment cessation, and many other AEs when compared to ibrutinib. While this study was performed in high-risk, relapsed CLL, it represents the first to provide support for broad use of acalabrutinib in CLL as the preferred BTKi based upon the favorable side effect profile observed in this trial. A third BTKi zanubrutinib has also been shown to have similar efficacy and improved safety over ibrutinib, but has not been formally approved for the treatment of CLL. We typically use zanubrutinib when patients are intolerant to acalabrutinib or when patients require proton pump inhibitor therapy.

Venetoclax in Untreated Chronic Lymphocytic Leukemia

The bcl-2 antiapoptotic protein is overexpressed in CLL and has been shown to disrupt apoptosis in this disease. Although the bcl-2 antisense molecule Genasense did not meet the bar for regulatory approval in CLL, attempts to target bcl-2 with small molecule inhibitors continued. Another potent inhibitor of bcl-2, navitoclax (formerly ABT263), demonstrated single agent activity in relapsed CLL with a target specific (BCL-XL) dose-limiting toxicity of profound thrombocytopenia. A second-generation oral bcl-2 inhibitor venetoclax (ABT-199) that lacked off-target effect on BCL-xL was subsequently developed. The initial clinical development of venetoclax was complicated by fulminant tumor lysis syndrome especially in patients with high burden of disease, and mitigation strategies were subsequently developed to improve its safety and reduce the incidence of hyperacute tumor lysis. It subsequently demonstrated impressive activity with deep remissions in patients with relapsed and refractory CLL including those with del17p resulting in its initial approval in that patient subgroup.

Given the impressive results seen in patients with relapsed CLL, venetoclax was combined with obinutuzumab and compared to chlorambucil and obinutuzumab in the CLL14 randomized clinical trial of 432 patients. Patients included in this trial would not be ordinarily considered for treatment with chemoimmunotherapy-based regimens and were treated with 12 months of treatment with venetoclax and chlorambucil while obinutuzumab was used at standard dose and schedule. This time-limited combination of venetoclax plus obinutuzumab (12 months) demonstrated a significantly higher PFS at 24 months of 88.2% (95% CI, 83.7 to 92.6) as compared with 64.1% (95% CI, 57.4 to 70.8) with chlorambucil and obinutuzumab. Most common AEs included neutropenia and infectious complications in both arms. There were no episodes of clinical tumor lysis syndrome in patients treated with venetoclax and only 3 patients had laboratory evidence of tumor lysis syndrome. AEs led to discontinuation of treatment in 16% of patients in the venetoclax and obinutuzumab arm and 15.4% of patients in the chlorambucil plus obinutuzumab arm. Treatment with venetoclax was also successful in achieving undetectable measurable residual disease (u-MRD) in 75% of patients in the peripheral blood and 56% of patients in the BM. With longer follow-up, patients treated with venetoclax-based treatment continued to demonstrate improved outcomes in all groups of patients after discontinuation of therapy. However, patients with del17p demonstrated much shorter PFS interval as compared to patients without del17p after discontinuation of venetoclax. Interestingly, patients treated on the venetoclax and obinutuzumab arm had a higher rate of second primary malignancy (17%) as compared to 10.3% in the chlorambucil plus obinutuzumab arm necessitating the need for ongoing routine health maintenance evaluations despite discontinuation of therapy. The resulting approval of venetoclax in combination with obinutuzumab as first-line treatment for patients with CLL therefore provides an effective, time-limited, and generally well-tolerated treatment, which results in sustained remissions despite drug discontinuation.

The choice of a specific novel therapeutic agent as first-line treatment is definitely a difficult decision in the current era and needs to be based on patient preferences, clinical scenarios, comorbid conditions, and cost and insurance issues. The completion of the Elevate R/R trial provides support for acalabrutinib if a BTK is used. While some view venetoclax-based options as attractive based upon providing an option to discontinue treatment after a defined time period, patients will definitely require repeat treatment and long-term disease-free or OS outcomes might be similar in patients with interrupted as compared to continuous treatment strategies. Our general approach is to start treatment with BTK inhibitors unless there is a clear contraindication to their use. We also prefer to utilize acalabrutinib and exclusively use it in older patients (>70 years of age) with documented cardiac comorbid conditions. The benefit of obinutuzumab addition to acalabrutinib represents a question to the field at this time although its use does deepen remission and offers the potential opportunity to discontinue therapy if CR is obtained. We strongly encourage participation in clinical trials comparing various drug combinations.

TREATMENT OF PATIENTS WITH RELAPSED CHRONIC LYMPHOCYTIC LEUKEMIA

The approach to reinitiating therapy for CLL patients who have relapsed after initial therapy is similar to that applied for initial therapy assessment. Patients need not receive therapy at the first sign of relapse. Rather, patients should have an indication for treatment, as discussed earlier. Patients should have repeat interphase FISH analysis of the peripheral blood or BM aspirate because patients may acquire additional cytogenetic abnormalities, most notably del(17p13.1) and TP53 mutational analysis, as their CLL becomes more advanced. The incidence of del(17p13.1) increases from 5% of patients at initial diagnosis to nearly half of heavily treated patients with advanced

CLL, and has been established as a consistent marker of poor outcomes. IGHV mutational analysis does not need to be performed if such information has been obtained previously because a patient's IGHV mutational status does not change with time. A BM should be performed if cytopenias are present to exclude other potential causes of cytopenias such as transformed lymphoma, prolonged BM toxicity from prior therapy, or development of a treatment-related myeloid neoplasia. Treatment-related myeloid neoplasia may be difficult to assess in CLL patients with extensive BM involvement but should be considered when either genomic mutations or cytogenetic aberrations present in the absence of CpG stimulation are present suggestive of this diagnosis in the setting of cytopenias. Patients with CLL should be treated according to the indications outlined in Table 76.5. Multiple novel agents have now been approved and more are in the advanced stages of development and the treatment for relapsed disease will continue to evolve with time. In general, therapy has moved away from chemoimmunotherapy to better tolerated targeted oral agents especially for patients older than 70 years of age and those with multiple comorbid conditions.

Ibrutinib for Relapsed Disease

The phase I study of ibrutinib evaluated two dose levels above where complete BTK inhibition occurred without obtaining a maximum tolerated dose. At the highest dose, toxicity was manageable, including grade 1/2 nausea, diarrhea, infections, rash, and fatigue. Activity was seen at all doses, including in nine of 16 CLL/SLL patients. A subsequent trial utilized the 420 mg daily oral dose of ibrutinib and demonstrated an ORR of 71% with an additional 20% of patients experiencing partial response with lymphocytosis (PR+L). The PR+L observed with ibrutinib in a percentage of patients does not appear to predict for inferior PFS. More importantly, the responses seen with ibrutinib were sustained and result in a PFS of 69% at 30 months. Responses to ibrutinib also tended to improve with time and after a median follow-up of 2 years, CR rates improved from 2% to 7%. Responses were also seen in patients with del17p who had an ORR of 55.9% with a median duration of response of 25 months. This compared favorably in historical comparison to either cyclin-dependent kinase inhibitors or other conventional therapies utilized in the past for patients with del17p. Patients with previously untreated CLL also appeared to benefit from ibrutinib monotherapy with an ORR of 71% and 13% PR+L. PFS at an early follow up was 96% at 2 years.

Ibrutinib was also well-tolerated in these trials and the most common side effects were diarrhea, nausea, and fatigue. A subsequent randomized study comparing ibrutinib to ofatumumab (RESONATE) in relapsed CLL confirmed the benefits of ibrutinib with improved response rate, PFS, and OS. With a median follow-up of 44 months, the median PFS was not achieved with ibrutinib and 46% of the patients were able to continue treatment. There was also a decreased incidence of grade 3 or higher AEs over time. Progressive disease resulted in discontinuation of treatment in 27% of patients on ibrutinib.

However, these studies also demonstrated a higher incidence of both minor and major bleeding episodes possibly due to a collagen-mediated platelet aggregation defect, and atrial fibrillation with ibrutinib. Ibrutinib should therefore not be used in patients on concurrent anticoagulation with warfarin and patients should be taken off ibrutinib for 3 to 7 days before and after minor or major surgical procedures respectively. Additional retrospective reports also established anti-platelet agent use as a risk factor for the increased bleeding diathesis observed in patients concurrently on ibrutinib. Given the substantial risk of bleeding with concurrent anticoagulant use with ibrutinib we generally avoid the use of anticoagulation in patients who develop atrial fibrillation after consultation with our cardio-oncology colleagues (see Chapter 158). For patients with a high risk of thrombotic or embolic events or deep venous thrombosis or pulmonary emboli, we generally utilize direct-acting oral anticoagulants (DOAC) like apixaban. We never use warfarin with ibrutinib due to an unusual and yet unexplained high frequency of subdural hematomas noted early in the development of this agent when these agents were combined. Ibrutinib use also results in an increased incidence of new or worsening hypertension requiring the addition and increased dose of antihypertensive therapy. It also increases the risk of major adverse cardiac events and ventricular arrhythmias and sudden cardiac death. Given the well-established cardiac toxicity risk with ibrutinib, we recommend a detailed cardiac evaluation prior to starting patients on ibrutinib. Patients with underlying cardiovascular disease should have formal consultation with experts familiar with these issues in the cardio-oncology field. We generally perform a baseline EKG and an echocardiogram prior to starting our patients on ibrutinib. Moreover, we also evaluate the list of their concomitant medications including antihypertensives, and maintain close communication with the primary care physician and the cardiologist throughout the treatment course.

Patients on ibrutinib experience a progressive decline in the incidence of infectious complications with continued use of ibrutinib and do not require routine anti-microbial prophylaxis. We do recommend prophylaxis with acyclovir or valacyclovir for all patients especially those with relapsed disease. There is also subjective improvements in stress, depressive symptoms, fatigue, and quality of life in patients.

Acalabrutinib for Relapsed Disease

Early data with the use of acalabrutinib for patients with relapsed CLL demonstrated very promising results. The initial phase IB/II study reported outcomes on 134 patients with relapsed and/or refractory CLL, with a median age of 66 years and median of 2 prior lines of therapy. Acalabrutinib was dosed at 100 mg twice a day and resulted in an overall response rate of 94% regardless of genomic features. With updated follow-up, the 45-month PFS was 62% (95% CI, 51% to 71%). Treatment was very well tolerated with most AEs being mild to moderate in intensity. The most common AE reported was diarrhea (52%) and headache (51%). Grade 3 or higher hypertension was reported in 7% of patients. Atrial fibrillation and major bleeding events occurred in 7% and 5% of patients respectively. The primary reason for discontinuation of treatment was progressive disease in 21% with the majority harboring BTK mutations, and AEs in 11%.

A subsequent large, phase III study of 310 patients (ASCEND) who were randomly assigned to acalabrutinib monotherapy ($n = 155$) or investigator choice therapy of idelalisib (a phosphoinositide 3-kinase inhibitor; more specifically, it blocks P110δ, the delta isoform of the enzyme phosphoinositide 3-kinase) and rituximab ($n = 119$), or bendamustine and rituximab ($n = 36$), revealed a significantly improved progression free survival advantage for acalabrutinib. Estimated 12-month PFS was 88% (95% CI, 81% to 92%) for acalabrutinib and 68% (95% CI, 59% to 75%) for investigator's choice treatment. AEs were as expected with the most common AEs in the acalabrutinib arm being neutropenia in 16%, anemia in 12%, and pneumonia in 5% of the patients. Most common AEs in patients treated with idelalisib and rituximab were neutropenia in 40%, diarrhea in 24%, and pneumonia and transaminitis in 9% of patients. Similarly, in patients treated with bendamustine and rituximab, neutropenia was the most common AE in 31% of the patients followed by anemia in 9% and constipation in 6%. Only 3% of patients who received acalabrutinib required a dose reduction compared to 24% who received idelalisib and rituximab, and 17% who received bendamustine and rituximab. Atrial fibrillation rate was 5% in the acalabrutinib arm compared to 3% in the control arm. Bleeding events were more commonly seen with the acalabrutinib arm at 26% versus 7% in the investigator's choice arm. The trial was important in establishing the superiority of acalabrutinib not just over chemoimmunotherapy but also over a PI3 kinase inhibitor in patients with relapsed and refractory disease. The toxicity profile was also favorable, and these results led to the approval for acalabrutinib in the relapsed/refractory setting.

Since treatment with ibrutinib or acalabrutinib does not result in CRs in the majority of patients, at this time it is recommended that treatment be continued indefinitely until disease progression or unacceptable toxicity, since discontinuation in heavily pre-treated patients often results in rapid disease progression. Development of resistance has been rare but whole exome sequencing has identified a cysteine-to-serine mutation in BTK at the binding site of ibrutinib and acalabrutinib, and distinct mutations in PLCγ2. Functional analysis revealed that the C481S mutation of BTK results in a protein that is only reversibly inhibited by ibrutinib. The R665W and L845F mutations in PLCγ2 are both potentially gain-of-function mutations that lead to autonomous B-cell-receptor activity. Gate-keeper mutations in the (481I or 481 M) are rarely noted with irreversible BTKi but may be observed more commonly as reversible BTKi are introduced into clinical practice.

Venetoclax in Relapsed Chronic Lymphocytic Leukemia

The utility of venetoclax was initially reported in the initial phase I/II clinical trial of 158 patients with predominantly relapsed, refractory disease. Use of venetoclax was associated with an ORR of 77% and 2-year PFS was 54%. Encouraging activity was also observed in patients who had failed prior kinase inhibitor-based treatments. Initial issues with tumor lysis syndrome were overcome with a graduated dose ramp up over a period of 4 weeks.

In the landmark open label, phase III MURANO trial of 389 patients, venetoclax treatment for 24 months was combined with rituximab for the first 6 months and compared to standard doses of bendamustine and rituximab for 6 months. With a median follow-up of approximately 2 years, PFS was significantly higher in patients treated with venetoclax and rituximab as compared to bendamustine and rituximab (84.9% vs. 36.3%, respectively HR 0.17; 95% CI, 0.11 to 0.25; $P < .001$). The improved outcome was seen across all subgroups of patients. Most common AE was neutropenia in 60.8% of patients in the venetoclax and rituximab group and 44.1% of patients in the bendamustine and rituximab group. Despite the higher incidence of neutropenia with venetoclax, the incidence of grade 3 to 4 febrile neutropenia and grade 3 or 4 infections were lower in the venetoclax and rituximab group. Clinical tumor lysis syndrome was only seen in one patient and was mitigated by the graduated ramp-up approach.

Importantly, patients who have failed to achieve a response to venetoclax had dismal outcomes with a median PFS of 5.4 months. Similarly, patients treated with prior kinase inhibitors, and with bulky disease and lymph nodes of ≥10 cm, appear to have a lower response rate. In addition, the addition of rituximab appeared to significantly improve the rate of CR.

Subsequent pooled efficacy analysis of four early-phase clinical trials of venetoclax including 436 patients in the relapsed and refractory setting demonstrated excellent efficacy of venetoclax with an ORR of 75% including a 22% CR rate. Undetectable MRD in the blood and marrow was achieved in 27% and 16% of patients respectively. Estimated median PFS was 30 months. As predicted, duration of response was longer in patients achieving undetectable MRD. PFS, but not response rate, was also inferior in patients with del17p or aTP53 mutation. A later pooled safety analysis of 350 patients who had received a median of 3 prior therapies, including kinase inhibitors in 42%, demonstrated manageable toxicity with the most common AEs being diarrhea (41%), neutropenia (40%), nausea (39%), anemia (31%), fatigue (28%), and upper respiratory tract infection (25%). The most common grade 3/4 AEs were neutropenia (37%), anemia (17%), and thrombocytopenia (14%). The incidence of clinical tumor lysis syndrome was nonexistent with the current graduated ramp-up of venetoclax dosing and laboratory evidence of tumor lysis syndrome was observed in less than 2% of patients. These studies not only resulted in the approval of venetoclax and rituximab for patients with relapsed/refractory CLL, but also established its efficacy and the safety of the graduated ramp-up dosing approach.

PI3-Kinase Inhibitors

Idelalisib is an orally bioavailable, isoform specific, PI3-kinase-δ inhibitor that, at therapeutic levels obtained in patients, does not inhibit other isoforms of PI3-kinase. Genetic mouse models knocking out PI3-kinase delta demonstrate predominantly a B-cell defect, absent B1 lymphocytes, and disrupted BCR signaling. Preclinical studies by our group and later confirmed by others demonstrated that idelalisib promotes the apoptosis of CLL cells via a PI3-kinase-δ pathway and inhibits signals from the microenvironment that protects CLL cells from apoptosis. Based in part on these data, a phase I study in healthy volunteers showed this agent to be well tolerated, and subsequently a large phase I study in NHL, CLL, multiple myeloma, and AML was undertaken. Idelalisib was active at all dose levels tested, with the dose-limiting toxicity being reversible transaminitis that generally occurred during the first 2 months of therapy in approximately 5% to 20% of patients, depending on histology. Although patients with NHL experienced a higher incidence of transaminitis, the cause of which is poorly understood, the incidence of transaminitis was less than 5% in CLL patients. In a phase I trial of 54 patients, with relapsed/refractory high-risk CLL patients, idelalisib resulted in an ORR of 72% (including PR+L). The median PFS was 15.8 months. Therapy was generally well tolerated with the most commonly observed Grade ≥3 AEs being pneumonia in 20%, neutropenic fever in 11%, and diarrhea in 6% of the patients. Subsequent studies have been undertaken with idelalisib combining it with rituximab, ofatumumab, obinutuzumab, and bendamustine/rituximab, showing that combination therapy with idelalisib is feasible. The combination of idelalisib and rituximab was also compared to rituximab and placebo in a phase III trial and resulted in an ORR of 81% versus 13% and PFS at 1 year in excess of 90% versus 5.5 months in the rituximab and placebo arm respectively. Serious toxicities observed with idelalisib were similar to ones observed with the single agent. Based on these results, idelalisib was approved in combination with rituximab for the treatment of patients with relapsed CLL. However, its use has been limited given the clinical activity and excellent tolerability of BTK inhibitors, and the serious immune mediated toxicities observed with the use of idelalisib including transaminitis, colitis, pneumonitis, and increased incidence of infectious complications. Colitis can also be a delayed complication and can be severe and manifest itself 4 to 6 months after starting treatment. Pneumonitis can also be particularly difficult to distinguish from infectious issues commonly seen in these patients. CMV reactivation also needs to be monitored closely. Since these toxicities can be particularly severe and potentially life-threatening in patients with previously untreated disease, we do not recommend the use of idelalisib in patients with previously untreated CLL. In addition, dose reductions and dose interruptions can actually result in improved tolerability, compliance, and PFS.

Duvelisib is another agent in this class that inhibits both PI3-kinase-δ and γ, an isoform expressed in T cells, myeloid cells, and other cell types. The initial phase I study of duvelisib in patients with relapsed refractory CLL established a maximum tolerated dose of 75 mg twice a day. However, the study also demonstrated a sustained maximum pharmacodynamic effect at a dose of 25 mg twice daily. The 55 patients with relapsed/refractory CLL/SLL that were treated had an ORR of 56% with a median progression free survival of 15.7 months and median duration of response of 21 months. Based on this study, a randomized phase III clinical trial was conducted in 319 patients with relapsed/refractory CLL/SLL who had received two or more prior therapies. Patients were randomized to duvelisib at 25 mg twice a day or ofatumumab. ORR was better with duvelisib as compared with ofatumumab (73.8% vs. 45.3%, $P < .0001$), with almost all responses being partial (72.5% duvelisib and 44.7% ofatumumab) and this translated into an improved median progression free survival as compared to ofatumumab (13.3 vs. 9.9 months; hazard ratio, 0.52; $P < .0001$). Outcomes for duvelisib were also better among del(17p) and/or TP53-mutated patients as compared to ofatumumab (median PFS of 12.7 vs. 9.0 months; hazard ratio, 0.40; $P = .0002$).

Most common AEs observed with duvelisib included neutropenia, anemia, and infectious complications. This included infections with opportunistic organisms. In this and other studies of duvelisib, immune mediated toxicities such as transaminitis, hepatitis, diarrhea, colitis, pneumonitis, and rash were also observed. This occurred in almost half of patients treated with duvelisib. As with idelalisib, this occurred secondary to increased CD8 positive effector cell infiltration of the organ experiencing the AE. This resulted in a significant proportion of patients requiring dose reductions and discontinuations. Despite the significant toxicity seen with the use of PI3-kinase inhibitors, they do have an important role to play in the management of patients with relapsed refractory CLL/SLL who would not ordinarily be considered candidates for BTK inhibitors or BCL-2 antagonists, either because of comorbid conditions or underlying organ dysfunction. They also appear to have some activity in patients who have failed prior BTK inhibitors although remission with these agents is relatively short. We do recommend close monitoring of patients on these agents especially for immune mediated AEs, and recommend employing dose reductions and drug holds in the setting of AEs. In particular, we caution continuing these agents when patients develop pneumonitis or colitis as sometimes significant morbidity can follow.

Role of Novel Agents in the Management of Patients With Del(17p13.1)

Conventional chemotherapy has limited benefit in the initial treatment of patients with del(17p13.1) and median PFS is in the range of 11 to 14 months. Over time del(17p13.1) and/or TP53 mutations have consistently emerged as reliable prognostic markers for inferior outcomes. While BTK inhibitors have significantly improved outcomes for patients with CLL, the outcome for patients with del(17p13.1) remains inferior to patients without this particular deletion or mutation. Ibrutinib's initial approval for patients with del(17p13.1) was based on limited data which demonstrated overall responses of up to 97% in this high-risk group of patients. These responses were rapid and improved over time and resulted in a progression free survival at 12 months of 82%. Given the significant improvement in outcomes in this patient cohort compared to historic controls, ibrutinib was approved for this indication across all lines of treatment. A subsequent integrated analysis of 230 patients with relapsed and refractory del(17p13.1) patients demonstrated an overall response rate of 85% with a 30-month progression-free and OS rates were 57% (95% CI 50 to 64) and 69% (95% CI 61 to 75), respectively. Similarly, patients treated with acalabrutinib in the phase I clinical trial demonstrated an overall response rate of 100% with PR in 89% and PR+lymphocytosis in 11% of patients. These results were similar to those observed with ibrutinib and response appears to be sustained over time. Venetoclax was also initially evaluated in 107 patients with del17p in the relapsed and refractory setting. Despite initial issues with tumor lysis syndrome, treatment with venetoclax was very effective and resulted in a 12-month PFS of 72%. Most common AEs were gastrointestinal including diarrhea, nausea, and cytopenias. Based on these results, and the comparison with historical data, chemoimmunotherapy-based regimens have limited use in the management of patients with del17p either in the untreated setting or in patients with relapsed/refractory disease. The choice of novel agent should be based on patient preferences, comorbid conditions, and clinical scenario.

Sequencing of Therapy and Management of Patients Relapsing After Novel Therapies

Early studies with ibrutinib demonstrated excellent tolerability and slow disease progression of patients who had to discontinue treatment because of AEs. This amounted to approximately 16% of patients over 6 years of follow-up in the RESONATE trial. However, real-world evidence suggests intolerance-related discontinuation with ibrutinib of approximately 30% to 50% over time. A substantial percentage of these patients who discontinue ibrutinib because of AEs do not require therapy immediately and can be monitored with active surveillance. However, in the event of progressive disease and the need for subsequent therapy acalabrutinib can be employed and has a demonstrated overall response rate of 76% with a 2-year progression free survival of 76% in these patients. In addition, the use of acalabrutinib resulted in recurrence of ibrutinib-attributable AEs in only a small minority. The vast majority of patients had either complete resolution or decrease in the severity of the AEs that resulted in the discontinuation of ibrutinib.

Management of patients in the relapsed setting in the recent chemotherapy free era can be challenging and dependent on the choice of first-line therapy. Patients progressing on BTK inhibitors can experience rapid tumor flare upon discontinuation for progressive disease, and hence we do not recommend abrupt discontinuation of these agents. BTK inhibitors should be overlapped with next-line agents as overlapping therapy, or alternatively bridging therapy such as steroids can be utilized for this purpose. In addition, it is important to rule out Richter transformation in the appropriate clinical situation. We also recommend repeat evaluation by FISH and karyotype to assess for clonal evolution along with testing for BTK and PLCγ2 mutations. In the absence of good quality clinical trial data, management of these patients progressing on BTK inhibitors is derived from real-world experiences which demonstrate that in patients progressing after prior BTK inhibitors, venetoclax-based treatment appears to have the best outcomes as compared to alternative treatments including PI3K inhibitors and chemoimmunotherapy. Similarly, patients progressing on venetoclax-based treatments should be tested for clonal evolution and BCL-2 resistance mutations. Venetoclax monotherapy in the setting of prior BTK failure resulted in an overall response rate of 65% in one study with a PFS and OS of 75% and 91% at 1 year respectively. This also allows an opportunity for deep MRD undetectable responses and fixed-duration therapy and is the preferred treatment modality in this setting. PI3K inhibitors have modest activity in this setting.

For patients who discontinue venetoclax-based treatment as a predefined option, retreatment with venetoclax either as a single agent or in combination with rituximab results in excellent outcomes. However, this retreatment approach is best suited for patients who experience a prolonged treatment free interval after discontinuation of venetoclax. Ibrutinib has also been shown to be effective in this setting with promising responses and prolonged disease control. For BTK inhibitor-naïve patients who progress after venetoclax-based treatment, the ORR to venetoclax varies from 84% to 100% and drops to around 54% with previous BTK inhibitor exposure. We therefore recommend that patients progressing after venetoclax-based therapy should be considered for retreatment with venetoclax especially if they have experienced a prolonged remission and treatment-free interval with initial venetoclax-based treatment. For patients who have previously failed BTK inhibitors or with demonstrable BTK resistance mutations, and demonstrate progression after venetoclax, BTK inhibitors should not be utilized, and patients should be considered for participation in clinical trials of alternative treatments, non-covalent BTK inhibitor, bispecific T-cell engagers (BiTEs), chimeric antigen receptor T-cell (CAR-T), and allogeneic hematopoietic stem cell transplantation (HSCT) in the appropriate setting. For patients progressing after prior chemoimmunotherapy use, we generally recommend the use of BTK inhibitors followed by venetoclax post relapse from BTK inhibitors.

Allogeneic HSC transplantation or CAR-T-cell therapy should also be considered for patients progressing after two prior lines of treatment with novel therapies or who demonstrate lack of response to novel agents. The prior use of ibrutinib does not appear to impact transplant-related outcomes. Noncovalent and reversible BTKi inhibitors are currently being tested in patients with documented resistant BTK C481S mutation. Early results with LOXO-305 demonstrated an impressive ORR of 57% with improvement in the depth of response over time in this high-risk patient population. The drug was also extremely well tolerated with a small proportion of patients experiencing fatigue and diarrhea. Similar promising early results and excellent tolerability has been shown in patients with ARQ531.

Other Emerging Therapeutic Modalities for Chronic Lymphocytic Leukemia

Several other promising therapeutics are in late phase II/III clinical trials for CLL. Those with documented activity in this disease that have potential for approval for use in CLL in the near future are described here.

Zanubrutinib is a potent and selective BTK inhibitor which utilizes the same C481 binding domain as ibrutinib and acalabrutinib. It is already approved for the treatment of patients with mantle cell lymphoma in the relapsed setting (see Chapter 86). It is also being compared to ibrutinib in a head-to-head, noninferiority, clinical trial in the relapsed and refractory setting. While its advantages over existing BTK inhibitors remain to be elucidated, its use does offer the potential advantage of a lower incidence of bleeding and cardiovascular toxicity, and the flexibility of twice a day or once a day dosing. Moreover, its bioavailability does not appear to be impacted by the use of concurrent proton pump inhibitors which can impact acalabrutinib use.

Umbralisib is a PI3K-δ and casein kinase-1 epsilon (CK1ε) inhibitor that has demonstrated promising activity in patients with CLL. It is currently approved for use in patients with marginal zone lymphoma and follicular lymphoma (see Chapters 82 and 83). In early results of 51 patients with relapsed/refractory CLL, 44 of whom were intolerant to BTK inhibitors and seven of whom were intolerant to PI3 kinase inhibitors, the median PFS was 23.5 months. Treatment was well-tolerated and most common AEs were neutropenia (18%), thrombocytopenia (12%), pneumonia (12%), diarrhea (8%). Treatment-related discontinuation occurred in 12% of patients treated with umbralisib. Combination of this drug with a new anti-CD20 monoclonal antibody ublituximab also demonstrated good tolerability with modest activity and an ORR of 46% with 17% CR in patients with relapsed/refractory CLL and other indolent lymphomas. Similar to the issue with the comparison of ibrutinib with alternative BTK inhibitors, while umbralisib appears to have a better toxicity profile as compared to other PI3 kinase inhibitors, head-to-head comparative data is currently lacking.

Multiple exciting agents are also in development, including BiTEs, various CAR-Ts, and other immunomodulatory agents portending an excellent prognosis for our patients.

Chimeric Antigen Receptor T-Cell Therapy

A major development over the last few years has been the approval of ex vivo transfected T cells with chimeric T-cell receptors bearing both CD3 and a B-cell–specific antigen (most often CD19) for various aggressive and indolent lymphoid malignancies. Trials for patients with CLL are currently ongoing but early results from studies evaluating patients with relapsed CLL following BTK inhibitors demonstrated an ORR of 87% with a CR and u-mRD rate of 47% in patients treated with lisocabtagene maraleucel. The use of ibrutinib in the peri-CAR-T phase appears to result in a higher ORR and improves ex vivo T-cell expansion. With improving understanding and management of toxicities, outcomes are continually improving and this treatment option has the potential to be a definitive treatment option for a number of patients with relapsed CLL. For patients progressing on BTK or BCL2 inhibitors, we recommend consultation with a CAR-T center to consider enrollment on a clinical trial.

Hematopoietic Stem Cell Transplantation

For many years, autologous and allogeneic HSCTs were generally used for treatment of CLL late in the course of the disease. Although autologous HSCT has been used historically as a treatment for CLL, nonmyeloablative allogeneic SCT is the preferred modality for most patients requiring a HSCT unless a suitable allogeneic donor is not available. Autologous SCT is no longer utilized in CLL because of a lack of a survival advantage, only modest benefit in PFS, and a high risk of treatment related mortality with this modality. With increasing recognition of poor prognostic factors, early HSCT has been recommended for some patients, including patients with a complex karyotype and who demonstrated early relapse after kinase inhibitor-based treatments. However, with the increasing use of venetoclax, availability of CAR-T-cell and additional therapeutic options, the optimal timing of HSCT is further open to debate, and the utility of HSCT as a salvage option for patients with CLL has declined considerably over the last decade. Nevertheless, we do recommend referral to a transplant center for patients exhibiting early relapse on kinase inhibitor-based therapies, or who fail to demonstrate response to venetoclax. These patients should ideally undergo HLA typing and identification of a suitable donor.

Initial use of TBI-containing myeloablative regimens demonstrated a lower transplant-related mortality (TRM) of 17% at 100 days compared to 57% in patients who received busulfan and cyclophosphamide (BuCy)-based conditioning regimen. Multiple retrospective registry evaluations continued to demonstrate unacceptably high TRM of greater than 30% and 5-year OS of less than 50% with these approaches. These dismal outcomes persisted despite the use of related and/or unrelated donor sources. Because of the lower toxicity of the TBI-based regimens, BuCy-based myeloablative conditioning regimens should not be routinely used in patients undergoing HSCT for relapsed CLL.

The goal of HSCT is to harness the GVL effect of allogeneic HSCT for patients with CLL while reducing TRM from acute graft-versus-host-disease (GVHD), acute infection, and organ toxicity associated with myeloablative conditioning. EBMT retrospectively examined 77 CLL patients who received a variety of nonmyeloablative conditioning regimens, followed by allogeneic HSCT. The 1-year TRM was 18%, and the 2-year probability of relapse was 31%. Two-year DFS and OS were 56% and 72%, respectively. Nineteen patients received donor lymphocyte infusions (DLIs) for relapse or incomplete donor chimerism, but only seven responded to DLI (37%). Unfortunately, this study was complicated by the heterogeneity of conditioning regimens and the use of antithymocyte globulin (ATG) or Campath-1H for T-cell depletion of the grafts in 40% of patients. A retrospective analysis of 73 CLL patients who underwent nonmyeloablative SCT and 82 patients who underwent myeloablative allogeneic SCT showed that the TRM was significantly reduced in the former group, with a hazard ratio of 0.4; however, there was no difference in EFS or OS between the two groups. Other studies employing low-dose TBI with fludarabine, or fludarabine with melphalan and alemtuzumab, demonstrated 2-year TRM of 22% to 27% OS of around 50%.

Given the decline in the utility of allogeneic HSCT in the era of novel therapies, data on optimal utilization of this treatment strategy is limited. In a recent retrospective series of 108 high-risk patients, allogeneic HSCT demonstrated a 3-year OS of 87% (95% CI, 68%, 95%), PFS of 72% (95% CI, 52%, 85%), cumulative incidence of NRM of 7% (95% CI, 1%, 19%), and cumulative incidence of relapse of 21% (95% CI, 8%, 38%). Of the 30 patients included in this cohort with a history of prior treatment with novel agents, transplant-related outcomes tended to be better as compared to patients treated with chemoimmunotherapy-based approaches but this was not statistically significant. In addition, the use of non-fludarabine and busulfan-containing regimens and presence of Richter transformation was associated with worse outcomes.

Management of Chronic Lymphocytic Leukemia in Specialized Centers

Given the recent advent of prognostic markers and treatment options in CLL, a study that examined the impact of physician expertise on patient outcomes in CLL has shown that disease-specific expertise made significant differences in outcomes across all aspects of patient care from prognostic evaluation to choice of therapy. These findings suggest that the expertise of the physician caring for the patient with CLL/SLL is an independent prognostic variable that may not be related to the number of patients he or she manages. We therefore recommend that all patients with newly diagnosed CLL/SLL should be managed in consultation with a CLL expert at a tertiary care center with access to the most recent molecular diagnostic tools.

SPECIAL CLINICAL SCENARIOS IN CHRONIC LYMPHOCYTIC LEUKEMIA

Young Patients (Younger Than 50 Years of Age) With Chronic Lymphocytic Leukemia

As mentioned previously, CLL is a disease of elderly adults, with only 10% of patients being younger than the age of 50 years at diagnosis. Although CLL patients live for a prolonged period of time, young patients without comorbid illnesses have a great potential to have their lives significantly shortened by the disease, irrespective of their cytogenetic abnormalities. Additionally, stress associated with job performance, insurance coverage maintenance, and disease-related symptoms are most significant in this age group. Special attention to psychosocial issues related to CLL should occur early in the course of the disease to allow patients to maintain or resume their normal lifestyles as soon as possible. Similarly, this group faces unique challenges concerning the choice of novel therapies. While kinase inhibitor-based treatments have the potential to control disease for a prolonged period of time, data beyond 10 years is limited. It also forces the patient to take a treatment indefinitely until progressive disease or intolerable toxicity. Similarly, long-term outcomes data after time-limited venetoclax treatment is lacking. In addition, if the young patient has mutated IGHV then treatment with FCR chemoimmunotherapy can be a consideration albeit at a higher cost of toxicity- and risk of treatment-related myeloid malignancies. Nevertheless, given the advent of multiple promising therapeutic options with a possibility of durable remissions, our approach is generally not to empirically pursue HLA typing or examine transplant options before the development of symptomatic disease. When therapy is initiated for this group of patients, aggressive intervention to promote prolonged remission duration is always compared to prolonged disease control state with the use of kinase inhibitors. HSCT and/or CAR-T-cell therapy is generally considered for patients in this age group who have high-risk cytogenetic abnormalities in second remission and for all patients who do not respond to initial novel agent-based therapy.

Richter Syndrome

Richter syndrome (RS), the development of high-grade lymphoma in patients with CLL, was described by Maurice Richter in 1928. Over the years, the classification of RS has expanded to include lymphoid malignancies such as Hodgkin disease, lymphoblastic lymphoma, PLL, and hairy cell leukemia (see Fig. 76.4A–C). Incidence estimates range from 2.8% to 10.7%. Studies have suggested that the development of RS may be related to the evolution of an abnormal clone unrelated to the underlying CLL clone (clonally unrelated ~30%) or arise from the underlying CLL (clonally—related ~70%). Clearly identifiable risk factors for the development of RS are lacking, and its development has been shown to be independent of disease stage, duration of disease, type of therapy, or response to therapy. However, the presence of diffuse lymphomatous involvement, advanced Rai stage, IGHV-unmutated disease, ZAP-70 expression, high LDH, del(17p13.1), high serum β_2M levels, and NOTCH1, TP53, CDKN2, and MYC gene mutations may predict the development of RS. However, these genetic abnormalities can be varied in patients progressing with RS while on treatment with novel therapies. It is notable that patients developing Richter transformation very often do not have mutations in BTK or PLCG2 after ibrutinib therapy. While there were initial concerns that treatment with novel agents might increase the incidence of RS, this has been refuted with longer-term follow-up from large, prospective clinical trials and the incidence appears to be similar to that with the use of chemoimmunotherapy. RS is characterized by sudden onset of B symptoms (fever, night sweats, weight loss) and rapidly progressive lymphadenopathy at any anatomic site. Rarely, the lymphomatous clone may arise from the bone or an extranodal site. Laboratory abnormalities including anemia, neutropenia, and thrombocytopenia may be due to large-cell transformation in the BM. A rapid increase in the serum LDH is seen in the majority of patients. The diagnosis is generally made after examining the histology of a rapidly enlarging lymph node, which typically reveals large-cell lymphoma. The most common histologic subtype of RS (~90%) is diffuse large B-cell lymphoma (DLBCL) and Hodgkin lymphoma is seen in approximately 10% of cases. PET scans can be helpful to localize the most hypermetabolic node for biopsy and also have a high specificity if the SUV max is ≥10. However, every attempt should be made to confirm the diagnosis through biopsy of the most FDG avid lymph node. Historically, outcomes for clonally related RS is poor, with most patients exhibiting chemorefractory disease. Clonally unrelated RS variants are treated similar to de novo diffuse large B-cell lymphoma and tend to have slightly better outcomes. Historically, RS has been treated with regimens similar to those used for the treatment of large-cell lymphomas involving multiple agents such as R-CHOP, OFAR, and R-EPOCH, etc. Outcomes remain dismal with any treatment modality with the best response rates of around 50% and median OS of less than 18 months seen with the use of venetoclax in combination with R-EPOCH, unless the patient can be salvaged with an allogeneic HSCT. This particular regimen is associated with substantial cytopenias but almost doubles the dismal survival seen with prior therapeutic modalities. Long-term remissions and survival have been reported in a few patients after allogeneic HSCT, but this approach is associated with a high TRM. When Richter transformation occurs, treatment on a clinical trial and consolidation with allogeneic HSCT is the preferred treatment. Newer treatments including kinase inhibitor combinations and checkpoint inhibitors have been used in small series with modest success.

Similar to RS, prolymphocytic transformation (PT) occurs in fewer than 10% of patients with CLL. PT is characterized by the appearance of large, immature prolymphocytes in the peripheral blood, which make up greater than 55% of the peripheral circulating malignant lymphoid cells. These patients may be older with advanced disease and have more pronounced lymphadenopathy and splenomegaly. PT is associated with a poor outcome, with limited survival beyond 1 year. These patients often have del(17p13.1) but do appear to respond to many of the novel therapies currently utilized for the treatment of CLL. Allogeneic HSCT or CART cell therapy may also be used as a potentially curative therapeutic option if targeted agents become ineffective.

Hodgkin lymphoma can also manifest as a form of Richter and may have a better outcome provided patients are treated appropriately. Identification of this diagnosis can sometimes be challenging due to its identification in BM, lower SUV readings on PET scan, and also more insidious onset as compared to DLBCL presentation of RS. Hodgkin lymphoma in the setting of CLL can be treated as de novo disease with some individuals attaining long-term remissions with ABVD or similar regimens (see Chapter 80). Unlike DLBCL, transformation of CLL to RS where reduced intensity allogeneic HSCT is recommended, we generally do not pursue this for individuals with Hodgkin lymphoma transformation.

Secondary Malignancies in Chronic Lymphocytic Leukemia

CLL is associated with an increased risk of secondary malignancies. These include not only hematologic malignancies such as MDS and AML associated with the use of chemotherapeutic agents but also solid tumors such as Kaposi sarcoma, malignant melanoma, and laryngeal and lung cancers. The increased incidence of secondary malignancies may be attributable to multiple reasons, including the immune dysfunction associated with CLL, the frequent infectious complications, the carcinogenic side effects of the various chemotherapeutic agents, and the increased and close medical surveillance that patients with CLL receive from trained oncologists. Hopefully, with the declining use of chemoimmunotherapeutic regimens the incidence of treatment-related myeloid malignancies will decline over time.

Hypersensitivity in Chronic Lymphocytic Leukemia to Mosquitoes and Insect Bites and Treatment

Patients with CLL commonly exhibit an exaggerated cutaneous response to insect bites. This was first reported in 1965 by Robert Weed, who documented a hypersensitivity reaction to insect bites in eight of 97 patients with CLL over a 13-year period. The reaction is characterized histologically by the presence of a dermal infiltrate composed of a mixed population of T and B cells, eosinophils, and eosinophilic granule protein. The extent of eosinophilic degranulation may also correlate with the severity of symptoms. Clinically, these patients present with recurrent, painful, bullous eruptions that may be traced to an insect bite in some instances. In limited cases, we have also observed that CLL patients have hypersensitivity to bed bugs, and this should be considered in the differential diagnosis. Identification and avoidance of known triggers may be useful in some cases, but most patients are unable to identify the inciting exposure. Treatment with a short course of steroids is usually effective, but these patients frequently relapse and may require multiple courses of therapy. Dapsone and chlorambucil may also be useful in severe, recurrent cases.

Other cutaneous conditions are also common in CLL patients, with up to 45% reporting some form of skin involvement. These include petechial, purpura, or ecchymotic lesions related to thrombocytopenia; infectious eruptions such as herpes simplex and zoster; and direct leukemic involvement in fewer than 10% of all patients with advanced disease.

INFECTIONS IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

Infectious complications remain the leading cause of morbidity and mortality in patients with CLL (see Chapter 94). The incidence of infectious complications has been estimated to be as high as 80% with a mortality rate of approximately 60%. Various factors contribute to the increased incidence of infectious complications in CLL, the most important being progressive disease affecting host immunity through an impaired antibody response and hypogammaglobulinemia; weakened host cellular immune responses, including impaired macrophage function; a decrease in T-regulatory cells; and, finally, the acquired defects after immunosuppressive chemotherapy.

Recent studies have examined predictors of severe infections in patients with CLL. In their retrospective analysis of infection-related mortality in 280 patients, advanced age, clinical stage B or C disease, unmutated IGHV, and positive CD38 status have been identified as independent predictors of both shorter time to first infection and infection-related mortality. Other risk factors that may also have an impact on development of infections include type of initial therapy and development of renal insufficiency.

Historically, sinopulmonary infections from encapsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae* have been the most common cause of infectious complications in patients with CLL. With the recent use of more potent cytotoxic chemotherapy and the resultant profound myelosuppression, an increased frequency of severe pulmonary infections, bacteremia, and gram-negative infections has been reported. Infections caused by atypical organisms such as *Listeria monocytogenes, Nocardia* spp., *Mycobacterium* spp., and *Neisseria meningitidis* are relatively infrequent in patients who receive conventional chemotherapy. Treatment for presumed infection should be initiated empirically in CLL patients who develop fever because fever in CLL patients usually indicates an active infection. Therapy should be tailored to the particular organ involved and the sensitivity of the organism. Prophylactic antibiotics can be initiated for debilitated patients with high-risk disease and significant immune dysfunction.

Viral infections are also commonly encountered in CLL patients (see Fig. 76.3D and E). Herpesvirus infections are especially common in patients treated with nucleoside analogs and alemtuzumab. Chronic, indolent oropharyngeal and circumoral herpes simplex virus (HSV) outbreaks are more frequent than aggressive, disseminated visceral disease. Reactivation of Epstein-Barr virus (EBV) has been implicated in some cases of Richter transformation. Other viruses may cause severe systemic disease in patients with CLL. Varicella zoster virus (VZV) can cause herpes zoster, herpetic neuralgia, and rarely meningoencephalitis; parvovirus B19 can cause severe polyarthritis and pure RBC aplasia; and JC polyoma virus has been implicated in the development of progressive multifocal leukoencephalopathy. Management of these infections depends on early recognition of disseminated viral disease, timely initiation of antiviral therapy in cases of HSV and EBV, and a low threshold for the introduction of prophylactic acyclovir and supportive therapy. All patients should be provided instruction to identify the signs and symptoms of herpes virus infection at the time of diagnosis of CLL.

Fungal infections are not typically observed in CLL patients in the absence of treatment with corticosteroids or other immunosuppressive therapy for autoimmune complications arising from CLL. Cryptococcal meningitis, pneumonia, and fungemia are well-recognized events in patients with CLL and are associated with significant morbidity and mortality. More cases of *Pneumocystis. jirovecii* pneumonia, systemic candidiasis, and aspergillosis were reported with the advent of combination nucleoside analog therapy with steroids. However, while these issues are still seen in patients treated with novel agents their incidence is much lower. Treatment of the infection is dictated by the identification of the particular organism. Trimethoprim–sulfamethoxazole is routinely used as effective prophylaxis against *P. jirovecii* infections, especially during and immediately after the use of nucleoside analogs. Fungal prophylaxis with posaconazole may also be used during protracted therapy with high-dose steroids to avoid invasive aspergillosis.

Prophylactic Strategies for Infections

Routine use of prophylactic antibiotics is generally not used for CLL despite the higher frequency of infections observed in patients with this disease. Early recognition of signs and symptoms of infection and prompt initiation of empiric broad-spectrum antibiotics is probably a more feasible and cost-effective approach. When chemoimmunotherapy is used that includes a nucleoside analog, prophylaxis for herpes simplex and varicella zoster should be used, particularly in older patients. Trimethoprim–sulfamethoxazole (or other alternative *P. jirovecii* pneumonia prophylaxis) should also be administered in this setting. Fortunately, kinase inhibitors result in continued decline in the incidence of infectious complications with ongoing therapy, primarily as a result of better disease control, and routine prophylactic antibiotics are either not required or can be weaned off in the majority of patients. We do however use acyclovir prophylaxis for all our patients on BTK and BCL2 inhibitors and both acyclovir and trimethoprim—sulfamethoxazole for patients on PI3K inhibitors.

Hypogammaglobulinemia is virtually always present in advanced CLL, and several studies have examined whether intravenous immunoglobulin (IVIG) replacement therapy can reduce the incidence and severity of infectious complications. Patients receiving IVIG in a double-blind, placebo-controlled trial experienced significantly fewer bacterial infections than the placebo group. The therapy was well tolerated with few adverse reactions, but there was no observed benefit in terms of preventing viral or fungal infections. However, other studies have shown an almost 50% reduction in the number of serious infections per year with IVIG infusions. Limited data support the use of IVIG at a higher dose of 600 mg/kg every 4 weeks to reduce the number and severity of respiratory infections. However, the prohibitive cost of IVIG therapy and the fact that it has not been shown to prolong survival argues against its empiric use in all patients. IVIG should be used judiciously and reserved for patients with advanced disease and recurrent infections. The usual dose used is 200 to 400 mg/kg every 4 to 6 weeks as needed, with the aim of keeping the trough serum IgG concentration greater than 500 mg/dL.

This is something our group routinely implements for refractory patients or those who have more than two infections requiring hospitalization in 1 year.

Among the strategies for preventing infections in this patient population is immunization. CLL patients, however, typically respond poorly to pneumococcal and influenza vaccines. Advanced age, advanced disease stage, hypogammaglobulinemia, and low levels of soluble CD23 influence the rate of responses to immunizations. Soluble CD23, a degradation product of membrane-bound CD23, is involved in several aspects of B-cell activation and proliferation and has a synergistic effect on histamine release. Histamine has a direct inhibitory effect on immunoglobulin production by B cells in vitro via histamine type-2 (H2) receptors and acts as an immune regulatory factor that can be modulated by H2-receptor antagonists. Thus responses to vaccines may be further enhanced by adjuvant treatment with H2 blockers. Studies have shown that response to protein-conjugated vaccines may be enhanced by ranitidine in CLL patients to as much as 90% compared with 43% in the control group. Unfortunately, this response is not seen with polysaccharide vaccines. Other studies have indicated that protein-conjugated vaccines may be more immunogenic, as shown by a more significant immune response to *H. influenzae* type b conjugate vaccine than to plain polysaccharide antigen. In light of the paucity of data on an appropriate immunization schedule, we suggest a modified immunization plan based on the recommendations of the Advisory Committee on Immunization Practices. This includes use of Prevnar-13 for pneumococcal prophylaxis and avoiding live vaccines, including the live attenuated VZV. The newer recombinant varicella vaccine is safe for use and modestly effective.

Growth factors such as G-CSF or granulocyte–macrophage colony-stimulating factor (GM-CSF) can be used prophylactically in high-risk, severely neutropenic patients to shorten the duration and severity of neutropenia.

AUTOIMMUNE COMPLICATIONS OF CHRONIC LYMPHOCYTIC LEUKEMIA

Patients with CLL have a greater predisposition to develop autoimmune hematologic complications, including AIHA, idiopathic thrombocytopenia purpura (ITP), and pure RBC aplasia. AIHA occurs in up to 37% of CLL patients at some time during the course of their disease. A small proportion (10% to 15%) of patients may present with AIHA at diagnosis (see Chapter 47). AIHA can have a varied presentation, with patients developing signs of anemia, including weakness, lethargy, dyspnea on exertion, and dizziness over a period of months. Examination may reveal pallor, jaundice, hepatosplenomegaly, and lymphadenopathy. Hemolysis can cause mild to moderate indirect hyperbilirubinemia, elevated LDH levels, and hemoglobinuria. The direct antiglobulin Coombs test result is positive in up to 74% of patients with CLL, but not all patients develop hemolysis. Most of the antibodies produced are warm reactive, but patients can occasionally present with cold agglutination syndrome. The antibodies are mostly polyclonal and usually a product of normal B cells rather than the leukemic clone.

In contrast to the high frequency of AIHA in CLL, ITP or ITP and AIHA, or Evans syndrome, occurs less frequently in CLL. These events can occur throughout the entire course of CLL and are much more difficult to diagnose because of the absence of multiple implicating laboratory features as seen with AIHA. Given the small number of patients with AIHA, there are no data from controlled trials to guide the management of AIHA. Glucocorticoids have been used since the 1940s and are considered the first line of therapy. Most patients will respond to prednisone at a dose of 1 mg/kg for 10 to 14 days followed by a slow taper over 2 to 3 months, depending on the extent of hemolysis. ORRs of up to 90%, with 65% CRs, have been reported with the use of steroids. Unfortunately, approximately 60% of patients relapse when steroid therapy is stopped. Rituximab 375 mg/m^2 weekly for 4 weeks is often effective for steroid-resistant AIHA or ITP and if steroid withdrawal is not possible. Because of the long-term morbidity of prolonged steroid administration in CLL and data demonstrating benefit to concurrent steroids and rituximab for rapid withdrawal of corticosteroids in ITP, our group gives these two concurrently at diagnosis of ITP or AIHA unless contraindicated (e.g., hepatitis B). IVIG, the next line of treatment, induces responses in 40% of patients for both AIHA and ITP. Thrombopoietin agonists are effective for refractory ITP in CLL and could also be considered at this time. Cyclosporin A (CSA) has been used for refractory AIHA and ITP of CLL at 5 mg/kg/day given in divided doses twice daily. The dose should be adjusted to maintain a serum level of around 100 to 150 μg/dL. Other treatment modalities include splenectomy or splenic irradiation, alkylating agents, or therapy (FCR or FR directed at the disease if active). Treatment of the underlying CLL is generally required for long-term control of autoimmune cytopenias. However, our practice is to first control the autoimmune process with steroids or other therapies before considering treatment of the underlying CLL. Supportive therapy with periodic RBC transfusions is also important in the management of AIHA. Ibrutinib has also been shown to reduce the incidence of autoimmune complications in patients who initiate therapy for progressive disease and provides an exciting option for these patients. Unfortunately, it has not been shown to effectively treat active AIHA.

Pure RBC aplasia (PRCA) is a relatively rare T cell–dependent complication associated with CLL, but an incidence as high as 6% has been reported (see Chapter 33). PRCA was first described by Dameshek and colleagues in 1967. It is characterized by a hypoproliferative anemia that can be detected even in early-stage CLL and is thought to be caused by cytotoxic effects of suppressor T cells on erythroid progenitor cells. Higher numbers of these CD3, CD8, and CD57 coexpressing cells have been shown to gradually accumulate in the BM of patients with PRCA. Abeloff and Waterbury described the first remission of PRCA to cyclophosphamide therapy in 1974, and Chikkappa and colleagues described the first case of response to CSA. Subsequent larger studies have shown a response rate as high as 63% with 300 mg/day of oral CSA. Mild reversible nephrotoxicity may warrant dose adjustment in some patients. Most patients exhibit a response by having reticulocytosis within the first 10 to 14 days, but maximal response may occur an average of 10 weeks after the start of therapy. However, steroid therapy at a dose of 1 mg/kg/day of prednisone remains the first line of treatment. If a response is not obtained in 4 weeks, CSA should be added to the regimen. Other agents that have shown promising activity in treating PRCA include rituximab, IVIG, alemtuzumab, and ATG. Packed RBC transfusions are usually indicated in patients who are clinically symptomatic from severe anemia.

A number of other complications, most of which are autoimmune in nature, have been reported in patients with CLL. These complications include paraneoplastic pemphigus, angioedema caused by acquired C1-inhibitor deficiency, and nephrotic syndrome. As a result of autoimmune involvement of various organs, patients with CLL may have abnormal serum chemistry profiles and liver function.

SARS-COV-2 AND CHRONIC LYMPHOCYTIC LEUKEMIA

The pandemic with the SARS-CoV-2 coronavirus that produces COVID-19 syndrome initially was devastating to CLL patients, with a mortality of over 30% (see Chapter 153). Knowledge about early management of infection with Spike protein directed neutralizing antibodies, dexamethasone, and other supportive care have improved outcome since the Spring of 2020. The advent of vaccine-based approaches for SARS-CoV-2 has dramatically reduced cases of COVID-19 in the US and other areas where this treatment approach is available. However, CLL patients have lower response to these vaccines as measured by development of Spike protein directed antibodies and therefore remain susceptible to infection, morbidity, and mortality from COVID-19 syndrome. This poor vaccine response seems to be enhanced by prior therapy with BTKi, CD20 antibodies, and venetoclax, with the latter two treatments almost uniformly predicting absent response to vaccine. Moving forward through the later part of the SARS-CoV-2 pandemic it is likely that this patient population will require other interventions such as neutralizing antibody therapy or enhanced vaccine strategies to prevent COVID-19 syndrome.

FUTURE DIRECTIONS

Advances in the molecular biology of CLL over the past two decades have translated into new diagnostic tests that allow better assessment of initial prognosis and also to assign treatment. Additionally, multiple new therapies have come forward, and progress is being made to extend remission duration, describe resistance mechanisms, develop stopping rules, and improve the quality of life of CLL patients. Additionally, medications and interventions to support complications that arise from CLL have also been introduced. Overall, the future for patients with CLL remains brighter based on these past efforts and likely will improve further with continued laboratory and clinical research ongoing in this disease. In particular, the kinase inhibitors have transformed treatment and outcome of patients with CLL and will continue to alter the natural history of the disease.

SUGGESTED READINGS

Bichi R, Shinton SA, Martin ES, et al. Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. *Proc Natl Acad Sci U S A*. 2002;99:6955–6960.

Burger JA. Treatment of chronic lymphocytic leukemia. *N Engl J Med*. 2020;383:460–473.

Byrd JC, Furman RR, Coutre SE, et al. Ibrutinib treatment for first-line and relapsed/refractory chronic lymphocytic leukemia: final analysis of the pivotal phase Ib/II PCYC-1102 Study. *Clin Cancer Res*. 2020;26:3918–3927.

Byrd JC, Wierda WG, Schuh A, et al. Acalabrutinib monotherapy in patients with relapsed/refractory chronic lymphocytic leukemia: updated phase II results. *Blood*. 2020;135:1204–1213.

Fischer K, Al-Sawaf O, Bahlo J, et al. Venetoclax and obinutuzumab in patients with CLL and coexisting conditions. *N Engl J Med*. 2019;380:2225–2236.

Hallek M, Cheson BD, Catovsky D, et al. iwCLL guidelines for diagnosis, indications for treatment, response assessment, and supportive management of CLL. *Blood*. 2018;131:2745–2760.

International CLL-IPI working group An international prognostic index for patients with chronic lymphocytic leukaemia (CLL-IPI): a meta-analysis of individual patient data. *Lancet Oncol*. 2016;17:779–790.

Kater AP, Levin MD, Niemann CU. Ibrutinib and venetoclax for first-line treatment of CLL. *N Engl J Med*. 2019;381:788–789.

Landau DA, Tausch E, Taylor-Weiner AN, et al. Mutations driving CLL and their evolution in progression and relapse. *Nature*. 2015;526:525–530.

Maity PC, Bilal M, Koning MT, et al. IGLV3-21*01 is an inherited risk factor for CLL through the acquisition of a single-point mutation enabling autonomous BCR signaling. *Proc Natl Acad Sci U S A*. 2020;117: 4320–4327.

Mato AR, Shah NN, Jurczak W, et al. Pirtobrutinib in relapsed or refractory B-cell malignancies (BRUIN): a phase I/II study. *Lancet*. 2021;397:892–901.

Montserrat E, Sanchez-Bisono J, Vinolas N, Rozman C. Lymphocyte doubling time in chronic lymphocytic leukaemia: analysis of its prognostic significance. *Br J Haematol*. 1986;62:567–575.

Oakes CC, Seifert M, Assenov Y, et al. DNA methylation dynamics during B cell maturation underlie a continuum of disease phenotypes in chronic lymphocytic leukemia. *Nat Genet*. 2016;48:253–264.

Sharman JP, Egyed M, Jurczak W, et al. Acalabrutinib with or without obinutuzumab versus chlorambucil and obinutuzmab for treatment-naive chronic lymphocytic leukaemia (ELEVATE TN): a randomised, controlled, phase III trial. *Lancet*. 2020;395:1278–1291.

Shanafelt TD, Wang XV, Kay NE, et al. Ibrutinib-rituximab or chemoimmunotherapy for chronic lymphocytic leukemia. *N Engl J Med*. 2019;381:432–443.

Stephens DM, Boucher K, Kander E, et al. Hodgkin lymphoma arising in patients with chronic lymphocytic leukemia: outcomes from a large multicenter collaboration. *Haematologica*. 2021;106(11):2845–2852.

Stephens DM, Byrd JC. How I manage ibrutinib intolerance and complications in patients with chronic lymphocytic leukemia. *Blood*. 2019;133: 1298–1307.

Turtle CJ, Hay KA, Hanafi LA, et al. Durable molecular remissions in chronic lymphocytic leukemia treated with CD19-specific chimeric antigen receptor-modified T cells after failure of ibrutinib. *J Clin Oncol*. 2017;35:3010–3020.

Woyach JA, Ruppert AS, Guinn D, et al. BTK(C481S)-mediated resistance to ibrutinib in chronic lymphocytic leukemia. *J Clin Oncol*. 2017;35: 1437–1443.

Woyach JA, Ruppert AS, Mandrekar SJ. Ibrutinib regimens in older patients with untreated CLL. Reply. *N Engl J Med*. 2019;380:1680–1681.

CHAPTER 77

HAIRY CELL LEUKEMIA

Farhad Ravandi

Hairy cell leukemia (HCL) is one of the diseases exemplifying the importance of the application of appropriate diagnostic techniques and the importance of treatment strategies to obtain the best treatment outcomes for the individual patient.[1] The disease was first described by Bouroncle and colleagues in 1958. The term *hairy cell leukemia* was first used to describe the disorder by Schreck and Donnelly in 1966 and was derived from the observation of hair-like projections from mononuclear cells, giving rise to a frayed cell surface appearance.

The evolution of therapeutic strategies in patients with HCL over the past 25 years has led to a significant change in the natural history of the disease. Using the currently available drugs, the majority of patients with this disease achieve complete response (CR), and the published survival curves from several large series are similar to those for appropriate age-matched individuals without the disease. At the same time, recent research efforts have led to a better understanding of the molecular mechanisms responsible for the disease pathogenesis. Several studies, using modern techniques, have demonstrated the persistence of minimal residual disease (MRD) after therapy with nucleoside analogs in the majority of patients, without consensus on the significance of such MRD. The role of monoclonal antibodies, naked or conjugated with toxins, in the management of HCL, as well as their ability to eradicate MRD, is under investigation. The possibility of such chemoimmunotherapy strategies, leading to further improvements in the outcome of patients with HCL, needs to be further investigated. More recently, the identification of the *BRAF* V600E mutation present almost universally has led to studies evaluating the role of BRAF-kinase inhibitors in this disease.

EPIDEMIOLOGY

HCL is an uncommon lymphoid malignancy, accounting for only 2% of lymphoid leukemias, with approximately 600 to 800 new patients diagnosed each year in the United States. The disease is more common in white people and occurs more frequently in men than women by a ratio of 4:1. The median age at diagnosis is reported by most studies to be 50 to 59 years. However, it is possible that the disease is underreported in the older population. The Swedish Cancer Registry has maintained records on the incidence of the disease for several decades and has shown a stable incidence since the 1980s. It reports the median age at diagnosis to be 62 years, suggesting less rigid diagnostic efforts in older patients.

HCL has been diagnosed in patients aged in their 20s and 30s but is exceptionally rare in children. Using data from the 17 population-based cancer registry areas in the United States, the National Cancer Institute's Surveillance Epidemiology and End Results program reported that the incidence of the disease was stable in the decades between 1978 and 2004, with a rapid rise in age-specific incidence ratios until approximately age 40 years and then at a slower pace beyond that age.

ETIOLOGY AND CELL OF ORIGIN

Several reports have suggested an association between development of HCL and exposure to benzene, organophosphorus insecticides, and other solvents. However, such association has not been confirmed by other reports. Exposure to radiation, wood dust, or agricultural chemicals, and a previous history of infectious mononucleosis have also been suggested as predisposing factors. However, a direct, causal association has not been established with any of these or other factors.

Morphologically and phenotypically, the cells in HCL have no resemblance to any of the normal stages of B-cell development and maturation; past studies have debated the cell of origin. Initially, because of morphologic and functional similarities between hairy cells and cells of the monocyte/macrophage system, HCL-tumor cells were thought to be derived from a cell transformed from the reticuloendothelium. Prior studies have demonstrated the rearrangement of the B-cell receptor (BCR) immunoglobulin genes in HCL, demonstrating the B-cell origin of the disease. Several studies have increased our understanding of the cell of origin in HCL, allowing for a better description of pathogenic mechanisms responsible for its development.[2]

In lymphoid cells, the analysis of the immunoglobulin variable region genes provides a tool for the delineation of the clonal history of cells at which lymphoid neoplasms originate, identifying whether antigen encountered by a normal mature B cell has resulted in somatic mutation (Fig. 77.1). This process occurs within the germinal centers (GCs) of lymph tissues and may be associated with isotype switching. The enzyme activation–induced cytidine deaminase (AICD) is critical for both processes. Tumor cells from various B-cell malignancies are arrested at a number of stages along normal B-cell differentiation, conserving the immunogenetic characteristics of the stage-specific cell. The analysis of immunoglobulin variable gene regions can provide information about whether the cell of origin has undergone somatic mutation and isotype switching. Several reports have clearly demonstrated that in more than 85% of patients with HCL, the tumor cells express switched immunoglobulin isotypes, and their rearranged variable region genes have undergone somatic mutations.[3] Furthermore, HCL cells express AICD, the enzyme that is critical for both processes.[3] This suggests that the cell of origin in the majority of HCL cases has transited through the GCs of peripheral lymphoid tissue, having undergone the GC reaction.[2] Of interest, in about 40% of cases of HCL, the leukemia cells express multiple immunoglobulin heavy (IGH)-chain isotypes with dominance of immunoglobulin G3 (IgG3) but only a single light chain, suggesting that clonally related, multiple isotypes coexist in single hairy cells. Therefore, this subset of the disease may be arrested at a point after isotype switching and before exit from the GC. However, other lines of evidence suggest a post-GC origin.[2]

Arons and colleagues have provided evidence that in the majority of patients with classic HCL (83% of 102 cases), the cell of origin is post-GC with a mutated IGH-chain variable region.[4] This is contrasted with historic data for patients with chronic lymphocytic leukemia, in which case, about half of the time the cell of origin is un-mutated. They also reported higher usage of certain immunoglobulin-gene families and a difference in mutational frequency among these genes.[4] Furthermore, by demonstrating that the mutations fulfilled predefined characteristics of a canonic and non-random event, Arons and colleagues provided further evidence suggestive of an antigen-driven process.

The post-GC origin of HCL is supported by gene-expression profiling studies, comparing HCL cells with cells from other lymphoid neoplasms and with naïve and memory B cells.[5] HCL patients displayed a homogeneous pattern of gene expression that was clearly

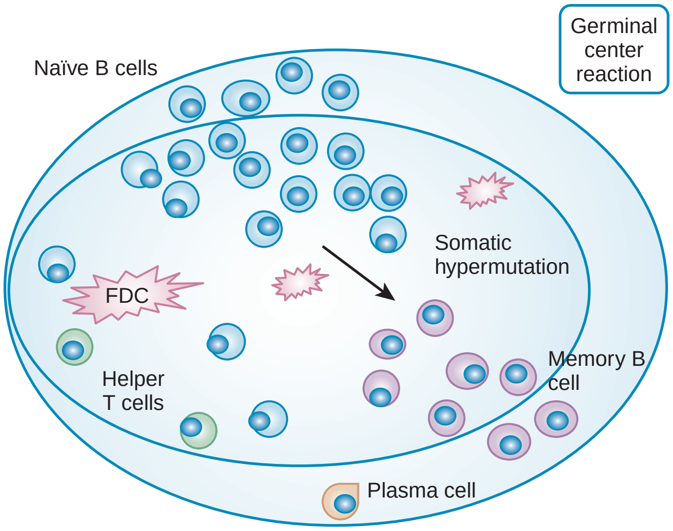


Figure 77.1 GERMINAL CENTER REACTION. The process of somatic hypermutation and isotypic switch in the germinal center. *FDC*, Follicular dendritic cells.

distinct from other B-cell lymphomas and was related to post-GC memory B cells.[5] Other investigators have reported this remarkably stable genome in HCL. Furthermore, when compared with memory B cells, HCL cells had a remarkable conservation of proliferation, apoptosis, and DNA metabolism programs but differed significantly in the expression of genes controlling cell adhesion and response to chemokines.[5] Against the hypothesis of a memory B-cell origin is the lack of expression by hairy cells of the memory B-cell marker, CD27. However, CD27-negative memory B cells have been described in humans, and hairy cells may lose this marker as a result of the neoplastic transformation.

As lymph node involvement in HCL is uncommon, the post-GC cell of origin is likely to originate from the spleen or the bone marrow, which are sites involved by the disease almost invariably. A number of reports have suggested that HCL may originate from the B cells of the splenic marginal zone (SMZ). Normal SMZ B cells are mainly memory B cells. Vanhentenrijk and colleagues, using comparative expressed sequence hybridization studies, demonstrated that hairy cells had an expression profile consistent with a splenic-expression signature that most likely reflected the expression profile of spleen-specific components, such as the sinusoidal-lining cells from the red pulp and the marginal-zone B cells from the white pulp.

Tiacci and colleagues reported the presence of *BRAF* V600E mutations in each of 47 patients with HCL and no mutations in the cells from patients with 195 peripheral B-cell lymphomas or leukemias.[6] Using whole-genome sequencing, in an index patient they identified five missense somatic clonal mutations, including a heterozygous mutation in *BRAF* that resulted in a *BRAF* V600E variant protein.[6] Since *BRAF* V600F is known to be oncogenic in other tumors, Tiacci and colleagues focused on this mutation and analyzed the subsequent 46 cases, as well as the patients with other lymphomas. They also demonstrated expression of phosphorylated MEK and extracellular signal-related kinase (ERK), showing constitutive activation of the RAF-MEK-ERK mitogen-activated, protein kinase pathway in HCL.[6] The presence of the *BRAF* V600E mutation in the hematopoietic stem cells (HSC) in patients with HCL, and the stable engraftment of *BRAF* V600E mutant HSC from a HCL patient into immunodeficient mice that results in a HCL-like disease are further indications of the central role of this mutation to the pathogenesis of HCL.[7] Other studies have further characterized the mutational landscape in HCL, with CDKN1B mutations found in one study to be the second most common mutated gene in HCL.[8]

CLINICAL PRESENTATION AND DIAGNOSIS

Typically, the majority of patients present with pancytopenia and splenomegaly with the associated fatigue, upper-left quadrant abdominal pain, fever, infections, and/or bleeding problems (Table 77.1). Common presenting features include significant anemia seen in up to 85% of patients, thrombocytopenia seen in about 60% to 80%, and leukopenia seen in 60% of patients; these cytopenias can be severe and life-threatening and are likely multifactorial, with hypersplenism and marrow infiltration being the more important contributors. Monocytopenia is a characteristic finding. Circulating hairy cells are typically scant in most patients or frequently absent. Hairy cells are small- and medium-sized lymphoid cells with an oval or indented (bean-shaped) nucleus and with less clumped homogeneous chromatin than in normal B cells (Fig. 77.2). Nucleoli are typically absent or inconspicuous, and the cytoplasm is typically abundant and pale blue in color with circumferential "hairy," projections. Electron micrographs of hairy cells clearly demonstrate their distinctive and complex surface features with multiple surface folds and clusters of short microvilli, creating an appearance unique to hairy cells (Fig. 77.3).

Bone marrow involvement can be interstitial or patchy. The infiltrate is characterized by widely spaced nuclei because of the abundant cytoplasm, giving rise to the commonly described "fried egg," appearance (Fig. 77.4). Occasionally, an increase in the bone marrow reticulin fibrosis, as well as significant loss of the hematopoietic elements, leads to a "dry tap." Bone marrow fibrosis is caused by the production and assembly of a fibronectin matrix by hairy cells and the

TABLE 77.1 Initial Workup of a Patient With Suspected Hairy Cell Leukemia

History and physical examination
Complete blood count with differential counts
Review of peripheral blood smear
Serum chemistries
Bone marrow aspirate and biopsy with immunostains
Immunophenotyping by flow cytometry of peripheral blood and bone marrow
? Serum soluble markers such as CD25 and CD22
? Immune status analysis with CD4/CD8 lymphocyte subsets
Appropriate imaging, if febrile, to rule out infections

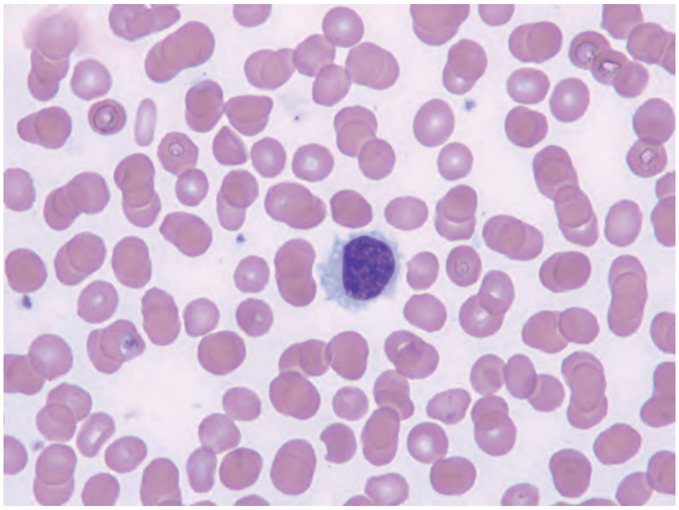


Figure 77.2 PHOTOMICROGRAPH OF A HAIRY CELL IN THE PERIPHERAL BLOOD. *(Provided by Jeffrey Jorgensen, Department of Hematopathology, UTMDACC.)*

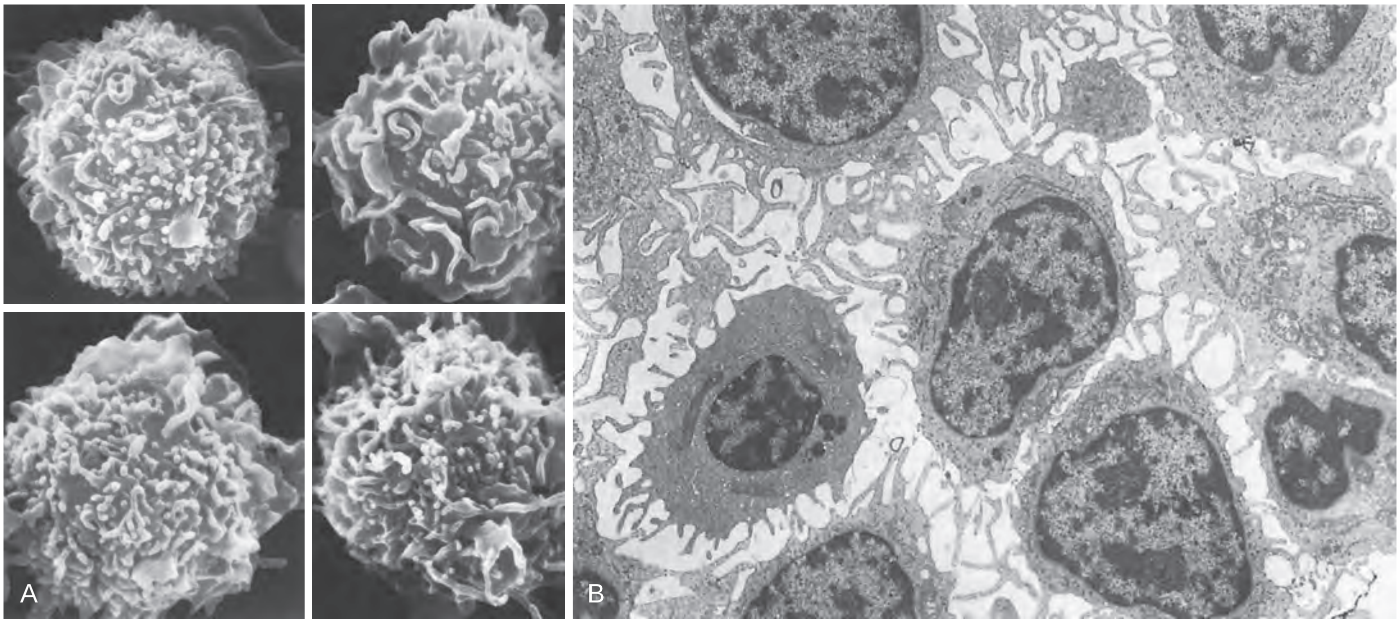


Figure 77.3 ELECTRON MICROGRAPH OF HAIRY CELLS. (A) Ruffles and folds on the surface. (B) Hair-like projections from the cytoplasm. *(Used with permission from Aaron Polliack, Emeritus Professor of Hematology, Hadassah University Hospital, Hebrew University Medical School, Jerusalem, Israel.)*

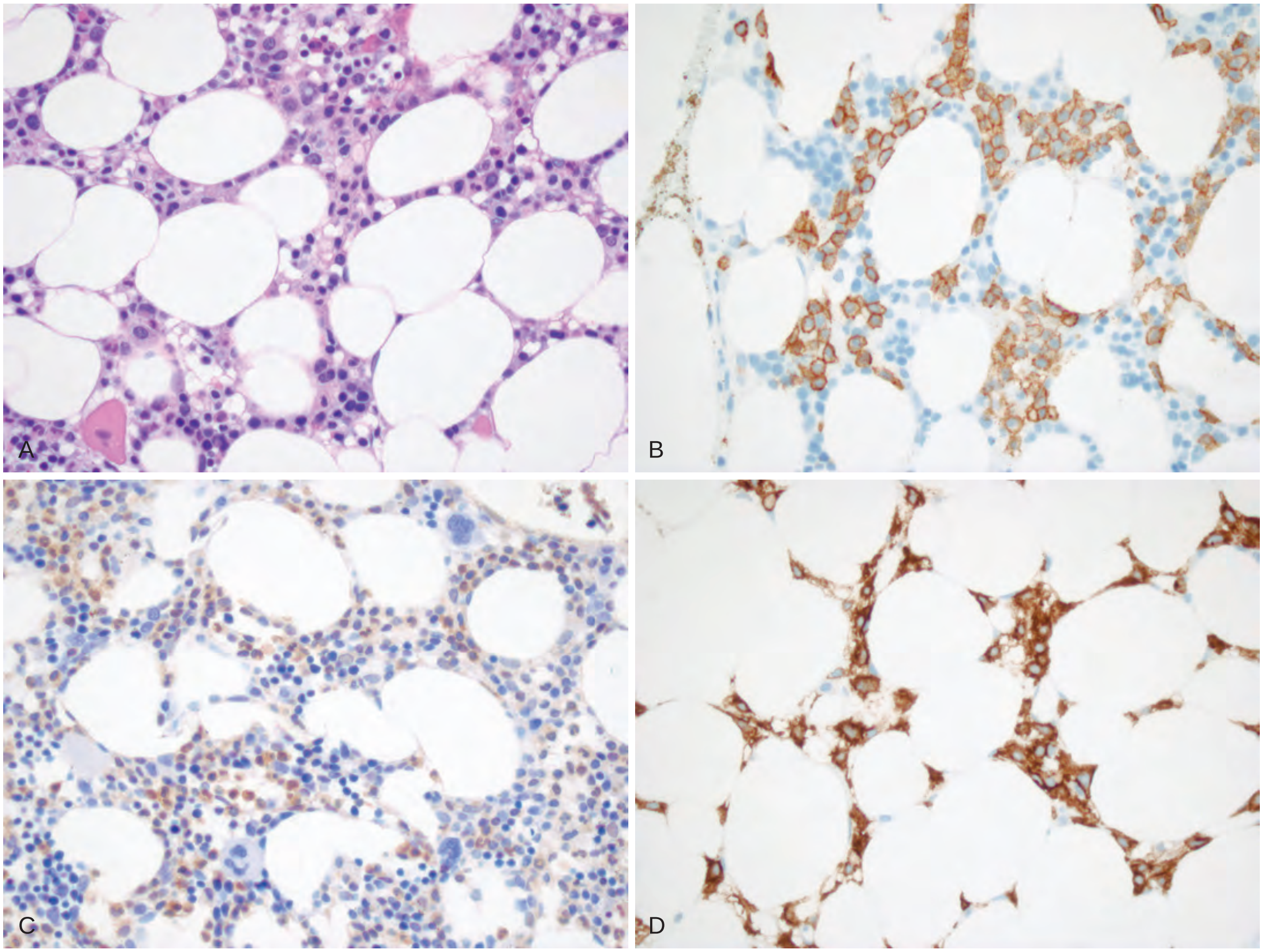


Figure 77.4 BONE MARROW FINDINGS IN HAIRY CELL LEUKEMIA. (A) Bone marrow biopsy (hematoxylin & eosin stain ×400). (B) Bone marrow biopsy (CD20 stain ×400). (C) Bone marrow biopsy (Annexin A1 stain ×400). (D) Bone marrow biopsy (CD11c stain ×400). *(Provided by Jeffrey Jorgensen, Department of Hematopathology, UTMDACC.)*

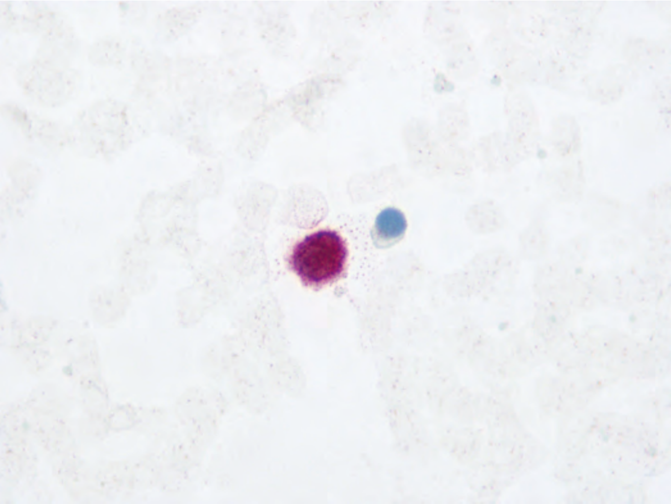


Figure 77.5 BONE MARROW ASPIRATE SMEAR (TRAP STAIN ×1000). *(Courtesy Jeffrey Jorgensen, Department of Hematopathology, UTMDACC.)*

deposition of fine reticulin fibers (mainly composed of type III collagen fibrils) by fibroblasts.[2] Hairy cells express isoenzyme 5 of acid phosphatase, which imparts resistance to treatment with tartaric acid, resulting in virtually all cases being positive for tartrate-resistant acid phosphatase (TRAP) (Fig. 77.5). Combined expression of DBA44 and TRAP by immunohistochemical analysis is highly specific and useful for arriving at the diagnosis. More recently, immunostaining for annexin A1 (ANAX1) has been reported to be very specific for HCL.[9] ANAX1 can be used to distinguish HCL from its variant form and from other lymphoid neoplasms, such as splenic marginal zone lymphoma (SMZL).[9] Cyclin D1 (encoded by *CCND1* gene) is frequently expressed, but this is not secondary to translocation involving *CCND1*, unlike mantle cell lymphoma. In a proportion of patients, the bone marrow is hypocellular with the loss of hematopoietic elements, which can result in an erroneous diagnosis of aplastic anemia. Immunostaining for antigens, such as CD20, may be helpful to detect the abnormal B-cell infiltrate, hence prompting more specific stains for HCL.

HCL cells have a characteristic immunophenotype, with flow cytometry being an important element of diagnostic evaluation in this disease (see Table 77.1). Hairy cells strongly express CD45 and gate within the monocytic region when analyzed by CD45 versus side scatter, which is typically devoid of monocytes. They exhibit a mature B-cell phenotype and commonly express one or more heavy chains and monotypic light chains (κ and λ light chains in equal numbers of patients). They express B-cell-associated antigens CD19, CD20, CD22, FMC7, and CD79b but typically lack CD5, CD10 (positive in about 10%), and CD23 (positive in about 20%) expression. No single marker is specific for distinguishing HCL from other B-cell neoplasms; however, the antigens CD11c, CD103, CD123, as well as the interleukin (IL)-2 receptor α-subunit (CD25), are typically expressed in HCL. Bright expression of CD22 and CD20 is also seen, which can be important therapeutically. Among the patients evaluated in one study, CD52 was also universally expressed.

Splenic enlargement is present in the majority of patients and can be massive in about 20% of cases. Splenic involvement is characterized by diffuse infiltration of the red pulp cords and sinuses, with atrophy and replacement of white pulp. Blood-filled sinuses lined by hairy cells (often referred to as *pseudosinuses* or *red blood lakes*) are often a prominent but not a pathognomonic finding (Fig. 77.6). Significant lymphadenopathy is uncommon and present only in the advanced stages of the disease. When involved, the lymph node enlargement is largely confined to the abdominal and retroperitoneal nodes. The infiltrates are distributed in the interfollicular and paracortical areas of the nodes and may extend through the capsule to the surrounding adipose tissue. Hepatomegaly is much less frequent, occurring in up to a third of patients. However, the liver is almost always involved with a mononuclear cell infiltrate in the sinusoids, portal areas, or both. Unusual sites of disease involvement have been reported, including mediastinal and paravertebral masses, skeletal lytic lesions, pleural effusions, and ascites, as well as involvement of the skin, the eye, the central nervous system, and the gastrointestinal tract. Other notable clinical features include a predisposition to infections and an uncommon association with autoimmune disorders, such as polyarteritis nodosa, vasculitis, and rheumatoid arthritis.

Several cytogenetic abnormalities have been reported in HCL, but no single abnormality is present consistently. Few cytogenetic studies have been reported because of the rarity of the disease, difficulty in obtaining marrow samples, and low responsiveness of hairy cells to common mitogens. In the reported series, chromosomes 1, 2, 5, 6, 11, 14, 19, and 20 are most frequently involved, with chromosome 5 and 14 abnormalities predominating. Deletions and mutations of p53, as well as overexpression of cyclin D1, have been reported. Lack of reciprocal, chromosomal translocations in HCL is consistent with a memory B-cell origin of the disease because these

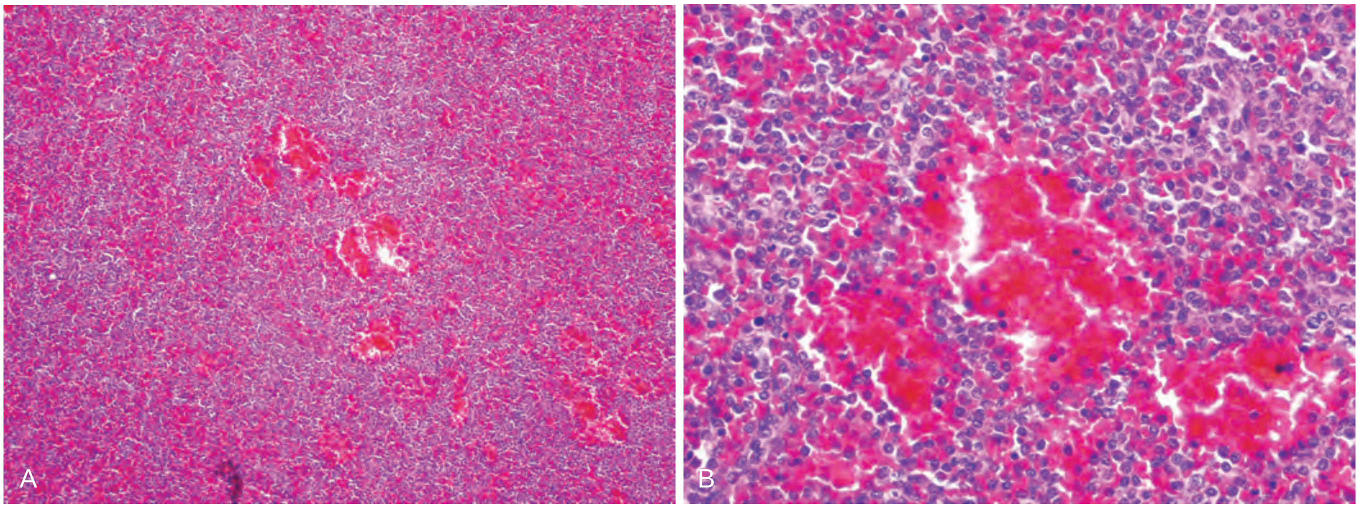


Figure 77.6 PATHOLOGIC FINDINGS IN THE SPLEEN. (A) Hematoxylin and eosin (H&E) stain (×100). (B) H&E stain (×400). *(Provided by Roberto Miranda, Department of Hematopathology, UTMDACC.)*

translocations are thought to arise from mistakes in the immunoglobulin remodeling mechanisms, which are believed to be turned off in memory B cells.[2]

DIFFERENTIAL DIAGNOSIS

HCL must be distinguished from other indolent lymphoid neoplasms, such as B-prolymphocytic leukemia and SMZL, and most notably, from HCLv, the variant form of the disease. HCLv is a rare disorder accounting for approximately 10% of cases and occurring in an older population, with the median age being 71 years.[10] No reports exist of an association with exposure to carcinogens, radiation, or viral infections, and no specific underlying cause has been described. Patients often have an elevated WBC count ($>10 \times 10^9$/L), including atypical hairy cells with prolymphocytic features and lack monocytopenia. Splenomegaly and cytopenias are present in the majority of patients. The patterns of bone marrow and splenic involvement is similar to HCL and is different from prolymphocytic leukemia and SMZL. The immunophenotypic expression of various lymphoid markers is also different in these disorders, with HCLv lacking CD25 expression, further assisting diagnosis (Table 78.2). Another aid in distinguishing HCL from HCLv is CD123 expression, which is positive in the former and negative in the latter. A number of chromosomal abnormalities, including translocations, have been reported in a few cases. Although HCLv has some similarities to HCL, the two conditions differ in a number of features, most notably being HCLv's lack of responsiveness to classic HCL therapies. As such, it is important to distinguish the two and consider them as separate diseases. HCL and HCLv have different IGH-chain gene repertoires and somatic hypermutation patterns.[4] A recent report also suggested that high expression levels of AICD can distinguish HCL from HCLv and from SMZL. However, the same group was unable to find distinct cytogenetic events to distinguish HCL from its variant, using high-resolution genomic profiling. Most recently, *BRAF* V600F mutations have been shown to occur exclusively in HCL samples and not in those from patients with HCLv and SMZL.

SMZL (previously referred to as *splenic lymphoma with villous lymphocytes [SLVL]*) exhibits some of the clinical and morphologic features of HCL but typically has a more prominent peripheral blood involvement, lacks TRAP expression, and has a different immunophenotype, including absence of expression of CD25 and CD103. HCL should be distinguished from other indolent lymphoid neoplasms, such as chronic lymphocytic leukemia, prolymphocytic leukemia, and follicular lymphomas, but this distinction is typically easily made using the characteristic, morphologic, and immunotypic findings of these disorders (see Table 77.2). Other disorders that should be included in the differential diagnosis include aplastic anemia, primary myelofibrosis, and systemic mast cell disorders.

TREATMENT

Indications for Therapy

International consensus guidelines for the diagnosis and management of patients with classical HCL has been recently published.[1] HCL has an indolent course. Some patients survive many years without receiving therapy. Others do not require further therapy despite persistence of residual morphologic evidence of the disease after the initial treatment.[11] Therefore a watch-and-wait strategy may be appropriate in the initial management of patients with limited or no manifestations of the disease. Disease progression that necessitates therapy is commonly evident by the development of progressive cytopenias and their associated complications, such as infections, bleeding, and progressive fatigue. No specific criteria for therapy have been established, but generally treatment is indicated when the patient has significant cytopenias, symptomatic organomegaly, adenopathy, infections, or constitutional symptoms, such as fever, night sweats, or fatigue. Typical blood counts warranting therapy include an absolute neutrophil count less than 1.0×10^9/L, a platelet count less than 100×10^9/L, and/or a hemoglobin less than 12.0 g/dL.

Historic Aspects of Therapy

Progress in the treatment of patients with HCL over the last 25 years has been significant, with survival curves now approaching those of age-matched cohorts without the disease. Before the introduction of interferon (IFN)-α, splenectomy was used effectively to treat patients with HCL. Although splenectomy does not result in morphologic remissions in the bone marrow, the peripheral blood counts are normalized in up to 70% of patients. Progression of disease can be expected in about 45% within 5 years. Furthermore, the procedure is associated with morbidity and mortality. As such, splenectomy is no longer performed in this disease except in rare, selected patients.

The first report of effective treatment of HCL, with IFN-α, described several patients with progressive disease who received daily doses of 3 million units. Three patients achieved CR with the other four having partial responses (PR). The activity of IFN-α in HCL was further confirmed by several large trials reporting CR rates of 4% to 30% and PR rates of 43% to 86%. Even in patients achieving a CR, careful morphologic examination of the bone marrow revealed residual hairy cells. After further follow-up of patients treated with IFN-α, it became clear that a significant proportion would relapse and require further therapy. However, the same authors reported that a number of patients remaining alive after a 10-year follow-up had not required further therapy with interferon. The median failure-free survival reported by these and other investigators ranges from 6 to 25 months. A number of predictors of outcome have been evaluated, with expression of CD5 reported as a predictor of poor response.

TABLE 77.2 **Immunophenotype of Hairy Cell Leukemia and Other Indolent Lymphoid Neoplasms**

Disease	sIg	CD5	CD10	CD11c	CD20	CD22	CD23	CD25	CD103
HCL	+/–	–/+	–	++	+	+	–/+	+	++
CLL	+/–	++	–	–/+	+/–	–/+	++	–/+	–
B-PLL	++	+	–	–/+	+/–	+	+/–	–	–
HCLv	+/–	–	–	++	+	+	–	–	–/+
MCL	+	++	–	–	+	+	–/+	–	–
SMZL	+	–/+	–/+	+	+	+/–	–/+	–	–
FL	+	–	+	–	++	+	–/+	–	–

B-PLL, B-prolymphocytic leukemia; *CLL*, chronic lymphocytic leukemia; *FL*, follicular lymphoma; *HCLv*, variant form of hairy cell leukemia; *MCL*, mantle cell lymphoma; *sIg*, surface immunoglobulin; *SMZL*, splenic marginal zone lymphoma.

The precise mechanism of action of IFN-α in HCL is unknown and may be related to the reduced production of a number of cytokines, such as granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, IL-3, and IL-6. Others have demonstrated that IFN-α can mediate apoptosis of hairy cells through the effects of tumor necrosis factor-α.

Treatment with IFN-α is associated with significant toxicity including flu-like symptoms, anorexia, fatigue, nausea, vomiting, diarrhea, skin rash, peripheral neuropathy, and dysfunction of the central nervous system (such as depression and memory loss). These adverse events and the introduction of nucleoside analogs that are generally more effective in achieving responses has led to the use of interferon being limited to rare cases and special circumstances.

Purine Nucleoside Analogs

Although there are no specific guidelines for the treatment of HCL, monotherapy with cladribine or pentostatin is the current established standard. Furthermore, despite the lack of a comparative study, a substantial amount of data suggests that both drugs are equally effective in terms of response rate and durability.[12] The use of nucleoside analogs in treating lymphoid neoplasms can be traced back to the observation that children with the deficiency of the enzyme adenine deaminase (ADA) developed severe combined immunodeficiency. The accumulation of the triphosphorylated form of deoxyadenosine (dependent on the action of the enzyme deoxycytidine kinase [DCK]) is thought to be responsible for the lack of lymphocyte development. Similarly, after treatment with purine nucleoside analogs, the accumulation of deoxyadenosine triphosphate results in DNA strand breaks, inhibition of DNA repair, and apoptosis preferentially in lymphoid cells that are rich in DCK and low in 5′ nucleotidase (the enzyme responsible for degrading deoxyadenosine monophosphate).

Cladribine is a purine nucleoside analog resistant to deamination by ADA. It accumulates in lymphocytes rich in DCK and inhibits ribonucleotide reductase, impairing DNA synthesis and repair. Cladribine has been very effective for the initial therapy of HCL. The overall response rates range from 75% to 100% after a single course of the drug (Table 77.3). Piro and colleagues first reported 12 patients with HCL who received cladribine 0.1 mg/kg per day by intravenous continuous infusion for 7 days.[13] Eleven patients achieved CR, and one had PR.[13] Since then, other doses, routes of administration, and schedules of the drug have been used effectively (see Tables 77.3 and 77.4). In a pilot study, weekly administration was evaluated in severely neutropenic patients in an effort to produce a less toxic strategy.[14] The authors reported a 73% CR rate and an overall response rate of 100%. However, only 16% developed severe neutropenia and only 8% developed infections.[14] In another report, 132 patients with untreated HCL were randomized to receive cladribine either on 5 consecutive days or a novel schedule of six weekly doses.[15] The response rates, overall response rates, progression-free, overall survival, and incidence of grade 3 and 4 infections were the same in the two arms.[15] The subcutaneous route was also evaluated after the initial demonstration that similar plasma levels of those seen with the intravenous route can be achieved subcutaneously. Juliusson and colleagues treated 73 patients with cladribine daily administered as a subcutaneous injection for 7 days and reported a CR rate of 75% after one course and 81% after two courses.[16]

Despite the very high response rate to cladribine, responses are not universal and a significant proportion of patients suffer from relapse. Saven and colleagues reported the long-term outcome of 358 patients with HCL who were followed for a median of 52 months.[17] Of these patients, 26% relapsed after a median of 29 months.[17] The same group reported on 209 patients treated with cladribine with a follow-up period of at least 7 years.[18] Although the overall response rate was 100%, 76 (37%) patients relapsed after their first course of cladribine with a median time to relapse of 42 months.[18] Notably, the time-to-treatment failure curve did not show a plateau, indicating that the treatment is probably not "curative."[18] Outcome of patients achieving CR with their initial therapy is better than those achieving PR (Fig. 77.7). Additional publications have confirmed the high response rate of patients with HCL treated with cladribine and have provided long-term follow-up data.[19] Jehn and colleagues described a 12-year follow-up of 44 patients (including 11 with prior therapy before receiving cladribine) who received cladribine at the originally

TABLE 77.3 Selected Published Reports of Cladribine Therapy for Hairy Cell Leukemia

Reference	Evaluable Patients (*n*)	CR (%)	PR (%)	OR (%)
Intravenous Daily Administration (Continuous or Pulsed)				
Saven[17]	349	91	7	98
Goodman[18]	207	95	5	100
Tallman	50	80	18	98
Chadha[19]	85	79	21	100
Hoffman	49	76	24	100
Seymour [35]	46	78	11	89
Dearden	45	84	16	100
Juliusson	16	75	0	75
Zinzani[a]	21	81	19	100
Jehn	44	98	2	100
Robak[b,15]	132	76	19	95
Intravenous Weekly Administration				
Lauria	30	73	27	100
Zinzani[a]	16	81	19	100
Robak[b,15]	57	72	19	91
Subcutaneous Administration				
Juliusson[16]	73	81	13	94
Forconi[22]	58	72	19	91

[a] Reports from the same publication.
[b] Numbers reported for the two arms of a randomized study.
CR, Complete response; *OR*, overall response; *PR*, partial response.

TABLE 77.4 Alternative Dose and Schedules of Cladribine Therapy Reported in the Literature

Study	Dosing	Route of Administration	Responses
Saven[17]	0.1 mg/kg/day × 7 days	Continuous IV infusion	CR: 91%; PR: 7%
Juliusson[16]	3.4 mg/m²/day × 7 days	Subcutaneous injection	CR: 75% after 1 cycle, 85% after 2 cycles
Robak[15]	0.12 mg/kg/day × 5 days	2 h IV bolus	CR: 76%; PR: 19%
Robak[15]	0.12 mg/kg/week × 6 weeks	2 h IV bolus	CR: 72%; PR: 19%
Chacko	0.15 mg/kg/week × 6 weeks	3 h IV infusion	CR: 100%
Lauria[14]	0.15 mg/kg/week × 6 weeks	2 h IV bolus	CR: 76%; PR: 24%
Von Rohr	0.14 mg/kg/day × 5 days	Subcutaneous injection	CR: 76%; PR: 21%

CR, Complete response; *IV*, intravenous; *PR*, partial response.

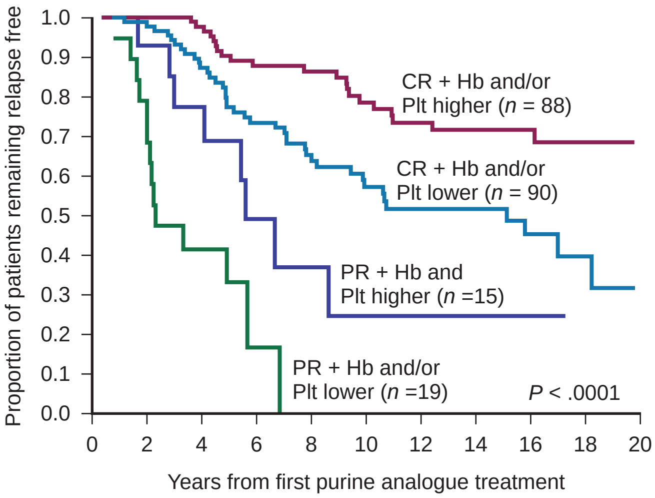


Figure 77.7 LONG-TERM FOLLOW-UP OF PATIENTS TREATED WITH NUCLEOSIDE ANALOG MONOTHERAPY. Excellent outcome for the majority, and significantly better relapse-free survival for patients achieving complete (CR) versus partial response (PR) with initial therapy. *(From Else M, Dearden CE, Matutes E, et al. Long-term follow-up of 233 patients with hairy cell leukaemia, treated initially with pentostatin or cladribine, at a median of 16 years from diagnosis. Br J Haematol. 2009;145:733.)*

reported dose and schedule. The CR rate was 98%, the median follow-up was 8.5 years (range 0.1 to 12.2), and 17 patients had relapsed. Eight of nine patients retreated with cladribine responded again. The overall survival at 12 years was 79%. Zinzani and colleagues reported the long-term outcome of 37 patients treated with one of two regimens of cladribine. Twenty-one patients received cladribine by a 2-hour infusion for 5 days, whereas 16 patients were treated with a once-weekly schedule for 5 weeks. A CR rate of 81% and an overall response rate of 100% was reported, with no difference between the two schedules. After a median follow-up of 122 months (range 54 to 156), the overall relapse rate was about 30% for both groups. The projected overall 13-year and relapse-free survival rates were 96% and 52%, respectively. Investigators at Northwestern University treated 86 consecutive patients with cladribine and reported a CR rate of 79%, as well as a PR rate of 21%.[19] The progression-free survival after 12 years was 54%. After a median follow-up of 9.7 years (range 0.3 to 13.8), 31 patients (36%) relapsed.[19] Of these, 23 were treated with a second course of cladribine, 12 (52%) achieved CR, and 7 (30%) achieved PR. The overall survival after 12 years was 87%. The authors suggested that the lower CR rate in this study was caused by their more stringent criteria for response, which included a requirement for resolution of splenomegaly and lymphadenopathy by computed tomography scan as criteria for CR.[19]

Similar excellent responses have been achieved using pentostatin (Table 77.5). Overall CR rates of 44% to 89% have been reported, with pentostatin administered intravenously at a dose of 2 to 4 mg/m^2 every 2 weeks. Spiers and colleagues were the first to report that a nucleoside analog (pentostatin) was capable of producing CRs in patients with HCL. The activity of pentostatin in HCL was confirmed in a number of larger studies by several investigators. Grever and colleagues conducted a large, randomized clinical trial comparing pentostatin with IFN-α in patients with previously untreated HCL.[20] Patients were randomized to receive either 3 million units of IFN-α subcutaneously three times per week or pentostatin 4 mg/m^2 intravenously every 2 weeks.[20] Patients who did not respond to initial treatment were crossed over. Confirmed complete and overall response rates were reported for 76% and 79% of patients treated with pentostatin, respectively, as compared with 11% and 38% of those treated with IFN-α.[20] Response rates were significantly higher ($P < .0001$), and relapse-free survival was significantly longer with pentostatin than with interferon ($P < .0001$).[20] Furthermore, patients who were initially assigned to receive interferon were frequently crossed over to pentostatin therapy, achieving a CR rate of 66%. In a follow-up report, Flinn and colleagues described the long-term outcome of 241 patients who were treated with pentostatin either as initial therapy or after failure of IFN-α.[21] The 5- and 10-year event-free survival rates were 85% and 67% respectively (Fig. 77.8). Other investigators have reported long-term follow-up data on patients treated with pentostatin. Maloisel and colleagues reported an outcome of 230 evaluable patients with HCL, treated with pentostatin, including 84 with pentostatin as the initial agent. They reported a CR rate of 79%, with an overall response rate of 96%. With a median follow-up of 63.5 months, 34 (15%) of 220 responding patients had relapsed. The estimated 5- and 10-year disease-free survival was 88% and 69%, respectively, and the estimated 5-year overall survival was 89%.

TABLE 77.5 **Selected Published Reports of Pentostatin Therapy for Hairy Cell Leukemia**

Reference	Evaluable Patients (*n*)	CR (%)	PR (%)	OR (%)
Kraut	23	87	4	91
Johnston	28	89	11	100
Dearden	165	82	15	97
Catovsky	148	74	22	96
Grever[20]	154	76	3	79
Rafel	78	72	16	88
Ribeiro	49	44	52	96
Maloisel	230	79	17	96

CR, Complete response; *PR*, partial response; *OR*, overall response.

There are no prospective trials comparing the efficacy and durability of response between cladribine and pentostatin. Dearden and colleagues examined the outcome of the patients with the two agents at their institution and reported that 82% of 165 patients treated with pentostatin achieved a CR, compared with 84% of 45 patients treated with cladribine. Relapse rates were 24% with pentostatin and 29% with cladribine, after a median follow-up of 71 and 45 months, respectively. These relapse rates suggest a longer duration of remission with pentostatin. However, with further follow-up, there appears to be no difference between the two agents, with regard to disease-free survival (Fig. 77.9A). Further follow-up data of these cohorts of patients have been reported.[12] With a median follow-up of 16 years, no significant difference existed in the outcome of the patients treated with the two drugs. After relapse or non-response, patients could be successfully retreated with pentostatin or cladribine but achieved lower response rates, with a decline in the proportion of patients achieving CR with second- and third-line therapy (see Fig. 77.9A).[12] However, CRs were equally durable after first, second, or third line of treatment. Complete responders and those with pretreatment hemoglobin greater than 10.0 g/dL and platelet count greater than 100 × 10^9/L, had the longest relapse-free survival ($P < .0001$) (Fig. 77.10).[12] Patients who were in CR after 5 years had only a 25% risk for relapsing by 15 years.[12] These data suggest that both pentostatin and cladribine are highly effective in treating patients with HCL but not curative in all patients. Few predictors of response and long-term outcome have been reported, but the demonstration by two groups that patients with unmutated IGH-chain variable *(IGHV)* region gene were less likely to respond to cladribine suggests that further insight into the biology of the disease may potentially clarify mechanisms of resistance (Fig. 77.11).[22,23]

Monoclonal Antibodies

Several therapeutic options are now available for patients with relapsed HCL. The efficacy of the monoclonal antibody, rituximab, in treating

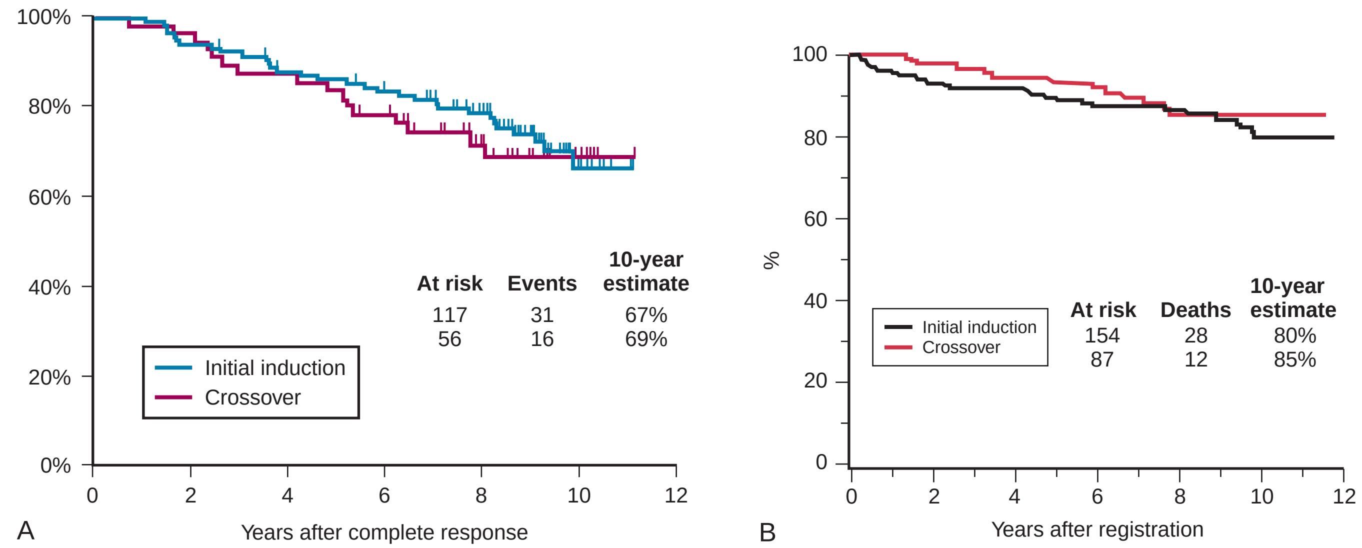


Figure 77.8 LONG-TERM FOLLOW-UP OF PATIENTS TREATED WITH PENTOSTATIN FOR THEIR INITIAL THERAPY OR FOLLOWING FAILURE OF INTERFERON-A AS A PART OF THE RANDOMIZED INTERGROUP STUDY. (A) Relapse-free survival by phase of treatment. (B) Overall survival by phase of treatment. *(From Flinn IW, Kopecky KJ, Foucar MK, et al. Long-term follow-up of remission duration, mortality, and second malignancies in hairy cell leukemia patients treated with pentostatin. Blood. 2000;96:2981.)*

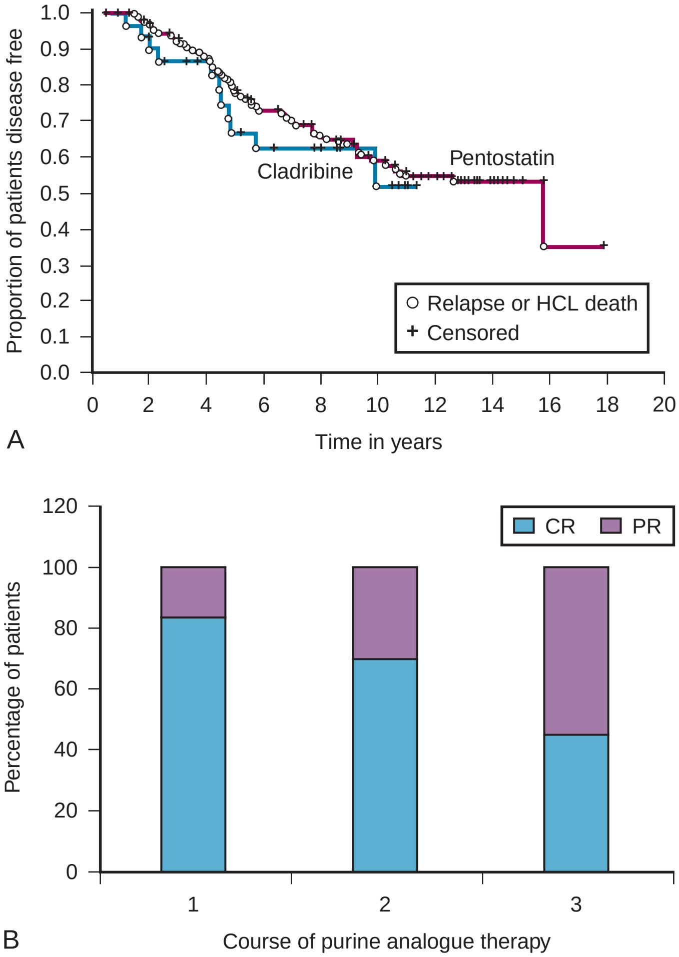


Figure 77.9 LONG-TERM FOLLOW-UP OF PATIENTS TREATED AT THE ROYAL MARSDEN HOSPITAL. (A) Patients who received either cladribine or pentostatin as their initial therapy. (B) Proportion of patients achieving CR and PR with first-, second-, and third-line therapy. *CR*, Complete response; *HCL*, hairy cell leukemia; *PR*, partial response. *(From Else MA, Ruchlemer R, Osuji N, et al. Long remissions in hairy cell leukemia with purine analogs. Cancer. 2005;104:2442.)*

these patients has been suggested by a number of studies (Table 77.6). Rituximab is a monoclonal antibody directed against the pan–B-cell antigen (CD20), which is heavily expressed on the surface of hairy cells. Nieva and colleagues reported their experience with 4 weekly doses of rituximab in 24 patients with HCL who had failed prior treatment with cladribine. The overall response rate was 26%, with 13% CR and 13% PR. No unusual toxicity was reported. Lauria and colleagues treated 10 patients with relapsed/progressed HCL, with 4 weekly doses of rituximab and reported one CR and four PR (overall response rate of 50%). Hagberg and colleagues treated 11 patients with HCL (including three previously untreated patients) with the same regimen of rituximab for 4 weeks. The response rate was 64%, with six CRs and one PR (including one CR in an untreated patient). The Swiss Group for Clinical Cancer Research reported their study of 4 weekly doses of rituximab in 26 patients with relapsed/progressed HCL, showing an 80% response rate, with 32% achieving CR. Thomas and colleagues used an extended regimen of rituximab (375 mg/m^2 weekly for eight doses) to treat 15 patients with relapsed/refractory HCL and reported a response rate of 80%, including eight (52%) CR, two (13%) CR with residual marrow disease, and two (13%) PR.[24]

Rituximab has been evaluated in a sequential strategy to improve responses in patients with HCL, after treatment with cladribine.[25] With a median follow-up of 60 months, a 100% CR rate in both untreated and relapsed HCL and a 5-year failure-free survival of 95% and 100%, respectively,[26] were reported. Rituximab has also been used concomitantly to treat patients with refractory disease, with reported excellent responses, suggesting a possible synergy. The investigators from the Royal Marsden NHS Trust treated 18 patients with either pentostatin ($n = 12$) or cladribine ($n = 6$) in combination with rituximab, after a median of two (range, 1 to 6) prior treatments and reported a CR rate of 89%. Toxicity with the combination was minimal and with a median follow-up of 36 months, all 16 patients who had achieved CR remained in CR. A randomized phase II study compared concomitant or delayed rituximab in 68 previously untreated patients with HCL. They were randomized to receive cladribine with 8 weekly doses of rituximab started on day 1 or at 6 months if the patients were MRD positive.[27] At 6 months, CR rates were 100% versus 88% in patients receiving concomitant therapy versus cladribine monotherapy. MRD-free CR rates were 97% versus 24%, respectively ($P < .0001$). Rituximab is also being evaluated in combination with BRAF inhibitors.

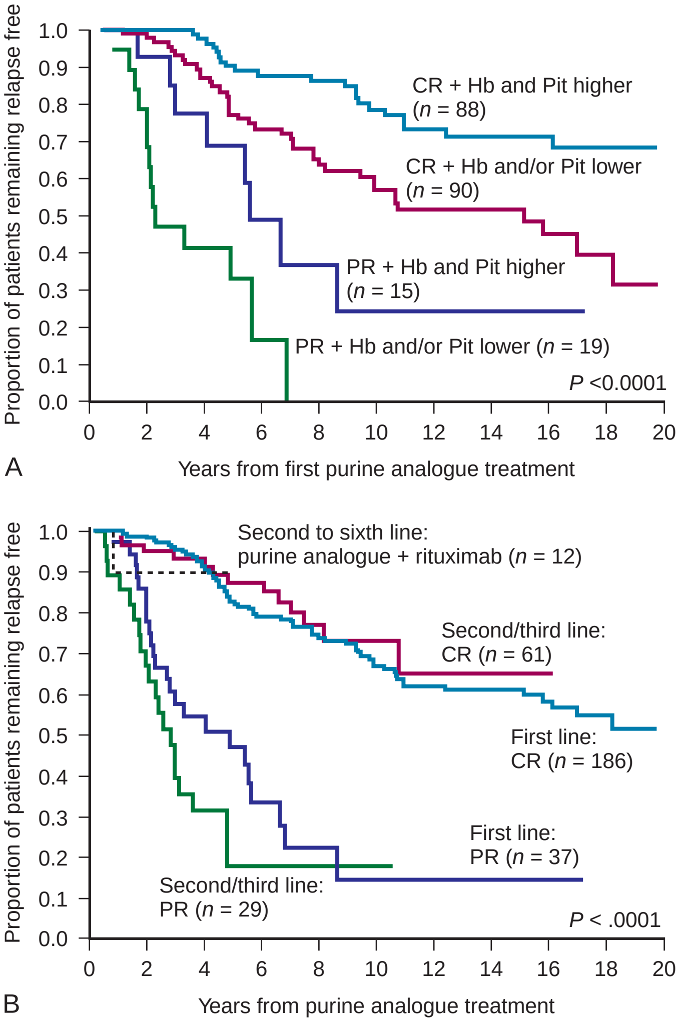
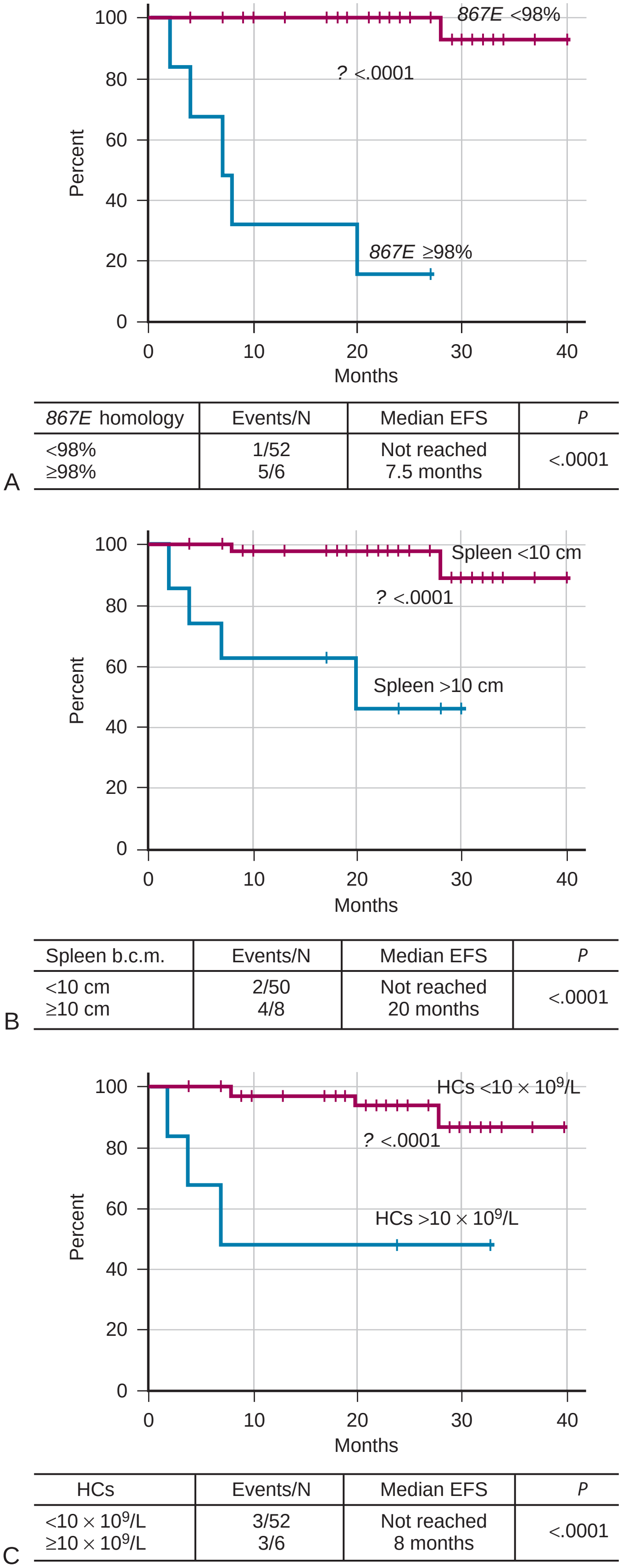


Figure 77.10 (A) Relapse-free survival after initial therapy with nucleoside analogs by initial response and blood counts. (B) Relapse-free survival analyzed by line of therapy and response to treatment. Complete responses (CR) were equally durable, whether achieved at first-, second-, or third-line single-agent treatment. *PR*, Partial response. *(From Else MA, Ruchlemer R, Osuji N, et al. Long remissions in hairy cell leukemia with purine analogs. Cancer. 2005;104:2442; and Else M, Dearden CE, Matutes E, et al. Long-term follow-up of 233 patients with hairy cell leukaemia, treated initially with pentostatin or cladribine, at a median of 16 years from diagnosis. Br J Haematol. 2009;145:733.)*

Other monoclonal antibody–based therapies have developed and are under investigation. BL22 is a recombinant immunotoxin containing the variable domains of the anti-CD22 monoclonal antibody RFB4, fused to a truncated pseudomonas endotoxin A.[28] In a dose-escalation study, 16 patients who were resistant to cladribine were treated with BL22 by intravenous infusion every other day for a total of three doses.[28] Response included 11 CR and 2 PR. The three nonresponders either had preexisting neutralizing antibodies or received low doses of the immunotoxin.[28] Toxicity included a cytokine-release syndrome and development of a reversible hemolytic-uremic syndrome in two patients.[28] In a larger phase I follow-up study of 46 patients with previously treated $CD22^+$ lymphoid malignancies, including 31 with HCL, 61% achieved a CR and 19% experienced a PR, further demonstrating the activity of this agent in HCL. Neutralizing antibodies occurred in 11 (24%) of 46 patients (all HCL), and a reversible hemolytic uremic syndrome, requiring plasmapheresis, was observed in 1 patient with NHL during cycle 1 and in 4 patients with HCL during cycle 2 or 3. The median duration for CR was 36 months (range, 5 to 66 months).



867E homology	Events/N	Median EFS	*P*
<98%	1/52	Not reached	<.0001
≥98%	5/6	7.5 months	

Spleen b.c.m.	Events/N	Median EFS	*P*
<10 cm	2/50	Not reached	<.0001
≥10 cm	4/8	20 months	

HCs	Events/N	Median EFS	*P*
$<10 \times 10^9$/L	3/52	Not reached	<.0001
$\geq 10 \times 10^9$/L	3/6	8 months	

Figure 77.11 PREDICTING RESPONSE AND OUTCOME OF THERAPY WITH CLADRIBINE BY ASSESSING (A) *IGHV* MUTATIONAL STATUS (AS WELL AS BY [B] SPLENOMEGALY AND [C] LEUKOCYTOSIS). (A) Mutational status of *IGHV*. (B) Spleen size. (C) Hairy cells. *EFS*, Event-free survival; *HCs*, hairy cells; *IGHV*, immunoglobulin heavy chain variable.

TABLE 77.6 Selected Published Reports of Rituximab Therapy for Hairy Cell Leukemia

Reference	Patients (*n*)	No Prior Therapy (*n*)	CR (%) (Untreated)	PR (%)	OR (%)
Lauria	10	0	10	40	50
Hagberg	11	3	55 (33)	10	65
Nieva	24	0	13	13	26
Thomas[24]	15	0	66	13	80
Zenhausern	25	0	32	48	80

CR, Complete response; *OR*, overall response; *PR*, partial response.

The maximum tolerated dose (MTD) was established at 40 μg/kg every other day × three dose. More recently, the results of a phase II study of the same drug in 36 patients with relapsed or refractory HCL was published.[29] Patients received BL22 at the MTD on cycle 1. Those achieving hematologic remission were observed while the others were retreated at 30 μg/kg every other day for three doses every 4 weeks beginning at least 8 weeks after cycle 1. The response after one cycle (CR, 25%; PR, 25%) improved when 56% were retreated (CR, 47%; PR, 25%).[29] A modified version of this agent, moxetumomab pasudotox (or HA22), is a recombinant immunotoxin targeting C22, fused to a truncated form of pseudomonas exotoxin. This immunotoxin has a superior affinity for CD22 and has been shown to be active in relapsed HCL. A multicenter study in 80 patients with relapsed HCL and two or more prior therapies, showed that moxetumomab pasudotox led to a CR rate of 41% and an objective response rate of 75%. Treatment-related severe adverse events included hemolytic-uremic syndrome (7.5%) and capillary-leak syndrome (5%).[30]

Minimal Residual Disease and Its Significance

Despite excellent responses, there is a definite relapse rate associated with therapy of HCL with both cladribine and pentostatin, and the relapse-free survival does not appear to plateau.[12] Wheaton and colleagues were the first to note that detection of MRD by immunohistochemistry (using anti-CD45RO, anti-CD20, and DBA.44) in paraffin-embedded bone-marrow sections of 39 patients with HCL, in CR, and after receiving cladribine, was predictive of relapse.[27] More sensitive methods of MRD detection, such as immunophenotyping with the use of multiparameter flow cytometry, as well as analysis of antigen-receptor genes by polymerase chain reaction (PCR) with the use of consensus or clone-specific primers, have been evaluated and can be used to monitor the disease course (Fig. 77.12).[25,31,32] Other markers of the disease, such as serum-soluble CD25, CD22, and CD307, have been evaluated and shown to be reliable for disease monitoring.[33]

Rituximab has been evaluated for eradication of MRD assessed by flow cytometry and consensus primer PCR; whether such elimination of MRD can translate to a longer relapse-free survival is unclear.[11,25] Sigal and colleagues reported that patients with HCL may harbor MRD and even other morphologically evident diseases many years after initial therapy, without experiencing overt relapse. This raises the question of whether eradication of MRD should be the goal of therapy in HCL.[11] However, several studies have suggested that patients who achieve a morphologic CR have a better outcome than those with lesser responses. In the study by Chihara et al. the investigators demonstrated that achieving MRD-negative CR after first line, cladribine is greatly enhanced by concurrent rituximab and less so by delayed rituximab. Whether this can be extrapolated to complete eradication of the detectable disease and to create a potential cure remains to be established.[34]

Treatment of Disease Relapse

Despite the success of the nucleoside analogs in achieving generally durable responses in the majority of patients, they are not considered to be curative, with about 40% of patients relapsing by 10 years after treatment.[12] Second and third courses of nucleoside analogs have been used, either with the same or the alternate agent used to treat relapsing disease. However, CRs are less frequent with subsequent courses. Because a single course of cladribine or pentostatin can suppress $CD4^+$ lymphocytes for a substantial period of time, concerns about the use of multiple courses of these agents have been raised.[35]

Rituximab has the advantage of sparing T-lymphocytes and has shown significant activity in patients with relapsed disease (see Table 77.6). However, the overall response rate is relatively low, particularly in studies using only 4 weekly doses of rituximab. Studies of combination therapy with rituximab and nucleoside analogs have reported high response rates, improved activity in variant HCL, and second CR durations longer than the first CR achieved with nucleoside analogs alone.[36,37] Immunotoxins, such as BL22 and HA22, have been studied in cases of relapsed disease, produce high responses, and have limited toxicity; however, they are not, as yet, widely available. Bendamustine, a novel molecule with structural features of both an alkylating agent and a purine nucleoside analog, has been used for the treatment of relapsed and refractory disease. Anecdotal reports have circulated about the use of other agents such as alemtuzumab. BCR signaling kinase inhibitors, such as ibrutinib, have shown significant promise in the treatment of a number of lymphoid neoplasms and are being investigated in patients with relapsed disease and HCLv.[38] A multisite phase II study of ibrutinib in patients with either relapsed classic or variant HCL has been completed.[39] The median duration of follow-up for 37 patients was 3.5 years. The overall response rate at 32 weeks was 24% which increased to 36% at 48 weeks. The estimated 36-month progression-free and overall survival were 73% and 85%, respectively. The most frequent adverse events were diarrhea (59%), fatigue (54%), myalgia (54%), and nausea (51%). Hematologic adverse events were common (anemia [43%], thrombocytopenia [41%], and neutropenia [35%]). The authors concluded that ibrutinib could be safely administered to HCL patients, with objective responses and prolonged disease control in patients that had not responded to frontline therapy.[39]

The identification of *BRAF* V600F as a universal aberration in patients with HCL and the availability of *BRAF* inhibitors for the treatment of solid tumors, such as melanoma, led to the investigation of this agent in patients with relapsed HCL, with promising early reports.[40] The *BRAF* mutation BRAFV600E constitutively activates the mitogen activated kinase pathway in virtually all patients with HCL. Two phase II trials of the BRAF inhibitor, vemurafenib, performed in Italy and the United States, were reported.[41] The treated patients had relapsed or were refractory to treatment with purine analogs. The drug, vemurafenib, was administered orally at a dose of 960 mg orally twice a day for 16 to 18 weeks. The overall response rate was 96% to 100% with a CR rate of 35% to 42%. The progression-free survival rate was 73% and the overall survival was 91%. Myelosuppression was minimal and the most common adverse effects were rash, arthralgia or arthritis.[41] In the United States trial, residual hairy cells in bone marrow, with their associated *BRAF* V600E allele burden, were consistently present at the end of treatment, even after a CR. In approximately half the patients who could be evaluated in the Italian study, these cells showed persistent expression of phosphorylated ERK despite prolonged, ongoing exposure to vemurafenib, which appeared to correlate with a higher burden of residual leukemia and shorter progression-free survival. These findings suggest that leukemic cells develop alternative mechanisms for reactivating MEK and ERK to bypass BRAF blockade retreatment with vemurafenib at the time of relapse was effective in the two trials, although to a lesser extent in the patients who relapsed after a PR than in those who had a relapse after a CR. Tiacci and coworkers have also reported promising results with dabrafenib, which is another oral reversible competitive BRAF inhibitor.[42] The patients participating in these studies had a median of 3.5 prior therapies, including a purine

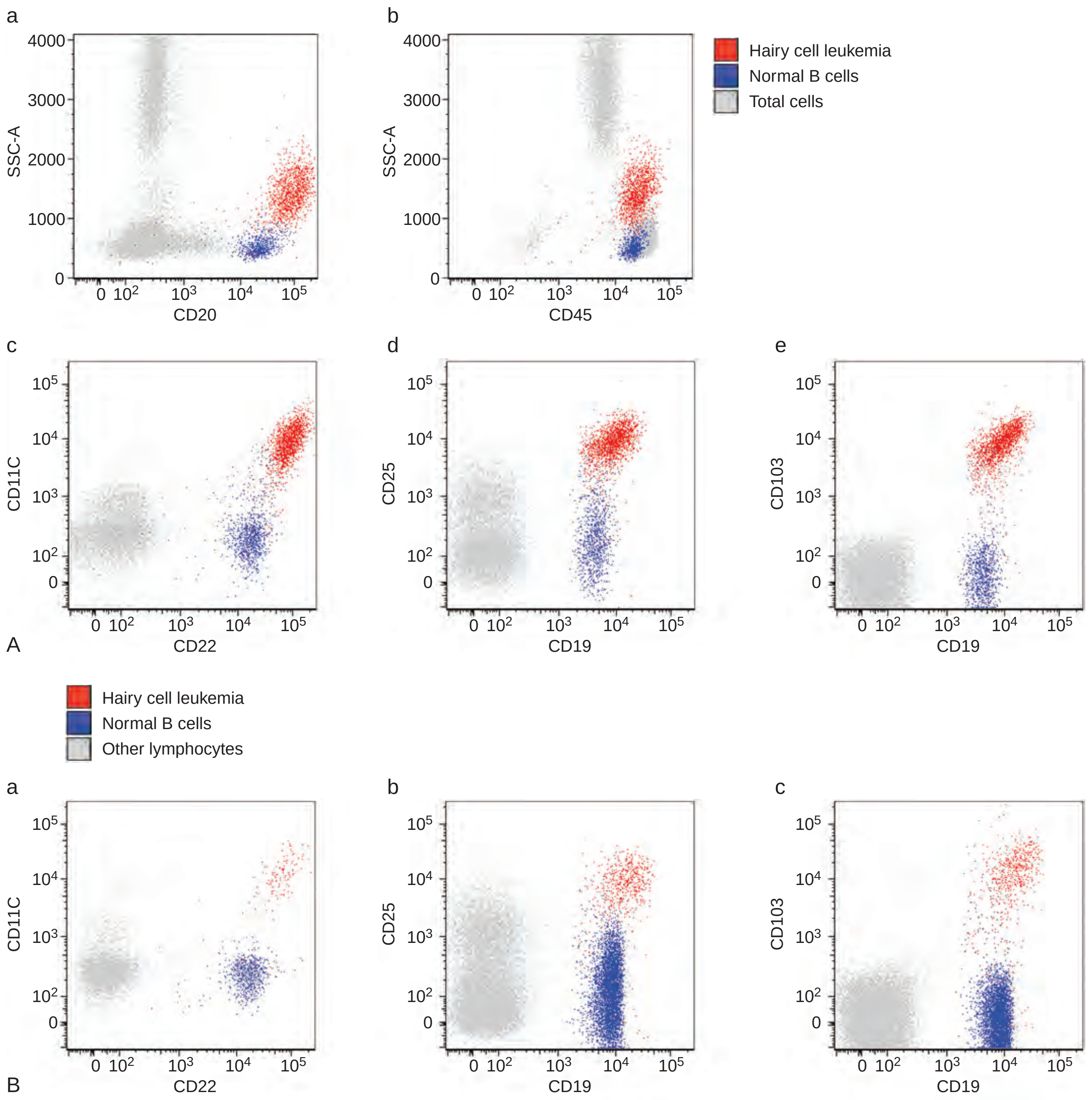


Figure 77.12 MONITORING MINIMAL RESIDUAL DISEASE IN HAIRY CELL LEUKEMIA. (A) Total nucleated cells in a hemodilute bone marrow aspirate (*a* and *b*); total lymphocytes (*c* to *e*). Hairy cells (*red*, about 6% of total cellularity and 12% of lymphocytes) show increased side scatter *(SSC)* because of their high cytoplasmic complexity, compared with normal B cells *(blue)*. Hairy cells often show increased expression of CD20 *(a)*. On CD45 versus SSC gating plots *(b)*, they may be mistaken for monocytes. Hairy cells usually show bright expression of CD11c and CD22 *(c)* and CD25 *(d)* and are positive for CD103 *(e)*. (B) Total lymphocytes from a posttherapy bone marrow aspirate, positive for minimal residual hairy cell leukemia, accounting for about 0.6% of total cellularity. Hairy cells *(red)* can be easily distinguished from normal B cells *(blue)*, particularly on the basis of their bright expression of CD11c *(a)* and CD25 *(b)* and positivity for CD103 *(c)*. In *a*, 30,000 total cells were collected; in *b* and *c*, 100,000. *(Courtesy Jeffrey Jorgensen, Department of Hematopathology, UTMDACC.)*

analogue in each case. The overall response rate was 80% with 30% achieving a CR. Remarkably, for those patients with a CR, the clinical benefit persisted for 21 to 60.5 months. As with vemurafenib, there was no hematological toxicity associated with dabrafenib. A prospective trial combining dabrafenib with the MEK inhibitor, trematinib, has also reported very high and durable responses in patients with relapsed HCL. Trials combining BRAF inhibitors with rituximab or with other CD20-directed monoclonal antibodies are ongoing. These studies suggest that BRAF inhibitors are promising agents for HCL patients who are unresponsive or refractory to purine analogs. The role of these agents alone or in combination with other novel agents will require further investigation.

Incidence of Second Malignancies

Early studies suggested an association between HCL and development of second malignancies, but this link is controversial and has been refuted by other reports. The reported second cancers include melanoma, prostate cancer, gastrointestinal cancers, and non-Hodgkin lymphomas. Whether these second malignancies are related to the primary disease itself or to its treatment has also been debated. A number of long-term studies of patients treated with pentostatin or cladribine have not shown a statistically significant increased risk for second malignancies. In a prospective long-term follow-up study of cladribine in 221 HCL patients performed at three different centers in Switzerland, 19.95% of patients developed a second primary malignancy. These malignancies were the main cause of death, but this rate was not higher than the incidence of primary tumors in a normal matched Swiss population. To date, the relationship between HCL treatment and second primary malignancies remains controversial.

FUTURE DIRECTIONS

Over the past decade, success in the treatment of HCL has led to diminished interest in developing new therapeutic strategies for treating this uncommon leukemia, with research confined to a few specialized centers interested in the biology and pathogenesis of this disease. Recent reports unraveling the biologic and molecular aspects of the disease have kindled a significant resurgence of interest.[2,7,43,44] Further studies into other aspects of the disease such as the role of microenvironment, BCR signaling, and the complex interaction of various signaling pathways will likely provide better therapeutic tools and strategies.[45]

REFERENCES

1. Grever MR, Abdel-Wahab O, Andritsos LA, et al. Consensus guidelines for the diagnosis and management of patients with classic hairy cell leukemia. *Blood*. 2017;129:553–560.
2. Tiacci E, Liso A, Piris M, et al. Evolving concepts in the pathogenesis of hairy-cell leukaemia. *Nat Rev Cancer*. 2006;6:437–448.
3. Forconi F, Sahota SS, Raspadori D, et al. Hairy cell leukemia: at the crossroad of somatic mutation and isotype switch. *Blood*. 2004;104: 3312–3317.
4. Arons E, Roth L, Sapolsky J, et al. Evidence of canonical somatic hypermutation in hairy cell leukemia. *Blood*. 2011;117:4844–4851.
5. Basso K, Liso A, Tiacci E, et al. Gene expression profiling of hairy cell leukemia reveals a phenotype related to memory B cells with altered expression of chemokine and adhesion receptors. *J Exp Med*. 2004;199: 59–68.
6. Tiacci E, Trifonov V, Schiavoni G, et al. BRAF mutations in hairy-cell leukemia. *N Engl J Med*. 2011;364:2305–2315.
7. Chung SS, Kim E, Park JH, et al. Hematopoietic stem cell origin of BRAFV600E mutations in hairy cell leukemia. *Sci Transl Med*. 2014;6 238ra71.
8. Dietrich S, Hüllein J, Lee SC, et al. Recurrent CDKN1B (p27) mutations in hairy cell leukemia. *Blood*. 2015;126:1005–1008.
9. Falini B, Tiacci E, Liso A, et al. Simple diagnostic assay for hairy cell leukaemia by immunocytochemical detection of annexin A1 (ANXA1). *Lancet*. 2004;363:1869–1870.
10. Matutes E, Wotherspoon A, Brito-Babapulle V, et al. The natural history and clinico-pathological features of the variant form of hairy cell leukemia. *Leukemia*. 2001;15:184–186.
11. Sigal DS, Sharpe R, Burian C, et al. Very long-term eradication of minimal residual disease in patients with hairy cell leukemia after a single course of cladribine. *Blood*. 2010;115:1893–1896.
12. Else M, Dearden CE, Matutes E, et al. Long-term follow-up of 233 patients with hairy cell leukaemia, treated initially with pentostatin or cladribine, at a median of 16 years from diagnosis. *Br J Haematol*. 2009;145:733–740.
13. Piro LD, Carrera CJ, Carson DA, et al. Lasting remissions in hairy-cell leukemia induced by a single infusion of 2-chlorodeoxyadenosine. *N Engl J Med*. 1990;322:1117–1121.
14. Lauria F, Bocchia M, Marotta G, et al. Weekly administration of 2-chlorodeoxyadenosine in patients with hairy-cell leukemia: a new treatment schedule effective and safer in preventing infectious complications. *Blood*. 1997;89:1838–1839.
15. Robak T, Jamroziak K, Gora-Tybor J, et al. Cladribine in a weekly versus daily schedule for untreated active hairy cell leukemia: final report from the Polish Adult Leukemia Group (PALG) of a prospective, randomized, multicenter trial. *Blood*. 2007;109:3672–3675.
16. Juliusson G, Heldal D, Hippe E, et al. Subcutaneous injections of 2-chlorodeoxyadenosine for symptomatic hairy cell leukemia. *J Clin Oncol*. 1995;13:989–995.
17. Saven A, Burian C, Koziol JA, et al. Long-term follow-up of patients with hairy cell leukemia after cladribine treatment. *Blood*. 1998;92:1918–1926.
18. Goodman GR, Burian C, Koziol JA, et al. Extended follow-up of patients with hairy cell leukemia after treatment with cladribine. *J Clin Oncol*. 2003;21:891–896.
19. Chadha P, Rademaker AW, Mendiratta P, et al. Treatment of hairy cell leukemia with 2-chlorodeoxyadenosine (2-CdA): long-term follow-up of the Northwestern University experience. *Blood*. 2005;106:241–246.
20. Grever M, Kopecky K, Foucar MK, et al. Randomized comparison of pentostatin versus interferon alfa-2a in previously untreated patients with hairy cell leukemia: an intergroup study. *J Clin Oncol*. 1995;13:974–982.
21. Flinn IW, Kopecky KJ, Foucar MK, et al. Long-term follow-up of remission duration, mortality, and second malignancies in hairy cell leukemia patients treated with pentostatin. *Blood*. 2000;96:2981–2986.
22. Forconi F, Sozzi E, Cencini E, et al. Hairy cell leukemias with unmutated IGHV genes define the minor subset refractory to single-agent cladribine and with more aggressive behavior. *Blood*. 2009;114:4696–4702.
23. Arons E, Suntum T, Stetler-Stevenson M, et al. VH4-34+ hairy cell leukemia, a new variant with poor prognosis despite standard therapy. *Blood*. 2009;114:4687–4695.
24. Thomas DA, O'Brien S, Bueso-Ramos C, et al. Rituximab in relapsed or refractory hairy cell leukemia. *Blood*. 2003;102:3906–3911.
25. Ravandi F, Jorgensen JL, O'Brien SM, et al. Eradication of minimal residual disease in hairy cell leukemia. *Blood*. 2006;107:4658–4662.
26. Chihara D, Kantarjian H, O'Brien S, et al. Long-term durable remission by cladribine followed by rituximab in patients with hairy cell leukaemia: update of a phase II trial. *Br J Haematol*. 2016;174:760–766.
27. Chihara D, Arons E, Stetler-Stevenson M, et al. Randomized phase II study of first-line cladribine with concurrent or delayed rituximab in patients with hairy cell leukemia. *J Clin Oncol*. 2020;38:1527–1538.
28. Kreitman RJ, Wilson WH, Bergeron K, et al. Efficacy of the anti-CD22 recombinant immunotoxin BL22 in chemotherapy-resistant hairy-cell leukemia. *N Engl J Med*. 2001;345:241–247.
29. Kreitman RJ, Stetler-Stevenson M, Margulies I, et al. Phase II trial of recombinant immunotoxin RFB4(dsFv)-PE38 (BL22) in patients with hairy cell leukemia. *J Clin Oncol*. 2009;27:2983–2990.
30. Kreitman RJ, Dearden C, Zinzani PL, et al. Moxetumomab pasudotox in relapsed/refractory hairy cell leukemia. *Leukemia*. 2018;32:1768–1777.
31. Arons E, Margulies I, Sorbara L, et al. Minimal residual disease in hairy cell leukemia patients assessed by clone-specific polymerase chain reaction. *Clin Cancer Res*. 2006;12:2804–2811.
32. Sausville JE, Salloum RG, Sorbara L, et al. Minimal residual disease detection in hairy cell leukemia. Comparison of flow cytometric immunophenotyping with clonal analysis using consensus primer polymerase chain reaction for the heavy chain gene. *Am J Clin Pathol*. 2003;119:213–217.
33. Ravandi F, O'Brien S, Jorgensen J, et al. Phase 2 study of cladribine followed by rituximab in patients with hairy cell leukemia. *Blood*. 2011;118:3818–3823.
34. Ravandi F. MRD in HCL: does it matter? *Blood*. 2018;131:2277–2278.
35. Seymour JF, Kurzrock R, Freireich EJ, et al. 2-chlorodeoxyadenosine induces durable remissions and prolonged suppression of CD4+ lymphocyte counts in patients with hairy cell leukemia. *Blood*. 1994;83:2906–2911.
36. Else M, Dearden CE, Matutes E, et al. Rituximab with pentostatin or cladribine: an effective combination treatment for hairy cell leukemia after disease recurrence. *Leuk Lymphoma*. 2011;52(Suppl 2):75–78.
37. Kreitman RJ, Wilson W, Calvo KR, et al. Cladribine with immediate rituximab for the treatment of patients with variant hairy cell leukemia. *Clin Cancer Res*. 2013;19:6873–6881.
38. Sivina M, Kreitman RJ, Arons E, et al. The bruton tyrosine kinase inhibitor ibrutinib (PCI-32765) blocks hairy cell leukaemia survival, proliferation and B cell receptor signalling: a new therapeutic approach. *Br J Haematol*. 2014;166:177–188.
39. Rogers KA, Andritsos LA, Wei L, et al. Phase 2 study of ibrutinib in classic and variant hairy cell leukemia. *Blood*. 2021;137:3473–3483.

40. Pettirossi V, Santi A, Imperi E, et al. BRAF inhibitors reverse the unique molecular signature and phenotype of hairy cell leukemia and exert potent antileukemic activity. *Blood*. 2015;125:1207–1216.
41. Tiacci E, Park JH, De Carolis L, et al. Targeting mutant BRAF in relapsed or refractory hairy-cell leukemia. *N Engl J Med*. 2015;373:1733–1747.
42. Tiacci E, De Carolis L, Simonetti E, et al. Safety and efficacy of the BRAF inhibitor dabrafenib in relapsed or refractory hairy cell leukemia: a pilot phase-2 clinical trial. *Leukemia*. 2021;35(11):3314–3318.
43. Durham BH, Getta B, Dietrich S, et al. Genomic analysis of hairy cell leukemia identifies novel recurrent genetic alterations. *Blood*. 2017;130:1644–1648.
44. Tiacci E, Pettirossi V, Schiavoni G, et al. Genomics of hairy cell leukemia. *J Clin Oncol*. 2017;35:1002–1010.
45. Falini B, Martelli MP, Tiacci E. BRAF V600E mutation in hairy cell leukemia: from bench to bedside. *Blood*. 2016;128:1918–1927.

CHAPTER 78

THE PATHOLOGIC BASIS FOR THE CLASSIFICATION OF NON-HODGKIN AND HODGKIN LYMPHOMAS

Girish Venkataraman, Elaine S. Jaffe, and Stefania Pittaluga

INTRODUCTION AND HISTORICAL BACKGROUND

The classification of malignant lymphomas has undergone significant changes over the past 50 years. The current approach is based on the integration of morphologic, phenotypic, genetic, and clinical features that allows the identification of distinct disease entities (see box on Principles of the Classification of Lymphomas). This practical approach to lymphoma categorization was initially proposed by the International Lymphoma Study Group in 1994 and formed the basis of the Revised European–American Classification of lymphoid neoplasm (REAL). It was then adopted by the World Health Organization (WHO) classification of neoplasm of the hematopoietic and lymphoid tissues published in 2001, updated in 2008, and revised again in 2017 (Table 78.1).[1–3] The WHO classification represents a significant achievement in terms of cooperation, communication, and consensus among pathologists, hematologists, and oncologists. Furthermore, it recognizes that for any classification system to be viable and applicable, it should evolve and incorporate new data resulting from emerging technologies in the field of hematopathology such as the results from genome-wide large-scale sequencing studies. These studies have led to the identifications of new prognostic and diagnostic categories, and provide insight into therapeutic targets based on a better understanding of molecular mechanisms of transformation. This chapter focuses on the classification of neoplasms derived from mature B cells, T cells, and natural killer (NK) cells with an emphasis on malignant lymphoma. We provide a framework for the subsequent chapters on Hodgkin and non-Hodgkin lymphomas in reviewing the major entities according to the WHO classification.

PRINCIPLES OF THE CLASSIFICATION OF LYMPHOMAS BASED ON THE REVISED EUROPEAN–AMERICAN CLASSIFICATION OF LYMPHOID NEOPLASM/WORLD HEALTH ORGANIZATION CLASSIFICATIONS

- Each disease is defined as a distinct entity based on a constellation of morphologic, clinical, and biologic features.
- The cell of origin is the starting point of disease definition.
- Some lymphoid neoplasms can be identified by routine morphologic approaches. However, for most diseases, knowledge of the immunophenotype and molecular genetics/cytogenetics plays an important role in differential diagnosis.
- A disease-based approach to classification facilitates the discovery of molecular pathogenesis.
- The sites of presentation and involvement are important clues to underlying biologic distinctions. Extranodal lymphomas differ in many respects from their nodal counterparts.
- Many lymphoma entities display a range in cytologic grade and clinical aggressiveness, making it difficult to stratify lymphomas according to clinical behavior. A number of prognostic factors influence clinical outcome, including stage, international prognostic index, cytologic grade, gene expression profile, secondary genetic events, and the host environment.

MATURE B-CELL NEOPLASMS

Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) usually presents in adults with generalized lymphadenopathy, frequent bone marrow and peripheral blood involvement, and often hepatosplenomegaly. Presentation as leukemia, that is, CLL, is more common than as lymphoma, SLL. Even in patients with a lymphomatous presentation, careful examination of the blood may reveal a circulating monoclonal B-cell component. Nevertheless, there are some patients who will present with generalized adenopathy, and whereas progression to CLL is frequent, it does not necessarily occur in all cases.

The increased sensitivity of immunophenotypic/molecular methodologies has resulted in the detection of clonal lymphoid proliferations with a CLL phenotype in the general population, even in the absence of clinical lymphocytosis, a condition now designated monoclonal B-cell lymphocytosis (MBL) (see box on Early Events in Lymphoid Neoplasia). The International Workshop on CLL proposed new diagnostic criteria that were then included in the WHO classification of 2008. Recent studies have distinguished between high count and low count MBL, with a count of monoclonal B cells greater than 5.0 × 10^3/L being more clinically significant.[4] Similar to peripheral blood, small clonal populations with a CLL phenotype can be detected in lymph nodes as an incidental finding and appear to represent a tissue counterpart of MBL.[5] Histologically, the lymph node involved by CLL/SLL shows diffuse architectural effacement (Fig. 78.1), although occasional residual naked germinal centers can be observed. The predominant cell type is a small lymphocyte with

EARLY EVENTS IN LYMPHOID NEOPLASIA

- In recent years, there has been a greater appreciation of early events in lymphoid neoplasia.
- These early lesions can in some ways be considered equivalent to benign neoplasms in the epithelial system and require special management approaches.
- These are clonal proliferations of B cells or T cells that carry genetic aberrations associated with specific forms of lymphoid neoplasia: CLL, multiple myeloma, follicular lymphoma, and mantle cell lymphoma.
- Examples include: MGUS, MBL, in situ follicular neoplasia, and in situ mantle cell neoplasia.
- Early lesions appear to lack the secondary and tertiary “hits” seen in lymphoid neoplasms that are clinically significant, and most patients have a very low risk of clinical progression.
- Challenges for the future are:
  - to define the precise genetic features that distinguish early lesions from lymphoma
  - to assess the risk of clinical progression
  - to determine how these patients should be managed clinically.

CLL, Chronic lymphocytic leukemia; *MBL*, monoclonal B-cell lymphocytosis; *MGUS*, monoclonal gammopathy of undetermined significance.

TABLE 78.1 World Health Organization Classification of Lymphomas[a]

Mature B-Cell Neoplasms

- ***Chronic lymphocytic leukemia/small lymphocytic lymphoma***
- B-cell prolymphocytic leukemia
- Splenic B-cell marginal zone lymphoma
- Hairy cell leukemia
- *Splenic B-cell lymphoma/leukemia, unclassifiable*
  - *Splenic diffuse red pulp small B-cell lymphoma*
  - *Hairy cell leukemia-variant*
- Lymphoplasmacytic lymphoma
  - Waldenström macroglobulinemia
- Heavy chain diseases
- ***Plasma cell myeloma***
  - Plasmacytoma
- ***Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)***
- Nodal marginal zone lymphoma
  - *Pediatric nodal marginal zone lymphoma*
- ***Follicular lymphoma***
  - *Pediatric-type follicular lymphoma*
- Primary cutaneous follicle center lymphoma
- ***Mantle cell lymphoma***
- ***Diffuse large B-cell lymphoma (DLBCL), NOS***
  - T-cell/histiocyte–rich large B-cell lymphoma
  - Primary DLBCL of the CNS
  - Primary cutaneous DLBCL, leg type
  - EBV-positive DLBCL, NOS
  - DLBCL associated with chronic inflammation
- Lymphomatoid granulomatosis
- Primary mediastinal (thymic) large B-cell lymphoma
- Intravascular large B-cell lymphoma
- ALK-positive large B-cell lymphoma
- Plasmablastic lymphoma
- Primary effusion lymphoma
- Burkitt lymphoma
- High-grade B-cell lymphomas, with *MYC* and *BCL2* and/or *BCL6* rearrangements
- B-cell lymphoma unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classic Hodgkin lymphoma

Mature T-Cell and NK-Cell Neoplasms

- T-cell prolymphocytic leukemia
- T-cell large granular lymphocytic leukemia
- *Chronic lymphoproliferative disorder of NK-cells*
- Aggressive NK-cell leukemia
- Systemic EBV-positive T-cell lymphoma of childhood
- Hydroa vacciniforme-like lymphoproliferative disorder
- Extranodal NK/T-cell lymphoma, nasal type
- Adult T-cell leukemia/lymphoma
- Enteropathy-associated T-cell lymphoma
- Monomorphic epitheliotropic intestinal T-cell lymphoma
- Hepatosplenic T-cell lymphoma
- Subcutaneous panniculitis-like T-cell lymphoma
- ***Mycosis fungoides***
- Sézary syndrome
- Primary cutaneous CD30-positive T-cell lymphoproliferative disorders
  - Lymphomatoid papulosis
  - Primary cutaneous anaplastic large cell lymphoma
- Primary cutaneous gamma-delta T-cell lymphoma
- Primary cutaneous CD8-positive aggressive epidermotropic cytotoxic T-cell lymphoma
- Primary cutaneous CD4-positive small/medium T-cell lymphoproliferative disorder
- ***Peripheral T-cell lymphoma, NOS***
- ***Angioimmunoblastic T-cell lymphoma***
- Nodal peripheral T-cell lymphoma with T-follicular helper phenotype
- Anaplastic large cell lymphoma, ALK-positive
- Anaplastic large cell lymphoma, ALK-negative

Hodgkin Lymphoma

- Nodular lymphocyte predominant Hodgkin lymphoma
- Classic Hodgkin lymphoma
  - ***Nodular sclerosis Hodgkin lymphoma***
  - Lymphocyte-rich classic Hodgkin lymphoma
  - Mixed-cellularity classic Hodgkin lymphoma
  - Lymphocyte-depleted classic Hodgkin lymphoma

[a] Most common entities are bold and italicized. Provisional entities are in non-bold italics. Some rare entities or variants are omitted (see reference 3 for complete list).
ALK, Anaplastic lymphoma kinase; *CNS*, central nervous system; *EBV*, Epstein-Barr virus; *NK*, natural killer; *NOS*, not otherwise specified.

clumped chromatin, but a spectrum of nuclear morphology is usually seen. Pseudofollicular growth centers or proliferation centers are present in the majority of cases and contain a spectrum of cells ranging from small lymphocytes to prolymphocytes and paraimmunoblasts. The prolymphocytes and paraimmunoblasts have more dispersed chromatin and more prominent nucleoli usually centrally placed. The presence of proliferation centers is also a helpful criterion in the differential diagnosis with mantle cell lymphoma (MCL), which may show otherwise some overlapping features with CLL. If needed, immunophenotypic studies can be helpful in this differential diagnosis.

CLL/SLL is characterized by $CD5^+$, $CD23^+$, $LEF1^+$ B cells expressing dim CD20, and usually dim surface immunoglobulin (sIg). Cyclin D1 is negative, in contrast to MCL. CLL has been shown to have a greater degree of heterogeneity biologically, and different subgroups have been identified based on immunoglobulin (Ig) heavy-chain mutational status, cytogenetics, ZAP-70 expression, and CD38 expression. The latter two have been used as partial surrogate markers for the mutational status. ZAP-70 expression correlates with an unmutated status and poorer prognosis (Fig. 78.1). In fact, ZAP-70 expression has been suggested to be more clinically relevant than mutation status when the two markers are discordant. The use of CD38 as surrogate marker for mutational status is less useful, but its high expression is also associated with a poor prognosis. Recently, recurrent somatic mutations have been identified in a subset of CLL patients using whole-genome and exome sequencing techniques, and some of them have been associated with clinical outcome and may be useful in the future for risk stratification including CLL-International

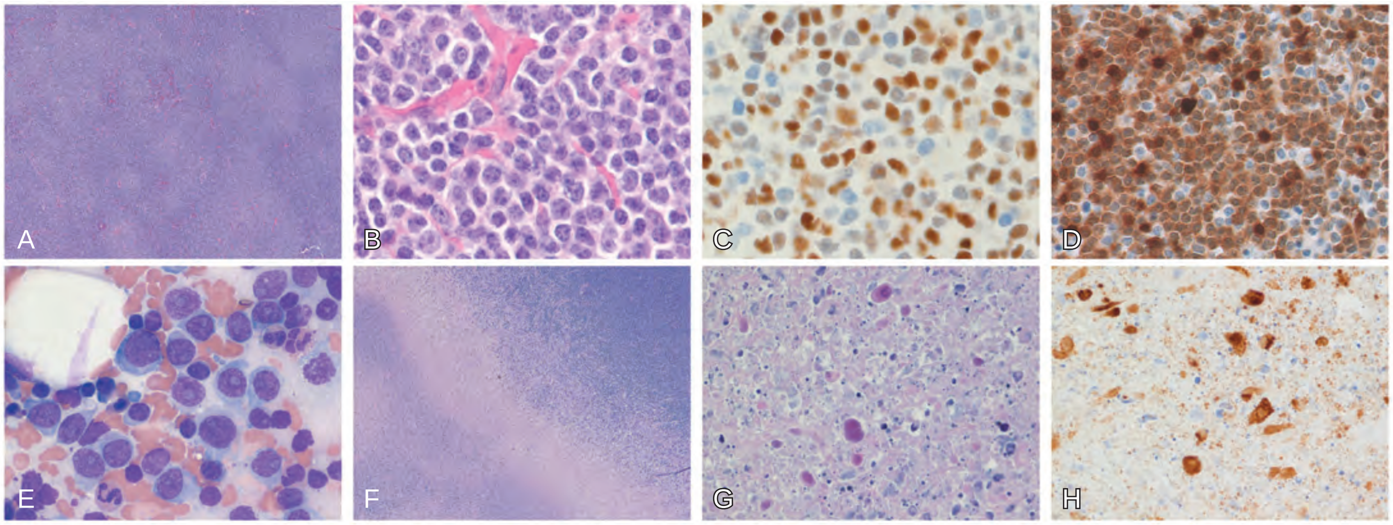


Figure 78.1 SMALL LYMPHOCYTIC LYMPHOMA. Low-power view (A) illustrates a diffuse effacement of the lymph node with scattered pale nodules corresponding to growth centers. A monotonous population of small lymphocytes is seen at higher power (B). The lymphoma cells are positive for LEF1 (C) and ZAP70 with scattered normal T-cells staining brightly for ZAP70 (D). Accelerated prolymphocytoid transformation shows increased numbers of large prolymphocytes in the marrow with prominent nucleoli (E). Patients, especially after fludarabine therapy, can develop worsening lymphadenopathy from viral infections such as herpes simplex virus, in which the node typically shows focal necrosis bottom left (F) with typical Cowdry nuclear inclusions in the necrotic areas at higher power (G) confirmed with immunohistochemistry for herpes simplex virus (H).

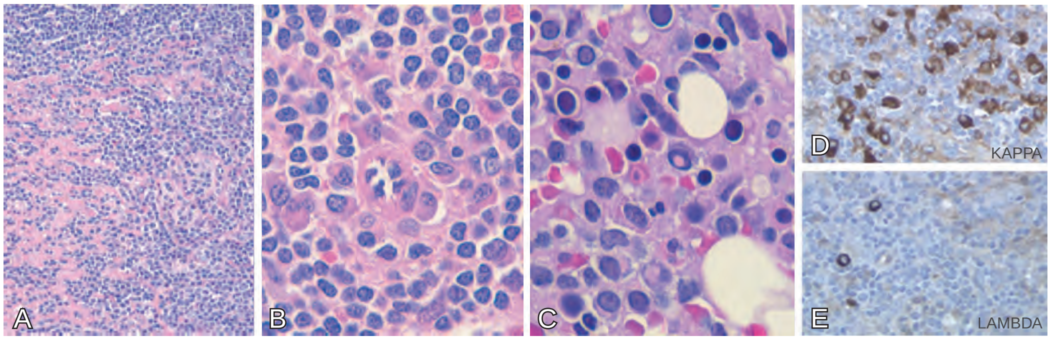


Figure 78.2 LYMPHOPLASMACYTIC LYMPHOMA (LPL). Lymphoplasmacytic lymphoma and Waldenström macroglobulinemia have nearly identical morphology. There is a diffuse infiltrate (A) of small lymphocytes that have plasmacytoid features or interspersed plasma cells (B). Intranuclear inclusions can sometimes be seen (C). Evaluation for κ and λ by immunohistochemical stains can demonstrate clonality in the plasma cells and plasmacytoid lymphocytes (D) and (E). This case shows kappa light chain restriction.

Prognostic Index (IPI). In addition, deletions at 17p, or mutations in TP53 correlate with a more aggressive clinical course.[6]

Histologic transformation over time may occur in CLL, a phenomenon known as Richter syndrome. Short of progression to diffuse large B-cell lymphoma (DLBCL), lymph nodes may show an increased number of prolymphocytes and paraimmunoblasts, sometimes referred to as "accelerated phase" or "prolymphocytoid" transformation (Fig. 78.1E). The two overt lymphomatous forms of Richter transformation include classic Hodgkin lymphoma (CHL) and more commonly, DLBCL. While a significant proportion of CHL cases are Epstein-Barr virus (EBV) associated, most DLBCLs are unrelated to EBV.[7] Treatment with immunosuppressive agents such as fludarabine appears to increase the risk of transformation.

Lymphoplasmacytic Lymphoma

The definition of lymphoplasmacytic lymphoma (LPL) and its relationship to other B-cell lymphomas associated with plasmacytoid differentiation and monoclonal gammopathy has been clarified in recent years. LPL is frequently associated with Waldenström macroglobulinemia (WM). The 2008 WHO classification adopted the approach advocated at the second international workshop on WM, which defined WM as the presence of an immunoglobulin M (IgM) monoclonal gammopathy of any concentration associated with bone marrow involvement by LPL.[8] Hence, LPL and WM are not synonymous, with WM defining a subset of LPL.

More recently, recurrent mutations in MYD88 (L265P) have been identified in greater than 90% of WM patients and are highly associated with LPL but infrequently seen in other B-cell lymphomas with plasmacytoid features, such as marginal zone lymphomas. In addition, nonsense and frameshift mutations involving CXC-chemokine receptor 4, similar to those occurring in warts hypogammaglobulinemia infections myelokathexis (WHIM) patients, were reported and are associated with heavy disease burden.

LPL is a disease of adult life that usually presents with bone marrow involvement and sometimes with nodal and splenic involvement (splenomegaly), vague constitutional symptoms, and anemia (see Chapter 92). The tumor consists of a diffuse proliferation of small lymphocytes, plasmacytoid lymphocytes, and plasma cells, with or without Dutcher bodies (Fig. 78.2). The growth pattern is often interfollicular, with sparing of the sinuses. The cells have surface and cytoplasmic Ig, usually of IgM type, usually lack IgD, and express B-cell-associated antigens (CD19, CD20, CD22, CD79a). They are usually negative for CD5 and CD10. CD25 or CD11c may be weakly expressed in some cases. The lack of CD5 and the presence of strong cytoplasmic Ig are useful in the distinction from CLL. The postulated normal counterpart is thought to be a postfollicular B-cell-based in part on the presence of somatic mutations in the Ig heavy and light-chain variable region genes.

Mantle Cell Lymphoma

MCL is a distinct entity that has been more precisely defined in recent years through the integration of immunophenotypic, molecular genetic, and clinicopathologic studies.[9] The molecular hallmark of MCL is the t(11;14)(q13;q32) involving Cyclin D1 (*CCND1*) and the *IGH* gene. Cyclin D1 overexpression is believed to be essential in the pathogenesis of MCL. However, rare variants negative for Cyclin D1 with similar immunomorphology and gene expression signature have been identified, and most often have translocations involving *CCND2*. Sox11 is overexpressed in most Cyclin D1 positive and negative cases.[10] The postulated normal counterpart is the $CD5^+$, $sIgM^+$ and $sIgD^+$, "naïve" B cell that can be found in the peripheral blood and mantle zones of reactive germinal centers. Mutational analysis of the rearranged Ig variable region genes shows few or no somatic mutations; however, similarly to CLL, a subset of MCL has mutated *IG* genes.

Recently, because of the widespread use of immunohistochemistry (IHC), early involvement of lymph node by cells carrying t(11;14) translocation with subsequent overexpression of Cyclin D1 has been documented in several cases, referred to initially in the WHO 2008 as "in situ MCL" but now termed "in situ MC Neoplasia" (in situ MCN) in the WHO 2017 update, reflecting that this is an early lesion. Most often, these represent an incidental finding, but some cases will eventually progress to overt MCL.[5] In some cases, in situ MCN is detected in a lymph node involved by another lymphoma type, such as follicular lymphoma (FL). The risk of progression of in situ MCN is difficult to ascertain, as the number of reported cases is few. In a recent multicentric retrospective study, it was noted that the expression of Sox11 was more frequently associated with progression to MCL since the majority of in situ cases lacked Sox11 expression. Also, similar to in situ follicular neoplasia (ISFN), a distinction should be made between partial involvement by MCL with a mantle zone pattern and in situ MCN. The latter refers to a reactive lymph node with Cyclin D1 positive cells limited to an otherwise normal-appearing follicle mantle.

Another newly identified variant is an indolent form of MCL characterized by a leukemic phase without nodal disease, but often with long-standing splenomegaly. These patients have an indolent clinical course and do not appear to require aggressive chemotherapy. These cases carry t(11;14) with few additional chromosomal abnormalities and lack the expression of Sox11 in contrast to conventional MCL.

MCL occurs in adults (median age 62), with a high male-to-female ratio. Most patients present with advanced stage at diagnosis. Common sites of involvement include lymph nodes, spleen, bone marrow, and lymphoid tissue of Waldeyer ring. Gastrointestinal (GI) tract involvement is frequent and is associated with the picture of lymphomatous polyposis.

The hallmark of MCL is a very monotonous cellular composition. In the typical case, the cells are slightly larger than a normal lymphocyte with finely clumped chromatin, scant cytoplasm, and inconspicuous nucleoli (Fig. 78.3). The nuclear contour is usually irregular or cleaved. Some cytologic variants, blastoid and pleomorphic, tend to be associated with a more aggressive course and adverse biologic features, such as tetraploidy or p53 mutation/deletion. The proliferation rate was previously identified as prognostically important based on the scoring of $Ki67^+$ cells (<10%, 10% to 30%, and >30%). More recently, gene expression profiling (GEP), using genes involved in cell cycle progression and DNA synthesis, has identified a proliferation signature that delineates cohorts with varied prognoses. These correlate to some extent with cytologic subtypes. For example, the blastoid variant has a high proliferation rate, utilizing both KI-67 and GEP signature. More recent data indicate that high P53 expression (>20%) has adverse prognostic significance independent of KI-67 or the MCL-IPI.[11]

Follicular Lymphoma

FL is the most common subtype of non-Hodgkin lymphoma within the United States and accounts for approximately 45% of all newly diagnosed cases. It has a peak incidence in the fifth and sixth decades and is rare under the age of 20. Men and women are equally affected. FL is less common in Black and Asian populations. Most patients have stage 3 or 4 disease at diagnosis, with generalized lymphadenopathy. Staging evaluation will usually detect bone marrow involvement. Approximately 10% of patients will have circulating malignant cells. However, careful immunophenotypic or molecular analyses may disclose peripheral blood involvement in a higher proportion of patients. A more accurate prognostic index than the IPI, the FL IPI, has been proposed for FL and has been widely adopted.

The natural history of the disease is associated with histologic progression in both pattern and cell type (Fig. 78.4). A heterogeneous cytologic composition is one of the hallmarks of FL. Usually, all of the follicle center cells are represented, but in varying proportions. It should be stressed that the variation in cytologic grade is a continuum, and therefore, precise morphologic criteria for subclassification are difficult to establish.

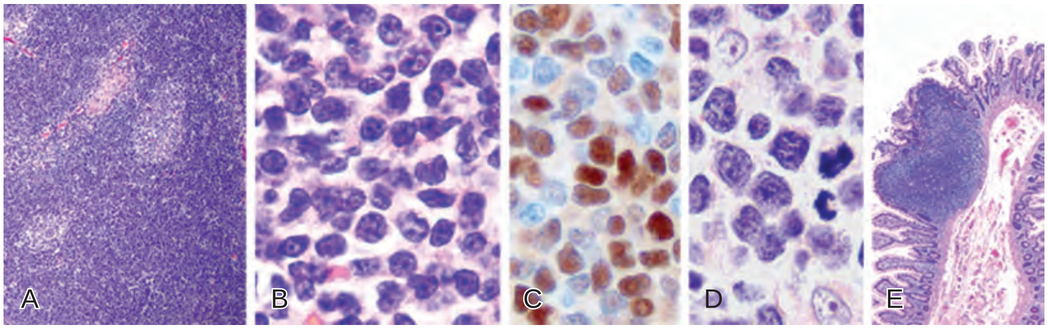


Figure 78.3 MANTLE CELL LYMPHOMA. At low power, mantle cell lymphoma (MCL) can show a diffuse, vaguely nodular, or mantle zone pattern. In the latter, the neoplastic mantle zones are expanded and can become confluent leaving "naked" germinal centers (A). At higher power, the lymphoma cells are small or slightly enlarged (B). They have irregular nuclear contours, especially compared with small lymphocytic lymphoma, and they have dense chromatin. Typically, cases are positive for cyclin D1 expression (C), which is related to the t(11;14) involving IgH and *CCND1*. Some cases can develop a "blastoid" transformation (D), although some cases can present as a "blastoid" variant. Such cases are characterized by cells with an intermediate size, a high mitotic rate, and finely dispersed "blastic" chromatin. Sometimes when the "blastoid" cases develop a leukemic phase, they can be difficult to distinguish morphologically from acute lymphoblastic leukemia. In such cases, flow immunophenotyping is needed to resolve the differential diagnosis. MCL can also present with gastrointestinal involvement (E) as in lymphomatous polyposis.

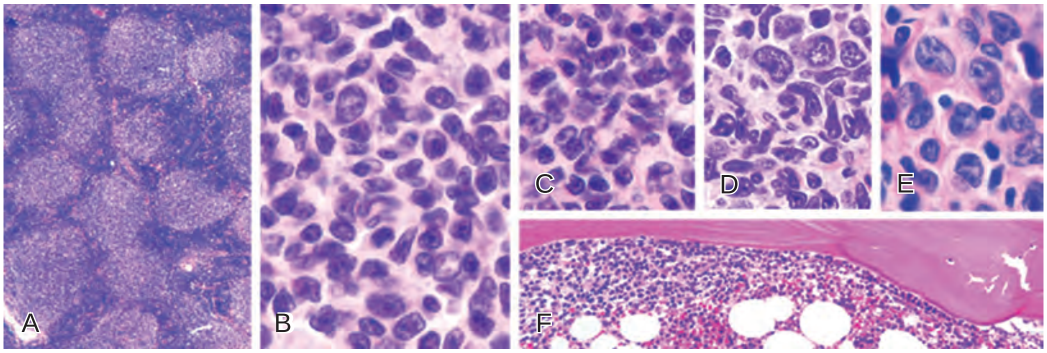


Figure 78.4 FOLLICULAR LYMPHOMA (FL). Follicular lymphoma (FL) shows effacement of the normal lymph node architecture because of an accumulation of neoplastic lymphoid follicles that lack the features of reactive follicles (A). They are crowded, show back-to-back localization, lack distinct mantle zones, and show no polarity. The lymphoma cells are highly irregular (B) with elongated, twisted, or clefted nuclear contours and dense chromatin. FL is typically graded into grade 1 or 2 (1/2; C, D), or 3 (E), depending on the number of large cells seen at higher power (see text). Large centrocytes should not be confused with centroblasts. FL typically involves the bone marrow with lymphoma cells spreading along the bone (F). This localization is termed "paratrabecular."

According to the WHO classification, all low-grade FL are combined into a single category, Grade 1–2, all containing overall a predominance of centrocytes with fewer than 15 centroblasts/high power field (hpf). FL grade 3 (with >15 centroblasts/hpf) is further subdivided into 3 A and 3B based on the presence or absence of centrocytes in the background.

The vast majority of FL (approximately 85%) are associated with a t(14;18) involving rearrangement of the *BCL2* gene. This translocation appears to result in constitutive expression of BCL2 protein, which is capable of inhibiting apoptosis in lymphoid cells. The cells of FL accumulate and are at risk to acquire secondary mutations, which may be associated with histologic progression. It has been postulated that the *BCL2/IGH* translocation occurs during Ig gene rearrangement in the bone marrow at the pre-B cell stage of development. This fact might contribute to the difficulty in eradicating the neoplastic clone with chemotherapy.

Biologically, the pathogenesis of most cases of FL grade 3B differs from that of FL grades 1–2/3 A, in lacking the *BCL2/IGH*, but also differs from DLBCL, in having a low incidence of *BCL6* aberrations.[12] These data provide a biologic explanation for the greater curability of grade 3B FL with aggressive therapy, although some studies have not found support for this hypothesis. Differences in diagnostic criteria might account for this apparent discrepancy, and the correlation between grade 3A versus 3B and molecular alterations is imprecise. Other phenotypic variants appear to have prognostic significance, such as FL negative for CD10 but positive for MUM1/IRF4. These cases are usually of higher grade and interestingly also lack the *BCL2* translocation. Evolution toward a molecularly defined classification of FL is a possibility for the future.

The phenomenon of localization of FL cells to isolated germinal centers within a lymph node has been termed ISFN.[5] The likelihood of evolution to clinically significant FL is low for these patients if there is no other evidence of disease at the time of diagnosis. Indeed, this translocation can be found in the peripheral blood and lymphoid organs of healthy individuals and suggests that the *BCL2/IGH* translocation is necessary but not sufficient for the development of FL. ISFN should be distinguished from partial involvement by FL. In the true "in situ" lesion, clusters of B cells strongly positive for CD10 and BCL2 are localized to germinal centers in an otherwise reactive lymph node. It often represents an incidental finding in a lymph node biopsied for other reasons.

The 2017 WHO classification recognizes other lymphomas of follicle center derivation that may resemble nodal FL but exhibit significant differences either clinically or biologically.[3] These include diffuse follicular FL, pediatric forms of FL, primary intestinal FL ("duodenal-type"), and cutaneous lymphomas of follicle center cell derivation. Intestinal FL, most often presenting in the duodenum, is associated with the *BCL2/IGH* translocation but usually presents as isolated mucosal polyps with a low risk of dissemination. FL in the pediatric age group is histologically diverse. Most nodal cases are cytologically high grade, composed of blastoid cells, but are usually localized, and may be cured in a number of instances with surgical excision and carry underlying *TNFRSF14* and *MAP2K1* mutations.[13] This so-called pediatric type of FL may also be seen in adults more rarely and shows a strong male predominance. Another form of FL seen in children and young adults is associated with translocations involving *IRF4* and shows overexpression of MUM1 by IHC. These cases frequently present in a Waldeyer ring.[14]

There are rare variants of FL with a mainly diffuse growth pattern. These often present as bulky localized inguinal masses and lack the *BCL2* translocation but often have deletions at 1p36. Primary cutaneous follicle center lymphoma, which also frequently lacks the *BCL2* translocation and BCL2 expression, is now considered by the WHO classification as a separate entity. They usually present in the head or upper trunk and can be managed conservatively with local approaches. However, when BCL2 expression is detected, the possibility that this may represent a secondary site of involvement should be considered.

Extranodal Marginal-Zone Lymphoma of Mucosa-Associated Lymphoid Tissue Type

Most lymphomas of marginal-zone derivation present in extranodal sites and have the histopathologic and clinical features identified by Isaacson and Wright as part of the spectrum of mucosa-associated lymphoid tissue (MALT) lymphomas. MALT lymphomas are characterized by a heterogeneous cellular composition that includes marginal-zone or centrocyte-like cells, monocytoid B cells, small lymphocytes, and plasma cells (Fig. 78.5A). In most cases, large transformed cells are infrequent. Reactive germinal centers are nearly always present. When follicular colonization occurs, the process may simulate FL. Clonality is confirmed by molecular and or immunohistochemical studies.

MALT lymphomas have been described in nearly every anatomic site but are most frequent in the stomach, lung, thyroid, salivary gland, and lacrimal gland. Other less common sites of involvement include the orbit, breast, conjunctiva, bladder and kidney, and thymus gland. Widespread nodal involvement is infrequent, as is marrow involvement. The clinical course is usually quite indolent, and many patients are asymptomatic. MALT lymphomas tend to relapse in other MALT-associated sites.

MALT lymphomas of the salivary gland and thyroid are usually associated with a history of autoimmune diseases. *Helicobacter* gastritis is frequent in most patients with gastric MALT lymphomas. Other infectious agents have been described in MALT lymphomas involving skin (*Borrelia burgdorferi*), ocular adnexae (*Chlamydia psittaci*), and small intestine (*Campylobacter jejuni*); however, in this latter group, a

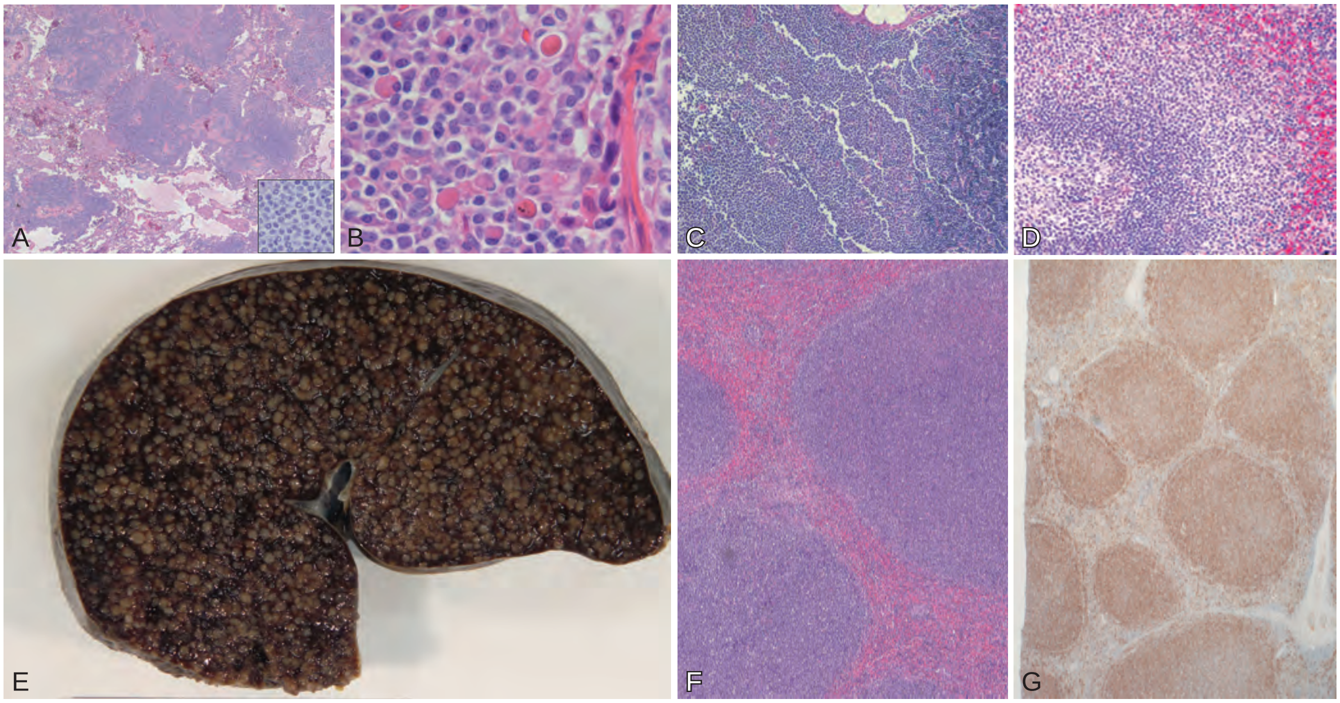


Figure 78.5 MARGINAL ZONE LYMPHOMA. Marginal zone lymphomas commonly occur at extranodal sites arising from mucosa-associated lymphoid tissue (MALT). MALT lymphomas typically infiltrate or invade into epithelial structures, resulting in "lymphoepithelial lesions" with multifocal nodular aggregates in the lung (A). They are composed of small to intermediate-sized cells with abundant clear cytoplasm (A, *detail bottom right*) with frequent plasmacytic differentiation and plasma cells filled with immunoglobulin ("Mott cells") (B). The normal lymph node does not have a marginal zone, but primary nodal marginal zone lymphomas (NMZL) can occur. They infiltrate the node in what would be a marginal zone pattern with an expansion of cells (C) peripheral to mantle zone seen at the right side. The spleen does however have a normal marginal zone, and this can give rise to a splenic marginal zone lymphoma (SMZL). Early on, these show expansion of the marginal zone areas (D). Advanced cases show marked splenomegaly with miliary nodular structures replacing the spleen (E). There is marked expansile nodular replacement of the entire white pulp (F) with expression of IgD (G), a feature more common in SMZL compared to NMZL.

causal relationship has not yet been demonstrated. Chronic antigen stimulation is critical to both the development of a MALT lymphoma and the maintenance of the neoplastic state. Indeed, in some cases, antibiotic therapy and the eradication of *Helicobacter pylori* have led to the spontaneous remission of gastric MALT lymphoma in cases lacking genetic aberrations.

Immunophenotype MALT lymphomas are positive for B-cell-associated antigens CD19, CD20, and CD22, but are negative for CD5 and CD10. The absence of cyclin D1 is useful in ruling out MCL, especially in intestinal disease. Rare cases of MALT lymphoma have been reported to be CD5$^+$, and in some but not all instances, this has been associated with more aggressive disease. The clinical significance of increased transformed cells is still uncertain, and no formal grading system exists for MALT lymphoma. The putative cell of origin of MALT lymphoma is a postgerminal center B-cell.

MALT lymphomas also have several recurring cytogenetic abnormalities, including t(11;18)(q21;q21), t(1;14)(p22;q32), t(14;18)(q32;q21), t(3;14)(q27;q32), and t(3;14)(p14.1;q32), which are observed with variable frequency, often depending upon the anatomic site. Although several genes are involved in these translocations, at least three of them, t(11;18), t(1;14), and t(14;18), share a common pathway, leading to the activation of NF-κB and its downstream targets. By genome-wide DNA profiling integrated with GEP, differences were detected among the three different main types of marginal zone lymphomas, lending support to the current WHO classification that separates these three entities.[15]

Nodal Marginal-Zone B-Cell Lymphoma

Nodal marginal zone lymphoma (NMZL) is a primary nodal disease that resembles other marginal zone lymphomas, extranodal or splenic types. These patients often present with bone marrow involvement and tend to have a more aggressive clinical course than those with extranodal MALT. The neoplastic proliferation is polymorphous and composed of monocytoid B cells, plasmacytoid cells, with interspersed large blast-like cells. There is an expansion of the marginal-zone area, often with preservation of the nodal architecture (see Fig. 78.5C). The mantle zone may be intact, attenuated, or effaced. The immunophenotype is similar to other MZL, that is, CD20$^+$, CD10$^-$, CD5$^-$, with variable expression of IgD (weak to negative). Because there are no precise immunophenotypic or genotypic markers of NMZL, the diagnosis is sometimes one of exclusion. The differential diagnosis with LPL may be problematic; however, the MYD88 (L265P) somatic mutation is detected infrequently in MZL lymphomas and its presence should raise the possibility of LPL. More stringent criteria are needed to separate these two entities. A variant of nodal MZL occurs in children; these cases show a striking male predominance; present with localized disease, exhibiting a morphologic pattern; and can be managed with local therapies.

Splenic Marginal-Zone Lymphoma

Splenic marginal-zone lymphoma (SMZL) presents in adults and is slightly more frequent in females than males. The clinical presentation is splenomegaly, usually without peripheral lymphadenopathy. The majority of patients have marrow involvement, but there is usually only a modest lymphocytosis, with elevations in the lymphocyte count usually less than that seen in CLL. Some evidence of plasmacytoid differentiation may be seen, and patients may have a small M component. The abundant pale cytoplasm evident in tissue sections may also be seen in blood smears. The course is indolent, and splenectomy may be followed by a prolonged remission.

Histologically, the spleen shows expansion of the white pulp, but usually some infiltration of the red pulp is also present (see Fig. 78.5D).

A characteristic biphasic pattern in the neoplastic white pulp has been described, with the neoplastic cells surrounding regressed follicles. The immunophenotype of these cells resembles that of other marginal-zone B-cell lymphomas; however, IgD expression is more frequently observed. Progression to DLBCL can be seen.

Although the molecular pathogenesis of SMZL has not been fully delineated, a frequent cytogenetic alteration involving deletions of the region 7q(22-32) has been reported.[16] Mutations in *NOTCH2* are the most common event, but other genes in the NOTCH pathway may be targeted. Many of the affected genes appear to play a role in marginal zone B-cell development. The differential diagnosis of SMZL includes other unspecified B-cell lymphomas of the spleen, including splenic lymphoma with villous lymphocytes (SLVL), and hairy cell variant. The latter have been grouped together under splenic B-cell lymphoma/leukemia unclassifiable, and the interrelationship among these disorders is not fully resolved.

Diffuse Large B-Cell Lymphoma, Not Otherwise Specified

DLBCL is one of the more common subtypes of non-Hodgkin lymphoma, representing up to 40% of cases. It has an aggressive natural history but responds well to chemotherapy. The complete remission rate with modern regimens is 75% to 80%, with long-term disease-free survival approaching 50% or more in most series. This lymphoma may present in lymph nodes or in extranodal sites. Frequent extranodal sites of involvement include bone, skin, thyroid, GI tract, and lung.

DLBCL represents one of the most heterogeneous categories in the WHO, and attempts to identify prognostic groups based on morphology and phenotype have shown limited usefulness and reproducibility (see box on Varied Basis for the Recognition of Diverse Entities). To address these issues, DLBCLs were among the first cases to be analyzed by complementary DNA (cDNA) array technology, and more recently also by genome-wide analysis. By GEP, three groups were identified based on the differential expression of a large set of genes, namely germinal center-like group (GCB), activated B-cell–like group (ABC), and primary mediastinal (thymic) large B-cell lymphoma (PMBL). PMBL is now recognized as a separate entity, and adaptations in GEP now allow profiling of formalin-fixed paraffin-embedded (FFPE) biopsies.[17] The ABC subtype frequently exhibits mutations in the BCR-signaling and NF-κB pathways providing new insight in the pathogenesis of DLBCL and new potential therapeutic targets.[18] Recurrent mutations in the GCB type of DLBCL appear to target histone-modifying genes.[19] Somatic mutations in *EZH2* also have been identified in FL, another tumor of germinal center derivation.

VARIED BASIS FOR THE RECOGNITION OF DIVERSE ENTITIES AMONG AGGRESSIVE B-CELL NEOPLASMS

- Cell of origin, in part as determined by gene expression profiling
  Activated B cell versus germinal center B cell
  Thymic B cell of PMBL
- Clinical factors
  Anatomic site, e.g., CNS, mediastinum, intravascular
  Advanced age, background of chronic inflammation
- Etiologic factors
  EBV, HHV-8
- Molecular pathogenesis
  ALK, BCL2, BCL6, C-MYC, MYD88 (translocations, amplification, mutation)

CNS, Central nervous system; *EBV*, Epstein–Barr virus; *HHV-8*, Human Herpesvirus-8; *PMBL*, Primary mediastinal large B-cell lymphoma.

DLBCLs are composed of large, transformed lymphoid cells with nuclei at least twice the size of a small lymphocyte (Fig. 78.6). The nuclei generally have vesicular chromatin, prominent nucleoli, and basophilic cytoplasm, resembling the centroblasts of the normal germinal center. The immunoblastic variant is characterized by cells with prominent central nucleoli and abundant deeply staining cytoplasm. Although there is no absolute correlation between morphology and GEP, the majority of centroblastic DLBCL falls into the GCB group, and the majority of immunoblastic fall into the ABC group.

Algorithms based on immunophenotype have been proposed as surrogates for cDNA microarray using CD10/BCL6 positivity for GCB and MUM1/IRF-4 for ABC with the addition of BCL2 in combination with IPI and may improve the stratification of DLBCL. Because of emerging differences in the treatment of GCB versus ABC (or non-GCB) DLBCL, it is recommended to include the subtype by

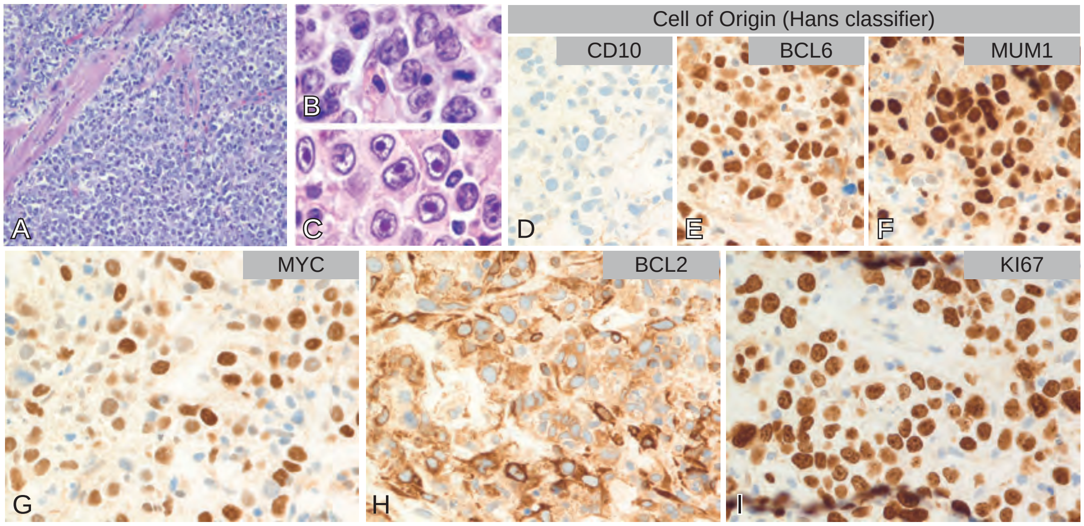


Figure 78.6 DIFFUSE LARGE B-CELL LYMPHOMA. The low-power illustration demonstrates the diffuse nature of the process (A). At high power (B,C), there are sheets of large cells. Those with a vesicular nuclear chromatin and variable numbers of nucleoli along the nuclear membrane are referred to as centroblasts (B) while cases with prominent central nucleoli are referred to as immunoblasts (C). The three markers used to assign cell of origin include CD10 (D), BCL6 (E), and MUM1 (F). This case corresponds to CD10-, BCL6+, MUM1+, non-germinal center phenotype. Additional prognostic markers include MYC (G), BCL2 (H), and KI67 (I). This case expresses 50% MYC (>40% considered positive), weak uniform BCL2 (>50% considered positive), and high proliferation of 90%. Based on the profile, this is a non-GCB, double-expressor of MYC and BCL2.

integrating GEP and/or IHC results in the report with clarification of the method used and the type of algorithm. As noted, newer GEP methods may be applicable to FFPE material.[17]

Diffuse Large B-Cell Lymphomas, Other Variants, and Subtypes

The spectrum of aggressive B-cell lymphomas has broadened in recent years, incorporating new entities based on unique clinical features such as age or anatomic site, viral pathogenesis (EBV, human herpesvirus [HHV]-8), or distinctive pathologic features.[20] *T-cell/histiocyte-rich large B-cell lymphoma (THRLBCL)* has distinctive morphologic and clinical features. It is associated with aggressive clinical behavior and often presents with advanced stage and bone marrow involvement. The relevance of the microenvironment and recruitment mechanism of the inflammatory cells, which are the main histologic component, has been the focus of recent studies.[21]

The WHO classification recognizes that some lymphomas arising in certain anatomic sites may have distinctive features both clinically and biologically. Among these are *primary DLBCL of the central nervous system (CNS)* and *DLBCL of the testis*. DLBCLs in these sanctuary sites differ at the genomic level from the usual nodal DLBCL. Both primary CNS DLBCL and intraocular large B-cell lymphomas commonly have mutations in MYD88.

Primary cutaneous DLBCL, leg type, has a GEP resembling the ABC type of DLBCL, presents most often in older adult females, and generally has an aggressive clinical course. As with nodal DLBCL, BCL2 expression is an adverse prognostic factor.

EBV-positive DLBCL was a provisional entity in the 2008 WHO classification and was accepted as a definitive entity in WHO 2017.[3] It was first recognized in the older adult.[22] More recent studies have shown a wider age distribution. Decreased immune surveillance in the older adult or a tolerogenic immune microenvironment may facilitate tumor development.[23] The morphologic spectrum is broad, but most cases have a prominent inflammatory background. The prognosis in the older adult is poor, but younger patients have a good outcome. EBV-positive DLBCL should be distinguished from EBV$^+$ mucocutaneous ulcer, which affects mainly cutaneous sites, and often has a self-limited clinical course. These lesions arise in a setting of decreased immune surveillance, most often in the elderly, and frequently with iatrogenic immune suppression.

Lymphomatoid granulomatosis is an EBV$^+$ B-cell lymphoproliferative disorder (LPD) associated with an inflammatory background rich in T cells. The lung is nearly always involved, with skin, kidney, liver, and brain being frequently affected as well. *DLBCL associated with chronic inflammation* was first described in association with chronic pyothorax but now has been associated with EBV-driven large B-cell proliferations in diverse clinical settings and is usually associated with a confined anatomic space and a background of chronic inflammation. These cases appear to have a good prognosis if successfully resected.

There are several LPDs associated with HHV-8/Kaposi sarcoma-associated herpesvirus (KSHV). These include *primary effusion lymphoma (PEL)* and *multicentric Castleman disease (MCD)*. The cells of PEL are usually coinfected with EBV, and the disease is most often diagnosed in the setting of human immunodeficiency virus (HIV) infection and immunosuppression. While pleural or peritoneal effusions are most common, extracavitary PEL can present as a tumor mass, usually in extranodal sites. PEL has a phenotype resembling that of terminally differentiated B-cells, i.e., plasmablastic (Fig. 78.7).

Two other lymphomas with a plasmablastic phenotype include *plasmablastic lymphoma (PBL)* and *anaplastic lymphoma kinase (ALK)-positive large B-cell lymphoma*. PBL is usually positive for EBV, most often extranodal, and associated with immunosuppression from either HIV infection or advanced age. Recent studies have identified a high incidence of *MYC* translocation in PBL.[24] ALK-positive large B-cell lymphomas show overexpression of ALK, usually as a consequence of translocation involving a partner gene other than *NPM1*. They mainly affect older individuals but can occur at any age. Interestingly, IgA is often expressed.

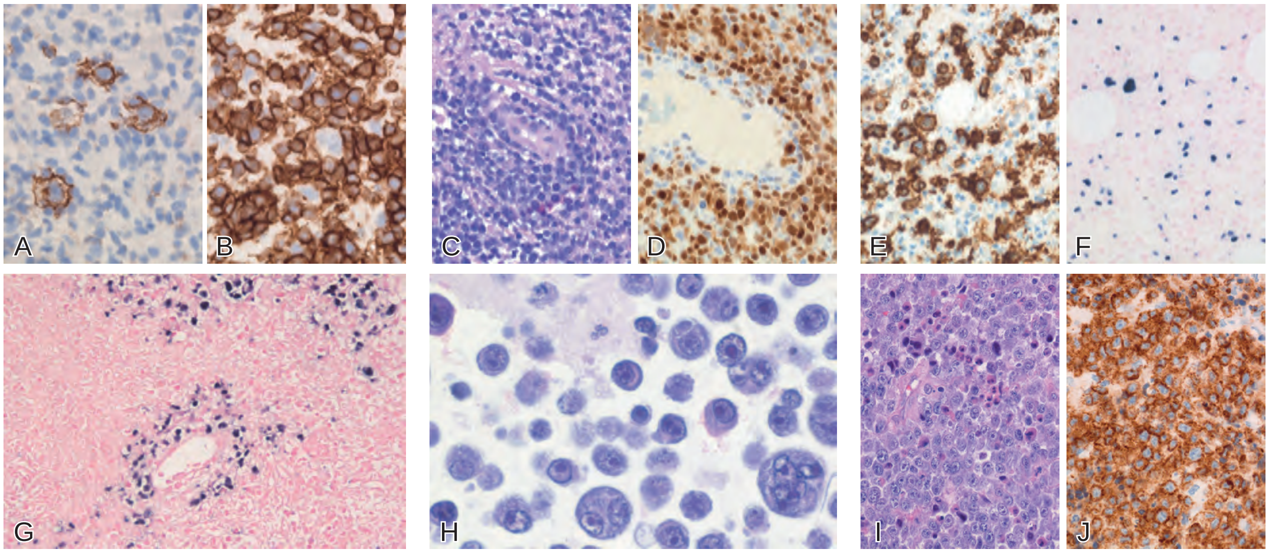


Figure 78.7 DIFFUSE LARGE B-CELL LYMPHOMA, VARIANTS, AND SUBTYPES. T-cell/histiocyte–rich large B-cell lymphoma is illustrated in (A, B), where a CD20 immunostain (A) identifies scattered large B cells, which are associated with a prominent background of small reactive T cells (CD3; B). Sometimes there are numerous histiocytes in the background or an admixture of reactive T cells and histiocytes. Primary diffuse large B-cell lymphoma (DLBCL) of the central nervous system usually shows a perivascular distribution (C) with frequent non-germinal center B-cell phenotype with expression of MUM1 (D). Epstein-Barr virus (EBV)–positive DLBCL can have variable morphologic features. The illustrated case is monomorphic and composed of large cells, which are positive for CD20 (E) and EBV-encoded RNA (EBER) (F). Note large Hodgkin-like EBER+ cells with a spectrum of size in positive cells. Lymphomatoid granulomatosis also has a perivascular distribution and is composed of a mix of malignant large EBV-positive B cells (G) and reactive T cells (not shown). Primary effusion lymphoma is usually diagnosed from cytologic preparations (H) and by flow cytometric and molecular techniques. Although the tumor cells do not generally form masses, extracavitary primary effusion lymphoma can present as a tumor mass, usually in extranodal sites. ALK$^+$ large B-cell lymphomas are rare and exhibit plasmablastic morphology (I) with ALK positivity as a consequence of a translocation of *ALK* (J). *ALK*, Anaplastic Lymphoma kinase; *RNA*, ribonucleic acid.

Intravascular Large B-Cell Lymphoma

Intravascular large B-cell lymphoma is a rare form of DLBCL characterized by the presence of lymphoma cells only in the lumens of small vessels, particularly capillaries (Fig. 78.8A). The neoplastic cells express B-cell related antigens including CD19 and CD20. The tumor cells are large, with vesicular nuclei and prominent nucleoli, resembling centroblasts or immunoblasts. Lymph node involvement is rare, and the tumor presents in extranodal sites, most readily diagnosed in the skin or bone marrow biopsy. Neurologic symptoms associated with the plugging of small vessels in the CNS are common. The disease is often not diagnosed until autopsy because of the lack of definitive radiologic or clinical evidence of disease and diverse symptomatology.

Primary Mediastinal (Thymic) Large B-Cell Lymphoma

PMBL has emerged in recent years as a distinct clinicopathologic entity, typically arising in young women, with a peak incidence in the fourth decade. Patients present with a mediastinal mass, with frequent superior vena cava syndrome. Regional lymph nodes may be involved but spread to distant nodal sites is uncommon. Frequent extranodal sites of involvement, particularly at relapse, include the liver, kidneys, adrenal glands, ovaries, GI tract, and CNS.

Histologically, PMBL is characterized by fine compartmentalizing sclerosis and large lymphoid cells with abundant pale cytoplasm (see Fig. 78.8C). An origin from medullary thymic B cells is proposed. The cells express CD20 and CD79a but do not express surface Ig. Recently, expression of the *MAL* gene has been detected in PMBL and not in other DLBCLs.[25] PMBL usually lack rearrangement for *BCL2*, *BCL6*; however, *REL* amplification is a common feature. A common cytogenetic abnormality seen in approximately 50% of cases includes gains in 9p, which may be associated with amplification of *JAK2* and *PDL1*/*PDL2* as well as translocations involving MHC class II transactivator (*CIITA*). Recently, GEP studies have found that PMBL bears a distinct molecular signature that differs from that of other DLBCLs and shares features of CHL; however, the same signature is not restricted to mediastinal sites since it can also be detected in other DLBCLs not otherwise specified (NOS) at nonmediastinal sites.

B-Cell Lymphoma, Unclassifiable, With Features Intermediate Between Diffuse Large B-Cell Lymphoma and Classic Hodgkin Lymphoma

This lymphoma is sometimes referred to as mediastinal gray zone lymphoma since the mediastinum is the most common site of presentation. A close relationship between PMBL and CHL was supported by GEP. TRAF1 expression and c-REL amplification were also seen in both types of neoplasms and could be detected with suitable immunohistochemical studies.

Gray zone lymphomas are more common in males than females, present with bulky mediastinal masses, and appear to have a more aggressive clinical course than PMBL or CHL. A recent study using methylation profiling identified gray zone lymphomas as having a signature distinct from both CHL and PMBL.[26] However, by fluorescence in situ hybridization, gray zone lymphomas, PBMCL and CHL share a number of common cytogenetic aberrations including gains at 2p16.1 (*REL*/*BCL11A* locus), 9p24.1 (*JAK2*/*PDL2*), and rearrangements of 16p13.13 (*CIITA*). It is not clear how these patients should be approached therapeutically, but they appear to benefit from combined modality therapy (systemic chemotherapy and radiation).

Burkitt Lymphoma

Burkitt lymphoma (BL) is most common in children and accounts for up to one-third of all pediatric lymphomas in the United States. It is the most rapidly growing of all lymphomas, with 100% of the cells in the cell cycle at any time. It usually presents in extranodal sites. In nonendemic regions, such as the United States, frequent sites of presentation are the ileocecal region, ovaries, kidneys, or breasts. Jaw presentations, as well as involvement of other facial bones, are common in African or endemic cases and are seen occasionally in nonendemic regions. Bone marrow involvement is a poor prognostic sign.

BL is one of the more common tumors associated with HIV. It can present at any time during the clinical course. In some patients with HIV infection, BL may be the initial acquired immunodeficiency syndrome-defining illness.

The pathogenesis of BL is related to the translocations involving the *MYC* oncogene, which are seen in virtually 100% of cases and often constitute the sole karyotypic abnormality. Most cases involve the IG heavy-chain gene on chromosome 14 and less frequently the light-chain genes on chromosomes 2 and 22. In endemic BL, genomic instability is thought to be promoted by *Plasmodium* infection.[27] EBV is closely linked to BL in endemic regions but is less frequently seen (15% to 20%) in sporadic cases. In other regions, characterized by low socioeconomic status and EBV infection at an early age, BL is often EBV$^+$, in the range of 50% to 70%. These data support the concept that the EBV is a cofactor for the development of BL. Cytologically, BL is monomorphic (Fig. 78.9). The cells are medium in size with round nuclei, moderately clumped chromatin, and multiple (2 to 5) basophilic nucleoli. The cytoplasm is deeply basophilic and moderately abundant. These cells contain cytoplasmic lipid vacuoles, which are probably a manifestation of the high rate of proliferation and high rate of spontaneous cell death. The starry sky pattern characteristic of BL is a manifestation of the numerous benign macrophages that have ingested karyorrhectic or apoptotic tumor cells.

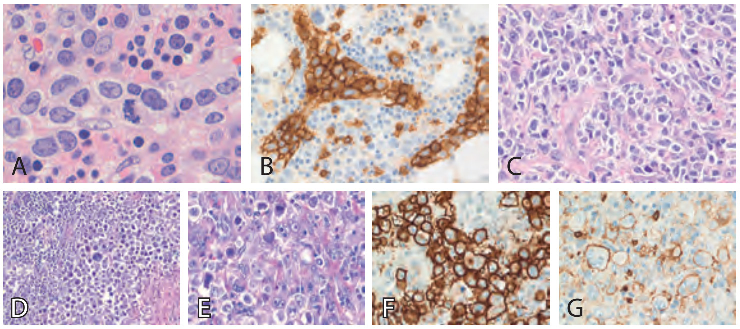


Figure 78.8 DIFFUSE LARGE B-CELL LYMPHOMA VARIANTS (INTRAVASCULAR, MEDIASTINAL, GRAY ZONE). In intravascular lymphoma, also known as angiotropic lymphoma, the large B cells are confined to the lumens of small vessels (A) and are sometimes very difficult to see in bone marrow biopsies and better seen on the CD20 stain (B). Paradoxically, they do not spread to the blood. Primary mediastinal (thymic) large B-cell lymphoma typically shows large B cells in a finely sclerotic background (C). So-called "gray zone lymphoma" has features intermediate between large B-cell lymphoma and Hodgkin lymphoma. In the case illustrated (D–G), the male patient presented with a mediastinal mass showing sheet-like DLBCL type areas (D) with other pleomorphic Hodgkin-like areas (E). The former areas are strongly CD20$^+$ (F) while the cHL-like areas express variable weak-negative CD20 (G). All cells were variably positive for CD30 in this case.

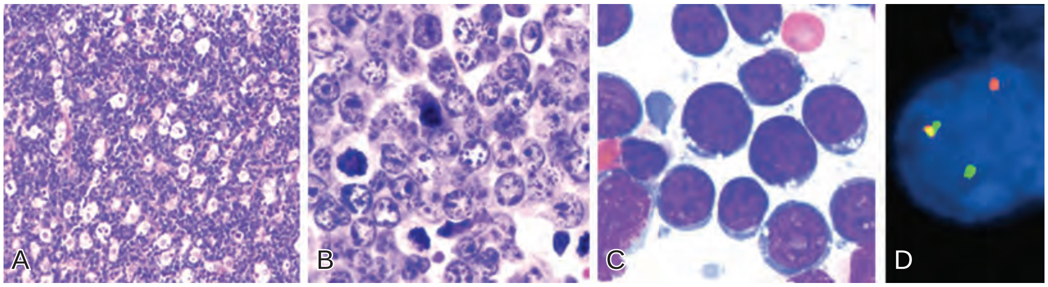


Figure 78.9 BURKITT LYMPHOMA. At low power, Burkitt lymphoma gives a classic "starry sky" appearance because of numerous histiocytes or tingible body macrophages with clear cytoplasm *(stars)*, in a background of darkly stained tumor cells (A). At high power, the cells exhibit a very high mitotic rate and are intermediate in size with finely clumped nuclear chromatin (B). On a Wright-stained touch preparation or in the blood or bone marrow aspirate, the cells also have a characteristic appearance with deep blue cytoplasm typically with vacuoles (C). Fluorescence in situ hybridization with a probe that spans *MYC* will show a break-apart signal (D) indicating that *MYC* has translocated to a partner chromosome.

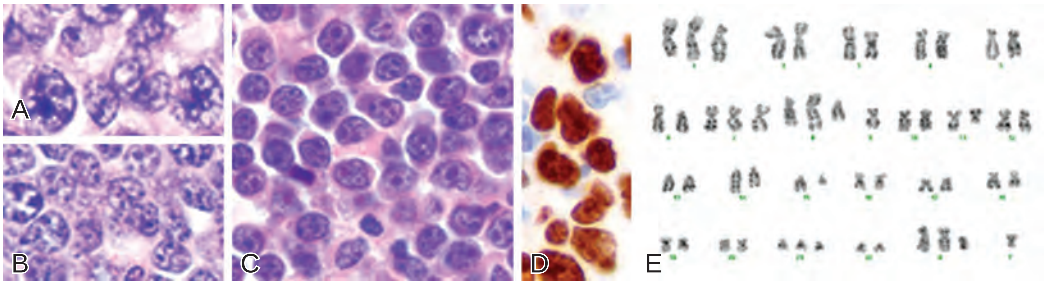


Figure 78.10 HIGH-GRADE B-CELL LYMPHOMA WITH *MYC* AND *BCL2* AND/OR *BCL6* REARRANGEMENTS. Examples of diffuse large B-cell lymphoma (DLBCL) (A) and Burkitt lymphoma (BL) (B) are for comparison. In (C), the lymphoma cells are intermediate in size and not as large as the DLBCL but without the typical characteristics of BL cells. The cells had a high Ki67 rate (D) and a B-cell phenotype with CD10 and BCL2 expression (not shown). The karyotype had a t(14;18) as in follicular lymphoma, MYC translocation as in BL, but was complex with multiple aberrations. The karyotype (E) was as follows: 51,XY,+X,+1,dup(1)(q32q44),der(1)del(1)(p21p36.3)dup(1)(q32q44),t(6;8)(p21.1;q24.1),+7,+del(?8) (p11.2p23),der(8)i(8)(q10)t(8;11)(q24.1;q13),9,der(11)t(8;11)(q24.1;q13), t(14;15)(q32;q15), t(14;18)(q32;q21.3),+21,+mar[13]/46,XY[1]. *(The karyotype was kindly provided by Dr. Yanming Zhan of Northwestern University.)*

BL has a mature B cell phenotype. The cells express CD19, CD20, CD22, CD79a, and monoclonal surface Ig, nearly always IgM. CD10 is positive in nearly all cases, and CD5, CD23, and BCL2 are consistently negative.

High-Grade B-Cell Lymphoma, with *MYC* and *BCL2* and/or *BCL6* Rearrangements

Historically, it has been difficult for pathologists to distinguish some DLBCL with a very high growth fraction from BL with atypical cytology. In addition, there are cases that carry a *C-MYC* translocation but carry additional cytogenetic abnormalities, most often involving *BCL2* or *BCL6*. These double-hit and triple-hit lymphomas have a very aggressive clinical course and have been separated out in the revised 2017 WHO classification (Fig. 78.10).[3] The clinical impact of *MYC* overexpression in DLBCL has not been fully resolved; in some series, it has been associated with a more aggressive clinical course. For the time being, an otherwise typical DLBCL with a *C-MYC* translocation should still be classified as DLBCL. Cases of BL with atypical cytologic features are retained under the heading of BL and have a better prognosis when treated appropriately.

T-CELL AND NATURAL KILLER-CELL LYMPHOMAS

Overview of the Classification of T-Cell Neoplasms

Although the definition of precursor T-cell or lymphoblastic neoplasms is straightforward, the classification of peripheral T-cell lymphomas has been controversial. These are uncommon, representing less than 15% of all non-Hodgkin lymphomas. Most previously published classification schemes for the malignant lymphomas in the United States or Europe have been based on B-cell malignancies, as these are far more common than their T-cell counterparts. T-cell and NK-cell lymphomas show significant variation in incidence in different geographic regions and racial populations.

The classification of T-cell and NK-cell neoplasms proposed by the WHO emphasizes a multiparameter approach, integrating morphologic, immunophenotypic, genetic, and clinical features. Clinical features play particular importance in the subclassification of these tumors, in part caused by the lack of specificity of other parameters (see box on Natural Killer and T-Cell Subsets).

NATURAL KILLER AND T-CELL SUBSETS AND THE CLASSIFICATION OF PERIPHERAL T-CELL AND NATURAL KILLER-CELL NEOPLASMS

Innate Immune System	Adaptive Immune System
Does not require antigen sensitization	Characterized by specificity and memory
NK-cells, NK/T-cells, gamma-delta T-cells	Effector and memory T-cells
Cell-mediated cytotoxicity	Act principally through cytokines and chemokines
Mainly cutaneous and other extranodal sites	Mainly nodal lymphomas
Children and adults	More often in adults

NK, Natural killer.

In contrast to B-cell lymphomas, specific immunophenotypic profiles are not associated with most T-cell lymphoma subtypes. Although certain antigens are commonly associated with specific disease entities, these associations are not entirely disease-specific. Presently, specific genetic features have not been identified for many of the T-cell and NK-cell neoplasms, although there are few exceptions.

Angioimmunoblastic T-Cell Lymphoma

Angioimmunoblastic T-cell lymphoma (AITL) was initially proposed as an abnormal immune reaction or form of atypical lymphoid hyperplasia with a high risk of progression to malignant lymphoma. Because the majority of cases show clonal rearrangements of T-cell receptor genes, it is now regarded as a variant of T-cell lymphoma. The median survival is generally less than 5 years.

AITL presents in adults; most patients have generalized lymphadenopathy, hepatosplenomegaly, skin rash, and prominent constitutional symptoms. There is usually polyclonal hypergammaglobulinemia and other hematologic abnormalities such as Coombs-positive hemolytic anemia. Rituximab has been used in some recent clinical trials in an attempt to control some of the effects of B-cell hyperactivity in this disease. Patients may also show evidence of immunodeficiency with recurrent opportunistic infections that may ultimately lead to their demise.

The nodal architecture is generally effaced, but peripheral sinuses are often open and even dilated. At low power, there is usually a striking proliferation of high endothelial venules (HEV) with prominent arborization (Fig. 78.11A). Follicles are typically regressed, but there is a proliferation of dendritic cells around HEV. The atypical T-cells have clear cytoplasm and are associated with small lymphocytes, immunoblasts, plasma cells, and histiocytes. The abnormal T-cells are usually positive for CD3, CD4, CD10, and CD279 (PD-1), a phenotype characteristic of follicular T-helper cells. This relationship is also confirmed by recent GEP data. CXC-chemokine ligand 13, a chemokine involved in B-cell trafficking into the germinal centers, is also expressed in AITL.

EBV$^+$ large B-cell blasts, sometimes with Reed–Sternberg (RS) like features, are nearly always present in the background, and progression to EBV-positive DLBCL has been reported in rare cases. Atypical B-cell proliferations that are negative for EBV also occur, presumably related to the T-helper follicular function of the neoplastic cells in promoting the activation and migration of bystander B cells. However, the exact role of EBV in AITL remains uncertain. Recently, recurrent mutations have been reported in AITL; these include mutations in *IDH2*, *TET2*, and *RHOA*.

The biologic spectrum of AITL is greater than previously thought and other entities related to follicular T-helper cells are considered within the same group. The updated version of the WHO 2017 classification recognizes AITL, follicular T-cell lymphoma, and nodal peripheral T-cell lymphoma of T-follicular helper phenotype, all within the umbrella of nodal T-cell neoplasm of T-helper follicular origin given their similar underlying molecular alterations.[3]

Adult T-Cell Leukemia/Lymphoma

Adult T-cell leukemia/lymphoma (ATL) is a distinct clinicopathologic entity associated with the retrovirus human T-lymphotropic virus-1 (HTLV-1), which is found clonally integrated into the T cells. HTLV-1 infection is endemic in Southwestern Japan and in the Caribbean basin. The disease has a long latency, and affected individuals are usually exposed to the virus very early in life. The virus may be transmitted in breast milk, and through exposure to blood and blood products. The cumulative incidence of ATL is estimated to be 2.5% among HTLV-1 carriers.

The median age of affected individuals is 45 years. Patients may present with leukemia or with generalized lymphadenopathy. Other clinical findings include lymphadenopathy, hepatosplenomegaly, lytic bone lesions, and hypercalcemia. Cutaneous involvement is seen in the majority of patients. The acute form of the disease is associated with a poor prognosis and a median survival of less than 2 years. Complete remissions may be obtained but the relapse rate is nearly 100%.

Chronic and smoldering forms of the disease are seen less commonly and are associated with minimal lymphadenopathy. The predominant clinical manifestation is skin rash, with only small numbers of atypical cells in the peripheral blood.

The cytologic spectrum of ATL is extremely diverse. The cells are often markedly polylobated and have been referred to as flower cells. (see Fig. 78.11B). Peripheral blood involvement is very common and occurs often in the absence of bone marrow disease. Immunophenotypically, the neoplastic cells are positive for mature T-cell antigens, such as CD2, CD3, and CD5; they are typically CD4/CD25$^+$, a phenotype that resembles regulatory T (Treg) cells. Some cases express FoxP3, but usually in a minority of tumor cells. The function of the tumor cells as Treg cells may correlate with the associated immunodeficiency.

Peripheral T-Cell Lymphomas, Not Otherwise Specified

PTCL, NOS is a diagnosis of exclusion and is admittedly a heterogeneous category with most cases being nodal in origin. Therefore, the cytologic spectrum is very broad (see Fig. 78.11C).[28] An inflammatory background is frequent, consisting of eosinophils, plasma cells, and histiocytes. If the epithelioid histiocytes are numerous and clustered, the neoplasm fulfills the criteria for the *lymphoepithelioid cell variant* of PTCL.

Clinically, PTCL, NOS most often presents in adults with generalized lymphadenopathy, hepatosplenomegaly, and frequent bone marrow involvement. Constitutional symptoms, including fever and night sweats, are common, as is pruritus. The clinical course is aggressive, although complete remissions may be obtained with

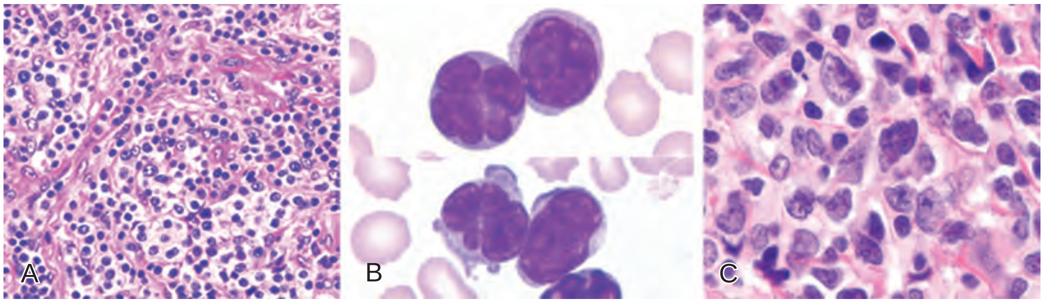


Figure 78.11 PERIPHERAL T-CELL LYMPHOMAS. In angioimmunoblastic T-cell lymphoma, the lymph node shows effacement with an arborizing vascular proliferation of postcapillary venules and clustered large cells with clear cytoplasm in the background of plasma cells, immunoblasts, and small lymphocytes (A). The peripheral blood in adult T-cell leukemia/lymphoma has classic "flower-cells" (B). Peripheral T-cell lymphoma, not otherwise specified is heterogeneous, but typically there is a mixture of small and large neoplastic T cells (C).

combination chemotherapy frequently including etoposide and brentuximab (anti-CD30 antibody conjugate). However, the relapse rate is higher than in aggressive B-cell lymphomas, including DLBCL.

PTCL, NOS, as defined in the WHO classification, remains heterogeneous. It is likely that individual clinicopathologic entities will be delineated in the future from this broad group of malignancies. Delineated separately from this category are tumors with a T-follicular helper (TFH) cell phenotype including the follicular T-cell lymphoma. Most cases have a mature T-cell phenotype and express one of the major subset antigens: CD4 more frequently than CD8 with loss of one of the pan T-cell antigens (CD3, CD5, CD2, or CD7) seen in 75% of cases, with CD7 most frequently being absent. GEP studies have shown some cases with a profile resembling AITL. Cases with a high proliferation signature appear to have a more aggressive clinical course, but GEP has not led to the delineation of distinctive subtypes as independent entities.

Anaplastic Large Cell Lymphoma, Anaplastic Lymphoma Kinase-Positive

Anaplastic large cell lymphoma (ALCL) is characterized by pleomorphic or monomorphic cells, which have a propensity to invade lymphoid sinuses (Fig. 78.12). Because of the sinusoidal location of the tumor cells and their lobulated nuclear appearance, this disease when first observed was suspected to be of histiocytic origin. A consistent feature is the strong expression of CD30 antigen, a diagnostic hallmark. However, CD30 expression is not specific for ALCL and can be seen in a variety of conditions, including, of course, CHL. Systemic ALCL is associated with a characteristic chromosomal translocation, t(2;5)(p23;q35) involving *NPM/ALK* genes, respectively. A number of variant translocations have been identified that involve partners other than *NPM*. All lead to overexpression of *ALK*, although the cellular distribution of ALK varies according to the gene partner.

The cells of classic ALCL have large, often lobulated nuclei with small basophilic nucleoli, so-called hallmark cells. The cytoplasm is usually abundant, amphophilic, and there are distinct cytoplasmic borders. A prominent Golgi region is generally visible. Small cell and lymphohistiocytic variants constitute part of the entity and appear to be associated with a more aggressive clinical course.

The cells exhibit an aberrant phenotype with loss of many of the T-cell-associated antigens. Both CD3 and CD5 are negative in greater than 50% of cases. CD2 and CD4 are positive in the majority of cases. CD8 is usually negative. ALCL cells, despite the $CD4^+/CD8^-$ phenotype, exhibit positivity for the cytotoxic associated antigens TIA-1, granzyme B, and perforin. In addition, clusterin is generally present in ALCL and represents another useful diagnostic marker. By molecular studies, in most of the cases, a clonal T-cell receptor rearrangement is found, supporting T-cell origin despite negative CD3.

ALK-positive ALCL is most common in children with a marked male predominance and incidence of ALK positivity drops sharply in young adults and older. Although most patients present with nodal disease, a high incidence of extranodal involvement has been reported (involving skin, bone, and soft tissue). Approximately 75% of cases present with advanced-stage and systemic symptoms. Although these lymphomas have an aggressive natural clinical history, they respond well to chemotherapy. Overall survival and disease-free survival are significantly better among ALK^+ versus ALK^- cases. Both ALK^+ and ALK^- ALCL have a better prognosis than other PTCLs, with a plateau in the survival curve seen in both groups.[29]

Anaplastic Large Cell Lymphoma, ALK-Negative

It has been controversial whether ALCL negative for ALK is a separate entity or part of the spectrum of PTCL, NOS. Part of the controversy relates to the lack of absolute criteria to recognize these cases. They should be morphologically (i.e., sinusoidal and cohesive growth pattern, presence of hallmark cells) and phenotypically similar to ALK^+ ALCL, with strong CD30 expression. A cytotoxic phenotype is common but is not essential. They occur in an older age group than the ALK^+ and, as noted earlier, appear to have a better prognosis than other PTCL, NOS. Recent molecular/cytogenetic studies have identified candidate genes *TP63* and *DUSP22*; the latter is associated with a more favorable outcome. Both ALK^+ and ALK^- ALCL appear to share activation of the JAK/STAT pathway.[30]

Primary Cutaneous Anaplastic Large Cell Lymphoma

The primary cutaneous form of ALCL is closely related to lymphomatoid papulosis (LYP) and differs clinically, immunophenotypically, and at the molecular level from the systemic form. LYP and cutaneous ALCL are part of the spectrum of $CD30^+$ cutaneous T-cell lymphoproliferative diseases. Small lesions are likely to regress. Patients with large tumor masses may develop disseminated disease with lymph node involvement. However, primary cutaneous ALCL is a more indolent disease than other T-cell lymphomas of the skin. Because the skin nodules may show spontaneous regression, usually a period of observation is warranted before the institution of any chemotherapy. Cutaneous ALCL is $CD30^+$ but ALK^-, lacking translocations involving the *ALK* gene. However, recent studies have identified rearrangements of *DUSP22* in a subset of cutaneous ALCL.

Mycosis Fungoides/Sézary Syndrome

Mycosis fungoides/Sézary syndrome (MF/SS) are now regarded as separate diseases but are closely related and often considered together from a clinical and biologic standpoint.[31] Both are primary cutaneous T-cell malignancies derived from mature CD4 positive skin-homing T cells. Skin involvement may be manifested as multiple cutaneous plaques or nodules. SS is characterized by erythroderma and a leukemic phase. Lymphadenopathy is usually not present at presentation

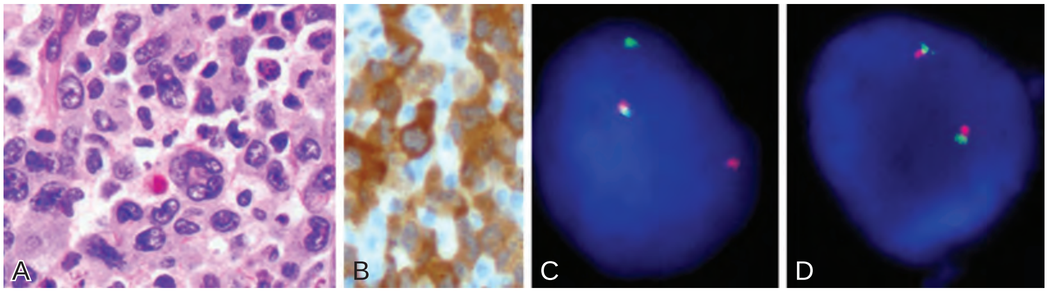


Figure 78.12 ANAPLASTIC LARGE CELL LYMPHOMA. Anaplastic large cell lymphoma can show a wide spectrum in cell size, and there is a small cell variant in addition to the more typical common form. The cells (A) include "wreath cells" *(center)* and "hallmark cells" *(bottom right)*. (B), The presence of ALK staining with nuclear and cytoplasmic localization *(right)* is associated with the t(2;5). A translocation of *ALK* can be identified with a break-apart probe that spans *ALK* but is split when *ALK* is translocated to one of a number of partners. A cell with a translocated *ALK* is pictured in (C) and a normal cell in (D). *ALK*, Anaplastic lymphoma kinase.

and, when identified, is associated with a poor prognosis. In early stages, enlarged lymph nodes may only show very minimal morphologic changes (Category I). If malignant cells are present in significant numbers and are associated with architectural effacement (Category II or III), the prognosis is significantly worse.

Cytologically, the small cells of MF demonstrate cerebriform nuclei with clumped chromatin, inconspicuous nucleoli, and sparse cytoplasm. Epidermotropism is usually a prominent feature (Fig. 78.13). SS presents with exfoliative erythroderma and circulating cerebriform lymphocytes known as Sézary cells. The typical phenotype is CD2$^+$, CD3$^+$, CD5$^+$, CD4$^+$, and CD8$^-$. However, CD8$^+$ variants of MF have been described and are more common in children. The absence of CD7 is a constant feature but may also be seen in reactive conditions and therefore is of limited diagnostic value. Aberrant expression of other T-cell antigens may be seen but mainly occurs in the advanced (tumor) stages. Inactivation of p16 (*CDKN2A*) and *PTEN* has been identified in some cases and may be associated with disease progression.

Subcutaneous Panniculitis-Like T-Cell Lymphoma

Subcutaneous panniculitis-like T-cell lymphoma (SPTCL) usually presents with subcutaneous nodules, primarily affecting the extremities and trunk. The nodules range in size from 0.5 cm to several centimeters in diameter. In its early stages, the infiltrate may appear deceptively benign, and lesions are often misdiagnosed as panniculitis. However, histologic progression usually occurs and subsequent biopsies show more pronounced cytologic atypia, permitting the diagnosis of malignant lymphoma.

Atypical lymphoid cells rim individual fat cells. Admixed reactive histiocytes are frequently present, particularly in areas of fat infiltration and destruction. Vascular invasion may be seen in some cases, and necrosis and karyorrhexis are common (see Fig. 78.13C).

The neoplastic cells are CD8$^+$ T alpha-beta cells, with tumors composed of gamma-delta T-cells now included under primary cutaneous gamma-delta T-cell lymphomas. The cells display an activated cytotoxic immunophenotype (positive for TIA-1, granzyme-B, and perforin). These proteins may be responsible for the cellular destruction seen in these tumors.

A hemophagocytic syndrome is less often seen in SPTCL than in panniculitis-like tumors of gamma-delta T-cell derivation, but whenever seen, it is associated with an adverse prognosis.[32] Patients present with fever, pancytopenia, and hepatosplenomegaly. The cause of the hemophagocytic syndrome appears related to cytokine production by the malignant cells with recent evidence indicating germline mutations in the T-cell Ig mucin 3 (*TIM-3*) gene in many cases.[33]

Primary Cutaneous Gamma-Delta T-Cell Lymphoma

Primary cutaneous gamma-delta T-cell lymphoma is considered a distinct entity, which can involve the subcutis, the dermis, or with epidermal infiltration. These are clinically aggressive tumors. The cells have a cytotoxic phenotype, and like normal gamma delta T-cells, lack CD5, are positive for TCR-gamma, and express cytotoxic molecules. They may be CD8$^+$ or more often double negative for CD4 and CD8. It is important to rule out MF and LYP before rendering a diagnosis of primary cutaneous gamma-delta T-cell lymphoma since there are newly recognized forms of LYP that share morphologic and phenotypic features. While skin is the most common presenting site, lymphomas of gamma delta T-cell origin can present in other mainly extranodal sites. The cells are invariably EBV$^-$ and show clonal rearrangement of T-cell receptor genes.

Primary Cutaneous CD8$^+$ Aggressive Epidermotropic Cytotoxic T-Cell Lymphoma and Primary Cutaneous CD4$^+$ Small/Medium T-Cell Lymphoproliferative Disorder

Primary cutaneous CD8$^+$ aggressive epidermotropic cytotoxic T-cell lymphoma is an aggressive cutaneous neoplasm that shares many clinical features with primary cutaneous gamma delta T-cell lymphomas but is derived from cytotoxic alpha-beta T cells. As the term implies, the neoplastic cells show prominent epidermotropism.

Primary cutaneous CD4$^+$ small/medium T-cell LPD most often presents with localized skin lesions. It is associated with an excellent prognosis and requires only limited localized therapy unless multiple skin lesions are present. The term LPD rather than lymphoma is used, reflecting the indolent nature of the proliferation. The lesions are rich

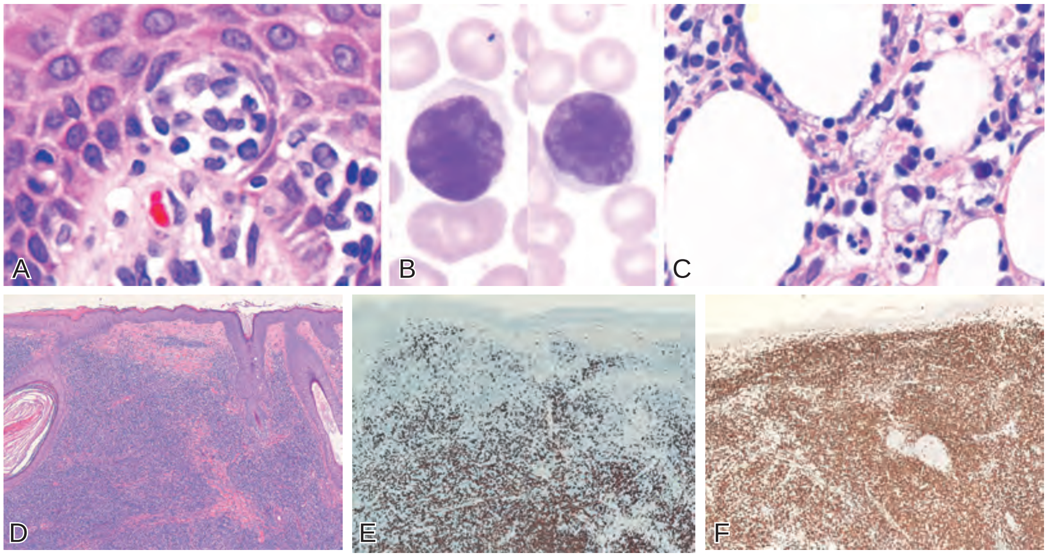


Figure 78.13 CUTANEOUS T-CELL LYMPHOMAS: MYCOSIS FUNGOIDES/SÉZARY SYNDROME AND SUBCUTANEOUS PANNICULITIS-LIKE T-CELL LYMPHOMA. In mycosis fungoides, there is a dermal infiltrate with some malignant T cells infiltrating into the epithelium (Pautrier microabscesses) (A). Sézary cells with convoluted nuclear folding are seen in (B). The peripheral nuclear outline is fairly rounded, but the internal nuclear detail shows complex nuclear folding giving rise to a convoluted and cerebriform look. A case of subcutaneous panniculitis-like T-cell lymphoma is illustrated and shows an abnormal lymphoid infiltrate in the subcutaneous fat (C). Fat necrosis is usually evident. Primary cutaneous small medium T-cell lymphoproliferative disorder is characterized by dense dermal infiltrate of small–medium sized lymphoid cells without epidermotropism (D). Numerous background CD20$^+$ B-cells (E) are noted besides CD3$^+$ T cells (F).

in B cells, and the proliferating T cells have a TFH phenotype, with clonality usually evident by molecular testing.

Enteropathy-Associated T-Cell Lymphoma

Two variants of enteropathy-associated T-cell lymphoma (EATL) were recognized in the WHO 2008, EATL Type I and Type II. The classic Type I form of EATL is associated with either overt or clinically silent gluten-sensitive enteropathy and is largely seen in patients of European extraction, whereas the Type II form has a more worldwide distribution. In recognition of its distinction from EATL Type I, the revised WHO classification designates these as "monomorphic epitheliotropic intestinal T-cell lymphoma." Patients usually present with abdominal symptoms, including pain, small bowel perforation, and associated peritonitis. The clinical course is aggressive, and most patients have multifocal intestinal disease.[28,34]

In EATL, Type I (Fig. 78.14A), the cytologic composition is somewhat varied; the neoplastic cells show prominent invasion of the mucosa and are cytotoxic T cells most often of alpha-beta origin. The cells also express the homing receptor CD103 (HML-1). Cells with anaplastic features positive for CD30 may be present. In EATL Type I, the adjacent small bowel usually shows villous atrophy associated with celiac disease. In EATL Type II, (now monomorphic epitheliotropic intestinal T-cell lymphoma), the infiltrate is monomorphic and composed of medium-sized cells with clear cytoplasm showing prominent epitheliotropism. They are CD8$^+$, CD56$^+$, and most often of gamma-delta T-cell derivation. An association with celiac disease is seen only rarely, and this form of intestinal lymphoma is relatively common in Asia. Both EATL Types I and II share some genetic aberrations, including chromosomal gains on 9q33–34 as well as activating mutations involving the JAK/STAT pathway.

Other PTCL can present with intestinal disease and should be distinguished from EATL. These include the EBV$^+$ extranodal T/NK-cell lymphomas, PTCL, NOS, and a rare indolent form of T-cell LPD of the GI tract recognized in the new WHO classification.[35]

Hepatosplenic T-Cell Lymphoma

Hepatosplenic T-cell lymphoma (HSTCL) presents with marked hepatosplenomegaly in the absence of lymphadenopathy. The great majority of cases are of gamma-delta T-cell origin. Most patients are male, with a peak incidence in young adults. Although patients may respond initially to chemotherapy, relapse has been seen in the vast majority of cases, and the median survival is less than 3 years. Rare long-term survival has been seen following allogeneic hematopoietic cell transplantation.

The cells of HSTCL are usually moderate in size, with a rim of pale cytoplasm. The nuclear chromatin is loosely condensed, with small inconspicuous nucleoli. The pattern of infiltration mimics the homing pattern of gamma-delta T cells with marked sinusoidal infiltration in the liver and spleen. Abnormal cells are usually present in the sinusoids of the bone marrow but may be difficult to identify without immunohistochemical stains (see Fig. 78.14B). The neoplastic cells also have a phenotype that resembles that of normal resting gamma-delta T cells. They are often negative for both CD4 and CD8, although CD8 may be expressed in some cases. CD56 is typically positive. The neoplastic cells express markers associated with cytotoxic T cells, such as TIA-1. However, perforin and granzyme B are usually negative, suggesting that these cells are not activated. Isochromosome 7q is a consistent cytogenetic abnormality and is often seen in association with trisomy 8. In addition, activating mutations involving *STAT5B*, *SETD2*, and less frequently *STAT3* have been described.[36]

Extranodal Natural Killer/T-Cell Lymphoma, Nasal Type

Extranodal NK/T-cell lymphoma, nasal type, is a distinct clinicopathologic entity highly associated with EBV. It is much more common in Asians than in Europeans.[34] Clusters of the disease also have been reported in Central and South America in individuals of Native American heritage, suggesting that ethnic background i.e., genetic risk factors, may play a role in the pathogenesis of these lymphomas. It affects adults (median age 50), and the most common clinical presentation is a destructive nasal or midline facial lesion. Palatal destruction, orbital swelling, and edema may be prominent. NK/T-cell lymphomas have been reported in other extranodal sites, including skin, soft tissue, testis, upper respiratory tract, and GI tract. The clinical course is usually aggressive, with a slightly improved median survival in patients with localized disease, in which local radiation therapy may be useful.[37] A hemophagocytic syndrome is a common clinical complication and adversely affects survival.

Extranodal NK/T-cell lymphoma, nasal type, is characterized by a broad cytologic spectrum. Although the cells express some T-cell-associated antigens, most commonly CD2, other T-cell markers, such as surface CD3, are usually absent. The cells express cytoplasmic CD3 but lack T-cell receptor gene rearrangement. In support of an NK-cell origin, the cells are usually CD56$^+$ but do not express CD57 or CD16. EBV is positive in 100% of cases by in situ hybridization (see Fig. 78.14C).

Aggressive NK-cell leukemia is a closely related entity. It presents at a younger age than extranodal NK/T-cell lymphoma, is associated with systemic disease, and a fulminant clinical course. It has a similar phenotype, EBV association, and epidemiology.

There are other EBV$^+$ T-cell and NK-cell proliferations, which are seen mainly in children. These include *systemic EBV$^+$ T-cell lymphoma, hydroa vacciniforme-like lymphoma, and severe mosquito bite allergy*, the latter usually being derived from NK-cells. All are seen most often in Asian children but are also reported in Central and South America in individuals of Native American origin. The latter two conditions affect mainly the skin and have a more indolent clinical course, whereas the systemic disease has a very aggressive clinical

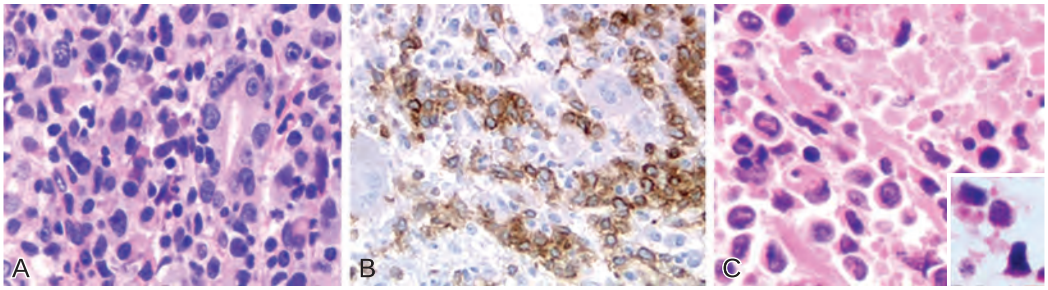


Figure 78.14 OTHER T-CELL AND NATURAL KILLER-CELL LYMPHOMAS. In enteropathy-associated T-cell lymphoma (A), there is an abnormal T-lymphoid proliferation with infiltration into the gastrointestinal glandular elements *(center right)*. Hepatosplenic gamma delta T-cell lymphoma in the bone marrow is illustrated with a CD2 stain showing the characteristic sinusoidal distribution (B). Extranodal natural killer cell/T-cell lymphoma typically has marked necrosis (C). The malignant cells are Epstein-Barr virus (EBV)–positive by in situ hybridization for EBV encoded RNA *(insert)*. *RNA*, Ribonucleic acid.

course with survival measured in weeks. Systemic EBV$^+$ T-cell lymphoma may arise in a background of chronic active EBV-infection.[3]

Hodgkin Lymphoma

Hodgkin and non-Hodgkin lymphoma have long been regarded as distinct disease entities based on their differences in pathology, phenotype, clinical features, and response to therapy. It is now accepted that the malignant cell of Hodgkin lymphoma (HL) is an altered B cell. Therefore, it is not surprising that both biologic and clinical overlaps should occur between these two lymphoma groups, as also shown by GEP in PMBL and cell lines derived from CHL. Although we have become aware of this closer relationship from the histogenetic point of view (hence the name HL), these disorders are still treated with different modalities. The preferred term of HL over Hodgkin disease reflects current knowledge concerning the nature of the neoplastic cell as a lymphocyte.

Sufficient evidence has emerged in recent years to warrant the recognition of nodular lymphocyte-predominant HL (NLPHL) as a distinct entity (discussed in the following section). Although it resembles other types of HL in having a minority of putative neoplastic cells on a background of benign inflammatory cells, it differs morphologically, immunophenotypically, and clinically from CHL. The diagnosis of CHL depends on the identification of Hodgkin and Reed-Sternberg (HRS) cells in an appropriate inflammatory background composed of small T lymphocytes, plasma cells, histiocytes, and granulocytes (often eosinophils). All cases of CHL share certain immunophenotypic and genotypic features. Neoplastic cells are CD30$^+$, CD15$^{+/-}$, CD45$^-$, and EMA$^-$. Expression of B-cell-associated antigens is seen in up to 75% of cases. However, when present, CD20 and PAX5 staining is weaker than that seen in normal B cells with variable intensity among individual tumor cells. CD79a and other B-lineage antigens (OCT2 and/or Bob.1) are downregulated and usually negative. IG and T-cell receptor genes are usually germline using whole sections, caused by the paucity of tumor cells in the inflammatory background; however, using microdissection and polymerase chain reaction (PCR) amplification clonal rearrangement of the Ig genes can generally be shown. In addition, the presence of somatic mutations indicates transit through the germinal center.

Nodular Lymphocyte-Predominant Hodgkin Lymphoma

NLPHL usually has a nodular growth pattern, with or without diffuse areas (Fig. 78.15). Nodularity may be more easily recognized using immunohistochemical stains with B-cell or follicular dendritic cell (FDC) specific antibodies. Concurrent progressively transformed germinal centers are often seen in partially involved lymph nodes or other lymph node sites. The atypical cells of NLPHL have vesicular, polylobated nuclei, and small nucleoli. These had been called lymphocytic and/or histiocytic (L&H) cells, or "popcorn" cells, but the term LP cell is now preferred. The background is predominantly lymphocytes with or without epithelioid histiocyte clusters. Plasma cells are infrequent, and eosinophils and neutrophils are rarely seen. Occasionally sclerosis may cause lesions to resemble nodular sclerosis.

The LP cells are CD45$^+$ expressing B-cell-associated antigens (CD19, CD20, CD22, CD79a), CDw75$^+$, EMA$^{+/-}$ CD15$^-$, CD30$^{-/+}$ and usually SIg$^-$ by routine techniques, although one study reported light-chain restriction. Neoplastic cells positive for IgD are more often found in young males. J chain has been demonstrated in many cases. Small lymphocytes in the nodules are predominantly B cells with a mantle-zone phenotype. However, numerous T cells are present, with T cells positive for CD57 and PD-1 (CD279) surrounding the LP cells. The proportion of T cells tends to increase over time in sequential biopsies. A prominent meshwork of FDC is present within the nodules. LP cells, when isolated by microdissection, have clonally rearranged Ig genes with evidence of somatic hypermutation.

NLPHL occurs at all ages, in adults more commonly than in children, and in males more than in females. It usually involves peripheral lymph nodes, with sparing of the mediastinum. It is usually localized at diagnosis but may be rarely disseminated. Survival is long, with or without treatment, for localized cases. However, when disseminated the prognosis is often poor. Patients with advanced-stage disease may benefit from treatment regimens used for aggressive B-cell lymphomas. Late relapses have been reported to be more common than in other types of HL; it may be associated with, or progress to, large B-cell lymphoma. Progression to a process resembling T-cell/histiocyte rich large B-cell lymphoma may also been seen, and recent data suggest that these diseases may be different ends of a spectrum, with a close biologic relationship,[38] and hence the new WHO 2017 recognized T-/HRLBCL-like transformation of NLPHL.[3]

Classic Hodgkin Lymphoma, Nodular Sclerosis

This variant is most common in adolescents and young adults, but can occur at any age; female cases equal or exceed those in males. The mediastinum is commonly involved; stage and bulk of disease have prognostic importance. Nodular sclerosis classic Hodgkin lymphoma (NSCHL) is often curable; however, in long-term survivors, the risk of secondary malignancies is increased, especially in those receiving both radiation and chemotherapy. NSCHL of the mediastinum is thought to be closely related to PMBL, and both types of tumors can be seen in the same patient, either as composite malignancy, or sequentially.[39]

The tumor has at least a partially nodular pattern, with fibrous bands separating the nodules in most cases (Fig. 78.16A). Diffuse areas may be present, as is necrosis. The characteristic cell is the

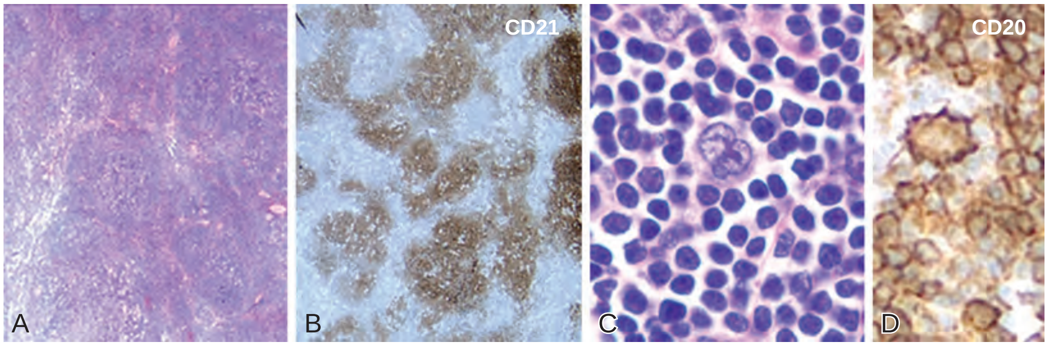


Figure 78.15 NODULAR LYMPHOCYTE PREDOMINANT HODGKIN LYMPHOMA. Low-power illustration shows vague expansile nodules that efface the lymph node architecture (A). The nodules can be accentuated with a stain for follicular dendritic cells, CD21 (B). The neoplastic cells are the so-called "LP" cells (previously called "L&H" cells or "popcorn" cells) (C). Unlike the neoplastic cells of classic Hodgkin lymphoma, these LP cells stain brightly for CD20 (D) and are typically CD45$^+$, CD30$^-$, and CD15$^-$. The current international consensus classification has indicated designation of NLPHL as nodular predominant B-cell lymphoma (NLPBL) to avoid confusion with classic Hodgkin lymphoma.

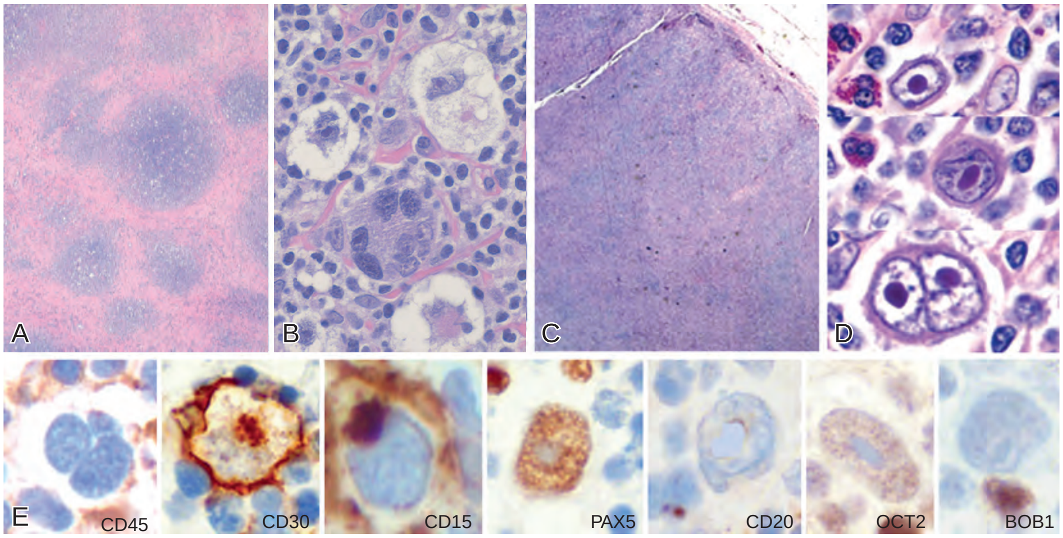


Figure 78.16 CLASSIC HODGKIN LYMPHOMA, NODULAR SCLEROSIS, AND MIXED CELLULARITY SUBTYPES. In nodular sclerosis Hodgkin lymphoma, broad bands of sclerosis typically divide the lymph node into cellular nodules (A). The nodules contain a mixed cellular infiltrate and scattered neoplastic cells with lobular nuclei and retracted cytoplasm (B). In mixed cellularity Hodgkin lymphoma, the lymph node is usually diffusely effaced with only fine fibrosis (C). Classic mononuclear, binuclear, and multinuclear Hodgkin and Reed-Sternberg cells are present (D). Hodgkin and Reed-Sternberg cells including lacunar forms are typically $CD45^-$, $CD30^+$, $CD15^+$, weakly $PAX5^+$, and $CD20^-$. The B-cell transcription factors OCT2 and BOB1 are variable but often negative (E).

lacunar-type RS cell, which may be very numerous. Diagnostic RS cells are usually also present. The background contains lymphocytes, histiocytes, plasma cells, eosinophils, and neutrophils. It can be graded according to the proportion of the tumor cells and the presence of necrosis: Grades I and II. However, grading is considered optional. The immunophenotype and genotype are characteristic of CHL. However, EBV is infrequently positive in less than 15% of cases.

Classic Hodgkin Lymphoma, Mixed Cellularity

Patients are usually adults; males outnumber females and the stage is often advanced. The course is moderately aggressive but is often curable. CHL, mixed cellularity (CHLMC) has a bimodal age distribution, with a peak in young children, and again in older adults. It is often EBV^+, seen in up to 75% of cases. Both CHLMC and the lymphocyte depleted form can be associated with underlying HIV infection. The infiltrate is diffuse, without band-forming sclerosis, although fine interstitial fibrosis may be present (see Fig. 78.16C). HRS cells are of the classic type.

Classic Hodgkin Lymphoma, Lymphocyte Depletion

This is the least common variant of CHL and is most common in older people, in HIV^+ individuals, and in non-industrialized countries. It frequently presents with abdominal lymphadenopathy, spleen, liver, and bone marrow involvement, without peripheral adenopathy. The stage is usually advanced at diagnosis. It shares many features with CHLMC and appears to represent a continuum with this variant and associated with more frequent neoplastic cells and fewer normal T cells. The infiltrate is diffuse and often appears hypocellular, owing to the presence of diffuse fibrosis and necrosis. Relative to the number of normal lymphocytes, there are large numbers of HRS cells and occasional bizarre “sarcomatous” variants, with a paucity of other inflammatory cells. The immunophenotype is characteristic of CHL. Since the histologic differential diagnosis often includes B or T-large-cell lymphoma or ALCL, IHC should be performed in most cases. EBV is positive in the majority of cases similar to the mixed cellularity subtype.

Classic Hodgkin Lymphoma, Lymphocyte-Rich

This type of CHL may be nodular or diffuse and contains relatively infrequent HRS cells, which are of the classic type, rather than the LP variants seen in NLPHL. There are infrequent eosinophils or plasma cells. In the nodular form, the HRS cells are seen at the periphery of B cell–rich nodules, mainly in the marginal zone. The neoplastic cells have the immunophenotype of classic HRS cells but morphologically may be difficult to distinguish from LP cells in some cases. Thus, in the past, many cases were misdiagnosed as NLPHL. The genetic features are similar to those of the other variants of CHL. Patients usually present with localized disease and tend to be older than patients with NLPHL.

ACKNOWLEDGMENT

The authors thank Manisha Goel, MBBS, MD, visiting hematopathologist, for proofing the final version and help with organizing the final images included in this chapter.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Attygalle AD, Cabeçadas J, Gaulard P, et al. Peripheral T-cell and NK-cell lymphomas and their mimics; taking a step forward - report on the lymphoma workshop of the XVIth meeting of the European Association for Haematopathology and the Society for Hematopathology. *Histopathology*. 2014;64(2):171–199.

Aukema SM, Hoster E, Rosenwald A, et al. Expression of TP53 is associated with the outcome of MCL independent of MIPI and Ki-67 in trials of the European MCL network. *Blood*. 2018;131(4):417–420.

Dojcinov SD, Venkataraman G, Pittaluga S, et al. Age-related EBV-associated lymphoproliferative disorders in the Western population: a spectrum of reactive lymphoid hyperplasia and lymphoma. *Blood*. 2011;117(18):4726–4735.

Eberle FC, Rodriguez-Canales J, Wei L, et al. Methylation profiling of mediastinal gray zone lymphoma reveals a distinctive signature with elements shared by classical Hodgkin's lymphoma and primary mediastinal large B-cell lymphoma. *Haematologica*. 2011;96(4):558–566.

Ganapathi KA, Pittaluga S, Odejide OO, et al. Early lymphoid lesions: conceptual, diagnostic and clinical challenges. *Haematologica*. 2014;99(9):1421–1432.

Hallek M. Chronic lymphocytic leukemia: 2020 update on diagnosis, risk stratification and treatment. *Am J Hematol*. 2019;94(11):1266–1287.

Harris NL. Shades of gray between large B-cell lymphomas and Hodgkin lymphomas: differential diagnosis and biological implications. *Mod Pathol*. 2013;26(Suppl 1):S57–S70.

Hartmann S, Döring C, Jakobus C, et al. Nodular lymphocyte predominant Hodgkin lymphoma and T cell/histiocyte rich large B cell lymphoma—endpoints of a spectrum of one disease? *PLoS One*. 2013;8(11):e78812.

Horn H, Schmelter C, Leich E, et al. Follicular lymphoma grade 3B is a distinct neoplasm according to cytogenetic and immunohistochemical profiles. *Haematologica*. 2011;96(9):1327–1334.

Hwang ST, Janik JE, Jaffe ES, et al. Mycosis fungoides and Sézary syndrome. *Lancet*. 2008;371(9616):945–957.

Jaffe ES, Harris NL, Stein H, et al. Classification of lymphoid neoplasms: the microscope as a tool for disease discovery. *Blood*. 2008;112(12):4384–4399.

Jares P, Colomer D, Campo E. Molecular pathogenesis of mantle cell lymphoma. *J Clin Invest*. 2012;122(10):3416–3423.

Louissaint Jr. A, Schafernak KT, Geyer JT, et al. Pediatric-type nodal follicular lymphoma: a biologically distinct lymphoma with frequent MAPK pathway mutations. *Blood*. 2016;128(8):1093–1100.

McKinney M, Moffitt AB, Gaulard P, et al. The genetic basis of hepatosplenic T-cell lymphoma. *Cancer Discov*. 2017;7(4):369–379.

Nicolae A, Pittaluga S, Abdullah S, et al. EBV-positive large B-cell lymphomas in young patients: a nodal lymphoma with evidence for a tolerogenic immune environment. *Blood*. 2015;126(7):863–872.

Parrilla Castellar ER, Jaffe ES, Said JW, et al. ALK-negative anaplastic large cell lymphoma is a genetically heterogeneous disease with widely disparate clinical outcomes. *Blood*. 2014;124(9):1473–1480.

Perry AM, Warnke RA, Hu Q, et al. Indolent T-cell lymphoproliferative disease of the gastrointestinal tract. *Blood*. 2013;122(22):3599–3606.

Robbiani DF, Deroubaix S, Feldhahn N, et al. Plasmodium infection promotes genomic instability and AID-dependent B cell lymphoma. *Cell*. 2015;162(4):727–737.

Salaverria I, Philipp C, Oschlies I, et al. Translocations activating IRF4 identify a subtype of germinal center-derived B-cell lymphoma affecting predominantly children and young adults. *Blood*. 2011;118(1):139–147.

Scott DW, Wright GW, Williams PM, et al. Determining cell-of-origin subtypes of diffuse large B-cell lymphoma using gene expression in formalin-fixed paraffin-embedded tissue. *Blood*. 2014;123(8):1214–1217.

Soldini D, Valera A, Solé C, et al. Assessment of SOX11 expression in routine lymphoma tissue sections: characterization of new monoclonal antibodies for diagnosis of mantle cell lymphoma. *Am J Surg Pathol*. 2014;38(1):86–93.

Steidl C, Gascoyne RD. The molecular pathogenesis of primary mediastinal large B-cell lymphoma. *Blood*. 2011;118(10):2659–2669.

Swerdlow SH, Campo E, Harris NL, et al. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. 4th ed. Lyon, France: IARC Press, 2008.

Swerdlow SH, Campo E, Harris NL, et al. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. revised*. 4th ed. Lyon: IARC Press, 2017.

Swerdlow SH, Jaffe ES, Brousset P, et al. Cytotoxic T-cell and NK-cell lymphomas: current questions and controversies. *Am J Surg Pathol*. 2014;38(10):e60–e71.

Valera A, Balagué O, Colomo L, et al. IG/MYC rearrangements are the main cytogenetic alteration in plasmablastic lymphomas. *Am J Surg Pathol*. 2010;34(11):1686–1694.

Van Loo P, Tousseyn T, Vanhentenrijk V, et al. T-cell/histiocyte-rich large B-cell lymphoma shows transcriptional features suggestive of a tolerogenic host immune response. *Haematologica*. 2010;95(3):440–448.

Vose J, Armitage J, Weisenburger D, et al. International peripheral T-cell and natural killer/T-cell lymphoma study: pathology findings and clinical outcomes. *J Clin Oncol*. 2008;26(25):4124–4130.

Willemze R, Jansen PM, Cerroni L, et al. Subcutaneous panniculitis-like T-cell lymphoma: definition, classification, and prognostic factors: an EORTC Cutaneous Lymphoma Group Study of 83 cases. *Blood*. 2008;111(2):838–845.

Xie Y, Pittaluga S, Jaffe ES. The histological classification of diffuse large B-cell lymphomas. *Semin Hematol*. 2015;52(2):57–66.

REFERENCES

1. Jaffe ES, Harris NL, Stein H, et al. Classification of lymphoid neoplasms: the microscope as a tool for disease discovery. *Blood*. 2008;112(12):4384–4399.
2. Swerdlow SH, Campo E, Harris NL, et al. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. 4th ed. Lyon, France: IARC Press; 2008.
3. Swerdlow SH, Campo E, Harris NL, et al. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, revised*. 4th ed. Lyon: IARC Press; 2017.
4. Strati P, Shanafelt TD. Monoclonal B-cell lymphocytosis and early-stage chronic lymphocytic leukemia: diagnosis, natural history, and risk stratification. *Blood*. 2015;126(4):454–462.
5. Ganapathi KA, Pittaluga S, Odejide OO, et al. Early lymphoid lesions: conceptual, diagnostic and clinical challenges. *Haematologica*. 2014;99(9):1421–1432.
6. Hallek M. Chronic lymphocytic leukemia: 2020 update on diagnosis, risk stratification and treatment. *Am J Hematol*. 2019;94(11):1266–1287.
7. Xiao W, Chen WW, Sorbara L, et al. Hodgkin lymphoma variant of Richter transformation: morphology, Epstein-Barr virus status, clonality, and survival analysis-with comparison to Hodgkin-like lesion. *Hum Pathol*. 2016;55:108–116.
8. Owen RG, Treon SP, Al-Katib A, et al. Clinicopathological definition of Waldenström's macroglobulinemia: consensus panel recommendations from the Second International Workshop on Waldenström's Macroglobulinemia. *Semin Oncol*. 2003;30(2):110–115.
9. Jares P, Colomer D, Campo E. Molecular pathogenesis of mantle cell lymphoma. *J Clin Invest*. 2012;122(10):3416–3423.
10. Soldini D, Valera A, Solé C, et al. Assessment of SOX11 expression in routine lymphoma tissue sections: characterization of new monoclonal antibodies for diagnosis of mantle cell lymphoma. *Am J Surg Pathol*. 2014;38(1):86–93.
11. Aukema SM, Hoster E, Rosenwald A, et al. Expression of TP53 is associated with the outcome of MCL independent of MIPI and Ki-67 in trials of the European MCL Network. *Blood*. 2018;131(4):417–420.
12. Horn H, Schmelter C, Leich E, et al. Follicular lymphoma grade 3B is a distinct neoplasm according to cytogenetic and immunohistochemical profiles. *Haematologica*. 2011;96(9):1327–1334.
13. Louissaint A Jr., Schafernak KT, Geyer JT, et al. Pediatric-type nodal follicular lymphoma: a biologically distinct lymphoma with frequent MAPK pathway mutations. *Blood*. 2016;128(8):1093–1100.
14. Salaverria I, Philipp C, Oschlies I, et al. Translocations activating IRF4 identify a subtype of germinal center-derived B-cell lymphoma affecting predominantly children and young adults. *Blood*. 2011;118(1):139–147.
15. Rinaldi A, Mian M, Chigrinova E, et al. Genome-wide DNA profiling of marginal zone lymphomas identifies subtype-specific lesions with an impact on the clinical outcome. *Blood*. 2011;117(5):1595–1604.
16. Watkins AJ, Huang Y, Ye H, et al. Splenic marginal zone lymphoma: characterization of 7q deletion and its value in diagnosis. *J Pathol*. 2010;220(4):461–474.
17. Scott DW, Wright GW, Williams PM, et al. Determining cell-of-origin subtypes of diffuse large B-cell lymphoma using gene expression in formalin-fixed paraffin-embedded tissue. *Blood*. 2014;123(8):1214–1217.
18. Wilson WH, Young RM, Schmitz R, et al. Targeting B cell receptor signaling with ibrutinib in diffuse large B cell lymphoma. *Nat Med*. 2015;21(8):922–926.
19. Morin RD, Mendez-Lago M, Mungall AJ, et al. Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. *Nature*. 2011;476(7360):298–303.
20. Xie Y, Pittaluga S, Jaffe ES. The histological classification of diffuse large B-cell lymphomas. *Semin Hematol*. 2015;52(2):57–66.
21. Van Loo P, Tousseyn T, Vanhentenrijk V, et al. T-cell/histiocyte-rich large B-cell lymphoma shows transcriptional features suggestive of a tolerogenic host immune response. *Haematologica*. 2010;95(3):440–448.
22. Dojcinov SD, Venkataraman G, Pittaluga S, et al. Age-related EBV-associated lymphoproliferative disorders in the Western population: a spectrum of reactive lymphoid hyperplasia and lymphoma. *Blood*. 2011;117(18):4726–4735.
23. Nicolae A, Pittaluga S, Abdullah S, et al. EBV-positive large B-cell lymphomas in young patients: a nodal lymphoma with evidence for a tolerogenic immune environment. *Blood*. 2015;126(7):863–872.
24. Valera A, Balagué O, Colomo L, et al. IG/MYC rearrangements are the main cytogenetic alteration in plasmablastic lymphomas. *Am J Surg Pathol*. 2010;34(11):1686–1694.
25. Steidl C, Gascoyne RD. The molecular pathogenesis of primary mediastinal large B-cell lymphoma. *Blood*. 2011;118(10):2659–2669.
26. Eberle FC, Rodriguez-Canales J, Wei L, et al. Methylation profiling of mediastinal gray zone lymphoma reveals a distinctive signature with elements shared by classical Hodgkin's lymphoma and primary mediastinal large B-cell lymphoma. *Haematologica*. 2011;96(4):558–566.
27. Robbiani DF, Deroubaix S, Feldhahn N, et al. Plasmodium infection promotes genomic instability and AID-dependent B cell lymphoma. *Cell*. 2015;162(4):727–737.
28. Attygalle AD, Cabecadas J, Gaulard P, et al. Peripheral T-cell and NK-cell lymphomas and their mimics; taking a step forward—report on the lymphoma workshop of the XVIth meeting of the European Association for Haematopathology and the Society for Hematopathology. *Histopathology*. 2014;64(2):171–199.
29. Vose J, Armitage J, Weisenburger D, et al. International peripheral T-cell and natural killer/T-cell lymphoma study: pathology findings and clinical outcomes. *J Clin Oncol*. 2008;26(25):4124–4130.
30. Parrilla Castellar ER, Jaffe ES, Said JW, et al. ALK-negative anaplastic large cell lymphoma is a genetically heterogeneous disease with widely disparate clinical outcomes. *Blood*. 2014;124(9):1473–1480.
31. Hwang ST, Janik JE, Jaffe ES, et al. Mycosis fungoides and Sézary syndrome. *Lancet*. 2008;371(9616):945–957.
32. Willemze R, Jansen PM, Cerroni L, et al. Subcutaneous panniculitis-like T-cell lymphoma: definition, classification, and prognostic factors: an EORTC Cutaneous Lymphoma Group Study of 83 cases. *Blood*. 2008;111(2):838–845.
33. Gayden T, Sepulveda FE, Khuong-Quang DA, et al. Germline HAVCR2 mutations altering TIM-3 characterize subcutaneous panniculitis-like T cell lymphomas with hemophagocytic lymphohistiocytic syndrome. *Nat Genet*. 2018;50(12):1650–1657.
34. Swerdlow SH, Jaffe ES, Brousset P, et al. Cytotoxic T-cell and NK-cell lymphomas: current questions and controversies. *Am J Surg Pathol*. 2014;38(10):e60–e71.
35. Perry AM, Warnke RA, Hu Q, et al. Indolent T-cell lymphoproliferative disease of the gastrointestinal tract. *Blood*. 2013;122(22):3599–3606.
36. McKinney M, Moffitt AB, Gaulard P, et al. The genetic basis of hepatosplenic T-cell lymphoma. *Cancer Discov*. 2017;7(4):369–379.
37. Au W-Y, Weisenburger DD, Intragumtornchai T, et al. Clinical differences between nasal and extranasal natural killer/T-cell lymphoma: a study of 136 cases from the international peripheral T-cell lymphoma project. *Blood*. 2009;113(17):3931–3937.
38. Hartmann S, Döring C, Jakobus C, et al. Nodular lymphocyte predominant Hodgkin lymphoma and T cell/histiocyte rich large B cell lymphoma–endpoints of a spectrum of one disease? *PLoS One*. 2013;8(11):e78812.
39. Harris NL. Shades of gray between large B-cell lymphomas and Hodgkin lymphomas: differential diagnosis and biological implications. *Mod Pathol*. 2013;26(Suppl 1):S57–S70.

CHAPTER 79

ORIGIN OF HODGKIN LYMPHOMA AND THERAPEUTIC TARGETS

Ralf Küppers

More than 150 years ago, Thomas Hodgkin described several cases of a lymphoproliferative disease, later named Hodgkin disease. For a long time this malignancy, now called *Hodgkin lymphoma* (HL), has been one of the most enigmatic forms of lymphomas. This is due to several key features of the disease: First, the pathognomonic and suspected tumor cells of HL, the mononuclear Hodgkin and the bi- or multinucleated large Reed-Sternberg cells, are very rare in the tumor tissue, often accounting for only about 1% of the cells. Thus, the molecular analysis of these cells was hampered until methods became available to isolate these cells by microdissection from tissue sections. Second, the Hodgkin and Reed-Sternberg (HRS) cells have a very unusual immunophenotype that does not resemble any normal cell in the hematopoietic system. Therefore, immunophenotyping, which was very informative in revealing the cellular derivation of most other lymphoid malignancies, did not uncover the cellular origin of HRS cells. Third, only a few cell lines could be established from patients with HL, these lines were rather heterogeneous, and for hardly any of these lines, their derivation from the HRS cells in the patient was unequivocally shown. Hence, it was initially difficult to draw firm conclusions from the study of such lines. Although HL still harbors many secrets, exciting novel insights into the cellular origin of the HRS cells and the pathogenetic processes in their generation have been obtained in recent years, which will be discussed in this chapter.

CHARACTERISTICS AND ORIGIN OF HODGKIN LYMPHOMA

Classification of Hodgkin Lymphoma

HL is subdivided into classic HL, which accounts for about 95% of cases, and nodular lymphocyte-predominant HL (NLPHL). The tumor cells are called *HRS cells* in classic HL and *lymphocyte predominant (LP) cells* in NLPHL (originally the tumor cells in NLPHL were called *lymphocytic and histiocytic [L&H] cells*). Classic HL and NLPHL differ in the histologic picture, the morphology and immunophenotype of the tumor cells, and multiple clinical features. Classic HL is further subdivided into four subforms: nodular sclerosis, mixed cellularity, lymphocyte-rich classic, and lymphocyte depletion HL. Again, differences in the histologic picture and HRS cell morphology are the basis for this subtyping (see Chapter 78, for a detailed description).

B-Cell Development and Differentiation

Because we now know that HRS and LP cells are derived from B cells (see later), a brief outline of B-cell development and differentiation is given first. B cells are generated in the bone marrow from hematopoietic stem cells in a multistep developmental process. The key determinant for B-cell development is the generation of a functional B-cell receptor (BCR) that is composed of two identical immunoglobulin (Ig) heavy chains and two identical light chains, the latter of which can be of the κ or λ type. B-cell development is initiated when common lymphoid progenitors undergo gene rearrangements at the Ig gene heavy chain locus. The variable part of the antibody heavy chain is composed of three gene segments: variable (V), diversity (D), and joining (J). First, a randomly selected D_H gene segment is rearranged to one J_H gene segment. In the next step, a V_H gene segment is rearranged to a D_H-J_H joint. A heavy chain can be expressed and the developmental stage of a pre-B cell is reached if the rearrangement is in-frame and productive. In the next step, V_κ to J_κ rearrangements occur to generate a κ light chain. If this is not successful, V gene rearrangement processes take place at the λ light chain locus. B cells expressing a functional (and nonautoreactive) BCR are released into the periphery and express their BCR as IgM and IgD receptors—that is, two different classes of the heavy chain constant region. The first D_H-J_H rearrangements are not completely specific for B-lineage cells, but $V_HD_HJ_H$ rearrangements and light chain gene rearrangements are highly specific for B cells. Thus, their detection in a cell unequivocally defines that cell as a B cell. Moreover, because of the availability of multiple V, D, and J gene segments and additional diversity generated at the joining sites of the rearranging gene segments, a V(D)J rearrangement (in particular for the heavy chain locus) is unique for each B cell and thus can be used as a clonal marker for B cells deriving from the same mature B cell.

If B cells are activated through the binding of antigen to their BCR and cognate help from T-helper cells, the cells undergo a T-dependent immune response in specific histologic structures, the germinal centers (GC), in lymph nodes or other secondary lymphoid organs. In these follicles, activated B cells undergo massive clonal expansion and further diversify their BCR through two processes: somatic hypermutation and class switching. The process of somatic hypermutation introduces point mutations and some deletions and duplications at a very high rate into the Ig heavy and light chain V region genes. This randomly modifies amino acids in the V regions, and in a selection process involving follicular dendritic cells and follicular T-helper cells, B cells expressing mutated BCR with increased affinity to the stimulating antigen are positively selected, whereas B cells acquiring unfavorable mutations undergo apoptosis within the GC microenvironment (Fig. 79.1). Unfavorable mutations include destructive mutations, such as nonsense mutations, that prevent the expression of a BCR as such. Other disadvantageous replacement mutations still allow BCR expression but reduce affinity to the antigen. After multiple rounds of proliferation, mutation, and selection, positively selected B cells expressing a high-affinity BCR differentiate into long-lived memory B cells or plasma cells and exit the GC. Many GC B cells also undergo class switching before they are selected into the memory or plasma cell pool. In class switching, the originally expressed Cμ and Cδ heavy chain constant region genes (encoding IgM and IgD, respectively) are replaced by downstream located Cγ, Cα, or Cε genes, encoding IgG, IgA, and IgE heavy chains, respectively, so that antibodies with altered effector functions are generated. At all stages of their development, B lineage cells are selected for expression of the appropriate BCR, and cells failing this selection are eliminated.

Cellular Origin of Lymphocyte Predominant Cells in Nodular Lymphocyte Predominant Hodgkin Lymphoma

LP cells in NLPHL express multiple typical B cell markers, such as the surface molecules CD20 and CD79 and the transcription factors PAX5, OCT-2, and BOB1 (Table 79.1).[1] LP cells mostly lack expression of markers of other hematopoietic cell lineages, thus their immunophenotype suggests a B-cell origin. Moreover, LP cells are located in follicular structures in close association with follicular dendritic

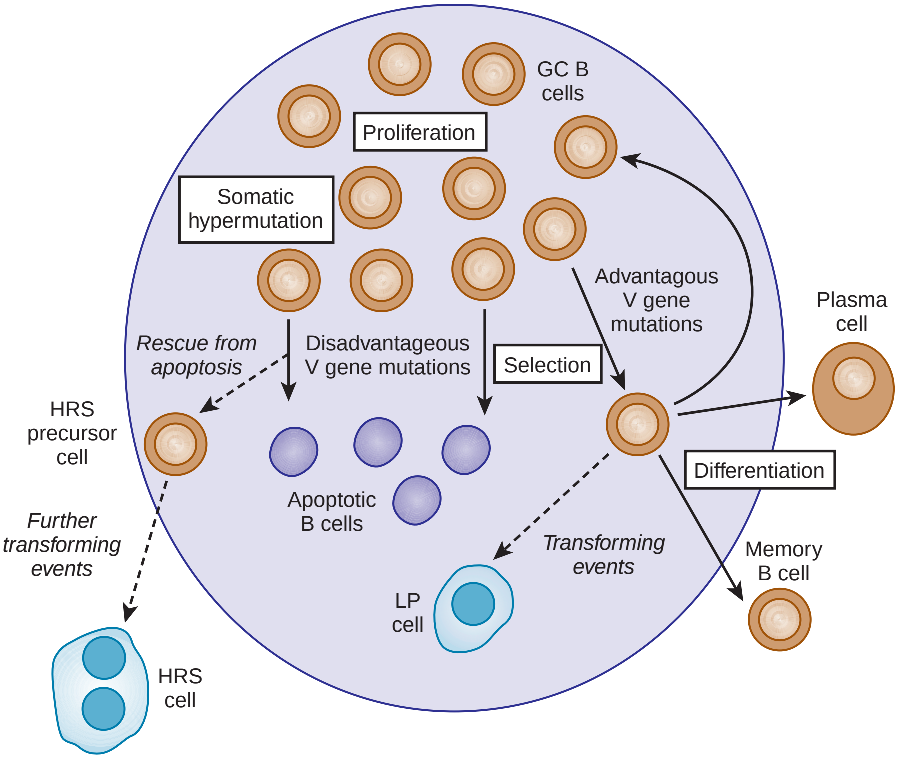


Figure 79.1 THE GERMINAL CENTER REACTION AND A SCENARIO FOR HODGKIN AND REED-STERNBERG AND LYMPHOCYTE PREDOMINANT CELL DERIVATION. Antigen-activated B cells that receive costimulatory signals from T-helper cells establish germinal center (GC) reactions in T-dependent humoral immune responses. In the GC, the activated B cells undergo massive clonal expansion in the dark zone. The proliferating GC B cells are called *centroblasts*. In the centroblasts, the process of somatic hypermutation is activated, which introduces somatic mutations at a very high rate into rearranged Ig V genes. Centroblasts migrate into the light zone and become resting centrocytes. Centrocytes are then selected based on their B-cell receptor (BCR). Most Ig V gene mutations will be disadvantageous (e.g., when they cause premature stop codons or lead to amino acid changes that reduce the affinity of the BCR to the immunizing antigen) and will cause apoptotic death of the respective B cells. GC B cells acquiring affinity-increasing mutations will be positively selected by interaction with T-helper cells and follicular dendritic cells (not shown). Positively selected centrocytes may either return to the dark zone for further rounds of proliferation, mutation, and selection, or they differentiate into memory B cells or plasma cells and exit the GC. Lymphocyte predominant (LP) cells of nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) express functional Ig V genes and proliferate in a follicular microenvironment resembling GC. Thus, these cells are likely derived from selected GC B cells. Hodgkin and Reed-Sternberg (HRS) cells in classic HL often carry destructive Ig V gene mutations, indicating that they derive from GC B cells that normally would have undergone apoptosis (i.e., pre-apoptotic GC B cells). Initial transforming events in HRS cell pathogenesis might have occurred already at a pre–GC B cell differentiation stage, or they might happen in the GC. In Epstein-Barr virus (EBV)-positive cases, EBV is a good candidate for a factor that allows GC B cells with destructive Ig V gene mutations to escape from apoptosis.

cells and GC-type T-helper cells, suggesting a close relationship with GC B cells. Indeed, LP cells express the transcription factor BCL6, the main regulator of the GC B-cell differentiation program. They also express activation-induced cytidine deaminase (AID), an enzyme that regulates somatic hypermutation and class switching in GC B cells. Thus, all of these phenotypic features point to a GC B cell origin of LP cells (see Table 79.1), and this was further corroborated at the genetic level when clonally rearranged and productive Ig V region genes were amplified from isolated LP cells. These rearrangements were always somatically mutated. In some cases, an indication for ongoing somatic hypermutation was observed. Therefore, the Ig V gene analysis further strongly supported a GC B cell origin of LP cells (see Fig. 79.1). The detection of the same V gene rearrangements in all LP cells of a given NLPHL case also firmly established for the first time the monoclonality of the LP cells, a hallmark for tumor cells. A gene expression profiling study of LP cells indicated a relationship of these cells to late GC B cells on the way to becoming post-GC memory B cells.[2]

Cellular Origin of Hodgkin and Reed-Sternberg Cells in Classic Hodgkin Lymphoma

As mentioned earlier, HRS cells show a peculiar immunophenotype that does not resemble any normal hematopoietic cell type. HRS cells express markers of different cell lineages in a fraction or even most cases, including B-cell genes (e.g., PAX5, IRF4), T-cell genes (e.g., CD3, GATA3), natural killer cell genes (ID2), myeloid genes (e.g., CD15, CSF1R), and dendritic cell markers (e.g., CCL17, fascin, restin) (see Table 79.1).[1,3,4] Therefore, immunohistochemical studies could not resolve the cellular origin of HRS cells. In addition, the first cell lines established from patients with HL and presumed to be derived from HRS cells were either of B-cell origin, T-cell, or nonlymphoid origin (discussed in further detail later). The origin of HRS cells was finally clarified when HRS cells microdissected from tissue sections were analyzed for rearranged Ig V region genes. These studies revealed that HRS cells in nearly all cases carry clonal Ig heavy and light chain gene rearrangements.[1,5] Because such rearrangements are highly specific for B cells, this established their B-cell nature. Moreover, with rare exceptions, the rearranged V region genes were highly mutated, pointing to a derivation from GC or post-GC B cells.[1,5] Intraclonal V gene diversity was not observed, showing that the process of somatic hypermutation is no longer active in these cells. There is also an indication that many HRS cell clones underwent class-switch recombination, a further antigen-driven and B cell-specific process. Thus, these genetic features unequivocally demonstrate that HRS cells are derived from mature B cells that had been activated by antigen.

Surprisingly, in about a quarter of the cases of classic HL, the Ig V gene rearrangements carried clearly destructive mutations that

TABLE 79.1 Comparison of Phenotypic and Genetic Features of Hodgkin and Reed-Sternberg Cells of Classic Hodgkin Lymphoma and Lymphocyte Predominant Cells of Nodular Lymphocyte Predominant Hodgkin Lymphoma

Feature	Hodgkin and Reed-Sternberg Cells	Lymphocyte Predominant Cells
Somatically mutated Ig V genes	Yes (very rare exceptions)	Yes
Crippling Ig V gene mutations	Yes (>25% of cases)	No
Ongoing somatic hypermutation	No	Yes (moderately)
Presumed cellular origin	Pre-apoptotic GC B cells	Positively selected, mutating GC B cells
Rare cases with a T-cell origin	Yes (<5%)	No
B-cell receptor expression	No	Yes
Expression of B-cell transcription factors (e.g., OCT2, BOB.1, PU.1, PAX5, E2A)	Rarely and/or at low levels	Yes
Expression of B-cell surface antigens (e.g., CD19, CD20)	No or rarely	Yes
Expression of GC B-cell markers (e.g., BCL6, AID, HGAL, GCET)	No or rarely	Yes
Expression of molecules involved in antigen presentation and interaction with T-helper cells (e.g., CD40, CD80, CD86, MHC class II)	Yes	Yes
Expression of markers of non-B cells (e.g., CCL17, NOTCH1, GATA3, ID2, CSFR1)	Yes	No
EBV infection of tumor cells	Yes (30%–40%)	No

AID, Activation-induced cytidine deaminase; *EBV*, Epstein-Barr virus; *GC*, germinal center.

rendered originally functional V region genes nonfunctional.[1,5] Such "crippling" mutations included deletions and insertions causing loss of the correct reading frame, as well as nonsense mutations. As discussed earlier, destructive mutations regularly happen in mutating GC B cells, but this normally results very efficiently in their removal by apoptosis. Thus, it is highly likely that the GC B cells that acquired the destructive mutations already carried some transforming events, allowing them to escape apoptosis (see Fig. 79.1). Of note, most disadvantageous mutations that cause apoptosis of normal GC B cells are likely replacement mutations that reduce affinity to the antigen or interfere with the proper folding and/or pairing of the Ig heavy and light chains. These mutations cannot be easily recognized by looking at the V gene sequences. Thus, the crippling mutations likely represent only the "tip of the iceberg," and we have speculated that HRS cells, as a rule, derive from the pool of pre-apoptotic GC B cells (see Fig. 79.1).[1,5]

Decisive steps in HL pathogenesis, therefore, become effective or take place in GC B cells. Thus, although some final transforming events may well occur when HRS precursor cells have already left the GC microenvironment, for the reasons discussed earlier, classic HL is considered as a GC B cell–derived malignancy. A global gene expression study comparing HRS cells to various mature B cell subsets revealed that the gene expression pattern of HRS cells is most similar to that of the rare normal CD30$^+$ B cells, so that HRS cell development may be closely linked to these normal B cells.[6]

T Cell–Derived Classic Hodgkin Lymphoma

The observation that in a fraction of cases the HRS cells express several T-cell markers (e.g., CD3, granzyme B, perforin, T cell intracellular antigen 1) prompted studies aimed to clarify whether in such cases the HRS cells derive from T cells. Analysis of HRS cells from several cases with T-cell marker expression showed that most of these cases nevertheless are B cell–derived. However, several cases were identified that lacked Ig V gene rearrangements and that showed clonal T-cell receptor gene rearrangements.[7,8] Thus, these cases have a T-cell origin. Because the cellular origin of a lymphoma clone is a key factor for current lymphoma classification, it is a matter of debate whether lymphomas with HRS cells of T-cell origin should be called *HL* or whether they should be considered a rare, separate type of T-cell lymphoma. Notably, in gene expression studies of HL cell lines, HDLM-2 (a T cell–derived HL cell line) clustered more closely to B cell–derived HL cell lines than to other T-cell lymphoma lines, suggesting that B cell– and T cell–derived HRS cells have a similar gene expression pattern.

Hodgkin Lymphoma Cell Lines

Tumor cell lines are valuable tools for detailed genetic, biochemical, and functional studies of a malignancy. Thus, there have been many attempts to establish such lines from patients with HL. However, this has proved to be very difficult, and less than 10 HL cell lines exist. The main reason for the difficulty in growing HRS or LP cells in culture is most likely their dependence on survival signals from the cellular microenvironment in the disease-affected lymph nodes. Of note, all of the existing HL lines are derived from patients at end stage and were not established from lymph nodes, but rather from peripheral blood, bone marrow, or pleural effusions. This suggests that only when the HRS cells have become independent from the lymph node microenvironment in the patient do they also have a chance to survive in suspension culture. The existing and available HL lines are L428, L540, L591, L1236, KM-H2, HDLM-2, UHO-1, SUP-HD1, and DEV (Table 79.2). Most lines are of B-cell origin, but HDLM2 and L540 are T cell–derived. L591 is the only HL line that is EBV-positive. Among primary cases of classic HL, approximately 30% to 40% show EBV infection of the HRS cells (see further details later). Notably, L591 cells do not have the typical EBV gene expression pattern of primary EBV-infected HRS cells, which should be kept in mind when using this line for gene expression or functional studies. The DEV line was originally reported as being derived from a case of classic HL, but subsequent more detailed phenotypic and gene expression studies indicated that this is indeed the only existing cell line from a patient with NLPHL. Although the phenotype and genotype of these lines fit well with their presumed origin from HRS cells (or LP cells in the case of DEV), L1236 is the only cell line for which the derivation from the HRS cell clone of the patient it was established from was unequivocally proven. Originally, the CO line was also published as an HL cell line. However, later studies showed that this line represents a cell culture contamination. Also, the HD-Myz line was for a long time considered to be a classic HL cell line. However, it was later discovered that the phenotype of the line differs in key aspects from the phenotype of the primary HRS cells of the respective patient. Even more important, HD-Myz lacks Ig- and T-cell receptor gene rearrangements and is presumably of myeloid origin. The nonlymphoid origin of HD-Myz argues against its derivation from HRS cells; thus, this line should not be used as a model for HL.

Although the HL cell lines are a valuable (and practically the only) tool for functional studies with HRS cells, it needs to be taken into consideration that the cells are adapted to growth in suspension culture in the absence of their normal microenvironment. Indeed, a comparison of the gene expression pattern of primary HRS cells and

TABLE 79.2 **Characteristics of Hodgkin Lymphoma Cell Lines**[a]

Cell Line	Hodgkin Lymphoma Subtype	Cellular Origin	Oncogenes or Tumor Suppressor Genes with Genetic Lesions	Remarks
L428	Classic	B cell	*B2M*, *NFKBIA*, *NFKBIE*, *PTPN1*, *SOCS1*, *TP53*	
L540	Classic	T cell	*PTPN1*	
L591	Classic	B cell		Only EBV-positive line
L1236	Classic	B cell	*CD95*, *PTPN1*, *SOCS1*, *TNFAIP3*, *TP53*	Only line with proven origin from HRS cells in the patient
KM-H2	Classic	B cell	*CIITA* (translocation), *CYLD*, *NFKBIA*, *TNFAIP3*	
HDLM-2	Classic	T cell	*SOCS1*, *TNFAIP3*, *TNFSF7*, *TNFSF9*, *TP53*, *UTX*	
UHO-1	Classic	B cell	*PTPN1*, *TNFAIP3*, *TRAF3*	
SUP-HD1	Classic	B cell	*PTPN1*, *PTPN2*, *TNFAIP3*	
DEV	NLPHL	B cell	*B2M*, *BCL6* (translocation), *SOCS1*, *NFKBIE*, *TNFAIP3*	

[a] A few additional lines have been published (HO, ZO, HD-70, HKB-1), but these have not been used in published studies in recent years, so it is unclear to the author whether they still exist, and very little is known about their phenotypic, functional, and genetic features.
EBV, Epstein-Barr virus; *HRS*, Hodgkin and Reed-Sternberg.

classic HL cell lines revealed more than one thousand differentially expressed genes, including many genes regulating the extracellular matrix, proliferation, and chemokine production and signaling.[9]

Relationship Between Hodgkin Cells and Reed-Sternberg Cells

The population of HRS cells is always composed of a mixture of mononuclear Hodgkin cells and bi- or multinucleated Reed-Sternberg cells. It is thus an intriguing question how these two types of cells are related to each other. It was proposed that the fusion of two independent cells might be involved in the generation of Reed-Sternberg cells from Hodgkin cells. It was also discussed whether the whole HRS cell clone could derive from a cell fusion (e.g., a fusion of a B cell with a non-B cell). Such a scenario was attractive because it might have provided an explanation for the mixed immunophenotype and the usually aneuploid karyotype of the HRS cells. However, generation of the HRS cell clone through the fusion of two separate cells was excluded by molecular studies.[10] Indeed, there is now firm evidence from studies of HL cell lines that Reed-Sternberg cells develop from mononuclear Hodgkin cells through a process of incomplete cytokinesis.[11] When Hodgkin cells attempt to undergo cell division, the final separation of the daughter cells often fails, and these cells undergo refusion, giving rise to a binucleated Reed-Sternberg cell.[11] Reed-Sternberg cells have little further proliferative potential, so that the mononuclear Hodgkin cells are the principal proliferative compartment of the HRS cell clone.

Potential Hodgkin and Reed-Sternberg Precursor or Stem Cells

Another interesting question is whether the rare CD30$^+$ HRS cells represent the whole tumor cell clone in the HL tissue or whether further tumor clone members are present among the smaller, CD30$^-$ cells in the microenvironment. In a single-cell Ig V gene analysis of EBV-positive HRS cells and EBV-infected CD30-negative cells, it was found that the HRS cells and the small CD30$^-$ EBV-infected B cells carried distinct V gene rearrangements in nearly all instances.[12] Because EBV infection of HRS cells is a clonal event, in that in EBV$^+$ HL cases all members of the tumor clone should carry the virus, this finding was taken as an argument that the HRS cell clone does not have additional members among CD30-negative lymphocytes.

In several types of tumors, cancer stem cells were identified. These cells are defined as rare cells that have a particular proliferative potential and that sustain the tumor clone, whereas the bulk of the tumor clone lacks the potential to regrow into a full tumor. One study described the existence of HRS precursor cells in HL that fulfill at least some key features of cancer stem cells. However, this finding was criticized because the markers for a clonal relationship between the HRS and non-HRS cells were unreliable and even argued, in part, against a relationship of the cells.[13] Thus there is currently no convincing evidence for the existence of CD20$^+$BCR$^+$ HRS stem cells. In any case, it needs to be considered that HRS cells carry clonal and somatically mutated Ig V gene rearrangements; thus, if HRS stem cells exist, they must be mature GC-derived B cells that carry the same Ig gene rearrangements and mutation pattern as the HRS cells.

The issue of potential subpopulations among the HRS cell clone with specific features in terms of proliferative potential and chemotherapy resistance was also addressed by searching for *side population cells*, which are defined as cells that extrude the Hoechst dye 33342, usually because they express drug transporters of the ABC family, such as the multidrug resistance 1 gene. In several types of tumors, side population cells were shown to share features with cancer stem cells (e.g., increased proliferative potential and chemotherapy resistance). Side population cells were indeed detected at low frequency (about 0.5% of cells) in some HL cell lines, and these cells showed increased resistance to chemotherapeutic drugs.[14,15] The side population cells had the phenotype of small Hodgkin cells and they were positive for CD30 and negative for CD19 and CD20. Hence, these cells are different from the cells identified earlier as potential HRS stem cells. Notably, side population cells were able to reestablish the full HRS cell clone upon subcloning. However, not all HL cell lines showed side population cells,[14,15] arguing against a general role of these cells in the maintenance of the HRS cell clone. Moreover, although side population cells were also identified in cell suspensions of HL lymph nodes, it remains to be clarified whether these cells belong to the HRS cell clone.

Lessons From Composite Lymphomas

Composite lymphomas are very rare lymphomas in which two distinct lymphomas, often an HL and a non-Hodgkin lymphoma (NHL), occur in the same patient. In a strict definition of composite lymphoma, this happens concurrently, but there are also cases where an HL may occur either before or after an NHL in the same patient. There are a few cases where the two lymphomas are not related to each other and hence represent the chance occurrence of two unrelated malignancies developing in parallel in a patient. However, in most composite and sequential HL and NHL that have been molecularly studied for their clonal relationship, it was found that the lymphomas share a common origin, even in instances where sequential lymphomas occurred several years apart from each other.[16] The detailed study of the rearranged Ig V genes of such cases revealed that, although, in many cases, the clonally related V region genes of the lymphomas share a number of somatic mutations, there are often additional mutations that were present only in the HRS cells and others only in the NHL B cells.[16] This showed that the two lymphomas have a common precursor, most likely a mutating GC B cell, and that the two lymphomas most likely developed from two distinct members of the GC B cell clone (Fig. 79.2). Thus, these composite lymphomas usually do not represent the transformation of one lymphoma into the other but, rather, the parallel development of the two malignant clones from a common, premalignant precursor cell. It is likely that some transforming events were already present in the common precursor, but distinct daughter cells of this GC B cell later acquired different mutations, leading to the generation of the two distinct B-cell malignancies (see Fig. 79.2). Therefore, composite lymphomas are intriguing models to study the multistep transformation process in lymphomagenesis. In initial studies of several composite lymphomas for shared and distinct transforming events, examples for such genetic lesions were indeed identified.

A further important aspect of the molecular studies of composite lymphomas is that they provide further evidence for the derivation of HRS cells in classic HL from GC B cells because the clonal relationship with shared and distinct somatic Ig V gene mutations of an HRS cell clone and a typical GC B cell-derived lymphoma (e.g., follicular lymphoma or diffuse large B-cell lymphoma) strongly supports a common GC B-cell origin of both malignancies.

The Role of Epstein-Barr Virus in Classic Hodgkin Lymphoma

In about 40% of cases of classic HL in the Western world, and about 90% of childhood HL cases in Central and Southern America, HRS cells are infected by EBV. Molecular analyses of EBV$^+$ cases showed that EBV infection of HRS cells is a clonal event (i.e., EBV infected or was already present in the founder cell of the HRS cell clone). EBV-positive HRS cells typically show a latency II gene expression pattern characterized by expression of the EBV nuclear antigen 1 (EBNA1) and the two latent membrane proteins 1 and 2a (LMP1 and LMP2a, respectively). EBNA1 is essential for the replication of the EBV episome in proliferating cells. LMP1 is an oncogene, and one of its main functions is to cause constitutive nuclear factor kappa B (NFκB) activation by mimicking an activated CD40 receptor. LMP2a has a cytoplasmic motif that resembles the signaling motif of the BCR. LMP2a can, on the one hand, diminish BCR signaling by recruiting BCR signaling components away from the BCR, but on the other hand, it mimics a tonic BCR signal. Strikingly, all HRS cell clones with clearly destructive Ig V gene mutations that prevent the expression of a BCR were found to be EBV-positive.[17] Hence, it appears that in GC B cell precursors of HRS cells that acquired BCR-destructive mutations, EBV is essential to rescuing these cells from apoptosis upon loss of BCR expression. This scenario is supported by studies showing that EBV can rescue BCR-deficient GC B cells from apoptosis and that LMP2a has an important role in this process.[18] Whether the BCR-mimicking function is still important in the established HRS cell clone is unclear. The downregulation of most components of BCR signaling in HRS cells indicates that LMP2a does not have this role in the established HRS cell clone.

Genetic Lesions in Hodgkin and Reed-Sternberg and Lymphocyte Predominant Cells

The molecular analysis of HRS and LP cells for genetic lesions is hampered by the rarity of these cells. Nevertheless, we are now aware of a number of transforming events, especially for HRS cells. The recognition of the constitutive activity of the NF-κB transcription factor, which is normally only transiently activated in lymphocytes, prompted studies of whether genetic mutations cause this deregulated activity. Inactivating mutations in the gene *NFKBIA*, which

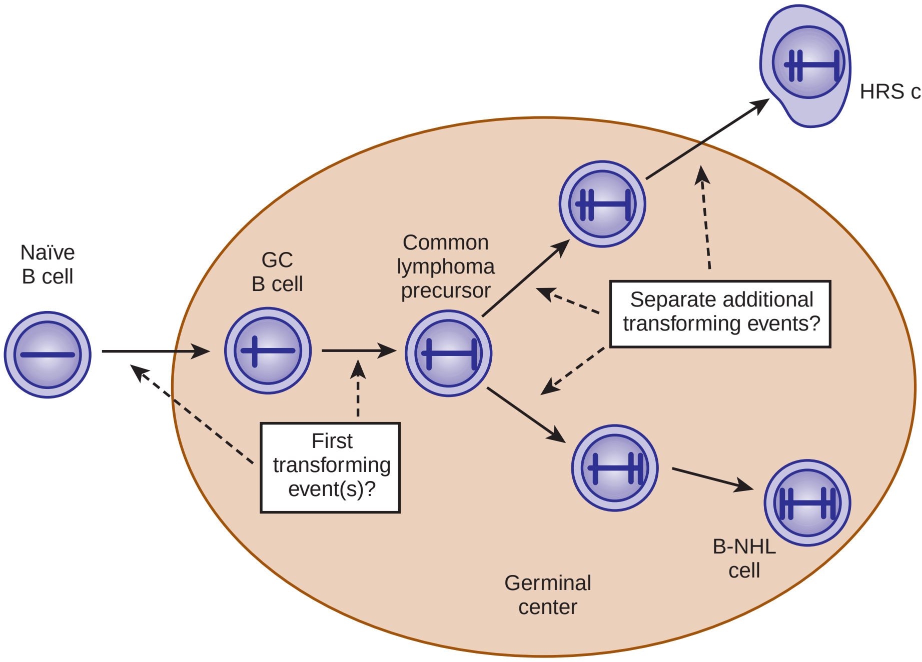


Figure 79.2 SCENARIO FOR THE GENERATION OF COMPOSITE LYMPHOMAS. Composite lymphomas are very rare combinations of two distinct lymphomas, often a classic Hodgkin lymphoma (HL) and a non-Hodgkin lymphoma (NHL). Detailed molecular analysis of rearranged Ig V genes in several combined HL and B-cell derived NHL revealed that, in most instances, the two lymphomas are clonally related. Notably, the pattern of both shared and distinct V gene mutations in the majority of these cases revealed that the two lymphomas share a common precursor, but developed separately from this precursor. The direct clonal relationship of Hodgkin and Reed-Sternberg (HRS) cells with typical germinal center (GC) B cell-derived NHL is a further strong argument for a GC derivation of HRS cells. The horizontal line within the cells indicates an Ig V region gene; the vertical lines indicate somatic Ig V gene mutations. (*Modified from Bräuninger A, Hansmann ML, Strickler JG, et al. Identification of common germinal center B-cell precursors in two patients with both Hodgkin's disease and non-Hodgkin's lymphoma. N Engl J Med. 1999; 340:1239.*)

encodes the main inhibitor of NFκB (i.e., IκBα) were detected in 10% to 20% of cases of classic HL (Fig. 79.3).[1] Also, mutations in the *NFKBIE* gene were found. Genomic gains of the *REL* gene, encoding one of the NFκB factors, are present in about 40% of classic HL. Inactivating mutations in *TNFAIP3*, encoding a further negative regulator of NFκB, were detected in 40% of HRS cell clones.[19] Interestingly, most cases with *TNFAIP3* mutations were EBV-negative,[19] suggesting that EBV infection and *TNFAIP3* mutations are alternative mechanisms in HL pathogenesis, thereby, further supporting a pathogenetic role of EBV in HL development. Reestablishing wild-type TNFAIP3 in HL cell lines reduced NFκB activity and survival, establishing that TNFAIP3 functions as a tumor suppressor gene in this lymphoma.[19] Rare mutations were also found in *TRAF3*, encoding a negative regulator of the noncanonical NFκB pathway. Finally, the NIK kinase gene (*MAP3K4*), encoding a major activating factor for the noncanonical NFκB pathway, shows gains in 20% of classic HL.

Although LP cells of NLPHL also show constitutive NFκB activity,[2] no destructive mutations were found in *TNFAIP3* and *NFKBIA* in these cells, and LP cells are not infected by EBV. In some NLPHL *REL* gains were found. Thus, the mechanisms causing NFκB activation in HRS and LP cells are largely different.

A second signaling pathway that shows constitutive activity in HRS cells and mutations in various pathway members is the JAK/STAT (Janus kinase/signal transducer and activator of transcription)pathway, the main pathway for cytokine signaling. Upon activation of cytokine receptors, JAK kinases bind to the receptors and become activated. The activated JAKs then phosphorylate and thereby activate STAT (Janus kinase/signal transducer and activator of transcription) factors, which upon phosphorylation and dimerization translocate into the nucleus and function as transcription factors. Frequent genomic gains of *JAK2* and also rare translocations involving this gene have been detected in HRS cells. SOCS1, a main inhibitor of this signaling pathway, is inactivated by somatic mutations in the HRS cells in about 40% of cases of classic HL. Destructive mutations in the *PTPN1* gene were detected in HRS cells in 20% of cases.[20] PTPN1 is a phosphatase that, among other functions, negatively regulates STAT activity. An exome sequencing study of HRS cells revealed activating *STAT6* mutations in about 30% of cHL cases.[21]

Notably, the genomic region on 9p24, which harbors the *JAK2* gene and shows gains in HRS cells, also encompasses the gene *JMJD2C* that encodes a histone demethylase, and the programmed death 1 (PD-1) ligand genes *PD-L1* and *PD-L2*.[22,23] PD-1 ligands inhibit PD-1–expressing T cells and may contribute to the immunosuppressive microenvironment in HL.[22] A functional role of JMJD2C in HRS cells is indicated from the finding that the downregulation of its expression in HL cell lines is toxic for these cells.[23] Thus, a single

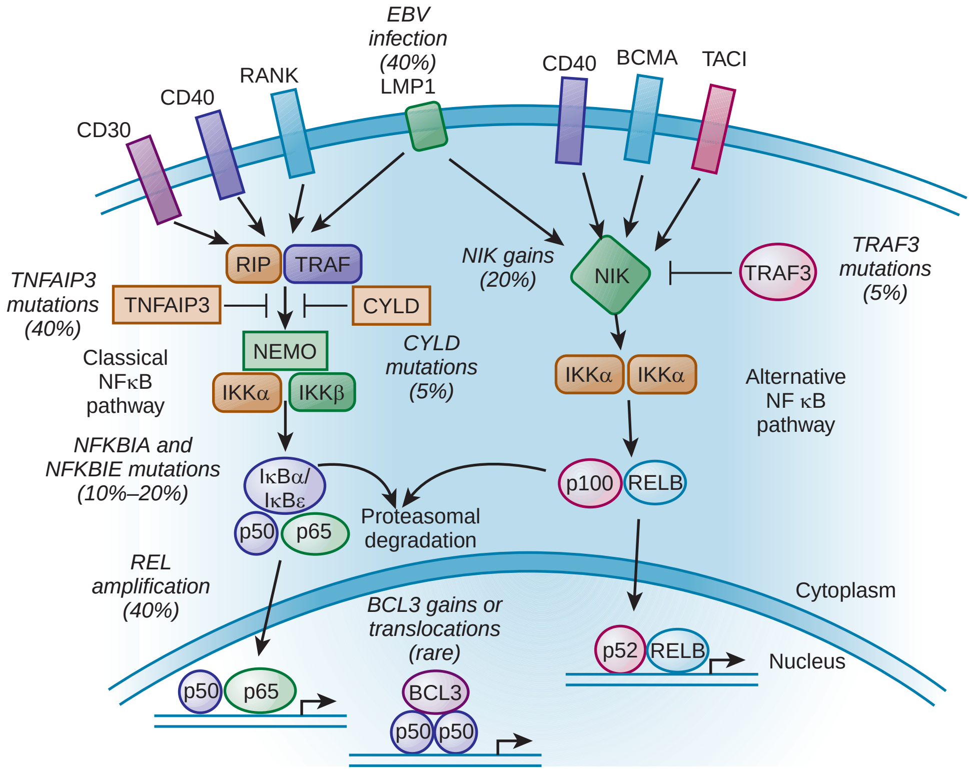


Figure 79.3 MECHANISM OF NFκB ACTIVATION IN HODGKIN AND REED-STERNBERG CELLS. A classic and an alternative NFκB signaling pathway are distinguished. In the classic NFκB pathway stimulation of numerous receptors (e.g., RANK, CD30, and CD40) leads via TNF receptor–associated factors (TRAFs) and other associated factors, such as the receptor-interacting protein (RIP), to activation of the IKK complex. The IKK complex is composed of IKKα, IKKβ, and NEMO. The activated IKK complex phosphorylates the NFκB inhibitors IκBα and IκBε which are encoded by the *NFKBIA* and *NFKBIE* genes, respectively. The phosphorylation marks the IκB factors for ubiquitinylation and subsequent proteasomal degradation. Thus, the NFκB transcription factors (e.g., p50/p65 or p50/REL heterodimers) are no longer retained in the cytoplasm and translocate into the nucleus where they activate multiple genes. The signal transduction from TRAFs/RIP to the IKK complex can be inhibited by A20/TNFAIP3, which removes activating ubiquitins from RIP and TRAFs and additionally links ubiquitins to these molecules to mark them for proteasomal degradation. In the alternative NFκB pathway, activation of receptors such as BCMA, CD40, and TACI causes stimulation of the kinase NIK (encoded by the *MAP3K4* gene), which then activates an IKKα complex. Activated IKKα processes p100 precursors to p52 molecules that translocate as active p52/RELB NFκB heterodimers into the nucleus. HRS cells show the constitutive activity of the classic and alternative NFκB signaling pathways. This activity is mediated by diverse mechanisms, including receptor signaling through CD40, RANK, BCMA, and TACI; genomic *REL* and *MAP3K4* amplifications; destructive mutations in the *TNFAIP3*, *NFKBIA*, and *NFKBIE* genes; and signaling through the EBV-encoded latent membrane protein 1. HRS cells may also harbor nuclear BCL3/$(p50)_2$ complexes, and in a few cases, the strong BCL3 expression appears to be mediated by genomic gains or chromosomal translocations of this gene. In rare instances, inactivating mutations in the inhibitory factors TRAF3 and CYLD have been found. (*Modified and updated from Küppers R. The biology of Hodgkin's lymphoma. Nat Rev Cancer. 2009; 9:15.*)

genetic lesion, the genomic gain on 9p24, can affect four genes with pathogenetic roles in HRS cells.

Because disturbed apoptosis seems to be a key aspect of classic HL pathogenesis, numerous regulators of apoptosis were also studied in HL cell lines and/or primary HRS cells. Mutations in the *TP53* gene were found in three HL cell lines and a few primary cases.[24] Mutations in the *CD95* gene were found in less than 10% of cases, and no mutations were found in the genes of the CD95 signaling components CASP8, CASP10, and FADD.[24] Moreover, no mutations were detected in the proapoptotic gene *BAD*; mutations in *ATM* are also very rare.[24]

Chromosomal translocations, usually involving one of the Ig loci and an oncogene, are a hallmark of most B cell lymphomas. Also in classic HL, chromosomal breaks involving the Ig loci were detected by fluorescence in situ hybridization in about 20% of cases. However, the translocation partners are in most cases unknown. In a few instances, *BCL2*, *BCL3*, *BCL6*, or *MYC* were identified as partners of the translocations. This also includes composite lymphomas where the clonally related classic HL and the B-cell NHL were found to carry the identical translocation—for example, a *BCL2*-IgH translocation in a composite classic HL/follicular lymphoma.[16] Notably, Ig locus–associated translocations usually function by causing deregulated expression of the translocated oncogene that has been brought under the control of the Ig regulatory elements, which are active in normal B cells and B-cell NHL. Because the Ig loci are largely silenced in HRS cells, it is still unclear whether the partner genes of Ig locus-associated translocations in HRS cells are still active and pathogenetically relevant. Perhaps the translocations were important during the early stages of HRS cell pathogenesis, when the lymphoma precursor cells still had a B-cell gene expression program but became less important during later stages of the multistep transformation process. Alternatively, it should also be considered that translocations might function by mechanisms other than Ig enhancer–driven oncogene overexpression (e.g., promoter replacement or tumor suppressor gene inactivation).

HRS cells show a heterogeneous pattern of major histocompatibility complex (MHC) class I and II expression. Recurrent translocations involving the MHC class II transactivator gene *CIITA* were found in about 15% of classic HL.[25] These translocations involved heterogeneous partner genes and seemed to function by impairing the CIITA function and hence dampening MHC class II expression. The gene of the MHC class I component β-2-microglobulin (*B2M*) is mutated in more than half of classic HL according to a recent study.[26] Down modulation of MHC class I is often a strategy of cancer cells to evade attack by cytotoxic T cells.

Recent exome or targeted sequencing analyses of isolated HRS cells or from circulating tumor DNA in plasma uncovered a number of additional genetic lesions in HRS cells.[21,26,27] These include mutations in the exportin 1 gene (*XPO1*), which encodes a factor regulating nuclear export of various molecules, the mutation in the gene encoding the G protein GNA13, and inactivating mutations in *ITPKB*, which encodes an inhibitory factor in AKT signaling. Moreover, recurrent mutations in *CSF2RB*, encoding a common component of the receptors for interleukin-3, interleukin-5, and granulocyte-macrophage colony-stimulating factor were observed in several studies.

For LP cells of NLPHL, few genetic lesions are known. *SOCS1* mutations occur frequently in LP cells, and translocations involving the *BCL6* gene are often found in these cells.[24] BCL6 encodes a transcription factor that orchestrates the GC B-cell differentiation program and that has an oncogenic function when constitutively expressed in B cells. A study published in 2016 identified highly recurrent mutations in the genes of the serine/threonine kinase SGK1, the phosphatase DUSP2, and the AP-1 transcription factor JUNB in LP cells.[28]

THERAPEUTIC TARGETS

For a long time, poly-chemotherapy and/or radiation therapy (or in advanced stages hematopoietic stem cell transplantation) were the only options for the treatment of HL patients. However, recently, several types of targeted immune therapies have been established, with very promising results. A main breakthrough was the development of a toxin-coupled anti-CD30 antibody, brentuximab vedotin, for the treatment of classic HL. CD30 seems to be an ideal molecule for a targeted therapy, because this surface receptor is, on the one hand, universally and most strongly expressed by HRS cells of classic HL, and, on the other hand, it is expressed by only a few normal cells in the body, reducing the risk for severe side effects. CD30 is currently also used as a target for an approach with gene-modified autologous T cells, which are engineered to express a chimeric anti-CD30 antibody and T-cell receptor. A further breakthrough was the recognition of high therapeutic effectiveness of antibodies against PD-1 or PD-L1.[29] PD-1 is expressed by various types of immune cells and signaling through this receptor has suppressive effects on the immune cells. HRS cells express PD-L1 and PD-L2, as already described above, and use this for immune evasion. The antibodies against PD-1 or PD-L1 interfere with this inhibitory effect and thereby allow reactivation of the immune cells that then can attack the HRS cells. Notably, it is not yet clear which immune cells are mainly responsible for the therapeutic effect of the anti-PD1 and anti-PD-L1 antibodies. As HRS cells often lack MHC class I expression, they should not be targetable by $CD8^+$ cytotoxic T cells, so that cytotoxic $CD4^+$ T cells and natural killer cells may play a role. Clinical trials are also ongoing with another checkpoint inhibitor, namely an antibody against CTLA4, which is a further immunosuppressive factor.

CONCLUSIONS AND FUTURE DIRECTIONS

It is now firmly established that HRS and LP cells stem from mature B cells. A few cases with features of classic HL and a T-cell origin of the HRS cells exist. HRS cells in classic HL appear to derive from crippled GC B cells, whereas LP cells likely originate from antigen-selected GC B cells. HRS cells show deregulated activation of multiple signaling pathways, and numerous genetic lesions have been identified that contribute to the deregulated activation of the NFκB and JAK/STAT pathways. HRS cells show a nearly complete loss of the B-cell gene expression program.[3] As normal GC B cells are stringently selected to express a functional, high-affinity BCR, the lost B-cell phenotype may be related to the origin of HRS cells from preapoptotic GC B cells: by losing the B cell–typical gene expression program, the HRS cell precursors may escape from the pressure to undergo apoptosis as "failed B cells." It has been speculated that transient hypoxic conditions in the GC may contribute to the initial "dedifferentiation" of the HRS precursor cells because exposing human B cells to hypoxia induces several phenotypic changes in these cells toward an HRS cell phenotype.

The recent molecular biologic findings about HRS cells also offer some explanations for the typical and rather unique immunophenotypic heterogeneity of the HRS cells between cases and also among HRS cells of a given clone. The heterogeneity is likely due in part to different combinations of transforming events between HL cases. Moreover, the coexpression of multiple master regulators of different hematopoietic cell lineages (e.g., PAX5, ID2, NOTCH1) within one HRS cell clone may cause major fluctuations in gene expression between clone members by the fluctuation of the competing transcription factors. Finally, because HRS cells are intimately interacting with multiple types of cells in their microenvironment, both by cellular interactions and through cytokines and chemokines, variations in the direct surrounding of HRS cells may cause differences in gene expression in these cells.

Further work is needed to fully understand the transforming events that lead to the generation of malignant HRS and LP cell clones, but with the availability of new methods, such as massive parallel sequencing of tumor genomes, it can be expected that major progress will be made in the near future.

Finally, the recent detection of cell-free DNA presumably derived from HRS cells in the blood plasma of patients with HL[30] offers exciting novel possibilities to monitor the disease, and this tumor-specific DNA may become a useful biomarker.

REFERENCES

1. Küppers R, Engert A, Hansmann M-L. Hodgkin lymphoma. *J Clin Invest*. 2012;122(10):3439–3447.
2. Brune V, Tiacci E, Pfeil I, et al. Origin and pathogenesis of nodular lymphocyte-predominant Hodgkin lymphoma as revealed by global gene expression analysis. *J Exp Med*. 2008;205(10):2251–2268.
3. Schwering I, Bräuninger A, Klein U, et al. Loss of the B-lineage-specific gene expression program in Hodgkin and Reed-Sternberg cells of Hodgkin lymphoma. *Blood*. 2003;101(4):1505–1512.
4. Lamprecht B, Walter K, Kreher S, et al. Derepression of an endogenous long terminal repeat activates the CSF1R proto-oncogene in human lymphoma. *Nat Med*. 2010;16(5):571–579.
5. Kanzler H, Küppers R, Hansmann ML, et al. Hodgkin and Reed-Sternberg cells in Hodgkin's disease represent the outgrowth of a dominant tumor clone derived from (crippled) germinal center B cells. *J Exp Med*. 1996;184(4):1495–1505.
6. Weniger MA, Tiacci E, Schneider S, et al. Human CD30+ B cells represent a unique subset related to Hodgkin lymphoma cells. *J Clin Invest*. 2018;128(7):2996–3007.
7. Müschen M, Rajewsky K, Bräuninger A, et al. Rare occurrence of classic Hodgkin's disease as a T cell lymphoma. *J Exp Med*. 2000;191(2):387–394.
8. Seitz V, Hummel M, Marafioti T, et al. Detection of clonal T-cell receptor gamma-chain gene rearrangements in Reed-Sternberg cells of classic Hodgkin disease. *Blood*. 2000;95(10):3020–3024.
9. Tiacci E, Döring C, Brune V, et al. Analyzing primary Hodgkin and Reed-Sternberg cells to capture the molecular and cellular pathogenesis of classic Hodgkin lymphoma. *Blood*. 2012;120(23):4609–4620.
10. Küppers R, Bräuninger A, Müschen M, et al. Evidence that Hodgkin and Reed-Sternberg cells in Hodgkin disease do not represent cell fusions. *Blood*. 2001;97(3):818–821.
11. Rengstl B, Newrzela S, Heinrich T, et al. Incomplete cytokinesis and re-fusion of small mononucleated Hodgkin cells lead to giant multinucleated Reed-Sternberg cells. *Proc Natl Acad Sci USA*. 2013;110 (51):20729–20734.
12. Spieker T, Kurth J, Küppers R, et al. Molecular single-cell analysis of the clonal relationship of small Epstein-Barr virus-infected cells and Epstein-Barr virus-harboring Hodgkin and Reed/Sternberg cells in Hodgkin disease. *Blood*. 2000;96(9):3133–3138.
13. Küppers R. Clonogenic B cells in classic Hodgkin lymphoma. *Blood*. 2009;114(18):3970–3971.
14. Nakashima M, Ishii Y, Watanabe M, et al. The side population, as a precursor of Hodgkin and Reed-Sternberg cells and a target for nuclear factor-κB inhibitors in Hodgkin's lymphoma. *Cancer Sci*. 2010;101(11):2490–2496.
15. Shafer JA, Cruz CR, Leen AM, et al. Antigen-specific cytotoxic T lymphocytes can target chemoresistant side-population tumor cells in Hodgkin lymphoma. *Leuk Lymphoma*. 2010;51(5):870–880.
16. Küppers R, Dührsen U, Hansmann ML. Pathogenesis, diagnosis, and treatment of composite lymphomas. *Lancet Oncol*. 2014;15(10):e435–e446.
17. Bräuninger A, Schmitz R, Bechtel D, et al. Küppers R. Molecular biology of Hodgkin and Reed/Sternberg cells in Hodgkin's lymphoma. *Int J Cancer*. 2006;118(8):1853–1861.
18. Mancao C, Hammerschmidt W. Epstein-Barr virus latent membrane protein 2A is a B-cell receptor mimic and essential for B-cell survival. *Blood*. 2007;110(10):3715–3721.
19. Schmitz R, Hansmann ML, Bohle V, et al. TNFAIP3 (A20) is a tumor suppressor gene in Hodgkin lymphoma and primary mediastinal B cell lymphoma. *J Exp Med*. 2009;206(5):981–989.
20. Gunawardana J, Chan FC, Telenius A, et al. Recurrent somatic mutations of PTPN1 in primary mediastinal B cell lymphoma and Hodgkin lymphoma. *Nat Genet*. 2014;46(4):329–335.
21. Tiacci E, Ladewig E, Schiavoni G, et al. Pervasive mutations of JAK-STAT pathway genes in classic Hodgkin lymphoma. *Blood*. 2018;131(22):2454–2465.
22. Green MR, Monti S, Rodig SJ, et al. Integrative analysis reveals selective 9p24.1 amplification, increased PD-1 ligand expression, and further induction via JAK2 in nodular sclerosing Hodgkin lymphoma and primary mediastinal large B-cell lymphoma. *Blood*. 2010;116(17):3268–3277.
23. Rui L, Emre NC, Kruhlak MJ, et al. Cooperative epigenetic modulation by cancer amplicon genes. *Cancer Cell*. 2010;18(6):590–605.
24. Schmitz R, Stanelle J, Hansmann M-L, et al. Pathogenesis of classic and lymphocyte-predominant Hodgkin lymphoma. *Annu Rev Pathol*. 2009;4:151–174.
25. Steidl C, Shah SP, Woolcock BW, et al. MHC class II transactivator CIITA is a recurrent gene fusion partner in lymphoid cancers. *Nature*. 2011;471(7338):377–381.
26. Reichel J, Chadburn A, Rubinstein PG, et al. Flow-sorting and exome sequencing reveals the oncogenome of primary Hodgkin and Reed-Sternberg cells. *Blood*. 2015;125(7):1061–1072.
27. Wienand K, Chapuy B, Stewart C, et al. Genomic analyses of flow-sorted Hodgkin Reed-Sternberg cells reveal complementary mechanisms of immune evasion. *Blood Adv*. 2019;3(23):4065–4080.
28. Hartmann S, Schuhmacher B, Rausch T, et al. Highly recurrent mutations of *SGK1*, *DUSP2* and *JUNB* in nodular lymphocyte predominant Hodgkin lymphoma. *Leukemia*. 2016;30(4):844–853.
29. Ansell SM. Nivolumab in the treatment of Hodgkin lymphoma. *Clin Cancer Res*. 2017;23(7):1623–1626.
30. Spina V, Bruscaggin A, Cuccaro A, et al. Circulating tumor DNA reveals genetics, clonal evolution, and residual disease in classic Hodgkin lymphoma. *Blood*. 2018;131(22):2413–2425.

CHAPTER 80

HODGKIN LYMPHOMA

Anas Younes, Ann S. LaCasce, Graham Collins, Bouthaina Dabaja, and Ahmet Dogan

Hodgkin lymphoma (HL) is an uncommon lymphoproliferative malignancy arising from B cells. It can affect all age groups but is most common in young adults. HL is the first adult malignancy to demonstrate the curative potential of combination chemotherapy. Today, more than 80% of patients with newly diagnosed HL can now expect to be cured of their disease.[1]

The challenge now, particularly since many affected patients are young, is not only to improve cure rates further, but also to minimize the risk of long-term complications of treatment, which can impact quality of life and survival. Accordingly, HL provides a very important clinical model for ongoing cancer research involving both the development of novel targeted agents and the study of late effects of cancer therapy.

Traditionally, the choice of frontline therapy for HL has been determined by clinical stage and prognostic factors. A combined modality approach with chemotherapy and radiotherapy (RT) remains the standard of care for those with early-stage disease, although the routine use of RT has been challenged recently. For those presenting with more advanced stage diseases, chemotherapy alone has been widely adopted. The availability of highly effective chemotherapy combinations and sensitive imaging tests has allowed the development of less toxic therapeutic strategies for those with limited disease. This includes using more limited radiation fields such as involved-field radiotherapy (IFRT), more recently involved-site radiotherapy (ISRT) and, in some centers, involved node radiotherapy (INRT) and a reduction in the number of chemotherapy cycles. This more focused treatment delivery has allowed efficacy to be preserved while exposure to unnecessary toxicity is reduced.

The management of HL continues to evolve ^{18}fluorodeoxyglucose (FDG). Positron emission tomography (PET) imaging has emerged as a useful tool for assessing response and to guide further therapy that may allow RT—free regimens in appropriate patients. This, coupled with ongoing research to identify better biologic prognostic factors, is likely to allow for a more accurate risk-adapted approach to management, with the future treatment of patients with HL being tailored to the needs of the individual. In addition, improved understanding of the molecular mechanisms underlying HL has hastened the development of more effective, and often less toxic, targeted therapies for use either as monotherapy or in combination with traditional chemotherapy to augment efficacy and decrease overall toxicity.

EPIDEMIOLOGY AND ETIOLOGY INCIDENCE AND AGE OF ONSET

HL is a rare B-cell malignancy accounting for less than 1% of all cancers with approximately 9200 new cases diagnosed in the United States and approximately 5500 new cases diagnosed in Europe each year.[2,3] The incidence of HL varies with economic status and geographic location. In developed countries it is associated with a bimodal age of onset distribution, with an early, larger, peak occurring in young adults aged between 20 and 40 years and a second, smaller, peak occurring in those over 55 years. In contrast, in developing countries, the disease predominantly occurs in childhood, with the incidence decreasing with age. Overall, men are affected slightly more frequently than women.

Etiology

The exact cause of HL remains unknown and clearly defined risk factors for the development of the disease are lacking. However, certain associations with its development have been identified. Although a clear genetic cause has not been established, familial susceptibility has been suggested by both an apparent increased risk among siblings of patients with HL, as well as concordance for HL observed in monozygotic twins. Increased maternal education, early birth order, low number of siblings and single-family dwellings in childhood have all also been positively associated with the occurrence of HL in younger patients.

Epstein-Barr virus (EBV)—positive Reed-Sternberg (RS) cells are found in approximately 40% of patients with HL using modern molecular techniques.[4] The incidence of EBV positive HL among those with a past history of serologically proven infectious mononucleosis appears to be higher than those without previous exposure.[5] EBV may play a role in promoting RS survival and has been associated with the increased production of molecules that are involved in mechanisms of immune escape, in turn influencing the microenvironment that supports HL development.

PATHOBIOLOGY OF HODGKIN LYMPHOMA

The World Health Organization (WHO) classifies HL into two distinct disease types: classic HL (cHL), representing 95% of all cases, and nodular lymphocyte-predominant HL (NLPHL), accounting for only 5%.[6] Both cHL and NLPHL are neoplasms composed of a minor component of atypical large neoplastic cells, usually accounting for less than 10% of all cells that are present in a reactive nonneoplastic background.[7,8] However, based on their distinct clinical and molecular genetic features, it is now evident that cHL and NLPHL are two biologically distinct entities. Within cHL, four histologic subtypes are recognized based on the morphology of the neoplastic cells, composition of the nonneoplastic infiltrate, and overall nodal architecture: nodular sclerosing (NSCHL), mixed cellularity (MCCHL), lymphocyte-depleted (LDCHL), and lymphocyte-rich (LRCHL). Of these, NSCHL predominates, accounting for 70% of cases of cHL in Europe and the United States (Table 80.1). The rate of NSCHL, however, varies with geographic location and socioeconomic status and is much lower in developing countries, where MCCHL predominates. Furthermore, NSCHL occurs less frequently in patients infected with HIV, among whom the more common presenting subtype, again, is MCCHL.

Nodular Lymphocyte-Predominant Hodgkin Lymphoma

Typical cases of NLPHL show partial or complete nodal architectural effacement by a macronodular proliferation (Fig. 80.1A), where the nodules are composed of scattered neoplastic cells termed *lymphocyte-predominant (LP) cells*, formerly known as L&H cells.[9] LP cells are large with single folded or multilobulated nucleus with distinct but smaller nucleoli than RS cells. Because of their highly complex nuclear lobation, they have also been widely referred to as “popcorn cells” (see Fig. 80.1B). The background nonneoplastic infiltrate is composed of predominantly small B cells and a variable number of histiocytes. Mixed inflammatory cells such as eosinophils and neutrophils are rare to absent.

LP cells typically express B-cell markers including CD20 (see Fig. 80.1C), PAX-5, and CD79a. In addition, LP cells consistently express CD45, and the B-cell transcriptional factors OCT-2 and BOB.1, that are usually not expressed by RS cells. In contrast to RS cells, LP cells typically lack CD15 and CD30 expressions.[10] In keeping with their germinal center B-cell origin, LP cells are consistently positive for BCL6, although CD10 is usually not expressed. They may express EMA (epithelial membrane antigen) in a subset of LP cells in approximately 50% of the cases, and variably express IRF4/MUM1. A subset of cases with expression of IgD, and unique pathological and clinical features has also been described.[11] EBV-encoded RNA (EBER) and latent membrane protein-1 (LMP1) are consistently negative.

TABLE 80.1 **Frequency of Histologic Subtypes of Hodgkin Lymphoma**

Classic Hodgkin lymphoma (cHL)	95%
• *Nodular sclerosis classic Hodgkin lymphoma (NSCHL)*	70%
• *Mixed cellularity classic Hodgkin lymphoma (MCCHL)*	20–25%
• *Lymphocyte-rich classic Hodgkin lymphoma (LRCHL)*	5%
• *Lymphocyte-depleted classic Hodgkin lymphoma (LDCHL)*	<1%
Nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL)	5%

The nodules in NLPHL are typically supported by an expanded meshwork of follicular dendritic cells that can be highlighted by CD21 and CD35, and populated by small B cells that are positive for immunoglobulin (Ig) M and IgD. There are a variable number of T cells, a significant proportion of which is positive for CD4, CD57, and programmed cell death protein 1 (PD-1) which frequently form rosettes around the LP cells (see Fig. 80.1D). Immunoarchitectural patterns as determined relative abundance and distribution of reactive B cells and T cells have been described. Limited data suggests that as the disease progresses the nodules acquire T-cell-rich features, at the end resembling T-cell histiocyte-rich large B-cell lymphoma. These histological variants are associated with increased chance of advanced disease and relapse.[12]

Genetic studies performed on microdissected LP cells show clonally rearranged Ig genes with high load of somatic mutations in the variable region, indicating the presence of ongoing mutations and consistent with germinal center B-cell derivation of NLPHL. In contrast to cHL, the Ig rearrangement is functional with detectable Ig mRNA transcripts. Acquired somatic mutations described in NLPHL include BCL6 gene rearrangements, mutations affecting PAX5, PIM1, RHOH, MYC, SGK1, DUSP2, and JUNB.[13]

Classic Hodgkin Lymphoma

cHL is characterized by the presence of RS cells and their morphologic variants in a reactive background composed of mixed inflammatory cells (except in lymphocyte-rich variant).[8] *Classic (diagnostic) RS cells* are large binucleated or multinucleated cells with pale chromatin, distinct nuclear membrane, single prominent eosinophilic,

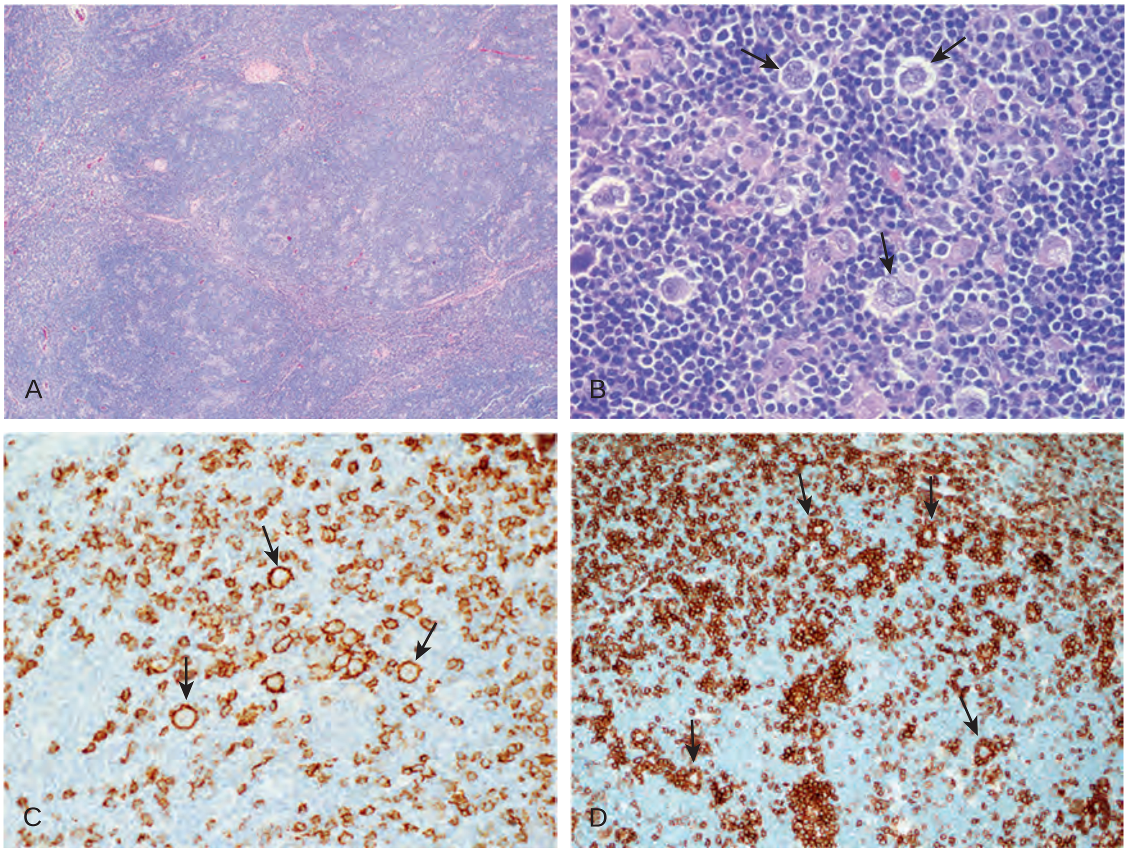


Figure 80.1 A CASE OF NODULAR LYMPHOCYTE-PREDOMINANT HODGKIN LYMPHOMA. The lymph node architecture is effaced by multiple expansile nodules with mottled appearance (A). Within the nodules there are scattered atypical large cells (lymphocyte-predominant *[LP]* cells) with single folded or multilobated nucleus with distinct but relatively small nucleoli (B). The LP cells are positive for CD20 *(arrows)* with similar strong expression intensity as background small reactive B cells (C). The background infiltrate is composed of numerous programmed cell death protein 1 *(PD-1)*—positive T cells that frequently form rosettes *(arrows)* around the LP cells (D).

inclusion-like nucleolus in each nuclear lobe, and abundant amphophilic cytoplasm (Fig. 80.2A). Mononuclear variants with otherwise similar cytonuclear features are termed *Hodgkin cells* (see Fig. 80.2B). *Mummified cells* are degenerated RS and Hodgkin cells with pyknotic nuclei and condensed cytoplasm (see Fig. 80.2C). These variants are usually seen in various proportions in all four subtypes of cHL. In addition, *lacunar cells* are characteristic of nodular sclerosis cHL but usually not other subtypes, which have abundant pale cytoplasm that frequently retracts in formalin fixed tissue, creating an empty space (lacunae) around the cells (see Fig. 80.2D).

NSCHL is characterized by sclerotic nodal capsule and presence of collagenous bands traversing through the nodal parenchyma, imparting a prominent nodular pattern (Fig. 80.3A). Within the nodules there are a variable number of RS cells and variants, especially lacunar cells, with a background of mixed inflammatory cells composed of a variable proportion of small lymphocytes, histiocytes, plasma cells, eosinophils, and neutrophils. The RS cells and variants may be singly dispersed or form confluent aggregates/sheets.

In *MCCHL* the lymph node architecture is usually diffusely obliterated, although an interfollicular pattern may be seen in early involvement. In contrast to NSCHL, the nodal capsule is not thickened and there are no collagenous bands of fibrosis (see Fig. 80.3B). RS cells and variants are usually easily identified and dispersed throughout the nodal tissue in a mixed inflammatory background. In comparison to NSCHL, MCCHL is more often associated with higher stage disease and EBV positivity and is more likely seen in the HIV-infected patient population.

LRCHL is a relatively recently defined subtype of cHL characterized by the presence of RS cells in a background of almost exclusively small lymphocytes, with a paucity or absence of eosinophils and neutrophils. The vast majority of the cases exhibit nodular growth pattern, although a rare diffuse variant has also been described. In the vast majority of the cases the affected lymph node is obliterated by multiple expansile nodules with expanded mantle zones and regressed, eccentrically located residual germinal centers (see Fig. 80.3C).

LDCHL is exceedingly rare (<1% of cHL) with a highly variable histologic appearance, but in all cases characterized by a relative predominance of RS cells in comparison to the background lymphocytes. Some cases are characterized by scattered RS cells in a diffusely fibrotic background containing histiocytes, fibroblasts, and few lymphocytes. In others, sheets of bizarre, pleomorphic, or anaplastic-appearing RS cells are present, imparting a sarcomatous appearance (see Fig. 80.3D).

The immunophenotypic profile of RS cells in all cHL subtypes is similar. RS cells are strongly positive for CD30 with membranous and Golgi pattern in nearly all cases (Fig. 80.4A), and CD15 with variable staining intensity in approximately 80% of the cases (see Fig. 80.4B). Consistent with their B-cell derivation, RS cells express PAX-5 in almost all cases (95%) but with weaker intensity when compared with the surrounding nonneoplastic small B cells (see Fig. 80.4C). However, in keeping with their defective B-cell program, RS cells lack Ig production as evidenced by absence of J chain, and are negative for most other B cell–associated antigens: CD20 (expressed in only 20% to 30% of cases; often only in a subset of RS cells with weak/variable intensity), CD19, and CD79a; as well as B-cell transcriptional factors octamer transcription factor-2 (OCT-2) and B-cell oct-binding protein 1 (BOB.1) (each expressed in 10% of cases; coexpression is rare). RS cells are almost always positive for IRF4/MUM1 and negative for CD45 and EMA, features that may

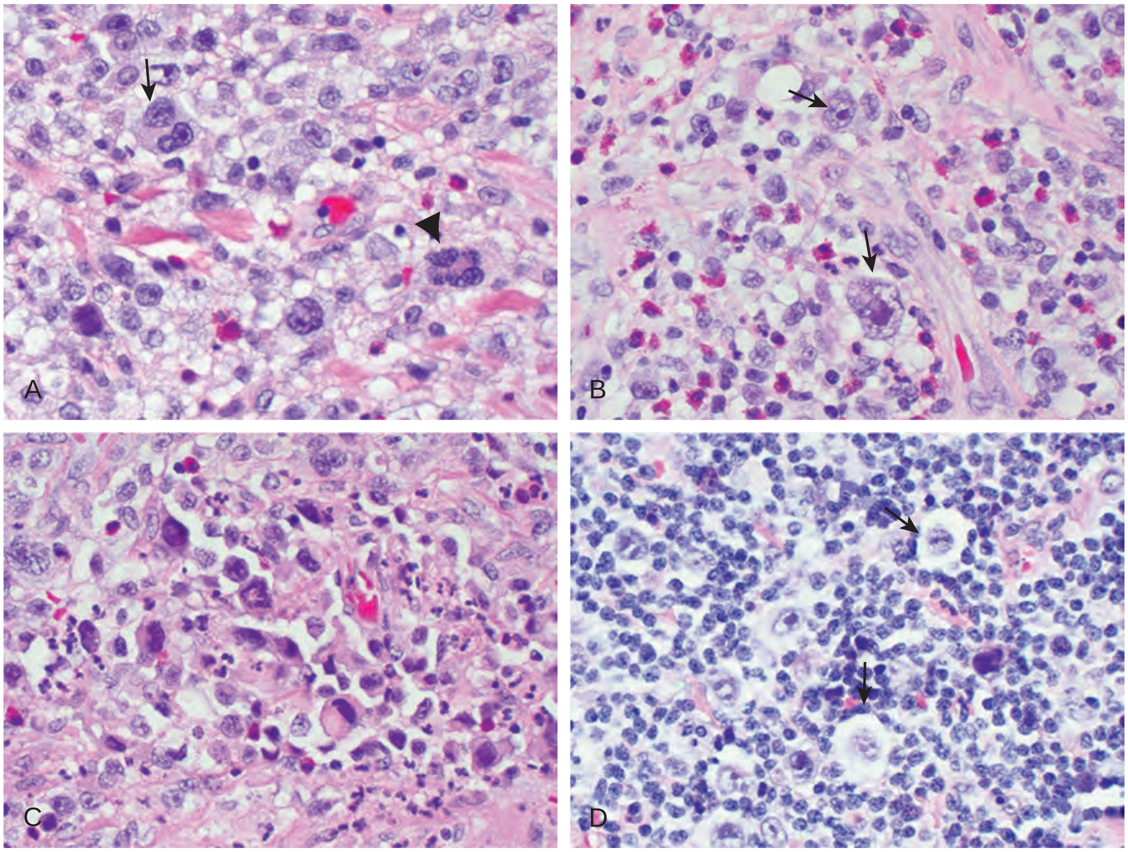


Figure 80.2 MORPHOLOGIC VARIATIONS OF REED-STERNBERG CELLS IN CLASSIC HODGKIN LYMPHOMA. The diagnostic Reed-Sternberg *(RS)* cells (A) are large binucleated *(arrow)* or multinucleated cells *(arrowhead)* with distinct nuclear membrane, prominent nucleolus in each nuclear lobe, and abundant amphophilic cytoplasm. Hodgkin cells are mononuclear variants of RS cells with similar cytonuclear features (B); numerous eosinophils and granulocytes are noted in the background. Mummified cells are degenerated RS and Hodgkin cells with pyknotic nuclei and condensed cytoplasm (C). Lacunar cells, characteristic of nodular sclerosis classic Hodgkin lymphoma (cHL), have abundant pale cytoplasm which frequently retracts in formalin fixed tissue (D).

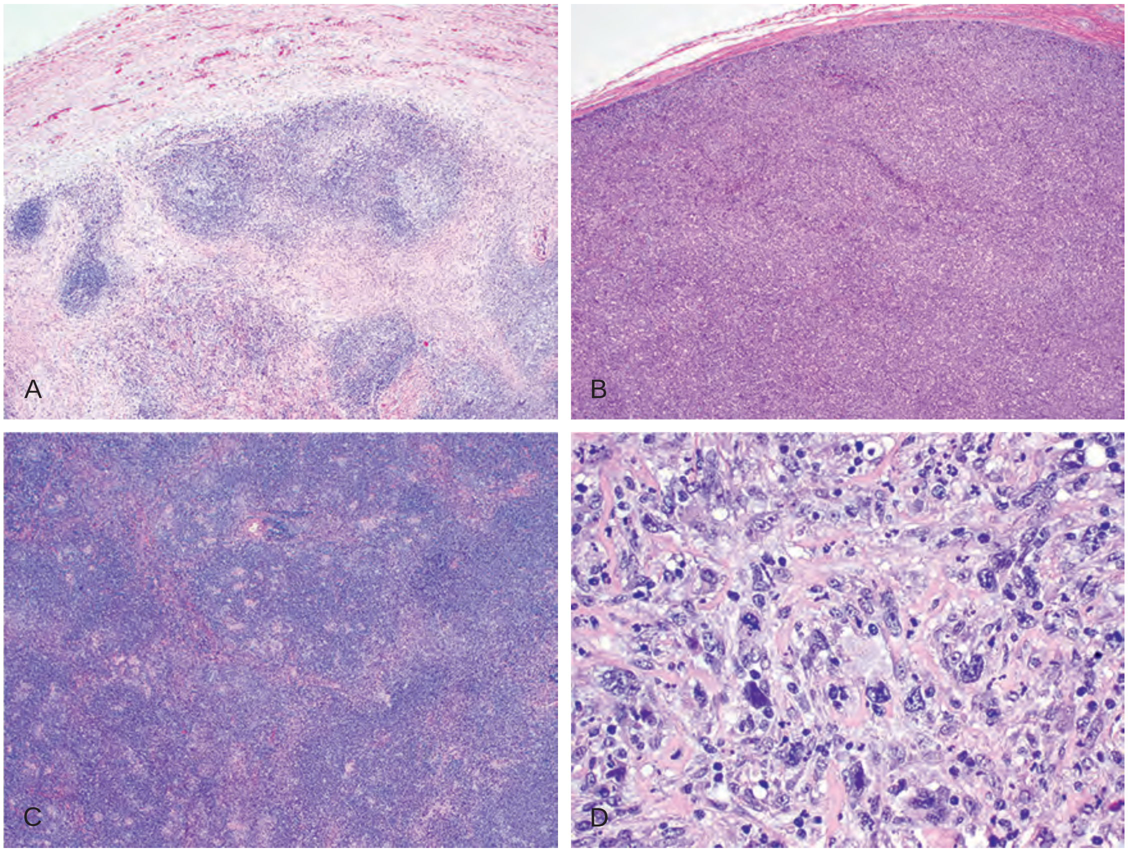


Figure 80.3 HISTOLOGIC PATTERNS OF CLASSIC HODGKIN LYMPHOMA SUBTYPES. Nodular sclerosis classic Hodgkin lymphoma (cHL) characteristically shows multinodular pattern on low magnification, where the nodules are surrounded by broad collagenous bands. The nodal capsule is markedly sclerotic (A). In mixed cellularity cHL, the lymph node architecture is diffusely obliterated by the neoplastic infiltrate. The nodal capsule is not sclerotic and there are no collagenous bands of fibrosis (B). A case of lymphocyte-rich cHL where the affected lymph node is obliterated by multiple expansile nodules, with strikingly similar morphologic appearance to nodular lymphocyte-predominant Hodgkin lymphoma (C). A case of lymphocyte-depleted cHL with presence of numerous pleomorphic/anaplastic appearing Reed-Sternberg (RS) cells (D).

help distinguish cHL from NLPHL. Expression of other hematopoietic lineage—associated markers, such as T cells (CD4, granzyme B), dendritic cells (fascin, CCL17), and myeloid cells (colony stimulating factor 1 receptor and α_1-antitrypsin) is also often present. EBV LMP1 and/or EBER expression (see Fig. 80.4D) by RS cells is seen in approximately 40% of cHL cases overall in Western countries, but mostly in MCCHL and LDCHL and less frequently in NSCHL and LRCHL. However, an association with EBV is seen in up to 90% of cHL cases in developing countries and nearly all cases in the HIV patient population.

The nonneoplastic background lymphocytes, with the exception of LRCHL, are composed of predominantly T cells with marked predominance of CD4-positive cells that coexpress CD25 and FOXP3, consistent with immunosuppressive regulatory T cells (TReg). In addition, there is a significant population of TH2 cells. TReg and TH2 cells are attracted by cytokines (CCL5, CCL17, and CCL22) secreted by RS cells. In HIV-infected patients there is often a predominance of CD8-positive T cells. In contrast to NLPHL, CD57-positive T cells are not increased in number in cHL.

Polymerase chain reaction studies performed on RS cells procured by microdissection demonstrated that in the vast majority of cHL cases (>98%), the RS cells harbor clonal IgH gene rearrangement. The rearranged IgH shows a high load of somatic hypermutation in the variable region without evidence of ongoing mutation, consistent with germinal center or postgerminal center B-cell derivation. Rare cases harboring T-cell receptor gene rearrangements have also been documented. Genetic structural alterations that modulate tumor cell biology and the tumor microenvironment have been recently reported. These include mutations leading to loss of MHC class I and class II expressions,[14,15] upregulation of PDL-1 expression by gene amplification or rearrangements,[16] deregulation of nuclear factor-kappa B (NF-κB) pathway,[17,18] and activation of janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway (Fig. 80.5).[19]

DIAGNOSIS AND STAGING

Diagnosis

The definitive diagnosis of HL must be made pathologically via excision biopsy of an affected lymph node or other suspected organ. Core needle biopsy is deemed inferior to excision biopsy because of the unique architecture of the lymph node in HL that comprises a relatively dilute population of malignant cells. The presence of RS cells in cHL, and LP cells in NLPHL in tissue biopsy, provides the basis for discriminating between these two major diagnostic subtypes, with immunophenotypic profiling representing a crucial adjunct for all new cases of HL.

Staging

Stage of disease is the major determinant of prognosis for patients with newly diagnosed HL, and accurate assessment is paramount when deciding upon optimal therapy.[20] The current staging system used for patients with HL is the Lugano classification, which was based on the Cotswold-modified Ann Arbor classification system (Table 80.2). Recently, a simplified staging and response assessment criteria were proposed.[21] An integrated PET/computed tomography

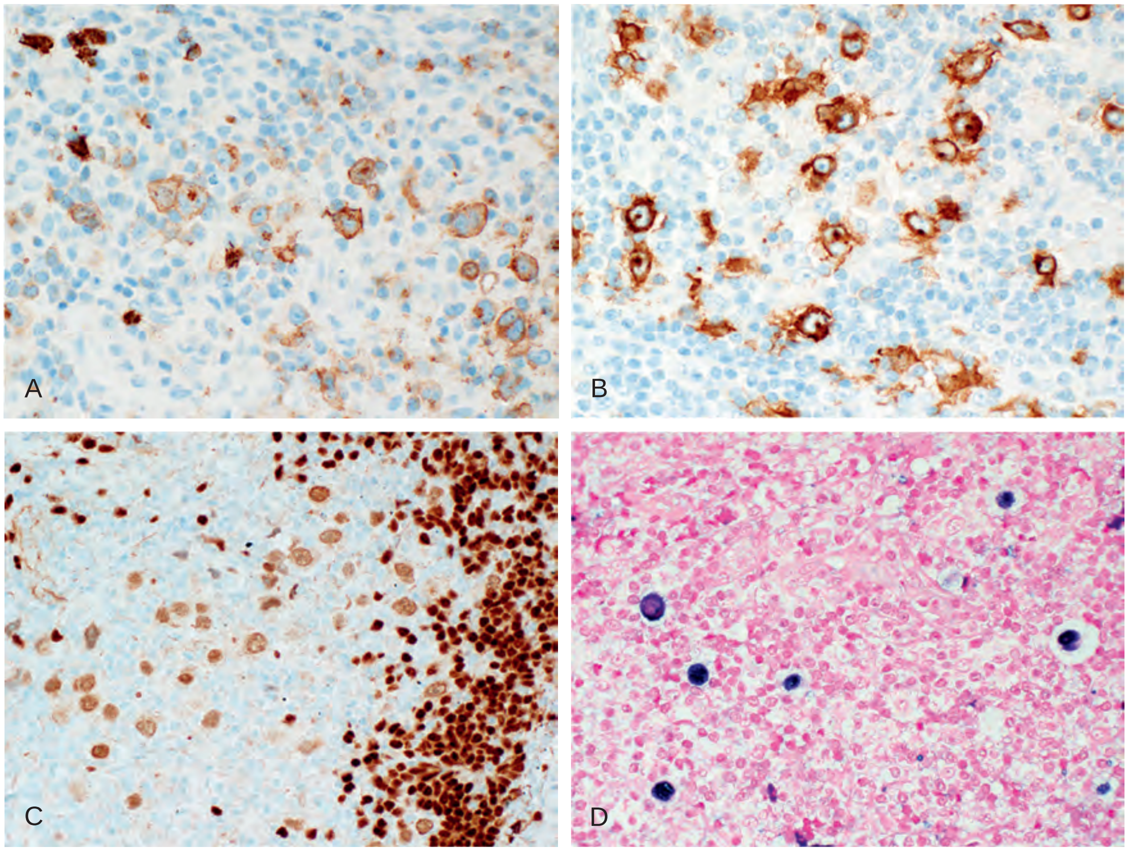


Figure 80.4 IMMUNOPHENOTYPIC PROFILE OF REED-STERNBERG (RS) CELLS IN CLASSIC HODGKIN LYMPHOMA. The RS cells and variants are positive for CD15 (A) and CD30 (B), with characteristic membranous staining and Golgi accentuation. These cells are positive for PAX-5, but with weaker intensity in comparison to background small reactive B cells (C). The RS cells are positive for Epstein-Barr virus (EBV) as demonstrated by in situ hybridization for EBV-encoded RNA/EBV-encoded RNA (EBER) (D).

(CT) is used for staging of ^{18}F-2-deoxyglucose (FDG)—avid lymphomas, and reinforced its recommendation as an effective response assessment tool both early on during treatment and at the end of treatment to establish remission status.[22]

Bone marrow involvement occurs in less than 5% of patients with HL at diagnosis. Until recently, following pathologic diagnosis with excisional node biopsy, a bone marrow biopsy was also required as part of routine staging for all patients with newly diagnosed HL. However, because of the high sensitivity of PET/CT for bone marrow involvement, bone marrow biopsy is no longer mandated for the routine staging of patients with newly diagnosed HL, unless PET/CT imaging is unavailable.[22]

In addition to excisional node biopsy and staging with PET/CT, assessment of the peripheral blood represents an important part of the diagnostic workup for patients with newly diagnosed HL with particular respect to risk stratification and treatment choice. Complete blood count, erythrocyte sedimentation rate (ESR), and serum biochemistry including C-reactive protein, alkaline phosphatase, lactate dehydrogenase, liver function tests, renal function tests, and albumin are required as part of standard care, and screening for HIV and hepatitis is strongly advised. In addition, given the potentially damaging effects of chemotherapy and RT, certain pretreatment investigations including cardiac and pulmonary function tests, thyroid function tests, reproductive counseling, and serum pregnancy testing, may also be warranted in selected patients.

CLINICAL FEATURES

The importance of an accurate clinical history in facilitating the management of patients with HL should not be underestimated. Certain symptoms may provide clues as to the likely stage of disease or lead to further investigations that might identify additional sites of disease. This may result in important treatment modifications. An accurate past medical history, particularly with regards to lung, heart, and kidney function, is also crucial in highlighting those organs that might benefit from further investigation before commencing therapy, to ensure that treatment choices are both effective and safe for the individual.

Systemic symptoms that are known to influence prognosis in patients with HL include night sweats, fever, and weight loss. These constitutional symptoms have come to be known as “B symptoms” and their importance in HL is reflected by their inclusion as key components of the Cotswold-modified Ann Arbor staging system. B symptoms are a presenting feature in approximately 30% of patients with advanced stage HL. A particular type of fever that is considered characteristic of HL historically, is the Pel-Ebstein fever, which typically follows a swinging pattern, occurring on a daily basis for many weeks, with intermittent afebrile periods occurring between episodes. Other well-described clinical features associated with HL include fatigue, chronic pruritus, which may be an early sign of disease in up to 15% of patients, and the presence of a pain localized to the site of involved lymphadenopathy that is precipitated by the consumption of alcohol.

PROGNOSTIC FACTORS, RISK STRATIFICATION, AND TREATMENT GROUPS

Prognostic factors have helped to predict the likely outcome for individual patients with HL at diagnosis. Clinical stage, presence of systemic symptoms, and tumor burden continue to be important prognostic factors in HL, and, in addition to disease histology and

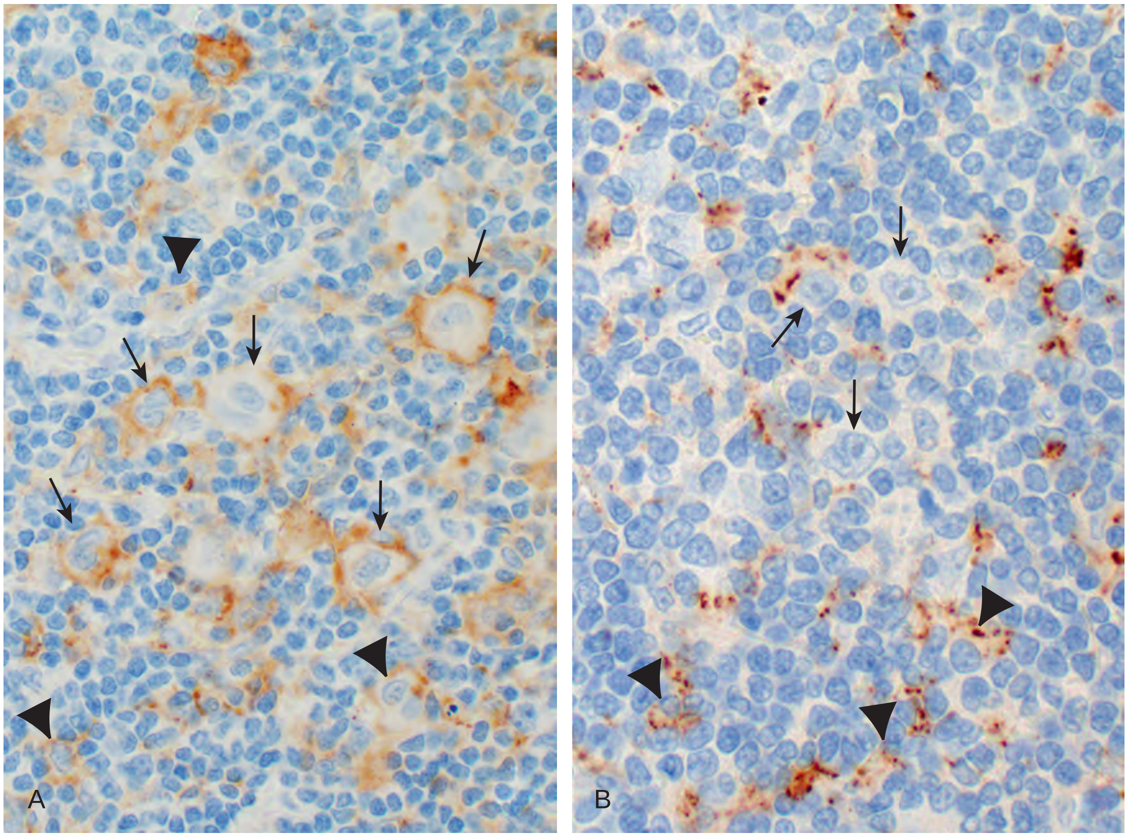


Figure 80.5 IMMUNOHISTOCHEMICAL ANALYSIS OF BETA-2-MICROGLOBULIN AND PDL-1 EXPRESSION IN CLASSIC HODGKIN LYMPHOMA. (A) A case of nodular sclerosis classic Hodgkin lymphoma (cHL) showing loss of beta-2-microglobulin in Reed_Stenberg cells, and (B) upregulation of PDL-1 secondary to rearrangement of *PDL-1/PDL-2* gene locus as demonstrated by fluorescence in situ hybridization (FISH) analysis *(inset)*. Blue probe represents centromeric region of chromosome 9 whereas green and red probes flank the *PDL-1/PDL-2* gene locus. The rearrangement affecting one of the three copies of the *PDL-1/PDL-2* locus lead to separation of green and red signals.

TABLE 80.2 Cotswold-Modified Ann Arbor Staging System for Hodgkin Lymphoma

Stage	Criteria
I	Disease affecting a single lymph node region or lymphoid structure (e.g., spleen, thymus, Waldeyer ring)
II	Disease affecting two or more discrete lymph node regions confined to the same side of the diaphragm
II	Disease affecting two or more discrete lymph node regions or lymphoid structures on both sides of the diaphragm
IV	Disease that has spread to one or more extranodal site (that does not meet the criteria for E) or extralymphatic structure including involvement of the bone marrow, liver, or lungs
Designation	**Criteria**
A	Absence of B symptoms[a]
B	Presence of B symptoms[a]
S	Involvement of the spleen
E	Single extranodal site or involvement of an extranodal site that is contiguous to an involved nodal region.
X	Bulky disease as defined as >1/3 mediastinum at its widest part or a nodal mass >10 cm at its greatest diameter.

[a] B symptoms: constitutional symptoms including night sweats, fevers, or weight loss (>10% over 6 months).

anatomic stage, are widely used for risk stratification and subsequent selection of appropriate initial therapy.[23]

HL patients have traditionally been divided into two distinct prognostic groups according to clinical stage at diagnosis: early-stage disease, accounting for 45% of newly diagnosed patients, and advanced-stage disease, accounting for 55% of newly diagnosed patients.

The category of early-stage HL includes patients with stages I or II. Early-stage disease may also include patients with B symptoms, bulky disease, or extension to adjacent sites. Additional prognostic factors were developed to stratify early-stage disease into early "favorable" and early "unfavorable" disease subgroups. Early stage I or II HL is considered "favorable" if it is limited to an area above the diaphragm and is not associated with other risk factors. Early stage I or II HL is considered "unfavorable" in the presence of other risk factors related to age, tumor burden, ESR, and number of involved nodal areas (Table 80.3).[24] Early-unfavorable disease is often referred to as Intermediate stage disease and these terms may be used interchangeably. Thus, early-stage HL is a heterogeneous group and treatment algorithms have been developed for different subgroups based on these prognostic factors and response criteria.

The prognostic criteria for advanced-stage HL again vary among different cooperative groups. The European Organisation for Research and Treatment of Cancer (EORTC) defines advanced stage as those patients with clinical stage III–IV disease only, whereas the German Hodgkin Study Group (GHSG) also includes those patients with clinical stage IIB disease and a large mediastinal mass and/or extranodal disease involvement in their definition (see Table 80.3).

Following the identification of more specific and more widely applicable prognostic factors, the International Prognostic Score (IPS) was developed as an internationally accepted means of distinguishing those patients with newly diagnosed advanced HL who might be cured

TABLE 80.3 Prognostic Factors in Early and Advanced-Stage Hodgkin Lymphoma

Prognostic Group	EORTC	GHSG	NCCN
Early-favorable	CS I-II without risk factors (supra-diaphragmatic)	CS I-II without risk factors	CS IA-IIA without risk factors
Early-unfavorable (Intermediate)	CS I-II with ≥1 risk factor (supra-diaphragmatic)	CS I-IIA with ≥1 risk factor C/D but not A/B	CS I-II with ≥1 risk factor
Advanced	CS III-IV	CS IIB with risk factors A/B CS III/IV	CS III-IV
Prognostic factors	(A) Bulky mediastinal mass[a] (B) Age ≥50 years (C) Elevated ESR (>50 mm/h without B symptoms; >30 mm/h with B symptoms[b]) (D) ≥4 nodal areas (out of 5 supra-diaphragmatic EORTC areas)	(A) Bulky mediastinal mass[a] (B) Extranodal disease (>1 lesion) (C) Elevated ESR (>50 mm/h without B symptoms; >30 mm/h with B symptoms[b]) (D) ≥3 nodal areas (out of 11 GHSG areas)	(A) Bulky mediastinal mass[a] (B) Bulk >10 cm (C) Elevated ESR (>50 mm/h without B symptoms) (D) B symptoms (E) ≥4 nodal areas (out of 17 Ann Arbor regions)

CS, Clinical stage; *EORTC*, European Organization for Research and Treatment of Cancer; *ESR*, estimated sedimentation rate; *GHSG*, German Hodgkin Study Group; *NCCN*, National Comprehensive Cancer Network.

[a]Bulky mediastinal mass: ratio ≥0.035 of the maximum horizontal chest diameter (EORTC); ratio ≥1/3 of the maximum horizontal chest diameter (GHSG); ratio greater than 1/3 of the maximum horizontal chest diameter (NCCN).

[b]B symptoms: night-sweats, fever, weight loss (unexplained, >10% over 6 months).

TABLE 80.4 International Prognostic Score for Advanced Hodgkin Lymphoma

No. of Prognostic Factors	% of Patients	5-year FFP (%)	5-year OS (%)
0–1 (low-risk)	29	79	90
2–3 (intermediate-risk)	52	64	80
4–7 (high-risk)	19	47	59

FFP, Freedom from progression; *OS*, overall survival.

by standard treatment, and, therefore, avoid overtreatment for those whom standard treatment might fail. In 1998, based on a multivariate analysis of survival data from 5141 patients with newly diagnosed advanced HL treated between 1983 and 1992, seven adverse prognostic factors were identified as being statistically meaningful when predicting 5-year freedom from progression (FFP) and overall survival (OS): age ≥45 years, male sex, albumin less than 40 g/L, hemoglobin less than 10.5 g/dL, Ann Arbor stage IV, leukocytosis ≥15 × 10^9/L, and lymphocyte count less than 0.6 × 10^9/L.[23] Five-year FFP was 84% for those patients with no adverse prognostic factors, and each additional factor reduced FFP by 7%, with four to seven factors representing an FFP of 40%. Three risk groups were established as a result, allowing therapy to be chosen according to these specific clinical characteristics, with the consensus being that higher risk patients should receive more intensive therapy (Table 80.4).

Treatment According to Prognostic Group

For patients with early stage disease, therapeutic options include combined modality therapy and chemotherapy alone.[24] In patients receiving combined modality therapy, the number of cycles of chemotherapy and dose of radiation are determined by whether patients present with favorable or unfavorable disease. Increasingly, however, given the desire to avoid late toxicities associated with radiation, PET adapted approaches with chemotherapy alone are being recommended.[25–27] The omission of RT is associated with a slightly higher risk of relapse compared to chemotherapy, only without an impact on OS (Table 80.5).

TREATMENT OF EARLY-STAGE HODGKIN LYMPHOMA

Early-Stage Nodular Lymphocyte-Predominant Hodgkin Lymphoma

Patients with NLPHL typically present with localized peripheral lymphadenopathy in the absence of B symptoms, bulky disease, and mediastinal involvement. The clinical behavior of NLPHL is more similar to that of indolent non-Hodgkin lymphoma, as opposed to cHL.

The optimal treatment for NLPHL remains poorly defined.[28,29] Although no randomized trials exist specifically for early stage NLPHL, ISRT is typically employed.[30] A single center series of 93 patients treated with RT alone reported a 10-year progression with free survival rates of 85% and 61% for stage I and stage II patients respectively, with OS rates of 94% and 97%.[31] For patients with stage I or contiguous stage II disease, ISRT is typically preferred, though observation for surgically excised patients is preferred by some physicians and is commonly used in pediatrics. Patients with more extensive disease or B symptoms, particularly those with splenic and/or upper abdominal involvement may have occult transformation to aggressive lymphoma. In the absence of transformation, optimal front-line therapy is unclear, and patients are often managed with chemoimmunotherapy including rituximab, as is used in advanced stage disease.

Early-Stage Classic Hodgkin Lymphoma

Recent strategies for the management of early-stage cHL focus on balancing long term control of disease with late effects of therapy and include combined modality therapy as well as chemotherapy only approaches.[32] Therapeutic options are guided by prognostic risk groups.[33]

In the GHSG HD10 study, 1370 patients with low-risk disease (one or two non-bulky sites of nodal disease), were randomized to 2 versus 4 cycles of ABVD (adriamycin, bleomycin, vinblastine, and dacarbazine) followed by 20 versus 30 Gy of IFRT. Outcomes were excellent in all groups with freedom from treatment failure over 90% and OS greater than 96% at 5 years, thus establishing 2 cycles of

TABLE 80.5 Standard Treatment Approach According to Prognostic Group

Early-favorable HL	Combined modality therapy • 2–4 cycles of chemotherapy followed by involved-field radiotherapy
Early-unfavorable HL (intermediate-stage)	Combined modality therapy • 4–6 cycles of chemotherapy followed by involved-field radiotherapy
Advanced HL	Extensive chemotherapy • 6–8 cycles of chemotherapy ± consolidation with localized radiotherapy

HL, Hodgkin lymphoma.

chemotherapy followed by 20 Gy as a standard approach in this group of favorable patients.[34] Subsequently, the HD13 study evaluated the elimination of bleomycin and dacarbazine in the ABVD regimen when given in combination with IRFT but demonstrated high rates of treatment failures with the omission of agents.[35]

In unfavorable early-stage patients, with the exception of the combination of bulky disease and B symptoms, the GHSG HD11 trial randomized 1397 patients between 4 cycles of ABVD and 4 cycles of baseline BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisolone), and each chemotherapy arm was followed by either 30 Gy or 20 Gy IFRT.[36] Four cycles of ABVD followed by IFRT of 30 Gy resulted in a 5-year FFTF of 85.3% and OS of 94.3% and was less toxic than baseline BEACOPP. In the subsequent trial, HD14, 2 cycles of escalated BEACOPP followed by 2 cycles of ABVD and 30 Gy IFRT (HD14 trial) was compared to 4 cycles of ABVD and IFRT. Although the FFTF was superior in the escalated BEACOPP arm at 95% versus 88% there was no difference in OS with median follow-up of 43 months. Toxicity in the intensified regimen was significant particularly in terms of myelosuppression and infection, and there were two cases of myelodysplastic syndrome (MDS)/acute myeloid leukemia (AML).

Given the risk of late toxicity associated with RT, the Canadian/ECOG HD6 study evaluated the use of chemotherapy alone in early stage HL in 405 patients.[37] The study which employed subtotal nodal radiation therapy (STNRT) was closed early after this approach was replaced by IRFT based on clinical trial results published during the course of the study. Patients with stage I-IIA disease treated in the experimental arm received 4 cycles of ABVD if interim CT scans demonstrated complete remission and all others were treated with 6 cycles. Patients in the standard arm with no risk factors were treated with STNRT and unfavorable disease defined as age greater than 40, great than four lymph node sites involved, presence of mixed cellularity or LD histology, ESR greater than 50, received STNRT plus 2 cycles of ABVD. With median follow-up of 11.3 years, freedom from disease progression was 87% in the chemotherapy only group versus 92% in those receiving RT. The 12-year OS rate, however, was superior at 94% in the chemotherapy only group compared to the 87% in those receiving RT. STNRT used in the study is likely associated with increased risk of late toxicity compared to modern RT approaches. Despite this, the study led to the increased use of chemotherapy alone, particularly in younger women with increased risk of breast cancer and others at higher risk for late adverse effects.

Radiation Therapy Planning and Treatment

Modern RT techniques for planning and treatment have significantly decreased the risk of side effects. The radiation fields using ISRT, based on published guidelines by the International Lymphoma Radiation Oncology Group (ILROG),[38,39] has educated radiation oncologists on how to target the sites of the disease while avoiding the surrounding normal tissues. Advances in computer-based planning, inverse planning technology (using mathematical iterations for planning with Intensity Modulated Radiation Therapy, IMRT) and proton therapy are now considered standard treatment.[40] Real-time verification of radiation delivery using onboard imaging increased the refined targeting of radiation. Finally, ILROG guidelines gives elaborate recommendations as to how to respect normal tissues tolerance, strict constraints to be applied to protect the heart, coronary vessels, breasts, lungs, thyroid, kidneys, and salivary glands.

Response-Adapted Treatment Approach

More recently, in the era of FDG-PET imaging for assessment of metabolic response to induction chemotherapy, risk adapted approaches have been tested in a number of clinical trials.[32,41] The EORTC H10 trial, which used modern- INRT techniques, randomized (both favorable and unfavorable risk) patients to chemotherapy alone (4 cycles total in the favorable and 6 cycles in the unfavorable) versus combined modality treatment (CMT) for those with a negative FDG-PET after 2 cycles of ABVD.[42] The outcomes were excellent in patients who received chemotherapy alone or CMT. This study enrolled 1137 patients and after 34 events, per study design, the interim analysis mandated closure of the chemotherapy only arms given excess relapse rates. Overall, approximately 81% of patients were PET2 negative. In the favorable group, the 5-year PFS was 99% in the CMT arm compared to 87% in the chemotherapy alone arm. The difference was less pronounced in the unfavorable arm at 92% versus 90% respectively. The addition of RT did not translate into an OS benefit. For the PET positive patients, 5-year PFS was 91% with escalation to BEACOPP plus INRT compared to 77% in the ABVD + INRT arm. There was a trend for improvement in OS in the intensified arm at 96% versus 89.3% with HR 0.4 ($P = .062$).[42]

The UK National Cancer Research Institute (NCRI) RAPID trial also tested a PET adapted strategy in early-stage HL patients.[26] 420 PET-negative patients (after 3 cycles of ABVD) were randomized to receive IFRT versus no further treatment. After a median follow-up of 45 months, this noninferiority trial revealed no difference in OS, but slightly, although not statistically significant, higher 3-year PFS (intention to treat analysis) in patients receiving IFRT (93.8% versus 90.7%), where a margin of ≤7% PFS difference was deemed acceptable.[5] When an “as-treated” analysis was performed, the 3-year PFS was 97% for CMT versus 90.7% for chemotherapy alone (hazard ratio [HR] = 2.39, $P = .03$).

In the HD16 GHSG study of patients with early favorable disease ($n = 1150$), patients were randomly assigned to standard therapy with 2 cycles of ABVD plus 20 Gy IFRT versus PET guided therapy.[43] PET2 negative patients received no further therapy. In PET2 negative patients ($n = 628$), 5-year PFS was 86% versus 93% in the chemotherapy only and CMT arms respectively. For patients treated with standard therapy the 5-year PFS was 93% in the PET2 negative and 88% in the PET2 positive groups.

In a US non-randomized PET directed study of 164 patients with early favorable and unfavorable patients without bulky disease, the Alliance cooperative group instead chose to administer 4 cycles of ABVD to PET2 negative patients.[27] PET2 positive patients were treated with 2 cycles of escalated BEACOPP plus IFRT. 91% of patients were PET2 negative with 3-year PFS of 91% (94% in those with Deauville scores of 1 to 2 and 77% in the Deauville 3 group). In the PET2 positive arm, the 3-year PFS was 66%.

TREATMENT OF ADVANCED-STAGE HODGKIN LYMPHOMA

The improved outlook for patients with advanced-stage HL (stages IIB–IV) over recent decades is largely attributed to the global drive to develop front-line chemotherapy regimens in which improved efficacy is coupled with reduced treatment-associated toxicity. Today, the most widely used regimens are ABVD (adriamycin, bleomycin, vinblastine, and dacarbazine), BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisolone) and AAVD (Adriamycin, brentuximab vedotin (Adcetris), vinblastine and dacarbazine) (Table 80.6).

ABVD

The ABVD regimen was developed in 1975 as a salvage regimen for patients with relapsed HL.[44] Subsequently, ABVD became a standard first-line therapy based on several randomized trials establishing its safety and efficacy (see Table 80.6). Although one of the main attractions of ABVD is its ability to maintain excellent OS without unnecessarily exposing the patient to the toxicity associated with alkylating agents, bleomycin-induced pulmonary toxicity remains an important problem for some individuals. The introduction of interim PET scanning in recent years has significantly reduced the use of bleomycin. The UK NCRI RATHL trial established that in those patients who had a negative PET scan (defined as a Deauville score of 1 to 3, Table 80.7) after 2 cycles of ABVD, 4 cycles of AVD were as effective and less toxic than 4 cycles of ABVD.[45] The 3-year PFS for interim PET negative patients in the ABVD arm was 85.7 (95% CI: 82.1% to 88.6%) compared with 84.4% (95% CI 80.7% to 87.5%) for those treated with AVD.[7]

A recent modification of the ABVD regimen has been to replace bleomycin with the anti-CD30 antibody drug conjugate Brentuximab vedotin (BV). Initially, BV was combined with standard ABVD in a phase-I trials, but this combination was associated with a high frequency of lung toxicity.[46] However, when bleomycin was omitted from the regimen, BV + AVD demonstrated no significant lung toxicity while maintaining its efficacy.[46–48] These encouraging results were the basis for a randomized phase 3 trials comparing standard 6 cycles of ABVD with 6 cycles of BV+AVD.[48] The 2 year modified PFS was 77.2% (95% CI: 73.7% to 80.4%) with ABVD and 82.4% (95% CI 78.8% to 85%) with AVD+BV.

TABLE 80.6 Standard Chemotherapy Regimens for the Treatment of Advanced Hodgkin Lymphoma

Regimen	Drugs	Route	Schedule
ABVD	Adriamycin 25 mg/m^2	IV	Day 1 and 15
	Bleomycin 10 mg/m^2	IV	Day 1 and 15
	Vinblastine 6 mg/m^2	IV	Day 1 and 15
	Dacarbazine 375 mg/m^2	IV	Day 1 and 15
			Every 28 days
BEACOPP (escalated)	Bleomycin 10 mg/m^2	IV	Day 8
	Etoposide 200 mg/m^2	IV	Day 1–3
	Adriamycin 35 mg/m^2	IV	Day 1
	Cyclophosphamide 1250 mg/m^2	IV	Day 1
	Vincristine 1.4 mg/m^2	IV	Day 8
	Procabazine 100 mg/m^2	PO	Days 1–7
	Prednisolone 40 mg/m^2	PO	Days 1–14
	G-CSF	SC	From day 8
			Every 21 days
A2VD (AVD with Brentuximab vedotin)	Adriamycin 25 mg/m^2	IV	Day 1 and 15
	Brentuximab vedotin 1.2 mg/kg	IV	Day 1 and 15
	Vinblastine 6 mg/m^2	IV	Day 1 and 15
	Dacarbazine 375 mg/m^2	IV	Day 1 and 15
	G-CSF	SC	
			Every 28 days

G-CSF, Granulocyte colony-stimulating factor.

BEACOPP

In 1992, the GHSG conducted a pilot study investigating whether treatment response and outcome could be improved by dose intensification in patients with newly diagnosed advanced HL. The BEACOPP regimen (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisolone) was developed with encouraging results in preliminary studies. In a subsequent randomized HD9 trial, standard-dose BEACOPP and escalated BEACOPP regimens were compared with COPP/ABVD.[49] The best results were achieved with escalated BEACOPP (OS 91% escalated BEACOPP vs. 88% standard BEACOPP vs. 83% COPP/ABVD, $P < .002$). The rate of early progression was also significantly lower in the escalated BEACOPP arm (2% escalated BEACOPP vs. 8% standard BEACOPP vs. 10% COPP/ABVD, $P < .001$). Because of the superior outcomes observed with both BEACOPP regimens, the COPP/ABVD arm in this study was closed at interim analysis. The improved outcome seen with escalated BEACOPP in the HD9 study was substantiated by long-term follow-up analysis at 10 years.

Despite the success demonstrated by escalated BEACOPP in the HD9 trial, longer-term complications including the development of myelodysplastic syndrome and secondary acute myeloid leukemia (AML) occurred more frequently when compared with standard BEACOPP and COPP/ABVD.[49,50] In addition, acute hematologic toxicity, including anemia and thrombocytopenia, was also greater in the escalated BEACOPP arm, with blood product support being required more frequently. The increased toxicity observed with escalated BEACOPP provided the rationale for the GHSG to investigate the efficacy and toxicity of lower doses of BEACOPP but given every 14 days, in a time-intensified manner (BEACOPP-14) and of reducing the number of cycles of escalated BEACOPP. This led to a multicenter, noninferiority randomized study (HD15 trial) in which 2182 patients with newly diagnosed advanced HL, aged between 18 and 60 years, were assigned to receive either 8 cycles of escalated BEACOPP, 6 cycles of escalated BEACOPP, or 8 cycles of BEACOPP-14, followed by PET-directed RT.[51] FFTF at 5 years was sequentially superior with 6 cycles of escalated BEACOPP (89.9%) compared to 8 cycles of BEACOPP-14 (85.4%) and with 8 cycles of escalated BEACOPP (84.4%). OS with 6× escBEACOPP was also superior that 8× escBEACOPP (95.3% for 6× escBEACOPP vs. 91.9% for 8× escBEACOPP).[51]

TABLE 80.7 Deauville 5-Point Scale for the Interpretation of Interim and End of Treatment FDG-PET Imaging in Patients With Hodgkin Lymphoma

FDG-PET Uptake	Deauville Score
No uptake above background	1
Uptake equal to or below mediastinum	2
Uptake higher than mediastinum but equal to or lower than liver	3
Uptake moderately higher than liver	4
Uptake significantly higher than liver and/or presence of new lesions	5
New areas of uptake/new FDG-PET avid lesions	X

FDG-PET, Fluorodeoxyglucose-positron emission tomography.

Furthermore, 8 cycles of escalated BEACOPP was associated with a higher mortality rate when compared to 6 cycles of escalated BEACOPP (7.5% vs. 4.6%, respectively), as well as an increased frequency of secondary malignancies (1.8% vs. 1.1%, respectively). It was concluded that overall, in patients younger than 60 years, 6 cycles of escalated BEACOPP followed by PET-directed RT was more effective and less toxic.

More recently, the HD18 trial compared PET directed therapy to further reduce the cycle number of escBEACOPP, with a standard 8 cycles of escBEACOPP (which was reduced to 6 cycles during the trial, when the HD15 results became known).[52] In this study, all patients with advanced HL received 2 cycles of escBEACOPP followed by a PET-CT scan. Patients with a negative scan (defined as a Deauville score of 1 and 2) were randomized to receive only 2 more cycles or 4 to 6 more (initially 6 but then reduced to 4 later in the trial) whereas those with a positive PET-CT were randomized to receive a full 6 to 8 cycles of escBEACOPP with or without rituximab. Rituximab use was not associated with significant differences in outcomes. In the interim PET negative group, 4 cycles of escBEACOPP in total was associated with a similar 5-year progression free survival (90.8% (95% CI: 87.9% to 93.7%) compared to 6 to 8 cycles (92.2% (89.4% to 95% for 4 cycles).[52]

ABVD Versus Escalated BEACOPP

ABVD and escalated BEACOPP are internationally accepted as appropriate first-line treatments for patients with newly diagnosed advanced HL, but which of these approaches is optimal remains under debate. Although ABVD is more widely used in the Unites States, escalated BEACOPP tends to be the more favored approach in many parts of Europe.

A number of studies have directly compared these two regimens with different approaches. A randomized study carried out by the EORTC in 2012 investigated whether patients with high-risk advanced disease (IPS ≥ 3) were likely to achieve greater benefit with a more intensive upfront regimen comprising 4 cycles of escalated BEACOPP followed by 4 cycles of standard BEACOPP or 8 cycles of ABVD.[53] There was no significant difference in event-free survival (EFS) or OS between the two treatment groups.[53] Relapse rates were higher with ABVD, but the frequency of early discontinuations was greater with escalated BEACOPP. In a parallel study investigating the same two regimens but in patients with advanced HL with lower-risk (IPS 0 to 2), the PFS was significantly better in the BEACOPP arm (93% vs. 75%, $P = .007$), but with no significant OS advantage (99% vs. 92%, $P = .06$). Again, fewer relapses were observed with BEACOPP.[54]

RELAPSED AND REFRACTORY HODGKIN LYMPHOMA

Although the vast majority of patients with newly diagnosed HL achieve a remission after first-line therapy, around 10% will have refractory disease (defined as progression or nonresponse during initial treatment or within 90 days of completing treatment). In addition, of those achieving initial cure, approximately 30% will relapse. Further remissions can be achieved for these patients with salvage therapy, for which the optimal choice depends on a combination of factors including the patient' s stage of disease at relapse, their prior therapies, and their risk factor profile.

Prognostic Factors in Relapsed or Refractory Hodgkin Lymphoma

A number of risk factors for refractory disease or relapse following initial therapy have been identified for patients with HL. In two large retrospective analyses conducted by the GHSG, poor performance score at the time of progression, age greater than 50 years, and failure to achieve a temporary remission on first-line treatment were highlighted as significant predictors of adverse outcome for patients with primary refractory HL: in addition, time to first relapse, clinical stage III or IV disease at relapse, and anemia (hemoglobin <12 g/L males or <10.5 females) were considered significant predictors of adverse outcome for patients with relapsed HL. Of these prognostic factors, performance status at relapse, advanced stage at relapse, and time to relapse appear to be consistently associated with poor risk across studies, but prospective confirmation of these is still needed.

Treatment of Relapsed or Refractory Hodgkin Lymphoma

Salvage Chemotherapy and Autologous Stem Cell Transplant

The treatment of choice for the majority of patients with relapsed HL, or with refractory disease that has not responded to initial chemotherapy, is cytoreduction with salvage chemotherapy followed by high-dose therapy and autologous stem cell transplant (ASCT).

The goal of cytoreduction with salvage chemotherapy and preparative high dose chemotherapy before ASCT is to achieve high response rates and a minimal disease state, while maintaining an acceptable toxicity profile, without compromising subsequent stem cell mobilization and engraftment. A number of second-line salvage chemotherapy combinations are currently in use, but because of a lack of randomized data comparing these regimens, there is no consensus as to which is optimal. Common regimens include DHAP (dexamethasone, cisplatin, cytarabine), ICE (ifosfamide, carboplatin, etoposide), GVD (gemcitabine, vinorelbine, liposomal doxorubicin), and ESHAP (etoposide, methylprednisolone, cytarabine, cisplatin), with patients typically receiving 2 to 3 cycles of therapy before proceeding to ASCT.[55–58]

The advantage of ASCT over standard chemotherapy alone has been demonstrated in a number of studies. In 1997, Yuen and colleagues compared the outcome of patients receiving high-dose etoposide, cyclophosphamide, and total body irradiation (TBI) or carmustine followed by ASCT, with a matched conventional salvage group who received chemotherapy alone.[59] At 4 years of follow-up a clear improvement was seen in those who underwent transplant with regards to OS, EFS, and FFP over chemotherapy alone (OS 54% vs. 47%, $P = .25$; EFS 53% vs. 27%, $P < .01$, FFP 62% vs. 32%, $P < .01$).

Two key randomized control trials have supported the superior role of ASCT over conventional salvage therapy and have been integral to establishing this approach as the standard of care for patients with relapsed or refractory HL: In a comparison of high-dose BEAM chemotherapy plus autologous transplant versus standard-dose mini-BEAM chemotherapy alone, the British National Lymphoma Investigation (BNLI) group demonstrated significant differences in EFS and PFS in favor of BEAM followed by autologous transplant (53% vs. 10%).[60] In a larger trial conducted by the GHSG/EBMT, 161 patients with relapsed or refractory HL were chosen at random to receive either 4 cycles of dexa-BEAM or 2 cycles of dexa-BEAM, followed by 2 further cycles of high-dose BEAM and ASCT. In chemosensitive patients, FFTF at 3 years was significantly better for those proceeding to HDCT and ASCT (55%) compared to standard chemotherapy alone (34%, $P = .019$), although there was no significant difference in OS between the two groups.[61]

Treatment Post Autologous Stem Cell Transplant

Three drugs have been approved by the US Food and Drug Administration (FDA) for the treatment of patients with relapsed HL after receiving ASCT; brentuximab vedotin, and two check point inhibitors, pembrolizumab and nivolumab.

Brentuximab Vedotin

The cell surface antigen CD30 is highly expressed on Hodgkin and Reed-Sternberg (HRS) cells.[62] The efficacy of agents targeting CD30 has been extensively investigated for patients with relapsed and refractory HL. Brentuximab vedotin (SGN-35) is an antibody-drug conjugate (ADC) comprising an anti-CD30 monoclonal antibody conjugated to antitubulin monomethyl auristatin E (MMAE).[63]

An initial phase I dose-escalation study in patients with relapsed or refractory CD30-positive hematologic cancers showed that BV is associated with a favorable safety profile and established 1.8 mg/kg every 3 weeks as the MTD. Significant efficacy was also observed in this study: tumor regression was demonstrated in 86% of patients, with a median durable response of 9.7 months.[64] In a pivotal phase II trial of 102 patients with CD30-positive relapsed or refractory HL who had failed ASCT the safety and efficacy of BV was evaluated further.[65] Overall response rate (ORR) was 75%, with 34% of patients achieving a complete response. The median duration of response was 20.5 months. Peripheral neuropathy, nausea, fatigue, neutropenia, and diarrhea were the most common treatment-related toxicities. As a result of this study, in 2011 BV was the first drug to be approved by the US FDA for the treatment of patients with relapsed or refractory HL following ASCT. BV was also evaluated as maintenance therapy following ASCT and was shown to significantly improve PFS compared to placebo in patients with high-risk relapsed or refractory HL.[66]

Checkpoint Inhibitors

PD-1 (programmed cell death protein 1) is a cell surface receptor that is expressed on T regulatory and activated T cells (CD4 and CD8), activated B cells and natural killer (NK) cells. PD-1 binds PDL-1 and PDL-2, two ligands that are expressed by antigen-presenting cells and several cancer cells, including RS cells in HL (see Fig. 80.5).[16,67] The PD-1/PDL-1/2 interaction inhibits kinases that are normally involved in T-cell activation. In this way the PD-1 pathway serves as a checkpoint to restrict T-cell–mediated immune responses, enabling tumor cells to evade immune detection. Inhibitors of PD-1, which block PD-1/PDL-1/2 engagement, may enhance T-cell immunity and prevent immune evasion by tumor cells, leading to effective cancer cell death.[68]

Remarkable single-agent activity in patients with relapsed HL has been reported with either monoclonal antibody directed against PD-1 nivolumab or pembrolizumab.[69,70] ORR is around 65% for either agent, with CR rate relatively low. Both agents are approved by the FDA for the treatment of relapsed and refractory HL.[69–71] Accordingly, anti-PD1 antibodies are investigated in combination with multiple regimens with promising activity. In particular, the combination of nivolumab and brentuximab vedotin showed a high ORR of 82% and CR rate of 61% in a second-line pre-transplant setting.[72]

SPECIAL CONSIDERATIONS

Hodgkin Lymphoma in the Elderly

The number of patients living with cancer over the age of 60 years is increasing. The outcome of elderly patients with HL may differ significantly because of the heterogeneity of this population with regards to life expectancy, premorbid performance status, and comorbidity. Establishing optimal treatment is therefore challenging, particularly because older patients do not tend to be well-represented in large clinical trials caused by tight exclusion criteria that often render them ineligible for enrollment. Data, therefore, are not necessarily representative of the general elderly population.[73]

In a comprehensive retrospective analysis of 4251 patients with HL, acute treatment-related toxicity was higher in older patients compared with those under the age of 60 years. In addition, elderly patients with HL had a worse outcome with respect to freedom from treatment failure (60% vs. 80%) and OS (65% vs. 90%), which was attributed to both increased mortality during treatment as well as the fact that significantly fewer elderly patients were able to tolerate the intended full dose of therapy (75% vs. 91%).[74]

It is recommended that elderly patients without significant comorbidity and with a good performance status should be treated according to the current standard of care for younger individuals, on a risk-adapted basis, according to stage, with curative intent. Treatment-related toxicity and mortality may, however, occur more frequently.[75,76]

Hodgkin Lymphoma During Pregnancy

Given that the early incidence peak of HL coincides with childbearing age its management during pregnancy should assume special consideration. Lymphoma is the fourth commonest malignancy presenting in pregnancy and, of these, HL occurs most frequently. The diagnosis of HL during pregnancy matches that of the background population; lymph node biopsy can be performed safely either under local or general anesthetic. Clearly, however, traditional staging methods for patients with newly diagnosed HL who are pregnant need to be adapted.[77] To avoid the risks associated with radiation exposure to the fetus, radiologic evaluation via chest X-ray with abdominal shielding, abdominal ultrasound, or magnetic resonance imaging, is recommended rather than CT or PET. Routine blood work and bone marrow biopsy are also indicated, as per standard practice. This modified approach to staging during pregnancy is considered sufficient to allow appropriate ongoing management decisions, at least until more comprehensive imaging can be completed following delivery.

Consensus regarding the optimal treatment for pregnant patients with HL varies. The risk of spontaneous abortion and congenital malformations secondary to cytotoxic treatments and/or RT is known to be highest in the first trimester. Although risk decreases as pregnancy continues, the exact extent of risk during this period has not been clearly established and long-term complications are unknown. This is largely caused by the lack of data that exists as a result of small patient numbers. Thus, whether chemotherapy should be started or not during pregnancy requires cautious evaluation of the risks to the unborn fetus versus the risks to maternal survival as a result of postponing treatment. Low-risk, asymptomatic patients may be monitored closely, with treatment being delayed until either the end of the first trimester or until delivery if diagnosis occurs later on during the pregnancy. For patients with bulky or symptomatic disease that requires treatment, however, single agent anthracyclines or vinca-alkaloids are deemed acceptable first choices, escalating to combined chemotherapy, usually with ABVD, following delivery or during the second or third trimester if there is evidence of progressive disease. In this latter subgroup of patients, however, the option of therapeutic abortion should be discussed with the patient and family to ensure all decisions made are informed.

LONG-TERM COMPLICATIONS OF TREATMENT IN HODGKIN LYMPHOMA

The high cure rate associated with HL translates to a significant proportion of patients who are at an increased risk for a range of long-term sequelae of treatment. Data on the cumulative incidence of cause-specific mortality in HL survivors show that although the rate of lymphoma-related deaths plateaus after the first 10 to 15 years following therapy, the rate of death because of late complications continues to rise.[78,79] The significance of long-term sequelae following treatment for HL has become more apparent over recent years given the growing number of young survivors.[80] However, it is important to remember that a significant proportion of the data surrounding late effects relate to patients who were treated with chemotherapy and RT approaches that no longer feature in current practice. Increased awareness of long-term complications can account for many of the important treatment modifications which have been made over the past two decades, and provides the current rationale for ongoing development with regards to novel agents as well as RT technique. Current and future follow-up approaches for these individuals requires vigilance, focusing on strategies that enable both early detection and primary prevention wherever possible for the impact of these sequelae to be reduced.[81]

The management of late toxicities associated with chemotherapy and/or RT is often more challenging than acute, with effects in some cases being irreversible and life threatening. Long-term treatment-related complications in patients previously treated for HL include hypothyroidism, peripheral neuropathy, gonadal dysfunction, secondary malignancy, and cardiovascular disease. Of these, secondary malignancy and cardiovascular complications occur most frequently, with risk continuing even beyond 25 years from diagnosis, and represent the two most common entities determining quality of life: long-term morbidity and mortality in this patient population.

It is well described that intensive RT with extended field size and certain chemotherapy agents, such as cyclophosphamide, nitrogen mustard, procarbazine, and etoposide may increase the risk of developing secondary malignancies. Solid tumors appear to account for the majority of cases of secondary malignancy, with breast cancer, lung cancer, and gastrointestinal cancer occurring most frequently.[78,79] This risk has been shown to be significantly greater among patients treated for HL during childhood or during teenage years. Among the female population, secondary breast cancer predominates, with extended radiation field size and the administration of RT to those under the age of 30 years representing the two most significant risk factors. The time to develop breast cancer is usually about 10 to 15 years. Therefore, for all female patients receiving radiation to the mediastinum or axillae, annual breast screening with mammography is recommended 8 to 10 years after treatment or from the age of 40 years, whichever occurs first.

The risk of secondary hematologic malignancy, including leukemia and myelodysplasia, has been particularly associated with prior exposure to alkylating chemotherapy, especially when delivered in combination with large-field RT. Although this risk has fallen with the use of more modern chemotherapy, the exact choice of regimen still appears to be significant, with the incidence of AML or myelodysplasia being significantly greater among those treated with more intensive chemotherapy, such as BEACOPP, than with ABVD. The time to develop treatment-related leukemia after HL is about 3 to 5 years, and the prognosis of these patients is poor, with an estimated OS of 7.2 months.

The risk of cardiovascular disease may be three- to fivefold increased in survivors of HL compared with the general population. Cardiac complications tend to present themselves approximately 10 to 15 years after exposure, although the actual disease onset may in fact be well before this, given the lack of early symptoms in many cases.[79,82,83] The association between anthracycline-based chemotherapy and cardiac toxicity is well established and is particularly relevant to the setting of HL given the chemotherapy combinations currently used in standard practice. Anthracycline-induced cardiac toxicity typically manifests as arrhythmias or congestive cardiac failure as a result of ventricular dysfunction secondary to direct myocardial damage. The severity of cardiac toxicity is directly correlated to the cumulative dose that is delivered and, as such, both individual dosages and the number of cycles are significant considerations when planning treatment. Radiation exposure has also been implicated in the development of late cardiac diseases including coronary artery disease, valvular dysfunction, myocardial dysfunction, pericarditis, and conduction defects, and radiation-related cardiac toxicity remains a major factor compromising overall outcome in HL survivors. Optimal screening methods for cardiovascular disease are not clearly established and therefore close monitoring of these patients is warranted, including strict control of cardiac risk factors (including hypertension, diabetes, hypercholesterolemia, and smoking).

A number of agents used in the treatment of HL can result in gonadal dysfunction, which is of particular relevance given the young age of this patient population.[84] Pelvic RT and intensive alkylating-based frontline chemotherapy regimens such as MOPP and escalated BEACOPP have been shown to induce male and female sterility whereas the risk is reduced with baseline BEACOPP and reduced even further with less aggressive combinations such as ABVD where it is usually only transient. Unsurprisingly, however, the risk rises significantly among patients with relapsed or refractory disease following high-dose salvage chemotherapy. It is therefore recommended that all patients with newly diagnosed HL, who are of reproductive age, should be offered fertility preservation before commencing therapy.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Ansell SM, Lesokhin AM, Borrello I, et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin' s lymphoma. *N Engl J Med*. 2015;372(4):311–319.

Armand P, Shipp MA, Ribrag V, et al. Programmed death-1 blockade with pembrolizumab in patients with classical Hodgkin lymphoma after brentuximab vedotin failure. *J Clin Oncol*. 2016;34(31): 3733–3739.

Bachanova V, Connors JM. Hodgkin lymphoma in pregnancy. *Curr Hematol Malig Rep*. 2013;8(3):211–217.

Carde P, Karrasch M, Fortpied C, et al. Eight cycles of ABVD versus four cycles of BEACOPPescalated plus four cycles of BEACOPPbaseline in Stage III to IV, International Prognostic Score ≥ 3, high-risk Hodgkin lymphoma: first results of the phase III EORTC 20012 Intergroup Trial. *J Clin Oncol*. 2016;34(17):2028–2036.

Castellino SM, Geiger AM, Mertens AC, et al. Morbidity and mortality in long-term survivors of Hodgkin lymphoma: a report from the Childhood Cancer Survivor Study. *Blood*. 2011;117(6):1806–1816.

Connors JM, Ansell SM, Fanale M, et al. Five-year follow-up of brentuximab vedotin combined with ABVD or AVD for advanced-stage classical Hodgkin lymphoma. *Blood*. 2017;130(11):1375–1377.

Connors JM, Jurczak W, Straus DJ, et al. Brentuximab vedotin with chemotherapy for stage III or IV Hodgkin' s lymphoma. *N Engl J Med*. 2018;378(4):331–344.

Fuchs M, Goergen H, Kobe C, et al. Positron emission tomography-guided treatment in early-stage favorable hodgkin lymphoma: final results of the international, randomized Phase III HD16 trial by the German Hodgkin study group. *J Clin Oncol*. 2019;37(31):2835–2845.

Hahn EE, Wu YL, Munoz-Plaza CE, et al. Use of recommended posttreatment services for adolescent and young adult survivors of Hodgkin lymphoma. *Cancer*. 2019;125(9):1558–1567.

Herrera AF, Moskowitz AJ, Bartlett NL, et al. Interim results of brentuximab vedotin in combination with nivolumab in patients with relapsed or refractory Hodgkin lymphoma. *Blood*. 2018;131(11):1183–1194.

Johnson P, Federico M, Kirkwood A, et al. Adapted treatment guided by interim PET-CT scan in advanced Hodgkin' s lymphoma. *N Engl J Med*. 2016;374(25):2419–2429.

Longley J, Johnson PWM. Current treatment paradigms for advanced stage Hodgkin lymphoma. *Br J Haematol*. 2019;184(1):60–71.

Mathas S, Hartmann S, Kuppers R. Hodgkin lymphoma: pathology and biology. *Semin Hematol*. 2016;53(3):139–147.

Meyer RM, Gospodarowicz MK, Connors JM, et al. ABVD alone versus radiation-based therapy in limited-stage Hodgkin' s lymphoma. *N Engl J Med*. 2012;366(5):399–408.

Moskowitz CH, Nademanee A, Masszi T, et al. Brentuximab vedotin as consolidation therapy after autologous stem-cell transplantation in patients with Hodgkin' s lymphoma at risk of relapse or progression (AETHERA): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet*. 2015;385(9980):1853–1862.

Radford J, Illidge T, Counsell N, et al. Results of a trial of PET-directed therapy for early-stage Hodgkin' s lymphoma. *N Engl J Med*. 2015;372(17): 1598–1607.

Reichel J, Chadburn A, Rubinstein PG, et al. Flow sorting and exome sequencing reveal the oncogenome of primary Hodgkin and Reed-Sternberg cells. *Blood*. 2015;125(7):1061–1072.

Roemer MG, Advani RH, Ligon AH, et al. PD-L1 and PD-L2 genetic alterations define classical Hodgkin lymphoma and predict outcome. *J Clin Oncol*. 2016;34(23):2690–2697.

Sasse S, Brockelmann PJ, Goergen H, et al. Long-term follow-up of contemporary treatment in early-stage Hodgkin lymphoma: updated analyses of the German Hodgkin Study Group HD7, HD8, HD10, and HD11 Trials. *J Clin Oncol*. 2017;35(18):1999–2007.

Spina V, Bruscaggin A, Cuccaro A, et al. Circulating tumor DNA reveals genetics, clonal evolution, and residual disease in classical Hodgkin lymphoma. *Blood*. 2018;131(22):2413–2425.

Straus DJ. Initial Hodgkin treatment of the frail elderly. *Blood*. 2017;130 (26):2813–2814.

Straus DJ, Jung SH, Pitcher B, et al. CALGB 50604: risk-adapted treatment of nonbulky early-stage Hodgkin lymphoma based on interim PET. *Blood*. 2018;132(10):1013–1021.

Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127(20):2375–2390.

van Leeuwen FE, Ng AK. Long-term risk of second malignancy and cardiovascular disease after Hodgkin lymphoma treatment. *Hematology Am Soc Hematol Educ Program*. 2016;2016(1):323–330.

Younes A, Connors JM, Park SI, et al. Brentuximab vedotin combined with ABVD or AVD for patients with newly diagnosed Hodgkin' s lymphoma: a phase 1, open-label, dose-escalation study. *Lancet Oncol*. 2013;14(13):1348–1356.

Younes A, Gopal AK, Smith SE, et al. Results of a pivotal phase II study of brentuximab vedotin for patients with relapsed or refractory Hodgkin' s lymphoma. *J Clin Oncol*. 2012;30(18):2183–2189.

Younes A, Santoro A, Shipp M, et al. Nivolumab for classical Hodgkin' s lymphoma after failure of both autologous stem-cell transplantation and brentuximab vedotin: a multicentre, multicohort, single-arm phase 2 trial. *Lancet Oncol*. 2016;17(9):1283–1294.

REFERENCES

1. Longley J, Johnson PWM. Current treatment paradigms for advanced stage Hodgkin lymphoma. *Br J Haematol*. 2019;184(1):60–71. https://doi.org/10.1111/bjh.15622. [published Online First: 2018/11/09].
2. Siegel RL, Miller KD, Fuchs HE, et al. Cancer Statistics, 2021. *CA Cancer J Clin*. 2021;71(1):7–33. https://doi.org/10.3322/caac.21654. [published Online First: 2021/01/13].
3. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021 https://doi.org/10.3322/caac.21660. [published Online First: 2021/02/05].
4. Araujo I, Bittencourt AL, Barbosa HS, et al. The high frequency of EBV infection in pediatric Hodgkin lymphoma is related to the classical type in Bahia, Brazil. *Virchows Arch*. 2006;449(3):315–319. https://doi.org/10.1007/s00428-006-0244-z. [published Online First: 2006/08/10].
5. Hjalgrim H, Smedby KE, Rostgaard K, et al. Infectious mononucleosis, childhood social environment, and risk of Hodgkin lymphoma. *Cancer Res*. 2007;67(5):2382–2388. https://doi.org/10.1158/0008-5472.CAN-06-3566. [published Online First: 2007/03/03].
6. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127(20):2375–2390. https://doi.org/10.1182/blood-2016-01-643569. [published Online First: 2016/03/17].
7. Brune V, Tiacci E, Pfeil I, et al. Origin and pathogenesis of nodular lymphocyte-predominant Hodgkin lymphoma as revealed by global gene expression analysis. *J Exp Med*. 2008;205(10):2251–2268. https://doi.org/10.1084/jem.20080809. [published Online First: 2008/09/17].
8. Mathas S, Hartmann S, Kuppers R. Hodgkin lymphoma: pathology and biology. *Semin Hematol*. 2016;53(3):139–147. https://doi.org/10.1053/j.seminhematol.2016.05.007. [published Online First: 2016/08/09].
9. Hartmann S, Eichenauer DA. Nodular lymphocyte predominant Hodgkin lymphoma: pathology, clinical course and relation to T-cell/histiocyte rich large B-cell lymphoma. *Pathology*. 2020;52(1):142–153. https://doi.org/10.1016/j.pathol.2019.10.003. [published Online First: 2019/12/02].
10. Gloghini A, Bosco A, Ponzoni M, et al. Immunoarchitectural patterns in nodular lymphocyte predominant Hodgkin lymphoma: pathologic and clinical implications. *Expert Rev Hematol*. 2015;8(2):217–223. https://doi.org/10.1586/17474086.2015.991388. [published Online First: 2014/12/10].
11. Prakash S, Fountaine T, Raffeld M, et al. IgD positive L&H cells identify a unique subset of nodular lymphocyte predominant Hodgkin lymphoma. *Am J Surg Pathol*. 2006;30(5):585–592. https://doi.org/10.1097/01.pas.0000194741.87798.45. [published Online First: 2006/05/16].
12. Hartmann S, Eichenauer DA, Plutschow A, et al. The prognostic impact of variant histology in nodular lymphocyte-predominant Hodgkin lymphoma: a report from the German Hodgkin Study Group (GHSG). *Blood*. 2013;122(26):4246–4252. https://doi.org/10.1182/blood-2013-07-515825. quiz 92. [published Online First: 2013/10/09].
13. Hartmann S, Schuhmacher B, Rausch T, et al. Highly recurrent mutations of SGK1, DUSP2 and JUNB in nodular lymphocyte predominant Hodgkin lymphoma. *Leukemia*. 2016;30(4):844–853. https://doi.org/10.1038/leu.2015.328. [published Online First: 2015/12/15].
14. Reichel J, Chadburn A, Rubinstein PG, et al. Flow sorting and exome sequencing reveal the oncogenome of primary Hodgkin and Reed-Sternberg cells. *Blood*. 2015;125(7):1061–1072. https://doi.org/10.1182/blood-2014-11-610436. [published Online First: 2014/12/10].
15. Steidl C, Shah SP, Woolcock BW, et al. MHC class II transactivator CIITA is a recurrent gene fusion partner in lymphoid cancers. *Nature*. 2011;471(7338):377–381. https://doi.org/10.1038/nature09754. [published Online First: 2011/03/04].
16. Roemer MG, Advani RH, Ligon AH, et al. PD-L1 and PD-L2 Genetic alterations define classical Hodgkin lymphoma and predict outcome. *J Clin Oncol*. 2016;34(23):2690–2697. https://doi.org/10.1200/JCO.2016.66.4482. [published Online First: 2016/04/14].
17. Emmerich F, Theurich S, Hummel M, et al. Inactivating I kappa B epsilon mutations in Hodgkin/Reed-Sternberg cells. *J Pathol*. 2003;201(3):413–420. https://doi.org/10.1002/path.1454. [published Online First: 2003/11/05].
18. Schmitz R, Hansmann ML, Bohle V, et al. TNFAIP3 (A20) is a tumor suppressor gene in Hodgkin lymphoma and primary mediastinal B cell lymphoma. *J Exp Med*. 2009;206(5):981–989. https://doi.org/10.1084/jem.20090528. [published Online First: 2009/04/22].
19. Spina V, Bruscaggin A, Cuccaro A, et al. Circulating tumor DNA reveals genetics, clonal evolution, and residual disease in classical Hodgkin lymphoma. *Blood*. 2018;131(22):2413–2425. https://doi.org/10.1182/blood-2017-11-812073. [published Online First: 2018/02/17].
20. Cheson BD, Fisher RI, Barrington SF, et al. Recommendations for initial evaluation, staging, and response assessment of Hodgkin and non-Hodgkin lymphoma: the Lugano classification. *J Clin Oncol*. 2014;32(27):3059–3068. https://doi.org/10.1200/JCO.2013.54.8800. [published Online First: 2014/08/13].
21. Younes A, Hilden P, Coiffier B, et al. International Working Group consensus response evaluation criteria in lymphoma (RECIL 2017). *Ann Oncol*. 2017;28(7):1436–1447. https://doi.org/10.1093/annonc/mdx097. [published Online First: 2017/04/06].
22. Cheson BD. Staging and response assessment in lymphomas: the new Lugano classification. *Chin Clin Oncol*. 2015;4(1):5 https://doi.org/10.3978/j.issn.2304-3865.2014.11.03. [published Online First: 2015/04/07].
23. Hasenclever D, Diehl V. A prognostic score for advanced Hodgkin' s disease. International prognostic factors project on advanced Hodgkin' s disease. *N Engl J Med*. 1998;339(21):1506–1514. https://doi.org/10.1056/NEJM199811193392104. [published Online First: 1998/11/20].
24. Younes A. Early-stage hodgkin' s lymphoma: in pursuit of perfection. *J Clin Oncol*. 2012;30(9):895–896. https://doi.org/10.1200/JCO.2011.40.1661. [published Online First: 2012/02/23].
25. Johnson PW. Management of early-stage Hodgkin lymphoma: is there still a role for radiation? *Hematology Am Soc Hematol Educ Program*. 2013;2013:400–405. https://doi.org/10.1182/asheducation-2013.1.400. [published Online First: 2013/12/10].
26. Radford J, Illidge T, Counsell N, et al. Results of a trial of PET-directed therapy for early-stage Hodgkin' s lymphoma. *N Engl J Med*. 2015;372(17):1598–1607. https://doi.org/10.1056/NEJMoa1408648. [published Online First: 2015/04/23].
27. Straus DJ, Jung SH, Pitcher B, et al. CALGB 50604: risk-adapted treatment of nonbulky early-stage Hodgkin lymphoma based on interim PET. *Blood*. 2018;132(10):1013–1021. https://doi.org/10.1182/blood-2018-01-827246. [published Online First: 2018/07/28].
28. Advani RH, Hoppe RT. How I treat nodular lymphocyte predominant Hodgkin lymphoma. *Blood*. 2013;122(26):4182–4188. https://doi.org/10.1182/blood-2013-07-453241. [published Online First: 2013/11/12].
29. Connors JM. To treat or not to treat, that is the NLPHL question. *Blood*. 2019;133(20):2113–2114. https://doi.org/10.1182/blood-2019-02-900811. [published Online First: 2019/05/18].
30. Ballas LK, Metzger ML, Milgrom SA, et al. Nodular lymphocyte predominant Hodgkin lymphoma: executive summary of the American radium society appropriate use criteria. *Leuk Lymphoma*. 2020:1–14. https://doi.org/10.1080/10428194.2020.1852559. [published Online First: 2020/12/05].
31. Chen RC, Chin MS, Ng AK, et al. Early-stage, lymphocyte-predominant Hodgkin' s lymphoma: patient outcomes from a large, single-institution series with long follow-up. *J Clin Oncol*. 2010;28(1):136–141. https://doi.org/10.1200/JCO.2009.24.0945. [published Online First: 2009/11/26].
32. Parsons SK, Kelly MJ, Cohen JT, et al. Early-stage Hodgkin lymphoma in the modern era: simulation modelling to delineate long-term patient outcomes. *Br J Haematol*. 2018;182(2):212–221. https://doi.org/10.1111/bjh.15255. [published Online First: 2018/05/01].
33. Engert A, Raemaekers J. Treatment of early-stage Hodgkin lymphoma. *Semin Hematol*. 2016;53(3):165–170. https://doi.org/10.1053/j.seminhematol.2016.05.004. [published Online First: 2016/08/09].
34. Sasse S, Brockelmann PJ, Goergen H, et al. Long-term follow-up of contemporary treatment in early-stage hodgkin lymphoma: updated analyses of the German Hodgkin Study Group HD7, HD8, HD10, and HD11 Trials. *J Clin Oncol*. 2017;35(18):1999–2007. https://doi.org/10.1200/JCO.2016.70.9410. [published Online First: 2017/04/19].
35. Boll B, Goergen H, Behringer K, et al. Bleomycin in older early-stage favorable Hodgkin lymphoma patients: analysis of the German Hodgkin Study Group (GHSG) HD10 and HD13 trials. *Blood*. 2016;127(18):2189–2192. https://doi.org/10.1182/blood-2015-11-681064. [published Online First: 2016/02/03].
36. Eich HT, Diehl V, Gorgen H, et al. Intensified chemotherapy and dose-reduced involved-field radiotherapy in patients with early unfavorable Hodgkin' s lymphoma: final analysis of the German Hodgkin Study Group HD11 trial. *J Clin Oncol*. 2010;28(27):4199–4206. https://doi.org/10.1200/JCO.2010.29.8018. [published Online First: 2010/08/18].
37. Meyer RM, Gospodarowicz MK, Connors JM, et al. ABVD alone versus radiation-based therapy in limited-stage Hodgkin' s lymphoma. *N Engl J Med*. 2012;366(5):399–408. https://doi.org/10.1056/NEJMoa1111961. [published Online First: 2011/12/14].
38. Yahalom J, Illidge T, Specht L, et al. Modern radiation therapy for extranodal lymphomas: field and dose guidelines from the International Lymphoma Radiation Oncology Group. *Int J Radiat Oncol Biol Phys*. 2015;92(1):11–31. https://doi.org/10.1016/j.ijrobp.2015.01.009. [published Online First: 2015/04/13].

39. Dabaja BS, Ng AK, Terezakis SA, et al. ILROG lymphoma mini-atlas part II, Hodgkin Lymphoma. *Int J Radiat Oncol Biol Phys*. 2020;108(4):977–978. https://doi.org/10.1016/j.ijrobp.2020.05.063. [published Online First: 2020/08/10].
40. Dabaja BS, Hoppe BS, Plastaras JP, et al. Proton therapy for adults with mediastinal lymphomas: the International lymphoma radiation oncology group guidelines. *Blood*. 2018;132(16):1635–1646. https://doi.org/10.1182/blood-2018-03-837633. [published Online First: 2018/08/16].
41. Barrington SF, Kirkwood AA, Franceschetto A, et al. PET-CT for staging and early response: results from the response-adapted therapy in advanced Hodgkin lymphoma study. *Blood*. 2016;127(12):1531–1538. https://doi.org/10.1182/blood-2015-11-679407. [published Online First: 2016/01/10].
42. Andre MPE, Girinsky T, Federico M, et al. Early positron emission tomography response-adapted treatment in stage I and II Hodgkin lymphoma: final results of the randomized EORTC/LYSA/FIL H10 trial. *J Clin Oncol*. 2017;35(16):1786–1794. https://doi.org/10.1200/JCO.2016.68.6394. [published Online First: 2017/03/16].
43. Fuchs M, Goergen H, Kobe C, et al. Positron emission tomography-guided treatment in early-stage favorable hodgkin lymphoma: final results of the international, randomized phase III HD16 trial by the German Hodgkin study group. *J Clin Oncol*. 2019;37(31):2835–2845. https://doi.org/10.1200/JCO.19.00964. [published Online First: 2019/09/10].
44. Bonadonna G, Zucali R, Monfardini S, et al. Combination chemotherapy of Hodgkin' s disease with adriamycin, bleomycin, vinblastine, and imidazole carboxamide versus MOPP. *Cancer*. 1975;36(1):252–259. doi: 10.1002/1097-0142(197507)36:1<252::aid-cncr2820360128>3.0.co;2-7 [published Online First: 1975/07/01].
45. Johnson P, Federico M, Kirkwood A, et al. Adapted treatment guided by interim PET-CT scan in advanced Hodgkin' s lymphoma. *N Engl J Med*. 2016;374(25):2419–2429. https://doi.org/10.1056/NEJMoa1510093. [published Online First: 2016/06/23].
46. Younes A, Connors JM, Park SI, et al. Brentuximab vedotin combined with ABVD or AVD for patients with newly diagnosed Hodgkin' s lymphoma: a phase 1, open-label, dose-escalation study. *Lancet Oncol*. 2013;14(13):1348–1356. https://doi.org/10.1016/S1470-2045(13)70501-1. [published Online First: 2013/11/19].
47. Connors JM, Ansell SM, Fanale M, et al. Five-year follow-up of brentuximab vedotin combined with ABVD or AVD for advanced-stage classical Hodgkin lymphoma. *Blood*. 2017;130(11):1375–1377. https://doi.org/10.1182/blood-2017-05-784678. [published Online First: 2017/07/25].
48. Connors JM, Jurczak W, Straus DJ, et al. Brentuximab vedotin with chemotherapy for stage III or IV Hodgkin' s lymphoma. *N Engl J Med*. 2018;378(4):331–344. https://doi.org/10.1056/NEJMoa1708984. [published Online First: 2017/12/12].
49. Engert A, Diehl V, Franklin J, et al. Escalated-dose BEACOPP in the treatment of patients with advanced-stage Hodgkin' s lymphoma: 10 years of follow-up of the GHSG HD9 study. *J Clin Oncol*. 2009;27(27):4548–4554. https://doi.org/10.1200/JCO.2008.19.8820. [published Online First: 2009/08/26].
50. Josting A, Wiedenmann S, Franklin J, et al. Secondary myeloid leukemia and myelodysplastic syndromes in patients treated for Hodgkin' s disease: a report from the German Hodgkin' s Lymphoma Study Group. *J Clin Oncol*. 2003;21(18):3440–3446. https://doi.org/10.1200/JCO.2003.07.160. [published Online First: 2003/04/02].
51. Engert A, Haverkamp H, Kobe C, et al. Reduced-intensity chemotherapy and PET-guided radiotherapy in patients with advanced stage Hodgkin' s lymphoma (HD15 trial): a randomised, open-label, phase 3 non-inferiority trial. *Lancet*. 2012;379(9828):1791–1799. https://doi.org/10.1016/S0140-6736(11)61940-5. [published Online First: 2012/04/07].
52. Borchmann P, Goergen H, Kobe C, et al. PET-guided treatment in patients with advanced-stage Hodgkin' s lymphoma (HD18): final results of an open-label, international, randomised phase 3 trial by the German Hodgkin Study Group. *Lancet*. 2017;390(10114):2790–2802. https://doi.org/10.1016/S0140-6736(17)32134-7. [published Online First: 2017/10/25].
53. Carde P, Karrasch M, Fortpied C, et al. Eight cycles of ABVD versus four cycles of BEACOPPescalated plus four cycles of BEACOPPbaseline in stage III to IV, International prognostic score ≥ 3, high-risk Hodgkin lymphoma: first results of the phase III EORTC 20012 intergroup trial. *J Clin Oncol*. 2016;34(17):2028–2036. https://doi.org/10.1200/JCO.2015.64.5648. [published Online First: 2016/04/27].
54. Mounier N, Brice P, Bologna S, et al. ABVD (8 cycles) versus BEACOPP (4 escalated cycles ≥4 baseline): final results in stage III-IV low-risk Hodgkin lymphoma (IPS 0-2) of the LYSA H34 randomized trial. *Ann Oncol*. 2014;25(8):1622–1628. https://doi.org/10.1093/annonc/mdu189. [published Online First: 2014/05/16].
55. Bartlett NL, Niedzwiecki D, Johnson JL, et al. Gemcitabine, vinorelbine, and pegylated liposomal doxorubicin (GVD), a salvage regimen in relapsed Hodgkin' s lymphoma: CALGB 59804. *Ann Oncol*. 2007;18(6):1071–1079. https://doi.org/10.1093/annonc/mdm090. [published Online First: 2007/04/12].
56. Choi CW, Paek CW, Seo JH, et al. ESHAP salvage therapy for relapsed or refractory non-Hodgkin' s lymphoma. *J Korean Med Sci*. 2002;17(5):621–624. https://doi.org/10.3346/jkms.2002.17.5.621. [published Online First: 2002/10/16].
57. Gisselbrecht C. Hodgkin' s lymphoma: what is next in salvage treatment? *Ann Oncol*. 2003;14(Suppl 1):i37–i38. https://doi.org/10.1093/annonc/mdg707. [published Online First: 2003/05/09].
58. Olivieri A, Brunori M, Capelli D, et al. Salvage therapy with an outpatient DHAP schedule followed by PBSC transplantation in 79 lymphoma patients: an intention to mobilize and transplant analysis. *Eur J Haematol*. 2004;72(1):10–17. https://doi.org/10.1046/j.0902-4441.2004.00171.x. [published Online First: 2004/02/14].
59. Yuen AR, Rosenberg SA, Hoppe RT, et al. Comparison between conventional salvage therapy and high-dose therapy with autografting for recurrent or refractory Hodgkin' s disease. *Blood*. 1997;89(3):814–822. [published Online First: 1997/02/01].
60. Linch DC, Vaughan Hudson B, Anderson L, et al. Impact of high-dose salvage therapy (BEAM) on overall survival in younger patients with advanced large-cell lymphomas entered into BNLI trials. *Ann Oncol*. 1997;8(Suppl 1):63–65. [published Online First: 1997/01/01].
61. Josting A, Muller H, Borchmann P, et al. Dose intensity of chemotherapy in patients with relapsed Hodgkin' s lymphoma. *J Clin Oncol*. 2010;28(34):5074–5080. https://doi.org/10.1200/JCO.2010.30.5771. [published Online First: 2010/10/27].
62. Younes A. CD30-targeted antibody therapy. *Curr Opin Oncol*. 2011;23(6):587–593. https://doi.org/10.1097/CCO.0b013e32834bb8a7. [published Online First: 2011/10/12].
63. Katz J, Janik JE, Younes A. Brentuximab vedotin (SGN-35). *Clin Cancer Res*. 2011;17(20):6428–6436. https://doi.org/10.1158/1078-0432.CCR-11-0488. [published Online First: 2011/10/18].
64. Younes A, Bartlett NL, Leonard JP, et al. Brentuximab vedotin (SGN-35) for relapsed CD30-positive lymphomas. *N Engl J Med*. 2010;363(19):1812–1821. https://doi.org/10.1056/NEJMoa1002965. [published Online First: 2010/11/05].
65. Younes A, Gopal AK, Smith SE, et al. Results of a pivotal phase II study of brentuximab vedotin for patients with relapsed or refractory Hodgkin' s lymphoma. *J Clin Oncol*. 2012;30(18):2183–2189. https://doi.org/10.1200/JCO.2011.38.0410. [published Online First: 2012/03/29].
66. Moskowitz CH, Nademanee A, Masszi T, et al. Brentuximab vedotin as consolidation therapy after autologous stem-cell transplantation in patients with Hodgkin' s lymphoma at risk of relapse or progression (AETHERA): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet*. 2015;385(9980):1853–1862. https://doi.org/10.1016/S0140-6736(15)60165-9. [published Online First: 2015/03/23].
67. von Keudell G, Younes A. Novel therapeutic agents for relapsed classical Hodgkin lymphoma. *Br J Haematol*. 2019;184(1):105–112. https://doi.org/10.1111/bjh.15695. [published Online First: 2018/12/12].
68. Vardhana S, Younes A. The immune microenvironment in Hodgkin lymphoma: T cells, B cells, and immune checkpoints. *Haematologica*. 2016;101(7):794–802. https://doi.org/10.3324/haematol.2015.132761. [published Online First: 2016/07/02].
69. Younes A, Santoro A, Shipp M, et al. Nivolumab for classical Hodgkin's lymphoma after failure of both autologous stem-cell transplantation and brentuximab vedotin: a multicentre, multicohort, single-arm phase 2 trial. *Lancet Oncol*. 2016;17(9):1283–1294. https://doi.org/10.1016/S1470-2045(16)30167-X. [published Online First: 2016/07/28].
70. Armand P, Shipp MA, Ribrag V, et al. Programmed death-1 blockade with pembrolizumab in patients with classical hodgkin lymphoma after brentuximab vedotin failure. *J Clin Oncol*. 2016;34(31):3733–3739. https://doi.org/10.1200/JCO.2016.67.3467. [published Online First: 2016/06/30].
71. Ansell SM, Lesokhin AM, Borrello I, et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin' s lymphoma. *N Engl J Med*. 2015;372(4):311–319. https://doi.org/10.1056/NEJMoa1411087. [published Online First: 2014/12/09].
72. Herrera AF, Moskowitz AJ, Bartlett NL, et al. Interim results of brentuximab vedotin in combination with nivolumab in patients with relapsed or refractory Hodgkin lymphoma. *Blood*. 2018;131(11):1183–1194. https://doi.org/10.1182/blood-2017-10-811224. [published Online First: 2017/12/13].

73. Erdkamp FL, Breed WP, Bosch LJ, et al. Hodgkin disease in the elderly. A registry-based analysis. *Cancer*. 1992;70(4):830–834. 10.1002/1097-0142(19920815)70:4<830::aid-cncr2820700418>3.0.co;2-x [published Online First: 1992/08/15].
74. Ballova V, Ruffer JU, Haverkamp H, et al. A prospectively randomized trial carried out by the German Hodgkin Study Group (GHSG) for elderly patients with advanced Hodgkin' s disease comparing BEACOPP baseline and COPP-ABVD (study HD9elderly). *Ann Oncol*. 2005;16(1):124–131. https://doi.org/10.1093/annonc/mdi023. [published Online First: 2004/12/16].
75. Borchmann S, Engert A, Boll B. Hodgkin lymphoma in elderly patients. *Curr Opin Oncol*. 2018;30(5):308–316. https://doi.org/10.1097/CCO.0000000000000464. [published Online First: 2018/07/12].
76. Straus DJ. Initial Hodgkin treatment of the frail elderly. *Blood*. 2017;130(26):2813–2814. https://doi.org/10.1182/blood-2017-10-812966. [published Online First: 2017/12/30].
77. Bachanova V, Connors JM. Hodgkin lymphoma in pregnancy. *Curr Hematol Malig Rep*. 2013;8(3):211–217. https://doi.org/10.1007/s11899-013-0163-4. [published Online First: 2013/06/12].
78. Sud A, Thomsen H, Sundquist K, et al. Risk of second cancer in Hodgkin lymphoma survivors and influence of family history. *J Clin Oncol*. 2017;35(14):1584–1590. https://doi.org/10.1200/JCO.2016.70.9709. [published Online First: 2017/04/07].
79. van Leeuwen FE, Ng AK. Long-term risk of second malignancy and cardiovascular disease after Hodgkin lymphoma treatment. *Hematology Am Soc Hematol Educ Program*. 2016;2016(1):323–330. https://doi.org/10.1182/asheducation-2016.1.323. [published Online First: 2016/12/04].
80. Kreissl S, Muller H, Goergen H, et al. Health-related quality of life in patients with Hodgkin lymphoma: a longitudinal analysis of the German Hodgkin Study Group. *J Clin Oncol*. 2020;38(25):2839–2848. https://doi.org/10.1200/JCO.19.03160. [published Online First: 2020/06/24].
81. Hahn EE, Wu YL, Munoz-Plaza CE, et al. Use of recommended posttreatment services for adolescent and young adult survivors of Hodgkin lymphoma. *Cancer*. 2019;125(9):1558–1567. https://doi.org/10.1002/cncr.31953. [published Online First: 2019/01/09].
82. Maraldo MV, Brodin NP, Aznar MC, et al. Estimated risk of cardiovascular disease and secondary cancers with modern highly conformal radiotherapy for early-stage mediastinal Hodgkin lymphoma. *Ann Oncol*. 2013;24(8):2113–2118. https://doi.org/10.1093/annonc/mdt156. [published Online First: 2013/04/27].
83. Castellino SM, Geiger AM, Mertens AC, et al. Morbidity and mortality in long-term survivors of Hodgkin lymphoma: a report from the Childhood Cancer Survivor Study. *Blood*. 2011;117(6):1806–1816. https://doi.org/10.1182/blood-2010-04-278796. [published Online First: 2010/11/03].
84. Behringer K, Mueller H, Goergen H, et al. Gonadal function and fertility in survivors after Hodgkin lymphoma treatment within the German Hodgkin Study Group HD13 to HD15 trials. *J Clin Oncol*. 2013;31(2):231–239. https://doi.org/10.1200/JCO.2012.44.3721. [published Online First: 2012/11/15].

CHAPTER 81

ORIGIN OF NON-HODGKIN LYMPHOMA AND THERAPEUTIC TARGETS

Matthew S. McKinney and Sandeep S. Dave

Over the past two decades, discoveries in basic immunology and the pathogenesis of malignancies have significantly advanced our understanding of the origin of lymphoid neoplasms. These diseases have been reexamined and grouped based on recurrent chromosomal rearrangements, histologic patterns, gene expression profiles, and more recently by patterns of gene mutations, gene fusions, and epigenetic alterations. The multiple revisions to the World Health Organization's classification schemes for lymphomas reflect our evolving understanding of groupable lymphoma subtypes. As with other cancers, lymphoma development is dependent upon acquisition of mutations, DNA copy number changes, recurrent cytogenetic rearrangements, and epigenetic dysregulation of gene expression involving oncogenic and tumor suppressor pathways. Many of these derangements occur as a result of disordered genetic recombination and somatic hypermutation (SHM) events intended to support adaptive immunity. Also, analysis of molecular features of lymphomas compared to normal lymphocyte compartments provides clues to the events driving their pathogenesis.

An evolving understanding of the steps involved in lymphoid development has provided further insights into the development of this diverse group of malignancies because most non-Hodgkin lymphomas (NHLs) reflect stages of lymphoid development. The application of gene expression profiling (GEP) and massively parallel high-throughput sequencing, as well as a better appreciation of the contribution of microRNAs (miRNAs) and epigenetic alterations to lymphoma pathogenesis, have shed new light on the mechanisms underlying lymphomagenesis. Understanding of clinical and epidemiologic features of non-Hodgkin lymphomas provides additional insight into the origin of these malignancies. In this review, we emphasize that a collaboration of genetic alterations (some of which are induced by the unique biology of the adaptive immune system) collaborated with host features including immune stimulation and lack of tumor surveillance to drive the formation of lymphomas that mirror the phenotypic characteristics of their underlying cell of origin (this can be conceptualized as in Fig. 81.1).

This chapter reviews the most common NHL subtypes, focusing on common B-cell lymphoma subtypes, with regard to classification using prevailing views regarding genetic or genomic classification of these disorders. Additionally, insights regarding pathogenesis, prognosis, and possible therapeutic targets gleaned from genomic approaches are discussed.

OVERVIEW OF B-CELL LYMPHOMAS

Greater than 85% of NHL cases have a B-cell phenotype. With rare exception, B-cell lymphomas represent immortalized, "frozen" stages of B-cell development and the underlying molecular features of B-cell tumors reflect biologic features found in analogous normal B-cell compartments. Understanding that regulation of B-cell compartments that mirror B-cell NHL subtypes is thus crucial to understanding the driving forces behind lymphoma development (Fig. 81.2).

The goal of early B-cell development is to produce mature B cells expressing surface immunoglobulin M (IgM) that can efficiently recognize cognate antigen. After this is accomplished, B cells develop along pathways involving germinal center (GC), mantle, and marginal zones in lymphoid organs where they encounter antigen in association with activated antigen-presenting cells (APCs). Differentiation of mature B cells into plasma cells after SHM is the final step in B-cell development and is critical to the development of effective humoral immunity. Antibody diversity is accomplished through the process of somatic V(D)J recombination at the pro–B-cell stage via recombinase activating genes (*RAG1* and *RAG2*) followed by trafficking to GCs, where they proliferate rapidly with dividing times of 6 to 8 hours. High-affinity B-cell clones produced in part via activation-induced cytidine deaminase (AID) expression and SHM are favored because of protection from apoptosis. Through this mechanism, protective antibodies are produced at the risk of exposing antigen-stimulated B cells to genetic lesions caused by the molecular machinery utilized to create V(D)J recombination and SHM. Many NHLs exhibit translocations of driver oncogenes to highly active Ig loci, implying that recombination events intended to produce high-affinity antibodies are misdirected and instead produce immortalized B cells representing stages of B-cell development. Furthermore, mutations in oncogenes or tumor suppressors frequently occur in a pattern consistent with SHM at these loci. When considered together with the recurrent chromosomal rearrangements noted in NHL, this confirms the notion that these neoplasms stem from mature B cells that have entered (or are poised to enter) GCs.

Subsets of NHL recapitulate this pattern of normal B-cell differentiation at the histologic, molecular, and genomic levels and serve as a template for the classification of these diseases. This template can be applied to the entire spectrum of B-cell neoplasms, suggesting that NHLs form as a result of genetic alterations developed along this process. B-cell differentiation must be carefully choreographed at the molecular and genomic level because the expression of the *RAG* genes and AID cause DNA strand breaks and put the nascent lymphocyte at risk of oncogene overexpression and tumor suppressor deletion. Moreover, a coordinated regulation of gene expression is required to allow such genomic revision without triggering reflexive, protective apoptotic pathways and to allow appropriate B-cell differentiation. GC B cells highly express BCL6, and its functions are critically important to regulation of cell survival and differentiation. Targets of BCL6 also include cell cycle regulators (p21, p27) and TP53, which may overall work to facilitate cell cycle progression in the face of ongoing AID-mediated DNA strand breaks. BCL6 also represses *PRDM1*/Blimp1 and serves to prevent plasmacytic differentiation. Plasma cell differentiation is mediated by upregulation of IRF4 and nuclear factor kappa-B (NFκB), which establish characteristic regulatory programs.

Genetic lesions altering these pathways are found recurrently in NHL. *BCL6* rearrangements occur in diffuse large B-cell lymphoma (DLBCL) and other lymphomas, and constitutively active NFκB signaling is associated specifically with aggressive phenotypes of DLBCL. Translocations and or mutations of *BCL2*, which is expressed at very low levels in GCs, occur in almost all follicular lymphomas (FLs) and illustrate the dangers of aberrant recombination events in the GCs because *BCL2* translocation appears to be a primary event in the formation of these tumors. Recent analyses of whole genome/exome/transcriptome sequencing data have confirmed the frequent presence of these genetic lesions and have illuminated other pathways driving lymphomagenesis. In GC-derived B-cell lymphomas, mutations in *EZH2*, a member of the polycomb group (PcG), occur frequently. In

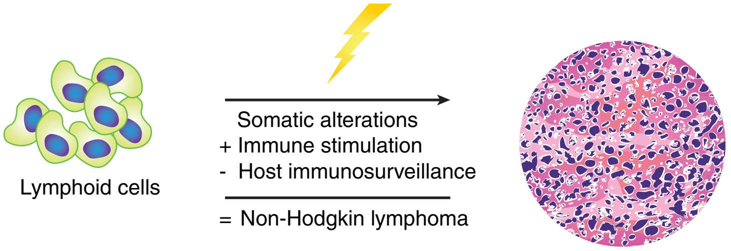


Figure 81.1 SCHEMATIC FOR COLLABORATION OF DNA DAMAGE/GENOME INSTABILITY AND IMMUNE STIMULATION. Non-Hodgkin lymphoma formation depends on several features that results in the transformation of normal lymphoid tissues to malignant lymphoma.

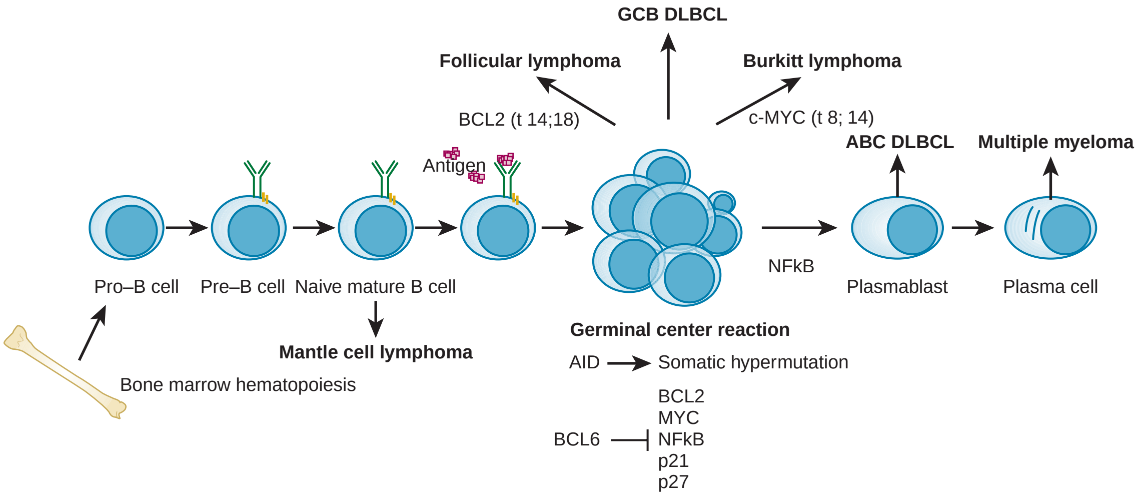


Figure 81.2 CLASSIFICATION OF COMMON NON-HODGKIN LYMPHOMAS (NHLs) IN RELATION TO B-CELL DEVELOPMENT IN GERMINAL CENTERS (GCs). Hematopoiesis results in B-cell precursors that ultimately produce immunoglobulin M (IgM) that recognizes cognate antigen via activation of recombinase activating genes (*RAG1* and *RAG2*) and a GC reaction occurs. A regulatory program relying on BCL6 regulation of cell proliferation and apoptosis occurs at this point, and rearrangements presumed to occur along this course result in post-GC lymphomas, including follicular lymphoma, Burkitt lymphoma, and diffuse large B-cell lymphoma (DLBCL). The underlying molecular biology of NHL subsets (and other lymphoid neoplasms) reflects the GC step and other stages of B-cell development. For example, gene profiling of DLBCL reveals that whereas activated B-cell–like (ABC) DLBCL reflects the plasmablast stage along normal B-cell development, multiple myeloma expression profiles more consistent with differentiated plasma cell origin.

these tumors, EZH2 mutations result in aberrant histone methylation with resultant dysregulated control of gene expression and result in GC hyperplasia in mouse models. This process appears to enhance lymphomagenesis due to an acquired inability of GC B cells to differentiate and appropriately complete maturation and thus become transformed in a state of reflecting GC B cells (Fig. 81.3). A similar role for GC regulation has been defined for genes in the sphingosine-1-phosphate receptor2 (S1PR-2) signaling pathway. Genes encoding mediators of this pathway such as *S1PR2*, *P2RY8*, and *GNA13* are frequently mutated in GC B-cell–derived lymphomas and result in dissemination of GC cells within lymph nodes. Thus, derangements in several different genes can result in abnormal GC homeostasis and drive lymphoma formation; this is a novel paradigm whereby dysregulation of B-cell differentiation leads to transformation and lymphoma.

Regulation of antigen stimulation of the B-cell receptor (BCR) is also important in both B-cell maturation and the development of NHL. The BCR consists of a multimeric signaling complex, including CD79A and CD79B, which acts through the immunoreceptor tyrosine activation motif on CD79B and leads to a cascade of molecular events involving SYK and BTK signaling. The end result of this cascade is an increase in cellular proliferation through activation of NFκB target genes and other cellular machinery. Almost all NHLs express surface Ig, suggesting that functional BCR signaling is important at some point in the process of lymphoma formation. Analysis of Ig SHM patterns in lymphoma samples further suggests that antigen-induced selective pressure is important in the pathogenesis of NHL. The antigen(s) involved in this process are unknown, although recent reports provide evidence that BCRs on lymphoma cells may recognize other malignant cells (stimulation in an autologous fashion) or antigens within the tumor microenvironment such as host stromal tissue or chronic infection with microbes.

Epidemiologic studies have bolstered the argument that chronic immune activation and inflammation are connected with NHL development; these data also suggest that immune surveillance also plays a role in the origin of NHL. DLBCL and Burkitt lymphoma (BL) occur more frequently in immunocompromised hosts and are often Epstein-Barr virus (EBV) positive. HIV/AIDS is a significant risk factor for post-GC neoplasms, and this risk is attenuated with the use of highly active antiretroviral therapy. For example, the risk of central nervous system lymphomas is increased more than 1000-fold in patients with AIDS. NHL also occurs more frequently in acquired or inherited immunodeficiencies such as common variable immunodeficiency and severe combined immunodeficiency and with solid organ and hemopoietic transplantation. Other clinical situations in which chronic infection or inflammation occurs have also been linked to NHL. Gastric MALT (*Helicobacter pylori*), hepatosplenic T-cell lymphoma (associated with infliximab use), and thyroid NHL (Hashimoto thyroiditis) are specific instances in which the development of lymphoid malignancy can be traced to chronic inflammation, either of microbial or autoimmune etiology. Finally, a critical association with EBV has been noted for posttransplant lymphoproliferative disorder, a disease that occurs in the setting of organ transplantation and immunosuppression targeting cellular immunity.

Beyond these factors, the contributions of other modifiable risk factors and heredity appear to play only a small role in NHL development. Several environmental factors (pesticides, Agent Orange,

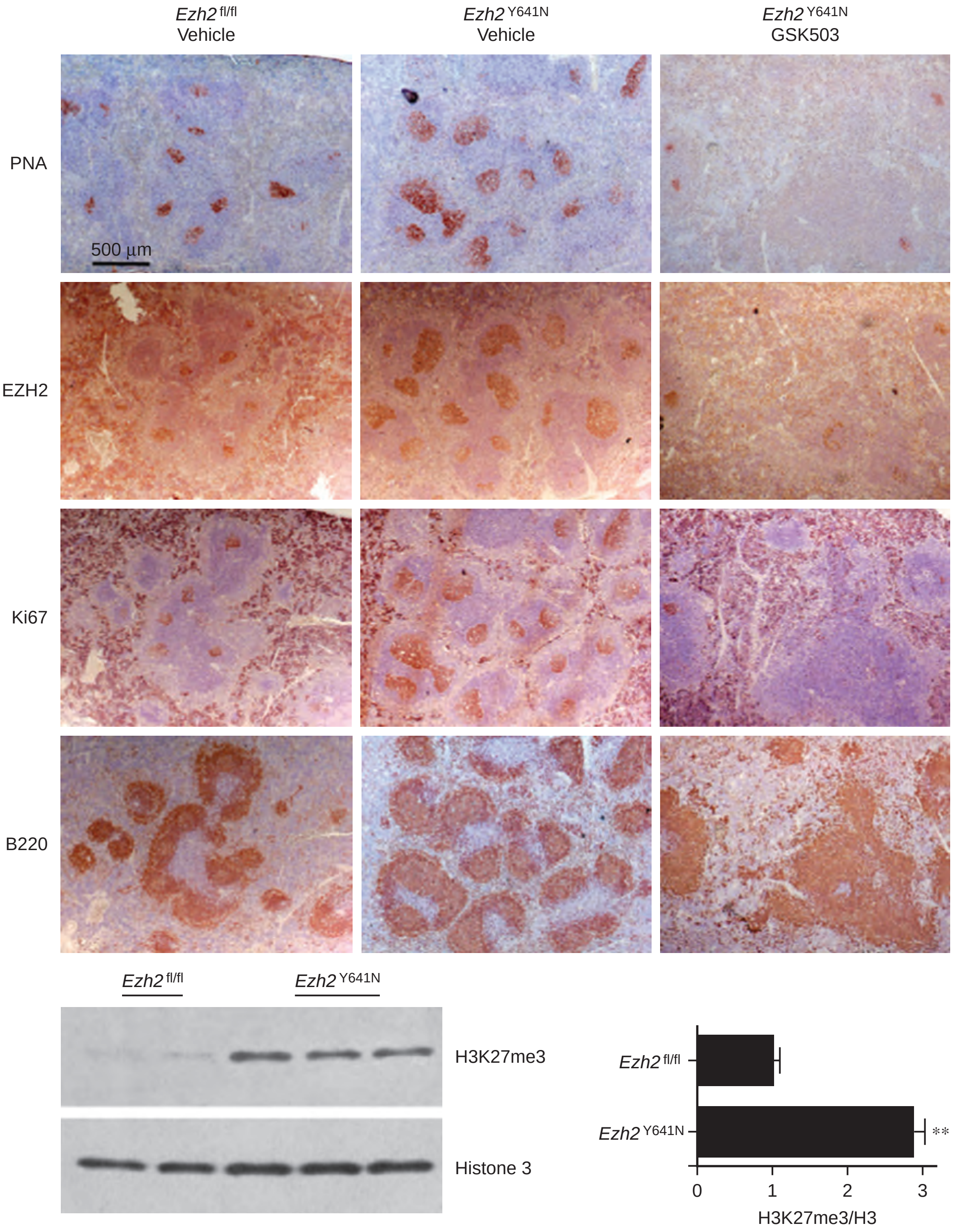


Figure 81.3 EXPANSION OF GERMINAL CENTERS WITH EZH2 MUTATION CONFERRING INCREASED HISTONE 3 LYSINE 36 TRIMETHYLATION (H3K36ME3). Upper color panels depict histologic staining as indicated by row (PNA, EZH2, Ki67, B220) of murine mouse model bearing wild-type versus mutant (Y641N) EZH2 with or without exposure to EZH2 inhibitor GSK503. Panels at bottom depict H3K36me3 levels according to EZH2 mutational status. *(From Béguelin W, Popovic R, Teater M, et al. EZH2 is required for germinal center formation and somatic EZH2 mutations promote lymphoid transformation. Cancer Cell. 2013;23(5):677–692.)*

radiation exposure) have been implicated in NHL, but the associations between these agents and lymphomagenesis are difficult to prove and the effect size of these factors appears to be relatively small. Pesticide exposure does appear to have a dose-effect relationship with regard to NHL pathogenesis and has been linked to recurrent t(14;18) cytogenetic rearrangements that are noted in DLBCL and FL, but the percentage of cases linked to pesticide exposure in these entities is very low. Likewise, several genetic linkage studies have found associations between several genes and the development of various lymphoma subtypes, but the role of variations in these pathways in lymphomagenesis has not been validated. Thus, there does appear to be a small role of inherited or modifiable risk factors that likely conspire with other acquired genetic/molecular lesions in the origin of lymphoma.

And so, factors related to abnormal immune surveillance, chronic inflammation, external exposures and host factors conspire to produce genetic/molecular lesions involved in lymphoma formation and progression. The remainder of this review will focus on current knowledge of biologic features of individual NHL subtypes.

DIFFUSE LARGE B-CELL LYMPHOMA

DLBCL is the most common subtype of NHL (≈25% of all NHL) and is responsible for more patient deaths than any other form of lymphoma. This entity often presents at an advanced stage, and around 50% of all patients fail to respond long-term to standard chemotherapy programs; the vast majority of those patients will eventually succumb to their disease. Even prior to the availability of GEP technology, it was clear clinically (based on tumor morphology, immunohistochemical staining, and patient outcomes) that DLBCL is a heterogeneous disease. Advances in molecular biology and genomic technologies have clarified the basis for some of this observed heterogeneity, and it has become clear that DLBCL comprises a diverse group of lymphomas that have distinct cellular origins, recurrent gene mutations, and chromosomal rearrangements that dictate their behavior. It is also clear that the stromal environment appears to play a key role in the development and progression of DLBCL, as with other cancers.

GENE EXPRESSION PROFILES DEFINE DIFFUSE LARGE B-CELL LYMPHOMA SUBTYPES (TABLE 81.1)

Histologic analysis and more recently high-throughput genomics assays such as GEP and massively parallel high-throughput sequencing for alterations (including somatic mutations, copy number changes, and gene fusions) have identified broad subtypes of DLBCL with molecular features that mimic their putative cell or origin; these studies have also shed light on the tremendous heterogeneity and complexity of the myriad features that drive lymphomagenesis.

GEP has identified GCB and activated B-cell–like (ABC) DLBCL subsets that express gene signatures that mimic GCs and activated B cells (Fig. 81.4). Roughly 30% of DLBCLs cannot be classified using this system and have been deemed unclassified DLBCL. There are thousands of genes that differ between these subtypes at the GEP level, which is a magnitude similar to that seen between acute myeloid and lymphoblastic leukemias, which have profoundly different mechanisms of origin and natural histories.

GCB DLBCLs express a characteristic spectrum of genes and have ongoing AID-mediated SHM as well as a histologic phenotype consistent with GC origin. BCL6 and LMO2 are both important to the transcriptional program of GCs and are commensurately overexpressed in GCB DLBCLs. Conversely, *PRDM1* is downregulated by BCL6, which results in decreased *PRDM1* activity in GCs, and GCB DLBCLs display a similar pattern. Histologically, GCB DLBCLs are characterized by CD10 expression and lack of IRF4 expression. Evaluation of Ig loci within GCB DLBCLs has also revealed evidence of ongoing SHM, which is further evidence that GCB DLBCLs reflect the biology of normal GC B cells and that dysregulation of pathways involved in GC formation might lead to this subset of DLBCL.

Alternatively, the ABC subtype of DLBCL expresses a post-GC phenotype but appears trapped just before differentiation into plasma cells. ABC DLBCLs are characterized by downregulation of many GC genes, including *BCL6* and *LMO2*, and by expression of genes associated with activated B cells and plasma cells. One of the hallmarks of the ABC DLBCL phenotype is upregulation of NFκB. NFκB is a transcription factor that targets a number of gene programs governing cell proliferation, immortality, and angiogenesis within NHLs. The effectors of NFκB signaling exist in inactive form in the cytosol and require signaling either through MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) signaling (the canonical NFκB pathway) or through ligation of CD40–LTbR–BAFF-R (B-cell activating factor receptor), which results in phosphorylation of the inactive complex and translocation of either the p50–RelA or p52–Rel B complexes to the nucleus and subsequent transcription of target genes. In ABC DLBCL cell lines, a signaling complex involving CARD11, BCL10, and MALT1 (the CBM complex) constitutively activates NFκB signaling and selective knock-down of any of the components of this complex using RNAi or small molecule inhibitors and is lethal to ABC cell lines. Interestingly, a few GCB cell lines also demonstrate activation of NFκB and mutations in the NFκB pathway, which might confer sensitivity to inhibition of this pathway in those tumors. The mechanisms by which DLBCLs acquire and maintain constitutive NFκB activity are still being elucidated and may provide insights into the origin and into possible treatments for this entity.

TABLE 81.1 **Frequency of Recurrent Genetic Aberrations in Diffuse Large B-Cell Lymphoma Subtypes**

Genetic Feature	ABC DLBCL	GCB DLBCL	PMBL	Notes
BCL2 translocation t(14;18)	0%	40%–50%	20%	Alternatively translocated to light chain loci 5%–10% of time
BCL2 amplification	34%	10%	16%	
BCL6 translocation t(3;V)(q27;V)	25%	10%	20%	Variable translocation partners (14q32, 2p11, 22q11, 4p11, 6p21, 11q23)
3q amplification	25%	0%	<5%	
9q24 amplification	5%	0%	45%	
PRDM1/ *PRDM1* deletion/ mutation	25%	0%	Unknown	
REL amplification	0	15%	25%	
Recurrent mutation	*CARD11*, *MYD88*, *CD79B*	*EZH2*, *GNA13*, *BCL2*	*PTPN1*, *SOCS1*, *STAT6*	
MYC rearrangement t(8;14) (q24;q32)	5%–10% of all DLBCLs	25%–30% occur with t(14;18) (q32;q21)		

ABC, Activated B-cell–like; *DLBCL*, diffuse large B-cell lymphoma; *GCB*, germinal center B-cell–like; *PMBL*, primary mediastinal B-cell lymphoma. Compiled from Iqbal J, Greiner TC, Patel K, et al. Distinctive patterns of BCL6 molecular alterations and their functional consequences in different subgroups of diffuse large B-cell lymphoma. *Leukemia*. 2007;21:2332; Iqbal J, Neppalli VT, Wright G, et al. BCL2 expression is a prognostic marker for the activated B-cell-like type of diffuse large B-cell lymphoma. *J Clin Oncol*. 2006;24:961; Iqbal J, Weisenburger DD, Greiner TC, et al. Molecular signatures to improve diagnosis in peripheral T-cell lymphoma and prognostication in angioimmunoblastic T-cell lymphoma. *Blood*. 2010;115:1026; Pasqualucci L, Compagno M, Houldsworth J, et al. Inactivation of the *PRDM1*/BLIMP1 gene in diffuse large B cell lymphoma. *J Exp Med*. 2006;203:311; and Tam W, Gomez M, Chadburn A, et al. Mutational analysis of *PRDM1* indicates a tumor-suppressor role in diffuse large B-cell lymphomas. *Blood*. 2006;107:4090.

Primary mediastinal B-cell lymphoma (PMBL) represents a distinct subset of patients that have sometimes been classified as

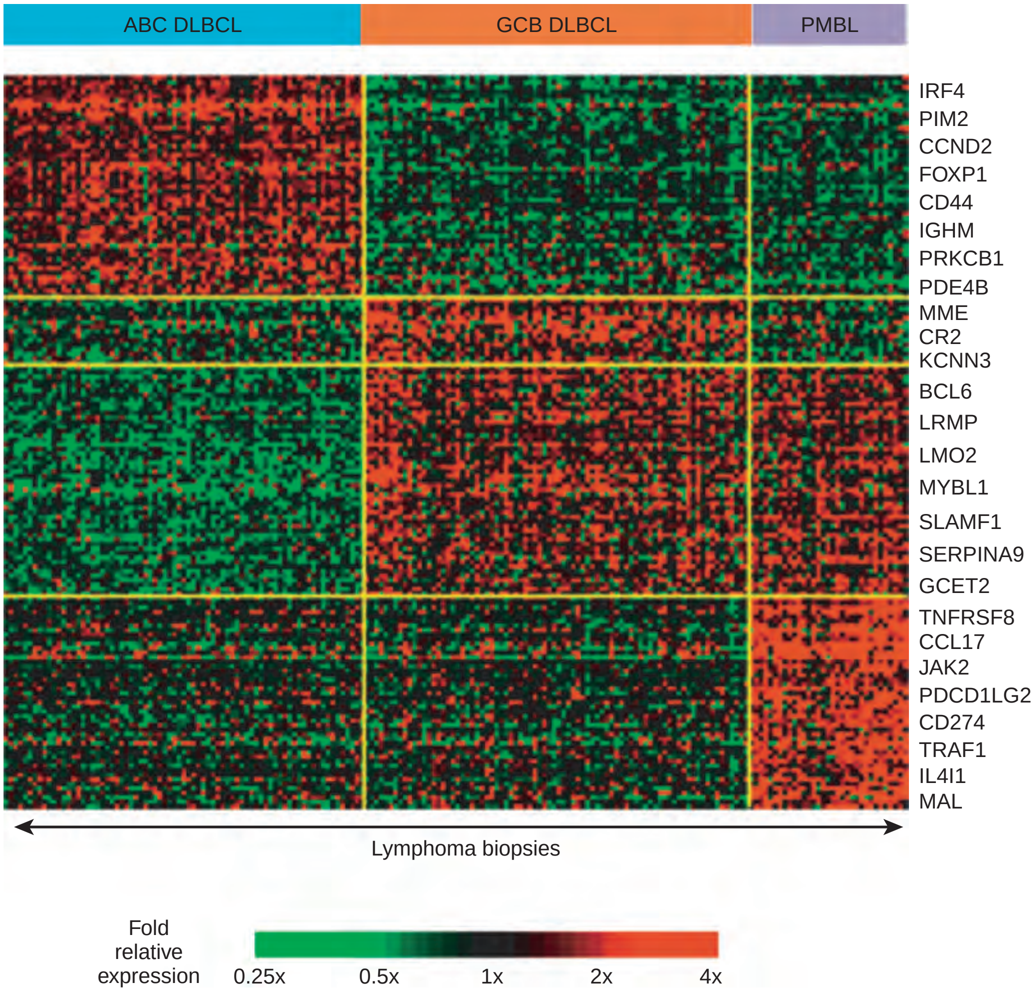


Figure 81.4 GENES CHARACTERISTICALLY EXPRESSED BY SUBGROUPS OF DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL). Activated B-cell–like (ABC) DLBCL, germinal center B-cell–like (GCB) DLBCL, and primary mediastinal B-cell lymphoma (PMBL). Each column represents gene expression data from a single DLBCL biopsy sample, and each row represents expression of a single gene. Relative gene expression is indicated according to the color scale shown. *(Adapted from Roschewski M, et al. Diffuse large B-cell lymphoma—treatment approaches in the molecular era. Nat Rev Clin Oncol. 2014;11:12-23.)*

DLBCL. PMBL was originally identified based on the clinical characteristics of the disease. Patients with PMBL are generally young (third or fourth decades of life), predominantly women, and present with a large mediastinal mass; outcomes with chemoimmunotherapy for PMBL are generally superior to those with other subtypes of DLBCL. GEP studies revealed that PMBL could be clearly delineated from ABC and GCB subtypes. Indeed, by gene expression, PMBL appears similar to Hodgkin lymphoma with many shared features in both diseases, including the expression of CD30 (Fig. 81.5). Both diseases also express genes associated with Janus-activated kinase (JAK)–signal transducer and activator of transcription (STAT) signaling as well as targets of the NFκB pathway.

Molecular Pathogenesis of Diffuse Large B-Cell Lymphoma

Investigation of the molecular landscape of DLBCL has revealed a set of recurrent cytogenetic and molecular abnormalities associated with pathogenesis. Chromosomal translocations involving *BCL6*, *BCL2*, and *MYC* (v-*myc* myelocytomatosis viral oncogene homolog) occur frequently in DLBCL, and these translocations appear to reflect cell-of-origin phenotype. 3q27 rearrangement with Ig gene partners is the most common rearrangement seen in DLBCL and occurs in about 30% of both the ABC and GCB subtypes. This genetic rearrangement upregulates the *BCL6* gene, resulting in an oncogenic phenotype. Additional mutations in the transcriptional regulatory program of *BCL6* may also occur with alternative translocation. Notably, *BCL6* translocations appear to not be sufficient for lymphoma formation as evidenced by mice bearing t(3;14) translocations that develop NHL at a low rate. *BCL2* translocations occur in a minority of GCB DLBCLs but are not seen in the ABC phenotype; this mirrors the situation in FL, in which *BCL2* overexpression results in disordered GC transit. *MYC* rearrangements additionally occur in DLBCL (≈5% of cases), sometimes with rearrangements of *BCL2, BCL6*, or both. These tumors, sometimes termed *double-hit lymphomas*, display an aggressive phenotype seemingly intermediate between BL and DLBCL.

Oncogenic mutations and areas of chromosomal amplification or deletion also appear important in the pathogenesis of DLBCL. Upregulation of c-REL occurs in about 20% of GCB tumors through amplification of the 2p locus. Amplification of *MYC* and *BCL2* may also occur through this mechanism. *TP53* mutations and SHM of other loci, including *PIM1, BCL6*, and *MYC*, also appear to contribute to lymphoma formation and progression. Mutations in *CARD11* causing constitutive NFκB activation have been described in a subset of both ABC and GCB DLBCLs and appear to drive oncogenesis in these tumors. Overall, the patterns of gene mutation and recurrent cytogenetic abnormalities add further complexity to the classification of DLBCLs.

Recurrent mutations of genes involved in DLBCL pathogenesis have also been reported. Most recently, several groups have reported

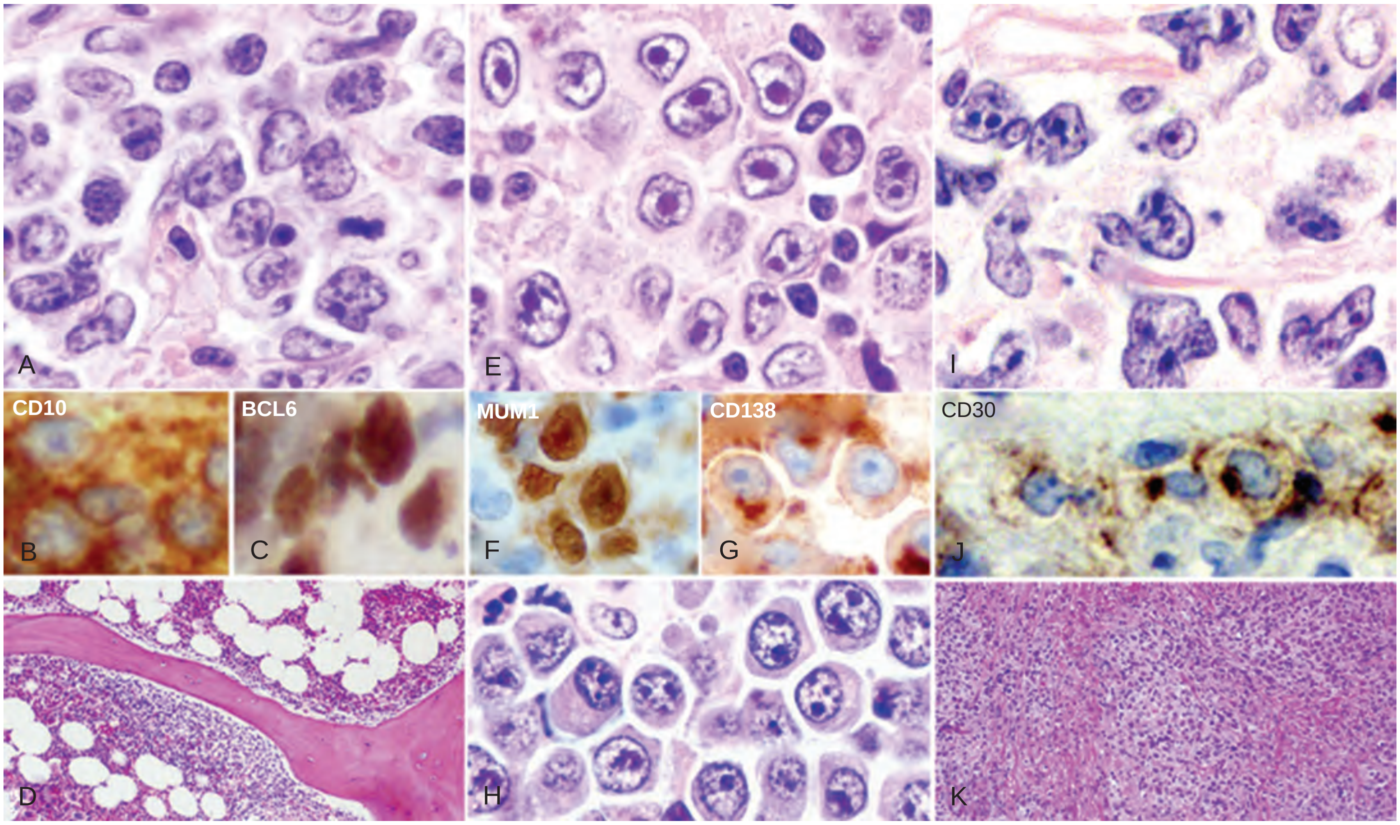


Figure 81.5 THREE TYPES OF DIFFUSE LARGE B-CELL LYMPHOMA DELINEATED BY HISTOLOGIC PATTERNS. Germinal center B-cell–like (GCB), activated B-cell–type, and primary mediastinal B-cell lymphoma (PMBCL) type. GCB, or germinal center (GC)–derived large B-cell lymphomas usually have a centroblastic morphology (A), are CD20$^+$, and express the GC markers CD10 (B) and BCL6 (C). In this case, the patient's staging bone marrow (D) showed paratrabecular involvement by small, cleaved cells (centrocytes), indicating that the large B-cell lymphoma likely arose from a lower-grade follicular lymphoma. Activated B-cell–like large B-cell lymphoma can sometime have an immunoblastic morphology (E) and can typically express MUM1 (F) and CD138 (G). These post-GC cell types of lymphomas can also have a plasmablastic morphology (H), as sometimes seen in HIV-related cases. PMBCLs are frequently composed of multilobated centroblasts (I). They are typically CD45$^+$, CD20$^+$, and usually CD30$^+$ (J) but CD15 negative. Surface immunoglobulin may be negative on such cases. PMBL is commonly associated with fibrosis, which is present in broad bands (K) or surrounding individual cells (as in I).

initial and now expanded/updated analyses of whole-genome, exome, or transcriptome data from the application of massively parallel sequencing in thousands of cases of DLBCL. DLBCL case clinical outcome data is now available in conjunction with recent data sets and allows for a refined analysis of features associated with treatment resistance and other clinical events in this entity (Fig. 81.6). These studies have identified hundreds of novel mutations in DLBCL and confirm previous descriptions of survival association with cell or origin and other phenotypic features. Two of these studies show DLBCL cases can be clustered into novel subgroups based on somatic alterations including SNVs, GEP, and CNVs, although these studies have somewhat different conclusions as to molecular grouping of DLBCL cases. It may be more appropriate to consider the contribution of various mutations with pathogenesis in a combinatorial factor as there is tremendous heterogeneity in the spectrum of mutations that participate. Interestingly, some of the molecular features of lymphoma subtypes such as activation of the NFκB signaling pathway identified through GEP have been shown to correlate with specific mutations in DLBCL cancer genes. Inactivating mutations of *PRDM1* and mutation in genes regulating NFκB signaling (*TNFAIP3, MYD88, CARD11*) have been noted in ABC DLBCL subtypes. *CD79B* and *BCL2* have also been found to harbor mutations in DLBCL and other NHLs. A series that performed RNA sequencing on 127 NHL samples (including 83 DLBCL tumors) found 651 coding single nucleotide variants (cSNVs), many of which were not previously documented in malignancies. Truncating deletions in tumor suppression genes (*TP53, TNFRSF14, CREBBP*) were among the most significantly mutated genes and other heavily mutated genes not previously known to be involved in DLBCL pathogenesis (including *MLL2, BTG1, EZH2*, and *GNA13*). *MLL2* contained cSNVs in 26% of tumors and most often occurred as a heterozygous defect, and sometimes multiple mutations occurred in trans fashion. MLL2 functions as a H3K4-specific methyltransferase responsible for regulation of transcription of developmental genes. Ostensibly, MLL2 might serve to deregulate genes involved in differentiation or development and act in conjunction with other oncogenes to facilitate NHL. Other groups have confirmed mutations in *MLL2* and other genes (*CREBBP/EP300*) that coordinate chromatin acetylation, leading to the hypothesis that mutations in histone-modifying genes lead to DLBCL and other NHLs. Further work is needed to dissect the mechanism(s) by which the various molecular lesions in individual tumors cause lymphoma formation and this will perhaps also improve our understanding of normal lymphocyte biology while providing new tools for lymphoma diagnosis and treatment.

The Tumor Microenvironment and Diffuse Large B-Cell Lymphoma

The complexities of antigen presentation and recognition and regulation of B-cell proliferation that occur in GCs suggest that the microenvironment may play a role in the origin and progression of DLBCL. Tumor-infiltrating T cells have been correlated to longer survival in patients with DLBCL, and failure of immune editing is likely responsible for the increase in NHL in HIV-positive and immunosuppressed persons. GEP has been used to define stromal elements related to DLBCL progression. In one series, hierarchical clustering was used to define three groups among DLBCL samples by GEP. Clusters identifying gene signature termed *oxidative phosphorylation,*

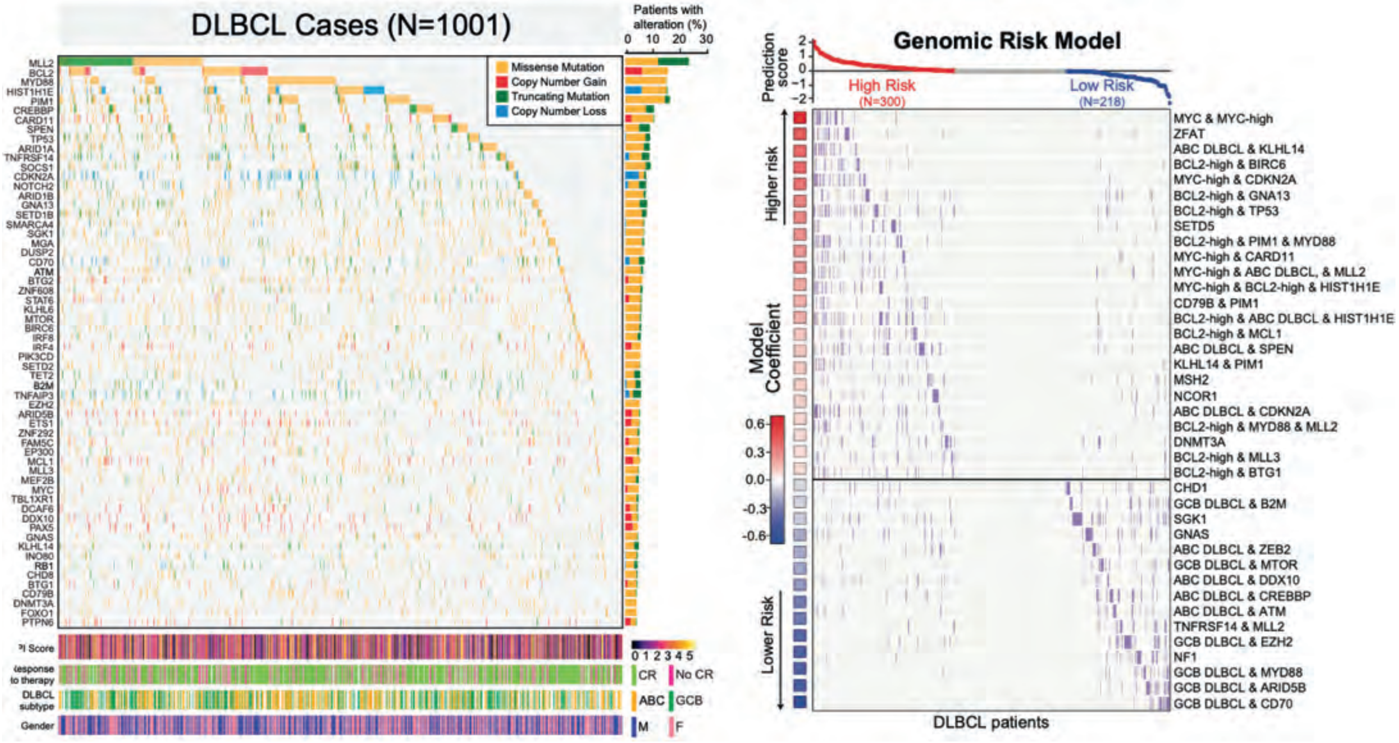


Figure 81.6 GENOMIC EVENTS IN DIFFUSE LARGE B CELL LYMPHOMA AND IMPACT OF MUTATIONS ON SURVIVAL WITH CHEMOIMMUNOTHERAPY. The left panel depicts the landscape of genetic mutations in diffuse large B-cell lymphoma (1001 cases) with clinical features such as International Prognostic Index (IPI) and chemotherapy response depicted for each case. The right panel depicts key molecular subgroups defined by collaborative mutation and gene expression profiling studies to identify key mediators of DLBCL pathogenesis and survival features. *(From Reddy A, Zhang J, Davis NS, et al: Genetic and functional drivers of diffuse large B cell lymphoma. Cell. 2017;171(2):481.–494.)*

BCR proliferation, and *host response* were identified; notably, the host response group exhibited gene expression profiles associated with T and natural killer cells, dendritic cells, and macrophages. This work illustrates that patterns of stromal involvement in DLBCL could play a key role in pathogenesis. In another series involving biopsy specimens from 414 patients treated with chemotherapy and chemoimmunotherapy, two gene signatures characterizing patterns of tumor stroma best predicted survival. Improved survival was noted with a pattern that included genes associated with the extracellular matrix and histiocytic infiltration. The converse was true for gene profiles correlating to angiogenesis because these were associated with far worse overall survival.

Although it is not known what roles distinct lymph node subsets play in the pathogenesis of lymphoma, it is clear from these studies that the tumor microenvironment plays a role in the origin of NHL.

Conclusions

DLBCL represents a heterogeneous group of B-cell neoplasms with a distinct underlying biology, prognosis, and response to therapy. Recent genomic approaches to studying DLBCL have identified a number of interesting potential targets for better prognostic stratification and treatment of this lymphoma entity. More work is needed to this end and to better understand the origin of DLBCL and the role of stromal factors in disease progression.

BURKITT LYMPHOMA

BL is a highly aggressive lymphoma entity characterized by a high mitotic rate, extranodal spread, and early death, but it is frequently curable with high-intensity multi-agent chemotherapy. It was first described more than 50 years ago as a disease in young Africans in association with EBV infection. Since then, the sporadic and immunodeficiency-related forms of the disease have been described. Subsequent work has shown that translocation of the *MYC* gene on chromosome 8q24 to the Ig locus occurs in virtually all cases of BL (Fig. 81.7). Given the high prevalence of *MYC* rearrangements and the high cure rates observed with chemotherapy, many have studied BL as a model for other B-cell lymphomas.

Overexpression of MYC occurs in association with a GC phenotype in BL. BL tumors typically display the immunophenotypic characteristics of GC B cells, and genetic and genomic studies have reinforced this notion of GC origin. In virtually all cases, rearrangements are found between the MYC locus and one of the Ig genes, most commonly with the IgH locus at 14q32.33 in more than 80% of cases. Alternative rearrangements involving MYC and the κ or λ light chain regions are also noted in a minority of cases. Two different patterns of rearrangement and SHM have been noted among the three types of BL. In endemic BL, chromosome 14 breakpoints can be mapped to J_H regions, indicating *RAG1*- or *RAG2*-mediated rearrangement occurring at the pro–B cell stage. In contrast, sporadic and immunodeficiency-related BL often demonstrate SHM and rearrangement at chromosome 14 breakpoints related in Ig switch regions; this is consistent with GC or memory B-cell origin.

Translocations placing MYC downstream of Ig genes result in massive gene upregulation and oncogenesis by dysregulation of multiple pathways. In normal B cells, MYC regulates B-cell proliferation and differentiation and is tightly regulated by multiple mechanisms, including posttranslational modifications and transcriptional autoregulation and by other factors, including a careful balance of interactions with regulatory proteins. Under normal circumstances, MYC also induces a number of proapoptotic cellular mechanisms, which are then perturbed when MYC is overexpressed by the robust Ig gene promoters existing in GC B cells. Constitutive induction of MYC expression in GCs via the upstream Ig gene promoter then confers a neoplastic phenotype. Highlighting the complexities of MYC in this setting, even brief reversal of MYC overexpression may induce a

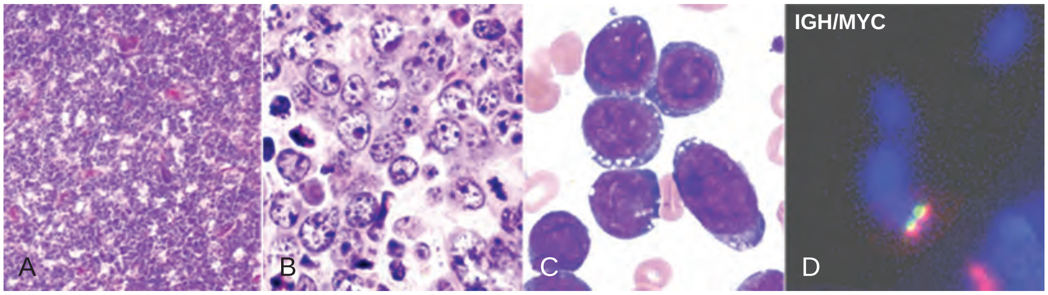


Figure 81.7 BURKITT LYMPHOMA (BL). (A) A case of BL illustrated at low power showing the "starry sky" appearance. This appearance is attributable to the dense proliferating cells producing the "dark sky," and the scattered lighter staining tingible body macrophages ("stars") phagocytizing dying cells. (B) Higher magnification image illustrating the syncytia of intermediate-sized cells with coarse chromatin and multiple nucleoli. Note the tingible body macrophage with abundant light cytoplasm and ingested debris *(center bottom)*. (C) Burkitt cells as seen on a Wright-stained bone marrow aspirate in a patient with BL. Notice deep blue cytoplasm with numerous vacuoles. (D) Fluorescence in situ hybridization with probes to MYC and immunoglobulin h (IgH) illustrate the IgH–MYC fusion. *(Courtesy Dr. Yanming Zhang, University of Chicago.)*

reversion of tumors to a benign phenotype; this implies that c-*MYC*–Ig rearrangements might serve as the "switch" that drives BL formation in the context of GCs.

Recently, GEP has further examined the role of MYC in BL and revealed insights into the similarities and differences between BL and other GCB-origin NHLs (Fig. 81.8). This work has further defined the differences between BL and DLBCLs carrying MYC rearrangements because these entities can be difficult to distinguish on histopathologic grounds alone. Although BL is characterized by gene expression profiles consistent with GC origin, a group of genes found in GCB DLBCL, including *LMO2, BCL2, CD80*, and *CD86*, are expressed only weakly in BL. BLs also express low amounts of NFκB and major histocompatibility class I molecules compared with DLBCL. These data also serve to allow the diagnostic distinction of BLs on molecular grounds. In two large studies, a fraction of DLBCLs were classified as BL in instances when there was DLBCL–BL overlap, causing disagreement among pathologists. The outcome with CHOP-like chemotherapy in these instances was associated with poorer survival. Furthermore, classic MYC t(8;14) rearrangements and intermediate molecular phenotypes were also associated with worse overall survival.

The development of a molecular diagnosis for BL aids in distinguishing BL from DLBCL and further illustrates the complexity of *MYC* rearrangements in NHL. Lymphoma entities containing MYC rearrangement in addition to rearrangements in *BCL2* or *BCL6* have been historically termed "double-hit" lymphomas. This subtype frequently has a clinical behavior distant from BL and is often associated with progressive disease, a high International Prognostic Index score, and poor response to intensive chemotherapy regimens. More work regarding this subtype of NHL is needed to understand its pathogenesis.

Aside from gene expression patterns related to GC origin, further genomic alterations may contribute to BL pathogenesis. As in DLBCL, whole genome/exome/transcriptome approaches have deepened our understanding of the importance of genetic lesions beyond *MYC* rearrangement in BL pathogenesis (Fig. 81.9). While *MYC* mutation (occurring in addition to *MYC* rearrangement) appears to be the most frequent mutation in BL, mutations in the transcription factor *TCF3* or its negative regulator *ID3* occur in the majority of cases of BL and appear to lead to phosphoinositol-3-kinase activation and other complex transcriptional changes with BL cells. *ID3* mutations may also serve as a regulator of *MYC* and directly influence cell cycle progression, thus fostering BL development. TP53 is frequently also mutated or deleted in a subset of BL tumors. High-throughput genomic techniques have revealed many other novel BL cancer mutations in genes such as *SMARCA2* and *CCND3* and recurrent copy number abnormalities including gains of 1q and 18q and deletion of 19p13. Thus these studies have revealed molecular complexity beyond the dysregulation of *MYC* through cytogenetic rearrangement.

EBV infection is also thought to play a role in the development of BL because virtually 100% of cases of endemic BL and 30% to 50% of all other BLs are associated with EBV positivity. The exact role of EBV in BL is unclear. Endemic BL is frequently associated with endemic malaria, which further suggests a complex interaction of the host immune response with microorganisms and other factors. Interestingly, *Plasmodium infection* appears to promote genomic instability and promote AID-dependent B-cell lymphomas and this mechanistically supports a link between chronic infection and resultant immune stimulation or dysregulation and lymphoma formation. Other host factors or genomic changes may further contribute to the development of BL.

Further work is needed to define what other genomic changes and host–environmental interactions contribute to the pathogenesis of BL. Ongoing studies seeking to define the entire spectrum of molecular, immunologic, genomic, and epigenetic changes associated with BL pathogenesis may further elucidate this process.

FOLLICULAR LYMPHOMA

FL is a low-grade B-cell neoplasm that is also characterized by recurrent chromosomal rearrangements involving the IgH locus. It is the most common indolent NHL with more than 16,000 adults diagnosed yearly in the United States. It is characterized by significant heterogeneity with regard to histologic grade and overall survival, with more than 10% of patients surviving at least 15 years after diagnosis. A spectrum of histologic grading based on the density of centroblasts is also characteristic of this disorder, with higher FL grades resembling DLBCL. There is no clear association between grade and clinical outcome in the disease. Some patients with FL experience an aggressive clinical course and transformation to aggressive lymphomas, but others may experience disease progression only after many years with the disease. This review focuses on the classic rearrangements of *BCL2* and the additional molecular changes associated with FL development and with histologic transformation of FL to more aggressive subtypes.

BCL2 Rearrangements and Other Genomic Alterations in Follicular Lymphoma

FL arises from GC B cells that have been immortalized at this stage of B-cell differentiation. Cytogenetic studies performed in the early 1970s defined a recurrent translocation involving the long arms of chromosomes 14 and 18 in more than 90% of FL cases. Further investigation revealed that this places the gene encoding the important antiapoptotic gene *BCL2* downstream of the Ig heavy chain transcriptional regulators. The BCL2 protein inhibits the release of cytochrome c from mitochondria in the process of apoptosis. In normal GCs, *BCL2* is essentially absent because selection of B-cell clones via apoptosis is an important step in the production of high-affinity Ig idiotypes. The t(14;18)(q32.3;q21.3) appears to

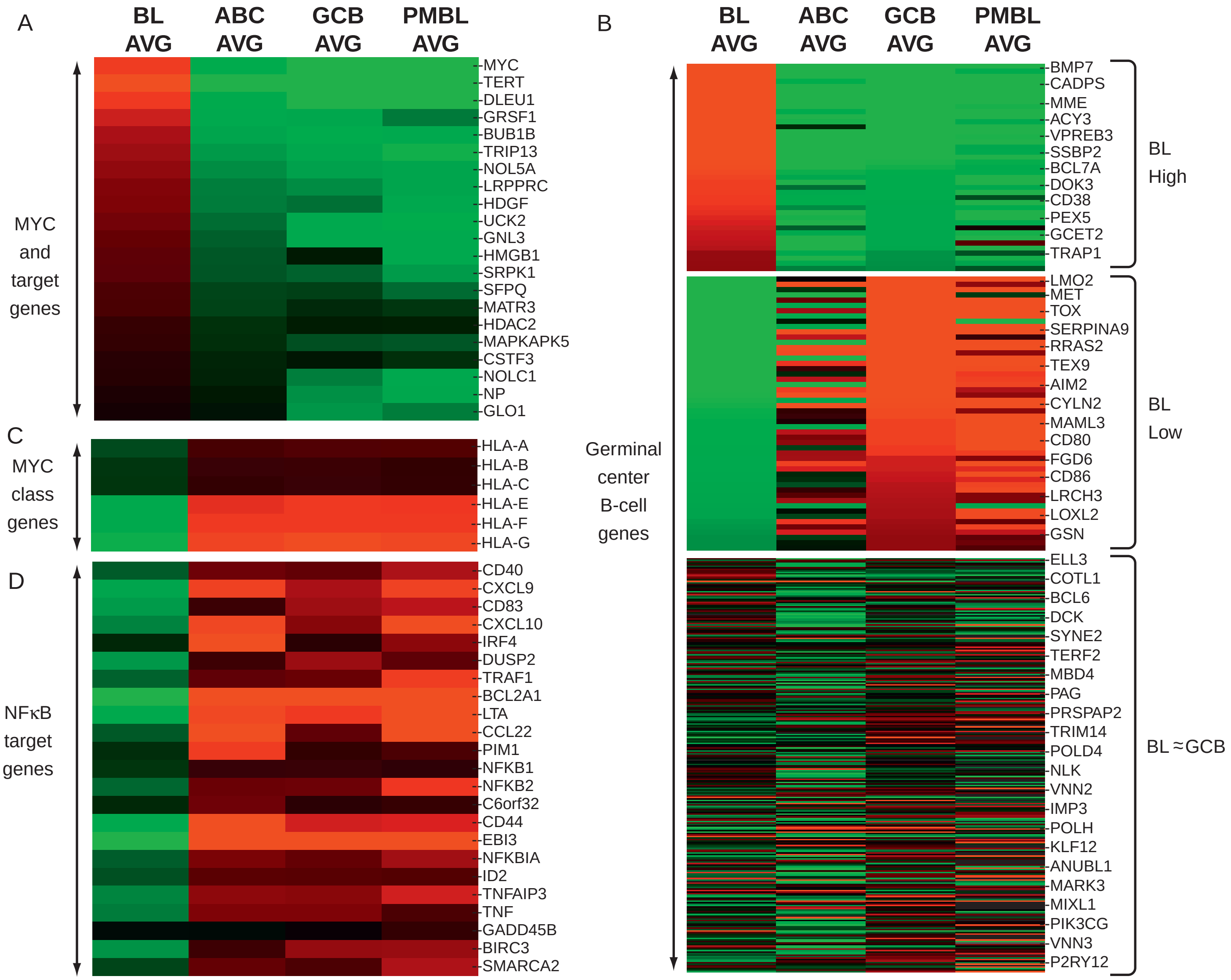


Figure 81.8 GENE EXPRESSION PROFILES IN BURKITT LYMPHOMA (BL) COMPARED TO DLBCL Relative expression of genes that distinguish BL from subgroups of diffuse large B-cell lymphoma (DLBCL)—activated B-cell–like (ABC) DLBCL, germinal center B (GCB) DLBCL, and primary mediastinal B-cell lymphoma (PMBL)—is categorized into gene expression signatures: c-myc and its target genes (A); genes that are expressed in normal germinal center (GC) B cells (B) and are expressed more highly (BL-high), less highly (BL-low), or equivalently (BL≈GCB) in BL than in GCB DLBCL; major histocompatibility class I genes (C); and genes targeted by the nuclear factor kappa-B (NFκB) signaling pathway (D). GC B-cell signature genes are those that were overexpressed in normal GC B cells, compared with blood B cells. The "BL-high" genes were expressed at levels twice as high in BL as in GCB DLBCL ($P < .001$). The "BL-low" genes were expressed at levels twice as high in GCB DLBCL as in BL ($P < .001$). The expression levels of the "BL≈GCB" genes did not differ significantly between the two lymphoma subtypes. *(Reproduced from Dave SS, Fu K, Wright GW, et al. Molecular diagnosis of Burkitt's lymphoma. N Engl J Med. 2006;354:2431.)*

be mediated by *RAG1/RAG2* in pre-GC B cells because *BCL2* is most often found juxtaposed to J chain exons. Isolated t(14;18) is not sufficient to induce FL because transgenic mice bearing this translocation develop lymphoid hyperplasia but require other genetic lesions to develop FL. Additionally, B cells with t(14;18) can be isolated from apparently normal individuals who never develop lymphoma, and *in situ* follicular hyperplasia with BCL2 overexpression has been noted on examination of otherwise normal lymph nodes. This suggests that other lesions must be acquired for development of FL; a preponderance of evidence suggests this occurs in GCs. Histologically, FL is characterized by an abnormal follicular architecture containing neoplastic cells. The follicles loosely resemble GCs but usually lack an appreciable mantle zone but generally occur in close proximity to follicular T-helper cells expressing CD3, CD4, CD57, PD1, and CXCL13. FL usually exhibits a CD10+BCL6+IRF8+ GC-like phenotype in association with ongoing SHM, confirming GC or post-GC origin (see Fig. 81.8). One obvious hypothesis is that BCL2 overexpression dysregulates apoptosis such that GC transition and SHM result in further genomic and epigenomic changes that result in FL. This framework might need to be reexamined in the small proportion of FL cases that do not express BCL2.

Although BCL2 is thought to have a critical role in development of FL, nearly 30% of grade 3A and a majority of grade 3B FLs are t(14;18) negative. Alternative rearrangements involving the κ or λ light chains and *BCL2* may occur, but many t(14;18) FLs do not express BCL2. These entities express a post-GC CD10-IRF4-phenotype with 3q27/*BCL6* rearrangements and are associated with lower chemotherapy response rates as well as worse overall survival compared with t(14;18)-positive FLs. It is not clear how this subset of FL evolves from normal B cells, but this immunophenotype suggests a late- or post-GC origin. GEP and analysis of miRNA profiles of FL confirm this association. Leich et al. analyzed gene expression profiles of 184 grade I-3A FLs, of which 17 were t(14;18) negative (six of these rearrangement-negative FLs still overexpressed BCL2). Analysis of this data showed enrichment of signatures associated with

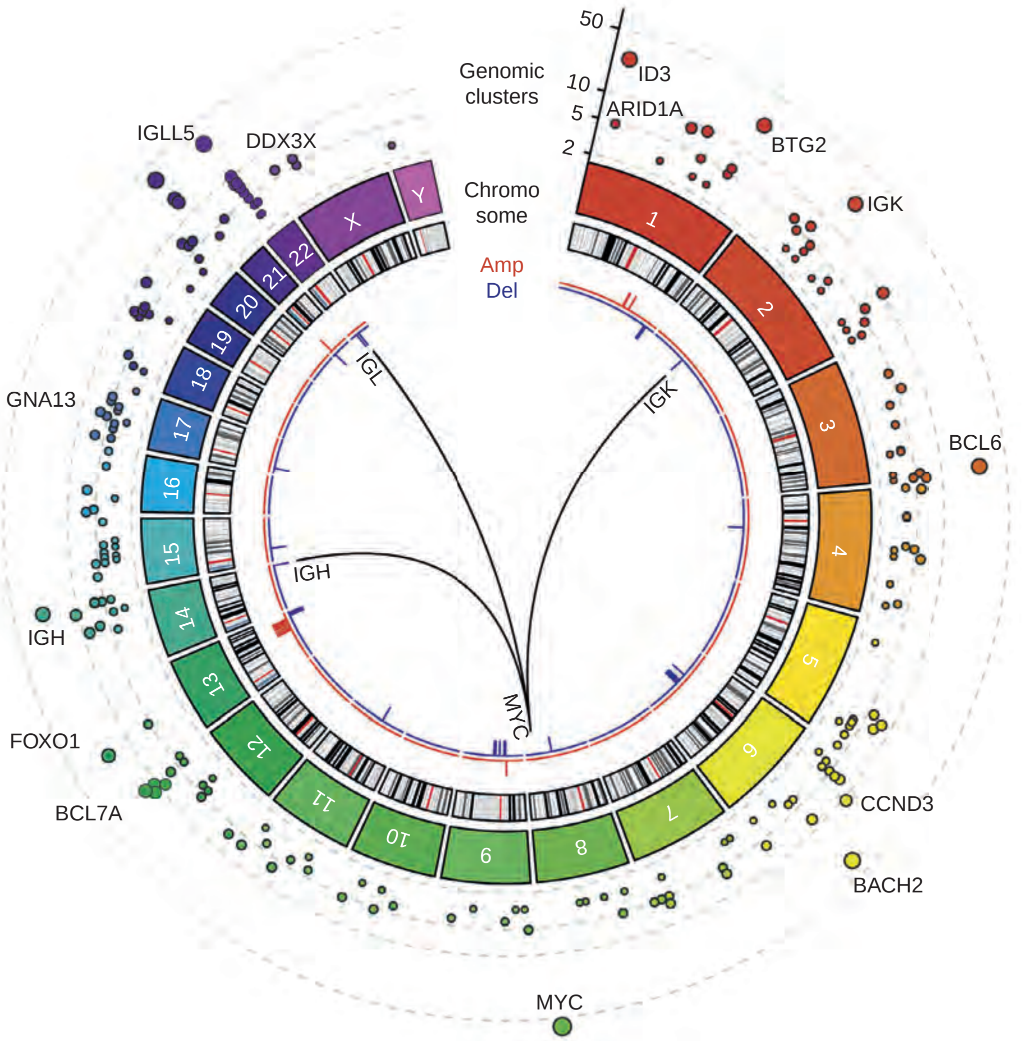


Figure 81.9 COMMON SOMATIC ALTERATIONS ACROSS THE ENTIRE BURKITT LYMPHOMA (BL) GENOME. CIRCOS diagram shows whole genome landscape of BL in regard to SNVs, gene amplifications and deletions, and gene fusions. Outer rings depict most common SNVs at gene level stratified by rate of mutation found in BL cases and chromosome coordinate locations are denoted by color/number. Inner ring and links show most common gene fusions including *MYC* and *IG* partners. *(Reproduced from Panea RI, et al. The whole-genome of Burkitt lymphoma. Blood. 2019;134(19):1598–1607.)*

activated B cells, including NFκB signaling, and those associated with cell cycle, proliferation, and the tumor microenvironment. A study involving a similar cohort showed that a pattern of 17 miRNAs was differentially expressed between translocation positive and negative phenotypes. A group of five miRNAs was found to be downregulated in the t(14;18) group, and this correlated to overexpression of genes related to proliferation, apoptosis, and differentiation. Thus evidence for a post-GCB origin of t(14;18) translocation–negative FLs exists at the gene expression level. Further work is needed to fully understand how BCL2-negative FL develops.

Similar work with BCL2-positive FL has revealed the additional steps required for FL development after t(14;18). Conventional cytogenetic studies in FL reveal recurrent duplications of 1p36 and 6q and gains of chromosomes 2, 8, 17, 21, and X. Additionally, SHM appears to affect glycosylation status of surface Ig, implicating BCR signaling in FL pathogenesis. As in DLBCL, FL is characterized by frequent mutations in genes involved in epigenetic regulation of gene expression including ARID1A, MLL3, and CREBBP as well as other genes involved in cellular proliferation and avoidance of apoptosis such as *TP53*, *MCL1*, and *TNFRSF14*.

Further genomic changes and host factors have also been delineated for DLBCL arising from FL. Histologic transformation is associated with further mutations or deletions in *TP53* and *p16* and appears to occur by distinct mechanisms involving either clonal evolution or by transformation of a putative progenitor FL stem cell. Acquired rearrangement of the MYC oncogene can also occur and has been associated with an aggressive plasmablastic phenotype. Stromal factors with FL tumors also likely play a key role in FL propagation and have been studied as having prognostic consequence in FL as well.

Tumor Microenvironment and Survival in Follicular Lymphoma

Just as the lymphoid microenvironment is integral to the formation and function of GCs and immune factors predict survival in DLBCL, FL development appears to depend on stromal factors and the host immune response. In the largest study examining this relationship Dave et al. performed gene expression analysis using whole-genome microarrays on 191 FL specimens with the goal of determining genomic predictors of survival. Genes identified were stratified into two groups based on correlation of expression with patient outcome (gene sets associated with good prognosis or poor prognosis). Hierarchical clustering then identified five survival gene sets within each of these groups; analysis revealed that a combination of two gene sets (immune response-1 and immune response-2) formed the best model for prediction of survival. Interestingly, immune response-1

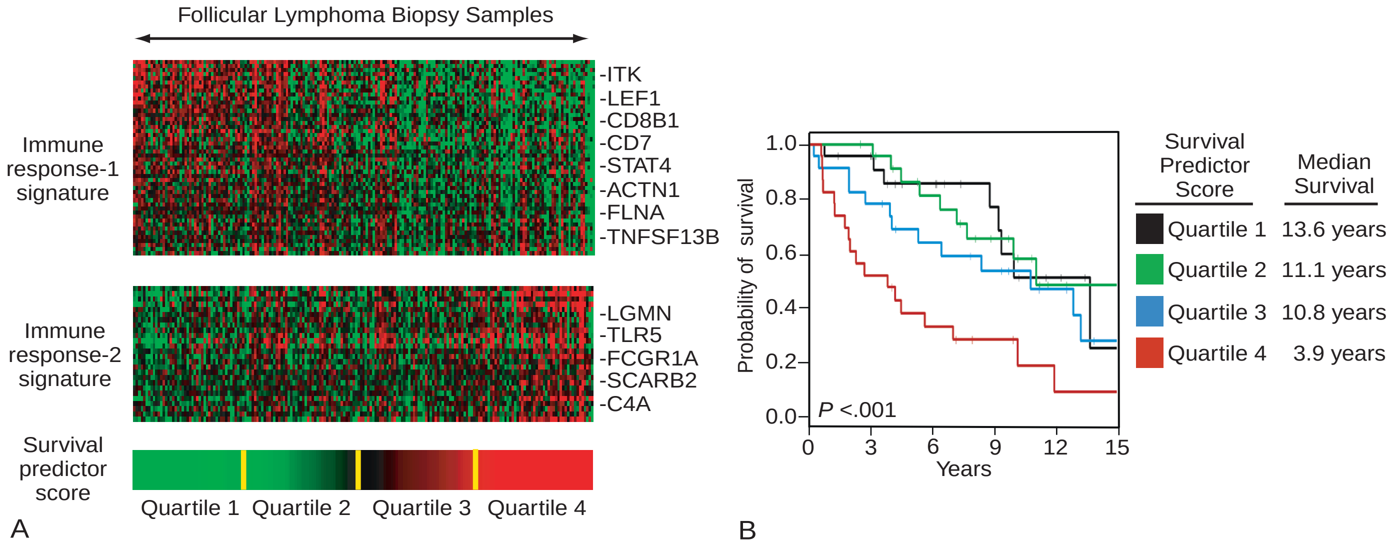


Figure 81.10 SURVIVAL IN FOLLICULAR LYMPHOMA (FL) CAN BE PREDICTED USING FEATURES OF THE TUMOR MICROENVIRONMENT. (A) Two sets of coordinately expressed genes, termed the *immune response-1* and *immune response-2 signatures*, are associated with survival in FL. The expression pattern of each gene in these two signatures is shown for FL biopsy samples. Expression of the immune response-1 signature is associated with favorable survival after diagnosis, and expression of the immune response-2 SIGNATURE is associated with adverse survival. These signatures are combined into a multivariate model of survival that generates a survival predictor score for each patient. Patients are ranked according to this survival predictor and divided into four equal quartiles as shown. (B) Kaplan-Meier plot of overall survival of patients in the four quartiles of the survival predictor. *(A, From Dave SS, Wright G, Tan B, et al. Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. N Engl J Med. 2004;351:2159.)*

consists of genes (*CD7, CD8B1, ITK, LEF1, STAT4*) associated with specific T-cell populations and macrophages. Conversely, immune response-2 consisted of genes found in dendritic cells and macrophages and was devoid of genes expressed by T-cell subsets (Fig. 81.10). Comparison with T-cell genes suggested a complex relationship with immune response-1 rather than a simple preponderance of T cells in the tumor biopsy.

Several hypotheses can be generated regarding the implication of these findings in FL biology. Given the relationship of genes involved in the immune response to survival, a direct impact of T-cell effector subsets in FL tumors could be one conclusion. Conversely, poor-risk tumors may be those that have become independent of FL follicles and therefore are more aggressive; this may be reflected in the variability of the immune response gene signatures seen across FLs. The impact of immune factors has been seen in other studies as well because survival may depend on FoxP3+ regulatory T-cell subsets, infiltration of macrophages, and the peripheral total monocyte count. Further work is needed to more clearly identify how the FL microenvironment impacts FL development and progression and to clarify whether manipulation of the immune compartment may be used therapeutically.

MANTLE CELL LYMPHOMA

Mantle cell lymphoma (MCL) is characterized by the chromosomal translocation t(11;14)q13;q32, which places cyclin D1 under the transcriptional control of the IgH promoter. MCL is predominantly a disease of elderly men and is characterized by a rather short median survival of 5 to 7 years. Histologic evaluation divides MCL into classic and blastoid variants, with the blastoid subtype having worse survival. Subgroups can also be defined on the basis of somatic mutations in the IgH loci because Ig-mutated tumors tend to have a more indolent disease as is the case with chronic lymphocytic leukemia (CLL), another CD5$^+$ neoplasm. This suggests two disparate origins for MCL with one subtype arising from pre-GC B lymphocytes and another stemming from cells that have encountered antigen and consequently have undergone SHM. Genomic approaches have also identified molecular profiles delineating MCL from histologically similar neoplasms and identified factors associated with survival. Notably, a minority of patients with MCL are cyclin D1-negative, and GEP has been useful in identifying this entity; further work in this area has identified numerous genomic lesions and molecular pathways involved in MCL. Herein the pathogenesis of MCL is reviewed focusing on the genomic and molecular basis of this disease.

Cyclin D1 and Mantle Cell Lymphoma

The genetic hallmark of MCL, the t(11;14) (q13; q32) translocation, juxtaposes the *CCND1* gene to the IgH locus, leading to overexpression of the cell cycle regulator cyclin D1. The three D-type cyclins (D1, D2, and D3) play an important role in cellular proliferation by propelling cells from G_1 to S phase of the cell cycle. Each forms heterodimers with the cyclin-dependent kinases CDK4 and CDK6, thus forming active kinase complexes. These complexes inactivate retinoblastoma protein (Rb) and bind to p27kip1, which functions to facilitate cell entry into the S phase of the cell cycle and consequent proliferation.

Cyclin D1 is not expressed in normal lymphocytes, and t(11;14) thus represents a pathogenic event in the origin of MCL. The gene encoding cyclin D1 *(CCND1)* consists of five exons that are alternatively spliced into two isoforms, cyclin D1a and D1b. Cyclin D1b does not appear to play a role in MCL. The proliferative capacity of MCL tumors appears closely tied to the degree of cyclin D1a overexpression. MCLs carry mutations in the 3′ untranslated region of *CCND1* that serve to stabilize cyclinD1a transcripts by removing miRNA (miR15/16) binding sites and deleting mRNA destabilizing elements. These sequences usually result in cyclin D1a being an unstable entity, with a half-life of less than 1 hour, but in MCL, these deletions or mutations result in cyclin D1a accumulation and increased cellular proliferation. Additional mutations in the translated regions of cyclinD1 result in protein stabilization through the blockade of GSK3β-mediated nuclear export of cyclin D1.

A minority of patients with MCL are cyclin D1 negative and are alternatively characterized by overexpression of cyclin D2 or D3. These derangements serve to deregulate the G_1–S transition in a manner similar to t(11;14) and highlight the theme that MCL is a disease created by cyclin complex–mediated cell cycle progression aided by upregulation of several molecular pathways associated with cellular proliferation and genomic instability.

Secondary Genomic Alterations in Mantle Cell Lymphoma

Beyond t(11;14), MCL is characterized by extensive genomic instability relative to other NHLs, and a variety of genetic lesions appears to drive proliferation of these tumors. Recurrent chromosomal losses, gains, and amplifications are seen in MCL, and many of these affect a large proportion of cases. Secondary genetic instability may occur as a result of aberrant DNA replication in the setting of deregulated S phase transition mediated by cyclinD1–CD4^{-}complexes. Acquired lesions in genes mediating cellular response to DNA damage and microtubule dynamics may contribute to the accumulation of these alterations as well.

Many (30% to 50%) of MCL cases are characterized by mutations or deletions in the DNA damage response pathway mediated by ATM and TP53 or modifiers of this pathway such as MDM2 and p14/ARF. *ATM* is often mutated with loss of the other chromosomal alleles. TP53 may also be downregulated in these tumors via similar mechanisms, and overexpression of TP53-negative regulators MDM2 and MDM4 also occurs via copy number amplification (examples of MCL histology with cyclin D1 and p53 staining are shown in Fig. 81.11). These overall serve to deregulate important cellular machinery involved in DNA damage repair, apoptosis, and cell cycle arrest, leading to increased cell cycle progression and proliferation in MCL. Further deletions at 9p21 occur frequently in MCL and serve to enhance cell cycle progression and block apoptosis. The 9p21 locus encodes p16(INK4a) and p14(ARF), which function as tumor suppressors. p16(INK4a) inhibits CDK 4/6 complex binding to cyclin D1, which results in decreased cell cycle progression. Deletion of p16(INK4a) in the setting of t(11;14) enhances proliferation in MCL and has been associated with worse overall survival. The function of p14(ARF) in MCL pathogenesis is less clear, but p14(ARF) blocks MDM2-mediated TP53 ubiquitination; p14(ARF) has other effects on cellular proliferation that usually work in a homeostatic pattern. Interestingly, cyclin D1 levels and INK4a/ARF deletions both correlate to a MCL proliferation signature created using GEP. Using a similar strategy, cyclin D1 expression levels and the presence of INK4a/ARF deletion have been used to create a predictor of overall survival using a cohort of MCL patients. Survival among groups stratified by this approach varies widely (from 6 to 7 years to <10 months), which confirms the importance of involvement of genes involved with the cell cycle and proliferation in MCL. This parallels the experience noted with histologic markers of tumor proliferation because Ki-67 (a marker of actively dividing cells) and the presence of blastoid features have previously been shown to correlate with worse outcomes.

When considered together, the recurrent deletions and mutations noted in MCL illustrate the importance of unchecked cell cycle progression in this disorder. DNA instability is another hallmark of MCL that appears to result from the blockade of cell cycle checkpoints in the setting of ongoing DNA damage. These factors are intertwined in a complicated fashion in MCL and further work to upregulate several diverse molecular pathways involved in cellular proliferation and survival.

Molecular Pathways and Profiles in Mantle Cell Lymphoma

A variety of molecular pathways have recently been described as being active in MCL. These include signaling pathways facilitating cellular proliferation, avoidance of apoptosis, evasion of immune editing, and cellular microenvironment interactions. As in other NHLs, GEP has been used to define a molecular diagnosis of MCL and identify genes and molecular pathways involved in its pathogenesis.

Genomic approaches identify a subset of MCL that is cyclin D1 negative and has features similar to other CD5+ B-cell neoplasms such as CLL. Although such cases are indistinguishable by histologic analysis, GEP demonstrates that these tumors express gene signatures that are otherwise nearly identical to cyclin D1+ MCL but lack the classic t(11;14) rearrangement. Overexpression of other D cyclins (cyclin D2 or D3) via rearrangement, amplification, or other mechanisms is found in many of these cases; this is consistent with the underlying theme that MCL is a disease of disordered cell cycle progression. Using a similar approach, *SOX11* has been identified as a sensitive and specific marker of MCL regardless of cyclinD1 expression status. *SOX11* encodes an HMG-box transcription factor that regulates embryogenesis and is important in neural development. Because greater than 90% of MCL specimens overexpress *SOX11* and this finding is rare in histologically similar NHLs, *SOX11* is a potentially useful marker for improving the diagnosis of MCL. *SOX11* has also been incorporated into prognostic models of MCL using histologic, clinical, and GEP data. Reports regarding the clinical significance of *SOX11* have been conflicting. Thus further work is needed to understand the molecular mechanisms underlying *SOX11* expression in MCL and the implications in this disease.

MCL has also been characterized by expression of several molecular pathways that enhance cellular proliferation and metabolism and are tightly intertwined with abnormalities in cell cycle regulation

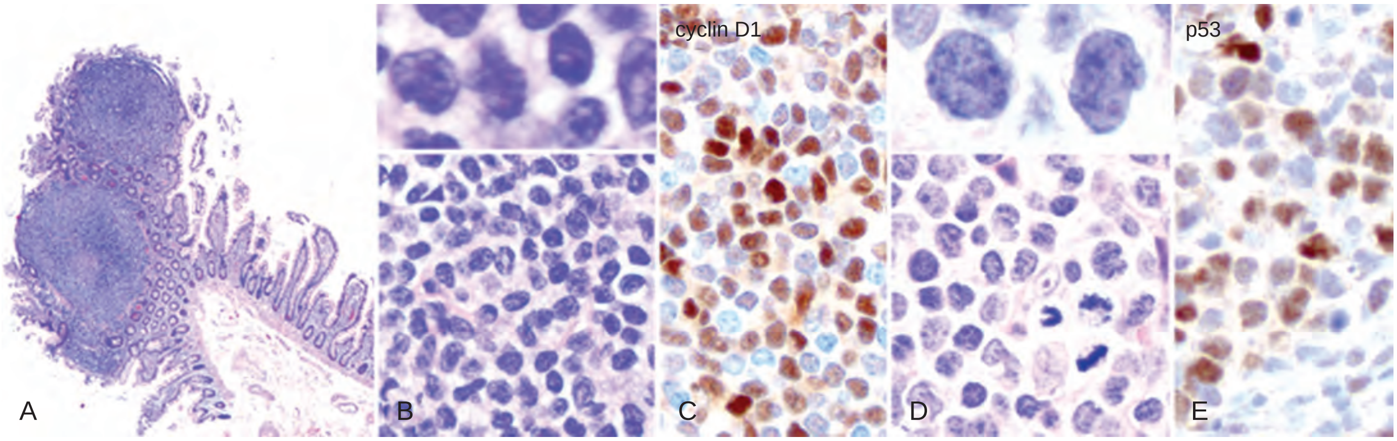


Figure 81.11 MANTLE CELL LYMPHOMA (MCL). (A) A case of MCL presenting as lymphomatoid polyposis. (B) The lymphoma cells are small to intermediate in size and have irregular nuclear contours and condensed nuclear chromatin. The cells were CD19^{+}, monoclonal B cells with λ light chain restriction, coexpression of CD5, FMC-7, and lack of CD23. (C) The cells showed cyclin D1 expression by immunohistochemical staining. The t(11;14)(q13;q32) was detected by conventional cytogenetic analysis. (D) In comparison, the blastoid variant of MCL has larger cells with a high mitotic rate and fine chromatin. (E) These cases frequently overexpress p53 and are associated with a complex karyotype, including t(11;14).

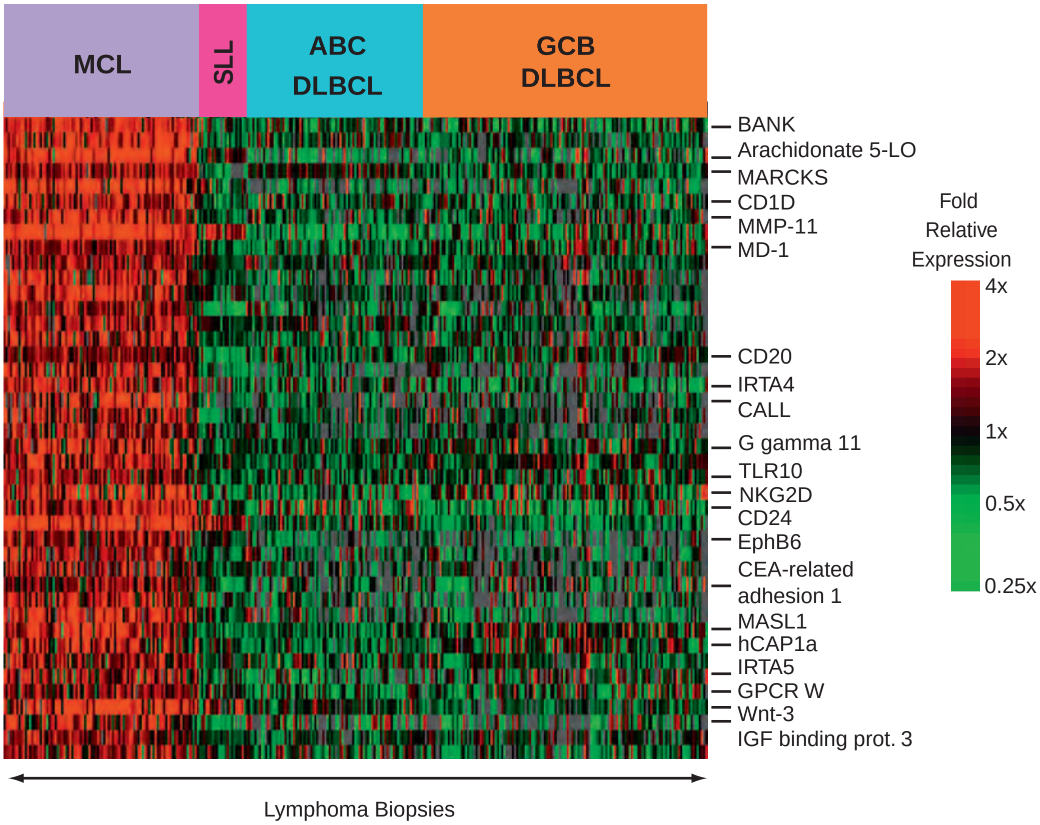


Figure 81.12 HIERARCHICAL CLUSTERING OF EXPRESSION MEASUREMENTS FROM 42 MANTLE CELL LYMPHOMA (MCL) SIGNATURE GENES THAT ARE MORE HIGHLY EXPRESSED IN MCL SAMPLES COMPARED WITH SMALL LYMPHOCYTIC LYMPHOMA (SLL), ACTIVATED B-CELL–LIKE (ABC) DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL), AND GERMINAL CENTER B (GCB) DLBCL SAMPLES. Each column represents a single lymphoma specimen, and each row represents expression of a single gene. *Red squares* indicate increased expression, and *green squares* indicate decreased expression relative to the median expression level according to the color scale shown. *(Reproduced from Fu K, Weisenburger DD, Greiner TC, et al. Cyclin D1-negative mantle cell lymphoma: a clinicopathologic study based on gene expression profiling. Blood. 2005;106:4315; and Rosenwald A, Wright G, Wiestner A, et al. The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma. Cancer Cell. 2003;3:185.)*

(Fig. 81.12). Whole exome/genome/transcriptome sequencing also reflects the idea that genomic alterations collaborate to upregulate activity of cell cycle machinery in MCL as loss of function alterations in *RB*, *ATM*, and *TP53* are found frequently in addition to cyclin D1 overexpression. *Wnt 3* is a consistently overexpressed gene in MCL, and abnormal Wnt signaling may be one way in which MCLs bypass negative feedback loops controlling differentiation. Proliferation in MCLs may also depend on Wnt–B-catenin signaling, but the impact of this pathway on patient survival or as a therapeutic target in MCL is unknown. Similar to ABC DLBCLs, the NFκB pathway is also active in MCL, and crosstalk between proliferation signaling between this pathway PI3K/AKT/mTOR and cell cycle signaling also appears to be important. Importantly, phosphatase and tensin homologue (PTEN), a molecule that has important roles in providing negative feedback to the mTOR pathway is also commonly deleted or acquires loss of function mutations in MCL. Taken together, these facts reinforce the fact that abnormalities in several diverse signaling pathways are required beyond t(11;14) for MCL to form. Further work is needed to define how to use these findings to build better prognostic models in this disease, and studies investigating the therapeutic utility of targeting these pathways are beginning.

OTHER NON-HODGKIN LYMPHOMAS

GEP and high-throughput sequencing have been applied to other lymphomas in manners similar to the subtypes already described. Important discoveries regarding other NHLs such as marginal zone lymphomas, lymphoplasmacytic lymphoma, and peripheral T-cell lymphomas (PTCLs), have refined our understanding of how these diseases develop (Fig. 81.13). Recurrent mutations in genes and involvement of molecular pathways governing control of apoptosis, cellular proliferation, and metabolism are recurrent themes in these NHL subtypes as well as those described earlier. In addition to being found in DLBCLs, *MYD88* L265P mutations have been reported in virtually all cases of lymphoplasmacytic lymphoma (Waldenström macroglobulinemia) and may serve (along with CXCR4 mutations) as a marker to differentiate this disease from other indolent lymphomas. *NOTCH2* mutations have been noted in a subset of marginal zone lymphomas and have been found in DLBCLs and other NHLs. Gene clusters have also been developed for T-cell lymphomas and identify PTCL subtypes such as angioimmunoblastic T-cell lymphoma (AITL) and hepatosplenic T-cell lymphoma as distinct molecular entities as compared to other PTCLs; this work also has shown upregulation of genes involved in NFκB signaling in AITL. A subset of PTCL is characterized by t(2;5) (p23;q35), which joins the anaplastic lymphoma kinase-1 gene *(ALK)* next to *NPM-1* at 5q35. This creates an aberrant tyrosine kinase that appears to have a number of downstream transforming effects. ALK positivity is generally associated with response to cytotoxic chemotherapy in PTCL; however, better molecular predictors of response are needed. Whereas NFκB gene signature expression also correlates to improved survival in PTCL, tumors that exhibit increased markers of proliferation are associated with poor outcomes, as in other NHLs. High-throughput genomic sequencing approaches have also revealed distinct patterns of somatic tumor mutations in PTCL subsets as compared to B-cell lymphomas. A recurrent mutation in *RHOA* (*RHOA* G17V), a small

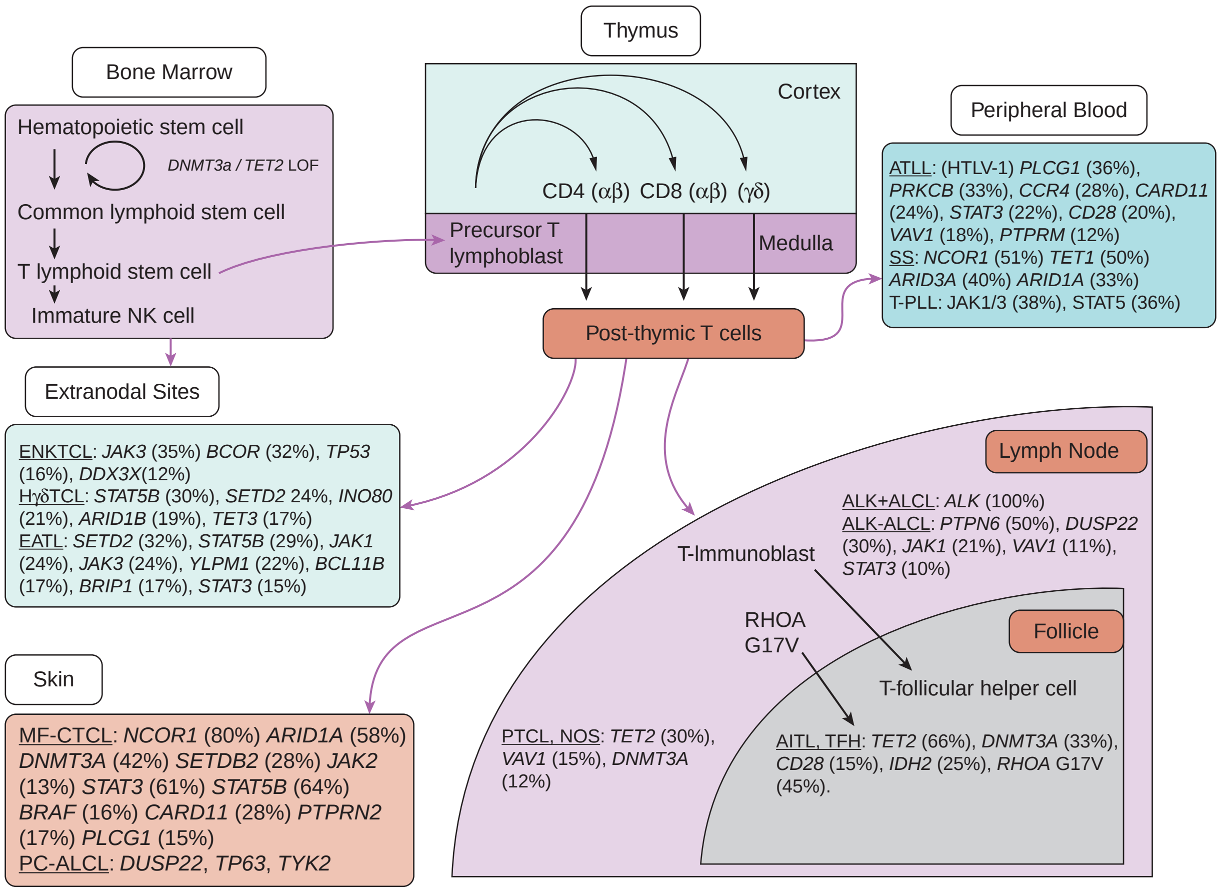

Bone Marrow
Hematopoietic stem cell
DNMT3a / TET2 LOF
Common lymphoid stem cell
T lymphoid stem cell
Immature NK cell
Thymus
Cortex
CD4 (αβ) CD8 (αβ) (γδ)
Precursor T lymphoblast
Medulla
Post-thymic T cells
Peripheral Blood
ATLL: (HTLV-1) PLCG1 (36%), PRKCB (33%), CCR4 (28%), CARD11 (24%), STAT3 (22%), CD28 (20%), VAV1 (18%), PTPRM (12%)
SS: NCOR1 (51%) TET1 (50%) ARID3A (40%) ARID1A (33%)
T-PLL: JAK1/3 (38%), STAT5 (36%)
Extranodal Sites
ENKTCL: JAK3 (35%) BCOR (32%), TP53 (16%), DDX3X(12%)
HγδTCL: STAT5B (30%), SETD2 24%, INO80 (21%), ARID1B (19%), TET3 (17%)
EATL: SETD2 (32%), STAT5B (29%), JAK1 (24%), JAK3 (24%), YLPM1 (22%), BCL11B (17%), BRIP1 (17%), STAT3 (15%)
Skin
MF-CTCL: NCOR1 (80%) ARID1A (58%) DNMT3A (42%) SETDB2 (28%) JAK2 (13%) STAT3 (61%) STAT5B (64%) BRAF (16%) CARD11 (28%) PTPRN2 (17%) PLCG1 (15%)
PC-ALCL: DUSP22, TP63, TYK2
Lymph Node
T-Immunoblast
ALK+ALCL: ALK (100%)
ALK-ALCL: PTPN6 (50%), DUSP22 (30%), JAK1 (21%), VAV1 (11%), STAT3 (10%)
RHOA G17V
Follicle
T-follicular helper cell
PTCL, NOS: TET2 (30%), VAV1 (15%), DNMT3A (12%)
AITL, TFH: TET2 (66%), DNMT3A (33%), CD28 (15%), IDH2 (25%), RHOA G17V (45%).

Figure 81.13 T-CELL ONTOGENESIS AND COMMON MOLECULAR DRIVERS IN T-CELL LYMPHOID NEOPLASIA SUBTYPES THAT MIRROR CELL TYPE OF ORIGIN. Analogous to B-cell development and lymphoid malignancies that mirror underlying cell subsets, T-cell lymphoid malignancies can be conceptualized as subtypes that arise of cells at various stages of T-cell development and differentiation with each having distinct major drivers and phenotypes. Most of these entities are thought to arise from cells that have completed peripheral thymic migration and differentiation. *(Reproduced from Van Arnam JS, Lim MS, Elenitoba-Johnson KSJ. Novel insights into the pathogenesis of T-cell lymphoma. Blood. 2018;131(121):2320–2330.)*

GTPase involved in regulation of actin polymerization and stress fiber formation occurs in both PTCL subtypes and AITL and appears to correlate with gene signatures of NF-κB and MAP kinase activation, although the exact oncogenic role of RHOA in these tumors is unknown. Additionally, activating mutations in mediators of T-cell receptor (TCR) signaling including FYN kinase have been reported and point toward an oncogenic role for constitutively active TCR signaling in these lymphomas. Further studies are needed to define the roles of these mutations in T-cell lymphogenesis.

FUTURE DIRECTIONS

NHL represents a complex group of related neoplasms that have quite disparate molecular characteristics. Given the lack of modifiable risk factors and varied prognosis with standard treatment (chemoimmunotherapy), a better understanding of the processes driving NHL is needed. Advances in technology have recently allowed whole-genomic surveys to identify the role of altered genetics, transcription, and epigenetic regulation as contributors to the observed lymphoma phenotypes. This work will serve as the starting point for the unraveling of new diagnostic and prognostic markers, as well as therapeutic targets in a group of diseases in which such options are urgently needed.

SUGGESTED READINGS

Alizadeh AA. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature*. 2000;403:503.

Bakhshi A, Jensen JP, Goldman P, et al. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell*. 1985;41:899.

Bea S, Zettl A, Wright G, et al. Diffuse large B-cell lymphoma subgroups have distinct genetic profiles that influence tumor biology and improve gene-expression-based survival prediction. *Blood*. 2005;106:3183.

Chapuy B, Stewart C, Dunford AJ, et al. Molecular subtypes of diffuse large B cell lymphoma are associated with distinct pathogenic mechanisms and outcomes. *Nat Med*. 2018;24(5):679–690.

Dave SS, Fu K, Wright GW, et al. Molecular diagnosis of Burkitt's lymphoma. *N Engl J Med*. 2006;354:2431.

Dave SS, Wright G, Tan B, et al. Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. *N Engl J Med*. 2004;351:2159.

de Boer CJ, van Krieken JH, Kluin-Nelemans HC, et al. Cyclin D1 messenger RNA overexpression as a marker for mantle cell lymphoma. *Oncogene*. 1995;10:1833.

Dölken G, Illerhaus G, Hirt C, et al. BCL-2/JH rearrangements in circulating B cells of healthy blood donors and patients with nonmalignant diseases. *J Clin Oncol*. 1996;14:1333.

Fisher SG, Fisher RI. The epidemiology of non-Hodgkin's lymphoma. *Oncogene*. 2004;23:6524.

Fu K, Weisenburger DD, Greiner TC, et al. Cyclin D1-negative mantle cell lymphoma: a clinicopathologic study based on gene expression profiling. *Blood*. 2005;106:4315.

Gracias DT, Katsikis PD. MicroRNAs: key components of immune regulation. *Adv Exp Med Biol*. 2011;780:15.

Iqbal J. Distinctive patterns of BCL6 molecular alterations and their functional consequences in different subgroups of diffuse large B-cell lymphoma. *Leukemia*. 2007;21:2332.

Iqbal J, Neppalli VT, Wright G, et al. BCL2 expression is a prognostic marker for the activated B-cell-like type of diffuse large B-cell lymphoma. *J Clin Oncol*. 2006;24:961.

Iqbal J, Wright G, Wang C, et al. Gene expression signatures delineate biological and prognostic subgroups in peripheral T-cell lymphoma. *Blood*. 2014;123(19):2915–2923.

Jardin F, Gaulard P, Buchonnet G, et al. Follicular lymphoma without t(14;18) and with BCL-6 rearrangement: a lymphoma subtype with distinct pathological, molecular and clinical characteristics. *Leukemia*. 2002;16:2309.

Jost PJ, Ruland J. Aberrant NF-kappaB signaling in lymphoma: mechanisms, consequences, and therapeutic implications. *Blood*. 2007;109:2700.

Küçük C, Jiang B, Hu X, Zhang W, et al. Activating mutations of STAT5B and STAT3 in lymphomas derived from γδ-T or NK cells. *Nat Commun*. 2015;6:6025.

Leich E, Zamo A, Horn H, et al. MicroRNA profiles of t(14;18)-negative follicular lymphoma support a late germinal center B-cell phenotype. *Blood*. 2011;118:5550.

Lenz G, Nagel I, Siebert R, et al. Aberrant immunoglobulin class switch recombination and switch translocations in activated B cell-like diffuse large B cell lymphoma. *J Exp Med*. 2007;204:633.

Lenz G, Wright G, Dave SS, et al. Stromal gene signatures in large-B-cell lymphomas. *N Engl J Med*. 2008;359:2313.

Magrath I. Epidemiology: clues to the pathogenesis of Burkitt lymphoma. *Br J Haematol*. 2012;156:744.

McHeyzer-Williams M, Okitsu S, Wang N, et al. Molecular programming of B cell memory. *Nat Rev Immunol*. 2011;12:24.

Morin RD, Mendez-Lago M, Mungall AJ, et al. Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. *Nature*. 2011;476:298.

Pasqualucci L. Inactivation of the PRDM1/BLIMP1 gene in diffuse large B cell lymphoma. *J Exp Med*. 2006;203:311.

Reddy A, Zhang J, Davis NS, Moffitt AB, et al. Genetic and functional drivers of diffuse large B cell lymphoma. *Cell*. 2017;171(2):481–494.

Rosebeck S, Rehman AO, Lucas PC, et al. From MALT lymphoma to the CBM signalosome: three decades of discovery. *Cell Cycle*. 2011;10:2485.

Rosenwald A. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. *N Engl J Med*. 2002;346:1937.

Rosenwald A, Wright G, Leroy K, et al. Molecular diagnosis of primary mediastinal B cell lymphoma identifies a clinically favorable subgroup of diffuse large B cell lymphoma related to Hodgkin lymphoma. *J Exp Med*. 2003;198:851.

Rosenwald A, Wright G, Wiestner A, et al. The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma. *Cancer Cell*. 2003;3:185.

Roschewski M, Staudt LM, Wilson WH. Diffuse large B-cell lymphoma-treatment approaches in the molecular era. *Nat Rev Clin Oncol*. 2014 Jan;11(1):12–23.

Schmitz R, Wright GW, Huang DW, et al. Genetics and pathogenesis of diffuse large B-cell lymphoma. *N Engl J Med*. 2018;378(15):1396–1407.

Schaffner C, Idler I, Stilgenbauer S, et al. Mantle cell lymphoma is characterized by inactivation of the ATM gene. *Proc Natl Acad Sci U S A*. 2000;97:2773.

Tam W. Mutational analysis of PRDM1 indicates a tumor-suppressor role in diffuse large B-cell lymphomas. *Blood*. 2006;107:4090.

Volpe G, Vitolo U, Carbone A, et al. Molecular heterogeneity of B-lineage diffuse large cell lymphoma. *Genes Chromosomes Cancer*. 1996;16:21.

Wang LD, Clark MR:. B-cell antigen-receptor signalling in lymphocyte development. *Immunology*. 2003;110:411.

Weinstock DM, Dalla-Favera R, Gascoyne RD, et al. A roadmap for discovery and translation in lymphoma. *Blood*. 2015;125(13):2175–2177.

CHAPTER 82

CLINICAL MANIFESTATIONS, STAGING, AND TREATMENT OF FOLLICULAR LYMPHOMA

Lucy Pickard and John G. Gribben

Non-Hodgkin lymphoma (NHL) refers to all malignancies of the lymphoid system with the exception of Hodgkin lymphoma. Development of the lymphoid system is a highly regulated process, characterized by differential expression of a number of cell-surface and intracytoplasmic proteins and antigen receptor gene rearrangements, somatic hypermutation, and class switching. Dysregulation of this orderly process can result in humoral deficiency, autoimmunity, or malignancy. The indolent B-cell lymphomas are mature peripheral B-cell neoplasms that exclude those diseases associated with an aggressive clinical course. Despite differences in cell of origin, molecular biology, clinical presentation, and clinical course, the indolent lymphomas share common features, including frequent localization to the principal lymphoid organs, a propensity for bone marrow (BM) infiltration and leukemic presentation, and generally an indolent clinical course. The classification of NHLs has been a challenge for pathologists as well as practicing physicians. A number of classifications have been proposed over the years, leading to considerable confusion and difficulty in comparison of outcomes of clinical trials performed using different pathologic classifications. The World Health Organization (WHO) lymphoma classification lists nearly 100 different types of lymphoid neoplasm.[1] This classification uses all available information such as morphology, cytochemistry, immunophenotype, and molecular genetics as well as clinical features. The WHO classification does not include the terminology *indolent lymphoma*. This is a clinical and not a pathologic term and defines those lymphomas that tend to grow and spread slowly and produce few symptoms. Indolent lymphomas represent 35% to 40% of NHLs, and follicular lymphoma (FL) is the most common of the indolent lymphomas.

EPIDEMIOLOGY

It is estimated that in 2021 more than 81,560 cases of NHL will be diagnosed in the United States, and more than 20,000 patients will die of their disease, making NHL the seventh most common cancer and the fifth most common cause of death from cancer (http://seer.cancer.gov/statfacts/html/nhl.html). NHL is extremely heterogeneous in its molecular pathophysiology, histology, and clinical course, and there are major differences in the incidence of subtypes in different geographic locations and among different racial and ethnic populations. This difference in geographic distribution is particularly striking for FL, and in the Western world, FL is the second most common lymphoma, comprising approximately 20% of all NHLs. The incidence of FL in the United States is approximately 2.7 cases per 100,000 persons per year and in Europe is approximately 3.14 cases per 100,000 persons per year.[2] The incidence appears stable over time. There is no strong sex preponderance. The incidence in Whites is approximately twice that of Black and Asian populations, and the disease appears less common in Central and South America. The incidence increases with age, and the median age of diagnosis is 65 years. Although most cases are sporadic, there is an increased incidence in family members of affected individuals, with a relative risk of 2.3 for siblings of patients.[3] Genome-wide association studies provide evidence for FL inheritance susceptibility. Multiple independent single nucleotide polymorphisms in human leucocyte antigen (HLA) class I and II genes on chromosome 6p21.3 were associated with susceptibility to FL.[4] Furthermore, FL risk was significantly associated with homozygosity at the HLA class II gene loci.[5]

Differentiation of complex environmental factors from true inherited factors remains difficult. The complexity of the epidemiology of NHL mirrors the complexity of the disease and that of the host immune system. Since lymphomas do not constitute a single disease, it should come as no surprise that there is no single etiologic factor. The influence on immune dysregulation of viruses, chemicals, radiation, diet, and aging remains unclear. Immune suppression leads to increased incidence of aggressive lymphomas but not usually indolent lymphomas.

PATHOGENESIS

FL (Fig. 82.1) is a neoplasm derived from germinal center (GC) B cells underlined by: histological appearance, with predominant follicular morphology and maintenance of GC architecture; immunophenotypical behavior, with FL B cells expressing GC markers BCL6$^+$ and CD10$^+$; and molecular characteristics, with evidence of ongoing class-switch recombination and somatic hyper mutation.[6] Bulk gene expression profiles of FL are closely related to normal GC light zone B cells,[7] therefore, the cell of origin is assumed to correspond with a GC B cell blocked at that stage of differentiation. A recent study examining cells at single cell resolution has shown that a continuum of dynamic cell states coexist within single patient biopsies, suggesting that FL cells have functional plasticity[8] as opposed to being “frozen” at a particular GC stage.

A hallmark of FL is the chromosomal translocation t(14;18)(q32;q21), placing the B cell lymphoma 2 (*BCL2*) gene under transcriptional control of the immunoglobulin heavy chain (*IGH)* locus, resulting in overexpression of the antiapoptotic protein BCL2.[9] Morphologically, the disease is composed of a mixture of centrocytes and centroblasts. The WHO third edition pathologic classification recommends grading in grades 1 to 3 according to the number of centroblasts (0 to 5, 6 to 15, and >15 per high-power field, respectively). Grade 3 was further subdivided into 3A (centrocytes still present) and 3B (sheets of centroblasts). An increased percentage of centroblasts is predictive of poor outcome. A problem with this classification is that it is poorly reproducible among pathologists. It is also clear that there are no major biologic or clinical differences between grades 1 and 2—whereas grade 3B FL is biologically distinct from grades 1 to 3A—and clinical and histological features suggest a closer relationship to diffuse large B cell lymphoma (DLBCL).[10,11] These considerations led to the recommendation to group together FL grade 1 to 3A as FL and create a new category called FL3B. The final classification combined FL grades 1 and 2 into one category (FL1–2 of 3) and made the distinction between FL3A and FL3B optional rather than mandatory.[12] The fourth edition of the WHO classification recognizes some distinctive clinical and genetic subtypes of FL, including primary duodenal FL and the pediatric type of FL that lacks the t(14;18) translocation and usually presents with localized disease. Several variants of FL that lack the t(14;18) have some distinctive

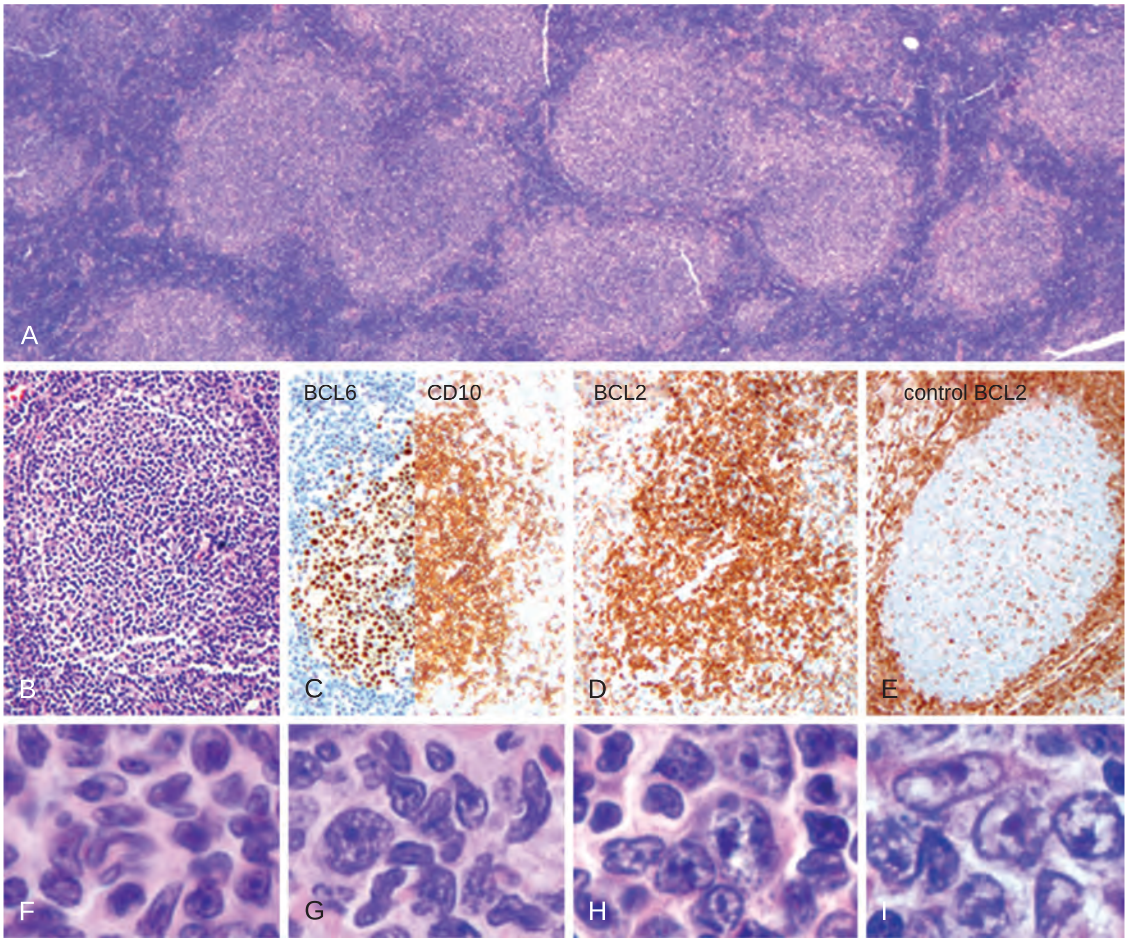


Figure 82.1 FOLLICULAR LYMPHOMA: MORPHOLOGIC AND IMMUNOPHENOTYPIC FINDINGS. (A) Low-power photomicrograph illustrates a lymph node involved by follicular lymphoma. The lymphoma cells grow in nodules or follicles that resemble the normal lymphoid follicles of a reactive lymph node. However, in the lymphomatous growth, the follicles are crowded, show back-to-back localization, and lack many of the features of their reactive counterparts. At higher power (B), the neoplastic follicles lack mantle zones, and the normal polarization of small and large germinal center cells (centrocytes and centroblasts, respectively), which occurs because of the cellular response to antigenic stimulation as it sweeps thorough the follicle. (C) The neoplastic follicles stain for the germinal center markers, BCL6 and CD10; however, they overexpress BCL2 (D) caused by the associated translocation t(14;18) involving the *IgH* gene and *BCL2*. BCL2 is not much expressed in normal germinal center B cells (E, control for comparison). Follicular lymphoma is graded by the number of large neoplastic cells (centroblasts) present among the smaller neoplastic cells (centrocytes) (F–I). The grading system is not entirely accurate, but provides some framework for subclassifying cases morphologically. Only 0 to 5 centroblasts per average field is grade 1 (F); between 6 and 15 is grade 2 (G); and greater than 15 is grade 3 A (H). Grades 1 and 2 are now considered together. When most of the cells in the neoplastic follicles are centroblasts without centrocytes the case is considered grade 3B (I) (see text).

features, e.g., predominantly diffuse FL with deletions of 1p36 that presents with localized bulky disease in the inguinal region.[13] A characteristic finding of t(14;18)-negative FL is an enrichment of activating mutations in the transcription factor *STAT6*, occurring in 57% of cases, compared with 10% in t(14;18) positive FL. *STAT6* mutations co-occurred with mutations in *TNFRSF14* and/or *CREBBP*, potentially revealing alternative oncogenic mechanisms in this FL subtype.[14]

FL lymphomagenesis is a complex multistep process with the acquisition of the t(14;18)(q32.3;q21.3) reciprocal translocation, generally considered the first hit in the oncogenic cascade.[15] However, there are multiple lines of evidence that t(14;18) in isolation is insufficient to propagate malignant transformation. Firstly, the translocation can be detected at low levels in the blood of healthy individuals.[16] Secondly, *BCL2* overexpression mouse models require additional genetic hits to promote overt tumor formation.[17] Finally, t(14;18) negative FL patients follow similar clinical trajectories.[13] Together, these data suggest GC B cells must acquire further oncogenic hits for complete transformation to FL.

The advent of next generation sequencing (NGS) technologies in the last decade—including whole genome, exome and targeted sequencing—have advanced our understanding of the genomic and epigenomic lesions that occur alongside the t(14;18) translocation. Compared to other malignancies, FL has an apparent “addiction” to epigenetic mutations, with greater than 90% of patients carrying one or more mutation in chromatin-modifying genes.[18–22] The histone lysine methyltransferase *KMT2D* is the most frequently mutated gene in FL affecting approximately 70% to 80% of cases.[18,20,21] Aberrations in the histone acetyltransferase (HATs) enzymes *CREBBP* and *EP300* occur in approximately 70% and 15% of FL cases, respectively.[18,20,22,23] The histone methyltransferase *EZH2* forms the catalytic component of the polycomb repressive complex 2 (PRC2) and is mutated in 20% to 25% of FL cases.[18,20,21] Other epimutations occur in genes involved in chromatin remodeling, such as *ARID1A* and the linker histones (*HIST1H1B-E*), occurring in approximately 10% and 30% of cases, respectively.[18,24]

Inactivating mutations of *KMT2D*, *CREBBP*, and *EP300*, and gain-of-function mutations of *EZH2*, result in a shift from active to repressive marks, leading to aberrant repression of gene transcription and interference with transcriptional programs that regulate normal GC B cell selection, differentiation, and exit.[25,26] Consequently, aberrant GC B cells accumulate resulting in exposure to further genomic instability, favoring their malignant transformation. Epimutations alone do not appear sufficient to initiate FL but require dysregulation of BCL2 expression to induce lymphomagenesis.[27–29] Other recurrently disrupted pathways in FL include: immune recognition (*TNFRSF14, CTSS*); BCR-NFκB (*CARD11, TNFAIP3, MYD88*); mTOR (*RRAGC, ATP6AP1, ATP6V1B2, SESTRIN1*); and JAK-STAT signaling (*STAT6, SOCS1*).[18,20–23,30–33] The major mutations seen in FL are listed in Table 82.1.[18–21,23,25,30,33–45]

TABLE 82.1 **Mutations in Follicular Lymphoma**

Gene Name	*Abbreviation*	Location	Frequency at Diagnosis (%)
Lysine methyltransferase 2D	*KMT2D*	12q	80–90
CREB binding protein	*CREBBP*	16p	33–70
Enhancer of zeste 2 polycomb repressive complex 2 subunit	*EZH2*	7q	7–25
TNF receptor superfamily member 14	*TNFRSF14*	1p	20–50
B cell lymphoma 2	*BCL2*	18q	80–90
TNF alpha induced protein 3	*TNFAIP3*	6q	5–26
Ras related GTP binding C	*RRAGC*	1p	17
ATPase H+ transporting V1 subunit B2	*ATP6V1B2*	8p	11
ATPase H+ transporting accessory protein 1	*ATP6AP1*	Xq	9.9
H1 linker histone, cluster member	*HIST1H1A-E*	6p	44
AT-rich interaction domain 1A	*ARID1A*	1p	15
Myocyte enhancer factor 2B	*MEF2B*	19p	13–15
Caspase recruitment domain family member 11	*CARD11*	7p	8–10
Signal transducer and activator of transcription 6	*STAT6*	12q	10
Suppressor of cytokine signaling 1	*SOCS1*	16p	8–27
MYD88 innate immune signal transduction adaptor	*MYD88*	3p	2–9
B cell lymphoma 6	*BCL-6*	3q	6–47
Cyclin D3	*CCND3*	6p	10–15
Interferon regulatory factor 8	*IRF8*	16q	10
Forkhead box O1	*FOXO1*	13q	8-15
Tumor protein P53	*TP53*	17p	8-31
MYC proto-oncogene, BHLH transcription factor	*MYC*	8q	17
REL proto-oncogene, NF-κB subunit	*REL*	2p	10–33
Cyclin dependent kinase inhibitor 2 A	*CDKN2A*	9p	46
Early B cell factor 1	*EBF1*	5q	7–17

FL cells exist within a tumor microenvironment milieu of nonmalignant immune, stromal, and extracellular components, and are critically reliant on micro-environmental signals for proliferation and survival. A detailed review of the complex interactions between these components is beyond the scope of this chapter, but readers can refer to specific reviews on this topic.[46,47]

CLINICAL PRESENTATION

The majority of patients present with lymphadenopathy in one or more sites. Patients with FL often present with a long history of having noted painless, enlarged lymph nodes (LN) over a number of years before presentation. Lymphadenopathy may wax and wane, and spontaneous remissions can occur, albeit rarely.[48] Disease transformation to a more aggressive histologic type confers inferior outcomes, especially early transformation.[49]

Extranodal disease is relatively common and can affect any organ. The most common sites of extranodal disease include the BM, skin, gastrointestinal tract, and bone. Symptoms may be nonspecific or related to the site of disease involvement. Many patients are asymptomatic, but some, particularly those with bulky disease may present with B symptoms defined as fever, drenching sweats, or weight loss of more than 10% of body weight. Patients may present with evidence of bowel obstruction from intraabdominal lymphadenopathy, and retroperitoneal disease may manifest as obstructive uropathy. Inguinal disease may cause compression of the venous system with deep venous thrombosis. Central nervous system involvement can occur, but is uncommon in FL.

DIAGNOSIS OF FOLLICULAR LYMPHOMA

Suggested guidelines for the diagnosis of lymphomas have been outlined by the National Comprehensive Cancer Network (guidelines available at http://www.nccn.org/) and by the European Society for Medical Oncology.[50] In all cases possible, diagnosis should be confirmed by excisional biopsy of an accessible LN and review by an expert hematopathologist with expertise in lymphoma diagnosis. In patients without easily accessible peripheral nodes, computed tomography (CT) or ultrasound-guided biopsies are typically well tolerated. Fine-needle aspiration is not appropriate for diagnosis in these conditions, and sufficient material must be obtained for adequate histologic review, immunophenotyping, and genetic studies, as required for diagnosis and assay for prognostic markers. When possible, consent should be obtained for the procurement, storage, and use of excess tissue from LN biopsies—at the time of presentation and at each subsequent relapse of disease—for research purposes to investigate the molecular biology of these diseases as new technologies and findings become available.

Initial essential investigations are shown in Table 82.2. Physical examination should include careful examination of all peripheral LN groups, including the cervical, supraclavicular, axillary, and inguinal chains, and examination of the Waldeyer ring. Abdominal examination should focus on evaluation of any intraabdominal masses, with particular attention paid to detection of enlargement of the liver or spleen. The skin should be carefully examined. Patients may present with pleural or pericardial effusions, although this is less common than in the aggressive lymphomas. Laboratory investigations should include a complete blood count to evaluate for cytopenias, which may be evidence of BM infiltration or of autoimmunity. A white blood count with differential and examination of the peripheral blood smear may elucidate leukemic involvement with disease. Baseline electrolytes including calcium and phosphate, creatinine, and liver function tests are important to determine organ dysfunction that may be related to direct infiltration by lymphoma. Elevation of lactate dehydrogenase (LDH) is an important prognostic factor and may be a useful indicator of transformation from indolent to aggressive lymphoma. Hepatitis B testing is essential as patients will likely require subsequent treatment with anti-CD20 monoclonal antibody therapy.

TABLE 82.2 Initial Evaluation of Follicular Lymphoma

- Physical examination with attention to peripheral nodes, abdomen
- Complete blood count, evaluation of peripheral blood
- Urea, creatinine and electrolytes
- Liver function tests; LDH; β_2-microglobulin
- Whole body PET-CT
- LN biopsy with review by an expert lymphoma histopathologist
- Bone marrow biopsy/aspirate
- Hepatitis B (surface antigen and core antibody), hepatitis C and human immunodeficiency virus (HIV)
- Other studies as indicated

CT, Computed tomography; *LDH*, lactate dehydrogenase; *LN*, lymph node; *PET*, positron emission tomography.

TABLE 82.3 Ann Arbor Staging

Stage	Criteria
I	Involvement of 1 lymph node (I) or 1 extralymphatic organ or site (IE)
II	Involvement of ≥2 lymph nodes on same side of diaphragm (II) or localized extralymphatic organ or site and ≥1 involved lymph node on same side of diaphragm (IIE)
III	Involvement of lymph nodes on both sides of diaphragm (III) or same side with localized involvement of extralymphatic site (IIIE), spleen (IIIS), or both (IIIS+E)
IV	Diffuse or disseminated involvement of 1 extralymphatic organ or tissues with or without lymph node enlargement

Staging workup also includes a CT scan of the chest, abdomen, and pelvis, and increasingly, a positron emission tomography (PET) scan to direct sites for biopsy and to help rule out histologic transformation. Particular attention should be paid to sites of bulk disease and to the number of involved sites. BM biopsy provides essential information and should be performed routinely as it is required for staging and prognostication of disease. Liver biopsy may be indicated based upon abnormal imaging or laboratory testing.

STAGING

The staging of NHL uses the Ann Arbor Classification (Table 82.3). Functional and anatomical imaging with positron emission tomography-computed tomography (PET-CT) is recommended as the standard for staging fluorodeoxyglucose (FDG)-avid nodal lymphomas.[51] FDG PET-CT may detect additional nodal and extranodal sites of disease when compared with standard CT. In a retrospective study, more nodal and extranodal lesions were detected by FDG PET-CT when compared with CT, and 11% of patients were upstaged from limited stage disease (I/II) to advanced stage disease (III/IV).[52] PET-CT staging may also contribute to improved prognosis in patients with stage I disease treated with involved field radiotherapy, likely due to more accurate identification of stage I disease.[53] However, PET-CT is of limited value for the detection of BM involvement. A BM aspirate and biopsy is still required if documentation of BM involvement by FL will have an impact on clinical management.[54]

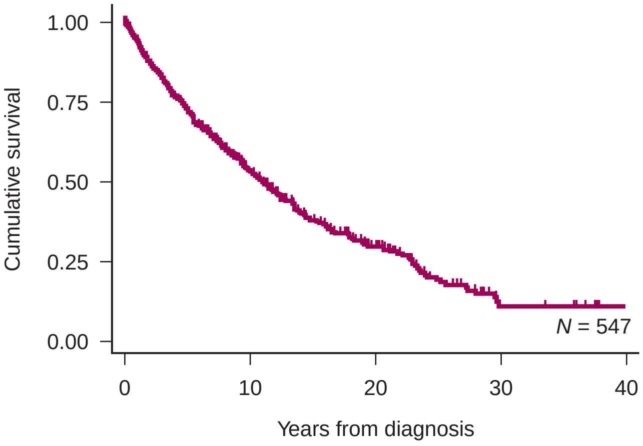


Figure 82.2 OVERALL PROBABILITY OF SURVIVAL OF PATIENTS WITH FOLLICULAR LYMPHOMA TREATED AT ST. BARTHOLOMEW'S HOSPITAL.

NATURAL HISTORY

Until recently, there was little evidence that the natural history of FL had changed over the last 30 years from the median survival of 10 years from diagnosis.[48] The survival of patients with FL presenting at St. Bartholomew's Hospital in London is shown in Fig. 82.2. The introduction of monoclonal antibodies, in combination with chemotherapy, has led to an improvement in survival. The median overall survival (OS) for FL now extends to 15 to 20 years.[55] However, it is still considered as an incurable malignancy. The natural history of FL is characterized by a protracted, relapse-remitting course, with each disease-free period becoming progressively shorter, leading to eventual treatment refractoriness. Importantly, significant clinical heterogeneity exists among patients, which poses dilemmas for treatment decision-making. Progression of disease within 24 months of initial immunochemotherapy (POD-24) occurs in 10% to 20% of patients and is associated with poor outcomes, with only 50% of patients alive after 5 years.[56] Similarly, transformation to high-grade lymphoma confers inferior outcomes, especially early transformation.[49]

There is, therefore, a need for prognostic markers that can help identify those patients who will have a good or poor prognosis. A number of prognostic scoring systems have been developed, with the aim of dichotomizing patients into groups to identify high-risk patients. The follicular lymphoma international prognostic index (FLIPI) is a five-factor prognostic index based upon the clinical characteristics age, stage, number of nodal sites, hemoglobin, and LDH level (Table 82.4). The index defines three prognostic risk groups of an almost equal numbers of patients.[57] This tool is useful in assessing the likely need for early treatment of patients and potential outcome, as well as in comparing the outcomes of different clinical trials. A revised FLIPI2 (incorporating β_2-microglobulin, diameter of largest LN, BM involvement, and hemoglobin level) may better discriminate the outcome for patients requiring treatment, with 5-year OS of 96% for low-risk patients, 80% for intermediate-risk patients, and 50% for high-risk patients.[58] Therefore, it is important to include the features required for calculation of FLIPI and FLIPI2 at the time of diagnosis.

The m7-FLIPI score combines clinical features (FLIPI and performance status) with the mutation status of seven genes *(EZH2, ARID1A, MEF2B, EP300, FOXO1, CREBBP, CARD11)*.[25] It was validated for patients receiving rituximab with CHOP or CVP chemotherapy. However, it was not prognostic for patients receiving immunotherapy in combination with bendamustine.[59,60] In addition, the m7-FLIPI was not prognostic for FL patients who received rituximab monotherapy.[61] GEP 23, a model derived from the expression profile of 23 genes, appears to be able to risk stratify FL patients, although this requires prospective validation.[62]. However, it remains uncertain by what means these prognostic tools can be used to inform clinical decisions on how and when to treat patients. These scores

TABLE 82.4 Factors Having Prognostic Significance in the Follicular Lymphoma International Prognostic Index (FLIPI)[57] and FLIPI2[58]

FLIPI

Parameter	Adverse Factor	HR
Age	≥60 years	2.38
Ann Arbor stage	III–IV	2.00
Hemoglobin level	<120 g/L	1.55
Serum LDH level	>ULN	1.50
Number of nodal sites	>4	1.39

FLIPI2

Parameter	Adverse Factor	HR (in Final Model)
β_2-microglobulin	>ULN	1.5
BM involvement	BM involvement with disease	1.59
Hemoglobin level	<120 g/L	1.51
Largest diameter of LN	>6 cm	1.66
Age	>60 years	1.38

BM, Bone marrow; *HR*, hazard ratio; *LDH*, lactate dehydrogenase; *LN*, lymph node; *ULN*, upper limit of normal.

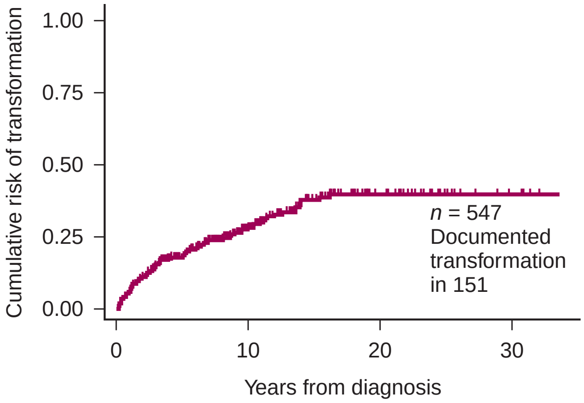


Figure 82.3 PROBABILITY OF RISK OF TRANSFORMATION OF PATIENTS WITH FOLLICULAR LYMPHOMA.

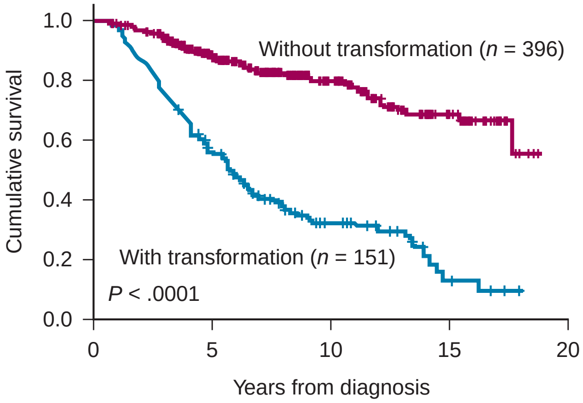


Figure 82.4 OVERALL PROBABILITY OF SURVIVAL OF PATIENTS WITH FOLLICULAR LYMPHOMA WHO HAVE AND HAVE NOT UNDERGONE HISTOLOGIC TRANSFORMATION.

need to be revalidated for use with new therapies, in an ever evolving treatment landscape. In addition, these scores cannot necessarily identify high-risk patients more reliably than simpler baseline assessments, such as total metabolic tumor volume (TMTV) by PET-CT[63] or PRIMA-PI, which uses serum B2M and the presence/absence of BM involvement.[64]

An important factor for the prognosis of FL is whether patients undergo histologic transformation.[65] The actuarial risk of FL undergoing histologic transformation in the database of the patients treated at St. Bartholomew's Hospital is shown in Fig. 82.3.The survival of patients with and without transformation is shown in Fig. 82.4. Despite a considerable body of information on the pathologic and molecular events associated with histologic transformation, the pathogenesis of transformation remains elusive, and the molecular events that have been identified have not been translated into changes in clinical practice. Our current, "one approach treats all" particularly underserves the high-risk FL population. A major research focus is attempting to identify patients at high risk of histologic transformation early in their clinical course, but this is not yet possible. The outcome of patients who undergo transformation after already having received multiple lines of therapy remains poor, but for those patients who undergo transformation and receive their first therapy for the transformed disease, the use of chemo-immunotherapy has led to a significant improvement in prognosis.

TREATMENT OF FOLLICULAR LYMPHOMA

For most cases of FL, the goal of therapy has been to maintain the best quality of life and treat only when patients develop symptoms. Any alteration to this approach requires demonstration of improved survival with early institution of therapy, or identification of criteria that define patients sufficiently "high-risk" to merit early therapy. There are many available therapies and no consensus on an optimal first-line or relapse treatment. Despite the lack of any data demonstrating benefit for early therapy, patients are being treated earlier in their disease course. There is no clear-cut treatment pathway for patients with FL, and although we have a good evidence base to decide on a particular treatment, there are little or no data regarding the optimal sequencing of treatment approaches in this disease. In the absence of such data, treatment choices remain empiric and should always involve discussion regarding patient choice and the goal of therapy. This is becoming even more complicated because many novel agents are either approved or are in clinical studies, particularly novel monoclonal antibodies and agents that alter the B cell receptor signaling pathways or anti-apoptotic pathways. The impact of these new agents on practice will be dependent upon the results of the ongoing clinical trials.

Multiple treatment approaches exist for advanced stage low-grade lymphomas, and these patients are best treated in the setting of clinical trials. Options range from a "watch and wait" expectant management approach, to a single agent chemotherapy (rarely used today) or a monoclonal antibody therapy with anti-CD20 antibodies, to a combination chemo-immunotherapy, with use of autologous or allogeneic stem cell transplantation (SCT) or chimeric antigen receptor (CAR) T cells (Table 82.5). Patients remaining on an expectant course should be documented every 3 to 6 months for 5 years, and then annually if stable, with history, physical exam, and blood counts including LDH. Special attention should be paid to any change in symptoms that might be suggestive of transformation, as these are an indication for a repeat biopsy to examine for histologic evidence to confirm transformation. Repeat scanning is not routinely performed unless this is indicated by symptoms or signs.

Since there is no clearly defined treatment algorithm for most patients with FL, eligible patients should be included whenever possible in clinical trials. This ensures delivery of optimal care and helps inform design of subsequent trials, hopefully leading to a cure. Information on available clinical trials can be found at http://www.clinicaltrials.gov. (See box on Management of Follicular Lymphoma.)

TABLE 82.5 Treatment Strategies for Indolent Lymphomas

Localized Disease
Radiotherapy
"Watchful waiting"
Advanced Stage Disease
"Watchful waiting"
Chemotherapy + monoclonal antibodies (chemoimmunotherapy)
Chemotherapy
Alkylating agents
Bendamustine
Purine analogs
Combination chemotherapy
Monoclonal antibodies
Unconjugated
Rituximab
Obinutuzumab
Conjugated—radioimmunoconjugates and immunotoxins
Agents approved for relapsed disease
Targeted therapy
Idelalisib
Duvalisib
Copanlisib
Tazemetostat
Lenalidomide
High-dose chemotherapy plus autologous/allogeneic stem cell transplantation
Reduced intensity conditioning allogeneic transplantation.
Chimeric antigen receptor T cells
Palliative radiotherapy

MANAGEMENT OF FOLLICULAR LYMPHOMA

Patients most often present with asymptomatic lymphadenopathy. The diagnosis should be made by excisional biopsy and reviewed by an expert hematopathologist. In the absence of symptoms requiring treatment or bulky lymphadenopathy, an expectant "watch and wait" approach is the treatment of choice. While in this phase of treatment, patients should be followed every 3–6 months for history, physical, and laboratory examination with radiologic restaging as clinically indicated. Once a decision to treat has been made, there is no clear treatment algorithm and a number of treatment options are available. The treatment goal, whether palliative or potentially with curative intent, is dependent upon the age and performance status of patients. Enrollment in a clinical trial should be the treatment of choice. For younger patients in whom high-dose therapy may be indicated later in their disease course, it is best to avoid profoundly myelotoxic regimens. Rituximab maintenance therapy in first remission given every 2 months for 2 years has been demonstrated to improve progression-free, but not overall survival. The role of maintenance therapy in first remission using interferon-α remains controversial. The choice of therapy after first relapse is also dependent upon the goal of therapy, but is also dependent upon the previous therapy given, response, and duration of response. Autologous or allogeneic stem cell transplantation has a role to play in selected younger patients with this disease, and chimeric antigen receptor (CAR) T cells are now licensed for treatment of multiply relapsed FL.

WHEN TO INSTITUTE THERAPY

Stage I–II Disease

Involved site radiation therapy is the preferred treatment for the approximately 10% of patients who present with limited stage, nonbulky disease. Treatment with photons, electrons, or protons may be appropriate depending on clinical circumstances. This treatment option is offered with curative potential[52,66] but must be weighed against the potential toxicity of the radiation therapy to other tissues. In patients with large tumor burden or with other adverse risk factors, systemic therapy is indicated. There is no proven role of radiation consolidation therapy.

Bulky Stage II and Stage II/IV Disease

Expectant management is the treatment of choice for asymptomatic patients with low-bulk disease until clear indications for initiation of treatment are seen, except for those patients enrolled in a clinical trial assessing the impact of early therapy. This approach is based upon the demonstration of no survival advantage for institution of immediate treatment, compared with deferred treatment until time of progression.[67] Three randomized trials, performed in the pre–rituximab era, confirmed no survival benefit for early therapy.[68–70] In the National Cancer Institute study, in 104 newly diagnosed patients with FL, deferred treatment was compared with immediate treatment with ProMACE-MOPP followed by total nodal irradiation. An updated analysis of this data is long overdue, but there was no difference in OS between the two arms at the time of the last analysis.[68] The Groupe pour l'Etude de Lymphome Folliculaire (GELF) used defined criteria for patients for whom immediate therapy was not felt to be indicated (Table 82.6) and randomized 193 patients to deferred treatment or to receive prednimustine 200 mg/m^2/day for 5 days per month for 18 months or IFN-α 5 million units (MU)/day for 3 months followed by 5 MU three times per week for 15 months.[69] The median OS time was not reached and was the same in all three arms of the study. The British National Lymphoma Investigation (BNLI)[70] compared treatment in 309 patients with asymptomatic advanced-stage, indolent lymphoma in whom 158 patients were randomized to receive immediate therapy with oral chlorambucil 10 mg/day continuously and 151 patients were randomized to deferred treatment until disease progression (see Table 82.6). In both arms, local radiotherapy to symptomatic nodes was allowed. There was no difference in OS or cause-specific survival between the two groups with median follow up of 16 years. A subsequent multicenter study examined whether rituximab could delay the need for chemotherapy or radiotherapy compared with watchful waiting.[72] A total of 463 patients were randomly assigned to watchful waiting, rituximab 375 mg/m^2 weekly for 4 weeks (rituximab induction), or rituximab induction followed by a maintenance schedule of 12 further infusions given at 2-monthly intervals for 2 years (maintenance rituximab). The rituximab induction arm was closed early, and the study continued to accrue to the other two arms. There was a significant difference in the time to start of next treatment, with 46% of watchful waiting patients still not needing treatment at 3 years compared with 88% in the maintenance rituximab group ($P < .0001$) and 78% in the rituximab induction group.

A major clinical trial question is whether identification of clinical or molecular risk factors can guide which patients are candidates for early therapy. A survival predictor score has also been developed from gene expression profiling studies.[73] This study demonstrated that gene expression from different immune cell components has a substantial impact on patient survival; FL cases enriched with genes expressed by macrophages and follicular dendritic cells showed an inferior outcome. Whereas it is to be hoped that in the future guidelines for treatment will be based upon clinical staging systems, genetic

TABLE 82.6 **Criteria for Delaying Treatment**

GELF[71]

All of the following

- Maximum diameter of disease <7 cm
- Fewer than 3 nodal sites
- Absence of systemic symptoms
- Spleen <16 cm on CT
- No significant effusions
- No risk of local compressive symptoms
- No circulating lymphoma cells or marrow compromise (Hb ≤10 g/dL, WBC <1.5 or platelets <100,000/dL)

BNLI[70]

Absence of all of the following

- B symptoms or pruritus
- Rapid generalized disease progression
- Marrow compromise (Hb ≤10 g/dL, WBC <3.0 or platelets <100,000/dL)
- Life-threatening organ involvement
- Renal infiltration
- Bone lesions

BNLI, British National Lymphoma Investigation; *CT*, computed tomography; *GELF*, Groupe pour l'Etude de Lymphome Folliculaire; *Hb*, hemoglobin; *WBC*, white blood cells.

profiles, and immune response signatures, it has not yet been established which factors help us to decide who should have immediate therapy. This may well have practical implications, since it has been demonstrated that patients who progress within 2 years of diagnosis have poorer outcome[56,74] and might well benefit from earlier institution of treatment.

Although there are little available data to suggest that we should change our practice of "watch and wait" for the asymptomatic low-bulk patients, data suggest that this practice is becoming much less common in the United States.[75] The National Lymphocare study is a prospective observational study designed to assess presentation, prognosis, treatment, and clinical outcomes in newly diagnosed FL. The treating physician determines management according to clinical judgment, with no prescribed treatment regimen, and data regarding histology, stage, therapy, response, relapse, and death are recorded. Among 2708 patients, enrolled at 265 centers, only 17.7% of patients were initially observed, and of these, 22% received active therapy within 1 year and 31% within 2 years, the majority with chemoimmunotherapy. This observation is in stark contrast to the data from the BNLI study, which demonstrated that, when censored for non-lymphoma death, 19% of patients and 40% of those older than 70 years who were randomized to expectant management still did not require therapy after 10 years.[70]

TREATMENT APPROACHES

Treatment is indicated in patients with symptomatic disease, bulky lymphadenopathy and/or splenomegaly, risk of local compressive disease, marrow compromise, or steady progression of disease. Once indicated, numerous treatment approaches are available (see Table 82.5). The concept that the treatment approach can be to "do nothing" or discuss an approach with considerable morbidity and mortality, including approaches such as SCT, is a confusing one for the newly diagnosed patient (as well as for the physician). Considerable consultation time is required to review available treatment approaches. Staging of response in FL is by the revised response criteria.[76] Depending on the treatment approach used, restaging after two to three cycles of therapy can be useful to ensure responsiveness, and the patient is then fully restaged after completion of therapy. Whereas curative approaches are being sought in aggressive lymphomas, the failure to achieve complete remission (CR) or complete eradication of disease does not have the same implication in FL as in aggressive lymphomas. Depending upon the goal of therapy, a partial remission (PR) may be a sufficient response to alleviate symptoms.[77–80]

In the absence of a clear standard of care with curative potential, the optimal first-line treatment remains enrollment in randomized clinical trials wherever possible. In the National Lymphocare study,[81] academic sites are more likely than community sites to treat patients on clinical trials (12% vs. 4%), but it is lamentable that only 6% of patients overall were enrolled in clinical trials. For patients who are not eligible for or who refuse entry into clinical trials, there is data demonstrating higher response rates and longer duration of responses, and perhaps improved survival with chemoimmunotherapy. Many investigators favor alkylator over fludarabine-based regimens for FL, based upon concerns regarding the ability to obtain stem cells for later use for autologous SCT (ASCT) or CAR-T therapy in fludarabine-treated patients.[82] It is suggested that more aggressive first-line therapy should be offered to patients who progress within 1 year of presentation, since these patients have a worse outcome.[71] Older adult patients or those with poor performance status may remain candidates for single-agent chlorambucil, although this is given most often in combination with rituximab. Single-agent rituximab therapy is appropriate for patients who choose to avoid chemotherapy and is a reasonable treatment choice based upon the results of clinical trials of standard or prolonged induction with or without maintenance therapy with rituximab. Although data suggest a survival advantage with the use of interferon (IFN)-α in combination with chemotherapy, this agent is more rarely used. Optimal results are seen when radioimmunoconjugates are used earlier in the disease course. On the basis of results showing no benefit for ASCT, there is no indication for the use of high-dose therapy and SCT in first remission in FL unless as part of a clinical trial.

Chemo-immunotherapy is now the treatment of choice for FL. No randomized trials demonstrate a benefit for the addition of anthracyclines, but cyclophosphamide, adriamycin, vincristine, prednisone, and rituximab (R-CHOP) is heavily favored over cyclophosphamide, vincristine, prednisone, and rituximab (CVP-R) or fludarabine-based regimens. Following demonstration of improved outcome using bendamustine plus rituximab (BR), compared with R-CHOP[83] or to fludarabine plus rituximab,[84] the use of BR has greatly increased. BR also has improved tolerability and can be given without the complication of hair loss. Choice to initiate therapy was associated with FLIPI, stage, and grade, but FLIPI was not associated with decision to use a specific treatment approach. In the Lymphocare study, significant regional and center differences are observed and strongly suggest that physician preference is the predominant factor that drives initial therapy. For example, initial "watch and wait" was used in 31% of patients in the Northeast, but in 13% of patients in the Southeast, whereas fludarabine-based chemoimmunotherapy was used in 18% of patients in the Southwest and in only 3% of patients in the Northeast.

Finally, the RELEVANCE study randomized previously untreated patients with advanced FL to receive either rituximab in combination with lenalidomide, or rituximab-chemotherapy, with both arms receiving maintenance rituximab. The 3-year PFS was 77% and 78%, respectively, which demonstrated the potential of a chemotherapy-free frontline therapy[85] (NCT01476787).

Alkylating Agents

The alkylating agents, chlorambucil and cyclophosphamide, with or without prednisone and CVP or CHOP, and other alkylator-based combination chemotherapy regimens have been the standard of therapy for decades. Single-agent alkylators at different doses and schedules produce overall response (OR) rates of 50% to 75% in

FL.[86,87] Comparable response rates, but higher CR rates with longer progression-free survival (PFS) are seen with CVP compared with chlorambucil, but have no survival advantage.[88] The addition of anthracyclines has not improved the response rate or duration of the response,[71,89] but its use may be associated with a lower risk of histologic transformation.[68,90]

Purine Analogues

The purine analogues have been studied extensively in various types of indolent lymphoma. Fludarabine monotherapy produces response rates of 65% to 84%, with 37% to 47% CR in previously untreated patients with FL.[91] In a randomized trial of 381 previously untreated indolent lymphoma patients, CR rates were higher with fludarabine than CVP.[92] Fludarabine combinations result in increased response rates, with 89% CR rate in an Eastern Cooperative Oncology Group (ECOG) trial combining fludarabine and cyclophosphamide.[93] Fludarabine and mitoxantrone (FM) produced a 91% overall response rate (ORR), 43% CR, and 2-year disease-free survival (DFS) of 63%.[94] A higher CR rate was seen with FM (68%) compared with CHOP (42%) in a randomized trial.[95] The use of alkylator-based regimens or purine analog–based regimens appears to vary geographically, suggesting personal preference for the use of regimens in which the clinician has experience, rather than alterations of practice based on the results of the published studies.

Bendamustine

Bendamustine is a potent alkylating agent that shows substantial efficacy in NHL patients, including those with FL. Bendamustine is highly effective in rituximab-refractory FL and in patients whose disease is refractory to other alkylating agents. It has also demonstrated considerable efficacy in previously untreated FL, both alone and in combination with rituximab or other chemotherapeutic agents.[96,97] Increased understanding of the mechanisms of action of bendamustine and the efficacy of bendamustine in combination with rituximab in newly diagnosed or relapsed/refractory FL[83,84] has led to investigation of other combinations. Ongoing studies are examining bendamustine with other agents.

Biologic Therapy

IFN-α is approved by the Food and Drug Administration (FDA) for the treatment of advanced stage FL in combination with anthracycline-based chemotherapy, based upon improved survival in clinical trials[57,98,99] and meta-analysis of phase III trial data.[100] IFN-α was more widely used in Europe than in the United States, where it is believed that its toxicity profile outweighs potential benefit. In the Southwest Oncology Group (SWOG) study,[101] 571 patients with stage III and IV indolent lymphoma were treated with ProMACE-MOPP, and 279 responding patients were randomized to 24 months of observation versus treatment with IFN-α. No statistically significant difference in PFS or OS was observed between observation and IFN-α groups after 4 years.

Monoclonal Antibody Therapy

Monoclonal antibodies remain the most exciting agents to emerge in the treatment of FL. The most widely used monoclonal antibody is rituximab, a chimeric unconjugated antibody against the CD20-antigen. It is licensed by the FDA[102] and the European Agency for the Evaluation of Medicinal Products[103] for treatment of patients with relapsed or refractory CD20-positive low-grade or FL; for the first-line treatment of CD20-positive FL in combination with CVP chemotherapy; and for the treatment of CD20-positive low-grade NHL in patients with stable disease or who achieve a PR or CR following first-line treatment with CVP chemotherapy. The use of this agent has a profound effect in finally leading to improvement in patient survival in FL.[104–106]

Following phase I studies,[107] rituximab at a dose of 375 mg/m^2 weekly for 4 weeks was selected for the pivotal phase II trial.[108] Although this remains the standard dose, the optimal dose and schedule of rituximab is still unknown. In relapsed patients with FL, OR to rituximab monotherapy was 60% with a median PFS for responders of 13 months. Factors associated with lower response rates include chemoresistant disease,[108] bulky disease,[109] and treatment late in the disease course.[110] OR was 73% in previously untreated patients with low-bulk disease,[111] and some of these patients needed no further treatment and had no evidence of polymerase chain reaction (PCR) detectable minimal residual disease (MRD) after 7 years.[112] Extended use with 8 weeks instead of 4 is associated with improvement in OR and duration of response.[113] Comparable or even longer durations of response have been observed with retreatment.[114]

A number of trials in front-line and in relapsed/refractory patients have investigated the potential benefits of extended or maintenance rituximab treatment,[80,115–119] and all demonstrated prolonged time to progression in patients receiving maintenance rituximab (Table 82.7). The results from the E1496 randomized trial from ECOG and Cancer and Leukemia Group B comparing CVP alone to CVP followed by rituximab in patients with advanced-stage FL demonstrated that addition of rituximab maintenance significantly improved OS[115] and led to FDA approval for rituximab therapy in patients responding to CVP chemotherapy. The results of the primary rituximab and maintenance (PRIMA) study demonstrated an advantage in PFS for maintenance rituximab therapy offered after initial chemoimmunotherapy.[119]

Chemoimmunotherapy

In a phase II study, 40 patients with indolent lymphoma were treated with six infusions of rituximab (375 mg/m^2 per dose) in combination with six doses of CHOP chemotherapy (R-CHOP),[121] and OR was 95%, with 55% CR. In a phase II study of 40 patients with indolent lymphomas, rituximab in combination with fludarabine produced OR of 90%, with 80% CR, with similar response rates in treatment-naive and previously treated patients.[122]

A number of randomized trials show a benefit for the use of rituximab with chemotherapy compared with chemotherapy alone (Table 82.8).[77,78,123,124] Each study showed an improvement in time

TABLE 82.7 **Studies of Rituximab Maintenance Therapy in Indolent Lymphomas**

Trial	Disease Setting	Diseases Included	Previous Therapy
ECOG[115]	1st line	Follicular Small lymphocytic	CVP
SAKK[80]	1st line Relapsed/refractory	Follicular Mantle cell	Rituximab
EORTC[120]	Relapsed/refractory	Follicular	CHOP vs R-CHOP
GLSG[116]	Relapsed/refractory	Follicular Mantle cell	FCM vs. R-FCM
LYM-5[117]	Relapsed/refractory	Follicular Small lymphocytic	Rituximab

CHOP, Cyclophosphamide, adriamycin, vincristine, prednisone; *CVP*, cyclophosphamide, vincristine, prednisone; *FCM*, fludarabine, cyclophosphamide, mitoxantrone; *R-CHOP*, rituximab plus CHOP; *R-FCM*, rituximab plus FCM.

TABLE 82.8 Randomized Trials of Chemotherapy Versus Chemo-immunotherapy

Study	Treatment, Number of Patients	Median FU (Months)	OR %	CR %	Median TTF (Months)	OS %
M39021[77]	CVP, 159	53	57	10	15	77
	R-CVP, 162		81	41	34	83
					$P < .0001$	$P = .0290$
GLSG[78]	CHOP, 205	18	90	17	29	90
	R-CHOP, 223		96	20	NR	95
					$P < .001$	$P = .016$
M39023[123]	MCP, 96	47	75	25	26	74
	R-MCP, 105		92	50	NR	87
					$P < .0001$	$P = .0096$
FL2000[124]	CHVP-IFN, 183	42	73	63	46	84
	R-CHVP-IFN 175		84	X	67	91
					$P < .0001$	$P = .029$

CHOP, Cyclophosphamide, adriamycin, vincristine, prednisone; *CVP*, cyclophosphamide, vincristine, prednisone; *CHVP-IFN*, cyclophosphamide, doxorubicin, teniposide, prednisone, IFN-α; *CR*, complete remission; *FU*, follow-up; *MCP*, MCP received a combination of mitoxantrone 8 mg/m^2 intravenously on days 1 and 2, chlorambucil 3 × 3 mg/m^2 orally on days 1 to 5, and prednisolone 25 mg/m^2 orally on days 1 to 5; *NR*, no response; *OR*, overall response; *R-CHOP*, rituximab plus CHOP; *R-CHVP-IFN*, rituximab plus CHVP-IFN; *R-CVP*, rituximab plus CVP; *R-MCP*, rituximab plus MCP; *TTF*, time to treatment failure.

to treatment failure and more recent follow-up data suggests improved OS in patients treated with chemo-immunotherapy compared with chemotherapy alone. A meta-analysis of these trials demonstrates that OS, OR, and disease control are significantly better in those on chemoimmunotherapy compared with chemotherapy for FL and mantle cell lymphoma (MCL).[125] Data from the German low grade study group (GLSG) suggest that it is the addition of rituximab that has led to the recent improvement in survival of patients with FL.[105] An independently assessed analysis of the clinical benefits provided by rituximab in relation to cost concluded that it is highly cost effective.[126]

The largest FL trial reported to date is the PRIMA study (NCT00140582).[119] This study enrolled 1217 patients to receive initial chemo-immunotherapy from three possible regimens, but of note, 75% received R-CHOP. All patients had fulfilled the criteria for treatment and 80% had FLIPI intermediate or high-risk features. Responding patients were randomized to receive no further therapy or to receive 12 doses (every 8 weeks) for 2 years.

Patients who received rituximab maintenance therapy had significantly better rates of 3-year PFS than did those who received observation (75% vs. 58%), and the benefit to maintenance was observed in all FLIPI groups. Time to next treatment was also longer in the maintenance group than in the observation group. There were increased toxicities in the maintenance group, with most being infections, but these were largely self-limiting.

The type II glycoengineered anti-CD20 monoclonal antibody obinutuzumab is approved for use in combination with chemotherapy for first line treatment of advanced disease and then alone as maintenance therapy. The Gallium study enrolled 1202 patients with advanced (stage III/IV or stage II bulky) FL who required treatment according to GELF criteria.[59] Patients were randomized to receive either rituximab- or obinutuzumab-based frontline therapy. The chemotherapy consisted either of CHOP, CVP, or bendamustine and was allocated by the treating study center (NCT01332968). Obinutuzumab-chemotherapy improved 3-year progression free survival (PFS) when compared to rituximab-chemotherapy (80% vs. 73.3%) but with higher rates of treatment-related toxicity and no improvement in OS. In this cohort, patients with *EZH2* mutant FL appeared to benefit more from CHOP/CVP, whereas patients without *EZH2* mutations had longer PFS with bendamustine based regimes, regardless of whether chemotherapy was combined with rituximab or obinutuzumab.[60] This observation requires prospective validation but demonstrates that genomic biomarkers could be used to stratify patient treatment.

Conjugated Radio-Labeled Monoclonal Antibody Therapy

Complexing a radioisotope to a monoclonal antibody (radio-immunoconjugate) might be expected to improve efficacy over antibody therapy alone. Tositumomab complexes[127] Iodine to the anti-B1 antibody and has been studied extensively in the treatment of heavily pretreated,[128] untreated,[129] and for retreatment of indolent lymphomas.[130] The best responses are seen in previously untreated patients, with FL treated in a single treatment course with tositumomab in cases where there was a 95% OR, 75% CR, and 80% of assessable patients achieved eradication of PCR detectable MRD patients.[129] Median PFS was 6.1 years, with 40 patients remaining in remission for 4.3 to 7.7 years and no cases of myelodysplastic syndrome were observed. A SWOG study investigated chemo-immunotherapy with six cycles of CHOP chemotherapy followed 4 to 8 weeks later by tositumomab in 90 patients with previously untreated, advanced-stage FL.[131] The OR was 91%, including 69% CR and at median follow-up time of 5.1 years, the estimated 5-year OS was 87%, and PFS 67%, 23% better than CHOP alone on previous SWOG protocols. Ibritumomab Tiuxetan is a ^{90}Y-labeled anti-CD20 antibody and produced OR of 74% and 15% CR in 57 FL patients refractory to rituximab.[132] Toxicity is primarily hematologic, with nadir counts occurring at 7 to 9 weeks and lasting approximately 1 to 4 weeks. The risk of hematologic toxicity increased with dose delivered and with degree of baseline BM lymphoma involvement.[127] An acceptable safety profile was observed in relapsed patients with less than 25% lymphoma marrow involvement, adequate marrow reserve, platelets greater than 100,000 cells/μL, and neutrophils greater than 1500 cells/μL.

High-Dose Therapy as Consolidation of First Remission

The role of high-dose therapy and ASCT in patients with FL during first remission was explored in phase II trials,[133,134] and in three phase III randomized trials.[135–137] The GLSG trial[137] recruited 307 previously untreated patients up to 60 years of age and patients who responded after induction chemotherapy with two cycles of CHOP or mitoxantrone-chlorambucil-prednisone (MCP) and were randomized to ASCT or IFN-α maintenance. Among 240 evaluable patients, the 5-year PFS was 64.7% for ASCT, and 33.3% in the IFN-α arm ($P < .0001$). Acute toxicity was higher in the ASCT group, but early mortality was below 2.5% in both study arms. Longer follow-up is necessary to determine the effect of ASCT on OS. In the Groupe Ouest-Est des Leucémies Aigües et des Maladies du Sang study, 172 patients newly diagnosed with advanced FL were randomized either to cyclophosphamide, doxorubicin, teniposide, prednisone (CHVP), and IFN-α or to high-dose therapy followed by purged ASCT.[136] Patients treated with high-dose therapy had a higher response rate than patients who received chemotherapy and IFN-α (81% vs. 69%, $P = .045$) and a longer median PFS (not reached vs. 45 months), but this did not translate into a better OS because of an excess of secondary malignancies after transplantation. A subgroup of patients with a significantly higher event-free survival rate ASCT could be identified using the FLIPI. The GELF94 study enrolled 401 previously untreated patients with advanced-stage FL who were randomized to receive CHVP plus IFN-α compared with four courses of CHOP followed by HDT with total body irradiation (TBI) and ASCT and OR rates were similar in both groups (79% and 78% respectively) and 87% of eligible patients underwent ASCT. Intent-to-treat analysis after a median follow up of 7.5 years showed no difference between the two arms for OS ($P = .53$) or PFS ($P = .11$). Long-term follow up demonstrated no statistically significant benefit in favor of first-line ASCT in patients with FL, which the investigators conclude, should be reserved for relapsed patients. A meta-analysis concluded that high-dose therapy and ASCT does not improve OS in FL in first remission.[138] In view of these results, ASCT should be used in first remission only in the setting of clinical trials.

TREATMENT OF RELAPSED FOLLICULAR LYMPHOMA

Relapsed asymptomatic disease is not necessarily an indication for treatment and patients can again be managed expectantly. A number of factors must be taken into account in planning therapy and it is not possible to define treatment at relapse without considering the goal of therapy (palliative vs. potentially curative) performance status, previous therapy, response, and duration of response. In addition to the agents approved for use in first line disease, and which can be repeated if there has been a long duration of response, there are a number of agents approved for treatment after relapse (see Table 82.5). There is no consensus on the order or sequencing of specific agents, and relapsed patients should ideally be treated in clinical trials.

Single-agent rituximab is approved for relapsed lymphoma and is still widely used in this setting. A multicenter randomized trial in relapsed patients has demonstrated a survival advantage for chemoimmunotherapy with CHOP-R or CHOP followed by rituximab compared with CHOP alone, and a further benefit for rituximab maintenance therapy.[120] Agents approved for relapsed or refractory disease include the PI3 kinase inhibitors, idelalisib, duvalisib, copanlisib, the immunomodulatory agent, lenalidomide and the EZH2 inhibitor tazemetostat. CAR-T cells are also FDA approved for patients with relapsed or refractory FL after two or more lines of prior therapy.

In rituximab refractory cases or remissions of less than 6 months, obinutuzumab bendamustine therapy with obinutuzumab maintenance has been shown to improve PFS and OS when compared to bendamustine monotherapy.[139] Lenalidomide in combination with rituximab is superior to rituximab monotherapy in the relapsed setting and may be considered for those patients with short remissions after chemotherapy.[140]

For younger patients who are suitable candidates for either ASCT or reduced intensity conditioning (RIC) allogeneic transplantation, referral to a transplant center should be considered early to discuss the potential role and timing of transplantation. Best results are seen when transplantation is considered sufficiently early in the course of disease that patients have not already become chemo-refractory. High-dose therapy and ASCT remains an effective treatment approach for younger patients with chemo-responsive relapsed disease, SCT approaches must be considered in the context of the improving results that are being seen with novel salvage therapies.

Novel Agents

There are a large number of novel approaches that are being studied in patients with FL.

The phosphoinositide 3-kinase (PI3K) inhibitors idelalisib, copanlisib, and duvelisib, have shown efficacy in a relapsed setting,[141] and are approved for the treatment of relapsed FL. Given the dependence of FL on B cell receptor (BCR) signaling, monotherapy with ibrutinib, a Bruton tyrosine kinase inhibitor in the relapsed setting has only shown moderate efficacy, with an ORR of 38%.[142] Since a hallmark of FL is overexpression of BCL, this protein is also a logical target for small molecule inhibitors that can inhibit the antiapoptotic activity of BCL2. Venetoclax, a selective BCL2 inhibitor has proved disappointing in the relapsed-refractory setting, as a monotherapy and in combination with treatments active in FL.[143,144] Histone deacetylase (HDAC) inhibitors remove acetylation marks imposed by *CREBBP* and *EP300* and are showing promise as a means of overcoming the loss of function effect of these mutations. In a phase II study, pan-HDAC inhibitor vorinostat demonstrated moderate efficacy in the relapsed refractory setting with an ORR of 49%.[145,146] EZH2 inhibition with the oral selective small molecule, tazemetostat, shows ORR of 69% in *EZH2*-mutated patients and 35% in *EZH2*-wild type patients[147] and has recently been FDA approved for the treatment of relapsed refractory FL.

Therapies Targeting the Tumour Microenvironment

FL cells are critically reliant on interaction with components of the immune microenvironment for growth and survival. For many years it has been evident that FL is sensitive to treatment strategies that harness the immune system, from monoclonal antibodies, such as rituximab, to hematopoietic SCT. There are now a number of established and emerging treatments that impact both the TME and the tumour cells. The oral immunomodulatory drug lenalidomide augments anti-tumor immunity via mechanisms including enhanced immune synapse formation with T and NK cells and stimulating proliferation and activation of NK cells, enhancing NK mediated cytotoxicity.[148] Immune checkpoint inhibitors (ICI) that target programmed death-1 (PD-1) such as nivolumab, are effective in classic Hodgkin lymphoma, which displays frequent copy-number gains of the chromosomal region 9p24.1, encoding programmed death-ligand 1 (PD-L1) and PD-L2.[149] There is a rich infiltrate of PD-1 expressing T cells in the FL TME. Despite this, a phase II study evaluating the use of nivolumab monotherapy in relapsed refractory FL demonstrated very limited activity, with a 4% ORR. Responders had significantly higher proportions of CD3+ T cells in the TME than non-responders, however no significant differences in PD-1 or PD-L1 expression were observed.[150] A range of predictive biomarkers of response and resistance to ICI in solid tumors have been identified, such as mutation burden and intra-tumoral T cell infiltration, however these require investigation and validation in FL.[151]

Other cells within the TME are also the target of novel therapies. Phagocytic cells including macrophages and dendritic cells express signal regulatory protein alpha (SIRPα). The CD47-SIRPα interaction provides a macrophage immune checkpoint pathway, resulting in inhibition of phagocytosis. Hu5F9-G4, a humanized monoclonal antibody against CD47, targets the inhibitory effects on macrophage phagocytosis, enhancing antibody dependent cellular phagocytosis.[152] Hu5F9-G4 in combination with rituximab induced an ORR of 71% and a CR of 43% in patients with relapsed refractory FL.[153]Another emerging group are the CD20-CD3 bispecific antibodies, that target tumor B-cells and engage T-cells, directing antigen-specific T cell–mediated cytotoxicity to the malignant cells. One such agent, mosunetuzumab, has demonstrated activity in relapsed refractory FL with an ORR of 68% in a phase I clinical trial, NCT 02500407.[154]

There are numerous other agents in this class being explored for use in the relapsed refractory setting.[155,156]

The Role of Transplant in Relapsed Indolent Lymphomas

Unlike aggressive lymphomas, the use of high-dose chemotherapy with ASCT in the treatment of FL has not yet been fully established. The rationale for considering transplantation is that the disease is incurable using standard approaches, young patients with FL will die of their disease, and promising results have been observed in a number of phase II studies.[157–159] Detection of MRD has been a useful surrogate marker for tracking long-term PFS in patients examining the autologous stem cells or serial samples after transplantation.[159–163] A major concern relates to the risk of development of secondary myelodysplasia/acute myeloid leukemia.[164] The European Bone Marrow Transplant Registry (EBMTR) sponsored CUP (conventional chemotherapy, unpurged, purged autograft) study is the only prospective randomized trial to assess the role of ASCT in patients with relapsed FL.[165] The results of the study suggest a PFS and OS advantage of ASCT over conventional chemotherapy, with 4-year OS of 46% for the chemotherapy arm, versus 71% for the unpurged and 77% for the purged ASCT arms. The study was closed early because of slow accrual with 140 of the planned 250 patients accrued and only 89 randomized.

Allogeneic Bone Marrow Transplant

There is a trend towards increasing use of allogeneic SCT (allo-SCT) in the management of indolent lymphomas. In a report of the International Bone Marrow Transplant Registry (IBMTR), results after SCT are described for 904 patients with FL.[166] Among these patients, 176 patients underwent allo-SCT, 131 patients underwent ASCT using purged stem cells and 597 using purged autologous stem cells. The treatment related mortality (TRM) in these three groups was 30%, 14% and 8% respectively. Disease recurrence occurred in 21%, 43%, and 58% and 5-year OS was 51%, 62%, and 55%, respectively. The use of TBI containing regimens was associated with increased TRM but decreased risk of relapse. The use of aSCT was associated with increased TRM but with significantly lower risk of disease recurrence in keeping with a graft versus lymphoma effect in this disease. Trends suggest that outcomes are improving, and this is highly likely to continue with the increased use of RIC regimens that have become used increasingly since the time this registry data was collated. Long-term PFS has been observed after aSCT even in patients with refractory FL.[167] In 29 patients with FL, 11 of whom had refractory disease, the nonrelapse mortality was 24% and there was a 23% incidence of relapse. The 5-year OS was 58% with 53% event-free survival. A group of patients with very poor outcome are those patients who have relapsed after previous ASCT. The outcome following myeloablative aSCT of 114 such patients has been reported from the IBMTR.[168] The TRM was 22% and the probability of disease progression was 52% at 3 years. The use of TBI conditioning regimens and achievement of CR at the time of aSCT were associated with improved outcome. The use of RIC regimens appears to be associated with improved outcome. In 20 such patients, there was only one TRM from fungal infection and the 3-year PFS was an excellent 95%.[169] The outcome following RIC transplant regimen incorporating alemtuzumab immunosuppressive therapy has been reported for 81 patients with lymphoma and included 41 with low-grade, 37 with high/intermediate-grade and 10 patients with MCL, 31 of whom had relapsed following previous ASCT.[170] Patients received a conditioning regimen consisting of alemtuzumab, fludarabine, and melphalan, and received short course cyclosporin as graft-versus-host disease (GVHD) prophylaxis. The use of this conditioning regimen was associated with a low incidence of GVHD and the TRM was decreased in patients with low-grade compared with higher grade histology. The 3-year PFS was 65% for patients with low-grade lymphoma, 50% for patients with MCL and 34% for high-grade lymphoma ($P = .002$). Donor lymphocyte infusion (DLI) was given to 36 patients, 21 for relapsed or persistent disease and 15 for persistence of mixed chimerism. The use of DLI to treat relapse after aSCT is solely dependent upon the existence of a graft versus lymphoma effect. In seven patients with FL and small lymphocytic lymphoma who had relapsed after prior aSCT, six patients responded and four maintained CR for 43 to 89 months. The effectiveness of DLI to treat relapse after aSCT provides very strong evidence for a graft versus lymphoma effect that can be exploited in indolent lymphomas.[170,171] The role of RIC aSCT has been evaluated by Cancer and Leukemia Group B in a phase II study to evaluate the safety and efficacy in patients with recurrent low-grade B-cell malignancies, including 16 patients with FL.[172] The 3-year TRM was 9% and the 3-year OS was 81%. The incidence of grade II–IV acute GVHD was 29%, and extensive chronic GVHD was 18%.

CAR-T Cells

Axicabtagene ciloleucel (axi-cel), an autologous anti-CD19 CAR-T-cell therapy, has demonstrated clinically meaningful responses among patients with relapsed/refractory FL in the pivotal single-arm ZUMA-5, a non-comparative phase II trial (NCT03105336) and this resulted the FDA granting accelerated approval for axi-cell for FL after two or more prior lines of therapy.[173] ZUMA-5 demonstrated a 95% objective response rate, 79% complete response, OS, and at a median follow up of 23 months the median PFS was not reached. Like other single-arm trials, this does not provide comparative measures with other available treatments. To contextualize the clinical benefit associated with axi-cel in relapsed refractory FL, the results were compared to an international, retrospective, observational cohort from leading clinical sites, named SCHOLAR-5.[174] For comparison of the ZUMA 5 and SCHOLAR 5 cohorts a propensity weighting score was used for the SCHOLAR 5 patient group to create an external comparator arm with similar clinical characteristics as the patients in ZUMA 5 and demonstrated a meaningful improvement in ORR, CR, PFS, and, importantly, OS for CAR-T treatment over standard of care offered to the patients in the weighted SCHOLAR 5 cohort. The SCHOLAR 5 data also highlighted the lack of international consensus on the treatment of FL, particularly in the relapsed and refractory setting. Tisagenlecleucel has been shown to have promising results in clinical trials and, is now approved for use in Europe a has been filed for approval in FL.[175] Lisocabtagene maraleucel is currently approved only for FL3B.[176]

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Carbone A, Roulland S, Gloghini A, et al. Follicular lymphoma. *Nat Rev Dis Primers*. 2019;5:83.

Dave SS, Wright G, Tab B, et al. Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. *N Engl J Med*. 2004;351:2159–2169.

Friedberg JW, Byrtek M, Link BK, et al. Effectiveness of first-line management strategies for stage I follicular lymphoma: analysis of the National LymphoCare Study. *J Clin Oncol*. 2012;30:3368–3375.

Kumar E, Pickard L, Okosun J. Pathogenesis of follicular lymphoma: genetics to the microenvironment to clinical translation. *Br J Haematol*. 2021;194(5):810–821.

Marcus R, Davies A, Ando K, et al. Obinutuzumab for the first-line treatment of follicular lymphoma. *N Engl J Med*. 2017;377(14):1331–1344.

Milpied P, Cervera-Marzal I, Mollichella ML, et al. Human germinal center transcriptional programs are de-synchronized in B cell lymphoma. *Nat Immunol*. 2018;19(9):1013–1024.

Morschhauser F, Fowler NH, Feugier P, et al. Rituximab plus lenalidomide in advanced untreated follicular lymphoma. *N Engl J Med*. 2018;379(10):934–947.

Okosun J, Bödör C, Wang J, et al. Integrated genomic analysis identifies recurrent mutations and evolution patterns driving the initiation and progression of follicular lymphoma. *Nat Genet*. 2014;46:176–181.

REFERENCES

1. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127(20):2375–2390.
2. Smith A, Crouch S, Lax S, et al. Lymphoma incidence, survival and prevalence 2004–2014: sub-type analyses from the UK's Haematological Malignancy Research Network. *Br J Cancer*. 2015;112(9):1575–1584.
3. Altieri A, Bermejo JL, Hemminki K. Familial risk for non-Hodgkin lymphoma and other lymphoproliferative malignancies by histopathologic subtype: the Swedish Family-Cancer Database. *Blood*. 2005;106(2):668–672.
4. Skibola CF, Bracci PM, Halperin E, et al. Genetic variants at 6p21.33 are associated with susceptibility to follicular lymphoma. *Nat Genet*. 2009;41(8):873–875.
5. Wang SS, Carrington M, Berndt SI, et al. HLA class I and II diversity contributes to the etiologic heterogeneity of non-Hodgkin lymphoma subtypes. *Cancer Res*. 2018;78(14):4086–4096.
6. Loeffler M, Kreuz M, Haake A, et al. Genomic and epigenomic co-evolution in follicular lymphomas. *Leukemia*. 2015;29(2):456–463.
7. Victora GD, Dominguez-Sola D, Holmes AB, et al. Identification of human germinal center light and dark zone cells and their relationship to human B-cell lymphomas (vol 120, pg 2240, 2012). *Blood*. 2015;126(10):1262 -.
8. Milpied P, Cervera-Marzal I, Mollichella ML, et al. Human germinal center transcriptional programs are de-synchronized in B cell lymphoma. *Nat Immunol*. 2018;19(9):1013–1024.
9. Tsujimoto Y, Cossman J, Jaffe E, et al. Involvement of the Bcl-2 Gene in Human Follicular Lymphoma. *Science*. 1985;228(4706):1440–1443.
10. Bosga-Bouwer AG, van Imhoff GW, Boonstra R, et al. Follicular lymphoma grade 3B includes 3 cytogenetically defined subgroups with primary t(14;18), 3q27, or other translocations: t(14;18) and 3q27 are mutually exclusive. *Blood*. 2003;101(3):1149–1154.
11. Horn H, Schmelter C, Leich E, et al. Follicular lymphoma grade 3B is a distinct neoplasm according to cytogenetic and immunohistochemical profiles. *Haematologica*. 2011;96(9):1327–1334.
12. Campo E, Swerdlow SH, Harris NL, et al. The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. *Blood*. 2011;117(19):5019–5032.
13. Leich E, Salaverria I, Bea S, et al. Follicular lymphomas with and without translocation t(14;18) differ in gene expression profiles and genetic alterations. *Blood*. 2009;114(4):826–834.
14. Nann D, Ramis-Zaldivar JE, Müller I, et al. Follicular lymphoma t(14;18)-negative is genetically a heterogeneous disease. *Blood Adv*. 2020;4(22):5652–5665.
15. Cleary ML, Sklar J. Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. *Proc Natl Acad Sci USA*. 1985;82(21):7439–7443.
16. Limpens J, Stad R, Vos C, et al. Lymphoma-associated translocation t(14;18) in blood B cells of normal individuals. *Blood*. 1995;85(9):2528–2536.
17. McDonnell TJ, Korsmeyer SJ. Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18). *Nature*. 1991;349(6306):254–256.
18. Okosun J, Bödör C, Wang J, et al. Integrated genomic analysis identifies recurrent mutations and evolution patterns driving the initiation and progression of follicular lymphoma. *Nat Genet*. 2014;46(2):176–181.
19. Pasqualucci L, Khiabanian H, Fangazio M, et al. Genetics of follicular lymphoma transformation. *Cell Rep*. 2014;6(1):130–140.
20. Pasqualucci L, Dominguez-Sola D, Chiarenza A, et al. Inactivating mutations of acetyltransferase genes in B-cell lymphoma. *Nature*. 2011;471(7337):189–195.
21. Kridel R, Chan FC, Mottok A, et al. Histological transformation and progression in follicular lymphoma: a clonal evolution study. *PLoS Med*. 2016;13(12):e1002197.
22. Green MR, Kihira S, Liu CL, et al. Mutations in early follicular lymphoma progenitors are associated with suppressed antigen presentation. *Proc Natl Acad Sci USA*. 2015;112(10):E1116–E1125.
23. Morin RD, Mendez-Lago M, Mungall AJ, et al. Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. *Nature*. 2011;476(7360):298–303.
24. Li H, Kaminski MS, Li Y, et al. Mutations in linker histone genes HIST1H1 B, C, D, and E; OCT2 (POU2F2); IRF8; and ARID1A underlying the pathogenesis of follicular lymphoma. *Blood*. 2014;123(10):1487–1498.
25. Pastore A, Jurinovic V, Kridel R, et al. Integration of gene mutations in risk prognostication for patients receiving first-line immunochemotherapy for follicular lymphoma: a retrospective analysis of a prospective clinical trial and validation in a population-based registry. *Lancet Oncol*. 2015;16(9):1111–1122.
26. Pasqualucci L. Molecular pathogenesis of germinal center-derived B cell lymphomas. *Immunol Rev*. 2019;288(1):240–261.
27. Ortega-Molina A, Boss IW, Canela A, et al. The histone lysine methyltransferase KMT2D sustains a gene expression program that represses B cell lymphoma development. *Nat Med*. 2015;21(10):1199–1208.
28. Zhang J, Dominguez-Sola D, Hussein S, et al. Disruption of KMT2D perturbs germinal center B cell development and promotes lymphomagenesis. *Nat Med*. 2015;21(10):1190–1198.
29. García-Ramírez I, Tadros S, González-Herrero I, et al. Crebbp loss cooperates with Bcl2 overexpression to promote lymphoma in mice. *Blood*. 2017;129(19):2645–2656.
30. Morin RD, Assouline S, Alcaide M, et al. Genetic landscapes of relapsed and refractory diffuse large B-cell lymphomas. *Clin Cancer Res*. 2016;22(9):2290–2300.
31. Dheilly E, Battistello E, Katanayeva N, et al. Cathepsin S regulates antigen processing and T cell activity in non-Hodgkin lymphoma. *Cancer Cell*. 2020;37(5):674–689. e12.
32. Okosun J, Wolfson RL, Wang J, et al. Recurrent mTORC1-activating RRAGC mutations in follicular lymphoma. *Nat Genet*. 2016;48(2):183–188.
33. Yildiz M, Li H, Bernard D, et al. Activating STAT6 mutations in follicular lymphoma. *Blood*. 2015;125(4):668–679.
34. Morin RD, Johnson NA, Severson TM, et al. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. *Nat Genet*. 2010;42(2):181–185.
35. Bödör C, Grossmann V, Popov N, et al. EZH2 mutations are frequent and represent an early event in follicular lymphoma. *Blood*. 2013;122(18):3165–3168.
36. Mottok A, Renné C, Seifert M, et al. Inactivating SOCS1 mutations are caused by aberrant somatic hypermutation and restricted to a subset of B-cell lymphoma entities. *Blood*. 2009;114(20):4503–4506.
37. Akasaka T, Lossos IS, Levy R. BCL6 gene translocation in follicular lymphoma: a harbinger of eventual transformation to diffuse aggressive lymphoma. *Blood*. 2003;102(4):1443–1448.
38. Kridel R, Mottok A, Farinha P, et al. Cell of origin of transformed follicular lymphoma. *Blood*. 2015;126(18):2118–2127.
39. Martinez-Climent JA, Alizadeh AA, Segraves R, et al. Transformation of follicular lymphoma to diffuse large cell lymphoma is associated with a heterogeneous set of DNA copy number and gene expression alterations. *Blood*. 2003;101(8):3109–3117.
40. Launay E, Pangault C, Bertrand P, et al. High rate of TNFRSF14 gene alterations related to 1p36 region in de novo follicular lymphoma and impact on prognosis. *Leukemia*. 2012;26(3):559–562.
41. Cheung KJ, Johnson NA, Affleck JG, et al. Acquired TNFRSF14 mutations in follicular lymphoma are associated with worse prognosis. *Cancer Res*. 2010;70(22):9166–9174.
42. Green MR, Gentles AJ, Nair RV, et al. Hierarchy in somatic mutations arising during genomic evolution and progression of follicular lymphoma. *Blood*. 2013;121(9):1604–1611.
43. Correia C, Schneider PA, Dai H, et al. BCL2 mutations are associated with increased risk of transformation and shortened survival in follicular lymphoma. *Blood*. 2015;125(4):658–667.
44. Honma K, Tsuzuki S, Nakagawa M, et al. TNFAIP3/A20 functions as a novel tumor suppressor gene in several subtypes of non-Hodgkin lymphomas. *Blood*. 2009;114(12):2467–2475.
45. Gunawardana J, Lee JN, Bednarska K, et al. Genetic aberrations of NLRC5 are associated with downregulated MHC-I antigen presentation and impaired T-cell immunity in follicular lymphoma. *eJHaem*. 2020;1(2):517–526.
46. Amé-Thomas P, Tarte K. The yin and the yang of follicular lymphoma cell niches: role of microenvironment heterogeneity and plasticity. *Semin Cancer Biol*. 2014;24:23–32.
47. Kumar E, Pickard L, Okosun J. Pathogenesis of follicular lymphoma: genetics to the microenvironment to clinical translation. *Br J Haematol*. 2021;194(5):810–821.
48. Horning SJ, Rosenberg SA. The natural history of initially untreated low-grade non-Hodgkin's lymphomas. *N Engl J Med*. 1984;311(23):1471–1475.
49. Federico M, Caballero Barrigón MD, Marcheselli L, et al. Rituximab and the risk of transformation of follicular lymphoma: a retrospective pooled analysis. *Lancet Haematol*. 2018;5(8):e359–e367.
50. Dreyling M, Ghielmini M, Rule S, et al. Newly diagnosed and relapsed follicular lymphoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2021;32(3):298–308.
51. Cheson BD, Fisher RI, Barrington SF, et al. Recommendations for initial evaluation, staging, and response assessment of Hodgkin and non-Hodgkin lymphoma: the Lugano classification. *J Clin Oncol*. 2014;32(27):3059–3068.
52. Brady JL, Binkley MS, Hajj C, et al. Definitive radiotherapy for localized follicular lymphoma staged by ^{18}F-FDG PET-CT: a collaborative study by ILROG. *Blood*. 2019;133(3):237–245.

53. Ng SP, Khor R, Bressel M, et al. Outcome of patients with early-stage follicular lymphoma staged with ^{18} F-Fluorodeoxyglucose (FDG) positron emission tomography (PET) and treated with radiotherapy alone. *Eur J Nucl Med Mol Imaging*. 2019;46(1):80–86.
54. Elstrom R, Guan L, Baker G, et al. Utility of FDG-PET scanning in lymphoma by WHO classification. *Blood*. 2003;101(10):3875–3876.
55. Bachy E, Houot R, Morschhauser F, et al. Long-term follow up of the FL2000 study comparing CHVP-interferon to CHVP-interferon plus rituximab in follicular lymphoma. *Haematologica*. 2013;98(7):1107–1114.
56. Casulo C, Byrtek M, Dawson KL, et al. Early relapse of follicular lymphoma after rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone defines patients at high risk for death: an analysis from the National LymphoCare Study. *J Clin Oncol*. 2015;33(23):2516–2522.
57. Solal-Céligny P, Roy P, Colombat P, et al. Follicular lymphoma international prognostic index. *Blood*. 2004;104(5):1258–1265.
58. Federico M, Bellei M, Marcheselli L, et al. Follicular lymphoma international prognostic index 2: a new prognostic index for follicular lymphoma developed by the international follicular lymphoma prognostic factor project. *J Clin Oncol*. 2009;27(27):4555–4562.
59. Marcus R, Davies A, Ando K, et al. Obinutuzumab for the first-line treatment of follicular lymphoma. *New Engl J Med*. 2017;377(14):1331–1344.
60. Jurinovic V PV, Oestergaard MZ, et al. Evaluation of the m7-FLIPI in patients with follicular lymphoma treated within the gallium trial: EZH2 mutation status may be a predictive marker for differential efficacy of chemotherapy #0122. Presented at the 61st Annual Meeting of the American Society of Haematology, Orlando, Florida. 2019.
61. Lockmer S, Ren W, Brodtkorb M, et al. M7-FLIPI is not prognostic in follicular lymphoma patients with first-line rituximab chemo-free therapy. *Br J Haematol*. 2020;188(2):259–267.
62. Huet S, Tesson B, Jais JP, et al. A gene-expression profiling score for prediction of outcome in patients with follicular lymphoma: a retrospective training and validation analysis in three international cohorts. *Lancet Oncol*. 2018;19(4):549–561.
63. Meignan M, Cottereau AS, Versari A, et al. Baseline metabolic tumor volume predicts outcome in high-tumor-burden follicular lymphoma: a pooled analysis of three multicenter studies. *J Clin Oncol*. 2016;34(30):3618–3626.
64. Bachy E, Maurer MJ, Habermann TM, et al. A simplified scoring system in de novo follicular lymphoma treated initially with immunochemotherapy. *Blood*. 2018;132(1):49–58.
65. Montoto S, Fitzgibbon J. Transformation of indolent B-cell lymphomas. *J Clin Oncol*. 2011;29(14):1827–1834.
66. MacManus MP, Hoppe RT. Is radiotherapy curative for stage I and II low-grade follicular lymphoma? Results of a long-term follow-up study of patients treated at Stanford University. *J Clin Oncol*. 1996;14(4):1282–1290.
67. Hoppe RT, Kushlan P, Kaplan HS, et al. The treatment of advanced stage favorable histology non-Hodgkin's lymphoma: a preliminary report of a randomized trial comparing single agent chemotherapy, combination chemotherapy, and whole body irradiation. *Blood*. 1981;58(3):592–598.
68. Young RC, Longo DL, Glatstein E, et al. The treatment of indolent lymphomas: watchful waiting v aggressive combined modality treatment. *Semin Hematol*. 1988;25(2 Suppl 2):11–16.
69. Brice P, Bastion Y, Lepage E, et al. Comparison in low-tumor-burden follicular lymphomas between an initial no-treatment policy, prednimustine, or interferon alfa: a randomized study from the Groupe d'Etude des Lymphomes Folliculaires. Groupe d'Etude des Lymphomes de l'Adulte. *J Clin Oncol*. 1997;15(3):1110–1117.
70. Ardeshna KM, Smith P, Norton A, et al. Long-term effect of a watch and wait policy versus immediate systemic treatment for asymptomatic advanced-stage non-Hodgkin lymphoma: a randomised controlled trial. *Lancet*. 2003;362(9383):516–522.
71. Dana BW, Dahlberg S, Nathwani BN, et al. Long-term follow-up of patients with low-grade malignant lymphomas treated with doxorubicin-based chemotherapy or chemoimmunotherapy. *J Clin Oncol*. 1993;11(4):644–651.
72. Ardeshna KM, Qian WD, Smith P, et al. Rituximab versus a watch-and-wait approach in patients with advanced-stage, asymptomatic, non-bulky follicular lymphoma: an open-label randomised phase 3 trial. *Lancet Oncol*. 2014;15(4):424–435.
73. Dave SS, Wright G, Tan B, et al. Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. *N Engl J Med*. 2004;351(21):2159–2169.
74. Casulo C, Le-Rademacher J, Dixon J, et al. Validation of POD24 as a robust early clinical endpoint of poor survival in follicular lymphoma: results from the Follicular Lymphoma Analysis of Surrogacy Hypothesis (FLASH) investigation using individual data from 5,453 patients on 13 clinical trials. *Blood*. 2017;130(Supplement 1):412.
75. Friedberg JW, Huang J, Dillon H, et al. Initial therapeutic strategy in follicular lymphoma (FL): An analysis from the National LymphoCare Study (NLCS). *J Clin Oncol*. 2006;24(18). 428s-s.
76. Cheson BD, Pfistner B, Juweid ME, et al. Revised response criteria for malignant lymphoma. *J Clin Oncol*. 2007;25(5):579–586.
77. Marcus R, Imrie K, Belch A, et al. CVP chemotherapy plus rituximab compared with CVP as first-line treatment for advanced follicular lymphoma. *Blood*. 2005;105(4):1417–1423.
78. Hiddemann W, Kneba M, Dreyling M, et al. Frontline therapy with rituximab added to the combination of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) significantly improves the outcome for patients with advanced-stage follicular lymphoma compared with therapy with CHOP alone: results of a prospective randomized study of the German Low-Grade Lymphoma Study Group. *Blood*. 2005;106(12):3725–3732.
79. Hochster H, Weller E, Gascoyne RD, et al. Maintenance rituximab after cyclophosphamide, vincristine, and prednisone prolongs progression-free survival in advanced indolent lymphoma: results of the randomized phase III ECOG1496 Study. *J Clin Oncol*. 2009;27(10):1607–1614.
80. Ghielmini M, Hsu Schmitz SF, Cogliatti SB, et al. Prolonged treatment with rituximab in patients with follicular lymphoma significantly increases event-free survival and response duration compared with the standard weekly x 4 schedule. *Blood*. 2004;103(12):4416–4423.
81. Friedberg JW, Taylor MD, Cerhan JR, et al. Follicular lymphoma in the United States: first report of the national LymphoCare study. *J Clin Oncol*. 2009;27(8). 1202-.
82. Ketterer N, Salles G, Moullet I, et al. Factors associated with successful mobilization of peripheral blood progenitor cells in 200 patients with lymphoid malignancies. *Br J Haematol*. 1998;103(1):235–242.
83. Rummel MJ, Niederle N, Maschmeyer G, et al. Bendamustine plus rituximab versus CHOP plus rituximab as first-line treatment for patients with indolent and mantle-cell lymphomas: an open-label, multicentre, randomised, phase 3 non-inferiority trial. *Lancet*. 2013;381(9873):1203–1210.
84. Rummel M, Kaiser U, Balser C, et al. Bendamustine plus rituximab versus fludarabine plus rituximab for patients with relapsed indolent and mantle-cell lymphomas: a multicentre, randomised, open-label, non-inferiority phase 3 trial. *Lancet Oncol*. 2016;17(1):57–66.
85. Morschhauser F, Fowler NH, Feugier P, et al. Rituximab plus lenalidomide in advanced untreated follicular lymphoma. *N Engl J Med*. 2018;379(10):934–947.
86. Portlock CS, Rosenberg SA, Glatstein E, et al. Treatment of advanced non-Hodgkin's lymphomas with favorable histologies: preliminary results of a prospective trial. *Blood*. 1976;47(5):747–756.
87. Gallagher CJ, Gregory WM, Jones AE, et al. Follicular lymphoma: prognostic factors for response and survival. *J Clin Oncol*. 1986;4(10):1470–1480.
88. Lister TA, Cullen MH, Beard MEJ, et al. Comparison of combined and single-agent chemotherapy in non-hodgkins lymphoma of favourable histological type. *Br Med J*. 1978;1(6112):533–537.
89. Peterson BA, Petroni GR, Frizzera G, et al. Prolonged single-agent versus combination chemotherapy in indolent follicular lymphomas: a study of the cancer and leukemia group B. *J Clin Oncol*. 2003;21(1):5–15.
90. Al-Tourah AJ, Gill KK, Hoskins PJ, et al. The impact of initial treatment of advanced stage indolent lymphoma on the risk of transformation. *J Clin Oncol*. 2006;24(18). 424s-s.
91. Zinzani PL, Magagnoli M, Moretti L, et al. Randomized trial of fludarabine versus fludarabine and idarubicin as frontline treatment in patients with indolent or mantle-cell lymphoma. *J Clin Oncol*. 2000;18(4):773–779.
92. Hagenbeek A, Eghbali H, Monfardini S, et al. Phase III intergroup study of fludarabine phosphate compared with cyclophosphamide, vincristine, and prednisone chemotherapy in newly diagnosed patients with stage III and IV low-grade malignant non-Hodgkin's lymphoma. *J Clin Oncol*. 2006;24(10):1590–1596.
93. Hochster HS, Oken MM, Winter JN, et al. Phase I study of fludarabine plus cyclophosphamide in patients with previously untreated low-grade lymphoma: Results and long-term follow-up—a report from the Eastern Cooperative Oncology Group. *J Clin Oncol*. 2000;18(5):987–994.
94. Velasquez WS, Lew D, Grogan TM, et al. Combination of fludarabine and mitoxantrone in untreated stages III and IV low-grade lymphoma: S9501. *J Clin Oncol*. 2003;21(10):1996–2010.
95. Zinzani PL, Pulsoni A, Perrotti A, et al. Fludarabine plus mitoxantrone with and without rituximab versus CHOP with and without rituximab as front-line treatment for patients with follicular lymphoma. *J Clin Oncol*. 2004;22(13):2654–2661.
96. Rummel MJ, Al-Batran SE, Kim SZ, et al. Bendamustine plus rituximab is effective and has a favorable toxicity profile in the treatment of mantle cell and low-grade non-Hodgkin's lymphoma. *J Clin Oncol*. 2005;23(15): 3383–3389.
97. Cheson BD, Friedberg JW, Kahl BS, et al. Bendamustine produces durable responses with an acceptable safety profile in patients with rituximab-refractory indolent non-Hodgkin lymphoma. *Clin Lymphoma Myeloma Leuk*. 2010;10(6):452–457.

98. Smalley RV, Weller E, Hawkins MJ, et al. Final analysis of the ECOG I-COPA trial (E6484) in patients with non-Hodgkin's lymphoma treated with interferon alfa (IFN-alpha2a) plus an anthracycline-based induction regimen. *Leukemia*. 2001;15(7):1118–1122.
99. Smalley RV, Andersen JW, Hawkins MJ, et al. Interferon alfa combined with cytotoxic chemotherapy for patients with non-Hodgkin's lymphoma. *N Engl J Med*. 1992;327(19):1336–1341.
100. Rohatiner AZS, Gregory WM, Peterson B, et al. Meta-analysis to evaluate the role of interferon in follicular lymphoma. *J Clin Oncol*. 2005;23(10): 2215–2223.
101. Fisher RI, Dana BW, LeBlanc M, et al. Interferon alpha consolidation after intensive chemotherapy does not prolong the progression-free survival of patients with low-grade non-Hodgkin's lymphoma: results of the Southwest Oncology Group randomized phase III study 8809. *J Clin Oncol*. 2000;18(10):2010–2016.
102. (rituximab) FaDAltfR. http://www.fda.gov/cder/foi/label/2006/103705s5230-s5231lbl.pdf. Accessed August 23, 2021.
103. Mabthera. EMAPARf. http://www.emea.eu.int/humandocs/Humans/EPAR/mabthera/mabthera.htm. Accessed August 23, 2021.
104. Fisher RI, LeBlanc M, Press OW, et al. New treatment options have changed the survival of patients with follicular lymphoma. *J Clin Oncol*. 2005;23(33):8447–8452.
105. Hiddemann W, Hoster E, Buske C, et al. Rituximab is the essential treatment modality that underlies the significant improvement in short and long term outcome of patients with advanced stage follicular lymphoma—A 10 year analysis of GLSG trials. *Blood*. 2006;108(11). 147a-a.
106. Swenson WT, Wooldridge JE, Lynch CF, et al. Improved survival of follicular lymphoma patients in the United States. *J Clin Oncol*. 2005;23(22):5019–5026.
107. Maloney DG, Grillo-López AJ, Bodkin DJ, et al. IDEC-C2B8: Results of a phase I multiple-dose trial in patients with relapsed non-Hodgkin's lymphoma. *J Clin Oncol*. 1997;15(10):3266–3274.
108. McLaughlin P, Grillo-López AJ, Link BK, et al. Rituximab chimeric Anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J Clin Oncol*. 1998;16(8):2825–2833.
109. Davis TA, White CA, Grillo-López AJ, et al. Single-agent monoclonal antibody efficacy in bulky non-Hodgkin's lymphoma: Results of a phase II trial of rituximab. *J Clin Oncol*. 1999;17(6):1851–1857.
110. McLaughlin P, Hagemeister FB, Grillo-López AJ. Rituximab in indolent lymphoma: the single-agent pivotal trial. *Semin Oncol*. 1999;26(5 Suppl 14):79–87.
111. Colombat P, Salles G, Brousse N, et al. Rituximab (anti-CD20 monoclonal antibody) as single first-line therapy for patients with follicular lymphoma with a low tumor burden: clinical and molecular evaluation. *Blood*. 2001;97(1):101–106.
112. Colombat P, Brousse N, Salles G, et al. Rituximab induction immunotherapy for first-line low-tumor-burden follicular lymphoma: survival analyses with 7-year follow-up. *Ann Oncol*. 2012;23(9):2380–2385.
113. Piro LD, White CA, Grillo-López AJ, et al. Extended Rituximab (anti-CD20 monoclonal antibody) therapy for relapsed or refractory low-grade or follicular non-Hodgkin's lymphoma. *Ann Oncol*. 1999;10(6):655–661.
114. Davis TA, Grillo-López AJ, White CA, et al. Rituximab anti-CD20 monoclonal antibody therapy in non-Hodgkin's lymphoma: safety and efficacy of re-treatment. *J Clin Oncol*. 2000;18(17):3135–3143.
115. Hochster HS, Weller E, Gascoyne RD, et al. Maintenance rituximab after CVP results in superior clinical outcome in advanced follicular lymphoma (FL): results of the E1496 phase III trial from the Eastern cooperative oncology group and the cancer and leukemia group. *Blood*. 2005;106(11). 106a-a.
116. Forstpointner R, Unterhalt M, Dreyling M, et al. Maintenance therapy with rituximab leads to a significant prolongation of response duration after salvage therapy with a combination of rituximab, fludarabine, cyclophosphamide, and mitoxantrone (R-FCM) in patients with recurring and refractory follicular and mantle cell lymphomas: Results of a prospective randomized study of the German Low Grade Lymphoma Study Group (GLSG). *Blood*. 2006;108(13):4003–4008.
117. Hainsworth JD, Litchy S, Shaffer DW, et al. Maximizing therapeutic benefit of rituximab: maintenance therapy versus re-treatment at progression in patients with indolent non-Hodgkin's lymphoma—a randomized phase II trial of the Minnie Pearl Cancer Research Network. *J Clin Oncol*. 2005;23(6):1088–1095.
118. Martinelli G, Schmitz SFH, Utiger U, et al. Long-term follow-up of patients with follicular lymphoma receiving single-agent rituximab at two different schedules in trial SAKK 35/98. *J Clin Oncol*. 2010;28(29): 4480–4484.
119. Salles G, Seymour JF, Offner F, et al. Rituximab maintenance for 2 years in patients with high tumour burden follicular lymphoma responding to rituximab plus chemotherapy (PRIMA): a phase 3, randomised controlled trial. *Lancet*. 2011;377(9759):42–51.
120. van Oers MHJ, Klasa R, Marcus RE, et al. Rituximab maintenance improves clinical outcome of relapsed/resistant follicular non-Hodgkin lymphoma in patients both with and without rituximab during induction: results of a prospective randomized phase 3 intergroup trial. *Blood*. 2006;108(10):3295–3301.
121. Czuczman MS, Grillo-López AJ, White CA, et al. Treatment of patients with low-grade B-cell lymphoma with the combination of chimeric anti-CD20 monoclonal antibody and CHOP chemotherapy. *J Clin Oncol*. 1999;17(1):268–276.
122. Czuczman MS, Koryzna A, Mohr A, et al. Rituximab in combination with fludarabine chemotherapy in low-grade or follicular lymphoma. *J Clin Oncol*. 2005;23(4):694–704.
123. Herold M, Haas A, Srock S, et al. Addition of rituximab to first-line MCP (mitoxantrone, chlorambucil, prednisolone) chemotherapy prolongs survival in advanced follicular lymphoma—4 year follow-up results of a phase III trial of the east German study group hematology and oncology (OSHO#39). *Blood*. 2006;108(11). 147a-a.
124. Foussard C, Mounier N, Van Hoof A, et al. Update of the FL2000 randomized trial combining rituximab to CHVP-Interferon in follicular lymphoma (FL) patients (pts). *J Clin Oncol*. 2006;24(18). 424s-s.
125. Schulz H, Bohlius J, Skoetz N, et al. Combined immunochemotherapy with rituximab improves overall survival in patients with follicular and mantle cell lymphoma: Updated meta-analysis results. *Blood*. 2006;108(11). 781a-a.
126. Lewis G, Marcus RE, Proctor SJ, et al. The cost-effectiveness of rituximab, cyclophosphamide, vincristine and prednisolone (RCVP) compared with CVP for the treatment of follicular non-Hodgkin's lymphoma (NHL) in the UK. *Blood*. 2006;108(11). 107a-a.
127. Witzig TE, White CA, Gordon LI, et al. Safety of yttrium-90 ibritumomab tiuxetan radioimmunotherapy for relapsed low-grade, follicular, or transformed non-Hodgkin's lymphoma. *J Clin Oncol*. 2003;21(7): 1263–1270.
128. Fisher RI, Kaminski MS, Wahl RL, et al. Tositumomab and iodine-131 tositumomab produces durable complete remissions in a subset of heavily pretreated patients with low-grade and transformed non-Hodgkin's lymphomas. *J Clin Oncol*. 2005;23(30):7565–7573.
129. Kaminski MS, Tuck M, Estes J, et al. 131I-tositumomab therapy as initial treatment for follicular lymphoma. *N Engl J Med*. 2005;352(5):441–449.
130. Kaminski MS, Radford JA, Gregory SA, et al. Re-treatment with I-131 tositumomab in patients with non-Hodgkin's lymphoma who had previously responded to I-131 tositumomab. *J Clin Oncol*. 2005;23(31):7985–7993.
131. Press OW, Unger JM, Braziel RM, et al. Phase II trial of CHOP chemotherapy followed by tositumomab/iodine I-131 tositumomab for previously untreated follicular non-Hodgkin's lymphoma: five-year follow-up of Southwest Oncology Group protocol S9911. *J Clin Oncol*. 2006;24(25):4143–4149.
132. Witzig TE, Flinn IW, Gordon LI, et al. Treatment with ibritumomab tiuxetan radioimmunotherapy in patients with rituximab-refractory follicular non-Hodgkin's lymphoma. *J Clin Oncol*. 2002;20(15): 3262–3269.
133. Freedman AS, Gribben JG, Neuberg D, et al. High-dose therapy and autologous bone marrow transplantation in patients with follicular lymphoma during first remission. *Blood*. 1996;88(7):2780–2786.
134. Horning SJ, Negrin RS, Hoppe RT, et al. High-dose therapy and autologous bone marrow transplantation for follicular lymphoma in first complete or partial remission: results of a phase II clinical trial. *Blood*. 2001;97(2):404–409.
135. Sebban C, Mounier N, Brousse N, et al. Standard chemotherapy with interferon compared with CHOP followed by high-dose therapy with autologous stem cell transplantation in untreated patients with advanced follicular lymphoma: the GELF-94 randomized study from the Groupe d'Etude des Lymphomes de l'Adulte (GELA). *Blood*. 2006;108(8): 2540–2544.
136. Deconinck E, Foussard C, Milpied N, et al. High-dose therapy followed by autologous purged stem-cell transplantation and doxorubicin-based chemotherapy in patients with advanced follicular lymphoma: a randomized multicenter study by GOELAMS. *Blood*. 2005;105(10):3817–3823.
137. Lenz G, Dreyling M, Schiegnitz E, et al. Myeloablative radiochemotherapy followed by autologous stem cell transplantation in first remission prolongs progression-free survival in follicular lymphoma: results of a prospective, randomized trial of the German Low-Grade Lymphoma Study Group. *Blood*. 2004;104(9):2667–2674.
138. Al Khabori M, de Almeida JR, Guyatt GH, et al. Autologous stem cell transplantation in follicular lymphoma: a systematic review and meta-analysis. *J Natl Cancer Inst*. 2012;104(1):18–28.

139. Cheson BD, Chua N, Mayer J, et al. Overall survival benefit in patients with rituximab-refractory indolent non-Hodgkin lymphoma who received obinutuzumab plus bendamustine induction and obinutuzumab maintenance in the GADOLIN study. *J Clin Oncol*. 2018;36(22):2259–2266.
140. Leonard JP, Trneny M, Izutsu K, et al. AUGMENT: a phase III study of lenalidomide plus rituximab versus placebo plus rituximab in relapsed or refractory indolent lymphoma. *J Clin Oncol*. 2019;37(14):1188–1199.
141. Gopal AK, Kahl BS, de Vos S, et al. PI3Kdelta inhibition by idelalisib in patients with relapsed indolent lymphoma. *N Engl J Med*. 2014;370(11):1008–1018.
142. Bartlett NL, Costello BA, LaPlant BR, et al. Single-agent ibrutinib in relapsed or refractory follicular lymphoma: a phase 2 consortium trial. *Blood*. 2018;131(2):182–190.
143. Zinzani PL, Flinn IW, Yuen SLS, et al. Venetoclax-rituximab with or without bendamustine vs bendamustine-rituximab in relapsed/refractory follicular lymphoma. *Blood*. 2020;136(23):2628–2637.
144. Davids MS, Roberts AW, Seymour JF, et al. Phase I first-in-human study of venetoclax in patients with relapsed or refractory non-Hodgkin lymphoma. *J Clin Oncol*. 2017;35(8):826–833.
145. Ogura M, Ando K, Suzuki T, et al. A multicentre phase II study of vorinostat in patients with relapsed or refractory indolent B-cell non-Hodgkin lymphoma and mantle cell lymphoma. *Br J Haematol*. 2014;165(6):768–776.
146. Mondello P, Tadros S, Teater M, et al. Selective inhibition of HDAC3 targets synthetic vulnerabilities and activates immune surveillance in lymphoma. *Cancer Discov*. 2020
147. Morschhauser F, Tilly H, Chaidos A, et al. Tazemetostat for patients with relapsed or refractory follicular lymphoma: an open-label, single-arm, multicentre, phase 2 trial. *Lancet Oncol*. 2020;21(11):1433–1442.
148. Gribben JG, Fowler N, Morschhauser F. Mechanisms of action of lenalidomide in B-cell non-Hodgkin lymphoma. *J Clin Oncol*. 2015;33(25):2803–2811.
149. Roemer MG, Advani RH, Redd RA, et al. Classical Hodgkin lymphoma with reduced β2M/MHC class I expression is associated with inferior outcome independent of 9p24.1 status. *Cancer Immunol Res*. 2016;4(11):910–916.
150. Armand P, Janssens A, Gritti G, et al. Efficacy and safety results from CheckMate 140, a phase 2 study of nivolumab for relapsed/refractory follicular lymphoma. *Blood*. 2021;137(5):637–645.
151. Batlevi CL, Matsuki E, Brentjens RJ, et al. Novel immunotherapies in lymphoid malignancies. *Nat Rev Clin Oncol*. 2016;13(1):25–40.
152. Chao MP, Alizadeh AA, Tang C, et al. Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. *Cell*. 2010;142(5):699–713.
153. Advani R, Flinn I, Popplewell L, et al. CD47 Blockade by Hu5F9-G4 and rituximab in non-Hodgkin's lymphoma. *N Engl J Med*. 2018;379(18): 1711–1721.
154. Assouline S KW, Sehn L et al. Mosunetuzumab shows promising efficacy in patients with multiply relapsed follicular lymphoma: updated clinical experience from a phase I dose-escalation trial presented at the 62nd annual meeting of the American Society of Haematology. 2020.
155. Bannerji R, Allan JN, Arnason JE, et al. Odronextamab (REGN1979), a human CD20 x CD3 bispecific antibody, induces durable, complete responses in patients with highly refractory B-cell non-Hodgkin lymphoma, including patients refractory to CAR T therapy. *Blood*. 2020:136.
156. Schuster SJ. Bispecific antibodies for the treatment of lymphomas: promises and challenges. *Hematol Oncol*. 2021;39(Suppl 1):113–116.
157. Freedman AS, Ritz J, Neuberg D, et al. Autologous bone marrow transplantation in 69 patients with a history of low-grade B-cell non-Hodgkin's lymphoma. *Blood*. 1991;77(11):2524–2529.
158. Freedman AS, Neuberg D, Mauch P, et al. Long-term follow-up of autologous bone marrow transplantation in patients with relapsed follicular lymphoma. *Blood*. 1999;94(10):3325–3333.
159. Apostolidis J, Gupta RK, Grenzelias D, et al. High-dose therapy with autologous bone marrow support as consolidation of remission in follicular lymphoma: long-term clinical and molecular follow-up. *J Clin Oncol*. 2000;18(3):527–536.
160. Gribben JG, Freedman AS, Neuberg D, et al. Immunologic purging of marrow assessed by PCR before autologous bone marrow transplantation for B-cell lymphoma. *N Engl J Med*. 1991;325(22):1525–1533.
161. Gribben JG, Neuberg D, Freedman AS, et al. Detection by polymerase chain reaction of residual cells with the bcl-2 translocation is associated with increased risk of relapse after autologous bone marrow transplantation for B-cell lymphoma. *Blood*. 1993;81(12):3449–3457.
162. Corradini P, Ladetto M, Zallio F, et al. Long-term follow-up of indolent lymphoma patients treated with high-dose sequential chemotherapy and autografting: evidence that durable molecular and clinical remission frequently can be attained only in follicular subtypes. *J Clin Oncol*. 2004;22(8):1460–1468.
163. Ladetto M, Vallet S, Benedetti F, et al. Prolonged survival and low incidence of late toxic sequelae in advanced follicular lymphoma treated with a TBI-free autografting program: updated results of the multicenter consecutive GITMO trial. *Leukemia*. 2006;20(10):1840–1847.
164. Stone RM. Myelodysplastic syndrome after autologous transplantation for lymphoma: the price of progress. *Blood*. 1994;83(12):3437–3440.
165. Schouten HC, Qian W, Kvaloy S, et al. High-dose therapy improves progression-free survival and survival in relapsed follicular non-Hodgkin's lymphoma: results from the randomized European CUP trial. *J Clin Oncol*. 2003;21(21):3918–3927.
166. van Besien K, Loberiza Jr. FR, Bajorunaite R, et al. Comparison of autologous and allogeneic hematopoietic stem cell transplantation for follicular lymphoma. *Blood*. 2003;102(10):3521–3529.
167. Toze CL, Barnett MJ, Connors JM, et al. Long-term disease-free survival of patients with advanced follicular lymphoma after allogeneic bone marrow transplantation. *Br J Haematol*. 2004;127(3):311–321.
168. Freytes CO, Loberiza FR, Rizzo JD, et al. Myeloablative allogeneic hematopoietic stem cell transplantation in patients who experience relapse after autologous stem cell transplantation for lymphoma: a report of the International Bone Marrow Transplant Registry. *Blood*. 2004;104(12):3797–3803.
169. Escalon MP, Champlin RE, Saliba RM, et al. Nonmyeloablative allogeneic hematopoietic transplantation: a promising salvage therapy for patients with non-Hodgkin's lymphoma whose disease has failed a prior autologous transplantation. *J Clin Oncol*. 2004;22(12):2419–2423.
170. Morris E, Thomson K, Craddock C, et al. Outcomes after alemtuzumab-containing reduced-intensity allogeneic transplantation regimen for relapsed and refractory non-Hodgkin lymphoma. *Blood*. 2004;104(13):3865–3871.
171. Gribben JG, Zahrieh D, Stephans K, et al. Autologous and allogeneic stem cell transplantations for poor-risk chronic lymphocytic leukemia. *Blood*. 2005;106(13):4389–4396.
172. Shea T, Johnson J, Westervelt P, et al. Reduced-intensity allogeneic transplantation provides high event-free and overall survival in patients with advanced indolent B cell malignancies: CALGB 109901. *Biol Blood Marrow Tr*. 2011;17(9):1395–1403.
173. Jacobson C, Chavez, J., Sehgal, A. et al. Primary analysis of Zuma-5: a phase 2 study of axicabtagene ciloleucel (Axi-Cel) in patients with relapsed/refractory (R/R) indolent non-Hodgkin lymphoma (iNHL) #700. Presented at the 62nd Annual Meeting of the American Society of Haematology 2020. 2020.
174. Paola Ghione AP, Sabella B, Kevin D. A comparison of clinical outcomes from ZUMA-5 and the international scholar-5 external control cohort in R/R FL. Presented at the EHA2021 Virtual Congress. 2021.
175. Fowler NH, Dickinson M, Dreyling M, et al. Efficacy and safety of tisagenlecleucel in adult patients with relapsed/refractory follicular lymphoma: interim analysis of the phase 2 Elara trial. *Blood*. 2020:136.
176. Abramson JS, Palomba ML, Gordon LI, et al. Lisocabtagene maraleucel for patients with relapsed or refractory large B-cell lymphomas (TRANSCEND NHL 001): a multicentre seamless design study. *Lancet*. 2020;396(10254):839–852.

CHAPTER 83

MARGINAL ZONE LYMPHOMAS (EXTRANODAL/MALT, SPLENIC, AND NODAL)

Samer Al Hadidi and Carlos A. Ramos

The term *marginal zone* refers to a histologic compartment located at the periphery of lymphoid follicles immediately outside their mantle zone.[1] The marginal zone is especially evident in the spleen, although identical areas have been observed in other lymphoid structures, including mesenteric lymph nodes and mucosa-associated lymphoid tissue (MALT) (Fig. 83.1).[2] Ordinarily, it is composed predominantly of B cells that are slightly larger than mantle zone lymphocytes and strongly positive for surface IgM, but weakly positive for IgD (in contrast to mantle zone cells, which are strongly positive for IgD).[3] Marginal zone B lymphocytes are thought to be involved in fast protective responses against pathogenic encapsulated bacteria that do not trigger classic T cell–dependent humoral immunity.[4] They are also assumed to be the physiologic counterpart of a group of indolent non-Hodgkin lymphomas (NHLs) that are currently referred to as *marginal zone lymphomas (MZLs)*.

Although MZLs share a common denomination, arising from histologic similarities, new genetic findings and longer follow-up studies have established that the currently recognized subtypes of MZLs, initially described in the Revised European-American Lymphoma (REAL) classification, are different diseases.[5] In the most recent 2016 WHO classification of tumors of the hematopoietic and lymphoid tissues,[6,7] MZLs comprise three distinct entities: extranodal MZL (ENMZL) of MALT, or MALT lymphoma; splenic MZL (SMZL) with or without villous lymphocytes; and nodal MZL (NMZL) with or without monocytoid B cells. Immunoproliferative small intestinal disease (IPSID), also known as alpha heavy chain disease, Seligmann disease, or Mediterranean lymphoma, has been recognized as a variant of ENMZL.[8]

As in other hematological malignancies, a precursor state for MZLs has been recognized. Clonal B-cell lymphocytosis of marginal zone origin (CBL-MZ) is a condition where clonal B cells appear in the blood before the onset of overt MZL.[9,10] The circulating B cells have phenotypic features consistent with a marginal zone origin, but there is absence of clinical symptoms, splenomegaly, hepatomegaly, or lymphadenopathy. In a subset of patients (15% to 20%), CBL-MZ will progress to an overt MZL, most often SMZL.[9,10]

In aggregate, MZLs represent approximately 10% of all NHL.[11] Clinically, they behave indolently and have a prolonged course. Therefore, management strategies share similarity with other low-grade lymphomas,[12] although specific biologic characteristics and particular pathophysiologic mechanisms determine unique therapeutic approaches in some of the subtypes. This chapter will summarize the clinical characteristics and current management strategies for the different types of MZLs.

INITIAL EVALUATION OF MARGINAL ZONE LYMPHOMA

Adequate biopsy of a lymph node or a suspicious mass, obtained by endoscopic or conventional means, is preferred. A review by a hematopathologist with expertise in the field is essential to establish the diagnosis. In patients without easily accessible nodes or masses, computed tomography (CT) or ultrasound-guided core needle biopsy is usually well tolerated and may be adequate for diagnosis. Sufficient tissue must be obtained for proper histologic examination and required immunophenotypic and genetic studies. Hence, fine-needle aspiration (FNA) is not appropriate for diagnosis.

Physical examination with special attention to peripheral lymph nodes and the abdomen to look for splenomegaly should be performed. Initial laboratory evaluation should include a complete blood count with evaluation of a peripheral blood smear and basic biochemical studies, including lactate dehydrogenase (LDH) level, which is an important prognostic factor and a potential indicator of transformation from indolent to aggressive lymphoma. Antiglobulin tests and reticulocyte count may be useful to rule out autoimmune hemolytic anemia. Serum protein electrophoresis and immunofixation may demonstrate a monoclonal immunoglobulin. β_2-microglobulin levels may have prognostic value[13] as in other indolent lymphomas. Viral hepatitis (B and C) and human immunodeficiency virus (HIV) studies to exclude co-existing infections that affect therapeutic approaches should be obtained.

Depending on the primary site of disease, specific procedures may be indicated. Gastrointestinal MZLs may require repeat staging endoscopies, during which an adequate number of biopsies should be obtained. For gastric ENMZL, the most commonly affected site, the European Gastro-Intestinal Lymphoma Study (EGILS) group recommends a mapping procedure with a minimum of 10 biopsies taken from visible lesions and additional ones from macroscopically normal mucosa; the same procedure should be repeated to assess treatment response.[14–20] Since endoscopic biopsies are not transmural, endoscopic ultrasound (EUS) is a useful way to assess the depth of involvement, which has prognostic implications.[12–17] Moreover, EUS can be helpful to assess regional lymph node involvement. For head primaries, such as ocular adnexal ENMZL, appropriate directed examination and imaging studies (CT or magnetic resonance) are indicated. Routine endoscopy is suggested for patients with non-gastric MZLs, especially females with primary involvement of upper airways, salivary glands, or lungs, and in patients with high β_2-microglobulin levels or *Helicobacter pylori (H. pylori)* infection regardless of primary MZL site.[21,22]

STAGING OF MARGINAL ZONE LYMPHOMA

Imaging studies of the chest, abdomen, and pelvis, usually CT scans, should be obtained to adequately stage the disease. A positron emission tomography (PET) scan has been increasingly used for staging and evaluation of response to therapy in NHL, but it may be less useful in MZL because up to 60% of these lymphomas may be PET-negative, especially in early-stage gastric ENZML.[23] Some MZL series, however, document a PET-positivity rate of up to 80%,[24–28] with better detection rates seen for head and neck and bronchial versus gastric or ocular MZL.[29] The use of PET scan is particularly important in patients with possible transformation to high-grade pathology. Also for staging, and occasionally for diagnostic purposes, bone marrow aspirate (with morphology and flow cytometry) and biopsy are usually advocated, even in cases in which the likelihood of systemic disease is low, because any positive result has important implications for therapy. The European Society for Medical Oncology (ESMO) clinical practice guidelines for diagnosis of MZLs mandate bone marrow examination for NMZL and SMZL, while they highly recommend it in ENMZL, especially in the non-gastric type and when only local therapy is planned.[30]

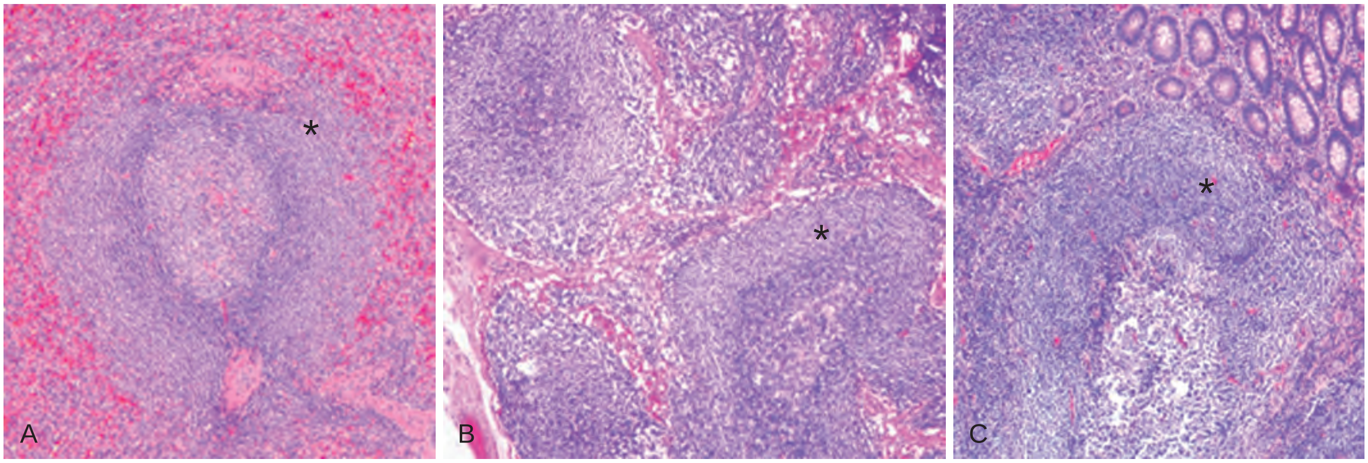


Figure 83.1 NORMAL MARGINAL ZONE CELLS. (A) Marginal zone cells are seen most readily in sections from the normal spleen. The splenic white pulp typically has three distinctive layers: the germinal center, the mantle zone, and external to this, the marginal zone (see *asterisk*). (B) Marginal zone cells are not usually seen in lymph nodes, but for some reason are sometimes present in mesenteric lymph nodes (see *asterisk*). They have a similar appearance to those in the spleen. (C) In the gastrointestinal tract, the lymphoid tissue in Peyer's patches is believed to have a marginal zone equivalent (see *asterisk*). The cells are again external to the mantle zone and are believed to traffic between the epithelium and the lymphoid follicle.

TABLE 83.1 **Staging Systems for Gastrointestinal Lymphomas**

Adapted Ann Arbor System[31]	Lugano System[32]	Paris System[33]	Areas Involved[a]
IE1	I_1	T1 N0 M0	Mucosa to submucosa
IE2	I_2	T2 N0 M0	To muscularis propria or subserosa
		T3 N0 M0	To serosa
	IIE	T4 N0 M0	To adjacent organs
IIE1	II_1	T1-4 N1 M0	Regional lymph nodes[b]
IIE2	II_2	T1-4 N2 M0	Non-regional abdominal lymph nodes
IIIE	IV	T1-4 N3 M0	Extra-abdominal lymph nodes
IV		T1-4 N0-3 M1	Distant organs
		B1	Bone marrow

[a] In case of more than one visible lesion synchronously originating in the gastrointestinal tract, select the characteristics of the more advanced lesion.
[b] Anatomic designation of lymph nodes as *regional* according to site: (1) stomach: perigastric nodes and those located along the ramifications of the celiac artery (i.e., left gastric artery, common hepatic artery, splenic artery); (2) duodenum: pancreaticoduodenal, pyloric, hepatic, and superior mesenteric nodes; (3) jejunum/ileum: mesenteric nodes and, for the terminal ileum only, the ileocolic as well as the posterior cecal nodes; (4) colorectum: pericolic and perirectal nodes and those located along the ileocolic, right, middle, and left colic, inferior mesenteric, superior rectal, and internal iliac arteries.

Staging of MZL is similar to that of other lymphomas; the Ann Arbor system, or an adapted version,[31] is used most frequently (Table 83.1). Nonetheless, specific staging systems have been adopted for particular sites. Gastrointestinal MZL is often staged according to a modified Ann Arbor scheme, commonly known as the Lugano staging system,[32] which incorporates indices corresponding to depth of mucosal invasion and proximity of affected lymph nodes to the primary lesion (see Table 83.1). Of note, the Lugano system has no stage III, and its stage IIE may refer to lesions that extend by contiguity to adjacent organs and not necessarily to secondary involvement of lymph nodes. Since the dissemination patterns of extranodal lymphomas are essentially different from those of primary nodal lymphomas, the EGILS group has proposed a new staging system (the Paris staging system)[33] that is based on the TNM scheme used for solid tumors (see Table 83.1). The International Society for Cutaneous Lymphomas (ISCL) and the cutaneous lymphoma task force of the European Organization of Research and Treatment of Cancer (EORTC) have also suggested a new, TNM-based staging system for cutaneous lymphomas other than mycosis fungoides and Sézary syndrome,[34] which may be used for the cutaneous forms of ENMZL (Table 83.2).

EXTRANODAL MARGINAL ZONE LYMPHOMA OF MUCOSA-ASSOCIATED LYMPHOID TISSUE TYPE

Epidemiology and Manifestations

ENMZL is the most frequent of the MZL subtypes, accounting for approximately 8% of all NHL and two-thirds of all MZLs.[11] An analysis of Surveillance, Epidemiology, and End Results (SEER) data quotes a yearly incidence rate of 1.59 per 100,000 adults in the United States.[35] The median age of presentation is around 60, with a wide range spanning from the third to the ninth decades, and there is a slight female predominance (55%).[36] In contrast to most other indolent lymphomas, ENMZL frequently presents at a localized stage (≈40% stage I and ≈30% stage II), and the risk for systemic dissemination is low (albeit variable depending on primary location), which has important implications for the choice of initial therapy.

The most commonly affected primary site is the mucosa of the gastrointestinal tract, in particular the stomach (approximately 44% of all ENMZL cases, with disease being usually multifocal) followed by the small intestine (≈7%). Ocular structures are also frequently involved (≈12%), namely the orbit (≈40% of all ocular adnexal ENMZL), the conjunctiva (35% to 40%), the lacrimal glands (10% to 15%), and the eyelids (≈10%).[37] Other commonly affected sites include the bronchial mucosa (≈11% of all ENMZL cases), the skin (≈9%), the salivary glands (≈6%), and the thyroid gland (≈6%). Sites that are more rarely reported include Waldeyer pharyngeal lymphoid ring, breast, liver, pancreas, urogenital tract, and central nervous system.[38–41] Findings at presentation depend on the specific organ affected. Gastric ENMZL may lead to dyspepsia, epigastric pain, nausea, anorexia, and manifestations of gastrointestinal bleeding.[42] Conjunctival ENMZL often forms a painless nodule or plaque that has a "salmon-pink patch" appearance and can be associated with erythema, chemosis, and foreign-body sensation.[43] Primary cutaneous

TABLE 83.2 International Society for Cutaneous Lymphoma/European Organization of Research and Treatment of Cancer Staging System for Cutaneous Lymphomas Other Than Mycosis Fungoides and Sézary Syndrome

Parameter[a]	Description
T	
T1	Solitary skin involvement
T1a	Lesion <5 cm diameter
T1b	Lesion >5 cm diameter
T2	Regional skin involvement: multiple lesions limited to 1 body region or 2 contiguous body regions[b]
T2a	All-disease-encompassing in a <15 cm diameter circular area
T2b	All-disease-encompassing in a >15 and <30 cm diameter circular area
T2c	All-disease-encompassing in a >30 cm diameter circular area
T3	Generalized skin involvement
T3a	Multiple lesions involving 2 noncontiguous body regions
T3b	Multiple lesions involving ≥3 body regions
N	
N0	No clinical or pathologic lymph node involvement
N1	Involvement of 1 peripheral lymph node region that drains an area of current or prior skin involvement
N2	Involvement of 2 or more peripheral lymph node regions or involvement of any lymph node region[c] that does not drain an area of current or prior skin involvement
N3	Involvement of central lymph nodes
M	
M0	No evidence of extracutaneous nonlymph node disease
M1	Extracutaneous nonlymph node disease present

[a] The ISCL/EORTC proposes to defer any stage groupings of the TNM classification until further information is available to validate specific stage grouping strategies.
[b] Definition of body regions: Head and neck: inferior border—superior border of clavicles, T1 spinous process. Chest: superior border—superior border of clavicles; inferior border—inferior margin of rib cage; lateral borders—midaxillary lines, glenohumeral joints (inclusive of axillae). Abdomen/genital: superior border—inferior margin of rib cage; inferior border—inguinal folds, anterior perineum; lateral borders—midaxillary lines. Upper back: superior border—T1 spinous process; inferior border—inferior margin of rib cage; lateral borders—midaxillary lines. Lower back/buttocks: superior border—inferior margin of rib cage; inferior border—inferior gluteal fold, anterior perineum (inclusive of perineum); lateral borders—midaxillary lines. Each upper arm: superior borders—glenohumeral joints (exclusive of axillae); inferior borders—ulnar/radial-humeral (elbow) joint. Each lower arm/hand: superior borders—ulnar/radial-humeral (elbow) joint. Each upper leg (thigh): superior borders—inguinal folds, inferior gluteal folds; inferior borders—mid-patellae, midpopliteal fossae. Each lower leg/foot: superior borders—mid-patellae, midpopliteal fossae.
[c] Definition of lymph node regions: Peripheral sites: antecubital, cervical, supraclavicular, axillary, inguinal-femoral, and popliteal. Central sites: mediastinal, pulmonary hilar, paraaortic, iliac.
From Kim YH, Willemze R, Pimpinelli N, et al. TNM classification system for primary cutaneous lymphomas other than mycosis fungoides and Sézary syndrome: a proposal of the International Society for Cutaneous Lymphomas (ISCL) and the Cutaneous Lymphoma Task Force of the European Organization of Research and Treatment of Cancer (EORTC). *Blood.* 2007;110(2):479–484.

ENMZL frequently presents as multiple red to violaceous papules, plaques, or nodules, most often on the trunk or extremities, in particular the arms, which very uncommonly ulcerate.[44] Salivary and lacrimal gland ENMZL is often preceded by sicca syndrome, with xerostomia or xerophthalmia. B symptoms are uncommon (≈15% of cases).[38] Bone marrow involvement has been reported in 2% to 20% of the cases and is usually more common in non-gastric ENMZL.[45]

IPSID usually affects young adults, with no gender predominance, and is seen most commonly in the Middle East and Northern Africa, usually in low socioeconomic status populations.[46] The disease affects the proximal small bowel diffusely and generally presents with a malabsorption syndrome, with steatorrhea, hypocalcemia, weight loss, abdominal pain, and fever. Cases involving the stomach, the colon, and very rarely the respiratory tract have been described.[8]

Pathobiology and Differential Diagnosis

Etiology

ENMZL is strongly associated with chronic antigenic stimulation, including that deriving from chronic bacterial infections[18,20,47–66] or autoimmune disorders[67–71] (Table 83.3), although the strength of this correlation for some primary disease sites is discordant among studies,

TABLE 83.3 Chronic Antigenic Stimulation and Extranodal Marginal Zone Lymphoma

	Bacterial Infections	
Organism	**Site**	**Prevalence (%)**
Helicobacter pylori[18,20,48–50]	Stomach	72–100
Campylobacter jejuni[51]	Intestine (IPSID)	≈70
Chlamydophila psittaci[52–62]	Conjunctiva	0–89
Borrelia burgdorferi[63–66]	Skin	0–42
Achromobacter xylosoxidans[273]	Lung	46

	Autoimmune Disorders	
Disease	**Site**	**Relative Risk**
Hashimoto thyroiditis[a][68,69]	Thyroid	67–80
Sjögren syndrome[67,70,71]	Salivary and lacrimal glands	6.6–30.6

[a] Estimated by assuming cases of thyroid histiocytic lymphoma were ENMZL because these were published before the REAL classification.

suggesting a possible geographic variation.[72] The common assumption about this association is that continual immune stimulation by bacterial or self-antigens leads to expansion of lymphoid elements in the connective tissue adjacent to the epithelium involved, initially leading to a process of reactive lymphoid hyperplasia. Persistent lymphocytic activation and proliferation predisposes to the accumulation of genetic errors that ultimately may result in antigen-independent growth and, consequently, lymphoma emergence.[73,74] The histologic distinction between the reactive inflammatory process associated with chronic infection (or autoimmunity) and lymphoma proper may be difficult, in which case demonstration of immunoglobulin gene monoclonality by molecular studies may aid in establishing the diagnosis of lymphoma.[75]

Histology

ENMZL is composed predominantly of morphologically heterogenous small B cells.[75] These resemble a spectrum spanning from small lymphocytes with scant cytoplasm to slightly larger cells with nuclei similar to those of centrocytes and having relatively abundant pale cytoplasm (leading possibly to a monocytoid appearance). These cells are located in the outer zone of reactive lymphoid follicles, extend into the interfollicular region, and may sometimes colonize the germinal centers. Larger, immunoblast- or centroblast-like cells may be present in small numbers, but an abundance of these should raise suspicion for diffuse large B-cell lymphoma (DLBCL), which requires different management. According to the most recent WHO classification, the term *high-grade MALT lymphoma*, denoting the presence of sheets of transformed cells, should not be used and instead these tumors should be diagnosed as DLBCL.

Often there are lymphoid infiltrates invading and destroying glandular structures, with eosinophilic degeneration of epithelial cells (so-called lymphoepithelioid lesions), which are strongly suggestive, albeit not pathognomonic, of progression to lymphoma in cases where the differential diagnosis with reactive lymphoid hyperplasia is in doubt (Fig. 83.2). Plasmacytic differentiation is frequent, especially in association with cutaneous, thyroid, and intestinal (IPSID) ENMZL and may pose differential diagnosis problems with lymphoplasmacytic lymphoma (LPL). These plasmacytoid cells often contain periodic acid–Schiff (PAS)–positive inclusions, known as Dutcher or Russell bodies, depending on whether they are located over the nucleus or in the cytoplasm, respectively.[76] Testing for MYD88 gene mutations, which are detected in the majority of LPLs but rarely found in MZLs, is helpful in these cases.[7]

In gastric ENMZL, histology also plays an important role in establishing the diagnosis of *H. pylori* infection. All biopsy samples should have sections appropriately stained for its detection (see Fig. 83.2D). Because proton pump inhibitors (PPIs) may decrease the sensitivity of detection, patients should stop taking these medications for at least 2 weeks before biopsies are obtained.[77,78]

Immunophenotype

ENMZL cells display common pan B-cell markers, such as CD19 and CD20. They are also usually positive for the complement receptors CD21 and CD35, antigens that are shared with follicular dendritic cells, and also for CD79a. Plasmacytoid cells can be CD138-positive. Helpful in the differential diagnosis with other indolent lymphomas, ENMZL are usually CD5-negative (in contrast to chronic lymphocytic leukemia/small lymphocytic lymphoma [CLL/SLL] and mantle cell lymphoma [MCL]), CD23-negative (in contrast to CLL/SLL), CD10-negative (in contrast to follicular lymphoma [FL]), and cyclin D1-negative (in contrast to MCL). They are also negative for BCL6, which may be helpful to exclude transformation to DLBCL. Except for IPSID, in which tumor cells express a truncated alpha heavy chain without any light chain, most ENMZL are typically positive for IgM or, less commonly, IgA or IgG, with light chain restriction. IgD expression is usually negative or very weak. These immunoglobulins may be secreted, especially when there is significant plasmacytic differentiation, and can give rise to a monoclonal band in the serum protein electrophoresis. The truncated heavy chains of IPSID usually do not appear as a monoclonal band because they co-migrate with other serum proteins but can be detected with anti-alpha heavy chain antibodies on immunofixation. ENMZL can be rarely associated with localized or systemic AL amyloidosis.[79]

Clinical practice guidelines published by ESMO propose that testing for CD20, CD5, CD10, and cyclin D1 by immunohistochemistry (IHC) is mandatory, whereas testing for CD23, IgD, and MYD88 mutation is suggested to aid in the diagnosis of MZLs.[30]

Genetics

Specific chromosomal aberrations have been associated with ENMZL, the frequency of which depends strongly on the primary site of disease (Table 83.4).[39,40,80] These abnormalities can be detected by conventional cytogenetics in metaphase plates or through fluorescent in situ hybridization (FISH) of interphase nuclei using specific

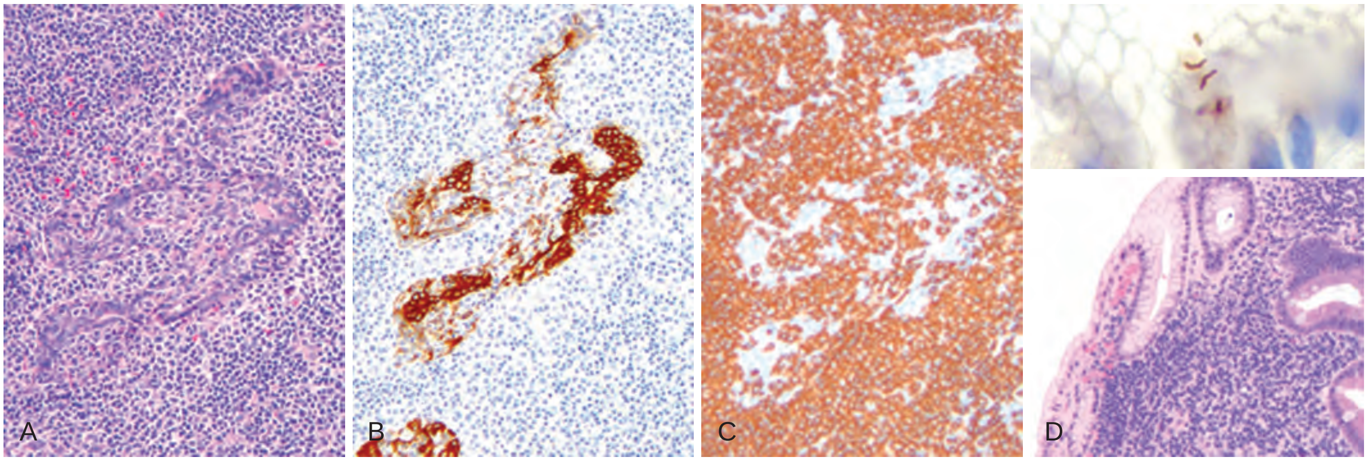


Figure 83.2 EXTRANODAL MARGINAL ZONE LYMPHOMAS OF MUCOSA-ASSOCIATED LYMPHOID TISSUE (MALT LYMPHOMAS). (A to C) An example of a MALT lymphoma in the parotid gland is illustrated with various stains. The glandular tissue is overrun by lymphoid cells, which disrupt and destroy the gland. The resulting structure is referred to as a *lymphoepithelial lesion* (A). These can be more clearly identified with a keratin stain (B) and with a B-cell stain such as CD20 (C), which illustrate the glandular remnant and the B-cell proliferation. (D) MALT lymphoma of the stomach is commonly associated with *H. pylori* infection, which can be identified by special stains *(top)*.

TABLE 83.4 **Frequency of Common Genetic Aberrations in Extranodal Marginal Zone Lymphoma According to Primary Site of Disease**

	Genetic Abnormality[39,40,80] and Genes Involved[89]					
	t(11;18)(q21;q21)	t(14;18)(q32;q21)	t(1;14)(p22;q32)	t(3;14)(p14.1;q32)	+3[a]	+18[a]
Primary Site	***BIRC3/MALT1***	***IGH@/MALT1***	***IGH@/BCL10***	***IGH@/FOXP1***	***NFKBIZ, BCL6, FOXP1, …***	***BCL2, NFATC1, …***
Lung	36–53	6–10	2–7	0	13–20	7
Intestine (non-IPSID)	13–56	0	0–13	0	75	13–25
Stomach	6–26	1–5	0	0	11–18	6–29
Ocular adnexa	3–10	0–25	0	0–20	30–38	14–26
Salivary glands	0–5	0–16	0–2	0	8–55	8–19
Skin	0–4	0–14	0	0–10	20	4
Thyroid	0–17	0	0	0–50	11–17	0–22

All values expressed as percentages of cases.

[a]Mostly partial trisomies. Data summarized based on references noted.

probes. The most commonly observed abnormality is the t(11;18)(q21;q21), which fuses the BIRC3 (baculoviral inhibitor of apoptosis repeat containing 3, also known as API2, or apoptosis inhibitor-2 protein) and MALT1 (MALT lymphoma translocation-1 protein) genes in chromosomes 11 and 18, respectively, leading to expression of a BIRC3-MALT1 chimeric protein.[81] The native MALT1 is part of a protein complex that includes the BCL10 protein (B-cell lymphoma protein 10) and that indirectly leads to nuclear factor κ-B (NFκB) activation, a process under strict control by several upstream factors. Expression of the fusion protein leads to constitutive activation of NFκB via canonical[82] and noncanonical[83] pathways, which in turn leads to resistance to apoptosis and uncontrolled proliferation. The t(11;18) has a special prognostic significance in gastric ENMZL, because its presence is associated with worse response to antibiotic therapy,[84] which is at least partly due to its higher prevalence in *H. pylori*–negative gastric ENMZL.[85]

Another, less frequently observed, abnormality is the t(14;18)(q32;q21). This translocation is different from that observed in follicular lymphoma, which involves the BCL2 gene, and instead brings the MALT1 gene under the influence of the immunoglobulin heavy chain gene promoter (IGH@), leading to overexpression of MALT1 and, through mechanisms akin to those of t(11;18), constitutive activation of NFκB. The t(1;14) (p22;q32) is seen even more rarely and causes overexpression of the BCL10 gene, which is placed under control of the IGH@ promoter, and, in turn, activation of the same pathways affected by MALT1. A fourth translocation, t(3;14)(p14.1;q32), described mostly in ocular, cutaneous, and thyroid ENMZL, involves the FOXP1 (forkhead box protein P1) transcription factor and the heavy chain promoter.[86] Although FOXP1 is overexpressed in these tumors, its exact significance in their biology is not fully understood.

Apart from the translocations described, all believed to be mutually exclusive, ENMZL has also been associated with gains of genetic material, in particular partial trisomies of chromosomes 3 (including regions affecting FOXP1, NFκBIZ [NFκB inhibitor zeta], and BCL6 [B-cell lymphoma protein 6]) and 18 (affecting NFATC1 [nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1], and BCL2). Gains at 6p25 and losses at 6q (affecting TNFAIP3 [tumor necrosis factor, α-induced protein 3])[87,88] and 1p have also been reported.[89]

Consistent with a postgerminal center B-cell origin, ENMZL have rearranged immunoglobulin genes that display somatic hypermutation of their variable regions.[90] In the case of IPSID, there are deletions of the alpha heavy chain gene in the VH and CH1 regions, which result in the production of an abnormal heavy chain that cannot bind light chains to form a complete immunoglobulin molecule.[91]

Therapy for Early-Stage (I/II) Disease

Given its rarity, there are no randomized controlled trials defining the optimal treatment for ENMZL. Often, MZL patients are included in trials that enroll multiple types of indolent NHL. Most recommendations arise from consensus panels based on data from retrospective or uncontrolled prospective trials. The most extensive body of data has been gathered on gastric ENMZL.

Gastric Extranodal Marginal Zone Lymphoma

This form of ENMZL has a strong association with active *H. pylori* infection. If histologic analysis of the gastric biopsies obtained during staging endoscopy fails to demonstrate *H. pylori*, noninvasive methods, such as breath tests, stool antigen test, or serology, should be used to exclude the infection. Although not necessarily a marker of active infection, the presence of antibodies against *H. pylori* in an individual not previously treated for this bacterium implicates it in the pathogenesis of the lymphoma.

All patients with gastric ENMZL should be given *H. pylori* eradication therapy regardless of stage.[30] The focus of therapy for *H. pylori*–positive disease is on eradication of the infection with one of the currently recommended regimens for this purpose according to local antibiotic resistance pattern. These commonly combine a 4-week therapy course of a PPI and a 2-week therapy course of clarithromycin plus a second antibiotic, usually amoxicillin or metronidazole (triple therapy). However, this is an issue in flux as resistance to clarithromycin is increasing in several regions.[92] Alternatively, quadruple therapy with a PPI, bismuth, tetracycline, and metronidazole can be used. Eradication of *H. pylori* should be confirmed with an appropriate test, such as the urea breath test, at least 4 to 6 weeks after finishing antibiotherapy and 2 weeks after discontinuing PPI.[30,92] Pooled data from several prospective and retrospective studies suggest more than 90% eradication rates after initial antibiotherapy.[93] Persistent infection should be treated with a different course of antibiotics, preferably guided by sensitivity tests, because the same data suggests an eradication rate close to 100% after second- or third-line treatments. Repeat endoscopy with biopsies should be obtained 3 months after completion of antibacterial therapy in order to assess tumor response and also to allow histologic confirmation of *H. pylori* eradication. If *H. pylori* is still detected, a different antibiotic combination should be tried and the patient reassessed as above.[12,14]

Responses to therapy are classified according to biopsy findings on endoscopy. Complete histologic response (complete remission [CR]) should be confirmed by a second endoscopy 3 months later and is managed by observation and regular follow-up thereafter as clinically indicated. The presence of small residual lymphoid aggregates early after *H. pylori* eradication, corresponding to a category of *probable minimal residual disease* in the French Study Group of Adult Lymphomas (GELA) grading system for posttreatment evaluation, should also be managed as CR.[94] In some cases, these lymphoid aggregates have been shown to harbor cells with the same monoclonal rearrangements of the original tumor.[95] Nevertheless, these patients do not seem to have an increased risk for relapse and most will have evidence of complete response in a subsequent evaluation.[96]

Patients with overt residual (partial remission [PR]) or stable disease, as long as asymptomatic, can also be managed conservatively, with observation or antibiotherapy as appropriate, for several months. Of interest, responses may occur as late as more than 18 months after completion of antibiotic therapy.[50,96] If a patient achieves clinical and endoscopic remission after eradication of *H. pylori* but continues to have persistent microscopic disease on histology, waiting at least 12 months before starting another treatment is reasonable.[14,30] Progressive or symptomatic disease should be managed with local therapy, with radiotherapy being preferred. Despite its established efficacy in disease control, gastrectomy, because of its immediate morbidity and long-term metabolic complications, is currently reserved for management of rare complications such as perforation or bleeding that cannot be controlled endoscopically.[12,14]

A meta-analysis of 1436 patients with early stage (IE-IIE1) *H. pylori*–positive disease on prospective or retrospective studies estimates a CR rate of 78% after eradication of *H. pylori*,[97] but with individual study remission rates anywhere from 47% to 100%.[18,20,48–50,95,98–122] On univariate analysis of available data from the same studies, adverse risk factors for achieving remission included the presence of t(11;18), stage greater than IE1, proximal (body or fundus) location of lesions, and Western (versus Asian) residence. No good evidence is available to support adjuvant chemotherapy after anti–*H. pylori* treatment as a means to prevent recurrence in localized gastric MALT lymphomas, although this has only been formally addressed with an additional single agent (chlorambucil).[123]

The management of early-stage *H. pylori*–negative disease is controversial, with some groups suggesting involved field radiation therapy or, if radiation therapy is contraindicated, systemic therapy with rituximab as initial treatment,[12] whereas others propose a trial of anti-*Helicobacter* therapy.[14,124] The rationale for the latter derives from anecdotal reports of CR in *H. pylori*–negative gastric ENMZL patients that were treated exclusively with antibiotherapy,[125] with a meta-analysis suggesting a response rate of up to 19%.[126] These patients are assumed to have been infected with *H. pylori* that was missed by diagnostic tests or with a different species of *Helicobacter*, several of which have been recognized.[127,128]

Response to involved field radiation therapy (IFRT) is excellent, with some series reporting CR rates of up to 100% with total doses as low as 24 to 30 Gy given over 3 to 4 weeks to the stomach and perigastric nodes.[129–133] Radiation therapy in early-stage gastric ENMZL has not been associated with increased risk of cardiac death.[134] The lower dose of radiation (24 Gy) was shown to be safe and effective in a phase 3 clinical trial of indolent NHL, including ENMZL.[135] Recurrence rate is very low in these patients, but follow-up is limited for most series reported. International Lymphoma Radiation Oncology Group (ILROG) guidelines provide different volumes of radiation to be used for treatment of each site of ENMZL, with newer definitions of involved-site radiotherapy (ISRT).[136]

The optimal follow-up schedule is unknown, although most recommend periodic endoscopies every 3 to 6 months for the first 5 years and yearly thereafter.[12,14] Long-term follow-up data in patients with complete remission after *H. pylori* eradication document a 7.2% relapse rate overall (2.2% per year).[97] Some of these relapses were associated with *H. pylori* recurrence, and responses to retreatment for *H. pylori* were seen. Whether most complete remissions equate to cures is a question that will require longer follow-up studies. An additional argument in favor of periodic endoscopies is that *H. pylori* may be associated with an increased risk for gastric adenocarcinoma.[137]

Molecular studies should not be done routinely in follow-up biopsy samples, outside of a research protocol. Several studies have shown that molecular disease, defined by the presence of residual t(11;18) or monoclonal immunoglobulin as evidenced by polymerase chain reaction (PCR) methods, may still be detected even with complete pathologic remissions.[109,138,139] However, this finding is not associated with an increased relapse rate and thus is not currently helpful for clinical management.

Ocular Extranodal Marginal Zone Lymphoma

Localized forms of ocular adnexal ENMZL are most frequently managed with radiation therapy, with treatment specifics dependent on the exact location of the tumor in the orbit. Reported responses are excellent, with CR rates of 83% to 100%.[140–149] Local recurrence rates vary between 0% and 17%, and distant recurrences can occur in up to 25% over 10 years, although disease-specific survival approaches 100% in most series. The exact site of presentation correlates with the risk for systemic recurrence, the lowest being for conjunctival and the highest for eyelid primaries.[37] Long-term complications related to the use and the dose of radiation therapy, such as cataract formation and xerophthalmia, occur in approximately half the patients.[150] Lower-dose fractionated radiation (4 Gy in two fractions) is associated with 5-year progression-free survival (PFS) of approximately 70% and can be considered in some patients to avoid long-term complications.[151] The fractionation of lower-dose radiotherapy in ocular ENMZL was associated with lower risk of cataract and high durable response rates (96% at 2 years).[152] Despite the growing evidence of the efficacy of lower doses of radiation, the standard recommended dose remains 24 Gy in most patients.[30]

Based on the success of antibiotherapy for *H. pylori*–associated ENMZL and reports of the presence of *Chlamydophila psittaci* (by PCR, immunofluorescence, or electron microscopy) in ocular tissues of patients affected by ocular adnexal ENMZL, a course of an antichlamydial antibiotic has been evaluated as initial management for these patients.[52–62] Some studies have reported PR or CR in a significant fraction of these patients, with a prospective study documenting an overall response rate (ORR) of 65% and suggesting improved response rate and PFS in patients in whom *C. psittaci* eradication was documented.[57] However, the recommendation to treat with a tetracycline at initial diagnosis is controversial because the reported rates of association with *C. psittaci* are highly variable—some studies suggest prevalence as high as 80%, whereas others are not able to detect the organism in any of the patients. A meta-analysis has suggested that the benefit of antibiotherapy may be restricted to specific geographic areas and, even then, is likely to be limited.[153]

A recent phase 2 study using intralesional rituximab in 20 patients who received previous therapy for ocular ENMZL showed ORR of 65% with a median follow up of 3.5 years.[154] Intralesional rituximab was well-tolerated and can be considered in patients with ocular ENMZL who relapse or are refractory to initial therapy.

Cutaneous Extranodal Marginal Zone Lymphoma

Results for both surgical excision and radiation therapy for limited disease are comparable, with CR rates approaching 100%. Both approaches have a relapse rate (usually limited to the skin) of around 45%. Encouraging results have also been obtained with intralesional injection of rituximab or α-interferon.[44]

Studies from Europe have suggested an association between *Borrelia burgdorferi* infection and ENMZL of the skin, although this has not been reproduced in Asian and American studies.[63–66] In view of this, similar to ocular ENMZL, it has been suggested that a course of a tetracycline may be a reasonable first approach, especially in locations where the association has been documented or when infection by *B. burgdorferi* is detected.

Immunoproliferative Small Intestinal Disease

Antibiotherapy (varying from single-agent tetracycline to triple-antibiotic therapy with ampicillin, metronidazole, and tetracycline, or an *H. pylori* regimen)[155–158] has long been recognized as being able to induce CR in early-stage disease. IPSID has been associated with chronic infection with *Campylobacter jejuni*.[51] Therefore treatment of early disease is directed at bacterial eradication. Historic rates of remission vary between 30% and 70%, depending on the study. Maximal responses may take more than 5 months of therapy, and relapses occur in a subset of patients.

Other Extranodal Marginal Zone Lymphoma

Early-stage ENMZL in other primary sites is managed in a similar way to ocular adnexal or cutaneous forms. When feasible, surgery can be potentially curative and often may be done with primary diagnostic intent. If full resection is achieved, these patients may be observed. Otherwise, for sites not amenable to surgery or if there is residual disease after surgical excision (i.e., positive margins), radiation therapy is the usual preferred approach. It is important to tailor the radiation amount and site to the clinical setting to avoid irradiation of wide areas of the body, which can potentially result in adverse events. For example, in thyroid ENMZL, the entire thyroid gland should be irradiated.[159] If radiation is contraindicated and the patient is asymptomatic, observation is an option. Otherwise, systemic therapy as for advanced stage, preferably one with minimal toxicity, is appropriate.[12,160]

Therapy for Advanced-Stage (III/IV) Disease

The data regarding management of advanced-stage disease are also limited, because most large treatment series with long-term follow-up aggregate all indolent lymphomas and include only a small fraction of patients with MZL among other more frequent histologies. Thus most treatment approaches have been modeled after those for FL (see Chapter 82), and indeed the most common recommendation is that advanced ENMZL be managed as advanced FL.[12] In any case, there is some suggestion that responses may be better, possibly because of a usually lower burden of disease. Eligible patients should be included whenever possible in clinical trials. It is important to consider that patients with indolent lymphomas, especially MZL, tend to relapse and may do so multiple times. Accordingly, sequencing treatment to avoid toxicity and preserve adequate organ function is necessary to achieve long survival time.[159]

As in other indolent lymphomas, extensive disease is likely incurable with current approaches, which together with a generally slow pace of progression means that systemic treatment is not always indicated. Since no benefit in survival has been demonstrated with early systemic treatment of asymptomatic disease, unless a treatable etiology has been identified, initial management of asymptomatic patients with expectant observation is acceptable. While on this watch-and-wait approach, patients should be reassessed approximately every 3 months with history, physical examination, complete blood counts, basic chemistry, and LDH. Any new symptoms or findings suggestive of transformation should be investigated with a repeat biopsy to rule it out. Routine repeat imaging studies are controversial and should be considered on a case-by-case basis.

Indications for initiating systemic treatment include symptomatic disease due to mass effect or effusion, risk for local compressive disease, bulky lymphadenopathy, symptomatic splenomegaly, B symptoms, cytopenias due to bone marrow involvement, or rapid disease progression. Although unlikely to contribute to regression of established advanced disease, treatment of an underlying infection associated with MZL (such as *H. pylori* for primary gastric ENMZL and *C. jejuni* for IPSID) is advisable in order to remove any inciting factor.[30] Otherwise, as already mentioned, the same treatment approaches used for FL (see Chapter 82) are usually followed for ENMZL. Nonetheless, a few studies specifically addressing MZL are worth reviewing here.

Single Agent Rituximab

Given its low toxicity, single-agent immunotherapy with rituximab has generated a lot of interest in the management of advanced disease. Several small phase 2 studies have reported ORRs of around 75%,[161–163] with up to 48% CR in patients without previous therapy. In one of the series, median time to treatment failure was 22 versus 12 months in chemotherapy-naive versus non-naive patients, respectively.[161] Rituximab has activity in t(11;18)–positive disease.[162] Maintenance rituximab is controversial, and no data are available to support its use in ENMZL. Previous studies failed to showed OS benefit of maintenance rituximab and accordingly it is not usually recommended.[30,164,165]

Alkylating Agents

Single-agent alkylators have been used in this setting. A study of 24 patients with gastric ENMZL (stages IE or IV) treated with daily oral chlorambucil or cyclophosphamide for 12 to 24 months showed a CR rate of 75%, with the remaining patients achieving PR.[166] Remissions were durable in approximately half of the patients after a median follow-up time of 45 months. Another study of 21 patients (stages I to IV) treated with continuous alkylating drugs documented a CR rate of 42% and 89% in t(11;18)–positive and t(11;18)–negative disease, respectively.[167] After a median follow-up of 7.5 years, CR was sustained in all patients with translocation-negative disease, but in only one patient with t(11;18).

The combination of rituximab and chlorambucil has produced an impressive 100% CR rate in 13 patients with t(11;18)–positive gastric ENMZL (stages I to IV).[168] No relapses were observed after a median follow-up of 24 months in this report but long-term data were not available. This combination has been studied in a randomized controlled trial versus each single agent in 393 patients not responding or not suitable for local therapy.[169,170] CR rate, and 5-year event-free and PFS were better with the combination, but 5-year overall survival (OS) was 90% in the whole population, without significant differences between the three arms.[171]

Responses to bendamustine (with or without rituximab) have been documented in patients with relapsed/refractory indolent lymphomas, a small fraction of which were ENMZL.[172,173] A retrospective series of 14 ENMZL patients reported 10 CR and 3 PR with the combination of bendamustine and rituximab.[174] MALT2008-01, a phase 2 study, reported excellent results in 60 patients treated with 4 cycles of bendamustine and rituximab, which resulted in 100% ORR and 98% CR/unconfirmed CR. The combination was active in patients with t(11;18) and most recently the results were sustained, with a median follow up of 7 years.[175,176]

Purine Analogues

The purine analogue cladribine has also been used as single-agent therapy in a series of 25 patients (stages I to IV), and CR was obtained in 84% of them. After a median follow-up time of 80 months, seven patients experienced disease relapse.[177] Of note, one of the patients treated developed a myelodysplastic syndrome immediately after the third infusion of the drug.[178] Another purine analogue, fludarabine, has been used in combination with rituximab to treat a series of 22 patients (stages I to IV).[179] At the end of treatment (three cycles, with some patients requiring six), 90% of patients achieved a CR. The PFS rate at 2 years in patients with gastric and extragastric ENMZL was 100% and 89%, respectively. Both purine analogues seem to have activity in t(11;18)–positive gastric ENMZL.

Other Agents

Other single chemotherapeutic agents whose use has been reported in ENMZL include oxaliplatin, bortezomib, and vorinostat. In a study of 16 patients (stages I to IV) treated with oxaliplatin, a CR rate of 56% with a median time to response of 4 months was observed.[180]

Other studies of patients with relapsed, multiply treated indolent lymphomas report encouraging responses to bortezomib (two cases with PR out of two patients)[181] and vorinostat (one CR and one PR out of nine patients).[182] The combination of lenalidomide with rituximab was found to be effective and safe in a phase 2 study that included previously untreated patients.[183] Long-term follow-up is not available; therefore, the role of these agents as first-line therapy has not been established.

Combination Chemotherapy

Combination chemotherapy regimens reported in ENMZL patients include CVP (cyclophosphamide, vincristine, and prednisone) followed by radiation therapy,[184] FM (fludarabine and mitoxantrone),[185] CHOP (cyclophosphamide, hydroxydaunomycin, vincristine [Oncovin], and prednisone) followed by CVP,[186] MCP (mitoxantrone, chlorambucil, and prednisone),[187] R-CHOP (rituximab, cyclophosphamide, hydroxydaunomycin, vincristine [Oncovin], and prednisone), and R-CNOP (rituximab, cyclophosphamide, mitoxantrone [Novantrone], vincristine [Oncovin], and prednisone).[188] These regimens have mostly been used in patients who were believed to have more aggressive disease. CR rates vary from 61% to 100%, with relapse rates reaching up to 36% on long-term follow-up. Advanced/transformed IPSID has usually been treated with combination chemotherapy, such as CHOP.[8,189]

Radioimmunotherapy

Radioisotope-conjugated forms of anti-CD20 antibodies (ibritumomab and tositumomab) have also been used both as first-line[190] therapy (eight CRs and one PR in nine patients with ocular adnexal ENMZL) and for relapsed disease[191,192] (three CRs and three PRs in six patients with ocular adnexal ENMZL; four CRs and one PR in six patients with a variety of primary sites). The combination of conventional radiation therapy and rituximab has been studied for early-stage follicular lymphoma.[193] There are no published studies of its use on ENMZL.

Therapy for Relapsed Disease

Limited relapses can be retreated, if feasible, with local therapy. Otherwise, treatment considerations are similar to those described for advanced disease. Symptomatic, relapsed extensive disease will usually require an alternative regimen to those previously used.

Ibrutinib, a Bruton tyrosine kinase (BTK) inhibitor, is Food and Drug Administration (FDA) approved for use in MZL patients who received at least one prior therapy containing rituximab. An updated analysis of a phase 2 study that used ibrutinib (560 mg daily) in 63 patients reported ORR of 58% and CR of 10% with a median follow-up of 33 months. The median duration of response was 27.6 months with a median PFS of 15.7 months. ORR was similar in ENMZL (63%) and splenic MZL (62%) and slightly lower in nodal MZL (47%).[194,195] The most common grade 3 adverse event was anemia (16%).

The combination of lenalidomide and rituximab is approved for the treatment of adult patients with previously treated marginal zone lymphoma based on results of two trials. One of these trials (AUGMENT) showed ORR of 65% (20/31 patients) with rituximab and lenalidomide versus 44% (14/32 patients) with rituximab alone in MZL patients.[196]

The phosphoinositide 3-kinase (PI3K) inhibitor copanlisib has demonstrated significant efficacy, with ORR of 70% in 23 patients with MZL, although most responses were partial.[197] The combination of copanlisib with rituximab was found to be better than single-agent rituximab in the most recent CHRONOS-3 study, which studied multiple subtypes of indolent NHL.[198] Approximately 20% of the enrolled patients had MZL and their responses seem to be consistent with the overall group of treated patients, with median PFS of 21.5 months (copanlisib with rituximab) versus 13.8 months (rituximab). Copanlisib was associated with more frequent serious treatment-related adverse events including hyperglycemia, hypertension, and pneumonia.[198] Other PI3K inhibitors, such as duvelisib and idelalisib, may have similar activity. A dual PI3K and casein kinase 1 (CK1) inhibitor, umbralisib, was FDA approved specifically for use in MZL patients who received at least one prior therapy containing a CD20 monoclonal antibody.[199] but approval has been later withdrawn for safety concerns.

The role of hematopoietic stem cell transplantation is controversial because most series do not directly address ENMZL and thus most recommendations are again extrapolated from studies of FL.[200–204] Given that most young patients with relapsed systemic ENMZL are likely to succumb to complications of their disease, considering them for allogeneic stem cell transplantation is reasonable, although this should preferably take place on a clinical trial. Encouraging results have also been reported with high-dose therapy and autologous stem cell transplantation specifically for MZL.[205]

Prognosis

Overall, prognosis of limited-stage ENMZL is excellent, as mentioned in each of the treatment subsections. A recent long-term report of 487 patients with early-stage ENMZL showed excellent outcomes with 5-year OS of 89%, with a median follow-up of 4.7 years.[206] The 5-year relapse free survival was 60%. Even for advanced disease, the expected lymphoma-specific survival at 5 years is close to 90%, with some series showing survivals similar to those of early disease. A retrospective analysis of SEER data lists the best 5-year OS for cutaneous (88%), ocular (83%), and thyroid (85%) forms, and worst for genitourinary (76%), intestinal (69%), and central nervous system (71%) forms.[35] As in other indolent lymphomas, transformation to aggressive forms (DLBCL) can occur, but this is thought to be a rare event (less than 10% of cases) associated with acquisition of additional genetic abnormalities.[41] Regardless, even patients in CR after first-line therapy can develop DLBCL. Main predictors for transformation, which is associated with worse survival, were reported to include elevated LDH, more than 4 nodal sites at the time of MZL diagnosis, and failure to achieve CR after initial therapy.[207] For instance, 0.05% of individuals with gastric ENMZL who underwent successful antibiotic treatment had evidence of DLBCL between 6 months and 2 years after achieving remission.[97] There is a sixfold increased risk of gastric adenocarcinoma in patients with gastric MZL, however the risk is low at 2.4%.[208] On the other hand, untreated IPSID tends to evolve to large-cell transformation.[8,189]

As for other lymphomas, the International Prognostic Index (IPI)[209] (see Chapter 84) predicts outcomes, with reported 5-year OS of > 90%, 70% to 80%, and 40% to 50% for patients with low, low-intermediate/high-intermediate, and high-risk scores, respectively.[36] Nonetheless, the utility of the IPI in ENMZL has been disputed.[210] One of the main criticisms to its use in patients with indolent lymphomas is that prognostic subgroups do not have a good discriminating power, because most patients are assigned to the favorable outcome groups and only very few patients are allocated to the adverse prognostic groups.[211] To circumvent this issue, modifications of the original IPI have been proposed in some cases.[212] A new prognostic score, MALT-IPI, was developed based on data of 401 newly diagnosed patients and subsequently validated in a separate cohort of 633 patients.[213] Three factors were found to be of prognostic value: age ≥70 years, Lugano stage III or IV, and elevated LDH. High risk was defined as two or more risk factors and was associated with 5-year event-free survival of 29% and 5-year OS of 64%. The utility of MALT-IPI was consistent in gastric and non-gastric ENMZL, and also across different treatment groups, which include rituximab-containing regimens.

The recent explosion of genomic analysis of these neoplasms has also led to a plethora of findings that have been shown to have prognostic significance in ENMZL. For instance, the expression of FOXP1 in tumors predicts poor prognosis and transformation to diffuse large B-cell lymphoma.[214] Disruption of NFκB signaling was found to be correlated to clinical outcome and can be helpful as a biomarker.[194] CARD11 and KMT2D mutations were associated with worse PFS.[194] Although these findings are not yet incorporated into clinical practice, as they become more widely available, they may be useful for risk stratification and therapeutic decisions (see box on Suggested Treatment Approach to Extranodal Marginal Zone Lymphoma).

SUGGESTED TREATMENT APPROACH TO EXTRANODAL MARGINAL ZONE LYMPHOMA

Stage I/II

Gastric H. pylori–positive

- Antibiotic therapy
- Repeat endoscopy in 3–6 months
- If still *H. pylori*–positive and no progression, alternative antibiotic therapy
- Repeat endoscopy in 3 months

Nongastric, gastric H. pylori–negative or not responding to antibiotic therapy

- Antibiotic trial for gastric *H. pylori*-negative, even in advanced disease
- Antibiotic trial for stage I ocular, or cutaneous?
- Involved field radiation therapy (24–30 Gy)

Stage III/IV or relapsed after antibiotic and radiation therapy

- Expectant observation until indication to treat
- Single-agent rituximab
- Rituximab + bendamustine, chlorambucil, cyclophosphamide or lenalidomide
- Ibrutinib (in relapsed disease after prior use of rituximab)
- PI3K inhibitors

SPLENIC MARGINAL ZONE LYMPHOMA

Epidemiology and Manifestations

SMZL (Fig. 83.3) is a rare disease, corresponding to <1% of all NHLs,[11] with a yearly incidence rate of 0.25 per 100,000 adults in the US according to SEER data.[35] The median age at diagnosis is around 65, but as with ENMZL, the age range is wide and there may be a slight female predominance. The vast majority of patients present with advanced-stage disease involving the spleen (with splenomegaly in more than 80% of patients), abdominal (mainly splenic hilar) lymph nodes, and in 83% to 94% of patients, the bone marrow.[215–218] Peripheral lymphadenopathy is, however, uncommon. Liver involvement is seen in up to one-fourth of patients, and rarely other nonhematopoietic sites can also be involved. B symptoms occur in approximately 25% to 60% of patients, depending on the series.[216,217]

Circulating villous lymphocytes (with short polar villi) can be seen in approximately two-thirds of patients (see Fig. 83.3B) and frank lymphocytosis in up to one-half. This has led to a prior designation of *splenic lymphoma with villous lymphocytes* being given to a specific presentation of this disorder and explains why the disease was called *SMZL with or without villous lymphocytes*. CBL-MZ may precede the onset of SMZL.[10] Anemia is seen in about one-half to two-thirds and thrombocytopenia in one-fifth of cases, which can be the result of bone marrow involvement or an autoimmune process (seen in approximately 15% of patients). Between 25% and 40% of patients have a low-level circulating monoclonal immunoglobulin (mostly IgM),[217] and in a few patients, especially in those with active hepatitis C virus (HCV) infection, mixed cryoglobulins can be demonstrated, which can be associated with vasculitis.[219,220] Autoimmune manifestations occur in 20% of the patients, including cold agglutinin disease, acquired von Willebrand disease, autoimmune hemolytic anemia, and immune thrombocytopenia. Ultrasonography can help define focal splenic lesions and may help in the diagnosis.[221]

Pathobiology and Differential Diagnosis

Etiology

Approximately 10% to 20% of SMZL patients from European series have evidence of HCV infection,[215,218] and a significant fraction of these patients can achieve CR after successful treatment of the infection.[220,222] This raises the possibility that, like ENMZL, this disease is associated with chronic antigenic stimulation. However, no other infections have been described in clear connection to SMZL, despite a suggested association with *Plasmodium*,[223] and thus the etiology of most cases is unknown.

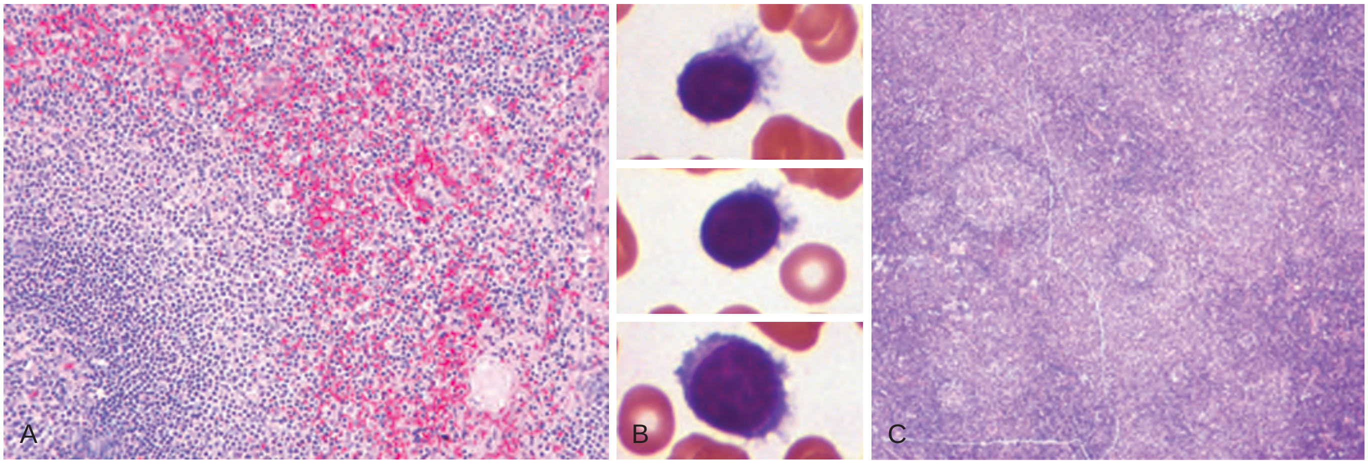


Figure 83.3 SPLENIC MARGINAL ZONE LYMPHOMA, VILLOUS LYMPHOCYTES, AND PRIMARY NODAL MARGINAL ZONE LYMPHOMA. (A) Splenic marginal zone lymphoma is characterized by an expansion of the marginal zone cells *(left)* and their spilling into the red pulp *(right)*. (B) These cells can also become leukemic and can be recognized on the peripheral blood smear. Note the polar distribution of the cytoplasmic projections. (C) Primary nodal marginal zone lymphoma is rare, and involvement of a node by extranodal disease must always be ruled out. NMZL is histologically characterized by an expansion of marginal zone cells (formerly referred to as monocytoid B cells) in between reactive germinal centers.

Histology

The classic histology of SMZL includes a population of small lymphocytes that surrounds or replaces the germinal centers of the lymphoid follicles of the white pulp, effacing their mantle zone, and progressively merging peripherally with larger, marginal zone–like cells.[224] These cells expand to the interfollicular zones and invariably invade the red pulp (see Fig. 83.3A). A few scattered lymphoblasts are usually present, and as in ENMZL, plasmacytic differentiation can occur. Bone marrow involvement usually gives rise to nodular interstitial lymphoid infiltrates that resemble the histology of the spleen, although the cell types are usually admixed, without distinct zones. Peripheral blood involvement is typically associated with lymphocytes that have short polar villi (Fig. 83.3B), as mentioned above, although the villi may be absent.

Immunophenotype

The phenotype of SMZL is similar to that of ENMZL (see earlier), but in contrast to the latter, SMZL is usually IgD-positive. The same differential diagnosis considerations apply; additionally, because this disease behaves as a chronic B-cell leukemia, it is of interest that SMZL is negative for annexin A1 and CD25 and usually negative for CD103 (in contrast to hairy cell leukemia).[224] Rare cases may be CD5-positive.[225]

Genetics

A substantial amount of genetic data on SMZL has been accumulated during the last decade. A review of 330 patients with SMZL documented del(7q) (affecting different loci in regions q21 to q36) as the most frequent cytogenetic abnormality (≈40% of patients).[218] Gains of genetic material from chromosomes 3 (≈25%), 8 (≈10%), and 12 (≈8%) were also seen frequently. Translocations involving 14q32, different from those seen in ENMZL, occurred in 12% of patients. The translocation t(11;18) was not seen in SMZL. More than 50% of cases had three or more cytogenetic aberrations. Deletions of 8p and 17p (TP53) have been seen in approximately 15% of cases analyzed with DNA microarrays.[89] The biologic significance of these chromosomal abnormalities is unclear.[226]

Approximately 50% to 60% of SMZL have evidence of somatic hypermutation of the immunoglobulin genes,[218,226–228] and there is some evidence that, similar to CLL, these cases may have better overall prognosis.[227] Both mutated and unmutated tumors display bias in variable region usage, with predominance of VH1-2 family genes. Intraclonal variation has also been described, which suggests the presence of ongoing mutational events in these lymphomas.[229]

Similar to hairy cell leukemia, the V600E BRAF variant has been reported in SMZL, but only in 16% of a small series of patients.[230,231] In addition, the L265P MYD88 mutation commonly observed in Waldenström macroglobulinemia/lymphoplasmacytic lymphoma is rare in SMZL.[232] These findings may help distinguish these disorders when the diagnosis is in doubt.

Therapy

Since SMZL behaves indolently, in asymptomatic patients without significant or progressive cytopenias, expectant observation is a reasonable approach. The 5-year OS rate of 32 patients with asymptomatic SMZL who never received treatment was 86% in one published series.[233] In another series, 10 out of 14 untreated patients were alive between 1 and 6 years after diagnosis.[234]

Indications for lymphoma treatment are similar to those for advanced-stage ENMZL, including symptomatic splenomegaly. As for other forms of MZL, there are no data from randomized trials guiding selection of therapy, and most recommendations come from consensus opinions of experts in the field.[12,30]

Anti-Hepatitis C Virus Therapy

In patients with evidence of active HCV infection, anti-HCV therapy is recommended. A study of the effects of interferon-α (IFNα) in SMZL showed eight complete and one partial hematologic responses in nine HCV-positive patients after clearance of HCV (with two of them requiring addition of ribavirin), versus no responses in six HCV-negative individuals.[222] One patient in CR treated initially only with IFNα had a relapse of the lymphoma with detectable levels of HCV RNA; treatment with IFNα and ribavirin resulted in second complete virologic and hematologic responses. Given that newer anti-HCV drugs are less toxic and more effective than IFNα, these may become first choice for these patients. Of note, evidence of monoclonal immunoglobulin gene rearrangement may persist after successful treatment of HCV and SMZL.[220]

Rituximab-Based Regimens

Rituximab has been used as single agent or in combination with chemotherapeutic drugs in symptomatic HCV-negative patients or in those whose disease did not respond to anti-HCV treatment. The response rate to single-agent rituximab in a retrospective subseries of 25 patients with splenic and nonsplenic MZL was 88%, which compared favorably with that of 11 patients who received chemotherapy alone (55%).[235] In two other retrospective studies, rituximab was found to be an effective therapy in 90% to 95% of patients with SMZL, with reduction in splenomegaly and improvement in blood counts.[236,237] Because of its low toxicity and improved survival outcomes, rituximab-containing regimens are often used as initial therapy for symptomatic SMZL.[238] Maintenance rituximab has a potential role in SMZL, with evidence of 64% improvement in 10-year progression free survival.[239] Rituximab may also be helpful in SMZL patients with autoimmune disorders such as autoimmune thrombocytopenia or autoimmune hemolytic anemia.

The combination of bendamustine and rituximab was investigated in a phase 2 study where 56 patients with newly diagnosed SMZL were given six cycles of therapy.[240] Outcomes were excellent with ORR of 91% and CR of 73% after a median follow up of 32 months.

Splenectomy

Before rituximab was widely available and studied in SMZL, splenectomy was frequently used as first-line therapy. A benefit of this procedure, which may be done for diagnostic purposes in patients with localized disease, is the usual improvement of cytopenias occurring as a result of hypersplenism. Precise response rates to splenectomy are difficult to ascertain. All of 25 patients in a series treated with splenectomy alone had responses, including 2 CRs.[13] Most responses were durable: only 8 of these 25 patients experienced disease progression on long-term follow-up, with a median time to progression of 32 months (with a range of 4 to 137 months).[241] Moreover, all of 16 patients treated with first-line splenectomy in an earlier series had good responses, most likely partial (complete responses could not be assessed because of a lack of bone marrow assessment after treatment); two patients progressed subsequently.[234] Finally, another series of 28 patients documented a 5-year OS of 71% after splenectomy; no CRs were reported.[233] Although an effective procedure with very low operative mortality, splenectomy is associated with a long-term increased risk of infection and sepsis, and possibly a hypercoagulable state with potential cardiovascular complications.[242,243] On the other hand, retrospective analysis of SEER data showed no advantage of splenectomy over other treatment approaches for SMZL.[244] Given these considerations, the role of therapeutic splenectomy in SMZL has been questioned.[245,246]

Ibrutinib

Ibrutinib is effective in MZLs including SMZL, with ORR of 62% and can be considered in patients who relapse after the use of rituximab.[194,195]

Chemotherapy

Single alkylating agents (chlorambucil or cyclophosphamide) have also been used for treatment of SMZL. Most series are very small, but all report a significant fraction of responses, albeit mostly partial. Fludarabine has produced encouraging results also,[247,248] but the efficacy of cladribine is controversial.[249,250] The use of combination chemotherapy (including CVP and CHOP) has also been reported, but differences between CHOP (or CHOP-like) therapy and other less-intensive regimens could not be demonstrated.[217] In a subseries of 19 patients treated with chemotherapy alone, the 5-year OS rate was 64%.[233]

Radiation Therapy

In patients ineligible for any of the aforementioned therapies, radiation therapy to the spleen can be used for symptomatic control. Even low-dose radiation (around 4 Gy) can result in resolution of splenomegaly and correction of cytopenias,[251] at least temporarily.

Although it is tempting to compare the results of observation, splenectomy, and systemic therapy, this is not prudent. All published series have considerable selection bias because patients who are believed to have more aggressive disease have been usually offered more-intensive therapies.

Prognosis

Reported 5-year OS rates vary from 65% to 78%,[217,233] with a median survival of more than 10 years.[13,252] The Italian Lymphoma Intergroup (ILI) has proposed a prognostic model using hemoglobin less than 12 g/dL, LDH higher than normal, and albumin less than 3.5 g/dL as risk factors.[215] Low-risk (no factors), intermediate-risk (1 factor), and high-risk (2 or more factors) patients have 5-year OS of 83%, 72%, and 56%, respectively. Shorter survival has been associated with CD38 expression, unmutated variable region immunoglobulin genes, and expression of a specific set of NFκB pathway genes (by gene expression array).[226] The presence of both del(8p) and del(17p) involving TP53 is associated with worse prognosis, although isolated deletion 17p is not.[89] An international retrospective survey conducted by the SMZL study group on 593 patients proposed a new prognostic model called HPLL (Hemoglobin level, Platelet count, LDH level, and extra-hilar Lymphadenopathy), which was subsequently simplified and validated.[253–255]

A recent SEER analysis reported no significant difference in SMZL outcomes by treatment strategy.[252] Transformation to aggressive lymphoma may occur in around 10% of cases (see box on Suggested Treatment Approach to Splenic Marginal Zone Lymphoma).[13]

SUGGESTED TREATMENT APPROACH TO SPLENIC MARGINAL ZONE LYMPHOMA

HCV-positive
- Anti-HCV therapy

HCV-negative (or not responding to anti-HCV therapy) or relapsed
- Expectant observation until indication to treat
- Single-agent rituximab
- Rituximab + bendamustine (or combination chemotherapy) ± maintenance rituximab.
- Ibrutinib (in relapsed disease after prior use of rituximab)
- Splenectomy (if other treatments fail)
- Splenic irradiation (if other treatments contraindicated)

NODAL MARGINAL ZONE LYMPHOMA

Epidemiology and Manifestations

NMZL represents approximately 2% of all NHLs, corresponding to a yearly incidence rate of 0.83 per 100,000 adults in the US according to SEER data. The reported incidence of NMZL has increased significantly over the previous decade,[35] but it is unclear whether this is the result of a true increase in the number of cases or of an increased recognition of this entity. The diagnosis requires the absence of extranodal or splenic disease, the presence of which makes ENMZL and SMZL more likely. The median age at presentation is around 60, and in most series, there is a slight female predominance. Most patients present with asymptomatic lymphadenopathy, most often in peripheral lymph nodes (especially cervical and inguinal), which is frequently associated with mediastinal or abdominal involvement. The percentage of B symptoms is extremely variable between different studies with most recent series reported in less than 20% of patients.[38,216,256–260] Except in three of the published series,[257–259] the majority of patients present with stage III or IV disease (approximately 70% to 80% of cases), with bone marrow involvement detected in up to two-thirds of cases. Anemia and thrombocytopenia have been described in up to 30% and 10% of cases, respectively. A monoclonal IgM is seen in approximately 10% of patients. A pediatric form, with excellent prognosis, has been recognized.[261]

Pathobiology and Differential Diagnosis

Etiology

As with SMZL, some series have reported an association with HCV in up to one-fourth of NMZL cases, but with a strong geographic variation (mostly Italian and Spanish series).[256,257,259,260,262] No other clear association has been described.

Histology and Immunophenotype

The histology and immunophenotype of NMZL resembles that of ENMZL or SMZL (see Fig. 83.3C).[263] The frequent presence of monocytoid B cells explains why this disease has been previously called *monocytoid B-cell lymphoma*[264] and *NMZL with or without monocytoid B cells*. A primary ENMZL should always be ruled out with appropriate studies (such as endoscopy) because 30% and 40% of cases presenting as NMZL may in fact represent nodal dissemination of an ENMZL of MALT type.[265,266]

Genetics

Complex genetic abnormalities are usually observed in NMZL, with the most frequent being partial trisomies of chromosomes 3 and 18, affecting the same regions as in ENMZL. None of the characteristic translocations of ENMZL are seen in NMZL, however.[89] Furthermore, del(7q) is also not observed. More than 75% of cases have mutated immunoglobulin genes.[257,267] Of interest, different VH immunoglobulin gene segments are predominantly involved in HCV-positive and HCV-negative patients, raising the possibility that distinct antigens drive different underlying chronic immune stimulation processes.[268] Recent gene sequencing work reported recurrent mutations in *KMT2D* (*MLL2*), *KLF2*, *PTTPRD*, and *NOTCH2*.[269]

Therapy

Few data are available to guide treatment of NMZL, and most recommendations are extrapolated from the management of FL.[12] As in other indolent lymphomas, expectant observation is appropriate for asymptomatic patients. A trial of anti-HCV can be considered in patients with HCV who do not require immediate treatment.[30] Radiation therapy may be curative for early disease. Symptomatic advanced disease can be managed with the same general approach described for advanced ENMZL, although there is usually a tendency to use combination chemoimmunotherapy identical to that for FL as first-line treatment because of the generally worse outcomes with NMZL as compared with ENMZL. Ibrutinib can be used in NMZL patients who relapse after use of rituximab; however, ORR was reported to be lower (47%) when compared to either ENMZL or SMZL (~63%).[194,195]

Prognosis

NMZL is usually associated with worse prognosis when compared with the other forms of MZL.[38] OS rates at 5 years vary from 55% to 70% in most series, except for those containing a greater proportion of early-stage disease, which report 5-year survivals of up to 80%. Five-year PFS is around 30%. Relapse in extranodal sites is rare. As in other lymphomas, the IPI correlates with outcomes, as does the Follicular Lymphoma International Prognostic Index (FLIPI)[270] (see Chapter 82). In a series of 47 patients, those with low-, intermediate-, and poor-risk FLIPI scores had a 5-year OS of approximately 90%, 70%, and 35%, respectively.[256] Patients with progression within 2 years of the initial treatment were reported to have worse OS.[271] Poor risk FLIPI, older age (>60 years), and high LDH were reported to be associated with inferior outcomes of 56 NMZL patients.[272]

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Al-Saleem T, Al-Mondhiry H. Immunoproliferative small intestinal disease (IPSID): a model for mature B-cell neoplasms. *Blood*. 2005;105:2274.

Armitage JO, Weisenburger DD. New approach to classifying non-Hodgkin's lymphomas: clinical features of the major histologic subtypes. Non-Hodgkin's lymphoma classification project. *J Clin Oncol*. 1998;16:2780.

Berger F, Felman P, Thieblemont C, et al. Non-MALT marginal zone B-cell lymphomas: a description of clinical presentation and outcome in 124 patients. *Blood*. 2000;95:1950.

Chacon JI, Mollejo M, Munoz E, et al. Splenic marginal zone lymphoma: clinical characteristics and prognostic factors in a series of 60 patients. *Blood*. 2002;100:1648.

Craig VJ, Arnold I, Gerke C, et al. Gastric MALT lymphoma B cells express polyreactive, somatically mutated immunoglobulins. *Blood*. 2010;115:581.

Escalon MP, Champlin RE, Saliba RM, et al. Nonmyeloablative allogeneic hematopoietic transplantation: a promising salvage therapy for patients with non-Hodgkin's lymphoma whose disease has failed a prior autologous transplantation. *J Clin Oncol*. 2004;22:2419.

Fischbach W, Goebeler ME, Ruskone-Fourmestraux A, et al. Most patients with minimal histological residuals of gastric MALT lymphoma after successful eradication of Helicobacter pylori can be managed safely by a watch and wait strategy: experience from a large international series. *Gut*. 2007;56:1685.

Hancock BW, Qian W, Linch D, et al. Chlorambucil versus observation after anti-Helicobacter therapy in gastric MALT lymphomas: results of the international randomised LY03 trial. *Br J Haematol*. 2009;144:367.

Hermine O, Lefrere F, Bronowicki JP, et al. Regression of splenic lymphoma with villous lymphocytes after treatment of hepatitis C virus infection. *N Engl J Med*. 2002;347:89.

Ho L, Davis RE, Conne B, et al. MALT1 and the API2-MALT1 fusion act between CD40 and IKK and confer NFκB-dependent proliferative advantage and resistance against FAS-induced cell death in B cells. *Blood*. 2005;105:2891.

Husain A, Roberts D, Pro B, et al. Meta-analyses of the association between *Chlamydia psittaci* and ocular adnexal lymphoma and the response of ocular adnexal lymphoma to antibiotics. *Cancer*. 2007;110:809.

Kalpadakis C, Pangalis GA, Angelopoulou MK, et al. Should rituximab replace splenectomy in the management of splenic marginal zone lymphoma? *Best Pract Res Clin Haematol*. 2018;31 65e72.

Lecuit M, Abachin E, Martin A, et al. Immunoproliferative small intestinal disease associated with *Campylobacter jejuni*. *N Engl J Med*. 2004;350:239.

Liu H, Ruskon-Fourmestraux A, Lavergne-Slove A, et al. Resistance of t(11;18) positive gastric mucosa-associated lymphoid tissue lymphoma to Helicobacter pylori eradication therapy. *Lancet*. 2001;357:39.

Malfertheiner P, Megraud F, O'Morain C, et al. Current concepts in the management of Helicobacter pylori infection: the Maastricht III Consensus Report. *Gut*. 2007;56:772.

Nathwani BN, Anderson JR, Armitage JO, et al. Marginal zone B-cell lymphoma: a clinical comparison of nodal and mucosa-associated lymphoid tissue types. Non-Hodgkin's Lymphoma Classification Project. *J Clin Oncol*. 1999;17:2486.

Olszewski AJ, Castillo JJ. Survival of patients with marginal zone lymphoma: analysis of the surveillance, epidemiology, and end results database. *Cancer*. 2013;119:629.

Rinaldi A, Mian M, Chigrinova E, et al. Genome-wide DNA profiling of marginal zone lymphomas identifies subtype-specific lesions with an impact on the clinical outcome. *Blood*. 2011;117:1595.

Rosebeck S, Madden L, Jin X, et al. Cleavage of NIK by the API2-MALT1 fusion oncoprotein leads to noncanonical NF-κB activation. *Science*. 2011;331:468.

Ruskone-Fourmestraux A, Fischbach W, Aleman BM, et al. EGILS consensus report. Gastric extranodal marginal zone B-cell lymphoma of MALT. *Gut*. 2011;60:747.

Salido M, Baro C, Oscier D, et al. Cytogenetic aberrations and their prognostic value in a series of 330 splenic marginal zone B-cell lymphomas: a multicenter study of the splenic B-cell lymphoma group. *Blood*. 2010;116:1479.

Schechter NR, Portlock CS, Yahalom J. Treatment of mucosa-associated lymphoid tissue lymphoma of the stomach with radiation alone. *J Clin Oncol*. 1998;16:1916.

Seligmann M, Danon F, Hurez D, et al. Alpha-chain disease: a new immunoglobulin abnormality. *Science*. 1968;162:1396.

Senff NJ, Noordijk EM, Kim YH, et al. European Organization for Research and Treatment of Cancer and International Society for Cutaneous Lymphoma consensus recommendations for the management of cutaneous B-cell lymphomas. *Blood*. 2008;112:1600.

Smedby KE, Vajdic CM, Falster M, et al. Autoimmune disorders and risk of non-Hodgkin lymphoma subtypes: a pooled analysis within the InterLymph Consortium. *Blood*. 2008;111:4029.

Stefanovic A, Lossos IS. Extranodal marginal zone lymphoma of the ocular adnexa. *Blood*. 2009;114:501.

Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. 2017*. Lyon: International Agency for Research on Cancer; 2017.

Weill JC, Weller S, Reynaud CA. Human marginal zone B cells. *Annu Rev Immunol*. 2009;27:267.

Xochelli A, Oscier D, Stamatopoulos K. Clonal B-cell lymphocytosis of marginal zone origin. *Best Pract Res Clin Haematol*. 2017 Mar-Jun;30(1-2):77–83.

Yahalom J, Illidge T, Specht L, et al. Modern radiation therapy for extranodal lymphomas: field and dose guidelines from the International Lymphoma Radiation Oncology Group International Lymphoma Radiation Oncology Group. *Int J Radiat Oncol Biol Phys*. 2015 May 1;92(1):11–31.

Zelenetz AD, Gordon LI, Abramson JS, et al. NCCN Guidelines Insights: B-Cell Lymphomas, Version 3.2019. *J Natl Compr Canc Netw*. 2019 Jun 1;17(6):650–661.

Zucca E, Bertoni F, Roggero E, et al. Molecular analysis of the progression from *Helicobacter pylori*-associated chronic gastritis to mucosa-associated lymphoid-tissue lymphoma of the stomach. *N Engl J Med*. 1998;338:804.

Zullo A, Hassan C, Cristofari F, et al. Effects of *Helicobacter pylori* eradication on early stage gastric mucosa-associated lymphoid tissue lymphoma. *Clin Gastroenterol Hepatol*. 2010;8:105.

REFERENCES

1. van Krieken JH, von Schilling C, Kluin PM, Lennert K. Splenic marginal zone lymphocytes and related cells in the lymph node: a morphologic and immunohistochemical study. *Hum Pathol*. 1989;20(4):320–325.
2. van den Oord JJ, de Wolf-Peeters C, Desmet VJ. The marginal zone in the human reactive lymph node. *Am J Clin Pathol*. 1986;86(4):475–479.
3. Martin F, Kearney JF. Marginal-zone B cells. *Nat Rev Immunol*. 2002;2(5):323–335.
4. Weill JC, Weller S, Reynaud CA. Human marginal zone B cells. *Annu Rev Immunol*. 2009;27:267–285.
5. Harris NL, Jaffe ES, Stein H, Banks PM, Chan JK, Cleary ML, et al. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. *Blood*. 1994;84(5):1361–1392.
6. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon: International Agency for Research on Cancer; 2017.
7. Swerdlow SH, Kuzu I, Dogan A, Dirnhofer S, Chan JK, Sander B, et al. The many faces of small B cell lymphomas with plasmacytic differentiation and the contribution of MYD88 testing. *Virchows Arch*. 2016;468(3).
8. Al-Saleem T, Al-Mondhiry H. Immunoproliferative small intestinal disease (IPSID): a model for mature B-cell neoplasms. *Blood*. 2005;105(6):2274–2280.
9. Xochelli AOD, Stamatopoulos K. Clonal B-cell lymphocytosis of marginal zone origin. *Best Pract Res Clin Haematol*. 2017;30(1-2):77–83.
10. Xochelli AKC, Gardiner A, Baliakas P, Vassilakopoulos TP, Mould S, Davis Z, et al. Clonal B-cell lymphocytosis exhibiting immunophenotypic features consistent with a marginal-zone origin: is this a distinct entity? *Blood*. 2014;123(8):1199–1206.
11. A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. The Non-Hodgkin"s Lymphoma Classification Project. *Blood*. 1997;89(11):3909-18.
12. Zelenetz AD, Abramson JS, Advani RH, Andreadis CB, Byrd JC, Czuczman MS, et al. NCCN Clinical Practice Guidelines in Oncology: non-Hodgkin's lymphomas. *J Natl Compr Canc Netw*. 2010;8(3):288–334.
13. Thieblemont C, Felman P, Berger F, Dumontet C, Arnaud P, Hequet O, et al. Treatment of splenic marginal zone B-cell lymphoma: an analysis of 81 patients. *Clin Lymphoma*. 2002;3(1):41–47.
14. Ruskone-Fourmestraux A, Fischbach W, Aleman BM, Boot H, Du MQ, Megraud F, et al. EGILS consensus report. Gastric extranodal marginal zone B-cell lymphoma of MALT. *Gut*. 2011;60(6):747–758.
15. Bayerdorffer E, Neubauer A, Rudolph B, Thiede C, Lehn N, Eidt S, et al. Regression of primary gastric lymphoma of mucosa-associated lymphoid tissue type after cure of *Helicobacter pylori* infection. MALT lymphoma study group. *Lancet*. 1995;345(8965):1591–1594.
16. Levy M, Copie-Bergman C, Traulle C, Lavergne-Slove A, Brousse N, Flejou JF, et al. Conservative treatment of primary gastric low-grade B-cell lymphoma of mucosa-associated lymphoid tissue: predictive factors of response and outcome. *Am J Gastroenterol*. 2002;97(2):292–297.
17. Montalban C, Castrillo JM, Abraira V, Serrano M, Bellas C, Piris MA, et al. Gastric B-cell mucosa-associated lymphoid tissue (MALT) lymphoma. Clinicopathological study and evaluation of the prognostic factors in 143 patients. *Ann Oncol*. 1995;6(4):355–362.
18. Ruskone-Fourmestraux A, Lavergne A, Aegerter PH, Megraud F, Palazzo L, de Mascarel A, et al. Predictive factors for regression of gastric MALT lymphoma after anti-*Helicobacter pylori* treatment. *Gut*. 2001;48(3):297–303.
19. Sackmann M, Morgner A, Rudolph B, Neubauer A, Thiede C, Schulz H, et al. Regression of gastric MALT lymphoma after eradication of *Helicobacter pylori* is predicted by endosonographic staging. MALT Lymphoma Study Group. *Gastroenterology*. 1997;113(4):1087–1090.
20. Steinbach G, Ford R, Glober G, Sample D, Hagemeister FB, Lynch PM, et al. Antibiotic treatment of gastric lymphoma of mucosa-associated lymphoid tissue. An uncontrolled trial. *Ann Intern Med*. 1999;131(2):88–95.
21. Mazloom A, Rodriguez A, Ha CS, Medeiros LJ, Wogan C, Shihadeh F, et al. Incidence of gastric involvement in patients with nongastrointestinal extranodal marginal zone lymphoma. *Cancer*. 2011;117(11):2461–2466.
22. Ogusa E, Tomita N, Ishii Y, Takasaki H, Hattori Y, Matsumoto C, Ishigatsubo Y. Clinical manifestations of primary pulmonary extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue in Japanese population. *Hematol Oncol*. 2013;31(1):18–21.
23. Perry C, Herishanu Y, Metzer U, Bairey O, Ruchlemer R, Trejo L, et al. Diagnostic accuracy of PET/CT in patients with extranodal marginal zone MALT lymphoma. *Eur J Haematol*. 2007;79(3):205–209.
24. Alinari L, Castellucci P, Elstrom R, Ambrosini V, Stefoni V, Nanni C, et al. 18F-FDG PET in mucosa-associated lymphoid tissue (MALT) lymphoma. *Leuk Lymphoma*. 2006;47(10):2096–2101.
25. Beal KP, Yeung HW, Yahalom J. FDG-PET scanning for detection and staging of extranodal marginal zone lymphomas of the MALT type: a report of 42 cases. *Ann Oncol*. 2005;16(3):473–480.
26. Hoffmann M, Kletter K, Becherer A, Jager U, Chott A, Raderer M. 18F-fluorodeoxyglucose positron emission tomography (18F-FDG-PET) for staging and follow-up of marginal zone B-cell lymphoma. *Oncology*. 2003;64(4):336–340.
27. Radan L, Fischer D, Bar-Shalom R, Dann EJ, Epelbaum R, Haim N, et al. FDG avidity and PET/CT patterns in primary gastric lymphoma. *Eur J Nucl Med Mol Imaging*. 2008;35(8):1424–1430.
28. Weiler-Sagie M, Bushelev O, Epelbaum R, Dann EJ, Haim N, Avivi I, et al. 18)F-FDG avidity in lymphoma readdressed: a study of 766 patients. *J Nucl Med*. 2010;51(1):25–30.
29. Treglia G, Zucca E, Sadeghi R, Cavalli F, Giovanella L, Ceriani L. Detection rate of fluorine-18-fluorodeoxyglucose positron emission tomography in patients with marginal zone lymphoma of MALT type: a meta-analysis. *Hematol Oncol*. 2014
30. Zucca E, Arcaini L, Buske C, Johnson PW, Ponzoni M, Raderer M, et al. Marginal zone lymphomas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up ESMO Guidelines Committee. *Ann Oncol*. 2020;31(1).
31. Radaszkiewicz T, Dragosics B, Bauer P. Gastrointestinal malignant lymphomas of the mucosa-associated lymphoid tissue: factors relevant to prognosis. *Gastroenterology*. 1992;102(5):1628–1638.
32. Rohatiner A, d'Amore F, Coiffier B, Crowther D, Gospodarowicz M, Isaacson P, et al. Report on a workshop convened to discuss the pathological and staging classifications of gastrointestinal tract lymphoma. *Ann Oncol*. 1994;5(5):397–400.
33. Ruskone-Fourmestraux A, Dragosics B, Morgner A, Wotherspoon A, De Jong D. Paris staging system for primary gastrointestinal lymphomas. *Gut*. 2003;52(6):912–913.
34. Kim YH, Willemze R, Pimpinelli N, Whittaker S, Olsen EA, Ranki A, et al. TNM classification system for primary cutaneous lymphomas other than mycosis fungoides and Sezary syndrome: a proposal of the International Society for Cutaneous Lymphomas (ISCL) and the Cutaneous Lymphoma Task Force of the European Organization of Research and Treatment of Cancer (EORTC). *Blood*. 2007;110(2):479–484.
35. Olszewski AJ, Castillo JJ. Survival of patients with marginal zone lymphoma: analysis of the Surveillance, epidemiology, and end results database. *Cancer*. 2013;119(3):629–638.
36. Armitage JO, Weisenburger DD. New approach to classifying non-Hodgkin's lymphomas: clinical features of the major histologic subtypes. Non-Hodgkin's Lymphoma Classification Project. *J Clin Oncol*. 1998;16(8):2780–2795.
37. Stefanovic A, Lossos IS. Extranodal marginal zone lymphoma of the ocular adnexa. *Blood*. 2009;114(3):501–510.
38. Nathwani BN, Anderson JR, Armitage JO, Cavalli F, Diebold J, Drachenberg MR, et al. Marginal zone B-cell lymphoma: a clinical comparison of nodal and mucosa-associated lymphoid tissue types. Non-Hodgkin's Lymphoma Classification Project. *J Clin Oncol*. 1999;17(8):2486–2492.
39. Remstein ED, Dogan A, Einerson RR, Paternoster SF, Fink SR, Law M, et al. The incidence and anatomic site specificity of chromosomal translocations in primary extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) in North America. *Am J Surg Pathol*. 2006;30(12):1546–1553.
40. Streubel B, Simonitsch-Klupp I, Mullauer L, Lamprecht A, Huber D, Siebert R, et al. Variable frequencies of MALT lymphoma-associated genetic aberrations in MALT lymphomas of different sites. *Leukemia*. 2004;18(10):1722–1726.
41. Thieblemont C, Bastion Y, Berger F, Rieux C, Salles G, Dumontet C, et al. Mucosa-associated lymphoid tissue gastrointestinal and nongastrointestinal lymphoma behavior: analysis of 108 patients. *J Clin Oncol*. 1997;15(4):1624–1630.
42. Koch P, del Valle F, Berdel WE, Willich NA, Reers B, Hiddemann W, et al. Primary gastrointestinal non-Hodgkin's lymphoma: I. Anatomic and histologic distribution, clinical features, and survival data of 371 patients registered in the German Multicenter Study GIT NHL 01/92. *J Clin Oncol*. 2001;19(18):3861–3873.
43. Decaudin D, de Cremoux P, Vincent-Salomon A, Dendale R, Rouic LL. Ocular adnexal lymphoma: a review of clinicopathologic features and treatment options. *Blood*. 2006;108(5):1451–1460.
44. Senff NJ, Noordijk EM, Kim YH, Bagot M, Berti E, Cerroni L, et al. European Organization for Research and Treatment of Cancer

and International Society for Cutaneous Lymphoma consensus recommendations for the management of cutaneous B-cell lymphomas. *Blood*. 2008;112(5):1600–1609.

45. Raderer MKB, Ferreri AJ. Clinicopathologic characteristics and treatment of marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma). *CA Cancer J Clin*. 2016;66(2):153–171.
46. Fine KD, Stone MJ. Alpha-heavy chain disease, Mediterranean lymphoma, and immunoproliferative small intestinal disease: a review of clinicopathological features, pathogenesis, and differential diagnosis. *Am J Gastroenterol*. 1999;94(5):1139–1152.
47. Wotherspoon AC, Ortiz-Hidalgo C, Falzon MR, Isaacson PG. *Helicobacter pylori*-associated gastritis and primary B-cell gastric lymphoma. *Lancet*. 1991;338(8776):1175–1176.
48. Akamatsu T, Mochizuki T, Okiyama Y, Matsumoto A, Miyabayashi H, Ota H. Comparison of localized gastric mucosa-associated lymphoid tissue (MALT) lymphoma with and without *Helicobacter pylori* infection. *Helicobacter*. 2006;11(2):86–95.
49. Chen LT, Lin JT, Tai JJ, Chen GH, Yeh HZ, Yang SS, et al. Long-term results of anti-*Helicobacter pylori* therapy in early-stage gastric high-grade transformed MALT lymphoma. *J Natl Cancer Inst*. 2005;97(18):1345–1353.
50. Fischbach W, Goebeler-Kolve ME, Dragosics B, Greiner A, Stolte M. Long term outcome of patients with gastric marginal zone B cell lymphoma of mucosa associated lymphoid tissue (MALT) following exclusive *Helicobacter pylori* eradication therapy: experience from a large prospective series. *Gut*. 2004;53(1):34–37.
51. Lecuit M, Abachin E, Martin A, Poyart C, Pochart P, Suarez F, et al. Immunoproliferative small intestinal disease associated with *Campylobacter jejuni*. *N Engl J Med*. 2004;350(3):239–248.
52. Chanudet E, Zhou Y, Bacon CM, Wotherspoon AC, Muller-Hermelink HK, Adam P, et al. *Chlamydia psittaci* is variably associated with ocular adnexal MALT lymphoma in different geographical regions. *J Pathol*. 2006;209(3):344–351.
53. Daibata M, Nemoto Y, Togitani K, Fukushima A, Ueno H, Ouchi K, et al. Absence of *Chlamydia psittaci* in ocular adnexal lymphoma from Japanese patients. *Br J Haematol*. 2006;132(5):651–652.
54. de Cremoux P, Subtil A, Ferreri AJ, Vincent-Salomon A, Ponzoni M, Chaoui D, et al. Re: Evidence for an association between *Chlamydia psittaci* and ocular adnexal lymphomas. *J Natl Cancer Inst*. 2006;98(5):365–366.
55. Ferreri AJ, Guidoboni M, Ponzoni M, De Conciliis C, Dell'Oro S, Fleischhauer K, et al. Evidence for an association between *Chlamydia psittaci* and ocular adnexal lymphomas. *J Natl Cancer Inst*. 2004;96(8):586–594.
56. Ferreri AJ, Ponzoni M, Guidoboni M, Resti AG, Politi LS, Cortelazzo S, et al. Bacteria-eradicating therapy with doxycycline in ocular adnexal MALT lymphoma: a multicenter prospective trial. *J Natl Cancer Inst*. 2006;98(19):1375–1382.
57. Ferreri AJ, Govi S, Pasini E, Mappa S, Bertoni F, Zaja F, et al. Chlamydophila psittaci eradication with doxycycline as first-line targeted therapy for ocular adnexae lymphoma: final results of an international phase II trial. *J Clin Oncol*. 2012;30(24):2988–2994.
58. Gracia E, Froesch P, Mazzucchelli L, Martin V, Rodriguez-Abreu D, Jimenez J, et al. Low prevalence of Chlamydia psittaci in ocular adnexal lymphomas from Cuban patients. *Leuk Lymphoma*. 2007;48(1):104–108.
59. Liu YC, Ohyashiki JH, Ito Y, Iwaya K, Serizawa H, Mukai K, et al. Chlamydia psittaci in ocular adnexal lymphoma: Japanese experience. *Leuk Res*. 2006;30(12):1587–1589.
60. Mulder MM, Heddema ER, Pannekoek Y, Faridpooya K, Oud ME, Schilder-Tol E, et al. No evidence for an association of ocular adnexal lymphoma with Chlamydia psittaci in a cohort of patients from the Netherlands. *Leuk Res*. 2006;30(10):1305–1307.
61. Rosado MF, Byrne Jr. GE, Ding F, Fields KA, Ruiz P, Dubovy SR, et al. Ocular adnexal lymphoma: a clinicopathologic study of a large cohort of patients with no evidence for an association with *Chlamydia psittaci*. *Blood*. 2006;107(2):467–472.
62. Vargas RL, Fallone E, Felgar RE, Friedberg JW, Arbini AA, Andersen AA, et al. Is there an association between ocular adnexal lymphoma and infection with *Chlamydia psittaci*? The University of Rochester experience. *Leuk Res*. 2006;30(5):547–551.
63. Li C, Inagaki H, Kuo TT, Hu S, Okabe M, Eimoto T. Primary cutaneous marginal zone B-cell lymphoma: a molecular and clinicopathologic study of 24 Asian cases. *Am J Surg Pathol*. 2003;27(8):1061–1069.
64. Wood GS, Kamath NV, Guitart J, Heald P, Kohler S, Smoller BR, et al. Absence of *Borrelia burgdorferi* DNA in cutaneous B-cell lymphomas from the United States. *J Cutan Pathol*. 2001;28(10):502–507.
65. Goodlad JR, Davidson MM, Hollowood K, Ling C, MacKenzie C, Christie I, et al. Primary cutaneous B-cell lymphoma and *Borrelia burgdorferi* infection in patients from the Highlands of Scotland. *Am J Surg Pathol*. 2000;24(9):1279–1285.
66. Cerroni L, Zochling N, Putz B, Kerl H. Infection by *Borrelia burgdorferi* and cutaneous B-cell lymphoma. *J Cutan Pathol*. 1997;24(8):457–461.
67. Anderson LA, Gadalla S, Morton LM, Landgren O, Pfeiffer R, Warren JL, et al. Population-based study of autoimmune conditions and the risk of specific lymphoid malignancies. *Int J Cancer*. 2009;125(2):398–405.
68. Holm LE, Blomgren H, Lowhagen T. Cancer risks in patients with chronic lymphocytic thyroiditis. *N Engl J Med*. 1985;312(10):601–604.
69. Kato I, Tajima K, Suchi T, Aozasa K, Matsuzuka F, Kuma K, et al. Chronic thyroiditis as a risk factor of B-cell lymphoma in the thyroid gland. *Jpn J Cancer Res*. 1985;76(11):1085–1090.
70. Smedby KE, Hjalgrim H, Askling J, Chang ET, Gregersen H, Porwit-MacDonald A, et al. Autoimmune and chronic inflammatory disorders and risk of non-Hodgkin lymphoma by subtype. *J Natl Cancer Inst*. 2006;98(1):51–60.
71. Smedby KE, Vajdic CM, Falster M, Engels EA, Martinez-Maza O, Turner J, et al. Autoimmune disorders and risk of non-Hodgkin lymphoma subtypes: a pooled analysis within the InterLymph Consortium. *Blood*. 2008;111(8):4029–4038.
72. Perry AM, Diebold J, Nathwani BN, MacLennan KA, Müller-Hermelink HK, Bast M, et al. Non-Hodgkin lymphoma in the developing world: review of 4539 cases from the International Non-Hodgkin Lymphoma Classification Project. *Haematologica*. 2016;101(10):1244–1250.
73. Suarez F, Lortholary O, Hermine O, Lecuit M. Infection-associated lymphomas derived from marginal zone B cells: a model of antigen-driven lymphoproliferation. *Blood*. 2006;107(8):3034–3044.
74. Zucca E, Bertoni F, Roggero E, Bosshard G, Cazzaniga G, Pedrinis E, et al. Molecular analysis of the progression from *Helicobacter pylori*-associated chronic gastritis to mucosa-associated lymphoid-tissue lymphoma of the stomach. *N Engl J Med*. 1998;338(12):804–810.
75. Isaacson PG, Chott A, Nakamura S, Muller-Hermelink HK, Harris NL, Swerdlow SH. Extranodal marginal zone lymphoma of mucosa-associated lymhpoid tissue (MALT lymphoma). In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid tissues*. Lyon, France: International Agency for Research on Cancer; 2008:185–187.
76. Bain BJ. Dutcher bodies. *Am J Hematol*. 2009;84(9):589.
77. Dickey W, Kenny BD, McConnell JB. Effect of proton pump inhibitors on the detection of *Helicobacter pylori* in gastric biopsies. *Aliment Pharmacol Ther*. 1996;10(3):289–293.
78. Graham DY, Opekun AR, Hammoud F, Yamaoka Y, Reddy R, Osato MS, et al. Studies regarding the mechanism of false negative urea breath tests with proton pump inhibitors. *Am J Gastroenterol*. 2003;98(5):1005–1009.
79. Basset M, Defrancesco I, Milani P, Nuvolone M, Rattotti S, Foli A, et al. Nonlymphoplasmacytic lymphomas associated with light-chain amyloidosis. *Blood*. 2020;135(4):293–296.
80. Kwee I, Rancoita PM, Rinaldi A, Ferreri AJ, Bhagat G, Gascoyne RD, et al. Genomic profiles of MALT lymphomas: variability across anatomical sites. *Haematologica*. 2011;96(7):1064–1066.
81. Murga Penas EM, Callet-Bauchu E, Ye H, Gazzo S, Berger F, Schilling G, et al. The t(14;18)(q32;q21)/IGH-MALT1 translocation in MALT lymphomas contains templated nucleotide insertions and a major breakpoint region similar to follicular and mantle cell lymphoma. *Blood*. 2010;115(11):2214–2219.
82. Ho L, Davis RE, Conne B, Chappuis R, Berczy M, Mhawech P, et al. MALT1 and the API2-MALT1 fusion act between CD40 and IKK and confer NFκB-dependent proliferative advantage and resistance against FAS-induced cell death in B cells. *Blood*. 2005;105(7):2891–2899.
83. Rosebeck S, Madden L, Jin X, Gu S, Apel IJ, Appert A, et al. Cleavage of NIK by the API2-MALT1 fusion oncoprotein leads to noncanonical NFκB activation. *Science*. 2011;331(6016):468–472.
84. Liu H, Ruskon-Fourmestraux A, Lavergne-Slove A, Ye H, Molina T, Bouhnik Y, et al. Resistance of t(11;18) positive gastric mucosa-associated lymphoid tissue lymphoma to *Helicobacter pylori* eradication therapy. *Lancet*. 2001;357(9249):39–40.
85. Ye H, Liu H, Raderer M, Chott A, Ruskone-Fourmestraux A, Wotherspoon A, et al. High incidence of t(11;18)(q21;q21) in *Helicobacter pylori*-negative gastric MALT lymphoma. *Blood*. 2003;101(7):2547–2550.
86. Streubel B, Vinatzer U, Lamprecht A, Raderer M, Chott A. T (3;14) (p14.1;q32) involving IGH and FOXP1 is a novel recurrent chromosomal aberration in MALT lymphoma. *Leukemia*. 2005;19(4):652–658.
87. Honma K, Tsuzuki S, Nakagawa M, Tagawa H, Nakamura S, Morishima Y, et al. TNFAIP3/A20 functions as a novel tumor suppressor gene in several subtypes of non-Hodgkin lymphomas. *Blood*. 2009;114(12):2467–2475.
88. Novak U, Rinaldi A, Kwee I, Nandula SV, Rancoita PM, Compagno M, et al. The NFκB negative regulator TNFAIP3 (A20) is inactivated by

somatic mutations and genomic deletions in marginal zone lymphomas. *Blood*. 2009;113(20):4918–4921.

89. Rinaldi A, Mian M, Chigrinova E, Arcaini L, Bhagat G, Novak U, et al. Genome-wide DNA profiling of marginal zone lymphomas identifies subtype-specific lesions with an impact on the clinical outcome. *Blood*. 2011;117(5):1595–1604.
90. Craig VJ, Arnold I, Gerke C, Huynh MQ, Wundisch T, Neubauer A, et al. Gastric MALT lymphoma B cells express polyreactive, somatically mutated immunoglobulins. *Blood*. 2010;115(3):581–591.
91. Seligmann M, Danon F, Hurez D, Mihaesco E, Preud'homme JL. Alpha-chain disease: a new immunoglobulin abnormality. *Science*. 1968;162(860):1396–1397.
92. Malfertheiner P, Megraud F, O'Morain C, Bazzoli F, El-Omar E, Graham D, et al. Current concepts in the management of *Helicobacter pylori* infection: the Maastricht III Consensus Report. *Gut*. 2007;56(6):772–781.
93. Zullo A, Hassan C, Andriani A, Cristofari F, De Francesco V, Ierardi E, et al. Eradication therapy for *Helicobacter pylori* in patients with gastric MALT lymphoma: a pooled data analysis. *Am J Gastroenterol*. 2009;104(8):1932–1937. quiz 8.
94. Copie-Bergman C, Gaulard P, Lavergne-Slove A, Brousse N, Flejou JF, Dordonne K, et al. Proposal for a new histological grading system for post-treatment evaluation of gastric MALT lymphoma. *Gut*. 2003;52(11):1656.
95. Wundisch T, Mosch C, Neubauer A, Stolte M. *Helicobacter pylori* eradication in gastric mucosa-associated lymphoid tissue lymphoma: results of a 196-patient series. *Leuk Lymphoma*. 2006;47(10):2110–2114.
96. Fischbach W, Goebeler ME, Ruskone-Fourmestraux A, Wundisch T, Neubauer A, Raderer M, et al. Most patients with minimal histological residuals of gastric MALT lymphoma after successful eradication of *Helicobacter pylori* can be managed safely by a watch and wait strategy: experience from a large international series. *Gut*. 2007;56(12):1685–1687.
97. Zullo A, Hassan C, Cristofari F, Andriani A, De Francesco V, Ierardi E, et al. Effects of *Helicobacter pylori* eradication on early stage gastric mucosa-associated lymphoid tissue lymphoma. *Clin Gastroenterol Hepatol*. 2010;8(2):105–110.
98. Agresta F, Trentin G, Mainente P, Della Libera D, Bianco A, Salvagno L. Gastric MALT lymphomas: the value of an endoscopic follow-up evaluation. *Surg Endosc*. 2000;14(12):1153–1155.
99. Andriani A, Miedico A, Tedeschi L, Patti C, Di Raimondo F, Leone M, et al. Management and long-term follow-up of early stage *H. pylori*-associated gastric MALT-lymphoma in clinical practice: an Italian, multicentre study. *Dig Liver Dis*. 2009;41(7):467–473.
100. Bertoni F, Conconi A, Capella C, Motta T, Giardini R, Ponzoni M, et al. Molecular follow-up in gastric mucosa-associated lymphoid tissue lymphomas: early analysis of the LY03 cooperative trial. *Blood*. 2002;99(7):2541–2544.
101. de Jong D, Vyth-Dreese F, Dellemijn T, Verra N, Ruskone-Fourmestraux A, Lavergne-Slove A, et al. Histological and immunological parameters to predict treatment outcome of *Helicobacter pylori* eradication in low-grade gastric MALT lymphoma. *J Pathol*. 2001;193(3):318–324.
102. Dong G, Liu C, Ye H, Gong L, Zheng J, Li M, et al. BCL10 nuclear expression and t(11;18)(q21;q21) indicate nonresponsiveness to *Helicobacter pylori* eradication of Chinese primary gastric MALT lymphoma. *Int J Hematol*. 2008;88(5):516–523.
103. El-Zahabi LM, Jamali FR, El II H, Naja M, Salem Z, Shamseddine A, et al. The value of EUS in predicting the response of gastric mucosa-associated lymphoid tissue lymphoma to *Helicobacter pylori* eradication. *Gastrointest Endosc*. 2007;65(1):89–96.
104. Hiyama T, Haruma K, Kitadai Y, Ito M, Masuda H, Miyamoto M, et al. c-myc gene mutation in gastric mucosa-associated lymphoid tissue (MALT) lymphoma and diffuse large B-cell lymphoma. *Oncol Rep*. 2001;8(2):289–292.
105. Hong SS, Jung HY, Choi KD, Song HJ, Lee GH, Oh TH, et al. A prospective analysis of low-grade gastric malt lymphoma after *Helicobacter pylori* eradication. *Helicobacter*. 2006;11(6):569–573.
106. Kawahara Y, Mizuno M, Yoshino T, Yokota K, Oguma K, Okada H, et al. HLA-DQA1*0103-DQB1*0601 haplotype and *Helicobacter pylori*-positive gastric mucosa-associated lymphoid tissue lymphoma. *Clin Gastroenterol Hepatol*. 2005;3(9):865–868.
107. Kim JS, Chung SJ, Choi YS, Cheon JH, Kim CW, Kim SG, et al. *Helicobacter pylori* eradication for low-grade gastric mucosa-associated lymphoid tissue lymphoma is more successful in inducing remission in distal compared to proximal disease. *Br J Cancer*. 2007;96(9):1324–1328.
108. Lee SK, Lee YC, Chung JB, Chon CY, Moon YM, Kang JK, et al. Low grade gastric mucosa associated lymphoid tissue lymphoma: treatment strategies based on 10 year follow-up. *World J Gastroenterol*. 2004;10(2):223–226.
109. Montalban C, Santon A, Redondo C, Garcia-Cosio M, Boixeda D, Vazquez-Sequeiros E, et al. Long-term persistence of molecular disease after histological remission in low-grade gastric MALT lymphoma treated with *H. pylori* eradication. Lack of association with translocation t(11;18): a 10-year updated follow-up of a prospective study. *Ann Oncol*. 2005;16(9):1539–1544.
110. Nobre-Leitao C, Lage P, Cravo M, Cabecadas J, Chaves P, Alberto-Santos A, et al. Treatment of gastric MALT lymphoma by *Helicobacter pylori* eradication: a study controlled by endoscopic ultrasonography. *Am J Gastroenterol*. 1998;93(5):732–736.
111. Ohashi S, Segawa K, Okamura S, Urano F, Kanamori S, Hosoi T, et al. Gastrin and *Helicobacter pylori* in low-grade MALT lymphoma patients. *Scand J Gastroenterol*. 2002;37(3):279–286.
112. Ono S, Kato M, Ono Y, Itoh T, Kubota K, Nakagawa M, et al. Characteristics of magnified endoscopic images of gastric extranodal marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue, including changes after treatment. *Gastrointest Endosc*. 2008;68(4):624–631.
113. Papa A, Cammarota G, Tursi A, Gasbarrini A, Gasbarrini G. *Helicobacter pylori* eradication and remission of low-grade gastric mucosa-associated lymphoid tissue lymphoma: a long-term follow-up study. *J Clin Gastroenterol*. 2000;31(2):169–171.
114. Pinotti G, Zucca E, Roggero E, Pascarella A, Bertoni F, Savio A, et al. Clinical features, treatment and outcome in a series of 93 patients with low-grade gastric MALT lymphoma. *Leuk Lymphoma*. 1997;26(5-6):527–537.
115. Raderer M, Osterreicher C, Machold K, Formanek M, Fiebiger W, Penz M, et al. Impaired response of gastric MALT-lymphoma to *Helicobacter pylori* eradication in patients with autoimmune disease. *Ann Oncol*. 2001;12(7):937–939.
116. Savio A, Franzin G, Wotherspoon AC, Zamboni G, Negrini R, Buffoli F, et al. Diagnosis and posttreatment follow-up of *Helicobacter pylori*-positive gastric lymphoma of mucosa-associated lymphoid tissue: histology, polymerase chain reaction, or both? *Blood*. 1996;87(4):1255–1260.
117. Savio A, Zamboni G, Capelli P, Negrini R, Santandrea G, Scarpa A, et al. Relapse of low-grade gastric MALT lymphoma after *Helicobacter pylori* eradication: true relapse or persistence? Long-term post-treatment follow-up of a multicenter trial in the north-east of Italy and evaluation of the diagnostic protocol's adequacy. *Recent Results Cancer Res*. 2000;156:116–124.
118. Takenaka R, Yokota K, Mizuno M, Okada H, Toyokawa T, Yamasaki R, et al. Serum antibodies to *Helicobacter pylori* and its heat-shock protein 60 correlate with the response of gastric mucosa-associated lymphoid tissue lymphoma to eradication of *H. pylori*. *Helicobacter*. 2004;9(3):194–200.
119. Vrieling C, de Jong D, Boot H, de Boer JP, Wegman F, Aleman BM. Long-term results of stomach-conserving therapy in gastric MALT lymphoma. *Radiother Oncol*. 2008;87(3):405–411.
120. Weston AP, Banerjee SK, Horvat RT, Zoubine MN, Campbell DR, Cherian R. Prospective long-term endoscopic and histologic follow-up of gastric lymphoproliferative disease of early stage IE low-grade B-cell mucosa-associated lymphoid tissue type following *Helicobacter pylori* eradication treatment. *Int J Oncol*. 1999;15(5):899–907.
121. Wundisch T, Thiede C, Morgner A, Dempfle A, Gunther A, Liu H, et al. Long-term follow-up of gastric MALT lymphoma after *Helicobacter pylori* eradication. *J Clin Oncol*. 2005;23(31):8018–8024.
122. Yamashita H, Watanabe H, Ajioka Y, Nishikura K, Maruta K, Fujino MA. When can complete regression of low-grade gastric lymphoma of mucosa-associated lymphoid tissue be predicted after *Helicobacter pylori* eradication? *Histopathology*. 2000;37(2):131–140.
123. Hancock BW, Qian W, Linch D, Delchier JC, Smith P, Jakupovic I, et al. Chlorambucil versus observation after anti-Helicobacter therapy in gastric MALT lymphomas: results of the international randomised LY03 trial. *Br J Haematol*. 2009;144(3):367–375.
124. Strati P, Lee ST, Teegavarupu P, Karri A, Anireddy S, Hagemeister FB, et al. Frontline antibiotic therapy for early-stage *Helicobacter pylori*-negative gastric MALT lymphoma. *Am J Hematol*. 2019;94(6):E150–E153.
125. Raderer M, Streubel B, Wohrer S, Hafner M, Chott A. Successful antibiotic treatment of *Helicobacter pylori* negative gastric mucosa associated lymphoid tissue lymphomas. *Gut*. 2006;55(5):616–618.
126. Park HS, Kim YJ, Yang WI, Suh CO, Lee YC. Treatment outcome of localized *Helicobacter pylori*-negative low-grade gastric MALT lymphoma. *World J Gastroenterol*. 2010;16(17):2158–2162.
127. De Groote D, Van Doorn LJ, Van den Bulck K, Vandamme P, Vieth M, Stolte M, et al. Detection of non-pylori *Helicobacter* species in "*Helicobacter heilmannii*"-infected humans. *Helicobacter*. 2005;10(5):398–406.
128. Morgner A, Lehn N, Andersen LP, Thiede C, Bennedsen M, Trebesius K, et al. *Helicobacter heilmannii*-associated primary gastric low-grade MALT lymphoma: complete remission after curing the infection. *Gastroenterology*. 2000;118(5):821–828.

129. Schechter NR, Portlock CS, Yahalom J. Treatment of mucosa-associated lymphoid tissue lymphoma of the stomach with radiation alone. *J Clin Oncol*. 1998;16(5):1916–1921.
130. Zullo A, Hassan C, Andriani A, Cristofari F, Bassanelli C, Spinelli GP, et al. Treatment of low-grade gastric MALT-lymphoma unresponsive to *Helicobacter pylori* therapy: a pooled-data analysis. *Med Oncol*. 2010;27(2):291–295.
131. Wirth A, Gospodarowicz M, Aleman BM, Bressel M, Ng A, Chao M, et al. Long-term outcome for gastric marginal zone lymphoma treated with radiotherapy: a retrospective, multi-centre, International Extranodal Lymphoma Study Group study. *Ann Oncol*. 2013;24(5):1344–1351.
132. Wirth A, Mikhaeel NG, Aleman BMP, Pinnix CC, Constine LS, Ricardi U, et al. Involved site radiation therapy in adult lymphomas: an overview of international lymphoma radiation oncology group guidelines. *Int J Radiat Oncol Biol Phys*. 2020;107(5):909–933.
133. Pinnix CC, Gunther JR, Milgrom SA, Cruz Chamorro RJ, Medeiros LJ, Khoury JD, et al. Outcomes after reduced-dose intensity modulated radiation therapy for gastric mucosa-associated lymphoid tissue (MALT) lymphoma. *Int J Radiat Oncol Biol Phys*. 2019;104(2):447–455.
134. De Leo AN, Bates JE, Lockney NA, Mendenhall NP, Shaikh ME, Morris CG, Hoppe BS. Radiotherapy in early-stage gastric MALT: improved survival without increased cardiac death. *Am J Clin Oncol*. 2020;43(11):770–775.
135. Lowry L, Smith P, Qian W, Falk S, Benstead K, Illidge T, et al. Reduced dose radiotherapy for local control in non-Hodgkin lymphoma: a randomised phase III trial. *Radiother Oncol*. 2011;100(1):86–92.
136. Yahalom J, Illidge T, Specht L, Hoppe RT, Li YX, Tsang R, Wirth A. Modern radiation therapy for extranodal lymphomas: field and dose guidelines from the International Lymphoma Radiation Oncology Group International lymphoma radiation oncology group. *Int J Radiat Oncol Biol Phys*. 2015;92(1):11–31.
137. Copie-Bergman C, Locher C, Levy M, Chaumette MT, Haioun C, Delfau-Larue MH, et al. Metachronous gastric MALT lymphoma and early gastric cancer: is residual lymphoma a risk factor for the development of gastric carcinoma? *Ann Oncol*. 2005;16(8):1232–1236.
138. Noy A, Yahalom J, Zaretsky L, Brett I, Zelenetz AD. Gastric mucosa-associated lymphoid tissue lymphoma detected by clonotypic polymerase chain reaction despite continuous pathologic remission induced by involved-field radiotherapy. *J Clin Oncol*. 2005;23(16):3768–3772.
139. Thiede C, Wundisch T, Alpen B, Neubauer B, Morgner A, Schmitz M, et al. Long-term persistence of monoclonal B cells after cure of *Helicobacter pylori* infection and complete histologic remission in gastric mucosa-associated lymphoid tissue B-cell lymphoma. *J Clin Oncol*. 2001;19(6):1600–1609.
140. Ejima Y, Sasaki R, Okamoto Y, Maruta T, Azumi A, Hayashi Y, et al. Ocular adnexal mucosa-associated lymphoid tissue lymphoma treated with radiotherapy. *Radiother Oncol*. 2006;78(1):6–9.
141. Fung CY, Tarbell NJ, Lucarelli MJ, Goldberg SI, Linggood RM, Harris NL, et al. Ocular adnexal lymphoma: clinical behavior of distinct World Health Organization classification subtypes. *Int J Radiat Oncol Biol Phys*. 2003;57(5):1382–1391.
142. Hasegawa M, Kojima M, Shioya M, Tamaki Y, Saitoh J, Sakurai H, et al. Treatment results of radiotherapy for malignant lymphoma of the orbit and histopathologic review according to the WHO classification. *Int J Radiat Oncol Biol Phys*. 2003;57(1):172–176.
143. Le QT, Eulau SM, George TI, Hildebrand R, Warnke RA, Donaldson SS, et al. Primary radiotherapy for localized orbital MALT lymphoma. *Int J Radiat Oncol Biol Phys*. 2002;52(3):657–663.
144. Lee JL, Kim MK, Lee KH, Hyun MS, Chung HS, Kim DS, et al. Extranodal marginal zone B-cell lymphomas of mucosa-associated lymphoid tissue-type of the orbit and ocular adnexa. *Ann Hematol*. 2005;84(1):13–18.
145. Suh CO, Shim SJ, Lee SW, Yang WI, Lee SY, Hahn JS. Orbital marginal zone B-cell lymphoma of MALT: radiotherapy results and clinical behavior. *Int J Radiat Oncol Biol Phys*. 2006;65(1):228–233.
146. Tanimoto K, Kaneko A, Suzuki S, Sekiguchi N, Watanabe T, Kobayashi Y, et al. Primary ocular adnexal MALT lymphoma: a long-term follow-up study of 114 patients. *Jpn J Clin Oncol*. 2007;37(5):337–344.
147. Tsang RW, Gospodarowicz MK, Pintilie M, Wells W, Hodgson DC, Sun A, et al. Localized mucosa-associated lymphoid tissue lymphoma treated with radiation therapy has excellent clinical outcome. *J Clin Oncol*. 2003;21(22):4157–4164.
148. Uno T, Isobe K, Shikama N, Nishikawa A, Oguchi M, Ueno N, et al. Radiotherapy for extranodal, marginal zone, B-cell lymphoma of mucosa-associated lymphoid tissue originating in the ocular adnexa: a multiinstitutional, retrospective review of 50 patients. *Cancer*. 2003;98(4):865–871.
149. Goda JS, Gospodarowicz M, Pintilie M, Wells W, Hodgson DC, Sun A, et al. Long-term outcome in localized extranodal mucosa-associated lymphoid tissue lymphomas treated with radiotherapy. *Cancer*. 2010;116(16):3815–3824.
150. Stafford SL, Kozelsky TF, Garrity JA, Kurtin PJ, Leavitt JA, Martenson JA, et al. Orbital lymphoma: radiotherapy outcome and complications. *Radiother Oncol*. 2001;59(2):139–144.
151. Hoskin PJ, Kirkwood AA, Popova B, Smith P, Robinson M, Gallop-Evans E, et al. 4 Gy versus 24 Gy radiotherapy for patients with indolent lymphoma (FORT): a randomised phase 3 non-inferiority trial. *Lancet Oncol*. 2014;15(4):457–463.
152. Fasola CE, Jones JC, Huang DD, Le QT, Hoppe RT, Donaldson SS. Low-dose radiation therapy (2 Gy × 2) in the treatment of orbital lymphoma. *Int J Radiat Oncol Biol Phys*. 2013;86(5):930–935.
153. Husain A, Roberts D, Pro B, McLaughlin P, Esmaeli B. Meta-analyses of the association between *Chlamydia psittaci* and ocular adnexal lymphoma and the response of ocular adnexal lymphoma to antibiotics. *Cancer*. 2007;110(4):809–815.
154. Ferreri AJM, Sassone M, Miserocchi E, Govi S, Cecchetti C, Corti ME, et al. Treatment of MALT lymphoma of the conjunctiva with intralesional rituximab supplemented with autologous serum. *Blood Adv*. 2020;4(6):1013–1019.
155. Akbulut H, Soykan I, Yakaryilmaz F, Icii F, Aksoy F, Haznedaroglu S, et al. Five-year results of the treatment of 23 patients with immunoproliferative small intestinal disease: a Turkish experience. *Cancer*. 1997;80(1):8–14.
156. Al-Bahrani ZR, Al-Mondhiry H, Bakir F, Al-Saleem T. Clinical and pathologic subtypes of primary intestinal lymphoma. Experience with 132 patients over a 14-year period. *Cancer*. 1983;52(9):1666–1672.
157. Ben-Ayed F, Halphen M, Najjar T, Boussene H, Jaafoura H, Bouguerra A, et al. Treatment of alpha chain disease. Results of a prospective study in 21 Tunisian patients by the Tunisian-French Intestinal Lymphoma Study Group. *Cancer*. 1989;63(7):1251–1256.
158. Fischbach W, Tacke W, Greiner A, Konrad H, Muller H. Regression of immunoproliferative small intestinal disease after eradication of *Helicobacter pylori*. *Lancet*. 1997;349(9044):31–32.
159. Broccoli A, Zinzani PL. How do we sequence therapy for marginal zone lymphomas? *Hematology Am Soc Hematol Educ Program*. 2020;2020(1):295–305.
160. Bertoni F, Zucca E. State-of-the-art therapeutics: marginal-zone lymphoma. *J Clin Oncol*. 2005;23(26):6415–6420.
161. Conconi A, Martinelli G, Thieblemont C, Ferreri AJ, Devizzi L, Peccatori F, et al. Clinical activity of rituximab in extranodal marginal zone B-cell lymphoma of MALT type. *Blood*. 2003;102(8):2741–2745.
162. Martinelli G, Laszlo D, Ferreri AJ, Pruneri G, Ponzoni M, Conconi A, et al. Clinical activity of rituximab in gastric marginal zone non-Hodgkin's lymphoma resistant to or not eligible for anti-*Helicobacter pylori* therapy. *J Clin Oncol*. 2005;23(9):1979–1983.
163. Raderer M, Jäger G, Brugger S, Püspök A, Fiebiger W, Drach J, Wotherspoon A, Chott A. Rituximab for treatment of advanced extranodal marginal zone B cell lymphoma of the mucosa-associated lymphoid tissue lymphoma. *Oncology*. 2003;65(4):306–310.
164. Stathis A, Gregorini A, Gressin R, Orsucci L, Perrot A, Tani M, et al. IELSG-38: a phase II study of chlorambucil in combination with rituximab followed by maintenance therapy with subcutaneous rituximab in patients with extranodal marginal zone B-cell lymphoma of mucosa associated lymphoid tissue (MALT). *Blood*. 2017;130:1506.
165. Mathias J, Rummel MK, Uwe Chow K, Knauf W, Lerchenmuller CA, Losem C, et al. Two years rituximab maintenance vs. observation after first line treatment with bendamustine plus rituximab (B-R) in patients with marginal zone lymphoma (MZL): results of a prospective, randomized, multicenter phase 2 study (the StiL NHL7-2008 MAINTAIN trial). *J Clin Oncol*. 2018;36:7515.
166. Hammel P, Haioun C, Chaumette MT, Gaulard P, Divine M, Reyes F, et al. Efficacy of single-agent chemotherapy in low-grade B-cell mucosa-associated lymphoid tissue lymphoma with prominent gastric expression. *J Clin Oncol*. 1995;13(10):2524–2529.
167. Levy M, Copie-Bergman C, Gameiro C, Chaumette MT, Delfau-Larue MH, Haioun C, et al. Prognostic value of translocation t(11;18) in tumoral response of low-grade gastric lymphoma of mucosa-associated lymphoid tissue type to oral chemotherapy. *J Clin Oncol*. 2005;23(22):5061–5066.
168. Levy M, Copie-Bergman C, Molinier-Frenkel V, Riou A, Haioun C, Gaulard P, et al. Treatment of t(11;18)-positive gastric mucosa-associated lymphoid tissue lymphoma with rituximab and chlorambucil: clinical, histological, and molecular follow-up. *Leuk Lymphoma*. 2010;51(2):284–290.
169. Zucca E, Conconi A, Laszlo D, Lopez-Guillermo A, Bouabdallah R, Coiffier B, et al. Addition of rituximab to chlorambucil produces superior event-free survival in the treatment of patients with extranodal marginal-zone B-cell lymphoma: 5-year analysis of the IELSG-19 Randomized Study. *J Clin Oncol*. 2013;31(5):565–572.

170. Zucca E, Conconi A, Martinelli G, Tucci A, Vitolo U, Russo E, et al. Chlorambucil plus rituximab produces better event-free and progression-free survival in comparison with chlorambucil or rituximab alone in extranodal marginal zone B-cell lymphoma (MALT lymphoma): final results of the IELSG-19 study. *Hematol Oncol*. 2013;31(Suppl. 1):97–98.
171. Zucca E, Conconi A, Martinelli G, Bouabdallah R, Tucci A, Vitolo U, et al. Results of the IELSG-19 randomized trial of Mucosa-associated lymphoid tissue lymphoma: improved event-free and progression-free survival with rituximab plus chlorambucil versus either chlorambucil or rituximab monotherapy. *J Clin Oncol*. 2017;35(17):1905–1912.
172. Kahl BS, Bartlett NL, Leonard JP, Chen L, Ganjoo K, Williams ME, et al. Bendamustine is effective therapy in patients with rituximab-refractory, indolent B-cell non-Hodgkin lymphoma: results from a Multicenter Study. *Cancer*. 2010;116(1):106–114.
173. Rummel MJ, Al-Batran SE, Kim SZ, Welslau M, Hecker R, Kofahl-Krause D, et al. Bendamustine plus rituximab is effective and has a favorable toxicity profile in the treatment of mantle cell and low-grade non-Hodgkin's lymphoma. *J Clin Oncol*. 2005;23(15):3383–3389.
174. Kiesewetter B, Mayerhoefer ME, Lukas J, Zielinski CC, Mullauer L, Raderer M. Rituximab plus bendamustine is active in pretreated patients with extragastric marginal zone B cell lymphoma of the mucosa-associated lymphoid tissue (MALT lymphoma). *Ann Hematol*. 2014;93(2):249–253.
175. Salar A, Domingo-Domenech E, Panizo C, Nicolás C, Bargay J, Muntañola A, et al. Long-term results of a phase 2 study of rituximab and bendamustine for mucosa-associated lymphoid tissue lymphoma. *Blood*. 2017;130(15):1772–1774.
176. Salar A, Domingo-Domenech E, Panizo C, Nicolás C, Bargay J, Muntañola A, et al. Grupo Español de Linfomas/Trasplante de Médula Ósea (GELTAMO). First-line response-adapted treatment with the combination of bendamustine and rituximab in patients with mucosa-associated lymphoid tissue lymphoma (MALT2008-01): a multicentre, single-arm, phase 2 trial. *Lancet Haematol*. 2014;1(3):e104–e111.
177. Jager G, Neumeister P, Quehenberger F, Wohrer S, Linkesch W, Raderer M. Prolonged clinical remission in patients with extranodal marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue type treated with cladribine: 6 year follow-up of a phase II trial. *Ann Oncol*. 2006;17(11):1722–1723.
178. Jager G, Hofler G, Linkesch W, Neumeister P. Occurrence of a myelodysplastic syndrome (MDS) during first-line 2-chloro-deoxyadenosine (2-CDA) treatment of a low-grade gastrointestinal MALT lymphoma. Case report and review of the literature. *Haematologica*. 2004;89(4). ECR01.
179. Salar A, Domingo-Domenech E, Estany C, Canales MA, Gallardo F, Servitje O, et al. Combination therapy with rituximab and intravenous or oral fludarabine in the first-line, systemic treatment of patients with extranodal marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue type. *Cancer*. 2009;115(22):5210–5217.
180. Raderer M, Wohrer S, Bartsch R, Prager G, Drach J, Hejna M, et al. Phase II study of oxaliplatin for treatment of patients with mucosa-associated lymphoid tissue lymphoma. *J Clin Oncol*. 2005;23(33):8442–8446.
181. O'Connor OA, Wright J, Moskowitz C, Muzzy J, MacGregor-Cortelli B, Stubblefield M, et al. Phase II clinical experience with the novel proteasome inhibitor bortezomib in patients with indolent non-Hodgkin's lymphoma and mantle cell lymphoma. *J Clin Oncol*. 2005;23(4):676–684.
182. Kirschbaum M, Frankel P, Popplewell L, Zain J, Delioukina M, Pullarkat V, et al. Phase II study of vorinostat for treatment of relapsed or refractory indolent non-Hodgkin's lymphoma and mantle cell lymphoma. *J Clin Oncol*. 2011;29(9):1198–1203.
183. Kiesewetter BWE, Willenbacher W, Egle A, Neumeister P, Voskova D, Mayerhoefer ME, et al. A phase 2 study of rituximab plus lenalidomide for mucosa-associated lymphoid tissue lymphoma. *Blood*. 2017;129(3):383–385.
184. Koch P, del Valle F, Berdel WE, Willich NA, Reers B, Hiddemann W, et al. Primary gastrointestinal non-Hodgkin's lymphoma: II. Combined surgical and conservative or conservative management only in localized gastric lymphoma—results of the prospective German Multicenter Study GIT NHL 01/92. *J Clin Oncol*. 2001;19(18):3874–3883.
185. Zinzani PL, Stefoni V, Musuraca G, Tani M, Alinari L, Gabriele A, et al. Fludarabine-containing chemotherapy as frontline treatment of nongastrointestinal mucosa-associated lymphoid tissue lymphoma. *Cancer*. 2004;100(10):2190–2194.
186. Aviles A, Nambo MJ, Neri N, Talavera A, Cleto S. Mucosa-associated lymphoid tissue (MALT) lymphoma of the stomach: results of a controlled clinical trial. *Med Oncol*. 2005;22(1):57–62.
187. Wohrer S, Raderer M, Kaufmann H, Hejna M, Chott A, Zielinski CC, et al. Effective treatment of indolent non-Hodgkin's lymphomas with mitoxantrone, chlorambucil and prednisone. *Onkologie*. 2005;28(2):73–78.
188. Raderer M, Wohrer S, Streubel B, Drach J, Jager U, Turetschek K, et al. Activity of rituximab plus cyclophosphamide, doxorubicin/mitoxantrone, vincristine and prednisone in patients with relapsed MALT lymphoma. *Oncology*. 2006;70(6):411–417.
189. Rambaud JC, Halphen M, Galian A, Tsapis A. Immunoproliferative small intestinal disease (IPSID): relationships with alpha-chain disease and "Mediterranean" lymphomas. *Semin Immunopathol*. 1990;12(2-3):239–250.
190. Esmaeli B, McLaughlin P, Pro B, Samaniego F, Gayed I, Hagemeister F, et al. Prospective trial of targeted radioimmunotherapy with Y-90 ibritumomab tiuxetan (Zevalin) for front-line treatment of early-stage extranodal indolent ocular adnexal lymphoma. *Ann Oncol*. 2009;20(4):709–714.
191. Hoffmann M, Troch M, Eidherr H, Traub-Weidinger T, Jonak C, Muellauer L, et al. 90Y-ibritumomab tiuxetan (Zevalin) in heavily pretreated patients with mucosa associated lymphoid tissue lymphoma. *Leuk Lymphoma*. 2011;52(1):42–45.
192. Leahy MF, Turner JH. Radioimmunotherapy of relapsed indolent non-Hodgkin lymphoma with 131I-rituximab in routine clinical practice: 10-year single-institution experience of 142 consecutive patients. *Blood*. 2011;117(1):45–52.
193. Witzens-Harig M, Hensel M, Unterhalt M, Herfarth K. Treatment of limited stage follicular lymphoma with Rituximab immunotherapy and involved field radiotherapy in a prospective multicenter Phase II trial-MIR trial. *BMC Cancer*. 2011;11:87.
194. Noy A, de Vos S, Coleman M, Martin P, Flowers CR, Thieblemont C, et al. Durable ibrutinib responses in relapsed/refractory marginal zone lymphoma: long-term follow-up and biomarker analysis. *Blood Adv*. 2020;4(22):5773–5784.
195. Noy A, de Vos S, Thieblemont C, Martin P, Flowers CR, Morschhauser F, et al. Targeting Bruton tyrosine kinase with ibrutinib in relapsed/refractory marginal zone lymphoma. *Blood*. 2017;129(16):2224–2232.
196. Leonard JP, Trneny M, Izutsu K, Fowler NH, Hong X, Zhu J, et al. AUGMENT trial investigators. AUGMENT: a phase III study of lenalidomide plus rituximab versus Placebo plus rituximab in relapsed or refractory indolent lymphoma. *J Clin Oncol*. 2019;37(14):1188–1199.
197. Dreyling M, Santoro A, Mollica L, Leppä S, Follows GA, Lenz G, et al. Phosphatidylinositol 3-kinase inhibition by copanlisib in relapsed or refractory indolent lymphoma. *J Clin Oncol*. 2017;35(35):3898–3905.
198. Matasar MJ, Capra M, Özcan M, Lv F, Li W, Yañez E, et al. Copanlisib plus rituximab versus placebo plus rituximab in patients with relapsed indolent non-Hodgkin lymphoma (CHRONOS-3): a double-blind, randomised, placebo-controlled, phase 3 trial. *Lancet Oncol*. 2021;S1470-2045:21.
199. Fowler NH, Samaniego F, Jurczak W, Ghosh N, Derenzini E, Reeves JA, Knopińska-Posłuszny W, et al. Umbralisib, a dual PI3Kδ/CK1ε inhibitor in patients with relapsed or refractory indolent lymphoma. *J Clin Oncol*. 2021 JCO2003433.
200. Corradini P, Ladetto M, Zallio F, Astolfi M, Rizzo E, Sametti S, et al. Long-term follow-up of indolent lymphoma patients treated with high-dose sequential chemotherapy and autografting: evidence that durable molecular and clinical remission frequently can be attained only in follicular subtypes. *J Clin Oncol*. 2004;22(8):1460–1468.
201. Escalon MP, Champlin RE, Saliba RM, Acholonu SA, Hosing C, Fayad L, et al. Nonmyeloablative allogeneic hematopoietic transplantation: a promising salvage therapy for patients with non-Hodgkin's lymphoma whose disease has failed a prior autologous transplantation. *J Clin Oncol*. 2004;22(12):2419–2423.
202. Freedman AS, Ritz J, Neuberg D, Anderson KC, Rabinowe SN, Mauch P, et al. Autologous bone marrow transplantation in 69 patients with a history of low-grade B-cell non-Hodgkin's lymphoma. *Blood*. 1991;77(11):2524–2529.
203. Freytes CO, Loberiza FR, Rizzo JD, Bashey A, Bredeson CN, Cairo MS, et al. Myeloablative allogeneic hematopoietic stem cell transplantation in patients who experience relapse after autologous stem cell transplantation for lymphoma: a report of the International Bone Marrow Transplant Registry. *Blood*. 2004;104(12):3797–3803.
204. Morris E, Thomson K, Craddock C, Mahendra P, Milligan D, Cook G, et al. Outcomes after alemtuzumab-containing reduced-intensity allogeneic transplantation regimen for relapsed and refractory non-Hodgkin lymphoma. *Blood*. 2004;104(13):3865–3871.
205. Li L, Bierman P, Vose J, Loberiza F, Armitage JO, Bociek RG. High-dose therapy/autologous hematopoietic stem cell transplantation in relapsed or refractory marginal zone non-Hodgkin lymphoma. *Clin Lymphoma Myeloma Leuk*. 2011;11(3):253–256.
206. Teckie S, Qi S, Chelius M, Lovie S, Hsu M, Noy A, Portlock C, Yahalom J. Long-term outcome of 487 patients with early-stage extra-nodal marginal zone lymphoma. *Ann Oncol*. 2017;28(5):1064–1069.

207. Alderuccio JP, Zhao W, Desai A, Gallastegui N, Ramdial J, Kimble E, et al. Risk factors for transformation to higher-grade lymphoma and its impact on survival in a large cohort of patients with marginal zone lymphoma from a single institution. *J Clin Oncol*. 2018 JCO1800138.
208. Capelle LG, de Vries VA, Looman CW, Casparie MK, Boot H, Meijer GA, Kuipers EJ. Gastric MALT lymphoma: epidemiology and high adenocarcinoma risk in a nation-wide study. *Eur J Cancer*. 2008;44(16).
209. A predictive model for aggressive non-Hodgkin's lymphoma. The International Non-Hodgkin's Lymphoma Prognostic Factors Project. *N Engl J Med*. 1993;329(14):987-94.
210. Castrillo JM, Montalban C, Abraira V, Carrion R, Cruz MA, Larana JG, et al. Evaluation of the international index in the prognosis of high grade gastric malt lymphoma. *Leuk Lymphoma*. 1996;24(1-2):159–163.
211. Bastion Y, Coiffier B. Is the international prognostic index for aggressive lymphoma patients useful for follicular lymphoma patients? *J Clin Oncol*. 1994;12(7):1340–1342.
212. Cortelazzo S, Rossi A, Oldani E, Motta T, Giardini R, Zinzani PL, et al. The modified International Prognostic Index can predict the outcome of localized primary intestinal lymphoma of both extranodal marginal zone B-cell and diffuse large B-cell histologies. *Br J Haematol*. 2002;118(1):218–228.
213. Thieblemont C, Cascione L, Conconi A, Kiesewetter B, Raderer M, Gaidano G, et al. A MALT lymphoma prognostic index. *Blood*. 2017;130(12):1409–1417.
214. Sagaert X, de Paepe P, Libbrecht L, Vanhentenrijk V, Verhoef G, Thomas J, et al. Forkhead box protein P1 expression in mucosa-associated lymphoid tissue lymphomas predicts poor prognosis and transformation to diffuse large B-cell lymphoma. *J Clin Oncol*. 2006;24(16):2490–2497.
215. Arcaini L, Lazzarino M, Colombo N, Burcheri S, Boveri E, Paulli M, et al. Splenic marginal zone lymphoma: a prognostic model for clinical use. *Blood*. 2006;107(12):4643–4649.
216. Berger F, Felman P, Thieblemont C, Pradier T, Baseggio L, Bryon PA, et al. Non-MALT marginal zone B-cell lymphomas: a description of clinical presentation and outcome in 124 patients. *Blood*. 2000;95(6):1950–1956.
217. Chacon JI, Mollejo M, Munoz E, Algara P, Mateo M, Lopez L, et al. Splenic marginal zone lymphoma: clinical characteristics and prognostic factors in a series of 60 patients. *Blood*. 2002;100(5):1648–1654.
218. Salido M, Baro C, Oscier D, Stamatopoulos K, Dierlamm J, Matutes E, et al. Cytogenetic aberrations and their prognostic value in a series of 330 splenic marginal zone B-cell lymphomas: a multicenter study of the Splenic B-Cell Lymphoma Group. *Blood*. 2010;116(9):1479–1488.
219. Farrell AM, Stern SC, El-Ghariani K, Frankel A, Woodrow D, Muller B, et al. Splenic lymphoma with villous lymphocytes presenting as leucocytoclastic vasculitis. *Clin Exp Dermatol*. 1999;24(1):19–22.
220. Saadoun D, Suarez F, Lefrere F, Valensi F, Mariette X, Aouba A, et al. Splenic lymphoma with villous lymphocytes, associated with type II cryoglobulinemia and HCV infection: a new entity? *Blood*. 2005;105(1):74–76.
221. Arcaini L, Rossi D, Paulli M. Splenic marginal zone lymphoma: from genetics to management. *Blood*. 2016;127(17):2072–2081.
222. Hermine O, Lefrere F, Bronowicki JP, Mariette X, Jondeau K, Eclache-Saudreau V, et al. Regression of splenic lymphoma with villous lymphocytes after treatment of hepatitis C virus infection. *N Engl J Med*. 2002;347(2):89–94.
223. Bates I, Bedu-Addo G. Chronic malaria and splenic lymphoma: clues to understanding lymphoma evolution. *Leukemia*. 1997;11(12):2162–2167.
224. Isaacson PG, Piris MA, Berger F, Swerdlow SH, Thieblemont C, Pittaluga S, et al. Splenic marginal zone lymphoma. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: International Agency for Research on Cancer; 2008:185–187.
225. Baseggio L, Traverse-Glehen A, Petinataud F, Callet-Bauchu E, Berger F, Ffrench M, et al. CD5 expression identifies a subset of splenic marginal zone lymphomas with higher lymphocytosis: a clinico-pathological, cytogenetic and molecular study of 24 cases. *Haematologica*. 2010;95(4):604–612.
226. Ruiz-Ballesteros E, Mollejo M, Rodriguez A, Camacho FI, Algara P, Martinez N, et al. Splenic marginal zone lymphoma: proposal of new diagnostic and prognostic markers identified after tissue and cDNA microarray analysis. *Blood*. 2005;106(5):1831–1838.
227. Algara P, Mateo MS, Sanchez-Beato M, Mollejo M, Navas IC, Romero L, et al. Analysis of the IgV(H) somatic mutations in splenic marginal zone lymphoma defines a group of unmutated cases with frequent 7q deletion and adverse clinical course. *Blood*. 2002;99(4):1299–1304.
228. Novara F, Arcaini L, Merli M, Passamonti F, Zibellini S, Rizzi S, et al. High-resolution genome-wide array comparative genomic hybridization in splenic marginal zone B-cell lymphoma. *Hum Pathol*. 2009;40(11):1628–1637.
229. Zhu D, Orchard J, Oscier DG, Wright DH, Stevenson FK. V(H) gene analysis of splenic marginal zone lymphomas reveals diversity in mutational status and initiation of somatic mutation in vivo. *Blood*. 2002;100(7):2659–2661.
230. Tiacci E, Trifonov V, Schiavoni G, Holmes A, Kern W, Martelli MP, et al. BRAF mutations in hairy-cell leukemia. *N Engl J Med*. 2011;364(24):2305–2315.
231. Pillonel V, Juskevicius D, Ng CKY, Bodmer A, Zettl A, Jucker D, Dirnhofer S, Tzankov A. High-throughput sequencing of nodal marginal zone lymphomas identifies recurrent BRAF mutations. *Leukemia*. 2018;32(11):2412–2426.
232. Gachard N, Parrens M, Soubeyran I, Petit B, Marfak A, Rizzo D, et al. IGHV gene features and MYD88 L265P mutation separate the three marginal zone lymphoma entities and Waldenstrom macroglobulinemia/lymphoplasmacytic lymphomas. *Leukemia*. 2013;27(1):183–189.
233. Troussard X, Valensi F, Duchayne E, Garand R, Felman P, Tulliez M, et al. Splenic lymphoma with villous lymphocytes: clinical presentation, biology and prognostic factors in a series of 100 patients. Groupe Francais d'Hematologie Cellulaire (GFHC). *Br J Haematol*. 1996;93(3):731–736.
234. Mulligan SP, Matutes E, Dearden C, Catovsky D. Splenic lymphoma with villous lymphocytes: natural history and response to therapy in 50 cases. *Br J Haematol*. 1991;78(2):206–209.
235. Tsimberidou AM, Catovsky D, Schlette E, O'Brien S, Wierda WG, Kantarjian H, et al. Outcomes in patients with splenic marginal zone lymphoma and marginal zone lymphoma treated with rituximab with or without chemotherapy or chemotherapy alone. *Cancer*. 2006;107(1):125–135.
236. Bennett M, Sharma K, Yegena S, Gavish I, Dave HP, Schechter GP. Rituximab monotherapy for splenic marginal zone lymphoma. *Haematologica*. 2005;90(6):856–858.
237. Kalpadakis C, Pangalis GA, Angelopoulou MK, Sachanas S, Kontopidou FN, Yiakoumis X, et al. Treatment of splenic marginal zone lymphoma with rituximab monotherapy: progress report and comparison with splenectomy. *Oncologist*. 2013;18(2):190–197.
238. Perrone S, D'Elia GM, Annechini G, Ferretti A, Tosti ME, Foà R, Pulsoni A. Splenic marginal zone lymphoma: prognostic factors, role of watch and wait policy, and other therapeutic approaches in the rituximab era. *Leuk Res*. 2016;44:53–60.
239. Kalpadakis C, Pangalis GA, Sachanas S, Tsirkinidis P, Kontopidou FN, Moschogiannis M, et al. Rituximab monotherapy in splenic marginal zone lymphoma: prolonged responses and potential benefit from maintenance. *Blood*. 2018;132(6):666–670.
240. Iannitto E, Belli M, Amorim S, Ferreri AJM, Marcheselli L, Cesaretti M, et al. Efficacy of bendamustine and rituximab in splenic marginal zone lymphoma: results from the phase II BRISMA/IELSG36 study. *Br J Haematol*. 2018;183(5):755–765.
241. Thieblemont C, Felman P, Callet-Bauchu E, Traverse-Glehen A, Salles G, Berger F, et al. Splenic marginal-zone lymphoma: a distinct clinical and pathological entity. *Lancet Oncol*. 2003;4(2):95–103.
242. Crary SE, Buchanan GR. Vascular complications after splenectomy for hematologic disorders. *Blood*. 2009;114(14):2861–2868.
243. Pata G, Damiani E, Bartoli M, Solari S, Anastasia A, Pagani C, Tucci A, Ragni F. Peri-operative complications and hematologic improvement after first-line splenectomy for splenic marginal zone lymphoma. *Leuk Lymphoma*. 2016;57(6):1467–1470.
244. Olszewski AJ. Survival outcomes with and without splenectomy in splenic marginal zone lymphoma. *Am J Hematol*. 2012;87(11):E119–E122.
245. Kalpadakis C, Pangalis GA, Vassilakopoulos TP, Sachanas S, Angelopoulou MK. Treatment of splenic marginal zone lymphoma: should splenectomy be abandoned? *Leuk Lymphoma*. 2014;55(7):1463–1470.
246. Martin P, Rutherford S, Leonard JP. Splenic lymphomas: is there still a role for splenectomy? *Oncology (Williston Park)*. 2012;26(2):204 6.
247. Bolam S, Orchard J, Oscier D. Fludarabine is effective in the treatment of splenic lymphoma with villous lymphocytes. *Br J Haematol*. 1997;99(1):158–161.
248. Lefrere F, Hermine O, Belanger C, Francois S, Tilly H, Lebas de La Cour JC, et al. Fludarabine: an effective treatment in patients with splenic lymphoma with villous lymphocytes. *Leukemia*. 2000;14(4):573–575.
249. Lefrere F, Hermine O, Francois S, Panelatti G, Valensi F, Grosbois B, et al. Lack of efficacy of 2-chlorodeoxyadenoside in the treatment of splenic lymphoma with villous lymphocytes. *Leuk Lymphoma*. 2000;40(1-2):113–117.
250. Virchis A, Mehta A. Splenic lymphoma with villous lymphocytes (SLVL) responding to 2-chlorodeoxyadenosine (2-CdA). *Br J Haematol*. 1998;100(3):609.
251. El Weshi A, Ribrag V, Girinski T, El Hajj M, Dhermain F, Bayle C. Low and medium dose spleen radiation therapy are able to induce long-term responses in splenic lymphoma with villous lymphocytes. *Br J Haematol*. 1998;103(4):1212–1213.

252. Florindez JA, Alderuccio JP, Reis IM, Lossos IS. Splenic marginal zone lymphoma: A US population-based survival analysis (1999-2016). *Cancer*. 2020;126(21):4706–4716.
253. Kalpadakis C, Pangalis GA, Angelopoulou MK, Sachanas S, Kontopidou F, Moschogiannis M, et al. Validation of the simplified prognostic score for splenic marginal zone lymphoma of the Splenic Marginal Zone Lymphoma Working Group. *Leuk Lymphoma*. 2014;55(11):2640–2642.
254. Montalbán C, Abraira V, Arcaini L, Domingo-Domenech E, Guisado-Vasco P, Iannitto E, et al. Risk stratification for splenic marginal zone lymphoma based on haemoglobin concentration, platelet count, high lactate dehydrogenase level and extrahilar lymphadenopathy: development and validation on 593 cases Splenic marginal zone lymphoma study group. *Br J Haematol*. 2012;159(2):164–171.
255. Montalban C, Abraira V, Arcaini L, Domingo-Domenech E, Guisado-Vasco P, Iannitto E, et al. Simplification of risk stratification for splenic marginal zone lymphoma: a point-based score for practical use Splenic Marginal Zone Lymphoma Study Group (SMZLSG). *Leuk Lymphoma*. 2014;55(4):929–931.
256. Arcaini L, Paulli M, Burcheri S, Rossi A, Spina M, Passamonti F, et al. Primary nodal marginal zone B-cell lymphoma: clinical features and prognostic assessment of a rare disease. *Br J Haematol*. 2007;136(2):301–304.
257. Camacho FI, Algara P, Mollejo M, Garcia JF, Montalban C, Martinez N, et al. Nodal marginal zone lymphoma: a heterogeneous tumor: a comprehensive analysis of a series of 27 cases. *Am J Surg Pathol*. 2003;27(6):762–771.
258. Kojima M, Inagaki H, Motoori T, Itoh H, Shimizu K, Tamaki Y, et al. Clinical implications of nodal marginal zone B-cell lymphoma among Japanese: study of 65 cases. *Cancer Sci*. 2007;98(1):44–49.
259. Oh SY, Ryoo BY, Kim WS, Kim K, Lee J, Kim HJ, et al. Nodal marginal zone B-cell lymphoma: analysis of 36 cases. Clinical presentation and treatment outcomes of nodal marginal zone B-cell lymphoma. *Ann Hematol*. 2006;85(11):781–786.
260. Traverse-Glehen A, Felman P, Callet-Bauchu E, Gazzo S, Baseggio L, Bryon PA, et al. A clinicopathological study of nodal marginal zone B-cell lymphoma. A report on 21 cases. *Histopathology*. 2006;48(2):162–173.
261. Taddesse-Heath L, Pittaluga S, Sorbara L, Bussey M, Raffeld M, Jaffe ES. Marginal zone B-cell lymphoma in children and young adults. *Am J Surg Pathol*. 2003;27(4):522–531.
262. Xiong W, Lv R, Li H, Li Z, Wang H, Liu W, Zou D, Qiu L, Yi S. Prevalence of hepatitis B and hepatitis C viral infections in various subtypes of B-cell non-Hodgkin lymphoma: confirmation of the association with splenic marginal zone lymphoma. *Blood Cancer J*. 2017;7(3):e548.
263. Campo E, Pileri SA, Jaffe ES, Muller-Hermelink HK, Nathwani BN. Nodal marginal zone lymphoma. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: International Agency for Research on Cancer; 2008:185–187.
264. Nathwani BN, Drachenberg MR, Hernandez AM, Levine AM, Sheibani K. Nodal monocytoid B-cell lymphoma (nodal marginal-zone B-cell lymphoma). *Semin Hematol*. 1999;36(2):128–138.
265. Campo E, Miquel R, Krenacs L, Sorbara L, Raffeld M, Jaffe ES. Primary nodal marginal zone lymphomas of splenic and MALT type. *Am J Surg Pathol*. 1999;23(1):59–68.
266. Nizze H, Cogliatti SB, von Schilling C, Feller AC, Lennert K. Monocytoid B-cell lymphoma: morphological variants and relationship to low-grade B-cell lymphoma of the mucosa-associated lymphoid tissue. *Histopathology*. 1991;18(5):403–414.
267. Traverse-Glehen A, Davi F, Ben Simon E, Callet-Bauchu E, Felman P, Baseggio L, et al. Analysis of VH genes in marginal zone lymphoma reveals marked heterogeneity between splenic and nodal tumors and suggests the existence of clonal selection. *Haematologica*. 2005;90(4):470–478.
268. Marasca R, Vaccari P, Luppi M, Zucchini P, Castelli I, Barozzi P, et al. Immunoglobulin gene mutations and frequent use of VH1-69 and VH4-34 segments in hepatitis C virus-positive and hepatitis C virus-negative nodal marginal zone B-cell lymphoma. *Am J Pathol*. 2001;159(1):253–261.
269. Spina V, Khiabanian H, Messina M, Monti S, Cascione L, Bruscaggin A, et al. The genetics of nodal marginal zone lymphoma. *Blood*. 2016;128(10):1362–1373.
270. Solal-Celigny P, Roy P, Colombat P, White J, Armitage JO, Arranz-Saez R, et al. Follicular lymphoma international prognostic index. *Blood*. 2004;104(5):1258–1265.
271. Luminari S, Merli M, Rattotti S, Tarantino V, Marcheselli L, Cavallo F, et al. Early progression as a predictor of survival in marginal zone lymphomas: an analysis from the FIL-NF10 study. *Blood*. 2019;134(10):798–801.
272. Starr AG, Caimi PF, Fu P, Massoud MR, Meyerson H, Hsi ED, et al. Dual institution experience of nodal marginal zone lymphoma reveals excellent long-term outcomes in the rituximab era. *Br J Haematol*. 2016:275–280.
273. Adam P, Czapiewski P, Colak S, Kosmidis P, Tousseyn T, Sagaert X, et al. Prevalence of Achromobacter xylosoxidans in pulmonary mucosa-associated lymphoid tissue lymphoma in different regions of Europe. *Br J Haematol*. 2014;164(6):804–810.

CHAPTER 84

DIFFUSE LARGE B-CELL LYMPHOMA OF THE CENTRAL NERVOUS SYSTEM

Syed A. Abutalib, Nilanjan Ghosh, Alexander Feldman[†], Karan S. Dixit, and Rimas V. Lukas

Diffuse large B-cell lymphoma (DLBCL) of the central nervous system (CNS) accounts for an overwhelming majority (95%) of cases of primary CNS lymphoma (PCNSL). According to the 2016 report of the World Health Organization (WHO), it is defined as DLBCL arising within the brain, spinal cord, leptomeninges, or eye.[1] The WHO definition[1] excludes lymphomas of the dura, intravascular large B-cell lymphomas (IVLBL), secondary CNS lymphomas (SCNSLs), and all immunodeficiency associated lymphomas from this category (Table 84.1). For the purpose of discussion, in this text, the terms PCNSL and DLBCL of the CNS are used interchangeably. The diagnosis and management of primary vitreoretinal lymphoma (PVRL) is also discussed, which is a subset of PCNSL. In the context of human immunodeficiency virus (HIV) infection, PCNSL is one of four acquired immunodeficiency syndrome (AIDS)-defining illnesses with distinct clinicopathologic characteristics and unique therapeutic challenges.[1–6] Secondary CNS lymphoma (SCNSL) may occur as a recurrence of previously diagnosed non-Hodgkin lymphoma (NHL) or may occur simultaneously as a manifestation of systemic disease. They can metastasize to the brain parenchyma, but more often, they spread to leptomeninges, causing symptoms across the entire neuraxis, including epidural cord compression in approximately 3% to 5% of patients.[7–9] Risk recognition and the prevention of CNS disease are important aspects of the overall management strategy in high-grade systemic DLBCLs.[9–11]

The primary origin of PCNSL is unclear, given the virtual absence of B cells in the normal brain tissue. The disease is categorized mainly as activated B cell (ABC) or non-germinal center B (non-GCB). However, the cell of origin (COO) distinction has uncertain therapeutic and prognostic merits in PCNSL.[12–15] More importantly, central to CNS lymphogenesis is the constitutive activation of B-cell receptor (BCR) signaling pathway, with its downstream target, nuclear factor-κB (NFκB), affected by frequent recurrent mutations, mainly *MYD*88 and CD79B.[16–18] From a diagnosis standpoint, the inherent challenges in timely diagnosis of PCNSL have paved the way for numerous minimally invasive diagnostic biomarkers, albeit with limited success,[19–21] in turn supporting biopsy of the affected area as the current diagnostic gold standard.

There is a paucity of high-quality comparative data to guide practitioners with level 1 evidence-based treatment recommendations for PCNSL or SCNSL. Nonetheless, unlike most other brain tumors, PCNSL often responds favorably to systemic therapy, specifically high-dose methotrexate (HD-MTX) based (poly) chemotherapy and/or whole-brain radiation therapy (WBRT). The latter modality, however, is plagued by unsustainability of responses and debilitating neurotoxicity, particularly in the older adults.[22–25] Following successful induction therapy, consolidation is administered to deepen and/or maintain the response. Overall, the treatment program of induction and consolidation attains long-term disease-free survival (DFS) in approximately 40% to 60% of the patients underscoring the fact that about half or more of the patients would relapse within the neuraxis.[26–28]

Most recently, the concept of maintenance therapy has emerged, especially for patients who are unsuitable for consolidation.[29] Novel approaches[30,31] and agents[32] are also being investigated and will be discussed throughout the chapter. The prognosis of patients who relapse, or are refractory, is dismal and treatment remains less optimally defined than for newly diagnosed PCNSL.[33] Whenever possible, patients with PCNSL and SCNSL should be guided immediately towards a well-designed, appropriately powered clinical trial.

EPIDEMIOLOGY

PCNSL accounts for less than 2% of all NHLs, 4% to 6% of all extranodal lymphomas, and 3% to 6% of all brain tumors, with an age-adjusted incidence rate of 4 cases per million persons per year.[34,35] Approximately 1500 new cases are diagnosed each year in the United States, a rate that is steadily increasing as the population ages.[35] The median age of diagnosis is 56 years in an immunocompetent host, with about an equal male-to-female ratio.[28,35] PVRL is the most common form of intraocular lymphoma (IOL). Approximately, 15% to 25% of patients with parenchymal PCNSL subsequently develop “secondary” vitreoretinal lymphoma (VRL), underscoring the importance of a bilateral eye exam at diagnosis and periodically thereafter. Conversely, about 65% to 90% of patients with PVRL consequently have or eventually develop parenchymal PCNSL, underscoring the importance of brain surveillance imaging. Primary choroidal lymphomas do not have association with CNS disease. Secondary choroidal lymphomas represent an extranodal manifestation of systemic lymphoma and are usually confined to the choroid. The most common systemic lymphoma subtype, involving the choroid, is DLBCL, followed by multiple myeloma, extramedullary plasmacytoma, Waldenström macroglobulinemia, and chronic lymphocytic leukemia (see Chapters 90, 92 and 67 respectively).

Besides advancing age, congenital and acquired immunodeficient conditions are other well-established risk factors for PCNSL (Table 84.2).[36] PCNSL, in immunocompromised individuals, occurs at an earlier age with a higher predilection for males than females.[37] It is less clear whether it follows a more aggressive course in this patient population.[37,38] In an HIV-infected individual, PCNSL is an AIDS-defining disease, associated with the Epstein-Barr virus (EBV) infection (see Chapters 78 and 87). The EBV association in an immunocompetent host with PCNSL is less frequent (<15%). The risk factors of PCNSL in HIV-infected individuals are a low CD4 count (<100,000 cells/mm ^{3}) and a high viral load. Due to effective antiretroviral therapy, the incidence of HIV-associated CNS lymphoma has been declining.

PCNSL may also present as a second malignancy.[36,39] For example, it should be considered in the differential diagnosis of patients with “recurrent disease of solid tumor” that is exclusively confined to the neuraxis or in situations where the primary systemic tumor is inactive and the patient develops a new brain lesion.

PATHOGENESIS

Despite significant progress in the elucidation of the genomic landscape of PCNSL, a specific genetic defect has not been reported. Due

---

[†]Deceased

TABLE 84.1 **2016 World Health Organization Classification of Lymphomas of the Nervous System**

- Diffuse large B-cell lymphoma of the CNS
- Immunodeficiency-associated CNS lymphomas
  - AIDS-related DLBCL
  - EBV-positive DLBCL, NOS
  - Lymphomatoid granulomatosis
  - Posttransplant lymphoproliferative disorders
- Intravascular large B-cell lymphomas
- Miscellaneous rare CNS lymphomas
  - Low-grade B-cell lymphomas
  - T-cell and NK-/T- cell lymphomas
  - Anaplastic large cell lymphoma (ALK+ and ALK−)
- MALT lymphoma of the dura

AIDS, Acquired immunodeficiency syndrome; *CNS*, central nervous system; *DLBCL*, diffuse large B-cell lymphoma; *EBV*, Epstein-Barr virus; *NK*, natural killer; *NOS*, not otherwise specified.

TABLE 84.2 **Immunodeficiency and Primary Central Nervous System Lymphoma Incidence**

Condition	Life-time risk of PCNSL[36]
Congenital • Wiskott-Aldrich syndrome • Ataxia telangiectasia • Severe combined immunodeficiency • X-linked immunoproliferative disorder	4%
Acquired – HIV/AIDS	2%–6% (Greater risk in patients with lowest CD4+ counts)
Acquired – Non-HIV • Immunosuppressive therapy • Organ transplantation • Autoimmune disorders	Up to 7%

AIDs, Acquired immunodeficiency syndrome; *HIV*, human immunodeficiency virus; *PCNSL*, primary central nervous system lymphoma.

to its rarity and location, access to the copious tissue for diagnosis (and research) is a major limitation. Furthermore, prediagnostic and nonjudicious use of steroids in a majority of immunocompetent patients with brain mass further restricts the quantity, as well as quality, of the examined neural tissue.[40,41]

Given the virtual absence of B cells in the healthy brain parenchyma, the origin of the malignant transformation of PCNSL remains a mystery. Similar to systemic DLBCL, PCNSL is also likely to originate from late GC-derived B cells as suggested by the ongoing somatic hypermutations (SHMs) in immunoglobulin (Ig) genes. Moreover, frequent (50% to 80%) usage of the V4–34 gene segment suggests that specific antigens trigger the development of PCNSL.[42,43] Presently, no cell adhesion molecule or chemokine predicting B cell homing selectively to the brain has been identified in the evolution of PCNSL.[44]

Interestingly, concomitant small-size monoclonal B-cell populations have been identified in the peripheral blood and/or marrow in a subset of patients (~15%) with PCNSL.[45–48] In a study by Fukumura et al.,[48] individuals with tumors positive for MYD88 mutations also harbored the same mutations, albeit at a low frequency in peripheral blood mononuclear cells, suggesting that MYD88 mutation-positive precancerous cells originate outside of the CNS. Hypothetically, these cells might develop additional genetic hits that confer adaptation to the CNS environment. However, such subclinical extra-CNS clonal positivity by itself does not imply presence (or absence) of systemic lymphoma.

Additionally, a plethora of highly sophisticated experimental, albeit small studies using comparative genomic hybridization (CGH) or single-nucleotide polymorphism (SNP) array karyotyping and whole-exome sequencing (WES) techniques have highlighted myriad and heterogeneous cellular and microenvironment abnormalities, forming the contexture of PCNSL.[16–21,48–60] These abnormalities involve genes influencing chromatin structure and modification, cell-cycle regulation, adhesion molecules and immune recognition with a remarkably high prevalence of oncogenic aberrations in BCR and Toll-like receptor (TLR), triggering brisk NF-κB signaling in the majority of patients with PCNSL (Fig. 84.1A,B) (Chapters 3, 6, 12, 14, and 22).

Genomic Landscape and Aberrant Pathways

The pathogenesis of this multicompartment disease is fragmented and unclear, with a few exceptions. The CGH has identified many nonspecific genetic abnormalities.[48,50,61,62] Chromosome 6q deletions, in particular the 6q21–23, may be the most frequent alteration. They occur in about 40% to 60% of PCNSL.[63] Candidate tumor suppressors linked to chromosome 6q include: PRDM1, a tumor suppressor and regulator of terminal B-cell differentiation;[52] PTPRK, a protein tyrosine phosphatase that participates in cell adhesion signaling events;[64] and A20 (TNFAIP3), a negative regulator of NFκB signaling.[63] Along with 6p21 deletion,[54] PCNSLs frequently exhibit 9p24.1/PD-L1/PD-L2 copy number alterations and translocations, a likely genetic explanation underlying immune evasion within this immunoprivileged site.[65] Interestingly, Four et al.[66] reported high levels of PD-L1 expression with nominal PD-L1/PD-L2 translocations in 32 immunocompetent PCNSL patients. Recurrent chromosomal losses have also been detected in the 9p21 site, which encodes loci involved in cell cycle regulation, including CDKN2A, a putative target for cyclin-dependent kinase inhibitors.[63] Recurrent gains have been detected on chromosome 12, specifically in the 12q region harboring STAT6, MDM2, CDK4 and GLI1 genes.[48] Abnormalities in the long arms of chromosomes 1, 7, and 18 have also been reported.[48,50,61,62]

The BCR, TLRs, and NF-κB pathways are frequently activated due to genetic alterations, affecting in particular (see Fig. 84.1A and B): MYD88, CD79A/B, INPP50 (also called SHIP), CBL, BLNK, CARD11, MALT1, and BCL2, which foster proliferation and prevent apoptosis.[44,58,59] Other genes that have been implicated in the pathogenesis include: IRF2BP2, TBL1XR1, ETV6, IRF4, IRF2BP2, EBF1, CCND3; and CDK20, CREBBP, MLL2, ARID1A/B, and SMARCA4.[59,60,63] A recent study by Bödör et al.[51] utilized NanoString Lymphoma Subtyping Test (LST)-assay gene expression in formalin-fixed paraffin-embedded (FFPE) tissue. They elegantly showed that the most frequently mutated genes in the PCNSL cohort ($n = 64$), included MYD88 (66%), PIM1 (41%), KMT2D (31%), and PRDM1 (30%). The mutation frequencies in the remaining genes were as follows: MYC (19%), IRF4 (19%), CD79B (17%), TP53 (11%), CCND3 (9%), CARD11 (8%), PAX5 (3%), CSMD2 (3%), and CSMD3 (3%). No mutation was found in PTPRD gene. In the SCNSL cohort ($n = 12$) the PRDM1 (50%), followed by MYD88 (42%) and PIM1 (25%) were the most frequently mutated target genes. The mutation frequencies in the remaining genes proved to be lower compared to PCNSL: KMT2D (17%), CD79B (8%), IRF4 (8%), CCND3 (8%), C-MYC (8%), TP53 (8%), and PAX5 (8%). No mutation was identified in CARD11, CSMD2, CSMD3, and PTPRD genes.

In another recent study by Nayyar et al.,[18] phylogenetic analysis of paired primary and relapsed specimens from patients with PCNSL

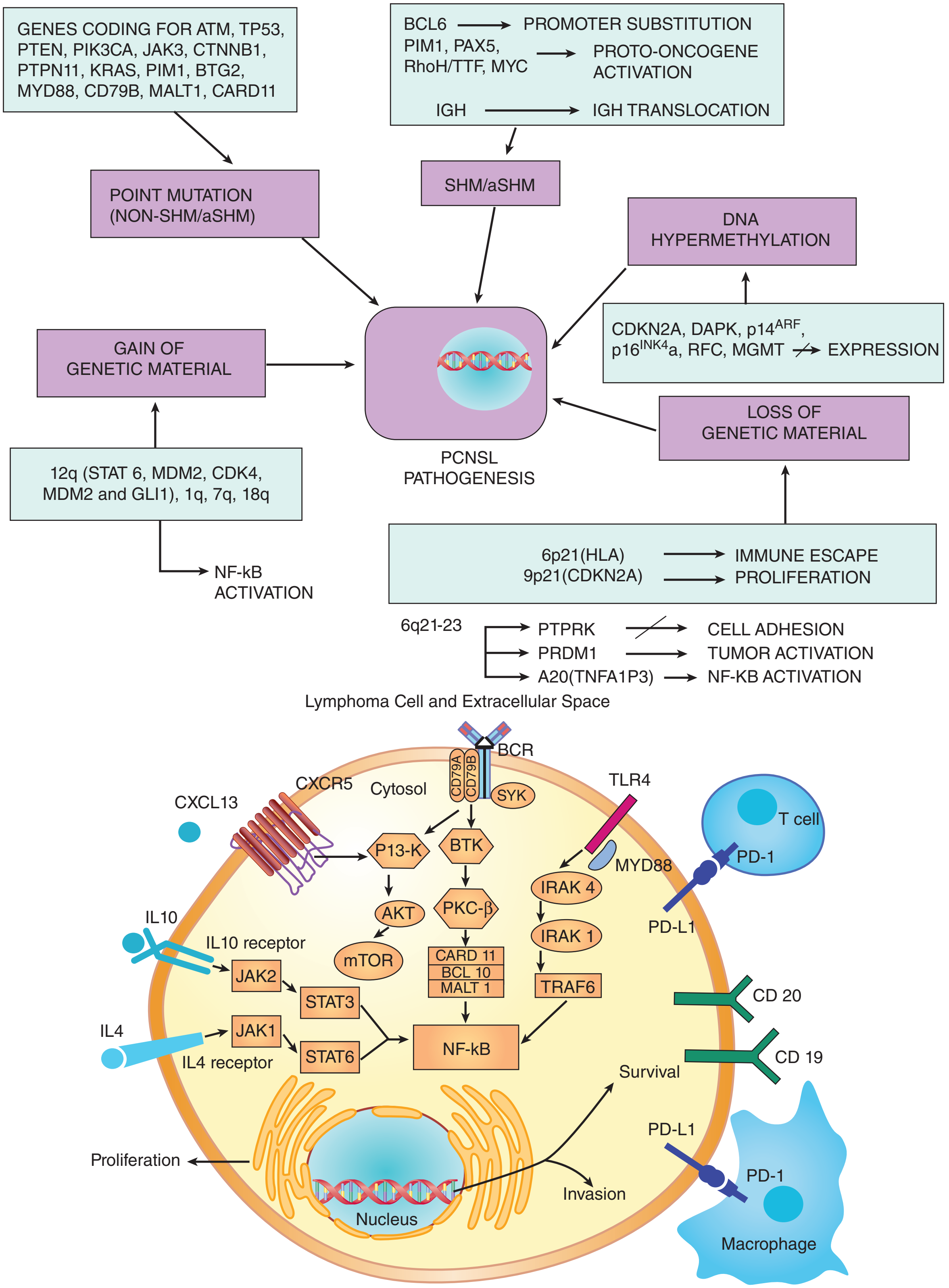

GENES CODING FOR ATM, TP53, PTEN, PIK3CA, JAK3, CTNNB1, PTPN11, KRAS, PIM1, BTG2, MYD88, CD79B, MALT1, CARD11
BCL6 → PROMOTER SUBSTITUTION
PIM1, PAX5, RhoH/TTF, MYC → PROTO-ONCOGENE ACTIVATION
IGH → IGH TRANSLOCATION
POINT MUTATION (NON-SHM/aSHM)
SHM/aSHM
DNA HYPERMETHYLATION
CDKN2A, DAPK, p14^ARF, p16^INK4a, RFC, MGMT ↛ EXPRESSION
GAIN OF GENETIC MATERIAL
LOSS OF GENETIC MATERIAL
PCNSL PATHOGENESIS
12q (STAT 6, MDM2, CDK4, MDM2 and GLI1), 1q, 7q, 18q
NF-kB ACTIVATION
6p21(HLA) → IMMUNE ESCAPE
9p21(CDKN2A) → PROLIFERATION
6q21-23
PTPRK ↛ CELL ADHESION
PRDM1 → TUMOR ACTIVATION
A20(TNFA1P3) → NF-KB ACTIVATION
Lymphoma Cell and Extracellular Space
BCR
CD79A
CD79B
SYK
CXCR5
Cytosol
CXCL13
TLR4
T cell
PD-1
MYD88
P13-K
BTK
IRAK 4
AKT
PKC-β
IRAK 1
PD-L1
IL10
IL10 receptor
mTOR
CARD 11
BCL 10
MALT 1
TRAF6
JAK2
STAT3
CD 20
IL4
JAK1
NF-kB
STAT6
CD 19
IL4 receptor
Survival
PD-L1
Proliferation
PD-1
Invasion
Nucleus
Macrophage

Figure 84.1 (A and B) The pathogenesis of primary central nervous system lymphoma (PCNSL). There is evidence for several pathways to be critically involved in the pathogenesis of PCNSL. These have emerged as a source of novel therapeutic targets. These aberrations affect a variety of genes, which become deregulated, ultimately leading to uncontrolled proliferation, impairment of apoptosis or B-cell differentiation, or may interfere with the sensitivity of the tumor cells to immune responses in the CNS. *AKT*, Serine/threonine kinase; *aSHM*, Aberrant somatic hypermutation; *BTK*, Bruton's tyrosine kinase; *CARD11*, caspase recruitment domain family member 11; *CSR*, class switch recombination; IL-10/4, interleukin 10/4; *CXCL-13*, chemokine (C-X-C motif) ligand 13; BCR, B-cell receptor; *TLR-4*, toll-like receptor-4; *IRAK1/4*, interleukin 1 receptor associated kinase 1/4; JAK-1/2, Janus tyrosine kinase 1/2; MALT1, mucosa-associated lymphoid tissue lymphoma translocation 1 gene; *mTOR*, mammalian target of rapamycin; MYD88, myeloid differentiation factor 88; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; *P13K*, phosphoinositide 3-kinases; *PKC-β*, protein kinase C-β; *PD-L1*, programmed death-ligand 1; *SHM*, somatic hypermutation; *STAT 3/6*, signal transducer and activator of transcription 3/6; SYK, spleen tyrosine kinase.

identified MYD88 mutation and loss of CDKN2A as early clonal events. In a study by Balint et al.,[67] the NGS analyses revealed that over 80% of potentially protein-changing mutations in lymphomagenesis were located in eight genes: CTNNB1, PIK3CA, PTEN, ATM, KRAS, PTPN11, TP53, and JAK3. The TP53 was the only gene harboring mutation seen in all 19 patients. Inactivation of $p14^{ARF}$ and $p16^{INK4a}$ genes has also been implicated in pathogenesis.[68] Somewhat at odds with these findings are results from the study by Chapuy et al.[65] which reported the rarity of TP53 mutations. However, the p53 pathway was frequently perturbed upstream by way of a mono- or bi- allelic loss of CDKN2A. As a consequence, it is suggested that certain patients with such a mutation may benefit from MDM2/4 inhibitors that may augment wild-type p53 activity[69] and CDK-blockade.[70]

MicroRNA Expression

The discovery of microRNAs (miRNAs) has opened a new field for unraveling and therapeutically targeting diseases (see Chapter 4).[71] These small noncoding RNAs regulate diverse biological processes through posttranscriptional gene expression modulation.[71–74] A study by Fischer et al.[73] demonstrated a distinct pattern of miRNA expression in PCNSL ($n = 11$) when compared to systemic DLBCL ($n = 10$), using paraffin-embedded biopsy specimens in immunocompetent patients. In all, 18 miRNAs were differentially expressed between PCNSL and systemic DLBCL. The upregulated miRNAs in PCNSL specimens were associated with the MYC pathway (miR-17-5p, miR-20a, miR-9), with the blocking of terminal B-cell differentiation (miR-9, miR-30b/c), or with upregulation by inflammatory cytokines (miR-155). Putative tumor-suppressor miRNAs (miR-199a, miR-214, miR-193b, miR-145) were downregulated in PCNSL. There was no overlap of miRNAs dysregulated in PCNSL with those differentially expressed between IHC, defined as GCB and non-GCB types, or in those apart from miR-9, with miRNAs known to be overexpressed in human brain. Notably, the results of another study by Robertus et al.[74] are somewhat contradictory, in which they reported that miR-155 showed the lowest expression level compared with other miRNAs involved in PCNSL. This could be possibly explained by a different sample preparation, varying RNA quality, and low sample size in both studies.[73–76]

Adhesion and Chemokine Molecules

The exact mechanism behind CNS tropism and dissemination within the CNS is not clear but may be facilitated by the increased expression of extracellular matrix molecules such as LFA-1/ICAM-1,[77] CD44,[78] Fas (CD95),[79] a transmembrane receptor protein, and B cell attracting chemokines such as CXCL-12 (SDF-1) and CXCL-13 that bind to CXCR4 and CXCR5, respectively,[80] on lymphoma cells (see Fig. 84.1B) (see Chapters 11, 12, 14 and 15).

Immune Evasion and Its Implications

Loss of both human leukocyte antigen (HLA) class I and class II expression in B cell lymphomas is a mechanism of escape from a cytotoxic T lymphocyte (CTL) immune response in lymphomas of immune-privileged sites. HLA-A, HLA-B, HLA-C, and HLA-DR are variably expressed, with approximately 50% of CNS DLBCLs showing loss of major histocompatibility complex (MHC) class I and/or II expression[1,57,81] (see Chapter 24). Interestingly, Alama et al.[57] retrospectively analyzed PCNSL transcriptomes in 54 samples (sequencing, $n = 20$; microarrays, $n = 34$) from PCNSL. Integrated correlation analysis and signaling pathway topology enabled them to infer intercellular interactions. Immunohistopathology and digital imaging were used to validate the bioinformatic results. Transcriptomics allowed them to classify the tumors into three immune subtypes: immune-rich, poor, and intermediate. The immune-rich subtype was associated with better survival and characterized by hyper-activation of STAT3 signaling and other proinflammatory signaling (e.g., interferon [IFN]-γ and tumor necrosis factor [TNF]-α), resembling the hot subtype described in primary testicular lymphoma and solid tumor. A group of developmental signaling pathways including WNT/β-catenin, HIPPO (Salvador-Warts-Hippo), and NOTCH were hyper-activated in the immune-poor subtype. HLA down-modulation was associated with a low or intermediate immune infiltration and the absence of T-cell activation. Moreover, HLA class I down regulation was also correlated with worse survival, with implications for immune-intermediate PCNSL that frequently feature reduced HLA expression. A ligand–receptor intercellular network revealed a high expression of two immune checkpoints (i.e., CTLA-4/CD86 and TIM-3/LAGLS9). Mucin-domain containing protein-3 (TIM-3) and galectin-9 proteins were clearly upregulated in PCNSL (see Chapter 58). Based on their observations, they suggest testing DNA methyltransferase (DNMT) inhibitors in PTPN6 methylated PCNSL (observed in 48.5% of their study samples) to restore STAT3 expression in immune-infiltrated PCNSL, ibrutinib in combination with immune checkpoint inhibitor in immune rich/MYD88-mutated PCNSL patients (ClinicalTrials.gov Identifier: NCT03770416), and histone deacetylase (HDAC) inhibitors that crosses blood brain barrier (BBB) to re-establish the HLA class II expression in immune-poor PCNSL subtype. Miyasato et al.[82] reported that the expression of PD-1 ligands on tumor-associated macrophages (TAM) was dependent on the activation of STAT3. In addition, the expression of indoleamine 2,3-dioxygenase (IDO1) by TAMs was involved in the immune evasion by lymphoma cells in patients with PCNSL, suggesting that combination therapy using dual inhibitors of IDO1 and immune-checkpoint may have a therapeutic value. The increased incidence of IDO expression in a normal "healthy" brain with aging may help explain the increased risk of PCNSL in older patients.[83] Marcelis et al.[84] showed high expression of PD-L1 in 28% of PCNSL without amplification of the 9p24.1 locus. The TME was predominantly composed of CD8+ T cells and macrophages. The presence of the former cells was associated with an improved overall survival (OS). On the contrary, there was a correlation between high expression of TIM-3 in T cells in the TME and inferior outcome.[84] The investigators reported marked intertumoral and intratumoral heterogeneity in the TME.

Cell of Origin and Its Implications

The precise implication of COO in PCNSL remains unclear. Data using the Hans classifier show both GCB and non-GCB subtypes, but unlike nodal DLBCL (see Chapter 85), the COO IHC classification does not affect progression-free survival (PFS) or OS.[12–14,85] Limitations of these studies include small sample sizes, retrospective nature, and incomplete treatment information that could impact survival analysis. On gene expression profiling (GEP), with the limitation of sample-introduced bias, the majority of PCNSL are non-GCB subtype.[1] Interestingly, Bödör et al.[51] demonstrated a higher proportion of GCB (13% vs. 5%) and a significantly lower proportion of ABC (80% vs. 95%) PCNSL cases when compared with parallel Hans characterization. These findings are in contradiction with previous reports.[12,13,15,43,86] Such discrepancies most likely can be explained by the heterogeneity in the type and depth of the sequencing approaches (whole genome/exome sequencing vs. targeted resequencing of selected genes), the type of the analyzed material (fresh frozen vs. FFPE), and the difference between the bioinformatics pipelines and variant calling methods. The same study[51] also showed that mutations of TP53 (15% vs. 6%) and PAX5 (15% vs. 2%) were more frequent in GC cases, compared to ABC cases, respectively. While mutations of CD79B, CARD11, CSMD2, and CSMD3 were exclusively detected in ABC cases, MYD88, PRDM1, and KMT2D genes showed similar mutational frequencies across the two subtypes.[51] This finding may be of potential therapeutic relevance, as the BTK inhibitor ibrutinib has demonstrated clinical efficacy in 37% (14 of 38 patients) of nodal ABC-DLBCLs, especially in cases with concurrent MYD88 and CD79B mutations.[87] In a phase I study by Grommes et al.,[88] clinical responses were seen in 10 of 13 patients with relapsed PCNSL who had MYD88 mutation. Importantly, this study[88] showed that CARD11 mutations were associated with ibrutinib resistance.

Moreover, Montesinos-Rongen et al.[89] analyzed tissues from 21 patients with PCNSL using a microarray-based expression profile. A comparison of the transcriptional profile of PCNSL with various normal and neoplastic B-cell subsets demonstrated PCNSL (i) to display gene expression patterns most closely related to late GCB cells, (ii) to display a GEP similar to systemic DLBCLs and (iii) to be in part assigned to the ABC or the GCB subtype of DLBCL. Interestingly, their study[89] supported the notion that PCNSL exhibits characteristics associated with both ABC (IGM expression with absence of IG class switch, high frequency of somatic mutations of IG genes, recurrent 18q21 gains, activation of the NF-κB pathway, and high expression of BCL2 mRNA and protein) and GCB type (expression of BCL6, ongoing somatic hypermutation) DLBCL.[89] These data, along with the data from Fukumura et al.[48] and Inoue et al.[85] support that PCNSL represents a distinct COO entity irrespective of the conventional Hans COO classification. Additionally, a more recent, large Canadian population-based study[49] of 155 patients, suggests that PCNSL has a unique molecular profile distinct from systemic DLBCL.

Epstein-Barr Virus–Associated (Tissue-Positive) Primary Central Nervous System Lymphoma and Its Implications

EBV is most frequently observed in HIV-associated PCNSL and early posttransplant PCNSL (see Chapter 87). A recent study by Gandhi et al.[2] showed that the genetic landscape typically observed in EBV^- HIV^- PCNSL is distinct from EBV-associated PCNSL. They used targeted sequencing and digital multiplex gene expression to compare the genetic landscape and TME of 91 DLBCL histology cases. Forty-seven patients were EBV tissue-negative: 45 EBV^- HIV^- PCNSL and 2 EBV^- HIV^+ PCNSL; and 44 were EBV tissue-positive: 23 EBV^+ HIV^+ PCNSL and 21 EBV^+ HIV^- PCNSL. As noted previously, EBV^- HIV^- PCNSL had frequent MYD88, CD79B, and PIM1 mutations, and enrichment for the ABC subtype.[90] In contrast, these mutations were absent in all EBV-associated PCNSL, and ABC subtype frequency was low. Furthermore, copy number loss in HLA class I/II and antigen-presenting/processing genes was rarely observed, indicating retained antigen presentation. To counter this, EBV^+ HIV^- PCNSL had a tolerogenic TME with elevated macrophage and immune-checkpoint gene expression, whereas AIDS-related PCNSL had low CD4 gene counts. Interestingly, the proportion of PD-L1 and PD-L2 gene amplification was similar between EBV^- HIV^- PCNSL and EBV^+ HIV^- PCNSL, suggesting that the increase in expression of these ligands in EBV^+ HIV^- PCNSL may be due to macrophage expression.[84] From a therapeutic standpoint, checkpoint blockade is potentially contraindicated in post-transplant lymphoproliferative disease (PTLD) due to risk of graft rejection and graft-versus-host disease. By contrast, checkpoint blockade might be a reasonable option in AIDS-related PCNSL. Adoptive transfer of EBV-specific third-party virus specific T cells chosen on the basis of the best HLA match and in vitro effector function see (Chapters 87 and 97) has previously been shown to induce high response rates in EBV^+ PTLD and can cross the blood-brain barrier (see Chapters 87 and 97).[91,92] Based on the cumulative biological data, a phase I Australasian Leukemia/Lymphoma Group clinical trial incorporating EBV-specific third-party virus specific T cells (ACTRN12618001541291) has commenced.

CLINICAL PRESENTATION

The clinical presentation of CNS lymphoma depends on the size, pattern, and location of the disease within the neuraxis.

Brain Parenchyma

The lesions vary in size from microscopic implants to large bulky masses. PCNSL often presents in a subacute manner with chronic lethargy, nonspecific decline in cognition, and psychomotor function (32% to 43%), often delaying the diagnosis.[93] Focal neurologic deficits (56% to 70%) can be observed immediately prior to the diagnosis. Presentation of headache, confusion, diplopia, nausea, and vomiting are usually late signs and symptoms of elevated intracranial pressure (ICP). Seizures are uncommon (14%), mainly due to non-cortical location of the disease. The classic B symptoms are rarely observed in PCNSL.

Primary Vitreoretinal Lymphoma

A proportion of PCNSL patients present with PVRL.[94] Overall, visual symptoms are rare at presentation, even though the ocular involvement is relatively high (15% to 20%).[94] Insidious and nonspecific onset of symptoms can often delay the diagnosis of PVRL by 6 to 24 months. PVRL symptoms can imitate a wide range of other ocular entities and have been described as a masquerade syndrome. Patients may be asymptomatic or complain of blurred vision (40% to 50%), reduced visual acuity (25% to 30%), and vitreous floaters (20% to 25%), often misdiagnosed as inflammatory eye conditions such as uveitis, vitritis, chorioretinitis, or normal degenerative changes. The clinical signs are bilateral in approximately 60% to 90% of individuals. A history of an older adult with noninfectious uveitis who failed to respond to anti-inflammatory therapy should raise a suspicion of VRL. Other important differential diagnoses include fungal or bacterial endophthalmitis, sarcoidosis, ocular syphilis, and tuberculosis. The classic B symptoms are rarely observed in PCNSL.

Leptomeninges

Simultaneous involvement of leptomeninges is observed in a small subset (18%) of PCNSL patients, but exclusive meningeal presentation is extremely unusual. The presence of leptomeningeal involvement at diagnosis should increase suspicion of SCNSL. Symptoms of lymphomatous spinal cord lesions are parallel to those observed with other intramedullary tumors and depend on tumor location.[95,96] In general, thoracic spine is the most common area of involvement by PCNSL.[95–97]

NEUROIMAGING

Up to 75% of all PCNSLs contact a cerebrospinal fluid (CSF) surface: either the ventricular ependymal or pia lesions are often deep-seated with a predilection for the periventricular white matter, especially the corpus callosum. The basal ganglia and thalami are the next most common locations. Occasionally, PCNSLs diffusely infiltrate large areas of the hemispheres, without forming a focal cohesive mass. Widespread infiltration of lymphoma cells in both gray and white matter is characteristic. This condition—also known as lymphomatosis cerebri—is uncommon, occurring in less than 5% of cases, and is a pattern, not a distinct disease entity.

Magnetic Resonance Imaging Brain With and Without Gadolinium

The cornerstone of diagnostic testing, whenever PCNSL is suspected, is MRI with gadolinium. Over three-quarters of DLBCLs in immunocompetent patients are iso- or slightly hypointense compared with gray matter on T1 and iso- or hyperintense on T2. FLAIR signal is also usually iso or hyperintense (Fig. 84.2A–C).

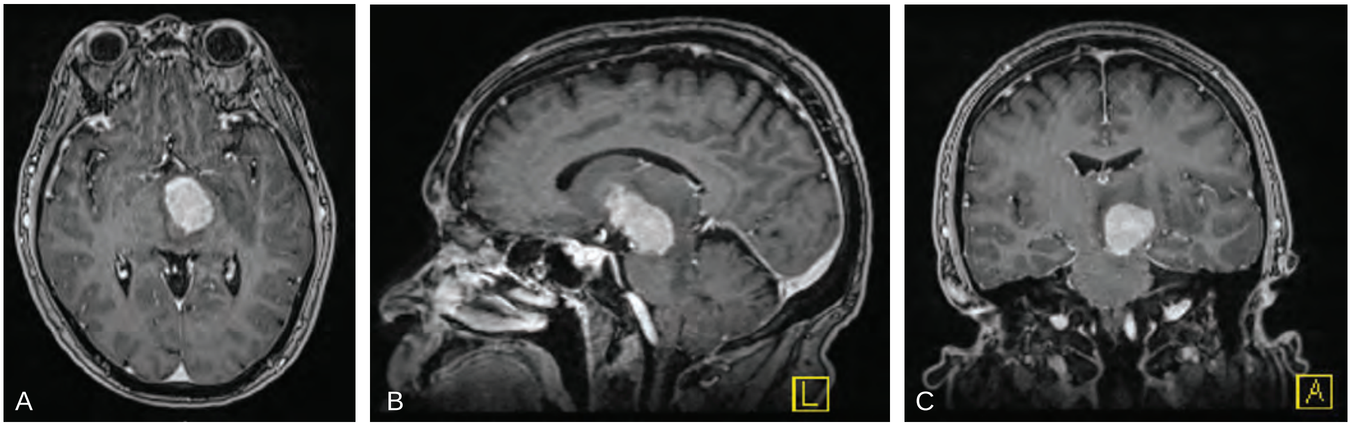


Figure 84.2 PRIMARY CENTRAL NERVOUS SYSTEM LYMPHOMA IN IMMUNOCOMPETENT INDIVIDUAL. (A) T1 postcontrast axial, (B) sagittal, and (C) coronal magnetic resonance imaging images demonstrate a homogenously enhancing lesion abutting the third ventricle.

On imaging, a single lesion is present in about 60% to 70% of the PCNSL. Keeping in mind that the disease is highly infiltrative and lymphomatous, dissemination in the brain and leptomeninges may occur in the absence of contrast enhancement on magnetic resonance imaging (MRI).[98,99] Multifocal disease is observed in a substantial proportion of patients, especially at relapse, and is somewhat more common in immunocompromised individuals. Moreover, in immunocompromised patients, the enhancement may be less robust, or completely lacking (Fig. 84.3A,B). About 60% of PCNSLs involve the supratentorial space, including the frontal (15%), temporal (8%), parietal (7%), and occipital (3%) lobes, basal ganglia, periventricular brain parenchyma (10%), and corpus callosum (5%). Less frequently involved sites include the posterior fossa (13%) and spinal cord (1%).[98,99] Furthermore, diffuse enhancing supra and/or infra tentorial pachymeninges and the extension along the (sub) ependymal surfaces can be seen with contrast-enhanced MRI in about 3% to 5% of patients, which is indicative of leptomeningeal infiltration. Because of their high cellularity, over 95% of PCNSLs show mild to moderate diffusion-restriction with apparent diffusion coefficient (ADC) and diffusion weighted imaging (DWI) sequences in a nonvascular distribution.[98]

Magnetic Resonance Spectroscopy

The findings on magnetic resonance spectroscopy (MRS) are *nonspecific* and include increased choline (a marker of cell membrane transition and proliferation of cells) and lipid (a marker of tumor necrosis) peaks, a moderately decreased N-acetyl-L-aspartic acid peak (a marker of mature neurons), and a slightly decreased creatine peak (negatively associated with energy metabolism).[98] There is interest in development of novel, advanced imaging approaches to detect and provide prognostic information on PCNSL more precisely.[99]

NEUROIMAGING AND DIFFERENTIAL DIAGNOSIS PRIOR TO BIOPSY

Brain imaging, with single or multiple enhancing lesion(s), raises a broad differential diagnosis other than PCNSL which includes infectious, vascular, autoimmune, and other nonlymphoid neoplastic entities. The comprehensive clinical history as well as several specific radiographic features can assist with contraction of the differential diagnoses. Even when a nonneoplastic etiology resides atop the differential diagnosis, a stereotactic biopsy may have substantial value in clarifying the diagnosis.[100]

Stroke

Certain acute and subacute vascular events can often be confused with CNS lymphoma. Acute/subacute strokes can be enhanced on postcontrast imaging due to BBB breakdown, particularly in the subacute timeframe. Typically, this enhancement is not homogenous and usually dissipates over time. Such contrast enhancement and associated restricted diffusion follow a vascular distribution with a wedge-like appearance in medium to large vessel distribution, or more punctate enhancement in small vessel distribution. As strokes can be in multiple distributions, particularly when secondary to a cardioembolic or vasculitis source, it is not surprising that their radiographic appearance can sometimes be conflated with CNS lymphoma. The clinical pace of symptom(s) in the setting of stroke is typically sudden; however, that of CNS lymphoma is usually subacute but this by itself does not exclude the possibility of underlying stroke (see Chapter 144).

Infection

Several CNS infections, including viral, bacterial, mycobacterial, fungal, and parasitic etiologies, are associated with contrast enhancement. While the radiographic appearance does not clearly delineate an infectious etiology, the clinical presentation can provide cues to an underlying, active infection.

Immune Mediated Diseases

Certain autoimmune entities can present similarly as CNS lymphomas with regard to their subacute presentation and brain enhancement on MRI scan. For example, multiple sclerosis (MS) can mimic CNS lymphoma. However, as opposed to PCNSL in immunocompetent hosts, the median age of diagnosis is earlier in MS. Also, MS rarely presents with multiple enhancing brain lesions. Unlike PCNSL, brain enhancement is in a relatively small area in MS. The classic enhancement pattern noted in MS is an "open C" with an incomplete ring of enhancement and the open portion facing medially. Other entities that can mimic CNS lymphoma include acute disseminated encephalomyelitis (ADEM) and neurosarcoidosis. ADEM is often monophasic and can present with single, or multiple, large areas of brain enhancement. Sarcoidosis can often be a chronic process, usually with systemic involvement, along with acute/subacute exacerbations. However, even in these settings, it is often necessary to obtain CNS tissue for pathological diagnosis. All of the autoimmune entities are less likely to exhibit restricted diffusion on ADC/DWI sequences.

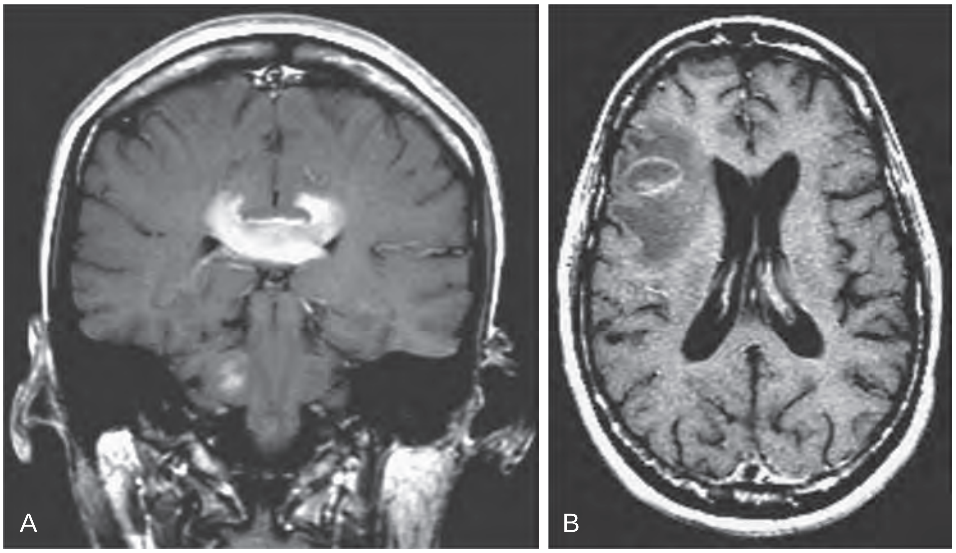


Figure 84.3 PRIMARY CENTRAL NERVOUS SYSTEM LYMPHOMA IN HUMAN IMMUNODEFICIENCY VIRUS INFECTED INDIVIDUALS. (A) A coronal T1 postcontrast magnetic resonance imaging (MRI) demonstrates a large homogenously enhancing lesion crossing the corpus callosum. A second lesion of patchier enhancement can be seen in the right cerebellar peduncle. (B) An axial T1 post-contrast MRI shows a right frontal lesion with a thin rim of enhancement and a larger area of surrounding cerebral edema.

Other Central Nervous System Cancers

Infiltrating gliomas may have a similar appearance to CNS lymphoma. Both tumor types can involve the corpus callosum with thickening of the anatomic structure as opposed to the atrophy seen in MS. CNS lymphoma often involves the deeper nuclear supratentorial structures as opposed to infiltrating gliomas, which often affect cortical to subcortical brain tissue. An exception to this rule is gliomas in patients who harbor the H3K27 mutation, which is associated with involvement of deeper midline structures. Low-grade infiltrating gliomas often do not typically enhance. The enhancement pattern of high-grade gliomas (including glioblastoma) is usually heterogeneous, in contrast to the more homogenous pattern of enhancement in CNS lymphoma. In addition, the pattern of restricted diffusion on DWI and ADC sequences is often heterogeneous, when present at all, in high-grade gliomas as opposed to being homogenous in CNS lymphoma.[100]

TISSUE DIAGNOSIS

Brain Stereotactic Biopsy

The diagnostic procedure of choice, with the least risk of morbidity and mortality, remains stereotactic biopsy of the brain lesion. If ocular (vitreous, and retina) lymphoma is suspected, then surgical intervention with *timely* (tissue procurement to laboratory testing) fluid assessment is absolutely essential. Of importance, unnecessary tests should be avoided in order not to jeopardize patient care. For example, analysis of CSF should not delay stereotactic brain biopsy in a non-HIV individual, and CSF fluid testing should be done promptly in cases of suspected isolated PVRL (discussed in detail later).

Misinterpretation of Stereotactic Biopsy

Administration of steroids prior to procurement of brain tissue may significantly decrease enhancement and the size of the tumor, which in turn can easily alter the diagnosis.[40,41,101,102] It is important to inform the neuropathologist of prior systemic steroid exposure to avoid misinterpretation of specimen as "encephalitis". Biopsy should be repeated in cases where initial biopsy is inconclusive, unsatisfactory, or the diagnosis does not align with the clinical and/or radiologic presentation. In selected cases, a distinct tumor mass is not evident on imaging and/or the biopsy may have been taken from the periphery of the tumor, resulting in the finding of an entirely interstitial pattern with isolated tumor cells intermingled between astrocytes. Sometimes cases with scant tissue may show a relatively monomorphic cell population with intermingled macrophages, mimicking Burkitt lymphoma (see Chapter 85).[1]

Risks With Brain Biopsy

Most brain biopsy procedures are safely performed, but the risk of complications is about 8.5%, consisting of hemorrhage, seizures, or brain edema.[103] Biopsy-related mortality is estimated to be 0.9%.[104] Of note, the risk of hemorrhage is 11.5% in individuals with AIDS, compared to only 1% in clinically stable immunocompetent individuals.[105] For this reason, in HIV-infected individuals stereotactic biopsy may be utilized once noninvasive tests are inconclusive or undiagnostic (discussed later).[106] Current evidence does not support diagnostic or therapeutic surgical resection unless there is an impending brain herniation, or rapidly deteriorating neurologic function, failing best conservative practices. Work by some investigators supports more extensive resection.[103,107,108] However, such an approach remains non-routine.

CEREBROSPINAL FLUID ASSESSMENT FOR DIAGNOSIS

Under most circumstances, cerebrospinal fluid (CSF) analysis is no longer required in patients suspected of PCNSL. The CSF findings in PCNSL, if performed, are nonspecific and include elevated protein and lymphocytic predominant pleocytosis with normal glucose levels.[95,96,109] A routine CSF examination can only provide definitive evidence of PCNSL in the presence of extensive leptomeningeal dissemination (Fig. 84.4A–D).[95,96.109] Importantly, a misinterpretation of a peak caused by a single B cell as a monoclonal population ("pseudomonoclonality") may result in a false-positive PCR.[96] False positive results can sometimes be minimized by repeating the test and/or

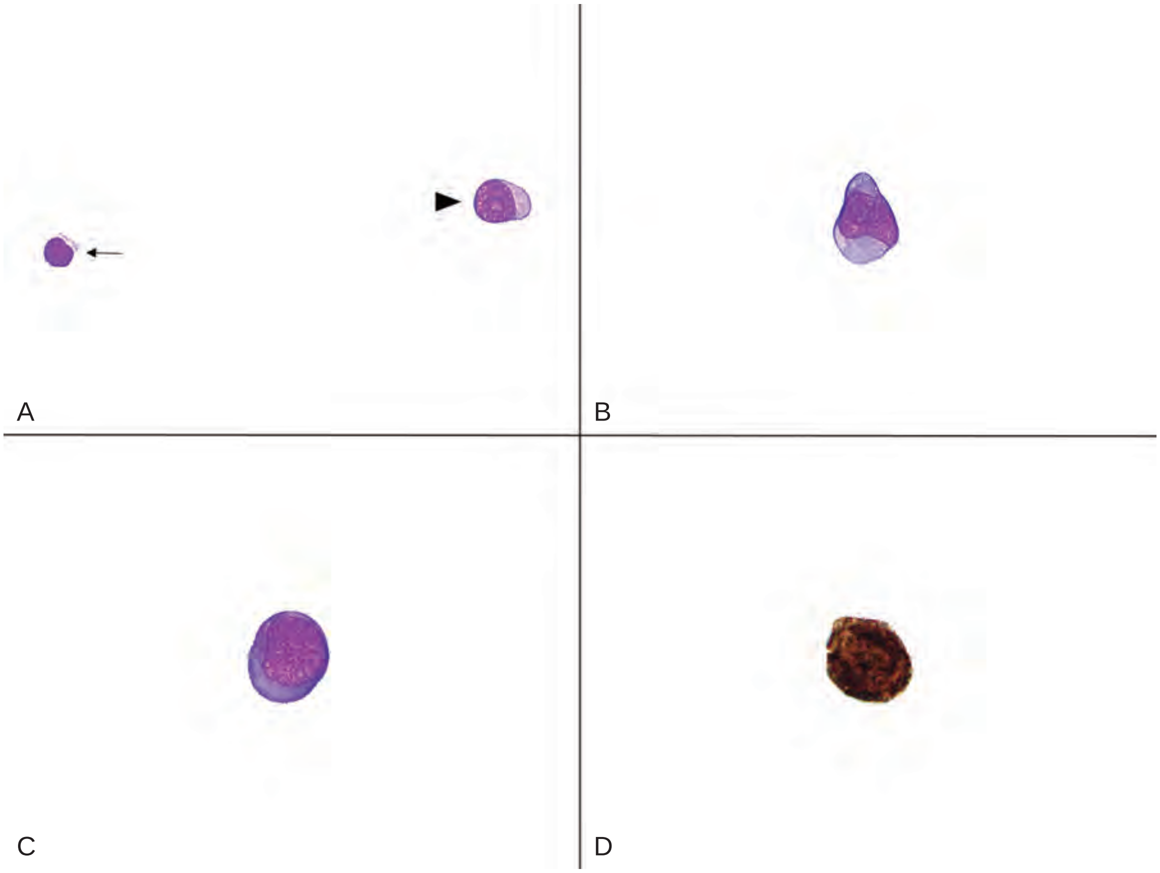


Figure 84.4 CEREBROSPINAL FLUID INVOLVEMENT BY DIFFUSE LARGE B-CELL LYMPHOMA OF THE CENTRAL NERVOUS SYSTEM. (A) In cytospin preparations the lymphoma cell (arrowhead) has a nucleus 3–4× larger than that of the nearby small lymphocyte (arrow). (B and C) Lymphoma cells show high nuclear to cytoplasmic ratios, vesicular chromatin and prominent, sometimes multiple, nucleoli. D, On cell block preparation, lymphoma cells show nuclear positivity for PAX-5, a marker of B-cell lineage. Concurrent flow cytometry of the cerebrospinal fluid confirmed a light-chain restricted large B-cell lymphoma.

through repeated PCR cycles in independent runs. Positive molecular findings in CSF should be interpreted in the context of clinical and tissue morphological data.

Cerebrospinal Fluid Testing in Human Immunodeficiency Virus–Infected Individuals

In selected cases where diagnostic brain biopsy is contraindicated and routine CSF cytology and cytometry are non-diagnostic, special tests may facilitate PCNSL diagnosis. Antinori et al.[110] reported that the combination of increased uptake on thallium-201 SPECT and a positive EBV by PCR in the CSF had 100% sensitivity and 100% negative predictive value. They suggested that patients with positive results could go directly to therapy, obviating the need for tissue biopsy. These findings are provocative and require further validation as few, if any, diagnostic studies have perfect sensitivity and specificity. In another study,[111] the sensitivity and specificity of PCR for EBV-DNA detection in CSF were 80% (95% CI: 60.9% to 91.6%) and 100% (95% CI: 92.6% to 100%), respectively.

OPHTHALMOLOGY EXAM AND VITREORETINAL SPECIMEN TESTING FOR DIAGNOSIS

In an appropriate clinical context, a comprehensive ophthalmology exam using dilated fundoscopy, fluorescent angiography, and/or optical coherent tomography is sufficient (without tissue procurement) to diagnose (primary or secondary) VRL, sparing the patient a more invasive vitreous biopsy. For example, the diagnosis of VRL can be established in an older patient with complaints of floaters, vitreous and/or subretinal cellular infiltration on exam, and positive CD20+ large cell cytology and/or flow cytometry for monoclonality in the CSF (Fig. 84.5A–C)[112]. However, in a patient with non-diagnostic CSF (as in the majority of cases) and/or brain biopsy, the gold standard for the diagnosis of VRL remains histologic identification of lymphoma cells in the vitreous and/or retina. The tissue diagnosis would require an invasive procedure, such as vitrectomy or vitreous aspirate, retinal or chorioretinal biopsy, or, rarely, diagnostic enucleation.[112,113]

The reported rates of sensitivity and specificity of cytology for the diagnosis of VRL vary widely, but cytology alone is able to confirm VRL in 45 to 60% of cases.[114,115] Alternatively, multicolor flow cytometry (MFC) has been successfully employed for phenotyping of vitreal aspirates, with a reported sensitivity of 82% and 100% specificity.[115,116] The sensitivity of clonality studies ranges between 65% and 95%, depending on the choice of primer sets and quality of material.[114] The limitations of molecular testing, particularly with the small sample size from the vitreous and/or retina, are that results could be false-positive or false-negative. In order to avoid misdiagnosis of minor clonal expansions as evidence for lymphoma, all tests should be run in duplicate to confirm the presence of a dominant clone, and results should be interpreted with caution and only in the context of clinical and morphological findings.[114] Therefore, microdissection identifying the area with most atypical lymphoid cells is critical, and if the patient has been placed on systemic or local corticosteroids, it is best to wait a few weeks to allow more of a vitreal infiltration before doing a tissue biopsy. If the biopsy is

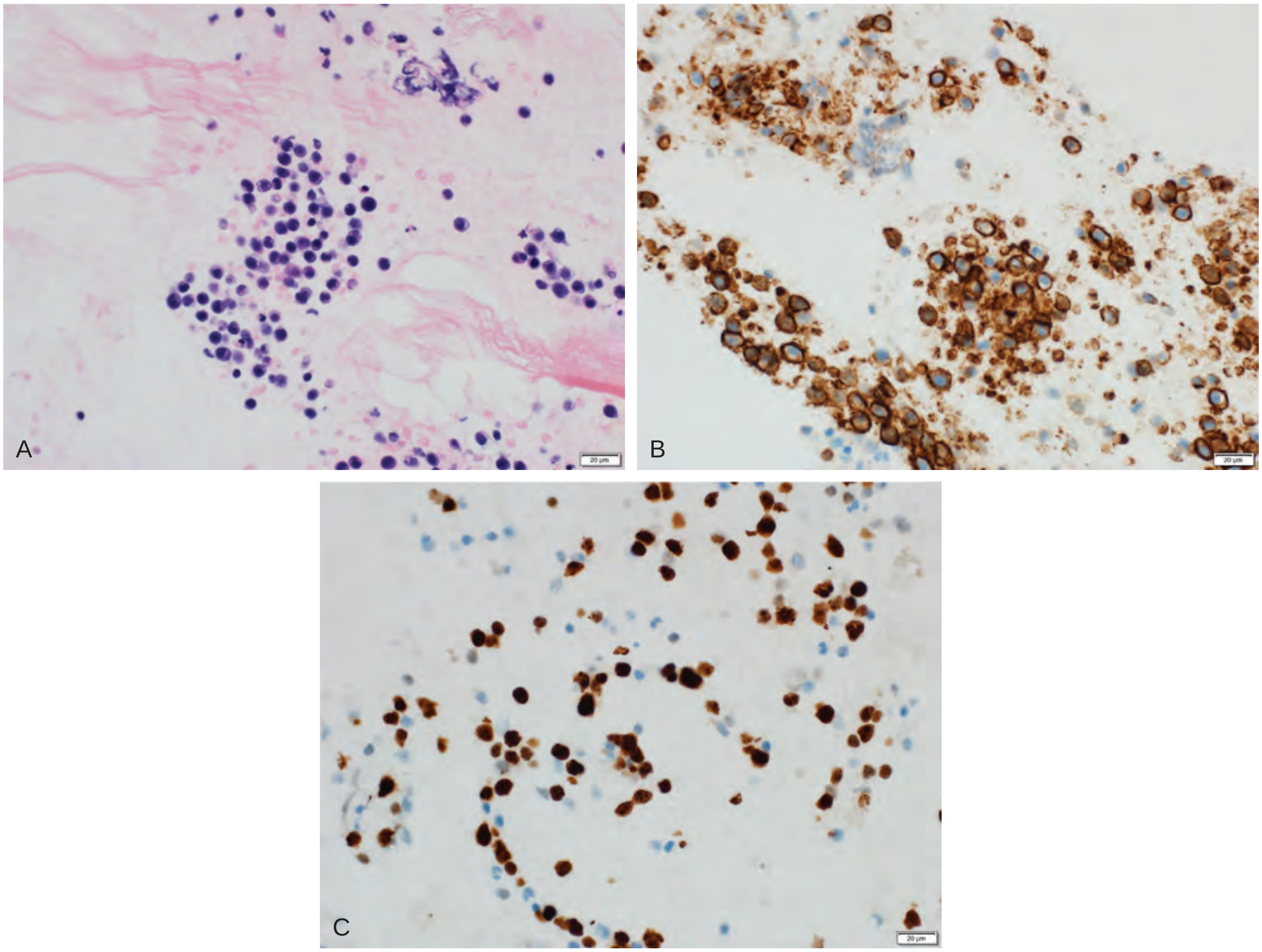


Figure 84.5 DIFFUSE LARGE B-CELL LYMPHOMA OF THE CENTRAL NERVOUS SYSTEM IN THE VITREOUS FLUID. (A) Cell block showing vitreous large B-cell lymphoma with numerous atypical large lymphocytes with irregular nuclear contours, small to moderate nuclei and scant cytoplasm (hematoxylin and eosin stain, original magnification ×400). (B) CD20 immunostain highlighting the large atypical lymphoid cells confirming B-cell lineage (CD20 immunohistochemical stain-original magnification 400×). (C) Ki-67 immunostain highlighting the large atypical lymphoid cells with a high proliferation index (Ki-67 immunohistochemical stain-original magnification 400×). *(Courtesy of Dr. Diva Salomao.)*

nondiagnostic and there is still strong suspicion of VRL, then the other vitreous should be biopsied; or if there are aggregates of subretinal cells, a retinal biopsy using the same vitrector technique with a lower cut rate and manual aspiration should be done. Additional adjuncts in diagnosing VRL exist, including elevation of interleukin-10 (IL-10) or IL-10:IL-6 ratio greater than 1 in ocular fluids, detection of *IgH*, as well as evaluation of mutant L265P MYD88 (positive in 60% to 80% of cases) and intraocular concentrations of the CD79B^{Y196} mutation.[114,117–119] In cases of mutant L265P MYD88 in the context of CD20+cells in the vitreous, a diagnosis of VRL can be confirmed.[114,117–119]

HISTOLOGY CHARACTERIZATION

Most CNS lymphomas are of B-cell origin, predominantly DLBCL. However, Burkitt lymphoma, acute lymphoblastic lymphoma, marginal zone lymphoma, extraosseous plasmacytoma, and intravascular B-cell lymphoma are rarely encountered. Moreover, less than 4% of PCNSL are of T-cell origin.[1,120] The morphology cannot discern between PCNSL and SCNSL; it is the role of staging to do so. Microscopically, the neoplastic cells are of medium to large size, with round, oval, irregular, or pleomorphic nuclei and distinct nucleoli, corresponding to centroblasts or immunoblasts, and grow in a characteristic perivascular pattern, forming concentric rings ("angiocentric" or "cuffing") (Fig. 84.6A–D).[1]

JAK/STAT signaling pathway activators such as IL-4 and IL-10 were found to be upregulated and create a favorable TME as evident by characteristic angiotropism. Rubenstein et al.[121] showed that a high expression of activated STAT6 in tumors was associated with short survival in an independent set of patients with PCNSL who were treated with HD-MTX therapy. Infiltration of cerebral blood vessels causes fragmentation of the argyrophilic fiber network. From these perivascular cuffs, tumor cells invade the neural parenchyma.[1] The TME is often accompanied by prominent microglial activation and a reactive inflammatory infiltrate, which consists of mature T cells and, sometimes, lipid-laden (foamy) histiocytes.[1] Notably, in a study by Ponzoni et al.[122], perivascular T cell infiltrates in PCNSL may predict a favorable outcome, suggesting that immunotherapeutic interventions that potentiate T-cell-mediated immune surveillance may be effective. Centrally, large areas of geographical necrosis are not uncommon making the histologic diagnosis sometimes cumbersome.[1] PCNSL does not have the degree of vascular proliferation commonly observed in glioblastoma. In the immunocompromised host, however, histologic findings of necrosis and hemorrhage are common.[1]

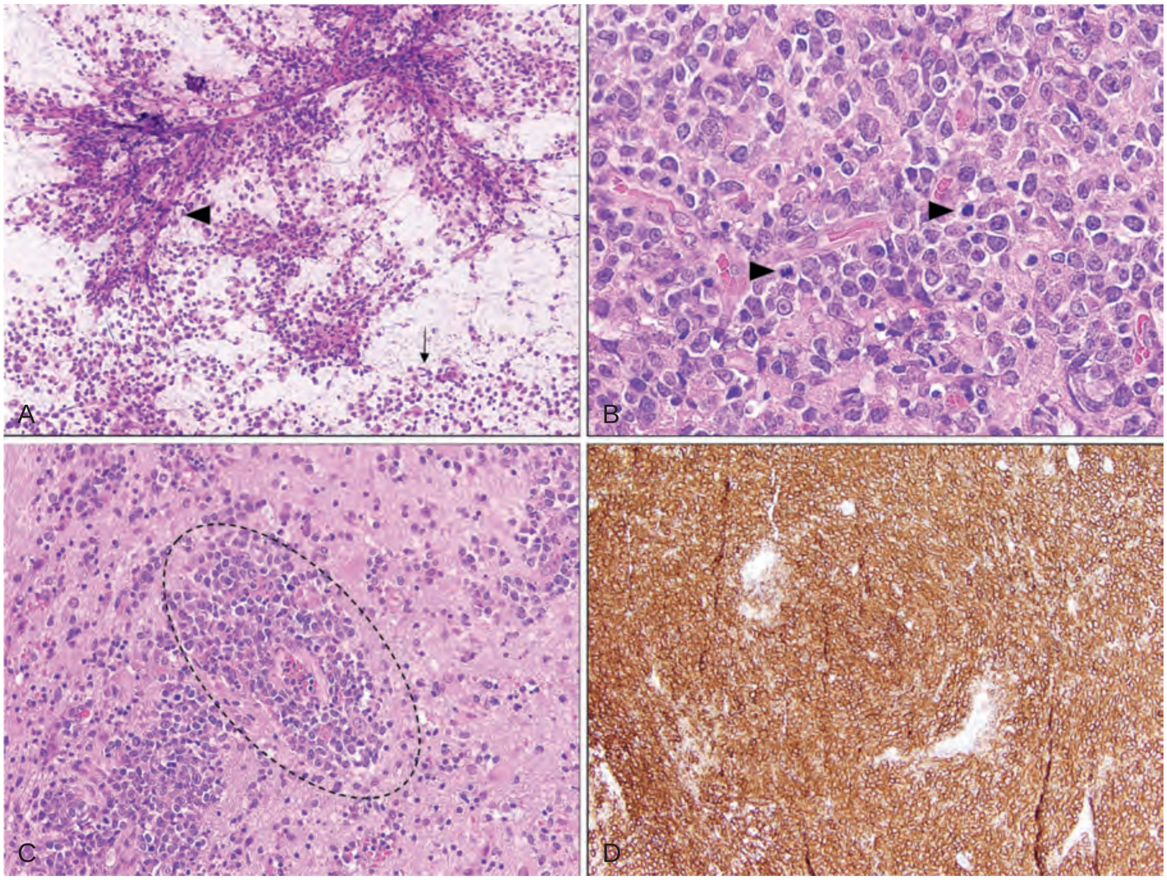


Figure 84.6 DIFFUSE LARGE B-CELL LYMPHOMA OF THE CENTRAL NERVOUS SYSTEM. (A) A squash preparation shows discohesive lymphoid cells *(arrow)* with perivascular accentuation of tumor *(arrowhead)*. (B) Hematoxylin, and eosin (H&E) sections show atypical lymphoid cells with high nuclear to cytoplasmic ratios, irregular nuclear boarders, centroblastic (multiple peripheral nucleoli) and immunoblastic (central prominent nucleoli) nuclear features and scattered mitotic figures *(arrowheads)*. (C) The periphery of the tumor is the optimal area to appreciate perivascular accentuation *(dotted oval)* and the discohesive nature of lymphoma cells. (D) The tumor cells show strong, complete membranous positivity for CD20, a marker of B-cell lineage.

The cells of PCNSL are mature B cells, with PAX5+, CD19+, CD20+, CD22+, and CD79a+ phenotypes (Fig. 84.6D).[1] IgM and IgD, but not IgG, are expressed, with either kappa or lambda light chain restriction. Most cases express BCL6 (60% to 80%) as well as MUM1 (90% to 100%), whereas plasma cell markers (i.e., CD38 and CD138) are usually not expressed. Expression of CD10 is unusual in PCNSL (<10%), and its positivity should prompt a search to exclude disseminated systemic DLBCL. Most cases (~80%) of PCNSL of the CNS are positive for BCL2 and MYC expression. However, MYC translocations are rare and BCL2 translocations are absent, suggesting that alternate mechanisms drive the protein expression in PCNSL[1,123] Conversely, BCL6 protein expression is strongly correlated with the presence of *BCL6* rearrangements. The Ki-67 is often markedly elevated; most lesions have a proliferative index that exceeds 70%. The presence of EBV should prompt evaluation for an underlying immunodeficiency. The V600E BRAF protein expression has not been detected in PCNSL.[1,124]

BIOMARKERS FOR DIAGNOSIS

Numerous biomarkers in the serum, CSF, and vitreous fluid have been evaluated with limited success[19–21] in an effort to possibly obviate the need for invasive diagnostic procedures and to expedite the diagnostic process.[125] Below are data of selected diagnostic (and in some cases prognostic) biomarkers that may be useful in clinical scenarios where diagnosis may be challenging.

Serologic Biomarkers

- **Cell-free DNA (cfDNA; liquid biopsy):** In a study by Fontanilles et al.,[126] serum cfDNA and matched tumor DNA (tDNA) from 25 PCNSL patients were sequenced. First, patient-specific targeted sequencing of identified somatic mutations in tDNA was performed. Then, a second sequencing targeting MYD88 c.T778C was performed and compared to serologic samples from 25 age-matched control patients suffering from other types of cancer. According to the patient-specific targeted sequencing, 8 patients (32% [95% CI 15 to 54%]) had detectable somatic mutations in cfDNA. Considering MYD88 sequencing, 6 patients had the specific c.T778C alteration detected. Using a control group, the sensitivity was 24% and the specificity was 100%.[126]
- **miR-21 levels:** Mao et al.[127] reported that the serum miR-21 was significantly increased in patients with PCNSL when compared with other brain tumors and normal controls in both the test and validation cohort.

Cerebrospinal Biomarkers

- **Circulating cell-free tumor DNA (ctDNA):** In the study by Grommes et al.,[128] of 9 relapsed/refractory PCNSL patients, tumor-specific ctDNA was found in the CSF of all patients. Sustained tumor responses were associated with clearance of ctDNA from the CSF.
- **IL-10:** Meta-analyses by Wang et al.[129] reported a sensitivity and a specificity of IL-10 in CSF for diagnosing CNSLs of 81%

(95% CI: 66% to 91%) and 97% (95% CI: 83% to 100%), respectively. Moreover, elevated IL-10 was associated with shorter PFS (hazard ratio: 2.89, 95% CI: 1.13 to 7.41, $P = .027$).

- **IL-10 and IL-10/IL-6 ratio:** Song et al.[19] reported a diagnostic sensitivity and a specificity of 95.5% and 96.1%, respectively (AUC, 0.957; 95% CI, 0.901 to 1.000) using an IL-10 cutoff value of 8.2 pg/mL in the CSF. In addition, the sensitivity and specificity of 95.5% and 100.0% (AUC, 0.976; 95% CI, 0.929 to 1.000), respectively, were reported using an IL-10/IL-6 cutoff value of 0.72. An increased CSF IL-10 level at diagnosis and post-treatment was associated with poor PFS for patients with PCNSL ($P = .0181$ and $P = .0002$, respectively).[19]
- **IL-10 and CXCL-13:** In the multicenter study by Rubenstein et al.[20] ($n = 220$), a bivariate elevation of IL-10 plus CXCL13 was 99.3% specific for primary and secondary CNS lymphomas, with a sensitivity two-fold greater than cytology or flow cytometry.
- **CXCL13 and CXCL9:** Masouris et al.[130] reported **a** sensitivity and a specificity of about 90% for the diagnosis of active CNSL using a cut-off value of 80 pg/mL for CXCL13. The sensitivity and specificity of CXCL9 were 61.5% and 87.1%, respectively, using a cut-off value of 84 pg/mL.
- **Soluble CD27 (sCD27):** Murase et al.[131] reported that sCD27 greater than 15 U/ml was associated with PCNSL, and inflammatory neurological diseases and not with other neurologic diseases or brain tumors.
- **Osteopontin:** This is a proinflammatory cytokine involved in immune cell activation and B-cell migration and proliferation. Strehlow et al.[132] reported significantly higher osteopontin levels (>620 ng/mL) in CNSLs compared to patients with inflammatory conditions and GBM or in healthy controls ($P < .01$). Multivariate analysis reported that high osteopontin levels were associated with shorter PFS (HR 1.61, 95 % CI 1.13 to 2.31; $P = .009$) and OS (HR 1.52, 95 % CI 1.04 to 2.21; $P = .029$).
- **Neopterin:** This is regarded as a good biomarker of immune activation mediated by the "lymphocyte-macrophage axis."[133] Viaccoz et al.[134] reported a sensitivity and a specificity of 96% and 93%, respectively, for the diagnosis of PCNSL at a neopterin cut-off value of 10 nmol/L. The positive and negative predictive values were 84% and 98%, respectively. Geng et al.[133] reported higher neopterin levels in PCNSL compared with other brain tumors and inflammatory conditions.
- **miR-21, miR-19, and miR-92a:** Baraniskin et al.[135] reported increased levels of miR-21, miR-19, and miR-92a in PCNSL. Receiver-operating characteristic analyses showed AUCs of 0.94, 0.98, and 0.97 for miR-21, miR-19, and miR-92a, respectively, in discriminating PCNSL from inflammatory and other neurologic conditions.
- **Proliferation-inducing ligand (APRIL) and B-cell activating factor (BAFF):** Mulazzani et al.[136] reported that high levels of APRIL and BAFF reliably differentiated CNSL from other focal brain lesions (including primary and metastatic brain tumors, autoimmune-inflammatory lesions, and neuroinfectious lesions) with a sensitivity of 62.3% (APRIL), 47.1% (BAFF), and a specificity of 93.7% (APRIL, BAFF).
- **Multimarker algorithm:** Maeyama et al.[137] reported a multimarker diagnostic model using CSF CXCL13, IL-10, β2-MG, and sIL-2R from the results of the case-control study and then applied the model to a prospective study ($n = 104$) to evaluate its utility. The multi-marker diagnostic algorithms had excellent diagnostic performance: the sensitivity, specificity, positive predictive value, and negative predictive value were 97%, 97%, 94%, and 99%, respectively.

Vitreous Biomarker for Vitreoretinal Lymphoma

- **miR-155:** Tuo et al.[138] reported significantly higher levels of miRNA-155 in uveitis compared to lymphoma.

Future studies with better understanding are required to validate and standardize the diagnostic utility of these and other[117,139,140] diagnostic biomarkers in primary and secondary CNSLs.

STAGING

The goal of staging in CNSL is to decipher (i) between PCNSL and SCNSL and (ii) in cases of PCNSL, to determine the extent of the disease within the neuraxis. Both of these goals can be accomplished with the tests displayed in Table 84.3, some of which are guided by individual's age, immune status, and specific signs and symptom(s), while others are performed routinely. For restaging, all the positive tests, including an ophthalmology exam, are repeated at a predetermined interval (e.g., induction and consolidation); response to therapy is categorized according to International PCNSL Collaborative Group (IPGLS) criteria[141] (described later). Cognitive testing may be performed prior to, during, and after therapy.[93] Correa et al.[142] proposed standardization of neuropsychological evaluation in PCNSL, which includes a battery of tests and a questionnaire to assess domains sensitive to disease and treatment effects such as attention, executive functions, memory, psychomotor speed, quality of life, and premorbid IQ.

TABLE 84.3 **Staging of Primary Diffuse Large B-Cell Lymphoma of the Central Nervous System**

All patients

- Brain MRI with gadolinium contrast (brain CT scan with contrast if MRI is contraindicated)
- Ophthalmologic examination including slit-lamp examination and fundoscopy to check cellular vitreal or subretinal infiltrates. Consider biomarker(s) testing in selected cases (refer to text)
- Lumbar puncture in selected cases (refer to text)
- PET-CT scan (Lugano criteria-contemporary studies demonstrate a detection of extra-CNS lymphoma in about 10% of cases (CT chest, abdomen, and pelvis if PET-CT scan unavailable)
- Bone marrow biopsy and aspirate in selected cases
- Gadolinium-enhanced MRI of the entire spine in patients with spinal cord-related sign(s) and symptom(s) or positive CSF or eye involvement.
- Testicular US in selected cases. In cases with a normal testicular exam and a negative PET-CT scan, its utility is minimal
- Infectious disease evaluation – HIV and hepatitis panel. Other infectious disease markers as clinically indicated
- Neuropsychological testing can be performed before the start of therapy and at periodic intervals. This should not delay the initiation of treatment
- Geriatric assessment in adults ≥60 years of age could be considered
- Pregnancy test (for women of reproductive potential)
- Sterility counseling/testing and referral to oncofertility for appropriate candidates

Special considerations in immunocompromised individual

- Similar to immunocompetent individuals plus
- CSF evaluation for EBV by PCR and cranial PET or single photon emission CT scan can be considered (refer to text)
- Selected cases may require additional infectious disease markers such as plasma EBV by PCR (e.g., PTLD)

CNS, Central nervous system; *CSF*, cerebrospinal fluid; *CT*, computed tomography; *EBV*, Epstein-Barr virus; *HIV*, human immunodeficiency virus; *MRI*, magnetic resonance imaging; *PCR*, polymerase chain reaction; *PET*, positron emission tomography; *PTLD*, post-transplant lymphoproliferative disease.

FRONTLINE THERAPY AND RESPONSE ASSESSMENT

Immediate Conservative Management: Best Practices

PCNSL patients who present with focal brain lesion(s) are often symptomatic at diagnosis, with an emergent to urgent requirement for therapy. Based on MRI findings, many clinicians preemptively start steroids. Priority should be given to immediate nonsteroid-based conservative management and neurosurgical consultation for immediate stereotactic biopsy. Prediagnosis steroids usually lyse the neoplastic B cells, leaving behind reactive cells that may cause false-negative interpretation of biopsy as "encephalitis", a phenomenon often referred to as "vanishing tumor". In the study by Brück et al.,[143] corticosteroids prevented diagnosis in as many as 50% of the patients. Therefore with the rare exception of impending or frank herniation, the use of prediagnostic steroids is strongly discouraged. Priority should be given to mannitol or hypertonic saline over steroids. There is no standard dosing of steroids for patients who must receive them. Also, there is no role for prophylactic anti-seizure therapy.

INDUCTION THERAPY

The treatment for patients who are deemed "medically fit" should be initiated with a HD-MTX-based regimen with the goal of achieving a complete radiologic response (CR or unconfirmed CR [CRu]), defined as a complete resolution of all enhancing diseases on the brain MRI, in the absence of corticosteroid use, for at least 2 weeks before imaging.[141] If the disease was initially noted in other CNS compartments, CR is similarly defined by the absence of disease in previously positive space(s). The consensus criteria for disease response were established by the International PCNSL Collaborative Group (IPCG),[141] and defined as follows: CR, CRu, partial response (PR), stable disease (SD), or progressive disease (PD). A CRu may still demonstrate radiographic abnormalities of uncertain significance, precluding the imaging from qualifying as a CR. Often, CRu findings are secondary to the initial diagnostic biopsy.

Commonly used induction regimens are displayed in Table 84.4. They include: HD-MTX/vincristine/procarbazine/rituximab (**MVP-R**[22]; CR of 78% [Cl not provided]); HD-MTX/temozolomide/rituximab (**MTR**[23]; CR rate of 66% [CI, 50% to 80%]); rituximab/HD-MTX/thiotepa/cytarabine (**MATRix**[24]; CR rate of 49% [95% CI 38 to 60]); and HD-MTX/BCNU/teniposide/prednisone/rituximab (**MBTP**[144]). Of high importance, the difference in induction CR rates must be interpreted in the context of the details of study design, number of patients, and specific toxicities related to each regimen. (See box on Treatment of Newly Diagnosed Adult Patient With Primary Central Nervous System Lymphoma.)

TABLE 84.4 **Commonly Utilized High-Dose Methotrexate-Based Induction Regimens in Diffuse Large B-Cell Lymphoma of the Central Nervous System**

Induction regimen	Schedule and Doses
MVP-R [22] *N* = 30	**Every 2 weeks** in patients with age between 30 and 76 years (median 57 years) **HD-MTX** 3.5 g/m^2 IV day #2 over 2 h with leucovorin rescue for 5–7 cycles. **Vincristine** 1.4 mg/m^2 (max 2.8 mg) IV day #2 **Procarbazine** 100 mg/m^2/day, orally day #7 through #13 (odd cycles only) **Rituximab** 500 mg/m^2 IV day #1 Prophylactic intrathecal chemotherapy was not administered Therapeutic intra-Ommaya MTX 12 mg was administered between days 5 and 12 of each cycle to patients with a positive CSF cytology. **Restage after the 5th cycle, and if <CR, then 2 additional cycles prior to consolidation**
MTR[23] (Alliance 50202) *N* = 43	**Every 2 weeks** in patients with age between 12 and 76 years (median 61 years) **HD-MTX** 8 g/m^2 IV day #1 over 4 h with leucovorin rescue. **Temozolomide** 150 mg/m^2 per day orally day #7 through #11 for the first 5 months (odd cycles only) **Rituximab** 375 mg/m^2 IV weekly, starting on day #3 of cycle 1, for 6 doses total No intrathecal chemotherapy was administered **Restage after 7th doses of HD-MTX with one additional HD-MTX dose in patients with CR/CRu prior to consolidation**.
MATRix[24] (IELSG32) *N* = 75	**Every 3 weeks** in patients with age between 18 and 70 years (median 57 years) **HD-MTX** 3.5 g/m^2 IV days 1 over approximately 3 h with leucovorin rescue × 4 courses **Cytarabine** 2 g/m^2 over 1 h, twice a day IV day 2, 3 **Thiotepa** 30 mg/m^2 over 30 min IV days 4 **Rituximab** 375 mg/m^2 IV days (−5), 0, Prophylactic intrathecal chemotherapy was not administered **Patients received 4 cycles prior to consolidation**
MBTP[144] (HOVON 105/ALLG NHL 24) *N* = 100	**Every 4 weeks** in patients with age between 18 and 70 years (median 61 years) **HD-MTX** 3 g/m^2 IV on days 1 and 15 with leucovorin rescue × 2 cycles **Carmustine** 100 mg/m^2 IV on day 4 **Teniposide** 100 mg/m^2/day IV days 2 and 3 **Prednisone** 60 mg/m^2/day, orally days 1 through 5 Prophylactic intrathecal chemotherapy was not administered Therapeutic IT chemotherapy in patients with positive CSF cytology after the 1st cycle **Patients received 2 cycles prior to consolidation**

CR, Complete remission; *CSF*, cerebrospinal fluid; *HD-MTX*, high-dose methotrexate; *IT MTX*, intrathecal methotrexate; *IT*, intrathecal; *IV*, intravenous; *N*, patients.

Prevention and Management of High-Dose Methotrexate Renal Toxicity

The most-feared toxicity of HD-MTX is nephropathy, caused by the precipitation of methotrexate and its metabolite 7-OH methotrexate within the renal tubules (see Chapter 58). Some protocols require a dose reduction for CrCl less than 100 mL/min, while most withhold HD-MTX for CrCl less than 50 mL/min. Delayed renal elimination can result in elevated plasma methotrexate levels for prolonged durations, with a resultant increase in systemic toxicity. Supportive measures to prevent this complication include hydration and urine alkalization with intravenous sodium bicarbonate (100 to 150 mEq/L) at 150 mL/h to maintain urine output of $\geq$100 mL/h and urine pH greater than 7.0, leucovorin rescue, and avoidance of medications that interact with methotrexate such as salicylates, nonsteroidal anti-inflammatory drugs (NSAIDs), beta-lactam antibiotics,

TREATMENT OF NEWLY DIAGNOSED ADULT PATIENT WITH PRIMARY CENTRAL NERVOUS SYSTEM LYMPHOMA

The patient is a 60-year-old male with a history of hypertension, diabetes, and gout. He initially developed malaise, nausea, and a feeling of lightheadedness. This was quickly followed by the subacute development of right-sided weakness. Initial workup included central nervous system imaging, which revealed a homogenously enhancing lesion involving the left thalamus and the midbrain (Fig. 84.2A–C). A biopsy was obtained, which demonstrated a diffuse large B-cell lymphoma. Molecular characterization was consistent with a nongerminal center B subtype. C-MYC expression was predominantly negative by immunohistochemistry. BCL-2 was positive in 40% on the large cells and PAX-5 was positive in all of the large cells. Ophthalmology exam was unremarkable.

The patient-initiated treatment was with curative intent. His induction regimen consisted of 2-weeks of inpatient cycles of high-dose methotrexate (HD-MTX) (8 g/m^2, day 1), temozolomide (150 mg/m^2, days 7–11, odd cycles only), and rituximab (375 mg/m^2, day 3, cycles 1–6 only) (methotrexate temozolomide and rituximab [MTR] regimen[23]). Intravenous bicarbonate was used to alkalinize the urine to facilitate excretion of methotrexate and decrease the risk of crystallization in the renal tubules, which could, in turn, impair renal function. Leucovorin was administered after methotrexate to diminish the systemic toxicity of the agent. The patient's allopurinol for his history of gout was held, as it can impair methotrexate renal clearance. Shortly after initiation of treatment, he returned to his mental (neurological) baseline. The patient received 10 doses of HD-MTX during his induction. Midway through this, an image was obtained through the magnetic resonance imaging (MRI) technique, which demonstrated a complete response by IPCNSLCG criteria.[141] This response was maintained on the postinduction MRI.

After the induction phase of the regimen he started consolidation in the inpatient setting. This consisted of high-dose etoposide (40 mg/kg continuously over 96 hours, days 1–4) and high-dose cytarabine (2 g/m^2 every 12 hours for 8 doses, days 1–[23]). The patient's course was complicated by a neutropenic fever. He was managed with growth factor support, antibiotics, antivirals, and antifungals. He was able to overcome this complication. Postconsolidation MRI demonstrated sustained CR. The patient is now a year and a half out since the completion of treatment and has no evidence of recurrence with an excellent quality of life. He is continuing with clinical and radiographic follow-up every 3 months.

sulfonamides, aminoglycosides, allopurinol, and proton pump inhibitors. Other risk factors contributing to delayed clearance of methotrexate and renal failure include high body mass index (BMI) and the presence of third-space fluid collection.

Initiate leucovorin rescue (20 to 25 mg every 6 hours) 24 hours after methotrexate infusion and continue for at least 72 hours, or until the serum MTX level is less than 0.1 μmol/L. Leucovorin is increased to 40 mg every 4 hours if serum MTX levels are in the toxic range (e.g., 24-h level $\geq$10 μmol/L, 48-h level >1 μmol/L, or 72-h level > 0.1 μmol/L). There might be a role of high-dose leucovorin, defined in one study as $\geq$4000 mg/day, given intravenously to patients who had delayed MTX clearance with levels $\geq$10 times that of toxic levels at 48 or 72 hours ($\geq$10 μmol/L at 48 hours and/or $\geq$1 μmol/L at 72 hours).[145] Additional interventions to mitigate delayed methotrexate clearance include carboxypeptidase–G2 (CPDG2, glucarpidase), a recombinant enzyme that rapidly reduces toxic serum methotrexate levels via direct hydrolysis of methotrexate, extracorporeal removal of methotrexate by means of peritoneal dialysis, and high-flux hemodialysis.[146] Finally, patients should avoid oral intake of acidic liquids while clearing HD-MTX.

CONSOLIDATION

Most patients, even those who achieve CR following HD-MTX-based induction, eventually relapse. The rationale behind consolidation therapy is to treat all residual, nonvisible (CR/CRu), and visible (PR/SD) diseases to attain, or maintain, long-term remissions. Age, performance status, comorbidities, and response to induction drive selection of the most suitable consolidation strategy. However, it must be reemphasized that there remains a lack of level 1 evidence on the choice of consolidation. Consolidation options include nonmyeloablative[23] to myeloablative[24] (autologous transplant) chemotherapy and WBRT, with doses ranging from 23.4 Gy to 45 Gy +/− chemotherapy.[22,24,144]

Whole Brain Radiation Therapy Consolidation

The timing and dose of WBRT are still unclear, given the significant risk of late neurotoxicity even at a dose of 30 Gy.[147,148] G-PCNSL-SG-1, the only phase III study in PCNSL, attempted to address the role of WBRT.[149] Patients who achieved a CR were randomized to receive 45 Gy WBRT, or observation alone, while those who did not achieve a CR were randomized to receive 45 Gy WBRT, or cytarabine. The median OS was 32.4 months (95% CI 25.8 to 39.0) in patients receiving 45 Gy WBRT ($n = 154$), and 37.1 months (27.5 to 46.7) in those not receiving WBRT ($n = 164$). The hazard ratio 1.06 (95% CI 0.80 to 1.40; $P = .71$). The median PFS was 18.3 months (95% CI 11.6 to 25.0) in patients receiving WBRT and 11.9 months (7.3 to 16.5; $P = .14$) in those not receiving WBRT (HR 0.83, Cl 0.65 to1.06, $P = .14$).[149] Treatment-related neurotoxicity in patients with sustained CR was more common in patients receiving WBRT (22 of 45, 49% by clinical assessment; 35 of 49, 71% by neuroradiology) than in those who were not (9 of 34, 26%; 16 of 35, 46%).[150] The predetermined primary endpoint for noninferiority was not met; one-third of the randomized trial population had major protocol violations; and the study was underpowered.[148,149] The question remains as to whether the elimination or deferral of WBRT is superior to a reduced and hyperfractionated dose schedule of WBRT approaches that maintain enhanced PFS without late neurocognitive toxicity. Most recently, the NRG Oncology RTOG 0227 study by Glass et al.[151] utilized the *hyperfractionated* schedule (1.2 Gy twice daily) at a lower dose of 36 Gy than what was piloted in a subset of patients who received treatment in RTOG 9310.[152] They also used temozolomide during induction (with rituximab) and 10 cycles of maintenance therapy. For patients alive at 3 years, albeit a short follow-up, none experienced a protocol-defined drop in their Mini Mental Status Examination (MMSE) score, a crude objective assessment of cognition. Sustained improvement in the quality of life (QoL) was consistent with this observation. This, at least in part, can be attributed to successful tumor treatment.[151] Another study by Morris et al.[153] used R-MVP, followed by cytarabine, and reduced-dose WBRT (23.4 Gy) for patients in whom CR was achieved. Patients who completed the regimen had a median PFS of 7.7 years and a 5-year OS of 80% (95% CI, 66% to 94%). At a short follow-up of 48 months, neuropsychological testing scores were stable. Based on these results, the RTOG is conducting a randomized phase II trial of this approach (ClinicalTrials.gov Identifier: NCT01399372). Currently, some centers do use WBRT as the consolidation in younger (age <60 years) patients. In general, lower doses are utilized in those who achieve CR, compared to patients with responses less than CR following induction (Table 84.5).[24,149–153]

Nonmyeloablative Therapy Consolidation-CALGB 50202 (Alliance 50202)

The Alliance 50202 study by Rubenstein et al.[23] did not utilize WBRT in their protocol with the objective of decreasing the risk of neurocognitive toxicity. Instead, chemotherapy was intensified with the addition of etoposide and cytarabine for patients who achieved a CR after 6 cycles (2-week cycles) of HD-MTX, temozolomide, and rituximab (MTR). Among 43 patients, 29 (66%) achieved a CR—implying that 34% of patients had radiographically visible residual disease after 6 cycles—and only 27 patients (52%) completed the entire regimen, a barrier to widespread adoption of this particular

TABLE 84.5 Selected Studies With Focus on Whole-Brain Radiation Therapy and Chemotherapy as Consolidation

Study	*N*	Median age	Induction	CR rate(s)	Consolidation	Important comments
Bromberg et al.[144]	199	61	MBVP with ($n = 99$ arm A) and without rituximab ($n = 100$ arm B [total six doses]) × 2 cycles in age up to 70 years	68% and 66%, respectively	Cytarabine × 2 cycles in patient with at least PR. Hyperfractionated WBRT (30 + 10 Gy boost to tumor site) in patients with at least PR and ≤age 60 years	HOVON 105/ALLG NHL 24 randomized phase III study • 1 years EFS (primary endpoint), where an event was no CR/CRu on protocol, relapse, or death. EFS did not differ between the arms: 1-year EFS was 49% without and 52% with rituximab • No clear benefit of addition of 6 doses of rituximab to MBTP; but longer follow-up is required. • Subgroup analysis showed that rituximab may benefit patients under 60 years of age
Glass et al.[151]	53	57	HD-MTX × 5 doses plus rituximab × 1 dose plus temozolomide daily for 5 days × 2 cycles in patient up to age 73	51%	Hyperfractionated WBRT (36 Gy) followed by temozolomide × 10 cycles	RTOG 0227 phase II study • Median follow-up for eligible patients was 3.6 years, and 2-year PFS and OS were 63.6% and 80.8%, respectively. • Cognitive function and quality of life improved, or stabilized, after hyperfractionated 36 Gy WBRT • 85% of the patients completed the entire regimen, compared to 52% in the Alliance 502020[23] study
Morris et al.[153]	52	60	R-MPV × 5–7 cycles up to age 79 years	79%	Low-dose WBRT (23.4 Gy; $n = 31$) followed by cytarabine × 2 in patients with CR	Multicenter phase II study • The 2-year PFS (primary end point) was 77% (95% CI, 63% to 92%) • Increased number of induction cycles yields higher CR rates. Also review Roth et al.[154] • Minimal neurotoxicity, albeit in a small number of survivors; no deaths from neurotoxicity have been reported • Median PFS was not reached in young patients (<60 years) and was 4.4 years in patients ≥60 years ($P = .02$) • No conclusions can be drawn about the effects of each drug when combined with HD-MTX (i.e., alkylating agent and rituximab)
Thiel et al.[149,154]	551	63	HD-MTX (4 mg/m^2) × 6 followed by HD-MTX × 6 ± ifosfamide	35%	WBRT (45 Gy; $n = 154$). Remaining patients: Cytarabine without WBRT in patients <CR	G-PCNSL-SG-1 phase III, randomized, non-inferiority trial • No significant difference in the OS (primary endpoint) was recorded when WBRT was omitted from chemotherapy • A post-hoc analysis of older adults ($n = 337$) treated with HD-MTX–based chemotherapy in the phase III trial (G-PCNSL-SG-1) reported that the median PFS and OS times after the achievement of a CR to be 16.1 and 26.7 months, respectively • Also refer to Korfel et al.[155]
Ferreri et al.[156]	79	58 and 59	HD-MTX (3.5 mg/m^2; $n = 40$) × 4 cycles vs. HD-MTX plus cytarabine ($n = 39$; 2 g/m^2 twice a day for 2 days) × 4 cycles in age up to age 75 years	18% vs. 46%, without and with cytarabine, respectively	WBRT in CR. Dose depended on age, response, and participating center (36 to 40 Gy ± boost)	Randomized phase II study • Superiority of polychemotherapy induction demonstrated in age ≤75 years • Overall response rate of 40% (CI 25–55) and 69% (CI 55–83), in HD-MTX without and with cytarabine respectively ($P = .009$) • 3-year PFS of 21% and 38%, respectively • 3-year OS of 32% and 46%, respectively

Continued

TABLE 84.5 **Selected Studies With Focus on Whole-Brain Radiation Therapy and Chemotherapy as Consolidation—cont'd**

Study	*N*	Median age	Induction	CR rate(s)	Consolidation	Important comments
Ferreri et al.[157]	41	57	MTX, cytarabine, idarubicin, thiotepa × 3 cycles up to age 70 years	76%	WBRT (30,36 and 45 Gy in CR, PR, and SD, respectively with 6 Gy boost)	Phase II study • After a median follow-up of 49 months, a plateau in the survival curve (5-year OS: 41% ± 7%) was obtained.
Fisher et al.[152]	82	NA	MPV × 5 cycles	39% after chemo	WBRT 45 Gy vs. hyperfractionated WBRT of 36 Gy if in CR. WBRT 45 Gy if not in CR after induction chemo	Secondary analysis of RTOG 93-10 study. • Although the fWBRT schedule represented a 25% reduction in biologically effective tumor dose, in comparison, PFS and OS were not significantly affected. • Neurotoxicity occurred later in the fWBRT group than in the conventional WBRT patients
Poortmans et al.[158]	52	51	MBVP × 2 cycles	69%	WBRT (30 Gy with 10 Gy boost to tumor bed) plus IT chemotherapy	• Median PFS - NR • Median OS—46 months • Neurotoxicity 12%
Bessell et al.[159]	26	59	Cyclophosphamide, doxorubicin, vincristine, and dexamethasone × 1 cycle followed by carmustine, vincristine, methotrexate, and cytarabine × 2 cycles (CHOD/BVAM)	77%	WBRT (30.6 Gy)	Reduction of the RT dose from 45 Gy to 30.6 Gy in patients younger than 60 years old with PCNSL who achieved CR resulted in an increased risk of relapse and lower OS
DeAngelis et al.[160]	98	56.5	MVP ×5 cycles and IT MTX	58%	WBRT (45 Gy) followed by cytarabine	RTOG 93-10 • First multicenter trial demonstrating improved survival with the combination of chemotherapy and WBRT, compared with previous reports of WBRT alone, but neurotoxicity (15%) was a delayed risk of this approach • Deaths ($n = 8$) from neurotoxicity • Median PFS was 24.0 months and OS was 37 months
Abrey et al.[161]	52	65	MPV × 5 cycles plus IT-MTX × 8 doses.	56%	WBRT (45 Gy; $n = 31$) plus cytarabine × 2 cycles ($n = 35$)	• Increasing the dose of MTX and adding procarbazine and vincristine improved disease control and overall survival • Median PFS 129 months • -Median OS 51 months • Neurotoxicity 30%

protocol, which may indicate a high rate of progression, toxicity, and/or intolerance. Estimated PFS at 1, 2, 3, and 4 years were 64% (95% CI, 48% to 76%), 57% (95% CI, 41% to 70%), 50% (95% CI, 34% to 64%), and 47% (95% CI). These results exceed those of other chemotherapy-alone studies[162,163] but may be inferior to the combined-modality approach with reduced-dose WBRT, albeit with less neurotoxicity.[153] The 2-year PFS for patients who completed consolidation was 0.77. Interestingly, age above 60 years ($n = 23$) did as well as younger patients ($n = 21$; $P = .48$), and the most significant clinical prognostic variable, as expected, was delay in treatments. The 3-year PFS was 0.59 (95% CI, 0.40 to 0.73) for those without a treatment delay and 0.2 (95% CI, 0.03 to 0.47) for those with a treatment delay.

Myeloablative Chemotherapy Versus Whole-Brain Radiation Therapy as Consolidation Therapy

High-dose myeloablative chemotherapy, followed by autologous hematopoietic stem cell transplantation (auto-HCT), appears to be superior relative to 45 Gy WBRT with favorable cognitive and QoL outcomes.[24,147,164] Phase II RCT PRECIS[164] compared these two approaches in 140 immunocompetent patients (age <60 years). The 2-year PFS was 87% (95% CI, 77% to 98%) versus 63% (95% CI, 49% to 81%) in auto-HCT (thiotepa, busulfan, and cyclophosphamide conditioning) and WBRT (40 Gy; 2 Gy/fraction), respectively. Cognitive impairment was observed after WBRT, whereas cognitive functions were preserved or improved after auto-HCT. Another phase II RCT-IELSG32[24] ($n = 227$ immunocompetent patients) also compared auto-HCT with carmustine and thiotepa conditioning with WBRT (age <70 years). However, with a caveat of examining three different induction regimens. The 2-year PFS rates were identical with either consolidation approach (76% ± 5%). Nonetheless, similar to the PRECIS study,[164] a significant impairment in attention and executive functioning was observed in patients who received WBRT (Table 84.6).[24]

Authors' Preference of Consolidation

At present, outside of a clinical trial, we prefer chemotherapy-based consolidation, including auto-HCT in appropriately selected

TABLE 84.6 Randomized Phase II Trials Comparing Autologous Transplant With Whole-Brain Radiation Therapy Consolidation

Study	*N*	Induction	CR rate(s)	Consolidation	Final results and comments
Houillier C et al. (2019)[164] Median age 55 years (22–60 years)	140	R-MBVP × 2 cycles ($n = 132$ intention to treat analysis; 66 patients in both consolidation arms) in age up to 60	43%	Rituximab and cytarabine × 2 cycles followed by WBRT (40 Gy; $n = 53$) or auto-HCT ($n = 44$; thiotepa, busulfan and cyclophosphamide) in all eligible patients regardless of response to induction	PRECIS • Noncomparative design of the study precludes firm conclusions • The CR rates were disappointing due to lower number and dose of HD-MTX cycles • Only 73% of the patients received consolidation after induction • The 2-year PFS rates (primary end-point) were 63% and 87% in the WBRT ($n = 38$) and auto-HCT ($n = 38$) arms, respectively • Auto-HCT mortality higher than expected due to multicenter setting and/or due to 3 drug conditioning • Neurocognitive impairment with WBRT was evident. The efficacy end points tended to favor the auto-HCT arm
Ferreri AJ et al.[24] (2017) Median age 58 years (18–70)	227	MATRix ($n = 75$) vs. MARix ($n = 69$) vs. MA ($n = 75$) × 4 cycles in age up to 70.	49%, 30%, and 23% with MATRix, MARix, and MA, respectively	WBRT ($n = 59$; 36 Gy ± 9) vs. auto-transplant ($n = 59$; thiotepa and carmustine conditioning) in patients with ≥stable disease	IELGS32 • Contribution of rituximab, and thiotepa with rituximab is evident on the backbone of methotrexate and cytarabine • MATRix was found to be superior, although with higher toxicity • At median follow-up of 30 months, patients treated with MATRix had a CR rate of 49% (95% CI 38–60), compared with 23% (14–31) of those treated with MA alone (hazard ratio 0.46, 95% CI 0.28–0.74) and 30% (CI 21–42) of those treated with MATRix (0.61, 0.40–0.94). • Randomization was stratified by IELSG risk score (low vs intermediate vs high). • The 2-year PFS rates were identical after WBRT and auto-HCT (76% ± 5%).

BCNU, carmustine; cyclophosphamide, cyclophosphamide; dox, doxorubicin; *EFS*, event-free survival; etop, etoposide; *Ifos*, ifosfamide; *IT*, intrathecal; methylpred, methylprednisolone; *MTX*, methotrexate; *NA*, not available; *OS*, overall survival; *pcb*, procarbazine; *PFS*, progression-free survival; *ritux*, rituximab; *tmz*, temozolomide; *TTP*, time to progression; *vinc*, vincristine; *WBRT*, whole-brain radiotherapy.

individuals.[24,164–167] An ongoing phase II RCT trial is comparing non-myeloablative to myeloablative (carmustine/thiotepa) consolidation in newly diagnosed patients (age 18 to 75 years) (ClinicalTrials.gov Identifier: NCT01511562; CALGB-51101) with a primary end-point of PFS.

PRIMARY CENTRAL NERVOUS SYSTEM LYMPHOMA IN OLDER ADULTS

Approximately 50% of patients with PCNSL are older than 65 years of age.[27,28,35] In general, older adults exhibit inferior outcomes relative to young patients, with the rare exception in the CALGB 50202 study ($n = 44$), in which outcomes were similar between the two groups.[24] The large US-based population database reported that survival of elderly patients has not improved, relative to younger patients.[28] Of prime relevance, the therapy for older adults should be similar to their younger counterparts, as described in previous sections, unless prohibited by comorbid conditions, poor performance status, or short life expectancy. Expectations, however, may need to be adjusted. Chronologic age by itself should not deter the practitioner from a curative approach, and many elderly patients are probably undertreated just because of their advanced age. Comprehensive comorbidity, social, and geriatric assessments are imperative prior to and during therapy[168]. The input from the multidisciplinary support team in clinical management is known to improve overall patient outcomes.[168,169]

INDUCTION IN OLDER ADULTS—SHOULD IT BE REDUCED?

Numerous studies have shown that older adults can tolerate HD-MTX regimens.[25,154,163,166–168,170–175] Surprisingly, even patients older than 80 years of age can tolerate and benefit from such an approach when adequate supportive measures are used and renal function is properly monitored.[175] HD-MTX-based regimens are not only effective and safe but also improve QoL in a significant proportion of older adults.[175,176] A meta-analysis by Kasenda et al.[177] of 783 older adults (defined as age ≥60 years) showed that 73% received HD-MTX with a median dose of 3 g/m^2. The KPS ≥70% was the strongest prognostic factor for mortality (HR 0.50, 95% Cl −0.41 to 0.62). After a median follow-up of 40 months, HD-MTX-based therapy was associated with improved OS (HR 0.70, 95% CI 0.53 to 0.93). There was no difference between HD-MTX plus oral procarbazine or temozolomide versus more aggressive HD-MTX-based intravenous therapies including cytarabine (HR 1.39, 95% CI 0.90 to 2.15).

The phase II ANOCEF-GOELAMS randomized trial[170] treating elderly patients aged above 60 years compared HD-MTX plus temozolomide (MT; $n = 48$) with HD-MTX in combination with prednisolone, vincristine, and cytarabine for consolidation (MPVA; $n = 47$). The 1-year PFS was similar at 36% (95% CI 22 to 50) in both groups. However, the median PFS was 6.1 months (95% CI 3.8 to 11.9) in the MT group and 9.5 months (95% CI 5.3 to 13.8) in the MPVA group. Quality-of-life evaluation showed improvements in most domains ($P = .01$ to .0001) compared with baseline in both groups. In the IELSG32 study[24,178] (Table 84.6), included patients up to the age of 70 years and showed a 2-year PFS of 61% (95% CI: 55% to 67%). Unremarkably, infectious complications were more frequent in patients older than 60 years.

From a practical standpoint, compromised performance status alone should not preclude HD-MTX induction as it often improves following initial treatment, allowing for subsequent intensification.[168] A less intensive initial treatment (e.g., rituximab plus HD-MTX (3 to 4 g/m^2) could mitigate toxicity in early cycles and improve the performance score, to achieve a high cumulative relative dose intensity in subsequent cycles. In patients, whose comorbidities, especially severely compromised renal and/or cardiac function, preclude HD-MTX, alternative non-WBRT-based therapy must be explored. The options include temozolomide,[179] pemetrexed,[180.] rituximab,[181] or steroids.[182] Subsequent strategies after induction (consolidation vs. maintenance vs. observation) are individualized based on the response and performance status.[166,167,183]

AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION IN OLDER ADULTS AS CONSOLIDATION THERAPY

Myeloablative approach may be considered in carefully selected "physiologically fit" adults with PCNSL.[166,167] A recent, small, single-arm pilot MARiTA study by Schorb et al.[167] ($n = 14$) demonstrated that high-dose chemotherapy (busulfan/thiotepa) followed by auto-HCT is safe and highly effective in older adults as well. MARiTA reported 2-year PFS and OS rates of 92.9% (95% CI: 80% to 100%) and 92.3% (95% CI: 78% to 100%), without TRM, in highly selected older adults. As expected, grade >3 hematological toxicity was more frequent, but infectious complications were similar to those reported in the PRIMAIN study (discussed in section below).[172] These results have led to ongoing phase II of MARTA trial that will investigate age-adjusted high-dose chemotherapy (rituximab/busulfan/thiotepa), followed by autologous hematopoietic cell rescue in the elderly "fit" patient population (DRKS-ID identifier: DRKS00011932).[178] However, to determine the precise role of auto-HCT in the elderly population, phase III trials comparing an age-adapted thiotepa-containing auto-HCT approach with non-intensive approaches (e.g., R-MP protocol) could provide clarification.[168] Adequate efficient accrual would be a primary consideration for conducting such a study.

MAINTENANCE THERAPY—EMERGING CONCEPT

There has been growing interest in maintenance therapy to improve outcomes.[29,184] This strategy mainly focuses on older adults who are unsuitable for chemotherapy-based consolidation,[172,174,185] but has been tested in younger adults following consolidation.[151] However, at present, the optimal agent, or regimen, remains uncertain. All maintenance agents or regimens are highly empirical in terms of optimal dose and schedule. Trials in this particular area are underway, as displayed in Table 84.7.

TABLE 84.7 **Selected Frontline Maintenance Therapy Trials in Primary Central Nervous System Lymphoma**

ClinicalTrials.gov Identifier	Phase	Maintenance Program	Eligibility Age
NCT02623010	II	Ibrutinib	60 to 85
NCT02313389	III	Rituximab-methotrexate -temozolomide versus Observation	≥ 60
NCT03495960	II	Lenalidomide versus Procarbazine (FIORELLA)	≥70
NCT02498951	II	Obinutuzumab versus Observation	≥18

Older Frail (or "Unfit") Adults Who Are Not Candidates for Consolidation

Early studies deferred WBRT consolidation and introduced the concept of maintenance by continuation of HD-MTX at protracted intervals.[186–188] A retrospective analysis by Ambady et al.[189] showed that maintenance therapy with rituximab increased the duration of survival compared to the surveillance group (HR 0.27, 95% CI 0.08 to 0.98, $P = .046$). However, the risk of progression remained similar across both groups (HR: 0.61, 95% CI 0.26 to 1.43, $P = .259$). To test this concept further, a phase II trial is underway evaluating the efficacy of obinutuzumab, a humanized anti-CD20 monoclonal antibody, as maintenance therapy in patients unsuitable for consolidation with either auto-HCT or WBRT (ClinicalTrials.gov Identifier: NCT02498951).

In the multicenter single arm, PRIMAIN study[172] patients ≥65 years (range: 66 to 85) received 3 cycles, repeated every 42 days of rituximab (375 mg/m^2, days 1, 15, 29), HD-MTX (3 g/m^2 days 2, 16, 30), and procarbazine (60 mg/m^2 days 2 to 11; R-MP,) followed by monthly 6 cycles of maintenance procarbazine (100 mg for 5 days) given to responders. In all, 38 of 107 patients achieved CR (35.5%) and 15 (14.0%) achieved PR. The CR rate was slightly lower in patients treated with R-MP, compared with R-MP plus lomustine (31.6% vs. 37.7%); lomustine was omitted from the study due to significant toxicity. Of importance, about 40% of patients were unable to finish all 3 cycles of HD-MTX. The 1- and 2-year PFS rates were 46.3% (95% CI: 36% to 55%) and 37.3% (95% CI: 28% to 46%), and OS rates after 1- and 2- years of 56.7% (95% CI: 47% to 66%) and 47% (95% CI: 37% to 56%), respectively. Omitting lomustine was associated with similar PFS and OS and less toxicity. The study was not designed to compare R-MP ($n = 38$) with R-MPL ($n = 69$). Also, fewer patients were treated with R-MP and their follow-up was shorter; therefore, any inference regarding comparative effectiveness should be considered exploratory.[172]

A comparable multicenter single-arm phase II EORTC 26925 study[163] enrolled 50 patients aged above 60 years (median age: 72 years). Treatment consisted of HD-MTX (1 g/m^2), procarbazine, lomustine, steroids, intrathecal therapy, and cytarabine for one induction cycle, followed by 5 maintenance cycles every 6 weeks. Regarding the best response, 48% achieved a CR and 1-year PFS was 40% (95% Cl, 26% to 53%). Most patients improved or preserved their cognitive functions. In the NORDIC trial[174] the 2-year PFS was 55.6% for patients ≥66 years ($n = 27$ age 66 to 75 years) who received HD-MTX (MTR, followed by temozolomide maintenance). Furthermore, only 28% of elderly patients receiving maintenance therapy had relapse after achieving CR compared to 63% in the younger cohort ($n = 39$; age 18 to 65 years) who did not receive maintenance treatment. Median duration of response was ten months in the younger subgroup, and not reached in the elderly patient subgroup ($P = .33$). The study highlights that maintenance therapy might be able to achieve sustained remission and allow deescalation of induction, thereby reducing cumulative toxicity in older adults. Other trials have examined, or are examining the prospect of ongoing treatment with an agent, not included in the induction regimen (Table 84.7).[185] Rituximab ± lenalidomide have been used in such a manner as well, but the data are mostly anecdotal and used in the setting of relapsed/refractory disease.[190–192] A retrospective series suggested the contribution of upfront maintenance lenalidomide in prolongation of PFS in 13 patients older than age 70.[185] Overall, these findings only suggest that the maintenance therapy might be a promising alternative in patients unsuitable for consolidation.

Maintenance Therapy After Consolidation

A phase II RTOG 0227 study by Glass et al.[151] ($n = 53$; median age 57 years) evaluated temozolomide maintenance after methotrexate, temozolomide, and rituximab and hyperfractionated WBRT (36 Gy). The 2-year PFS and OS were 63.6% and 80.8%, respectively.

TREATMENT OF PRIMARY VITREORETINAL LYMPHOMA - A SUBSET OF PRIMARY CENTRAL NERVOUS SYSTEM LYMPHOMA

The optimal therapy for PVRL also remains unsettled. The therapy requires the combined effort of an oncologist, an ophthalmologist, and a pathologist and includes localized and/or systemic therapy Table 84.8).[94] Suboptimal concentration of chemotherapy in the eye with high local relapse rates provides the rationale for ocular directed strategies.[193] Localized therapy includes radiation and/or intraocular methotrexate and rituximab.[194–202] However, some patients have resolution of intraocular lymphoma with systemic therapy alone.[162] There is an absence of randomized trials comparing the efficacy of localized radiation, intraocular therapy, and systemic therapy.

TABLE 84.8 **Consensus Therapy Recommendations[a] for Primary Vitreoretinal Lymphoma From the 2011 Report of International Primary Central Nervous System Lymphoma Collaborative Group[94]**

Without Central Nervous System or Systemic Involvement	With Central Nervous System Involvement
(a) If only one eye is involved, use local therapy. Whether it is local intravitreal methotrexate and rituximab given alone, or external beam radiation therapy (b) If both eyes are involved, there is still a preference toward local therapy, although systemic treatment should not be excluded. The addition of intravitreal chemotherapeutic agents to systemic therapy should be considered	(a) High-dose methotrexate-based therapy (possibly with systemic rituximab) is recommended in conjunction with local therapy, given limited penetration of systemic agents into the vitreous cavity (b) Whole brain radiation therapy, in conjunction with ocular radiotherapy, should be considered in those who have failed systemic therapy and are debilitated or do not meet the criteria for more aggressive therapy such as autologous transplant

[a] Low level of evidence.

Intraocular Lymphoma Without Brain Parenchyma Involvement

In a multicenter retrospective collaborative European series by Riemens et al.,[203] which included patients ($n = 78$) with isolated PVRL, the use of systemic chemotherapy did not prevent CNS lymphoma and was associated with more severe adverse effects compared with local treatment. CNS lymphoma developed in 32% of patients who received localized ocular therapy with radiation and/or ocular chemotherapy, 43% of patients who received systemic chemotherapy, and 39% of patients who received both systemic and localized therapies. Intravitreal rituximab has been used alone or in combination with intravitreal methotrexate.[197–202] In patients with PVRL, where intravitreal rituximab was used as a single agent after discontinuation of intravitreal methotrexate due to corneal epitheliopathy, all patients responded after 1 dose of intravitreal rituximab.[199] Although 55% of patients experienced disease recurrence, the lesions resolved with a course of intravitreal rituximab plus methotrexate.[199] Mayo clinic reported their experience (1990 to 2018) of 33 patients with PVRL.[202] Interestingly, combined systemic and intraocular therapy led to higher failure-free-survival ($P = .002$) and CNS relapse-free survival ($P = .003$) with no difference in OS.[202]

Intraocular Lymphoma With Brain Parenchyma Involvement

A large retrospective study by Grimm et al.[112] reported the outcomes of HIV-negative PCNSL patients with intraocular involvement. All patients ($n = 176$) received systemic therapy for PCNSL, and 102 patients also received localized therapy (ocular radiotherapy in 79 patients and intravitreal methotrexate in 22 patients). Median PFS was prolonged in patients who received ocular-directed therapy (19 months) compared to those who did not receive it (15 months; $P = .01$). However, the initial treatment approach did not impact the OS. Multivariate analysis identified receipt of dedicated local ocular therapy as the only factor predictive of PFS ($P = .01$, HR = 1.69, 95% CI 1.12 to 2.54). Localized therapy did not lower the risk of intraocular relapse compared to patients who received systemic therapy alone ($P = .7$).

Concerns and Adverse Events With Local Therapy for Primary Vitreoretinal Lymphoma

The major concern when managing PVRL with local treatment alone is the risk of CNS dissemination, which is a devastating event that occurs in half of the patients, resulting in a 4-year OS of 32%.[112] Conversely, patients with PVRL, receiving systemic chemotherapy, had a CNS relapse at the rate of 43%, but the reported 4-year OS was superior at 85%.[194] Adverse events of ocular radiation therapy include xerophthalmia and chemosis. Radiation retinopathy can be seen with higher doses of radiation.[194] Complications of intravitreal methotrexate reported include cataract, corneal epitheliopathy, maculopathy, vitreous hemorrhage, optic atrophy, and sterile endophthalmitis. Only mild and reversible ocular side effects have been reported with intravitreal rituximab.[197–201]

REAL-WORLD TREATMENT PATTERNS AND OUTCOMES—DATA FROM POPULATION-BASED STUDIES

United States

Fallah et al.[204] analyzed 9165 HIV-negative PCNSL cases reported to the US National Cancer Database from the period of 2004 to 2013. During this time, the proportion of patients receiving chemotherapy significantly increased from 65.6% to 78.8% (P for trend <.0001), whereas the proportion receiving WBRT decreased from 37.6% to 18.8% ($P < .0001$). The OS at 3 years was 37.7% (95% CI, 36.6 to 38.8) and ranged from 14.1% for patients treated with radiation therapy alone, to 51.8% for those who received multiagent chemotherapy. There was evidence of improved survival over time (P for trend =.0002). Adjusting for the varying distribution of clinical and sociodemographic characteristics by type of treating facility, the risk of not receiving chemotherapy was significantly (31%) lower in academic/research cancer programs compared with community programs (adjusted relative risk, 0.69; 95% CI, 0.62 to 0.76; $P < .0001$). The disparities in application of chemotherapy for PCNSL underscore the need for education, especially in the community setting, where most older patients receive their care (Table 84.9).

France

Houillier et al.[27] analyzed the data of 1002 immunocompetent PCNSL patients (72% aged >60 years, median KPS 60) on behalf of French oculo-cerebral lymphoma network (LOC) from the period of 2011 to 2016. They found that younger (age <60 years) patients received consolidation treatment in 77% of cases, consisting of WBRT (54%) or auto-HCT (23%). Among older (age >60 years) patients, WBRT and auto-HCT consolidation were administered at only 9% and 2%, respectively. Despite involving a minority of patients (6% in the first line and 17% at relapse), the use of auto-HCT in first-line treatment or at relapse was observed with a promising 5-year OS of 76% in the first line and a 3-year OS of 57% after relapse; this is in line with previously published studies of auto-HCT.[165,166,205,206]

Netherlands

Van der Meulen et al.[207] reported a Dutch population-based cohort of 1673 patients with PCNSL diagnosed between 1989 and 2015. An increasing incidence of PCNSL in patients aged over 60 was noted. The OS improved over time, but only in the patients less than 70 years, probably because approximately 40% of the elderly patients did not receive any antineoplastic therapy, even in the more recent period from 2009 to 2015. The 5-year OS was 56% in patients less than 60 years of age from 2009 to 2015.

United Kingdom

Martinez-Calle et al.[208] analyzed 244 PCNSL cases of older (age ≥65 years) adults from 14 UK centers between the period of 2012 and 2017. Patients received palliative care ($n = 52$), methotrexate ± rituximab ± alkylators ($n = 74$) and methotrexate and cytarabine combinations ($n = 118$). The median methotrexate cumulative dose was 8.8 g/m^2 (range 1.5 to 21) over a median of three cycles. Higher relative dose intensity of methotrexate was associated with an improved PFS and OS after adjusting for age, performance status, and baseline parameters. The 2-year PFS and OS for patients receiving four or more induction cycles followed by consolidation ($n = 36$) were 65% and 70%, respectively (see Table 84.9).

TABLE 84.9 Real World Population-Based Studies and Outcomes in Primary Central Nervous System Lymphoma

Country	Database	Year	*N*	Survival
US[204]	National cancer database	2004–2013	9165 (HIV-negative)	OS at 3 years was 37.7%, and ranged from 14.1% for patients treated with radiation therapy alone, to 51.8% for those who received multiagent chemotherapy
France[27]	LOC network	2011–2016	1002 (HIV-negative)	The median, 2-year, and 5-year OS was 25.3 months, 51%, and 38%, respectively (<60 years: not reached, 70%, and 61%; > 60 years: 15.4 months, 44%, and 28%)
Netherlands[207]	Netherlands Cancer Registry	1989–2015	1673 (HIV-negative)	The overall 5-year age-standardized relative survival (95% confidence interval) among adult patients with PCNSL was 30% (27%–34%) in the period 2009–2015
UK[208]	UK centers × 14	2012–2017	244 (age ≥ 65 years and HIV-negative)	2-year PFS and OS for patients receiving ≥ 4 induction cycles followed by consolidation ($n = 36$), were 65% and 70%, respectively
US[209]	SEER	1992–2011	4158 (36% HIV-infected)	Among HIV-uninfected cases, the 5-year OS increased from 19.1% (1992–1994) to 30.1% (2004–2006).5-year OS among HIV-infected cases was 9.0%

HIV, Human immunodeficiency virus, *LOC*, French oculo-cerebral lymphoma network; *OS*, overall survival; *PFS*, progression free survival; *SEER*, 10 Surveillance, Epidemiology and End Results cancer registries.

PRIMARY CENTRAL NERVOUS SYSTEM LYMPHOMA IN THE IMMUNOCOMPROMISED HOST

Human Immunodeficiency Virus: An Acquired Immunodeficiency Syndrome–Defining Illness

PCNSL has been designated an AIDS-defining disease since 1983 and accounts for 15% of NHLs in HIV-infected individuals.[4–6]

TABLE 84.10 **Primary Central Nervous System Lymphoma in Human Immunodeficiency Virus–Infected Host Versus in Immunocompetent Host**

- Worse performance status at diagnosis
- Higher LDH levels at diagnosis
- Almost universally associated with Epstein-Barr virus infection
- Radiologic features are usually strikingly different: strong rim enhancement, bleeding within the lesion, and more likely to have multiple lesions
- Unfortunately, more likely to be treated with supportive care or whole-brain radiation therapy and excluded from most clinical trials
- Higher risk of opportunistic infections

It typically affects patients with CD4 counts less than 50 cells per microliter and is rarely seen in patients receiving ART. It is important to recall that even limited interruptions of antiretroviral therapy can lead to CD4 cell nadirs and HIV viremia, along with an increased risk of lymphoma. In the late course of the infection, many patients succumb to opportunistic infections. The treatment principles have evolved as in the immunocompetent host and mirror, as described previously, for the immunocompetent host, in addition to immediate optimization of ART therapy.[4–6,210–215] The prognosis of these patients is usually inferior, compared to the immunocompetent host, due to the presence of greater adverse features as displayed in Table 84.10. Registry data on behalf of Uldrick et al.[213] reported that survival had improved over time among cases diagnosed between 1990 and 2000, but remained poor, particularly among African-Americans. Another analysis by Olszewski et al.[216] reviewed the National Cancer Center database for patients with HIV-related PCNSL, diagnosed between 2004 and 2012. The median OS was only 3.6 months. In a multicenter study evaluating outcomes in HIV positive-patients treated at academic medical centers, the outcomes were similar to those in the HIV-negative population. Survival gains in patients with PCNSL parallel an increase in chemotherapy use. However, it was less likely to be received by patients who were older, Black, or without private insurance.[216] Like immunocompetent patients, these patients should also be given the opportunity to access the best available therapy and should be included in clinical trials.

POST-TRANSPLANT LYMPHOPROLIFERATIVE DISEASE–RELATED PRIMARY CENTRAL NERVOUS SYSTEM LYMPHOMA

In the setting of PTLD of the CNS, the primary approach, provided EBV is driving the disease, is to reconstitute the immune function, which may be achieved in a few cases with the reduction, or cessation, of immunosuppressive therapy. If this does not work, then the treatment is individualized on a case-by-case basis. Rituximab, (systemic or intrathecal [IT]) alone, which targets the EBV-infected CD20+ cells, is a reasonable option.[217,218] Chemotherapy should be reserved for patients who (i) fail rituximab, (ii) have EBV-negative lymphomas, (iii) need a rapid response, or (iv) have high-grade monomorphic PTLD. In a retrospective study of 22 patients treated with rituximab, the ORR was 68%.[218] The median time to treatment failure was not reached at 19 months and the estimated OS was 31 months. None of the patients died of rituximab toxicity, and all patients who progressed underwent further treatment with chemotherapy. A total of 23 patients who received chemotherapy had an ORR of 74%. The median time to treatment failure was 10.5 months and the estimated OS was 42 months. However, 26% of the patients receiving chemotherapy died of toxicity. It is unclear if this high degree of treatment-related toxicity reflects real-world clinical practice (see Chapter 87).

AUTOIMMUNE DISEASE–RELATED PRIMARY CENTRAL NERVOUS SYSTEM LYMPHOMA

Immunosuppressive drugs given to treat autoimmune conditions *may* increase PCNSL risk, but these associations cannot explain the observed temporal increase in PCNSL rates, given the low prevalence of these conditions.[219] A recent systematic review (39 records) and meta-analysis ($n = 50$) by Kaulen et al.[220] revealed DLBCL subtype (80%) and EBV positivity (75%) in most autoimmune (AD)-PCNSL. Data, with its inherent limitations, suggests that aggressive polychemotherapy can accomplish long-term OS in AD-PCNSL, comparable to immunocompetent patients. Age above 60 years ($P = .014$) was identified as a significant prognostic factor.[220]

EXPERIMENTAL THERAPEUTICS IN PRIMARY CENTRAL NERVOUS SYSTEM LYMPHOMA

Osmotic Blood-Brain Barrier Disruption With Cerebral Intra-Arterial Chemotherapy

Osmotic disruption of the BBB is an alternative approach to achieving higher concentrations of chemotherapy in the CNS.[221–226] Angelov et al.[224] reported a multiinstitutional experience of treating 149 patients with newly diagnosed PCNSL with mannitol BBB disruption and cerebral intraarterial methotrexate-based chemotherapy. The CR rate was 58%, with a median OS of 3.1 years and a median PFS of 1.8 years. Partial seizures were reported in about 9% of patients. Most recently, Iorio-Morin et al.[225] reported retrospective analysis using a similar type of approach in 44 patients. The median follow-up was 38 months. CR was achieved in 34 patients (79%) at a median of 7.3 months. Actuarial median survival and PFS were 45 months and 24 months, respectively. Age, performance status, and tumor location did not impact the outcome. Complications included thrombocytopenia (39%), neutropenia (20%), anemia (5%), seizures (11%), stroke (2%), and others (20%; anaphylaxis to carboplatin, sepsis, pulmonary embolism, and pneumonia). This procedurally intensive approach requires institutional expertise and has not demonstrated improved outcomes compared to systemic therapies.

NGR-Human Tumor Necrosis Factor With R-CHOP

NGR-human tumor necrosis factor (NGR-hTNF) targets CD13+ (tumor) vessels, enhances vascular permeability and CNS access of anticancer drugs, and provides the rationale for the treatment of PCNSL with R-CHOP. A phase II study by Ferreri et al.[30] reported activity and safety of R-CHOP preceded by NGR-hTNF in patients with PCNSL relapsed/refractory to HD-MTX-based chemotherapy enrolled in a phase II trial. A sample size of 28 patients was considered necessary to demonstrate improvement from 30% to 50% ORR (primary endpoint). NGR-hTNF/R-CHOP would be declared active if ≥12 responses were recorded. Treatment was well tolerated; there were no cases of unexpected toxicities, dose reductions, or interruptions. NGR-hTNF/R-CHOP was active, with confirmed tumor response in 21 patients (75%; 95% Cl, 59% to 91%), which was completed in 11 patients. Seventeen of the 21 patients with a response to treatment received consolidation (auto-HCT, WBRT, and/or lenalidomide maintenance). At a median follow-up of 21 (range, 14 to 31) months, five patients remained relapse-free and six were alive.[227] Further research on this innovative approach in CNS lymphomas is warranted.

FRONT-LINE TREATMENT CHALLENGES AND CONTROVERSIES

Therapeutic Role of Surgical Resection Followed by Induction Therapy?

In most studies, no survival benefit from subtotal or gross total resection has been observed prior to induction therapy.[227–229] However, this traditional view has been rechallenged.[108,230,231] A retrospective subset analysis of the phase III German PCNSL Study Group-1[108] trial showed improved clinical outcomes in patients undergoing subtotal ($n = 70$) or gross total resection ($n = 67$) compared with patients in the biopsy cohort ($n = 379$; 48 open and 331 stereotactic). When controlled for the number of lesions, the HR of biopsy versus subtotal, or gross total, resection remained unchanged for PFS (HR = 1.37; $P = .009$) but was smaller for OS (HR = 1.27; $P = .085$). The proponents of the biopsy approach believe that routine gross surgical resection unnecessarily delays effective systemic therapy and increases the risk of permanent neurologic deficit(s). Currently, there is insufficient evidence to routinely recommend subtotal, or gross total, surgical resection in PCNSL. However, in future, the debate will continue in favor of surgical resection, especially in patients with favorable presurgical parameters (e.g., superficial isolated lesion), when preoperative diagnosis is uncertain (PCNSL vs. glioma), or when there is substantial symptomatic mass effect.[108,230,231]

Definition of High-Dose Methotrexate, its Frequency, and Definition of Failure of High-Dose Methotrexate?

The definition of “HD-MTX” is unclear and typically ranges from 3 to 8 g/m^2. Doses of 1 g/m^2 have also been called “HD-MTX”. A direct comparison of different “HD-MTX” doses has not been performed. Also, the standardized frequency of HD-MTX (i.e., every 2 weeks vs. every 3 weeks) and which parameters precisely define the term “failure of HD-MTX” are not settled.

High-Dose Methotrexate-Based Monotherapy Versus Combination Therapy?

Retrospective and prospective phase II studies have investigated the efficacy of HD-MTX (8 mg/m^2) monotherapy and similarly demonstrated a shorter PFS when compared with HD-MTX-based combinations, especially with alkylating agent(s), vina alkaloids, and cytarabine.[22–24,144,153,156] A standardized, or perfect, recipe that goes along with HD-MTX has not been established.

Induction Cycles, Response Assessment, and Timing of Consolidation?

The optimal number of induction cycles is unclear. However, studies have shown that a longer duration of induction of HD-MTX (6 to 8 cycles) results in higher CR rates, which must be balanced with toxicity.[153,162] Interestingly, there is a lack of a standard number of induction cycles that define induction success, or failure, in addition to a lack of standardized eligibility criteria to begin consolidation. For example, phase II RCT-IELGS32 study[24] allowed consolidation therapy only in patients with ≥SD, whereas phase II RCT-PRECIS[157] allowed all patients to proceed to consolidation randomization regardless of their response to induction.

Intravenous Rituximab?

There is a controversy about whether rituximab improves outcomes in patients with PCNSL. Despite limited CSF penetration, radiographic responses have been observed with rituximab monotherapy in relapsed PCNSL patients.[181] In a newly diagnosed disease, a retrospective study from Johns Hopkins compared HD-MTX with and without rituximab.[232] The addition of rituximab led to a significant improvement in the response rate, PFS, and OS. Another retrospective study from China indicated the benefit of adding rituximab to conventional HD-MTX-based polychemotherapy.[233]

Subsequently, two randomized studies comparing rituximab plus chemotherapy to chemotherapy alone in PCNSL have been reported. First, the IELSG-32 study,[24] which compared three different induction regimens (HD-MTX and high-dose cytarabine with and without rituximab and rituximab, HD-MTX, high-dose cytarabine, and thiotepa). Responding patients were randomized to WBRT or high-dose chemotherapy followed by auto-HCT. In this phase II RCT trial, addition of rituximab to chemotherapy improved PFS [HR, 0.52 (95% CI, 0.32 to 0.86)] but not OS [HR, 0.63 (95% CI, 0.42 to 1.02)]. The comparison of rituximab versus non-rituximab arms is confounded by the presence of two different chemotherapy regimens and a second randomization after induction therapy.[24] Second, the HOVON105/ALLG NHL24 study[144] compared the MBVP regimen (HD-MTX, carmustine [BCNU] to teniposide [a drug not available in United States], and prednisone) with or without rituximab. Induction therapy was followed by consolidation with high-dose cytarabine (× 2 cycles) for patients who achieved CR or PR and, in patients aged 60 years or younger, WBRT. In this open-label randomized phase III trial, the addition of 6 doses of rituximab to induction chemotherapy showed no advantage of PFS [HR, 0.77 (95% CI, 0.52 to 1.13], or OS [HR, 0.93 (95% CI, 0.59 to 1.44]. It should be noted that both studies were not powered to measure the effect of rituximab on PFS and OS. Hence a meta-analysis was performed, which suggests a marginal benefit for PFS but not for OS. The same analysis also demonstrated lack of additional grade 3 and 4 toxicities or treatment-related mortality (TRM; RR 0.53; 95% CI, 0.20 to 1.37; low certainty) with the addition of rituximab to chemotherapy.[144] In clinical practice, rituximab is routinely administered with chemotherapy.

Prophylactic Intrathecal Chemotherapy?

There has been a notable trend away from the use of IT chemotherapy treatment in PCNSL. HD-MTX (≥3 g/m^2) yields cytotoxic levels in the CSF when infused rapidly over 2 to 3 hours, obviating the need for intrathecal chemotherapy, especially for the prevention of leptomeningeal disease.[234] The proponent of prophylactic IT therapy argues that the IT approach achieves longer drug exposures at cytotoxic concentrations in the CSF compared to HD-MTX. High-dose cytarabine can also yield significant drug concentrations in the CSF; however, levels are maintained for short duration and remain unpredictable. The lack of parenchymal brain penetration with IT therapies limits its efficacy. In a multicenter retrospective series of 370 patients with PCNSL,[235] the addition of IT chemotherapy to HD-MTX did not improve survival, even in patients with positive CSF cytology. Additionally, there was a higher incidence of neurotoxicity within IT chemotherapy. Similarly, a case-controlled retrospective study[236] reported no difference in survival between the two cohorts, that is, HD-MTX with or without IT methotrexate. In practice, there are limitations and concerns about infection in using IT chemotherapy in patients with PCNSL. Also, approximately one-third of patients present with increased intracranial pressure, limiting lumbar punctures or placement of Ommaya reservoirs.[237–239] In the absence of more definitive studies showing a survival benefit, the routine use of prophylactic IT chemotherapy remains controversial and is rarely used in PCNSL.

Therapeutic Intrathecal Chemotherapy in PCNSL?

This is also an area of controversy, except when leptomeningeal disease is the sole presentation of CNSL. In cases of CNS, parenchymal, and leptomeningeal diseases, precedence is given to systemic therapy over IT therapy in most (but not all) contemporary studies. With regard to the IT approach, methotrexate is the most widely used agent, given twice a week until clearance of CSF lymphoma cells (with multiple negative flow cytometry), followed by a minimum of two additional IT injections. Other IT chemotherapy agents include cytarabine ± steroids.[240] Triple therapy with methotrexate, cytarabine, and hydrocortisone is used.[241] There are no prospective studies guiding the choice of intrathecal agent(s) or duration of therapy.

High-Dose Methotrexate–Related Neurotoxicity

HD-MTX has been implicated in both preclinical and clinical studies and has been linked with neurotoxicity.[242] However, few studies have assessed pre- and post-treatment psychometric evaluation. Data regarding the long-term impact of HD-MTX neurotoxicity is sparse.[243,244] An international observational study by Doolittle et al., using standardized psychometric assessments with a long median follow-up of 5.5 years, demonstrated multidomain cognitive impairment in 19% of survivors and up to 47% of patients treated with WBRT.[243] Fliessbach et al.[245] performed a prospective neuropsychological and neuroimaging study in a series of 20 patients treated with HD-MTX-based (poly) chemotherapy. There was no gross cognitive decline in long-term survivors, despite therapy-induced white matter changes on posttherapy serial imaging in five patients. In comparison, after WBRT, abnormal areas of white matter abnormalities corresponded with a worse performance of patients in multiple neurocognitive domains.[164,246,247] Interestingly, some functional polymorphisms interfering with the methionine metabolism, in addition to male gender, might also influence methotrexate associated white matter changes (see box on Therapy-Related Neurotoxicity).[248]

THERAPY-RELATED NEUROTOXICITY

The patient was a 66-year-old male who would become lost while performing his job as a pizza delivery driver. This led to evaluation with MRI of the brain, which revealed multifocal homogenously enhancing lesions. The patient underwent a lumbar puncture, revealing a diffuse large B cell lymphoma (see Fig. 84.4A–D). Staging did not reveal the presence of lymphoma elsewhere. He was treated in a National Cancer Institute cooperative group study (Alliance 51101) where he received induction with high-dose methotrexate (HD-MTX), temozolomide, and rituximab. He was randomized to consolidation with a myeloablative regimen of carmustine (BCNU) and thiotepa, followed by an autologous hematopoietic cell transplant (auto-HCT).

The patient has remained disease-free for approximately 5 years. However, he has developed the insidious onset of cognitive symptoms. These include symptoms of frontal network dysfunction such as decreased attention, increased impulsivity, inappropriate behavior, and perseveration. These symptoms have impaired his activities of daily living, including his ability to drive. Neuropsychological testing and evaluation by Cognitive Neurology confirmed these deficits as well as mild memory impairment. Central nervous system imaging with magnetic resonance imaging revealed some diffuse atrophy and extensive white signal abnormalities (Fig. 84.7). FDG PET did not demonstrate the typical findings associated with Alzheimer disease. While whole-brain radiation therapy in older patients receiving HD-MTX for primary central nervous system lymphoma is very frequently associated with a fairly rapidly progressive leukoencephalopathy, methotrexate alone can be associated with a substantial nervous system injury. This is predominantly subcortical in nature and is observed most frequently in older patients. It is, however, extremely difficult to differentiate between treatment-related cognitive deficits and an underlying or concomitant neurodegenerative dementia such as Alzheimer disease.

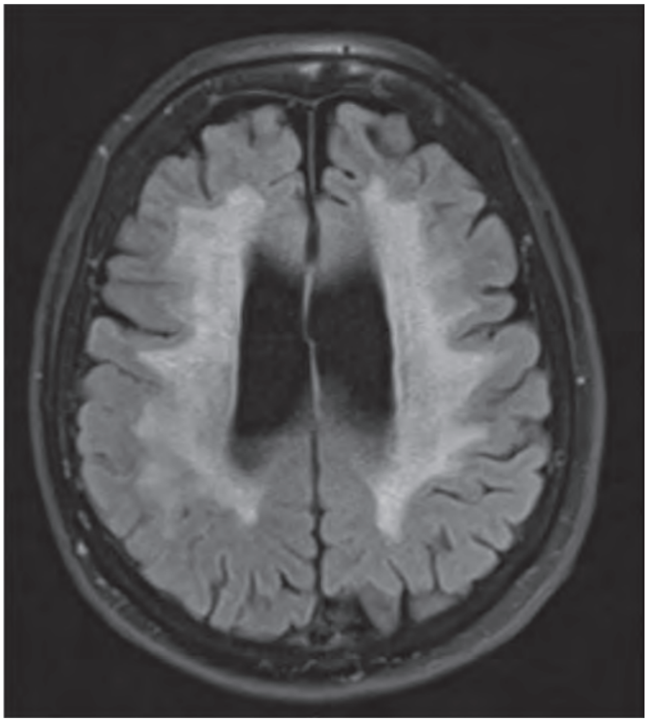


Figure 84.7 An axial magnetic resonance imaging FLAIR sequence demonstrating extensive subcortical increase in signal accompanied by clinically evident cognitive symptomatology in a patient treated with a high-dose methotrexate regimen without radiation.

Whole-Brain Radiation Therapy–Related Neurotoxicity

Patients who survive after conventional doses of WBRT are at high risk of developing potentially fatal neurotoxicity, raising the question of how to balance optimum therapy with side effects. It can affect patients of all ages, but the risk sharply increases with advancing age.[246,249,250] HD-MTX is currently the most effective and widely used chemotherapeutic agent for treatment of PCNSL, and when combined with WBRT (≥30 Gy), can manifest as early (sometimes as early as 3 months) or delayed neurotoxicity in 60% to 90% of patients.[161,247] The time course to symptomatic onset is much shorter than what is seen with focal RT for high-grade gliomas or WBRT for brain metastases, implicating that the combination with methotrexate is a likely contributing factor. The pathophysiology of radiation-induced leukoencephalopathy appears to be multifactorial and poorly understood. Depletion of oligodendrocyte progenitor cells, oxidative stress, fibrotic thickening of small vessels in the deep white matter, atherosclerosis of large intracranial vessels, as well as impaired hippocampal neurogenesis are implicated.[249,251,252] Such effects are dose-dependent and cumulative over time, making long-term survivors most vulnerable to this debilitating complication.

Common clinical presentations include attention deficit, memory impairment, psychomotor slowing, executive dysfunction, behavioral changes, urinary incontinence, and gait ataxia, consistent with diffuse subcortical dementia. These clinical findings comport with radiographic changes of progressive leukoencephalopathy (e.g., confluent periventricular and subcortical white matter abnormalities), consistent with demyelination as well as global cortical atrophy.[252] It is important to acknowledge late treatment-related retinopathy as well.[253] This complication appears to be most closely related to the combination of HD-MTX and WBRT (despite eyeball shielding), particularly in the long-term survivors. In one study, 18.5% of patients who received HD-MTX and WBRT frontline treatment developed clinically significant retinopathy by 5 years.[253]

Lower Dose of Whole-Brain Radiation Therapy?

Therapeutic doses of WBRT (40 to 45 Gy + 9 to 20 Gy boost to tumor bed) as a single modality result in neurotoxicity and disappointing PFS and median OS, in the range of 12 to 18 months with recurrences within and outside the tumor bed, leaving very few (1% to 2%) long-term survivors.[182,254,255] The addition of chemotherapy to WBRT improves CR rates and median OS but at the cost of debilitating neurotoxicity. In a multicenter phase II study, Morris et al.[153] demonstrated efficacy and better neurologic safety profile, using lower dose of WBRT (23.4 Gy) with 2 cycles of cytarabine in patients who achieved CR after 5 to 7 cycles of induction therapy with MPV-R (methotrexate, procarbazine, and vincristine-rituximab). This led to an RTOG randomized clinical trial using a similar therapeutic platform in newly diagnosed PCNSL patients who achieved CR or CRu; the final results of this trial are eagerly awaited (ClinicalTrials.gov Identifier: NCT01399372; Table 84.11). The interim results showed that the study had reached its primary endpoint. Median OS was not reached in either arm, with data still maturing.[256,257] In a retrospective series from South Korea,[258] after HD-MTX treatment, patients were treated with a reduced dose of WBRT (23.4 Gy) and a local boost or standard dose WBRT (>23.4 Gy) in cases of incomplete remission. The 3-year PFS and OS among these patients ($n = 45$) were 53.3% and 77.8% with reduced dose WBRT, and 45.8% and 58.3% with standard dose WBRT without significant difference. At present, it remains questionable whether there is a "safe and effective" dose of WBRT.[259,260]

Conditioning Regimen for Autologous Hematopoietic Stem Cell Transplantation?

Although non-thiotepa-based conditioning regimens (e.g., cyclophosphamide, carmustine; etoposide, melphalan, cyclophosphamide, dexamethasone; carmustine, etoposide, cytarabine, and melphalan) have been utilized in PCNSL and SCNSL settings,[261–265] thiotepa-based regimens (e.g., thiotepa, carmustine and etoposide[266] or thiotepa, busulfan, cyclophosphamide[165,267–269] or thiotepa and busulfan[270] or thiotepa and carmustine)[271,272] have gained more widespread clinical appreciation. Most recently, in line with previous reports the CIBMTR[273] and Japanese Society Hematopoietic Cell Transplantation Registry[274] data confirmed superior outcomes with thiotepa based regimens (Table 84.11).

Incorrect or Ambiguous Definition of the Term "Elderly"

The definition of elderly in PCNSL is nonuniform and, importantly, age cannot be used as the sole determinant to exclude patients from highly effective chemotherapy.[275–278] Despite evidence of HD-MTX showing survival benefit in older adults, spurious (in most instances) concerns of iatrogenic toxicity have led to a decreased use of effective chemotherapy.[28,275–278] An analysis of the National Cancer Database (NCDB) Participant Use File Registry among patients between the ages of 61 and 70 years old who survived longer than 3 months from diagnosis found that 14.4% did not receive any chemotherapy.[204] This fraction increased to 23.3% in patients between the ages of 71 and 80 years old and escalated to 44.1% in patients older than 80 years of age. Another recently reported retrospective analysis from NCDB, evaluating non-HIV patients with PCNSL aged above 65 years from 2004 to 2015, found that 16% of patients received no treatment.[279] Moreover, in the population-based Medicare study of 579 patients diagnosed with PCNSL from 1994 to 2002, age was inversely correlated with the chance of treatment with HD-MTX, and up to 82% of patients ≥80 years of age received either WBRT alone or no treatment at all.[280] International consensus is required to better define the biologic criteria of fitness for both induction and chemotherapy-based consolidation, including auto-HCT in older adults, to assist harmonize the design of clinical studies.[168]

TABLE 84.11 **Randomized Clinical Trials Comparing Three Consolidation Approaches (Autologous Transplantation, Reduced Dose Whole-Brain Radiation Therapy and Nonmyeloablative Chemotherapy) in Primary Central Nervous System Lymphoma**

Trial	ClinicalTrials.gov Identifier	Phase	Randomization
MATRix/ IELSG 43	NCT02531841	III	Arm 1: Rituximab, methotrexate, cytarabine, and thiotepa (4 cycles) followed by thiotepa and carmustine or busulfan-based auto-HCT Arm 2: Rituximab, methotrexate, cytarabine, and thiotepa (4 cycles) followed by dexamethasone, ifosfamide, etoposide, carboplatin (DEViC) × 2 cycles
RTOG1114[256]	NCT01399372	II	Arm 1: Rituximab, methotrexate, vincristine, and procarbazine followed by low-dose (23.40 Gy) WBRT and cytarabine × 2 cycles Arm 2: Rituximab, methotrexate, vincristine, and procarbazine followed by consolidation with single agent cytarabine × 2 cycles only
Alliance 51101	NCT01511562	II	Arm 1: Patients undergo induction therapy for five cycles as defined in the protocol followed by thiotepa and carmustine-based autologous transplant (thiotepa and carmustine conditioning regimen) Arm 2: Patients undergo induction therapy for five cycles as defined in the protocol, followed by etoposide and cytarabine consolidation therapy

PROGNOSTIC AND PREDICTIVE MARKERS

In population-based studies, the outcomes vary based upon many variables, including year and type of therapy, setting where the therapy was administered, access to care, and patient's age and immune status (Table 84.9).[27,204,207–209] It is important to reemphasize that many of the prognostic variables are modifiable and their correction would improve long-term outcomes; this has been discussed in the chapter previously. The two universally established and unmodifiable diagnostic prognostic factors include age and performance status. Both of these factors are part of two widely adopted prognostic models for PCNSL: (1) the International Extranodal Lymphoma Study Group (IESLG)[281] and (2) the Memorial Sloan-Kettering Cancer Center (MSKCC).[282]

International Extranodal Lymphoma Study Group Model[281]

This system was developed from multicenter retrospective data in immunocompetent patients ($n = 105$) treated from 1980 to 1999 and considers age (>60 years), performance status (>1), abnormal lactate dehydrogenase (LDH), high CSF protein level as defined by the study (arbitrary cutoffs of 45 mg/dL in patients ≤60 years old and 60 mg/dL in patients more than 60 years old), and radiographic location of tumor in deep regions (periventricular regions, basal ganglia, brainstem, and/or cerebellum) as unfavorable features. Points are added to create a score which estimates 2-year OS with and without HD-MTX therapy. The IESLG model reports 2-year survival rates, ranging from 15% to 85%, allowing for more nuanced discussions with patients and caregivers regarding goals of clinical management and expected outcomes. The 2-year OS ± standard deviation (SD) was 80% ± 8%, 48% ± 7%, and 15% ± 7% ($P = .00001$) for patients with 0 to 1, 2 to 3, and 4 to 5 unfavorable feature(s), respectively. The prognostic role of this model was confirmed by limiting the analysis to assessable patients treated with HD-MTX-based chemotherapy ($n = 75$; 2-year OS ± SD: 85% ± 8%, 57% ± 8%, and 24% ± 11%; $P = .0004$). Besides being a retrospective study, other limitations of this model include short follow-up of 3 years and the difficulty of attaining all the data points, particularly CSF protein, as lumbar punctures have diminishing value and/or are not always feasible at diagnosis.

Memorial Sloan-Kettering Cancer Center Model[282]

This model has greater simplicity with regard to collection of data points. However, it reflects retrospective data of immunocompetent patients ($n = 338$) treated at a single center during the period from 1983 to 2003. An external validation set obtained from three prospective RTOG PCNSL clinical trials ($n = 282$) was used to test the recursive partitioning analysis classification. It exclusively uses age and performance status to estimate median OS and failure-free survival. This system obtained three prognostic classes: class 1, patients with age ≤50 (median OS, 5.2 years; 95% CI, 3.1 to not reached); class 2, patients older than 50 with a KPS of ≥70 (median OS, 2.1 years; 95% CI, 1.4 to 2.6); and class 3, patients older than 50 plus KPS lower than 70 (median OS, 0.8 years; 95% CI, 0.5 to 1.2).

Differences Between the Two Models

Contrary to the IESLG model,[281] the MSKCC model[282] does not account specifically for therapeutic intervention and uses an age cut-off point of 50 years. As the outcome measures between the two models are different, it is difficult to make direct comparisons between them. Notably, the outcomes of older adults (>60 years to age 76 years) in the Alliance 50202 study[23] were similar to their younger (age >12 years) counterparts, a result that suggests that the optimal age cut-off may be linked to treatment variables (e.g., absence of radiation therapy) and exclusion criteria of a particular study.

Prognosis of Vitreoretinal Lymphoma With and Without Central Nervous System Parenchymal Disease

Retrospective data on behalf of IPCG ($n = 221$)[112] suggests that the natural history of patients with HIV-negative PCNSL with ocular dissemination is unaffected compared with patients with isolated parenchymal brain lymphoma. Furthermore, IESLG[281] and MSKCC[282] prognostic models, with their inherent biases, failed to identify ocular dissemination as an independent risk factor. A recent multicenter retrospective study failed to show HIV as an independent factor of disease progression and mortality. When adjusted for other variables, clinical outcomes were associated with primary treatment modalities and not with HIV status. IESLG[281] and MSKCC[282] prognostic models cannot be reliably used in PVRL.

ADDITIONAL PROGNOSTIC AND PREDICTIVE MARKERS

The following markers should also be interpreted in context of variation in treatment regimens, tissue sample sizes, and analytical procedures which may explain discrepancies between studies.

BCL-6 Overexpression and Rearrangement

In the Alliance 50202 study, BCL-6 overexpression, defined as ≥ 30%, was associated with a shorter time to progression and OS.[23] While few additional studies agree with this finding,[14,49,283] several small retrospective analyses refute it.[284,285]

c-MYC and/or BCL2 Overexpression

The study by Villa et al.[49] used tissue microarray and reported that overexpression of either of these makers is not prognostic of clinical outcomes. On the contrary, two retrospective reports using different methodologies have reported inferior outcomes with their overexpression.[286,287]

O^6-Methylguanine-DNA Methyltransferase (MGMT) Gene Promoter Methylation

In a study by Toffolatti et al.,[288] the presence of methylated MGMT promoter sequences correlated with a better OS (>60 months) in three of four patients who received high-dose chemotherapy. In another study by Adachi et al.,[289] quantitative methylation-sensitive high resolution melting analysis for the detection of MGMT promoter methylation predicted response to temozolomide. MGMT promoter methylation was detected in tumors from 23 (51%) of 45 PCNSL patients, 11 of which were considered to have a high (more than 70.0%) methylation status. Of the 5 recurrent PCNSLs treated with temozolomide, 4 cases responded, with 3 achieving CR and 1 with PR. All 4 responsive PCNSLs had methylated MGMT promoters, compared to none of the nonresponsive patients with recurrent PCNSL.

PD-1 and PD-L1

Genomic studies confirm tumor microenvironment dysfunctions in PCNSL, such as immune evasion mechanisms (e.g., *HLA* [6p21] or *B2M* [15q21.2]) copy loss, *PD-L1* (9p24.1) copy number gain, and immune communication impairments (e.g., *IL17REL* [22q13.33] copy gain or *IFNGR1* [6p23.3-q24] copy loss).[57,290] In a study by Ou et al.[291] involving patients with PCNSL, in a 42-patient cohort, high PD-L1 expression (>5% staining) was seen in 18 patients (37.5%) and intermediate expression (1% to 5% staining) was noted in 14 patients (29.2%). Sixteen patients (33.3%) lacked PD-L1 expression, suggesting that immune checkpoint therapy could be efficient against PCNSL. The presence of PD-1+ TILs and the expression of PD-L1 on malignant B cells correlated with patient outcomes.[57,65,292] A study by Four et al.[293] analyzed 32 immunocompetent patients for PD-1 and PD-L1 protein expression in both TILs and tumor cells, respectively. Presence of PD-1-positive TILs was associated with inferior overall survival ($P = .011$). Patients with PD-L1 overexpression tended to better response to chemotherapy ($P = .23$) (see Chapter 97).

MicroRNA Signature

Roth et al.[294] analyzed blood samples from patients enrolled in the G-PCNSL-SG1 trial, a phase III study for patients with newly diagnosed PCNSL. The short-term survival group comprised 20 patients with a median OS of 3 months and was compared to 20 long-term survival patients with a median OS of 55 months. The cohorts were balanced for age and performance status. Twelve annotated miRNAs were significantly deregulated between the two groups. Among them, miR-151a-5p and miR-151b exhibited the most prominent differences. Importantly, the combination of several miRNA allowed for a good separation between short- and long-term survivors with a maximal AUC above 0.75. Takashima et al.[295] analyzed 847 miRNAs expressed in 27 PCNSL specimens using microarray profiling and surveyed miRNA signatures for prognostic prediction. They reported poor prognosis with lower expression of miR-93. Yang et al.[296] reported that CSF miRNA-21 be a predictive marker to chemotherapy. Further research is warranted to maximize the clinical potential of miRNA-based diagnostics and therapeutics in PCNSL.

RELAPSED AND REFRACTORY DISEASE

Despite advances in the initial therapy, about 50% of patients relapse. The median time to relapse is between 10 and 18 months.[33] Approximately 10% to 15% of patients are refractory to initial therapy.[33] Without additional therapy, the median OS is about 2 months. The best second-line regimen for patients with relapsed and refractory PCNSL is unknown.[297] Current salvage treatments include HD-MTX rechallenge and other chemotherapy agents, auto-HCT, targeted therapies, immunomodulatory agents, immunotherapy, radiation, and allogeneic HCT. Table 84.12 highlights selected studies using a variety of agents in R/R primary and secondary CNSLs. The selection of an appropriate agent, or regimen, is a challenge given lack of prospective data.

Salvage Chemotherapy and Rituximab

Rechallenge with HD-MTX ($\geq 3\,g/m^2$) with and without other agents has shown excellent ORR of 85% to 90% with a median OS of 41 to 62 months. This is considered a reasonable approach with an acceptable toxicity profile in patients who have previously responded to methotrexate with at least 12 months of duration response.[310] Rituximab monotherapy demonstrated modest activity with a response rate of 36% and a median OS of 21 months.[181] Pemetrexed, a multi-targeted antifolate-like methotrexate, has been combined with low-dose dexamethasone. This combination yielded an ORR of 55% and a median OS of 10.1 months (range, 1.5 to 46.2 months).[300] Also, pemetrexed has been safely combined with rituximab, demonstrating an ORR of 63% and a median OS of 11.2 (95% months (CI, 9.1 to 13.4).[299] Temozolomide with or without rituximab (± steroid maintenance therapy) has demonstrated modest activity and good tolerability with response rates of 14% to 53% and 1-year OS ranging from 38% to 71%.[301,302,311,312] Topotecan is a semisynthetic camptothecin derivative that selectively inhibits topoisomerase I in the S-phase of the cell cycle and can induce long-term remissions with acceptable toxicity.[303,304] Bendamustine demonstrated a median PFS of 3.5 months (range 1 to 14 months) and a 6-month PFS of 33% in HD-MTX refractory disease.[313] Other chemotherapy combinations include procarbazine, carmustine, and vincristine (PCV)[314] and etoposide, ifosfamide, and cytarabine (VIA).[315]

Autologous Transplant

This is an attractive option in appropriately selected patients with chemosensitive disease.[206,271,316] Soussain et al.[206] utilized high-dose cytarabine and etoposide followed by a conditioning regimen of thiotepa, busulfan, and cyclophosphamide. Of 43 patients enrolled, 27 underwent transplant, with all but one patient achieving a CR with a median OS of 58.6 months. The 2-year PFS probability was 43% among all the patients and 58% in the auto-HCT subpopulation.

NON-CHEMOTHERAPEUTIC TARGETS AND AGENTS

Non-chemotherapeutic targeted agents are being increasingly tested in clinical trials. They inhibit different aspects of pathologically activated cellular pathways, unbuckle immune checkpoint inhibition, and disrupt lymphoma-nurturing TME.

Bruton Tyrosine Kinase Inhibitors

The most notable advancement is improved understanding of the Bruton tyrosine kinase (BTK) pathway in CNSLs.[65,317] Ibrutinib monotherapy (560 or 840 mg daily) showed promising clinical response with a median OS of 19.2 months.[318] Ibrutinib is also being studied in combination with chemotherapy. One such sequential combination starts with ibrutinib monotherapy followed by dose-adjusted (DA) temozolomide, etoposide, liposomal doxorubicin, dexamethasone, ibrutinib (560 to 840 mg daily) and rituximab (DA-TEDDi-R). This unique maneuvering of ibrutinib with chemotherapy and rituximab demonstrated responses in the order of 86%, with the majority of the patients achieving CR. Median PFS for the relapsed/refractory cohort was 15.3 months, with 51.3% alive at 1-year. Unfortunately, ibrutinib alone and in combination was associated with significant toxicity, including pulmonary and cerebral aspergillosis.[319,320] Additional studies are needed to improve the safety of ibrutinib. One means of doing so may be the elimination of steroids from ibrutinib-containing regimens. Ibrutinib has also been combined with HD-MTX and rituximab without significant toxicity, with an ORR of 80%, with 71% of patients alive at 1-year.[128] In this study, ibrutinib was held on days of HD-MTX and resumed 5 days after HD-MTX or after methotrexate was serologically cleared. Daily ibrutinib was administered following completion of induction therapy till disease progression, intolerability, toxicity, or death. Second-generation BTKi, tirabrutinib[321] and zanibrutinib[322] are also being investigated. In March 2020, oral tirabrutinib was approved in Japan for the treatment of recurrent or refractory PCNSL.

Immunomodulatory Agents

Lenalidomide, an oral second-generation immunomodulatory agent with CNS penetrating capability, has been evaluated as monotherapy (15 mg daily) and in combination with rituximab.[191,306] In a dose-escalating phase I study of heavily pretreated and older adults, 50% of patients achieved a response, allowing six patients to maintain responses for ≥9 months and four to maintain responses ≥18 months.[191,323] The study provided some clinical evidence for the activation of the indoleamine 2,3 dioxygenase (IDO) pathway and showed that changes in CSF IL-10 at 1 month correlated with clinical response and duration (refer to pathogenesis section). Another study demonstrated response in 64% of patients, including those with CSF and intraocular disease.[192] Lenalidomide was also safely combined with rituximab ($R^2 \times 8$ cycles followed by 12 months of maintenance).[306] This combination showed end of induction (primary-endpoint), an overall response rate of 35.6% (95% CI 21.9 to 51.2) in assessable patients, and 32.0% (95% CI 21.9 to 51.2) in the intent-to-treat analysis, including 13 CRs/CRu 29% and 3 PR (7%). With a median follow-up of 19.2 months (range 1.5 to 31), the median PFS and OS were 7.8 months (95% CI 3.9 to 11.3) and 17.7 months (95% CI 12.9 to not reached), respectively.[306] No unexpected toxicity was observed. Pomalidomide (5 mg daily for 21 days of a 28-day cycle), also an immunomodulatory agent, coupled with dexamethasone (40 mg weekly × 2 months), was studied in

TABLE 84.12 Selected Studies in Relapsed and Refractory Central Nervous System Lymphomas

Reference	Regimen	Dose and Schedule	Patients, *N*	Median Age (y)	Complete Response *N* (%)	Partial Response *N* (%)	mPFS(months)	mOS (months)
Dietrich et al. [298]	Pemetrexed	600–1200 mg/m^2 every 2 weeks (MTD: 900 mg/m^2 every 2 weeks)	17 (14 evaluable for response)	64	3 (21)	5 (36)	42	44.5
Zhao et al.[299]	Pemetrexed, rituximab	Pemetrexed, 900 mg/m^2 every 21 days; rituximab, 375 mg/m^2 on day 0	27	53	6 (22)	11 (41)	6.9	11.2
Raizer et al.[300]	Pemetrexed, dexamethasone	Pemetrexed, 900 mg/m^2 every 21 days with low-dose dexamethasone	11	70	4 (36)	2 (18)	5.7	10.1
Batchelor et al.[181]	Rituximab	375 mg/m^2 IV weekly × 8	12	64	3 (25)	1 (8)	57 days	20.9
Nayak et al.[301]	TMZ, rituximab maintenance steroids	Rituximab, 750 mg/m^2 on days 1, 8, 15, and 22 TMZ, 100–200 mg/m^2 days 1–7 and 15–21	16	63	2 (13)	3 (19)	7 weeks	NR
Reni et al.[302]	TMZ	150 mg/m^2 for .5 days every 28 days	36	60	9 (25)	2 (6)	2.8	3.9
Voloschin et al[303]	Topotecan	1.5 mg/m^2 days 1–5 every 3 weeks	15	56	3 (20)	3 (20)	2	32.2
Fischer et al.[04]	Topotecan	1.5 mg/m^2 days 1–5 every 3 weeks	27	51	5 (19)	4 (15)	2	8.4
Grommes et al.[128]	HD-MTX, ibrutinib	HD-MTX, 3.5 g/m^2 and ibrutinib, 560–840 mg daily	15 (9 PCNSL, 6 SCNSL)	62	8 (53)	4 (27)	9.2	NR
Soussain et al[305]	Ibrutinib	560 mg daily	52	69	10 (19)	17 (33)	4.8	19.2
Grommes et al[88]	Ibrutinib	560, 700, or 840 mg daily	13	69	5 (39)	5 (39)	4.6	15
Ghesquieres et al.[306]	Lenalidomide, rituximab	Lenalidomide, 20 mg/d days 1–21 for cycle 1; 25 mg/d days 1–21 for subsequent cycles; rituximab, 375 mg/m^2 on day 1 of each 28-day cycle; maintenance phase: lenalidomide alone, 10 mg/d for up to 12 28-day cycles	45	69	12 CR/ CRu (29)	3 (7)	7.8	17.7
Tun et al.[307]	Pomalidomide, dexamethasone	Pomalidomide, 3, 5, 7, or 10 mg days 1–21 per 28-day cycle and dexamethasone, 40 mg every week for 8 weeks; then pomalidomide alone	25	NR	8 (32) 6 CR, 2 CRu	3 (16)	5.3	NR
Korfel et al.[308]	Temsirolimus	25 or 75 mg IV weekly	37	70	CR 5 (14) CRu 3 (8)	12 (32)	2.1	3.7
Ambady 2019[309]	Pembrolizumab or nivolumab plus rituximab	Pembrolizumab: every 3 weeks; nivolumab: every 4 weeks	6	65	3 (50)	0 (0)	NR	NR

HD-MTX, High-dose methotrexate *mPFS*, median progression free survival; *mOS*, median overall survival; *NR*, not reported; *TMZ*, temozolomide.

relapsed/refractory PCNSL (n = 23) and PRVL (n = 2), showing ORRs of 48% (95% Cl, 27.8% to 68.7%).[307] Lenalidomide is being investigated in a phase I NCI trial in frontline setting with HD-MTX, rituximab with and without nivolumab (ClinicalTrials.gov Identifier: NCT04609046). In another trial, pomalidomide has been combined with nivolumab in patients with relapsed/refractory PCNSL. (ClinicalTrials.gov Identifier: NCT03798314)

mTOR Inhibitor

Temsirolimus showed some activity (dose of 75 mg weekly) with an ORR of 54% and median PFS of 2.1 months (95% CI, 1.1 to 3.0 months) at the expense of 13.5% treatment-related mortality with severe infections in 18.9% of patients.[308]

Intrathecal Rituximab as Monotherapy

Schulz et al.[241] reported their experience using direct intraventricular/intrathecal rituximab (10 to 40 mg) in six patients with recurrent PCNSL/SCNSL. Relevant toxicity was acute reversible (4-hour duration) paraparesis in one patient. Responses were seen in all four patients with leptomeningeal disease, while no responses were observed in two patients with parenchymal CNSL. Subsequently, a phase I study (n = 10) with rituximab monotherapy in a recurrent setting reported cytologic responses in six patients; four patients exhibited CR. Two patients experienced improvement in IOL and one exhibited resolution of parenchymal disease.[324] Most recently, Kadoch et al.[325] provided the first evidence of C3 (complement 3) activation within the neuraxis with intraventricular rituximab and suggested that the complement system may contribute to immunotherapeutic responses of rituximab in CNS lymphoma. They suggested that the presence of tumor-infiltrating CD14+ macrophages may portend a better treatment response as these cells provide complement and Fc receptors needed for rituximab to be effective.

Intrathecal Rituximab in Combination

Rubenstein et al.[326] (n = 14) conducted a phase I study in recurrent PCNSL/SCNSL and found that intraventricular methotrexate, combined with rituximab (10 or 25 mg twice weekly for 4 weeks), is feasible and effective. They reported 75% rate of CSF cytologic CRs and a 43% rate of overall CRs in the CSF and/or brain parenchyma. Direct injection of rituximab monotherapy (during the first treatment each week) or in combination with methotrexate (during the second treatment each week) was associated with responses in both recurrent leptomeningeal and subependymal disease.

Immunotherapy Agents

In a study using nivolumab, a PD-1 immune checkpoint inhibitor, all five patients responded with four CRs and one PR (four relapsed/refractory PCNSL and one CNS relapse of primary testicular lymphoma), with the caveat that two patients received radiation immediately prior to nivolumab.[323,327] A small retrospective study[309] of six patients assessed the combination of PD-1 checkpoint inhibition, using either pembrolizumab or nivolumab, and rituximab, which showed a 50% response rate, all of which being in CR, durable for at least 7 months.

Adoptive Immunotherapy

Chimeric antigen receptor T (CAR T) cell therapy is another promising therapy.[328] Presently, limited studies analyzing the clinical efficiency of anti-CD19 CAR-T cells in patients with primary or secondary CNS lymphoma are available.[329–331] One study by Li et al.[332] used dual targets against CD19 and CD22. In 2017, a first case report on lisocabtagene maraleucel efficacy in secondary CNS lymphoma was published, showing activity in a patient with secondary CNS involvement of refractory systemic DLBCL.[329] Subsequently, Frigault et al.[330] treated and reported on a retrospective cohort of 8 patients with secondary CNS involvement of the brain, spine, and leptomeninges. Response assessment on day 28 after tisagenlecleucel showed CR in 2 patients, PR in 2 patients, and PD in 4 patients (including two fatalities due to PD). Further follow-up on day 90 revealed ongoing disease control in three of four patients who initially responded to CAR-T cells, and long-term follow-up on day 180 was available in one of those patients showing CR. In the study by Li et al.,[332] two of five patients achieved CR and the other three, PR. Four patients had PD after remission. In addition, one CR patient and one PD patient accepted CAR-T cell infusion following HCT in the third month and were in ongoing remission for 14 and 6 months of follow-up, respectively. Several clinical trials are underway in this area as well (reviewed in detail in Karschnia et al.[328]).

Allogeneic Hematopoietic Stem Cell Transplantation

The intent of therapy in relapsed disease, especially after auto-HCT, is rarely curative.[317,333] Perhaps selected patients in this high-risk group may be considered for allogeneic HCT. Small case reports do hint (not definitively) toward a graft-versus-lymphoma (GvL) effect in the CNS compartment.[334–338] For example, Varadi et al.[337] observed continuous PCNSL shrinkage in parallel to an overt graft-versus-host disease (GvHD) following a nonmyeloablative preparative regimen. In a recent retrospective analysis by Mika et al.,[338] six patients with PCNSL were treated with a myeloablative regimen, five of whom were early relapses, and none of them had prior WBRT. The mean PFS after first-line therapy was only 8.3 months, and four of six patients relapsed within 12 months, indicating highly aggressive disease. All patients were in CRu prior to allo-HCT hence direct assessment GvL effect was not feasible. Overall, four of six patients are alive without further evidence of PCNSL at a minimum of 3.75 years.

Salvage Radiation Therapy

Salvage WBRT may be a reasonable option for radiation, especially for naïve patients who are too frail to receive any other therapy. In a study by Nguyen et al.[339] (n = 27), WBRT (median dose, 36 Gy) resulted in a median estimated survival of 10.9 months (range, 0.3 to 63.7 months). In another study by Hottinger et al.[340] (n = 48), WBRT achieved PR in 21%, SD in 6%, and 15% progress. The median survival from the initiation of WBRT was 16 months. The median time to PCNSL progression was 10 months; 15 patients (31%) had no subsequent disease recurrence after WBRT. Treatment-related neurotoxicity was observed in 22% of patients. Patients older than 60 years and those treated less than 6 months after HD-MTX therapy were at an increased risk of the development of neurotoxicity.

Salvage Stereotactic Radiosurgery

Palmer et al.[341] performed a literature review on the use of stereotactic radiosurgery (SRS) in refractory or recurrent lesions in PCNSL, along with a report of their six patients treated with SRS. They found 16 small series, of which only 1 was prospective and included a control group. Mostly single doses from 12 to 21 Gy were used. As a result, 183 out of 256 evaluated lesions (69%) responded completely to treatment, and in 13 of 204 of the patients (6%), lesions recurred within the treatment area at last follow-up. Overall, the treatment was well tolerated. Additional data is warranted.

PREVENTION OF CNS RELAPSE FOR AGGRESSIVE SYSTEMIC LYMPHOMAS AND CONTROVERSIES

Central Nervous System International Prognostic Index

Even in rituximab era, CNS relapse is an important concern in the subset of patients with systemic high-grade DLBCL and grade 3 B follicular lymphoma. It must be noted that the role of CNS-directed prophylaxis is controversial in the absence of randomized controlled trials. Median OS after CNS relapse is usually short and is in the order of less than 12 months.[342–346] For this reason, it is best to prevent such complications after the recognition of a high-risk population. The CNS IPI model[345] estimates the risk of CNS relapse/progression in patients (18 to 80 years old) with aggressive B-cell lymphomas (80% were DLBCL) who were treated with R-CHOP-like chemotherapy ($n = 2{,}164$) . The factors that contribute significantly to the risk of CNS relapse include age above 60 years, LDH higher than normal range, ECOG PS greater than 1, Ann Arbor stage III or IV, greater than 1 extranodal disease site, or involvement of the kidney or adrenal gland. If present, each factor is given one point. Based on the total number of points, patients are characterized into low- (0 to 1 points), intermediate- (2 to 3 points), or high- (4 to 6 points) risk groups. The respective 2-year rates for the development of CNS disease are 0.6% (95% CI, 0% to 1.2%), 3.4% (95% CI, 2.2% to 4.4%), and 10.2% (95% CI, 6.3% to 14.1%) for low-, intermediate- and high-risk groups, respectively. The resulting risk model was validated in an independent dataset of 1,597 patients with DLBCL identified in the British Columbia Cancer Agency (BCCA) Lymphoid Cancer database. Patients from the validation BCCA dataset showed similar rates of CNS disease for low-risk (0.8%; 95% CI, 0.0% to 1.6%), intermediate-risk (3.9%; 95% CI, 2.3% to 5.5%), and high-risk (12.0%; 95% CI, 7.9% to 16.1%) groups. Close to 90% of patients with DLBCL belong to the low- and intermediate-risk groups and have a CNS relapse risk of less than 5%; they may be spared CNS-directed diagnostic and therapeutic intervention. In contrast, those in the high-risk group have a greater than 10% risk of CNS relapse and may be considered for CNS-directed investigations and prophylactic interventions.

Controversies

A study, by Gleeson et al.[346] called into question the benefit of using prophylactic IT chemotherapy or intravenous HD-MTX, although small numbers in some individual groups limits interpretation. Interestingly, two recent studies by Eyre et al. noted that (i) stand-alone IT CNS prophylaxis provides unclear benefit in reducing the CNS relapse risk in elderly DLBCL patients treated with R-CHOP and is associated with increased infection-related toxicity,[347] and (ii) there are no convincing published data between the period 01/2002 and 01/2019, adjusted for well-described confounding variables, that clearly suggest that stand-alone IT chemotherapy CNS prophylaxis reduces the risk of CNS relapse in patients treated with anthracycline-based front-line immunochemotherapy using an anti-CD20 antibody.[348] It must also be recognized, however, that no individual study provides strong evidence for the lack of benefit of stand-alone IT prophylaxis.[348] Dual expression of MYC and BCL2 (double expressor) by IHC is a high-risk group for CNS disease and relapse and should be considered for CNS-directed evaluation and prophylaxis.[349]

Group With Highest Risk for CNS Disease and in Need of Prophylactic CNS-Directed Therapy

Studies have demonstrated that patients with testicular involvement,[350] double-hit lymphoma (DHT),[351] and intravascular DLBCL,[352] portend the highest risk of CNS disease and relapse. Therefore these patients might derive the most benefit from CNS-directed prophylactic therapy (see Chapter 85).

Preventive Interventions

The use of IT chemotherapy and HD-MTX during or after R-CHOP therapy are the two most commonly used approaches. There are no prospective clinical trials to address the choice of CNS prophylaxis. An international phase II IELSG 10 study[353] showed that R-CHOP with 4 doses of IT methotrexate decreased the rate of CNS relapse, compared with historical controls,[350] in 50 patients with primary testicular DLBCL. In a retrospective series of 95 patients with high-risk (defined as IPI ≥3, elevated LDH, and more than one extranodal site or specific extranodal sites being sinus, epidural, testicular, and breast) DLBCL and grade 3B FL, the use of HD-MTX led to a significantly lower rate of CNS relapse of 5%, compared to the 26% in patients who did not receive CNS prophylaxis.[342] Concerns also exist regarding inability of IT therapy to prevent parenchymal relapses. Therefore systemic therapy with HD-MTX is increasingly used with two different approaches. One approach is to interpose HD-MTX with R-CHOP, to ensure early CNS prophylaxis. A second approach is to deliver HD-MTX after 6 cycles of R-CHOP. A recent multicenter retrospective study compared these two approaches ($n = 204$ interposed vs. $n = 130$ at the end of therapy).[354] The group which received HD-MTX interposed with R-CHOP had delays in systemic therapy that were directly related to HD-MTX. The rate of CNS relapse was 5.7% for the entire group and there were no significant differences in the rate of CNS relapse between the 2 cohorts.

PERSPECTIVE AND FUTURE DIRECTIONS

PCNSL is a challenging disease to study. In this chapter, we have extensively highlighted almost every aspect of DLBCL of the CNS, along with the current challenges, controversies, trials, and future perspectives. Where appropriate, we have also provided our current practice patterns and opinions. As noted, the progress in PCNSL compared to systemic DLBCL has been arduous, and hampered by numerous factors. Improvement in the understanding of genomic landscape has provided rationale for a few precision-based approaches. Investigations are also underway to identify novel diagnostic, predictive, and prognostic biomarkers. New concepts are being explored to prevent iatrogenic encephalopathy. Advances in the field of cellular therapy for PCNSL are also promising.[328,329,338,355] In parallel with the ongoing experimental and clinical advances, it is equally important to further consolidate the global collaborative efforts.

ACKNOWLEDGMENTS

We extend our gratitude toward Arjun G. Patel, PharmD, BCOP (CTCA/affiliate of City of Hope), for diligent review of the tables' accuracy and Thomas Schiera for artwork assistance.

In memory of Dr. Alex Feldman (1984–2022), who was a delightful colleague and an excellent pathologist.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Abramson JS, McGree B, Noyes S, et al. Anti-CD19 CAR T Cells in CNS Diffuse Large-B-cell Lymphoma. *N Engl J Med*. 2017;377(8):783–784.

Abrey LE, Batchelor TT, Ferreri AJ, et al. Report of an international workshop to standardize baseline evaluation and response criteria for primary CNS lymphoma. *J Clin Oncol*. 2005;23(22):5034–5043.

Abrey LE, Ben-Porat L, Panageas KS, et al. Primary central nervous system lymphoma: the Memorial Sloan-Kettering Cancer Center prognostic model. *J Clin Oncol*. 2006;24(36):5711–5715.

Alame M, Cornillot E, Cacheux V, et al. The immune contexture of primary central nervous system diffuse large B cell lymphoma associates with patient survival and specific cell signaling. *Theranostics*. 2021;11(8):3565–3579.

Alame M, Pirel M, Costes-Martineau V, et al. Characterisation of tumour microenvironment and immune checkpoints in primary central nervous system diffuse large B cell lymphomas. *Virchows Arch*. 2020;476(6): 891–902.

Bodor C, Alpar D, Marosvari D, et al. Molecular subtypes and genomic profile of primary central nervous system lymphoma. *J Neuropathol Exp Neurol*. 2020;79 (2):176–183.

Bromberg JEC, Issa S, Bakunina K, et al. Rituximab in patients with primary CNS lymphoma (HOVON 105/ALLG NHL 24): a randomised, open-label, phase 3 intergroup study. *Lancet Oncol*. 2019;20(2):216–228.

Calimeri T, Ferreri AJM. Prevention of CNS relapse in diffuse large B-cell lymphoma: common sense prevails where science fails. *Br J Haematol*. 2020;190(5):645–647.

Chan CC, Rubenstein JL, Coupland SE, et al. Primary vitreoretinal lymphoma: a report from an International Primary Central Nervous System Lymphoma Collaborative Group symposium. *Oncologist*. 2011;16(11):1589–1599.

Chapuy B, Roemer MG, Stewart C, et al. Targetable genetic features of primary testicular and primary central nervous system lymphomas. *Blood*. 2016;127 (7):869–881.

Correa DD, Maron L, Harder H, et al. Cognitive functions in primary central nervous system lymphoma: literature review and assessment guidelines. *Ann Oncol*. 2007;18(7):1145–1151.

DeAngelis LM, Seiferheld W, Schold SC, et al. Combination chemotherapy and radiotherapy for primary central nervous system lymphoma: Radiation Therapy Oncology Group Study 93-10. *J Clin Oncol*. 2002;20(24): 4643–4648.

Elstrom RL, Andreadis C, Aqui NA, et al. Treatment of PTLD with rituximab or chemotherapy. *Am J Transplant*. 2006;6(3):569–576.

Eyre TA, Kirkwood AA, Wolf J, et al. Stand-alone intrathecal central nervous system (CNS) prophylaxis provide unclear benefit in reducing CNS relapse risk in elderly DLBCL patients treated with R-CHOP and is associated increased infection-related toxicity. *Br J Haematol*. 2019;187(2):185–194.

Fallah J, Qunaj L, Olszewski AJ. Therapy and outcomes of primary central nervous system lymphoma in the United States: analysis of the National Cancer Database. *Blood Adv*. 2016;1(2):112–121.

Ferreri AJ, Blay JY, Reni M, et al. Prognostic scoring system for primary CNS lymphomas: the International Extranodal Lymphoma Study Group experience. *J Clin Oncol*. 2003;21(2):266–272.

Ferreri AJ, Cwynarski K, Pulczynski E, et al. Chemoimmunotherapy with methotrexate, cytarabine, thiotepa, and rituximab (MATRix regimen) in patients with primary CNS lymphoma: results of the first randomisation of the International Extranodal Lymphoma Study Group-32 (IELSG32) phase 2 trial. *Lancet Haematol*. 2016;3(5):e217–e227.

Ferreri AJM, Cwynarski K, Pulczynski E, et al. Whole-brain radiotherapy or autologous stem-cell transplantation as consolidation strategies after high-dose methotrexate-based chemoimmunotherapy in patients with primary CNS lymphoma: results of the second randomisation of the International Extranodal Lymphoma Study Group-32 phase 2 trial. *Lancet Haematol*. 2017;4(11):e510–e523.

Gandhi MK, Hoang T, Law SC, et al. EBV-associated primary CNS lymphoma occurring after immunosuppression is a distinct immunobiological entity. *Blood*. 2021;137(11):1468–1477.

Graham MS, DeAngelis LM. Improving outcomes in primary CNS lymphoma. *Best Pract Res Clin Haematol*. 2018;31(3):262–269.

Grimm SA, Pulido JS, Jahnke K, et al. Primary intraocular lymphoma: an International Primary Central Nervous System Lymphoma Collaborative Group Report. *Ann Oncol*. 2007;18(11):1851–1855.

Grommes C, Pastore A, Palaskas N, et al. Ibrutinib unmasks critical role of bruton tyrosine kinase in primary CNS lymphoma. *Cancer Discov*. 2017; 7(9):1018–1029.

Grommes C, Rubenstein JL, DeAngelis LM, et al. Comprehensive approach to diagnosis and treatment of newly diagnosed primary CNS lymphoma. *Neuro Oncol*. 2019;21(3):296–305.

Ho KG, Grommes C. Molecular profiling of primary central nervous system lymphomas—predictive and prognostic value? *Curr Opin Neurol*. 2019; 32(6):886–894.

Holdhoff M, Wagner-Johnston N, Roschewski M. Systemic approach to recurrent primary CNS lymphoma: perspective on current and emerging treatment strategies. *Onco Targets Ther*. 2020;13:8323–8335.

Houillier C, Soussain C, Ghesquières H, et al. Management and outcome of primary CNS lymphoma in the modern era: An LOC network study. *Neurology*. 2020;94(10):e1027–e1039.

Inoue A, Ohnishi T, Kohno S, et al. Prognostic significance of immunohistochemical subtypes based on the stage of B-cell differentiation in primary CNS lymphoma. *Int J Clin Exp Pathol*. 2019;12(4):1457–1467.

Jahnke K, Korfel A, Martus P, et al. High-dose methotrexate toxicity in elderly patients with primary central nervous system lymphoma. *Ann Oncol*. 2005;16 (3):445–449.

Kakkassery V, Heindl LM, Rokohl AC, et al. Primary vitreoretinal lymphoma therapy monitoring: significant vitreous haze reduction after intravitreal rituximab. *Neurosignals*. 2021;29(S1):1–7.

Kalogeropoulos D, Vartholomatos G, Mitra A, et al. Primary vitreoretinal lymphoma. *Saudi J Ophthalmol*. 2019;33(1):66–80.

Kam KL, Brooker SM, Mao Q, et al. Newly diagnosed enhancing lesions: steroid initiation may impede diagnosis of lymphoma involving the central nervous system. *J Clin Neurosci*. 2020;81:61–64.

Karmur BS, Philteos J, Abbasian A, et al. Blood-brain barrier disruption in neuro-oncology: strategies, failures, and challenges to overcome. *Front Oncol*. 2020;10:563840.

Karschnia P, Blobner J, Teske N, et al. CAR T-cells for CNS lymphoma: driving into new terrain? *Cancers (Basel)*. 2021;13(10):2503.

Kiewe P, Martus P, Thiel E, Korfel A. Meningeal dissemination in primary CNS lymphoma: diagnosis, treatment, and survival in a large monocenter cohort. *Neuro Oncol*. 2010;12(4):409–417.

Kluin PM, Deckert M, Ferry JA. Primary diffuse large B-cell lymphoma of the CNS. In: Swerdlow SH, Campo E, Harris NL, eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon France: International Agency for Research in Cancer; 2017:300–302.

Kuitunen H, Kaprio E, Karihtala P, et al. Impact of central nervous system (CNS) prophylaxis on the incidence of CNS relapse in patients with high-risk diffuse large B cell/follicular grade 3B lymphoma. *Ann Hematol*. 2020; 99(8):1823–1831.

Mahale P, Herr MM, Engels EA, et al. Autoimmune conditions and primary central nervous system lymphoma risk among older adults. *Br J Haematol*. 2020;188(4):516–521.

Marcelis L, Antoranz A, Delsupehe AM, et al. In-depth characterization of the tumor microenvironment in central nervous system lymphoma reveals implications for immune-checkpoint therapy. *Cancer Immunol Immunother*. 2020;69(9):1751–1766. Erratum in: *Cancer Immunol Immunother*. 2021;70(4):1163.

Martinez-Calle N, Isbell L, Cwynarski K, Schorb E. Treatment of elderly patients with primary CNS lymphoma. *Ann Lymph*. 2021;5:2.

Martinez-Calle N, Poynton E, Alchawaf A, et al. Outcomes of older patients with primary central nervous system lymphoma treated in routine clinical practice in the UK: methotrexate dose intensity correlates with response and survival. *Br J Haematol*. 2020;190(3):394–404.

Mendez JS, Ostrom QT, Gittleman H, et al. The elderly left behind-changes in survival trends of primary central nervous system lymphoma over the past 4 decades. *Neuro Oncol*. 2018;20(5):687–694.

Narita Y, Nagane M, Mishima K, et al. Phase I/II study of tirabrutinib, a second-generation Bruton tyrosine kinase inhibitor, in relapsed/refractory primary central nervous system lymphoma. *Neuro Oncol*. 2021;23(1):122–133.

Nayak L, Iwamoto FM, LaCasce A, et al. PD-1 blockade with nivolumab in relapsed/refractory primary central nervous system and testicular lymphoma. *Blood*. 2017;129(23):3071–3073.

Nayyar N, White MD, Gill CM, et al. MYD88 L265P mutation and CDKN2A loss are early mutational events in primary central nervous system diffuse large B-cell lymphomas. *Blood Adv*. 2019;3(3):375–383.

Noy A. Optimizing treatment of HIV-associated lymphoma. *Blood*. 2019; 134(17):1385–1394.

Omuro A, Correa DD, DeAngelis LM, et al. R-MPV followed by high-dose chemotherapy with TBC and autologous stem-cell transplant for newly diagnosed primary CNS lymphoma. *Blood*. 2015;125(9):1403–1410.

Panageas KS, Elkin EB, Ben-Porat L, et al. Patterns of treatment in older adults with primary central nervous system lymphoma. *Cancer*. 2007;110(6): 1338–1344.

Rae AI, Mehta A, Cloney M, et al. Craniotomy and survival for primary central nervous system lymphoma. *Neurosurgery*. 2019;84(4):935–944.

Riemens A, Bromberg J, Touitou V, et al. Treatment strategies in primary vitreoretinal lymphoma: a 17-center European collaborative study. *JAMA Ophthalmol*. 2015;133(2):191–197.

Rubenstein JL, Fridlyand J, Abrey L, et al. Phase I study of intraventricular administration of rituximab in patients with recurrent CNS and intraocular lymphoma. *J Clin Oncol*. 2007;25(11):1350–1356.

Rubenstein JL, Hsi ED, Johnson JL, et al. Intensive chemotherapy and immunotherapy in patients with newly diagnosed primary CNS lymphoma: CALGB 50202 (Alliance 50202). *J Clin Oncol*. 2013;31(25):3061–3068.

Rubenstein JL, Wong VS, Kadoch C, et al. CXCL13 plus interleukin 10 is highly specific for the diagnosis of CNS lymphoma. *Blood*. 2013;121(23): 4740–4748.

Samhouri Y, Moaath Mustafa A, Jayakrishnan T, et al. Patterns of treatment and survival in elderly patients with primary CNS lymphoma: an 11-year population-based analysis. *Blood*. 2020;136(Suppl 1):2–3.

Schmitz N, Zeynalova S, Nickelsen M, et al. CNS international prognostic index: a risk model for CNS relapse in patients with diffuse large B-cell lymphoma treated with R-CHOP. *J Clin Oncol*. 2016;34(26):3150–3156.

Schorb E, Fox CP, Kasenda B, et al. Induction therapy with the MATRix regimen in patients with newly diagnosed primary diffuse large B-cell lymphoma of the central nervous system—an international study of feasibility and efficacy in routine clinical practice. *Br J Haematol*. 2020;189(5):879–887.

Schorb E, Kasenda B, Ihorst G, et al. High-dose chemotherapy and autologous stem cell transplant in elderly patients with primary CNS lymphoma: a pilot study. *Blood Adv*. 2020;4(14):3378–3381.

Scordo M, Wang TP, Ahn KW, et al. Outcomes associated with thiotepa-based conditioning in patients with primary central nervous system lymphoma after autologous hematopoietic cell transplant. *JAMA Oncol*. 2021;7(7): 993–1003.

Seidel C, Viehweger C, Kortmann RD. Is there an indication for first line radiotherapy in primary CNS lymphoma? *Cancers (Basel)*. 2021; 13(11):2580.

Shah GD, Yahalom J, Correa DD, et al. Combined immunochemotherapy with reduced whole-brain radiotherapy for newly diagnosed primary CNS lymphoma. *J Clin Oncol*. 2007;25(30):4730–4735.

Siddiqi T, Wang X, Blanchard MS, et al. CD19-directed CAR T-cell therapy for treatment of primary CNS lymphoma. *Blood Adv*. 2021;5(20):4059–4063.

Soussain C, Hoang-Xuan K, Taillandier L, et al. Intensive chemotherapy followed by hematopoietic stem-cell rescue for refractory and recurrent primary CNS and intraocular lymphoma: Société Française de Greffe de Moëlle Osseuse-Thérapie Cellulaire. *J Clin Oncol*. 2008;26(15):2512–2518.

van der Meulen M, Dinmohamed AG, Visser O, et al. Improved survival in primary central nervous system lymphoma up to age 70 only: a population-based study on incidence, primary treatment and survival in the Netherlands, 1989-2015. *Leukemia*. 2017;31(8):1822–1825.

Vater I, Montesinos-Rongen M, Schlesner M, et al. The mutational pattern of primary lymphoma of the central nervous system determined by whole-exome sequencing. *Leukemia*. 2015;29(3):677–685.

Villalonga JF, Alessandro L, Farez MF, et al. The role of surgery in primary central nervous system lymphomas. *Arq Neuropsiquiatr*. 2018;76(3):139–144.

Weller M, Martus P, Roth P, et al. German PCNSL Study Group. Surgery for primary CNS lymphoma? Challenging a paradigm. *Neuro Oncol*. 2012; 14(12):1481–1484.

Wilson MR, Eyre TA, Martinez-Calle N, et al. Timing of high-dose methotrexate CNS prophylaxis in DLBCL: an analysis of toxicity and impact on R-CHOP delivery. *Blood Adv*. 2020;4(15):3586–3593.

REFERENCES

1. Kluin PM, Deckert M, Ferry JA. Primary diffuse large B-cell lymphoma of the CNS. In: Swerdlow SH, Campo E, Harris NL, eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon France: International Agency for Research in Cancer; 2017:300–302.
2. Gandhi MK, Hoang T, Law SC, et al. EBV-associated primary CNS lymphoma occurring after immunosuppression is a distinct immunobiological entity. *Blood*. 2021 Mar 18;137(11):1468–1477. https://doi.org/10.1182/blood.2020008520. PMID: 33202420; PMCID: PMC7976507.
3. Gasser O, Bihl FK, Wolbers M, et al. Swiss HIV Cohort Study. HIV patients developing primary CNS lymphoma lack EBV-specific CD4+ T cell function irrespective of absolute CD4+ T cell counts. *PLoS Med*. 2007 Mar 27;4(3):e96. https://doi.org/10.1371/journal.pmed.0040096. PMID: 17388662; PMCID: PMC1831733.
4. Dandachi D, Ostrom QT, Chong I, et al. Primary central nervous system lymphoma in patients with and without HIV infection: a multicenter study and comparison with U.S national data. *Cancer Causes Control*. 2019 May;30(5):477–488. https://doi.org/10.1007/s10552-019-01144-8. Epub 2019 Mar 19. PMID: 30888569.
5. Noy A. Optimizing treatment of HIV-associated lymphoma. *Blood*. 2019 Oct 24;134(17):1385–1394. https://doi.org/10.1182/blood-2018-01-791400. PMID: 30992269; PMCID: PMC7493463.
6. Brandsma D, Bromberg JEC. Primary CNS lymphoma in HIV infection. *Handb Clin Neurol*. 2018;152:177–186. https://doi.org/10.1016/B978-0-444-63849-6.00014-1. PMID: 29604975.
7. Savage KJ. Secondary CNS relapse in diffuse large B-cell lymphoma: defining high-risk patients and optimization of prophylaxis strategies. *Hematology Am Soc Hematol Educ Program*. 2017 Dec 8;2017(1):578–586. https://doi.org/10.1182/asheducation-2017.1.578. PMID: 29222307; PMCID: PMC6142549.
8. Savage KJ, Slack GW, Mottok A, et al. Impact of dual expression of MYC and BCL2 by immunohistochemistry on the risk of CNS relapse in DLBCL. *Blood*. 2016 May 5;127(18):2182–2188. https://doi.org/10.1182/blood-2015-10-676700. Epub 2016 Feb 1. PMID: 26834242.
9. McKay P, Wilson MR, Chaganti S, Smith J, Fox CP, Cwynarski K. British Society of Haematology. The prevention of central nervous system relapse in diffuse large B-cell lymphoma: a British Society for Haematology good practice paper. *Br J Haematol*. 2020 Sep;190(5):708–714. https://doi.org/10.1111/bjh.16866. Epub 2020 Jul 15. PMID: 32433789.
10. Calimeri T, Ferreri AJM. Prevention of CNS relapse in diffuse large B-cell lymphoma: common sense prevails where science fails. *Br J Haematol*. 2020 Sep;190(5):645–647. https://doi.org/10.1111/bjh.16719. Epub 2020 May 12. PMID: 32394433.
11. Deng L, Song Y, Zhu J, et al. Secondary central nervous system involvement in 599 patients with diffuse large B-cell lymphoma: are there any changes in the rituximab era? *Int J Hematol*. 2013 Dec;98(6):664–671. https://doi.org/10.1007/s12185-013-1458-x. PMID: 24234713.
12. Raoux D, Duband S, Forest F, et al. Primary central nervous system lymphoma: immunohistochemical profile and prognostic significance. *Neuropathology*. 2010;30(3):232–240.
13. Liu J, Wang Y, Liu Y, et al. Immunohistochemical profile and prognostic significance in primary central nervous system lymphoma: analysis of 89 cases. *Oncol Lett*. 2017;14(5):5505–5512.
14. Momota H, Narita Y, Maeshima AM, et al. Prognostic value of immunohistochemical profile and response to high-dose methotrexate therapy in primary CNS lymphoma. *J Neurooncol*. 2010;98(3):341–348.
15. Camilleri-Broet S, Criniere E, Broet P, et al. A uniform activated B-cell-like immunophenotype might explain the poor prognosis of primary central nervous system lymphomas: analysis of 83 cases. *Blood*. 2006;107(1):190–196.
16. Nakamura T, Tateishi K, Niwa T, et al. Recurrent mutations of CD79B and MYD88 are the hallmark of primary central nervous system lymphomas. *Neuropathol Appl Neurobiol*. 2016;42(3):279–290.
17. Kraan W, Horlings HM, van Keimpema M, et al. High prevalence of oncogenic MYD88 and CD79B mutations in diffuse large B-cell lymphomas presenting at immune-privileged sites. *Blood Cancer J*. 2013;3(9):e139.
18. Nayyar N, White MD, Gill CM, et al. MYD88 L265P mutation and CDKN2A loss are early mutational events in primary central nervous system diffuse large B-cell lymphomas. *Blood Adv*. 2019;3(3):375–383.
19. Song Y, Zhang W, Zhang L, et al. Cerebrospinal fluid IL-10 and IL-10/IL-6 as accurate diagnostic biomarkers for primary central nervous system large B-cell lymphoma. *Sci Rep*. 2016 Dec 7;6:38671 https://doi.org/10.1038/srep38671. PMID: 27924864; PMCID: PMC5141427.
20. Rubenstein JL, Wong VS, Kadoch C, et al. CXCL13 plus interleukin 10 is highly specific for the diagnosis of CNS lymphoma. *Blood*. 2013 Jun 6;121(23):4740–4748. https://doi.org/10.1182/blood-2013-01-476333. Epub 2013 Apr 9. PMID: 23570798; PMCID: PMC3674672.
21. van Westrhenen A, Smidt LCA, Seute T, et al. Diagnostic markers for CNS lymphoma in blood and cerebrospinal fluid: a systematic review. *Br J Haematol*. 2018 Aug;182(3):384–403. https://doi.org/10.1111/bjh.15410. Epub 2018 May 29. PMID: 29808930; PMCID: PMC6099264.
22. Shah GD, Yahalom J, Correa DD, et al. Combined immunochemotherapy with reduced whole-brain radiotherapy for newly diagnosed primary CNS lymphoma. *J Clin Oncol*. 2007 Oct 20;25(30):4730–4735. https://doi.org/10.1200/JCO.2007.12.5062. Erratum in: *J Clin Oncol*. 2008 Jan 10;26(2):340. PMID: 17947720.
23. Rubenstein JL, Hsi ED, Johnson JL, et al. Intensive chemotherapy and immunotherapy in patients with newly diagnosed primary CNS lymphoma: CALGB 50202 (Alliance 50202). *J Clin Oncol*. 2013 Sep 1;31(25):3061–3068. https://doi.org/10.1200/JCO.2012.46.9957. Epub 2013 Apr 8. PMID: 23569323; PMCID: PMC3753699.
24. Ferreri AJ, Cwynarski K, Pulczynski E, et al. International Extranodal Lymphoma Study Group (IELSG). Chemoimmunotherapy with methotrexate, cytarabine, thiotepa, and rituximab (MATRix regimen) in patients with primary CNS lymphoma: results of the first randomisation of the International Extranodal Lymphoma Study Group-32 (IELSG32) phase 2 trial. *Lancet Haematol*. 2016 May;3(5):e217–e227. https://doi.org/10.1016/S2352-3026(16)00036-3. Epub 2016 Apr 6. PMID: 27132696.
25. Seidel S, Kowalski T, Margold M, et al. HDMTX-based polychemotherapy including intraventricular therapy in elderly patients with primary CNS lymphoma: a single center series. *Ther Adv Neurol Disord*. 2020 Oct 7 13 1756286420951087. doi: 10.1177/1756286420951087. PMID: 33101460; PMCID: PMC7549157.
26. Yu J, Du H, Ye X, Zhang L, Xiao H. High-dose methotrexate-based regimens and post-remission consolidation for treatment of newly diagnosed primary CNS lymphoma: meta-analysis of clinical trials. *Sci Rep*. 2021 Jan 22;11(1):2125 https://doi.org/10.1038/s41598-020-80724-0. PMID: 33483528; PMCID: PMC7822904.
27. Houillier C, Soussain C, Ghesquières H, et al. Management and outcome of primary CNS lymphoma in the modern era: An LOC network study. *Neurology*. 2020 Mar 10;94(10):e1027–e1039. https://doi.org/10.1212/WNL.0000000000008900. Epub 2020 Jan 6. PMID: 31907289; PMCID: PMC7238921.
28. Mendez JS, Ostrom QT, Gittleman H, et al. The elderly left behind: changes in survival trends of primary central nervous system lymphoma over the past 4 decades. *Neuro Oncol*. 2018 Apr 9;20(5):687–694. https://doi.org/10.1093/neuonc/nox187. PMID: 29036697; PMCID: PMC5892148.
29. Bairey O, Siegal T. The possible role of maintenance treatment for primary central nervous system lymphoma. *Blood Rev*. 2018 Sep;32(5):378–386. https://doi.org/10.1016/j.blre.2018.03.003. Epub 2018 Mar 11. PMID: 29551465.
30. Ferreri AJM, Calimeri T, Ponzoni M, et al. Improving the antitumor activity of R-CHOP with NGR-hTNF in primary CNS lymphoma: final results of a phase 2 trial. *Blood Adv*. 2020 Aug 11;4(15):3648–3658. https://doi.org/10.1182/bloodadvances.2020002270. PMID: 32766857; PMCID: PMC7422104.
31. Tu S, Zhou X, Guo Z, et al. CD19 and CD70 dual-target chimeric antigen receptor T-cell therapy for the treatment of relapsed and refractory primary central nervous system diffuse large B-cell lymphoma. *Front Oncol*. 2019 Dec 4;9:1350 https://doi.org/10.3389/fonc.2019.01350. PMID: 31867275; PMCID: PMC6904344.
32. Schaff LR, Ambady P, Doolittle ND, Grommes C. Primary central nervous system lymphoma: a narrative review of ongoing clinical trials and goals for future studies. *Ann Lymphoma*. 2021 Mar;5:8 https://doi.org/10.21037/aol-20-47. PMID: 33912868; PMCID: PMC8078860.
33. Jahnke K, Thiel E, Martus P, Herrlinger U, Weller M, Fischer L, Korfel A. Relapse of primary central nervous system lymphoma: clinical features, outcome and prognostic factors German Primary Central Nervous System Lymphoma Study Group. *J Neurooncol*. 2006 Nov;80(2):159–165. https://doi.org/10.1007/s11060-006-9165-6. Epub 2006 May 13. PMID: 16699873.
34. Villano JL, Koshy M, Shaikh H, Dolecek TA, McCarthy BJ. Age, gender, and racial differences in incidence and survival in primary CNS lymphoma. *Br J Cancer*. 2011;105(9):1414–1418.
35. Olson JE, Janney CA, Rao RD, et al. The continuing increase in the incidence of primary central nervous system non-Hodgkin lymphoma: a surveillance, epidemiology, and end result analysis. *Cancer*. 2002;95(7):1504–1510.

36. Schabet M. Epidemiology of primary CNS lymphoma. *J Neurooncol*. 1999 Jul;43(3):199–201. https://doi.org/10.1023/a:1006290032052. PMID: 10563423.
37. Ostrum QT, Gittleman H, Truitt G, et al. CBTRUS statistical report: primary brain and other central nervous system tumors diagnosed in the United States in 2011–2015. *Neuro Oncol*. 2018;20(Suppl_4):iv1–iv86.
38. Karmali R, Nabhan C, Petrich AM, et al. Impact of treatment variability on survival in immune-competent and immune-compromised patients with primary central nervous system lymphoma. *Br J Haematol*. 2017;177(1): 72–79.
39. DeAngelis LM. Primary central nervous system lymphoma as a secondary malignancy. *Cancer*. 1991 Mar 1;67(5):1431–1435. doi:10.1002/1097-0142(19910301)67:5<1431::aid-cncr2820670527>3.0.co;2-k. PMID: 1991309.
40. Kam KL, Brooker SM, Mao Q, et al. Newly diagnosed enhancing lesions: Steroid initiation may impede diagnosis of lymphoma involving the central nervous system. *J Clin Neurosci*. 2020 Nov;81:61–64. https://doi.org/10.1016/j.jocn.2020.09.014. Epub 2020 Sep 29. PMID: 33222970.
41. Lukas RV, Riedell P, Horowitz PM, Pytel P, Kamson DO. Diagnostic evaluation in primary CNS lymphoma. *Neurologist*. 2018 Mar;23(2):53–54. https://doi.org/10.1097/NRL.0000000000000169. PMID: 29494436.
42. Montesinos-Rongen M, Küppers R, Schlüter D, Spieker T, Van Roost D, Schaller C, et al. Primary central nervous system lymphomas are derived from germinal-center B cells and show a preferential usage of the V4-34 gene segment. *Am J Pathol*. 1999;155(6):2077–2086.
43. Thompsett AR, Ellison DW, Stevenson FK, Zhu DV(H). gene sequences from primary central nervous system lymphomas indicate derivation from highly mutated germinal center B cells with ongoing mutational activity. *Blood*. 1999;94(5):1738–1746.
44. Deckert M, Montesinos-Rongen M, Brunn A, Siebert R. Systems biology of primary CNS lymphoma: from genetic aberrations to modeling in mice. *Acta Neuropathol*. 2014;127(2):175–188.
45. McCann KJ, Ashton-Key M, Smith K, et al. Primary central nervous system lymphoma: tumor-related clones exist in the blood and bone marrow with evidence for separate development. *Blood*. 2009;113(19):4677–4680.
46. Jahnke K, Hummel M, Korfel A, et al. Detection of subclinical systemic disease in primary CNS lymphoma by polymerase chain reaction of the rearranged immunoglobulin heavy-chain genes. *J Clin Oncol*. 2006; 24(29):4754–4757.
47. Malecka A, Tierens A, Østlie I, Schmitz R, Trøen G, Spetalen S, et al. Primary diffuse large B cell lymphoma associated with clonally-related monoclonal B lymphocytosis indicates a common precursor cell. *Haematologica*. 2015
48. Fukumura K, Kawazu M, Kojima S, et al. Genomic characterization of primary central nervous system lymphoma. *Acta Neuropathol*. 2016; 131(6):865–875.
49. Villa D, Tan KL, Steidl C, et al. Molecular features of a large cohort of primary central nervous system lymphoma using tissue microarray. *Blood Adv*. 2019 Dec 10;3(23):3953–3961. https://doi.org/10.1182/bloodadvances.2019000989. PMID: 31805190; PMCID: PMC6963243.
50. Harada K, Nishizaki T, Kubota H, Harada K, Suzuki M, Sasaki K. Distinct primary central nervous system lymphoma defined by comparative genomic hybridization and laser scanning cytometry. *Cancer Genet Cytogenet*. 2001;125(2):147–150.
51. Bödör C, Alpar D, Marosvari D, et al. Molecular subtypes and genomic profile of primary central nervous system lymphoma. *J Neuropathol Exp Neurol*. 2020;79(2):176–183.
52. Courts C, Montesinos-Rongen M, Brunn A, et al. Recurrent inactivation of the PRDM1 gene in primary central nervous system lymphoma. *J Neuropathol Exp Neurol*. 2008;67(7):720–727.
53. Montesinos-Rongen M, Schmitz R, Brunn A, et al. Mutations of CARD11 but not TNFAIP3 may activate the NF-kappa B pathway in primary CNS lymphoma. *Acta Neuropathol*. 2010;120(4):529–535.
54. Gonzalez-Aguilar A, Idbaih A, Boisselier B, et al. Recurrent mutations of MYD88 and TBL1XR1 in primary central nervous system lymphomas. *Clin Cancer Res*. 2012;18(19):5203–5211.
55. Montesinos-Rongen M, Siebert R, Deckert M. Primary lymphoma of the central nervous system: just DLBCL or not? *Blood*. 2009;113(1):7–10.
56. Sethi TK, Kovach AE, Grover NS, et al. Clinicopathologic correlates of MYD88 L265P mutation and programmed cell death (PD-1) pathway in primary central nervous system lymphoma. *Leuk Lymphoma*. 2019; 60(12):2880–2889.
57. Alame M, Cornillot E, Cacheux V, et al. The immune contexture of primary central nervous system diffuse large B cell lymphoma associates with patient survival and specific cell signaling. *Theranostics*. 2021;11(8):3565–3579.
58. Lohr J.G., Stojanov P., Lawrence M.S., et al. Discovery and prioritization of somatic mutations in diffuse large B-cell lymphoma (DLBCL) by whole-exome sequencing. *Proc Natl Acad Sci U S A*. 109(10):3879–3884.
59. Vater I, Montesinos-Rongen M, Schlesner M, et al. The mutational pattern of primary lymphoma of the central nervous system determined by whole-exome sequencing. *Leukemia*. 2015;29(3):677–685.
60. Bruno A, Boisellier B, Labreche K, et al. Mutational analysis of primary central nervous system lymphoma. *Oncotarget*. 2014;5(13): 5065–5075.
61. Schwindt H, Vater I, Kreuz M, et al. Chromosomal imbalances and partial uniparental disomies in primary central nervous system lymphoma. *Leukemia*. 2009 Oct;23(10):1875–1884.
62. Weber T, Weber RG, Kaulich K, et al. Characteristic chromosomal imbalances in primary central nervous system lymphomas of the diffuse large B-cell type. *Brain Pathol*. 2000 Jan;10(1):73–84.
63. Braggio E, McPhail ER, Macon W, et al. Primary central nervous system lymphomas: a validation study of array-based comparative genomic hybridization in formalin-fixed paraffin embedded tumor specimens. *Clin Cancer Res*. 2011;17(13):4245–4253.
64. Nakamura M, Kishi M, Sakai T, et al. Novel tumor suppressor loci on 6q22-23 in primary central nervous system lymphomas. *Cancer Res*. 2003; 63(4):737–741.
65. Chapuy B, Roemer MG, Stewart C, et al. Targetable genetic features of primary testicular and primary central nervous system lymphomas. *Blood*. 2016 Feb 18;127(7):869–881. https://doi.org/10.1182/blood-2015-10-673236. Epub 2015 Dec 23. PMID: 26702065; PMCID: PMC4760091.
66. Four M, Cacheux V, Tempier A, et al. PD1 and PDL1 expression in primary central nervous system diffuse large B-cell lymphoma are frequent and expression of PD1 predicts poor survival. *Hematol Oncol*. 2017;35(4): 487–496.
67. Todorovic Balint M, Jelicic J, Mihaljevic B, et al. Gene mutation profiles in primary diffuse large B cell lymphoma of central nervous system: next generation sequencing analyses. *Int J Mol Sci*. 2016 May 6;17(5):683 https://doi.org/10.3390/ijms17050683. PMID: 27164089; PMCID: PMC4881509.
68. Nakamura M, Sakaki T, Hashimoto H, Nakase H, Ishida E, Shimada K, Konishi N. Frequent alterations of the p14(ARF) and p16(INK4a) genes in primary central nervous system lymphomas. *Cancer Res*. 2001 Sep 1; 61(17):6335–6339. PMID: 11522621.
69. Chang YS, Graves B, Guerlavais V, et al. Stapled α-helical peptide drug development: a potent dual inhibitor of MDM2 and MDMX for p53-dependent cancer therapy. *Proc Natl Acad Sci U S A*. 2013 Sep 3; 110(36):E3445–54. https://doi.org/10.1073/pnas.1303002110. Epub 2013 Aug 14. PMID: 23946421; PMCID: PMC3767549.
70. Monti S, Chapuy B, Takeyama K, et al. Integrative analysis reveals an outcome-associated and targetable pattern of p53 and cell cycle deregulation in diffuse large B cell lymphoma. *Cancer Cell*. 2012 Sep 11;22(3):359–372. https://doi.org/10.1016/j.ccr.2012.07.014. PMID: 22975378; PMCID: PMC3778921.
71. Sebestyén E, Nagy A, Marosvári D, et al. Distinct microRNA expression signatures of primary and secondary central nervous system lymphomas. *medRxiv*. 2021 https://doi.org/10.1101/2021.02.05.21249862. 02.05.21249862.
72. Zheng J, Xu J, Ma S, Sun X, Geng M, Wang L. Clinicopathological study of gene rearrangement and microRNA expression of primary central nervous system diffuse large B-cell lymphomas. *Int J Clin Exp Pathol*. 2013; 6(10):2048–2055.
73. Fischer L, Hummel M, Korfel A, Lenze D, Joehrens K, Thiel E. Differential micro-RNA expression in primary CNS and nodal diffuse large B-cell lymphomas. *Neuro Oncol*. 2011 Oct;13(10):1090–1098. https://doi.org/10.1093/neuonc/nor107. Epub 2011 Jul 29. PMID: 21803762; PMCID: PMC3177663.
74. Robertus JL, Harms G, Blokzijl T, et al. Specific expression of miR-17-5p and miR-127 in testicular and central nervous system diffuse large B-cell lymphoma. *Mod Pathol*. 2009 Apr;22(4):547–555. https://doi.org/10.1038/modpathol.2009.10. Epub 2009 Mar 13. PMID: 19287466.
75. Yu X, Li Z, Shen J, Chan MT, Wu WK. Role of microRNAs in primary central nervous system lymphomas. *Cell Proliferation*. 2016 Apr;49(2): 147–153. https://doi.org/10.1111/cpr.12243.
76. Cai Q, Fang Y, Young KH. Primary central nervous system lymphoma: molecular pathogenesis and advances in treatment. *Transl Oncol*. 2019 Mar;12(3):523–538. https://doi.org/10.1016/j.tranon.2018.11.011. Epub 2019 Jan 4. PMID: 30616219; PMCID: PMC6371000.
77. Bashir R, Coakham H, Hochberg F. Expression of LFA-1/ICAM-1 in CNS lymphomas: possible mechanism for lymphoma homing into the brain. *J Neurooncol*. 1992 Feb;12(2):103–110. https://doi.org/10.1007/BF00172658. PMID: 1348529.
78. Aho R, Kalimo H, Salmi M, Smith D, Jalkanen S. Binding of malignant lymphoid cells to the white matter of the human central nervous system: role of different CD44 isoforms, beta 1, beta 2 and beta 7 integrins, and

L-selectin. *J Neuropathol Exp Neurol*. 1997 May;56(5):557–568. https://doi.org/10.1097/00005072-199705000-00012. PMID: 9143269.

79. Baiocchi RA, Khatri VP, Lindemann MJ, et al. Phenotypic and functional analysis of Fas (CD95) expression in primary central nervous system lymphoma of patients with acquired immunodeficiency syndrome. *Blood*. 1997 Sep 1;90(5):1737–1746. PMID: 9292506.
80. Smith JR, Braziel RM, Paoletti S, Lipp M, Uguccioni M, Rosenbaum JT. Expression of B-cell-attracting chemokine 1 (CXCL13) by malignant lymphocytes and vascular endothelium in primary central nervous system lymphoma. *Blood*. 2003 Feb 1;101(3):815–821. https://doi.org/10.1182/blood-2002-05-1576. Epub 2002 Oct 3. PMID: 12393412.
81. Riemersma SA, Oudejans JJ, Vonk MJ, et al. High numbers of tumour-infiltrating activated cytotoxic T lymphocytes, and frequent loss of HLA class I and II expression, are features of aggressive B cell lymphomas of the brain and testis. *J Pathol*. 2005 Jul;206(3):328–336. https://doi.org/10.1002/path.1783. PMID: 15887291.
82. Miyasato Y, Takashima Y, Takeya H, et al. The expression of PD-1 ligands and IDO1 by macrophage/microglia in primary central nervous system lymphoma. *J Clin Exp Hematop*. 2018;58(2):95–101. https://doi.org/10.3960/jslrt.18001. PMID: 29998979; PMCID: PMC6413151.
83. Ladomersky E, Zhai L, Lauing KL, et al. Advanced age increases immunosuppression in the brain and decreases immunotherapeutic efficacy in subjects with glioblastoma. *Clin Cancer Res*. 2020 Oct 1;26(19):5232–5245. https://doi.org/10.1158/1078-0432.CCR-19-3874. Epub 2020 Jun 16. PMID: 32546647; PMCID: PMC7541490.
84. Marcelis L, Antoranz A, Delsupehe AM, et al. In-depth characterization of the tumor microenvironment in central nervous system lymphoma reveals implications for immune-checkpoint therapy. *Cancer Immunol Immunother*. 2020 Sep;69(9):1751–1766. https://doi.org/10.1007/s00262-020-02575-y. Epub 2020 Apr 25. Erratum in: *Cancer Immunol Immunother*. 2021 Apr;70(4):1163. PMID: 32335702.
85. Inoue A, Ohnishi T, Kohno S, et al. Prognostic significance of immunohistochemical subtypes based on the stage of B-cell differentiation in primary CNS lymphoma. *Int J Clin Exp Pathol*. 2019 Apr 1;12(4):1457–1467. PMID: 31933963; PMCID: PMC6947071.
86. Larocca LM, Capello D, Rinelli A, et al. The molecular and phenotypic profile of primary central nervous system lymphoma identifies distinct categories of the disease and is consistent with histogenetic derivation from germinal center-related B cells. *Blood*. 1998;92(3):1011–1019.
87. Wilson WH, Young RM, Schmitz R, et al. Targeting B cell receptor signaling with ibrutinib in diffuse large B cell lymphoma. *Nat Med*. 2015;21(8):922–926.
88. Grommes C, Pastore A, Palaskas N, et al. Ibrutinib unmasks critical role of bruton tyrosine kinase in primary CNS lymphoma. *Cancer Discov*. 2017 Sep;7(9):1018–1029. https://doi.org/10.1158/2159-8290.CD-17-0613. Epub 2017 Jun 15. PMID: 28619981; PMCID: PMC5581705.
89. Montesinos-Rongen M, Brunn A, et al. Gene expression profiling suggests primary central nervous system lymphomas to be derived from a late germinal center B cell. *Leukemia*. 2008 Feb;22(2):400–405. https://doi.org/10.1038/sj.leu.2405019. Epub 2007 Nov 8. PMID: 17989719; PMCID: PMC6053313.
90. Dubois S, Viailly PJ, Bohers E, et al. Biological and clinical relevance of associated genomic alterations in MYD88 L265P and non-L265P-mutated diffuse large B-cell lymphoma: analysis of 361 cases. *Clin Cancer Res*. 2017 May 1;23(9):2232–2244. https://doi.org/10.1158/1078-0432.CCR-16-1922. Epub 2016 Dec 6. PMID: 27923841.
91. Haque T, Wilkie GM, Jones MM, et al. Allogeneic cytotoxic T-cell therapy for EBV-positive posttransplantation lymphoproliferative disease: results of a phase 2 multicenter clinical trial. *Blood*. 2007 Aug 15;110(4):1123–1131. https://doi.org/10.1182/blood-2006-12-063008. Epub 2007 Apr 27. PMID: 17468341.
92. Gandhi MK, Wilkie GM, Dua U, et al. Immunity, homing and efficacy of allogeneic adoptive immunotherapy for posttransplant lymphoproliferative disorders. *Am J Transplant*. 2007 May;7(5):1293–1299. https://doi.org/10.1111/j.1600-6143.2007.01796.x. Epub 2007 Apr 8. PMID: 17425621.
93. Deutsch MB, Mendez MF. Neurocognitive features distinguishing primary central nervous system lymphoma from other possible causes of rapidly progressive dementia. *Cogn Behav Neurol*. 2015 Mar;28(1):1–10. https://doi.org/10.1097/WNN.0000000000000048. PMID: 25812125; PMCID: PMC4427341.
94. Chan CC, Rubenstein JL, Coupland SE, et al. Primary vitreoretinal lymphoma: a report from an International Primary Central Nervous System Lymphoma Collaborative Group symposium. *Oncologist*. 2011;16(11):1589–1599. https://doi.org/10.1634/theoncologist.2011-0210. Epub 2011 Nov 1. PMID: 22045784; PMCID: PMC3233294.
95. Rohden B, Röth A, Storek B, Hummel M, Nägele T, Thiel E, Korfel A. Meningeal dissemination in primary CNS lymphoma: prospective evaluation of 282 patients. *Neurology*. 2008 Sep 30;71(14):1102–1108. https://doi.org/10.1212/01.wnl.0000326958.52546.f5. PMID: 18824675.
96. Kiewe P, Martus P, Thiel E, Korfel A. Meningeal dissemination in primary CNS lymphoma: diagnosis, treatment, and survival in a large monocenter cohort. *Neuro Oncol*. 2010 Apr;12(4):409–417. https://doi.org/10.1093/neuonc/nop053. Epub 2010 Jan 25. PMID: 20308318; PMCID: PMC2940610.
97. Hegde U, Filie A, Little RF, et al. High incidence of occult leptomeningeal disease detected by flow cytometry in newly diagnosed aggressive B-cell lymphomas at risk for central nervous system involvement: the role of flow cytometry versus cytology. *Blood*. 2005 Jan 15;105(2):496–502. https://doi.org/10.1182/blood-2004-05-1982. Epub 2004 Sep 9. PMID: 15358629.
98. Nabavizadeh SA, Vossough A, Hajmomenian M, Assadsangabi R, Mohan S. Neuroimaging in central nervous system lymphoma. *Hematol Oncol Clin North Am*. 2016 Aug;30(4):799–821. https://doi.org/10.1016/j.hoc.2016.03.005. PMID: 27443998.
99. Rubenstein JL. Biology of CNS lymphoma and the potential of novel agents. *Hematology Am Soc Hematol Educ Program*. 2017 Dec 8;2017(1):556–564. https://doi.org/10.1182/asheducation-2017.1.556. PMID: 29222305; PMCID: PMC6053314.
100. Lukas RV, Stupp R, Gondi V, Raizer JJ. Primary central nervous system lymphoma. PART 1: epidemiology, diagnosis, staging, and prognosis. *Oncology*. 2018 Jan 15;32(1):17–22. PMID: 29447417.
101. Pirotte B, Levivier M, Goldman S, Brucher JM, Brotchi J, Hildebrand J. Glucocorticoid-induced long-term remission in primary cerebral lymphoma: case report and review of the literature. *J Neurooncol*. 1997 Mar;32(1):63–69. https://doi.org/10.1023/a:1005733416571. PMID: 9049864.
102. Singh A, Strobos RJ, Singh BM, Rothballer AB, Reddy V, Puljic S, Poon TP. Steroid-induced remissions in CNS lymphoma. *Neurology*. 1982 Nov;32(11):1267–1271. https://doi.org/10.1212/wnl.32.11.1267. PMID: 6890161.
103. Rae AI, Mehta A, Cloney M, et al. Craniotomy and survival for primary central nervous system lymphoma. *Neurosurgery*. 2019 Apr 1;84(4):935–944. https://doi.org/10.1093/neuros/nyy096. PMID: 29660011; PMCID: PMC6500886.
104. Khatab S, Spliet W, Woerdeman PA. Frameless image-guided stereotactic brain biopsies: emphasis on diagnostic yield. *Acta Neurochir (Wien)*. 2014 Aug;156(8):1441–1450. https://doi.org/10.1007/s00701-014-2145-2. Epub 2014 Jun 5. PMID: 24898761.
105. Luzzati R, Ferrari S, Nicolato A, et al. Stereotactic brain biopsy in human immunodeficiency virus-infected patients. *Arch Intern Med*. 1996 Mar 11;156(5):565–568. PMID: 8604963.
106. Josephson SA, Papanastassiou AM, Berger MS, et al. The diagnostic utility of brain biopsy procedures in patients with rapidly deteriorating neurological conditions or dementia. *J Neurosurg*. 2007 Jan;106(1):72–75. https://doi.org/10.3171/jns.2007.106.1.72. PMID: 17236490.
107. Yun J, Yang J, Cloney M, et al. Assessing the safety of craniotomy for resection of primary central nervous system lymphoma: a nationwide inpatient sample analysis. *Front Neurol*. 2017 Sep 12;8:478 https://doi.org/10.3389/fneur.2017.00478. PMID: 28955300; PMCID: PMC5600910.
108. Weller M, Martus P, Roth P, Thiel E, Korfel A. German PCNSL Study Group. Surgery for primary CNS lymphoma? Challenging a paradigm. *Neuro Oncol*. 2012 Dec;14(12):1481–1484. https://doi.org/10.1093/neuonc/nos159. Epub 2012 Sep 14. PMID: 22984018; PMCID: PMC3499010.
109. Chamberlain MC. Neoplastic meningitis. *Oncologist*. 2008 Sep;13(9):967–977. https://doi.org/10.1634/theoncologist.2008-0138. Epub 2008 Sep 5. PMID: 18776058.
110. Antinori A, De Rossi G, Ammassari A, et al. Value of combined approach with thallium-201 single-photon emission computed tomography and Epstein-Barr virus DNA polymerase chain reaction in CSF for the diagnosis of AIDS-related primary CNS lymphoma. *J Clin Oncol*. 1999 Feb;17(2):554–560. https://doi.org/10.1200/JCO.1999.17.2.554. PMID: 10080599.
111. Cingolani A, De Luca A, Larocca LM, et al. Minimally invasive diagnosis of acquired immunodeficiency syndrome-related primary central nervous system lymphoma. *J Natl Cancer Inst*. 1998 Mar 4;90(5):364–369. https://doi.org/10.1093/jnci/90.5.364. PMID: 9498486.
112. Grimm SA, Pulido JS, Jahnke K, et al. Primary intraocular lymphoma: an International Primary Central Nervous System Lymphoma Collaborative Group Report. *Ann Oncol*. 2007 Nov;18(11):1851–1855. https://doi.org/10.1093/annonc/mdm340. Epub 2007 Sep 5. PMID: 17804469.
113. Mastropasqua R, Thaung C, Pavesio C, et al. The role of chorioretinal biopsy in the diagnosis of intraocular lymphoma. *Am J Ophthalmol*. 2015 Dec;160(6):1127–1132. e1. doi:10.1016/j.ajo.2015.08.033. Epub 2015 Sep 4. PMID: 26344582.

114. Fend F, Ferreri AJ, Coupland SE. How we diagnose and treat vitreoretinal lymphoma. *Br J Haematol*. 2016 Jun;173(5):680–692. https://doi.org/10.1111/bjh.14025. Epub 2016 May 2. Erratum in: *Br J Haematol*. 2018 Jun;181(5):712. PMID: 27133587.
115. Kimura K, Usui Y, Goto H. Japanese Intraocular Lymphoma Study Group. Clinical features and diagnostic significance of the intraocular fluid of 217 patients with intraocular lymphoma. *Jpn J Ophthalmol*. 2012 Jul;56(4):383–389. https://doi.org/10.1007/s10384-012-0150-7. Epub 2012 Jun 5. PMID: 22661396.
116. Missotten T, Tielemans D, Bromberg JE, et al. Multicolor flowcytometric immunophenotyping is a valuable tool for detection of intraocular lymphoma. *Ophthalmology*. 2013 May;120(5):991–996. https://doi.org/10.1016/j.ophtha.2012.11.007.
117. Bonzheim I, Giese S, Deuter C, et al. High frequency of MYD88 mutations in vitreoretinal B-cell lymphoma: a valuable tool to improve diagnostic yield of vitreous aspirates. *Blood*. 2015 Jul 2;126(1):76–79. https://doi.org/10.1182/blood-2015-01-620518. Epub 2015 Apr 21. PMID: 25900979.
118. Yonese I, Takase H, Yoshimori M, et al. CD79B mutations in primary vitreoretinal lymphoma: diagnostic and prognostic potential. *Eur J Haematol*. 2019 Feb;102(2):191–196. https://doi.org/10.1111/ejh.13191. Epub 2018 Nov 23. PMID: 30390359.
119. Cani AK, Hovelson DH, Demirci H, Johnson MW, Tomlins SA, Rao RC. Next generation sequencing of vitreoretinal lymphomas from small-volume intraocular liquid biopsies: new routes to targeted therapies. *Oncotarget*. 2017 Jan 31;8(5):7989–7998. https://doi.org/10.18632/oncotarget.14008. PMID: 28002793; PMCID: PMC5352376.
120. Shenkier TN, Blay JY, O'Neill BP, et al. Primary CNS lymphoma of T-cell origin: a descriptive analysis from the international primary CNS lymphoma collaborative group. *J Clin Oncol*. 2005 Apr 1;23(10):2233–2239. https://doi.org/10.1200/JCO.2005.07.109. PMID: 15800313.
121. Rubenstein JL, Fridlyand J, Shen A, et al. Gene expression and angiotropism in primary CNS lymphoma. *Blood*. 2006 May 1;107(9):3716–3723. https://doi.org/10.1182/blood-2005-03-0897. Epub 2006 Jan 17. PMID: 16418334; PMCID: PMC1895776.
122. Ponzoni M, Berger F, Chassagne-Clement C, et al. International Extranodal Lymphoma Study Group. Reactive perivascular T-cell infiltrate predicts survival in primary central nervous system B-cell lymphomas. *Br J Haematol*. 2007 Aug;138(3):316–323. https://doi.org/10.1111/j.1365-2141.2007.06661.x. Epub 2007 Jun 7. PMID: 17555470.
123. Cobbers JM, Wolter M, Reifenberger J, et al. Frequent inactivation of CDKN2A and rare mutation of TP53 in PCNSL. *Brain Pathol*. 1998 Apr;8(2):263–276. https://doi.org/10.1111/j.1750-3639.1998.tb00152.x. PMID: 9546285.
124. Berghoff AS, Capper D, Preusser M. Lack of BRAF V600E protein expression in primary central nervous system lymphoma. *Appl Immunohistochem Mol Morphol*. 2013 Jul;21(4):351–353. https://doi.org/10.1097/PAI.0b013e3182688e59. PMID: 23235345.
125. Ho KG, Grommes C. Molecular profiling of primary central nervous system lymphomas—predictive and prognostic value? *Curr Opin Neurol*. 2019 Dec;32(6):886–894. https://doi.org/10.1097/WCO.0000000000000759. PMID: 31592789; PMCID: PMC7141780.
126. Fontanilles M, Marguet F, Bohers É, et al. Non-invasive detection of somatic mutations using next-generation sequencing in primary central nervous system lymphoma. *Oncotarget*. 2017 Jul 18;8(29):48157–48168. https://doi.org/10.18632/oncotarget.18325. PMID: 28636991; PMCID: PMC5564634.
127. Mao X, Sun Y, Tang J. Serum miR-21 is a diagnostic and prognostic marker of primary central nervous system lymphoma. *Neurol Sci*. 2014 Feb;35(2):233–238. https://doi.org/10.1007/s10072-013-1491-9. Epub 2013 Jul 6. PMID: 23832112.
128. Grommes C, Tang SS, Wolfe J, et al. Phase 1b trial of an ibrutinib-based combination therapy in recurrent/refractory CNS lymphoma. *Blood*. 2019 Jan 31;133(5):436–445. https://doi.org/10.1182/blood-2018-09-875732. Epub 2018 Dec 19. PMID: 30567753; PMCID: PMC6356986.
129. Wang L, Luo L, Gao Z, et al. The diagnostic and prognostic value of interleukin-10 in cerebrospinal fluid for central nervous system lymphoma: a meta-analysis. *Leuk Lymphoma*. 2017 Oct;58(10):2452–2459. https://doi.org/10.1080/10428194.2017.1289523. Epub 2017 Feb 20. PMID: 28278715.
130. Masouris I., Manz K., Pfirrmann M., et al. CXCL13 and CXCL9 CSF levels in central nervous system lymphoma-diagnostic, therapeutic, and prognostic relevance. *Front Neurol. 2021 Mar 26*;12:654543 https://doi.org/10.3389/fneur.2021.654543. PMID: 33841320; PMCID: PMC8032970.
131. Murase S, Saio M, Andoh H, et al. Diagnostic utility of CSF soluble CD27 for primary central nervous system lymphoma in immunocompetent patients. *Neurol Res*. 2000 Jul;22(5):434–442. https://doi.org/10.1080/01616412.2000.11740697. PMID: 10935213.
132. Strehlow F, Bauer S, Martus P, et al. Osteopontin in cerebrospinal fluid as diagnostic biomarker for central nervous system lymphoma. *J Neurooncol*. 2016 Aug;129(1):165–171. https://doi.org/10.1007/s11060-016-2162-5. Epub 2016 Jun 13. PMID: 27294357.
133. Geng M, Xiao H, Liu J, et al. The diagnostic role and dynamic changes in cerebrospinal fluid neopterin during treatment of patients with primary central nervous system lymphoma. *Cancer Med*. 2018 Aug;7(8):3889–3898. https://doi.org/10.1002/cam4.1581. Epub 2018 Jul 7. PMID: 29982995; PMCID: PMC6089159.
134. Viaccoz A, Ducray F, Tholance Y, et al. CSF neopterin level as a diagnostic marker in primary central nervous system lymphoma. *Neuro Oncol*. 2015 Nov;17(11):1497–1503. https://doi.org/10.1093/neuonc/nov092. Epub 2015 May 25. PMID: 26014047; PMCID: PMC4648303.
135. Baraniskin A, Kuhnhenn J, Schlegel U, et al. Identification of microRNAs in the cerebrospinal fluid as marker for primary diffuse large B-cell lymphoma of the central nervous system. *Blood*. 2011 Mar 17;117(11):3140–3146. https://doi.org/10.1182/blood-2010-09-308684. Epub 2011 Jan 3. PMID: 21200023.
136. Mulazzani M, Huber M, Borchard S, et al. APRIL and BAFF: novel biomarkers for central nervous system lymphoma. *J Hematol Oncol*. 2019 Oct 15;12(1):102 https://doi.org/10.1186/s13045-019-0796-4. PMID: 31615554; PMCID: PMC6792247.
137. Maeyama M, Sasayama T, Tanaka K, et al. Multi-marker algorithms based on CXCL13, IL-10, sIL-2 receptor, and β2-microglobulin in cerebrospinal fluid to diagnose CNS lymphoma. *Cancer Med*. 2020 Jun;9(12):4114–4125. https://doi.org/10.1002/cam4.3048. Epub 2020 Apr 20. PMID: 32314548; PMCID: PMC7300423.
138. Tuo J, Shen D, Yang HH, Chan CC. Distinct microRNA-155 expression in the vitreous of patients with primary vitreoretinal lymphoma and uveitis. *Am J Ophthalmol*. 2014 Mar;157(3):728–734. https://doi.org/10.1016/j.ajo.2013.12.014. Epub 2013 Dec 15. PMID: 24345320; PMCID: PMC3961580.
139. Zajdel M, Rymkiewicz G, Sromek M, et al. Tumor and cerebrospinal fluid micrornas in primary central nervous system lymphomas. *Cancers (Basel)*. 2019 Oct 25;11(11):1647 https://doi.org/10.3390/cancers11111647. PMID: 31731456; PMCID: PMC6895823.
140. Hildebrandt B, Müller C, Pezzutto A, Daniel P, Dörken B, Scholz C. Assessment of free light chains in the cerebrospinal fluid of patients with lymphomatous meningitis—a pilot study. *BMC Cancer*. 2007 Oct 3;7:185 https://doi.org/10.1186/1471-2407-7-185. PMID: 17915026; PMCID: PMC2194780.
141. Abrey LE, Batchelor TT, Ferreri AJ, et al. International Primary CNS Lymphoma Collaborative Group. Report of an international workshop to standardize baseline evaluation and response criteria for primary CNS lymphoma. *J Clin Oncol*. 2005 Aug 1;23(22):5034–5043. https://doi.org/10.1200/JCO.2005.13.524. Epub 2005 Jun 13. PMID: 15955902.
142. Correa DD, Maron L, Harder H, et al. Cognitive functions in primary central nervous system lymphoma: literature review and assessment guidelines. *Ann Oncol*. 2007 Jul;18(7):1145–1151. https://doi.org/10.1093/annonc/mdl464. Epub 2007 Feb 6.
143. Brück W, Brunn A, Klapper W, et al. [Differential diagnosis of lymphoid infiltrates in the central nervous system: experience of the Network Lymphomas and Lymphomatoid Lesions in the Nervous System]. *Pathologe*. 2013 May;34(3):186–197 [in German]. https://doi.org/10.1007/s00292-013-1742-9. PMID: 23471726.
144. Bromberg JEC, Issa S, Bakunina K, et al. Rituximab in patients with primary CNS lymphoma (HOVON 105/ALLG NHL 24): a randomised, open-label, phase 3 intergroup study. *Lancet Oncol*. 2019 Feb;20(2):216–228. https://doi.org/10.1016/S1470-2045(18)30747-2. Epub 2019 Jan 7. PMID: 30630772.
145. Flombaum CD, Liu D, Yan SQ, et al. Management of patients with acute methotrexate nephrotoxicity with high-dose leucovorin. *Pharmacotherapy*. 2018 Jul;38(7):714–724. https://doi.org/10.1002/phar.2145. Epub 2018 Jun 27. PMID: 29863765; PMCID: PMC6279619.
146. Schwartz S, Borner K, Müller K, et al. Glucarpidase (carboxypeptidase g2) intervention in adult and elderly cancer patients with renal dysfunction and delayed methotrexate elimination after high-dose methotrexate therapy. *Oncologist*. 2007 Nov;12(11):1299–1308. https://doi.org/10.1634/theoncologist.12-11-1299. PMID: 18055849.
147. Sun A, Bae K, Gore EM, et al. Phase III trial of prophylactic cranial irradiation compared with observation in patients with locally advanced non-small-cell lung cancer: neurocognitive and quality-of-life analysis. *J Clin Oncol*. 2011 Jan 20;29(3):279–286. https://doi.org/10.1200/JCO.2010.29.6053. Epub 2010 Dec 6. PMID: 21135267; PMCID: PMC3056463.

148. Grommes C, Rubenstein JL, DeAngelis LM, Ferreri AJM, Batchelor TT. Comprehensive approach to diagnosis and treatment of newly diagnosed primary CNS lymphoma. *Neuro Oncol*. 2019 Feb 19;21(3):296–305. https://doi.org/10.1093/neuonc/noy192. PMID: 30418592; PMCID: PMC6380418.
149. Thiel E, Korfel A, Martus P, et al. High-dose methotrexate with or without whole brain radiotherapy for primary CNS lymphoma (G-PCNSL-SG-1): a phase 3, randomised, non-inferiority trial. *Lancet Oncol*. 2010 Nov;11 (11):1036–1047. https://doi.org/10.1016/S1470-2045(10)70229-1. Epub 2010 Oct 20. PMID: 20970380.
150. DeAngelis LM, Yahalom J, Thaler HT, Kher U. Combined modality therapy for primary CNS lymphoma. *J Clin Oncol*. 1992 Apr;10(4): 635–643. https://doi.org/10.1200/JCO.1992.10.4.635. PMID: 1548527.
151. Glass J, Won M, Schultz CJ, et al. Phase I and II. study of induction chemotherapy with methotrexate, rituximab, and temozolomide, followed by whole-brain radiotherapy and postirradiation temozolomide for primary CNS lymphoma: NRG Oncology RTOG 0227. *J Clin Oncol*. 2016 May 10;34(14):1620–1625. https://doi.org/10.1200/JCO.2015.64.8634. Epub 2016 Mar 28. PMID: 27022122; PMCID: PMC4872318.
152. Fisher B, Seiferheld W, Schultz C, et al. Secondary analysis of Radiation Therapy Oncology Group study (RTOG) 9310: an intergroup phase II combined modality treatment of primary central nervous system lymphoma. *J Neurooncol*. 2005 Sep;74(2):201–205. https://doi.org/10.1007/s11060-004-6596-9. PMID: 16193393.
153. Morris PG, Correa DD, Yahalom J, et al. Omuro A. Rituximab, methotrexate, procarbazine, and vincristine followed by consolidation reduced-dose whole-brain radiotherapy and cytarabine in newly diagnosed primary CNS lymphoma: final results and long-term outcome. *J Clin Oncol*. 2013 Nov 1;31(31):3971–3979. https://doi.org/10.1200/JCO.2013.50.4910. Epub 2013 Oct 7. PMID: 24101038; PMCID: PMC5569679.
154. Roth P, Martus P, Kiewe P, et al. Outcome of elderly patients with primary CNS lymphoma in the G-PCNSL-SG-1 trial. *Neurology*. 2012 Aug 28; 79(9):890–896. https://doi.org/10.1212/WNL.0b013e318266fcb2. Epub 2012 Aug 15. PMID: 22895585.
155. Korfel A, Thiel E, Martus P, et al. Randomized phase III study of whole-brain radiotherapy for primary CNS lymphoma. *Neurology*. 2015 Mar 24;84(12):1242–1248. https://doi.org/10.1212/WNL.0000000000001395. Epub 2015 Feb 25. PMID: 25716362.
156. Ferreri AJ, Reni M, Foppoli M, et al. International Extranodal Lymphoma Study Group (IELSG). High-dose cytarabine plus high-dose methotrexate versus high-dose methotrexate alone in patients with primary CNS lymphoma: a randomised phase 2 trial. *Lancet*. 2009 Oct 31;374 (9700):1512–1520. https://doi.org/10.1016/S0140-6736(09)61416-1. Epub 2009 Sep 18. PMID: 19767089.
157. Ferreri AJ, Dell'Oro S, Foppoli M, et al. MATILDE regimen followed by radiotherapy is an active strategy against primary CNS lymphomas. *Neurology*. 2006 May 9;66(9):1435–1438. https://doi.org/10.1212/01.wnl.0000210464.94122.e1. PMID: 16682682.
158. Poortmans PM, Kluin-Nelemans HC, Haaxma-Reiche H, et al. High-dose methotrexate-based chemotherapy followed by consolidating radiotherapy in non-AIDS-related primary central nervous system lymphoma: European Organization for Research and Treatment of Cancer Lymphoma Group Phase II Trial 20962. *J Clin Oncol*. 2003 Dec 15;21(24):4483–4488. https://doi.org/10.1200/JCO.2003.03.108. Epub 2003 Nov 3. PMID: 14597741.
159. Bessell EM, López-Guillermo A, Villá S, et al. Importance of radiotherapy in the outcome of patients with primary CNS lymphoma: an analysis of the CHOD/BVAM regimen followed by two different radiotherapy treatments. *J Clin Oncol*. 2002 Jan 1;20(1):231–236. https://doi.org/10.1200/JCO.2002.20.1.231. PMID: 11773174.
160. DeAngelis LM, Seiferheld W, Schold SC, Fisher B, Schultz CJ. Study 93-10. Combination chemotherapy and radiotherapy for primary central nervous system lymphoma: Radiation Therapy Oncology Group Study 93-10 Radiation Therapy Oncology Group. *J Clin Oncol*. 2002 Dec 15;20 (24):4643–4648. https://doi.org/10.1200/JCO.2002.11.013. PMID: 12488408.
161. Abrey LE, Yahalom J, DeAngelis LM. Treatment for primary CNS lymphoma: the next step. *J Clin Oncol*. 2000 Sep;18(17):3144–3150. https://doi.org/10.1200/JCO.2000.18.17.3144. PMID: 10963643.
162. Batchelor T, Carson K, O'Neill A, et al. Treatment of primary CNS lymphoma with methotrexate and deferred radiotherapy: a report of NABTT 96-07. *J Clin Oncol*. 2003;21:1044–1049.
163. Hoang-Xuan K, Taillandier L, Chinot O, et al. Chemotherapy alone as initial treatment for primary CNS lymphoma in patients older than 60 years: a multicenter phase II study (26952) of the European Organisation for Research and Treatment of Cancer Brain Tumor Group. *J Clin Oncol*. 2003;21:2726–2731.
164. Houillier C, Taillandier L, Dureau S, et al. Radiotherapy or autologous stem-cell transplantation for primary CNS lymphoma in patients 60 years of age and younger: results of the Intergroup ANOCEF-GOELAMS randomized phase II PRECIS study. *J Clin Oncol*. 2019;37(10):823–833. https://doi.org/10.1200/JCO.18.00306. Epub 2019 Feb 20. PMID: 30785830.
165. Omuro A, Correa DD, DeAngelis LM, et al. MPV followed by high-dose chemotherapy with TBC and autologous stem-cell transplant for newly diagnosed primary CNS lymphoma. *Blood*. 2015 Feb 26;125(9): 1403–1410. https://doi.org/10.1182/blood-2014-10-604561. Epub 2015 Jan 7. PMID: 25568347; PMCID: PMC4342354.
166. Schorb E, Fox CP, Fritsch K, et al. High-dose thiotepa-based chemotherapy with autologous stem cell support in elderly patients with primary central nervous system lymphoma: a European retrospective study. *Bone Marrow Transplant*. 2017 Aug;52(8):1113–1119. https://doi.org/10.1038/bmt.2017.23. Epub 2017 Apr 24. PMID: 28436974.
167. Schorb E, Kasenda B, Ihorst G, et al. High-dose chemotherapy and autologous stem cell transplant in elderly patients with primary CNS lymphoma: a pilot study. *Blood Adv*. 2020 Jul 28;4(14):3378–3381. https://doi.org/10.1182/bloodadvances.2020002064. PMID: 32722778; PMCID: PMC7391148.
168. Martinez-Calle N, Isbell L, Cwynarski K, Schorb E. Treatment of elderly patients with primary CNS lymphoma. *Ann Lymphoma*. 2021:5 https://doi.org/10.21037/aol-20-30. Epub 2021 Mar 30.
169. Fox CP, Phillips EH, Smith J, et al. British Society for Haematology. Guidelines for the diagnosis and management of primary central nervous system diffuse large B-cell lymphoma. *Br J Haematol*. 2019 Feb;184(3): 348–363. https://doi.org/10.1111/bjh.15661. Epub 2018 Nov 23. PMID: 30467845.
170. Omuro A, Chinot O, Taillandier L, et al. Methotrexate and temozolomide versus methotrexate, procarbazine, vincristine, and cytarabine for primary CNS lymphoma in an elderly population: an intergroup ANOCEF-GOELAMS randomised phase 2 trial. *Lancet Haematol*. 2015;2:e251–e259.
171. Illerhaus G, Marks R, Müller F, et al. High-dose methotrexate combined with procarbazine and CCNU for primary CNS lymphoma in the elderly: results of a prospective pilot and phase II study. *Ann Oncol*. 2009;20: 319–325.
172. Fritsch K, Kasenda B, Schorb E, et al. High-dose methotrexate-based immuno-chemotherapy for elderly primary CNS lymphoma patients (PRIMAIN study). *Leukemia*. 2017;31:846–852.
173. Olivier G, Clavert A, Lacotte-Thierry L, et al. A phase 1 dose escalation study of idarubicin combined with methotrexate, vindesine, and prednisolone for untreated elderly patients with primary central nervous system lymphoma. The GOELAMS LCP 99 trial. *Am J Hematol*. 2014; 89:1024–1029.
174. Pulczynski EJ, Kuittinen O, Erlanson M, et al. Successful change of treatment strategy in elderly patients with primary central nervous system lymphoma by de-escalating induction and introducing temozolomide maintenance: results from a phase II study by the Nordic Lymphoma Group. *Haematologica*. 2015 Apr;100(4):534–540. https://doi.org/10.3324/haematol.2014.108472. Epub 2014 Dec 5. PMID: 25480497; PMCID: PMC4380727.
175. Welch MR, Omuro A, Deangelis LM. Outcomes of the oldest patients with primary CNS lymphoma treated at Memorial Sloan-Kettering Cancer Center. *Neuro Oncol*. 2012 Oct;14(10):1304–1311. https://doi.org/10.1093/neuonc/nos207. Epub 2012 Sep 5. PMID: 22952196; PMCID: PMC3452344.
176. Pels H, Juergens A, Schirgens I, et al. Early complete response during chemotherapy predicts favorable outcome in patients with primary CNS lymphoma. *Neuro Oncol*. 2010 Jul;12(7):720–724. https://doi.org/10.1093/neuonc/noq010. Epub 2010 Feb 16. PMID: 20159882; PMCID: PMC2940662.
177. Kasenda B, Ferreri AJ, Marturano E, et al. First-line treatment and outcome of elderly patients with primary central nervous system lymphoma (PCNSL)—a systematic review and individual patient data meta-analysis. *Ann Oncol*. 2015 Jul;26(7):1305–1313. https://doi.org/10.1093/annonc/mdv076. Epub 2015 Feb 20. PMID: 25701456; PMCID: PMC4735103.
178. Schorb E, Finke J, Ihorst G, Kasenda B, Fricker H, Illerhaus G. Age-adjusted high-dose chemotherapy and autologous stem cell transplant in elderly and fit primary CNS lymphoma patients. *BMC Cancer*. 2019 Mar 29;19(1):287 https://doi.org/10.1186/s12885-019-5473-z. PMID: 30925912; PMCID: PMC6440161.
179. Kurzwelly D, Glas M, Roth P, et al. Primary CNS lymphoma in the elderly: temozolomide therapy and MGMT status. *J Neurooncol*. 2010 May; 97(3):389–392. https://doi.org/10.1007/s11060-009-0032-0. Epub 2009 Oct 20.
180. Han S, Wang M, Liu B, Yu J. Pemetrexed for primary central nervous system lymphoma in the elderly. *Clin Transl Oncol*. 2016 Feb;18(2):

138–143. https://doi.org/10.1007/s12094-015-1345-4. Epub 2015 Jul 14. PMID: 26169215.

181. Batchelor TT, Grossman SA, Mikkelsen T, et al. Rituximab monotherapy for patients with recurrent primary CNS lymphoma. *Neurology*. 2011; 76:929–930.
182. Laack NN, Ballman KV, Brown PB, O'Neill BP. North Central Cancer Treatment Group. Whole-brain radiotherapy and high-dose methylprednisolone for elderly patients with primary central nervous system lymphoma: results of North Central Cancer Treatment Group (NCCTG) 96-73-51. *Int J Radiat Oncol Biol Phys*. 2006 Aug 1;65(5):1429–1439. https://doi.org/10.1016/j.ijrobp.2006.03.061. PMID: 16863926.
183. Ferreri AJM, Cwynarski K, Pulczynski E, et al. International Extranodal Lymphoma Study Group (IELSG). Whole-brain radiotherapy or autologous stem-cell transplantation as consolidation strategies after high-dose methotrexate-based chemoimmunotherapy in patients with primary CNS lymphoma: results of the second randomisation of the International Extranodal Lymphoma Study Group-32 phase 2 trial. *Lancet Haematol*. 2017 Nov;4(11):e510–e523. https://doi.org/10.1016/S2352-3026(17)30174-6. Epub 2017 Oct 17. PMID: 29054815.
184. Graham MS, DeAngelis LM. Improving outcomes in primary CNS lymphoma. *Best Pract Res Clin Haematol*. 2018 Sep;31(3):262–269. https://doi.org/10.1016/j.beha.2018.07.006. Epub 2018 Jul 21. PMID: 30213395.
185. Vu K, Mannis G, Hwang J, Geng H, Rubenstein JL. Low-dose lenalidomide maintenance after induction therapy in older patients with primary central nervous system lymphoma. *Br J Haematol*. 2019 Jul; 186(1):180–183. https://doi.org/10.1111/bjh.15787. Epub 2019 Feb 3. PMID: 30714128.
186. Cher L, Glass J, Harsh GR, Hochberg FH. Therapy of primary CNS lymphoma with methotrexate-based chemotherapy and deferred radiotherapy: preliminary results. *Neurology*. 1996 Jun;46(6):1757–1759. https://doi.org/10.1212/wnl.46.6.1757. PMID: 8649588.
187. Yang SH, Lee KS, Kim IS, et al. Long-term survival in primary CNS lymphoma treated by high-dose methotrexate monochemotherapy: role of STAT6 activation as prognostic determinant. *J Neurooncol*. 2009 Mar; 92(1):65–71. https://doi.org/10.1007/s11060-008-9736-9. Epub 2008 Nov 22. PMID: 19030780.
188. Chamberlain MC, Johnston SK. High-dose methotrexate and rituximab with deferred radiotherapy for newly diagnosed primary B-cell CNS lymphoma. *Neuro Oncol*. 2010 Jul;12(7):736–744. https://doi.org/10.1093/neuonc/noq011. Epub 2010 Feb 8. PMID: 20511181; PMCID: PMC2940660.
189. Ambady P, Fu R, Szidonya L, Peereboom DM, Doolittle ND, Neuwelt EA. Impact of maintenance rituximab on duration of response in primary central nervous system lymphoma. *J Neurooncol*. 2020 Mar;147(1): 171–176. https://doi.org/10.1007/s11060-020-03411-0. Epub 2020 Feb 5. PMID: 32026432.
190. Ney DE, Abrey LE. Maintenance therapy for central nervous system lymphoma with rituximab. *Leuk Lymphoma*. 2009 Sep;50(9):1548–1551. https://doi.org/10.1080/10428190903128645. PMID: 19637086.
191. Houillier C, Choquet S, Touitou V, et al. Lenalidomide monotherapy as salvage treatment for recurrent primary CNS lymphoma. *Neurology*. 2015 Jan 20;84(3):325–326. https://doi.org/10.1212/WNL.0000000000001158. Epub 2014 Dec 19. PMID: 25527263.
192. Rubenstein JL, Geng H, Vu K, et al. Maintenance lenalidomide in primary CNS lymphoma. *Ann Oncol*. 2019 Aug 1;30(8):1397–1398. https://doi.org/10.1093/annonc/mdz142. PMID: 31046114.
193. Batchelor TT, Kolak G, Ciordia R, Foster CS, Henson JW. High-dose methotrexate for intraocular lymphoma. *Clin Cancer Res*. 2003 Feb; 9(2):711–715. PMID: 12576439.
194. Berenbom A, Davila RM, Lin HS, Harbour JW. Treatment outcomes for primary intraocular lymphoma: implications for external beam radiotherapy. *Eye (Lond)*. 2007 Sep;21(9):1198–1201. https://doi.org/10.1038/sj.eye.6702437. Epub 2006 May 26. PMID: 16732210.
195. Frenkel S, Hendler K, Siegal T, Shalom E, Pe'er J. Intravitreal methotrexate for treating vitreoretinal lymphoma: 10 years of experience. *Br J Ophthalmol*. 2008 Mar;92(3):383–388. https://doi.org/10.1136/bjo.2007.127928. PMID: 18303160.
196. Smith JR, Rosenbaum JT, Wilson DJ, et al. Role of intravitreal methotrexate in the management of primary central nervous system lymphoma with ocular involvement. *Ophthalmology*. 2002 Sep;109(9):1709–1716. https://doi.org/10.1016/s0161-6420(02)01125-9. PMID: 12208721.
197. Kitzmann AS, Pulido JS, Mohney BG, et al. Intraocular use of rituximab. *Eye*. 2007;21:1524–1527.
198. Larkin KL, Saboo US, Comer GM, et al. Use of intravitreal rituximab for treatment of vitreoretinal lymphoma. *Br J Ophthalmol*. 2014;98:99–103.
199. Hashida N, Ohguro N, Nishida K. Efficacy and complications of intravitreal rituximab injection for treating primary vitreoretinal lymphoma. *Transl Vis Sci Technol*. 2012 Oct 22;1(3):1 https://doi.org/10.1167/tvst.1.3.1. PMID: 24049708; PMCID: PMC3763885.
200. Ohguro N, Hashida N, Tano Y. Effect of intravitreous rituximab injections in patients with recurrent ocular lesions associated with central nervous system lymphoma. *Arch Ophthalmol*. 2008;126:1002–1003.
201. Kakkassery V, Heindl LM, Rokohl AC, et al. Primary vitreoretinal lymphoma therapy monitoring: significant vitreous haze reduction after intravitreal rituximab. *Neurosignals*. 2021 May 5;29(S1):1–7. https://doi.org/10.33594/000000367. PMID: 33945240.
202. Castellino A, Pulido JS, Johnston PB, et al. Role of systemic high-dose methotrexate and combined approaches in the management of vitreoretinal lymphoma: a single center experience 1990–2018. *Am J Hematol*. 2019 Mar;94(3):291–298. https://doi.org/10.1002/ajh.25350. Epub 2018 Dec 5. PMID: 30516868.
203. Riemens A, Bromberg J, Touitou V, et al. Treatment strategies in primary vitreoretinal lymphoma: a 17-center European collaborative study. *JAMA Ophthalmol*. 2015 Feb;133(2):191–197. https://doi.org/10.1001/jamaophthalmol.2014.4755. PMID: 25412269.
204. Fallah J, Qunaj L, Olszewski AJ. Therapy and outcomes of primary central nervous system lymphoma in the United States: analysis of the National Cancer Database. *Blood Adv*. 2016 Nov 30;1(2):112–121. https://doi.org/10.1182/bloodadvances.2016000927. PMID: 29296804; PMCID: PMC5737162.
205. Kasenda B, Schorb E, Fritsch K, Finke J, Illerhaus G. Prognosis after high-dose chemotherapy followed by autologous stem-cell transplantation as first-line treatment in primary CNS lymphoma—a long-term follow-up study. *Ann Oncol*. 2015 Mar;26(3):608–611. https://doi.org/10.1093/annonc/mdv002. Erratum for: *Ann Oncol*. 2012 Oct;23(10):2670-5. PMID: 25725080.
206. Soussain C, Hoang-Xuan K, Taillandier L, et al. Intensive chemotherapy followed by hematopoietic stem-cell rescue for refractory and recurrent primary CNS and intraocular lymphoma: Société Française de Greffe de Moëlle Osseuse-Thérapie Cellulaire. *J Clin Oncol*. 2008 May 20;26 (15):2512–2518. https://doi.org/10.1200/JCO.2007.13.5533. Epub 2008 Apr 14. PMID: 18413641.
207. van der Meulen M, Dinmohamed AG, Visser O, Doorduijn JK, Bromberg JEC. Improved survival in primary central nervous system lymphoma up to age 70 only: a population-based study on incidence, primary treatment and survival in the Netherlands, 1989–2015. *Leukemia*. 2017 Aug;31(8): 1822–1825. https://doi.org/10.1038/leu.2017.128. Epub 2017 Apr 28. PMID: 28450731.
208. Martinez-Calle N, Poynton E, et al. Outcomes of older patients with primary central nervous system lymphoma treated in routine clinical practice in the UK: methotrexate dose intensity correlates with response and survival. *Br J Haematol*. 2020 Aug;190(3):394–404. https://doi.org/10.1111/bjh.16592. Epub 2020 Mar 31. PMID: 32232989.
209. Shiels MS, Pfeiffer RM, Besson C, et al. Trends in primary central nervous system lymphoma incidence and survival in the U.S. *Br J Haematol*. 2016 Aug;174(3):417–424. https://doi.org/10.1111/bjh.14073. Epub 2016 Mar 28. PMID: 27018254; PMCID: PMC4961566.
210. Hoffmann C, Tabrizian S, Wolf E, et al. Survival of AIDS patients with primary central nervous system lymphoma is dramatically improved by HAART-induced immune recovery. *AIDS*. 2001 Nov 9;15(16):2119–2127. https://doi.org/10.1097/00002030-200111090-00007. PMID: 11684931.
211. Newell ME, Hoy JF, Cooper SG, et al. Human immunodeficiency virus-related primary central nervous system lymphoma: factors influencing survival in 111 patients. *Cancer*. 2004 Jun 15;100(12):2627–2636. https://doi.org/10.1002/cncr.20300. PMID: 15197806.
212. Skiest DJ, Crosby C. Survival is prolonged by highly active antiretroviral therapy in AIDS patients with primary central nervous system lymphoma. *AIDS*. 2003 Aug 15;17(12):1787–1793. https://doi.org/10.1097/00002030-200308150-00007. PMID: 12891064.
213. Uldrick TS, Pipkin S, Scheer S, Hessol NA. Factors associated with survival among patients with AIDS-related primary central nervous system lymphoma. *AIDS*. 2014 Jan 28;28(3):397–405. https://doi.org/10.1097/QAD.0000000000000030. PMID: 24076659; PMCID: PMC3966974.
214. Gupta NK, Nolan A, Omuro A, et al. Long-term survival in AIDS-related primary central nervous system lymphoma. *Neuro Oncol*. 2017 Jan; 19(1):99–108. https://doi.org/10.1093/neuonc/now155. Epub 2016 Aug 30. PMID: 27576871; PMCID: PMC5193026.
215. Moulignier A, Lamirel C, Picard H, et al. Long-term AIDS-related PCNSL outcomes with HD-MTX and combined antiretroviral therapy. *Neurology*. 2017 Aug 22;89(8):796–804. https://doi.org/10.1212/WNL.0000000000004265. Epub 2017 Jul 26. PMID: 28747447.
216. Olszewski AJ, Fallah J, Castillo JJ. Human immunodeficiency virus-associated lymphomas in the antiretroviral therapy era: Analysis of the

National Cancer Data Base. *Cancer*. 2016 Sep 1;122(17):2689–2697. https://doi.org/10.1002/cncr.30112. Epub 2016 Jun 23. PMID: 27337679.
217. van de Glind G, de Graaf S, Klein C, Cornelissen M, Maecker B, Loeffen J. Intrathecal rituximab treatment for pediatric post-transplant lymphoproliferative disorder of the central nervous system. *Pediatr Blood Cancer*. 2008 Apr;50(4):886–888. https://doi.org/10.1002/pbc.21297. PMID: 17668865.
218. Elstrom RL, Andreadis C, Aqui NA, Ahya VN, Bloom RD, Brozena SC, Olthoff KM, Schuster SJ, Nasta SD, Stadtmauer EA, Tsai DE. Treatment of PTLD with rituximab or chemotherapy. *Am J Transplant*. 2006 Mar;6(3):569–576. https://doi.org/10.1111/j.1600-6143.2005.01211.x. PMID: 16468968.
219. Mahale P, Herr MM, Engels EA, Pfeiffer RM, Shiels MS. Autoimmune conditions and primary central nervous system lymphoma risk among older adults. *Br J Haematol*. 2020 Feb;188(4):516–521. https://doi.org/10.1111/bjh.16222. Epub 2019 Oct 17. PMID: 31625136; PMCID: PMC7012721.
220. Kaulen LD, Karschnia P, Dietrich J, Baehring JM. Autoimmune disease-related primary CNS lymphoma: systematic review and meta-analysis. *J Neurooncol*. 2020 Aug;149(1):153–159. https://doi.org/10.1007/s11060-020-03583-9. Epub 2020 Jul 18. PMID: 32683530.
221. Neuwelt EA, Goldman DL, Dahlborg SA, et al. Primary CNS lymphoma treated with osmotic blood-brain barrier disruption: Prolonged survival and preservation of cognitive function. *J Clin Oncol*. 1991;9:1580–1590.
222. McAllister LD, Doolittle ND, Guastadisegni PE, et al. Cognitive outcomes and long-term follow-up results after enhanced chemotherapy delivery for primary central nervous system lymphoma. *Neurosurgery*. 2000;46:51–60. discussion 60-61.
223. Jahnke K, Doolittle ND, Muldoon LL, et al. Implications of the blood-brain barrier in primary central nervous system lymphoma. *Neurosurg Focus*. 2006;21:E11.
224. Angelov L, Doolittle ND, Kraemer DF, et al. Blood-brain barrier disruption and intra-arterial methotrexate-based therapy for newly diagnosed primary CNS lymphoma: a multi-institutional experience. *J Clin Oncol*. 2009 Jul 20;27(21):3503–3509. https://doi.org/10.1200/JCO.2008.19.3789. Epub 2009 May 18. PMID: 19451444; PMCID: PMC2717756.
225. Iorio-Morin C, Gahide G, Morin C, et al. Management of primary central nervous system lymphoma using intra-arterial chemotherapy with osmotic blood-brain barrier disruption: retrospective analysis of the Sherbrooke cohort. *Front Oncol*. 2021 Jan 20;10:543648 https://doi.org/10.3389/fonc.2020.543648. PMID: 33552946; PMCID: PMC7855856.
226. Karmur BS, Philteos J, Abbasian A, et al. Blood-brain barrier disruption in neuro-oncology: strategies, failures, and challenges to overcome. *Front Oncol*. 2020 Sep 18;10:563840 https://doi.org/10.3389/fonc.2020.563840. PMID: 33072591; PMCID: PMC7531249.
227. Bataille B, Delwail V, Menet E, et al. Primary intracerebral malignant lymphoma: report of 248 cases. *J Neurosurg*. 2000 Feb;92(2):261–266. https://doi.org/10.3171/jns.2000.92.2.0261. PMID: 10659013.
228. Bellinzona M, Roser F, Ostertag H, Gaab RM, Saini M. Surgical removal of primary central nervous system lymphomas (PCNSL) presenting as space occupying lesions: a series of 33 cases. *Eur J Surg Oncol*. 2005 Feb;31(1):100–105. https://doi.org/10.1016/j.ejso.2004.10.002. PMID: 15642434.
229. Morell AA, Shah AH, Cavallo C, et al. Diagnosis of primary central nervous system lymphoma: a systematic review of the utility of CSF screening and the role of early brain biopsy. *Neurooncol Pract*. 2019;6:415–423.
230. Villalonga JF, Alessandro L, Farez MF, et al. The role of surgery in primary central nervous system lymphomas. *Arq Neuropsiquiatr*. 2018 Mar;76(3):139–144. https://doi.org/10.1590/0004-282x20180002. PMID: 29809232.
231. Jelicic J, Todorovic Balint M, Raicevic S, et al. The possible benefit from total tumour resection in primary diffuse large B-cell lymphoma of central nervous system—a one-decade single-centre experience. *Br J Neurosurg*. 2016;30(1):80–85. https://doi.org/10.3109/02688697.2015.1071328. Epub 2015 Sep 4. PMID: 26337736.
232. Holdhoff M, Ambady P, Abdelaziz A, et al. High-dose methotrexate with or without rituximab in newly diagnosed primary CNS lymphoma. *Neurology*. 2014 Jul 15;83(3):235–239. https://doi.org/10.1212/WNL.0000000000000593. Epub 2014 Jun 13. PMID: 24928128; PMCID: PMC4117362.
233. Chen C, Sun P, Cui J, et al. High-dose Methotrexate plus temozolomide with or without rituximab in patients with untreated primary central nervous system lymphoma: A retrospective study from China. *Cancer Med*. 2019 Apr;8(4):1359–1367. https://doi.org/10.1002/cam4.1906. Epub 2019 Mar 1. PMID: 30821418; PMCID: PMC6488123.
234. Ferreri AJ, Guerra E, Regazzi M, et al. Area under the curve of methotrexate and creatinine clearance are outcome-determining factors in primary CNS lymphomas. *Br J Cancer*. 2004 Jan 26;90(2):353–358. https://doi.org/10.1038/sj.bjc.6601472. PMID: 14735176; PMCID: PMC2409565.
235. Ferreri AJ, Reni M, Pasini F, et al. A multicenter study of treatment of primary CNS lymphoma. *Neurology*. 2002 May 28;58(10):1513–1520. https://doi.org/10.1212/wnl.58.10.1513. PMID: 12034789.
236. Khan RB, Shi W, Thaler HT, DeAngelis LM, Abrey LE. Is intrathecal methotrexate necessary in the treatment of primary CNS lymphoma? *J Neurooncol*. 2002 Jun;58(2):175–178. https://doi.org/10.1023/a:1016077907952. PMID: 12164690.
237. Ferreri AJ, Reni M, Villa E. Primary central nervous system lymphoma in immunocompetent patients. *Cancer Treat Rev*. 1995 Sep;21(5):415–446. https://doi.org/10.1016/0305-7372(95)90028-4. PMID: 8556717.
238. Sierra Del Rio M, Ricard D, Houillier C, et al. Prophylactic intrathecal chemotherapy in primary CNS lymphoma. *J Neurooncol*. 2012 Jan;106(1):143–146. https://doi.org/10.1007/s11060-011-0649-7. Epub 2011 Jul 8. PMID: 21739169.
239. Glantz MJ, Cole BF, Recht L, et al. High-dose intravenous methotrexate for patients with nonleukemic leptomeningeal cancer: is intrathecal chemotherapy necessary? *J Clin Oncol*. 1998 Apr;16(4):1561–1567. https://doi.org/10.1200/JCO.1998.16.4.1561. PMID: 9552066.
240. Wilson WH, Bromberg JE, Stetler-Stevenson M, et al. Detection and outcome of occult leptomeningeal disease in diffuse large B-cell lymphoma and Burkitt lymphoma. *Haematologica*. 2014 Jul;99(7):1228–1235. https://doi.org/10.3324/haematol.2013.101741. Epub 2014 Apr 11. PMID: 24727817; PMCID: PMC4077085.
241. Schulz H, Pels H, Schmidt-Wolf I, Zeelen U, Germing U, Engert A. Intraventricular treatment of relapsed central nervous system lymphoma with the anti-CD20 antibody rituximab. *Haematologica*. 2004 Jun;89(6):753–754. PMID: 15194546.
242. Herrlinger U, Küker W, Uhl M, et al. Neuro-Oncology Working Group of the German Society. NOA-03 trial of high-dose methotrexate in primary central nervous system lymphoma: final report. *Ann Neurol*. 2005 Jun;57(6):843–847. https://doi.org/10.1002/ana.20495. PMID: 15929034.
243. Doolittle ND, Dósa E, Fu R, et al. Preservation of cognitive function in primary CNS lymphoma survivors a median of 12 years after enhanced chemotherapy delivery. *J Clin Oncol*. 2013 Nov 1;31(31):4026–4027. https://doi.org/10.1200/JCO.2013.52.7747. Epub 2013 Oct 7. PMID: 24101051; PMCID: PMC3805933.
244. Fliessbach K, Helmstaedter C, Urbach H, et al. Neuropsychological outcome after chemotherapy for primary CNS lymphoma: a prospective study. *Neurology*. 2005 Apr 12;64(7):1184–1188. https://doi.org/10.1212/01.WNL.0000156350.49336.E2. PMID: 15824344.
245. Fliessbach K, Urbach H, Helmstaedter C, et al. Cognitive performance and magnetic resonance imaging findings after high-dose systemic and intraventricular chemotherapy for primary central nervous system lymphoma. *Arch Neurol*. 2003 Apr;60(4):563–568. https://doi.org/10.1001/archneur.60.4.563. PMID: 12707070.
246. Correa DD, Shi W, Abrey LE, et al. Cognitive functions in primary CNS lymphoma after single or combined modality regimens. *Neuro Oncol*. 2012 Jan;14(1):101–108. https://doi.org/10.1093/neuonc/nor186. Epub 2011 Oct 19. PMID: 22013168; PMCID: PMC3245999.
247. Harder H, Holtel H, Bromberg JE, et al. Cognitive status and quality of life after treatment for primary CNS lymphoma. *Neurology*. 2004 Feb 24;62(4):544–547. https://doi.org/10.1212/wnl.62.4.544. PMID: 14981168.
248. Linnebank M, Moskau S, Jürgens A, et al. Association of genetic variants of methionine metabolism with methotrexate-induced CNS white matter changes in patients with primary CNS lymphoma. *Neuro Oncol*. 2009 Feb;11(1):2–8. https://doi.org/10.1215/15228517-2008-082. Epub 2008 Sep 19. PMID: 18806228; PMCID: PMC2718955.
249. Omuro AM, Ben-Porat LS, Panageas KS, et al. Delayed neurotoxicity in primary central nervous system lymphoma. *Arch Neurol*. 2005 Oct;62(10):1595–1600. https://doi.org/10.1001/archneur.62.10.1595. PMID: 16216945.
250. Gavrilovic IT, Hormigo A, Yahalom J, DeAngelis LM, Abrey LE. Long-term follow-up of high-dose methotrexate-based therapy with and without whole brain irradiation for newly diagnosed primary CNS lymphoma. *J Clin Oncol*. 2006 Oct 1;24(28):4570–4574. https://doi.org/10.1200/JCO.2006.06.6910. PMID: 17008697.
251. Monje ML, Palmer T. Radiation injury and neurogenesis. *Curr Opin Neurol*. 2003 Apr;16(2):129–134. https://doi.org/10.1097/01.wco.0000063772.81810.b7. PMID: 12644738.
252. Lai R, Abrey LE, Rosenblum MK, DeAngelis LM. Treatment-induced leukoencephalopathy in primary CNS lymphoma: a clinical and autopsy study. *Neurology*. 2004 Feb 10;62(3):451–456. https://doi.org/10.1212/01.wnl.0000106941.51340.a2. PMID: 14872029.
253. Grimm SA, Yahalom J, Abrey LE, DeAngelis LM. Retinopathy in survivors of primary central nervous system lymphoma. *Neurology*. 2006 Dec 12;67(11):2060–2062. https://doi.org/10.1212/01.wnl.0000247679.87738.1e. PMID: 17159122.

254. Nelson DF, Martz KL, Bonner H, et al. Non-Hodgkin's lymphoma of the brain: can high dose, large volume radiation therapy improve survival? Report on a prospective trial by the Radiation Therapy Oncology Group (RTOG): RTOG 8315. *Int J Radiat Oncol Biol Phys*. 1992;23(1):9–17.
255. Shibamoto Y, Ogino H, Hasegawa M, et al. Results of radiation monotherapy for primary central nervous system lymphoma in the 1990s. *Int J Radiat Oncol Biol Phys*. 2005;62(3):809–813.
256. Omuro AMP, DeAngelis LM, Karrison T, et al. Randomized phase II study of rituximab, methotrexate (MTX), procarbazine, vincristine, and cytarabine (R-MPV-A) with and without low-dose whole-brain radiotherapy (LD-WBRT) for newly diagnosed primary CNS lymphoma (PCNSL). *J Clin Oncol*. 2020;38:2501. https://doi.org/10.1200/JCO.2020.38.15_suppl.2501.
257. Seidel C, Viehweger C, Kortmann RD. Is there an indication for first line radiotherapy in primary CNS lymphoma? *Cancers (Basel)*. 2021 May 25;13(11):2580 https://doi.org/10.3390/cancers13112580. PMID: 34070309; PMCID: PMC8197540.
258. Lee TH, Lee JH, Chang JH, et al. Reduced-dose whole-brain radiotherapy with tumor bed boost after upfront high-dose methotrexate for primary central nervous system lymphoma. *Radiat Oncol J*. 2020 Mar;38(1):35–43. https://doi.org/10.3857/roj.2020.00052. Epub 2020 Mar 25. PMID: 32229807; PMCID: PMC7113152.
259. Panagiotakos G, Alshamy G, Chan B, et al. Long-term impact of radiation on the stem cell and oligodendrocyte precursors in the brain. *PLoS One*. 2007 Jul 11;2(7):e588 https://doi.org/10.1371/journal.pone.0000588. PMID: 17622341; PMCID: PMC1913551.
260. Mizumatsu S, Monje ML, Morhardt DR, et al. Extreme sensitivity of adult neurogenesis to low doses of X-irradiation. *Cancer Res*. 2003 Jul 15;63(14):4021–4027. PMID: 12874001.
261. Alvarnas JC, Negrin RS, Horning SJ, et al. High-dose therapy with hematopoietic cell transplantation for patients with central nervous system involvement by non-Hodgkin's lymphoma. *Biol Blood Marrow Transplant*. 2000;6(3A):352–358. https://doi.org/10.1016/s1083-8791(00)70060-7. PMID: 10905773.
262. Miyao K, Sakemura R, Imai K, et al. Upfront autologous stem-cell transplantation with melphalan, cyclophosphamide, etoposide, and dexamethasone (LEED) in patients with newly diagnosed primary central nervous system lymphoma. *Int J Hematol*. 2014 Aug;100(2):152–158. https://doi.org/10.1007/s12185-014-1608-9. Epub 2014 Jun 20. PMID: 24947495.
263. Brevet M, Garidi R, Gruson B, Royer B, Vaida I, Damaj G. First-line autologous stem cell transplantation in primary CNS lymphoma. *Eur J Haematol*. 2005 Oct;75(4):288–292. https://doi.org/10.1111/j.1600-0609.2005.00508.x. PMID: 16146534.
264. Abrey LE, Moskowitz CH, Mason WP, et al. Intensive methotrexate and cytarabine followed by high-dose chemotherapy with autologous stem-cell rescue in patients with newly diagnosed primary CNS lymphoma: an intent-to-treat analysis. *J Clin Oncol*. 2003 Nov 15;21(22):4151–4156. https://doi.org/10.1200/JCO.2003.05.024. PMID: 14615443.
265. Colombat P, Lemevel A, Bertrand P, et al. High-dose chemotherapy with autologous stem cell transplantation as first-line therapy for primary CNS lymphoma in patients younger than 60 years: a multicenter phase II study of the GOELAMS group. *Bone Marrow Transplant*. 2006 Sep;38(6):417–420. https://doi.org/10.1038/sj.bmt.1705452. PMID: 16951691.
266. Korfel A, Elter T, Thiel E, et al. Phase II study of central nervous system (CNS)-directed chemotherapy including high-dose chemotherapy with autologous stem cell transplantation for CNS relapse of aggressive lymphomas. *Haematologica*. 2013 Mar;98(3):364–370. https://doi.org/10.3324/haematol.2012.077917. Epub 2012 Dec 14. PMID: 23242601; PMCID: PMC3659948.
267. Soussain C, Suzan F, Hoang-Xuan K, et al. Results of intensive chemotherapy followed by hematopoietic stem-cell rescue in 22 patients with refractory or recurrent primary CNS lymphoma or intraocular lymphoma. *J Clin Oncol*. 2001 Feb 1;19(3):742–749. https://doi.org/10.1200/JCO.2001.19.3.742. PMID: 11157026.
268. Cote GM, Hochberg EP, Muzikansky A, et al. Autologous stem cell transplantation with thiotepa, busulfan, and cyclophosphamide (TBC) conditioning in patients with CNS involvement by non-Hodgkin lymphoma. *Biol Blood Marrow Transplant*. 2012 Jan;18(1):76–83. https://doi.org/10.1016/j.bbmt.2011.07.006. Epub 2011 Jul 13. PMID: 21749848.
269. Young PA, Gaut D, Kimaiyo DK, et al. Durable survival outcomes in primary and secondary central nervous system lymphoma after high-dose chemotherapy and autologous stem cell transplantation using a thiotepa, busulfan, and cyclophosphamide conditioning regimen. *Clin Lymphoma Myeloma Leuk*. 2020 Jul;20(7):468–479. https://doi.org/10.1016/j.clml.2020.02.009. Epub 2020 Feb 20. PMID: 32229199; PMCID: PMC7138188.
270. Montemurro M, Kiefer T, Schüler F, et al. Primary central nervous system lymphoma treated with high-dose methotrexate, high-dose busulfan/thiotepa, autologous stem-cell transplantation and response-adapted whole-brain radiotherapy: results of the multicenter Ostdeutsche Studiengruppe Hamato-Onkologie OSHO-53 phase II study. *Ann Oncol*. 2007 Apr;18(4):665–671. https://doi.org/10.1093/annonc/mdl458. Epub 2006 Dec 21. PMID: 17185743.
271. Kasenda B, Ihorst G, Schroers R, et al. High-dose chemotherapy with autologous haematopoietic stem cell support for relapsed or refractory primary CNS lymphoma: a prospective multicentre trial by the German Cooperative PCNSL study group. *Leukemia*. 2017 Dec;31(12):2623–2629. https://doi.org/10.1038/leu.2017.170. Epub 2017 May 31. PMID: 28559537.
272. Illerhaus G, Marks R, Ihorst G, et al. High-dose chemotherapy with autologous stem-cell transplantation and hyperfractionated radiotherapy as first-line treatment of primary CNS lymphoma. *J Clin Oncol*. 2006 Aug 20;24(24):3865–3870. https://doi.org/10.1200/JCO.2006.06.2117. Epub 2006 Jul 24. PMID: 16864853.
273. Scordo M, Wang TP, Ahn KW, et al. Outcomes associated with thiotepa-based conditioning in patients with primary central nervous system lymphoma after autologous hematopoietic cell transplant. *JAMA Oncol*. 2021 May 6. https://doi.org/10.1001/jamaoncol.2021.1074. Epub ahead of print. PMID: 33956047.
274. Kondo E, Ikeda T, Izutsu K, et al. Adult Lymphoma Working Group of the Japan Society for Hematopoietic Cell Transplantation. High-dose chemotherapy with autologous stem cell transplantation in primary central nervous system lymphoma: data from the Japan Society for Hematopoietic Cell Transplantation registry. *Biol Blood Marrow Transplant*. 2019 May;25(5):899–905. https://doi.org/10.1016/j.bbmt.2019.01.020. Epub 2019 Jan 18. PMID: 30664936.
275. Jahnke K, Korfel A, Martus P, et al. German Primary Central Nervous System Lymphoma Study Group (G-PCNSL-SG). High-dose methotrexate toxicity in elderly patients with primary central nervous system lymphoma. *Ann Oncol*. 2005 Mar;16(3):445–459. https://doi.org/10.1093/annonc/mdi075. Epub 2005 Jan 14. PMID: 15653703.
276. Ng S, Rosenthal MA, Ashley D, Cher L. High-dose methotrexate for primary CNS lymphoma in the elderly. *Neuro Oncol*. 2000 Jan;2(1):40–44. https://doi.org/10.1093/neuonc/2.1.40. PMID: 11302253; PMCID: PMC1920699.
277. Siegal T, Bairey O. Primary CNS lymphoma in the elderly: the challenge. *Acta Haematol*. 2019;141(3):138–145. https://doi.org/10.1159/000495284. Epub 2019 Feb 15. PMID: 30783026.
278. Ferreri AJ. How I treat primary CNS lymphoma. *Blood*. 2011 Jul 21;118(3):510–522. https://doi.org/10.1182/blood-2011-03-321349. Epub 2011 May 25. PMID: 21613254.
279. Yazan Samhouri, Mustafa Ali Moaath, Jayakrishnan T, et al. Patterns of treatment and survival in elderly patients with primary CNS lymphoma. An 11-year population-based analysis. *Blood*. 2020;136(Supplement 1):2–3.
280. Panageas KS, Elkin EB, Ben-Porat L, Deangelis LM, Abrey LE. Patterns of treatment in older adults with primary central nervous system lymphoma. *Cancer*. 2007 Sep 15;110(6):1338–1344. https://doi.org/10.1002/cncr.22907. PMID: 17647247.
281. Ferreri AJ, Blay JY, Reni M, et al. Prognostic scoring system for primary CNS lymphomas: the International Extranodal Lymphoma Study Group experience. *J Clin Oncol*. 2003 Jan 15;21(2):266–272. https://doi.org/10.1200/JCO.2003.09.139. PMID: 12525518.
282. Abrey LE, Ben-Porat L, Panageas KS, et al. Primary central nervous system lymphoma: the Memorial Sloan-Kettering Cancer Center prognostic model. *J Clin Oncol*. 2006 Dec 20;24(36):5711–5715. https://doi.org/10.1200/JCO.2006.08.2941. Epub 2006 Nov 20. PMID: 17116938.
283. Chang CC, Kampalath B, Schultz C, et al. Expression of p53, c-Myc, or Bcl-6 suggests a poor prognosis in primary central nervous system diffuse large B-cell lymphoma among immunocompetent individuals. *Arch Pathol Lab Med*. 2003 Feb;127(2):208–212. https://doi.org/10.5858/2003-127-208-EOPMOB. PMID: 12562237.
284. Levy O, Deangelis LM, Filippa DA, Panageas KS, Abrey LE. BCL-6 predicts improved prognosis in primary central nervous system lymphoma. *Cancer*. 2008 Jan 1;112(1):151–156. https://doi.org/10.1002/cncr.23149. PMID: 17999414.
285. Braaten KM, Betensky RA, de Leval L, et al. BCL-6 expression predicts improved survival in patients with primary central nervous system lymphoma. *Clin Cancer Res*. 2003 Mar;9(3):1063–1069. PMID: 12631608.
286. Son SM, Ha SY, Yoo HY, Oh D, Kim SJ, Kim WS, Ko YH. Prognostic impact of MYC protein expression in central nervous system diffuse large B-cell lymphoma: comparison with MYC rearrangement and MYC mRNA

expression. *Mod Pathol*. 2017 Jan;30(1):4–14. https://doi.org/10.1038/modpathol.2016.56. Epub 2016 Sep 30. PMID: 27687005.Son.

287. Kim S, Nam SJ, Kwon D, et al. MYC. and BCL2 overexpression is associated with a higher class of Memorial Sloan-Kettering Cancer Center prognostic model and poor clinical outcome in primary diffuse large B-cell lymphoma of the central nervous system. *BMC Cancer*. 2016 Jun 10;16:363 https://doi.org/10.1186/s12885-016-2397-8. PMID: 27286976; PMCID: PMC4903010.
288. Toffolatti L, Scquizzato E, Cavallin S, et al. MGMT promoter methylation and correlation with protein expression in primary central nervous system lymphoma. *Virchows Arch*. 2014 Nov;465(5):579–586. https://doi.org/10.1007/s00428-014-1622-6. Epub 2014 Jul 17. PMID: 25031012.
289. Adachi J, Mishima K, Wakiya K, et al. O^6-methylguanine-DNA methyltransferase promoter methylation in 45 primary central nervous system lymphomas: quantitative assessment of methylation and response to temozolomide treatment. *J Neurooncol*. 2012 Mar;107(1):147–153. https://doi.org/10.1007/s11060-011-0721-3. Epub 2011 Oct 4. PMID: 21968944.
290. Hayano A, Komohara Y, et al. Programmed cell death ligand 1 expression in primary central nervous system lymphomas: a clinicopathological study. *Anticancer Res*. 2017 Oct;37(10):5655–5666. https://doi.org/10.21873/anticanres.12001. PMID: 28982883.
291. Ou A, Sumrall A, Phuphanich S, et al. Primary CNS lymphoma commonly expresses immune response biomarkers. *Neurooncol Adv*. 2020 Jan-Dec;2(1):vdaa018 https://doi.org/10.1093/noajnl/vdaa018. Epub 2020 Feb 19. PMID: 32201861; PMCID: PMC7067145.
292. Alame M, Pirel M, Costes-Martineau V, et al. Characterisation of tumour microenvironment and immune checkpoints in primary central nervous system diffuse large B cell lymphomas. *Virchows Arch*. 2020 Jun;476(6):891–902. https://doi.org/10.1007/s00428-019-02695-6. Epub 2019 Dec 6. PMID: 31811434.
293. Four M, Cacheux V, Tempier A, et al. PD1. and PDL1 expression in primary central nervous system diffuse large B-cell lymphoma are frequent and expression of PD1 predicts poor survival. *Hematol Oncol*. 2017 Dec;35(4):487–496. https://doi.org/10.1002/hon.2375. Epub 2016 Dec 13. PMID: 27966264.
294. Roth P, Keller A, Hoheisel JD, et al. Differentially regulated miRNAs as prognostic biomarkers in the blood of primary CNS lymphoma patients. *Eur J Cancer*. 2015 Feb;51(3):382–390. https://doi.org/10.1016/j.ejca.2014.10.028. Epub 2014 Dec 17.
295. Takashima Y, Kawaguchi A, Iwadate Y, et al. MicroRNA signature constituted of miR-30d, miR-93, and miR-181b is a promising prognostic marker in primary central nervous system lymphoma. *PLoS One*. 2019 Jan 7;14(1):e0210400 https://doi.org/10.1371/journal.pone.0210400. PMID: 30615673; PMCID: PMC6322780.
296. Yang K, Wang S, Cheng Y, et al. Role of miRNA-21 in the diagnosis and prediction of treatment efficacy of primary central nervous system lymphoma. *Oncol Lett*. 2019 Mar;17(3):3475–3481. https://doi.org/10.3892/ol.2019.9941. Epub 2019 Jan 17. PMID: 30867786; PMCID: PMC6396200.
297. Reni M, Ferreri AJ, Villa E. Second-line treatment for primary central nervous system lymphoma. *Br J Cancer*. 1999 Feb;79(3-4):530–534. https://doi.org/10.1038/sj.bjc.6690083. PMID: 10027325; PMCID: PMC2362452.
298. Dietrich J, Versmee L, Drappatz J, et al. Pemetrexed in recurrent or progressive central nervous system lymphoma: a phase i multicenter clinical trial. *Oncologist*. 2020 Sep;25(9):747–e1273. https://doi.org/10.1634/theoncologist.2020-0489. Epub 2020 Jul 1. PMID: 32520407; PMCID: PMC7485351.
299. Zhao HT, Chen J, Shi SB, Tian J, Tao RJ. Pemetrexed plus rituximab as second-line treatment for primary central nervous system lymphoma. *Med Oncol*. 2015 Jan;32(1):351 https://doi.org/10.1007/s12032-014-0351-7. Epub 2014 Nov 27. PMID: 25428379.
300. Raizer JJ, Rademaker A, Evens AM, et al. Pemetrexed in the treatment of relapsed/refractory primary central nervous system lymphoma. *Cancer*. 2012 Aug 1;118(15):3743–3748. https://doi.org/10.1002/cncr.26709. Epub 2011 Dec 16. PMID: 22179954.
301. Nayak L, Abrey LE, Drappatz J, et al. North American Brain Tumor Consortium. Multicenter phase II study of rituximab and temozolomide in recurrent primary central nervous system lymphoma. *Leuk Lymphoma*. 2013 Jan;54(1):58–61. https://doi.org/10.3109/10428194.2012.698736. Epub 2012 Jul 9. PMID: 22656234; PMCID: PMC4802006.
302. Reni M, Zaja F, Mason W, et al. Temozolomide as salvage treatment in primary brain lymphomas. *Br J Cancer*. 2007 Mar 26;96(6):864–867. https://doi.org/10.1038/sj.bjc.6603660. Epub 2007 Feb 27. PMID: 17325700; PMCID: PMC2360092.
303. Voloschin AD, Betensky R, Wen PY, Hochberg F, Batchelor T. Topotecan as salvage therapy for relapsed or refractory primary central nervous system lymphoma. *J Neurooncol*. 2008 Jan;86(2):211–215. https://doi.org/10.1007/s11060-007-9464-6. Epub 2007 Sep 21. PMID: 17896078.
304. Fischer L, Thiel E, Klasen HA, Birkmann J, Jahnke K, Martus P, Korfel A. Prospective trial on topotecan salvage therapy in primary CNS lymphoma. *Ann Oncol*. 2006 Jul;17(7):1141–1145. https://doi.org/10.1093/annonc/mdl070. Epub 2006 Apr 7. PMID: 16603598. 307.
305. Soussain C, Choquet S, Blonski M, et al. Ibrutinib monotherapy for relapse or refractory primary CNS lymphoma and primary vitreoretinal lymphoma: Final analysis of the phase II 'proof-of-concept' iLOC study by the Lymphoma study association (LYSA) and the French oculo-cerebral lymphoma (LOC) network. *Eur J Cancer*. 2019 Aug;117:121–130. https://doi.org/10.1016/j.ejca.2019.05.024. Epub 2019 Jul 3. PMID: 31279304.
306. Ghesquieres H, Chevrier M, Laadhari M, et al. Lenalidomide in combination with intravenous rituximab (REVRI) in relapsed/refractory primary CNS lymphoma or primary intraocular lymphoma: a multicenter prospective 'proof of concept' phase II study of the French Oculo-Cerebral lymphoma (LOC) Network and the Lymphoma Study Association (LYSA). *Ann Oncol*. 2019 Apr 1;30(4):621–628. https://doi.org/10.1093/annonc/mdz032. PMID: 30698644.
307. Tun HW, Johnston PB, DeAngelis LM, et al. Phase 1 study of pomalidomide and dexamethasone for relapsed/refractory primary CNS or vitreoretinal lymphoma. *Blood*. 2018 Nov 22;132(21):2240–2248. https://doi.org/10.1182/blood-2018-02-835496. Epub 2018 Sep 27. PMID: 30262659; PMCID: PMC6265643.
308. Korfel A, Schlegel U, Herrlinger U, et al. Phase II trial of temsirolimus for relapsed/refractory primary CNS lymphoma. *J Clin Oncol*. 2016 May 20;34(15):1757–1763. https://doi.org/10.1200/JCO.2015.64.9897. Epub 2016 Mar 14. PMID: 26976424.
309. Ambady P, Szidonya L, Firkins J, et al. Combination immunotherapy as a non-chemotherapy alternative for refractory or recurrent CNS lymphoma. *Leuk Lymphoma*. 2019 Feb;60(2):515–518. https://doi.org/10.1080/10428194.2018.1480771. Epub 2018 Jul 22. PMID: 30033836; PMCID: PMC6342672.
310. Pentsova E, Deangelis LM, Omuro A. Methotrexate re-challenge for recurrent primary central nervous system lymphoma. *J Neurooncol*. 2014 Mar;117(1):161–165. https://doi.org/10.1007/s11060-014-1370-0. Epub 2014 Jan 31. PMID: 24481997; PMCID: PMC5256683.
311. Makino K, Nakamura H, Hide T, Kuratsu J. Salvage treatment with temozolomide in refractory or relapsed primary central nervous system lymphoma and assessment of the MGMT status. *J Neurooncol*. 2012 Jan;106(1):155–160. https://doi.org/10.1007/s11060-011-0652-z. Epub 2011 Jul 1. PMID: 21720808.
312. Enting RH, Demopoulos A, DeAngelis LM, Abrey LE. Salvage therapy for primary CNS lymphoma with a combination of rituximab and temozolomide. *Neurology*. 2004 Sep 14;63(5):901–903. https://doi.org/10.1212/01.wnl.0000137050.43114.42. PMID: 15365145.
313. Chamberlain MC. Salvage therapy with bendamustine for methotrexate refractory recurrent primary CNS lymphoma: a retrospective case series. *J Neurooncol*. 2014 May;118(1):155–162. https://doi.org/10.1007/s11060-014-1411-8. Epub 2014 Mar 1. PMID: 24584709.
314. Herrlinger U, Brugger W, Bamberg M, Küker W, Dichgans J, Weller M. PCV salvage chemotherapy for recurrent primary CNS lymphoma. *Neurology*. 2000 Apr 25;54(8):1707–1708. https://doi.org/10.1212/wnl.54.8.1707. PMID: 10762527.
315. Arellano-Rodrigo E, López-Guillermo A, Bessell EM, Nomdedeu B, Montserrat E, Graus F. Salvage treatment with etoposide (VP-16), ifosfamide and cytarabine (Ara-C) for patients with recurrent primary central nervous system lymphoma. *Eur J Haematol*. 2003 Apr;70(4):219–224. https://doi.org/10.1034/j.1600-0609.2003.00045.x. PMID: 12656744.
316. Welch MR, Sauter CS, Matasar MJ. Autologous stem cell transplant in recurrent or refractory primary or secondary central nervous system lymphoma using thiotepa, busulfan and cyclophosphamide. *Leuk Lymphoma*. 2015 Feb;56(2):361–367. https://doi.org/10.3109/10428194.2014.916800. Epub 2014 Jun 27. PMID: 24745937.
317. Holdhoff M, Wagner-Johnston N, Roschewski M. Systemic approach to recurrent primary cns lymphoma: perspective on current and emerging treatment strategies. *Onco Targets Ther*. 2020 Aug 20;13:8323–8335. https://doi.org/10.2147/OTT.S192379. PMID: 32903865; PMCID: PMC7445492.
318. Chamoun K, Choquet S, Boyle E, et al. Ibrutinib monotherapy in relapsed/refractory CNS lymphoma: a retrospective case series. *Neurology*. 2017 Jan 3;88(1):101–102. https://doi.org/10.1212/WNL.0000000000003420. Epub 2016 Nov 18. PMID: 27864520.
319. Lionakis MS, Dunleavy K, Roschewski M, et al. Inhibition of B cell receptor signaling by ibrutinib in primary CNS lymphoma. *Cancer Cell*. 2017 Jun 12;31(6):833–843.e5. https://doi.org/10.1016/j.ccell.2017.04.012. Epub 2017 May 25. PMID: 28552327; PMCID: PMC5571650.

320. Grommes C, Younes A. Ibrutinib in PCNSL: the curious cases of clinical responses and aspergillosis. *Cancer Cell*. 2017 Jun 12;31(6):731–733. https://doi.org/10.1016/j.ccell.2017.05.004. Epub 2017 May 25. PMID: 28552326; PMCID: PMC5572136.
321. Narita Y, Nagane M, Mishima K, et al. Phase I/II study of tirabrutinib, a second-generation Bruton's tyrosine kinase inhibitor, in relapsed/refractory primary central nervous system lymphoma. *Neuro Oncol*. 2021 Jan 30; 23(1):122–133. https://doi.org/10.1093/neuonc/noaa145. PMID: 32583848; PMCID: PMC7850159.
322. Cheng Q, Wang J, Lv C, Xu J. Successful management of a patient with refractory primary central nervous system lymphoma by zanubrutinib. *Onco Targets Ther*. 2021 May 24;14:3367–3372. https://doi.org/10.2147/OTT.S309408. PMID: 34079282; PMCID: PMC8163630.
323. Rubenstein JL, Geng H, Fraser EJ, et al. Phase 1 investigation of lenalidomide/rituximab plus outcomes of lenalidomide maintenance in relapsed CNS lymphoma. *Blood Adv*. 2018 Jul 10;2(13):1595–1607. https://doi.org/10.1182/bloodadvances.2017014845. PMID: 29986852; PMCID: PMC6039666.
324. Rubenstein JL, Fridlyand J, Abrey L, et al. Phase I study of intraventricular administration of rituximab in patients with recurrent CNS and intraocular lymphoma. *J Clin Oncol*. 2007 Apr 10;25(11):1350–1356. https://doi.org/10.1200/JCO.2006.09.7311. Epub 2007 Feb 20. PMID: 17312328.
325. Kadoch C, Li J, Wong VS, et al. Complement activation and intraventricular rituximab distribution in recurrent central nervous system lymphoma. *Clin Cancer Res*. 2014 Feb 15;20(4):1029–1041. https://doi.org/10.1158/1078-0432.CCR-13-0474. Epub 2013 Nov 4. PMID: 24190981; PMCID: PMC3944388.
326. Rubenstein JL, Li J, Chen L, et al. Multicenter phase 1 trial of intraventricular immunochemotherapy in recurrent CNS lymphoma. *Blood*. 2013 Jan 31;121(5):745–751. https://doi.org/10.1182/blood-2012-07-440974. Epub 2012 Nov 29. PMID: 23197589; PMCID: PMC3563362.
327. Nayak L, Iwamoto FM, LaCasce A, et al. PD-1 blockade with nivolumab in relapsed/refractory primary central nervous system and testicular lymphoma. *Blood*. 2017 Jun 8;129(23):3071–3073. https://doi.org/10.1182/blood-2017-01-764209. Epub 2017 Mar 29. PMID: 28356247; PMCID: PMC5766844.
328. Karschnia P, Blobner J, Teske N, et al. CAR T-cells for CNS lymphoma: driving into new terrain? *Cancers (Basel)*. 2021 May 20;13(10):2503 https://doi.org/10.3390/cancers13102503. PMID: 34065471; PMCID: PMC8161128.
329. Abramson JS, McGree B, Noyes S, et al. Anti-CD19 CAR T cells in CNS diffuse large-B-cell lymphoma. *N Engl J Med*. 2017 Aug 24;377(8): 783–784. https://doi.org/10.1056/NEJMc1704610. PMID: 28834486.
330. Frigault MJ, Dietrich J, Martinez-Lage M, et al. Tisagenlecleucel CAR T-cell therapy in secondary CNS lymphoma. *Blood*. 2019 Sep 12;134 (11):860–866. https://doi.org/10.1182/blood.2019001694. Epub 2019 Jul 18. PMID: 31320380; PMCID: PMC7022436.
331. Siddiqi T, Wang X, Palmer J, et al. CD19-targeting CAR-T cell therapy in CNS lymphoma. *Blood*. 2019;134:4075. https://doi.org/10.1182/blood-2019-123765.
332. Li T, Zhao L, Zhang Y, et al. CAR T-cell therapy is effective but not long-lasting in B-cell lymphoma of the brain. *Front Oncol*. 2020 Aug 4;10:1306 https://doi.org/10.3389/fonc.2020.01306. PMID: 32903866; PMCID: PMC7438944.
333. Gaut D, Schiller GJ. Hematopoietic stem cell transplantation in primary central nervous system lymphoma: a review of the literature. *Int J Hematol*. 2019 Mar;109(3):260–277. https://doi.org/10.1007/s12185-019-02594-1. Epub 2019 Jan 22. PMID: 30671909.
334. Waterhouse M, Bartsch I, Bertz H, Duyster J, Finke J. Cerebrospinal fluid chimerism analysis in patients with neurological symptoms after allogeneic cell transplantation. *Bone Marrow Transplant*. 2016 Jan;51(1):127–131. https://doi.org/10.1038/bmt.2015.226. Epub 2015 Oct 5. PMID: 26437058.
335. Spruit JL, Fagoaga OR, Savaşan S. Donor cell chimerism evaluation in cerebrospinal fluid by short tandem repeat analysis following allogeneic hematopoietic stem cell transplantation. *Hum Immunol*. 2018 Jul;79(7): 537–538. https://doi.org/10.1016/j.humimm.2018.04.001. Epub 2018 Apr 11. PMID: 29654803; PMCID: PMC6854661.
336. Atilla E, Sahin U, Atilla PA, et al. Allogeneic stem cell transplantation for relapsed primary central nervous system lymphoma: Is it feasible? *Hematol Oncol Stem Cell Ther*. 2019 Dec;12(4):220–225. https://doi.org/10.1016/j.hemonc.2018.02.001. Epub 2018 Mar 16. PMID: 29559300.
337. Varadi G, Or R, Kapelushnik J, et al. Graft-versus-lymphoma effect after allogeneic peripheral blood stem cell transplantation for primary central nervous system lymphoma. *Leuk Lymphoma*. 1999 Jun;34(1-2):185–190. https://doi.org/10.3109/10428199909083396. PMID: 10350348.
338. Mika T, Ladigan S, Baraniskin A, et al. Allogeneic hematopoietic stem cell transplantation for primary central nervous system lymphoma. *Haematologica*. 2020 Apr;105(4):e160–e163. https://doi.org/10.3324/haematol.2019.227199. Epub 2019 Aug 8. PMID: 31399528; PMCID: PMC7109742.
339. Nguyen PL, Chakravarti A, Finkelstein DM, Hochberg FH, Batchelor TT, Loeffler JS. Results of whole-brain radiation as salvage of methotrexate failure for immunocompetent patients with primary CNS lymphoma. *J Clin Oncol*. 2005 Mar 1;23(7):1507–1513. https://doi.org/10.1200/JCO.2005.01.161. PMID: 15735126.
340. Hottinger AF, DeAngelis LM, Yahalom J, Abrey LE. Salvage whole brain radiotherapy for recurrent or refractory primary CNS lymphoma. *Neurology*. 2007 Sep 11;69(11):1178–1782. https://doi.org/10.1212/01.wnl.0000276986.19602.c1. PMID: 17846417.
341. Palmer JD, Bhamidipati D, Shukla G, Epperla N, Glass J, Kim L, Shi W. Outcomes after stereotactic radiosurgery for CNS lymphoma. *J Neurooncol*. 2020 Apr;147(2):465–476. https://doi.org/10.1007/s11060-020-03444-5. Epub 2020 Feb 27. PMID: 32108296.
342. Kuitunen H, Kaprio E, Karihtala P, et al. Impact of central nervous system (CNS) prophylaxis on the incidence of CNS relapse in patients with high-risk diffuse large B cell/follicular grade 3B lymphoma. *Ann Hematol*. 2020 Aug;99(8):1823–1831. https://doi.org/10.1007/s00277-020-04140-0. Epub 2020 Jun 23. PMID: 32577843; PMCID: PMC7340636.
343. Kridel R, Dietrich PY. Prevention of CNS relapse in diffuse large B-cell lymphoma. *Lancet Oncol*. 2011 Dec;12(13):1258–1266. https://doi.org/10.1016/S1470-2045(11)70140-1. Epub 2011 Sep 18. PMID: 21933751.
344. Miyazaki K, Yamaguchi M, Suzuki R, et al. CD5-positive diffuse large B-cell lymphoma: a retrospective study in 337 patients treated by chemotherapy with or without rituximab. *Ann Oncol*. 2011 Jul;22(7):1601–1607. https://doi.org/10.1093/annonc/mdq627. Epub 2011 Jan 3. PMID: 21199885.
345. Schmitz N, Zeynalova S, Nickelsen M, et al. CNS International Prognostic Index: a risk model for CNS relapse in patients with diffuse large B-cell lymphoma treated with R-CHOP. *J Clin Oncol*. 2016 Sep 10;34(26): 3150–3156. https://doi.org/10.1200/JCO.2015.65.6520. Epub 2016 Jul 5. PMID: 27382100.
346. Gleeson M, Counsell N, Cunningham D, et al. Central nervous system relapse of diffuse large B-cell lymphoma in the rituximab era: results of the UK NCRI R-CHOP-14 versus 21 trial. *Ann Oncol*. 2017 Oct 1;28 (10):2511–2516. https://doi.org/10.1093/annonc/mdx353. PMID: 28961838; PMCID: PMC5834096.
347. Eyre TA, Kirkwood AA, Wolf J, et al. Stand-alone intrathecal central nervous system (CNS) prophylaxis provide unclear benefit in reducing CNS relapse risk in elderly DLBCL patients treated with R-CHOP and is associated increased infection-related toxicity. *Br J Haematol*. 2019 Oct;187 (2):185–194. https://doi.org/10.1111/bjh.16070. Epub 2019 Jun 20. PMID: 31222719.
348. Eyre TA, Djebbari F, Kirkwood AA, Collins GP. Efficacy of central nervous system prophylaxis with stand-alone intrathecal chemotherapy in diffuse large B-cell lymphoma patients treated with anthracycline-based chemotherapy in the rituximab era: a systematic review. *Haematologica*. 2020 Jul;105(7):1914–1924. https://doi.org/10.3324/haematol.2019.229948. Epub 2019 Sep 5. PMID: 31488560; PMCID: PMC7327624.
349. Savage KJ, Slack GW, Mottok A, et al. Impact of dual expression of MYC and BCL2 by immunohistochemistry on the risk of CNS relapse in DLBCL. *Blood*. 2016 May 5;127(18):2182–2188. https://doi.org/10.1182/blood-2015-10-676700. Epub 2016 Feb 1. PMID: 26834242.
350. Zucca E, Conconi A, Mughal TI, et al. International Extranodal Lymphoma Study Group. Patterns of outcome and prognostic factors in primary large-cell lymphoma of the testis in a survey by the International Extranodal Lymphoma Study Group. *J Clin Oncol*. 2003 Jan 1;21(1):20–27. https://doi.org/10.1200/JCO.2003.11.141. PMID: 12506165.
351. Oki Y, Noorani M, Lin P, et al. Double hit lymphoma: the MD Anderson Cancer Center clinical experience. *Br J Haematol*. 2014 Sep;166(6): 891–901. https://doi.org/10.1111/bjh.12982. Epub 2014 Jun 18. PMID: 24943107.
352. Shimada K, Murase T, Matsue K, et al. IVL Study Group in Japan. Central nervous system involvement in intravascular large B-cell lymphoma: a retrospective analysis of 109 patients. *Cancer Sci*. 2010 Jun;101(6): 1480–1486. https://doi.org/10.1111/j.1349-7006.2010.01555.x. Epub 2010 Mar 6. PMID: 20412122.
353. Vitolo U, Chiappella A, Ferreri AJ, et al. First-line treatment for primary testicular diffuse large B-cell lymphoma with rituximab-CHOP, CNS prophylaxis, and contralateral testis irradiation: final results of an international phase II trial. *J Clin Oncol*. 2011 Jul 10;29(20):2766–2772. https://doi.org/10.1200/JCO.2010.31.4187. Epub 2011 Jun 6. PMID: 21646602.

354. Wilson MR, Eyre TA, Martinez-Calle N, et al. Timing of high-dose methotrexate CNS prophylaxis in DLBCL: an analysis of toxicity and impact on R-CHOP delivery. *Blood Adv*. 2020 Aug 11;4(15):3586–3593. https://doi.org/10.1182/bloodadvances.2020002421. PMID: 32761231; PMCID: PMC7422120.

355. Siddiqi T, Wang X, Blanchard MS, et al. CD19-directed CAR. T-cell therapy for treatment of primary CNS lymphoma. *Blood Adv*. 2021 Oct 26;5(20):4059–4063. https://doi.org/10.1182/bloodadvances.2020004106. PMID: 34492703.

CHAPTER 85

HIGH-GRADE B-CELL LYMPHOMAS

Kieron Dunleavy and Stephen Douglas Smith

High-grade lymphomas are aggressive hematopoietic malignancies derived predominantly from transformed B cells. Notable subtypes include diffuse large B-cell lymphoma (DLBCL), high-grade B-cell lymphoma with MYC and BCL2 and/or BCL6 rearrangements (HGBCL), and Burkitt lymphoma (BL). Although these entities share many clinical and biologic features, the approach to their management is different; therefore an accurate histologic diagnosis is of utmost importance. There have been several recent therapeutic advances in the management of these diseases, and treatment is curative in a large proportion of patients. As a result, prompt diagnosis, evaluation, and institution of appropriate therapy are imperative.

DIFFUSE LARGE B-CELL LYMPHOMA

Epidemiology

DLBCL is the most prevalent histologic subtype of non-Hodgkin lymphoma (NHL) (of which there are approximately 77,000 new cases per year in the United States) and comprises 30% to 40% of all NHL. Although the median age at diagnosis is in the seventh decade of life, DLBCL affects children and adults of all ages, and it is slightly more common in males than females. Although the etiology of DLBCL is unknown in most cases, it can arise from transformation of an indolent lymphoma. A history of immunodeficiency is a significant risk factor, and individuals who are human immunodeficiency virus (HIV) positive have a much higher incidence of DLBCL compared with those who do not have HIV.

Pathobiology

The pathobiology of DLBCL is heterogeneous, and within DLBCL, there are several morphologic variants that include centroblastic, immunoblastic, T-cell rich or histiocyte-rich (see E-Slide VM03959), and anaplastic subtypes. Progress using techniques such as molecular profiling and other high-resolution genomic technologies are further advancing this taxonomy. There are also several clinical-pathologic variants of DLBCL. Primary mediastinal B-cell lymphoma (PMBL), for example, commonly presents in young females and frequently remains localized to the mediastinum (Fig. 85.1). Primary central nervous system lymphoma (PCNSL) is another rare subtype of DLBCL that rarely disseminates to extraneural sites and is observed with higher frequency in HIV-positive individuals (see Chapter 84). It is important to recognize that DLBCL can also arise as a result of histologic transformation from an indolent lymphoma. Although this differentiation may not affect treatment choice initially, it may impact natural history and prognosis of the lymphoma and therefore needs to be recognized at diagnosis.

The neoplastic cells of DLBCL express pan B-cell markers, including CD20, and surface or cytoplasmic immunoglobulin is often demonstrated. CD10, BCL6, and IRF4/MUM1 are variably expressed, and the proliferation index as measured by Ki67 staining is typically high in the range of 70% to 90%. Approximately 30% of cases show abnormalities involving the *BCL6* gene, and translocation of the *BCL2* gene, a hallmark of follicular lymphoma, is present in 20% to 30% of cases. Approximately 10% of cases of DLBCL harbor a t(8:14) *MYC* translocation, which also characterizes BL. The presence of certain translocation combinations has been associated with inferior outcomes following standard therapies. In particular, a new entity called "high-grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* translocations" (HGBCL; colloquially "double hit" lymphoma) has been defined.[1]

Gene expression profiling has defined a new molecular taxonomy for DLBCL. This is continuously evolving as molecular insights into B-cell lymphoma biology become more refined. Morphologically indistinguishable tumors can show marked heterogeneity in gene expression, and these patterns of expression may be classified into signatures that correspond to the cellular origin of the lymphoma according to its stage of B-cell differentiation; on the basis of these signatures, most cases of DLBCL can be divided into a germinal center B-cell (GCB)-like or activated B-cell (ABC)-like subtype. Recently, the identification of genetic heterogeneity within these subtypes has led to further molecular subclassifications.[2,3] Molecular subclassifications of DLBCL are important in that they may define diseases with disparate outcomes and permit development of therapeutic approaches rooted in disease biology, which address the heterogeneity of DLBCL.

Clinical Features

The clinical presentation of DLBCL is variable and depends on a number of factors, including histology, patient age, and immune status. The disease typically presents with lymphadenopathy that can range from relatively asymptomatic to causing pain (Fig. 85.2), causing organ compromise such as ureteral obstruction or spinal cord compression. The involvement of bone marrow (BM) is much less frequent than with indolent lymphomas and is present in approximately 20% of cases.

Patients may have constitutional manifestations from the production of inflammatory molecules and a variety of other cytokines and chemokines produced by the lymphoma cells or host tissues. Such manifestations include weight loss, malaise, fevers, night sweats, and loss of appetite. Of these, unexplained weight loss of more than 10% of body weight, temperature higher than 38° C, and drenching night sweats are referred to as "B symptoms."

Investigation

History and Physical Examination

Patients should be questioned about systemic symptoms, and their performance status should be assessed (Table 85.1). It is important to determine if there is a history of potential causative factors such as prior malignancy, chemotherapy or radiation treatment, or autoimmune or immunodeficiency diseases. A history of infection with or exposure to various pathogens, including HIV and hepatitis B and C, should be excluded. A detailed physical examination should be performed with particular attention to lymph node (LN) regions. Skin involvement by DLBCL is rare but may consist of violaceous papules or larger masses (Fig. 85.3).

An accurate histologic diagnosis is imperative to determine the patient's prognosis and treatment; therefore the single most important diagnostic test is a technically adequate and properly evaluated tissue biopsy—optimally an excisional surgical biopsy. Although core needle biopsy may suffice in some cases, tissue may be inadequate to assess nodal architecture or perform ancillary studies, leading to delay in definitive diagnosis and subclassification. With few exceptions, fine-needle aspiration is inadequate for diagnosis. Aggressive lymphoma should be diagnosed by an experienced hematopathologist familiar with the nuances and pitfalls of lymphoma diagnosis.

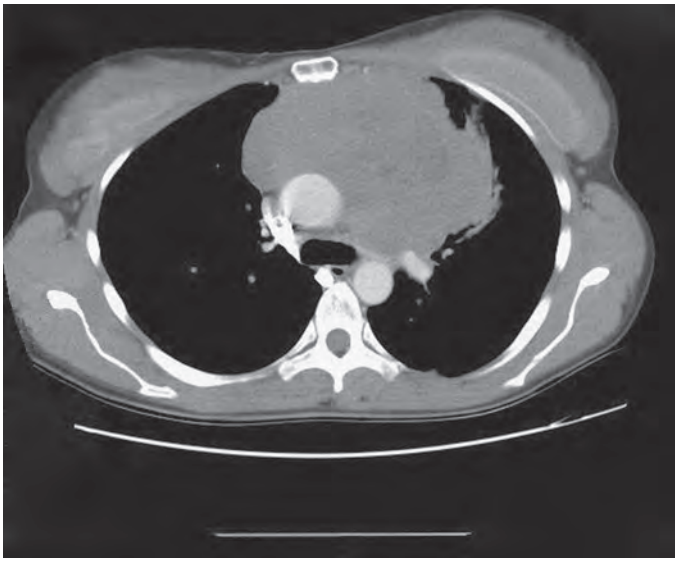


Figure 85.1 COMPUTED TOMOGRAPHY SCAN OF THE CHEST SHOWING A LARGE 17-CM ANTERIOR MEDIASTINAL MASS. A biopsy was consistent with primary mediastinal B-cell lymphoma.

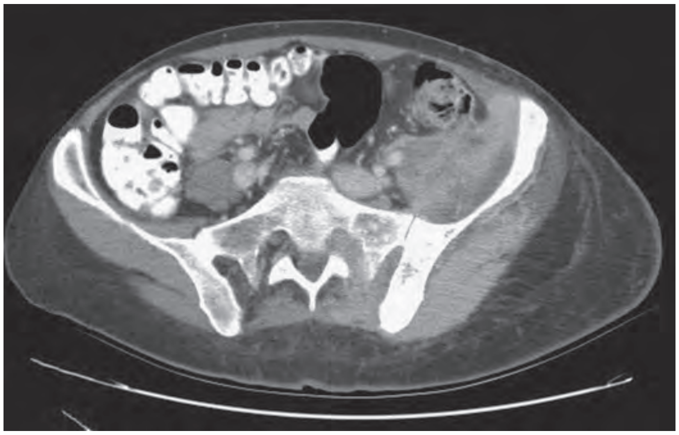


Figure 85.2 COMPUTED TOMOGRAPHY SCAN OF THE ABDOMEN SHOWING A LARGE LEFT-SIDED PSOAS MASS. A biopsy was consistent with diffuse large B-cell lymphoma.

TABLE 85.1 **Eastern Cooperative Oncology Group Performance Scale**

Performance Status	Definition
0	Asymptomatic
1	Symptomatic but fully ambulatory
2	Symptomatic and in bed <50% of the day
3	Symptomatic and in bed >50% of the day
4	Bedridden

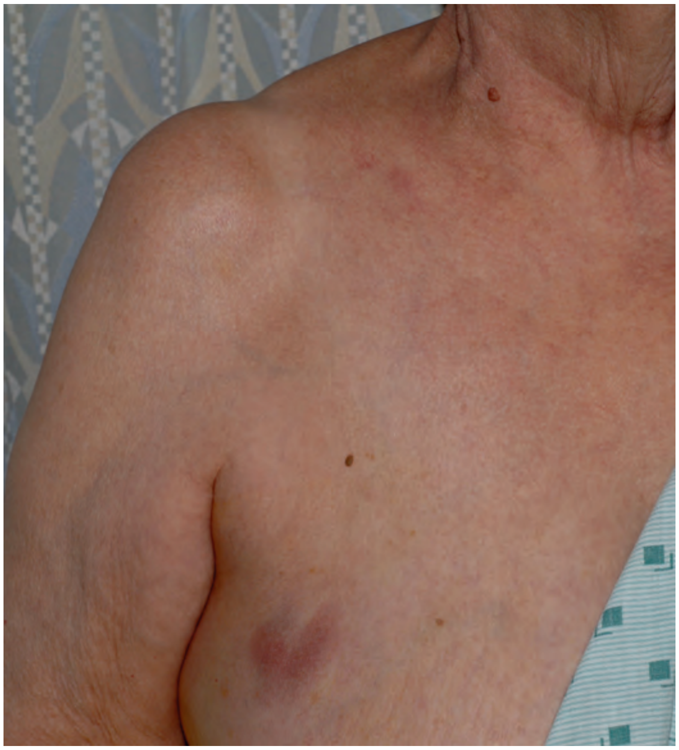


Figure 85.3 INFILTRATION OF THE RIGHT LOWER ANTERIOR CHEST WALL WITH DIFFUSE LARGE B-CELL LYMPHOMA.

Laboratory Investigations

Laboratory tests should include a complete blood count; serum chemistry, including lactate dehydrogenase (LDH); and HIV and hepatitis serology tests (Table 85.2). The latter should be included due to the importance of identifying patients with active hepatitis and a history of hepatitis B, because they will likely require treatment with antivirals, viral load monitoring, or both (see box on Hepatitis B Prophylaxis and Therapy During Lymphoma Treatment). Epstein-Barr virus (EBV) viral loads may also be useful in specific lymphomas such as posttransplant lymphoproliferative disorders (PTLDs) and certain types of EBV-positive DLBCL. An elevated LDH level has adverse prognostic implications for patients with DLBCL.

TABLE 85.2 **Staging Evaluation for Diffuse Large B-Cell Lymphoma**

All Patients	As Clinically Indicated
History and physical examination	CT or MRI of head
CBC and chemistry (including LDH)	Other additional imaging
HIV and hepatitis B and C serology	CSF evaluation by cytology or flow cytometry
CT scan of chest, abdomen and pelvis	Upper endoscopy or colonoscopy
Body FDG-PET scan	Other tests indicated by results of staging
BM aspirate and biopsy	

BM, Bone marrow; *CBC*, complete blood count; *CSF*, cerebrospinal fluid; *CT*, computed tomography; *FDG*, fluorodeoxyglucose; *HIV*, human immunodeficiency virus; *LDH*, lactate dehydrogenase; *MRI*, magnetic resonance imaging; *PET*, positron emission tomography.

HEPATITIS B PROPHYLAXIS AND THERAPY DURING LYMPHOMA TREATMENT

There is a risk of hepatitis B reactivation both from chemotherapy and rituximab, and this is a potentially fatal complication. We check hepatitis serology (hepatitis B surface antigen [HBsAg], hepatitis B surface antibody [anti-HBs], and hepatitis B core antibody [anti-HBc]) in all patients at the time of lymphoma diagnosis. Patients with active hepatitis B receive antiviral medication, and liver function tests (LFTs) and hepatitis B viral loads are monitored closely. Patients with a history of hepatitis B infection (anti-HBc positive but HBsAg negative) should either receive antiviral prophylaxis or have the hepatitis B viral load closely monitored with a low threshold to commence antiviral medications.

Imaging and Staging

It is important to determine sites of disease involvement; therefore imaging studies should include imaging, ideally incorporating fluorodeoxyglucose positron emission tomography (FDG-PET) scanning which is more sensitive than contrast-enhanced computed tomography (CT), particularly for detecting extranodal disease. The use of other imaging modalities depends on the clinical presentation and sites of disease. For example, if central nervous system (CNS) involvement is highly suspected, evaluation of the head by magnetic resonance imaging (MRI) and diagnostic lumbar puncture with flow cytometry may be indicated. Involvement of the bone is best evaluated by PET scans or MRI scans in particular clinical scenarios.

When involvement of the BM may impact management, it should be assessed with DLBCL by a BM aspirate and biopsy, although FDG-PET may detect DLBCL marrow involvement in most cases. Patients at increased risk of CNS involvement should undergo lumbar puncture with evaluation of the cerebrospinal fluid (CSF) by cytology and flow cytometry.[4] Specifically, certain clinical presentations with several extranodal sites and elevated LDH level, as well as particular sites such as the testis, kidney/adrenal gland, breast, and BM, are associated with an increased risk of CNS disease. In high-risk patients, CNS evaluation and prophylaxis should be considered (see box on Central Nervous System Prophylaxis in Diffuse Large B-Cell Lymphoma).

DLBCL is staged according to the Ann Arbor staging system (Table 85.3), which was originally developed for Hodgkin lymphoma (HL). However, because of the heterogeneity and hematogenous pattern of dissemination in NHL, in contrast to contiguous LN spread with HL, the staging system has more limited value. At the same time, important modifications to the Ann Arbor staging system made at the Cotswold Conference have made it more applicable to NHL.

TABLE 85.3 **Ann Arbor Staging System for Lymphomas**

Stage[a]	Cotswold Modification of Arbor Classification
I	Involvement of a single LN region or lymphoid structure
II	Involvement of two or more LN regions on the same side of the diaphragm (the mediastinum is considered a single site, but the hilar LNs are considered bilaterally); the number of atomic sites should be indicated by a subscript (e.g., II_3)
III	Involvement of LN regions on both sides of the diaphragm: III_1 (with or without involvement of splenic hilar, celiac, or portal nodes) and III_2 (with involvement of paraaortic, iliac, and mesenteric nodes)
IV	Involvement of one or more extranodal sites in addition to a site for which the designation E has been used

[a] All cases are subclassified to indicate the absence (A) or presence (B) of the systemic symptoms of significant fever (>38.0°C [100.4(F]), night sweats, and unexplained weight loss exceeding 10% of normal body weight within the previous 6 months. The clinical stage (CS) denotes the stage as determined by all diagnostic examinations and a single diagnostic biopsy only. In the Ann Arbor classification, the term *pathologic stage* (PS) is used if a second biopsy of any kind has been obtained, whether the result was negative or positive. In the Cotswold modification, the PS is determined by laparotomy; X designates bulky disease (widening of the mediastinum by more than one-third or the presence of a nodal mass >10 cm), and E designates involvement of a single extranodal site that is contiguous or proximal to the known nodal site.
LN, Lymph node.

CENTRAL NERVOUS SYSTEM PROPHYLAXIS IN DIFFUSE LARGE B-CELL LYMPHOMA

In DLBCL, the role of central nervous system (CNS) prophylaxis to prevent CNS recurrence is controversial, and several different approaches are followed. There is not widespread agreement on which patients should receive prophylaxis—the CNS International Prognostic Index (CNS-IPI) has been helpful in this respect. All patients at high risk (score 4–6) for CNS involvement undergo a lumbar puncture at diagnosis, and CSF is checked by cytology and flow cytometry; if the results are positive, patients receive active treatment of the CNS. If results are negative, consideration of prophylaxis using intrathecal and/or intravenous chemotherapy is required, although data are unclear on the optimal agent, route, dose, and duration of such therapy. If the results are negative, we strongly consider the use of prophylaxis in high-risk patients, particularly those with more than one extranodal disease site. In addition, certain extranodal locations such as the testis or bone marrow are considered to be associated with a high risk of CNS dissemination, and CNS prophylaxis should be considered.

The type and dosing schedule of CNS prophylaxis is controversial because prospective data are lacking. We frequently use intrathecal methotrexate at a dose of 12 mg. Prophylaxis is started on cycle 3 for a total of 4 cycles. The reason for delaying institution of prophylaxis is to avoid potential increased toxicity from immunochemotherapy in the early cycles. (See Chapter 84.)

Prognosis

To identify prognostic factors in NHL, an international project to correlate clinical variables and outcome in 2031 patients with untreated aggressive lymphoma was undertaken. The following parameters were associated with inferior outcome: age older than 60 years, Ann Arbor stage III or IV disease, serum LDH level above normal range, Eastern Cooperative Oncology Group (ECOG) performance status of two or higher, and involvement of two or more extranodal sites. A clinical prognostic model, termed the International Prognostic Index (IPI), was developed using these five factors. In this model, one point was allocated for each feature and nicely stratified patients into four groups with 5-year survivals of 73%, 51%, 43%, and 26% for zero or one, two, three, and four or five risk factors, respectively, with cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP)-based treatment. Based on this model, the IPI has become the standard in DLBCL for assessing clinical prognosis and treatment stratification within and comparison between clinical trials. A revised prognostic model for R-CHOP, termed the *Revised-IPI*, was also developed (Table 85.4).[5]

Although not yet routinely performed in high-grade B-cell lymphoma, gene expression profiling with assessment of tumor genomic characteristics is emerging as an important prognostic tool. In many studies in DLBCL, outcome following standard therapy has been superior in patients with the GCB compared with the ABC subtype (Fig. 85.4). In routine clinical practice, it has not been feasible to perform gene expression profiling or complex mutational assays and immunohistochemistry algorithms (e.g., Hans algorithm) to predict cell of origin have been applied. Although these are helpful, they have demonstrated varying degrees of concordance with microarray results, limiting their usefulness. Alternative assays to more accurately predict cell of origin are needed, and many studies are investigating limited gene predictor assays.[6] Similarly, DLBCL with simultaneous MYC and BCL2 protein overexpression by immunohistochemistry is associated with inferior outcomes, but reproducibility of IHC in this setting is a concern.[7]

TABLE 85.4 Revised International Prognostic Index[a]

No. of IPI Factors	IPI Score	Outcome	Overall Survival (%)
0	0	Very good	94
1–2	1–2	Good	79
3, 4, or 5	3–5	Poor	55

[a] One point is given for the presence of each of the following characteristics: age older than 60 years, elevated serum LDH level, ECOG performance status 2, Ann Arbor stage III or IV, and more than two extranodal sites.

ECOG, Eastern Cooperative Oncology Group; *IPI*, International Prognostic Index; *LDH*, lactate dehydrogenase.

From Sehn LH, Berry B, Chhanabhai M, et al. The revised International Prognostic Index (R-IPI) is a better predictor of outcome than the standard IPI for patients with diffuse large B-cell lymphoma treated with R-CHOP. *Blood.* 2007;109:1857.

Improved elucidation of the heterogeneous biology of DLBCL has led to the investigation of strategies and novel agents that have selective activity within molecular subtypes and sets the stage for a future era of precision medicine in front-line DLBCL therapeutics, where therapy could be ascribed based on molecular characteristics. Recent molecular classifications of DLBCL have relied significantly on the presence or absence of distinct mutations within cell of origin subtypes, and many of these mutations are “targetable” with small molecule inhibitors.

Treatment

The mainstay of treatment for DLBCL is systemic chemotherapy; radiation treatment alone is inadequate and associated with high recurrence rates. For early-stage disease, whether or not radiation treatment adds benefit to chemotherapy has been controversial. Based on a randomized study that showed a survival advantage of limited course CHOP plus involved field radiation compared with full-course CHOP in early stage (I/II) aggressive lymphoma, combined modality therapy became the standard. However, longer patient follow-up showed a convergence of the overall survival (OS) curves because of late systemic relapses in the combined modality arm, necessitating further trials conducted in the modern R-CHOP era to clarify the role of radiation. Recently, a series of prospective trials in early-stage DLBCL without bulky adenopathy has shown consistently excellent outcomes using four to six cycles of R-CHOP without radiation therapy.[8–10] These trials demonstrate that early-stage patients lacking high-risk features by the IPI, bulk, or achieving FDG-PET negativity after 3 cycles of R-CHOP may be treated with 4 cycles of chemotherapy alone. Additional R-CHOP (to 6 cycles) or incorporation of radiation therapy remains a reasonable strategy in select patients, based on baseline disease risk and anticipated or observed tolerance of therapy.

Patients with advanced-stage DLBCL typically receive 6 cycles of rituximab in combination with CHOP chemotherapy.[11] This backbone has been used since the early 1970s, when doxorubicin was added to cyclophosphamide, vincristine, and prednisone (CVP) and CHOP became the first curative regimen in DLBCL, highlighting the critical role of anthracyclines. Later on, in an attempt to improve upon the results with CHOP, studies focused on the empiric addition of drugs to the regimen. However, this did not improve survival but increased toxicity. The addition of rituximab to CHOP by the GELA group (Groupe d’Etude des Lymphomes de l’Adulte) 20 years ago demonstrated a significant survival advantage in elderly patients with DLBCL, and this advantage was also demonstrated in young patients (younger than the age of 60 years), leading to its acceptance as the standard first-line approach currently. Due to promising activity in single-arm trials in DLBCL, DA-EPOCH-R (dose-adjusted etoposide, prednisone, vincristine, cyclophosphamide, hydroxydaunorubicin, and rituximab) was compared with R-CHOP in a prospective randomized study. The efficacy was similar in both groups, but toxicity was higher in DA-EPOCH-R; this study was not powered to compare outcomes of subsets such as PMBCL and MYC-rearranged DLBCL, where the regimen has demonstrated good activity in single-arm studies. Whether younger patients (more likely to tolerate and benefit from dose escalation inherent to DA-EPOCH-R) or certain biologic subsets (more susceptible to continuous infusion chemotherapy exposure) may benefit remains a matter of active consideration and debate. A small proportion of aggressive B-cell lymphoma cases harbor rearrangements of MYC and BCL2 and/or BCL6—these tumors were previously referred to as “double-hit” or “triple-hit” lymphomas but the World Health Organization (WHO) Lymphoid Classification has put them into a distinct category called “high-grade B-cell lymphoma with rearrangements of MYC and BCL2 and/or BCL6” (Fig.85.5).[1] They are clinically aggressive diseases that have been associated with inferior outcomes compared with DLBCL-NOS (not otherwise specified), and currently, their optimal management has not been defined. High-intensity approaches such as those used in BL are favored by many.[12]

PMBL was once considered to be a subtype of DLBCL but is now considered a distinct clinicopathologic entity. It is approached therapeutically with immunochemotherapy, but this is frequently followed by consolidation mediastinal radiation depending on the regimen used. In early studies with approaches such as the MACOP-B (methotrexate, ARA-C [Cytarabine], cyclophosphamide, vincristine, prednisone, and bleomycin) regimen followed by radiation, 66% of patients had persistently positive gallium scans after chemotherapy, suggesting active disease; however, following consolidation radiotherapy, just 19% had a positive gallium scan, and 80% were event free at 39 months of median follow-up. This study suggested that radiotherapy was necessary after chemotherapy. Although the addition of rituximab to CHOP has improved outcome in DLBCL, there remains a significant proportion of patients with PMBCL who require consolidation radiation. More intensive regimens than R-CHOP, such as dose-adjusted EPOCH-R (DA-EPOCH-R) or R-ACVBP (rituximab, doxorubicin, cyclophosphamide, vindesine, bleomycin, and prednisone), challenge the need for radiation in PMBL; regarding the use of DA-EPOCH-R, in a prospective study of 53 patients, 93% were event free without radiation (see box on Treatment of Primary Mediastinal B-Cell Lymphoma).[13,14] These results suggest that obviating the need for radiation is possible in this disease with more intensive approaches, thus eliminating the risk of long-term toxicities such as secondary malignancies and heart disease. An ongoing study by the International Extranodal Lymphoma

TREATMENT OF PRIMARY MEDIASTINAL B-CELL LYMPHOMA

Patients with a diagnosis of PMBL undergo routine body CT and/or FDG-PET imaging and staging. We administer six cycles of DA-EPOCH-R. After four cycles of therapy, the CT is repeated, and after six cycles an FDG-PET scan is performed. If patients have responded and the posttherapy PET scan result is negative, surveillance with clinical evaluation including history, physical, and laboratory studies—and in select cases, periodic CT imaging—is initiated. If the FDG-PET result is overtly positive, a biopsy is frequently performed, and if there is residual disease, patients undergo mediastinal radiation treatment or further systemic treatment. If the FDG-PET result is suspicious, which may be due to varied benign causes including thymic rebound, posttreatment inflammatory or infectious lesions, or brown fat uptake in young patients, the FDG-PET scan is repeated at intervals of 6–8 weeks. If at this time, there is evidence of improvement or the scan is negative, patients go into routine follow-up; and if the changes are worsening or remain abnormal, a biopsy is attempted, and salvage therapy initiated if residual disease is proven. In light of complexities around FDG-PET restaging, the variable relapse pattern (which can be local or include extranodal sites), the rarity and young affected age, and a rapidly evolving treatment landscape, thorough restaging and consideration of tertiary care center referral are critical in PMBCL beyond first-line therapy.

CT, Computed tomography; *DA-EPOCH-R*, dose adjusted etoposide, prednisone, vincristine, cyclophosphamide, hydroxydaunorubicin, and rituximab; *FDG-PET*, fluorodeoxyglucose positron emission tomography; *PMBL*, primary mediastinal B-cell lymphoma.

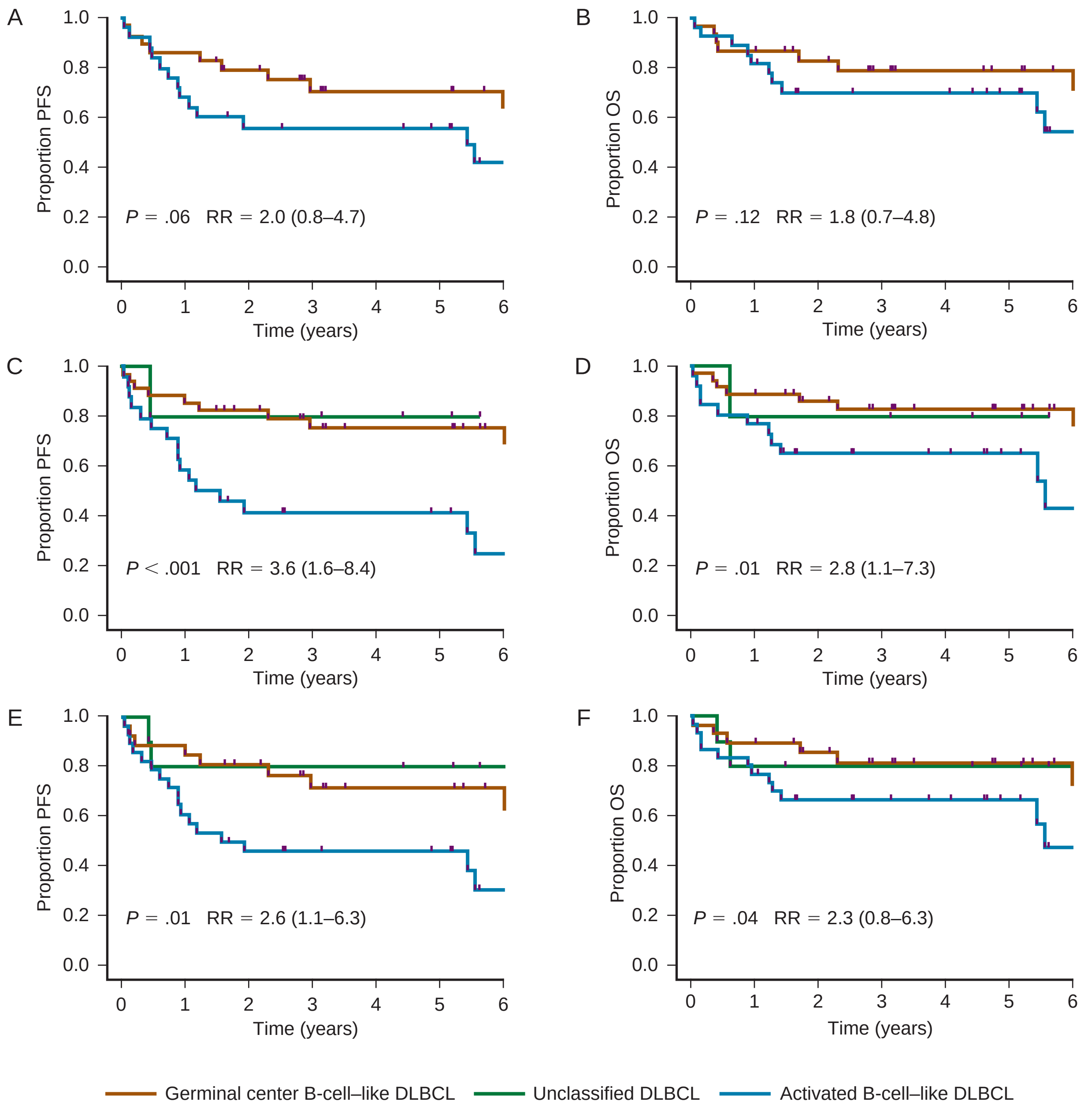


Figure 85.4 PATIENTS WITH DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL) OUTCOMES FOLLOWING R-CHOP ACCORDING TO CELL ORIGIN (COO; IN THE INDEPENDENT VALIDATION COHORT). (A) Progression-free survival (PFS) for COO assignment using the "Hans" algorithm. (B) Overall survival (OS) for COO assignment using the "Hans" algorithm. (C) PFS in the COO groups as determined by the Lymph2Cx assay. (D) OS in the COO groups as determined by the Lymph2Cx assay. (E) PFS in the COO groups determined by the gold standard method applying the previously described model to gene expression on frozen tissue (FT). (F) OS in the COO groups determined by the gold standard method. The *P*-values are from log-rank tests comparing the activated B-cell (ABC) and germinal center B-cell (GCB) groups. The log-rank tests are one sided in the direction of greater hazard for ABC. *RR*, Relative risk (with the 95% confidence interval in brackets) associated with the ABC group compared with the GCB group. *(Republished with permission of the American Society of Hematology from Scott DW, Wright GW, Williams PM, et al. Determining cell-of-origin subtypes of diffuse large B-cell lymphoma using gene expression in formalin-fixed paraffin-embedded tissue. Blood. 2014;123:1214.)*

Study Group (IELSG) is testing if mediastinal radiation can be omitted in patients who have a negative end of therapy (EOT) FDG-PET scan.[15] Clinically and biologically, PMBCL shares many features with classical HL, and in particular, overexpression of programmed death receptor 1/programmed death ligand 1 (PD-1/PD-L1) is commonly found. New approaches such as immune checkpoint inhibitors have demonstrated high activity in HL and are also effective in relapsed/refractory cases of PMBCL and are currently being investigated in the upfront setting.

The appreciation of disease heterogeneity within DLBCL and the existence of subtypes that can be differentially targeted due to distinct oncogenic mechanisms has led to recent novel drug development in the disease. In particular, a number of studies have investigated strategies that target the nuclear factor (NF)-(B pathway in ABC DLBCL. Bruton tyrosine kinase inhibition has demonstrated good but short-lived activity in ABC DLBCL in the relapsed/refractory setting; based on this activity, the recently published PHOENIX study (NCT01855750) compared ibrutinib with R-CHOP to R-CHOP alone, but the addition of ibrutinib was not associated with a better overall outcome.[16] The immunomodulatory agent lenalidomide is also activated in relapsed/refractory ABC-DLBCL, but when lenalidomide was added to R-CHOP, it was not superior to R-CHOP alone in newly diagnosed DLBCL when tested in the ABC subtype or all-comers. Over the past few

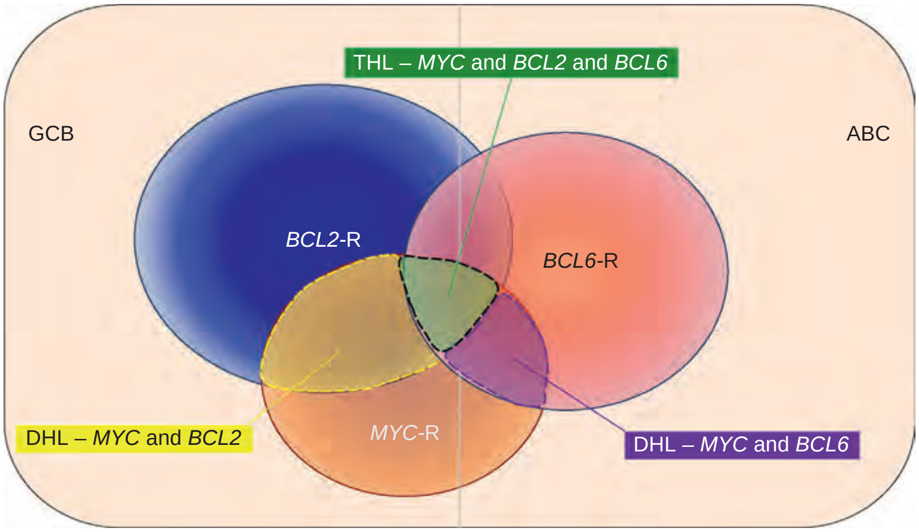


Figure 85.5 GRAPHIC REPRESENTATION OF THE NEW CATEGORY OF AGGRESSIVE B-CELL LYMPHOMAS—"HIGH-GRADE B-CELL LYMPHOMAS WITH MYC AND BCL2 AND/OR BCL6 REARRANGEMENTS." This was described in the 2016 revision to the World Health Organization Classification of Tumors of Hematopoietic and Lymphoid Tumors (2008). Most cases with MYC and BCL2 rearrangements are of germinal center B-cell origin, whereas most cases with BCL6 rearrangements are of activated B-cell origin. This category includes double-hit (DH) lymphomas that involve MYC and BCL2 or MYC and BCL6 and triple hit (TH) lymphomas that involve MYC, BCL2, and BCL6.

decades, there have been significant improvements in the outcome of patients with HIV-associated DLBCL, and these can be attributed to the widespread availability of combination antiretroviral therapy (CART) and to advances in the therapeutics of these diseases. The AIDS Malignancy Consortium in the United States reported excellent results for patients with newly diagnosed aggressive B-cell lymphoma with the DA-EPOCH-R that were similar to survival outcomes in HIV-negative cases.

PRIMARY CENTRAL NERVOUS SYSTEM LYMPHOMA

PCNSL is a rare and highly aggressive lymphoma confined to the CNS and is of diffuse large B-cell histology in more than 90% of cases. Its unique radiographic findings present challenges in evaluation. The incidence of PCNSL is particularly high in the setting of HIV infection, in which it often presents with multifocal disease and is virtually always associated with EBV. In contrast, PCNSL in HIV-negative patients often presents with solitary intracranial masses and is almost never associated with EBV (Fig. 85.6).

Treatment of PCNSL differs from systemic DLBCL because many chemotherapy agents that are standard for treatment of the latter do not adequately penetrate the blood-brain barrier. Radiotherapy has been a mainstay of treatment because it is effective and sidestepped the limitations of chemotherapy, but responses are usually short lived, and virtually all patients relapse. In contrast, high-dose methotrexate (HD-MTX) is a cytotoxic agent with good CNS penetration, but when used alone, progression-free survival (PFS) is relatively short and prolonged dosing or consolidation are considered. A logical step was to administer HD-MTX followed by whole-brain radiotherapy, and this resulted in high complete response (CR) rates and median PFS rates of 32 to 40 months. Unfortunately, such combined modality treatment is associated with severe long-term neurotoxicity, and recently, a high incidence of late relapses has been identified for this disease entity. For this reason, there has been much interest in developing regimens that obviate or defer the need for radiation until relapse. Many intensive approaches have been investigated, frequently followed by autologous stem-cell transplantation, particularly in younger patients. Interestingly, most DLBCLs that arise in the CNS have biologic features that resemble the systemic ABC subtype and a high proportion have mutations in the B-cell receptor (BCR) and MYD88. Therefore there is interest in pursuing strategies that target BCR signaling as well as using immunomodulatory agents in these diseases.[17] See Chapter 84 for more comprehensive discussion of PCNSL.

BURKITT LYMPHOMA

Epidemiology

BL mostly occurs in the first two decades of life, is more common in males, and accounts for some 2% of all lymphomas. There are

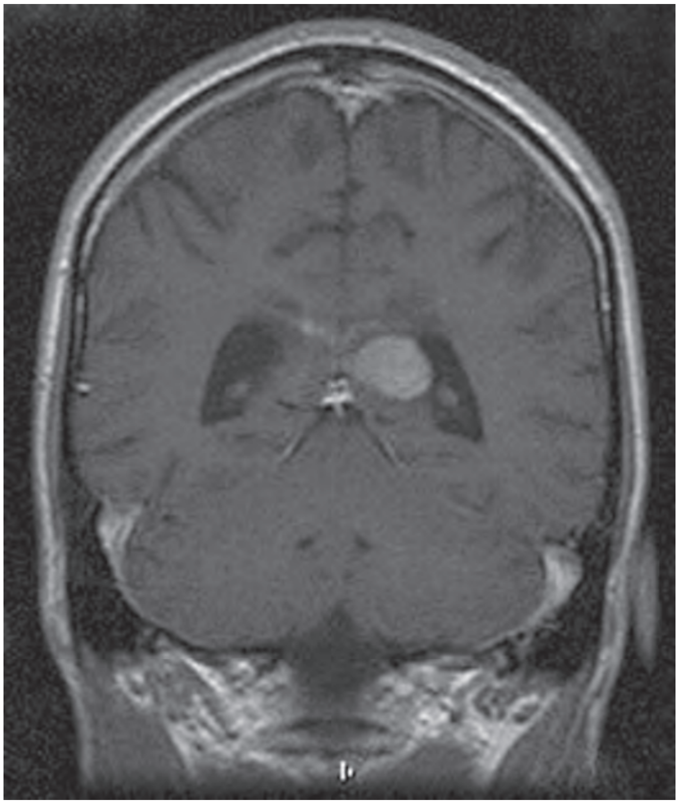


Figure 85.6 THIS GADOLINIUM-ENHANCED MAGNETIC RESONANCE IMAGING SCAN OF THE BRAIN SHOWS AN ENHANCING INFILTRATIVE MASS IN THE MAJOR FORCEPS OF THE CORPUS CALLOSUM. A biopsy was consistent with primary central nervous system lymphoma.

three recognized clinical variants, and they vary in whom they affect and how they present, and they also have morphologic and biologic differences. Endemic BL occurs in equatorial Africa and Papua New Guinea, peaks in incidence in 4- to 7-year-old children, and is predominantly a male disease. Sporadic BL presents worldwide and is the most common variant in the Western world. It typically affects children and young adults and is more commonly observed in boys. Immunodeficiency-associated BL occurs in association with HIV infection and is approximately 1000 times more common in HIV-infected individuals.

Pathobiology

BL is highly aggressive and characterized by an extremely high proliferation rate approaching 100% and a high fraction of apoptosis, and this accounts for its “starry sky” appearance (see E-Slide VM03958). Although a leukemic phase of BL can occur in patients with advanced disease, it is very rare for BL to present purely as acute leukemia. Biologically, BL is derived from a GCB cell as indicated by its CD20$^+$, CD10$^+$, and terminal deoxynucleotidyl transferase (TdT)-negative immunohistochemical profile and gene expression profiling. The neoplastic cells are usually negative or weakly positive for BCL2. Although EBV is virtually always detected in endemic BL, it is only present in 25% to 40% of sporadic and immunodeficiency-associated cases.

Although virtually all cases of BL have MYC translocations, usually at 8q24 to the immunoglobulin heavy chain region, MYC translocations are not specific for BL and can be found in other aggressive B-cell lymphomas (Fig. 85.7). BL has a unique gene expression signature that is molecularly distinct from that of DLBCL. Studies have demonstrated that some cases of DLBCL by histology have gene expression profiles consistent with BL. Given that BL does not respond well to CHOP-based treatments, this distinction by molecular profiling is important, and gene expression profiling may be useful in rare cases that would otherwise be diagnosed as DLBCL. In addition, there are cases with a profile intermediate between that of DLBCL and BL; these cases typically harbor MYC, and most would fall into the new aforementioned category “high-grade B-cell lymphoma with MYC and BCL2 and/or BCL6 translocations.”

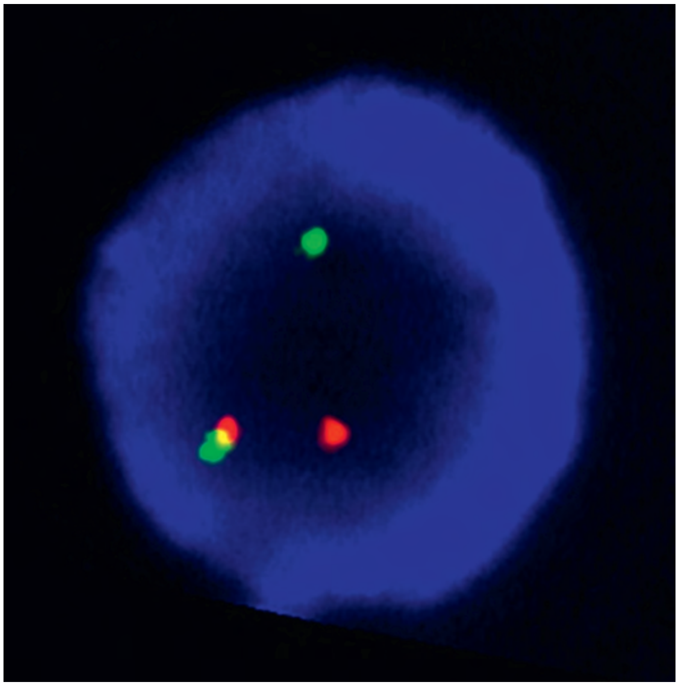


Figure 85.7 FLUORESCENT IN SITU HYBRIDIZATION SHOWING AN MYC TRANSLOCATION IN A PATIENT WITH DIFFUSE LARGE B-CELL LYMPHOMA. This is a break apart probe (LSI-MYC) with one normal (fused) signal and one abnormal (separated green and red) signal indicating a translocation involving MYC.

Clinical Features

The clinical presentation of BL is variable and depends on the epidemiologic subtype as well as other factors. In endemic BL, it is common for patients to present with jaw and other facial disease, and other extranodal sites of involvement include the ileocecum, gonads, kidneys, and breasts. The ileocecal area is the most common site of disease involvement in sporadic BL, and occasionally umbilical involvement can be clinically detected (Fig. 85.8). Jaw involvement is very rare. In immunodeficiency-associated BL, involvement of the ileocecum, LNs, and BM is commonly observed. Patients often present with advanced-stage and bulky disease caused by the short doubling time of the tumor, and it is common for patients to develop tumor lysis syndrome (TLS) after the institution of therapy.

Investigation

History and Physical Examination and Laboratory Investigations

Similar to patients with DLBCL, a detailed history and physical examination are required, and the diagnosis of BL should be made by an experienced hematopathologist. Given its association with HIV infection, it is imperative to perform an HIV test at diagnosis and to check hepatitis serologies.

Imaging and Staging

As with DLBCL, imaging studies should include chest radiography and FDG-PET or CT scanning of the chest, abdomen, and pelvis. The need for additional imaging studies depends on the clinical presentation. A BM aspirate and biopsy should be performed in all patients, and select, high-risk patients should undergo a lumbar puncture with evaluation of the CSF by cytology and flow cytometry. Although BL in adults is staged according to the Ann Arbor staging system, the Murphy staging system is often used in children.

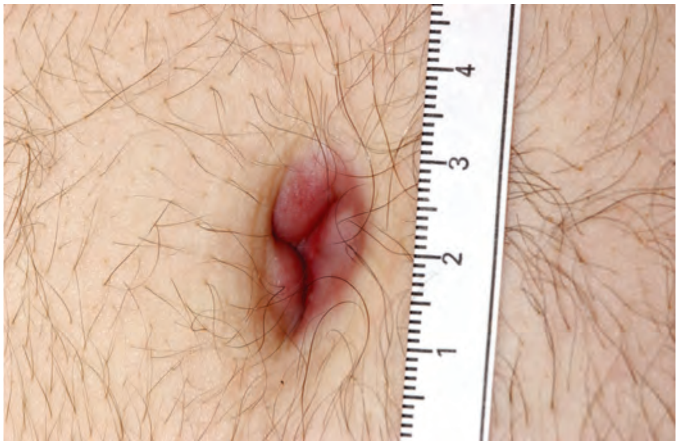


Figure 85.8 BURKITT LYMPHOMA (BL) INVOLVING THE UMBILICUS. This is an 18-year-old man who presented with a history of abdominal pain. Computed tomography scan demonstrated a large intra-abdominal mass extending up to the umbilicus, and a biopsy revealed it to be BL.

Prognosis

Age, large tumor volume, and CNS involvement have been associated with a poor prognosis in the past. Although early studies demonstrated that HIV-positive patients with BL had a worse outcome, this has not been the case with newer treatment approaches. Recently, an analysis of a large cohort of BL patients led to identification of four independent prognostic factors: age 40 years or older, LDH elevation greater than three times the upper limit of normal, ECOG performance status 2 or higher, and CNS involvement; presence of two to four factors (seen in approximately half of BL included patients) comprised the high-risk group, portending a 3-year PFS of approximately 50%.[18]

Treatment

BL is a systemic disease and requires chemotherapy for all disease stages. Importantly, locoregional radiation does not improve survival and should be avoided. Early treatment strategies for BL were modeled on acute lymphoblastic leukemia (ALL) regimens that used dose-intense and prolonged treatment with induction, consolidation, and maintenance phases. Although dose intensity and dose density are important treatment components for BL, later studies indicated that shorter treatment durations were equally effective. Later, risk adaptive approaches allowed further reductions in treatment for patients with early-stage disease. Several biologic characteristics of BL have helped guide treatment strategies, including its high proliferative fraction.

The risks of TLS and propensity for CNS dissemination in BL also have important treatment implications. All patients should receive TLS prophylaxis during the first cycle and undergo close electrolyte monitoring. The high risk of CNS involvement has prompted the use in the past of relatively high-dose intravenous methotrexate and cytarabine, both of which have CNS penetration, as well as intrathecal administration of these drugs. An important advance has been to reduce intrathecal treatment and eliminate whole-brain radiation for prophylaxis, which has significantly reduced CNS toxicity. A study published by the FAB/LMB (French American British/Lymphomes Malins B) group demonstrated that patients with early-stage BL had a high cure rate and very low rate of CNS relapse without the use of intrathecal chemotherapy.[17]

There are multiple highly effective regimens for BL; however, because of the rarity of the disease (there are only 1200 new cases in the United States each year), there are no good comparative studies of different therapies in BL. A variety of dose-intense short-duration regimens have achieved durable CRs in a high proportion of patients. Included in these are the French LMB and German Berlin-Frankfurt-Münster (BFM) protocols and the National Cancer Institute CODOX-M/IVAC (cyclophosphamide, vincristine, doxorubicin, and HD-MTX alternating with ifosfamide, etoposide, and high-dose cytarabine; intrathecal methotrexate and cytarabine are also administered) regimen. These regimens are similar in their drug composition, short cycle length, and CNS prophylaxis. Although most BL occurs in children, Magrath and colleagues demonstrated that adults have a similar disease outcome when treated with the same approaches and reported high cure rates. Other groups have confirmed the efficacy of this regimen, albeit with lower survival rates. In the United Kingdom, Mead and colleagues reported an overall event-free survival (EFS) of 65% at 2 years. The hyper-CVAD (cyclophosphamide, vincristine, doxorubicin, and dexamethasone) regimen has also been tested in BL, with good results (recently with the addition of rituximab). Rituximab is typically used in this disease, and randomized studies in both children and adults demonstrate that it adds a survival advantage.

Toxicity is an important clinical limitation of these regimens in adults, particularly in older patients and in patients who are immunosuppressed, in whom severe morbidity and even mortality occur. Therefore one of the major therapeutic challenges in BL is to develop therapies that are as effective in achieving high cure rates as "standard" regimens but that also improve the therapeutic index and reduce toxicity complications. This approach has been investigated using EPOCH-R–based therapy in BL. The approach was associated with a high PFS of more than 90% in an early study and validated in a multicenter study in low- and high-risk groups.[19] There were low rates of TLS and other toxicities compared with conventional BL regimens. In the setting of HIV infection, a concern with standard BL regimens has been toxicity, and less toxic appear to be more appropriate.

SALVAGE THERAPY

The salvage treatment of relapsed DLBCL should be approached in an individual manner because the choice of treatment is influenced by the time to recurrence, prior therapy, patient's performance status and comorbidities, and the potential for cure. Although most relapsed aggressive lymphomas require combination chemotherapy for adequate disease control, a subset of patients with local disease may be salvaged with radiation therapy. Examples include primary mediastinal DLBCL, which can remain local even at relapse, and PTLDs, which may have an isolated resistant EBV clone after chemotherapy.

A variety of active salvage chemotherapy regimens are available for relapsed or refractory DLBCL. Platinum-containing regimens, such as ESHAP (etoposide, methylprednisolone, cytosine arabinoside, and platinum), DHAP (dexamethasone, cisplatin, cytarabine arabinoside), GDP (gemcitabine, dexamethasone, cisplatin), and ICE (ifosfamide, carboplatin, and etoposide), combined with rituximab are widely used.

Patients with chemotherapy-sensitive disease have the best outcome with autologous stem cell transplant (ASCT), and this is recommended at initial relapse; in the pre-rituximab era, this approach yielded OS and EFS rates in the range of 40% to 50% and 30% to 40%, respectively. However, the improvement in upfront curability of DLBCL because of immunochemotherapy has diminished the efficacy of ASCT at relapse. In addition, recent randomized data suggest that some patients with primary refractory/relapsed DLBCL have improved outcomes with CD19-directed chimeric antigen receptor T cell (CAR-T cell) therapy.[20]

There are a number of novel agents and strategies that have demonstrated good activity in relapsed/refractory DLBCL (Fig. 85.9), and some of these are now being investigated in front-line DLBCL studies. Anti-CD19 CAR-T-cell therapy has led to long-term remissions in a significant proportion of patients with relapsed/refractory disease, and this has led to the US Food and Drug Administration (FDA) approval of anti-CD19 CAR-T-cell therapy in relapsed/refractory DLBCL after two lines of therapy.[21–23] CD19 can additionally be targeted by monoclonal antibodies, and recently one such antibody, tafasitamab, showed adequate activity in combination with lenalidomide to lead to FDA approval in relapsed/refractory DLBCL.[24] Polatuzumab is an antibody-drug conjugate directed against CD79B and based on a randomized study of the drug in combination with bendamustine-rituximab (B-R); it was FDA approved for relapsed/refractory disease.[25] Another strategy that inhibits XP01-mediated nuclear transport nuclear export, selinexor, was also recently FDA approved. Notably, none of these strategies have been compared with one another, and differences in trial populations and nuanced eligibility criteria make application to the clinical setting a challenge. Among newer experimental approaches, antibodies targeting several unique B-cell antigens and others targeting multiple antigens simultaneously (including bispecific T-cell engagers [BITEs]) are being investigated in this DLBCL.

With regard to BL, the outcome for patients who relapse after or progress during initial therapy—which generally is observed within a year of first diagnosis—is extremely poor, and there are no standard approaches that have been associated with good outcomes. Experimental approaches should be strongly considered in this setting.

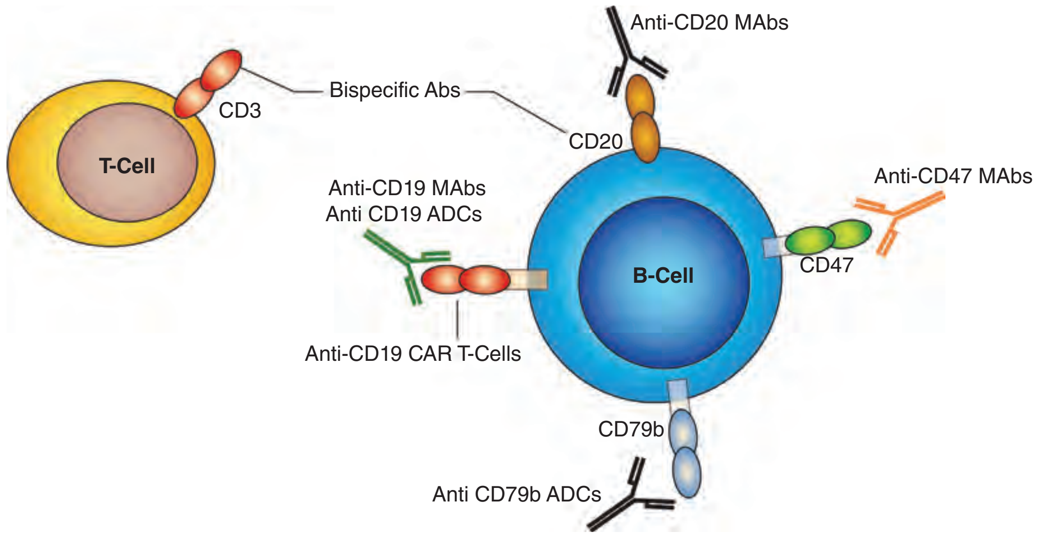


Figure 85.9 SELECT NOVEL TARGETS AND AGENTS IN DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL). Recently, a number of new strategies have been developed to target DLBCL. These include novel monoclonal antibodies and antibody-drug conjugates against CD19, CD79b, and CD47. Anti-chimeric antigen T-cell therapy (CAR-T) has been developed against antigens including CD19 and bispecific antibodies against CD20 (B cells) and CD3 (T cells).

Late Complications of Treatment and Follow-Up

It is important to recognize that successful treatment may be associated with late complications that may not appear for decades. Among the major late-term complications are secondary malignancies, ischemic heart disease, anthracycline-related cardiotoxicity, and radiation- or bleomycin-induced pulmonary toxicity. The risk of developing myelodysplastic disorders and acute myeloid leukemia is related to alkylator and topoisomerase inhibitor use and is enhanced by radiation. Radiation therapy increases the risk of malignancy in the treatment region, particularly breast cancer in women and lung cancer in smokers.

Surveillance for DLBCL for patients in remission can vary but generally involves visits every 3 or 4 months for 2 years, and every 6 months or annually thereafter. During these visits, examination of the LN areas, abdomen, thyroid, and skin is important. CT scans are recommended in early follow-up, particularly for high-risk patients or those with uncertain posttreatment imaging findings, but do not commonly detect relapse in asymptomatic patients and have not been shown to improve survival. FDG-PET scans are not recommended for routine follow-up because the high rate of false-positive scans is unlikely to offset the value of early detection. Routine laboratory studies with blood counts, liver function tests, and LDH should be performed. Thyroid-stimulating hormone levels should be monitored annually in patients who received neck radiotherapy. Women who received thoracic irradiation may require enhanced breast cancer screening, depending on age of exposure, and may include annual mammography and breast MRI starting 10 years after radiation therapy. Mammography for females should begin 10 years from the diagnosis of lymphoma or at age 40 years, whichever comes first.

FUTURE DIRECTIONS

Over recent years, we have made significant advances in elucidating the molecular biology of DLBCL and BL, and this has led to an era of enhanced and exciting drug discovery. In DLBCL in particular, many critical pathways and novel mutations have been identified, and many new agents are being investigated. The promising activity of novel strategies such as anti-CD19 CAR-T cells, novel antibodies and bispecific antibodies in the relapsed/refractory setting has led to their investigation in the upfront setting which is currently underway, principally in high-risk groups of patients.

REFERENCES

1. Alsharif R, Dunleavy K. Burkitt lymphoma and other high-grade B-cell lymphomas with or without MYC, BCL2, and/or BCL6 rearrangements. *Hematol Oncol Clin North Am*. 2019;33(4):587–596.
2. Chapuy B, Stewart C, Dunford AJ, et al. Molecular subtypes of diffuse large B cell lymphoma are associated with distinct pathogenic mechanisms and outcomes. *Nat Med*. 2018;24(5):679–690.
3. Schmitz R, Wright GW, Huang DW, et al. Genetics and pathogenesis of diffuse large B-cell lymphoma. *N Engl J Med*. 2018;378(15):1396–1407.
4. Wilson WH, Bromberg JE, Stetler-Stevenson MV, et al. Detection and outcome of occult leptomeningeal disease in diffuse large B-cell lymphoma and Burkitt lymphoma. *Haematologica*. 2014;99(7):1228–1235.
5. Zhou Z, Sehn LH, Rademaker AW, et al. An enhanced International Prognostic Index (NCCN-IPI) for patients with diffuse large B-cell lymphoma treated in the rituximab era. *Blood*. 2014;123(6):837–842.
6. Wright GW, Huang DW, Phelan JD, et al. A probabilistic classification tool for genetic subtypes of diffuse large B cell lymphoma with therapeutic implications. *Cell*. 2020;37(4):551–568.
7. Johnson NA, Slack GW, Savage KJ, et al. Concurrent expression of MYC and BCL2 in diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. *J Clin Oncol*. 2012;30(28):3452–3459.
8. Lamy T, Damaj G, Soubeyran P, et al. R-CHOP 14 with or without radiotherapy in nonbulky limited-stage diffuse large B-cell lymphoma. *Blood*. 2018;131(2):174–181.
9. Poeschel V, Held G, Ziepert M, et al. Four versus six cycles of CHOP chemotherapy in combination with six applications of rituximab in patientswith aggressive B-cell lymphoma with favourable prognosis (FLYER): a randomised, phase 3, non-inferiority trial. *Lancet*. 2019;394(10216).:2271–228.
10. Persky DO, Li H, Stephens DM, Park SI, et al. Positron emission tomography-directed therapy for patients with limited-stage diffuse large B-cell lymphoma: results of Intergroup National Clinical Trials Network Study S1001. *J Clin Oncol*. 2020;38(26):3003–3301.
11. Bartlett NL, Wilson WH, Jung S-H, et al. Dose-adjusted EPOCH-R compared with R-CHOP as frontline therapy for diffuse large B-cell lymphoma: clinical outcomes of the phase III intergroup trial alliance/CALGB 50303. *J Clin Oncol*. 2019;37(21):1790–1799.
12. Dunleavy K, Fanale MA, Abramson JS, et al. Dose-adjusted EPOCH-R (etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin, and rituximab) in untreated aggressive diffuse large B-cell lymphoma with MYC rearrangement: a prospective, multicentre, single-arm phase 2 study. *Lancet Haematol*. 2018;5(12):e609–e617.
13. Dunleavy K, Pittaluga S, Maeda LS, et al. Dose-adjusted EPOCH-rituximab therapy in primary mediastinal B-cell lymphoma. *New Engl J Med*. 2013;368(15):1408–1416.

14. Giulino-Roth L, O'Donohue T, Chen Z, et al. Outcomes of adults and children with primary mediastinal B-cell lymphoma treated with dose-adjusted EPOCH-R. *Br J Haematol*. 2017;179(5):739–747.
15. Martelli M, Ceriani L, Zucca E, et al. [18F]fluorodeoxyglucose positron emission tomography predicts survival after chemoimmunotherapy for primary mediastinal large B-cell lymphoma: results of the International Extranodal Lymphoma Study Group IELSG-26 Study. *J Clin Oncol*. 2014;32(17):1769–1775.
16. Younes A, Sehn LH, Johnson P, et al. Randomized phase III trial of ibrutinib and rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone in non-germinal center B-cell diffuse large B-cell lymphoma. *J Clin Oncol*. 2019;37(15):1285–1295.
17. Lionakis MS, Dunleavy K, Roschewski M, et al. Inhibition of B cell receptor signaling by ibrutinib in primary CNS lymphoma. *Cancer Cell*. 2017;31(6):833–843. e5.
18. Evens AM, Danilov A, Jagadeesh D, et al. Burkitt lymphoma in the modern era: real-world outcomes and prognostication across 30 US cancer centers. *Blood*.2021;137(3):374–386.
19. Roschewski M, Dunleavy K, Abramson JS, et al. Multicenter study of risk-adapted therapy with dose-adjusted EPOCH-R in adults with untreated burkitt lymphoma. *J Clin Oncol*. 2020;38(22):2519–2529.
20. Locke FL, Miklos DB, Jacobson CA, et al. Axicabtagene ciloleucel as second-line therapy for large B-cell lymphoma.. *N Engl J Med*. 2022;386:640–654.
21. Neelapu SS, Locke FL, Bartlett NL, et al. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. *N Engl J Med*. 2017; 377(26):2531–2544.
22. Schuster SJ, Bishop MR, Tam CS, et al. Tisagenlecleucel in adult relapsed or refractory diffuse large B-cell lymphoma. *N Engl J Med*. 2019;380(1): 45–56.
23. Abramson JS, Palomba ML, Gordon LI, et al. Lisocabtagene maraleucel for patients with relapsed or refractory large B-cell lymphomas (TRANSCEND NHL 001): a multicentre seamless design study. *Lancet*. 2020; 396(10254):839–852.
24. Salles G, Duell J, González Barca E, et al. Tafasitamab plus lenalidomide in relapsed or refractory diffuse large B-cell lymphoma (L-MIND): a multicentre, prospective, single-arm, phase 2 study. *Lancet Oncol*. 2020;21(7):978–988.
25. Sehn LH, Herrera AF, Flowers CR, et al. Polatuzumab vedotin in relapsed or refractory diffuse large B-cell lymphoma. *J Clin Oncol*. 2020;38(2): 155–165.

CHAPTER 86

MANTLE CELL LYMPHOMA

Julie M. Vose

Mantle cell lymphoma (MCL) is a distinct subtype of mature B-cell non-Hodgkin lymphoma (NHL) that accounts for 5% to 10% of all NHL. Although a subset of patients with MCL may have an indolent course, MCL is generally an aggressive NHL. In the 1970s, the Kiel classification used the terminology *centrocytic lymphoma* to describe MCL, whereas Berard and Dorfman categorized it as *lymphocytic lymphoma of intermediate differentiation*. In the 1980s, the term *mantle-zone lymphoma* was used to denote a distinct subtype of MCL, characterized by the proliferation of atypical, small lymphoid cells with wide mantles around benign germinal centers (GCs). In the Revised European–American Lymphoma classification in 1994, 2001, 2008, and 2016, the WHO classification of tumors of hematopoietic and lymphoid tissues recognized MCL as a distinct disease under mature B-cell lymphomas. In recent years, with the recognition as a distinct entity, significant progress has been made toward understanding the underlying pathogenesis of MCL and translating that knowledge to design more effective therapies.[1]

EPIDEMIOLOGY

MCL represents 6% to 10% of all lymphomas and is two- to sevenfold more common in men than women and almost twofold more common in people of European ancestry than African Americans. The average age at diagnosis varies between 60 and 70 years. The annual incidence is approximately four to eight cases per million population in the United States and Europe.[2]

PATHOBIOLOGY

MCL, characterized by an alteration in the regulation of the cell cycle, demonstrates cyclin D1 overexpression and increased replication. The tumor cells also demonstrate decreased response to DNA damage and resistance to apoptosis. The tumor cells originate from pre-B cells in the bone marrow that can follow at least two different molecular pathways of development.

The classic MCL originates from a naïve B cell that has not entered the follicular GC but has SOX11 expression, lambda light chain restriction, and limited or no immunoglobulin heavy chain variable (IgVH) somatic mutations. SOX11 expression is associated with the activation of histone marks and absence of DNA methylation. The second subset of MCL, an indolent form typically limited to peripheral blood, bone marrow, and spleen, originates from post-GC B cells. This MCL subset has frequent IgVH mutations, typically lacks SOX11 expression, and has kappa light chain restriction. A few studies have demonstrated a correlation between SOX11 expression and aggressive tumors with high serum levels of lactate dehydrogenase (LDH) and high Ki-67 index. Conversely, SOX11-negative MCL may occasionally acquire complex karyotype and TP53 alterations, transforming into an aggressive tumor (Fig. 86.1).[3]

Genetic Alteration of Proliferative Pathways

The three most common recurrent mutations in MCL involve *CCND1* (also known as cyclin D1, BCL1, or PRAD1), *TP53*, and ataxia-telangiectasia mutated (*ATM*) genes. The vast majority of MCL have the reciprocal translocation, t(11;14) (q13;q23), which juxtaposes *CCND1* gene to the immunoglobin heavy-chain locus. The consequent overexpression of *CCND1* gene, located at 11q13, encodes cyclin D1 protein. Cyclin D1 protein, which is not expressed in normal lymphocytes, is expressed in virtually all MCL and at a lower level in Burkitt lymphoma and acute lymphoblastic lymphoma (ALL). A few cells with t(11;14) have been found in the blood of 1% to 2% of healthy individuals. Therefore, cyclin D1 overexpression is not sufficient for the transformation of normal B cells to MCL, rather it is thought to represent an early genetic event. Interestingly, the close physical proximity of the chromosomal regions of the IgVH and CCND1 loci in the nucleus of immature lymphoid B cells may facilitate the occurrence of recurrent translocations. Furthermore, colocalization and interaction of IgVH-CCND1 chromosomal segment and a transcription factor called *nucleolin* near the perinucleolar area leads to the transcription of cyclin D1 protein. Cyclin D1 protein then regulates the G1 phase by binding to cyclin-dependent kinase (CDK) 4 and CDK6. The cyclin D1-CDK complex phosphorylates retinoblastoma 1 (RB1), resulting in a release of E2F transcription factor and transition of a cell from G1 to S phase. Additionally, the *CDKN2A* gene on chromosome 9p, which regulates INK4a protein, is deleted in 20% to 30% of highly proliferating MCL. The INK4a protein inhibits CDK4 and CDK6 and thus maintains the RB1 protein in its active, antiproliferative state. INK4a is frequently deleted in MCL and cooperates with *CCND1* gene. Therefore, the CDKN2A-INK4a-CDK4-RB1 pathway is dysregulated in patients with MCL, particularly those with blastoid histology.[3–5]

Recent studies have described cyclin D1-negative MCL cases that demonstrate pathologic features and gene expression profile, similar to cyclin D1–positive MCL. Cyclin D1–negative MCL is associated with the dysregulation in the expression of the following genes (often due to epigenetic changes): overexpression of cyclin D2 or D3; decreased CDK inhibitor 1B (CDKI1B or p27); upregulation of cyclin E; or inactivation of *RB* gene. CDKI1B protein regulates the cell progression from G1 to S phase by inhibiting the cyclin E/CDK2 complex (the latter also inactivates the RB protein). Although the diagnosis of cyclin D1–negative MCL is uncommon and requires caution, these cases highlight the relevance of the oncogenic dysregulation of the cell cycle, especially of the G1 phase in the pathogenesis of MCL.[4]

Antiapoptotic and Prosurvival Pathways

Some patients with aggressive MCL, particularly the blastoid variant, have an increased number of chromosomal abnormalities, such as complex karyotype and tetraploidy. The accumulation of chromosomal abnormalities suggests alterations in the DNA damage response pathways. *TP53* mutation and deletion or point mutation of the *ATM* gene (located at 11q22–q23) are recurrent mutations in MCL. *TP53* mutations, associated with alterations of 17p chromosomal segment, are present in 10% to 28% of MCL cases. These point mutations are frequently missense mutations that compromise normal p53 expression. The *TP53* tumor suppressor gene normally regulates the cell cycle, DNA repair, apoptosis, senescence, and autophagy through transcription-dependent and independent pathways. Disruption in TP53-dependent apoptosis has also been linked to deletion, mutation, or silencing of *BIM* and *NOXA* genes. The latter two genes transcribe proapoptotic proteins important in Bax/Bak-mediated

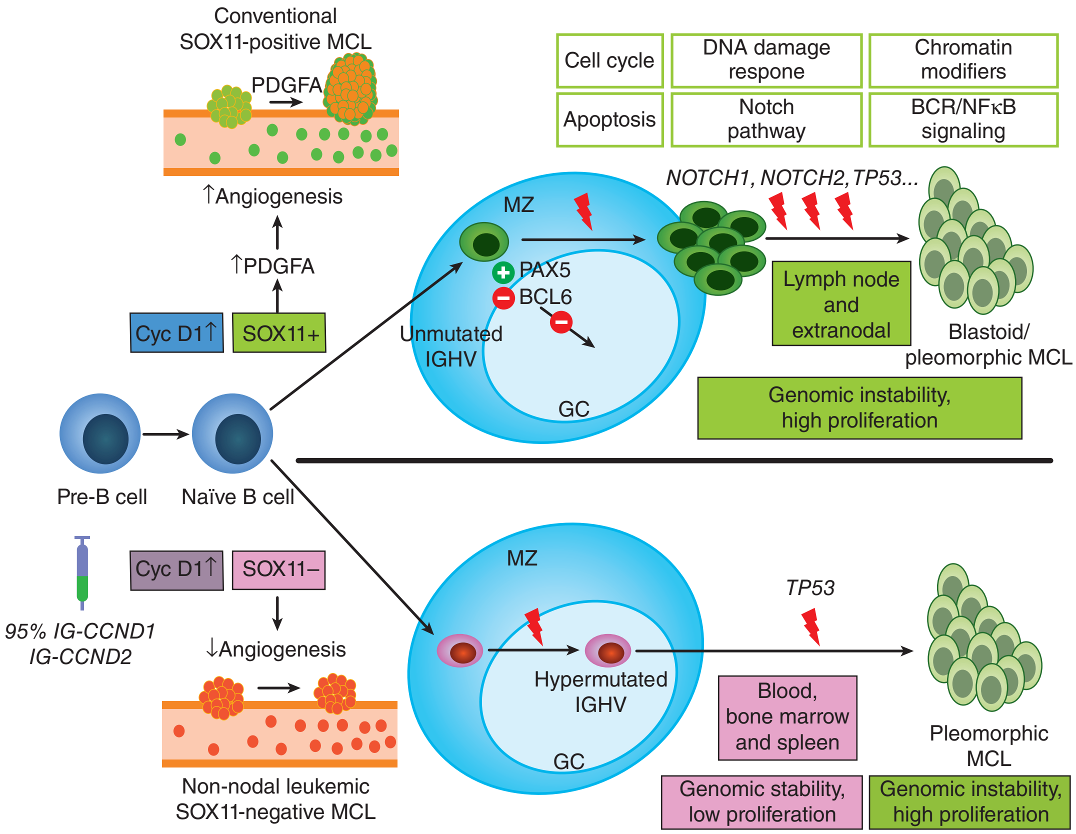

Conventional
SOX11-positive MCL
PDGFA
↑Angiogenesis
↑PDGFA
Cyc D1↑
SOX11+
Cell cycle
DNA damage respone
Chromatin modifiers
Apoptosis
Notch pathway
BCR/NFκB signaling
NOTCH1, NOTCH2, TP53...
MZ
PAX5
BCL6
Unmutated IGHV
GC
Lymph node and extranodal
Blastoid/ pleomorphic MCL
Genomic instability, high proliferation
Pre-B cell
Naïve B cell
95% IG-CCND1
IG-CCND2
Cyc D1↑
SOX11–
↓Angiogenesis
Non-nodal leukemic
SOX11-negative MCL
MZ
Hypermutated IGHV
GC
TP53
Blood, bone marrow and spleen
Pleomorphic MCL
Genomic stability, low proliferation
Genomic instability, high proliferation

Figure 86.1 PROPOSED MODEL OF MOLECULAR PATHOGENESIS IN THE DEVELOPMENT AND PROGRESSION OF MAJOR SUBTYPES OF MANTLE CELL LYMPHOMA (MCL). Role of *SOX11* and genetic events cooperating with cyclin D1 in mantle cell lymphoma. *GC*, Germinal center; *NFκB*, nuclear factor kappaB; PDGFA, platelet-derived growth factor subunit A. *(From Beà S, Amador V. Role of SOX11 and genetic events cooperating with cyclin D1 in mantle cell lymphoma. Curr Oncol Rep. 2017;19(6):43.)*

cytochrome C release, which is essential to apoptosis. TP53 expression is also inhibited by the binding of PAX5 transcription factor to the TP53 promoter. PAX5 is upregulated by SOX11 in a significant number of patients with MCL. PAX5 represses PRDM1/BLIMP1 and negatively regulates differentiation of early lymphoid progenitors to plasma cells. Therefore, the SOX11-PAX5-PRDM1/BLIMP1 axis suppresses B-cell differentiation to plasma cells and plays an important role in the pathogenesis of MCL. MDM2 is another protein overexpressed in about 16% of MCL and mediates polyubiquitination and degradation of p53. This function of MDM2 protein is prevented by ARF protein, also known as p14 (encoded by *CDKN2A* gene). In MCL, however, the homozygous deletion of the *CDKN2A* gene allows MDM2-mediated degradation of TP53 and dysregulation of apoptotic pathways.[3,5]

Inactivation of the *ATM* gene, detected in about 40% of MCL, facilitates genomic instability by impairing responses to DNA damage. Intriguingly, the inactivation of *ATM*, perhaps an early phenomenon in MCL, is not commonly seen in other lymphomas—except in T-cell prolymphocytic leukemia. *ATM* encodes the PI3K protein, which phosphorylates p53, promoting its stabilization and transcriptional activation, therefore leading to cell-cycle arrest, DNA repair, or apoptosis. Consequently, inactivation of *ATM* causes genomic instability by inactivation of p53. Despite having similar pathogenic significance, *ATM* mutations do not impact overall survival, whereas *TP53* mutations do predict for worse survival. In addition, *ATM* may play a role in activation of the nuclear factor kappaB (NFκB) by phosphorylation of the NFκB inhibitor, IkappaB. The NFκB-signaling regulates transcription factors that activate numerous genes involved in cell survival, apoptosis, and cell migration. Its persistent activity is associated with tumor formation, tumor growth, metastasis, and drug resistance, in B-cell lymphomas and other cancers. Notably, the PI3K-AKT pathway is upstream of the mammalian target of rapamycin (mTOR), which in turn is upstream of cyclin D1, CDKI1B, and other regulatory proteins. Conversely, CHK2 and CHK1 are kinases downstream of ATM and prevent cell-cycle progression in response to ATM. Downregulation or mutations of CHK2 and CHK1 are also commonly seen in MCL.

Wnt canonical and Sonic Hedgehog (SHH) pathways are also altered in MCL. The Wnt canonical pathway, via β-catenin and important for normal cell growth and development, is constitutively activated in a subset of MCL and may promote tumor growth. Similarly, SHH-GLI-signaling molecules such as PTCH and SMO receptors, as well as GLI1 and GLI2 transcription factors, are expressed in MCL and may contribute to the proliferation of MCL.

Lastly, dysregulation of the B-cell receptor (BCR), a key player of normal B-cell differentiation and development, is involved in the pathogenesis of MCL. Activation of BCR-associated kinases (spleen tyrosine kinase [SYK], Bruton tyrosine kinase [BTK], and protein kinase C-beta [PKC-β]) is observed in MCL. BTK, a member of the Tec family of nonreceptor protein tyrosine kinases, has a well-defined role in the constitutively activated BCR signaling pathway in MCL. Amplification and overexpression of SYK have been found in a subset of MCL. SYK, LYN, and BTK are frequently phosphorylated in MCL, suggesting activation of the BCR pathway. Therefore, the BCR pathway appears to contribute to the growth and survival of MCL cells. The signaling pathways that contribute to the pathogenesis are summarized in Fig. 86.2.

CLINICAL MANIFESTATIONS

The early symptoms sometimes include fever, night sweats, unexplained weight loss, lymphadenopathy, and splenomegaly. Typically, the majority of patients present at an advanced stage (III or IV) with lymphadenopathy, hepatomegaly, splenomegaly, and bone marrow

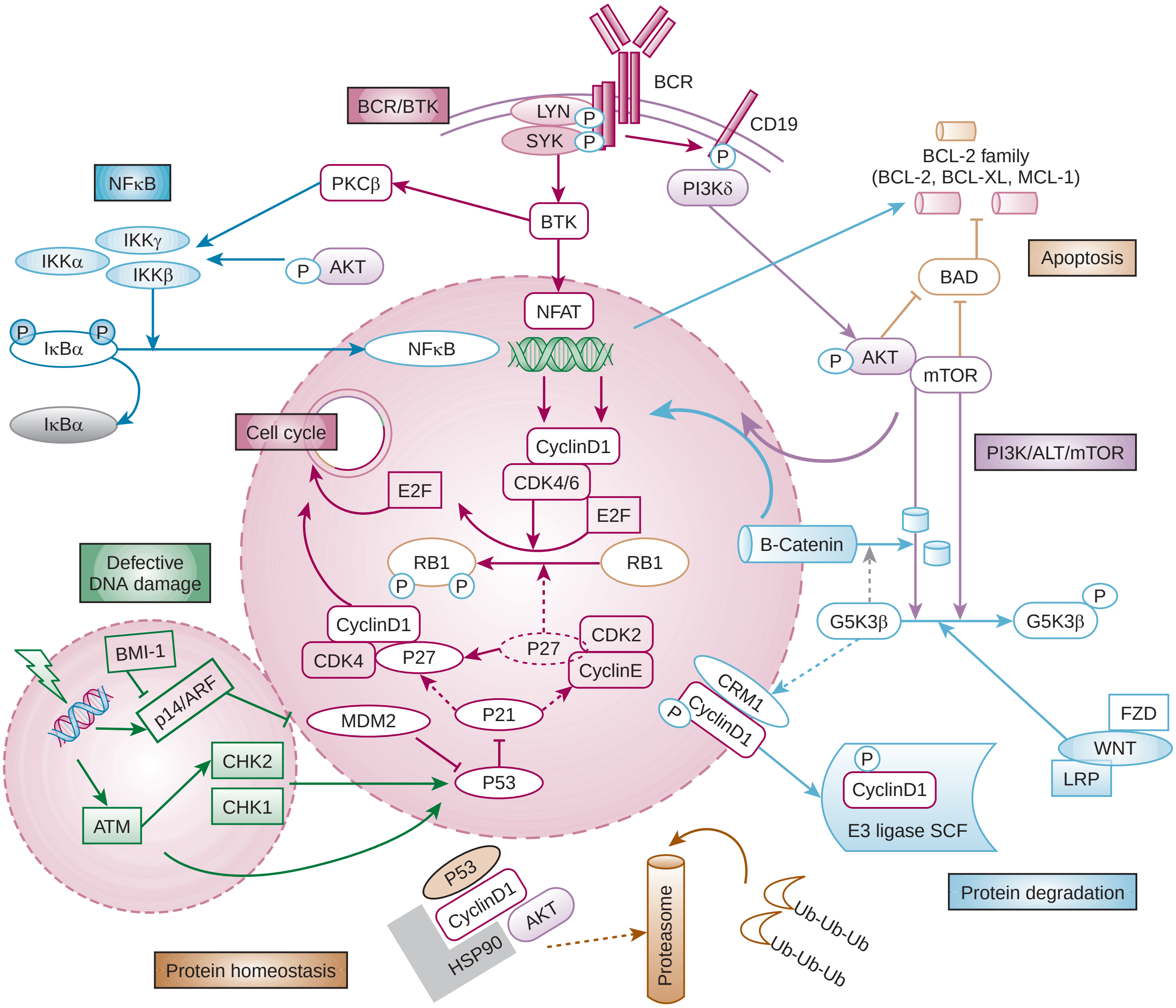

BCR
BCR/BTK
LYN
SYK
P
CD19
PI3Kδ
BCL-2 family
(BCL-2, BCL-XL, MCL-1)
NFκB
PKCβ
BTK
IKKγ
IKKα
IKKβ
AKT
Apoptosis
BAD
IκBα
NFκB
NFAT
mTOR
IκBα
Cell cycle
CyclinD1
CDK4/6
E2F
PI3K/ALT/mTOR
B-Catenin
RB1
Defective DNA damage
G5K3β
CyclinD1
CDK2
CDK4
P27
CyclinE
BMI-1
p14/ARF
CRM1
CyclinD1
MDM2
P21
FZD
WNT
LRP
CHK2
CHK1
P53
E3 ligase SCF
ATM
Protein degradation
P53
CyclinD1
AKT
HSP90
Proteasome
Ub-Ub-Ub
Ub-Ub-Ub
Protein homeostasis

Figure 86.2 THE SIGNALING PATHWAYS CONTRIBUTING TO MANTLE CELL LYMPHOMA PATHOGENESIS. *CCND1* translocation and mutation of *TP53* and ataxia-telangiectasia mutated (*ATM*) genes are common genetic alterations involved in the pathogenesis of mantle cell lymphoma (MCL). Cyclin D1 protein regulates the G1 phase by binding to cyclin-dependent kinase (CDK) 4 and CDK6. The cyclin D1-CDK complex phosphorylates retinoblastoma 1 (RB1) resulting in release of E2F transcription factor and transition of a cell from G1 to S phase. Other major abnormalities include inactivation of the ATM, and cell cycle checkpoint kinase (CHK 1 and CHK2) genes; MDM2-mediated degradation of TP53; activation of B cell receptor-associated kinases and constitutive activation of nuclear factor kappaB (NFκB), PI3K/AKT/mammalian target of rapamycin (mTOR), Wnt canonical, and Sonic Hedgehog (SHH) pathways.

involvement. Splenomegaly is noted in 40% of patients and approximately 50% of patients present with circulating lymphoma cells. MCL also tends to involve extranodal sites, especially in the gastrointestinal (GI) tract. Involvement of the GI tract, frequently in the form of lymphomatous polyposis, can be detected in up to 90% of patients; however, many patients do not have any pertinent GI symptoms. During disease progression, MCL can infiltrate other organs including the respiratory tract, breast, or orbit. Involvement of the central nervous system (CNS) is rare but may be relatively more common with blastoid MCL and relapsed disease.

LABORATORY MANIFESTATIONS

Cytopenias are typically present secondary to bone marrow involvement and/or splenomegaly, whereas autoimmune cytopenias are rare. Elevation of LDH and uric acid correlates with tumor burden. Liver function tests may be abnormal if there is hepatic involvement. Other findings include elevated beta 2-microglobulin and, rarely, hypogammaglobulinemia or monoclonal gammopathy.

Histologically, the 2016 WHO classification[4] describes classic MCL as a monomorphic proliferation of small- to medium-sized lymphoid cells, with an irregular nuclear border, dispersed chromatin, and inconspicuous nucleoli. The architectural patterns may include a vaguely nodular and diffuse mantle zone or, rarely, follicular growth pattern. Infrequent cases, referred to as in situ MCL, may exclusively involve the inner mantle zones or narrow mantles. Histologic transformation to large B-cell lymphoma does not occur; however, MCL can have several morphologic variants, including aggressive blastoid and pleomorphic variants (Fig. 86.3).

1. Blastoid variant: lymphoma cells have a high mitotic rate (20 to 30 per 10 high-power fields) and dispersed chromatin; frequently resembles lymphoblasts.
2. Pleomorphic variant: cells have different forms including frequent large cells, pale cytoplasm, oval to irregular nuclear border, and often prominent nucleoli.

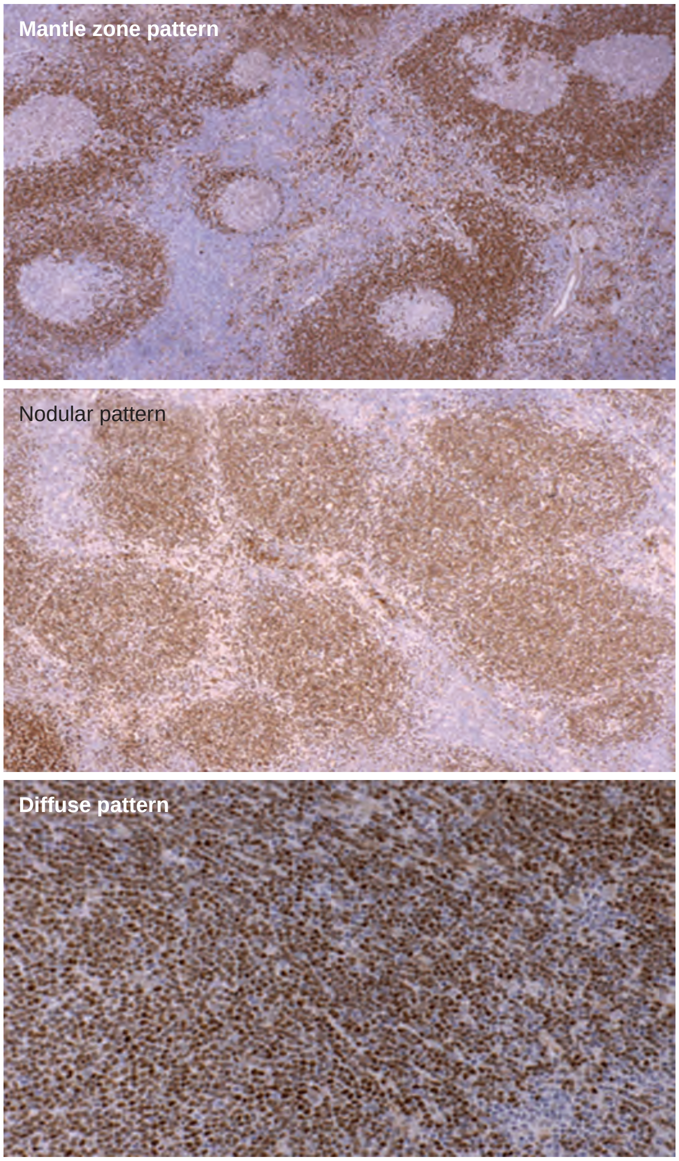


Figure 86.3 HISTOPATHOLOGIC SUBTYPES OF MANTLE CELL LYMPHOMA.

3. Small cell variant: cells are small and round with clumped chromatin; frequently resembles small lymphocytic lymphoma (SLL).
4. Marginal zone-like variant: this variant has clusters of marginal zone-like or monocytoid B cells, with abundant pale cytoplasm.

DIAGNOSIS

The diagnosis of MCL is based on the morphologic, immunophenotypic, and genetic features evaluated on biopsied tissue. The inclusion of immunophenotyping increased the diagnostic consensus between expert hematopathologists in the International NHL Classification project from 77% (based on histology) to 87%. The characteristic, immunophenotypic features of MCL include an expression of the B cell–associated antigens CD19, CD20, CD22, and CD79a, an aberrant expression of the T cell–associated antigens CD5 and CD43, and a lack of expression of CD3, CD10, and CD23. The identification of t(11;14)(q13;q32) and CCND1 oncogene can further aid to the diagnostic accuracy.[4,5] The presence of CCND1 oncogene is revealed most accurately in stains of formalin-fixed, paraffin-embedded lymph nodes or in soft tissue. Nonetheless, the cyclin D1 overexpression can also be seen in a subset of patients with ALL, Burkitt lymphoma, hairy cell leukemia, and multiple myeloma. The t(11;14) is seen in more than half of patients on karyotyping and in virtually all the patients screened with fluorescence in situ hybridization. In patients with cyclin D1–negative MCL, overexpression of cyclin D2, cyclin D3, or SOX11 can help in establishing the diagnosis. However, cyclin D2 is also overexpressed in patients with chronic lymphocytic leukemia and lymphoplasmacytic lymphoma, and cyclin D3 can be overexpressed in the vast majority of B-cell malignancies. Overexpression of SOX11, present in more than 90% of the patients with MCL, can also be seen in ALL, T-cell prolymphocytic leukemia, and Burkitt lymphoma. However, these other entities are usually easier to differentiate by clinicopathologic and other criteria; therefore, SOX11 is a very useful tool in cyclin D1–negative cases.

Differential Diagnosis

MCL should be differentiated from other B-cell NHL, particularly SLL/chronic lymphocytic leukemia, lymphoplasmacytic lymphoma, marginal-zone lymphoma, and follicular lymphoma. Additionally, with minimal differentiation, the blastoid variant MCL may resemble B-cell ALL and acute myeloid leukemia. Immunophenotypic markers are helpful in differentiating MCL (CD5$^+$, CD10$^-$, CD19$^+$, CD23$^-$, FMC$^+$) from SLL/chronic lymphocytic leukemia (CD5$^+$, CD10$^-$, CD19$^+$, CD23$^+$, FMC$^-$) and follicular lymphoma (CD5$^-$, CD10$^+$, CD19$^+$). In CD5-negative or cyclin D1–negative MCL, careful exclusion of other lymphomas, via detection of overexpression of cyclin D2, cyclin D3 or SOX11 and gene expression profiling, may help in the diagnosis.

Staging

Staging procedures include a complete blood count, comprehensive metabolic panel, LDH, evaluation of a bone marrow biopsy, and computed tomography (CT) of chest, abdomen, and pelvis. Although endoscopies of upper and lower GI tracts are frequently positive, such evaluation did not change management in a retrospective study. MCL has low to intermediate avidity for fluorodeoxyglucose (FDG); however, the blastoid variant is more FDG-avid. Nonetheless, an integrated FDG positron emission tomography (PET) and CT scan is frequently utilized for staging of patients with MCL. Lumbar puncture is not routinely performed but should be done in patients with neurologic symptoms or blastoid subtype. Hepatitis B serologies are performed prior to the use of rituximab because of a risk of reactivation.

THERAPY

MCL often combines the worst features of both follicular lymphoma (noncurability) and diffuse, large B-cell lymphoma (aggressive course). A paucity of randomized controlled trials, consequent to disease rarity, has resulted in a lack of standard therapy. Therapy options range from wait-and-watch strategy in select asymptomatic patients to intensified chemotherapy followed by upfront high-dose chemotherapy (HDT) and autologous hematopoietic stem cell transplant (ASCT) in young fit patients.[1]

Wait-and-Watch Strategy

The approach of watchful waiting, as utilized in other indolent lymphomas, has been advocated by some investigators in select patients with MCL. This recommendation is supported by a study from Weill Cornell Medical College among 97 patients with MCL. The study compared the outcomes between early treatment and observation groups. Among the observation group, the median time to treatment was 12 months (4 to 128 months). Patients in the observation group had better performance status (PS) and were more likely to have lower-risk standard International Prognostic Index scores than the early treatment group. The study demonstrated that a delay in initiation of therapy did not influence overall survival (OS) in a multivariate analysis.[6] Good outcomes with initial wait-and-watch approach were demonstrated in other studies from the United Kingdom and Sweden. Although these retrospective studies may have limitations, the wait-and-watch strategy is reasonable in asymptomatic older patients, who emphasize on delaying the use of noncurative therapy to preserve the quality of life.

Therapy Options: Non-Transplant Directed

When a decision has been made to start treatment, several chemo-immunotherapy regimens are available: alkylator-based (cyclophosphamide, vincristine, and prednisone [CVP]), anthracycline-based (cyclophosphamide, doxorubicin, vincristine and prednisone [CHOP]), fludarabine-based (fludarabine, cyclophosphamide, and mitoxantrone [FCM]), bendamustine-based (bendamustine and rituximab [BR]), cytarabine- and/or methotrexate-based (cyclophosphamide, vincristine, doxorubicin, and dexamethasone alternating with cytarabine and methotrexate [hyper-CVAD/MA]) regimens, cytarabine-based regimens without methotrexate (cisplatin, cytarabine, and dexamethasone [DHAP]), or alternating CHOP with DHAP; all are typically combined with rituximab in the current era.[7–16] More recently, attempts to incorporate agents used for relapsed MCL, such as bortezomib, ibrutinib, or lenalidomide in upfront therapy, have shown promising results.[17–20] With conventional chemotherapy such as rituximab and CHOP (R-CHOP), the median remission duration is 1.5 to 3 years.[7–9] Table 86.1 outlines front-line studies for combination therapies, without autologous stem-cell transplantation (ASCT).

Therapy Options: Upfront Intensified With or Without Consolidation Autologous Stem Cell Transplantation

Several studies have demonstrated improved outcomes with upfront intensified therapy, either alone or followed by high-dose chemotherapy and ASCT, in young symptomatic patients with MCL. In a randomized trial conducted in the pre-rituximab era by the European MCL network, the use of myeloablative radiochemotherapy and ASCT, versus α-interferon maintenance, following CHOP-like regimen, improved median PFS (39 vs. 17 months, $P = .01$) This occurred in 122 MCL patients without OS benefit (83% vs. 77% at 3 years, $P = 0.18$) and who were also ≤ 65 years old in the first complete remission.[21] In an MDACC study, 97 treatment-naïve MCL patients received rituximab-HyperCVAD and R-methotrexate/cytarabine alternating. The overall response rate was high with 87% of the patients having a complete response.[22] In the rituximab era, several nonrandomized prospective and retrospective studies have demonstrated better survival outcomes through intensified therapy

TABLE 86.1 **Outcomes of Non-Intensive Rituximab-Based Therapy Utilized in Mantle Cell Lymphoma**

Author, Year	Number of Patients	Therapy	CR (%)	EFS/PFS (%)	OS (%)
[7]Howard, 2002	40	R-CHOP	48%	16.6 months, median	NR
[8]Forstpointer, 2004	48	R-FCM vs. FCM	29% vs. 0%	4 vs. 8 months median ($P=.38$)	Not reached at a median follow-up of 18 vs. 11 months ($P=.004$)
[9]Lenz, 2005	122	R-CHOP vs. CHOP	34% vs. 7%, $P < .001$	Median time to treatment failure (21 vs. 14 months, $P=.013$, but similar PFS ($P=.31$)	76% at 2 years ($P=.93$)
[10]Ruan, 2011	36	R-CHOP + bortezomib	72%	44% at 2 years	86% at 2 years
[11]Grant, 2011	26	DA-R-EPOCH and idiotype vaccine	92%	24 months median	104 months median
[12]Kluiin-Nelemans, 2012	485	R-CHOP vs. FCR	34% vs. 40% ($P=0.1$)	-	47% vs. 62% at 4 years, $P=.005$
[13]Dunleavy, 2012	43	Bortezomib + DA-R-EPOCH	-	50% at 4 years	80% at 4 years
[14]Rummel, 2013	94 MCL/54 NHL	BR vs. R-CHOP	40% vs. 30% ($P=.02$)	Median of 35 vs. 22 months ($P=.004$)	Similar
[15]Visco, 2013	20	Rituximab, bendamustine, cytarabine	95%	95% at 2 years	Not provided
[16]Flinn, 2019	74 MCL/447 NHL	BR vs. R-CHOP/R-CVP	50% BR vs. 27% R-CHOP	65.5% BR vs. 55.8% R-CHOP ($P=.0035$)	81.7% BR vs. 85.0% R-CHOP at 5 years
[17]Gressin, 2019	74	Rituximab, bendamustine, bortezomib	56.5%	70% at 2 years	86.6 % at 4 years
[18]Ruan, 2018	38	Lenalidomide + rituximab	60.5%	80% at 3 years	90% at 3 years
[19]Albertsson-Linblad, 2016		Lenalidomide, bendamustine, rituximab	64%	Median PFS–42 months	73% at 3 years
[20]Jain, 2019	49	Ibrutinib with rituximab	60%	Median not reached at 28 months	Median not reached at 28 months

CHOP, Cyclophosphamide, doxorubicin, vincristine and prednisone; *EFS*, event-free survival; *NHL*, non-Hodgkin lymphoma; *R-CHOP*, rituximab and CHOP.

TABLE 86.2 Outcomes of Intensive Rituximab-Based Therapy Followed by Autologous Stem Cell Transplantation in Mantle Cell Lymphoma

Author, Year	Number of Patients	Therapy	CR (%)	EFS/PFS (%)	OS (%)
[21]Dreyling, 2005	122	R-CHOP/auto PSCT	34%	50% at 2 years	75% at 3 years
[22]Romaguera, 2005	65	R-HyperCVAD/R-MTX-AraC	87%	Median 5.5 years	Median OS 13.4 years
[23]Geisler, 2008	160	Maxi-R-CHOP/cytarabine/BEAM/auto PSC	54%	65.9% at 6 years	81% at 4 years
[24]Damon, 2009	79	R-M-CHOP, EAR, CVB, R maintenance	69%	56% at 5 years	64% at 5 years
[25]Merli, 2012	63	R-Hyper-CVAD/R-MA	72%	61% at 5 years	73% at 5 years
[26]Delarue, 2013	60	CHOP-R/R-DHAP, then HDT/ASCT	57%	64% at 5 years	75% at 5 years
[27]Budde, 2011	118	R-HyperCVAD, R-CHOP, or R-EPOCH + auto PSCT	N/A	47.9% at 5 years	57.2% at 5 years
[28]Eskelund, 2017	160	R-maxi-CHOP/HiDAC, ASCT	90%	Median PFS 8.5 years	Median OS 12.7 years
[29]Hermine, 2016	497 total	R-CHOP /Alternating R-DHAP	83%	65% at 5 years	76% at 5 years
[30]Widmer, 2018	43	Chemoimmunotherapy then Auto PSCT	95.5%	33.1% at 10 years	76% at 10 years

ASCT, Autologous stem cell transplantation; *CHOP*, cyclophosphamide, doxorubicin, vincristine and prednisone; *EFS*, event-free survival; *HDT*, high-dose chemotherapy; *PSCT*, peripheral stem cell transplant; *R-CHOP*, rituximab and CHOP.

and HDT/ASCT, compared with historical controls. The second Nordic MCL trial, among untreated patients with MCL <66 years ($n = 160$), utilized a phase II protocol with dose-intensified induction immunochemotherapy with maxi-CHOP-R (dose-intensified R-CHOP), alternating with rituximab and high-dose cytarabine, followed by HDT and rituximab-in vivo purged ASCT among the responders. This resulted in a 6-year OS and event-free survival (EFS) of 70% and 56%, respectively, with a higher proportion of molecular response rate (92% vs. 38%, $P < .001$), compared with a historical control in the first Nordic MCL trial.[23] Cancer and Leukemia Group B (CALGB) 59909 study demonstrated a 5-year PFS and OS of 56% and 64%, respectively, among patients with MCL who were up to the age of 69 years ($n = 78$) and who were treated with cytarabine- and methotrexate-based immunochemotherapy, followed by HDT/ASCT.[24] Several other studies have demonstrated excellent outcomes with rituximab-based chemotherapy, such as R-Hyper-CVAD, R-CHOP/RDHAP (rituximab, cisplatin, cytarabine and dexamethasone), or BR and HDT/ASCT (Table 86.2).[25–30] It is currently not clear if the choice of induction therapy makes a difference in the long-term outcomes of patients going on to receive HDT/ASCT, as long as the patient is in a good CR at the time of transplant.

Although these studies demonstrate improved progression-free survival (PFS) with HDT/ASCT consolidation, the optimal induction therapy is unclear. In order to determine the value of induction therapy, an NCCN NHL-database study compared the outcomes of patients with MCL <65 years, treated with several chemotherapy regimens. This study demonstrated a worse 3-year PFS with R-CHOP alone (18%), compared with R-Hyper-CVAD (58%, $P < .001$), R-CHOP and HDT/ASCT (56%, $P < .001$), and R-Hyper-CVAD and HDT/ASCT (55%, $P = .004$). PFS did not differ between the latter three groups. The 3-year OS was similar between R-CHOP alone (69%), R-Hyper-CVAD (85%, $P < .07$), and R-CHOP and HDT/ASCT (87%, $P < .20$). Multivariable analysis revealed that R-CHOP alone had worse OS compared with R-Hyper-CVAD but not worse compared to R-CHOP and HDT/ASCT.[31] Similarly, the type of induction therapy or the timing of therapy did not predict OS in 118 MCL patients who were treated with HDT/ASCT in another study. The use of R-Hyper-CVAD, compared with R-CHOP-like therapy, improved PFS for the entire MCL cohort ($P = .01$) but not among transplant recipients ($P = .26$). These studies indicate that intensive induction therapy, such as R-Hyper-CVAD, may be preferred to R-CHOP among patients who are not planned to undergo HDT/ASCT consolidation; however, the choice of initial therapy may not make a significant difference in OS of patients who are scheduled to undergo HDT/ASCT.[32]

In one study, the use of pre-emptive rituximab therapy (375 mg/m^2 weekly for 4 weeks) for molecular relapse, after upfront HDT/ASCT, resulted in re-induction of molecular remission in 92% and median molecular and clinical relapse-free survivals of 1.5 and 3.7 years, respectively.[33] Rituximab maintenance, after R-Hyper-CVAD or upfront HDT/ASCT, may also improve PFS.[34] The use of rituximab frequently increases the risk of neutropenia and hypogammaglobulinemia; however, this may not necessarily translate to a substantially increased risk of major infections.

Since we do not know the need for HDT/ASCT in MCL patients with negative minimal residual disease (MRD) after induction therapy, a United States intergroup study, EA4151, is randomizing patients who are MRD-negative after their induction to either rituximab maintenance × 3 years versus HDT/ASCT and then rituximab maintenance × 3 years (NCT3267433).

Central Nervous System Prophylaxis

The incidence of CNS involvement at diagnosis and the role of primary CNS prophylaxis in MCL are controversial. In relapsed MCL, CNS involvement is noted in up to 5% to 20% of patients, particularly those with blastoid variant and those that portend a poor prognosis. CNS-penetrating agents such as high-dose methotrexate and cytarabine, with or without intrathecal chemotherapy, may provide sufficient CNS prophylaxis in patients undergoing intensive therapies. In other patients with high-risk features such as blastoid morphology or presence of neurologic symptoms, some centers perform lumbar puncture and utilize intrathecal methotrexate or high-dose systemic methotrexate. Ibrutinib and lenalidomide have both been found to cross the blood-brain barrier and may be useful in some cases of CNS disease.

Relapsed or Refractory Mantle-Cell Lymphoma

Despite induction therapy, most patients with MCL will relapse and require subsequent therapies. The choice of therapy in relapsed or refractory MCL depends on the upfront chemotherapy regimen, outcomes with initial therapy, PS, comorbidities, and desired goals. Several options are FDA approved and available bortezomib,

TABLE 86.3 **Outcomes of Therapy Utilized in Relapsed/Refractory Mantle Cell Lymphoma**

Author, Year	Number of Patients	Therapy	ORR (%)	EFS/PFS (%)	OS (%)
[35]Goy, 2009	141	Bortezomib	32%	5 months	22 months
[36]Rummel, 2005	63	Bendamustine + rituximab	75%	Median 18 months	55% at 4 years
[37]Wang, 2012	44	Lenalidomide + rituximab	57%	11 months	24 months
[38]Rule, 2019	370	Ibrutinib	69.7%	12.5 months median	26.7 months median
[39]Wang, 2018	124	Acalabrutinib	81%	67% at 12 months	87% at 12 months
[40]Song, 2018	86	Zanubrutinib	84%	82% at 6 months	N/A
[43]Eyre, 2019	20	Venetoclax	53%	3 months	9.4 months
[44]Wang, 2020	74	KTE-X19 CAR-T cell	93%	61% at 12 months	83% at 12 months

CAR, Chimeric antigen receptor; *EFS*, event-free survival.

bendamustine, or lenalidomide, which are often combined with rituximab.[35–37] BTK inhibitors, such as ibrutinib, acalabrutinib, or zanubrutinib, have more recently been approved and used extensively for relapsed MCL.[38–40] Ibrutinib received an accelerated approval in November of 2013 based on a phase II study of relapsed or refractory MCL ($n = 111$), which demonstrated an overall response rate of 68%, with an OS of 58% at 18 months.[38] Acalabrutinib is an oral irreversible BTKi, with less off-target inhibition of similar kinases in comparison to ibrutinib.[41] The safety profile of acalabrutinib and a more recent approval of zanubrutinib may have less atrial fibrillation and bleeding risks than ibrutinib, but only a head-to-head comparison will show this definitively.[42] Other agents that are FDA approved for relapsed MCL include venetoclax or brexucabtagene autoleucel (Table 86.3).[43,44]

A new class of BTKi targets BTK via noncovalent, reversible inhibition. Unlike the irreversible BTKi, this class does not bind BTK at the C481 residue and thus would be expected to be unaffected by BTK binding site mutations. Mutations within the BTK binding site (most commonly C481S) are a well-characterized resistance mechanism to second-generation BTKi.[45] Preliminary results from phase I/II trials, with several agents in this class, show activity in B-cell malignancies, including BTKi relapsed MCL.[46,47]

Cellular Therapy in Relapsed/Refractory Mantle Cell Lymphoma

Patients who have not undergone upfront HDT/ASCT may benefit from HDT/ASCT, after salvage therapy. In one study, the use of HDT/ASCT demonstrated a median EFS and OS of 36% and 65%, respectively.[48]

With the advent of CD19-directed chimeric antigen receptor (CAR) T-cell therapy for the treatment of other B-cell malignancies, the ZUMA-2 trial tested brexucabtagene autoleucel (KTE-X19, an autologous CD19 CAR-T-cell construct).[44] Of the 74 patients enrolled on the trial, 68 had the product infused. Of the eligible patients, the overall response rate (ORR) was 93% and the complete response (CR) rate was 68%. Cytokine release syndrome and neurotoxicity, at a grade three or higher, was 15% and 31%, respectively. Infectious adverse events occurred twice at grade level five.[44] This therapy is now FDA approved and also undergoing further combination or sequential studies.

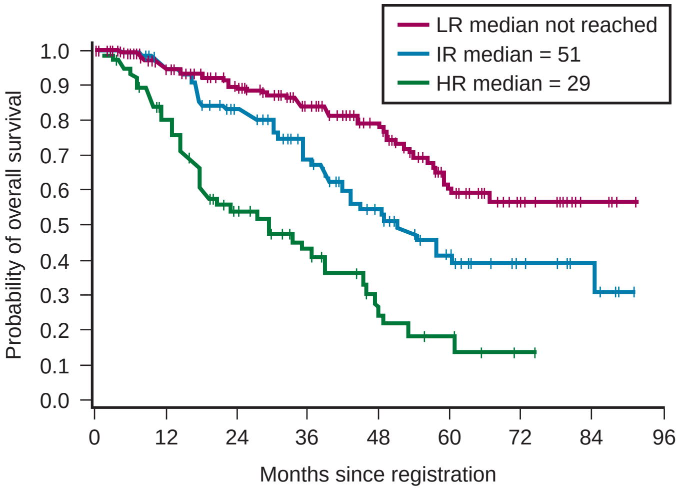
Although allogeneic stem cell transplantation (alloSCT) is potentially curative, lack of large-scale randomized trials and high transplant-related mortality (TRM) has limited enthusiasm toward the use of alloSCT to treat MCL. Nonetheless, alloSCT should be considered in young and fit MCL patients with multiple relapsed disease or ASCT failure. The outcomes of alloSCT have been compared with those of ASCT in a few studies. In a Center for International Blood and Marrow Transplant Research (CIBMTR), retrospective study of chemosensitive patients with MCL ($n = 519$) and reduced intensity conditioning (RIC) alloSCT resulted in lower relapse/progression, compared with ASCT. This was offset by a higher non-relapse mortality (NRM); hence, the 5-year OS was similar between the two groups. ASCT in first complete remission was associated with the highest OS and PFS, confirming the results of prior studies of upfront HDT/ASCT.[49] In these studies, the comparable OS with alloSCT, compared with ASCT, also demonstrates the efficacy of disease control with alloSCT in patients with multiple relapses. In clinical practice, therefore, alloSCT may be utilized in heavily pretreated patients.

RIC has been explored with a hope to reduce TRM and expand alloSCT to older patients or patients with other comorbidities. In a study, 70 patients with relapsed and refractory MCL, including prior ASCT failures (34%), underwent RIC alloSCT, mainly with alemtuzumab-containing regimens. NRM, cumulative risk of relapse, PFS, and OS were 21%, 65%, 14%, and 37%, respectively, at 5 years. Among patients who relapsed, donor lymphocyte infusion (DLI) or a second RIC alloSCT resulted in a complete remission rate of 73%.[50] Hence, RIC and nonmyeloablative alloSCT, along with DLI for relapse, may be reasonable options in patients who are not candidates for myeloablative alloSCT.

PROGNOSIS

As discussed previously, complete remission is seen in up to two-thirds of patients with MCL, particularly with upfront intensive therapy; however, almost all patients will relapse and develop resistance to chemotherapy over time. Although the prognosis for newly diagnosed patients has improved over the last decades, the OS compares unfavorably with the more frequent NHLs, such as follicular lymphoma and diffuse, large B-cell lymphoma. A small subset of patients may follow a more indolent clinical course in the beginning, and these patients may have a clinical history that resembles a chronic lymphocytic leukemia.

Prior studies have identified several adverse prognostic factors, which include eastern cooperative oncology group (ECOG) PS ≥ 2, age >65 years, elevated LDH, elevated serum beta$_2$-microglobulin, hemoglobin level <12 g/dL, advanced stage, involvement of peripheral blood or extranodal sites ≥ 2, high cell proliferation rate (Ki-67, mitotic index or proliferation signature), blastoid variant histology, deletion or mutation of TP53, and secondary chromosomal aberrations (the presence of gain 3q, 12q, and 13q and losses of 6q, 9p, 11q, and 17p).[51] More recent studies have demonstrated that the MCL international prognostic index (MIPI), based on age, ECOG PS, LDH, and white-blood cell count, is one of the most important prognostic factors in MCL. In a large study, MIPI categorized MCL patients into low risk (0 to 3 scores; OS not reached at a median follow-up of 32 months), intermediate risk (4 to 5 scores; median OS of 51 months), and high risk (6 to 11 scores; median OS of 29 months) categories, with a significant difference in OS (Fig. 86.4).



Numbers of patients at risk

LR	180	153	131	99	69	39	15	4
IR	145	116	83	57	37	19	9	5
HR	84	58	29	19	8	5	1	0

Figure 86.4 PROGNOSTIC GROUPS STRATIFIED ACCORDING TO THE MANTLE CELL INTERNATIONAL PROGNOSTIC INDEX. *HR*, High risk; *IR*, intermediate risk; *LR*, low risk.

MIPI separated the survival curves better than IPI utilized for diffuse large B-cell lymphoma or follicular lymphoma (FLIPI).[52] Cell proliferation (Ki-67), but not number of mitoses per square millimeter, was an independent prognostic score.[53] Ki-67 and MIPI have been shown to independently predict PFS and OS in other studies also. Prognosis may also be influenced by disease status at ASCT or alloSCT, achievement of molecular remission after therapy.[54] In the modern era, molecular diagnostics such as gene expression profiling, genome-wide miRNA profiling, as well as a limited-gene model (based on five genes *RAN*, *MYC*, *TNFRSF10B*, *POLE2*, and *SLC29A2*), may also provide meaningful prognostic information; however, the lack of commercial testing and standardization, as well as high cost, limit their use in clinical practice.

FUTURE DIRECTIONS

Since the recognition of MCL as a separate entity, significant advancement has been made in the understanding of tumor biology, diagnostic, prognostic, and treatment approaches. At a population level, this has resulted in a significant improvement in OS of patients with MCL, with approximately half of the patients in the United States surviving beyond 5 years, in recent years. A few studies have demonstrated poor prognosis of blastoid-variant MCL. Intensified therapy, including upfront ASCT, may overcome this poor prognosis, which needs further exploration in prospective studies. The roles of radiotherapy and CNS prophylaxis are currently not well established and should be investigated. Integration of clinical parameters and gene expression profiling has a potential to improve prognostication. Such prognostication may identify an indolent subgroup that can be observed, as well as an aggressive subgroup that may be better served, with upfront intensive therapy. A development of prognosis-directed therapy is an ideal goal. Molecular markers such as clonal immunoglobulin heavy chain gene rearrangements and *SOX11* gene expression may be used to identify MRD. Although the correlation is not consistent, molecular remission has been correlated with long-term remission and PFS. Molecular relapse may precede clinical relapse and has been utilized to guide preemptive therapy, as discussed above. If confirmed in other prospective trials, MRD-based therapies may be utilized to improve PFS in the future. Elderly patients and patients with comorbidities generally have poor outcomes and should be enrolled in clinical trials of novel, well-tolerated agents or combinations. Improved prognostic tools, incorporation of MRD-based preemptive therapy, translation of molecular tumor biology to bedside in the form of novel targeted therapies, and integration of such therapies to upfront traditional chemoimmunotherapy regimens offer a potential to change the therapeutic landscape of MCL.

REFERENCES

1. Vose JM. Mantle cell lymphoma: 2017 Update on diagnosis, risk-stratification, and clinical management. *Am J Hematol*. 2017;92(8):806–813.
2. The Non-Hodgkin's Lymphoma Classification Project A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. *Blood*. 1997;89:3909–3918.
3. Beà S, Amador V. Role of SOX11 and genetic events cooperating with cyclin D1 in mantle cell lymphoma. *Curr Oncol Rep*. 2017;19(6):43.
4. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127:2375–2390.
5. Veloza L, Ribera-Cortada I, Campo E. Mantle cell lymphoma pathology update in the 2016 WHO classification. *Annals Lymphoma*. 2019;3(3):1–17.
6. Martin P, Chadburn A, Christos P, et al. Outcome of deferred initial therapy in mantle-cell lymphoma. *J Clin Oncol*. 2009;27(8):1209–1213.
7. Howard OM, Gribben JG, Neuberg DS, et al. Rituximab and CHOP induction therapy for newly diagnosed mantle-cell lymphoma: molecular complete responses are not predictive of progression-free survival. *J Clin Oncol*. 2002;20(5):1288–1294.
8. Forstpointner R, Dreyling M, Repp R, et al. The addition of rituximab to a combination of fludarabine, cyclophosphamide, mitoxantrone (FCM) significantly increases the response rate and prolongs survival as compared with FCM alone in patients with relapsed and refractory follicular and mantle cell lymphomas: results of a prospective randomized study of the German Low-Grade Lymphoma Study Group. *Blood*. 2004;104(10):3064–3071.
9. Lenz G, Dreyling M, Hoster E, et al. Immunochemotherapy with rituximab and cyclophosphamide, doxorubicin, vincristine, and prednisone significantly improves response and time to treatment failure, but not long-term outcome in patients with previously untreated mantle cell lymphoma: results of a prospective randomized trial of the German Low Grade Lymphoma Study Group (GLSG). *J Clin Oncol*. 2005;23(9):1984–1992.
10. Ruan J, Martin P, Furman RR, et al. Bortezomib plus CHOP-rituximab for previously untreated diffuse large B-cell lymphoma and mantle cell lymphoma. *J Clin Oncol*. 2011;29(6):690–697.
11. Grant C, Neelapu SS, Kwak LW, et al. Eleven-year follow-up of idiotype vaccine and DA-EPOCH-rituximab in untreated mantle cell lymphoma: correlation of survival with idiotype immune response. *Blood*. 2011;118(21):2707.
12. Kluin-Nelemans HC, Hoster E, Hermine O, et al. Treatment of older patients with mantle-cell lymphoma. *N Engl J Med*. 2012;367(6):520–531.
13. Dunleavy K, Grant C, Hessler J, et al. Bortezomib + DA-EPOCH-R induction therapy followed by maintenance bortezomib versus observation in newly diagnosed mantle cell lymphoma. *Blood*. 2012;120(21):3672.
14. Rummel MJ, Niederle N, Maschmeyer G, et al. Bendamustine plus rituximab versus CHOP plus rituximab as first-line treatment for patients with indolent and mantle-cell lymphomas: an open-label, multicentre, randomised, phase 3 non-inferiority trial. *Lancet*. 2013;381(9873):1203–1210.
15. Visco C, Finotto S, Zambello R, et al. Combination of rituximab, bendamustine, and cytarabine for patients with mantle-cell non-Hodgkin lymphoma ineligible for intensive regimens or autologous transplantation. *J Clin Oncol*. 2013;31(11):1442–1449.
16. Flinn IW, van der Jagt R, Kahl BS, et al. First-line treatment of patients with indolent non-Hodgkin lymphoma or mantle-cell lymphoma with bendamustine plus rituximab versus R-CHOP or R-CVP: results of the BRIGHT 5-year follow-up study. *J Clin Oncol*. 2019;37:984–991.
17. Gressin R, Daguindau N, Tempescul A, et al. A phase 2 study of rituximab, benadmustine, bortezomib and dexamethasone for first-line treatment of older patients with mantle cell lymphoma. *Haematologica*. 2019;104:138–146.
18. Ruan J, Martin P, Christos P, et al. Five-year follow-up of lenalidomide plus rituximab as initial treatment of mantle cell lymphoma. *Blood*. 2018;132:2016–2025.
19. Albertsson-Lindblad A, Kolstad A, et al. Lenalidomide-bendamustine-rituximab in patients older than 65 years with untreated mantle cell lymphoma. *Blood*. 2016;128:1814–1820.
20. Jain P, Lee HJ, Steiner RE, et al. Frontline treatment with ibrutinib with rituximab (IR) combination is highly effective in elderly (≥65 years)

patients with mantle cell lymphoma (MCL)—results from a phase II trial. *Blood*. 2019;134:3988.

21. Dreyling M, Lenz G, Hoster E, et al. Early consolidation by myeloablative radiochemotherapy followed by autologous stem cell transplantation in first remission significantly prolongs progression-free survival in mantle-cell lymphoma: results of a prospective randomized trial of the European MCL Network. *Blood*. 2005;105(7):2677–2684.
22. Romaguera JE, Fayad L, Rodriguez MA, et al. High rate of durable remissions after treatment of newly diagnosed aggressive mantle-cell lymphoma with rituximab plus hyper-CVAD alternating with rituximab plus high-dose methotrexate and cytarabine. *J Clin Oncol*. 2005;23(28):7013–7023.
23. Geisler CH, Kolstad A, Laurell A, et al. Long-term progression-free survival of mantle cell lymphoma after intensive front-line immunochemotherapy with in vivo-purged stem cell rescue: a nonrandomized phase 2 multicenter study by the Nordic Lymphoma Group. *Blood*. 2008;112(7):2687–2693.
24. Damon LE, Johnson JL, Niedzwiecki D, et al. Immunochemotherapy and autologous stem-cell transplantation for untreated patients with mantle-cell lymphoma: CALGB 59909. *J Clin Oncol*. 2009;27(36):6101–6108.
25. Merli F, Luminari S, Ilariucci F, et al. Rituximab plus HyperCVAD alternating with high dose cytarabine and methotrexate for the initial treatment of patients with mantle cell lymphoma, a multicentre trial from Gruppo Italiano Studio Linfomi. *Br J Haematol*. 2012;156(3):346–353.
26. Delarue R, Haioun C, Ribrag V, et al. CHOP and DHAP plus rituximab followed by autologous stem cell transplantation in mantle cell lymphoma: a phase 2 study from the Groupe d'Etude des Lymphomes de l'Adulte. *Blood*. 2013;121(1):48–53.
27. Budde LE, Guthrie KA, Till BG, et al. Mantle cell lymphoma international prognostic index but not pretransplantation induction regimen predicts survival for patients with mantle-cell lymphoma receiving high-dose therapy and autologous stem-cell transplantation. *J Clin Oncol*. 2011;29(22):3023–3029.
28. Eskelund CW, Kolstad A, Jerkeman M, et al. 15-year follow-up of the second Nordic Mantle Cell Lymphoma trial (MCL2): prolonged remissions without survival plateau. *Br J Haematol*. 2016;175(3):410–418.
29. Hermine O, Hoster E, Walewski J, et al. Addition of high-dose cytarabine to immunochemotherapy before autologous stem-cell transplantation in patients aged 65 years or younger with mantle cell lymphoma (MCL Younger): a randomised open-label, phase 3 trial of the European Mantle Cell Lymphoma network. *Lancet*. 2016;388(10044):565–575.
30. Widmer F, Balabanov S, Soldini D, et al. R-hyper-CVAD versus R-CHOP/cytarabine with high-dose therapy and autologous haematopoietic stem cell support in fit patients with mantle cell lymphoma: 20 years of single-center experience. *Ann Hematol*. 2018;97(2):277–287.
31. LaCasce AS, Vandergrift JL, Rodriguez MA, et al. Comparative outcome of initial therapy for younger patients with mantle cell lymphoma: an analysis from the NCCN NHL database. *Blood*. 2012;119(9):2093–2099.
32. Sawalha Y, Radivoyevitch T, Tullio K, et al. The role of upfront autologous hematopoietic cell transplantation in the treatment of mantle cell lymphoma, a population based study using the National Cancer Data Base (NCDB). *Blood*. 2017;130(S1).
33. Ferrero S, Monitillo L, Mantoa B, et al. Rituximab-based pre-emptive treatment of molecular relapse in follicular and mantle cell lymphoma. *Ann Hematol*. 2013;92:1503–1511.
34. Le Gouill S, Thieblemont C, Oberic L, et al. Rituximab after autologous stem-cell transplantation in mantle-cell lymphoma. *N Engl J Med*. 2017;377(13):1250–1260.
35. Goy A, Bernstein SH, Kahl BS, et al. Bortezomib in patients with relapsed or refractory mantle cell lymphoma: updated time-to-event analyses of the multicenter phase 2 PINNACLE study. *Ann Oncol*. 2009;20(3):520–525.
36. Rummel MJ, Al-Batran SE, Kim S-Z, et al. Bendamustine plus rituximab is effective and has a favorable toxicity profile in the treatment of mantle cell and low-grade non-Hodgkin's lymphoma. *J Clin Oncol*. 2005;23(15):3383–3389.
37. Wang M, Fayad L, Wagner-Bartak N, et al. Lenalidomide in combination with rituximab for patients with relapsed or refractory mantle-cell lymphoma: a phase 1/2 clinical trial. *Lancet. Oncol*. 2012;13(7):716–723.
38. Rule S, Dreyling M, Goy A, et al. Ibrutinib for the treatment of relapsed/refractory mantle cell lymphoma: extended 3.5-year follow up from a pooled analysis. *Haematolgica*. 2019;104(5):211–214.
39. Wang M, Rule S, Zinzani PL, et al. Acalabrutinib in relapsed or refractory mantle cell lymphoma (ACE-LY-004): a single-arm, multicentre, phase 2 trial. *Lancet*. 2018;391 659-6.
40. Song Y, Zhou K, Zou D. Safety and activity of the investigational Bruton tyrosine kinase inhibitor zanubrutinib (BGB-3111) in patients with mantle cell lymphoma from a phase 2 trial. *Blood*. 2018;132:148a.
41. Byrd JC, Owen R, O'Brien S. Pooled analysis of safety data from clinical trials evaluating acalabrutinib monotherapy in hematologic malignancies. *Blood*. 2017;130:4326.
42. Tam CS, Wang M, Simpson D. Updated safety and activity of the investigational Bruton tyrosine kinase inhibitor zanubrutinib (BGB-3111) in patients with mantle cell lymphoma. *Blood*. 2018;132:1592.
43. Eyre TA, Walter HS, Lyengar S, et al. Efficacy of venetoclax monotherapy in patients with relapsed, refractory mantle cell lymphoma after Bruton tyrosine kinase inhibitor therapy. *Haematologica*. 2019;104(2):e68–e71.
44. Wang M, Munoz J, Goy A, et al. KTE-X19 CAR T-cell therapy in relapsed or refractory mantle-cell lymphoma. *N Engl J Med*. 2020;382(14):1331–1342.
45. Jain P, Kanagal-Shamanna R, Zhang S, et al. Long-term outcomes and mutation profiling of patients with mantle cell lymphoma (MCL) who discontinued ibrutinib. *Br J Haematol*. 2018;183(4):578–587.
46. Woyach J, Stephens DM, Flinn IW, et al. Final results of phase 1, dose escalation study evaluating ARQ 531 in patients with relapsed or refractory B-cell lymphoid malignancies. *Blood*. 2019;134:4298.
47. Wang M, Shah NN, Alencar AJ, et al. LOXO-35, a next generation, highly selective, non-covalent BTK inhibitor in previously treated mantle cell lymphoma, Waldenstrom's macroglobulinemia, and other non-Hodgkin lymphomas: result from the phase 1/2 BRUIN Study. ASH 2020, Abstr 117.
48. Ganti AK, Bierman PJ, Lynch JC, et al. Hematopoietic stem cell transplantation in mantle cell lymphoma. *Ann Oncol*. 2005;16(4):618–624.
49. Fenske TS, Zhang M-J, Carreras J, et al. Autologous or reduced-intensity conditioning allogeneic hematopoietic cell transplantation for chemotherapy sensitive mantle-cell lymphoma: analysis of transplantation timing and modality. *J Clin Oncol*. 2014;32(4):273–281.
50. Cook G, Smith GM, Kirkland K, et al. Outcomes following reduced-intensity allogeneic stem cell transplantation (RIC AlloSCT) for relapsed and refractory mantle cell lymphoma (MCL): a study of the British Society for Blood and Marrow Transplantation. *Biol Blood Marrow Transplant*. 2010;16(10):1419–1427.
51. Caballero D, Campo E, López-Guillermo A, et al. Clinical practice guidelines for diagnosis, treatment, and follow-up of patients with mantle cell lymphoma. Recommendations from the GEL/TAMO Spanish Cooperative Group. *Ann Hematol*. 2013;92(9):1151–1179.
52. Hoster E, Dreyling M, Klapper W, et al. A new prognostic index (MIPI) for patients with advanced-stage mantle cell lymphoma. *Blood*. 2008;111(2):558–565.
53. Hoster E, Rosenwald A, Berger F, et al. Prognostic value of Ki-67 index, cytology, and growth pattern in mantle-cell lymphoma: results from randomized trials of the European Mantle Cell Lymphoma Network. *J Clin Oncol*. 2016;34:1386–1394.
54. Pott C. Minimal residual disease detection in mantle cell lymphoma: technical aspects and clinical relevance. *Semin Hematol*. 2011;48:172–184.

CHAPTER 87

VIRUS-ASSOCIATED LYMPHOMA

Katherine C. Rappazzo, Jennifer A. Kanakry, and Richard F. Ambinder

There are five well-characterized human viruses that are generally accepted as important in lymphomagenesis (Table 87.1). These viruses may infect tumor cells (or their progenitors) or may act at a distance. The genomes of Epstein-Barr virus (EBV), Kaposi sarcoma–associated herpesvirus (KSHV, also known as human herpesvirus 8 [HHV-8]), and human T-lymphotropic virus-1 (HTLV-1) are present in tumor cells. The viral genes expressed in tumor cells modulate cellular metabolism, proliferation, and cell death. By contrast, the human immunodeficiency virus (HIV) genome is generally not detected in tumor cells. Whether hepatitis C virus (HCV) genomes are present in lymphoma cells remains a subject of controversy.

Although viral infection plays a role in the pathogenesis of some lymphomas, lymphomagenesis is unusual. Only a small subset of infected people develops lymphoma, and most lymphomas arise years or decades after primary infection. Geography and associated environmental exposures, host genetic factors, and immune status all modify risk.

Aspects of the biology and epidemiology of each of these viruses and their relationship with lymphomagenesis are reviewed in this chapter. In addition, clinically important and distinctive features of diagnosis and treatment of the associated lymphomas are presented.

EPSTEIN-BARR VIRUS

Viral Biology

EBV is a gammaherpesvirus transmitted mainly through saliva.[1,2] Infected B cells are driven to proliferate until an immune response controls infection. Thereafter, the viral genome persists for life in memory B lymphocytes. These cells elude immune surveillance in part because of very restricted viral gene expression and antigen presentation. Occasionally there is activation of viral lytic gene expression, but T cell–mediated immune function keeps such proliferation in check.

In vitro, EBV immortalizes B cells such that they grow indefinitely as lymphoblastoid cell lines (LCLs) (Fig. 87.1). LCLs are tumorigenic in immunodeficient mice. In LCLs, viral genomes are present as circular, double-stranded deoxyribonucleic acid (DNA) episomes within the nucleus. The viral proteins required for immortalization include Epstein-Barr virus nuclear antigen-1 (EBNA1), a sequence-specific DNA-binding protein important in the maintenance of the viral episome; EBNA2, a transcription factor that has many effects similar to those of activated Notch receptors; and latent membrane protein-1 (LMP1), a constitutively activated member of the tumor necrosis factor receptor superfamily, which most closely resembles CD40.[3] LMP1 activates the nuclear factor-κB (NFκB) pathway, which modulates cell proliferation and apoptosis.[4] Several other EBV proteins are also required for immortalization. Although EBV immortalization of B cells in vitro may offer some insights into tumorigenesis, some caution is required in using LCL as a tumor model. Most EBV tumors, including tumors of B-lineage cells, do not express many of the viral genes required for lymphocyte immortalization. The only tumors that express the full complement of viral proteins required for immortalization are those that arise in the most profoundly immunocompromised patients. It has been suggested that there is an inverse relationship between cellular mutations and viral gene expression in tumors.[5]

EBV gene expression may directly drive proliferation or inhibit apoptotic pathways, as illustrated by lymphocyte immortalization. However, viral gene expression may also perturb normal lymphocyte biology. Thus LMP1 expression upregulates activation induced (cytidine) deaminase expression, which facilitates somatic hypermutation and immunoglobulin class switching.[6] LMP1 expression may also be important in the conversion of naive B cells to postgerminal center memory B cells. LMP2A allows B cells that lack normal immunoglobulin expression to escape regulatory checkpoints and survive.[7]

Epidemiology of Viral Infection

EBV infection is ubiquitous. The vast majority of adults are infected worldwide. Primary infection most often occurs in childhood and is asymptomatic, but it may present as infectious mononucleosis in older children and adults.[8] Other possible determinants of symptomatic primary infection include genetic factors and possibly the size of the viral inoculum.

Strain differences in EBV are well recognized.[9] There is general agreement that the type 1 strain EBV is most common worldwide and in tumors. However, the importance of these strain differences with regard to lymphomagenesis remains poorly understood. In addition, variations in the regulatory regions or coding regions of a variety of other genes, including *EBNA1, LMP1*, and *ZTA*, have been recognized and suggested to play a role in lymphomagenesis. A simple classification of latent viral gene expression recognizes three patterns, as shown in Table 87.2.

Epstein-Barr Virus Detection in Clinical Specimens

Detection of virus in tumor cells requires demonstration of viral DNA, RNA, or protein in tumor cells.[10] Although polymerase chain reaction (PCR) makes detection of viral DNA straightforward, the ubiquity of EBV infection and its persistence in a tiny fraction of B cells that harbor EBV means that EBV DNA is readily detected in many specimens that include normal lymphocytes. In pathology laboratories, immunohistochemistry for LMP1 is commonly employed and is sensitive for the detection of EBV in Hodgkin lymphoma (HL).[11] In a variety of other EBV-associated B- and T-cell malignancies, expression of LMP1 is more variable. Thus failure to detect LMP1 expression does not exclude the presence of EBV, except perhaps in HL. In situ hybridization to detect viral RNA in tissue specimens has emerged as the pathology laboratory standard. The viral RNAs targeted are the EBV-encoded RNA (EBER) RNAs that encode short non-coding RNA transcripts expressed at high copy number per cell. Whereas viral DNA is typically present at less than 100 copies/cell in tumor tissue, EBER RNAs are present in very high numbers (millions of copies).

TABLE 87.1 **Viruses and Lymphomagenesis**

Virus	Viral Genome in Tumor Cell	Lymphoma Type
EBV	Episomal	B, T, NK
KSHV	Episomal	B
HTLV-1	Integrated	T
HIV-1	Absent	B
HCV	Uncertain	B

EBV, Epstein-Barr virus; *HCV*, hepatitis C virus; *HIV-1*, human immunodeficiency virus type 1; *HTLV-1*, human T-lymphotropic virus-1; *KSHV*, Kaposi sarcoma–associated herpesvirus; *NK*, natural killer.

TABLE 87.2 **Patterns of Epstein-Barr Virus Gene Expression in Latency**

Latency	EBNA1	EBNA2, EBNA3A, EBNA3B, EBNA3C	LMP1	LMP2A
I	+			
II	+		+	+
III	+	+	+	+

EBNA, Epstein-Barr virus nuclear antigen; *EBV*, Epstein-Barr virus; *LMP*, latent membrane protein.

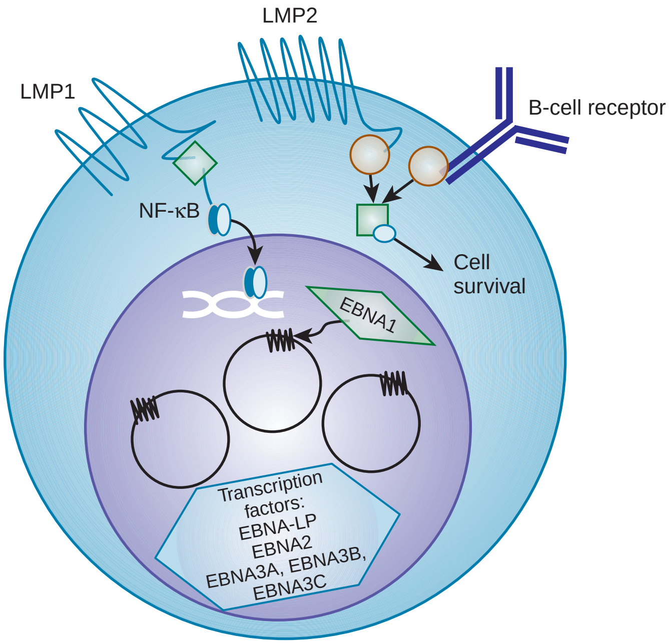


Figure 87.1 EPSTEIN-BARR VIRUS–IMMORTALIZED B CELL. Normal B cells are readily immortalized in vitro with EBV. These cells express EBV nuclear and membrane proteins. The nuclear proteins include a protein expressed in all EBV-associated tumors, Epstein-Barr virus nuclear antigen 1 (EBNA1). This protein is required for episomal maintenance. Other viral nuclear proteins expressed are transcription factors. These include EBNA-LP, EBNA2, EBNA3A, EBNA3B, and EBNA3C. Two membrane proteins are expressed: latent membrane protein 1 (LMP1), which activates nuclear factor-κB (NFκB) pathways, and LMP2A, which mimics B-cell receptor (immunoglobulin) signaling.

EBV-positive cancers typically have detectable viral DNA in the blood, making cell-free or plasma EBV DNA an attractive tumor marker.[12–14] Detection of EBV DNA in whole blood may reflect the presence of viral DNA in cells or in plasma. In cells the viral DNA may be present as linear double-stranded DNA molecules replicated by the viral DNA polymerase (to be packaged into virions) or as latent circular double stranded DNA plasmids associated with nuclear cell DNA and replicated by cellular enzymes with cell cycle. Virion DNA and viral DNA from latently infected cells that has been released following cell death is also measured in plasma. When blood has been fractionated and EBV DNA in peripheral blood mononuclear cells (PBMC) compared with EBV DNA in plasma, the correlation with EBV-associated disease is stronger with plasma.[15] It should be noted that many patients had detectable EBV DNA in PBMC and plasma without any EBV-associated disease (i.e., no EBV tumor nor evidence of infectious mononucleosis or other virus-related syndrome). Virion DNA is never methylated and recent reports have suggested that the presence of viral DNA in the absence of CpG methylation may make it less likely that a malignancy is the source of the viral DNA.[16,17] Further refinement of diagnostic tools that characterize EBV DNA methylation may be useful for improving the specificity of plasma EBV DNA for EBV-associated malignancies.

Association with Particular Types of Lymphoma

Some lymphoma types are nearly 100% EBV associated, including endemic Burkitt lymphoma (BL), extranodal natural killer (NK)/T-cell lymphoma of the nasal type, early posttransplantation lymphoproliferative disorder (PTLD), lymphomatoid granulomatosis, diffuse large B-cell lymphoma (DLBCL) associated with chronic inflammation, EBV-positive DLBCL of older adults, and AIDS primary central nervous system (CNS) lymphoma (PCNSL).[18–20] Other lymphoma types are variably EBV associated. These include classic HL, PTLDs occurring many months or years after transplantation, and systemic AIDS-related lymphoma.

Some lymphoma types appear never or almost never to be EBV associated, including most indolent B-cell lymphomas, although there is growing evidence that exceptions do exist, particularly in the setting of immunocompromise.[21–23] Thus, the spectrum of EBV-associated lymphomas continues to expand, and the presence of EBV might be an indication to consider an underlying immune defect in the patient.

Other lymphomas are typically not EBV associated but have the interesting feature of being associated with EBV upon transformation or upon development of de novo secondary lymphomas. For example, chronic lymphocytic leukemia (CLL) can transform into EBV-positive HL, and patients with angioimmunoblastic T-cell lymphoma (AITL) can rarely develop EBV-positive DLBCL.[24,25]

Table 87.3 lists the lymphomas that have been associated with EBV, associated cofactors, viral antigen expression, and an estimate of the percentage of tumors within each lymphoma subtype that harbor viral genomes. The table serves to illustrate the range of EBV lymphomas as well as host features that increase risk, but it is not meant to be comprehensive.

Posttransplantation Lymphoproliferative Disorder

PTLD is a group of lymphoproliferative disorders ranging from polyclonal lymphoid hyperplasia to lymphomas that arise in patients after solid organ or hematopoietic stem cell transplant (HSCT).[26] PTLD, especially in the first few months after transplant, is highly associated with EBV (Fig. 87.2A and B). EBV gene expression in PTLD most commonly corresponds to latencies II and III.[27] Broad expression of viral proteins is seen only in immunosuppressed hosts, reflecting that many of these proteins are immunogenic and commonly targeted by cytotoxic T cells.

B cells that harbor EBV are able to proliferate in the setting of posttransplant immunosuppression, at least in part because of decreased T-cell surveillance.[28] HSCT patients who receive grafts that have been

TABLE 87.3 Epstein-Barr Virus–Associated Lymphoma

Type	Cofactors	Viral Gene Expression	Approximate Percentage EBV Associated	Comment
PTLD	Immunosuppression, allograft	Latency II or III	50%–95%	Early days/months after transplantation are more commonly associated with EBV
Sporadic BL		Latency I	20% in the United States	Higher in Latin America
Endemic BL	Malaria	Latency I	>95%	
AIDS BL	HIV	Latency I	30%	
HL		Latency II	30% in the United States	Higher percentage in mixed cellularity, in males, in Hispanics
AIDS PCNSL	HIV	Latency II or III	>95%	
Extranodal NK/T-cell lymphoma, nasal type	More common in Asia	Latency II	>95%	
AIDS PEL	HIV and KSHV	Latency I	>75%	Rare cases of PEL in HIV-negative patients are typically EBV-negative
Lymphomatoid granulomatosis	Immunocompromise	Latency II	>90%	
Methotrexate-associated lymphoma	Methotrexate treatment	Latency III	>95%	May regress with withdrawal of methotrexate

AIDS, Acquired immunodeficiency syndrome; *BL*, Burkitt lymphoma; *EBV*, Epstein-Barr virus; *HIV*, human immunodeficiency virus; *HL*, Hodgkin lymphoma; *KSHV*, Kaposi sarcoma–associated herpesvirus; *NK*, natural killer; *PCNSL*, primary central nervous system lymphoma; *PEL*, primary effusion lymphoma; *PTLD*, posttransplantation lymphoproliferative disorder.

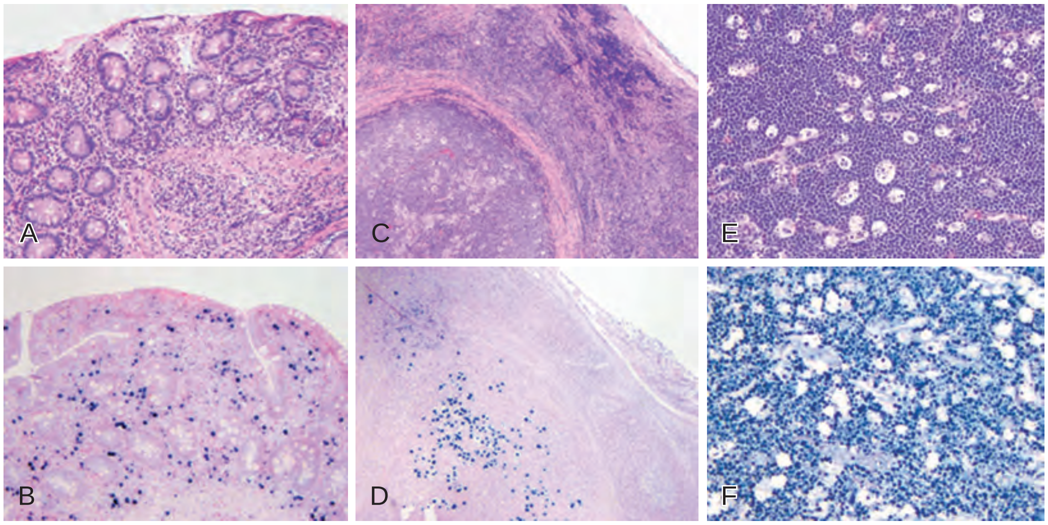


Figure 87.2 EXAMPLES OF EPSTEIN-BARR VIRUS–RELATED LYMPHOMAS. Posttransplant lymphoproliferative disorder (PTLD), polymorphic type, EBV-positive occurring in the gastrointestinal tract of a 15-month-old girl following an orthotopic liver transplant (A and B). There was a mildly atypical lymphocytic infiltrate in the duodenal mucosa composed of small lymphocytes, plasma cells, and occasional large cells (A). The infiltrate was EBV-positive, as demonstrated by in situ hybridization for Epstein-Barr–encoded RNA (EBER) (B). Hodgkin lymphoma, nodular sclerosing type, EBV-positive (C and D). The hematoxylin and eosin–stained section shows a portion of a lymph node from a cervical lymph node biopsy of an 8-year-old girl. There are bands of sclerosis forming a cellular nodule, and within the nodule there are a mixed inflammatory infiltrate and scattered large cells with contracted cytoplasm consistent with lacunar cells (C). The immunophenotype of these cells was that of classic Hodgkin lymphoma, and the cells were EBER-positive (D). EBV is seen more frequently in mixed-cellularity Hodgkin lymphoma but can be seen in 10% to 40% of cases of nodular sclerosing type. It is even more frequent in cases associated with human immunodeficiency virus (HIV; see text) and in resource-poor regions. Burkitt lymphoma, sporadic type, EBV-positive (E and F). A section of the cervical lymph node biopsy of a 9-year-old girl with a rapidly enlarging neck mass is shown. The section illustrates the classic morphologic features of Burkitt lymphoma with a “starry sky” appearance, sheets of intermediate-sized cells with multiple small nucleoli and high mitotic rate. The cells were virtually all EBER-positive (F). EBV can be seen in about 20% to 30% of cases of sporadic Burkitt lymphoma and is essentially always positive in endemic cases.

T-cell depleted develop EBV-associated PTLD at very high rates. Treatment of rejection in solid organ transplant recipients with agents such as the monoclonal antibody OKT3, which targets CD3$^+$ cells, is associated with markedly increased risk for PTLD.[29] Treatment strategies such as reduction of immunosuppression, use of rituximab, and infusion of EBV-specific cytotoxic T lymphocytes (CTL) have been effective in treating or preventing PTLD. A recent publication describing “off-the-shelf” third-party adoptive cell therapy for EBV-PTLD found overall response rates of 68% (for HSCT patients) and 54% (for solid organ transplantation), which is comparable to

EPSTEIN-BARR VIRUS–ASSOCIATED POSITIVE POSTTRANSPLANT LYMPHOPROLIFERATIVE DISORDER

A 55-year-old renal transplant patient presents with acute renal failure 5 months after transplant. She is found on imaging to have an obstructing mass in the transplanted kidney. She undergoes kidney biopsy, and EBV-PTLD involving the transplanted organ is diagnosed. Treatment options include decreasing immunosuppression (acknowledging the associated risk for organ rejection); changing immunosuppressive agents—switching a calcineurin inhibitor for a mammalian target of rapamycin (mTOR) inhibitor; rituximab followed by combination chemotherapy if remission is not achieved; adoptive T-cell therapy with autologous or third party EBV-specific T cells; or investigational therapies.

rates reported for donor-derived EBV-CTL (see box on Epstein-Barr Virus–Associated Positive Posttransplant Lymphoproliferative Disorder).[30]

Hodgkin Lymphoma

Approximately 30% of classic HL tumors in the United States and Europe are EBV-associated (see Fig. 87.2C and D).[31,32] Epidemiologic studies suggest that individuals with a history of symptomatic infectious mononucleosis are at increased risk for EBV-associated HL, but not for EBV-negative HL or other lymphomas.[33] The period of risk peaks at about 2 years but continues to be elevated for at least 10 years after symptomatic mononucleosis. In addition, specific HLA genes have been associated with an increased risk for EBV-associated HL.[34]

Higher EBV associations are seen in Latin America, Africa, and parts of Asia. Factors associated with EBV tumor positivity include mixed cellularity and lymphocyte-depleted classic HL histologic subtypes, male sex, low socioeconomic background, history of symptomatic infectious mononucleosis, and Hispanic ethnicity. Organ and hematopoietic stem cell transplant recipients, patients with primary immunodeficiencies, and HIV-positive patients are more likely to develop HL than the general population, and approximately 90% of the tumors in these settings are EBV-associated.[35]

The EBV gene expression pattern in HL is latency II, even when HL occurs in immunocompromised populations.[36] LMP1 and LMP2A may mimic signaling of B-cell receptors and thus protect B cells lacking functional immunoglobulin expression from apoptotic signaling. Approximately 20% of HLs lack productive immunoglobulin gene rearrangements. These tumors appear to be exclusively EBV-associated.

Patients with EBV-positive HL quite reliably have EBV DNA detected in cell-free blood (plasma or serum) in the setting of active disease.[13,37] Failure to clear EBV DNA from plasma or serum with therapy has been associated with inferior failure-free survival.

Currently, treatment regimens for EBV-positive HL do not differ from those used for EBV-negative HL. However, novel treatment strategies involving adoptive therapy with autologous EBV-specific T cells targeting type II latency LMP antigens appear to be associated with durable complete responses and low toxicity in some patients with EBV-positive HL, even in the setting of relapsed/refractory disease.[38,39]

Burkitt Lymphoma

Endemic BL is nearly 100% associated with EBV, whereas sporadic and HIV-associated BL are much more variably EBV-associated (see Table 87.3 and Fig. 87.2E and F).[40] Viral expression is latency I (i.e., EBNA1 is the only viral protein consistently expressed). The defining feature of BL is a translocation between c-Myc on chromosome 8 and one of the immunoglobulin genes on chromosome 2, 14, or 22. It has been generally presumed that *falciparum* malaria is a cofactor in endemic BL, and the distribution of BL in Africa corresponds to the distribution of holoendemic malaria. However, little is understood of the pathogenesis or interaction between these infectious cofactors.[41] The characteristic presentation of BL is different in the endemic versus sporadic settings, but there is no evidence to link these presentations specifically with the virus. Data has been presented suggesting that EBV DNA measurements in plasma may have utility in facilitating diagnosis, assessing prognosis, and monitoring tumor response in East Africa.[42] The virus-tumor association does not currently guide therapy.

KAPOSI SARCOMA–ASSOCIATED HERPESVIRUS

Virus and Tumor Epidemiology

KSHV (HHV-8) is a gammaherpesvirus that, unlike EBV, has a low prevalence worldwide.[43] The virus is endemic in certain areas, such as in sub-Saharan Africa and the Middle East, and has an intermediate prevalence in Mediterranean countries. Transmission is believed to be predominately through saliva. Similar to EBV, KSHV latently infects B cells, and viral genes that promote cell survival are implicated in lymphomagenesis.[44]

KSHV was discovered in Kaposi sarcoma but is also present in rare lymphoproliferative diseases, including primary effusion lymphoma

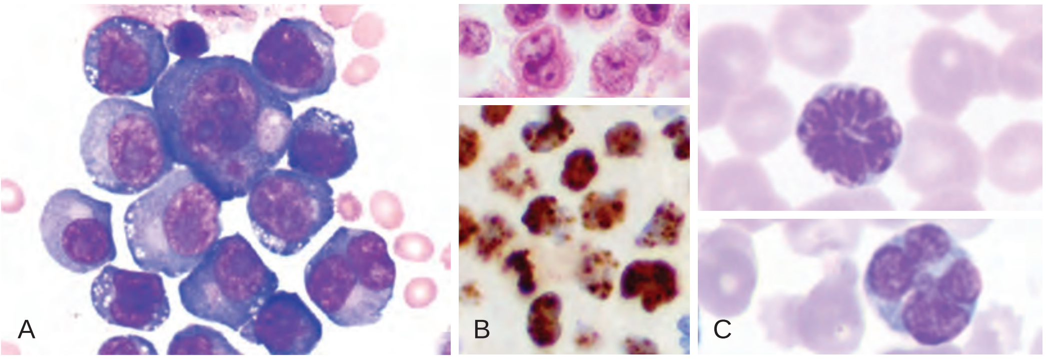


Figure 87.3 EXAMPLES OF KAPOSI SARCOMA–ASSOCIATED HERPESVIRUS AND HUMAN T-LYMPHOTROPIC VIRUS-1 (HTLV-1)–ASSOCIATED LYMPHOPROLIFERATIVE DISEASE. Primary effusion lymphoma (A and B). (A) The pleural tap had a high cell count, and the cytospin preparation revealed a markedly pleomorphic cell population with large and giant cells with deep-blue cytoplasm. (B) A cell block was prepared *(top)* so that in situ hybridization studies could be performed. These studies showed the cells to be KSHV positive by immunohistochemistry for latency associated nuclear antigen-1 (LANA-1) *(bottom)* and EBV positive by EBER in situ hybridization (not shown). (C) Adult T-cell leukemia/lymphoma in a patient who was HTLV-1 positive. The peripheral smear showed the classic "flower" cells. *EBER*, Epstein-Barr–encoded RNA. *(A and B, Courtesy Dr. Elizabeth Hyjek, University of Chicago.)*

(PEL) (Fig. 87.3A and B) and multicentric Castleman disease (MCD).[45] Other KSHV-positive lymphomas have been characterized, all of which tend to occur in severely immunocompromised individuals and carry a poor prognosis. PEL occurs almost exclusively in HIV-positive patients, typically when CD4 counts are less than 100/mm^3. However, PEL has been reported very rarely in solid organ transplant patients and elderly men with some degree of immunocompromise.[46] KSHV is always present in PEL. In HIV patients with PEL, tumor cells are usually dually infected with EBV as well. By contrast, HIV-negative PEL is usually EBV-negative. KSHV-associated MCD, although much more common in HIV-infected populations, also occurs in the general population.

MCD is a nonclonal lymphoproliferative disorder typically involving the mantle zone of lymph nodes and the spleen.[47] MCD can be a KSHV-associated or idiopathic, without detectable KSHV. In KSHV-associated MCD, the virus appears to drive λ light chain rearrangement in the lymphocytes that give rise to Castleman disease.[48] These cells express a broad range of KSHV lytic antigens, and high KSHV copy numbers are present in blood. Evolution into or coassociation with an aggressive lymphoma, often of plasmablastic phenotype, is not uncommon. Within the HIV population, nearly all cases of MCD are KSHV-associated; this viral association is not as strong among HIV-negative MCD patients. High expression of viral interleukin-6 (IL-6) is thought to contribute to the systemic inflammation seen in this disorder.[49] The KSHV-associated lymphomas that arise in association with MCD are not nodal equivalents of PEL; rather, these plasmablastic lymphomas (see E-Slide VM03957) are uniformly EBV-negative and express IgM λ.[50]

Diagnostic and Therapeutic Considerations

The characteristic finding in PEL is a lymphomatous effusion, which can be pleural, pericardial, or peritoneal, without associated lymphadenopathy or masses, arising in the setting of immunocompromise.[45] Often, patients with HIV/AIDS will present with PEL in addition to other KSHV-associated diseases, such as Kaposi sarcoma and MCD, so thorough evaluation and staging should be undertaken at diagnosis. On cytologic examination, the PEL tumor cells are large with prominent nucleoli. The effusion cells are clonal B cells with CD45 positivity but typically lack other specific B-cell lineage markers, although CD30 and CD38 positivity can be seen. B-cell lymphoma 6 (BCL6) mutations are frequently detected. Tumor cells always harbor KSHV, as demonstrated by staining for KSHV-associated latency-associated nuclear antigen-1. In MCD, there is characteristically λ light chain restriction. This does not reflect clonality as assessed by study of immunoglobulin DNA rearrangements. Rather, it reflects a tendency for the virus to selectively drive B lymphocytes to proliferate and induce immunoglobulin class switching.[51]

Treatment of PEL is most commonly combination chemotherapy and outcomes remain poor. Treatment of KSHV-associated MCD has been more successful with rituximab alone or in combination with chemotherapy.[47] Antivirals such as ganciclovir and valganciclovir have shown clinical activity in MCD because viral lytic replication is a feature of this disease.[52] Tocilizumab, an IL-6 receptor antibody, has transient activity in KSHV-associated MCD, but siltuximab, an antibody IL-6, has not been studied due to concerns that it will not bind to viral IL-6.[53]

HUMAN T-LYMPHOTROPIC VIRUS-1

Viral Biology

HTLV-1 is a retrovirus with a single-stranded RNA genome. Following infection there is reverse transcription and integration of proviral DNA into the host genome. HTLV-1 infects a variety of cell types but persists in the subset of CD4$^+$ T lymphocytes that are regulatory T cells.[54] Viral infection within the host is spread from cell to

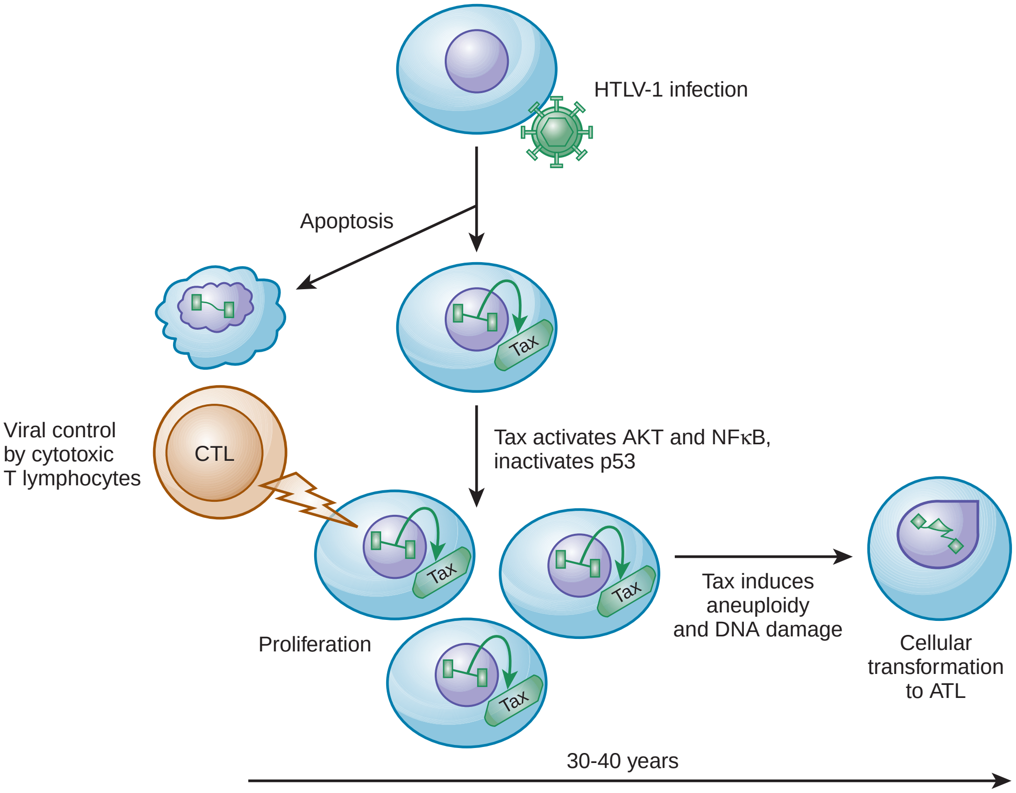

HTLV-1 infection
Apoptosis
Tax
Viral control by cytotoxic T lymphocytes
CTL
Tax activates AKT and NFκB, inactivates p53
Tax
Tax
Tax induces aneuploidy and DNA damage
Proliferation
Tax
Cellular transformation to ATL
30-40 years

Figure 87.4 HUMAN T-LYMPHOTROPIC VIRUS-1 (HTLV-1) AND THE EVOLUTION OF ADULT T-CELL LEUKEMIA/LYMPHOMA. Following HTLV-1 infection, many cells undergo apoptosis, but some infected CD4$^+$ T cells are driven to proliferate by the effects of Tax on the nuclear factor-κB (NFκB) pathway and on the AKT pathway. Tax is also suggested to result in inactivation of p53, aneuploidy, and deoxyribonucleic acid (DNA) damage. Over many decades, malignancy evolves.

cell through direct cell-to-cell contact. As with EBV, the proliferation of HTLV-1–infected lymphocytes plays a central role in ensuring viral persistence.

The HTLV-1 protein Tax is also a mediator of immune dysregulation, T-lymphocyte immortalization, and transformation (Fig. 87.4).[55] Tax affects NFκB and the serine/threonine kinase protein kinase B (AKT) pathways with diverse proliferative and antiapoptotic effects. As with some of the immunodominant EBV antigens expressed in proliferating lymphocytes, Tax expression is targeted by cytotoxic T cells. Another viral protein, Hbz, suppresses Tax expression, allowing transformed T cells to elude immune surveillance. Tax interferes with various DNA repair pathways and induces reactive oxygen species, facilitating the development of aneuploidy.[56] Tax leads to functional inactivation of p53 and may interfere with the spindle assembly checkpoint that normally operates in mitosis to preserve euploidy.

Epidemiology of Viral Infection and Adult T-Cell Leukemia/Lymphoma

HTLV-1 is endemic in particular regions of Japan, Africa, South America, and some Caribbean islands.[57] As assessed by seroprevalence, rates up to 37% are found on the southwestern Japanese islands of Shikoku, Kyushu, and Okinawa, whereas most other areas of Japan have an intermediate prevalence of 1% to 5%. In the United States, the incidence in blood donors is 0.025%. It has been estimated that about 20 million individuals are infected worldwide. The major mode of transmission in endemic areas is from mother to child in breast milk, although infection is also transmitted through sexual intercourse, transfusion of cellular blood products, and the sharing of needles and syringes. Evidence has been presented that HTLV-1 infection persists in association with certain human leukocyte antigen (HLA) types and may be more readily transmitted from mother to child when these HLA types are shared.

Adult T-cell leukemia (ATL) is more common in men than in women and typically presents in the fourth or fifth decade of life. Perhaps as a function of the long latency period, cases of ATL following blood transfusion or needle sharing are vanishingly rare. The lifetime risk for ATL has been estimated to be in excess of 6% in men who are HTLV-1 carriers in an endemic region of Japan, although in other settings the risk may be much lower.[58] As with EBV, the subset of the infected population that develops lymphoma is quite small.

Adult T-Cell Leukemia/Lymphoma Diagnostic Considerations

There is a spectrum of ATL. In the acute leukemic subtype of ATL, comprising around 60% of cases, leukocytosis, diffuse lymphadenopathy, and hypercalcemia are common. The classic findings on a peripheral blood smear are lymphocytes with flower-shaped nuclei (see Fig. 87.3C). Twenty percent of patients with ATL present with a lymphomatous subtype dominated by lymphadenopathy and hepatosplenomegaly. Cutaneous infiltration, lytic bone lesions, malignant effusions, and involvement of the CNS and other extranodal sites are not uncommon.[59] There are also chronic and smoldering subtypes that behave much more indolently and may not even require treatment upon diagnosis. Across all subtypes, presentations reflecting immune dysfunction such as strongyloidiasis with dissemination, *Pneumocystis jiroveci* pneumonia (PJP), mycobacterium, or cryptococcal infection are common. The immune dysfunction seen in patients with ATL may be due to the fact that the leukemic cells have a regulatory T-cell phenotype that fosters an immunosuppressive environment.

Histologically ATL shows lymph node effacement by large, atypical T cells usually expressing $CD4^+$, $CD25^+$, and $CD52^+$, with variable CD30 and CD15 expression. Aneuploidy is consistent, although characteristic cytogenetic abnormalities have not been identified. Serologic analysis confirms the presence of HTLV-1 infection.[60]

Therapies Specific to Human T-Lymphotropic Virus-1 Adult T-Cell Leukemia

Smoldering and favorable chronic-type variants of ATL may initially be observed, offered skin-directed therapies, or given a trial of antivirals. Several antivirals used in the treatment of HIV infection have activity against HTLV-1. Zidovudine and IFN can be used in smoldering, chronic, or leukemic subtypes, but patients with lymphoma-type ATL do not benefit from this combination.[61,62]

The leukemic and lymphomatous forms of ATL are aggressive and are associated with poor responses to chemotherapy, high relapse rates, and low overall survival. There is a 10% to 20% risk of CNS involvement, and all patients should receive CNS prophylaxis. Various combinations of chemotherapy are used depending on local availability.[63] The humanized anti–CC chemokine receptor 4 antibody, mogamulizumab, is approved in Japan, but is associated with increased mortality when given days to weeks prior to allogeneic hematopoietic stem cell transplantation.[64,65] Importantly, transplantation is increasingly being used in the front-line setting.[66,67] Brentuximab vedotin with combination chemotherapy has recently been included for treatment of CD30+PTCL, including ATL.[68] The PD-1 inhibitor nivolumab appears to lead to rapid progression of disease although only 3 patients were enrolled in a cooperative group trial.[69]

Several other agents have shown activity in the recurrent or refractory setting based on limited data: lenalidomide, proteasome and histone deacetylase inhibitors, alemtuzumab, an anti-CD52 monoclonal antibody, arsenic trioxide combined with IFN-α, all-*trans* retinoic acid, and pralatrexate.[70,71]

HUMAN IMMUNODEFICIENCY VIRUS-ASSOCIATED LYMPHOMAS

Viral Biology and Pathogenesis

HIV-1 is a retrovirus that infects $CD4^+$ T cells and monocytes. It establishes a lifelong reservoir in $CD4^+$ T cells. Viral infection may lead to cell death or establishment of latency in resting cells. The lymphomas that are increased in HIV-infected patients (see Table 87.4) are of B-cell lineage, and many are associated with EBV, KSHV, or both (see Table 87.4).

HIV infection compromises cellular immunity, decreasing immune surveillance of EBV- and KSHV-infected B cells.[72] In addition, HIV infection stimulates proliferation of B lymphocytes and perhaps genetic aberrations in the proliferating cells.[73] Many possible mechanisms have been invoked, including direct stimulation of B cells by HIV antigens or antigens associated with opportunistic infection; (2) stimulation of B cells by cellular proteins (CD40 ligand) incorporated into the HIV virion, leading to expression of activation-induced deaminase, an enzyme that mediates double-stranded DNA

TABLE 87.4 **Human Immunodeficiency Virus–Associated Lymphoma**

Lymphoma	CD4 Association (cells/mm^3)	EBV Association (%)	Other Cofactors
PCNSL	<50	>95%	
DLBCL	Variable	40%	
BL	>100	30%	
HL	>100	90%	
PEL	<100	>75%	KSHV

BL, Burkitt lymphoma; *DLBCL*, diffuse large B-cell lymphoma; *EBV*, Epstein-Barr virus; *HIV*, human immunodeficiency virus; *HL*, Hodgkin lymphoma; *KSHV*, Kaposi sarcoma–associated herpesvirus; *PCNSL*, primary central nervous system lymphoma; *PEL*, primary effusion lymphoma.

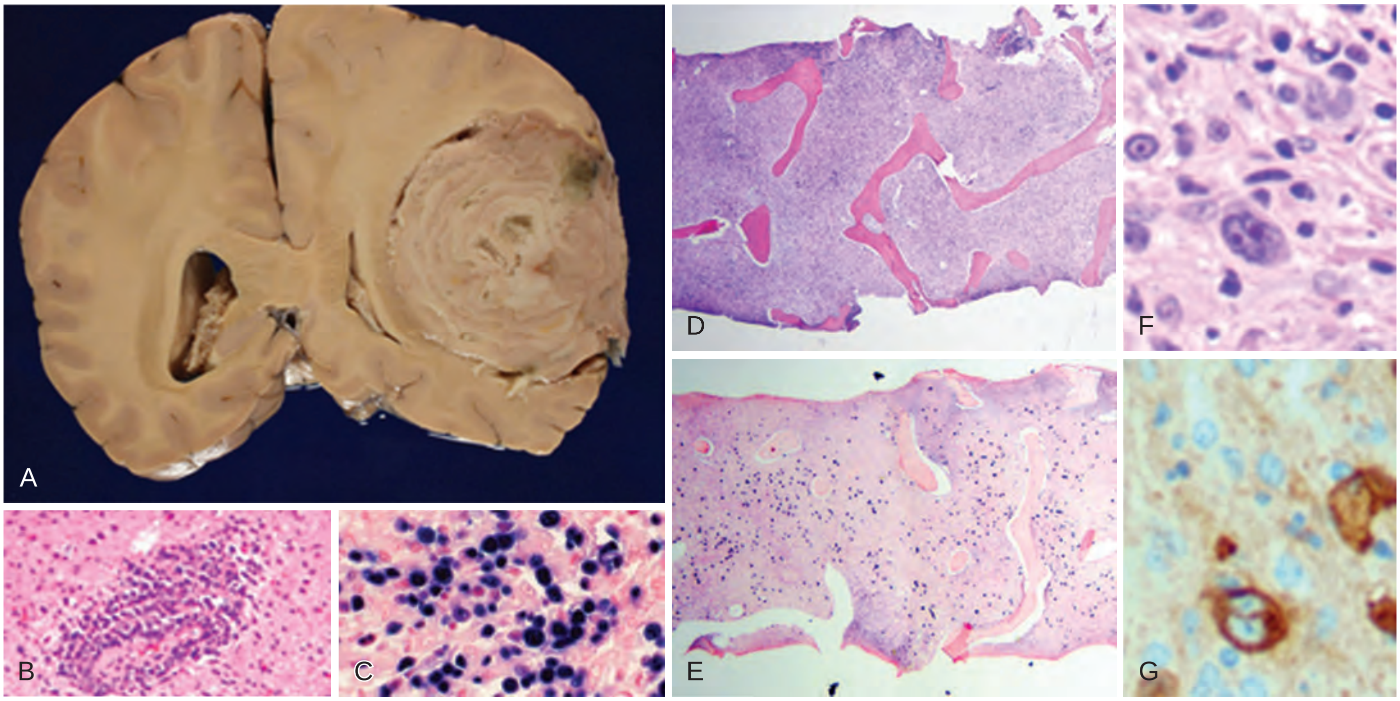


Figure 87.5 EXAMPLES OF HUMAN IMMUNODEFICIENCY VIRUS (HIV)–RELATED LYMPHOMAS. Primary central nervous system (CNS) lymphoma in HIV-positive patients (A–C). Gross appearance (coronal section) of the brain from an autopsy of a 24-year-old HIV-positive female patient with temporoparietal mass due to a primary CNS lymphoma (A). The patient died as a result of uncal and cingulate herniation. (B) Biopsy section from another patient showing a perivascular infiltrate of large lymphoma cells. This is the typical pattern of involvement by CNS lymphoma. The cells were shown to be CD20$^+$ B cells and were EBER-positive (C). A diagnosis can be made without biopsy when magnetic resonance imaging studies show characteristic features and EBV is demonstrated in the cerebrospinal fluid by polymerase chain reaction (see box on AIDS Primary Central Nervous System Lymphoma). Hodgkin lymphoma extensively involving the bone marrow in an HIV-positive patient with stage IVB disease (D–G). The bone marrow biopsy was entirely replaced with Hodgkin lymphoma associated with dense sclerosis (D). An EBER study shows scattered positive cells throughout the marrow (E), corresponding to the Hodgkin and Reed-Sternberg cells (F), which were CD30$^+$ as illustrated (G). Hodgkin lymphoma infrequently involves the bone marrow in HIV-negative cases, but some HIV-positive patients can first present with extensive bone marrow disease (see box on HIV Associated Hodgkin Lymphoma). *(A, Courtesy Dr. Peter Pytel, University of Chicago.)*

AIDS PRIMARY CENTRAL NERVOUS SYSTEM LYMPHOMA

In AIDS PCNSL, EBV PCR of the cerebrospinal fluid is positive approximately 90% of the time and rarely positive in patients with AIDS but without PCNSL. When EBV is detected in the cerebrospinal fluid of a patient with AIDS, coupled with characteristic magnetic resonance imaging findings, this is sufficient to diagnose AIDS PCNSL without a confirmatory brain biopsy.

HIV-ASSOCIATED HODGKIN LYMPHOMA

A 42-year-old HIV-positive patient, on highly active ART with a CD4 count of 324 cells/mm^3 and undetectable viral load, presents with fever, weight loss, and a palpable axillary lymph node. HL is diagnosed on excisional biopsy, and EBV positivity is demonstrated by EBER in the Reed-Sternberg tumor cells. Although he lacks additional lymphadenopathy, a bone marrow biopsy is performed, and it shows involvement by HL. He has stage IVB disease. *Pneumocystis jiroveci* pneumonia prophylaxis is started despite an adequate CD4 count in anticipation of chemotherapy. His ART regimen is reviewed for potential antiviral-chemotherapy drug interactions. He receives six cycles of full-dose, first-line chemotherapy and achieves a complete remission.

breaks; and (3) dysregulation of B cells as a consequence of T-cell dysfunction. There is also some evidence that host biology may contribute to lymphomagenesis.[74]

Epidemiology

Lymphoma is increased in all HIV risk groups, in contrast to Kaposi sarcoma, which is rarely seen in injection drug users (or in hemophiliacs in an earlier era).[75] There is a well-established relationship between CD4$^+$ cells per cubic millimeter overall and the risk for lymphoma, but the relationship is complex and differs among lymphomas (Table 87.4).

The incidence of non-Hodgkin lymphoma (NHL) in the HIV population, particularly PCNSL (Fig. 87.5A–C), has decreased with the widespread use of highly active antiretroviral therapy (ART). However, the incidence of HL has not declined.[76] CD4$^+$ counts can help guide evaluation insofar as CD4$^+$ counts greater than 300 cells/mm^3 are typically associated with BL or HL, whereas these diagnoses would be unlikely in patients with CD4$^+$ counts less than 50 cells/mm^3 (see box on AIDS Primary Central Nervous System Lymphoma).[77,78]

Patients with HIV are now living much longer because of effective antiretroviral regimens and decreased rates of opportunistic infection. As a result, malignancy has emerged as the major cause of mortality in HIV populations with access to antiretroviral therapy.[79,80]

Diagnostic Considerations Specific to Lymphoma in Patients With Human Immunodeficiency Virus

Lymphomas in patients with HIV infection are more likely to present with B symptoms. Therefore, unexplained fever and sweats, even in the absence of lymphadenopathy, are sufficient to warrant consideration of HL. Bone marrow–only presentations of

HL are not uncommon, and contiguous spread so characteristic of classic HL in other settings is less common in HIV HL (see Fig. 87.5D–G). Patients with HIV-associated NHL have higher rates of extranodal involvement, including bone marrow and CNS disease, as well as higher-stage disease and more aggressive tumors on average.[81] All HIV-seropositive patients with aggressive NHL should undergo a diagnostic lumbar puncture. Imaging must be interpreted with caution because metabolically active lymph nodes on positron emission tomography–computed tomography (PET-CT) may represent HIV infection itself, opportunistic infection, immune reconstitution following the initiation of antiretroviral therapy, or lymphoma.

Therapy

Prior to effective antiretroviral therapy, HIV patients experienced increased mortality from chemotherapy due to their underlying immunocompromised state.[82] However, with ART and the advancement of supportive care, dose-reduced chemotherapy is no longer standard practice.[83,84] Dose reductions can be considered with low $CD4^+$ count (<100 cells/mm^3), history of ongoing opportunistic infection, performance status below 75%, or compromised organ function. There is general agreement that the inclusion of rituximab with multiagent chemotherapy improves outcomes, at least in patients with $CD4^+$ counts of greater than 50 cells/mm^3.[85,86]

Antiretroviral Therapy

A few key special issues in considering concurrent antiretroviral and lymphoma therapy include concerns about shared toxicities, drug–drug interactions, and risk for inability to comply with consistent antiretroviral dosing. Antiretrovirals that interact with the cytochrome P-450 CYP3A4 pathway are of particular concern due to multiple drug-drug interactions and modification of ART may need to be considered. Collaboration with HIV or oncology pharmacists is recommended. In addition, nausea and vomiting associated with chemotherapy may interfere with regular antiviral dosing, which raises the concern for the development of a resistant strain of HIV.[87] Trials have demonstrated that patients continued on their previously established antiretroviral regimen during chemotherapy have acceptable outcomes and more rapid immune reconstitution after chemotherapy compared with those not on antiretrovirals.[88,89] Clinicians should only hold ART during chemotherapy when alternative ART regimens without drug interactions are not available.

Supportive Care

Although PJP prophylaxis is recommended only when the $CD4^+$ count is less than 200 cells/mm^3 for patients with HIV not receiving cytotoxic chemotherapy, prophylaxis is often universally used for patients with HIV receiving cytotoxic chemotherapy. The following are also commonly used: fungal prophylaxis with fluconazole; herpes simplex and varicella prophylaxis with acyclovir, valacyclovir, or famciclovir; quinolone prophylaxis when neutrophil counts fall below 1000 cells/mm^3; and granulocyte growth factors.

Bone Marrow Transplant in Patients With Human Immunodeficiency Virus

Autologous bone marrow transplant has been successful in HIV-seropositive patients with NHL, with these patients having adequate stem cell mobilization, nonrelapse mortality rates comparable to those for HIV-negative patients, count recovery within 2 weeks of stem cell rescue, and maintained control of HIV viral loads and $CD4^+$ counts after high-dose chemotherapy[90] (see box on Autologous Bone Marrow Transplant in an HIV-Seropositive Hodgkin Lymphoma Patient). There have also been successful reduced-intensity allogeneic bone marrow transplants in HIV-seropositive patients.[91,92] The first national trial of allogeneic bone marrow transplant for patients with HIV and hematologic malignancy demonstrated the safety and efficacy of transplantation for all HIV-positive patients who otherwise meet standard inclusion criteria.[93] The role of transplant in curing HIV is an area of active investigation, including cell engineering strategies to render cells resistant to HIV.[94,95] To date, there have been two patients who obtained sustained viral remissions after CCR5Δ32 homozygous hematopoietic stem-cell transplant.[96–98]

AUTOLOGOUS BONE MARROW TRANSPLANT IN AN HIV-SEROPOSITIVE PATIENT WITH HODGKIN LYMPHOMA

A 38-year-old HIV-positive patient with a $CD4^+$ count of 485 cells/mm^3 is diagnosed with classic Hodgkin lymphoma (HL) and treated with doxorubicin (Adriamycin), bleomycin, vinblastine, and dacarbazine (ABVD), achieving a complete remission. Two years later he presents with retroperitoneal lymphadenopathy and is found on biopsy to have relapsed HL. He is treated with salvage chemotherapy and has a complete response, as well as good performance status and no active infections. His HIV remains well controlled on antiretroviral therapy. He is deemed an excellent candidate for high-dose therapy and undergoes consolidation with autologous bone marrow transplant.

HEPATITIS C VIRUS

Viral Biology and B-Lymphocyte Proliferation

HCV is an enveloped, positive-stranded RNA virus.[99,100] Infection involves interactions between E2, a viral structural protein with two hypervariable regions, and a cellular protein CD81 present on hepatocytes and B lymphocytes. A polyprotein is translated from viral RNA and is cleaved by cellular and viral proteases, including NS3, to yield proteins required for viral replication. The RNA-dependent RNA polymerase that replicates the viral genome lacks proofreading capacity, thus generating genetic heterogeneity among viral progeny. Viral replication occurs predominantly in hepatocytes, but viral RNA and NS3 have also been detected in B cells, although HCV replication in B cells remains controversial.[101]

Chronic infection can be associated with mixed cryoglobulinemia, a systemic immune disease that results from clonal expansion of B cells producing an IgM autoantibody against IgG, leading to deposition of immune complexes on endothelial surfaces and resulting in inflammation.[102] In this setting of B-cell proliferation, some patients develop B-cell NHL, which is classically of low-grade histology. Several hypotheses have been advanced with regard to how HCV might drive B-cell proliferation. There is controversy as to whether infection of B cells plays any role in this process.[103] A lymphoma cell line that produces infectious HCV has been reported. Even in the absence of infection of B cells, interaction of the HCV E2 protein with CD81 on B cells may drive B-cell proliferation or lower the threshold for other B-cell stimuli to drive proliferation. Immunoglobulin signaling may be activated by immunoglobulin-virus complexes, and Toll-like receptor 7 signaling may be activated by viral RNA. Finally, it is noted that E2 binding triggers expression of activation-induced deaminase, an enzyme that is important in generating somatic hypermutation and that has also been implicated in mediating mutations thought to play a role in DLBCL lymphomagenesis.[104]

Epidemiology of Viral Infection and Associated Lymphoma

In systematic reviews, approximately 13% to 18% of B-cell lymphomas are associated with HCV infection.[105–107] Most commonly, these lymphomas are histologically indolent subtypes, with splenic marginal zone lymphoma (MZL), nongastric MALT, and lymphoplasmacytic lymphoma more often seen in association with HCV than aggressive histologies such as DLBCL.[108–110] The HCV–lymphoma association is more apparent in some countries than in others, with the association being established most clearly in Italy, Taiwan, and Japan. Several studies in regions or countries where HCV infection is less prevalent have failed to identify any association with lymphoma.[111–114] In the United States, there is a small but significant increase in B-NHL risk with HCV infection.[115,116]

Further evidence in support of an etiologic relationship comes from studies in which successful treatment of HCV was followed by lymphoma regression.[117] Furthermore, clearance of HCV infection has been shown to reduce the incidence of B-NHL, with cumulative incidence of lymphoma among patients with a sustained virologic response of 0% at 15 years, as compared with 2.6% at 15 years among both nonresponders and untreated patients.[118]

Diagnostic and Prognostic Considerations

In contrast to EBV-, KSHV-, or HTLV-1-associated tumors, there is no established role for studies demonstrating HCV nucleic acid or protein in tumor cells. An HCV prognostic score comprised of poor performance status, hypoalbuminemia, and high HCV-RNA viral load effectively risk stratified patients with DLBCL into three groups with significantly different progression-free and overall survival outcomes.[119]

Therapy

In patients with indolent lymphomas and untreated HCV infection, antiviral treatment may obviate the need for cytotoxic chemotherapy and should be considered as an initial therapeutic strategy.[117,120]

Rituximab has been associated with rises in HCV viral load and increased rates of hepatotoxicity, and there is the possibility that elimination of B cells for several months following treatment may compromise humoral responses to the evolution of HCV quasispecies. However, in studies to date, overall survival is not inferior.[119,121] Unlike in cases of indolent lymphoma, where antiviral therapy alone may be sufficient, rituximab-containing multiagent chemotherapy regimens are recommended for HCV-associated DLBCL. Concomitant treatment with direct acting antivirals is safe and effective.[122]

FUTURE DIRECTIONS

In this chapter, a variety of virus-associated lymphomas and lymphoproliferative diseases have been reviewed. For most of these lymphomas, standard antiviral drugs do not have a role in treatment. There are several exceptions, however, and these are worth highlighting. Antiviral therapy for HCV-associated splenic lymphoma with villous lymphocytes is accepted as a standard approach and likely has a role in the treatment of other HCV-associated indolent lymphomas. Similarly, ganciclovir or valganciclovir may have a role in the management of MCD associated with KSHV in patients with HIV. Of course, there is an established role for antiretroviral therapy in the treatment of patients with HIV and malignancy. There are virus-targeted therapies that are broadly accepted as standard, including adoptive immunotherapy with EBV-specific T cells for PTLD. The use of targeted T cells also has promise in other settings, including EBV-associated HL. Other virus-targeted therapies are being developed. Some involve vaccination; others involve induction of viral genes in tumor cells, rendering them more susceptible to pharmacologic treatment. Finally, it may ultimately be possible to prevent some kinds of lymphoma by preventing viral infection or altering the host response to viral infection.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Ambinder RF, Wu J, Logan B, et al. Allogeneic hematopoietic cell transplant for HIV patients with hematologic malignancies: the BMT CTN-0903/AMC-080 trial. *Biol Blood Marrow Transplant*. 2019;25(11):2160–2166.

Barta SK, Lee JY, Kaplan LD, et al. Pooled analysis of AIDS malignancy consortium trials evaluating rituximab plus CHOP or infusional EPOCH chemotherapy in HIV-associated non-Hodgkin lymphoma. *Cancer*. 2012;118(16):3977–3983.

Bollard CM, Tripic T, Cruz CR, et al. Tumor-specific T-cells engineered to overcome tumor immune evasion induce clinical responses in patients with relapsed Hodgkin lymphoma. *J Clin Oncol*. 2018;36(11):1128–1139.

Bower M, Newsom-Davis T, Naresh K, et al. Clinical features and outcome in HIV-associated multicentric Castleman's disease. *J Clin Oncol*. 2011;29:2481.

Carbone A, Cesarman E, Spina M, et al. HIV-associated lymphomas and gamma-herpesviruses. *Blood*. 2009;113:1213.

Choi I, Tanosaki R, Uike N, et al. Long-term outcomes after hematopoietic SCT for adult T-cell leukemia/lymphoma: results of prospective trials. *Bone Marrow Transplant*. 2011;46:116.

Cook LB, Fuji S, Hermine O, et al. Revised adult T-cell leukemia-lymphoma international consensus meeting report. *J Clin Oncol*. 2019;37(8):677–687.

Cook LB, Phillips AA. How I treat adult T-cell leukemia/lymphoma. *Blood*. 2021;137(4):459-470.

Guech-Ongey M, Simard EP, Anderson WF, et al. AIDS-related Burkitt lymphoma in the United States: what do age and CD4 lymphocyte patterns tell us about etiology and/or biology? *Blood*. 2010;116:5600.

Henrich TJ. Second example reported of a stem-cell transplant in the clinic leading to HIV remission. *Nature*. 2019;568:175–176.

Horwitz S, O'Connor OA, Pro B, et al. Brentuximab vedotin with chemotherapy for CD30-positive peripheral T-cell lymphoma (ECHELON-2): a global, double-blind, randomised, phase 3 trial. *Lancet*. 2019;393(10168):229–240.

Levine AM. HIV-associated lymphoma. *Blood*. 2010;115:2986.

Murray PG, Young LS. An etiological role for the Epstein-Barr virus in the pathogenesis of classical Hodgkin lymphoma. *Blood*. 2019;134(7):591–596.

Noy A. Optimizing treatment of HIV-associated lymphoma. *Blood*. 2019; 134(17):1385–1394.

Phillips AA, Harewood JCK. Adult T cell leukemia-lymphoma (ATL): state of the art. *Curr Hematol Malig Rep*. 2018;13(4):300–307.

Prockop S, Doubrovina E, Suser S, et al. Off-the-shelf EBV-specific T cell immunotherapy for rituximab-refractory EBV-associated lymphoma following transplantation. *J Clin Invest*. 2020;130(2):733–747.

Ramaswami R, Lurain K, Peer CJ, et al. Tocilizumab in patients with symptomatic Kaposi sarcoma herpesvirus-associated multicentric Castleman disease. *Blood*. 2020;135(25):2316–2319.

Ratner L, Waldmann TA, Janakiram M, et al. Rapid progression of adult T-cell leukemia–lymphoma after PD-1 inhibitor therapy. *N Engl J Med*. 2018;378(20):1947–1948.

Salgado M, Kwon M, Gálvez C, et al. Mechanisms that contribute to a profound reduction of the HIV-1 reservoir after allogeneic stem cell transplant. *Ann Intern Med*. 2018;169(10):674–683.

Tan CRC, Barta SK, Lee J, et al. Combination antiretroviral therapy accelerates immune recovery in patients with HIV-related lymphoma treated with EPOCH: a comparison within one prospective trial AMC034. *Leuk Lymphoma*. 2018;59(8):1851–1860.

Uldrick TS, Ison G, Rudek MA, et al. Modernizing clinical trial eligibility criteria: recommendations of the American society of clinical oncology–friends of cancer research HIV working group. *J Clin Oncol*. 2017;35(33):3774–3780.

Uldrick TS, Polizzotto MN, Aleman K, et al. High-dose zidovudine plus valganciclovir for Kaposi sarcoma herpesvirus-associated multicentric Castleman disease: a pilot study of virus-activated cytotoxic therapy. *Blood*. 2011;117:6977.

Xu Lei, Jun Wang Yulin Liu, et al. CRISPR-edited stem cells in a patient with HIV and acute lymphocytic leukemia. *N Engl J Med*. 2019;381(13):1240–1247.

REFERENCES

1. Young LS, Yap LF, Murray PG. Epstein–Barr virus: more than 50 years old and still providing surprises. *Nat Rev Cancer*. 2016;16(12):789.
2. Thorley-Lawson DA, Hawkins JB, Tracy SI, Shapiro M. The pathogenesis of Epstein-Barr virus persistent infection. *Curr Opin Virol*. 2013;3(3):227–232.
3. Fish K, Longnecker R. EBV germinates lymphoma from the germinal center in a battle with T and NK cells. *Proc Natl Acad Sci U S A*. 2017;114(18):4571–4573.
4. Kanda T. EBV-encoded latent genes. *Adv Med Exp Biol*. 2018;1045: 377-394.
5. Vereide DT, Sugden B. Lymphomas differ in their dependence on Epstein-Barr virus. *Blood*. 2011;117(6):1977–1985.
6. Epeldegui M, Hung Yee P, McQuay A, Ambinder Richard F, Martínez-Maza O. Infection of human B cells with Epstein-Barr virus results in the expression of somatic hypermutation-inducing molecules and in the accrual of oncogene mutations. *Mol Immunology*. 2007;44(5). 934–442.
7. Rovedo M, Longnecker R. Epstein-Barr virus latent membrane protein 2A preferentially signals through the Src family kinase Lyn. *J Virol*. 2008;82(17):8520–8528.
8. Grimm JM, Schmeling DO, Dunmire SK, Knight JA, Mullan BD, Ed JA, et al. Prospective studies of infectious mononucleosis in university students. Clin Trans Immunol. 2016;5(8):e94.
9. Correia S, Bridges R, Wegner F, Venturini C, Palser A, Middeldorp JM, et al. Sequence variation of Epstein-Barr virus: viral types, geography, codon usage, and diseases. *J Virol*. 2018;92(22).
10. Weiss LM, Chen Y-Y. EBER in situ hybridization for Epstein-Barr virus. *Methods Mol Biol*. 2013;999: 223–230.
11. Glaser SL, Gulley ML, Borowitz MJ, Craig FE, Mann RB, Stewart SL, et al. Inter-and intra-observer reliability of Epstein-Barr virus detection in Hodgkin lymphoma using histochemical procedures. *Leuk Lymphoma*. 2004;45(3):489–497.
12. Westmoreland KD, Montgomery ND, Stanley CC, El-Mallawany NK, Wasswa P, van der Gronde T, et al. Plasma Epstein-Barr virus DNA for pediatric Burkitt lymphoma diagnosis, prognosis and response assessment in Malawi. *Intl J Cancer*. 2017;140(11):2509–2516.
13. Kanakry JA, Li H, Gellert LL, Lemas MV, Hsieh WS, Hong F, et al. Plasma Epstein-Barr virus DNA predicts outcome in advanced Hodgkin lymphoma: correlative analysis from a large North American cooperative group trial. *Blood*. 2013;121(18):3547–3553.
14. Chan KCA, Woo JKS, King A, Zee BCY, Lam WKJ, Chan SL, et al. Analysis of plasma Epstein-Barr virus DNA to screen for nasopharyngeal cancer. *N Engl J Med*. 2017;377(6):513–522.
15. Kanakry JA, Hegde AM, Durand CM, Massie AB, Greer AE, Ambinder RF, et al. The clinical significance of EBV DNA in the plasma and peripheral blood mononuclear cells of patients with or without EBV diseases. *Blood*. 2016;127(16):2007–2017.
16. Shamay M, Kanakry JA, Low JS, Horowitz NA, Journo G, Ahuja A, et al. CpG methylation in cell-free Epstein–Barr virus DNA in patients with EBV-Hodgkin lymphoma. *Blood Adv*. 2020;4(8):1624–1627.
17. Lam WJ, Jiang P, Chan KA, Peng W, Shang H, Heung MM, et al. Methylation analysis of plasma DNA informs etiologies of Epstein-Barr virus-associated diseases. *Nat Commun*. 2019;10(1):3256.
18. MacMahon EM, Glass JD, Hayward SD, Mann RB, Becker PS, Charache P, et al. Epstein-Barr virus in AIDS-related primary central nervous system lymphoma. *Lancet.*. 1991;338(8773):969–973.
19. Campo E, Swerdlow SH, Harris NL, Pileri S, Stein H, Jaffe ES. The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. *Blood.*. 2011;117(19):5019–5032.
20. Lurain K, Uldrick TS, Ramaswami R, Polizzotto MN, Goncalves PH, Widell A, et al. Treatment of HIV-associated primary CNS lymphoma with antiretroviral therapy, rituximab, and high-dose methotrexate. *Blood*. 2020;136(19):2229–2232.
21. Huppmann AR, Nicolae A, Slack GW, Pittaluga S, Davies-Hill T, Ferry JA, et al. EBV may be expressed in the LP cells of nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) in both children and adults. *Am J Surg Pathol*. 2014;38(3):316–324.
22. Bennett JA, Bayerl MG. Epstein-Barr virus–associated extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT Lymphoma) arising in the parotid gland of a child with ataxia telangiectasia. *J Pediatr Hematol Oncol*. 2015;37(2):e114–e117.
23. Gong S, Crane GM, McCall CM, Xiao W, Ganapathi KA, Cuka N, et al. Expanding the spectrum of EBV-positive marginal zone lymphomas-a lesion associated with diverse immunodeficiency settings. *Am J Surg Pathol*. 2018;42(10):1306.
24. Davies-Hill T, Raffeld M, Jaffe ES. Hodgkin Lymphoma variant of Richter transformation: morphology, EBV status, clonality and survival analysis—with comparison to Hodgkin-like lesion. *Hum Pathol*. 2016;55:108-116. 2016.
25. Hoffmann JC, Chisholm KM, Cherry A, Chen J, Arber DA, Natkunam Y, et al. An analysis of MYC and EBV in diffuse large B-cell lymphomas associated with angioimmunoblastic T-cell lymphoma and peripheral T-cell lymphoma not otherwise specified. *Hum Pathol*. 2016;48:9–17.
26. Evens AM, Roy R, Sterrenberg D, Moll MZ, Chadburn A, Gordon LI. Post-transplantation lymphoproliferative disorders: diagnosis, prognosis, and current approaches to therapy. *Curr Oncol Rep*. 2010;12(6):383–394.
27. Murray PG, Deacon E, Young LS, Barletta JM, Mann RB, Ambinder RF, et al. Localization of Epstein-Barr virus in Castleman's disease by in situ hybridization and immunohistochemistry. *Hemato Pathol*. 1995;9(1): 17–26.
28. Bollard CM, Heslop HE. T cells for viral infections after allogeneic hematopoietic stem cell transplant. *Blood*. 2016;127(26):3331–3340.
29. Swinnen LJ, Costanzo-Nordin MR, Fisher SG, Jeanne O'Sullivan E, Johnson MR, Heroux AL, et al. Increased incidence of lymphoproliferative disorder after immunosuppression with the monoclonal antibody OKT3 in cardiac-transplant recipients. *N Engl J Med*. 1990;323(25):1723–1728.
30. Prockop S, Doubrovina E, Suser S, Heller G, Barker J, Dahi P, et al. Off-the-shelf EBV-specific T cell immunotherapy for rituximab-refractory EBV-associated lymphoma following transplantation. *J Clin Invest*. 2020;130(2):733–747.
31. Glaser SL, Lin RJ, Stewart SL, Ambinder RF, Jarrett RF, Brousset P, et al. Epstein-Barr virus-associated Hodgkin's disease: epidemiologic characteristics in international data. *Int J Cancer*. 1997;70(4):375–382.
32. Keegan TH, Glaser SL, Clarke CA, Gulley ML, Craig FE, Digiuseppe JA, et al. Epstein-Barr virus as a marker of survival after Hodgkin's lymphoma: a population-based study. *J Clin Oncol*. 2005;23(30):7604–7613.
33. Hjalgrim H, Askling J, Rostgaard K, Hamilton-Dutoit S, Frisch M, Zhang JS, et al. Characteristics of Hodgkin's lymphoma after infectious mononucleosis. *N Engl J Med*. 2003;349(14):1324–1332.
34. Murray PG, Young LS. An etiological role for the Epstein-Barr virus in the pathogenesis of classical Hodgkin lymphoma. *Blood*. 2019 blood. 2019000568.
35. Glaser SL, Clarke CA, Gulley ML, Craig FE, DiGiuseppe JA, Dorfman RF, et al. Population-based patterns of human immunodeficiency virus-related Hodgkin lymphoma in the greater San Francisco Bay area, 1988–1998. *Cancer*. 2003;98(2):300–309.
36. Siebert JD, Ambinder RF, Napoli VM, Quintanilla-Martinez L, Banks PM, Gulley ML. Human immunodeficiency virus-associated Hodgkin's disease contains latent, not replicative, Epstein-Barr virus. *Hum Pathol*. 1995;26(11):1191–1195.
37. Welch JJG, Schwartz CL, Higman M, Chen L, Buxton A, Kanakry JA, et al. Epstein-Barr virus DNA in serum as an early prognostic marker in children and adolescents with Hodgkin lymphoma. *Blood Adv*. 2017;1(11):681–684.
38. Bollard CM, Gottschalk S, Torrano V, Diouf O, Ku S, Hazrat Y, et al. Sustained complete responses in patients with lymphoma receiving autologous cytotoxic T lymphocytes targeting Epstein-Barr virus latent membrane proteins. *J Clin Oncol*. 2014;32(8):798–808.
39. Bollard CM, Tripic T, Cruz CR, Dotti G, Gottschalk S, Torrano V, et al. Tumor-specific T-cells engineered to overcome tumor immune evasion induce clinical responses in patients with relapsed hodgkin lymphoma. *J Clin Oncol*. 2018;36(11):1128–1139.
40. Haque T, Wilkie GM, Jones MM, Higgins CD, Urquhart G, Wingate P, et al. Allogeneic cytotoxic T cell therapy for EBV-positive post transplant lymphoproliferative disease: results of a phase II multicentre clinical trial. *Blood*. 2007.
41. Peprah S, Ogwang MD, Kerchan P, Reynolds SJ, Tenge CN, Were PA, et al. Risk factors for Burkitt lymphoma in East African children and minors: A case-control study in malaria-endemic regions in Uganda, Tanzania and Kenya. *Int J Cancer*. 2020;146(4):953–969.
42. Westmoreland KD, Montgomery ND, Stanley CC, El-Mallawany NK, Wasswa P, van der Gronde T, et al. Plasma Epstein-Barr virus DNA for pediatric Burkitt lymphoma diagnosis, prognosis and response assessment in Malawi. *Int J Cancer*. 2017;140(11):2509–2516.
43. Moore PS, Chang Y. KSHV: forgotten but not gone. *Blood*. 2011;117(26):6973–6974.
44. Park A, Oh S, Jung KL, Choi UY, Lee H-R, Rosenfeld MG, et al. Global epigenomic analysis of KSHV-infected primary effusion lymphoma identifies functional MYC superenhancers and enhancer RNAs. *Proc Natl Acad Sci U S A*. 2020;117(35):21618–21627.
45. Vega F, Miranda RN, Medeiros LJ. KSHV/HHV8-positive large B-cell lymphomas and associated diseases: a heterogeneous group of lymphoproliferative processes with significant clinicopathological overlap. *Mod Pathol*. 2020;33(1):18–28.

46. Sasaki Y, Isegawa T, Shimabukuro A, Yonaha T, Yonaha H. Primary effusion lymphoma in an elderly HIV-negative patient with hemodialysis: importance of evaluation for pleural effusion in patients receiving hemodialysis. *Case Rep Nephrology Urol*. 2014;4(2):95–102.
47. Lurain K, Yarchoan R, Uldrick TS. Treatment of Kaposi sarcoma herpesvirus–associated multicentric Castleman disease. *Clin Hematol Oncol*. 2018;32(1):75–88.
48. Totonchy J, Osborn JM, Chadburn A, Nabiee R, Argueta L, Mikita G, et al. KSHV induces immunoglobulin rearrangements in mature B lymphocytes. *PLoS Pathog*. 2018;14(4):e1006967.
49. Cannon JS, Nicholas J, Orenstein JM, Mann RB, Murray PG, Browning PJ, et al. Heterogeneity of viral IL-6 expression in HHV-8-associated diseases. *J Infect Dis*. 1999;180(3):824–828.
50. Carbone A, Cesarman E, Spina M, Gloghini A, Schulz TF. HIV-associated lymphomas and gamma-herpesviruses. *Blood*. 2009;113(6):1213–1224.
51. Alomari N, Totonchy J. Cytokine-targeted therapeutics for KSHV-associated disease. *Viruses*. 2020;12(10).
52. Uldrick TS, Polizzotto MN, Aleman K, O'Mahony D, Wyvill KM, Wang V, et al. High-dose zidovudine plus valganciclovir for Kaposi sarcoma herpesvirus-associated multicentric Castleman disease: a pilot study of virus-activated cytotoxic therapy. *Blood*. 2011;117(26):6977–6986.
53. Ramaswami R, Lurain K, Peer CJ, Serquiña A, Wang V, Widell A, et al. Tocilizumab in patients with symptomatic Kaposi sarcoma herpesvirus-associated multicentric Castleman disease. *Blood*. 2020;135(25):2316–2319.
54. Yamano Y, Cohen CJ, Takenouchi N, Yao K, Tomaru U, Li H-C, et al. Increased expression of human T lymphocyte virus type I (HTLV-I) tax11-19 Peptide–human histocompatibility leukocyte antigen A*201 complexes on CD4 CD25 T cells detected by peptide-specific, major histocompatibility complex–restricted antibodies in patients with HTLV-I–associated neurologic disease. *J Exp Med*. 2004;199(10):1367–1377.
55. Mahgoub M, Yasunaga J-i, Iwami S, Nakaoka S, Koizumi Y, Shimura K, et al. Sporadic on/off switching of HTLV-1 Tax expression is crucial to maintain the whole population of virus-induced leukemic cells. *Proc Natl Acad Sci U S A*. 2018;115(6):E1269–E1278.
56. Matsuoka M, Jeang KT. Human T-cell leukemia virus type 1 (HTLV-1) and leukemic transformation: viral infectivity, tax, HBZ and therapy. *Oncogene*. 2011;30(12):1379–1389.
57. Sonoda S, Li HC, Tajima K. Ethnoepidemiology of HTLV-1 related diseases: ethnic determinants of HTLV-1 susceptibility and its worldwide dispersal. *Cancer Sci*. 2011;102(2):295–301.
58. Koga Y, Iwanaga M, Soda M, Inokuchi N, Sasaki D, Hasegawa H, et al. Trends in HTLV-1 prevalence and incidence of adult T-cell leukemia/lymphoma in Nagasaki, Japan. *J Med Virol*. 2010;82(4):668–674.
59. Matsuoka M, Jeang K-T. Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. *Nat Rev Cancer*. 2007;7(4):270–280.
60. National Comprehensive Cancer Network. T-cell Lymphomas (Version 1.2021).
61. Hodson A, Crichton S, Montoto S, Mir N, Matutes E, Cwynarski K, et al. Use of zidovudine and interferon alfa with chemotherapy improves survival in both acute and lymphoma subtypes of adult T-cell leukemia/lymphoma. *J Clin Oncol*. 2011;29(35):4696–4701.
62. Cook LB, Phillips AA. How I treat adult T-cell leukemia/lymphoma. *Blood*. 2021;137(4):459-470.
63. Tsukasaki K, Utsunomiya A, Fukuda H, Shibata T, Fukushima T, Takatsuka Y, et al. VCAP-AMP-VECP compared with biweekly CHOP for adult T-cell leukemia-lymphoma: Japan clinical oncology group study JCOG9801. *J Clin Oncol*. 2007;25(34):5458–5464.
64. Ishida T, Jo T, Takemoto S, Suzushima H, Uozumi K, Yamamoto K, et al. Dose-intensified chemotherapy alone or in combination with mogamulizumab in newly diagnosed aggressive adult T-cell leukaemia-lymphoma: a randomized phase II study. *Br J Haematology*. 2015;169(5):672–682.
65. Fuji S, Inoue Y, Utsunomiya A, Moriuchi Y, Uchimaru K, Choi I, et al. Pretransplantation anti-CCR4 antibody mogamulizumab against adult T-cell leukemia/lymphoma is associated with significantly increased risks of severe and corticosteroid-refractory graft-versus-host disease, nonrelapse mortality, and overall mortality. *J Clin Oncol*. 2016;34(28):3426–3433.
66. Choi I, on behalf of the Aa-HSG Tanosaki R, Uike N, Utsunomiya A, Tomonaga M, et al. Long-term outcomes after hematopoietic SCT for adult T-cell leukemia/lymphoma: results of prospective trials. *Bone Marrow Transplant*. 2011;46(1):116–118.
67. Ishida T, Hishizawa M, Kato K, Tanosaki R, Fukuda T, Taniguchi S, et al. Allogeneic hematopoietic stem cell transplantation for adult T-cell leukemia-lymphoma with special emphasis on preconditioning regimen: a nationwide retrospective study. *Blood*. 2012;120(8):1734–1741.
68. Horwitz S, O'Connor OA, Pro B, Illidge T, Fanale M, Advani R, et al. Brentuximab vedotin with chemotherapy for CD30-positive peripheral T-cell lymphoma (ECHELON-2): a global, double-blind, randomised, phase 3 trial. *Lancet*. 2019;393(10168):229–240.
69. Ratner L, Waldmann TA, Janakiram M, Brammer JE. Rapid progression of adult T-cell leukemia–lymphoma after PD-1 inhibitor therapy. *N Engl J Med*. 2018;378(20):1947–1948.
70. Jiang L, Yuan CM, Hubacheck J, Janik JE, Wilson W, Morris JC, et al. Variable CD52 expression in mature T cell and NK cell malignancies: implications for alemtuzumab therapy. *Br J Haematol*. 2009;145(2):173–179.
71. Phillips AA, Harewood JCK. Adult T cell leukemia-lymphoma (ATL): state of the art. *Curr Hematol Malig Rep*. 2018;13(4):300–307.
72. Dolcetti R, Gloghini A, Caruso A, Carbone A. A lymphomagenic role for HIV beyond immune suppression? *Blood*. 2016;127(11):1403–1409.
73. Gaidano G, Pasqualucci L, Capello D, Berra E, Deambrogi C, Rossi D, et al. Aberrant somatic hypermutation in multiple subtypes of AIDS-associated non-Hodgkin lymphoma. *Blood*. 2003;102(5):1833–1841.
74. Thorball CW, Oudot-Mellakh T, Ehsan N, Hammer C, Santoni FA, Niay J, et al. Genetic variation near CXCL12 is associated with susceptibility to HIV-related non-Hodgkin lymphoma. *Haematologica*. 2020
75. Levine AM. HIV-associated lymphoma. *Blood*. 2010;115(15):2986–2987.
76. Bruyand M, Ryom L, Shepherd L, Fatkenheuer G, Grulich A, Reiss P, et al. Cancer risk and use of protease inhibitor or nonnucleoside reverse transcriptase inhibitor-based combination antiretroviral therapy: the D: A: D study. *J Acquir Immune Defic Syndr*. 2015;68(5):568–577.
77. Guech-Ongey M, Simard EP, Anderson WF, Engels EA, Bhatia K, Devesa SS, et al. AIDS-related Burkitt lymphoma in the United States: what do age and CD4 lymphocyte patterns tell us about etiology and/or biology? *Blood*. 2010;116(25):5600–5604.
78. Ambinder RF, Bhatia K, Martinez-Maza O, Mitsuyasu R. Cancer biomarkers in HIV patients. *Curr Opin HIV AIDS*. 2010;5(6):531–537.
79. Engels EA, Pfeiffer RM, Landgren O, Moore RD. Immunologic and virologic predictors of AIDS-related non-Hodgkin lymphoma in the highly active antiretroviral therapy era. *J Acquir Immune Defic Syndr*. 2010;54(1):78–84.
80. Noy A. Optimizing treatment of HIV-associated lymphoma. *Blood*. 2019;134(17):1385–1394.
81. Gopal S, Martin KE, Richards KL, Eron JJ. Clinical presentation, treatment, and outcomes among 65 patients with HIV-associated lymphoma treated at the university of North Carolina, 2000–2010. *AIDS Res Hum Retroviruses*. 2012;28(8):798–805.
82. Kaplan LD, Straus DJ, Testa MA, Von Roenn J, Dezube BJ, Cooley TP, et al. Low-dose compared with standard-dose m-BACOD chemotherapy for non-Hodgkin's lymphoma associated with human immunodeficiency virus infection. National Institute of Allergy and Infectious Diseases AIDS clinical trials group. *N Engl J Med*. 1997;336(23):1641–1648.
83. Sparano JA, Lee JY, Kaplan LD, Levine AM, Ramos JC, Ambinder RF, et al. Rituximab plus concurrent infusional EPOCH chemotherapy is highly effective in HIV-associated B-cell non-Hodgkin lymphoma. *Blood*. 2010;115(15):3008–3016.
84. Barta SK, Lee JY, Kaplan LD, Noy A, Sparano JA. Pooled analysis of AIDS malignancy consortium trials evaluating rituximab plus CHOP or infusional EPOCH chemotherapy in HIV-associated non-Hodgkin lymphoma. *Cancer*. 2012;118(16):3977–3983.
85. Alwan F, He A, Montoto S, Kassam S, Mee M, Burns F, et al. Adding rituximab to CODOX-M/IVAC chemotherapy in the treatment of HIV-associated Burkitt lymphoma is safe when used with concurrent combination antiretroviral therapy. *AIDS*. 2015;29(8):903–910.
86. National Comprehensive Cancer Network. Cancer in People Living With HIV (Version 2.2020) [Available from: https://www.nccn.org/professionals/physician_gls/pdf/hiv.pdf.
87. Rudek MA, Flexner C, Ambinder RF. Use of antineoplastic agents in patients with cancer who have HIV/AIDS. *Lancet Oncol*. 2011;12(9):905–912.
88. Tan CRC, Barta SK, Lee J, Rudek MA, Sparano JA, Noy A. Combination antiretroviral therapy accelerates immune recovery in patients with HIV-related lymphoma treated with EPOCH: a comparison within one prospective trial AMC034. *Leuk Lymphoma*. 2018;59(8):1851–1860.
89. Lawn SD, Estée Török M, Wood R. Optimum time to start antiretroviral therapy during HIV-associated opportunistic infections. *Curr Opin Infect Dis*. 2011;24(1):34–42.
90. Balsalobre P, Díez-Martín JL, Re A, Michieli M, Ribera JM, Canals C, et al. Autologous stem-cell transplantation in patients with HIV-related lymphoma. *J Clin Oncol*. 2009;27(13):2192–2198.
91. Durand CM, Ambinder RF. Hematopoietic stem cell transplantation in HIV-1-infected individuals: clinical challenges and the potential for viral eradication. *Curr Opin Oncol*. 2013;25(2):180–186.
92. Uldrick TS, Ison G, Rudek MA, Noy A, Schwartz K, Bruinooge S, et al. Modernizing clinical trial eligibility criteria: recommendations of the

American Society of clinical oncology-friends of cancer research HIV working group. *J Clin Oncol*. 2017;35(33):3774–3780.

93. Ambinder RF, Wu J, Logan B, Durand CM, Shields R, Popat UR, et al. Allogeneic hematopoietic cell transplant for HIV patients with hematologic malignancies: the BMT CTN-0903/AMC-080 trial. *Biol Blood Marrow Transplant*. 2019;25(11):2160-2166.
94. Salgado M, Kwon M, Gálvez C, Badiola J, Nijhuis M, Bandera A, et al. Mechanisms that contribute to a profound reduction of the HIV-1 Reservoir after allogeneic stem cell transplant. *Ann Intern Med*. 2018 169(10):674-683.
95. Xu L, Wang J, Liu Y, Xie L, Su B, Mou D, et al. CRISPR-edited stem cells in a patient with HIV and acute lymphocytic leukemia. *N Engl J Med*. 2019;381(13):1240–1247.
96. Abbasi J. HIV remission after hematopoietic stem cell transplant. *JAMA*. 2019;321(14):1343.
97. Gupta RK, Abdul-Jawad S, McCoy LE, Mok HP, Peppa D, Salgado M, et al. HIV-1 remission following CCR5Delta32/Delta32 haematopoietic stem-cell transplantation. *Nature*. 2019;568(7751):244–248.
98. Henrich TJ. Second example reported of a stem-cell transplant in the clinic leading to HIV remission. *Nature*. 2019;568:175–176.
99. Rosen HR. Chronic hepatitis C infection. *N Engl J Med*. 2011;364(25): 2429–2438.
100. Ito M, Kusunoki H, Mochida K, Yamaguchi K, Mizuochi T. HCV infection and B-cell lymphomagenesis. *Adv Hematol*. 2011;2011:1–8.
101. Pawełczyk A, Kubisa N, Jabłońska J, Bukowska-Ośko I, Cortes KC, Fic M, et al. Detection of hepatitis C virus (HCV) negative strand RNA and NS3 protein in peripheral blood mononuclear cells (PBMC): CD3, CD14 and CD19. *Virology J*. 2013;10(1):346.
102. Monti G, Pioltelli P, Saccardo F, Campanini M, Candela M, Cavallero G, et al. Incidence and characteristics of non-Hodgkin lymphomas in a multicenter case file of patients with hepatitis C virus–related symptomatic mixed cryoglobulinemias. *Arch Intern Med*. 2005;165(1):101.
103. Marcucci F, Mele A. Hepatitis viruses and non-Hodgkin lymphoma: epidemiology, mechanisms of tumorigenesis, and therapeutic opportunities. *Blood*. 2011;117(6):1792–1798.
104. An X, Wang FH, Ding PR, Deng L, Jiang WQ, Zhang L, et al. Plasma Epstein-Barr virus DNA level strongly predicts survival in metastatic/ recurrent nasopharyngeal carcinoma treated with palliative chemotherapy. *Cancer*. 2011;117(16):3750–3757.
105. Gisbert JP, García-Buey L, Pajares JMa, Moreno-Otero R. Prevalence of hepatitis C virus infection in B-cell non-Hodgkin's lymphoma: systematic review and meta-analysis. *Gastroenterology*. 2003;125(6):1723–1732.
106. Amin J, Dore GJ, O'Connell DL, Bartlett M, Tracey E, Kaldor JM, et al. Cancer incidence in people with hepatitis B or C infection: a large community-based linkage study. *J Hepatology*. 2006;45(2):197–203.
107. Negri E, Little Da, Boiocchi M, La Vecchia C, Franceschi S. B-cell non-Hodgkin's lymphoma and hepatitis C virus infection: a systematic review. *Int J Cancer*. 2004;111(1):1–8.
108. Anderson LA, Engels EA. Hepatitis C virus infection and non-Hodgkin lymphoma: Interesting association or causal relationship? *Int J Cancer*. 2008;122(8):x–xii.
109. Libra M, et al. Extrahepatic disorders of HCV infection: a distinct entity of B-cell neoplasia? *Int J Oncol*. 2010;36(6).
110. Besson C, Canioni D, Lepage E, Pol S, Morel P, Lederlin P, et al. Characteristics and outcome of diffuse large B-cell lymphoma in hepatitis C virus–positive patients in LNH 93 and LNH 98 groupe d'Etude des lymphomes de l'adulte programs. *J Clin Oncol*. 2006;24(6):953–960.
111. Varma S, Menon MC, Garg A, Malhotra P, Sharma A, Chawla YK, et al. Hepatitis C virus infection among patients with non-Hodgkin's lymphoma in northern India. *Hepatology Int*. 2011;5(2):688–692.
112. Chuang SS, Liao YL, Chang ST, Hsieh YC, Kuo SY, Lu CL, et al. Hepatitis C virus infection is significantly associated with malignant lymphoma in Taiwan, particularly with nodal and splenic marginal zone lymphomas. *J Clin Pathol*. 2010;63(7):595–598.
113. Omland LH, Jepsen P, Krarup H, Christensen PB, Weis N, Nielsen L, et al. Liver cancer and non-Hodgkin lymphoma in hepatitis C virus-infected patients: Results from the DANVIR cohort study. *Int J Cancer*. 2012;130(10):2310–2317.
114. Isikdogan A, Ayyildiz O, Dursun M, Tiftik N, Batun S, Muftuoglu E. Hepatitis C virus in patients with non-Hodgkin's lymphoma in Southeastern Anatolia region of Turkey: a prospective case-control study of 119 patients. *Leuk Lymphoma*. 2003;44(10):1745–1747.
115. Giordano TP, Henderson L, Landgren O, Chiao EY, Kramer JR, El-Serag H, et al. Risk of non-Hodgkin lymphoma and lymphoproliferative precursor diseases in US veterans with hepatitis C virus. *JAMA*. 2007;297(18):2010–2017.
116. Engels EA, Chatterjee N, Cerhan JR, Davis S, Cozen W, Severson RK, et al. Hepatitis C virus infection and non-hodgkin lymphoma: results of the NCI-seer multi-center case-control study. *Int J Cancer*. 2004;111(1):76–80.
117. Hermine O, Lefrere F, Bronowicki JP, Mariette X, Jondeau K, Eclache-Saudreau V, et al. Regression of splenic lymphoma with villous lymphocytes after treatment of hepatitis C virus infection. *N Engl J Med*. 2002;347(2):89–94.
118. Kawamura Y, Ikeda K, Arase Y, Yatsuji H, Sezaki H, Hosaka T, et al. Viral elimination reduces incidence of Malignant lymphoma in patients with hepatitis C. *Am J Med*. 2007;120(12):1034–1041.
119. Merli M, Visco C, Spina M, Luminari S, Ferretti VV, Gotti M, et al. Outcome prediction of diffuse large B-cell lymphomas associated with hepatitis C virus infection: a study on behalf of the Fondazione Italiana Linfomi. *Haematologica*. 2014;99(3):489–496.
120. Arcaini L, Vallisa D, Rattotti S, Ferretti VV, Ferreri AJM, Bernuzzi P, et al. Antiviral treatment in patients with indolent B-cell lymphomas associated with HCV infection: a study of the Fondazione Italiana Linfomi. *Ann Oncol*. 2014;25(7):1404–1410.
121. Ennishi D, Maeda Y, Niitsu N, Kojima M, Izutsu K, Takizawa J, et al. Hepatic toxicity and prognosis in hepatitis C virus–infected patients with diffuse large B-cell lymphoma treated with rituximab-containing chemotherapy regimens: a Japanese multicenter analysis. *Blood*. 2010; 116(24):5119–5125.
122. Persico M, Aglitti A, Caruso R, De Renzo A, Selleri C, Califano C, et al. Efficacy and safety of new direct antiviral agents in hepatitis C virus–infected patients with diffuse large B-cell non-Hodgkin's lymphoma. *Hepatology*. 2018;67(1):48–55.

CHAPTER 88

MALIGNANT LYMPHOMAS IN CHILDHOOD

Kara M. Kelly, Birgit Burkhardt, and Catherine M. Bollard

Malignant lymphomas are the third most common malignancy among children and adolescents.[1–3] Among children less than 15 years of age, non-Hodgkin Lymphoma (NHL) is more frequent; however, in patients up to 18 years of age, Hodgkin lymphoma (HL) is predominant. NHLs in children are usually extranodal diffuse high-grade tumors, whereas low- and intermediate-grade nodal lymphomas predominate in adults. These differences are speculated to reflect maturational changes in the function and composition of the immune system.[2] The different histologies explain in part the differing clinical features, disease course, and treatment strategies used in adults and children.

The differences in treatment approach and disease subtypes are less striking in adults and children with HL. However, there are significant challenges in the management of children with HL. These primarily comprise the sequelae of therapy, such as radiation-induced bone growth abnormalities, endocrine dysfunction, and chemotherapy-related sterility. Of greater concern are the radiation- and chemotherapy-related second malignancies and late cardiac deaths. Current trials are examining ways to reduce the toxicity of therapy without compromising the excellent outcome generally achieved.

NON-HODGKIN LYMPHOMA

Epidemiology

The incidence of NHL increases steadily throughout life, in contrast to Hodgkin disease, which has a bimodal age distribution peaking in early and late adulthood.[2] Although NHLs may occur at any age in childhood, they are infrequent among children younger than 3 years of age; the median age at presentation is approximately 10 years. NHL is two to three times more frequent in boys than in girls and is almost twice as common in Whites as in African Americans—the reasons for these differences have yet to be determined.[4]

Specific populations at risk for the development of NHL include those with either congenital or acquired immunodeficiency conditions.[5] Inherited immunodeficiency syndromes include Wiskott-Aldrich syndrome, X-linked lymphoproliferative syndrome (XLP), and ataxia-telangiectasia (AT).[6] The recognition of these syndromes in children with newly diagnosed NHL is important for appropriate therapeutic design. For example, involved field irradiation and radiomimetics should be avoided in children with AT. Additionally, children with AT are at increased risk for the development of cyclophosphamide-induced hemorrhagic cystitis; therefore, they should receive vigorous hydration and uroprotectants (e.g., Mesna) when administering any dose of cyclophosphamide. XLP should be considered in any male with a high-grade B-cell lymphoma who either develops a late recurrence (second occurrence) of a high-grade B-cell lymphoma or has a brother with either a high-grade B-cell lymphoma or fatal infectious mononucleosis. Children with acquired immunodeficiency conditions predisposing to the development of NHL include those who have received post-transplant immunosuppressive therapy and those with acquired immunodeficiency syndrome (AIDS).

There are differences in both the incidence and proportion of histologic subtypes in distinct parts of the world. For example, the NHLs are rare in Japan but occur quite frequently in equatorial Africa. Burkitt lymphoma, which accounts for about one-half of all childhood cancers in equatorial Africa, is the predominant NHL histologic subtype in both equatorial Africa and northeast Brazil.[7] There are also geographic differences in both the clinical and biologic features of certain NHL subtypes.[8,9]

Classification

After Thomas Hodgkin described the disease bearing his name in 1832, various schemes emerged to classify the tumors now collectively referred to as the NHLs. Several classification schemes were developed based on histopathologic features and the putative cell of origin. In an attempt to reduce the confusion of multiple classification schemes, the National Cancer Institute (NCI) sponsored a workshop to design a single classification scheme for clinical usage. This scheme published in 1982 and referred to as the NCI Working Formulation was widely accepted for almost two decades. In the Working Formulation, the NHLs of childhood are predominantly diffuse high-grade tumors and can be divided among three major subgroups: lymphoblastic, small noncleaved cell, and large cell lymphomas.

In the past decade, additional classification schemes were designed to improve upon the NCI Working formulation. Because of the problems associated with attempts to classify lymphoid neoplasms into categories based on presumed normal cell counterparts, the International Lymphoma Study Group proposed a classification system for lymphoid neoplasms[10] predicated on a practical approach to categorizing these diseases using available immunologic and molecular genetic techniques in addition to the standard morphologic criteria. This Revised European-American Classification of Lymphoid Neoplasms (REAL Classification) has been endorsed by many of the world's leading lymphoma pathologists and has served as the basis for the World Health Organization (WHO) classification of hematopoietic and lymphoid tumors.[11] Both the REAL and WHO classification systems include related lymphoid leukemias and recognize that NHL and acute lymphoblastic leukemia (ALL) represent different stages of evolution within specific morphologically and immunologically defined disease categories—an observation recognized by clinicians caring for children with lymphoid neoplasms and reflected in current clinical practice which prescribes similar therapies for lymphomas and leukemias of related phenotype.

In the REAL and WHO classification systems, NHLs are classified based on phenotype (B-lineage versus T-lineage versus natural killer (NK) cell lineage) and differentiation (precursor vs. mature). Hence, NHLs that occur commonly in children appear in three major categories: Lymphoblastic lymphoma (LBL; precursor B-cell lymphoma and precursor T-cell lymphoma), mature B-cell NHL (Burkitt and Burkitt-like lymphoma and diffuse large B-cell lymphoma [DLBCL]), and anaplastic large cell lymphoma (ALCL; mature T-cell or null-cell types). The current version of the WHO classification introduced pediatric follicular lymphoma, marginal zone lymphoma, and grey zone lymphoma.[10]

The clinical and biological characteristics of NHL in children are summarized in Table 88.1 and illustrated in Fig. 88.1A-C.

TABLE 88.1 Characteristics of Non-Hodgkin Lymphoma in Children

Subtype	Proportion of Cases in BFM Studies (%)[14]	Phenotype	Primary Site
Lymphoblastic	26	T-cell; B-cell	Mediastinum or head and neck; lymph nodes, skin, soft tissue, bone
Burkitt	49	B-cell	Abdomen or head and neck
DLBCL	13	B-cell	Lymph nodes, mediastinum, abdomen, head and neck
ALCL	13	T-cell indeterminate	Mediastinum, abdomen, head and neck, bone, soft tissue, or skin

ALCL, Anaplastic large cell lymphoma, *BFM*, Berlin-Frankfurt-Münster; *Ig*, immunoglobulin.

Lymphoblastic Lymphoma

Introduction

It is an ongoing discussion whether LBL and ALL are two clinical presentations of the same disease or two different diseases.[12,13] Patients with a mass and fewer than 25% bone marrow lymphoblasts are designated as LBL, whereas patients with at least 25% bone marrow involvement have ALL. In contrast to ALL, more than 75% of LBL demonstrate a precursor T-cell immunophenotype (T-LBL), with the remainder showing a precursor B-cell immunophenotype (pB-LBL).[14–16]

Epidemiology

LBL constitutes 22% to 28% of childhood NHL. There is a 2:1 male predominance for LBL, but the incidence of LBL remains constant across the pediatric age group for both boys and girls.[17]

Pathobiology

LBL arises from precursor T- or B-lymphoblasts at varying stages of differentiation. Morphology is similar to that of ALL, with lymphoblasts of small or medium size and with scant cytoplasm, round or convoluted nuclei, fine chromatin, and indistinct or small nucleoli. Immunophenotyping shows terminal deoxynucleotidyl transferase (TdT) positivity. T-LBL is usually positive for CD7 and surface or cytoplasmic CD3, with variable expression of CD2, CD5, CD1a, CD4, and CD8.[18] Stains for TdT, CD3, MPO, and a B-cell marker like pax5 or CD79a are recommended parts of a resource-saving diagnostic staining panel.[19] CD10 expression is more frequent in T-LBL (40%) than T-ALL (less than 10%), possibly related to the maturational stage, with T-ALL more frequently demonstrating an immature phenotype.[16,20,21] B-lineage markers are positive in B-LBL. Similar to ALL, cases with mixed phenotypes are reported but interestingly not associated with inferior outcome.[22] Unlike ALL, there are no known cytogenetic prognostic factors for LBL. Recurrent cytogenetic anomalies are seen in about half of childhood T-ALL but have not been well-defined in T-LBL. Literature is scarce regarding typical chromosomal aberrations for T-LBL.[23] The commonest chromosomal translocations for both T-LBL and T-ALL involve the T-cell receptor (*TCR*) gene loci at chromosome 14q11 or 7q34. Certain molecular genetic alterations were analyzed in relevant patient series of pediatric T-LBL including *NOTCH1*, *FBXW7*, *PTEN* alterations, PIM1 variants, alterations of chromosome 9p containing *CDKN2A/CDKN2B* loci, and chromosome 6q.[24–28] A recent targeted sequencing study of a relevant series of pediatric T-LBL confirmed *NOTCH1* as the putative driver for T-LBL resulting in an activated NOTCH/PI3K-AKT signaling axis. In addition, alterations in cell cycle regulators constitute the core oncogenic program for T-LBL. The mutational status of *KMT2D* was identified as a prognostic marker. The ongoing international clinical trial LBL 2018 (NCT04043494) introduced a

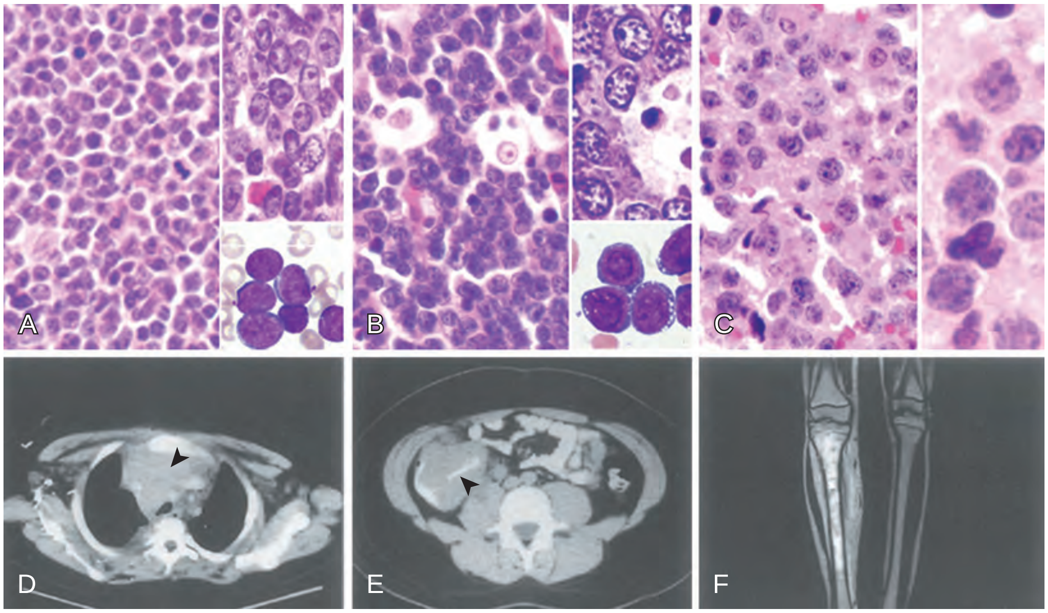


Figure 88.1 HISTOLOGIC AND CLINICAL FEATURES OF THE NON-HODGKIN LYMPHOMAS OF CHILDHOOD. The upper panels demonstrate the appearance in the H&E section of Burkitt lymphoma (Panel A); lymphoblastic lymphoma (Panel B); and anaplastic large cell lymphoma (Panel C). The inserts in Panels A and B demonstrate the appearance of Wright's stained specimens of the L3 blasts of Burkitt lymphoma and the L1 blasts of lymphoblastic lymphoma, respectively. The lower panels show common clinical presentations of the two histologic subtypes of lymphoma: airway compression by lymphoblastic lymphoma of the anterior mediastinum on computed tomography of the chest (Panel D) and encasement of the bowel lumen by Burkitt lymphoma on abdominal computed tomography (Panel E). *(Reproduced, in part, from Sandlund JT, Downing JR, Crist WM, et al. Non-Hodgkin's lymphoma in childhood. N Engl J Med. 1996;334[19]:1238–1248.)*

TABLE 88.2 Murphy Staging of Non-Hodgkin Lymphoma

Stage	Criteria
Stage I	A single tumor (extranodal) or involvement of a single anatomic area (nodal), with the exclusion of the mediastinum and abdomen.
Stage II	A single tumor (extranodal) with regional node involvement.
	Two or more nodal areas on the same side of the diaphragm.
	Two single (extranodal) tumors, with or without regional node involvement on the same side of the diaphragm.
	A primary gastrointestinal tract tumor (usually in the ileocecal area), with or without the involvement of associated mesenteric nodes, that is completely resectable.
Stage III	Two single tumors (extranodal) on opposite sides of the diaphragm.
	Two or more nodal areas above and below the diaphragm.
	Any primary intrathoracic tumor (mediastinal, pleural, or thymic).
	Extensive primary intraabdominal disease.
	Any paraspinal or epidural tumor, whether or not other sites are involved.
Stage IV	Any of the above findings with initial involvement of the central nervous system, bone marrow, or both.

*Based on the classification proposed by Murphy SB, Fairclough DL, Hutchison RE, et al. Non-Hodgkin's lymphomas of childhood: an analysis of the histology, staging, and response to treatment of 338 cases at a single institution. *J Clin Oncol.* 1989;7(2):186–193.

TABLE 88.3 International Pediatric Non-Hodgkin Lymphoma Staging System

Stage	Criteria for Extent of Disease
I	A single tumor with exclusion of mediastinum and abdomen
II	A single tumor with regional node involvement
	≥2 nodal areas on the same side of the diaphragm
	A primary gastrointestinal tract tumor (usually in the ileocecal area), with or without involvement of associated mesenteric nodes, that is completely resectable (if malignant ascites or extension of tumor to adjacent organs, it should be regarded as stage III)
III	≥2 extranodal tumors (including EN-B or EN-S) above and/or below the diaphragm
	≥2 nodal tumors above and below the diaphragm
	Any intrathoracic tumor (mediastinal, hilar, pulmonary, pleural, or thymic)
	Intraabdominal and retroperitoneal disease, including liver, spleen, kidney, and/or ovary localizations, regardless of degree of resection (except primary gastrointestinal tract tumor [usually in ileocecal region] and/or involvement of associated mesenteric nodes that is completely resectable)
	Any paraspinal or epidural tumor, regardless of whether other sites are involved
	A single bone lesion with concomitant involvement of extranodal and/or non-regional nodal sides
IV	Any of the above findings with:
	Initial involvement of CNS (stage IV CNS)
	Initial involvement of the bone marrow (stage IV BM)
	Initial involvement of CNS and bone marrow (stage IV combined) based on conventional methods

B, Bone; *BM*, bone marrow; *EN*, extranodal; *N*, nodal; *S*, skin; *EN-B*, extranodal bone; *EN-S*, extranodal skin.
From Rosolen A, Perkins SL, Pinkerton CR, et al. Revised international pediatric non-Hodgkin lymphoma staging System. *J Clin Oncol.* 2015;33(18):2112–2118.

new stratification system taking into account the mutational status of *NOTCH1* and *FBXW7*. In parallel, the trial will allow prospective systematic validation of the prognostic relevance of individual markers or marker combinations to be translated into future trials.

Clinical Manifestations

The clinical features at presentation vary with primary site and extent of disease spread. Almost all patients with T-LBL frequently present with an anterior mediastinal mass that may cause respiratory symptoms, airway compromise, dysphagia, or superior vena cava syndrome (see Fig. 88.1D). Pleural effusions are common (75%), and lymphadenopathy above the diaphragm is frequent. Bone, skin, bone marrow, central nervous system (CNS), abdominal organs, other lymph nodes, and occasionally testes may also be involved. Children with B-LBL are less likely to present with a mediastinal mass, but there is a higher frequency of cutaneous, soft tissue, or bone involvement with pB-LBL.[29–32] CNS involvement at diagnosis is seen in 4% to 5% of patients with LBL.[31,33]

Differential Diagnosis

LBL is distinguished from ALL by having less than 25% bone marrow involvement defined by cytomorphology of bone marrow smears and from myeloid malignancies being positive for TdT and T- or B-cell markers and negative for myeloperoxidase and additional myeloid markers. T-LBL and B-LBL are differentiated by flow cytometry or immunohistochemistry. In cases of insufficient biopsy material or incomplete diagnostic staining, LBL is sometimes misdiagnosed (e.g., as Ewing sarcoma or other small-, round-, blue-cell tumors).

Prognosis (Staging)

After obtaining malignant effusion or tissue diagnosis, staging is performed with imaging (ultrasound of the abdomen, lymph nodes and testes, and magnetic resonance imaging or computed tomography of involved sites), bilateral bone marrow evaluation, and lumbar puncture. Bone scans are only done if clinically indicated. Childhood NHL, including LBL, is most commonly staged using the Murphy classification previously and now by the International Pediatric Non-Hodgkin Lymphoma Staging System (IPNHLSS) (Table 88.2 and 88.3).[34,35]

With current therapies based on ALL protocols, LBL has a long-term survival greater than 90% in low-stage disease and greater than 80% in advanced-stage disease (Table 88.4).[36] The Children's Oncology Group demonstrated that minimal disseminated disease at diagnosis has prognostic value, as indicated by flow cytometric evidence of tumor cells in bone marrow.[37] The prognostic impact of minimal disseminated disease at diagnosis and minimal residual disease is under evaluation.[38–40] On NHL-BFM trials, adolescent females with T- LBL had poorer outcomes than adolescent males despite similar presenting characteristics.[41] One adult study for T-cell ALL has demonstrated poorer outcomes in females than males,[42] but a prognostic impact of gender has not been found in other pediatric or adult studies for LBL. While adolescent age itself has not been established as a poor prognostic factor as it has for ALL, adult outcomes for LBL are inferior to pediatric outcomes.[43]

Therapy

Two potentially life-threatening situations requiring urgent intervention must be considered in children with LBL: (1) mediastinal tumor with airway obstruction or superior vena cava syndrome, and (2) tumor lysis syndrome. Due to the cardiac and respiratory risks of anesthesia or sedation in children with a large mediastinal mass, the least invasive procedure should be chosen to establish a diagnosis. In children with relevant pleural effusion, pleural puncture and morphologic combined with immunologic diagnostics by

TABLE 88.4 Outcomes for Lymphoblastic Lymphoma

Trial	Age	Stage	Treatment	No. of Pts	pEFS
LMT81[184]	9 years (0.9–16)	I–IV	mod. LSA2-L2	84	75±3%
CCG502[185]	9 years (0.5–19)	I–IV	mod. LSA2-L2 vs.	143	74%
			ADCOMP	138	64%
POG8704[186]	10 years (5–15)	III/IV	L-Asp–vs.	83	64±6%
			L-Asp+	84	78±5%
	9 years (1–16)	I–IV	ALL-BFM	105	90%
NHL-BFM95[28]	8 years (0.2–19)	III/IV	BFM	169	78±3%
EORTC 58881[187]	8 years (0–16)	I–IV	BFM-based	119	78±3%
COG Pilot[188]	n.d.	III/IV	mod. LSA2-L2	85	78±5%
LNH92[189]	8 years (0–<16)	I–IV	mod. LSA2-L2	55	69±6%
St. Jude 13[47]	n.d.	III//V	T-ALL	41	83±6%
pB EORTC[31]	7 years	I–IV	mod. LMT, BFM	53	82%
POG 9404[45]	50% <10 years	III/IV	mod. DFCI ALL with HDMTX	66	82±5%
			w/o HDMTX	71	88±4%
COG A 5971[190]	7 years (1–25)	I/II	CCG BFM	56	90%
COG A 5971[191]	10 years	III/IV	NHL-BFM95 MTX	total 257	85±4%
			w/o HDMTX		83-4%
			intensification		83±4%
			w/o intensification		83–4%
EURO-LB[46]	8 years (0–18)	I–IV	Dexa vs. Pred 18 vs. 24 months treatment duration	319	82±2%
AALL0434[48]	11 years (1–31)	III–IV	Nelarabine ± in high-risk patients	299	85±2%

ALL, Acute lymphoblastic leukemia; *Asp*, asparaginase; *BFM*, Berlin-Frankfurt-Münster; *CCG*, Children's Cancer Group; *DFCI*, Dana-Farber Cancer Institute; *EFS*, event-free survival; *EORTC*, European Organisation for Research and Treatment of Cancer; *HDMTX*, high-dose methotrexate; *mod*, modified; *NHL*, non-Hodgkin lymphoma; *n.d.*, no data; *pB-LBL*, precursor B-cell lymphoblastic lymphoma; *POG*, Pediatric Oncology Group; *T-LBL*, T-cell lymphoblastic lymphoma; *vs.*, versus.

flow cytometry allow adequate diagnosis. In children with peripheral lymphadenopathy, lymph node biopsy under local anesthesia may be possible. In children who cannot tolerate a procedure, pretreatment with steroids may be necessary to stabilize the patient. Since pretreatment may diminish the ability to accurately diagnose a patient, a tissue diagnosis should be obtained as soon as it is possible to do so safely. Tumor lysis syndrome is characterized by metabolic consequences of the breakdown of lymphoma cells, causing renal failure if severe. Hyperuricemia, hyperkalemia, and hyperphosphatemia must be aggressively managed by hyperhydration, rasburicase and/or allopurinol, and close monitoring. Children with NHL can also present with epidural masses and associated neurologic deficits caused by spinal cord compression. If the diagnosis is known, chemotherapy should be started as soon as possible. If the diagnosis is not known, or if there is a sluggish response to chemotherapy, low-dose radiation therapy may be considered in consultation with a radiation oncologist.

ALL treatment strategies achieved favorable outcomes in LBL and are accepted as standard treatments as (e.g., the Berlin-Frankfurt-Münster [BFM]) combination chemotherapy with induction, consolidation, and maintenance phases lasting a total of 24 months with 90% 5-year event-free survival (EFS).[36] Even patients with stage III and IV LBL had good outcomes on ALL-type therapy.[44,45] This ALL-like therapy has now become standard for LBL (see Table 88.4).[46] CNS prophylaxis is needed for LBL, but chemotherapy prophylaxis has not proven inferior to prophylactic cranial irradiation in CNS-negative patients, even with advanced-stage disease.[28,31,47] Additionally, recent studies did not demonstrate a survival advantage of high-dose methotrexate for T-LBL.[45,48] Unfortunately, outcome is still poor for patients suffering LBL relapse.[49]

Burkitt Lymphoma

Introduction

Burkitt lymphoma (BL) was first described by Dennis Burkitt in the 1950s in Uganda.[50] First thought to be endemic to equatorial Africa, it was subsequently observed in Europe and North America. The WHO classification recognizes three variants: (1) sporadic BL, occurring throughout the world and more common in children, adolescents, and young adults; (2) endemic BL, occurring primarily in sub-Saharan Africa and New Guinea, with some unique clinical features but morphologically identical to sporadic BL; and (3) immunodeficiency-associated BL, observed primarily in HIV patients and less commonly in the setting of other immunodeficiencies. Burkitt-like lymphoma with 11q aberration is introduced as a provisional lymphoma entity in the 2017 revision of the WHO classification.[51] When there is marrow involvement of more than 25%, it is designated Burkitt leukemia (FAB L3 leukemia) but is treated similarly to BL.[10,11]

Epidemiology

Sporadic BL constitutes approximately 50% of childhood NHL and is much more common in boys than girls (ratio 4:1), with a peak incidence between 5 and 14 years of age.[14,17] Endemic BL associated with Epstein-Barr virus (EBV) in more than 85% of cases accounts for approximately half of all childhood cancers in equatorial Africa. In contrast, sporadic BL is most common in the United States and Europe and is associated with EBV in only 10% to 15% of cases.[52,53]

Pathobiology

BL is composed of monomorphic, small, noncleaved cells with round nuclei, clumped chromatin, and basophilic cytoplasm. A high uniform proliferation index is seen, with the Ki-67 positivity approaching 100%. The classic "starry sky" appearance of BL seen under low power microscopy is caused by tingible body macrophages scattered among malignant cells. BL cells show mature B-cell features and usually express surface immunoglobulins. B-cell markers such as CD19, CD20, and CD22 are usually present, and the majority express CD10 (common acute lymphoblastic leukemia antigen, CALLA). BL is negative for TdT and in most cases for B-cell lymphoma (BCL)-2. CD21, the EBV receptor, is more commonly seen in endemic BL than in sporadic BL.

Characteristic chromosomal translocations suggest that BL develops from genetic aberrations during somatic hypermutation or attempted immunoglobulin class switching. These translocations, usually t(8;14) or infrequently t(8;22) or t(2;8), juxtapose the c-*myc* gene (involved in cellular proliferation) with immunoglobulin locus regulatory elements, resulting in c-*myc* overexpression. In sporadic cases, the predominant chromosome 8 breakpoints usually occur within the c-*myc* gene, whereas they are upstream of the gene in endemic cases. For the rare event of *c-myc*-negative BL, characteristic aberrations of chromosome 11q characterized by interstitial gains including 11q23.2-q23.3 and telomeric losses of 11q24.1-qter were reported.[51,54] Other cytogenetic abnormalities, such as gain of 7q and deletion of 13q, are uncommon.[55,56] Recently published large-scale next-generation sequencing studies unveiled sets of recurrently mutated genes in tumor cells of pediatric and adult B-NHL patients and introduced functionally related inhibitor of DNA 3 (*ID3)*, transcription factor 3 (*TCF3)*, and cyclin D3 (*CCND3)* as potential drivers of BL lymphomagenesis.[35,57–59]

Clinical Manifestations

BL is an extremely fast-growing malignancy. The most common primary sites of sporadic BL are the abdomen and the lymph nodes of the head and neck.[60,61] Abdominal disease presentation often associated with nausea, vomiting, and abdominal pain carries a risk of intestinal perforation, obstruction, and gastrointestinal bleeding. Abdominal lymphoma often arises from the distal ileum causing intestinal obstruction secondary to intussusception or compression by an expanding mass encasing the bowel (see Fig. 88.1E). BL can involve lymph nodes, testes, bone, bone marrow, and CNS. CNS involvement at diagnosis occurring in about 9% of patients is associated with a worse outcome.[33] Endemic BL frequently involves the abdomen, jaw, paraspinal region, orbit, and CNS.

Differential Diagnosis

In DLBCL, another mature B-cell lymphoma, the cells are usually larger and less basophilic. Cytogenetic abnormalities such as BCL-6 gene rearrangements or t(14;18) may be seen in individual cases, although these abnormalities are more common in adult DLBCL. Ki-67 staining in less than 95% of the cells or positivity for BCL-2 are helpful in excluding a diagnosis of BL. TdT negativity is helpful in distinguishing BL from B-LBL. Recent studies addressed B-cell malignancies with immature phenotype but c-*myc* translocation. While biologically more closely related to ALL, treatment with BL regimen resulted in acceptable outcome.[62,63]

Prognosis (Staging)

The risk group classification developed by the French Society of Pediatric Oncology (SFOP) is widely used on current protocols incorporating risk-adapted therapy (Table 88.5). This system accounts for the adverse prognosis associated with CNS or bone marrow involvement and whether localized disease has been resected. Localized or CNS-negative advanced-stage BL have greater than 90% long-term survival with current therapies.[64] Combined CNS and BM involvement and suboptimal response to initial cytoreduction is also associated with worse prognosis, and poor responders are often stratified to more intensive chemotherapy on current regimens.[65] In recent trials, the French stratification system was extended by including LDH, as high LDH at diagnosis also carries a worse prognosis and is frequently incorporated in risk classification schemes (e.g., that of the BFM) (Table 88.6).[66] Age greater than 15 years has a worse outcome for mature B-cell malignancies, but this may be primarily due to DLBCL rather than BL. Recent studies on clonal evolution in BL underlined the prognostic role of *TP53* mutations.[36]

TABLE 88.5 French Society of Pediatric Oncology Risk Group Classification of Mature B-Cell Lymphomas

Group	Extent of Disease
Group A	Completely resected Stage I Completely resected abdominal Stage II
Group B	Nonresected Stage I and II Any Stage III CNS-negative Stage IV with BM involvement 5%–25%
Group C	CNS-positive Stage IV Stage IV with BM involvement ≥25% (mature B-cell ALL)

ALL, Acute lymphoblastic leukemia; *BM*, bone marrow; *CNS*, central nervous system; *SFOP*, French Society of Pediatric Oncology.

Therapy

Tumor lysis syndrome must be anticipated as a major risk in newly diagnosed BL or Burkitt leukemia. To help prevent this complication, children at risk should be vigorously hydrated. The urine pH should be maintained at about 7.0; at a more alkaline pH, phosphorus is less soluble, and at a more acidic pH, uric acid is less soluble. In some cases, furosemide is required to maintain urine output. Uricolytic agents (e.g., uricozyme), which have been used for many years in Europe, directly cleave the uric acid molecule and result in a precipitous drop in serum uric acid levels within a few hours. The use of uricolytic agents (e.g., urate oxidase, uricase, rasburicase) has proven to be superior to allopurinol to reduce rapidly the level of serum uric acid and improve renal function. Rasburicase has significantly reduced the need for hemodialysis consequent upon tumor lysis syndrome. Recent protocols incorporate a prophase of reduced intensity to decrease the risk of severe tumor lysis syndrome.[67] In the setting of abdominal BL, intestinal perforation, obstruction, or gastrointestinal bleeding may not occur until after the initiation of chemotherapy as the lymphoma begins to regress.

Localized resected mature B-cell NHL (including both BL and DLBCL) could be cured without significant toxicity by two courses

TABLE 88.6 Berlin-Frankfurt-Münster Risk Group Classification of Mature B-Cell Lymphomas

Risk Group	Resection Status	Stage and Initial Serum LDH Level
R1	Complete	
R2	Incomplete	Stage I+II Stage III and LDH <500 U/L
R3	Incomplete	Stage III and LDH ≥500 U/L but <1000 U/L Stage IV/B-AL and LDH <1000 U/L and CNS-negative
R4	Incomplete	Stage III and LDH ≥1000 U/L Stage IV/B-AL and LDH ≥1000 U/L and CNS-negative
R4 CNS+	Incomplete	Stage IV/B-AL and CNS-positive

B-AL, Burkitt leukemia with ≥25% blasts in the bone marrow; *BFM*, Berlin-Frankfurt-Münster; *BM*, bone marrow; *CNS*, central nervous system; *LDH*, lactate dehydrogenase; *U/L*, unit per liter.

of chemotherapy.[37,41–43,66,68] Advanced-stage BL requires aggressive combination chemotherapy with CNS prophylaxis (Table 88.7). Cyclophosphamide, methotrexate, and cytarabine at high doses have been used most recently, with or without anthracyclines and epipodophyllotoxins. Therapy should be started as quickly as possible upon presentation since this tumor grows very rapidly, and subsequent cycles should be administered in an intensive fashion as soon as recovery occurs from the last cycle. CNS prophylaxis is needed because without it approximately 30% to 50% of patients will relapse in the CNS,[69] while 6% to 11% develop CNS relapse if adequate prophylaxis is given.[33] CNS prophylaxis includes high-dose methotrexate and cytarabine to penetrate the CNS, together with intensive intrathecal chemotherapy. Risk-adapted therapy used in the French-American-British (FAB)/LMB-96 study and the NHL-BFM95 study conferred an excellent EFS in advanced-stage patients.[65–67] Treatment intensity was escalated based on tumor burden and response to therapy. Both regimens have significant hematologic toxicity. Therefore, both the FAB/LMB-96 study and the NHL-BFM 95 study successfully reduced therapy for low- and intermediate-risk patients without a compromise in survival. However, both studies failed to safely reduce the intensity of therapy for patients with advanced stages of disease.

Rituximab is a mouse/human chimeric monoclonal antibody against CD20 that is highly expressed in BL and DLBCL. In adults, the addition of Rituximab to CHOP chemotherapy is beneficial for DLBCL and has been given safely in combination with intensive BL therapy. In children, single-agent rituximab showed activity for BL in a phase II window study for newly diagnosed patients,[70] and the Children's Oncology Group showed the safety of adding rituximab to the LMB backbone for the treatment of BL.[71,72] Current clinical trials address the question of whether rituximab added to standard chemotherapy can improve the EFS of patients with advanced disease and showed superior outcome with rituximab.[47] The rituximab window prior to standard NHL-BFM chemo and additional doses in high-risk patients are currently under evaluation in the ongoing NHL-BFM and Nordic Society of Pediatric Haematology and Oncology (NOPHO) trial B-NHL 2013 along with the kinetics of immune reconstitution (NCT03206671). This trial enrolls low, standard, and high-risk patients.

Local radiation therapy has no role in BL because it is chemosensitive and often diffuse. CNS radiation has been used in the past for CNS involvement at diagnosis but did not show an impact on outcome in this group. Surgery is no longer routinely used for BL.

TABLE 88.7 **Outcomes for Burkitt Lymphoma and Diffuse Large B-Cell Lymphoma**

Trial	Arm	Patients	Number of Courses	EFS (%)
Limited Disease				
LMB89[64]	A	10%	2	98%
FAB-LMB96[192]	A	144	2	98%
NHL-BFM95[66]	R1	10%	2	94%
AIEOP-LNH92[69]	R1	9%	3	100%
JACLS NHL-98[68]	A	19%	2	100%
Intermediate Disease				
LMB89[64]	B	69%	6	92%
FAB-LMB96[67]	B arm 1	164	5	n.a
	B arm 1	163	4	n.a
	B arm 3	167	5	n.a
	B arm 3	163	4	n.a
NHL-BFM95[66]	R2	46%	5	94%
	R3	16%	6	85%
AIEOP LNH92[69]	R2	38%	5	87%
	R2/R3 with insufficient response	30% of R2 and 55% of R3	7	n.a.
JACLS NHL98[68]	B	25%	5	100%
	C	30%	7	75%
Advanced Disease				
LMB89[64]	C	22%	9	84%
	C CNS− stand.	52	9	94%
	C CNS− red.	51	6	86%
	C CNS+ stand.	44	9	84%
	C CNS+ red.	43	6	72%
NHL-BFM95[66]	R4	28%	7	81%
AIEOP LNH92[69]	R3	53%	7	75%
	R2/R3 with insufficient response	30% of R2 and 55% of R3		n.a.
JACLS NHL98[68]	D	26% (18/69)	7	66%
Inter B-NHL 2010[193]	B high and C	all	Group B high or group C treatment w/o rituximab	EFS 3 years 94% vs. 82%

AIEOP, Associazione Italiana di Ematologia e Oncologia Pediatrica; *CCG*, Children's Cancer Group; *EFS*, event-free survival; *JACLS*, Japan Association of Childhood Leukemia Study; *LMB*, Lymphome Malins de Burkitt; *n.a.*, not available; *NHL-BFM*, non-Hodgkin lymphoma Berlin-Frankfurt-Münster;.

However, patients with localized disease resected at diagnosis are eligible for reduced chemotherapy, as noted previously.

Diffuse Large B-Cell Lymphoma

Introduction

DLBCL includes a heterogeneous group of neoplasms of transformed B cells, accounting for 10% to 13% of pediatric NHL.[14] DLBCL has some distinctive biologic and clinical features, described in more detail below.[73] DLBCL involving the mediastinum need to be distinguished from the primary mediastinal large B-cell lymphoma (PMBCL) that represents a distinct subtype of B-NHL in the WHO classification. In addition, the current version of the WHO classification introduced pediatric follicular lymphoma, marginal zone lymphoma, and grey zone lymphoma.

Epidemiology

The incidence of DLBCL increases with age, being more common in the second decade of life.[17,74] It is rare in children younger than 4 years of age. The gender difference is far less than in BL. PMBCL is more common in adolescents, with no gender difference.[14,73]

Pathobiology

Most pediatric DLBCL cases have a germinal center mature B-cell phenotype.[74] Nuclei are usually more than twice the size of normal lymphocytes. They express pan-B-cell antigens, including CD19, CD20, CD22, and CD79a with or without surface immunoglobulin. Most express CD10 and BCL6, and approximately 40% express BCL2. However, breaks or translocations in *BCL2* and *BCL6* are rare in pediatric DLBCL. Unlike adult DLBCL, pediatric cases rarely demonstrate the t(14;18) translocation.[73,74] Gene profiling has led to the subclassification of adult cases into germinal center B-cell like, activated B-cell like, and type 3 (not belonging to the first two groups) phenotypes. The great majority of children have the germinal center B-cell like phenotype.[74,75] Interestingly, this phenotype has a better prognosis in adults, and childhood DLBCL outcomes are better than adult outcomes. Morphologic variants of DLBCL include centroblastic, immunoblastic, anaplastic, and T-cell/histiocyte rich variants.[76] Although DLBCL is clearly heterogeneous, the clinical value of these distinctions is unclear in children.

PMBCL is thought to originate from medullary thymic B cells. B-lineage antigens and CD30 are often positive, but surface immunoglobulin and HLA class I and II molecules are absent or incompletely expressed.[73] *MYC*, *BCL2*, and *BCL6* genes are not rearranged. PMBCL is associated with gains in chromosome 9p (involving *JAK2*) and 2p (involving c-*rel*). They commonly demonstrate the inactivation of *SOCS1*.[77,78] Variable degrees of sclerosis occur in PMBCL,[79] as well as occasional lymphocytes, eosinophils, and Reed-Sternberg-like cells, sometimes leading to confusion with HL.

Clinical Manifestations

DLBCL is often more localized than BL and is less likely to involve the bone marrow or CNS.[33] Nodal disease is more common in DLBCL than in BL, but extranodal disease is frequent. Common sites of involvement include the head and neck, abdomen, mediastinum, and bone. PMBCL is locally invasive and frequently associated with SVC syndrome or airway compression. Pleural or pericardial effusions are present in approximately 40% of PMBCL cases,[79] and kidney metastases are frequent.[73]

Differential Diagnosis.

The differential diagnosis of DLBCL encompasses BL, pB-LBL, and nodular lymphocyte-predominant HL. BL is distinguished from DLBCL by morphology, although atypical BL shows more pleomorphism than typical BL. BCL2 negativity, high cell proliferation, and translocations involving c-*myc* may suggest BL but are not definitive. DLBCL with its mature phenotype differs from pB-LBL by a lack of TdT expression. DLBCL and nodular lymphocyte-predominant HL share some similar features and derive from a common B-cell clone. DLBCL may represent the clonal progression of nodular lymphocyte-predominant HL. PMBCL can also be confused with HL, and an adequate biopsy is essential for diagnosis. PMBCL and other DLBCL are distinguished clinically, as PBMCL typically resides within the thymic area while mediastinal DLBCL usually involves mediastinal lymph nodes.

Prognosis (Staging)

Murphy classification and the revised IPNHLSS are used to stage DLBCL.[16,80] There is no difference in outcomes between BL and DLBCL based on histology, and the treatment is identical in children and adolescents.[73] Outcomes for pediatric DLBCL are superior to adults, with 5-year overall survival (OS) of 90% for current therapies. Adolescent females fared worse than adolescent males with DLBCL on NHL-BFM protocols.[41] BCL2 expression is not an unfavorable prognostic factor in children with DLBCL, in contrast to adults with DLBCL.[74] Prognosis is poorer for children and adolescents with PMBCL (5-year EFS 65% to 75%).[55,79,81] In the setting of PMBCL, LDH of 500 U/l or more has a worse prognosis.[79] For adult patients with PMBCL, favorable outcome was reported using a dose-adjusted EPOCH-rituximab regimen with continuous infusions of etoposide, doxorubicin, vincristine, and bolus[66,67] administrations of cyclophosphamide and prednisone.[82] In a small pediatric series similar excellent results were observed but require systematic prospective validation.[83]

Therapy

In children and adolescents, all mature B-cell lymphomas are usually treated similarly with good results. Despite biologic and clinical differences, BL therapy is effective in DLBCL (see Table 88.7).[73] Risk-adapted therapy with LMB (SFOP) or B-NHL (BFM) backbones consists of short, dose-intense courses of chemotherapy including steroids, vincristine, high-dose methotrexate, cyclophosphamide, doxorubicin, cytarabine, etoposide, and intrathecal chemotherapy.[64,73] As described for BL, risk-adapted therapy based on FAB/LMB-96 and NHL-BFM 95 are also the current standard of care for childhood DLBCL.[41,64,66,67] Rituximab added to standard chemo regimen improved the outcomes for patients with advanced disease.[10,47] The impact of rituximab for limited-stage B-NHL is currently being evaluated in the NHL-BFM and NOPHO trial B-NHL 2013 (NCT03206671).

Having evolved from BL regimens, DLBCL treatment has traditionally included intrathecal chemotherapy for CNS prophylaxis. Since the risk of CNS involvement is lower for DLBCL than for BL, it is unclear if CNS-directed therapy should be as intensive.[33,73] Local radiation has no role in front-line DLBCL therapy.[73] As mentioned, the optimal treatment strategy for PMBCL has not been established yet, but favorable results with DA- EPOCH-R were reported for a small series of the NHL-BFM group and in a retrospective analysis.[84] However, in the first prospective multisite international study evaluating children and adolescents with PMBCL ($n = 46$), DA-EPOCH-R did not improve the EFS compared with historical controls. (ClinicalTrials.gov identifier: NCT01516567).[85] Hence, further studies are required to determine the optimum therapy for children and adolescents with PMBCL including evaluating the impact of checkpoint inhibitors, which is currently the focus of prospective trials (NCT04759586).

Anaplastic Large Cell Lymphoma

Introduction and Epidemiology

Most childhood NHLs previously classified as large cell lymphomas fall into the category of ALCL in the REAL and WHO classification. This entity was first described in 1985 as a clinicopathologic variant of large cell lymphoma with a predilection for young patients.[86]

Pathobiology

ALCLs are characterized by the proliferation of large, pleomorphic cells with one or more prominent nucleoli. The cells preferentially involve lymph node sinuses and extranodal sites (notably skin, bone, and soft tissue) where they grow in a cohesive pattern. The cells express epithelial membrane antigen (EMA) and CD30 (Ki-1) antigen—a 120kd membrane-bound molecule a member of the tumor necrosis factor (TNF) receptor superfamily—previously found in association with Hodgkin disease. A soluble (88kd) form of the CD30 molecule is found in high levels in the serum of nearly all patients with ALCL.[87] Marked elevation of soluble CD30 levels in patients with ALCL correlates with a higher risk of relapse, and soluble CD30 levels correlate with clinical disease status, returning to normal with the attainment of complete remission and increasing with disease recurrence.

The ALCLs are associated with chromosomal rearrangements involving the long arm of chromosome 5 at position q35.[88] In most cases, this translocation includes material from chromosome 2p23 [t(2;5)(p23;q35)] resulting in the fusion of the NPM nucleolar phosphoprotein gene on chromosome 5q35 to anaplastic lymphoma kinase (ALK), a tyrosine kinase gene on chromosome 2p23. The hybrid protein produced from the translocation links the amino terminus of nucleophosmin (NPM) with the catalytic domain of ALK.[89] Deregulated expression of the truncated ALK may contribute to malignant transformation. The chimeric NPM-ALK protein is clearly oncogenic, perhaps through the triggering of antiapoptotic signals via phosphatidylinositol 3-kinase/Akt, although secondary molecular events may be required for lymphomagenesis.

Immunologic and molecular biologic studies reveal that most cases of Ki-1+ ALCLs are derived from activated T-cells, although non-T, non-B-cell (null-cell) cases and more rarely B-cell cases occur.[90] Based on extended testing for T-cell antigens and examination of the configuration of TCR genes, many "null" cell cases are, in fact, T-lineage neoplasms, although a minority may be derived from NK cells.[91] The diagnosis of ALCL can be difficult, and many cases are initially misdiagnosed as Hodgkin disease. Molecular techniques (reverse transcriptase-polymerase chain reaction [RT-PCR]) to detect the fusion gene produced of the t(2;5) in many cases of ALCL confirms the diagnosis. Variant translocations where *ALK* is involved with other partner genes on other chromosomes limit the application of RT-PCR for diagnosis. However, the t(2;5) results in the expression of the NPM-ALK fusion protein, while the variant translocations result in the up-regulation of *ALK*. The ALK1 monoclonal antibody recognizing the formalin resistant epitope of both the NPM-ALK chimeric protein and normal ALK thus serves as a useful diagnostic reagent for identifying cases of ALCL. It should be noted that the distribution of ALK staining varies depending on the translocation. Further, ALK expression is a feature of inflammatory myofibroblastic tumors (IMT); however, confusion with ALCL can be minimized by testing for other hematopoietic markers, and the distinction is not usually difficult for experienced pathologists.

Clinical Manifestations

ALCL is rare, accounting for 8% to 13% of childhood NHLs and approximately 30% to 40% of the pediatric large cell lymphomas. Approximately one-third of the cases present with localized disease, while the majority have advanced disease at presentation, although bone marrow and CNS involvement is uncommon.[92] A rare leukemic presentation of ALCL possibly associated with the small cell variant of ALCL has been described. Systemic symptoms (fevers, weight loss) are frequently present in advanced-stage disease. Cutaneous (spontaneously regressing) lesions sometimes accompany disease at other sites, but skin involvement is not universal.[86] A variety of presenting sites—both nodal and extranodal—can occur, including the mediastinum, gastrointestinal tract, and bone. Further, tumors may invade adjacent structures and be associated with ascites and other intraabdominal sites of disease, including kidney, liver, and lymph nodes. The outcome of children with ALCL in most series has been good (with survivals ranging from 70% to 85%), albeit inferior to that of children with Burkitt and diffuse large B-cell lymphomas (Table 88.8).

Diagnosis and Differential Diagnosis

Immunophenotyping and immunohistochemistry are critical for the definitive diagnosis of ALCL. The typically ALCL immunophenotype is: CD30$^+$, CD15$^-$, CD45$^+$, in contrast to HL which is typically CD30$^+$ and CD15$^+$. Over 60% of cases of ALCL express 1 or more T-cell antigens (CD3$^+$, CD43, or CD45RO), and ALK protein is detected in most cases (over 60%).

Prognosis (Staging)

The disease stage of ALCL is determined according to the staging system described by Murphy (see Table 88.2).[35] As for other types of childhood NHL, stages I and II are considered limited-stage disease, whereas stage III represents advanced-stage disease. Stage IV is reserved for children with bone marrow or CNS involvement. For

TABLE 88.8 **Outcomes for Anaplastic Large Cell Lymphoma**

Protocol	*n*	Stage	Regimen	EFS
POG 8314 and 8719[98]	72	I–II (resected)	CHOP ± maintenance	88% (5-year)[a]
CCG 5941[95]	86	Non-localized	Multi-agent, + maintenance	68% (5-year)
NHL-BFM 90[99]	9	I–II (resected)	Multi-agent, risk-adapted (short-pulse B-NHL type therapy)	100% (5-year)
	65	II (non-resected)–III		73% (5-year)
	14	IV and multifocal bone involvement		79% (5-year)
ALCL-99[94]	225	I–IV	Multi-agent, risk-adapted, 3–6 cycles chemotherapy	81% (3-year) (100% EFS in completed resected patients
COG ANHL0131[104]	125	III or IV	APO versus APV	74% (3-year) in APO group 79% (3-year) in APV group
COG ANHL12P1[108]	68	II–IV	ALCL-99 plus brentuximab vedotin	79% (2-year) overall 100% stage I 76.7% stage II 66.7% stage III

[a]ALCL and DLBCL combined.
ALCL, Anaplastic large cell lymphoma; *APO*, doxorubicin, prednisone, vincristine, methotrexate, 6-mercaptopurine; *APV*, doxorubicin, prednisone, vinblastine, methotrexate, 6-mercaptopurine; *BFM*, Berlin-Frankfurt-Münster; *CCG*, Children's Cancer Group; *CHOP*, cyclophosphamide, doxorubicin, vincristine, prednisone; *EFS*, event-free survival; *NHL*, non-Hodgkin lymphoma; *POG*, Pediatric Oncology Group.

elderly patients with cutaneous CD30$^+$ ALCL, the 5-year disease-free survival appears to be determined by the extent of limb involvement; however, this has not been shown in children, possibly due to the fact that primary cutaneous ALCL is rare in the pediatric population.[93]

For systemic ALCL, studies have suggested that the stage of disease may be more important than the expression of the ALK protein. The European Intergroup for Childhood Non-Hodgkin Lymphoma defined three factors (mediastinal involvement, visceral involvement, and skin lesions) associated with poorer prognosis in childhood ALCL.[94] This group conducted a multivariate analysis (merging preexisting databases from BFM, SFOP, and United Kingdom Children's Cancer Study Group studies) and identified poor prognosis (1 or more risk factors) and standard risk groups with 5-year PFS of 61% and 89%, respectively. However, it is important to note that this study was not done as a prospective collaborative study. In contrast, the Children's Cancer Group Study 5941 reported that only bone marrow involvement significantly changed the 5-year survival rate.[95]

The presence of ALK autoantibodies appears to be associated with decreased clinical risk factors and lower clinical stage, resulting in a lower cumulative incidence of relapses.[96] There is no correlation between outcome and the ALK translocation type. However, recently the SFOP group has shown that for patients with ALK+ ALCL, the presence of a small cell or lymphohistiocytic component is associated with a worse prognosis.[97]

Therapy

The optimal treatment approach for patients with ALCL has yet to be determined, as evidenced by a wide range of successful treatment strategies. In the United States, children with advanced-stage CD30+ ALCL are generally treated with non-ALCL large cell lymphoma regimens, with long-term EFS rates ranging from 60% to 75% (see Table 88.8).[94,95,98,99] In a randomized trial performed by the pediatric oncology group (POG), the addition of intermediate-dose methotrexate and high-dose cytarabine did not improve upon the 70% 4-year EFS achieved with APO alone.[100] The BFM reported 3-year EFSs of ~80% using a BL-based strategy.[99] Regimens designed specifically for children with CD30+ ALCL have been developed by the French (SFOP) and German Groups.[101] The SFOP also reported the successful salvage of ALCL patients with the weekly administration of single-agent vinblastine.[102]

The impact of incorporating vinblastine into two different frontline treatments for ALCL (the BFM B-cell approach [multi-national European trial]) and APO (Children's Oncology Group [COG] trial in the United States) was then studied in a randomized fashion. In the European study, patients receiving the vinblastine plus chemotherapy regimen had a better EFS in the first year after therapy (91%) than those not receiving vinblastine (74%); however, at 2 years of follow-up, EFS was 73% for both groups.[103] Similarly, The COG ANHL0131 study evaluated whether a maintenance regimen including vinblastine compared to APO (doxorubicin, prednisone, vincristine, methotrexate, 6-mercaptopurine) alone would result in superior EFS. Post induction patients were randomized to receive APO with vincristine every 3 weeks or a regimen that substituted vincristine with weekly vinblastine (APV). In this study of 125 patients, no difference was observed between the patients randomized to the APO versus APV arms in either EFS (3-year EFS 74% vs. 79% $P = .68$) or OS 84% vs. 86%, $P = .87$).[104]

In addition, the European study also showed that infusing methotrexate 1 g/m^2 over 24 hours was comparable to 3 g/m^2 over 3-hour infusions without intrathecal methotrexate.[105] However, 3 g/m^2 of methotrexate had less toxicity. Other studies have come to no firm conclusions. For example, (1) the POG-9317 trial demonstrated no benefit of adding methotrexate and high-dose cytarabine to 52 weeks of their APO (doxorubicin, prednisone, and vincristine) regimen,[100] and (2) the CCG-5941 study evaluated a more intensive induction and consolidation with maintenance for 1-year total duration of therapy again and had similar outcomes but with significant hematologic toxicity.[95]

Targeted therapy options are important areas of investigation for future studies since crizotinib, an ALK inhibitor, has had outstanding results in pediatric phase I studies in children with ALCL showing a nearly 100% response rate and minimal toxicity.[106] In addition, with the remarkable activity of brentuximab vedotin (tubulin-inhibitor conjugated monoclonal anti-CD30) in ALCL,[107] this led the COG to pursue testing the efficacy and toxicity of adding these two biologic targeted agents with standard ALCL-99 chemotherapy in newly diagnosed pediatric ALCL patients (ANHL12P1). In this study, 68 children with ALK+ ALCL were enrolled and received brentuximab vedotin (from 2013 to 2017) on day 1 of each of the chemotherapy 6 cycles. The addition of brentuximab vedotin to standard chemotherapy did not add significant toxicity, and with 14 relapses, the 2-year EFS was 79.1% (95% confidence interval [CI], 67.2 to 87.1) with a 2-year OS of 97.0% (95% CI, 88.1 to 99.2) that compared favorably to results obtained using conventional chemotherapy.[108] The results of the crizotinib arm have not been published yet (NCT01979536).

Relapsed Non-Hodgkin Lymphoma-Management

There is no standard treatment for relapsed NHL. Treatment usually consists of intensive chemotherapy to induce a complete or partial response, followed by autologous or allogeneic stem cell transplant.[49] Recent studies suggest that allogeneic transplant may be more effective than autologous transplant for relapsed NHL, particularly for LBL.[109] Outcomes are appreciably better for patients with a complete remission prior to transplant. Radiation may be useful prior to transplant in selected patients with an incomplete response to chemotherapy. However, with the increased area of study comparing commercial CD19 chimeric antigen receptor (CAR) T-cell therapy versus standard of care (including autologous stem cell transplant) in the adult NHL setting (NCT03391466 and NCT03570892), the utility of this therapy in the relapsed setting for pediatric patients is being actively evaluated. Pediatric and adolescent patients with relapsed/refractory B-NHL (including BL) were infused with tisagenlecleucel in the Novartis sponsored BIANCA trial demonstrating the feasibility and manageable safety in this population with aggressive disease (NCT03610724).

While the efficacy data of CD19-CAR-T therapy for pediatric B-NHL still needs to be determined, many other targeted immunotherapeutic modalities are being developed for relapsed/refractory B-NHL in adults, but few are being tested in pediatric patients. However, a pediatric phase II trial combining obinutuzumab with ifosfamide/carboplatin/etoposide (ICE) is ongoing (NCT02393157). The CD19/CD3 bispecific antibody Blinatumomab is active in adults and children with relapsed/refractory ALL and was also active in a small trial in adults with heavily pretreated relapsed/refractory DLBCL but is yet to be comprehensively evaluated in the pediatric setting.[110]

Outside of immune-based therapies, outcomes of relapsed disease typically vary by histologic subtype. Relapsed BL and DLBCL are often chemoresistant, and survival is only 10% to 20%. The COG used rituximab, ifosfamide, carboplatin, and etoposide (R-ICE) for salvage therapy in this population, achieving a 60% response rate (complete and partial).[111] Patients who achieve a complete remission should proceed to stem cell transplant.

Relapsed LBL is also frequently chemoresistant, and reported survival is 10% to 40%.[112–114] Salvage regimens used for ALL may be employed for LBL, and Nelarabine has demonstrated a 40% response rate in this setting for T-ALL and T-LBL in a phase II COG study.[115] Encouragingly, novel immunotherapeutic agents such as CD5-directed CAR-T cells (NCT03081910) are also being evaluated in pediatric patients with relapsed/refractory T-LBL.

Survival for relapsed ALCL is better than for other NHLs, reaching 40% to 60%.[6,112,116] Vinblastine monotherapy as salvage therapy resulted in a complete remission rate of 83% in one study.[117] Brentuximab vedotin, an antibody against CD30, has been used successfully in adults and in pediatrics with relapsed ALCL.[118,119] Crizotinib, an ALK inhibitor, has also shown promise in adults in early clinical trials for relapsed ALCL patients and has shown remarkable results in phase I studies in children.[106] Autologous transplant has been used successfully for some patients, but allogeneic transplant may have better outcomes and is preferred for patients with bone marrow or CNS involvement, early relapse, and /or CD3-positive relapsed ALCL.[41,109,120,121]

Post-Transplant Lymphoproliferative Disorder

Post-transplant lymphoproliferative disease (PTLD) is discussed in other Chapters. Therefore, this section merely serves to summarize the issues specific to the pediatric population. The major pediatric populations where PTLD is most frequently seen are patients with congenital immune deficiencies or patients after bone marrow or solid organ transplant. Further, stem cell transplant recipients with underlying immunodeficiencies such as Wiskott-Aldrich syndrome (WAS) represent an independent risk group for EBV-associated lymphoproliferative disease (LPD).[122] Patients with primary, congenital immunodeficiencies such as X-linked agammaglobulinemia and AT have an incidence of EBV-associated LPD ranging from 0.7% to 15%.[123] The incidence of PTLD after solid organ transplant is higher in children than in the adult population. The disease is heterogeneous, but in children, it is most frequently of B-cell origin and EBV associated. In the United States, 150 new cases are diagnosed in children each year.

Numerous therapeutic approaches to PTLD have been explored in children, but there has been a paucity of multicenter collaborative studies for this disease. Withdrawal or reduction of immunosuppression is often considered as first-line therapy for PTLD but depends on whether the patient has a recovering immune system sufficient to eradicate EBV-infected B cells and depends on stage and disease histology. For the pediatric hematopoietic stem cell transplant (HSCT) patient, strategies have included reduction in immune suppression, rituximab,[124] donor lymphocyte infusions,[125] and EBV-specific T-cell directly therapy.[126] The advantages of T-cell-based therapies over antibody therapy is that EBV-specific T-cell immune reconstitution is restored, thus reducing the risk for disease recurrence. Responses to donor-derived EBV-specific T-cell therapies developed at multiple centers for pediatric patients range from 70% to 85%.[126,127] Immune-based therapies are generally preferred in this population since the use of chemotherapy in patients with LPD post HSCT is associated with high mortality rates secondary to increased infectious complications.[128] However, hydroxyurea and immune-based therapies may be an option for patients with CNS disease.[129] After solid organ transplant (SOT), modalities such as radiotherapy or surgical resection for localized LPD can result in complete remissions. One study evaluated the efficacy of low-dose cyclophosphamide and prednisone for pediatric patients with PTLD after SOT. All patients had failed reduction of immune suppression and received 6 cycles. The 2-year event-free and OS was 67% and 73%, respectively.[130] Subsequently, the COG was evaluated in a phase II study (ANHL0221) where the CD20 monoclonal antibody was added to the previously reported regimen of low-dose cyclophosphamide and prednisone regimen (http://clinicaltrials.gov/show/NCT00066469). All patients had refractory PTLD after SOT, CD20+, EBV+ PTLD in pediatric patients following solid organ transplantation. The study showed that the addition of the CD20 monoclonal antibody to the previously reported regimen of low-dose cyclophosphamide and prednisone demonstrated an EFS and OS of 71% and 83%, respectively.[131] Therefore, to build on the success of this first cooperative group trial for PTLD, the COG have just completed a feasibility and safety study (NCT02900976) administering "off the shelf" third-party allogeneic EBV/latent membrane protein (LMP)– cytotoxic T lymphocytes (CTLs)[132] to determine the safety and efficacy in patients with PTLD post SOT in combination with rituximab. This represented the first multicenter study of its kind combining antibody therapy with "off the shelf" antigen-specific T-cell therapy for this disease.

Finally, for patients refractory to these low-dose regimens or patients with definitive features of malignancy, standard lymphoma chemotherapy regimens are used depending on histological subtype (e.g., BL vs. DLBCL vs. HL directed therapy).

HODGKIN LYMPHOMA

Epidemiology

HL comprises 10% of all lymphomas and approximately 10% of pediatric cancers[2] and has a bimodal incidence, with one peak at 15 to 34 years of age, and a second peak in the 6th decade. In children, the highest peak is among 15 to 19-year-olds (29 per million per year) and is least frequent in children under 5 years. There is an age-dependent sex predominance, with a male predominance in children under 5 years (male:female ratio of 5.3:1) and a slight female predominance in children aged 15 to 19 years (male:female ratio of 0.8:1). HL (especially the nodular sclerosing subtype) is classically associated with higher socioeconomic status, increased incidence in single-family homes, smaller family sizes, and a higher level of maternal education. In contrast, mixed cellularity HL is inversely related to socioeconomic status. It is commonly postulated that delayed exposure to some environmental antigen may trigger the development of HL, and higher rates of certain childhood infections (e.g. varicella, measles, mumps, rubella) are negatively associated with HL development.[133]

HL has been shown to have a genetic predisposition, although this is incompletely understood. Siblings of HL patients have a 5-fold (50-fold for monozygotic twins) increased risk of developing HL.[134] There is also an increased risk among parent-child pairs, though not between spouses, supporting a genetic rather than a uniquely environmental predisposition. Genome wide association studies have identified HL susceptibility loci,[135] implicating dysfunction of the germinal center reaction, disruption of T-cell differentiation and function, and constitutive activation of nuclear factor kappa B (NFκB) as mechanistic factors associated with elevated risk for HL.

Environmental factors are nevertheless involved in HL.[136] Approximately 40% of all HL cases in economically developed countries harbor EBV in the Hodgkin and Reed–Sternberg (HRS) cells. EBV positivity of the malignant HRS populations is more common in children under 10 years of age and is highly associated with childhood HL with mixed cellularity subtype (80% of cases) characteristically seen in developing countries. The EBV protein latent membrane protein 1 (LMP1) may be the key in the oncogenic process since it can elicit B lymphocyte transformation *in vitro*.[137]

There is also a relationship between the development of HL and cell-mediated immune deficiency with an increased incidence in patients with HIV,[138] patients on chronic immunosuppressive therapy following solid organ transplantation, and common variable immunodeficiency (CVID). It remains unclear whether the underlying immune deficiency commonly seen in HL is primary, secondary, or both.[137]

Pathobiology

HL is considered to be slow growing with a tendency to spread to contiguous lymph nodes. Only in advanced stages is there evidence of blood vessel invasion and spread to more distant organs.[139] Over the past 30 years, significant advances have been made in the treatment of pediatric HL, such that over 85% of children are cured with chemotherapy and/or radiation even with stage III/IV disease.

HL is notable for the characteristic B-cell-derived HRS cells found in a background of an inflammatory microenvironment usually comprising Tregs, Th2 T cells, macrophages, and eosinophils. The REAL/WHO classification identifies two main subtypes: classical HL and lymphocyte-predominant HL. Classical HL accounts for most cases in adolescents and young adults and is characterized by HRS cells which are usually CD15+ and CD30+. HRS cells do not express B-cell markers such as CD19 and CD79A, although CD20 is expressed in 5% to 10% of cases. Mixed cellularity HL accounts for 1/3 of cases diagnosed in children less than 10 years. The histopathology shows frequent HRS cells on a background of normal reactive immune cells including T lymphocytes, plasma cells, eosinophils, macrophages, and histiocytes. Lymphocyte-predominant HL and lymphocyte-rich classical HL may both have nodular appearances, but the former is more commonly CD15 negative and usually has strong CD20 and CD45 positivity, thus distinguishing it from classical HL.[140]

Multiple prognostic markers related to HRS cells, the microenvironment or both, have been evaluated in patients with HL, although none have been found to be superior to current clinical or radiographic factors.[141] More recently, circulating tumor DNA analysis by next-generation sequencing has shown early promise for the diagnosis and disease monitoring after initiation of therapy, including studies in pediatric HL[142,143]

Clinical Manifestations

The majority of pediatric patients with HL present with painless, firm, "rubbery" lymphadenopathy, most commonly in the cervical/supraclavicular regions. Over 70% of adolescents and young adults present with mediastinal disease, which can be asymptomatic. This presentation is less frequent in younger children and may be related to a lower incidence of nodular sclerosis HL and a greater frequency of mixed cellularity or lymphocyte-predominant HL in this group. Approximately one-quarter of patients present with systemic "B" symptoms including fevers, weight loss, and drenching night sweats. The majority of pediatric patients presenting with HL have stage I–III disease (involvement of lymph nodes and/or the spleen only) with a minority (approximately 15%) presenting with stage IV disease (noncontiguous extranodal involvement such as bone marrow, lung, liver, and/or bone).

Staging

Pediatric HL is staged using the Ann Arbor staging system (with subsets A and B used for the absence or presence of B symptoms, respectively) (Table 88.9).[144] Briefly, stage I involves a single lymph node region. Stage II involves two or more lymph node regions on the same side of the diaphragm. Stage III involves lymph node regions above and below the diaphragm. For stages I–III, if there is an extension to an adjacent extralymphatic region/organ, then this is designated E (e.g., stage IIE). For stage III, if there is splenic involvement, this is designated stage IIIS. Finally, for stage IV disease, there is noncontiguous involvement of one or more extralymphatic organs or tissues +/− involvement of associated lymph nodes.

TABLE 88.9 **Ann Arbor Staging System for Hodgkin Lymphoma**

Stage I
Involvement of a single lymph node region (I) or of a single extralymphatic organ or site (I_E)
Stage II
Involvement of two or more lymph node regions on the same side of the diaphragm (II) or localized involvement of an extralymphatic organ or site and one or more lymph node regions on the same side of the diaphragm (II_E)
Stage III
Involvement of lymph node regions on both sides of the diaphragm (III), which may be accompanied by involvement of the spleen (III_S) or by localized involvement of an extralymphatic organ or site (III_E) or both (III_{SE})
Stage IV
Diffuse or disseminated involvement of one or more extralymphatic organs or tissues with or without associated lymph node involvement

Note: The absence or presence of fever higher than 38°C for three consecutive days, drenching night sweats, or unexplained loss of 10% or more of body weight in the 6 months preceding admission are to be denoted in all cases by the suffix letters A or B, respectively. Suffix letter E denotes extralymphatic extension from contiguous nodal disease. Disease involving the spleen is designated a suffix letter S, and mediastinal bulk is often designated with an X.
Adapted from Carbone PP, et al. Report of the Committee on Hodgkin's Disease Staging Classification. *Cancer Res.* 1971;31:1860-1861; Rosenberg SA. Report of the Committee on the Staging of Hodgkin's Disease. *Cancer Res.* 1966;26:1310; Lister TA, Crowther D, Sutcliffe SB, et al. Report of a committee convened to discuss the evaluation and staging of patients with Hodgkin's disease: Cotswolds meeting. *J Clin Oncol.* 1989;7(11):1630–1636.

Treatment

Despite the excellent outcomes for children with HL, there is still no ideal therapeutic approach. Consensus guidelines from the National Comprehensive Cancer Network (NCCN) provide specific pediatric HL treatment options based on risk status and disease staging using results from recent clinical trials.[145] Generally, combination chemotherapy with or without low-dose involved field radiation is used with varying intensities and duration depending usually on disease stage and prognostic factors such as disease bulk and B symptoms. However, studies of late effects in these patients[146] have fueled cooperative groups in particular to explore regimens with decreased radiation and/or chemotherapy doses, especially for children with low-risk disease. Implementation of risk-adapted treatment approaches for pediatric HL has resulted in a 20% reduction in risk for serious or fatal chronic health conditions.[147] Risk stratification for treatment assignment varies considerably between the pediatric HL research groups, thus limiting comparisons between trial results. The general consensus, however, is that chemotherapy should be given to all HL patients, with or without radiation. The exception to this approach is patients with single node stage I, completely resected, nodular lymphocyte-predominant HL, who can achieve cure with surgery alone.[148,149]

Chemotherapeutic agents used for the initial treatment of pediatric HL are similar to those used in adults and include alkylating agents, corticosteroids, vinca alkaloids, antimetabolites, doxorubicin, bleomycin, dacarbazine, and etoposide. However, doxorubicin (or Adriamycin) is associated with cardiac toxicity and bleomycin can lead to pulmonary fibrosis and alkylators may impair fertility. Therefore, many cooperative group studies have developed hybrid regimens using lower cumulative doses of alkylators, doxorubicin, and bleomycin, especially for low-risk (stages I–IIA; no bulk; no B symptoms) and intermediate-risk (all stage I and II patients not classified as early stage; and variably stage IIIA and stage IVA) disease. Results of clinical trials by the major international pediatric HL study groups are summarized in Table 88.10. Trials differ by the use of chemotherapy regimens of varying dose intensity, as well as the criteria for the omission of radiotherapy. The German Society of Pediatric Oncology and now in conjunction with other European pediatric oncology centers (Euronet consortium) has investigated OEPA (vincristine, etoposide, prednisone, doxorubicin) for low risk and OEPA with COPDac (cyclophosphamide, vincristine, prednisone, dacarbazine) for intermediate- and high-risk groups.[150] In North America, the COG has primarily evaluated ABVE-PC (doxorubicin, bleomycin, vincristine, etoposide, prednisone, cyclophosphamide) and its derivatives across the risk groups.[151,152]

Radiotherapy usage varies considerably. The COG reported that radiotherapy may be safely omitted in intermediate-risk patients who have a rapid reduction in tumor dimensions by CT after 2 cycles of chemotherapy, in the only published large randomized trial to evaluate omission of radiotherapy.[153] The European consortium has omitted radiotherapy for low-risk patients achieving a CR after 2 cycles of OEPA.[150] The utility of response by positron emission tomography (PET) is being evaluated in trials recently completed or in progress by all the major groups. In general, pediatric radiotherapy approaches utilize lower doses (15 to 25 Gy) and fields (involved field or node). The use of contemporary modalities such as Intensity-modulated radiotherapy, deep inspiration breath hold, and proton beam therapy, may further reduce long-term toxicities.

Therapies targeting CD30, a transmembrane glycoprotein that is present on Reed-Sternberg cells but is not expressed in most normal tissue, have been an area of significant interest as a means of

TABLE 88.10 Treatment Outcomes for Pediatric Hodgkin Lymphoma Clinical Trials

Study	*N*	Risk Group	Rx	Radiation (Gy)	EFS or DFS; OS (yr)
Children's Oncology Group					
CCG5942[194]	215	IA, IB, IIA without adverse features[a]	COPP/ABV × 4	CR after cycle 4: randomized to 21, IFvs. none; PR: 21, IF	IF: 97.1% None: 89.1% (*P* = .001); IF: 100%, None: 95.9% (*P* = 0.5) (10 years)
POG9426[195]	294	IA, IIA, IIIA (no bulk)	DBVE × 2–4 (based on response after cycle 2)	25.5, IF	86.2%; 97.4% (8-year)
AHOD0431[155,196]	287	IA, IIA(no bulk)	AVPC × 3	CR after cycle 3: none PR: 21, IF	Nodular sclerosis: 75.8%; Mixed cellularity 95.2%; 99.6% (4 years)
German Society of Pediatric Oncology			Mauz 2010		
GPOH[150]	195	IA, IB, IIA	OEPA (males) OPPA (females) × 2	CR after cycle 2: no RT; PR after cycle 2: 20–30, IF	92%; 99.5% (5 years)
	239	IIB$_E$, IIIA$_E$, IIIB, IV	2 OEPA/OPPA+ 4COPP/COPADC	20–35 IF	87%; 97% (5 years)
French Society of Pediatric Oncology (SFOP)					
MDH-90[197]	202	IA, IB, IIA,IIB	VBVP × 4 (*OPPA × 1–2 if PR after cycle 4)	20–40, IF	91.1%, 97.5% (5 years)
Stanford, Dana-Farber and St Jude Consortium[198]					
	110	IA, IB, IIA, IIB no bulk, no E	VAMP × 4	15–22.5, IF	89.4%; 96.1% (10 years)
HOD99[199]	88	IA, IIA, <3 nodal sites, no bulk, no E	VAMP × 4	CR after 2 cycles: no RT; PR after cycle 2: 25.5 IF	CR: 89.4%, PR: 92.5% (2 years)
HLHR13[153]	77	IIB, IIIB, IV	2 AEPA + 4 CAPDac	CR at ERA: None; <CR: 25.5 Gy RN	97.4%; 98.7% (3 year)

ABVE-PC, Adriamycin, bleomycin, vincristine, etoposide, prednisone and cyclophosphamide ([a]indicates escalated dose cyclophosphamide); *AEPA*, brentuximab vedotin, etoposide, prednisone, doxorubicin; *AVPC*, Adriamycin, vinblastine, prednisone and cyclophosphamide; *BEACOPP*, bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisone; CCG, Children's Cancer Group; *COG*, Children's Oncology Group; *COPP/ABV*, cyclophosphamide, oncovin, procarbazine, prednisone/Adriamycin, bleomycin, vinblastine; *DBVE*, doxorubicin, bleomycin, vincristine, and etoposide; *DFS*, disease-free survival; *EF*, extended field; *EFS*, event-free survival; *GPOH-HD*, German Pediatric Oncology-Hematology Hodgkin Lymphoma Study Group; *IF*, involved field; *MDH*, maladie de Hodgkin; *OEPA*, oncovin, etoposide, prednisone, Adriamycin; *OPPA*, oncovin, procarbazine, prednisolone and Adriamycin: *VAMP*, vinblastine, doxorubicin, methotrexate, and prednisone; *POG*, Pediatric Oncology Group; *RFS*, relapse-free survival; *VBVP*, vinblastine, bleomycin, etoposide, prednisone.

improving efficacy and replacing agents with long-term toxicities. Brentuximab vedotin (Bv) is an antibody-drug conjugate composed of a monoclonal anti-CD30 antibody linked to the anti-mitotic agent monomethyl auristatin E (MMAE). In a phase II study evaluating Bv in relapsed HL, the ORR was 75% with complete remission in 34%.[154] Replacement of vincristine with Bv in the OEPA/COPDac regimen was investigated in a phase 2 trial of 77 pediatric patients with high-risk HL by the Stanford, St. Jude, Dana-Farber Cancer Institute Consortium.[153] Approximately 1/3 of patients avoided radiotherapy based on attaining complete metabolic and anatomic response after two cycles, and high EFS and OS rates were observed at 3 years. A randomized phase 3 trial by the COG investigating the replacement of vincristine with Bv in the ABVE-PC has completed accrual with results anticipated soon (NCT02166463).

Management of Relapsed Hodgkin Lymphoma

For patients who relapse, response depends on whether or not they had favorable disease at diagnosis as well as the extent of disease at the time of relapse. Time to relapse and response to re-induction therapy are strong predictors of outcome.[155] The EuroNet Pediatric Hodgkin Lymphoma group has issued evidence and consensus-based guidelines for the management of relapse/refractory HL.[156] Low-risk patients can generally be salvaged with chemotherapy and involved-site radiation therapy and even without hematopoietic stem cell transplant results are very acceptable.

For all other patients, treatment of refractory, progressive, or relapsed disease includes induction chemotherapy with multiple chemotherapeutic agents, not generally used in the initial therapy (e.g., gemcitabine, vinorelbine, carboplatin/cisplatin, ifosfamide, and more recently brentuximab vedotin, alone or in combination with gemcitabine or bendamustine), followed by high-dose chemotherapy and autologous stem cell rescue. Conditioning regimens are generally alkylator based,[155] and the frequently reported regimens are CBV (cyclophosphamide [CY], carmustine, and etoposide) and BEAM (carmustine, etoposide, cytosine arabinoside, and melphalan).[155] None of these conditioning regimens produce a superior outcome in pediatric patients. The role of local radiation therapy either before or after HSCT is still unclear, although TBI is now generally not used.[156] Overall outcomes following HSCT have been estimated using a prognostic model for PFS derived from retrospective analyses of 606 pediatric and young adult patients undergoing HSCT using data from the CIBMTR from 1995 to 2010.[157] By stratifying patients into low-, intermediate- and high-risk groups, 5-year PFS probabilities are predicted as 72% (95% CI: 64 to 80), 53% (95% CI: 47

to 59), and 23% (95% CI: 9 to 36), respectively. Post HSCT Bv maintenance therapy has led to an approximate 30% reduction in PFS events among adults with no pre-HSCT use of Bv and high-risk factors for subsequent relapse.[158] Two-thirds of patients experienced peripheral neuropathy, although 90% showed improvement (including 73% with resolution) by 5 years. Pediatric data on this strategy for pediatric are lacking.

The role of allogeneic HSCT has also been investigated for patients with relapsed/refractory HL, although never in a prospective, randomized manner.[159] The use of submyeloablative regimens (generally fludarabine based) may reduce transplant-related mortality rates while still achieving a graft-versus lymphoma (GVL) effect in lymphoma patients receiving allografts and warrants further investigation in the pediatric population.[160,161]

Among the more promising approaches to activating therapeutic antitumor immunity in HL is the blockade of the immune checkpoint, programmed cell death protein 1 (PD-1) pathway. Classical HL is characterized by HRS cells surrounded by an extensive but ineffective inflammatory cell infiltrate. Increased PD-1 expression by T-lymphocytes in the microenvironment and increased PD-1 ligand expression by HRS cells allow evasion of T-cell-mediated destruction of HRS cells. Blocking the interaction between PD-1 and its ligands through the administration of PD-1 blocking antibodies can result in T-cell activation and a more florid tissue inflammatory response.[162] Nivolumab is associated with a high objective response rate of 87% among heavily pretreated patients with HL.[163] Another anti–PD-1 drug, pembrolizumab, was associated with a 60% response rate in a similar cohort of pediatric HL patients.[164] Studies are now investigating these agents in conjunction with other targeted therapies, including brentuximab vedotin. Preliminary results of a pediatric study of Bv with nivolumab, followed by Bv with bendamustine for those with an inadequate response have been reported in abstract format.[165] Overall, the complete metabolic response (CMR) rate was 86%, including 64% achieving a CMR after Bv with nivolumab alone.

Early investigations of CAR-T cells have been associated with durable responses and low toxicity among heavily pretreated patients.[166] Autologous cytotoxic, *ex-vivo* expanded T cells targeting EBV or other tumor-associated antigens are being explored for pediatric HL patients associated with EBV either as adjuvant therapy after transplant or for relapsed disease.[132,167]

In summary, most children with HL are initially treated with risk-adapted chemotherapy alone or in combination with low-dose involved site or node radiotherapy involving carefully designed radiation fields to achieve local disease control while minimizing bystander organ toxicity. Especially for low-risk patients, some studies suggest that the OS for patients receiving chemotherapy alone may be similar to that for patients receiving chemotherapy plus radiotherapy, despite possible differences in EFS. This is due to the fact that it is usually possible to salvage relapse after initial therapy.[168] If salvage therapy for such patients can be targeted and relatively nontoxic (e.g., using novel agents such as brentuximab),[169] then using a less intense initial regimen may be appropriate. Currently, however, salvage therapy for the majority of patients is typically more toxic and can lead to an unacceptable incidence of late events such as cardiac toxicities and secondary malignancies.[147,170] Therefore, strategies using a less intense upfront regimen should only be investigated within the context of a clinical trial. Future clinical studies therefore attempt to address this important issue by evaluating the prognostic significance of achieving PET negative disease after 2 cycles of chemotherapy and the use of upfront Bv and the checkpoint inhibitors, nivolumab, and pembrolizumab to replace more toxic modalities such as bleomycin and radiation therapy.[171]

RARE SUBTYPES OF LYMPHOMA

Other subtypes, which are observed rarely in children, deserve mention, but the rarity of these neoplasms in children makes generalizations and therapeutic recommendations difficult. Follicular lymphomas (characterized by the arrangement of malignant cells in aggregates separated by normal cells) that account for approximately 30 percent of adult NHLs are extremely rare in children. Children with follicular lymphomas tend to present with cervical lymph node involvement, (although primary tumors of the testis have been reported)[170] with early-stage disease, and have an excellent prognosis.[172,173] Unlike most cases of follicular lymphoma in adults where aberrant expression of bcl-2 (usually as a result of the t(14;18) translocation) is thought to play an important role in lymphomagenesis, the majority of cases of follicular lymphomas in children demonstrate neither the t(14;18) nor bcl-2 expression. Bcl-2 expression appears to occur more frequently in older children and is associated with advanced-stage disease at presentation and a more aggressive clinical course.[170–172]

Marginal Zone B-cell lymphomas arising in mucosa-associated lymphoid tissue (MALT) can arise in extranodal sites. They tend to present with localized disease and infrequently disseminate. Natural Killer Cell Lymphoma and NK-like T-cell lymphomas usually involve the upper aerodigestive tract (midline lethal granuloma, angiocentric T-cell lymphoma) but can present in the skin as well.[174–176] They are rare in children and follow a very aggressive clinical course, are often fatal, but may respond to high-dose chemotherapy with stem cell transplantation.[176] Peripheral T-cell lymphomas include a variety of neoplasms that have not yet been further specified. These lymphomas are rare in children, but advanced stage at presentation, often in association with systemic symptoms and hemophagocytic syndrome, is common, and an aggressive course is the rule.

FUTURE DIRECTIONS

Although there have been dramatic improvements in the treatment of children with NHL and HL over the past 25 years, approximately 25% of children with these tumors still relapse or fail to respond to initial therapy. Additionally, late effects such as anthracycline-related cardiomyopathy, secondary malignancies such as epipodophyllotoxin-related acute myeloid leukemia, and endocrine abnormalities such as cyclophosphamide-related azoospermia remain a concern.[170,177] Thus, a major task is to develop treatment strategies that provide a cure for the remaining 25% while reducing treatment-related morbidity. Several approaches appear promising.

The identification of both clinical and biologic features at the time of diagnosis that predict treatment failure will enable investigators to refine existing risk-adapted therapeutic approaches. Strategies to be considered for children at elevated risk for treatment failure include the intensification of existing regimens and the incorporation of new active or novel agents. More intensive therapy may require either autologous or allogeneic hematopoietic stem cell support. The administration of colony-stimulating factors may be necessary in some cases, although their role in therapy remains controversial. Novel approaches include the incorporation of immunotherapeutic agents into multi-agent chemotherapy regimens. For example, there is increasing experience with the anti-CD20 antibody (Rituximab) in pediatric patients with B-cell lymphomas.[70] A radiolabeled form of this product (Yttrium-90 Zevalin) has also yielded promising preliminary results in children.[178] CD30+ lymphomas such as ALCL, mediastinal B-large cell, and HL are also candidates for CD30 antibody therapy. Novel T-cell therapy approaches including CAR-T-cell therapies targeting CD19, CD5, and CD30 are being evaluated in phase I clinical trials in the pediatric setting. Moreover, antigen-specific T cells, especially T cells targeting EBV antigens have shown early evidence of efficacy in the prevention and treatment of EBV-related PTLD, NHL and HL.[126,132] Checkpoint inhibitors have high response rates in patients with HL.[164] Agents targeting specific molecular lesions (e.g., the ALK inhibitor for ALCL) are promising in children, and the implementation of anti-idiotype and anti-sense strategies are also being studied.

The continued investigation of molecular abnormalities and pathogenic mechanisms of malignant transformation associated with childhood lymphomas is essential. Micro-chip gene arrays have already identified clinically relevant subtypes of large B-cell lymphomas in adults.[179] Gene array analyses of BL, ALCL, and T-lymphoblastic disease have also been reported.[180] Similar studies are ongoing for childhood lymphomas, and preliminary results for T-lymphoblastic leukemia/lymphoma have been published.[181] Flow-sorting tissue for HRS and intratumor T cells and optimizing low-input exome sequencing is beginning to overcome the limitations of genomic evaluation in HL.[182] Comprehensive molecular characterization of childhood lymphomas may help to further refine disease classification, provide a means of detecting minimal residual disease (mrd) during clinical remission and enhance our assessment of early response.[183] Monitoring is facilitated by the finding that the level of mrd in the peripheral blood is comparable to that in the bone marrow in children presenting with advanced-stage lymphomas. Additionally, a more complete understanding of the molecular pathogenesis of pediatric HL and NHLs, will provide clues to new and better treatments directed toward tumor-specific molecular lesions.

SUGGESTED READING

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Au-Yeung RKH, Arias Padilla L, Zimmermann M, et al. Experience with provisional WHO-entities large B-cell lymphoma with IRF4-rearrangement and Burkitt-like lymphoma with 11q aberration in paediatric patients of the NHL-BFM group. *Br J Haematol*. 2020;190(5):753–763.

Burke GAA, Minard-Colin V, Auperin A, et al. Dose-adjusted etoposide, doxorubicin, and cyclophosphamide with vincristine and prednisone plus rituximab therapy in children and adolescents with primary mediastinal B-cell lymphoma: a multicenter phase II trial. *J Clin Oncol*. 2021;39(33):3716–3724.

Camus V, Viennot M, Lequesne J, et al. Targeted genotyping of circulating tumor DNA for classical Hodgkin lymphoma monitoring: a prospective study. *Haematologica*. 2021;106(1):154–162.

Desch AK, Hartung K, Botzen A, et al. Genotyping circulating tumor DNA of pediatric Hodgkin lymphoma. *Leukemia*. 2020;34(1):151–166.

Dunleavy K, Pittaluga S, Maeda LS, et al. Dose-adjusted EPOCH-rituximab therapy in primary mediastinal B-cell lymphoma. *N Engl J Med*. 2013;368(15):1408–1416.

Gambacorti-Passerini C, Messa C, Pogliani EM. Crizotinib in anaplastic large-cell lymphoma. *N Engl J Med*. 2011;364(8):775–776.

Giulino-Roth L, O'Donohue T, Chen Z, et al. Outcomes of adults and children with primary mediastinal B-cell lymphoma treated with dose-adjusted EPOCH-R. *Br J Haematol*. 2017;179(5):739–747.

Hayashi RJ, Winter SS, Dunsmore KP, et al. Successful outcomes of newly diagnosed T lymphoblastic lymphoma: results from Children's Oncology Group AALL0434. *J Clin Oncol*. 2020 Sep 10;38(26):3062–3070.

Jaglowski SM, Linden E, Termuhlen AM, et al. Lymphoma in adolescents and young adults. *Semin Oncol*. 2009;36(5):381–418.

Love C, Sun Z, Jima D, et al. The genetic landscape of mutation. *Nature Genet*. 2012;44(12):1321–1325.

Lowe EJ, Reilly AF, Lim MS, et al. Brentuximab vedotin in combination with chemotherapy for pediatric patients with ALK+ ALCL: results of COG trial ANHL12P1. *Blood*. 2021;137(26):3595–3603.

Mariotto AB, Rowland JH, Yabroff KR, et al. Long-term survivors of childhood cancers in the United States. *Cancer Epidemiol Biomarkers Prev*. 2009;18(4):1033–1040.

Minard-Colin V, Auperin A, Pillon M, et al. Rituximab for high-risk, mature B-cell non-Hodgkin's lymphoma in children. *N Engl J Med*. 2020;382(23):2207–2219.

Mottok A, Steidl C. Biology of classical Hodgkin lymphoma: implications for prognosis and novel therapies. *Blood*. 2018;131(15):1654–1665.

Patte C, Auperin A, Michon J, et al. The Societe Francaise d'Oncologie Pediatrique LMB89 protocol: highly effective multiagent chemotherapy tailored to the tumor burden and initial response in 561 unselected children with B-cell lymphomas and L3 leukemia. *Blood*. 2001;97(11):3370–3379.

Reiter A, Schrappe M, Tiemann M, et al. Improved treatment results in childhood B-cell neoplasms with tailored intensification of therapy: a report of the Berlin-Frankfurt-Münster Group Trial NHL-BFM 90. *Blood*. 1999;94(10):3294–3306.

Rosolen A, Perkins SL, Pinkerton CR, et al. Revised international pediatric non-Hodgkin lymphoma staging system. *J Clin Oncol*. 2015;33(18):2112–2118.

Sandlund JT, Downing JR, Crist WM. Non-Hodgkin's lymphoma in childhood. *N Engl J Med*. 1996;334(19):1238–1248.

Schmitz R, Young RM, Ceribelli M, et al. Burkitt lymphoma pathogenesis and therapeutic targets from structural and functional genomics. *Nature*. 2012;490(7418):116–120.

Wagener R, Lopez C, Kleinheinz K, et al. IG-MYC (+) neoplasms with precursor B-cell phenotype are molecularly distinct from Burkitt lymphomas. *Blood*. 2018;132(21):2280–2285.

Woessmann W, Lisfeld J, Burkhardt B, et al. Therapy in primary mediastinal B-cell lymphoma. *N Engl J Med*. 2013;369(3):282.

REFERENCES

1. Mariotto AB, Rowland JH, Yabroff KR, et al. Long-term survivors of childhood cancers in the United States. *Cancer Epidemiol Biomarkers Prev*. 2009;18(4):1033–1040. https://doi.org/10.1158/1055-9965.EPI-08-0988.
2. Jaglowski SM, Linden E, Termuhlen AM, et al. Lymphoma in adolescents and young adults. *Semin Oncol*. 2009;36(5):381–418. https://doi.org/10.1053/j.seminoncol.2009.07.009.
3. Gurney JG, Severson RK, Davis S, et al. Incidence of cancer in children in the United States. Sex-, race-, and 1-year age-specific rates by histologic type. *Cancer*. 1995;75(8):2186–2195. https://doi.org/10.1002/1097-0142.(19950415)75:8<2186::aid-cncr2820750825>3.0.co;2-f.
4. Murphy SB, Fairclough DL, Hutchison RE, et al. Non-Hodgkin's lymphomas of childhood: an analysis of the histology, staging, and response to treatment of 338 cases at a single institution. *J Clin Oncol*. 1989;7(2):186–193. https://doi.org/10.1200/JCO.1989.7.2.186.
5. Filipovich AH, Heinitz KJ, Robison LL, et al. The Immunodeficiency Cancer Registry. A research resource. *Am J Pediatr Hematol Oncol*. 1987;9(2):183–184. https://doi.org/10.1097/00043426-198722000-00017.
6. Bienemann K, Burkhardt B, Modlich S, et al. Promising therapy results for lymphoid malignancies in children with chromosomal breakage syndromes (Ataxia teleangiectasia or Nijmegen-breakage syndrome): a retrospective survey. *Br J Haematol*. 2011;155(4):468–476. https://doi.org/10.1111/j.1365-2141.2011.08863.x.
7. Sandlund JT, Fonseca T, Leimig T, et al. Predominance and characteristics of Burkitt lymphoma among children with non-Hodgkin lymphoma in northeastern Brazil. *Leukemia*. 1997;11(5):743–746. https://doi.org/10.1038/sj.leu.2400609.
8. Magrath IT. African Burkitt's lymphoma. History, biology, clinical features, and treatment. *Am J Pediatr Hematol Oncol*. 1991;13(2):222–246.
9. Shad A, Magrath IT. *Malignant Non-Hodgkin's Lymphomas in Children*. 3rd ed. Philadelphia: Lippincott-Raven; 1997.
10. World Health Organization (WHO). *Classification of Tumours of Haematopoietic and Lymphoid Tissue*. Lyon: IARC; 2008.
11. Campo E, Swerdlow SH, Harris NL, et al. The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. *Blood*. 2011;117(19):5019–5032. https://doi.org/10.1182/blood-2011-01-293050.
12. Raetz EA, Perkins SL, Bhojwani D, et al. Gene expression profiling reveals intrinsic differences between T-cell acute lymphoblastic leukemia and T-cell lymphoblastic lymphoma. *Pediatric Blood & Cancer*. 2006;47(2):130–140. https://doi.org/10.1002/pbc.20550.
13. Burkhardt B. Paediatric lymphoblastic T-cell leukaemia and lymphoma: one or two diseases? *British Journal of Haematology*. 2010;149(5):653–668. https://doi.org/10.1111/j.1365-2141.2009.08006.x.
14. Burkhardt B, Zimmermann M, Oschlies I, et al. The impact of age and gender on biology, clinical features and treatment outcome of non-Hodgkin lymphoma in childhood and adolescence. *British Journal of Haematology*. 2005;131(1):39–49. https://doi.org/10.1111/j.1365-2141.2005.05735.x.
15. Reiter A, Schrappe M, Parwaresch R, et al. Non-Hodgkin's lymphomas of childhood and adolescence: results of a treatment stratified for biologic subtypes and stage—a report of the Berlin-Frankfurt-Münster Group. *J Clin Oncol*. 1995;13(2):359–372. https://doi.org/10.1200/JCO.1995.13.2.359.
16. Weiss LM, Bindl JM, Picozzi VJ, et al. Lymphoblastic lymphoma: an immunophenotype study of 26 cases with comparison to T cell acute lymphoblastic leukemia. *Blood*. 1986;67(2):474–478.
17. Percy C, O'Conor G, Ries LG, et al. Non-Hodgkin's lymphomas. Application of the International Classification of Diseases for Oncology (ICD-O) to the Working Formulation. *Cancer*. 1984;54(7):1435–1438. https://doi.org/10.1002/1097-0142. (19841001)54:7<1435::aid-cncr2820540734>3.0.co;2-o.
18. Patel JL, Smith LM, Anderson J, et al. The immunophenotype of T-lymphoblastic lymphoma in children and adolescents: a Children's Oncology Group report. *British Journal of Haematology*. 2012;159(4):454–461. https://doi.org/10.1111/bjh.12042.
19. Oschlies I, Burkhardt B, Chassagne-Clement C, et al. Diagnosis and immunophenotype of 188 pediatric lymphoblastic lymphomas treated within a randomized prospective trial: experiences and preliminary recommendations from the European childhood lymphoma pathology panel. *Am J Surg Pathol*. 2011;35(6):836–844. https://doi.org/10.1097/PAS.0b013e318213e90e.
20. Bernard A, Boumsell L, Reinherz EL, et al. Cell surface characterization of malignant T cells from lymphoblastic lymphoma using monoclonal antibodies: evidence for phenotypic differences between malignant T cells from patients with acute lymphoblastic leukemia and lymphoblastic lymphoma. *Blood*. 1981;57(6):1105–1110.
21. Ritz J, Nadler LM, Bhan AK, et al. Expression of common acute lymphoblastic leukemia antigen (CALLA) by lymphomas of B-cell and T-cell lineage. *Blood*. 1981;58(3):648–652.
22. Martin-Guerrero I, Salaverria I, Burkhardt B, et al. Non-leukemic pediatric mixed phenotype acute leukemia/lymphoma: genomic characterization and clinical outcome in a prospective trial for pediatric lymphoblastic lymphoma. *Genes Chromosomes Cancer*. 2019;58(6):365–372. https://doi.org/10.1002/gcc.22726. [published Online First: 2018/12/24].
23. Lones MA, Heerema NA, Le Beau MM, et al. Chromosome abnormalities in advanced stage lymphoblastic lymphoma of children and adolescents: a report from CCG-E08. *Cancer GenetCytogenet*. 2007;172(1):1–11.
24. De Smedt R, Peirs S, Morscio J, et al. Pre-clinical evaluation of second generation PIM inhibitors for the treatment of T-cell acute lymphoblastic leukemia and lymphoma. *Haematologica*. 2019;104(1):e17–e20. https://doi.org/10.3324/haematol.2018.199257. [published Online First: 2018/08/05].
25. Basso K, Mussolin L, Lettieri A, et al. T-cell lymphoblastic lymphoma shows differences and similarities with T-cell acute lymphoblastic leukemia by genomic and gene expression analyses. *Genes Chromosomes Cancer*. 2011;50(12):1063–1075. https://doi.org/10.1002/gcc.20924. [published Online First: 2011/10/12].
26. Park MJ, Taki T, Oda M, et al. FBXW7 and NOTCH1 mutations in childhood T cell acute lymphoblastic leukaemia and T cell non-Hodgkin lymphoma. *British Journal of Haematology*. 2009;145(2):198–206. https://doi.org/10.1111/j.1365-2141.2009.07607.x. [published Online First: 2009/02/28].
27. Baleydier F, Decouvelaere AV, Bergeron J, et al. T cell receptor genotyping and HOXA/TLX1 expression define three T lymphoblastic lymphoma subsets which might affect clinical outcome. *Clin Cancer Res*. 2008;14(3):692–700. https://doi.org/10.1158/1078-0432.CCR-07-1927. [published Online First: 2008/02/05].
28. Burkhardt B, Woessmann W, Zimmermann M, et al. Impact of cranial radiotherapy on central nervous system prophylaxis in children and adolescents with central nervous system-negative stage III or IV lymphoblastic lymphoma. *J Clin Oncol*. 2006;24(3):491–499. https://doi.org/10.1200/JCO.2005.02.2707.
29. Bernard A, Murphy SB, Melvin S, et al. Non-T, non-B lymphomas are rare in childhood and associated with cutaneous tumor. *Blood*. 1982;59(3):549–554.
30. Link MP, Roper M, Dorfman RF, et al. Cutaneous lymphoblastic lymphoma with pre-B markers. *Blood*. 1983;61(5):838–841.
31. Ducassou S, Ferlay C, Bergeron C, et al. Clinical presentation, evolution, and prognosis of precursor B-cell lymphoblastic lymphoma in trials LMT96, EORTC 58881, and EORTC 58951. *Br J Haematol*. 2011;152(4):441–451. https://doi.org/10.1111/j.1365-2141.2010.08541.x.
32. Muljono A, Graf NS, Arbuckle S. Primary cutaneous lymphoblastic lymphoma in children: series of eight cases with review of the literature. *Pathology*. 2009;41(3):223–228. https://doi.org/10.1080/00313020902756246.
33. Salzburg J, Burkhardt B, Zimmermann M, et al. Prevalence, clinical pattern, and outcome of CNS involvement in childhood and adolescent non-Hodgkin's lymphoma differ by non-Hodgkin's lymphoma subtype: a Berlin-Frankfurt-Münster Group Report. *J Clin Oncol*. 2007;25(25):3915–3922. https://doi.org/10.1200/JCO.2007.11.0700.
34. Rosolen A, Perkins SL, Pinkerton CR, et al. Revised international pediatric non-Hodgkin lymphoma staging system. *J Clin Oncol*. 2015;33(18):2112–2118. https://doi.org/10.1200/JCO.2014.59.7203. [published Online First: 2015/05/06].
35. Murphy SB. Classification, staging and end results of treatment of childhood non-Hodgkin's lymphomas: dissimilarities from lymphomas in adults. *Semin Oncol*. 1980;7(3):332–339.
36. Reiter A, Schrappe M, Ludwig WD, et al. Intensive ALL-type therapy without local radiotherapy provides a 90% event-free survival for children with T-cell lymphoblastic lymphoma: a BFM group report. *Blood*. 2000;95(2):416–421.
37. Coustan-Smith E, Sandlund JT, Perkins SL, et al. Minimal disseminated disease in childhood T-cell lymphoblastic lymphoma: a report from the children's oncology group. *J Clin Oncol*. 2009;27(21):3533–3539. https://doi.org/10.1200/JCO.2008.21.1318.
38. Mussolin L, Buldini B, Lovisa F, et al. Detection and role of minimal disseminated disease in children with lymphoblastic lymphoma: the AIEOP experience. *Pediatr Blood Cancer*. 2015;62(11):1906–1913. https://doi.org/10.1002/pbc.25607. [published Online First: 2015/06/26].
39. Stark B, Avigad S, Luria D, et al. Bone marrow minimal disseminated disease (MDD) and minimal residual disease (MRD) in childhood T-cell lymphoblastic lymphoma stage III, detected by flow cytometry (FC) and real-time quantitative polymerase chain reaction (RQ-PCR). *Pediat Blood Cancer*. 2009;52(1):20–25. https://doi.org/10.1002/pbc.21823. [published Online First: 2008/11/14].

40. Coustan-Smith E, Sancho J, Hancock ML, et al. Clinical importance of minimal residual disease in childhood acute lymphoblastic leukemia. *Blood*. 2000;96(8):2691–2696. [published Online First: 2000/10/07].
41. Burkhardt B, Oschlies I, Klapper W, et al. Non-Hodgkin's lymphoma in adolescents: experiences in 378 adolescent NHL patients treated according to pediatric NHL-BFM protocols. *Leukemia*. 2011;25(1):153–160. https://doi.org/10.1038/leu.2010.245.
42. Marks DI, Paietta EM, Moorman AV, et al. T-cell acute lymphoblastic leukemia in adults: clinical features, immunophenotype, cytogenetics, and outcome from the large randomized prospective trial (UKALL XII/ ECOG 2993). *Blood*. 2009;114(25):5136–5145. https://doi.org/10.1182/blood-2009-08-231217.
43. Hoelzer D, Gokbuget N, Digel W, et al. Outcome of adult patients with T-lymphoblastic lymphoma treated according to protocols for acute lymphoblastic leukemia. *Blood*. 2002;99(12):4379–4385. https://doi.org/10.1182/blood-2002-01-0110.
44. Reiter A, Schrappe M, Tiemann M, et al. Improved treatment results in childhood B-cell neoplasms with tailored intensification of therapy: a report of the Berlin-Frankfurt-Münster Group Trial NHL-BFM 90. *Blood*. 1999;94(10):3294–3306.
45. Asselin BL, Devidas M, Wang C, et al. Effectiveness of high-dose methotrexate in T-cell lymphoblastic leukemia and advanced-stage lymphoblastic lymphoma: a randomized study by the children's oncology group (POG 9404). *Blood*. 2011;118(4):874–883. https://doi.org/10.1182/blood-2010-06-292615.
46. Landmann E, Burkhardt B, Zimmermann M, et al. Results and conclusions of the European Intergroup EURO-LB02 trial in children and adolescents with lymphoblastic lymphoma. *Haematologica*. 2017;102(12):2086–2096. https://doi.org/10.3324/haematol.2015.139162. [published Online First: 2017/10/07].
47. Sandlund JT, Pui CH, Zhou Y, et al. Effective treatment of advanced-stage childhood lymphoblastic lymphoma without prophylactic cranial irradiation: results of St Jude NHL13 study. *Leukemia*. 2009;23(6):1127–1130. https://doi.org/10.1038/leu.2008.400. [pii]10.1038/leu.2008.400 [published Online First: 2009/02/06].
48. Hayashi RJ, Winter SS, Dunsmore KP, et al. Successful outcomes of newly diagnosed T lymphoblastic lymphoma: results from Children's Oncology Group AALL0434. *J Clin Oncol*. 2020;38(26):3062–3070. https://doi.org/10.1200/JCO.20.00531. [published Online First: 2020/06/20].
49. Burkhardt B, Taj M, Garnier N, et al. Treatment and outcome analysis of 639 relapsed non-Hodgkin lymphomas in children and adolescents and resulting treatment recommendations. *Cancers (Basel)*. 2021;13(9). https://doi.org/10.3390/cancers13092075. [published Online First: 2021/05/01].
50. Burkitt D. A children's cancer dependent on climatic factors. *Nature*. 1962;194:232–234. https://doi.org/10.1038/194232a0.
51. Au-Yeung RKH, Arias Padilla L, Zimmermann M, et al. Experience with provisional WHO-entities large B-cell lymphoma with IRF4-rearrangement and Burkitt-like lymphoma with 11q aberration in paediatric patients of the NHL-BFM group. *Br J Haematol*. 2020;190(5):753–763. https://doi.org/10.1111/bjh.16578. [published Online First: 2020/04/03].
52. Gutierrez MI, Bhatia K, Barriga F, et al. Molecular epidemiology of Burkitt's lymphoma from South America: differences in breakpoint location and Epstein-Barr virus association from tumors in other world regions. *Blood*. 1992;79(12):3261–3266.
53. Karajannis MA, Hummel M, Oschlies I, et al. Epstein-Barr virus infection in Western European pediatric non-Hodgkin lymphomas. *Blood*. 2003;102(12):4244.
54. Salaverria I, Martin-Guerrero I, Wagener R, et al. A recurrent 11q aberration pattern characterizes a subset of MYC-negative high-grade B-cell lymphomas resembling Burkitt lymphoma. *Blood*. 2014;123(8):1187–1198. https://doi.org/10.1182/blood-2013-06-507996.
55. Poirel HA, Cairo MS, Heerema NA, et al. Specific cytogenetic abnormalities are associated with a significantly inferior outcome in children and adolescents with mature B-cell non-Hodgkin's lymphoma: results of the FAB/LMB 96 international study. *Leukemia*. 2009;23(2):323–331. https://doi.org/10.1038/leu.2008.312.
56. Nelson M, Perkins SL, Dave BJ, et al. An increased frequency of 13q deletions detected by fluorescence in situ hybridization and its impact on survival in children and adolescents with Burkitt lymphoma: results from the Children's Oncology Group study CCG-5961. *Br J of Haematol*. 2010;148(4):600–610. https://doi.org/10.1111/j.1365-2141.2009.07967.x.
57. Schmitz R, Young RM, Ceribelli M, et al. Burkitt lymphoma pathogenesis and therapeutic targets from structural and functional genomics. *Nature*. 2012;490(7418):116–120. https://doi.org/10.1038/nature11378.
58. Richter J, Schlesner M, Hoffmann S, et al. Recurrent mutation of the ID3 gene in Burkitt lymphoma identified by integrated genome, exome and transcriptome sequencing. *Nature Genet*. 2012;44(12):1316–1320. https://doi.org/10.1038/ng.2469.
59. Love C, Sun Z, Jima D, et al. The genetic landscape of mutations in Burkitt lymphoma. *Nature Genet*. 2012;44(12):1321–1325. https://doi.org/10.1038/ng.2468.
60. Sandlund JT, Downing JR, Crist WM. Non-Hodgkin's lymphoma in childhood. *N Engl J Med*. 1996;334(19):1238–1248. https://doi.org/10.1056/NEJM199605093341906.
61. Mbulaiteye SM, Biggar RJ, Bhatia K, et al. Sporadic childhood Burkitt lymphoma incidence in the United States during 1992–2005. *Pediat Blood Cancer*. 2009;53(3):366–370. https://doi.org/10.1002/pbc.22047.
62. Herbrueggen H, Mueller S, Rohde J, et al. Treatment and outcome of IG-MYC(+) neoplasms with precursor B-cell phenotype in childhood and adolescence. *Leukemia*. 2020;34(3):942–946. https://doi.org/10.1038/s41375-019-0606-6.
63. Wagener R, Lopez C, Kleinheinz K, et al. IG-MYC (+) neoplasms with precursor B-cell phenotype are molecularly distinct from Burkitt lymphomas. *Blood*. 2018;132(21):2280–2285. https://doi.org/10.1182/blood-2018-03-842088.
64. Patte C, Auperin A, Michon J, et al. The Societe Francaise d'Oncologie Pediatrique LMB89 protocol: highly effective multiagent chemotherapy tailored to the tumor burden and initial response in 561 unselected children with B-cell lymphomas and L3 leukemia. *Blood*. 2001;97(11):3370–3379. https://doi.org/10.1182/blood.v97.11.3370.
65. Cairo MS, Gerrard M, Sposto R, et al. Results of a randomized international study of high-risk central nervous system B non-Hodgkin lymphoma and B acute lymphoblastic leukemia in children and adolescents. *Blood*. 2007;109(7):2736–2743. https://doi.org/10.1182/blood-2006-07-036665. [pii]10.1182/blood-2006-07-036665 [published Online First: 2006/12/02].
66. Woessmann W, Seidemann K, Mann G, et al. The impact of the methotrexate administration schedule and dose in the treatment of children and adolescents with B-cell neoplasms: a report of the BFM Group Study NHL-BFM95. *Blood*. 2005;105(3):948–958. https://doi.org/10.1182/blood-2004-03-0973.
67. Patte C, Auperin A, Gerrard M, et al. Results of the randomized international FAB/LMB96 trial for intermediate risk B-cell non-Hodgkin lymphoma in children and adolescents: it is possible to reduce treatment for the early responding patients. *Blood*. 2007;109(7):2773–2780. https://doi.org/10.1182/blood-2006-07-036673.
68. Fujita N, Kobayashi R, Takimoto T, et al. Results of the Japan association of childhood leukemia study (JACLS) NHL-98 protocol for the treatment of B-cell non-Hodgkin lymphoma and mature B-cell acute lymphoblastic leukemia in childhood. *Leukemia Lymphoma*. 2011;52(2):223–229. https://doi.org/10.3109/10428194.2010.537794.
69. Pillon M, Di Tullio MT, Garaventa A, et al. Long-term results of the first Italian association of pediatric hematology and oncology protocol for the treatment of pediatric B-cell non-Hodgkin lymphoma (AIEOP LNH92). *Cancer*. 2004;101(2):385–394.
70. Meinhardt A, Burkhardt B, Zimmermann M, et al. Phase II window study on rituximab in newly diagnosed pediatric mature B-cell non-Hodgkin's lymphoma and Burkitt leukemia. *J Clin Oncol*. 2010;28(19):3115–3121. https://doi.org/10.1200/JCO.2009.26.6791. [published Online First: 2010/06/03].
71. Goldman S, Smith L, Anderson JR, et al. Rituximab and FAB/LMB 96 chemotherapy in children with Stage III/IV B-cell non-Hodgkin lymphoma: a Children's Oncology Group report. *Leukemia*. 2013;27(5):1174–1177. https://doi.org/10.1038/leu.2012.255.
72. Goldman S, Smith L, Galardy P, et al. Rituximab with chemotherapy in children and adolescents with central nervous system and/or bone marrow-positive Burkitt lymphoma/leukaemia: a Children's Oncology Group Report. *Br J Haematol*. 2014;167(3):394-401. https://doi.org/10.1111/bjh.13040.
73. Reiter A, Klapper W. Recent advances in the understanding and management of diffuse large B-cell lymphoma in children. *Br J Haematol*. 2008;142(3):329–347. https://doi.org/10.1111/j.1365-2141.2008.06988.x.
74. Oschlies I, Klapper W, Zimmermann M, et al. Diffuse large B-cell lymphoma in pediatric patients belongs predominantly to the germinal-center type B-cell lymphomas: a clinicopathologic analysis of cases included in the German BFM (Berlin-Frankfurt-Münster) Multicenter Trial. *Blood*. 2006;107(10):4047–4052. https://doi.org/10.1182/blood-2005-10-4213.
75. Miles RR, Raphael M, McCarthy K, et al. Pediatric diffuse large B-cell lymphoma demonstrates a high proliferation index, frequent c-Myc protein expression, and a high incidence of germinal center subtype: report of the French-American-British (FAB) international study group. *Pediat Blood Cancer*. 2008;51(3):369–374. https://doi.org/10.1002/pbc.21619.
76. Lim MS, Beaty M, Sorbara L, et al. T-cell/histiocyte-rich large B-cell lymphoma: a heterogeneous entity with derivation from germinal center B cells. *Am J Surg Pathol*. 2002;26(11):1458–1466. https://doi.org/10.1097/00000478-200211000-00008.

77. Bea S, Zettl A, Wright G, et al. Diffuse large B-cell lymphoma subgroups have distinct genetic profiles that influence tumor biology and improve gene-expression-based survival prediction. *Blood*. 2005;106(9):3183–3190. https://doi.org/10.1182/blood-2005-04-1399.
78. Mestre C, Rubio-Moscardo F, Rosenwald A, et al. Homozygous deletion of SOCS1 in primary mediastinal B-cell lymphoma detected by CGH to BAC microarrays. *Leukemia*. 2005;19(6):1082–1084. https://doi.org/10.1038/sj.leu.2403741.
79. Seidemann K, Tiemann M, Lauterbach I, et al. Primary mediastinal large B-cell lymphoma with sclerosis in pediatric and adolescent patients: treatment and results from three therapeutic studies of the Berlin-Frankfurt-Munster Group. *J Clin Oncol*. 2003;21(9):1782–1789. https://doi.org/10.1200/JCO.2003.08.151.
80. Steinfeld R, Grapp M, Kraetzner R, et al. Folate receptor alpha defect causes cerebral folate transport deficiency: a treatable neurodegenerative disorder associated with disturbed myelin metabolism. *Am J Hum Genet*. 2009;85(3):354–363. https://doi.org/10.1016/j.ajhg.2009.08.005.
81. Burkhardt B, Bruch J, Zimmermann M, et al. Loss of heterozygosity on chromosome 6q14-q24 is associated with poor outcome in children and adolescents with T-cell lymphoblastic lymphoma. *Leukemia*. 2006;20(8):1422–1429. https://doi.org/10.1038/sj.leu.2404275. [published Online First: 2006/06/02].
82. Dunleavy K, Pittaluga S, Maeda LS, et al. Dose-adjusted EPOCH-rituximab therapy in primary mediastinal B-cell lymphoma. *N Engl J Med*. 2013;368(15):1408–1416. https://doi.org/10.1056/NEJMoa1214561.
83. Woessmann W, Lisfeld J, Burkhardt B, et al. Therapy in primary mediastinal B-cell lymphoma. *N Engl J Med*. 2013;369(3):282. https://doi.org/10.1056/NEJMc1305983#SA1.
84. Giulino-Roth L, O'Donohue T, Chen Z, et al. Outcomes of adults and children with primary mediastinal B-cell lymphoma treated with dose-adjusted EPOCH-R. *Br J Haematol*. 2017;179(5):739–747. https://doi.org/10.1111/bjh.14951. [published Online First: 2017/10/31].
85. Burke GAA, Minard-Colin V, Auperin A, et al. Dose-adjusted etoposide, doxorubicin, and cyclophosphamide with vincristine and prednisone plus rituximab therapy in children and adolescents with primary mediastinal B-cell lymphoma: a multicenter phase II trial. *J Clin Oncol*. 2021 https://doi.org/10.1200/JCO.21.00920. JCO2100920[published Online First: 2021/09/28].
86. Kadin ME. Ki-1/CD30+ (anaplastic) large-cell lymphoma: maturation of a clinicopathologic entity with prospects of effective therapy. *J Clin Oncol*. 1994;12(5):884–887. https://doi.org/10.1200/JCO.1994.12.5.884.
87. Nadali G, Vinante F, Stein H, et al. Serum levels of the soluble form of CD30 molecule as a tumor marker in CD30+ anaplastic large-cell lymphoma. *J Clin Oncol*. 1995;13(6):1355–1360. https://doi.org/10.1200/JCO.1995.13.6.1355.
88. Mason DY, Bastard C, Rimokh R, et al. CD30-positive large cell lymphomas ('Ki-1 lymphoma') are associated with a chromosomal translocation involving 5q35. *Br J Haematol*. 1990;74(2):161–168. https://doi.org/10.1111/j.1365-2141.1990.tb02560.x.
89. Morris SW, Xue L, Ma Z, et al. Alk+ CD30+ lymphomas: a distinct molecular genetic subtype of non-Hodgkin's lymphoma. *Br J Haematol*. 2001;113(2):275–295. https://doi.org/10.1046/j.1365-2141.2001.02574.x.
90. Rodriguez J, Pugh W, Romaguera J, et al. Anaplastic Ki-1 + large-cell lymphoma. *Cancer Invest*. 1993;11(5):554–558. https://doi.org/10.3109/07357909309011673.
91. Stein H, Foss HD, Durkop H, et al. CD30(+) anaplastic large cell lymphoma: a review of its histopathologic, genetic, and clinical features. *Blood*. 2000;96(12):3681–3695.
92. Murphy SB. Pediatric lymphomas: recent advances and commentary on Ki-1-positive anaplastic large-cell lymphomas of childhood. *Ann Oncol*. 1994;5(Suppl 1):31–33. https://doi.org/10.1093/annonc/5.suppl_1.s31.
93. Woo DK, Jones CR, Vanoli-Storz MN, et al. Prognostic factors in primary cutaneous anaplastic large cell lymphoma: characterization of clinical subset with worse outcome. *Arch Dermatol*. 2009;145(6):667–674. https://doi.org/10.1001/archdermatol.2009.74.
94. Le Deley MC, Reiter A, Williams D, et al. Prognostic factors in childhood anaplastic large cell lymphoma: results of a large European intergroup study. *Blood*. 2008;111(3):1560–1566. https://doi.org/10.1182/blood-2007-07-100958.
95. Lowe EJ, Sposto R, Perkins SL, et al. Intensive chemotherapy for systemic anaplastic large cell lymphoma in children and adolescents: final results of Children's Cancer Group Study 5941. *Pediatric Blood & Cancer*. 2009;52(3):335–339. https://doi.org/10.1002/pbc.21817.
96. Ait-Tahar K, Damm-Welk C, Burkhardt B, et al. Correlation of the autoantibody response to the ALK oncoantigen in pediatric anaplastic lymphoma kinase-positive anaplastic large cell lymphoma with tumor dissemination and relapse risk. *Blood*. 2010;115(16):3314–3319. https://doi.org/10.1182/blood-2009-11-251892.
97. Lamant L, McCarthy K, d'Amore E, et al. Prognostic impact of morphologic and phenotypic features of childhood ALK-positive anaplastic large-cell lymphoma: results of the ALCL99 study. *J Clin Oncol*. 2011;29(35):4669–4676. https://doi.org/10.1200/JCO.2011.36.5411.
98. Link MP, Shuster JJ, Donaldson SS, et al. Treatment of children and young adults with early-stage non-Hodgkin's lymphoma. *N Engl J Med*. 1997;337(18):1259–1266. https://doi.org/10.1056/NEJM199710303371802.
99. Seidemann K, Tiemann M, Schrappe M, et al. Short-pulse B-non-Hodgkin lymphoma-type chemotherapy is efficacious treatment for pediatric anaplastic large cell lymphoma: a report of the Berlin-Frankfurt-Munster Group Trial NHL-BFM 90. *Blood*. 2001;97(12):3699–3706. https://doi.org/10.1182/blood.v97.12.3699.
100. Laver JH, Kraveka JM, Hutchison RE, et al. Advanced-stage large-cell lymphoma in children and adolescents: results of a randomized trial incorporating intermediate-dose methotrexate and high-dose cytarabine in the maintenance phase of the APO regimen: a Pediatric Oncology Group phase III trial. *J Clin Oncol*. 2005;23(3):541–547. https://doi.org/10.1200/JCO.2005.11.075.
101. Brugieres L, Deley MC, Pacquement H, et al. CD30(+) anaplastic large-cell lymphoma in children: analysis of 82 patients enrolled in two consecutive studies of the French Society of Pediatric Oncology. *Blood*. 1998;92(10):3591–3598.
102. Brugieres L, Quartier P, Le Deley MC, et al. Relapses of childhood anaplastic large-cell lymphoma: treatment results in a series of 41 children—a report from the French Society of Pediatric Oncology. *Ann Oncol*. 2000;11(1):53–58. https://doi.org/10.1023/a:1008352726155.
103. Le Deley MC, Rosolen A, Williams DM, et al. Vinblastine in children and adolescents with high-risk anaplastic large-cell lymphoma: results of the randomized ALCL99-vinblastine trial. *J Clin Oncol*. 2010;28(25):3987–3993. https://doi.org/10.1200/JCO.2010.28.5999.
104. Alexander S, Kraveka JM, Weitzman S, et al. Advanced stage anaplastic large cell lymphoma in children and adolescents: results of ANHL0131, a randomized phase III trial of APO versus a modified regimen with vinblastine: a report from the children's oncology group. *Pediat Blood Cancer*. 2014;61(12):2236–2242. https://doi.org/10.1002/pbc.25187.
105. Wrobel G, Mauguen A, Rosolen A, et al. Safety assessment of intensive induction therapy in childhood anaplastic large cell lymphoma: report of the ALCL99 randomised trial. *Pediat Blood Cancer*. 2011;56(7):1071–1077. https://doi.org/10.1002/pbc.22940.
106. Gambacorti-Passerini C, Messa C, Pogliani EM. Crizotinib in anaplastic large-cell lymphoma. *N Engl J Med*. 2011;364(8):775–776. https://doi.org/10.1056/NEJMc1013224.
107. Pro B, Advani R, Brice P, et al. Brentuximab vedotin (SGN-35) in patients with relapsed or refractory systemic anaplastic large-cell lymphoma: results of a phase II study. *J Clin Oncol*. 2012;30(18):2190–2196. https://doi.org/10.1200/JCO.2011.38.0402.
108. Lowe EJ, Reilly AF, Lim MS, et al. Brentuximab vedotin in combination with chemotherapy for pediatric patients with ALK+ ALCL: results of COG trial ANHL12P1. *Blood*. 2021;137(26):3595–3603. https://doi.org/10.1182/blood.2020009806. [published Online First: 2021/03/09].
109. Gross TG, Hale GA, He W, et al. Hematopoietic stem cell transplantation for refractory or recurrent non-Hodgkin lymphoma in children and adolescents. *Biol Blood Marrow Transplant*. 2010;16(2):223–230. https://doi.org/10.1016/j.bbmt.2009.09.021.
110. Harker-Murray PD, Pommert L, Barth MJ. Novel therapies potentially available for pediatric B-cell non-Hodgkin lymphoma. *J Natl Compr Canc Netw*. 2020;18(8):1125–1134. https://doi.org/10.6004/jnccn.2020.7608. [published Online First: 2020/08/07].
111. Griffin TC, Weitzman S, Weinstein H, et al. A study of rituximab and ifosfamide, carboplatin, and etoposide chemotherapy in children with recurrent/refractory B-cell (CD20+) non-Hodgkin lymphoma and mature B-cell acute lymphoblastic leukemia: a report from the children's oncology group. *Pediat Blood Cancer*. 2009;52(2):177–181. https://doi.org/10.1002/pbc.21753.
112. Attarbaschi A, Dworzak M, Steiner M, et al. Outcome of children with primary resistant or relapsed non-Hodgkin lymphoma and mature B-cell leukemia after intensive first-line treatment: a population-based analysis of the Austrian cooperative study group. *Pediat Blood Cancer*. 2005;44(1):70–76. https://doi.org/10.1002/pbc.20121.
113. Mitsui T, Mori T, Fujita N, et al. Retrospective analysis of relapsed or primary refractory childhood lymphoblastic lymphoma in Japan. *Pediat Blood Cancer*. 2009;52(5):591–595. https://doi.org/10.1002/pbc.21941.
114. Burkhardt B, Reiter A, Landmann E, et al. Poor outcome for children and adolescents with progressive disease or relapse of lymphoblastic lymphoma: a report from the berlin-frankfurt-muenster group. *J Clin Oncol*. 2009;27(20):3363–3369. https://doi.org/10.1200/JCO.2008.19.3367.

115. Berg SL, Blaney SM, Devidas M, et al. Phase II study of nelarabine (compound 506U78) in children and young adults with refractory T-cell malignancies: a report from the Children's Oncology Group. *J Clin Oncol*. 2005;23(15):3376–3382. https://doi.org/10.1200/JCO.2005.03.426.
116. Mori T, Takimoto T, Katano N, et al. Recurrent childhood anaplastic large cell lymphoma: a retrospective analysis of registered cases in Japan. *Br J Haematol*. 2006;132(5):594–597. https://doi.org/10.1111/j.1365-2141.2005.05910.x.
117. Brugieres L, Pacquement H, Le Deley MC, et al. Single-drug vinblastine as salvage treatment for refractory or relapsed anaplastic large-cell lymphoma: a report from the French Society of Pediatric Oncology. *J Clin Oncol*. 2009;27(30):5056–5061. https://doi.org/10.1200/JCO.2008.20.1764.
118. Pro B, Advani R, Brice P, et al. Five-year results of brentuximab vedotin in patients with relapsed or refractory systemic anaplastic large cell lymphoma. *Blood*. 2017;130(25):2709–2717. https://doi.org/10.1182/blood-2017-05-780049. [published Online First: 2017/10/05].
119. Younes A, Bartlett NL, Leonard JP, et al. Brentuximab vedotin (SGN-35) for relapsed CD30-positive lymphomas. *N Engl J Med*. 2010;363(19):1812–1821. https://doi.org/10.1056/NEJMoa1002965.
120. Knorr F, Brugieres L, Pillon M, et al. Stem cell transplantation and vinblastine monotherapy for relapsed pediatric anaplastic large cell lymphoma: results of the international, prospective ALCL-Relapse Trial. *J Clin Oncol*. 2020;38(34):3999–4009. https://doi.org/10.1200/JCO.20.00157. [published Online First: 2020/07/31].
121. Woessmann W, Peters C, Lenhard M, et al. Allogeneic haematopoietic stem cell transplantation in relapsed or refractory anaplastic large cell lymphoma of children and adolescents—a Berlin-Frankfurt-Münster group report. *Br J Haematol*. 2006;133(2):176–182. https://doi.org/10.1111/j.1365-2141.2006.06004.x.
122. Bhatia S, Ramsay NK, Steinbuch M, et al. Malignant neoplasms following bone marrow transplantation. *Blood*. 1996;87(9):3633–3639.
123. Oertel SH, Riess H. Immunosurveillance, immunodeficiency and lymphoproliferations. *Recent Results Cancer Res*. 2002;159:1–8. https://doi.org/10.1007/978-3-642-56352-2-1.
124. Kuehnle I, Huls MH, Liu Z, et al. CD20 monoclonal antibody (rituximab) for therapy of Epstein-Barr virus lymphoma after hemopoietic stem-cell transplantation. *Blood*. 2000;95(4):1502–1505.
125. O'Reilly RJ, Lacerda JF, Lucas KG, et al. Adoptive cell therapy with donor lymphocytes for EBV-associated lymphomas developing after allogeneic marrow transplants. *Important Adv Oncol*. 1996:149–166.
126. Heslop HE, Slobod KS, Pule MA, et al. Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood*. 2010;115(5):925–935. https://doi.org/10.1182/blood-2009-08-239186.
127. Doubrovina E, Oflaz-Sozmen B, Prockop SE, et al. Adoptive immunotherapy with unselected or EBV-specific T cells for biopsy-proven EBV+ lymphomas after allogeneic hematopoietic cell transplantation. *Blood*. 2012;119(11):2644–2656. https://doi.org/10.1182/blood-2011-08-371971.
128. Swinnen LJ. Diagnosis and treatment of transplant-related lymphoma. *Ann Oncol*. 2000;11(Suppl 1):45–48.
129. Pakakasama S, Eames GM, Morriss MC, et al. Treatment of Epstein-Barr virus lymphoproliferative disease after hematopoietic stem-cell transplantation with hydroxyurea and cytotoxic T-cell lymphocytes. *Transplantation*. 2004;78(5):755–757. https://doi.org/10.1097/01.tp.0000129813.54517.25.
130. Gross TG, Bucuvalas JC, Park JR, et al. Low-dose chemotherapy for Epstein-Barr virus-positive post-transplantation lymphoproliferative disease in children after solid organ transplantation. *J Clin Oncol*. 2005;23(27):6481–6488. https://doi.org/10.1200/JCO.2005.08.074.
131. Gross TG, Orjuela MA, Perkins SL, et al. Low-dose chemotherapy and rituximab for posttransplant lymphoproliferative disease (PTLD): a Children's Oncology Group Report. *Am J Transplant*. 2012;12(11):3069–3075. https://doi.org/10.1111/j.1600-6143.2012.04206.x.
132. Bollard CM, Gottschalk S, Torrano V, et al. Sustained complete responses in patients with lymphoma receiving autologous cytotoxic T lymphocytes targeting Epstein-Barr virus latent membrane proteins. *J Clin Oncol*. 2014;32(8):798–808. https://doi.org/10.1200/JCO.2013.51.5304.
133. Montella M, Maso LD, Crispo A, et al. Do childhood diseases affect NHL and HL risk? A case-control study from northern and southern Italy. *Leuk Res*. 2006;30(8):917–922. https://doi.org/10.1016/j.leukres.2005.11.020.
134. Goldin LR, Bjorkholm M, Kristinsson SY, et al. Highly increased familial risks for specific lymphoma subtypes. *Br J Haematol*. 2009;146(1):91–94. https://doi.org/10.1111/j.1365-2141.2009.07721.x. [published Online First: 2009/05/15].
135. Sud A, Thomsen H, Orlando G, et al. Genome-wide association study implicates immune dysfunction in the development of Hodgkin lymphoma. *Blood*. 2018;132(19):2040–2052. https://doi.org/10.1182/blood-2018-06-855296. [published Online First: 2018/09/09].
136. Grufferman S, Delzell E. Epidemiology of Hodgkin's disease. *Epidemiol Rev*. 1984;6:76–106. https://doi.org/10.1093/oxfordjournals.epirev.a036276. [published Online First: 1984/01/01].
137. Kennedy-Nasser AA, Hanley P, Bollard CM. Hodgkin disease and the role of the immune system. *Pediatr Hematol Oncol*. 2011;28(3):176–186. https://doi.org/10.3109/08880018.2011.557261.
138. Biggar RJ, Jaffe ES, Goedert JJ, et al. Hodgkin lymphoma and immunodeficiency in persons with HIV/AIDS. *Blood*. 2006;108(12):3786–3791. https://doi.org/10.1182/blood-2006-05-024109.
139. Schnitzer B. Hodgkin lymphoma. *Hematol Oncol Clin North Am*. 2009;23(4):747–768. https://doi.org/10.1016/j.hoc.2009.04.013.
140. Pileri SA, Ascani S, Leoncini L, et al. Hodgkin's lymphoma: the pathologist's viewpoint. *J Clin Pathol*. 2002;55(3):162–176. https://doi.org/10.1136/jcp.55.3.162.
141. Mottok A, Steidl C. Biology of classical Hodgkin lymphoma: implications for prognosis and novel therapies. *Blood*. 2018;131(15):1654–1665. https://doi.org/10.1182/blood-2017-09-772632. [published Online First: 2018/03/04].
142. Camus V, Viennot M, Lequesne J, et al. Targeted genotyping of circulating tumor DNA for classical Hodgkin lymphoma monitoring: a prospective study. *Haematologica*. 2021;106(1):154–162. https://doi.org/10.3324/haematol.2019.237719. [published Online First: 2020/02/23].
143. Desch AK, Hartung K, Botzen A, et al. Genotyping circulating tumor DNA of pediatric Hodgkin lymphoma. *Leukemia*. 2020;34(1):151–166. https://doi.org/10.1038/s41375-019-0541-6. [published Online First: 2019/08/23].
144. Lister TA, Crowther D, Sutcliffe SB, et al. Report of a committee convened to discuss the evaluation and staging of patients with Hodgkin's disease: Cotswolds meeting. *J Clin Oncol*. 1989;7(11):1630–1636. https://doi.org/10.1200/JCO.1989.7.11.1630.
145. The Lancet Haematology. New guidelines for paediatric Hodgkin lymphoma. *Lancet Haematol*. 2020;7(12):e851 https://doi.org/10.1016/S2352-3026(20)30371-9. [published Online First: 2020/11/27].
146. Dunleavy K, Bollard CM. Sobering realities of surviving Hodgkin lymphoma. *Blood*. 2011;117(6):1772–1773. https://doi.org/10.1182/blood-2010-11-317131.
147. Oeffinger KC, Stratton KL, Hudson MM, et al. Impact of risk-adapted therapy for pediatric Hodgkin lymphoma on risk of long-term morbidity: a report from the childhood cancer survivor study. *J Clin Oncol*. 2021;39(20):2266–2275. https://doi.org/10.1200/JCO.20.01186. [published Online First: 2021/02/26].
148. Appel BE, Chen L, Buxton AB, et al. Minimal treatment of low-risk, pediatric lymphocyte-predominant Hodgkin lymphoma: a report from the children's oncology group. *J Clin Oncol*. 2016;34(20):2372–2379. https://doi.org/10.1200/JCO.2015.65.3469. [published Online First: 2016/05/18].
149. Mauz-Korholz C, Gorde-Grosjean S, Hasenclever D, et al. Resection alone in 58 children with limited stage, lymphocyte-predominant Hodgkin lymphoma-experience from the European network group on pediatric Hodgkin lymphoma. *Cancer*. 2007;110(1):179–185. https://doi.org/10.1002/cncr.22762.
150. Mauz-Körholz C, Hasenclever D, Dörffel W, et al. Procarbazine-free OEPA-COPDAC chemotherapy in boys and standard OPPA-COPP in girls have comparable effectiveness in pediatric Hodgkin's lymphoma: the GPOH-HD-2002 study. *J Clin Oncol*. 2010;28:3680–3686.
151. Kelly KM, Cole PD, Pei Q, et al. Response-adapted therapy for the treatment of children with newly diagnosed high risk Hodgkin lymphoma (AHOD0831): a report from the children's oncology group. *Br J Haematol*. 2019;187(1):39–48. https://doi.org/10.1111/bjh.16014. [published Online First: 2019/06/11].
152. Keller FG, Castellino SM, Chen L, et al. Results of the AHOD0431 trial of response adapted therapy and a salvage strategy for limited stage, classical Hodgkin lymphoma: a report from the children's oncology group. *Cancer*. 2018;124(15):3210–3219. https://doi.org/10.1002/cncr.31519. [published Online First: 2018/05/09].
153. Metzger ML, Link MP, Billett AL, et al. Excellent outcome for pediatric patients with high-risk Hodgkin lymphoma treated with brentuximab vedotin and risk-adapted residual node radiation. *J Clin Oncol*. 2021;39(20):2276–2283. https://doi.org/10.1200/JCO.20.03286. [published Online First: 2021/04/08].

154. Younes A, Gopal AK, Smith SE, et al. Results of a pivotal phase II study of brentuximab vedotin for patients with relapsed or refractory Hodgkin's lymphoma. *J Clin Oncol*. 2012;30(18):2183–2189. https://doi.org/10.1200/JCO.2011.38.0410.
155. Harker-Murray PD, Drachtman RA, Hodgson DC, et al. Stratification of treatment intensity in relapsed pediatric Hodgkin lymphoma. *Pediat Blood Cancer*. 2014;61(4):579–586. https://doi.org/10.1002/pbc.24851. [published Online First: 2014/02/08].
156. Daw S, Hasenclever D, Mascarin M, et al. Risk and response adapted treatment guidelines for managing first relapsed and refractory classical Hodgkin lymphoma in children and young people. recommendations from the EuroNet pediatric Hodgkin lymphoma group. *Hemasphere*. 2020;4(1):e329 https://doi.org/10.1097/HS9.0000000000000329. [published Online First: 2020/02/20].
157. Satwani P, Ahn KW, Carreras J, et al. A prognostic model predicting autologous transplantation outcomes in children, adolescents and young adults with Hodgkin lymphoma. *Bone Marrow Transplant*. 2015;50(11):1416–1423. https://doi.org/10.1038/bmt.2015.177.
158. Moskowitz CH, Walewski J, Nademanee A, et al. Five-year PFS from the AETHERA trial of brentuximab vedotin for Hodgkin lymphoma at high risk of progression or relapse. *Blood*. 2018;132(25):2639–2642. https://doi.org/10.1182/blood-2018-07-861641. [published Online First: 2018/09/30].
159. Peniket AJ, Ruiz de Elvira MC, Taghipour G, et al. An EBMT registry matched study of allogeneic stem cell transplants for lymphoma: allogeneic transplantation is associated with a lower relapse rate but a higher procedure-related mortality rate than autologous transplantation. *Bone Marrow Transplant*. 2003;31(8):667–678. https://doi.org/10.1038/sj.bmt.1703891.
160. Gutman JA, Bearman SI, Nieto Y, et al. Autologous transplantation followed closely by reduced-intensity allogeneic transplantation as consolidative immunotherapy in advanced lymphoma patients: a feasibility study. *Bone Marrow Transplant*. 2005;36(5):443–451. https://doi.org/10.1038/sj.bmt.1705081.
161. Claviez A, Klingebiel T, Beyer J, et al. Allogeneic peripheral blood stem cell transplantation following fludarabine-based conditioning in six children with advanced Hodgkin's disease. *Ann Hematol*. 2004;83(4):237–241. https://doi.org/10.1007/s00277-003-0814-y.
162. Pardoll DM. Immunology beats cancer: a blueprint for successful translation. *Nat Immunol*. 2012;13(12):1129–1132. https://doi.org/10.1038/ni.2392.
163. Ansell SM, Lesokhin AM, Borrello I, et al. PD-1 Blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. *N Engl J Med*. 2014;372(4):311–319. https://doi.org/10.1056/NEJMoa1411087.
164. Geoerger B, Kang HJ, Yalon-Oren M, et al. Pembrolizumab in paediatric patients with advanced melanoma or a PD-L1-positive, advanced, relapsed, or refractory solid tumour or lymphoma (KEYNOTE-051): interim analysis of an open-label, single-arm, phase 1-2 trial. *Lancet Oncol*. 2020;21(1):121–133. https://doi.org/10.1016/S1470-2045(19)30671-0. [published Online First: 2019/12/10].
165. Harker-Murray P, Leblanc T, Mascarin M, et al. Response-adapted therapy with nivolumab and brentuximab vedotin (BV), followed by BV and bendamustine for suboptimal response, in children, adolescents, and young adults with standard-risk relapsed/refractory classical Hodgkin lymphoma. *Blood*. 2018;132(Supplement 1):927–928. https://doi.org/10.1182/blood-2018-99-111279.
166. Ramos CA, Grover NS, Beaven AW, et al. Anti-CD30 CAR-T cell therapy in relapsed and refractory Hodgkin lymphoma. *J Clin Oncol*. 2020;38(32):3794–3804. https://doi.org/10.1200/JCO.20.01342. [published Online First: 2020/07/24].
167. Bollard CM, Gottschalk S, Leen AM, et al. Complete responses of relapsed lymphoma following genetic modification of tumor-antigen presenting cells and T-lymphocyte transfer. *Blood*. 2007;110(8):2838–2845. https://doi.org/10.1182/blood-2007-05-091280.
168. Ruhl U, Albrecht M, Dieckmann K, et al. Response-adapted radiotherapy in the treatment of pediatric Hodgkin's disease: an interim report at 5 years of the German GPOH-HD 95 trial. *Int J Radiat Oncol Biol Phys*. 2001;51(5):1209–1218. https://doi.org/10.1016/s0360-3016(01)01798-9.
169. Younes A. CD30-targeted antibody therapy. *Curr Opin Oncol*. 2011;23(6):587–593. https://doi.org/10.1097/CCO.0b013e32834bb8a7.
170. Castellino SM, Geiger AM, Mertens AC, et al. Morbidity and mortality in long-term survivors of Hodgkin lymphoma: a report from the childhood cancer survivor study. *Blood*. 2011;117(6):1806–1816. https://doi.org/10.1182/blood-2010-04-278796.
171. Kelly KM, Hodgson D, Appel B, et al. Children's Oncology Group's 2013 blueprint for research: Hodgkin lymphoma. *Pediat Blood Cancer*. 2013;60(6):972–978. https://doi.org/10.1002/pbc.24423. [published Online First: 2012/12/21].
172. Lorsbach RB, Shay-Seymore D, Moore J, et al. Clinicopathologic analysis of follicular lymphoma occurring in children. *Blood*. 2002;99(6):1959–1964.
173. Attarbaschi A, Beishuizen A, Mann G, et al. Children and adolescents with follicular lymphoma have an excellent prognosis with either limited chemotherapy or with a "watch and wait" strategy after complete resection. *Ann Hematol*. 2013;92(11):1537–1541. https://doi.org/10.1007/s00277-013-1785-2.
174. Mraz-Gernhard S, Natkunam Y, Hoppe RT, et al. Natural killer/natural killer-like T-cell lymphoma, CD56+, presenting in the skin: an increasingly recognized entity with an aggressive course. *J Clin Oncol*. 2001;19(8):2179–2188.
175. Natkunam Y, Smoller BR, Zehnder JL, et al. Aggressive cutaneous NK and NK-like T-cell lymphomas: clinicopathologic, immunohistochemical, and molecular analyses of 12 cases. *Am J Surg Pathol*. 1999;23(5):571–581.
176. Shaw PH, Cohn SL, Morgan ER, et al. Natural killer cell lymphoma: report of two pediatric cases, therapeutic options, and review of the literature. *Cancer*. 2001;91(4):642–646.
177. Haddy TB, Adde MA, McCalla J, et al. Late effects in long-term survivors of high-grade non-Hodgkin's lymphomas. *J Clin Oncol*. 1998;16(6):2070–2079. https://doi.org/10.1200/JCO.1998.16.6.2070.
178. Cooney-Qualter E, Krailo M, Angiolillo A, et al. A phase I study of 90yttrium-ibritumomab-tiuxetan in children and adolescents with relapsed/refractory CD20-positive non-Hodgkin's lymphoma: a children's oncology group study. *Clin Cancer Res*. 2007;13(18 Pt 2). https://doi.org/10.1158/1078-0432.CCR-07-1060. 5652s-60s.
179. Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature*. 2000;403(6769):503–511. https://doi.org/10.1038/35000501.
180. Dave BJ, Nelson M, Sanger WG. Lymphoma cytogenetics. *Clin Lab Med*. 2011;31(4):725–761. https://doi.org/10.1016/j.cll.2011.08.001. x-xi.
181. Yu L, Slovak ML, Mannoor K, et al. Microarray detection of multiple recurring submicroscopic chromosomal aberrations in pediatric T-cell acute lymphoblastic leukemia. *Leukemia*. 2011;25(6):1042–1046. https://doi.org/10.1038/leu.2011.33.
182. Reichel J, Chadburn A, Rubinstein PG, et al. Flow sorting and exome sequencing reveal the oncogenome of primary Hodgkin and Reed-Sternberg cells. *Blood*. 2015;125(7):1061–1072. https://doi.org/10.1182/blood-2014-11-610436.
183. Shiramizu B, Goldman S, Kusao I, et al. Minimal disease assessment in the treatment of children and adolescents with intermediate-risk (Stage III/IV) B-cell non-Hodgkin lymphoma: a children's oncology group report. *Br J Haematology*. 2011;153(6):758–763. https://doi.org/10.1111/j.1365-2141.2011.08681.x.
184. Patte C, Kalifa C, Flamant F, et al. Results of the LMT81 protocol, a modified LSA2L2 protocol with high dose methotrexate, on 84 children with non-B-cell (lymphoblastic) lymphoma. *Med Pediatr Oncol*. 1992;20(2):105–113.
185. Tubergen DG, Krailo MD, Meadows AT, et al. Comparison of treatment regimens for pediatric lymphoblastic non-Hodgkin's lymphoma: a children's cancer group study. *J Clin Oncol*. 1995;13(6):1368–1376.
186. Amylon MD, Shuster J, Pullen J, et al. Intensive high-dose asparaginase consolidation improves survival for pediatric patients with T cell acute lymphoblastic leukemia and advanced stage lymphoblastic lymphoma: a pediatric oncology group study. *Leukemia*. 1999;13(3):335–342.
187. Uyttebroeck A, Suciu S, Laureys G, et al. Treatment of childhood T-cell lymphoblastic lymphoma according to the strategy for acute lymphoblastic leukaemia, without radiotherapy: long term results of the EORTC CLG 58881 trial. *Eur J Cancer*. 2008;44(6):840–846. https://doi.org/10.1016/j.ejca.2008.02.011.
188. Abromowitch M, Termuhlen A, Chang M, et al. High-dose methotrexate and early intensification of therapy do not improve 3 year EFS in children and adolescents with disseminated lymphoblastic lymphoma. Results of the Randomized Arms of COG A5971. *Blood*. 2008;112(11):3610. https://doi.org/10.1182/blood.V112.11.3610.3610.
189. Pillon M, Piglione M, Garaventa A, et al. Long-term results of AIEOP LNH-92 protocol for the treatment of pediatric lymphoblastic lymphoma: a report of the Italian association of pediatric hematology and oncology. *Pediat Blood Cancer*. 2009;53(6):953–959. https://doi.org/10.1002/pbc.22162.
190. Termuhlen AM, Smith LM, Perkins SL, et al. Outcome of newly diagnosed children and adolescents with localized lymphoblastic lymphoma treated on children's oncology group trial A5971: a report from the children's oncology group. *Pediat Blood Cancer*. 2012;59(7):1229–1233. https://doi.org/10.1002/pbc.24149.
191. Termuhlen AM, Smith LM, Perkins SL, et al. Disseminated lymphoblastic lymphoma in children and adolescents: results of the COG A5971 trial: a report from the Children's Oncology Group. *Br J Haematol*. 2013;162(6):792–801. https://doi.org/10.1111/bjh.12460.

192. Gerrard M, Cairo MS, Weston C, et al. Excellent survival following two courses of COPAD chemotherapy in children and adolescents with resected localized B-cell non-Hodgkin's lymphoma: results of the FAB/LMB 96 international study. *Br J Haematol*. 2008;141(6):840–847. https://doi.org/10.1111/j.1365-2141.2008.07144.x.
193. Minard-Colin V, Auperin A, Pillon M, et al. Rituximab for high-risk, mature B-cell non-Hodgkin's lymphoma in children. *N Engl J Med*. 2020;382(23):2207–2219. https://doi.org/10.1056/NEJMoa1915315. [published Online First: 2020/06/04].
194. Wolden SL, Chen L, Kelly KM, et al. Long-term results of CCG 5942: a randomized comparison of chemotherapy with and without radiotherapy for children with Hodgkin's lymphoma—a report from the children's oncology group. *J Clin Oncol*. 2012;30(26):3174–3180. https://doi.org/10.1200/JCO.2011.41.1819.
195. Tebbi CK, Mendenhall NP, London WB, et al. Response-dependent and reduced treatment in lower risk Hodgkin lymphoma in children and adolescents, results of P9426: a report from the Children's Oncology Group, 2012. *Pediatr Blood Cancer*. 2012;59(7):1259–1265.
196. Keller FG, Nachman J, Constine L, et al. A phase III study for the treatment of children and adolescents with newly diagnosed low risk Hodgkin lymphoma (HL). *Blood*. 2010;116(21):767.
197. Landman-Parker J, Pacquement H, Leblanc T, et al. Localized childhood Hodgkin's disease: response-adapted chemotherapy with etoposide, bleomycin, vinblastine, and prednisone before low-dose radiation therapy-results of the French society of pediatric oncology study MDH90. *J Clin Oncol*. 2000;18(7):1500–1507. https://doi.org/10.1200/JCO.2000.18.7.1500.
198. Donaldson SS, Link MP, Weinstein HJ, et al. Final results of a prospective clinical trial with VAMP and low-dose involved-field radiation for children with low-risk Hodgkin's disease. *J Clin Oncol*. 2007;25(3):332–337. https://doi.org/10.1200/JCO.2006.08.4772. [published Online First: 2007/01/20].
199. Metzger ML, Weinstein HJ, Hudson MM, et al. Association between radiotherapy vs no radiotherapy based on early response to VAMP chemotherapy and survival among children with favorable-risk Hodgkin lymphoma. *JAMA*. 2012;307(24):2609–2616. https://doi.org/10.1001/jama.2012.5847.

CHAPTER 89

T-CELL LYMPHOMAS

Alessandro Broccoli and Pier Luigi Zinzani

T-cell lymphomas are a highly heterogeneous group of diseases sharing common origin from mature T-lymphocytes. Heterogeneity is appreciated on biologic and clinical grounds, both in terms of presentation (leukemic, nodal, extranodal, and cutaneous forms) and clinical behavior (aggressive *versus* indolent). Historically, peripheral T-cell lymphomas (PTCL) have always been separated by cutaneous T-cell lymphomas (CTCL), mostly because the former has a nodal and aggressive presentation, while the latter display relatively indolent (although progressive in most of the times) course.

Treatment approaches are mostly inadequate for the treatment of highly aggressive disease subtypes. New molecules hitting specific biologic targets have emerged as opportunities in patients with relapsed disease, yet with very short response durations. On the other hand, combinations of newer agents with conventional chemotherapy in untreated patients or strategies within chemo-free combination is an active field of research.

All the most frequent and clinically relevant leukemic T-cell lymphomas, PTCL, and CTCL will be treated in detail in the following sections of this chapter, with a focus on their epidemiology, morphology, biopathology, clinical manifestations, and prognosis. Current treatment approaches and future directions in the treatment of these diseases are discussed separately for leukemic T-cell lymphomas, PTCL, and CTCL at the end of the corresponding sections of the chapter.

ADVANCES IN MATURE T-CELL LYMPHOMA CLASSIFICATION

The significant advances occurred in the classification of both nodal and extranodal T-cell and natural killer-cell neoplasms have led to revisions: many of these changes are the result of genomic studies using approaches to examine the gene expression profile and the genetic landscape of these neoplasms. This box highlights the innovations with greater impact on clinical and therapeutic grounds. More details are found in the text.

- Peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS). Subsets are recognized based on phenotype and molecular abnormalities as that may have clinical implications. Stratification however is mostly not a part of routine practice at present.
- Nodal T-cell lymphomas with T_{FH}phenotype. This is an umbrella category that encompasses the spectrum of nodal lymphomas with a T_{FH} phenotype. It includes AITL, follicular T-cell lymphoma, and other nodal PTCL with a T_{FH} phenotype and it serves to highlight the specific clinical and pathologic differences among these subtypes. Overlapping recurrent molecular and cytogenetic abnormalities are shared by these entities.
- Anaplastic large-cell lymphoma (ALCL), ALK^-. It is now a regarded as a definite entity that includes cytogenetic subsets with possible prognostic implications (e.g., rearrangements at *IRF4/DUSP22* locus).
- Breast implant–associated anaplastic large cell lymphoma (BIA-ALCL). New provisional entity distinguished from other ALK-ALCL; noninvasive disease associated with excellent outcome.
- Enteropathy-associated T-cell lymphoma (EATL). This denomination is only to be used for cases formerly known as type I EATL. It is typically associated with celiac disease.
- Monomorphic epitheliotropic intestinal T-cell lymphoma (MEITL). Formerly type II EATL, it is segregated from type I EATL and it is given a new definition due to its distinctive nature and lack of association with celiac disease.
- Lymphomatoid papulosis. New subtypes are described with similar clinical behavior but atypical histologic/immunophenotypic features.

CLASSIFICATION AND CELL OF ORIGIN

In 2017, the classification of hematologic and lymphoid neoplasm was updated, and the World Health Organization (WHO) now recognizes 29 established and provisional entities of mature T-cell non-Hodgkin lymphomas (Table 89.1). Provisional entities are lymphoma categories for which scientific data are not fully validated by large studies, thus forthcoming additional biologic elements are required to confirm—or not—these entities as established varieties.

This complex classification underscores the complexity and the great heterogeneity of these neoplasms, as it emerges from the advances in the molecular partitioning of these diseases. On the other hand, a more appropriate classification—based on morphological, immunophenotypical, clinical, and molecular grounds—is aimed at better stratifying patients in terms of prognosis and outcomes, at guiding physicians' decisions before and throughout treatment, and at providing new insights into potentially more effective therapies or drug combinations (see box on Advances in Mature T-Cell Lymphoma Classification).[1,2]

T-cell lymphomas are mature T-cell neoplasms, given that the postulated cell of origin is always a mature, post-thymic T-lymphocyte; this means that all mature T-cell lymphomas originate from a T cell born in the bone marrow that has already undergone T-cell receptor (TCR) gene rearrangement and has completed its pre-antigenic maturation (Fig. 89.1).[2] Mature T cells consist of α/β TCR (more than 90%) and γ/δ TCR lymphocytes: the former derive from precursor T cells that colonize the thymus, whereas the latter may originate from thymic or extrathymic tissues. The final maturation step of T-lymphocytes into either $CD4^+$ or $CD8^+$ T cells is determined by the kinetics and strength of TCR signals and the specific *milieu* of cytokines that dictates the selective expression of transcription factors.

Under the heading *mature T-cell lymphomas*, four broad groups of diseases can be distinguished according to their presentation (Table 89.2): leukemic, nodal, extranodal, and cutaneous. This partition has limitations, however, as in many instances nodal diseases may display a concomitant extranodal involvement, a cutaneous presentation, or a peripheral blood leukemic dissemination.[1]

Nodal Forms: Among the nodal forms, peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS), angioimmunoblastic T-cell lymphoma (AITL), and anaplastic large-cell lymphoma (ALCL) and its two variants (distinguished according to the expression of the anaplastic lymphoma kinase [ALK]) are the most relevant entities. PTCL-NOS includes a heterogeneous group of neoplasms derived either from mature $CD4^+$ or $CD8^+$ α/β TCR lymphocytes and does not fit into any of the specifically defined entities. AITL originates from T follicular helper (T_{FH}) cells, a subset of T-lymphocytes that derive from naïve T cells homing to the germinal center of the lymph node and differentiating under the pressure of a strong TCR signal through the interaction with dendritic cells, cytokines, and B-lymphocytes. T_{FH} cells are putatively the normal counterpart of follicular T-cell lymphoma and PTCL with T_{FH} phenotype. ALCL-ALK$^+$ has been suggested to derive from thymocytes, whereas the origin of ALCL-ALK$^-$ is still debated.[1]

Extranodal Forms: Among the extranodal forms, NK/T-cell lymphoma nasal type (ENKL), subcutaneous panniculitis-like T-cell lymphoma (SPLTCL), hepatosplenic T-cell lymphoma (HSTCL), and primary intestinal T-cell lymphomas—which in turn are distinguished into enteropathy-associated T-cell lymphoma (EATL) and monomorphic epitheliotropic intestinal T-cell lymphoma (MEITL)—are the most clinically relevant subtypes. All nodal PTCL and most extranodal PTCL display an aggressive behavior, as they usually present with systemic symptoms and may cause a rapid clinical decay if not promptly treated. ENKL derives from the transformation of NK cells or cytotoxic innate cells, probably under the oncogenic pressure of the Epstein-Barr virus (EBV). HSTCL, EATL, and MEITL may derive from either innate cells (NK/T cells or γ/δ TCR CD3$^+$ lymphocytes), adaptive cells (CD4$^+$ or CD8$^+$ α/β TCR lymphocytes), or γ/δ TCR CD3$^+$ intestinal intraepithelial lymphocytes.[1,2]

Cutaneous Forms: Mycosis fungoides (MF) and primary cutaneous CD30$^+$ T-cell lymphoproliferative disorders are cutaneous diseases with a prolonged natural history and treatment may be unnecessary in the first stages of the disease. MF, however, invariably and progressively evolves into more aggressive stages, requiring an adequate treatment, and it is ultimately fatal. Sézary syndrome (SS) can be considered as the leukemic phase of MF, since the tumor arises in an extranodal organ—i.e., the skin—but also displays a characteristic leukemic picture: this is an aggressive form of CTCL, requiring specific treatment and characterized by a dismal prognosis. MF, SS, and CD30$^+$ T-cell lymphoproliferative disorders derive from CD4$^+$ T-lymphocytes with a specific tropism for the skin.

Leukemic Forms: These are mainly represented by T-cell prolymphocytic leukemia (T-PLL), T-cell large granular lymphocytic leukemia (T-LGL), aggressive natural killer (NK)-cell leukemia, and adult T-cell leukemia/lymphoma (ATLL). These diseases always arise in the bone marrow and then evolve with a peripheral blood leukemic presentation. T-PLL is discussed in more detail in this chapter.

TABLE 89.1 The 2017 World Health Organization Classification of Mature T- and NK-cell Neoplasms*

T-cell prolymphocytic leukemia
T-cell large granular lymphocytic leukemia
Chronic lymphoproliferative disorder of NK cells
Aggressive NK-cell leukemia
Systemic EBV$^+$ T-cell lymphoma of childhood (**)
Hydroa vacciniforme-like lymphoproliferative disorder (**)
Adult T-cell leukemia/lymphoma
Extranodal NK-/T-cell lymphoma, nasal type
Enteropathy-associated T-cell lymphoma
Monomorphic epitheliotropic intestinal T-cell lymphoma (**)
Indolent T-cell lymphoproliferative disorder of the GI tract (**)
Hepatosplenic T-cell lymphoma
Subcutaneous panniculitis-like T-cell lymphoma
Mycosis fungoides
Sézary syndrome
Primary cutaneous CD30$^+$ T-cell lymphoproliferative disorders
Lymphomatoid papulosis
Primary cutaneous anaplastic large cell lymphoma
Primary cutaneous γ/δ T-cell lymphoma
Primary cutaneous CD8$^+$ aggressive epidermotropic cytotoxic T-cell lymphoma
Primary cutaneous acral CD8$^+$ T-cell lymphoma (**)
Primary cutaneous CD4$^+$ small/medium T-cell lymphoproliferative disorder (**)
Peripheral T-cell lymphoma, NOS
Angioimmunoblastic T-cell lymphoma
Follicular T-cell lymphoma (**)
Nodal peripheral T-cell lymphoma with TFH phenotype (**)
Anaplastic large-cell lymphoma, ALK$^+$
Anaplastic large-cell lymphoma, ALK$^-$ (**)
Breast implant-associated anaplastic large-cell lymphoma (**)

*Entities in bold type are explained in the text. Provisional entities are listed in italics.
**Changes from the previous WHO classification (2008).

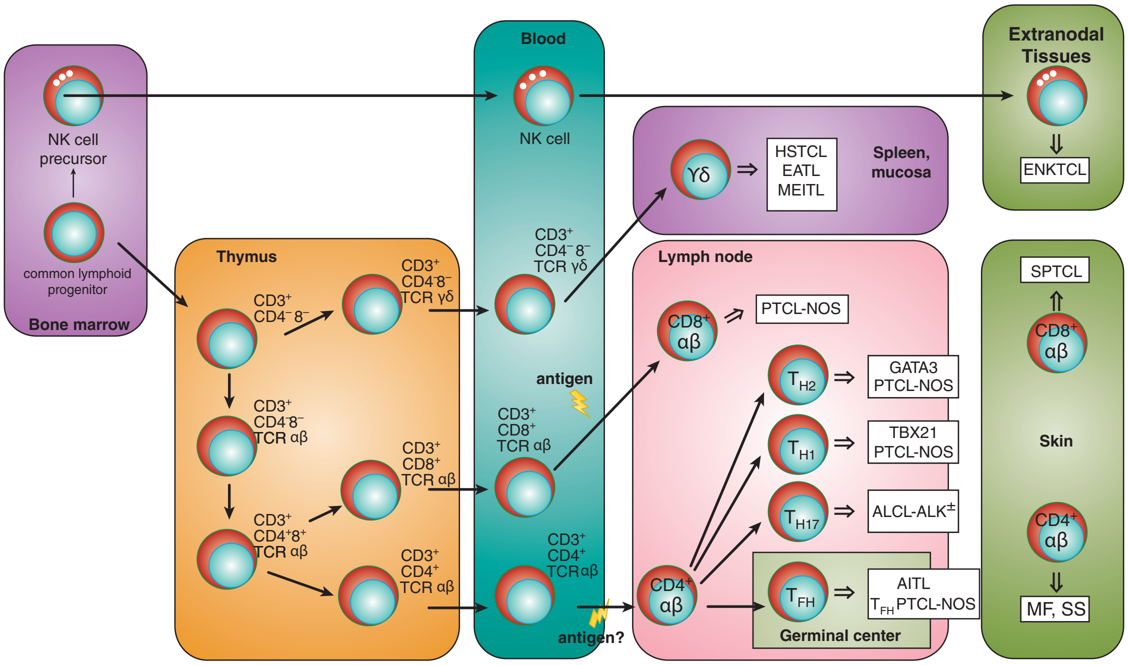


Figure 89.1 THE ONTOGENY OF MATURE T-CELL LYMPHOMAS IN THE CONTEXT OF NORMAL T-CELL DEVELOPMENT. See text for abbreviations.

TABLE 89.2 Clinical Classification of Mature T- and NK-cell Neoplasms*

Predominantly nodal	Peripheral T-cell lymphoma, NOS
	Angioimmunoblastic T-cell lymphoma
	Follicular T-cell lymphoma (**)
	Nodal peripheral T-cell lymphoma with TFH phenotype (**)
	Anaplastic large-cell lymphoma, ALK^{+}
	Anaplastic large-cell lymphoma, ALK^{-} (**)
Predominantly extranodal	Extranodal NK-/T-cell lymphoma, nasal type
	Enteropathy-associated T-cell lymphoma
	Monomorphic epitheliotropic intestinal T-cell lymphoma (**)
	Indolent T-cell lymphoproliferative disorder of the GI tract (**)
	Hepatosplenic T-cell lymphoma
	Breast implant-associated anaplastic large-cell lymphoma (**)
	Systemic EBV^{+} T-cell lymphoma of childhood (**)
	Hydroa vacciniforme-like lymphoproliferative disorder (**)
	Subcutaneous panniculitis-like T-cell lymphoma
Leukemic	T-cell prolymphocytic leukemia
	T-cell large granular lymphocytic leukemia
	Chronic lymphoproliferative disorder of NK cells
	Aggressive NK-cell leukemia
	Adult T-cell leukemia/lymphoma
Cutaneous	Mycosis fungoides
	Sézary syndrome
	Primary cutaneous CD30^{+} T-cell lymphoproliferative disorders
	Lymphomatoid papulosis
	Primary cutaneous anaplastic large cell lymphoma
	Primary cutaneous γ/δ T-cell lymphoma
	Primary cutaneous CD8^{+} aggressive epidermotropic cytotoxic T-cell lymphoma
	Primary cutaneous acral CD8^{+} T-cell lymphoma (**)
	Primary cutaneous CD4^{+} small/medium T-cell lymphoproliferative disorder (**)

*Provisional entities are listed in italics.
**Changes from the previous WHO classification (2008).

CHRONIC T-CELL LEUKEMIAS

T-Cell Prolymphocytic Leukemia

T-PLL is a rare, post-thymic, mature T-cell neoplasm, accounting for 2% of mature lymphoid leukemias. It generally displays an aggressive clinical course and only a minority of patients are asymptomatic at presentation. Disease progression occurs rapidly, even in asymptomatic patients. Older adults are generally affected (median age of 61 years), with a male predominance.

Morphology and Pathobiology

Prolymphocytes are medium-sized cells, displaying a high nuclear-cytoplasmic ratio, with round, oval, irregular, or convoluted nuclei and a single prominent nucleolus. Their cytoplasm is agranular, frequently with blebs. In up to 20% of cases, prolymphocytes may resemble small lymphocytes or show a cerebriform nuclear appearance (Fig. 89.2). Bone marrow is constantly infiltrated, with a diffuse and interstitial pattern, as well as the spleen, which shows an expansion of both red and white pulp.

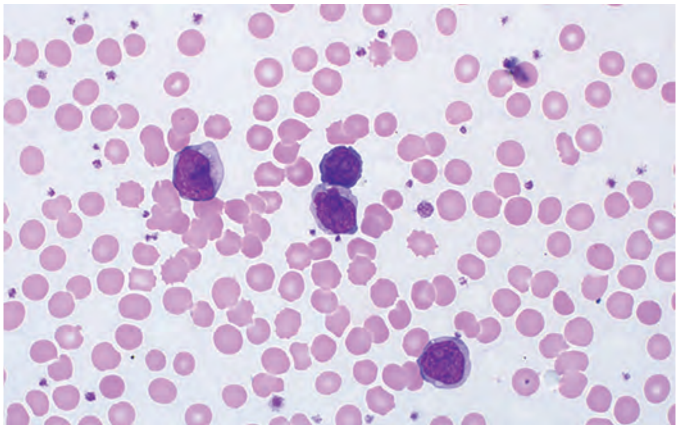


Figure 89.2 T-CELL PROLYMPHOCYTIC LEUKEMIA, PERIPHERAL BLOOD SMEAR. Prolymphocytes show a convoluted nucleus with an evident nucleolus and an irregular cytoplasmic contour.

In virtually all cases, the Xq28 (*MTCP1* locus) or 14q32.1 (*TCL1* and *TCL1b* loci) regions are involved in translocations or inversions with *TCRA/D* at 14q11: this determines the activation of all the three genes and their consequent overexpression, all being implied in T-PLL leukemogenesis by conferring resistance to cell death and determining cell growth arrest. Other complex karyotype alterations may be detected, above all chromosome 8 unbalanced rearrangements, like trisomy, monosomy, t(8;8)(p12;q11), or other translocations with another chromosomal partner. Mutations of the *ATM* gene at 11q21–q23 are also demonstrated in virtually all sporadic cases of T-PLL, reinforcing the association of T-PLL with ataxia-telangiectasia, regarded as a predisposing condition.

Clinical Manifestations

Splenomegaly, seen in almost two thirds of patients, and marked peripheral blood lymphocytosis (usually much higher than 100,000/mmc), with circulating prolymphocytes, are distinctive clinical aspects. Hepatomegaly and lymphadenopathies are seen in up to half of patients, as well as skin nodules, maculopapular rash, or erythroderma. Peripheral edema (periorbital or conjunctival), pleuroperitoneal effusions, or central nervous system involvement may also be present, especially during disease progression. Anemia and thrombocytopenia are usually documented, along with lactate dehydrogenase (LDH) elevation.

Diagnosis

It is established on both morphological and immunophenotyping tests. Tissue histology, however, is not mandatory for diagnosis. Immunophenotyping by flow cytometry demonstrates a positivity for CD7 (strong), CD5, and CD2, with lack of expression of terminal deoxynucleotidyl transferase (TdT) and CD1a. Most cases are CD4^{+} and CD8^{-} (60%), but the phenotype may also be CD4^{+}/CD8^{+} (15% to 25% of the cases) or CD4^{-}/CD8^{+} (10% to 15% of the cases). CD52 is expressed at high density. TCR α/β genes are rearranged in most of the cases, and just in a minority of instances a γ/δ rearrangement is detected.[3]

Table 89.3 displays the requirements to establish the diagnosis of T-PLL: the diagnosis is confirmed when all the three major criteria are met or if the first two major criteria and one minor criterion are met.

TABLE 89.3 **Criteria to Establish the Diagnosis of T-Cell Prolymphocytic Leukemia**

Major Criteria	Minor Criteria
• More than 5000/mmc cells of T-PLL phenotype in peripheral blood or bone marrow. • T-cell clonality (by polymerase chain reaction for *TCRB/TCRG*, or by flow cytometry). • Abnormalities of 14q32 or Xq28 or expression of *TCL1A/B* or *MTCP1*.	• Abnormalities involving chromosome 11 (11q22.3; *ATM*). • Abnormalities in chromosome 8. • Abnormalities in chromosomes 5, 12, 13, 22, or complex karyotype. • Involvement of T-PLL specific site (splenomegaly, effusions).

Differential Diagnosis

T-cell neoplasms with leukemic presentation other than T-PLL can be ruled out by morphology evaluation and flow cytometry assays. The most relevant diseases that can resemble T-PLL are represented by: T-cell acute lymphoblastic leukemia (T-ALL), leukemic dissemination of PTCL, T-LGL, SS, and ATLL. Table 89.4 summarizes the criteria to accomplish a correct differential diagnosis.

Therapy

Since the disease is usually symptomatic, clinically aggressive, and rapidly progressive, a prompt initiation of a systemic treatment is required. On the other hand, 20% to 30% of patients demonstrate an initially stable or slowly progressive disease ("inactive" T-PLL). In these cases, a period of close observation is recommended. However, almost all "inactive" T-PLL turn into "active" (symptomatic) disease within 1 to 2 years.

Symptomatic T-PLL is defined as (at least one of the following): presence of constitutional symptoms (fatigue, unintentional weight loss of >10% of normal body weight within 6 months, drenching night sweats or fever >38°C without signs of infection); symptomatic bone marrow failure (hemoglobin <10 g/dL and/or platelet count <100,000/mmc); rapidly enlarging lymph nodes, spleen, and liver (>50% enlargement within 2 months, or diameter doubling within 6 months, or symptomatic organomegaly); increasing lymphocytosis (>50% within 2 months when lymphocytes are >30,000/mmc or doubling time shorter than 6 months); extranodal involvement (organ infiltration, peritoneal or pleural effusion, central nervous system involvement).

Intravenous alemtuzumab, a humanized anti-CD52 antibody, is the first-line treatment of choice, yielding up to an 80% of complete response (CR) in treatment-naïve patients.[4] Of note, this treatment is frequently complicated by cytomegalovirus (CMV) reactivations, sometimes requiring adequate antiviral treatment. Thus, CMV-DNA monitoring during the courses is strongly recommended.[5] Prophylaxis for *Pneumocystis jirovecii* is also indicated.

Although prolonged responses to alemtuzumab have been described, all patients have a tendency to relapse; therefore, a consolidation with an allogeneic stem cell transplantation in first remission may offer a chance for a better long-term survival, when feasible. Autologous stem-cell transplantation may also be beneficial for patients, but it does not result in a cure. Retreatment with alemtuzumab at disease relapse is feasible if remission duration is longer than 6 months. However, the emergence of a CD52$^-$ phenotype clearly indicates a mechanism of resistance to alemtuzumab. Purine-analogues, such as pentostatin, cladribine, nelarabine, or fludarabine, may be a good treatment alternative, with some evidence of activity in T-PLL.[6,7] Pentostatin, in particular, is the agent of choice when suboptimal responses to alemtuzumab are seen.

Experiences with targeted agents, like the combination of venetoclax and ibrutinib, are now ongoing. Reports exist on salvage treatment with JAK inhibitors in patients with multi-treated T-PLL harboring JAK mutations.

TABLE 89.4 **Most Relevant Differential Diagnoses Among T-Cell Neoplasms With Leukemic Presentation**

Disease	Morphology	Immunophenotype
T-PLL	High nuclear-cytoplasmic ratio, round or oval and irregular or convoluted nuclei, single prominent nucleolus, agranular cytoplasm, frequently with blebs	TCL1$^+$ (>90%), CD3$^+$ (>80%), CD4$^+$ (60%), CD5$^+$ (100%), CD7$^+$ (>90%), CD8$^+$ (15%), CD4$^+$CD8$^+$ (25%)
T-ALL	Blasts with high nuclear-cytoplasmic ratio, rather condensed chromatin, inconspicuous nuclei, scarce agranular cytoplasm	TdT$^+$, CD1a$^+$
Leukemic PTCL	Highly pleomorphic mature lymphocytes	TCL1$^-$
T-LGL	Round eccentric nucleus with dense chromatin, abundant pale cytoplasm with numerous azurophilic granules	CD8$^+$, CD57$^+$, CD16$^+$
SS	Convoluted (cerebriform) nucleus with condensed chromatin and sometimes evident nucleoli, agranular cytoplasm, sometimes with a ring of vacuoles	CD7$^-$, CD4$^+$, CD25$^+$
ATLL	Highly pleomorphic mature lymphocytes, sometimes with a characteristic lobated nucleus (flower cells)	CD4$^+$, CD25$^+$, HTLV1$^+$

Treatment response criteria are outlined in Table 89.5: both clinical and laboratory parameters need to be considered to define the depth of the response. A general recommendation on adequate timing to assess a response cannot be made, as response kinetics appear different depending on the agents used (chemotherapy *versus* antibodies).

Prognosis

Response to alemtuzumab is the main outcome predictor: non-responders have a median survival of only 4 months. Five-year overall survival (OS) is nearly 21%, with high white cell counts, older age, and short lymphocyte doubling time being adverse prognostic factors. Reports exist on the possible development of EBV-driven non-Hodgkin lymphomas in patients receiving alemtuzumab for T-PLL: an aggressive clinical course and rapid development of nodal lesions in an otherwise responding T-PLL patient always deserve attention (with imaging studies and nodal biopsy).

PERIPHERAL T-CELL LYMPHOMAS

This section reviews some general aspects regarding the epidemiology of PTCL and the shared mechanisms underlying the lymphomagenesis of these entities. Specific disease subtypes are reviewed especially in terms of their biopathology, clinical peculiarities, and prognostic tools. First-line treatment approaches are discussed at the end, with a particular focus on the management of some specific subtypes, bearing in mind that the vast heterogeneity of these diseases, coupled

TABLE 89.5 Treatment Response Criteria in T-PLL

Group and Parameter	Complete Response (All Criteria to Be Met)	Partial Response (≥2 Criteria in A and ≥1 in B)	Stable Disease (All Criteria to Be Met)	Progression (≥1 Criterion in A or B to Be Met)
Group A				
Lymph nodes	Long axis <1 cm	Decrease ≥30% in SLD	Reduction <30% or increase ≤20%	Increase >20% in SLD
Spleen	Spleen size <13 cm	Decrease ≥50% in vertical length	Reduction or increase <50%	Increase ≥50%
Symptoms	None	Any	Any	Any
Circulating lymphocytes	<4000/mmc	≤30,000/mmc with decrease ≥50%	>30,000/mmc or change <50%	Increase ≥50%
Marrow	T-PLL cells <5%	Any	Any	Any
Any other site	No involvement	Any	Any	Any
Group B				
Platelets	≥100,000/mmc	≥100,000/mmc or increase ≥50%	Change <50%	Decrease ≥50%
Hemoglobin	≥11 g/dL	≥11 g/dL or increase ≥50%	<11 g/dL or change <50% or reduction <2 g/dL	Decrease ≥2 g/dL
Neutrophils	≥1500/mmc	≥1500/mmc or increase ≥50%	Change <50%	Decrease ≥50%

SLD, Sum of long-axis diameters of up to three target lesions.

with their rarity, has always hampered the design and development of adequately powered clinical studies able to provide strong treatment evidence. Recommended strategies for the treatment of relapsed and refractory disease, along with a focus on newly available single agents and innovative combinations, is also provided.

Epidemiology of Peripheral T-Cell Lymphomas

The most common entity is PTCL-NOS, which includes a heterogenous group of mature T-cell lymphomas representing up to a third of all PTCL. AITL is the second most common subtype, accounting for 15% to 30% of all PTCL, according to different case series. ALCL represents nearly 15% of all PTCL. Among extranodal entities, ENKL accounts for nearly 10% of all PTCL, followed by primary intestinal T-cell lymphomas (5% to 6% of all PTCL), HSTCL (2% of all PTCL), and SPLTCL (<2%). Some lymphomas display a peculiar geographical distribution: the broad category of PTCL-NOS is uniformly distributed in Western countries, where it represents the most frequent category. Similarly, AITL is more frequently diagnosed in Europe, although incidence varies across regions.[8,9] It should be noted, however, that many cases previously characterized as PTCL-NOS can be reclassified into more recent 2017 WHO-recognized categories. Such update of classification may affect the real epidemiology of AITL and PTCL-NOS. Part of PTCL-NOS cases display a T_{FH} phenotype, and thus require to be more correctly classified as T_{FH}-derived PTCL.[10] ENKL is mostly observed in Asia and Central-South America and shows higher prevalence among Hispanic people, American Indians, Asians, and Pacific Islanders (with a frequency of 15% among all PTCL). Likewise, ALCL seems to be more frequent in Hispanic people and American Indians than in Black or non-Hispanic people.

A family history of hematologic disorders, genetic background, viral infections, occupational and environmental exposure, dietary intake, immune-mediated diseases, and persistent immunosuppression have all been considered relevant risk factors for the development of PTCL.

Shared Pathogenetic Mechanisms

As it happens with all lymphoid neoplasms, the accumulation of genomic defects over time leads to an altered balance between genomically aberrant lymphocytes and normal host cells, ultimately determining the neoplastic transformation of the tissue and the clinical manifestation of the disease. Multiple mechanisms have been described as responsible for the T-cell transformation into an overt neoplasm.[2] Given the wide heterogeneity of PTCL, not all the following mechanisms apply to each disease category, although some shared pathogenetic steps may be identified; most PTCL, in fact, demonstrate intrinsic defects which end up with the deregulation of multiple signaling pathways. Six shared mechanisms are briefly described in this paragraph; peculiar genomic or epigenomic changes specifically correlated with well-defined disease entities are instead described separately.[2]

1. **Role of the TCR signaling**. Some PTCL acquire defects that uncouple the TCR signaling from the TCR engagement. Somatic mutations in co-stimulatory molecules (such as CD28, as seen in AITL) or downstream components of the signaling cascade (PI3K-AKT signaling, NFκB signaling), structural alterations (as it happens with ITK-SYK fusion protein in PTCL-NOS and with ALK-chimeric proteins in ALCL-ALK^{+}) or loss of TCR-negative regulators (like the phosphatase DUSP22, which works as an onco-suppressor) all contribute to the uncontrolled TCR activation, fostering a neoplastic phenotype.
2. **Epigenomic changes**. Epigenetic dysregulation is a hallmark of several PTCL, mainly those arising from T_{FH} cells, where *TET2*, *IDH2*, and *DMT3A* mutations are characteristically detected. Epigenetic alterations lead to a deregulated expression of genes that promote T_{FH} differentiation and sustain a transformed phenotype synergistically with additional defects. Moreover, they contribute to an increased self-renewal capacity of stem cells and hematopoietic progenitors and alter their terminal differentiation. Of note, mutations of *TET2* and *DNMT3A* are also detected in hematopoietic progenitors of ageing people and in clonal hematopoiesis processes, putting those individuals at greater risk of neoplasm, mainly myeloproliferative diseases. Importantly, patients with *TET2* or *DNMT3A*-mutated AITL who later developed a myelodysplastic or myeloproliferative syndrome showed the same mutation in their hematopoietic stem cells and in the neoplastic myeloid cells as well, thus leading to postulate that these mutations constitute a pre-malignant status that precedes the malignant transformation of both myeloid and lymphoid cells.
3. **Deregulated activation of the JAK-STAT pathway**. The JAK-STAT pathway controls cell growth arrest and apoptosis and its

constitutive activation results in cells that are independent of (or at least less dependent on) extracellular signals. Together with the loss of negative regulators (e.g., due to mutations of *PTPN2* or *PTPN6*-encoded phosphatases), cells become insensitive to inhibitory signals, thus being prone to a malignant transformation. The JAK-STAT pathway is commonly deregulated in PTCL and mutations in the JAK and STAT system in T-cell neoplasms have been detected in the SH2 domain of STAT3 and STAT5 and in the pseudokinase domain of JAK1 and 3. This has been particularly observed in ALCL, PTCL-NOS, CTCL, ENKL, and rarer extranodal entities.[11]

4. **Metabolic dysregulation**. Metabolic reprogramming of cancer cells also contributes to tumorigenesis and to the maintenance of the tumor itself: high glucose uptake, high glycolytic activity, nucleotide, and membrane phospholipids synthesis promote proliferation and sustain PTCL cells under stress conditions. These mechanisms have been particularly highlighted in ALCL, where they seem to be correlated to the activity of ALK-fusion proteins along with the constitutive activation of certain regulatory proteins (e.g., STAT3).
5. **Microenvironmental interactions**. Neoplastic cells in PTCL highly depend on their microenvironment: TCR activation and secretion of cytokines through paracrine and autocrine stimulations (e.g., IL-4, IL-5, IL-13, and VEGF) are of great importance for tumor progression and maintenance and explain the majority of systemic symptoms patients affected by PTCL complain of. Moreover, the successful growth and survival of neoplastic cells is modulated by pro-tumorigenic signals from surrounding elements (e.g., functional recruitment of surrounding suppressor regulatory T_{reg} cells operated by PTCL-NOS and ALCL) and by decreased immunogenicity due to loss of HLA class I protein or via the interaction between programmed death-1 (PD-1) and its ligand PD-L1, the latter being highly expressed on the surface of ALCL-ALK$^+$ and ENKL cells.
6. **EBV-mediated oncogenesis**. Apart from B cells, EBV may infect an early precursor lymphoid cell which ultimately can differentiate into a B, T, or NK cell. A spectrum of EBV$^+$ T-cell lymphoproliferative neoplasms is now recognized, ranging from aggressive ENKL to more indolent cutaneous lymphoproliferative diseases. The pathogenesis of these disease entities and how EBV$^+$ T and NK cells progress and ultimately escape from immunosurveillance is not entirely clear. It can be postulated that in ENKL EBV$^+$ T and NK cells derive from the clonal expansion of a pre-malignant EBV-infected normal T or NK cell, which likely occurs in a unique genomic background (this may be an explanation of the much higher incidence of this lymphoma in Asia).

Diagnosis and Staging

The diagnosis of PTCL is always established on the biopsy of the involved tissue, which is mainly represented by a lymph node. However, virtually any extranodal sites may also be the site of biopsy: liver, small intestine, and skin are among the mostly involved extralymphatic tissues. The review of all slides and of formalin-fixed paraffin embedded tissue by a pathologist with expertise in T-cell lymphoma is always encouraged. Generally, diagnostic accuracy is very good for ALCL-ALK$^+$, but agreement is lost in case of other lymphoma subtypes, with a rate of concordance of about 75% for the most common subtype, PTCL-NOS.

Molecular studies may be helpful under certain circumstances to clarify or refine the diagnosis: at present, however, no molecular markers specifically dictate or guide treatment decisions. The sole demonstration of T-cell clonality through the assessment of TCR rearrangement alone is not sufficient for diagnosis, as this may be seen with reactive and inflammatory processes.

The clinical approach to the patient is described in the box on Clinical Approach to a Patient Affected by PTCL and Staging Recommendations.

CLINICAL APPROACH TO A PATIENT AFFECTED BY PTCL AND STAGING RECOMMENDATIONS

1. Accurate past medical history: concomitant diseases (e.g., celiac disease or concomitant hematological neoplasms); occupational exposure.
2. Recent medical history, particularly focused on lymphoma-related symptoms (recent weight loss, fever, night sweats).
3. Performance status assessment.
4. Physical examination, which must include the evaluation of Waldeyer's ring, nasopharynx, node-bearing areas, liver and spleen, thoracic auscultation, and a full skin inspection.
5. Laboratory: complete blood count with differential counts (including the examination of a peripheral blood smear, when indicated), liver and renal function tests, lactate dehydrogenase. Reticulocytes and bilirubin (complete and fractionated) are useful markers in case of suspect hemolysis, which may be associated with some PTCL cases (mainly AITL). Direct Coombs test is also necessary to establish a diagnosis of autoimmune hemolytic anemia.
6. Staging procedures: computed tomography (CT) scan of neck, chest, abdomen, and pelvis with intravenous contrast and unilateral bone marrow trephine biopsy. ^{18}F-fluorodeoxyglucose positron emission tomography (PET) scan is not mandatory but may be helpful (see box on The Role of PET Scan as a Staging Tool in Peripheral T-Cell Lymphomas).

THE ROLE OF PET SCAN AS A STAGING TOOL IN PERIPHERAL T-CELL LYMPHOMAS

- ^{18}F-fluorodeoxyglucose (FDG) PET scan is not mandatory for disease staging, although it has proven to be helpful in detecting FDG-avid nodal or extranodal lesions that can be missed by a CT scan evaluation.
- PET scan may change the disease stage in no more than 5% of patients at diagnosis as compared to CT. This change generally does not translate into treatment modification or intensification.
- PET positivity found at the end of induction treatment and in patients who have received autologous stem cell transplantation is a strong predictor of reduced survival.
- Maximum standard uptake values in patients with PTCL are lower (and much more heterogeneous) than in aggressive B-cell counterparts, and usually less pronounced for extranodal lesions than for lymph node localizations of the disease.

Like nodal B-cell lymphoma, PTCL are staged according to the Ann Arbor staging system. Computed tomography (CT) scan of neck, chest, abdomen, and pelvis with contrast and a bone marrow trephine biopsy are requested to accomplish disease staging. The role of positron emission tomography (PET) during staging and response assessment is briefly reviewed in the box on The Role of PET Scan as a Staging Tool in Peripheral T-Cell Lymphomas.

Peripheral T-Cell Lymphoma, Not Otherwise Specified

PTCL-NOS remains a category of biologically and clinically heterogeneous diseases which cannot be further classified into any other of the existing entities defined by the current WHO classification. As a consequence, this disease is heterogeneous, as it displays a broad cytological spectrum and a multiplicity of molecular aspects. Gene expression profile studies have shown that many of these cases resemble ALCL, adult T-cell leukemia/lymphoma, AITL, or ENKL; in some cases, molecular markers help define a peculiar T_{FH} origin.[1,10,12]

PTCL-NOS is the most common subtype, accounting for at least 25% to 30% of PTCL. Its prevalence is geographically variable, given that it is equally spread across North America and Europe (34% of PTCL cases), and slightly less diffused in Asia, accounting for 22% of PTCL cases.

Morphology and Pathobiology

The normal architecture of the lymph node appears effaced and neoplastic elements show a variable morphology, ranging from small cells with irregular nuclei to large cells with prominent nucleoli and mitotic figures. An inflammatory background is often present including small reactive lymphocytes, eosinophils, plasma cells, and epithelioid histiocytes. Epithelioid histiocytes are particularly numerous in the *lymphoepithelioid variant* (also termed Lennert lymphoma). Neoplastic cells stain positively for CD2, CD3, CD5, and CD7, although CD5 and/or CD7 may be frequently lost. The expression of CD4 and CD8 is also variable. The ALK protein is always negative and given that CD30 can be expressed in more than 75% of the neoplastic cells, the differential diagnosis with an ALCL-ALK$^-$ constitutes a diagnostic challenge. The proliferation index according to Ki-67 is usually high and rates exceeding 70% are associated with a worse prognosis (Fig. 89.3).

Rare cases of PTCL-NOS express the cytotoxic granule-associated proteins (perforin, granzyme-B, and TIA-1), and this specific pattern has been observed in the lymphoepithelioid variant.

Significant improvements have been made in delineating specific biological and prognostic subgroups within the PTCL-NOS spectrum: in particular, two major molecular clusters have recently been delineated: one subgroup shows a high expression of *GATA3* and an enrichment of gene signatures related to cell proliferation (*MYC*), mammalian target of rapamycin (mTOR), and β-catenin; the other significantly expresses *TBX21* (*T-bet*) and is enriched of interferon (IFN)- γ and NFκB-induced gene signatures. *GATA3* regulates T-helper 2 cell differentiation, whereas *TBX21* polarizes cells to a T-helper 1 and cytotoxic T-cell phenotype. This distinction has an important prognostic correlate, as *GATA3* expression confers a poor prognosis, with a 5-year OS of 19%. On the contrary, cases with a *TBX21* signature display more favorable outcomes, and may display a 5-year OS of 38%.[13]

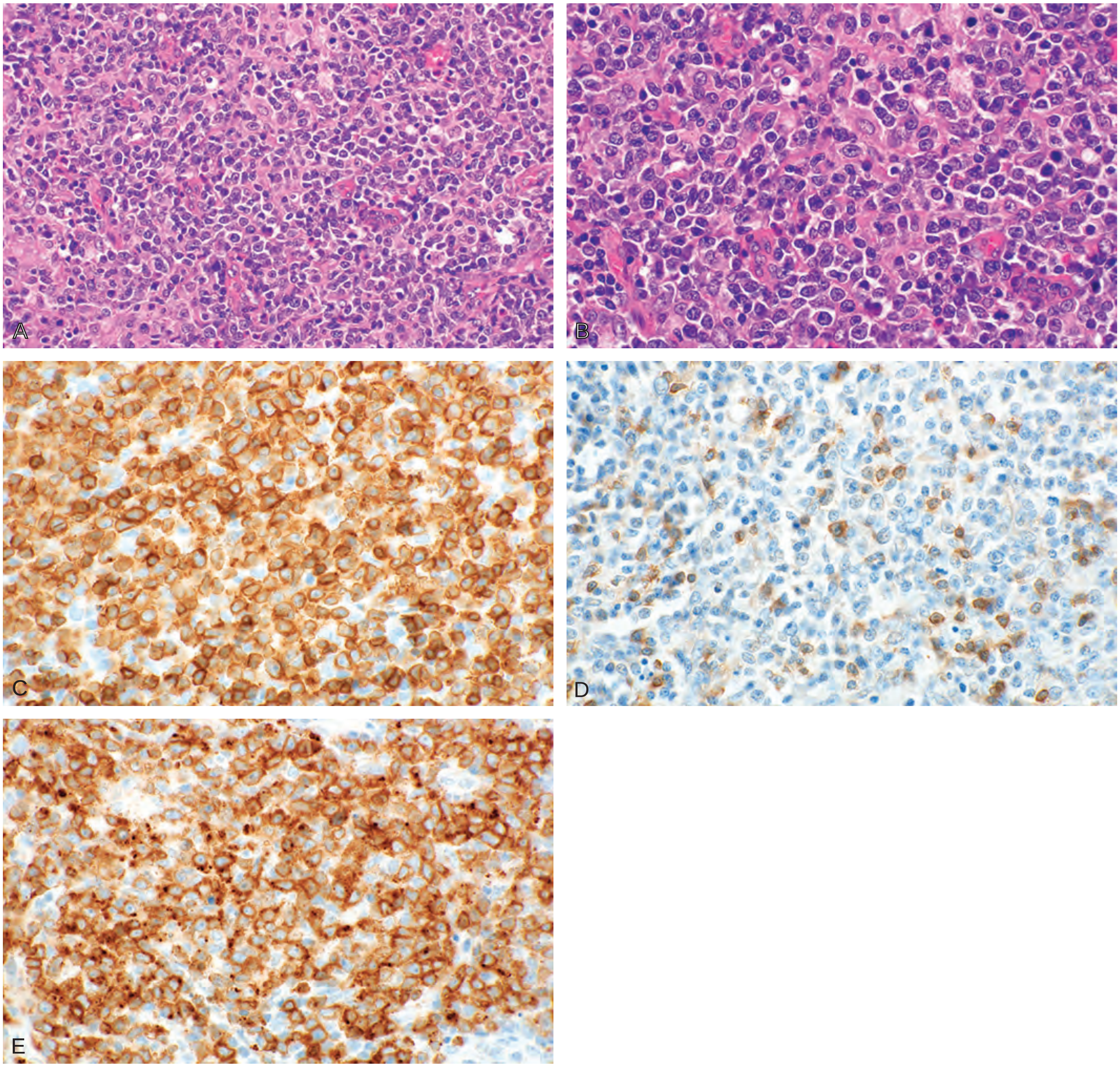


Figure 89.3 Peripheral T-cell lymphoma, not otherwise specified, lymph node biopsy, hematoxylin-eosin staining at lower (40×, panel A) and higher (50×, panel B) magnification. Immunohistochemical staining for CD4 (panel C), T-cell receptor βF1 (panel D), and δ (panel E).

Recurrent genetic abnormalities of *TET2*, *IDH2*, *DNMT3A*, *RHOA*, and *CD28* mutations may be observed in cases of PTCL-NOS that manifest a T_{FH} phenotype, as it happens with AITL: for this reason, the former *follicular variant* of PTCL-NOS has been moved to the new T_{FH} lymphoma category in the most recent (2017) WHO classification.[14]

Clinical Manifestations

Adult patients are mostly affected, as median age at presentation is 60 years. Children can be affected, albeit rarely. The disease shows predilection for males, with a male-to-female ratio of 2:1. Nodal involvement is the most relevant clinical feature at diagnosis, although any organ can be involved, including bone marrow, liver, spleen, gastrointestinal tract, and skin (the latter being the most widely involved, 16% of the cases). Synchronous nodal and extranodal involvement is not infrequent. Nearly 70% of cases present with advanced stage (III-IV according to the Ann Arbor staging system); lymphoma-related symptoms, LDH elevation and poor performance status may be documented in half of the patients.

Prognosis

The International Prognostic Index (IPI), widely applied in aggressive B-cell lymphomas, retains its association with treatment outcomes in T-cell neoplasms:[15] patients with low score (0 to 1 prognostic factors) have a 5-year OS of 50%, in contrast with those with high score (4 to 5 prognostic factors), whose 5-year OS is only 11%. Newer scores have been specifically built up for PTCL-NOS patients: the Prognostic Index for PTCL-NOS (PIT) and the modified PIT (m-PIT): they share age (>60 years), Eastern Cooperative Oncology Group (ECOG) performance status (>1) and LDH elevation with IPI; PIT also takes into account bone marrow infiltration, while m-PIT integrates the expression of the proliferation-associated protein Ki-67.[16,17] A fourth prognostic index derived from the International T-cell Lymphoma Project (ITCLP) indicates age (>60 years), ECOG (>1), and thrombocytopenia (<150,000/mmc) as the three most relevant parameters for prognostic stratification (Table 89.6).[18] Importantly, all these prognostic scores have been validated in subpopulations treated with cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) or at least an anthracycline-containing regimen. The four scores divide PTCL-NOS patients in 3 (m-PIT, ITCLP) or 4 (IPI, PIT) prognostic categories: the lower-risk group, whatever score is used (score 0 to 1 for IPI and m-PIT, score 0 for PIT and ITCLP) emerges as a clearly separated category and shows better outcomes than all the other risk groups (Table 89.7.)

TABLE 89.6 **Variables Used in the Calculation of Relevant Prognostic Scores**

	IPI	PIT	m-PIT	ITCLP
Age (>60 years)	•	•	•	•
ECOG (>1)	•	•	•	•
LDH (elevated values)	•	•	•	
Ann Arbor stage (III-IV)	•			
Extranodal involvement (≥2 sites)	•			
Bone marrow involvement		•		
Platelet count (<150,000/mmc)				•
Ki-67 (≥80%)			•	

IPI, International Prognostic Index; *PIT*, Prognostic Index for PTCL-NOS; *m-PIT*, modified PIT; *ITCLP*, International T-cell Lymphoma Project; *ECOG*, Eastern Cooperative Oncology Group; *LDH*, lactate dehydrogenase.

Angioimmunoblastic T-Cell Lymphoma

AITL is a rare disease, which accounts for only 1% to 2% of non-Hodgkin lymphomas and 15% to 20% of PTCL. Disease incidence is higher in Europe (29% of all cases of PTCL), followed by Asia (18%) and North America (16%); the reasons for this heterogeneity in different parts of the world is largely unexplained.[19]

AITL is a T_{FH}-derived neoplasm that originates from follicular helper T-lymphocytes: these cells are normally present in lymphoid follicles and are mainly implicated in the germinal center reaction in close collaboration with germinal center B-lymphocytes. Given that a number of recurrent mutations and a characteristic gene signature characterize a significant proportion of AITL cases, as well as some cases of PTCL-NOS, which manifest a T_{FH} phenotype, the 2017 revision of the WHO classification unifies under a common heading AITL, follicular T-cell lymphoma and nodal PTCL with T_{FH} phenotype.[1,2]

AITL is a disease of the elderly, with a median age of 65 years at presentation, with no gender predilection.

Morphology and Pathobiology

The architecture of the affected lymph node is effaced by a T-cell infiltrate of polymorphous lymphocytes that extends beyond the nodal capsule and characteristically spares the subcapsular sinus, which appears open and dilated. Regressed follicles may be appreciated, especially in earlier stages of the disease, indicating that the disease arises in association with germinal centers, extending to extrafollicular regions as it progresses. Neoplastic cells are admixed with reactive small lymphocytes, eosinophils, plasma cells, and an abundant amount of follicular dendritic cells, which represent the accompanying non-neoplastic populations. Scattered large $CD20^+$ immunoblastic cells, usually staining positively for EBV-encoded RNA (EBNA), are found in most cases. The prominent proliferation of high endothelial venules with a tendency to arborization is

TABLE 89.7 **Prognosis of Aggressive Peripheral T-Cell Lymphomas According to the Most Widely Used Prognostic Scores***

Disease	Score	5-year OS (%)		5-year FFS (%)	
		Low Score	High Score	Low Score	High Score
PTCL-NOS	IPI	50	11	36	9
	PIT	50	11	34	8
AITL	IPI	56	25	34	16
	PIAI	44	24	28	15
ALCL-ALK$^+$	IPI	90	33	80	25
ALCL-ALK$^-$		74	13	62	13
ENKL, nasal	IPI	57	0	53	0
ENKL, extranasal		17	20	21	20
EATL	(**)	20		4–17	
HSTCL	(**)	0–43		0–20	
SPTCL	(**)	60		30–40	

OS, overall survival; *FFS*, failure-free survival. See text for disease and prognostic scores abbreviations.

*"Low score" means score 0 to 1 for IPI, score 0 (group 1) for PIT, and score 0 to 1 for PIA. "High score" means score 4 to 5 for IPI, score 3 to 4 (group 4) for PIT, and score ≥2 for PIA.

**IPI (or any other prognostic score) not relevant for prognostic stratification of patients.

a characteristic feature of AITL. T-cell-associated antigens are demonstrated by immunohistochemistry, although neoplastic cells may show aberrant phenotypes: CD3, CD4, CD10, PD-1, and sometimes BCL6 are the most frequently encountered antigens, whereas CD5 and CD7 are frequently defective (Fig. 89.4).

Several genes overexpressed in AITL (like *CD200*, *PDCD1*, *CD40L*, *NFATC1*, *LIF*) have been reported as overexpressed in T_{FH} compared to other T-cell subsets, thus supporting the AITL derivation from T_{FH} cells. The detection of at least three T_{FH}-associated molecules by immunohistochemistry (CD10, BCL6, PD-1, CXCL13, CCR5, SAP, ICOS), which is a surrogate for gene expression profiling, is considered sufficient to suggest the derivation of a given T-cell neoplasm from T_{FH}. This is generally true for AITL, but also for a proportion of PTCL-NOS, as said. The cellular derivation of AITL from T_{FH} cells provides an explanation to the peculiar pathological and biological features of the disease, like the expansion of B-lymphocytes, the intimate association with germinal centers in early disease stages, the associated immune deficiency and the deregulated immunoinflammatory response that characterizes its clinical course.[20]

The AITL mutational landscape is complex: *TET2*, *IDH2*, *DNMT3A*, and *RHOA* are among the most relevant mutated genes, possibly with a role in lymphomagenesis.[21–25] Mutations of *TET2* are the most prevalent among AITL (half to three quarters of the cases) and generally correlate with advanced-stage disease, high IPI, thrombocytopenia, elevated LDH, and shortened survival. *TET2* and *DNMT3A* mutations seem to occur at an early stage of hematopoietic differentiation and may represent early events of lymphomagenesis. Mutations of *IDH2* at R172 are also characteristic of AITL and tend to co-occur with *TET2* mutations: they define a unique subgroup of AITL with distinct gene expression, and *IDH2*/*TET2* double-mutant cases display a significantly more polarized T_{FH} phenotype. Somatic *RHOA* mutations have also been

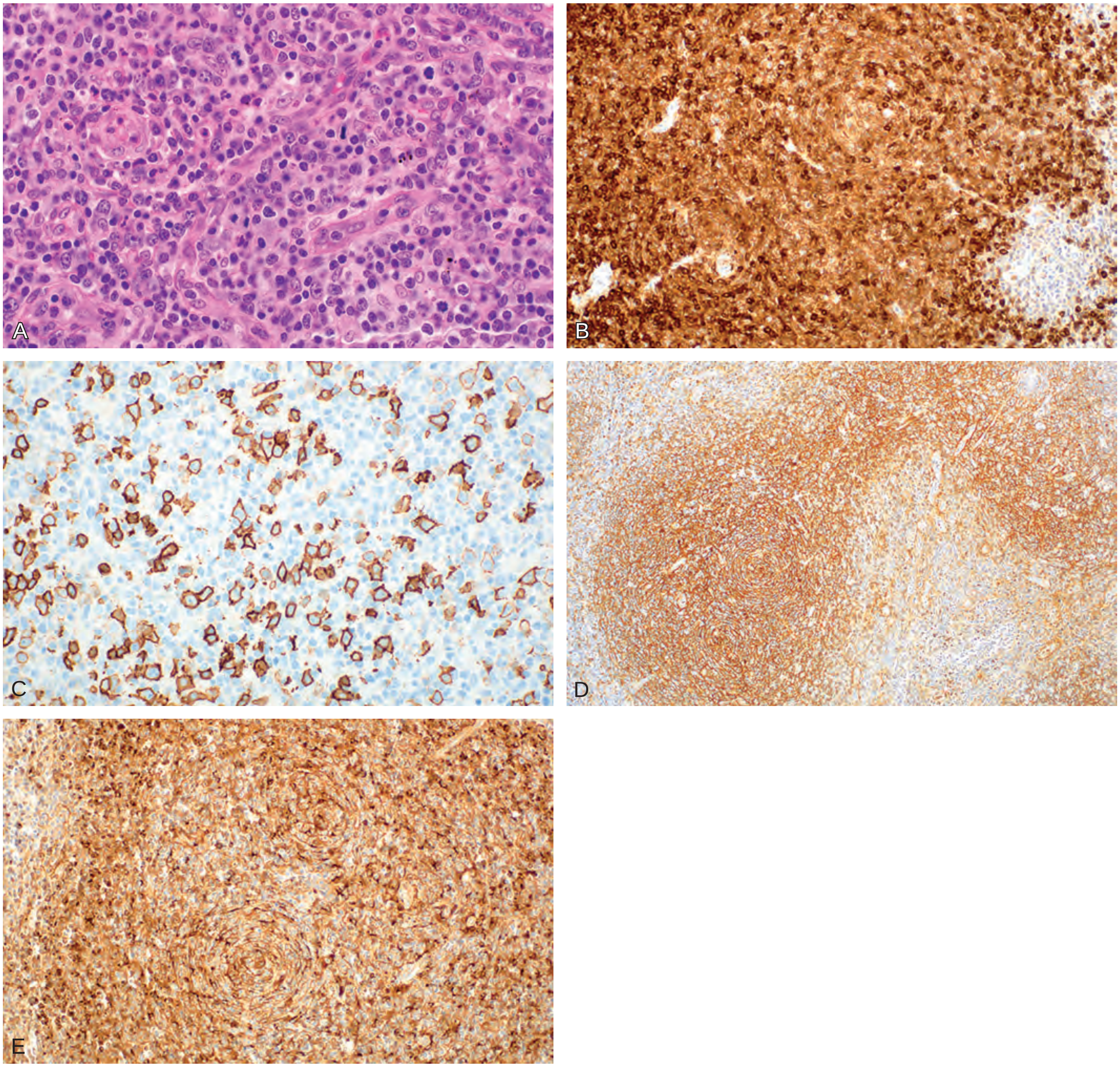


Figure 89.4 Angioimmunoblastic T-cell lymphoma, lymph node biopsy, hematoxylin-eosin staining (panel A). Positive immunostaining for CD10 (panel B). Immunohistochemistry for CD20 reveals several scattered B cells (panel C). Follicular dendritic cell meshwork highlighted with CD21 immunostaining (panel D). The immunohistochemical expression of CXCL13 on neoplastic elements suggest their derivation from T_{FH} elements (panel E).

described in nearly 70% of AITL, constantly co-occurring with *TET2* and very often with *DNMT3A* mutations: RHOA is activated downstream of TCR engagement in mature T-lymphocytes, and when mutated it shows an impaired GTP-ase function that also inhibits its wild-type form. The coexistence of these mutations occurring at different times of a multistep model of lymphomagenesis, the co-operation of the aberrant encoded proteins, along with other mutations affecting genes related to TCR signaling that deregulate its activation may co-operate in the pathogenesis of AITL and other T_{FH}-derived PTCL.

Clinical Manifestations

This disease behaves aggressively: at least 70% of patients display systemic symptoms at onset, including fever and weight loss, and the disease presents with advanced stage in about 75% of cases. Generalized lymphadenopathy along with a fluctuating skin rash (due to cutaneous dissemination) constitute a distinctive clinical presentation. Hepato-splenomegaly may be found in one third of cases and the bone marrow is infiltrated in nearly half of patients. Peripheral edema, pleural or peritoneal effusion, joint pain, or cold agglutinin disease can be associated phenomena. Autoimmune cytopenia(s)—above all Coombs-positive autoimmune hemolytic anemia—are also a common feature of the disease: they often respond to lymphoma-directed therapies, and their evolution parallels the one of the lymphoma itself.

Laboratory Manifestations

A specific laboratory abnormality associated with AITL is the detection of a polyclonal hypergammaglobulinemia in 30% to 50% of cases, sometimes with the presence of a monoclonal serum immunoglobulin. Hypergammaglobulinemia is probably related to the fact that the tumor cells are derived from T cells with a specific B-cell helper capacity. This also explains the frequent detection of autoimmune markers, such as rheumatoid factor, circulating immunocomplexes, and anti-smooth muscle antibodies.

Prognosis

According to retrospective series, when patients are treated frontline with anthracycline-containing combination chemotherapy, the 5-year OS for those with an IPI score of 0 to 1 is 56% and 25% for patients with a score of 4 to 5. An alternative prognostic model based on age (>60 years), performance status (>2), extranodal site involvement (≥2 sites), presence of B-symptoms, and platelet count (<150,000/mmc) has been recently proposed (prognostic index for AITL, PIAI) and was more predictive of OS than IPI in patients with lower (score 0 to 1) and higher (score 3 to 5) risk.[19] Anemia (hemoglobin <12 g/dL), mediastinal lymphadenopathy, white blood cell count (>10,000/mmc), and immunoglobulin A concentration (>400 mg/dL) (see Table 89.4) have also shown a significant impact on prognosis.

Anaplastic Large-Cell Lymphoma

ALCL should be conceived as a group of T-cell non-Hodgkin lymphomas with unifying pathological characteristics although displaying heterogeneous clinical and genetic features. The current WHO classification includes four distinct ALCL entities.[1] Two of them are of nodal origin and are distinguished by the expression (or not) of the ALK protein into an ALCL-ALK$^+$ and an ALCL-ALK$^-$, the latter being no longer conceived as a provisional subcategory, but rather defined as a specific disease entity. This distinction is of importance, as the two entities display a heterogeneous clinical behavior, a different prognosis when treated frontline with conventional therapy, and are managed differently. The third entity has an exquisitely cutaneous presentation, and thus it is termed primary cutaneous ALCL (pcALCL): for this reason, it is discussed in the cutaneous T-cell lymphoma section, among the primary cutaneous CD30$^+$ T-cell lymphoproliferative disorders. The last entity is inherently associated with breast prostheses and therefore termed breast implant-associated (BIA) ALCL, nowadays considered as a new provisional category.

ALCL represents nearly 15% of all PTCL and accounts for approximately 2% of all adult non-Hodgkin lymphomas and 10% to 30% of childhood non-Hodgkin lymphomas.[8,9] Taken as a whole, it shows a bimodal age of presentation with a peak in children and young adults and a second peak around the age of 60. More precisely, ALCL-ALK$^+$ is mostly diagnosed in the first three decades of life; on the other hand, ALCL-ALK$^-$ usually affects adults, with a median age of 54 to 61 years. BIA-ALCL typically presents approximately one decade after implant placement, with a median age of 52.5 years.

Morphology and Pathobiology

ALCL is histologically characterized by the presence of sheets of *hallmark cells*: these are large anaplastic cells with round to kidney-shaped nucleus, central nucleolus, and variably basophilic cytoplasm. Different morphological variants of ALCL-ALK$^+$ are recognized: the *common type* (diffuse growth and sinus invasion), the *lymphohistiocytic type* (with hallmark cells hidden within a reactive lymphohistiocytic background), the *small cell type* (the neoplastic cells are mainly of small size), and the *Hodgkin-like type* (characterized by nodular growth and fibrosis which suggest a nodular sclerosis variant of a classical Hodgkin lymphoma). All morphologic variants can be represented in the same lymph node. ALCL-ALK$^-$ more commonly presents with a *common type* pattern of growth.

The phenotype is often aberrant, lacking the common leukocyte antigen (CD45) and some or all the T-cell associated markers (CD3, CD2, CD5 e CD7) in up to 30% of cases. ALCL are more frequently of CD4 origin, but CD8$^+$ or CD4$^-$/CD8$^-$ cases can occur. The most peculiar immunophenotypic feature is the wide and strong expression of the lymphoid activation molecule CD30. Additionally, ALCL-ALK$^+$ shows a nuclear positivity for the ALK protein (Fig. 89.5).

ALCL-ALK$^+$ harbors the t(2;5)(p23;q35) translocation, which juxtaposes the *ALK* gene to the *NPM* (nucleophosmin) gene on chromosome 5.[26] The *ALK* gene encodes for a tyrosine-kinase receptor belonging to the insulin receptor superfamily, which is usually silent in normal lymphoid cells and absent in normal (but few cerebral) cells. The NPM protein is expressed at a nuclear level in normal cells and acts as a shuttle protein from the nucleus to the cytoplasm. This translocation produces an *ALK-NPM* fusion gene capable of encoding for a novel chimerical aberrant protein absent in normal tissues. The NPM side of the newly produced molecule resides both in the nucleus and the cytoplasm, where it can be detected with specific commercially available antibody. Translocation variants have been described, which always involve the *ALK* gene on chromosome 2. The aberrant NPM-ALK protein, as well as the fusion proteins resulting from variant translocations, constitutively activate downstream effectors, including phospholipase Cγ, PI3K-AKT, Ras-ERK, JAK3-STAT3, and the Sonic hedgehog signaling pathways, all of which lead to prolonged survival and proliferative advantage of the neoplastic cells.

The molecular features of ALCL-ALK$^-$ are far less known. Approximately 30% of cases harbor rearrangements of *DUSP22* as a consequence of the t(6;7) translocation: this alteration identifies a subset of patients with more favorable outcomes, closely resembling ALCL-ALK$^+$, as the 5-year OS approximates 90%.[27] Conversely, rearrangements of the *TP63* gene, marked by inv(3) and identified in 8% of the cases, are associated with very poor outcomes, as the 5-year OS is only 17%. Other genetic aberrations, including *PDRM1/BLIMP1*

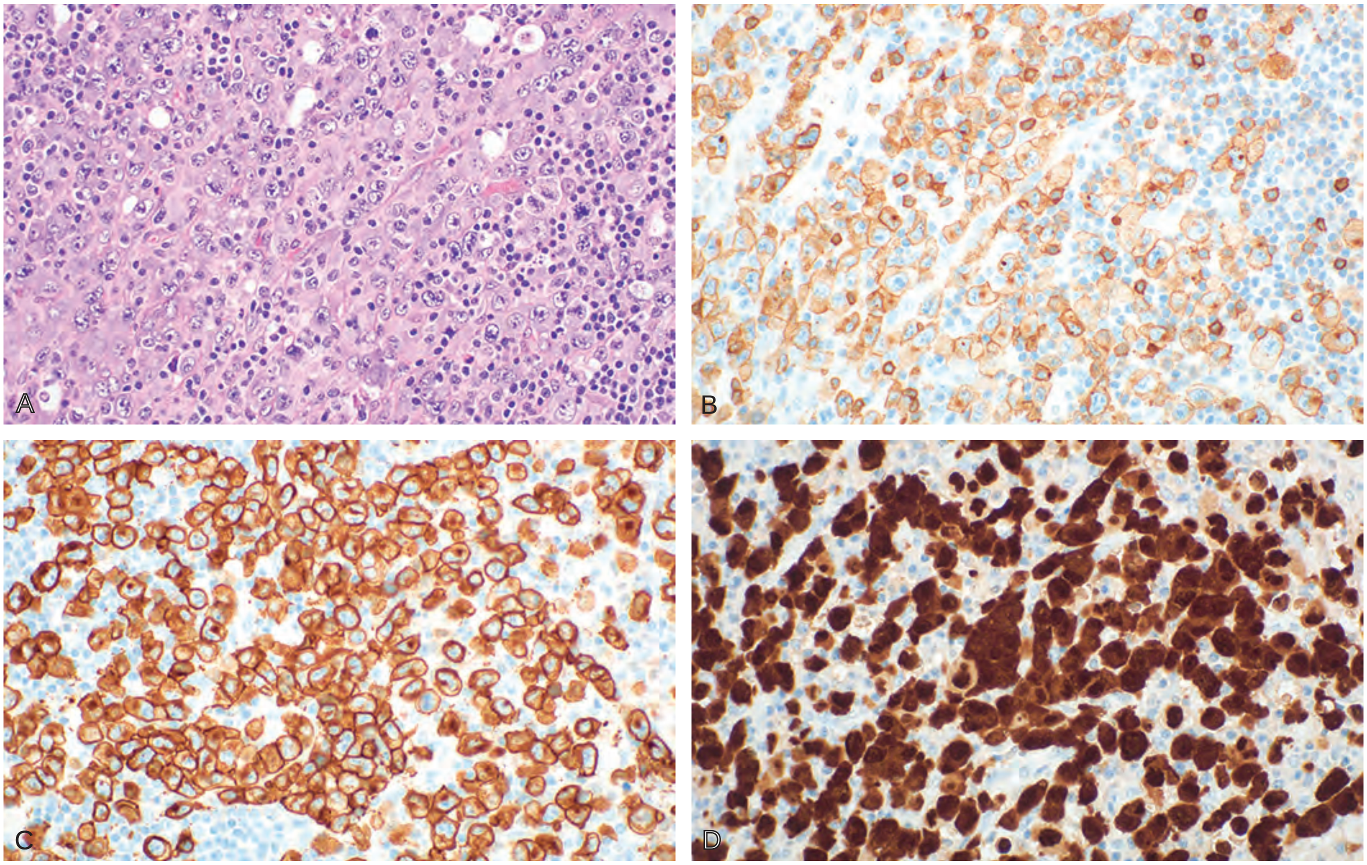


Figure 89.5 Anaplastic large-cell lymphoma, lymph node biopsy, hematoxylin-eosin staining (panel A). Immunohistochemistry for CD5 (panel B). CD30 is highly expressed on neoplastic elements (panel C). Expression of the anaplastic lymphoma kinase (ALK) by immunohistochemistry (panel D).

gene inactivation and the 17p13/*TP53* genetic loss, define a poor prognostic clinical entity.

BIA-ALCL does not harbor recurrent translocations found in other ALCL-ALK$^-$; conversely, activation of STAT3 is common and recurrent mutations of *STAT3*, *JAK1*, or *SOCS1* have been described. There is a close association of this disease with textured rather than smooth breast implants; implant surface texturing may promote bacterial proliferation, with lymphocyte activation and chronic inflammation due to a bacterial biofilm infection around the implant. The associated chronic T-cell stimulation could promote lymphomagenesis.[28]

Clinical Manifestations

Clinical presentation of ALK$^-$ and ALK$^+$ subtypes is similar. About 70% of patients with ALCL present with advanced-stage disease at diagnosis. In one third of patients, peripheral or abdominal nodal disease is associated with bone marrow infiltration or extranodal involvement, more commonly at the skeleton, lungs, and liver. Advanced-stage disease is documented in at least 60% of cases, and lymphoma-related symptoms—mainly fever—can be often encountered at onset.

BIA-ALCL first presents as a seroma fluid that accumulates between the implant and the surrounding fibrous capsule. More than 90% of the cases are localized at the time of diagnosis (stage I–II), sometimes with regional adenopathy, thus suggesting that surgical capsulectomy may be curative. Alternatively, the disease may develop as a growing mass, eventually with distant dissemination. The clinical course varies widely from apparent spontaneous resolution to systemic treatment-resistant disease and death.[29]

Prognosis

Outcomes are highly variable depending on the specific disease subset: this indicates why a clear cut among subtypes is necessary. The 5-year OS rate ranges between 70% and 86% for ALCL-ALK$^+$ and between 30% and 49% for ALCL-ALK$^-$. The 5-year OS of ALCL-ALK$^+$ however gets poorer in patients with advanced age and more advanced disease stage at presentation (see Table 89.7). BIA-ALCL displays a good prognosis if properly managed, with a 5-year OS rate of 89%.

Hepatosplenic T-Cell Lymphoma

HSTCL is an aggressive subtype of extranodal lymphoma, accounting for less than 1% of all non-Hodgkin lymphomas and less than 5% of all PTCL. A strong association with immunosuppressive treatments, such as those related with solid organ transplantation, as well as with some immune-compromised states, as it happens with pregnancy, inflammatory bowel disease, and other systemic autoimmune disorders, has been noted.[30]

Morphology and Pathobiology

HSTCL results from a proliferation of non-activated cytotoxic T cells, usually monomorphic and medium-sized, that exhibit a unique sinusoidal pattern of infiltration of the splenic red pulp, liver, and bone marrow. For this reason, diagnosis is usually confirmed on liver and bone marrow biopsy, and more rarely upon splenectomy. Neoplastic cells generally express CD2, CD3, CD7, CD16, and CD56, and lack

CD4, CD5, CD8, and CD57. Positivity for TIA-1 and granzyme-M is usually documented, but cells tend to be negative for perforin and granzyme-B. The majority (80%) of cases express the γ/δ dimer of the TCR (for this reason, the disease was previously known as hepatosplenic γ/δ T-cell lymphoma, a term now abandoned), but importantly the remaining 20% of cases express an α/β dimer.

Given the rarity of HSTCL, precise mechanisms underlying pathogenesis and disease evolution remain unclear. The most frequent known genetic abnormalities in HSTCL are isochromosome 7q and trisomy 8 but the role of somatic mutations and other genomic alterations in HSTCL has yet to be defined. Mutations in genes like *SETD2*, *INO80*, *STAT5B*, *SMARCA2*, *TET3*, and *PIK3CD* are frequently encountered. Importantly, it has been demonstrated that disruptions of *SETD2* mediate the increased cellular proliferation rate in HSTCL. On the other hand, mutations seen in other T-cell lymphomas and involving genes like *RHOA*, *CD28*, and *CCR4* are characteristically absent or occur at much lower frequency.[31]

Clinical Manifestations

HSTCL demonstrates a predilection for young males. Median age at presentation is 34 years, although epidemiology data are rather scarce. Most patients present with B-symptoms and organomegaly. Massive splenomegaly may be encountered in nearly 80% of cases; nodal involvement is not a characteristic feature, being reported in less than 25% of the cases. Hepatic involvement is the rule, but despite parenchymal infiltration elevated transaminases may only be seen in 50% of cases. Pancytopenia is common, as the bone marrow is often infiltrated. Peripheral blood lymphocytosis is uncommon and when it occurs it may mimic acute leukemia.

Prognosis

The present treatments are largely unsatisfactory, and this disease displays a severe prognosis, with a 5-year OS of 7% and progression-free survival (PFS) of 0%. Prolonged survival intervals have been anecdotally reported after an induction with alemtuzumab or cladribine or after allogeneic stem cell transplantation.

Primary Intestinal T-Cell Lymphomas

Primary intestinal T-cell lymphomas are neoplasms with an aggressive behavior that comprise approximately 20% of all intestinal lymphomas and less than 10% of all PTCL. EATL and MEITL are regarded as the most common entities and together they encompass more than 90% of all cases. Since EATL and MEITL have overlapping clinical and pathologic features, they were considered disease variants in the prior WHO classification and termed EATL types I and II, respectively. Biomolecular studies performed over the last decade have revealed biological differences that required their distinct categorization in the revised 2017 edition of the WHO classification. Both entities are described in this paragraph, but their features are presented separately.

Enteropathy-Associated T-cell lymphoma

EATL is a neoplasm derived from intraepithelial lymphocytes that develops in individuals with celiac disease, a common immune-mediated gluten-sensitive enteropathy with an incidence of nearly 1% in Western populations. This lymphoma accounts for slightly less than 5% of all gastrointestinal lymphomas and 5% of PTCL, but it is acknowledged as the most common primary intestinal T-cell lymphoma in Western countries, accounting for 66% to 80% of all cases, with a median age at onset of 64 years.

EATL is more frequently observed in Europe and the United States where the celiac risk alleles HLA DQA1*0501 and DQB1*0201 are more common, compared to Eastern regions. Homozygosity for HLA-DQ2.5 (encoded by HLA DQA1*0501 and DQB1*0201), poor adherence to a gluten-free diet, and older age are all risk factors for the development of this disease.[32]

Morphology and Pathobiology

The histopathologic features of EATL are highly variable, either among different patients or within a single case. The neoplastic lymphocytes morphologically vary between highly polymorphic elements to a more monomorphic appearance, sometimes resembling immunoblastic elements. In some cases, a mixed inflammatory infiltrate composed of histiocytes, eosinophils, and plasma cells may obscure the malignant cells. The histiocytic granulomatous aggregates may lead to the mistaken impression of Crohn disease. The neoplastic cells frequently spread within the epithelial compartment thus migrating adjacently to or distantly from the initial tumor mass. Portions of small intestine away from the tumor mass frequently show typical histopathologic features of celiac disease; importantly, however, mucosal changes are more prominent proximally and tend to improve distally.

EATL typically expresses CD103, CD2, cytoplasmic CD3, CD7, TIA-1, perforin, and granzyme-B, and lack CD4, CD5, and CD56 expression. Most cases lack surface CD3 and TCR. The Ki-67 proliferation index is generally higher than 50%.

Virtually all EATL cases have clonal TCR rearrangements, mainly of β and γ chain genes, with an association to HLA DQA1*0501 and DQB1*0201, as said. Genome-wide copy number analyses have revealed recurrent gains of chromosomes 9q, 7q, 1q, 5q, and losses of 16q, 8p, 13q, 9p, but a clear identification of the altered genes remains largely unknown. Recurrent genetic mutations have been detected in members of the JAK-STAT pathway (*JAK1, JAK3, STAT3 STAT5B, SOCS1*), MAPK pathway (*BRAF*, *KRAS*, *NRAS*), and epigenetic modifiers (*TET2*, *SETD2, YLPM1*); mutations in DNA damage response and repair and tumor suppressor genes (*TP53*, *BRIP1*, *TERT*, *BCL11b*, *DAPK3*, *PRDM1*) have also been reported.[33]

Clinical Manifestations

EATL may be associated with abdominal discomfort and pain at onset, along with fatigue, anorexia, diarrhea, nausea and vomiting, and possibly B-symptoms, especially weight loss. Symptoms may be present for weeks to years prior to the diagnosis of EATL or may arise acutely in individuals without known celiac disease. Likewise, reappearance of malabsorption in a patient with a history of celiac disease favorably responding to a gluten-free diet or, alternatively, the sudden onset of gluten-insensitive severe malabsorption in an otherwise healthy individual can be key symptoms of disease at its onset. More than half of the patients with EATL (58%) have no prior diagnosis of celiac disease and in these cases celiac disease is diagnosed either concurrently with EATL or afterward.

Nearly half of the patients present with an acute abdomen secondary to intestinal perforation and hemorrhage, because of disease penetration into the intestinal wall. The jejunum is the mostly affected site, although any segment can be involved. When multiple segments of the small intestine have been involved, it is likely that the disease has disseminated to nearby and distant organs (mesenteric nodes, liver, spleen, lungs, and bone marrow).

Refractory Celiac Disease

In some celiac disease patients, development of EATL is preceded by a precursor disease state known as refractory celiac disease (RCD): this is defined as the persistence—or recurrence—of severe gastrointestinal symptoms and villous atrophy despite adherence to a gluten-free diet for at least 12 months, after excluding any secondary etiologies for the symptoms. RCD has been categorized into two subtypes (RCD-I and RCD-II): RCD-I represents a polyclonal expansion of intraepithelial lymphocytes that display a normal immunophenotype, whereas RCD-II (also known as intraepithelial T-cell lymphoma or *cryptic* EATL), is a clonal proliferation of immunophenotypically abnormal intraepithelial lymphocytes. Both RCD-I and RCD-II patients experience recurrent or persistent episodes of malabsorption, but the severity of malnutrition in RCD-II is usually more profound than in RCD-I. Also, the risk of developing EATL is different between the two subpopulations, being 3% to 14% within 5 years in patients with RCD-I compared to nearly 50% in RCD-II.

Prognosis

The clinical course of this disease is very unfavorable and roughly 20% of patients are alive at 5 years. Surgical resection of the affected intestinal tract may offer longer remission rates only in case of a single focal lesion. In most instances, however, the disease involves multiple segments of the gastrointestinal tract or has disseminated to lymph nodes or distant organs.

Monomorphic Epitheliotropic Intestinal T-Cell Lymphoma

MEITL is the second most common primary intestinal T-cell lymphoma. It occurs in older adults, with a median age of 61 years and shows male predominance (male-to-female ratio of 2:1). In contrast to EATL, there is no definitive association with celiac disease.

Morphology and Pathobiology

The neoplastic infiltrate is composed of a monotonous (thus the term *monomorphic*) population of small- to medium-sized T-lymphocytes lacking neoplastic features but characterized by the presence of a rim of clear or pale pink cytoplasm. At the periphery of the tumor, the neoplastic cells often demonstrate epitheliotropism. The immunophenotype overlaps with one of the normal intraepithelial lymphocytes (CD103$^+$, CD3$^+$, and CD8$^+$), although CD56 is commonly expressed, along with cytotoxic markers (TIA-1 and granzyme-B). MEITL expresses TCR γ and β chains in 23% to 61% and 16% to 46% of the cases, respectively; 6% to 33% of the cases are TCR silent and 16% are positive for both TCR γ and β chains. Megakaryocyte-associated tyrosine kinase (MATK) is expressed almost constantly (87% of the cases), being particularly helpful in distinguishing MEITL from EATL.[34]

Similar to EATL, gains at 9q (48% to 70%) and losses at 16q (23% to 56%) represent the most common copy number alterations; gains of chromosome 8q24 (with the involvement of the *MYC* locus) are also frequent, but *MYC* rearrangements are rare. The most common mutations occur in members of the JAK-STAT (76% to 83%) and MAPK (32% to 56%) pathway and deletions or mutations that inactivate *SETD2* are observed in the majority of MEITL (up to 80% of the cases). Finally, *SYK* and *NCAM1* (CD56) are more frequently overexpressed in MEITL compared to EATL.

Clinical Manifestations

Approximately half of MEITL patients are asymptomatic, whereas a few patients complain of non-specific gastrointestinal symptoms, like abdominal pain, weight loss, nausea or vomiting, and diarrhea. In a significant proportion of cases, however, the disease has an abrupt onset with an acute abdomen due to intestinal perforation. The disease is usually multifocal within the intestinal tract, with jejunum and ileum being the mostly affected sites; also, the colon and the stomach may be affected (10% to 28% and 5% of the cases, respectively). Extraintestinal dissemination to mesenteric lymph nodes or pelvic organs is common, whereas extra-abdominal diffusion is rare.

Extranodal NK/T-Cell Lymphomas

ENKL is a rare neoplasm occurring with higher prevalence among Asians, Mexicans, and South Americans of Native American ethnic origin. It accounts for approximately 6% of all non-Hodgkin lymphomas in these ethnic groups (20% to 40% of all PTCL), as opposed to nearly 1.5% in all other populations (up to 4% to 5% of all PTCL). This neoplasm affects adults almost exclusively, with a median age at presentation of 49 to 53 years and a male-to-female ratio ranging from 2:1 to 3:1. The nasal cavity is the most common site of involvement and it was the prototypic site when this disease was initially characterized; for this reason, the neoplasm is generally termed *nasal type*, although extranasal localizations—even with lack of nasal/paranasal involvement—do exist.

EBV seems to play an important role in lymphomagenesis, as it is invariably detected in nasal ENKL cases, irrespective of patients' origins, and in the great majority of lymphomas presenting outside the nasal cavity. Iatrogenic immunosuppression may facilitate the development of this neoplasm, e.g., in renal transplant recipients.

Morphology and Pathobiology

The affected tissue appears diffusely infiltrated by packed lymphoma cells. Infiltrating cells are small-to-medium in size, sometimes larger, with irregularly folded nuclei and a variable amount of cytoplasm. Necrosis is common. The presence of tumor cells is accentuated around blood vessels (*angiocentric growth*), with their walls being infiltrated and destructed and their lumen frequently thrombosed. The immunophenotype of neoplastic cells is prototypically CD2$^+$, CD3$^-$, CD56$^+$; CD43 and CD45RO are commonly positive; the expression of CD7 is variable. Other T-cell-associated antigens, such as CD4, CD5, CD8, and α/β-γ/δ TCR are negative (TCR is in germline configuration in virtually all cases). Cytotoxic molecules like perforin, granzyme-B, and TIA-1 are usually positive. CD30 is positive in about one half of cases; its staining is focal and not as intense as in other PTCL. The proliferative fraction, according to Ki-67, is usually higher than 50%; the higher the rate, the worse the prognosis, especially with nasal presentation.

Overexpression of TP53 is found in 45% to 86% of the cases, along with *TP53* gene mutations in a quarter to two-thirds of the cases. Mutations of β-catenin, *KRAS*, and *c-KIT* genes can be detected in 6% to 20% of cases, although their biologic role is unclear. Demonstration of killer cell immunoglobulin-like receptor (KIR) repertoire restriction along with germline TCR genes is useful to support the monoclonal nature of the process and to confirm its NK-cell origin.

Clinical Manifestations

Nasal ENKL arises in the nasal cavity, nasopharynx, or upper aerodigestive tract, causing both obstructive symptoms and destructive and ulcerative lesions. The disease shows a tendency to spread to nearby tissues and structures, such as the orbits, the paranasal sinuses, the oral cavity, and the palate, usually determining bone erosions; it may also extend to the oropharynx. Extranasal sites are far less involved than nasal cavity and paranasal spaces; the skin is the most frequently affected site, along with the gastrointestinal tract, the testis, and the lung. Eye and facial soft tissues can also be involved, as it happens during the dissemination of primary nasal type disease.

Early-stage disease (I–II) characterizes patients with nasal involvement; clinical symptoms are mostly related to facial destruction and bone marrow involvement is exceedingly rare. On the contrary, patients with extranasal disease frequently display advanced stage (III–IV) at presentation and often complain of systemic symptoms. Skin nodule ulceration, bowel obstruction, or perforation and testicular masses characterize the extranasal spread of this lymphoma.

Prognosis

High response rates and favorable long-term survival rates can be obtained in patients with localized disease with radiotherapy or chemotherapy + radiotherapy approaches, as discussed below, and 5-year OS rates range from 40% to 78%. Relapse is common, however. Extranodal or advanced disease is much more aggressive and difficult to treat. Long-term survival rates for patients with advanced disease are dismal, and median OS is only 4.3 months.

Subcutaneous Panniculitis-Like T-Cell Lymphoma

SPLTCL is a mature lymphoma of cytotoxic α/β T-cell derivation that involves the subcutaneous fat similarly to panniculitis. It is a rare disease involving predominantly young individuals (both young adults and children), with a median age at onset of 35 years and no clear sex predilection. This is acknowledged as an extranodal T-cell lymphoma, but more correctly included in the classification of CTCL.

Morphology and Pathobiology

Atypical lymphoid T cells of variable size admixed to benign histiocytes infiltrate the subcutaneous tissue, sparing the overlying dermis and epidermis. Neoplastic lymphocytes rim adipose spaces, and fat necrosis is frequently encountered. Vascular invasion is a common feature. Tumor cells characteristically express CD3 and CD8 but lack CD4. Perforin, granzyme-B, and TIA-1 are present. Positive stain for βF1, an antibody that identifies the α/β dimer of the TCR, is characteristic.

Clinical Manifestations

Solitary nodules or plaques are the hallmark of the disease, usually involving the lower extremities, but sometimes spread to both arms and the trunk. Nodules display variable dimensions; they have an indurated appearance and do not ulcerate. Systemic symptoms, like fever and weight loss, may also be present.[35]

Prognosis

The 5-year disease-specific survival is 80%, as the disease rarely disseminates to lymph nodes and distant organs; recurrences may be observed over periods of several years, but they tend to remain confined to subcutaneous tissue. Very rare cases associated with hemophagocytic syndrome display an aggressive behavior and a poor outcome.

First-Line Therapy

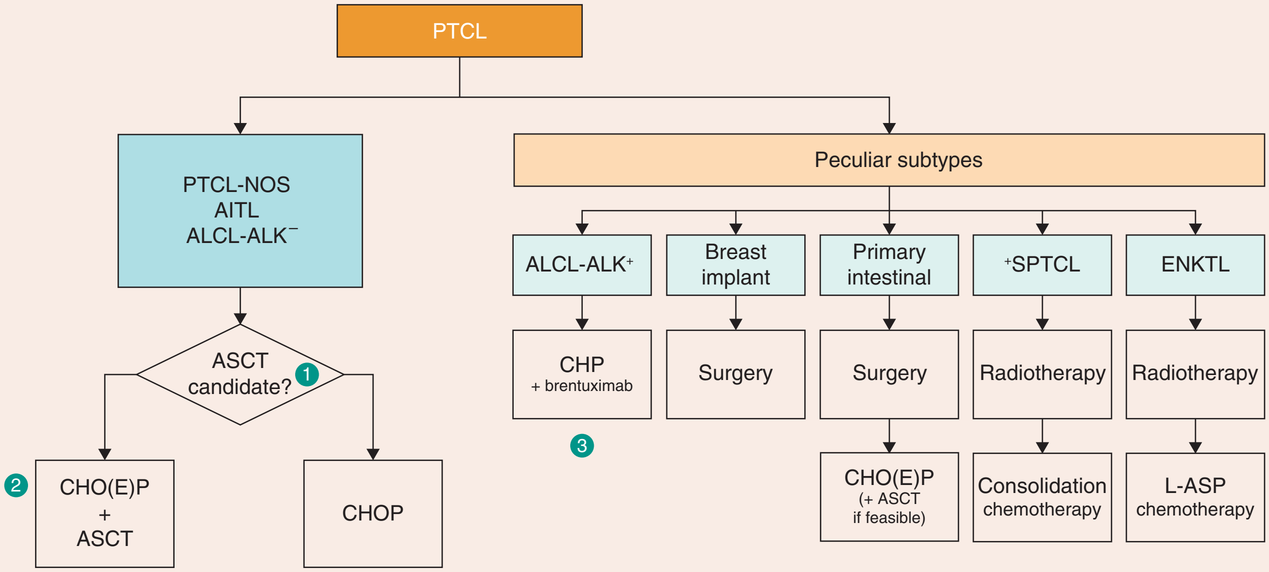
The main goal of first-line treatment should be the achievement of at least a partial remission, in order to apply a consolidative autologous stem cell transplantation (ASCT), where appropriate (described later in the chapter), and to enhance the opportunity of a good control of the disease in the long term.

The treatment strategies most widely adopted in aggressive PTCL are the same applied in aggressive B-cell lymphomas; however, as PTCL are biologically and clinically different from the B-cell counterparts, a significant prognostic gap exists between lymphoma of B- and T-cell origin when the same (or at least very similar) approaches are applied.

Data regarding treatment results can be difficult to analyze given that most of—if not all—the older studies report on all entities of PTCL treated together as a single entity. For this reason, treatment modalities for the initial treatment of nodal aggressive PTCL are presented together. The growing knowledge about the heterogeneity of PTCL has in part led to separate certain entities from others as far as treatment approaches are concerned. This is particularly true for extranodal PTCL, in which surgery or radiotherapy may play a major role, and for ENKL, given the peculiar sensitivity of this disease to selected classes of cytostatic agents. The treatment of specific disease subtypes is discussed below and in the box on Frontline Management of PTCL.

FRONTLINE MANAGEMENT OF PTCL

- Systemic chemotherapy is the mainstay of frontline treatment in all nodal forms. Anthracycline-containing regimens (like CHOP) are generally acknowledged as the gold standard. Etoposide may be added (CHOEP regimen).
- Autologous stem cell transplantation (ASCT) is generally regarded as the most suitable consolidation treatment, and for this reason it is given frontline, provided the patient is eligible (review patient's comorbidities, disease status, performance status, age).
- Some peculiar PTCL subtypes may require a different treatment because of: (a) lower risk of disease progression or relapse over time (ALCL-ALK$^+$); (b) curability with loco-regional procedures (e.g., surgery in breast implant-associated ALCL; radiotherapy in SPTCL); c) clear extranodal presentation (e.g., primary intestinal T-cell lymphomas).



Controversial Points

1. Can new drugs be added to the standard CHOP backbone (e.g., brentuximab vedotin in CD30$^+$ disease, lenalidomide in AITL)?
2. Can frontline ASCT be avoided in patients achieving a complete remission?
3. Do we need ASCT consolidation in ALCL-ALK$^+$ patients with higher-risk disease (e.g., IPI ≥ 2)?

ASCT is regarded as a key step in the consolidation of an initial response. However, its role is still debated, as we lack evidence of its benefit in patients in first CR. Experiences with ASCT in PTCL is discussed later.

Induction Regimens

Anthracycline-based regimens are the mainstay of induction treatment of PTCL patients, as demonstrated in several reported experiences in which the CHOP combination was adopted in a range of patients variable from 60% to 85%. The overall response rates (ORR) ranged from 70% to 80% for ALCL-ALK$^-$ patients, with CR rates in up to 50% of the cases. On the contrary, ALCL-ALK$^+$ patients responded to first-line CHOP in up to 90% of cases. Nearly 40% of patients with ALCL-ALK$^+$ disease and roughly 60% of those with ALCL-ALK$^-$ disease however failed to maintain their response over time with induction only. The role of anthracyclines as part of the induction schedule is much more debated in AITL and PTCL-NOS. On the one hand, it should be noted that according to the ITCLP the CR rate observed in patients receiving an anthracycline-based induction was 61% for AITL patients and 56% for PTCL-NOS patients, however with no significant survival advantage for those receiving anthracyclines when compared to patients treated with anthracycline-free schedules. On the other, different data sets indicate that anthracycline-containing regimens can improve OS and PFS in all patients affected by PTCL, particularly in those with AITL and PTCL-NOS subtypes, and that the benefit is reinforced when ASCT is applied as first-line consolidation.[36–40]

A retrospective analysis of German T-cell lymphoma trials suggested that the addition of etoposide to CHOP can be more effective than CHOP alone, at least in PTCL-NOS and ALCL patients; the CHOEP regimen, given either every 14 or 21 days, improved response and event-free survival (EFS) rates in young patients with normal LDH levels (3-year EFS was 70.5% after CHOEP and 51.0% after CHOP), although 3-year OS did not significantly differ between the two groups. In addition, CHOEP failed to enhance clinical outcomes in patients older than 60 years, for whom CHOP should remain the standard first-line approach.[41]

Combination of New Drugs With Standard Chemotherapy

Given that CHOP is regarded as the reference regimen for the induction treatment of patients with PTCL, it has been used as a backbone for new first-line combination regimens which incorporate drugs that have demonstrated their efficacy when used as single agents in relapsed or refractory patients (refer to "Treatment of relapsed and refractory disease" paragraph for a more detailed description of these agents.) First-line combination treatments are intended to enhance CR rates, to allow a higher proportion of patients to undergo ASCT in remission and to prolong survival (Table 89.8).

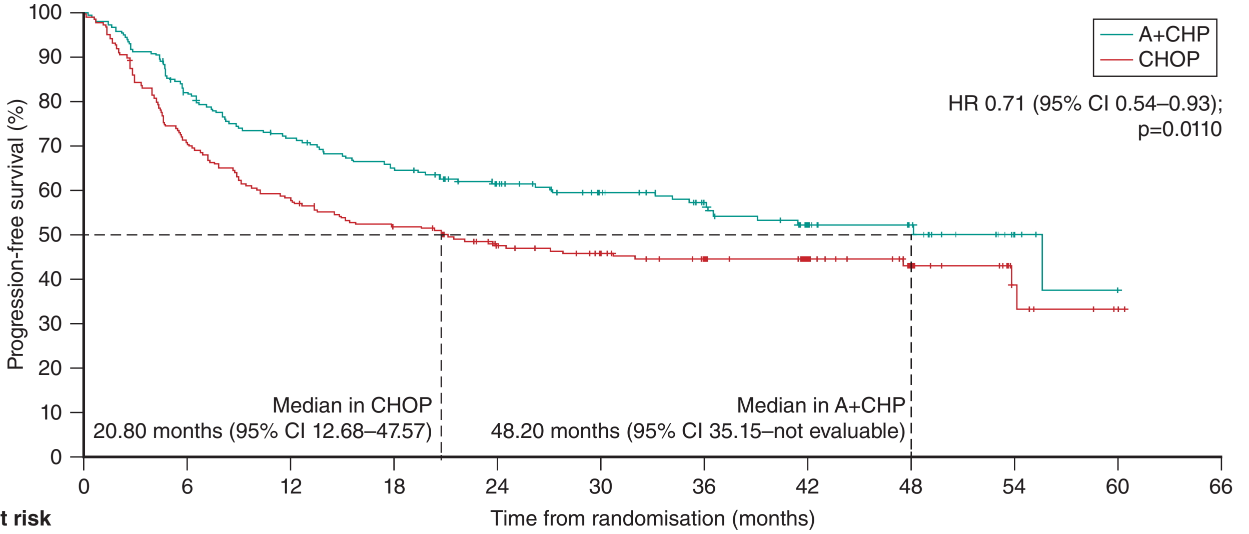
Phase 1/2 trials combining pralatrexate, romidepsin, belinostat, and alemtuzumab with CHOP and CHOP-like regimens have shown the feasibility of the proposed combination. Nevertheless, published studies are relatively small and sometimes displayed a high incidence of toxic events. Randomized trials exploring the combination of alemtuzumab with CHOP have been completed but have failed to show the superiority of the combination regimen, both in terms of disease control and as far as OS and PFS are concerned. The combination of romidepsin and CHOP has not demonstrated any superiority over standard CHOP.

The only clearly positive study was ECHELON-2, a large double-blind multicenter randomized phase 3 trial. This trial tested the combination of brentuximab vedotin (BV) plus cyclophosphamide, doxorubicin, and prednisone (BV-CHP regimen) versus standard CHOP in previously untreated subjects with CD30-expressing PTCL (at least 10% of the cells). The experimental arm did not include vincristine in order to minimize the incidence of peripheral neuropathy, given the concomitant administration of BV. Consolidative transplantation, in case of response, was not mandatory, but its application was at discretion of treating physicians. Each arm enrolled 226 patients, with a median age of 58 years and with an advanced stage (Ann Arbor III-IV) in at least 80% of cases. Patients with systemic ALCL represented the majority of patients (72% in the BV+CHP arm and 68% in the CHOP arm), followed by PTCL-NOS (13% and 19%, respectively), AITL (13% and 11%, respectively), and rarer entities. An overall response was seen in 83% of patients in the experimental arm and in 72% of those treated with CHOP, with CR in 68% and 56% of cases, respectively. The 3-year PFS was 57% for the BV+CHP arm and 44% for the CHOP arm (Fig. 89.6); importantly, this difference remained statistically significant even when patients were censored for PFS immediately before they received stem cell transplantation or radiation therapy, thus indicating that the combination of BV and chemotherapy retains its effectiveness regardless of any subsequent consolidation.[42]

Based on these results, in November 2018 the Food and Drug Administration (FDA) approved BV for the treatment of adult patients with previously untreated systemic anaplastic large cell lymphoma or other CD30$^+$ PTCL in combination with CHP. However, most of the patients in the study had ALCL and the benefit for the BV-CHP

TABLE 89.8 **CHOP Combinations With New Agents in the Frontline Treatment of Peripheral T-Cell Lymphomas**

Combination	Phase	Randomization	Pts.	ORR, %	CR, %	PFS, %	OS, %
Pralatrexate + CHOP	1	None	31	89	67	—	—
Belinostat + CHOP	1	None	23	89	72	—	—
Romidepsin + CHOP	1/2	None	37	68	51	77 (18 months)	—
Pralatrexate + CEOP	2	None	33	70	52	39 (2 years)	60 (2 years)
Alemtuzumab + CHOP	2	None	24	75	71	48 (2 years)	53 (2 years)
	2	None	20	90	65	30 (2 years)	55 (2 years)
Romidepsin + CHOP	3	Ro-CHOP	211	63	41	43 (2 years)	64 (2 years)
		CHOP	210	61	37	36 (2 years)	63 (2 years)
Brentuximab + CH(O)P	3	BV-CHP	226	83	68	57 (3 years)	NR (median)
		CHOP	226	72	56	44 (3 years)	NR (median)
Alemtuzumab + CHOP	3	A-CHOP (+ ASCT)	65	—	52	37 (3 years)	52 (3 years)
		CHOP (+ ASCT)	66	—	42	26 (3 years)	50 (3 years)
	3	A-CHOP	58	—	60	26 (3 years)	38 (3 years)
		CHOP	58		43	29 (3 years)	56 (3 years)



Number at risk (number censored)

	0	6	12	18	24	30	36	42	48	54	60	66
A+CHP	226 (0)	175 (39)	149 (61)	134 (75)	108 (82)	81 (85)	64 (88)	38 (93)	24 (93)	9 (94)	3 (95)	0 (95)
CHOP	226 (0)	157 (65)	129 (93)	112 (107)	87 (116)	75 (119)	63 (121)	44 (121)	26 (122)	7 (123)	2 (124)	0 (124)

Figure 89.6 PROGRESSION-FREE SURVIVAL IN PATIENTS TREATED IN THE BV$^+$CHP ARM COMPARED TO THOSE IN THE CHOP ARM. *(Reprinted with permission from Elsevier, Horwitz S, et al. Brentuximab vedotin with chemotherapy for CD30-positive peripheral T-cell lymphoma (ECHELON-2): a global, double-blind, randomized, phase 3 trial. Lancet. 2019; 393: 229–240.)*

regimen was not statistically significant in non-ALCL subtypes. Therefore, the evidence is not as strong as with ALCL to recommend BV-CHP in the frontline treatment of CD30$^+$ non-ALCL PTCL.

Frontline Autologous Stem Cell Transplantation

Patients responding to first-line chemotherapy treatment generally display a short duration of remission and a high risk of relapse; for this reason, a frontline consolidation with ASCT has been considered a valid therapeutic opportunity for patients achieving a response to induction. This strategy seems to be particularly important for those with intermediate to high IPI score, while it is not indicated for ALCL-ALK$^+$. This is mainly because ALCL-ALK$^+$ cases have not been enrolled in prospective clinical trials evaluating the role of upfront ASCT, mainly because of their overall better prognosis compared to the remaining nodal entities.

The first multicenter prospective trial aimed at establishing the benefit of frontline ASCT in PTCL patients has involved 111 patients, treated with 6 to 8 CHOP induction cycles followed by mobilizing chemotherapy and total-body-irradiation + cyclophosphamide myeloablative conditioning, with the rescue of autologous stem cells. Overall, 68% could successfully undergo ASCT, while failure to do so was mainly related to early disease progression. Patients obtained a CR in 59% of the cases after myeloablative conditioning, with 5-year OS, disease-free survival (DFS), and PFS rates of 44%, 54%, and 39%, respectively, for the entire intention-to-treat population.[43]

The Nordic Lymphoma Group applied a CHOEP induction strategy (given every 14 days), although omitting etoposide in patients over 60 years, in 160 PTCL patients. The fifth or sixth cycle was used as a mobilizing therapy, while the upfront ASCT was conditioned by carmustine, etoposide, cytarabine, and melphalan (or high-dose cyclophosphamide). The reported CR rate was 51%; the 5-year OS and PFS were 51% and 44% for the entire patient population, respectively.[44]

These trials indicate that ASCT consolidation is able to offer a chance of long-term survival in PTCL patients. Nevertheless, it should be noted that a substantial proportion of patients (16% to 41%) had evidence of disease progression during induction or immediately before transplantation, thus precluding an effective consolidation in a relevant proportion of cases.

A systematic review and meta-analysis of 27 publications covering 1368 patients reported a PFS rate of 33% for the prospective studies analyzed, with an OS of 54% and a transplant-related mortality (TRM) of 2%. It is worthwhile to note that these studies are inherently biased by the selection of better outcome patients (i.e., only patients with good quality responses are candidates for ASCT), and that about one-third of the enrolled patients have failed to receive the planned ASCT either because they did not achieve a remission or due to early progression.[45]

Results from the Comprehensive Oncology Measure in Peripheral T-cell Lymphoma Treatment (COMPLETE) study have recently been reported. This observational study identified 119 patients with nodal PTCL in first CR; 36 of them received ASCT whereas 83 did not. In multivariate analyses, ASCT was independently associated with improved OS (49); in particular, ASCT was associated with superior survival for patients with advanced-stage disease or intermediate-to-high IPI. In terms of PTCL histology, ASCT significantly improved OS and PFS for patients with AITL but not for patients with other PTCL subtypes.[46]

This issue has been recently analyzed by some registry studies, which collectively failed to show an advantage for ASCT when the analysis focused on patients in CR after induction. In a French study of over 500 patients, 57% of responders had the same outcome regardless of whether they received ASCT or not, with a 5-year OS and PFS of 60% and 45%, respectively, for both groups.[47]

In conclusion, no randomized trials have specifically clarified whether upfront ASCT should be regarded superior to conventional chemotherapy only or, more specifically, if patients in CR after induction strictly require a high-dose chemotherapy consolidation. However, upfront consolidation with ASCT is still recommended by current guidelines for patients with chemo-sensitive PTCL-NOS, AITL, and other rare subtypes when in first CR after induction (see box on Frontline Management of PTCL).

Treatment of Specific Disease Subtypes

Much more disappointing results have been achieved with standard chemotherapy in patients affected by extranodal disease subtypes, like EATL, HSTCL, SPTCL, and ENKL, which nowadays still represent a clearly unmet medical need. Alternative treatment strategies, other than standard chemotherapy, are urgently required.

Low-risk ALCL-ALK$^+$ (IPI 0 to 1)

Given the overall better prognosis of this disease entity, ASCT is not generally considered as part of initial therapy in this category of patients. Those with low IPI score, and more in general those whose IPI score is lower than 3, may take advantage of an anthracycline-containing induction (either CHOP or CHOEP, as discussed above), without the need of consolidation with ASCT. Of note, the most relevant trials of ASCT in PTCL have excluded ALCL-ALK$^+$ patients, therefore no definitive conclusions may be drawn for this category of patients on the role of ASCT.[39,40]

BIA-ALCL

Complete surgical excision, with removal of the implant, total capsulectomy, and complete avulsion of any associated mass with negative margins upon pathological evaluation, is recommended in any patients either presenting with effusion or tumor masses. Systemic chemotherapy alone is insufficient unless applied after surgical resection. At least 30% of patients undergoing chemotherapy showed unsatisfactory responses or progressed in a published series of 87 patients with BIA-ALCL, whereas only 4% of patients had recurrence or progression when treated by complete resection of the implant, tumor tissue, and fibrous capsule. Better OS and PFS rates were described for patients undergoing complete surgical excision in comparison to those who received chemotherapy, radiotherapy, or a limited/partial surgical approach (partial capsulectomy, implant removal or replacement, excisional biopsy of capsule or tumor mass). Multi-agent chemotherapy is justified in cases with a tumor mass presentation at onset rather than with effusion only, as these patients are thought to display a more aggressive clinical behavior. Chemotherapy is also required when disease disseminates to nearby and distant nodes; however, any systemic approach needs to be preceded by a radical surgical resection.[48,49]

EATL and MEITL

Patients with EATL and MEITL display one of the poorest prognoses among all patients with PTCL, with a 5-year OS and PFS of only 20% and 4%, respectively, even in case of favorable IPI score. An initial surgical approach is rather common in EATL and MEITL patients, as many of them show acute abdominal symptoms (bowel perforation, hemorrhage, obstruction) requiring prompt intervention. An intensified induction with an ifosfamide-containing regimen and methotrexate, followed by ASCT has demonstrated better outcomes in terms of OS and PFS (60% and 50% at 5 years, respectively) if compared with historical controls receiving standard anthracycline-containing inductions. Nevertheless, overall toxicity is not negligible, and most patients are unable to tolerate this aggressive approach or fail to proceed to ASCT consolidation because of poor performance status after surgery, rapid disease progression, or clinical decay.[50,51]

SPTCL

Radiation therapy (20 to 30 Gy), possibly preceded by reductive surgery, should be recommended in patients with localized lesions. Multiple, non-contiguous lesions may be treated with pulse steroids (prednisone or equivalents), at the dose of 0.6 to 0.7 mg/kg/day × 10 days/month. Systemic chemotherapy with gemcitabine, or multiagent chemotherapy (CHOP, CHOP-like) is advisable in case of systemic dissemination or progressive disease.

ENKL

Management strictly depends on stage at presentation and local *versus* distant disease spread. Patients with localized ENKL, which means a disease that involves the nose and the nasal sinuses, may benefit of external radiation therapy (at a dose of at least 50 Gy), which is sometimes the only required treatment. In a cooperative study from Japan, concurrent systemic chemotherapy has also been administered in nearly three-quarters of patients, the dexamethasone, etoposide, ifosfamide, and carboplatin (DeVIC) regimen being the most widely used. Patients with primary disease localization other than nose and nasal sinuses rarely show localized disease and require systemic multiagent approaches. ENKL has proved to be particularly sensitive to L-asparaginase, which has been incorporated in several induction schemes, like SMILE (dexamethasone, methotrexate, ifosfamide, L-asparaginase, etoposide), which produced higher response rates (79% of ORR, with 45% of CR) if compared to standard anthracycline-containing inductions.[52] Toxicity is severe, however, and this regimen should be applied with a careful supportive treatment. An asparaginase-containing regimen, the GELOX (gemcitabine, L-asparaginase, oxaliplatin), has also been applied with concomitant radiotherapy in localized-stage ENKL, yielding an ORR of 96%, with at least 74% of patients achieving a CR.[53]

Relapsed and Refractory Disease

Survival outcomes for patients with PTCL who relapse or progress after first-line treatment are very poor. In a series of 153 patients with relapsed/refractory PTCL diagnosed between 1976 and 2010 from the British Columbia Cancer Agency, it was shown that the median PFS and OS after first recurrence or disease progression were only 3.1 and 5.5 months, respectively, without stem cell transplant. Those receiving systemic chemotherapy after progression or relapse did not have significantly improved survival compared to those who did not. Only a small number of patients survived a relatively long period without transplant with 3-year OS of 18%. There was no significant difference in PFS and OS after first recurrence or disease progression by PTCL subtypes. Similar results have been reported by other research groups. Interestingly, however, patients with ALCL seem to display better OS outcomes with first salvage treatments if compared to AITL and PTCL-NOS, with 47.3 - and 10.8-months median OS for ALCL-ALK$^+$ and ALCL-ALK$^-$, respectively.[39,54]

These results clearly indicate that PTCL patients failing an initial treatment are generally hard to be saved with conventional chemotherapy, thus representing a clear unmet medical need. New agents targeting specific molecular hallmarks of these disease entities may have a rationale in the treatment of relapsed and refractory patients. Licensed agents and off-label compounds are discussed in detail in the next paragraphs (Table 89.9).

TABLE 89.9 **Selected Experiences With Food and Drug Administration (FDA)-Approved Single Agents and Off-Label Compounds in Relapsed and Refractory Peripheral T-Cell Lymphomas Patients**

Treatment	AITL		PTCL-NOS		ALCL	
	ORR, %	CR, %	ORR, %	CR, %	ORR, %	CR, %
Pralatrexate[a]	8	—	32	—	35	—
Romidepsin[a]	30	19	29	14	24	19
Belinostat[a]	46	—	23	—	15	—
Brentuximab vedotin[b]	54	38	33	14	86	57
Lenalidomide[c]	33	11	43	14	10	0
Mogamulizumab[c]	50	25	19	6	100	100*

ORR, overall response rate; *CR*, rate of complete responses (if available).

[a] The drug has received a Food and Drug Administration approval in patients with relapsed and refractory peripheral T-cell lymphoma.

[b] The drug has received a Food and Drug Administration approval in patients with CD30$^+$ relapsed or refractory anaplastic large cell lymphoma.

[c] This compound is used off-label for the treatment of relapsed and refractory PTCL.

*Response is established on 1 ALK-negative patient.

Licensed Agents

As of the writing of this chapter, 5 compounds have been approved by the FDA for their specific use as single agents in the setting of patients with relapsed or refractory PTCL: pralatrexate, a novel antifolate agent; romidepsin and belinostat, both histone deacetylase (HDAC) inhibitors; BV, an anti-CD30 drug-conjugated monoclonal antibody; and mogamulizumab, an anti-CC chemokine receptor 4 (CCR4) monoclonal antibody. Although available in the United States, the European approval of both pralatrexate and romidepsin has been rejected due to a lack of evident clinical benefit; belinostat is not licensed in Europe, but it has been granted orphan designation status by the European Medicines Agency. In fact, their approval by the FDA was based on response rates alone and not on a documented increase in OS. BV has granted approval for the treatment of relapsed or refractory systemic ALCL and now it is moving to frontline PTCL treatment according to the results of the ECHELON-2 trial (see above). Mogamulizumab is now approved for ATLL in Japan and in pretreated MF and SS patients (see section on CTCL).

Pralatrexate

Pralatrexate is an aminopterin derivative administered intravenously with high affinity for the reduced folate carrier-1 and for folypolyglutamate synthase. This causes the retention of the drug in the cytoplasm and a potent inhibition of dihydrofolate reductase, which ends up with blocking the reduction of dihydrofolate to tetrahydrofolate. Given that tetrahydrofolate works as a cofactor for the metabolism of some amino acids (such as methionine, serine, and glycine) and for the synthesis of pyrimidines and purines, pralatrexate ultimately causes a depletion of thymidine monophosphate and other nucleotides, thus determining cell-cycle arrest and apoptosis.

The phase 2 registration trial was PROPEL, a single-arm study that involved 115 patients with relapsed or refractory PTCL, 111 of whom received at least 1 dose. The majority of patients had PTCL-NOS followed by systemic ALCL and AITL (53%, 15% and 12%, respectively), all heavily pretreated. Pralatrexate was administered intravenously at the dose of 30 mg/m^2/week for 6 weeks, followed by 1 week of rest, until progressive disease or unacceptable toxicity, as determined in a previous phase 1 study. The ORR was 29%, including 11% of patients with CR and 18% with partial response (PR). A quarter of patients without a response to the most recent prior treatment responded to pralatrexate, as well as 19% of those who had never achieved a response. Responses were seen across all histologies, although patients with AITL responded less well than those with PTCL-NOS or ALCL (8% vs. 32% and 35%, respectively). ORR raised to 35% among less treated patients. The median PFS for the entire population was 3.5 months, with a median OS of 14.5 months. Mucositis was the most relevant adverse event, which determined dose reductions in 23% of patients and caused withdrawal from treatment in 6% of cases.[55]

Histone Deacetylase Inhibitors

HDAC inhibitors alter the acetylation pattern of histone lysine residues. They mainly work through chromatin remodeling and restoration of normal gene expression in cancer cells, which results in cell growth inhibition, cell cycle regulation, and induction of apoptosis. However, HDAC can target substrates other than histones and may play a role in the post-translational acetylation of proteins. HDAC inhibitors therefore interfere with several other cellular mechanisms, like autophagy, cell differentiation and cell cycle progression, modulation of immune responses, and suppression of angiogenesis. Romidepsin and belinostat are the two HDAC inhibitors tested in PTCL patients that received FDA registration for their use as single agents in relapsed and refractory PTCL patients.

Romidepsin is a cyclic tetrapeptide (depsipeptide) that selectively inhibits class 1 HDAC after its interaction with the zinc atom in the binding pocket of the enzyme. It has been shown that romidepsin is capable of downregulating the pro-survival NFκB, PI3K-AKT and β-catenin pathways in PTCL patients; it can also increase the production of reactive oxygen species, decrease the mitochondrial membrane potential and put malignant T cells under significant biologic stress.

The phase 2 registration study involved 130 relapsed or refractory and advanced-stage PTCL patients, among which there were 69 PTCL-NOS, 27 AITL, and 21 ALCL-ALK$^-$. The trial demonstrated an ORR of 25%, with 19% CR and with 29% of patients responding despite being refractory to their most recent prior systemic therapy. The median PFS was 4 months for the entire cohort of patients, raising to 18 months for patients in CR, and OS was 11.3 months. Adverse events were predominantly hematologic and included neutropenia, lymphopenia, thrombocytopenia, and anemia. Nausea, fatigue, vomiting, diarrhea, anorexia, and dysgeusia were among the most prevalent—albeit not severe—nonhematologic adverse events, reported in at least 20% of the patients enrolled in the trial.[56]

Belinostat is a hydroxamic acid-derived pan-HDAC inhibitor. Like other HDAC inhibitors, it regulates apoptosis and cell cycle arrest of transformed cancer cells. It is administered intravenously over 30 minutes at the fixed dose of 1000 mg/m^2 on days 1 to 5 every 21 days, as established in phase 1 escalation dose studies in patients with advanced malignancies, and treatment is maintained until unacceptable toxicity or progression.

The phase 2 registration trial (BELIEF) involved 129 patients with relapsed or refractory PTCL, more than half affected by PTCL-NOS. The ORR was 23%, most impressively in AITL patients (for whom ORR was 46%), whereas none of those affected by extranodal T-cell variants (intestinal T-cell lymphomas and HSTCL) achieved a response. Of note, meaningful responses were seen in 16% of patients refractory to their last prior systemic treatment, although patients who displayed a CR to their last prior treatment could obtain a higher ORR (48%). Median PFS was however only 1.6 months and the median OS was 7.9 months. In terms of safety, relevant adverse events were nausea, fatigue, and pyrexia; anemia, neutropenia, and thrombocytopenia were overall the most common grade 3 to 4 toxicities, although belinostat seemed apparently less myelotoxic than romidepsin.[57]

Brentuximab Vedotin

BV is an antibody-drug conjugate which enables the delivery of a cytotoxic agent directly to its malignant target, composed by an anti-CD30 antibody conjugated through a protease-cleavable linker with monomethylauristatin E (MMAE), a potent microtubule-disrupting agent. Once the antibody has bound to CD30 on the cell surface, the complex is internalized and trafficked to lysosomes, where MMAE is released by proteolytic cleavage. The cytotoxic effects of MMAE on the microtubule network cause cell cycle arrest and induce apoptotic death. It is plausible that a certain amount of MMAE is uptaken by the tumor microenvironment and subsequently released to tumor cells regardless of antibody binding. This would explain in part the efficacy of BV also in context of low (or sometimes even absent) CD30 expression.

BV is now approved in the treatment of relapsed and refractory ALCL, according to the results of the pivotal phase 2 trial. This study involved 58 systemic ALCL patients with a confirmed CD30$^+$ status by immunohistochemistry. An objective response was obtained in 86% of the cases, with a CR in 57%. With a median follow-up of 58 months, the 5-year OS rate was 60% for the entire patient population, with a median OS not reached. Patients in CR fared better, with a 5-year OS of 79%; on the contrary, patients not achieving a CR had a 5-year OS rate of 25%. The 5-year PFS rate for the entire population was 39%, with a median of 20 months; among CR cases, the median PFS rate was not reached, and at 5 years the estimated rate was 57%. Patients with ALCL-ALK$^-$ had similar response and survival rates to those with ALCL-ALK$^+$. Importantly, patients who achieved a CR and who received subsequent consolidative stem cell transplantation had comparable outcomes to those who did not undergo consolidation. Peripheral neuropathy was the most relevant safety concern regarding the treatment with BV. Fifty-seven percent of patients in the study experienced this adverse event, which resolved in 67% of cases or improved (regressed to at least grade 1 or less) in 24%, mainly due to drug withdrawal.[58]

Besides ALCL, a phase 2 study involved 35 patients with mature T-cell lymphoma with variable CD30 expression, among which there were 22 subjects with PTCL-NOS and 13 with AITL. Patients were treated with BV at the standard dose of 1.8 mg/kg every 3 weeks, as reported for Hodgkin lymphoma and ALCL patients. Responses were seen in 41% of cases, with an ORR of 33% for PTCL-NOS and 54% for AITL, and a CR rate of 14% and 38%, respectively. No apparent correlation between CD30 expression and depth of response was documented, as responses could be seen in cases of undetectable CD30 upon centralized pathological review. This means that it is plausible that not much CD30 expression may be related for an effective drug targeting.[59]

Mogamulizumab

Mogamulizumab is a humanized anti-CCR4 monoclonal antibody characterized by a defucosylated Fc region that stimulates antibody-dependent cellular cytotoxicity, with a potent antitumor activity toward T-cell lymphoma cell lines and ATLL mouse models. CCR4 is a marker for type 2 helper T cells and T_{reg} and is expressed on tumor cells in 30% to 65% of patients with PTCL, conferring a worse prognosis when expressed.

The drug was granted approval for ATLL in Japan based on the results of a multicenter phase 2 study conducted on 28 patients relapsed after at least one previous systemic line. In this study, mogamulizumab administered intravenously at the dose of 1.0 mg/kg (as established from the previous phase 1 trial) once a week for 8 weeks determined an ORR of 50%, including 31% CR.[60]

Data on relapsed and refractory $CCR4^+$ PTCL patients are derived from two phase 2 studies conducted in Japan and Europe. The Japanese study showed a higher ORR than observed in the European trial (34% vs. 11%), without an apparent explanation, although PFS was comparable between the two experiences (2.0 vs. 2.1 months, respectively). Toxic effects were also similar, with peripheral cytopenias, skin rash, pyrexia, and infusion reactions being the most frequently observed.[61,62]

Off-Label Compounds

Besides approved agents, other drugs have demonstrated efficacy in patients with various subtypes of PTCL who have failed one or more previous treatment lines. The studies in which these agents have been evaluated have generally involved patients with mixed diagnoses, so that it is difficult to clearly establish if a peculiar PTCL histology may respond more favorably to a specific single agent. Taken together, the results obtained with investigational and off-label compounds in terms of ORR, CR rates, and survival rates are close to what has been already described for the FDA-approved drugs pralatrexate, romidepsin, belinostat, and BV. Nevertheless, most of the trials in which these agents were involved have not been designed to follow patients for survival; therefore, it is difficult to draw definite conclusions on how these molecules effectively impact on disease history, and apart from some favorable response rates, none of these agents has yet proved to ameliorate survival functions in relapsed or refractory patients with PTCL.

Lenalidomide

It is an immunomodulatory drug, working in several hematologic malignancies, including multiple myeloma, with a mechanism of action that mainly rests on 3 principles: (i) antiproliferative effect, mediated by its binding to cereblon ($CRL4^{CRBN}$), which in turns induces the downregulation of IKZF1 and 3 and leads to a decreased expression of NFκB, MYC, and IRF4, an enhanced expression of $p21^{WAF1}$, and the suppression of cell cycle via degradation of cyclin-dependent kinases; (ii) immunomodulation, depending on the increased levels of IL-2 as a consequence of lenalidomide binding to $CRL4^{CRBN}$ in T cells, which consists of an improvement in T-cell and NK-cell antitumor activity; (iii) inhibition of angiogenesis, with a consequent reduced microvessel density within the tumor.

A first phase 2 study evaluating lenalidomide monotherapy in PTCL enrolled 29 patients with relapsed or refractory disease. The drug produced an ORR of 29%, with a median duration of response (DOR) of 5 months.[63] A second phase 2 study focusing on patients with PTCL-NOS showed an ORR of 30%.[64] In a multicenter phase 2 trial with 54 patients conducted in France, the ORR was 22% with a CR rate of 11%. Non-significant slightly higher response was seen in patients with AITL, with an ORR of 33%. The median PFS and the median DOR were 2.5 and 3.6 months, respectively. The most common side effects were hematologic toxicities, with grade 3 to 4 thrombocytopenia and neutropenia observed in 20% and 15% of patients, respectively.[65]

Bendamustine

Bendamustine is a bifunctional compound, structurally consisting of chloroethylamine, an alkylating group, attached to a benzimidazole ring, a purine analog, and a butyric acid side chain that imparts water solubility. Its role in relapsed and refractory PTCL was evaluated at the dose of 120 mg/m^2 (administered intravenously on days 1 to 2 every 3 weeks) in the phase 2 BENTLY trial, which enrolled 60 patients (58 affected by PTCL and 2 by MF). Although 33% of patients could not receive more than 3 cycles mainly due to disease progression, the ORR was 50% with a CR rate of 28%. Responses were seen across multiple histologies, but their duration was short (3.5 months), lasting more than 1 year in only 7% of patients. Grade 3/4 neutropenia and thrombocytopenia were seen in 30% and 24% of patients, respectively.[66]

Gemcitabine

Given as a single agent in a population of older (median age 54 years, up to 78 years) and pretreated (median of three previous treatment lines) patients with relapsed and refractory PTCL-NOS, gemcitabine allowed a very good disease control yielding an ORR of 55% and a CR rate of 50%, with a median response duration of 28 months. No treatment related grade 3 to 4 adverse events were seen; the most frequently reported grade 1 to 2 toxicities were neutropenia, thrombocytopenia, and elevation of liver enzymes.[67] A combination of gemcitabine, cisplatin, and methylprednisolone (GEM-P) was previously tested in an English experience with 16 PTCL patients, yielding a 69% of ORR and a 19% of PR across various disease subtypes.[68]

New Drugs

Demethylating Agents

Recurrent mutations in *TET2*, *DNMT3A*, *IDH2* are known in a relevant percentage of patients with T_{FH} PTCL. For this reason, hypomethylating agents could rationally demonstrate a clinical effect in T_{FH}-derived PTCL, including AITL.[21,22] Recently, the French group reported the outcomes of 12 patients with AITL (all but one with relapsed and refractory disease and in 5 cases with a concomitant myeloid neoplasm), with a median age of 70.5 years, treated with subcutaneous azacitidine at the dose of 75 mg/m^2 given daily for 7 consecutive days, every 4 weeks. Nine patients responded, with a CR in half of the cases and a PR rate of 25%. At a median follow-up of 27 months, the median PFS was 15 months, and the median OS was 21 months, with 5 patients showing a sustained CR lasting more than 2 years.[69]

Ruxolitinib

The JAK-STAT pathway is commonly deregulated in PTCL and mutations in the JAK and STAT system in T-cell neoplasms have been detected in the SH2 domain of STAT3 and STAT5 and in the pseudokinase domain of JAK1 and 3.[11] It can be hypothesized that JAK-STAT pathway alterations, detected as elevated phospho-STAT3 expression or presence of JAK or STAT mutations, will predict the activity of ruxolitinib, a JAK1/2 inhibitor, in patients with PTCL and CTCL. This has been tested in a phase 2 clinical trial in which ruxolitinib, at the dose of 20 mg bid, was given to patients with relapsed and refractory PTCL and CTCL failing at least one previous systemic therapy. The ORR obtained so far are 38% and 40%, respectively in patients bearing JAK or STAT mutations (cohort 1) and functional activation of the JAK-STAT system without concomitant mutations (cohort 2). The observed rate of CR is still low, albeit accrual is ongoing; nevertheless, the observation of objective responses or disease stability for more than 6 months while on treatment provides the rationale of JAK-STAT targeting in T-cell lymphomas.[70]

Duvelisib

PI3K inhibitors block mitogen and prosurvival signals from the tumor and its microenvironment and concomitantly activate an antilymphoma immune response. Duvelisib, an oral dual inhibitor of PI3Kδ and PI3Kγ, was applied as a single agent in a phase 1 trial involving 16 PTCL and 19 CTCL patients with relapsed and refractory disease. The drug was administered on a dose-escalation basis, starting from 25 mg twice daily up to 100 mg twice daily on a 28-day cycle; the maximum tolerated dose was set at 75 mg twice daily, and 77% of the enrolled patients were treated at this dose. In the PTCL population, the ORR was 50%, with 3 CR and 5 PR, all occurring within the first 2 to 4 months of treatment. Responses were seen across multiple histologies, including PTCL-NOS, EATL, and AITL. Median OS and PFS were 8.4 and 8.3 months, respectively. Transaminase elevation was the most relevant adverse event (57% of cases), with grade 3 to 4 toxicity described in 40% of patients. Pharmacodynamic results showed that a down-modulation of serum cytokines and chemokines related to T-cell activation and migration and to the microenvironment (such as CCL22, CXCL13, tumor necrosis factor-α, matrix metallopeptidase-9, and IL-16) occurred within 8 days of starting treatment.[71]

Immunotherapy

The development of checkpoint inhibitors, targeting the interaction of PD-1 with its ligands (namely PD-L1) with subsequent repression of T-cell activation has changed the therapeutic landscape in multiple malignancies. In PTCL, increased expression of PD-L1 has been described in both malignant and stromal cells across various subtypes, thus supporting the rationale of a therapeutic checkpoint-inhibition. Indeed, a PR rate of 40% was described in PTCL patients enrolled in a phase 1b study with nivolumab. Albeit preliminary, this trial suggests further efforts in order to clarify the role of PD-1 blockers in PTCL, alone or in combination with other agents.[72]

Concepts for chimeric antigen receptor (CAR) T cells are being developed for PTCL. However, as pan-T-cell aplasia (the *"fratricide" effect*) causes severe immunodeficiency, the application of CAR-T cells in this context is challenged by the lack of specific antigens discriminating malignant *versus* normal T-lymphocytes. The mutually exclusive expression pattern of the TCR β-chain constant domains 1 and 2 (TRBC1 and TRBC2) might be a promising approach to target neoplastic T cells harboring either TRBC1 or TRBC2 while preserving a substantial proportion of normal T cells.[73]

Allogeneic Hematopoietic Stem Cell Transplantation

Reports on allogeneic hematopoietic stem cell transplantation (allo-HSCT) in patients with T-cell lymphomas are just a few and tend to group together patients with very advanced and often refractory disease and several histology subgroups. Therefore, it is hard to draw conclusions about its clinical impact and no clear distinctions can be operated for different disease subtypes.

The rationale behind allo-HSCT rests on the fact that allogeneic hematopoietic stem cells are free of tumor contamination and that donor-derived immune cells are potentially capable of mediating an antitumor (i.e., graft-versus-lymphoma) effect.[74] This is demonstrated by responses to donor lymphocyte infusions. Disease status at transplantation and chemosensitivity are outcome predictors. Patients in remission at the time of transplantation may achieve a cure and OS curves reach a plateau at approximately 18 months. On the contrary, only 25% to 30% of refractory patients may take advantage of the procedure.

The French group has reported the outcome of 77 patients who received allo-HSCT: the 5-year TRM was 33%, and the 5-year OS and PFS were 57% and 53%, respectively. In this study, most patients received myeloablative conditioning regimen and relatively high TRM was a concern.[74]

Reduced-intensity conditioning (RIC) regimen has become a more standard approach. A phase 2 Italian study of RIC allo-HSCT in 17 patients with relapsed and refractory PTCL showed 3-year OS and PFS rates of 81% and 64%, respectively, with lower TRM rates (6% at 2 years).[75] In a similar study from the United States, again with 17 patients, the 3-year OS and PFS were 59% and 53%, respectively, and the 3-year TRM was 19%.[76] In a Japanese study, 354 patients (200 PTCL-NOS, 77 AITL, and 77 ALCL patients) who received allo-HSCT were retrospectively analyzed: the 3-year TRM rates and the 3-year OS rates in younger patients (16 to 49 years) who received myeloablative regimen were 22% and 43%, respectively, while in those who received a RIC regimen they were 14% and 56%, respectively.[77]

At present, allo-HSCT is feasible in just a few patients with relapsed PTCL, as most of cases are characterized by rapid progression, clinical decay, and chemoresistance, all factors that may preclude a timely and effective application of this approach. Better OS and PFS results are obtained when allo-HSCT is performed early in the course of the disease and after a few treatment lines: this suggests that allo-HSCT should be performed as early as possible in patients with recurrent disease and preferably in chemosensitive individuals.

Future Directions

Advances in the molecular pathology of PTCL have provided the rationale for better classification of patients, more effective prognostic stratification, and ultimately more powerful treatment approaches. Current first-line and salvage therapies for PTCL patients are still largely unsatisfactory. New drugs have offered some improvement in frontline setting and, more importantly, in patients with relapsed and refractory disease. However, response durations are short, and many patients still develop progressive disease while under treatment, thus having no chance for long-term good quality outcomes.

Preclinical models have suggested potent synergy between novel drugs, especially between HDAC inhibitors and pralatrexate, PI3K inhibitors and azacytidine. This means that we have initial evidence that some non-chemo drugs may complement their activity when combined with other drugs active in PTCL. The addition of checkpoint inhibitors to doublets may reinforce the synergistic paradigm in the future, pointing toward the development of lineage-specific and disease-specific chemo-free combinations.

CUTANEOUS T-CELL LYMPHOMAS

CTCL are defined as T-cell lymphomas with exclusive cutaneous involvement, lacking any nodal or extracutaneous disease at the time of their diagnosis. They represent 75% of all primary cutaneous non-Hodgkin lymphomas, with the rest being represented by cutaneous B-cell counterparts. The current classification of CTCL is part of the joint WHO-European Organization for Research and Treatment of Cancer (EORTC) classification of primary CTCL (containing both B- and T-cell lineage diseases). It was first published in 2005 and then included in the 2006 WHO classification of skin tumors, ultimately incorporated in the 2008 WHO classification of tumors of hematopoietic and lymphoid tissues and its subsequent 2017 revision. In August 2018, an updated version of the WHO-EORTC classification (Table 89.10) was published in the fourth edition of the WHO classification of skin tumors, mostly maintaining the same terminology currently applied in the 2017 WHO classification of hematopoietic neoplasms.[78]

MF and SS are the prototypical forms of CTCL: they are closely related to one another, as composed by neoplastic T cells with the capability of infiltrating the skin and variably circulating in peripheral blood or homing to lymph nodes. Although displaying overlapping presentations and a continuous spectrum of evolution from an initial phase of skin-limited disease to a more disseminated and systemic

TABLE 89.10 The 2018 Update of the World Health Organization-European Organization for Research and Treatment of Cancer (WHO-EORTC) Classification of Cutaneous T-cell Lymphomas*

- **Mycosis fungoides**
- Mycosis fungoides variants
    - Folliculotropic mycosis fungoides
    - Pagetoid reticulosis
    - Granulomatous slack skin
- **Sézary syndrome**
- Adult T-cell leukemia/lymphoma (cutaneous presentation)
- **Primary cutaneous CD30⁺ T-cell lymphoproliferative disorders**
    - **Lymphomatoid papulosis**
    - **Primary cutaneous anaplastic large cell lymphoma**
- **Subcutaneous panniculitis-like T-cell lymphoma (**)**
- **Extranodal Natural Killer-/T-cell lymphoma, nasal type (**)**
- Primary cutaneous peripheral T-cell lymphomas, rare subtypes
    - Primary cutaneous γ/δ T-cell lymphoma
    - *Primary cutaneous CD8⁺ aggressive epidermotropic cytotoxic T-cell lymphoma*
    - *Primary cutaneous acral CD8⁺ T-cell lymphoma*
    - *Primary cutaneous CD4⁺ small/medium T-cell lymphoproliferative disorder*
- Primary cutaneous T-cell lymphoma, not otherwise specified

*Provisional entities are listed in italics. Entities in bold type are explained in the text.
**This entity is described in the peripheral T-cell lymphoma section.

TABLE 89.11 The T (skin), N (nodes), M (Visceral Involvement), and B (Blood Involvement) Parameters Required for the Staging of Mycosis Fungoides

Skin	T_1	Limited patches, papules, and/or plaques covering <10% of the skin surface. Further stratify into T_{1a} (patches only) and T_{1b} (plaques ± patches).
	T_2	Patches, papules, or plaques covering ≥10% of the skin surface. Further stratify into T_{2a} (patches only) and T_{2b} (plaques ± patches).
	T_3	One or more tumors, ≥1 cm in diameter.
	T_4	Confluence of erythema, covering ≥80% of body surface area.
Nodes	N_0	No clinically abnormal peripheral lymph nodes: biopsy not required.
	N_1–N_3	Clinically and histologically abnormal peripheral lymph nodes (firm, irregular, clustered, fixed nodes, ≥1.5 cm in diameter).
	N_X	Clinically abnormal peripheral lymph nodes; no histological confirmation.
Viscera	M_0	No visceral organ involvement.
	M_1	Visceral involvement (requires histological confirmation; involved organ should be specified).
Blood	B_0	Absence of significant blood involvement: ≤ 5% of peripheral blood lymphocytes are atypical (Sézary) cells.
	B_1	Low blood tumor burden: >5% of peripheral blood lymphocytes are atypical (Sézary) cells, but criteria for B_2 are not met.
	B_2	High blood tumor burden: ≥1000/mmc Sézary cells clonally positive.

TABLE 89.12 The TNMB Staging System for Mycosis Fungoides

	T	N	M	B
I A	1	0	0	0–1
I B	2	0	0	0–1
II A	1–2	1–2	0	0–1
II B	3	0–2	0	0–1
III A	4	0–2	0	0
III B	4	0–2	0	1
IV A_1	1–4	0–2	0	2
IV A_2	1–4	3	0	0–2
IV B	1–4	0–3	1	0–2

presentation, the WHO-EORTC classification considers these two entities separately (see Table 89.10). MF and SS are presented under the same heading in this paragraph, as the diagnostic approach and the disease staging are the same for both conditions. The treatment of these two conditions is strictly based on the stage at presentation. For this reason, it will be discussed by focusing on the extent of the disease (limited stage MF *versus* advanced stage MF/SS).

Primary cutaneous CD30⁺ T-cell lymphoproliferative disorders are the second most common group of CTCL, which includes pcALCL and LyP as two extremities of a disease spectrum. Histologic and phenotypic features are mostly overlapping; thus the clinical presentation of the disease and its clinical course are decisive to discriminate clearly between pcALCL and LyP and to decide treatment.

CTCL other than MF, SS, and primary cutaneous CD30⁺ T-cell lymphoproliferative disorders are very rare, and together account for less than 10% of all CTCL cases. These entities are listed in Table 89.10, but their discussion is beyond the scope of this chapter.

A multidisciplinary approach, requiring the involvement of hematologists, experienced hemopathologists, and dermatologists, is strongly encouraged for the management of patients with CTCL, both at diagnosis and at relapse. Cooperation between specialists is of utmost importance, as most CTCL cases are seen by dermatologists first, who need to establish a correct diagnosis and to manage the patient properly according to the extent of the disease. Skin-directed therapies are suitable in case of limited disease, whereas systemic approaches need to be considered when the disease is diffuse or as soon as it progresses under (or after) an initial conservative approach. Systemic treatments are generally administered in a hematology setting. For this reason, they are treated in more detail in this section of the chapter.

Mycosis Fungoides and Sézary Syndrome

MF is characterized by the presence of mature T-lymphocytes, in most cases with a T-helper phenotype, that infiltrate the epidermis in early stages of disease (giving rise to flat or slightly scaly lesions, or patches) or have acquired the capacity to proliferate and invade the dermis (plaques and tumor stages), with possible dissemination to internal organs. SS is the leukemic counterpart of MF. Its classic features are represented by erythroderma (i.e., diffuse reddening of the skin) with hyperkeratosis of palms and soles, lymphadenopathy, and presence of atypical circulating T-lymphocytes with grooved and lobulated cerebriform nuclei (Sézary cells). Patients initially presenting with classic MF lesions—i.e., patches or plaques—may eventually evolve to an erythrodermic and leukemic phase, thus meeting the clinical and diagnostic criteria for SS (being referred to as having leukemic MF or secondary SS). Alternatively, SS may arise *de novo* as a disease independent from a previous MF phase.

Although biologically different (discussed later in the chapter), MF and SS represent a continuous disease spectrum, as clearly emerges from staging criteria (Tables 89.11 and 89.12). In other words, all patients with a high blood burden of disease—if Sézary cells are at least 1000/mmc (B_2 disease, stage IVA) or represent at least 20% of circulating lymphocytes (some of the B1 disease cases, stage IIIB)—are diagnosed with SS, whereas in all other instances the diagnosis is MF (with stage IVA and IVB MF being defined as nodal or visceral MF, respectively).

MF has an incidence of 5.6 cases per million people in Western countries, whereas the age-adjusted incidence of SS is 0.1 per million. Importantly, the incidence of these diseases in a well-defined population strictly depends on the awareness of dermatologists in recognizing such entities and in performing a correct diagnosis and staging workup. They are largely a disease of the middle-aged and elderly people, with highest incidence above 70 years; nevertheless, increased awareness of the disease has led to earlier diagnosis even in much younger patients. Blacks have a higher incidence rate than Caucasians; their onset seems to be earlier and the clinical outcomes appear much worse.

Morphology

Early patches of MF display small lymphocytes scattered in the papillary dermis or around the venules of the superficial plexus; a few of them are within the epidermis. As patches develop, the papillary dermis becomes fibrotic and the lymphocytic infiltrates become denser and band-like, at least in foci. Cells also begin to show atypical features, mostly irregular cerebriform nuclei, which helps in the differential diagnosis with inflammatory conditions. MF cells typically colonize the basal layer of the epidermis and diffuse along the dermoepidermic junction, with a tendency to climb upward (epidermotropism), sometimes forming small lymphocytic aggregates within the epidermis, known as Pautrier microabscesses (Fig. 89.7). Plaque stage is characterized by a significant number of lymphocytes with atypical nuclei and sometimes by prominent collections of lymphocytes in the epidermis. Tumors of MF are consistent with a diffuse replacement of the reticular dermis by atypical lymphocytes, sometimes with aspects of transformation into larger cells with abundant cytoplasm, large oval vesicular nuclei, and prominent nucleoli, as happens in ALCL. The typical phenotype of MF cells is $CD3^+$, $CD4^+$, $CD8^-$, $CD45R0^+$, and most often negative for CD7 and PD1. CD30 is expressed in some MF cells, mostly in tumors enriched with large cells; its expression displays wide variability among lesions even in the same patient.

The histological features of SS overlap with those of MF as far as the cytological details are concerned. SS lymphocytes are generally smaller than MF ones and tend to localize around the small vessels in the superficial dermis, sometimes forming small Pautrier abscesses. On genetic grounds, complex karyotypes are found in many cases, especially in those presenting with more advanced stages.

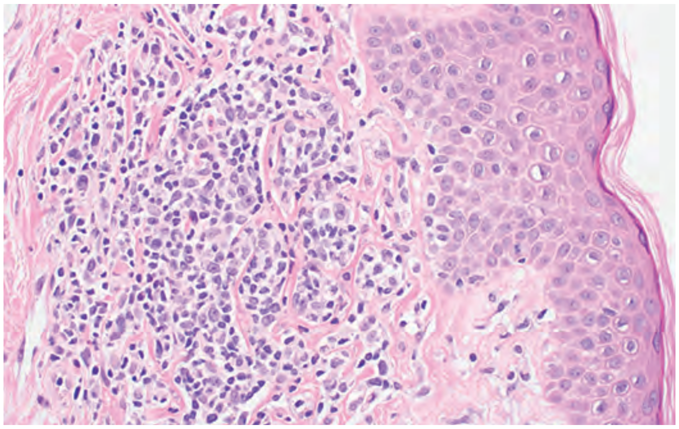


Figure 89.7 MYCOSIS FUNGOIDES, SKIN BIOPSY, HEMATOXYLIN-EOSIN STAINING.

Pathobiology

MF and SS classically arise from skin-tropic memory $CD4^+$ cells; $CD8^+$ or $CD4^-/CD8^-$ cells can also be encountered. The demonstration of different T-cell immunophenotypes and molecular profiles, however, supports the hypothesis that these two malignancies originate from distinct memory T-cell subtypes. More precisely, MF seems to originate from skin resident memory T cells (T_{RM}), whereas SS is a neoplasm of skin-tropic central memory T cells (T_{CM}). This distinction may also explain their peculiar clinical features, as it refers to their tendency to migrate from lymph nodes to blood and skin, and vice versa.[79,80]

When naive T cells residing in skin-draining lymph nodes encounter their cognate antigen, they proliferate clonally as effector T cells and ultimately to memory T cells. Effector T cells express the cutaneous lymphocyte antigen (CLA), a skin-homing receptor that interacts with E-selectin on endothelial and epidermal cells, which favors their translocation from blood to skin and their specific skin tropism. Moreover, they express the C-C chemokine receptor 4 (CCR4), which in turn cooperates in their migration by binding to chemokines produced by the skin itself, such as CCL17 and CCL22. Skin T_{CM} cells also express CCR7 and L-selectin, which is responsible for their circulation between skin and blood and blood and lymph nodes. Once they have migrated to the skin, in other words, they are able to turn back to the blood stream and then to lymph nodes through high endothelial venules, following the interaction between CCR7 and its ligands CCL19 and CCL21 (Fig. 89.8). Skin-resident T_{RM} cells, on the other hand, lack L-selectin and CC7, albeit displaying CLA and CCR4. For this reason, they rarely circulate out of the skin. A third subset of cells, termed migratory memory T cells (T_{MM}), expresses CCR7 but not L-selectin and perhaps has the capability of recirculating more slowly out of the skin toward blood and lymph nodes, compared to T_{CM} cells.[79,80]

Given that T_{RM} cells are non-migratory populations, they constitute fixed skin lesions with well-defined borders; this is what happens in MF patients. On the contrary, the tendency of T_{CM} to recirculate between several compartments explains the diffuse erythroderma of SS patients, which is accompanied by lymph node enlargement and leukemic presentation. Patients with a T_{MM} phenotype have ill-defined but discrete skin lesions.

Clinical Manifestations

MF follows a stagewise progression from patches and plaques to thicker tumor lesions to diffuse erythroderma. Patches are generally round or oval, sometimes they may be digitated, and they vary in size (from 1 cm to more than 10 cm). They can be confined to a limited area—predominantly in sites protected by sunlight—or may show a tendency to disseminate in the former case; the disease generally behaves indolently, requiring no treatment for long periods of time (Fig. 89.9). Plaques present as red-to-brown flat or raised indurated and sometimes ulcerated areas that can further evolve into tumors; the latter are nodular lesions developed on pre-existing patches or plaques, with a high tendency to ulceration and bleeding (Fig. 89.10). The evolution of the disease into progressive stages indicates its increased aggressiveness over time.

Blood involvement is the main characteristic of SS. Erythroderma is the key feature of SS, characterized by a diffusely erythematous and desquamating skin (Fig. 89.11) along with hyperkeratosis of palms and soles, skin fissuration, and loss or dystrophy of nails.

Diagnosis and Staging

Diagnosis is established upon skin biopsy within a compatible clinical context. Immunohistochemistry is also useful to confirm the diagnosis, as well as detection of TCR rearrangements. These elements help discriminate MF from drug reactions, connective tissue diseases, lichen *sclerosus et atrophicus*, and inflammatory dermatoses.

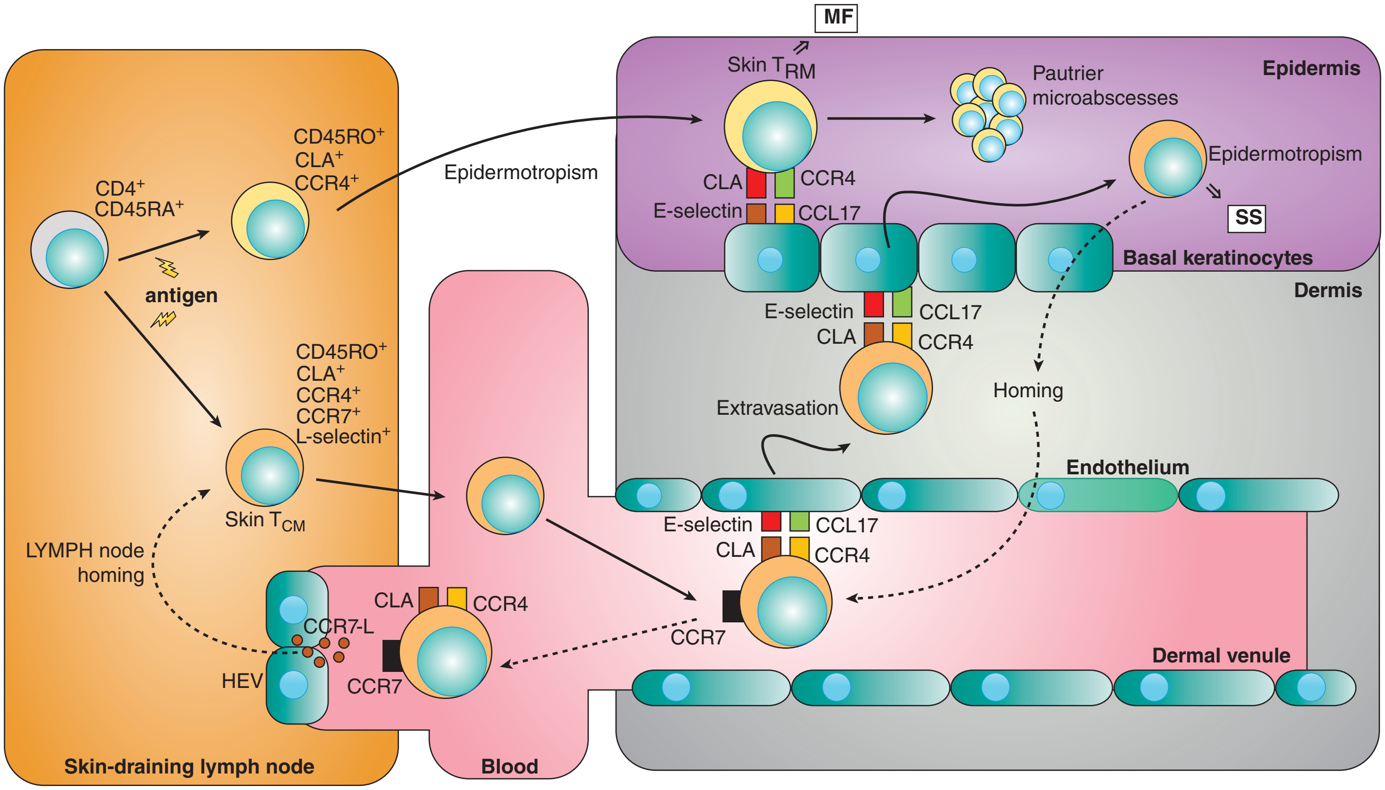

MF
Skin T_{RM}
Pautrier microabscesses
Epidermis
Epidermotropism
SS
Basal keratinocytes
Dermis
CLA
CCR4
E-selectin
CCL17
E-selectin
CCL17
CLA
CCR4
Extravasation
Homing
Endothelium
E-selectin
CCL17
CLA
CCR4
CCR7
Dermal venule
CD4+
CD45RA+
CD45RO+
CLA+
CCR4+
Epidermotropism
antigen
CD45RO+
CLA+
CCR4+
CCR7+
L-selectin+
Skin T_{CM}
LYMPH node homing
CCR7-L
HEV
CLA
CCR4
CCR7
Skin-draining lymph node
Blood

Figure 89.8 THE ONTOGENY OF MYCOSIS FUNGOIDES (MF) AND SÉZARY SYNDROME (SS). Epidermotropism and homing to blood and lymph nodes are the main charactF and SS cells, respectively, which reflect onto clinical features of these diseases. See text for abbreviations and explanation.

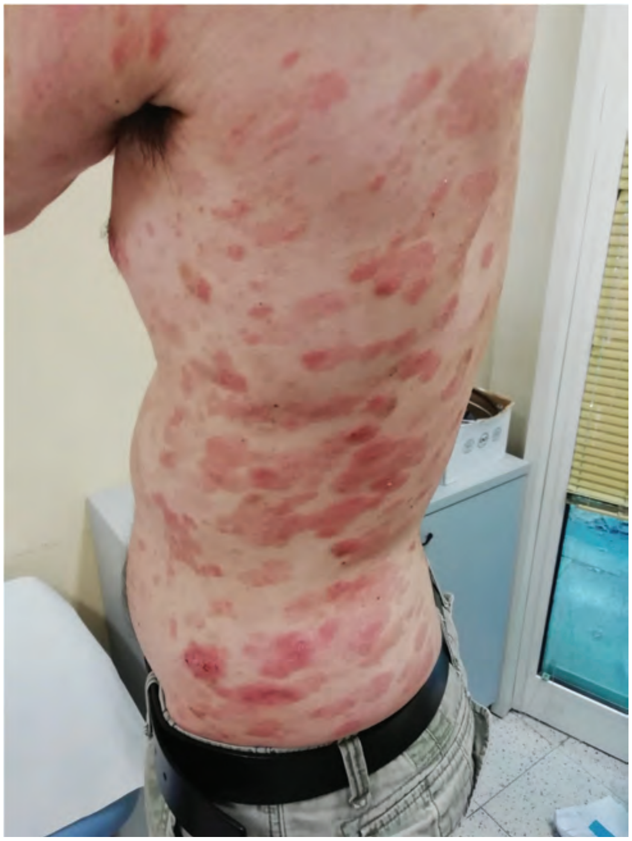


Figure 89.9 MYCOSIS FUNGOIDES, DISSEMINATED PATCHES AND PLAQUES.

SS is diagnosed when Sézary cells are ≥1000/mmc or represent at least 20% of circulating lymphocytes. Detection of TCR rearrangement is required to establish the diagnosis of SS, along with the flow cytometric demonstration of the typical phenotype (generally with a CD4/CD8 ratio ≥10). SS should be placed in differential diagnosis with erythrodermic allergic contact dermatitis and drug eruptions. The clinical approach to the patient is described in the box on Clinical Approach to a Patient Affected by MF or SS and Staging Recommendations.

Prognosis

Outcomes are related to the extent of skin, blood, lymph node, and visceral involvement, according to the TNMB system. Patients with patches of MF covering less than 10% of the body surface area (T_1) have a very indolent disease and their life expectancy is generally unaffected. Those with more diffuse involvement are more likely to develop plaques or tumors (T_3, stage II B and higher). In general, patients with stages I to II A (T_1–T_2) have a "limited-stage" disease and their lifespan is measured in decades. Patients with tumors, blood involvement (B_1–B_2), erythroderma (T_4), nodal and visceral (M_1) involvement have an "advanced-stage" disease, and their survival is severely affected (median OS of 1 to 5 years).

Treatment of Limited-Stage MF

Limited-stage disease is mostly asymptomatic, especially if limited body surface is affected by patches or plaques. For this reason, it is generally managed conservatively. A "watch and wait" policy is considered adequate in many cases, whereas skin-directed therapies are the mainstay of treatment in cases of symptomatic disease or in patients who

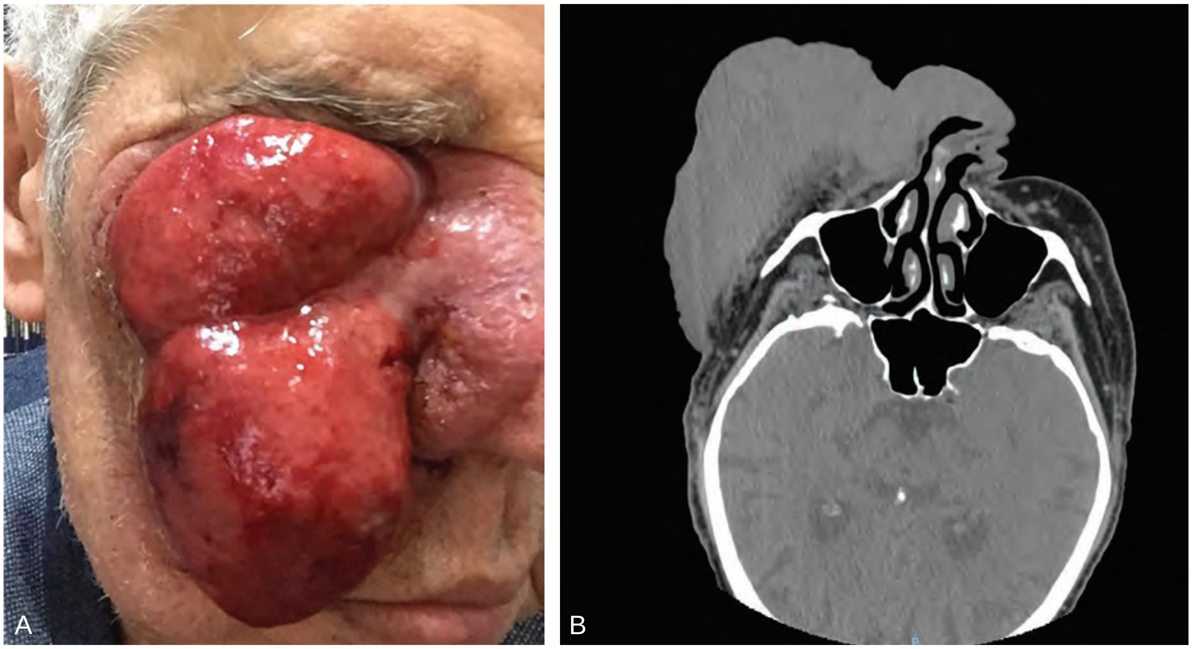


Figure 89.10 MYCOSIS FUNGOIDES. (A) Tumor stage. (B) Computed tomography scan of the head of the same patient showing marked compression and deviation of the nasal pyramid.

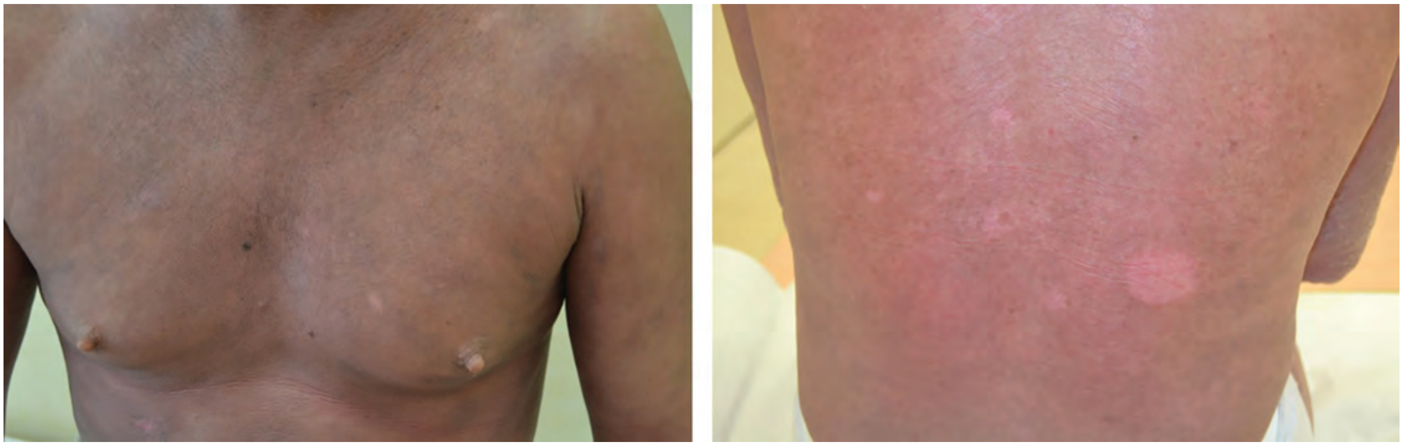


Figure 89.11 ERYTHRODERMA IN A PATIENT WITH SÉZARY SYNDROME.

develop extensive skin involvement. Patient management is addressed at the control of local symptoms and preserving a good quality of life, avoiding treatment-related toxicities. (See box on Treatment Approach to MF and SS Patients According to Stage at Presentation.)

Dermatologists and radiation oncologists mainly guide treatment at this stage. For this reason, only the most widely applied treatment strategies are briefly reviewed here.

Topical Treatments

Topical steroids represent the most widely applied first-line treatment in localized disease. Clobetasol propionate (cream or ointment) is able to produce high response rates, up to 90% in patients with T_1 disease and 80% in those with T_2 disease, with side effects (like purpura or irritant dermatitis) emerging in case of prolonged treatment.[81]

Topical toll-like receptor (TLR) agonists stimulate the innate immune system to harness an anti-lymphoma effect by the production of cytokines such as IFN α and IL-12, which are capable of inducing cell death and promoting host anti-tumor immunity. Topical 5% imiquimod, a TLR7 agonist, has shown activity in limited-stage MF. In a cohort of 20 patients treated with this agent, responses were observed in 80% of the cases, with a CR rate of 45%. Local toxicities were limited to localized pain and redness, pruritus, or ulceration, whereas systemic symptoms were just occasional.[82]

Mechlorethamine gel, 0.02%, demonstrated its efficacy in 58% of MF patients (76 patients out of 130) after at least 6 months of treatment compared with 48% of patients (62 out of 130) who used the ointment. A CR was obtained in 14% of treated patients. The most commonly reported side effects were dermatitis (skin inflammation with reddening, rash, pain or burning sensation), skin infection, and itching.[83]

Phototherapy

It is used alone or in combination with other agents (such as topical therapies, interferon, or bexarotene) in patients with limited-stage disease and with extensive skin involvement. Narrowband ultraviolet B (NB-UVB) has been used on both patches and plaques in limited-stage disease until clearance or plateau in response, with reported CR rates ranging from 54% to 90%, 84% on average. Patients with patch-only disease seem to perform better than those with plaques.

Relapse-free intervals reported in literature vary between 5.9 to 14.5 months, but they can be extended up to 26 months if any form of NB-UVB maintenance is applied. Treatment with 8-methoxypsoralene (8-MOP) plus ultraviolet A (PUVA) has produced very variable response rates in patients with limited-stage disease, with CR rates ranging between 65% and 85%. Patches and thin plaques are known to respond better to PUVA than thick and more infiltrated plaques, according to the experience of experts. However, PUVA should be preferred to NB-UVB treatment in plaque-stage disease because of the superior penetration of skin of ultraviolet A. Time-to-response is generally 2 to 3 months and seems to be largely stage-independent.[84]

CLINICAL APPROACH TO A PATIENT AFFECTED BY MF OR SS AND STAGING RECOMMENDATIONS

1. Skin examination with determination of type(s) of skin lesions (patches, plaques, tumors, erythroderma), along with skin ulceration or fissuration, hyperkeratosis or nail changes. Estimate the percentage of body surface area involved.
2. Complete physical examination: identification of any palpable lymph node, especially those larger than 1.5 cm or firm, irregular, clustered, or fixed; palpation of any organomegaly.
3. Skin biopsy. Most indurated area if only one biopsy is performed. Special immunohistochemistry staining: CD30, to be considered if lymphomatoid papulosis, anaplastic lymphoma, or large-cell transformation is suspected; CCR4 if treatment with mogamulizumab is planned. Evaluate for clonality of T-cell receptor gene rearrangement.
4. Laboratory: complete blood count with differential counts (including the examination of a peripheral blood smear to evaluate for Sézary cells), liver and renal function tests, lactate dehydrogenase. Flow cytometry (including $CD4^+/CD7^-$ or $CD4^+/CD26^-$).
5. Staging procedures: computed tomography (CT) scan of neck, chest, abdomen, and pelvis with intravenous contrast (it can be omitted in patients with T_1 and limited T_2 disease, with N_0 and B_0, and replaced by chest X-ray and abdomen ultrasonography); unilateral bone marrow trephine biopsy (mandatory in case of concomitant peripheral blood abnormalities).
6. Lymph node biopsy: excisional biopsy is indicated in those patients with a lymph node either larger than 1.5 cm in diameter and/or is firm, irregular, clustered, or fixed.

Total Skin Electron Beam Irradiation

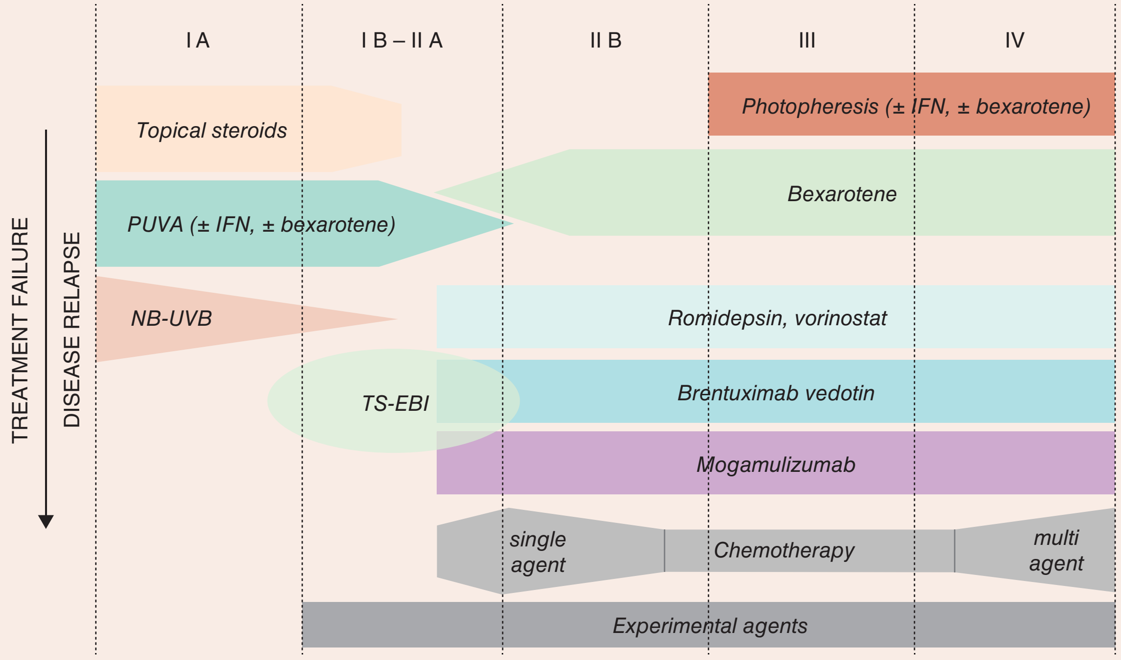
Total skin electron beam irradiation (TS-EBI) is the single most effective skin-directed therapy for MF. Conventional TS-EBI doses range from 24 to 36 Gy, as the rate of CR improves according with dose increase. Toxicity is however dose-dependent and common adverse events consist of erythema, dry desquamation, temporary anhidrosis, and temporary loss of fingernails and toenails. Alopecia may be irreversible at doses exceeding 25 Gy. Most patients display disease relapse, even after 36 Gy. However, repeated courses of high-dose TS-EBI are administered reluctantly because of the significant risk of skin atrophy and xerosis. For this reason, TS-EBI has been applied at lower doses in order to perform shorter treatment courses, reduce radiation-related adverse events, and possibly apply the treatment more than once as soon as patients relapse. It has been demonstrated that low-dose TS-EBI in the ranges of 10 to 20 Gy or 20 to 30 Gy were comparable with doses of 30 Gy or more. Indeed, an ORR of 88% was documented in a pooled analysis of 3 clinical trials of 12 Gy TS-EBI, with transient and manageable side effects, although the CR rate was significantly lower than with 36 Gy.[85]

Treatment of Advanced-Stage MF and SS and Management of Relapsed Disease

The management of advanced-stage disease patients is based on the close cooperation between hematologists, dermatologists, and radiation oncologists. The same happens in patients with relapsed or

TREATMENT APPROACH TO MF AND SS PATIENTS ACCORDING TO STAGE AT PRESENTATION

- Skin-directed treatments are applied in limited-stage disease and may be repeated as soon as disease relapses.
- Systemic treatments need to be considered in case of advanced-stage disease at presentation or relapse.
- Enrollment in clinical trials is always encouraged, especially for patients with multiple relapsing disease.



refractory disease. Goals of therapy are to control symptoms, maintain cosmesis, and improve survival by maximally reducing the tumor burden.

Biologic-response modifiers, like bexarotene, and HDAC inhibitors are applied as early treatment lines in advanced disease, while systemic treatments with BV or mogamulizumab are appropriate in patients lacking proper responses and with relapsed disease. Systemic chemotherapy may also play a role, especially in patients with disseminated visceral disease and bulky masses. However, published experience is scarce and responses are not durable, as response durations are generally measured in months.

Despite the availability of several active systemic therapeutic strategies, there is still an unmet need for treatments with favorable therapeutic indices. As treatment of advanced-stage MF and SS is largely palliative, a stage-based approach applying sequential therapies in an escalated fashion is preferred. Participation in a well-designed clinical trial is encouraged, as the introduction of novel agents will continue to expand the therapeutics options available in the management of CTCL.

Here we review the agents approved for the treatment of advanced-stage and relapsed or refractory MF and SS. (See box on Treatment Approach to MF and SS Patients According to Stage at Presentation.)

Bexarotene

It is an orally available synthetic retinoid capable of binding and activating retinoid X receptors (RXR), which are in turn responsible for changes in gene expression that lead to cell differentiation, decreased cell proliferation, and apoptosis. Bexarotene was approved by the FDA for the treatment of CTCL in 1999; later, a topical gel formulation of bexarotene was also approved, even though it is not available everywhere.

Bexarotene was tested in a multicenter phase 2/3 trial in 94 patients with biopsy-confirmed CTCL in advanced stages (IIB-IVB) who had received previous treatment for their disease; 56 patients received an initial dose of 300 mg/m^2/day and 38 started at a higher dose (>300 mg/m^2/day). Clinical responses were reported in 45% of patients (25 out of 56) enrolled in the 300 mg/m^2/day group, although with only 2% of them achieving a CR. Among those who received a higher dose, 55% (21 of 38) responded, including 13% clinical CR. Despite this, dose-limiting toxicity was far more common in patients receiving doses higher than 300 mg/m^2/day, thus the 300 mg/m^2/day dose appeared to provide the optimal risk-to-benefit ratio. Importantly, hypertriglyceridemia and central hypothyroidism were noted as the most frequent adverse events. Therefore, patients on bexarotene treatment need to have their basal free T4, thyroid-stimulating hormone, and serum lipid concentrations measured and monitored throughout treatment. Moreover, the use of lipid-lowering drugs (as fenofibrate) and adequately titrated doses of L-thyroxine is suggested before treatment initiation. In the 300 mg/m^2/day initial dose group, the rate of relapse after response was 36% and the projected median DOR was 299 days. Responses are generally seen within 2 to 3 months of treatment. Treatment should be carried on for at least 6 months and maintained until disease progression or unacceptable toxicity.[86]

Combination of bexarotene with PUVA or extracorporeal photopheresis (see below) can also be an option.

Histone Deacetylase Inhibitors

Vorinostat (suberoylanilide hydroxamic acid, SAHA) and romidepsin (depsipeptide) are class I and II HDAC inhibitors active in CTCL. Their mechanism of action is summarized in the section on treatment of relapsed and refractory PTCL.

Following the initial dose-finding study, which established 400 mg daily of vorinostat as the optimal dose to be further investigated, this drug was evaluated as a single agent in a phase 2b multicenter trial that included 74 patients with stage I B-IV A MF or SS who had received at least two prior systemic therapies including bexarotene (unless this drug was not tolerated). Disease stage was at least II B in 61 patients. Patients were treated with 400 mg of oral vorinostat daily until disease progression or intolerable toxicity. The ORR was approximately 30% in patients with advanced-stage disease, and all the initial responses were PR, with one CR. Up to 36% of patients with SS achieved a response. The median time to response in stage IIB or higher patients was 56 days, with a median response duration longer than 185 days. Median time to progression (TTP) was 4.9 months overall and longer than 9.8 months for stage IIB or higher responders. Among patients who failed to achieve a clinical response, pruritus relief, decreased lymphadenopathy, and reduction of leukemic tumor burden could be appreciated.[87]

Romidepsin was evaluated in two phase 2 international multicenter trials. The first trial involved 71 patients who had undergone a median of four prior treatments. Advanced-stage disease (higher than II B) was documented in 62 patients (87%), with 41 patients with stage IV. Romidepsin was administered at the dose of 14 mg/m^2 on days 1, 8, and 15 every 28 days. CR were observed in 4 patients and PR in 20, yielding an ORR of 34%. The median DOR was 13.7 months.[88] The second study involved 96 MF and SS patients with stage IB to IVA. Most patients (71%) had advanced-stage disease. The ORR was 34%, including six patients with CR. Among patients with advanced-stage disease, the ORR was 38%, including 5 CR. The median time to response was 2 months and the median DOR was 15 months. A clinically relevant improvement in pruritus was seen in 43% of cases even if lacking a clinical response.[89]

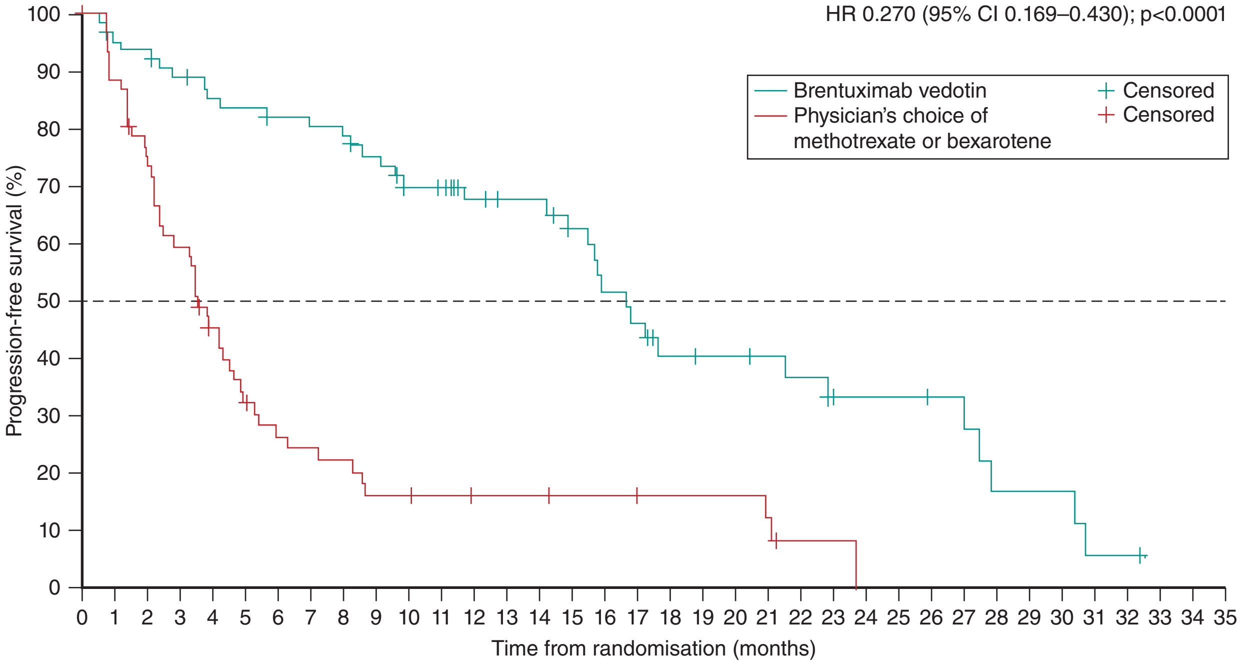
Brentuximab Vedotin

An initial phase 2 study of BV in CTCL reported its activity in CTCL and LyP, with an ORR of 73% and a CR rate of 35%. Fifteen of 28 patients with MF responded (54%), across a wide range of CD30 expression. Importantly, the ORR was 50% in patients with low CD30 expression (<10%), 58% in those with a medium expression (10% to 50%), and 50% in case of high expression (>50%). Time to response was 12 weeks (range, 3 to 39 weeks), and DOR was 32 weeks.[90] Another phase 2 study reported an ORR of 70% in advanced MF and SS with a wide range of CD30 expression levels, again underlying that responses were seen independently of CD30 expression within the affected tissue.[91]

In patients with previously treated MF and cutaneous ALCL, BV used as a single agent at the standard dose of 1.8 mg/kg every 21 days has demonstrated superior activity in disease control over standard treatments (oral methotrexate, 5 to 50 mg/week or oral bexarotene, 300 mg/m^2 once daily). In a phase 3, multicenter, randomized trial (ALCANZA), 64 patients received BV and 64 standard treatment according to physician's discretion (population evaluable for efficacy). The primary endpoint of the study was the proportion of patients who achieved an objective global response (skin + nodes + blood) lasting at least 4 months (termed ORR4) per independent review facility. At a median follow-up of 23 months, the ORR4 was 56.3% in patients treated with BV *versus* 12.5% in those receiving standard treatments ($P < .0001$). Median PFS was 16.7 months in the BV group and 3.5 months in the physician's choice group (Fig. 89.12). These results granted BV approval in patients with MF and cutaneous ALCL who have received at least one previous systemic treatment line.[92]

Mogamulizumab

A phase 1/2 open-label multicenter randomized clinical trial demonstrated an ORR of 47.1% in SS patients and 28.6% in MF patients. Importantly, the treatment determined a rapid clearance of malignant cells as measured by flow cytometry in 18 of 19 patients with blood involvement.[93] In the Japanese phase 2 study, also including PTCL patients, a 50% ORR was reported in the CTCL cohort.[67]



Number at risk																																			
Brentuximab vedotin	64	59	58	54	51	50	48	47	46	43	38	38	29	27	27	23	19	17	13	12	12	11	10	8	7	7	7	6	3	3	3	1	1	-	-	-
Physician's choice of methotrexate or bexarotene	64	54	42	34	24	17	13	12	11	8	8	7	7	6	6	5	5	5	4	4	4	3	1	1	0	0	0	0	0	0	0	0	0	-	-	-

Figure 89.12 PROGRESSION-FREE SURVIVAL IN PATIENTS TREATED IN THE BRENTUXIMAB VEDOTIN ARM COMPARED TO THOSE IN THE PHYSICIAN'S CHOICE ARM. *(Reprinted with permission from Elsevier, Prince HM, et al. Brentuximab vedotin or physician's choice in CD30-positive cutaneous T-cell lymphoma (ALCANZA): an international, open-label, randomised, phase 3, multicentre trial . Lancet. 2017;390:555–566.)*

In a phase 3 randomized trial (MAVORIC), mogamulizumab was compared to vorinostat in 372 patients with relapsed or refractory MF or SS. Mogamulizumab was administered at the dose of 1.0 mg/kg intravenously on a weekly basis for the first 28-day cycle, then on days 1 and 15 of subsequent cycles, while vorinostat was given orally at the dose of 400 mg/day. Patients treated with mogamulizumab showed a statistically significant superior PFS compared to those receiving vorinostat (7.7 *vs*. 3.1 months, respectively). Of note, mogamulizumab was also superior to vorinostat in terms of response rate and duration by disease compartment, i.e., blood, skin, and lymph nodes, as it produced a best global overall response of 35% (*versus* 6% obtained with vorinostat) and at least a 50% improvement in skin response in 44% of cases (*versus* 22% with vorinostat). Importantly, among the 136 patients initially treated with vorinostat who crossed over to mogamulizumab due to lack of efficacy or intolerable toxicity of the drug, 31% achieved an overall response. These findings led to the FDA approval of mogamulizumab for MF and SS patients who have failed at least one prior systemic therapy.[94]

Extracorporeal Photopheresis

Extracorporeal photopheresis (ECP) is based on the extracorporeal irradiation of patient's leukapheresis and plasmapheresis products which are subsequently reinfused. The whole blood of the patient is initially collected, then leukocytes are separated from plasma and non-nucleated cells. Leukocytes are then exposed to ultraviolet A (UVA) irradiation in the presence of a photosensitizing agent, 8-MOP, through a 1 mm thick disposable cassette, prior to reinfusion to the patient.

Psoralen binds covalently to DNA after UVA exposure, thus forming DNA crosslinks. Originally, the primary hypothesis stated that the formation of DNA crosslinks was responsible for apoptosis in the treated immune cells by mechanisms involving the BCL-2 family members, the disruption of mitochondrial membrane potential, and the activation of extrinsic cell death pathways. More recently, this view was enriched by evidence that ECP is capable of dendritic cell initiation, modification of the cytokine profile, and stimulation of several T-cell lineages, in particular regulatory T cells. This ultimately favors antigen presentation and initiation of a host immune response.

The first experience with ECP in patients with SS showed a response rate of 55.7% and a CR rate of 17.6%. A better response rate was found in patients with lower counts of Sézary cells and low CD4/CD8 ratio.[95] Following these results, ECP was approved by the FDA in 1988 for the frontline treatment of SS and erythrodermic MF with blood involvement. Since that preliminary experience, no randomized clinical trials showing the superiority of ECP over other treatment modalities have been performed. However, experience in more than 500 patients from several case series confirms its efficacy with a range of responses between 33% and 74%, with CR rates of approximately 20%. According to current guidelines, ECP should be performed for 2 consecutive days every 4 weeks. More accelerated regimens showed no clear evidence of increase in response rates. It is important to remember that long-term ECP may cause iron deficiency anemia due to a small residual blood volume that is not returned to the patient after each procedure.[96]

Systemic Chemotherapy

Gemcitabine (1000 mg/m^2 once per week for 3 weeks followed by one week of rest, up to 6 cycles) produced an ORR of 68% in 25 patients with advanced-stage and refractory MF and proved to be particularly active in patients with cutaneous tumors.[97] In a retrospective series of 19 MF patients, the same schedule determined an ORR of 48% (with CR and PR rates of 16% and 32%, respectively) with durable responses (15 to 120 months).[64]

Pegylated liposomal doxorubicin was first evaluated more than 20 years ago in 10 patients with MF (stage IB to IVA), who were treated at the dose of 20 mg/m^2. The drug produced an ORR of

80% as a single agent, with a CR rate of 60%. Mean DFS was 13.3 months. In a prospective phase 2 trial enrolling 49 MF patients with stage IIB, IVA, or IVB, refractory or recurrent after at least two previous systemic therapies, responses to liposomal doxorubicin (20 mg/m^2 on days 1 and 15, every 28 days) were seen in 40.8% of the cases; continuous complete remissions were obtained in 6.1% of the patients. A 50% or greater reduction of cutaneous manifestations was observed in 60.5% of treated patients. The median TTP and median DOR were 7.4 and 6 months, respectively.[98]

Primary Cutaneous CD30$^+$ T-Cell Lymphoproliferative Disorders

They are a wide spectrum of indolent T-cell lymphoproliferative disorders, primarily localized to the skin. They account for nearly 30% of CTCL, thus representing the most frequent disease entities after MF and SS, and overall they account for nearly 10% of cutaneous lymphomas. Clinically, they display a chronic clinical course, with cutaneous lesions sometimes showing a relapsing-remitting behavior or spontaneous regressions, and always feature the presence of large and atypical CD30$^+$ cells variably represented within the histological specimen. Primary cutaneous anaplastic large-cell lymphoma (pcALCL) and LyP represent the two extremities of the spectrum and are discussed in more detail. Diagnosis always relies on skin biopsy, which always needs to be reviewed by an expert hematopathologist and then correlated with the clinical presentation. Differential diagnosis is with insect bites and drug eruptions, as well as folliculitis, contact dermatitis, or molluscum contagiosum, all of which may show significant proportions of CD30$^+$ atypical cells on skin biopsies.[99,100]

Primary Cutaneous Anaplastic Large-Cell Lymphoma

pcALCL is the second most common type of CTCL, being mostly represented in middle-aged men (median age at presentation is 60 years, with a male-to-female ratio of 2 to 3:1). pcALCL needs to be distinguished from secondary involvement of systemic ALCL: this is relevant, as the two conditions are treated differently and their prognosis is markedly different, with the former displaying a 10-year disease-specific survival rate of nearly 90%, while the other is a much more aggressive and severe disease (see related paragraph).

Morphology

Tumor cells are large, with an anaplastic (abundant cytoplasm, round to oval or irregularly shaped nuclei with prominent nucleoli), pleomorphic, or immunoblastic cytomorphology. The infiltrate can be either nodular or diffuse; it involves the dermis, sometimes the upper subcutaneous tissue, without epidermotropism. Cells mostly display a CD4$^+$ T-cell phenotype, being CD8$^+$ in a minority of cases, with possible loss of CD2, CD5, or CD3. CD30 is expressed by the majority (>75%) of the neoplastic cells. Multinucleated giant cells found within the neoplastic tissue may mimic Reed-Sternberg cells, thus causing possible confusion with Hodgkin lymphoma; CD15, however, is negative. Cytotoxicity markers, such as TIA-1, perforin, and granzyme B are frequently expressed. The ALK antigen is usually negative, in contrast with ALCL. Clonal rearrangements of the TCR are common and can be found in nearly 90% of cases.

Clinical Manifestations

It clinically presents with solitary and localized papules, nodules, or tumors, frequently ulcerated, involving the skin of trunk, face, extremities, and buttocks. Mucosal surfaces can also be affected. Multifocal involvement is rather uncommon, as no more than 20% of patients have multiple or disseminated lesions. Lesions may show a spontaneous complete or partial regression; however skin relapses are frequent. Extracutaneous dissemination is rare, accounting for less than 10% of the cases, with regional lymph nodes being the most affected tissue other than the skin.

Disease staging differs from the conventional Ann Arbor staging system used for nodal lymphomas and from the TNMB system applied in MF and SS (Table 89.13). This peculiar staging system, also used for LyP, has clinical validity in pcALCL as it clearly separates patients with limited skin involvement from those with more diffuse involvement, with relevant prognostic implications.

Therapy

Localized lesions favorably respond to radiation therapy (with CR rates up to 95%) and topical steroids. Surgical excision may be curative in some cases. pcALCL with regional lymph node involvement may benefit from BV treatment, as do patients with widespread localized (T_2) or generalized (T_3) lesions and relapsed disease. In the phase 3 ALCANZA study, among 16 patients with pcALCL, an ORR of 89% with 44% CR rate was obtained in patients with skin-limited disease, and an ORR of 57% was seen in patients with extracutaneous disease as well.[92] These results appear much more significant than those obtained with bexarotene and oral methotrexate.[99,100]

Prognosis

The prognosis is good, with a 5-year DFS higher than 90%, although relapses are frequent. Patients with multifocal skin lesions and patients with involvement of regional nodes show a similar prognosis to those presenting with a unique skin lesion. Exceptions are represented by patients with extensive lower limb or generalized skin involvement, older age, lack of spontaneous remission, and disease arising in the context of immunosuppression after solid organ transplantation.

TABLE 89.13 **The Proposed TNM Staging System for Patients with Primary Cutaneous ALCL and Lymphomatoid Papulosis**

Skin	T_1	Solitary skin involvement. Further stratify into T_{1a} (solitary lesion <5 cm in diameter) and T_{1b} (solitary lesion >5 cm in diameter).
	T_2	Regional skin involvement, with multiple lesions limited to one body region or two contiguous body regions.[a] Further stratify into T_{2a} (all lesions confined within a 15 cm diameter circular area), T_{2b} (all lesions confined within a >15 cm but <30 cm diameter circular area), and T_{2c} (lesions spread over a >30 cm diameter circular area).
	T_3	Generalized skin involvement. Further stratify into T_{3a} (multiple lesions involving two con–contiguous body regions) and T_{3b} (multiple lesions involving ≥3 body regions).
Nodes	N_0	No clinical or pathologic lymph node involvement.
	N_1	Involvement of one peripheral lymph node region[b] that drains an area of current or prior skin involvement.
	N_2	Involvement of ≥2 peripheral lymph node regions or involvement of any lymph node region that does not drain an area of current or prior skin involvement.
	N_3	Involvement of central lymph nodes.
Viscera	M_0	No evidence of extracutaneous non–lymph node disease.
	M_1	Extracutaneous non–lymph node disease present.

[a] Body regions: head and neck, chest, abdomen/genital, upper back, lower back/buttocks and perineum, upper arms, lower arms/hands, upper legs (thighs), lower legs/feet.
[b] Lymph node regions: a) peripheral sites: antecubital, cervical, supraclavicular, axillary, inguinal-femoral, and popliteal; b) central sites: mediastinal, pulmonary hilar, paraaortic, iliac.

Lymphomatoid Papulosis

LyP is a chronic, recurrent, self-healing skin disease, which mainly affects middle-aged men, with a male-to-female ratio of 2 to 3:1. Its pathogenesis is unclear, but a correlation with a chronic inflammatory status can be hypothesized. No viral correlates have been found in affected skin cells.

Approximately 80% of skin lesions (ranging from 22% to 100%) display clonal TCR rearrangements; however, the presence of a T-cell clone is not required to establish the diagnosis, nor the absence of the clonal rearrangement excludes LyP.

Histological information is required to accomplish the diagnosis, but it needs to be correlated with the overall clinical scenario, given that the morphologic appearance may sometimes closely resemble significantly different diseases.[99,100]

Morphology

LyP has a very heterogeneous appearance upon examination of skin biopsy. For this reason, it has been classified into 6 distinct subtypes, termed A to E, plus a newly recognized subtype harboring the *DUSP22-IRF4* rearrangement. Type A is the most common, accounting for about 75% of the cases, which is characterized by a wedge-shaped dermal infiltrate of large atypical $CD4^+/CD30^+$ lymphocytes within an inflammatory background. Type C, accounting for 10% of the cases, closely resembles type A but it lacks the accompanying inflammatory *pabulum* of neutrophils, eosinophils, and histiocytes. Type B is histologically different, as it is characterized by small $CD4^+/CD8^-/CD30^+$ lymphocytes with cerebriform nuclei and epidermotropism. Importantly, this subtype widely overlaps with the presentation of MF, thus a clinical correlation is always required to establish the correct diagnosis. Type D disease displays epidermotropic infiltrates of small- to medium-sized pleomorphic lymphocytes, usually expressing cytotoxic markers such as granzyme, perforin, and TIA-1. As this subtype largely overlaps with aggressive epidermotropic $CD8^+$ cytotoxic lymphoma, primary cutaneous γ/δ T-cell lymphoma or pagetoid reticulosis in terms of both histology and immunophenotype, it is important to correlate the histologic information with the clinical picture: type D LyP behaves indolently, despite the aggressive appearance upon histology, differently than the aforementioned disease entities which, in contrast, require a more intensive management. Type E shows an angioinvasive infiltrate of atypical lymphocytes in superficial and deep dermis, in a mixed inflammatory background. Histologic and phenotypic peculiarities, however, do not translate into clinically meaningful differences. In other words, the clinical manifestations of the disease are independent of the phenotypic presentation.

Clinical Manifestations

It is a chronic and benign lymphoproliferative disorder, clinically characterized by the presence of papular, papulonecrotic, or nodular skin lesions, mainly affecting the trunk and the extremities. Lesions occur in crops of a few to hundreds, sometimes with long lesion-free intervals. Patients report outbreaks of self-resolving papules, with a relapsing-and-remitting course within 3 to 12 weeks. New lesions may develop while others are regressing. When they coalesce, they may undergo ulceration. Upon disappearance, they leave behind superficial scars or postinflammatory pigmentary changes. The waxing and waning course may last from months to decades before remission; some physiological events—like menses or pregnancy—may modulate the appearance of LyP papules. Transformation into an aggressive lymphoma does not occur; however, in 4% to 25% of the patients, LyP can be associated—or followed by—another malignant lymphoma, such as MF, pcALCL, or Hodgkin disease. For this reason, the onset of persistent lymphadenopathy or systemic symptoms (malaise, fever, weight loss) should prompt the investigation for a systemic lymphoproliferative disease.

Therapy

LyP tends to regress spontaneously, thus no treatment is required. Topical treatments, such as steroids, do not prevent the outbreak of new lesions or disease recurrence; however, they may be useful in relieving associated symptoms, such as pruritus or tenderness. Low-dose methotrexate or PUVA phototherapy may be suitable in case of disseminated disease. BV has an approval for the treatment of LyP, although its exceedingly rarely required. Moreover, given the not negligible risk of peripheral neuropathy correlated with this drug, harm largely outweighs benefit.

Prognosis

The prognosis of LyP is excellent, with a 5-year OS of 100%. For those who develop a secondary overt lymphoma, prognosis overlaps with that of primary disease.

Future Directions

CTCL still remain difficult to manage in cases of advanced-stage disease, as current agents can control the disease temporarily but do not prevent further recurrences or progression. This means that newer agents are needed, and that enrollment in clinical trials is of paramount importance especially of patients with heavily pre-treated disease.

The programmed death-1 (PD-1)- PD-1 ligand (PD-L1) interaction represents a potential target for the treatment of CTCL. Through this interaction, T-cell responses against the tumor are attenuated, thus representing a strategy by which tumors escape to immune surveillance. PD-1 and both its ligands, PD-L1 and PD-L2, are expressed in some hematological malignancies, including MF and SS. In a multicenter phase 2 study, pembrolizumab, a monoclonal antibody which binds to PD-1 receptor inhibiting its interaction with PD-L1 and PD-L2, has demonstrated clinical activity in patients with previously treated MF and SS. Responses were durable and were not associated with any identifiable clinical or pathologic characteristics. Treatment was overall well tolerated, with a toxicity profile consistent with prior pembrolizumab studies, though 40% of patients with SS developed a notable skin flare reaction. The ORR was 38% with 1 CR and 8 PR. Of the responding patients, 6 patients had 90% or greater improvement in skin disease burden.[101]

IPH4102 is a first-in-class humanized IgG_1 monoclonal antibody that binds to KIR3DL2-expressing tumor cells. KIR3DL2 (CD158) is a killer-cell immunoglobulin-like receptor that binds to its cognate HLA-class I ligands negatively modulating immune cell function. This receptor is expressed across most CTCL subtypes. Mechanistically, it recruits human NK cells and macrophages and exerts killing of $KIR3DL2^+$ cells via antibody-dependent cell cytotoxicity and antibody-dependent phagocytosis. A first-in-human, open-label, phase 1 clinical trial with dose-escalation and cohort-expansion parts of IPH4102 enrolled 44 CTCL patients, mostly with SS (80%). Intravenous infusions ranging from 0.0001 mg/kg to 10 mg/kg were assessed. No dose-limiting toxicities were detected in the dose-escalation phase of the trial and the recommended flat dose of 750 mg was considered amenable for the dose-expansion phase of the study. A global ORR of 36.4% was observed; patients affected by SS had an ORR of 43%. Given these promising results, IPH4102 is now being evaluated within phase 2 trials in SS patients and in other subtypes of T-cell lymphomas.[102]

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Coiffier B, Pro B, Prince HM, et al. Results from a pivotal, open-label, phase II study of romidepsin in relapsed or refractory peripheral T-cell lymphoma after prior systemic therapy. *J Clin Oncol.* 2012;30:631–636.

d'Amore F, Relander T, Lauritzsen GF, et al. Up-front autologous stem-cell transplantation in peripheral T-cell lymphoma: NLG-T-01. *J Clin Oncol*. 2012;30:3093–3099.

El-Asmar J, Reljic T, Ayala E, et al. Efficacy of high-dose therapy and autologous hematopoietic cell transplantation in peripheral T cell lymphomas as front-line consolidation or in the relapsed/refractory setting: a systematic review/meta-analysis. *Biol Blood Marrow Transplant*. 2016;22:802–814.

Ellin F, Landström J, Jerkeman M, Relander T. Real-world data on prognostic factors and treatment in peripheral T-cell lymphomas: a study from the Swedish Lymphoma Registry. *Blood*. 2014;124:1570–1577.

Fiore D, Cappelli LV, Broccoli A, et al. Peripheral T cell lymphomas: from the bench to the clinic. *Nat Rev Cancer*. 2020;20:323–342.

Horwitz S, O'Connor OA, Pro B, et al. Brentuximab vedotin with chemotherapy for CD30-positive peripheral T-cell lymphoma (ECHELON-2): a global, double-blind, randomised, phase 3 trial. *Lancet*. 2019;393:229–240.

Iqbal J, Wright G, Wang C, et al. Gene expression signatures delineate biological and prognostic subgroups in peripheral T-cell lymphoma. *Blood*. 2014;123:2915–2923.

Kim YH, Bagot M, Pinter-Brown L, et al. Mogamulizumab versus vorinostat in previously treated cutaneous T-cell lymphoma (MAVORIC): an international, open-label, randomised, controlled phase 3 trial. *Lancet*. 2018;19:1192–1204.

Le Gouill S, Milpied N, Buzyn A, et al. Graft-versus-lymphoma effect for aggressive T-cell lymphomas in adults: a study by the Societe Francaise de Greffe de Moelle et de Therapie Cellulaire. *J Clin Oncol*. 2008;26:2264–2271.

Mak V, Hamm J, Chhanabhai M, et al. Survival of patients with peripheral T-cell lymphoma after first relapse or progression: spectrum of disease and rare long-term survivors. *J Clin Oncol*. 2013;31:1970–1976.

O'Connor OA, Horwitz S, Masszi T, et al. Belinostat in patients with relapsed or refractory peripheral T-cell lymphoma: results of the pivotal phase II BELIEF (CLN-19) study. *J Clin Oncol*. 2015;33:2492–2499.

O'Connor OA, Pro B, Pinter-Brown L, et al. Pralatrexate in patients with relapsed or refractory peripheral T-cell lymphoma: results from the pivotal PROPEL study. *J Clin Oncol*. 2011;29:1182–1189.

Prince HM, Kim YH, Horwitz SM, et al. Brentuximab vedotin or physician's choice in CD30-positive cutaneous T-cell lymphoma (ALCANZA): an international, open-label, randomised, phase 3, multicentre trial. *Lancet*. 2017;390:555–566.

Pro B, Advani R, Brice P, Bartlett NL, et al. Five-year results of brentuximab vedotin in patients with relapsed or refractory systemic anaplastic large cell lymphoma. *Blood*. 2017;130:2709–2717.

Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016(127):2375–2390.

Vose J, Armitage J, Weisenburger D. International T-Cell Lymphoma Project. International peripheral T-cell and natural killer/T-cell lymphoma study: pathology findings and clinical outcomes. *J Clin Oncol*. 2008;26:4124–4130.

Wilhelm M, Smetak M, Reimer P, et al. First-line therapy of peripheral T-cell lymphoma: extension and long-term follow-up of a study investigating the role of autologous stem cell transplantation. *Blood Cancer J*. 2016;6:e452.

Willemze R, Cerroni L, Kempf W, et al. The 2018 update of the WHO-EORTC classification for primary cutaneous lymphomas. *Blood*. 2019;133:1703–1714.

REFERENCES

1. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016; 127:2375–2390.
2. Fiore D, Cappelli LV, Broccoli A, et al. Peripheral T cell lymphomas: from the bench to the clinic. *Nat Rev Cancer*. 2020;20:323–342.
3. Staber PB, Herling M, Bellido M, et al. Consensus criteria for diagnosis, staging, and treatment response assessment of T-cell prolymphocytic leukemia. *Blood*. 2019;134:1132–1143.
4. Staber PB, et al. Consensus criteria for diagnosis, staging, and treatment response assessment of T-cell prolymphocytic leukemia. *Blood*. 2019;134(14) :1132 Epub 2019 Jul 10.
5. Dearden CE, Matutes E, Cazin B, et al. High remission rate in T-cell prolymphocytic leukemia with CAMPATH-1H. *Blood*. 2001;98: 1721–1726.
6. Mercieca J, Matutes E, Dearden C, et al. The role of pentostatin in the treatment of T-cell malignancies: analysis of response rate in 145 patients according to disease subtype. *J Clin Oncol*. 1994;12:2588–2593.
7. Gomez-Arteaga A, Margolskee E, Wei MT, et al. Combined use of tofacitinib (pan-JAK inhibitor) and ruxolitinib (a JAK1/2 inhibitor) for refractory T-cell prolymphocytic leukemia (T-PLL) with a JAK3 mutation. *Leuk Lymphoma*. 2019;60(7):1626–1631. https://doi.org/10.1080/10428194.2019.1594220. Epub 2019 Apr 18. PMID: 30997845.
8. Vose J, Armitage J, Weisenburger D. International T-Cell Lymphoma Project. International peripheral T-cell and natural killer/T-cell lymphoma study: pathology findings and clinical outcomes. *J Clin Oncol*. 2008;26:4124–4130.
9. Ellin F, Landström J, Jerkeman M, Relander T. Real-world data on prognostic factors and treatment in peripheral T-cell lymphomas: a study from the Swedish Lymphoma Registry. *Blood*. 2014;124:1570–1577.
10. Iqbal J, Wright G, Wang C, et al. Gene expression signatures delineate biological and prognostic subgroups in peripheral T-cell lymphoma. *Blood*. 2014;123:2915–2923.
11. Waldmann TA, Chen J. Disorders of the JAK/STAT pathway in T cell lymphoma pathogenesis: implications for immunotherapy. *Annu Rev Immunol*. 2017;35:533–550.
12. Broccoli A, Zinzani PL. Peripheral T-cell lymphoma, not otherwise specified. *Blood*. 2017;129:1103–1112.
13. Wang T, Feldman AL, Wada DA, et al. GATA-3 expression identifies a high-risk subset of PTCL, NOS with distinct molecular and clinical features. *Blood*. 2014;123:3007–3015.
14. Lemonnier F, Couronné L, Parrens M, et al. Recurrent TET2 mutations in peripheral T-cell lymphomas correlate with TFH-like features and adverse clinical parameters. *Blood*. 2012;120:1466–1469.
15. A predictive model for aggressive non-Hodgkin's lymphoma. The International Non-Hodgkin's Lymphoma Prognostic Factors Project. *N Engl J Med*. 1993;329:987–994.
16. Gallamini A, Stelitano C, Calvi R, et al. Intergruppo Italiano Linfomi. Peripheral T-cell lymphoma unspecified (PTCL-U): a new prognostic model from a retrospective multicentric clinical study. *Blood*. 2004;103:2474–2479.
17. Went P, Agostinelli C, Gallamini A, et al. Marker expression in peripheral T-cell lymphoma: a proposed clinical-pathologic prognostic score. *J Clin Oncol*. 2006;24:2472–2479.
18. Weisenburger DD, Savage KJ, Harris NL, et al. International Peripheral T-cell Lymphoma Project. Peripheral T-cell lymphoma, not otherwise specified: a report of 340 cases from the International Peripheral T-cell Lymphoma Project. *Blood*. 2011;117:3402–3408.
19. Federico M, Rudiger T, Bellei M, et al. Clinicopathologic characteristics of angioimmunoblastic T-cell lymphoma: analysis of the International Peripheral T-cell lymphoma project. *J Clin Oncol*. 2013;31:240–246.
20. de Leval M, Rickman DS, Thielen C, et al. The gene expression profile of nodal peripheral T-cell lymphoma demonstrates a molecular link between angioimmunoblastic T-cell lymphoma (AITL) and follicular helper T (T_{FH}) cells. *Blood*. 2007;109:4952–4963.
21. Lemonnier F, Couronné L, Parrens M, et al. Recurrent TET2 mutations in peripheral T-cell lymphomas correlate with TFH-like features and adverse clinical parameters. *Blood*. 2012;120:1466–1469.
22. Schwartz FH, Cai Q, Fellmann E, et al. TET2 mutations in B cells of patients affected by angioimmunoblastic T-cell lymphoma. *J Pathol*. 2017;242:129–133.
23. Cairns RA, Iqbal J, Lemonnier F, et al. IDH2 mutations are frequent in angioimmunoblastic T-cell lymphoma. *Blood*. 2012;119:1901–1903.
24. Palomero T, Couronné L, Khiabanian H, et al. Recurrent mutations in epigenetic regulators, RHOA and FYN kinase in peripheral T cell lymphomas. *Nat Genet*. 2014;46:166–170.
25. Sakata-Yanagimoto M, Enami T, Yoshida K, et al. Somatic RHOA mutation in angioimmunoblastic T cell lymphoma. *Nat Genet*. 2014;46:171–175.
26. Boddicker RL, Feldman AL. Progress in the identification of subgroups in ALK-negative anaplastic large-cell lymphoma. *Biomark Med*. 2015;9:719–722.
27. Parrilla Castellar ER, Jaffe ES, Said JW, et al. ALK-negative anaplastic large cell lymphoma is a genetically heterogeneous disease with widely disparate clinical outcomes. *Blood*. 2014;124:1473–1480.
28. Oishi N, Brody GS, Ketterling RP, et al. Genetic subtyping of breast implant-associated anaplastic large cell lymphoma. *Blood*. 2018;132:544–547.
29. Thompson PA, Lade S, Webster H, et al. Effusion-associated anaplastic large cell lymphoma of the breast: time for it to be defined as a distinct clinico-pathological entity. *Haematologica*. 2010;95:1977–1979.
30. Yabe M, Miranda RN, Medeiros LJ. Hepatosplenic T-cell Lymphoma: a review of clinicopathologic features, pathogenesis, and prognostic factors. *Hum. Pathol*. 2018;74:5–16.
31. McKinney M, Moffitt AB, Gaulard P, et al. The genetic basis of hepatosplenic T-cell lymphoma. *Cancer Discov*. 2017;7:369–379.
32. Delabie J, Holte H, Vose JM, et al. Enteropathy-associated T-cell lymphoma: clinical and histological findings from the International Peripheral T-Cell Lymphoma Project. *Blood*. 2011;118:148–156.
33. Moffitt AB, Ondrejka SL, McKinney M, et al. Enteropathy-associated T cell lymphoma subtypes are characterized by loss of function of SETD2. *J Exp Med*. 2017;214:1371–1386.
34. Tan SY, Chuang SS, Tang T, et al. Type II EATL (epitheliotropic intestinal T-cell lymphoma): a neoplasm of intra-epithelial T-cells with predominant CD8αα phenotype. *Leukemia*. 2013;27:1688–1696.
35. Willemze R, Jansen PM, Cerroni L, et al. Subcutaneous panniculitis-like T-cell lymphoma: definition, classification, and prognostic factors: an EORTC Cutaneous Lymphoma Group Study of 83 cases. *Blood*. 2008;111:838–845.
36. Abouyabis AN, Shenoy PJ, Sinha R, et al. A systematic review and meta-analysis of front-line anthracycline-based chemotherapy regimens for peripheral T-cell lymphoma. *ISRN Hematol*. 2011;2011:623924.
37. Abramson JS, Feldman T, Kroll-Desrosiers AR, et al. Peripheral T-cell lymphomas in a large US multicentre cohort: prognostication in the modern era including impact of frontline therapy. *Ann Oncol*. 2014;25:2211–2217.
38. Briski R, Feldman AL, Bailey NG, et al. The role of front-line anthracycline-containing chemotherapy regimens in peripheral T-cell lymphomas. *Blood Cancer J*. 2014;4:e214.
39. Chihara D, Fanale MA. Management of anaplastic large cell lymphoma. *Hematol Oncol Clin North Am*. 2017;31:209–222.
40. Savage KJ, Harris NL, Vose JM, et al. ALK—anaplastic large-cell lymphoma is clinically and immunophenotypically different from both ALK$^+$ ALCL and peripheral T-cell lymphoma, not otherwise specified: report from the International Peripheral T-Cell Lymphoma Project. *Blood*. 2008;111: 5496–5504.
41. Schmitz N, Trümper L, Ziepert M, et al. Treatment and prognosis of mature T-cell and NK-cell lymphoma: an analysis of patients with T-cell lymphoma treated in studies of the German high-grade non-hodgkin lymphoma study group. *Blood*. 2010;116:3418–3425.
42. Horwitz S, O'Connor OA, Pro B, et al. Brentuximab vedotin with chemotherapy for CD30-positive peripheral T-cell lymphoma (ECHELON-2): a global, double-blind, randomised, phase 3 trial. *Lancet*. 2019;393:229–240.
43. Wilhelm M, Smetak M, Reimer P, et al. First-line therapy of peripheral T-cell lymphoma: extension and long-term follow-up of a study investigating the role of autologous stem cell transplantation. *Blood Cancer J*. 2016;6:e452.
44. d'Amore F, Relander T, Lauritzsen GF, et al. Up-front autologous stem-cell transplantation in peripheral T-cell lymphoma: NLG-T-01. *J Clin Oncol*. 2012;30:3093–3099.
45. El-Asmar J, Reljic T, Ayala E, et al. Efficacy of high-dose therapy and autologous hematopoietic cell transplantation in peripheral T cell lymphomas as front-line consolidation or in the relapsed/refractory setting: a systematic review/meta-analysis. *Biol Blood Marrow Transplant*. 2016;22:802–814.
46. Park SI, Horwitz SM, Foss FM, et al. The role of autologous stem cell transplantation in patients with nodal peripheral T-cell lymphomas in first complete remission: report from COMPLETE, a prospective, multicenter cohort study. *Cancer*. 2019;125:1507–1517.
47. Fossard G, Broussais F, Coelho I, et al. Role of up-front autologous stem-cell transplantation in peripheral T-cell lymphoma for patients in response after induction: an analysis of patients from LYSA centers. *Ann Oncol*. 2018;29:715–723.
48. Clemens MW, Medeiros J, Butler CE, et al. Complete surgical excision is essential for the management of patients with breast implant-associated anaplastic large-cell lymphoma. *J Clin Oncol*. 2016;34:160–168.

49. Miranda RN, Aladily TN, Prince HM, et al. Breast implant-associated anaplastic large-cell lymphoma: long-term follow-up of 60 patients. *J Clin Oncol*. 2014;32:114–1120.
50. Phillips EH, Lannon MM, Lopes A, et al. High-dose chemotherapy and autologous stem cell transplantation in enteropathy-associated and other aggressive T-cell lymphomas: a UK NCRI/Cancer Research UK Phase II study. *Bone Marrow Transplant*. 2019;54:465–468.
51. Sieniawski M, Angamuthu N, Boyd K, et al. Evaluation of enteropathy-associated T-cell lymphoma comparing standard therapies with a novel regimen including autologous stem cell transplantation. *Blood*. 2010;115:3664–3670.
52. Yamaguchi M, Kwong YL, Kim WS, et al. Phase II study of SMILE chemotherapy for newly diagnosed stage IV, relapsed, or refractory extranodal natural killer (NK)/T-cell lymphoma, nasal type: the NK-cell tumor study group study. *J Clin Oncol*. 2011;29:4410–4416.
53. Wang L, Wang Z, Chen X, et al. First-line combination of gemcitabine, oxaliplatin, and L-asparaginase (GELOX) followed by involved-field radiation therapy for patients with stage IE/IIE extranodal natural killer/T-cell lymphoma. *Cancer*. 2013;119:348–355.
54. Mak V, Hamm J, Chhanabhai M, et al. Survival of patients with peripheral T-cell lymphoma after first relapse or progression: spectrum of disease and rare long-term survivors. *J Clin Oncol*. 2013;31:1970–1976.
55. O'Connor OA, Pro B, Pinter-Brown L, et al. Pralatrexate in patients with relapsed or refractory peripheral T-cell lymphoma: results from the pivotal PROPEL study. *J Clin Oncol*. 2011;29:1182–1189.
56. Coiffier B, Pro B, Prince HM, et al. Results from a pivotal, open-label, phase II study of romidepsin in relapsed or refractory peripheral T-cell lymphoma after prior systemic therapy. *J Clin Oncol*. 2012;30:631–636.
57. O'Connor OA, Horwitz S, Masszi T, et al. Belinostat in patients with relapsed or refractory peripheral T-cell lymphoma: results of the pivotal phase II BELIEF (CLN-19) study. *J Clin Oncol*. 2015;33:2492–2499.
58. Pro B, Advani R, Brice P, et al. Five-year results of brentuximab vedotin in patients with relapsed or refractory systemic anaplastic large cell lymphoma. *Blood*. 2017;130:2709–2717.
59. Horwitz S, Advani RH, Bartlett NL, et al. Objective responses in relapsed T-cell lymphomas with single-agent brentuximab vedotin. *Blood*. 2014;123:3095–3100.
60. Ishida T, Joh T, Uike N, et al. Defucosylated anti-CCR4 monoclonal antibody (KW-0761) for relapsed adult T-cell leukemia-lymphoma: a multicenter phase II study. *J Clin Oncol*. 2012;30:837–842.
61. Ogura M, Ishida T, Hatake K, et al. Multicenter phase II study of mogamulizumab (KW-0761), a defucosylated anti-cc chemokine receptor 4 antibody, in patients with relapsed peripheral T-cell lymphoma and cutaneous T-cell lymphoma. *J Clin Oncol*. 2014;32:1157–1163.
62. Zinzani PL, Karlin L, Radford J, et al. European phase II study of mogamulizumab, an anti-CCR4 monoclonal antibody, in relapsed/refractory peripheral T-cell lymphoma. *Haematologica*. 2016;101:e407–e410.
63. Dueck G, Chua N, Prasad A, et al. Interim report of a phase 2 clinical trial of lenalidomide for T-cell non-Hodgkin lymphoma. *Cancer*. 2010;116:4541–4548.
64. Zinzani PL, Pellegrini C, Broccoli A, et al. Lenalidomide monotherapy for relapsed/refractory peripheral T-cell lymphoma not otherwise specified. *Leuk Lymphoma*. 2011;52:1585–1588.
65. Morschhauser F, Fitoussi O, Haioun C, et al. A phase 2, multicentre, single-arm, open-label study to evaluate the safety and efficacy of single-agent lenalidomide (Revlimid®) in subjects with relapsed or refractory peripheral T-cell non-Hodgkin lymphoma: the EXPECT trial. *Eur J Cancer*. 2013;49:2869–2876.
66. Damaj G, Gressin R, Bouabdallah K, et al. Results from a prospective, open-label, phase II trial of bendamustine in refractory or relapsed T-cell lymphomas: the BENTLY trial. *J Clin Oncol*. 2013;31:104–110.
67. Zinzani PL, Venturini F, Stefoni V, et al. Gemcitabine as single agent in pretreated T-cell lymphoma patients: evaluation of the long-term outcome. *Ann Oncol*. 2010;21:860–863.
68. Arkenau HT, Chong G, Cunningham D, et al. Gemcitabine, cisplatin and methylprednisolone for the treatment of patients with peripheral T-cell lymphoma: the Royal Marsden Hospital experience. *Haematologica*. 2007;92:271–272.
69. Lemonnier F, Dupuis J, Sujobert P, et al. Treatment with 5-azacytidine induces a sustained response in patients with angioimmunoblastic T-cell lymphoma. *Blood*. 2018;132:2305–2309.
70. Moskowitz AJ, Jacobsen E, Ruan J, Schatz JH, Obadi O, Motylinski K, et al. Durable responses observed with JAK inhibition in T-cell lymphomas. *Blood (ASH annual meeting abstracts)*. 2018;132 2922abstr.
71. Horwitz SM, Koch R, Porcu P, et al. Activity of the PI3K-δ,γ inhibitor duvelisib in a phase 1 trial and preclinical models of T-cell lymphoma. *Blood*. 2018;131:888–898.
72. Lesokhin AM, Ansell SM, Armand P, et al. Nivolumab in patients with relapsed or refractory hematologic malignancy: preliminary results of a phase Ib study. *J Clin Oncol*. 2016;34:2698–2704.
73. Maciocia PM, Wawrzyniecka PA, Philip B, et al. Targeting the T cell receptor beta-chain constant region for immunotherapy of T cell malignancies. *Nat Med*. 2017;23:1416–1423.
74. Le Gouill S, Milpied N, Buzyn A, et al. Graft-versus-lymphoma effect for aggressive T-cell lymphomas in adults: a study by the Societe Francaise de Greffe de Moelle et de Therapie Cellulaire. *J Clin Oncol*. 2008;26:2264–2271.
75. Corradini P, Dodero A, Zallio F, et al. Graft-versus-lymphoma effect in relapsed peripheral T-cell non-Hodgkin's lymphomas after reduced-intensity conditioning followed by allogeneic transplantation of hematopoietic cells. *J Clin Oncol*. 2004;22:2172–2176.
76. Shustov AR, Gooley TA, Sandmaier BM, et al. Allogeneic haematopoietic cell transplantation after nonmyeloablative conditioning in patients with T-cell and natural killer-cell lymphomas. *Br J Haematol*. 2010;150:170–178.
77. Aoki K, Suzuki R, Chihara D, Suzuki T, et al. Reduced-intensity conditioning of allogeneic transplantation for nodal peripheral T-cell lymphomas. *Blood (ASH annual meeting abstracts)*. 2014;124:2585.
78. Willemze R, Cerroni L, Kempf W, et al. The 2018 update of the WHO-EORTC classification for primary cutaneous lymphomas. *Blood*. 2019;133:1703–1714.
79. Campbell JJ, Clark RA, Watanabe R, et al. Sézary syndrome and mycosis fungoides arise from distinct T-cell subsets: a biologic rationale for their distinct clinical behaviors. *Blood*. 2010;116:767–771.
80. Watanabe R, Gehad A, Yang C, et al. Human skin is protected by four functionally and phenotypically discrete populations of resident and recirculating memory T cells. *Sci Transl Med*. 2015;7 279ra239.
81. Zackheim HS, Kashani-Sabet M, Amin S. Topical corticosteroids for mycosis fungoides. Experience in 79 patients. *Arch Dermatol*. 1998;134:949–954.
82. Shipman AR, Scarisbrick J. New treatment options for mycosis fungoides. *Indian J Dermatol*. 2016;61:119.
83. Lessin SR, Duvic M, Guitart J, et al. Topical chemotherapy in cutaneous T-cell lymphoma: positive results of a randomized, controlled, multicenter trial testing the efficacy and safety of a novel mechloretamine, 0.02%, gel in mycosis fungoides. *JAMA Dermatol*. 2013;149:25–32.
84. Olsen EA, Hodak E, Anderson T, et al. Guidelines for phototherapy of mycosis fungoides and Sézary syndrome: a consensus statement of the United States cutaneous lymphoma consortium. *J Am Acad Dermatol*. 2016;74:27–58.
85. Hoppe RT, Harrison C, Tavallaee M, et al. Low-dose total skin electron beam therapy as an effective modality to reduce disease burden in patients with mycosis fungoides: results of a pooled analysis from 3 phase-II clinical trials. *J Am Acad Dermatol*. 2015;72:286–292.
86. Duvic M, Hymes K, Heald P, et al. Bexarotene is effective and safe for treatment of refractory advanced-stage cutaneous T-cell lymphoma: multinational phase II-III trial results. *J Clin Oncol*. 2001;19:2456–2471.
87. Olsen EA, Kim YH, Kuzel TM, et al. Phase IIb multicenter trial of vorinostat in patients with persistent, progressive, or treatment refractory cutaneous T-cell lymphoma. *J Clin Oncol*. 2007;25:3109–3115.
88. Piekarz RL, Frye R, Turner M, et al. Phase II multi-institutional trial of the histone deacetylase inhibitor romidepsin as monotherapy for patients with cutaneous T-cell lymphoma. *J Clin Oncol*. 2009;27:5410–5417.
89. Whittaker SJ, Demierre MF, Kim EJ, et al. Final results from a multicenter, international, pivotal study of romidepsin in refractory cutaneous T-cell lymphoma. *J Clin Oncol*. 2010;28:4485–4491.
90. Duvic M, Tetzlaff MT, Gangar P, et al. Results of a phase II trial of brentuximab vedotin for CD30$^+$ cutaneous T-cell lymphoma and lymphomatoid papulosis. *J Clin Oncol*. 2015;33:3759–3765.
91. Kim YH, Tavallaee M, Sundram U, et al. Phase II investigator-initiated study of brentuximab vedotin in mycosis fungoides and Sézary syndrome with variable CD30 expression level: a multi-institution collaborative project. *J Clin Oncol*. 2015;33:3750–3758.
92. Prince HM, Kim YH, Horwitz SM, et al. Brentuximab vedotin or physician's choice in CD30-positive cutaneous T-cell lymphoma (ALCANZA): an international, open-label, randomised, phase 3, multicentre trial. *Lancet*. 2017;390:555–566.
93. Duvic M, Pinter-Brown LC, Foss FM, et al. Phase 1/2 study of mogamulizumab, a defucosylated anti-CCR4 antibody, in previously treated patients with cutaneous T-cell lymphoma. *Blood*. 2015;125:1883–1889.
94. Kim YH, Bagot M, Pinter-Brown L, et al. Mogamulizumab versus vorinostat in previously treated cutaneous T-cell lymphoma (MAVORIC): an international, open-label, randomised, controlled phase 3 trial. *Lancet*. 2018;19:1192–1204.
95. Edelson R, Berger C, Gasparro F, et al. Treatment of cutaneous T-cell lymphoma by extracorporeal photochemotherapy. Preliminary results. *N Engl J Med*. 1987;316:297–303.

96. Zic JA. Extracorporeal Photopheresis in the treatment of mycosis fungoides and Sézary syndrome. *Dermatol Clin*. 2015;33:765–776.
97. Duvic M, Talpur R, Wen S, et al. Phase II Evaluation of Gemcitabine Monotherapy for Cutaneous T-Cell Lymphoma. *Clin Lymphoma Myeloma*. 2006;7:51–58.
98. Dummer R, Quaglino P, Becker JC, et al. Prospective international multicenter phase II trial of intravenous pegylated liposomal doxorubicin monochemotherapy in patients with stage IIB, IVA, or IVB advanced mycosis fungoides: final results from EORTC 21012. *J Clin Oncol*. 2012;30:4091–4097.
99. Shinohara MM, Shustov A. How I treat primary cutaneous CD30$^+$ lymphoproliferative disorders. *Blood*. 2019;134:515–524.
100. Di Raimondo C, Parekh V, Song JY, et al. Primary cutaneous CD30+ lymphoproliferative disorders: a comprehensive review. *Curr Hematol Malig Rep*. 2020;15:333–342.
101. Khodadoust MS, Rook AH, Porcu P, et al. Pembrolizumab in relapsed and refractory mycosis fungoides and Sézary syndrome: a multicenter phase II study. *J Clin Oncol*. 2020;38:20–28.
102. Bagot M, Porcu P, Marie-Cardine A, et al. IPH4102, a first-in-class anti-KIR3DL2 monoclonal antibody, in patients with relapsed or refractory cutaneous T-cell lymphoma: an international, first-in-human, open-label, phase 1 trial. *Lancet Oncol*. 2019;20:1160–1170.

CHAPTER 90

MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE AND SMOLDERING MULTIPLE MYELOMA

S. Vincent Rajkumar and Shaji Kumar

Monoclonal gammopathy of undetermined significance (MGUS) is a premalignant clonal plasma cell disorder.[1] It is characterized by the presence of a monoclonal (M) protein in the serum or urine that is commonly discovered during laboratory work-up of a variety disorders. MGUS is a precursor to various hematologic disorders including multiple myeloma (MM), Waldenström macroglobulinemia (WM), solitary plasmacytoma, and immunoglobulin light chain amyloidosis (AL). Smoldering MM (SMM) is a clinically-defined disorder similar to MGUS, but has a much higher risk of progression to MM or a related disorder.[2] SMM is not a unique intermediate biologic stage between MGUS and MM, but it consists of a heterogeneous mixture of patients, some of whom have a biologic premalignancy and have a clinical course resembling MGUS, and some who have an asymptomatic malignancy that will develop end-organ damage typical of MM within a few years. Unfortunately, at the current time, there is no single pathologic or molecular feature that reliably distinguishes patients with SMM who have premalignancy from those with asymptomatic malignancy. MGUS and SMM are usually asymptomatic but can be causally related to a variety of systemic disorders. They also carry a lifelong risk of progression to MM (see Chapter 91) or a related malignancy (see box on Diagnosis of MGUS and SMM: Role of Screening.)

MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE

MGUS is defined by the presence of a serum monoclonal (M) protein of <3 g/dL (or increased serum free light chain (FLC) level with abnormal free light chain (FLC ratio), $<10\%$ clonal bone marrow plasma cells (BMPC) with no evidence of SMM, end-organ damage, or other myeloma defining events (MDEs) (Table 90.1).[3] There are three major subtypes of MGUS: IgM MGUS, non-IgM MGUS, and light-chain MGUS (LC-MGUS). IgM MGUS carries a risk of progression to WM (see Chapter 92), although patients can rarely develop IgM MM. Non-IgM MGUS is associated with a risk of progression to MM, and LC-MGUS is a precursor of light chain MM. All forms of MGUS can lead to AL amyloidosis (see Chapter 93).

Epidemiology

Prevalence

With conventional testing, MGUS can be detected in approximately 4% of normal persons 50 years of age and older. Age-adjusted rates are greater in men than in women. The prevalence increases with age, exceeding 7.5% among those 85 years or older. Sensitive mass spectrometry studies show that the overall prevalence of MGUS is higher and is estimated at approximately 5% of the general population age 50 years and older.[6] The prevalence of MGUS is higher in Blacks compared to Whites. In a large population-based study ($n = 12{,}482$) of persons 50 years of age and older, using samples from the National Health and Nutrition Examination Survey (NHANES), the adjusted prevalence of MGUS was significantly higher in Blacks (3.7%) compared to Whites (2.3%) or Hispanics (1.8%) ($P < .001$).[7] A subsequent NHANES study found that the racial disparity in prevalence occurs even at younger ages, with MGUS having an onset approximately a decade earlier in Blacks compared with Whites.[8] Table 90.2 gives the estimated prevalence of MGUS by race and age based on NHANES studies.

The increased risk of MGUS seen in African Americans has also been found in Black people living in Ghana. Furthermore, studies show that the racial disparity between Blacks and Whites persists even after adjusting for socio-economic status, suggesting that the disparity in incidence is more likely due to genetics rather than environmental factors. Recent studies show that the t(11;14) translocation type of MGUS may be responsible for most of the increased disparity in MGUS between Blacks and Whites. The probability of having one of three specific subtypes, namely t(11;14), t(14;16), or t(14;20) was significantly higher in the 120 individuals with the highest African ancestry ($\geq 80\%$) compared with the 235 individuals with lowest African ancestry ($<0.1\%$) (51% vs. 33%, respectively, P value $= 0.008$).[9] Studies in other racial groups are limited, but so far an increased risk has not been seen in Japanese individuals, or in Hispanics living in the United States when compared to the White population of Olmsted County.

Incidence

The annual incidence of MGUS in males is estimated to be 120/100,000 at age 50, and rises to 530/100,000 at age 90 years.[10] The rates for women are 60/100,000 at age 50, and 370/100,000 at age 90. When MGUS is first diagnosed, it is estimated that the condition has already been present in an undiagnosed form for a median of over 10 years.

Other Risk Factors

First-degree relatives of patients with MGUS and MM have a two- or threefold higher risk of MGUS compared to those with no known affected relatives.[11] Obesity, exposure to pesticides, and immunosuppression are also known risk factors for MGUS.

DIAGNOSIS OF MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE AND SMOLDERING MULTIPLE MYELOMA: ROLE OF SCREENING

Routine screening is not recommended for MGUS since there are no randomized trials to support such a strategy. There are also no interventions to offer to patients who are diagnosed with MGUS. However, we do not have interventions to prevent end organ damage for patients with high-risk SMM. Since these patients are asymptomatic, diagnosis is incidental. Thus many patients who can potentially benefit from early intervention will go unidentified. This dilemma has led to consideration of screening in high-risk populations, namely Blacks with one affected first-degree relative with MM, and in all others with two or more affected first-degree relatives. In these subsets of the population, the prevalence of MGUS is likely greater than 10%–15%, and the probability of finding patients with high-risk SMM is higher. In our practice, we recommend such selected screening, until we have results of a large, randomized trial in Iceland, which is investigating the role of screening as well as the role of more aggressive follow-up of monoclonal gammopathies.

TABLE 90.1 **International Myeloma Working Group Diagnostic Criteria for Monoclonal Gammopathy of Undetermined Significance, Multiple Myeloma and Related Disorders**

Disorder	Disease Definition
IgM monoclonal gammopathy of undetermined significance (IgM MGUS)	All three criteria must be met: • Serum IgM monoclonal protein <3 g/dL • Bone marrow lymphoplasmacytic infiltration <10% • No evidence of anemia, constitutional symptoms, hyperviscosity, lymphadenopathy, or hepatosplenomegaly that can be attributed to the underlying lymphoproliferative disorder
Non-IgM monoclonal gammopathy of undetermined significance (MGUS)	All three criteria must be met: • Serum monoclonal protein (non-IgM type) <3 g/dL • Clonal bone marrow plasma cells <10%[a] • Absence of end-organ damage such as hypercalcemia, renal insufficiency, anemia, and bone lesions (CRAB) that can be attributed to the plasma cell proliferative disorder
Light chain MGUS	All criteria must be met: • Abnormal FLC ratio (<0.26 or >1.65) • Increased level of the appropriate involved light chain (increased kappa free light chain (FLC) in patients with ratio >1.65 and increased lambda FLC in patients with ratio <0.26) • No immunoglobulin heavy chain expression on immunofixation • Absence of end-organ damage that can be attributed to the plasma cell proliferative disorder • Clonal bone marrow plasma cells <10% • Urinary monoclonal protein <500 mg/24 h
Smoldering multiple myeloma	Both criteria must be met: • Serum monoclonal protein (IgG or IgA) ≥3 g/dL, or urinary monoclonal protein ≥500 mg/24 h and/or clonal bone marrow plasma cells 10%–60% • Absence of myeloma defining events or amyloidosis

Multiple myeloma	Both criteria must be met:
	• Clonal bone marrow plasma cells ≥10% or biopsy-proven bony or extramedullary plasmacytoma
	• Any one or more of the following myeloma defining events:
	  • Evidence of end organ damage that can be attributed to the underlying plasma cell proliferative disorder, specifically:
	  • Hypercalcemia: serum calcium >0·25 mmol/L (>1 mg/dL) higher than the upper limit of normal or >2·75 mmol/L (>11 mg/dL)
	  • Renal insufficiency: creatinine clearance <40 mL per minute or serum creatinine >177 µmol/L (>2 mg/dL)
	  • Anemia: hemoglobin value of >2 g/dL below the lower limit of normal, or a hemoglobin value <10 g/dL
	  • Bone lesions: one or more osteolytic lesions on skeletal radiography, computed tomograph (CT), or positron emission tomography-CT (PET-CT)
	• Clonal bone marrow plasma cell percentage ≥60%
	• Involved:uninvolved serum FLC ratio ≥100 (involved FLC level must be ≥100 mg/L)
	• >1 focal lesions on magnetic resonance imaging (MRI) studies (at least 5 mm in size)

Smoldering Waldenström macroglobulinemia (also referred to as indolent or asymptomatic Waldenström's macroglobulinemia)	Both criteria must be met: • Serum IgM monoclonal protein ≥3 g/dL and/or bone marrow lymphoplasmacytic infiltration ≥10% • No evidence of anemia, constitutional symptoms, hyperviscosity, lymphadenopathy, or hepatosplenomegaly that can be attributed to the underlying lymphoproliferative disorder.
Waldenström macroglobulinemia	All criteria must be met: • IgM monoclonal gammopathy (regardless of the size of the M protein) • ≥10% bone marrow lymphoplasmacytic infiltration (usually intertrabecular) by small lymphocytes that exhibit plasmacytoid or plasma cell differentiation and a typical immunophenotype (e.g., surface IgM^{+}, $CD5^{+/-}$, $CD10^{-}$, $CD19^{+}$, $CD20^{+}$, $CD23^{-}$) that satisfactorily excludes other lymphoproliferative disorders including chronic lymphocytic leukemia and mantle cell lymphoma • Evidence of anemia, constitutional symptoms, hyperviscosity, lymphadenopathy, or hepatosplenomegaly that can be attributed to the underlying lymphoproliferative disorder
Solitary plasmacytoma	All 4 criteria must be met • Biopsy proven solitary lesion of bone or soft tissue with evidence of clonal plasma cells • Normal bone marrow with no evidence of clonal plasma cells • Normal skeletal survey and MRI (or CT) of spine and pelvis (except for the primary solitary lesion) • Absence of end-organ damage such as hypercalcemia, renal insufficiency, anemia, or bone lesions (CRAB) that can be attributed to a lympho-plasma cell proliferative disorder
Solitary plasmacytoma with minimal marrow involvement[b]	All four criteria must be met • Biopsy proven solitary lesion of bone or soft tissue with evidence of clonal plasma cells • Clonal bone marrow plasma cells <10% • Normal skeletal survey and MRI (or CT) of spine and pelvis (except for the primary solitary lesion) • Absence of end-organ damage such as hypercalcemia, renal insufficiency, anemia, or bone lesions (CRAB) that can be attributed to a lympho-plasma cell proliferative disorder

Continued

TABLE 90.1 International Myeloma Working Group Diagnostic Criteria for Monoclonal Gammopathy of Undetermined Significance, Multiple Myeloma and Related Disorders—cont'd

Disorder	Disease Definition
Systemic AL amyloidosis	All four criteria must be met: • Presence of an amyloid-related systemic syndrome (such as renal, liver, heart, gastrointestinal tract, or peripheral nerve involvement) • Positive amyloid staining by Congo red in any tissue (e.g., fat aspirate, bone marrow, or organ biopsy) • Evidence that amyloid is light-chain related established by direct examination of the amyloid using mass spectrometry (MS)-based proteomic analysis, or immuno-electron microscopy • Evidence of a monoclonal plasma cell proliferative disorder (serum or urine M protein, abnormal FLC ratio, or clonal plasma cells in the bone marrow) **Note:** Approximately 2%–3% of patients with AL amyloidosis will not meet the requirement for evidence of a monoclonal plasma cell disorder listed above; the diagnosis of AL amyloidosis must be made with caution in these patients. Patients with AL amyloidosis who also meet criteria for multiple myeloma are considered to have both diseases.
POEMS syndrome	All four criteria must be met • Polyneuropathy • Monoclonal plasma cell proliferative disorder (almost always lambda) • Any one of the following three other major criteria: 1 Sclerotic bone lesions 2 Castleman's disease 3 Elevated levels of vascular endothelial growth factor (VEGF)[†] • Any one of the following six minor criteria 1 Organomegaly (splenomegaly, hepatomegaly, or lymphadenopathy) 2 Extravascular volume overload (edema, pleural effusion, or ascites) 3 Endocrinopathy (adrenal, thyroid, pituitary, gonadal, parathyroid, pancreatic)[‡] 4 Skin changes (hyperpigmentation, hypertrichosis, glomeruloid hemangiomata, plethora, acrocyanosis, flushing, white nails) 5 Papilledema 6 Thrombocytosis/polycythemia **Note:** Not every patient meeting the above criteria will have POEMS syndrome; the features should have a temporal relationship to each other and no other attributable cause. Anemia and/or thrombocytopenia are distinctively unusual in this syndrome unless Castleman disease is present. [†]The source data do not define an optimal cut off value for considering elevated VEGF level as a major criterion. We suggest that VEGF measured in the serum or plasma should be at least three- to fourfold higher than the normal reference range for the laboratory that is doing the testing to be considered a major criterion. [‡]In order to consider endocrinopathy as a minor criterion, an endocrine disorder other than diabetes or hypothyroidism is required since these two disorders are common in the general population.

[a] A bone marrow can be deferred in patients with low-risk MGUS (IgG type, M protein <15 g/L, normal free light chain ratio) in whom there are no clinical features concerning for myeloma.

[b] Solitary plasmacytoma with 10% or more clonal plasma cells is considered as multiple myeloma.

From Rajkumar SV, Dimopoulos MA, Palumbo A, et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. *Lancet Oncol.* 2014;15:e538–e548, with permission from Elsevier.

TABLE 90.2 Prevalence of Monoclonal Gammopathy of Undetermined Significance (%), by Race/Ethnicity and Age

Age Group (Years)	Blacks % (95%CI)	Whites % (95%CI)	Hispanic American % (95%CI)	Total[a] % (95%CI)
10–19	0.00 (NA)	0.00 (NA)	0.11 (0.02–0.84)	0.01 (0.00–0.07)
20–29	0.26 (0.08–0.84)	0.00 (NA)	0.00 (NA)	0.03 (0.01–0.11)
30–39	0.81 (0.43–1.53)	0.32 (0.11–0.99)	0.23 (0.06–0.79)	0.49 (0.27–0.91)
40–49	3.26 (2.04–5.18)	0.53 (0.20–1.37)	2.20 (1.22–3.95)	0.88 (0.53–1.47)
50–59	2.19 (1.32–3.60)	1.01 (0.62–1.66)	0.85 (0.33–2.23)	1.20 (0.83–1.72)
60–69	3.48 (2.26–5.33)	2.43 (1.72–3.43)	2.41 (1.69–3.44)	2.45 (1.85–3.23)
70–79	5.67 (3.66–8.70)	3.44 (2.55–4.63)	2.51 (1.36–4.58)	3.43 (2.64–4.47)
80+	8.56 (4.92–14.47)	4.42 (3.31–5.87)	3.97 (1.33–11.22)	4.58 (3.54–5.90)

[a] Includes "Other" race/ethnicity group.

Modified from Landgren O, Graubard BI, Kumar S, et al. Prevalence of myeloma precursor state monoclonal gammopathy of undetermined significance in 12372 individuals 10–49 years old: a population-based study from the National Health and Nutrition Examination Survey. *Blood Cancer J.* 2017;7:e618.

Pathobiology

MGUS likely originates when plasma cells are dividing in response to antigenic stimulation. During this period of antigenic stimulation they acquire a primary cytogenetic abnormality that established the MGUS clone (Fig. 90.1).[12] The primary cytogenetic abnormalities associated with the onset of MGUS consist of two major types: trisomies and immunoglobulin heavy chain (IgH) translocations (Table 90.3). IgH translocations involve the immunoglobulin heavy chain switch region on chromosome 14q32, and one of five common partner chromosomes loci: 11q13 (CCND1 [cyclin D1 gene]), 4p16.3 (FGFR-3 and MMSET), 6p21 (CCND3 [cyclin D3 gene]), 16q23 (c-MAF), and 20q11 (MAF-B).

MGUS may stay dormant or acquire secondary cytogenetic abnormalities and progress to MM or a related malignancy. The rate of progression of MGUS is approximately 1% per year regardless of the known duration of antecedent MGUS, indicating a random hit model of progression.[13] Although the precise pathogenetic steps are unclear, there appear to be two main pathways involved, namely, *MYC* translocations and mutations in the MAP kinase pathway. The presence of *MYC* structural variants is associated with a significantly shorter median time to progression (TTP) compared to the absence of such abnormalities. Other abnormalities associated with progression include chromosome 1q21 (gain 1q), p16 methylation, p53 mutations, and changes in the bone marrow microenvironment, including induction of angiogenesis and suppression of cell-mediated immunity, affecting things such as T-cell production.

Progression is also associated with increase in receptor activator of nuclear factor κB ligand (RANKL) expression by osteoblasts and possibly plasma cells accompanied by decreased stromal cell secretion of the RANKL decoy receptor, osteoprotegerin (OPG). There is also release of macrophage inflammatory protein–1α (MIP-1α) and MIP-1β by myeloma cells. Collectively these changes promote osteoclast activation through the nuclear factor κB (NF-κB) pathway. In addition, there is simultaneous inhibition of osteoblasts mediated by increased dickkopf 1 (DKK-1) expression by myeloma cells. The combination of osteoclast activation and inhibition of osteoblast differentiation is thought to be the mechanism behind the development of osteolytic lesions in MM, and may also explain increased risk of fractures seen in MGUS even in absence of progression to MM.

Clinical Manifestations

MGUS is an asymptomatic condition.[14] It is typically detected as an incidental finding when electrophoresis and immunofixation of the serum and/or urine, or the serum FLC assay are performed during the work-up of suspected MM or WM, including symptoms such as unexplained weakness or fatigue, increased erythrocyte sedimentation rate, anemia, unexplained back pain, osteoporosis, osteolytic lesions or fractures, hypercalcemia, proteinuria, renal insufficiency, or recurrent infections. MGUS may also be detected during work-up of patients with symptoms suggestive of AL amyloidosis such as unexplained sensorimotor peripheral neuropathy, carpal tunnel syndrome, refractory congestive heart failure, nephrotic syndrome, orthostatic hypotension, malabsorption, weight loss, change in the tongue or voice, paresthesias, numbness, increased bruising, bleeding, and steatorrhea.

Association of Monoclonal Gammopathy of Undetermined Significance With Other Diseases

Although most patients diagnosed with MGUS are asymptomatic, some can have disorders that are causally related to the M protein, without any evidence of overt progression to MM or related malignancy (Table 90.4).[14] In fact, increasingly MGUS is diagnosed in such situations, during work-up of unexplained renal, neuropathic, or other organ dysfunction. Over a hundred different medical conditions have been reported to be associated with MGUS, but most such reported associations are coincidental rather than causal. The important causal related disorders are discussed below.

Monoclonal Gammopathy Associated Neuropathy

Approximately 3% to 5% of patients with monoclonal gammopathy have a peripheral neuropathy, and approximately 3% to 5% of patients with a peripheral neuropathy have evidence of an M protein. IgM MGUS associated neuropathy presents as a chronic

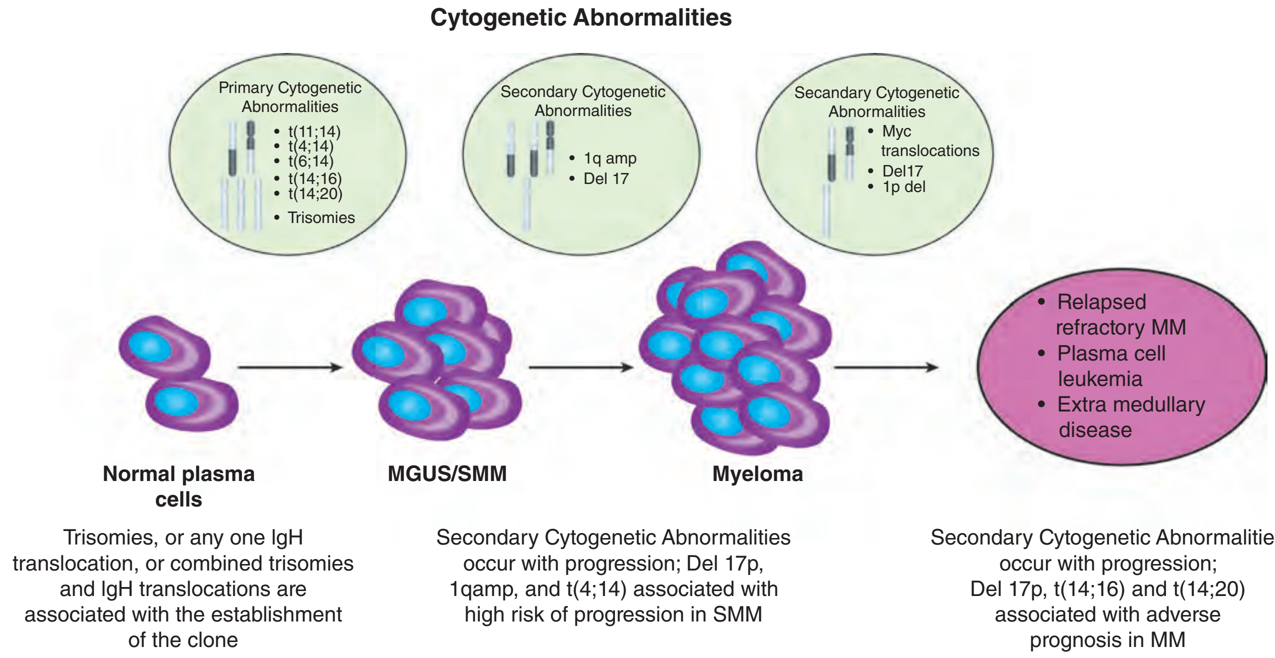

Cytogenetic Abnormalities
Primary Cytogenetic Abnormalities
• t(11;14)
• t(4;14)
• t(6;14)
• t(14;16)
• t(14;20)
• Trisomies
Secondary Cytogenetic Abnormalities
• 1q amp
• Del 17
Secondary Cytogenetic Abnormalities
• Myc translocations
• Del17
• 1p del
• Relapsed refractory MM
• Plasma cell leukemia
• Extra medullary disease
Normal plasma cells
MGUS/SMM
Myeloma
Trisomies, or any one IgH translocation, or combined trisomies and IgH translocations are associated with the establishment of the clone
Secondary Cytogenetic Abnormalities occur with progression; Del 17p, 1qamp, and t(4;14) associated with high risk of progression in SMM
Secondary Cytogenetic Abnormalities occur with progression; Del 17p, t(14;16) and t(14;20) associated with adverse prognosis in MM

Figure 90.1 PATHOGENESIS OF MONOCLONAL GAMMOPATHIES. Primary cytogenetic abnormalities occur early, when the normal plasma cell transitions to a clonal, premalignant stage, termed monoclonal gammopathy of undetermined significance. Subsequently, secondary cytogenetic abnormalities occur associated with and leading to malignant transformation. *(From Rajan AM, Rajkumar SV. Interpretation of cytogenetic results in multiple myeloma for clinical practice. Blood Cancer J. 2015;5:e365.)*

TABLE 90.3 **Primary Cytogenetic Abnormalities in Monoclonal Gammopathy of Undetermined Significance**

Subtype	Gene(s) Affected
Trisomies	Usually involving odd-numbered chromosomes with the exception of chromosomes 1, 13, and 21. Multiple genes affected
Primary immunoglobulin rearrangements[a]	
t(11;14) (q13;q32)	*CCND1* (cyclin D1)
t(4;14) (p16;q32)	*FGFR-3* and MMSET
t(14;16) (q32;q23)	*C-MAF*
t(14;20) (q32;q11)	*MAFB*
t(6;14) (p21;q32)	*CCND3* (cyclin D3)
Other IgH translocations	The genes affected vary depending on the partner chromosome

[a] Other rare partner chromosomes have been described. Some patients have combined IgH translocations and trisomies, but they should be classified based on the IgH translocation.

distal, acquired, demyelinating, symmetric sensory neuropathy (DADS-M).[15] In approximately half of these patients, the IgM M protein binds to myelin-associated glycoprotein (MAG). In contrast, patients with non-IgM associated neuropathy have a varied presentation, but the clearest association is in patients who present with features similar to chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) with predominantly motor involvement with distal and proximal involvement. MGUS neuropathy must be differentiated from two other plasma cell disorders that cause neuropathy that have been well characterized and have strict diagnostic criteria, namely immunoglobulin light chain (AL) amyloidosis, and neuropathy associated with POEMS (polyneuropathy, organomegaly).

Monoclonal Gammopathy Associated Renal Disorders

A substantial proportion of cases with "idiopathic" immune-complex mediated membranoproliferative glomerulonephritis (MPGN) are now felt to be related to MGUS.[16] MGUS can cause proliferative glomerulonephritis by direct deposition of M protein in the mesangium and along capillary walls, resulting in an immune complex-mediated proliferative glomerulonephritis. It can also cause glomerulonephritis by, indirectly, activating the alternative pathway of complement resulting in deposition of complement factors in the glomeruli, resulting in C3 glomerulopathy. The term monoclonal gammopathy of renal significance (MGRS) is used to describe renal diseases such as those caused by a premalignant MGUS clone, and the aforementioned M protein-related disorders such as AL amyloidosis and monoclonal immunoglobulin deposition disease.

Monoclonal Gammopathy Associated Dermatologic Diseases

A variety of dermatologic diseases have been associated with MGUS including lichen myxedematosus (papular mucinosis, scleromyxedema), scleredema (Buschke disease), pyoderma gangrenosum, diffuse plane xanthomatosis, and subcorneal pustular dermatosis. Other skin disorders associated with MGUS are necrobiotic xanthogranuloma and Schnitzler syndrome, a rare disorder characterized by chronic urticaria and an IgM monoclonal gammopathy.

Other Associations of Monoclonal Gammopathy of Undetermined Significance

MGUS has been associated with an increased risk of fractures and deep vein thrombosis, and has been linked to chronic lymphocytic leukemia, hairy cell leukemia, T-cell lymphoma, and other malignancies, but the significance of these associations is not known. MGUS has also been associated with acquired von Willebrand disease, Gaucher disease, pure red cell aplasia, connective tissue disorders, various neurologic disorders. and can occasionally be the cause of systemic capillary leak syndrome.

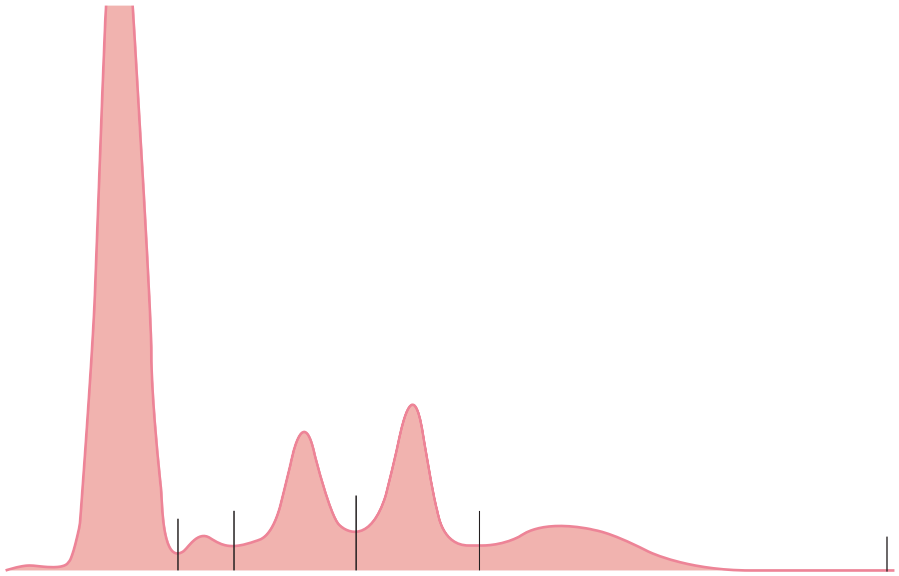
TABLE 90.4 **Non-Malignant Diseases Associated With Monoclonal Gammopathy of Undetermined Significance and May Respond to Lymphoplasmacytic Cell-Directed Therapy**

Primary Organ Involved	Clinical Presentation	Role of Monoclonal Protein/ Pathophysiology
Schnitzler syndrome	Chronic urticaria, fever, bone pain, IgM-MGUS	Unclear
Insulin autoimmune syndrome	Episodic confusion, diaphoresis, dizziness, lethargy, palpitation, seizure	Antibody to insulin causing its inactivation
TEMPI	Telangiectasias, erythrocytosis, elevated erythropoietin level, MGUS, perinephric fluid collections, and intrapulmonary shunting	Unclear
Rheumatologic scleromyxedema	Waxy papules or plaques, arthralgia, restrictive lung disease, seizure	Unclear
Progressive glomerulonephritis with monoclonal immunoglobulin deposits	Hematuria, hypertension, proteinuria, renal impairment, mostly IgG3κ-MGUS	Granular deposition of immunoglobulin in glomeruli
Sporadic late-onset nemaline myopathy	Muscular weakness and atrophy frequently resulting in "head drop," respiratory insufficiency, congestive heart failure	Unclear
Crystalline keratopathy	Decreased visual acuity, corneal opacity, IgGκ-MGUS	Corneal deposition of antibody forming a crystalline structure
Crystal-storing histiocytosis	Mass or tissue infiltration, which may involve the bone marrow, breast, gastrointestinal tract, kidneys, lymph node, skin, or spleen	Accumulation of light chain crystals in histiocytes

MGUS, Monoclonal gammopathy of undetermined significance.
From Go RS, Rajkumar SV. How I manage monoclonal gammopathy of undetermined significance. *Blood*. 2018;131;163–173. © American Society of Hematology.

Laboratory Manifestations

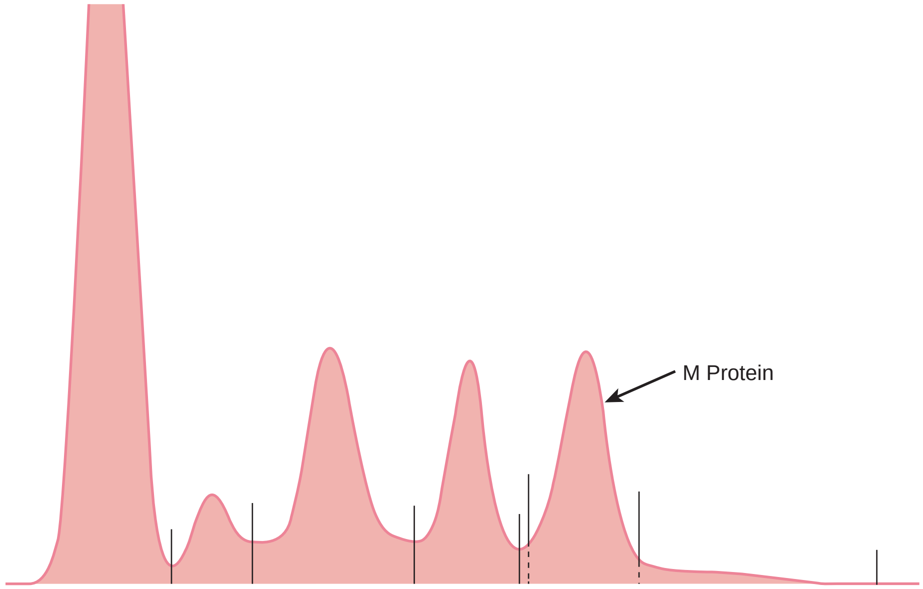
The hallmark of MGUS is the presence of an M protein in the serum and/or urine. M proteins are detected using protein electrophoresis of the serum and urine. The M protein appears as a narrow peak (like a church spire) on the densitometer tracing or as a dense, discrete band on agarose gel (Fig. 90.2). Protein electrophoresis also permits quantitation of M proteins. Urine electrophoresis requires a 24-hour urine collection. Immunofixation of the serum and urine is more sensitive than protein electrophoresis and is used to identify the type of M protein heavy and light chains (IgM kappa, IgG lambda, etc.).



Fraction	%	Units
Albumin	56.26	3.43
Alpha 1	2.78	0.17
Alpha 2	13.93	0.85
Beta	15.50	0.95
Gamma	11.54	0.70
Total Protein		6.10

Ratio	
A/G	1.29

SPEP Normal



Fraction	%	Units
Albumin	45.72	2.97
Alpha 1	5.10	0.33
Alpha 2	18.85	1.23
Beta	12.77	0.83
Gamma	17.56	1.14
Total Protein		6.50

Restricted Band	%	Units
Gamma	14.93	0.97

Figure 90.2 SERUM PROTEIN ELECTROPHORESIS. (*top panel*) Normal serum protein electrophoresis result with albumin, alpha 1, alpha 2, beta, and gamma fractions (*from left to right*). (*bottom panel*) Abnormal serum protein electrophoresis with a monoclonal (M) protein in the gamma region.

The FLC assay is a nephelometric assay that measures free kappa (κ) and lambda (λ) light chains that are not bound to intact immunoglobulin. Patients with abnormally low kappa/lambda FLC ratios are considered to have a monoclonal lambda FLC, and those with abnormally high ratios are defined as having a monoclonal kappa FLC. The serum FLC assay is more sensitive than electrophoresis or immunofixation in detecting free monoclonal light chains, and must be done at diagnosis in conjunction with those tests.

At diagnosis, osteolytic bone disease must be excluded in order to distinguish MGUS from MM. The preferred method to detect bone disease is a whole-body low-dose computed tomography (CT).[17] Positron emission tomography-CT (PET-CT) is an equally sensitive alternative to whole body low-dose CT. A unilateral bone marrow aspiration and biopsy, if indicated, must show less than 10% clonal BMPCs. For flow cytometry, plasma cells in MGUS typically stain positive for CD38 and CD138, and are positive only for one of the two light chain isotypes, indicating they are monoclonal (either kappa or lambda). Baseline cytogenetic abnormalities are best ascertained by fluorescent in situ hybridization (FISH) testing on the bone marrow sample at diagnosis (Fig. 90.3). In patients suspected of monoclonal protein related disorders, confirmatory testing is needed, such as renal biopsy in patients with monoclonal gammopathy associated proliferative glomerulonephritis (Fig. 90.4).

Differential Diagnosis

MGUS is differentiated from MM and SMM by the size of the M protein, the BMPC percentage, and the presence or absence of anemia, renal failure, hypercalcemia, or lytic bone lesions (see Table 90.1). Because anemia and renal insufficiency are relatively common in the elderly population with MGUS, the causes of these conditions should be carefully investigated with adequate laboratory studies.

Prognosis

MGUS is associated with a life-long risk of progression to MM or a related disorder. In a study of 1384 patients from Southeastern Minnesota, the risk of progression of MGUS to MM or related malignancy was 1% per year.[13] The true life-time probability of progression is lower when competing causes of death are taken into account, approximately 11% at 25 years.[18] The risk of progression with MGUS does not diminish with time, even after over 30 years of follow-up.[1] Progression events include MM, WM, solitary plasmacytoma, and AL amyloidosis. The risk of progression from MGUS has been estimated in several other studies, and the results mirror those seen in the Southeastern Minnesota study. No findings from the diagnosis of MGUS can reliably distinguish patients whose condition will remain stable indefinitely from those in whom MM or related malignancy develops. However, there are several known prognostic factors that assist in estimation of the risk of progression for appropriate counseling and management.

Size of M Protein

The size of the M protein at recognition of MGUS is one of the most important predictors for the risk of progression. In the study of 1384 patients from Southeastern Minnesota, the risk of progression to MM or a related disorder 10 years after diagnosis of MGUS was 6% for patients with an initial M-protein level of 0.5 g/dL or less, 7% for 1 g/dL, 11% for 1.5 g/dL, 20% for 2 g/dL, 24% for 2.5 g/dL, and 34% for 3.0 g/dL.[13]

Type of M Protein

Patients with an IgM or IgA M protein have a higher risk of progression compared with those with an IgG M protein.[13] IgM MGUS is a unique subtype of MGUS in which patients are at risk of progression to WM rather than MM. Rarely patients with IgM MGUS can evolve into IgM MM.

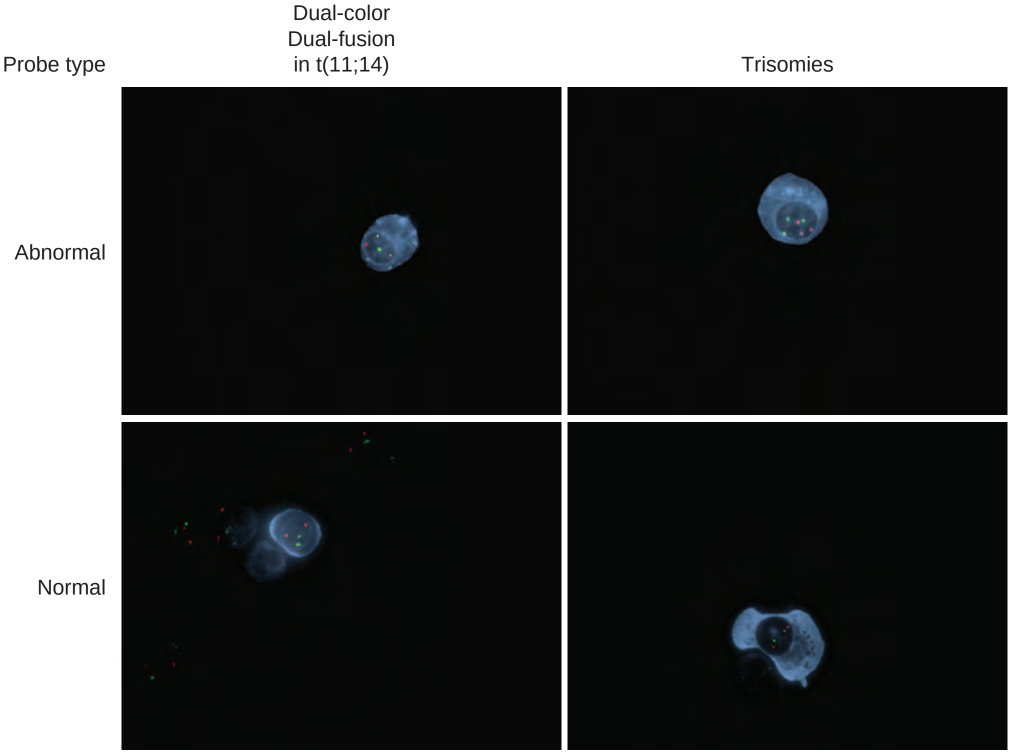


Figure 90.3 FLUORESCENT IN SITU HYBRIDIZATION (FISH). Cytoplasmic immunoglobulin (cIg) stain antibodies for kappa and lambda are used to detect plasma cells in unsorted bone marrow. Representative FISH images of cIg-positive plasma cells using the following probes: CCND1/IGH (CCND1-XT[11q13](R)/IGH-XT[14q32](G), Abbott Molecular) to detect t(11;14)(q13;q32); D3Z1/D7Z1 (D3Z1[CEN3](R)/D7Z1[CEN7](G), Abbott Molecular) for trisomy 3 and/or 7 (G = green, R = red). Abnormal (*top from left to right*): showing representative patients with a t(11;14) translocation showing a fusion signal (yellow), and trisomies 3 and 7 (three red and three green signals seen instead of two each). Normal (*bottom from left to right*): representative patient with a normal signal pattern for the listed probes. *(Images provided by the Mayo Clinic Genomics Laboratory.)*

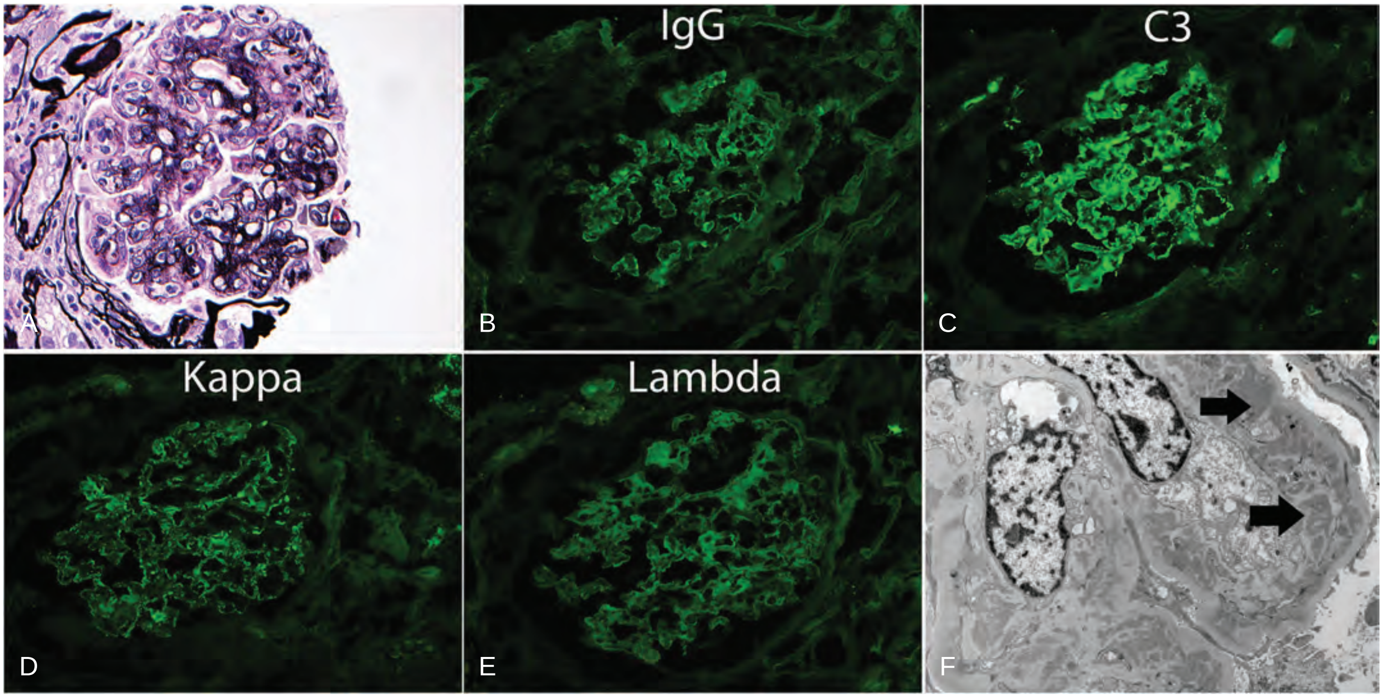


Figure 90.4 PROLIFERATIVE GLOMERULONEPHRITIS DUE TO MONOCLONAL GAMMOPATHY. (A) Light microscopy showing membranoproliferative glomerulonephritis pattern of injury (silver 40×). (B–E) Immunofluorescence studies showing granular (B) IgG, (C) C3, (D) kappa light chains along capillary walls, and (E) negative lambda light chains. (F) Electron microscopy showed sub-endothelial electron dense deposits *(arrows)*.

Abnormal Serum-Free Light Chain Ratio

An abnormal FLC ratio is an independent risk factor for progression of MGUS, and is present in approximately one-third of patients with MGUS.[18] The risk of progression in patients with an abnormal FLC ratio was significantly higher than that in patients with a normal ratio (hazard ratio 3.5; $P < .001$).

Other Risk Factors

Patients with MGUS with 5% to 9% BMPCs had a higher risk of progression than those with <5% BMPCs. The risk of progression of MGUS is also increased if there is associated suppression of the other uninvolved immunoglobulins.

Risk Stratification

A risk-stratification model, based on the size and type of the M protein and the FLC ratio, is used to predict risk of progression of MGUS (Table 90.5).[18] Patients with all three risk factors consisting of an abnormal serum FLC ratio, IgA or IgM MGUS, and an increased serum M-protein value (≥1.5 g/dL) have a risk of progression at 20 years of 58%, whereas the risk is 5% when none of the risk factors are present.

TABLE 90.5 **Risk-Stratification Model to Predict Progression of Monoclonal Gammopathy of Undetermined Significance to Myeloma or Related Disorders**

Risk Group	No. of Patients	Relative Risk	Absolute Risk of Progression at 20 Years	Absolute Risk of Progression at 20 Years Accounting for Death as a Competing Risk
Low-risk (Serum M protein <1.5 g/dL, IgG subtype, normal FLC ratio (0.26–1.65)	449	1	5%	2%
Low-intermediate-risk (Any 1 factor abnormal)	420	5.4	21%	10%
High-intermediate-risk (Any 2 factors abnormal)	226	10.1	37%	18%
High-risk (All 3 factors abnormal)	53	20.8	58%	27%

From Rajkumar SV, Kyle RA, Therneau TM, et al. Serum free light chain ratio is an independent risk factor for progression in monoclonal gammopathy of undetermined significance. *Blood*. 2005; 106;812–817. © American Society of Hematology.

Life Expectancy

In a population-based study of 1384 patients with MGUS in Southeastern Minnesota, a lower survival was seen compared to age-matched control population, even after excluding all persons who progressed to MM or related disorders.[1] However, it is not clear whether the excess risk of death from MGUS was due to the conditions that led to testing for MGUS, or whether there is a true adverse impact on survival that is independent of malignant progression.

Therapy

The approach to management of MGUS is given in Fig. 90.5. All patients diagnosed with MGUS need baseline complete blood count (CBC), serum calcium, and serum creatinine. If available, peripheral blood flow cytometry for circulating plasma cells should also be done. Radiographic studies of the bones can be omitted in asymptomatic patients with IgM MGUS or low-risk MGUS (see Table 90.5). A bone marrow biopsy can be deferred in patients with a clinical picture otherwise consistent with MGUS, and the patient is classified as low-risk MGUS based on the risk-stratification model shown in

Table 90.5.[14] A bone marrow biopsy can also be deferred in patients with a small (<1.5 g/dL) IgM MGUS and normal blood counts.

No therapy is needed for MGUS. In all patients, the CBC, serum calcium, creatinine and serum protein electrophoresis, and serum FLC must be repeated in 6 months. If stable, further follow-up can be deferred in patients with low-risk MGUS until worrisome symptoms for progression develop. In all other patients with MGUS, lifelong annual follow-up of the M protein is recommended (see box on Approach to Monoclonal Gammopathy of Undetermined Significance).

Clinical trials are ongoing to determine if the risk of progression can be reduced by low-risk interventional approaches. However, since the overall risk of progression is low, any intervention will have to be highly effective with very low risk to justify incorporation into clinical practice. A promising clinical trial in Iceland is testing if more aggressive monitoring can detect MM early in patients with MGUS, and can provide clinical benefit.

MGUS Associated Disorders

The results of treatment of peripheral neuropathy and monoclonal gammopathy have been disappointing. For patients with IgM monoclonal gammopathy associated neuropathy, a trial of intravenous immunoglobulin (IVIG) is reasonable.[15] Rituximab can be considered if IVIG is not of benefit. If patients are refractory to rituximab, or other treatments used for WM can be tried on a case-by-case basis. For patients with non-IgM monoclonal gammopathy associated neuropathy who present similar to CIDP, therapy should

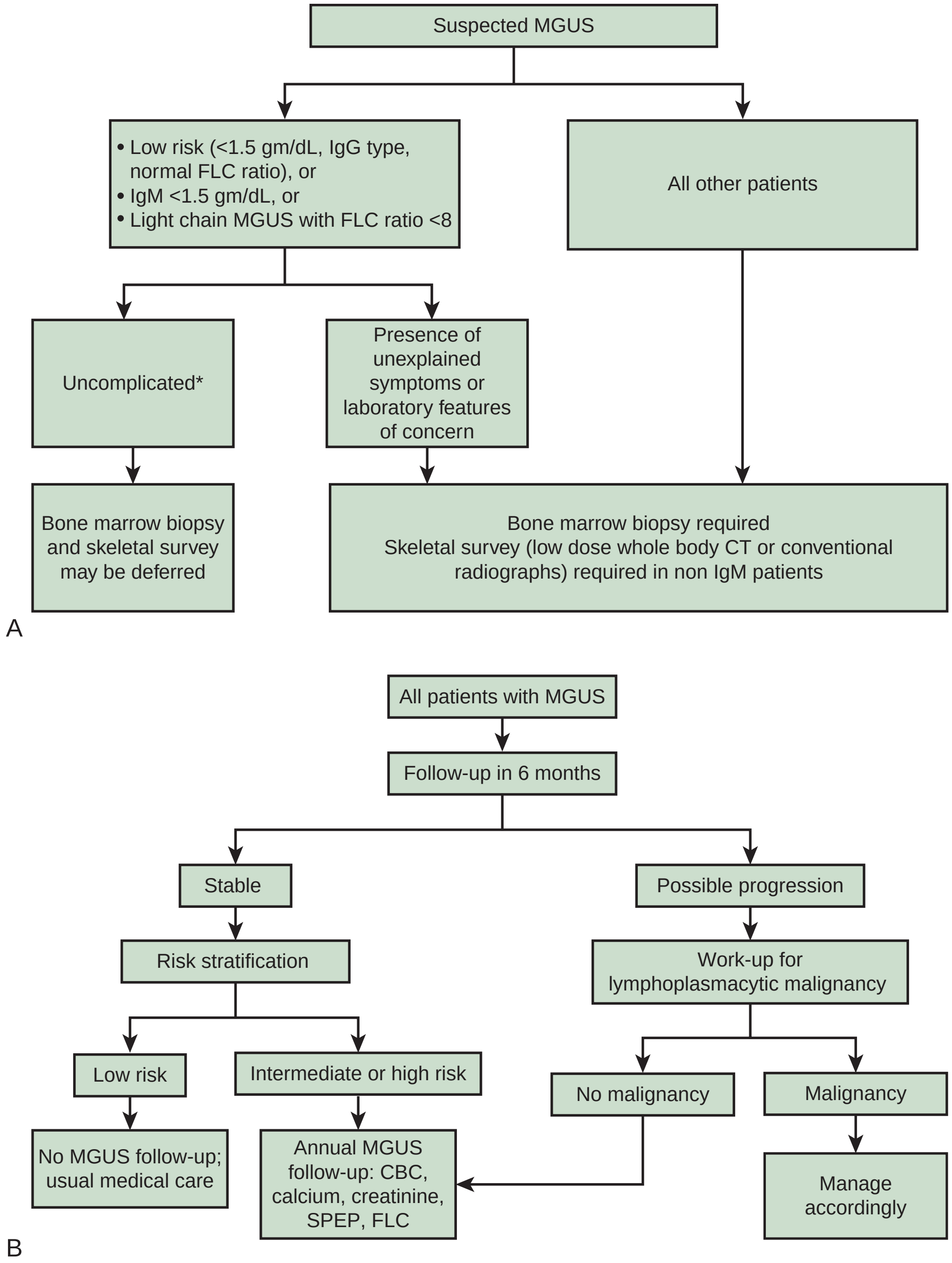

Suspected MGUS
• Low risk (<1.5 gm/dL, IgG type, normal FLC ratio), or
• IgM <1.5 gm/dL, or
• Light chain MGUS with FLC ratio <8
All other patients
Uncomplicated*
Presence of unexplained symptoms or laboratory features of concern
Bone marrow biopsy and skeletal survey may be deferred
Bone marrow biopsy required
Skeletal survey (low dose whole body CT or conventional radiographs) required in non IgM patients
A
All patients with MGUS
Follow-up in 6 months
Stable
Possible progression
Risk stratification
Work-up for lymphoplasmacytic malignancy
Low risk
Intermediate or high risk
No malignancy
Malignancy
No MGUS follow-up; usual medical care
Annual MGUS follow-up: CBC, calcium, creatinine, SPEP, FLC
Manage accordingly
B

Figure 90.5 APPROACH TO MANAGEMENT OF MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE (MGUS). (A) Approach to diagnostic work-up. (B) Approach to follow-up. *CT*, Computed tomography; *FLC*, free light chain; *SPEP*, serum protein electrophoresis. *(From Go RS, Rajkuamr SV. How I manage monoclonal gammopathy of undetermined significance. Blood. 2018;131(2):163–173. © American Society of Hematology.)*

APPROACH TO MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE

The first step in the evaluation of MGUS is to exclude multiple myeloma, AL amyloidosis, or related malignancy. At the same time, even if anemia, hypercalcemia, or renal failure was present, a diagnosis of myeloma cannot be assumed. Other causes need to be excluded. In patients with uncomplicated low-risk MGUS by risk stratification (see Table 90.5), we usually omit both the bone marrow biopsy and bone imaging. A repeat measurement of the M protein in 6 months is sufficient. A bone marrow biopsy can also be omitted in small (<1.5 g/dL) IgM MGUS. Further patients with IgM MGUS of any size do not need bone imaging unless they have concerning symptoms. This strategy reduces the number of unnecessary investigations and reduces cost. The yield of these procedures in such patients has been shown to be extremely low. All other patients need a baseline bone marrow biopsy and bone imaging.

be similar to that used for the treatment of classic CIDP with plasmapheresis, IVIG, and steroids. Patients with monoclonal gammopathy associated glomerulonephritis may benefit from attempts to lower or eradicate the M protein, with treatments used in MM such as bortezomib, cyclophosphamide, dexamethasone (VCd), or daratumumab.[16]

VARIANTS OF MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE

Secondary Monoclonal Gammopathy of Undetermined Significance

Secondary MGUS refers to new M proteins that arise in myeloma patients following therapy that is of an isotype distinct from the original clone. In a study of 1942 patients with MM, 128 (6.6%) developed a secondary MGUS, at a median time of 12 months from the diagnosis of MM.[19] Secondary MGUS is usually transient and disappears at a median duration of 6 months. In some, there can be multiple secondary MGUS's of varying isotypes. Secondary MGUS occurs more commonly following stem cell transplantation, and most of these M proteins are small and detectable by immunofixation only. Interestingly, overall survival of MM is significantly superior among patients who developed secondary MGUS compared with those who do not, especially if the M protein resolves over time.

Biclonal and Triclonal Gammopathy

Occasionally two or three unique M proteins can be detected in one person at the same time and are referred to as biclonal and triclonal gammopathies. In some patients complete class switching in a single plasma cell clone results in the production of two M proteins, but in others, two or more M proteins can arise from separate plasma cell clones.

SMOLDERING MULTIPLE MYELOMA

Introduction

SMM is defined by the presence of a serum M protein of ≥ 3 g/dL (or urine M protein ≥ 500 mg/24 h), and/or 10% to 60% clonal BMPC with no evidence of end-organ damage (i.e., CRAB criteria), or other MDE (see Table 90.1).[3] It is distinguished from MGUS based on the level of serum M protein and the percentage of clonal BMPCs.[2] The current International Myeloma Working Group (IMWG) diagnostic criteria for SMM excludes patients with clonal BMPC $\geq$ 60%, serum involved/uninvolved FLC ratio of ≥ 100, and those with two or more focal bone lesions on magnetic resonance imaging (MRI).[3] Such patients have an approximately 80% risk of progression to end-organ damage and are considered as MM. Light chain SMM is a unique subtype of SMM in which there is monoclonal FLC excess with no expression of IgH.[20] This entity is characterized by excess secretion (≥ 500 mg/24 hours) of monoclonal FLC in the urine (Bence Jones proteinuria).

Epidemiology

SMM accounts for approximately 15% of all cases of newly diagnosed MM. An estimate of the true prevalence of SMM on the basis of updated IMWG criteria is not available. Furthermore, since SMM is asymptomatic and requires bone marrow examination for diagnosis, prevalence in the general population is unknown. Details on racial disparity are not available, but it is likely that as with MGUS and MM, the prevalence of SMM is likely to be two- to threefold higher in Blacks compared to Whites.

Pathobiology

SMM is not a unique biologic entity. It is a heterogeneous entity created primarily for clinical purposes to identify a group of patients with an asymptomatic plasma cell dyscrasia, who have a much higher risk of progression than MGUS, so that they can be monitored more closely.[3] SMM includes patients with premalignancy (biological MGUS), as well as patients with early asymptomatic malignancy (MM) who will develop clinical symptoms and end-organ damage within a few years. Unfortunately, at present, histopathologic and other laboratory methods cannot distinguish SMM patients with premalignant MGUS from those who have early MM, since there is no clear marker of malignancy that can distinguish a clonal premalignant plasma cell from a clonal malignant MM cell.

Clinical Manifestations

As with MGUS, SMM is asymptomatic and is diagnosed during the routine work-up for a variety of symptoms and signs. In some patients SMM may be diagnosed in conjunction with one of the monoclonal gammopathy associated disorders that are listed in Table 90.4. Unlike MGUS, which has a low 1% per year risk of progression, most patients with SMM progress eventually to MM or related disorder. Therefore, the distinction between MGUS and SMM has major implications for baseline testing, follow-up, and consideration for clinical trials.

Laboratory Manifestations

The diagnostic work-up of suspected SMM should include CBC, biochemical studies including creatinine and calcium, albumin, serum FLCs, and quantitative immunoglobulins in addition to protein electrophoresis and immunofixation of the serum and urine. Any abnormality that is detected on CBC or chemistry examination must have an alternative explanation, and must be felt to be unrelated to the underlying clonal plasma cell disorder. Bone imaging is mandatory, and should show no evidence of osteolytic lesions. Whole body CT imaging is more sensitive for the detection of bone disease compared to whole body X-ray.[17] Low-dose whole body CT can detect osteolytic lesions in approximately 20% of patients with negative skeletal surveys. Bone marrow examination is required, and by definition should reveal 10% to 60% clonal plasma cells (Fig. 90.6). Bone marrow FISH studies must be done to ascertain the primary cytogenetic subtype of SMM which has prognostic significance. The primary cytogenetic classification of SMM is the same as MGUS (and MM), as shown on Table 90.3. BMPC immunophenotyping by multiparametric flow cytometry is needed to enable accurate risk stratification.

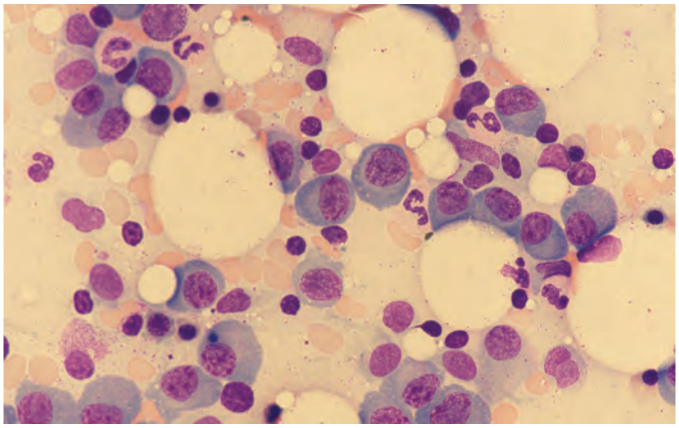


Figure 90.6 BONE MARROW ASPIRATE. Bone marrow aspirate at 600× magnification showing increased plasma cells in smoldering multiple myeloma.

If after the above work-up SMM is considered as the likely diagnosis, additional imaging with whole body MRI is needed to definitively exclude MM. By IMWG diagnostic criteria, patients with >1 focal lesion on MRI are now categorized as having MM. However, whole body MRI is not available in all practice settings. In a study that compares spine and pelvis MRIs to a whole-body MRI in MGUS patients, 90% of lesions were detected by MRI of the spine and pelvis, making this a reasonable alternative. PET-CT for evaluation of SMM is recommended by the IMWG in the event whole body MRI is not available.[17]

Differential Diagnosis

SMM must be distinguished from MGUS, MM, AL amyloidosis, and other related disorders using the criteria listed in Table 90.1. Patients with SMM must then be risk stratified into low-, intermediate-, and high-risk SMM based on the criteria discussed below (Table 90.6).

Prognosis

The risk of progression in SMM is 10% per year, compared to 1% per year in MGUS.[21] The risk of progression is affected by a number of variables, which essentially help identify the subset of SMM patients who have asymptomatic malignancy (high-risk SMM) from those with biologic MGUS (low-risk SMM). No one variable is adequate. However, by combining variables, we can get a reasonably accurate identification of patients with high-risk SMM who are candidates for early intervention and clinical trials.

Size and Type of M Protein

The size and type of the serum M protein are significant risk factors for progression in SMM.[21] The TTP is significantly shorter in patients with IgA M protein compared with IgG M protein, median 27 months versus 75 months, respectively, *P* = .004. The risk of progression in light-chain SMM is lower, with median TTP of approximately 160 months in one study.

Extent of Bone Marrow Involvement

Even after IMWG diagnostic criteria were updated to reclassify patients with ≥60% clonal BMPCs as having MM, the risk of progression in SMM is still influenced by the extent of bone marrow involvement. In a Mayo Clinic study, median TTP was 117 months for patients with <20% BMPCs versus 26 months for patients with 20% to 50% bone marrow involvement, *P* < .001.[21] BMPC estimates should be done on either the bone marrow aspirate or biopsy examination, and in the event of a discrepancy the higher of the two values should be used.[3] BMPC estimates must not be estimated from flow cytometry or from immunostaining.

TABLE 90.6 Definition of High-Risk Smoldering Multiple Myeloma[a]

Bone marrow clonal plasma cells ≥10% and any one or more of the following:
Serum M protein ≥30 g/L
IgA SMM
Immunoparesis with reduction of two uninvolved immunoglobulin isotypes
Serum involved/uninvolved free light chain ratio ≥8 (but <100)
Progressive increase in M protein level (Evolving type of SMM)[b]
Bone marrow clonal plasma cells 50%–60%
Abnormal plasma cell immunophenotype (≥95% of bone marrow plasma cells are clonal) and reduction of one or more uninvolved immunoglobulin isotypes
t (4;14) or del 17p or 1q gain
Increased circulating plasma cells
MRI with diffuse abnormalities or 1 focal lesion
PET-CT with focal lesion with increased uptake without underlying osteolytic bone destruction

[a] Note that the term smoldering multiple myeloma excludes patients without end-organ damage who meet revised definition of multiple myeloma, namely clonal bone marrow plasma cells ≥60% or serum free light chain (FLC) ratio ≥100 (plus measurable involved FLC level ≥100 mg/L), or more than one focal lesion on magnetic resonance imaging. The risk factors listed in this table are not meant to be indications for therapy; they are variables associated with a high risk of progression of SMM, and identify patients who need close follow-up and consideration for clinical trials.

[b] Increase in serum monoclonal protein by ≥25% on two successive evaluations within a 6 month period*M*, Monoclonal; *MRI*, magnetic resonance imaging; *PET-CT*, positron emission tomography-computed tomography; *SMM*, smoldering multiple myeloma.

From Rajkumar SV, Landgren O, Mateos MV. Smoldering multiple myeloma. *Blood*. 2015;125:3069–75. © American Society of Hematology.

Suppression of Uninvolved Immunoglobulins

In one study, median TTP was 159 months in patients with normal levels of uninvolved immunoglobulins, 89 months in those with a reduction in one isotype, and 32 months in patients with reduction in two isotypes of uninvolved immunoglobulins, *P* = .001.[21] Other studies have confirmed these results.

Magnetic Resonance Imaging

Patients with one focal lesion and those with solely diffuse (non-focal) abnormalities on MRI are at increased risk of progression to MM, and require more frequent follow-ups and repeat imaging in 3 to 6 months.[22] Patients with two or more focal lesions are considered to have MM.

Positron Emission Tomography-Computed Tomography

The finding of an increased uptake on PET-CT without bone destruction is not adequate to be considered as a MDE. The patients with the absence of other MDEs should be considered to have high-risk SMM. Zamagni and colleagues studied 120 SMM patients who had undergone PET-CT imaging, to assess the impact of increased uptake without underlying osteolysis.[23] They found increased PET-CT uptake without evidence of osteolysis in 16% of patients. The median TTP was significantly shorter for patients with increased PET-CT uptake compared to patients with negative PET-CT, median TTP 1.1 versus 4.5 years, *P* = .001. The probability of progression within 2 years was 58% for PET-CT positive versus the 33% for PET-CT negative patients.

Serum Free Light Chain Assay

Patients with a markedly abnormal involved/uninvolved FLC ratio (≥100) have been redefined as having MM if they meet other criteria required for the diagnosis. Elevations that are less marked are a risk factor for progression. In a study of 273 patients with SMM, an abnormal involved/uninvolved FLC ratio ≥8 were at increased risk of progression (HR, 2.3; 95% CI, 1.6 to 3.2) with a 25% per year risk of progression in the first 2 years.[24]

Multiparametric Flow Cytometry

Immunophenotyping with multiparametric flow cytometry is useful in determining prognosis in SMM by accurately distinguishing and quantitating BMPCs with malignant potential from normal PCs. Abnormal plasma cell immunophenotype can be defined as expression of CD56, lack of expression of CD19 and/or CD45, or weak expression of CD38. The risk of progression in SMM is significant if >95% plasma cells in the bone marrow have an abnormal immunophenotype.

Cytogenetic Abnormalities

A Mayo Clinic series of 351 patients with SMM found that patients with t(4;14) and/or del(17p) have a higher risk of progression.[25] Similar results have also been reported by a German study as well. Gene expression profiling (GEP) signatures may offer additional prognostic information, but clinical utility is limited.

Change in Monoclonal Protein Level

One of the most important variables associated with a high risk of progression in SMM is change in M protein levels over time. In a Mayo Clinic study of 90 patients with SMM, a rise in the M protein level over 6 months (evolving M protein) was associated with a significant increase in risk of progression.[26] The 2-year progression rate was over 80% in persons with evolving M protein levels and a decrease in hemoglobin of 0.5 g/dL or more.

RISK STRATIFICATION

Multiple risk stratification models are available for risk stratification of SMM. The Mayo Clinic 2018/IMWG 2019 risk stratification model is given in Table 90.7.[27]

The three high-risk factors in this model are if the BMPCs >20, if the serum M protein level >2 g/dL, and if the serum involved/uninvolved FLC ratio >20. Presence of two or three of these factors is associated with a median TTP to MM of approximately 2 years and is considered high-risk SMM.

TABLE 90.7 **Risk Stratification of Smoldering Multiple Myeloma**[a]

Mayo 2018 Criteria
High-Risk SMM (Median Time to Progression 2 Years)
Any 2–3 of the following high-risk factors:
Serum M protein > 2 g/dL
Serum free light chain ratio (involved/uninvolved) >20
Serum M protein ≥30 g/L
Bone marrow plasma cells >20%
Intermediate-Risk SMM (Median Time to Progression 5 Years)
Any 1 high-risk factor
Low-Risk SMM (Median Time to Progression 10 Years)
No high-risk factor
Other High-Risk Factors
Progressive increase in M protein level (evolving type of SMM)[b]
Bone marrow clonal plasma cells 50%–60%
t (4;14) or del 17p or 1q gain
Increased circulating plasma cells
MRI with diffuse abnormalities or with one focal lesion
PET-CT with focal lesion with increased uptake but without underlying osteolytic bone destruction

[a] Patients who are high risk by Mayo 2018 criteria are candidates for prophylactic therapy with lenalidomide or lenalidomide plus dexamethasone in the absence of clinical trials.

[b] Increase in serum monoclonal protein by ≥25% on two successive evaluations within a 6 month.

M, Monoclonal; *MRI*, magnetic resonance imaging; *PET-CT*, positron emission tomography-computed tomography; *SMM*, smoldering multiple myeloma.

Therapy

Multiple studies have been conducted to determine if early therapy can delay progression and improve overall survival in SMM.[2] Many early studies failed to show significant benefits. In a randomized trial, Witzig et al. compared thalidomide plus zoledronic acid versus zoledronic acid alone in patients with SMM.[28] The TTP was superior for patients treated with thalidomide plus zoledronic acid ($n = 35$) compared with zoledronic acid alone ($n = 33$); median TTP 2.4 years (95% CI: 1.4 to 3.6) versus 1.2 years (95% CI: 0.7 to 2.5), respectively, $P = .02$. However, there were no significant differences in overall survival, and there was toxicity from therapy with thalidomide, especially neuropathy.

More recently, studies have found that early therapy with lenalidomide offers significant clinical benefit for patients with high-risk SMM (see box on Approach to Smoldering Multiple Myeloma). In a randomized trial in high-risk SMM conducted by the Spanish Myeloma group, TTP was significantly longer in patients treated with lenalidomide plus dexamethasone (Rd) compared with observation, $P < .001$.[29] Overall survival was also significantly longer with Rd compared with observation. An ECOG trial comparing lenalidomide alone versus observation in high-risk SMM has confirmed the findings of the Spanish trial.[30] In this trial, early therapy with lenalidomide significantly prolonged time to end-organ damage in patients with intermediate and high-risk SMM, hazard ratio, 0.28; 95% CI, 0.12 to 0.62; $P = .002$). The 3-year PFS was 91% for the lenalidomide arm versus the 66% for the observation arm. The benefit was highest in patients with high-risk SMM by the Mayo 2018/IMWG 2019 (20-2-20) risk-stratification model, hazard ratio, 0.09, 95% CI, 0.02 to 0.44). Based on these two trials, patients with newly diagnosed high-risk SMM patients should be considered for early intervention with lenalidomide or Rd given for a time-limited duration of approximately 2 years (Fig. 90.7). An ongoing ECOG randomized trial is testing whether a standard myeloma therapeutic triplet daratumumab, lenalidomide, dexamethasone (DRd) will be superior to prophylactic doublet therapy with Rd.

The role of bisphosphonates to delay bony events in SMM is still unclear.[2] In a randomized trial a reduction in skeletal-related events (SREs) was noted with pamidronate administered monthly for 1 year, compared with observation. Similar results have also been seen in a randomized trial of zoledronic acid (4 mg once a month for 12 months) versus observation. Based on these studies, bisphosphonates every 3 to 4 months can be considered for selected high-risk SMM patients.

Other trials are exploring agents such as daratumumab and isatuximab in an attempt to delay progression of SMM to symptomatic MM. Patients with high-risk SMM are also candidates for clinical trials testing during early intervention, some of which are testing intensive therapy with curative intent.

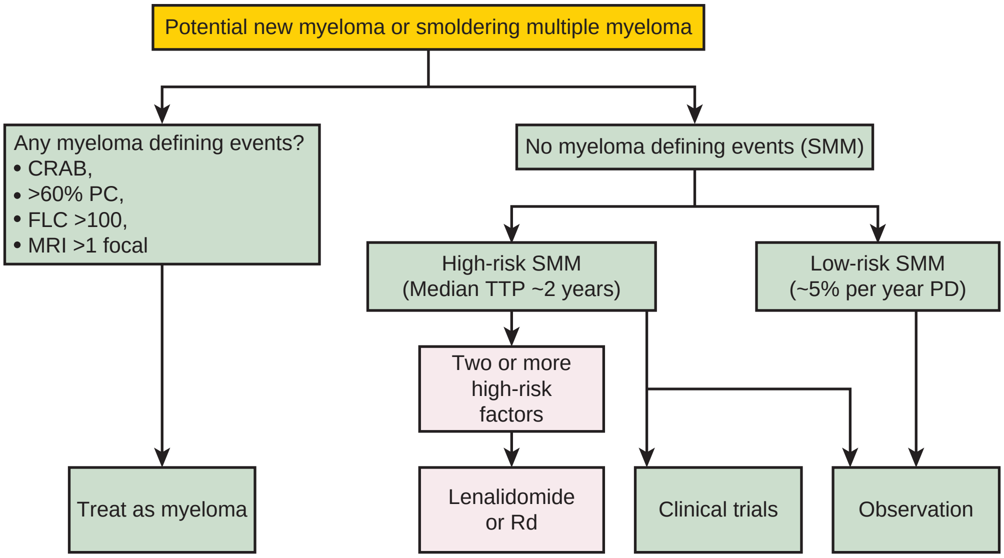


Figure 90.7 APPROACH TO THE MANAGEMENT OF SMOLDERING MULTIPLE MYELOMA. *CRAB*, Hypercalcemia, renal failure, anemia, bone lesions attributable to the plasma cell disorder; *FLC*, free light chain; *MRI*, magnetic resonance imaging; *PD*, progressive disease; *TTP*, time to progression; *Rd*, lenalidomide plus dexamethasone; *SMM*, smoldering multiple myeloma.

APPROACH TO SMOLDERING MULTIPLE MYELOMA

In patients with low- and intermediate-risk SMM, follow-up is needed every 3–4 months, on a long-term basis. In patients with high-risk SMM, therapy with lenalidomide alone, or with lenalidomide plus low dose dexamethasone is recommended. These patients are also candidates for clinical trials evaluating more aggressive treatment options, especially addition of monoclonal antibodies. There are limited data on duration of therapy. In general, we would recommend therapy for a limited duration of 2 years, and then observe. Therapy can be resumed if there is evidence of rise in M protein levels. Patients with high-risk SMM must be followed with annual bone imaging, to detect bone progression. We would also recommend pamidronate or zoledronic acid once a month for 12 months in these patients for bone prophylaxis.

FUTURE DIRECTIONS

SMM is a heterogeneous entity and often misunderstood as an intermediate stage between MGUS and MM. Our current paradigm recognizes that SMM actually comprises of two distinct groups of patients who, on the surface, meet the same diagnostic criteria, but are quite different from a biological standpoint. Thus approximately half of the patients with SMM have asymptomatic malignancy (high-risk SMM) that is destined to progress in a matter of time, and the other half have true biologic premalignancy (MGUS), which has a random, but low risk of progression. This knowledge has helped advance risk stratification and therapy. Thus using the current risk-stratification models, we are able to identify patients who have asymptomatic malignancy (high-risk SMM) and offer them intervention. In the future, we need to identify additional biomarkers to accurately identify this group. High-risk patients with SMM have a very similar genetic landscape as MM, and any new risk factor we identify will need to show incremental value to currently available clinical biomarkers. In addition, we must also study the role of the microenvironment and the immune system in the progression of SMM to determine if such factors can increase the predictive value of existing biomarkers, which are mainly focused on the plasma cell.

The other area of ongoing investigation is focused on improving outcome of high-risk SMM beyond therapy with lenalidomide or with Rd. Trials are going on with daratumumab as a single-agent, as well as comparing Rd (preventive therapy) to daratumumab plus Rd (myeloma-like therapy). More important is the question whether early therapy with aggressive regimens can cure a subset of patients with SMM. These issues need well-designed clinical trials. The long natural history of SMM makes interpretation of these trials in a timely manner difficult, hence why we also need to validate surrogate endpoints such as sustained minimal residual disease (MRD) negative state.

Finally we also need studies to determine whether screening for SMM can be beneficial, particularly in high-risk groups such as Blacks, as well as first-degree relatives of patients with MM.

REFERENCES

1. Kyle RA, Larson DR, Therneau TM, et al. Long-term follow-up of monoclonal gammopathy of undetermined significance. *N Engl J Med*. 2018;378:241–249.
2. Rajkumar SV, Landgren O, Mateos MV. Smoldering multiple myeloma. *Blood*. 2015;125:3069–3075.
3. Rajkumar SV, Dimopoulos MA, Palumbo A, et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. *Lancet Oncol*. 2014;15:e538–e548.
4. Kyle RA, Therneau TM, Rajkumar SV, et al. Prevalence of monoclonal gammopathy of undetermined significance. *N Engl J Med*. 2006;354: 1362–1369.
5. Dispenzieri A, Katzmann JA, Kyle RA, et al. Prevalence and risk of progression of light-chain monoclonal gammopathy of undetermined significance: a retrospective population-based cohort study. *Lancet*. 2010;375:1721–1728.
6. Murray D, Kumar SK, Kyle RA, et al. Detection and prevalence of monoclonal gammopathy of undetermined significance: a study utilizing mass spectrometry-based monoclonal immunoglobulin rapid accurate mass measurement. *Blood Cancer J*. 2019;9:102.
7. Landgren O, Graubard BI, Katzmann JA, et al. Racial disparities in the prevalence of monoclonal gammopathies: a population-based study of 12,482 persons from the National Health and Nutritional Examination Survey. *Leukemia*. 2014;28:1537–1542.
8. Landgren O, Graubard BI, Kumar S, et al. Prevalence of myeloma precursor state monoclonal gammopathy of undetermined significance in 12,372 individuals 10–49 years old: a population-based study from the National Health and Nutritional Examination Survey. *Blood Cancer J*. 2017;7:e618.
9. Baughn LB, Pearce K, Larson D, et al. Differences in genomic abnormalities among African individuals with monoclonal gammopathies using calculated ancestry. *Blood Cancer J*. 2018;8:96.
10. Therneau TM, Kyle RA, Melton 3rd LJ, et al. Incidence of monoclonal gammopathy of undetermined significance and estimation of duration before first clinical recognition. *Mayo Clin Proc*. 2012;87:1071–1079.
11. Greenberg AJ, Rajkumar SV, Vachon CM. Familial monoclonal gammopathy of undetermined significance and multiple myeloma:

epidemiology, risk factors, and biological characteristics. *Blood*. 2012;119:4771–4779.

12. Kumar SK, Rajkumar SV. The multiple myelomas—current concepts in cytogenetic classification and therapy. *Nat Rev Clin Oncol*. 2018;15:409–421.
13. Kyle RA, Therneau TM, Rajkumar SV, et al. A long-term study of prognosis in monoclonal gammopathy of undetermined significance. *N Engl J Med*. 2002;346:564–569.
14. Go RS, Rajkumar SV. How I manage monoclonal gammopathy of undetermined significance. *Blood*. 2018;131(2):163–173.
15. Chaudhry HM, Mauermann ML, Rajkumar SV. Monoclonal gammopathy-associated peripheral neuropathy: diagnosis and management. *Mayo Clin Proc*. 2017;92:838–850.
16. Sethi S, Rajkumar SV. Monoclonal gammopathy-associated proliferative glomerulonephritis. *Mayo Clin Proc*. 2013;88:1284–1293.
17. Hillengass J, Usmani S, Rajkumar SV, et al. International myeloma working group consensus recommendations on imaging in monoclonal plasma cell disorders. *Lancet Oncol*. 2019;20:e302–e312.
18. Rajkumar SV, Kyle RA, Therneau TM, et al. Serum free light chain ratio is an independent risk factor for progression in monoclonal gammopathy of undetermined significance. *Blood*. 2005;106:812–817.
19. Wadhera RK, Kyle RA, Larson DR, et al. Incidence, clinical course, and prognosis of secondary monoclonal gammopathy of undetermined significance in patients with multiple myeloma. *Blood*. 2011;118:2985–2987.
20. Kyle RA, Larson DR, Therneau TM, et al. Clinical course of light-chain smouldering multiple myeloma (idiopathic Bence Jones proteinuria): a retrospective cohort study. *Lancet Haematol*. 2014;1:e28–e36.
21. Kyle RA, Remstein ED, Therneau TM, et al. Clinical course and prognosis of smoldering (asymptomatic) multiple myeloma. *N Engl J Med*. 2007;356:2582–2590.
22. Merz M, Hielscher T, Wagner B, et al. Predictive value of longitudinal whole-body magnetic resonance imaging in patients with smoldering multiple myeloma. *Leukemia*. 2014;28:1902–1908.
23. Zamagni E, Nanni C, Gay F, et al. 18F-FDG PET/CT focal, but not osteolytic, lesions predict the progression of smoldering myeloma to active disease. *Leukemia*. 2016;30:417–422.
24. Dispenzieri A, Kyle RA, Katzmann JA, et al. Immunoglobulin free light chain ratio is an independent risk factor for progression of smoldering (asymptomatic) multiple myeloma. *Blood*. 2008;111:785–789.
25. Rajkumar SV, Gupta V, Fonseca R, et al. Impact of primary molecular cytogenetic abnormalities and risk of progression in smoldering multiple myeloma. *Leukemia*. 2013;27:1738–1744.
26. Ravi P, Kumar S, Larsen JT, et al. Evolving changes in disease biomarkers and risk of early progression in smoldering multiple myeloma. *Blood Cancer J*. 2016;6:e454.
27. Lakshman A, Rajkumar SV, Buadi FK, et al. Risk stratification of smoldering multiple myeloma incorporating revised IMWG diagnostic criteria. *Blood Cancer J*. 2018;8:59.
28. Witzig TE, Laumann KM, Lacy MQ, et al. A phase III randomized trial of thalidomide plus zoledronic acid versus zoledronic acid alone in patients with asymptomatic multiple myeloma. *Leukemia*. 2013;27:220–225.
29. Mateos M-V, Hernández M-T, Giraldo P, et al. Lenalidomide plus dexamethasone versus observation in patients with high-risk smouldering multiple myeloma (QuiRedex): long-term follow-up of a randomised, controlled, phase 3 trial. *Lancet Oncol*. 2016;17:1127–1136.
30. Lonial S, Jacobus SJ, Weiss M, et al. E3A06: Randomized phase III trial of lenalidomide versus observation alone in patients with asymptomatic high-risk smoldering multiple myeloma. *J Clin Oncol*. 2019;38:1126–1137.

CHAPTER 91

MULTIPLE MYELOMA

Sydney X. Lu, Even H. Rustad, Saad Z. Usmani, and C. Ola Landgren

Clonal plasma cell (PC) neoplasms are comprised of a number of interrelated and partially overlapping entities, ranging from benign to fully malignant, which are variously defined and categorized by burden of disease, presence of organ injury, anatomic location, and in some cases, unique constellations of signs, symptoms, and pathologic findings (see box on Rare Disorders Associated With Monoclonal Proteins and/or Plasma Cells, and also Chapters 90 and 93). As the majority of neoplastic PCs are functional with respect to secreting clonal immunoglobulins and/or their fragments (i.e., free light or heavy chains), as well as cytokines, growth factors, and other signaling molecules, these abnormal proteins can become relevant both in the pathophysiology of PC disorders, manifesting (e.g., as kidney injury, neuropathy, cardiac impairment, hyperviscosity, etc.) and can also frequently serve as useful biomarkers for evaluating disease burden and response to treatment. This chapter highlights continued advances in both knowledge regarding the underlying pathophysiology of PC neoplasms, and their therapy, with a focus on multiple myeloma (MM) as the prototypical malignant PC disorder. In this context we emphasize minimal residual disease (MRD) testing for risk stratification, disease management, and decision making, and also highlight novel therapies with mechanisms-of-action beyond existing proteasome inhibitors (PIs), immunomodulatory agents, and monoclonal antibodies.

SCREENING, EPIDEMIOLOGY, AND RISK FACTORS

MM, the dominant malignant PC disorder, accounts for 1.8% of all malignancies and is the second most common hematologic malignancy. It is estimated that in 2016, there were about 138,500 incident cases and 98,500 deaths attributed to MM globally.[1,2]

Age is a major risk factor for MM as well as its precursor states, smoldering myeloma (SMM) and monoclonal gammopathy of undetermined significance (MGUS) (see Chapter 90). Generally, MM is a disease affecting a relatively older population with a median age at diagnosis of 69 years (Fig. 91.1A), and less than 2% to 5% of patients are younger than 40 years old at diagnosis. Myeloma is more frequent in men than in women, and in African Americans than in White persons in the United States (see Fig. 91.1B). The incidence of MM in African American men is approximately 15.7 per 100,000 per year as compared with 11.5 per 100,000 per year in African American women; the corresponding incidence rates are 7.7 and 4.5 per 100,000 in White men and women, respectively. In addition, African Americans also generally present with MM earlier in life than Whites. Although there has been an increase in the incidence of MM over time, this can be attributed to better detection and surveillance of the disease, overall aging of the population worldwide, together with increased prevalence attributable to markedly longer median overall survival (OS) of patients in the era of modern therapies, with the current median OS for undifferentiated newly diagnosed MM patients likely longer than 6 years.[3]

In addition to the precursor neoplasms MGUS and SMM (see Chapter 90),[4] which are believed to be obligatory precursors to MM, increasing age and certain racial groups are known predisposing conditions. Other risk factors for MM include human immunodeficiency virus (HIV) infection[5] (ameliorated in the era of effective antiviral therapy), obesity, and certain occupations,[6] as well as exposure to specific chemical carcinogens, including Agent Orange (linked to MGUS[7]). Although clusters of familial MM have been described in the literature, they are rare and are not believed to contribute significantly to the overall epidemiology of MM. Recent genetic studies have identified mutations of LSD1 (KDM1A),[8] DIS3,[9] and Lynch syndrome (deoxyribonucleic acid [DNA] mismatch repair) genes as being associated with familial myeloma. In addition, Gaucher syndrome,[10] a lysosomal storage disorder, is also associated with an elevated risk of developing MM. As malignant PC disorders are rare overall, there are currently no guidelines from national organizations or scientific societies endorsing screening for PC disorders in the asymptomatic general population.

PATHOGENESIS

The transformative event which initiates the trajectory toward MM occurs in a germinal center B cell that has undergone somatic hypermutation and class-switch recombination (with the exception of rare cases of immunoglobulin M [IgM]-myeloma).[11] Epidemiological and genomic studies have indicated that initial transformation often occurs in the first three decades of life.[12–15] Years after immortalization of the first plasma cell, when the clonal population has expanded sufficiently, they produce detectable serum monoclonal proteins. Based on clinical features, the condition may be classified as MGUS or SMM.[16,17] In a minority of patients, clonal plasma cells acquire additional driver, leading to the development of MM and eventually relapsed/refractory disease with a highly aggressive phenotype.[11,16]

Genomic Landscape of Multiple Myeloma at Presentation

Large-scale studies mapping the driver mutational landscape of MM at presentation have focused on three types of somatic alterations: recurrent translocations involving the immunoglobulin gene loci, copy number alterations (CNAs), and single nucleotide variants (SNVs) (Fig. 91.2A) (see Chapter 57). Moreover, these early studies focused on alterations detectable by conventional cytogenetics, fluorescence in situ hybridization (FISH), array-based copy number analysis, and whole exome sequencing. More recently, whole-genome sequencing (WGS) studies have added another level of complexity and highlighted areas that remain poorly understood. In one recent WGS study, the median number of SNVs in a tumor was 5377 (range 982 to 15738), with a median of 26 (range 0 to 129) structural variants (SVs), including translocations, inversions, deletions, and tandem duplications, which often combine to form complex SVs. Patients who were highly similar in their recurrent driver profile often displayed striking differences in mutational burden and SV landscape (see Fig. 91.2B). Extracting the biologically important information from this complex panorama of somatic alterations is a major goal of ongoing research.

In the following sections we summarize the main pillars and recent advances in MM genomics, focusing on overall principles and potential genomic biomarkers. We have taken a chronological perspective, starting with the earliest events which led to initiation of monoclonal gammopathy, followed by the key mutational processes and genomic alterations which appear to drive the evolution to overt MM and beyond.

RARE DISORDERS ASSOCIATED WITH MONOCLONAL PROTEINS AND/OR PLASMA CELLS

- POEMS[221, 222]: polyneuropathy, organomegaly, endocrinopathy, monoclonal protein, skin changes. Typically presents with osteosclerotic rather than frankly lytic bone lesions, and may also co-occur with Castleman disease. POEMS is associated with elevated vascular endothelial growth factor (VEGF) levels, which are helpful in diagnosis and assessing therapy response. Assessing for endocrinopathy at diagnostic evaluation and co-management with an expert endocrinologist may be helpful. POEMS carries a better prognosis than conventional multiple myeloma.
- TEMPI[223, 224]: telangiectasias, erythrocytosis, monoclonal protein, perinephric fluid collections, intrapulmonary shunt. A rare disorder which can be efficaciously treated with bortezomib and daratumumab. Autologous transplant is rarely required.
- AESOP[225–227]: adenopathy and extensive skin patch overlying a plasmacytoma. This is considered a paraneoplastic syndrome which can co-occur with POEMS, Castleman disease, plasma cell disorders as well as lymphoplasmacytic lymphoma.
- Schnitzler syndrome[228, 229]: an autoinflammatory and rheumatologic disorder associated with M-protein, which may also be associated with splenomegaly, lymphadenopathy, and chronic urticaria. Managed primarily with the IL-1 receptor antagonist anakinra rather than anti-neoplastic therapy per se.
- Cryoglobulinemia type I[230]: monoclonal IgG, IgM, IgA, or free light chains which can be associated with hyperviscosity syndrome and vasculitis.
- Heavy chain diseases: a rare assortment of B lymphoproliferative disorders, typically associated with indolent lymphomas, which produce heavy chains (generally α, γ, μ) which are deposited in tissues, without intact light chains. Variable clinical course. Managed with observation, supportive care (e.g., antibiotics for infections), and anti-lymphoma or anti-myeloma therapy.
- Primary amyloidosis—see Chapter 93 on AL amyloidosis.
- Chronic neutrophilic leukemia associated with plasma cell dyscrasias (see Chapter 72).[231, 232]
- Monoclonal gammopathy of renal significance13: these disorders are characterized by low-level clonal plasmacytosis and paraproteinemia causing renal injury. Although they may not meet criteria for MM, therapy may be considered.

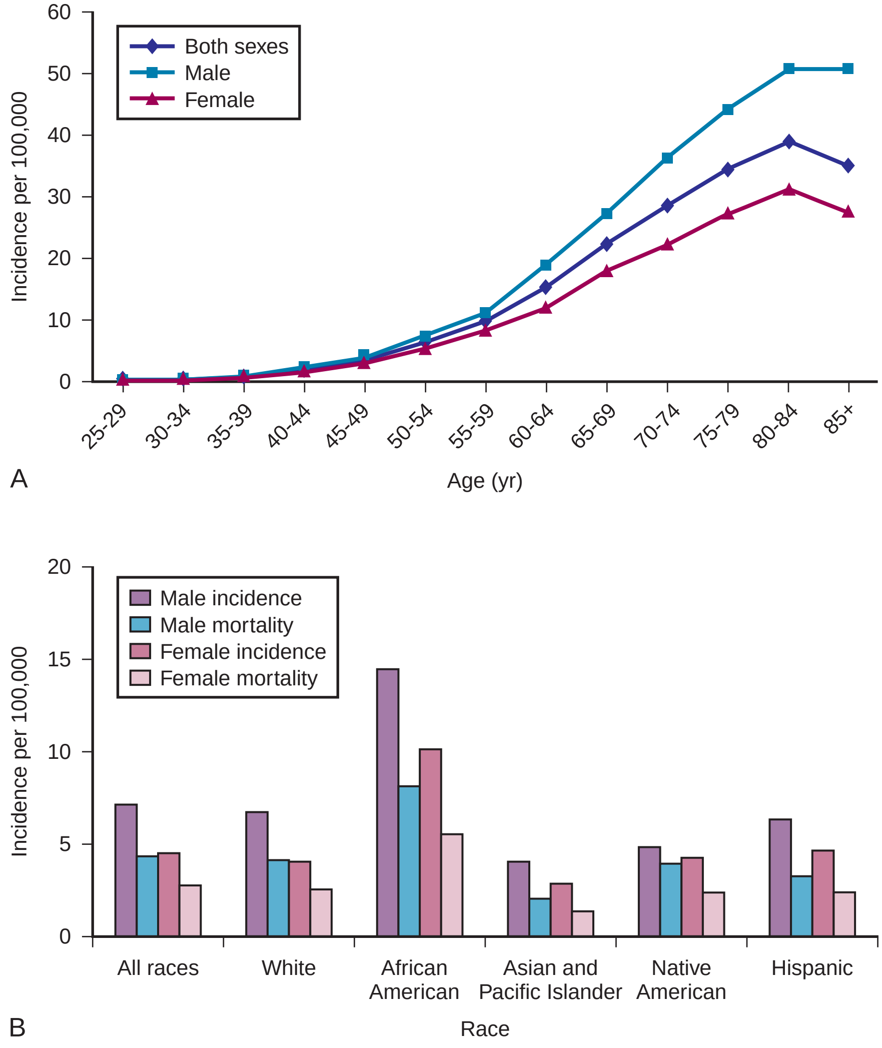


Figure 91.1 MULTIPLE MYELOMA EPIDEMIOLOGY. (A) Multiple myeloma (MM) average annual age- and gender-specific incidence per 100,000 in the United States in 2009. (B) MM average annual race-specific incidence per 100,000 in the United States in 2009. Increase in incidence is noted with advancing age; the incidence is greater in males than in females; and a higher incidence is observed in African American than in White populations.

Primary Genomic Driver Events

Traditionally, MM has been divided into molecular subgroups based on the presumed initiating driver event. Forty percent of patients have a translocation involving the immunoglobulin heavy chain (*IGH*) locus on chromosome 14 and one of seven canonical partner oncogenes: *CCND1* (t11;14), *CCND2* (t12;14), *CCND3* (t6;14), *MMSET* (t4;14), *MAF* (t14;16), *MAFA* (t8;14), and *MAFB* (t14;20).[18]

These events drive disease development by placing key oncogenes under the transcriptional control of the *IGH* enhancers, a phenomenon known as super-enhancer hijacking. Sequencing analysis of the *IGH* locus in primary translocations most commonly reveals a breakpoint in the class-switch recombination regions, consistent with DNA double-strand breaks introduced by activation-induced cytidine deaminase (AID) in the germinal center. Sixty percent of patients have a hyperdiploid genome (HRD), defined by three or more trisomies of odd-numbered chromosomes. Chromosomes 9, 15, 19, and 21 are usually acquired first, which may be followed by gain of additional copies of the same or different chromosomes, typically 3, 5, and 7.[19,20] Although not considered part of hyperdiploidy, gain of chromosome 1q may occur in the same early time-window and play a role in disease initiation. The etiology of whole chromosome gains in MM remains unknown. Approximately 5% of patients have both HRD and a canonical *IGH* translocation and <10% of patients have neither. Some patients may be driven by other rare genomic rearrangements, including translocations involving *IGH* and rare partners such as the B-cell master regulator *PAX5* or the co-stimulatory molecule *CD40*.[18] Gene expression profiling (GEP) studies have confirmed the importance of primary *IGH*-translocations and HRD as drivers of MM biology, while providing additional refinement.[21–23] Clinically, *IGH* translocations involving *MMSET*, *MAF*, and *MAFB* have been associated with poor prognosis. However, the penetrance of this "high-risk" genotype at the individual patient level is variable, with many of these patients achieving long-term disease control using modern highly effective therapies. Furthermore, the prognostic impact of primary driver events is most likely modulated by the combination of secondary drivers present in each individual patient, although these higher-order interactions are thus far poorly understood.

Genomic Drivers of Progression From Precursor to Myeloma and Beyond

There is currently no single genomic abnormality that separates MM from its precursors, or from advanced relapsed/refractory disease. Instead, there is a gradual increase in genomic complexity over time, characterized by the accumulation of somatic alterations of all types. This evolutionary process follows preferred trajectories, where the early drivers determine which additional hits will confer the greatest advantage and therefore end up being selected (some such common interactions are highlighted by the clusters in Fig. 91.2A). There are also more general temporal patterns, where oncogene activation tends to occur earlier (e.g., copy number gains, super-enhancer hijacking and gain of function SNVs), while tumor suppressor gene inactivation occurs later (e.g., copy number loss and truncating SNVs).

Key gain-of-function drivers in MM include activation of *MYC* through a variety of mechanisms including translocation with an immunoglobulin locus (i.e., *IGH*, *IGL*, or *IGK*), which is particularly enriched in the HRD subgroup, as well as gain of one or more copies of chromosome 1q. *MYC* is a transcriptional master regulator in MM along with *IRF4*, and knock–down of both factors in vitro lead to cell death. Gain of 1q usually involves the whole chromosome arm, but a minimally amplified region on 1q21.3 has led to identification of the putative driver gene *CKS1B*. Nuclear factor-kappa B (NFκB) pathway activation is a recurrent feature where different types of genomic alterations converge, including recurrent deletions of the NFκB inhibitor (16p.12), while the MAPK pathway genes, *NRAS*, *KRAS*, and *BRAF*, are predominantly affected by SNVs. Given that MAPK mutations in MM are acquired relatively late and most commonly are subclonal, the rationale for targeted therapy using MEK and/or BRAF inhibitor is

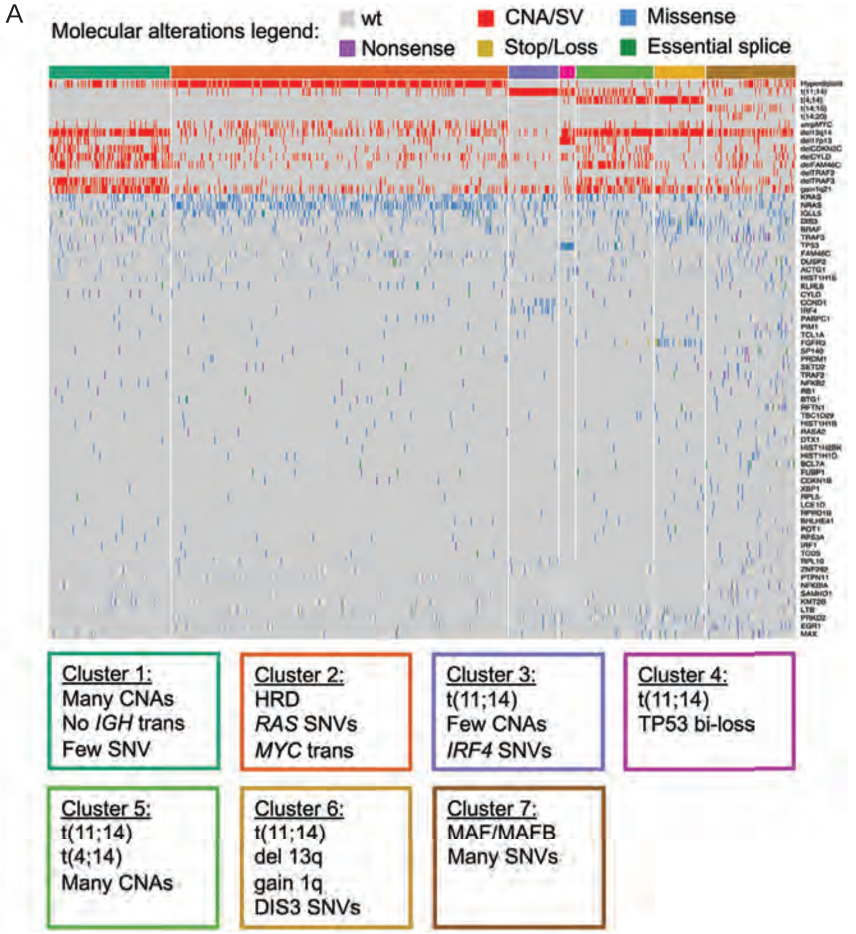


Figure 91.2 GENOMIC CLASSIFICATION OF MULTIPLE MYELOMA. (***Continued***)

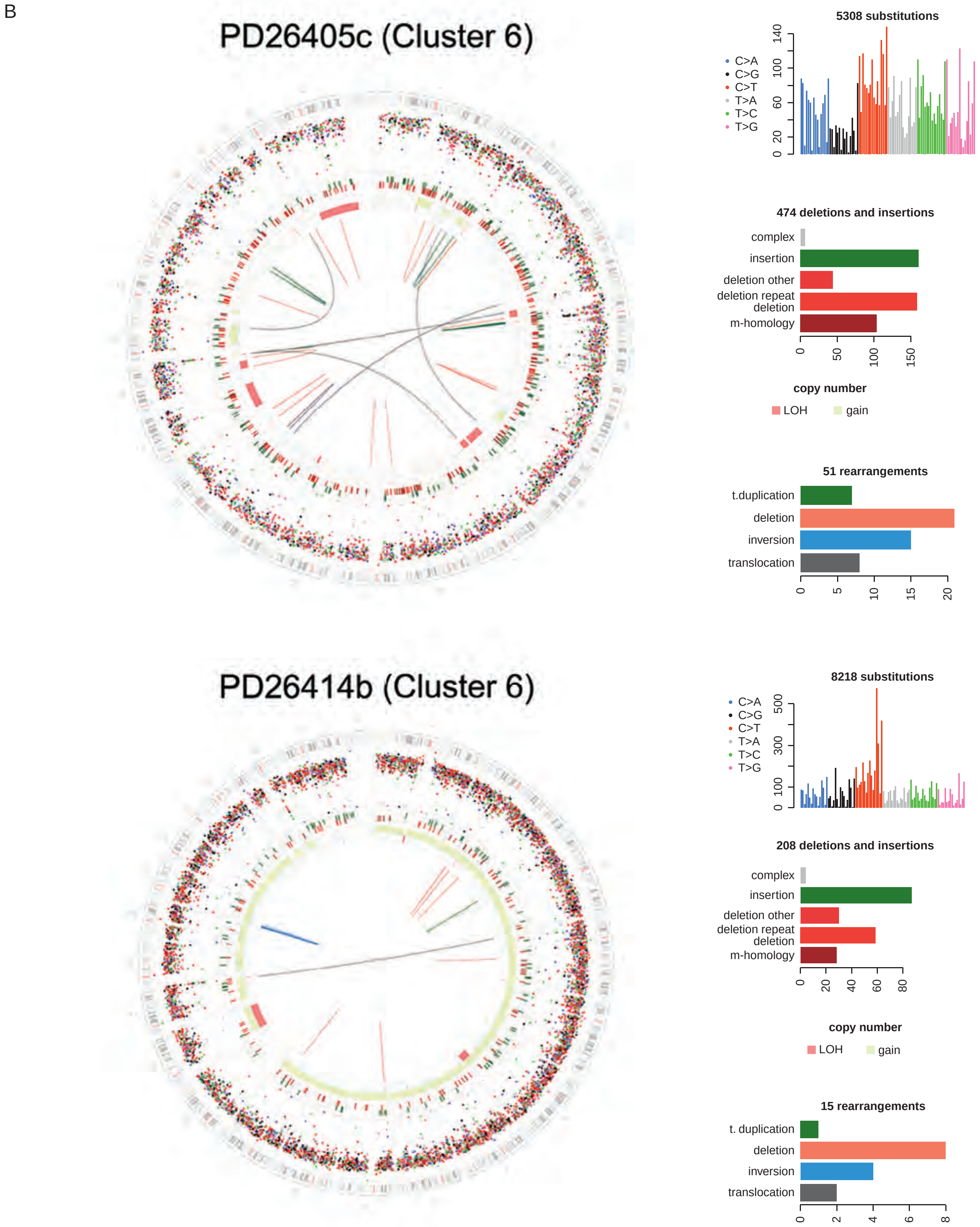


Figure 91.2–cont'd Genomic classification of multiple myeloma. (A) Heatmap of 724 patients with newly diagnosed multiple myeloma assigned to one of seven genomic subclasses of multiple myeloma defined by recurrent translocations, copy number alterations *(CNAs)*, and single nucleotide variants *(SNVs)*. Each column represents a patient and each row a molecular alteration (color legend above heatmap). Defining features of each cluster (color coded on the top row) are displayed in boxes below the heatmap. (B) Circos plots showing the genome-wide landscape of genomic alterations in two patients belonging to cluster 6 (*orange*) in panel (A), with legend and summary data to the right. Chromosomes are plotted around the outer plot margin, followed by concentric circles representing SNVs, CNAs, and structural variants *(SVs)*. Although both cases harbor defining features of cluster 6 (e.g., t[11;14] and del 13q), they differ strikingly in SNV burden and genome-wide profile of CNAs and SVs. *(The molecular classification and heatmap has been adapted from Maura F, Bolli N, Angelopoulos N, et al. Genomic landscape and chronological reconstruction of driver events in multiple myeloma. Nat Commun. 2019;10[1]:3835.)*

weaker than in diseases where MAPK inactivation is an early or initiating feature, such as hairy cell leukemia and *BRAF*-mutant melanoma.

Loss-of-function events in MM tend to converge on established tumor suppressor genes involved in cell cycle regulation, such as *TP53* (17p13), *RB1* (13q14), and *CDKN2C* (1p.32). Tumor suppressor genes more specific to MM include *FAM46C* (1p12), a regulator of messenger RNA (mRNA) polyadenylation, and *DIS3* (13q21), encoding the catalytic subunit of the human exosome complex.[24,25]

Multiple studies have investigated the prognostic impact of secondary genomic drivers, often with conflicting results. Two alterations have been thoroughly established as independent prognostic factors across several studies: bi-allelic inactivation of *TP53* and amplification of 1q21. Deletion of 17p has long been considered a marker of poor prognosis, but recent studies including SNV data has shown that this phenotype depends on inactivating mutation of *TP53* on the remaining allele. For gains of 1q21, there appears to be a dose response relationship between the number of copies and prognosis, where having four or more copies is associated with particularly poor outcomes.

Recent efforts have begun to reveal the mutational process shaping the evolving genome of MM. We will highlight here three findings of particular clinical importance. First, a hyper-mutator phenotype in MM has been identified in <5% of patients, driven almost entirely by the APOBEC family of cytidine deaminases. High burden of APOBEC-induced mutations is associated with poor prognosis independently from other genomic features, despite many of these patients also having *IGH*-translocations involving *MAF* or *MAFB*. The mechanisms underlying aberrant APOBEC activation in MM and how this might be exploited therapeutically is subject to ongoing investigations. Second, complex SVs termed chromothripsis have been identified as key driver events in up to 24% of patients with MM and are strongly associated with poor prognosis. Chromothripsis is the result of shattering and re-joining of one or more chromosomes, resulting in widespread genomic rearrangements. This is particularly important because it means that a high level of genomic complexity including multiple driver events can be acquired by a single cell at a single point in time, which may contribute to the dramatic changes in clinical behavior that can sometimes be observed in MM. Finally, recent studies have identified mutational signatures specifically associated with prior exposure to melphalan and platinum-containing chemotherapies used as an anti-myeloma therapy. This is perhaps not surprising, since the mechanism of action of these agents is cell-cycle arrest through the introduction of DNA damage. Nonetheless, having identified the specific mutational footprint of these therapies, one can begin to explore their role in development of treatment resistance as well as secondary malignancies. In particular, myelodysplastic syndromes and acute myeloid leukemia (AML) are strongly enriched in MM patients following melphalan therapy. With life expectancies exceeding 10 years for many patients with MM receiving state-of-the-art therapies, long-term toxicity is becoming an increasing concern which may warrant more restrictive use of cytotoxic agents.

In summary, the panorama of genomic drivers in MM is characterized by a limited number of frequent alterations followed by a long tail of rare drivers. Evolution from precursor stages to MM and acquisition of an increasingly aggressive and treatment-refractory phenotype usually results from accumulation of multiple genomic hits over time, converging on pathways responsible for cell proliferation and survival. In some cases, accelerated or "punctuated" evolution may occur as a result of complex SVs introducing multiple drivers in a single genomic event. We anticipate that improved understanding of the processes shaping tumor evolution and how multiple genomic drivers interact to determine tumor biology will lead to the emergence of clinically meaningful MM molecular subgroups.

CLINICAL IMPLICATIONS OF CLONAL HETEROGENEITY AND EVOLUTION

In any given patient with MM, a number of distinct subclones of disease are present which differentially populate the bone marrow and soft tissue lesions inside and outside of the skeleton. Tumor phylogenies reconstructed from multiple biopsies at presentation indicate early seeding from a common disease site followed by local expansion.[26,27] Presence of high-risk features in a single lesion conferred the same adverse prognosis as if every site shared the same high-risk profile. At the extreme end, in multi-regional biopsies obtained at autopsy, there is evidence of accelerated seeding where tumor sites throughout the body have been re-populated by descendants of a single cell which survived intensive therapy.[27] Although the number of patients analyzed with high spatiotemporal resolution remains low, the current evidence indicates that high-risk subclones determine the final outcome of disease but may take years and multiple lines of therapy before emerging as dominant. This time lag may reflect the need of tumor subclones to acquire multiple cooperating driver lesions to achieve their full malignant potential and the gradual increase of immunosuppression resulting from both chronic disease and multiple lines of therapy.

IMMUNE AND BONE MARROW MICROENVIRONMENT

In addition to cell-intrinsic genomic changes and other aberrancies within neoplastic PCs, MM also exhibits a strong dependence on the microenvironment as a feature of disease pathogenesis.[2] Neoplastic PCs both rely on, and in turn promote, an abnormal bone marrow and immune microenvironment as a key feature of oncogenesis (Fig. 91.3).[28] The complex interplay between malignant PCs and their heterogeneous microenvironment, which includes endothelium, osteoblasts, osteoclasts, and a variety of other mesenchymal and stromal cell types, as well as myeloid and lymphoid hematopoietic and immune cell subsets,[29] soluble molecules including growth factors, cytokines, and secreted receptor molecules, as well as diverse extracellular matrix components including collagen and other structural proteins, explains in part some of the clinical features of PC disorders, and has also been increasingly targeted, directly or indirectly, in therapy of these disorders.

The effect of PCs within their environment can reflect disease evolution. For example, in precursor states prior to the development of frank MM, the PETHEMA and Scandinavian[30] groups have shown that biomarkers such as the ratio of abnormal to total plasma cells in the marrow, which reflects in part the paracrine suppression of normal B cell to plasma cell maturation and function, as well as the reciprocal suppression of uninvolved immunoglobulins (termed "immunoparesis" [e.g., low IgA and IgM] in a case of IgG plasma cell neoplasm) can risk-stratify patients for risk of progression to MM, with immunoparesis and high ratios of abnormal to total plasma cells associated with increased risk.[31] With respect to MM and anemia as a defining criterion, this is in large part also believed to be mediated via paracrine effects of malignant PCs suppressing erythropoiesis.[32] Finally, imbalanced osteoblastic vs. osteoclastic activity with resultant bone lesions and hypercalcemia are arguably the *sine qua non* features of MM. Finally, although not considered a myeloma-defining criterion, impaired cellular and humoral immunity in patients with PC disorders, with resultant infectious complications, arguably also reflects the deleterious effects of neoplastic PCs on the immune microenvironment. Given this, dysregulated B and plasma cell maturation and function, disordered erythropoiesis, and imbalanced osteoblastic versus osteoclastic activity represent some commonly observed clinical examples of neoplastic PCs acting to create an abnormal marrow microenvironment. In turn, MM is a disease most stereotypically confined to the bone marrow ("medullary disease"), with extramedullary manifestations or circulating disease in the blood, sometimes representing progression[33] as a late phenomenon, reflecting the strong dependence of both normal and neoplastic PCs on the marrow microenvironment and its many complex cell-cell interactions and paracrine signaling molecules, for their survival.

With respect to therapy, many current and investigational therapies for MM target the microenvironment, directly or indirectly. Although the many United States Food & Drug Administration (FDA)-approved agents discussed below have direct, cell-autonomous

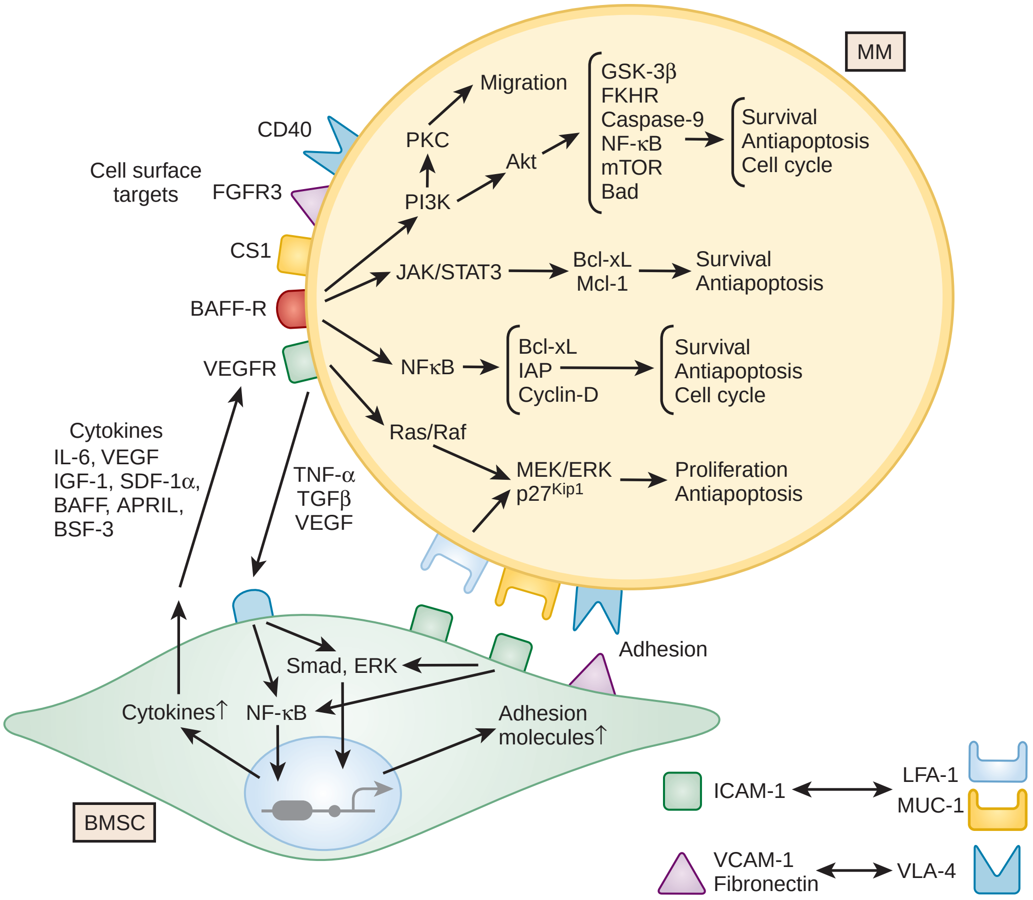


Figure 91.3 INTERACTION AND ADHESION OF MULTIPLE MYELOMA CELLS TO BONE MARROW STROMAL CELLS (BMSCS). Binding of multiple myeloma *(MM)* cells to BMSCs induces the activation of p42/p44 mitogen-activated protein kinase *(MAPK)* and nuclear factor-κB *(NFκB)* in BMSCs. The activation of NFκB upregulates adhesion molecules on BMSCs. Cytokines secreted through this interaction, including interleukin 6 *(IL-6)*, tumor necrosis factor-α *(TNF-α)*, and vascular endothelial growth factor *(VEGF)*, activate the main signaling pathways (p42/p44 MAPK, Janus kinase *[JAK]*/signal transducer and activator of transcription 3 *[STAT3]* and/or phosphatidylinositol 3-kinase *[PI3K]/AKT*) and their downstream targets, which triggers MM cell growth, survival, and migration. The RAS/RAF/mitogen-activated protein kinase kinase *(MEK)*/MAPK pathway mediates proliferation of MM cells. JAK/STAT3, along with upregulation of B-cell lymphoma extra large *(BCL-XL)* and myeloid cell leukemia-1 *(MCL1)*, mediates survival. PI3K/AKT, through downstream activation of BAD and NFκB and/or inactivation of caspase-9, mediates antiapoptosis. NFκB and forkhead in rhabdomyosarcoma *(FKHR)* modulate cyclin D and KIP1, thereby regulating cell-cycle progression. Signaling through PI3K induces downstream protein kinase C *(PKC)* activity and MM cell migration. *APRIL*, A proliferation-inducing ligand; *BAFF-R*, B cell–activating factor receptor; *BSF-3*, B-cell stimulating factor 3; *ERK*, Extracellular signal-related kinase; *FGFR3*, fibroblast growth factor receptor 3; *GSK-3β*, glycogen synthase kinase 3β; *IAP*, inhibitor of apoptosis protein; *ICAM-1*, intercellular adhesion molecule 1; *IGF-1*, insulin-like growth factor 1; *IL*, interleukin; *LFA-1*, lymphocyte function-associated antigen 1; *MM*, multiple myeloma; *mTOR*, mammalian target of rapamycin; *MUC-1*, mucin 1; *SDF-1α*, stromal cell–derived factor 1α; *TNF-α*, tumor-necrosis factor-α; *VCAM-1*, vascular cell adhesion molecule 1; *VEGF*, vascular endothelial growth factor; *VLA-4*, very late antigen 4. *(Adapted from Hideshima T, Mitsiades C, Tonon G, et al. Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. Nat Rev Cancer. 2007;7[8]:585–598.)*

effects on neoplastic PCs, these drugs can also render the microenvironment less hospitable to PC growth. The relative importance of microenvironmental vs. direct anti-neoplastic effects of many commonly used agents are difficult to investigate in preclinical models due to the difficulty of modeling the microenvironment, and this remains an area of interest for investigators.

For example, the immunomodulatory drugs[34] (e.g., thalidomide, lenalidomide) have a particularly complex mechanism of action: in addition to acting directly on PCs to induce apoptosis by mediating the degradation of the essential transcription factors *IZKF1* and *IZKF3*[35] by coopting the endogenous ubiquitin ligase machinery to degrade these proteins as neo-substrates, lenalidomide also decreases the generation and activity of osteoclasts necessary for myeloma survival,[36–38] decreases the expression of cell adhesion molecules expressed by other marrow stromal cells which ordinarily promote PC survival, and stimulates T- and natural killer (NK)-cell anti-tumor activity by increasing immune synapse formation, increasing direct NK-mediated killing and CD8$^+$ T-cell effector function. Likewise, thalidomide is known to have anti-angiogenesis[39] and anti-VEGF[40] signaling properties which also likely account in part for its therapeutic efficacy. The other major class of novel agents is the PIs with bortezomib as the prototype agent. Again, the mechanism of action of this drug class is complex: in addition to direct anti-PC activity by inhibition of the proteasome and thus indirect downregulation of NFκB signaling,[41] among a plethora of other cell-autonomous mechanisms, bortezomib also modulates the bone marrow microenvironment,[42] inducing endothelial cell and osteoclast apoptosis,[43] and inhibiting the secretion of PC growth-promoting molecules by the microenvironment, such as IL-6, vascular endothelial growth factor (VEGF), and IGF-1. Similar effects on the balance between osteoblasts vs. osteoclasts has been observed with the irreversible proteasome inhibitor carfilzomib.[44] The newest class of FDA approved agents, the monoclonal antibodies elotuzumab (targeting signaling lymphocytic activation molecule F7

[SLAMF7], aka CS1 or CD319) and daratumumab and isatuximab (targeting CD38) also have both direct anti-neoplastic effects as well as indirect effects by modulating the immune microenvironment. For example, while elotuzumab binding to PCs mediates cell killing, its ligation of SLAMF7 on NK cells may activate NK cells,[45–47] leading to improved NK-directed killing of PCs. Daratumumab[48] not only inhibits the ectoenzymatic activity of CD38 and induces antibody-dependent cellular cytotoxicity (ADCC) of tumor cells, but has also been suggested to deplete CD38$^+$ regulatory B and T cells and myeloid-derived suppressor cells, thereby favorably altering the tumor immune microenvironment and biasing it toward effective anti-tumor immunity.[49,50] Finally, daratumumab is also believed to result in immune activation by targeting CD38$^+$ NK cells.[51] The more recently approved anti-CD38 antibody isatuximab is also believed to have similar immune-activating effects.[52] Finally, modulation of osteoclast activity, via bisphosphonates or inhibiting the RANK/RANKL axis with denosumab,[53] also inhibit osteoclast activity and osteoclastogenesis, and additionally also have been suggested to mediate an anti-angiogenic and thereby anti-PC effect.

With respect to other investigational agents, interactions between stromal cells which express the co-stimulatory ligands B-cell activating factor (BAFF) and a proliferation inducing ligand (APRIL) and malignant PCs which express the cognate receptor B-cell maturation antigen (BCMA)[54] are believed to be important for the survival of normal and neoplastic PCs, thus representing selection pressure for expression of BCMA. This requirement has been exploited with chimeric antigen receptor T cell (CAR-T cells[55]) as well as monoclonal antibody-drug conjugate-based therapies targeting BCMA (e.g., belantamab mafodotin[56]), many of which have shown promising efficacy in multiply relapsed MM patients. Other investigational efforts targeting paracrine IL-6 signaling, aberrant VEGF signaling, and secreted or cell surface molecules which mediate interactions between neoplastic PCs and the marrow microenvironment are ongoing and represent additional promising therapeutic venues.

TABLE 91.1 Clinical Features of Multiple Myeloma

Bone Destruction
Pain, fractures
Cord compression with bowel and bladder dysfunction
Radicular pain and cranial nerve abnormalities secondary to nerve compression
Hypercalcemia
Polyuria, polydipsia, nausea, vomiting, constipation
Lethargy and altered mental status
Renal Failure
Nausea, vomiting, weakness, malaise
Refractory electrolyte abnormalities, volume status abnormalities
Amyloidosis
Peripheral neuropathy, autonomic neuropathy (e.g., orthostasis)
Dependent edema
Organomegaly and organ dysfunction (e.g., heart failure, jaundice)
Marrow Infiltration
Anemia, thrombocytopenia (late event)
Bleeding tendency
Reduced Immunoglobulins
Recurrent infections
Pneumonia
Cryoglobulins
Raynaud phenomenon
Acrocyanosis
Hyperviscosity
Shortness of breath
Transient ischemic attacks
Deep vein thrombosis
Retinal hemorrhage
Epistaxis

CLINICAL MANIFESTATIONS

Patients with MM may present with signs and symptoms that are most commonly related to either marrow infiltration by plasma cells and paracrine disruption of physiologic hematopoiesis, or end-organ damage manifested by renal dysfunction, bone lesions, hypercalcemia, and/or immune dysfunction.[2] However, in a minority of patients, MM may present asymptomatically or even without frank end-organ damage at all, with this group of patients diagnosed on the basis of laboratory or radiologic abnormalities, with investigations generally prompted by initial abnormalities in routine studies performed for other indications. In some patients, signs and symptoms may also be related either to deposition of paraproteins in various organs, such as the heart, kidneys, or neural tissue, as either light chain, amyloid, or very rarely, heavy chain deposits or secondary to the humoral effects of cytokines or other secreted molecules such as IL-6 or VEGF produced by the myeloma cells and/or the bone marrow microenvironment (see box on Rare Disorders Associated With Monoclonal Proteins and/or Plasma Cells). Various clinical features of myeloma are summarized in Table 91.1.

Bone Disease

Almost two-thirds of patients with MM present with bone pain and/or destructive, generally lytic bone lesions as a major symptom. Arguably, bony injury and the resulting pain and functional limitations from skeletal injury are the cardinal symptoms of myeloma which affects quality of life. As discussed above, bone disease is believed to primarily represent unbalanced hyperactivity of osteoclasts and suppression of osteoblastic activity, although direct infiltration of bone by myeloma cells also causes bone destruction. Bone modifying agents including bisphosphonates and RANKL inhibitors can reduce the number of skeletal-related events (SREs) in MM (see Skeletal Protection, below), although risks of renal injury and complications such as atypical fractures and jaw osteonecrosis must be considered. Previous studies have shown a modest OS benefit for the use of these bone-modifying agents, but they were largely performed in the era when markedly less potent induction regimens were used—the survival benefit for these agents in the modern era of potent induction therapies and relapsed/refractory disease therapies is uncertain at present, with the use of bone resorptive agents primarily viewed as adjunctive therapy by most practitioners.

Skeletal injury of the axial and appendicular skeleton can additionally result in hypercalcemia (discussed below) and may contribute secondarily to renal injury, and the functional limitations and immobility associated with skeletal injury can predispose to additional complications, such as loss of muscle mass (sarcopenia) and decreased overall physiologic reserve, increased infection risk, neurologic injury, or thrombotic events. Management is primarily focused on anti-PC therapy and anti-resorptive therapy, although pain control, physical therapy, and local therapies (e.g., kyphoplasty for vertebral fractures, surgery or radiation for cord compression or impending fractures) must be prioritized as clinically appropriate. Finally, note that healing or recovery of existing bone lesions is variable even with effective anti-PC therapy, and the lack of radiologic resolution of a lytic lesion does not necessarily indicate treatment resistance.

Hypercalcemia

Hypercalcemia is observed in approximately 25% to 30% of patients with MM and is usually a manifestation of higher disease burden. In contrast to solid tumors where hypercalcemia is generally a terminal event late in the disease course, hypercalcemia in MM more commonly occurs early, oftentimes prior to initiating effective therapy, and is eminently reversible in essentially all cases. In a minority of cases, hypercalcemia can be a manifestation of relapsing disease. Its occurrence is related to direct bone involvement by PCs with local bony injury as well as production of various cytokines that lead to increased bone resorption and calcium release at distant sites. Hypercalcemia manifests as mental status changes, lethargy, nausea and vomiting, and constipation, and can also induce renal failure directly by injuring the parenchyma, as well as by causing volume depletion. Symptomatic hypercalcemia should be managed as a hematologic emergency: adequate hydration, the use of calcitonin and steroids can temporize hypercalcemia for several days while definitive therapy, generally in the form of anti-PC therapy and/or bone modifying agents are initiated. The use of bisphosphonates or a RANKL inhibitor (denosumab) (often more appropriate when patients present with concomitant renal failure) requires the careful serial monitoring of serum total and ionized calcium as well as phosphorus levels and appropriate correction, as *hypo*calcemia after treatment with these agents has been reported.[57,58] Risks and rewards of using anti-resorptive agents in patients who have not yet had dental clearance must be weighed and cautiously considered.

Renal Failure

Renal insufficiency in the context of PC malignancies is a frequent and serious complication with a frequently multifactorial etiology, with significant implications for therapy. Monoclonal gammopathy of renal significance (MGRS)[59] (see box on Rare Disorders Associated With Monoclonal Proteins and/or Plasma Cells) may represent a *forme fruste* of classical MM and therapy may be contemplated for such patients. Up to 20% of newly diagnosed MM patients can present with renal injury, ranging from mild laboratory abnormalities through the need for renal replacement therapy (dialysis). With respect to frank MM, patients with milder renal failure may nonetheless be asymptomatic. Despite this, as renal failure can frequently be partially or fully reversible, and because of the profound implications of kidney dysfunction for both overall outcomes and options for MM disease management, new renal failure, whether at presentation or later in the disease course should be considered a medical emergency and treated promptly and aggressively in consultation with an expert nephrologist.

The most common and reversible cause of renal failure in MM is light chain tubular cast deposition and/or light chain deposition disease,[60] though monoclonal proteins may also be deposited as amyloidosis[61] (often associated with nephrotic syndrome range proteinuria) (see Chapter 93). Another common cause is hypercalcemia leading to osmotic diuresis and prerenal dysfunction associated with volume depletion—these are readily managed with intravenous volume repletion. Additional mechanisms of renal failure in MM include renal calcium deposition with interstitial nephritis, overuse of nonsteroidal anti-inflammatory drugs, hyperuricemia, chemotherapy-induced nephrotoxicity, and bisphosphonates.

Renal failure both at disease onset and during its course can significantly complicate management, as two of the three immunomodulatory drugs (lenalidomide, pomalidomide), which form the backbone of most modern therapies, are challenging or impossible to use with severe renal failure or in dialysis patients. Additionally, cytotoxic chemotherapy backbones which use cisplatin and etoposide (such as PACE [Platinol cisplatin, Adriamycin, cyclophosphamide, etoposide] or DCEP [dexamethasone, cyclophosphamide, etoposide, Platinol]) may also require therapeutically meaningful dose reductions with renal injury. Finally, because serum light chains are partially catabolized or cleared renally, in patients with kidney injury, levels of both the involved and un-involved free light chain may be elevated, and these tests must be interpreted with caution.

Anemia

Anemia, typically normocytic, is another presenting feature of myeloma. A small fraction of MM patients may present with solitary but profound anemia in the absence of other end-organ damage, and in such scenarios, a thorough evaluation including bone marrow sampling to exclude other causes of anemia is warranted. With respect to anemia secondary to PC disorders, causes are again multifactorial, including anemia of chronic inflammation, direct marrow replacement by PCs in the setting of high disease burden, paraneoplastic suppression of physiologic erythropoiesis even in patients with low marrow PC burdens, as well as inadequate erythropoietin production in patients with concomitant renal injury. After therapy with cytotoxic chemotherapy or autologous transplant, therapy-related anemia is expected and may persist, although moderate anemia can also result from treatment with novel agents, particularly in combination. Supportive management with transfusions may be initially required in newly diagnosed patients, but in most instances, disease-associated anemia is highly reversible with anti-PC therapy in the modern era and is rarely if ever a limiting toxicity when patients are treated with novel agents. Erythropoietin administration in MM patients should be used sparingly and judiciously and only in patients with renal injury, in consultation with an expert nephrologist, as significant risks are present.

Neurologic Symptoms

Patients with MM may present with a number of neurologic symptoms related either to direct involvement of the nervous system or the impact of cytokines and/or paraproteins. The most common overt abnormality is compression of the spinal cord and/or nerve roots, causing neurologic dysfunction, including paraplegia with bladder and bowel dysfunction. Cord compression is a neurologic emergency requiring urgent intervention: in this setting, spine magnetic resonance imaging (MRI) (with contrast if renal function allows) and high-dose glucocorticoids, followed by urgent radiation and/or neurosurgical intervention is generally warranted.

Peripheral neuropathy is another common manifestation and is also a key manifestation of light-chain (AL) amyloidosis (see Chapter 93). It may also develop as a result of use of a number of therapeutic agents, such as the immunomodulatory drugs (IMiDs) (most prominently thalidomide), bortezomib and ixazomib, cisplatin, and the vinca alkaloids. Anti-myelin-associated glycoprotein (MAG) peripheral neuropathy can also occur in rare cases in association with paraproteinemias. Moreover, POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes; see box on Rare Disorders Associated With Monoclonal Proteins and/or Plasma Cells) includes a prominent sensory neuropathy associated with sclerotic myeloma. As with other signs and symptoms, polyneuropathy in MM may be multifactorial, including amyloid deposits, infiltrative processes with other protein deposits, metabolic causes related to hypercalcemia and/or hyperviscosity, immune processes, or cytokines effects. Management is generally focused on treating the underlying PC disorder and minimizing further exposure to therapies with the risk of neuropathy.

Finally, although leptomeningeal disease (LMD) has traditionally been considered rare in myeloma, with the advent of novel agents and associated prolonged patient survival, this still-rare complication is nonetheless increasing in prevalence. It is a late complication of disease with no standard of care and carries a grim prognosis if present. Diagnosis can be secured with total brain and spine MRI, with and without contrast together with lumbar puncture for cytology and flow cytometry. Even more rarely, true central nervous system (CNS) intraparenchymal plasmacytomas have been reported in advanced disease – again, with no standard of care.

Hyperviscosity

Hyperviscosity in myeloma is uncommon: in general, an IgG more than 10 g/dL, IgA more than 7 g/dL, and IgM more than 5 g/dL are required to cause symptoms, and disease burdens correlated with such profound paraproteinemias would generally come to attention by manifesting other signs or symptoms. Serum viscosity can be measured to confirm hyperviscosity-associated symptoms. However, although symptomatic patients may demonstrate levels greater than 4 centipoise (cP), symptoms may also occur at lower viscosity levels. These can include headache, visual symptoms, shortness of breath, altered mental status, or an acquired bleeding diathesis associated with decreased effective levels of coagulation factor X. Therapy should be initiated more on the basis of symptomatology than on absolute measured levels of viscosity and can require prompt institution of plasmapheresis in addition to anti-PC therapy. Because of their corresponding volumes of distribution, plasmapheresis is generally more effective at rapidly resolving hyperviscosity from large pentameric IgM molecules, which are confined to the circulation, than hyperviscosity due to smaller monomeric IgG or dimeric IgA molecules.

Immune Deficiency

Infection is one of the most important causes of morbidity and a common cause of mortality in myeloma. Myeloma is associated with significant dysfunction across multiple facets of innate and adaptive immunity, with T-, B-, NK-, and myeloid-cell dysfunction all contributing to the immunodeficiency syndrome which affects both the humoral and cell-mediated immune responses. Patients with MM are at increased risk of bacterial, viral, and fungal infections and in some cases of secondary malignancies, which are a consequence of both immune dysfunction from the plasma cell disorder, as well as cytopenias and immunosuppression and the mutagenizing effects of some MM therapies. Most notably, patients receiving PIs require prophylaxis with acyclovir and valacyclovir against varicella zoster reactivation and use of cytotoxic chemotherapy combinations can be associated with prolonged periods of neutropenia, requiring growth factor support, as well as the use of prophylactic antimicrobials. With respect to steroids, the schedule and dose given in most regimens using novel agents typically does not warrant routine *Pneumocystis jirovecii* prophylaxis for the majority of patients. However, neutropenia can become a limiting toxicity of many therapeutic regimens; indeed PIs, IMiDs, and anti-CD38 monoclonal antibody therapy as well as a number of agents used late in treatments such as panobinostat and selinexor can all be associated with neutropenia—an individualized approach to antimicrobial prophylaxis may be required. Finally, with effective anti-PC therapy, most patients develop hypogammaglobulinemia. However, routine IVIG administration in the absence of recurrent infections is not a common practice for most practitioners who treat MM.

Coagulation Disorders

Coagulation abnormalities are observed in MM patients and can be a consequence of treatment. A disease-related hypercoagulable state is observed in many patients, secondary to hyperviscosity, acquired activated protein C resistance, lupus-like anticoagulants with thromboembolic complications, acquired protein S deficiency, and a therapy-related hypercoagulability associated specifically with the use of IMiDs. As with other cancers, venous thromboembolic events dominate over arterial clots. Given this, aspirin or direct oral anticoagulant prophylaxis (factor Xa inhibitors) is routine when patients are prescribed IMiD-based therapeutic regimens, and a high index of suspicion is warranted for patients who present with potential clinical signs or symptoms consistent with venous thromboembolism. Although in newly diagnosed patients, thrombocytosis is more frequently associated with myeloma than thrombocytopenia, as a consequence of extensive bone marrow infiltration by myeloma cells, and more commonly repeated cycles of chemotherapy over multiple lines of treatment, patients may become significantly thrombocytopenic during the advanced stages of disease. The dangers of bleeding tendency in combination with weakness and debility and the potential for falls and traumatic injury in advanced patients is particularly worrisome. Furthermore, much like neutropenia, thrombocytopenia can become a dose-limiting toxicity for effective disease-directed therapy, particularly in later lines.

Amyloidosis

Monoclonal proteins, specifically light chains, can be deposited in various organs as an insoluble fibrillar protein, amyloid, affecting organ dysfunction (see Chapter 93). Around 5% to 20% of patients with AL amyloidosis also have a concurrent diagnosis of MM, and by definition, all patients with AL amyloid have clonal light-chain production. Although clinically overt amyloidosis is observed less frequently in MM, intense investigation to identify amyloid deposits using fat pad biopsies (for immunohistochemistry, electron microscopy, or mass spectrometry), concurrent staining of bone marrow with Congo Red, and obtaining biopsies of other tissues (such as the GI tract) can identify some level of amyloid deposit in almost one-third of such patients. Identification of cardiac amyloidosis can have implications for treatment planning in MM patients with respect to use of regimens with potential cardiotoxicity, such as carfilzomib, and screening patients with cardiac troponins, B-type natriuretic peptide (BNP), or N-terminal pro-B-type natriuretic peptide (NT-proBNP) is recommended. EKG as well as both echocardiogram and cardiac MRI (to demonstrate an infiltrative cardiomyopathy) may be helpful non-invasive modalities which can suggest cardiac amyloidosis. Patients with both amyloid deposits and MM can present with a number of features primarily related to organ damage, including renal and cardiac dysfunction and neuropathy. Such patients may be challenging to treat, but therapeutic intervention is generally similar to those with MM without amyloidosis, with the necessary concessions to modifications of regimens for cardiomyopathy, renal dysfunction, etc.

Laboratory and Radiologic Studies for the Diagnosis and Monitoring of Multiple Myeloma and Related Entities

A thorough diagnostic evaluation is required to secure the diagnosis of MM and to assess for disease sequelae and organ dysfunction, related to the plasma cell neoplasm or otherwise. The findings can have significant impact on the precise entity diagnosed (e.g., MM vs. solitary plasmacytoma), disease risk stratification and associated selection of appropriate treatment regimens, and of course patient counseling and prognostication. A number of investigations are also useful to assess for treatment response and to restage patients after therapy. For a summary of the workup of new patients, see box on Investigations for Patients Suspected or Confirmed to Have Newly Diagnosed Multiple Myeloma.

Investigations to Detect Clonality

Protein Electrophoresis

Serum protein electrophoresis is performed for screening, and to quantitate the monoclonal proteins present in plasma cell disorders (see box on Investigations for Patients Suspected or Confirmed to Have Newly Diagnosed Multiple Myeloma). In approximately 70% of the patients, the monoclonal protein is IgG; in 20%, it is IgA; and in 5% to 10% of patients, it is light chains only without an intact immunoglobulin molecule. Less than 1% of patients have a monoclonal protein that is IgD, IgE, or IgM or even more rarely, a true nonsecretory myeloma. The identification of the precise paraprotein(s) in both serum and urine currently requires immunofixation (Fig. 91.4), and this test should always be paired with protein electrophoresis studies. Of note, because of the rarity of clonal IgD and IgE disorders, immunofixation studies do not routinely assess for IgE or IgD paraproteins; on initial evaluation for a new patient, measurement of total serum levels of IgE and IgD is recommended.

INVESTIGATIONS FOR PATIENTS SUSPECTED OR CONFIRMED TO HAVE NEWLY DIAGNOSED MULTIPLE MYELOMA

Studies of Blood

- Complete blood count with differential
- Comprehensive metabolic panel and GFR calculation
- Magnesium
- Phosphorus
- Serum protein electrophoresis
- Serum immunofixation electrophoresis
- Serum free light chains and ratio
- Quantitative IgG, IgM, IgA, IgD, IgE
- Lactate dehydrogenase
- β_2 microglobulin
- Erythrocyte sedimentation rate
- C-reactive protein
- Additional studies:
  - Type and screen, if patient will require transfusions (notify laboratory if daratumumab planned)
  - Serum viscosity if M-spike is extremely large, or concerned for hyperviscosity symptoms
  - VEGF and hormone studies (TSH, free T4, serum cortisol, sex hormone levels) if concerned for POEMS
  - Serum coagulation factor X and cardiac biomarker evaluation if concerned for AL amyloidosis (see also *cardiac markers*, below)

Urine Studies

- 24 urine collection for:
- Total protein
- Total creatinine and creatinine clearance
- Protein/creatinine ratio
- Urine free light chains
- Urine immunofixation electrophoresis

Unilateral Marrow Sampling

- Bone marrow biopsy and aspirate
  - Plasma cell percentage (highest as determined by biopsy, aspirate, or flow cytometry)
  - Plasma cells are quantified by morphology on an aspirate smear, typically by counting 100–200 consecutive cells
  - Plasma cells are quantified by CD138 immunohistochemistry on core biopsy; this percentage is typically the highest of the three studies
  - By flow cytometry, plasma cells (by characteristic cell surface markers and/or light chain restriction) as a percentage of total white blood cells is calculated; flow cytometry drastically underestimates the percentage in nearly all cases
- Demonstration of clonality: κ and λ light chain restriction (excess), typically with immunostaining on core biopsy, or intracellular light chain studies by flow cytometry. An aberrant flow cytometric cell surface phenotype may also suggest
- Genomic profiling by next-generation sequencing (NGS) or other assays as available, performed on $CD138^+$ enriched cells from an aspirate sample, including as a minimum the following validated markers of high-risk disease:
  - Translocations of *IGH* (chromosome 14) with a number of partners
    - t(4;14) – *MMSET*
    - t(11;14) – *CCND1*
    - t(14;16) - *MAF*
    - t(14;20) – *MAFB*
  - Chromosome 17p deletion (loss of *TP53*)
  - Chromosome 1q gains
  - Somatic mutation analysis of *TP53*
- Baseline profiling of tumor cells for MRD tracking by flow cytometry and/or NGS-based assays
  - Flow cytometry: per local guidelines (not mandatory)
  - NGS: immunoglobulin gene sequencing mandatory
- Additional studies:
- Congo Red staining if suspecting amyloid
- Gene expression profiling or extended genomic sequencing (investigational)
- Banking of marrow samples for clinical trials, including subsequent assessment of BCMA, GPRC5D, etc.

Imaging Studies

- 18F-FDG/PET with associated low dose CT
- Total body MRI without contrast—if not available, total spine and pelvis MRI is sufficient

Note: there is no role for plain film skeletal survey in the evaluation of patients in practice scenarios where MRI and/or PET/CT are available.

Cardiac Studies

- EKG
- Troponin T or I
- BNP, NT-proBNP
- Consider echocardiogram if symptomatic, prior cardiac history, ruling out AL amyloidosis, or using carfilzomib-based therapy
- Consider cardiac MRI in consultation with cardiologist, if high suspicion for amyloidosis

Tissue Biopsy

- Abdominal fat pad biopsy, if ruling out AL amyloidosis. Possible studies to identify amyloidosis include immunohistochemistry, electron microscopy, and/or mass spectrometry

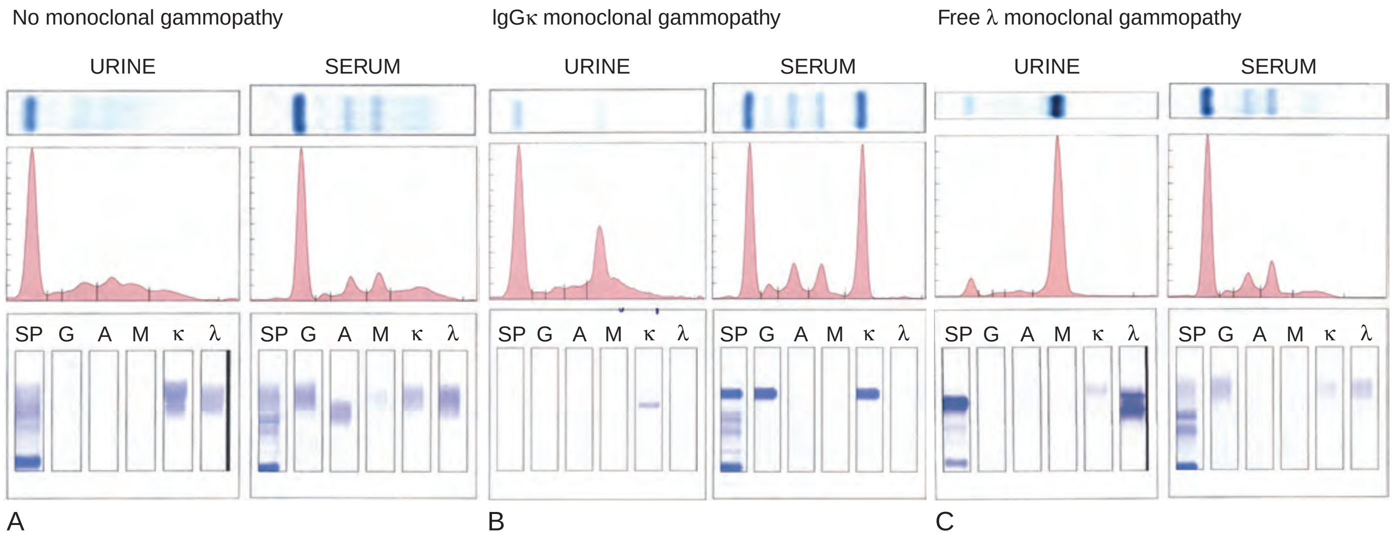


Figure 91.4 REPRESENTATIVE PATTERNS OF SERUM PROTEIN ELECTROPHORESIS AND IMMUNOFIXATION ELECTROPHORESIS. In each figure, the *upper panels* represent immunofixation patterns, the *middle panels* are the densitometric tracings of the gels, and the *lower panels* are agarose gels of urine sample *(left)* and serum *(right)*. (A) Normal pattern of serum and urine protein on electrophoresis. Because there are many different immunoglobulins in the serum, their differing mobilities in an electric field produce a broad peak. (B) In monoclonal gammopathies, the predominance of a product of a single cell produces a sharp peak. The immunofixation identifies the type of immunoglobulin, such as κ light chain in urine and IgGκ in serum. (C) In patients with light chain–only disease, a clonal band is observed only in urine, with no clear peak in serum, such as γ light chain in urine with no distinct immunofixation positivity in serum. *(Courtesy Dr. Neal I. Lindeman; used with permission.)*

As noted above, patients who produce intact immunoglobulins can also produce excess free light chain; for example, a patient's PCs can secrete an intact IgG κ protein as well as free κ light-chains. Notably, in such cases, with the selection pressures of anti-myeloma therapy, sequential evaluation may reveal a predominance of either the intact immunoglobulin or the light chain as the disease biomarker (e.g., only IgG κ, or only a κ light-chain myeloma—the so called "light chain escape" phenomenon).

Finally, the presence of a monoclonal protein can cause "reciprocal" suppression of levels of the uninvolved immunoglobulins. This can be observed both in precursor states such as SMM as well as MM: for example, patients with IgG myeloma can have suppressed IgA and IgM. In newly diagnosed patients if all three immunoglobulins are suppressed, one should suspect either light-chain disease and/or the possibility of IgD or IgE or even true nonsecretory MM, although light chain disease is the far more common entity. More notably, suppression of all three immunoglobulins is common with successful anti-PC therapy. More rarely, a biclonal or triclonal pattern of immunoglobulins is observed, more often with the same light chain but rarely with a different heterotypic light chain. This may suggest truly separate clones, especially if distinct (κ vs. λ) light chains are seen. In other rare clinical instances, a PC neoplasm may co-exist in the same patient with an indolent B cell lymphoma (clonally related or otherwise), which may also secrete paraproteins, leading to unusual electrophoresis results.

Quantitation of Bence Jones proteins in urine is still important, both for diagnosis of myeloma and for follow-up. However, it is important to note that quantitative free light-chain measurements or ratios in the urine is rarely informative. Rare patients with a monoclonal protein detectable only in the urine but no paraproteinemia in the blood, may require more frequent 24-hour urine collections during treatment and follow-up.

Therapeutically, MM patients with each of the various types of immunoglobulins are treated with the identical therapy at present; however, IgA myeloma was previously associated an inferior survival for a given level of blood proteinemia. This has been attributed to more rapid catabolism and turnover of serum IgA, such that an equivalent numeric level of serum IgA relative to monoclonal IgG may reflect a higher total disease burden.

The immunoglobulin isotype remains constant in a given patient over the natural history of the disease; however, occasionally, a patient producing one immunoglobulin at the time of diagnosis, at relapse, or with advanced disease may present with only the same light chain as initially observed with the original immunoglobulin (light chain escape) or more rarely may become "hyposecretory" (with aberrantly low paraprotein levels for the actual total body disease burden) or even truly nonsecretory. Such changes are believed to reflect changes in neoplastic plasma cells to a more aggressive or undifferentiated form. As a result of the observed light-chain escape, such patients without Bence Jones proteins initially detected in the urine can require periodic 24-hour urine Bence Jones protein measurements during follow-up.

Finally, although not directly evaluated by electrophoresis or immunofixation, the unique genetic sequences that are observed with the idiotype of each paraprotein (the third complementarity determining region (or CDR3) have been used as a marker that specifically identifies a tumor cell clone and have been applied to next generation sequencing (NGS) or allele-specific oligo-polymerase chain reaction (PCR)—based methodology to detect MRD with high sensitivity (see Minimal Residual Disease).

Serum Free Light Chains

This test specifically measures serum light chains that are *not* associated with heavy chains, i.e., those that are not part of an intact immunoglobulin molecule (Fig. 91.5). The presence of excess serum free light chains provides an additional marker and measurement of plasma cell burden, and its quantitation has been used to assess disease burden levels in a number of patients who were previously considered oligosecretory or nonsecretory. For example, up to 80% of patients with the previous label of "nonsecretory myeloma" actually have measurable serum free light chains.[62] Routine use of serum free light chain measurements is indicated for diagnosis, response evaluation, and prognosis in essentially all patients.

Bone Marrow Examination

Except for patients with solitary plasmacytoma, the presence of clonal plasma cells (>5%) is usually observed in all plasma cell disorders starting with MGUS up through MM, with the phenotype of malignant plasma cells generally distinguished from normal plasma cells by monoclonal antibody staining and flow cytometry (Table 91.2).[63–65] Clonal plasma cell populations comprising 10% or greater of the nucleated cells within a bone marrow aspirate or biopsy (whichever is higher) are required to differentiate MGUS from SMM and for the diagnosis of MM (Table 91.3). The quantitation of plasma cells should be performed with at least a 200-cell count on aspirate smear, and confirmation of clonality is essential for diagnosis, which can be done either by immunostaining using κ/λ staining on core biopsy for excesses of PCs expressing κ or λ, or alternatively by flow cytometry for intracellular light chain restriction and/or the expression of aberrant cell surface proteins (Fig. 91.6 and box on Investigations for Patients Suspected or Confirmed to Have Newly Diagnosed Multiple Myeloma). In the absence of clear bone marrow involvement, a

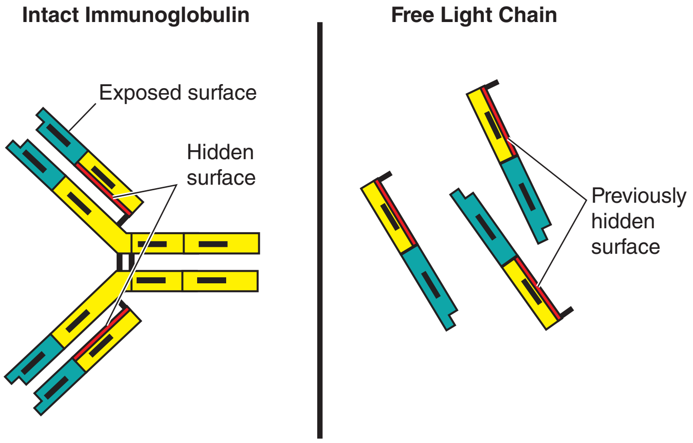


Figure 91.5 PRINCIPLE FOR MEASUREMENT OF FREE LIGHT CHAINS. The free light chain assay measures light chains on the basis of a part of the light chain molecule which is hidden when the light chains are in complex with heavy chains as part of an intact immunoglobulin molecule *(left)*. This hidden surface is exposed when light chains circulate as free proteins, allowing their specific measurement *(right)*.

TABLE 91.2 Phenotypic Characterization of Plasma Cells

Adhesion Molecule	Normal Plasma Cells	Multiple Myeloma Cells
CD138	+	+
CD19	+	–
CD28	–	–
CD38	+	+
CD40	+	+[a]
CD45	+	–[b]
CD27	–	+
CD11a	+	–
CD11b	–	–
CD44	+	+
CD54	+	+
CD56	–	+
CD58	–	+
LFA-1	–	–/+
RHAMM	–	+
VLA-4	+	+
VLA-5	+	+

[a] CCD40 expression is enhanced on plasma cell leukemia cells relative to normal plasma cells and MM cells.
[b] CD45 on immature myeloma cells.
LFA-1, Lymphocyte function-associated antigen 1; *MM*, multiple myeloma; *RHAMM*, receptor for hyaluronan-mediated motility; *VLA-4*, very late antigen 4; *VLA-5*, very late antigen 5.

TABLE 91.3 Diagnostic Criteria for Multiple Myeloma, Myeloma Variants, and Monoclonal Gammopathy of Unknown Significance

Monoclonal Gammopathy of Undetermined Significance (MGUS) or Monoclonal Gammopathy, Unattributed/Unassociated (MG[u])
M protein in serum <30 g/L
Bone marrow clonal plasma cells <10%
No evidence of other B-cell proliferative disorders
No myeloma-related organ or tissue impairment (no end-organ damage, including bone lesions)
Asymptomatic Myeloma (Smoldering Myeloma)
M protein in serum >30 g/L and/or
Bone marrow clonal plasma cell ≥10%– 60%
No related organ or tissue impairment (no end-organ damage, including bone lesions) or symptoms
Symptomatic Multiple Myeloma
M protein in serum and/or urine[a]
Bone marrow (clonal) plasma cells[a] or plasmacytoma
Related organ or tissue impairment (end-organ damage, including bone lesions)
Bone marrow clonal plasma cells >60%
Involved/Uninvolved light chain ratio >100, with the involved light chain >100 mg/L
More than one characteristic lesion in the bone or bone marrow by MRI
Solitary Plasmacytoma of Bone
No M protein in serum and/or urine[b]
Single area of bone destruction caused by clonal plasma cells
Bone marrow not consistent with MM
Normal skeletal survey (and MRI of spine and pelvis if done)
No related organ or tissue impairment (no end-organ damage other than solitary bone lesion)[b]
Nonsecretory Myeloma
No M protein in serum and/or urine with immunofixation
Bone marrow clonal plasmacytosis ≥10% or plasmacytoma
Related organ or tissue impairment (end-organ damage, including bone lesions)
Extramedullary Plasmacytoma
No M protein in serum and/or urine[c]
Extramedullary tumor of clonal plasma cells
Normal bone marrow
Normal skeletal survey
No related organ or tissue impairment (end-organ damage including bone lesions)
Multiple Solitary Plasmacytomas (Recurrent or Not)
No M protein in serum and/or urine[d]
More than one localized area of bone destruction or extramedullary tumor of clonal plasma cells that may be recurrent
Normal bone marrow
Normal skeletal survey and MRI of spine and pelvis if done
No related organ or tissue impairment (no end-organ damage other than the localized bone lesions)
Myeloma-Related Organ or Tissue Impairment (End-Organ Damage)
Calcium levels increased: serum calcium >0–25 mmol/L above the upper limit of normal or >2–75 mmol/L
Renal insufficiency: creatinine >173 mmol/L (>2 mg/dL)
Anemia: Hemoglobin 2 g/dL below the lower limit of normal or hemoglobin <10 g/dL
Bone lesions: Lytic lesions or osteoporosis with compression fractures (MRI or CT may clarify)
Other: Symptomatic hyperviscosity, amyloidosis, recurrent bacterial infections (more than two episodes a year)

CT, Computed tomography; *MRI*, magnetic resonance imaging.
[a] If flow cytometry is performed, most plasma cells (>90%) will show a neoplastic phenotype.
[b] A small M component may sometimes be present.
[c] A small M component may sometimes be present.
[d] A small M component may sometimes be present.

biopsy-proven soft tissue or bony plasmacytoma is also adequate for diagnosis (Fig. 91.7). Genomic profiling of $CD138^+$ enriched marrow aspirate samples should be performed at the time of diagnosis and may also need to be repeated at the time of relapse or progression. Of note, although important for prognostication in MM, genomic abnormalities *do not* differentiate MM from precursor states and are also currently not predictive of response to therapy in the up-front setting. Eight to ten-color flow cytometry panels are now also used with bone marrow samples to detect MRD,[65] and establishment of the initial cell-surface phenotype at diagnosis can be important if flow-cytometry based MRD monitoring is elected. Baseline characterization of immunoglobulin gene sequences is mandatory for NGS-based MRD tracking.

Investigations to Detect End-Organ Damage

The diagnosis of a plasma cell disorder is based on the presence of a monoclonal protein and clonal plasma cells. However, the diagnosis of MGUS/SMM, for which there is currently insufficient evidence to warrant therapeutic intervention, versus active symptomatic MM,

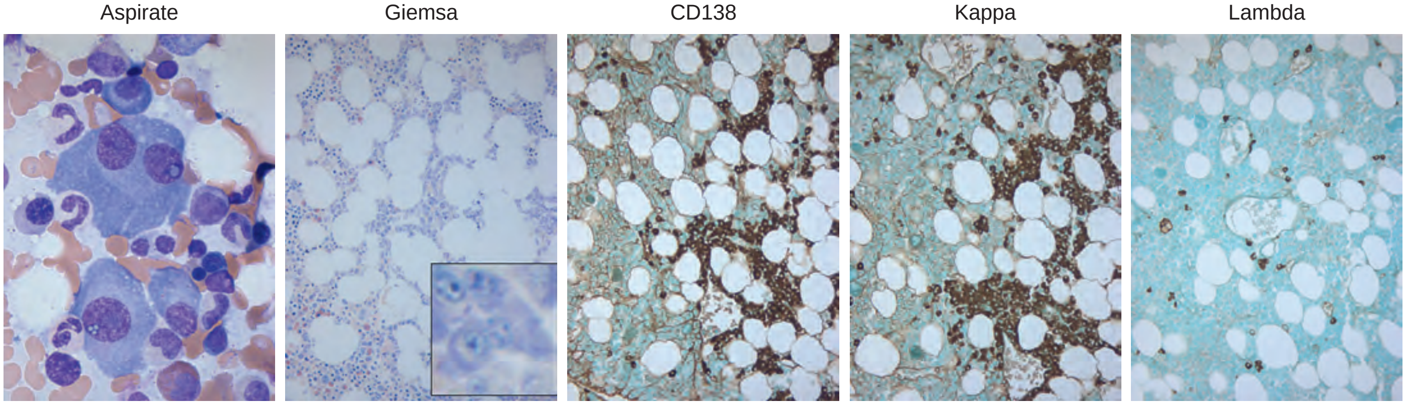


Figure 91.6 BONE MARROW EXAMINATION FROM A PATIENT WITH IGG κ MYELOMA, SHOWING NEOPLASTIC PLASMA CELLS. The cells are CD138$^+$, and κ light chain–positive but λ-negative. *(Courtesy Dr. Ruben Carrasco, M.D.; used with permission.)*

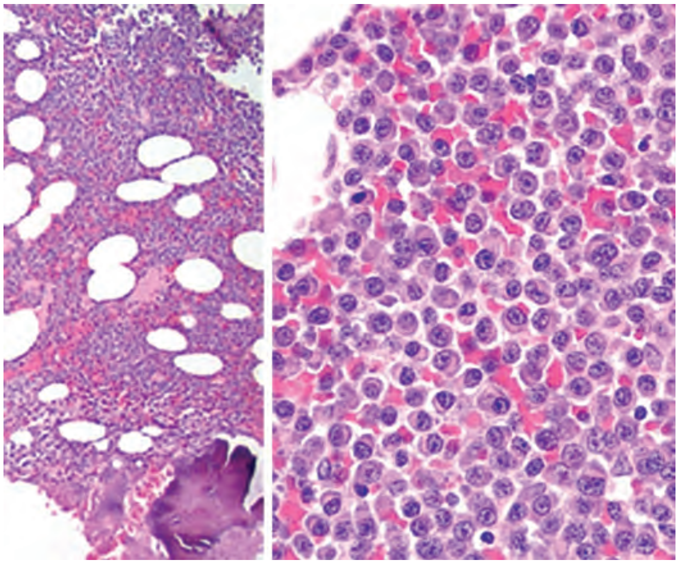


Figure 91.7 MORPHOLOGY OF NEOPLASTIC PLASMA CELLS IN MULTIPLE MYELOMA. There is significant morphologic heterogeneity in the plasma cells, as seen on bone marrow biopsy.

which needs treatment, is largely predicated on the demonstration of end-organ damage, which includes bone lesions, anemia, renal dysfunction, and hypercalcemia. The details of the diagnostic criteria are listed in Table 91.3 and diagnostically useful tests can be found in the box on Investigations for Patients Suspected or Confirmed to Have Newly Diagnosed Multiple Myeloma.

Radiographic Evaluation

Skeletal evaluation is mandatory in the workup of patients with possible MM. Arguably the previous standard for evaluation of bone lesions, the whole body plain X-ray skeletal survey has been supplanted by 18F-FDG-PET (fluorodeoxyglucose-positron emission tomography) combined with low-dose whole body computed tomography (CT), or total body (occasionally total spine and hip) MRI without contrast due to markedly improved sensitivity and specificity in clinical practice settings where these advanced imaging modalities are available.[66,67] The presence of characteristic bony lytic lesions is considered diagnostic for myeloma (Fig. 91.8). Of note, although useful for assessing disease burden and location at the time of initial diagnostic workup, bone lesions do not always resolve following effective therapy, although if patients are followed by serial positron emission tomography (PET) imaging, there is oftentimes resolution of FDG avidity. Given this, cross-sectional imaging is frequently most useful for initial assessment and restaging and at time of disease progression, rather than for response assessment per se. Lastly, because of the absence or suppression of osteoblastic activity and therefore lack of radiotracer uptake, the bone scan is generally not a useful investigative tool in myeloma. Details of various imaging modalities and their role in myeloma diagnostics are summarized in Table 91.4.

Indeed, based on superior test performance characteristics, MRI, CT, and PET are increasingly being used for patients with MM. In particular, MRI[68] without contrast is a useful modality. In the initial diagnostic workup of patients with overlap syndromes which span indolent lymphomas vs. MM, modern MRI protocols can allow for assessment of lymphadenopathy and organomegaly, while also allowing for detailed evaluation of the degree of bone marrow involvement and involvement of the spinal cord. Note that even in the absence of frank lytic bone disease, the presence of two or more characteristic marrow-based lesions each at least 5 mm in diameter is consistent with MM. Moreover, MRI is indicated in all patients with a suspected diagnosis of a solitary plasmacytoma and is indicated in patients with putative SMM to exclude any occult bone or marrow involvement. Finally, in the rare patient with true nonsecretory myeloma, MRI without contrast is an important tool for response evaluation and assessing disease burden, as it is a noninvasive modality that facilitates serial imaging without concerns for cumulative radiation exposure or other toxicities.

CT scanning, typically not involving the use of contrast, has also been used to localize focal lesions for tissue biopsy. CT scanning has superior sensitivity and specificity for lytic bone disease as compared with plain film skeletal series and may be superior for imaging of the hard bone itself relative to MRI, although the bone marrow cannot be usefully evaluated. In areas where 18F-FDG-PET scanning is available, many practitioners combine low-dose CT with PET. Combined PET/CT can be used to define extramedullary disease (EMD) as well as medullary lesions, and also complement MRI for follow-up of patients with nonsecretory myeloma (Fig. 91.9). Conversion of target lesions from 18F-FDG-PET positivity to negativity with effective locoregional or systemic therapy, as appropriate, also has prognostic significance. A prospective evaluation of MRI and PET/CT at diagnosis and before maintenance therapy was performed in a subgroup of 134 patients with MM in the IFM/DFCI 2009 trial. At diagnosis, MRI was positive in 127 (94.7%) of 134 patients and PET-CT was positive in 122 (91%) of 134 patients, respectively ($P = .33$). Few patients achieved a normal MRI after 3 cycles of VRD before maintenance. Normalization of the MRI following 3 cycles of VRD and before maintenance was not predictive of both progression-free survival (PFS) and OS. Normalization of PET/CT was more frequently observed: 45% following 3 cycles of VRD and 79% before maintenance. Importantly, normalization of PET-CT was associated with an improvement in PFS, and normalization before maintenance was a predictor for improved OS.

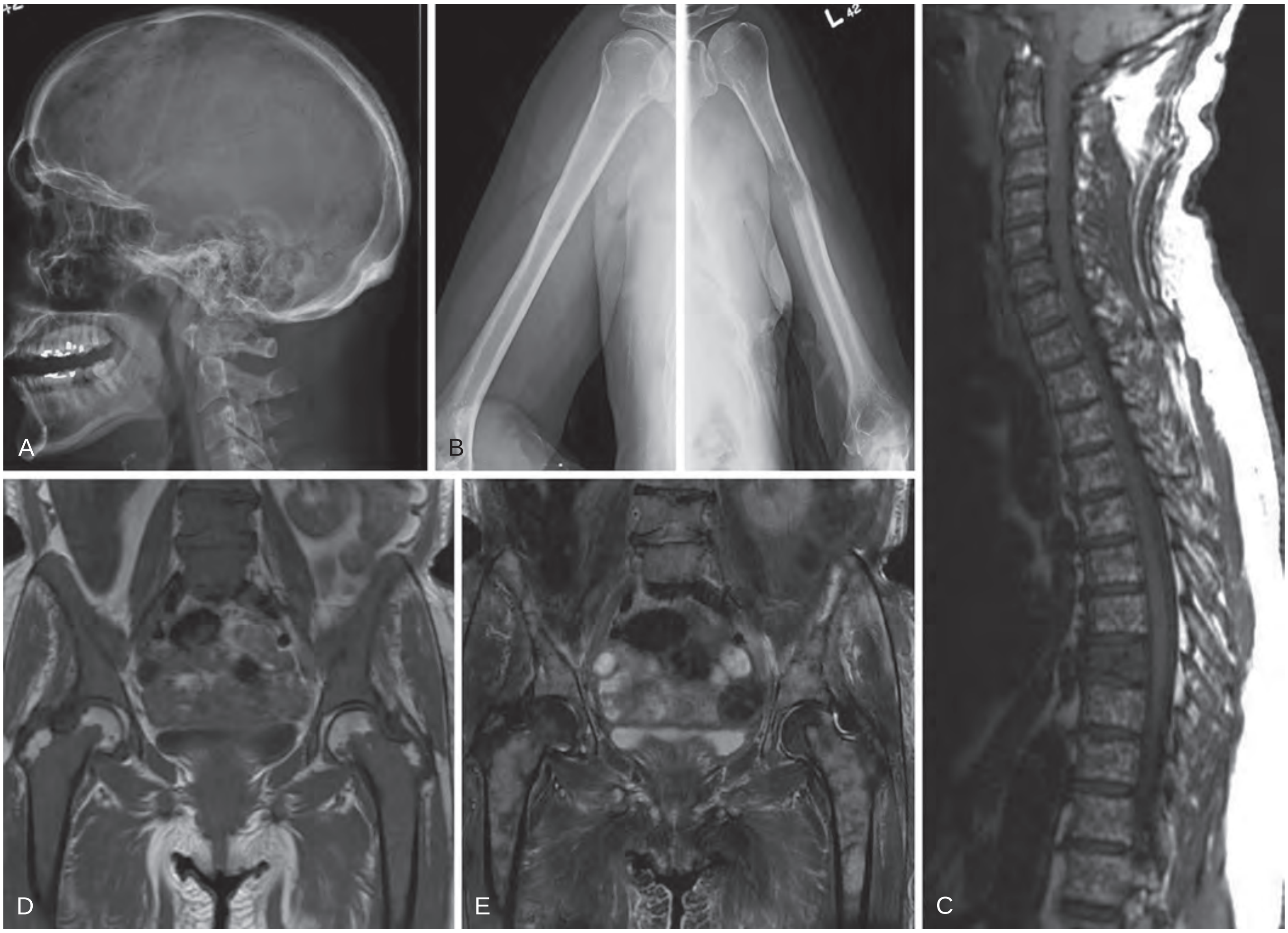


Figure 91.8 TYPICAL SKELETAL CHANGES OF MULTIPLE MYELOMA. (A, B) Examples of "punched-out" lytic lesions in skull and humerus. (C–E) Magnetic resonance imaging pattern in multiple myeloma in spine and pelvis showing diffuse involvement with focal lesions.

TABLE 91.4 Imaging Modalities for Disease Assessment in Myeloma

	Use	Sensitivity/Specificity	False-Negatives	False-Positives
Bone scan	• For diagnostic screening, except for multiple myeloma	Varies	Pure osteolytic lesions	• Trauma • Inflammation • Benign tumor • Healing
X-ray	• Can clarify nonspecific findings on bone scan • Assesses risk of fracture • Possible follow-up of tumor response, but evidence of response takes considerable time to appear	Low sensitivity	Low disease burden Osteopenia	• Trauma • Inflammation • Benign tumor • Healing
CT	• For anatomic detail in axial skeleton • Possible follow-up of tumor response, but role is still undefined	High sensitivity	Low disease burden	• Trauma • Inflammation • Benign tumor • Healing
MRI	• Detection of spinal cord compression • Can help distinguish benign from malignant vertebral compression fracture • Possible follow-up of tumor response, but role is still undefined	High sensitivity and specificity	Lesion only in cortex	• Edema
PET scan	• May eventually become first-line screening test for bone metastases • Possible follow-up of tumor response, but role is still undefined	High specificity	Lesion only in cortex	• After chemotherapy
Bone density	• Measure osteoporosis • Response to bisphosphonates	High specificity and sensitivity		• Age-related osteoporosis

CT, Computed tomography; *MRI*, magnetic resonance imaging; *PET*, positron emission tomography.

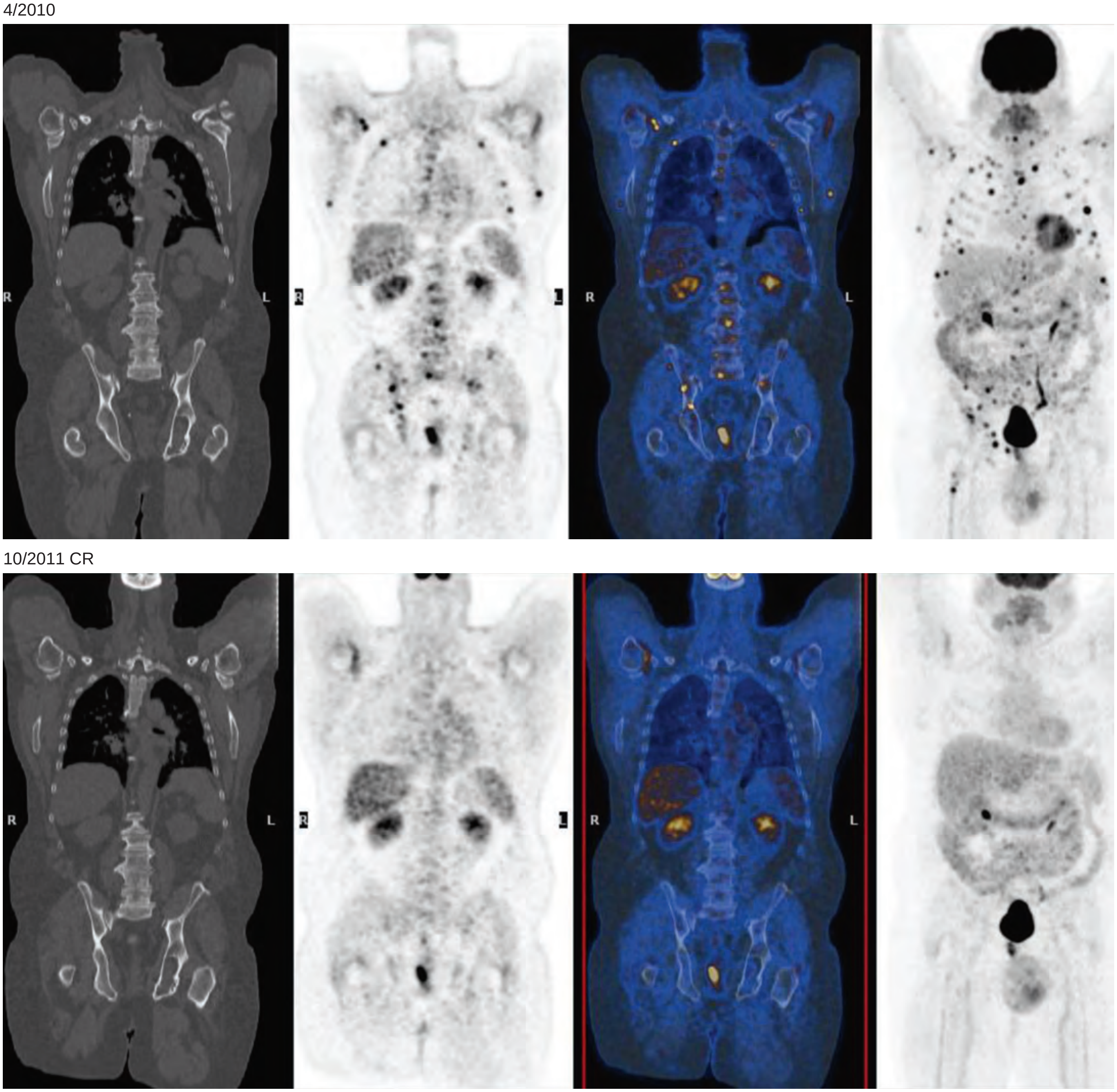


Figure 91.9 POSITRON EMISSION TOMOGRAPHY / COMPUTED TOMOGRAPHY SCANS IN MULTIPLE MYELOMA. Upper panels show multiple 18F-fluorodeoxyglucose (FDG)-avid lesions throughout the skeleton. The lower panels show their resolution at the time of achieving complete remission.

Renal Function

A serum creatinine greater than 173 mmol/L (2 mg/dL) is considered to represent end-organ damage from myeloma. However, as absolute creatinine values do not incorporate the patient's age, race, or body mass, they may under- or over-represent the extent of renal dysfunction. Conversely, because patients with myeloma are older and may have other comorbidities such as diabetes and hypertension which can also impair renal function, it may be necessary to establish the causal relationship between renal dysfunction and the plasma cell disorder. This is particularly critical in patients who would otherwise be classified as MGUS or SMM but have renal dysfunction. In the majority of cases, a 24-hour urine collection with analysis of creatinine, total protein, and identification of paraproteins in the urine is sufficient. However, a renal biopsy in consultation with an expert nephrologist can occasionally be required when the clinician is truly is uncertain about the cause of the renal dysfunction. On the other hand, patients who present with renal impairment but also other manifestations of myeloma end-organ damage primarily require careful monitoring and optimization of kidney function. In such a scenario precise diagnostic workup to assign attribution of the precise subtype of PC-associated pathophysiologic process causing renal injury rarely causes changes in management.

Blood Counts and Serum Calcium

A standard complete blood count with differential is performed to detect anemia. The level of anemia considered diagnostic of myeloma is a hemoglobin 2 g/dL below the lower limit of normal or the patient's baseline level or hemoglobin <10 g/dL. It is always necessary to exclude other causes of anemia, especially in the elderly population where MM is more common. The white blood cell differential is important for assessing primarily neutropenia or thrombocytopenia secondary to disease or treatment, although in some cases circulating plasma cells may also be observed on automatic or manual differential count. Likely because of their strong dependence on the microenvironment in general, circulating plasma cells are infrequently observed in patients with MM. If present even at low level in the blood that do not meet criteria for plasma cell leukemia (PCL), this finding is associated with a poor prognosis.[69,70] A serum calcium level should be measured and corrected to the level of albumin. An ionized calcium level can sometimes be useful, particularly in patients receiving therapy for hypercalcemia.

Ancillary Studies

As part of initial evaluation, serum IgD and IgE levels are useful in order to screen all patients for rare cases of myeloma secreting these immunoglobulins, as serum or urine immunofixation electrophoresis does not routinely assess for monoclonal IgD or IgE. Lactate dehydrogenase (LDH) and serum β_2 microglobulin levels are useful measures of disease burden and required for accurate use of the revised international staging system (R-ISS). For patients who present with hypercalcemia, ionized calcium, an intact PTH, PTH related peptide, 25-OH vitamin D and 1,25-$(OH)_2$ vitamin D levels, as well as 24-hour urine calcium studies, may be helpful if there is diagnostic uncertainty about the etiology of hypercalcemia.

EKG, cardiac troponin T or I, BNP, and/or NT-proBNP are useful at diagnosis to screen for cardiac amyloidosis as well as to assess baseline cardiac function in anticipation of therapy, especially if use of agents with potential cardiac toxicities, such as carfilzomib or anthracycline containing c chemotherapy regimens are anticipated. A coagulation factor X level can be useful if assessing coagulopathy secondary to AL amyloidosis. For selected patients where POEMS is suspected or diagnosed, a VEGF level, and endocrine studies (e.g., thyroid stimulating hormone (TSH), free T_4, adrenocorticotropic hormone (ACTH), cortisol, sex hormone studies) may have utility at diagnosis and during follow-up. Finally, in our practice, we routinely check the erythrocyte sedimentation rates (ESR) and C-reactive protein (CRP) levels in newly diagnosed patients.

Although tumor lysis syndrome is rare it has been observed with effective myeloma therapy in both the newly diagnosed and relapsed/refractory settings. Measurement of uric acid levels and a glucose-6-phosphate dehydrogenase screen (for rasburicase therapy if needed) should be considered in patients judged to be at high risk for developing tumor lysis syndrome, although routine pharmacologic tumor lysis prophylaxis is not generally warranted for most patients.

Minimal Residual Disease

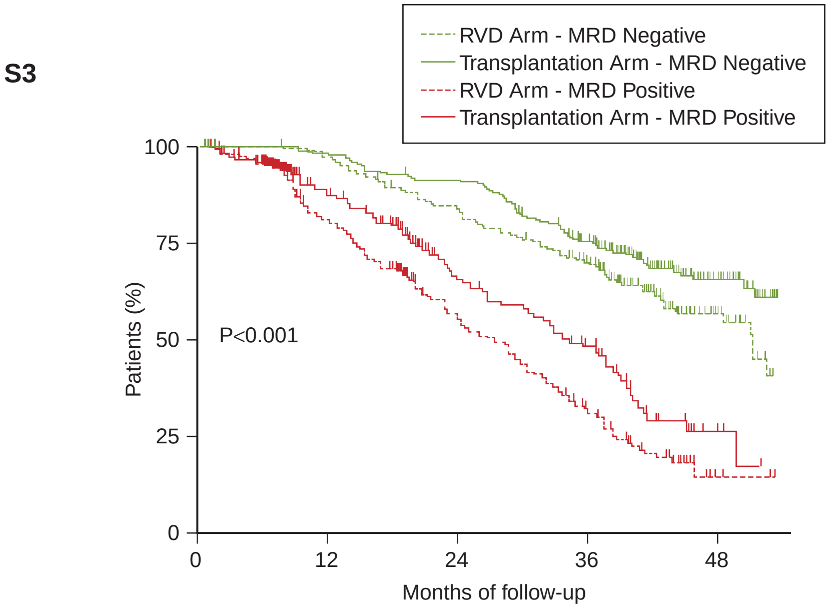
Minimal (or measurable) residual disease (MRD) testing refers to dynamic assessment of the tumor mass remaining after treatment and during follow-up. Current International Myeloma Working Group (IMWG) guidelines define MRD negativity as fewer than one tumor cell in 100,000 bone marrow cells (10^{-5}) as assessed by flow cytometry or NGS in a patient who fulfills the conventional criteria for a complete response.[71] Achieving MRD negativity has been established as a strong prognostic biomarker in several recent meta-analyses (Table 91.5).[72,73] Indeed, becoming MRD negative more strongly affects MM PFS than which therapeutic regimen was used to get there, as evidenced by recent randomized clinical trials in the newly diagnosed setting (Fig. 91.10)[74,75] and relapse setting.[76–78] Furthermore, in these studies, MRD status had a stronger impact on PFS than conventional biomarkers of high-risk disease such as the International Staging System (ISS), t(4;14), and del(17p), raising the possibility that MRD status may be used prospectively to guide treatment.[77,79] Investigators from the University of Arkansas have followed over 600 patients treated with Total Therapy–type regimens (described in the Treatment section) who were MRD-negative to a depth of 10^{-5} by flow cytometry for a median of 10 years, with serial MRD assessment.[80] In this population, 60% of patients achieved sustained MRD negativity, with the remaining 40% converting to the MRD positive state on average 5.7 years after high dose chemotherapy and autologous stem cell transplant (ASCT). Conversion to MRD positivity was associated with inferior PFS and OS, as has been observed in other studies. These data are similar to results of phase II data with combinations of novel agents such as KRd (carfilzomib, lenalidomide, dexamethasone), where the median duration of MRD negativity was also close to 5 years[81] in one series, with an arguably significantly less toxic modern regimen. The duration of sustained MRD negativity with modern therapies is likely to be an area of continued investigation and may be one yardstick to measure their efficacy as the PFS and OS of MM patients continues to lengthen.

There have been considerable advances in MRD testing since the latest IMWG criteria were developed in 2014. Recent studies using highly sensitive assays have demonstrated a dose-response relationship between the concentration of residual tumor cells and patient outcomes.[79,82] The strongest impact on PFS was seen with a sensitivity of

TABLE 91.5 Association of Minimal Residual Disease Status With Progression-Free Survival and Overall Survival

Study	Design	MRD Testing Strategy	N (PFS)	N (OS)	PFS Benefit, HR (95% CI)	PFS Benefit, Median	OS Benefit, HR (95% CI)	OS Benefit, Median
Landgren 2016	Meta-analysis of 4 clinical trials	MFC or ASO-PCR; sensitivity $>10^{-4}$	659	555	0.35 (0.27–0.46); $P < .001$		0.48 (0.33–0.70); $P < 0.001$	
Lahuerta 2016	Pooled analysis of 3 clinical trials	MFC; sensitivity $>10^{-4}$	609	609	0.42 (0.34–0.51); $P < .001$	63 m (MRD-) vs 27 m (MRD+ CR); $P < .001$	0.33 (0.25–0.44); $P < .001$	Not reached (MRD-) vs 59 m (MRD+ CR); $P < .001$
Munshi 2017	Meta-analysis of 21 clinical trials	MFC, ASO-qPCR, or NGS; sensitivity $>10^{-4}$	1593	1541	0.41 (0.36–0.48); $P < .001$	56 m (MRD-) vs 34 m (MRD+); $P < .001$	0.57 (0.46–0.71); $P < .001$	112 m (MRD-) vs 82 m (MRD+); $P < .001$
Munshi 2019	Meta-analysis of 86 studies (only published as abstract)	MFC, ASO-qPCR, or NGS; sensitivity of 10^{-4}, 10^{-5} or 10^{-6}	8590	3392	All; 0.35 (0.31–0.39); 10^{-4}; 0.36 (0.31–0.42); 10^{-5}; 0.35 (0.30–0.41); 10^{-6}; 0.26 (0.17–0.39); $P < .001$ for all		All; HR, 0.48 (0.41–0.55); 10^{-4}; 0.49 (0.42–0.57); 10^{-5}; 0.47 (0.34–0.65); $P < .001$ for all	

ASO-qPCR, Allele-specific oligonucleotide quantitative polymerase chain reaction; *CR*, complete response; *HR*, hazard ratio; *NGS*, next-generation sequencing; m, months; *MFC*, multiparameter flow cytometry; *MRD*, minimal residual disease; *N*, number; *OS*, overall survival; *PFS*, progression-free survival.



No. at Risk					
RVD Arm – MRD Negative	0	136	145	115	28
Transplantation Arm – MRD Negative	0	171	202	155	45
RVD Arm – MRD Positive	350	158	83	42	4
Transplantation Arm – MRD Positive	350	137	62	41	5

Figure 91.10 The association of minimal residual disease *(MRD)* negative status with outcomes from the IFM/DFCI 2009 trial. MRD status is a stronger predictor of disease progression than the treatment modality to which patients were exposed (i.e., transplant vs. no-transplant).

10^{-6}, which is the highest that can be achieved with current methods. Higher sensitivity than 10^{-6} may be technically possible but is practically limited by the number of bone marrow cells available for analysis (e.g., a sensitivity of 10^{-6} requires at least 3 million cells; 10^{-7} would require 30 million). Based on current data, there is strong evidence that 10^{-6} should be the future standard for MRD negativity in the bone marrow; MRD testing with a sensitivity of 10^{-4} or lower is not to be informative.

The optimal time to test for MRD in the bone marrow during the course of treatment depends on the dynamics of response to a particular therapeutic strategy. Historically, MM therapies resulted in gradual reduction of tumor burden over the course of several months, mirroring the slow clearance of serum monoclonal immunoglobulins (the half-life of IgG is ~25 days).[83,84] In this setting, it is logical to only apply invasive MRD testing when monoclonal immunoglobulins have become undetectable, which was the basis for current IMWG response criteria.[71] This strategy of MRD assessment has been challenged by novel highly effective therapies (e.g., CAR-T cells and four-drug combinations) capable of eradicating all measurable tumor cells from the bone marrow long before monoclonal proteins have cleared from the serum.[85,98] Most of these patients go on to achieve a conventional CR up to several months later, indicating that early MRD negativity in the bone marrow is an early predictor of deep and durable responses irrespective of the serum protein levels at the time of assessment.

The most commonly used laboratory techniques to assess MRD status in the bone marrow are (1) flow cytometry for the aberrant cell-surface phenotype of myeloma cells and (2) NGS[86] for tracking of tumor-specific immunoglobulin V(D)J rearrangement sequences.[87,88] There is currently no evidence that one methodology is superior to the other, but clinical trials which include head-to-head comparison of both approaches are ongoing. There are however important practical considerations for choosing an assay. Flow cytometry is more widely accessible, but there is great variability in assay performance between centers; interpretation relies on local expertise; and fresh bone marrow aspirates are required for analysis. By contrast, highly standardized NGS assays are offered commercially and can be performed using stored samples but require a pre-treatment sample with high degree of tumor infiltration which is required to identify tumor-specific immunoglobulin sequences for tracking. Importantly, flow cytometry assays require 3 to 10 million bone marrow cells to reach 10^{-6} sensitivity, while NGS assays generally require only 3 million cells. Both assays are highly dependent on sample quality and initial bone marrow aspirate pulls are recommended for use in order to avoid false negatives due to hemodilution.

In keeping with the view of MRD negativity as a surrogate marker for cure, there is a constant drive to develop more sensitive MRD testing strategies. As mentioned above, there are practical limitations to the sensitivity of bone marrow MRD testing. Moreover, a single bone marrow aspirate represents a random sampling of cells from a single site from a patient with MM which is a patchy disease. Current research efforts are directed to develop non-invasive methodologies that may complement bone marrow-based MRD testing. These include highly sensitive mass spectrometry assays for monoclonal proteins in serum, circulating tumor cells, circulating tumor DNA, and novel imaging modalities. First-in-human studies have shown highly promising results from targeted PET-CT, using radiolabeled monoclonal antibody (anti-CD38) to increase sensitivity and specificity over conventional FDG-based PET-CT.[89,90]

Staging, Prognosis, and Risk Stratification

MM is a heterogeneous disease with a varied outcome, with 20% of patients having a survival of less than 2 years, whereas over 40% of patients have more than a 10–year survival. Therefore, it is critically important to identify disease features that allow one to identify low versus high-risk disease to counsel the patient, and in some cases to better plan and sequence therapeutic interventions. Moreover, accurate risk stratification defines precise patient populations by which clinical trial results can be appropriately evaluated. Given these considerations and needs, various prognostic variables have been identified,[91] including measurement of disease burden related factors (such as β_2-microglobulin, LDH, number or nature of lytic bone lesions, hypercalcemia), tumor biology–related factors (such as genomic changes,[92] plasma cell labeling index[93]), tumor microenvironment–related factors, patient-related factors, such as age, renal failure, albumin, and performance status, and treatment-related factors, such as achieving a CR, or MRD negative status.

The history of risk stratification (i.e., staging) and prognostication for MM began with systems which assessed the extent of end-organ damage, then moved to systems which predominantly assessed disease burden. However, in an era of improved understanding of underlying disease pathogenesis and improving novel therapies which may be relatively agnostic to (high) disease burden, MRD detection is increasingly moving to systems which attempt to capture disease biology in its many facets. Of note, among cancers the staging of MM and related disorders is relatively unique in being entirely unrelated to anatomic considerations, in distinction to staging systems for solid tumors and indeed even for most lymphomas.

TABLE 91.6 **Standard Risk Factors for Multiple Myeloma and the Revised International Staging System**

Prognostic Factor	Criteria
International Staging System (ISS) Stage	
• I	Serum β_2-microglobulin <3.5 mg/L, serum albumin ≥ 3.5 g/dL
• II	Not ISS stage I or III
• III	Serum β_2-microglobulin ≥ 5.5 mg/L
Chromosomal Abnormalities (CA) by Interphase Fluorescence in Situ Hybridization	
• High risk	Presence of del(17p) and/or translocation t(4;14) and/or translocation t(14;16)
• Standard risk	No high-risk CA
LDH	
• Normal	Serum LDH below the upper limit of normal
• High	Serum LDH above the upper limit of normal
A New Model for Risk Stratification for Multiple Myeloma Revised International Staging System Stage	
• I	ISS stage I and standard-risk CA by iFISH and normal LDH
• II	Not R-ISS stage I or III
• III	ISS stage III and either high-risk CA by iFISH or high LDH

CA, Chromosomal abnormalities; *iFISH*, interphase fluorescence in situ hybridization; *ISS*, International Staging System; *LDH*, lactate dehydrogenase; *MM*, multiple myeloma; *R-ISS*, revised International Staging System.
From Greipp PR, San Miguel J, Durie BG, et al. International staging system for multiple myeloma. *J Clin Oncol.* 2005;23(15):3412.

The Durie-Salmon staging system[94] was published in 1975 on the basis of only 71 patients that were assessed for severity of anemia, presence of hypercalcemia, number/level of skeletal lesions, the size of the M-protein in blood and urine, and presence or absence of renal injury. The subsequent ISS was published in 2005 and was a simple system which assessed the overall patient by means of serum albumin levels, and also crudely measured disease burden as defined by serum β_2-microglobulin levels.[95] With the advent of novel therapies, the discriminating power of the ISS had decreased over time, and currently the most commonly used system for clinical trials and most practitioners is the R-ISS (Table 91.6, Fig. 91.11),[96] which adds to the ISS by beginning to account for the biology of MM. The LDH is a crude measure of the proliferation rate and turnover of malignant cells, and the R-ISS also adds a handful of the more highly validated cytogenetic/FISH changes which reflect the biology of each patient's MM in order to make staging more accurate. R-ISS staging is able to predict both event-free survival (EFS) and OS and is valid regardless of age, geographic region, study site, use of standard-dose therapy or high-dose melphalan (HDM), and use of novel agents. Also of note, although presumably such tests reflect disease biology as described above in the Pathogenesis section, GEP-based scoring systems have also been described by a number of groups. Although useful, these remain investigational and have not entered routine clinical practice.

In addition, newer genomic correlates of outcome have been validated in a number of studies and are considered standard predictive markers. Continuing efforts are focused on further refining risk stratification, with improved understanding of MM pathogenesis and genomic changes translating to meaningful risk stratification tools. For example, emerging data suggest that biallelic *TP53* gene loss[92] or more than three copies of chromosome 1q are associated with poor outcomes,[97] and investigators have combined recurrent molecular adverse features with the ISS and proposed an ISS mutation score to identify high-risk patients.[92]

Finally, it has become increasingly evident that disease sensitivity to therapy, which likely reflects the underlying biology of a given patient's neoplastic PCs, is critical for predicting a patient's prognosis. As discussed in the Minimal Residual Disease section, achievement of very low disease levels (MRD negativity) at the conclusion of therapy is predictive of improved outcomes such as PFS and likely even OS. Although chronic myeloid leukemia (CML) management currently leads the way with respect to the evidence relating the speed

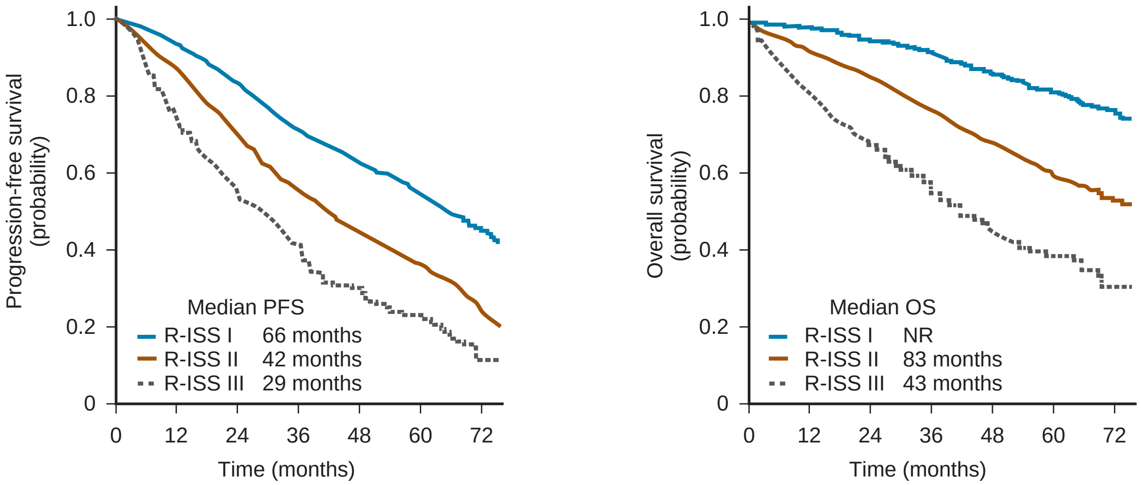


Figure 91.11 Revised International Staging System *(R-ISS)* is able to predict both event-free survival *(left panel)* and overall survival *(OS; right panel)*. *NR*, Not reached; *PFS*, progression-free survival.

and depth of response achieved following treatment to patient outcomes, a number of other hematologic neoplasms, including acute lymphoblastic leukemia (ALL), acute promyelocytic leukemia (APL), and even other types of AML and chronic lymphocytic leukemia (CLL) are also moving in this direction. We speculate that MM therapy and monitoring may follow an analogous path, with both the speed and depth of response being shown to be prognostically important for building the risk profile of a given patient's disease. Indeed, the ADVANCE trial as well as other future clinical trials in MM are incorporating MRD-directed endpoints.

GENOMIC BIOMARKERS

Genomic alterations identified in MM cells before treatment can improve prognostic risk stratification over the use of clinical features alone. Historically, cytogenetic analysis and fluorescence in-situ hybridization to identify *IGH* translocations and large chromosomal aneuploidies have been the standard approach. This was supplemented in some centers with array-based methods capable of identifying genome-wide copy number changes with high accuracy. Recently, NGS has emerged as a new standard of care technology capable of identifying relevant genomic alterations with a single assay. Today, the most relevant technological platforms for routine clinical use are targeted hybridization-capture sequencing panels and low-coverage WGS. Both of these strategies can replace older methods like FISH, while providing additional information about SNVs, small insertions/deletions, SVs, and CNAs, depending on the specific method used.

Currently the main genomic biomarkers used to predict adverse prognosis are the following: *IGH* translocations involving *MMSET* (t4;14), *MAF* (t14;16), or *MAFB* (t14;20); bi-allelic inactivation of *TP53* (i.e., deletion and mutation); and gain of chromosome 1q21 (three or more copies). Genomic information has been incorporated in the revised R-ISS prognostic score, which is simple and commonly used. There is however a great need for prognostic scores which incorporate the wide array of genomic alterations that can now be identified using increasingly accessible NGS assays.

Transcriptome-based biomarkers are appealing because they have the potential to assess the functional state of the tumor. Several prognostic risk scoring systems based on GEP have been proposed, but none have been widely adopted.[21–23] This is partially due to challenges with reproducibility, but also the greater availability of cytogenetic analyses. More recently, RNA sequencing-based models have been developed to predict patient benefit from different therapies including IMIDs, Pis, and ASCT.[98,99] The role of RNA-based biomarkers in the routine care of patients with MM remains to be determined.

DIFFERENTIAL DIAGNOSIS

The diagnosis of MM does not often present a dilemma and is based largely on laboratory and imaging investigations together with the clinical presentation. However, no single symptom or group of symptoms is pathognomonic, including even the highly suggestive triad of anemia, hypercalcemia, and lytic lesions. For example, though less prevalent than MM, adult T-cell leukemia/lymphoma (ATLL), a mature T-cell neoplasm, can present with a similar clinical picture (see Chapter 89),[100] as can certain aggressive mast cell neoplasms (see Chapter 75).[101,102] However, tissue biopsy easily resolves any confusion, as differentiating T- or mast-cell neoplasms versus plasma cells is usually pathologically straightforward. On the other hand, pathologic entities related to MM which can share certain (mmune)histologic features include plasmablastic lymphoma[103,104] and lymphoplasmacytic lymphoma.[105] Fortunately, these entities can generally be clinically distinguished from MM, for example with a history of HIV infection or splenomegaly, respectively.

With respect to the diagnosis of MM, a significant number of patients present with minimal symptoms, delaying diagnosis. Most frequently, in a relatively asymptomatic patient, investigations are carried out because of increased total protein levels, proteinuria, renal dysfunction, and/or bone pain. An older patient with any of these features or unexplained back pain, anemia, or recurrent infections should be evaluated for MM. In general, a diagnostic workup is pursued in two steps: first, the demonstration of a monoclonal protein and monoclonal plasma cells, and second, identification of end-organ damage or biomarker criteria that define MM. This is essential to differentiate early-stage plasma cell disorders such as MGUS or SMM (see Chapter 90) from active symptomatic MM (see Table 91.3). Once a diagnosis of MM is suspected, investigations should be carried out as detailed in the box on Investigations for Patients Suspected or Confirmed to Have Newly Diagnosed Multiple Myeloma. Detection of a monoclonal protein requires the performance of a protein electrophoresis and immunofixation as well as a 24-hour urine collection and serum-free light chain measurements. Both serum protein electrophoresis and quantitative immunoglobulins are required, and an immunofixation is important to identify the type of paraprotein present. The bone marrow is examined for the detection of clonal plasma cells, mainly by histology but also importantly by immunostaining or flow cytometry using κ/λ staining and by demonstrating aberrant cell surface molecules (see Table 91.2). Determination and quantitation of clonal marrow plasma cell numbers are required to differentiate SMM from MGUS, from active myeloma; patients with greater than 10% and less than 60% marrow plasma cells are considered to have SMM, with MGUS and active myeloma respectively bookending plasma cell burdens below and above this range. Detailed investigations searching for evidence of organ damage should be undertaken to look for bony lesions, renal dysfunction, anemia, and hypercalcemia. It should again be reiterated that in addition to bloodwork, bone marrow aspirate and biopsy to establish a clonal neoplastic PC disorder, as well as cross-sectional imaging with total body MRI (without contrast) and/or PET/CT are mandatory for the initial diagnostic evaluation of myeloma. Detailed diagnostic criteria are summarized in Table 91.3.

INDICATIONS FOR TREATMENT

Therapy for MM is warranted when an organ function is compromised or will become imminently compromised, and this forms the crux of the IMWG 2014 diagnostic criteria for MM. To that end, the acronym *CRAB* (hypercalcemia, renal impairment, anemia, and bone disease) defines the major manifestations of end-organ damage. Additionally, symptomatic hyperviscosity, amyloidosis, monoclonal immunoglobulin deposition disease, recurrent bacterial infections, and progressive peripheral neuropathy attributed to a plasma cell disorder can also be indications for treatment. Additionally, the IMWG has defined the following events, which strongly predict early progression to end-organ damage, thereby warranting systemic therapy: (1) clonal bone marrow plasma cell percentage (aspirate, biopsy, or flow cytometry whichever is higher) of 60% or above, (2) elevated serum free light chain ratio (involved/uninvolved) of 100 or higher, with the associated light chain at least 100 mg/L, and (3) more than one characteristic focal lesion, in the bone or bone marrow, visualized by MRI.[106] We emphasize that MRI lesions may be bone *marrow*-based and not lytic lesions per se. Of course, one or more osteolytic lesions using essentially any imaging modality also represents symptomatic bone disease and justifies the initiation of therapy. Of note however, the presence of clonal plasma cells coupled *solely* with isolated osteoporosis, in the absence of meeting other criteria for MM defining events, do not secure the diagnosis of MM, and cannot be used to justify beginning treatment.

Response Criteria

The most recent IMWG response criteria[71] from 2016 define both "standard" criteria as well as MRD-based criteria. In brief, serial monitoring and comparison of the size of the M-protein in blood and/or urine, free light chains, and size of plasmacytomas allows for numeric calculations from a reference value, typically the most recent peak value. Although the actual criteria are highly detailed to account for unusual entities such as pure free light chain myeloma and true nonsecretary myelomas, we highlight below the most commonly used aspects of these criteria, reflecting increasing depths of response.

A "minimal response" (MR) is defined as 25% to 49% decrease in size of the serum M-spike, and a 50% to 89% reduction in urine M-spike if measured. Additionally, the size of any measurable (soft tissue) plasmacytomas should have decreased by at least 50%. A "partial response" (PR) is defined as 50 to 89% decrease in size of the serum M-spike, and a 90% or greater response in urine M-spike. If only free light chains are present, a 50% reduction in the difference between involved vs. uninvolved light chains is required. A "very good partial response" (VGPR) is defined as greater than 90% decrease in serum and urine M-spike, with urine M-protein <100 mg/day. A "complete response" (CR) is defined by absence of an M-spike in the serum and urine, negative immunofixation studies, total radiographic resolution of any previously identified plasmacytomas, and <5% plasma cells in bone marrow sampling. A "stringent complete response" (sCR) adds the absence of marrow PC clonality by immunohistochemistry as well as a normal free light chain ratio to the criteria for the achievement of CR.

On the other hand, "progressive disease" (PD) would be defined by a 25% increase (from the nadir) of the serum M-spike (absolute increase >0.5 g/dL), urine M-spike (absolute increase > 200 mg/day), a 25% increase in the difference between involved and uninvolved free light chains (absolute increase >10 mg/dL), growth of new anatomic lesions, or >50% increase in the size of an existing lesion or plasmacytoma. "Stable disease" (SD) in an MM patient is defined as a clinical condition which fulfills none of the above criteria for PD, MR, PR, VGPR, CR, or sCR.

Lastly, the MRD-based response criteria presuppose levels of response at least equivalent to sCR including absence of clonal PCs by immunohistochemistry, and then further assesses for clonal plasma cells by flow cytometry, NGS, or other approaches to declare MRD positivity or negativity. As discussed above in the Minimal Residual Disease section, the details of MRD response criteria including the ideal cutoff (10^{-5} or 10^{-6}), as well as related considerations for the significance of MRD positivity vs. negativity remain areas of active investigation.

Solitary Plasmacytoma: Medullary and Extramedullary

Solitary plasmacytomas are rather uncommon and account for 6% of plasma cell neoplasms. The incidence is 0.3 per 100,000 person-years in the United States. A diagnosis of a solitary plasmacytoma requires meeting all of the following criteria:[106] histologic confirmation of clonal plasma cells at a single anatomic site; a negative bone marrow with absence of clonal plasmacytosis; no distant bone involvement; and no anemia, hypercalcemia, or renal impairment (see Table 91.3). In particular, note that presence of two plasmacytomas, or more than 10% (clonal) bone marrow PCs in association with a single plasmacytoma meet the diagnostic criteria for MM. Solitary plasmacytomas may present as a single bony lesion (medullary) or as a soft tissue lesion outside the bone (extramedullary), with solitary plasmacytomas involving the bone being more common.

Patients presenting with solitary plasmacytomas require a complete workup to confirm the diagnosis and to thoroughly exclude MM, as management differs drastically for patients with a solitary plasmacytoma (locoregional therapy) vs. MM (systemic therapy). Given this, patients should undergo serum protein electrophoresis, serum immunofixation, serum free light chain assay, UPEP, urine immunofixation, a diagnostic bone marrow aspiration and biopsy with sensitive flow cytometry analysis to detect clonal plasma cells, and detailed skeletal imaging that should include PET/CT and/or total body MRI. In our opinion, because of insufficient sensitivity, a negative plain film skeletal series alone is not sufficient to rule out MM in resource-abundant practice settings.

Presumably because of the burden of plasma cells, one-third of patients with solitary plasmacytoma may present with a detectable monoclonal paraprotein in the serum, urine, or both. However, persistence of the monoclonal paraprotein after definitive locoregional therapy is predictive of recurrence, as it reflects the presence of viable neoplastic PCs which continue to secrete the paraprotein. Of note, patients with clonal but less than 10% plasma cells in a bone marrow biopsy specimen[107] (termed "solitary plasmacytoma with minimal marrow involvement") may be managed with locoregional therapies directed against the solitary lesion initially. However, these patients by definition have clonal neoplastic cells outside the field of treatment, and have a higher risk of progression than those without any marrow involvement. Indeed many of these patients do progress to MM over subsequent years of follow-up.

Solitary plasmacytomas are generally treated with local radiation therapy, although some solitary plasmacytomas of the bone may require surgical intervention for stabilization followed by local radiation therapy. Depending upon their location, small extramedullary soft tissue plasmacytomas may be treated with excisional biopsy alone. Because of the risk of recurrence or progression to MM, after definitive locoregional therapy, at a minimum yearly imaging studies for at least 5 years is recommended. Serial appropriate blood work and urine studies including studies for paraproteins (generally every 3 to 6 months) are warranted. The disease-free survival at 10 years is approximately 50% for patients with solitary plasmacytomas. For those patients who are not cured, progression to MM generally occurs within 5 years from initial diagnosis. Patients presenting with medullary plasmacytomas, with persistence of a monoclonal paraprotein after treatment for the solitary plasmacytoma, those with detectable low levels of clonal plasma cells in the bone marrow, patients between 40 and 60 years of age, and patients of African American descent are at higher risk for progression to MM. Such patients should be considered for potentially even closer surveillance for disease progression.

Plasma Cell Leukemia

PCL[108] is a rare and highly aggressive malignant plasma cell disorder, currently defined by the IMWG as greater than 0.5 to 2×10^9 circulating plasma cells per liter, or greater than 5% to 20% of nucleated cells being PCs in the peripheral blood.[109] The precise numeric threshold to be used to establish the diagnosis varies by investigator and has not been prospectively validated, but most agree that satisfying one of the two criteria mentioned above are sufficient.[110] Signs and symptoms are similar to those of classical MM, although primary PCL patients are generally younger than their MM counterparts, manifest high disease burdens, have hepatosplenomegaly, and more frequently display other extramedullary signs and symptoms than patients with MM.

There are no pathognomonic gene mutations or cytogenetic abnormalities of PCL, yet the t(11;14) translocation is common in this disease entity, with up to half of patients having this abnormality, as compared to 15% to 20% of MM patients. Why this translocation, which is generally viewed as standard-risk or even favorable in MM, is present more often in PCL patients is unclear. Since the BCL-2 inhibitor venetoclax has shown efficacy in t(11;14) MM and in mantle cell lymphoma, there is significant interest presently in studying its use in PCL.

Most primary PCL patients present with a high tumor burden[109] corresponding to R-ISS 3, and this entity should be managed in a similar fashion as extremely high-risk newly diagnosed MM patients as described in detail below; urgent and aggressive combination therapy is warranted, employing the most potent regimens appropriate for a given patient. For transplant-eligible patients, high-dose therapy is generally warranted. All patients, regardless of transplant eligibility, should receive maintenance therapy, as relapses are unfortunately common with PCL. Secondary PCL is a rare complication of treated MM and occurs in under 5% of patients. Its incidence may be increasing as the OS of MM patient's increases. It is generally a late manifestation of multiply relapsed/refractory MM and treated as such. It often carries a poor prognosis.

Overview of Therapy for Multiple Myeloma

Patients with diagnosis of MM should be treated with systemic medical therapy. To warrant the diagnosis of MM, patients generally present with symptoms caused by the myeloma tumor mass, such as anemia, lytic bone disease, hypercalcemia, or renal impairment (the "CRAB" criteria). Other MM-defining events that warrant systemic therapy include a clonal bone marrow plasma cell percentage as a percentage of nucleated cells (on aspirate, biopsy, or flow cytometry, whichever is higher) of 60% or above, elevated serum free light chain levels of at least 100 mg/L associated with an involved/uninvolved free light chain ratio of 100 or higher, and/or more than one focal lesion, each at least 5 mm in diameter on MRI. The presence of any one of these three "biomarker criteria" portend a high risk of early progression to frank end-organ damage as defined by the CRAB criteria and therefore such patients are also classified as having active MM. In addition, patients with a low tumor burden per se but with organ dysfunction caused by a paraprotein or immunodeficiency such as monoclonal immunoglobulin deposition disease or AL amyloidosis of an organ, progressive peripheral neuropathy or recurrent serious infections may also require systemic therapy (see box on Rare Disorders Associated With Monoclonal Proteins and/or Plasma Cells).[111]

Front-line therapy for MM as well as entry onto clinical trials has traditionally been determined by whether the patient is sufficiently medically robust to be able to proceed with HDM chemotherapy followed by ASCT, the so called "transplant eligibility." This longstanding paradigm, however, may be changing in the era of increasingly potent upfront therapies and the gradual adoption of MRD testing and MRD-driven decision-making.[112] Transplant eligibility in MM[113,114] is largely a clinical decision based on organ function, comorbidities, patient age, and social support, although formal scoring systems exist (see Chapter 157). The treatment given before HDM chemotherapy and ASCT, if performed, is termed induction therapy. The term consolidation can be confusing: it traditionally referred to HDM and ASCT itself, although various investigators also use the term consolidation to refer to additional cycles of combination therapy after HDM, or induction therapy in transplant ineligible patients. Regardless of whether patients undergo consolidation or high-dose chemotherapy, in the United States all patients treated by expert myeloma clinicians receive subsequent, generally lower-intensity maintenance therapy until progression, with the goal of deepening treatment responses, prolonging time to the next active treatment, and improving OS.

TREATMENT OF NEWLY DIAGNOSED MYELOMA

During the past two decades, tremendous advances have been made in the treatment of MM patients, with concomitant dramatic improvements in OS,[115] driven largely by the availability of the so-called novel agents belonging to a number of drug classes (Fig. 91.12). Therapeutic advances have resulted in an overall response rate (ORR) of essentially 100% and increasingly high rates of CR and MRD negativity. Modern advances in induction regimens are focused on increasing the CR rate and MRD negative rate. These modern regimens have largely supplanted older regimens centered around traditional cytotoxic chemotherapeutic agents. Although not all the drugs mentioned below are FDA-approved or United States National Comprehensive Cancer Center (NCCN) compendium-listed for induction therapy per se, the commonly used modern induction agents include (1) the IMiDs thalidomide, lenalidomide, and pomalidomide; (2) the PIs bortezomib, ixazomib, and carfilzomib; (3) the anti-SLAMF7 monoclonal antibody elotuzumab; and (4) the anti-CD38 monoclonal antibodies daratumumab and isatuximab. These drug classes, together with steroids, traditionally either dexamethasone or prednisone (MP), are generally combined, in some cases with the addition of older alkylating drugs, to form the backbone of most modern induction regimens. Such regimens typically include three, four, or even more drugs in combination (see box on Treatment of Newly Diagnosed Multiple Myeloma).

Improvements in regimens with non-overlapping mechanisms of action, and non-overlapping toxicities, as reviewed below, have now advanced to the point where the most modern of therapies[116] achieve an ORR of essentially 100%, and even a VGPR rate of nearly 100%, with high rates of MRD negativity at the end of induction therapy.[117] Because of these successes, the rapid achievement of deep responses, and achieving CRs and MRD-negative status is becoming

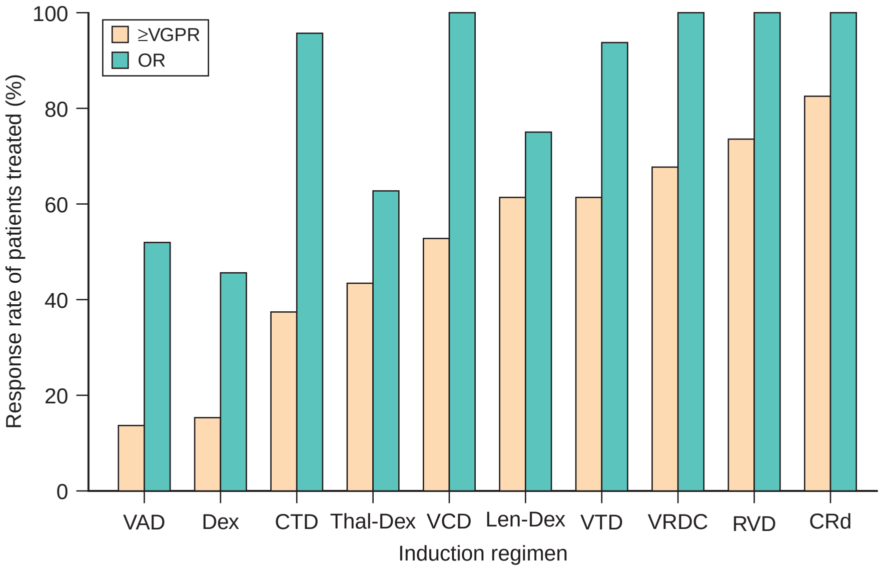


Figure 91.12 RESPONSE RATES IN NEWLY DIAGNOSED MYELOMA PATIENTS WITH COMMONLY USED INDUCTION REGIMENS. *CRd*, Carfilzomib, lenalidomide and dexamethasone; *CTD*, cyclophosphamide, thalidomide and dexamethasone; *Dex*, dexamethasone; *Len–Dex*, lenalidomide and dexamethasone; *MM*, multiple myeloma; *OR*, overall response; *RVD*, revlimid, bortezomib and dexamethasone; *Thal–Dex*, thalidomide and dexamethasone; *VAD*, vincristine, doxorubicin and dexamethasone; *VCD*, bortezomib, cyclophosphamide and dexamethasone; *VGPR*, very good partial response; *VRDC*, bortezomib, lenalidomide, dexamethasone and cyclophosphamide; *VTD*, bortezomib, thalidomide and dexamethasone.

TREATMENT OF NEWLY DIAGNOSED MULTIPLE MYELOMA

- Confirm patient has symptomatic myeloma by CRAB criteria, or greater than 60% marrow plasma cells, or serum involved/uninvolved free light chain ratio greater than 100 with the involved free light chain greater than 10 mg/dL, or two or more lesions, each at least 5 mm in diameter, on total body magnetic resonance imaging (MRI). Patients with monoclonal gammopathy of unknown significance or smoldering multiple myeloma should not be treated outside a clinical trial.
- Risk stratify and assess disease burden using the Revised International Staging System, quantitative studies of paraproteinemia, lactate dehydrogenase and β_2 microglobulin, bone marrow plasma cell burden, genomic profiling, and flow cytometric features. These are helpful for prognostication and to develop a long-term treatment plan. Although initial treatment is generally not affected by risk category per se, consideration of more aggressive consolidation approaches in eligible patients could be warranted.
- Induction: most transplant-eligible and transplant-ineligible patients can be treated with a three or four-drug regimen, typically composed of a steroid (prednisone or dexamethasone), an immunomodulatory drug, and a proteasome inhibitor, with addition or substitution of an alkylating agent (cyclophosphamide, melphalan) and/or monoclonal antibody.
- Selection of therapy should be influenced by patient characteristics.
- Renal dysfunction: safe agents include daratumumab, bortezomib, carfilzomib, cyclophosphamide, thalidomide, and steroids.
- Neuropathy: agents that can be considered are daratumumab, lenalidomide, carfilzomib cyclophosphamide, and steroids.
- Elderly or frail patients: consider dose or schedule attenuation or use of fewer agents. Daratumumab-based regimens are well-tolerated in general.
- Convenience: use of an all-oral versus intravenous agent regimen.
- Assess treatment responses after each cycle of induction therapy and treat to best response (generally up to 8 cycles with modern therapies). For transplant-eligible patients, stem cell collection is generally performed no later than cycle 6 of induction due to cumulative stem cell toxicity from both novel agents and conventional cytotoxic drugs. For patients who achieve serologic very good partial response (VGPR) or better and have no clinical evidence of disease at the end of induction, assess minimal residual disease status (typically by repeat bone marrow biopsy).
- Patients not achieving VGPR, or minimal residual disease (MRD) positive patients, can undergo additional cycles of induction therapy to achieve MRD negativity. Eligible patients whose best response to treatment is still with disease present should be considered for a single autologous transplant with high-dose melphalan. A second autologous transplant (so-called “tandem transplant”) or autologous transplant followed by allogeneic transplant (“auto-allo”) should not be performed outside the confines of a clinical trial.
- Maintenance: following transplant or optimal induction therapy (to best response), all patients should receive maintenance therapy, most commonly with an oral immunomodulatory agent and less commonly with proteasome inhibitor, or anti-CD38 antibody. For high-risk patients or suboptimal responders including those not achieving MRD negativity after high-dose melphalan and autologous transplant, combination maintenance therapy can be considered. Maintenance delays disease progression and time to next treatment and can further improve the depth of response.
- Achieving an MRD negative status is associated with excellent patient outcomes. Conversion to MRD negativity can occur in the setting of induction, autologous transplant, or maintenance.

a benchmark for the evaluation of further improvements in therapy. As described above, MRD negative status predicts a better PFS and is associated with improved patient outcomes.

In general, treatment should be tailored to both patient characteristics (comorbidities such as pre-existing diabetes or hyperglycemia, organ dysfunction, performance status, venous thromboembolism risk, preferences with respect to toxicity) and disease characteristics, primarily with respect to the clinician’s assessment of a particular malignancy’s biologic aggressiveness, as reflected by disease presentation, genomic abnormalities, cell surface markers, and disease burden (as measured by paraprotein levels, number and size of lesions on imaging, bone marrow PC percentage, etc.). For the practicing clinician, these considerations are further influenced by region-specific access to medications, healthcare and insurance coverage of drugs, patient logistical considerations (e.g., the ability to make frequent clinic visits for subcutaneous injections or IV infusions), and patient preferences with respect to specific toxicities, such as neuropathy.

Chemotherapy with Cytotoxic Agents

In the United States and a number of other countries, combinations of novel agents, including the IMiDs, PIs, and oftentimes monoclonal antibodies now form the standard-of-care backbone for the induction therapy of MM patients. However, because of challenges due to cost and access to novel agents in some regions as well as unique toxicities which may occur with some novel agents, traditional cytotoxic agents still play a role in the treatment of patients with both newly diagnosed and relapsed disease, as well as during high-dose therapy during consolidation.

Alkylating agents, primarily cyclophosphamide and melphalan, have been most frequently used as cytotoxic therapy for MM. This has been followed by the anthracyclines (primarily doxorubicin) and a handful of other cytotoxic drugs such as platinum chemotherapy, the vinca alkaloids, and topoisomerase II inhibitors. The majority of these other classes of agents are used in modern practice only as part of a multi-day infusional combination cytotoxic chemotherapy regimens and are given primarily in the salvage setting.

Melphalan and cyclophosphamide were introduced in the management of MM in the early 1960s. To this day, they continue to play a pivotal role in treatment of MM. Melphalan + MP and cyclophosphamide + dexamethasone (CyD) are flexible and well-tolerated regimens which can be given orally or intravenously, and remain useful both in low resource environments, and to treat patients too frail for, or with contraindications to novel agents or biologics. Melphalan remains the agent of choice for high-dose therapy with stem cell rescue, and both melphalan and cyclophosphamide also continue to be used in combination with novel agents and other cytotoxic drugs, both during induction and the management of relapsed disease (summarized in Table 91.7).

Although primarily historical of interest, clinicians seeing heavily pretreated patients may continue to encounter patients with longstanding MM who were previously treated with VAD (vincristine, Adriamycin [doxorubicin], and dexamethasone), a common regimen used widely prior to the widespread adoption of novel agents. In some less frequently used regimens, the anthracycline doxorubicin or liposomal doxorubicin are also combined with novel agents. Finally, infusional chemotherapy using combinations of cytotoxic agents in the form of PACE or DCEP, with or without additional novel agents, has also been used previously in the newly diagnosed setting, most prominently by investigators at the University of Arkansas in a series of high-intensity regimens centered around cytotoxic chemotherapy under the rubric of “Total Therapy.”[118–120] In modern practice, such regimens are largely reserved for relapsed patients due to their dramatically higher toxicity and requirement for hospitalization for several days to administer these therapies as well as the intensive subsequent outpatient follow-up necessary to monitor for and ameliorate resulting toxicities. As an ancillary note, although studies in lymphoma have successfully replaced cisplatin with carboplatin or oxaliplatin in many (salvage) regimens to reduce nephrotoxicity, we are not aware of similar data in myeloma. When a platinum drug is required, cisplatin appropriately dosed for renal function remains the standard of care. Additionally, in some infusional cytotoxic chemotherapy regimens, it may be possible to replace standard doxorubicin with its liposomal formulation (Doxil) in order to minimize the risk of cardiotoxicity.

TABLE 91.7 Selected Regimens Utilizing Cytotoxic Agents

Regimen Abbreviation	Agents
MP	Melphalan, prednisone
CyD	Cyclophosphamide, dexamethasone
CTD	Cyclophosphamide, thalidomide, dexamethasone
CyPR	Cyclophosphamide, prednisone, Revlimid (lenalidomide)
CYKLONE	Cyclophosphamide, Kyprolis (carfilzomib), thalidomide, dexamethasone
KRDC	Kyprolis (carfilzomib), Revlimid (lenalidomide), dexamethasone, cyclophosphamide
MPR	Melphalan, prednisone, Revlimid (lenalidomide)
MPT	Melphalan, prednisone, thalidomide
RVDC	Revlimid (lenalidomide), Velcade (bortezomib), dexamethasone, cyclophosphamide
VAD	Vincristine, Adriamycin (doxorubicin), dexamethasone
CVAD	Cyclophosphamide, vincristine, Adriamycin (doxorubicin), dexamethasone
TAD	Thalidomide, Adriamycin (doxorubicin), dexamethasone
TVAD	Thalidomide, vincristine, Adriamycin (doxorubicin), dexamethasone
CyBorD	Cyclophosphamide, bortezomib, dexamethasone
VMP	Velcade (bortezomib), melphalan, prednisone
VMPT	Velcade (bortezomib), melphalan, prednisone, thalidomide
PACE	Platinol (cisplatin), Adriamycin (doxorubicin), cyclophosphamide, etoposide
DCEP	Dexamethasone, cyclophosphamide, etoposide, cisplatin
BBD	Bendamustine, bortezomib, dexamethasone

Combinations of Three, Four, or More Classes of Novel Agents

It has long been established that it is possible to combine drugs with different mechanisms of action with non-overlapping toxicities without compromising the ability to deliver an effective dose in order to maximize anti-myeloma effects, eliminate potentially resistant clones, deepen the depth of response, and prolong remission duration. Optimizing the precise combination drug, schedules, and dosing of a steroid, IMiD, PI, monoclonal antibody, and in some cases alkylating agent for each population of newly diagnosed MM patient continues to be a challenge. Since the toxicities and route of drug elimination characteristic of commonly used agents differ sometimes even within the same drug class, it is useful to consider patients as belonging to several distinct subpopulations with respect to the feasible drugs that can be administered to each group.

- *Renal failure:* Since such patients are ineligible for lenalidomide or pomalidomide therapy, regimens containing alkylating agents, thalidomide, PIs, and monoclonal antibodies are reasonable options. This includes several combination therapies: CyBorD, IRd, VTD, daratumumab-VTD, carfilzomib, cyclophosphamide, dexamethasone (KCyD), and the CYKLONE regimen. The regimen of daratumumab + Cyclophosphamide, bortezomib, and dexamethasone (CyBorD) is now NCCN compendium-listed and is also a suitable choice. Older regimens such as MP or cyclophosphamide + dexamethasone may also be appropriate if there is difficulty in gaining access to novel agents.
- *Severe heart failure with reduced ejection fraction, concomitant amyloid cardiomyopathy, or related entities:* Such patients should likely avoid carfilzomib-based induction regimens, instead receiving regimens with bortezomib as the PI (e.g., RVd, daratumumab/lenalidomide/dex, daratumumab-VTD). Daratumumab can be used in this patient population as long as the fluid status of the patient is carefully monitored. Liposomal doxorubicin-based combination therapy may be considered in special circumstances.
- *Pre-existing neuropathy or high concerns for neuropathy:* In this situation one should avoid bortezomib and thalidomide. By contrast the next-generation PI carfilzomib carries essentially no neuropathy risk. If bortezomib is used, a subcutaneous administration is favored over intravenous[121] in order to decrease neurotoxicity. Given this, regimens such as KRd and DRd are associated with less neuropathy. Clinical trial regimens are being evaluated which add daratumumab to an appropriate triplet of IMiD + PI + steroid.
- *Otherwise frail and/or transplant-ineligible, but without severe cardiac or renal disease:* consider DRd, RVd-lite, or Rd
- *Medically fit, and without significant organ dysfunction:* KRd is an NCCN compendium-listed regimen in the United States and covered by most insurers, yielding excellent responses for patients who can tolerate this regimen. Although initially studied with carfilzomib as twice-weekly infusions on back-to-back days, in modern schedules once-weekly carfilzomib is equally acceptable. In rare instances with very aggressive and symptomatic disease in otherwise fit and younger patients, combinations of infusional chemotherapy with novel agents such as VTD-PACE or KRd-PACE can be considered. Again, clinical trials adding daratumumab to an IMiD/PI/steroid backbone are attractive both for advancing the field and expected patient outcomes.

Selected Trials of Induction Therapy With Novel Agents

Although the large number of high-quality trials using these agents are impossible to summarize comprehensively, below are highlighted a number of seminal studies which have moved the MM treatment field forward. The phase 3 SWOG 0777[122] trial compared the doublet of lenalidomide and dexamethasone versus the triplet of lenalidomide, bortezomib, and dexamethasone (RVd) in transplant-eligible patients, and demonstrated superior PFS and OS with acceptable toxicities with a PI and IMiD containing triplet over doublet. Next, the EVOLUTION trial compared RVd vs. a triplet of bortezomib, cyclophosphamide, and dexamethasone (CyBorD or VCd) or all four agents together (VCRd). Deeper responses were reported in regimens which contained lenalidomide vs. those that did not, with RVd and VCRd performing the best, demonstrating again the importance of IMiDs as a component of induction therapy. Finally, a study by Moreau and colleagues comparing subcutaneous vs. intravenous bortezomib administration, albeit in relapsed/refractory disease, demonstrated equal efficacy but markedly less neuropathy with subcutaneous bortezomib.[121] This route of administration is standard of care in essentially all settings where bortezomib is given. Also of note, the oral proteasome inhibitor ixazomib has similar pharmacologic properties as bortezomib. Ixazomib together with lenalidomide and dexamethasone (IRd[123,124]) is a regimen with pharmacologic properties highly similar to RVd, and represents a convenient all-oral NCCN compendium–listed regimen for both transplant-eligible and ineligible patients who have difficulty with frequent medical appointments.

Most modern three-drug induction regimens do not include an alkylating agent, and investigations to find the superior IMiD and PI combination are ongoing. To that end, multiple phase II studies have demonstrated superior performance and a favorable toxicity profile for a triplet based on the next-generation PI carfilzomib[125–128] in combination with lenalidomide and dexamethasone (KRd). KRd therapy is associated with the rapid achievement of deep responses, down to the level of MRD negativity prior to the delivery of consolidation therapy, if utilized. In addition, a phase III trial exploring the need for high-dose melphalan consolidation therapy in the context of KRd or KCyD induction (the FORTE trial, NCT02203643) reported outstanding results with respect to MRD negativity rates, with or without consolidation therapy.[129] KRd is a NCCN compendium-listed regimen for upfront treatment in the United States and is a preferred regimen. Of note, although it has been compared to RVd in the phase III ENDURANCE trial[130] and found to result in comparable outcomes, the design of this trial and the large number of patients in both arms opting for "alternative therapy" (not defined) likely prevented superior outcomes from being demonstrated with KRd. In addition, the KRd regimen also differs from RVd with respect to a significantly decreased neuropathy risk, although adverse cardiovascular events can be more common with the use of KRd. For patients who will receive this regimen, a baseline EKG and echocardiogram as well as cardiac biomarkers including troponin, BNP, and/or NT-proBNP are warranted. Furthermore, in otherwise-fit patients with renal injury, carfilzomib-based platforms can also be utilized: the CYKLONE[131] regimen (cyclophosphamide, carfilzomib, thalidomide and dexamethasone) achieves deep responses, with excellent PFS and OS and minimal neuropathy, and the regimen of KCyD is also appropriate in selected instances.

Patients with newly diagnosed MM and renal injury represent a challenging and arguably underserved patient population. Since lenalidomide is renally cleared, its use is not feasible in most newly diagnosed patients who have significant kidney injury. CyBorD, also known as VCd, has been used for many years in this patient population. Akin to the EVOLUTION trial, the IFM 2013–04 study[132] asked whether a triplet containing an alkylating agent or an IMiD was superior, and compared bortezomib, thalidomide, and dexamethasone (VTD) to VCd. This trial showed superiority for VTD with respect to ORR and VGPR rates; VCd caused more hematologic toxicity while VTD caused significantly more neuropathy, as thalidomide carries significant neuropathy risk along with bortezomib. Both regimens are acceptable for induction therapy, as are the KCyD and the CYKLONE regimens described above.

Recently investigators have added the anti-CD38 monoclonal antibody daratumumab to existing induction regimens. Daratumumab (Dara) is a human anti-CD38 IgGκ monoclonal antibody that was initially FDA-approved in 2015 for myeloma patients who have had more than three relapses. Daratumumab binds CD38-expressing malignant plasma cells with high affinity and induces tumor cell death through diverse mechanisms of action, which include complement-dependent cytotoxicity; antibody-dependent, cell-mediated cytotoxicity; antibody-dependent cellular phagocytosis; and induction of apoptosis. Moreover, daratumumab suppresses Tregs and the actor enzyme activity-driven suppression of T cells via the adenosine signaling pathway. The phase III ALCYONE trial[133] studied a bortezomib, melphalan, and prednisone (VMP) regimen (which is safe in patients with renal impairment), with or without daratumumab in transplant-ineligible patients. This trial showed superior PFS and OS with the quadruplet regimen of daratumumab + VMP over VMP alone. Of note, also in transplant-ineligible patients, the MAIA trial[134] studied the doublet of lenalidomide and dexamethasone, with or without daratumumab (Rd, vs. DRd). This study showed higher CR rates, higher MRD negativity rates, and higher PFS rates for the triplet containing daratumumab, and this regimen is also now FDA-approved. Also of note is the ongoing LYRA study[135] for both transplant-eligible and -ineligible patients, which is evaluating the regimen of daratumumab with CyBorD (daratumumab + VCd) as induction with daratumumab maintenance for one year. Currently, the choice of DRd vs. daratumumab + VMP for transplant-ineligible patients is largely based on the patient's renal function, as well as access to medications and the drug toxicity profile.

More recently, the phase III CASSIOPEIA trial[75] has explored whether adding daratumumab to VTD would further improve outcomes in transplant-eligible patients, and showed that daratumumab + VTD therapy led to increased depths of response and PFS and was associated with a good safety profile. This regimen is now FDA-approved as the first four-drug induction therapy combining monoclonal antibody with IMiD, PI, and steroid, and is additionally also suitable for patients with renal insufficiency.

A number of groups are exploring the combination of daratumumab with even newer IMiD or PI platforms. To this end, the GRIFFIN trial[136] is studying RVd with or without daratumumab. The latest update as of this writing showed superior response rates and depth of response, including MRD negativity rates, for the daratumumab-containing quadruplet therapy arm. Even more exciting, an induction regimen, combining once-weekly KRd with daratumumab,[117] showed not only rapid responses, but also a VGPR rate greater than 97%, with nearly 80% of patients achieving an MRD negative state *without* consolidation high-dose therapy. Indeed, the MASTER trial,[137] which also studied daratumumab + KRd and a number of related regimens with the addition of HDM/ASCT consolidation, reported comparable MRD negativity rates, without obvious benefit from the HDM consolidation. These outstanding results have prompted the initiation of the multi-institutional ADVANCE trial (NCT04268498) to further investigate the cutting-edge quadruplet regimen of daratumumab + KRd. Finally, in May 2020 the FDA approved subcutaneous daratumumab (Darzalex Faspro) in MM based on results of the COLUMBA noninferiority trial; subcutaneous daratumumab is currently approved in combination with VMP, Rd, Vd, and as monotherapy. As it is priced comparably to intravenous daratumumab, the markedly improved convenience of drug administration and additionally improved safety of subcutaneous daratumumab is anticipated to further accelerate the use of this agent.

Because of daratumumab's excellent safety and toxicity profile and non-overlapping mechanism of action with that of other novel agents, the field is gradually shifting toward quadruplet induction therapy, adding daratumumab to the triplet of PI + IMiD + steroid, since these regimens are safe, and lead to rapid and deep responses, including MRD-negative CRs for newly diagnosed patients. As MRD negativity is an important proxy for a variety of important patient outcomes, we anticipate that the profound improvements in induction will also impact the use of HDM/ASCT as consolidation therapy in the future, as discussed below.

Indeed, with these modern regimens, properly diagnosed treated newly diagnosed MM has essentially a 100% ORR and an increasingly high CR and MRD negativity rate as well. True de novo primary refractory or primary progressive MM patients are extremely unusual even in high-volume myeloma centers, and such presentations warrant repeat pathologic confirmation of the underlying diagnosis. Rare newly diagnosed MM patients who are truly unresponsive to induction therapy have a poor prognosis, and the reader is referred to the section on Relapsed Disease for consideration of further treatment options.

Practical Considerations of Induction Therapy

Although definitive anti-myeloma therapy as described above should begin promptly, some patients may require "prephasing" or temporizing maneuvers. These maneuvers include pulses of steroids for hypercalcemia, pain, and other complications of disease for rapid palliation while the patient's diagnostic evaluation is completed and or emergent locoregional maneuvers, such as surgery or radiation, are delivered. While not ideal, the administration of high-dose steroids even prior to bone marrow sampling may be required in medical emergencies, which may include cord compression or related neurologic conditions, severe hypercalcemia, or fulminant renal failure.

In addition, for patients who will receive skeletal prophylaxis and will be exposed to anti-resorptive agents with induction therapy (see "skeletal protection"), dental "clearance" is useful. We refer patients to our dentistry colleagues with the request that major dental work (extractions, implants, etc.) which may increase the risk of osteonecrosis of the jaw and/or infectious complications be completed prior to initiation of anti-resorptive therapy if possible, and prior to initiation of systemic medical therapy for MM. This approach not only minimizes the risk of osteonecrosis of the jaw but may also reduce the risk of infectious complications following dental interventions during induction therapy when the patient is immunosuppressed. Indeed, while it is beneficial to start skeletal prophylaxis promptly, it is by no means mandatory to start this adjunctive therapy concomitantly with systemic medical therapy.

Finally, although tumor lysis syndrome is not commonly observed with the systemic therapy of myeloma, it can be useful to check a baseline serum uric acid level and glucose 6 phosphate dehydrogenase level (for administering rasburicase, if required) if the clinician judges the patient to have a high disease burden. In the authors' clinical practice, however, we do not routinely prescribe prophylactic allopurinol.

With respect to monitoring disease response, assessing toxicity, and treatment planning, it is common practice to obtain a complete blood count, comprehensive metabolic panel, magnesium, phosphorus, uric acid, LDH, β_2 microglobulin, serum protein electrophoresis (SPEP), serum immunofixation electrophoresis (SIFE), serum free light chains and ratio, and quantitative serum IgG/IgM/IgA such that the results are available on day one of each new therapy cycle which allows these results to be reviewed with the patient. The vast majority of patients can be assessed during therapy with spot urine studies for protein/creatinine ratio rather than repeated 24-hour collections, although an initial 24-hour urine collection for the protein/creatinine ratio, urine protein electrophoresis (UPEP), and immunofixation electrophoresis (UIFE), as well as analysis of urine light chains (if present) can be useful to establish a baseline. Finally, although the number of cycles of induction therapy varies across clinical trials and practitioners during routine practice, 3 to 8 cycles are commonly used in many MM trials. In general it is recommended to administer sufficient numbers of cycles of induction therapy to achieve the best response rather than identifying a defined number of cycles that are to be administered.

For transplant-eligible patients, early referral to a transplant center for counseling and planning is especially helpful. Most practitioners will plan for stem cell mobilization and collection by cycle 4 to 6 of induction therapy and/or after achieving at least VGPR. Importantly, continued lenalidomide exposure beyond 4 to 6 cycles of induction therapy may make peripheral blood stem cell collection more difficult. As a general rule, responding patients who plateau (e.g., at PR, VGPR) with measurable disease in the blood can be referred for HDM if required and eligible. There is no true upper limit on number of cycles that should be administered during induction therapy save for cumulative toxicities (generally decreased bone marrow reserve, cytopenias and neuropathy from a number of agents). In most cases, however, the patient is unlikely to obtain additional incremental benefit past 10 to 12 cycles of induction therapy with a given regimen.

For those patients who do achieve CR in the blood and urine, stem cell grafts for transplant-eligible patients should be collected. At this juncture, a repeat bone marrow examination with the addition of high-sensitivity MRD studies is warranted, if available. Such patients will typically have either very low levels of morphologically identifiable disease, or are in morphologic CR and are either MRD-positive or negative using the sensitive techniques described above. In the authors' practice, patients who have low but measurable amounts of morphologic disease in the marrow, and/or those who are MRD positive, can be offered 2 additional cycles of the same induction therapy to attempt to "clear" the marrow in order to achieve an MRD-negative status. Patients who fail to clear their marrow of disease and who are eligible may be considered for HDM/ASCT.

Although this is a practice which is being re-evaluated, most patients who achieve MRD negativity following induction therapy and who are transplant-eligible are offered the opportunity to collect and bank peripheral blood stem cells for possible early HDM/ASCT (see discussion below). Finally, with the current four-drug regimens, there are reports of very early MRD negative responses, as early as the end of cycle one or two of induction therapy. In these cases, there may still be measurable monoclonal proteins in the blood due to the long half-life of complete immunoglobulin molecules. Therefore, particularly when using highly effective induction regimens, MRD testing may be warranted in patients who have not reached serologic CR (i.e., are in VGPR). There is no clear guidance on how many induction cycles of therapy are truly needed for such patients, but we speculate that it may be possible to model therapy after PET-directed approaches for the management of classical Hodgkin lymphoma, where treatment with a defined number of cycles past demonstration of CR has been successfully used in a number of trials, with excellent outcomes.

Lastly, with respect to repeat imaging studies, we note that repeat cross-sectional imaging at the end of (successful) induction therapy, absent the development of new symptoms, is rarely necessary or useful and can be confusing, since not all lesions resolve even with highly successful therapy. In our practice, we repeat imaging typically with disease progression and/or the development of new or unexplained symptoms.

Skeletal Protection

Aminobisphosphonates, pamidronate, zoledronate, and more recently denosumab have all been extensively investigated in patients with MM and bone disease and have been shown to reduce skeletal complications and bone pain. Denosumab is a human monoclonal antibody that inhibits this maturation of osteoclasts by binding to and inhibiting RANKL. Researchers in a number of older randomized studies assessed their efficacy in patients with existing bone lesions and evaluated the development of new SREs. In this regard, pamidronate 90 mg and zoledronic acid 4 mg were equipotent in reducing bone-related complications.[138–140] In a large study, Medical Research Council (MRC) IX, investigators also evaluated the efficacy of zoledronic acid in a randomized comparison with clodronate and demonstrated the superiority of zoledronic acid in preventing new SREs in patients both with and without existing bone lesions. This trial also provided evidence that its continued use beyond 2 years was beneficial[141]; importantly, the study also suggested that zoledronic acid compared with clodronate, a first-generation bisphosphonate, improves OS, although it must be noted that the survival advantage was demonstrated with the use of older, less potent MM therapies. Pamidronate administration is also beneficial and has also been shown to produce responses or delays in disease progression in occasional patients. Whether a survival benefit for anti-resorptive bone agents remains in the modern era of potent therapies with combinations of novel agents is unknown.

Despite these benefits, two major toxicities of bisphosphonates have limited their use: renal impairment and osteonecrosis of the jaw. Osteonecrosis of the jaw is associated with dental infections for those having procedures following long-term use as well as the use of higher doses of anti-resorptive drugs that are delivered to MM patients. Patients with poor nutritional status, poor baseline dentition, and immunodeficiency may be at a higher risk. The frequency of these adverse events are between 3% and 5%. Given this, patients receiving bisphosphonates or denosumab require a thorough dental checkup before starting therapy with completion of all major dental work if possible (extractions, implants, etc.) and should have frequent dental follow-up with careful and conservative use of dental procedures. With these precautions, the frequency of osteonecrosis of the jaw has been reduced.

Similarly, the measurement of renal function before each bisphosphonate dose and the adjustment of the dose based on renal function also make use of bisphosphonates safer. For patients with renal insufficiency from any cause, denosumab is likely the safer agent. Finally, in the authors' clinical practice, a meaningful fraction of patients encounter significant and sometimes intolerable constitutional side effects associated with IV bisphosphonate therapy, including myalgias, arthralgias, fatigue, bone pain, and flu-like symptoms. A trial of both zoledronate and pamidronate may be warranted. However, patients who are intolerant of either may be candidates for denosumab. Indeed, in a large recent phase III trial,[142] denosumab was non-inferior to zoledronate for time to skeletal-related event in MM patients. Denosumab still carries the potential to cause osteonecrosis of the jaw but is associated with less renal toxicity and is much better tolerated in patients. With use of either class of anti-resorptive drugs, initial serial monitoring of serum calcium, phosphorus, and ionized calcium levels may be prudent, as hypocalcemia has been reported following the initial administration of anti-resorptive agents.

High-Dose Melphalan Therapy and Consolidation

In the era before the introduction of novel agents, and routine use of maintenance therapy, induction regimens, such as high dose dexamethasone, melphalan-prednisone, or VAD chemotherapy, rarely achieved CRs. In such a scenario, HDM/ASCT played a critical role in increasing CR and VGPR rates and prolonging the durability of unmaintained responses. The availability of novel agents and the use of maintenance therapy have dramatically changed this paradigm, with markedly improved rates of VGPR, CR, and even the MRD negative states at the end of induction therapy.

Because of the ever-shifting changes in induction regimens and use of maintenance therapy, many have questioned if modern regimens using novel agents can replace HDM and ASCT in the treatment of MM patients. This is an important question that has yet to be answered with a randomized trial, although critical trial results to date are reviewed below. Leading investigators are already asking whether patients who are MRD negative at the end of induction can defer HDM and proceed directly to maintenance, although most still advocate for stem cell collection and banking, thereby keeping HDM/ASCT available as an option as a later line of therapy. Nonetheless, at most centers in the United States HDM/ASCT is currently considered the standard-of-care "consolidation" for medically eligible patients after induction therapy. It is worth noting that although many efforts have been made to add agents to HDM to improve outcomes, convincing data at present showing that melphalan plus another agent is better than HDM alone is not available, and such regimens should not be used outside the context of a clinical trial.

Despite current practice by the overall MM treatment community, which offers HDM to essentially all eligible patients at the end of induction therapy, its role is changing in the modern era with increasing potent novel agents and high-sensitivity MRD monitoring, as well as greater awareness of both the acute and chronic toxicities associated with HDM therapy. The immediate down sides of HDM include both the cost and inconvenience of a relatively long inpatient hospital stay, mucositis and infection risk, risk of acute organ toxicities, as well as an arguably low but not-zero transplant-related mortality (TRM) rate.[143,144] Chronic effects include long-standing cytopenias, immunodeficiency, and pulmonary fibrosis among others, but most prominent are secondary malignancies,[145] particularly myeloid neoplasms[146,147] but also solid tumors. This secondary malignancy risk is compounded with the use of lenalidomide maintenance[148] and has gained even greater importance as a drawback of HDM therapy, as the OS of MM patients continues to improve.

The reader should note that although previously in fashion for certain high-risk patient populations, double or "tandem" autologous transplantation,[149] which represented prior interest in high-intensity induction therapy with a focus on cytotoxic agents, is no longer routinely practiced in the United States, due to the even greater toxicity associated with this approach, as well as improved response rates and depths of CR being achieved with induction regimens using the newer agents obviating the need for multiple rounds of HDM. Similarly, *allogeneic* stem cell transplantation alone, or after ASCT (the so-called "auto-allo"), carries a markedly higher rate of TRM than autologous transplantation, and is *not* the standard of care after induction therapy for newly diagnosed patients, essentially regardless of the depth of response. In the relapsed/refractory setting, highly selected patients may be considered for allogeneic stem cell transplantation,[150,151] if performed at a high-volume stem cell transplant center by specialized clinicians with expertise in allogeneic transplant for myeloma, ideally in the context of a clinical trial.

With respect to HDM and autologous transplant, the use of peripheral blood stem/progenitor cells mobilized with chemotherapy, granulocyte colony-stimulating factor (G-CSF), and/or plerixafor is the standard-of-care in MM, and bone marrow stem cells are rarely if ever used in the modern era. Surprisingly, multiple studies agree that the degree of tumor cell contamination in the collected stem cell product has no influence on transplant outcomes, and consistent with this, ex vivo manipulations to eliminate tumor cells within the graft have never demonstrated improvement in the depth of response, PFS or OS. Despite these observations, most practitioners will collect stem cells at or near the achievement of best antitumor response to induction therapy to minimize tumor cell contamination and the theoretical risks associated with re-introduction of neoplastic PCs. Because of potential difficulties with stem cell mobilization with cumulative use of alkylating agent exposure and lenalidomide e should be limited to insure an adequate stem cell harvests and complete hematopoietic recovery post-transplant. As a practical matter, achievement of VGPR or better response, and/or approximately 6 cycles of induction therapy are practical points at which stem cell collection can be performed, with additional cycles of induction therapy added after stem cell collection if appropriate.

Specific Trials of High-Dose Melphalan With Autologous Stem Cell Transplant

Because of the early discovery of melphalan's anti-myeloma activity and its evident dose-response relationship, HDM with ASCT has been studied extensively as consolidation therapy in newly diagnosed patients. The literature is confounded by the use of disparate and now oftentimes outdated induction regimens, inconsistent post-transplant maintenance therapy, and oftentimes durations of maintenance therapy that are now considered too short, and until very recently, lack of MRD assessments as part of the decision-making process and disease monitoring. Given this, we highlight only a few randomized clinical trials in the era of modern triplet therapy of IMiD, PI, and steroid.

The EMN02/HO95 trial[152] was a large multicenter randomized phase III study that used a proteasome (but not IMiD)-based backbone induction regimen, with randomization to ASCT (single or tandem) or VMP intensification, and an additional randomization to either RVD consolidation or no consolidation. This trial demonstrated PFS but no OS benefit for HDM/ASCT, but was limited by the use of relatively weak induction regimens.

The IFM/DFCI 2009 trial[82] randomized patients either to 8 cycles of RVd without high-dose therapy, as compared to 3 cycles of RVd, followed by HDM/ASCT, followed by 2 additional cycles of RVd post-transplant (for a total of 5 cycles). Both groups received lenalidomide maintenance, but its duration was limited to one year. This trial showed that for newly diagnosed transplant-eligible patients, HDM/ASCT provided a PFS but not an OS benefit, but interpretation of even these results is limited due to the short duration of lenalidomide maintenance (current practice is to continue maintenance therapy until disease progression). Additionally, this trial revealed that for MRD-negative patients at the beginning of maintenance therapy, PFS was excellent regardless of whether patients received HDM therapy; conversely, MRD-positive patients, including those who received ASCT, had inferior outcomes. What drives the difference in overall PFS is the *percentage* of patients achieving MRD-negative status at the beginning of maintenance therapy. The IFM/DFCI 2009 trial showed that in induction therapy with RVd, arguably already now a somewhat outdated regimen, HDM may help to improve the MRD negativity rates. Also of note, the ongoing phase III DETERMINATION trial has an identical study design except for administering lenalidomide maintenance therapy to progression; results are pending but as of early 2020 this trial has yet to meet its primary endpoint, which is to demonstrate a PFS or OS advantage for HDM/ASCT. This suggests that in addition to MRD status, prolonged maintenance therapy with lenalidomide, which is known to convert some MRD-positive patients to negativity, may abrogate the benefit of HDM/ASCT.

The second major randomized phase III trial of transplant with modern regimens is the FORTE trial.[129,153] This three-armed study used the potent PI carfilzomib, comparing 12 cycles of KRd without transplant ("KRd12"), versus 4 cycles of KRd plus HDM/ASCT and 4 additional cycles (total of 8), versus 4 cycles of KCyD plus HDM/ASCT and 4 additional cycles (total of 8). Patients were further randomized to maintenance therapy with carfilzomib monotherapy, or the doublet of carfilzomib + lenalidomide. When comparing the two most interesting arms, KRd12 versus KRd with HDM/ASCT, rates of MRD negativity were similar but PFS and OS data are still pending.

Lastly, the BMT CTN 0702 (STAMINA) trial[154] randomized patients to single ASCT tandem ASCT, or single ASCT followed by RVD × 4 cycles as consolidation, with all three arms proceeding with lenalidomide maintenance. This trial revealed single ASCT followed by lenalidomide maintenance to be non-inferior to the other two higher-intensity approaches with respect to PFS or OS.

In summary, the lack of benefit for HDM/ASCT as compared to the most potent commonly used current induction regimens (KRd), the advent of four-drug induction regimens which are expected to be even more potent than KRd induction, as well as evidence based on IFM/DFCI 2009 that MRD negativity during maintenance therapy is a strong predictor of favorable patient outcomes, and conversely the worrisome acute and chronic toxicities of HDM/ASCT, has given clinicians the impetus to pause and re-evaluate the current paradigm of offering HDM/ASCT as consolidation to all eligible patients. In the authors' practice, only patients who are morphologically positive or MRD positive at the end of induction therapy are encouraged to consider HDM/ASCT. For MRD-negative patients, it is reasonable to have an informed discussion with the patient and stem cell transplant colleagues, review existing data, and make an individualized decision as whether to proceed to HDM/ASCT.

Maintenance

Maintenance therapy is the use of ongoing lower-intensity therapy, most traditionally with a single agent, to eliminate or suppress remaining PC disease over a prolonged period. Previously, the standard of care for MM patients in first remission was observation with disease monitoring. However, a handful of practice-changing trials[155–158] published around 2012 and thereafter demonstrated clear benefits of oral lenalidomide maintenance therapy in both transplant-eligible and -ineligible MM patients, with all trials showing a PFS benefit and one trial showing an OS benefit.[156] Subsequent meta-analyses also demonstrated an OS benefit in multiple patient populations and treatment scenarios.[159–161]

Therefore, where available, maintenance therapy, with lenalidomide as the default drug, should be offered to patients, regardless of whether they received consolidation therapy and/or HDM/ASCT.[161] In the post-transplant setting, maintenance therapy is generally started after three months or on day 100, whereas patients not receiving HDM/ASCT can proceed directly to maintenance therapy. Ideally, maintenance should be initiated when the disease is in remission, at either undetectable or comparatively low levels, and in this clinical scenario, maintenance therapy can further deepen responses. For example, conversion of MRD positive to negative status can be observed, and this supports the notion that an additional antitumor response during the maintenance phase can be beneficial. Such observations call into question whether HDM/ASCT is in fact mandatory for patients who have a low disease burden at the end of induction therapy.

As a historical note, previous maintenance agents have included interferon alfa-2b and thalidomide, although these two drugs are no longer routinely used and will not be discussed further. Indeed, although many agents have been studied, data is strongest with lenalidomide as a maintenance agent (and lenalidomide is NCCN category 1 for both transplant-eligible and ineligible patients), with bortezomib probably being the next most-commonly used.

Immunomodulatory molecules are well suited for maintenance therapy because they can be administered orally at low doses for a prolonged period, with most patients tolerating such therapy well without undue side effects or cumulative toxicities. There have been three large randomized trials exploring the role of lenalidomide as maintenance therapy. Two of these studies were maintenance therapy after HDM/ASCT (CALGB100104[156,162] and IFM2005–02[157]), and the third study was in elderly patients receiving MP. All three studies showed improvement in PFS by 18 months; only the CALGB100104 randomized study showed improvement in OS. Of note, while there is general consensus as to the positive benefits of lenalidomide maintenance, its use after HDM/ASCT has been associated with higher rates of secondary malignancies, both solid tumors and hematologic, but most prominently MDS and AML. The relative risks and benefits of lenalidomide maintenance are worth discussing with patients who have received HDM/ASCT. This increase in secondary malignancies is *not* meaningfully observed with lenalidomide maintenance in patients not exposed to HDM/ASCT, whether due to medical ineligibility or preference.

Bortezomib has also been used in the maintenance setting. Generally, bortezomib maintenance has been restricted to clinical trials in which bortezomib was also used during the induction phase of the treatment. In the phase III HOVON-65/GMMG-HD4 trial, bortezomib maintenance seemingly improved the outcome in patients with high-risk karyotypic abnormalities.[163] As compared with induction regimens, bortezomib is usually given less frequently in the maintenance setting; different studies have used varying schedules of administration. In clinical practice, those patients who are intolerant of even low-dose lenalidomide, frequently due to constitutional side effects, may be considered for bortezomib maintenance. However, use of this agent is less convenient as it requires subcutaneous drug administration in the clinic. Based on the TOURMALINE-MM3 trial,[164] ixazomib oral therapy has also been used by some practitioners for patients on bortezomib maintenance who have irregular access to subcutaneous injections. The TOURMALINE-MM3 trial demonstrated a PFS but not OS benefit to the use of ixazomib maintenance therapy. Lastly, as a newer-generation PI with greater efficacy and lack of cumulative neuropathy, carfilzomib maintenance, either as monotherapy or combination therapy, is also highly attractive. In a phase II trial,[165] transplant-ineligible patients were induced with carfilzomib, cyclophosphamide, and dexamethasone (CCyD) with carfilzomib maintenance. This regimen showed excellent two-year PFS and OS rates. Another phase II trial by Zimmerman and colleagues[166] studied combination therapy in transplant-eligible patients, with KRd induction, HDM/ASCT, KRd consolidation, and then 10 cycles of KRd maintenance, for a total of up to 18 cycles of KRd. At the conclusion of this admittedly intensive regimen, an impressive 74% to 97% of patients were assessed to be MRD negative.

Finally, as the FDA has now approved induction regimens which include daratumumab, a number of investigators have naturally considered continuing this agent as first-line maintenance therapy, particularly for patients who are ineligible for or decline HDM/ASCT. Data confirming the efficacy of daratumumab monotherapy, albeit in the relapsed/refractory setting, come from the SIRIUS and GEN501 trials.[167,168] Daratumumab is an attractive maintenance agent with minimal toxicity, and a relatively convenient schedule being administered every 28 days. Although previously given solely by intravenous infusion, subcutaneous daratumumab administration was investigated in the COLUMBA trial,[169] and was recently approved by the FDA. This markedly more convenient route of administration is also associated with lower rates of reactions, further enhancing the feasibility of long-term daratumumab administration. As one of the newest agents to be used in this fashion, prospective data on outcomes with daratumumab maintenance after first-line therapy remain limited at present, although the phase 2 LYRA study[135] is administering daratumumab maintenance to both transplant-eligible and -ineligible patients after induction, with preliminary results showing deepening of responses throughout the maintenance phase. Finally, although single-agent maintenance or doublet maintenance (with steroid) is commonly used, investigators have also studied multi-agent combination therapy in the maintenance setting. Investigators at Emory have studied RVd as a combination consolidation/maintenance regimen[170] in high-risk transplant-eligible patients after HDM/ASCT. For this patient population, this combination regimen achieved high rates of VGPR and good results for PFS and OS.

Having considered all of the above, most patients at present should be treated with single-agent maintenance in the United States, with lenalidomide the default choice. Bortezomib, carfilzomib, daratumumab, or other agents can be used but generally on an individualized basis. Multi-agent combination therapy in the maintenance setting should likely be reserved for the highest-risk patients. Finally, although many patients will stop maintenance therapy due to biochemical or symptomatic relapse of MM, a fraction of patients, most on lenalidomide maintenance, remain MRD negative for a prolonged period of time, often many years. The optimal duration of maintenance therapy in such patients is unknown, and therapeutic discontinuation with close monitoring, built on the model of tyrosine kinase inhibitor discontinuation in chronic myeloid leukemia with highly sensitive disease monitoring, is now an emerging area of investigator interest for this fortunate patient population.

RELAPSED DISEASE

Managing Relapsed Multiple Myeloma

Despite the significant improvements in PFS/OS outcomes for MM with the introduction of PIs and IMiDs, the vast majority of patients experience relapse during their disease course.[171,172] Relapse is defined as the reappearance of signs and symptoms of the disease or signs of increasing disease and/or end-organ dysfunction that are related to the underlying MM.[173] When patients present with overt clinical symptoms, the relapse is referred to as *clinical* relapse whereas if the relapse is determined by increase in MM protein studies in the absence of new symptoms, then it is termed a *biochemical* relapse. Upon disease relapse, there are a number of factors to be weighed before selecting therapies (Table 91.8).

Disease-Related Factors

The most important question to ask is when to start new therapy in a relapsing MM patient. The current approach for newly diagnosed MM is to provide continuous therapy in the form of maintenance for both transplant-eligible and -ineligible patients.[160,174] Since maintenance therapy continues until relapse or the development of unacceptable toxicities in most cases, patients may relapse either on therapy or during a drug holiday—this will influence the choice of regimen and the timing to initiate the next line of treatment. The duration of maintenance therapy and/or time off maintenance therapy, as well as the duration of time since completion of the initial induction therapy and/or HDM/ASCT should also be considered when making this decision. Regardless of whether the relapse occurs on or off therapy, the timing of starting therapy can also pose a challenge: if the relapse is associated with clinical features (e.g., symptomatic anemia, new bone pain, expanding or new plasmacytomas, hyperviscosity, etc.), immediately starting the next line of treatment is clearly indicated. Conversely, for a biochemical relapse, the pace of relapse is important and the IMWG has defined criteria indicating the need for treatment initiation[71]: the doubling of monoclonal protein characterized by an increase in the absolute levels of monoclonal protein of greater than 1 g/dL in serum or of greater than 500 mg per 24 hours in urine, or involved free light chain level by more than or equal to 20 mg/dL (plus an abnormal FLC ratio) in two consecutive measurements separated by ≤2 months. Keeping these criteria in mind, the decision to start a patient with a biochemical relapse on therapy should be individualized, and made based on a risk-benefit analysis keeping in mind the patient-related and therapy-related factors discussed below. If, however, a patient is rapidly progressing and has symptomatic disease, the therapy should match disease intensity. High-risk karyotypic abnormalities, EMD, circulating plasma cells, high R-ISS stage at relapse, poor response to earlier therapies, rapid progression while on maintenance or after earlier lines of therapy, and related factors may all justify the use of aggressive therapies for rapid disease control regardless of whether the relapse is clinical or biochemical.

As a general rule, in the typical patient the majority of relapse regimens can be initiated at the full doses and continued until progression or intolerance occur. However, treatment holidays or dose reductions for toxicity are not uncommon for many patients, particularly in later lines of therapy, and practitioners may commonly need to modify regimens via dose reductions, temporary treatment holidays, or occasionally deviating from prescribed dosing schedules, thereby customizing a given regimen for each patient. This can maximize the benefits of a relapse regimen while minimizing toxicities, and help the patient to obtain the maximum potential benefit from each drug or drug combination before the next line of treatment is needed.

Finally, on occasion a symptomatic plasmacytoma in the setting of relapsed disease may require a short course of radiation therapy or in rare cases, surgical intervention; however, it is important to remember that the goal of local therapy in this setting is palliation of local symptoms only and should ideally not unduly delay systemic therapy.

TABLE 91.8 Factors to Consider in Selecting Therapy for Patients With Relapsed Myeloma

Patient-Related	Disease-Related	Therapy-Related
• Age • Performance status • Renal insufficiency • Poor marrow reserve • Neuropathy • Frailty and comorbidities • Social support for clinic and infusion visits	• Cytogenetics: presence of high-risk features such as del (17p), t(4;14), t(14;16) • Nature of relapse • Clinical vs. biochemical • Presence and extent of end organ damage • Extramedullary disease • Plasma cell leukemia	• Previous therapy • Depth of response • Duration of response • Route of administration (IV, SC, PO) • Cost • Residual toxicity from previous therapies • Myelosuppression • Neuropathy • Thrombosis • Risk of second primary malignancies

IV, Intravenous; *PO*, per oral; *SC*, subcutaneous.

Patient-Related Factors

The individual patient's performance status and comorbidities can play a critical role in determining optimal therapy (see Chapters 157 and 158). Although age can be a factor determining tolerance to therapy, frailty appears to be more important in predicting survival in MM.[175] Indeed, patient-related factors play a very important in the decision-making for optimal therapy for both newly diagnosed and relapsed MM. In either setting, the goal of therapy is to achieve a deep and durable response, and to alleviate or prevent sequelae of the disease. The clinician also must bear in mind patient preferences, socioeconomic considerations, and logistics in choosing between oral and parenteral therapies, as well as dosing schedule. For example, a patient who has a longer commute to the clinic to receive parenteral therapies may elect to pursue an all-oral regimen when efficacy and safety are comparable. Similarly, medication co-pays and insurance considerations may influence some patients' choice of therapy.

Treatment-Related Factors

There are several issues related to prior therapies received that need to be taken into consideration when approaching a relapsed and/or refractory MM patient. First, it is important to decide whether a patient's MM is refractory to a certain agent or class of drugs, and whether they can be retreated with that same agent or drug class. Refractory disease is defined as MM that is nonresponsive while on therapy or progresses within 60 days of last therapy as per IMWG criteria.[71,173] However, it is also important to consider the dosing and schedule of the agents or drug classes used at the time. For example, one patient progressing on standard RVd induction dose and schedule may be different than another progressing on either dose-attenuated lenalidomide and/or bortezomib maintenance. For example, whether a patient who has progressed on standard RVd with lenalidomide at 25 mg daily, versus another patient progressing on lower doses of a drug (e.g., lenalidomide 10 mg daily as monotherapy, arguably the most common maintenance regimen) should both be considered "lenalidomide refractory" needs to be considered on a case-by-case basis. Some practitioners may choose to rechallenge a patient progressing on attenuated doses of a drug with the full dose of that same agent, oftentimes in combination, before concluding that the disease is truly refractory.

In general, if the patient has previously responded well to an agent or drug class without major toxicities, one might consider retreatment with the same agent or another in the same drug class. Response rates with re-treatment, however, are generally lower, and the duration of response is commonly decreased. For example, in a phase II study of bortezomib with or without dexamethasone[176] retreatment for patients relapsing ≥ 6 months after prior bortezomib-based therapy, the ORR was 40% and median time to progression (TTP) merely 8.4 months. In addition, retreatment may be a suboptimal approach in a patient with aggressive disease where other novel effective therapies with differing mechanisms-of-action are available. Given this, a lack of response or a short duration of response to a particular agent or class of drugs in the past would generally lead to the use of non-cross-resistant agents or drug classes. Next, toxicities to previous drug regimens should be considered when deciding future therapies. For example, someone who had severe GI side effects or neuropathy (especially persistent symptoms after drug discontinuation) would not be an ideal candidate for a bortezomib-based or thalidomide-based salvage regimen. In the case of overlapping toxicities, it is helpful to ascertain where possible if the toxicity was due to multiple agents or one particular agent that can be avoided in the future. The depth and duration of response following a previous HDM/ASCT is also important when considering a salvage transplant (discussed below).[177]

Clinical Trials in Relapsed Multiple Myeloma

Several large phase II and III randomized clinical trials have been conducted in patients with relapsed myeloma. These studies have resulted in the approval of several new regimens for the treatment of relapsed myeloma since 2012, including eight new drugs (carfilzomib, daratumumab, isatuximab, ixazomib, panobinostat, pomalidomide, Selinexor, belantamab mafodotin) for relapsed MM patients with one or more prior lines of therapies (Tables 91.9 and 91.10). There are nuances in the patient populations that enrolled on each of the trials and the tables summarize the clinical trial patient populations to better inform the optimal treatment choice for a given patient.

Carfilzomib is a selective and irreversible PI which is administered intravenously. In a large, open-label, single-arm phase II study,[178] 266 patients with relapsed myeloma progressing on their prior therapy were treated with carfilzomib at 20 mg/m^2 during cycle 1 and thereafter at 27 mg/m^2 intravenously twice weekly for three of four weeks. Patients were refractory or intolerant to both bortezomib and lenalidomide. The ORR was 23.7% with a median duration of response of 7.8 months and a median OS of 15.6 months. Common adverse events included fatigue, anemia, and thrombocytopenia. A small proportion of patients (<5%) experienced grade 3 or 4 dyspnea, acute renal failure, and heart failure.

In the ENDEAVOR trial,[167,179] dose-intense carfilzomib/dexamethasone (Kd) was more efficacious than bortezomib/dexamethasone (Vd) with improvement in the ORR (77% vs. 63%) as well as doubling of CR rate (13% vs. 6%) and median PFS (18.7 months vs. 9.4 months). Because the median follow-up time was short (1 year), there was no survival advantage noted. Anemia, dyspnea, and cardiac failure were the most common grade 3/4 adverse events in the Kd group, whereas diarrhea and peripheral neuropathy were more common in the Vd arm.

ASPIRE was a large, randomized phase III trial[180] comparing carfilzomib, lenalidomide, and dexamethasone (KRd; 396 patients) with lenalidomide and dexamethasone (Rd; 396 patients) in patients with relapsed MM who had received one to three prior treatments. PFS was significantly improved in the KRd arm compared with the Rd arm (26.3 months vs. 17.6 months; hazard ratio for progression or death, 0.69; $P = .0001$). The KRd arm had an ORR of 87.1% compared with 66.7% in the Rd arm ($P < .001$). There was an increased incidence of grade 3/4 adverse events such as hypokalemia, thrombocytopenia, hypertension, and cardiac failure in the KRd arm. The median OS was not reached in either group at the time of the interim analysis, however after 67.1-month follow-up[181] the median OS was 48.3 months (95% CI, 42.4 to 52.8 months) for KRd versus 40.4 months (95% CI, 33.6 to 44.4 months) for Rd (hazard ratio, 0.79; 95% CI, 0.67 to 0.95; one-sided $P = .0045$).

PANORAMA-1[182] was a large, multicenter, international, randomized, placebo-controlled, double-blind phase III trial for patients with relapsed MM who had received between one and three prior regimens. Patients were randomly assigned to receive panobinostat + Vd (387 patients) or placebo + Vd (381 patients). The median PFS was 12 months for the panobinostat group and 8 months for the control group. The median follow-up was 6 months, and there was no difference in the OS at the time of publication of the results. There was an increased incidence of grade 3/4 adverse events such as

TABLE 91.9 **Lenalidomide- and Pomalidomide-Based Regimens for Relapsed Multiple Myeloma**

Regimen	% Len Exposed	% PI Ref	% PI Exposed	% With High-Risk Cytogenetics	ORR (%) vs SOC arm	PFS (months) vs SOC arm	OS vs SOC arm
KRd	19.8	No	66 vs 66	12 vs 13	87 vs 67	26.3 vs 17.6 ($P = .0001$)	73% vs 65% at 24 months
IRd	12	No	69 vs 69	17 vs 21	78 vs 72	20.6 vs 14.7 ($P = .012$)	--
EloRd	6	22	68 vs 71	41 vs 42	79 vs 66	19.4 vs 14.9 ($P = .014$)	43.7 months vs 39.6 months ($P = .026$)
Dara-Rd	18	21	84 vs 84	17 vs 25	93 vs 76	44.5 vs 17.5 ($P <.0001$)	65% vs 57% at 42-months
Dara-Pomd (single arm study)	100	80	98	25	60	8.8 months	17.5 months
Isa-Pomd	100	77	100	16 vs 24	60 vs 35	11.53 vs 6.47	72% vs 63% at 12-months ($P = .0631$)
Elo-Pomd	98	100	78	22 vs 25	53 vs 26	10.3 vs 4.7	–

Len, Lenalidomide; *PFS*, progression free survival; *PI*, proteasome inhibitor; *ORR*, overall response rate; *OS*, overall survival; *Ref*, refractory; *SOC*, standard of care.

TABLE 91.10 **Bortezomib- and Carfilzomib-Based Regimens for Relapsed Multiple Myeloma**

Regimen	% PI Exposed	% IMiD Ref	% IMiD Exposed	% With High-Risk Cytogenetics	ORR (%) vs SOC arm	PFS (months) vs. SOC arm	OS vs SOC arm
Pano-Vd	44	–	19	–	60.7 vs 54.6	12 vs 8	–
Dara-Vd	67	33	71	23 vs 21	83 vs 63	16.7 vs 7.1	NR
Kd	54	–	45	21 vs 24	77 vs 63	18.7 vs 9.4	NR
Dara-Kd	92	32	39	15.4 vs 16.9	84 vs 69	NR vs 15.8	NR
Isa-Kd	92.7	32	43.6	23.5 vs 25.2	86.6 vs 82.9	NR vs 19.15	NR

IMiD, Immunomodulatory drugs; *NR*, not reached; *ORR*, overall response rate; *OS*, overall survival; *PFS*, progression-free survival; *Ref*, refractory; *SOC*, standard of care.

thrombocytopenia, diarrhea, asthenia, or fatigue and peripheral neuropathy in the panobinostat arm.

TOURMALINE-MM-1[183] was a phase III study comparing the oral PIs ixazomib, lenalidomide, and dexamethasone (IRd; 360 patients) with placebo, lenalidomide, and dexamethasone (Rd; 362 patients) in patients with relapsed/refractory myeloma who failed one to three prior regimens. After a median follow-up of 14.7 months, the IRd group had a PFS of 20.6 months compared with the placebo group, which had a PFS of 14.7 months (hazard ratio for disease progression or death in the ixazomib group, 0.74; $P = .01$). The ORRs were 78% in the ixazomib group and 72% in the placebo group. The median OS has not been reached in either group after 23 months. Diarrhea, rash, and thrombocytopenia were the more common grade 3/4 adverse reactions associated with the IRd group compared with the placebo group.

Pomalidomide (Pom) is a third-generation IMiD that is more potent than lenalidomide. In a multicenter, open-label, randomized phase II study,[184] the efficacy and safety of pomalidomide with or without low-dose dexamethasone (Pomd) was studied in 221 patients who had relapsed MM after two or more lines of prior therapy and were progressing on the last therapy. The ORR was 33% for patients receiving Pomd and 18% for patients receiving pomalidomide alone with median response durations of 8.3 and 10.7 months, median PFS rates of 4.2 and 2.7 months, and OS of 16.5 and 13.6 months, respectively. Refractoriness to lenalidomide or resistance to both lenalidomide and bortezomib did not affect the outcomes with pomalidomide and low-dose dexamethasone. Grade 3/4 neutropenia (41%), anemia (22%), and thrombocytopenia (19%), as well as pneumonia (22%), were the predominant adverse events encountered. An international, randomized, open-label phase III trial compared Pomd (302 subjects) with high-dose dexamethasone (153 subjects) in patients with refractory MM after two lines of chemotherapy containing bortezomib and lenalidomide.[185] After a median follow-up of 10 months, the median PFS was 4 months for patients receiving pomalidomide and dexamethasone versus 1.9 months for high-dose dexamethasone alone. The most common grade 3/4 hematologic adverse events in the pomalidomide arm were neutropenia, anemia, and cytopenias. Nonhematologic adverse events (grade 3/4) included pneumonia and fatigue. The OPTIMISMM trial[186] was a randomized, open-label phase III study comparing pomalidomide, bortezomib, dexamethasone (Pom-Vd; 281 patients) compared to Vd (278 patients) for patients who have received 1 to 3 prior lines of therapies. The study met its primary endpoint after a 15.9-month follow-up, showing improved PFS in favor of Pom-Vd with median 11.2 months compared to 7.1 months for Vd arm (hazard ratio 0.61, 95% CI 0.49 to 0.77; $P < 0{\cdot}0001$). The ORR was 82.2% for the Pom-Vd arm whereas it was 50% for the VD arm. The most common grade 3 or 4 treatment emergent AEs were neutropenia (42% vs 9%), infections (31% vs 18%), and thrombocytopenia (27% vs 29%).

Elotuzumab (Elo) is an immunostimulatory monoclonal antibody targeting SLAMF7. Elotuzumab directly activates natural killer cells through both the SLAMF7 pathway and Fc receptors; it also targets SLAMF7 on myeloma cells and facilitates the interaction with natural killer cells to mediate the killing of MM cells through antibody-dependent cellular cytotoxicity ADCC. The drug showed activity in combination with lenalidomide and dexamethasone in a phase IBM-II study of patients with relapsed/refractory myeloma.[187] Elo was further evaluated in an open-label, international, randomized, multicenter phase III trial known as the ELOQUENT-2 trial.[188] The two arms of the trial consisted of Elo, lenalidomide, and dexamethasone (Elo-Rd; 321 patients) and lenalidomide and dexamethasone (Rd; 325 patients). The rate of PFS at 1 year (after a median follow-up of 24.5 months) in the Elo-Rd group was 68%, as compared with 57% in the Rd group. Moreover, at 2 years, there was a 41% PFS advantage in the Elo-Rd group compared with 27% in the Rd group. The median PFS was 19.4 months in the Elo-Rd group versus 14.9 months in the Rd group (hazard ratio for progression or death in the elotuzumab group, 0.70; $P < .001$). ORRs varied between the two groups: 79% in the Elo-Rd group and 66% in the Rd group. Prespecified interim analysis for OS indicated a strong trend ($P = .0257$) in favor of the Elo-Rd arm compared with the Rd arm with early separation sustained over time (43.7 months vs. 39.6 months, respectively; hazard ratio, 0.77). Of the patients in the Elo-Rd group, 10% had infusion reactions. Other grade 3/4 adverse events in the Elo-Rd arm were lymphocytopenia and fatigue. A combination of elotuzumab with pomalidomide and dexamethasone (Elo-Pomd) was also compared with pomalidomide dexamethasone in a randomized phase II trial,[189] leading to the FDA approval of this combination.

Daratumumab (Dara) is a human anti-CD38 IgGκ monoclonal antibody that was initially FDA-approved in 2015 for myeloma patients who have had more than three relapses.[190] The ORR was 31% among 148 patients treated with daratumumab at 16 mg/kg; the median response duration was 7.4 months, PFS was 3.7 months, and OS was 20.5 months.[190] Dara was well tolerated; fatigue and anemia were the most common adverse events.

The POLLUX trial[77] was a phase III study comparing daratumumab, lenalidomide, and dexamethasone (Dara-Rd; 286 patients) with lenalidomide and dexamethasone (Rd; 283 patients) in patients with at least one prior line of therapy. After a median follow-up of 13.5 months, the estimated median PFS was not reached in the Dara-Rd group compared with 18 months in the lenalidomide and dexamethasone group. The ORR was superior in the Dara-Rd arm (92.9%) compared with Rd (76.4%), as was MRD negativity via NGS at the 10^{-5} threshold (22.4% vs. 4.6%). The most common ≥grade 3 AEs during treatment were neutropenia (Dara-Rd = 51.9%, Rd = 37%), thrombocytopenia (Dara-Rd = 12.7%, Rd = 13.5%), and anemia (Dara-Rd = 12.4%, Rd = 19.6%). The CASTOR trial[76] was a phase III study comparing daratumumab, bortezomib, and dexamethasone (Dara-Vd; 251 patients) with Vd (247 patients) in patients with at least one prior line of therapy. The primary endpoint of PFS was met after a 7.4-month follow-up, showing median PFS of 16 months on the Dara-Vd arm, whereas that of the Vd arm was 7.2 months. The ORR was higher in the Dara-Vd arm compared to the Vd arm (82.9% vs. 63.2%, $P < .001$). The most common ≥grade 3 AEs in the Dara-Vd and the Vd arms were thrombocytopenia (45.3% and 32.9%, respectively), anemia (14.4% and 16.0%, respectively), and neutropenia (12.8% and 4.2%, respectively).

The EQUULEUS trial[78] was an open-label multicenter trial that studied the combination of daratumumab, pomalidomide, and dexamethasone (Dara-Pomd) in patients who have received two more prior lines of therapy (103 patients). The safety profile of Dara-Pomd was similar to that of pom-dex alone, with the exception of a higher incidence of neutropenia without an increase in infection rate. The ORR was 60% and among patients who achieved a CR, 29% were MRD negative at the 10^{-5} threshold. After a median follow-up of 13.1 months, the median PFS was 8.8 months and median OS was 17.5 months. The CANDOR trial[191] was a randomized, phase III study that compared daratumumab, carfilzomib, and dexamethasone (Dara-Kd; 312 subjects) with Kd (154 subjects) in patients who have received at least one prior line of therapy, with PFS as the primary endpoint. After a median follow-up of 16 months, the primary endpoint was met with Kd showing PFS of 15.8 months whereas that for Dara-Kd was not reached. The ORR and MRD negative CR rates were superior in the Dara-Kd arm (84.3% vs 74.7%, 13.8% vs 3.2%, respectively).

Isatuximab (Isa) is a chimeric IgG kappa monoclonal antibody that targets CD38[192,193] that has been examined both as a single agent and in combination therapies in relapsed MM patients. Two large, randomized phase III trials have recently been reported with this monoclonal antibody in combination with Pomd and Kd. ICARIA was a randomized phase III trial[194] that compared Isa-Pomd (307 subjects) with Pomd (153 subjects) for patients with one prior line of therapy, which met its primary endpoint of median PFS improvement with Isa-Pomd (11.5 months vs. 6.5 months) after a median follow-up of 11.6 months. IKEMA was a randomized phase III trial[195,196] comparing Isa-Kd (179 patients) with Kd (123 patients) in patients with 1 to 3 prior lines of therapy. After a follow-up of 20 months, the primary endpoint was reached showing PFS not being reached in the Isa-Kd arm whereas it was 19.15 months for the Kd arm. There were no differences seen between ORR between the two arms of this study (86.6% vs 82.9%), and the safety profile appeared similar as well.

Selinexor is a first-in-class selective inhibitor of nuclear exportin protein "exportin-1 inhibitor" (XPO1, CaRM1). It works by binding to exportin 1 and blocking the transport of a number of regulatory proteins that are involved in MM cell growth from the nucleus to the cytoplasm. This blockade leads to cell cycle arrest and apoptosis.) Selinexor t has shown preclinical activity in several solid and hematologic cancer models.[197] Selinexor was combined with dexamethasone in the STORM trial,[198] a single arm phase IIb study (122 patients), in patients who had received at least III prior therapies including PI, IMiD, and an anti-CD38 monoclonal antibody. The ORR was 26%, median PFS was 3.7 months, and median OS was 8.6 months.

Belantamab mafodotin is a first-in-class antibody drug conjugate (ADC) consisting of an anti- BCMA antibody conjugated to the cytotoxic payload of monomethyl auristatin F, an anti-neoplastic that blocks tubulin polymerization. BCMA is a protein that is almost universally expressed by MM cells. In the DREAMM-2 trial[56] of single-agent belantamab mafodotin in MM patients refractory to PI, IMiD, and anti-CD38 therapy, it showed a response rate of 31% to 34% ($n = 196$, median 6 prior lines of therapy) with two dosing strategies (2.5 mg/kg, 3.4 mg/kg). However, the drug moiety appears to cause ≥grade 3 keratopathy in 42% to 46% of the patients and lower doses are being explored in drug combination clinical trials.

Role of Salvage Autologous and Allogeneic Stem Cell Transplant

The role of salvage ASCT is less clear compared with the data in the front-line setting. However, the American Society for Transplantation and Cellular Therapy, the European Society of Blood and Marrow Transplantation, and the IMWG have developed consensus guidelines based on best evidence regarding use of salvage HDM/ASCT in relapsed MM.[177] They recommend that salvage transplant be considered for relapsed MM patients who are transplant eligible but have not received a transplant as part of first-line therapy, or in patients who relapsed after first-line therapy including HDM/ASCT and who achieved an at least 18-month PFS. In one phase III study performed in the UK,[199] patients with relapsed MM after at least 18 months after prior HDM/ASCT were randomized to induction therapy with bortezomib, doxorubicin, and dexamethasone followed by either ASCT (melphalan 200 mg/m^2 preparative regimen) or oral cyclophosphamide (400 mg/m^2 per week for 12 weeks). The study showed an improved PFS in the auto-SCT group compared to oral cyclophosphamide (19 months vs. 11 months) but has been criticized for the choice of oral cyclophosphamide as a control since it is not widely used as a single agent in this setting. It appears that PFS duration after the first ASCT is predictive of outcomes after the second, salvage ASCT.[200–202] Although the PFS benefit being achieved with many regimens utilizing novel agents in the relapsed setting appears better than that of a second ASCT, this maneuver may still have a role in select cases. Furthermore, it remains relevant globally as many regions either do not have access to most novel agents or patients are unable to afford them.

The role of allogenic stem cell transplant is yet more controversial and requires more prospective studies in the era of modern MM therapy, given the numerous novel agents now available. There is one single arm phase II trial that treated 49 patients with melphalan (140 mg/m^2), fludarabine (90 mg/m^2), and antithymocyte globulin (ATG; 60 mg/kg body weight) conditioning followed by an unrelated donor transplant graft.[203] Although the ORR was 95% (including 46% CRs), the 1-year treatment related mortality (TRM) was 25%. At 43 months of follow up, the 5-year PFS and 5-year OS were reported at 20% and 26%, respectively. Given the efficacy of the novel regimens and the high TRM of this therapeutic modality, allogeneic stem cell transplant in relapsed/refractory myeloma should primarily be considered for young, fit patients with higher-risk disease, ideally in the context of a clinical trial, and such patients should regardless be treated by expert transplant physicians with substantial experience in allogeneic transplantation for myeloma.

Practical Approach to the First Relapse

The clinical trials summarized above included patients with a variety of patient-specific, disease and treatment features. One needs to take into account each of these factors in order to select the best therapeutic options for a given patient. The following are general guidelines on which options may be considered for each category of MM patient relapse below:

Bortezomib and Lenalidomide Sensitive: Essentially, all combination regimens in Tables 91.9 and 91.10 are applicable for these patients.

Bortezomib Sensitive/Lenalidomide Resistant: Most of the bortezomib or carfilzomib-based options (such as Dara-Vd, Dara/Pom/Dex Pom-Vd, Kd, etc.) would be reasonable options based on availability and patient preferences.

Bortezomib Resistant/Lenalidomide Sensitive: Any of the lenalidomide-based regimens (KRd, IRd, Elo-Rd, DRd, etc.) would be options for these patients, with perhaps the PI/IMiD-based combinations being preferred for patients with high-risk cytogenetics. Patients with resistance to bortezomib may nonetheless respond favorably to the newer-generation agent carfilzomib.

Bortezomib and Lenalidomide Resistant: This is a challenging population and commonly represents patients who achieved relatively unsatisfactory responses to, or those who progressed quickly on common PI/IMiD-based first-line therapies. Here, several regimens (Dara-Pomd, Isa-Pomd, Dara-Kd, Elo-Pomd, Kd, etc.) may be active. Belantamab mafodotin-blmf, a novel anti-BCMA ADC, is now also approved for patients who have been treated with at least four anti-myeloma agents, including PI, IMID, and anti-CD38 antibody. In addition to the above, a salvage second ASCT may be an option where access to novel agents is a practical impediment (see below). Moreover, salvage ASCT may be considered for a young, fit, high-risk patient with bortezomib-/lenalidomide-resistant disease.

Approach to Second Relapse and Beyond

A majority of patients who are relapsing for the second time have been exposed to both PIs and IMiDs, and a significant fraction in the modern era will also have been challenged with an anti-CD38 monoclonal antibody. As mentioned above, patients who are refractory to PI, IMiD, anti-CD38 antibody, and a fourth agent are eligible for belantamab mafodotin. However, the following are general guidelines of picking the most appropriate regimen for these patients.

Carfilzomib, Pomalidomide and anti-CD38 Monoclonal Antibody Sensitive: Most non-bortezomib-/non-lenalidomide-based regimens would be useful in this situation (Dara-Kd, Dara-Pomd, Elo-Pomd, Isa-Pomd, Isa-Kd, Kd, Dara monotherapy, etc.).

Carfilzomib Refractory/Pomalidomide and Anti-CD38 Monoclonal Antibody Sensitive: Here, most pomalidomide-based regimens would be applicable (Dara-Pomd, Elo-Pomd, Isa-Pomd, etc.) except Pom-Vd as these patients are now PI refractory.

Pomalidomide Refractory/Carfilzomib and Anti-CD38 Monoclonal Antibody Sensitive: Here, most carfilzomib-based regimens would be applicable (Dara-Kd, Isa-Kd, Kd etc.).

Carfilzomib and Pomalidomide Refractory/anti-CD38 Monoclonal Antibody Sensitive: Dara as monotherapy would be a relevant option in this scenario, as well as participation in clinical trials.

Carfilzomib, Pomalidomide Refractory, Anti-CD38 Monoclonal Antibody Refractory: Seli-Dex would be the most relevant approved option in this scenario, although clinical trial participation with novel immunotherapies may be the best option. Selinexor is approved for patients who have received at least four prior therapies, and are refractory to at least two PIs, two IMiDs, and an anti-CD38 antibody. Finally, a salvage ASCT may be an option where access to novel agents is a practical impediment. In addition, an allogeneic stem cell transplant may be considered for a young, fit, high-risk relapsed MM.

Investigational Therapies in Relapsed and/or Refractory Disease

A number of small molecules and immune therapeutics in clinical trials are showing promising results in terms of both safety and efficacy for relapsed myeloma (Table 91.11). Therapeutic targeting is geared toward the malignant plasma cell biology as well as modulation of the immune repertoire and the bone marrow microenvironment. More recently, focus of the field has shifted from predominantly small molecule development to more immunotherapy-based approaches. However, among the small molecule options, the drug classes that are further ahead in development include novel PIs, cereblon E3 ligase modulators (or CELMoDs), and BCL-2/MCL1 pathway inhibitors. CELMoDs are functionally different from the IMiDs. They are larger molecules and work by quickly degrading a specific set of proteins (Ikaros and Aiolos). They are potent modulators of the cereblon E3 ligase complex. They also stimulate the immune system and kill myeloma cells directly, even when myeloma has become resistant to other therapies. Among the novel PIs, early phase data with both oprozomib[204,205] and marizomib[206] show clinical activity but safety has been a concern, with gastrointestinal adverse events for oprozomib and central nervous system side effects for marizomib being dose-limiting toxicities. The oral CELMoDs CC-220[207] and CC92480[208] have recently been reported to be active even in patients who have become refractory to both lenalidomide and pomalidomide. These and other CELMoDs are now being studied in combination with other novel agents in clinical trials for relapsed MM. Lastly, the Bcl-2 inhibitor venetoclax has demonstrated both single-agent activity[209] and good synergy with bortezomib and dexamethasone,[210] especially in the patients with the t(11;14) translocation and Bcl-2 overexpression. A randomized phase III trial is underway to compare venetoclax-Vd versus Vd in relapsed MM patients with one or more prior lines of therapy.

With respect to immune therapy for MM, chimeric antigen receptor T cells have been examined in early phase clinical development targeting BCMA for relapsed MM patients. An initial proof-of-concept study from the NCI reported clinical activity in heavily pretreated patients[211] but observed high grades of cytokine release syndrome (CRS) and neurotoxicity. Subsequently, the bb2121 construct (idecabtagene vicleucel) has shown impressive clinical activity in advanced relapsed/refractory MM patients ($n = 128$, median 5 prior lines of therapy), with ORR of 73%, median PFS of 8.8 months, and median DoR of 10.7 months[212] [46]. CRS was seen in 84% of the patients (≥ grade 3 = 5%). Similarly, neurotoxicity was seen in 18% of patients (≥ grade 3 = 3%). The JCARH125 construct[213] (orvacabtagene autoleucel) phase I/II trial reported an ORR of 92% in heavily pretreated relapsed MM patients ($n = 62$), with 36% CR or better rates. The CRS rate was 89% (≥ grade 3 = 3%), while the neurotoxicity rate was 13% (≥ grade 3 = 3%). The JNJ-4528 construct[214–216] phase I recently reported on ORR of 100% ($n = 29$) with very high sCR rate (86%), but did not report the median PFS due to the short follow-up. The CRS rate was 93%, grade 3 or higher making up 7%, whereas neurotoxicity was observed in 10% (≥ grade 3 = 3%). Idecabtagene vicleucel (Abecma) was approved in March of 2021 for the treatment of adult patients with relapsed or refractory MM after four or more prior lines of therapy, including an immunomodulatory agent, a proteasome inhibitor, and an anti-CD38 monoclonal antibody. Other surface marker targets are also being explored as immunotherapeutic targets (see Table 91.11).

The antibody-based approaches being explored in clinical trials for relapsed MM include naked antibodies, ADCs, and bispecific antibodies. As mentioned above, the BCMA-ADC belantamab mafodotin has received accelerated FDA approval with a number of other drug conjugates in trials. There are several BCMA-directed bispecific monoclonal antibodies that have been recently evaluated. A phase I

TABLE 91.11 **Investigational Therapies**

B-Cell Maturation Antigen (BCMA) Directed Therapies

- Naked antibodies: SEA-BCMA
- Antibody-drug conjugates: MEDI2228, CC-99712, HDP-101,
- Bispecific antibodies: AMG-420, AMG-701, Teclistamab, PF-06863135, TNB-383B, REGN5458, CC93269
- Chimeric antigen receptor T cells: BB2121, BB21217, JNJ-4528, JCARH125, ALLO-715, LCAR-B38M, P-BCMA-101, CT-053, MCARH171, BRD015, CT103A
- Chimeric antigen receptor NK cells: CAR-NK92

GPRC5D Directed Therapies

- Bispecific antibodies: talquetamab, GPRC5D TRAB
- Chimeric antigen receptor T cells:

Signaling Lymphocytic Activation Molecule F7 (SLAMF7) Directed Therapies

- Antibody-drug conjugates: ABBV-838
- Bispecific antibodies: CS1-NKG2D BsAb
- Chimeric antigen receptor T cells: UCARTCS1, CS1-CAR T

CD38 Directed Therapies

- Naked antibodies: TAK-079
- Immune conjugates: TAK-169, TAK-573, 1053-PE38
- Radio-immune conjugates: Actinium-225-Daratumumab,
- Bispecific antibodies: AMG-424, GBR-1342

Small Molecules

- Exportin-1 inhibitors: selinexor, verdinexor
- CelMods: CC-220, CC-92480
- BCL2 inhibitors: venetoclax, S65487
- MCL1 inhibitors: MIK665, ABV-467, AMG-176, AZD5991
- Proteasome inhibitors: marizomib, oprozomib
- HDAC inhibitors: ACY-1215, bisthianostat
- Bromodomain inhibitors: CPI-0610, RO6870810, ARV825
- MDM2 inhibitors: AMG232, DS3032b, idasanutlin

trial of AMG-420,[217] a short half-life bispecific antibody, was the first proof-of-concept study in relapsed MM patients, showing a 31% ORR (*n* = 42, median 5 prior lines of therapy) for all treated patients but an impressive 70% ORR for those treated with the 400 ug/day, and only a 38% CRS rate (grade 3 = 1%). However, AMG-420 is administered as a continuous infusion over 4 weeks due to its short half-life. An improved half-life product, AMG-701, is now under investigation.[218] Teclistamab, another humanized BCMAx CD3 bispecific antibody, has shown clinical activity in relapsed MM[219, 220] with an ORR of 31% (*n* = 78, median 6 prior lines of therapy), CRS rate of 62% (no ≥ grade 3). A third bispecific monoclonal antibody, CC-93269,[220] reported an ORR of 42% (*n* = 30, median 5 prior lines of therapy) with a CRS rate of 77% (≥ grade 3 = 3%). As these BCMA-directed bispecific monoclonal antibodies get farther along in their clinical development, they may become more attractive, off-the-shelf options for relapsed MM compared to the BCMA CART therapies.

FUTURE DIRECTIONS

Significant advances have occurred in the understanding of the pathobiology of MM, which have translated to development of novel therapeutics. Predictive in vitro and in vivo models have been developed to study growth and survival characteristics of MM cells in the context of the bone marrow microenvironment, but, more important, to preclinically evaluate the efficacy of novel targeted agents. Similarly, there has been an explosion of genomic data covering various genomic correlates including expression profiling, CNA, alternate splicing, miRNA, and now whole-genome and RNA sequencing with mutational analysis of expressed genes as well as noncoding regions. Understanding of mutational landscape, molecular drivers of the disease and patterns, and surrogates of genomic evolution are providing newer clues to disease biology as well as novel therapeutic approaches. Evolving understanding of the epigenomic alterations that affect cell growth, survival, and development of drug resistance is providing clues for novel interventions. For example, there is already an HDAC inhibitor approved for therapy of relapsed myeloma. Moreover, MM cell proliferation is now considered to be dependent on the bromodomain and extraterminal (or "BET") domain family of bromodomain-containing proteins (BRD2, BRD3, and BRD4), and are considered novel therapeutic targets.

Indeed, improved understanding of disease biology has led to new drug development, with 10 new agents approved for myeloma in the last 12 years and over 15 other novel agents being in phase I or II studies. The genomic information is being integrated to develop more accurate risk stratification models and to develop personalized medicine to optimize response while avoiding toxicities. With these advances and use of combinations of agents, there is a significant improvement in the achievement of CR, over 60% to 70% in some studies. This has led to validation of MRD as an important new endpoint in MM with its incorporation into all clinical studies as well as exploration as an ultimate endpoint to achieve cure. Finally, because of these advances, the median survival of patients with MM has increased from 3 years to over 8–10 years, and in a number of individuals, it becomes a chronic disease with curative outcome predicted in a proportion of patients in the near future.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Attal M, et al. Lenalidomide maintenance after stem-cell transplantation for multiple myeloma. *N Engl J Med*. 2012;366:1782–1791.

Attal M, et al. Lenalidomide, bortezomib, and dexamethasone with transplantation for myeloma. *N Engl J Med*. 2017;376:1311–1320.

Attal M, et al. Isatuximab plus pomalidomide and low-dose dexamethasone versus pomalidomide and low-dose dexamethasone in patients with relapsed and refractory multiple myeloma (ICARIA-MM): a randomised, multicentre, open-label, phase 3 study. *Lancet*. 2019;394:2096–2107.

Chng WJ, et al. IMWG consensus on risk stratification in multiple myeloma. *Leukemia*. 2014;28:269–277.

Dimopoulos MA, et al. Daratumumab, lenalidomide, and dexamethasone for multiple myeloma. *N Engl J Med*. 2016;375:1319–1331.

Dimopoulos M, et al. Carfilzomib, dexamethasone, and daratumumab versus carfilzomib and dexamethasone for patients with relapsed or refractory multiple myeloma (CANDOR): results from a randomised, multicentre, open-label, phase 3 study. *Lancet*. 2020;396:186–197.

Dimopoulos MA, et al. Elotuzumab plus pomalidomide and dexamethasone for multiple myeloma. *N Engl J Med*. 2018;379:1811–1822.

Giralt S, et al. American Society of Blood and Marrow Transplantation, European Society of Blood and Marrow Transplantation, Blood and Marrow Transplant Clinical Trials Network, and International Myeloma Working Group Consensus Conference on Salvage Hematopoietic Cell Transplantation in Patients with Relapsed Multiple Myeloma. *Biol Blood Marrow Transplant*. 2015;21:2039–2051.

Korde N, et al. Treatment with carfilzomib-lenalidomide-dexamethasone with lenalidomide extension in patients with smoldering or newly diagnosed multiple myeloma. *JAMA Oncol*. 2015;1:746–754.

Krönke J, et al. Lenalidomide causes selective degradation of IKZF1 and IKZF3 in multiple myeloma cells. *Science*. 2014;343:301–305.

Kumar S, et al. International Myeloma Working Group consensus criteria for response and minimal residual disease assessment in multiple myeloma. *Lancet Oncol*. 2016;17:e328–e346.

Landgren O, et al. Efficacy and safety of carfilzomib-lenalidomide-dexamethasone (KRd) in newly diagnosed multiple myeloma: pooled analysis of 4 single-arm studies. *Blood*. 2019;134 1891–1891.

Lohr JG, et al. Widespread genetic heterogeneity in multiple myeloma: implications for targeted therapy. *Cancer Cell*. 2014;25:91–101.

Lokhorst HM, et al. Targeting CD38 with daratumumab monotherapy in multiple myeloma. *N Engl J Med*. 2015;373:1207–1219.

Lonial S, et al. Daratumumab monotherapy in patients with treatment-refractory multiple myeloma (SIRIUS): an open-label, randomised, phase 2 trial. *Lancet*. 2016;387:1551–1560.

Lonial S, et al. Belantamab mafodotin for relapsed or refractory multiple myeloma (DREAMM-2): a two-arm, randomised, open-label, phase 2 study. *Lancet Oncol*. 2020;21:207–221.

Maura F, et al. Moving from cancer burden to cancer genomics for smoldering myeloma: a review. *JAMA Oncol*. 2020;6(3):425–432.

McCarthy PL, et al. Lenalidomide after stem-cell transplantation for multiple myeloma. *N Engl J Med*. 2012;366:1770–1781.

Moreau P, et al. Oral ixazomib, lenalidomide, and dexamethasone for multiple myeloma. *N Engl J Med*. 2016;374:1621–1634.

Moreau P, et al. Bortezomib, thalidomide, and dexamethasone with or without daratumumab before and after autologous stem-cell transplantation for newly diagnosed multiple myeloma (CASSIOPEIA): a randomised, open-label, phase 3 study. *Lancet*. 2019;394:29–38.

Nair S, et al. Clonal immunoglobulin against lysolipids in the origin of myeloma. *N Engl J Med*. 2016;374:555–561.

Palumbo A, et al. Continuous lenalidomide treatment for newly diagnosed multiple myeloma. *N Engl J Med*. 2012;366:1759–1769.

Palumbo A, et al. Autologous transplantation and maintenance therapy in multiple myeloma. *N Engl J Med*. 2014;371:895–905.

Palumbo A, et al. Daratumumab, bortezomib, and dexamethasone for multiple myeloma. *N Engl J Med*. 2016;375:754–766.

Palumbo A, et al. Revised international staging system for multiple myeloma: a report from international myeloma working group. *J Clin Oncol*. 2015;33:2863–2869.

Raje N, et al. Anti-BCMA CAR T-cell therapy bb2121 in relapsed or refractory multiple myeloma. *N Engl J Med*. 2019;380:1726–1737.

Rajkumar SV, et al. International myeloma working group updated criteria for the diagnosis of multiple myeloma. *Lancet Oncol*. 2014;15:e538–e548.

Rustad EH, et al. Timing the initiation of multiple myeloma. *Nat Commun*. 2020;11:1917.

Usmani SZ, et al. Daratumumab monotherapy in patients with heavily pretreated relapsed or refractory multiple myeloma: final results from the phase 2 GEN501 and SIRIUS trials. *Lancet Haematol*. 2020;7:e447–e455.

REFERENCES

1. Cowan AJ, et al. Global burden of multiple myeloma: a systematic analysis for the global burden of disease study 2016. *JAMA Oncol*. 2018;4:1221–1227. https://doi.org/10.1001/jamaoncol.2018.2128.
2. Kumar SK, et al. Multiple myeloma. *Nat Rev Dis Primers*. 2017;3:17046. https://doi.org/10.1038/nrdp.2017.46.
3. Nandakumar B, et al. Continued improvement in survival in multiple myeloma (MM) including high-risk patients. *J Clin Oncol*. 2019;37:8039. https://doi.org/10.1200/JCO.2019.37.15_suppl.8039.
4. Landgren O, Kyle RA, Rajkumar SV. From myeloma precursor disease to multiple myeloma: new diagnostic concepts and opportunities for early intervention. *Clin Cancer Res*. 2011;17:1243–1252. https://doi.org/10.1158/1078-0432.CCR-10-1822.
5. Dezube BJ, Aboulafia DM, Pantanowitz L. Plasma cell disorders in HIV-infected patients: from benign gammopathy to multiple myeloma. *AIDS Read*. 2004;14:377–379. 372–374.
6. Sergentanis TN, et al. Risk factors for multiple myeloma: a systematic review of meta-analyses. *Clin Lymphoma Myeloma Leuk*. 2015;15:563–577. https://doi.org/10.1016/j.clml.2015.06.003.
7. Landgren O, et al. Agent orange exposure and monoclonal gammopathy of undetermined significance: an operation ranch hand veteran cohort study. *JAMA Oncol*. 2015;1:1061–1068. https://doi.org/10.1001/jamaoncol.2015.2938.
8. Wei X, et al. Germline lysine-specific demethylase 1 (LSD1/KDM1A) mutations confer susceptibility to multiple myeloma. *Cancer Res*. 2018;78:2747–2759. https://doi.org/10.1158/0008-5472.CAN-17-1900.
9. Pertesi M, et al. Exome sequencing identifies germline variants in DIS3 in familial multiple myeloma. *Leukemia*. 2019;33:2324–2330. https://doi.org/10.1038/s41375-019-0452-6.
10. Nair S, et al. Clonal Immunoglobulin against lysolipids in the origin of myeloma. *N Engl J Med*. 2016;374:555–561. https://doi.org/10.1056/NEJMoa1508808.
11. Pawlyn C, Morgan GJ. Evolutionary biology of high-risk multiple myeloma. *Nat Rev Cancer*. 2017;17:543–556. https://doi.org/10.1038/nrc.2017.63.
12. Rustad EH, et al. Timing the initiation of multiple myeloma. *Sneak Peak (pre-print)*. 2019 https://doi.org/10.2139/ssrn.3409453.
13. Landgren O, et al. Prevalence of myeloma precursor state monoclonal gammopathy of undetermined significance in 12372 individuals 10–49 years old: a population-based study from the National Health and Nutrition Examination Survey. *Blood Cancer J*. 2017;7:e618. https://doi.org/10.1038/bcj.2017.97.
14. Murray, D. et al. 18th International Myeloma Workshop, September 8-11, 2021, Vienna, Austria.
15. Rustad EH, et al. Timing the initiation of multiple myeloma. *Nat Commun*. 2020;11:1917. https://doi.org/10.1038/s41467-020-15740-9.
16. Maura F, et al. Moving from cancer burden to cancer genomics for smoldering myeloma: a review. *JAMA Oncol*. 2020;6(3):425–432. https://doi.org/10.1001/jamaoncol.2019.4659.
17. Landgren O, et al. Association of immune marker changes with progression of monoclonal gammopathy of undetermined significance to multiple myeloma. *JAMA Oncol*. 2019;5:1293–1301. https://doi.org/10.1001/jamaoncol.2019.1568.
18. Barwick BG, et al. Multiple myeloma immunoglobulin lambda translocations portend poor prognosis. *Nat Commun*. 2019;10:1911. https://doi.org/10.1038/s41467-019-09555-6.
19. Maura F, et al. Genomic landscape and chronological reconstruction of driver events in multiple myeloma. *Nat Commun*. 2019;10:3835. https://doi.org/10.1038/s41467-019-11680-1.
20. Aktas Samur A, et al. Deciphering the chronology of copy number alterations in multiple myeloma. *Blood Cancer J*. 2019;9:39. https://doi.org/10.1038/s41408-019-0199-3.
21. Zhan F, et al. Global gene expression profiling of multiple myeloma, monoclonal gammopathy of undetermined significance, and normal bone marrow plasma cells. *Blood*. 2002;99:1745–1757. https://doi.org/10.1182/blood.v99.5.1745.
22. Weinhold N, et al. Clinical value of molecular subtyping multiple myeloma using gene expression profiling. *Leukemia*. 2016;30:423–430. https://doi.org/10.1038/leu.2015.309.
23. Broyl A, et al. Gene expression profiling for molecular classification of multiple myeloma in newly diagnosed patients. *Blood*. 2010;116:2543–2553. https://doi.org/10.1182/blood-2009-12-261032.
24. Mroczek S, et al. The non-canonical poly(A) polymerase FAM46C acts as an onco-suppressor in multiple myeloma. *Nat Commun*. 2017;8:619. https://doi.org/10.1038/s41467-017-00578-5.
25. Lohr JG, et al. Widespread genetic heterogeneity in multiple myeloma: implications for targeted therapy. *Cancer Cell*. 2014;25:91–101. https://doi.org/10.1016/j.ccr.2013.12.015.
26. Rasche L, et al. Spatial genomic heterogeneity in multiple myeloma revealed by multi-region sequencing. *Nat Commun*. 2017;8:268. https://doi.org/10.1038/s41467-017-00296-y.
27. Landau H, et al. Accelerated single cell seeding in relapsed multiple myeloma. *bioRxiv*. 2020 https://doi.org/10.1101/2020.02.25.963272. 2020.2002.2025.963272.
28. Kawano Y, et al. Targeting the bone marrow microenvironment in multiple myeloma. *Immunol Rev*. 2015;263:160–172. https://doi.org/10.1111/imr.12233.
29. Romano A, et al. Immunological dysregulation in multiple myeloma microenvironment. *Biomed Res Int*. 2014;2014:198539. https://doi.org/10.1155/2014/198539.
30. Turesson I, et al. Monoclonal gammopathy of undetermined significance and risk of lymphoid and myeloid malignancies: 728 cases followed up to 30 years in Sweden. *Blood*. 2014;123:338–345. https://doi.org/10.1182/blood-2013-05-505487.
31. Mateos MV, Landgren O. MGUS and smoldering multiple myeloma: diagnosis and epidemiology. *Cancer Treat Res*. 2016;169:3–12. https://doi.org/10.1007/978-3-319-40320-5_1.
32. Silvestris F, Tucci M, Quatraro C, Dammacco F. Recent advances in understanding the pathogenesis of anemia in multiple myeloma. *Int J Hematol*. 2003;78:121–125. https://doi.org/10.1007/bf02983379.
33. Ryu D, et al. Alterations in the transcriptional programs of myeloma cells and the microenvironment during extramedullary progression affect proliferation and immune evasion. *Clin Cancer Res*. 2020;26:935–944. https://doi.org/10.1158/1078-0432.CCR-19-0694.
34. Kotla V, et al. Mechanism of action of lenalidomide in hematological malignancies. *J Hematol Oncol*. 2009;2:36. https://doi.org/10.1186/1756-8722-2-36.
35. Krönke J, et al. Lenalidomide causes selective degradation of IKZF1 and IKZF3 in multiple myeloma cells. *Science*. 2014;343:301–305. https://doi.org/10.1126/science.1244851.
36. Qu X, et al. Lenalidomide regulates osteocytes fate and related osteoclastogenesis via IL-1β/NF-κB/RANKL signaling. *Biochem Biophys Res Commun*. 2018;501:547–555. https://doi.org/10.1016/j.bbrc.2018.05.035.
37. Guo J, et al. Lenalidomide restores the osteogenic differentiation of bone marrow mesenchymal stem cells from multiple myeloma patients via deactivating Notch signaling pathway. *Oncotarget*. 2017;8:55405–55421. https://doi.org/10.18632/oncotarget.19265.
38. Bolomsky A, et al. Immunomodulatory drugs thalidomide and lenalidomide affect osteoblast differentiation of human bone marrow stromal cells in vitro. *Exp Hematol*. 2014;42:516–525. https://doi.org/10.1016/j.exphem.2014.03.005.
39. D'Amato RJ, Loughnan MS, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. *Proc Natl Acad Sci USA*. 1994;91:4082–4085. https://doi.org/10.1073/pnas.91.9.4082.
40. Yabu T, et al. Thalidomide-induced antiangiogenic action is mediated by ceramide through depletion of VEGF receptors, and is antagonized by sphingosine-1-phosphate. *Blood*. 2005;106:125–134. https://doi.org/10.1182/blood-2004-09-3679.
41. Scalzulli E, Grammatico S, Vozella F, Petrucci MT. Proteasome inhibitors for the treatment of multiple myeloma. *Expert Opin Pharmacother*. 2018;19:375–386. https://doi.org/10.1080/14656566.2018.1441287.
42. Accardi F, et al. Mechanism of action of bortezomib and the new proteasome inhibitors on myeloma cells and the bone microenvironment: impact on myeloma-induced alterations of bone remodeling. *Biomed Res Int*. 2015;2015:172458. https://doi.org/10.1155/2015/172458.
43. Mohty M, et al. The effects of bortezomib on bone disease in patients with multiple myeloma. *Cancer*. 2014;120:618–623. https://doi.org/10.1002/cncr.28481.
44. Hurchla MA, et al. The epoxyketone-based proteasome inhibitors carfilzomib and orally bioavailable oprozomib have anti-resorptive and bone-anabolic activity in addition to anti-myeloma effects. *Leukemia*. 2013;27:430–440. https://doi.org/10.1038/leu.2012.183.
45. Campbell KS, Cohen AD, Pazina T. Mechanisms of NK cell activation and clinical activity of the therapeutic SLAMF7 antibody, elotuzumab in multiple myeloma. *Front Immunol*. 2018;9:2551. https://doi.org/10.3389/fimmu.2018.02551.
46. Pazina T, et al. Enhanced SLAMF7 homotypic interactions by elotuzumab improves NK cell killing of multiple myeloma. *Cancer Immunol Res*. 2019;7:1633–1646. https://doi.org/10.1158/2326-6066.CIR-18-0579.
47. Balasa B, et al. Elotuzumab enhances natural killer cell activation and myeloma cell killing through interleukin-2 and TNF-α pathways. *Cancer Immunol Immunother*. 2015;64:61–73. https://doi.org/10.1007/s00262-014-1610-3.
48. Costa F, Dalla Palma B, Giuliani N. CD38 Expression by myeloma cells and its role in the context of bone marrow microenvironment: modulation by therapeutic agents. *Cells*. 2019;8:1632. https://doi.org/10.3390/cells8121632.

49. Krejcik J, et al. Daratumumab depletes CD38+ immune regulatory cells, promotes T-cell expansion, and skews T-cell repertoire in multiple myeloma. *Blood*. 2016;128:384–394. https://doi.org/10.1182/blood-2015-12-687749.
50. van de Donk NWCJ, Usmani SZ. CD38 Antibodies in multiple myeloma: mechanisms of action and modes of resistance. *Front Immunol*. 2018;9:2134. https://doi.org/10.3389/fimmu.2018.02134.
51. Viola D, et al. Daratumumab induces mechanisms of immune activation through CD38+ NK cell targeting. *Leukemia*. 2021;35:189–200. https://doi.org/10.1038/s41375-020-0810-4.
52. Moreno L, et al. The Mechanism of action of the anti-CD38 monoclonal antibody isatuximab in multiple myeloma. *Clin Cancer Res*. 2019;25: 3176–3187. https://doi.org/10.1158/1078-0432.CCR-18-1597.
53. Terpos E, Ntanasis-Stathopoulos I, Dimopoulos MA. Myeloma bone disease: from biology findings to treatment approaches. *Blood*. 2019;133:1534–1539. https://doi.org/10.1182/blood-2018-11-852459.
54. Cho SF, Anderson KC, Tai YT. Targeting B cell maturatioa antigen (BCMA) in multiple myeloma: potential uses of BCMA-based immunotherapy. *Front Immunol*. 2018;9:1821. https://doi.org/10.3389/fimmu.2018.01821.
55. Raje N, et al. Anti-BCMA CAR T-cell therapy bb2121 in relapsed or refractory multiple myeloma. *N Engl J Med*. 2019;380:1726–1737. https://doi.org/10.1056/NEJMoa1817226.
56. Lonial S, et al. Belantamab mafodotin for relapsed or refractory multiple myeloma (DREAMM-2): a two-arm, randomised, open-label, phase 2 study. *Lancet Oncol*. 2020;21:207–221. https://doi.org/10.1016/S1470-2045(19)30788-0.
57. Li Y, Fan CY, Manni A, Simonds WF. Pitfalls of using denosumab preoperatively to treat refractory severe hypercalcaemia. *BMJ Case Rep*. 2020;13:e233665. https://doi.org/10.1136/bcr-2019-233665.
58. White PS, Dennis M, Jones EA, Weinberg JM, Sarosiek S. Risk factors for development of hypocalcemia in patients with cancer treated with bone-modifying agents. *J Natl Compr Canc Netw*. 2020;18:420–427. https://doi.org/10.6004/jnccn.2019.7370.
59. Zuo C, Zhu Y, Xu G. An update to the pathogenesis for monoclonal gammopathy of renal significance. *Ann Hematol*. 2020;99:703–714. https://doi.org/10.1007/s00277-020-03971-1.
60. Adapa S, Konala VM, Naramala S, Nast CC. Multiple morphological phenotypes of monoclonal immunoglobulin disease on renal biopsy: significance of treatment. *Clin Nephrol Case Stud*. 2020;8:17–24. https://doi.org/10.5414/CNCS110052.
61. Vaxman I, Gertz M. When to suspect a diagnosis of amyloidosis. *Acta Haematol*. 2020;143:304–311. https://doi.org/10.1159/000506617.
62. Drayson M, et al. Serum free light-chain measurements for identifying and monitoring patients with nonsecretory multiple myeloma. *Blood*. 2001;97: 2900–2902. https://doi.org/10.1182/blood.v97.9.2900.
63. Takamatsu H, et al. Comparison of minimal residual disease detection in multiple myeloma by SRL 8-color single-tube and EuroFlow 8-color 2-tube multiparameter flow cytometry. *Int J Hematol*. 2019;109:377–381. https://doi.org/10.1007/s12185-019-02615-z.
64. Roshal M, et al. MRD detection in multiple myeloma: comparison between MSKCC 10-color single-tube and EuroFlow 8-color 2-tube methods. *Blood Adv*. 2017;1:728–732. https://doi.org/10.1182/bloodadvances.2016003715.
65. Flores-Montero J, et al. Next generation flow for highly sensitive and standardized detection of minimal residual disease in multiple myeloma. *Leukemia*. 2017;31:2094–2103. https://doi.org/10.1038/leu.2017.29.
66. Hillengass J, et al. International myeloma working group consensus recommendations on imaging in monoclonal plasma cell disorders. *Lancet Oncol*. 2019;20:e302–e312. https://doi.org/10.1016/S1470-2045(19)30309-2.
67. Ormond Filho AG, et al. Whole-body imaging of multiple myeloma: diagnostic criteria. *Radiographics*. 2019;39:1077–1097. https://doi.org/10.1148/rg.2019180096.
68. Dutoit JC, Verstraete KL. Whole-body MRI, dynamic contrast-enhanced MRI, and diffusion-weighted imaging for the staging of multiple myeloma. *Skelet Radiol*. 2017;46:733–750. https://doi.org/10.1007/s00256-017-2609-6.
69. Granell M, et al. Prognostic impact of circulating plasma cells in patients with multiple myeloma: implications for plasma cell leukemia definition. *Haematologica*. 2017;102:1099–1104. https://doi.org/10.3324/haematol.2016.158303.
70. Bae MH, et al. Increased circulating plasma cells detected by flow cytometry predicts poor prognosis in patients with plasma cell myeloma. *Cytometry B Clin Cytom*. 2018;94:493–499. https://doi.org/10.1002/cyto.b.21606.
71. Kumar S, et al. International Myeloma Working Group consensus criteria for response and minimal residual disease assessment in multiple myeloma. *Lancet Oncol*. 2016;17:e328–e346. https://doi.org/10.1016/S1470-2045(16)30206-6.
72. Landgren O, Devlin S, Boulad M, Mailankody S. Role of MRD status in relation to clinical outcomes in newly diagnosed multiple myeloma patients: a meta-analysis. *Bone Marrow Transplant*. 2016;51:1565–1568. https://doi.org/10.1038/bmt.2016.222.
73. Avet-Loiseau H, et al. Minimal residual disease in multiple myeloma: final analysis of the IFM2009 trial. *Blood*. 2017;130 435-435.
74. Perrot A, et al. Development and validation of a cytogenetic prognostic index predicting survival in multiple myeloma. *J Clin Oncol*. 2019;37: 1657–1665. https://doi.org/10.1200/JCO.18.00776.
75. Moreau P, et al. Bortezomib, thalidomide, and dexamethasone with or without daratumumab before and after autologous stem-cell transplantation for newly diagnosed multiple myeloma (CASSIOPEIA): a randomised, open-label, phase 3 study. *Lancet*. 2019;394:29–38. https://doi.org/10.1016/S0140-6736(19)31240-1.
76. Palumbo A, et al. Daratumumab, bortezomib, and dexamethasone for multiple myeloma. *N Engl J Med*. 2016;375:754–766. https://doi.org/10.1056/NEJMoa1606038.
77. Dimopoulos MA, et al. Daratumumab, lenalidomide, and dexamethasone for multiple myeloma. *N Engl J Med*. 2016;375:1319–1331. https://doi.org/10.1056/NEJMoa1607751.
78. Chari A, et al. Daratumumab plus pomalidomide and dexamethasone in relapsed and/or refractory multiple myeloma. *Blood*. 2017;130:974–981. https://doi.org/10.1182/blood-2017-05-785246.
79. Perrot A, et al. Minimal residual disease negativity using deep sequencing is a major prognostic factor in multiple myeloma. *Blood*. 2018;132: 2456–2464. https://doi.org/10.1182/blood-2018-06-858613.
80. Mohan M, Kendrick S, Yarlagadda N, Atwal D, Pandey Y, Roy A, Parikh P, Lopez J, Thanendrarajan S, Schinke CD, Alapat D, Van Rhee F, Kendrick S, Zangari M. In *ASCO Annual Meeting*.
81. Kazandjian D, et al. Remission and progression-free survival in patients with newly diagnosed multiple myeloma treated with carfilzomib, lenalidomide, and dexamethasone: 5-year follow-up of a phase 2 clinical trial. *JAMA Oncol*. 2018;4:1781–1783. https://doi.org/10.1001/jamaoncol.2018.5457.
82. Attal M, et al. Lenalidomide, bortezomib, and dexamethasone with transplantation for myeloma. *N Engl J Med*. 2017;376:1311–1320. https://doi.org/10.1056/NEJMoa1611750.
83. Mankarious S, et al. The half-lives of IgG subclasses and specific antibodies in patients with primary immunodeficiency who are receiving intravenously administered immunoglobulin. *J Laboratory Clin Med*. 1988;112:634–640.
84. Landgren O, Lu SX, Hultcrantz M. MRD testing in multiple myeloma: the main future driver for modern tailored treatment. *Semin Hematol*. 2018;55: 44–50. https://doi.org/10.1053/j.seminhematol.2018.03.001.
85. Landgren, O. et al. In *American Society of Hematology Annual Meeting*.
86. Yao Q, Bai Y, Orfao A, Chim CS. Standardized minimal residual disease detection by next-generation sequencing in multiple myeloma. *Front Oncol*. 2019;9:449. https://doi.org/10.3389/fonc.2019.00449.
87. Bai Y, Orfao A, Chim CS. Molecular detection of minimal residual disease in multiple myeloma. *Br J Haematol*. 2018;181:11–26. https://doi.org/10.1111/bjh.15075.
88. Takamatsu H. Comparison of minimal residual disease detection by multiparameter flow cytometry, ASO-qPCR, droplet digital PCR, and deep sequencing in patients with multiple myeloma who underwent autologous stem cell transplantation. *J Clin Med*. 2017;6:91. https://doi.org/10.3390/jcm6110106.
89. Ulaner G, et al. Preclinical development and first-in-human imaging of 89Zr-daratumumab for CD38 targeted imaging of myeloma. *J Nucl Med*. 2019;60:203.
90. Ulaner GA, et al. CD38-targeted immuno-PET of multiple myeloma: from xenograft models to first-in-human imaging. *Radiology*. 2020;295:606–615. https://doi.org/10.1148/radiol.2020192621.
91. Chng WJ, et al. IMWG consensus on risk stratification in multiple myeloma. *Leukemia*. 2014;28:269–277. https://doi.org/10.1038/leu.2013.247.
92. Walker BA, et al. A high-risk, double-hit, group of newly diagnosed myeloma identified by genomic analysis. *Leukemia*. 2019;33:159–170. https://doi.org/10.1038/s41375-018-0196-8.
93. Steensma DP, et al. A high bone marrow plasma cell labeling index in stable plateau-phase multiple myeloma is a marker for early disease progression and death. *Blood*. 2001;97:2522–2523. https://doi.org/10.1182/blood.v97.8.2522.
94. Durie BG, Salmon SE. A clinical staging system for multiple myeloma. Correlation of measured myeloma cell mass with presenting clinical features, response to treatment, and survival. *Cancer*. 1975;36:842–854. 10.1002/1097-0142(197509)36:3<842::aid-cncr2820360303>3.0.co;2-u.
95. Greipp PR, et al. International staging system for multiple myeloma. *J Clin Oncol*. 2005;23:3412–3420. https://doi.org/10.1200/JCO.2005.04.242.
96. Palumbo A, et al. Revised international staging system for multiple myeloma: a report from international myeloma working group. *J Clin Oncol*. 2015;33:2863–2869. https://doi.org/10.1200/JCO.2015.61.2267.

97. Yellapantula V, et al. Comprehensive detection of recurring genomic abnormalities: a targeted sequencing approach for multiple myeloma. *Blood Cancer J*. 2019;9:101. https://doi.org/10.1038/s41408-019-0264-y.
98. Ubels J, et al. Predicting treatment benefit in multiple myeloma through simulation of alternative treatment effects. *Nat Commun*. 2018;9:2943. https://doi.org/10.1038/s41467-018-05348-5.
99. Chapman MA, et al. RNA-Seq of newly diagnosed patients in the PADIMAC study leads to a bortezomib/lenalidomide decision signature. *Blood*. 2018;132:2154–2165. https://doi.org/10.1182/blood-2018-05-849893.
100. Hermine O, Ramos JC, Tobinai K. A review of new findings in adult T-cell leukemia-lymphoma: a focus on current and emerging treatment strategies. *Adv Ther*. 2018;35:135–152. https://doi.org/10.1007/s12325-018-0658-4.
101. Ozturk K, et al. Non-hematologic diagnosis of systemic mastocytosis: collaboration of radiology and pathology. *Blood Rev*. 2021;100693 https://doi.org/10.1016/j.blre.2020.100693.
102. Tefferi A, Pardanani A. Systemic mastocytosis: current concepts and treatment advances. *Curr Hematol Rep*. 2004;3:197–202.
103. Chen BJ, Chuang SS. Lymphoid neoplasms with plasmablastic differentiation: a comprehensive review and diagnostic approaches. *Adv Anat Pathol*. 2020;27:61–74. https://doi.org/10.1097/PAP.0000000000000253.
104. Lopez A, Abrisqueta P. Plasmablastic lymphoma: current perspectives. *Blood Lymphat Cancer*. 2018;8:63–70. https://doi.org/10.2147/BLCTT.S142814.
105. Wang W, Lin P. Lymphoplasmacytic lymphoma and waldenstrom macroglobulinaemia: clinicopathological features and differential diagnosis. *Pathology*. 2020;52:6–14. https://doi.org/10.1016/j.pathol.2019.09.009.
106. Rajkumar SV, et al. International myeloma working group updated criteria for the diagnosis of multiple myeloma. *Lancet Oncol*. 2014;15:e538–e548. https://doi.org/10.1016/S1470-2045(14)70442-5.
107. Pham A, Mahindra A. Solitary plasmacytoma: a review of diagnosis and management. *Curr Hematol Malig Rep*. 2019;14:63–69. https://doi.org/10.1007/s11899-019-00499-8.
108. Fernández de Larrea C, et al. Plasma cell leukemia: consensus statement on diagnostic requirements, response criteria and treatment recommendations by the International Myeloma Working Group. *Leukemia*. 2013;27:780–791. https://doi.org/10.1038/leu.2012.336.
109. Gundesen MT, Lund T, Moeller HEH, Abildgaard N. Plasma cell leukemia: definition, presentation, and treatment. *Curr Oncol Rep*. 2019;21:8. https://doi.org/10.1007/s11912-019-0754-x.
110. Mina R, D'Agostino M, Cerrato C, Gay F, Palumbo A. Plasma cell leukemia: update on biology and therapy. *Leuk Lymphoma*. 2017;58:1538–1547. https://doi.org/10.1080/10428194.2016.1250263.
111. International Myeloma Working Group Criteria for the classification of monoclonal gammopathies, multiple myeloma and related disorders: a report of the International Myeloma Working Group. *Br J Haematol*. 2003;121:749–757.
112. Landgren O, Giralt S. MRD-driven treatment paradigm for newly diagnosed transplant eligible multiple myeloma patients. *Bone Marrow Transplant*. 2016;51:913–914. https://doi.org/10.1038/bmt.2016.24.
113. LeBlanc TW, et al. Risk stratification, treatment selection, and transplant eligibility in multiple myeloma: a qualitative study of the perspectives and self-reported practices of oncologists. *Curr Oncol*. 2016;23:e598–e604. https://doi.org/10.3747/co.23.3298.
114. Gandolfi S, et al. The evolving role of transplantation in multiple myeloma: the need for a heterogeneous approach to a heterogeneous disease. *Clin Adv Hematol Oncol*. 2018;16:564–574.
115. Kristinsson SY, Anderson WF, Landgren O. Improved long-term survival in multiple myeloma up to the age of 80 years. *Leukemia*. 2014;28:1346–1348. https://doi.org/10.1038/leu.2014.23.
116. Mailankody S, et al. Minimal residual disease in multiple myeloma: bringing the bench to the bedside. *Nat Rev Clin Oncol*. 2015;12:286–295. https://doi.org/10.1038/nrclinonc.2014.239.
117. Landgren O, et al. Weekly carfilzomib, lenalidomide, dexamethasone and daratumumab (wKRd-D) combination therapy provides unprecedented MRD negativity rates in newly diagnosed multiple myeloma: a clinical and correlative phase 2 study. *Blood*. 2019;134:862. https://doi.org/10.1182/blood-2019-126378.
118. Barlogie B. High-dose therapy and innovative approaches to treatment of multiple myeloma. *Semin Hematol*. 2001;38:21–27. https://doi.org/10.1016/s0037-1963(01)90091-5.
119. Usmani SZ, et al. Improvement in long-term outcomes with successive Total Therapy trials for multiple myeloma: are patients now being cured? *Leukemia*. 2013;27:226–232. https://doi.org/10.1038/leu.2012.160.
120. Barillé-Nion S, et al. Advances in biology and therapy of multiple myeloma. *Hematol Am Soc Hematol Educ Program*. 2003:248–278.
121. Moreau P, et al. Subcutaneous versus intravenous administration of bortezomib in patients with relapsed multiple myeloma: a randomised, phase 3, non-inferiority study. *Lancet Oncol*. 2011;12:431–440. https://doi.org/10.1016/S1470-2045(11)70081-X.
122. Durie BGM, et al. Bortezomib with lenalidomide and dexamethasone versus lenalidomide and dexamethasone alone in patients with newly diagnosed myeloma without intent for immediate autologous stem-cell transplant (SWOG S0777): a randomised, open-label, phase 3 trial. *Lancet*. 2017;389:519–527. https://doi.org/10.1016/S0140-6736(16)31594-X.
123. Kumar SK, et al. Ixazomib, lenalidomide, and dexamethasone in patients with newly diagnosed multiple myeloma: long-term follow-up including ixazomib maintenance. *Leukemia*. 2019;33:1736–1746. https://doi.org/10.1038/s41375-019-0384-1.
124. Richardson PG, et al. Twice-weekly ixazomib in combination with lenalidomide-dexamethasone in patients with newly diagnosed multiple myeloma. *Br J Haematol*. 2018;182:231–244. https://doi.org/10.1111/bjh.15394.
125. Korde N, et al. MRD response-driven phase i/ii study for newly diagnosed multiple myeloma patients using higher doses of twice-weekly carfilzomib (45 and 56mg/m^2) in combination with lenalidomide and dexamethasone. *Blood*. 2017;130 3133-3133, https://doi.org/10.1182/blood.V130.Suppl_1.3133.3133.
126. Korde N, et al. Treatment with carfilzomib-lenalidomide-dexamethasone with lenalidomide extension in patients with smoldering or newly diagnosed multiple myeloma. *JAMA Oncol*. 2015;1:746–754. https://doi.org/10.1001/jamaoncol.2015.2010.
127. Landgren O, et al. Carfilzomib with immunomodulatory drugs for the treatment of newly diagnosed multiple myeloma. *Leukemia*. 2019;33:2127–2143. https://doi.org/10.1038/s41375-019-0517-6.
128. Landgren O, et al. Efficacy and safety of carfilzomib-lenalidomide-dexamethasone (KRd) in newly diagnosed multiple myeloma: pooled analysis of 4 single-arm studies. *Blood*. 2019;134 https://doi.org/10.1182/blood-2019-127076. 1891-1891.
129. Gay F, et al. Carfilzomib-lenalidomide-dexamethasone (KRd) induction-autologous transplant (ASCT)-Krd consolidation Vs KRd 12 cycles vs carfilzomib-cyclophosphamide-dexamethasone (KCd) induction-ASCT-KCd consolidation: analysis of the randomized forte trial in newly diagnosed multiple myeloma (NDMM). *Blood*. 2018;132 https://doi.org/10.1182/blood-2018-99-112093. 121-121.
130. Kumar SK, et al. Carfilzomib or bortezomib in combination with lenalidomide and dexamethasone for patients with newly diagnosed multiple myeloma without intention for immediate autologous stem-cell transplantation (ENDURANCE): a multicentre, open-label, phase 3, randomised, controlled trial. *Lancet Oncol*. 2020;21:1317–1330. https://doi.org/10.1016/S1470-2045(20)30452-6.
131. Mikhael JR, et al. Phase Ib/II trial of CYKLONE (cyclophosphamide, carfilzomib, thalidomide and dexamethasone) for newly diagnosed myeloma. *Br J Haematol*. 2015;169:219–227. https://doi.org/10.1111/bjh.13296.
132. Moreau P, et al. VTD is superior to VCD prior to intensive therapy in multiple myeloma: results of the prospective IFM2013-04 trial. *Blood*. 2016;127:2569–2574. https://doi.org/10.1182/blood-2016-01-693580.
133. Mateos MV, et al. Daratumumab plus bortezomib, melphalan, and prednisone for untreated myeloma. *N Engl J Med*. 2018;378:518–528. https://doi.org/10.1056/NEJMoa1714678.
134. Facon T, et al. Daratumumab plus lenalidomide and dexamethasone for untreated myeloma. *N Engl J Med*. 2019;380:2104–2115. https://doi.org/10.1056/NEJMoa1817249.
135. Yimer H, et al. Daratumumab, bortezomib, cyclophosphamide and dexamethasone in newly diagnosed and relapsed multiple myeloma: LYRA study. *Br J Haematol*. 2019;185:492–502. https://doi.org/10.1111/bjh.15806.
136. Voorhees PM, et al. Depth of response to daratumumab (DARA), lenalidomide, bortezomib, and dexamethasone (RVd) improves over time in patients (pts) with Transplant-Eligible Newly Diagnosed Multiple Myeloma (NDMM): Griffin study update. *Blood*. 2019;134:691. https://doi.org/10.1182/blood-2019-123465.
137. Costa LJ, et al. Daratumumab, carfilzomib, lenalidomide, and dexamethasone (Dara-KRd) induction, autologous transplantation and post-transplant, response-adapted, measurable residual disease (MRD)-based dara-krd consolidation in patients with newly diagnosed multiple myeloma (NDMM). *Blood*. 2019;134 https://doi.org/10.1182/blood-2019-123170. 860-860.
138. Rosen LS, et al. Zoledronic acid versus pamidronate in the treatment of skeletal metastases in patients with breast cancer or osteolytic lesions of multiple myeloma: a phase III, double-blind, comparative trial. *Cancer J*. 2001;7:377–387.
139. Terpos E, et al. International myeloma working group recommendations for the treatment of multiple myeloma-related bone disease. *J Clin Oncol*. 2013;31:2347–2357. https://doi.org/10.1200/JCO.2012.47.7901.
140. Rosen LS, et al. Long-term efficacy and safety of zoledronic acid compared with pamidronate disodium in the treatment of skeletal complications in patients with advanced multiple myeloma or breast carcinoma: a

randomized, double-blind, multicenter, comparative trial. *Cancer*. 2003;98: 1735–1744. https://doi.org/10.1002/cncr.11701.

141. Morgan GJ, et al. First-line treatment with zoledronic acid as compared with clodronic acid in multiple myeloma (MRC Myeloma IX): a randomised controlled trial. *Lancet*. 2010;376:1989–1999. https://doi.org/10.1016/S0140-6736(10)62051-X.
142. Raje N, et al. Denosumab versus zoledronic acid in bone disease treatment of newly diagnosed multiple myeloma: an international, double-blind, double-dummy, randomised, controlled, phase 3 study. *Lancet Oncol*. 2018;19:370–381. https://doi.org/10.1016/S1470-2045(18)30072-X.
143. Gertz MA, et al. Autologous stem cell transplant in 716 patients with multiple myeloma: low treatment-related mortality, feasibility of outpatient transplant, and effect of a multidisciplinary quality initiative. *Mayo Clin Proc*. 2008;83:1131–1138. https://doi.org/10.4065/83.10.1131.
144. Al Hamed R, Bazarbachi AH, Malard F, Harousseau JL, Mohty M. Current status of autologous stem cell transplantation for multiple myeloma. *Blood Cancer J*. 2019;9:44. https://doi.org/10.1038/s41408-019-0205-9.
145. Thomas A, et al. Second malignancies after multiple myeloma: from 1960s to 2010s. *Blood*. 2012;119:2731–2737. https://doi.org/10.1182/blood-2011-12-381426.
146. Govindarajan R, et al. Preceding standard therapy is the likely cause of MDS after autotransplants for multiple myeloma. *Br J Haematol*. 1996;95: 349–353. https://doi.org/10.1046/j.1365-2141.1996.d01-1891.x.
147. Cuzick J, Erskine S, Edelman D, Galton DA. A comparison of the incidence of the myelodysplastic syndrome and acute myeloid leukaemia following melphalan and cyclophosphamide treatment for myelomatosis. A report to the Medical Research Council's working party on leukaemia in adults. *Br J Cancer*. 1987;55:523–529. https://doi.org/10.1038/bjc.1987.107.
148. Jones JR, et al. Second malignancies in the context of lenalidomide treatment: an analysis of 2732 myeloma patients enrolled to the Myeloma XI trial. *Blood Cancer J*. 2016;6:e506. https://doi.org/10.1038/bcj.2016.114.
149. Attal M, et al. Single versus double autologous stem-cell transplantation for multiple myeloma. *N Engl J Med*. 2003;349:2495–2502. https://doi.org/10.1056/NEJMoa032290.
150. Bashir Q, Qazilbash MH. Allogeneic hematopoietic cell transplantation for myeloma: when and in whom does it work. *Curr Hematol Malig Rep*. 2017;12:126–135. https://doi.org/10.1007/s11899-017-0374-1.
151. Chhabra S, et al. Relapse after allogeneic hematopoietic cell transplantation for multiple myeloma: survival outcomes and factors influencing them. *Biol Blood Marrow Transpl*. 2020;26:188–1297. https://doi.org/10.1016/j.bbmt.2020.02.020.
152. Cavo M, et al. Autologous haematopoietic stem-cell transplantation versus bortezomib-melphalan-prednisone, with or without bortezomib-lenalidomide-dexamethasone consolidation therapy, and lenalidomide maintenance for newly diagnosed multiple myeloma (EMN02/HO95): a multicentre, randomised, open-label, phase 3 study. *Lancet Haematol*. 2020;7:e456–e468. https://doi.org/10.1016/S2352-3026(20)30099-5.
153. Gay F, et al. Efficacy of carfilzomib lenalidomide dexamethasone (KRd) with or without transplantation in newly diagnosed myeloma according to risk status: Results from the FORTE trial. *J Clin Oncol*. 2019;37:8002. https://doi.org/10.1200/JCO.2019.37.15_suppl.8002.
154. Stadtmauer EA, et al. Autologous transplantation, consolidation, and maintenance therapy in multiple myeloma: results of the BMT CTN 0702tTrial. *J Clin Oncol*. 2019;37:589–597. https://doi.org/10.1200/JCO.18.00685.
155. Palumbo A, et al. Continuous lenalidomide treatment for newly diagnosed multiple myeloma. *N Engl J Med*. 2012;366:1759–1769. https://doi.org/10.1056/NEJMoa1112704.
156. McCarthy PL, et al. Lenalidomide after stem-cell transplantation for multiple myeloma. *N Engl J Med*. 2012;366:1770–1781. https://doi.org/10.1056/NEJMoa1114083.
157. Attal M, et al. Lenalidomide maintenance after stem-cell transplantation for multiple myeloma. *N Engl J Med*. 2012;366:1782–1791. https://doi.org/10.1056/NEJMoa1114138.
158. Palumbo A, et al. Autologous transplantation and maintenance therapy in multiple myeloma. *N Engl J Med*. 2014;371:895–905. https://doi.org/10.1056/NEJMoa1402888.
159. Gay F, et al. Maintenance treatment and survival in patients with myeloma: a systematic review and network meta-analysis. *JAMA Oncol*. 2018;4: 1389–1397. https://doi.org/10.1001/jamaoncol.2018.2961.
160. McCarthy PL, et al. Lenalidomide maintenance after autologous stem-cell transplantation in newly diagnosed multiple myeloma: a meta-analysis. *J Clin Oncol*. 2017;35:3279–3289. https://doi.org/10.1200/JCO.2017.72.6679.
161. Ludwig H, et al. IMWG consensus on maintenance therapy in multiple myeloma. *Blood*. 2012;119:3003–3015. https://doi.org/10.1182/blood-2011-11-374249.
162. Holstein SA, et al. Updated analysis of CALGB (Alliance) 100104 assessing lenalidomide versus placebo maintenance after single autologous stem-cell transplantation for multiple myeloma: a randomised, double-blind, phase 3 trial. *Lancet Haematol*. 2017;4:e431–e442. https://doi.org/10.1016/S2352-3026(17)30140-0.
163. Sonneveld P, et al. Bortezomib induction and maintenance treatment in patients with newly diagnosed multiple myeloma: results of the randomized phase III HOVON-65/GMMG-HD4 trial. *J Clin Oncol*. 2012;30: 2946–2955. https://doi.org/10.1200/JCO.2011.39.6820.
164. Dimopoulos MA, et al. Oral ixazomib maintenance following autologous stem cell transplantation (TOURMALINE-MM3): a double-blind, randomised, placebo-controlled phase 3 trial. *Lancet*. 2019;393:253–264. https://doi.org/10.1016/S0140-6736(18)33003-4.
165. Bringhen S, et al. Carfilzomib, cyclophosphamide, and dexamethasone in patients with newly diagnosed multiple myeloma: a multicenter, phase 2 study. *Blood*. 2014;124:63–69. https://doi.org/10.1182/blood-2014-03-563759.
166. Zimmerman T, et al. Final results of a phase 2 trial of extended treatment (tx) with carfilzomib (CFZ), lenalidomide (LEN), and dexamethasone (KRd) plus autologous stem cell transplantation (ASCT) in newly diagnosed multiple myeloma (NDMM). *Blood*. 2016;128:675. https://doi.org/10.1182/blood.V128.22.675.675.
167. Lonial S, et al. Daratumumab monotherapy in patients with treatment-refractory multiple myeloma (SIRIUS): an open-label, randomised, phase 2 trial. *Lancet*. 2016;387:1551–1560. https://doi.org/10.1016/S0140-6736(15)01120-4.
168. Lokhorst HM, et al. Targeting CD38 with daratumumab monotherapy in multiple myeloma. *N Engl J Med*. 2015;373:1207–1219. https://doi.org/10.1056/NEJMoa1506348.
169. Mateos M-V, et al. Efficacy and safety of the randomized, open-label, non-inferiority, phase 3 study of subcutaneous (SC) versus intravenous (IV) daratumumab (DARA) administration in patients (pts) with relapsed or refractory multiple myeloma (RRMM): COLUMBA. *J Clin Oncol*. 2019;37:8005. https://doi.org/10.1200/JCO.2019.37.15_suppl.8005.
170. Nooka AK, et al. Consolidation and maintenance therapy with lenalidomide, bortezomib and dexamethasone (RVD) in high-risk myeloma patients. *Leukemia*. 2014;28:690–693. https://doi.org/10.1038/leu.2013.335.
171. Kumar SK, et al. Natural history of relapsed myeloma, refractory to immunomodulatory drugs and proteasome inhibitors: a multicenter IMWG study. *Leukemia*. 2017;31:2443–2448. https://doi.org/10.1038/leu.2017.138.
172. Kumar SK, et al. Risk of progression and survival in multiple myeloma relapsing after therapy with IMiDs and bortezomib: a multicenter international myeloma working group study. *Leukemia*. 2012;26:149–157. https://doi.org/10.1038/leu.2011.196.
173. Rajkumar SV, et al. Consensus recommendations for the uniform reporting of clinical trials: report of the International Myeloma Workshop Consensus Panel 1. *Blood*. 2011;117:4691–4695. https://doi.org/10.1182/blood-2010-10-299487.
174. Palumbo A, et al. Continuous therapy versus fixed duration of therapy in patients with newly diagnosed multiple myeloma. *J Clin Oncol*. 2015;33: 3459–3466. https://doi.org/10.1200/JCO.2014.60.2466.
175. Palumbo A, et al. Geriatric assessment predicts survival and toxicities in elderly myeloma patients: an International Myeloma Working Group report. *Blood*. 2015;125:2068–2074. https://doi.org/10.1182/blood-2014-12-615187.
176. Petrucci MT, et al. A prospective, international phase 2 study of bortezomib retreatment in patients with relapsed multiple myeloma. *Br J Haematol*. 2013;160:649–659. https://doi.org/10.1111/bjh.12198.
177. Giralt S, et al. American society of blood and marrow transplantation, european society of blood and marrow transplantation, blood and marrow transplant clinical trials network, and international myeloma working group consensus conference on salvage hematopoietic cell transplantation in patients with relapsed multiple myeloma. *Biol Blood Marrow Transpl*. 2015;21:2039–2051. https://doi.org/10.1016/j.bbmt.2015.09.016.
178. Siegel DS, et al. A phase 2 study of single-agent carfilzomib (PX-171-003-A1) in patients with relapsed and refractory multiple myeloma. *Blood*. 2012;120:2817–2825. https://doi.org/10.1182/blood-2012-05-425934.
179. Dimopoulos MA, et al. Carfilzomib and dexamethasone versus bortezomib and dexamethasone for patients with relapsed or refractory multiple myeloma (ENDEAVOR): a randomised, phase 3, open-label, multicentre study. *Lancet Oncol*. 2016;17:27–38. https://doi.org/10.1016/S1470-2045(15)00464-7.

180. Stewart AK, et al. Carfilzomib, lenalidomide, and dexamethasone for relapsed multiple myeloma. *N Engl J Med*. 2015;372:142–152. https://doi.org/10.1056/NEJMoa1411321.
181. Siegel DS, et al. Improvement in overall survival with carfilzomib, lenalidomide, and dexamethasone in patients with relapsed or refractory multiple myeloma. *J Clin Oncol*. 2018;36:728–734. https://doi.org/10.1200/JCO.2017.76.5032.
182. San-Miguel JF, et al. Panobinostat plus bortezomib and dexamethasone versus placebo plus bortezomib and dexamethasone in patients with relapsed or relapsed and refractory multiple myeloma: a multicentre, randomised, double-blind phase 3 trial. *Lancet Oncol*. 2014;15:1195–1206. https://doi.org/10.1016/S1470-2045(14)70440-1.
183. Moreau P, et al. Oral Ixazomib, lenalidomide, and dexamethasone for multiple myeloma. *N Engl J Med*. 2016;374:1621–1634. https://doi.org/10.1056/NEJMoa1516282.
184. Richardson PG, et al. Pomalidomide alone or in combination with low-dose dexamethasone in relapsed and refractory multiple myeloma: a randomized phase 2 study. *Blood*. 2014;123:1826–1832. https://doi.org/10.1182/blood-2013-11-538835.
185. Miguel JS, et al. Pomalidomide plus low-dose dexamethasone versus high-dose dexamethasone alone for patients with relapsed and refractory multiple myeloma (MM-003): a randomised, open-label, phase 3 trial. *Lancet Oncol*. 2013;14:1055–1066. https://doi.org/10.1016/S1470-2045(13)70380-2.
186. Richardson PG, et al. Pomalidomide, bortezomib, and dexamethasone for patients with relapsed or refractory multiple myeloma previously treated with lenalidomide (OPTIMISMM): a randomised, open-label, phase 3 trial. *Lancet Oncol*. 2019;20:781–794. https://doi.org/10.1016/S1470-2045(19)30152-4.
187. Lonial S, et al. Elotuzumab in combination with lenalidomide and low-dose dexamethasone in relapsed or refractory multiple myeloma. *J Clin Oncol*. 2012;30:1953–1959. https://doi.org/10.1200/JCO.2011.37.2649.
188. Lonial S, et al. Elotuzumab therapy for relapsed or refractory multiple myeloma. *N Engl J Med*. 2015;373:621–631. https://doi.org/10.1056/NEJMoa1505654.
189. Dimopoulos MA, et al. Elotuzumab plus pomalidomide and dexamethasone for multiple myeloma. *N Engl J Med*. 2018;379:1811–1822. https://doi.org/10.1056/NEJMoa1805762.
190. Usmani SZ, et al. Daratumumab monotherapy in patients with heavily pretreated relapsed or refractory multiple myeloma: final results from the phase 2 GEN501 and SIRIUS trials. *Lancet Haematol*. 2020;7:e447–e455. https://doi.org/10.1016/S2352-3026(20)30081-8.
191. Dimopoulos M, et al. Carfilzomib, dexamethasone, and daratumumab versus carfilzomib and dexamethasone for patients with relapsed or refractory multiple myeloma (CANDOR): results from a randomised, multicentre, open-label, phase 3 study. *Lancet*. 2020;396:186–197. https://doi.org/10.1016/S0140-6736(20)30734-0.
192. Martin T, et al. Phase I trial of isatuximab monotherapy in the treatment of refractory multiple myeloma. *Blood Cancer J*. 2019;9:41. https://doi.org/10.1038/s41408-019-0198-4.
193. Mikhael J, et al. A phase 1b study of isatuximab plus pomalidomide/dexamethasone in relapsed/refractory multiple myeloma. *Blood*. 2019;134:123–133. https://doi.org/10.1182/blood-2019-02-895193.
194. Attal M, et al. Isatuximab plus pomalidomide and low-dose dexamethasone versus pomalidomide and low-dose dexamethasone in patients with relapsed and refractory multiple myeloma (ICARIA-MM): a randomised, multicentre, open-label, phase 3 study. *Lancet*. 2019;394:2096–2107. https://doi.org/10.1016/S0140-6736(19)32556-5.
195. Moreau P, et al. Isatuximab plus carfilzomib/dexamethasone versus carfilzomib/dexamethasone in patients with relapsed/refractory multiple myeloma: IKEMA Phase III study design. *Future Oncol*. 2020;16:4347–4358. https://doi.org/10.2217/fon-2019-0431.
196. Moreau P, Dimopoulos M.-A., Mikhael J, Yong K, Capra M, Facon T, Hajek R, Spicka I, Risse ML, Asset G, Macé S, Martin T. in *EHA 2020 Conference*. LB2603.
197. Tai YT, et al. CRM1 inhibition induces tumor cell cytotoxicity and impairs osteoclastogenesis in multiple myeloma: molecular mechanisms and therapeutic implications. *Leukemia*. 2014;28:155–165. https://doi.org/10.1038/leu.2013.115.
198. Chari A, et al. Oral selinexor-dexamethasone for triple-class refractory multiple myeloma. *N Engl J Med*. 2019;381:727–738. https://doi.org/10.1056/NEJMoa1903455.
199. Cook G, et al. High-dose chemotherapy plus autologous stem-cell transplantation as consolidation therapy in patients with relapsed multiple myeloma after previous autologous stem-cell transplantation (NCRI Myeloma X Relapse [Intensive trial]): a randomised, open-label, phase 3 trial. *Lancet Oncol*. 2014;15:874–885. https://doi.org/10.1016/S1470-2045(14)70245-1.
200. Sellner L, et al. Autologous retransplantation for patients with recurrent multiple myeloma: a single-center experience with 200 patients. *Cancer*. 2013;119:2438–2446. https://doi.org/10.1002/cncr.28104.
201. Michaelis LC, et al. Salvage second hematopoietic cell transplantation in myeloma. *Biol Blood Marrow Transpl*. 2013;19:760–766. https://doi.org/10.1016/j.bbmt.2013.01.004.
202. Gonsalves WI, et al. Second auto-SCT for treatment of relapsed multiple myeloma. *Bone Marrow Transpl*. 2013;48:568–573. https://doi.org/10.1038/bmt.2012.183.
203. Kröger N, et al. Unrelated stem cell transplantation after reduced intensity conditioning for patients with multiple myeloma relapsing after autologous transplantation. *Br J Haematol*. 2010;148:323–331. https://doi.org/10.1111/j.1365-2141.2009.07984.x.
204. Hari P, et al. Efficacy and safety results from a phase 1b/2, multicenter, open-label study of oprozomib and dexamethasone in patients with relapsed and/or refractory multiple myeloma. *Leuk Res*. 2019;83:106172. https://doi.org/10.1016/j.leukres.2019.106172.
205. Shah J, et al. Oprozomib, pomalidomide, and dexamethasone in patients with relapsed and/or refractory multiple myeloma. *Clin Lymphoma Myeloma Leuk*. 2019;19 https://doi.org/10.1016/j.clml.2019.05.017. 570-578 e571.
206. Richardson PG, et al. Phase 1 study of marizomib in relapsed or relapsed and refractory multiple myeloma: NPI-0052-101 Part 1. *Blood*. 2016;127:2693–2700. https://doi.org/10.1182/blood-2015-12-686378.
207. Lonial S, et al. First clinical (phase 1b/2a) study of iberdomide (CC-220; IBER), a CELMoD, in combination with dexamethasone (DEX) in patients (pts) with relapsed/refractory multiple myeloma (RRMM). *J Clin Oncol*. 2019;37 8006-8006, doi:10.1200/JCO.2019.37.15_suppl.8006.
208. Richardson PG, et al. First-in-human phase I study of the novel CELMoD agent CC-92480 combined with dexamethasone (DEX) in patients (pts) with relapsed/refractory multiple myeloma (RRMM). *J Clin Oncol*. 2020;38 https://doi.org/10.1200/JCO.2020.38.15_suppl.8500. 8500-8500.
209. Kumar S, et al. Efficacy of venetoclax as targeted therapy for relapsed/refractory t(11;14) multiple myeloma. *Blood*. 2017;130:2401–2409. https://doi.org/10.1182/blood-2017-06-788786.
210. Moreau P, et al. Promising efficacy and acceptable safety of venetoclax plus bortezomib and dexamethasone in relapsed/refractory MM. *Blood*. 2017;130:2392–2400. https://doi.org/10.1182/blood-2017-06-788323.
211. Ali SA, et al. T cells expressing an anti-B-cell maturation antigen chimeric antigen receptor cause remissions of multiple myeloma. *Blood*. 2016;128:1688–1700. https://doi.org/10.1182/blood-2016-04-711903.
212. Munshi NC, et al. Idecabtagene vicleucel (ide-cel; bb2121), a BCMA-targeted CAR T-cell therapy, in patients with relapsed and refractory multiple myeloma (RRMM): Initial KarMMa results. *J Clin Oncol*. 2020;38 https://doi.org/10.1200/JCO.2020.38.15_suppl.8503. 8503-8503.
213. Mailankody S, et al. Orvacabtagene autoleucel (orva-cel), a B-cell maturation antigen (BCMA)-directed CAR T cell therapy for patients (pts) with relapsed/refractory multiple myeloma (RRMM): update of the phase 1/2 EVOLVE study (NCT03430011). *J Clin Oncol*. 2020;38 https://doi.org/10.1200/JCO.2020.38.15_suppl.8504. 8504-8504.
214. Madduri D, et al. Results from CARTITUDE-1: a phase 1b/2 Study of JNJ-4528, a CAR-T cell therapy directed against B-cell maturation antigen (BCMA), in patients with relapsed and/or refractory multiple myeloma (R/R MM). *Blood*. 2019;134 https://doi.org/10.1182/blood-2019-121731. 577-577.
215. Berdeja JG, et al. Update of CARTITUDE-1: a phase Ib/II study of JNJ-4528, a B-cell maturation antigen (BCMA)-directed CAR-T-cell therapy, in relapsed/refractory multiple myeloma. *J Clin Oncol*. 2020;38 https://doi.org/10.1200/JCO.2020.38.15_suppl.8505. 8505-8505.
216. Usmani SZ, Berdeja JG, Singh I, Zudaire E, Yeh TM, Allred AJ, Olyslager Y, Banerjee A, Goldberg JD, Schecter JM, Geng D, Wu X, Carrasco-Alfonso MJ, Rizvi S, Fan F, Jakubowiak A, Jagannath S. In *EHA 2020*. EP926.
217. Topp MS, et al. Anti-B-cell maturation antigen BiTE molecule AMG 420 induces responses in multiple myeloma. *J Clin Oncol*. 2020;38:775–783. https://doi.org/10.1200/JCO.19.02657.
218. Cho SF, et al. AMG 701 potently induces anti-multiple myeloma (MM) functions of T cells and IMiDs further enhance its efficacy to prevent mm relapse in vivo. *Blood*. 2019;134 https://doi.org/10.1182/blood-2019-128528. 135-135.
219. Usmani SZ, et al. Phase I study of teclistamab, a humanized B-cell maturation antigen (BCMA) x CD3 bispecific antibody, in relapsed/refractory multiple myeloma (R/R MM). *J Clin Oncol*. 2020;38 https://doi.org/10.1200/JCO.2020.38.15_suppl.100. 100-100.
220. Costa LJ, et al. First clinical study of the B-cell maturation antigen (BCMA) 2+1T cell engager (TCE) CC-93269 in patients (Pts) with relapsed/refractory multiple myeloma (RRMM): interim results of a phase 1 multicenter trial. *Blood*. 2019;134 143-143, doi: 10.1182/blood-2019-122895.

221. Wang C. Recent advances in POEMS syndrome. *Eur J Haematol*. 2021 https://doi.org/10.1111/ejh.13522.
222. Dispenzieri A. POEMS Syndrome: 2019 update on diagnosis, risk-stratification, and management. *Am J Hematol*. 2019;94:812–827. https://doi.org/10.1002/ajh.25495.
223. Zhang X, Fang M. TEMPI syndrome: erythrocytosis in plasma cell dyscrasia. *Clin Lymphoma Myeloma Leuk*. 2018;18:724–730. https://doi.org/10.1016/j.clml.2018.07.284.
224. Sykes DB, O'Connell C, Schroyens W. The TEMPI syndrome. *Blood*. 2020;135:1199–1203. https://doi.org/10.1182/blood.2019004216.
225. Dagrosa AT, et al. Adenopathy and extensive skin patch overlying a plasmacytoma with unusual histologic findings in a patient with polyneuropathy, organomegaly, endocrinopathy, monoclonal protein and skin changes syndrome and Castleman disease. *J Cutan Pathol*. 2019;46:784–789. https://doi.org/10.1111/cup.13514.
226. Lipsker D, Rondeau M, Massard G, Grosshans E. The AESOP (adenopathy and extensive skin patch overlying a plasmacytoma) syndrome: report of 4 cases of a new syndrome revealing POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal protein, and skin changes) syndrome at a curable stage. *Med (Baltimore)*. 2003;82:51–59. https://doi.org/10.1097/00005792-200301000-00005.
227. Cardenas-de la Garza JA, Esquivel-Valerio JA, Arvizu-Rivera RI, Colunga-Pedraza PR, Galarza-Delgado DA. Flushing out a plasmacytoma in a patient with POEMS and AESOP syndromes. *Lancet*. 2020;396:e21. https://doi.org/10.1016/S0140-6736(20)31756-6.
228. Gusdorf L, Lipsker D. Schnitzler syndrome: a review. *Curr Rheumatol Rep*. 2017;19:46. https://doi.org/10.1007/s11926-017-0673-5.
229. de Koning HD. Schnitzler's syndrome: lessons from 281 cases. *Clin Transl Allergy*. 2014;4:41. https://doi.org/10.1186/2045-7022-4-41.
230. Roccatello D, et al. Cryoglobulinaemia. *Nat Rev Dis Primers*. 2018;4:11. https://doi.org/10.1038/s41572-018-0009-4.
231. Standen GR, Jasani B, Wagstaff M, Wardrop CA. Chronic neutrophilic leukemia and multiple myeloma. An association with lambda light chain expression. *Cancer*. 1990;66:162–166. https://doi.org/10.1002/1097-0142(19900701)66:1<162::aid-cncr2820660129>3.0.co;2-z.
232. Cehreli C, Undar B, Akkoc N, Onvural B, Altungoz O. Coexistence of chronic neutrophilic leukemia with light chain myeloma. *Acta Haematol*. 1994;91:32–34. https://doi.org/10.1159/000204241.

CHAPTER 92

WALDENSTRÖM MACROGLOBULINEMIA/LYMPHOPLASMACYTIC LYMPHOMA

Jorge J. Castillo and Steven P. Treon

Waldenström macroglobulinemia (WM) is a lymphoid neoplasm resulting from the accumulation, predominantly in the bone marrow (BM), of a clonal population of lymphocytes, lymphoplasmacytic cells, and plasma cells, which secrete a monoclonal immunoglobulin (Ig)M.[1] WM corresponds to lymphoplasmacytic lymphoma (LPL) as defined in the World Health Organization classification system.[2] Most cases of LPL are WM; less than 5% of cases secrete IgA, IgG, kappa, lambda, or can be non-secretory LPL.

In 1944, Jan Waldenström, a Swedish physician-scientist, reported in *Acta Medica Scandinavica* three cases of a disease he presciently thought was related to myeloma, but for the absence of skeletal bone involvement and the scarcity of plasma cells in a marrow infiltrated with small lymphocytic cells. He noted an increase in plasma protein concentration, marked increased serum viscosity, exaggerated bleeding, and retinal hemorrhages. In collaboration with a colleague, he showed, using ultracentrifugation and electrophoresis, that the abundant abnormal protein had a molecular weight of approximately 1 million and was not an aggregate of smaller proteins. The disease, which he described with such thoroughness, was later named in his honor.

EPIDEMIOLOGY

The age-adjusted incidence rate of WM in the United States is 3.4 per 1 million among males and 1.7 per 1 million among females. The incidence of WM increases with age.[3,4] The incidence rate is higher among Americans of European descent. Americans of African descent represent approximately 5% of all patients.

Genetic factors play a role in the pathogenesis of WM. Approximately 20% of patients with WM are of Ashkenazi Jewish ethnic background.[5] Familial disease has been reported commonly, including multigenerational clustering of WM and other B-cell lymphoproliferative diseases.[6–9] Approximately 20% of 257 sequential patients with WM presenting to a tertiary referral center had a first-degree relative with either WM or another B-cell disorder.[8] Familial clustering of WM with other immunologic disorders, including hypogammaglobulinemia and hypergammaglobulinemia (particularly polyclonal IgM), autoantibody production (particularly to the thyroid), and manifestation of hyperactive B cells, has also been reported in relatives without WM.[8,9] Increased expression of the *BCL2* gene with enhanced survival has been observed in B cells from familial patients and their family members.[9]

The role of environmental factors is uncertain; however, chronic antigenic stimulation from infections and certain drug or chemical exposures have been considered but have not reached a level of scientific certainty. Hepatitis C virus (HCV) infection was implicated in WM causality in some series, but no association was found in a study of 100 consecutive patients with WM in whom serologic and molecular diagnostic studies for HCV infection were performed.[10–12]

PATHOGENESIS

Nature of the Waldenström Macroglobulinemia Clone

Examination of the B-cell clone(s) found in the BM of patients with WM reveals a range of differentiation, from small lymphocytes with large focal deposits of surface immunoglobulins, to lymphoplasmacytic cells, to mature plasma cells that contain intracytoplasmic IgM (Fig. 92.1).[13] Circulating clonal B cells are often detectable in patients with WM, though lymphocytosis is uncommon.[14,15] WM cells express the monoclonal IgM, and some clonal cells also express surface IgD.[16] The characteristic immunophenotypic profile of WM lymphoplasmacytic cells includes the expression of the pan–B-cell markers CD19, CD20 (including FMC7), CD22, and CD79.[16,17] Expression of CD5, CD10, and CD23 can be present in 10% to 20% of cases, and their presence does not exclude the diagnosis of WM.[18] In addition, multiparameter flow cytometric analysis has also identified CD25 and CD27 as being characteristic of the WM clone, and that a $CD22^{dim}/CD25^{+}/CD27^{+}/IgM^{+}$ population can be observed among clonal B lymphocytes in patients with IgM monoclonal gammopathy of undetermined significance (MGUS) who ultimately progress to WM.[19]

Somatic mutations in immunoglobulin genes are present with increased frequency of nonsynonymous versus silent mutations in complementarity-determining regions, along with somatic hypermutation, thereby supporting a postgerminal center derivation for the WM B-cell clone in most patients.[20,21] A strong preferential use of VH3/JH4 gene families without intraclonal variation, and without evidence for any isotype-switched transcripts, has also been shown.[22,23] Taken together, these data support an IgM^{+} and/or $IgM^{+}IgD^{+}$ memory B-cell origin for most cases of WM.

In contrast to myeloma plasma cells, no recurrent translocations have been described in WM, which can help one distinguish IgM myeloma cases that often exhibit t(11;14) from WM.[24,25] Despite the absence of IgH translocations, recurrent chromosomal abnormalities are present in WM cells. These include deletions in chromosome 6q21–23 in 40% to 60% of patients with WM, with concordant gains in 6p in 41% of patients with 6q deletion.[26–29] In a series of 174 untreated patients with WM, 6q deletions, followed by trisomy 18, 13q deletions, 17p deletions, trisomy 4, and 11q deletions, were observed.[29] Deletion of 6q and trisomy 4 were associated with an adverse prognosis in this series. Because 6q deletions represent the most recurrent cytogenetic finding in WM cases, there has been great interest in identifying the region of minimal deletion and possible target genes within this region. Two putative gene candidates within this region include *TNFAIP3*, a negative regulator of nuclear factor-κB signaling (NFκB), and *PRDM1*, a master regulator of B-cell differentiation.[28,30] The removal of an NFκB-negative regulator is of particular interest because the phosphorylation and translocation of NFκB into the nucleus is a crucial event for WM cell survival.[31] The success of proteasome inhibitor therapy in WM has been postulated to occur because the degradation of negative regulators of NFκB, such as inhibitor of κB (IκB), is blocked.[32,33]

MUTATION IN *MYD88*

A highly recurrent somatic mutation ($\mathit{MYD88}^{L265P}$) was first identified in patients with WM by whole-genome sequencing (WGS) and confirmed by multiple studies through Sanger sequencing and/or allele-specific polymerase chain reaction (PCR) assays.[34–39] $\mathit{MYD88}^{L265P}$ is expressed in 90% to 95% of WM cases when more

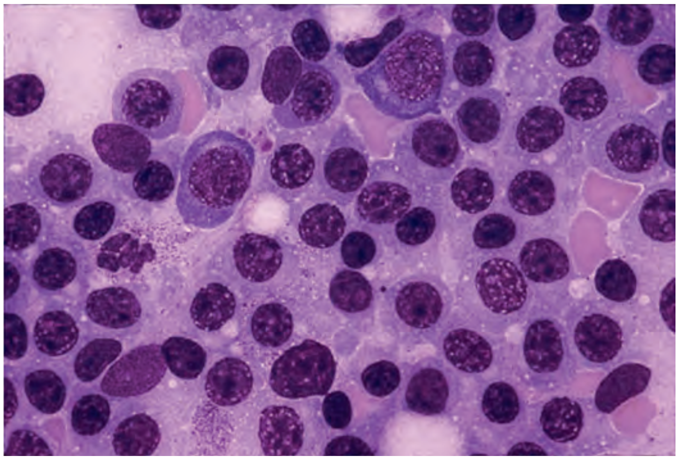


Figure 92.1 MARROW FILM FROM A PATIENT WITH WALDENSTRÖM MACROGLOBULINEMIA. Note infiltrate of mature lymphocytes, lymphoplasmacytic cells, and plasma cells. *(Used with permission from Marvin J. Stone, MD.)*

sensitive allele-specific PCR is employed using both CD19-sorted and unsorted BM cells.[35–39] By comparison, $MYD88^{L265P}$ was absent in myeloma samples, including IgM myeloma, and was expressed in a small subset (6% to 10%) of patients with marginal zone lymphoma, who surprisingly have WM-related features.[35–37,40] By PCR assays, 50% to 80% of patients with IgM MGUS also express $MYD88^{L265P}$, and expression of this mutation was associated with increased risk for malignant progression.[35–37,41] The presence of $MYD88^{L265P}$ in patients with IgM MGUS suggests a role for this mutation as an early oncogenic driver, and other mutations and/or copy number alterations leading to abnormal gene expression are likely to promote disease progression.[28]

The impact of $MYD88^{L265P}$ to growth and survival signaling in WM cells has been addressed in several studies (Fig. 92.2). Knockdown of *MYD88* decreased survival of $MYD88^{L265P}$-expressing WM cells, whereas survival was enhanced by knock-in of $MYD88^{L265P}$ versus wild-type (WT) *MYD88*.[42] The discovery of a mutation in MYD88 is of significance, given its role as an adaptor molecule in Toll-like receptor (TLR) and interleukin-1 receptor (IL-1R) signaling.[43] All TLRs except for TLR3 use MYD88 to facilitate their signaling. Following TLR or IL-1R stimulation, MYD88 is recruited to the activated receptor complex as a homodimer, which then complexes with interleukin receptor-associated kinase 4 (IRAK4) and activates IRAK1 and IRAK2.[44–46] Tumor necrosis factor receptor-associated factor 6 is then activated by IRAK1, leading to NFκB activation via IκBα phosphorylation.[47] Use of inhibitors of the MYD88 pathway led to decreased IRAK1 and IκBα phosphorylation, as well as to survival of $MYD88^{L265P}$-expressing WM cells. These observations are of particular relevance to WM because NFκB signaling is important for WM growth and survival.[48] Bruton's tyrosine kinase (BTK) is also activated by $MYD88^{L265P}$.[42] Activated BTK coimmunoprecipitates with MYD88, which could be abrogated by use of a BTK inhibitor,

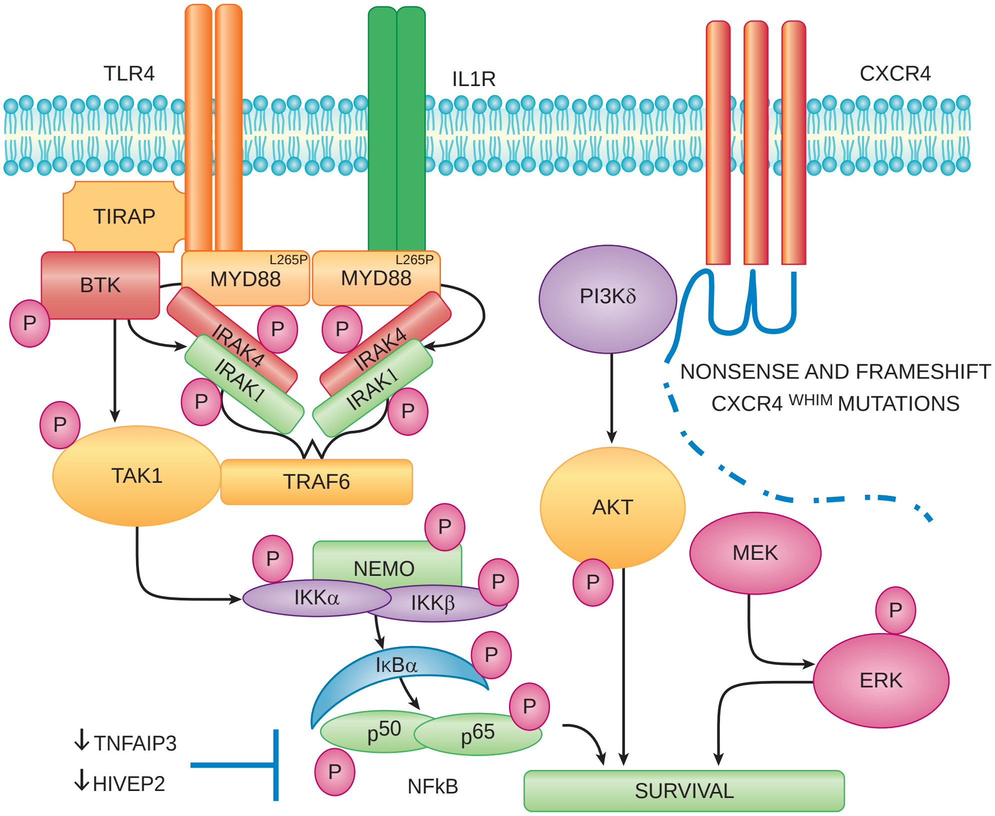


Figure 92.2 *$MYD88^{L265P}$* AND *$CXCR4^{WHIM}$* MUTATIONS ARE HIGHLY PREVALENT IN PATIENTS WITH WALDENSTRÖM MACROGLOBULINEMIA AND TRIGGER TRANSCRIPTIONAL FACTORS THAT INCLUDE NUCLEAR FACTOR-κB (NFκB), AKT, AND EXTRACELLULAR SIGNAL-REGULATED KINASE (ERK) THAT SUPPORT THE GROWTH AND SURVIVAL OF LYMPHOPLASMACYTIC CELLS. *BTK*, Bruton's tyrosine kinase; *CXCR4*, C-X-C chemokine receptor type 4; *HIVEP2*, human immunodeficiency virus type I enhancer binding protein 2; *IKBα*, nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor α; *IKK*, inhibitor of nuclear factor-κB kinase; *IL1R*, interleukin-1 receptor; *IRAK*, interleukin receptor-associated kinase; *MEK*, mitogen-activated protein kinase kinase; *NEMO*, nuclear factor-κB essential modulator; *NFκB*, nuclear factor-κB; *PI3Kδ*, phosphoinositide 3-kinase δ; *TAK1*, transforming growth factor β-activated kinase 1; *TIRAP*, Toll-interleukin 1 receptor domain-containing adaptor protein; *TLR4*, Toll-like receptor 4; *TNFAIP3*, tumor necrosis factor α-induced protein 3; *TRAF6*, tumor necrosis factor α receptor-associated factor 6; *WHIM*, warts, hypogammaglobulinemia, infections, and myelokathexis syndrome.

and overexpression of *MYD88*^{L265P} but not WT *MYD88* triggers BTK activation. Knockdown of *MYD88* by lentiviral transfection or use of a MYD88 homodimerization inhibitor also abrogated BTK activation in *MYD88*^{L265P}-mutated WM cells.

CXCR4 WHIM MUTATIONS

The second most common somatic mutation after *MYD88*^{L265P} revealed by WGS was found in the C-terminus of the C-X-C chemokine receptor type 4 (CXCR4) receptor. These mutations are present in 30% to 35% of patients with WM, and they impact serine phosphorylation sites that regulate CXCR4 signaling by its only known ligand, SDF-1α (CXCL12).[28,49–51] The location of somatic mutations found in the C-terminus of *CXCR4* in WM are similar to those observed in the germline of patients with WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis, retention of neutrophils within the marrow) syndrome, a congenital immunodeficiency disorder characterized by chronic noncyclic neutropenia.[52] Patients with WHIM syndrome exhibit impaired CXCR4 receptor internalization following SDF-1α stimulation, which results in persistent CXCR4 activation and myelokathexis.[53]

In patients with WM, two classes of *CXCR4* mutations occur in the C-terminus. These include nonsense *(CXCR4*^{NS}*)* mutations that truncate the distal 15– to 20–amino acid region and frameshift *(CXCR4*^{FS}*)* mutations that comprise a region of up to 40 amino acids in the C-terminal domain.[28,49] Nonsense and frameshift mutations are almost equally divided among patients with WM with *CXCR4* somatic mutations, and over 30 different types of *CXCR4* mutations have been identified in patients with WM.[28,49] Preclinical studies with WM cells engineered to express nonsense and frameshift *CXCR4*-mutated receptors have shown enhanced and sustained AKT and extracellular signal-regulated kinase signaling following SDF-1α relative to wild type CXCR4 (*CXCR4*^{WT}) (see Fig. 92.2), as well as increased cell migration, adhesion, growth, and survival, and also drug resistance of WM cells.[50,54,55]

Other Somatic Events

Many copy number alterations have been revealed in patients with WM that impact growth and survival pathways. Frequent loss of *HIVEP2* (80%) and *TNAIP3* (50%) genes that are negative regulators of NFκB expression (see Fig. 92.2), as well as *LYN* (70%) and *IBTK* (40%) that modulate B-cell receptor signaling have been revealed by WGS.[28] WGS has also revealed common defects in chromatin remodeling, with somatic mutations in ARID1A present in 17% and loss of *ARID1B* in 70% of patients with WM. Both *ARID1A* and *ARID1B* are members of the SWI/SNF family of proteins, and are thought to exert their effects via p53 and cyclin-dependent kinase inhibitor 1A regulation. TP53 is mutated in 7% of sequenced WM genomes, whereas *PRDM2* and *TOP1* that participate in TP53-related signaling are deleted in 80% and 60% of patients with WM, respectively.[28] Taken together, somatic events that contribute to impaired DNA damage response are also common in WM.

IMPACT OF WM GENOMICS ON CLINICAL PRESENTATION

The importance of *MYD88* and *CXCR4* mutations in the clinical presentation of patients with WM has been established. Significantly higher BM involvement, serum IgM levels, hyperviscosity syndrome, and acquired von Willebrand disease were observed in the patients with *MYD88*^{L265P}*CXCR4*^{NS} mutations.[49,56] Patients with *MYD88*^{L265P}*CXCR4*^{FS} or *MYD88*^{L265P}*CXCR4*^{WT} had intermediate BM and serum IgM levels; those with *MYD88*^{WT}*CXCR4*^{WT} showed the lowest BM disease burden. Fewer patients with *MYD88*^{L265P} and *CXCR4*$^{FS\ or\ NS}$ than with *MYD88*^{L265P}*CXCR4*^{WT} presented with adenopathy, further delineating differences in disease tropism based on *CXCR4* status. Despite the more aggressive presentation associated with *CXCR4*^{NS} genotype, risk of death was not impacted by *CXCR4* mutation status. Risk of death and histological transformation to more aggressive lymphoma were found to be higher in patients with the *MYD88*^{WT} versus the *MYD88*^{L265P} genotype.[49,57]

MARROW MICROENVIRONMENT

In patients with WM, increased numbers of mast cells are found in the BM, where they are usually admixed with tumor cell aggregates (Fig. 92.3).[13,17,58] The role of mast cells in WM has been investigated in one study in which coculture of primary autologous or mast cell lines with WM lymphoplasmacytic cells resulted in dose-dependent WM cell proliferation and/or tumor colony formation through CD40 ligand (CD40L) signaling.[58] WM cells release soluble CD27 (sCD27), which may be triggered by cleavage of membrane bound CD27 by matrix metalloproteinase 8.[59] sCD27 levels are elevated in the serum of patients with WM and follow disease burden in mice engrafted with WM cells, as well as in patients with WM.[60] sCD27 triggers the upregulation of CD40L as well as a proliferation-inducing ligand on mast cells derived from patients with WM, and mast cell lines through its receptor CD70. Modeling in mice treated with a CD70-blocking antibody shows inhibition of tumor cell growth, suggesting that WM cells require a microenvironmental support system for their growth and survival.[61] High levels of CXCR4 and very late antigen-4 (VLA-4) are expressed by WM cells.[60] In blocking experiment studies, CXCR4 was shown to support migration of WM cells, whereas VLA-4 contributed to adhesion of WM cells to BM stromal cells.[60]

CLINICAL FEATURES

Table 92.1 provides the clinical and laboratory features at the time of diagnosis of patients with WM in one large institutional study.[15] Unlike most indolent lymphomas, splenomegaly and lymphadenopathy are uncommon (≤15%). Purpura is frequently associated with

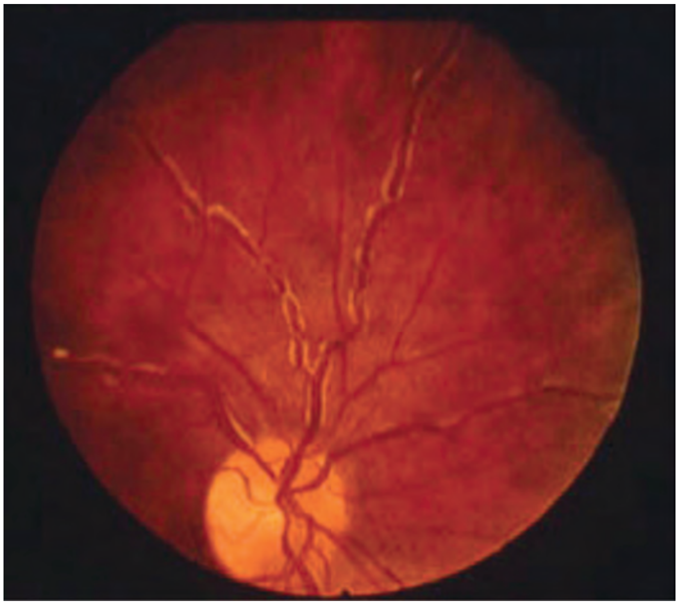


Figure 92.3 FUNDUSCOPIC EXAMINATION OF A PATIENT WITH WALDENSTRÖM MACROGLOBULINEMIA WITH HYPERVISCOSITY-RELATED CHANGES, INCLUDING DILATED RETINAL VESSELS, HEMORRHAGES, AND "VENOUS SAUSAGING." The white material at the edge of the veins may be cryoglobulin. *(Used with permission from Marvin J. Stone, MD.)*

TABLE 92.1 **Clinical and Laboratory Findings for 356 Consecutive Newly Diagnosed Patients With Waldenström Macroglobulinemia**

	Median	Range	Normal Reference Range
Age (years)	58	32–91	NA
Sex (male/female)	215/141		NA
Marrow involvement (% of area on slide)	30	5–95	NA
Adenopathy (% of patients)	15		NA
Splenomegaly (% of patients)	10		NA
IgM (mg/dL)	2620	270–12,400	40–230
IgG (mg/dL)	674	80–2770	700–1600
IgA (mg/dL)	58	6–438	70–400
Serum viscosity (cp)	2.0	1.1–7.2	1.4–1.9
Hematocrit (%)	35	17–45	35–44
Platelet count ($\times 10^9$/L)	275	42–675	155–410
White cell count ($\times 10^9$/L)	6.4	1.7–22	3.8–9.2
β_2-M (mg/dL)	2.5	0.9–13.7	0–2.7
LDH (U/mL)	313	61–1701	313–618

β_2 M, β_2-Microglobulin; *cp*, centipoise; *Ig*, immunoglobulin; *LDH*, lactate dehydrogenase; *NA*, not applicable.
Data from patients seen at the Dana-Farber Cancer Institute, Boston, MA.

cryoglobulinemia and in rare circumstances with light-chain (AL) amyloidosis. Hemorrhagic and neuropathic manifestations are multifactorial (see IgM-Related Neuropathy section below). The morbidity associated with WM is caused by the co-occurrence of two main components: tissue infiltration by neoplastic cells and, importantly, the physicochemical and immunologic properties of the monoclonal IgM. As shown in Table 92.2, the monoclonal IgM can produce clinical manifestations through several different mechanisms related to its physicochemical properties, nonspecific interactions with other proteins, antibody activity, and tendency to deposit in tissues.[62–64]

MORBIDITY MEDIATED BY THE EFFECTS OF IMMUNOGLOBULIN M

Hyperviscosity Syndrome

The increased plasma IgM levels lead to increased blood hyperviscosity and complications.[65] The mechanisms behind the marked increase in the resistance to blood flow and the resulting impaired transit through the microcirculatory system are complex.[65–68] The main determinants are (1) a high concentration of monoclonal IgMs, which may form aggregates and may bind water through their carbohydrate component; and (2) the interaction of IgMs with blood cells. Monoclonal IgM increases red cell aggregation (rouleau formation) and red cell internal viscosity while reducing red cell deformability. The presence of cryoglobulins contributes to increasing blood viscosity, as well as the tendency to induce erythrocyte aggregation. Serum viscosity is negligible up to an IgM concentration up to 30 g/L, then increases sharply at higher levels, with a median time to symptomatic hyperviscosity of 3 months when IgM levels surpass 60 g/L.[69] Increased plasma viscosity may also contribute to inappropriately low erythropoietin production, which is the major reason for anemia in these patients.[68] Renal synthesis of erythropoietin is inversely correlated with plasma viscosity. Clinical manifestations are related to circulatory disturbances that can best be appreciated by ophthalmoscopy, which shows distended and tortuous retinal veins, hemorrhages, and papilledema (Fig. 92.4).[70] Symptoms usually occur when the monoclonal IgM concentration exceeds 50 g/L or when serum viscosity is greater than 4.0 centipoises (cp), but there is individual variability, with some patients showing no evidence of hyperviscosity even at 10 cp.[65] The most common symptoms are oronasal mucosal bleeding, visual disturbances because of retinal bleeding, and dizziness that rarely may lead to stupor or coma. Heart failure can be aggravated, particularly in the elderly, owing to increased blood viscosity, expanded plasma volume, and anemia. Inappropriate red cell transfusions can exacerbate hyperviscosity and may precipitate cardiac failure.

Cryoglobulinemia

The monoclonal IgM can behave as a type I cryoglobulin in up to 20% of patients, and it leads to no symptoms in most cases.[15,64,71,72] Cryoprecipitation is dependent mainly on the concentration of monoclonal IgM; for this reason, plasmapheresis or plasma exchange

TABLE 92.2 **Physicochemical and Immunologic Properties of the Monoclonal Immunoglobulin M Protein in Waldenström Macroglobulinemia**

Properties of IgM Monoclonal Protein	Diagnostic Condition	Clinical Manifestations
Pentameric structure	Hyperviscosity	Headaches, blurred vision, epistaxis, retinal hemorrhages, leg cramps, impaired mentation, intracranial hemorrhage
Precipitation on cooling	Cryoglobulinemia (type I)	Raynaud phenomenon, acrocyanosis, ulcers, purpura, cold urticaria
Autoantibody activity to myelin-associated glycoprotein, ganglioside M_1, sulfatide moieties on peripheral nerve sheaths	Peripheral neuropathies	Sensorimotor neuropathies, painful neuropathies, ataxic gait, bilateral foot drop
Autoantibody activity to IgG	Cryoglobulinemia (type II)	Purpura, arthralgia, renal failure, sensorimotor neuropathies
Autoantibody activity to red blood cell antigens	Cold agglutinins	Hemolytic anemia, Raynaud phenomenon, acrocyanosis, livedo reticularis
Tissue deposition as amorphous aggregates	Organ dysfunction	Skin: bullous skin disease, papules, Schnitzler syndrome Gastrointestinal: diarrhea, malabsorption, bleeding Kidney: proteinuria, renal failure (light-chain component)
Tissue deposition as amyloid fibrils (light-chain component most commonly)	Organ dysfunction	Fatigue, weight loss, edema, hepatomegaly, macroglossia, organ dysfunction of involved organs (heart, kidney, liver, peripheral sensory and autonomic nerves)

IgM, Immunoglobulin M.

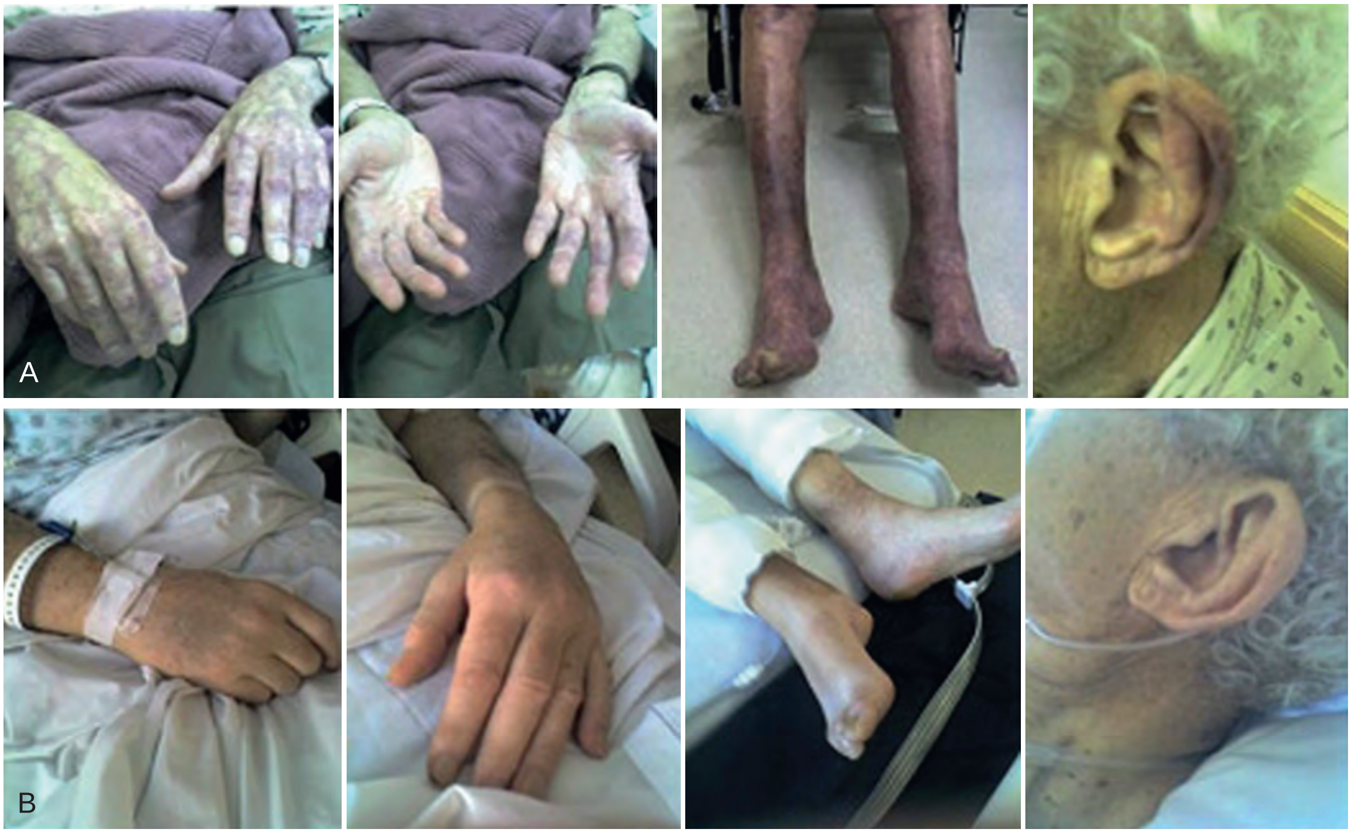


Figure 92.4 Cryoglobulinemia manifesting with severe acrocyanosis in a patient with Waldenström macroglobulinemia (A) before and (B) following warming and plasmapheresis.

is commonly effective in this condition. Symptoms result from impaired blood flow in small vessels and include Raynaud's phenomenon; acrocyanosis; necrosis of the regions most exposed to cold, such as the tips of the nose, ears, fingers, and toes (Fig. 92.5); malleolar ulcers; purpura; and cold urticaria. Renal manifestations are infrequent. Mixed cryoglobulins (type II) consisting of IgM-IgG complexes may be associated with HCV infection.[73]

Autoantibody Activity

Monoclonal IgM may exert its pathogenic effects through specific recognition of autologous antigens, the most notable being nerve constituents, immunoglobulin determinants, and red blood cell antigens.

Immunoglobulin M–Related Neuropathy

IgM-related peripheral neuropathy is common in patients with WM, with estimated prevalence rates of 5% to 40%.[73–75] Approximately 8% of idiopathic neuropathies are associated with a monoclonal gammopathy, with a preponderance of IgM (60%), followed by IgG (30%) and IgA (10%).[76,77] The nerve damage is mediated by diverse pathogenetic mechanisms: (1) IgM antibody activity toward nerve constituents causing demyelinating polyneuropathies; (2) endoneurial granulofibrillar deposits of IgM without antibody activity, associated with axonal polyneuropathy; (3) occasionally by tubular deposits in the endoneurium associated with IgM cryoglobulin; and, rarely, (4) amyloid deposits or neoplastic cell infiltration of nerve structures.[75,78]

Half of the patients with IgM neuropathy have a distinctive clinical syndrome that is associated with antibodies against a minor 100 kDa glycoprotein component of nerve known as the *myelin-associated glycoprotein* (MAG). Anti-MAG antibodies are generally monoclonal IgMκ and usually also exhibit reactivity with other glycoproteins or glycolipids that share antigenic determinants with MAG.[79–81] The anti–MAG-related neuropathy is typically distal and symmetrical, affecting both motor and sensory functions; it is slowly progressive with a long period of stability.[74,82] Most patients present with sensory complaints (paresthesias, aching discomfort, dysesthesias, or lancinating pains); imbalance and gait ataxia, owing to lack proprioception; and muscle atrophy in advanced stage. Patients with predominantly demyelinating sensory neuropathy in association with monoclonal IgM to gangliosides with disialosyl moieties, such as GD1b, GD3, GD2, GT1b, and GQ1b, have also been reported.[83,84] Anti-GD1b and anti-GQ1b antibodies were associated with sensory ataxic neuropathy. These antiganglioside monoclonal IgMs present core clinical features of chronic ataxic neuropathy, sometimes associated with ophthalmoplegia and/or red blood cell cold agglutinating activity. The disialosyl epitope is also present on red blood cell glycophorins, thereby accounting for the red cell cold agglutinin activity of anti-Pr2 specificity.[85,86] Monoclonal IgM proteins that bind to gangliosides with a terminal trisaccharide moiety, including ganglioside M_2 and GalNac-GD1A, are associated with chronic demyelinating neuropathy and severe sensory ataxia, unresponsive to glucocorticoids.[87] Antiganglioside IgM proteins may also cross-react with lipopolysaccharides of *Campylobacter jejuni*, an infection known to precipitate the Miller Fisher syndrome, a variant of Guillain-Barré syndrome.[85] Thus, molecular mimicry may play a role in this condition. Anti-sulfatide monoclonal IgM proteins, associated with sensory-sensorimotor neuropathy, have been detected in 5% of patients with IgM monoclonal gammopathy and neuropathy.[88] Motor neuron disease has been reported in patients with WM and monoclonal IgM with anti-GM_1 and sulfoglucuronyl paragloboside activity.[89] Polyneuropathy, organomegaly, endocrinopathy, M protein, and skin changes (the POEMS syndrome) are rare in patients with WM.[90]

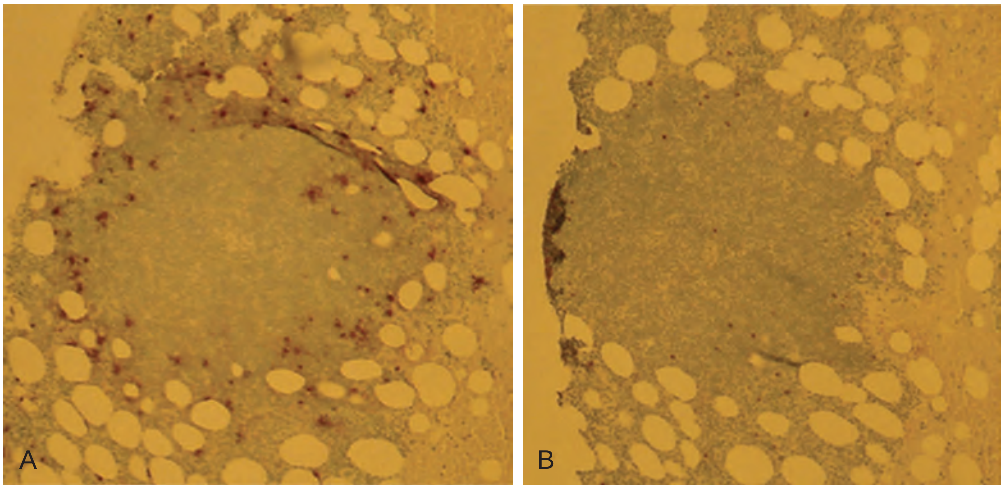


Figure 92.5 MARROW CLOT SECTION. (A) Tryptase-staining mast cells surrounding a nodule of lymphoplasmacytic cells in a patient with Waldenström macroglobulinemia. (B) Mast cells in the same section exhibit strong CD40 ligand signaling, which has been shown to support (at least in part) the growth and survival of lymphoplasmacytic cells.

Cold Agglutinin Hemolytic Anemia

Monoclonal IgMs may have cold agglutinin activity; they recognize specific red cell antigens at temperatures below 37°C, producing a chronic hemolytic anemia (see Chapter 47). This disorder occurs in less than 10% of patients with WM and is associated with cold agglutinin titers greater than 1:1000 in most cases.[91] The monoclonal component is usually an IgMκ and reacts most commonly with red cell I/i antigens, resulting in complement fixation and activation.[92,93] Mild to moderate chronic hemolytic anemia can be exacerbated after cold exposure. Hemoglobin levels usually remain above 70 g/L. The hemolysis is usually extravascular, mediated by the removal of C3b opsonized red cells by the mononuclear phagocytes, primarily in the liver. Intravascular hemolysis from complement destruction of red blood cell membrane is infrequent. The agglutination of red cells in the skin circulation also causes Raynaud syndrome, acrocyanosis, and livedo reticularis. Macroglobulins with the properties of both cryoglobulins and cold agglutinins with anti-Pr blood group specificity can occur. These properties may have as a common basis the binding of the sialic acid–containing carbohydrate present on red blood cell glycophorins, which involve the Pr blood group and Ig molecules. Several other macroglobulins with antibody activity toward autologous antigens (e.g., phospholipids, tissue and plasma proteins), and foreign ligands have also been described.

Immunoglobulin M Tissue Deposition

The monoclonal IgM can be deposited into several tissues as amorphous aggregates. Linear deposition of monoclonal IgM along the skin basement membrane is associated with bullous skin disease.[94] Amorphous IgM deposits in the dermis result in IgM storage papules on the extensor surface of the extremities, referred to as *macroglobulinemia cutis*.[95] Deposition of monoclonal IgM in the lamina propria and/or submucosa of the intestine may be associated with diarrhea, malabsorption, and gastrointestinal bleeding.[96,97] Kidney involvement is less common and less severe in WM than in myeloma, probably because the amount of light chain that is excreted in the urine is generally less in WM than in myeloma and because of the absence of contributing factors, such as hypercalcemia. Urinary cast nephropathy, however, has been reported in WM.[98] On the other hand, the IgM macromolecule is more susceptible to being trapped in the glomerular loops, where ultrafiltration presumably contributes to its precipitation, forming subendothelial deposits of aggregated IgM proteins that occlude the glomerular capillaries.[99] Mild and reversible proteinuria may result, and most patients are asymptomatic. The deposition of monoclonal light chain as fibrillar amyloid deposits (AL amyloidosis) (Chapter 93) is uncommon in patients with WM.[100] The clinical manifestations and prognosis are similar to those of other patients with AL amyloidosis, with involvement occurring in the heart (44%), kidneys (32%), liver (14%), lungs (10%), peripheral or autonomic nerves (38%), and soft tissues (18%). The incidence of cardiac and pulmonary involvement is higher in patients with monoclonal IgM than in those with other immunoglobulin isotypes. The association of WM with reactive or ATTR amyloidosis has been documented rarely.[101–103] Simultaneous occurrence of fibrillary glomerulopathy, characterized by glomerular deposits of wide noncongophilic fibrils and amyloid deposits, has been described.[104]

Manifestations Related to Tissue Infiltration by Neoplastic Cells

Tissue infiltration by neoplastic cells is uncommon but can involve various organs and tissues, including the liver, spleen, lymph nodes, lungs, gastrointestinal tract, kidneys, skin, eyes, and central nervous system.

Lung

Pulmonary involvement in the form of masses, nodules, diffuse infiltrate, or pleural effusions is uncommon; the overall incidence of pulmonary and pleural findings is approximately 4%.[105–107] Cough is the most common presenting symptom, followed by dyspnea and chest pain. Chest radiographic findings include parenchymal infiltrates, confluent masses, and effusions.

Gastrointestinal Tract

Malabsorption, diarrhea, bleeding, or obstruction may indicate involvement of the gastrointestinal tract at the level of the stomach, duodenum, or small intestine.[108–111]

Renal System

In contrast to myeloma, infiltration of the kidney interstitium with lymphoplasmacytoid cell can occur in WM, and renal or perirenal masses are not uncommon.[112,113]

Skin

The skin can be the site of dense lymphoplasmacytic infiltrates, similar to those seen in the liver, spleen, and lymph nodes, forming cutaneous plaques and, rarely, nodules.[114] Chronic urticaria and IgM gammopathy are the two cardinal features of the Schnitzler syndrome, which is not usually associated initially with clinical features of WM, although evolution to WM is not uncommon.[115] Thus close follow-ups of such patients are important.

Joints

Invasion of articular and periarticular structures by WM malignant cells is rarely reported.[116]

Eye

The neoplastic cells can infiltrate the periorbital structures, lacrimal gland, and retro-orbital lymphoid tissues, resulting in ocular nerve palsies.[117,118]

Central Nervous System

Direct infiltration of the central nervous system by monoclonal lymphoplasmacytic cells as infiltrates or as tumors constitutes the rarely observed Bing-Neel syndrome, characterized clinically by confusion, memory loss, disorientation, and sensory or motor dysfunction.[120,121]

LABORATORY FINDINGS

Blood Abnormalities

Anemia is the most common finding in patients with symptomatic WM and is caused by a combination of factors: decrease in red cell survival, impaired erythropoiesis, moderate plasma volume expansion, hepcidin production leading to an iron reuse defect, and blood loss from the gastrointestinal tract.[15,121,122] Blood films are usually normocytic and normochromic, and rouleau formation is often pronounced. Mean red cell volume may be elevated spuriously owing to erythrocyte aggregation. In addition, the hemoglobin estimate can be inaccurate (i.e., falsely high) because of interaction between the monoclonal protein and the diluent used in some automated analyzers.[118] Leukocyte and platelet counts are usually within their reference ranges at presentation, although patients may occasionally present with severe thrombocytopenia. Monoclonal B lymphocytes expressing surface IgM and late-differentiation B-cell markers are uncommonly detected in blood by flow cytometry. A raised erythrocyte sedimentation rate is almost always present and may be the first clue to the presence of macroglobulinemia. The clotting abnormality detected most frequently is the prolongation of thrombin time. AL amyloidosis (Chapter 93) should be suspected in all patients with nephrotic syndrome, cardiomyopathy, hepatomegaly, or peripheral neuropathy. Diagnosis requires the demonstration of green birefringence under polarized light of amyloid deposits stained with Congo red with further confirmation of light chain amyloidosis by mass spectrometry.[123]

MARROW FINDINGS

Central to the diagnosis of WM is the demonstration, by trephine biopsy, of marrow infiltration by a lymphoplasmacytic cell population characterized by small lymphocytes with evidence of plasmacytoid and plasma cell maturation (see Fig. 92.1).[1,13] The pattern of marrow infiltration may be diffuse, interstitial, or nodular, usually with an intertrabecular pattern of infiltration. A solely paratrabecular pattern of infiltration is unusual and should raise the possibility of follicular lymphoma.[1] The marrow cell immunophenotype should be confirmed by flow cytometry and/or immunohistochemistry. The cell immunoprofile sIgM$^+$CD19$^+$CD20$^+$CD22$^+$CD79$^+$ is characteristic of WM.[13,124,125] Up to 20% of cases may express either CD5, CD10, or CD23.[18] In these cases, chronic lymphocytic leukemia and mantle cell lymphoma should be excluded. “Intranuclear” periodic acid-Schiff–positive inclusions (Dutcher-Fahey bodies)[126] consisting of IgM deposits in the perinuclear space, and sometimes in intranuclear vacuoles, may be seen occasionally in lymphoid cells. An increased number of mast cells, usually in association with the lymphoid aggregates, is commonly found, and their presence may help in differentiating WM from other B-cell lymphomas (see Fig. 92.3).[13] *MYD88*^{L265P} testing of BM samples has been incorporated into many clinical laboratories and may help in clarifying the diagnosis of WM from other IgM-secreting entities, such as IgM multiple myeloma or marginal zone lymphoma.[34–38] The use of peripheral blood B cells may also permit determination of *MYD88*^{L265P} status by allele-specific PCR assays, particularly in untreated patients with WM.

IMMUNOLOGIC ABNORMALITIES

High-resolution electrophoresis combined with immunofixation of serum and urine is recommended for identification and characterization of the IgM monoclonal protein. The light chain of the monoclonal IgM is κ in 75% to 80% of patients. More than one M component may be present. The concentration of the serum monoclonal protein is very variable, but in most cases, it lies within the range of 15 to 45 g/L. Densitometry should be adopted to determine IgM levels for serial evaluations because nephelometry is unreliable and shows large laboratory variation. The presence of cold agglutinins or cryoglobulins may affect determination of IgM levels, and, therefore, testing for cold agglutinins and cryoglobulins should be performed at diagnosis. If present, subsequent serum samples should be analyzed at 37°C for determination of serum monoclonal IgM level. Although Bence Jones proteinuria is frequently present, it exceeds 1 g/24 h in only 3% of cases. Whereas IgM levels are elevated in patients with WM, IgA and IgG levels are most often depressed and might not recover after successful treatment.[127]

SERUM VISCOSITY

Because of their large size (molecular weight almost 1 million), most IgM molecules are retained within the intravascular compartment and can exert an undue effect on serum viscosity.[63] Serum viscosity can be measured if the patient has signs or symptoms of hyperviscosity syndrome, though levels are often slow to be reported and erratic owing to a lack of standardization in most clinical laboratories.[15] As such, serum IgM levels may be more expedient and relied upon. Patients typically become symptomatic at serum viscosity levels of 4.0 cp and above, which correspond to serum IgM levels above 6,000 mg/dL.[128,129] Patients may be symptomatic at lower serum viscosity and IgM levels, and in these patients cryoglobulins may be present. Recurring nosebleeds, headaches, and visual disturbances are common symptoms in patients with symptomatic hyperviscosity.[15] Funduscopy is an important indicator of clinically relevant hyperviscosity. Among the first clinical signs of hyperviscosity are the appearance of peripheral and midperipheral dot- and blot-like hemorrhages in the retina, which are best appreciated with indirect ophthalmoscopy and scleral depression.[70] In more severe cases of hyperviscosity, dot, blot, and flame-shaped hemorrhages can appear in the macular area along with markedly dilated and tortuous veins with focal constrictions resulting in “venous sausaging,” as well as papilledema (see Fig. 92.4).

IMAGING

Magnetic resonance imaging (MRI) of the spine in conjunction with computed tomography (CT) of the abdomen and pelvis are useful in evaluating disease status.[130] Marrow involvement can be documented by MRI studies of the spine in more than 90% of patients; CT of the abdomen and pelvis demonstrates enlarged nodes in approximately 20% of patients with WM at diagnosis, but this proportion may be higher at relapse.[130]

LYMPH NODE BIOPSY

Lymph node biopsy may show preserved architecture or replacement by infiltration of neoplastic cells with lymphoplasmacytoid, lymphoplasmacytic, or polymorphous cytologic patterns.

TREATMENT

Initiating Treatment

As part of the Second International Workshop on Waldenström Macroglobulinemia, a consensus panel was organized to recommend criteria for the initiation of therapy in patients with WM.[131] The panel recommended that initiation of therapy should not be based on the IgM level per se, because this may not correlate with the clinical manifestations of WM. The consensus panel did, however, agree that initiation of therapy is appropriate for patients with constitutional symptoms, such as recurrent fever, night sweats, fatigue as a consequence of anemia, or weight loss. Progressive symptomatic lymphadenopathy and/or splenomegaly provide additional reasons to begin therapy. Anemia with a hemoglobin value ≤10 g/dL or a platelet count ≤100 × 10^9/L owing to marrow infiltration also justifies treatment. Certain complications, such as hyperviscosity syndrome, symptomatic sensorimotor peripheral neuropathy, systemic amyloidosis, renal insufficiency, or symptomatic cryoglobulinemia, may also be indications for therapy.[15,131,132]

Initial Therapy

The International Workshops on Waldenström Macroglobulinemia have formulated consensus recommendations for both initial therapy and therapy for refractory disease based on the best available evidence. The most recent recommendations emerged from the Tenth International Workshop on Waldenström Macroglobulinemia.[133] Individual patient considerations, including the presence of cytopenias, need for more rapid disease control, age, and candidacy for autologous transplant therapy, should be taken into account in making the choice of the drugs to use. For patients who are candidates for autologous stem cell transplant (SCT), which typically is reserved for those patients younger than 70 years of age, the panel recommended that exposure to alkylating agents or nucleoside analogues should be limited. The use of nucleoside analogues should be approached cautiously in patients with WM because there appears to be an increased risk for the development of disease transformation as well as myelodysplasia and acute myeloid leukemia. For patients who present with symptomatic hyperviscosity, cryoglobulinemia or cold agglutinin anemia, rapid control of the disease can be achieved with plasmapheresis. However, plasmapheresis should be considered a temporary control measure and should be immediately followed by definitive therapy. A proposed algorithm for initial treatment selection based on genomic profile in WM patients is shown in Fig. 92.6.[134]

Oral Alkylating Agents

Oral alkylating drugs, alone and in combination therapy with glucocorticoids, have been evaluated extensively in the treatment of WM. Chlorambucil has been administered on both a continuous (i.e., daily dose schedule) and an intermittent schedule. In a prospective, randomized study, a significant difference in the overall response rate (ORR) between these schedules was not observed,[135] although the median

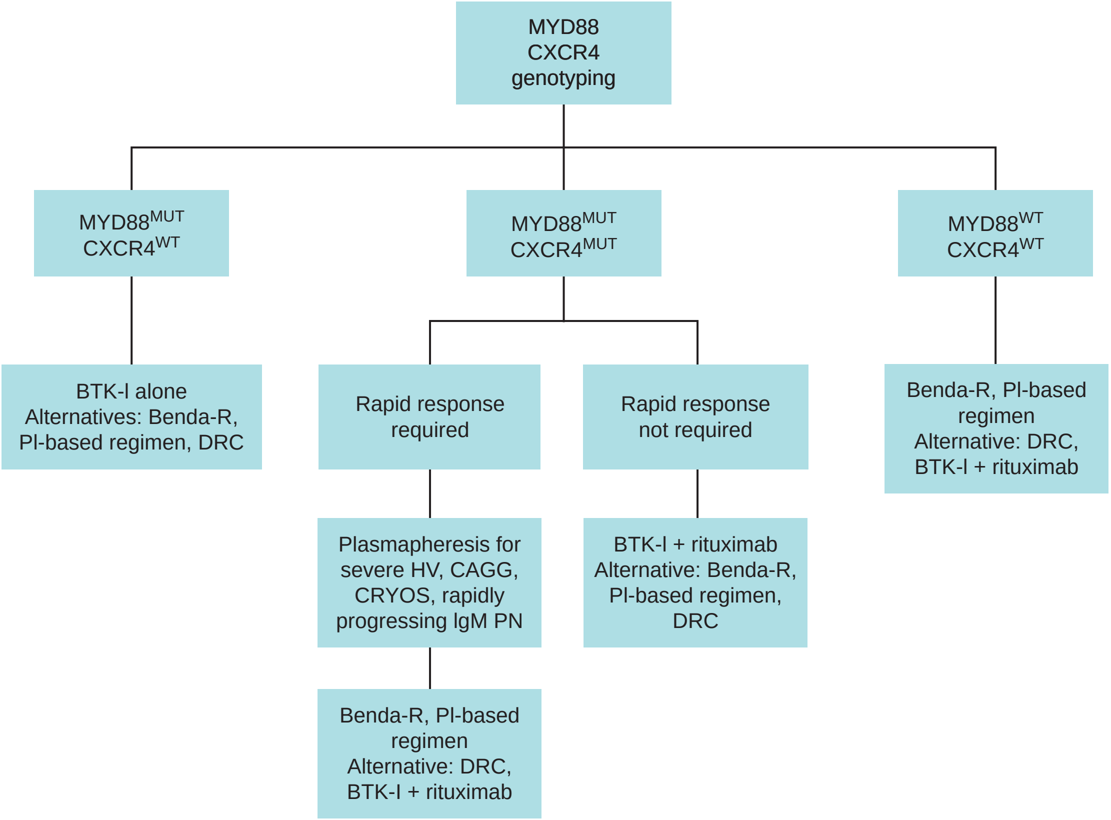

MYD88
CXCR4
genotyping
MYD88^{MUT} CXCR4^{WT}
MYD88^{MUT} CXCR4^{MUT}
MYD88^{WT} CXCR4^{WT}
BTK-I alone
Alternatives: Benda-R, PI-based regimen, DRC
Rapid response required
Rapid response not required
Benda-R, PI-based regimen
Alternative: DRC, BTK-I + rituximab
Plasmapheresis for severe HV, CAGG, CRYOS, rapidly progressing IgM PN
BTK-I + rituximab
Alternative: Benda-R, PI-based regimen, DRC
Benda-R, PI-based regimen
Alternative: DRC, BTK-I + rituximab

Figure 92.6 GENOMIC-BASED TREATMENT ALGORITHM FOR SYMPTOMATIC TREATMENT-NAÏVE PATIENTS WITH WALDENSTRÖM MACROGLOBULINEMIA (WM). *Benda-R*, bendamustine and rituximab; *BTK-I*, Bruton tyrosine kinase inhibitor; *CAGG*, cold agglutinin disease; *CRYOS*, cryoglobulins; *DRC*, dexamethasone, rituximab, cyclophosphamide; *HV*, hyperviscosity; *PI*, proteasome inhibitor; *PN*, progressive neuropathy. *(From Treon SP, Xu L, Guerrera ML, et al. Genomic landscape of Waldenström macroglobulinemia and its impact on treatment strategies. J Clin Oncol. 2020;38(11):1198–1208.)*

response duration was greater for patients receiving intermittent- versus continuous-dose chlorambucil (46 vs. 26 months). Despite the favorable median response duration in this study for use of the intermittent schedule, differences in the median overall survival were not observed. Moreover, an increased incidence of myelodysplasia and acute myeloid leukemia was observed with the intermittent (3 of 22 patients) versus the continuous (0 of 24 patients) chlorambucil schedule prompted the preference for use of continuous chlorambucil dosing. The use of glucocorticoids in combination with alkylating agent therapy has also been explored, resulting in a major response (i.e., reduction of IgM by more than 50%) in 72% of patients.[136] Alkylating agent regimens employing melphalan and cyclophosphamide in combination with glucocorticoids have also been examined.[137,138] This approach produced slightly higher ORRs and response durations, although the benefit of these more complex regimens over chlorambucil remains to be demonstrated. Pretreatment factors associated with shorter survival of patients receiving single-agent chlorambucil were age greater than 60 years, male sex, hemoglobin less than 10 g/dL, leukocytes less than 4×10^9/L, and platelets less than 150×10^9/L. Organomegaly, signs of hyperviscosity, renal failure, monoclonal IgM level, blood lymphocytosis, and percentage of marrow lymphoid cells were not significantly correlated with survival.[139] Additional factors to be taken into account in considering alkylating agent therapy for patients with WM include necessity for more rapid disease control, given the slow response, as well as consideration for preserving stem cells in patients who are candidates for autologous SCT. A large, randomized study showed an inferior response rate and time to progression in patients with WM receiving chlorambucil versus fludarabine, as well as a higher incidence of secondary malignancies in the former. Neutropenia was more pronounced, however, in those patients on fludarabine.[140]

Nucleoside Analogue Therapy

Cladribine has resulted in major responses in 40% to 90% of WM patients who received primary therapy, whereas in the previously treated patients, responses have ranged from 38% to 54%.[141–147] Median time to achievement of response in responding patients following cladribine ranged from 1.2 to 5 months. The ORR with daily infusion of fludarabine, administered mainly on 5-day schedules, in previously untreated and treated patients ranged from 38% to 100% and from 30% to 40%, respectively,[148–153] similar to the responses to cladribine. Median time to achievement of a response with fludarabine (3 to 6 months) was also similar to cladribine. In general, response rates and durations of responses have been greater for patients receiving nucleoside analogues as initial therapy, although no difference in the ORR was reported in several studies in which both untreated and previously treated patients were enrolled.

Myelosuppression commonly occurs following prolonged exposure to either of the nucleoside analogues. A sustained decrease in both $CD4^+$ and $CD8^+$ T lymphocytes, measured 1 year following initiation of therapy, is notable.[141–143] Treatment-related mortality as a consequence of myelosuppression and/or opportunistic infections attributable to immunosuppression occurred in up to 5% of all treated patients in some series with nucleoside analogues.

Factors predicting a better response to nucleoside analogues include younger age at start of treatment (<70 years), higher pretreatment hemoglobin (>9.5 g/dL), higher platelet count ($>75 \times 10^9$/L), disease relapsing off-therapy, and a long interval between first line therapy and initiation of a nucleoside analogue in relapsing patients.[141,146,152] There are limited data on the use of an alternate nucleoside analogue in previously treated patients in whom disease relapsed or who had resistance when not on cladribine or fludarabine therapy.[154,155] Three (75%) of four patients responded to cladribine after progression following an unmaintained remission with fludarabine, whereas only 1 (10%) of 10 with disease resistant to fludarabine responded to cladribine.[154] A response to fludarabine has been reported in two (33%) of six patients and disease stabilization in the remaining patients, in spite of an inadequate response or progressive disease, following cladribine therapy.[155]

Harvesting autologous blood stem cell grafts was successful on the first attempt in 14 of 15 patients who had not received nucleoside analogue therapy, as compared with 2 of 6 patients who received a nucleoside analogue.[156] A sevenfold increase in patients transforming to an aggressive lymphoma and a threefold increase in the development of myelodysplasia or acute myeloid leukemia was observed among patients who received a nucleoside analogue versus other therapies for their WM.[157] A meta-analysis of several trials in which patients with WM were treated with nucleoside analogues included patients who had previously received an alkylating agent, and the results showed a crude incidence of approximately 8% of developing a disease transformation event and approximately 5% for developing myelodysplasia or acute myeloid leukemia.[158] None of the risk factors—that is, sex, age, family history of WM, or B-cell malignancies, typical markers of tumor burden and prognosis, type of nucleoside analogue therapy (cladribine vs. fludarabine), time from diagnosis to nucleoside analogue use, nucleoside analogue treatment as primary or salvage therapy, or treatment with an oral alkylator (i.e., chlorambucil)—predicted the occurrence of transformation or development of myelodysplasia or acute myeloid leukemia in patients treated with a nucleoside analogue.[157]

CD20-Directed Antibody Therapy

Rituximab is a chimeric monoclonal antibody that targets CD20, a widely expressed antigen on lymphoplasmacytic cells in WM.[159] Several retrospective and prospective studies have indicated that rituximab, when used at standard doses (i.e., 4 weekly infusions of 375 mg/m^2/week) induced major responses in approximately 30% of previously treated and untreated patients.[160,161] Even patients who achieved minor responses benefited from rituximab by improved hemoglobin and platelet counts, as well as reduction of lymphadenopathy and/or splenomegaly.[160] The median time to treatment failure in these studies was found to range from 8 to 27+ months. Patients on an extended rituximab schedule consisting of 4 weekly courses at 375 mg/m^2/week, repeated 3 months later by another 4-week course have demonstrated major response rates of approximately 45%, with time to progression estimates of 16+ to 29+ months.[162,163]

In many patients with WM, a transient increase or flare of the serum IgM may occur immediately following initiation of rituximab treatment.[162,164,165] Such an increase does not herald treatment failure, and most patients will return to their baseline serum IgM level by 12 weeks. Some patients continue to show a prolonged increase in IgM despite an apparent reduction in their marrow tumor cells. However, patients with baseline serum IgM levels greater than 50 g/dL or serum viscosity greater than 3.5 cp may be particularly at risk for a hyperviscosity-related event, and plasmapheresis should be considered in these patients in advance of rituximab therapy.[164] Because of the decreased likelihood of response in patients with higher IgM levels, as well as the possibility that serum IgM and blood viscosity levels may abruptly rise, rituximab monotherapy should not be used as sole therapy for the treatment of patients at risk for hyperviscosity symptoms.[133,162,163]

About 5% to 10% of WM patients can develop rituximab intolerance, characterized by increasingly severe infusion reactions.[166]

The time to respond after rituximab is slow and exceeds 3 months on average. The time to best response in one study was 18 months.[163] Patients with baseline serum IgM levels less than 60 g/dL are more likely to respond, regardless of the underlying degree of marrow involvement by tumor cells.[162,163] An analysis of 52 patients who were treated with single-agent rituximab found the objective response rate was significantly lower in patients who had either low serum albumin (<35 g/L) or a serum monoclonal protein greater than 40 g/L.[167] The presence of both adverse prognostic factors was associated with a short time to progression (3.6 months). Patients who had normal serum albumin and relatively low serum monoclonal protein levels derived a substantial benefit from rituximab, with a time to progression exceeding 40 months.

The fully human anti-CD20 monoclonal antibody ofatumumab is effective on inducing responses in WM patients.[168] Ofatumumab-induced ORR of 59% with partial response rates of 41%. Very good partial or complete responses were not attained. The most common grade ≥3 adverse events included infusions reactions, chest pain, hemolysis and neutropenia. Ofatumumab can be used in patients who are intolerant to rituximab.[166]

Proteasome Inhibitors

In a retrospective study, 10 patients with refractory or relapsed WM were treated with bortezomib administered intravenously at a dose of 1.3 mg/m^2 on days 1, 4, 8, and 11 in a 21-day cycle for a total of 4 cycles. Most patients had been exposed to all other active agents for WM available at that time, and eight patients had received three or more regimens. Six of these patients achieved a partial response that occurred after a median of 1 month. The median time to progression in the responding patients is expected to exceed 11 months. Peripheral neuropathy occurred in three patients, and one patient developed severe paralytic ileus.[169] In a prospective study including 27 relapsed or refractory patients who received up to 8 cycles of bortezomib at 1.3 mg/m^2 on days 1, 4, 8, and 11, median serum IgM levels declined significantly, from 4.7 g/dL to 2.1 g/dL.[32] The ORR was 85%, with 10 and 13 patients achieving a minor (<25%) and major (<50%) decrease in IgM level, respectively. Responses occurred after a median of 1.4 months. The median time to progression for all responding patients in this study was 7.9 (range, 3 to 21.4+) months, and the most common grade III/IV toxicities were sensory neuropathies (22.2%), leukopenia (18.5%), neutropenia (14.8%), dizziness (11.1%), and thrombocytopenia (7.4%). Sensory neuropathies resolved or improved in nearly all patients following cessation of therapy. Twenty-seven patients with both untreated (44%) and previously treated (56%) disease received bortezomib, using the standard schedule until they either demonstrated progressive disease or 2 cycles beyond a complete response or stable disease.[170] The ORR was 78%, with major responses observed in 44% of patients. Sensory neuropathy occurred in 20 patients following 2 to 4 cycles of therapy. Among the 20 patients who developed a neuropathy, 14 showed resolution or improvement 2 to 13 months after therapy.

Combination Therapies

Because rituximab is not myelosuppressive, its use in combination with chemotherapy has been explored. A regimen of rituximab, cladribine, and cyclophosphamide in 17 previously untreated patients resulted in a partial response in 94% of patients with WM, including a complete response in 18%.[171] No patient had relapsed with a median follow-up of 21 months. The combination of rituximab and fludarabine was used in 43 patients, of whom 32 (75%) were previously untreated, which led to an ORR of 95.3%, with 83% of patients achieving a major response (i.e., 50% reduction in disease burden).[172] The median time to progression was 51.2 months in this series, and it was longer for those patients who were previously untreated and for those achieving a very good partial remission (i.e., 90% reduction in disease) or better. Hematologic toxicity was common: Grade 3 neutropenia and thrombocytopenia were observed in 27 and four patients, respectively. Two deaths occurred in this study as a result of pneumonia. Secondary malignancies, including transformation to aggressive lymphoma and development of myelodysplasia or acute myeloid leukemia, were observed in six patients. The addition of rituximab to fludarabine and cyclophosphamide has also been explored in previously treated patients, and four of five patients had a response.[173] In another combination study, rituximab along with pentostatin and cyclophosphamide was given to 13 patients with untreated and previously treated WM or LPL and resulted in a major response in 77% of patients.[174] The combination of rituximab, dexamethasone, and cyclophosphamide was used as primary therapy to treat 72 patients with WM, among whom a major response was observed in 74% of patients, with a 2-year progression-free survival rate of 67%.[175] Therapy was well tolerated, although one patient died as a result of interstitial pneumonia.

Two studies have examined cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) therapy in combination with rituximab (R-CHOP). In a randomized trial involving 69 patients, most of whom had WM, the addition of rituximab to CHOP resulted in a higher ORR (94% vs. 67%) and median time to progression (63 vs. 22 months) in comparison to patients treated with CHOP alone.[176] R-CHOP was also used in 13 patients with WM, 10 of whom had relapsed or refractory disease.[177] Among 13 evaluable patients, 10 patients achieved a major response (77%), including three complete and seven partial remissions. Two other patients achieved a minor response. Patients in a retrospective study of symptomatic WM received either R-CHOP; rituximab, cyclophosphamide, vincristine, and prednisone (R-CVP); or cyclophosphamide, prednisone, and rituximab (R-CP). The patients were similar in most pretreatment variables, and the ORRs to therapy were comparable among all three treatment groups—R-CHOP (96%), R-CVP (88%), and R-CP (95%)—although there was a trend for more complete remissions among patients treated with R-CVP and R-CHOP.[178] Adverse events attributed to therapy showed a higher incidence for neutropenic fever and treatment-related neuropathy for R-CHOP and R-CVP versus R-CP. The results of this study suggest that in patients with WM, the use of R-CP may provide treatment responses analogous to those of more intense cyclophosphamide-based regimens while minimizing treatment-related complications. The extended alkylator bendamustine has also been evaluated in combination with rituximab in both untreated and previously treated patients with WM. A randomized study by the German STiL Group examined bendamustine plus rituximab (Benda-R) versus R-CHOP in patients with untreated, indolent B-cell lymphomas including WM.[179] Patients with WM in this study showed similar overall responses (96% vs. 94%), though progression-free survival was significantly longer (69 vs. 29 months) in patients who received Benda-R versus R-CHOP. Treatment was also better tolerated in patients receiving Benda-R. In the relapsed or refractory setting, an ORR of 83% was observed with bendamustine in combination with a CD20 monoclonal antibody.[180] The median time to progression was 13 months in this study. Prolonged myelosuppression was more common in patients who had received prior nucleoside analogue therapy.

The use of two cycles of oral cyclophosphamide along with subcutaneous cladribine to 37 patients with previously untreated WM led to a partial response in 84% of patients, and the median duration of response was 36 months.[171] Fludarabine in combination with intravenous cyclophosphamide resulted in partial responses in 6 (55%) of 11 patients with WM with either primary refractory disease or who had relapsed on treatment.[181] The combination of fludarabine plus cyclophosphamide was also evaluated in 49 patients, 35 of whom were previously treated. Seventy-eight percent of the patients achieved a response, and the median time to treatment failure was 27 months.[182] Hematologic toxicity was frequent, and three patients died as a result of treatment-related toxicities. Two important findings in this study were the development of acute leukemia in two patients, histologic transformation to diffuse large B-cell lymphoma in one patient, and two cases of solid tumors (prostate and melanoma), as well as failure to be able mobilize stem cells for use as grafts for transplantation in four of six patients.

The combination of bortezomib, dexamethasone, and rituximab (BDR) as primary therapy in patients with WM resulted in an ORR of 96% and a major response rate of 83%.[183] The incidence of grade 3 neuropathy was approximately 30%, but this was reversible in most patients following discontinuation of therapy. An increased incidence of herpes zoster was also observed, prompting the prophylactic use of antiviral therapy. Alternative schedules for administration of bortezomib (i.e., once weekly at higher doses) in combination with rituximab in patients with WM have achieved ORRs of 80% to 90%.[184,185] The European Myeloma Network showed that transitioning bortezomib from twice-weekly intravenous dosing during the first cycle to weekly administration thereafter reduced grade 3 neuropathy to under 10% in patients treated with BDR.[186] There have been no studies addressing the safety and efficacy of subcutaneous bortezomib use in WM.

Carfilzomib, which is associated with a low risk of treatment-related peripheral neuropathy, has been evaluated in combination with rituximab and dexamethasone in patients with WM.[33] Carfilzomib was administered intravenously at 20 mg/m^2 (cycle 1), then 36 mg/m^2 (cycles 2 to 6), together with dexamethasone (20 mg) on days 1, 2, 8, and 9 as part of a 21-day cycle. As part of this regimen, rituximab 375 mg/m^2 was given on days 2 and 9 every 21 days. Maintenance therapy was given 8 weeks following induction therapy with intravenous carfilzomib (36 mg/m^2) and dexamethasone (20 mg) administered on days 1 and 2 and with rituximab 375 mg/m^2 on day 2 every 8 weeks for up to 8 cycles. The ORR with this regimen was 87.1% (one complete response, 10 very good partial responses, 10 partial responses, and 6 minimal responses) and was not impacted by *MYD88*^{L265P} or *CXCR4*^{WHIM} mutation status. With a median follow-up of 15.4 months, 20 patients remained progression-free. Grade ≥2 toxicities included asymptomatic hyperlipasemia (41.9%), reversible neutropenia (12.9%), and cardiomyopathy in 1 patient (3.2%) with multiple risk factors. Grade 2 treatment-related neuropathy occurred in one patient (3.2%). Declines in serum IgA and IgG were common, and some patients required intravenous immunoglobulin therapy for recurring sinus and bronchial infections.

The oral proteasome inhibitor ixazomib, in combination with dexamethasone and rituximab, was evaluated in 26 previously untreated patients with WM.[187,188] Ixazomib 4 mg PO and dexamethasone 20 mg PO were administered once weekly on days 1, 8, 15, with rituximab 375 mg/m2 IV on day 1, every 28 days for 6 cycles, and then every 56 weeks for 6 cycles. Rituximab was omitted from the first 2 cycles to minimize risk of IgM flare. ORR was 93%, major response rate was 77% and VGPR rate was 19%. The median PFS was 40 months. The adverse event profile of IDR was benign without grade 4 adverse events. The most common grade 3 adverse events were infusion reactions, hyperglycemia and infections.

Novel Therapeutics

The use of ibrutinib was recently approved by the US Food and Drug Administration for the treatment of symptomatic patients with WM. Ibrutinib targets BTK, a target of ibrutinib that is activated by MYD88^{L265P}.[42] A multicenter study examined the role of ibrutinib 420 mg PO once daily until disease progression or unacceptable toxicity in previously treated patients with WM (median 2 prior therapies, 40% refractory).[189,190] The ORR and major response rates were 91% and 79%, respectively. Decreased or resolved adenopathy was observed in 60% of patients with extramedullary disease, and five of nine patients with IgM-related peripheral neuropathy had symptomatic improvement. The 5-year PFS and OS rates were 54% and 93%, respectively. Although major response rates were lower in patients with *MYD88*^{WT} or with *CXCR4* mutations, it is not recommended that genotyping be used to select which patients should be placed on ibrutinib until further data are available. Grade ≥2 treatment-related toxicities included neutropenia (24%) and thrombocytopenia (13%) that were more common in heavily pretreated patients, atrial fibrillation associated with a prior history of arrhythmia (10%), and bleeding associated with procedures (3%). Treatment-related infections were infrequent.

Similar results were observed in two separate studies, one in 31 WM patients who were refractory to rituximab (INNOVATE-C), and in 30 previously untreated WM patients. In both studies, ibrutinib was administered at a dose of 420 mg PO once daily until disease progression or unacceptable toxicity. In the INNOVATE-C study, ORR was 90%, major response rate was 71% and VGPR rate was 13%, with an 18-month PFS rate of 86%.[191] In previously untreated patients, the ORR was 100%, major response rate was 83% and VGPR rate was 20%, with an 18-month PFS rate of 92%.[192] *CXCR4* mutations were associated with longer time to response and lower rate of response to ibrutinib monotherapy. The adverse event profile in these studies was similar to what had been previously reported. These results consolidated ibrutinib as a standard of care in WM patients.

In the randomized portion of the INNOVATE study in which 75 WM patients were randomized to ibrutinib and rituximab (ibrutinib-R) and 75 to placebo and rituximab (placebo-R).[193] Ibrutinib was administered at 420 mg PO once daily until disease progression or unacceptable toxicity, and rituximab at a dose of 375 mg/m^2 IV given once weekly for 4 weeks followed by a second 4-week round 4 months later. The combination of ibrutinib-R was associated with higher rates of major response (72% vs. 32%) and 30-month PFS rates (82% vs. 28%) than placebo-R. The rate of IgM flare and infusion reactions to rituximab were lower in the patients who received ibrutinib-R. *CXCR4* mutations also seemed to adversely affect depth and duration of response in WM patients treated with ibrutinib-R. Given the lack of an ibrutinib-placebo arm, the benefit of the addition of rituximab to ibrutinib is currently unclear.

Acalabrutinib and zanubrutinib are novel BTK inhibitors with higher specificity towards BTK than ibrutinib. A phase II study evaluated acalabrutinib 100 mg PO BID in 106 (14 treatment naïve and 92 previously treated) WM patients.[194] Acalabrutinib therapy was associated with an ORR of 93% in both treatment-naïve and previously treated patients. The 2-year PFS rate was 90% and 82%, respectively. The most common serious adverse events included respiratory infections (14%), cellulitis (3%) and sepsis (3%). One treatment-related death caused by intracranial hematoma was reported.

The ASPEN study randomized 201 (37 treatment-naïve and 164 previously treated) WM patients to zanubrutinib 160 mg PO BID (n = 102) versus ibrutinib 420 mg PO QD[195] (n = 99). The primary end point of the study was to show a superiority of zanubrutinib over ibrutinib on very good partial response rates assessed by an independent review committee. The rates of very good partial response for zanubrutinib and ibrutinib were 28% and 19%, respectively (P = .09). No patients attained a complete response. There were no differences between groups in ORR or PFS. Zanubrutinib was associated with lower incidence of atrial fibrillation (2% vs. 15%) but higher incidence of neutropenia (29% vs. 13%) than ibrutinib.

Maintenance Therapy

The outcome of rituximab-naïve patients who were either observed or received maintenance rituximab categorical responses has been examined in several retrospective studies, which suggested PFS and OS benefits.[196] A prospective study by the German STiL Group examining the role of maintenance rituximab is ongoing.[197] Preliminary results suggest that there was no PFS benefit from maintenance rituximab compared with observation after induction therapy with Benda-R (101 vs. 82 months, respectively; P = .32). In subset analyses, patients older than 65 and with intermediate/high-risk disease seemed to benefit from maintenance rituximab. Also, patients who attained minor response to induction were not included in the randomization portion of the study.

High-Dose Therapy and Stem Cell Transplant

The European Bone Marrow Transplant Registry (EBMT) reported the largest experience for both autologous as well as allogeneic SCT in WM.[198,199] Among 158 patients with WM receiving an autologous SCT, which included primarily relapsed or refractory patients, the 5-year progression-free and overall survival rates were 39.7% and 68.5%, respectively.[198] Nonrelapse mortality at 1 year was 3.8%. Chemorefractory disease and the number of prior lines of therapy at the time of the autologous SCT were the most important prognostic factors for progression-free and overall survival. In the allogeneic SCT experience from the EBMT, the long-term outcomes of 86 patients with WM were reported.[199] A total of 86 patients received an allograft following either myeloablative or reduced-intensity conditioning. The median age of patients in this series was 49 years, and 47 patients had undergone three or more previous lines of therapy. Eight patients had failed prior autologous SCT. Fifty-nine patients (68.6%) had chemotherapy-sensitive disease at the time of allogeneic SCT. Nonrelapse mortality rates at 3 years were 33% for patients receiving a myeloablative transplant and 23% for those who received reduced-intensity conditioning. The ORR was 75.6%. The relapse rates at 3 years were 11% for myeloablative and 25% for those recipients receiving reduced-intensity conditioning. Five-year progression-free and overall survival for WM patients who received a myeloablative allogeneic SCT were 56% and 62%, respectively, and for patients who received reduced intensity conditioning, the rates were 49% and 64%, respectively. The occurrence of chronic graft-versus-host disease was associated with improved progression-free survival and suggested the existence of a clinically relevant graft-versus-WM effect in this study. A consensus document on the role of transplantation in WM was presented at the 2017 ASH Annual Meeting.[200] Consensus was reached, among other topics, on the following: (1) Autologous SCT is not appropriate as part of first line therapy in patient responding to induction therapy; (2) Autologous SCT is appropriate following second or subsequent relapses in high-risk patients with chemosensitive disease; (3) SCT should not be considered in BTK inhibitor-naïve patients, provided that BTK inhibitors are available. Allogeneic SCT should be considered in highly selected WM cases and preferably in the context of a clinical trial.

Treatment Recommendations During the COVID-19 Pandemic

The SARS-CoV-2-associated coronavirus disease 2019 (COVID-19) pandemic has affected individuals across the globe, including patients with blood cancers. There are mounting data suggesting an increased risk of contracting and severe complications with COVID-19. The International Workshop for WM provided recommendations for the management of WM patients during the COVID-19 pandemic.[201] Some of the recommendations include, but are not limited to:

- Increasing the threshold for treatment initiation and preferentially pursue watchful waiting whenever possible.
- If therapy is needed, BTK inhibitors or chemoimmunotherapy are reasonable options.
- For patients who attained an excellent response with chemoimmunotherapy, a reduced number of cycles could be considered.
- The use of maintenance rituximab should be avoided.
- Proactive support with growth factors, antibiotics, intravenous immunoglobulins and vaccines is highly encouraged.

Response Criteria in Waldenström Macroglobulinemia

Table 92.3 summarizes the response categories and criteria for progressive disease in WM based on the most recent consensus recommendations.[202] The term *overall response* is used to characterize all responses, including minor responses. *Major responses* include partial, very good partial, and complete responses. The attainment of very good partial or complete responses is associated with improved progression-free survival.[161,172,183,186,203] Response assessments in WM rely primarily on serum IgM or IgM paraprotein levels, though complete responses require disappearance of the IgM monoclonal protein, and resolution of evidence of BM and/or extramedullary disease.[202] An important concern with the use of IgM as a surrogate marker of disease is that it can fluctuate independent of tumor cell killing with some agents. By way of example, rituximab can induce a flare in serum IgM levels, whereas bortezomib and ibrutinib can suppress IgM levels independent of tumor cell killing in some patients, a phenomenon referred to as *IgM discordance*.[164,165,169,196,204,205] Moreover, with selective B cell–depleting agents such as rituximab, residual IgM-producing plasma cells are spared and persist, thus potentially skewing the relative response and assessment to treatment.[206]

Course and Prognosis

WM typically presents as an indolent disease. The presence of 6q deletions may have prognostic significance, although this is disputed.[19,20] Age is an important prognostic factor (>65 years),[207–209] but it is influenced by comorbidities. Anemia that reflects both marrow involvement and the serum level of the IgM monoclonal protein (because of the impact of IgM on intravascular fluid retention) has emerged as a strong adverse prognostic factor with hemoglobin levels of less than 9 to 12 g/dL associated with decreased survival in several series.[207–209] Other cytopenias also may be significant predictors of survival.[208] The precise level of cytopenias with prognostic significance has not been determined. Some series have identified a platelet count of less than 100 to 150 × 10^9/L and a granulocyte count of less than

TABLE 92.3 **Summary of Consensus Response Criteria for Waldenström Macroglobulinemia**

Response Type	Abbreviation	Criteria
Complete response	CR	Absence of serum monoclonal IgM protein by immunofixation Normal serum IgM level Complete resolution of extramedullary disease (i.e., lymphadenopathy/splenomegaly if present at baseline) Morphologically normal bone marrow aspirate and trephine biopsy
Very good partial response	VGPR	Monoclonal IgM protein is detectable 90% reduction in serum IgM level from baseline or normalization of serum IgM level Complete resolution of extramedullary disease (i.e., lymphadenopathy/splenomegaly if present at baseline) No new signs or symptoms of active disease
Partial response	PR	Monoclonal IgM protein is detectable ≥50% but <90% reduction in serum IgM level from baseline Reduction in extramedullary disease (i.e., lymphadenopathy/splenomegaly if present at baseline) No new signs or symptoms of active disease
Minor response	MR	Monoclonal IgM protein is detectable ≥25% but <50% reduction in serum IgM level from baseline No new signs or symptoms of active disease
Stable disease	SD	Monoclonal IgM protein is detectable <25% reduction and <25% increase in serum IgM level from baseline No progression in extramedullary disease (i.e., lymphadenopathy/splenomegaly) No new signs or symptoms of active disease
Progressive disease	PD	>25% increase in serum IgM level from lowest nadir (requires confirmation) and/or progression in clinical features attributable to the disease

IgM, Immunoglobulin M.
From Owen RG, Kyle RA, Stone MJ, et al. Response assessment in Waldenström macroglobulinemia. *Br J Haematol.* 2013;160:171.

1.5×10^9/L as independent prognostic factors.[208,209] The number of cytopenias in a given patient has been proposed as a prognostic factor.[208] Serum albumin levels also correlate with survival in patients with WM in some studies, using multivariate analyses.[208,210] Elevated serum β_2-microglobulin levels (>3 to 3.5 g/dL) have also been shown to have strong prognostic correlation in WM.[208–211] Several scoring systems have been proposed that are based on these analyses (Table 92.4), including the International Prognostic Scoring System for Waldenström macroglobulinemia (IPSSWM), which incorporates five adverse covariates: advanced age (>65 years), hemoglobin $\leq$11.5 g/dL, platelet count $\leq 100 \times 10^9$/L, β_2-microglobulin greater than 3 mg/L, and serum monoclonal protein concentration greater than 7.0 g/dL.[212] Among 537 patients with WM evaluated in the development of IPSSWM, low-risk patients (27%) presented with no or one of the adverse characteristics and advanced age; intermediate-risk patients (38%) presented with two adverse characteristics or only advanced age; and high-risk patients (35%) presented with more than two adverse characteristics. Five-year survival rates for these three patient groups were 87%, 68%, and 36%, respectively. Importantly, the IPSSWM retained its prognostic significance in subgroups defined by age and treatment with alkylating agent and nucleoside analogues. Despite its prognostic value in WM patients treated with chemotherapy agents, the prognostic value of the IPSSWM has not been validated in WM patients on BTK inhibitors. Furthermore, the IPSSWM is not used to inform time to treat or treatment selection. Recent data from the Surveillance, Epidemiology, and End Results (SEER) database involving 7744 patients with WM showed that the relative survival of patients with WM has improved over time. Patients diagnosed during 2001–2010 had higher 5-year (78% vs. 67%) and 10-year (66% vs. 49%) relative survival rates versus patients diagnosed during 1980–2000. A Greek study that included 345 patients with WM failed to show any overall or cause-specific survival improvement in recent years, though the study might have been underpowered to detect any expected benefit.[211] A Swedish study of 1555 patients diagnosed with WM between 1980 and 2005 showed that the 5-year relative survival rate improved from 57% in 1980–1985 to 78% in 2001–2005.[213]

FUTURE DIRECTIONS

WM remains an incurable disease and, therefore, novel approaches that would improve response rates and survival while decreasing toxicity are being actively investigated.

A number of clinical trials are currently evaluating BTK inhibitors in combination with chemotherapy, proteasome inhibitors and other targeted agents, such as the BCL2 antagonist venetoclax, the anti-CXCR4 inhibitor mavorixafor and the anti-CD38 monoclonal antibody daratumumab. Preliminary data from a phase II study evaluating single agent venetoclax have reported an ORR of 90% with an 18-month PFS rate of 82% in previously treated WM patients.[214] Single agent daratumumab was associated with a lower-than-expected ORR of 23% in previously treated WM patients but preclinical data have suggested synergism with BTK inhibitors.[215,216]

The emergence of *BTK C481S* mutations has been associated with the development of resistance to covalent BTK inhibitors. Noncovalent BTK inhibitors that do not interact with C481, such as pirtobrutinib and ARQ531, are being investigated in WM patients progressing or intolerant to covalent BTK inhibitors.[217,218]

Other targets of clinical interest include IRAK/IRAK4, HCK and CD19.

TABLE 92.4 Prognostic Scoring Systems in Waldenström Macroglobulinemia

Study	Adverse Prognostic Factors	Number of Groups	Survival
Gobbi et al.[207]	Hgb <9 g/dL	0–1 prognostic factors	Median: 48 months
	Age >70 years	2–4 prognostic factors	Median: 80 months
	Weight loss		
	Cryoglobulinemia		
Morel et al.[208]	Age ≥65 years	0–1 prognostic factors	5-year: 87% of patients
	Albumin <4 g/dL	2 prognostic factors	5-year: 62%
	Number of cytopenias:	3–4 prognostic factors	5-year: 25%
	Hgb <12 g/dL		
	Platelets $<150 \times 10^9$/L		
	WBC $<4 \times 10^9$/L		
Dhodapkar et al.[209]	β_2M ≥3 g/dL	β_2M <3 mg/dL + Hgb ≥12 g/dL	5-year: 87% of patients
	Hgb <12 g/dL	β_2M <3 mg/dL + Hgb <12 g/dL	5-year: 63%
	IgM <4 g/dL	β_2M ≥3 mg/dL + IgM ≥4 g/dL	5-year: 53%
		β_2M ≥3 mg/dL + IgM <4 g/dL	5-year: 21%
Dimopoulos et al.[210]	Albumin ≤3.5 g/dL	Albumin ≥3.5 g/dL + β_2M <3.5 mg/dL	Median: NR
	β_2M ≥3.5 mg/L	Albumin ≤3.5 g/dL + β_2M <3.5 or	Median: 116 months
		β_2M 3.5–5.5 mg/dL	Median: 54 months
		β_2M >5.5 mg/dL	
Morel et al.[212]	Age >65 years	0–1 prognostic factors (excluding age)	5-year: 87% of patients
	Hgb <11.5 g/dL	2 prognostic factors (or age >65 years)	5-year: 68%
	Platelets $<100 \times 10^9$/L	3–5 prognostic factors	5-year: 36%
	β_2M >3 mg/L		
	IgM >7 g/dL		

β_2M, β_2-microbloulin; *Hgb*, hemoglobulin; *IgM*, immunoglobulin M; *NR*, not reported; *WBC*, white blood cell count.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Castillo JJ, Olszewski AJ, Cronin AM, et al. Survival trends in Waldenström macroglobulinemia: an analysis of the surveillance, epidemiology and end results database. *Blood*. 2014;123:3999.
Castillo JJ, Garcia-Sanz R, Hatjiharissi E, et al. Recommendations for the diagnosis and initial evaluation of patients with Waldenström macroglobulinaemia: A Task Force from the 8th International Workshop on Waldenström Macroglobulinaemia. *Br J Haematol*. 2016;175(1):77–86.
Castillo JJ, Meid K, Flynn CA, et al. Ixazomib, dexamethasone, and rituximab in treatment-naive patients with Waldenström macroglobulinemia: long-term follow-up. *Blood Adv*. 2020;4(16):3952–3959.
Castillo JJ, Advani RH, Branagan AR, et al. Consensus treatment recommendations from the tenth International Workshop for Waldenström Macroglobulinaemia. *Lancet Haematol*. 2020;7(11):e827–e837.
Dimopoulos MA, García-Sanz R, Gavriatopoulou M, Morel P, Kyrtsonis MC, Michalis E, et al. Primary therapy of Waldenstrom macroglobulinemia (WM) with weekly bortezomib, low-dose dexamethasone, and rituximab (BDR): long-term results of a phase 2 study of the European Myeloma Network (EMN). *Blood*. 2013;122(19):3276–3282.
Dimopoulos MA, Tedeschi A, Trotman J, et al. Phase 3 trial of ibrutinib plus rituximab in Waldenström's macroglobulinemia. *N Engl J Med*. 2018;378(25):2399–2410.
Hunter ZR, Xu L, Yang G, et al. The genomic landscape of Waldenstrom macroglobulinemia is characterized by highly recurring MYD88 and WHIM-like CXCR4 mutations, and small somatic deletions associated with B-cell lymphomagenesis. *Blood*. 2014;123:1637.
Kyle RA, Treon SP, Alexanian R, et al. Prognostic markers and criteria to initiate therapy in Waldenstrom's macroglobulinemia: consensus panel recommendations from the Second International Workshop on Waldenstrom's Macroglobulinemia. *Semin Oncol*. 2003;30:116.
Morel P, Duhamel A, Gobbi P, et al. International prognostic scoring system for Waldenstrom macroglobulinemia. *Blood*. 2009;113:4163.
Owen RG, Treon SP, Al-Katib A, et al. Clinicopathological definition of Waldenstrom's macroglobulinemia: consensus panel recommendations from the Second International Workshop on Waldenstrom's Macroglobulinemia. *Semin Oncol*. 2003;30:110.
Owen RG, Kyle RA, Stone MJ, et al. Response assessment in Waldenström macroglobulinaemia: update from the VIth International Workshop. *Br J Haematol*. 2013;160(2):171–176.
Owen RG, McCarthy H, Rule S, et al. Acalabrutinib monotherapy in patients with Waldenström macroglobulinemia: a single-arm, multicentre, phase 2 study. *Lancet Haematol*. 2020;7(2):e112–e121.
Rummel MJ, Niederle N, Maschmeyer G, et al. Bendamustine plus rituximab versus CHOP plus rituximab as first-line treatment for patients with indolent and mantle-cell lymphomas: an open-label, multicentre, randomised, phase 3 non-inferiority trial. *Lancet*. 2013;381:1203.
Stone MJ, Bogen SA. Evidence-based focused review of management of hyperviscosity syndrome. *Blood*. 2012;119:2205.
Tam CS, Opat S, D'Sa S, Jurczak W, Lee HP, Cull G, et al. A randomized phase 3 trial of zanubrutinib vs ibrutinib in symptomatic Waldenström macroglobulinemia: the ASPEN study. *Blood*. 2020;136(18):2038–2050.
Treon SP, Xu L, Yang G, et al. MYD88 L265P somatic mutation in Waldenström's macroglobulinemia. *N Engl J Med*. 2012;367:826.
Treon SP, Tripsas CK, Meid K, et al. Carfilzomib, rituximab and dexamethasone (CaRD) is active and offers a neuropathy-sparing approach for proteasome-inhibitor based therapy in Waldenström's macroglobulinemia. *Blood*. 2014;124:503.
Treon SP, Tripsas CK, Meid K, et al. Ibrutinib in previously treated Waldenstrom's macroglobulinemia. *N Engl J Med*. 2015;372:1430.
Treon SP, Gustine J, Meid K, et al. Ibrutinib monotherapy in symptomatic, treatment-naïve patients with Waldenström macroglobulinemia. *J Clin Oncol*. 2018;36(27):2755–2761.
Treon SP, Meid K, Gustine J, et al. Long-term follow-up of ibrutinib monotherapy in symptomatic, previously treated patients with Waldenström macroglobulinemia. *J Clin Oncol*. 2021;39(6):565–575.
Yang G, Zhou Y, Liu X, et al. A mutation in MYD88 (L265P) supports the survival of lymphoplasmacytic cells by activation of Bruton tyrosine kinase in Waldenström macroglobulinemia. *Blood*. 2013;122:1222.

REFERENCES

1. Owen RG, Treon SP, Al-Katib A, et al. Clinicopathological definition of Waldenstrom's macroglobulinemia: consensus panel recommendations from the Second International Workshop on Waldenstrom's Macroglobulinemia. *Semin Oncol*. 2003;30:110.
2. Harris NL, Jaffe ES, Diebold J, et al. The World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting, Airlie House, Virginia, November, 1997. *Ann Oncol*. 1999;10:1419.
3. Groves FD, Travis LB, Devesa SS, et al. Waldenström's macroglobulinemia: incidence patterns in the United States, 1988–1994. *Cancer*. 1998;82:1078.
4. Herrinton LJ, Weiss NS: Incidence of Waldenström's macroglobulinemia. *Blood*. 1993;82:3148.
5. Hanzis C, Ojha RP, Hunter Z, et al. Associated malignancies in patients with Waldenström's macroglobulinemia and their kin. *Clin Lymphoma Myeloma Leuk*. 2011;11:88.
6. Björnsson OG, Arnason A, Gudmunosson S, et al. Macroglobulinaemia in an Icelandic family. *Acta Med Scand*. 1978;203:283.
7. Renier G, Ifrah N, Chevailler A, et al. Four brothers with Waldenstrom's macroglobulinemia. *Cancer*. 1989;64:1554.
8. Treon SP, Hunter ZR, Aggarwal A, et al. Characterization of familial Waldenstrom's macroglobulinemia. *Ann Oncol*. 2006;17:488.
9. Ogmundsdóttir HM, Sveinsdóttir S, Sigfússon A, et al. Enhanced B cell survival in familial macroglobulinaemia is associated with increased expression of Bcl-2. *Clin Exp Immunol*. 1999;117:252.
10. Santini GF, Crovatto M, Modolo ML, et al. Waldenström macroglobulinemia: a role of HCV infection? *Blood*. 1993;82:2932.
11. Silvestri F, Barillari G, Fanin R, et al. Risk of hepatitis C virus infection, Waldenström's macroglobulinemia, and monoclonal gammopathies. *Blood*. 1996;88:1125.
12. Leleu X, O'Connor K, Ho AW, et al. Hepatitis C viral infection is not associated with Waldenström's macroglobulinemia. *Am J Hematol*. 2007;82:83.
13. Swerdlow SH, Campo E, Harris NL, et al. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. ed 4 Lyon, France: IARC Press; 2008.
14. Smith BR, Robert NJ, Ault KA: In Waldenstrom's macroglobulinemia the quantity of detectable circulating monoclonal B lymphocytes correlates with clinical course. *Blood*. 1983;61:911.
15. Treon SP: How I treat Waldenström macroglobulinemia. *Blood*. 2009;114:2375.
16. Preud'homme JL, Seligmann M. Immunoglobulins on the surface of lymphoid cells in Waldenström's macroglobulinemia. *J Clin Invest*. 1972;51:701.
17. San Miguel JF, Vidriales MB, Ocio E, et al. Immunophenotypic analysis of Waldenstrom's macroglobulinemia. *Semin Oncol*. 2003;30:187.
18. Hunter ZR, Branagan AR, Manning R, et al. CD5, CD10, and CD23 expression in Waldenstrom's macroglobulinemia. *Clin Lymphoma*. 2005;5:246.
19. Paiva B, Montes MC, García-Sanz R, et al. Multiparameter flow cytometry for the identification of the Waldenström's clone in IgM-MGUS and Waldenström's macroglobulinemia: new criteria for differential diagnosis and risk stratification. *Leukemia*. 2013;28:166.
20. Wagner SD, Martinelli V, Luzzatto L. Similar patterns of V kappa gene usage but different degrees of somatic mutation in hairy cell leukemia, prolymphocytic leukemia, Waldenstrom's macroglobulinemia, and myeloma. *Blood*. 1994;83:3647.
21. Aoki H, Takishita M, Kosaka M, et al. Frequent somatic mutations in D and/or JH segments of Ig gene in Waldenström's macroglobulinemia and chronic lymphocytic leukemia (CLL) with Richter's syndrome but not in common CLL. *Blood*. 1995;85:1913.
22. Shiokawa S, Suehiro Y, Uike N, et al. Sequence and expression analyses of mu and delta transcripts in patients with Waldenström's macroglobulinemia. *Am J Hematol*. 2001;68:139.
23. Sahota SS, Forconi F, Ottensmeier CH, et al. Typical Waldenstrom macroglobulinemia is derived from a B-cell arrested after cessation of somatic mutation but prior to isotype switch events. *Blood*. 2002;100:1505.
24. Ackroyd S, O'Connor SJM, Owen RG: Rarity of IgH translocations in Waldenström macroglobulinemia. *Cancer Genet Cytogenet*. 2005;163:77.
25. Avet-Loiseau H, Garand R, Lodé L, et al. 14q32 translocations discriminate IgM multiple myeloma from Waldenstrom's macroglobulinemia. *Semin Oncol*. 2003;30:153.
26. Braggio E, Keats JJ, Leleu X, et al. High-resolution genomic analysis in Waldenström's macroglobulinemia identifies disease-specific and common abnormalities with marginal zone lymphomas. *Clin Lymphoma Myeloma*. 2009;9:39.
27. Schop RFJ, Kuehl WM, Van Wier SA, et al. Waldenström macroglobulinemia neoplastic cells lack immunoglobulin heavy chain locus translocations but have frequent 6q deletions. *Blood*. 2002;100:2996.
28. Hunter ZR, Xu L, Yang G, et al. The genomic landscape of Waldenstrom macroglobulinemia is characterized by highly recurring MYD88 and WHIM-like CXCR4 mutations, and small somatic deletions associated with B-cell lymphomagenesis. *Blood*. 2014;123:1637.
29. Nguyen-Khac F, Lambert J, Chapiro E, et al. Chromosomal aberrations and their prognostic value in a series of 174 untreated patients with Waldenström's macroglobulinemia. *Haematologica*. 2013;98:649.
30. Braggio E, Keats JJ, Leleu X, et al. Identification of copy number abnormalities and inactivating mutations in two negative regulators of nuclear factor-kappaB signaling pathways in Waldenström's macroglobulinemia. *Cancer Res*. 2009;69:3579.
31. Leleu X, Eeckhoute J, Jia X, et al. Targeting NF-kappaB in Waldenstrom macroglobulinemia. *Blood*. 2008;111:5068.
32. Treon SP, Hunter ZR, Matous J, et al. Multicenter clinical trial of bortezomib in relapsed/refractory Waldenstrom's macroglobulinemia: results of WMCTG Trial 03-248. *Clin Cancer Res*. 2007;13:3320.
33. Treon SP, Tripsas CK, Meid K, et al. Carfilzomib, rituximab and dexamethasone (CaRD) is active and offers a neuropathy-sparing approach for proteasome-inhibitor based therapy in Waldenström's macroglobulinemia. *Blood*. 2014;124:503.
34. Treon SP, Xu L, Yang G, et al. MYD88 L265P somatic mutation in Waldenström's macroglobulinemia. *N Engl J Med*. 2012;367:826.
35. Xu L, Hunter ZR, Yang G, et al. MYD88 L265P in Waldenström macroglobulinemia, immunoglobulin M monoclonal gammopathy, and other B-cell lymphoproliferative disorders using conventional and quantitative allele-specific polymerase chain reaction. *Blood*. 2013;121:2051.
36. Varettoni M, Arcaini L, Zibellini S, et al. Prevalence and clinical significance of the MYD88 (L265P) somatic mutation in Waldenstrom's macroglobulinemia and related lymphoid neoplasms. *Blood*. 2013;121:2522.
37. Jiménez C, Sebastián E, Chillón MC, et al. MYD88 L265P is a marker highly characteristic of, but not restricted to, Waldenström's macroglobulinemia. *Leukemia*. 2013;27:1722.
38. Poulain S, Roumier C, Decambron A, et al. MYD88 L265P mutation in Waldenstrom macroglobulinemia. *Blood*. 2013;121:s4504.
39. Ansell SM, Hodge LS, Secreto FJ, et al. Activation of TAK1 by MYD88 L265P drives malignant B-cell growth in non-Hodgkin lymphoma. *Blood Cancer J*. 2014;4:e183.
40. Ngo VN, Young RM, Schmitz R, et al. Oncogenically active MYD88 mutations in human lymphoma. *Nature*. 2011;470:115.
41. Landgren O, Staudt L. MYD88 L265P somatic mutation in IgM MGUS. *N Engl J Med*. 2012;367:2255.
42. Yang G, Zhou Y, Liu X, et al. A mutation in MYD88 (L265P) supports the survival of lymphoplasmacytic cells by activation of Bruton tyrosine kinase in Waldenström macroglobulinemia. *Blood*. 2013;122:1222.
43. Watters TM, Kenny EF, O'Neill LAJ: Structure, function and regulation of the Toll/IL-1 receptor adaptor proteins. *Immunol Cell Biol*. 2007;85:411.
44. Cohen L, Henzel WJ, Baeuerie PA: IKAP is a scaffold protein of the IkappaB kinase complex. *Nature*. 1998;395:292.
45. Loiarro M, Gallo G, Fantò N, et al. Identification of critical residues of the MYD88 death domain involved in the recruitment of downstream kinases. *J Biol Chem*. 2009;284:28093.
46. Lin SC, Lo YC, Wu H. Helical assembly in the MYD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. *Nature*. 2010;465:885.
47. Kawagoe T, Sato S, Matsushita K, et al. Sequential control of Toll-like receptor dependent responses by IRAK1 and IRAK2. *Nat Immunol*. 2008;9:684.
48. Leleu X, Eeckhoute J, Jia X, et al. Targeting NF-kappaB in Waldenstrom macroglobulinemia. *Blood*. 2008;111:5068.
49. Treon SP, Cao Y, Xu L, et al. Somatic mutations in MYD88 and CXCR4 are determinants of clinical presentation and overall survival in Waldenstrom macroglobulinemia. *Blood*. 2014;123:2791.
50. Roccaro AM, Sacco A, Jiminez C, et al. C1013G/CXCR4 acts as a driver mutation of tumor progression and modulator of drug resistance in lymphoplasmacytic lymphoma. *Blood*. 2014;123:4120.
51. Poulain S, Roumier C, Doye E, et al. Genomic landscape of CXCR4 mutations in Waldenström's macroglobulinemia [abstract 1610]. *Blood*. 2014;124:1627.
52. Busillo JM, Amando S, Sengupta R, et al. Site-specific phosphorylation of CXCR4 is dynamically regulated by multiple kinases and results in differential modulation of CXCR4 signaling. *J Biol Chem*. 2010;285:7805.
53. Dotta L, Tassone L, Badolato R. Clinical and genetic features of warts, hypogammaglobulinemia, infections and myelokathexis (WHIM) syndrome. *Curr Mol Med*. 2011;11:317.
54. Cao Y, Hunter ZR, Liu X, et al. The WHIM-like CXCR4(S338X) somatic mutation activates AKT and ERK, and promotes resistance to ibrutinib and other agents used in the treatment of Waldenstrom's macroglobulinemia. *Leukemia*. 2015;29:169.

55. Cao Y, Hunter ZR, Liu X, et al. CXCR4 WHIM-like frameshift and nonsense mutations promote ibrutinib resistance but do not supplant MYD88(L265P) -directed survival signalling in Waldenström macroglobulinaemia cells. *Br J Haematol*. 2015;168:701.
56. Castillo JJ, Gustine JN, Meid K, et al. Low levels of von Willebrand markers associate with high serum IgM levels and improve with response to therapy, in patients with Waldenström macroglobulinaemia. *Br J Haematol*. 2018;184(6):1011–1014.
57. Treon SP, Gustine J, Xu L, et al. MYD88 wild-type Waldenstrom macroglobulinaemia: differential diagnosis, risk of histological transformation, and overall survival. *Br J Haematol*. 2018;180(3):374–380.
58. Tournilhac O, Santos DD, Xu L, et al. Mast cells in Waldenstrom's macroglobulinemia support lymphoplasmacytic cell growth through CD154/CD40 signaling. *Ann Oncol*. 2006;17:1275.
59. Zhou Y, Liu X, Xu L, et al. Matrix metalloproteinase-8 is overexpressed in Waldenström's macroglobulinemia cells, and specific inhibition of this metalloproteinase blocks release of soluble CD27. *Clin Lymphoma Myeloma Leuk*. 2011;11:172.
60. Ngo HT, Leleu X, Lee J, et al. SDF-1/CXCR4 and VLA-4 interaction regulates homing in Waldenstrom macroglobulinemia. *Blood*. 2008;112:150.
61. Ho AW, Hatjiharissi E, Ciccarelli BT, et al. CD27-CD70 interactions in the pathogenesis of Waldenstrom macroglobulinemia. *Blood*. 2008;112:4683.
62. Merlini G, Farhangi M, Osserman EF: Monoclonal immunoglobulins with antibody activity in myeloma, macroglobulinemia and related plasma cell dyscrasias. *Semin Oncol*. 1986;13:350.
63. Farhangi M, Merlini G. The clinical implications of monoclonal immunoglobulins. *Semin Oncol*. 1986;13:366.
64. Marmont AM, Merlini G. Monoclonal autoimmunity in hematology. *Haematologica*. 1991;76:449.
65. Mackenzie MR, Babcock J. Studies of the hyperviscosity syndrome. II. Macroglobulinemia. *J Lab Clin Med*. 1975;85:227.
66. Gertz MA, Kyle RA: Hyperviscosity syndrome. *J Intensive Care Med*. 1995;10:128.
67. Kwaan HC, Bongu A. The hyperviscosity syndromes. *Semin Thromb Hemost*. 1999;25:199.
68. Singh A, Eckardt KU, Zimmermann A, et al. Increased plasma viscosity as a reason for inappropriate erythropoietin formation. *J Clin Invest*. 1993;91:251.
69. Gustine JN, Meid K, Dubeau T, et al. Serum IgM level as predictor of symptomatic hyperviscosity in patients with Waldenstrom macroglobulinaemia. *Br J Haematol*. 2017;177(5):717–725.
70. Menke MN, Feke GT, McMeel JW, et al. Hyperviscosity-related retinopathy in Waldenström's macroglobulinemia. *Arch Ophthalmol*. 2006;124:1601.
71. Merlini G, Baldini L, Broglia C, et al. Prognostic factors in symptomatic Waldenstrom's macroglobulinemia. *Semin Oncol*. 2003;30:211.
72. Stone MJ: Waldenström's macroglobulinemia: hyperviscosity syndrome and cryoglobulinemia. *Clin Lymphoma Myeloma*. 2009;9:97.
73. Dellagi K, Dupouey P, Brouet JC, et al. Waldenström's macroglobulinemia and peripheral neuropathy: a clinical and immunologic study of 25 patients. *Blood*. 1983;62:280.
74. Nobile-Orazio E, Marmiroli P, Baldini L, et al. Peripheral neuropathy in macroglobulinemia: incidence and antigen-specificity of M proteins. *Neurology*. 1987;37:1506.
75. Treon SP, Hanzis CA, Ioakimidis LI, et al. Clinical characteristics and treatment outcome of disease-related peripheral neuropathy in Waldenström's macroglobulinemia (WM) [abstract 8114]. *J Clin Oncol*. 2010;28(15_Suppl):8114.
76. Nemni R, Gerosa E, Piccolo G, et al. Neuropathies associated with monoclonal gammapathies. *Haematologica*. 1994;79:557.
77. Ropper AH, Gorson KC. Neuropathies associated with paraproteinemia. *N Engl J Med*. 1998;338:1601.
78. Vital A. Paraproteinemic neuropathies. *Brain Pathol*. 2001;11:399.
79. Latov N, Braun PE, Gross RB, et al. Plasma cell dyscrasia and peripheral neuropathy: identification of the myelin antigens that react with human paraproteins. *Proc Natl Acad Sci USA*. 1981;78:7139.
80. Chassande B, Léger JM, Younes-Chennoufi AB, et al. Peripheral neuropathy associated with IgM monoclonal gammopathy: correlations between M-protein antibody activity and clinical/electrophysiological features in 40 cases. *Muscle Nerve*. 1998;21:55.
81. Weiss MD, Dalakas MC, Lauter CJ, et al. Variability in the binding of anti-MAG and anti-SGPG antibodies to target antigens in demyelinating neuropathy and IgM paraproteinemia. *J Neuroimmunol*. 1999;95:174.
82. Latov N, Hays AP, Sherman WH. Peripheral neuropathy and anti-MAG antibodies. *Crit Rev Neurobiol*. 1988;3:301.
83. Dalakas MC, Quarles RH. Autoimmune ataxic neuropathies (sensory ganglionopathies): are glycolipids the responsible autoantigens? *Ann Neurol*. 1996;39:419.
84. Eurelings M, Ang CW, Notermans NC, et al. Antiganglioside antibodies in polyneuropathy associated with monoclonal gammopathy. *Neurology*. 2001;57:1909.
85. Ilyas AA, Quarles RH, Dalakas MC, et al. Monoclonal IgM in a patient with paraproteinemic polyneuropathy binds to gangliosides containing disialosyl groups. *Ann Neurol*. 1985;18:655.
86. Willison HJ, O'Leary CP, Veitch J, et al. The clinical and laboratory features of chronic sensory ataxic neuropathy with anti-disialosyl IgM antibodies. *Brain*. 2001;124:1968.
87. Lopate G, Choksi R, Pestronk A. Severe sensory ataxia and demyelinating polyneuropathy with IgM anti-GM2 and GalNAc-GD1A antibodies. *Muscle Nerve*. 2002;25:828.
88. Nobile-Orazio E, Manfredini E, Carpo M, et al. Frequency and clinical correlates of anti-neural IgM antibodies in neuropathy associated with IgM monoclonal gammopathy. *Ann Neurol*. 1994;36:416.
89. Gordon PH, Rowland LP, Younger DS, et al. Lymphoproliferative disorders and motor neuron disease: an update. *Neurology*. 1997;48:1671.
90. Pavord SR, Murphy PT, Mitchell VE. POEMS syndrome and Waldenström's macroglobulinaemia. *J Clin Pathol*. 1996;49:181.
91. Crisp D, Pruzanski W. B-cell neoplasms with homogeneous cold-reacting antibodies (cold agglutinins). *Am J Med*. 1982;72:915.
92. Pruzanski W, Shumak KH. Biologic activity of cold-reacting autoantibodies (first of two parts). *N Engl J Med*. 1977;297:538.
93. Pruzanski W, Shumak KH. Biologic activity of cold-reacting autoantibodies (second of two parts). *N Engl J Med*. 1977;297:583.
94. Whittaker SJ, Bhogal BS, Black MM. Acquired immunobullous disease: a cutaneous manifestation of IgM macroglobulinaemia. *Br J Dermatol*. 1996;135:283.
95. Daoud MS, Lust JA, Kyle RA, et al. Monoclonal gammopathies and associated skin disorders. *J Am Acad Dermatol*. 1999;40:507.
96. Gad A, Willén R, Carlén B, et al. Duodenal involvement in Waldenström's macroglobulinemia. *J Clin Gastroenterol*. 1995;20:174.
97. Amrein PC, Compton CC. Case 3-1990: a 66-year-old woman with Waldenström's macroglobulinemia, diarrhea, anemia, and persistent gastrointestinal bleeding. *N Engl J Med*. 1990;322:183.
98. Isaac J, Herrera GA. Cast nephropathy in a case of Waldenström's macroglobulinemia. *Nephron*. 2002;91:512.
99. Morel-Maroger L, Basch A, Danon F, et al. Pathology of the kidney in Waldenstrom's macroglobulinemia. Study of sixteen cases. *N Engl J Med*. 1970;283:123.
100. Gertz MA, Kyle RA, Noel P. Primary systemic amyloidosis: a rare complication of immunoglobulin M monoclonal gammopathies and Waldenström's macroglobulinemia. *J Clin Oncol*. 1993;11:914.
101. Møyner K, Sletten K, Husby G, et al. An unusually large (83 amino acid residues) amyloid fibril protein AA from a patient with Waldenström's macroglobulinaemia and amyloidosis. *Scand J Immunol*. 1980;11:549.
102. Gardyn J, Schwartz A, Gal R, et al. Waldenström's macroglobulinemia associated with AA amyloidosis. *Int J Hematol*. 2001;74:76.
103. Singh A, Geller HI, Alexander KM, et al. True, true unrelated? Coexistence of Waldenström macroglobulinemia and cardiac transthyretin amyloidosis. *Haematologica*. 2018;103(8):e374–e376.
104. Dussol B, Kaplanski G, Daniel L, et al. Simultaneous occurrence of fibrillary glomerulopathy and AL amyloid. *Nephrol Dial Transplant*. 1998;13:2630.
105. Rausch PG, Herion JC. Pulmonary manifestations of Waldenstrom macroglobulinemia. *Am J Hematol*. 1980;9:201.
106. Fadil A, Taylor DE. The lung and Waldenstrom's macroglobulinemia. *South Med J*. 1998;91:681.
107. Kyrtsonis MC, Angelopoulou MK, Kontopidou FN, et al. Primary lung involvement in Waldenström's macroglobulinaemia: report of two cases and review of the literature. *Acta Haematol*. 2001;105:92.
108. Kaila VL, el Newihi HM, Dreiling BJ, et al. Waldenström's macroglobulinemia of the stomach presenting with upper gastrointestinal hemorrhage. *Gastrointest Endosc*. 1996;44:73.
109. Yasui O, Tukamoto F, Sasaki N, et al. Malignant lymphoma of the transverse colon associated with macroglobulinemia. *Am J Gastroenterol*. 1997;92:2299.
110. Rosenthal JA, Curran Jr WJ, Schuster SJ. Waldenström's macroglobulinemia resulting from localized gastric lymphoplasmacytoid lymphoma. *Am J Hematol*. 1998;58:244.
111. Recine MA, Perez MT, Cabello-Inchausti B, et al. Extranodal lymphoplasmacytoid lymphoma (immunocytoma) presenting as small intestinal obstruction. *Arch Pathol Lab Med*. 2001;125:677.
112. Veltman GA, van Veen S, Kluin-Nelemans JC, et al. Renal disease in Waldenström's macroglobulinaemia. *Nephrol Dial Transplant*. 1997;12:1256.

113. Moore Jr DF, Moulopoulos LA, Dimopoulos MA. Waldenström macroglobulinemia presenting as a renal or perirenal mass: clinical and radiographic features. *Leuk Lymphoma*. 1995;17:331.
114. Mascaro JM, Montserrat E, Estrach T, et al. Specific cutaneous manifestations of Waldenström's macroglobulinaemia. A report of two cases. *Br J Dermatol*. 1982;106:17.
115. Schnitzler L, Schubert B, Boasson M, et al. Urticaire chronique, lésions osseuses, macroglobulinémie IgM: maladie de Waldenström? *Bull Soc Fr Dermatol Syphiligr*. 1974;81:363.
116. Roux S, Fermand JP, Brechignac S, et al. Tumoral joint involvement in multiple myeloma and Waldenström's macroglobulinemia—report of 4 cases. *J Rheumatol*. 1996;23:2175.
117. Orellana J, Friedman AH. Ocular manifestations of multiple myeloma, Waldenström's macroglobulinemia and benign monoclonal gammopathy. *Surv Ophthalmol*. 1981;26:157.
118. Ettl AR, Birbamer GG, Philipp W. Orbital involvement in Waldenström's macroglobulinemia: ultrasound, computed tomography and magnetic resonance findings. *Ophthalmologica*. 1992;205:40.
119. Castillo JJ, D'Sa S, Lunn MP, et al. Central nervous system involvement by Waldenström macroglobulinaemia (Bing–Neel syndrome): a multi-institutional retrospective study. *Br J Haematol*. 2016;172(5):709–715.
120. Simon L, Fitsiori A, Lemal R, et al. Bing-Neel syndrome, a rare complication of Waldenström macroglobulinemia: analysis of 44 cases and review of the literature. A study on behalf of the French Innovative Leukemia Organization (FILO). *Haematologica*. 2015;100(12):1587–1594.
121. Ciccarelli BT, Patterson CJ, Hunter ZR, et al. Hepcidin is produced by lymphoplasmacytic cells and is associated with anemia in Waldenström's macroglobulinemia. *Clin Lymphoma Myeloma Leuk*. 2011;11:160.
122. Treon SP, Tripsas CK, Ciccarelli BT, et al. Patients with Waldenström macroglobulinemia commonly present with iron deficiency and those with severely depressed transferrin saturation levels show response to parenteral iron administration. *Clin Lymphoma Myeloma Leuk*. 2013;13:241.
123. Merlini G, Dispenzieri A, Sanchorawala V, et al. Systemic immunoglobulin light chain amyloidosis. *Nat Rev Dis Primers*. 2018;4(1):38.
124. Owen RG, Barrans SL, Richards SJ, et al. Waldenström macroglobulinemia. Development of diagnostic criteria and identification of prognostic factors. *Am J Clin Pathol*. 2001;116:420.
125. Feiner HD, Rizk CC, Finfer MD, et al. IgM monoclonal gammopathy/Waldenström's macroglobulinemia: a morphological and immunophenotypic study of the bone marrow. *Mod Pathol*. 1990;3:348.
126. Dutcher TF, Fahey JL. The histopathology of the macroglobulinemia of Waldenström. *J Natl Cancer Inst*. 1959;22:887.
127. Hunter ZR, Manning RJ, Hanzis C, et al. IgA and IgG hypogammaglobulinemia in Waldenström's macroglobulinemia. *Haematologica*. 2010;95:470.
128. Stone MJ, Bogen SA. Evidence-based focused review of management of hyperviscosity syndrome. *Blood*. 2012;119:2205.
129. Menke MN, Treon SP. Hyperviscosity syndrome. In: Sekeres MA, Kalaycio M, Bolwell BJ, eds. *Clinical Malignant Hematology*. New York: McGraw-Hill; 2007:937.
130. Moulopoulos LA, Dimopoulos MA, Varma DG, et al. Waldenström macroglobulinemia: MR imaging of the spine and CT of the abdomen and pelvis. *Radiology*. 1993;188:669.
131. Kyle RA, Treon SP, Alexanian R, et al. Prognostic markers and criteria to initiate therapy in Waldenström's macroglobulinemia: consensus panel recommendations from the Second International Workshop on Waldenstrom's Macroglobulinemia. *Semin Oncol*. 2003;30:116.
132. Castillo JJ, Advani RH, Branagan AR, et al. Consensus treatment recommendations from the tenth International Workshop for Waldenström Macroglobulinaemia. *Lancet Haematol*. 2020;7(11):e827–e837.
133. Dimopoulos MA, Kastritis E, Owen RG, et al. Treatment recommendations for patients with Waldenström macroglobulinemia (WM) and related disorders: IWWM-7 consensus. *Blood*. 2014;124:1404.
134. Castillo JJ, Treon SP. Management of Waldenström macroglobulinemia in 2020. *Hematology Am Soc Hematol Educ Program*. 2020;2020(1):372–379.
135. Kyle RA, Greipp PR, Gertz MA, et al. Waldenström's macroglobulinaemia: a prospective study comparing daily with intermittent oral chlorambucil. *Br J Haematol*. 2000;108:737.
136. Dimopoulos MA, Alexanian R. Waldenström's macroglobulinemia. *Blood*. 1994;83:1452.
137. Petrucci MT, Avvisati G, Tribalto M, et al. Waldenström's macroglobulinaemia: results of a combined oral treatment in 34 newly diagnosed patients. *J Intern Med*. 1989;226:443.
138. Case Jr DC, Ervin TJ, Boyd MA, et al. Waldenström's macroglobulinemia: long-term results with the M-2 protocol. *Cancer Invest*. 1991;9:1.
139. Facon T, Brouillard M, Duhamel A, et al. Prognostic factors in Waldenström's macroglobulinemia: a report of 167 cases. *J Clin Oncol*. 1993;11:1553.
140. Leblond V, Johnson S, Chevret S, et al. Results of a randomized trial of chlorambucil versus fludarabine for patients with untreated Waldenström macroglobulinemia, marginal zone lymphoma, or lymphoplasmacytic lymphoma. *J Clin Oncol*. 2013;31:301.
141. Dimopoulos MA, Kantarjian H, Weber D, et al. Primary therapy of Waldenström's macroglobulinemia with 2-chlorodeoxyadenosine. *J Clin Oncol*. 1994;12:2694.
142. Delannoy A, Ferrant A, Martiat P, et al. 2-Chlorodeoxyadenosine therapy in Waldenström's macroglobulinaemia. *Nouv Rev Fr Hematol*. 1994;36:317.
143. Fridrik MA, Jäger G, Baldinger C, et al. First-line treatment of Waldenström's disease with cladribine. *Ann Hematol*. 1997;74:7.
144. Liu ES, Burian C, Miller WE, et al. Bolus administration of cladribine in the treatment of Waldenström macroglobulinaemia. *Br J Haematol*. 1998;103:690.
145. Hellmann A, Lewandowski K, Zaucha JM, et al. Effect of a 2-hour infusion of 2-chlorodeoxyadenosine in the treatment of refractory or previously untreated Waldenström's macroglobulinemia. *Eur J Haematol*. 1999;63:35.
146. Betticher DC, Hsu Schmitz SF, Ratschiller D, et al. Cladribine (2-CDA) given as subcutaneous bolus injections is active in pretreated Waldenström's macroglobulinaemia. Swiss Group for Clinical Cancer Research (SAKK). *Br J Haematol*. 1997;99:358.
147. Dimopoulos MA, Weber D, Delasalle KB, et al. Treatment of Waldenstrom's macroglobulinemia resistant to standard therapy with 2-chlorodeoxyadenosine: identification of prognostic factors. *Ann Oncol*. 1995;6:49.
148. Dimopoulos MA, O'Brien S, Kantarjian H, et al. Fludarabine therapy in Waldenström's macroglobulinemia. *Am J Med*. 1993;95:49.
149. Foran JM, Rohatiner AZ, Coiffier B, et al. Multicenter phase II study of fludarabine phosphate for patients with newly diagnosed lymphoplasmacytoid lymphoma, Waldenström's macroglobulinemia, and mantle-cell lymphoma. *J Clin Oncol*. 1999;17:546.
150. Thalhammer-Scherrer R, Geissler K, Schwarzinger I, et al. Fludarabine therapy in Waldenström's macroglobulinemia. *Ann Hematol*. 2000;79:556.
151. Dhodapkar MV, Jacobson JL, Gertz MA, et al. Prognostic factors and response to fludarabine therapy in patients with Waldenström macroglobulinemia: results of United States intergroup trial (Southwest Oncology Group S9003). *Blood*. 2001;98:41.
152. Zinzani PL, Gherlinzoni F, Bendandi M, et al. Fludarabine treatment in resistant Waldenstrom's macroglobulinemia. *Eur J Haematol*. 1995;54:120.
153. Leblond V, Ben-Othman T, Deconinck E, et al. Activity of fludarabine in previously treated Waldenström's macroglobulinemia: a report of 71 cases. *J Clin Oncol*. 1998;16:2060.
154. Dimopoulos MA, Weber DM, Kantarjian H, et al. 2-Chlorodeoxyadenosine therapy of patients with Waldenström macroglobulinemia previously treated with fludarabine. *Ann Oncol*. 1994;5:288.
155. Lewandowski K, Halaburda K, Hellmann A. Fludarabine therapy in Waldenström's macroglobulinemia patients treated previously with 2-chlorodeoxyadenosine. *Leuk Lymphoma*. 2002;43:361.
156. Popat U, Saliba R, Thandi R, et al. Impairment of filgrastim-induced stem cell mobilization after prior lenalidomide in patients with multiple myeloma. *Biol Blood Marrow Transplant*. 2009;15:718.
157. Leleu X, Manning R, Soumerai J, et al. Increased incidence of transformation and myelodysplasia/acute leukemia in patients with Waldenström macroglobulinemia treated with nucleoside analogs. *J Clin Oncol*. 2009;27:250.
158. Leleu X, Tamburini J, Roccaro A, et al. Balancing risk versus benefit in the treatment of Waldenström's macroglobulinemia patients with nucleoside analogue-based therapy. *Clin Lymphoma Myeloma*. 2009;9:71.
159. Treon SP, Kelliher A, Keele B, et al. Expression of serotherapy target antigens in Waldenstrom's macroglobulinemia: therapeutic applications and considerations. *Semin Oncol*. 2003;30:248.
160. Treon SP, Agus DB, Link B, et al. CD20-directed antibody-mediated immunotherapy induces responses and facilitates hematologic recovery in patients with Waldenstrom's macroglobulinemia. *J Immunother*. 2001;24:272.
161. Gertz MA, Rue M, Blood E, et al. Multicenter phase 2 trial of rituximab for Waldenström macroglobulinemia (WM): an Eastern Cooperative Oncology Group study (E3A98). *Leuk Lymphoma*. 2004;45:2047.
162. Dimopoulos MA, Zervas C, Zomas A, et al. Treatment of Waldenstrom's macroglobulinemia with rituximab. *J Clin Oncol*. 2002;20:2327.
163. Treon SP, Emmanouilides C, Kimby E, et al. Extended rituximab therapy in Waldenström's macroglobulinemia. *Ann Oncol*. 2005;16:132.
164. Treon SP, Branagan AR, Hunter Z, et al. Paradoxical increases in serum IgM and viscosity levels following rituximab in Waldenstrom's macroglobulinemia. *Ann Oncol*. 2004;15:1481.
165. Ghobrial IM, Fonseca R, Greipp PR, et al. Initial immunoglobulin M 'flare' after rituximab therapy in patients diagnosed with Waldenström macroglobulinemia: an Eastern Cooperative Oncology Group study. *Cancer*. 2004;101:2593.

166. Castillo JJ, Kanan S, Meid K, Manning R, Hunter ZR, Treon SP. Rituximab intolerance in patients with Waldenstrom macroglobulinaemia. *Br J Haematol*. 2016;174(4):645–648.
167. Dimopoulos MA, Anagnostopoulos A, Zervas C, et al. Predictive factors for response to rituximab in Waldenstrom's macroglobulinemia. *Clin Lymphoma*. 2005;5:270.
168. Furman RR, Eradat HA, DiRienzo CG, et al. Once-weekly ofatumumab in untreated or relapsed Waldenstrom's macroglobulinaemia: an open-label, single-arm, phase 2 study. *Lancet Haematol*. 2017;4(1):e24–e34.
169. Dimopoulos MA, Anagnostopulos A, Kyrtsonis MC, et al. Treatment of relapsed or refractory Waldenström's macroglobulinemia with bortezomib. *Haematologica*. 2005;90:1655.
170. Chen CI, Kouroukis CT, White D, et al. Bortezomib is active in patients with untreated or relapsed Waldenström's macroglobulinemia: a phase II study of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol*. 2007;25:1570.
171. Weber DM, Dimopoulos MA, Delasalle K, et al. 2-Chlorodeoxyadenosine alone and in combination for previously untreated Waldenström's macroglobulinemia. *Semin Oncol*. 2003;30:243.
172. Treon SP, Branagan AR, Ioakimidis L, et al. Long term outcomes to fludarabine and rituximab in Waldenström's macroglobulinemia. *Blood*. 2009;113:3673.
173. Tam CS, Wolf MM, Westerman D, et al. Fludarabine combination therapy is highly effective in first-line and salvage treatment of patients with Waldenström's macroglobulinemia. *Clin Lymphoma Myeloma*. 2005;6:136.
174. Hensel M, Villalobos M, Kornacker M, et al. Pentostatin/cyclophosphamide with or without rituximab: an effective regimen for patients with Waldenström's macroglobulinemia/lymphoplasmacytic lymphoma. *Clin Lymphoma Myeloma*. 2005;6:131.
175. Dimopoulos MA, Anagnostopoulos A, Kyrtsonis MC, et al. Primary treatment of Waldenström's macroglobulinemia with dexamethasone, rituximab and cyclophosphamide. *J Clin Oncol*. 2007;25:3344.
176. Buske C, Hoster E, Dreyling MH, et al. The addition of rituximab to front-line therapy with CHOP (R-CHOP) results in a higher response rate and longer time to treatment failure in patients with lymphoplasmacytic lymphoma: results of a randomized trial of the German Low-Grade Lymphoma Study Group (GLSG). *Leukemia*. 2009;23:153.
177. Treon SP, Hunter Z, Branagan A. CHOP plus rituximab therapy in Waldenström's macroglobulinemia. *Clin Lymphoma Myeloma*. 2005;5:273.
178. Ioakimidis L, Patterson CJ, Hunter ZR, et al. Comparative outcomes following CP-R, CVP-R and CHOP-R in Waldenström's macroglobulinemia. *Clin Lymphoma Myeloma*. 2009;9:62.
179. Rummel M, Niederle N, Maschmeyer G, et al. Bendamustine plus rituximab versus CHOP plus rituximab as first-line treatment for patients with indolent and mantle-cell lymphomas: an open-label, multicentre, randomised, phase 3 non-inferiority trial. *Lancet*. 2013;381:1203.
180. Treon SP, Hanzis C, Tripsas C, et al. Bendamustine therapy in patients with relapsed or refractory Waldenström's macroglobulinemia. *Clin Lymphoma Myeloma Leuk*. 2011;211:133.
181. Dimopoulos MA, Hamilos G, Efstathiou E, et al. Treatment of Waldenström's macroglobulinemia with the combination of fludarabine and cyclophosphamide. *Leuk Lymphoma*. 2003;44:993.
182. Tamburini J, Levy V, Chateilex C, et al. Fludarabine plus cyclophosphamide in Waldenström's macroglobulinemia: results in 49 patients. *Leukemia*. 2005;19:1831.
183. Treon SP, Ioakimidis L, Soumerai JD, et al. Primary therapy of Waldenström's macroglobulinemia with bortezomib, dexamethasone and rituximab. *J Clin Oncol*. 2009;27:3830.
184. Ghobrial IM, Matous J, Padmanabhan S, et al. Phase II trial of combination of bortezomib and rituximab in relapsed and/or refractory Waldenström's macroglobulinemia. *Blood*. 2008;112:832.
185. Agathocleous A, Rohatiner A, Rule S, et al. Weekly versus twice weekly bortezomib given in conjunction with rituximab in patients with recurrent follicular lymphoma, mantle cell lymphoma, and Waldenström macroglobulinemia. *Br J Haematol*. 2010;151:346.
186. Dimopoulos MA, García-Sanz R, Gavriatopoulou M, et al. Primary therapy of Waldenström macroglobulinemia (WM) with weekly bortezomib, low-dose dexamethasone, and rituximab (BDR): long-term results of a phase 2 study of the European Myeloma Network (EMN). *Blood*. 2013;122:3276.
187. Castillo JJ, Meid K, Gustine JN, et al. Prospective Clinical Trial of Ixazomib, Dexamethasone, and Rituximab as Primary Therapy in Waldenstrom Macroglobulinemia. *Clin Cancer Res*. 2018;24(14):3247–3252.
188. Castillo JJ, Meid K, Flynn CA, et al. Ixazomib, dexamethasone, and rituximab in treatment-naive patients with Waldenström macroglobulinemia: long-term follow-up. *Blood Adv*. 2020;4(16):3952–3959.
189. Treon SP, Meid K, Gustine J, et al. Long-term follow-up of ibrutinib monotherapy in symptomatic, previously treated patients with Waldenström macroglobulinemia. *J Clin Oncol*. 2021;39(6):565–575.
190. Treon SP, Palomba ML, Tripsas CK, et al. Ibrutinib in previously treated patients with Waldenström's macroglobulinemia. Paper presented at the 8th International Workshop on Waldenström's Macroglobulinemia, London, UK, August 14–16, 2014. Abstract 91.
191. Dimopoulos MA, Trotman J, Tedeschi A, et al. Ibrutinib for patients with rituximab-refractory Waldenstrom's macroglobulinaemia (iNNOVATE): an open-label substudy of an international, multicentre, phase 3 trial. *Lancet Oncol*. 2017;18(2):241–250.
192. Treon SP, Gustine J, Meid K, et al. Ibrutinib monotherapy in symptomatic, treatment-naive patients with Waldenstrom macroglobulinemia. *J Clin Oncol*. 2018;36(27):2755–2761.
193. Dimopoulos MA, Tedeschi A, Trotman J, et al. Phase 3 trial of ibrutinib plus rituximab in Waldenstrom's macroglobulinemia. *N Engl J Med*. 2018;378(25):2399–2410.
194. Owen RG, McCarthy H, Rule S, et al. Acalabrutinib monotherapy in patients with Waldenström macroglobulinemia: a single-arm, multicentre, phase 2 study. *Lancet Haematol*. 2020;7(2):e112–e121.
195. Tam CS, Opat S, D'Sa S, et al. A randomized phase 3 trial of zanubrutinib vs ibrutinib in symptomatic Waldenström macroglobulinemia: the ASPEN study. *Blood*. 2020;136(18):2038–2050.
196. Treon SP, Hanzis C, Manning RJ, et al. Maintenance rituximab is associated with improved clinical outcome in rituximab naïve patients with Waldenström's macroglobulinemia who respond to a rituximab containing regimen. *Br J Haematol*. 2011;154:357.
197. Rummel MJ, Lerchenmüller C, Hensel M, et al. Two years rituximab maintenance vs. observation after first line treatment with bendamustine plus rituximab (B-R) in patients with Waldenström's macroglobulinemia (MW): results of a prospective, randomized, multicenter phase 3 study (the StiL NHL7-2008 MAINTAIN trial). *Blood*. 2019;134(suppl 1):343.
198. Kyriakou C, Canals C, Sibon D, et al. High-dose therapy and autologous stem-cell transplantation in Waldenström macroglobulinemia: the Lymphoma Working Party of the European Group for Blood and Marrow Transplantation. *J Clin Oncol*. 2010;28:2227.
199. Kyriakou C, Canals C, Cornelissen JJ, et al. Allogeneic stem-cell transplantation in patients with Waldenström macroglobulinemia: report from the Lymphoma Working Party of the European Group for Blood and Marrow Transplantation. *J Clin Oncol*. 2010;28:4926.
200. Kyriakou C, Advani RH, Ansell SM, et al. Indications for hematopoietic stem cell transplantation in patients with Waldenstrom's macroglobulinemia: a Consensus Project of the EBMT Lymphoma Working Party (LWP)/European Consortium for Waldenstrom's macroglobulinemia (ECWM)/International Waldenstrom's macroglobulinemia Foundation (IWMF). *Blood*. 2017;130(Supplement 1):2026.
201. Talaulikar D, Advani RH, Branagan AR, et al. Consensus statement on the management of Waldenström macroglobulinemia patients during the COVID-19 pandemic. *Hemasphere*. 2020;4(4):e433.
202. Owen RG, Kyle RA, Stone MJ, et al. Response assessment in Waldenström macroglobulinemia. *Br J Haematol*. 2013;160:171.
203. Treon SP, Yang G, Hanzis C, et al. Attainment of complete/very good partial response following rituximab based therapy is an important determinant to progression-free survival and is impacted by polymorphisms in FCGR3A in Waldenström macroglobulinaemia. *Br J Haematol*. 2011;154:223.
204. Ghobrial IM, Witzig TE, Gertz M, et al. Long-term results of the phase II trial of the oral mTOR inhibitor everolimus (RAD001) in relapsed or refractory Waldenström macroglobulinemia. *Am J Hematol*. 2014; 89:237.
205. Strauss SJ, Maharaj L, Hoare S, et al. Bortezomib therapy in patients with relapsed or refractory lymphoma: potential correlation of in vitro sensitivity and tumor necrosis factor alpha response with clinical activity. *J Clin Oncol*. 2006;24:2105.
206. Varghese AM, Rawstron AC, Ashcroft AJ, et al. Assessment of bone marrow response in Waldenström's macroglobulinemia. *Clin Lymphoma Myeloma*. 2009;9:53.
207. Gobbi PG, Bettini R, Montecucco C, et al. Study of prognosis in Waldenström's macroglobulinemia: a proposal for a simple binary classification with clinical and investigational utility. *Blood*. 1994;83:2939.
208. Morel P, Monconduit M, Jacomy D, et al. Prognostic factors in Waldenström macroglobulinemia: a report on 232 patients with the description of a new scoring system and its validation on 253 other patients. *Blood*. 2000;96:852.
209. Dhodapkar MV, Jacobson JL, Gertz MA, et al. Prognostic factors and response to fludarabine therapy in patients with Waldenström macroglobulinemia: results of United States intergroup trial (Southwest Oncology Group S9003). *Blood*. 2001;98:41.
210. Dimopoulos M, Gika D, Zervas K, et al. The international staging system for multiple myeloma is applicable in symptomatic Waldenström's macroglobulinemia. *Leuk Lymphoma*. 2004;45:1809.

211. Anagnostopoulos A, Zervas K, Kyrtsonis M, et al. Prognostic value of serum beta 2-microglobulin in patients with Waldenström's macroglobulinemia requiring therapy. *Clin Lymphoma Myeloma*. 2006;7:205.
212. Morel P, Duhamel A, Gobbi P, et al. International prognostic scoring system for Waldenström macroglobulinemia. *Blood*. 2009;113:4163.
213. Kastritis S, Kyrtsonis MC, Hatjiharissi E, et al. No significant improvement in the outcome of patients with Waldenström's macroglobulinemia treated over the last 25 years. *Am J Hematol*. 2011;86:479.
214. Castillo J, Allan J, Siddiqi T, et al. Multicenter prospective phase II study of venetoclax in patients with previously treated Waldenstrom macroglobulinemia. *Clin Lymphoma Myeloma Leuk*. 2019;19(10, Supplement):e39–e40.
215. Castillo JJ, Libby EN, Ansell SM, et al. Multicenter phase 2 study of daratumumab monotherapy in patients with previously treated Waldenström macroglobulinemia. *Blood Adv*. 2020;4(20):5089–5092.
216. Paulus A, Manna A, Akhtar S, et al. Targeting CD38 with daratumumab is lethal to Waldenstrom macroglobulinaemia cells. *Br J Haematol*. 2018;183(2):196–211.
217. Mato AR, Shah NN, Jurczak W, et al. Pirtobrutinib in relapsed or refractory B-cell malignancies (BRUIN): a phase 1/2 study. *Lancet*. 2021;397(10277):892–901.
218. Woyach J, Stephens DM, Flinn IW, et al. Final results of phase 1, dose escalation study evaluating ARQ 531 in patients with relapsed or refractory B-cell lymphoid malignancies. *Blood*. 2019;134(suppl 1):4298.

CHAPTER 93

IMMUNOGLOBULIN LIGHT-CHAIN AMYLOIDOSIS (PRIMARY AMYLOIDOSIS)

Morie A. Gertz, Francis K. Buadi, Martha Q. Lacy, and Suzanne R. Hayman

Amyloidosis is defined as the clinical syndrome associated with deposition of amyloid. Amyloid in tissue is defined by its tinctorial properties of a homogeneous, eosinophilic, hyaline material when viewed by hematoxylin and eosin staining. Amyloid stains specifically with Congo red, demonstrating a deep pink amorphous composition. Although Congo red staining is the sine qua non for the diagnosis of amyloid, many pathology laboratories prefer the use of sulfated Alcian blue or crystal violet for screening. A second requirement is that the Congo red-positive histologic finding must demonstrate apple-green birefringence when observed under polarized light.[1] A positive crystal violet or sulfated Alcian blue stain should be confirmed with Congo red staining.

The term *amyloid* is a misnomer. The term was first used in 1838 by a German botanist because the tissue stained blue with iodine and was incorrectly thought to be starch-like, thus the terminology of *amyloid*. In Vienna, Rokitansky believed that the material was not starch but rather made of lipid composition and used the term *lardaceous degeneration* for amyloid. Both were incorrect because all forms of amyloid appear to be protein-derived.[2]

By electron microscopy, amyloid has been shown to be fibrillar in origin with an approximate diameter of 9.5 nm, and linear nonbranching and comprised of protofilaments (Fig. 93.1). The Congo red binding occurs because the protein misfolds from the physiologic configuration of the α-helix into a β-pleated sheet, which is directly responsible for its insolubility and resistance to proteolysis. Amyloid is highly resistant to solubilization unless it is subjected to the harshest denaturation conditions. This insolubility in the physiologic solution likely contributes to the amyloid's ability to disrupt normal organ function and leads to the clinical disease amyloidosis, the clinical syndrome associated with deposition of amyloid.[3]

EPIDEMIOLOGY

The incidence of immunoglobulin light-chain amyloidosis is estimated to be approximately 1.2 per 100,000 person years. The rate of the disease is not increasing. One would expect 3852 new cases of amyloid light-chain (AL) amyloidosis in the United States each year. It is the cause of death in 0.58 of 1000 recorded deaths and is responsible for 0.8% of end-stage renal disease. At the Mayo Clinic, AL amyloidosis represents 9% of all patients seen with monoclonal gammopathies.[4,5] Half of these patients have over 10% plasma cells in the bone marrow fulfilling criteria for smoldering multiple myeloma. At the time of diagnosis of amyloidosis, if multiple myeloma is not present it will only be developed subsequently in 0.4% of patients.[4] At the time of diagnosis of light-chain amyloidosis 8% have been found to have coexisting multiple myeloma.[5] AL amyloidosis represents 52% of all patients seen with amyloidosis. However, hereditary amyloidosis now accounts for 5% of patients, localized amyloidosis 9% of patients, *wild-type TTR* (TTRwt) (senile systemic amyloidosis) 32% of patients, and secondary amyloidosis 1% of patients. All these forms of amyloidosis have the same tinctorial properties in the histologic section and must be distinguished using other techniques. The reported overall mean age-adjusted mortality rate from amyloidosis as the underlying cause of death has doubled from 1.77 to 3.96 per 100,000 population.[6]

The cause of the plasma cell dyscrasia that underlies AL amyloidosis remains unknown. There does not appear to be a linkage to any specific occupation or toxic chemical exposure. No environmental agents have been linked to the development of amyloidosis. However, the Veterans Administration in the United States considers amyloidosis in a veteran who was exposed to Agent Orange (dioxin) during the Vietnam era to have a service-connected illness.[7]

Amyloidosis may be systemic or localized. The localized forms do not require systemic chemotherapy. The distinction is, therefore, important so that patients for whom chemotherapy will not be of benefit are not inappropriately subjected to this form of therapy. Localized amyloidosis can be suspected, usually based on the location of the deposits, as discussed in the "Clinical Manifestations" section below.[8]

Familial amyloidosis is, for most patients, caused by an inherited mutation of transthyretin (TTR). It is difficult to distinguish from light-chain amyloidosis because the clinical manifestations may be quite similar. Other inherited forms of amyloidosis have been associated with apolipoprotein A1 mutations, fibrinogen A α mutations, and mutations of gelsolin.[9]

Secondary systemic amyloidosis is amyloidosis that is usually a consequence of a sustained inflammatory process. The frequency of symptomatic secondary systemic amyloidosis has been in a sharp decline for the past decade. Previously, secondary systemic amyloidosis was a consequence of an uncontrolled inflammatory process, typically inflammatory polyarthropathies, such as juvenile rheumatoid arthritis, psoriatic arthritis, or ankylosing spondylitis. Another cause was long-standing inflammatory bowel disease. Amyloidosis as a consequence of these inflammatory processes has fallen sharply with the introduction of biologic agents that function as inhibitors of interleukin-1, interleukin-6, and inhibitors of tumor necrosis factor α (TNF-α). Sporadic patients are seen with multiple cutaneous abscesses, usually as a result of subcutaneous injection of contaminated narcotics. Patients can also be seen with chronic infections related to paraplegia or quadriplegia. Rare patients with lifelong bronchiectasis related to cystic fibrosis can develop systemic amyloidosis. There are also forms of inherited secondary amyloidosis associated with familial inflammatory syndromes such as familial Mediterranean fever or mutations of the TNF receptor gene (so-called TNF receptor-associated periodic syndrome).[10]

PATHOBIOLOGY OF THE DISEASE

In immunoglobulin light-chain amyloidosis, the immunoglobulin fragments have specific thermodynamic instability that causes them to misfold into the insoluble amyloid configuration. The injection of immunoglobulin light chains purified from the urine of patients with multiple myeloma will not produce amyloid when injected into mice. However, when the light chains are extracted from the urine of patients with light-chain amyloidosis and injected into mice, it will produce amyloid deposits and organ dysfunction, suggesting that the specifics of the immunoglobulin light chain are important in determining its propensity to misfold into amyloids. Monoclonal immunoglobulin light chains can be converted in vitro to amyloid by in vitro digestion with pepsin. Historically, before it was clearly

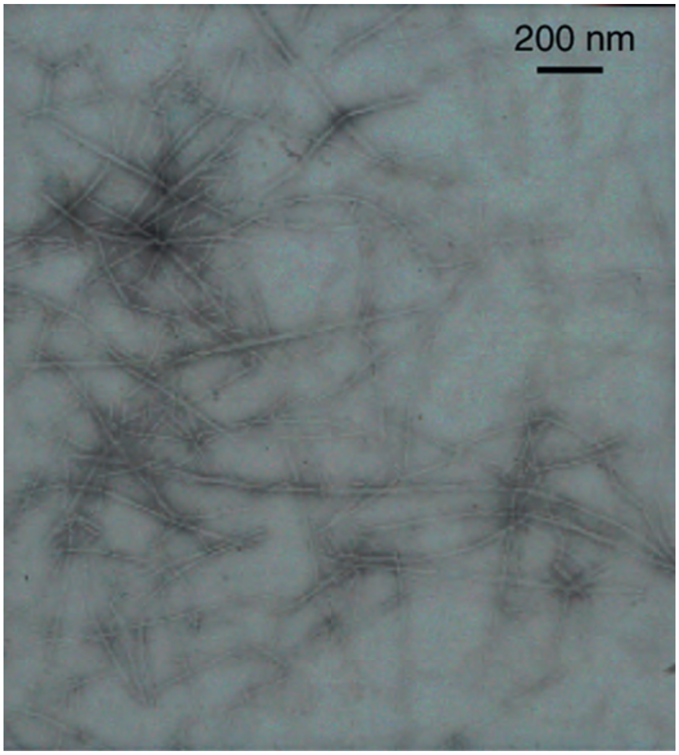


Figure 93.1 ELECTRON MICROGRAPH DEMONSTRATING THE CLASSIC FIBRILS OF AMYLOID.

understood that amyloidosis was derived from a plasma cell dyscrasia with the development of “toxic” immunoglobulin light chains, the term *primary amyloidosis* was used as a consequence of the lack of understanding of the pathophysiology. This term should be abandoned, and the term *immunoglobulin light-chain amyloidosis* or *AL amyloidosis* (amyloid light chain) should be used. Many forms of localized amyloidosis are also derived from immunoglobulin light chains, but there is no evidence of a systemic plasma cell dyscrasia, and anti-plasma cell chemotherapy is contraindicated. Virtually all patients with systemic AL amyloidosis have a demonstrable clonal plasma cell disorder. In monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma, two-thirds of the immunoglobulin light chains are κ type. In AL amyloidosis, nearly three-fourths of the light chains are of λ origin (Fig. 93.2), suggesting that there is a greater propensity for λ light chains to misfold into amyloid configuration. The λ_6 subgroup of light chains is exclusively associated with amyloid deposition. There are also abnormalities in amyloid-associated germline gene use. A preferential use of variable lambda (VL) germline genes is noted for ALκ and AL λ patients. There is a significant correlation between the use of V λ_6 germline, 6a, and renal involvement as well as the λ_3 gene, 3r, with soft tissue AL. Identification of the clonal VL gene in AL has important implications regarding clinical outcome.[11]

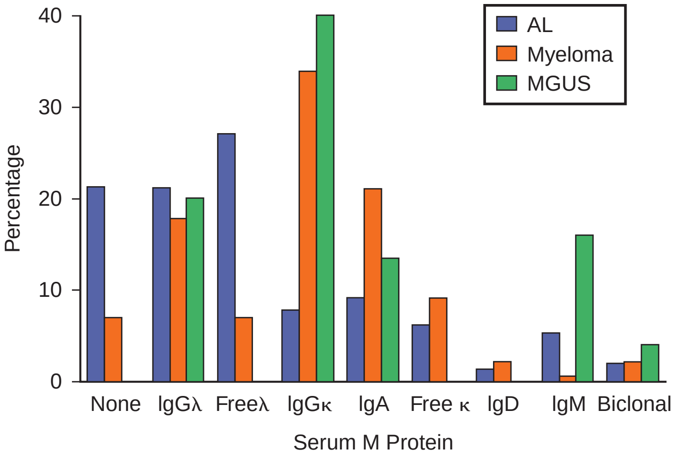


Figure 93.2 DISTRIBUTION OF HEAVY- AND LIGHT-CHAIN PROTEINS IN MYELOMA, MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE, AND AMYLOID LIGHT-CHAIN AMYLOIDOSIS. *AL*, Amyloid light chain; *Ig*, Immunoglobulin; *MGUS*, monoclonal gammopathy of undetermined significance.

Chromosomal aberrations of the plasma cells in AL amyloidosis have also been recognized. Interphase fluorescence in situ hybridization (FISH) has detected a gain of 1q21, predicting a poor overall survival, and is an independent prognostic factor. Patients with t(11;14) have a longer median overall survival. The t(11;14) is associated with lower response rates to bortezomib-containing regimens. This translocation is seen in 50% of AL patients compared to 15% of myeloma patients. This high prevalence has led to the exploration of venetoclax as a possible therapy for AL amyloidosis. Translocation (11;14) suppresses the emergence of sub-clones and likely accounts for the lower frequency of −17p and 1q+ in AL amyloidosis. Deletion 13q14 and hyperdiploidy are prognostically neutral. Because the incidence of t(11;14) is so high in AL amyloidosis the possibility that venetoclax would be active is rational. Clinical trials are underway to test venetoclax but pending results of these trials it would be reasonable to consider venetoclax in patients who failed the first- or second-line therapies. There are case reports of venetoclax-induced complete response in patients with AL amyloidosis. The combination of daratumumab and venetoclax has been reported to produce both hematological complete response and organ response in a patient with cardiac, renal, peripheral, and autonomic nervous system involvement.[12]

The pathobiology of organ dysfunction associated with amyloidosis has long been assumed to be caused by the direct effect of amyloid protein deposition in tissues. However, there is increasing evidence that the light chains themselves have a toxic effect on tissues as a soluble mediator. A zebrafish model of human light-chain cardiotoxicity has demonstrated that the injection of light chains results in impaired cardiac function, pericardial edema, and increased cell death. Mitogen-activated protein (MAP) kinase activation may mediate this cardiotoxicity, and p38 MAP kinase inhibition reduces cell death and improves cardiac function.[13]

Amyloidosis and multiple myeloma both share the presence in the bone marrow of a clonal population of plasma cells. The number of plasma cells and the immunoglobulin light chains that they produce are both important for prognosis. Patients who have an increased number of plasma cells in the bone marrow with light-chain amyloidosis have a worse prognosis and shorter overall survival, regardless of whether they manifest any of the clinical criteria associated with multiple myeloma, such as hypercalcemia, renal insufficiency, anemia, or destructive bone lesions. There appears to be no survival difference between patients who have an elevated plasma cell count over 10% in their bone marrow and whether they have overt (CRAB—hyperCalcemia, Renal insufficiency, Anemia, Bone lesions) symptoms of myeloma. The therapeutic import has led our group to recommend induction chemotherapy for patients with AL amyloidosis at a diagnosis prior to consolidative, autologous stem cell transplantation.[14]

CLINICAL MANIFESTATIONS

Patient 1

Patient 1 is a 46-year-old man who, 10 months prior to diagnosis, began to develop cramping abdominal pain associated with an unexplained 45-lb weight loss. Four months later, he began to develop episodes of orthostatic syncope and was documented to have an orthostatic drop in blood pressure from systolic 110 mmHg in supine to 66 mmHg when standing. As a consequence of his abdominal cramping, the patient underwent endoscopy and colonoscopy. Biopsies were obtained from the duodenum, proximal jejunum, colon, and rectum, all of which demonstrated amyloid deposits. Subsequently, a bone marrow biopsy was performed that showed 4% clonal plasma cells. A subcutaneous fat aspirate was positive for amyloid. The plasma cells showed a CCND1/IgH fusion t(11;14). The patient's hemoglobin level was normal, as were his creatinine, alkaline phosphatase, and urinary protein. The patient had a λ free light chain level of 92.8 mg/L, κ free light chain of 10.9 mg/L, and a ratio of 0.12.

Comment on Patient 1

This patient presented with amyloid autonomic neuropathy, leading to gastrointestinal (GI) tract dysmotility and pain as well as orthostatic hypotension. There was clear-cut evidence of a low-volume plasma cell dyscrasia. Orthostatic hypotension causing lightheadedness is a very nonspecific complaint, and syncope owing to amyloid orthostatic hypotension is easily overlooked because of its infrequency.

Patient 2

A 61-year-old man with a known immunoglobulin G λ MGUS of 0.6 mg/dL was monitored on an annual basis with no change in the level of the monoclonal protein over a period of 3 years. He developed progressive fatigue and shortness of breath. He was evaluated by a cardiologist, and his echocardiogram showed thickening that was interpreted to be related to hypertension, with a normal ejection fraction. The patient was placed on lisinopril and did not improve. He returned and underwent catheterization with coronary angiography. His coronary arteries were normal, and he was diagnosed with deconditioning. His dyspnea continued, and he was referred to a pulmonologist, who recognized the long-standing monoclonal gammopathy and obtained light chain studies that showed a λ light chain of 410 mg/L and κ of 10 mg/dL. The patient underwent a subcutaneous fat aspiration that demonstrated amyloid deposits, which, by mass spectroscopy, were found to be of λ origin. At this point, the patient had New York Heart Association class IV heart failure; he died 3 months thereafter.

Comment on Patient 2

This case is an example of a classic failure to recognize that patients with monoclonal gammopathies should have AL amyloidosis included in the differential diagnosis. The fact that the monoclonal protein was stable would certainly argue against the development of multiple myeloma, but light-chain amyloidosis regularly occurs without any change in the M component over time. The failure to recognize the patient's progressive fatigue and shortness of breath as cardiac amyloid is not unusual. The echocardiographic findings of thickening were interpreted as being caused by hypertension, but, in fact, they were caused by infiltrative cardiomyopathy, and the cardiac catheterization and angiogram without an endomyocardial biopsy led to a missed diagnosis. Only an increased index of suspicion in patients with a known monoclonal protein allowed this diagnosis to be confirmed.

One of the major difficulties in the diagnosis of amyloidosis is that there is virtually no blood test or imaging study that is diagnostic of the disease. The most common symptoms associated with amyloidosis are fatigue, lower extremity edema (may be cardiac or renal), unexplained weight loss (often leading to search for occult malignancy), exertional dyspnea, orthostatic hypotension, and paresthesias. These symptoms are quite vague, and in a general medical practice, there are hundreds of disorders that are far more common and are responsible for light-chain amyloidosis. As noticed in Patient 2, the fatigue, which is often caused by early cardiac amyloid but is not associated with overt congestive heart failure, can be missed. Cardiac amyloidosis is a classic form of heart failure with a preserved ejection fraction owing to the restrictive physiology associated with amyloidosis.[15] The typical echocardiographic findings include thickening of the interventricular septum due to infiltration with amyloid (usually >12 mm), a decline in global longitudinal strain (normal is greater than −18%) and the slowing of filling of the ventricles during diastole due to non-compliant walls.

Edema in amyloidosis may be a manifestation of high right-sided filling pressures, leading to lower extremity edema, or it may be a consequence of renal involvement with the nephrotic syndrome (peripheral edema, hypoalbuminemia, hyperlipidemia, and proteinuria). Even when nephrotic range proteinuria is seen, amyloidosis, which is known to cause nephrotic syndrome in 10% of adults who are nondiabetic, is infrequently considered in the differential diagnosis, which would include minimal change glomerulopathy, as well as membranous and membranoproliferative glomerulopathy.[16,17]

The physical findings that are known to be associated with light-chain amyloidosis include enlargement of the tongue (15%) (Fig. 93.3) and periorbital purpura (12%). These findings are highly specific when recognized. However, although they are highly specific, they lack sensitivity because nearly 80% of patients with amyloidosis lack both of these physical features. It is common for an enlarged tongue in amyloid to be misdiagnosed as suspected glossal cancer or a manifestation of acromegaly. Many patients with significant tongue enlargement needlessly undergo a painful and often hemorrhagic tongue biopsy, which could be avoided if the diagnosis was considered. Patients with enlargement of the tongue frequently have major indentations on the underside of their tongue from their teeth and the continuous pressure that the tongue exerts against their lower jaw. The purpura is also quite specific, and the patient often will note the

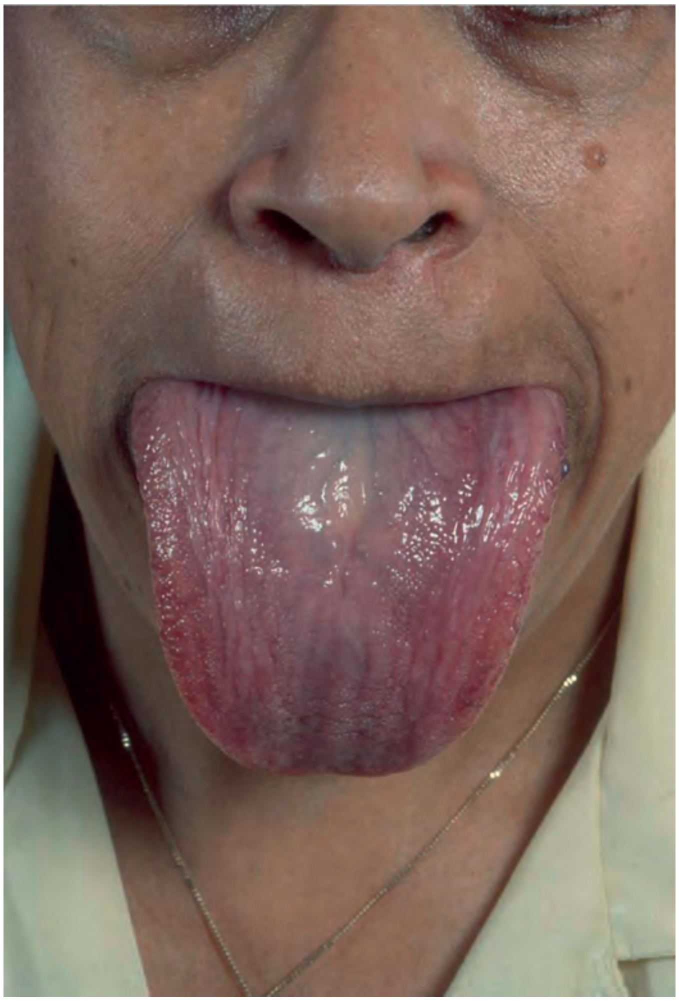


Figure 93.3 ENLARGED TONGUE INFILTRATED BY AMYLOID DEPOSITS.

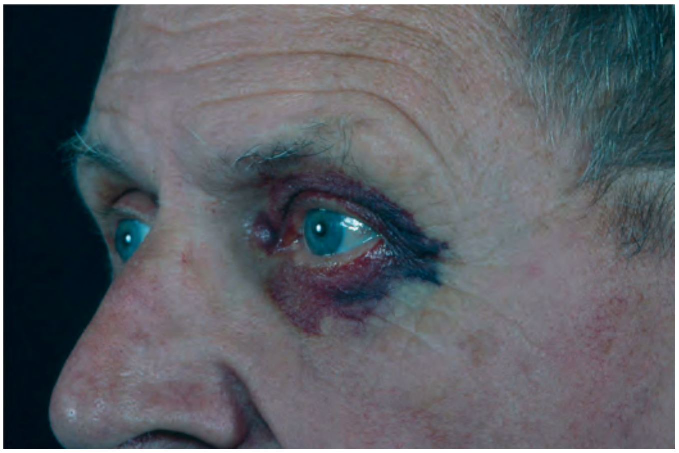


Figure 93.4 CLASSIC AMYLOID PURPURA.

development of purpura simply by rubbing of the eyelids. However, these patients often undergo an evaluation for a coagulation disorder or are simply reassured that purpura is benign, and the physical findings do not trigger an investigation for light-chain amyloidosis (Fig. 93.4).[18]

Hepatomegaly is seen in approximately 10% of patients with amyloidosis, but it is nonspecific, and an imaging of the liver will show homogeneous enlargement without filling defects. Occasionally, patients will have a widespread vascular amyloid that will result in claudication of the calf, buttock, upper extremities, and jaw. Occasionally, these patients will undergo temporal artery biopsy, which, when appropriately stained, will show amyloid. More often, these patients will be given an empiric diagnosis of polymyalgia rheumatica and be placed on corticosteroids without appropriate screening. The rarest physical finding in amyloidosis is periarticular infiltration of the synovium in the upper extremities, leading to the "shoulder pad" sign (Fig. 93.5). This causes a continuous, chronic, low-grade pain caused by the periarticular infiltration. Diagnosis in this setting requires arthrocentesis and the demonstration of Congo red-positive deposits in the synovial fluid.[19]

LABORATORY MANIFESTATIONS

If the symptoms of amyloidosis are so vague as not to be helpful and the physical findings are specific but not sensitive, when should a clinician suspect amyloidosis and aggressively pursue this rare disorder? Amyloidosis should be considered in any patient who presents with (1) nephrotic range proteinuria; (2) fatigue, which may have a cardiac basis, including heart failure with preserved ejection fraction or restrictive cardiomyopathy; (3) unexplained hepatomegaly; (4) a peripheral neuropathy resembling chronic inflammatory demyelinating polyneuropathy (CIDP); and (5) "atypical" smoldering multiple myeloma or MGUS with unexplained fatigue, weight loss, and edema. Consulting patients with any one of these five syndromes should lead to the placement of light-chain amyloid in the differential diagnosis, and screening should commence.[20]

Because all patients with systemic immunoglobulin light-chain amyloidosis have a plasma cell dyscrasia, the initial step should be screening by performing immunofixation of the serum, immunofixation of the urine, and an immunoglobulin free light chain assay. Results of one of these three tests will be abnormal in 99% of patients with light-chain amyloidosis. If results of these three tests are negative, the likelihood is that one of the following conditions exists: (1) The patient does not have amyloidosis; (2) the chance of immunoglobulin light-chain amyloidosis is only 1%; (3) the patient has systemic amyloidosis, but it is not of immunoglobulin light-chain origin (familial amyloid or senile systemic amyloid); or (4) the amyloidosis is localized.[21]

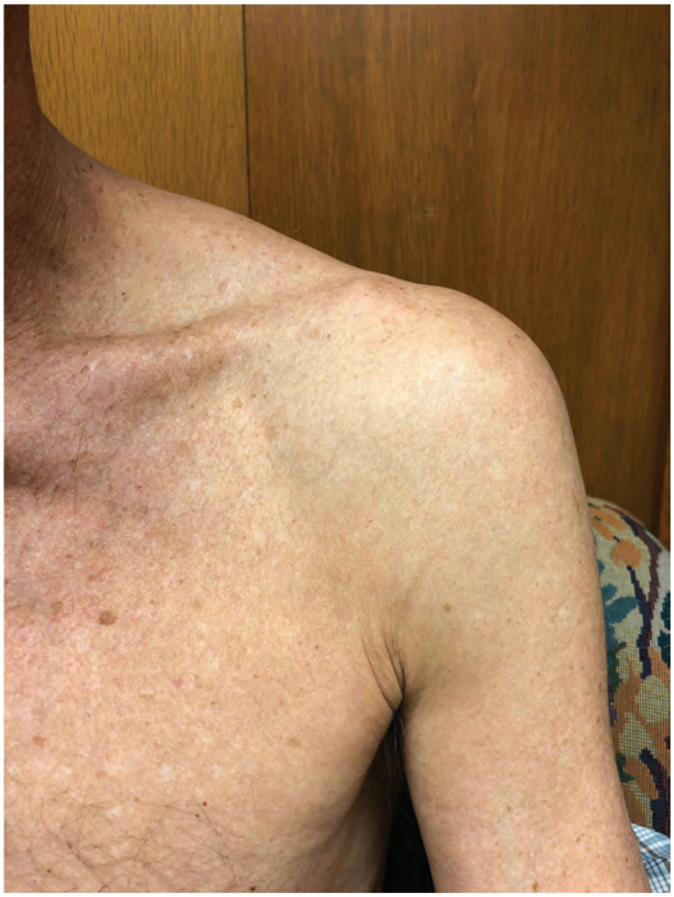


Figure 93.5 SHOULDER PAD SIGN.

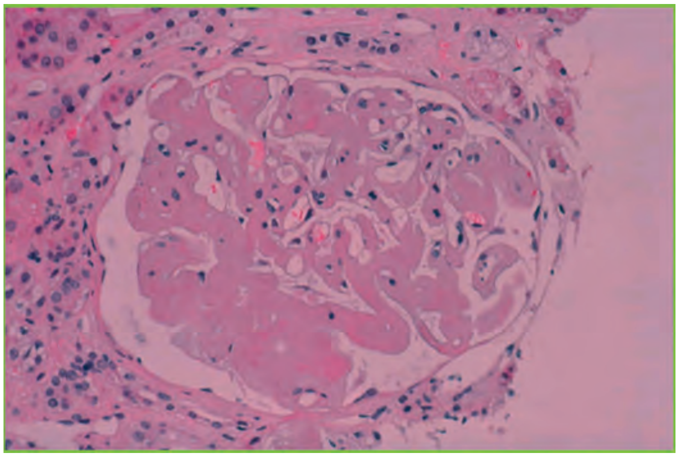


Figure 93.6 RENAL BIOPSY SHOWING AMORPHOUS HYALINE MATERIAL CONSISTENT WITH AMYLOID. (Original magnification, ×1000.)

Alternatively, if a patient with nephrotic range proteinuria has a monoclonal protein or an abnormal free light chain ratio, the diagnosis, which often includes minimal change glomerulopathy and membranoproliferative glomerulopathy, suddenly shifts to either (1) myeloma cast nephropathy, (2) immunoglobulin light-chain amyloidosis of the kidney (Fig. 93.6), or (3) κ light-chain deposition disease of the kidney (5% of patients may exhibit lambda deposition). In this situation, a kidney biopsy could be avoided by doing less invasive diagnostic testing.[16]

In a patient with fatigue, weight loss, or dyspnea upon exertion, the finding of an immunoglobulin abnormality raises the suspicion of amyloid. Evaluation can be redirected to include echocardiography with the specific inclusion of Doppler studies that accurately measure the restricted filling that occurs with amyloid infiltration (stiff heart syndrome). It may also include magnetic resonance imaging of the heart, which can show distinct endomyocardial enhancement following gadolinium injection, as well as myocardial nulling, which is specific for light-chain amyloidosis.[22] Myocardial nulling refers to an inversion recovery pulse sequence that is used to null the signal from the desired tissue to accentuate surrounding pathology. The nulled normal myocardium will be dark in contrast to the enhanced abnormal myocardium.

A patient with unexplained hepatomegaly who has a monoclonal protein can often avoid a liver biopsy, which can be associated with bleeding and occasionally with hepatic rupture. Fat aspiration or excisional fat biopsy is positive in over 75% of patients with hepatic amyloidosis. Minor salivary gland biopsy can demonstrate amyloid deposits in 68% of patients with amyloidosis and is more sensitive than a blind skin biopsy.[23] Salivary gland biopsy is complementary to fat pad biopsy in selected cases of negative fat pad biopsy, thereby avoiding liver, kidney, or endocardial biopsy.

In a patient with peripheral neuropathy who is found to have a monoclonal protein, consideration of amyloidosis in the differential diagnosis can prevent interventions such as plasma exchange or intravenous immunoglobulin infusions, which are ineffective in patients with light-chain amyloidosis but are often attempted in patients who have a monoclonal gammopathy and peripheral neuropathy (presumed CIDP) if amyloidosis is not considered in the differential diagnosis.[24] One needs to distinguish between IgM amyloidosis and IgM-mediated peripheral neuropathy, so-called DADS (distal acquired demyelinating symmetric neuropathy), a form of CIDP.

All patients with MGUS need to be monitored for life for the development of myeloma and AL amyloidosis. Over 25 years, approximately 25% of patients will go on to develop a more serious plasma cell dyscrasia. In 21%, this represents multiple myeloma, but 4% of patients with MGUS will develop light-chain amyloidosis during the course of observation. If screening is limited to detection of changes consistent with multiple myeloma, such as anemia, bone pain, and hypercalcemia, amyloidosis will be overlooked because all three of these findings are unusual in light-chain amyloidosis. Patient 2, as described above, was being followed by a hematologist for a monoclonal gammopathy, and when he developed fatigue (cardiac) in the absence of progressive anemia, amyloidosis was not considered.[25]

Any patient with one of the five compatible clinical syndromes listed in Table 93.1 should be screened for monoclonal protein. If a monoclonal protein is not found, the likelihood of light-chain amyloidosis is very small. However, if a monoclonal gammopathy exists with an appropriate clinical syndrome, histologic demonstration of the amyloid should be sought.

In patients who have renal, cardiac, hepatic, and peripheral nerve amyloid, the biopsy of the affected organs has a very high sensitivity of demonstrating the amyloid. However, these biopsies are not required if the amyloid is considered in the differential diagnosis.[26]

The first diagnostic studies that should be performed in patients with a compatible clinical syndrome and an immunoglobulin light-chain abnormality are a subcutaneous fat aspirate and a bone marrow biopsy. Subcutaneous fat aspirate in an experienced laboratory will demonstrate amyloid deposits in 75% of patients tested (Fig. 93.7). An excellent YouTube video has been produced by the Boston Medical Center on the technique (https://www.youtube.com/watch?v=tctYTmxd9gQ). In a patient with light-chain amyloidosis, staining of the bone marrow biopsy for amyloid deposition in blood vessels is positive 50% of the time. A second advantage of the bone marrow biopsy is it provides the percentage of plasma cells in the bone marrow, which is prognostic and is an essential evaluation in patients with immunoglobulin light-chain abnormalities. When combining the subcutaneous fat aspirate and the bone marrow biopsy, 85% of patients with light-chain amyloidosis will be identified. For the remaining 15%, a direct biopsy of the involved organ would be indicated if the index of suspicion for light-chain amyloidosis remained high. Other centers have reported success with biopsies of the minor submandibular salivary gland, skin biopsies, and endoscopic biopsies of the stomach. These procedures would be best undertaken in laboratories where there is extensive experience in the processing and staining of amyloid tissues.[27]

TABLE 93.1 **Syndromes in Primary Amyloidosis**

Syndrome	Patients (%)
Nephrotic or nephrotic and renal failure	30
Hepatomegaly	24
Congestive heart failure	22
Carpal tunnel	21
Neuropathy	17
Orthostatic hypotension	12

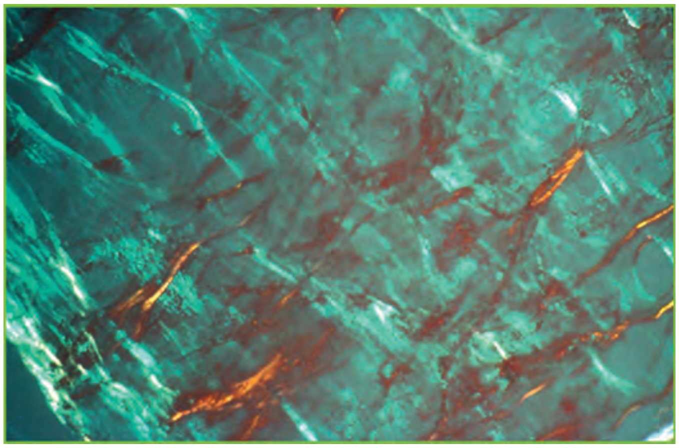


Figure 93.7 FAT ASPIRATION. (Congo red stain; original magnification, ×1000.) Note preserved fat cell interstices.

Once the amyloid has been demonstrated in histologic sections, it is imperative that the amyloid be typed to ensure it is of immunoglobulin light-chain origin because of the critical therapeutic implications. Although immunoglobulin light-chain amyloidosis is always associated with a monoclonal gammopathy, it is important to recognize that monoclonal gammopathies are common in the elderly population. On screening, 3% of adult patients over the age of 50 will have a monoclonal protein, and 5% of those over the age of 80 will have a monoclonal protein. Therefore, it is possible to have a positive biopsy for amyloid and a coincidental monoclonal protein when the amyloid itself is unrelated to immunoglobulin light-chain amyloidosis. Thus, when a pathologist reports amyloid-laden tissue, the task will not be complete until the specific protein subunit of amyloid is identified (Table 93.2). Historically, immunohistochemistry was used to classify amyloids. This technique is being used less. First, the type of amyloid can be identified only if the appropriate antibodies are used. In patients with amyloid deposition, it would be very challenging to diagnose dialysis-related amyloid (β_2-microglobulin type), insulin-type amyloid (found at the sites of insulin injection in diabetics), keratin amyloid (seen in skin biopsies), or TTR amyloid (seen in senile systemic amyloid, TTRwt). Most laboratories are not equipped with such a broad panel of antibodies. Second, even when light-chain amyloidosis exists, the use of anti-κ and anti-λ antibodies frequently will not be able to identify the type of amyloid in the tissue section. There are two widely held hypotheses for this lack of sensitivity of immunohistochemistry in AL amyloidosis.[28]

The first hypothesis is a reflection of the immunoglobulin in amyloid deposits. The light chain in amyloid is usually not the intact immunoglobulin light chain but usually represents a fragment. The average AL protein subunit is 12 kDa, approximately half the molecular weight of an intact immunoglobulin light chain. In most of these proteins, the constant portion of the light chain has been deleted.

TABLE 93.2 Nomenclature of Amyloidosis

Protein	Precursor	Clinical Characteristics
AL or AH	Immunoglobulin light or heavy chain	Primary or localized; myeloma or macroglobulinemia associated
AA	SAA	Secondary or familial Mediterranean fever, familial periodic fever syndromes
ATTR	Transthyretin	Familial and senile
A fibrinogen	Fibrinogen	Familial renal amyloidosis (Ostertag type)
$A\beta_2M$	β_2-Microglobulin	Dialysis associated; carpal tunnel syndrome
Aβ	ABPP	Alzheimer disease
A Apo A-I/A-II	Apolipoprotein A-I	Proteinuria
	Apolipoprotein A-II	Cardiac
		Neuropathy
A lysozyme	Lysozyme	GI tract
		Liver
		Renal
ALECT2	Renal	

AA, Amyloid A; *Aβ*, amyloid-β; *ABPP*, amyloid-β precursor protein; *$A\beta_2M$*, β_2-microglobulin-related amyloid; *AH*, amyloid heavy chain; *AL*, amyloid light chain; *ALECT2*, leukocyte chemotactic factor 2 amyloidosis; *Apo*, apolipoprotein; *ATTR*, transthyretin-related hereditary amyloidosis; *GI*, gastrointestinal; *SAA*, serum amyloid A.

BOX 93.1 Recommended Diagnostic Evaluation for Amyloidosis

- CBC
- Sodium, potassium, alkaline phosphatase, calcium, phosphorus, AST, bilirubin, creatinine, β_2-microglobulin, glucose, cholesterol, uric acid, thyroid profile
- Immunofixation; serum; immunofixation urine; nephelometric assay if immunoglobulin free light chains; immunoglobulins G, A, and M
- Troponin, NT-proBNP
- Factor X and prothrombin time
- Chest X-ray, EKG, echocardiogram with Doppler and strain imaging
- Cardiac MRI (optional selected situations)
- Bone marrow with FISH genetics, Congo red stain of marrow
- Fat aspiration
- Creatinine clearance

AST, Aspartate transaminase; *CBC*, complete blood count; *EKG*, electrocardiogram; *FISH*, fluorescence in situ hybridization; *NT-proBNP*, N-terminal pro-B-type natriuretic peptide; *MRI*, magnetic resonance imaging.

Most commercial antisera used in immunohistochemistry recognize the constant portion of the immunoglobulin light chain and, therefore, have no recognizable binding sites to AL, owing to the deletions it undergoes as it is deposited.[29]

The second hypothesis is that the immunoglobulin light chain, by definition, has undergone misfolding as it assumes the amyloid configuration in tissues. This misfolding can lead to the loss of available epitopes on the protein surface that commercial antisera bind to. It is for these reasons that it is common for immunohistochemistry, even in light-chain amyloidosis, to be equivocal or difficult to distinguish from background staining. At the Mayo Clinic, immunohistochemistry has been abandoned as a modality to diagnose the type of amyloidosis. Currently, laser capture microdissection with mass spectroscopic analysis of amyloid deposits has supplanted immunohistochemistry in our practice. Amyloid deposits can be excised by laser microdissection directly from a glass slide and then can undergo mass spectroscopic sequencing. The results are compared with a library of proteins stored in a database and then identified. Virtually all amyloid proteins contain serum amyloid P protein, apolipoprotein E, and vitronectin. These findings are confirmatory of the amyloid and serve as a good positive control but are ancillary proteins and are not the specific primary protein. In patients with amyloidosis, sequencing will identify the specific protein composition. In a survey of over 4000 tissues, light chains were detected by mass spectroscopic analysis in 61.68%, but 24.5% were transthyretin-related amyloidosis, 3.7% were amyloid A, 3.6% were leukocyte chemotactic factor 2 amyloidosis (ALECT2) (renal amyloidosis in Mexican and Indian patients), and insulin 1% (localized in diabetic). It is important to recognize that sites of subcutaneous insulin injection may form deposits of amyloid due to crystalline insulin. A subcutaneous fat aspiration at this site will demonstrate amyloid and mass spectroscopy is essential to distinguish localized insulin amyloid from a systemic form of amyloidosis., and the remaining comprised less than 1%. Due to its high sensitivity and specificity, mass spectroscopy is now our technique of choice.[30] The advantage of mass spectroscopic analysis is that it does not require any large panel of antibodies and can be done on fixed tissue on a glass slide. In addition, many commercial antibodies will not recognize the light chain immunoglobulin epitope either because of protein misfolding with loss of exposure or fragmentation of the immunoglobulin so that constant sequences are no longer present in the amyloid deposit.

In a survey of 143 heart biopsies, of which 81 were TTR (familial or senile systemic amyloid), an M protein was found in 20 patients, and free light chain abnormality was found in 8 of the 81 patients, indicating that the finding of an immunoglobulin protein does not prove that amyloidosis is AL in origin. Because senile systemic amyloidosis is a disease that causes heart failure in the elderly, the finding of a high prevalence of immunoglobulin abnormalities as a coincidental observation is not unusual.[31] Box 93.1 provides a list of the diagnostic tests needed to evaluate patients for whom amyloidosis is established as a diagnosis.

Other required diagnostic studies in patients with amyloidosis include cardiac biomarkers, which are extensively discussed in the section below on prognosis and screening measurements of the coagulation system. A unique and highly specific finding, albeit limited to no more than 5% of patients with light chain amyloidosis, is the development of deficiency of coagulation factor X. This is usually recognized as prolongation of the prothrombin time. The underlying mechanism of factor X deficiency in immunoglobulin light-chain amyloidosis is the direct binding of factor X to the amyloid deposit itself, which can occasionally be associated with serious clinical bleeding and can complicate the systemic therapy of this disorder. If a prothrombin time is completely normal, it is not our practice to routinely screen for factor X deficiency, but any abnormality of the prothrombin time or international normalized ratio should lead to a screening for factor X deficiency, given its unique association with light chain amyloid. In our experience, factor X deficiency is most commonly seen in patients with hepatosplenic deposits of AL amyloid.[32]

DIFFERENTIAL DIAGNOSIS

The path to the diagnosis of amyloid depends on the initial presentation. Many patients will be presented to a hematologist because of a monoclonal gammopathy and greater than 10% plasma cells in the bone marrow, which will fulfill the diagnostic criteria for myeloma, even though the clinical manifestations and the drivers of outcome could all be related to AL amyloidosis. It, therefore, is incumbent upon practicing hematologists who see a patient with smoldering multiple myeloma or MGUS to inquire about progressive fatigue out of proportion to any anemia, intractable edema, unexplained elevation of the serum alkaline phosphatase, unexplained weight loss, or the presence of peripheral neuropathy. The index of suspicion should be heightened if the monoclonal gammopathy is of λ type relative to κ type because of the stronger association with light chain amyloidosis. Any of these findings should lead to staining of the bone marrow biopsy for Congo red and the performance of subcutaneous fat aspiration. If the fatigue is significant, echocardiography, specifically to look for amyloid, and measurement of troponin and the N-terminal pro-brain natriuretic peptide (NT-proBNP) is indicated.[33] Conversely, any patient with a known nonischemic cardiomyopathy should be screened with serum and urine immunofixation and immunoglobulin light-chain assay because a positive result will redirect the evaluation toward AL amyloidosis. Any patient with albuminuria and a light chain should not be assumed to have myeloma cast nephropathy. Rather, the pattern of the urinary protein, whether albumin-dominant or globulin-dominant, can help distinguish between the two syndromes. In a patient with a peripheral neuropathy and a monoclonal protein, it should not be assumed that this is MGUS neuropathy; rather, screening for amyloidosis, in the presence of associated autonomic neuropathy (rare in MGUS neuropathy, 20% of AL amyloidosis) or concomitant carpal tunnel syndrome (50% of amyloid neuropathy patients, uncommon in MGUS neuropathy) is required. Congo red staining of tenosynovium tissue detected amyloid deposits in 10.2% of patients.[34] Six percent of patients with light chain amyloidosis have an IgM monoclonal protein. These patients' light chains are produced by lymphoplasmacytic cells in the bone marrow rather than plasma cells. This subtype of amyloidosis is characterized by a higher frequency of pulmonary involvement and a lower frequency of cardiac involvement. These patients also have a higher incidence of amyloid peripheral neuropathy. Therapy is directed at the lymphoplasmacytic clone rather than standard anti-plasma cell chemotherapy.

Other monoclonal immunoglobulin deposition diseases should be considered in the differential diagnosis. The most common would be light-chain deposition disease in the kidney where non-fibrillary granular deposits occur on the basement membrane of the glomerulus. Clinically, this manifests as a nephrotic syndrome that is hard to distinguish from renal amyloidosis. In most instances, renal biopsy is required.

Excluding Localized Amyloidosis

Localized forms of amyloidosis are characterized by the presence of amyloid deposits in biopsy tissue without systemic organ dysfunction. The typical sites associated with localized amyloidosis include skin, vocal cord, tracheobronchial tree, ureter, bladder, and urethra. Occasionally, GI biopsies, particularly polyps, or the edges of ulcers will show amyloid deposits and not be reflective of a systemic form of amyloidosis.[35]

In some instances, mass spectroscopic analysis will demonstrate that the amyloid is not of immunoglobulin light-chain origin. However, there are a number of forms of localized amyloidosis that are of immunoglobulin light-chain origin but are not part of a systemic plasma cell dyscrasia. When a deposit is seen and is of light-chain origin, but there is no evidence of a clonal plasma cell disorder in the bone marrow, and serum and urine immunofixation and light chain assays are normal, the index of suspicion should be that this is a localized form of amyloid for which systemic therapy is not indicated. Most forms of cutaneous amyloidosis do not require therapeutic intervention. Amyloidosis involving the vocal cords is most commonly treated by endoscopic resection of the deposits or yttrium-aluminum-garnet (YAG) laser vaporization of the deposits. Tracheobronchial amyloid deposits can be treated with laser vaporization of the deposits or, if the deposits extend beyond the reach of the bronchoscope, with external beam radiation, which has been reported to control the amyloid deposits successfully. Amyloids involving the ureter, bladder, and urethra often carry a preoperative diagnosis of urothelial malignancy. Surgeons have often treated patients with ureteral resections for suspected transitional cell carcinoma only to find that amyloid is present. Bladder amyloids most commonly manifest with gross hematuria, and endoscopic resection will demonstrate amyloid deposits. Most patients with localized bladder amyloid can be controlled via cystoscopic resection and then surveillance. There are occasional patients who require subtotal cystectomy. There is experience with the use of instillation of dimethyl sulfoxide into the urinary bladder. Urethral amyloid can usually be treated with resection to prevent obstruction.[36]

Systemic Forms of Amyloidosis Unrelated to Immunoglobulin Light Chain

The aging of the population is leading to increased recognition of TTRwt (senile systemic amyloidosis formally known as *senile cardiac amyloidosis*). Autopsy studies in patients over the age of 90 shows that nearly one-third of patients have cardiac amyloid deposits, and approximately half of those deposits were responsible for clinically significant cardiac dysfunction. With the rising age of the population and increased application of echocardiography, it is expected that the recognition of senile systemic amyloid, also known as *native TTR amyloid* or *wild-type TTR amyloid (TTRwt)*, will increase. Patients with this form of cardiac amyloidosis can develop congestive heart failure and commonly have conduction system abnormalities, including atrial fibrillation, first-degree heart block, and bundle branch block. The echocardiographic features are similar to those of light-chain amyloidosis, although extreme degrees of infiltration can be seen (septal thicknesses >20 mm), which are uncommon in light-chain amyloidosis. It should never be assumed that a patient with suspected cardiac amyloidosis has light-chain amyloidosis, particularly if the patient is over the age of 70. However, we have seen patients as young as 57 with TTRwt. Moreover, when patients are recognized by mass spectroscopy to have TTR cardiac amyloidosis, sequencing of the *TTR* gene is essential to exclude the possibility of a late-onset inherited form of amyloidosis. This is particularly the case because a specific mutation, ILE-122, is found in 4% of the African-American population in the United States and is associated with the development of late-onset cardiac amyloidosis, and we have seen patients with inherited cardiac amyloidosis who have been inappropriately treated with systemic chemotherapy. Technetium pyrophosphate (Tc-PYP) scanning can be diagnostic of transthyretin-related hereditary amyloidosis (ATTR) cardiac amyloidosis, both wild type and mutant.[37]

Familial amyloidosis represents a small but important subset of patients with systemic amyloidosis. The majority of cases are caused by mutations of TTR and can be recognized by identifying the TTR subunit by mass spectroscopy and detection of the mutation by sequencing of the *TTR* gene using polymerase chain reaction amplification techniques (Fig. 93.8). Clinically, most patients present either with cardiac amyloid or with amyloid peripheral neuropathy and are not easily distinguished clinically from immunoglobulin light-chain amyloid. Most of these patients will have no evidence of systemic plasma cell dyscrasia. Only half of the patients in our practice actually have a positive family history for this autosomal dominantly inherited disorder. In many instances, this is simply because of a failure to recognize in a prior generation and misattribution of the etiology of cardiac death or neural disability. Recognition of inherited TTR amyloidosis is important because there are now two approved gene silencers for inherited TTR neuropathy and one TTR stabilizer approved for TTR cardiomyopathy.[38]

There are forms of renal amyloidosis that are not immunoglobulin light chain in origin. The two most common are fibrinogen A α and ALECT2. Again, this requires mass spectroscopic identification of these subunit proteins to validate the diagnosis. The distinction of other forms of inherited amyloidosis is important. In TTR amyloidosis, multiple effective therapies have been US Food and Drug Administration (FDA) approved. In non-TTR inherited amyloidosis, organ transplant (renal transplant for apolipoprotein A1 amyloid renal failure) has been used for the management of renal and cardiac failure.[39]

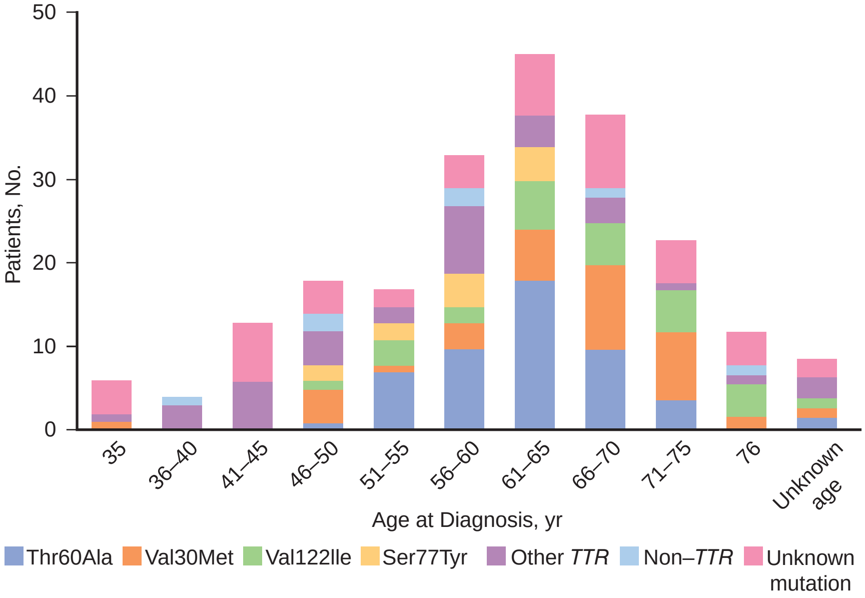


Figure 93.8 TRANSTHYRETIN MUTATIONS SEEN AT MAYO CLINIC, BY AGE. *TTR*, Transthyretin.

PROGNOSIS

The prognosis of immunoglobulin light-chain amyloidosis has been associated with abnormalities in lactate dehydrogenase, β_2-microglobulin, genetics, circulating plasma cells, the fraction of plasma cells in S-phase, and the serum amyloid P-scanning technique. The simplest and most powerful assessments of prognosis revolve around the importance of cardiac amyloid in determining the outcome.[40]

The most common cause of death in patients with cardiac amyloidosis is cardiac failure or sudden death, presumed to be caused by arrhythmia. However, the most common cause of death in renal, hepatic, and peripheral nerve amyloid is also cardiac amyloid. The presence of cardiac amyloid predicts for the fraction of patients unable to complete 3 months of therapy and remains responsible for the 1-year mortality of AL amyloidosis of 40%, which has not changed in 30 years, is the major driver of the outcome, and emphasizes why an earlier diagnosis of cardiac amyloid is essential to the improvement of outcomes for patients with this disease. In addition, the burden of plasma cells, as measured both in the bone marrow and by the levels of involved and uninvolved immunoglobulin light chains in the serum, is key in assessing prognosis. Box 93.2 gives the outcomes for the four stages of immunoglobulin light-chain amyloidosis, based on the measurement of cardiac troponin T, NT-proBNP, and the difference between the involved (iFLC) and uninvolved (dFLC) serum free light chain levels (see Box 93.2). These three blood tests have been converted into a very convenient staging system with dramatic differences in prognosis and almost equal distribution of patients into the four stages. Fig. 93.9 shows post-transplant overall survival for the four stages in 444 patients. Other important prognostic features, which are not currently part of the staging system, include echocardiographic Doppler studies of diastolic performance and mitral deceleration time (normal 140 to 240 ms). Echocardiographic strain at the apex, which measures the rate at which myocardial shortening occurs, has also been shown to be a powerful measure of outcome. Others have described a systolic blood pressure of less than 90 mmHg as being an adverse prognostic factor, but this has not been effectively incorporated into the current staging system for amyloidosis. At presentation, an NT-proBNP level greater than 8500 ng/L carries a particularly grave prognosis. A comparison of staging systems has been performed. The European model incorporating NT-proBNP of greater than 8500 has a better prediction for 1-year mortality. The Mayo staging system incorporating the free light chain increases the ability to predict long-term survival.[41,42]

BOX 93.2 Amyloid Staging

Patients are assigned a score of 1 point for each of the following:

1. FLC-diff ≥18 mg/dL
2. High sensitivity cTnT ≥40 ng/L
3. NT-proBNP ≥1800 pg/mL

This creates stages I–IV with scores of 0–3 points, respectively

Median survival (months):

I. 94.1
II. 40.3
III. 14
IV. 5.8

cTnT, Cardiac troponin T; *FLC-diff*, difference between involved and uninvolved serum free light chains; *NT-proBNP*, N-terminal pro-brain natriuretic peptide.

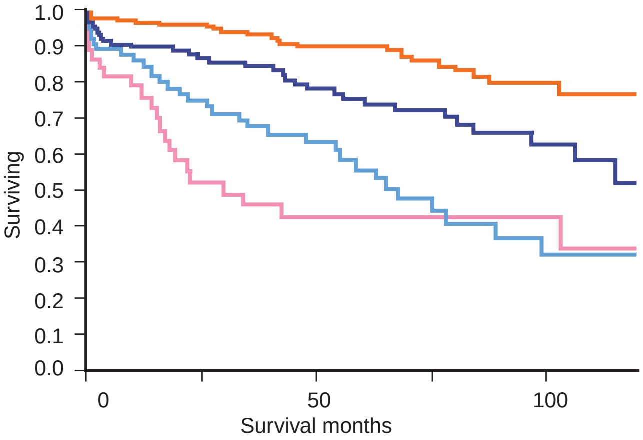


Figure 93.9 SURVIVAL BY STAGE FOLLOWING STEM CELL TRANSPLANT. *Orange*, stage I; *dark blue*, stage II; *light blue*, stage III; *pink*, stage IV.

Patients who present with an involved free light chain of less than 5 milligram/deciliter have an inherently better prognosis and longer survival than those who present with an involved free light chain level greater than 10. The optimal response is assessed by the difference between the involved and uninvolved light chain and is superior to using the light chain ratio. We believe the involved free light chain should be reduced as low as possible, preferably to less than 2 mg/dL which is associated with a higher organ response rate and improved progression-free and overall survival[43]).

Response Assessments and Importance of Supportive Care in Amyloid Light-Chain Amyloidosis

Before one can understand the various therapies, it is important to understand how to interpret response in patients with amyloidosis. In amyloidosis, because there is both a plasma cell dyscrasia and organ dysfunction related to amyloidosis, there are now response criteria for both hematologic and organ responses. Hematologic responses parallel response criteria that have been established for multiple myeloma and rely heavily on the measurement of the involved and uninvolved immunoglobulin free light chain.[44]

The immunoglobulin free light chain assay has been summarized earlier as being critical in the recognition of the disease; it has been described as being important in prognosis, and its serial measurement is the key to assessing hematologic response. In fact, when both an intact immunoglobulin is present and an abnormal involved free light chain is detected, the latter is more important prognostically than changes in the intact immunoglobulin and the light chain value is given preference to intact immunoglobulin levels when interpreting responses in treated patients and for assessment of the role of salvage therapies. A complete hematologic response is defined as a negative serum and urine immunofixation, a normal free light chain ratio, and a normal bone marrow examination. A very good partial response is now defined as a difference between involved and uninvolved free light chain of less than 40 mg/L. A partial response is a 50% reduction in the difference between involved and uninvolved serum free light chain levels, and less than a 50% decrease is considered a stable disease. A cardiac response in amyloidosis is defined not by changes in the echocardiogram but by changes in the NT-proBNP. A decrease of NT-proBNP of 30% and at least 300 ng/L is considered a response. However, the NT-proBNP level must be greater than 650 ng/L prior to therapy to be considered measurable for cardiac organ response. The definition of renal response is a 50% reduction in the amount of urinary protein loss over a 24-hour period. This reduction is associated with longer survival. A 75% reduction in proteinuria is associated with markedly prolonged survival.[45]

The therapy for amyloidosis is both supportive care and systemic chemotherapy against the plasma cell dyscrasia. Supportive care for cardiac and renal amyloidosis includes diuretic therapy to help reduce the elevated filling pressures in both the right and left sides of the heart and reduce pulmonary edema, as well as to relieve symptomatic lower extremity edema. Diuretics are also required for the management of the lower extremity edema associated with nephrotic syndrome. Many cardiologists at the Mayo Clinic believe that torsemide is a superior diuretic to furosemide in patients with amyloidosis. For refractory edema, the addition of metolazone can be useful, but it can result in serious hypokalemia when combined with furosemide or torsemide. There is no evidence that the use of lisinopril, as has been applied in the nephrotic syndrome of diabetic nephropathy, plays any role in the management of renal amyloidosis. In addition, afterload reduction, shown to provide a survival benefit in patients with ischemic cardiomyopathy, has not been shown to be beneficial in patients with amyloidosis and can significantly aggravate hypotension in patients with poor filling during diastole. Finally, a number of patients in our practice have shown significant deterioration with the addition of β-blockade, and these agents are avoided in our patients as well. For cardiac AL amyloidosis, an implantable cardiac defibrillator (ICD) has been reported in 15 patients. The median time to implantation was 13 days from the first presentation. Thirteen of the 15 received oral amiodarone after implantation. One patient had ventricular fibrillation after 8 days and was successfully reverted to sinus rhythm but died 10 days later with intractable ventricular fibrillation. Only two of the four patients achieved longer-term survival with an ICD.[46,47]

The arthropathy of amyloidosis can be challenging but most patients benefit from low doses of prednisone such as 10 mg daily. Amyloid neuropathy can be managed with gabapentin, pregabalin and scrambler therapy. In scrambler therapy, electrocardiographic-like pads are placed around the area of pain. The device then synthesizes different types of nerve action potentials that deliver nonpain information through cutaneous nerves to the pain site.

Management of amyloid orthostatic hypotension involves the use of both fludrocortisone and midodrine, which can help reduce orthostatic syncope but are associated with both fluid retention and supine hypertension, making their use a challenge. The diarrhea associated with GI amyloid is also challenging. It is common for these patients to fail imodium therapy and loperamide. We frequently will use tincture of opium to manage this situation. In refractory instances where fecal incontinence is common, the placement of a diverting sigmoid colostomy has no impact on the diarrhea but can provide the patient important improvement in quality of life and social relief.[48]

Treatment for Light-Chain Amyloidosis

The treatment of light-chain amyloidosis requires a determination of whether the patient is a candidate for high-dose therapy with a stem cell transplant or is best served with conventional regimens.[49]

It is now 40 years since melphalan and prednisone treatment was shown to benefit patients with AL amyloidosis, and two randomized clinical trials comparing melphalan and prednisone with colchicine regimens demonstrated a response rate of 15% and a 50% increase in median survival in responders. Melphalan has a significant impact on survival in patients without heart failure. The best responses to melphalan-containing regimens are in patients with renal amyloid nephrotic syndrome and preserved renal function.[50]

Dexamethasone in high doses of 40 mg on days 1 to 4, 9 to 12, and 17 to 20 as demonstrated in three individual studies to result in organ improvement in amyloid patients for whom melphalan-containing regimens had failed. Subsequently, the combination of melphalan and weekly dexamethasone was introduced, showing a 50% 5-year survival rate, a hematologic complete response rate of 24%, and organ improvement of 43% (Table 93.3). Oral melphalan and dexamethasone can be administered to virtually any patient with AL amyloidosis and should be considered the standard with which all other therapies are compared. There is a strong correlation between organ improvement and hematologic response. In patients who achieve a hematologic complete response, organ improvement has been seen in 87% of patients. Organ response rates are less than 15% in the patients who do not achieve a hematologic partial response. Bendamustine, an agent with alkylating type properties, was studied in 122 patients. In this relapsed population the

TABLE 93.3 Current Regimens for Light-Chain Amyloidosis Therapy

Melphalan dexamethasone	50% response rate can be used with renal and cardiac failure
Cyclophosphamide bortezomib dexamethasone	Most common triplet regimen in use
Daratumumab cyclophosphamide bortezomib dexamethasone	Appears to triple the ≥VGPRrate in newly diagnosed patients with NT-proBNP <8500 ng/mL
Bortezomib lenalidomide dexamethasone	Lenalidomide poorly tolerated; aggravates cardiac congestion; initial lenalidomide dose 5–10 mg
Pomalidomide	Active second line regimen. Caution with cardiac amyloidosis
Venetoclax	Potential when t(11;14) present

NT-proBNP, N-terminal pro-B-type natriuretic peptide.

overall hematologic response rate was 35%. Median survival was 48 months but was significantly longer for IgM amyloidosis and represents ineffective salvage therapy.[51]

The introduction of proteasome inhibitors and immunomodulatory agents has broadened the horizon for patients with AL amyloidosis. Initial experience with thalidomide demonstrated that the administration of immunomodulatory drugs to patients with amyloidosis cannot be implemented solely on the basis of experience with multiple myeloma. In an early study of 16 patients with AL amyloidosis treated with thalidomide, grade 3 or greater adverse events were noticed in 50% of patients, including exacerbation of neuropathy and heart failure. When thalidomide was combined with cyclophosphamide and dexamethasone, the hematologic response rates were high, but the toxicity was significant.[52]

Lenalidomide has demonstrated activity in AL as a single agent and combination with steroids and the alkylating agents, cyclophosphamide and melphalan. Lenalidomide appears to be better tolerated than thalidomide but produces significant increases in the NT-proBNP and symptomatic exacerbations of cardiac symptoms. In a phase II trial of 35 patients, 8 of 13 evaluable subjects had a hematologic response, but the maximum tolerated dose of lenalidomide was only 15 mg. Lenalidomide has been used as salvage therapy in patients who have progressed after melphalan, dexamethasone, and bortezomib combinations. Melphalan, lenalidomide, and dexamethasone in a relapsed setting produced hematologic complete response rates in 42% of patients. A phase II trial at the Mayo Clinic of cyclophosphamide, lenalidomide, and dexamethasone also produced response rates of 50%, with 7% showing a complete response. Myelosuppression with the combination of melphalan and lenalidomide is significant. Cardiac arrhythmias were seen in 33%.[53]

As a salvage therapy in relapsing disease, lenalidomide has been combined with cyclophosphamide and dexamethasone in 35 patients. The median number of treatment cycles was six, and the hematologic response rate was 60%; in those receiving at least four cycles, the response rate was 87%. A median overall survival of 37.8 months has been reported. In patients with amyloidosis, lenalidomide has been reported to worsen renal function even in patients whose amyloidosis spares the kidney. Recovery of renal function was reported in 44%. Lenalidomide salvage therapy after bortezomib achieves a hematologic response rate of 41%. Pomalidomide is a third-generation immunomodulatory drug with a toxicity profile that compares favorably with the other immunomodulatory drugs. In a single phase II trial, 29 patients previously treated received pomalidomide with a response rate of 38%. Seventy-five percent of patients were alive at 1-year, and 56% were free of progression. The majority of these patients had previously received stem cell transplants, bortezomib, and thalidomide, indicating that this immunomodulatory drug is active and capable of producing durable remissions, albeit with significant toxicity. Twenty-nine patients were treated with pomalidomide at the National Amyloidosis Centre in the United Kingdom. Thirty-five percent of patients achieved a very good partial response, in 31% a partial response. Median overall survival was 27 months; median progression-free survival was 15 months. Pomalidomide represents an effective second-line therapy for AL amyloidosis. The quality of responses in the second-line is in large part predicted by the quality of response to first-line therapy.[54]

Bortezomib

Bortezomib, which was the first proteasome inhibitor used for the treatment of amyloidosis, has the advantage of a rapid time to response and no modification required for renal dysfunction. In the first phase II trial, 14 of 18 patients with relapsed refractory AL amyloidosis had a hematologic response, with 16% achieving complete response. Schedules of weekly and twice-weekly bortezomib have been investigated with similar response rates and similar 1-year progression-free survival. The weekly use of bortezomib, however, does appear to have lower rates of treatment discontinuation and peripheral neuropathy. Bortezomib has been combined with dexamethasone with a hematologic response rate of 71%, complete in 25%. In previously untreated patients, bortezomib produces a complete response rate of 47%, although in one study the progression-free survival was only 5 months and the overall survival was 18.7 months, raising questions about how durable these responses are. Bortezomib has also been combined with stem cell transplant as induction therapy, as a post-transplant consolidation therapy, and as a part of the conditioning regimen. In a report of 19 patients for whom bortezomib was used after stem cell transplant to deepen the response, 67% of patients achieved a complete response, with organ responses in 60% using post-transplant consolidation with bortezomib. Data from 33 national centers were combined, with 94 patients having received bortezomib. A cardiac response was seen in 29% of patients. The NT-proBNP predicted survival. The median time to response was 7.5 weeks. Authors of a survey of European centers with 428 evaluable patients reported that bortezomib therapy achieved a lower difference between involved and uninvolved free light chains at the end of therapy compared with cyclophosphamide-thalidomide-dexamethasone, melphalan-dexamethasone, stem cell transplant, and cyclophosphamide-lenalidomide-dexamethasone.[55]

Bortezomib had been combined with cyclophosphamide and dexamethasone. In a study of 17 patients, 10 of whom were therapy-naive, a response rate of 94% was achieved, 71% complete. A second cohort of 43 patients achieved a hematologic response rate of 81%, with 42% achieving a complete response and a 2-year progression-free survival of 67% for newly diagnosed patients.[56]

Despite the promising published results with bortezomib, a prospective randomized trial conducted in Europe of bortezomib, melphalan, and dexamethasone (BMD) compared with melphalan and dexamethasone failed to show any difference in organ response rates or in progression-free or overall survival in the bortezomib-containing regimen; yet grade 3 toxicity was seen in 27% of patients receiving BMD compared with only 12% treated with melphalan and dexamethasone. Moreover, day 100 all-cause mortality was 7.5% in the BMD arm and only 2.5% in the melphalan–dexamethasone arm. The hematologic response rate was higher in the BMD arm at 77% versus 52% ($P = .045$). A compilation of 230 patients in Europe who received cyclophosphamide, bortezomib, and dexamethasone showed a high level of effectiveness in low-risk amyloidosis patients with an at least very good partial response rate of 56% and no deaths seen in stage I patients. In a trial of 915 patients treated with bortezomib, the overall response rate (intent-to-treat) was 65%, with 49% complete response (CR)/very good partial response/low dFLC response and with a stringent dFLC response, dFLC 10–40 mg/L, and dFLC greater than 40 mg/L was 30%, 22%, and 48%, respectively. Median overall survival was 72 months.[57]

A European collaborative study of treatment outcomes in 346 patients with cardiac stage III AL amyloidosis was reported. In this cohort of patients with advanced cardiac amyloidosis, the median overall survival was 7 months with a 2-year overall survival of 29%. As is typical for this disease, 42% of patients died before their first response evaluation, and the overall hematologic response rate was 33% (12% complete). The overall survival rate was 88% at 2 years for complete responders, 53% for partial responders, and 22% for nonresponders; 45% of responders achieved an organ response. The only factors predictive of survival were an NT-proBNP >8500 ng/L and a systolic blood pressure less than 100 mmHg. The regimens administered included melphalan and dexamethasone, alkylating agents, thalidomide and dexamethasone, and bortezomib as well as lenalidomide combinations. Although this was not a prospective trial, the overall response rate for melphalan–dexamethasone–thalidomide–bortezomib combinations ranged from 60% to 64%. The response rate to lenalidomide combination therapy was 41% but included only 13 patients. The most common regimen administered was melphalan and dexamethasone, administered to 154 of the 286 patients evaluable for response.[58]

Carfilzomib has been used particularly in the treatment of light-chain amyloid neuropathy. However in view of the very high incidence of cardiac involvement with amyloidosis and toxicity of carfilzomib to cause hypertension and dyspnea its use has been limited due to toxicity.

Daratumumab

Daratumumab is an anti-CD 38 monoclonal antibody with an established role as monotherapy in multiple myeloma. Daratumumab as a single agent in relapsed refractory amyloidosis has provided impressive preliminary results. Among five case series published on 142 patients the median time to response ranges from 1 to 2.2 months and the overall response rate ranged from 63 to 100%. The response depth greater than or equal to very good partial responders range from 46 to 86%. In a series reported from Mayo Clinic, the median follow-up was 10.2 months the complete response rate was 17% and the median time response was 2.2 months. This is a highly active agent and a phase III trial that is completed accrual has compared daratumumab-cyclophosphamide-bortezomib-dexamethasone to cyclophosphamide-bortezomib-dexamethasone. In the final publication daratumumab increased the complete response rate 3-fold and doubled both the renal and cardiac organ response rates. Daratumumab is the only FDA-approved agent for the treatment of AL amyloidosis. The combination of daratumumab, cyclophosphamide, bortezomib, and dexamethasone is now considered the standard of care for newly diagnosed patients with AL amyloidosis.[59,60]

Autologous Hematopoietic Stem Cell Transplantation

Unlike multiple myeloma, where visceral organ function tends to be normal with the exception of the kidney, and the bone marrow tends to be heavily involved, the converse is true in AL amyloidosis. In amyloidosis, the marrow usually shows minimal involvement, but organ dysfunction increases the morbidity as well as the mortality of high-dose chemotherapy and stem cell transplant. Ablative therapy can be more effective, and the response durability of patients who achieve a complete response is greater in AL amyloidosis than it is in multiple myeloma, the number one indication for autologous stem cell transplant in the United States. Stem cell transplant is not a suitable technique for patients with advanced cardiac disease, because these patients decompensate if they develop neutropenic infection during the phase of maximal myelosuppression. Careful patient selection becomes critical in ensuring a safe outcome for patients who undergo stem cell transplants. Over the past two decades, the treatment-related mortality associated with stem cell transplants has fallen from 10% to 2% because of improved supportive care and optimization of patient selection. As a general guideline, it has been our experience that patients whose systolic BP is less than 90 mmHg or whose troponin T is greater than 70 ng/L at diagnosis have too high a mortality associated with transplant and are better managed with standard-dosage chemotherapy, as outlined in the previous section. Systolic blood pressure less than 90 mmHg reflects insufficient diastolic compliance to improve cardiac output during the stress of stem cell transplant. Patients who have stage III and stage IV cardiac amyloidosis are candidates for stem cell transplant if they fulfill the other criteria outlined. Reports from the Boston University Medical Center performing stem cell transplantation in patients who had significant elevation of cardiac biomarkers (NT-proBNP >5000) demonstrated a treatment-related mortality of only 5% despite advanced cardiac involvement.[61]

At the Mayo Clinic, approximately 20% of patients with newly diagnosed amyloidosis are eligible for high-dose melphalan. Melphalan 200 mg/m^2 and 140 mg/m^2 can both be used, but the response rates associated with lower-dose melphalan are decreased and may not be superior to the response rates reported with standard chemotherapies. The ability of stem cell transplant to produce a durable response is unmatched in the literature, with 43% of patients surviving 10 years or longer. Patients with more than two organs involved are oftentimes not considered adequate candidates for stem cell transplant, but some judgment is warranted because patients can have very mild involvement of three organs and be candidates, yet have advanced involvement of two organs and not be candidates (Fig. 93.10). The serum creatinine is predictive of the risk of requiring dialysis during the transplant, a major complication leading to serious morbidity. We are disinclined to perform the transplant in renal amyloid patients whose serum creatinine is ≥2.0 mg/dL. Although age is not an absolute criterion, additional scrutiny is warranted above the age of 65. From January 1, 2013, through December 31, 2014, 85 amyloid patients underwent transplants; 35 were older than 65 years of age, and 11 were over age 70 years.[62] Median overall survival for the entire cohort was 122 months. Treatment-related mortality declined over time to 2.4%. When careful selection is applied, these results are comparable to younger patients

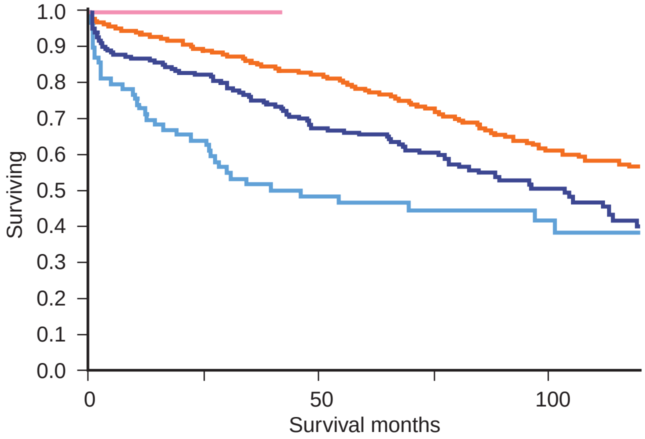


Figure 93.10 SURVIVAL AFTER STEM CELL TRANSPLANT, BASED ON NUMBER OF ORGANS INVOLVED. *Pink*, Soft tissue only, no visceral involvement; *orange*, 1 organ; *dark blue*, 2 organs; *light blue*, >2 organs.

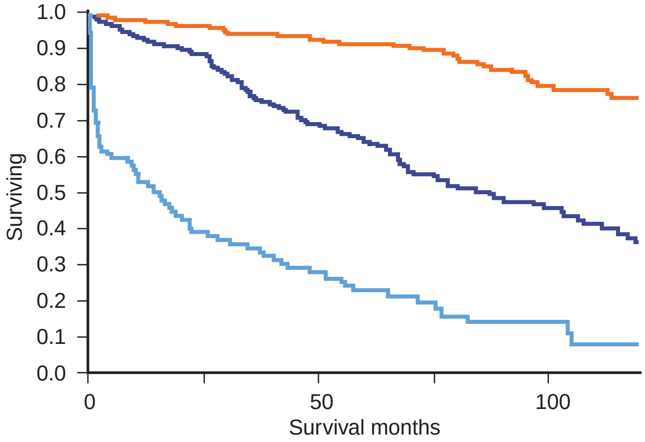


Figure 93.11 SURVIVAL BY RESPONSE DEPTH. *Orange*, Complete response; *dark blue*, partial response; *light blue*, no response.

As with conventional chemotherapy, hematologic response is a predictor of survival, with complete responders having improved outcomes compared with very good partial responders and partial responders (Fig. 93.11). The depth of the hematologic response required to achieve optimal outcomes is not well defined in amyloidosis, because highly amyloidogenic light chains can continue to deposit even if the levels are sharply reduced. Unlike myeloma (where the monoclonal protein is a surrogate for tumor mass), in amyloidosis, the monoclonal protein is itself the problem, and to completely disrupt deposition, one may hypothesize that nearly complete eradication of the protein from the serum is required. In amyloidosis, hematologic response is used as a surrogate for organ response, which is the only outcome that improves survival by the prevention of progressive organ failure.[63]

Most published information on high-dose therapy and stem cell transplant in amyloidosis have used melphalan in doses ranging from 100 to 200 mg/m^2 as the standard. The Boston Medical Center reported 250 patients with AL amyloidosis who underwent high-dose melphalan and autologous stem cell rescue. Two-thirds were alive at a mean follow-up of 23 months, with a 3-month all-cause mortality of 14%. Febrile neutropenia, GI tract hemorrhage, and dialysis-dependent renal failure were unique toxicities associated with transplants. However, the Boston group has demonstrated an improved quality of life for patients who respond. We have published our experience with

434 patients with amyloidosis. The hematologic response rate among those patients was 75%. The complete response rate was 38%, and the organ response rate was 46%. The most important predictor of outcome was the depth of hematologic response. Median survival was not reached for patients who achieved a complete response, whereas it was 107 months for those with partial remission and 32 months for nonresponders (Fig. 93.11). There is a significant impact of pretreatment NT-proBNP levels on outcome. We do not use chemo-mobilization to obtain stem cells. The low number of plasma cells in the bone marrow allows growth factor-only mobilization to achieve the requisite goal. Stem cell mobilization (SCM) is associated with unusual morbidity in AL amyloidosis compared to multiple myeloma, even when performed without chemotherapy. In a large monocentric series, 11% of patients SCM with granulocyte colony-stimulating factor (G-CSF) did not proceed to stem cell transplantation due to complications and 4% died during SCM. Caution must be exercised when selecting patients with AL amyloidosis for stem cell mobilization. Extreme caution must be used in patients that are not fit. Hypoxia and hypotension are rare life-threatening events during SCM with G-CSF and may be the result of a capillary leak syndrome triggered by the growth factor. Plerixafor is not routinely used to mobilize stem cells; it is used only in patients who do not achieve a peripheral blood CD34 count greater than 10/μL. We also do not routinely use granulocyte colony-stimulating factor to support engraftment following transplant, owing to fluid retention that is seen, and we have observed no increase in bacteremia or hospital days after ceasing use of post-transplant growth factor support. Among AL amyloid patients, approximately 30% will complete the procedure without requiring hospitalization. Adjuvant therapy has been used in patients who are the recipients of stem cell transplants. Both thalidomide–dexamethasone and bortezomib–dexamethasone has been used to consolidate a response after transplant, and it has been demonstrated that the deepening of the response can be achieved.[64]

It is difficult to obtain category A evidence in support of high-dose therapy and stem cell transplant for AL amyloidosis. The only prospective controlled trial enrolled 100 patients and did not demonstrate survival differences between the two groups, whether in a landmark analysis or on an intention-to-treat basis. This study has been called into question because of a 24% treatment-related mortality in the transplant group. A subsequent meta-analysis of high-dose therapy in patients with AL amyloidosis also could not identify a survival advantage with stem cell transplant. Boston University has reported a 10-year survival of 25% in their transplant population, compared with 4% prior to the advent of high-dose therapy. A case-matched control study done at the Mayo Clinic matched 63 transplanted patients with 63 non-transplanted patients and showed a survival advantage for patients who received a stem cell transplant. A retrospective comparison of stem cell transplantation with chemotherapy alone was performed in 232 patients. The median event-free and overall survivals were significantly better in the transplant group than in the chemotherapy group even when patients with severe findings were excluded. The hazard ratio for event-free survival was 0.41 and for overall survival was 0.31.[65]

In a report from MD Anderson, including 128 patients that received high-dose therapy, induction therapy within immunomodulatory drug or proteasome inhibitor was associated with better overall survival and was safe and feasible. The 2-year progression-free survival with combination chemotherapy induction was 73%. In a report from Mayo Clinic Arizona, 63 patients underwent stem cell transplants, and 34 received bortezomib-based therapy before transplant. Bortezomib-based induction resulted in improved hematologic response organ response and overall survival.[66]

MAYO CLINIC TREATMENT APPROACH IN NEWLY DIAGNOSED AMYLOID LIGHT-CHAIN AMYLOIDOSIS

Organ Transplantation

Both cardiac and renal transplants have been performed in patients with AL amyloidosis. Criteria to be considered in selecting patients for solid organ transplant include amyloidosis confined only to the organ that is to be transplanted. Also, the patient should either have achieved a complete hematologic response with therapy or be a candidate for therapy that has a high likelihood of producing a complete hematologic response and thereby obviate the possibility of recurrent amyloid in the transplanted organ.[67]

Three patients were reported with AL amyloidosis who had cardiac involvement, and the time from symptomatic onset to the diagnosis of AL amyloidosis ranged from 12 to 24 months. The times from the onset of heart failure to orthotopic heart transplant in these three patients were 4, 5, and 7 months, respectively. The patients then underwent a stem cell transplant at 13, 16, and 13 months, respectively; all three are alive at 23+, 35+, and 39 months, demonstrating that in highly selected patients, heart transplants in those with dominant cardiac involvement followed by subsequent stem cell transplant can result in survival benefit. The authors of a report of six patients who underwent a heart transplant for cardiac amyloidosis at Stanford stated that all patients received chemotherapy in the interval between heart transplant and autologous stem cell transplant. Five patients were alive up to 25 months after heart transplant without evidence of recurrent cardiac amyloid deposition, but chemotherapy began as early as 32 days after the cardiac transplant. The three patients are alive 19, 24, and 25 months post-transplant, respectively.[68,69]

Renal transplant has also been reported. Nineteen patients with AL underwent renal transplant, comprising living donor transplants in eighteen and a cadaveric transplant in one, with a median follow-up of 41.4 months; 79% were alive, and five patients had cellular rejection. Recurrent amyloidosis was diagnosed by a kidney biopsy in only one patient, indicating that renal transplant can be successfully performed in patients with AL amyloidosis in complete hematologic response without a significant risk of recurrent amyloid when the primary hematologic disorder is controlled. A recent report noted a median overall survival of 10.5 years from renal transplantation and a 5-year graft survival of 81%. Patients with a complete your very good partial response to therapy had significant overall survival from diagnosis and a lower rate of recurrence of amyloidosis in the graft. During a median follow-up of over 7 years, no lymphoproliferative disorder or second cancer developed. In this cohort, renal transplantation occurred at a median of 2.4 years after hematologic response and was considered reasonable to proceed with renal transplantation when hematologic response was present of at least 6 to 12 months.[70]

Nine cardiac transplants were reported from Boston, eight of whom subsequently received a stem cell transplant. Six of seven evaluable patients achieved a complete hematologic remission. One achieved a partial remission. At a median follow-up of 56 months, five of seven patients were alive without recurrent amyloid, comparable to patients who received heart transplants for non-amyloid heart disease. At the Mayo Clinic, the 5-year survival of patients who have received cardiac transplants for amyloid is inferior to patients who receive hearts for cardiomyopathy without a systemic disorder. Eleven patients underwent heart transplants followed by autologous peripheral blood stem cell transplant. Two patients died as a result of transplant-related complications. Nine survived, but three died because of progressive amyloidosis. The 1- and 5-year survival rates were 82% and 65%, respectively. The median survival was 76 months from a heart transplant and 57 months from stem cell transplant. A report on 32 patients with AL cardiac amyloidosis reported a significantly lower survival than patients with non-amyloid cardiomyopathy after heart transplantation. Outcomes improved substantially in the bortezomib era. The 2-year survival was 80%.[71]

Organ transplant is a viable option for patients in whom complete suppression of light chain production can be achieved. Because of the shortage of organs, critical decisions regarding allocation must be made. Organ transplant has also been applied to familial amyloid polyneuropathy amyloidosis, usually combined with liver transplant. We have performed three cardiac transplants for familial amyloidosis, heart transplantation for TTRwt amyloid in 2 patients, heart–liver transplant for familial amyloid in 18 patients, and heart–liver–kidney transplant for familial amyloid in 4 patients.[72]

In general, organ transplantation should not be considered unless the underlying plasma cell clone can be well controlled to prevent recurrent deposition into the transplanted organ. Virtually all recipients have single organ involvement. It would not be appropriate to do renal transplantation in a patient with advanced cardiac amyloidosis. In patients with cardiac amyloidosis, the heart failure is usually too severe to sustain intensive chemotherapy. These patients receive an organ and then are aggressively managed to suppress the plasma cell clone after approximately 6 months posttransplant.

> A 44-year-old male was found to have nephrotic range proteinuria. A renal biopsy was performed and the patient developed a massive hematoma that required surgical evacuation. Postoperatively, there was oozing from the incision site. The renal biopsy demonstrated amyloidosis and mass spectroscopic analysis revealed lambda type. The patient's INR was 2.4 and the partial thromboplastin time (PTT) was 85 seconds. Specialized coagulation studies demonstrated factor X level of 8%. The patient received 4 months of bortezomib–cyclophosphamide–dexamethasone with no change in the factor X level despite normalization of elevated free light chains. The patient was taken to autologous stem cell transplant. Factor X levels were maintained by the use of factor X concentrate daily through the thrombocytopenic period. By day 100, factor X was 35% and the PTT and INR were both normal.

CONCLUSIONS

Amyloidosis should always be considered in the differential diagnosis of a patient who presents with proteinuria and is nondiabetic. Any patient with unexplained fatigue or restrictive cardiomyopathy or heart failure with preserved ejection fraction should be screened for amyloidosis. Patients who fulfill criteria for chronic inflammatory demyelinating peripheral neuropathy, unexplained hepatomegaly, or atypical multiple myeloma should all be considered for the possibility of amyloidosis. When a patient with one of these five compatible syndromes is seen, screening with immunofixation of the serum or urine in a free light chain assay should be performed (Fig. 93.12). If an immunoglobulin light-chain abnormality is found, it would be appropriate to do biopsies of the bone marrow and subcutaneous fat to stain for Congo red. Only if the index of suspicion is high should further biopsies be performed if the results of bone marrow and fat biopsies are both negative. It is critical to ensure that all amyloid deposits are typed. The gold standard is laser capture mass spectroscopy in an effort to ensure that all chemotherapy-treated amyloid is of immunoglobulin light-chain origin. The prognosis of amyloidosis can be determined by measurements of the immunoglobulin free light chain, serum troponin, and NT-proBNP. We believe that stem cell transplant is the preferred technique for patients in whom it can be performed safely, but this should not be more than 20% of patients. For non-transplant candidates, melphalan–dexamethasone, melphalan–dexamethasone–bortezomib, and cyclophosphamide–bortezomib–dexamethasone are all legitimate options for induction

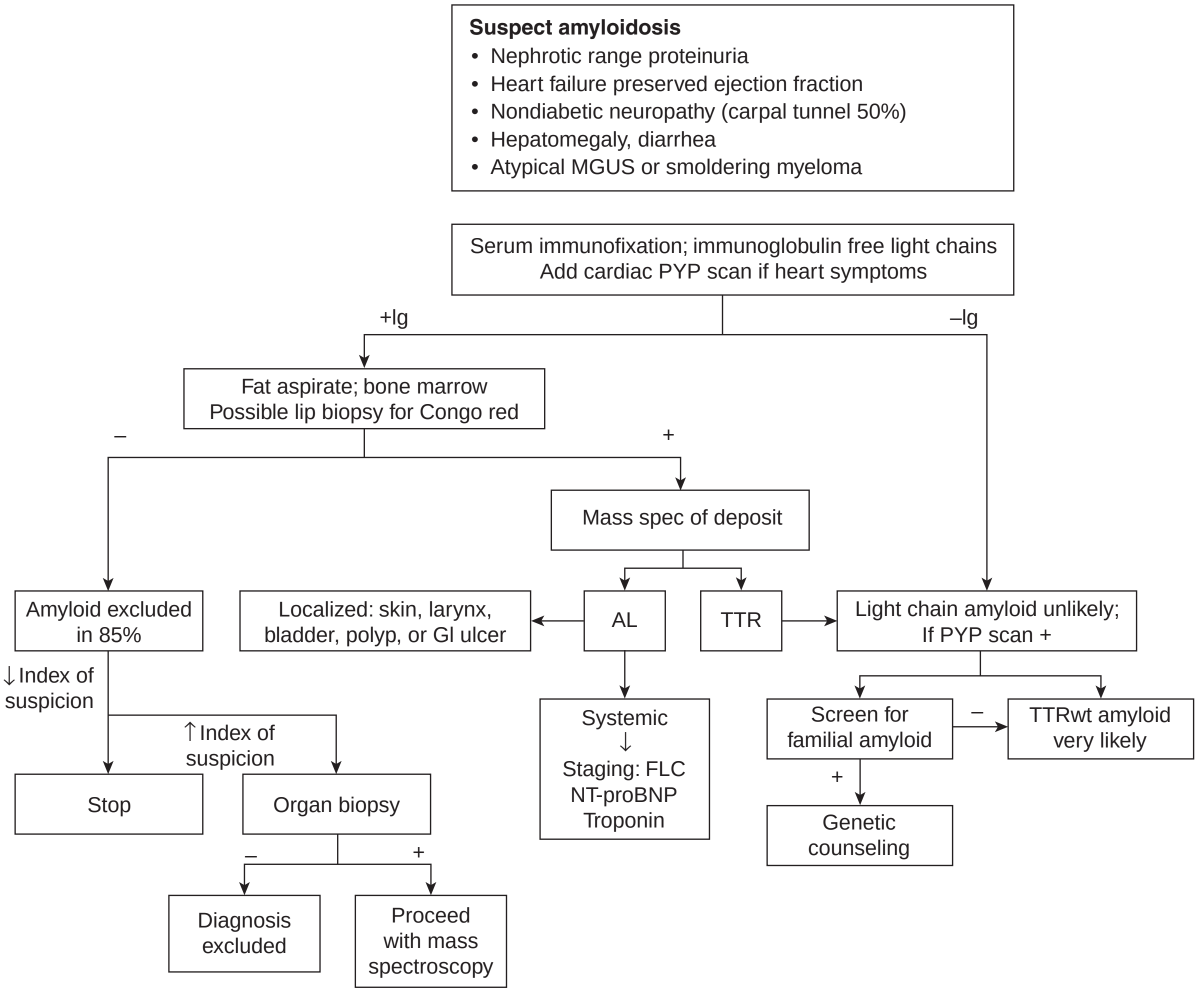

Suspect amyloidosis
• Nephrotic range proteinuria
• Heart failure preserved ejection fraction
• Nondiabetic neuropathy (carpal tunnel 50%)
• Hepatomegaly, diarrhea
• Atypical MGUS or smoldering myeloma
Serum immunofixation; immunoglobulin free light chains
Add cardiac PYP scan if heart symptoms
+Ig
–Ig
Fat aspirate; bone marrow
Possible lip biopsy for Congo red
–
+
Mass spec of deposit
Amyloid excluded in 85%
Localized: skin, larynx, bladder, polyp, or GI ulcer
AL
TTR
Light chain amyloid unlikely; If PYP scan +
↓Index of suspicion
↑Index of suspicion
Systemic
↓
Staging: FLC
NT-proBNP
Troponin
Screen for familial amyloid
–
TTRwt amyloid very likely
+
Genetic counseling
Stop
Organ biopsy
–
+
Diagnosis excluded
Proceed with mass spectroscopy

Figure 93.12 DIAGNOSTIC ALGORITHM FOR USE WHEN AMYLOIDOSIS IS SUSPECTED. *AL*, Amyloid light-chain; *FLC*, free light chain; *GI*, gastrointestinal; *Ig*, Immunoglobulin; *MGUS*, monoclonal gammopathy of undetermined significance; *NT-proBNP*, N-terminal pro-B-type natriuretic peptide; *PYP*, pyrophosphate; *TTRwt*, wild type TTR.

TABLE 93.4 Active Trials for Amyloidosis

Drug	Mechanism	Amyloidosis Type	Trial Registration Number	Comments
Isatuximab	Anti-CD38 antibody	AL	NCT03499808	Monoclonal antibody
Elotuzumab lenalidomide dexamethasone with or without cyclophosphamide	Anti-plasma cell chemotherapy	AL	NCT03252600	Phase 3 ImID based therapy
Daratumumab ixazomib dexamethasone	Chemo-immunotherapy	AL newly diagnosed and relapsed	NCT03283917	Newly diagnosed and treated patients
Doxycycline vs standard supportive care	Anti-misfolding strategy	Cardiac AL	NCT03474458	Prevention of fibril formation
Ixazomib	Anti-plasma cell	AL maintenance	NCT03618537	Post-induction therapy
Vultirsiran	Gene silencing	TTRm	NCT04153149	siRNA against TTR

AL, Light-chain amyloid; *TTR*, transthyretin.

therapy. For patients without a response to their primary therapy, immunomodulatory drug-containing therapies such as melphalan–dexamethasone–lenalidomide, lenalidomide–dexamethasone, and cyclophosphamide–lenalidomide–dexamethasone are all appropriate combinations.[53]

FUTURE DIRECTIONS

Some of the research-based initiatives for amyloidosis treatment are listed in Table 93.4. Antibodies, small interfering RNA, and antisense oligonucleotides are all being explored in TTR amyloidosis. Combinations of anti-light-chain synthesis and therapies to prevent misfolding are likely the next generation of therapies. The future focus for patients with light-chain amyloidosis is to direct therapy not at the plasma cell clone but fully formed amyloid fibrils. Even with effective therapy persistent amyloid fibrils deposited prior to the diagnosis lead to significant morbidity and impair quality of life. Explorations are underway to look at antibodies that bind to amyloid fibrils and activate phagocytosis. The ability to use both anti-plasma cell-directed chemotherapy and anti-fibril antibodies will open a new dimension into the therapy of amyloidosis.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Falk RH, Alexander KM, Liao R, Dorbala S. AL (Light-chain) cardiac amyloidosis: a review of diagnosis and therapy. *J Am Coll Cardiol*. 2016;68(12):1323–1341.

Fermand JP, Bridoux F, Kyle RA, et al. How I treat monoclonal gammopathy of renal significance (MGRS). *Blood*. 2013;122(22):3583–3590.

Gertz MA. Immunoglobulin light chain amyloidosis: 2013 update on diagnosis, prognosis, and treatment. *Am J Hematol*. 2013;88(5):416–425.

Gertz MA, Dispenzieri A, Sher T. Pathophysiology and treatment of cardiac amyloidosis. *Nat Rev Cardiol*. 2015;12(2):91–102.

Gillmore JD, Hawkins PN. Pathophysiology and treatment of systemic amyloidosis. *Nat Rev Nephrol*. 2013;9(10):574–586.

Graziani MS, Merlini G. Serum free light chain analysis in the diagnosis and management of multiple myeloma and related conditions. *Expert Rev Mol Diagn*. 2014;14(1):55–66.

Jazbeh S, Said A, Haddad RY, Hamad A, Lerma EV. Renal amyloidosis. *Dis Mon*. 2014;60(10):489–493.

Mahmood S, Palladini G, Sanchorawala V, Wechalekar A. Update on treatment of light chain amyloidosis. *Haematologica*. 2014;99(2):209–221.

Milani P, Merlini G, Palladini G. Light chain amyloidosis. *Mediterr J Hematol Infect Dis*. 2018;10(1):e2018022.

Mollee P, Renaut P, Gottlieb D, Goodman H. How to diagnose amyloidosis. *Intern Med J*. 2014;44(1):7–17.

Sachchithanantham S, Wechalekar AD. Imaging in systemic amyloidosis. *Br Med Bull*. 2013;107:41–56.

Sanchorawala V. High dose melphalan and autologous peripheral blood stem cell transplantation in AL amyloidosis. *Hematol Oncol Clin North Am*. 2014;28(6):1131–1144.

Sayed RH, Hawkins PN, Lachmann HJ. Emerging treatments for amyloidosis. *Kidney Int*. 2015;87(3):516–526.

Shah G, Kaul E, Fallo S, et al. Bortezomib subcutaneous injection in combination regimens for myeloma or systemic light-chain amyloidosis: a retrospective chart review of response rates and toxicity in newly diagnosed patients. *Clin Ther*. 2013;35(10):1614–1620.

Sher T, Gertz MA. Recent advances in the diagnosis and management of cardiac amyloidosis. *Future Cardiol*. 2014;10(1):131–146.

Ueda M, Ando Y. Recent advances in transthyretin amyloidosis therapy. *Transl Neurodegener*. 2014;3:19.

Vaxman I, Dispenzieri A, Muchtar E, Gertz M. New developments in diagnosis, risk assessment and management in systemic amyloidosis. *Blood Rev*. 2019:100636.

Weber N, Mollee P, Augustson B, et al. Management of systemic AL amyloidosis: recommendations of the Myeloma Foundation of Australia Medical and Scientific Advisory Group. *Intern Med J*. 2015;45(4):371–382.

Wechalekar AD, Gillmore JD, Hawkins PN. Systemic amyloidosis. *Lancet*. 2016;387(10038):2641–2654.

Yusuf SW, Solhpour A, Banchs J, et al. Cardiac amyloidosis. *Expert Rev Cardiovasc Ther*. 2014;12(2):265–277.

REFERENCES

1. Desport E, Bridoux F, Sirac C, et al. Al amyloidosis. *Orphanet J Rare Dis*. 2012;7:54 https://doi.org/10.1186/1750-1172-7-54. Published 2012 Aug 21.
2. Kyle RA. Amyloidosis: a brief history [published correction appears in Amyloid. 2011 Jun;18 Suppl 1:7]. *Amyloid*. 2011;18(Suppl 1):6–7. https://doi.org/10.3109/13506129.2011.574354001.
3. Yakupova EI, Bobyleva LG, Vikhlyantsev IM, Bobylev AG. Congo red and amyloids: history and relationship. *Biosci Rep*. 2019;39(1). https://doi.org/10.1042/BSR20181415. BSR20181415. Published 2019 Jan 15.
4. Rajkumar SV, Gertz MA, Kyle RA. Primary systemic amyloidosis with delayed progression to multiple myeloma. *Cancer*. 1998;82:1501–1505.
5. Mendelson L, Shelton A, Brauneis D, et al. AL amyloidosis in myeloma: red flag symptoms. *Clin Lymphoma Myeloma Leuk* 20:S2152-S2650, 2020. https://doi.10.1016/j.clml.2020.05.023.
6. Wechalekar AD, Gillmore JD, Hawkins PN. Systemic amyloidosis. *Lancet*. 2016;387(10038):2641–2654. https://doi.org/10.1016/S0140-6736(15)01274-X. Prepublished on 2016/01/01 as (PMID 26719234).
7. Landgren O, Shim YK, Michalek J, et al. Agent orange exposure and monoclonal gammopathy of undetermined significance: an operation ranch hand veteran cohort study. *JAMA Oncol*. 2015;1(8):1061–1068. https://doi.org/10.1001/jamaoncol.2015.2938.
8. Kourelis TV, Kyle RA, Dingli D, et al. Presentation and outcomes of localized immunoglobulin light chain amyloidosis: the Mayo Clinic experience. *Mayo Clin Proc*. 2017;92(6):908–917. https://doi.org/10.1016/j.mayocp.2017.02.016.
9. Gertz MA, Mauermann ML, Grogan M, Coelho T. Advances in the treatment of hereditary transthyretin amyloidosis: a review. *Brain Behav*. 2019;9(9):e01371. https://doi.org/10.1002/brb3.1371.
10. Papa R, Lachmann HJ. Secondary, AA, amyloidosis. *Rheum Dis Clin North Am*. 2018;44(4):585–603. https://doi.org/10.1016/j.rdc.2018.06.004.
11. Abraham RS, Geyer SM, Price-Troska TL, et al. Immunoglobulin light chain variable (V) region genes influence clinical presentation and outcome in light chain-associated amyloidosis (AL). *Blood*. 2003;101(10):3801–3808. https://doi.org/10.1182/blood-2002-09-2707. Epub 2002 Dec 19.Blood. 2003. PMID: 12515719.
12. Gertz MA, Dispenzieri A, Muchtar E. Importance of FISH genetics in light chain amyloidosis. *Oncotarget*. 2017;8(47):81735–81736. https://doi.org/10.18632/oncotarget.21052. Published 2017 Sep 19.
13. Mishra S, Joshi S, Ward JE, et al. Zebrafish model of amyloid light chain cardiotoxicity: regeneration versus degeneration. *Am J Physiol Heart Circ Physiol*. 2019;316(5):H1158–H1166. https://doi.org/10.1152/ajpheart.00788.2018.
14. Vaxman I, Dispenzieri A, Muchtar E, Gertz M. New developments in diagnosis, risk assessment and management in systemic amyloidosis. *Blood Rev*. 2019:100636 https://doi.org/10.1016/j.blre.2019.100636. Prepublished on 2019/11/11 as (PMID 31706583).
15. Sachchithanantham S, Wechalekar AD. Imaging in systemic amyloidosis. *Br Med Bull*. 2013;107:41–56. https://doi.org/10.1093/bmb/ldt021. Prepublished on 2013/07/31 as (PMID 23896486).
16. Fermand JP, Bridoux F, Kyle RA, et al. How I treat monoclonal gammopathy of renal significance (MGRS). *Blood*. 2013;122(22):3583–3590. https://doi.org/10.1182/blood-2013-05-495929. Prepublished on 2013/10/11 as (PMID 24108460).
17. Jazbeh S, Said A, Haddad RY, Hamad A, Lerma EV. Renal amyloidosis. *Dis Mon*. 2014;60(10):489–493. https://doi.org/10.1016/j.disamonth.2014.08.003. Prepublished on 2014/10/05 as (PMID 25280992).
18. Gertz MA. Immunoglobulin light chain amyloidosis: 2018 update on diagnosis, prognosis, and treatment. *Am J Hematol*. 2018;93(9):1169–1180. https://doi.org/10.1002/ajh.25149.
19. Gertz MA. Immunoglobulin light chain amyloidosis: 2013 update on diagnosis, prognosis, and treatment. *Am J Hematol*. 2013;88(5):416–425. https://doi.org/10.1002/ajh.23400. Prepublished on 2013/04/23 as (PMID 23605846).
20. Kapoor P, Thenappan T, Singh E, Kumar S, Greipp PR. Cardiac amyloidosis: a practical approach to diagnosis and management. *Am J Med*. 2011;124(11):1006–1015. https://doi.org/10.1016/j.amjmed.2011.04.013.
21. Kumar S, Dispenzieri A, Lacy MQ, et al. Revised prognostic staging system for light chain amyloidosis incorporating cardiac biomarkers and serum free light chain measurements. *J Clin Oncol*. 2012;30(9):989–995. https://doi.org/10.1200/JCO.2011.38.5724.
22. Pagourelias ED, Mirea O, Duchenne J, et al. Echo parameters for differential diagnosis in cardiac amyloidosis: a head-to-head comparison of deformation and nondeformation parameters. *Circ Cardiovasc Imaging*. 2017;10(3):e005588. https://doi.org/10.1161/CIRCIMAGING.116.005588.
23. Lecadet A, Bachmeyer C, Buob D, Cez A, Georgin-Lavialle S. Minor salivary gland biopsy is more effective than normal appearing skin biopsy for amyloid detection in systemic amyloidosis: a prospective monocentric study. *Eur J Intern Med*. 2018;57:e20–e21. https://doi.org/10.1016/j.ejim.2018.07.026.
24. Kaku M, Berk JL. Neuropathy associated with systemic amyloidosis. *Semin Neurol*. 2019;39(5):578–588. https://doi.org/10.1055/s-0039-1688994.
25. Go RS, Rajkumar SV. How I manage monoclonal gammopathy of undetermined significance. *Blood*. 2018;131(2):163–173. https://doi.org/10.1182/blood-2017-09-807560.
26. Quarta CC, Gonzalez-Lopez E, Gilbertson JA, et al. Diagnostic sensitivity of abdominal fat aspiration in cardiac amyloidosis. *Eur Heart J*. 2017;38(24):1905–1908. https://doi.org/10.1093/eurheartj/ehx047.
27. Iida T, Yamano H, Nakase H. Systemic amyloidosis with gastrointestinal involvement: diagnosis from endoscopic and histological views. *J Gastroenterol Hepatol*. 2018;33(3):583–590. https://doi.org/10.1111/jgh.13996.
28. Vrana JA, Gamez JD, Madden BJ, Theis JD, Bergen 3rd HR, Dogan A. Classification of amyloidosis by laser microdissection and mass spectrometry-based proteomic analysis in clinical biopsy specimens. *Blood*. 2009;114(24):4957–4959. https://doi.org/10.1182/blood-2009-07-230722.
29. Kebbel A, Röcken C. Immunohistochemical classification of amyloid in surgical pathology revisited. *Am J Surg Pathol*. 2006;30(6):673–683. https://doi.org/10.1097/00000478-200606000-00002.
30. Winter M, Tholey A, Kristen A, Röcken C. MALDI Mass Spectrometry Imaging: a novel tool for the identification and classification of amyloidosis. *Proteomics*. 2017;17(22):1700236. https://doi.org/10.1002/pmic.201700236.
31. Maleszewski JJ, Murray DL, Dispenzieri A, et al. Relationship between monoclonal gammopathy and cardiac amyloid type. *Cardiovasc Pathol*. 2013;22(3):189–194. https://doi.org/10.1016/j.carpath.2012.09.001.
32. Mollee P, Renaut P, Gottlieb D, Goodman H. How to diagnose amyloidosis. *Intern Med J*. 2014;44(1):7–17. https://doi.org/10.1111/imj.12288. Prepublished on 2013/09/13 as (PMID 24024789).
33. Palladini G, Merlini G. What is new in diagnosis and management of light chain amyloidosis. *Blood*. 2016;128(2):159–168. https://doi.org/10.1182/blood-2016-01-629790.
34. Sperry BW, Reyes BA, Ikram A, et al. Tenosynovial and cardiac amyloidosis in patients undergoing carpal tunnel release. *J Am Coll Cardiol*. 2018;72(17):2040–2050. https://doi.org/10.1016/j.jacc.2018.07.092.
35. Ebert EC, Nagar M. Gastrointestinal manifestations of amyloidosis. *Am J Gastroenterol*. 2008;103(3):776–787. https://doi.org/10.1111/j.1572-0241.2007.01669.x.
36. Sirohi D, Gandhi J, Amin MB, Luthringer DJ. Amyloidosis of the bladder and association with urothelial carcinoma: report of 29 cases. *Hum Pathol*. 2019;93:48–53. https://doi.org/10.1016/j.humpath.2019.08.011.
37. Falk RH, Alexander KM, Liao R, Dorbala S. AL (light-chain) cardiac amyloidosis: a review of diagnosis and therapy. *J Am Coll Cardiol*. 2016;68(12):1323–1341. https://doi.org/10.1016/j.jacc.2016.06.053. Prepublished on 2016/09/17 as (PMID 27634125).
38. Hayashi Y, Jono H. Recent advances in oligonucleotide-based therapy for transthyretin amyloidosis: clinical impact and future prospects. *Biol Pharm Bull*. 2018;41(12):1737–1744. https://doi.org/10.1248/bpb.b18-00625.
39. Ueda M, Ando Y. Recent advances in transthyretin amyloidosis therapy. *Transl Neurodegener*. 2014;3:19 https://doi.org/10.1186/2047-9158-3-19. Prepublished on 2014/09/18 as (PMID 25228988).
40. Bhogal S, Ladia V, Sitwala P, et al. Cardiac amyloidosis: an updated review with emphasis on diagnosis and future directions. *Curr Probl Cardiol*. 2018;43(1):10–34. https://doi.org/10.1016/j.cpcardiol.2017.04.003.
41. Sher T, Gertz MA. Recent advances in the diagnosis and management of cardiac amyloidosis. *Future Cardiol*. 2014;10(1):131–146. https://doi.org/10.2217/fca.13.85. Prepublished on 2013/12/19 as (PMID 24344669).
42. Yusuf SW, Solhpour A, Banchs J, et al. Cardiac amyloidosis. *Expert Rev Cardiovasc Ther*. 2014;12(2):265–277. https://doi.org/10.1586/14779072.2014.876363. Prepublished on 2014/01/07 as (PMID 24386926).
43. Nguyen VP, Rosenberg A, Mendelson LM, Comenzo RL, Varga C, Sanchorawala V. Outcomes of patients with AL amyloidosis and low serum free light chain levels at diagnosis. *Amyloid*. 2018;25(3):156–159. https://doi.org/10.1080/13506129.2018.1490261.
44. Muchtar E, Dispenzieri A, Leung N, et al. Depth of organ response in AL amyloidosis is associated with improved survival: grading the organ response criteria. *Leukemia*. 2018;32(10):2240–2249. https://doi.org/10.1038/s41375-018-0060-x.

45. Muchtar E, Therneau TM, Larson DR, et al. Comparative analysis of staging systems in AL amyloidosis. *Leukemia*. 2019;33(3):811–814. https://doi.org/10.1038/s41375-018-0370-z.
46. Gertz MA, Dispenzieri A, Sher T. Pathophysiology and treatment of cardiac amyloidosis. *Nat Rev Cardiol*. 2015;12(2):91–102. https://doi.org/10.1038/nrcardio.2014.165. Prepublished on 2014/10/15 as (PMID 25311231).
47. Gillmore JD, Hawkins PN. Pathophysiology and treatment of systemic amyloidosis. *Nat Rev Nephrol*. 2013;9(10):574–586. https://doi.org/10.1038/nrneph.2013.171. Prepublished on 2013/08/28 as (PMID 23979488).
48. Cibeira MT, Ortiz-Pérez JT, Quintana LF, Fernádez de Larrea C, Tovar N, Bladé J. Supportive care in AL amyloidosis [published online ahead of print, 2020 Apr 1]. *Acta Haematol*. 2020:1–8. https://doi.org/10.1159/000506760.
49. Mahmood S, Palladini G, Sanchorawala V, Wechalekar A. Update on treatment of light chain amyloidosis. *Haematologica*. 2014;99(2):209–221. https://doi.org/10.3324/haematol.2013.087619. Prepublished on 2014/02/06 as (PMID 24497558).
50. Milani P, Merlini G, Palladini G. Light chain amyloidosis. *Mediterr J Hematol Infect Dis*. 2018;10(1):e2018022 https://doi.org/10.4084/MJHID.2018.022. Prepublished on 2018/03/14 as (PMID 29531659).
51. Milani P, Schönland S, Merlini G, et al. Treatment of AL amyloidosis with bendamustine: a study of 122 patients. *Blood*. 2018;132(18):1988–1991. https://doi.org/10.1182/blood-2018-04-845396.
52. Jelinek T, Kufova Z, Hajek R. Immunomodulatory drugs in AL amyloidosis. *Crit Rev Oncol Hematol*. 2016;99:249–260. https://doi.org/10.1016/j.critrevonc.2016.01.004.
53. Weber N, Mollee P, Augustson B, et al. Management of systemic AL amyloidosis: recommendations of the Myeloma Foundation of Australia Medical and Scientific Advisory Group. *Intern Med J*. 2015;45(4):371–382. https://doi.org/10.1111/imj.12566. Prepublished on 2014/08/30 as (PMID 25169210).
54. Sharpley FA, Manwani R, Mahmood S, et al. Real world outcomes of pomalidomide for treatment of relapsed light chain amyloidosis. *Br J Haematol*. 2018;183(4):557–563. https://doi.org/10.1111/bjh.15541.
55. Manwani R, Cohen O, Sharpley F, et al. A prospective observational study of 915 patients with systemic AL amyloidosis treated with upfront bortezomib. *Blood*. 2019;134(25):2271–2280. https://doi.org/10.1182/blood.2019000834.
56. Sayed RH, Hawkins PN, Lachmann HJ. Emerging treatments for amyloidosis. *Kidney Int*. 2015;87(3):516–526. https://doi.org/10.1038/ki.2014.368. Prepublished on 2014/12/04 as (PMID 25469850).
57. Shah G, Kaul E, Fallo S, et al. Bortezomib subcutaneous injection in combination regimens for myeloma or systemic light-chain amyloidosis: a retrospective chart review of response rates and toxicity in newly diagnosed patients. *Clin Ther*. 2013;35(10):1614–1620. https://doi.org/10.1016/j.clinthera.2013.08.015. Prepublished on 2013/10/01 as (PMID 24075726).
58. Wechalekar AD, Schonland SO, Kastritis E, et al. A European collaborative study of treatment outcomes in 346 patients with cardiac stage III AL amyloidosis. *Blood*. 2013;121(17):3420–3427. https://doi.org/10.1182/blood-2012-12-473066.
59. Chung A, Kaufman GP, Sidana S, et al. Organ responses with daratumumab therapy in previously treated AL amyloidosis. *Blood Adv*. 2020;4(3):458–466. https://doi.org/10.1182/bloodadvances.2019000776.
60. Palladini G, Kastritis E, Maurer MS, et al. Daratumumab plus CyBorD for patients with newly diagnosed AL amyloidosis: safety run-in results of ANDROMEDA [published online ahead of print, 2020 Apr 3]. *Blood*. 2020:2019004460. https://doi.org/10.1182/blood.2019004460.
61. Sanchorawala V. High dose melphalan and autologous peripheral blood stem cell transplantation in AL amyloidosis. *Hematol Oncol Clin North Am*. 2014;28(6):1131–1144. https://doi.org/10.1016/j.hoc.2014.08.013. Prepublished on 2014/12/03 as (PMID 25459183).
62. Sidiqi MH, Aljama MA, Buadi FK, et al. Stem cell transplantation for light chain amyloidosis: decreased early mortality over time. *J Clin Oncol*. 2018;36(13):1323–1329. https://doi.org/10.1200/JCO.2017.76.9554.
63. Sidana S, Sidiqi MH, Dispenzieri A, et al. Fifteen year overall survival rates after autologous stem cell transplantation for AL amyloidosis. *Am J Hematol*. 2019;94(9):1020–1026. https://doi.org/10.1002/ajh.25566.
64. White PS, Phull P, Brauneis D, et al. High-dose melphalan and stem cell transplantation in AL amyloidosis with elevated cardiac biomarkers. *Bone Marrow Transplant*. 2018;53(12):1593–1595. https://doi.org/10.1038/s41409-018-0242-1.
65. Gertz MA, Lacy MQ, Dispenzieri A, et al. Stem cell transplantation compared with melphalan plus dexamethasone in the treatment of immunoglobulin light-chain amyloidosis. *Cancer*. 2016;122(14):2197–2205. https://doi.org/10.1002/cncr.30051.
66. Afrough A, Saliba RM, Hamdi A, et al. Impact of induction therapy on the outcome of immunoglobulin light chain amyloidosis after autologous hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2018;24(11):2197–2203. https://doi.org/10.1016/j.bbmt.2018.07.010.
67. Angel-Korman A, Stern L, Sarosiek S, et al. Long-term outcome of kidney transplantation in AL amyloidosis [published correction appears in Kidney Int. 2019 Sep;96(3):796]. *Kidney Int*. 2019;95(2):405–411. https://doi.org/10.1016/j.kint.2018.09.021.
68. Sousa M, Monohan G, Rajagopalan N, Grigorian A, Guglin M. Heart transplantation in cardiac amyloidosis. *Heart Fail Rev*. 2017;22(3):317–327. https://doi.org/10.1007/s10741-017-9601-z.
69. Kristen AV, Kreusser MM, Blum P, et al. Improved outcomes after heart transplantation for cardiac amyloidosis in the modern era. *J Heart Lung Transplant*. 2018;37(5):611–618. https://doi.org/10.1016/j.healun.2017.11.015.
70. Stern L, Havasi A. Renal transplantation in amyloidosis and MIDD. *Front Biosci (Elite Ed)*. 2015;7:149–157. https://doi.org/10.2741/724. Published 2015 Jan 1.
71. Chen Y, Shlofmitz E. Should patients with cardiac amyloidosis be prioritized for heart transplantation? *J Card Fail*. 2019;25(9):772–773. https://doi.org/10.1016/j.cardfail.2019.07.001.
72. Ruberg FL, Grogan M, Hanna M, Kelly JW, Maurer MS. Transthyretin amyloid cardiomyopathy: JACC state-of-the-art review. *J Am Coll Cardiol*. 2019;73(22):2872–2891. https://doi.org/10.1016/j.jacc.2019.04.003.

PART VIII COMPREHENSIVE CARE OF PATIENTS WITH HEMATOLOGIC MALIGNANCIES

CHAPTER 94

KEY CONSIDERATIONS FOR MANAGING INFECTIONS IN THE COMPROMISED HOST

Samuel A. Shelburne, Russell E. Lewis, and Dimitrios P. Kontoyiannis

Advances in the supportive care during the treatment of hematologic malignancies have improved the life expectancy of afflicted patients. However, this progress is increasingly at the expense of developing a wider range of infectious complications caused by drug-resistant organisms. The clinical approach to infections occurring among hematology patients involves consideration of host immune system defects, and skin or mucosal barrier disruptions that predispose patients to infection (Fig. 94.1). This chapter reviews host defense defects specific to hematologic diseases, their associated infections (Table 94.1), and the differential diagnoses of these syndromes (Table 94.2). We also demonstrate how predictable timelines of immunosuppression associated with a hematopoietic stem cell transplant (HSCT) can be anticipated to devise effective prevention, diagnosis, and management strategies of infectious complications.

HEMATOLOGIC CONDITIONS PREDISPOSING TO INFECTION

Malignant Hematologic Disorders

Antineoplastic Therapy

During antineoplastic treatment, cytotoxic agents frequently are administered in combination with other immunosuppressive therapies, such as corticosteroids or radiation therapy. A diverse array of cytotoxic agents, such as methotrexate, cyclophosphamide, 6-mercaptopurine, and azathioprine, impair cell-mediated immunity. Many of the drugs themselves (e.g., cyclophosphamide) also impair humoral responses and profoundly suppress lymphocyte counts particularly when administered at high doses in multiple cycles.[1] Fludarabine, a purine analogue used for chronic lymphocytic leukemia (CLL), myeloid leukemias, lymphoma, and conditioning chemotherapy for HSCT produces prolonged and profound defects in cell-mediated immunity, thereby increasing susceptibility to *Pneumocystis, Mycobacterium*, cryptococcus, *Listeria monocytogenes* and herpes group viruses (herpes simplex virus [HSV], varicella-zoster virus [VZV], and cytomegalovirus [CMV]).[2]

The use of monoclonal antibody therapy for hematologic disorders results in dysfunction of particular aspects of the immune system.[3,4] Rituximab results in a sustained depletion of B lymphocytes for 6 to 9 months and has been specifically associated with reactivation of hepatitis B virus infection.[3] Blinatumomab is a bi-specific T cell engaging (BiTE) anti-CD19 antibody used for the treatment of Philadelphia-chromosome negative relapsed or refractory B-cell precursor acute lymphoblastic leukemia. Following infusion, blinatumomab causes B-cell aplasia, prolonged hypogammaglobulinemia, neutropenia, and may be associated with a cytokine release syndrome (CRS) similar to other T-cell activating therapies. Consideration for immunoglobulin substitution should be considered in patients with severe infections.[5] Alemtuzumab administration causes profound lymphopenia and an increased risk for a variety of viral and fungal infections.

Exogenous administration of glucocorticoids leads to increased susceptibility to infection. The degree of immunosuppression and the relative risk for infection depend on the dose and duration of use. The major effect of steroids on granulocyte function is a decrease in chemotactic activity. This explains, in part, the clinical observation that the signs and symptoms of severe infections may be masked or greatly reduced in patients receiving steroids. Steroids may enhance susceptibility to infection by means of negative effects on glucose homeostasis, wound healing, skin fragility, monocyte and lymphocyte function, production of cytokines, and humoral immune responses.[5]

Radiation therapy has been associated with granulocyte dysfunction and delayed wound healing. Defects in cell-mediated immunity may persist for more than 1 year after intensive radiation therapy or after HSCT.

Acute Leukemias

In patients with acute leukemias, a major cause of morbidity is infection due to drug-associated mucositis and therapy-induced neutropenia. Most infections occurring during neutropenia are bacterial, but patients with prolonged neutropenia are at additional risk for development of yeast and mold infections. Patients with acute leukemia who progress to hematopoietic cell transplant have added risk for infections associated with acquired deficiencies in cell-mediated and humoral immunity, such as *Pneumocystis jirovecii* and CMV infections.[6]

Chronic Leukemias

Infections in patients with chronic myeloid leukemia are most commonly associated with aggressive chemotherapy or HSCT. Host defense defects with tyrosine kinase inhibitors, such as imatinib or dasatinib, have not been well defined.[7] Chemotherapy for blast crisis resembles therapy for acute leukemia. Patients with CLL are predisposed to infection because of immunodeficiency related to the leukemia itself (humoral and cellular immune dysfunction) and to therapy-related immunosuppression.[4] In early B-cell CLL, the infectious risk is mainly related to unbalanced immunoglobulin chain synthesis and resultant hypogammaglobulinemia. In patients with advanced CLL, particularly after the introduction of therapy with purine analogues and monoclonal agents (e.g., rituximab, alemtuzumab), neutropenia

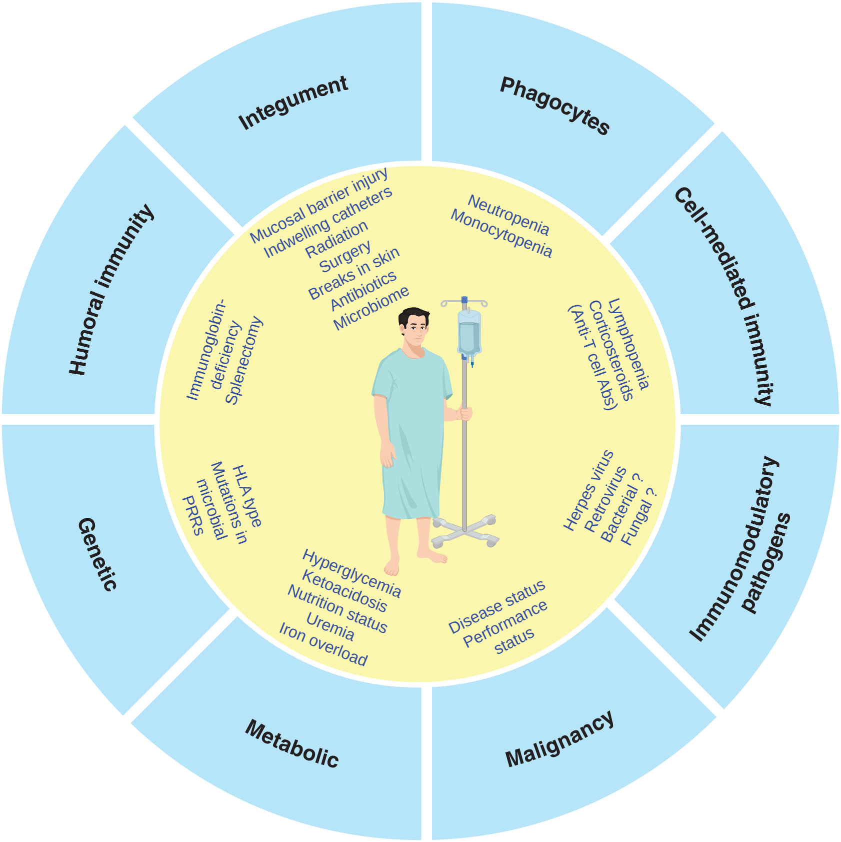

Integument
Phagocytes
Cell-mediated immunity
Immunomodulatory pathogens
Malignancy
Metabolic
Genetic
Humoral immunity
Mucosal barrier injury
Indwelling catheters
Radiation
Surgery
Breaks in skin
Antibiotics
Microbiome
Neutropenia
Monocytopenia
Lymphopenia
Corticosteroids
(Anti-T cell Abs)
Herpes virus
Retrovirus
Bacterial ?
Fungal ?
Disease status
Performance status
Hyperglycemia
Ketoacidosis
Nutrition status
Uremia
Iron overload
HLA type
Mutations in microbial PRRs
Immunoglobin-deficiency
Splenectomy

Figure 94.1 CONSTELLATION OF FACTORS CONTRIBUTING TO INCREASED RISK FOR INFECTION IN IMMUNOCOMPROMISED HOSTS.

TABLE 94.1 **Malignant and Select Nonmalignant Hematologic Diseases and Their Associated Infection-Predisposing Host Defects**

Hematologic Condition	Infection-Predisposing Host Defects
Acute myeloid leukemia	Neutropenia; therapies such as dose-intensive chemotherapy and hematopoietic stem cell transplant may result in additional anatomic disruptions, cell-mediated defects, and humoral defects
Acute lymphocytic leukemia	Neutropenia; therapy effects similar to acute myeloid leukemia
Hairy cell leukemia	Neutropenia (also monocytopenia); abnormal humoral immunity; T-cell suppressing therapy
Chronic lymphocytic leukemia	Hypogammaglobulinemia; abnormal cell-mediated immunity
Chronic myeloid leukemia	No prominent host defects unless aggressive therapy, advanced stage, or postsplenectomy
Multiple myeloma	Hypogammaglobulinemia; other host defects may occur with aggressive therapy or advanced stage
Hodgkin/non-Hodgkin lymphomas	Abnormal cell-mediated immunity, therapy-related neutropenia, splenic dysfunction (if splenectomy or radiation)
Myelodysplastic syndromes	Functional or absolute neutropenia
Aplastic anemia	Neutropenia; abnormal cell-mediated immunity from immunosuppressive therapies (e.g., steroids, antithymocyte globulin, cyclosporine, hematopoietic stem cell transplantation)
Paroxysmal nocturnal hemoglobinuria	Deficient Fc receptor may contribute to abnormal cell-mediated immunity
Hemolytic states (thalassemia)	Gallstones may serve as a nidus for infection; splenic dysfunction or splenectomy
Sickle cell disease	Can be neutropenic with aplastic crisis; bone infarcts may serve as a nidus for infection; splenic dysfunction with poor complement activation and opsonization from autosplenectomy

and defects in cell-mediated immunity are other factors predisposing to infection. The risk for infectious complications increases with the duration of CLL, reflecting the cumulative immunosuppression related to its treatment. The incidence of infection correlates with the serum levels of immunoglobulins (particularly IgG), which may be further impaired by use of anti-CD20 therapies.

Ibrutinib, the first Bruton tyrosine kinase inhibitor approved for the treatment of CLL, is associated with hypogammaglobulinemia and inhibits B-cell signaling. In clinical trials one-third of patients developed respiratory tract infections, of which 15% were categorized as severe pneumonia requiring hospitalization and intravenous antibiotics.[8] Other opportunistic infections including *P. jirovecii* pneumonia, cryptococcosis, histoplasmosis, and fusariosis, have been reported. *Aspergillus* infections with high rates of central nervous system involvement have been observed in patients receiving ibrutinib for CLL or for primary central nervous system lymphoma.[9–11] At

TABLE 94.2 **Host Defense Impairments and Their Associated Infectious Pathogens**

Host Defense Defect	Pathogen Categories
Neutropenia	Enteric gram-negative organisms
	Gram-positive staphylococci and streptococci
	Anaerobes
	Yeast, particularly *Candida* species
	Molds, particularly *Aspergillus* species
Abnormal cell-mediated immunity	Atypical bacteria: *Legionella, Nocardia*
	Salmonella species
	Mycobacteria (*M. tuberculosis* and atypical mycobacteria)
	Disseminated infection from live bacillus Calmette-Guérin (BCG) vaccine
	Environmental fungi, including *Cryptococcus neoformans, Histoplasma capsulatum, Coccidioides immitis*
	Endogenous yeast, particularly *Candida* species
	Herpesviruses
	Infections from live-virus vaccines
	Pneumocystis jirovecii
	Toxoplasma gondii
	Cryptosporidium
	Strongyloides stercoralis
Immunoglobulin abnormalities	Gram-positive *Streptococcus pneumoniae, Staphylococcus aureus*
	Gram-negative *Haemophilus influenzae*, *Neisseria* species, enteric organisms
	Enteroviruses
	Disseminated infections from live-virus vaccines
	Giardia lamblia
Complement abnormalities C3, C5	Gram-positive *S. pneumoniae*, staphylococci
	Gram-negative *H. influenzae, Neisseria* species, enteric organisms
Complement abnormalities C5–C9	*Neisseria* species
Anatomic disruption	Pathogen categories
Oral cavity	α-Hemolytic streptococci, oral anaerobes
	Candida species
	Herpes simplex virus
Esophagus	*Candida* species,
	Herpes simplex virus, cytomegalovirus
Lower gastrointestinal tract	Enterococcus, gram-negative enteric organisms,
	Anaerobes *(Bacteroides fragilis, Clostridium perfringens)*,
	Candida species, *Strongyloides stercoralis*
Skin (IV catheter)	Gram-positive staphylococci and streptococci, *Corynebacterium, Bacillus*
	Atypical mycobacteria
Urinary tract	Enterococcus
	Gram-negative enteric organisms
	Candida species
Splenectomy	Encapsulated organisms: *S. pneumoniae, H. influenzae, Neisseria, Capnocytophaga canimorsus*
	Salmonella (especially sickle cell disease),
	Babesia

present there are no standard recommendations for antimicrobial or antifungal prophylaxis with ibrutinib therapy.

Idelalisib and duvelisib inhibit B-cell receptor signaling by targeting the delta isoform of phosphoinositide 3-kinase (PI3K). Serious infections have been reported in 20% to 30% of patients with CLL receiving these agents alone or in combination with rituximab, including pneumocystis pneumonia and CMV reactivation. Therefore, a high index of suspicion is important for these infections in patients receiving these agents.

Lymphomas

Hodgkin and non-Hodgkin lymphoma are commonly associated with impaired cell-mediated immunity. The degree of immune impairment may correlate with the extent of disease and often is compounded by administration of immunosuppressive therapy. The intrinsic impairment of cell-mediated immunity in Hodgkin lymphoma can persist even after an apparent cure. Splenectomy-related infections occur with sepsis caused by encapsulated bacterial organisms at a median of 22 months, but sometimes many years after surgery. The risk of viral and fungal infection is elevated in patients who have undergone extensive treatment and are undergoing salvage chemoimmunotherapy after relapsing (typically rituximab-based regimens) followed by autologous HCT or allogeneic HSCT following the relapse.

Myelodysplastic Syndrome

Neutrophils and band forms from patients with myelodysplastic syndrome are functionally defective and probably are derived from a malignant clone of myeloid precursor cells. Neutrophils from patients with myelodysplastic syndrome have deficiencies in myeloperoxidase, elastase, and integrins. A significant portion of myelodysplastic syndrome patients die within 3 years of diagnosis from infections, bleeding complications, or progression to acute leukemia.

Multiple Myeloma

Malignant plasma cells produce a variety of immunomodulatory molecules such as transforming growth factor beta (TGF-β) that suppress B-cell function, which contribute to defects in humoral immunity.[11] Patients having myeloma with IgG paraprotein have an increased rate of catabolism of normal and clonal IgG. They also may have defects in complement and granulocyte function. Cell-mediated immunity is not impaired by the disease but is compromised by corticosteroids or cytotoxic therapy. Prolonged and intense multiple myeloma therapy, including autologous stem cell transplant, can eventually result in a profoundly immunocompromised state resembling that seen in acute leukemia or allogenic stem cell transplant recipients.

Uncommon Malignancies

Patients with hairy cell leukemia frequently develop infections with atypical mycobacteria. Effective treatment of hairy cell leukemia can reverse host cellular immune defects with eradication of mycobacterial infection.

Defects in cell-mediated immunity have been postulated to explain the incidence of infection caused by intracellular pathogens in patients with the relatively rare T-cell malignancies mycosis fungoides and T-cell CLL.

Nonmalignant Hematologic Disorders

Aplastic Anemia

Chronic neutropenia is the main cause of recurrent bacterial and fungal infections among patients. Periodontal infections are particularly common. Treatment of the underlying hematologic disease is required to stop recurrent infections and cure some chronic infections.

Paroxysmal Nocturnal Hemoglobinuria

Patients with paroxysmal nocturnal hemoglobinuria are at some increased risk for bacterial infection due to a deficiency of decay-accelerating factor on the membrane of neutrophils. Modest and progressive granulocytopenia, progression to aplasia or leukemia may compound these risks. Vaccination against meningococcus is required before treatment with the terminal complement inhibitor eculizumab.

Granulocytic Phagocyte Disorders

The clinical approach to infections in patients with granulocytic phagocyte disorders is specific to each of these disorders and is beyond the scope of this chapter. However, chronic granulomatous disease is discussed because patients with this congenital immunodeficiency who survive into adulthood are at risk for severe infections. Chronic granulomatous disease is a heterogeneous group of disorders resulting from defective or malfunctioning oxidative metabolism capacity of phagocytes. Recurrent infections with bacteria and fungi are common and occasionally life threatening, despite optimal antimicrobial therapy. Infections with *Staphylococcus* species and *Aspergillus* species can be particularly aggressive.[12] Granulomata may form in response to infection, especially in the gastrointestinal (GI) and genitourinary tracts.

Erythrocyte Disorders

Glucose-6-phosphate dehydrogenase deficiency is a sex-linked disorder. Deficiency of this enzyme limits glucose metabolism through the hexose monophosphate shunt, resulting in an abnormal respiratory burst in neutrophils. Bacterial infections can occur if the deficiency is severe.

Hemoglobin Gene Variants

Patients with chronic hemolytic states may develop bilirubin gallstones, which can serve as a nidus for infection. Defects in cell-mediated immunity have been described in patients with thalassemia. Patients with sickle cell disease have an increased susceptibility to bacterial infections.[13] Defective alternative complement pathway function, especially in conjunction with asplenia, contributes to the propensity to bacterial infection. Splenic involution results in depressed synthesis of the alternate pathway factor(s) of complement and decreased phagocytic clearance of bacteria. Phagocytosis of *Streptococcus pneumoniae* is abnormal, in part because of an inability to use the alternate pathway for C3 fixation as a means of opsonization. An increased risk for *Salmonella* infection appears to be unique to the sickle cell population. Suppurative arthritis can occur after repeated episodes of hemarthrosis among patients with sickle cell disease.

Coagulation Disorders

Hemophilias are sex-linked deficiencies of clotting factor VIII or IX. Septic arthritis should be considered in the differential diagnosis of any hemophiliac with repeated episodes of hemarthrosis whose articular signs and symptoms fail to improve quickly after administration of appropriate coagulation factor replacement.

Blood Groups

The Duffy blood group antigen serves as a receptor for *Plasmodium vivax* to invade erythrocytes. Blood group O is associated with *Helicobacter pylori* infection and an associated increase in peptic ulceration because the Lewis (b) blood group antigen mediates *H. pylori* attachment to human gastric mucosa.

Host Defense Impairment and Associated Infection Issues

Neutropenia

Infection rates increase when neutrophil counts fall below 1000/mm^3, but the patient is most at risk for spontaneous infection when the count is below 100/mm^3 (Fig. 94.2). The patient who is neutropenic from cytotoxic therapy can serve as a basic model for predicting infections that could occur in other patients with qualitative or quantitative granulocyte defects.

Neutropenia predisposes to the development of bacterial and fungal infections but does not appear to increase the incidence or severity

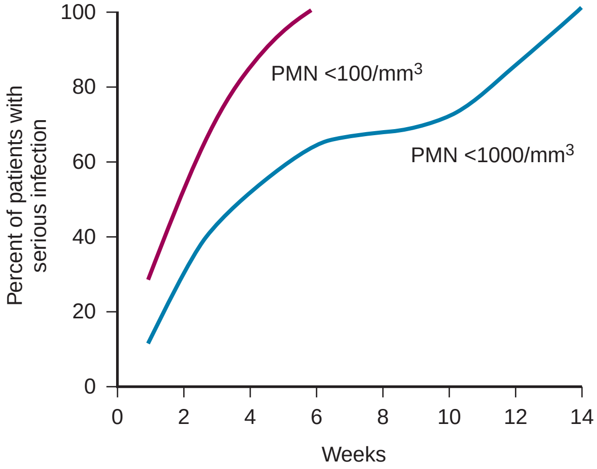


Figure 94.2 RELATIONSHIP OF SEVERITY AND DURATION OF NEUTROPENIA TO THE RISK FOR DEVELOPING A SERIOUS INFECTION. *PMN*, Polymorphonuclear neutrophil. *(Courtesy GE Body.)*

of viral and parasitic infections. Patients with profound and prolonged neutropenia who are at particular high risk for infection (e.g., cytopenic patients with acute myelogenous leukemia and severe mucositis following induction therapy) are likely to benefit from receiving prophylactic antimicrobials.[14,15] However, patients with moderate granulocytopenia (i.e., absolute granulocyte counts in the range from 500 to 2000/mm^3 that are not falling precipitously) should not receive prophylactic antimicrobial agents. Colony-stimulating factors (granulocyte colony-stimulating factor [G-CSF; pegylated filgrastim], granulocyte-macrophage colony-stimulating factor [GM-CSF, sargramostim]) are routinely used in the management of hematologic disorders. A number of functional granulocyte parameters may be enhanced by G-CSF or GM-CSF, including enhanced per-cell phagocytosis, oxidative metabolism, microbicidal activity, and antibody-dependent cytotoxicity.[16] However, G-CSF or GM-CSF administration may decrease the motility of granulocytes, impair in vivo migration, and decrease bacteria-induced chemotaxis. The clinical significance of any of these effects remains to be established but is generally believed to be insignificant in light of the potency of these agents in shortening the depth and duration of therapy-induced neutropenia.

Defects in Cell-Mediated Immunity

Cellular immune dysfunction occurs in patients with lymphoid malignancies, in those with hematologic malignancies undergoing HSCT, and in patients with AIDS. The most frequently encountered pathogens are intracellular organisms because they can survive and even replicate inside macrophages in a non-immune individual or in the absence of T-cell immunity. Specific pathogens associated with infection in patients with cell-mediated immunity include bacteria (including *Mycobacterium tuberculosis*, atypical mycobacteria, *Salmonella*, streptococci, *Legionella, Nocardia*, and *Listeria*); fungi (including the yeasts *Candida* and *Cryptococcus;* molds such as *Aspergillus;* endemic dimorphic fungi such as *Histoplasma* and *Coccidioides;* and *Pneumocystis*); viruses (including herpes group viruses [HSV, VZV, CMV] and respiratory viruses); and protozoa (*Toxoplasma gondii, Cryptosporidium*, and *Strongyloides stercoralis*).

Patients with defects in cell-mediated immunity or who are about to receive HSCT should undergo risk factor assessment for reactivation of tuberculosis. Patients with a known history of an untreated but positive Mantoux test or interferon-gamma release assay should receive prophylactic isoniazid to prevent reactivation and potential dissemination of tuberculosis. Patients without a prior Mantoux test but with risk factors for tuberculosis should undergo a screening test for latent tuberculosis before starting the conditioning regimen for HSCT.

Patients with defects in cell-mediated immunity are at risk for the development of disseminated infection due to live vaccines, even when the vaccine contains attenuated organisms. Accordingly, these patients should not receive vaccines containing bacillus Calmette-Guérin (BCG), live varicella, measles, mumps, rubella, yellow fever, or oral polio. HSCT recipients are eligible for revaccination with some of the live virus vaccines 2 years after transplantation, provided the patient does not have graft-versus-host disease (GVHD), or is not receiving ongoing pharmacologic immunosuppression (see Chapter 110, Table 110.4).

Defects in the Humoral Immune System

Patients with primary or secondary defects, or deficiencies in humoral immunity, such as deficiencies in immunoglobulin or complement, are susceptible to recurrent pyogenic infections from polysaccharide-encapsulated bacteria, such as *S. pneumoniae, Haemophilus influenzae*, and *Neisseria* species. HSCT recipients who continue to receive immunosuppressive therapy more than 100 days after transplantation should be given antimicrobial prophylaxis with coverage for encapsulated bacteria until immunosuppression is discontinued. Patients are also at risk for infections from enteroviruses and *Giardia lamblia*. Patients with low levels of circulating IgG may benefit from intravenous Ig (IVIg) infusions, although the benefit of using of IVIg infusions for routine prophylaxis must be weighed against the expense of this approach.

Abnormalities in Splenic Function

A number of hematologic disorders are complicated by either intrinsic splenic impairment or splenectomy. The spleen removes organisms from the blood that have been ineffectively opsonized by complement, serving an adjunctive role in fighting infection. Alternate complement pathway defects may be particularly important in patients with splenic dysfunction associated with sickle cell disease.

Asplenic or splenectomized patients are at increased risk for serious, frequently fulminant, bacterial infections, primarily caused by *S. pneumoniae, H. influenzae, Neisseria, Babesia*, and *Capnocytophaga canimorsus*. The initial presentation of even overwhelming infection can be subtle, with fever often the only sign. Accordingly, all asplenic patients with underlying hematologic disease who present with fever should be managed initially as potentially septic. Overwhelming infection after splenectomy occurs in approximately 7% of postsplenectomy patients, with 50% of infection-related deaths occurring in the first 3 months.[17] Prophylaxis against pneumococcal infection is used for asplenic patients who are small children or for those with increased immune impairment from malignant disease.

Pneumococcal, *H. influenzae* type b, and meningococcal vaccines should be administered to asplenic patients. Patients with an intact spleen may respond better to pneumococcal polysaccharide vaccine than do splenectomized patients, so immunization is recommended as early as possible before elective surgery. Additionally, immunization before splenectomy can result in protective pneumococcal antibody titers immediately after the operation. For patients with Hodgkin lymphoma, the antibody response to pneumococcal vaccine may not be affected by the timing of immunization relative to splenectomy. Immunizations reduce, but do not eliminate, the risk for serious infection with encapsulated bacteria.

Infections Associated With Immunotherapy

The use of immunotherapeutic agents for hematological malignancies, such as checkpoint inhibitors and chimeric antigen receptor T cell (CAR-T) cells, have shown considerable promise in the treatment of a wide spectrum of malignancies. A comprehensive workup of autoimmune/organ-specific disease, endocrinopathy, or infectious diseases is important before immunotherapy is initiated, as inflammatory responses are strongly induced against these conditions during checkpoint therapy. Although any organ system can be affected, immune-related adverse events associated with checkpoint inhibitors most commonly involve the GI tract, endocrine glands, skin, and liver.[18] Less frequently, the central nervous system and cardiovascular, pulmonary, musculoskeletal, and hematologic systems are involved. Whenever symptoms of immune-related adverse events are present, infections involving these organ systems must be ruled out, as infections can mimic or co-exist alongside immune-driven processes. In more severe cases, glucocorticoids or an anti-TNF-α antibody, infliximab are used, which can increase the patient's risk for developing opportunistic infections including invasive pulmonary aspergillosis, CMV, hepatitis, and pneumocystis pneumonia.

The most commonly observed toxicity with CAR-T therapy is CRS, which ranges in severity from low-grade constitutional symptoms to life-threatening multiorgan dysfunction.[19] Less frequently, severe CRS can evolve into fulminant hemophagocytic lymphohistiocytosis/macrophage activation syndrome (HLH/MAS). CAR-T-cell–related encephalopathy syndrome (CRES) can also develop concurrently or after CRS.[19] CRS is typically treated initially with the anti-IL6 agent tocilizumab, but in more severe cases (Grade III/IV) requires treatment with corticosteroids.

Patients receiving therapy with CAR-T cells are considered to be at high risk for infection due to cumulative immunosuppression associated with the underlying malignant disease and its treatment, as well as the cytotoxic and lymphodepleting chemotherapy administered prior to the cell infusion.[20] In addition, the infusion of CAR-T cells depletes normal CD19$^+$ B cells, which results in prolonged hypogammaglobulinemia that can persist over time.[21] Patients are at high risk for recurrent and severe bacterial and viral respiratory infections. Invasive fungal disease, including invasive candidiasis and invasive aspergillosis have been reported in patients requiring steroid treatment for CRS.[22]

Anatomic Alterations in Host Defense

Immunocompromised patients frequently have disruptions in the skin and mucosa, which are important primary physical barriers against endogenous and exogenous sources of infections (see Fig. 94.1). Disruption of skin and mucosa may result from invasion by malignant cells, the effects of chemotherapy or radiation therapy, the use of invasive diagnostic or therapeutic procedures (e.g., intravenous catheters), and from the effects of local infections, such as oral HSV. Such alterations may provide a nidus for microbial colonization, a focus for localized infection, and a portal of entry for systemic invasion. Organisms associated with defects in skin or mucosal surfaces depend on the site of the breakdown, local colonizing flora, and other factors. Gram-positive organisms are associated with isolated disruption of the skin from an indwelling intravenous catheter, usually with coagulase-negative staphylococci, but also with *Corynebacterium jeikeium, Bacillus* species, and occasionally atypical mycobacteria.

GI mucosal integrity is frequently disrupted by chemotherapeutic agents. Because the GI tract normally is colonized by a multitude of organisms, this state can lead to infection by streptococci, aerobic gram-negative enteric and anaerobic bacteria, and yeast. Mucosal damage can allow normal flora to invade and become pathogens. Lower GI ulcerations permit infections by *Bacteroides fragilis* or *Streptococcus bovis*. Oral lesions are associated with HSV reactivation, ulceration, and possible bloodstream infection with other common oral flora such as α-hemolytic streptococci. Mucosal integrity also depends on resident gut bacteria production of a number of metabolites and bioproducts necessary to maintain homeostasis in the host. When chemotherapy and/or antibiotic therapy are administered, the associated dysbiosis of the gut microbiome may trigger local inflammation, or alternation of the host immune response against malignant cells and pathogens.[23] Strategies aimed at modulation of host-microbiome interactions to improve cancer treatment, or to reduce infection risk have become a major research focus.

The genitourinary tract mucosa may be disrupted by tumors, invasive procedures, or cytotoxic therapy, with subsequent colonization and the potential for local or invasive infection. The most common urinary tract pathogens are enteric gram-negative bacilli, enterococci, and *Candida albicans*. Viral reactivation is common, predominantly from adenovirus and polyomavirus (BK virus), but also from the herpesviruses (HSV and CMV).

The lung, genitourinary tract, biliary tract, and auditory canal are potential sites of mechanical obstruction, increasing the risk for localized infection. Obstruction may lead to stasis of local body fluids, with resultant overgrowth of potentially pathogenic colonizing organisms. Patients with chronic hemolytic states are prone to gallstones that can become a nidus for infection.

Infection in Patients With Acute Neutropenia or Lymphopenia Following Chemotherapy or Transplantation

This section outlines predictable infections that can present during acute profound neutropenia/lymphopenia.

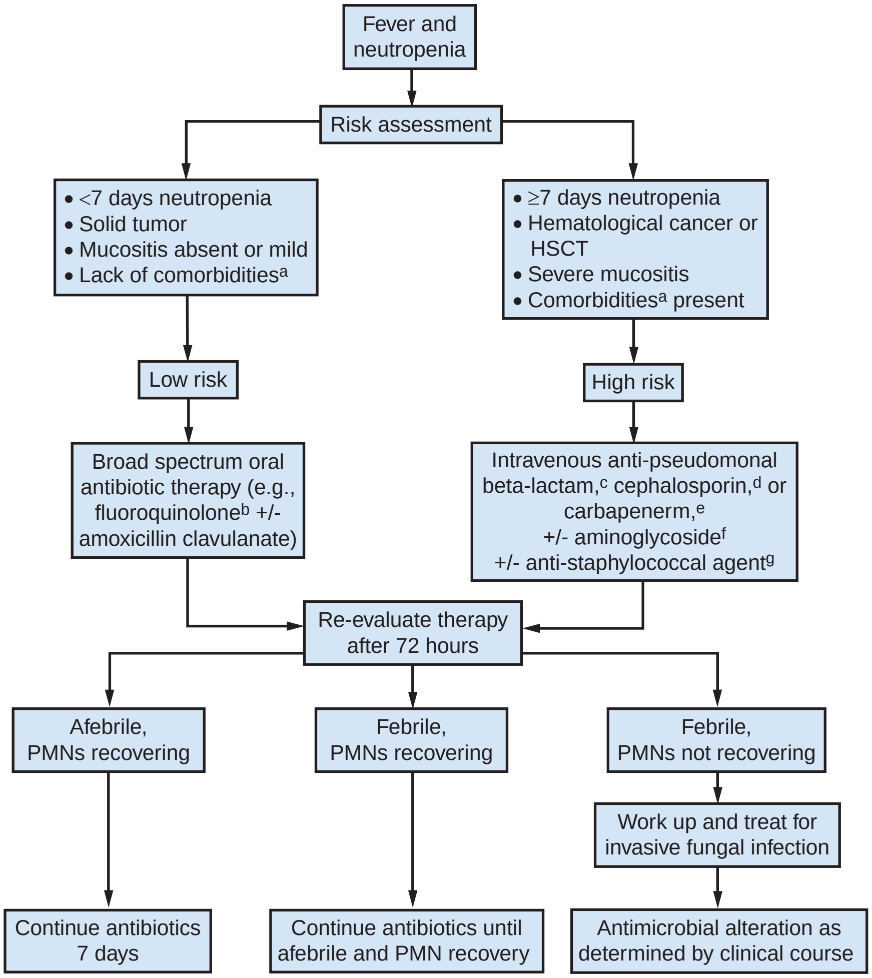
Fever

Despite the specific prophylactic measures directed against common pathogens, many fevers occur in neutropenic patients after transplantation or chemotherapy. Fevers can be divided into three categories: infectious fever with an obvious source, infectious fever without an obvious source, and noninfectious fever. Risk factor assessment should include knowledge of the temporal relationship to blood product infusions; recent exposure to contagious infection; temperature of the fever and whether the fever is accompanied by chills, rigors, or diaphoresis; and response to antipyretics. Symptom assessment should include evaluation to assess sinus drainage, sore throat, ear pain, cough, sputum production, shortness of breath, abdominal pain, diarrhea, rash, and dysuria.

The initial workup of a fever in a patient with neutropenia, regardless of whether or not an infectious or non-infectious source is suspected, is identical and includes blood culture, culture of symptom-related sites (e.g., sputum, urine, with/without stool, with/without cerebrospinal fluid), review of the medication list for potential contributors to drug fever, review of recent transfusions, chest radiograph, and computed tomography (CT) scan of any symptom-related body systems. When feasible, blood cultures should be drawn through an existing indwelling catheter as well as peripherally because this can facilitate the diagnosis of a catheter-related bloodstream infection compared to a bloodstream infection arising from a different source (e.g., the GI tract).[24]

Consensus guidelines on the management of patients with febrile neutropenia have been published.[24,25] Empiric antibiotics are recommended for all febrile patients with neutropenia, but the type of antibiotics and the site of administration (i.e., hospital vs. outpatient) depends on the severity of immunosuppression, expected duration of neutropenia, and factors related to the local epidemiology and resistance patterns (Fig. 94.3). Patients defined as low-risk using the Multinational Association for Supportive Care in Cancer (MASCC) index can be treated as outpatients, whereas other patients are generally admitted for intravenous therapy.[26,27] Typical pathogens causing infection in this situation include Enterobacteriaceae*, Pseudomonas, Streptococcus*, and *Staphylococcus* spp. Thus, antipseudomonal β-lactam antibiotics such as ceftazidime or cefepime, piperacillin-tazobactam, and carbapenems are commonly employed as empiric therapy. The selection of antibiotic therapy depends on local susceptibility patterns and whether the fever developed after previous antibiotic treatment. Vancomycin is administered if staphylococcal disease is suspected, or if the patient is clinically unstable while cultures are pending. Severely ill patients are often also treated with an aminoglycoside for the first 48 to 72 hours of illness, although there is limited evidence supporting this approach.[27]

Therapeutic changes to the antimicrobial regimens are made in response to culture results, but cultures are negative about 50% of the time (see Fig. 94.3). If the patient becomes afebrile, therapy is


Fever and neutropenia
Risk assessment
• <7 days neutropenia
• Solid tumor
• Mucositis absent or mild
• Lack of comorbidities[a]
• ≥7 days neutropenia
• Hematological cancer or HSCT
• Severe mucositis
• Comorbidities[a] present
Low risk
High risk
Broad spectrum oral antibiotic therapy (e.g., fluoroquinolone[b] +/- amoxicillin clavulanate)
Intravenous anti-pseudomonal beta-lactam,[c] cephalosporin,[d] or carbapenerm,[e] +/- aminoglycoside[f] +/- anti-staphylococcal agent[g]
Re-evaluate therapy after 72 hours
Afebrile, PMNs recovering
Febrile, PMNs recovering
Febrile, PMNs not recovering
Work up and treat for invasive fungal infection
Continue antibiotics 7 days
Continue antibiotics until afebrile and PMN recovery
Antimicrobial alteration as determined by clinical course

a Hypotension, altered mental status, neurologic changes, respiratory failure, abdominal pain, hemorrhage, cardiac compromise or new arrythmia, catheter tunnel infection, extensive cellulitis, acute renal or liver failure
b Institution sensitivity–dependent, ciprofloxacin, levofloxacin, moxifloxacin
c Drug selection and dosing institution-specific: piperacillin tazobactam, ticarcillin/clavulanate
d Drug selection and dosing institution-specific: ceftazidime
e imipenem, cefepime/cilastatin, meropenem, doripenem
f Gentamin, tobramycin, or amikacin
g Drug selection and dosing institution-specific: vancomycin, linezolid, daptomycin, ceftaroline

Figure 94.3 APPROACH TO PATIENT WITH FEVER AND NEUTROPENIA. *HSCT*, Hematopoietic stem cell transplant; *PMN*, polymorphonuclear neutrophil.

usually continued for 7 days. Persistent fever after more than 72 hours of empiric therapy suggests an untreated infection. If the patient is recovering his or her neutrophil count, no additional changes in antibiotics are typically needed. However, the persistently neutropenic and febrile patient may have an occult fungal infection such as candidiasis or aspergillosis. Thus, empiric antifungal therapy with anti-mold activity (e.g., liposomal amphotericin B or voriconazole or isavuconazole) is recommended.

Two common anaerobic infections occur during neutropenia at sites where biopsy is difficult or contraindicated. Neutropenic enterocolitis, also known as typhlitis, manifests as fever, abdominal pain, and tenderness. A CT scan of the abdomen shows signs of right-sided colonic and ileal inflammation.[16] Excessive soft tissue swelling of the neck during mucositis can be present as a Ludwig angina variant. Broadly active anti-anaerobic, aerobic, and possibly antifungal antimicrobial agents should be added for either of these clinical findings.

Pulmonary Infiltrates

Pulmonary infections are common in the immunocompromised host (see box on Approach to Pulmonary Infiltrates). Plain chest radiography is a good initial screening test but lacks the sensitivity of CT, which generally provides more useful information in terms of characterizing the nature of an infiltrate, and assists the pulmonologist in determining where to direct the bronchoscope for highest yield. A specific infectious and noninfectious differential diagnosis exists for certain radiologic signs (Table 94.3). For example, consolidative focal airspace disease is associated most typically with a bacterial pneumonia. A halo of interstitial changes around a pulmonary nodule or an air crescent above a pulmonary nodule is suggestive of invasive aspergillosis (Fig. 94.4).[28] Although 90% of pulmonary nodules are due to fungal pneumonia (mainly aspergillosis), 10% have various etiologies, including septic bacterial emboli, *Nocardia, Legionella*, mycobacterial infection (with cavitation), Epstein-Barr virus (EBV)–related

APPROACH TO PULMONARY INFILTRATES

Pulmonary infiltrates can be divided into three general categories: consolidative, interstitial, and nodular. A consolidative infiltrate may be bacterial, even polymicrobic, so sampling of the infiltrate through sputum or endotracheal tube suctioning for bacterial and fungal cultures is required. If either of these methods does not provide an adequate sample, “early” (within 72 h) bronchoscopy is indicated. If interstitial or nodular infiltrates are not peripheral (i.e., within the range of the bronchoscope), they should also be evaluated with timely bronchoscopy. An advantage of bronchoscopy, in addition to the ability to obtain a deep specimen, is that the samples usually are sent for a broad range of diagnostic tests. These tests usually are ordered from preprinted order sheets, which reduce errors. Most preprinted order sheets for bronchoalveolar lavage fluid will test for the following:

- Cytology, with the attendant Fite and Gomori methenamine silver stains
- Bacterial (Gram) stain and aerobic culture
- Mycobacterial (acid-fast bacillus) stain and culture
- Fungal (potassium hydroxide or calcofluor) stain and culture
- *Aspergillus* galactomannan antigen
- *Nocardia* culture
- *Legionella* culture
- Viral cultures for herpes viruses and respiratory viruses
- Rapid test for cytomegalovirus (polymerase chain reaction [PCR])
- Rapid test for respiratory viruses during respiratory virus season (pooled respiratory virus shell vial, respiratory syncytial virus antigen, influenza A/B antigen, severe acute respiratory syndrome coronavirus 2)

Optional tests that can be requested from bronchoalveolar lavage fluid, some at significant extra expense, include the following:

- Mycobacterial stain (requires separate 5 mL volume that cannot be used later for culture)
- *Mycoplasma pneumoniae* PCR
- *Chlamydia pneumoniae* PCR
- Human herpesvirus type 6 PCR
- Human metapneumovirus PCR
- Any of the above first-tier tests that your institution does not send on a routine basis (e.g., *Nocardia* or *Legionella*)

Invasive sampling may be the only definitive means for making a diagnosis of peripheral nodules. The two main options are percutaneous fine-needle aspiration and open lung biopsy (usually obtained through video-assisted thoracoscopic surgical procedure). In toto sampling of a lung nodule permits enough material for the whole range of the above diagnostic tests as well as histopathology. Of note, fewer lobectomies of unilateral pulmonary nodular infiltrates have been reported in recent years, probably a result of the frequent use of a broad range of less toxic antifungal therapies.

TABLE 94.3 Pulmonary Infiltrates and Their Association With Specific Infectious and Noninfectious Etiologies

Radiologic Sign	Differential Diagnosis
Interstitial infiltrates	Pulmonary edema
	Diffuse alveolar damage
	Idiopathic pneumonia syndrome
	Respiratory viruses: respiratory syncytial virus, parainfluenza, influenza, adenovirus, enterovirus
	Herpes viruses: cytomegalovirus, herpes simplex virus, varicella zoster virus, human herpes virus type 6
	Pneumocystis pneumonia
Focal airspace disease	Bacterial pneumonia
	Fungal pneumonia
Nodules	Fungal pneumonia (aspergillosis)
	Nocardia
	Legionella
	Septic bacterial emboli
	Mycobacterial infection (with cavitation)
	Epstein-Barr virus lymphoproliferative disorder
	Relapsed malignancy
	Pulmonary embolism (pleural based)

lymphoproliferative disease, relapsed malignancy, and pulmonary embolism (pleural based). Interstitial infiltrates can be caused by either respiratory viruses during the winter season (except for parainfluenza virus, which is nonseasonal), herpes viruses, *P. jirovecii*, edema, or an idiopathic etiology. A complete differential of causes of interstitial pneumonitis (a pulmonary syndrome often associated with HSCT) is given in Chapter 110.

Pulmonary and sinus infections from inhaled molds are more likely to occur as the duration of neutropenia extends beyond 21 days.[29] For this reason, among HSCT recipients without graft failure, invasive tissue mold infections occur at a low rate (less than 3%) before engraftment because of the environmental preventive measures taken for air filtration. However, if endogenous occult aspergillosis infections are present before HSCT, the infection can rapidly escalate when the immune system is profoundly suppressed by the conditioning regimen. This phenomenon manifests as early invasive aspergillosis (before day 40). Therefore, patients with hematologic malignancy at risk for occult mold infections in the lungs, sinuses, and at times, the oral cavity should have CT scans of the lungs and sinuses before the onset of any profoundly immunosuppressive regimens, such as HSCT and select nontransplantation regimens. High-risk patients should be given mold-active antifungal prophylaxis to either prevent or suppress invasive mold infections.

Bacteria

Because bacterial infections cause significant morbidity and mortality in neutropenic patients, broad-spectrum antibacterial prophylaxis is often used during periods of profound neutropenia with the best data available for levofloxacin. Institutional protocols and resistance patterns drive selection of specific prophylactic agents. Oral prophylaxis regimens are generally discontinued and substituted with intravenous antibacterials with enhanced activity against *Pseudomonas aeruginosa* with the first onset of fever. The use of oral antibiotics among low-risk, neutropenic patients with fever is guided by the use of standardized scoring systems (e.g., MASCC index).

The most common serious infections occurring in neutropenic patients with mucositis are bacteremias due to viridans group streptococci and gram-negative bacilli. Such infections are particularly common during the first several weeks following HSCT, so broad-spectrum antimicrobial prophylaxis is generally given during this time period. The propensity of viridans streptococci to develop resistance to quinolones, and gram-negative bacilli to be resistant to quinolones and β-lactams, means that there is no perfect preventive regimen.

Central venous catheter placement through the skin can lead to blood infection from colonizing gram-positive skin organisms, despite sterile, operative placement of these catheters and antisepsis cleansing procedures.[30] Because gram-positive bacteremias, including those caused by coagulase-negative *Staphylococcus* species, usually are not immediately life threatening, antimicrobials with expected activity against typical gram-positive organisms are not routinely recommended for all febrile patients with a catheter. Indications for empirically adding an anti-Gram-positive antimicrobial (e.g., vancomycin) include evidence of infection at the catheter site and septic shock, The use of antimicrobial-coated bloodstream catheters can be effective in reducing rates of catheter-related bloodstream infections.

Once a gram-positive bacterial infection is identified, vancomycin therapy is often used empirically until the susceptibility profile

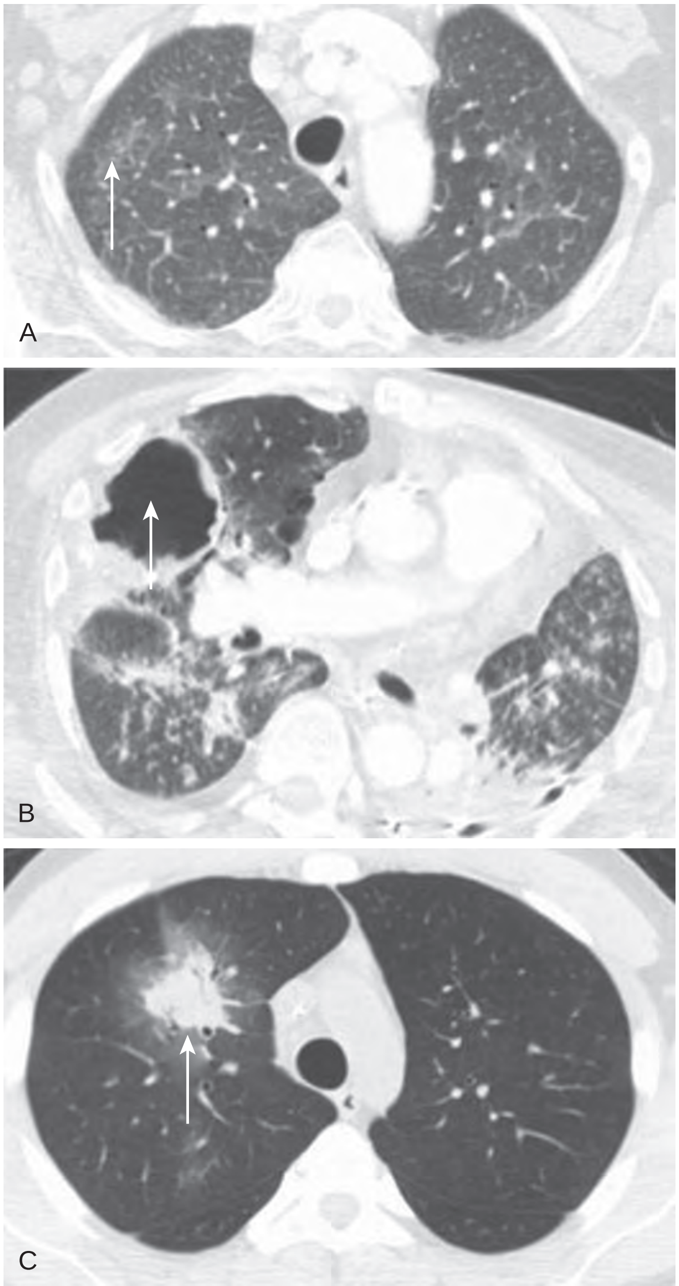


Figure 94.4 COMPUTED TOMOGRAPHY SCAN EXAMPLES OF DIFFERENT PULMONARY INFILTRATES IN IMMUNOCOMPROMISED PATIENTS. (A) Diffuse ground-glass opacities in a patient with *Pneumocystis jirovecii* pneumonia. (B) Cavitary lung lesion in a patient with *Pseudomonas aeruginosa* pneumonia. (C) Lung nodule with halo sign due to *Aspergillus*. For each panel, the *arrow* points to involved pulmonary parenchyma.

is available and targeted therapy can be provided. There are concerns about the efficacy of vancomycin in treating serious *S. aureus* infections because of relative or frank vancomycin resistance and risk of nephrotoxicity in the host. Several alternative therapies to vancomycin should be considered[31] depending on the site of infection and clinical indication, warranting infectious diseases consultation.[32] Similarly, if the patient is known to be colonized with vancomycin-resistant enterococcus and the Gram stain indicates gram-positive organisms in chains, therapy for empiric vancomycin-resistant enterococcus is appropriate until the bacterial identification and susceptibility profile is available, usually with daptomycin or linezolid.

Once a gram-negative infection is identified, empiric therapy that includes *Pseudomonas* coverage is continued until the susceptibility profile is available. In severely neutropenic hosts, serious gram-negative infections are often treated with combination therapy consisting of a β-lactam plus an aminoglycoside depending on the antimicrobial sensitivity pattern and patient comorbidities. However, data demonstrating the superiority of combination therapy over β-lactam monotherapy are lacking.[27] The emergence of extended-spectrum β-lactamase and carbapenemase producing gram-negative bacilli is a particular concern as these pathogens can rapidly evolve into pan-resistant organisms, such as multidrug-resistant *Acinetobacter baumannii* (see box on Treatment of Drug-Resistant Gram-Negative Bacilli). For most infections, the antimicrobial regimen can be tailored to a single agent and continued for 2 weeks after the last positive culture in clinically responding patients. Therapy with a carbapenem resistant to β-lactamase hydrolysis is required if an organism is isolated that is known to have an inducible β-lactamase enzyme (e.g., *Enterobacter*).

For bloodstream infections, repeat blood cultures should be drawn after 2 to 3 days of effective therapy to ensure that sterilization of the bloodstream has been achieved. Persistently positive blood cultures suggest the development of a deep-seated or endovascular site of infection, such as infective endocarditis or suppurative thrombophlebitis. When a bloodstream catheter is proven to be the source of the infection,[30] removal of the catheter may be required for most, but not all, infections. At the end of a 14-day treatment course beyond the last positive culture, the patient should be transitioned to an oral gram-negative prophylaxis regimen if still neutropenic.

Antimicrobial agents with anaerobic activity should be added for neutropenic enterocolitis, enlargement of neck soft tissues during mucositis (Ludwig angina variant), or culture-documented anaerobic infection. Infrequently, tuberculous and nontuberculous mycobacteria are responsible for infections in the bloodstream, catheters, and pulmonary tree. Infections of central catheters or catheter tunnels caused by rapidly growing atypical mycobacterial infections require a high index of suspicion, as well as specific culture media.[34] Organism identification and drug sensitivities may take several weeks. Tuberculous diseases can be empirically treated based on risk factors. For patients without risk factors for tuberculosis, recovery of acid-fast bacilli will prompt therapy for suspected nontuberculous mycobacteria, usually with clarithromycin and either a quinolone or ethambutol until specific susceptibility information is available. Tailored therapy is often continued for a minimum of 3 to 6 months.

Nocardiosis is an uncommon gram-positive bacterial infection caused by over 30 species of aerobic acid-fast actinomycetes in the genus *Nocardia*. The infection has a predisposition to causing disseminated infections involving the CNS and frequently relapses in the setting of intensive immunosuppression, particularly high-dose corticosteroid therapy. Molecular methods used to identify *Nocardia* species have resulted in the reclassification and renaming of many isolates. *Nocardia* isolates that, in the past, were identified as *N. asteroides* complex have now been reclassified to other, or new, species. Some *Nocardia* species (*N. farcinica*) exhibit unique patterns of virulence and variable susceptibility to antibiotics. Trimethoprim-sulfamethoxazole (TMP-SMX) remains the drug of choice, although breakthrough infections especially in the setting of low-dose (TMP-SMX) prophylaxis may occur but often will still respond to treatments with higher-dose TMP-SMX regimens (e.g., 15 mg/kg/day in three to four divided doses) in combination with a second agent (imipenem). Other treatment options may include linezolid, amikacin, amoxicillin-clavulanate, clarithromycin. Nocardiosis in severely immunocompromised patients requires prolonged treatment (6 months) therefore susceptibility testing plays an important role in therapy selection, particularly in patients who cannot tolerate TMP-SMX.

Viruses

Neutropenia per se is not a major risk factor for developing viral infections, but concomitant lymphopenia is common and does predispose to infections with a range of viruses.[35] Respiratory virus infections are

TREATMENT OF DRUG-RESISTANT GRAM-NEGATIVE BACILLI

The widespread use of antibiotic prophylaxis among immunocompromised patients has been associated with the development of antimicrobial drug resistance, which is especially problematic among gram-negative bacilli. Drug-resistant organisms can colonize the GI tract for prolonged periods of time and emerge to cause serious infections during periods of neutropenia or other medical stress (e.g., following admission to the intensive care unit).

- *Pseudomonas aeruginosa* is the paradigm for drug resistance among gram-negative bacilli and can develop pan-resistance to antimicrobials. Serious pseudomonal infections are generally treated with two antimicrobial agents, although convincing data supporting this approach are lacking. Meropenem or ceftolozane-tazobactam (a newer advanced generation cephalosporin combined with a beta-lactamase inhibitor) should be considered as initial therapy in centers where the prevalence of multi-drug resistant (MDR) *P. aeruginosa* exceeds 10%. Carbapenem-resistant strains may be treatable with newer combinations regimens that inhibit serine carbapenemase (e.g., ceftazidime-avibactam, meropenem-vaborbactam or imipenem-cilastatin-relebactam). Metallo-carbapenemase-producing strains of *P. aeruginosa* may be treatable with ceftazidime-avibactam plus aztreonam, or if no response, polymyxin B. Confirmation of the in vitro antibiotic susceptibility is critical for these multidrug-resistant isolates and infectious diseases consultation is strongly recommended.
- Extended-spectrum β-lactamase (ESBL) production among Enterobacterales such as *Escherichia coli* and *Klebsiella pneumoniae* renders these organisms resistant to non-carbapenem β-lactam antibiotics. Treatment is generally with a carbapenem (e.g., meropenem), although tigecycline is sometimes used.
- The presence of a *Klebsiella pneumoniae* carbapenemase (KPC) can occur among species other than *K. pneumoniae* and creates resistance to most β-lactam antibiotics including carbapenems. Antibiotic combinations that include newer carbapenemase inhibitors (e.g., ceftazidime-avibactam, meropenem-vaborbactam, imipenem-cilastatin-relebactam) are preferred over polymyxin B-based combination regimens (e.g., with tigecycline or gentamicin) as polymyxins are less effective and carry a higher risk of nephrotoxicity and neurotoxicity. Plazomicin is a newer semi-synthetic aminoglycoside that may retain activity against Enterobacterales resistant to beta-lactams; but it should not be administered as monotherapy.
- Like *P. aeruginosa, A. baumannii* can develop resistance to all known antimicrobials via a variety of mechanisms. This generally occurs among critically ill patients following prolonged stays in the intensive care unit. Treatment options for MDR and extensively-drug resistant (XDR) strains may include polymyxins, minocycline and sulbactam, a β-lactamase inhibitor that possesses some activity against drug-resistant *Acinetobacter*. Cefiderocol, a novel siderophore cephalosporin that uses iron transport systems in bacteria to accumulate in the periplasmic space of bacteria, has been used successfully to treat XDR strains of *A. baumannii*. The selection of a treatment regimen relies on in vitro susceptibility testing.
- *Stenotrophomonas maltophilia* is an intrinsically MDR gram-negative bacteria (GNB) that is the most common carbapenem-resistant bacteria isolated in patients with hematological malignancies. Infections often begin insidiously as slowly progressing breakthrough pneumonia on carbapenem therapy that leads to bacteremia. Occasionally *S. maltophila* are associated with central venous catheter infections. High-dose trimethoprim-sulfamethoxazole (e.g., 15 mg/kg/day intravenously or orally in divided doses based on trimethoprim component) is the best established therapy but can be problematic to use because of bone marrow-suppressive effects and hypersensitivity reactions. More recent guidelines have recommended use of lower doses of trimethoprim-sulfamethoxazole (8–12 mg/kg/day IV or oral in divided doses) with possible addition of a second agent (minocycline, tigecycline, levofloxacin or cefiderocol).[33] Other regimens that may have some efficacy include minocycline or tigecycline, ceftazidime-avibactam + aztreonam, or cefiderocol.
- *Enterobacter, Serratia*, and *Citrobacter* species can elaborate an AmpC β-lactamase following initiation of β-lactam therapy, which can result in a relapsing infection following an initial response. Thus clinicians treating such infections with penicillins or cephalosporins should closely monitor patients for the emergence of resistance. If resistance does emerge, AmpC-producing organisms can be treated with a carbapenem.
- Patients with MDR gram-negative infections should be cared for in such a manner as to minimize spread of these dangerous organisms to other patients. Compliance with infection control protocols is paramount.
- Knowledge of the local epidemiology and antibiogram are important for preemptive and empiric antibiotic choices, especially against gram-negative rods.
- A carefully designed and executed antibiotic stewardship program could curtail unnecessary antibiotic use, thereby decreasing antibiotic selection pressure with a resulting decrease in bacterial resistance rates.

seasonal, except for parainfluenza. A frequent pathogen with clinical significance is respiratory syncytial virus, although influenza, parainfluenza, adenovirus, enteroviruses, the herpesviruses (including human herpesvirus type 6 [HHV-6]), human metapneumovirus, and rhinovirus also produce diffuse interstitial infiltrates and pneumonitis.[20] HHV-6 and human metapneumovirus are not recovered with the usual tests ordered at the time of bronchoscopy, so a high suspicion for infection is required to order specific polymerase chain reaction (PCR) testing. Documented infection with respiratory syncytial virus (RSV) prompts contact and droplet isolation precautions. Treatment components may include ribavirin (typically orally taken instead of the inhaled version), IVIg, and in some cases, monoclonal antibody therapy. However, proof of efficacy of these treatments remains elusive, and the disease can cause significant morbidity and mortality.[36] Secondary graft failure or diffuse alveolar hemorrhage associated with parainfluenza virus infection has been sporadically reported following transplantation.

Infections with novel coronavirus (e.g., severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2]) requires immediate contact and droplet isolation precautions and ideally antiviral therapy such as remdesivir. The role of dexamethasone or other immunosuppressive agents to reduce inflammation in the lungs is less clear in this population, but it may predispose patients to a higher risk for opportunistic fungal infections including invasive aspergillosis.

Patients with lymphopenia are susceptible to a range of herpes virus family infections. Herpes simplex reactivation infections typically manifest as oral or genital ulcers, although more widespread involvement can occur, and are treated with low doses of acyclovir adjusted for renal function (e.g., 5 mg/kg IV every 8 hours) or oral valacyclovir (1 g bid). Reactivation of VZV can be severe in immunosuppressed persons and is treated with higher doses of acyclovir (e.g., 10 mg/kg IV every 8 hours or oral valacyclovir, 1 g three times a day). Use of acyclovir to prevent reactivation of HSV will also prevent VZV reactivation, although a higher dose (1000 mg daily for VZV vs. 500 mg for HSV) is needed. In the absence of prior acyclovir exposure, acyclovir-resistant HSV is very rare. However, HSV infections that appear or persist through acyclovir prophylaxis should be considered acyclovir resistant until viral sensitivity testing can be performed, and consideration should be given to initiating treatment with foscarnet or cidofovir.[37,38] Reports of acyclovir-resistant varicella are extremely rare. Foscarnet infusions affect calcium homeostasis, so monitoring of ionized calcium and phosphorus levels is required during clinical use of the drug. The major side effect of cidofovir is renal toxicity, which can be reduced to some degree with probenecid and hydration. Clinically significant CMV infection can occur during neutropenia, with DNA PCR based methods used for diagnosis and monitoring response to therapy.

HHV-6 is ubiquitous, and reactivation commonly occurs in patients with significant immunosuppression. However, clinically significant disease due to HHV-6 occurs in only a small percentage of patients who have HHV-6 detected in their blood.[23] HHV-6 infection can manifest as fever or pancytopenia. Other recognized clinical syndromes include pneumonitis and encephalitis. The infection is diagnosed by quantitative PCR, although the exact levels of the virus that indicate clinically significant disease are not known. Detection of the virus in the first 3 weeks following transplantation may be associated with early skin maculopapular rash and acute GVHD, but it can also present without overt clinical symptoms. Prospective large-scale studies are needed to determine the role of HHV-6 infection. HHV-6 has 60% DNA homology with CMV, and treatment of documented infection usually is initiated with induction doses of foscarnet or ganciclovir. Responses to antiviral therapy are not universal, and benefits of foscarnet versus ganciclovir have not been determined.

Cidofovir, when combined with aggressive supportive measures, is considered an adjunct in treatment of BK and adenovirus-associated hemorrhagic cystitis. Cidofovir is recommended for the treatment of disseminated adenovirus infections and is typically administered at 1 mg/kg intravenously 3 times a week with probenecid and saline hydration to reduce nephrotoxicity.

Fungi

Most yeast infections in immunosuppressed patients are caused by *Candida*.[39] Candidal organisms that colonize the mouth and gut proliferate when antibacterial agents suppress the coexisting bacterial flora population. Guidelines for the treatment of candidiasis have been issued.[39] Patients who are clinically stable generally can be treated with fluconazole unless there has been previous azole exposure or mold infection is also a concern. Unstable patients or those who have been receiving an azole are usually treated with an echinocandin. Prevention of candidemia among allogeneic HSCT recipients is generally achieved with fluconazole. However, fluconazole does not have activity against some non-albicans *Candida* species (e.g., *C. glabrata, C. krusei*) and all molds (e.g., *Aspergillus, Fusarium*, the Mucorales species). Among patients colonized with yeast species other than *C. albicans*, and among heavily pretreated patients who may have incubating mold infection, prophylaxis using a mold-active triazole (e.g., posaconazole) or an echinocandin is preferred.[40]

Although less common than *Candida* infections, mold infections in immunocompromised hosts are a significant source of mortality, with aspergillosis being the most common (Fig. 94.5). Because such infections are difficult to diagnose using standard microbiologic techniques, there has been significant interest in developing more effective screening tests. When used regularly, the serum *Aspergillus* galactomannan enzyme-linked immunosorbent assay (ELISA) may allow for earlier diagnosis of invasive aspergillosis in some patients. The test is compromised by difficulties with both false-positive and false-negative results and thus needs to be interpreted as part of the overall clinical picture rather than as a stand-alone diagnostic tool. PCR assays that distinguish common *Aspergillus* species and triazole resistance mechanisms are frequently used in European centers in conjunction with galactomannan, but they are not currently approved in the United States.

The development of a fungal infection in a patient receiving voriconazole should raise concern for mucormycosis, which is the second-most common mold infection in many transplant centers. Mucormycosis can rapidly disseminate through the lungs, sinuses, GI tract, and skin, which is uniformly fatal in patients with hematologic malignancies if not diagnosed early and aggressively treated.[41]

Although in vitro antifungal susceptibility testing for yeasts is not as well established as antibacterial testing, susceptibility from bloodstream or invasive isolates can be helpful in the management of infections, especially if the patient will require transition to longer-term oral therapy. Long-term treatment with newer azoles may be valuable in responding patients. Echinocandins cannot be used as monotherapy to treat *Cryptococcus*, other rare yeast (e.g., *Rhodotorula, Geotrichum, Saccharomyces or Saprochaeta* spp.) or Mucorales. Mucorales species are generally susceptible to amphotericin B and posaconazole and isavuconazole, although there are interspecies variations in susceptibility. At present, the benefits of combination therapy outside of cryptococcal meningitis are inconclusive, but one prospective randomized trial suggested that combination therapy with voriconazole and anidulafungin was associated with reduced aspergillosis-associated mortality in patients diagnosed by serum galactomannan.[42] Clinical experience may support the value of extended combination antifungal therapy, especially when a breakthrough invasive mold disease with a spectrum of possible pathogens is wider and the potential for antifungal resistance increased. Molds that often are resistant to amphotericin B but may be susceptible to voriconazole or posaconazole include *Fusarium, Scedosporium* or *Pseudallescheria*, and *Trichosporon*.

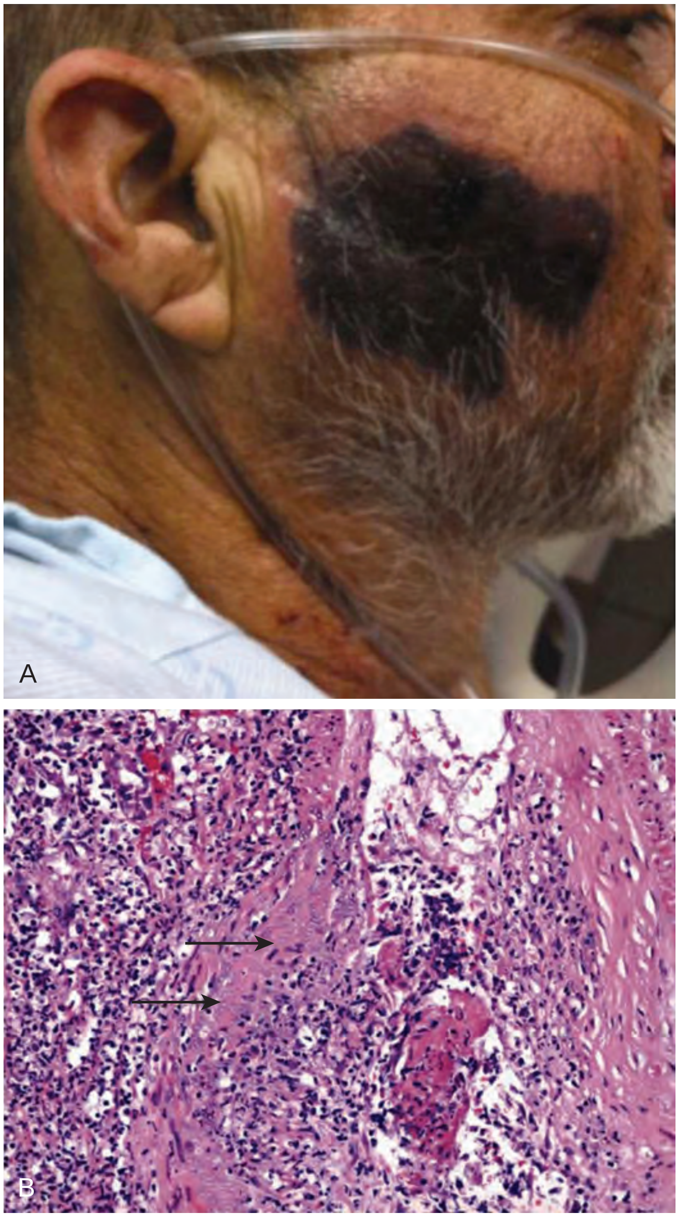


Figure 94.5 EXAMPLES OF ASPERGILLOSIS. (A) Cutaneous aspergillosis in a patient with lymphoma treated with high-dose steroids. (B) Invasive aspergillosis with organism invading into blood vessel wall *(black arrows)*.

Malignancy-Associated Fever and Drug Fever

Although fever usually indicates the presence of infection, relapsing malignancy, autoimmune-granulomatous, or collagen vascular diseases, immunologic drug reactions can also be sources of fever in the immunocompromised host.[43] Malignancy or drug-associated fevers are the most common causes of persistent noninfectious fevers in cancer patients, but these are "diagnoses of exclusion" that require extensive clinical, radiographic, and microbiologic workup for occult infection until a noninfectious cause is considered most likely. All too often, patients receive escalating empiric broad-spectrum antibiotic therapy during this workup, which should be discouraged in clinically stable patients. Several clinical clues may favor a diagnosis of malignancy-associated fever, including (1) a prior history of fever at the time of initial malignancy diagnosis, (2) clinical stability despite prolonged fever, (3) response of the fever to a trial of naproxen.[2] Patients with drug-associated fever often appear clinically well with relative bradycardia during periods of fever. Drug-induced fevers typically develop 1 to 3 weeks after the start of a drug (β-lactams, sulfas, vancomycin, and phenytoin are among the most common inciting agents) and resolve, on average, within 48 hours of drug discontinuation unless the patient also has an accompanying rash. Drug-associated fever with rash and liver function tests or complete blood count abnormalities (e.g., thrombocytopenia and eosinophilia) are often indicative of more severe reactions that should prompt immediate discontinuation or substitution of the likely offending medications.

Cutaneous eruptions, particularly maculopapular exanthema, may be caused by a number of conditions during the treatment of hematological malignancies (e.g., Sweet syndrome, leukemia cutis, engraftment syndrome, viral infection, skin graft versus host disease) that may be erroneously documented as a beta-lactam or sulfonamide drug allergy. More than one-third of immunocompromised patients admitted to US hospitals from 2010 to 2015 had an antibiotic-associated allergy label (AAL) that was be associated with higher readmission rates, increased utilization of restricted antibiotics and longer antibiotic course duration.[42] In similar Australian studies, skin-testing performed in immunocompromised patients allowed for “de-labeled” of the allergy designation in 84% of patients resulting in a 12-fold improvement in appropriate antibiotic prescriptions.[44] Although immunosuppression has the theoretical potential to cause a “false-negative” skin prick or intradermal test primarily in delayed testing, recent clinical experience has not supported this concern and the vast majority of patients will test negative prior to the onset of immunosuppressive therapy.[45] Therefore, beta-lactam and sulfonamide allergy testing, ideally before the start of chemotherapy, could improve the care of the immunocompromised host.

INFECTION MANAGEMENT IN THE HEMATOPOIETIC STEM CELL TRANSPLANT RECIPIENT: A MODEL OF SEVERE IMMUNE DEFICIENCY

HSCT patients are susceptible to a wide range of infections, but the risk for a particular infection depends on a multitude of factors, including the type of transplantation, the length of time since transplantation, and the development of GVHD. A summary of common infections occurring in HSCT recipients is shown in Fig. 94.6.

Pretransplantation Prophylactic Techniques in Hematopoietic Stem Cell Transplant Recipients

Over the four decades during which HSCT has evolved, antimicrobial prophylaxis regimens have advanced to prevent common or high-risk infections.[3] This is balanced by problems associated with the preventive regimen, including toxicity, overgrowth of resistant organisms, and sometimes high cost. Algorithms for these preventive regimens evolve with changes in epidemiology, diagnostic methods, and new treatment agents for infections. Despite an overall preventive approach, infections still occur when a severe immune defect persists, when the infecting inoculum of organisms is large, when diagnostic methods are not sensitive enough for early detection, or when infecting agents overcome the effect of the antimicrobial agents. Measures taken to prevent infection in the pre-engraftment HSCT patient include pretransplantation serostatus blood workup, environmental measures to prevent infection (including frequent handwashing), and common sense.

Assessment of Pretransplantation Serostatus

Current pretransplant serologic testing of donor and recipient includes assaying for latent viruses (herpesvirus antibodies, hepatitis panels, and human T-cell lymphotropic virus antibodies [human T-lymphotropic virus I/II and HIV 1/2]) and syphilis. Antibody tests that are checked variably among individual transplantation centers include VZV, EBV, and *Toxoplasma*.

Herpes Simplex Virus

Most centers routinely administer prophylactic medication (e.g., acyclovir or oral valacyclovir), which targets HSV for the neutropenic phase of transplantation. HSV lesions can appear as black scabs on the outside of the lips, white- to yellow-based ulcers when found on

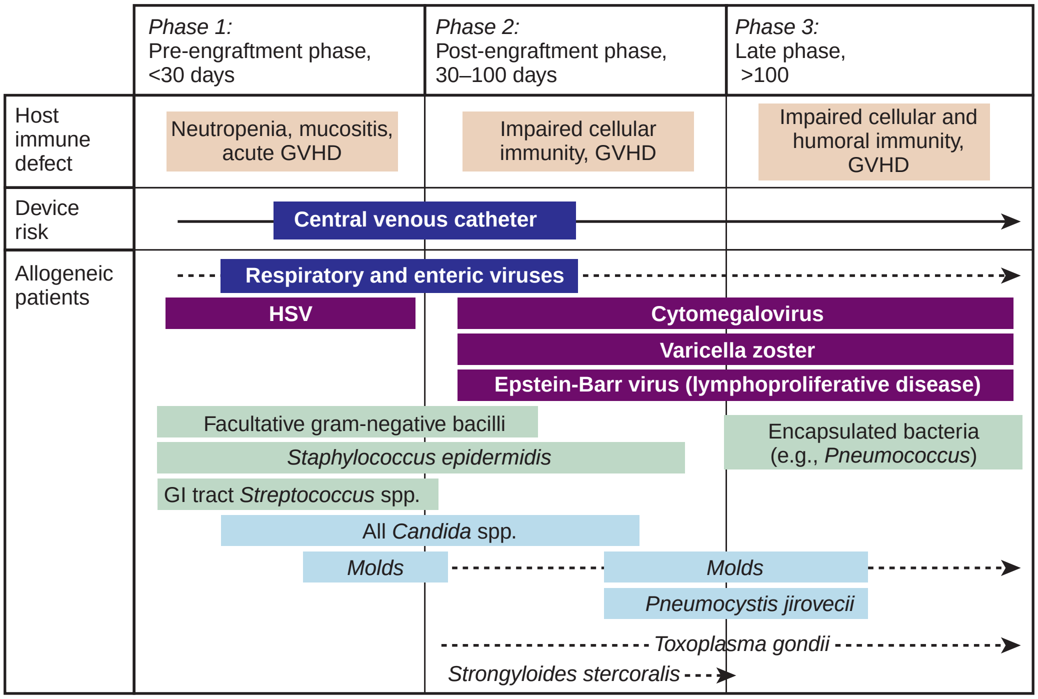

Phase 1:
Pre-engraftment phase,
<30 days
Phase 2:
Post-engraftment phase,
30–100 days
Phase 3:
Late phase,
>100
Host immune defect
Neutropenia, mucositis, acute GVHD
Impaired cellular immunity, GVHD
Impaired cellular and humoral immunity, GVHD
Device risk
Central venous catheter
Allogeneic patients
Respiratory and enteric viruses
HSV
Cytomegalovirus
Varicella zoster
Epstein-Barr virus (lymphoproliferative disease)
Facultative gram-negative bacilli
Staphylococcus epidermidis
Encapsulated bacteria (e.g., Pneumococcus)
GI tract Streptococcus spp.
All Candida spp.
Molds
Molds
Pneumocystis jirovecii
Toxoplasma gondii
Strongyloides stercoralis

Figure 94.6 TIMING OF INFECTIONS FOLLOWING HEMATOPOIETIC STEM CELL TRANSPLANT. *GI*, Gastrointestinal; *GVHD*, graft-versus-host disease; *HSV*, herpes simplex virus.

oral mucosa, or as an unusually severe exacerbation of mucositis or esophagitis. HSV prophylaxis administered until recovery from neutropenia may be helpful even in patients receiving nontransplantation chemotherapy such as acute leukemia induction therapy.

Cytomegalovirus

If the transplant recipient is CMV-seropositive before transplantation, they will have a 60% to 70% probability of CMV reactivation and should be followed with periodic (usually weekly) diagnostic PCR monitoring tests for 10 to 20 weeks. If the patient is CMV-seronegative before transplantation but the donor is seropositive, the risk of primary CMV ranges from 20% to 30% and the same monitoring algorithm used for the CMV-seropositive patient is needed.

If the donor and recipient are CMV-seronegative before transplantation, infection through blood products is possible; therefore, exclusive use of CMV-seronegative blood products or other means of preventing CMV seroconversion, such as leukocyte depletion by filtration, are recommended. CMV monitoring should be followed weekly, but the duration of this testing can be shortened by 50%, or by 6 to 10 weeks, because late infection in seronegative recipients is uncommon.

Anti-CMV prophylaxis is often used in high-risk patients. Differences in the mechanism of action of the antiviral drugs should be considered in the interpretation of CMV PCR results. Ganciclovir and valganciclovir, and foscarnet inhibit replication of CMV DNA by interfering with the function of CMV DNA polymerase. Letermovir inhibits terminase complex subunit pUL56 at a stage of maturation and packaging of viral particles distal to viral DNA synthesis. Consequently, free CMV DNA fragments may remain detectable in the blood, and breakthrough CMV DNAemia may not necessarily represent breakthrough infection. However, preemptive therapy with ganciclovir or valganciclovir should be considered for patients on letermovir prophylaxis if CMV DNAeamia continues to arise or is above 150 IU/mL, especially in higher-risk transplants.[46]

Varicella, Human Herpes Virus Type 6, and Epstein-Barr Virus

A history of chickenpox is an adequate surrogate for performing the VZV antibody test. As an alternative to ordering varicella serology on every patient, the test could be ordered in patients with no history of varicella infection or vaccination.

Acyclovir used to prevent reactivation of HSV during neutropenia will also prevent occurrence of clinical VZV infection in most transplantation recipients. Later after transplantation, when acyclovir prophylaxis may have been discontinued, VZV reactivations usually are recognized by their characteristic dermatomal distribution, and treated using acyclovir or valacyclovir.

Serology for HHV-6 is not tested before transplantation because more than 95% of adults are seropositive for the virus. The dose of acyclovir and derivatives used to prevent HSV/VZV is unlikely to have effects of HHV-6 and the clinical significance of detection of HHV-6 in the blood following HSCT is unknown. Meningoencephalitis is the most well-established syndrome caused by HHV-6.

The vast majority of adult patients undergoing transplantation and their donors are EBV seropositive. EBV reactivation leading to post-transplant lymphoproliferative disease is in the differential diagnosis of any new mass, such as enlarged nodes or lung nodules after transplantation (see Chapter 55). Now that sensitive quantitative EBV viral load tests and effective treatments such as rituximab are available, investigators are monitoring high-risk recipients with quantitative viral load studies.[47]

Hepatitis B and C

Hepatitis B (core antibody, surface antibody, and surface antigen) and hepatitis C serologies are tested in donor and recipient before transplantation. Hepatic dysfunction from either hepatitis B or C after HSCT can lead to life-threatening liver complications, including hepatic sinusoidal obstructive syndrome (veno-occlusive disease) and acute hepatic necrosis. Short-term complications are usually due to hepatitis B, whereas the long-term complication of cirrhosis is due to hepatitis C. The risk for hepatitis in the recipient can be reduced by antiviral therapy for recipients and donors who have detectable viral loads, and by transfer of immunity from donor to recipient. A hepatitis-infected individual can be used as a donor if no alternative donor is available or if the intended recipient already is seropositive.

The risk for transmission is small when the hepatitis B–seropositive donor has an undetectable viral load; however, careful follow-up with the recipients is recommended. A surface antigen-positive hepatitis B donor with a high viral load should be treated with lamivudine or another agent to reduce the circulating viral load before transplantation. High-circulating hepatitis C viral load in the seropositive donor for a seronegative recipient is an indication for antiviral therapy of the donor before HSCT.

If the potential recipient has serologic evidence of infection with hepatitis B or C before transplantation, viral load levels should be checked before and monitored after HSCT. High hepatitis B viral load ($>10^5$ copies/mL) is the most important risk factor for reactivation in patients positive for surface antigen undergoing HSCT. High-circulating hepatitis B viral load in the intended recipient is an indication for lamivudine or entecavir. There is no evident correlation between hepatitis C genotype and type or severity of liver disease after transplantation.

Human Immunodeficiency Virus

A positive donor HIV test for a recipient candidate not known to be seropositive is a contraindication for donation. If the screening test for HIV is positive, a Western blot study should be completed to confirm the result because of the potential for false-positive testing.

Syphilis

If the indirect screening test for syphilis returns positive and is confirmed by a direct test, high-dose penicillin treatment should be given for 10 days after transplantation.

Toxoplasma

Historically, 15% of patients who undergo transplantation in the United States are seropositive for *Toxoplasma*, but this percentage may be higher for European centers. The risk for reactivation among seropositive patients is ~2%, for an overall incidence of less than 1% of HSCT recipients. PCR based blood monitoring of seropositive patients prior to starting prophylactic may help with early identification of infection. It is suspected that low-dose sulfa-based regimens, such as those used to prevent *Pneumocystis* pneumonia, may be effective in preventing *Toxoplasma*.

Review of Commonsense Measures to Prevent Infection

Commonsense measures that should be discussed before transplantation or aggressive nontransplantation chemotherapy include attention to diet, travel, crowds, and pets. Additionally, a history of family or social exposure to tuberculosis should be used to guide whether or not an interferon-gamma release assay is applied before therapy.

Diet should be reviewed for herbal supplements or restricted foods. Patients may not recognize that most supplements will have to be discontinued after HSCT. Ground meat products need to be thoroughly cooked so that bacteria, distributed onto meat in the grinding process, are killed. Any fruits or vegetables that cannot be peeled should be washed. Salad bars are associated with occasional transmission of infections. Food products or supplements that inherently contain infectious organisms should be avoided, including undercooked eggs. Blue cheeses have molds spiked into the cheese wheel as they are curing and should be avoided. Soft cheeses carry the potential risk for *Listeria* infection. Yogurt contains *Lactobacillus* that, rather than causing gut problems, has been found to cause infection in other sites, including the lungs after aspiration events.

There are no special travel restrictions, but strategies to minimize transmission of infectious diseases have been summarized. Some social situations, such as sitting in a crowded movie theater or classroom, increase the risk for acquiring a viral illness. Turning away from individuals who are coughing or sneezing may be helpful in preventing the transmission of infections. Patients need instruction to remember to complete the cycle of infection prevention by washing their hands as soon as possible after being close to such an individual. Given outbreaks of noroviruses (Norwalk-like viruses) on cruise ships and other types of outbreaks (e.g., *Staphylococcus*) commonly associated with the close living quarters during this type of travel, cruise ships should be avoided.

Healthy dogs and cats are considered acceptable pets. However, the immunosuppressed patient should not be responsible for scooping cat litter because of potential *Toxoplasma* cyst exposure. Similarly, the patient should not play in sandboxes because these areas are concentrated sites that feral outdoor cats may use as litter boxes. Because reptiles of many sorts have been reported to be infected with *Salmonella*, patients should not touch these animals or the insides or outsides of their cages or tanks. The heated water of tropical fish tanks carry *Mycobacterium marinum*. *Cryptococcus* and *Chlamydophila* (formerly *Chlamydia*) *psittaci* can be transmitted from large pet birds.

Additionally, patients should be counseled about the importance of avoiding activities that may involve exposure to high fungal spore inoculums, such as gardening or composting, vacuuming, smoking tobacco or cannabis, and occupational exposures (farming, demolition/construction, etc.).

Environmental Measures to Prevent Infection

Persons entering the patient's room to perform an examination or touch the patient (including visitors as well as health care workers) should wash their hands outside the room. Ideally, the institution will have handwashing sinks in the hallways outside patient rooms for this purpose. During respiratory virus season, the infection control department often adds extra signs to doorways and other places in the wards to remind visitors of the importance of handwashing. Staff and visitors with symptoms of infection (e.g., cough, fever) should not be permitted to have direct patient contact.

Some infectious situations require special isolation procedures. Contact isolation (gloves and gowns) is used for patients with adenovirus, methicillin-resistant *S. aureus*, or *Clostridium difficile* infection. Droplet precautions are added to contact precautions for respiratory virus or varicella infection. Carriers of vancomycin-resistant *Enterococcus* are placed in contact isolation until they meet defined criteria for discontinuation of isolation.

Laminar airflow is a cumbersome and expensive isolation technique that has been largely outmoded with advances in airflow and isolation technology as well as current antimicrobial therapy. Historically, it has been commonly used for patients with aplastic anemia or those receiving T cell-depleted transplants.

High-efficiency particulate air (HEPA) filtration has replaced laminar airflow as the means for preventing infection through ventilation. With at least 12 air exchanges per hour, HEPA filters can remove particles greater than 0.2 μm in diameter, such as mold spores. Patients often ask whether they should purchase portable HEPA filters for the home or apartment they will occupy after hospitalization. In the broadest sense, this is an extra measure that can be used on an individual basis. If portable HEPA filters are used, they should be placed in each room that the patient will occupy during the day and night, and each unit should be sized for the individual room. A beneficial effect of HEPA filtration in the outpatient setting has not been demonstrated.

Infection in the Hematopoietic Stem Cell Transplant Recipient Preengraftment

The major risk factors for infection in the preengraftment period include drug-induced mucositis, profound neutropenia, and the presence of indwelling catheters. Thus, the major infections observed in this period are due to bacteria that colonize the skin and GI tract (e.g., viridans group streptococci and gram-negative bacilli), *Candida*, and respiratory viruses, especially in the winter months (see Fig. 94.6). Reactivation of latent or partially treated fungal infections can also occur, with *Aspergillus* being well recognized. The propensity of patients to develop such infections has led to widespread use of prophylaxis with an antiviral, antibacterial, and antifungal agent during this time period.

Infection in the Hematopoietic Stem Cell Transplant Recipient After Engraftment

Once engraftment has occurred, the major risk factors for infection include immunosuppression used to treat GVHD and mechanical disruption of mucosal barriers, particularly indwelling venous catheters. Viral infections that reactivate after engraftment often are related to defective T cell-mediated immunity and include CMV, adenovirus, and hepatitis viruses. Outpatient HSCT recipients inhale mold spores and *Pneumocystis* cysts from the environment, so common exogenously acquired infections include aspergillosis, mucormycosis, and *P. jirovecii* (previously *carinii*). Bacterial infections associated with defective cell-mediated immunity such as pneumococcosis, nocardiosis, and atypical mycobacterial disease can also occur in association with significant immunosuppression (see Fig. 94.6).

Cytomegalovirus

CMV causes significant morbidity and mortality in transplant patients both directly and indirectly because of its immunosuppressive effects that predispose patients to concomitant or sequential infection with bacterial and fungal pathogens. Use of prophylactic ganciclovir at engraftment usually is avoided because it leads to myelosuppression and possibly a higher incidence of late CMV disease. Alternatively, letermovir lacks myelotoxic and the effects are shown to reduce CMV-related mortality compared to a placebo.[48] Instead, the patient is treated with ganciclovir when weekly PCR DNAemia monitoring test meets a positive threshold. Initial viral load levels do not predict disease. CMV infections are treated with an induction ganciclovir regimen followed by approximately 6 weeks of a maintenance regimen (half the induction dose). The duration of induction (1 to 3 weeks) varies by institution, but in general 1 week is used for low-grade infection, 2 weeks for high-grade infection, and 3 weeks for end-organ disease. A rising viral load, when checked weekly during the first month of preemptive therapy, signals the need for continued induction dosing or repeat induction dosing.

When the end-organ manifestation of CMV is pneumonitis (recovery of CMV from a deep lung specimen along with an interstitial infiltrate on chest radiograph), IVIg is added on an every-other-day basis for the duration of induction. When CMV manifests in an end organ other than the lungs, use of IVIg is not as clearly useful. Some centers add IVIg when the patient's total IgG level falls below 400 mg/dL. Dosing schedules vary from IVIg given once weekly for 3 weeks to every other day for the duration of induction, similar to therapy for pneumonitis. Ganciclovir resistance is uncommon, but when it occurs, foscarnet or cidofovir may be used.

Varicella-Zoster Virus

VZV reactivations from latency (zoster) usually are recognized by their characteristic dermatomal distribution. No temporal pattern is seen, viremia can occur concurrently, and multiple episodes are possible but uncommon. For patients who have been treated for a zoster episode after HSCT, some centers provide acyclovir prophylaxis until 1 year after HSCT. Less common VZV clinical manifestations that can result in severe infection and may require molecular testing or viral culture for diagnosis include hemorrhagic pneumonia, hepatitis, central nervous system disease, thrombocytopenia, and retinal necrosis.

HSCT recipients with a negative or unknown VZV disease history and a significant exposure to active varicella are susceptible to primary VZV infection. For these patients, varicella-zoster immune globulin, if available, can be provided within 96 hours of exposure. Patients with positive serology can become clinically reinfected after exposure and should be provided with acyclovir prophylaxis; however, such patients do not require varicella-zoster immune globulin. Recombinant VZV vaccination is being increasingly identified as effective in preventing VZV reactivation even in transplant recipients

Epstein-Barr Virus

EBV causes a spectrum of scenarios after stem cell transplantation, ranging from asymptomatic but detectable viremia, hemophagocytic syndrome, or posttransplant lymphoproliferative disorder (see Chapter 55). The incidence of EBV-related complications is higher in mismatched donors, patients with T-cell–depleted transplants, and patients receiving intensive immunosuppression (e.g., antithymocyte globulin). Posttransplant lymphoproliferative disorder after allogeneic stem cell transplantation most often is of donor origin. Quantitative EBV viral load diagnostic testing is not standardized, so the algorithms for monitoring and initiation of the treatment vary. Recognition of greater than 1000 viral copies/mL of blood requires investigation, repeated testing, and possibly treatment, especially in high-risk patients. Direct antiviral agents have limited impact on reducing detectable EBV viral loads. General treatment approaches involve reduction of immunosuppression and rituximab or donor lymphocyte infusion (see Chapter 55).

Invasive Mold Infections

Invasive fungal infections are among the most feared complications of HSCT both because of their high mortality rates and the difficulty in establishing a diagnosis. Among the HSCT recipients that were studied at autopsy, yeast and mold infections were common, seen in more than 25% of deaths. The probability of survival is higher in recent years, likely due to newer and more effective agents, as well as nonmyeloablative conditioning. Posaconazole has been shown to be effective in preventing invasive fungal infections during GVHD.[49] Mold spores may initiate a localized infection in the lungs or sinuses that, after intensive immunosuppression for GVHD, may disseminate to the skin, abdominal organs, or central nervous system. Treatment of mold central nervous system (CNS) infections is problematic due to low CNS penetration of many anti-fungals, with voriconazole achieving the most reliable CNS levels. Infection with Mucorales organisms tends to have later onset than infection with *Aspergillus* (after day 90).

Pneumocystis

The most common presenting symptoms of *Pneumocystis* infection are dyspnea, cough, and fever. Definitive diagnosis requires demonstration of the organism in silver-stained specimens (bronchoalveolar lavage or lung biopsy) although PCR testing of bronchoalveolar lavage fluid is used despite concerns of being overly sensitive for low-positive results. Additionally, serum beta-D-glucan has been reported to have excellent sensitivity and specificity for ruling out *Pneumocystis* infection.[50] However, the interpretation should still require clinical context of the clinical condition of the patient. Most patients present between day 40 and day 80 after HSCT, but cases as early as day 12 and as late as 42 months after HSCT have been reported. Once lymphocyte function is reconstituted, *Pneumocystis* infections are rare. Prophylaxis options include trimethoprim-sulfamethoxazole, aerosol or intravenous pentamidine, dapsone, and atovaquone. Among patients treated with dapsone after transplantation, increased red blood cell and platelet transfusion requirements are noted. Prophylaxis is generally discontinued 1 to 2 years after HSCT, or later if immunosuppression is ongoing.

Parasitic Infections

Similar to the early transplant period, toxoplasmosis remains a concern after engraftment, particularly if there is ongoing immunosuppression.

Infection Issues in the Late Posttransplantation Period

Encapsulated Organism Prophylaxis

Penicillin prophylaxis has decreased the incidences of infection-related morbidity and mortality from polysaccharide-encapsulated bacteria (*S. pneumoniae, H. influenzae* type b, *Neisseria meningitidis*). Penicillin-resistant pneumococcal infection has been reported, prompting consideration of alternate prophylaxis such as a change from penicillins to quinolones. Prophylaxis is generally discontinued 2 years after HSCT, or later if GVHD and immunosuppression are ongoing after the 2-year time-lapse. Once the patient is ready for vaccinations, they are given the conjugate pneumococcal, meningococcal, and *H. influenzae* type b.

Vaccination

Recipients of HSCT frequently lose antibody responses to viral and bacterial pathogens previously targeted by childhood vaccination. Although practice varies among transplantation centers, killed-virus vaccines are often given at 1 year after HSCT and live-virus vaccines approximately 2 years after HSCT for patients without GVHD.[6] For adults, Tdap (formulation of reduced-antigen, combined diphtheria-tetanus-acellular pertussis vaccine) has replaced Td (diphtheria-tetanus booster vaccine) as a means for decreasing the adult reservoir of pertussis. For protection against hepatitis, the combined vaccine providing protection against both hepatitis A and B can be used. The efficacy of vaccination is influenced by the time elapsed since transplantation, the nature of the hematopoietic graft, the presence of GVHD, and the use of serial immunization. Guidelines have been published (see Chapter 110, Table 110.4).

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Brudno JN, Kochenderfer JN. Toxicities of chimeric antigen receptor T cells: recognition and management. *Blood*. 2016;127(26):3321–3330.

Byrd JC, Furman RR, Coutre SE, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N Engl J Med*. 2013;369(1):32–42.

Chamilos G, Lionakis MS, Kontoyiannis DP. Call for action: invasive fungal infections associated with ibrutinib and other small molecule kinase inhibitors targeting immune signaling pathways. *Clin Infect Dis*. 2018;66(1):140–148.

Drgona L, Gudiol C, Lanini S, et al. ESCMID Study group for infections in compromised hosts (ESGICH) consensus document on the safety of targeted and biological therapies: an infectious diseases perspective (Agents targeting lymphoid or myeloid cells surface antigens [II]: CD22, CD30, CD33, CD38, CD40, SLAMF-7 and CCR4). *Clin Microbiol Infect*. 2018;24(Suppl 2):S83–S94.

Einsele H, Ljungman P, Boeckh M. How I treat CMV reactivation after allogeneic hematopoietic stem cell transplantation. *Blood*. 2020;135(19):1619–1629.

Engelhard D, Akova M, Boeckh MJ, et al. Bacterial infection prevention after hematopoietic cell transplantation. *Bone Marrow Transplant*. 2009;44(8):467–470.

Falagas ME, Vardakas KZ, Samonis G. Decreasing the incidence and impact of infections in neutropenic patients: evidence from meta-analyses of randomized controlled trials. *Curr Med Res Opin*. 2008;24(1):215–235.

Freifeld AG, Bow EJ, Sepkowitz KA, et al. Clinical practice guideline for the use of antimicrobial agents in neutropenic patients with cancer: 2010 update by the Infectious Diseases Society of America. *Clin Infect Dis*. 2011;52(4):e56–e93.

Fried S, Avigdor A, Bielorai B, et al. Early and late hematologic toxicity following CD19 CAR-T cells. *Bone Marrow Transplant*. 2019;54(10): 1643–1650.

Gulley ML, Tang W. Using Epstein-Barr viral load assays to diagnose, monitor, and prevent posttransplant lymphoproliferative disorder. *Clin Microbiol Rev*. 2010;23(2):350–366.

Helmink BA, Khan MAW, Hermann A, et al. The microbiome, cancer, and cancer therapy. *Nat Med*. 2019;25(3):377–388.

Huang K-HG, Cluzet V, Hamilton K, Fadugba O. The impact of reported beta-lactam allergy in hospitalized patients with hematologic malignancies requiring antibiotics. *Clin Infect Dis*. 2018;67(1):27–33.

Kontoyiannis DP, Lewis RE. How I treat mucormycosis. *Blood*. 2011;118(5):1216–1224.

Kumar D. Emerging viruses in transplantation. *Curr Opin Infect Dis*. 2010;23(4): 374–378.

Luu S, Spelman D, Woolley IJ. Post-splenectomy sepsis: preventative strategies, challenges, and solutions. *Infect Drug Resist*. 2019;12:2839–2851.

Mikulska M, Lanini S, Gudiol C, et al. ESCMID Study group for infections in compromised hosts (ESGICH) consensus document on the safety of targeted and biological therapies: an infectious diseases perspective (Agents targeting lymphoid cells surface antigens [I]: CD19, CD20 and CD52). *Clin Microbiol Infect*. 2018;24(Suppl 2):S71–S82.

NCCN. *NCCN Guidelines Prevention and Treatment of Cancer-Related Infections*, Ver. 2.2020. June 2020. https://www.nccn.org/professionals/physician_gls/pdf/infections.pdf.

Neelapu SS, Tummala S, Kebriaei P, et al. Chimeric antigen receptor T-cell therapy—assessment and management of toxicities. *Nat Rev Clin Oncol*. 2018;15(1):47.

O'Grady NP, Alexander M, Burns LA, et al. Summary of recommendations: guidelines for the prevention of intravascular catheter-related infections. *Clin Infect Dis*. 2011;52(9):1087–1099.

Pappas PG, Kauffman CA, Andes DR, et al. Clinical practice guideline for the management of candidiasis: 2016 update by the Infectious Diseases Society of America. *Clin Infect Dis*. 2016;62(4):e1–e50.

Park JH, Romero FA, Taur Y, et al. Cytokine release syndrome grade as a predictive marker for infections in patients with relapsed or refractory B-cell acute lymphoblastic leukemia treated with chimeric antigen receptor T cells. *Clin Infect Dis*. 2018;67(4):533–540.

Paul M, Dickstein Y, Schlesinger A, et al. Beta-lactam versus beta-lactam-aminoglycoside combination therapy in cancer patients with neutropenia. *Cochrane Database Syst Rev*. 2013(6). https://doi.org/10.1002/14651858.CD003038.pub2.

Postow MA, Sidlow R, Hellmann MD. Immune-related adverse events associated with immune checkpoint blockade. *N Engl J Med*. 2018;378(2):158–168.

Pratt G, Goodyear O, Moss P. Immunodeficiency and immunotherapy in multiple myeloma. *Br J Haematol*. 2007;138(5):563–579.

Reinwald M, Silva JT, Mueller NJ, et al. ESCMID Study Group for Infections in Compromised Hosts (ESGICH) consensus document on the safety of targeted and biological therapies: an infectious diseases perspective (Intracellular signaling pathways: tyrosine kinase and mTOR inhibitors). *Clin Microbiol Infect*. 2018;24:S53–S70.

Shah JN, Chemaly RF. Management of RSV infections in adult recipients of hematopoietic stem cell transplantation. *Blood*. 2011;117(10):2755–2763.

Sullivan K, Dykewicz C. Preventing opportunistic infections after hematopoietic stem cell transplantation: the Centers for Disease Control and Prevention, Infectious Diseases Society of America, and American Society for Blood and Marrow Transplantation practice guidelines and beyond. *Hematology Am Soc Hematol Educ Program*. 2001;1:392–421.

Turner NA, Sharma-Kuinkel BK, Maskarinec SA, et al. Methicillin-resistant *Staphylococcus aureus*: an overview of basic and clinical research. *Nat Rev Microbiol*. 2019;17(4):203–218.

Yu HH, Yang YH, Chiang BL. Chronic granulomatous disease: a comprehensive review. *Clin Rev Allergy Immunol*. 2020;61:101–113.

REFERENCES

1. Mackall CL. T-cell immunodeficiency following cytotoxic antineoplastic therapy: a review. *Stem Cell*. 2000;18:1–10.
2. Lukenbill J, Kalaycio M. Fludarabine: a review of the clear benefits and potential harms. *Leuk Res*. 2013;37(9):986–994.
3. Mikulska M, Lanini S, Gudiol C, et al. ESCMID study group for infections in compromised hosts (ESGICH) Consensus Document on the safety of targeted and biological therapies: an infectious diseases perspective (Agents targeting lymphoid cells surface antigens [I]: CD19, CD20 and CD52). *Clin Microbiol Infect*. 2018;24(Suppl 2):S71–S82.
4. Drgona L, Gudiol C, Lanini S, et al. ESCMID Study group for infections in compromised hosts (ESGICH) consensus document on the safety of targeted and biological therapies: an infectious diseases perspective (Agents targeting lymphoid or myeloid cells surface antigens [II]: CD22, CD30, CD33, CD38, CD40, SLAMF-7 and CCR4). *Clin Microbiol Infect*. 2018;24(Suppl 2):S83–S94.
5. Coutinho AE, Chapman KE. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. *Mol Cell Endocrinol*. 2011;335(1):2–13.
6. Sullivan K, Dykewicz C. Preventing opportunistic infections after hematopoietic stem cell transplantation: the Centers for Disease Control and Prevention, Infectious Diseases Society of America, and American Society for Blood and Marrow Transplantation practice guidelines and beyond. *Hematol Am Soc Hematol Educ Program*. 2001;1:392–421.
7. Reinwald M, Silva JT, Mueller NJ, et al. ESCMID Study Group for Infections in Compromised Hosts (ESGICH) consensus document on the safety of targeted and biological therapies: an infectious diseases perspective (intracellular signaling pathways: tyrosine kinase and mTOR inhibitors). *Clin Microbiol Infect*. 2018;24:S53–S70.
8. Byrd JC, Furman RR, Coutre SE, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N Engl J Med*. 2013;369(1):32–42.
9. Ghez D, Calleja A, Protin C, et al. Early-onset invasive aspergillosis and other fungal infections in patients treated with ibrutinib. *Blood*. 2018;131(17):1955–1959.
10. Chamilos G, Lionakis MS, Kontoyiannis DP. Call for Action: Invasive fungal infections associated with ibrutinib and other small molecule kinase inhibitors targeting immune signaling pathways. *Clin Infect Dis*. 2018;66(1):140–148.
11. Pratt G, Goodyear O, Moss P. Immunodeficiency and immunotherapy in multiple myeloma. *Br J Haematol*. 2007;138(5):563–579.
12. Yu H-H, Yang Y-H, Chiang B-L. Chronic granulomatous disease: a comprehensive review. *Clin Rev Allergy Immunol*. 2020;61:101–113.
13. Ramakrishnan M, Moïsi JC, Klugman KP, et al. Increased risk of invasive bacterial infections in African people with sickle-cell disease: a systematic review and meta-analysis. *Lancet Infect Dis*. 2010;10(5):329–337.
14. Falagas ME, Vardakas KZ, Samonis G. Decreasing the incidence and impact of infections in neutropenic patients: evidence from meta-analyses of randomized controlled trials. *Curr Med Res Opin*. 2008;24(1):215–235.
15. Engelhard D, Akova M, Boeckh MJ, et al. Bacterial infection prevention after hematopoietic cell transplantation. *Bone Marrow Transpl*. 2009;44(8):467–470.
16. Clark OAC, Lyman GH, Castro AA, et al. Colony-stimulating factors for chemotherapy-induced febrile neutropenia: a meta-analysis of randomized controlled trials. *J Clin Oncol*. 2005;23(18):4198–4214.
17. Luu S, Spelman D, Woolley IJ. Post-splenectomy sepsis: preventative strategies, challenges, and solutions. *Infect Drug Resist*. 2019;12:2839–2851.
18. Postow MA, Sidlow R, Hellmann MD. Immune-related adverse events associated with immune checkpoint blockade. *N Engl J Med*. 2018;378(2):158–168.
19. Neelapu SS, Tummala S, Kebriaei P, et al. Chimeric antigen receptor T-cell therapy—assessment and management of toxicities. *Nat Rev Clin Oncol*. 2018;15(1):47.
20. Brudno JN, Kochenderfer JN. Toxicities of chimeric antigen receptor T cells: recognition and management. *Blood*. 2016;127(26):3321–3330.
21. Fried S, Avigdor A, Bielorai B, et al. Early and late hematologic toxicity following CD19 CAR-T cells. *Bone Marrow Transpl*. 2019;54(10):1643–1650.
22. Park JH, Romero FA, Taur Y, et al. Cytokine release syndrome grade as a predictive marker for infections in patients with relapsed or refractory B-cell acute lymphoblastic leukemia treated with chimeric antigen receptor T cells. *Clin Infect Dis*. 2018;67(4):533–540.
23. Helmink BA, Khan MAW, Hermann A, et al. The microbiome, cancer, and cancer therapy. *Nat Med*. 2019;25(3):377–388.
24. NCCN. NCCN Guidelines Prevention and Treatment of Cancer-Related Infections, Ver. 2.2020. June 2020. https://www.nccn.org/professionals/physician_gls/pdf/infections.pdf.
25. Freifeld AG, Bow EJ, Sepkowitz KA, et al. Clinical practice guideline for the use of antimicrobial agents in neutropenic patients with cancer: 2010 update by the Infectious Diseases Society of America. *Clin Infect Dis*. 2011;52(4):e56–e93.
26. Baskaran ND, Gan GG, Adeeba K. Applying the multinational association for supportive care in cancer risk scoring in predicting outcome of febrile neutropenia patients in a cohort of patients. *Ann Hematol*. 2008;87(7):563–569.
27. Paul M, Dickstein Y, Schlesinger A, et al. Beta-lactam versus beta-lactam-aminoglycoside combination therapy in cancer patients with neutropenia. *Cochrane Database Syst Rev*. 2013(6)). https://doi.org/10.1002/14651858.CD003038.pub2.
28. Greene RE, Schlamm HT, Oestmann J-W, et al. Imaging findings in acute invasive pulmonary aspergillosis: clinical significance of the halo sign. *Clin Infect Dis*. 2007;44(3):373–379.
29. Gerson SL, Talbot GH, Hurwitz S, et al. Prolonged granulocytopenia: the major risk factor for invasive pulmonary aspergillosis in patients with acute leukemia. *Ann Intern Med*. 1984;100(3):345–351.
30. O'Grady NP, Alexander M, Burns LA, et al. Summary of recommendations: guidelines for the prevention of intravascular catheter-related infections. *Clin Infect Dis*. 2011;52(9):1087–1099.
31. Turner NA, Sharma-Kuinkel BK, Maskarinec SA, et al. Methicillin-resistant *Staphylococcus aureus*: an overview of basic and clinical research. *Nat Rev Microbiol*. 2019;17(4):203–218.
32. Tissot F, Calandra T, Prod'hom G, et al. Mandatory infectious diseases consultation for MRSA bacteremia is associated with reduced mortality. *J Infect*. 2014;69(3):226–234.
33. Tamma PD, Aitken SL, Bonomo RA, et al. Infectious Diseases Society of America guidance on the treatment of AmpC β-lactamase-producing Enterobacterales, carbapenem-resistant *Acinetobacter baumannii*, and *Stenotrophomonas maltophilia* infections. *Clin Infect Dis*. 2021. https://doi.org/10.1093/cid/ciab1013.
34. Redelman-Sidi G, Sepkowitz KA. Rapidly growing mycobacteria infection in patients with cancer. *Clin Infect Dis*. 2010;51(4):422–434.
35. Kumar D. Emerging viruses in transplantation. *Curr Opin Infect Dis*. 2010;23(4):374–378.
36. Shah JN, Chemaly RF. Management of RSV infections in adult recipients of hematopoietic stem cell transplantation. *Blood*. 2011;117(10):2755–2763.
37. Piret J, Boivin G. Resistance of herpes simplex viruses to nucleoside analogues: mechanisms, prevalence, and management. *Antimicrob Agents Chemother*. 2011;55(2):459–472.
38. Kontoyiannis DP, Marr KA, Park BJ, et al. Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001–2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. *Clin Infect Dis*. 2010;50(8):1091–1100.
39. Pappas PG, Kauffman CA, Andes DR, et al. Clinical practice guideline for the management of candidiasis: 2016 update by the Infectious Diseases Society of America. *Clin Infect Dis*. 2016;62(4):e1–e50.
40. Ullmann AJ, Lipton JH, Vesole DH, et al. Posaconazole or fluconazole for prophylaxis in severe graft-versus-host disease. *N Engl J Med*. 2007;356(4):335–347.
41. Kontoyiannis DP, Lewis RE. How I treat mucormycosis. *Blood*. 2011;118(5):1216–1224.
42. Huang K-HG, Cluzet V, Hamilton K, Fadugba O. The impact of reported beta-lactam allergy in hospitalized patients with hematologic malignancies requiring antibiotics. *Clin Infect Dis*. 2018;67(1):27–33.
43. Zell JA, Chang JC. Neoplastic fever: a neglected paraneoplastic syndrome. *Support Care Cancer*. 2005;13(11):870–877.
44. Trubiano JA, Thursky K, Stewardson AJ, et al. The impact of an integrated antibiotic allergy testing program on antimicrobial stewardship: A multicentre evaluation. *J Allergy Clin Immunol*. 2017;139(2):AB377.
45. Trubiano JA, Slavin MA, Thursky KA, et al. Beta-lactam and sulfonamide allergy testing should be a standard of care in immunocompromised hosts. *J Allergy Clin Immunol Pract*. 2019;7(7):2151–2153.
46. Einsele H, Ljungman P, Boeckh M. How I treat CMV reactivation after allogeneic hematopoietic stem cell transplantation. *Blood*. 2020;135(19):1619–1629.
47. Gulley ML, Tang W. Using Epstein-Barr viral load assays to diagnose, monitor, and prevent posttransplant lymphoproliferative disorder. *Clin Microbiol Rev*. 2010;23(2):350–366.
48. Marty FM, Ljungman P, Chemaly RF, et al. Letermovir prophylaxis for cytomegalovirus in hematopoietic-cell transplantation. *N Engl J Med*. 2017;377:2433–2444.
49. Ullmann AJ, Lipton JH, Vesole DH, et al. Posaconazole or fluconazole for prophylaxis in severe graft-versus-host disease. *N Engl J Med*. 2007;356:335–347.
50. Karageorgopoulos DE, Qu J-M, Korbila IP, et al. Accuracy of β-D-glucan for the diagnosis of *Pneumocystis jirovecii pneumonia*: a meta-analysis. *Clin Microbiol Infect*. 2013;19(1):39–49.

CHAPTER 95

PRINCIPLES OF RADIATION THERAPY FOR HEMATOLOGIC DISEASE

Idalid Franco, Daphne Haas-Kogan, and Andrea K. Ng

The approach and utilization of radiation therapy (RT) for the treatment of hematologic malignancies have seen many changes throughout the years. Advances in imaging and technology have allowed for more precise delineation and targeting of disease. This, along with a reduction in radiation doses and treatment volume, have resulted in increased sparing of normal tissues and dramatically reduced short- and long-term toxicities previously associated with RT. With the majority of hematologic malignancies being highly responsive to radiation, RT plays a critical role in disease management. Use of RT ranges from definitive primary treatment to adjuvant therapy in combination with systemic therapies, as well as salvage or palliative therapy in the relapsed/refractory setting. This chapter will detail the historical changes in the application of RT for hematologic malignancies and focus on the current role of RT based on modern staging, systemic therapies, response assessment, and RT delivery. We will provide current recommendations based on clinical evidence and published guidelines. The goal is to equip the hematologist and oncologist with an evidence-based approach to understanding the overall role and clinical applications, including new concepts in modern RT for a wide variety of hematologic malignancies.

HISTORY AND BACKGROUND

Technological advances that are associated with the diagnosis, treatment response evaluation, and delivery of RT in the last several decades have allowed for more precise and personalized treatment applications for patients. Even within the last 30 years, advances in the components of RT delivery have allowed for higher precision in dose composition. The use of more advanced imaging acquisition for staging and planning has allowed for better patient selection and smaller treatment volumes, resulting in high rates of long-term tumor control with reduced normal tissue exposure and the associated short- and long-term complications. The use of combined systemic treatments with RT, including chemotherapy and more recent targeted and immunotherapy, have resulted in improved locoregional control and overall survival (OS). This chapter aims to provide a framework through which the image of radiation will shift from a one-size-fits-all, to the individualized targeted therapy that is currently used in most radiation oncology practices.

Hodgkin Lymphoma and the Changing Landscape

In 1950, Vera Peters was the first physician to definitively show that RT could be used to cure early-stage Hodgkin lymphoma (HL).[1] Until that time, HL was thought to be incurable. Dr. Peters not only showed that high doses of fractionated RT resulted in a 5- and 10-year survival rate of 88% and 79%, but also proposed the importance of a staging system in prognosis. Historically, RT alone was used to treat early-stage HL, through the use of 2D imaging set up to bony landmarks. Extended-field radiation therapy (EFRT) included total nodal irradiation (TNI), mantle and paraaortic/splenic RT, mantle field RT, and inverted-Y field RT. These large-field treatments were associated with significant long-term toxicities, including risks of second malignancy and cardiac diseases. In the 1990s, in the context of combined modality therapy, EFRT was replaced by involved-field radiation therapy (IFRT), which included only involved nodal regions. However, similar to EFRT, IFRT still relied on 2D planning using bony landmarks to set field borders, with largely anterior-posterior beam arrangements, resulting in unnecessary exposure of a large amount of normal tissue. Involved-site radiation therapy (ISRT), the current standard treatment field, uses 3D-based simulation to incorporate the prechemotherapy volume into the clinical target volume, while excluding uninvolved areas and previously displaced organs based on tumor response. Involved-node radiation therapy (INRT), is a special case of ISRT where staging imaging is acquired with the patient in the RT position, using the same breathing instructions and immobilization devices. This typically results in further reduction in the irradiated volume by allowing more accurate target delineation. Pictorial representations of the changes in HL treatment fields over the years are useful in visualizing the substantial changes that RT has encountered over the years (Fig. 95.1). Detailed guidelines on ISRT/INRT approaches for HL have been published and recently updated by the International Lymphoma Radiation Oncology Group (ILROG), available at https://www.ilrog.org/main-st. A Mini-Atlas of case examples, focusing on the technical aspect of RT, has also recently been published by the group.[2]

MODERN RADIATION PLANNING AND TREATMENT TECHNIQUES

There have been significant technological advances since the days of 2D planning and predominantly anterior-posterior beam arrangements to large treatment fields. With the advent of 3D planning, advanced radiation planning and treatment delivery including intensity-modulated radiation therapy (IMRT), proton beam therapy, image-guided radiation therapy (IGRT), and motion management techniques, highly conformal treatment with significant sparing of normal tissue can be achieved while maintaining disease control. IMRT improves conformality of radiation dose to target structures, while limiting doses to surrounding normal tissues by using multiple beams of varying intensities and shapes that change throughout the treatment to precisely irradiate a target. For mediastinal cases, the use of deep-inspiration breath-hold technique displaces the lungs and heart away from the radiation fields and has been shown to result in significantly reduced lung and heart doses. IGRT via daily cone-beam CT imaging results in further reduced doses to critical structures by allowing a narrower expansion of the clinical target volume to planning target volume. More recently, the use of a Magnetic Resonance Linear Accelerator (MR Linac), with its better image quality, its use of non-ionizing radiation, and its ability to adapt the daily radiation as tumor responds, has the potential to further reduce RT volume and normal tissue exposure. For proton-beam therapy, its dose profile with a sharper fall off can decrease unwanted dose to critical cardiac structures. Guidelines on the use of proton therapy for mediastinal lymphoma have been published by ILROG. Ultimately, it is critical that these various advanced radiation treatment approaches be adapted to provide the greatest benefit by accounting for patient characteristic, needs, and treatment goals.

Numerous dosimetric and modeling studies have estimated and quantified the reduction in doses to normal organs, including heart,

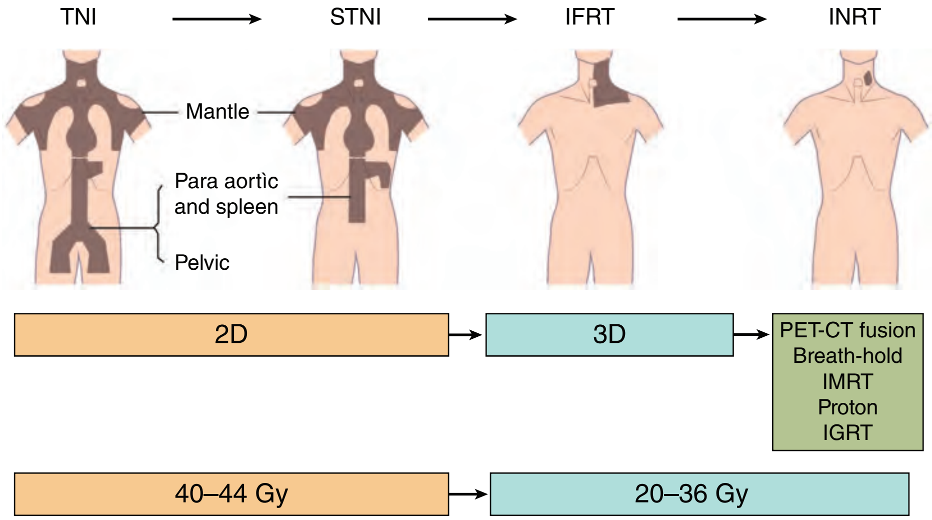
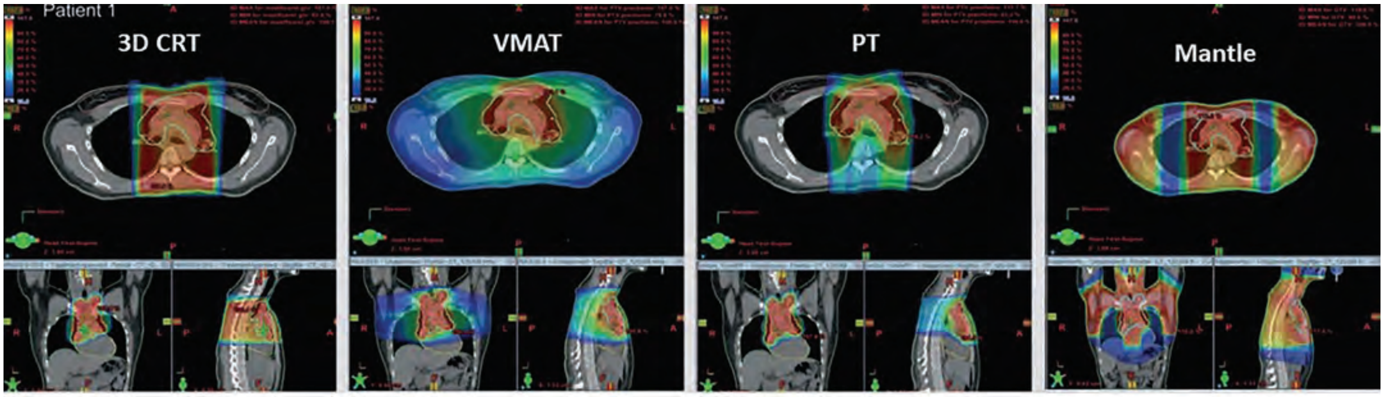


Figure 95.1 HISTORICAL EVOLUTION OF TREATMENT FIELDS IN HODGKIN LYMPHOMA. *IFRT*, Involved field radiation therapy; *IGRT*, image guided radiation therapy; *IMRT*, intensity modulated radiation therapy; *INRT*, involved node radiation therapy; *PET-CT fusion*, positron emission tomography with correlative anatomic information from computed tomography; *STNI*, subtotal nodal irradiation; *TNI*, total nodal irradiation.

lung, and breasts, using modern fields, doses, and techniques. Fig. 95.2 is an example of a modeling study showing significantly reduced estimated breast cancer, lung cancer, and cardiovascular mortality risks when comparing the historical mantle field with more limited radiation fields and modern radiation technology.[3] There is a growing effort to track volumetric dose estimates to critical normal organs, including cardiac substructures such as left anterior descending artery, heart valves, and cardiac chambers. This will facilitate work on correlating dose-volume exposure of normal organs with late effects, which in turn will guide future treatment and follow-up.

Optimal Use of Imaging

Guidance on optimal use of modern imaging techniques in the staging, planning, treatment delivery, and follow-up of hematologic malignancies was recently published by ILROG. A baseline pre-treatment, [18 F]-fluorodeoxyglucose (FDG) positron emission tomography-computed tomography (PET/CT) is best practice for not only staging but also for RT planning and assessment of response to systemic treatment. Magnetic resonance imaging (MRI) is recommended in tumors of the central nervous system (CNS), head and neck (orbits, paranasal sinuses, and skull base), and skeletal sites to determine and distinguish disease from inflammatory response. Thin CT slices (2 to 3 mm) with IV contrast are also recommended for treatment planning CT to assist in nodal and soft-tissue target delineation. IGRT for verification of accurate delivery of planned dose allows for smaller volume expansion and steeper dose gradients and is recommended for sites where critical organs at risk are in close proximity to the planning target volume, including mediastinum and head and neck regions. Adaptive IGRT with real time shifts are used when there are large changes anticipated in the anatomy or when small changes will result in significant impact on dose distribution.



Risk Estimates	3D-CRT	VMAT	Proton	Mantle
Breast Ca	3.7%	8.0%	1.4%	23%
Lung Ca	4.4%	6.0%	3.3%	10.5%
CV Mortality	1%	1.1%	0.9%	2.9%

Figure 95.2 MODELING OF DATA ON LATE EFFECT RISKS TO ORGANS AT RISK WITH THE USE OF MODERN RADIOTHERAPY TECHNIQUES. *CRT*, Conformal radiation therapy; *PT*, proton therapy; *VMAT*, volumetric modulated arc therapy. *(Adapted from Maraldo MV, Brodin NP, Aznar MC, et al. Estimated risk of cardiovascular disease and secondary cancers with modern highly conformal radiotherapy for early-stage mediastinal Hodgkin lymphoma. Ann Oncol. 2013;24[8]:2113–2118.)*

RADIATION THERAPY BY DIAGNOSIS

Hodgkin Lymphoma

HL may present as limited or advanced-stage disease.[4] Limited stage is typically further categorized into favorable and unfavorable prognostic groups. Table 95.1 summarizes the definition of favorable-prognosis disease, according to the German Hodgkin Study Group (GHSG) and European Organization for Research and Treatment of Cancer (EORTC) (see the box with Case 1).

Table 95.2 summarizes the results of the GHSG HD10 and HD11 trials, conducted prior to the incorporation of FDG-PET in guiding treatment decisions.[5] These two trials evaluated treatment de-escalation for early-stage, low-risk, and high-risk patients, respectively. The GHSG HD10 compared 4 versus 2 cycles of adriamycin, bleomycin, vinblastine, and dacarbazine (ABVD), followed by 30 versus 20 Gy IFRT. At a median follow-up of 98 months, no differences occurred in freedom from treatment failure (FFTF) and OS among the four arms, leading to a recommendation of 20 Gy after 2 cycles of ABVD in patients with limited stage, low-risk HL.[6] The GHSG HD11 trial compared 4 cycles of ABVD versus 4 cycles of baseline bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone (baseline BEACOPP), followed by 20 Gy versus 30 Gy of IFRT. At a median follow-up of 106 months, there were no differences in outcome between 20 Gy versus 30 Gy after BEACOPP, but inferiority of 20 Gy after 4 cycles of ABVD could not be excluded. A radiation dose of 30 Gy therefore remains the standard in limited stage, unfavorable HL, after ABVD chemotherapy.[7]

More recent trials have explored using PET-guided response to chemotherapy to further de-escalate or escalate therapy. Table 95.3 summarizes results of these trials, focusing on the elimination of RT, based on PET-guided response or early PET-response to chemotherapy. The RAPID trial compared combined modality therapy with chemotherapy alone in stage I-IIA non-bulky patients with complete PET-response (Deauville 1–2) after 3 cycles of ABVD.[8] At a median follow-up time of 60 months, by intent-to-treat analysis, the 3-year, progression-free survival (PFS) rate was 94.6% in the RT group and 90.8% in the non-RT group ($P = .16$). Although the difference was not statistically significant, the pre-specified, non-inferiority margin of 7% points was not met and hence, non-inferiority of chemotherapy alone could not be concluded. Moreover, by per-protocol analysis, after excluding patients who were randomized to the RT arm but did not receive the treatment, there was a significant difference between the arms in favor of RT (3-year PFS 97.1% vs. 90.8%, $P = .02$). The EORTC/LYSA/FIL H10 trial examined adapting therapy in early-stage patients, according to early PET response to ABVD. For patients with favorable prognosis disease who achieved early complete PET response to ABVD, the 5-year PFS of the combined modality therapy arm was significantly higher than that of the chemotherapy alone arm (99%, vs. 87.1%). For patients with unfavorable prognosis disease, the corresponding 5-year PFS of the two arms were 92.1% and 89.6%, respectively. The difference was not significant but the pre-specified, non-inferiority margin was not met; hence, non-inferiority of chemotherapy alone could not be concluded.[9] For patients with interim PET-positive disease, compared to the standard arm of ABVD followed by INRT, the 5-year PFS of intensifying therapy to dose-escalated BEACOPP or BEACOPP-14 followed by INRT was significantly higher (90.6% vs. 77.8%). However, escalating treatment to BEACOPP was associated with significantly higher rates of grade 3 and 4 hematologic toxicities. The GHSG HD16 trial randomized limited-stage, low-risk HL to 2 cycles of ABVD followed by 20 Gy IFRT, versus the PET-guided treatment arm of 2 cycles of ABVD, with omission of RT if PET negativity was attained after chemotherapy. At a median follow-up time of 45 months, among patients with PET-negative scan at the end of chemotherapy, 5-year PFS was 93.4% for the RT arm and 86.1% for the non-RT arm, leading to the conclusion, similar to the RAPID and EORTC/LYSA/FIL H10 trials, that RT cannot be safely eliminated despite complete PET response to ABVD chemotherapy.[10] The GHSG HD17 trial randomized limited-stage, high-risk HL patients to 2 cycles of escalated BEACOPP and 2 cycles of ABVD, followed by 30 Gy IFRT, versus the PET-guided treatment arm of the same chemotherapy, with omission of RT if PET negative after the chemotherapy and 30 Gy INRT if PET positive. At a median follow-up time of 46 months, the 5-year PFS of the standard arm of combined modality therapy was 97.3% versus 95.1% in the PET-directed therapy arm, demonstrating non-inferiority for the PET-stratified treatment approach. This is the first randomized trial in early-stage HL that showed that RT can be eliminated if complete response to chemotherapy, though the chemotherapy that was used included escalated BEACOPP, which is not a commonly used regimen, especially among early-stage patients in the United States. Of note, the GHSG HD17 is also the first study comparing INRT with IFRT in a prospective randomized fashion.[11]

TABLE 95.1 **Limited Stage Hodgkin Lymphoma, Definition of "Favorable-Prognosis" Disease According to the German Hodgkin Study Group and European Organization for Research and Treatment of Cancer**

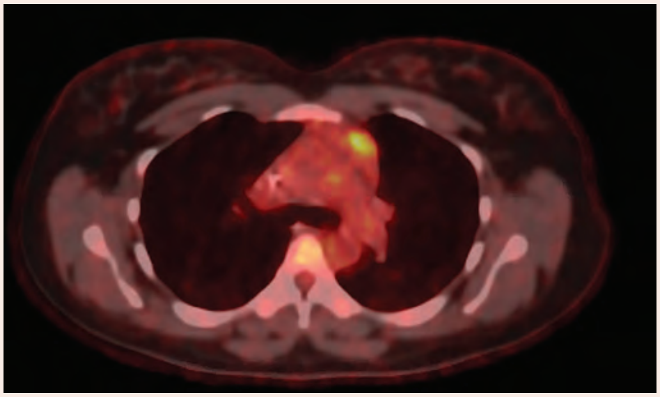
	GHSG	EORTC
Age	—	<50
Histology	—	—
Erythrocyte Sedimentation Rate (ESR)/B Symptoms	<30 or <50 with no B Symptoms	<30 or <50 with no B Symptoms
Large Mediastinal Adenopathy (LMA)	No	No
Extranodal Lesions	None	—
Number of Sites	<3	<4

EORTC, European Organization for Research and Treatment of Cancer; *GHSG*, German Hodgkin Study Group.

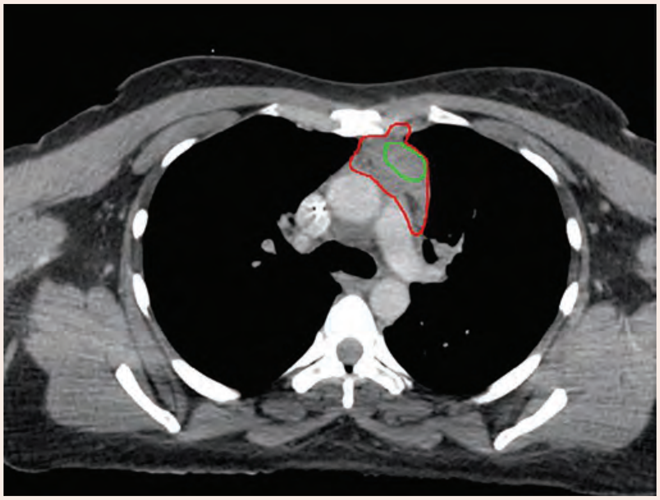
The role of RT for patients with advanced-stage HL is less well defined. The GHSG HD12 trial is a four-arm study for patients with bulky-stage IIB and stages III-IV disease, comparing two different BEACOPP variants with or without RT. Among the patients with initial bulky disease (>5 cm) or residual disease after chemotherapy (>1.5 cm), patients randomized to the non-RT arm had a significantly inferior 10-year PFS in comparison to patients on the RT arm (83.5% vs. 88.6%). In the subgroup of patients with residual disease (>1.5 cm), patients on the non-RT arm had both a significantly inferior PFS (83.4% vs. 89.7%) and OS (88.4% vs. 94.4%). Using PET-guided response to chemotherapy for RT decision in advanced HL was addressed in several trials. In the GHSG HD15 trial, after dose-escalated BEACOPP and its variants, RT was limited to patients with residual disease of 2.5 cm and with residual PET avidity. The 4-year PFS in patients with complete response or those with PET-negative disease/partial response, after chemotherapy and non-RT, were 92.6% and 92.1%, respectively, showing that in patients with complete metabolic response to BEACOPP, excellent results can be achieved without RT. In the GITIL/FIL HD 0607 trial, patients with a negative PET scan after 2 cycles of ABVD continued on to a total of 6 cycles of ABVD; those with a large nodal mass at diagnosis (≥ 5 cm), who continued to be PET-negative, were randomly assigned to RT or no further treatment. At a median follow-up of 3.6 years, there was no difference in 3-year PFS between the two arms (97% vs. 93%; $P = .29$). When the analysis was limited to the subgroup of patients with large nodal mass of greater than 10 cm, the 3-year PFS rates were 94% versus 86% ($P = .34$), although the comparison in this subgroup may be limited by inadequate power. Routine addition of RT, after chemotherapy, in patients with advanced-stage HL is not supported by available data but should be considered in patients who lack complete metabolic response to modern chemotherapy.

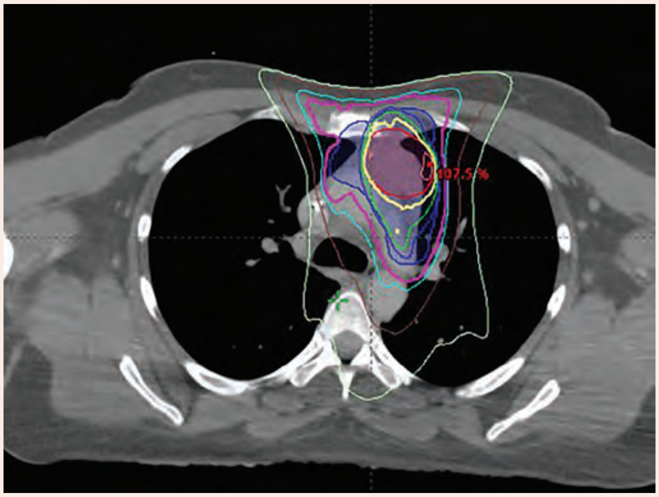
CASE 1

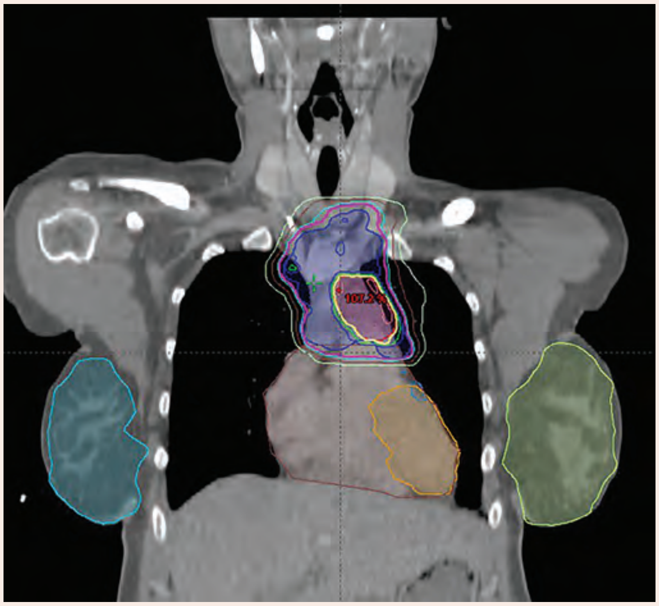
A 28-year-old female with classic Hodgkin lymphoma with Deauville 4 response to chemotherapy:



The patient received involved site radiation therapy (ISRT) to 30 Gy (in 1.5 Gy per fraction, CTV_30 contoured in red) with simultaneous integrated boost to residual fluorodeoxyglucose avid site to 40 Gy (in 2 Gy per fraction, CTV_40 contoured in green). Patient was planned in arms down position on 10-degree incline board to displace breast tissue inferiorly. Intensity modulated radiation therapy (IMRT) with butterfly technique was used (limiting arcs to narrow angles anteriorly and posteriorly to reduce low-dose bath to breasts and lungs):







Continued

Nodular Lymphocyte Predominant Hodgkin Lymphoma

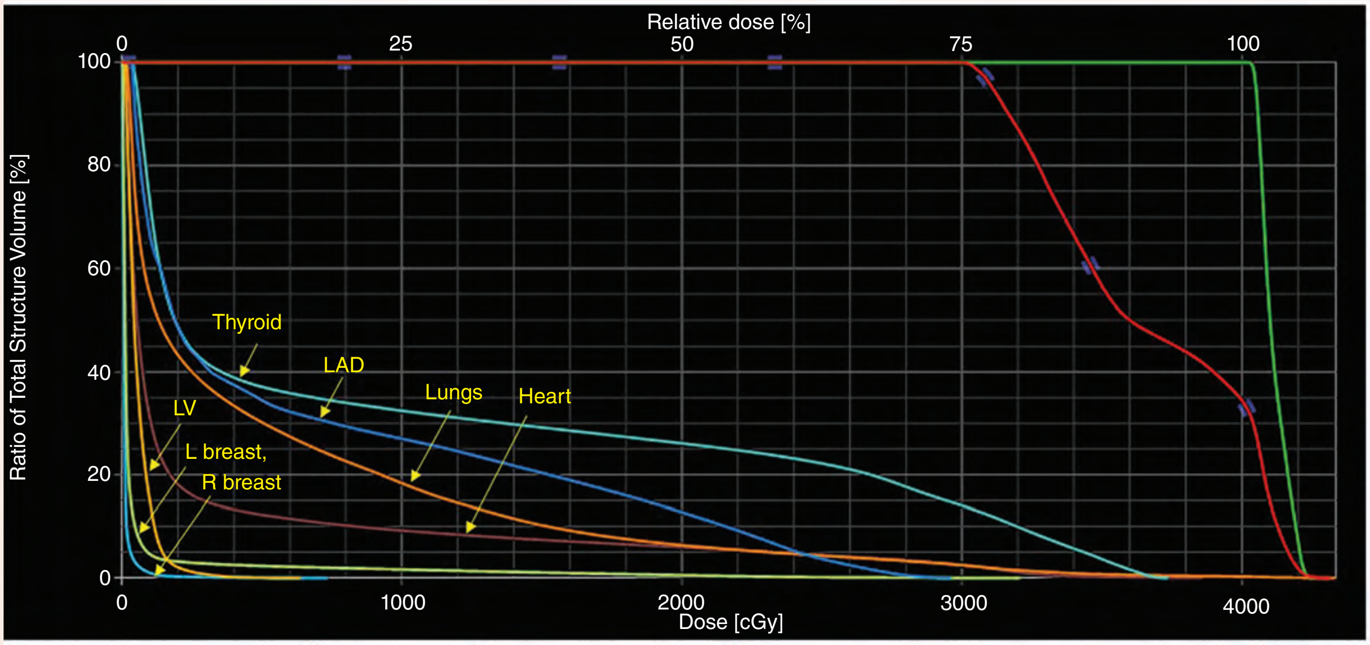
Nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) is rare, accounting for only 5% of all HL cases, half of which present at an early stage. In these early, localized, stage I, or contiguous stage II cases, ISRT may be preferred single-modality treatment. Although the rarity of the disease limits prospective randomized data to guide treatment, large-database studies have shown excellent outcome with the use of RT. A recent large multi-center study, conducted by ILROG, showed a 5-year PFS rate of 91.1% for stage I–II NLPHL treated with RT. Treatment should focus on treating the entirety of the disease, while limiting unnecessary exposure of normal tissues. ILROG guidelines are available and recommend a generous clinical target volume to encompass microscopic subclinical disease and a gross tumor volume (GTV) that is readily visualized. Typical dosing is 30 to 36 Gy in 15 to 18 fractions. Additionally, biopsy confirmation of relapsed disease is strongly advised to rule out transformed lymphoma.

Relapsed/Refractory Hodgkin Lymphoma

ILROG guidelines outlined key indications for incorporating peritransplantation RT, including localized sites of relapsed/refractory disease, bulky disease, incomplete response to salvage therapy, and involvement of sites where local control is critical. Specific emergent situations, secondary to disease progression, include spinal cord compression and superior vena cava syndrome. Depending on the response to salvage therapy, doses of 30 to 40 Gy are recommended. A frequently used strategy is to treat responsive disease sites to 30 Gy and boosting partially responding sites to 36 to 40 Gy. The target volume should be determined on a case-by-case basis, depending on disease location and distribution, but at a minimum should include the site(s) of relapsed/refractory disease, and if feasible, should include adjacent and previously involved sites. Many systemic agents used in HL, either in the upfront or salvage setting, are associated with increased pulmonary toxicity, including bleomycin, carmustine, brentuximab, and immune checkpoint inhibitors. Close attention to

CASE 1—CONT'D

Dose-volume histogram showing doses to organs-at-risk (OAR), and summary table comparing mean doses to OAR with this current ISRT, compared to the typical mean doses to the same OAR in a historical mantle field:



OAR	Mean Dose (Gy)	*Mean Dose with Mantle Field (Gy)*
Lungs	5.1	15–20 Gy
Heart	3.0	20–25 Gy
LAD	6.5	
LV	0.6	
L breast	0.5	15–20 Gy
R breast	0.1	15–20 Gy

lung metrics is especially important in salvage RT for mediastinal HL, and as much as possible, volume of lungs receiving 5 Gy (lung V_5) should be kept below 55%.

Non-Hodgkin Lymphoma

Non-Hodgkin lymphoma is a heterogeneous disease. The two most common histologic subtypes are diffuse, large B-cell lymphoma (DLBCL) and follicular lymphoma (FL). The role of RT in DLBCL is evolving, especially in the era of PET-guided therapy. In FL, RT alone may play a curative role in patients with limited-stage disease. Marginal zone lymphoma or mucosa-associated lymphoid tumor (MALT) is highly responsive to RT; similar to FL, RT alone can be curative in patients with limited-stage disease.

Diffuse Large B Cell Lymphoma

Current standard of care for DLBCL is systemic treatment with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) and has typically consisted of 6 cycles of chemotherapy. However, in patients with limited-stage disease, results of the FLYER trial, randomizing patients aged 18 to 60 years with limited stage, low-risk (normal serum lactate dehydrogenase concentration, ECOG performance status 0 to 1, and without bulky disease [<7.5 cm]) DLBCL to 6 versus 4 cycles of R-CHOP, showed that 4 cycles of chemotherapy plus two doses of rituximab are noninferior to 6 cycles. Indications for adjuvant RT are controversial and have evolved over time. Table 95.4 summarizes studies that explored the role of RT after R-CHOP chemotherapy, prior to the routine use of PET, to assess response to chemotherapy. Findings from these studies suggest a role for adjuvant RT in patients with initial skeletal involvement or bulky disease (≥7.5 cm). However, it is notable that two of the studies were retrospective subgroup analyses of prospective trials and thus prone to selection biases. Importantly, there were no PET assessment at the end of chemotherapy.

More recently, a number of studies have become available, addressing the role of RT in the setting of complete PET response to chemotherapy. The LYSA/GOELAMS 02–03 trial randomized patients with non-bulky (<7 cm), limited-stage DLBCL to 4 to 6 cycles of R-CHOP, delivered once every 2 weeks with or without RT, to a dose of 40 Gy if a complete PET response was achieved after 4 cycles and at the end of chemotherapy. At a median follow-up of 64 months, 5-year EFS (89% vs. 92%) and OS (92% vs. 96%) was not statistically significantly different between the R-CHOP arm versus R-CHOP + RT.[12] S1001, a phase II study of non-bulky (<10 cm), limited-stage DLBCL, treated patients with 3 cycles of R-CHOP, followed by interim PET-CT, with PET-negative patients proceeding with 1 additional cycle of R-CHOP and PET-positive patients receiving IFRT, followed by ibritumomab

TABLE 95.2 Trials on Radiation Dose Reduction in Early-Stage Hodgkin Lymphoma

	GHSG HD10[5,6]	GHSG HD11[5,7]
Number of Patients	1370	1395
Patient Characteristics	Low risk: 1–2 sites, non-bulky, no E-lesions, ESR <50 or <30 with B symptoms	High risk: ≥ 3 sites, bulky med, E-lesions or ESR >50 or >30 with B symptoms
Treatment Arms	ABVD x 4+30 Gy IFRT	BEACOPP x 4+30 Gy IFRT
	ABVD x 4+20 Gy IFRT	BEACOPP x 4+20 Gy IFRT
	ABVD x 2+30 Gy IFRT	ABVD x 4+30 Gy IFRT
	ABVD x 2+20 Gy IFRT	ABVD x 4+20 Gy IFRT
Median Follow-Up	90 months	82 months
8-year Freedom from Tumor Failure (FFTF)	87%	87%
	90%	87%
	86%	85%
	86%	81%
8-year Overall Survival (OS)	95%	95%
	95%	95%
	94%	94%
	95%	94%
10-year Progression Free Survival (PFS)	ABVD × 4 + 30 Gy IFRT [87.4%]	ABVD × 4 + 30 Gy IFRT [83.9%]
	ABVD × 2 + 20 Gy IFRT [87.2%]	ABVD × 4 + 20 Gy IFRT [75.6%]

ABVD, Adriamycin, bleomycin, vinblastine, and dacarbazine; *BEACOPP*, bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone; *GHSG*, German Hodgkin Study Group; *IFRT*, involved field radiation therapy; *PFS*, progression-free survival.

TABLE 95.3 Trials on Omission of Radiation Therapy in Patients With Complete Positron Emission Tomography Response to Chemotherapy in Early-Stage Hodgkin Lymphoma

	RAPID	EORTC/GELA/FIL		GHSG HD16[10]	GHSG HD17[11]
		H10F	H10U		
Number of Patients	420	551	594	628	1100
Patient Characteristics	Stage I-IIA, non-bulky	Favorable per EORTC	Unfavorable per EORTC	Favorable per GHSG	Unfavorable per GHSG
PET Negative Definition	Deauville 1–2	≤ Mediastinal blood pool level		Deauville 1–2	Deauville 1–2
Chemo Alone	ABVD × 3	ABVD × 4	ABVD × 6	ABVD × 2	eBEACOPP × 2, ABVD × 2
5 y PFSPer - Protocol	88.6%	87%	89.6%	86.1%	95.1%
	90.8%				
Combined Modality Therapy	ABVD × 3 + RT	ABVD × 3 + RT	ABVD × 4 + RT	ABVD × 2 + RT	eBEACOPP × 2, ABVD × 2 + RT
5 y PFSPer-Protocol	92.3%	99%	92.1%	93.4%	97.3%
	97.1%				

ABVD, Adriamycin, bleomycin, vinblastine and dacarbazine; *eBEACOP*, dose-escalated: bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone; *EORTC*, European Organization for Research and Treatment of Cancer; *GHSG*, German Hodgkin Study Group; *PET*, positron emission tomography; *PFS*, progression-free survival; *RT*, radiation therapy.

tiuxetan radioimmunotherapy. With a median follow-up of 4.5 years, the 5-year PFS was 87% and OS was 89%, with only 11% of patients having a positive PET and requiring RT. The authors concluded that R-CHOP × 4 alone, in patients with complete PET response, should be standard of care for limited-stage, non-bulky DLBCL in the majority of patients.[13] A retrospective series from British Columbia Cancer Agency reported on the outcome PET-guided RT in 723 patients with advanced stage DLBCL, treated with at least 6 cycles of R-CHOP, with observation of those with a negative PET-CT at the end of chemotherapy. In patients with PET positive scans, consolidative RT was offered, when feasible. At a median follow-up of 4.3 years, the 3-year time to progression was 83% among PET-negative patients who were treated with chemotherapy alone. The excellent results suggest that RT may not be needed if a complete metabolic response is achieved. In patients with PET-positive disease at the end of chemotherapy, treated with or without RT, the 3-year time to progression rates were 69% and 33%, respectively, reflecting the important role of RT in those who lack complete metabolic response. Among 285 patients with bulky disease (≥10 cm) and among 142 patients with skeletal involvement, the 3-year time to progression rates were similar to those of non-bulky patients, despite omission of RT. A longer follow-up may be needed to confirm the efficacy of chemotherapy alone in these subgroup of patients who

TABLE 95.4 **Studies in Diffuse Large B-Cell Lymphoma that Explored the Role of Radiation Therapy After R-CHOP (Rituximab, Cyclophosphamide, Doxorubicin, Vincristine and Prednisone) Chemotherapy Prior to the Routine Use of Positron Emission Tomography Imaging to Assess Response to Chemotherapy**

Study	RT Decision	Results
Retrospective subgroup analysis of patients with skeletal involvement from MInT and RICOVER-60 trials	RT to skeletal sites per treating physician discretion	*3-year EFS* RT: 75% No RT: 36%, $P < .001$
Retrospective subgroup analysis of patients with bulky disease (≥7.5 cm) from RICOVER-60 trial	Before amendment: RT for bulky disease After amendment: No RT	*EFS* RT: 80% No RT: 54%, $P = .001$ *PFS* RT: 88% No RT: 62%, $P < .001$ *OS* RT: 90% No RT: 65%, $P = .001$
UNFOLDER	For patients with bulky disease ≥7.5 cm, RT assessed in a randomized fashion after R-CHOP-14/21	Second planned interim analysis: *3-year EFS* RT: 84% No RT: 68%, $P = .001$ No difference in 3-year PFS, OS

EFS, Event-free survival; *OS*, overall survival; *PFS*, progression-free survival; *RT*, radiation therapy.

may be at higher risk for local recurrence. Based on these recent results, the role of RT in patients with DLBCL is largely limited to patients who lack complete PET response to R-CHOP or R-CHOP-like chemotherapy, although cases with initial bulky disease or dominant skeletal sites of involvement, as well as receipt of suboptimal chemotherapy due to age, comorbidities, or other circumstances might still necessitate or benefit from RT as part of treatment.

In terms of treatment dose, a trial by Lowry et al. showed that at a median follow-up of 5.6 years, there was no difference in freedom from local progression and OS in patients treated with 30 Gy in 15 fractions, compared to 40 to 45 Gy in 20 to 23 fractions.[14] A phase II trial from Duke, using 20 Gy in patients with complete PET response to chemotherapy, showed that with a median follow-up of 51 months, the 5-year freedom from local recurrence was 98%, and the 5-year PFS and OS were 83% and 90%, respectively.[15] This lower dose is currently being studied in a multi-center phase II trial.

Indolent Lymphoma

For patients with limited stage FL, RT alone has been shown in retrospective studies to result in a 10-year DFS of 40% to 50%. An international multicenter study, conducted by ILROG that included only PET-staged patients, found a 5-year freedom from progression (FFP) of 68.9%. Additionally, among patients with stage I disease, the 5-year FFP was 74.1%, suggesting potentially better results of RT in patients who have been more accurately staged.[16] In terms of optimal dose, the prospective, randomized trial by Lowry et al. found no differences in local progression, PFS, or OS, with 24 Gy in 12 fractions compared to the traditional 40 to 45 Gy in 20 to 30 fractions in patients with indolent lymphoma. This resulted in the adoption of 24 Gy as the standard radiation dose for early-stage, indolent lymphomas. TROG 99.03 compared IFRT alone versus IFRT and cyclophosphamide, vincristine, and prednisone (CVP), with rituximab added later in the trial. The addition of systemic therapy to IFRT showed a PFS benefit but no improvement in OS.[17]

The majority of patients with FL present with advanced-stage disease. In these patients, and in patients with recurrent disease, low-dose RT of 4 Gy in 2 fractions provides effective palliation in those with localized symptomatic sites, is associated with minimal toxicity, and can potentially delay the need to start systemic therapy. Multiple retrospective series demonstrated overall response rates of 80% to 90%. Results from the FoRT trial, comparing 4 Gy in 2 fractions versus the 24 Gy in 12 fractions, showed a statistically significant difference in overall response rate between the lower and higher doses (71.4% vs. 81%; $P = .006$), as well as worse PFS (80.4% vs. 93.7%; $P < .001$), indicating that the low-dose approach is generally not appropriate for patients with limited-stage disease as curative intent (see the box with Case 2).[18]

Marginal zone B-cell lymphoma (MZL or MALT) comprises 10% of NHL. The majority of cases are localized at presentation and tend to remain localized for long periods of time. This subtype of lymphoma is highly responsive to RT. Doses of around 30 Gy to involved nodal regions or extranodal sites will yield a local control rate of close to 100%, with a chance of long-term cure in about three-quarters of the patients. Results from the previously described randomized trial by Lowry et al. in which MALT lymphomas comprise 14% of the cases, suggested that 24 Gy is adequate. Relapses tend to occur at other extranodal sites in which MALT lymphomas tend to present or in a non-irradiated contralateral paired organ. The likelihood of achieving a second remission in patients with limited relapses remains excellent with further local RT.

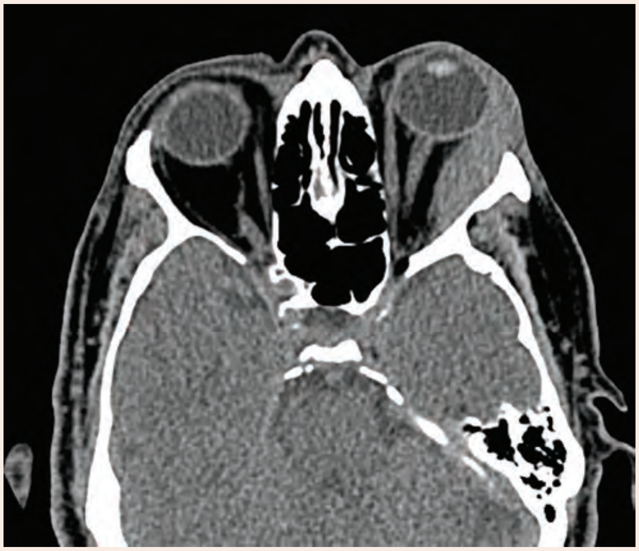
Relapsed/Refractory Diffuse Large B-Cell Lymphoma

In patients with relapsed/refractory DLBCL, results of the landmark PARMA trial, published in 1995, established high-dose therapy and autologous transplantation as the standard for patients with chemotherapy-sensitive disease. The indications of and general approaches to peri-transplant RT are similar to that for relapsed/refractory HL and have been detailed in guidelines put forth by ILROG. One key difference is that for chemotherapy-refractory DLBCL, a higher dose of up to 50 Gy in conventional fractionation, or hyperfractionated approach of 1.3 to 1.5 Gy/fraction twice daily to 35 to 40 Gy, may be needed.

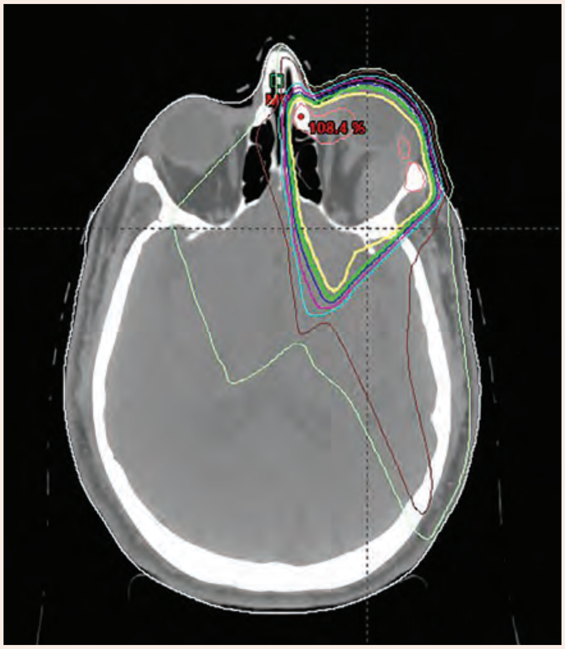
The advent of chimeric antigen receptor T-cell (CAR-T) therapy has changed the landscape in management of relapsed/refractory DLBCL. There is an emerging role for RT as bridging therapy as

CASE 2

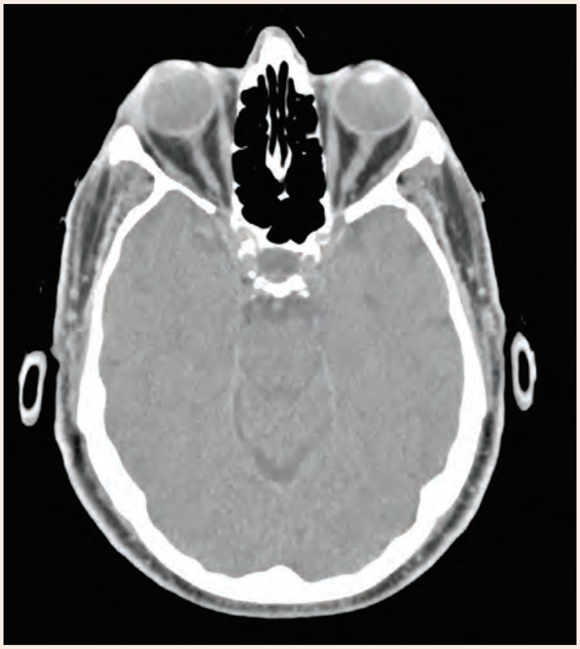
A 70-year-old man with stage IV follicular lymphoma, grade 1 and 2, presented with slowly progressive proptosis and diplopia. Imaging revealed a left lateral rectus mass. Biopsy was consistent with underlying follicular lymphoma:



Low-dose palliative radiation therapy, 4 Gy in 2 fractions was recommended. Aquaplast mask was used for immobilization, and the clinical target volume (CTV) included the entire orbital content. 3D conformal radiation therapy (RT) was used:



The patient noted resolution of proptosis and diplopia approximately 2 weeks after the 2 doses of RT. He did not experience any side effects other than slight fatigue. Follow-up CT scan at 1-year post-treatment showed continued local disease control:



consolidation after CAR-T therapy or as salvage for further relapses after CAR-T therapy. Several small, single institution series, have recently been published, highlighting these approaches. These studies provide early results showing that incorporation of RT to CAR-T therapy is generally safe and provides excellent local response and symptom control, prior to the infusion of CAR-T-cell product. RT may also lead to less severe cytokine release syndrome and neurotoxicity by providing disease debulking. The optimal timing, dose, fractionation schedule, long-term toxicity, and efficacy remain to be determined.

Multiple Myeloma and Plasmacytoma

For patients with multiple myeloma, RT plays an important role in the palliation of painful bone lesions or less commonly soft-tissue disease, lytic lesions at risk for pathologic fractures in weight-bearing regions, and symptoms of cord or nerve root compressions for patients. A dose of 20 Gy in 5 fractions can provide effective palliation, although in cases of cord compression, a higher dose of 30 Gy in 10 fractions may be needed. Solitary plasmacytoma accounts for 5% to 10% of all plasma-cell dyscrasias. For solitary plasmacytoma, RT to doses of 40 to 50 Gy can provide effective local control of over 80%. In cases of solitary plasmacytoma of the bone, about half of the patients will progress to multiple myeloma at 10 years; by 15 years, most will develop multiple myeloma. Extraosseous solitary plasmacytoma is associated with a lower risk of progression to multiple myeloma and has a more favorable disease-free survival. Guidelines from ILROG provide recommendations on optimal RT management in these patients.

Leukemia

Myeloid sarcoma, also known as chloroma, is a rare tumor most commonly seen in the setting of acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML). ILROG guidelines are available on the use of radiation in the setting of CNS relapses, isolated chloroma with inadequate response to chemotherapy, or after hematopoietic stem cell transplant. RT is also used as palliation, including in emergent symptomatic situations, such as cord or nerve root compression. A dose 24 Gy in 2 Gy per fraction can be used in most cases, resulting in excellent and rapid responses, with overall durable control and symptom relief. Doses as low as 6 Gy can provide effective palliation in patients with limited life expectancy.

Total body irradiation (TBI) is used as part of the conditioning regimen for myeloablative or non-myeloablative hematopoietic stem cell transplantation in selected patients with ALL, AML, and other hematopoietic conditions. Technique is variable and based on treatment center. ILROG guidelines list the most common TBI schedules, delivering 12 to 13.5 Gy total over 3 to 4.5 days in 1.2 to 3.0 Gy per fraction. To reduce short- and long-term effects of TBI, interest has been increasing in total marrow and lymphoid irradiation (TMLI), using tomotherapy or volumetric modulated arc therapy (VMAT) techniques to limit target doses to bone marrow, lymphoid tissue, and sanctuary sites.

Skin Lymphoma

Primary cutaneous lymphoma is highly radiation sensitive, with RT playing a major role in its management. The most common type of cutaneous T-cell lymphomas (CTCL) is mycosis fungoides (MF). Total skin electron beam therapy (TSEBT) can provide effective palliation for this disease, and doses of 8 to 36 Gy have been used, with higher doses allowing more durable control. However, a lower TSEBT dose of 10 to 12 Gy is increasingly accepted because of the shorter treatment course, fewer side effects, and opportunity for repeated treatment if needed. The most commonly encountered cutaneous B-cell lymphoma (CBCL), seen in a radiation oncology practice, are primary cutaneous follicle center lymphoma and cutaneous marginal zone lymphoma. In patients with solitary or localized disease, a dose of 24 to 30 Gy can be potentially curative. Otherwise, low doses of 4 Gy in 2 fractions or 8 Gy in 1 to 2 fractions in CBCL and CTCL, respectively, can provide effective local palliation, with complete response rates of 70% to 90%. Details on technique, field, and dose guidelines for the various histologies and clinical scenarios have been published by ILROG.

Rare/Miscellaneous and Special Situations

Primary central nervous system lymphoma (PCNSL) most commonly presents in the brain parenchyma, followed by leptomeninges, spinal cord, and ocular via vitreous and retinal seeding. High-dose methotrexate (HD-MTX)-based induction chemotherapy is considered standard for newly diagnosed PCNSL. The role of RT as part of upfront treatment has been evaluated by several randomized trials. Thiel et al. conducted the first phase III trial, comparing whole brain radiation therapy (WBRT) to a dose of 45 Gy versus non-RT, after HD-MTX-based therapy in PCNSL, showing improved PFS (HR = 0.79, $P = .041$) in the WBRT group but at the expense of higher neurotoxicity; no OS differences occurred between the two arms.[19] More recently, a randomized phase II trial on patients with PCNSL, aged 18 to 60 years, compared WBRT (40 Gy) versus autologous stem-cell transplantation (ASCT) as consolidation treatment after induction chemotherapy. The 2-year PFS rates were 63% and 87% in the WBRT and ASCT arms, respectively, with increased cognitive impairment seen on the WBRT arm.[20] The use of a lower WBRT dose of 23.4 Gy in patients with complete response to HD-MTX was explored in a phase II study from Memorial Sloan Kettering Cancer Center (MSKCC), showing high response rates, long-term disease control, and minimal neurotoxicity. Results of the NRG-RTOG 1114 randomizing patients to low-dose WBRT of 23.4 Gy versus non-RT after chemotherapy were recently presented in abstract form. At a median follow-up of 55 months, the median PFS was 25 months in the chemotherapy alone arm, and it was not reached in the chemotherapy and WBRT arm ($P = .015$). The 2-year PFS were 54% and 78%, respectively. Neurotoxicity rates were not significantly different between the two arms. However, additional neuropsychological testing and neuroimaging analyses are still ongoing to fully characterize cognitive decline associated with the WBRT treatment.[21]

Extranodal natural killer (NK)/T-cell lymphoma, nasal type, is commonly seen in East Asia but rare in North America and Europe. Approximately, 80% of cases involve the nasal cavity and present as erosive and destructive lesions. In patients with localized disease, the treatment approach is non-anthracycline-based chemotherapy regimens, including etoposide and L-asparaginase, in combination with RT. A commonly used regimen in the United States is RT concurrently with 2/3 dexamethasone, etoposide, ifosfamide, and carboplatin (2/3DeVIC). To facilitate radiation target volume delineation, in addition to PET-CT, MRI is important for an accurate determination of soft-tissue disease extent in radiation planning. Radiation dose of 50 to 54 Gy in conventional fractionation is recommended. In the setting of locally-advanced or stage IV disease, the dexamethasone, methotrexate, ifosfamide, L-asparaginase, and etoposide (SMILE) regimen, with sequential RT as indicated, can provide effective disease control.[22]

Primary mediastinal B-cell lymphoma (PMBL) is a distinct clinicopathological entity of lymphoid malignancies, originating from B-cells of the thymus. A phase II trial from the National Cancer Institute found excellent outcome, using 6 cycles of dose-adjusted etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin, and rituximab (EPOCH-R) but without RT. At a 5-year median follow-up, the EFS and OS rates were 93% and 97%, respectively. In a recent abstract reporting on the subgroup of 131 PMBL from the previously described UNFOLDER trial, patients assigned to the RT arm after R-CHOP chemotherapy had a superior 4-year EFS (94% vs. 78%; $P = .007$), mostly due to a higher partial response rate in the non-RT arm, triggering RT but no difference in PFS (95% vs. 90%) or OS (98% vs. 96%).[23] The role of mediastinal RT, after a complete PET response to rituximab-containing chemotherapy regimens in patients with PMBL, is currently being addressed by the International Extranodal Lymphoma Study Group (IELSG) 37 trial.

Primary testicular lymphoma, usually pathologically diagnosed as an extranodal DLBCL of the testis, affects primarily men over the age of 50. There is a high propensity for CNS relapses, and as such CNS prophylaxis with either intrathecal or intravenous methotrexate is included as part of upfront treatment. In addition, there is an increased risk of contralateral testicular relapse, occurring in 15% of patients at 3 years, with an increase to 42% at 15 years. Prophylactic scrotal irradiation of 25 to 30 Gy in 10 to 15 fractions can significantly reduce the risk. A phase II study by the IELSG showed that with the addition of intrathecal methotrexate to R-CHOP chemotherapy and 30 Gy of RT to the contralateral testis the cumulative risk of CNS relapse was reduced to 6%. No contralateral relapse was seen in the 53 treated patients.[24]

DOSE CONSTRAINTS AND TOLERANCES

When considering dose constraints and tolerances in the setting of hematologic malignancies, it is important to keep in mind the young age of a significant proportion of patients, often with highly curable disease and a long-life expectancy. Minimization of doses to surrounding organs-at-risk is therefore especially important, aiming for the lowest possible dose to normal structures, while maintaining adequate target coverage using modern radiation technology. Moreover, because of the modest doses of radiation typically used in the treatment of hematologic malignancies, dose constraints used in solid tumors do not apply to most lymphoma patients. Another important consideration is that many hematologic malignancy patients presenting for RT may have previously been exposed to one or more lines of systemic therapies, with toxicities to specific organs, including heart, lungs, gastrointestinal track, CNS, and bone marrow. Depending on the site of irradiation, special attention to organs that are at risk for overlapping toxicity needs to be made. Table 95.5 summarizes dose constraints recommended by ILROG for mediastinal lymphoma RT.

SUMMARY OF GUIDELINES

Tables 95.6 and 95.7 list the current recommended dose and fractionation for curative and palliative RT, respectively, based on ILROG published guidelines. References to the ILROG published guidelines are listed under suggested readings.

FUTURE DIRECTIONS

As advances in technology continue to improve the prognosis of lymphoma, special attention will be necessary to optimize outcomes, while reducing unnecessary harms. The importance of patient selection and evaluation of individualized de-escalation or intensification approaches based on treatment response, will require a team-based approach. Emphasis on individualized treatment recommendations will continue to require the integration of multidisciplinary teams, with consideration of individual patients' presentation, clinical picture, and goals of care.

TABLE 95.5 Dose Constraints for Organs at Risk Based on Proton Therapy Use in Mediastinal Lymphoma

	Dose Constraint		
Organ	**Ideal**	**Unacceptable**	**Avoid Max Dose To**
Heart: left ventricle, coronary arteries, valves[a]	Mean <5 Gy	Mean >30 Gy	Coronary vessels
Lung[a]	Mean <10 Gy V_5 <55% V_{20} <30%	Mean >13.5 Gy V_5 >60%	—
Breast (age dependent) [a]	Mean <4 Gy	Mean >30 Gy	Glandular tissue
Thyroid[a]	V_{25} <62.5%	—	Whole thyroid

[a] Doses obtained from *Guide to acceptable dose, volume, and field considerations* (Table 2) in Dabaja BS, Hoppe BS, Plastaras JP, et al. Proton therapy for adults with mediastinal lymphomas: the International Lymphoma Radiation Oncology Group guidelines. *Blood*. 2018;132(16):1635–1646.

TABLE 95.6 Summary of the International Lymphoma Radiation Oncology Group Guideline Recommendations for Hematologic Malignancy Cases Treated with Curative Intent

Histology and Stage	Systemic Tx	Total Dose (Gy)	Number of Fractions
Hodgkin Lymphoma—Limited Stage, Favorable	ABVD × 2	20	10
Hodgkin Lymphoma—Limited Stage, Unfavorable	ABVD × 3–4	30	15
Relapsed/Refractory Hodgkin Lymphoma	Salvage chemotherapy, ASCT	Peri-transplant RT: If CR, 30–36 Boost to PR site(s): 36–40	20
Nodular Lymphocyte Predominant Hodgkin Lymphoma—Limited Stage	None	30	15
Diffuse Large B-Cell Lymphoma (DLBCL)	R-CHOP × 3–6	If CR, 30 If PR, boost to 40–45	15 20–25
Indolent Lymphoma—Limited Stage	None	24	12
Natural Killer (NK)/T-cell Lymphoma	Non-anthracycline-based chemotherapy, e.g., DeVIC SMILE	Concurrent or sequential with chemotherapy: 45–54	25–30
Cutaneous Lymphoma, Solitary	None	24–30	12–15
Solitary Plasmacytoma	None	40–50	20–25

ABVD, Adriamycin, bleomycin, vinblastine and dacarbazine; *ASCT*, autologous stem cell transplantation; *DeVIC*, dexamethasone, etoposide, ifosfamide and carboplatin; *RT*, radiation therapy; *SMILE*, dexamethasone, methotrexate, ifosfamide, L-asparaginase and etoposide.

TABLE 95.7 Summary of the International Lymphoma Radiation Oncology Group Guideline Recommendations for Hematologic Malignancy Cases Treated with Palliative Intent Radiation Therapy

Histology	Total Dose (Gy)	Number of Fractions
Aggressive Non-Hodgkin Lymphoma (NHL)	8–30 (depending on performance status, life expectancy, treatment site, prior radiation therapy history)	1–10
Multiple Myeloma	20 30 (if cord compression)	5 10
Follicular Lymphoma, Marginal Zone Lymphoma	4	2
Myeloid Sarcoma/Leukemia	24 Limited life expectancy: 6	12 3

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Bakst RL, Dabaja BS, Specht LK, et al. Use of radiation in extramedullary leukemia/chloroma: guidelines from the International Lymphoma Radiation Oncology Group. *Int J Radiat Oncol Biol Phys*. 2018;102(2):314–319.

Constine LS, Yahalom J, Ng AK, et al. The role of radiation therapy in patients with relapsed or refractory Hodgkin lymphoma: guidelines from the International Lymphoma Radiation Oncology Group. *Int J Radiat Oncol Biol Phys*. 2018;100(5):1100–1118.

Dabaja BS, Hoppe BS, Plastaras JP, et al. Proton therapy for adults with mediastinal lymphomas: the International Lymphoma Radiation Oncology Group guidelines. *Blood*. 2018;132(16):1635–1646.

Dabaja BS, Ng AK, Terezakis SA, et al. Making every single gray count: involved site radiation therapy delineation guidelines for hematological malignancies. *Int J Radiat Oncol Biol Phys*. 2020;106(2):279–281.

Dabaja BS, Specht L, Yahalom J. Lymphoblastic Lymphoma: Guidelines from the International Lymphoma Radiation Oncology Group (ILROG). *Int J Radiat Oncol Biol Phys*. 2018;102(3):508–514.

Illidge T, Specht L, Yahalom J, et al. Modern radiation therapy for nodal non-Hodgkin lymphoma—target definition and dose guidelines from the International Lymphoma Radiation Oncology Group. *Int J Radiat Oncol Biol Phys*. 2014;89(1):49–58.

Mikhaeel NG, Milgrom SA, Terezakis S, et al. The optimal use of imaging in radiation therapy for lymphoma: guidelines from the International Lymphoma Radiation Oncology Group (ILROG). *Int J Radiat Oncol Biol Phys*. 2019;104(3):501–512.

Ng AK, Yahalom J, Goda JS, et al. Role of radiation therapy in patients with relapsed/refractory diffuse large B-cell lymphoma: guidelines from the International Lymphoma Radiation Oncology Group. *Int J Radiat Oncol Biol Phys*. 2018;100(3):652–669.

Pinnix CC, Yahalom J, Specht L, et al. Radiation in central nervous system leukemia: guidelines from the International Lymphoma Radiation Oncology Group. *Int J Radiat Oncol Biol Phys*. 2018;102(1):53–58.

Specht L, Dabaja B, Illidge T, et al. Modern radiation therapy for primary cutaneous lymphomas: field and dose guidelines from the International Lymphoma Radiation Oncology Group. *Int J Radiat Oncol Biol Phys*. 2015;92(1):32–39.

Specht L, Yahalom J, Illidge T, et al. Modern radiation therapy for Hodgkin lymphoma: field and dose guidelines from the International Lymphoma Radiation Oncology Group (ILROG). *Int J Radiat Oncol Biol Phys*. 2014;89(4):854–862.

Tsang RW, Campbell BA, Goda JS, et al. Radiation therapy for solitary plasmacytoma and multiple myeloma: Guidelines from the International Lymphoma Radiation Oncology Group. *Int J Radiat Oncol Biol Phys*. 2018;101(4):794–808.

Wirth A, Mikhaeel NG, Aleman BMP, et al. Involved site radiation therapy in adult lymphomas: an overview of International Lymphoma Radiation Oncology Group guidelines. *Int J Radiat Oncol Biol Phys*. 2020;107(5):909–933.

Wong JYC, Filippi AR, Dabaja BS, et al. Total body irradiation: guidelines from the International Lymphoma Radiation Oncology Group (ILROG). *Int J Radiat Oncol Biol Phys*. 2018;101(3):521–529.

Yahalom J, Illidge T, Specht L, et al. Modern radiation therapy for extranodal lymphomas: field and dose guidelines from the International Lymphoma Radiation Oncology Group. *Int J Radiat Oncol Biol Phys*. 2015;92(1):11–31.

REFERENCES

1. Cowan DH. Vera Peters and the curability of Hodgkin disease. *Curr Oncol*. 2008;15(5):206.
2. Dabaja BS, Ng AK, Terezakis SA, et al. ILROG Lymphoma Mini-Atlas Part II, Hodgkin Lymphoma. *Int J Radiat Oncol Biol Phys*. 2020;108(4): 977–978.
3. Maraldo MV, Brodin NP, Aznar MC, et al. Estimated risk of cardiovascular disease and secondary cancers with modern highly conformal radiotherapy for early-stage mediastinal Hodgkin lymphoma. *Annals of Oncology*. 2013;24(8):2113–2118.
4. Cheson BD, Fisher RI, Barrington SF, et al. Recommendations for initial evaluation, staging, and response assessment of Hodgkin and non-Hodgkin lymphoma: the Lugano classification. *J Clin Oncol*. 2014;32(27):3059.
5. Sasse S, Bröckelmann PJ, Goergen H, et al. Long-term follow-up of contemporary treatment in early-stage Hodgkin lymphoma: updated analyses of the German Hodgkin Study Group HD7, HD8, HD10, and HD11 trials. *J Clin Oncol*. 2017;35(18):1999–2007.
6. Engert A, Plütschow A, Eich HT, et al. Reduced treatment intensity in patients with early-stage Hodgkin's lymphoma. *N Engl J Med*. 2010;363(7):640–652.
7. Eich HT, Diehl V, Görgen H, et al. Intensified chemotherapy and dose-reduced involved-field radiotherapy in patients with early unfavorable Hodgkin's lymphoma: final analysis of the German Hodgkin Study Group HD11 trial. *J Clin Oncol*. 2010;28(27):4199–4206.
8. Radford J, Illidge T, Counsell N, et al. Results of a trial of PET-directed therapy for early-stage Hodgkin's lymphoma. *N Engl J Med*. 2015;372(17):1598–1607.
9. André MPE, Girinsky T, Federico M, et al. Early positron emission tomography response-adapted treatment in stage I and II Hodgkin lymphoma: final results of the randomized EORTC/LYSA/FIL H10 trial. *J Clin Oncol*. 2017;35(16):1786–1794.
10. Fuchs M, Goergen H, Kobe C, et al. Positron emission tomography-guided treatment in early-stage favorable Hodgkin lymphoma: final results of the international, randomized phase III HD16 trial by the German Hodgkin study group. *J Clin Oncol*. 2019;37(31):2835–2845.
11. Borschmann P, et al. Positron emission tomography guided omission of radiotherapy in early-stage unfavorable Hodgkin lymphoma: final results of the international, randomized phase III HD17 trial by the GHSG. EHA25 Virtual Abstract. 2020;S101
12. Lamy T, Damaj G, Soubeyran P, et al. R-CHOP 14 with or without radiotherapy in nonbulky limited-stage diffuse large B-cell lymphoma. *Blood*. 2018;131(2):174–181.
13. Persky DO, Li H, Stephens DM, et al. Positron emission tomography–directed therapy for patients with limited-stage diffuse large B-cell lymphoma: results of Intergroup National Clinical Trials Network Study S1001. *J Clin Oncol*. 2020;38(26):3003–3011.
14. Lowry L, Smith P, Qian W, et al. Reduced dose radiotherapy for local control in non-Hodgkin lymphoma: a randomised phase III trial. *Radiotherapy and Oncology*. 2011;100(1):86–92.
15. Kelsey CR, Broadwater G, James O, et al. Phase 2 study of dose-reduced consolidation radiation therapy in diffuse large B-cell lymphoma. *Int J Radiat Oncol Biol Phys*. 2019;105(1):96–101.
16. Brady JL, Binkley MS, Hajj C, et al. Definitive radiotherapy for localized follicular lymphoma staged by ^{18}F-FDG PET-CT: a collaborative study by ILROG. *Blood*. 2019;133(3):237–245.
17. MacManus M, Fisher R, Roos D, et al. Randomized trial of systemic therapy after involved-field radiotherapy in patients with early-stage follicular lymphoma: TROG 99.03. *J Clin Oncol*. 2018;36(29):2918–2925.
18. Hoskin PJ, Kirkwood AA, Popova B, et al. 4 Gy versus 24 Gy radiotherapy for patients with indolent lymphoma (FORT): a randomised phase 3 non-inferiority trial. *Lancet Oncol*. 2014;15(4):457–463.
19. Thiel E, Korfel A, Martus P, et al. High-dose methotrexate with or without whole brain radiotherapy for primary CNS lymphoma (G-PCNSL-SG-1): a phase 3, randomised, non-inferiority trial. *Lancet Oncol*. 2010;11(11): 1036–1047.
20. Houillier C, Taillandier L, Dureau S, et al. Radiotherapy or autologous stem-cell transplantation for primary CNS lymphoma in patients 60 years of age and younger: results of the intergroup ANOCEF-GOELAMS randomized phase II PRECIS study. *J Clin Oncol*. 2019;37(10):823–833.
21. Omuro AMP, DeAngelis LM, Karrison T, et al. Randomized phase II study of rituximab, methotrexate (MTX), procarbazine, vincristine, and cytarabine (R-MPV-A) with and without low-dose whole-brain radiotherapy (LD-WBRT) for newly diagnosed primary CNS lymphoma (PCNSL). *J Clin Oncol*. 2020;38(15). 2501-2501.
22. Yamaguchi M, Suzuki R, Oguchi M. Advances in the treatment of extranodal NK/T-cell lymphoma, nasal type. *Blood*. 2018;131(23): 2528–2540.
23. Held G, Thurner L, Poeschel V, et al. Role of radiotherapy and dose-densification of R-CHOP in primary mediastinal B-cell lymphoma: a subgroup analysis of the unfolder trial of the German Lymphoma Alliance (GLA). *J Clin Oncol*. 2020;38(15). 8041-8041.
24. Vitolo U, Chiappella A, Ferreri AJM, et al. First-line treatment for primary testicular diffuse large B-cell lymphoma with rituximab-CHOP, CNS prophylaxis, and contralateral testis irradiation: final results of an international phase II trial. *J Clin Oncol*. 2011;29(20):2766–2772.

CHAPTER 96

GRADING AND TOXICITY MANAGEMENT AFTER IMMUNE EFFECTOR THERAPY

Emily C. Ayers, Noelle V. Frey, and Daniel W. Lee

The growing use of novel immunotherapies has revolutionized the management of hematologic malignancies over the past several years. While historically cytotoxic chemotherapy has been the standard treatment approach, newer cellular therapies have demonstrated promising activity in patients with relapsed and refractory hematologic malignancies and have significantly improved outcomes in this setting.[1,2] The increased use of these innovative therapies has highlighted a set of unique toxicities which are distinct from previously well-understood side effects associated with standard chemotherapy, monoclonal antibodies, or targeted agents. In order to optimize the use of immune effector therapy, clinicians need to understand how best to manage these newly recognized toxicities.

Immunotherapies and cellular therapies, which harness the host immune system to battle tumor cells, include chimeric antigen receptor (CAR) T-cell therapy, bispecific antibodies and their derivatives, T-cell receptor (TCR) modified gene therapies, and CAR-transduced natural killer (CAR-NK) cell therapy. While many of their toxicities are similar, each of these therapies has a different risk profile. For example, genetically unmanipulated viral-specific T cells are generally much better tolerated than effective CAR-T-cell therapies. Despite these differences, this group of innovative therapies share a toxicity profile which should be graded and managed similarly.

CYTOKINE RELEASE SYNDROME

Cytokine release syndrome (CRS) is the most commonly seen toxicity with immune effector therapies and is a term which describes a pattern of clinical signs and symptoms that develop as a result of robust immune activation resultant from a cell-mediated immune response after therapy. This clinical syndrome was recognized in pediatric acute lymphoblastic leukemia (ALL) patients treated with CD19-targeted CAR-T cells who developed fever, tachycardia, and hypotension consistent with a systemic inflammatory response and was subsequently seen following immune effector cell therapy in the adult population. The pathophysiology of CRS originates from the robust and widespread on-target activation and proliferation of engineered T cells and surrounding bystander inflammatory cells followed by the release of inflammatory cytokines and chemokines including interleukin-6 (IL-6), IL-2, IL-8, IL-10, interferon-γ (IFNγ), monocyte chemoattractant protein-1, and macrophage inflammatory protein-1b among others. Data suggests serum IL-6 is likely derived from macrophages, and peak CAR-T-cell and serum IL-6 levels correlate strongly with severity of CRS in clinical studies. The delayed onset of CRS supports the hypothesis that in the days after cell infusion CAR-T cells proliferate and recruit bystander immune effectors, including macrophages, ultimately resulting in the clinical features of CRS.

CRS of any grade has been reported in 37% to 93% of patients receiving CAR-T cells on clinical trials.[1,2] Clinical manifestations must include fever that can be accompanied by other signs and symptoms ranging from mild flu-like symptoms to a severe inflammatory syndrome with hypotension and a capillary leak syndrome causing pulmonary edema, hypoxemia, and if left unchecked, eventually multisystem organ failure. Time of onset may vary depending on the product used but typically occurs within one week of CAR-T-cell infusion and peaks within 1 to 2 weeks after infusion. Timing of CRS onset and duration after bispecific antibody engager therapies are often much faster with the risk period beginning at the start of the product infusion, while those from TCR, CAR-NK, or other novel engineered cells remain to be determined.

GRADING OF CYTOKINE RELEASE SYNDROME

Prior to the development of the consensus grading established by the American Society for Transplantation and Cellular Therapy (ASTCT) in 2019, the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) versions 3 and 4 were used to grade CRS both on clinical trials and in the standard of care practice setting.[3] However, both CTCAE v3 and v4 demonstrated significant shortcomings with regard to grading CRS resultant from cellular therapies and were perhaps more applicable to toxicities from monoclonal antibody therapies. For example, grading outlined in CTCAE v3 assumes CRS onset within 24 hours of T-cell infusion and fails to account for the delayed onset of symptoms seen in clinical practice. The CTCAE v4 scale is dependent upon infusion interruption, which is not applicable to CAR-T-cell treatments.

In response to inadequate CRS grading by the CTCAE, there were a number of attempts to standardize both the definition and grading of CRS across institutions. Lee et al. developed a structured grading system in which CRS severity was categorized by the degree of hypoxia, hypotension, and/or organ dysfunction and linked to a treatment algorithm.[4] The group at the University of Pennsylvania, likewise, devised a grading system in which CRS severity was scaled according to the degree of hypoxia and/or hypotension and was associated with the intensity of therapy required for treatment of these toxicities.[5] Additional grading systems were developed by Memorial Sloan Kettering Cancer Center as well as a multi-institutional group of investigators who adopted the Lee criteria but developed a unique scale for neurotoxicities called the CAR-T-Cell Therapy–Associated TOXicity (CARTOX) score. These CRS grading scales differed from one another in several ways. For example, the Penn scale categorized patients requiring any intravenous fluids or low-dose vasopressors as well as any supplemental oxygen as grade 3 CRS, while many of these same patients would be classified as grade 2 CRS under the Lee criteria. Due to classification differences such as this, the development of a standardized grading system was paramount to allow for comparison of patients treated across institutions.

Most recently, the ASTCT published international consensus grading guidelines in an effort to standardize the definition and grading scale of CRS (Table 96.1). This group defines CRS as “a supraphysiologic response following any immune therapy that results in the activation or engagement of endogenous or infused T cells and/or other immune effector cells. Symptoms can be progressive, must include fever at onset, and may include hypotension, capillary leak (hypoxia), and end organ dysfunction.” In this simplified consensus grading schema, fever is a prerequisite and CRS grade is determined by the degree of intervention necessary to address hypotension and/or hypoxia in patients. An added feature is that grading can be rapidly assessed by any practitioner at the bedside.

TABLE 96.1 Cytokine Release Syndrome Grading

CRS Parameter	Grade 1	Grade 2	Grade 3	Grade 4
Fever	Temperature ≥38°C	Temperature ≥38°C	Temperature ≥38°C	Temperature ≥38°C
		With		
Hypotension	None	Not requiring vasopressors	Requiring a vasopressor with or without vasopressin	Requiring multiple vasopressors (excluding vasopressin)
		And/Or		
Hypoxia	None	Requiring low-flow nasal cannula or blow-by	Requiring high-flow nasal cannula, facemask, nonrebreather mask, or Venturi mask	Requiring positive pressure (e.g., CPAP, BiPAP, intubation and mechanical ventilation)

BiPAP, Bilevel positive airway pressure; *CPAP*, continuous positive airway pressure.
Adapted from Lee DW, Santomasso BD, Locke FL, et al. ASTCT consensus grading for cytokine release syndrome and neurologic toxicity associated with immune effector cells. *Biol Blood Marrow Transplant.* 2019;25:625–638.

MANAGEMENT OF CYTOKINE RELEASE SYNDROME

Patients with all grades of CRS require close monitoring in an inpatient setting with daily physical exam and laboratory tests including a complete blood count, comprehensive metabolic panel, magnesium, phosphorus, coagulation profile, fibrinogen, C-reactive protein, and ferritin. All patients should receive a thorough infectious workup and strong consideration should be given to administering prophylactic or empiric broad spectrum anti-microbials.

The management of CRS ranges from best supportive care, including antipyretics or intravenous fluids, in mild cases to intervention with anti-cytokine-directed therapy such as the US Food and Drug Administration (FDA) approved anti-IL6-receptor monoclonal antibody tocilizumab[4,6] or corticosteroids. Historically, the first wave of clinical trials with CAR-T cells reserved tocilizumab for more severe stages of CRS due to concern that earlier intervention may negatively impact outcomes. However, there is limited data to inform the optimal timing of intervention for CRS, but earlier intervention strategies are the current norm in the commercial setting with ongoing clinical trials investigating the effect of prophylactic tocilizumab.[7]

The use of tocilizumab varies between treatment centers, the population being treated, and the CAR-T-cell product used. Neelapu et al. suggest the use of tocilizumab for grade 1 CRS in the case of a persistent fever or for grade 2 CRS prior to the use of vasopressors if hypotension is refractory to two fluid boluses.[8] In contrast, Lee et al. advocate the use of tocilizumab in grade 2 CRS for elderly patients or patients with significant medical comorbidities, or for any patient with grade 3 CRS with the express aim to prevent grade 4 toxicity.[4] While there are minor differences between treatment algorithms for earlier grades of CRS published by these different groups, the overall consensus recommends the use of tocilizumab with or without corticosteroids as first-line therapy for grade 3 or grade 4 CRS. While final consensus guidelines are still pending, the National Comprehensive Cancer Network (NCCN) recommends use of anti-IL-6 therapy for prolonged grade 1 CRS (>3 days) in adults with comorbidities or for any adult patient with grade 2 or higher CRS (as per ASTCT criteria). The NCCN recommends corticosteroids for patients with persistent symptoms of grade 2 CRS despite 1 to 2 doses of anti-IL-6 therapy and for any patient with grade 3 or 4 CRS.

Other potential therapeutic interventions include corticosteroids or other cytokine-directed therapies. Initial concerns regarding direct toxicity to T cells associated with corticosteroid use, while not validated in the literature, together with the often-rapid improvement seen with tocilizumab have led experts to disfavor steroids as first-line therapy in treatment of CRS for fear of decreasing the efficacy of the CAR-T cells.[9] Corticosteroids should be added to tocilizumab, however, for refractory or severe CRS. Siltuximab is another monoclonal antibody which binds IL-6 directly and, while it is not FDA approved for this indication, it has been used by some centers to treat CRS refractory to tocilizumab and corticosteroids. Other agents that have been used in the salvage setting include anakinra, dasatinib, and cytotoxic therapy such as cyclophosphamide.

Known risk factors for the development of CRS include tumor burden, higher CAR-T-cell dose, type of CAR-T-cell product, as well has high baseline levels of IL-6.[10,11] In addition, CAR-T-cell products using CD28 costimulatory domains may be associated with increased incidence of CRS compared to products with 4-1BB costimulatory molecules, although conclusive evidence is lacking. Our current ability to predict the risk of CRS accurately, however, is not yet refined, and ongoing research aims to identify predictive biomarkers of severe CRS using cytokine profiles.

IMMUNE EFFECTOR CELL–ASSOCIATED NEUROTOXICITY SYNDROME

The second most commonly observed toxicity from immune effector therapy is immune effector cell–associated neurotoxicity syndrome (ICANS), which is seen in 20% to 60% of people following immune effector therapy. In addition to CRS, the ASTCT also published a consensus definition and grading scale for ICANS (Table 96.2). While some early reports included neurologic events as part of systemic CRS, the current consensus approach advocates for distinct grading and management

The pathophysiology of ICANS is less well understood compared to that of CRS but is still thought to be, at least in part, related to supraphysiologic expression of inflammatory cytokines and chemokines. One potential mechanism involves the expression of IL-1 with subsequent activation of by-standing monocytes, cytokine production and T-cell activation, and endothelial cell activation and disruption of the blood-brain barrier.[12] Known risk factors for severe ICANS include increased tumor burden, higher CAR-T-cell doses, and higher T-cell peak expansion values. Some data suggests increased rates of ICANS with CAR structures containing CD28 costimulatory domains compared to 4-1BB, although there is no conclusive evidence to confirm this association.

The clinical phenotype of ICANS can vary widely (Table 96.3). Early symptoms include encephalopathy, dysgraphia, tremor, and expressive aphasia, which is thought to be most specific for ICANS, but can range in severity to include seizure, akinesis, increased intracranial pressure (ICP), and alterations in consciousness including obtundation and coma.[13] While headaches can be associated with ICANS, this symptom is not sufficient for ICANS as the cause can be multifactorial. The onset of ICANS typically occurs after but sometimes concurrently with CRS and can still occur up to 4 weeks after infusion.

TABLE 96.2 Immune Effector Cell–Associated Neurotoxicity Syndrome Grading

Neurotoxicity Domain	Grade 1	Grade 2	Grade 3	Grade 4
ICE Score	7–9	3–6	0–2	0 (unarousable patient and unable to perform assessment)
Depressed level of consciousness	Awakens spontaneously	Awakens to voice	Awakens only to tactile stimulus	Patient is unarousable or requires vigorous or repetitive tactile stimuli to arouse. Stupor or coma
Seizure	None	None	Any clinical seizure focal or generalized that resolves rapidly or nonconvulsive seizures on EEG that resolve with intervention	Life-threatening prolonged seizure (>5 min); or repetitive clinical or electrical seizures without return to baseline in between
Motor findings	None	None	None	Deep focal motor weakness such as hemiparesis or paraparesis
Elevated ICP/cerebral edema	None	None	Focal/local edema on neuroimaging	Diffuse cerebral edema on neuroimaging; decerebrate or decorticate posturing; or cranial nerve VI palsy; or papilledema; or Cushing triad

EEG, Electroencephalography; *ICE*, immune effector cell–associated encephalopathy; *ICP*, intracranial pressure.

TABLE 96.3 Neurologic and Psychiatric Adverse Reactions Reported With Chimeric Antigen Receptor T-Cell Products

Domain	Clinical Signs/Symptoms
Encephalopathy	Cognitive disorder, confusional state, depressed level of consciousness, disturbance in attention, lethargy, memory impairment, leukoencephalopathy, mental status changes, paranoia, somnolence, and automatism
Delirium	Agitation, hallucination (auditory and visual), disorientation, hyperactivity, restlessness, irritability
Headache	Headache or migraine
Anxiety	
Sleep disorders	Insomnia, nightmares
Motor dysfunction	Muscle spasms, muscular weakness, tremor, myoclonus
Cerebellar dysfunction	Ataxia
Seizure	

Lee DW, Santomasso BD, Locke FL, et al. ASTCT consensus grading for cytokine release syndrome and neurologic toxicity associated with immune effector cells. *Biol Blood Marrow Transplant*. 2019;25:625–638.

GRADING OF IMMUNE EFFECTOR CELL–ASSOCIATED NEUROTOXICITY SYNDROME

As with CRS, early clinical trials graded neurotoxicity associated with immune effector therapy using the CTCAE system, which included overlapping terms and was vulnerable to a high degree of subjectivity. Prior to the published consensus grading scale by the ASTCT, the CARTOX criteria were developed by a multi-institutional group of investigators with experience in CAR-T-cell therapy in an attempt to overcome the lack of objectivity in the CTCAE grading system. This group created a CARTOX-10 score to evaluate several neurologic domains associated with neurotoxicity including orientation, naming, writing, and attention, as well as guidelines for grading increased ICP and seizures. One limitation of this grading criteria, however, included the need to measure directly the CSF opening pressure or papilledema in patients, which is difficult to do in a standard-of-care setting.

TABLE 96.4 Immune Effector Cell–Associated Encephalopathy (ICE) Score

Domain	Task	Points
Orientation	Orientation to year, month, city, hospital	4
Naming	Ability to name 3 objects	3
Following commands	Ability to follow simple commands (close your eyes and stick out tongue)	1
Writing	Ability to write a standard sentence	1
Attention	Ability to count backwards from 100 by 10	1

Lee DW, Santomasso BD, Locke FL, et al. ASTCT consensus grading for cytokine release syndrome and neurologic toxicity associated with immune effector cells. *Biol Blood Marrow Transplant*. 2019;25:625–638.

The ASTCT consensus modified the CARTOX scale to develop an easily applicable and comprehensive grading system for ICANS. The group altered the CARTOX-10 score to include a measurement of receptive aphasia and termed this tool the Immune Effector Cell–Associated Encephalopathy (ICE) score which specifically evaluates the level of encephalopathy (Table 96.4). However, full grading of ICANS requires assessment of level of consciousness, seizures, motor findings, and ICP in addition to an evaluation of the patient's level of encephalopathy. Of note, the ASTCT consensus grading includes neuroimaging as a means of evaluating for increased ICP to facilitate grading toxicity.

MANAGEMENT OF IMMUNE EFFECTOR CELL–ASSOCIATED NEUROTOXICITY SYNDROME

Similar to CRS, management of ICANS depends upon the severity of the toxicity and varies across institutions. Patients with any grade ICANS should be monitored in a hospital setting and undergo a comprehensive workup including basic laboratories with a complete blood count, comprehensive metabolic panel, magnesium, phosphorus, prothrombin time (PT) and partial thromboplastin time (PTT), fibrinogen, and infectious workup as indicated, including lumbar puncture when feasible. When possible, patients should also receive radiological imaging with either a CT scan or MRI of the brain to assess for any other causes of neurological symptoms. Electroencephalogram should be performed to rule out electrical or subclinical seizures which may upgrade the severity of ICANS and change management.

If grade 1 ICANS is diagnosed, patients can be managed with supportive measures, close monitoring, and a diagnostic workup as described above. While some centers will start prophylactic anti-epileptics, usually levetiracetam, at the onset of early ICANS, this approach has not yet been standardized. For grade 2 or greater, therapy with corticosteroids is the first-line treatment of choice and is generally agreed to be necessary. Though dosing and choice of steroid is actively being debated still, initial management should likely include 10 mg dexamethasone (or the pediatric equivalent) every 6 to 12 hours, given better CNS penetration compared to other corticosteroids, until resolution of symptoms and can increase to doses of 1000 mg methylprednisolone (or the pediatric equivalent) for grade 4 ICANS. Intermediate grades or refractory symptoms to initial steroid dosing should prompt an escalation of dosing or a change to a different corticosteroid or alternative agent such as anakinra. Once ICANS begins to improve, steroids should be rapidly tapered as tolerated. In general, ICANS is frequently slower to resolve even with treatment compared to CRS. It is not uncommon for ICANS to persist for weeks.

Tocilizumab has not been shown to be effective in the treatment of ICANS, likely because it does not cross the blood-brain barrier. There is also a hypothetical concern for increased risk of severe ICANS due to a transient increase in circulating levels of IL-6 with tocilizumab administration. For this reason, tocilizumab is not recommended for the initial treatment of isolated ICANS in the absence of CRS, though it should be used if the severity of CRS demands it. Other potential interventions for severe ICANS include siltuximab and anakinra, a small molecule IL-1 receptor antagonist, although these have not yet been extensively studied.

NCCN guidelines recommend the use of dexamethasone for adults with grade 2 or higher ICANS along with ICU admission for those patients with grade 3 or 4 ICANS. For patients with concurrent CRS, the NCCN suggests tocilizumab for grade 1 ICANS regardless of duration and corticosteroids as previously described.

OTHER TOXICITIES FROM IMMUNE EFFECTOR THERAPY

Less commonly observed toxicities from immune effector therapies include delayed cytopenias, which can persist for more than 30 days in up to 30% of patients.[2,14,15] The etiology is unclear but is potentially related to ongoing CAR-T-cell activity and subsequent disturbance of normal hematopoiesis. The majority of cytopenias resolve with time and are managed supportively with growth factor support for grade 3 to 4 neutropenia (outside the CRS window) and transfusion support for anemia and/or thrombocytopenia.

Cardiac toxicities observed with CAR-T-cell therapies include sinus tachycardia, other arrhythmias (supraventricular tachycardia, atrial fibrillation, and atrial flutter), left ventricular systolic dysfunction, ST-segment changes, elevated biomarkers (NT-proBNP, troponin, and lactate), and profound hypotension requiring inotropic support. For these reasons, echocardiogram and electrocardiography should be utilized during the risk period.

Other notable laboratory and clinical side effects with these therapies include coagulation abnormalities (elevated D-dimer, PT/PTT and decreased fibrinogen levels) associated with both bleeding and clotting events mainly seen within 2 to 3 weeks of T-cell infusion. Frequent monitoring and aggressive plasma and/or cryoprecipitate supplementation may be required to prevent complications.

Electrolyte abnormalities are also seen in association with and without acute kidney injury and include hypophosphatemia, hypokalemia, and hyponatremia. Hypophosphatemia in particular can be profound and often requires aggressive replacement multiple times a day.

Hemophagocytic lymphohistiocytosis (HLH) or macrophage activation syndrome (MAS) has also been seen with CAR-T-cell therapies although the incidence and pathogenesis of this syndrome is unclear given the overlapping laboratory and clinical findings of both CRS and HLH/MAS. Steroids, and managing severe CRS with tocilizumab, remain the standard of care for CAR-T-cell associated HLH/MAS. Though etoposide is used in other forms of HLH/MAS, there is presently no role for its use here.

Additional, and expected, toxicities of CD19 and CD22-targeted CAR-T-cell therapies are B-cell aplasia and resultant hypogammaglobulinemia, particularly in products which have long in vivo persistence. Patients should have immunoglobulin levels monitored periodically and IVIG replacement therapy should be considered depending on age and institutional practices.

CONCLUSIONS

The CAR-T-cell era has brought a new appreciation for the impressive power of the immune system. Exaggerated and superphysiologic CRS events not previously seen on this scale as well as an entirely new constellation of neurologic toxicities, ICANS, have challenged the successful implementation and availability of widespread CAR-T-cell therapy. Seminal discoveries in the management of CRS with tocilizumab followed by development of a broad consensus (ASTCT) for CRS and ICANS definition and grading were necessary steps to commercialization. There are likely other effective approaches to managing, preventing, or mitigating CRS, ICANS, and the other toxicities discussed, so additional study is needed in these domains.

KEY POINTS

1. American Society for Transplantation and Cellular Therapy (ASTCT) consensus cytokine release syndrome (CRS) and immune effector cell–associated neurotoxicity syndrome (ICANS) definitions and grading criteria should be used for all clinical trial and commercial products that engage T cells moving forward.
2. Tocilizumab +/– steroids should be used as the initial intervention for clinically significant CRS. Early rather than late intervention for CRS is preferred.
3. Steroids should be added if a second dose of tocilizumab is needed. Additional agents (provider's choice) should be utilized if more than 2–3 doses of tocilizumab with steroids are required.
4. Steroids, not tocilizumab, should be used for ICANS. Tocilizumab can be added if significant CRS coexists with ICANS.
5. ICANS may present suddenly and after CRS resolves, so all patients need a caregiver at all times for at least the first four weeks after infusion.

REFERENCES

1. Schuster SJ, et al. Chimeric antigen receptor T cells in refractory B-cell lymphomas. *N Engl J Med*. 2017;377:2545–2554.
2. Neelapu SS, et al. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. *N Engl J Med*. 2017;377:2531–2544.
3. Lee DW, et al. ASTCT consensus grading for cytokine release syndrome and neurologic toxicity associated with immune effector cells. *Biol Blood Marrow Transpl*. 2019;25:625–638.
4. Lee DW, et al. Current concepts in the diagnosis and management of cytokine release syndrome. *Blood*. 2014;124:188–195.
5. Porter D, Frey N, Wood PA, et al. Grading of cytokine release syndrome associated with the CAR T cell therapy tisagenlecleucel. *J Hematol Oncol*. 2018;11:35.
6. Grupp S, et al. Chimeric antigen receptor–modified T cells for acute lymphoid leukemia. *N Engl J Med*. 2013;368:1509–1518.
7. Ahmed N, et al. Prophylactic tocilizumab in patients with relapsed refractory lymphoma treated with anti CD19 chimeric antigen receptor T-cell therapy. *Biol Blood Marrow Transpl*. 2020;26:S275–S276.
8. Neelapu SS, et al. Chimeric antigen receptor T-cell therapy—assessment and management of toxicities. *Nat Rev Clin Oncol*. 2017;15:47–62.
9. Davila M, et al. Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. *Sci Transl Med*. 2014;6 224ra25-224ra25.

10. Hay K, et al. Kinetics and biomarkers of severe cytokine release syndrome after CD19 chimeric antigen receptor–modified T-cell therapy. *Blood*. 2017;130:2295–2306.
11. Teachey D, et al. Identification of predictive biomarkers for cytokine release syndrome after chimeric antigen receptor T-cell therapy for acute lymphoblastic leukemia. *Cancer Discov*. 2016;6:664–679.
12. Norelli M, et al. Monocyte-derived IL-1 and IL-6 are differentially required for cytokine-release syndrome and neurotoxicity due to CAR T cells. *Nat Med*. 2018;24:739–748.
13. Santomasso B, et al. Clinical and biologic correlates of neurotoxicity associated with CAR T cell therapy in patients with B-cell acute lymphoblastic leukemia (B-ALL). *Cancer Discov*. 2018;8:958–971.
14. Schuster SJ, et al. Tisagenlecleucel in adult relapsed or refractory diffuse large B-cell lymphoma. *N Engl J Med*. 2018;380:45–56.
15. Neelapu SS. Managing the toxicities of CAR T-cell therapy. *Hematol Oncol*. 2019;37:48–52.

CHAPTER 97

IDENTIFICATION AND MANAGEMENT OF CHECKPOINT INHIBITION TOXICITY

Evgeniya Kharchenko and John W. Sweetenham

Checkpoint inhibitors revolutionized cancer therapy and the field of oncology when the first drug of this class was introduced for melanoma treatment. But their therapeutic benefit comes at a cost of immunotherapy-related adverse events (IrAEs).

Checkpoint inhibitors are immunomodulatory antibodies that are used to activate the immune system to detect and destroy malignant cells. Several immune checkpoint receptors are targeted by different immunotherapy agents, such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed cell death protein ligand 1 (PD-L1). Different mechanisms of action explain diverse toxicity profiles: patients who are treated with anti-CTLA-4 therapy generally have more severe IrAEs than PD-1 inhibitors,[1] which inhibit T cells in peripheral tissues at later stages of the immune response. The pathophysiological mechanisms of immunotherapy-related toxicity is still unclear, but translational studies suggest that T cells, antibodies, and cytokine responses may be involved.[2]

Immunotherapy agents are currently widely used in patients with hematologic malignancies. Toxicity profiles for these drugs are generally well studied and include dermatologic, gastrointestinal, hepatic, endocrine, hematologic, and other less common complications (Fig. 97.1).[3] It is important to note that at the moment there are no prospective trials on IrAEs management, and all available data are based on clinical experience and retrospective analysis.

Although rare, severe and sometimes fatal toxicities may occur with checkpoint inhibitors.[4] Therefore, there is a strong need for predictive biomarkers for IrAEs. Such biomarkers can potentially identify the group of patients who will tolerate immunotherapy. Biomarkers that are being studied include serum proteins, factors in the tumor microenvironment, as well as genomic predictors of toxicity.

IMMUNOTHERAPY-RELATED FATIGUE AND SYSTEMIC TOXICITIES

One of the most common IrAEs is fatigue. According to different studies it accounts for 16% to 24% of all IrAEs.[5] In general, fatigue is relatively mild and does not have serious negative impact on daily activity and quality of life. Therefore, for most patients, no specific treatment is needed. Severe fatigue is rare for immunotherapy agents. If it does occur it is rational to exclude other fatigue-related causes like cancer progression or endocrine IrAEs.

Systemic toxicities such as fever and chills can be caused by cytokine release syndrome.[6] Usually it is mild and can be treated by anti-inflammatory agents and acetaminophen. Management of an infusion reaction depends on its grade and requires the use of antihistamines and/or corticosteroids, as well as a dose reduction or infusion discontinuation. Life-threatening consequences of this IrAE occur in less than 2% of patients.

DERMATOLOGIC/MUCOSAL TOXICITY

Rash or pruritus appears in approximately 40% of patients receiving PD-1 inhibitors. Usually it develops early in the course of treatment.[7] Rash can present in various forms such as spongiotic dermatitis-like eczema described as maculopapular, faintly erythematous, and pruritic vitiligo. Less common manifestations include lichenoid, eczematous, follicular, or psoriasiform, dermatitides, and bullous pemphigoid.[8] A clinician must rule out other etiologies like infection, an autoimmune condition, or side effect of a different drug. Diagnostic work-up includes patient history and physical examination, blood cell count, liver and kidney tests. In some situations, skin biopsy should be considered. Management of this IrAE depends on the type, affected body surface area (BSA), signs, response to initial treatment, etc. Diagnostic and treatment approaches are well summarized in the American Society of Clinical Oncology (ASCO) guidelines.[9]

1. Grade 1 implies close weekly monitoring without treatment discontinuation. Topical emollients, oral antihistamines, and/or mild-strength topical corticosteroids can be used.
2. Grade 2 also does not require interruption of immunotherapy, but close monitoring is necessary, and therapy should be stopped if symptoms are not resolving to grade 1. Therapeutic strategies include topical emollients, oral antihistamines, and median-to-high-strength topical steroids.
3. Grade 3 requires immediate pause in immunotherapy until the symptoms resolve to grade 1. Management includes the use of emollients, oral antihistamines, and high-potency topical corticosteroids. Initiating methylprednisolone (or equivalent) 1 to 2 mg/kg should be considered, depending on the severity of the symptoms.
4. Grade 4 demands discontinuation of immune checkpoint inhibitors, immediate hospital admission, and dermatologist consultation (with infectious disease specialist if necessary), as well as intravenous corticosteroids.[9]

Oral mucositis associated with immunotherapy can be a therapeutic dilemma especially in patients who received oral corticosteroids for management of other IrAEs. Oral candidiasis remains an important consideration in the differential diagnosis. In some patients improvement can be achieved with oral corticosteroid rinses and lidocaine.

DIARRHEA/COLITIS

Diarrhea and colitis (symptoms include abdominal pain, fever, nausea, vomiting, bloody stool) or radiographic findings of bowel inflammation are well-documented toxicities of immunotherapy agents. These toxicities are more common and better described for anti-CTLA-4 antibodies than for anti-PD-1 agents. Severe colitis occurs with anti-CTLA-4 drugs in up to 7% of patients compared to 1.8% of those receiving anti-PD-1 therapy.[6] Diagnostic work-up includes testing stool sample for *Clostridium difficile*, Salmonella, and other bacterial enteropathogens. Other causes of colitis may include viral infection like cytomegalovirus.[8] Patients with severe, stable, or progressive course should undergo computed tomography.[10] In case of uncertain diagnosis colonoscopy with biopsy should be considered.[2]

Treatment of immunotherapy-related diarrhea and colitis depends on the grade of toxicity.

1. Grade 1 with mild symptoms that last for less than one week can be managed symptomatically: antidiarrheals, bowel rest, adequate fluid, and electrolyte replacement. Short delay in immune checkpoints inhibitors therapy could be considered in individual cases.

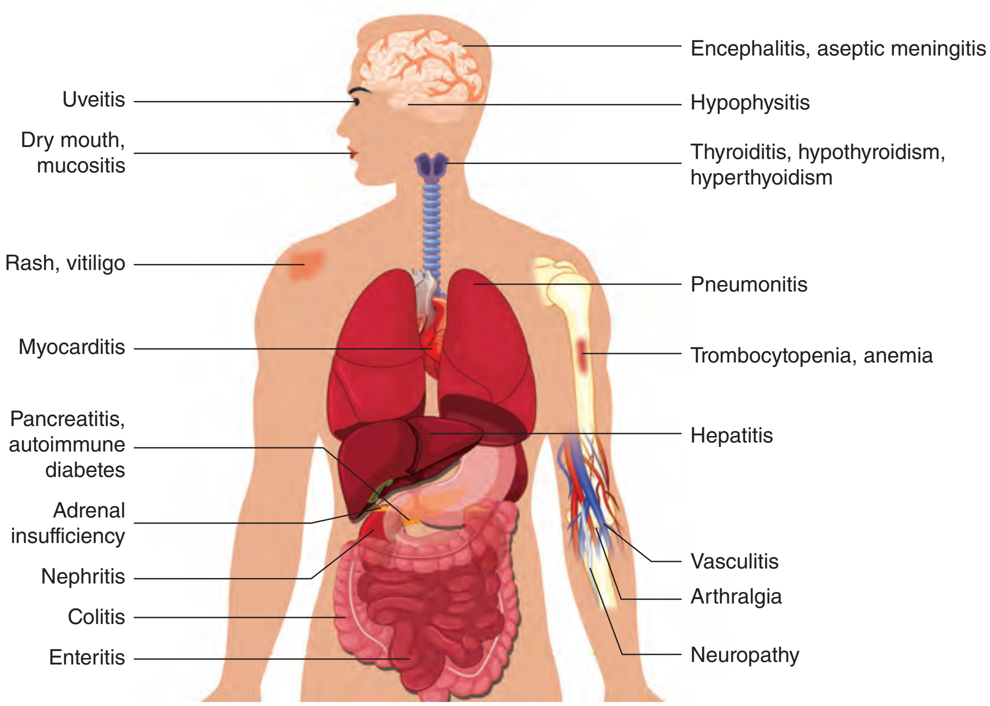


Figure 97.1 Adverse effects of immune checkpoint inhibitors.

2. Grade 2 (4 to 6 liquid stools per day over baseline or abdominal pain, or blood in stool, or nausea, or nocturnal episodes) usually does not require hospital admission, but sometimes is the reason to stop therapy, until the symptoms resolve to grade 1.
3. For severe forms (grade 3 or 4) described as more than 7 liquid stools per day or a life-threatening condition, therapy interruption, hospitalization, gastroenterologist consultation, and corticosteroids 1 to 2 mg/kg are mandatory. The tumor necrosis factor inhibitor infliximab can be used in patients who do not respond to intravenous corticosteroids within 48 to 72 hours.

In a D.H. Johnson et al. analysis, treatment with infliximab was associated with faster symptom resolution compared with corticosteroids alone. In the infliximab group higher-grade colitis were observed (grade 3/4: 86% vs. 34%; $P < .001$), but despite that, median time to diarrhea resolution (3 vs. 9 days; $P < .001$), steroid titration (4 vs. 13 days; $P < .001$), and total steroid duration (median 35 vs. 51 days; $P = .150$) were shorter than in the corticosteroid group (Fig. 97.2).[11]

HEPATITIS

Generally, hepatitis occurs within 8 to 12 weeks after the therapy initiation. According to different reports, immunotherapy-related hepatitis is more common for CTLA-4 than for PD-1 inhibitors (10% vs. 5% respectively).[12] It presents with asymptomatic elevation of aspartate aminotransferase, alanine aminotransferase (AST/ALT). An increase in bilirubin levels and fever are less common.[6,9,13] The grade of toxicity depends on AST/ALT or total bilirubin elevation. Diagnostic work-up includes complete blood count, comprehensive metabolic panel, and stool sampling (culture, *C. difficile* assay, ova and parasite exam). Testing for lactoferrin and calprotectin is optional. The differential diagnosis includes viral hepatitis, alcohol use, medication toxicities, and metastatic liver disease which should be ruled out by appropriate testing which may include[14] computed tomography and liver biopsy. Endoscopic evaluation is highly recommended for Grade 2 cases or higher to stratify patients for early treatment with infliximab based on the endoscopic findings and to determine the safety of resuming anti-PD-1/PD-L1 therapy.

Treatment options for grade 2 hepatotoxicity include withholding immunotherapy until symptoms resolution to grade 1, repeated liver function tests after 1 week, and, if rising, starting prednisone at a dose of 1 mg/kg. In the case of grade 3 toxicity with immune checkpoint inhibitors, therapy should be discontinued, with possible resuming if symptoms resolve to grade 1. If symptoms persist ≥3 to 5 days on oral corticosteroids, consider administering intravenous steroids. Monitoring of AST/ALT and total bilirubin should be done every 48 hours, switching to weekly testing following improvement, until they normalize. Patients refractory to intravenous corticosteroids are rare and they should be treated with mycophenolate mofetil (500 mg every 12 hours). Infliximab should be avoided in patients with elevated AST/ALT since infliximab can worsen hepatotoxicity.[9]

PNEUMONITIS

Pneumonitis is a rare but potentially fatal complication of checkpoint inhibitors. It is usually a therapeutic dilemma, because drug-induced pneumonitis is a diagnosis of exclusion, and other diagnoses, like infection and tumor growth, need to be ruled out.[12,15,16] This IrAE is reported more frequently with anti-PD-1 agents than with anti-CTLA-4 therapy. The median time of symptom manifestation is almost 3 months from initiation of therapy. Clinical presentation is diverse: patients can be asymptomatic or have progressive dyspnea, cough, wheezing, or even be in need of supplemental oxygen.[6] In the diagnostic algorithm, computed tomography is preferred over chest radiography.[12] In moderate and life-threating cases, bronchoscopy should be considered for diagnostic evaluation.

Treatment of immunotherapy-related pneumonitis depends on severity:

1. Mild pneumonitis may only require withholding of immune checkpoint inhibitors and subsequent computed tomography in 2 to 4 weeks.
2. Moderate cases demand immunotherapy interruption and prescription of corticosteroids (0.5 to 1 mg/kg daily).
3. Grade 4 requires complete discontinuation of immunotherapy, intravenous corticosteroids, and possible additional immunosuppression with infliximab, mycophenolate mofetil, intravenous

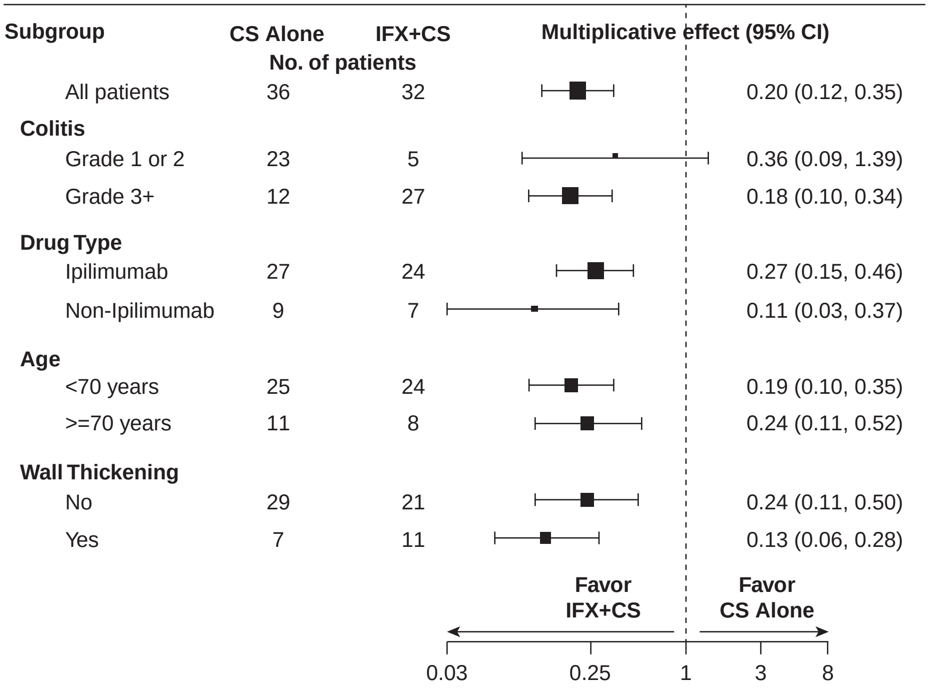


Figure 97.2 Univariate subgroup analysis for the multiplicative effect of irEC treatment (IFX plus CS vs. CS alone) on time to diarrhea resolution. *irEC*, immune-related enterocolitis; *CS*, corticosteroids; *IFX*, infliximab.

immunoglobulin, or cyclophosphamide. Up to 12% of patients with such severe IrAE are resistant to the treatment despite immunosuppression.[12]

ENDOCRINOPATHIES

Several types of endocrinopathies associated with immune checkpoint inhibitors have been reported, such as thyroid dysfunction, hypophysitis, rare primary adrenal insufficiency, and type 1 diabetes mellitus. These conditions could potentially require lifelong hormone replacement, so endocrinologist consultation is essential.[6]

Endocrinopathies manifest as inflammation of pituitary, thyroid, or adrenal glands and result in symptoms like fatigue, nausea, headache, and vision changes. The non-specific course makes these toxicities difficult to diagnose and appropriate treatment can be delayed. Based on available data, endocrinopathies occur in 10% of patients treated with checkpoint inhibitors, with hypothyroidism, hyperthyroidism, and hypophysitis being the most common. Hypophysitis is very rare in patients treated with anti-PD-1 and anti-PD-L1 agents.[17]

Patients who develop hypothyroidism are usually asymptomatic or have mild symptoms like fatigue. Hypothyroidism has an overall incidence of 6.6% and occurs more often with anti-PD-1 than with anti-CTLA-4 drugs.[18] Clinical signs of thyroid dysfunction can be noted with a median of 42 days after therapy initiation.[19] Thyrotropin levels should be monitored, and abnormal numbers are the signs to perform free triiodothyronine and thyroxine testing.[8,20] Management of symptomatic patients with thyrotropin levels greater than 10 mIU/L includes replacement therapy with levothyroxine and endocrinologist consultation. In some cases carbimazole or steroids can be required along with immunotherapy interruption until the symptoms resolve.

Type 1 Diabetes Mellitus

This is a rare complication of immunotherapy which occurs in less than 1% of patients. This IrAE is associated mostly with PD-1 and PDL-1 inhibitors.[21] Blood glucose should be routinely assessed prior to every cycle of therapy. Patients can have different clinical course, ranging from asymptomatic hyperglycemia to diabetic ketoacidosis.[22] Management of immunotherapy-related diabetes includes endocrinologist consultation, lifelong insulin therapy, and temporary withholding of immune checkpoint inhibitors until adequate glucose control is achieved.[9] Benefits of steroids are controversial: they *can* negatively influence diabetes control in these patients.

Rare Immune-Related Toxicities

Some of the IrAEs are extremely rare but worth mentioning due to their potential debilitating effects. These side effects include neurological, cardiac, rheumatological, renal, ocular, and hematological toxicities.

Neurological Adverse Events

Neurological adverse events are reported in 1% of patients receiving immune checkpoint inhibitors and include polyneuropathy, facial nerve palsy, demyelination, transverse myelitis, enteric neuropathy, myasthenia gravis, Guillain-Barré syndrome, and aseptic meningitis. Clinicians should follow general principles for immune-related neurological toxicity: for grade 1 checkpoint inhibitor therapy should be withheld until the nature of the adverse event is defined. For grade 2 to 3 oral corticosteroids should be considered and in case of significant neurological toxicity a switch to intravenous form is required.[23]

Cardiac Toxicity

The incidence of cardiac toxicity is less than 1% and it can present as myocarditis, pericarditis, arrhythmias or cardiomyopathy.[24] It is extremely important to involve a cardiologist into the decision-making process. Early administration of high-dose corticosteroids and immunosuppressive drugs in case of resistance-escalation should be considered.[23]

Rheumatologic Toxicity

Rheumatologic toxicity, which occurs in 2% to 12% of patients, includes myalgias and arthralgias, vasculitis, polymyositis, myositis, and temporal arteritis. Management of grade 1 to 2 (mild or moderate) toxicity is normally limited to paracetamol and/or NSAIDs. Moderate and severe symptoms require prednisolone at a dose of 10 to 20 mg/day or equivalent, and rheumatologist consultation in some clinical cases.[25,26]

Renal Toxicity

Renal dysfunction is rare and occurs in less than 1%. Usually renal toxicity develops 3 to 10 months into therapy.[27] The clinical presentation is not specific and may vary from asymptomatic to oliguria, hematuria, and peripheral edema. Serum sodium, potassium, creatinine, and urea should be measured before every cycle of immunotherapy.[6] Differential diagnosis should rule out contrast nephrotoxicity, volume depletion, or medications. In mild symptoms administration of corticosteroids is usually enough, but in some cases, immunotherapy should be temporarily discontinued. Severe forms of this IrAE require permanent immune checkpoint inhibitor discontinuation and nephrologist consultation.[9]

Hematologic Toxicities

Hematologic toxicity related to immune checkpoint inhibitors includes anemia, neutropenia, thrombocytopenia as well as rare complications like pure red cell aplasia, immune thrombocytopenic purpura, and disseminated intravascular coagulopathy. Cryoglobulinemia and acquired hemophilia A have also been reported.[14] Complete blood cell count should be taken before every treatment cycle. During diagnostics other secondary causes of cytopenias such as bone marrow involvement, hemolysis, medication-induced toxicity, gastrointestinal bleeding, etc., should be ruled out.[28] In case of clinical uncertainty, a bone marrow biopsy should be performed.[6] Common medical approaches used to manage hematologic toxicities are appropriate. This includes initial corticosteroid treatment and use of other immunosuppressive agents in patients refractory to steroids. A detailed algorithm of hematologic toxicities management is well summarized in the ASCO guidelines.[9]

Summary of Toxicity Management

Treatment of the immune-related adverse events is based on the severity of the observed toxicity.

- For grade 1 (mild) toxicity, patients should be carefully monitored during immunotherapy.
- For grade 2 (moderate) toxicity, immunotherapy should be withheld until the symptoms resolve to grade 1 or less. Prednisone 0.5 mg/kg/day or equivalent should be started if a patient is not getting better within a week.
- For grade 3 or 4 (severe or life-threatening) immune-related toxicity, treatment should be permanently discontinued, and patients should be started on high doses of corticosteroids (including intravenous form). In case of resistance to corticosteroid adding immunosuppressive agents is the next step. A proper specialist consultation as well as multidisciplinary evaluation are highly encouraged. Summary of recommendations for immune-related toxicities are well presented in Table 97.1.[23]

Patients-candidates for retreatment after prior immune-related toxicity should be carefully selected. Primarily, the choice should be

TABLE 97.1 Summary of Recommendations for Immune-Related Toxicities

Immune-Related Skin Toxicity

- For grade 1–2 skin AEs, continue (at least 1 week) with ICPis. Start topical emollients, antihistamines in the case of pruritus and/or topical (mild strength) corticosteroid creams. Reinitiate ICPi when grade 1.
- For grade 3 skin AEs, interrupt ICPi and start immediate treatment with topical emollients, antihistamines, and high-strength corticosteroid creams [II, B].
- For grade 4 skin AEs, discontinue ICPi (permanently), consider admitting patient and always consult dermatologist immediately. Start i.v. corticosteroids [1–2 mg/kg (methyl)prednisone] and taper based on response of AE [II, B].

Immune-Related Endocrinopathies

- In symptomatic hyperthyroidism patients, usually grade 1 or 2, interrupt ICPi, start beta-blocker therapy (propranolol or atenolol/metoprolol). Restart ICPi when asymptomatic [IV–V, B].
- In the case of hypothyroidism, rarely > grade 2, start HRT depending on the severity (50–100 lg/day). Increase the dose until TSH is normal. In the case of inflammation of the thyroid gland, start prednisone orally 1 mg/kg. Taper based on recovery of clinical symptoms. Consider interruption of ICPi treatment when symptomatic [IV–V, B].
- In the case of hypophysitis (rarely > grade 2), when headache, diplopia, or other neurological symptoms are present, start (methyl)prednisone 1 mg/kg orally and taper over 2–4 weeks. Start HRT depending on the affected hormonal axis (levothyroxine, hydrocortisol, testosterone) [V, B].
- In patients with type I DM grade 3 to 4 [ketoacidotic (sub)coma], admit to hospital immediately and start treatment of newly onset type I DM [I, A]. Role of corticosteroids in preventing complete loss of insulin producing cells is unknown and not recommended.

Immune-Related Hepatotoxicity

- For grade 2 hepatitis, withhold ICPi and monitor AST/ALT levels closely (1–2 times/week). When no improvement over 1 week, start (methyl)prednisone (0.5–1 mg/kg). Taper over several weeks under close monitoring of AST/ALT and bilirubin [IV–V, B].
- For grade 3 hepatitis, discontinue ICPi and immediately start with (methyl)prednisone 1–2 mg/kg. When no improvement in 2–3 days, add MMF (1000 mg 3_ daily). Taper immunosuppression over 4–6 weeks under close monitoring of AST/ALT and bilirubin [IV–V, B].
- For grade 4 hepatitis, permanently discontinue ICPi, admit patient to the hospital, and initiate (methyl)prednisone 2 mg/kg i.v. Add MMF if no improvement is observed within 2–3 days. Consult hepatologist if no improvement under double immunosuppression. Other immunosuppressive drugs to consider are ATG and tacrolimus. Consult or refer patient to an experienced center. Taper over 6 weeks under close monitoring of liver tests [IV–V, B].

Gastrointestinal Hepatotoxicity

- In patients with non-severe diarrhea (grade 1), ICPi can be continued. Treatment with antidiarrheal medication (e.g., loperamide) should be prescribed [IV–V, B].
- In grade 2 diarrhea, ICPi should be interrupted and the patient should start with corticosteroids depending on the severity and other symptoms (either budesonide or oral corticosteroids 1 mg/kg). In the case of no improvement within 3–5 days, colonoscopy should be carried out and, in the case of colitis, infliximab 5 mg/kg should be administered [IV–V, B].
- In patients with severe diarrhea (grade 3 to 4), permanently discontinue ICPi. Admit patient to the hospital and initiate (methyl)prednisone 2 mg/kg i.v.
- Add MMF if improvement is observed within 2–3 days. Consult a hepatologist if no improvement under double immunosuppression. Other immunosuppressive drugs to consider are ATG and tacrolimus. Consult or refer patient to an experienced center. Taper over 6 weeks under close monitoring of liver tests [IV–V, B].

Immune-Related Pneumonitis

- In grade 1 and 2 pneumonitis, interrupt ICPi therapy, try to rule out infection, and start with prednisone 1–2 mg/kg orally. Taper over 4–6 weeks [IV–V, B].
- In grade 3 and 4 pneumonitis, discontinue ICPi permanently, admit the patient to the hospital, even ICU if necessary, and immediately start high-dose (methyl)prednisone 2–4 mg/kg i.v. Add infliximab, MMF or cyclophosphamide in the case of deterioration under steroids. Taper over a period of 4–6 weeks [IV–V, B].

Continued

TABLE 97.1 Summary of Recommendations for Immune-Related Toxicities–cont'd

Immune-Related Skin Toxicity
Neurological Toxicity
• In the case of mild neurological AEs, withhold ICPi and perform work-up (MRI scan, lumbar puncture) to define nature of neurotoxicity. In the case of deterioration or severe neurological symptoms, admit the patient and start (methyl)prednisone 1–2 mg/kg orally or i.v. In the case of Guillain-Barré or myasthenia-like symptoms, consider adding plasmapheresis or i.v. Ig [V, B].
Cardiac Toxicity
• When a myocarditis is suspected, admit the patient and immediately start high-dose (methyl)prednisone (1–2 mg/kg). In the case of deterioration, consider adding another immunosuppressive drug (MMF or tacrolimus) [V, B].
Rheumatological Toxicity
• For mild arthralgia, start NSAIDs, and in the case of no improvement, consider low-dose steroids (10–20 mg prednisone). In the case of severe polyarthritis, refer patient to or consult a rheumatologist and start prednisone 1 mg/kg. Sometimes infliximab or another anti-TNFa drug is required for improvement of arthritis [V, B].
Renal Toxicity
• In case of nephritis, rule out other causes of renal failure first. Interrupt or permanently discontinue ICPi depending on the severity of the renal insufficiency. • Stop other nephrotoxic drugs. Start (methyl)prednisone 1–2 mg/kg. Consider renal biopsy to confirm diagnosis [V, B].

AE, Adverse event; *ALT*, alanine transaminase; *AST*, aspartate transaminase; *ATG*, anti-thymocyte globulin; *DM*, diabetes mellitus; *HRT*, hormone replacement therapy; *ICPi*, immune checkpoint inhibitor; *ICU*, intensive care unit; *Ig*, immunoglobulin; *i.v.*, intravenous; *MMF*, mycophenolate mofetil; *MRI*, magnetic resonance imaging; *NSAID*, nonsteroidal anti-inflammatory drug; *TNFa*, tumor necrosis factor alpha; *TSH*, thyroid-stimulating hormone.

based on the type and severity of previously developed toxicity as well as on responsiveness to systemic immunosuppression. Careful clinical evaluation is mandatory. In case of anamnesis of life-threatening adverse events (like myocarditis) during immunotherapy, the patient should not be re-offered retreatment. It was reported that retreatment is discouraged in those receiving prednisone 10 mg daily (or equivalent) or higher for treatment of an initial IrAE, because of prednisone association with reduced immunotherapy efficacy.[29] Dose reductions of immunotherapy agents are not recommended for retreatment, as this approach has not been assessed in clinical trials.

Peritransplant Period, Impact on Graft-Versus-Host Disease

Immunotherapy has been used as a bridge for allogeneic hematopoietic stem cell transplantation (allo-HSCT) and in the post-transplant period. Hodgkin lymphoma is the most common indication in patients receiving immune checkpoint inhibitors before and after allo-HSCT. According to available information, administration of immunotherapy in the pre-transplant period is associated with high rates of hyperacute (7%), acute (56%), and chronic (29%) graft-versus-host disease (GVHD). In the post-transplant period immunotherapy shows high efficacy but also increases the risk of GVHD (14%-acute, 9%-chronic).[30]

PATIENTS WITH AUTOIMMUNE DISEASE

In patients with a history of autoimmune disease or a prior reported IrAE, there is a concern of exacerbation of autoimmune disease, return of previously developed IrAEs, or appearance of new side effects. A small number of reports regarding patients with preexisting autoimmune disease are available, because they were excluded from clinical trials. There is an urgent need for prospective clinical trials for this special population. Decision-making should be based on the nature and severity of the autoimmune disease, the organ affected, goals of treatment, therapeutic alternatives, and multidisciplinary team discussion with a proper specialist.[31]

CONCLUSIONS

Immunotherapy has transformed the treatment landscape not only for solid tumors but also for hematologic malignancies. Immune-checkpoint inhibitors provide durable responses and bridge patients to stem cell transplantation. But this group of drugs has its unique toxicity profile which requires special diagnostic and therapeutic considerations. Serious adverse events associated with immunotherapy always need evaluation by the multidisciplinary team and with proper specialists. The following problems remain unsolved: A predictive model for immunotherapy-related toxicity, retreatment after preexisting toxicity, and the effect of immunosuppression on antitumor efficacy. Prospective clinical trials for special groups of patients (such as those with preexisting autoimmune disease) will give us new data and potentially create new therapeutic options for these cancer patients. Ongoing clinical trials expand indications for treatment and future perspectives look promising.

REFERENCES

1. Khoja L, Day D, Wei-Wu Chen T, et al. Tumour- and class-specific patterns of immune-related adverse events of immune checkpoint inhibitors: a systematic review. *Ann Oncol*. 2017;28:2377–2385.
2. Postow MA, Sidlow R, Hellmann MD. Immune-related adverse events associated with immune checkpoint blockade. *N Engl J Med*. 2018;378(2):158–168.
3. Boussiotis VA. Molecular and biochemical aspects of the PD-1 checkpoint pathway. *N Engl J Med*. 2016;375:1767–1778.
4. Wang DY, Salem JE, Cohen JV, et al. Fatal toxic effects associated with immune checkpoint inhibitors: a systematic review and meta-analysis. *JAMA Oncol*. 2018;4(12):1721.
5. Naidoo J, Page DB, Li BT, et al. Toxicities of the anti-PD-1 and anti-PD-L1 immune checkpoint antibodies. *Ann Oncol*. 2015;26(12):2375–2391. [published correction appears in Ann Oncol. 2016;27(7):1362].
6. Puzanov I, Diab A, Abdallah K, et al. Managing toxicities associated with immune checkpoint inhibitors: consensus recommendations from the Society for Immunotherapy of Cancer (SITC) Toxicity Management Working Group. *J Immunother Cancer*. 2017;5(1):95.
7. Belum VR, Benhuri B, Postow MA, et al. Characterisation and management of dermatologic adverse events to agents targeting the PD-1 receptor. *Eur J Cancer*. 2016;60:12–25.
8. Friedman CF, Proverbs-Singh TA, Postow MA. Treatment of the immune-related adverse effects of immune checkpoint inhibitors: a review. *JAMA Oncol*. 2016;2(10):1346–1353.
9. Brahmer JR, Lacchetti C, Schneider BJ, et al. Management of immune-related adverse events in patients treated with immune checkpoint inhibitor therapy: American Society of Clinical Oncology Clinical Practice Guideline. *J Clin Oncol*. 2018;36(17):1714–1768.
10. Marin-Acevedo JA, Harris DM, Burton MC. Immunotherapy-induced colitis: an emerging problem for the hospitalist. *J Hosp Med*. 2018;13(6):413–418.
11. Johnson DH, Zobniw CM, Trinh VA, et al. Infliximab associated with faster symptom resolution compared with corticosteroids alone for the

management of immune-related enterocolitis. *J Immunother Cancer*. 2018;6(1):103 Epub 2018 Oct 11.
12. Naidoo J, Wang X, Woo KM, et al. Pneumonitis in patients treated with anti-programmed death-1/programmed death ligand 1 therapy. *J Clin Oncol*. 2017;35:709.
13. Thompson JA. New NCCN guidelines: recognition and management of immunotherapy-related toxicity. *J Natl Compr Canc Netw*. 2018;16(5S):594–596.
14. Michot JM, Bigenwald C, Champiat S, et al. Immune-related adverse events with immune checkpoint blockade: a comprehensive review. *Eur J Cancer*. 2016;54:139–148.
15. Nishino M, Giobbie-Hurder A, Hatabu H, et al. Incidence of programmed cell death 1 inhibitor-related pneumonitis in patients with advanced cancer: a systematic review and meta-analysis. *JAMA Oncol*. 2016;2:1607.
16. Nishino M, Ramaiya NH, Awad MM, et al. PD-1 inhibitor-related pneumonitis in advanced cancer patients: radiographic patterns and clinical course. *Clin Cancer Res*. 2016;22:6051.
17. Torino F, Corsello SM, Salvatori R. Endocrinological side-effects of immune checkpoint inhibitors. *Curr Opin Oncol*. 2016;28:278–287.
18. Barroso-Sousa R, Barry WT, Garrido-Castro AC, et al. Incidence of endocrine dysfunction following the use of different immune checkpoint inhibitor regimens: a systematic review and meta-analysis. *JAMA Oncol*. 2018;4(2):173–182.
19. Osorio JC, Ni A, Chaft JE, et al. Antibody-mediated thyroid dysfunction during T-cell checkpoint blockade in patients with non-small-cell lung cancer. *Ann Oncol*. 2017;28(3):583–589.
20. Postow MA. Managing immune checkpoint-blocking antibody side effects. *Am Soc Clin Oncol Educ Book*. 2015:76–83.
21. Mellati M, Eaton KD, Brooks-Worrell BM, et al. Anti-PD-1 and anti-PDL-1 monoclonal antibodies causing type 1 diabetes. *Diabetes Care*. 2015;38:e137–e138.
22. Hughes J, Vudattu N, Sznol M, et al. Precipitation of autoimmune diabetes with anti-PD-1 immunotherapy. *Diabetes Care*. 2015;38(4):e55–e57.
23. Haanen JBAG, Carbonnel F, Robert C, et al. Management of toxicities from immunotherapy: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2017;28(suppl_4):iv119–iv142.
24. Johnson DB, Balko JM, Compton ML, et al. Fulminant myocarditis with combination immune checkpoint blockade. *N Engl J Med*. 2016;375:1749–1755.
25. Zimmer L, Goldinger SM, Hofmann L, et al. Neurological, respiratory, musculoskeletal, cardiac and ocular side-effects of anti-PD-1 therapy. *Eur J Cancer*. 2016;60:210–225.
26. Spain L, Diem S, Larkin J. Management of toxicities of immune checkpoint inhibitors. *Cancer Treat Rev*. 2016;44:51–60.
27. Wanchoo R, Karam S, Uppal NN, et al. Cancer and Kidney International Network Workgroup on Immune Checkpoint Inhibitors. Adverse renal effects of immune checkpoint inhibitors: a narrative review. *Am J Nephrol*. 2017;45(2):160–169.
28. Bulbul A, Mustafa A, Chouial S, Rashad S. Idiopathic thrombocytopenic purpura and autoimmune neutropenia induced by prolonged use of nivolumab in Hodgkin's lymphoma. *Ann Oncol*. 2017;28:1675.
29. Arbour KC, Mezquita L, Long N, et al. Impact of baseline steroids on efficacy of programmed cell death-1 and programmed death-ligand 1 blockade in patients with non-small-cell lung cancer. *J Clin Oncol*. 2018;36:2872.
30. Ijaz A, Khan AY, Malik SU, et al. Significant risk of graft-versus-host disease with exposure to checkpoint inhibitors before and after allogeneic transplantation. *Biol Blood Marrow Transplant*. 2019;25(1):94–99.
31. Kennedy LB, Salama AKS. A review of cancer immunotherapy toxicity. *CA Cancer J Clin*. 2020;70(2):86–104.

CHAPTER 98

PSYCHOSOCIAL ASPECTS OF HEMATOLOGIC DISORDERS

Hermioni L. Amonoo, Cynthia S. Peng, Rebecca M. Hammond, and Roxanne Sholevar

Major advances in cancer diagnostics and therapeutics have led to increased survival and improved health outcomes for people diagnosed with hematologic malignancies.[1] However, high levels of psychological distress (e.g., depression) and diminished positive psychological well-being factors (e.g., optimism) exist in this population for myriad reasons, including the cancer diagnosis itself, uncertain prognosis, prolonged treatment course that entails numerous hospitalizations with extended periods of isolation, and potential toxicities of treatment.[2] The life-threatening nature of hematologic malignancies also results in existential, social, and economic stressors which can also negatively impact psychological well-being (Fig. 98.1).[3]

Hence, this chapter describes factors that impact psychosocial well-being in patients with hematologic malignancies, the psychologic responses experienced by patients throughout the illness and treatment trajectories, the risk factors for psychological distress, and current evidence for psychosocial interventions that promote psychological well-being.

CLINICAL COURSE OF HEMATOLOGIC MALIGNANCIES

The incidence, course, treatment, and survival for various hematologic malignancies vary widely, yet dramatic improvements in survival rates have been seen almost universally.[1] In contrast to many solid cancers, treatment of hematologic malignancies often involves intense regimens, highly technical therapies, lengthy hospitalizations, high infection risk, periods of unpredictability, and frequent outpatient monitoring of the patient's condition.[2] Robust psychological well-being is essential for each unique aspect of the illness or treatment trajectory.[3,4] Although some patients with hematologic malignancies have the potential for cure via treatments such as hematopoietic stem cell transplantations (HSCTs), others have an initial reduction in tumor burden only to face relapsed disease. Yet, others do not respond to treatment, resulting in a progressive decline despite best efforts with various therapeutic measures. In a minority of patients, the cancer is so advanced at the time of diagnosis that they experience a rapid progression of their disease which results in death.

Time of Diagnosis

Being diagnosed with a hematologic malignancy can be a devastating time of crisis. The time of diagnosis has been described by a cancer survivor as "a lightning bolt through a stop sign" or an existential plight.[5,6] It is a time of intense distress and likened to a personal disaster in the patient's and family's life.[6] Often these patients have experienced vague symptoms such as fatigue for weeks or even months prior to diagnosis with treatment and reassurance for presumed non–life-threatening illnesses. Oftentimes, a large majority of patients may have had their concerns dismissed for many months, and then subsequently the ultimate diagnosis of an unexpected, catastrophic illness can feel even more blindsiding. Moreover, there are multiple personal and cultural factors that may profoundly influence the patient's response to their cancer diagnosis.[7,8] Being aware of the patient's exposure to cancer in the past (e.g., history of loved ones with a cancer diagnosis) and the meaning of their cancer experience may provide insights into their initial response.[8] Despite the inevitable psychosocial challenge of getting diagnosed with a hematological malignancy, it is well documented that many patients ultimately manage effectively with their diagnosis by employing a variety of coping mechanisms and skills.[9]

Although distress at diagnosis can sometimes translate into potential psychiatric diagnoses (e.g., mood disorders), most patients will not develop full-blown psychiatric disorders unless they likely had undiagnosed psychiatric symptoms prior to their cancer diagnosis.[10] Risk factors for a psychiatric disorder at the time of diagnosis include prior psychiatric history, gender, social and psychologic factors, disability, and avoidant coping.[10-14] Careful assessment of the patient's reaction is important, particularly as initial psychological reactions are often predictive of later adaptation and coping in survivorship.[15] Early assessment by clinicians can help to identify patients at risk for adjustment challenges and psychiatric disorders—these patients, in particular, will benefit from early psychological support (Box 98.1).[16–18]

Compared to patients with solid malignancies, patients with hematologic malignancies often need immediate oncologic treatment. The period from the time of diagnosis through the initiation of treatment is characterized by fast-paced medical evaluations and treatment planning—the rushed development of new relationships with unfamiliar medical personnel, and the need to integrate a barrage of information can be at best frightening and confusing.[2] During this time of diagnosis, many providers convey large amounts of information as part of treatment planning. It is not always easy for patients (especially those with limited health literacy) to assimilate the information, prioritize their problem-solving behaviors, and articulate their preferences. Consequently, providers often repeat information at each contact and inquire about the patient's and caregivers' understanding of facts and treatment options. Patients and their caregivers describe a state of numbness during this phase and that information is not thoroughly processed or understood—clinicians must consider this added barrier to communication when counseling patients and their caregivers.[19]

Clinicians have an important role in monitoring and possibly mediating psychosocial adjustment in presenting an initial diagnosis of cancer to patients. If patients have a clear explanation of their condition while encouraged to maintain hope, the initial reaction of shock, fear, and desperation can foster optimism about treatment. Clinician awareness of the unique meaning that patients ascribe to their cancer diagnosis is also vital. Patients who have a pervasive and unyielding negative affect that persists long after the crisis of diagnosis may require ongoing psychosocial monitoring and referral for services and supportive interventions throughout their treatment and disease course.[20]

Caregiver distress assessment during this diagnosis phase is also essential, especially as caregivers are a significant source of social support for patients during treatment and in recovery.[21] Studies have shown that caregivers share similar rates of psychosocial distress.[20,22] Psychological distress is easy to assess in patients and caregivers.[23] Hence, timely evaluation of distress and referral for psychological support during this period is critical to adequate coping throughout the patient's course of illness.

Treatment

Active treatment of cancer usually initiates another acute phase of the cancer experience. Psychosocial factors are critical parameters in

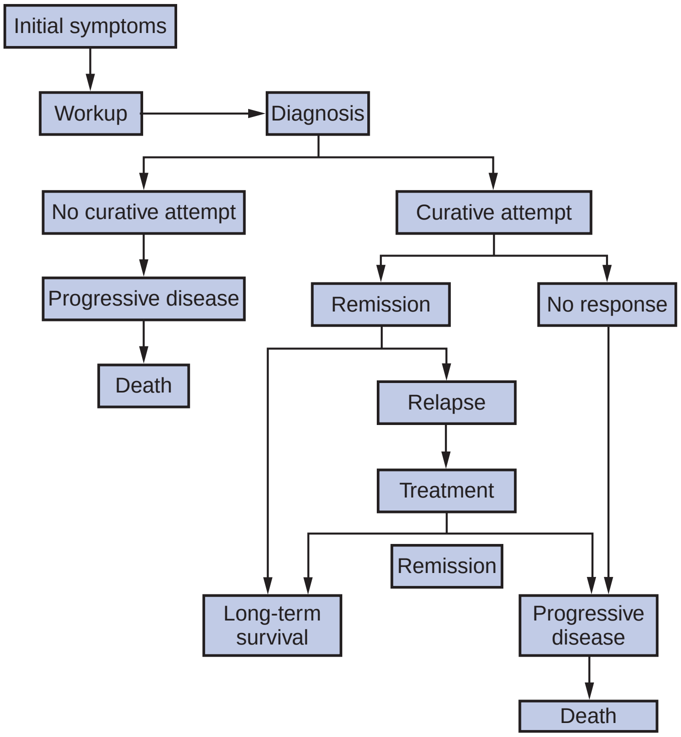


Figure 98.1 CLINICAL COURSE OF A PATIENT WITH A HEMATOLOGIC MALIGNANCY. *(Modified from Lesko LM. Hematopoietic dyscrasias. In: Holland JC, ed. Psychooncology. New York: Oxford University Press; 1998: 408.)*

BOX 98.1 Factors That May Predict Poor Coping in Patients With Cancer (Hematologic Malignancies)

Advanced disease
Higher regimen-related toxicity
Uncontrolled symptoms
Past psychiatric history
Avoidant coping (e.g., escape-avoidance, distancing, and denial)
Substance abuse history
Recent history of smoking
Compliance issues
Demographic factors (e.g., younger age and female sex)
Limited social support or difficult social relationships
Recent losses
Pessimistic outlook on life
Family or interpersonal obligations (e.g., childcare, eldercare)
Lower functional status

deciding which treatment is best for an individual patient and should be considered alongside all aspects of medical/surgical treatments in the development of the treatment plan. Empowering patients in their own treatment by involving them in decision-making can help to mitigate some aspects of the psychosocial distress. Often, patients react to a diagnosis of cancer with feelings of fear and helplessness. In this vulnerable state, patients may believe that complete reliance on the oncologist is necessary. A patient may look to the primary oncologist for a curative treatment that also can preserve their quality of life. Combating feelings of helplessness during this period can help patients alleviate anxiety and possible depression.[12] A member of the clinical team who has established treatment alliance with the patient is best poised to explore distress that could accompany treatment. A concerted effort to partner with patients while balancing patient autonomy during treatment planning can be beneficial. Providing patients and their caregivers with accessible information about their treatment options often alleviates anxiety and uncertainty in empowering them to feel more in control.

It is also important to engage caregivers and families during treatment planning. Caregivers play a crucial role during the treatment phase of cancer care since patients rely on their caregivers to adhere to different aspects of their treatment including follow-up visits, medication management, and maintaining quarantine status.[24] Assessing for economic, social, and relationship stressors among caregivers is also important as these external factors can exacerbate psychological stressors during the treatment phase. Patients and their caregivers should be made aware of the plethora of resources available for support, including patient and caregiver support groups.[25]

HEMATOPOIETIC STEM CELL TRANSPLANTATION

HSCT is a potentially curative treatment for many high-risk hematologic malignancies and nonmalignant diseases either as part of initial treatment or after relapsed disease. HSCT is complex with its own staggering morbidity and mortality, accompanied by significant distress on patients and their caregivers. The transplantation itself is followed by a variably extended recovery period which includes a prolonged 3- to 4-week hospitalization, periods of isolation, significant lifestyle changes, permanent loss of function in some cases, and changes in physical appearance. Psychological distress in HSCT patients is associated with worse health outcomes (e.g., increased mortality and higher risk of graft versus host disease [GVHD]) and psychological stress-related factors are thought to modulate cell engraftment and recovery following transplantation via the hypothalamic-pituitary axis and sympathetic nervous system.[26]

Because HSCT is an intense and distinctive experience for patients and caregivers, it has the potential to cause short- and long-term psychological distress. In recovery, patients face unique medical challenges, including recurrent infections and repeated hospitalizations for weeks to months during their initial recovery.[18] Consequently, a thorough pretransplant psychosocial evaluation is recommended to establish a psychiatric baseline, identify those patients at risk for development of psychological morbidity, and initiate timely psychological support and interventions during the immediate and long-term recovery following HSCT.

Identified risk factors for psychosocial morbidity associated with HSCT include psychiatric history, pretransplant non-adherence to medical treatment, non–transplant-related medical comorbidities, younger age, female sex, recent smoking cessation, lower functioning status on admission, quality of life deficits, perception of limited social support, and the presence of difficult relationships.[16,18] In particular, assessment of substance use and prior adherence with medical care are important in predicting patients' ability to consistently and reliably adhere to the demands and requirements of aftercare during HSCT recovery.

The decision to undergo HSCT can be its own source of psychological distress to patients due to the complexity and demands of the recovery. The existential issues of the anticipated loss of freedom and choice become immediately prominent when patients are presented with the option of HSCT. Despite best efforts by HSCT clinicians to explain probabilities of outcomes, patients often feel overwhelmed with the decision that is ultimately theirs alone. The patient's support network, not limited to family and/or friends may contribute to the burden of this decision-making anxiety, unknowingly or not, through expressing their opinions. Patients may also use their caregivers as objects on which to project their own anxieties and grief. An increased awareness of these factors by HSCT clinicians provides the patient a comfortable and safe space to process these anxieties.

Psychological Considerations in Hematopoietic Stem Cell Transplantation

Anxiety is usually heightened during the conditioning phase (or minus days) of HSCT due to anticipation of how the transplant would be tolerated.[10] The infusion of stem cells can be a special moment for patients, some of whom refer to the date of transplant as a "birthday" or special anniversary date. Some institutions offer a "blessing" of the stem cells by a member of the spiritual care team prior to the transfusion. Patients are sometimes surrounded by family, friends, and caregivers for the transplant. Attention should be paid to any special relationship between the patient and the marrow donor (i.e., related donors in allogeneic transplantations) as that can impact distress levels going into the transplant.

Patients are particularly vulnerable to psychological distress during the engraftment phase due to several factors. First, patients experience a high physical symptom burden including fatigue, nausea, anorexia, pain, diarrhea, and fevers, most of which are side effects from the conditioning regimen. Although some patients attribute these physical symptoms to "healthy" bone marrow, these physical symptoms heighten distress during the transplant hospitalization. Patients are most vulnerable to infection while awaiting engraftment, requiring strict isolation, limiting interactions with friends and family, all of which undoubtedly contribute to distress. Body image (e.g., hair loss, cachexia, steroid-related changes) and functional changes (e.g., severe fatigue, bedridden status, cognitive impairment in setting of delirium) are also sources of significant psychological distress; though these changes (at least in their present severity) are temporary, patients may experience them as a herald of permanent change. Hence, gentle reminders of the typical physical symptom burden associated with the transplant, especially those symptoms that are usually temporary, could reduce patient distress during the engraftment phase.

Khan and colleagues identified the following common psychiatric diagnoses among patients hospitalized for HSCT: adjustment disorder (40%), depression (23%), generalized anxiety disorder (10%), acute psychotic disorder (10%), delirium (10%), and depressive psychosis (7%).[27] Notably, undiagnosed and untreated pre-transplant psychiatric disorders constitute a significant proportion of psychiatric diagnoses identified during the transplantation process.[10] The prevalence of psychological distress that does not meet diagnostic criteria has been identified as even higher: depressive symptoms in 35%, delirium in 35%, and posttraumatic stress disorder (PTSD) in 20%.[10] Kishi and colleagues compared inpatient psychiatric consultations among transplant and nontransplant patients.[28] Transplant patients differed on several characteristics: more frequently White, less likely to have a previous psychiatric history, longer period between admission to consult, more delirium, and more socioeconomic and health-related distress.

Typically, patients perceive their quality of life to be worse during the first year post-transplant compared to their quality of life pre-transplant.[29] The psychological distress patients experience in the acute and chronic post-transplant phases may differ. The first 100 days after transplantation are demanding, with at least twice weekly outpatient visits, and isolation requirements (e.g., restriction from contact with large numbers of people or areas with crowds, including restaurants). Factors associated with distress in the acute post-transplant phase include persistent physical symptoms, slow physical recovery, increased treatment-related toxicity, body image disturbance, and lower performance status.[10,30] In addition, patients often grapple with the fear of relapse and/or secondary malignancies. The post-transplant period could also entail complex medication regimens (e.g., steroids, tacrolimus) that have neuropsychiatric sequelae including but not limited to mood disturbance, anxiety, psychosis, neurocognitive dysfunction, and posterior reversible leukoencephalopathy syndrome. A comprehensive discussion of the neuropsychiatric complications of HSCT is beyond the scope of this chapter.[31]

The chronic post-transplant phase comes with its own set of complications. Although most patients gradually improve their performance status, there is often discordance between the patients' pre-HSCT expectations and the everyday symptoms that limit their physical abilities. Distress associated with cognitive dysfunction is also repeatedly reported by patients as they return to the workplace or reenter school.[25] Educating patients about common short-term neurocognitive side effects can reduce anxiety. Further, neurocognitive side effects of treatment (e.g., diminished concentration, short-term memory loss, decreased speed of information processing, and loss of effective problem-solving abilities) can be long term in high-risk patients but are mostly temporary. Ongoing physical and cognitive impairment often impact longstanding roles in relationships and vocations that can unleash existential anxieties related to isolation and meaning.

Sexual dysfunction is a common long-term side effect of HSCT in the setting of high-dose cytotoxic chemotherapy and total-body irradiation.[32,33] Commonly reported sexual symptoms among HSCT patients include decreased libido, vaginal alterations, erectile and ejaculatory dysfunction, premature menopause, dysregulation of sexual hormones, dyspareunia, and infertility.[34,35] Despite these known symptoms, sexual side-effects are inadequately assessed and managed in patients undergoing HSCT. Clinicians' proactive approach to exploring these problems will help reduce patients' embarrassment about discussing sexual issues and function. Barriers for discussion and lack of referrals for supportive services in this area may be related to the patient's embarrassment, the clinician's lack of knowledge, or the focus on other issues that may be interpreted as more critical.

The financial burden for patients who undergo HSCT can be overwhelming, including medical expenses for the patient and marrow donor in the case of an allogeneic HSCT, potential travel expenses, and loss of income for other caregiving family members.[25] Patients and their immediate families are often geographically distant from their usual support systems due to the distance from their home to the HSCT center. In some cases, family members who have not been close in the past may be forced to interact with each other, leading to additional stress.

Researchers have described the following factors as predictors of poorer quality of life in patients 1 to 5 years after transplant: diagnoses of anxiety and/or depression, younger age, long-term sequelae, chronic GVHD, unemployment, lower income, poor functional status, family/caregiver distress, and short follow-up by the treatment center.[29,36,37] Psychosocial factors have been shown to directly impact medical outcomes. Patients considered higher psychosocial risk had higher readmission rates 90 days after transplant. Patients who experience depression in the first year also have a higher mortality rate.[38]

The American Society for Blood and Marrow Transplantation (ASBMT) consensus statement recommends annual evaluation of patients' psychological status. Health care providers must have a high level of vigilance to assess depression in both the patient and caregiver years after transplant, with clinical assessments recommended annually after transplant.[39] One study reported that 43% of long-term HSCT survivors with an average of 3.4 years after transplant had clinically significant global psychologic distress.[40] Despite this distress, only 50% of the patients received mental health services.[37] Patients who have undergone HSCT are at a higher risk of PTSD, which has recently been reclassified to include life-threatening medical illness and treatment as qualifying stressors. The prevalence of PTSD in patients who have undergone HSCT is 20% and does not correlate to patient age. PTSD symptoms are similar to patients who do not have cancer, and frequently include nightmares and emotional hypersensitivity.[20]

The concept of posttraumatic growth (PTG) after transplantation has been evolving. By definition, the potential for PTG requires that patients experience a stressful event and then subsequently experience positive psychologic outcomes or benefits. As early as 1996, Fromm identified that positive sequelae are possible after transplantation, including the development of a new philosophy of life, greater appreciation of life,[41] making changes in personal characteristics, and improving relationships with family and friends.[42] Potential predictors of PTG among patients who are post-HSCT include good social support, little avoidant coping, younger age, less education, greater use of positive reinterpretation, problem solving, seeking alternative rewards, more stressful appraisal of the experience, and more negatively biased recall of pretransplant levels of psychologic distress.[17,43] Discussing the PTG potential with patients and making referrals for counseling to experienced clinicians who are aware of the potential for PTG are essential.

Although patients report long-term psychosocial effects after transplantation, they may be reluctant to accept help and fail to access psychological resources and social support.[44] These patients must be encouraged to use resources and seek psychological support because this experience may impair the patients' and families' ability to cope with life after transplant. In an article about HSCT patients' experiences with a support group, Sherman and colleagues identified the themes of meaning and changing of perspectives as patients expressed their struggles with redefining themselves, their priorities, and their values.[25] Some patients wanted to change their former values and behaviors. The support group experience may be therapeutic for patients who often do not have physical signs of transplant to the untrained eye but continue to experience increasing or unresolved psychological and physical issues.[16,24] Sharing a common experience may encourage patients to believe that their symptoms and feelings are not unique and may decrease their feelings of isolation.

Immediate Period Following Diagnosis and Treatment

As the treatment and acute side effects improve and subside, patients often feel that the whirlwind has passed, only to be confronted with an uneasy silence. Weeks and months of clinic and physician appointments, infusions, and admissions stop or trickle to a small stream of appointments. Families who have been functioning on a grinding schedule of crisis mode find the change almost paralyzing. Adding to this halt of activity, health care providers have a tendency to limit their contacts when the patients' physical status has stabilized. This is a critical time when psychosocial interventions and supportive services from other members of the health care team must be instituted for patients and family members to deal with the uncertainty and anxiety of waiting. Fears and anxieties change from fighting the disease to returning to life.[45] Due to the decreased amount of contact with the primary treatment hematologists, patients and families may perceive a withdrawal of support from the medical team. Long-term psychosocial health of the patient and family is affected by the meaning ascribed to the cancer experience, fear of recurrence, support of the family, demographic factors, and financial stressors. This transition time is pivotal for patients' long-term quality of life. Surveillance with specific questions to access the need for referrals can have lasting positive outcomes.

Unlike other patients with cancer, patients with hematologic malignancies who have undergone HSCT are especially vulnerable in the early chronic recovery phase because most patients have to still grapple with integrating into life with some restrictions about activities as their immune system fully recovers. Additionally, some patients still experience anxiety about returning to their baseline level of activity and interacting with their environment due to persistent fear around the higher risk of infection.

Time of Relapse

For many patients, disease relapse represents a more aggressive malignancy than anticipated and can be more threatening and psychologically tasking than the initial diagnosis with cancer.

With the progression of the disease, patients often report upsetting experiences of uncertainty, frequent pain, diminished function, decreased independence, and disability.[46] The medical workup itself can be difficult and anxiety provoking, and psychosocial problems experienced at the time of diagnosis frequently resurface, often with greater intensity.[46] The shock and depression that can accompany disease relapse forces patients and their caregivers to reevaluate the future. In spite of the overwhelming nature of the psychosocial responses, however, most patients cope effectively with disease relapse and intense emotions do not necessarily equate with maladaptive coping.

An essential goal in the psychosocial treatment of patients with advanced cancer is optimal symptom management with robust evidence suggesting symptom management is positively correlated with quality of life.[47,48] However, symptom management can be less prioritized as despairing patients and their families prioritize fighting the disease with aggressive treatment. Patients and families often request participation in experimental protocols, even when there is little likelihood of extending survival. Controversy continues about the efficacy of such therapies and the role health care providers can play in facilitating patients' choices about treatment options. Clear communication about treatment goals and expectations is essential between patients and their care team. Robust evidence suggests that most patients and their families prefer timely and realistic information about their prognosis. However, most patients with aggressive malignancies do not initiate conversations about their treatment intent and end-of-life care goals.[49]

Survivorship

Successful treatment of hematologic malignancies has resulted in a cure for many patients and progressively longer lives for others. Overwhelming evidence from the literature involving survivors with hematologic malignancies suggests that, overall, most do very well after the initial adjustment in the first 1 to 3 years after treatment.[29,50] Although most HSCT survivors express satisfaction with their quality of life, psychosocial challenges certainly exist, and it behooves clinicians to consider these challenges in order to deliver the best care possible and to help patients know what to expect in this phase.[51,52]

The initial transition from patient to survivor can be difficult. Individuals undergoing this transition express common themes in their distress.[53] Fear of recurrence is the most commonly identified concern that colors the transition to survivorship and beyond.[54] It can dominate the lives of both patients and their families and has been shown to be among the strongest predictors of quality of life 1 to 5 years after treatment.[36,47,55-57] Concurrent family dynamics, family stressors, and patient's employment status have also been shown to be strong predictors of quality of life in this period, underscoring the importance of addressing the patient's broader psychosocial context.[58]

Other psychologic challenges of survivorship may include concern over termination of treatment, preoccupation with somatic symptoms, reentry into previous life roles, existential distress pertaining to one's mortality, and difficulties with finances, employment, and insurance. Many patients may be apprehensive about reduced interaction with their clinical team after the constant support they received during chemotherapy and radiation—they may feel suddenly left "on their own" after treatment ends. The nature of social support may also impact patient's perceptions of their anxieties regarding care in the survivorship phase. Patients who have less social support are more likely to feel overwhelmed by the prospect of not having frequent interaction with their clinical teams. Clinicians should proactively discuss these transitions with patients to help patients with realistic expectations of what their needs should be.

Lingering physical symptoms or side effects of treatment may cause further distress as cancer ceases to be the focus of survivors' lives but rather functional limitations that result from the treatment. Acceptance of a new "normal" in terms of level of physical ability and function can be a source of distress—patients should be educated about what to expect as a way to help them adjust to their new "normal." Despite advances in cancer therapeutics, surgical, chemotherapeutic, and radiation therapies can leave permanent damage to organs and physiologic functioning and disfigurement across the different hematologic diagnoses (Table 98.1). Cancer-associated treatments may have resulted in persisting unintended physical side effects such as infertility, treatment-related toxicity, and organ system failure.[47,48,59] In younger cancer survivors, concerns about infertility are particularly distressing and should be addressed adequately.[60] In a study of 250 patients with non-Hodgkin's lymphoma, Hammond and colleagues report that over half of study subjects felt they have not received adequate information about fertility issues, underlying the importance of educating patients regarding what to expect from the sequelae of their treatment.[61]

Psychological distress symptoms persist into short- and long-term survivorship.[50,62–66] As many as 9% to 30% of long-term survivors of hematologic malignancies experience significant symptoms of distress (e.g., anxiety, depression, and PTSD symptoms).[63,65,67] Patient,

TABLE 98.1 Long-Term Consequences of Diagnosis and Treatment of Hematologic Cancers

Anxiety
Depression
Fear of recurrence
Conditioned nausea and vomiting
Disruption in sexual functioning
Fertility issues
Disfigurement
Unemployment
Denial of life insurance
Increase in life insurance rates
Denial of health benefits
Difficulty changing health care coverage
Breakdown of marriage or relationship
Diminution of support from others
Decline in participation in leisure activities

disease, and medication factors contribute to the risk of psychiatric comorbidities in patients with hematologic malignancies.[68] Among patient-related factors, age predisposes patients to certain psychiatric illnesses such as delirium—older patients are more at risk compared to younger patients.[69] Women are at increased risk of mood disorders such as depression and anxiety.[70] A history of mental illness also contributes to the risk of developing psychiatric comorbidities.[71] With respect to PTSD in particular, studies have consistently described a higher incidence of this disorder in patients with hematologic malignancies than in the average population.[67] Higher distress levels and avoidance coping styles have been found to be useful predictors of PTSD severity.[17,43,72] Social support impacts various health outcomes such as quality of life and functioning for patients with hematologic malignancies—inadequate social support can also contribute to distress.[73,74] Sociodemographic factors such as lower educational levels and overall lower socioeconomic status result in a greater predisposition to emotional problems.[71]

For disease and treatment factors, a more aggressive hematologic malignancy diagnosis (characterized by biological and genetic markers) and relapsed disease results in a higher risk for psychological distress (especially anxiety) and psychiatric comorbidities.[75] A higher burden of medical comorbidities and complications (e.g., hospitalizations) also results in a higher level of emotional distress.[76-78] With the progression of disease, patients often report distressing experiences of uncertainty, frequent pain, diminished functional ability, increased dependence, and disability.[46] Among patients with hematologic malignancies, those who undergo HSCT, are especially vulnerable to psychiatric comorbidities due to the intense nature of the treatment, prolonged hospitalizations, and potential medical complications (e.g., GVHD) in the short- and long-term recovery from the transplant.[10] Unfortunately, symptoms of distress often go untreated in this population. Hence, routine screening and assessment of psychiatric symptoms in this population are essential to ensuring that patients receive adequate support.

Terminal Stage and End-of-Life Care

Technological advances in health care have improved the potential for a cure of many previously fatal hematologic malignancies. However, many patients still have an incurable disease. When a cure is acknowledged to be impossible and alternative efforts to combat the progression of disease are exhausted, patients are considered terminally ill or dying. While some clinicians advocate for palliative and end-of-life care at this phase of the treatment with the emphasis on forgoing active treatment and emphasizing symptom management,[55,79,80] the paradigm has shifted in the field of palliative care to initiating palliative care alongside curative treatment. In fact, palliative care should be implemented across the illness trajectory—the ultimate goal is to improve the quality of life and facilitate relief of suffering over the course of one's illness.[81] The new World Health Organization definition states: "Palliative care is an approach to care which improves the quality of life of patients and their families facing life-threatening illness, through prevention, assessment, and treatment of pain and other physical, psychologic, and spiritual problems."[82] While evidence supports that palliative care can reduce mortality in addition to improving quality of life, the majority of research is for patients with solid malignancies.[83] Increasingly, research has demonstrated the feasibility, and efficacy of integrating palliative care to improve the quality of life and care of patients with hematologic malignancies and their caregivers.[84]

Despite the high burden of physical and psychological symptoms in patients with hematologic malignancies because of the disease and intensive treatments, there is poor integration of palliative care services in their treatment course. Indeed, as compared with patients with solid malignancies, palliative care clinicians are seldomly consulted for patients with hematologic malignancies.[84] The unmet palliative care needs in this population also extend to survivors who often struggle with post-treatment complications and symptoms of psychological distress which jeopardizes function and quality of life. Evidence supports that illness-specific, cultural, and system-based barriers to optimal end-of-life care and palliative care assimilation exist.[84] Traditionally, patients referred to palliative care are not eligible for curative therapies. Hence, since most patients with hematologic malignancies are candidates for curative therapies such as HSCT or chimeric antigen receptor T-cell (CAR-T) therapy, a clear transition between the curative and palliative phases of treatment is lacking—this ambiguity is a barrier for the timely referral to palliative care.[84] Some of the cultural barriers to palliative care in this population include transplant clinicians' limited knowledge about the benefits of palliative care and their perception that coordination of patient care across all phases of their illness and treatment trajectories should be managed by the oncologist.[85] Overall, the lack of data to support the role of palliative care for this patient population also contributes to transplant clinicians' hesitation to partner with palliative care clinicians for symptom management and end-of-life care conversations. Further, hospice care has not been integrated into the care of patients dying with hematologic malignancies.[86,87] Despite the robust evidence that most terminal patients prefer to spend their final days of life and dying at home, a significant proportion of patients with hematologic malignancies still die in healthcare facilities other than a hospice.[88-90] Respecting patient and family wishes, appropriately managing and alleviating distressing symptoms, and providing care tailored to meeting patients' needs can help patients dying of hematologic malignancies reach the end of life with peace and dignity.

FACTORS THAT INFLUENCE PSYCHOSOCIAL ADJUSTMENT

Psychosocial responses to cancer vary widely and are influenced by several factors that clinicians should bear in mind when considering the responses of individual patients. These factors can be conceptualized as falling into the broad categories of patient, environmental, and disease-related factors.

Patient Factors

Patient demographics, comorbid conditions (including psychiatric disease history), and coping strategies have been consistently documented as important predictors for psychosocial adjustment. Specific personality characteristics, coping strategies, and life experiences also enhance or inhibit positive adjustment to cancer.[91,92] A person's coping style is a useful predictor of adjustment to cancer, as coping style tends to remain stable over time and across situations. Coping strategies and personality attributes can also positively impact long-term survival.[93-95] Active coping strategies including having a "fighting spirit," hopefulness and acceptance of a challenging situation, belief that life has purpose and coherence, and having a feeling of control over events have been positively associated with active participation in treatment and engagement in daily life. By contrast, poor adjustment has been associated with avoidant coping strategies, anxious preoccupation and high distraction, prior negative sexual experiences, body image problems, and inhibition in discussing personal and sexual problems.[96]

Psychologic stability before a diagnosis is a key predictor of psychosocial adjustment to cancer. People with a history of poor psychosocial adjustment before the development of cancer are at the highest risk for psychologic decompensation and should be monitored closely throughout all phases of treatment.[18,97] This is particularly true of people with a history of major psychiatric disorders, psychiatric hospitalization, or both.[18]

Demographics also appear to impact psychosocial adjustment, though the interrelation of individual patient factors can make it difficult to draw conclusions regarding the effect of any specific patient factor on how a given patient will adjust psychosocially to a hematologic malignancy. Nonetheless, demographic factors can significantly impact psychosocial adjustment and should be considered in treatment decision-making.[98,99] In patients diagnosed with hematologic malignancies, female sex and younger age are both associated with worse psychosocial adjustment and poorer quality of life.[16,100-102] This association could at least in part be due to the interrelated factor of anxiety level—females tend to have higher anxiety scores than males. In patients receiving treatment, on the other hand, quality of life seems to be worse with higher age of the recipient at the time of the transplant, poorer self-image, decreased cognitive functioning, and inability to return to work.[103,104]

Finally, smoking status can also impact psychosocial adjustment. One study showed that patients who smoke are at higher risk for psychiatric morbidity, perhaps due to the potential development of depression and/or anxiety with withdrawal symptoms.[105] It is therefore important to include smoking history in the patient's assessment of substance control use when preparing patients for treatment options.

Environmental Factors

Social support contributes significantly to the environment of a patient with cancer. The quality of spousal and familial relationships has a substantial impact on clinical outcomes in the cancer population.[74,106] Social support has consistently been found to influence a person's psychosocial adjustment to cancer.[88,89] Patients who are able to maintain close connections with family and friends during the course of illness are more likely to cope effectively with the disease than those who are not able to maintain such relationships.[86,95,107]

Often the transition out of the hospital post-transplant can be a fearful time for patients and their caregivers. Caregivers frequently serve as the major providers of care outside the hospital, and this can be an overwhelming responsibility.[21] In these situations, referral for home care can provide significant relief of the burden on these family members, in turn helping strained relationships and improving the environmental circumstances for the patient.

Disease-Related Factors

Disease-related factors including the type of disease, side effects of treatment, and residual symptoms all greatly affect psychosocial adjustment. Most disease-related factors are mediated via treatment modalities which introduce varying degrees of side effects, symptoms, and impact on quality of life. Patients who undergo HSCT have a high burden of distress symptoms and adjustment difficulties due to the toxic side-effects of treatment, prolonged hospitalizations, and high risk of life-threatening complications in both acute and long-term recovery. Predictably, HSCT recipients have more psychiatric morbidity if they experience lower functional status and higher regimen-related toxicity.[18] Time interval after treatment is also an important predictor for psychosocial adjustment. Particularly, time since HSCT or completion of treatment appears to be an important factor for facilitating psychosocial adjustment and improving quality of life. In general, during the first year after HSCT, patients perceive their physical and overall well-being as being at its worst, during which time they experience more anxiety and total mood disturbance, as well as report the highest degree of illness intrusiveness in every aspect of life.[29,59,108-112] With the passage of time, improvements in functional status, quality of life, levels of anxiety, depression, and satisfaction with life are often observed.

DIFFERENTIATING PSYCHIATRIC COMPLICATIONS FROM EXPECTED PSYCHOLOGIC RESPONSE

Most patients with hematologic malignancies experience transient symptoms of distress that are responsive to support, reassurance, and information about what to expect regarding the cancer course and its treatment. Living with a chronic illness often requires continuing care and management by a team of specialists. The use of supportive care services has been related to improved quality of life; therefore, it is in the patient's best interest to access services needed for psychologic distress early in the continuum of care.[107] Differentiating a psychiatric complication from expected psychologic responses is imperative. Although the crisis of a cancer diagnosis may result in symptoms of distress, most symptoms do not meet the criteria for a psychiatric disorder. Collaborating with mental health clinicians is essential to the timely diagnosis and treatment of psychiatric comorbidities in this population. Unfortunately, non–mental health providers often miss clinically relevant and severe psychiatric syndromes. Table 98.2 provides a quick reference to common symptoms of distress; also refer to the *Diagnostic and Statistical Manual of Mental Disorders*.

A history of severe mental illness including schizophrenia, bipolar disorder, schizoaffective disorder, and obsessive-compulsive disorder also increases the risk for psychiatric comorbidity in the care continuum. More crucially, cancer mortality is two to four times higher among individuals with mental illness than in the general population.[113] Patients with severe mental illness often report a history of disregard and neglect from providers.[114] Hence, ongoing collaboration between the oncological and psychiatric clinical teams is critical in caring for patients with active psychiatric illness—first to stabilize the psychiatric symptoms and then to treat the cancer.[115,116]

The following guidelines can help identify patients who are especially at risk of developing psychiatric symptoms that require aggressive psychotherapeutic management by a psychiatrist.

1. History of psychiatric hospitalization or significant psychiatric/personality disorder.
2. Persistent refusal, indecisiveness, or noncompliance with regard to needed treatment.
3. Persistent symptoms of anxiety and depression that are unresponsive to usual support from health care providers or family members; symptoms may present in the form of constant fear associated with treatment and procedures or excessive crying and feelings of hopelessness that worsen rather than improve with time.
4. Abrupt, unexplained change in mood or behavior.
5. Insomnia, anorexia, diminished energy out of proportion to expected treatment effects.
6. Persistent suicidal ideation.
7. Unusual or eccentric behavior or confusion (may be indicative of an organic mental disorder).
8. Excessive guilt and self-blame for illness.
9. Evidence of dysfunctional family coping or complex family issues.

TABLE 98.2 Common Psychiatric Disorders

Psychiatric Disorder	Etiology	DSM-5 Criteria
Adjustment Disorder	The development of emotional or behavioral symptoms in response to an identifiable stressor; occurs within 3 months of the stressor and lasts less than 6 months after the stressor has ended; does not represent normal bereavement	1. Marked distress that is in excess of what would be expected for exposure to the stressor 2. Significant impairment in social or occupational functioning
Major Depressive Disorder (MDD)	Symptoms that are present for a 2-week period and a change from previous functioning; symptoms cause clinically significant distress or impairment in functioning; not attributable to the physiological effects of a substance or other medical condition	Five or more of the following in the same 2-week period: 1. Depressed mood most of the day 2. Marked diminished interest or pleasure 3. Significant weight loss or decrease or increase in appetite 4. Insomnia or hypersomnia 5. Psychomotor agitation or retardation 6. Fatigue or loss of energy 7. Feelings of worthlessness or excessive guilt 8. Diminished ability to think or concentrate 9. Recurrent thoughts of death, suicidal ideation, or a plan for suicide
Generalized Anxiety Disorder (GAD)	Excessive worry and anxiety for at least 6 months about a number of activities; the person finds it difficult not to worry; causes impairment in function; not attributable to another mental disorder, substances, or medical condition	Anxiety and worry are associated with three of the six following symptoms: 1. Restlessness 2. Fatigue 3. Difficulty concentrating 4. Irritability 5. Muscle tension 6. Sleep disturbance
Posttraumatic Stress Disorder (PTSD)	Exposure to traumatic events (actual or threatened death, serious injury, violence) with presence of intrusion symptoms associated with the traumatic event; persistent avoidance of stimuli associated with the traumatic events; with significant impairment in function	Experiencing the following symptoms for >1 month after the traumatic event 1. Intrusion symptoms (e.g., nightmares, flashbacks, emotional distress, physical reactivity) 2. Avoidance of trauma-related stimuli 3. Negative thoughts or feelings that began or worsened after the trauma 4. Marked alterations in arousal and reactivity (e.g., irritable behavior, angry outbursts, reckless/self-destructive behavior, exaggerated startle response, sleep disturbance)

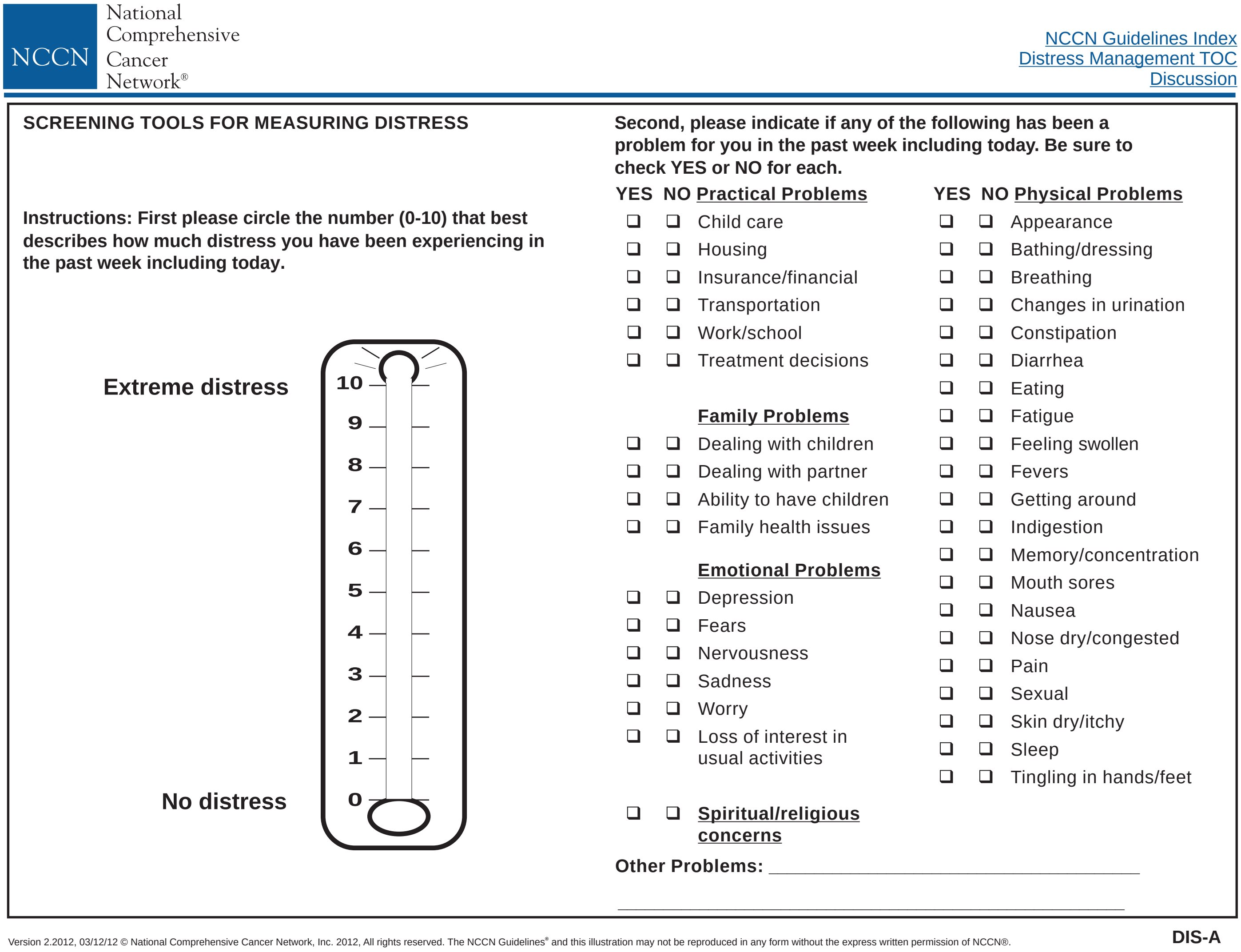
Data from American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders*. 5th ed. (DSM-5). Washington, DC: APA; 2013.

SCREENING FOR PSYCHOLOGIC DISTRESS

A number of tools have been developed to screen for psychological distress, but adoption and consistent incorporation into clinical care has been slow.[117,118] The National Comprehensive Cancer Network (NCCN) guidelines have been updated to work towards implementing standard distress screening. These guidelines now clearly advocate for distress to be recognized, monitored, documented, and treated promptly at all stages of disease.[119] In a strong show of support for distress screening, the American College of Surgeons Commission on Cancer has required cancer centers to implement screening programs for psychosocial distress as a criterion for further accreditation.[120]

One tool that is easy to administer and that patients have reported as satisfactorily capturing their problems is the *Distress Thermometer*.[121] The term "*distress*" was selected as it was felt to be more acceptable and less stigmatizing than other related terms (e.g., psychiatric, psychosocial, emotional). The tool, which consists of two parts, is similar to pain measurement scales that ask patients to rate their pain on a scale from 0 to 10. The first part is a picture of a thermometer, upon which patients are asked to mark their level of distress. A rating of 4 or above indicates that a patient has symptoms indicating a need for evaluation by a mental health professional and potential referral for support services. On the second part, the patient marks items that relate to his or her distress from a six-item problem list (Fig. 98.2).[121,122] Multiple studies have validated the use of this distress thermometer in various oncologic settings including HSCT, and it has been found to be a useful tool for screening HSCT recipients before their transplant hospitalization.[123,124] Pre-transplant distress appears to be highly predictive of distress following transplant, though controversy remains as to whether screening for pre-transplant distress and structuring care accordingly makes a difference in post-transplant distress.[125]

A diagnosis of major depression in medically ill patients relies heavily on the presence of affective symptoms such as hopelessness, crying spells, and guilt; preoccupation with death or suicide; feelings of diminished self-worth; and loss of pleasure in most activities, such as being with friends and loved ones. The neurovegetative symptoms that usually characterize depression in physically healthy individuals are not good predictors of depression in the medically ill because disease and treatment can also result in similar symptoms. Additionally, patients who are at risk for suicide become more energized after



NCCN National Comprehensive Cancer Network®

NCCN Guidelines Index
Distress Management TOC
Discussion

SCREENING TOOLS FOR MEASURING DISTRESS

Instructions: First please circle the number (0-10) that best describes how much distress you have been experiencing in the past week including today.

Extreme distress

10
9
8
7
6
5
4
3
2
1
0

No distress

Second, please indicate if any of the following has been a problem for you in the past week including today. Be sure to check YES or NO for each.

YES NO Practical Problems

☐ ☐ Child care
☐ ☐ Housing
☐ ☐ Insurance/financial
☐ ☐ Transportation
☐ ☐ Work/school
☐ ☐ Treatment decisions

Family Problems

☐ ☐ Dealing with children
☐ ☐ Dealing with partner
☐ ☐ Ability to have children
☐ ☐ Family health issues

Emotional Problems

☐ ☐ Depression
☐ ☐ Fears
☐ ☐ Nervousness
☐ ☐ Sadness
☐ ☐ Worry
☐ ☐ Loss of interest in usual activities

☐ ☐ **Spiritual/religious concerns**

YES NO Physical Problems

☐ ☐ Appearance
☐ ☐ Bathing/dressing
☐ ☐ Breathing
☐ ☐ Changes in urination
☐ ☐ Constipation
☐ ☐ Diarrhea
☐ ☐ Eating
☐ ☐ Fatigue
☐ ☐ Feeling swollen
☐ ☐ Fevers
☐ ☐ Getting around
☐ ☐ Indigestion
☐ ☐ Memory/concentration
☐ ☐ Mouth sores
☐ ☐ Nausea
☐ ☐ Nose dry/congested
☐ ☐ Pain
☐ ☐ Sexual
☐ ☐ Skin dry/itchy
☐ ☐ Sleep
☐ ☐ Tingling in hands/feet

Other Problems: ____________________

____

Version 2.2012, 03/12/12 © National Comprehensive Cancer Network, Inc. 2012, All rights reserved. The NCCN Guidelines® and this illustration may not be reproduced in any form without the express written permission of NCCN®.

DIS-A

Figure 98.2 NATIONAL COMPREHENSIVE CANCER NETWORK (NCCN) DISTRESS MANAGEMENT GUIDELINE—A DISTRESS THERMOMETER. (*From National Comprehensive Cancer Network*. The National Comprehensive Cancer Network 1.2005 Distress Management, the Complete Library of NCCN Clinical Practice Guidelines in Oncology (CD-ROM). *Jenkintown, Pennsylvania: National Comprehensive Cancer Network*; 2005.)

immediate treatment and appear to look better long before their depressive feelings and suicidal thoughts are relieved. Hence, careful monitoring of suicidal ideation should continue for weeks after the patient appears improved.

SCREENING FOR POSITIVE PSYCHOLOGICAL WELLBEING

Although most of the emphasis on achieving psychological health among cancer patients has focused on distress and the alleviation of distress, positive psychological factors such as optimism, gratitude, and meaning also significantly contribute to the psychological wellbeing in patients with serious illnesses. Indeed, the presence of positive psychological wellbeing constructs is not merely the opposite of distress. Optimism and depression, for example, are only modestly inversely correlated—and strong evidence suggests that the beneficial effects of positive psychological wellbeing on clinical outcomes exceed the adverse effects of negative psychological syndromes like depression.[126-128] Hence, it is imperative for positive psychological wellbeing factors to be explored and promoted when assessing for the psychological wellbeing of patients with hematologic malignancies.

MANAGEMENT OF PSYCHOSOCIAL PROBLEMS

Pharmacological and non-pharmacological interventions have both been successfully used to manage distress in patients with hematologic disorders. Because of the increasing complexity of patient care, a multidisciplinary approach is imperative. Psychologic distress that can be managed effectively includes anxiety and depression, sexual dysfunction, body image disturbances, noncompliance, pain, neurologic complications such as delirium and dementia induced by brain metastasis or treatment, anticipatory and posttreatment nausea and vomiting, anorexia and feeding problems, and marital and family difficulties.

Pharmacologic Interventions

Different psychotherapeutic agents (including antidepressants, antipsychotics, and anxiolytics) effectively manage psychiatric symptoms (e.g., anxiety, depression, delirium) in patients with hematologic malignancy.[129] Consideration of drug-drug reactions, physical symptom burden and toxic side effects (especially those pertaining to the bone marrow environment for HSCT recipients), and overall medical comorbidities is essential to the selection of pharmacological agents.[31]

Antidepressants from various classes such as selective serotonin reuptake inhibitors (SSRIs) or selective norepinephrine reuptake inhibitors (SNRIs) can effectively manage symptoms of depression and anxiety. SSRIs and SNRIs, however, can reduce platelet serotonin resulting in platelet dysfunction and increased risk of bruising and bleeding, especially for patients with hematologic disorders.[130] As such, fluoxetine, paroxetine, and sertraline should be used with caution since they have the highest degree of serotonin reuptake inhibition and are more frequently associated with bleeding problems.[10,131] Mirtazapine, an atypical antidepressant used primarily to treat depression, can also be an effective anxiolytic, sedative, antiemetic, and appetite stimulant. However, on rare occasions, mirtazapine can cause neutropenia and agranulocytosis.[132,133] Though antidepressants are the gold standard for managing anxiety as a whole, benzodiazepines can effectively manage acute or intermittent anxiety that may arise at different stages of the care continuum. Benzodiazepines rarely cause agranulocytosis but clonazepam and diazepam have been associated with thrombocytopenia, anemia, and leukopenia.[134]

Antipsychotic medications effectively treat neuropsychiatric and behavioral concerns that accompany delirium, the sudden change in mental status characterized by disorientation, and different psychiatric symptoms including psychosis. Antipsychotics also have anxiolytic (e.g., quetiapine, olanzapine, haloperidol), antiemetic (e.g., olanzapine), appetite stimulation (e.g., olanzapine), and sedative properties. However, several antipsychotic medications including olanzapine, clozapine, chlorpromazine, and haloperidol should be used cautiously in patients with hematological disorders due to their associations with agranulocytosis and neutropenia.[10,134-136] Mood stabilizers and antiepileptics (e.g., valproic acid) used for patients with comorbid psychiatric disorders such as bipolar disorder can also be used to manage agitation in patients with delirium whose medical problems prohibit the use of antipsychotic medications.[137]

Patients must be monitored closely by a consistent provider during the initiation and modification of psychopharmacologic regimens. In patients with a limited prognosis shorter than a few weeks, recent evidence suggests that ketamine may be useful for the rapid treatment of depression. The average time-to-response is on the order of a few hours.[138] It is important to note that although sadness may be present in the end-of-life period, major depression is not common and should be addressed. It is beyond the scope of this chapter to provide a comprehensive overview of all psychopharmacological agents—the threshold to collaborate with psychiatric providers in the management of these symptoms is crucial. A comprehensive discussion of psychotropic drugs is summarized in the quick pocketbook reference for oncology clinicians.[139]

Psychotherapeutic Interventions

"Returning to normal" is a prominent theme and desired goal but is not always possible for patients with hematologic malignancies.[109,140] Survivors of cancer continue to face challenges with long-term physical and psychologic symptoms long after treatment has ended, and data indicate that they report more contact with mental health providers than do people without cancer.[141] The ASBMT released a joint statement recommending screening and preventive practices for long-term survivors of HSCT. The recommendation states that "a high level of vigilance for psychologic symptoms should be maintained. Clinical assessment is recommended throughout the recovery period, at six months, one year, and annually thereafter, with mental health professional counseling recommended for those with recognized deficits."[39] The number of intervention studies designed with the hematologic population is limited; however, growing interest in survivorship is changing the research climate in this area. Psychological interventions should be tailored to a patient's need—it may take the form of group therapy, individual psychotherapy, family therapy, marital therapy, cognitive or behaviorally-oriented therapy, or some combination.[142]

Support groups can provide a therapeutic experience for patients and their caregivers. Social support has been associated with a variety of important patient-reported outcomes including quality of life.[74] Patients often feel alone in their distress and report that sharing a common experience normalizes their feelings, provides an avenue for emotional support, facilitates dialogue to problem solve, and offers opportunities to learn from other patients. Support groups facilitated by professionals such as social workers, nurses, or psychologists can provide a forum for health and psychological education and provide patients with printed literature and online community-based resources. Sherman and colleagues found that psychoeducational support groups facilitated by professionals were a helpful strategy for psychologic recovery in patients' post-HSCT course.[25]

Other modalities have been shown to be effective with patients.[91,109] Studies have consistently shown that relaxation training with or without guided imagery or hypnosis is associated with better quality of life, improved symptom management, and decreased symptoms of anxiety and depression.[140,143,144] Although relaxation training is not time consuming, it requires patients to be motivated to learn and practice new techniques as a way of coping. Cognitive therapy/reappraisal, problem solving, and stress management training are also helpful in the general cancer population.[140,145,146] Fritzsche and colleagues identified that a considerable need for the psychotherapeutic treatment of hospitalized patients with hematologic malignancies is best handled by mental health professionals.[143] They described a consultation-liaison (CL) psychiatry service that provides psychosocial support for patients with hematologic malignancies, including patients going through transplant. The service screened patients for anxiety, depression, poor coping, quality of life, and psychosocial openness for support, and provided psychotherapy, relaxation training, or group therapy as interventions. Although all transplant patients received support from the team, 23% of the hematology patients on the general ward received additional psychosocial interventions. There is also increasing evidence for integrated palliative care services in the care of patients with hematologic malignancies. In a randomized clinical trial of 160 adults with acute myeloid leukemia, El-Jawahri and colleagues showed that integrated palliative care improved patient-reported quality of life, as well as depression, anxiety, and posttraumatic stress symptoms during intensive chemotherapy and for up to 24 weeks.[147] Also, among patients who died, patients who received integrated palliative care were more likely to have reported discussing their end-of-life care preferences and less likely to have received chemotherapy near the end of life.

Other evidence-based interventions found to be helpful in the general oncology population include individual and group counseling, family therapy, and music and art therapy. It has been estimated that one of six cancer survivors with mental health problems is unable to access mental health services due to economic constraints and high cost.[141] If cost is prohibitive and referral to a psychologist or psychiatrist is not feasible, both patients and providers must investigate other potential providers, such as chaplains, art therapists, music therapists, social workers, and psychiatric advanced practice nurses who may be more affordable. Other possibilities include online support groups and advocacy organizations, including local wellness communities, although data on their impact on alleviating symptoms of distress is limited.

Increasing evidence supports the use of an aerobic exercise program for patients with hematologic malignancies.[148-150] Researchers have concluded that fatigue and loss of physical performance in patients undergoing HSCT may improve with exercise.[149] Others have found increased muscle strength with supervised weight bearing and lifting after allogeneic HSCT.[148] Table 98.3 outlines the major psychotherapeutic modalities and their advantages, goals, and indications.

FUTURE DIRECTIONS

The psychosocial issues faced by patients with hematologic malignancies are influenced by individual, environmental, and disease-related factors. Most patients undergoing cancer treatment, as well as their caregivers, experience expected periods of psychologic turmoil that occur at transition points along the clinical course of cancer. In a small

TABLE 98.3 Non-Pharmacological Interventions Successfully Used for Patients and Caregivers with Hematologic Malignancies

Modality	Selected Indications	Goals and Advantages	Comments
Individual Psychotherapy	Prolonged adverse reactions to diagnosis, treatment, and other aspects of chronic illness (e.g., anxiety, depression)	Supports patients and enhances the ability to cope with distressing feelings Short-term therapy; focused and goal directed	Pharmacology and family involvement are useful adjuncts in some cases
Support Groups	Patients who desire contact with others who are experiencing chronic illness	Supports patient and enhances coping ability Patients benefit by observing coping strategies of others Usually does not involve a fee	Expands social network of patients with limited support systems
Family and Marital Therapy	Relationship problems secondary to illness (e.g., family tension, role changes, conflict, sexual problems)	Assists couples with clarifying problems and facilitates solving them together Addresses role changes in the family system	Problems, issues, and concerns about relationships including children can be addressed
Mind-body Therapies (e.g., progressive muscle relaxation, yoga, guided imagery, Reiki, meditation, hypnosis, and biofeedback)	Patients who desire assistance with control of pain, anxiety, anticipatory and posttreatment nausea and vomiting, fears associated with medical procedures	Increases sense of control and participation in treatment Individualized to meet patients' preferences and circumstances Time limited and goal directed Evaluated in terms of observable changes in symptoms and self-efficacy	Realistic goals should be stated explicitly (Some patients may view these therapies as curative for cancer)

proportion of patients, more severe psychiatric complications may occur, warranting a referral to a psychiatric specialist, including psychiatrists, social workers, psychologists, and psychiatric nurses. Screening for ongoing psychiatric problems must become standard of care. A variety of psychotherapeutic interventions are useful for helping patients work through the psychological challenges that may accompany their diagnosis and treatment.[6] Supportive psychotherapeutic measures should be used routinely because they minimize distress and enhance feelings of control and mastery over self and environment.

Throughout the clinical course of cancer, the patient's relationship with health care providers and the presence of supportive social networks are essential to ensuring successful management of the many physical and psychosocial demands imposed by a cancer diagnosis and treatment. As scientific inquiry continues to produce vast but sometimes conflicting information regarding etiology and treatment of cancers, concurrent research regarding the psychosocial aspects of hematologic malignancies is needed to ensure that patients with hematologic malignancy and their caregivers have optimal psychological wellbeing as they navigate their cancer diagnosis and treatment as well as in survivorship. Robust studies on the psychosocial aspects of cancer may provide some "missing links" regarding psychosocial adaptation and patient-reported outcomes in the immediate and long-term recovery from treatment. The Institute of Medicine report that recommends that all cancer care should ensure the provision of appropriate psychosocial health services states that at a minimum, patients must be screened for emotional distress and evaluated for additional services.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Albrecht TA, Rosenzweig M. Management of cancer related distress in patients with a hematological malignancy. *J Hosp Palliat Nurs*. 2012;14(7):462–468.

Amonoo HL, Massey CN, Freedman ME, et al. Psychological considerations in hematopoietic stem cell transplantation. *Psychosomatics*. 2019;60(4):331–342.

Applebaum AJ, Bevans M, Son T, et al. A scoping review of caregiver burden during allogeneic HSCT: lessons learned and future directions. *Bone Marrow Transplant*. 2016;51(11):1416–1422.

Back AL, Arnold RM, Baile WF, et al. Approaching difficult communication tasks in oncology. *CA Cancer J Clin*. 2005;55(3):164–177.

Banks E, Byles JE, Gibson RE, et al. Is psychological distress in people living with cancer related to the fact of diagnosis, current treatment or level of disability? Findings from a large Australian study. *Med J Aust*. 2010;193(S5):S62–S67.

Beattie S, Lebel S. The experience of caregivers of hematological cancer patients undergoing a hematopoietic stem cell transplant: a comprehensive literature review. *Psychooncology*. 2011;20(11):1137–1150.

Bushkin E. The Mara Mogensen Flaherty memorial lecture. Signposts of survivorship. *Oncol Nurs Forum*. 1993;20(6):869–875.

Cooke L, Gemmill R, Kravits K, Grant M. Psychological issues of stem cell transplant. *Semin Oncol Nurs*. 2009;25(2):139–150.

Cooke L, Grant M, Eldredge DH. Hematopoietic cell transplantation: the trajectory of quality of life. In: Ezzone S, ed. *Blood and Marrow Stem Cell Transplantation: Principles, Practice, and Nursing Insights*. Sudbury, MA: Jones & Bartlett Learning; 2007:391.

Edwards B, Clarke V. The psychological impact of a cancer diagnosis on families: the influence of family functioning and patients' illness characteristics on depression and anxiety. *Psychooncology*. 2004;13(8):562–576.

Ernst J, Hinz A, Niederwieser D, et al. Dyadic coping of patients with hematologic malignancies and their partners and its relation to quality of life—a longitudinal study. *Leuk Lymphoma*. 2017;58(3):655–665.

Gil F, Costa G, Hilker I, Benito L. First anxiety, afterwards depression: psychological distress in cancer patients at diagnosis and after medical treatment. *Stress Health*. 2012;28(5):362–367.

Halbert CH, Wrenn G, Weathers B, et al. Sociocultural determinants of men's reactions to prostate cancer diagnosis. *Psychooncology*. 2010;19(5):553–560.

Haylock PJ. The shifting paradigm of cancer care. *Cancer Nurs*. 2006;29(2 Suppl):16–19.

Heinonen H, Volin L, Uutela A, et al. Quality of life and factors related to perceived satisfaction with quality of life after allogeneic bone marrow transplantation. *Ann Hematol*. 2001;80(3):137–143.

Jacobsen PB, Sadler IJ, Booth-Jones M, et al. Predictors of posttraumatic stress disorder symptomatology following bone marrow transplantation for cancer. *J Consult Clin Psychol*. 2002;70(1):235–240.

Jones WC, Parry C, Devine S, et al. Prevalence and predictors of distress in posttreatment adult leukemia and lymphoma survivors. *J Psychosoc Oncol*. 2015;33(2):124–141.

Kagawa-Singer M, Dadia AV, Yu MC, et al. Cancer, culture, and health disparities: time to chart a new course? *CA Cancer J Clin*. 2010;60(1):12–39.

Keyzer-Dekker CM, de Vries J, Mertens MC, et al. The impact of diagnosis and trait anxiety on psychological distress in women with early stage breast cancer: a prospective study. *Br J Health Psychol*. 2014;19(4):783–794.

Khan AG, Irfan M, Shamsi TS, Hussain M. Psychiatric disorders in bone marrow transplant patients. *J Coll Physicians Surg Pak*. 2007;17(2):98–100.

Kishi Y, Meller WH, Swigart SE, Kathol RG. Are the patients with post-transplant psychiatric consultation different from other medical-surgical consultation inpatients? *Psychiatry Clin Neurosci*. 2005;59(1):19–24.

Knight JM, Moynihan JA, Lyness JM, et al. Peri-transplant psychosocial factors and neutrophil recovery following hematopoietic stem cell transplantation. *PLoS One*. 2014;9(6):e99778.

Morselli M, Bandieri E, Zanin R, et al. Pain and emotional distress in leukemia patients at diagnosis. *Leuk Res*. 2010;34(2):e67–e68.

Mosher CE, Redd WH, Rini CM, et al. Physical, psychological, and social sequelae following hematopoietic stem cell transplantation: a review of the literature. *Psychooncology*. 2009;18(2):113–127.

Prieto JM, Blanch J, Atala J, et al. Stem cell transplantation: risk factors for psychiatric morbidity. *Eur J Cancer*. 2006;42(4):514–520.

Pulte D, Jansen L, Castro FA, Brenner H. Changes in the survival of older patients with hematologic malignancies in the early 21st century. *Cancer*. 2016;122(13):2031–2040.

Sherman RS, Cooke E, Grant M. Dialogue among survivors of hematopoietic cell transplantation support-group themes. *J Psychosoc Oncol*. 2005;23(1):1–24.

Syrjala KL, Langer SL, Abrams JR, et al. Late effects of hematopoietic cell transplantation among 10-year adult survivors compared with case-matched controls. *J Clin Oncol*. 2005;23(27):6596–6606.

Tremblay N, Roy O, Lecocq A. Systemic screening of distress: Portraits and cross-examination of the various stakeholders' perceptions, clinical perspectives, and research-Part 1. *Can Oncol Nurs J*. 2017;27(3):224–235.

Weisman AD, Worden JW. The existential plight in cancer: significance of the first 100 days. *Int J Psychiatry Med*. 1976;7(1):1–15.

REFERENCES

1. Pulte D, Jansen L, Castro FA, Brenner H. Changes in the survival of older patients with hematologic malignancies in the early 21st century. *Cancer*. 2016;122(13):2031–2040.
2. Jones WC, Parry C, Devine S, et al. Prevalence and predictors of distress in posttreatment adult leukemia and lymphoma survivors. *J Psychosoc Oncol*. 2015;33(2):124–141.
3. Albrecht TA, Rosenzweig M. Management of cancer related distress in patients with a hematological malignancy. *J Hosp Palliat Nurs*. 2012;14(7):462–468.
4. Haylock PJ. The shifting paradigm of cancer care. *Cancer Nurs*. 2006;29(2 Suppl):16–19.
5. Bushkin E. The Mara Mogensen Flaherty memorial lecture. Signposts of survivorship. *Oncol Nurs Forum*. 1993;20(6):869–875.
6. Weisman AD, Worden JW. The existential plight in cancer: significance of the first 100 days. *Int J Psychiatry Med*. 1976;7(1):1–15.
7. Halbert CH, Wrenn G, Weathers B, et al. Sociocultural determinants of men's reactions to prostate cancer diagnosis. *Psychooncology*. 2010;19(5):553–560.
8. Kagawa-Singer M, Dadia AV, Yu MC, et al. Cancer, culture, and health disparities: time to chart a new course? *CA Cancer J Clin*. 2010;60(1):12–39.
9. Ernst J, Hinz A, Niederwieser D, et al. Dyadic coping of patients with hematologic malignancies and their partners and its relation to quality of life—a longitudinal study. *Leuk Lymphoma*. 2017;58(3):655–665.
10. Amonoo HL, Massey CN, Freedman ME, et al. Psychological considerations in hematopoietic stem cell transplantation. *Psychosomatics*. 2019;60(4):331–342.
11. Banks E, Byles JE, Gibson RE, et al. Is psychological distress in people living with cancer related to the fact of diagnosis, current treatment or level of disability? Findings from a large Australian study. *Med J Aust*. 2010;193(S5):S62–S67.
12. Gil F, Costa G, Hilker I, Benito L. First anxiety, afterwards depression: psychological distress in cancer patients at diagnosis and after medical treatment. *Stress Health*. 2012;28(5):362–367.
13. Keyzer-Dekker CM, de Vries J, Mertens MC, et al. The impact of diagnosis and trait anxiety on psychological distress in women with early stage breast cancer: a prospective study. *Br J Health Psychol*. 2014;19(4):783–794.
14. Morselli M, Bandieri E, Zanin R, et al. Pain and emotional distress in leukemia patients at diagnosis. *Leuk Res*. 2010;34(2):e67–e68.
15. Syrjala KL, Langer SL, Abrams JR, et al. Late effects of hematopoietic cell transplantation among 10-year adult survivors compared with case-matched controls. *J Clin Oncol*. 2005;23(27):6596–6606.
16. Cooke L, Gemmill R, Kravits K, Grant M. Psychological issues of stem cell transplant. *Semin Oncol Nurs*. 2009;25(2):139–150.
17. Jacobsen PB, Sadler IJ, Booth-Jones M, et al. Predictors of posttraumatic stress disorder symptomatology following bone marrow transplantation for cancer. *J Consult Clin Psychol*. 2002;70(1):235–240.
18. Prieto JM, Blanch J, Atala J, et al. Stem cell transplantation: risk factors for psychiatric morbidity. *Eur J Cancer*. 2006;42(4):514–520.
19. Back AL, Arnold RM, Baile WF, et al. Approaching difficult communication tasks in oncology. *CA Cancer J Clin*. 2005;55(3):164–177.
20. Edwards B, Clarke V. The psychological impact of a cancer diagnosis on families: the influence of family functioning and patients' illness characteristics on depression and anxiety. *Psychooncology*. 2004;13(8):562–576.
21. Beattie S, Lebel S. The experience of caregivers of hematological cancer patients undergoing a hematopoietic stem cell transplant: a comprehensive literature review. *Psychooncology*. 2011;20(11):1137–1150.
22. Tremblay N, Roy O, Lecocq A. Systemic screening of distress: portraits and cross-examination of the various stakeholders' perceptions, clinical perspectives, and research-Part 1. *Can Oncol Nurs J*. 2017;27(3):224–235.
23. Applebaum AJ, Bevans M, Son T, et al. A scoping review of caregiver burden during allogeneic HSCT: lessons learned and future directions. *Bone Marrow Transplant*. 2016;51(11):1416–1422.
24. Cooke L, Grant M, Eldredge DH. Hematopoietic cell transplantation: the trajectory of quality of life. In: Ezzone S, ed. *Blood and Marrow Stem Cell Transplantation: Principles, Practice, and Nursing Insights*. Sudbury, MA: Jones & Bartlett Learning; 2007:391.
25. Sherman RS, Cooke E, Grant M. Dialogue among survivors of hematopoietic cell transplantation support-group themes. *J Psychosoc Oncol*. 2005;23(1):1–24.
26. Knight JM, Moynihan JA, Lyness JM, et al. Peri-transplant psychosocial factors and neutrophil recovery following hematopoietic stem cell transplantation. *PLoS One*. 2014;9(6):e99778.
27. Khan AG, Irfan M, Shamsi TS, Hussain M. Psychiatric disorders in bone marrow transplant patients. *J Coll Physicians Surg Pak*. 2007;17(2):98–100.
28. Kishi Y, Meller WH, Swigart SE, Kathol RG. Are the patients with post-transplant psychiatric consultation different from other medical-surgical consultation inpatients? *Psychiatry Clin Neurosci*. 2005;59(1):19–24.
29. Heinonen H, Volin L, Uutela A, et al. Quality of life and factors related to perceived satisfaction with quality of life after allogeneic bone marrow transplantation. *Ann Hematol*. 2001;80(3):137–143.
30. Mosher CE, Redd WH, Rini CM, et al. Physical, psychological, and social sequelae following hematopoietic stem cell transplantation: a review of the literature. *Psychooncology*. 2009;18(2):113–127.
31. Nakamura ZM, Nash RP, Quillen LJ, et al. Psychiatric care in hematopoietic stem cell transplantation. *Psychosomatics*. 2019;60(3):227–237.
32. Syrjala KL, Martin PJ, Lee SJ. Delivering care to long-term adult survivors of hematopoietic cell transplantation. *J Clin Oncol*. 2012;30(30):3746–3751.
33. Wong FL, Francisco L, Togawa K, et al. Longitudinal trajectory of sexual functioning after hematopoietic cell transplantation: impact of chronic graft-versus-host disease and total body irradiation. *Blood*. 2013;122(24):3973–3981.
34. Yoo KH, Kang D, Kim IR, et al. Satisfaction with sexual activity and sexual dysfunction in hematopoietic stem cell transplantation survivors and their partners: a couple study. *Bone Marrow Transplant*. 2018;53(8):967–976.
35. Claessens JJ, Beerendonk CC, Schattenberg AV. Quality of life, reproduction and sexuality after stem cell transplantation with partially T-cell-depleted grafts and after conditioning with a regimen including total body irradiation. *Bone Marrow Transplant*. 2006;37(9):831–836.
36. Baker F, Denniston M, Smith T, West MM. Adult cancer survivors: how are they faring? *Cancer*. 2005;104(11 Suppl):2565–2576.
37. Mosher CE, DuHamel KN, Rini C, Corner G, Lam J, Redd WH. Quality of life concerns and depression among hematopoietic stem cell transplant survivors. *Support Care Cancer*. 2011;19(9):1357–1365.
38. Loberiza Jr FR, Rizzo JD, Bredeson CN, et al. Association of depressive syndrome and early deaths among patients after stem-cell transplantation for malignant diseases. *J Clin Oncol*. 2002;20(8):2118–2126.
39. Rizzo JD, Wingard JR, Tichelli A, et al. Recommended screening and preventive practices for long-term survivors after hematopoietic cell transplantation: joint recommendations of the European Group for Blood and Marrow Transplantation, Center for International Blood and Marrow Transplant Research, and the American Society for Blood and Marrow Transplantation (EBMT/CIBMTR/ASBMT). *Bone Marrow Transplant*. 2006;37(3):249–261.
40. Rusiewicz A, DuHamel KN, Burkhalter J, et al. Psychological distress in long-term survivors of hematopoietic stem cell transplantation. *Psychooncology*. 2008;17(4):329–337.
41. Adelstein KE, Anderson JG, Taylor AG. Importance of meaning-making for patients undergoing hematopoietic stem cell transplantation. *Oncol Nurs Forum*. 2014;41(2):E172–E184.
42. Fromm K, Andrykowski MA, Hunt J. Positive and negative psychosocial sequelae of bone marrow transplantation: implications for quality of life assessment. *J Behav Med*. 1996;19(3):221–240.
43. Widows MR, Jacobsen PB, Booth-Jones M, Fields KK. Predictors of posttraumatic growth following bone marrow transplantation for cancer. *Health Psychol*. 2005;24(3):266–273.
44. Gruber U, Fegg M, Buchmann M, Kolb HJ, Hiddemann W. The long-term psychosocial effects of haematopoetic stem cell transplantation. *Eur J Cancer Care (Engl)*. 2003;12(3):249–256.
45. Mullan F. Seasons of survival: reflections of a physician with cancer. *N Engl J Med*. 1985;313(4):270–273.
46. Lesko L. Hematopoietic dyscrasias. In: Holland JC, ed. *Psycho-Oncology*. New York: Oxford University Press; 1998:406–416.
47. Ferrell B, Grant M, Schmidt GM, et al. The meaning of quality of life for bone marrow transplant survivors. Part 2. Improving quality of life for bone marrow transplant survivors. *Cancer Nurs*. 1992;15(4):247–253.
48. Ferrell B, Grant M, Schmidt GM, et al. The meaning of quality of life for bone marrow transplant survivors. Part 1. The impact of bone marrow transplant on quality of life. *Cancer Nurs*. 1992;15(3):153–160.
49. Lamont EB, Christakis NA. Prognostic disclosure to patients with cancer near the end of life. *Ann Intern Med*. 2001;134(12):1096–1105.
50. Bush NE, Haberman M, Donaldson G, Sullivan KM. Quality of life of 125 adults surviving 6-18 years after bone marrow transplantation. *Soc Sci Med*. 1995;40(4):479–490.
51. Raphael D, Frey R, Gott M. Psychosocial distress in haematological cancer survivors: an integrative review. *Eur J Cancer Care (Engl)*. 2017;26(6).
52. Lee CJ, Muffly LS. Coordination of care in survivorship after treatment of hematological malignancies-the journey is not over yet. *Curr Hematol Malig Rep*. 2017;12(4):317–323.

53. Raphael D, Frey R, Gott M. The nature and timing of distress among post-treatment haematological cancer survivors. *Eur J Cancer Care (Engl)*. 2019;28(1):e12951.
54. Swash B, Hulbert-Williams N, Bramwell R. Unmet psychosocial needs in haematological cancer: a systematic review. *Support Care Cancer*. 2014;22(4):1131–1141.
55. Emanuel EJ, Emanuel LL. The promise of a good death. *Lancet*. 1998;351(Suppl 2). SII21-29.
56. Dow KH. The enduring seasons in survival. *Oncol Nurs Forum*. 1990;17(4):511–516.
57. Sarkar S, Scherwath A, Schirmer L, et al. Fear of recurrence and its impact on quality of life in patients with hematological cancers in the course of allogeneic hematopoietic SCT. *Bone Marrow Transplant*. 2014;49(9):1217–1222.
58. Mellon S, Northouse LL. Family survivorship and quality of life following a cancer diagnosis. *Res Nurs Health*. 2001;24(6):446–459.
59. Zittoun R, Suciu S, Watson M, et al. Quality of life in patients with acute myelogenous leukemia in prolonged first complete remission after bone marrow transplantation (allogeneic or autologous) or chemotherapy: a cross-sectional study of the EORTC-GIMEMA AML 8A trial. *Bone Marrow Transplant*. 1997;20(4):307–315.
60. Waimey KE, Smith BM, Confino R, Jeruss JS, Pavone ME. Understanding fertility in young female cancer patients. *J Womens Health (Larchmt)*. 2015;24(10):812–818.
61. Hammond CT, Beckjord EB, Arora NK, Bellizzi KM, Jeffery DD, Aziz NM. Non-Hodgkin's lymphoma survivors' fertility and sexual function-related information needs. *Fertil Steril*. 2008;90(4):1256–1258.
62. Bokemeyer C. Late damage of curative oncologic therapy. Results of a patient sample with Hodgkin's disease and testicular tumors at the Hannover Medical School. *Med Klin (Munich)*. 1996;91(Suppl 3):60–67.
63. Schimmer AD, Elliott ME, Abbey SE, et al. Illness intrusiveness among survivors of autologous blood and marrow transplantation. *Cancer*. 2001;92(12):3147–3154.
64. Persson L, Hallberg IR, Ohlsson O. Survivors of acute leukaemia and highly malignant lymphoma–retrospective views of daily life problems during treatment and when in remission. *J Adv Nurs*. 1997;25(1):68–78.
65. Wellisch DK, Centeno J, Guzman J, Belin T, Schiller GJ. Bone marrow transplantation vs. high-dose cytorabine-based consolidation chemotherapy for acute myelogenous leukemia. A long-term follow-up study of quality-of-life measures of survivors. *Psychosomatics*. 1996;37(2):144–154.
66. Molassiotis A, van den Akker OB, Milligan DW, et al. Quality of life in long-term survivors of marrow transplantation: comparison with a matched group receiving maintenance chemotherapy. *Bone Marrow Transplant*. 1996;17(2):249–258.
67. Wettergren L, Langius A, Björkholm M, Björvell H. Post-traumatic stress symptoms in patients undergoing autologous stem cell transplantation. *Acta Oncol*. 1999;38(4):475–480.
68. Lotfi-Jam K, Gough K, Schofield P, Aranda S, Jefford M. A longitudinal study of four unique trajectories of psychological distress in cancer survivors after completing potentially curative treatment. *Acta Oncol*. 2019;58(5):782–789.
69. Fong TG, Tulebaev SR, Inouye SK. Delirium in elderly adults: diagnosis, prevention and treatment. *Nat Rev Neurol*. 2009;5(4):210–220.
70. Seney ML, Sibille E. Sex differences in mood disorders: perspectives from humans and rodent models. *Biol Sex Differ*. 2014;5(1):17.
71. Rueda-Lara M, Lopez-Patton MR. Psychiatric and psychosocial challenges in patients undergoing haematopoietic stem cell transplants. *Int Rev Psychiatry*. 2014;26(1):74–86.
72. Andrykowski MA, Cordova MJ, Studts JL, Miller TW. Posttraumatic stress disorder after treatment for breast cancer: prevalence of diagnosis and use of the PTSD Checklist-Civilian Version (PCL-C) as a screening instrument. *J Consult Clin Psychol*. 1998;66(3):586–590.
73. Ehrlich KB, Miller GE, Scheide T, et al. Pre-transplant emotional support is associated with longer survival after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant*. 2016;51(12):1594–1598.
74. Amonoo HL, Johnson PC, Dhawale TM, et al. Sharing and caring: the impact of social support on quality of life and health outcomes in hematopoietic stem cell transplantation. *Cancer*. 2021;127(8):1260–1265.
75. Köhler N, Mehnert A, Götze H. Psychological distress, chronic conditions and quality of life in elderly hematologic cancer patients: study protocol of a prospective study. *BMC Cancer*. 2017;17(1):700.
76. Kenzik K, Huang IC, Rizzo JD, Shenkman E, Wingard J. Relationships among symptoms, psychosocial factors, and health-related quality of life in hematopoietic stem cell transplant survivors. *Support Care Cancer*. 2015;23(3):797–807.
77. Sherman AC, Simonton S, Latif U, Spohn R, Tricot G. Psychosocial adjustment and quality of life among multiple myeloma patients undergoing evaluation for autologous stem cell transplantation. *Bone Marrow Transplant*. 2004;33(9):955–962.
78. Prieto JM, Blanch J, Atala J, et al. Psychiatric morbidity and impact on hospital length of stay among hematologic cancer patients receiving stem-cell transplantation. *J Clin Oncol*. 2002;20(7):1907–1917.
79. Patrick DL, Engelberg RA, Curtis JR. Evaluating the quality of dying and death. *J Pain Symptom Manage*. 2001;22(3):717–726.
80. Steinhauser KE, Christakis NA, Clipp EC, McNeilly M, McIntyre L, Tulsky JA. Factors considered important at the end of life by patients, family, physicians, and other care providers. *JAMA*. 2000;284(19):2476–2482.
81. Coyle N. Introduction to palliative nursing care. In: Ferrel B, Coyle N, eds. *Textbook of Palliative Nursing*. New York: Oxford University Press; 2006:1.
82. Paice J, Fine P. Pain at the end of life. In: Ferrel B, Coyle N, eds. *Textbook of Palliative Nursing*. New York: Oxford University Press; 2006:131.
83. Temel JS, Greer JA, Muzikansky A, et al. Early palliative care for patients with metastatic non-small-cell lung cancer. *N Engl J Med*. 2010;363(8):733–742.
84. El-Jawahri A, Nelson AM, Gray TF, Lee SJ, LeBlanc TW. Palliative and end-of-life care for patients with hematologic malignancies. *J Clin Oncol*. 2020;38(9):944–953.
85. El-Jawahri A, LeBlanc TW, Burns LJ, et al. What do transplant physicians think about palliative care? A national survey study. *Cancer*. 2018;124(23):4556–4566.
86. McGrath P. Update on psychosocial research on leukaemia for social work practitioners. *Soc Work Health Care*. 1999;29(2):1–20.
87. Maddocks I, Bentley L, Sheedy J. Quality of life issues in patients dying from haematological diseases. *Ann Acad Med Singap*. 1994;23(2):244–248.
88. Institute of Medicine (US) Committee on Care at the End of Life Field MJ, Cassel CK, eds. *Approaching Death: Improving Care at the End of Life*. Washington, DC: National Academies Press (US); 1997. Copyright 1997 by the National Academy of Sciences. All rights reserved. 1997.
89. Tang ST, McCorkle R. Determinants of place of death for terminal cancer patients. *Cancer Invest*. 2001;19(2):165–180.
90. Manitta VJ, Philip JA, Cole-Sinclair MF. Palliative care and the hemato-oncological patient: can we live together? A review of the literature. *J Palliat Med*. 2010;13(8):1021–1025.
91. Johnson Vickberg SM, Duhamel KN, Smith MY, et al. Global meaning and psychological adjustment among survivors of bone marrow transplant. *Psychooncology*. 2001;10(1):29–39.
92. Jenkins PL, Lester H, Alexander J, Whittaker J. A prospective study of psychosocial morbidity in adult bone marrow transplant recipients. *Psychosomatics*. 1994;35(4):361–367.
93. Andrykowski MA. Psychosocial factors in bone marrow transplantation: a review and recommendations for research. *Bone Marrow Transplant*. 1994;13(4):357–375.
94. Tschuschke V, Hertenstein B, Arnold R, Bunjes D, Denzinger R, Kaechele H. Associations between coping and survival time of adult leukemia patients receiving allogeneic bone marrow transplantation: results of a prospective study. *J Psychosom Res*. 2001;50(5):277–285.
95. Molassiotis A, Van Den Akker OB, Milligan DW, Goldman JM. Symptom distress, coping style and biological variables as predictors of survival after bone marrow transplantation. *J Psychosom Res*. 1997;42(3):275–285.
96. Hoppe RT, Advani RH, Bierman PJ, et al. Hodgkin disease/lymphoma. Clinical practice guidelines in oncology. *J Natl Compr Canc Netw*. 2006;4(3):210–230.
97. Misono S, Weiss NS, Fann JR, Redman M, Yueh B. Incidence of suicide in persons with cancer. *J Clin Oncol*. 2008;26(29):4731–4738.
98. Extermann M. Measuring comorbidity in older cancer patients. *Eur J Cancer*. 2000;36(4):453–471.
99. Yancik R, Ries LA. Aging and cancer in America. Demographic and epidemiologic perspectives. *Hematol Oncol Clin North Am*. 2000;14(1):17–23.
100. Bishop MR, Logan BR, Gandham S, et al. Long-term outcomes of adults with acute lymphoblastic leukemia after autologous or unrelated donor bone marrow transplantation: a comparative analysis by the National Marrow Donor Program and Center for International Blood and Marrow Transplant Research. *Bone Marrow Transplant*. 2008;41(7):635–642.
101. Evan EE, Zeltzer LK. Psychosocial dimensions of cancer in adolescents and young adults. *Cancer*. 2006;107(7 Suppl):1663–1671.
102. Bishop M, Logan B, Gandham S, et al. Long-term outcomes of adults with acute lymphoblastic leukemia after autologous or unrelated donor bone marrow transplantation: a comparative analysis by the National Marrow Donor Program and Center for International Blood and Marrow Transplant Research. *Bone Marrow Transplant*. 2008;41(7):635–642.
103. McGrath P. End-of-life care for hematological malignancies: the "technological imperative" and palliative care. *J Palliat Care*. 2002;18(1):39–47.
104. Knobel H, Loge JH, Nordøy T, et al. High level of fatigue in lymphoma patients treated with high dose therapy. *J Pain Symptom Manage*. 2000;19(6):446–456.

105. Li XH, Sun L, Yuan J. [Application of WHO classification and NCCN guidelines in diagnosis of lymphoma]. *Zhonghua Bing Li Xue Za Zhi*. 2009;38(4):217–220.
106. Frick E, Motzke C, Fischer N, Busch R, Bumeder I. Is perceived social support a predictor of survival for patients undergoing autologous peripheral blood stem cell transplantation? *Psychooncology*. 2005;14(9):759–770.
107. Lim JW, Zebrack B. Social networks and quality of life for long-term survivors of leukemia and lymphoma. *Support Care Cancer*. 2006;14(2):185–192.
108. Leigh S, Wilson KC, Burns R, Clark RE. Psychosocial morbidity in bone marrow transplant recipients: a prospective study. *Bone Marrow Transplant*. 1995;16(5):635–640.
109. Andrykowski MA, Brady MJ, Greiner CB, et al. "Returning to normal" following bone marrow transplantation: outcomes, expectations and informed consent. *Bone Marrow Transplant*. 1995;15(4):573–581.
110. Loge JH, Abrahamsen AF, Ekeberg Kaasa S. Fatigue and psychiatric morbidity among Hodgkin's disease survivors. *J Pain Symptom Manage*. 2000;19(2):91–99.
111. Hjermstad MJ, Evensen SA, Kvaløy SO, Fayers PM, Kaasa S. Health-related quality of life 1 year after allogeneic or autologous stem-cell transplantation: a prospective study. *J Clin Oncol*. 1999;17(2):706–718.
112. Kopp M, Schweigkofler H, Holzner B, et al. Time after bone marrow transplantation as an important variable for quality of life: results of a cross-sectional investigation using two different instruments for quality-of-life assessment. *Ann Hematol*. 1998;77(1-2):27–32.
113. Hwong AR, Irwin KE. Reducing global disparities in cancer screening for people with mental illness. *Lancet Psychiatry*. 2020;7(1):4–6.
114. Miovic M, Block S. Psychiatric disorders in advanced cancer. *Cancer*. 2007;110(8):1665–1676.
115. Golomb BA, Pyne JM, Wright B, Jaworski B, Lohr JB, Bozzette SA. The role of psychiatrists in primary care of patients with severe mental illness. *Psychiatr Serv*. 2000;51(6):766–773.
116. Inagaki T, Yasukawa R, Okazaki S, et al. Factors disturbing treatment for cancer in patients with schizophrenia. *Psychiatry Clin Neurosci*. 2006;60(3):327–331.
117. Pirl W. Screening instruments. In: Holland J, Greenberg D, Hughes M, eds. *Quick Reference for Oncology Clinicians: The Psychiatric and Psychological Dimensions of Cancer Symptom Management*. Charlottesville, VA: IPOS Press; 2006:6.
118. Jacobsen PB, Donovan KA, Trask PC, et al. Screening for psychologic distress in ambulatory cancer patients. *Cancer*. 2005;103(7):1494–1502.
119. Holland JC, Andersen B, Breitbart WS, et al. Distress management. *J Natl Compr Canc Netw*. 2013;11(2):190–209.
120. American College of Surgeons Commission on Cancer (ACoSCo C). *Cancer Program Standards 2012: Ensuring Patient-Centered Care*. Chicago, IL: American College of Surgeons; 2012.
121. Holland JC, Jacobsen PB, Riba MB. NCCN: Distress management. *Cancer Control*. 2001;8(6 Suppl 2):88–93.
122. Trask PC, Paterson A, Riba M, et al. Assessment of psychological distress in prospective bone marrow transplant patients. *Bone Marrow Transplant*. 2002;29(11):917–925.
123. Ransom S, Jacobsen PB, Booth-Jones M. Validation of the distress thermometer with bone marrow transplant patients. *Psychooncology*. 2006;15(7):604–612.
124. Lee SJ, Loberiza FR, Antin JH, et al. Routine screening for psychosocial distress following hematopoietic stem cell transplantation. *Bone Marrow Transplant*. 2005;35(1):77–83.
125. Meijer A, Roseman M, Delisle VC, et al. Effects of screening for psychological distress on patient outcomes in cancer: a systematic review. *J Psychosom Res*. 2013;75(1):1–17.
126. Kubzansky LD, Huffman JC, Boehm JK, et al. Positive psychological well-being and cardiovascular disease: JACC Health Promotion Series. *J Am Coll Cardiol*. 2018;72(12):1382–1396.
127. Huffman JC, Beale EE, Celano CM, et al. Effects of optimism and gratitude on physical activity, biomarkers, and readmissions after an acute coronary syndrome: the gratitude research in acute coronary events study. *Circ Cardiovasc Qual Outcomes*. 2016;9(1):55–63.
128. Ronaldson A, Molloy GJ, Wikman A, Poole L, Kaski JC, Steptoe A. Optimism and recovery after acute coronary syndrome: a clinical cohort study. *Psychosom Med*. 2015;77(3):311–318.
129. Fann JR. Neurological effects of psychopharmacological agents. *Semin Clin Neuropsychiatry*. 2002;7(3):196–205.
130. de Abajo FJ. Effects of selective serotonin reuptake inhibitors on platelet function: mechanisms, clinical outcomes and implications for use in elderly patients. *Drugs Aging*. 2011;28(5):345–367.
131. Halperin D, Reber G. Influence of antidepressants on hemostasis. *Dialogues Clin Neurosci*. 2007;9(1):47–59.
132. Hartmann PM. Mirtazapine: a newer antidepressant. *Am Fam Physician*. 1999;59(1):159–161.
133. Ahmed A. Neutropenia associated with mirtazapine use: is a drop in the neutrophil count in a symptomatic older adults a cause for concern? *J Am Geriatr Soc*. 2002;50(8):1461–1463.
134. Oyesanmi O, Kunkel EJ, Monti DA, Field HL. Hematologic side effects of psychotropics. *Psychosomatics*. 1999;40(5):414–421.
135. Pick AM, Nystrom KK. Nonchemotherapy drug-induced neutropenia and agranulocytosis: could medications be the culprit? *J Pharm Pract*. 2014;27(5):447–452.
136. Pirmohamed M, Park K. Mechanism of clozapine-induced agranulocytosis: current status of research and implications for drug development. *CNS Drugs*. 1997;7(2):139–158.
137. Sher Y, Miller Cramer AC, Ament A, Lolak S, Maldonado JR. Valproic acid for treatment of hyperactive or mixed delirium: rationale and literature review. *Psychosomatics*. 2015;56(6):615–625.
138. Irwin SA, Iglewicz A. Oral ketamine for the rapid treatment of depression and anxiety in patients receiving hospice care. *J Palliat Med*. 2010;13(7):903–908.
139. Valentine A. Common psychiatric disorders. In: Holland J, Greenberg D, Hughes M, eds. *Quick reference for oncology clinicians: The psychiatric and psychological dimensions of cancer symptom management*. 39. Charlottesville, VA: IPOS Press; 2006.
140. McQuellan R, Danhauer S. Psychosocial rehabilitation in cancer care. In: Chang A, Ganz P, Hayes D, eds. *Oncology: An Evidence-Based Approach*. New York: Springer Verlag; 2006:1942.
141. Hewitt M, Rowland JH. Mental health service use among adult cancer survivors: analyses of the National Health Interview Survey. *J Clin Oncol*. 2002;20(23):4581–4590.
142. Institute of Medicine Committee on Psychosocial Services to Cancer Patients/Families in a Community Setting (Adler NE, Page AEK, eds.). *Cancer Care for the Whole Patient: Meeting Psychosocial Health Needs*. Washington, DC: National Academies Press (US); 2008.
143. Fritzsche K, Struss Y, Stein B, Spahn C. Psychosomatic liaison service in hematological oncology: need for psychotherapeutic interventions and their realization. *Hematol Oncol*. 2003;21(2):83–89.
144. Cheung YL, Molassiotis A, Chang AM. The effect of progressive muscle relaxation training on anxiety and quality of life after stoma surgery in colorectal cancer patients. *Psychooncology*. 2003;12(3):254–266.
145. McGrath P. Taking control: findings of a prospective study of an educational course for patients with leukaemia. *Support Care Cancer*. 2000;8(5):377–384.
146. Ronson A. Psychiatric disorders in oncology: recent therapeutic advances and new conceptual frameworks. *Curr Opin Oncol*. 2004;16(4):318–323.
147. El-Jawahri A, LeBlanc TW, Kavanaugh A, et al. Effectiveness of integrated palliative and oncology care for patients with acute myeloid leukemia: a randomized clinical trial. *JAMA Oncol*. 2021;7(2):238–245.
148. Mello M, Tanaka C, Dulley FL. Effects of an exercise program on muscle performance in patients undergoing allogeneic bone marrow transplantation. *Bone Marrow Transplant*. 2003;32(7):723–728.
149. Courneya KS, Keats MR, Turner AR. Physical exercise and quality of life in cancer patients following high dose chemotherapy and autologous bone marrow transplantation. *Psychooncology*. 2000;9(2):127–136.
150. Dimeo F, Bertz H, Finke J, Fetscher S, Mertelsmann R, Keul J. An aerobic exercise program for patients with haematological malignancies after bone marrow transplantation. *Bone Marrow Transplant*. 1996;18(6):1157–1160.

CHAPTER 99

PAIN MANAGEMENT AND ANTIEMETIC THERAPY IN HEMATOLOGIC DISORDERS

Thomas W. LeBlanc

Relieving pain in patients with hematologic disorders requires a multifaceted approach.[1,2] This chapter will provide the clinician with the tools to perform a systematic evaluation of the pain complaint and propose a rational, evidence-based, strategy. After the source and type of the pain has been identified and assessed, appropriate nonpharmacologic and pharmacologic therapies can be initiated. The approach to managing pain in hematologic disorders is largely based on the approach for managing cancer-related pain. We do not address the neurosurgical or anesthetic procedures for managing pain; those interested in these techniques are referred to several excellent reviews.[1–5] We will also review the pharmacologic strategies for managing nausea in patients with hematologic disorders undergoing treatment.

TAXONOMY OF PAIN

Although there is no one standardized classification system for cancer pain, several systems have been proposed. Cancer pain syndromes, and thus by analogy, pain syndromes in hematologic disorders, may be classified temporally, pathophysiologically, and etiologically, according to distinct clinical–anatomical entities, or any combination thereof. It is important to determine both the etiology and inferred pathophysiology in the assessment of the pain complaint because this may suggest the use of specific therapies. Pain can be categorized as *nociceptive* (somatic or visceral), *neuropathic*, or *idiopathic*.[6] Nociceptive pain is pain that is sustained predominantly by tissue injury or inflammation. Nociceptive somatic pain is described as sharp, aching, stabbing, throbbing, or pressure-like. Nociceptive visceral pain is poorly localized and is usually described as crampy pain (e.g., obstruction of hollow viscus) or as aching and stabbing (e.g., pain secondary to splenomegaly). Neuropathic pain is sustained by abnormal somatosensory processing in the peripheral or central nervous system (CNS). Sensations described as “burning,” “shock-like,” and “electrical” typically suggest neuropathic pain. On physical examination, patients may have allodynia (pain induced by nonpainful stimuli) and hyperalgesia (increased perception of painful stimuli). In the absence of evidence sufficient to label pain as either nociceptive or neuropathic, we may use the term *idiopathic*. However, in patients with hematologic disorders, this term should lead to additional workup and a search for an underlying etiology and pathophysiology.

EVALUATION OF THE PAIN COMPLAINT

Initial Evaluation

Effective pain management requires a comprehensive assessment of the patient’s pain. The clinical presentation of a patient with chronic pain is very different from that of a patient in acute pain. The patient with chronic pain does not present with the common autonomic manifestations of acute pain (e.g., tachycardia, sweating, elevated blood pressure) or facial grimacing, but often appears withdrawn, quiet, depressed, or irritable; moves very little spontaneously; and experiences discomfort when moved. When the pain is relieved, these patients often exhibit completely different behaviors, becoming mobile, engaged, and involved with other people. The first component in the assessment is to believe the patient’s complaint.

Patient reports of pain are valid, reliable, and reproducible.[1] A variety of assessment tools are available, and most can be completed in 5 to 10 minutes or less.[1,2] A commonly-used example is the “Brief Pain Inventory.”[3] The pain complaint should be characterized by a number of descriptors, including the pain location, intensity, quality, onset, and duration; location and patterns of radiation; and what relieves or exacerbates the pain and its functional consequences (including how the pain affects the patient’s function, including their ability to sleep or eat and how it affects physical activity, relationships with others, emotions, and concentration). Most patients with chronic cancer pain also experience periodic flares of pain, or “breakthrough pain.”[2,4] An important subtype of breakthrough pain is “incident pain,” which is caused by voluntary activity. The initial evaluation should determine the extent to which the patient has breakthrough pain and if it is provoked by movement (nociceptive) or tends to be paroxysmal in nature (neuropathic). This subjective information, combined with the physical examination and diagnostic studies, may identify a specific pain syndrome and its implied pathophysiology.

In a patient with a hematologic disorder, the cause of pain may be the disease itself, the specific therapy for the disease, diagnostic procedures related to the disease, or unrelated disorders (Table 99.1). Splenomegaly, bone injury (e.g., infarction, infection, hemarthroses, and infiltration), leptomeningeal infiltration, and spinal cord compression frequently accompany hematologic diseases. Chemotherapy and radiation therapy can cause mucositis, typhlitis, hemorrhagic cystitis, and peripheral neuropathy; corticosteroid withdrawal may cause myalgias. Immunosuppression, caused by the diseases themselves or by the therapies used to treat them, may lead to painful infections such as perirectal abscesses, herpetic or candidal esophagitis, and herpes zoster. Patients with sickle cell disease have a number of causes for both acute and chronic pain (Table 99.2).

Distress, however, may arise from nonanatomic sources. The pain complaint may represent the patient’s only means of expressing nonspecific feelings of distress to the clinician. Chapman recognized three categories of this distress: anxiety, arising from fear of disfigurement or of uncontrollable pain, fear of loss of social position or of self-control, or fear of death; anger at the failure of the physicians to provide a cure; and depression from the loss of physical ability, a sense of helplessness, and the impact of financial problems. In addition to these psychological, social, and financial contributions, spiritual concerns may exacerbate any concomitant painful sensations.[5] Alleviating them may significantly reduce distress and decrease the need for pain medications or other interventions.

Sometimes there may be a disparity between the patient’s expression of pain and the patient’s family or friends’ appreciation of the impact of the pain. The following script may help to begin a conversation around this issue: “You seem to show a different perspective about the pain from those of your family and friends. Help me to understand *what the pain is like for you* and why you think your family and friends feel differently.” Differences in cultural backgrounds may also affect the expression of pain, and thus may vary within a family as well as in different families.[5]

TABLE 99.1 Common Pain Syndromes in Hematologic Malignancies

Procedure-Related Pain	
Deep somatic pain	Bone marrow aspiration, biopsy, and harvest
	Headache following lumbar puncture
Superficial somatic pain	Venipuncture (needle insertions)
	Central catheter placement/positioning
Therapy-Related Pain	
Deep somatic pain	Bone marrow expansion and/or sensitization by granulocyte colony-stimulating factor, osteoporosis (e.g., from corticosteroids use), myalgias (e.g., from corticosteroid withdrawal), myopathy
Superficial somatic pain	Oropharyngeal mucositis (e.g., from chemotherapy or radiotherapy)
Visceral pain	Enteritis, typhlitis, hemorrhagic cystitis
Neuropathic pain	Drug-related neuropathies (e.g., from chemotherapy agents)
Headache	Drug-related (e.g., due to tretinoin)
Pain From Hematologic Malignancy	
Somatic pain	Bone infarct or necrosis, osteomyelitis, compression fracture, hemarthrosis
Visceral pain	Tumor involvement, splenomegaly, lymphadenopathy or lymphadenitis
Neuropathic pain	Paraproteins with antimyelin properties, amyloid infiltration, peripheral nerve compression, spinal cord compression
Mixed pain	Headache, meningeal infiltration or infection, brain metastasis, or primary tumor

TABLE 99.2 Classification of Pain Syndromes in Sickle Cell Disease

- Pain secondary to the disease itself
  - Acute pain syndromes
    - Acute chest syndrome
    - Calculus cholecystitis (pigment stones)
    - Hand–foot syndrome (in children)
    - Hepatic crisis
    - Priapism
    - Pulmonary infarction
    - Recurrent acute painful episode
    - Splenic sequestration (in children)
  - Chronic pain syndromes
    - Arthropathies or arthritis
      - Avascular necrosis
      - Chronic osteomyelitis
      - Intractable chronic pain
      - Leg ulcers
    - Neuropathic pain
    - Pain secondary to therapy
    - Loose prosthesis (in patients after arthroplasty for avascular necrosis)
    - Opioid withdrawal
  - Postoperative pain
- Pain caused by comorbid conditions

From Ballas SK. Pain management of sickle cell disease. *Hematol Oncol Clin North Am.* 2005;19:785.

Continued Assessment

A standardized measurement tool, such as a verbal rating scale (VRS) or a visual analog scale (VAS), should be consistently used during follow-up visits.[1,2,6] These measurements are thought to be more accurate than mere qualitative descriptors, such as: "The pain is better." Using, for example, a scale of 0 (no pain) to 10 (the worst pain one can imagine), a decrease in pain intensity from 10 to 8 indicates that the patient could benefit from additional dose titration, but a decrease from 10 to 3 suggests that the current dose is effective. A pain goal should be assessed by asking the patient, "What level of pain would be acceptable to you?" Negotiating this goal together is often necessary, and helpful, as the complete elimination of pain is often not possible. The ongoing assessment should also pay attention to changes in the phenomenology of the pain, new occurrences of pain, or a change in the location of pain—all of which may suggest progression of disease in the patient with a hematologic disorder.

The successful outcome of pain therapy should be more than simply the lowering of pain intensity scores, but the impact on function, the minimization of side effects from treatment, and the prevention of opioid misuse. Passik and Weinreb developed a useful mnemonic device for the assessment of pain therapy in chronic nonmalignant pain known as the "4 As": analgesia, activities of daily living, adverse events, and aberrant drug-taking behaviors (e.g., repeated dose escalation or nonadherence, hoarding medications, or acquiring controlled substances from other medical sources or even non-medical avenues).[7] By focusing on these relevant domains in the continued assessment of the pain therapy, the clinician is able to determine if the therapy makes a true difference in the patient's life, stabilizes or improves psychosocial functioning, manages side effects, and provides a means of assessing for aberrant behaviors. Ultimately, the goal should be to lower the pain *to a level acceptable to the patient* and to improve the patient's level of functioning.

THERAPY DIRECTED AT THE UNDERLYING ETIOLOGY

A major component of pain therapy is to ameliorate the underlying cause of the pain, when possible. Surgery, chemotherapy, radiation therapy, immunosuppression, and antibiotics may all be used. Attention to patient comfort should be maintained regardless of the diagnostic or treatment interventions proposed. Generally speaking, the most effective pain management strategy for cancer-related pain is to effectively treat the cancer. This is not always possible, unfortunately.

NONPHARMACOLOGIC METHODS OF PAIN MANAGEMENT

Cognitive-Behavioral Interventions

Education and Reassurance

Patients with serious hematologic disorders are often required to undergo extensive diagnostic testing, which can include painful procedures. A rehearsal of the planned test or procedure, including a discussion (or view) of the appearance of the room and the length of time to be spent in the test apparatus, can minimize the patient's anxiety. Such explanations, offered preoperatively, lessen the need for postoperative medication and shorten the patient's hospital stay. If conscious sedation is not planned, a pleasant distraction may be helpful to divert attention from certain procedures (e.g., bone marrow aspiration or biopsy) that take place in the clinician's office or in the patient's room. For example, a clinician might present a patient with an electronic tablet–guided relaxation training session while the physician is preparing for a procedure. The patient can utilize the relaxation techniques recently acquired during the procedure in order to diminish pain.[8]

Hypnosis

Hypnosis can be a useful adjunct in the management of pain, including for patients undergoing painful procedures.[5] The hypnotic trance, a state of heightened and focused concentration, allows one to manipulate the perception of pain and diminish sleeplessness, anxiety, and anticipation of discomfort. Hypnotic training of patients with sickle cell disease or hemophilia decreases the frequency and pain intensity of painful crises or bleeding episodes, respectively. In a controlled trial comparing hypnosis with cognitive-behavioral therapy in relieving mucositis after a bone marrow transplant, patients using hypnosis reported a significant reduction in pain control compared with patients who used cognitive-behavioral techniques.[8]

In the absence of a formal hypnotic induction, words used by a practitioner to describe procedures are very important. For example, suggesting that the numbness and coolness of a local anesthetic will persist may diminish the discomfort of a bone marrow biopsy. Using the phrase: "You will feel something; I'm not sure what you will feel because everyone feels this a little differently" in place of "This is going to hurt a lot!" gives the patient permission to alter the sensation and may also diminish the experience of pain.[9]

Meditation

Meditation has long been used as a method for controlling pain. Given the wide range of meditation practices and methods, it has historically been difficult to conduct evidence-based research into the effectiveness of meditation. Some research has shown that using meditation to increase focus and clarity does improve pain symptoms and appears to correlate with decreased pharmacologic requirements.[10]

Cognitive-Behavioral Techniques and Counseling

The cognitive-behavioral approach addresses several psychosocial and behavioral factors that contribute to patients' experience of pain.[2,4,8] These techniques have demonstrated clinical utility for patients with a wide range of chronic pain syndromes. Psychological counseling as part of a multidisciplinary approach to pain treatment provides education, support, and skill development for patients with pain. It can improve patients' abilities to communicate their pain to health care personnel and may be effective in overcoming anxiety and depression. Spiritual counseling may help patients who have lost hope, can find no meaning in their lives, or believe they are being punished or have been forsaken by God.[5] They may interpret their pain in light of these feelings. Through counseling, they can regain a sense of worth and belonging. As they recast the pain in its true light, its intensity is often diminished.

Cutaneous Techniques

Acupuncture, massage, vibration, and applying cold or heat to the skin over injured areas are often very effective. Cold wraps, ice packs, or cold massage using a cup filled with water that has frozen into a solid piece of ice relieve the pain of muscles that are in spasm from nerve injury. Heat from heating pads, hot wraps, or paraffin treatments can soothe injured joints but should not be used over areas of vascular insufficiency.

EMLA, a cream containing two topical anesthetics (2.5% lidocaine and 2.5% prilocaine) is used, especially in children, to decrease the pain of superficial cutaneous procedures (e.g., venous cannulation or skin anesthesia before lumbar puncture, bone marrow aspiration, or biopsy).[5] In adults, it is sometimes used before access of implanted vascular access devices or CNS ports. To achieve anesthesia, the EMLA cream must be applied 1 to 1.5 hours before the planned procedure in a mound under a semipermeable dressing such as Opsite or Tegaderm. When EMLA is used as directed, methemoglobinemia has not been a problem even in infants as young as 3 months old. Skin blanching occurs, sometimes exceeding or equaling the frequency of that found with placebo moisturizing cream placed under the occlusive dressing. ELA-Max, a cream containing 4% lidocaine, is available over the counter and is an alternative to EMLA cream. Because it does not contain prilocaine, there is no risk of methemoglobinemia.

Lidocaine patches can be used over areas of hyperesthesia, as can occur in patients with postherpetic neuralgia (PHN) or nerve entrapment by vertebral body collapse.[5] The patch is applied to the affected area for no more than 12 consecutive hours a day and can be cut to size. Use should be avoided over areas of broken skin and in patients undergoing radiation therapy. Lidocaine patches have been safely applied for up to 24 hours/day for up to 4 days with minimal systemic absorption in healthy volunteers and in patients with PHN.

Neuromodulation

Transcutaneous electrical nerve stimulation (TENS) devices are indicated for patients with dermatomal pain, such as PHN or radiculopathy from spinal cord compression.[5,11] For optimal effect, a physiatrist or physical therapist familiar with the device should train the patient in its use. The efficacy of TENS therapy for patients with cancer pain remains controversial.

Radiation Therapy

Radiation therapy is commonly used in the management of painful bone lesions, spinal cord compression, bulky lymphadenopathy, and symptomatic splenomegaly in patients with hematologic malignancies.[5,12] Radiotherapy is the treatment of choice for local metastatic bone pain in most situations, although patients with underlying pathologic fractures may require surgical fixation before radiotherapy. Randomized trials have shown that single-fraction radiotherapy is as effective as multifraction radiotherapy in relieving pain caused by metastases. However, there are higher rates of retreatment, and single-fraction radiotherapy may not prevent pathological fractures or spinal cord compression.[12] In patients with poor performance status or a short life expectancy, a single dose (8 Gy) of radiation or a hypofractionated course (20 Gy/5 fractions) may be preferable and less burdensome. Some hematologic malignancies can be more sensitive to radiation therapy than are most solid organ tumors, with effective palliation of cancer-related symptoms occurring at lower radiation doses in many cases of lymphoma, for example.

Vertebroplasty and Kyphoplasty

Vertebroplasty and kyphoplasty are both minimally invasive techniques used to stabilize vertebral compression fractures and reduce pain. Vertebroplasty is a procedure in which bone cement, usually polymethylmethacrylate, is injected into the vertebral body. With kyphoplasty, a balloon is first inserted into the vertebral body followed by inflation and then deflation before cement is added. Balloon kyphoplasty has been shown to stabilize pathologic vertebral fractures caused by multiple myeloma and significantly reduce pain, though there remains some debate about its efficacy amid other negative studies.[13]

Anesthetic Techniques

Trigger-point injections, nerve blocks, and neurolytic procedures are useful for acute and chronic localized pain. After excisional biopsy of an axillary lymph node, for example, a burning, constricting pain in the posterior arm and chest wall may develop; this pain is often promptly relieved by trigger-point injection.

Lymphoma or multiple myeloma may involve the spine and lead to vertebral collapse or pain from progressive disease that is refractory to antineoplastic therapy. Such pain is often particularly difficult to manage. Insertion of temporary or permanent indwelling epidural or intrathecal catheters to deliver opioids, local anesthetic agents, clonidine, or combinations of these and other agents can be very effective, especially in relieving lower thoracic or lumbar spine pain, as well as pelvic and lower extremity pain.[14] Reviews of the indications for and techniques of the anesthetic and neurolytic procedures are available.[15] Consultation with an expert in anesthesiology and/or palliative medicine can be helpful in these situations, to obtain expert guidance about appropriate indications for these procedures.

PHARMACOTHERAPY

Drugs useful for pain relief include nonopioid analgesics, opioids, and adjuvant analgesics. Most patients require a combination of medications for optimal pain relief (Fig. 99.1).[16]

Management of Mild-to-Moderate Pain

Nonopioid analgesics should be given to patients with mild-to-moderate pain.[1,2] Aspirin and nonsteroidal antiinflammatory drugs (NSAIDs), including cyclooxygenase-2 (COX-2) inhibitors, are especially useful as antiinflammatory agents because they decrease local prostaglandin release through the inhibition of COX (although the mechanisms for their analgesic properties are not as clear).[17] Acetaminophen is an effective analgesic but only a weak antiinflammatory agent. Daily intake of acetaminophen should not exceed 3 to 4 g because of the potential hepatic toxicity. It is important to prescribe an adequate dose of acetaminophen or NSAID at regular intervals, switching to another nonopioid analgesic only when maximal doses of the first have become ineffective.[17]

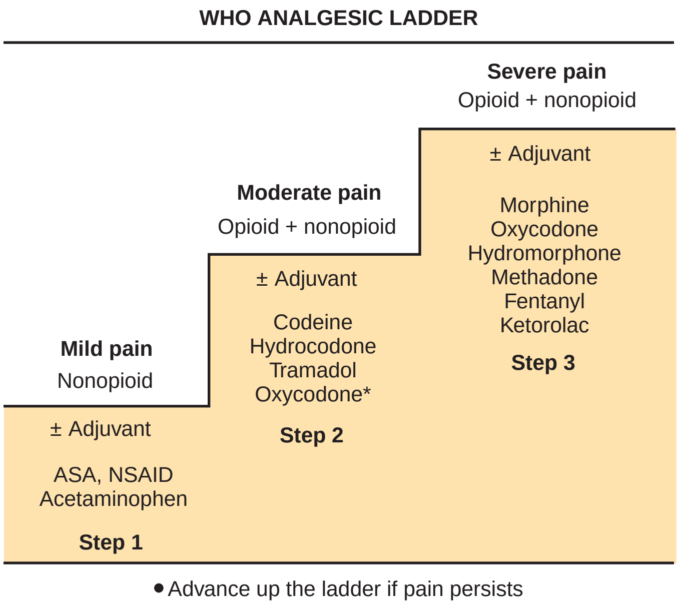


Figure 99.1 STRATEGY FOR PHARMACOLOGIC MANAGEMENT OF PAIN USING A MODIFIED (FOUR-STEP) WORLD HEALTH ORGANIZATION ANALGESIC LADDER. Multiagent therapy is usually required for optimal pain management. Patients with mild pain should be started on a nonopioid analgesic, and those with moderate pain should be started on a step 2 opioid. Many patients can benefit from the addition of a nonopioid to the opioid (e.g., for bone pain) or an adjuvant agent to the opioid (e.g., for neuropathic pain). If this combination does not produce adequate relief or the patient presents with severe pain, step 3 opioids should be begun initially. Toradol (Ketorolac) is a nonsteroidal antiinflammatory drug (NSAID) with the pain-relieving potency of a step 3 opioid. Many patients can benefit from the addition of nonopioid analgesics or adjuvants, if indicated. *Oxycodone in combination with products. It has been suggested that if opioids with nonopioid analgesics and adjuvants is unsuccessful in providing relief that an additional "4th step" of interventional/intraspinal delivery systems should be initiated.[16] *ASA*, Aspirin; *NSAID*, nonsteroidal antiinflammatory drug; *WHO*, World Health Organisation.

Ketorolac tromethamine (Toradol) is an NSAID of particular value in relieving moderate-to-severe acute pain.[18] A parenteral dose of 30 mg of ketorolac equals the pain-relieving potency of a parenteral dose of 15 mg of morphine, and acute toxicity is minimal if the total daily dose is under 100 mg. Oral ketorolac is considered less potent. Ketorolac has all of the side effects of the NSAIDs and is not recommended for use beyond 5 days because of an increased risk of renal toxicity. If that degree of pain relief is needed chronically, an opioid agent should be substituted.

Because NSAIDs can cause renal insufficiency in a significant number of patients, renal function should be assessed 1 to 2 weeks after initiation of any of these agents. NSAIDs should be used with caution in patients with a history of aspirin allergy or asthma because they can precipitate bronchospasm in as many as 20%. Significant edema can occur in patients with cirrhosis or congestive heart failure.[17] The relatively selective COX-2 inhibitors, such as meloxicam, celecoxib, and nabumetone, have been shown to cause similar gastrointestinal side effects as nonselective NSAIDs.[19] If NSAIDs are required in patients with a history of significant gastritis or ulcer disease, or who are older than 70 years, COX-2 inhibitors or a concomitant proton pump inhibitor should be considered. Cardiotoxicity is well described in the literature for COX-2 selective inhibitors.[20] Recently, the cardiac risk associated with traditional NSAIDs has been questioned. In a meta-analysis that included naproxen, ibuprofen, diclofenac, and several COX-2–specific inhibitors, ibuprofen and diclofenac demonstrated an increased risk of stroke. Diclofenac was also associated with an increased risk of cardiovascular death. Naproxen was not noted to increase risk in this study.[20] Nonopioid analgesics should be continued when opioid analgesics are added because they can potentiate the pain-relieving effect of the opioid (see Fig. 99.1).[21] However, when aspirin or acetaminophen is included in a fixed drug combination (e.g., Percodan, Percocet), toxicities may develop if the patient uses the medication more frequently than prescribed. The metabolism of salicylates is limited by the capacity of the hepatic microsomal system.[5] After it is saturated, salicylate levels are dependent on renal clearance. Small increases in maintenance doses can lead to serious salicylism. Patients with low albumin levels or acid urine are particularly susceptible to the development of salicylate toxicity.

Tramadol, which both weakly inhibits norepinephrine and serotonin reuptake, and weakly binds to μ-opioid receptors and has opioid-like side effects, can be used for mild-to-moderate pain. A dose of 100 mg is more effective than 60 mg of codeine. Tapentadol (Nucynta) also has both μ-receptor agonism and norepinephrine reuptake inhibition. Studied in moderate-to-severe acute postoperative pain, osteoarthritis, and low back pain, doses of 50 to 100 mg every 4 to 6 hours are comparable to oxycodone 10 to 15 mg every 4 to 6 hours with less nausea, vomiting, and constipation.

Management of Moderate-to-Severe Pain

Opioid therapy is the cornerstone of management of patients with moderate-to-severe pain.

In patients with acute moderate-to-severe pain (i.e., ≥4/10 on VRS), intravenous dosing should be started with the goal of rapid titration (see, for example, National Comprehensive Cancer Network [NCCN] guidelines for Adult Cancer Pain).[22] In opioid-naïve patients, morphine 2 to 5 mg or its equivalent (see box on Relative Potencies of Commonly Used Opioids) should be dosed every 15 minutes (peak effect ~15 minutes). The patient should be reassessed for analgesic effect and side effects. If pain is not relieved, then the dose can be increased by 50% to 100%; if the pain score decreases to an acceptable level, then the same dose can be continued and given as a standing dose "around the clock." Alternatively, a patient-controlled analgesia (PCA) can also be used in these situations and can provide the patient with significant control over their pain experience. Similarly, the PCA demand dose can be calculated over a 24-hour period to determine the "basal dose" of opioid the patient will need for stable analgesia. This can be provided as a basal rate on the PCA, or given the clinical situation, as a long-acting formulation equivalent to the amount of demand doses received. In opioid tolerant patients, a dose equivalent to 10% to 20% of the patient's total 24-hour oral requirement should be administered as an intravenous bolus (see box on Relative Potencies of Commonly Used Opioids) and titrated, as above, every 15 minutes until pain is controlled and acceptable to the patient. For example, a patient receiving oxycodone sustained release 60 mg twice daily, should be prescribed morphine 5 to 10 mg intravenously every 15 minutes as needed (oxycodone 120 mg is approximately equivalent to 180 mg of oral morphine, or 60 mg of intravenous morphine; take 10% to 20% of this dose). The long-acting opioid should be continued or converted to a continuous infusion, or given as a basal rate via a PCA.

Once the patient has an acceptable level of analgesia, this dose can be converted to a sustained release formulation, or to another equivalent long-acting opioid. If morphine is used, for example, the patient will need three times the parenteral dose that was effective. For example, a patient who requires 10 mg of morphine per hour (i.e., 240 mg/24 hours given intravenously) will need 720 mg/day of the oral sustained-release agent (240 mg every 8 hours). This can also be given orally as 360 mg every 12 hours. Short-acting immediate-release morphine should be available for rescue dosing at 10% of the total daily dose. For this patient, 60 to 90 mg every 3 to 4 hours is recommended. If the amount of opioid taken as a rescue dose is significant (>25% of the daily dose) for 1 or 2 days, the total dose of long-acting agent is adjusted upward accordingly.

In the outpatient setting, patients with chronic, moderate-to-severe pain can be treated with oral pain medications, e.g., morphine 5 to 15 mg (or an equianalgesic dose, e.g., oxycodone 5 to 10 mg), and titrated in a similar fashion. The peak effect of most oral opioids is approximately 60 minutes, so the patient should be reassessed 60 minutes following the dose for analgesic efficacy and side effects. The same strategy as above can be used for opioid-tolerant patients, i.e., 10% to 20% of the basal dose can be given for additional analgesia and titrated to effect.

Agents to prevent side effects should be started once opioids are initiated. All patients should be prescribed a stimulant laxative, for example, senna (one or two tablets orally daily to twice daily, up to a maximum of eight pills per day), plus or minus a stool softener, for example, Colace 100 mg three times per day. If a more potent laxative effect is needed, lactulose (15 to 30 mL) or polyethylene glycol (17 g) is added. In opioid-naïve patients, prochlorperazine (Compazine 10 mg taken orally two or three times daily) is prescribed as needed to treat nausea. Other antiemetics may be prescribed instead, depending on the desired side effect profile; 5HT3 blocking medications, for example, are constipating and can cause QTc prolongation and headaches, but are not sedating like prochlorperazine.

Opioid Analgesics

Patient Education and Risk Assessment

To ensure patient adherence with an opioid prescription, education of members of the health care team, the patient, and the family is often required to dispel the many misconceptions associated with opioid therapy.[5]

Fear of addiction is a common cause of inadequate prescribing of opioids and a barrier to their acceptance by patients.[5] Patient adherence can be improved by providing a full explanation of the differences between addiction and physical dependence, education regarding the appropriate use, storage, and disposal of opioid medications, and the risk factors for opioid misuse. Patients may also fear that if they take opioid medications for moderate pain, the medications will no longer be effective if more severe pain occurs. Because this fear, if unexpressed, can lead to undertreatment, the topic should be addressed even if the patient does not raise the question. A functional goal of therapy, such as returning to a favorite activity or reinstituting normal activities of everyday life, may enable the patient and the family to accept the opioid. Misconceptions about religious teachings may prevent health care personnel, patients, and their families from giving or accepting adequate pain medication. Roman Catholics, for example, may not be aware of the church's position, as stated in the current Catechism, that opioids may be used at the approach of death even if their use to manage pain may unintentionally shorten the patient's life. The Church does not consider this use of pain medication to be a means of suicide or euthanasia.[5] Regardless, it is important to perform a risk assessment each time an opioid is being considered, to inform decision-making about risk of abuse. There are several validated tools available to facilitate this, such as the Opioid Risk Tool (ORT). Many states now also mandate checking a state-wide prescription monitoring system before prescribing an opioid, to ensure that a patient is not obtaining multiple prescriptions from various clinicians. As part of a high-quality opioid risk assessment, one must also consider the additive risk of the patient's entire medication list, as the combination of an opioid plus other medication classes can increase the risk of a serious or fatal adverse event (e.g., an opioid plus a benzodiazepine).

Choice of Medication

Because a wide variety of medications are available, pharmacokinetic considerations and side-effect profiles should be weighed when choosing opioid agents. Intermittent moderate-to-severe pain lasting hours to several days is amenable to oral analgesics with short half-lives (3 to 4 hours) with appropriate potency (e.g., immediate-release oxycodone, morphine, hydromorphone [Dilaudid], or oxymorphone [Opana] when available). Severe pain of relatively constant intensity should be treated with oral sustained-release morphine or oxycodone taken every 8 or 12 hours,[1,2] hydromorphone (Exalgo) taken every 24 hours, oxymorphone (Opana ER) taken every 12 hours, hydrocodone (Zohydro ER, Hysingla ER) taken every 12 hours, methadone taken every 6 to 8 hours, or transdermal fentanyl renewed every 48 to 72 hours. Twelve- to 24-hour formulations of oral morphine (e.g., Kadian, Avinza) are also available; for patients unable to take pills, the capsule can be opened and the pellets sprinkled on food or suspended in water and given through a feeding tube (see box on Relative Potencies of Commonly Used Opioids).[5]

Practical Considerations When Using Opioids

Drugs with short half-lives should be used for "rescue doses" given for incident pain (i.e., pain with movement) and for between-dose pain exacerbations often referred to as "breakthrough pain." The dose of the rescue medication is usually calculated as 10% to 20% of the total 24-hour dose, although there is no evidence for this heuristic.[23,24] For example, if a patient is receiving 300 mg of oral sustained-release morphine twice each day, the rescue dose is 10% to 20% of 600 mg, which is 60 to 120 mg of short-acting morphine. Agents with short half-lives should also be used in older patients and in patients with impaired renal or hepatic function.[5] For patients with a history of drug abuse, agents with longer half-lives, such as methadone, are preferred.

There is considerable variability with respect to the side effect profile of the various opioids in each patient. Therefore it is often useful to switch to another agent if a patient is experiencing dose-limiting side effects with the initial opioid chosen, or inadequate analgesia despite escalating doses amid prior efficacy of this agent. This practice is often called "opioid rotation." For example, if the patient on morphine experiences persistent or debilitating nausea, the rotation to another opioid at an equianalgesic dose should be considered (see box on Relative Potencies of Commonly Used Opioids). Because of incomplete crosstolerance, the initial dose for patients taking higher doses of opioids should be reduced by approximately 25% to 50%. The short-acting rescue medication can provide relief if this initial dose does not prove adequate.

Methadone is increasingly used for patients with moderate-to-severe pain. It is by far the least expensive of the opioids; can be given

RELATIVE POTENCIES OF COMMONLY USED OPIOIDS

Drug	Epidural	SC or IV (mg)	PO (mg)
Morphine	1	10	30
Codeine		130	200
Oxycodone		N/A	20
Hydromorphone	0.15	1.5	7.5
Methadone[a]		[a]	
Oxymorphone		1	10
Levorphanol		2	4
Fentanyl		0.1	N/A
Meperidine (Demerol)[b]		75	300

[a] Methadone is approximately half as potent orally as it is intravenously. It is usually not given SC because of local irritation. Standard equianalgesic tables do not reflect methadone's potency when used in repeated doses.

[b] Not recommended for patients with chronic pain.

IV, Intravenous; *N/A*, not applicable; *PO*, oral; *SC*, subcutaneous.

by the oral, sublingual, rectal, intravenous, and epidural routes; and may have particular usefulness in patients with poor tolerance to other opioids. Methadone has anecdotally been used for neuropathic pain; however, recent clinical trials have not demonstrated this benefit.[25] Methadone is structurally unrelated to morphine and fentanyl and can be used in the rare case of true allergy to these medications. It is also helpful when patients have neurotoxic side effects of other opioids. Methadone is a potent μ-opioid receptor agonist. The l-isomer has higher binding affinity for delta opioid receptors, which reduces tolerance, while the d-isomer has *N*-methyl-D-aspartate (NMDA) receptor antagonism and 5-hydroxytryptamine and norepinephrine reuptake blockade, which may be helpful in providing analgesia.[25] Patients with neuropathic pain and patients taking opioids chronically have increased levels of NMDA receptors in the dorsal horn of the spinal cord. NMDA antagonizes the activity of the opiate receptors. Blocking the NMDA receptors therefore enhances the analgesic effect of externally administered opioids. Ketamine, a pure NMDA antagonist, similarly enhances opioid efficacy but is associated with more cognitive side effects than methadone.

Methadone interacts with inducers and inhibitors of the cytochrome P450 system. It is extensively metabolized by CYP1A2, CYP3A4, and CYP2D6; the first two are induced by several drugs and other substances (e.g., cigarette smoke), and the last enzyme has a genetic polymorphism. Drug levels of desipramine and zidovudine increase when patients are receiving methadone. Drugs that lower the levels of methadone include phenytoin (by 50%), phenobarbital, carbamazepine, rifampicin, and risperidone, each of which has precipitated withdrawal symptoms.[26] Drugs that raise the serum methadone levels include ketoconazole, fluconazole, fluoxetine, and fluvoxamine. The selective serotonin reuptake inhibitors (SSRIs) (except venlafaxine) may raise methadone levels in CYP2D6 rapid metabolizers. Owing to these complexities, it is recommended to consult with an anesthesia pain specialist, palliative care specialist, and/or pharmacist when prescribing methadone.

Methadone can cause prolongation of the QT interval. A mean methadone dose of 400 mg/day (standard deviation: 283 mg) was found in 17 patients with torsades de pointes. In an evaluation of reports of methadone-related adverse events to the US Food and Drug Administration (FDA), approximately 1% of the greater than 5000 reports were of QT prolongation or torsades de pointes. The median dose was 345 mg with a range of 29 to 1680 mg. Drugs that also prolong the QT interval, such as metoclopramide and olanzapine, should be used with caution in patients receiving significant doses of methadone. Gabapentin, the drug used most often as a neuropathic pain adjuvant, does not interact with methadone metabolism. There is insufficient evidence to recommend a screening interval or dose threshold for detecting methadone-induced QTc prolongation, so overall caution and attention are warranted.[27]

Other difficulties with using methadone lie in its variable and long biologic half-life, and the controversy about its equianalgesic dosing range. Some studies have reported that the equianalgesic dose of morphine to methadone varies as the dose of morphine increases.[5] Dose ratios vary from 4:1 at morphine doses of 30 to 90 mg, to 6:1 at 90 to 300 mg, and to 8:1 at doses of more than 300 mg of morphine. Other studies report a ratio of 20:1 for doses of oral morphine equivalents greater than 1000 mg. Some physicians advise a 3-day conversion to methadone. On the first day, the dose of the old opioid is reduced by one-third, and one-third of the calculated dose of methadone is given; the second day, the dose of the remaining opioid is reduced by half, and the dose of methadone is only increased if the patient is experiencing moderate-to-severe pain; the third day, the old opioid is discontinued, and the standing dose of methadone is adjusted to reflect the rescue doses.[28] When converting patients directly from intravenous fentanyl to intravenous methadone, a conversion ratio of 25 μg/h of fentanyl to 0.1 mg/h of methadone has been found in a pilot study to be a safe and effective initial infusion rate.[28]

A relatively new opioid to the US market that is now available in both extended release and immediate release is oxymorphone. Oxymorphone extended release has been found to provide safe and effective pain relief for cancer pain.[29] Oxymorphone extended release is dosed twice daily and is about twice as potent as oxycodone.[30]

Levorphanol, which is chemically similar to dextromethorphan (an NMDA antagonist and cough suppressant), is a potent opioid that may be considered for patients with severe cancer pain. It was originally synthesized as an alternative to morphine more than 40 years ago. It has a greater potency than morphine, approximately five times as potent in its oral formulation. Analgesia is achieved through its agonistic activity at μ-, δ-, and κ-opioid receptors, as well as by its antagonism of NMDA receptors. Levorphanol can be given orally, intravenously, and subcutaneously.

Buprenorphine has long been used to treat patients with addiction as an alternative to methadone. It is available buccal/sublingually, intravenously, and now as a transdermal patch. Buprenorphine has been shown to be very effective in treating cancer pain (including neuropathic) and provides several important advantages over other opioids: it is associated with less cardiotoxicity (does not significantly prolong the QTc interval), respiratory depression, cognitive impairment, and constipation, and is one of the safer opioids to use in older patients or patients with renal dysfunction.[31]

Meperidine is 8 to 10 times less potent than morphine and has a short duration of action, approximately 2 to 3 hours. Normeperidine, an active metabolite which induces dysphoria, is excitatory to the CNS and can cause agitation, tremors, myoclonus, and seizures, especially in high doses, with prolonged use or in renal failure.[5] Normeperidine has a half-life of 13 to 24 hours, which can lengthen with renal failure. The seizure incidence is further increased if the opioid antagonist naloxone (Narcan) must be given. Therefore meperidine is not recommended for use in patients with long-lasting moderate-to-severe pain.[1,2]

Routes of Delivery

Opioids can be delivered noninvasively (orally, rectally, transmucosally, or transdermally) or invasively (subcutaneously, intravenously, or by spinal infusion). For patients switched from one route to another such as oral or rectal to parenteral or spinal medication, or vice versa, the dose must be converted accordingly to avoid overdose or undertreatment (see box on Relative Potencies of Commonly Used Opioids).

Oral Route

Most patients can achieve excellent pain relief with short-acting and/or sustained-release oral opioid preparations. Close attention, frequent follow-up, and ongoing adjustment and titration are often needed to achieve this, however. The typical onset of short-acting opioids via the oral route is 45 minutes to 1 hour, with a typical duration of action around 3 to 4 hours. When tablets and capsules are not feasible, many liquid forms are available in various concentrations. Some solutions do contain alcohol, which can be irritating to patients with oral lesions.

Rectal Route

Rectal opioids (i.e., morphine, oxymorphone, and hydromorphone) replace subcutaneous injections in patients who are suddenly unable to take oral medications. They have approximately the same potency and half-life as orally administered agents[5] and must therefore be administered frequently. Oxycodone has been shown to have a similar mean bioavailability but with a large interpatient variability but longer duration of activity (8 hours). Although not approved by the FDA, in single-dose bioavailability studies of sustained-release morphine preparations, despite delayed absorption from the rectal route, total morphine absorption over 24 hours was equivalent, whether the drug was given orally or rectally.[5]

Transdermal Route

The transdermal fentanyl patch delivers the lipophilic fentanyl into the fat-containing areas of the skin. The drug diffuses continuously from the patch's reservoir through a rate-controlling membrane and is absorbed from the skin depot into the bloodstream, where it is rapidly metabolized.[5] The onset of pain relief is delayed about 12 hours, and a relatively constant plasma concentration of fentanyl is not reached until about 14 to 20 hours after the initial patch is placed.[5] Liberal rescue medication must therefore be provided during the first 24 hours of use of the patch. Similarly if a patient develops signs of fentanyl overdose, naloxone (Narcan) must be given until the skin reservoir has become depleted. About 50% of the drug is still present 24 hours after patch removal. Converting patients from oral or parenteral medication to the patch is easily accomplished[5] (see box on Conversions Between the Transdermal Fentanyl Patch and Morphine). A new patch is applied every 72 hours, although up to 25% of patients require a new patch every 48 hours, thus it is important to ask patients specifically about their pain relief in the last 24 hours of that 72-hour interval.

The transdermal system is an effective method of delivering pain relief for patients with a stable level of chronic moderate-to-severe pain, no oral route available, or no desire to take pills. Side effects include those caused by the contact adhesive along with those commonly associated with other opioids, but they may be better tolerated than those caused by morphine.

CONVERSIONS BETWEEN THE TRANSDERMAL FENTANYL PATCH AND MORPHINE

	Morphine (mg/24 h)	
Fentanyl (μg/h)	Oral	IM or IV
25	50	17
50	100	33
75	150	50
100	200	67
125	250	83
150	300	100

IM, Intramuscular; *IV*, intravenous.
Data from Miaskowski C, Cleary J, Burney R, et al. *Guideline for the Management of Cancer Pain in Adults and Children, Aps Clinical Practice Guidelines Series, No.3.* Glenview, IL: American Pain Society; 2005.

The transdermal system should not be used in patients with sepsis, those experiencing acute pain, those with markedly fluctuating opioid requirements, cachectic patients, or individuals with significant dermatologic insults (i.e., skin graft-versus-host disease [GVHD] or diffuse varicella). When the patient's temperature rises to 40°C, drug absorption from the skin can increase by as much as 35%. If hepatic function is impaired or sepsis or shock develops and blood flow to the liver decreases, plasma concentrations may rise sharply. Patients with cachexia lack the subcutaneous tissue necessary for formation of a drug reservoir. Lower doses may also be required in older patients and in those with respiratory insufficiency.

Transdermal buprenorphine (Butrans) patches have been shown to be safe and effective in patients with cancer pain.[32] They can be used alone or in combination with other opioids without reported problems, and unlike other opioids, which can cause a hyperalgesia effect from long-term use, buprenorphine has been described to exert an antihyperalgesic effect. A lack of immunosuppression and safety in renal insufficiency make buprenorphine ideal for the treatment of cancer patients. Due to the risk of QTc prolongation the transdermal patch carries an FDA warning against exceeding 20 μg/h.

Transmucosal Route

Transmucosally administered fentanyl induces rapid analgesia with a short duration of effect (≈1 hour) and is an effective treatment in the management of breakthrough pain.[24] Oral transmucosal fentanyl citrate (Actiq), fentanyl buccal tablet (Fentora), fentanyl buccal soluble film (Onsolis), fentanyl sublingual tablets (Abstral), and fentanyl sublingual liquid spray (Subsys), as well as a fentanyl nasal spray (Lazanda) are the available transmucosal fentanyl products. They have been found to be both efficacious and safe in the treatment of cancer-related breakthrough pain.

Subcutaneous and Intravenous Routes

Subcutaneous or intravenous administration of opioids can provide pain relief in the shortest amount of time with minimal oversedation. The drugs can be delivered by portable infusion pump that is initiated or continued in the home.[4] Guidelines for their use are available. PCA systems for subcutaneous or intravenous drug delivery have the advantage of responding to the individual patient's threshold for pain while eliminating delays when nurses must administer supplemental medication. The pumps can administer a continuous fixed infusion of the opioid chosen and allow the patient to self-administer boluses of additional medication at frequencies chosen by the ordering clinician. By recording the additional amounts of self-administered medication, the devices also facilitate the adjustment of the continuous dose required for pain relief.

Spinal Route

Epidural or intrathecal opioid infusions can be helpful for select patients. The infused opioids block pain transmission by binding to receptors in the dorsal horn of the spinal cord. Because the drug is being infused in close proximity to the spinal cord, only a small amount of opioid is needed, and the systemic side effects are reduced. Problems with this delivery system in patients who are not opioid naive include pruritus, respiratory depression, and sedation. If tolerance to the opioid develops and higher doses are required for relief, the incidence of side effects may approach that of systemically administered opioids.[33,34] Addition of local anesthetic or α-adrenergic agent (e.g., clonidine) or other agents[14] to the epidural opioid infusion allows for fairly rapid lowering of the opioid concentration and reestablishing opioid sensitivity but can cause hypotension.[35]

Management of Opioid-Related Side Effects

Sedation

The addition of 2.5 to 7.5 mg of dextroamphetamine or methylphenidate[5] (taken orally twice daily) has been shown to reduce opioid-induced sedation and at times allow for escalation of opioid doses without sedation. These medications also improve cognitive function and symptoms of depression. Methylphenidate may also enhance the analgesic effects of opioids.[5] They should be avoided in patients with anxiety, moderate-to-severe hypertension, agitation, thyrotoxicosis, tachyarrhythmias, severe angina pectoris, and closed-angle glaucoma. Modafinil (Provigil), a novel psychostimulant with a mechanism of action different than the amphetamine derivatives, which is approved for narcolepsy and fatigue related to multiple sclerosis, has also been found to be effective for opioid-related sedation.[5]

Constipation

Constipation is the most common opioid-induced side effect.[2] Laxatives should therefore be given routinely, not on an as-needed (PRN) basis,[1,2,5] to patients treated with any of the drugs listed in the box on Relative Potencies of Commonly Used Opioids. Detailed bowel preparation recommendations can be found, but no regimen has been studied in a controlled fashion. Commonly used stool softeners and stimulants include docusate sodium, senna, lactulose, and polyethylene glycol. A combination of a stool softener and laxative seems to be a rational choice for patients taking chronic opioids (e.g., docusate sodium with senna). Promotility agents most directly counter the mechanism of opioid-induced constipation. Bulk-forming laxatives such as psyllium and methylcellulose should be avoided because they increase stool volume without promoting peristaltic action. For refractory opioid-induced constipation, a trial of oral naloxone, methylnaltrexone, or alvimopan may be initiated.[5] These μ-opioid receptor antagonists (RAs) act locally to reverse the effects of opioids on the gut. There is minimal systemic absorption with oral naloxone and subsequently a low risk of precipitating opioid withdrawal or worsening pain at low-to-moderate doses (1.6 to 12 mg/day). Methylnaltrexone (administered subcutaneously)[5] and alvimopan (oral) do not cross the blood–brain barrier and therefore do not cause opioid withdrawal or worsening pain. There is now an oral formulation of methylnaltrexone available as well.

Nausea

Prochlorperazine (10 mg taken two or three times daily) or metoclopramide (10 mg taken three to four times daily) can prevent the nausea that occurs in most patients during the first days of opioid therapy. Relieving constipation or changing the opioid (e.g., from morphine to oxycodone) often eliminates the later development of nausea. Rarely, patients need oral or intravenous ondansetron (8 mg taken two or three times daily). Attention may be needed to QTc prolongation with all of these medications, especially if there is concomitant prescribing of multiple QT-prolonging agents.

Respiratory Depression

Naloxone (Narcan), given intravenously, reverses opioid-induced respiratory depression, although repeated doses are often required.[5] Respiratory depression can occur in patients with mild-to-moderate pain during the initial use of opioids, although it is rare in patients with severe pain and in those chronically receiving opioids. Caution should be exercised before administering naloxone to patients who are chronically receiving opioids to avoid precipitation of severe pain and withdrawal. In such cases, it is inadvisable to administer the usual 0.4 mg/mL dose. Rather, 0.4 mg of naloxone should be diluted with 9 mL of saline and 1 to 2 mL (0.04 to 0.08 mg) of this dilute mixture given every 2 to 3 minutes until the patient is rousable and breathing at least 10 times/min. Do not give enough to fully waken the patient or withdrawal is likely to ensue.[1] In a comatose patient, endotracheal tube placement is recommended to prevent aspiration from the salivation and bronchial spasm that will be induced.[1] Naloxone should not be administered to an alert patient. Concomitant prescribing of multiple sedating medications may increase the risk of respiratory depression and related complications, so attention is needed to the patient's entire medication list; common medications of concern include benzodiazepines and sedative/hypnotic agents.

Adjuvant Analgesics

Adjuvant analgesics are a diverse class of medications, which typically have indications for conditions other than pain. They have analgesic properties and are often used when an opioid regimen alone is unable to provide sufficient analgesia or is associated with dose-limiting side effects.

Neuropathic Pain

Adjuvant agents for patients with neuropathic pain include anticonvulsants, antidepressants, α_2-adrenergic agonists, corticosteroids, topical agents, γ-aminobutyric acid (GABA) agonists, and NMDA RAs.[1,2,5,36] However, the analgesic antidepressants and anticonvulsants are typically preferred for treating neuropathic pain secondary to cancer.[37]

The anticonvulsants gabapentin (Neurontin) and pregabalin (Lyrica) have the fewest side effects and are very effective for patients with neuropathic pain from tumor, peripheral neuropathy from tumor or treatment, and PHN.[36] Despite their names, they have no effect on GABA receptors, but rather bind to the α-2-delta subunit of the N-type calcium channels in neurons within the dorsal horn, thus inhibiting calcium influx and diminishing neuronal hyperactivity. To minimize sedation, doses should be low at first (e.g., gabapentin 100 mg three times daily or 300 mg at bedtime; pregabalin 50 mg twice a day) and should be increased as tolerated every 3 to 5 days until analgesia is achieved. The effective dose of gabapentin varies between 900 and 3600 mg/day in divided doses and that of pregabalin is 150 to 300 mg twice a day. The pharmacokinetics of gabapentin are unique in that it has a ceiling effect related to a saturable transport mechanism in the gut, such that the effects of this drug may plateau during dose escalation.[37] The most common dose-limiting side effect is sedation. Gabapentin and pregabalin need to be renally dosed in patients with decreased creatinine clearance. Peripheral edema related to gabapentin or pregabalin may require diuretics. Pregabalin's gastrointestinal absorption is proportional to the dose throughout the effective dose range, making titration simpler. Other, generally less effective anticonvulsants used for neuropathic adjuvants include phenytoin, carbamazepine, lamotrigine, topiramate, and tiagabine.

The tricyclic antidepressants (TCAs; e.g., amitriptyline, nortriptyline) are effective agents for neuropathic pain.[5] The TCAs, when used as adjuvant analgesics, are effective faster and at lower doses than when they are used as antidepressants (e.g., amitriptyline is effective within 2 to 3 days at 50 to 100 mg/day). However, because of their anticholinergic side effects they should be started at doses of 10 to 25 mg at bedtime and used with caution in older patients and in patients who have cardiac conduction abnormalities or bladder outlet obstruction. Combination therapy with gabapentin and nortriptyline has been shown to be more effective than either drug alone in patients with diabetic neuropathy and PHN.[38]

Selective serotonin and norepinephrine reuptake inhibitors (SSNRIs; e.g., venlafaxine and duloxetine) have been shown to be analgesic for a number of neuropathic pain syndromes. There is less evidence supporting the use of SSRIs for neuropathic pain.

Corticosteroids given epidurally, intravenously, or orally are useful as antineoplastics (e.g., in leukemia, lymphoma, and myeloma) and can also provide nonspecific relief for patients with spinal cord compression and plexus infiltrations. Doses of 16 to 100 mg of dexamethasone are needed to reduce vasogenic edema in spinal cord compression,[5] but lesser doses (6 to 20 mg/day) can be helpful in patients with plexus injuries. Patients must be monitored for the development of oral or esophageal candidiasis and steroid-induced delirium.

Bone Pain

Adjuvants for bone pain include NSAIDs, corticosteroids, bisphosphonates, receptor activator of nuclear factor κ-B ligand inhibitors (e.g., denosumab), and the radiopharmaceuticals strontium chloride (^{89}Sr) and samarium153-lexidronan.[5] Multiple studies have demonstrated the efficacy of bisphosphonates in reducing skeletal complications and pain from bone metastases.[5] Pamidronate and zoledronate are recommended for patients with multiple myeloma and other hematologic malignancies with painful bone lesions. Calcium and sometimes vitamin D supplementation (especially for denosumab) are often needed.[39] Also, the long-term use of bisphosphonates is associated with a small but meaningful risk of osteonecrosis of the jaw.[39] The limitations of radiopharmaceuticals include cost, logistical issues related to administration, and cytopenias.[5] Given the limited evidence available, a recent Cochrane review did not support the use of calcitonin for control of pain from bone metastases.

Cannabinoids

Long used empirically for their analgesic and antiemetic properties in a wide range of illnesses, cannabis and cannabinoid therapies have been subjected to an increasingly large number of controlled clinical trials (of varying size and quality) over the last several decades.[40] Oral cannabis, used for breakthrough pain, was studied in a recent multicenter randomized controlled trial, involving 360 patients, who were started on a long-acting opioid. The study showed analgesic efficacy in the low and medium dose ranges, which were also well tolerated.[41] Inhaled cannabis, used for nausea and vomiting secondary to active cancer chemotherapy, was studied in three randomized controlled trials, involving 43 subjects in total, which demonstrated inhaled cannabis to be an efficacious antiemetic. Additional studies have provided evidence for its effect on spasticity, appetite stimulation, and insomnia. Cannabis is not available medically in some states. There is also potential risk of fungal pneumonias in patients with hematologic malignancies who smoke cannabis, so patients should be advised about this possibility, and consider alternate administration routes in areas where cannabis is legally available for medical use.

SPECIFIC CLINICAL PROBLEMS

Oral Complications

Oral complications of chemotherapeutic and bone marrow transplant regimens can be frequent causes of pain. A thorough dental evaluation and prompt treatment of infections can minimize the discomfort arising from underlying periodontal disease and caries; secondary bacterial, viral, and fungal infections; and mucositis.[42] Preventive regimens include saline, sodium bicarbonate, chlorhexidine gluconate rinses, acyclovir, antifungals, and ice. Palifermin (recombinant human keratinocyte growth factor [KGF-1]) is used for the prevention and treatment of mucositis induced by conditioning regimens for hematopoietic stem cell transplants (SCTs).[5] Amifostine is a thiol compound that is a selective cytoprotective agent approved for salivary gland protection in patients receiving radiation therapy.[43] The benefit of using colony-stimulating factors in the treatment of oral mucositis has been confirmed in at least four controlled trials.[44] They have not achieved widespread use, possibly because of their high cost.

Anesthetic cocktails composed of agents such as viscous lidocaine (Xylocaine), dyclonine hydrochloride, or a slurry of sucralfate provide temporary relief from oral mucositis-related oral pain. A variety of mucosal-coating agents have been used to protect mucosal surfaces of the oral cavity including Orabase, Episil, oral antacids, and Gelclair. There is little evidence from randomized trials to support any benefit from these preparations. Gelclair, a bioadherent gel that adheres to the oral surface, creating a protective barrier for irritated tissue, showed a reduction in oral discomfort within 5 to 7 hours of initial treatment in an uncontrolled, open-label study of 30 hospice patients (only three of whom had chemotherapy-related mucositis).[45] The more severe cases, occurring in bone marrow transplant recipients, usually require infusional opioid therapy delivered by standard drip or PCA. Pilocarpine (5 to 10 mg three times daily 1 hour before meals) may improve xerostomia from neck irradiation. However, caution is warranted because of reported side effects of glaucoma and cardiac problems. Sugar-free hard candy is also useful for opioid-induced xerostomia and dysgeusia. Doxepin oral rinse has been shown in several studies to be effective in reducing oral mucositis pain in patients receiving radiation for head and neck cancer, or with mucositis due to stem cell transplantation.[46]

Postherpetic Neuralgia

PHN, defined as pain persisting beyond 4 months from the initial onset of the rash, can be a difficult problem for patients with hematologic disorders and has been the subject of several reviews. The anticonvulsant medications gabapentin, pregabalin, and valproic acid are especially useful in reducing the lancinating component of the various pain syndromes generated by this infection. A 2011 systemic review identified four placebo-controlled randomized trials, with two trials evaluating immediate-release gabapentin at doses between 1800 and 3600 mg daily showing benefit compared with placebo for the outcome of "much or very much improved" (38% vs. 20%).[47] Pregabalin in randomized studies with patients with PHN has shown improvement in sleep and decrease in pain at doses of 150 to 600 mg daily.[48] Because of the risks associated with physical dependence, tolerance, addiction, and overdose, many experts consider opioids as second- or third-line options for PHN.[49]

TCAs are effective for PHN.[50] One trial concluded that nortriptyline was better tolerated than amitriptyline.[51] Older patients, however, often do not tolerate the anticholinergic side effects, principally sedation and dry mouth, and therefore nortriptyline (Pamelor), a less anticholinergic TCA, may be useful in these patients. Topical lidocaine (5%) has been used for the relief of pain associated with PHN; however, its role has not been established.[52] There are limited data on the efficacy of topical capsaicin (0.075%).[53] Available evidence of botulinum toxin injection for PNH has not been well studied, but available evidence suggests it is effective and well tolerated.[54] Patients with severe pain refractory to these therapies may benefit from a combination of intrathecal methylprednisolone and lidocaine.[55]

Sickle Cell Anemia

Patients with sickle cell anemia have chronic and episodic pain despite optimal medical therapy, and 60% of patients with sickle cell anemia will have an episode of severe pain each year. Chronic arthritic pain can be treated with physical therapy and full doses of antiarthritic medication, but some patients require low doses of chronic opioid therapy to maintain independent functioning. Several studies have confirmed the safety and efficacy of long-term opioids in the treatment of pain of nonmalignant origin. In some cases, joint replacement may be required. The best approach to managing acute pain in sickle cell anemia is to reduce or prevent painful vasoocclusive crises to begin with, as is discussed in the relevant chapter on this topic. Here we discuss only symptomatic management of pain once it occurs.

When a patient with sickle cell anemia experiences pain, it is important to attempt to define the precise cause of the pain before attributing it to a vasoocclusive crisis. Acute vasoocclusive pain may occur along with the chronic pain caused by the long-term complications of compression fractures, avascular necrosis, arthropathies, fractures, avascular necroses, and leg ulcers.[56]

Treating patients with sickle cell pain is complex and requires understanding that much of the pain in adults with this illness is chronic with intermittent, recurring painful episodes. For mild pain, nonopioid therapy such as NSAIDs or acetaminophen with oxycodone or hydrocodone should be considered. However, because of possible compromise of renal blood flow in these patients and the risk of acute renal failure, NSAIDs should probably not be used beyond 5 days.[2] Uncontrolled severe pain accounts for more than 90% of hospital admissions in adults with sickle cell disease. Using short-acting analgesics on an “as-needed” basis exposes the patient to periods of insufficient analgesia, anticipation, and anxiety. Their repeated requests for medication to relieve their ongoing pain may be mistakenly interpreted as “drug-seeking behavior,” and they may be unfairly stigmatized. Thus, intravenous analgesics should be started as a continuous infusion or with PCA. When adequate analgesia is obtained, a long-acting opioid or a sustained-release opioid may be initiated with intermittent use of rescue medication. In adult patients with frequent episodes of painful crisis, the use of long-acting opioid medications reduced visits to the emergency department and hospitalizations, and shortened the lengths of stay in hospital. Meperidine should be avoided in this population and has been associated with seizures in 1% to 12% of these patients.

Patients With Opioid Addiction

In order to identify and treat patients with opioid addiction, the Food and Drug Administration Amendments Act (FDAAA) of 2007 established the requirement for postmarketing studies and mandated the implementation of Risk Evaluation and Mitigation Strategies (REMS).[57] Statewide Prescription Drug Monitoring Programs (PDMP) were established to track patients with prescriptions for controlled substances from multiple practitioners (e.g., I-STOP in New York State). These systems allow the practitioner to easily reference which scripts a patient has filled from which practitioner. Many guidelines for treating non-malignant chronic pain with opioids exist. Patients with cancer are not immune to the risks of opioid misuse and some patients may have a history of substance abuse. It is important to screen all patients for a history of or ongoing substance misuse, including with a formal opioid risk assessment tool, several of which are available. Patients with risk factors should be managed more strictly with the use of medication management agreements, urine toxicology screening, pill counts, and possibly more frequent visits, with prescriptions for shorter durations.

Hematopoietic Stem Cell Transplantation

While some studies have reported pain after hematopoietic stem cell transplantation, thought to be secondary to bone marrow expansion during the engraftment phase,[58] other studies have incidentally reported improvement in pain in patients with sickle cell disease after completion of successful transplantation. While there are no studies that specifically look at pain outcomes, several studies report positive results. One study, which involved 50 children (26 with long-term follow-up) with SCTs, reported that 22 of the children experienced no further episodes of pain after transplantation. It is notable that the age range was 4 to 14 years of age, which is likely to be too young to start developing the serious long-term effects of sickle cell disease.[59] Subsequent studies have demonstrated similar results in children who undergo SCT.[60] A recent study of adult patients with sickle cell disease who underwent SCT demonstrated that while these patients do continue to take opioids, they require lower doses. Pain in these patients is thought to be secondary to sickle cell disease injury prior to SCT.[61]

Graft-Versus-Host Disease

For patients who have undergone stem cell transplantation, GVHD can be a significant problem. The usual triad of GVHD includes hepatitis, dermatitis, and gastroenteritis. Patients are often unable to take oral, rectal, subcutaneous, or transdermal opioids because of the effects on the skin (GVHD), lining of the gastrointestinal tract (mucositis, infection, and GVHD), and thrombocytopenia. Polyarthritis and musculoskeletal manifestations can cause significant pain (Table 99.3). Therefore pain management with intravenous opioids is often a necessity. The intestinal involvement that usually causes the most physical pain includes abdominal cramping and voluminous diarrhea. Treatment often begins with intravenous opioids given through a PCA pump. Addition of octreotide continuous infusion at 50 to 100 μg/h intravenously or intermittent dosing at 500 μg subcutaneously every 8 hours may be effective in decreasing the volume of diarrhea and level of abdominal pain.[5]

Peripheral Neuropathy Caused by Chemotherapy Agents

Several chemotherapy agents used in the treatment of hematologic malignancies can cause painful sensory peripheral neuropathy. The vinca alkaloids, most notably vincristine, are neurotoxic, but they are not always associated with painful neuropathy. Thalidomide, lenalidomide, pomalidomide, bortezomib, carfilzomib, cisplatin, oxaliplatin, and paclitaxel are all commonly used agents that carry a significant risk of causing painful peripheral neuropathy. The most common mechanism of neuropathy is damage to the axons starting with the most distal branches and is a result of chemotherapy’s ability to damage DNA replication, leading to apoptosis. The major manifestations are burning paresthesias of the hands and feet, and loss of reflexes. Paclitaxel also causes a motor neuropathy, which predominately affects proximal muscles.

Studies have evaluated numerous agents for prevention of painful peripheral neuropathy, although there have not been any magic bullets. Most of the research has focused on taxane and platinum-based chemotherapy agents. The American Society of Clinical Oncology (ASCO) has published a review of the available literature for prevention and treatment of chemotherapy-induced peripheral neuropathy (CIPN). Many vitamins and dietary supplements have been studied; however, most have not consistently shown benefit in larger randomized trials.[62] Specifically, the ASCO review indicates that acetyl-L-carnitine, vitamin E, all-*trans*

TABLE 99.3 **Classification of Transplant-Related Pain Syndromes**

Deep somatic pain	Bone marrow expansion and/or sensitization related to the engraftment, pneumonia, pleuritic, deep abscess
Superficial somatic pain	Oral ulcers and skin lesions associated with acute and chronic GVHD; superficial abscess, ocular lesions associated with chronic GVHD
Visceral pain	Gastrointestinal GVHD, neutropenic enterocolitis, visceral involvement by HZV and CMV
Neuropathic pain	HZV outbreak and PHN

CMV, Cytomegalovirus; *GVHD*, graft-versus-host disease; *HZV*, herpes zoster virus; *PHN*, postherpetic neuralgia.
From Niscola P, Romani C, Scaramucci L, et al. Pain syndromes in the setting of haematopoietic stem cell transplantation for haematological malignancies. *Bone Marrow Transplant.* 41:757, 2008. doi:10.1038/bmt.2008.3.

retinoic acid, glutathione, and calcium and magnesium infusions should not be utilized in the prevention of CIPN. Chemoprotectants have also not withstood scrutiny; amifostine, nimodipine, and others are not recommended. Although amitriptyline has not been shown to prevent CIPN, carbamazepine, oxcarbazepine, and venlafaxine have shown promise, although further evaluation is warranted.

Treatment of chemotherapy-induced painful peripheral neuropathy includes the usual agents used for patients with neuropathic pain from any etiology (i.e., anticonvulsants, TCAs, and occasionally tramadol or opioids). Duloxetine, an SNRI, has been shown to improve CIPN at a dose of 60 mg daily.[63] In addition to treating these painful symptoms of neuropathy, the doses of the chemotherapy often require reduction or even discontinuation of therapy.

Problems in Older Patients

Pain management in older patients is a highly prevalent problem. It is complicated by difficulties in pain assessment and by the altered pharmacokinetics of opioids and of psychotropic adjuvant medications. Older patients may underreport pain because of misconceptions that pain is part of the aging process or fears of addiction.[64] Physicians may ascribe observed limitations in social contacts and physical activities to age-related changes when they are pain-induced limitations.

Older patients are particularly susceptible to the side effects of NSAIDs and opioids, and patients taking them should be monitored closely.[65] In patients aged 70 to 89 years old, the volume of distribution for opioids is generally smaller, the drugs have longer plasma half-lives, and renal and hepatic clearances are decreased, all of which can prolong the duration of effect. The effective doses for these patients are half to one-fourth of those needed in younger patients. Drugs with short half-lives (e.g., morphine, oxycodone, hydromorphone) should be used, and initial doses should be low. Patients should be monitored carefully for the development of sedation or confusion, especially if they are receiving antihistaminic agents (e.g., famotidine, diphenhydramine), drugs with anticholinergic activity, or hypnotics.

Neuropathic pain syndromes are common in older adults, and the adjuvant analgesics gabapentin and pregabalin are often used. Common side effects include somnolence, dizziness, fatigue, and weight changes that can be problematic in the geriatric population.[66] Treatment should be initiated at 100 mg of gabapentin or 50 mg of pregabalin at bedtime, and close monitoring for side effects should occur before dose escalation. Acute urinary retention caused by opioids (especially in patients with prostatic hypertrophy) and the hypotension and tachycardia caused by tricyclic compounds can occur more frequently and be more clinically severe in this population. The starting dose of nortriptyline should be low (usually 10 mg at bedtime), and the dose should be slowly increased as tolerated. Treatment of opioid-related urinary retention may include generic Proscar (5 mg/day) in patients with benign prostatic hypertrophy and bethanechol (10 to 50 mg three times daily) to help increase bladder smooth muscle tone.

PATHOPHYSIOLOGY OF NAUSEA AND VOMITING

Nausea is the subjective sensation that precedes vomiting. It is caused by stimulation of one or more of four sites—the gastrointestinal tract, the vestibular system, the chemoreceptor trigger zone in the area postrema of the floor of the fourth ventricle, or higher centers in the CNS (Fig. 99.2). The gastrointestinal tract can activate the vomiting center by stimulation of mechanoreceptors or chemoreceptors on glossopharyngeal or vagal afferents (cranial nerves IX and X), or by release of serotonin from gut enterochromaffin cells, which in turn stimulates serotonin (5-HT3) receptors on vagal afferents. The vestibular system activates the vomiting center when stimulated by motion or disease (e.g., labyrinthitis) or when sensitized

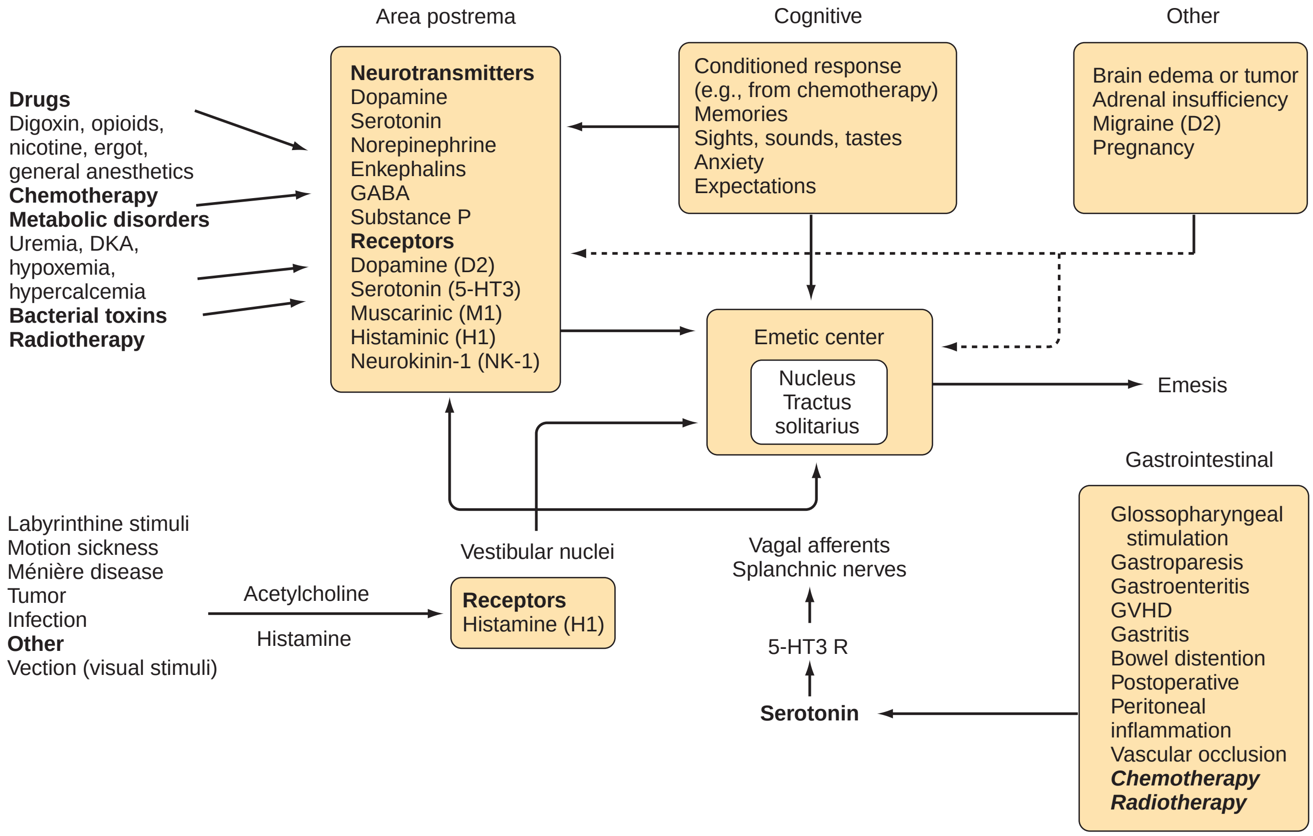

Area postrema
Neurotransmitters
Dopamine
Serotonin
Norepinephrine
Enkephalins
GABA
Substance P
Receptors
Dopamine (D2)
Serotonin (5-HT3)
Muscarinic (M1)
Histaminic (H1)
Neurokinin-1 (NK-1)
Cognitive
Conditioned response (e.g., from chemotherapy)
Memories
Sights, sounds, tastes
Anxiety
Expectations
Other
Brain edema or tumor
Adrenal insufficiency
Migraine (D2)
Pregnancy
Drugs
Digoxin, opioids, nicotine, ergot, general anesthetics
Chemotherapy
Metabolic disorders
Uremia, DKA, hypoxemia, hypercalcemia
Bacterial toxins
Radiotherapy
Emetic center
Nucleus Tractus solitarius
Emesis
Gastrointestinal
Glossopharyngeal stimulation
Gastroparesis
Gastroenteritis
GVHD
Gastritis
Bowel distention
Postoperative
Peritoneal inflammation
Vascular occlusion
Chemotherapy
Radiotherapy
Labyrinthine stimuli
Motion sickness
Ménière disease
Tumor
Infection
Other
Vection (visual stimuli)
Acetylcholine
Histamine
Vestibular nuclei
Receptors
Histamine (H1)
Vagal afferents
Splanchnic nerves
5-HT3 R
Serotonin

Figure 99.2 PATHOPHYSIOLOGY OF NAUSEA AND VOMITING. *DKA*, Diabetic ketoacidosis; *GABA*, γ-aminobutyric acid; *GVHD*, graft-versus-host-disease; *5-HT3*, serotonin; *5-HT3 R*, serotonin receptor.

by medication (e.g., opioids). Histamine (H1) and acetylcholine (M1) receptors are present on vestibular afferents. Endogenous or exogenous blood-borne toxins may activate chemoreceptors in the area postrema of the floor of the fourth ventricle via dopamine type 2 receptors. Finally, higher CNS centers may activate or inhibit the vomiting center. In addition, there may be direct activation of H1 receptors in the meninges secondary to increased intracranial pressure.

The means by which chemotherapy agents induce vomiting are still incompletely understood, but the most likely mechanism is believed to include stimulation of the chemoreceptor trigger zone.[5] Other causes of nausea and vomiting in hematologic patients include stimulation of the cerebral cortex, gastritis and gastroesophageal reflux disease, delayed gastric emptying, radiation enteritis, constipation, esophageal candidiasis, inner ear processes, hypoadrenalism, hypercalcemia, changes in taste and smell, and anticipatory nausea.[5]

Although the sites of emetic action of the chemotherapeutic agents have not been identified, blocking agents directed against type 3 serotonin receptors (5-HT3 receptors), dopamine (D2) receptors, and neurokinin (NK-1) receptors have been effective in inhibiting chemotherapy-induced nausea and vomiting (CINV).[5] Higher centers in the brain, such as the cortex, are also believed to be involved in producing anticipatory nausea and vomiting (ANV). Cognitive therapy, as well as antianxiety and amnesic agents, may provide effective antiemesis.

PATIENT ASSESSMENT

Risk factors for developing CINV include age younger than 60 years, female gender, history of motion sickness, and hyperemesis gravidarum.[5] Patients who have a history of alcohol intake of more than five alcoholic drinks per day (>100 g of alcohol) tend to have less nausea and vomiting. This has been studied carefully in patients receiving high-dose cisplatin therapy but has been anecdotally observed in patients receiving other agents.

Anticipatory Nausea and Vomiting

ANV is believed to be a classic conditioned response.[5] Chemotherapy administration (the unconditioned stimulus) results in nausea and vomiting (the unconditioned response). Clinic sights, smells, and sounds are the conditioned stimuli. After frequent pairings of chemotherapy administration and the clinic's sights, smells, and sounds, the responses (nausea and vomiting) can be triggered in the absence of any chemotherapy by the clinic's sights, smells, or sounds, or simply by seeing clinic personnel even at a location distant from the site of treatment.

Patients who are at risk for developing ANV are those who have already experienced posttreatment nausea and vomiting, therefore primary prevention of CINV is the best strategy. The risk of developing ANV increases with the increasing frequency, severity, and duration of the symptoms. Other possible predisposing factors include susceptibility to motion sickness, awareness of tastes or odors during infusions, younger age, lengthier infusions, greater autonomic sensitivity, and general anxiety or emotional distress.[5]

Acute and Delayed Chemotherapy-Induced Nausea and Vomiting

Acute CINV is defined as nausea, vomiting, or both occurring within 24 hours of administration of the agent. Although most drugs produce emesis 1 to 2 hours after they are given (in patients who have never received chemotherapy), the onset of emesis from high-dose intravenous cyclophosphamide is delayed until 9 to 18 hours after treatment, and nausea and vomiting from high total doses of cisplatin can occur 24 to 72 hours later (delayed CINV).

THERAPY

Antiemetic

Several effective antiemetics are used to prevent the nausea and vomiting induced in patients by treatment of their hematologic disorders with chemotherapy or radiation therapy. These antiemetic therapies markedly improve patients' quality of life. Most patients can expect complete control of vomiting,[67] and most patients also are free of nausea.

Most studies of the potent antiemetic agents have been conducted in patients receiving therapy with cisplatin, a drug of high emetic potential. Although cisplatin is not generally used to treat patients with hematologic malignancies, the data are included in the following discussion because the findings can be extrapolated to other drugs of equivalent or less emetogenic potential. When available, data regarding efficacy in patients receiving radiation therapy or emetogenic drugs commonly used to treat hematologic disorders are reviewed. The choice of antiemetic agents depends on the emetogenic potential of the chemotherapy or radiation therapy regimen, the side effect profiles of the antiemetic agents, and patient preferences and characteristics.

The emetogenic potentials of commonly used chemotherapeutic agents are outlined in the NCCN Clinical Practice Guidelines in Oncology Antiemesis Guideline (www.nccn.org). For patients being treated with chemotherapeutic agents with low or minimal emetogenic potential, no antiemetic therapy may be needed, or a single agent such as dexamethasone or metoclopramide may be sufficient. For drugs with higher emetogenic potential, standard antiemetic treatment usually includes combinations of several antiemetic agents along with agents designed to treat anxiety.

Combination Antiemetic

These combinations include a 5-HT3 RA such as ondansetron and a corticosteroid such as dexamethasone for moderately emetogenic regimens.[67] Recent NCCN guideline updates have re-classified some chemotherapy agents or regimens from moderately emetogenic to highly emetogenic, and also added recommendations for the use of NK-1 inhibitors in preventing CINV even for some moderately emetogenic regimens. Guideline updates now also include the option to use olanzapine as part of a triplet antiemetic strategy, since studies show it can be a helpful adjunct for reducing delayed CINV when used in combination with a 5-HT3 RA and a steroid (olanzapine is given 5 to 10 mg by mouth daily on days 1 to 3; the 10 mg dose can be quite sedating, and 5 mg may be just as effective). For highly emetogenic combination chemotherapy or regimens that include two moderately emetogenic agents, such as cyclophosphamide and Adriamycin, an NK-1 inhibitor such as aprepitant should be added to the antiemetic combination.[67] Optional lorazepam may be added for anxiety-related symptoms. It is important to give antiemetic therapy before administering chemotherapy agents and to continue for about 24 to 72 hours after the drugs have been given to prevent emesis. Patient-specific factors and preferences regarding degree of alertness can help the clinician choose an antiemetic regimen, as can the level of anxiety observed in the patient. For instance, younger patients have a higher incidence of CINV and of metoclopramide-related acute dystonic reactions; trismus or torticollis was seen in only 2% of patients older than 30 years of age but in 27% of younger patients. Therefore even if they are receiving only moderately emetogenic therapy, they may require an antiemetic regimen for high-emetogenic drugs.[67] Younger patients find cannabinoids (e.g., Marinol) more effective than do older patients because they can better tolerate the side effects associated with the therapeutic doses of these agents. Older patients also have high risks of extrapyramidal side effects and are more susceptible to anticholinergic and sedating side effects. 5-HT3 RAs are therefore preferred to regimens containing metoclopramide and diphenhydramine.

The antiemetic evidence behind the utilization of different medications changes over time. We recommend utilizing the ASCO clinical practice guidelines for recommendations on combination antiemetic regimens: http://www.instituteforquality.org/practice-guidelines.

Serotonin Receptor Antagonists: Ondansetron, Granisetron, and Palonosetron

Ondansetron, granisetron, and palonosetron are the best studied of the 5-HT3 RAs. This class also includes the drugs dolasetron and tropisetron. Several studies of patients receiving regimens that included cyclophosphamide, methotrexate, or doxorubicin showed significant efficacy and the superiority of ondansetron over placebo.

Ondansetron also demonstrates efficacy in patients with emesis (induced by non-cisplatin-containing chemotherapy) that has been refractory to standard antiemetics, with complete control achieved in 50% of these patients. When dexamethasone is added, the rate of control is as high as 91%. More recent evidence suggests, however, that although vomiting is rare with appropriate use of modern combination antiemetic regimens, breakthrough nausea is more common than previously realized. This has led to the shift from doublet to triplet antiemetic regimens for some chemotherapies previously considered only moderately emetogenic, and the increasing use of NK-1 inhibitors and/or olanzapine.[68]

Ondansetron effectively prevents nausea and vomiting in patients treated with highly emetogenic agents for acute leukemia or multiple myeloma (i.e., high-dose melphalan) or who are being prepared for bone marrow transplantation with cyclophosphamide and total-body irradiation.[67,69] In the transplantation recipients, 83% of patient-days were without any vomiting or retching, and in another 10%, there were no more than two emetic episodes. Ondansetron is also effective in preventing emesis induced by single- or multiple-fraction radiation therapy.[5] Ondansetron is as effective as high-dose metoclopramide in preventing nausea induced by cisplatin, cyclophosphamide alone, or doxorubicin chemotherapy.

Oral granisetron has shown equivalence to ondansetron in studies of patients receiving cisplatin- or carboplatin-based regimens. In patients receiving moderately emetogenic therapy, oral granisetron with dexamethasone was as effective as intravenous ondansetron and dexamethasone. Oral or intravenous granisetron and dexamethasone were effective in a bone marrow transplantation program in which patients were treated with Cytoxan and total-body irradiation, and these agents, along with oral prochlorperazine, were also effective in patients undergoing peripheral blood SCT for which patients received Cytoxan, VP-16 or thiotepa, and cisplatin. Granisetron is now available in an extended-release formulation for one-time subcutaneous administration on day 1 of chemotherapy.

Palonosetron is a second-generation 5-HT3 RA with higher potency, stronger receptor-binding activity, and longer half-life than the other drugs in this class.[5] Palonosetron 0.25 mg intravenously has been studied in moderately and highly emetogenic chemotherapy (MEC and HEC). Several subgroup analyses of the registrational noninferiority trials have been reported in which palonosetron was superior. Palonosetron has been identified as the preferred 5-HT3 RA in prevention of nausea and vomiting from moderately emetogenic regimens. Palonosetron is now available in an oral formulation along with netupitant, for single-day dosing on day 1 of MEC and HEC (to be given with oral steroids as well, with the steroid sometimes dosed orally on days 2 to 4 as well, depending on the emetogenicity of the regimen). Ondansetron and the other 5-HT3 RAs have far fewer side effects than high-dose metoclopramide. Reports of extrapyramidal reactions are rare,[5] and sedation, dystonic reactions, akathisia (e.g., severe restlessness, "ants-in-the-pants" feeling), and tardive dyskinesias do not occur. Patients can develop constipation, mild headache, QT prolongation, and elevated levels of transaminases.

Corticosteroids

The mechanism of the antiemetic action of corticosteroids remains undefined. Corticosteroids are effective when used alone to prevent emesis induced by agents of moderate or low emetogenic potential.[67,69] They are also a useful component of antiemetic therapy regimens that include ondansetron, granisetron, aprepitant, or metoclopramide and add efficacy in randomized controlled trials.[67,69]

The recommended specific corticosteroid drug, dose, and duration of therapy has not been determined. Dexamethasone and methylprednisolone are the best-studied agents, but no trials have demonstrated the superiority of one corticosteroid over another. Using anecdotal evidence recommendations for dexamethasone 4 to 8 mg orally (PO) two to three times per day, methylprednisolone 16 to 32 mg PO two to three times per day, or prednisone 20 to 30 mg PO two to three times per day have all been suggested. It is important to recognize that steroids are often part of the combination antiemetic regimens that have been shown effective in published trials, yet the steroid is often forgotten or omitted in real-world practice after day 1 of treatment; one should consult a published guideline and attend to whether an oral steroid should be prescribed on days 2 to 4 of the chosen antiemetic regimen.

Metoclopramide

Metoclopramide is a substituted benzamide that promotes gastric motility and reduces the emetic activity of chemotherapy agents by blocking dopamine receptors (at low-to-moderate doses) and 5-HT3 receptors (at high doses) in the chemoreceptor trigger zone. Because of a more favorable side effect profile, 5-HT3 antagonists have replaced high-dose metoclopramide. At lower doses (5 to 10 mg orally or intravenously every 6 hours) metoclopramide is useful in treating mild-to-moderate and delayed nausea and vomiting. Delivery of the drug on a schedule that maintains adequate levels during expected emesis appears to be important.

The side effects, which may be caused by the interaction of metoclopramide with dopamine receptors, can be quite troublesome. They include akathisia, dystonic reactions (age related), sedation, and diarrhea. Benzodiazepines such as lorazepam and β-blockers such as propranolol can prevent or reverse the akathisia, and diphenhydramine or benztropine can prevent or reverse the dystonias. However, these agents induce additional side effects, including dry mouth and sedation. Short-term, high-dose metoclopramide or long-term use at usual doses has been associated with persistent and disabling movement disorders, especially tardive dyskinesias.

Neurokinin-1 Inhibitors

Substance P can cause emesis, and it appears to play a role in chemotherapy-related nausea and vomiting. Its effects are mediated through NK-1 receptors.[5] Agents that cross the blood-brain barrier and inhibit NK-1 activity (e.g., aprepitant, netupitant) are more effective than a 5-HT3 RA and dexamethasone alone in moderating acute chemotherapy-related nausea and vomiting, and they are particularly effective in decreasing delayed nausea and vomiting occurring after HEC and MEC.[5,67] Aprepitant is an inhibitor of CYP3A4 and therefore may cause elevation of chemotherapy agents primarily metabolized by this route; however, these elevations are not considered clinically significant, and there are no recommended dose adjustments. Aprepitant can cause significant decreases in the prolongation of the international normalized ratio induced by warfarin and increases the area under the curve of dexamethasone. The maximum dose of dexamethasone in combination with aprepitant is 12 mg. Fosaprepitant, the intravenous prodrug of aprepitant, has demonstrated equivalent efficacy to the 3-day oral regimen as a single dose at 150 mg on day 1. Oral netupitant has been studied in HEC and MEC regimens as part of a single-dose combination product with oral palonosetron. These studies have demonstrated similar safety and efficacy when compared with combination antiemetic therapy including aprepitant.[70]

Olanzapine

Olanzapine has been shown to improve control of both acute and delayed nausea and vomiting in HEC and MEC when used in combination with dexamethasone and a 5-HT3 RA. Olanzapine is also shown to be superior to standard antiemetics in the breakthrough CINV setting.[71] Its primary side effect is sedation, and some evidence suggests that a 5 mg dose may be effective, with fewer side effects than the 10 mg dose.

Anticipatory Nausea and Vomiting

The treatment of ANV is best achieved through prevention with aggressive antiemetic therapy and treatment of anxiety with the appropriate agents (see "Benzodiazepines"). However, in patients for whom this approach has not been successful and in whom ANV develops, a variety of behavioral techniques have been helpful.[5] These include hypnosis, progressive muscle relaxation with guided imagery, systemic desensitization, and distraction. Results may be optimized if a therapist trained in these techniques works with the patient, but patients can use progressive muscle relaxation with guided imagery on their own.

Benzodiazepines

The benzodiazepine lorazepam has only mild antiemetic activity when used as a single agent. It is used frequently in the treatment and prevention of nausea and vomiting, particularly when anxiety is associated with the nausea and vomiting. It markedly decreases anxiety and the akathisia associated with metoclopramide therapy, and induces dose-related memory loss and marked sedation.

Cannabinoids

Currently only one pharmaceutical tetrahydrocannabinol-containing agent is available via prescription (Cesamet or nabilone has been discontinued in the US market). Dronabinol is FDA approved for the treatment of CINV. Synthetic cannabinoids are mostly reserved for patients with difficult-to-treat, refractory nausea and vomiting or as a rescue agent. Younger patients find cannabinoids more effective than do older patients, likely due to better tolerated side effects associated with the therapeutic doses of these agents. The cannabinoids cause ataxia, dry mouth, orthostatic hypotension and dizziness, euphoria (or dysphoria), and a feeling of being "high."[72]

Other Drugs

Other agents that are more active than placebo include the butyrophenones haloperidol and droperidol, and the phenothiazine prochlorperazine. These agents are less effective drugs than the agents previously mentioned, and all cause sedation. The butyrophenones produce dystonic reactions, akathisia, and occasionally hypotension. Scopolamine, a centrally acting anticholinergic, can be effective for patients with a vertiginous component to their nausea, but can also cause significant anticholinergic side effects. It is typically used as a topical medicated patch placed behind the ear, and is anecdotally more helpful in patients who have CINV and a history of motion sickness. Caution must be used when handling the patch, as it can transfer medication to the hands and then cause pupillary abnormalities if the eyes are rubbed, which may be mistaken as signs of a serious medical issue like a stroke.

CONCLUSION

Pain remains one of the most distressing and debilitating symptoms in patients with hematological disorders. Whether secondary to the disease process, iatrogenic, diagnostic, or therapeutic interventions, or related complications, pain is frequently underappreciated and undertreated. A methodological approach to evaluate and manage pain is recommended. Knowledge of the wide range of pharmacologic and nonpharmacological interventions is essential for the clinician managing pain. Pain refractory to standard approaches should be referred to pain or palliative care specialists.

Nausea and vomiting are common side effects of chemotherapy and should be treated aggressively to assure patient comfort during the treatment of their hematologic illness. Several targeted therapies exist and should be used based on the emetogenic potential of the agents used and the patient's clinical condition and comorbidities. Severe or refractory nausea should involve palliative care consultation.

ACKNOWLEDGMENTS

Many thanks to the prior authors of this chapter for their excellent work and their willingness to share its content for this version. This includes Dr. Janet Abrahm, as well as Drs. Shane E. Peterson, Kathy J. Selvaggi, Bridget Fowler Scullion, and Craig D. Blinderman.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians

Abrahm JL. A physician's guide to pain and symptom management in cancer patients, ed 2. Baltimore: Johns Hopkins University Press; 2005.

Berenson J, Pfugmacher R, Jarzem P, et al. Balloon kyphoplasty versus non-surgical fracture management for treatment of painful vertebral body compression fractures in patients with cancer: a multicentre, randomised controlled trial. *Lancet Oncol*. 2011;12:225.

Bhala N, Emberson J, Merhi A, et al. Vascular and upper gastrointestinal effects of non-steroidal anti-inflammatory drugs: meta-analyses of individual participant data from randomised trials. *Lancet*. 2013;382(9894):769–779.

Bruera E, Sweeney C. Methadone use in cancer patients with pain: A review. *J Pall Med*. 2002;5:127.

Blinderman CD. Management of cancer pain: optimal pharmacotherapy and the role of interventions. *Adv Pain Manage*. 2008;1(4):122–140.

Gorin SS, Krebs P, Badr H, et al. Meta-analysis of psychosocial interventions to reduce pain in patients with cancer. *J Clin Oncol*. 2012;30(5):539–547.

Hurlow A, et al. Transcutaneous electric nerve stimulation (TENS) for cancer pain in adults. *Cochrane Database Syst Rev*. 2012;3:CD006276.

Joishy SK, Walsh D. The opioid-sparing effects of intravenous ketorolac as an adjuvant analgesic in cancer pain: application in bone metastases and the opioid bowel syndrome. *J Pain Symptom Manage*. 1998;16:334.

LeBlanc TW. Management of cancer pain. In: DeVita VT, Lawrence TS, Rosenberg SA, eds. *Devita, Hellman, and Rosenberg's Cancer: Principles and Practice of Oncology*. ed 10. Philadelphia: Lippincott Williams & Wilkins; 2014.

McNicol E, et al. NSAIDS or paracetamol, alone or combined with opioids, for cancer pain. *Cochrane Database Syst Rev*. 2005;1:CD005180.

National Cancer Institute: "Pain." N.p., 2015. Web. 12 Apr. 2015. http://www.cancer.gov/cancertopics/pdq/supportivecare/pain/HealthProfessional.

Nicholson AB. Methadone for cancer pain. *Cochrane Database Syst Rev*. 2007;4:CD003971.

Strada EA, Portenoy RK. Psychological, rehabilitative, and integrative therapies for cancer pain. UpToDate online, Topic 14249 Version 19.0.

Jensen MP. The validity and reliability of pain measures in adults with cancer. *J Pain*. 2003;4:2–21.

Kaplan R, Portenoy RK, Cancer pain management: interventional therapies. UpToDate online, Topic 14248, Version 23.0.

Merskey H, Bogduk N. *Classification of Chronic Pain*, (revised). *International Association for the Study of Pain Taxonomy*. Seattle: IASP Press; 2012. Updated May 22.

Miaskowski C, Cleary J, Burney R, et al. *Guideline for the Management of Cancer Pain in Adults and Children, APS Clinical Practice Guidelines Series, No. 3*. Glenview, IL: American Pain Society; 2005.

Miguel R. Interventional treatment of cancer pain: the fourth step in the World Health Organization analgesic ladder? *Cancer Control*. 2000;7(2):149–156.

Montgomery GH, et al. Hypnosis for cancer care: over 200 years young. *CA Cancer J Clin*. 2013;63(1):31–44.

Pani PP, et al. QTc interval screening for cardiac risk in methadone treatment of opioid dependence. *Cochrane Database Syst Rev*. 2013;6:CD008939.

Passik SD, et al. Managing chronic nonmalignant pain: overcoming obstacles to the use of opioids. *Adv Ther*. 2000;17(2):70–83.

Portenoy RK. Cancer pain management. *Clin Adv Hematol Oncol*. 2005;3:30.

Rawlins MD, et al. Non-opioid analgesics. In: Hanks G, Cherny NI, Christakis NA, eds. *Oxford Textbook of Palliative Medicine*. ed 3. Oxford, UK: Oxford University Press; 2010:355.

Santiago-Palma J, et al. Intravenous methadone in the management of chronic cancer pain: safe and effective starting doses when substituting methadone for fentanyl. *Cancer*. 2001;92(7):1919–1925.

Sze WM, Shelley MD, Held I, et al. Palliation of metastatic bone pain: single fraction versus multifraction radiotherapy—a systematic review of randomised trials. *Clin Oncol*. 2003;15:345.

Stearns L, Boortz-Marx R, Du Pen S, et al. Intrathecal drug delivery for the management of cancer pain: a multidisciplinary consensus of best clinical practices. *J Support Oncol*. 2005;3:399.

Swarm RA, Abernethy AP, et al. Adult cancer pain. *J Natl Compr Canc Netw*. 2013;11(8):992–1022.

Thomas JW, Cohen M. A methodological review of meditation research. *Front Psychiatry*. 2014;5:74.

Trelle S, Reichenbach S, Wandel S, et al. Cardiovascular safety of non-steroidal anti-inflammatory drugs: network meta-analysis. *BMJ*. 2011;342:c7086.

Zeppetella G, et al. Opioids for the management of breakthrough pain in cancer patients. *Cochrane Database Syst Rev*. 2013;10:CD004311.

REFERENCES

1. LeBlanc TW. Management of cancer pain. In: DeVita VT, Lawrence TS, Rosenberg SA, eds. *Devita, Hellman, and Rosenberg's Cancer: Principles and Practice of Oncology*. ed 10. Philadelphia: Lippincott Williams & Wilkins; 2014.
2. Miaskowski C, Cleary J, Burney R, et al. *Guideline for the Management of Cancer Pain in Adults and Children, APS Clinical Practice Guidelines Series, No.3*. Glenview, IL: American Pain Society; 2005.
3. Cleeland CS, Ryan KM. Pain assessment: global use of the Brief Pain Inventory. *Ann Acad Med Singapore*. 1994;23(2):129–138.
4. Zeppetella G, et al. Opioids for the management of breakthrough pain in cancer patients. *Cochrane Database Syst Rev*. 2013;10:CD004311.
5. Abrahm JL. *A Physician's Guide to Pain and Symptom Management in Cancer Patients*. ed 2, Baltimore: Johns Hopkins University Press; 2005.
6. Jensen MP. The validity and reliability of pain measures in adults with cancer. *J Pain*. 2003;4:2–21.
7. Passik SD, et al. Managing chronic nonmalignant pain: overcoming obstacles to the use of opioids. *Adv Ther*. 2000;17(2):70–83.
8. Gorin SS, et al. Meta-analysis of psychosocial interventions to reduce pain in patients with cancer. *J Clin Oncol*. 2012;30(5):539–547.
9. Montgomery GH, et al. Hypnosis for cancer care: over 200 years young. *CA Cancer J Clin*. 2013;63(1):31–44.
10. Thomas JW, Cohen M. A methodological review of meditation research. *Front Psychiatry*. 2014;5:74.
11. Hurlow A, et al. Transcutaneous electric nerve stimulation (TENS) for cancer pain in adults. *Cochrane Database Syst Rev*. 2012;3:CD006276.
12. Sze WM, Shelley MD, Held I, et al. Palliation of metastatic bone pain: Single fraction versus multifraction radiotherapy—a systematic review of randomised trials. *Clin Oncol*. 2003;15:345.
13. Berenson J, Pfugmacher R, Jarzem P, et al. Balloon kyphoplasty versus non-surgical fracture management for treatment of painful vertebral body compression fractures in patients with cancer: A multicentre, randomised controlled trial. *Lancet Oncol*. 2011;12:225.
14. Stearns L, Boortz-Marx R, Du Pen S, et al. Intrathecal drug delivery for the management of cancer pain: A multidisciplinary consensus of best clinical practices. *J Support Oncol*. 2005;3:399.
15. Kaplan R, Portenoy RK, Cancer pain management: interventional therapies. UpToDate online, Topic 14248, Version 23.0.
16. Miguel R. Interventional treatment of cancer pain: the fourth step in the World Health Organization analgesic ladder? *Cancer Control*. 2000;7(2):149–156.
17. Rawlins MD, et al. Non-opioid analgesics. In: Hanks G, Cherny NI, Christakis NA, eds. *Oxford Textbook of Palliative Medicine*, ed 3. Oxford: Oxford University Press; 2010:355.
18. Joishy SK, Walsh D. The opioid-sparing effects of intravenous ketorolac as an adjuvant analgesic in cancer pain: Application in bone metastases and the opioid bowel syndrome. *J Pain Symptom Manage*. 1998;16:334.
19. Bhala N, Emberson J, Merhi A, et al. Vascular and upper gastrointestinal effects of non-steroidal anti-inflammatory drugs: meta-analyses of individual participant data from randomised trials. *Lancet*. 2013;382(9894):769–779.
20. Trelle S, Reichenbach S, Wandel S, et al. Cardiovascular safety of non-steroidal anti-inflammatory drugs: network meta-analysis. *BMJ*. 2011;342:c7086.
21. McNicol E, et al. NSAIDS or paracetamol, alone or combined with opioids, for cancer pain. *Cochrane Database Syst Rev*. 2005;1:CD005180.
22. Swarm RA, Abernethy AP, et al. Adult cancer pain. *J Natl Compr Canc Netw*. 2013;11(8):992–1022.
23. Portenoy RK. Cancer pain management. *Clin Adv Hematol Oncol*. 2005;3:30.
24. Zeppetella G, Ribeiro MDC. Opioids for the management of breakthrough pain in cancer patients. *Cochrane Database Syst Rev*. 2006;1:CD004311.
25. Nicholson AB. Methadone for cancer pain. *Cochrane Database Syst Rev*. 2007;4:CD003971.
26. Bruera E, Sweeney C. Methadone use in cancer patients with pain: A review. *J Pall Med*. 2002;5:127.
27. Pani PP, et al. QTc interval screening for cardiac risk in methadone treatment of opioid dependence. *Cochrane Database Syst Rev*. 2013;6:CD008939.
28. Santiago-Palma J, et al. Intravenous methadone in the management of chronic cancer pain: safe and effective starting doses when substituting methadone for fentanyl. *Cancer*. 2001;92(7):1919–1925.
29. Prommer E. Oxymorphone: a review. *Support Care Cancer*. 2006;14:109.
30. Gabrail NY, Dvergsten C, Ahdieh H. Establishing the dosage equivalency of oxymorphone extended release and oxycodone controlled release in patients with cancer pain: A randomized controlled study. *Curr Med Res Opin*. 2004;20:911.
31. Davis MP. Twelve reasons for considering buprenorphine as a frontline analgesic in the management of pain. *J Support Oncol*. 2012;10(6):209–219.
32. Schmidt-Hansen M, et al. Buprenorphine for treating cancer pain. *Cochrane Database Syst Rev*. 2015;3:CD009596.
33. Smith TJ, et al. Use of an implantable drug delivery system for refractory chronic sickle cell pain. *Am J Hematol*. 2005;78(2):153–154.
34. Smith TJ, et al. Implantable drug delivery systems (IDDS) after failure of comprehensive medical management (CMM) can palliate symptoms in the most refractory cancer pain patients. *J Palliat Med*. 2005;8(4):736–742.
35. Swarm RA, Cousins MJ. Anaesthetic techniques for pain control. In: Hanks G, Cherny NI, Christakis NA, eds. *Oxford Textbook of Palliative Medicine*, ed 3. Oxford, UK: Oxford University Press; 2010:390.
36. Portenoy RK. Adjuvant analgesics in pain management. In: Hanks G, Cherny NI, Christakis NA, eds. *Oxford Textbook of Palliative Medicine*, ed 3. Oxford, UK: Oxford University Press; 2010:361.
37. McDonald A, Portenoy RK. How to use antidepressants and anticonvulsants as adjuvant analgesics in the treatment of neuropathic cancer pain. *J Support Oncol*. 2006;4:43.
38. Gilron I, Bailey JM, Tu D, et al. Nortriptyline and gabapentin, alone and in combination for neuropathic pain: a double-blind, randomised controlled crossover trial. *Lancet*. 2009;374(9697):1252.
39. Kyle RA, Yee GC, Somerfield MR, et al. American Society of Clinical Oncology clinical practice guideline update on the role of bisphosphonates in multiple myeloma. *J Clin Oncol*. 2007;25:2464.
40. Hazekamp A, Grotenhermen F. Review on clinical studies with cannabis and cannabinoids 2005–2009. *Cannabinoids*. 2010;5:1–21.
41. Portenoy RK, et al. Nabiximols for opioid-treated cancer patients with poorly-controlled chronic pain: a randomized, placebo-controlled, graded-dose trial. *J Pain*. 2012;13:438–449.
42. Eliezer S, Fall-Dickson JM, Berger AM. Oral complications. In: DeVita VT, Lawrence TS, Rosenberg SA, eds. *Cancer: Principles and Practice of Oncology*, ed 9. Philadelphia: Wolters Kluwer/Lippincott Williams and Wilkins; 2011:2337.
43. Sasse AD, Clark LG, Sasse EC, et al. Amifostine reduces side effects and improves complete response rate during radiotherapy: results of a meta-analysis. *Int J Radiat Oncol Biol Phys*. 2006;64:784.
44. Crawford J, Tomita DK, Mazanet R, et al. Reduction of oral mucositis by filgrastim (r-metHuG-CSF) in patients receiving chemotherapy. *Cytokines Cell Moll Ther*. 1999;5:187.
45. Innocenti M, Moscatelli G, Lopez S. Efficacy of gelclair in reducing pain in palliative care patients with oral lesions: preliminary findings from an open pilot study. *J Pain Symptom Manage*. 2002;24:456.
46. Leenstra JL, Miller RC, Qin R, et al. Doxepin rinse versus placebo in the treatment of acute oral mucositis pain in patients receiving head and neck radiotherapy with or without chemotherapy: a phase III, randomized, double-blind trial (NCCTG-N09C6 [Alliance]). *J Clin Oncol*. 2014;32(15):1571–1577. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4026580/.
47. Moore RA, Wiffen PJ, Derry S, et al. Gabapentin for chronic neuropathic pain and fibromyalgia in adults. *Cochrane Database Syst Rev*. 2011;3:CD007938.
48. Sabatowski R, Galvez R, Cherry DA, et al. Pregabalin reduces pain and improves sleep and mood disturbances in patients with post-herpetic neuralgia: results of a randomised, placebo-controlled clinical trial. *Pain*. 2004;109:26.
49. Johnson RW, Rice AS. Clinical practice. Postherpetic neuralgia. *N Engl J Med*. 2014;371:1526.
50. Argoff CE. Review of current guidelines on the care of postherpetic neuralgia. *Postgrad Med*. 2011;123:134.
51. Watson CP, Vernich L, Chipman M, et al. Nortriptyline versus amitriptyline in postherpetic neuralgia: a randomized trial. *Neurology*. 1998;51:1166.
52. Wolff RF, Bala MM, Westwood M, et al. 5% lidocaine-medicated plaster vs other relevant interventions and placebo for post-herpetic neuralgia (PHN): a systematic review. *Acta Neurol Scand*. 2011;123:295.
53. Edelsberg JS, Lord C, Oster G. Systematic review and meta-analysis of efficacy, safety, and tolerability data from randomized controlled trials of drugs used to treat postherpetic neuralgia. *Ann Pharmacother*. 2011;45:1483.
54. Apalla Z, Sotiriou E, Lallas A, et al. Botulinum toxin A in postherpetic neuralgia: A parallel, randomized, double-blind, single-dose, placebo-controlled trial. *Clin J Pain*. 2013;29:857.
55. Kotani N, Kushikata T, Hashimoto H, et al. Intrathecal methylprednisolone for intractable postherpetic neuralgia. *N Engl J Med*. 2000;343:1514.
56. Ballas SK. Pain management of sickle cell disease. *Hematol Oncol Clin North Am*. 2005;19:785.
57. Nelson L, et al. Curbing the opioid epidemic in the United States: the risk evaluation and mitigation strategy (REMS). *JAMA*. 2012;308(5):457–458.

58. Niscola P, et al. Pain syndromes in the setting of haematopoietic stem cell transplantation for haematological malignancies. *Bone Marrow Transplant*. 2008;41:757–764.
59. Walters MC, et al. Impact of bone marrow transplantation for symptomatic sickle cell disease: an interim report. *Blood*. 2000;95:1918–1924.
60. Walters MC, et al. Pulmonary, gonadal, and central nervous system status after bone marrow transplantation for sickle cell disease. *Biol Blood Marrow Transplant*. 2010;16(2):263–272.
61. Hsieh MM, et al. Nonmyeloablative HLA-matched sibling allogeneic hematopoietic stem cell transplantation for severe sickle cell phenotype. *JAMA*. 2014;312(1):48–56.
62. Hershman D, Lacchetti C, Dworkin RH, et al. Prevention and management of chemotherapy-induced peripheral neuropathy in survivors of adult cancers: American Society of Clinical Oncology practice guideline. *J Clin Oncol*. 2014;32:1941–1967.
63. Lavoie Smith EM, Pang H, Cirrincione C, et al. Effect of duloxetine on pain, function, and quality of life among patients with chemotherapy-induced painful peripheral neuropathy: a randomized clinical trial. *JAMA*. 2013;309(13):1359–1367.
64. Culberson JW, Ziska M. Prescription drug misuse/abuse in the elderly. *Geriatrics*. 2008;63:22.
65. Gloth 3rd FM. Pharmacological management of persistent pain in older persons: focus on opioids and nonopioids. *J Pain*. 2011;12:S14.
66. Moore RA, Straube S, Wiffen PJ, et al. Pregabalin for acute and chronic pain in adults. *Cochrane Database Syst Rev*. 2009;3:CD007076.
67. Basch E, Prestrud AA, Hesketh PJ, et al. Antiemetics: American Society of Clinical Oncology clinical practice guideline update. *J Clin Oncol*. 2011;29:4189–4198.
68. Chiu L, Chow R, Popovic M, et al. Efficacy of olanzapine for the prophylaxis and rescue of chemotherapy-induced nausea and vomiting (CINV): a systematic review and meta-analysis. *Support Care Cancer*. 2016;24:2381–2392. https://doi.org/10.1007/s00520-016-3075-8.
69. National Comprehensive Cancer Network: Clinical practice guidelines in oncology: Antiemesis, v.1.2015. Available at www.ncc.org/professionals/physician_gls/pdf/antiemesis.pdf.
70. Gralla RJ, Bosnjak SM, Hontsa A, et al. A phase III study evaluating the safety and efficacy of NEPA, a fixed-dose combination of netupitant and palonosetron, for prevention of chemotherapy-induced nausea and vomiting over repeated cycles of chemotherapy. *Ann Oncol*. 2014;25:1333–1339.
71. Hocking CM, Kichenadasse G. Olanzapine for chemotherapy-induced nausea and vomiting: a systematic review. *Support Care Cancer*. 2014;22(4):1143–1151.
72. Machado Rocha FC, et al. Therapeutic use of *Cannabis sativa* on chemotherapy-induced nausea and vomiting among cancer patients: systematic review and meta-analysis. *Eur J Cancer Care (Engl)*. 2008;17(5):431–443.
73. National Cancer Institute: “Pain.” N.p., 2015. Web. 12 Apr. 2015. http://www.cancer.gov/cancertopics/pdq/supportivecare/pain/HealthProfessional.
74. Strada EA, Portenoy RK: Psychological, rehabilitative, and integrative therapies for cancer pain. UpToDate online, Topic 14249 Version 19.0.
75. Blinderman CD. Management of cancer pain: optimal pharmacotherapy and the role of interventions. *Advances in Pain Management*. 2008;1(4):122–140.
76. Merskey H, Bogduk N. *Classification of Chronic Pain* (revised). International Association for the Study of Pain Taxonomy. Seattle: IASP Press; 2012. Updated May 22.

CHAPTER 100

PALLIATIVE CARE

Kathleen A. Lee, Hilary McGuire, Barbara Reville, and Janet L. Abrahm

Palliative care for seriously ill children and adults is provided by an interdisciplinary team of clinicians who focus on communication about values and goals of care and ameliorating suffering from physical, social, spiritual, existential, and psychological concerns.[1] Members of the interdisciplinary palliative care team include registered and advanced practice nurses, physician assistants, social workers, pharmacists, chaplains, and adult and pediatric physicians, many of whom are ABIM certified in Hospice and Palliative Medicine. Palliative care teams augment rather than replace the active treatment of illness, striving to enhance patients' quality of life in concert with patient and family goals.

The Institute of Medicine (IOM) recommends improved access to palliative care, expanded education of all healthcare professionals in palliative care domains with an emphasis on communication, and expanded research in the areas of caregiver support, innovative payment models to incentivize team-based palliative care, and use of health information technology in care coordination and communication.[2]

Palliative care is not reserved for patients at the end of life; it should be available as well to patients of any age receiving curative or life-prolonging treatments.[2] Recent expert consensus practice guidelines, including those by the American Society of Clinical Oncology (ASCO), recommend palliative care concurrent with oncological care for patients with advanced cancer and/or high symptom burden.[3] Early palliative care integration alongside treatment of solid tumors improves patient quality of life and symptom management and may extend lives.[4] While palliative care subspecialists provide clinical support in more complex situations and palliative care education, innovation, and research, all clinicians should integrate high-quality palliative care into their care of patients with serious illness because there are not now nor in the future will there be enough palliative care providers to meet the need.[5]

National Comprehensive Cancer Network (NCCN) guidelines advise palliative care referral for patients with refractory or relapsing hematologic malignancies (HMs) and intractable cancer pain.[2] However, although patients living with HM and hematopoietic stem cell transplant (HSCT) have palliative care needs and an especially high symptom burden,[3,4] they are less likely than solid tumor patients to receive palliative care referrals.[5] Unique barriers to palliative care and hospice involvement in HM care include significant prognostic uncertainty related to many HM diagnoses and the benefit of palliative blood transfusions. HM patients are more likely to receive more aggressive measures (e.g., chemotherapy, hospital admissions, intensive care unit admissions) in the last 30 days of life and are less likely to enroll in hospice at the end of life.[6] Furthermore, many hematologic oncologists still view palliative care as end-of-life care.[7] HSCT physicians report concerns about palliative care specialists' lacking adequate knowledge about HSCT and that patients perceive a palliative care referral signifies the end of further cancer-directed therapies.[8]

This chapter reviews core elements of palliative care for children and adults, with specific attention to the growing understanding of palliative care needs in patients with HM/HSCT and newer models of palliative care integration within HM/HSCT care.

PEDIATRIC PALLIATIVE CARE: SPECIAL ISSUES

Pediatric palliative care is an emerging frontier in the comprehensive care of children. Specific issues related to palliative care for pediatric patients and their families include the following[13]:

- Because there are smaller numbers of dying children than adults, there are fewer palliative care pediatric professionals and children are under-represented in palliative care protocols.
- The heterogeneity of serious illnesses, many rare, requires the involvement of many disciplines and specialists in the palliative care team.
- Many children have genetic diseases, so there may be more than one affected child in a family.
- Pediatric palliative care may extend over years, even decades. A broad developmental spectrum is represented, including changes in the individual child through time.
- The underlying principles and ethics of how to care for patients with serious illness are universal across the lifespan. A child or adolescent diagnosis of life-threatening or life-limiting illness, however, throws an assumed sequence out of order. Children expect to care for dying parents. When parents instead find themselves watching their child face death, a sense of tragic absurdity prevails. Not only is time shortened, but its order is shattered. A child or adolescent with a life-threatening illness represents a premature separation from the family. Even before the child has become a differentiated individual through a natural developmental sequence, that child is wrenched away. When a psychological separation from parents has not yet been affected, the child and the parents have little preparation for the separation caused by death. The adolescent who is beginning to negotiate independent existence is often the hardest to face when that "moving forward" is irreversibly halted, or at least disrupted; a younger child has not even had the time to begin to form life goals.
- One of the foremost goals is to initiate palliative care proactively for children earlier in the illness trajectory, prioritizing the care of the family, with a particular focus on the young siblings. The timing of palliative care consultation and the clinical approach may differ depending on the prognosis for the child, the decisions that must be made in choosing treatment options, and the need for improved management of pain and suffering to provide optimal quality of life.

COMMUNICATION

Prognosis and Decision Making

Pediatric Population

Surveys of bereaved parents indicate that physician communication about prognosis is not optimal, and bereaved parents consider high-quality communication to be the most important element of quality physician care.[2] Compared with physicians, parents are overly optimistic about the child's chances of cure, and this is especially true when the prognosis is uncertain. Parents value clear information that is communicated sensitively and includes the child, when developmentally appropriate. Although they find prognostic information about their child upsetting, a majority of parents want as much information about the prognosis as possible.[9] Being aware of these beliefs may help physicians discuss the prognosis with greater frequency and clarity.

During the last decade, there has been increased recognition of the importance of the child's participation in making treatment decisions. Crucial to this process is an assessment of the child's or adolescent's ability to appreciate the nature and consequences of a specific medical decision. This becomes particularly complex when the wishes of the child differ from those of the parents. Because actual assessment tools are only in the early stages of development, professionals must rely exclusively on their clinical judgment to assess children's understanding of the contingencies they are facing. This is often a juncture when input from members of the interdisciplinary palliative care team can be crucial: children often express their understanding, awareness, and thoughts about treatment options and living or dying to individuals other than their parents or primary physician.

Adult Population

Similarly, adult patients living with serious illness and their families value clear information about the prognosis when communicated sensitively.

Although most patients report wanting prognostic information from their oncologist, patients with advanced refractory disease may not ask about their prognosis, and oncologists often do not initiate the discussion. In one recent observational study of HM patients after their first oncologic visit,[10] only half of the patients were told unambiguous prognostic estimates for mortality or cure. This is likely due in part to significant prognostic uncertainty associated with HM diagnoses.

When first diagnosed, HM patients often need to start treatment immediately. Though great advances in the treatment of adult HM have improved survival rates, the clinical courses of these diseases vary significantly from patient to patient. Some HM patients respond to curative treatment attempts with long-term remission and after a period of time are considered cured. Others have an initial response to curative-intended therapies only to relapse later at an unpredictable time; some then respond to further treatments for eventual remission while others succumb. For some unfortunate HM patients, their cancer is so advanced at the time of diagnosis that they experience a rapid clinical decline and die. This unpredictability of an HM patient's course can make decisions about when to stop cancer-directed therapies and when to start end-of-life therapies challenging.

Communication in Serious Illness: Strategies

Effective communication can allow clinical providers to align therapeutically with patients and family members. Discussing prognosis, breaking bad news, and discussing late goals of care with patients with advanced disease are all times when clinicians can demonstrate their commitment to an ongoing collaborative partnership. Conversations should demonstrate respect for cultural differences and the belief that psychosocial and spiritual growth can occur, even at the end of life. If these conversations are done well, they can lay the groundwork for further discussions about patients' hopes, fears, goals, values, and spiritual concerns as they make decisions about their medical care, including their thoughts about resuscitation and life-sustaining therapies. These conversations should be documented in the electronic medical record and recorded as a physician order when possible (e.g., in a Medical Order for Life sustaining Treatment [MOLST] or Physician Order for Life-Sustaining Treatment [POLST] form).[2]

Children with serious hematologic disorders have usually lived with the illness over months or years. Their knowledge, understanding, and awareness of their precarious life situation are often profound at physical, cognitive, and emotional levels. In the past, disclosure to the child of his or her prognosis (and even, in some instances, the diagnosis) was thought to be harmful.[11] Since the 1980s, there has been a shift toward open communication. To shield the child from the truth may only heighten anxiety and cause the child to feel isolated, lonely, and unsure of whom to trust.

Clinicians interested in improving their communication skills when caring for patients with serious illness can refer to online resources, such as the Center to Advance Palliative Care (capc.org), the Serious Illness Conversation Guide (https://www.ariadnelabs.org/areas-of-work/serious-illness-care/), and VitalTalk (vitaltalk.org). A few key communication skills strategies are described as follows.

Cultivating Prognostic Awareness

Even when patients are provided with clear statistical information about their prognosis by their oncologist, they may have an inaccurate impression of the goals of their treatment and of their prognosis. If they cannot process this difficult information, they are unlikely to be able to make appropriate personal or medical decisions.

Improving a patient's prognostic awareness is an important component of early palliative care. Using advanced communication techniques,[12] palliative care clinicians can facilitate patients' prognostic awareness, and support patients as they swing between hope and the nightmare of awareness of how serious their illness is. After improving a patient's prognostic awareness, palliative care clinicians can help patients with serious illnesses identify their hopes, values, and realistic goals.

Breaking Bad News

Table 100.1 contains an outline of the suggested steps to take when breaking bad news.[13] For patients with advanced disease, the goal is to establish or strengthen trust and reassure them that the clinician is committed to caring for them, even though their disease cannot be cured.

REMAP: A Framework to Facilitate Effective Late Goals of Care Conversations

Table 100.2 contains an outline of the suggested steps of REMAP, a technique useful for goals-of-care conversations with patients and/or family members when current treatments for the disease have not worked and the medical plan needs to be re-evaluated.[14]

TABLE 100.1 **Breaking Bad News**

1. Make yourself, the patient, and the family comfortable.
2. Find out what they know.
3. Indicate that you are planning to tell them something that is serious (give a "warning shot").
4. Find out whether they want to be told or whether they want someone else to be told.
5. Find out how much they want to know (i.e., the big picture versus all the details).
6. Tell them in words they can understand, allowing time for questions along the way (i.e., use less medical jargon).
7. Empathically respond to their emotions.
8. Let them know that this is only the first of many discussions with you.
9. Ask them to summarize what they heard you say; ask if they have further questions.
10. Arrange your next meeting with them.

Based on Abrahm JL. Update in palliative medicine and end-of-life care. *Annu Rev Med.* 2003;54:53; and Back A, et al. *Mastering Communication With Seriously Ill Patients: Balancing Honesty With Empathy and Hope.* New York: Cambridge University Press; 2009.

TABLE 100.2 **REMAP Framework for Late Goals of Care Conversations**

Reframe the situation: Explain that the status quo related to current medical plan is not working anymore. "We're in a different place."

Expect emotions: Talking begins to make this transition real.

Map values before talking about treatments or code status.

Align yourself with the patient's values-show that you heard.

Plan medical treatments that match your patient's values.

From Childers JW, Back AL, Tulsky JA, et al. REMAP: A framework for goals of care conversations. *J Oncol Pract.* 2017;13(10):e844–e850.

Caregiver Distress

Caregivers, whether close relatives or friends of the patient with an HM, suffer significant collateral distress in psychosocial, physical, financial, and informational domains during the course of their loved one's illness.[15] Emerging evidence shows parents, partners, relatives, and friends experience high levels of emotional distress and diminished quality of life at predictable illness milestones including diagnosis, onset of treatment toxicities, treatment regimen changes, and care transitions.[16]

The overlay of uncertainty about the future heightens both caregivers' stress level and sense of being out of control. The innate coping resources and social, psychological, and medical health of the caregiver are predictive of whether the caregiving experience will simply traumatize them or also be a source of personal growth. The interdisciplinary team should therefore assess caregivers' emotional health routinely and provide support and resources from diagnosis through survivorship. Integrating palliative care within HM teams to assess and support caregivers early in the treatment course led to reduced psychosocial distress and early identification of stress symptoms.[17,18] As novel treatment regimens shift from acute care to ambulatory settings, the impact on caregivers' efficacy, well-being, and sequelae will be an important variable of treatment outcomes and program development.[19]

RELIEF OF SUFFERING

Suffering includes physical, social, spiritual or existential, and psychological dimensions.

Management of Physical Distress in Children

Maintaining patient comfort is of critical importance throughout treatment, as well as during the end stages of life. Although effective pain control is a hallmark of palliative care, pain is only one of many distressing symptoms. The spectrum of physical symptoms includes (although it is not limited to) dyspnea, fatigue, seizures, loss of appetite, nausea and vomiting, constipation, and diarrhea. Early studies among bereaved parents of children who died of cancer indicate that optimal comfort is far from being achieved, even in major pediatric teaching centers.[20] Failure to relieve a child's end-of-life distress may have long-lasting implications for bereaved parents, who are negatively affected by the child's experience of pain years beyond the death.

Psychological symptoms such as depression and anxiety are also prevalent in children at the end of life. Children also experience existential concerns. Creating opportunities for communication around these sources of distress involves using creative strategies that incorporate the developmental stage of the child. Strategies may involve verbal communication using open-ended questions such as "What are you hoping for?" and "What are you worried about?"

However, many children communicate best through nonverbal means such as artwork and music. Children may be more willing to "talk things over" with puppets or stuffed animals rather than real people. Importantly, euphemistic expressions about death can be confusing or even frightening (for instance, equating death with sleep may result in the child's being afraid of going to bed).

Management of Physical Distress in Adults

Patients with HM experience a high physical symptom burden similar to patients with metastatic solid tumors, especially during treatment, when hospitalized, or when they develop advanced disease.[7] Intense HM treatment modalities (HSCT) and chimeric antigen receptor (CAR) T-cell therapy are likely to worsen an HM patient's existing symptom burden and neuropsychiatric outcomes.[7,21]

Pain management and antiemetic therapy for adults are reviewed elsewhere in this textbook (see Chapters 97, 99, and 110). Anxiety, depression, delirium, and control of symptoms occurring in the last days of life are reviewed later in this chapter.

Social Concerns

Among social sources of distress are financial concerns and, with increasing disability, loss of independence and decreasing sense of contribution to the family, work, or other pursuits. Worries about burdening the family or that the family will fail them when they really need them can lead patients to request Medical Aid in Dying. Social workers are key team members who can help alleviate or at least ameliorate these sources of distress and can help the caregivers cope. They can also assess and address the contributions to the distress of developmental issues, meaning and impact of the illness, the patient's coping style, the illness' impact on the patient's sense of self, relationships, stressors, economic, circumstances, and physician-patient relationships.

Spiritual Concerns

Clinicians should also explore a seriously ill patient's religious and spiritual concerns and understand what rituals will be important at the end of life. Spiritual and existential distress occurs when individuals are unable to find sources of meaning, hope, love, peace, comfort, strength, and connection in life, or when there is dissonance between their beliefs and what is happening to them. Patients who use "positive" religious coping (e.g., prayer, feeling a sense of connectedness to a religious community, having a positive relationship with a god) have been found to have better mental health status, growth in the spiritual dimension with stress, and a better overall quality of life than those with "negative" religious coping (e.g., ascribing their illness to a punishing god or one who has abandoned them).[22]

Psychological Concerns

Clinicians must assess and attend to patients' and families' psychological distress and must be able to distinguish normal human reactions of grief, sadness, despair, fear, anxiety, loss, and loneliness in patients living with serious illness from clinical anxiety and depression.

Anxiety and Depression

It is noteworthy that the symptoms of depression and anxiety are often not recognized in children and in many instances are inadequately addressed. Significant anxiety is found in approximately 25% of adult patients with cancer,[18] and anxiety symptoms can interfere with their ability to receive care. Patients with panic disorder, agitated depression, phobias, obsessive-compulsive disorder, delirium, post-traumatic stress disorder, or adjustment disorders can all present with anxiety. Anxiety in dying patients may arise from worries about the future (uncontrolled symptoms, family concerns, concerns about death), isolation from loved ones, metabolic abnormalities, withdrawal from alcohol, opioids, benzodiazepines, drug reactions (e.g., akathisia from metoclopramide, paradoxical agitation from benzodiazepines and olanzapine), and uncontrolled pain.

Non-pharmacological treatments, such as relaxation training, hypnosis, supportive psychotherapy, and counseling are very effective. Pharmacologic treatments usually include benzodiazepines (e.g., short-acting lorazepam, starting dose 0.5 to 2 mg every 8 hours orally [PO] or parenterally [IV] as needed; or long-acting clonazepam, starting dose 0.25 to 0.5 mg PO two times daily), selective serotonin reuptake inhibitors (SSRIs, see later), and when there is evidence of delirium, neuroleptics (see later).

It is estimated that 5% to 26% of patients with advanced cancer meet the criteria for major depressive disorder; patients with high symptom burden, such as patients facing HSCT, are at higher risk.[23] It can be difficult to discern which patients with advanced disease are grieving rather than being depressed. The usual somatic signs of depression are common in this population and are not helpful in making this distinction. Grieving patients are very sad, and tearful, but there are things that make them happy, and they look forward to the future. Depressed patients, however, have anhedonia and can feel worthless, guilty, hopeless, or helpless. Pain, a past or family history of substance abuse, depression, or bipolar illness are major risk factors for depression. When asked the screening question “Are you depressed?,” most seriously ill patients who respond “Yes” are very likely to be confirmed as depressed in a more comprehensive evaluation.

As part of depression treatment, pain must be brought under control. Counseling can explore patients’ fears, provide emotional support, and help patients review their lives and find meaning and areas of accomplishment in them. A variety of models of therapy are used, and none has been shown to be superior to others. The psychostimulants, dextroamphetamine, and methylphenidate (2.5 to 5 mg PO at 8 AM and noon; maximum dose 20 mg PO daily) often act within a few days. The SSRIs are the first choice when immediate onset is not needed because they usually take several weeks to show effect. Useful agents include citalopram (Celexa; 10 mg PO daily initially; maximum 40 mg PO daily); escitalopram (Lexapro; 10 mg PO daily initially; maximum 20 mg PO daily); sertraline (Zoloft; 50 mg PO daily initially, maximum 200 mg PO daily); fluoxetine (Prozac; 5 to 10 mg PO daily initially; maximum 50 mg PO daily); and the serotonin norepinephrine reuptake inhibitor venlafaxine (Effexor; 37.5 PO twice daily initially; maximum 225 mg PO daily). Venlafaxine inhibits norepinephrine serotonin and dopamine reuptake, and patients have severe withdrawal symptoms if it is stopped abruptly. Major side effects of the SSRIs include hyponatremia, sexual dysfunction or loss of libido, and gastrointestinal complaints (e.g., nausea, diarrhea, foul-smelling flatus). Modafinil may reduce SSRI-related sedation. The exact mechanism of action of mirtazapine (Remeron; 15 mg PO at bedtime initially; maximum 45 mg PO at bedtime) is unknown.

If the patient is expected to live longer than weeks to a few months, a stimulant and an SSRI should be started simultaneously. The stimulant can then be titrated off several weeks later. Tricyclic antidepressants are less useful in these patients because of their side effect profile.

If the patient does not respond to first-line agents, a psychiatrist should be consulted. Referral to a psychiatrist is also necessary when the psychiatric diagnosis is unclear.

Delirium

Delirium occurs in up to 80% of patients dying from advanced cancer and can cause distress and anxiety for caregivers.[20] Delirious patients can be agitated, hypo-active, or vacillate between the two. Symptoms of delirium include insomnia and daytime somnolence, nightmares, restlessness or agitation, irritability, distractibility, hyper-sensitivity to light and sound, anxiety, difficulty in concentrating or marshalling thoughts, fleeting delusions, hallucinations, emotional lability, attention deficits, and memory disturbances.

Validated delirium screening severity tools are available, but comprehensive psychiatric evaluation is recommended to exclude other disorders, such as anxiety, depression, anger, dementia, or psychosis. The cause of delirium is often never determined and frequently multi-factorial. Medications, especially opioids, H2-blockers, high dose corticosteroids, and use of multiple anti-cholinergic agents commonly contribute. Opioid-induced central nervous system toxicities are more common in patients with renal dysfunction, on high doses of opioids for longer durations, with impaired cognition before starting the opioids, with dehydration, or taking other psychoactive drugs. Other causes include metabolic abnormalities (hypercalcemia, hyperglycemia, or uremia), malnutrition, hypoxia, fever, infection, uncontrolled pain, hepatic failure, primary brain tumor, and brain metastases.

Treatment for delirium should begin while the underlying causes are being treated. In addition to the medications listed in Table 100.3, it is helpful to make the patient’s surroundings as familiar as possible,

TABLE 100.3 **Treatment of Delirium (Adult Patient)**

Drug	Dose	Comment
Typical and Atypical Antipsychotics		
Haloperidol	1–4 mg PO, or 0.5–2 mg SQ, IV qhs or bid to tid	Do not exceed 20 mg in 24 h
Quetiapine	25–200 mg PO qhs	Particularly useful in elderly patients with evening delirium Start 25 mg qhs for 3–4 days
Olanzapine	2.5–5 mg PO/SL/IV	Start 2.5–5 mg PO/SL/IV qhs to bid (2.5 mg for elderly patients); can use q4–6 h prn agitation Maintain the patient on the effective dose (divided into a bid dose), then taper over 2 weeks, as tolerated; also antiemetic
Aripiprazole	5 mg PO qd	Do not exceed 30 mg Does not prolong and may shorten QTc
Chlorpromazine	12.5–1000 mg PO/IV/PR	Sedating; may cause significant hypotension
Benzodiazepines*		
Lorazepam	0.5–1 mg PO/IV q1–2 h 5–10 mg PO bid	Avoid using alone (i.e., without an antipsychotic) for patients with a hyperactive delirium
Diazepam	5–10 mg PO bid	Useful PR for patients unable to take oral medication
Clonazepam	0.5–5 mg PO/SL bid to tid	Tablets have been used PR for terminal delirium; do not exceed 20 mg/24 h
Midazolam	30–100 mg IV / SQ over 24 h	IV drip or subcutaneous infusion for terminal delirium

*Caution: Any benzodiazepine may exacerbate delirium, especially in older adults (>70 years of age).

bid, Twice a day; *d*, day; *hs*, at bedtime; *IM*, intramuscularly; *IV*, intravenously; *PO*, orally; *PR*, rectally; *prn*, as needed; *q*, every; *SL*, sublingual; *SQ*, subcutaneously; *tid*, three times a day.

Modified from Abrahm JL. *A Physician’s Guide to Pain and Symptom Management in Cancer Patients*. 3rd ed. Baltimore: Johns Hopkins University Press; 2014; and Miovic M, Block S. Psychiatric disorders in advanced cancer. *Cancer*. 2007;110:1665–1676, 2007.

restore aids to hearing and sight if they are needed, re-orient the patient frequently, and have family members, friends, or well-known caregivers present.

MANAGEMENT CONCERNS DURING THE LAST DAYS OF LIFE

Children

Children with advanced cancer who receive concurrent home-based palliative care have improved quality of life at the end of life and are more likely to die at home. Families can plan ahead and choose a setting for their child's death: home, hospice, or hospital, with their child's input. Clear information about how the child is likely to die and professional support to validate the family's choice are crucial. Even more important is the explicit stated "permission" from all members of the professional team that the family may change their choice freely at any time, that all options remain open, and that no decision is irrevocable. In the past, siblings were rarely included in these discussions and were often inadequately prepared for the eventuality of a child dying at home. Recently, however, their voices are beginning to be heard.

Adults

Common physical symptoms that occur in the last weeks before an adult's death from cancer include pain, noisy or moist breathing, urinary incontinence or retention, dyspnea, and nausea and vomiting. Patients may also experience fatigue. Hunger and thirst are unusual. Treatments for problems at the end of life are reviewed in Table 100.4. Patient and family wishes and options about the setting for end-of-life care should be explored. Some evidence suggests that patients with cancer who die at home have better quality of life and that their caregivers have better bereavement outcomes than cancer patients who die in the hospital. Given the potentially high symptom burden of HM patients in the final moments of life, such as severe bleeding or dyspnea, more patients with HM die in the hospital and are less likely to be enrolled in hospice services (discussed further later).

HOSPICE PROGRAMS

Table 100.5 contains a detailed comparison of palliative care and hospice programs. Hospice programs focus on providing end-of-life care to patients with a prognosis of 6 months or less (for adults). Hospice enrollment improves the quality of life for patients with advanced cancer and reduces the risk of depression for caregivers. In the United States, the four levels of hospice care include: (1) home care, the most common setting; (2) respite care, during which patients can be admitted to a nursing home or hospice house for brief periods each month to provide respite for the family caregivers; (3) continuous crisis care, in which hospice nurses/staff provide intensive continuous (often symptom management) care in the home; and (4) general inpatient hospice ("GIP"), requiring frequent adjustment of parenteral medications for intensive symptom control that cannot be managed in another care setting (GIP is typically given in a hospice facility or hospital setting). The Medicare Hospice benefit does not require a "do not resuscitate" status, but it does require that the attending physician and the Hospice medical director certify that the patient has a prognosis of 6 months or less to live if the disease follows its usual course.

Despite the clear benefits of hospice care among cancer patients, patients with HM have the lowest rates of enrollment. When patients with HM do enroll, they are more likely to do so within days of death than are patients with non-hematological malignancies. Though multiple factors exist for low and late hospice use in this population, a significant barrier is limited access to blood transfusions in hospice programs because of cost and logistical concerns. Since transfusion dependence is common in patients with blood cancers, recent efforts have advocated for combining palliative transfusions with hospice services in efforts to optimize end-of-life care for patients with HM.[19]

TABLE 100.4 **Treatment of Common Problems in the Final Days (Adult Patient)**

Problem	Agent(s)	Routes, Doses
Baseline pain	Concentrated oxycodone or morphine solution Morphine or hydromorphone tablets Fentanyl[a] Methadone liquid Acetaminophen, naproxen Dexamethasone	PO/SL q4h around the clock; individualized PR q4h; individualized Transdermal; individualized PO, per gastric tube, individualized PO, PR tid to qid PO, PR (requires compounding) daily to bid
Breakthrough pain	Concentrated oxycodone or morphine solution Fentanyl[a]	PO or per gastric tube q4h around the clock; individualized Transmucosal (buccal, sublingual); individualized
Excessive oropharyngeal secretions "Noisy breathing"	Scopolamine Hyoscyamine Glycopyrrolate Atropine	Scopolamine Transderm Scop patch 1mgq3 days 0.125–0.25mg SL tid to qid 0.2–0.4 mg IV tid to qid or 1–2 mg PO bid to tid 0.4 mg SL, IV q4–6 h
Dyspnea (anxiety)	Lorazepam Chlorpromazine	1 mg PO, SL, q2–4 h; IV q1–2 h 25 mg PO, PR q4–12 h; or 12.5 mg IV q4–8 h
Dyspnea (other)	Morphine	10–15 mg SL oral concentrate q4h
Nausea	Combinations of lorazepam, metoclopramide, dexamethasone, and/or haloperidol	PR q6h; compounded suppositories with desired agents (depending on presumed cause of nausea)
Anxiety	Lorazepam Diazepam suppository	1 mg PO, SL, q2–4 h; IV q1–2 h 5–10 mg PR daily
Delirium	Haloperidol Olanzapine	2–4 mg PO, SQ, IV q30 min prn (max 20 mg/24 h) 2.5–5mg PO, SL/IV qhs to bid plus PRN q4h

[a] Fentanyl only for opioid tolerant patients.

All liquid PO medications can be given per gastric tube.

h, Hour; *IV*, intravenously; *PO*, orally; *PR*, rectally; *PRN*, as needed; *q*, every; *qhs*, nightly at bedtime; *qid*, four times a day; *SL*, sublingually; *tid*, three times a day.

Modified from Abrahm JL. *A Physician's Guide to Pain and Symptom Management in Cancer Patients.* 3rd ed. Baltimore: Johns Hopkins University Press; 2014.

Notably, many children are not referred to hospice because their illness experience is inconsistent with hospice specifications: their prognosis is uncertain; there is a blending of goals, which can result in more costly health care; and providers lack pediatric expertise. Importantly, the Patient Protection and Affordable Care Act now requires state Medicaid programs to allow children (under the age of 21) with a life-limiting illness to receive both Hospice care and

TABLE 100.5 Specialty Level Palliative Care Versus Hospice Care

Palliative Care	Hospice Care
Interdisciplinary Model of Care	**Interdisciplinary Program of Care**
Clinical specialty, offers expert: Symptom management and communication Psychosocial and spiritual care Inpatient, outpatient, and home care consultations to the primary team Coordination of care among treating teams	Medicare Hospice benefit, delivers: Symptom management and communication All medications needed for the terminal illness; DME; oxygen Psychosocial and spiritual care Home, inpatient, or respite care in a nursing home/hospice facility under the direction of the patient's physician delivered by the interdisciplinary hospice team Continuity with referring care team Bereavement care for a year after the death
Eligibility	**Eligibility**
Any patient with serious or life-threatening illness Any stage of the illness Concurrent with curative or disease-directed therapies	Estimated 6 months or less prognosis. Eligible for Medicare or secondary insurance. Some free care is available Focus is quality of life, rather than life prolongation
Interdisciplinary Consult Team	**Interdisciplinary Hospice Care Team**
Core team: Palliative care physicians, advanced practice nurses, physician assistants, registered nurses, social workers, chaplains, pharmacists. Encouraged: art, music, and massage therapists	Hospice medical director (physician), advanced practice nurses, physician assistants, registered nurses, social workers, home health aides, chaplains, volunteers, Administrative personnel, medical consultants, occupational therapists, physical therapists, speech therapists, and bereavement counselors. Encouraged: art, music, and massage therapists

curatives treatments concurrently (Section 2302, "Concurrent Care for Children" Requirement, CCCR). The full effect of this change remains to be seen.

BEREAVEMENT

Bereavement follow-up by the hospice or palliative care team is an intrinsic component of comprehensive pediatric palliative care. Many families expressed feeling a double loss: loss of their child and loss of their oncology team whom they have known and trusted, often over months and years. Parental grief has been recognized as more intense and longer lasting and other types of grief. Contact from a team member after the child's death can lessen the family's sense of abandonment and the palliative care team can serve a crucial preventive role by identifying families at particular risk for prolonged grief disorder and identifying resources for them.

Each bereaved person's loss is unique, but many people manifest similar symptoms of grief, and some of which become less persistent as they rebuild their lives. Recurrent intense symptoms typically occur at the anniversary of the patient's death but can occur at unpredictable times, induced by reminders of the deceased. Survivors appreciate calls or letters from the patient's physicians and nurses. For patients enrolled in Hospice programs, a formal bereavement program is offered for the family throughout the first year after the patient dies. After the formal program ends, the bereaved are welcome to continue to participate in any bereavement activities that have been meaningful to them.

At the time of death, survivors may seem numb, confused, or dazed. By the second month after the death, yearning predominates. During the next months, disbelief, depressed mood, and yearning decline gradually, and by 6 months after the death, most people have accepted its reality and are beginning to think about re-engaging in relationships and work, discovering new meaning and purpose. Siblings are especially vulnerable in the year following a child's death. By a year or 2, most survivors are accommodated to their loss. They become aware of the changes that must be made if they are to resume old relationships and responsibilities or to establish new ones and risk recurrent loss.

However, about 10% to 20% of survivors suffer either from depression or from a symptom complex previously called complicated grief, now identified as prolonged grief disorder.[20] Those with depression manifest symptoms of sadness, anhedonia, and psychomotor retardation but they are not yearning for the deceased or unable to accept the death. Depressed survivors benefit from counseling and consideration of pharmacologic treatment. Bereaved persons with prolonged grief disorder, in contrast, have grief symptoms that last beyond 6 months and cause functional impairments. Such patients are at increased risk for medical and psychiatric illness and should be referred for psychiatric or spiritual counseling. Persons at higher risk for the disorder include those with a history of attachment disorders, aversion to lifestyle changes, being unprepared for the death and unsupported afterward, and having a particularly interdependent relationship with the deceased.

Likely because of the treatment intensity and high symptom burden, parents of children who received HSCT reported higher levels of depression, anxiety, and stress compared to parents of children with HM who did not receive HSCT; those parents whose children died in the hospital after receipt of HSCT had a greater likelihood of meeting the criteria for prolonged grief disorder.[24] Though caregivers of both pediatric and adult patients with HM experience significant stressors during their loved one's illness which could increase the risk of developing prolonged grief disorder, there is currently a lack of studies focusing on the bereavement of family members of adult patients who die of HM.

CONCLUSION AND FUTURE DIRECTIONS

Palliative Care Integration with Hematologic Malignancy Care

Patients with HM and their families face unique challenges compared with patients with solid tumors. These challenges include the need to undergo high risk, intensive treatments with significant symptom burden, and prolonged hospitalization to achieve cure; times of high infection risk; significant prognostic uncertainty; difficult decisions about the benefits and burdens of continuing transfusion products when the prognosis of the cancer is weeks to months; increased likelihood of receiving more aggressive care in the last month of life; and increased likelihood of dying in the hospital.

Encouragingly, there is growing evidence that palliative care integration into the care of patients with HM and those receiving HSCT is achievable and can improve patient and family outcomes.[22,25]

Recent evidence suggests that HSCT recipients in both pediatric and adult settings benefit from collaborative efforts between HSCT and palliative care services, particularly when initiated early in the transplantation course.[22,26] With transparent communication, respect for roles, and a collaborative approach, HSCT and palliative care can collectively provide high-quality, multidisciplinary care for these highly vulnerable patients and their families.[24,26] Various pediatric models of palliative care and HSCT care exist to meet the differing needs of individual settings. In all, the incorporation of palliative care in HSCT does not change the focus of therapy or the goals of care. Rather, palliative care involvement provides an extra layer of support for HM/HSCT patients and their families, helping them hold their concurrent goals of cure/remission while maintaining their hope to live as well as possible.[26–29]

Adult inpatient integrated HM/HSCT/palliative care models are aimed at building collaboration and improving HM/HSCT providers' perceptions of palliative care. In a quality improvement project, Selvaggi et al. showed the feasibility of a specialty palliative team integration model that was well-received by hematologic oncologists within an HM/HSCT inpatient unit.[30] In a single-center randomized controlled trial of HM patients admitted for HSCT, after 2 weeks, those receiving inpatient palliative care concurrently with standard transplant care had less decrease in quality of life, decreased anxiety, less increase in depression, and less increase in overall symptom burden.[22]

Because HSCT patients experience a high burden suffering from both physical and emotional symptoms, patient and caregiver outcomes can be improved with early palliative care involvement.[18,19] To address this need, starting in September 2018 at Brigham and Women's Hospital/Dana Farber Cancer Institute (BWH/DFCI), we instituted a pilot "HemePal" quality improvement project. HemePal was an integrated palliative care co-rounding model that helped the inpatient BWH/DFCI bone marrow transplant physician assistant (PA) team control patient symptoms. A palliative care specialist (physician or nurse practitioner) co-rounded with the team once weekly. They also communicated daily with the PA team to identify patients needing more palliative care assistance for symptom management, which was provided either by "curbside" recommendations or a full consultation. This co-rounding model provided both palliative care specialists and the HM/HSCT providers an invaluable opportunity to learn from each other's perspectives and to build collaborative relationships to improve HSCT patients' care. Data evaluating the efficacy of this model is in process.

Though the benefits of PC integration within inpatient HSCT/HM care are known,[22] more research is needed to characterize the symptom burden of HM patients at all points in their illness trajectories and to evaluate further integrated palliative care interventions within this population.

REFERENCES

1. Center to Advance Palliative Care (CAPC). *What Is Palliative Care?* http://getpalliativecare.org/whatis/
2. Institute of Medicine (IOM) Report. *Dying in America: Improving Quality and Honoring Individual Preferences Near the End of Life*. http://www.ahaphysicianforum.org/resources/appropriate-use/ICU/resources/IOM-Dying-in-America.pdf.
3. Ferrell BR, Temel JS, Temin S, et al. Integration of palliative care into standard oncology care: American Society of Clinical Oncology clinical practice guideline update. *J Clin Oncol*. 2017;35:96–112.
4. Temel JS, Greer JA, Muzikansky A, et al. Early palliative care for patients with metastatic non-small-cell lung cancer. *N Engl J Med*. 2010;363:733–742.
5. Quill TE, Abernethy AP. Generalist plus specialist palliative care—creating a more sustainable model. *N Engl J Med*. 2013;368:1173–1175.
6. National Comprehensive Cancer Network (NCCN). *Palliative Care Report*. https://www.nccn.org/professionals/physician_gls/pdf/palliative.pdf.
7. Manitta V, Zordan R, Cole-Sinclair M, et al. The symptom burden of patients with hematological malignancy: a cross-sectional observational study. *J Pain Symptom Manag*. 2011;42:432–442.
8. Fadul NA, El Osta B, Dalal S, et al. Comparison of symptom burden among patients referred to palliative care with hematologic malignancies versus those with solid tumors. *J Palliat Med*. 2008;11:422–427.
9. LeBlanc TW. In the sandbox. palliative care and hematologic malignancies. *J Commun Support Oncol*. 2014;12:44–45.
10. Howell DA, Shellens R, Roman E, et al. Haematological malignancy: are patients appropriately referred for specialist palliative and hospice care? A systematic review and meta-analysis of published data. *Palliat Med*. 2011;25:630–641.
11. LeBlanc TW, O'Donnell JD, Crowley-Matoka M, et al. Perceptions of palliative care among hematologic malignancy specialists: a mixed-methods study. *J Oncol Pract*. 2015;11:e230–e238.
12. El-Jawahri A, LeBlanc TW, Burns LJ, et al. What do transplant physicians think about palliative care? A national survey study. *Cancer*. 2018;124:4556–4566.
13. Ullrich C, Duncan J, Joselow M, Wolfe J. Pediatric palliative care. In: Kliegman RM, St. Geme JW, Blum NJ, Shah SS, Tasker RC, Wilson KM, eds. *Nelson Textbook of Pediatrics*. 21st ed. Philadelphia, PA: Elsevier; 2020. [ch. 7].
14. Mack JW, Wolfe J, Grier HE, et al. Communication about prognosis between parents and physicians of children with cancer: parent preferences and the impact of prognostic information. *J Clin Oncol*. 2006;24:5265–5270.
15. Alexande SC, Sullivan AM, Back AL, et al. Information giving and receiving in hematological malignancy consultations. *Psychooncology*. 2012;21:297–306.
16. De Vos MA, Bos AP, Plotz FB, et al. Talking with parents about end-of-life decisions for their children. *Pediatrics*. 2015;135:e465–e476.
17. Jackson VA, Jacobsen J, Greer JA, et al. The cultivation of prognostic awareness through the provision of early palliative care in the ambulatory setting: a communication guide. *J Palliat Med*. 2015;16:894–900.
18. Webb JA, LeBlanc TW, El-Jawahri A. Integration of palliative care into acute myeloid leukemia care. *Semin Oncol Nurs*. 2019;35:1–4.
19. El-Jawahri A, LeBlanc TW, VanDusen H, et al. Effect of inpatient palliative care on quality of life 2 weeks after hematopoietic stem cell transplantation: A randomized clinical trial. *JAMA*. 2016;316:2094–2103.
20. Abrahm JL. *A Physician's Guide to Pain and Symptom Management in Cancer Patients*. 3rd ed. Baltimore: Johns Hopkins University Press; 2014.
21. Childers JW, Back AL, Tulsky JA, Arnold RM. REMAP: A framework for goals of care conversations. *J Oncol Pract*. 2017;13:e844–e850.
22. El-Jawahri A, Nelson AM, Gray TF, et al. Palliative and end-of-life care for patients with hematologic malignancies. *J Clin Oncol*. 2020;38:944–953.
23. Miovic M, Block S. Psychiatric disorders in advanced cancer. *Cancer*. 2007;110:1665–1676.
24. Wolf J, Grier H, Klar N, et al. Symptoms and suffering at the end of life in children with cancer. *N Engl J Med*. 2000;342:326–333.
25. Ruark J, Mullane E, Cleary N, et al. Patient-reported neuropsychiatric outcomes of long-term survivors after chimeric antigen receptor T cell therapy. *Biol Blood Marrow Transplant*. 2020;26:34–43.
26. Levine DR, Baker JN, Wolfe J, et al. Strange bedfellows no more: how integrated stem-cell transplantation and palliative care programs can together improve end-of-life care. *J Oncol Pract*. 2017;13:569–577.
27. Balboni TA, Paulk ME, Balboni MJ, et al. Provision of spiritual care to patients with advanced cancer: Associations with medical care and quality of life near death. *J Clin Oncol*. 2010;28:445–452.
28. Odejide OO, Steensma DP. Patients with haematological malignancies should not have to choose between transfusions and hospice care. *Lancet Haematol*. 2020;7:e418–e424.
29. Drew D, Goodenough B, Maurice L, et al. Parental grieving after a child dies from cancer: is stress from stem cell transplant a factor? *Int J Palliat Nurs*. 2005;11:266–273.
30. Selvaggi KJ, Vick JB, Jessell SA, et al. Bridging the gap: a palliative care consultation service in a hematological malignancy-bone marrow transplant unit. *J Commun Support Oncol*. 2014;12:50–55.

CHAPTER 101

THERAPY-RELATED LATE EFFECTS OF HEMATOLOGIC MALIGNANCIES

Wendy Landier and Smita Bhatia

The past several decades have seen a marked improvement in survival for patients with hematologic malignancies. The 5-year survival is as follows: leukemia, 66%; Hodgkin lymphoma (HL), 89%; non-Hodgkin lymphoma (NHL), 75%; and multiple myeloma (MM), 54%. As a consequence, the population of long-term cancer survivors continues to grow. As of January 1, 2019, there were 451,700 leukemia survivors living in the United States, 234,890 HL survivors, and 757,710 NHL survivors.[1,2]

Attendant with this success is an increasing awareness of the occurrence of long-term morbidity and mortality associated with the very treatments responsible for the improvement in survival. The subject of long-term morbidity suffered by cancer survivors has been the topic of numerous reports. These reports demonstrate that survivors are at risk for developing adverse outcomes, including premature death, subsequent neoplasms, organ dysfunction (e.g., cardiac, pulmonary, gonadal), reduced growth, decreased fertility, impaired intellectual function, difficulties obtaining employment and insurance, and overall reduced quality of life.

Hematopoietic cell transplantation (HCT) is the treatment of choice for patients with hematologic malignancies experiencing disease recurrence after conventional regimens and for those with disease characteristics associated with poor prognosis if treated with conventional chemotherapy and radiation regimens. Complications observed after HCT often have a multifactorial origin encompassing issues related to prior cancer therapy, intensity of the preparative regimen, graft-versus-host disease (GVHD), and other posttransplantation complications.

This chapter summarizes select adverse outcomes among individuals treated for hematologic malignancies with conventional therapy alone or with HCT. Recommendations for providing ongoing follow-up care to this population are also reviewed.

CARDIOVASCULAR DISEASE

One of the more serious adverse events encountered in survivors of hematologic malignancies is late-occurring cardiovascular disease (CVD), which include arterial disease (coronary artery disease [CAD]: myocardial infarction, atherosclerotic heart disease, and angina pectoris; and cerebrovascular disease [stroke]) and cardiac disease (cardiomyopathy and congestive heart failure [CHF], valvular heart disease (VHD), conduction abnormalities, and constrictive pericarditis). These complications are more common than expected,[3,4] and often occur at an earlier age than observed in the general population.[5–7]

Mediastinal radiotherapy (RT) is associated with an increased risk of CAD, VHD, and CHF. Anthracycline chemotherapy increases the risks of VHD and CHF. Anthracyclines are directly toxic to the myocardium through a variety of mechanisms, including free radical-mediated oxidative damage and induction of cellular apoptosis. Joint effects of mediastinal RT, anthracyclines, and smoking appear to be additive.[8] Outcome is poor, with less than 50% of patients surviving 5 years after a diagnosis of CHF.[9]

Among adult-onset HL survivors, the risk of CVD increases with heart radiation dose and cumulative anthracycline dose.[10,11] Throughout their lives, HL survivors treated as adolescents or adults are at high risk for CVD. For patients treated before 25 years of age, the cumulative incidence at 60 years or older is 20%, 31%, and 11% for CAD, VHD, and CHF as first events, respectively.

Cardiac Disease

The anthracycline class of drugs is a well-known cause of late-onset cardiomyopathy leading to CHF. There is a strong dose-dependent relation between anthracycline exposure and risk of CHF. Among childhood cancer survivors, the incidence of CHF is less than 5% with cumulative anthracycline exposure of less than 250 mg/m^2, approaches 10% at doses between 250 mg/m^2 and 600 mg/m^2, and exceeds 30% for doses higher than 600 mg/m^2.[12–14] However, cumulative anthracycline exposure as low as 101 to 150 mg/m^2 may be associated with a 3.9-fold increased risk for cardiomyopathy when compared with those unexposed to anthracyclines.[15]

Among adult-onset cancer survivors, the 5-year cumulative risk of CHF is 10%.[16] The incidence of CHF has been estimated to exceed 25% with a dosage of 550 mg/m^2.[17] Among survivors older than 65 years of age, the risk of late-onset CHF is 29% higher among those exposed to doxorubicin, compared with those not treated with doxorubicin, and an increase in the number of cycles of doxorubicin-containing chemotherapy is significantly associated with increasing risk of CHF.[18]

Among blood or marrow transplant (BMT) recipients, the risk of CHF is highest after autologous BMT, approaching 10% at 15 years after BMT; (21976673) autologous BMT survivors are at a nearly fivefold higher risk of CHF when compared with age- and sex-matched individuals from the general population. The risk of late-occurring CHF is primarily due to pre-BMT exposure to anthracyclines.[4,9] Doxorubicin dose $\geq$300 mg/m^2 and cardiac RT dose greater than 30 Gy are independent risk factors for left ventricular (LV) systolic dysfunction.[19]

Young age at exposure is a significant modifier of anthracycline-related cardiotoxicity.[20] Females treated in childhood are at greater risk of developing doxorubicin-induced ventricular dysfunction.[12] The combined use of doxorubicin and chest radiation has been associated with a greater risk of late cardiac toxicity than either treatment given alone.[21,22] The presence of one or more conventional cardiovascular risk factors (CVRFs; diabetes, dyslipidemia, and hypertension) has a significant effect on the incidence of heart disease among HL survivors and NHL survivors.[18,21,23] The risk of post-BMT anthracycline-related cardiotoxicity also increases significantly among individuals with one or more conventional cardiac risk factors.[3,4,24,25]

Arterial Disease

Chest radiation produces intimal thickening of the coronary arteries and microvascular damage that causes reduced myocardial perfusion. Patients who received mediastinal radiation for HL have an increased risk of CAD compared with the general population.[26–28]

The risk of radiation-related CAD is generally higher among men and among younger patients. The risk of deaths related to myocardial infarction is 2.5-fold higher among HL patients when compared with an age- and sex-matched general population; the increased risk of myocardial infarction mortality persisted through to 25 years after first treatment. Risks are increased for patients treated with supradiaphragmatic radiation, anthracyclines, or vincristine. Among those exposed to supra-diaphragmatic radiation and vincristine and followed for ≥20 years after first treatment, the risk is 15-fold that of the general population.[29]

Using medically ascertained data, the cumulative burden metric was used to compare chronic cardiovascular health conditions among childhood HL survivors and general population controls. At 50 years of age, the cumulative incidence of survivors experiencing at least one grade 3 to 5 cardiovascular condition was 45.5%. The survivor cohort experienced, on average, 430 grade 1 to 5 and 101 grade 3 to 5 cardiovascular conditions per 100 survivors. At age 50, the grade 1 to 5 and 3 to 5 cumulative burdens in community-controls were appreciably lower at 227/100 and 17.0/100, respectively. Myocardial infarction and structural heart defects were the major contributors to the excess grade 3 to 5 cumulative burden among survivors. Higher cardiac radiation dose (≥35 Gy) was associated with higher grade 3 to 5 cardiovascular burden.[30]

Arterial disease in the transplant setting is related to accelerated atherosclerosis, attributed to pre-BMT and conditioning-related RT, and is compounded by the presence of CVRFs in the early post-BMT period.[24,31] The cumulative incidence of arterial events such as CAD or stroke among allogeneic BMT recipients is 10% at 15 years and the risk exceeds 20% at 20 years.[6,7] In this population, the median age at first myocardial infarction is as low as 53 years (range: 35 to 66),[25,32] which is earlier than would be expected for the general population (67 years)[33] or that reported in survivors of autologous BMT (61 years).[25,32] The risk of developing CVRFs is higher in allogeneic BMT recipients when compared with autologous BMT recipients, as well as age- and sex-matched general population.[25,34] The 10-year cumulative incidence of hypertension, diabetes, and dyslipidemia in allogeneic BMT recipients is 38%, 18%, and 47%, respectively; the risk for multiple (>2) CVRFs approaches 40% (compared with 26% in autologous BMT survivors). Conditioning with total body irradiation (TBI) has been associated with an increased risk of dyslipidemia and diabetes in survivors of BMT.[25,34] Abdominal RT possibly contributes to insulin resistance and/or metabolic syndrome, suggesting a role for radiation-induced pancreatic or hepatic injury.[35] The increased risk of diabetes and dyslipidemia among BMT survivors with prior exposure to TBI could potentially be due to the combined effects of abdominal RT and post-BMT gonadal dysfunction.[36]

Prevention of Cardiotoxicity

Given the known cardiovascular complications of cancer therapy, prevention of CVD is a focus of active investigation. There is strong evidence for dexrazoxane as a cardioprotectant. Dexrazoxane decreases oxygen free radicals through intracellular iron chelation.[37] In two meta-analyses, dexrazoxane was associated with 60% to 80% fewer clinical and subclinical cardiac events during and after anthracycline-based therapy.[38,39] Overall, toxicity and measures of tumor response were similar between patients exposed and unexposed to dexrazoxane.[38] Currently, the Food and Drug Administration approves dexrazoxane for women with metastatic breast cancer who have received 300 mg/m^2 of doxorubicin and who need additional anthracycline-based therapy. The American Society of Clinical Oncology also recommends considering dexrazoxane for adults with any history of cancer who have already received 300 mg/m^2 of doxorubicin-based therapy.[40]

Intermediate or surrogate endpoints (cardiac troponin T) in randomized clinical trials show that dexrazoxane reduces cardiotoxicity in children exposed to anthracyclines.[33,41,42] Longer-term follow-up data demonstrate that various echocardiographic indices of LV structure and function are worse in those not receiving dexrazoxane.[41] Girls benefit from dexrazoxane more than boys, particularly with respect to changes in the LV end-diastolic thickness-to-dimension ratio, a marker of pathologic LV remodeling. Further, there is no impact on late mortality among HL patients randomized to dexrazoxane.[43] However, comprehensive longer-term follow-up is required to document that dexrazoxane does indeed have a cardioprotective effect while maintaining comparable event-free survival.[43,44]

Liposome-encapsulated anthracyclines have been explored, using the premise that liposome-encapsulated anthracyclines escape the capillaries with wide endothelial gaps in the tumor, thus reaching high concentrations in the interstitial fluid of the tumor bed, but are less likely to escape the tight capillary junctions of the heart. Biopsy results have confirmed the relative safety in clinical use.[45,46] Data on the potential cardioprotection associated with liposomal formulations of anthracyclines is limited. A phase I study of liposomal daunorubicin in 48 children reported no cardioprotection.[47] A primary cardioprotection strategy (angiotensin-converting enzyme inhibitors and/or beta blockers as well as liposomal doxorubicin) in NHL patients with a high risk of anthracycline cardiotoxicity was found to result in fewer events (cardiovascular mortality and heart failure occurrence) when compared with historical controls.[48]

Specific recommendations for monitoring have been developed for childhood cancer survivors (http://www.survivorshipguidelines.org)[49,50] as well as for survivors of adult-onset cancer[51] and BMT (Table 101.1).[52]

PULMONARY EFFECTS

Pulmonary function compromise has been reported after conventional therapy in survivors of hematologic malignancies.[5,53–55] Pulmonary function abnormalities include reductions in total lung capacity (TLC), forced vital capacity (FVC), forced expiratory volume in the first second of expiration (FEV$_1$), and gas transfer (diffusing capacity of lung for carbon monoxide [DLCO]), suggesting obstructive and restrictive defects. Risk factors include exposure to certain chemotherapeutic agents (particularly bleomycin), radiation to the chest, underlying lung disease, female sex, and a younger age at exposure to the pulmonary-toxic therapeutic agents. Patients present with chronic cough, emphysema, lung fibrosis, oxygen requirements, and recurrent pneumonia. The cumulative incidence of any pulmonary disease after 35 years of follow-up exceeds 20% in childhood cancer survivors.[56] Childhood cancer survivors are 6.5-fold more likely to develop restrictive disease and 5.2-fold more likely to develop diffusion abnormalities when compared with healthy noncancer controls. Higher radiation dose (>20 Gy) and younger age at exposure (<16 years) is associated with restrictive disease. Female sex and higher chest radiation dose are associated with diffusion abnormalities. Over a period of 5 years, diffusion capacity declines among females and those exposed to high doses of chest radiation.[57]

Pulmonary complications, both infectious and noninfectious, are common after BMT. Multiple factors are thought to contribute to pulmonary complications in this population, including the type and duration of immunological defects produced by the underlying disease and treatment, the development of chronic GVHD, and the conditioning regimens employed. These complications are classified as early or late, depending on whether they occur before or after 100 days from transplantation. Early noninfectious pulmonary complications typically include pulmonary edema, upper airway complications, diffuse alveolar hemorrhage, and pleural effusion. Bronchiolitis obliterans occurs later after BMT. Idiopathic pneumonia syndrome, GVHD, and radiation-induced lung injury can occur in the early or late periods after BMT (reviewed in Khurshid and Anderson).[58]

The following pulmonary function tests are noted to decline after BMT: FEV$_1$/FVC, forced mid-expiratory flow, TLC, DLCO, residual volume (RV), functional residual capacity, and RV/TLC. Older age at allogeneic BMT is associated with lower FEV$_1$/FVC and DLCO, and higher RV/TLC. Bronchiolitis obliterans syndrome is a progressive, insidious lung disease occurring after allogeneic BMT and results in progressive circumferential fibrosis and ultimate cicatrization of the small terminal airways, manifesting as new fixed airflow obstruction (reviewed in Williams et al.).[59–64] Bronchiolitis obliterans has a strong correlation with chronic GVHD and has been reported in up to 6% of BMT recipients.[59] Most patients present when the degree

TABLE 101.1 Monitoring for Potential Late Effects

Potential Late Effects	Therapeutic Exposure	Recommended Monitoring	Suggested Interventions
Adverse psychosocial effects (e.g., depression, anxiety, posttraumatic stress disorder, limitations in healthcare access, relationship problems, risky behaviors, psychosocial disability due to pain, sleep problems, fatigue)	Diagnosis and treatment for hematologic malignancy	Clinical interview: yearly	Psychologic or social work consultation if indicated
Dental abnormalities (abnormal tooth development, increased susceptibility to caries and gum disease)	Chemotherapy Cranial irradiation TBI	Dental examination: every 6 months	Professional dental cleaning every 6 months; use of fluoridated toothpaste; topical fluoride applications as indicated; Panorex radiograph before orthodontic or dental procedures for patients treated before completion of tooth development
Peripheral neuropathy Raynaud phenomenon	Vincristine Vinblastine	Neurologic examination if symptomatic	Physical therapy if indicated; advise patient to protect against precipitating factors (e.g., cold environment)
Neurocognitive deficits	Methotrexate (intrathecal, high-dose systemic) Cytarabine (high-dose systemic) Cranial irradiation TBI	Formal neuropsychologic testing: baseline on entry to long-term follow-up; repeat as clinically indicated if evidence of impaired performance Clinical interview yearly, including assessment of educational and vocational progress	Referral for specialized educational services, curricular modifications, or vocational training programs if indicated
Clinical leukoencephalopathy	Methotrexate (intrathecal, high-dose systemic) Cranial irradiation	Clinical evaluation: yearly Brain MRI or CT if clinically indicated	Neurology consultation if clinically indicated
Cataracts	Corticosteroids Busulfan Cranial irradiation TBI	Funduscopic examination and visual acuity evaluation: yearly Ophthalmologic examination: yearly	Ophthalmology consultation if abnormalities detected
Xerophthalmia	Chronic GVHD related to HCT	History and eye examination yearly	Artificial tears, ophthalmology consultation if indicated
Xerostomia	Cranial irradiation Chronic GVHD related to HCT	Dental evaluation: every 6 months	Meticulous oral hygiene Artificial saliva products if indicated
Hearing loss	Platinum chemotherapy Aminoglycoside antibiotics Cranial irradiation	History and physical examination: yearly Audiogram: every 2–5 years for survivors who received platinum chemotherapy and/or cranial radiation dose $\geq$ 30 Gy	Audiology consultation if indicated
Hypothyroidism Thyroid nodules Thyroid malignancy	Cranial, cervical, spinal, mantle, or mediastinal irradiation; TBI	Free T_4, TSH, thyroid examination: yearly	Endocrine or surgical referral as indicated
Cardiomyopathy Arrhythmias Subclinical left ventricular dysfunction	Anthracyclines Chest or thoracic irradiation (e.g., mantle, mediastinal)	Detailed history of exertional tolerance (e.g., dyspnea on exertion, chest pain): yearly ECG (for evaluation of QT interval): baseline on entry into long-term follow-up; ECHO: Every 2–5 years as indicated based on age at therapy and total anthracycline or radiation dose	Cardiology consultation if indicated; additional cardiology evaluation of patients who are pregnant or planning to become pregnant if patient received $\geq$ 250 mg/m^2 of an anthracycline, radiation dose $\geq$ 35 Gy, or any dose of anthracycline combined with radiation $\geq$ 15 Gy
Pericarditis Pericardial fibrosis Valvular disease Premature atherosclerotic heart disease	Chest or thoracic irradiation (e.g., mantle, mediastinal)	Consider cardiology consultation 5–10 years after irradiation to evaluate risk for coronary artery disease in patients who received doses $\geq$35 Gy or $\geq$15 Gy plus anthracyclines. Optimize cardiovascular risk factors, including blood pressure, lipid profile, and blood glucose.	Cardiology consultation if indicated; additional cardiology evaluation in patients who are pregnant or planning to become pregnant if patient received $\geq$ 250 mg/m^2 anthracycline, anthracycline combined with radiation $\geq$15 Gy, or radiation dose $\geq$35 Gy

Continued

TABLE 101.1 **Monitoring for Potential Late Effects—cont'd**

Potential Late Effects	Therapeutic Exposure	Recommended Monitoring	Suggested Interventions
Pulmonary dysfunction (fibrosis, interstitial pneumonitis)	Bleomycin Busulfan Chest/thoracic irradiation TBI Chronic GVHD related to HCT	Pulmonary function testing: baseline at entry into long-term follow-up and as clinically indicated for patients with progressive dysfunction	Pulmonary consultation for symptomatic patients; influenza and pneumococcal vaccine; counsel patients to avoid smoking and avoid SCUBA diving
Thrombosis Vascular insufficiency Infection of retained cuff or line tract	Central venous catheter	History and physical examination: yearly as clinically indicated	Surgical referral as indicated
Hepatic dysfunction	Mercaptopurine, thioguanine, methotrexate (systemic), abdominal radiation, HCT	ALT, AST, bilirubin: baseline on entry into long-term follow-up; repeat if clinically indicated	If abnormal results for baseline studies, obtain prothrombin time (to assess hepatic synthetic function) and viral hepatitis screening
Chronic viral hepatitis	Blood products	Hepatitis C antibody and HCV RNA by PCR: once if transfused before universal screening of blood supply (1992 in the United States) Hepatitis B surface antigen and core antibody: once if transfused before universal screening of blood supply (1972 in the United States)	Gastroenterology or hepatology consultation and annual AFP for patients with chronic hepatitis; hepatitis A and B immunizations in patients lacking immunity
HIV infection	Blood products	HIV-1 and HIV-2 antibodies: once if transfused before universal screening of blood supply (1985 in the United States)	Infectious disease consultation for patients with confirmed infection
Life-threatening infection	Splenectomy Abdominal/splenic radiation ≥ 40 Gy Chronic active GVHD	Physical examination at time of febrile illness to evaluate degree of illness and for potential source of infection	Counsel patients regarding risk of life-threatening infections and indication for medical alert bracelet. Administer parenteral antibiotics and continue close medical observation in patients with temperature ≥ 38.3°C (101°F) or other signs of serious infection; immunize with pneumococcal, meningococcal, and HIB vaccines
Iron overload	HCT (and patients requiring multiple red blood cell transfusions)	Serum ferritin: at entry into long-term follow-up	If abnormal, consider chelation, or repeat as clinically indicated until within normal limits
Bowel obstruction Chronic enterocolitis Fistulas and strictures	Abdominal surgery Abdominal or pelvic irradiation Chronic GVHD related to HCT (esophageal strictures, vaginal stenosis)	History and physical examination: yearly and as clinically indicated Serum protein and albumin levels: yearly in patients with chronic diarrhea or fistula	Surgical and gastroenterology consultations as clinically indicated
Renal insufficiency	TBI Abdominal or splenic irradiation Ifosfamide Platinum chemotherapy	Blood pressure: yearly BUN, creatinine, electrolytes, calcium, magnesium, phosphorus baseline and repeat as clinically indicated	Nephrology consultation for proteinuria, hypertension, progressive renal insufficiency
Hemorrhagic cystitis Bladder fibrosis Dysfunctional voiding Bladder malignancy	Cyclophosphamide Ifosfamide Irradiation of abdomen, pelvis, iliac, inguinal sites	Voiding history: yearly	Urinalysis and urology consultation as clinically indicated for incontinence, dysfunctional voiding, macroscopic hematuria (culture negative)
Growth hormone deficiency	Cranial irradiation TBI	Height, weight: every 6 months during puberty until growth is complete Obtain bone age in poorly growing children	Endocrine referral for patients failing to follow normal growth curve

Continued

TABLE 101.1 Monitoring for Potential Late Effects—cont'd

Potential Late Effects	Therapeutic Exposure	Recommended Monitoring	Suggested Interventions
Overweight/obesity	Cranial irradiation	BP, growth percentile, BMI: yearly	Endocrine referral as indicated
Dyslipidemia	TBI	Lipid profile every 2 years	
Hypogonadism/Infertility	Alkylating agents Cranial irradiation Abdominal or pelvic irradiation Testicular irradiation Spinal irradiation > 25 Gy TBI	Menstrual history, sexual function, height, weight: yearly Pubertal history, Tanner stage: yearly until maturity	FSH, estradiol: (females); Testosterone: (males); for failure of pubertal progression or clinical symptoms of estrogen or testosterone deficiency Semen analysis: as indicated or requested by patient. Endocrine referral for hypogonadal patients (for hormone-replacement therapy); reproductive endocrinology referral for patients desiring evaluation of fertility options
Precocious puberty	Cranial irradiation	Physical examination, height, weight, Tanner stage: yearly until maturity LH, FSH, estradiol (females), or LH, FSH, testosterone (males): as clinically indicated in patients with accelerated pubertal progression Obtain bone age in rapidly growing prepubertal children	Endocrine referral as indicated for accelerated puberty (<age 8 years in females, <age 9 years in males)
Adverse pregnancy outcomes (e.g., spontaneous abortion, premature delivery, low-birthweight infant)	Irradiation of abdomen, pelvis, iliac, inguinal, paraaortic sites TBI	History: yearly and as clinically indicated	High-risk obstetric care
Osteopenia, osteoporosis	Corticosteroids Methotrexate (high-dose systemic) HCT	Bone-density study (DXA scan): baseline at entry into long-term follow-up, repeat as clinically indicated	Calcium and vitamin D supplementation; weight-bearing exercise; treatment of exacerbating conditions (e.g., hypogonadism); consider pharmacologic intervention (e.g., bisphosphonates)
Avascular necrosis	Corticosteroids Methotrexate (systemic) HCT	History: yearly; MRI if clinically indicated	Orthopedic consultation if indicated
Scoliosis/kyphosis	Irradiation of trunk (e.g., mantle, spine, abdomen, pelvis)	Physical examination of spine: yearly (every 6 months during pubertal growth spurt) Radiologic imaging of the spine if clinical evidence of scoliosis or kyphosis	Orthopedic referral
Joint contractures	Chronic GVHD related to HCT	Physical examination yearly	Orthopedic referral if indicated
Chronic infection	Chronic GVHD related to HCT	History: yearly	Prophylactic anti-infective agents; infectious disease consultation if indicated
Vitiligo Scleroderma Joint contractures Nail dysplasia Dysplastic nevi Skin cancer Secondary benign or malignant neoplasms in radiation field	Chronic GVHD related to HCT Irradiation (any field)	Physical examination: yearly Inspection and palpation of irradiated skin and soft tissues: yearly	Dermatology or rehabilitation consultation if clinically indicated Dermatology or surgical referral and radiographs if indicated for any suspicious lesions
Bone malignancies Brain tumor	Cranial irradiation	History and physical examination: yearly Neurologic exam: yearly	Neurosurgical consultation as indicated
Breast cancer	Chest or axillary radiation TBI	Clinical breast examination: yearly beginning at puberty until age 25, then every 6 months Mammogram and breast MRI: yearly beginning at age 25 or 8 years after irradiation (whichever comes last)	Teach breast self-examination; instruct patient to perform monthly self-examination beginning at puberty and report changes immediately; surgical consultation if clinically indicated

Continued

TABLE 101.1 **Monitoring for Potential Late Effects—cont'd**

Potential Late Effects	Therapeutic Exposure	Recommended Monitoring	Suggested Interventions
Gastrointestinal malignancy	Abdominal, pelvic, spinal irradiation, TBI	Colonoscopy every 5 years beginning 5 years after radiation or at age 30, whichever occurs last; more frequently as clinically indicated; or multitarget stool DNA test every 3 years; other options may be considered based on informed decision-making between patient and provider	Surgical consultation if indicated
AML (preceding myelodysplastic phase associated with alkylating agents)	Anthracyclines Epipodophyllotoxins Alkylating agents Stem cell priming with etoposide Autologous transplantation for NHL or Hodgkin lymphoma	Physical examination: yearly for 10 years after therapy CBC and differential and bone marrow evaluation if clinically indicated	Counsel patient to report fatigue, bruising, bleeding, bone pain

AFP, α-Fetoprotein; *ALT*, alanine aminotransferase; *AML*, acute myeloid leukemia; *AST*, aspartate aminotransferase; *BMI*, body mass index; *BP*, blood pressure; *BUN*, blood urea nitrogen; *CBC*, complete blood cell count; *CT*, computed tomography; *DXA*, dual-energy X-ray absorptiometry; *ECG*, electrocardiogram; *ECHO*, echocardiogram; *FSH*, follicle-stimulating hormone; *GFR*, glomerular filtration rate; *GVHD*, graft-versus-host disease; *HCT*, hematopoietic cell transplantation; *HCV*, hepatitis C virus; hemoglobin A1c, glycosylated hemoglobin; *HIB*, *Haemophilus influenzae* type B; *HIV*, human immunodeficiency virus; *LH*, luteinizing hormone; *MRI*, magnetic resonance imaging; *NHL*, non-Hodgkin lymphoma; *PCR*, polymerase chain reaction; *RNA*, ribonucleic acid; T_4, thyroxine; *TBI*, total body irradiation; *TSH*, thyroid-stimulating protein.
Modified from Children's Oncology Group: *Long-term follow-up guidelines for survivors of childhood, adolescent, and young adult cancers*, version 5.0, 2018, Children's Oncology Group. http://www.survivorshipguidelines.org

of airflow is severe, causing significant dyspnea on exertion and a persistent nonproductive cough. Lung biopsy findings demonstrating damage to the bronchiolar epithelium, obliteration of bronchiolar lumens, inflammation between the epithelium and the smooth muscle, and pulmonary fibrosis are characteristic. The National Institutes of Health definition of bronchiolitis obliterans requires the following: (1) absence of active infection, (2) decreased FEV_1 (<75% of predicted value), (3) evidence of airway obstruction with a ratio of FEV_1 to FVC of less than 0.7, (4) elevated RV of air (>120% of predicted normal), or (5) an expiratory chest computed tomographic (CT) scan or lung biopsy that reveals air trapping or bronchiectasis. Recommended therapy includes high-dose systemic steroids for a protracted course, with or without the addition of other immune suppressants.[64] Leukotriene inhibitors have emerged as a potential therapy because of the elevated levels of leukotrienes implicated in bronchiolitis obliterans. Late-onset severe restrictive lung defect is also observed after allogeneic BMT, usually ~3 months post-BMT. Major CT findings include pleuroparenchymal thickening with volume loss, and evidence for fibrosis, predominantly in upper lobes–a finding that is consistent with pleuroparenchymal fibroelastosis. Other findings include interstitial pneumonia. Pulmonary function tests reveal diffusion capacity abnormalities.[65]

Specific recommendations for monitoring for pulmonary complications have been developed for childhood cancer survivors (http://www.survivorshipguidelines.org)[49] and for survivors of BMT (see Table 101.1).[66,67]

ENDOCRINOLOGIC EFFECTS

Thyroid

Patients with hematologic malignancies treated with cranial, craniospinal, or mantle irradiation are at increased risk for thyroid complications. Among survivors of HL, and to a lesser extent leukemia, abnormalities of the thyroid gland, including hypothyroidism, hyperthyroidism, and thyroid neoplasms, have been reported to occur at rates significantly higher than those found in the general population.[68] Hypothyroidism is the most common nonmalignant late effect involving the thyroid gland. Survivors of HL are at particularly increased risk; after exposure to radiation at doses of 30 to 45 Gy to the thyroid gland the cumulative incidence of hypothyroidism is 30% at 20 years, with the cumulative incidence increasing to 50% for radiation doses above 45 Gy.[69] Hyperthyroidism is infrequently seen, but is also associated with a history of neck radiation.[69] Thyroid nodules are common in patients treated with neck radiation for HL, but the majority of these do not undergo malignant transformation.[70]

Monitoring for survivors at risk for thyroid complications is reviewed in Table 101.1.[69]

Obesity

An increased prevalence of obesity has been reported among survivors of childhood ALL.[71,72] In an analysis from the Childhood Cancer Survivor Study, Oeffinger et al.[73] compared the distribution of the body mass index (BMI) of 1765 adult survivors of childhood ALL with that of 2565 adult siblings of childhood cancer survivors. Survivors were significantly more likely to be overweight (BMI of 25 to <30) or obese (BMI ≥30). Risk factors for obesity were cranial irradiation, female sex, and age 0 to 4 years at diagnosis of leukemia. Females diagnosed under the age of 4 years who received a cranial radiation dose of more than 20 Gy were found to have a 3.8-fold increased risk for obesity. In a recent report of survivors of standard-risk childhood ALL treated without cranial radiation, there was no increased risk of overweight or obesity compared with general population health data.[74] Obesity has the potential to adversely impact the overall health status in survivors and is associated with insulin resistance, diabetes mellitus, hypertension, and dyslipidemia. Yearly monitoring of BMI is recommended (see Table 101.1).

Gonadal Dysfunction

Treatment-related gonadal dysfunction has been well documented in male and female patients after therapy for hematologic malignancies and there is a reasonable body of research that provides a basis for counseling patients regarding the long-term gonadal effects of radiation and chemotherapy.

Radiation effects on the ovary are age and dose dependent. The ovaries are particularly sensitive to radiation, and it has been estimated that there is a 50% depletion in oocytes after exposure of the ovaries to 2 Gy,[75] and premature menopause is associated with even low doses of ovarian radiation.[76] The majority of females who receive ovarian radiation doses greater than 20 Gy develop acute ovarian failure; at lower radiation doses, post-pubertal adolescent and adult women are more susceptible to acute ovarian failure than are pre-pubertal girls.[77] Spinal irradiation for the treatment of childhood leukemia appears to result in clinically significant ovarian damage in some survivors,[78] and cranial irradiation in young girls is associated with an increased risk for premature puberty.[79]

The effects of radiation on testicular function, including germ cell number and Leydig cell function, have been investigated. Reduced sperm production has been observed after testicular doses of 1 to 6 Gy and follows a dose-dependent pattern.[80] Azoospermia has been reported among HL patients with calculated testicular irradiation exposures ranging from 1 to 3 Gy, which may be reversible.[81] Testicular doses between 4 to 6 Gy have been associated with prolonged azoospermia and decreased testicular volume, whereas doses greater than 6 to 8 Gy are associated with irreversible injury.[81] Spermatogenesis is impaired in 75% to 100% of male childhood HL survivors in whom radiation and chemotherapy were combined.[82] Most pre-pubertal males treated with fractionated radiation doses less than 12 Gy to the testis will undergo normal pubertal maturation. Males treated for testicular leukemia with radiation doses greater than 20 Gy who pre- or peri-pubertal are at high risk of delayed sexual maturation as well as significant germ cell depletion. In males who have reached puberty and undergo testicular radiation, only about 50% develop Leydig cell failure at doses greater than 30 Gy.[80]

Ovarian and testicular damage can also result from chemotherapeutic agents, with alkylating agents showing the strongest association. Effects of chemotherapy on gonadal function are typically sex-, age-, and dose-dependent. Ovarian dysfunction has been well documented in HL patients treated with alkylating agents, singly or in combination (e.g., MOPP regimen consisting of mechlorethamine, vincristine [Oncovin], procarbazine, and prednisolone, or a COPP regimen consisting of cyclophosphamide, vincristine [Oncovin], procarbazine, and prednisolone).[83] MOPP and COPP regimens have been reported to result in azoospermia in more than 90% of exposed males, with a negative correlation seen between cumulative doses of alkylating agents and sperm concentration.[84] Even with a reduction in the dose of cyclophosphamide in the hybrid COPP/adriamycin, bleomycin, and vinblastine (ABV) regimen for HL, the majority of young males are infertile, likely due to the procarbazine component in this regimen.[85] In a study of 6224 male survivors of childhood cancer between 15 and 44 years of age, those with a diagnosis of hematologic malignancy were significantly less likely to sire a pregnancy compared with sibling controls; those with a diagnosis of HL were least likely to sire a pregnancy (RR of fertility: 0.34; 95% confidence interval [CI]: 0.28–0.41), followed by NHL (RR: 0.60; 95% CI: 0.48–0.74), and leukemia (RR: 0.70; 95% CI: 0.59–0.84); $P < .001$ for all comparisons.[81]

Young boys and adolescent males with aplastic anemia who receive standard-dose cyclophosphamide alone (200 mg/kg) as the pretransplant conditioning regimen appear to retain normal Leydig cell function, as do males who receive busulfan and cyclophosphamide, with normal plasma concentrations of luteinizing hormone and testosterone, and normal progression through puberty.[86] Semen analyses have been normal in approximately two-thirds of men after high-dose cyclophosphamide and several men have fathered normal children. Men treated with TBI-based regimens who have not received prior testicular irradiation generally retain normal Leydig cell function, regardless of their age at treatment.[87] Germ cell dysfunction occurs in all men treated with TBI-based regimens and azoospermia is the rule; however, some younger males (age <25years at HCT) without chronic GVHD have showed some degree of spermatogenesis following standard-dose TBI.[88]

The effect of alkylating agents alone on ovarian function in females is dependent upon dose and age at exposure.[76] Females treated with busulfan and cyclophosphamide are at very high risk for ovarian failure and premature menopause, and procarbazine is also particularly gonadotoxic.[89] The outcome of ovarian function after TBI appears to be determined by the age at exposure. Approximately 50% of prepubertal girls receiving fractionated TBI enter puberty spontaneously and premature ovarian failure is seen in all patients who are older than 10 years of age when treated with TBI.[86] Pregnancies among survivors who received TBI are at an increased risk for spontaneous abortion, premature labor, and low-birthweight offspring.[90]

Monitoring for gonadal dysfunction is reviewed in Table 101.1.[80,91]

MUSCULOSKELETAL EFFECTS

Osteonecrosis is a painful and debilitating condition that develops when the blood supply to the bone is disrupted, usually in areas of terminal circulation. The condition is believed to be the result of vascular compromise with resultant death of bone and cell tissues or disruption of bone-repair mechanisms.[92] Osteonecrosis has been reported after conventional therapy for hematologic malignancies, particularly after exposure to dexamethasone between the ages of 10 and 20 years. This complication usually develops during or shortly after completion of therapy but may progress over time.[93]

Osteonecrosis is increasingly being reported among HCT recipients.[94,95] Campbell et al.[96] conducted a retrospective cohort study and described the cumulative incidence of osteonecrosis to be 2.9% among autologous HCT recipients, 5.4% among allogeneic HCT recipients, and 15% among unrelated donor HCT recipients. Among allogeneic HCT recipients, male sex, presence of chronic GVHD, and exposure to cyclosporine, tacrolimus, prednisone, and mycophenolate mofetil rendered patients at increased risk, in particular among patients with a history of exposure to three or more drugs. The mean latency period was 18 months. The hip joint was the most commonly involved joint (80%); however, the knee, wrist, and ankle joints were also affected. The cumulative incidence of surgery (mainly arthroplasty) approached 31% at 1 year from osteonecrosis diagnosis. Li et al.[95] conducted a case-control study of children who underwent allogeneic HCT and found that children at highest risk included those who underwent transplant during the period of rapid pubertal growth and those who received myeloablative conditioning and immunosuppression for treatment of GVHD.

Osteopenia (bone density 1 to 2.5 standard deviations below mean) or osteoporosis (bone density >2.5 standard deviations below mean) is commonly seen in survivors of hematologic malignancies.[69,97] Risk factors include therapy with corticosteroids, methotrexate (at higher doses), and cranial irradiation with resultant pituitary insufficiency or gonadal dysfunction. Survivors of HCT are also at increased risk for reduced bone mineral density; identified risk factors in these patients include treatment with corticosteroids for chronic GVHD, prior cranial irradiation (resulting in growth hormone deficiency), and gonadal failure.[86] Lifestyle factors that increase the risk for osteopenia include lack of regular weight-bearing exercise, inadequate calcium and vitamin D intake, smoking, and excessive alcohol consumption.[98]

Detection and diagnosis of musculoskeletal sequelae depend largely on anticipating these issues in vulnerable hosts, on taking a careful history, and on performing a thorough physical examination. Monitoring recommendations are reviewed in Table 101.1.[49,52]

COGNITIVE IMPAIRMENT

Cognitive impairment affects over 10 million cancer survivors in the US. Cognitive impairment can range from subtle to severe and last for months or years after treatment. Many survivors of hematologic malignancies also experience memory loss, distractibility, and difficulty performing multiple tasks. These patients may also concurrently suffer from mood disturbances and symptoms including fatigue and pain; these may compromise their ability to function adequately.[99] Decreased cognitive function is associated with poorer quality of life, inability to achieve work and educational goals, inability to drive or read, and decreased social connectedness (reviewed in Williams et al., 2016[100]). There is evidence to suggest that impaired working memory and executive functioning is prevalent in older patients with hematologic malignancies and may have an impact on survival.[101]

Many non-therapy-related factors can influence cognition in patients with a hematological malignancy. These include demographics (e.g., age and cognitive reserve) and comorbidities (physical comorbidities, as well as anxiety, depression, and fatigue). Cancer and its treatment may exacerbate normal aging and increase cognitive impairment in the cancer survivors.[102] A National Health and Nutrition Examination Survey (NHANES) analysis showed that survivors aged 60 years and older scored worse on a test of processing speed, attention, working memory, and executive function domains compared to non-cancer survivors.[103] Non-neurotoxic exposures, such as thoracic radiation, can adversely impact survivors' cognitive function through chronic conditions. Management of chronic diseases may mitigate neurocognitive outcomes among aging survivors of childhood cancer.[104] Cognitive impairment and its downstream effects become a major issue in survivors of hematological malignancies, given that many of these diagnoses are more common in older patients.

Cranial radiation has long been associated with cognitive impairment in childhood cancer survivors,[105–107] although antimetabolite chemotherapy and corticosteroids have also been implicated as potential contributors.[108–111] Additional risk factors include higher treatment intensity, younger age at treatment exposure, and female sex.[109,112–116] Cognitive deficits usually become evident within 1 to 2 years following cranial radiation and are progressive in nature. The decline over time is typically reflective of the child's failure to acquire new abilities or information at a rate similar to peers, rather than because of a progressive loss of skills and knowledge. The cognitive problems seen in childhood ALL survivors are generally characterized by reduced attention, processing speed, executive function and global intellectual function.[114] Affected children with information-processing deficits exhibit academic difficulties and are prone to problems with receptive and expressive language, attention span, and visual and perceptual motor skills. The deficits observed after chemotherapy alone are restricted to attention, executive function, and complex fine-motor functioning; global intellectual function is relatively preserved. Cognitive function in long-term survivors of childhood cancer appears particularly vulnerable to the effects of fatigue and sleep disruption.[117,118] The cognitive impairment observed in survivors of childhood ALL persists into adulthood; this impairment is associated with reduced educational attainment and unemployment,[119] and challenges with independent living, and health care use.[120]

A meta-analysis of existing evidence for the mechanisms underlying changes in the central nervous system (CNS) and cognitive function in ALL survivors after treatment compared with controls found that ALL survivors develop: (i) cognitive sequelae in intelligence, academics, attention, memory, processing speed, and executive function domains; (ii) decreased grey and white matter volume in cortical and several subcortical brain regions, with functional changes particularly in frontal regions and the hippocampus; (iii) cognitive impairments related to CNS changes; and (iv) reduction, but not resolution, of late cognitive sequelae in patients in whom prophylactic irradiation was replaced by systemic/intrathecal chemotherapy. Thus, ALL survivors treated with chemotherapy alone perform worse in processing speed, verbal selective reminding, and academics and display significant IQ deficits of 6 to 8 points compared with population norms but perform better than survivors treated with cranial RT on verbal selective reminding, processing speed, and memory span.[111,121]

A spectrum of neuropathological syndromes related to leukoencephalopathy may occur in survivors of childhood hematologic malignancies, including radionecrosis, necrotizing leukoencephalopathy, mineralizing microangiopathy and dystrophic calcification, cerebellar sclerosis, and spinal cord dysfunction, manifesting clinically as ataxia, spasticity, dysarthria, hemiparesis, or seizures. Leukoencephalopathy has been primarily associated with methotrexate-induced injury of white matter. However, cranial irradiation may play an additive role through disruption of the blood-brain barrier, allowing greater exposure of the brain to systemic therapy.[122] Acute leukoencephalopathy during chemotherapy treatment without cranial radiation for childhood ALL predicts higher risk for long-term neurobehavioral problems. Survivors of ALL may benefit from preventative cognitive and/or behavioral interventions, particularly those who develop acute leukoencephalopathy.[123]

Long-term survivors of HL may also be at risk for cognitive impairment. HL survivors followed for an average of 10 to 15 years from diagnosis show impaired cognitive functioning, with reduced attention, memory, processing speed, and executive function.[124] The cognitive impairment is associated with radiologic indices suggestive of reduced brain integrity and occurs in the presence of symptoms of cardiopulmonary dysfunction.[125]

NHL is a heterogeneous group of diseases with diverse treatments including radiation, immunotherapy, and chemotherapy. Adult survivors of childhood NHL experience impaired neurocognitive function, which is associated with lower social attainment and poor health-related quality of life (HRQOL).[126] Small studies of adult-onset B-cell NHL survivors find a higher prevalence of self-endorsed cognitive problems when compared with the general population. The domains affected include attention and executive function. The 18- to 59-year-olds endorse more problems than the 60- to 85-year-olds.[127,128] Treatment of primary CNS lymphoma with cranial radiation and high-dose methotrexate has resulted in median overall survivals that exceed 5 years.[129] Longitudinal studies in this population indicate that although there is some recovery over time, cognitive impairment does persist in the domains of attention, executive function, verbal fluency, memory, and psychomotor function 5 years or more after treatment.[130–132]

BMT survivors are at risk for cognitive impairment. Among adults undergoing BMT, myeloablative allogeneic BMT recipients showed significant cognitive decline compared with healthy controls. Reduced-intensity allogeneic BMT recipients showed evidence of delayed decline. Cognitive functioning in autologous BMT recipients is generally spared. Older age, male sex, and lower education, income, and cognitive reserve are associated with post-BMT cognitive impairment. At 3 years post-BMT, global cognitive impairment is present in 19% of autologous and 36% of allogeneic BMT recipients.[133] BMT recipients endorse more cognitive problems at all timepoints when compared with healthy controls. Fatigue is associated with greater endorsement of cognitive problems at 1 year after BMT. Overall, there is a modest correlation between self-endorsed cognitive problems and objective cognitive impairment. Higher self-endorsed cognitive problems are associated with higher odds of not returning to work at 3 years after BMT. These findings suggest that self-endorsed cognitive problems can identify vulnerable patient subpopulations in need of detailed cognitive assessment and possible cognitive remediation.[134]

There is emerging interest in attempting to understand whether interpatient variability in cognitive outcomes can be explained by polymorphisms in candidate genes conferring susceptibility to cognitive decline. Among childhood cancer survivors, inferior cognitive or behavioral outcomes have been associated with polymorphisms in genes related to oxidative stress (*NOS3*, *SLCO2A1*) and/or neuroinflammation (*COMT*).[135] Among BMT recipients, after adjusting for BMT type, age at BMT, sex, race/ethnicity, and cognitive reserve, SNPs in the blood-brain barrier, telomere homeostasis, and DNA repair genes are significantly associated with cognitive impairment. Inclusion of candidate genetic variants enhances the prediction of risk of post-BMT cognitive impairment beyond that offered by demographic/clinical characteristics and represents a step toward a personalized approach to managing patients at high risk for cognitive impairment after BMT.[136]

Monitoring recommendations for cognitive dysfunction are reviewed in Table 101.1.[49,52]

Psychosocial Effects

Treatment for hematologic malignancies may result in adverse psychosocial effects, including mental health disorders, such as anxiety, depression, and posttraumatic stress; psychosocial disability due to pain, sleep problems, and fatigue; relationship problems; fear of recurrence; and limitations in healthcare access (see Table 101.1). Survivors of HL reported more impairments in physical (particularly fatigue), professional, and cognitive domains, as well as more financial limitations, when compared with age-matched healthy controls.[124] In NHL survivors, treatment-related neurocognitive impairment (related to cranial radiation, intrathecal chemotherapy, and high-dose methotrexate and cytarabine), was found to be associated with lower educational attainment, occupational status, and unemployment.[126] In those with diffuse large B-cell lymphoma, younger survivors (18 to 59 years of age) were found to have more financial and social functioning problems compared with the normative population; however, in older survivors the proportion experiencing these problems was comparable to that reported by the normative population.[128] Survivors of primary CNS lymphoma treated with high-dose methotrexate and whole-brain radiation were more likely to report poorer self-perceived quality of life outcomes compared with survivors treated without radiation.[130]

Monitoring for psychosocial adverse effects of cancer treatment in survivors of hematologic malignancies should include a yearly clinical interview, with referral for psychologic or social work consultation if problems are identified.

OTHER TOXICITIES

Ocular Effects

Survivors of hematologic malignancies are at risk for the development of cataracts as a consequence of therapy with corticosteroids,[137] cranial irradiation,[138] TBI,[139] or busulfan.[140] In a cohort of childhood ALL survivors who were treated with chemotherapy with or without cranial radiation and received a mean cumulative prednisone equivalent dose of 5.2 gm/m^2, cumulative incidence of cataracts was 26 ± 8.1% at 25 years; cranial radiation was the only significant risk factor.[137] Belkacemi et al.[141] studied 1063 patients who underwent HCT for acute leukemia; the overall 10-year incidence of cataracts in this group of patients was 50%. Single-dose TBI was associated with a 60% incidence of cataracts, fractionated TBI was associated with a 43% incidence for those receiving six or fewer fractions, and a 7% incidence was reported for those receiving more than six fractions. Factors independently associated with an increased risk for cataract formation in this cohort were older age (>23 years), allogeneic bone marrow transplantation, higher dose rate (>0.04 Gy/min), and steroid administration for longer than 100 days. Xerophthalmia may also occur as a late complication because of decreased lacrimation resulting from damage to the lacrimal gland during radiation or, in HCT patients, from chronic GVHD.[142] Westeneng et al.[142] followed 101 adults up to 24 months after allogeneic HCT and reported ocular GVHD in 54% of patients, manifesting mainly as xerophthalmia and conjunctivitis; blepharitis and uveitis were encountered less often. Tabbara et al.[143] reported major ocular complications in 13% of 620 patients (age range: 9 to 65 years) after allogeneic HCT; complications included chronic ocular GVHD, corneal ulcers, cataracts, glaucoma, cytomegalovirus retinitis, fungal endophthalmitis, and the acquisition of allergic conjunctivitis from atopic donors.

Audiologic Effects

Survivors of hematologic malignancies who received platinum chemotherapy, those who had cranial irradiation at a young age (especially during infancy), and those who required supportive therapy with aminoglycoside antibiotics are at risk for therapy-related hearing loss.[144] Hearing loss associated with ototoxic agents is generally sensorineural in origin and is usually irreversible. Although a low incidence of hearing loss has been reported in survivors of HCT performed in childhood, the risk is elevated threefold to fourfold over that in the general population.[145] Data are beginning to emerge regarding genetic polymorphisms associated with increased susceptibility to platinum-related hearing loss; these data may prove useful in identifying future patients who are at increased risk for hearing loss as a consequence of platinum-based chemotherapy.[146]

Second and Subsequent Neoplasms

Second or subsequent neoplasms are defined as histologically distinct cancers developing after the occurrence of a first cancer. Subsequent neoplasms are conventionally categorized into two major types: therapy-related myelodysplastic syndrome/acute myeloid leukemia (t-MDS/AML) and solid tumors. The latency between diagnosis of the primary cancer and development of t-MDS/AML is generally short, whereas solid tumors have a longer latency, and the risk continues to rise for three or more decades. Using the World Health Organization classification, two types of t-MDS/AML are recognized, related closely to the therapeutic exposure: alkylating agent/radiation and topoisomerase II inhibitor.[147] The alkylating agent-related t-MDS/AML typically develops 4 to 7 years after exposure. Cytopenias are common, and ~65% of the patients present with myelodysplasia; the remaining present with AML but carry myelodysplastic features. Abnormalities involving chromosomes 5 (−5/del[5q]) and 7 (−7/del[7q]) are frequent. AML secondary to topoisomerase II inhibitors presents as overt leukemia, without a preceding myelodysplastic phase. The latency is brief, ranging from 6 months to 5 years, and is associated with balanced translocations involving chromosome bands 11q23 or 21q22. Solid tumors are related to the RT used to treat the primary cancer, typically have a long latency, and the risk is high among those exposed to irradiation at a young age.

Adult-onset cancer survivors diagnosed before 2000 have a less than twofold increased risk of subsequent neoplasms when compared with the general population (in particular among those diagnosed with their primary cancer at age <50 years).[148] Among individuals diagnosed with a primary cancer between 1992 and 2008 in the US, 8% to 11% develop a subsequent neoplasm.[149,150] The risk is highest among those diagnosed with their primary cancer at age below 50 years.[150] Cancer types sharing common risk factors, such as smoking or alcohol consumption (e.g., oropharynx, larynx, and lung), are the most common subsequent neoplasms. Over 50% of the patients with a subsequent neoplasm die of their second cancer.

The risk of subsequent neoplasms among childhood cancer survivors has been studied extensively. Childhood cancer survivors are at a 3-fold to 12-fold higher risk of subsequent neoplasms when compared with the general population.[151–156] Breast cancer is the most common subsequent neoplasm.[151] The risk of subsequent neoplasms remains elevated for more than 20 years from diagnosis of the primary cancer. Survivors are twice as likely as the general population to receive a diagnosis of subsequent neoplasm after age 40 years. The risk is increased for breast cancer, renal cancer, soft tissue sarcoma, and thyroid cancer in this age group.[157] Subsequent neoplasms are the leading cause of non-relapse late mortality.[158] Multiple subsequent neoplasms are common among aging survivors of childhood cancer. Cumulative incidence of a second subsequent neoplasm was reported as 47% at 20 years after the first subsequent neoplasm. A first subsequent neoplasm of a non-melanoma skin cancer identifies a population at high risk for a subsequent invasive neoplasm.[159]

Treatment with RT and specific chemotherapeutic agents are associated with a higher risk of subsequent neoplasms; female sex, older age at diagnosis, and earlier treatment era modify that association.[151] There is a dose-dependent increased risk for solid tumors after RT,[160] with the highest risks for tumors occurring in or near the treatment field. In addition to the well-known association with t-MDS/AML, alkylating agents and anthracycline chemotherapies also increase the risk of solid tumors.[161] Alkylating agents are associated with an increased risk for GI, thyroid, lung, breast, and bladder cancer, as well as sarcoma. Anthracyclines are associated with an increased risk for breast cancer. An evaluation of the association between temporal treatment dosing changes and subsequent neoplasm risk among childhood cancer survivors reveals that the risk of subsequent neoplasms at 15 years after initial cancer diagnosis remains increased for those diagnosed in the 1990s, although the risk is lower compared with those diagnosed in the 1970s. This lower risk of subsequent neoplasms is associated with reduction in therapeutic radiation dose.[162]

BMT performed across the age spectrum is associated with a 1.7-fold higher risk of subsequent neoplasms when compared with the general population.[163] The most common subsequent neoplasms include head and neck cancers, melanoma, non-melanoma skin cancers (both basal cell and squamous cell), bone tumors, breast and thyroid cancer, and lung cancer. Several host and clinical factors are associated with an increased risk for subsequent neoplasms after BMT, such as age at BMT, pre-BMT exposure to chemotherapy and radiation, exposure to TBI as part of conditioning, infection with oncogenic viruses (Epstein-Barr virus and hepatitis B and C viruses), and chronic GVHD.

Subsequent Malignancies Among Survivors of Acute Lymphoblastic Leukemia

Evaluation of large cohorts of patients with childhood ALL treated on therapeutic trials has shown that the cumulative incidence of subsequent neoplasms approaches 4% at 15 years from diagnosis of ALL.[164–168] Radiation-related subsequent neoplasms observed in ALL survivors include high-grade gliomas (glioblastomas and malignant astrocytomas), peripheral neuroectodermal tumors, ependymomas and meningiomas, thyroid cancer, and non-melanoma skin cancers.[167–169] The cumulative incidence of t-MDS/AML after therapy for ALL is generally low (<2%), except among those patients treated with epipodophyllotoxin therapy, where a cumulative incidence of 3.8% at 6 years has been reported.[170,171]

Subsequent Malignancies in Hodgkin Lymphoma Survivors

Childhood HL survivors demonstrate the highest risk of subsequent neoplasms; the risk ranges from 8.7-fold[151] to 14-fold[172] higher than the general population. The cumulative incidence of subsequent neoplasms among HL survivors ranges from 8% at 20 years to 18% at 30 years.[170,173,174] With follow-up extending to 40 years from HL diagnosis, the cumulative incidence of any solid subsequent neoplasm exceeds 25%.[172] Extended follow-up of early cohorts finds an excess risk for lung and gastrointestinal cancers, breast cancer, thyroid cancer, and bone/soft tissue sarcomas.[175–180] Risk factors for breast cancer among females includes receipt of chest RT between ages 10 years and 16 years and males treated with chest RT at age below 10 years are at highest risk of developing lung cancer. HL survivors who were treated with abdominal/pelvic RT and high-dose alkylating agents are at highest risk of developing colorectal cancer and females exposed to neck RT at age below 10 years are at highest risk of thyroid cancer. Among HL survivors at highest risk, the cumulative incidence of breast, thyroid, colorectal, and lung cancer is 45%, 17%, 9%, and 4%, respectively by age 50 years.[172]

The risk of subsequent neoplasms in survivors of adult-onset HL ranges from 2.4-fold[181] to 4.6-fold[182] when compared to the general population. The risk remains elevated 35 years or more after treatment. The 30-year cumulative incidence of breast cancer in women diagnosed with HL at age below 35 years is 13.8%.[181] The risk of any subsequent neoplasms is 1.3-fold higher for HL survivors with a first-degree relative with cancer; the corresponding excess risk is 3.3-fold, 2.1-fold, and 1.8-fold for lung, colorectal, and breast cancers, respectively.[181] A cumulative procarbazine dose of 4.3 g or more per square meter of body-surface area is associated with a significantly lower risk of breast cancer but a higher risk of gastrointestinal cancer.[182]

Early studies of HL identified the increased risk for t-MDS/AML among patients treated with MOPP-based therapy, which included mechlorethamine and cyclophosphamide.[183,184] These alkylating agent-associated t-MDS/AMLs are characterized by the presence of chromosomal abnormalities involving chromosomes 5 and/or 7, and are often preceded by myelodysplasia; the risk extends for a period of 10 to 15 years after exposure.

Subsequent Malignancies in Multiple Myeloma Survivors

The overall risk of any subsequent neoplasm in patients with MM ranges from 0.98- to 1.26-fold when compared with the general population.[185,186] The risk for t-MDS/AML is higher than that for the solid tumors, although some solid tumors are observed at a higher risk than the general population; these include gastrointestinal cancers, kidney cancer, bladder cancer, and melanoma and non-melanoma skin cancers. Population-based studies inclusive of patients treated prior to the widespread use of immunomodulatory agents (IMiDs) and proteasome inhibitors (PIs) find a 6.5-fold to 11.5-fold higher risk for t-MDS/AML when compared with the general population.[187–193] Prolonged alkylator therapy and conditioning with melphalan are associated with an increased risk of t-MDS/AML. There is a reduction in the incidence of t-MDS/AML noted as the use of prolonged upfront alkylating agent declines. The outcome after a diagnosis of t-MDS/AML is dismal, with a median overall survival between 8 and 14 months.[194–196] A modest increase in risk of subsequent neoplasms is observed in patients treated with lenalidomide. The risk is predominantly observed in patients who were conditioned with melphalan for an autologous BMT.[197]

Subsequent Malignancies in Non-Hodgkin Lymphoma Survivors

Subsequent neoplasms have been increasingly reported in the post-rituximab era in patients diagnosed with diffuse large B-cell lymphoma. The risk of subsequent neoplasms of t-MDS/AML has nearly doubled in the post-rituximab era (8.7-fold vs. 4.4-fold). Second thyroid cancer risk has also increased (2.3-fold vs. 0.7-fold). However, these findings are based on registry data, and there is a lack of therapeutic data at the patient level.[198]

Subsequent Breast Cancer

The incidence of breast cancer among young women treated for childhood cancer with chest RT ranges from 5% to 14% by age 40 years.[175,178,199–201] The latency after chest RT ranges from 7 years to 10 years, and the risk of breast cancer increases in a linear fashion with radiation dose, with an estimated relative risk of 6.4 at a dose of 20 Gy and 11.8 at a dose of 40 Gy.[202,203] Moreover, 40% of identified cases develop contralateral disease. The highest risk group is survivors of childhood HL treated with high doses of RT to an extended mantle field.[175,204,205] However, HL survivors treated with contemporary therapy using reduced volumes and lower doses continue to demonstrate an increased risk.[204,206] Women treated for other childhood cancers with high-dose RT to breast tissue also have an elevated risk.[151,178,199,201] Further, women treated for childhood cancer with whole lung irradiation have a greater risk of breast cancer than previously recognized, demonstrating the importance of radiation volume.[207] Finally, there is an increased risk of breast cancer in childhood sarcoma or leukemia survivors not exposed to chest radiation; in these patients, alkylators and anthracyclines are associated with increased risk of breast cancer.[208] A combination of radiation and anthracyclines may further increase breast cancer risk.[177]

The 25-year cumulative incidence of breast cancer is 11% after allogeneic BMT.[209] Allogeneic BMT survivors are at a 2.2-fold increased risk for developing breast cancer when compared with age and sex-matched general population. The median latency from BMT to diagnosis of breast cancer is 12.5 years. The incidence is higher among those exposed to TBI (17%) than among those who did not receive TBI (3%). The risk is higher among those exposed to TBI at a younger age.

There appears to be a protective effect of early menopause either because of alkylating agents or radiation dose above 5 Gy to the ovaries, suggesting that ovarian hormones play an important role in promoting tumorigenesis once an initiating event has been produced by radiation.[203,210,211] Breast cancer risk is 2.9-fold higher for survivors with $\geq$10 years of ovarian function after chest RT *vs.* less than 10 years and 1.8-fold higher for RT given within 1 year of menarche *vs* greater than 1 year from menarche. Risk decreases with decreasing age at menopause ($P_{trend} = 0.01$). Survivors with an age at menopause less than 20 years treated with hormone therapy had a lower breast cancer risk than premenopausal survivors. Over half (52%) of the radiation-related breast cancers are estrogen-receptor positive (ER^+); risk factors do not differ for ER^+ breast cancer. These findings suggest that endogenous hormones are key contributors to breast cancer observed among childhood cancer survivors. Hormone therapy given for premature ovarian insufficiency does not fully replace the function that endogenous hormones have in breast cancer development.[212]

Estimates of cumulative absolute risk for use in counseling patients have been developed.[205] For example, the cumulative absolute risks for an HL survivor who was treated at age 25 years with a chest radiation dose of 40 Gy or more without alkylating agents are estimated to be 1.4% after 10 years, 11% after 20 years, and 29% after 30 years.

Breast cancer-specific mortality at 5 years and 10 years is 12% and 19%, respectively.[207] Overall 10-year survival after breast cancer is 73%. Subsequent risk of all-cause mortality is 2.4-fold higher among childhood cancer survivors than among controls after adjusting for breast cancer treatment. While breast cancer-specific mortality is modestly elevated among childhood cancer survivors (HR, 1.3; 95% CI, 0.9 to 2.0), survivors are five times more likely to die as a result of other health-related causes, including other subsequent neoplasms and cardiovascular or pulmonary disease. The cumulative incidence of a second asynchronous breast cancer is also elevated significantly compared with controls ($P < .001$).[213]

Subsequent Thyroid Cancer

Thyroid cancer is observed after neck radiation for HL, ALL, and brain tumors, and after TBI for BMT,[151,164,167,170,214] and typically develops 10 years or more after RT exposure. The risk of thyroid cancer is 18-fold that of the general population.[215] RT at a young age is a major risk factor for the development of thyroid cancer. A linear dose-response relationship between thyroid cancer and radiation is observed up to 20 Gy, with a decline in the risk at higher doses, demonstrating evidence for a cell kill effect.[216,217] Female sex, younger age at RT exposure, and longer time since exposure are significant modifiers of the radiation-related risk of thyroid cancer.[218] Thyroid cancer develops after a latency of 8.5 years and is associated with an excellent outcome.

The risk of subsequent thyroid cancer is 1.2-fold to 3.5-fold higher among adult-onset cancer survivors when compared with the general population. The risk is highest after primary pancreatic cancer, soft tissue sarcoma, melanoma, chronic lymphocytic leukemia, diffuse large B-cell lymphoma, laryngeal cancer, kidney cancer, and central nervous system tumors.[219] However, use of registry data has precluded examination of association with treatment exposures or identification of high-risk groups.

Therapy-Related Myelodysplasia/Acute Myeloid Leukemia

Several studies have described an increased risk for t-MDS/AML after autologous BMT, particularly for HL or NHL. The cumulative probability of t-MDS/AML ranges from 1% at 30 months to 11.7% at 6 years after autologous BMT, with a median latency of 12 to 24 months after BMT (range: 4 months to 6 years).[220] The risk for t-MDS/AML usually does not extend beyond the first 10 to 15 years after therapeutic exposure. t-MDS/AML is the major cause of nonrelapse mortality in patients undergoing autologous BMT for HL or NHL.[221,222] t-MDS/AML after autologous BMT is the result of cumulative toxicity resulting from pre-transplantation therapeutic exposures (alkylating agents, topoisomerase II inhibitors, and radiation), stem cell mobilization and manipulation (exposure to etoposide for mobilization, and difficult stem cell harvests yielding smaller number of $CD34^+$ cells), conditioning with TBI, and multiple transplants.[221,223] The diagnosis of t-MDS/AML after autologous BMT confers a uniformly poor prognosis, with a median survival of 6 months in patients treated with conventional chemotherapy.

Therapy-related AML associated with topoisomerase II inhibitors is characterized typically by a shorter latency period (3 to 5 years from therapeutic exposure) than that seen for t-MDS/AML after alkylating agents, lack of a myelodysplastic phase, and the presence of 11q23 rearrangements with mutations in the mixed-lineage leukemia (*MLL*) gene. Epipodophyllotoxin-associated secondary AML depends more on the schedule of drug administration than total cumulative dose.[224]

Molecular Basis for Subsequent Neoplasms

Mutations in high-penetrance genes (e.g., Li-Fraumeni syndrome, *RB* [retinoblastoma], *NF1* [neurofibromatosis], *PTCH1* [Gorlin or nevoid basal cell carcinoma syndrome], *WT1* [Wilms' tumor] and *ATM* [ataxia telangiectasia]) could possibly modify the association between therapeutic exposures and subsequent neoplasms. Many genes associated with familial cancer syndromes are responsible for mediating cellular response to DNA damage (e.g., *ATM, BRCA*) induced by genotoxic insults such as radiation and chemotherapy. Cancer survivors who carry a deleterious high-penetrance mutation are likely to be at increased risk for subsequent neoplasms. However, the low frequency of these mutations in the general population suggests the attributable risk to the development of subsequent neoplasms is likely very small. The inter-individual variability in risk of subsequent neoplasms is more likely related to common polymorphisms in low-penetrance genes that regulate drug metabolism/disposition, or those responsible for DNA repair. Between 20% and 95% of the variability in cytotoxic drug disposition can possibly be explained by genetic variation,[225] and polymorphisms in genes involved in drug metabolism/disposition contribute to disease-free survival and drug toxicity.[226] Variation in DNA repair could possibly modify subsequent neoplasm risk among cancer patients exposed to DNA-damaging agents, such as radiation and chemotherapy. Finally, it is conceivable that gene-environment (therapeutic exposure) interactions could magnify functional impact of the polymorphisms.[227] There is some evidence that hematopoietic stem progenitor cells (HSPC) carrying age-related *TP53* mutations are resistant to chemotherapy and expand preferentially after treatment. The early acquisition of *TP53* mutations in the founding HSPC clone probably contributes to the frequent cytogenetic abnormalities and poor responses to chemotherapy that are typical of patients with t-AML/t-MDS.[228] Clonal hematopoiesis of indeterminate potential (CHIP) is an age-related condition characterized by somatic mutations in the blood of otherwise healthy adults. In patients undergoing autologous BMT for HL or NHL, CHIP at the time of transplantation is associated with increased risk of t-MDS/AML.[229]

Recommendations for screening and follow-up of survivors at risk for subsequent malignant neoplasms are reviewed in Table 101.1.[49,52,230,231]

Late Mortality

Late mortality has been described in detail for childhood cancer survivors and for survivors of adult-onset hematologic

il

malignancies treated with BMT. Childhood cancer survivors remain at risk for late mortality, and the excess mortality persists long after diagnosis.[158,232,233] All-cause mortality among survivors is eightfold that of the general population. Deaths are primarily due to recurrence/persistence of primary cancer or due to treatment-related complications, such as subsequent neoplasms, and cardiac or pulmonary causes. Extended follow-up of these cohorts demonstrates changing patterns of cancer-specific mortality with increasing time from diagnosis.[232,234] Thus excess risk for deaths from recurrence declines with time, while the excess risk for deaths from subsequent neoplasms and cardiovascular causes continues to increase; subsequent to 45 years from diagnosis, recurrence accounts for only 7% of the excess number of deaths while subsequent neoplasms and cardiovascular causes accounted for 51% and 26% of the excess number of deaths respectively (20628130). Childhood cancer survivors are at a 15-fold increased risk of subsequent neoplasm-related deaths, 9-fold increased risk of pulmonary deaths, and 7-fold increased risk of death due to cardiac causes when compared with the general population.[158] A recent study finds that the strategy of lowering therapeutic exposure over the past three decades has successfully translated to an observed decline in late mortality among 5-year survivors of childhood cancer.[235]

The high burden of morbidity carried by BMT recipients can result in late mortality. Mortality rates are fourfold to ninefold higher than in the expected population for at least 30 years after BMT, producing an estimated lower life expectancy of 30% compared with the general population, regardless of current age.[236] Relapse of primary disease is the main cause of death. Among BMT recipients, non-relapse-related mortality is greater among patients who are over 18 years of age when undergoing BMT, as well as among those with chronic GVHD. Compared with the general population, allogeneic BMT recipients are 3.6 times more likely to die of subsequent neoplasms, 15 times more likely to die of pulmonary complications, and 2 times more likely to die of a cardiac compromise. However, the conditional survival probability at 10 to 20 years after allogeneic BMT exceeds 80% for those individuals who were alive and disease free 2 to 5 years after BMT.[237,238] Autologous BMT recipients demonstrate a conditional 5-year survival that approaches 75% among those who have survived 2 years or more and approaches 90% among those who have survived 10 years after BMT. Among those who survive the first 10 years, non-relapse-related mortality exceeds relapse-related mortality.[239] (See box on Evaluating Survivors for Potential Late Effects.)

EVALUATING SURVIVORS FOR POTENTIAL LATE EFFECTS

To diminish the incidence and severity of untoward late effects and to improve the quality of survival for patients with hematopoietic malignancies, systematic evaluation of outcomes with subsequent modification of current and future therapies is required. To decrease late morbidity and mortality rates and to meet the specialized healthcare needs of this group of patients, ongoing comprehensive follow-up care with attention to early detection and intervention for late effects are essential.

Patients are generally eligible to enter formal long-term follow-up care when the risk for relapse of their primary disease is minimal. For most hematologic malignancies, this occurs when a patient is at least 2 years off therapy. When a patient enters long-term follow-up, the focus of care shifts from vigilant surveillance for disease recurrence to a survivorship model of health maintenance or promotion and management of treatment-related late effects. Effective management of these late effects requires ongoing surveillance, early intervention, and when possible, prevention.

The long-term complications of treatment for which an individual survivor is at risk are determined by several factors, including the patient's diagnosis, age at treatment, specific chemotherapeutic agents received (including cumulative doses), specific radiation fields and doses, therapy-related complications, degree of psychosocial support received, genetic predisposition, and current health-related behaviors (e.g., diet, physical activity, tobacco, and alcohol use).

POTENTIAL LATE EFFECTS BY DIAGNOSIS

Therapeutic approaches to hematologic malignancies vary widely depending on the patient's age at diagnosis, biologic subtype and staging of disease, year (era) of diagnosis, initial response to therapy, and physician/institutional preference. Even though two patients may share an identical diagnosis, their risks for late effects may differ significantly because of differences in therapy, age at exposure to therapy, or pharmacogenetics. General associations of late effects with conventional treatment for common hematologic malignancies are reviewed in Tables 101.2–101.4. The risk for specific late effects in survivors who have undergone HCT depends on the conditioning regimen, donor source, complications experienced during the transplantation process, presence or absence of GVHD, and prior cancer therapy. Transplantation-related sequelae have been reviewed throughout the text of this chapter.

Acute Lymphoblastic Leukemia

ALL is a heterogeneous disease. Therapy for ALL ranges from a relatively innocuous, antimetabolite-based approach for low-risk childhood ALL[240] to marrow-ablative therapy followed by HCT for very high-risk disease in all age groups.[241] The risk for long-term complications for individual survivors varies widely and is dependent on the specific therapy received as well as the patient's age at time of treatment.[242,243] Potential late effects that may occur as a consequence of conventional therapy for ALL are listed in Table 101.2.

TABLE 101.2 **Late Effects Associated With Conventional Therapy for Acute Lymphoblastic Leukemia and Non-Hodgkin Lymphoma**

Common Therapeutic Exposures	Potential Late Effects
Vincristine	Peripheral neuropathy, Raynaud phenomenon
Corticosteroids	Cataracts, osteopenia, osteoporosis, avascular necrosis
Asparaginase	No known late effects
Mercaptopurine	Hepatic dysfunction (rare)
Thioguanine	Portal hypertension, hepatotoxicity (when used continuously in maintenance therapy)
Methotrexate (systemic)	Osteopenia, osteoporosis, osteonecrosis, hepatic dysfunction (rare)
Methotrexate (intrathecal, high dose)	Neurocognitive deficits, clinical leukoencephalopathy
Cytarabine (high dose)	Neurocognitive deficits
Cranial or craniospinal irradiation	Neurocognitive deficits, clinical leukoencephalopathy, cataracts, hypothyroidism, second malignant neoplasm in radiation field (e.g., skin, thyroid, brain), short stature, scoliosis or kyphosis, obesity
Anthracyclines	Cardiomyopathy, arrhythmias, subclinical left ventricular dysfunction, secondary AML
Cyclophosphamide	Hypogonadism, hemorrhagic cystitis, dysfunctional voiding, bladder malignancy, secondary AML or MDS
Blood products	Chronic viral hepatitis, HIV

AML, Acute myeloid leukemia; *HIV*, human immunodeficiency virus; *MDS*, myelodysplastic syndrome.

Acute Myeloid Leukemia

Therapy for AML is generally more intense and of shorter duration than that used for treatment of ALL. Higher doses of anthracycline chemotherapy are often employed, as is consolidation therapy with HCT.[243] Typically patients with AML receive less CNS-directed therapy than those with ALL. Examples of potential late effects associated with conventional therapy for AML are listed in Table 101.3.

Hodgkin Lymphoma

Therapy for HL relies on the use of alkylating agents, antitumor antibiotics (including anthracyclines and bleomycin), corticosteroids, and RT. Some patients may be asplenic as a consequence of the staging procedures performed in the earlier era.[244] Examples of potential late effects associated with conventional therapy for HL are listed in Table 101.4.

TABLE 101.3 Late Effects Associated With Conventional Therapy for Acute Myeloid Leukemia

Common Therapeutic Exposures	Potential Late Effects
Anthracyclines	Cardiomyopathy, arrhythmias, subclinical left ventricular dysfunction, secondary acute myeloid leukemia
Corticosteroids	Cataracts, osteopenia, osteoporosis, avascular necrosis
Asparaginase	No known late effects
Cytarabine (high dose)	Neurocognitive deficits
Blood products	Chronic viral hepatitis, human immunodeficiency virus infection

TABLE 101.4 Late Effects Associated With Conventional Therapy for Hodgkin Lymphoma

Common Therapeutic Exposures	Potential Late Effects
Anthracyclines	Cardiomyopathy, arrhythmias, subclinical left ventricular dysfunction, secondary AML or MDS
Corticosteroids	Cataracts, osteopenia, osteoporosis, avascular necrosis
Bleomycin	Pulmonary dysfunction
Vincristine, vinblastine	Peripheral neuropathy, Raynaud phenomenon
Procarbazine, mechlorethamine, dacarbazine	Hypogonadism, infertility, secondary AML or MDS
Cyclophosphamide	Hypogonadism, infertility, hemorrhagic cystitis, dysfunctional voiding, bladder malignancy, secondary AML or MDS
Mantle irradiation	Hypothyroidism, premature cardiovascular disease, cardiac valvular disease, cardiomyopathy, arrhythmias, carotid artery disease, scoliosis or kyphosis, second malignant neoplasm in radiation field (e.g., thyroid, breast), pulmonary dysfunction
Inverted Y irradiation	Hypogonadism, infertility, adverse pregnancy outcome, second malignant neoplasm in radiation field (e.g., gastrointestinal)
Splenectomy	Acute life-threatening infections
Blood products	Chronic viral hepatitis, HIV

AML, Acute myeloid leukemia; *HIV*, human immunodeficiency virus; *MDS*, myelodysplastic syndrome.

Non-Hodgkin Lymphoma

The potential late effects after therapy for NHL are therapy specific and similar to those experienced by survivors of ALL (see Table 101.2).[244] Those patients whose treatment included HCT are also at risk for transplantation-related sequelae.

Chronic Myeloid Leukemia

Long-term survivors of CML have usually either undergone HCT or are receiving tyrosine kinase inhibitors long term. Among HCT recipients, there is a significant risk for late effects as a result of the transplantation-conditioning regimen, as well as treatment for and sequelae of GVHD.[245] With the transition in the early 2000s to tyrosine kinase inhibitor therapy for CML as the predominant treatment modality, the long-term effects of these agents are beginning to emerge in the growing cohort of CML survivors (see box on Late Effects Research: What Is Needed and the section Providing Clinical Care to Survivors).[246]

PROVIDING CLINICAL CARE TO SURVIVORS

A summary of cancer treatment and a survivorship care plan are critical components of care provision for survivors of hematologic

LATE EFFECTS RESEARCH: WHAT IS NEEDED

Medical Issues Faced by This Population

- Premature death.
- Second malignancies.
- Organ dysfunction (e.g., cardiac, pulmonary, gonadal).
- Impaired growth and development.
- Decreased fertility.
- Neurocognitive impairment.
- Difficulties obtaining employment and insurance.
- Overall reduced quality of life.

Issues to Be Considered by Physicians Providing Care to This Population

- Providing long-term follow-up care for cancer survivors.
- Models of care delivery.
- Guidelines for ongoing screening and management.

Major Clinical and Research Challenges

- Cancer survivorship research continually changing because of new:
- Therapeutic agents or combinations of agents (including targeted therapies).
- Radiation oncology techniques.
- Surgical procedures.
- Supportive care techniques.

Future Directions

Research is needed to:

- More clearly define survivors at greatest risk for specific outcomes.
- Identify genetic predispositions to certain key outcomes and the role of gene–environment interactions.
- Identify the role of lifestyle choices (e.g., alcohol, tobacco, diet, exercise) in modification of risks for late adverse outcomes.
- Understand the potential long-term impact of cancer therapy in order to effectively counsel survivors.
- Develop effective intervention strategies to prevent or minimize the impact of adverse late effects.
- Develop scientifically valid, evidence-based recommendations for clinical follow-up of survivors, which should include screening for potential late effects and application of proven approaches for health promotion.
- Much of the current information available relates to outcomes within the first decade after treatment, and only minimal data address the longer-term outcomes that may subsequently occur. Large sample sizes within the context of well-characterized cohorts with complete long-term follow-up remain the greatest challenge to sound survivorship research.

SURVIVORSHIP CARE PLANS

Survivorship care plans have been identified by the Institute of Medicine[248,249] as an important tool to facilitate follow-up care. Survivorship care plans should include both a summary of cancer treatment and a follow-up plan that can be used to enhance communication between care providers, coordinate care, and encourage health monitoring and promotion.[250,251] Specific elements of the survivorship care plan should include:

- Diagnosis, including histologic subtype and stage if relevant.
- Contact information for all cancer care providers and institutions where care was received.
- Surgical procedure(s) with dates.
- Chemo/biotherapy drugs received and completion dates for each.
- Radiation treatment dates with anatomical area(s) treated.
- Ongoing toxicities or treatment side effects.
- Genetic/hereditary risk factors or predisposing conditions, if relevant, including genetic testing results if performed.
- List of potential late and/or long-term effects.
- List of possible symptoms of cancer recurrence and surveillance testing schedule.
- Follow-up visit schedule, including who will provide care, and schedule of recommended screening/testing as indicated.
- Recommendations regarding general health maintenance/promotion.
- Information regarding common survivorship issues (e.g., emotional, financial, work/employment).

malignancies (see box on Survivorship Care Plans). Guidelines for long-term follow-up of survivors of hematologic malignancies and those who underwent HCT in childhood, adolescence, or young adulthood have been developed by the Children's Oncology Group[49] and are available at http://www.survivorshipguidelines.org; HCT long-term follow-up guidelines have also been developed by an international group of transplant experts.[52] In order to provide risk-based guideline-directed follow-up care, a comprehensive treatment summary is needed (Table 101.5) and a copy should be given to each survivor with instructions to share this information with all healthcare providers. Survivors should undergo annual comprehensive, multidisciplinary health evaluations with special attention to the detection of potential late effects specific to the patient's diagnosis and treatment history (see Table 101.1). Because certain late effects have prolonged asymptomatic intervals before becoming clinically evident (e.g., late-onset CHF as a result of anthracycline-induced cardiomyopathy), ongoing evaluation is important to identify and provide early intervention for these potential complications. Health education regarding potential health risks and risk-reduction measures should be provided to each survivor. Targeted health education materials related to potential late complications of therapy during childhood, adolescence, or young adulthood have been developed by the Children's Oncology Group[247] and are available at http://www.survivorshipguidelines.org. To optimize future follow-up care for all survivors, patients should be invited to participate in any relevant research studies for which they are eligible (see box on Late Effects Research: What Is Needed).

FUTURE DIRECTIONS

Cancer outcomes research enables researchers to study testable hypotheses and determine the effects of host- and therapy-related factors in the development of adverse outcomes ranging from carcinogenesis and organ dysfunction to psychosocial consequences. Opportunities also exist to explore gene and environment interactions that may modify susceptibility to develop adverse outcomes, thus providing insights into the identification of high-risk populations.

Better understanding of treatment-related toxicity has not only guided the design of less toxic therapies, but also the development of treatment summaries,[49] survivorship plans,[251] and efforts to harmonize survivorship guidelines worldwide.[252] It is important that

TABLE 101.5 Comprehensive Treatment Summary

Topic	Specific Information to Include
Demographics	Name
	Record number or patient identification number
	Date of birth
	Sex
	Race or ethnicity
Diagnosis	Date or age at diagnosis
	Referring physician or institution
	Treating physician or institution
	Presenting symptoms
	Past medical history
	Family history (including cancer in first- or second-degree relatives)
	Physical examination findings at presentation
	Initial diagnostics (complete blood cell count, chemistry panel, radiographic studies)
	Diagnostic procedures (biopsies, cytologic studies)
	Pathology (morphology, histology, cytochemistry, flow cytometry)
	Cytogenetics
	Central nervous system status (if applicable)
	Stage (if applicable)
	Metastatic sites (if applicable)
	Initial response to therapy (e.g., RER, SER, date first complete remission achieved)
	Relapse(s) dates, age at relapse(s), relapse site(s)
Treatment	Date of initial treatment (initiated and completed)
	Date(s) for treatment of relapse (initiated and completed)
	Final off-therapy date
	Chemotherapy agents received, including route of administration (list all)
	Cumulative doses (in mg/m^2) and age at treatment for all alkylators, anthracyclines, and heavy metals
	Dose ranges for cytarabine and methotrexate (e.g., standard dose vs. high dose $>1000\,mg/m^2$)
	Radiation fields, doses, shielding, age at treatment
	Surgical procedure(s)
	Transfusion(s), including all blood or serum products
	Stem cell transplantation(s), including donor source, preparative regimen, GVHD prophylaxis or treatment
	Other therapeutic modalities (e.g., immunotherapy, CAR-T)
Acute complications	Significant therapy-related complications (e.g., tumor lysis, septic shock, typhlitis, acute GVHD)
	Significant treatment required for complications (e.g., hemodialysis, amphotericin, aminoglycosides)
Complications after therapy	Significant complications after completion of therapy (e.g., herpes zoster, acute life-threatening infection after splenectomy)

CAR-T, Chimeric antigen receptor T-cell therapy; *GVHD*, graft-versus-host disease; *RER*, rapid early response; *SER*, slow early response.
Modified from Children's Oncology Group Summary of Cancer Treatment, 2018. http://www.survivorshipguidelines.org.

we continue to follow contemporary cohorts of cancer survivors to determine how therapy modifications affect the prevalence and spectrum of late effects. It is important to apply known interventions that can reduce the impact of cancer and treatment-related late effects on quality of life, morbidity, and mortality, as well as to develop and test new interventions. For example, survivors of HL treated with chest radiation have an increased risk of developing lung cancer, and tobacco use increases this risk 20-fold. Successful smoking prevention and cessation strategies among survivors can decrease the risk of this prevalent and highly morbid cancer of adulthood, while also decreasing the development and progression of atherosclerosis and other subsequent malignant neoplasms (SMNs). Screening of young women at increased risk of breast cancer due to chest radiation has already been mentioned. Given the role of endogenous estrogen in radiation-related breast cancer risk, it is important to study whether chemoprevention using anti-estrogen agents would reduce the risk of breast cancer. Survivors of childhood cancer, particularly those who received radiation to the hypothalamic–pituitary axis, are also at risk of obesity. Obesity can exacerbate the increased risks of CVD associated with anthracycline chemotherapy and RT to the chest. Modifiable CVRFs, such as hypertension, can potentiate the risk of major cardiac events and both behavioral and medical interventions can potentially reduce these risks. However, interventions need to be targeted to those at highest risk for developing these complications. There is therefore a need to develop risk prediction models that integrate the clinical/demographic profile of the patients with their genetic profile such as those developed to identify BMT survivors at risk for cognitive outcomes.[136]

The growing population of cancer survivors carries a significant burden of morbidity, necessitating comprehensive long-term follow-up of these survivors.[253] This follow-up should ideally begin at the completion of active therapy, with a documented summarization of therapeutic exposures and recommendations for follow-up, thus ensuring standardization of care received by the survivors.[248,249,251] However, many barriers prevent effective follow-up, the most fundamental being the lack of knowledge regarding survivorship issues demonstrated by both the long-term survivors and the primary care physicians caring for them. Shortcomings of the healthcare system also pose potential barriers and include logistical issues such as a lack of capacity within centers, training and educational deficiencies, inadequate communication between pediatric oncologists, and primary care physicians that subsequently provide the large bulk of follow-up.

Despite these unique opportunities, conduct of survivorship research faces several challenges. Cancer survivorship research is expected to evolve during the next several years as better treatment options, new agents, and combinations of agents are developed for the cancer patients who do not now survive. Targeted therapies (e.g., bispecific T-cell engager [BiTE] antibodies, chimeric antigen receptor T-cell therapy [CAR-T], gemtuzumab ozogamicin)[246] will contribute to increased survivorship. Evaluation of their late effects will need to keep in step with their increased usage. Refinements in RT and minimally invasive surgeries are intended to minimize late effects. Furthermore, the influence of genetic profiles on susceptibility to late effects, as well as their interaction with lifestyle exposures such as tobacco, alcohol, and diet, has not been fully explored. However, the multifactorial etiology of the adverse effects, coupled with the heterogeneous nature of the patient population, necessitates large sample sizes within the context of well-characterized cohorts with complete long-term follow-up, and this remains the greatest challenge to sound survivorship research.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Baker KS, Ness KK, Weisdorf D, et al. Late effects in survivors of acute leukemia treated with hematopoietic cell transplantation: a report from the Bone Marrow Transplant Survivor Study. *Leukemia*. 2010;24:2039.

Bhatia S. Caring for the long-term survivor after allogeneic stem cell transplantation. *Hematology Am Soc Hematol Educ Program*. 2014;2014(1):495–503.

Chemaitilly W, Cohen LE, Mostoufi-Moab S, et al. Endocrine late effects in childhood cancer survivors. *J Clin Oncol*. 2018;36(21):2153–2159.

Children's Oncology Group http://www.survivorshipguidelines.org. *Children's Oncology Group Long-Term Follow-Up Guidelines for Survivors of Childhood, Adolescent, and Young Adult Cancers, Version 5.0*. Monrovia, CA: Children's Oncology Group; 2018.

Chow EJ, Antal Z, Constine LS, et al. New agents, emerging late effects, and the development of precision survivorship. *J Clin Oncol*. 2018;36(21):2231–2240.

de Moor JS, Mariotto AB, Parry C, et al. Cancer survivors in the United States: prevalence across the survivorship trajectory and implications for care. *Cancer Epidemiol Biomarkers Prev*. 2013;22:561–570.

Guy Jr. GP, Ekwueme DU, Yabroff KR, et al. Economic burden of cancer survivorship among adults in the United States. *J Clin Oncol*. 2013;31(30):3749–3757.

Hewitt ME, Greenfield DM, Stovall E, eds. *From Cancer Patient to Cancer Survivor: Lost in Transition*. Washington, DC: The National Academies Press; 2006.

Hewitt ME, Weiner SL, Simone JV, eds. *Childhood cancer survivorship: improving care and quality of life*. Washington, DC: The National Academies Press; 2003.

Hudson MM, Oeffinger KC, Jones K, et al. Age-dependent changes in health status in the Childhood Cancer Survivor cohort. *J Clin Oncol*. 2015;33(5):479–491.

Landier W, Armenian SH, Gramatges MM, Chow EJ, Bhatia S. Late effects of childhood cancer and its treatment. In: Blaney SM, Helman LJ, Adamson PC, eds. *Pizzo & Poplack's Principles and Practice of Pediatric Oncology*. 8th ed. Philadelphia: Lippincott Raven; 2020.

Landier W, Skinner R, Wallace WH, et al. Surveillance for late effects in childhood cancer survivors. *J Clin Oncol*. 2018;36(21):2216–2222.

Majhail NS, Rizzo JD, Lee SJ, et al. Recommended screening and preventive practices for long-term survivors after hematopoietic cell transplantation. *Bone Marrow Transplant*. 2012;47(3):337–341.

Mayer DK, Nekhlyudov L, Snyder CF, et al. American Society of Clinical Oncology clinical expert statement on cancer survivorship care planning. *J Oncol Pract*. 2014;10(6):345–351.

Oeffinger KC, Mertens AC, Sklar CA, et al. Chronic health conditions in adult survivors of childhood cancer. *N Engl J Med*. 2006;355(15):1572–1582.

Phillips SM, Padgett LS, Leisenring WM, et al. Survivors of childhood cancer in the United States: prevalence and burden of morbidity. *Cancer Epidemiol Biomarkers Prev*. 2015;24(4):653–663.

Sun C-L, Kersey JH, Francisco L, et al. Burden of morbidity in 10+ year survivors of hematopoietic cell transplantation: report from the bone marrow transplantation survivor study. *Biol Blood Marrow Transplant*. 2013;19(7):1073–1080.

Vanderwalde AM, Sun C-L, Laddaran L, et al. Conditional survival and cause-specific mortality after autologous hematopoietic cell transplantation for hematological malignancies. *Leukemia*. 2013;27(5):1139–1145.

REFERENCES

1. American Cancer Society *Cancer Treatment and Survivorship Facts and Figures 2019–2021*. Atlanta: American Cancer Society; 2019.
2. American Cancer Society *Cancer Treatment and Survivorship Facts and Figures 2014–2015*. Atlanta: American Cancer Society; 2014.
3. Chow EJ, Mueller BA, Baker KS, et al. Cardiovascular hospitalizations and mortality among recipients of hematopoietic stem cell transplantation. *Ann Intern Med*. 2011;155(1):21–32.
4. Armenian SH, Sun C-L, Shannon T, et al. Incidence and predictors of congestive heart failure after autologous hematopoietic cell transplantation. *Blood*. 2011;118(23):6023–6029.
5. Socié G, Salooja N, Cohen A, et al. Nonmalignant late effects after allogeneic stem cell transplantation. *Blood*. 2003;101(9):3373–3385.
6. Tichelli A, Bucher C, Rovó A, et al. Premature cardiovascular disease after allogeneic hematopoietic stem-cell transplantation. *Blood*. 2007;110(9):3463–3471.
7. Tichelli A, Passweg J, Wójcik D, et al. Late cardiovascular events after allogeneic hematopoietic stem cell transplantation: a retrospective multicenter study of the Late Effects Working Party of the European Group for Blood and Marrow Transplantation. *Haematologica*. 2008;93(8):1203–1210.
8. van Nimwegen FA, Schaapveld M, Janus CP, et al. Cardiovascular disease after Hodgkin lymphoma treatment: 40-year disease risk. *JAMA Intern Med*. 2015;175(6):1007–1017.
9. Armenian SH, Sun C-L, Francisco L, et al. Late congestive heart failure after hematopoietic cell transplantation. *J Clin Oncol*. 2008;26(34):5537–5543.
10. Maraldo MV, Giusti F, Vogelius IR, et al. Cardiovascular disease after treatment for Hodgkin's lymphoma: an analysis of nine collaborative EORTC-LYSA trials. *Lancet Haematol*. 2015;2(11):e492–e502.
11. van Nimwegen FA, Ntentas G, Darby SC, et al. Risk of heart failure in survivors of Hodgkin lymphoma: effects of cardiac exposure to radiation and anthracyclines. *Blood*. 2017;129(16):2257–2265.
12. Mulrooney DA, Yeazel MW, Kawashima T, et al. Cardiac outcomes in a cohort of adult survivors of childhood and adolescent cancer: retrospective analysis of the Childhood Cancer Survivor Study cohort. *BMJ*. 2009;339:b4606.
13. van Dalen EC, van der Pal HJ, Kok WE, et al. Clinical heart failure in a cohort of children treated with anthracyclines: a long-term follow-up study. *Eur J Cancer*. 2006;42(18):3191–3198.
14. Pein F, Sakiroglu O, Dahan M, et al. Cardiac abnormalities 15 years and more after adriamycin therapy in 229 childhood survivors of a solid tumour at the Institut Gustave Roussy. *Br J Canc*. 2004;91(1):37–44.
15. Blanco JG, Sun C-L, Landier W, et al. Anthracycline-related cardiomyopathy after childhood cancer: role of polymorphisms in carbonyl reductase genes—a report from the Children's Oncology Group. *J Clin Oncol*. 2012;30:1415–1421.
16. Limat S, Daguindau E, Cahn J-Y, et al. Incidence and risk-factors of CHOP/R-CHOP-related cardiotoxicity in patients with aggressive non-Hodgkin's lymphoma. *J Clin Pharm Ther*. 2014;39(2):168–174.
17. Swain SM, Whaley FS, Ewer MS. Congestive heart failure in patients treated with doxorubicin: a retrospective analysis of three trials. *Cancer*. 2003;97(11):2869–2879.
18. Hershman DL, McBride RB, Eisenberger A, et al. Doxorubicin, cardiac risk factors, and cardiac toxicity in elderly patients with diffuse B-cell non-Hodgkin's lymphoma. *J Clin Oncol*. 2008;26(19):3159–3165.
19. Murbraech K, Smeland KB, Holte H, et al. Heart failure and asymptomatic left ventricular systolic dysfunction in lymphoma survivors treated with autologous stem-cell transplantation: a National Cross-Sectional Study. *J Clin Oncol*. 2015;33(24):2683–2691.
20. Kremer LCM, van Dalen EC, Offringa M, Voûte PA. Frequency and risk factors of anthracycline-induced clinical heart failure in children: a systematic review. *Ann Oncol*. 2002;13(4):503–512.
21. Aleman BM, van den Belt-Dusebout AW, De Bruin ML, et al. Late cardiotoxicity after treatment for Hodgkin lymphoma. *Blood*. 2007;109(5):1878–1886.
22. Myrehaug S, Pintilie M, Tsang R, et al. Cardiac morbidity following modern treatment for Hodgkin lymphoma: supra-additive cardiotoxicity of doxorubicin and radiation therapy. *Leuk Lymphoma*. 2008;49(8):1486–1493.
23. Salz T, Zabor EC, de Nully Brown P, et al. Preexisting cardiovascular risk and subsequent heart failure among non-Hodgkin lymphoma survivors. *J Clin Oncol*. 2017;35(34):3837–3843.
24. Armenian SH, Bhatia S. Cardiovascular disease after hematopoietic cell transplantation–lessons learned. *Haematologica*. 2008;93(8):1132–1136.
25. Armenian SH, Sun C-L, Vase T, et al. Cardiovascular risk factors in hematopoietic cell transplantation survivors: role in development of subsequent cardiovascular disease. *Blood*. 2012;120(23):4505–4512.
26. Hull MC, Morris CG, Pepine CJ, Mendenhall NP. Valvular dysfunction and carotid, subclavian, and coronary artery disease in survivors of Hodgkin lymphoma treated with radiation therapy. *JAMA*. 2003;290(21):2831–2837.
27. Adams MJ, Lipsitz SR, Colan SD, et al. Cardiovascular status in long-term survivors of Hodgkin's disease treated with chest radiotherapy. *J Clin Oncol*. 2004;22(15):3139–3148.
28. King V, Constine LS, Clark D, et al. Symptomatic coronary artery disease after mantle irradiation for Hodgkin's disease. *Int J Radiat Oncol Biol Phys*. 1996;36(4):881–889.
29. Swerdlow AJ, Higgins CD, Smith P, et al. Myocardial infarction mortality risk after treatment for Hodgkin disease: a collaborative British cohort study. *J Natl Cancer Inst*. 2007;99(3):206–214.
30. Bhakta N, Liu Q, Yeo F, et al. Cumulative burden of cardiovascular morbidity in paediatric, adolescent, and young adult survivors of Hodgkin's lymphoma: an analysis from the St Jude Lifetime Cohort Study. *Lancet Oncol*. 2016;17(9):1325–1334.
31. Tichelli A, Bhatia S, Socié G. Cardiac and cardiovascular consequences after haematopoietic stem cell transplantation. *Br J Haematol*. 2008;142(1):11–26.
32. Armenian SH, Sun C-L, Mills G, et al. Predictors of late cardiovascular complications in survivors of hematopoietic cell transplantation. *Biol Blood Marrow Transpl*. 2010;16(8):1138–1144.
33. Greenland P, Alpert JS, Beller GA, et al. 2010 ACCF/AHA guideline for assessment of cardiovascular risk in asymptomatic adults: executive summary: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *Circulation*. 2010;122(25):2748–2764.
34. Baker KS, Ness KK, Steinberger J, et al. Diabetes, hypertension, and cardiovascular events in survivors of hematopoietic cell transplantation: a report from the bone marrow transplantation survivor study. *Blood*. 2007;109(4):1765–1772.
35. de Vathaire F, El-Fayech C, Ben Ayed FF, et al. Radiation dose to the pancreas and risk of diabetes mellitus in childhood cancer survivors: a retrospective cohort study. *Lancet Oncol*. 2012;13(10):1002–1010.
36. Chemaitilly W, Sklar CA. Endocrine complications of hematopoietic stem cell transplantation. *Endocrinol Metab Clin North Am*. 2007;36(4):983–998. ix.
37. Minotti G, Recalcati S, Menna P, et al. Doxorubicin cardiotoxicity and the control of iron metabolism: quinone-dependent and independent mechanisms. *Methods Enzymol*. 2004;378:340–361.
38. Hensley ML, Hagerty KL, Kewalramani T, et al. American Society of Clinical Oncology 2008 clinical practice guideline update: use of chemotherapy and radiation therapy protectants. *J Clin Oncol*. 2009;27(1):127–145.
39. Kalam K, Marwick TH. Role of cardioprotective therapy for prevention of cardiotoxicity with chemotherapy: a systematic review and meta-analysis. *Eur J Cancer*. 2013;49(13):2900–2909.
40. van Dalen EC, Caron HN, Dickinson HO, Kremer LC. Cardioprotective interventions for cancer patients receiving anthracyclines. *Cochrane Database Syst Rev*. 2011(6):CD003917.
41. Lipshultz SE, Scully RE, Lipsitz SR, et al. Assessment of dexrazoxane as a cardioprotectant in doxorubicin-treated children with high-risk acute lymphoblastic leukaemia: long-term follow-up of a prospective, randomised, multicentre trial. *Lancet Oncol*. 2010;11(10):950–961.
42. Lipshultz SE, Rifai N, Dalton VM, et al. The effect of dexrazoxane on myocardial injury in doxorubicin-treated children with acute lymphoblastic leukemia. *N Engl J Med*. 2004;351(2):145–153.
43. Chow EJ, Asselin BL, Schwartz CL, et al. Late mortality after dexrazoxane treatment: a report from the Children's Oncology Group. *J Clin Oncol*. 2015
44. Bryant J, Picot J, Baxter L, et al. Clinical and cost-effectiveness of cardioprotection against the toxic effects of anthracyclines given to children with cancer: a systematic review. *Br J Cancer*. 2007;96(2):226–230.
45. Gabizon AA, Lyass O, Berry GJ, Wildgust M. Cardiac safety of pegylated liposomal doxorubicin (Doxil/Caelyx) demonstrated by endomyocardial biopsy in patients with advanced malignancies. *Cancer Invest*. 2004;22(5):663–669.
46. Marina NM, Cochrane D, Harney E, et al. Dose escalation and pharmacokinetics of pegylated liposomal doxorubicin (Doxil) in children with solid tumors: a pediatric oncology group study. *Clin Cancer Res*. 2002;8(2):413–418.
47. Lowis S, Lewis I, Elsworth A, et al. A phase I study of intravenous liposomal daunorubicin (DaunoXome) in paediatric patients with relapsed or resistant solid tumours. *Br J Cancer*. 2006;95(5):571–580.
48. Długosz-Danecka M, Gruszka AM, Szmit S, et al. Primary cardioprotection reduces mortality in lymphoma patients with increased risk of

anthracycline cardiotoxicity, treated by R-CHOP regimen. *Chemotherapy*. 2018;63(4):238–245.
49. Children's Oncology Group *Long-Term Follow-Up Guidelines for Survivors of Childhood, Adolescent, and Young Adult Cancers, Version 5.0*. Monrovia, CA: Children's Oncology Group; 2018. www.survivorshipguidelines.org.
50. Armenian SH, Hudson MM, Mulder RL, et al. Recommendations for cardiomyopathy surveillance for survivors of childhood cancer: a report from the International Late Effects of Childhood Cancer Guideline Harmonization Group. *Lancet Oncol*. 2015;16(3):e123–e136.
51. Armenian SH, Lacchetti C, Barac A, et al. Prevention and monitoring of cardiac dysfunction in survivors of adult cancers: American Society of Clinical Oncology Clinical Practice Guideline. *J Clin Oncol*. 2017;35(8):893–911.
52. Majhail NS, Rizzo JD, Lee SJ, et al. Recommended screening and preventive practices for long-term survivors after hematopoietic cell transplantation. *Bone Marrow Transpl*. 2012;47(3):337–341.
53. Leiper AD. Non-endocrine late complications of bone marrow transplantation in childhood: part I. *Br J Haematol*. 2002;118(1):3–22.
54. Bredenfeld H, Franklin J, Nogova L, et al. Severe pulmonary toxicity in patients with advanced-stage Hodgkin's disease treated with a modified bleomycin, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisone, and gemcitabine (BEACOPP) regimen is probably related to the combination of gemcitabine and bleomycin: a report of the German Hodgkin's Lymphoma Study Group. *J Clin Oncol*. 2004;22(12):2424–2429.
55. Dietz AC, Chen Y, Yasui Y, et al. Risk and impact of pulmonary complications in survivors of childhood cancer: a report from the Childhood Cancer Survivor Study. *Cancer*. 2016;122(23):3687–3696.
56. Kasteler R, Weiss A, Schindler M, et al. Long-term pulmonary disease among Swiss childhood cancer survivors. *Pediatric Blood Cancer*. 2018;65(1).
57. Armenian SH, Landier W, Francisco L, et al. Long-term pulmonary function in survivors of childhood cancer. *J Clin Oncol*. 2015;33(14): 1592–1600.
58. Khurshid I, Anderson LC. Non-infectious pulmonary complications after bone marrow transplantation. *Postgrad Med J*. 2002;78(919):257–262.
59. Bergeron A, Cheng GS. Bronchiolitis obliterans syndrome and other late pulmonary complications after allogeneic hematopoietic stem cell transplantation. *Clin Chest Med*. 2017;38(4):607–621.
60. Rhee CK, Ha JH, Yoon JH, et al. Risk factor and clinical outcome of bronchiolitis obliterans syndrome after allogeneic hematopoietic stem cell transplantation. *Yonsei Med J*. 2016;57(2):365–372.
61. Uhlving HH, Andersen CB, Christensen IJ, et al. Biopsy-verified bronchiolitis obliterans and other noninfectious lung pathologies after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transpl*. 2015;21(3):531–538.
62. Duque-Afonso J, Ihorst G, Waterhouse M, et al. Impact of lung function on bronchiolitis obliterans syndrome and outcome after allogeneic hematopoietic cell transplantation with reduced-intensity conditioning. *Biol Blood Marrow Transpl*. 2018;24(11):2277–2284.
63. Williams KM, Chien JW, Gladwin MT, Pavletic SZ. Bronchiolitis obliterans after allogeneic hematopoietic stem cell transplantation. *JAMA*. 2009;302(3):306–314.
64. Williams KM. How I treat bronchiolitis obliterans syndrome after hematopoietic stem cell transplantation. *Blood*. 2017;129(4):448–455.
65. Namkoong H, Ishii M, Mori T, et al. Clinical and radiological characteristics of patients with late-onset severe restrictive lung defect after hematopoietic stem cell transplantation. *BMC Pulm Med*. 2017;17(1):123.
66. Majhail NS, Rizzo JD, Lee SJ, et al. [Recommended screening and preventive practices for long-term survivors after hematopoietic cell transplantation]. *Rinsho Ketsueki*. 2014;55(6):607–632.
67. Yoon J-S, Chun YH, Lee JW, et al. Value of screening spirometry for early diagnosis of bronchiolitis obliterans syndrome in children after allogeneic hematopoietic stem cell transplantation. *J Pediatr Hematol Oncol*. 2015;37(8):e462–e467.
68. Hancock SL, Cox RS, McDougall IR. Thyroid diseases after treatment of Hodgkin's disease. *N Engl J Med*. 1991;325(9):599–605.
69. Chemaitilly W, Cohen LE, Mostoufi-Moab S, et al. Endocrine late effects in childhood cancer survivors. *J Clin Oncol*. 2018;36(21):2153–2159.
70. Greenfield DM, Wright J, Brown JE, et al. High incidence of late effects found in Hodgkin's lymphoma survivors, following recall for breast cancer screening. *Br J Cancer*. 2006;94(4):469–472.
71. Chow EJ, Pihoker C, Hunt K, et al. Obesity and hypertension among children after treatment for acute lymphoblastic leukemia. *Cancer*. 2007;110(10):2313–2320.
72. Asner S, Ammann RA, Ozsahin H, Beck-Popovic M, von der Weid NX. Obesity in long-term survivors of childhood acute lymphoblastic leukemia. *Pediatr Blood Cancer*. 2008;51(1):118–122.
73. Oeffinger KC, Mertens AC, Sklar CA, et al. Obesity in adult survivors of childhood acute lymphoblastic leukemia: a report from the Childhood Cancer Survivor Study. *J Clin Oncol*. 2003;21(7):1359–1365.
74. Lindemulder SJ, Stork LC, Bostrom B, et al. Survivors of standard risk acute lymphoblastic leukemia do not have increased risk for overweight and obesity compared to non-cancer peers: a report from the Children's Oncology Group. *Pediatr Blood Cancer*. 2015;62(6):1035–1041.
75. Wallace WH, Thomson AB, Kelsey TW. The radiosensitivity of the human oocyte. *Hum Reprod*. 2003;18(1):117–121.
76. Levine JM, Whitton JA, Ginsberg JP, et al. Nonsurgical premature menopause and reproductive implications in survivors of childhood cancer: a report from the Childhood Cancer Survivor Study. *Cancer*. 2018;124(5):1044–1052.
77. Chemaitilly W, Mertens AC, Mitby P, et al. Acute ovarian failure in the childhood cancer survivor study. *J Clin Endocrinol Metab*. 2006;91(5):1723–1728.
78. Hamre MR, Robison LL, Nesbit ME, et al. Effects of radiation on ovarian function in long-term survivors of childhood acute lymphoblastic leukemia: a report from the Childrens Cancer Study Group. *J Clin Oncol*. 1987;5(11):1759–1765.
79. Quigley C, Cowell C, Jimenez M, et al. Normal or early development of puberty despite gonadal damage in children treated for acute lymphoblastic leukemia. *N Engl J Med*. 1989;321(3):143–151.
80. Kenney LB, Cohen LE, Shnorhavorian M, et al. Male reproductive health after childhood, adolescent, and young adult cancers: a report from the Children's Oncology Group. *J Clin Oncol*. 2012;30(27):3408–3416.
81. Green DM, Kawashima T, Stovall M, et al. Fertility of male survivors of childhood cancer: a report from the Childhood Cancer Survivor Study. *J Clin Oncol*. 2010;28(2):332–339.
82. van Dorp W, van Beek RD, Laven JS, et al. Long-term endocrine side effects of childhood Hodgkin's lymphoma treatment: a review. *Hum Reprod Update*. 2012;18(1):12–28.
83. Green DM, Sklar CA, Boice Jr. JD, et al. Ovarian failure and reproductive outcomes after childhood cancer treatment: results from the Childhood Cancer Survivor Study. *J Clin Oncol*. 2009;27(14):2374–2381.
84. Green DM, Liu W, Kutteh WH, et al. Cumulative alkylating agent exposure and semen parameters in adult survivors of childhood cancer: a report from the St Jude Lifetime Cohort Study. *Lancet Oncol*. 2014;15(11):1215–1223.
85. Mostoufi-Moab S, Seidel K, Leisenring WM, et al. Endocrine abnormalities in aging survivors of childhood cancer: a report from the Childhood Cancer Survivor Study. *J Clin Oncol*. 2016;34(27):3240–3247.
86. Sklar C, Boulad F, Small T, Kernan N. Endocrine complications of pediatric stem cell transplantation. *Front Biosci*. 2001;6:G17–G22.
87. Sanders JE. The impact of marrow transplant preparative regimens on subsequent growth and development. The Seattle Marrow Transplant Team. *Semin Hematol*. 1991;28(3):244–249.
88. Rovó A, Tichelli A, Passweg JR, et al. Spermatogenesis in long-term survivors after allogeneic hematopoietic stem cell transplantation is associated with age, time interval since transplantation, and apparently absence of chronic GvHD. *Blood*. 2006;108(3):1100–1105.
89. Afify Z, Shaw PJ, Clavano-Harding A, Cowell CT. Growth and endocrine function in children with acute myeloid leukaemia after bone marrow transplantation using busulfan/cyclophosphamide. *Bone Marrow Transpl*. 2000;25(10):1087–1092.
90. Sanders JE, Hawley J, Levy W, et al. Pregnancies following high-dose cyclophosphamide with or without high-dose busulfan or total-body irradiation and bone marrow transplantation. *Blood*. 1996;87(7):3045–3052.
91. Metzger ML, Meacham LR, Patterson B, et al. Female reproductive health after childhood, adolescent, and young adult cancers: guidelines for the assessment and management of female reproductive complications. *J Clin Oncol*. 2013;31(9):1239–1247.
92. Lafforgue P. Pathophysiology and natural history of avascular necrosis of bone. *Jt Bone Spine*. 2006;73(5):500–507.
93. Biddeci G, Bosco G, Varotto E, et al. Osteonecrosis in children and adolescents with acute lymphoblastic leukemia: early diagnosis and new treatment strategies. *Anticancer Res*. 2019;39(3):1259–1266.
94. Socié G, Cahn JY, Carmelo J, et al. Avascular necrosis of bone after allogeneic bone marrow transplantation: analysis of risk factors for 4388 patients by the Société Française de Greffe de Moëlle (SFGM). *Br J Haematol*. 1997;97(4):865–870.
95. Li X, Brazauskas R, Wang Z, et al. Avascular necrosis of bone after allogeneic hematopoietic cell transplantation in children and adolescents. *Biol Blood Marrow Transpl*. 2014;20(4):587–592.
96. Campbell S, Sun C-L, Kurian S, et al. Predictors of avascular necrosis of bone in long-term survivors of hematopoietic cell transplantation. *Cancer*. 2009;115(18):4127–4135.

97. Redman JR, Bajorunas DR, Wong G, et al. Bone mineralization in women following successful treatment of Hodgkin's disease. *Am J Med*. 1988;85(1):65–72.
98. Kaste SC, Rai SN, Fleming K, et al. Changes in bone mineral density in survivors of childhood acute lymphoblastic leukemia. *Pediatr Blood Cancer*. 2006;46(1):77–87.
99. Meyers CA. Neurocognitive dysfunction in cancer patients. *Oncol (Williston Park)*. 2000;14(1):75–79. discussion 79, 81-72, 85.
100. Williams AM, Zent CS, Janelsins MC. What is known and unknown about chemotherapy-related cognitive impairment in patients with haematological malignancies and areas of needed research. *Br J Haematol*. 2016;174(6):835–846.
101. Hshieh TT, Jung WF, Grande LJ, et al. Prevalence of cognitive impairment and association with survival among older patients with hematologic cancers. *JAMA Oncol*. 2018;4(5):686–693.
102. Mandelblatt JS, Jacobsen PB, Ahles T. Cognitive effects of cancer systemic therapy: implications for the care of older patients and survivors. *J Clin Oncol*. 2014;32(24):2617–2626.
103. Williams AM, Janelsins MC, van Wijngaarden E. Cognitive function in cancer survivors: analysis of the 1999–2002 National Health and Nutrition Examination Survey. *Support Care Cancer*. 2016;24(5):2155–2162.
104. Cheung YT, Brinkman TM, Li C, et al. Chronic health conditions and neurocognitive function in aging survivors of childhood cancer: a report from the Childhood Cancer Survivor Study. *J Natl Cancer Inst*. 2018;110(4):411–419.
105. Moleski M. Neuropsychological, neuroanatomical, and neurophysiological consequences of CNS chemotherapy for acute lymphoblastic leukemia. *Arch Clin Neuropsychol*. 2000;15(7):603–630.
106. Butler RW, Haser JK. Neurocognitive effects of treatment for childhood cancer. *Ment Retard Dev Disabil Res Rev*. 2006;12(3):184–191.
107. Campbell LK, Scaduto M, Sharp W, et al. A meta-analysis of the neurocognitive sequelae of treatment for childhood acute lymphocytic leukemia. *Pediatr Blood Cancer*. 2007;49(1):65–73.
108. Peterson CC, Johnson CE, Ramirez LY, et al. A meta-analysis of the neuropsychological sequelae of chemotherapy-only treatment for pediatric acute lymphoblastic leukemia. *Pediatr Blood Cancer*. 2008;51(1):99–104.
109. Kadan-Lottick NS, Brouwers P, Breiger D, et al. Comparison of neurocognitive functioning in children previously randomly assigned to intrathecal methotrexate compared with triple intrathecal therapy for the treatment of childhood acute lymphoblastic leukemia. *J Clin Oncol*. 2009;27(35):5986–5992.
110. Conklin HM, Krull KR, Reddick WE, et al. Cognitive outcomes following contemporary treatment without cranial irradiation for childhood acute lymphoblastic leukemia. *J Natl Cancer Inst*. 2012;104(18):1386–1395.
111. Iyer NS, Balsamo LM, Bracken MB, Kadan-Lottick NS. Chemotherapy-only treatment effects on long-term neurocognitive functioning in childhood ALL survivors: a review and meta-analysis. *Blood*. 2015;126(3):346–353.
112. Jain N, Brouwers P, Okcu MF, et al. Sex-specific attention problems in long-term survivors of pediatric acute lymphoblastic leukemia. *Cancer*. 2009;115(18):4238–4245.
113. Kadan-Lottick NS, Zeltzer LK, Liu Q, et al. Neurocognitive functioning in adult survivors of childhood non-central nervous system cancers. *J Natl Cancer Inst*. 2010;102(12):881–893.
114. Krull KR, Brinkman TM, Li C, et al. Neurocognitive outcomes decades after treatment for childhood acute lymphoblastic leukemia: a report from the St Jude lifetime cohort study. *J Clin Oncol*. 2013;31(35):4407–4415.
115. Buizer AI, de Sonneville LM, Veerman AJ. Effects of chemotherapy on neurocognitive function in children with acute lymphoblastic leukemia: a critical review of the literature. *Pediatr Blood Cancer*. 2009;52(4):447–454.
116. Spiegler BJ, Kennedy K, Maze R, et al. Comparison of long-term neurocognitive outcomes in young children with acute lymphoblastic leukemia treated with cranial radiation or high-dose or very high-dose intravenous methotrexate. *J Clin Oncol*. 2006;24(24):3858–3864.
117. Clanton NR, Klosky JL, Li C, et al. Fatigue, vitality, sleep, and neurocognitive functioning in adult survivors of childhood cancer: a report from the Childhood Cancer Survivor Study. *Cancer*. 2011;117(11):2559–2568.
118. Cheung YT, Brinkman TM, Mulrooney DA, et al. Impact of sleep, fatigue, and systemic inflammation on neurocognitive and behavioral outcomes in long-term survivors of childhood acute lymphoblastic leukemia. *Cancer*. 2017;123(17):3410–3419.
119. Kirchhoff AC, Krull KR, Ness KK, et al. Physical, mental, and neurocognitive status and employment outcomes in the childhood cancer survivor study cohort. *Cancer Epidemiol Biomarkers Prev*. 2011;20(9): 1838–1849.
120. Krull KR, Annett RD, Pan Z, et al. Neurocognitive functioning and health-related behaviours in adult survivors of childhood cancer: a report from the Childhood Cancer Survivor Study. *Eur J Cancer*. 2011;47(9):1380–1388.
121. Zhou C, Zhuang Y, Lin X, et al. Changes in neurocognitive function and central nervous system structure in childhood acute lymphoblastic leukaemia survivors after treatment: a meta-analysis. *Br J Haematol*. 2020;188(6):945–961.
122. Edelmann MN, Krull KR, Liu W, et al. Diffusion tensor imaging and neurocognition in survivors of childhood acute lymphoblastic leukaemia. *Brain*. 2014;137(Pt 11):2973–2983.
123. Cheung YT, Sabin ND, Reddick WE, et al. Leukoencephalopathy and long-term neurobehavioural, neurocognitive, and brain imaging outcomes in survivors of childhood acute lymphoblastic leukaemia treated with chemotherapy: a longitudinal analysis. *Lancet Haematol*. 2016;3(10):e456–e466.
124. Joly F, Henry-Amar M, Arveux P, et al. Late psychosocial sequelae in Hodgkin's disease survivors: a French population-based case-control study. *J Clin Oncol*. 1996;14(9):2444–2453.
125. Krull KR, Sabin ND, Reddick WE, et al. Neurocognitive function and CNS integrity in adult survivors of childhood Hodgkin lymphoma. *J Clin Oncol*. 2012;30(29):3618–3624.
126. Ehrhardt MJ, Mulrooney DA, Li C, et al. Neurocognitive, psychosocial, and quality-of-life outcomes in adult survivors of childhood non-Hodgkin lymphoma. *Cancer*. 2018;124(2):417–425.
127. Zimmer P, Mierau A, Bloch W, et al. Post-chemotherapy cognitive impairment in patients with B-cell non-Hodgkin lymphoma: a first comprehensive approach to determine cognitive impairments after treatment with rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone or rituximab and bendamustine. *Leuk Lymphoma*. 2015;56(2):347–352.
128. van der Poel MWM, Oerlemans S, Schouten HC, et al. Quality of life more impaired in younger than in older diffuse large B cell lymphoma survivors compared to a normative population: a study from the population-based PROFILES registry. *Ann hematol*. 2014;93(5):811–819.
129. Schäfer N, Glas M, Herrlinger U. Primary CNS lymphoma: a clinician's guide. *Expert Rev Neurother*. 2012;12(10):1197–1206.
130. Doolittle ND, Korfel A, Lubow MA, et al. Long-term cognitive function, neuroimaging, and quality of life in primary CNS lymphoma. *Neurology*. 2013;81(1):84–92.
131. Doolittle ND, Dósa E, Fu R, et al. Preservation of cognitive function in primary CNS lymphoma survivors a median of 12 years after enhanced chemotherapy delivery. *J Clin Oncol*. 2013;31(31):4026–4027.
132. Juergens A, Pels H, Rogowski S, et al. Long-term survival with favorable cognitive outcome after chemotherapy in primary central nervous system lymphoma. *Ann Neurol*. 2010;67(2):182–189.
133. Sharafeldin N, Bosworth A, Patel SK, et al. Cognitive functioning after hematopoietic cell transplantation for hematologic malignancy: results from a Prospective Longitudinal Study. *J Clin Oncol*. 2018;36(5):463–475.
134. Murdaugh DL, Bosworth A, Patel SK, et al. Self-endorsed cognitive problems versus objectively assessed cognitive impairment in blood or bone marrow transplantation recipients: a longitudinal study. *Cancer*. 2020;126(10):2174–2182.
135. Cole PD, Finkelstein Y, Stevenson KE, et al. Polymorphisms in genes related to oxidative stress are associated with inferior cognitive function after therapy for childhood acute lymphoblastic leukemia. *J Clin Oncol*. 2015;33(19):2205–2211.
136. Sharafeldin N, Richman J, Bosworth A, et al. Clinical and genetic risk prediction of cognitive impairment after blood or marrow transplantation for hematologic malignancy. *J Clin Oncol*. 2020;38(12):1312–1321.
137. Alloin AL, Barlogis V, Auquier P, et al. Prevalence and risk factors of cataract after chemotherapy with or without central nervous system irradiation for childhood acute lymphoblastic leukaemia: an LEA study. *Br J Haematol*. 2014;164(1):94–100.
138. Chodick G, Sigurdson AJ, Kleinerman RA, et al. The risk of cataract among survivors of childhood and adolescent cancer: a report from the Childhood Cancer Survivor Study. *Radiat Res*. 2016;185(4):366–374.
139. Horwitz M, Auquier P, Barlogis V, et al. Incidence and risk factors for cataract after haematopoietic stem cell transplantation for childhood leukaemia: an LEA study. *Br J Haematol*. 2015;168(4):518–525.
140. Holmström G, Borgström B, Calissendorff B. Cataract in children after bone marrow transplantation: relation to conditioning regimen. *Acta Ophthalmol Scand*. 2002;80(2):211–215.
141. Belkacemi Y, Labopin M, Vernant JP, et al. Cataracts after total body irradiation and bone marrow transplantation in patients with acute leukemia in complete remission: a study of the European Group for Blood and Marrow Transplantation. *Int J Radiat Oncol Biol Phys*. 1998;41(3): 659–668.

142. Westeneng AC, Hettinga Y, Lokhorst H, et al. Ocular graft-versus-host disease after allogeneic stem cell transplantation. *Cornea*. 2010;29(7):758–763.
143. Tabbara KF, Al-Ghamdi A, Al-Mohareb F, et al. Ocular findings after allogeneic hematopoietic stem cell transplantation. *Ophthalmology*. 2009;116(9):1624–1629.
144. Landier W. Ototoxicity and cancer therapy. *Cancer*. 2016;122(11):1647–1658.
145. Gurney JG, Ness KK, Rosenthal J, et al. Visual, auditory, sensory, and motor impairments in long-term survivors of hematopoietic stem cell transplantation performed in childhood: results from the Bone Marrow Transplant Survivor study. *Cancer*. 2006;106(6):1402–1408.
146. Travis LB, Fossa SD, Sesso HD, et al. Chemotherapy-induced peripheral neurotoxicity and ototoxicity: new paradigms for translational genomics. *J Natl Cancer Inst*. 2014;106(5):dju044.
147. Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*. 2009;114(5):937–951.
148. Dong C, Hemminki K. Second primary neoplasms in 633,964 cancer patients in Sweden, 1958–1996. *Int J Cancer*. 2001;93(2):155–161.
149. Donin N, Filson C, Drakaki A, et al. Risk of second primary malignancies among cancer survivors in the United States, 1992 through 2008. *Cancer*. 2016;122(19):3075–3086.
150. Feller A, Matthes KL, Bordoni A, et al. Correction to: the relative risk of second primary cancers in Switzerland: a population-based retrospective cohort study. *BMC Cancer*. 2020;20(1):87.
151. Friedman DL, Whitton J, Leisenring W, et al. Subsequent neoplasms in 5-year survivors of childhood cancer: the Childhood Cancer Survivor Study. *J Natl Cancer Inst*. 2010;102(14):1083–1095.
152. Ishida Y, Qiu D, Maeda M, et al. Secondary cancers after a childhood cancer diagnosis: a nationwide hospital-based retrospective cohort study in Japan. *Int J Clin Oncol*. 2016;21(3):506–516.
153. Olsen JH, Möller T, Anderson H, et al. Lifelong cancer incidence in 47,697 patients treated for childhood cancer in the Nordic countries. *J Natl Cancer Inst*. 2009;101(11):806–813.
154. Teepen JC, van Leeuwen FE, Tissing WJ, et al. Long-term risk of subsequent malignant neoplasms after treatment of childhood cancer in the DCOG LATER study cohort: role of chemotherapy. *J Clin Oncol*. 2017;35(20):2288–2298.
155. Scholz-Kreisel P, Kaatsch P, Spix C, et al. Second malignancies following childhood cancer treatment in Germany from 1980 to 2014. *Dtsch Arztebl Int*. 2018;115(23):385–392.
156. Zakaria D, Shaw A, Xie L. Risk of a second cancer in Canadians diagnosed with a first cancer in childhood or adolescence. *EClinicalMedicine*. 2019;16:107–120.
157. Turcotte LM, Whitton JA, Friedman DL, et al. Risk of subsequent neoplasms during the fifth and sixth decades of life in the Childhood Cancer Survivor Study Cohort. *J Clin Oncol*. 2015;33(31):3568–3575.
158. Mertens AC, Liu Q, Neglia JP, et al. Cause-specific late mortality among 5-year survivors of childhood cancer: the Childhood Cancer Survivor Study. *J Natl Cancer Inst*. 2008;100(19):1368–1379.
159. Armstrong GT, Liu W, Leisenring W, et al. Occurrence of multiple subsequent neoplasms in long-term survivors of childhood cancer: a report from the childhood cancer survivor study. *J Clin Oncol*. 2011;29(22):3056–3064.
160. Inskip PD, Sigurdson AJ, Veiga L, et al. Radiation-related new primary solid cancers in the childhood cancer survivor study: comparative radiation dose response and modification of treatment effects. *Int J Radiat Oncol Biol, Phys*. 2016;94(4):800–807.
161. Turcotte LM, Neglia JP, Reulen RC, et al. Risk, risk factors, and surveillance of subsequent malignant neoplasms in survivors of childhood cancer: a review. *J Clin Oncol*. 2018;36(21):2145–2152.
162. Turcotte LM, Liu Q, Yasui Y, et al. Temporal trends in treatment and subsequent neoplasm risk among 5-year survivors of childhood cancer, 1970–2015. *JAMA*. 2017;317(8):814–824.
163. Heydari K, Shamshirian A, Lotfi-Foroushani P, et al. The risk of malignancies in patients receiving hematopoietic stem cell transplantation: a systematic review and meta-analysis. *Clin Transl Oncol*. 2020;22(10):1825–1837.
164. Neglia JP, Meadows AT, Robison LL, et al. Second neoplasms after acute lymphoblastic leukemia in childhood. *N Engl J Med*. 1991;325(19):1330–1336.
165. Schmiegelow K, Levinsen MF, Attarbaschi A, et al. Second malignant neoplasms after treatment of childhood acute lymphoblastic leukemia. *J Clin Oncol*. 2013;31(19):2469–2476.
166. Schmiegelow K, Al-Modhwahi I, Andersen MK, et al. Methotrexate/6-mercaptopurine maintenance therapy influences the risk of a second malignant neoplasm after childhood acute lymphoblastic leukemia: results from the NOPHO ALL-92 study. *Blood*. 2009;113(24):6077–6084.
167. Bhatia S, Sather HN, Pabustan OB, et al. Low incidence of second neoplasms among children diagnosed with acute lymphoblastic leukemia after 1983. *Blood*. 2002;99(12):4257–4264.
168. Hijiya N, Hudson MM, Lensing S, et al. Cumulative incidence of secondary neoplasms as a first event after childhood acute lymphoblastic leukemia. *JAMA*. 2007;297(11):1207–1215.
169. Pui CH, Cheng C, Leung W, et al. Extended follow-up of long-term survivors of childhood acute lymphoblastic leukemia. *N Engl J Med*. 2003;349(7):640–649.
170. Neglia JP, Friedman DL, Yasui Y, et al. Second malignant neoplasms in five-year survivors of childhood cancer: childhood cancer survivor study. *J Natl Cancer Inst*. 2001;93(8):618–629.
171. Pui CH, Ribeiro RC, Hancock ML, et al. Acute myeloid leukemia in children treated with epipodophyllotoxins for acute lymphoblastic leukemia. *N Engl J Med*. 1991;325(24):1682–1687.
172. Holmqvist AS, Chen Y, Berano Teh J, et al. Risk of solid subsequent malignant neoplasms after childhood Hodgkin lymphoma-identification of high-risk populations to guide surveillance: a report from the Late Effects Study Group. *Cancer*. 2018
173. Bhatia S, Robison LL, Oberlin O, et al. Breast cancer and other second neoplasms after childhood Hodgkin's disease. *N Engl J Med*. 1996;334(12):745–751.
174. Metayer C, Lynch CF, Clarke EA, et al. Second cancers among long-term survivors of Hodgkin's disease diagnosed in childhood and adolescence. *J Clin Oncol*. 2000;18(12):2435–2443.
175. Bhatia S, Yasui Y, Robison LL, et al. High risk of subsequent neoplasms continues with extended follow-up of childhood Hodgkin's disease: report from the Late Effects Study Group. *J Clin Oncol*. 2003;21(23):4386–4394.
176. Dores GM, Metayer C, Curtis RE, et al. Second malignant neoplasms among long-term survivors of Hodgkin's disease: a population-based evaluation over 25 years. *J Clin Oncol*. 2002;20(16):3484–3494.
177. Veiga LH, Curtis RE, Morton LM, et al. Association of breast cancer risk after childhood cancer with radiation dose to the breast and anthracycline use: a report from the Childhood Cancer Survivor Study. *JAMA Pediatr*. 2019;173(12):1171–1179.
178. Kenney LB, Yasui Y, Inskip PD, et al. Breast cancer after childhood cancer: a report from the Childhood Cancer Survivor Study. *Ann Intern Med*. 2004;141(8):590–597.
179. Ng AK, Patricia Bernardo MV, Weller E, et al. Second malignancy after Hodgkin disease treated with radiation therapy with or without chemotherapy: long-term risks and risk factors. *Blood*. 2002;100(6):1989–1996.
180. Swerdlow AJ, Barber JA, Hudson GV, et al. Risk of second malignancy after Hodgkin's disease in a collaborative British cohort: the relation to age at treatment. *J Clin Oncol*. 2000;18(3):498–509.
181. Sud A, Thomsen H, Sundquist K, et al. Risk of second cancer in Hodgkin lymphoma survivors and influence of family history. *J Clin Oncol*. 2017;35(14):1584–1590.
182. Schaapveld M, Aleman BM, van Eggermond AM, et al. Second cancer risk up to 40 years after treatment for Hodgkin's lymphoma. *N Engl J Med*. 2015;373(26):2499–2511.
183. Tucker MA, Coleman CN, Cox RS, et al. Risk of second cancers after treatment for Hodgkin's disease. *N Engl J Med*. 1988;318(2):76–81.
184. Kaldor JM, Day NE, Clarke EA, et al. Leukemia following Hodgkin's disease. *N Engl J Med*. 1990;322(1):7–13.
185. Maclachlan K, Diamond B, Maura F, et al. Second malignancies in multiple myeloma; emerging patterns and future directions. *Best Pract Res Clin Haematol*. 2020;33(1):101144.
186. Musto P, Anderson KC, Attal M, et al. Second primary malignancies in multiple myeloma: an overview and IMWG consensus. *Ann Oncol*. 2018;29(4):1074.
187. Razavi P, Rand KA, Cozen W, et al. Patterns of second primary malignancy risk in multiple myeloma patients before and after the introduction of novel therapeutics. *Blood Cancer J*. 2013;3:e121.
188. Mailankody S, Pfeiffer RM, Kristinsson SY, et al. Risk of acute myeloid leukemia and myelodysplastic syndromes after multiple myeloma and its precursor disease (MGUS). *Blood*. 2011;118(15):4086–4092.
189. Dong C, Hemminki K. Second primary neoplasms among 53 159 haematolymphoproliferative malignancy patients in Sweden, 1958–1996: a search for common mechanisms. *Br J Cancer*. 2001;85(7):997–1005.
190. Engelhardt M, Ihorst G, Landgren O, et al. Large registry analysis to accurately define second malignancy rates and risks in a well-characterized cohort of 744 consecutive multiple myeloma patients followed-up for 25 years. *Haematologica*. 2015;100(10):1340–1349.

191. Radivoyevitch T, Dean RM, Shaw BE, et al. Risk of acute myeloid leukemia and myelodysplastic syndrome after autotransplants for lymphomas and plasma cell myeloma. *Leuk Res*. 2018;74:130–136.
192. Chen T, Fallah M, Brenner H, et al. Risk of second primary cancers in multiple myeloma survivors in German and Swedish cancer registries. *Sci Rep*. 2016;6:22084.
193. Mahindra A, Raval G, Mehta P, et al. New cancers after autotransplantations for multiple myeloma. *Biol Blood Marrow Transpl*. 2015;21(4):738–745.
194. Guru Murthy GS, Hamadani M, Dhakal B, et al. Incidence and survival of therapy related myeloid neoplasm in United States. *Leuk Res*. 2018;71:95–99.
195. Fianchi L, Pagano L, Piciocchi A, et al. Characteristics and outcome of therapy-related myeloid neoplasms: report from the Italian network on secondary leukemias. *Am J Hematol*. 2015;90(5):E80–E85.
196. Smith SM, Le Beau MM, Huo D, et al. Clinical-cytogenetic associations in 306 patients with therapy-related myelodysplasia and myeloid leukemia: the University of Chicago series. *Blood*. 2003;102(1):43–52.
197. Palumbo A, Bringhen S, Kumar SK, et al. Second primary malignancies with lenalidomide therapy for newly diagnosed myeloma: a meta-analysis of individual patient data. *Lancet Oncol*. 2014;15(3):333–342.
198. Tao L, Clarke CA, Rosenberg AS, et al. Subsequent primary malignancies after diffuse large B-cell lymphoma in the modern treatment era. *Br J Haematol*. 2017;178(1):72–80.
199. Guibout C, Adjadj E, Rubino C, et al. Malignant breast tumors after radiotherapy for a first cancer during childhood. *J Clin Oncol*. 2005;23(1):197–204.
200. Taylor AJ, Winter DL, Stiller CA, et al. Risk of breast cancer in female survivors of childhood Hodgkin's disease in Britain: a population-based study. *Int J Cancer*. 2007;120(2):384–391.
201. Henderson TO, Amsterdam A, Bhatia S, et al. Systematic review: surveillance for breast cancer in women treated with chest radiation for childhood, adolescent, or young adult cancer. *Ann Intern Med*. 2010;152(7):444–455. W144-454.
202. Castellino SM, Geiger AM, Mertens AC, et al. Morbidity and mortality in long-term survivors of Hodgkin lymphoma: a report from the Childhood Cancer Survivor Study. *Blood*. 2011;117(6):1806–1816.
203. Inskip PD, Robison LL, Stovall M, et al. Radiation dose and breast cancer risk in the childhood cancer survivor study. *J Clin Oncol*. 2009;27(24):3901–3907.
204. De Bruin ML, Sparidans J, van't Veer MB, et al. Breast cancer risk in female survivors of Hodgkin's lymphoma: lower risk after smaller radiation volumes. *J Clin Oncol*. 2009;27(26):4239–4246.
205. Travis LB, Hill D, Dores GM, et al. Cumulative absolute breast cancer risk for young women treated for Hodgkin lymphoma. *J Natl Cancer Inst*. 2005;97(19):1428–1437.
206. Swerdlow AJ, Cooke R, Bates A, et al. Breast cancer risk after supradiaphragmatic radiotherapy for Hodgkin's lymphoma in England and Wales: a National Cohort Study. *J Clin Oncol*. 2012;30(22):2745–2752.
207. Moskowitz CS, Chou JF, Wolden SL, et al. Breast cancer after chest radiation therapy for childhood cancer. *J Clin Oncol*. 2014;32(21):2217–2223.
208. Henderson TO, Moskowitz CS, Chou JF, et al. Breast cancer risk in childhood cancer survivors without a history of chest radiotherapy: a report from the Childhood Cancer Survivor Study. *J Clin Oncol*. 2016;34(9):910–918.
209. Friedman DL, Rovo A, Leisenring W, et al. Increased risk of breast cancer among survivors of allogeneic hematopoietic cell transplantation: a report from the FHCRC and the EBMT-Late Effect Working Party. *Blood*. 2008;111(2):939–944.
210. Travis LB, Hill DA, Dores GM, et al. Breast cancer following radiotherapy and chemotherapy among young women with Hodgkin disease. *JAMA*. 2003;290(4):465–475.
211. van Leeuwen FE, Klokman WJ, Stovall M, et al. Roles of radiation dose, chemotherapy, and hormonal factors in breast cancer following Hodgkin's disease. *J Natl Cancer Inst*. 2003;95(13):971–980.
212. Moskowitz CS, Chou JF, Sklar CA, et al. Radiation-associated breast cancer and gonadal hormone exposure: a report from the Childhood Cancer Survivor Study. *Br J Cancer*. 2017;117(2):290–299.
213. Moskowitz CS, Chou JF, Neglia JP, et al. Mortality after breast cancer among survivors of childhood cancer: a report from the Childhood Cancer Survivor Study. *J Clin Oncol*. 2019;37(24):2120–2130.
214. Bhatia S, Sklar C. Second cancers in survivors of childhood cancer. *Nat Rev Cancer*. 2002;2(2):124–132.
215. Sklar C, Whitton J, Mertens A, et al. Abnormalities of the thyroid in survivors of Hodgkin's disease: data from the Childhood Cancer Survivor Study. *J Clin Endocrinol Metab*. 2000;85(9):3227–3232.
216. Sigurdson AJ, Ronckers CM, Mertens AC, et al. Primary thyroid cancer after a first tumour in childhood (the Childhood Cancer Survivor Study): a nested case-control study. *Lancet*. 2005;365(9476):2014–2023.
217. Ronckers CM, Sigurdson AJ, Stovall M, et al. Thyroid cancer in childhood cancer survivors: a detailed evaluation of radiation dose response and its modifiers. *Radiat Res*. 2006;166(4):618–628.
218. Bhatti P, Veiga LHS, Ronckers CM, et al. Risk of second primary thyroid cancer after radiotherapy for a childhood cancer in a large cohort study: an update from the childhood cancer survivor study. *Radiat Res*. 2010;174(6):741–752.
219. Schonfeld SJ, Morton LM, Berrington de González A, et al. Risk of second primary papillary thyroid cancer among adult cancer survivors in the United States, 2000–2015. *Cancer Epidemiol*. 2020;64:101664.
220. Danylesko I, Shimoni A. Second malignancies after hematopoietic stem cell transplantation. *Curr Treat Options Oncol*. 2018;19(2):9.
221. Krishnan A, Bhatia S, Slovak ML, et al. Predictors of therapy-related leukemia and myelodysplasia following autologous transplantation for lymphoma: an assessment of risk factors. *Blood*. 2000;95(5):1588–1593.
222. Bhatia S, Robison LL, Francisco L, et al. Late mortality in survivors of autologous hematopoietic-cell transplantation: report from the Bone Marrow Transplant Survivor Study. *Blood*. 2005;105(11):4215–4222.
223. Bhatia S, Ramsay NK, Steinbuch M, et al. Malignant neoplasms following bone marrow transplantation. *Blood*. 1996;87(9):3633–3639.
224. Smith MA, Rubinstein L, Anderson JR, et al. Secondary leukemia or myelodysplastic syndrome after treatment with epipodophyllotoxins. *J Clin Oncol*. 1999;17(2):569–577.
225. Kalow W, Ozdemir V, Tang BK, et al. The science of pharmacological variability: an essay. *Clin Pharmacol Ther*. 1999;66(5):445–447.
226. Evans WE, McLeod HL. Pharmacogenomics—drug disposition, drug targets, and side effects. *N Engl J Med*. 2003;348(6):538–549.
227. Bhatia S. Genetic variation as a modifier of association between therapeutic exposure and subsequent malignant neoplasms in cancer survivors. *Cancer*. 2015;121(5):648–663.
228. Wong TN, Ramsingh G, Young AL, et al. Role of TP53 mutations in the origin and evolution of therapy-related acute myeloid leukaemia. *Nature*. 2015;518(7540):552–555.
229. Gibson CJ, Lindsley RC, Tchekmedyian V, et al. Clonal hematopoiesis associated with adverse outcomes after autologous stem-cell transplantation for lymphoma. *J Clin Oncol*. 2017;35(14):1598–1605.
230. Mulder RL, Kremer LCM, Hudson MM, et al. Recommendations for breast cancer surveillance for female survivors of childhood, adolescent, and young adult cancer given chest radiation: a report from the International Late Effects of Childhood Cancer Guideline Harmonization Group. *Lancet Oncol*. 2013;14(13):e621–e629.
231. Saslow D, Boetes C, Burke W, et al. American Cancer Society guidelines for breast screening with MRI as an adjunct to mammography. *CA Cancer J Clin*. 2007;57(2):75–89.
232. Reulen RC, Winter DL, Frobisher C, et al. Long-term cause-specific mortality among survivors of childhood cancer. *JAMA*. 2010;304(2):172–179.
233. Garwicz S, Anderson H, Olsen JH, et al. Late and very late mortality in 5-year survivors of childhood cancer: changing pattern over four decades–experience from the Nordic countries. *Int J Cancer*. 2012;131(7):1659–1666.
234. Armstrong GT, Liu Q, Yasui Y, et al. Late mortality among 5-year survivors of childhood cancer: a summary from the Childhood Cancer Survivor Study. *J Clin Oncol*. 2009;27(14):2328–2338.
235. Armstrong GT, Chen Y, Yasui Y, et al. Reduction in late mortality among 5-year survivors of childhood cancer. *N Engl J Med*. 2016;374(9):833–842.
236. Martin PJ, Counts Jr. GW, Appelbaum FR, et al. Life expectancy in patients surviving more than 5 years after hematopoietic cell transplantation. *J Clin Oncol*. 2010;28(6):1011–1016.
237. Wingard JR, Majhail NS, Brazauskas R, et al. Long-term survival and late deaths after allogeneic hematopoietic cell transplantation. *J Clin Oncol*. 2011;29(16):2230–2239.
238. Socié G, Stone JV, Wingard JR, et al. Long-term survival and late deaths after allogeneic bone marrow transplantation. Late Effects Working Committee of the International Bone Marrow Transplant Registry. *N Engl J Med*. 1999;341(1):14–21.
239. Vanderwalde AM, Sun C-L, Laddaran L, et al. Conditional survival and cause-specific mortality after autologous hematopoietic cell transplantation for hematological malignancies. *Leukemia*. 2013;27(5):1139–1145.
240. Pui CH, Yang JJ, Hunger SP, et al. Childhood acute lymphoblastic leukemia: progress through collaboration. *J Clin Oncol*. 2015;33(27):2938–2948.

241. Oh H, Gale RP, Zhang MJ, et al. Chemotherapy vs HLA-identical sibling bone marrow transplants for adults with acute lymphoblastic leukemia in first remission. *Bone Marrow Transpl*. 1998;22(3):253–257.
242. Fulbright JM, Raman S, McClellan WS, August KJ. Late effects of childhood leukemia therapy. *Curr Hematol Malig Rep*. 2011;6(3):195–205.
243. Baker KS, Ness KK, Weisdorf D, et al. Late effects in survivors of acute leukemia treated with hematopoietic cell transplantation: a report from the Bone Marrow Transplant Survivor Study. *Leukemia*. 2010;24(12):2039–2047.
244. Hudson MM, Neglia JP, Woods WG, et al. Lessons from the past: opportunities to improve childhood cancer survivor care through outcomes investigations of historical therapeutic approaches for pediatric hematological malignancies. *Pediatr Blood Cancer*. 2012;58(3):334–343.
245. Baker KS, Gurney JG, Ness KK, et al. Late effects in survivors of chronic myeloid leukemia treated with hematopoietic cell transplantation: results from the Bone Marrow Transplant Survivor Study. *Blood*. 2004;104(6):1898–1906.
246. Chow EJ, Antal Z, Constine LS, et al. New agents, emerging late effects, and the development of precision survivorship. *J Clin Oncol*. 2018;36(21):2231–2240.
247. Eshelman D, Landier W, Sweeney T, et al. Facilitating care for childhood cancer survivors: integrating children's oncology group long-term follow-up guidelines and health links in clinical practice. *J Pediatr Oncol Nurs*. 2004;21(5):271–280.
248. Hewitt ME, Greenfield S, Stovall E, eds. *From Cancer Patient to Cancer Survivor: Lost in Transition*. Washington, DC: National Academies Press; 2006.
249. Hewitt ME, Weiner SL, Simone JV, eds. *Childhood Cancer Survivorship: Improving Care and Quality of Life*. Washington, DC: The National Academies Press; 2003.
250. Mayer DK, Alfano CM. Personalized risk-stratified cancer follow-up care: its potential for healthier survivors, happier clinicians, and lower costs. *J Natl Cancer Inst*. 2019;111(5):442–448.
251. Mayer DK, Nekhlyudov L, Snyder CF, et al. American Society of Clinical Oncology clinical expert statement on cancer survivorship care planning. *J Oncol Pract*. 2014;10(6):345–351.
252. Kremer LCM, Mulder RL, Oeffinger KC, et al. A worldwide collaboration to harmonize guidelines for the long-term follow-up of childhood and young adult cancer survivors: a report from the International Late Effects of Childhood Cancer Guideline Harmonization Group. *Pediatr Blood Cancer*. 2013;60(4):543–549.
253. de Moor JS, Mariotto AB, Parry C, et al. Cancer survivors in the United States: prevalence across the survivorship trajectory and implications for care. *Cancer Epidemiol Biomarkers Prev*. 2013;22(4):561–570.

PART IX TRANSPLANTATION AND OTHER CELL-BASED THERAPIES

CHAPTER 102

PRACTICAL ASPECTS OF HEMATOPOIETIC STEM CELL HARVESTING AND MOBILIZATION

Abba C. Zubair and Scott D. Rowley

Hematopoietic stem cell (HSC) products for autologous or allogeneic transplantation are available from bone marrow (BM), peripheral blood, or umbilical cord blood (UCB) sources. Bone marrow was the original source of cells for transplantation because of the ease and reliability of collecting adequate numbers of cells for transplantation, and it remains the standard with which other sources of HSCs are compared.

Peripheral blood stem cell (PBSC) products have virtually replaced bone marrow as the HSC component for autologous transplantation and are frequently used for allogeneic transplantation. The rapid engraftment kinetics of PBSCs compared with bone marrow is widely recognized. Median times to achieve an absolute neutrophil count greater than 500/μL and platelet transfusion independence after PBSC transplantation typically are approximately 11 to 14 days. The allogeneic donor has a wide range of options, including marrow, PBSC, or UCB products from human leukocyte antigen (HLA)-compatible or partially compatible related or unrelated donors. The availability of HLA-matched related or unrelated donors is the primary consideration in the selection of a donor, but donor health or donation preferences may restrict what products will be available to the recipient. The patient's physician may select a stem cell source based on the expected transplant outcomes. PBSC products, for example, have the greatest quantity of HSCs and will result in faster hematologic recovery compared with marrow or UCB transplants. Bone marrow transplantation has a higher risk of graft failure, resulting in a twofold higher probability of second harvest request compared with PBSC donation. In some reports, PBSC transplantation also results in a survival advantage. The transplant recipient may request a source of cells, but the donor has the right to decide about the method of donation. Although graft-versus-host disease (GVHD) prophylaxis with posttransplant methotrexate will slow engraftment, the kinetics of engraftment for the allogeneic PBSC recipient is similar to that experienced by the autologous PBSC recipient. A number of phase III studies involving either autologous or allogeneic HSC transplantation confirmed the more rapid engraftment kinetics for recipients of PBSCs,[1,2] and this effect is not limited to HSCs collected from the peripheral blood, because cytokine administration to the patient or donor before marrow harvesting will also increase the number of HSCs collected and result in quicker hematologic recovery. The disadvantages to using PBSC components compared with bone marrow or UCB for autologous or allogeneic transplantation include the possible need for multiple days of collection (especially for autologous transplantation), the inability to collect adequate components from all patients and donors, and a possibly higher risk for chronic GVHD or the occurrence of chronic GVHD that is more difficult to control (see box on Choice of Hematologic Stem Cell Product for Transplantation).

CHOICE OF HEMATOPOIETIC STEM CELL PRODUCT FOR TRANSPLANTATION

Virtually all patients undergoing autologous hematopoietic stem cell (HSC) transplantation will have peripheral blood stem cells (PBSCs) as the source of HSC, based on the following advantages: ease of collection, greater quantities of HSCs (resulting in faster hematologic recovery and shorter and less costly hospital stays), and potentially lower risks for tumor cell contamination of the graft.

The allogeneic donor has a wider range of options, including marrow, PBSCs, or umbilical cord blood (UCB) products from human leukocyte antigen-compatible or partially compatible related or unrelated donors. The transplant recipient may request a source of cells, but the donor has the right to decide about the method of donation. PBSC products have the greatest quantity of HSCs and will result in faster hematologic recovery compared with marrow or UCB transplants. In some reports, PBSC transplantation resulted in a survival advantage. However, PBSC transplantation is also associated with a higher risk for difficult-to-control chronic graft-versus-host disease (GVHD) and may not be appropriate for use in patients who would not benefit from a robust graft-versus-leukemia effect, such as those treated for nonmalignant disease. Infusion of two cord blood units may achieve a greater graft-versus-tumor effect (this concept has not been proven), even though one unit will be rejected. The much smaller quantity of HSCs in the cord blood product results in slower hematologic recovery, and the adult patient, in particular, may be at greater risk for post-transplant infection because of the relative immature immune system of the donor.

UCB from public banks has the advantage of being immediately available, reducing the time to transplantation. Targeting collection of UCB products from ethnic populations not well represented in donor registries will facilitate treatment of ethnic minority patients. The relative immunologic naïveté of the cord blood donor allows use of HLA-mismatched products without an undue increase in GVHD risk.[3] The much smaller quantity of HSCs in the cord blood product results in slower hematologic recovery and a higher risk for primary engraftment failure, which may be partially offset by infusion of multiple products; and the older adult patient, in particular, may also be at greater risk for posttransplant infections because of the relative immature immune system of the donor.

STEM CELL DONOR

Selection of Autologous Donor

The primary selection criterion for the patient undergoing autologous HSC collection and transplantation is the diagnosis of an illness amenable to treatment with a dose-intense regimen requiring HSC support. Extensive prior treatments, especially with marrow-toxic chemotherapy regimens or with radiotherapy, may prohibit collection of adequate quantities of autologous HSCs, which would exclude a patient from this treatment option. Proper management of patients with a disease amenable to dose-intensive therapy should include provisions for HSC collection before extensive marrow-toxic agents are administered. In general, however, any serious comorbid illnesses that would preclude either marrow or PBSC collection would also disqualify the patient from treatment with dose-intensive regimens used in preparation for autologous HSC transplantation.

Selection Allogeneic Donor

The selection of the allogeneic HSC donor is more complex. The HLA major histocompatibility complex is the primary consideration in selection of a donor for allogeneic HSC transplantation, since its loci contribute significantly to host-versus-graft (leading to immunologic rejection of donor HSCs) and to graft-versus-host (leading to GVHD and graft-versus-leukemia) reactions. Donor age, gender, and parity are secondary considerations in the selection of an allogeneic HSC donor. Mismatching for killer-cell immunoglobulin-like receptor ligands may reduce the risk for posttransplant relapse of disease. More than 30% of allogeneic HSC transplants from related or unrelated donors will involve ABO-disparate donors and recipients, and donor and recipient pairs may also differ for other red blood cell antigens, with no clear evidence of deleterious effect on engraftment, survival, or GVHD. Cancer, autoimmune disorders, and genetic diseases such as the hemoglobinopathies can be transmitted to the allograft recipient; thus, donor health is an important consideration in donor selection and determination of donor eligibility.

Evaluation of Hematopoietic Stem Cell Donor Suitability and Eligibility

The immediate pre-collection evaluation of a patient or donor is intended to address the risks of the collection procedure to the donor ("donor suitability") and the risks for transmission of disease from the donor to the recipient ("donor eligibility"). The same general health criteria apply to both bone marrow and PBSC donors. Patients undergoing autologous HSC collection and transplantation are not at risk for transmitting disease to themselves, and determination of donor eligibility is not medically required or economically justifiable, but these patients must be evaluated for their suitability for the collection procedures. All allogeneic and syngeneic HSC donors must be evaluated for donor eligibility, as well as suitability, using the same criteria currently applied to blood or other tissue donors, including a targeted history regarding behaviors exposing the donor to infection, recent or concurrent illnesses, and medication use. This evaluation of the allogeneic donor suitability and eligibility must be clearly documented in the donor medical record, with appropriate additional documentation of donor eligibility placed in the intended recipient's medical record before initiation of the transplant conditioning regimen. Older donors have a greater probability of comorbid medical conditions, which will increase the risks of the collection procedures, and the risks to the allogeneic donor with underlying health problems must be fully considered before subjecting the donor to HSC collection. Published standards describe evaluation of the donor for the risk of the donation process, as well as the risk for transmission of disease to the recipient.[4–6] Evaluation by appropriate consultants may be required before autologous or allogeneic donor approval is finalized. Procedures involving donors with acute infectious illnesses should be delayed, if at all possible, because of the risk for disease transmission. Genetic disorders, such as hemoglobinopathies, will be transmitted to the recipient as a direct consequence of stem cell engraftment. Cancer can be transmitted, as illustrated by the transmission of donor leukemia not detected during initial evaluation of the donor, and donors previously treated for cancer should be evaluated for the probability of recurrent disease that could be transferred to the immunocompromised recipient.

Collection of PBSCs is generally an outpatient procedure conducted in the clinic setting. In contrast, marrow harvesting has the luxury of the intensive support capability of the operating room. PBSC collection, therefore, should never be viewed as a safer alternative to marrow harvesting for the donor with underlying health problems. Pediatric donors present different challenges, based on the smaller size and varying ages (and ability to cooperate) of the donors (see box on Evaluation of the Allogeneic or Syngeneic Marrow or Peripheral Blood Stem Cell Donor).

EVALUATION OF THE ALLOGENEIC AND SYNGENEIC MARROW OR PERIPHERAL BLOOD STEM CELL DONOR

Hematopoietic stem cell (HSC) transplantation involves the infusion of a "blood product," and allogeneic and syngeneic donors must be evaluated for risks for disease transmission as per the current criteria for blood or tissue donation ("donor eligibility"). Exemptions from criteria that specifically address the risk for disease transmission are permissible, if the risks of excluding an otherwise appropriate donor outweigh the risks for disease transmission to the transplant recipient, who may not have an alternate donor. Informed consent must be obtained for the evaluation and collection procedures. Informed consent also must be specifically obtained for the release of protected donor health information to the transplant recipient, allowing proper informed consent for the transplant to be obtained. Minors and donors not competent to provide consent must be represented by a third party not involved in the care of the recipient. Ideally, similar courtesy will be provided to the adult competent donor.

Donors must also be evaluated for health issues that would increase the risks resulting from the collection procedures ("donor suitability"). For marrow donors, this includes the risks of anesthesia and harvesting in the prone position; for peripheral blood stem cell (PBSC) donors, evaluation should include the risks of mobilization medications and apheresis, including the need for venous catheter placement.

The donor collection facility's standard operating procedures for evaluation of HSC donors must meet the Foundation for the Accreditation of Cellular Therapy/Joint Accreditation Committee or AABB standards and United States Food and Drug Administration (or other equivalent regulatory agency) regulations, and include policies and procedures for the following:

- Education of donor, including education regarding procedures, risks and alternatives, and possible request for future donations;
- Medical history, including special attention to history of autoimmune disorders, arthritis, cardiac and vascular disease, and history of cancer;
- History of high-risk behaviors, such as recent tattoos, body piercing, sexual practices, and travel;
- Physical examination, including vein assessment (PBSC donors) and oral examination (marrow donors undergoing inhalational anesthesia);
- Laboratory studies, including verification of human leukocyte antigen typing, ABO typing, complete blood count, chemistry panel, infectious disease panel, urinalysis, ECG, CXR;
- Consent for the collection procedures and the release of protected health information to the stem cell recipient;
- Documentation of both donor eligibility and suitability before initiation PBSC mobilization and the transplant conditioning regimen.

Use of a donor who does not meet eligibility criteria and who poses a risk for transmission of disease requires appropriate informed consent both from the donor (for disclosure of this confidential health information to the recipient and for counseling of the recipient) and from the recipient (for use of the stem cell product). The potential conflict of interest between protecting donor confidentiality and patient needs must be recognized by the personnel caring for each person, and preferably, the donor and patient should be represented by different physicians.

Determination of Suitability for Peripheral Blood Stem Cell Donation

The PBSC donor is exposed to the risks of cytokine (and chemokine) administration and the risks related to the apheresis procedure, including the risks of central venous catheter insertion and use. No long-term health consequences have been associated with granulocyte colony-stimulating factor (G-CSF) administration, and the specific toxicities with these agents are described later. G-CSF may lead to a flare of autoimmune disorders and may increase the risk for blood clots, particularly for donors who are sedentary or who may be traveling shortly after the donation procedures.[7] The PBSC donor must be assessed for venous access before the patient receives conditioning, and consent for use of a central venous catheter must be obtained if the venous access is deemed inadequate for the apheresis procedure.

Determination of Suitability for Bone Marrow Donation

Anesthesia and blood loss present the greatest risks for serious complications to the bone marrow donor. Most marrow harvesting is performed under general anesthesia, which requires intubation for control of the airway for a surgical procedure being performed on a prone patient. Regional (spinal or epidural) anesthesia may not be effectively established, so patients and donors who express a preference for this form of anesthesia must be counseled about the potential need for general anesthesia. The health assessment must include questioning about a history of joint disease of the cervical spine and mandible, and examination of the mouth if general anesthesia requiring intubation is chosen. Patients and donors with comorbid conditions, such as aortic stenosis sensitive to changes in blood volume and blood pressure, may require anesthesia consultation and plans for invasive monitoring during the surgical procedure. A history of marrow fibrosis, pelvic irradiation, or pelvic tumor involvement may exclude a patient from marrow harvesting, although unilateral harvesting from the posterior and iliac crests and aspiration of the sternum may achieve adequate quantities of cells for transplantation.

Suitability and Eligibility for Umbilical Cord Blood Donation

Evaluation of the donor for UCB donation begins with a history of maternal and paternal illnesses and exposures to infectious diseases. Although linkage between the infant and the product is currently maintained, an update of infant health is not obtained at the time of transplantation, which may be several years after collection. Therefore, parental medical history includes specific questions addressing the risks for transmission of hereditary or acquired blood-borne diseases. A comprehensive genetic and family history should be obtained. Testing for infectious diseases is obtained from the mother at the time of collection to minimize loss of product through such testing.

Public UCB banks set criteria for the storage of units in order to avoid the collection and storage of UCB units that would not be acceptable for transplantation. Exclusion criteria for potential donors may include multiple gestation; premature delivery; active chorioamnionitis or sepsis; mother being the recipient of an organ transplant; mother with history of cancer; mother with high-risk behaviors or previously diagnosed with human immunodeficiency virus (HIV), hepatitis, or syphilis; and mother having an active venereal disease, such as vaginal herpes simplex and delivering vaginally.

There are also private cord blood banks that usually provide service to parents that enable collection, processing, and storage of cord blood units for future use by their children when they reach adult years. This is performed as an investment in the unknown potential for cord blood to be used to treat serious illnesses in the future. In general there is less than 1% chance of any child to receive HSC transplant in their lifetime, especially if the child has no family history of leukemia or lymphoma. Therefore, the general consensus is that routine cord blood storage in healthy babies is unnecessary because it is highly unlikely even if the child needs transplant in the future, the child's own cord blood will be suitable for the child's transplant due to possible risk for contamination with inherently cancerous cells. Also if the indication for the transplant is a genetic condition, the child's own cord blood will not be suitable because the cord blood will contain the same genetic problem.

In the absence of a suitable matched sibling or unrelated donor, finding a suitable UCB unit or haploidentical donor will be an important consideration. The choice between UCB and haploidentical transplant depends on the urgency of the transplantation, cell dose needed and the expertise at the transplant center. In terms of relapse rate, leukemia-free survival and transplant-related mortality, no significant difference was observed when UCB was compared with haploidentical transplant.

STEM CELL COLLECTION

Bone Marrow Collection Techniques

Bone marrow is typically harvested from the posterior iliac crests using virtually the same techniques used to obtain diagnostic samples in the clinic. The primary differences between obtaining diagnostic specimens and cell quantities adequate for transplantation are the volume of blood and marrow removed, which requires attention to fluid and blood component replacement during the procedure, and the need for appropriate anesthesia. Bone marrow harvesting from healthy donors presents little risk for serious morbidity, permitting the ethical recruitment of allogeneic and syngeneic donors, including pediatric bone marrow donors and donors not related to the recipient. Multiple aspirations are performed with collection of approximately 5 mL of marrow from each puncture site. If properly spaced, no more than two or three skin-puncture sites per side usually are required. Other harvest sites, such as the anterior iliac crests or sternum, can be used, but at increased risk for complications from accidental laceration or perforation of contiguous anatomic structures. For patients with a history of radiation or tumor involvement of one pelvic crest, adequate cells can be harvested from the anterior and posterior crests of the other side.

The prescription for marrow collection will define the desired quantity of nucleated cells per kg recipient weight to be collected. Ideally, this quantity of cells will be collected in a minimal total volume and procedure duration. Although transplant registries may require physicians to be experienced in marrow harvesting, defined as the number of procedures performed, few published studies report a correlation between such experience and harvest yields or donor complications.[8] The nucleated cell yield (cells per volume aspirated) appears greater for needles with side aspiration ports. Smaller quantities aspirated per "pull" also improves cell yield. Warming of the donor may improve cell yield. Quality-assurance management should review for each harvest team the nucleated cell yield per volume of marrow, total volume aspirated, use of blood replacement, and duration of anesthesia.

Marrow is collected in the day surgery suite using either general or regional anesthesia. With proper fluid and blood replacement, overnight hospitalization should not be required. Bone marrow harvesting necessitates placing the donor into the prone position, which has specific considerations to avoid complications directly resulting from this positioning. Donors must be supported, at a minimum, by positioning on chest rolls. For the healthy donor, the risks for serious complications from either general or regional anesthesia are minimal, although a multivariate analysis of adverse events performed by the National Marrow Donor Program for unrelated donors reported a higher risk for serious adverse events for donors receiving regional anesthesia.[9] Use of spinal or epidural anesthesia avoids the nausea that may occur with general anesthesia, especially for younger women, but hypotension from loss of vascular tone in the lower extremities often occurs as the volume of marrow is collected. General anesthesia is preferable for the donor with comorbid disorders, such as cardiovascular or cerebral vascular disease, because of the better control of donor airway and lower risk for hypotension during the harvest procedures. Local anesthesia is acceptable only if a very limited harvest is being performed, because local anesthesia does not achieve anesthesia of the marrow space and because large quantities of lidocaine, for example, are cardiotoxic.

Both heparin and acid-citrate-dextran-A (ACD-A) can be used for anticoagulation of bone marrow products. ACD-A decreases the accumulation of lactic acid and may be preferable, especially for products that will be transported or stored for longer periods before infusion or cryopreservation.[10]

Complications of Bone Marrow Collection

Anesthesia complications present the major health risk to the donor; marrow aspiration is generally well tolerated, although postharvest discomfort is experienced to some extent by all donors. Complications include hemorrhage and infections at skin-puncture sites. Severe hematomas and neuralgias rarely occur, and training regarding pelvic anatomy is required to decrease the risk for damage to vessels and nerves lying under or adjacent to the iliac crest harvest sites. Irritation of the sacral nerves may result from needle penetration through the pelvic bone or from blood tracking into the nerve roots, and requires several months of convalescence. Localized pain is common, may last for several days, and may require a brief period of opioid medication. In a survey of over 9000 donors for unrelated bone marrow transplantation, 82% reported collection site pain, with a median time to recovery of 3 weeks (see Figs. 102.1 and 102.2). Pain associated with the anesthesia procedures (throat pain, 33%; postanesthesia headache, 17%) was reported by a large proportion of the donors. Fatigue was reported by 59% of donors. Serious adverse effects were reported for 125 donors (1.35%), with 116 donors reporting serious complications considered to be a consequence of the collection procedures. Most of the serious complications ($n = 69$) were mechanical injury to tissue, bone, or nerve; and a smaller number ($n = 45$) were related to anesthesia. Infection and grand mal seizure were reported for one donor each. A retrospective survey of donor events reported by the European Group for Blood and Marrow Transplantation described almost 28,000 bone marrow donors, with one death from pulmonary embolism.[11] An additional 12 donors experienced severe adverse events, including four cardiac arrests (three during anesthesia), two episodes of severe hypertension, one pulmonary embolism from heparin-induced thrombocytopenia, one episode of pulmonary edema, one donor with a subdural hematoma, and three events not otherwise specified. This retrospective survey did not include all donors and may have underreported adverse events. This report, furthermore, did not report the experiences of related and unrelated donors separately. The adverse events reported by unrelated donor registries will underestimate the risks faced by donors for related recipients, who may undergo collection despite comorbid illnesses that would preclude participation in an unrelated donor registry. Most donors are able to return to routine activities 1 to 2 days after harvesting, The total recovery time for the 67 donors reporting serious mechanical injury was a median of 10 months (range: 1 to 96 months). Toxicity was

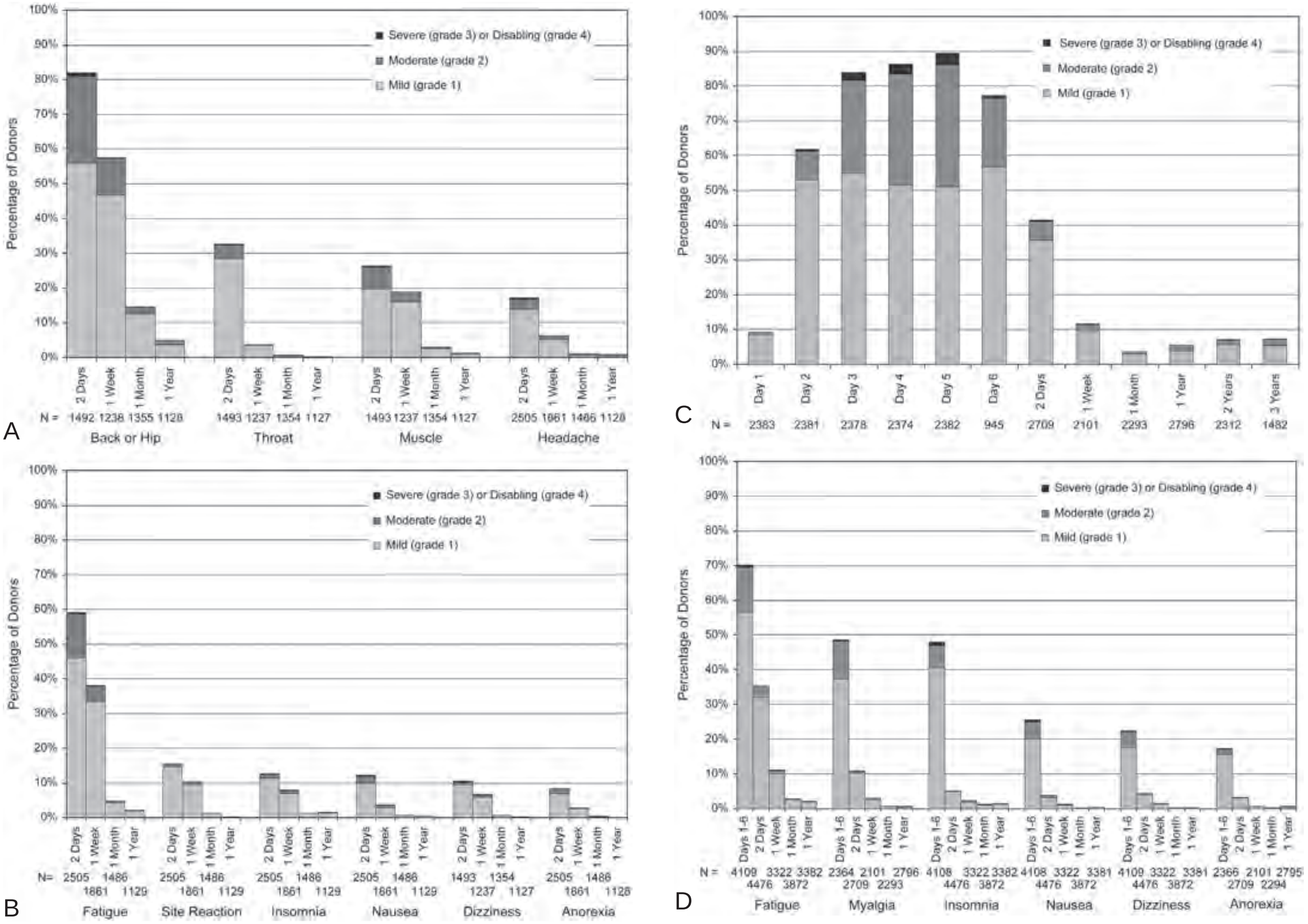


Figure 102.1 (A) Percentage of BM donors reporting pain at selected sites over time. Reports of pain and pain severity were collected at the indicated time points post-donation. Throat pain is largely restricted to donors receiving general anesthesia, whereas headache is more common in donors receiving epidural or spinal regional anesthesia. (B) Six most frequently reported body symptoms experienced by BM donors at the indicated time points post-donation. (C) Percentage of peripheral blood stem cell (PBSC) donors reporting bone pain over time. Reports of bone pain and severity of pain were collected at the indicated time points during mobilization, collection, and post-donation. Day 1 is the first day of filgrastim administration; day 5 is the first day of apheresis. Bone pain represents pain in at least one of the following sites: general bone pain, back, head, limb, joint, hip, and neck. The severity of bone pain is defined as the maximum grade among these pain sites. (D) Six most frequently reported body symptoms experienced by PBSC donors during mobilization and collection, and at the indicated time points post-donation. The percentages for day 1 to day 6 represent the frequencies of the highest grade of symptoms during mobilization and collection. *(From Miller JP, Perry EH, Price TH, et al. Recovery and safety profiles of marrow and PBSC donors: experience of the National Marrow Donor Program. Biol Blood Marrow Transplant. 2008; 14(9 Suppl):29–36. doi: 10.1016/j.bbmt.2008.05.018.)*

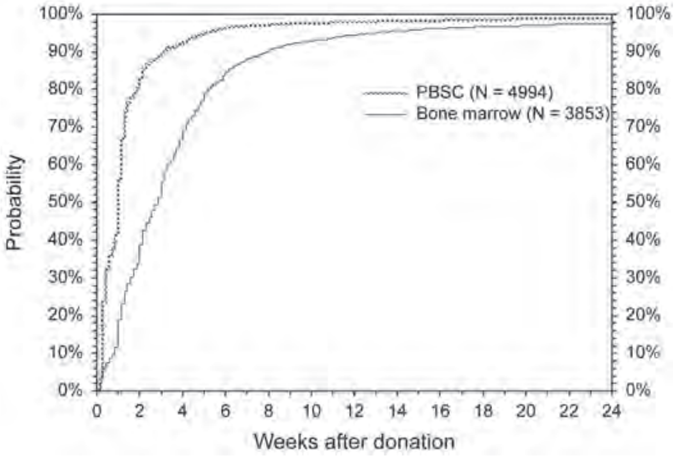


Figure 102.2 Kaplan–Meier plots of time to recovery from stem cell donation (first donations performed from November 2001 through March 2006). *(From Miller JP, Perry EH, Price TH, et al. Recovery and safety profiles of marrow and PBSC donors: experience of the National Marrow Donor Program. Biol Blood Marrow Transplant. 2008;14[9 Suppl]:29–36. doi: 10.1016/j.bbmt.2008.05.018.)*

more likely to occur for less experienced harvest teams, and harvest teams must address training and maintenance of harvest skills in their quality-assurance plans. Any team associated with a second severe mechanical injury should specifically be evaluated, in light of the very low probability of these events.

The usual volume harvested from healthy donors is approximately 10 to 15 mL of marrow per kilogram of recipient bodyweight to achieve the desired nucleated cell and CD34$^+$ cell doses. This results in a blood loss of 800 to 1000 mL for donors providing marrow for an average-sized adult recipient. The quantity of marrow harvested from autologous patients may be greater, reflecting previous chemotherapy given to these patients causing decreased marrow cellularity. Donors for pediatric recipients will lose proportionately less blood. Most patients and donors receive blood transfusions to alleviate symptoms of volume depletion. With proper pre-harvest autologous blood storage, use of homologous blood for healthy first-time allogeneic donors should be extremely rare. For a blood loss of less than 10 mL/kg of donor weight, salt solutions are acceptable for volume replacement. Colloid solutions, such as hydroxyethyl starch, can be used to avoid homologous blood transfusion for blood losses between 10 and 20 mL/kg donor weight. Blood transfusion will be required for larger blood losses (>20 mL/kg) or for patients with comorbid illnesses. Homologous blood transfusions must be irradiated to prevent transfusion-associated GVHD in the transplant recipient caused by “passenger lymphocytes” from the third-party blood donor. Donors undergoing a second harvest shortly after the first harvest are more likely to require homologous blood.[12] Oral iron supplements should be considered for healthy donors, particularly for female donors or donors from whom a proportionately large blood volume is to be harvested.

Collection of Umbilical Cord Blood Stem Cells

Advantages of this source of HSCs include the ability of public cord blood banks to target collections from ethnic-minority populations not well represented in the various unrelated donor registries and the relative immaturity of the donor immune system allowing transplantation of HLA-mismatched units without overwhelming GVHD. The primary obstacle to the widespread use of cord blood cells is the limited quantity of HSCs collected, and one public UCB bank predicted that from less than 5% to less than 38% (depending on the cell dose criterion for transplantation) of the units stored in that bank would be acceptable for transplantation of an 80-kg adult patient. The speed and success of engraftment are predicted by the total nucleated cell dose and, more important, by the quantity of CD34$^+$ cells or infused colony-forming units (CFUs).

CB Collection Techniques

UCB is collected from the placental vein after delivery of the infant and transection of the cord, either before delivery of the placenta by the obstetrician or by laboratory personnel after delivery of the placenta. Published reports conflict regarding the volume of UCB collected and the likelihood of obtaining a product inadequate for storage with either in utero or ex utero collection techniques. The timing of cord clamping after delivery of the infant is associated with the volume of cord blood collected, and greater volumes are collected with earlier clamping. Greater cell quantities were found for infants with greater birth weight, but no difference was found based on gender or gestational age. Ethnic background appears to predict the cell quantities, with smaller quantities of cells collected from persons from other ethnic populations compared with Whites. UCB is usually collected by cannulation of the umbilical cord veins with aspiration of the blood into a collection bag. Collection of cord blood into open containers results in an unacceptable rate of bacterial contamination. Perfusion of the placenta with salt solutions may increase the cell number collected, but this technique has not been widely adopted. Many cord blood banks reduce the volume of the product by red cell and plasma depletion to minimize storage space and to reduce possible infusion-related toxicities from mature blood cells contained in unfractionated cord blood units. Bacterial contamination of UCB products is of concern, especially in the collection of products for related donor transplantation by obstetricians with limited or no experience in HSC collection and processing. The identification and evaluation of the donor and the collection techniques used should be viewed as the first steps in a manufacturing process with adequately validated procedures, personnel training, quality control, and performance improvement oversight.

COLLECTION PERIPHERAL BLOOD STEM CELLS

The presence of HSCs in the peripheral circulation was suggested by animal studies as early as 1951.[13] Although the nature of the survival agent was not recognized at that time, parabiosis experiments demonstrated that some factor in the blood of a healthy animal was able to rescue another animal from the effects of lethal irradiation. Subsequently, a number of animal models demonstrated the presence of HSCs in the peripheral blood and the successful use of these cells to rescue animals from the marrow-lethal effects of radiation. The concentration of HSCs in the peripheral blood is normally very low, requiring the processing of large quantities of blood to collect the quantity of HSCs equivalent to what could be collected in a bone marrow harvest. For this reason, PBSC transplantation was initially used by a few transplant programs that explored this source of HSCs for patients who otherwise were ineligible for marrow harvesting, collecting cells during steady-state hematopoiesis or during the transient increase in circulating HSCs that occurred during recovery from marrow hypoplasia-producing chemotherapy. These early reports noted that engraftment could be achieved sooner after infusion of PBSC components compared with marrow cell transplantation. However, because of the occasionally limited quantity of HSCs that was collected from the peripheral blood, the kinetics of engraftment for some patients was considerably slower. The effective mobilization of HSCs achieved by cytokine or chemokine administration, and the reliability of same-day flow cytometric analysis in assessing the quality of the collection, are the direct bases for the rapid and widespread adoption of PBSCs as a source of HSCs for transplantation (see box on Mobilization and Collection of Peripheral Blood Stem Cell for Autologous Transplantation).

Mobilization

The self-renewal and differentiation of HSCs is controlled by the surrounding microenvironment of the stem cell niche(s) in which the HSCs reside. These niches are composed of a complex three-dimensional architecture of a variety of cell types, including sinusoidal

MOBILIZATION AND COLLECTION OF PERIPHERAL BLOOD STEM CELL FOR AUTOLOGOUS TRANSPLANTATION

Five important considerations when prescribing a mobilization regimen for collection of peripheral blood stem cells (PBSCs) for autologous transplantation are as follows: (1) A regimen of chemotherapy followed by granulocyte colony-stimulating factor (G-CSF) results in higher numbers of circulating $CD34^+$ cells than will be achieved with G-CSF alone. (2) The choice of chemotherapy (or cytokine alone mobilization) should be appropriate to the disease and stage of disease for the patient. (3) Each cycle of prior chemotherapy and any previous treatment with radiotherapy will decrease the response to mobilization therapy. (4) Tumor infiltration of the marrow will increase the probability of circulating tumor cells and will decrease the response to mobilization therapy. (5) Some patients will benefit from tandem cycles of dose-intense therapy, and the prescription should target adequate quantities of $CD34^+$ cells for these patients. With these considerations in mind, elective collection of PBSCs either before extensive treatment or after a limited number of cycles of debulking chemotherapy should be considered. Additional cycles of chemotherapy can be given after PBSC collection is completed, for those patients who require further tumor reduction before proceeding to transplantation. The timing of apheresis after chemotherapy and G-CSF mobilization is best guided by measurement of the level of peripheral blood $CD34^+$ cells. Daily or every-other-day quantification of these cells can be initiated after the white blood cell count reaches 1000/μL. Patients with poor mobilization of $CD34^+$ cells should be considered for addition of a chemokine such as plerixafor to improve $CD34^+$ cell yield and reduce the costs associated with daily doses of G-CSF, laboratory testing, apheresis procedures, and cryopreservation. Patients who fail to mobilize may have successful collections if given a short drug "holiday" before undergoing mobilization with high-dose G-CSF with plerixafor.

endothelial cells, sympathetic nerve fibers, cells of the osteoblastic lineage, macrophages, and mesenchymal stem cells that are responsible for controlling the balance between HSC quiescence, self-renewal, and differentiation. A number of pathways with mutually recognized cellular adhesion molecules and their respective ligands responsible for the spontaneous migration of HSCs from the stem cell niche, as well as the multistep process of homing back into the niche, have been identified. The mechanisms by which G-CSF and other cytokines promote mobilization of HSCs are being elucidated and appear to be an indirect effect (HSCs do not express receptors for G-CSF) on the CXC-chemokine receptor 4 (CXCR4)/stromal cell-derived factor-1 (SDF-1) axis mediated by monocytes and the sympathetic nervous system. The mechanisms of mobilization by chemotherapy and CXCR4 antagonists such as plerixafor are also being determined, and the elucidation of the mechanisms of mobilization and homing may result in more effective harvesting and transplantation techniques.

Cytokine Mobilization

The ability of recombinant hematopoietic cytokines to increase the level of myeloid progenitor cells in the blood, as well as mature blood cells, was reported in 1988 by different groups for both G-CSF and granulocyte–macrophage colony-stimulating factor (GM-CSF).[14,15] Subsequently, a number of different investigators reported the collection of PBSCs from patients using a variety of mobilization regimens, including cytokines alone, cytokine combinations, and combinations of chemotherapy with cytokines. Various other recombinant human hematopoietic cytokines, including erythropoietin and fusion molecules, increase the quantity of $CD34^+$ cells in the peripheral blood but have not been developed for clinical transplantation.

Granulocyte Colony-Stimulating Factors Including Biosimilars

G-CSF is the cytokine most commonly used because of its efficacy compared with other cytokines and its relatively benign toxicity profile. Recombinant methionyl human G-CSF (filgrastim) and recombinant human G-CSF (lenograstim) were the two forms of this cytokine initially available for clinical use. There are slight differences between these two similar cytokines in their ability to mobilize PBSCs. Watts and colleagues[16] studied 20 healthy volunteers and found that peak levels of colony-forming unit–granulocyte-macrophages (CFU-GMs) in the peripheral blood were 28% higher after treatment with the glycosylated molecule (lenograstim), attributed to the higher specific activity of this form. De Arriba and colleagues[17] treated 30 women with breast cancer in a randomized study of these two drugs, using dosages containing bioequivalent units of activity and found no difference in mobilization of $CD34^+$ cells. The two forms have otherwise similar biologic activity and are not further distinguished in this discussion.

Regulatory agencies, including the United States Food and Drug Administration (FDA), the European Medicines Agency, and others, established regulatory pathways leading to approval of biologic medicines that are highly similar to an already-approved biologic product ("biosimilar"). In the United States, the Biologics Price Competition and Innovation Act of 2009 created an abbreviated licensure pathway for biological products shown to be "biosimilar" to or "interchangeable" with an FDA-licensed biologic product, known as the "reference product." This abbreviated licensure pathway permits reliance on existing scientific knowledge about the safety and effectiveness of the reference product, and enables a biosimilar biologic product to be licensed based on less than a full complement of product-specific preclinical and clinical data usually required before marketing a new drug. The biosimilar must show it has no clinically meaningful differences in terms of safety and effectiveness from the reference product. Only minor differences in clinically inactive components are allowable. Filgrastim-sndz is the first biosimilar drug approved by the FDA and is marketed with the same indication for use as filgrastim. Tbo-filgrastim, however, was licensed under a different mechanism, and does not have FDA approval for use in PBPC collection. The use of a biosimilar G-CSF in mobilization of PBPCs must be extrapolated from laboratory data such as mobilization of $CD34^+$ cells, and it is conceivable, although considered very unlikely, that differences in the mobilization of stem and other cells, as well as in toxicity to the donor, may occur. Clinical experience describing similar mobilization results are now being reported for these drugs.[18]

Mobilization of Hematopoietic Stem Cells Using Granulocyte Colony-Stimulating Factor

G-CSF is the most potent cytokine currently available for mobilization of HSCs. In a randomized study of healthy volunteers comparing G-CSF, GM-CSF, and the combination of both, Lane and colleagues[3] reported an average 0.99% $CD34^+$ cells in the peripheral blood of healthy donors treated with 10 μg/kg/day of G-CSF compared with 0.25% for donors treated with the same dose of GM-CSF. The quantity of $CD34^+$ cells in the peripheral blood before treatment averaged 1.6/μL. After GM-CSF treatment, the level increased to 3/μL, but with G-CSF, the level increased to 61/μL. Each group underwent one leukapheresis on the fifth day of treatment, and the collections from donors treated with G-CSF averaged 119×10^6 $CD34^+$ cells compared with 12.6×10^6 for the donors treated with GM-CSF.

The appearance of $CD34^+$ cells during administration of G-CSF follows a distinct time course, with the maximal level of $CD34^+$ cells occurring on day 5 after daily G-CSF administration. Smaller numbers of $CD34^+$ are present on days 4 and 6, and the level falls rapidly on subsequent days despite a continual rise in white blood cell (WBC) count.

The number of $CD34^+$ cells collected after G-CSF treatment is proportional to the number of these cells in the peripheral blood before initiation of the cytokine. Although doses as low as 5 mcg/kg/day have been used, there is a dose response to G-CSF, with higher average levels of $CD34^+$ cells achieved with 10 mcg/kg/day compared with 5 mcg/kg/day. With appropriate dosing of allogeneic donors, adequate numbers of $CD34^+$ cells can be collected in one procedure for transplantation of most patients. A similar dose response is observed in autologous patients and may extend to doses as high as 40 mcg/kg/day. An advantage to twice-daily dosing of G-CSF has been suggested but not confirmed. Anderlini and colleagues[19] compared administration of 6 mcg/kg given twice daily with 12 mcg/kg given once daily and found no differences in CD34$^+$ cells per liter of blood processed during the apheresis procedure or the total yield of CD34$^+$ cells per kilogram collected. In contrast, a second trial enrolling primarily pediatric patients noted better results with the twice-daily schedule.[20] Patients, especially those previously treated with chemotherapy or radiotherapy, will generally have lower quantities of CD34$^+$ cells mobilized.

Toxicity and Complications of Granulocyte Colony-Stimulating Factor

The toxicity of G-CSF has been most clearly defined in studies of allogeneic donors. The autologous patient will experience a similar toxicity profile, but with the added complications of the underlying malignancy and its treatment.

Almost all recipients of G-CSF will develop somatic complaints, of which skeletal pain is most prominent (see Fig. 102.1), but also fatigue, insomnia, and nausea. The somatic complaints are generally tolerable, and few donors will require reduction in dose or discontinuation of the medicine. At present, there appear to be minimal, if any, long-term health risks for the donor. Few serious complications of the mobilization regimen and donation process have been reported. G-CSF increases spleen size, with a rare patient or donor experiencing splenic rupture. G-CSF may induce a hypercoagulable state, which is of concern for donors (and patients) requiring central venous catheter placement or for those who may have other risk factors for the development of deep venous thrombosis (such as air travel immediately after the collection procedures). Patients with autoimmune disorders may experience a flare-up of their disease during administration of G-CSF, and a variety of case reports of ophthalmologic and other adverse events have been reported for healthy donors or patients treated with G-CSF.

Of concern is the possibility that cytokine administration will increase the risk for marrow dysplasia or malignancy. Although this is a theoretical concern in that these cytokines are known to stimulate growth of leukemia cells, no clinical evidence from large registry reviews of healthy donor experiences indicates that these agents will induce abnormalities in the HSC.

G-CSF administration results in a number of changes in blood counts and chemistries in addition to the coagulation factor changes. Alanine aminotransferase, lactate dehydrogenase, and alkaline phosphatase levels increase, and the levels of blood urea nitrogen and bilirubin may decrease. The elevation in alkaline phosphatase level is primarily of bone origin; γ-glutamyl transferase levels remain normal. These abnormalities of serum chemistries resolve within 2 weeks after discontinuation of the medication. G-CSF administration also will result in a decrease in platelet count, especially if the cytokine is administered over 5 to 10 days. WBC counts fall rapidly after discontinuation of G-CSF. In approximately 10% of donors, the WBC count may fall to abnormal levels (but generally still remain above 1000/μL), reaching a nadir 10 to 14 days after discontinuation of the cytokine before stabilizing at normal levels.

Plerixafor

Chemokines (chemoattractant cytokines) are a family of approximately 40 related, small proteins that influence leukocyte (and malignant cell) migration and function. Chemokines with varying effects on different WBC populations have been identified, as have a number of chemokine receptors. The roles of chemokines and chemokine receptors in the trafficking of HSCs into and from the bone marrow compartment are under active investigation.

In a preclinical study, administration to both mice and rhesus monkeys of a modified CXC chemokine growth-related oncogene-β after 4 days of G-CSF resulted in a fivefold increase in the number of circulating stem and progenitor cells compared with G-CSF alone, also with a much shorter time course of release, measured in hours. Furthermore, more rapid recovery of hematopoietic function was observed for animals given similar quantities of cells collected after administration of the chemokine and cytokine combination. Interleukin (IL)-8, a related ligand for the CXCR2, mobilizes stem cells within 15 to 30 minutes of injection into mice, which appears to involve increased matrix metalloproteinase-9 activity detectable immediately before the appearance of HSCs in the peripheral circulation.[21] Murine studies demonstrate a mobilizing effect of the chemokine SDF-1 and its receptor CXCR4 that can be blocked by neutralizing antibodies. The bicyclam molecule plerixafor disrupts SDF-1/CXCR4 binding and has been shown to result in mobilization of HSC in murine, canine, and nonhuman transplant models.

Similar signaling pathways are used for mobilization and homing of normal and malignant hematopoietic cells. Although PBSC products collected after G-CSF mobilization generally contain fewer detectable tumor cells than does bone marrow harvested from the same patient, the effects of chemokine treatment on the degree of PBSC contamination and on disease relapse after autologous transplantation remain to be established. For example, acute myelogenous leukemia cells express CXCR4 in varying levels, and homing of primary human acute myeloid leukemia cells into nonobese diabetic/severe combined immunodeficiency mice is CXCR4 dependent, similar to normal human stem cells. Similar studies of chemokine control on tumor cell growth, migration, and metastasis are being reported from a number of laboratories. There are now reports of a possibly increased risk of posttransplant myelodysplasia or acute leukemia after transplantation using plerixafor-mobilized cells, although others have reported that poor mobilizers (who are more likely to receive this chemokine) have a higher risk of posttransplant myelodysplasia, complicating determination of causation of this complication of transplantation.

COMBINATION REGIMEN

Plerixafor Plus Granulocyte Colony-Stimulating Factor

Plerixafor (AMD3100) is a small-molecule inhibitor of CXCR4. Clinical studies of HSC mobilization using plerixafor with or without G-CSF priming are being reported in both healthy donors and patients undergoing autologous or allogeneic HSC transplantation. In a study involving normal volunteers, a single dose of plerixafor was equal in mobilization of CD34$^+$ cells to administration of a standard 5-day course of G-CSF.[22] However, a single dose of plerixafor given on day 5 of a daily course of G-CSF administration resulted in a further 3.8-fold increase in circulating CD34$^+$ cells (as well as B and T cells, which may be important in allogeneic transplantation). Randomized phase III studies conducted in patients undergoing autologous PBSC transplantation in the treatment of multiple myeloma or non-Hodgkin lymphoma demonstrated a clear increase in CD34$^+$ cells collected by the addition of plerixafor to filgrastim.[23,24] Plerixafor was also effective in remobilization attempts for patients who failed initial collection goals. Plerixafor clearance is proportional to the degree of renal function in patients with renal failure. Minor gastrointestinal symptoms appear to be the most common toxicities of this medication.

Chemotherapy Plus Cytokine Mobilization

The number of HSCs in the peripheral blood is moderately increased during the early hematologic recovery phase after marrow hypoplasia-producing chemotherapy. Chemotherapy plus cytokine generally mobilizes greater numbers of PBSCs than either agent alone. This finding was confirmed in a randomized study comparing cyclophosphamide followed by G-CSF versus G-CSF alone, in which higher numbers of CD34 cells were found for the patients treated with the chemotherapy-based regimen. However, no differences in the degree of tumor cell contamination of PBSC components, speed of hematological recovery after transplantation, or survival probability were found. A wide variety of different chemotherapy regimens has been used successfully for mobilization of HSCs into the blood, with cyclophosphamide- or ifosfamide-based regimens most commonly used. The primary consideration is that the choice of chemotherapy used for mobilization must also meet the treatment needs of the patient. Demirer and colleagues[25] studied the effect of different chemotherapy regimens for mobilization of HSCs for patients with breast cancer. Four regimens were used, all involving cyclophosphamide (CY), but including etoposide with or without cisplatin, or paclitaxel. All patients also received G-CSF. The median quantity of $CD34^+$ cells collected on the first day of apheresis after cyclophosphamide mobilization was 0.9×10^6 per kilogram of patient weight. The addition of etoposide and then of etoposide and cisplatin increased the first-day yield to 8.1×10^6 and 3.5×10^6 $CD34^+$ cells/kg, respectively, in separate cohorts of patients. The median number of $CD34^+$ cells harvested on the first day of apheresis after cyclophosphamide plus paclitaxel was 11.1×10^6/kg, and more than 50% of the women mobilized with this last regimen achieved the target dose of $CD34^+$ cells in one apheresis procedure. Of the 100 women studied, 94 achieved the target dose of greater than 5×10^6 $CD34^+$ cells/kg. Only four patients failed to reach a lower but acceptable dose of 2.5×10^6 $CD34^+$ cells/kg.

Granulocyte-Macrophage Colony-Stimulating Factor Plus Granulocyte Colony-Stimulating Factor

Much of the early experience with the use of hematopoietic cytokines for mobilization of HSCs involved GM-CSF and chemotherapy. GM-CSF is not as potent as G-CSF, although as with G-CSF, there is a dose response in mobilization of PBSCs with GM-CSF over a range from 0.3 to 30 mcg/kg/day, without a plateau. In this dose-ranging study, however, the average increase in CFU-GM in the blood at this highest dose level again was only 8.4-fold. In a randomized study comparing GM-CSF with G-CSF, or both drugs used in sequence after chemotherapy administration, patients had faster recovery of counts, required less supportive care including transfusions, and achieved greater collections of $CD34^+$ cells if given one of the G-CSF–containing regimens.

Administration of GM-CSF results in somatic complaints and hepatic function abnormalities similar to those reported after G-CSF administration. In addition, 44% to 80% of patients experience fever, sometimes after each dose, as well as generalized or local skin reactions. Doses greater than 20 mcg/kg/day are poorly tolerated because of fluid retention, pleural and pericardial inflammation, and venous thrombosis. A "first-dose reaction," characterized by hypoxia and hypotension occurring within 3 hours of administration, has been described for some recipients, especially after intravenous administration.

Other Hematopoietic Cytokines

Recognition of the mobilization potential of G-CSF led many investigators to study other hematopoietic cytokines, including erythropoietin, monocyte–colony-stimulating factor, IL-3, PIXY 321 (a fusion molecule), and stem cell factor (SCF) for their capacity to mobilize HSCS into the peripheral blood. Used as single agents, these cytokines resulted in only an approximately 5- to 10-fold increase in circulating CFU-GM or $CD34^+$ cells.

Strategies for the Patient Who Is Difficult to Mobilize

Most patients achieve the targeted dose of $CD34^+$ cells after processing 20 to 30 L of blood in one to three apheresis procedures. However, approximately 5% to as many as 30% of patients in various series have inadequate collections because only small numbers of HSCs are present in the peripheral blood despite the administration of hematopoietic cytokines. With wide use of plerixafor in combination with G-CSF, the rate of poor mobilization has significantly diminished to less than 5%. Of note, the number of days of apheresis also predicts the kinetics of neutrophil engraftment independent of the $CD34^+$ cell dose infused, indicating an undefined measurement of graft quality, probably a reflection of the various quantities of $CD34^+$ cell subsets and $CD34^-$ stem cells, as well as the characteristics of the stem cell niche. Patient-specific factors predictive of poor mobilization include older age, marrow disease, prior radiotherapy, and prior chemotherapy. Approximately 50% of patients who fail to achieve the targeted dose of $CD34^+$ cells will achieve this goal on a second attempt. High-dose (15 mg/kg twice daily) G-CSF after a 2- to 4-week drug holiday to allow marrow recovery is one strategy. Combination cytokine therapy is also of potential value in this situation, and the combination of SCF with G-CSF may be effective for patients who reside in countries where SCF is available. The addition of GM-CSF to G-CSF is not of proven value. The addition of plerixafor will often be effective when used in combination with G-CSF for collection of PBSCs from patients who failed a prior mobilization attempt. Treatment with the use of cyclophosphamide- or ifosfamide-based mobilizing chemotherapy plus a cytokine regimen also will be effective, but will be associated with increased toxicity. Bone marrow collection is very unlikely to achieve an acceptable product in that the poor mobilization of PBSCs predicts for a poor marrow harvest. Patients who fail initial collection attempts will frequently fail subsequent attempts, and transplantation of these patients with a lower dose of $CD34^+$ cells (as low as 1×10^6 $CD34^+$ cells/kg) may be an option.

It is, therefore, strongly preferable to avoid collection failure. Consideration should be given to the prophylactic collection of PBSCs early in the course of treatment for patients who later may be candidates for autologous HSC transplantation but who are advised to receive multiple courses of therapy or therapy involving alkylating agents or radiation therapy. HSCs can be collected and cryopreserved before extensive therapy while the patient has good marrow function and stored for years without obvious progressive loss of engraftment potential.

COLLECTION OF PERIPHERAL BLOOD STEM CELLS BY APHERESIS

Apheresis Technology

A number of apheresis devices are available for separating mononuclear cells (MNCs) containing HSCs from the peripheral blood. The devices may be classified as continuous flow (e.g., Fenwal CS3000, COBE Spectra, Spectra Optia [CMNC platform only], Fenwal Amicus) or discontinuous flow (e.g., Haemonetics family of equipment). Discontinuous-flow devices have the advantage of requiring only a single venous access. Continuous-flow devices require two access lines for aspiration and return of blood, but they process much larger volumes of blood in a shorter period of time. All apheresis devices collect MNCs. Continuous-flow devices are more efficient in the collection of PBSCs and are, accordingly, preferred over discontinuous-flow devices (see box on Physician Prescription for Apheresis and Peripheral Blood Stem Cell Processing). The ideal apheresis device will have: a high efficiency of $CD34^+$ cell collection with minimal contamination of the product by mature blood cells such as granulocytes, which complicate subsequent processing and can increase infusion toxicity; rapid blood processing with minimal anticoagulant use reducing toxicity to the donor; and minimal depletion of platelets. The various apheresis devices, in general, have

PHYSICIAN PRESCRIPTION FOR APHERESIS AND PERIPHERAL BLOOD STEM CELL PROCESSING

The attending physician should be aware of apheresis and laboratory procedures in order to maximize the value of peripheral blood stem cell (PBSC) products. The choices of venous access, anticoagulant(s), blood volume processed, target dose of CD34$^+$ cells, and cryopreservation volumes can be individualized. Apheresis unit staff may ask for guidance regarding pain medications, concurrent medications, and blood transfusions (although it is advisable to avoid transfusion during the apheresis procedure because of citrate or other reactions, and because changes in the hematocrit may affect the efficiency of the collection). Adequate numbers of CD34$^+$ cells can be collected for more than one cycle of chemotherapy. It is important to communicate with the cryopreservation facility if PBSC components will be used to support more than one cycle of chemotherapy or if dimethyl sulfoxide toxicity is of concern, in order to facilitate appropriate packaging of each product. Current cytometric techniques for quantification of CD34$^+$ cells require at least 1 h of processing, so it may not be practical to prescribe the number of CD34$^+$ cells to be frozen in each bag. However, it is possible to divide the component into the number of bags equaling the number of anticipated infusions so that equal numbers of CD34$^+$ cells will be available for each.

similar efficacies in collecting CD34$^+$ cells from the peripheral blood, but may vary in processing time, and in final product volume and mature blood cell contamination.

Apheresis technology is widely used for collection of platelets and other blood products from healthy donors and is considered to be without major risk to the donor. The important safety considerations for PBSC collection are the same as for platelet collection and include the venous access to be used for the procedure, the extracorporeal volume of blood during the procedure, and the solutions administered to the donor. Of note, however, PBSC collection for autologous transplantation involves patients with underlying medical conditions who may require considerable nursing care during the procedure. In a prospective evaluation of 2408 healthy unrelated volunteer donors, apheresis-associated adverse events were reported by 20% of female donors ($n = 964$) and 7% of male donors ($n = 1444$) on the first day of apheresis, falling to 10% and 4%, respectively, on the second day of collection (if performed). Most (51%) of reported adverse events were related to citrate infusion. A smaller proportion of donors (22%) reported problems with venous access. Rare (1% to 6% of events) adverse events included hypertension or hypotension, allergic reactions, fatigue, and syncope. The placement of a central venous catheter was required by 17% of female donors and 4% of male donors. Also, larger-volume and repetitive exchanges will result in platelet depletion. The platelet count may reach its nadir several days after completion of the apheresis collections and discontinuation of G-CSF, and donors should be counseled in this regard.

Important caveats are that the donors in this analysis from the unrelated donor registry met strictly defined health criteria and were between 18 and 69 years of age. A higher probability of adverse events may be expected in patients and in donors of older (or younger) age. For example, in an older retrospective publication, Goldberg and colleagues[12] studied the complications occurring during 554 PBSC collections from 75 consecutive patients. Patient diagnoses and the mobilization treatment regimens were varied. All but one patient had subclavian or jugular venous system catheters placed for apheresis. A median of nine collections per patient were performed using a discontinuous-flow apheresis device. The most common problems were related to the venous catheters: 50% of patients developed at least one occlusion. Hypocalcemia occurred in 14.6% of patients and hypotension in 13.3%. Sixteen percent of patients experienced infectious complications during the PBSC collection period.

Staffing of the apheresis unit should be appropriate for the medical condition of the patients undergoing apheresis. Staffing must include nurses familiar with the care of the oncologic patient who may be recovering from marrow hypoplasia complicated by neutropenic fever requiring multiple medications and the care of a central venous catheter. Collection of PBSCs by apheresis in the outpatient setting should be performed only after careful review of the medical support requirements for the individual patient or donor, and it should never be assumed to be a safer alternative for the donor or patient with a serious comorbid illness than marrow harvesting conducted in the intensive care setting of the operating room.

Timing of Apheresis Using Granulocyte Colony-Stimulating Factor and Chemotherapy

A major problem with chemotherapy-based mobilization regimens is the difficulty in determining the optimal time to commence HSC collection. Apheresis devices can collect only those CD34$^+$ cells actually being released into and circulating in the peripheral blood. It is possible to estimate the quantity of CD34$^+$ cells that will be collected during apheresis by multiplying the quantity of CD34$^+$ cells in the blood by the total volume of blood processed during the apheresis procedure and by the efficiency of the apheresis device in collecting these cells. If the device has an efficiency of 50% and the patient undergoes a 10-L exchange, approximately 5×10^7 CD34$^+$ cells will be collected for a peripheral blood level of 10 CD34$^+$ cells/μL and 5×10^8 CD34$^+$ cells for a blood level of CD34$^+$ cells that is 10-fold higher. Although many protocols call for initiation of apheresis after chemotherapy mobilization when the WBC count has recovered to greater than 1000/μL, there is a poor, if any, correlation between the peripheral blood white cell or MNC counts and the CD34$^+$ cells in the peripheral blood. Characteristics that suggest a higher CD34$^+$ cell level are a rapidly rising WBC count, shift in differential to immature myeloid cells, circulating nucleated red cells, and platelet transfusion independence. However, it is much more cost-effective to obtain an actual measurement of CD34$^+$ cells in the blood and to time the apheresis collection when these cells are present in adequate numbers. Fig. 102.3 shows such a relationship for patients with lower concentrations of CD34$^+$ cells in the peripheral blood and illustrates the very poor collections obtained when the peripheral blood CD34$^+$ count is less than 10/μL. Although there is considerable error in the enumeration of CD34$^+$ cells at levels less than 5/μL, this error is not clinically relevant because even a doubling of the CD34$^+$ cells in this low range still results in a very poor apheresis yield.

The level of CD34$^+$ cells in the peripheral blood at which to start apheresis is a clinical decision. Although levels in the range of 50 to 100/μL or greater will reduce the number of apheresis procedures necessary to achieve a target goal of CD34$^+$ cells, each day's delay in initiating apheresis incurs the costs of additional cytokine administration and blood testing. In some patients who previously

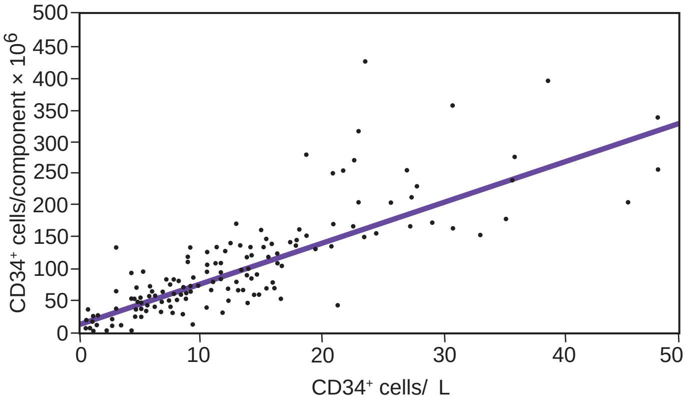


Figure 102.3 RELATIONSHIP BETWEEN QUANTITY OF CD34$^+$ CELLS IN THE PERIPHERAL BLOOD AND NUMBER COLLECTED BY APHERESIS USING APHERESIS AND FLOW CYTOMETRIC TECHNIQUES. Data shown are limited to peripheral blood CD34$^+$ cell numbers <50.0/μL ($n = 157$, $r = 0.82$, $P < .001$).

have undergone extensive treatment and who may show a slowly rising WBC count, multiple apheresis procedures may be necessary to achieve the target goal. No patient should undergo apheresis if the peripheral blood CD34$^+$ cell count is less than 5/μL (see Fig. 102.3). For patients with CD34$^+$ cell counts in the range of 10 to 20/μL, it is possible to process more blood per day using large-volume leukapheresis (LVL) techniques, thereby reducing the numbers of days the patients are required to return to the apheresis unit. Most patients mobilized with chemotherapy have a rising CD34$^+$ cell count in the peripheral blood, so starting apheresis the day after the patient has achieved a desirable CD34$^+$ level generally is feasible.

The timing of apheresis after G-CSF mobilization differs from the timing after chemotherapy and cytokine mobilization. For both patients and healthy donors, the peak concentration of CD34$^+$ cells occurs on day 5 of G-CSF administration (after four daily doses). Lower levels are present on day 4, and the concentration continues to fall after day 6, even if cytokine administration is continued and despite a possible continued rise in WBC count. Thus, PBSC collection should be initiated on day 4 or 5 of G-CSF administration. The kinetics of CD34$^+$ cells in the peripheral blood for patients and donors mobilized with G-CSF alone are so reliable that monitoring of peripheral blood levels is not necessary unless there is concern that the patient has failed to mobilize cells and it is practical to obtain the cell count rapidly enough to initiate apheresis on the same day.

Timing of Apheresis Using Granulocyte Colony-Stimulating Factor and Plerixafor

Plerixafor administration results in a rapid mobilization of HSCs into the peripheral blood, with peak CD34$^+$ cell and CFU-GM concentrations occurring about 6 to 10 hours but with a wide range of activity extending to over 24 hours after administration of a dose of 240 mcg/kg. The initial phase III studies demonstrating the efficacy of this agent in patients failing to mobilize adequate cells with G-CSF alone required administration of plerixafor about 10 hours before apheresis, and then daily administration of plerixafor on the same schedule until completion of collections. The broad duration of activity may allow alternate timing schedules and, conceivably, two apheresis procedures after one dose of plerixafor (particularly for patients with renal impairment). In a study of 11 patients who failed two previous attempts to collect PBSCs using G-CSF alone, Lefrère et al.[26] found an early rise in circulating CD34$^+$ cells, as early as 3 hours, with a fall in circulating CD34$^+$ cells occurring after 8 to 12 hours. Plerixafor allows for rapid mobilization of CD34$^+$ PBSC especially when used in combination with G-CSF. However, according to the manufacturer the peak CD34$^+$ PBSC in the blood is achieved in 10 to 11 hours and therefore leukapheresis is recommended when the peak is likely to be reached. This creates a logistical challenge because if the collection is to start during regular working hours, the Plerixafor needs to be given in the evening the day before. Even though about 10 hours is needed to reach the peak CD34$^+$ cell level in the blood, CD34$^+$ cell level begin to rise as early as 1 hour post-plerixafor administration. Same day plerixafor and leukapheresis had been explored with some success, though it took more days to achieve CD34$^+$ PBSC collection goals.

Large-Volume Leukapheresis

The apheresis device has a uniform and fairly reproducible efficiency of collection. Thus, for a consistent quantity of blood processed through the machine, the quantity of CD34$^+$ cells collected is directly related to the number present in the peripheral blood. Greater quantities of CD34$^+$ cells can be collected by increasing the number of these cells in the peripheral circulation or by increasing the volume of blood processed in each procedure (LVL). For patients with lower CD34$^+$ cell levels, multiple apheresis procedures will be required to achieve the target dose of CD34$^+$ cells needed for transplantation. An alternate approach is to process the same total quantity of blood but in fewer, longer procedures. LVL is not standardly defined, but in general usage it refers to processing of more than two or three times the patient's blood volume. Typically, the quantity of blood processed is six or more times the patient's blood volume, often 25 to 36 L of blood. The advantage of LVL is that it reduces the number of days of cytokine administration and apheresis, with associated reduced costs of laboratory processing and testing. The apheresis techniques are the same as those used for processing of smaller volumes of blood, although blood flow rates may be increased to reduce the time required. The risks of LVL are the increased time required and the higher risk for citrate (or other anticoagulant) toxicity. Patients will also incur a proportional drop in platelet counts and may become profoundly thrombocytopenic.

Most reports of LVL describe the collection of more CD34$^+$ cells than are calculated to be present in the peripheral blood at the initiation of the apheresis procedure. This results from the ongoing release of cells from the marrow replacing those cells removed by apheresis (or returning to the marrow space). Apheresis of CD34$^+$ cells is a three-compartment system consisting of the extracorporeal circuit of the apheresis device (including the collection bag), the peripheral blood, and the marrow. It is not obvious that the apheresis technique itself "mobilizes" CD34$^+$ cells. Apheresis-induced mobilization, if it occurs, may be related to a decrease in divalent cations resulting from the citrate anticoagulant, possibly affecting cell adhesion forces.

Studies at the Fred Hutchinson Cancer Research Center demonstrated a continuous release of CD34$^+$ cells from the marrow (and, presumably, return to the marrow space).[27] Patients having higher levels of CD34$^+$ cells in the peripheral blood appeared to have a greater number of these cells circulating between the marrow and peripheral blood compartments. In this model the apheresis device merely serves as a siphon, removing these cells from the blood as they are released from the marrow. If this description of CD34$^+$ cell kinetics is accurate, it may be possible to deplete these cells from the blood and marrow by prolonged processing, but probably only if limited numbers of them are present in the marrow compartment. Also, the model suggests that higher blood flow rates used to shorten the apheresis procedure may be counterproductive for patients with low CD34$^+$ cell levels in the blood because of the slower rate of release of CD34$^+$ cells in these patients.

Anticoagulation During Apheresis

Anticoagulants are added to the blood during apheresis to prevent clotting in the extracorporeal circuit and clumping of cells in the product. Citrate anticoagulants have a proven record of safety in the apheresis of healthy platelet donors. The major drawback is the risk for a symptomatic decrease in the level of ionized calcium ("citrate toxicity"), especially during processing of large volumes of blood. Citrate ions chelate calcium ions (and other divalent cations such as magnesium), making them unavailable for Ca^{2+}-dependent metabolic reactions. ACD-A contains 10.67 g of citrate per 500-mL volume in the form of trisodium citrate and citric acid. Citrate is diffused throughout the extravascular space, and this diffusion is the first defense against citrate toxicity. The body size and difference in muscle mass between men and women results in an increased risk for citrate toxicity for women in particular and smaller donors in general. Metabolism by liver, kidney, and muscle also reduces the concentration of citrate. Metabolism of citrate becomes an important clinical consideration during processing of larger blood volumes or at higher rates of blood flow. The initial signs of citrate toxicity include circumoral or acral paresthesias and may progress to nausea, vomiting, loss of consciousness, tetany, and seizures. Because pediatric patients may not be able to relate the initial symptoms of the condition, citrate toxicity should be considered as the cause of any change of behavior, such as crying, during the apheresis procedure. Citrate toxicity is prevented by limiting the quantity of citrate infused, either by decreasing the blood flow rate through the apheresis device or changing the blood-to-citrate ratio. The processing of blood from patients experiencing the initial symptoms of citrate toxicity should be temporarily halted until the symptoms abate, and then resumed at a slower rate. The benefit of oral calcium supplements for these patients is not proven and may cause abdominal discomfort. Heparin can be used as a replacement for some or all of the citrate, although additional citrate should be added to the component bag to prevent clumping of platelets. Some centers that use citrate anticoagulants also administer intermittent or continuous infusions of calcium gluconate during the procedure, especially if large volumes of blood are being processed. However, excessive calcium replacement can induce cardiac dysfunction.

Venous Access

Adequate venous access is required for optimal apheresis technique. Continuous-flow apheresis devices require two-lumen access with a stable blood flow capacity generally greater than 20 mL/min. Single-lumen access may be used with discontinuous-flow apheresis devices, although at a much slower rate of blood processing. The great majority (≈95%) of adult male allogeneic PBSC donors have adequate arm veins for the procedure to be conducted "vein to vein," with female donors more likely to require alternate venous access. Some donors, especially those with small veins and undergoing several daily procedures, may require placement of a temporary venous catheter. Venous access for the patient undergoing collection for autologous PBSC transplantation is much more heterogeneous. Vein-to-vein procedures can be performed, even on several consecutive days, with proper phlebotomy technique and post-collection care of the phlebotomy site. Most patients received previous chemotherapy or are proceeding directly to transplantation, conditions for which tunneled access is commonly placed. Ideally, this venous access should be appropriate both for the apheresis procedures and the subsequent transplant. Length, lumen size, and wall stiffness all affect the blood flow that can be achieved through a catheter. For this reason, the commonly used dual-lumen Hickman or Broviac catheters are usually unsuitable for apheresis, as are all subcutaneously placed ports. Most triple-lumen catheters are inadequate because of the small lumen size. If such access is already in place, consideration can be given to replacement with a shorter, stiffer tunneled catheter or to placement of a temporary percutaneous dialysis/apheresis catheter. The catheters designed for dialysis and apheresis have adequate wall thickness to prevent collapse during aspiration of blood, as well as a tip design that decreases local recirculation of blood and the resulting decrease in apheresis efficiency. Catheters of 10 F or larger size are appropriate for adult patients. Pediatric patients, whose blood flow rates are considerably slower, may use catheter sizes of 5 to 7 F.

Pediatric Donors and Patients

PBSCs can be collected from pediatric patients, including infants. The special challenges of the pediatric patient arise from the fixed extracorporeal blood volume of the apheresis device, the need for venous catheters for blood access, and the management of a patient who may be unwilling or unable to rest quietly for the period of apheresis. It is especially important in management of the pediatric patient that timing of apheresis be optimal to minimize the number of procedures required to achieve the desired quantity of PBSCs. Given these considerations, a number of centers have reported successful collection of PBSCs from pediatric patients and donors.

Almost all pediatric patients undergo insertion of a venous catheter adequate for the flow rates expected, although older patients (>12 years) may tolerate vein-to-vein procedures. The whole blood flow rate for the pediatric patient is much reduced compared with that of adult patients, and catheters as small as 5 F may be adequate.

Appropriate management of fluid balance during the apheresis procedure is critical for the smaller patient. The volume of red blood cells contained in the extracorporeal circuit of the continuous-flow apheresis device could represent 30% to 50% of the red cell mass of a pediatric donor. Although discontinuous-flow devices are appealing because of the feasibility of performing apheresis with a single-lumen venous access, they may result in even higher extracorporeal volumes and should be avoided in the smallest patients. The obvious solution to this problem is to prime the apheresis device with ABO-compatible, irradiated red blood cells (leukocyte-depleted and cytomegalovirus-negative blood is also desirable) when the blood in the extracorporeal circuit is expected to exceed 15% of the patient's blood volume. Packed red blood cell units can be diluted with saline or albumin (to reduce the loss of plasma protein that may occur). The red cells remaining in the extracorporeal circuit at the completion of the run need not be returned ("rinse-back"), although if performed slowly with monitoring of vital signs, rinse-back may actually increase the hematocrit after the procedure and otherwise reduce the need for red cell transfusions for these patients. For the intermediate-size pediatric patient (weight 25 to 50 kg), the apheresis device can be primed with a 5% albumin solution. This step will reduce the albumin loss that otherwise would occur. However, clotting proteins and other proteins not contained in this solution may decrease with repetitive apheresis.

The pediatric patient may not exhibit or relate the prodromal symptoms associated with citrate toxicity. Continuous calcium gluconate infusion can be incorporated into the procedure, or heparin can be added to the citrate anticoagulant solution or used as the sole anticoagulant. Sedation of the pediatric patient is usually not necessary and hinders the ability to recognize the symptoms of citrate toxicity. (Some patients may require antihistamine premedication if the apheresis device is primed with red blood cells.) Centers routinely performing pediatric PBSC collection should design an environment conducive to the management of pediatric patients and develop support procedures that recognize the unique physical and cognitive features of pediatric patients.

The range in blood volumes for pediatric donors of differing ages is greater than the range for adult donors. Therefore, most centers set a goal for volume processed based on the individual's blood volume instead of a set volume (e.g., two blood volumes vs. 6 L of blood) for all patients. The pediatric patient may undergo LVL to achieve the target goal of HSCs with fewer procedures. Blood flow rates for pediatric patients are slower than for adults to minimize the risk for citrate reaction. As with adults, the timing of apheresis can be optimized by monitoring the quantity of CD34$^+$ cells in the peripheral blood.

Complications of Apheresis Collection

Even though most of the complications that occur during leukapheresis procedure to collect PBSC are due to anticoagulant use and/or central line, there are complications that may arise during the procedure that are related to mechanical or technical problems, and problems related to extracorporeal volume. In addition, cytopenia related to cells and platelets depletion may lead to bleeding problems. The mechanical and technical problems that may occur during collection include obstruction or leakage in the sets and hemolysis resulting from kinking or poorly placed tubing. Even though most of the apheresis machines are designed to trap air pockets, air embolism may still occur. Volume shift occurs when blood is drawn to fill the extracorporeal circuit. This may lead to development of vaso-vagal reaction that is typically manifested by nausea, diaphoresis, bradycardia, and pallor. Also, hypovolemic reaction can occur as a result of the volume shift that is characterized by tachycardia, hypotension, syncope, and more rarely, seizure-like reaction. Management includes placing the donor in Trendelenburg position and intravenous hydration.

HEMATOPOIETIC STEM CELL PRODUCT QUALITY

Hematopoietic Stem Cell Product Composition

The composition of the graft depends on its source (BM, PBSC, or CB) and whether HSC mobilization was involved and the type of mobilizing agent(s) used. Even though CD34$^+$ cell content in the graft is most important factor in the product, other cell types play important roles. Specifically, T cell subsets play a major role in facilitating GVHD, graft versus leukemia, and HSC homing and engraftment. Compared to BM, PBSC has the most T cells that may constitute about 30% of all the total nucleated cell content in the graft. The composition of T cells in the BM is about 10% and for CBU is about 20%. Because the T cells in CBU tend to be more immature, the risk of GVHD development is lower than in both BM and PBSC transplants.

The use and nature of mobilizing agent affect BM and PBSC composition. The use of G-CSF as mobilizing agent significantly increases the content of $CD14^+$ monocytes and $CD3^+CD4^-CD8^-$ natural suppressor T cells in a graft. Addition of plerixafor to chemomobilization plus G-CSF further increases the proportion of primitive $CD34^+$ cell subsets, total T and NK cell content in the graft.

$CD34^+$ Cell Enumeration and Dose

Quantification of CD34 antigen-positive cells by flow cytometry has become the standard of care for management of the PBSC donor because it provides a rapid and clinically relevant assessment of HSC content in the peripheral blood or in the PBSC product. This antigen is found on HSCs (including a variety of subpopulations) and limited populations of other blood cells, and can be identified using a variety of commercially available antibodies. If antibodies directly conjugated with dyes are used, the technique requires only about 1 hour of preparation time. Cell viability using propidium iodide or 7-aminoactinomycin D exclusion can simultaneously be determined if the cells are analyzed while still fresh, or the cells can be fixed after staining for analysis at a later date. Other antibodies can be added for analysis of CD34 subsets if desired (and if the flow cytometer has proper detectors to detect the different emission wavelengths of the fluorochromes used). A strong correlation exists between the numbers of $CD34^+$ cells and CFU-GM in the sample, but with ratios of about 5:1 to 20:1. Thus, CD34 analysis will provide data similar to that obtainable with cell cultures, except that the latter demonstrates the functional viability of the progenitor cells. Mobilized PBPC products contain a heterogeneous mixture of cells including $CD34^+$ cells belonging to different cell subsets. Subset analysis of $CD34^+$ cells will provide additional information regarding early and sustained engraftment after autologous PBPC transplantation but does not appear to be clinically useful for the patient who easily meets the PBSC collection goal.

The major difficulty with analysis of $CD34^+$ cells is the low frequency of these cells. Clinical decisions to initiate apheresis are being made for $CD34^+$ cell levels as low as 10/μL. This may represent a cell frequency that is 0.01% or less of the nucleated cells in the specimen. This enumeration is possible because of multidimensional measurements obtained by flow cytometry. Most cytometers can measure at least five characteristics of each cell, including size, granularity, and the presence of up to three different fluorochromes. Thus, the cells of interest can be separated in five-dimensional space, achieving discrimination of cells as rare as 1:10,000. The difficulties arise from developing an adequate technique that makes optimal use of the cytometer to measure these rare cells. Sources of errors include: (1) sampling of the HSC product, (2) cell counting, (3) cytometer calibration and operation, (4) choice of antibody and fluorochrome, (5) lysis technique, and (6) gating strategy. Moreover, cytometry provides a proportion of cells that must be multiplied by the cell count to obtain an absolute number. The steps involved in preparing a specimen for cytometry may alter the proportion of cells in the sample, and this error will be translated into an error in the absolute number. Clinically, this imprecision may explain some of the range in engraftment kinetics observed for patients receiving low doses of $CD34^+$ cells.

Bone Marrow

Total nucleated cell count is normally used as a surrogate for the stem cell content of the marrow product because the definition of adequate HSC products predated the availability of flow cytometric analysis of HSC content, and nucleated cell counting is the only quality-control measure easily performed during the collection procedure. For autologous transplantation, cell doses of 1×10^8 nucleated cells/kg are adequate. Based on early reports that smaller quantities increased the risk for engraftment failure, most centers target 3×10^8 nucleated cells/kg of recipient weight for allogeneic transplantation. However, those early reports were of patients being treated for aplastic anemia, in which engraftment failure is a more common event. A review of unrelated donor transplantation found that recipients of higher marrow cell doses experienced faster neutrophil and platelet engraftment, as well as better leukemia-free survival.[28] Pretreatment of the marrow donor with either GM-CSF or G-CSF may increase the number of myeloid progenitor cells harvested and decrease the period of posttransplant aplasia to that achievable by PBSC transplantation. $CD34^+$ cell dose is now being correlated with transplant outcomes—with more rapid engraftment kinetics, possibly lower transplant-related mortality, and better overall survival, for example, in recipients of allogeneic bone marrow products containing higher quantities of $CD34^+$ cells. $CD34^+$ cell count is now a routine component of bone marrow product quality control.

Peripheral Blood Stem Cells

The quantity of $CD34^+$ cells in a PBSC component varies greatly and is dependent on the number in the peripheral blood at the time of apheresis, the volume of blood processed, and the efficiency of the apheresis device. Therefore, any definition of an adequate component cannot include a set number of $CD34^+$ cells to be contained in any single apheresis component. Instead, one or more components will be collected to meet the appropriate dose of these cells for transplantation. The dose of $CD34^+$ cells required for infusion depends on the intended treatment regimen. For marrow-ablative regimens, increasingly higher $CD34^+$ doses results in greater likelihood of rapid engraftment (Figs. 102.4 and 102.5).[29] Lower doses of $CD34^+$ cells appear satisfactory for nonablative regimens.

Patients who receive a dose of $CD34^+$ cells above a certain threshold will engraft. At lower doses of $CD34^+$ cells, considerable heterogeneity occurs in engraftment speed, especially for platelet engraftment. Why this heterogeneity exists is not known, but it may reflect a weakness in the correlation between $CD34^+$ cells and the cells responsible for engraftment, a greater heterogeneity of $CD34^+$ subsets collected, or perhaps simply a greater degree of error in the measurement of $CD34^+$ cells at the lower cell concentrations. As the dose of $CD34^+$ cells increases, the engraftment kinetics becomes both more rapid and more consistent for the population studied.

Subset analysis of $CD34^+$ cells may improve the accuracy of predicting neutrophil and platelet engraftment kinetics but does not appear to predict engraftment failure enough to be of clinical utility. Pecora and colleagues[30] reported that the quantity of $CD34^+CD33^-$ cells infused was identified as an independent factor predictive of engraftment kinetics. Dercksen and colleagues[7] reported better correlation between the number of $CD34^+CD33^-$ cells and time to granulocyte engraftment, and between the number of $CD34^+CD41^+$ cells and time to platelet engraftment than found with the overall number of $CD34^+$ cells. Coexpression of adhesion molecules is also predictive of hematopoietic recovery, with the same pathways important for both mobilization and homing of HSCs. Given the limited range in recovery times when adequate numbers of $CD34^+$ cells are collected and infused, however, this additional information is currently of limited clinical value.

$CD34^+$ cell dose is also predictive of outcome of allogeneic PBSC (and marrow) transplantation. Higher $CD34^+$ cell doses result in better neutrophil and platelet engraftments after either related or unrelated donor transplantation and may correlate with better survival after bone marrow transplantation, but may also be associated with the development of more chronic GVHD.

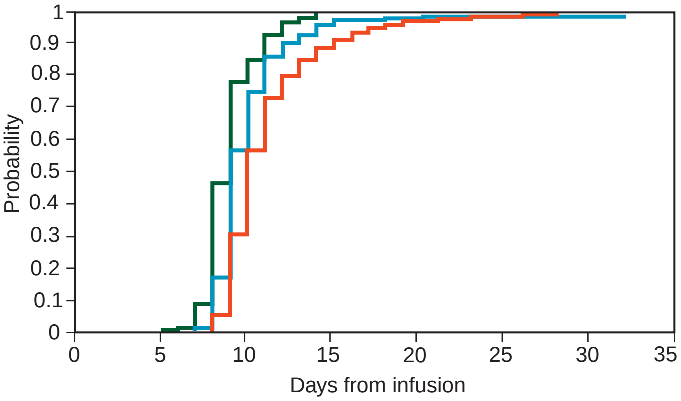


Figure 102.4 Kaplan–Meier probability of achieving ≥0.5 × 10^9 neutrophils/L for <5.0 × 10^6 *(red)*, >5.0–10.0 × 10^6 *(blue)*, and >10 × 10^6 *(green)* CD34$^+$ cells per kilogram (P = .0001). *PBSC*, Peripheral blood stem cell. *(From Weaver CH, Hazelton B, Birch R, et al. An analysis of engraftment kinetics as a function of the CD34 content of peripheral blood progenitor cell collections in 692 patients after the administration of myeloablative chemotherapy. Blood. 1995;86:3961.)*

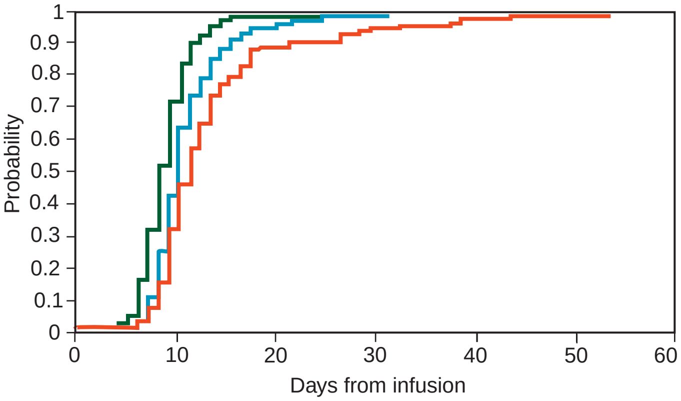


Figure 102.5 Kaplan–Meier probability of achieving ≥20.0 × 10^9 platelets/L for <5.0 × 10^6 *(red)*, >5.0–10.0 × 10^6 *(blue)*, and >10 × 10^6 *(green)* CD34$^+$ cells/kg (P = .0001). *(From Weaver CH, Hazelton B, Birch R, et al. An analysis of engraftment kinetics as a function of the CD34 content of peripheral blood progenitor cell collections in 692 patients after the administration of myeloablative chemotherapy. Blood. 1995;86:3961.)*

Cord Blood

Similar to bone marrow, total nucleated cell count is normally used as a surrogate for the stem cell content of the UCB product because the definition of adequate CD34$^+$ cell dose for engraftment is yet to be well defined. However, CD34$^+$ cell content has been correlated with engraftment, and CD34$^+$ cell count is now a routine component of UCB product quality control. It was also proposed that the HSC in the cord blood are more primitive than marrow and PBPC products and therefore may not express CD34 antigen well.

Tumor Contamination

The probability that tumor cell contamination of HSC product could contribute to relapse was demonstrated by Brenner and colleagues[31] in studies involving the autologous transplantation of genetically marked marrow cells, as well as in individual case reports describing the transmission of malignancy to allogeneic recipients from donors with occult disease at time of harvesting. Sensitive immunocytostaining techniques, clonal assays, flow cytometric analysis, and polymerase chain reaction amplification of malignant genetic material detect tumor cells in the autologous PBSC components of many patients with a variety of malignancies. In general, the incidence of contamination (number of patients with positive components) and the level of contamination (number of tumor cells per number of normal cells) is much less for PBSCs than for marrow products.

The presence of tumor cells at the time of collection or persisting after ex vivo processing may correlate with the extent of systemic disease or the chemotherapy sensitivity of disease at the time of cell collection. Whether patients transplanted with autologous PBSCs have a lower relapse rate compared with patients receiving bone marrow is not well defined, and appropriate phase III studies of this question will be difficult to design and enroll with patients in light of the other well-defined advantages of PBSC transplantation. In a retrospective study, Sharp and colleagues[32] demonstrated similar probabilities of relapse-free survival in recipients of PBSCs or marrow components if the components were free of lymphoma cells. In that study, patients with marrow involvement by lymphoma were assigned to transplantation with PBSCs. The authors concluded that PBSC transplantation is a sensible approach to the patient with overt marrow involvement. However, Brugger and colleagues[33] demonstrated that patients with breast cancer involvement of the marrow at the time of chemotherapy mobilization were likely to mobilize tumor cells into the blood. The most disturbing finding of this study is that the tumor cells were detected in the peripheral blood at the same time as CD34$^+$ cells. Similarly, Pecora and colleagues[34] found a relationship between the ability to detect tumors in PBSC components and in bone marrow samples, but they also found a higher incidence of positive PBSC components for patients who required greater numbers of apheresis procedures to achieve the target dose of CD34$^+$ cells. Investigators at the Johns Hopkins Oncology Center found no difference in the incidence of tumor contamination of PBSC components between patients treated with chemotherapy and cytokines and those treated with cytokines alone.[35] Ex vivo purging of tumor cells from PBPC products has not been shown to reduce the risk for relapse after autologous transplantation, either because the techniques are not adequate in depleting minimal residual disease or because patients relapse primarily from endogenous disease surviving the pretransplant conditioning regimen. Patients with marrow involvement may benefit from several cycles of debulking (in vivo purging) chemotherapy before collection of HSCs, with the caveat that extensive chemotherapy will also decrease the subsequent yield of PBSCs. Tumor cells in the HSC inoculum likely will not benefit the patient, but until further data demonstrating a deleterious effect on transplant outcomes are available, it is advisable that reports of tumor contamination in the collections for individual patients be interpreted with caution.

Microbial Contamination

Bacterial culture is an essential quality-control component and it is used to identify errors and breakdowns in manufacturing technique. Skin flora are the bacteria usually isolated, and infusion of culture-positive HSC products is generally without clinical sequelae, although serious infections have occurred after infusion of HSC products contaminated during processing. Culture-positive products need not be automatically destroyed. Any decision regarding the disposition of a culture-positive HSC product must be made by the patient's transplant physician after considering the type of contamination, the anticipated risks from use of the component, and the ability to replace the culture-positive product(s) in a timely manner.

The incidence of culture-positive PBSC components is considerably less than that for marrow. The actual incidence of contamination for all HSC products is likely several times higher than reported, however, because most laboratories will culture only a very small volume of product (≈1 mL). In a retrospective review of 2935 HSC products processed and transplanted at one center, positive microbial products were reported for 1.3% of bone marrow products, 0.7% of PBSC products, and 2.0% of UCB products. Coagulase-negative

Staphylococcus and *Bacillus* species accounted for 23 of the 38 positive cultures, but *Escherichia coli*, *Klebsiella pneumonia*, and *Pseudomonas cepacia* were cultured from one product each. The recipient of the *Pseudomonas*-contaminated product subsequently died of complications of *Pseudomonas* sepsis, but no adverse sequelae could be documented for any of the other 34 recipients of culture-positive products. The highest rate of contamination occurred in the UCB products collected for related donor transplantation (5 out of 18, 27.8%), likely a reflection of the collection techniques in place at the time, and illustrating the need for strict quality control in the collection and processing of HSC products.

REFERENCES

1. Bensinger WI, Martin PJ, Storer B, et al. Transplantation of bone marrow as compared with peripheral-blood cells from HLA-identical relatives in patients with hematologic cancers. *N Engl J Med*. 2001;344(3):175–181.
2. Schmitz N, Beksac M, Hasenclever D, et al. Transplantation of mobilized peripheral blood cells to HLA-identical siblings with standard-risk leukemia. *Blood*. 2002;100(3):761–767.
3. Lane TA, Law P, Maruyama M, et al. Harvesting and enrichment of hematopoietic progenitor cells mobilized into the peripheral blood of normal donors by granulocyte-macrophage colony-stimulating factor (GM-CSF) or G-CSF: potential role in allogeneic marrow transplantation. *Blood*. 1995;85(1):275–282.
4. Horowitz MM, Confer DL. Evaluation of hematopoietic stem cell donors. *Hematology Am Soc Hematol Educ Program*. 2005:469–475.
5. Sacchi N, Costeas P, Hartwell L, et al. Haematopoietic stem cell donor registries: World Marrow Donor Association recommendations for evaluation of donor health. *Bone Marrow Transplant*. 2008;42(1):9–14.
6. Worel N, Buser A, Greinix HT, et al. Suitability criteria for adult related donors: a consensus statement from the Worldwide Network for Blood and Marrow Transplantation Standing Committee on Donor Issues. *Biol Blood Marrow Transplant*. 2015;21(12):2052–2060.
7. Dercksen MW, Rodenhuis S, Dirkson MK, et al. Subsets of CD34$^+$ cells and rapid hematopoietic recovery after peripheral-blood stem-cell transplantation. *J Clin Oncol*. 1995;13(8):1922–1932.
8. Shaw BE, Logan BR, Kiefer DM, et al. Analysis of the effect of race, socioeconomic status, and center size on unrelated National Marrow Donor Program donor outcomes: donor toxicities are more common at low-volume bone marrow collection centers. *Biol Blood Marrow Transplant*. 2015;21(10):1830–1838. https://doi.org/10.1016/j.bbmt.2015.06.013.
9. Pulsipher MA, Chitphakdithai P, Logan BR, et al. Acute toxicities of unrelated bone marrow versus peripheral blood stem cell donation: results of a prospective trial from the National Marrow Donor Program. *Blood*. 2013;121:197–206.
10. Kao GS, Kim HT, Daley H, et al. Validation of short-term handling and storage conditions for marrow and peripheral blood stem cell products. *Transfusion*. 2011;51(1):137–147.
11. Halter J, Kodera Y, Ispizua AU, et al. Severe events in donors after allogeneic hematopoietic stem cell donation. *Haematologica*. 2009;94(1):94–101.
12. Goldberg SL, Mangan KF, Klumpp TR, et al. Complications of peripheral blood stem cell harvesting: review of 554 PBSC leukaphereses. *J Hematother*. 1995;4(2):85–90.
13. Brecher G, Cronkite EP. Post-radiation parabiosis and survival in rats. *Proc Soc Exp Biol Med*. 1951;77(2):292–294.
14. Dührsen U, Villeval J-L, Boyd J, et al. Effects of recombinant human granulocyte colony-stimulating factor on hematopoietic progenitor cells in cancer patients. *Blood*. 1988;72(6):2074–2081.
15. Socinski MA, Cannistra SA, Elias A, et al. Granulocyte-macrophage colony stimulating factor expands the circulating haemopoietic progenitor cell compartment in man. *Lancet*. 1988;1(8596):1194–1198.
16. Watts MJ, Addison I, Long SG, et al. Crossover study of the haematological effects and pharmacokinetics of glycosylated and non-glycosylated G-CSF in healthy volunteers. *Br J Haematol*. 1997;98(2):474–479.
17. De Arriba F, Lozano ML, Ortuño F, et al. Prospective randomized study comparing the efficacy of bioequivalent doses of glycosylated and nonglycosylated rG-CSF for mobilizing peripheral blood progenitor cells. *Br J Haematol*. 1997;96(2):418–420.
18. Elayan MM, Horowitz JG, Magraner JM, et al. Tbo-filgrastim versus filgrastim during mobilization and neutrophil engraftment for autologous stem cell transplantation. *Biol Blood Marrow Transplant*. 2015;21(11): 1921–1925. https://doi.org/10.1016/j.bbmt.2015.05.024.
19. Anderlini P, Donato M, Lauppe MJ, et al. A comparative study of once-daily versus twice-daily filgrastim administration for the mobilization and collection of CD34$^+$ peripheral blood progenitor cells in normal donors. *Br J Haematol*. 2000;109(4):770–772.
20. Lee V, Li CK, Shing MM, et al. Single vs twice daily G-CSF dose for peripheral blood stem cells harvest in normal donors and children with non-malignant diseases. *Bone Marrow Transplant*. 2000;25(9): 931–935.
21. Pruijt JF, van Kooyk Y, Figdor CG, et al. Anti-LFA-1 blocking antibodies prevent mobilization of hematopoietic progenitor cells induced by interleukin-8. *Blood*. 1998;91(11):4099–4105.
22. Liles WC, Rodger E, Broxmeyer HE, et al. Augmented mobilization and collection of CD34$^+$ hematopoietic cells from normal human volunteers stimulated with granulocyte-colony-stimulating factor by single-dose administration of AMD3100, a CXCR4 antagonist. *Transfusion*. 2005;45(3):295–300.
23. DiPersio JF, Stadtmauer EA, Nademanee A, et al. Plerixafor and G-CSF versus placebo and G-CSF to mobilize hematopoietic stem cells for autologous stem cell transplantation in patients with multiple myeloma. *Blood*. 2009;113(23):5720–5726.
24. DiPersio JF, Micallef IN, Stiff PJ, et al. Phase III prospective randomized double-blind placebo-controlled trial of plerixafor plus granulocyte colony-stimulating factor compared with placebo plus granulocyte colony-stimulating factor for autologous stem-cell mobilization and transplantation for patients with non-Hodgkin's lymphoma. *J Clin Oncol*. 2009;27(28):4767–4773.
25. Demirer T, Buckner CD, Storer B, et al. Effect of different chemotherapy regimens on peripheral-blood stem-cell collections in patients with breast cancer receiving granulocyte colony-stimulating factor. *J Clin Oncol*. 1997;15(2):684–690.
26. Lefrère F, Mauge L, Réa D, et al. A specific time course for mobilization of peripheral blood CD34$^+$ cells after plerixafor injection in very poor mobilizer patients: impact on the timing of the apheresis procedure. *Transfusion*. 2013;53:564–569.
27. Rowley SD, Yu J, Gooley T, et al. Trafficking of CD34$^+$ cells into the peripheral circulation during collection of peripheral blood stem cells by apheresis. *Bone Marrow Transplant*. 2001;28(7):649–656.
28. Sierra J, Storer B, Hansen JA, et al. Transplantation of marrow cells from unrelated donors for treatment of high-risk acute leukemia: the effect of leukemic burden, donor HLA-matching, and marrow cell dose. *Blood*. 1997;89(11):4226–4235.
29. Weaver CH, Hazelton B, Birch R, et al. An analysis of engraftment kinetics as a function of the CD34 content of peripheral blood progenitor cell collections in 692 patients after the administration of myeloablative chemotherapy. *Blood*. 1995;86(10):3961–3969.
30. Pecora AL, Preti RA, Gleim GW, et al. CD34$^+$CD33$^-$ cells influence days to engraftment and transfusion requirements in autologous blood stem-cell recipients. *J Clin Oncol*. 1998;16(6):2093–2104.
31. Brenner MK, Rill DR, Moen RC, et al. Gene-marking to trace origin of relapse after autologous bone-marrow transplantation. *Lancet*. 1993;341(8837):85–86.
32. Sharp JG, Kessinger A, Mann S, et al. Outcome of high-dose therapy and autologous transplantation in non-Hodgkin's lymphoma based on the presence of tumor in the marrow or infused hematopoietic harvest. *J Clin Oncol*. 1996;14(1):214–219.
33. Brugger W, Bross KJ, Glatt M, et al. Mobilization of tumor cells and hematopoietic progenitor cells into peripheral blood of patients with solid tumors. *Blood*. 1994;83(3):636–640.
34. Pecora AL, Lazarus HM, Jennis AA, et al. Breast cancer cell contamination of blood stem cell products in patients with metastatic breast cancer: predictors and clinical relevance. *Biol Blood Marrow Transplant*. 2002;8(10):536–543.
35. Passos-Coelho JL, Ross AA, Kahn DJ, et al. Similar breast cancer cell contamination of single-day peripheral-blood progenitor-cell collections obtained after priming with hematopoietic growth factor alone or after cyclophosphamide followed by growth factor. *J Clin Oncol*. 1996;14(9):2569–2575.

CHAPTER 103

GRAFT ENGINEERING AND CELL PROCESSING

Adrian P. Gee

It has been more than 70 years since allogeneic hematopoietic stem cells (HSC) were first transplanted to human recipients in the form of bone marrow. Since that time, the source of HSC has widened to include autologous bone marrow, growth factor mobilized peripheral blood progenitor cells (PBPCs), umbilical cord blood, and more. In parallel, there has been the development of other cellular therapies to treat a variety of diseases (such as the use of T cells genetically modified to express a chimeric antigen receptor (CAR) for the treatment of leukemias)[1] and mesenchymal stromal cells (MSCs) to treat or prevent graft-versus-host disease (GVHD).[2] This chapter provides a general overview of how these cells are prepared ex vivo, tested, and released for clinical use. Preparation procedures range from the simple depletion of plasma or incompatible erythrocytes from HSC grafts to complex genetic modifications of other cells using viral vectors to induce tumor-associated antigen specificity.

The rapid development of cellular administrations for therapeutic applications has attracted the interest of regulatory authorities (e.g., the Food and Drug Administration [FDA] in the United States). Understanding the relevant regulations is critically important when undertaking the manufacture of these types of products.

REGULATORY ISSUES WITH CELL PROCESSING

In response to the increase in the use of therapeutic cell products, the FDA, and other national regulatory authorities, have had to develop a relevant regulatory strategy to cover these products. The approach used by the FDA, and some other agencies, has been to develop a risk-based method (Table 103.1). This is based on an assessment of the relative risks posed broadly by the collection of the initial cells, their ex vivo manipulation and storage, and their administration to the recipient. Good Manufacturing Practices (GMP) regulations have existed for some time and are applied in the drug field, primarily in the preparation of small molecules. This was extended to certain cellular therapy products when these were classified as drugs for regulatory purposes. The applicable GMP regulations appear in Title 21 of the Code of Federal Regulations, primarily in Parts 210/211.[3] They cover all aspects of manufacturing, testing, packaging, storing, releasing, and distributing drugs. Recognizing that these regulations were probably overkill for experimental cellular therapeutics being developed by an academic institution, the FDA published a guide indicating the level of GMP compliance required for products to be used in Phase 1 clinical trials.[4] Simultaneously, the Agency recognized that there were certain products whose preparation and use posed a substantially lower risk (e.g., PBPC) since their collection was straightforward, their ex vivo manipulation was simple and their clinical use did not result in adverse events, and was of clinical benefit. As a result, in 2005 they published Good Tissue Practice (GTP) regulations as a part of 21 CFR Part 1271 (Human Cells, Tissues, and Cellular and Tissue-Based Products [HCT/Ps])[5] to cover the preparation of these products. These were established nominally to prevent the introduction, transmission, and spread of communicable diseases by HCT/Ps. This was based on the presumption that most post-transplant infections and admissions to intensive care units were attributable to the administration of contaminated products. The validity of this assumption is open to question. GTP regulations provide a framework for screening, performing ex vivo processing, storing and distributing HCT/Ps, and providing the FDA with an overview of current activities by requiring an annual registration of collection and processing facilities. The Part 1271 regulations effectively filled a gap in the law that had left unregulated HCT/Ps that were minimally manipulated (e.g., were not cultured ex vivo, genetically modified, activated ex vivo), intended for homologous use, or not combined with another article (e.g., a matrix or scaffold) for administration. Minimally manipulated HCT/Ps should not exert a systemic effect and should not be dependent on the metabolic activity of living cells for them to function. If this was not the case, then the cells should be for autologous use only, for use in a first- or second-degree blood relative, or in reproductive use. Cellular products that fall into this classification are Type 361 products. The Part 1271 regulations do not apply to vascularized organs for transplantation, whole blood, blood components, or minimally manipulated BM for homologous use that is not combined with another article. This means that traditional marrow-derived hematopoietic stem cell transplants (HSCT) do not fall under FDA regulations and that these procedures are currently considered a practice of medicine.[5] Products that fall under IND regulations are referred to as Type 351 products. These are cells that have been cultured ex vivo and transduced or activated ex vivo and, therefore, are more-than-minimally manipulated. The facility processing these cells is required to operate under GMP.[3] These regulations were developed to ensure that products are prepared under a controlled and auditable process that ensures their safety, purity, and potency. As indicated previously, the agency has recognized the differences between products prepared for a phase 1 clinical trial[4] and, therefore, the application of GMP to cellular therapy products follows a continuum, such that as the trial proceeds, the expectation is that implementation of GMP will become more rigorous, such that a phase III product would be extensively characterized and manufactured using a fully validated process.

Implementation of Part 1271 regulations has impacted the "routine" laboratory that prepares cells primarily for HSCT. Laboratories that use cells other than bone marrow (BM) now must register annually with the FDA; ensure that donors meet eligibility requirements (or document why non-eligible donors are used); and manufacture, store, and distribute the cells under GTP. If a laboratory has previous experience manufacturing products under GMP conditions, it will already be familiar with most of the features of GTP. Generally, these cover personnel, procedures, facilities, environmental control and monitoring, equipment, supplies and reagents, recovery, processing and process controls, process changes, process validation, labeling controls, storage, receipt, pre-distribution shipment and distribution, records, tracking, and complaints. Implementation of the components of GMP and GTP operations is a time-consuming process that requires the development, implementation, and maintenance of numerous components and generates a considerable volume of documentation. Professional societies, such as the Foundation for the Accreditation of Cellular Therapy (FACT)[6,7] and AABB (formerly the American Association of Blood Banks),[8] have developed standards and an accreditation process that takes into account GTP/GMP regulations and provides a framework around which compliance can be built.

In the United States, cord blood is a licensed product, and facilities that prepare and bank cord blood are required to obtain a Biologics License. This allows the manufacturer to introduce, or deliver for introduction, a biologic product into interstate commerce (21 CFR 601.2). There are professional standards that address cord blood collection, manufacturing, and use.[9]

Central to both GMP and GTP regulations is the establishment and maintenance of a quality program. This quality program must ensure that the appropriate regulations are being followed on an

TABLE 103.1 **Characteristics of Products Regulated Under Good Tissue Practices and Good Manufacturing Practice Regulations and Associated Guidance Documents**

Good Tissue Practices (GTP) (Type 361 Products) 21CFR Part 1271	Good Manufacturing Practices (GMP) (Type 351 Products) 21CFR Parts 210 & 211	Food and Drug Administration (FDA) Guidance
Minimally-Manipulated	More-than-Minimally Manipulated	Regulatory Considerations for Human Cells, Tissues, and Cellular and Tissue-Based Products: Minimal Manipulation and Homologous Use. December 2017
Homologous use only	Includes non-homologous use	
No combination products	Combination (e.g., with matrix or scaffold) products	Current Good Manufacturing Practice Requirements for Combination Products. January 2017 Requesting FDA Feedback on Combination Products. December 2019
No systemic effects unless for: • Autologous use • Allogeneic use in 1st- or 2nd-degree relative • Reproductive use	Systemic effects	
Donor Eligibility Assessment Register establishment with FDA annually	Investigational New Drug/Device Application required Annual IND Report	Eligibility Determination for Donors of Human Cells, Tissues, and Cellular & Tissue-Based Products (GTP) August 2007. Investigational New Drug Applications Prepared and Submitted by Sponsor-Investigators (GMP) May 2015 IND Applications for Clinical Investigations: Regulatory and Administrative Components (GMP) February 2018

ongoing basis, that mechanisms are in place for detecting, reviewing, and remediating errors and deviations from regulations, policies, and procedures, and that an audit program will be developed, implemented, and maintained. Activities performed by the quality program must be documented, and the program should be staffed by individuals who are not involved in the hands-on manufacturing of products.[10]

Holders of INDs must provide the agency with an annual report on the protocol, including a listing of the products administered and those that have been prepared but not used. In addition, cell processing facilities should be prepared to assist the IND sponsor by providing information on products that have been associated with severe adverse reactions in the recipients and whether these were attributable to product quality. For Type 361 products, the facility must, as Biological Product Deviations, report any contaminated products that have been administered to a patient.

PROFESSIONAL STANDARDS

The two major accrediting organizations in the United States for cellular therapies are FACT and AABB. Whereas FACT offers accreditation for collection, processing, and clinical use of cellular therapy products,[6,7] the AABB focuses on collection and laboratory processing.[8] Both organizations inspect based on standards that are published every 18 months to 3 years. In the case of FACT, the standards are published in collaboration with the Joint Committee on Accreditation in Europe (JACIE). FACT also publishes separate standards that cover cord blood banking (in collaboration with NetCord)[9] and for Immune Effector Cells. Both organizations have worked to harmonize their standards with American, Canadian, Australasian, and European regulatory agencies; therefore, accreditation by either organization is of great assistance on the pathway to regulatory compliance. Several other professional organizations accredit particular aspects of operations within the cell processing facility. These include the College of American Pathologists (CAP), which accredits general laboratories and hematology and flow cytometry facilities, the American Society for Histocompatibility and Immunogenetics (ASHI), and the European Federation for Immunogenetics (EFI), which accredit histocompatibility testing laboratories. Some organizations, such as CAP and StemCell Technologies, also provide proficiency testing services for laboratory staff.

MANIPULATION OF HEMATOPOIETIC STEM CELL TRANSPLANTATION PRODUCTS

Hematopoietic grafts are manipulated in vivo to remove an unwanted component that may cause adverse effects or to enrich the desired population, such as HSC.[10,11] As discussed previously, the degree of manipulation may determine the regulations under which the product is manufactured and handled. The FDA defines minimal manipulation as processing that does not alter the relevant biologic characteristics of the cells or tissues. This includes procedures such as RBC, plasma depletion, and cell selection using an approved device. By contrast, more-than-minimal manipulation would include activities such as culture ex vivo, genetic modification, and ex vivo activation. As stated previously, manufacturers of minimally manipulated products would follow GTP regulations,[5] whereas products that are more-than-minimally manipulated would fall under GMP regulations.[3]

Routine Minimal Manipulation for Volume Reduction or ABO Incompatibility

The most widely used form of manipulation in the hematopoietic progenitor cell (HPC) processing facility probably is the removal of erythrocytes and plasma to overcome incompatibility between donor and recipient (Table 103.2). This process is performed using techniques that were developed by the blood banking industry.

Plasma depletion to remove donor antibodies that may react with recipient cells is achieved by centrifugation of the graft, usually in a transfer pack, at approximately 2000 g for 10 minutes at ambient temperature. The pack is then placed in a plasma expresser, which compresses the product bag so that plasma can be forced out and into a separate collection bag. Plasma depletion can also be used to reduce the volume of ABO-compatible grafts when the donor is larger than the recipient.

Erythrocyte depletion removes incompatible donor erythrocytes that would stimulate a reaction by the recipient upon administration. Most facilities establish a maximum volume of incompatible RBCs that can be infused with HPC; exceeding this limit can result in hemolysis and a transfusion reaction. Depletion of erythrocytes can be achieved most simply by centrifugation at approximately 3000 g for approximately 10 minutes at ambient temperature. The leukocyte-rich buffy coat is then collected from the interface between the plasma layer and the RBCs.

Erythrocyte depletion can also be achieved by sedimenting erythrocytes using hydroxyethyl starch (Hetastarch). This promotes RBC sedimentation by the formation of erythrocyte rouleaux. The hematocrit of the product is first adjusted to ~25% by the addition of normal saline, and 6% Hetastarch (Hespan) is added at a volume:volume ratio of 1:6 to 1:7. Sedimentation can be performed under gravity or accelerated by centrifugation.

A more rigorous erythrocyte depletion is achieved by centrifugation of the collection on a Ficoll-Hypaque density gradient. The cells are layered onto a density gradient cushion of Ficoll-Hypaque and centrifuged at 400 g for approximately 30 minutes. The mononuclear cells collect at the interface and are removed and washed before use. This process depletes erythrocytes, platelets, and granulocytes. The overall nucleated cell recovery is lower than with other techniques in which granulocytes are retained.

Automated devices are available for preparing buffy coats and density gradient-enriched cells. For larger volumes, the COBE 2991 Cell Processor from Terumo can be used. This requires a minimum volume of 150 mL RBCs for operation and may therefore not be suitable for pediatric processing. It is capable of preparing buffy coats and density-separated cell preparations using a functionally closed disposable set.

The COBE Spectra from Terumo is commonly used to collect PBPC by apheresis. The device is also less widely used in the processing facility to enrich mononuclear cells from BM.

For smaller starting volumes, the Sepax device from Cytiva (formerly GE Cell Therapy) (Fig. 103.1A) can be used. It has found widespread application in cord blood banks for buffy coat preparation (with or without the addition of hydroxyethyl starch) and has also been used for volume reduction of PBPC collections, density gradient separation of BM, and cell washing. The device has a small footprint, uses functionally closed disposables, and provides a printout of operations.

The AXP II System for cord blood processing is available from Thermogenesis. The device is designed for enriching mononuclear cells from cord blood that are transferred to the processing set, which is then placed into the AXP device. This fits into a centrifuge bucket, and during spinning, the red and mononuclear cells are collected into separate bags, and the plasma is retained in the processing set. The PXP System performs a similar procedure on marrow harvests. Both devices provide closed sterile systems, and each system comes with software that enables data tracking to assist with regulatory compliance.

TABLE 103.2 **Processing Performed to Address ABO Incompatibilities Between Hematopoietic Stem Cell Donors and Recipients**

Recipient ABO Type	Donor ABO Type	Type of Processing
ABO identical	ABO identical	No special processing required
A or B	AB	RBC depletion
O	A/B/AB	RBC depletion
A	B	RBC + plasma depletion
B	A	RBC + plasma depletion
Antibody to RBCs	N/A	RBC depletion
A/B/AB	O	Plasma depletion
AB	A or B	Plasma depletion

N/A, Not applicable; *RBC*, red blood cell.

Purging of Autologous Grafts

Autologous HPC can be used for recipients lacking a human leukocyte antigen (HLA)-matched related or unrelated donor. It has been proposed that occult viable tumor cells collected with the graft and returned to the patient could act as a source for disease relapse. Gene marking studies have supported this hypothesis. As a result, much effort has been exerted to develop methods for the ex vivo detection and removal of tumor cells from autologous grafts (Table 103.3). Techniques have included incubation with chemotherapeutic drugs, such as 4-hydroperoxy cyclophosphamide (4-HC), photosensitizing agents, and antisense oligonucleotides. Alternatively, tumor-directed monoclonal antibodies (MAb) can be used to identify the cells and effect their removal. The MAb-coated tumor cells can be eliminated by the addition of serum complement or by capturing them on a solid phase, such as a column matrix, a plastic sheet, or magnetic particles. These particles may be large (5 μm diameter—Dynabeads, Invitrogen), so they can be collected with the attached tumor cells in a standard magnetic field. The matrix material may be much smaller, such as iron-dextran nanoparticles (Miltenyi Biotec

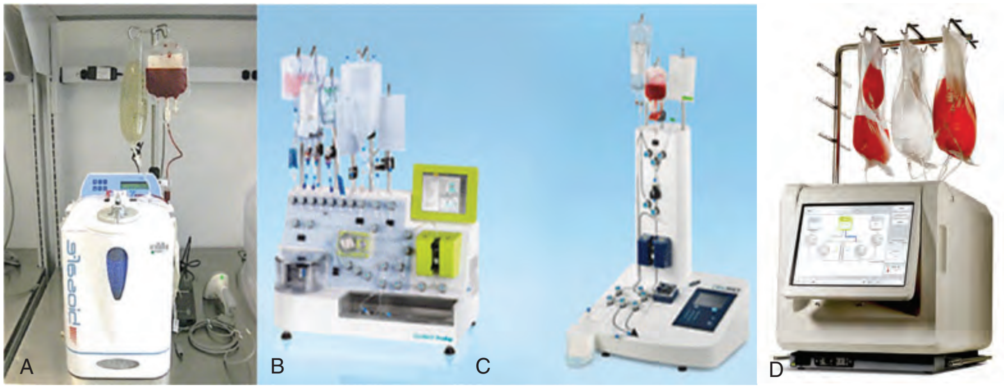


Figure 103.1 AUTOMATED CELL SEPARATION AND CULTURE DEVICES. (A) The Sepax Device from Cytiva. (B) The Prodigy Device from Mitenyi Biotec. (C) The CliniMACS Device from Miltenyi Biotec. (D) ©Terumo BCT, Inc.

TABLE 103.3 Methods Used for Manipulation of Hematopoietic Grafts

Destruction in Situ	Physical Separation
Tumor: Cytotoxic drugs (e.g., 4-HC[a])	**T cells:** Rosetting with sheep erythrocytes
	Lectins (e.g., soybean agglutinin)
	Centrifugal elutriation
Monoclonal Antibody-Based	
Tumor or T cells:	Immunomagnetic separation
Antibody + Complement	• **T cells:** Negative selection (Pan T-cell and T-cell subset removal)
Immunotoxins (e.g., ricin)	• **Tumor:** Negative selection (using tumor-associated antigens)
Panning and immunoaffinity columns	• **Positive selection (Tumor and T cells)** (CD34 enrichment) CliniMACS device

[a]4-Hydroperoxycyclophosphamide.

Bergisch Gladbach, Germany) or ferrofluids (Ferrotec Santa Clara, CA), which coat the cells. These are then collected on a metal matrix placed in a field generated by permanent magnets. Such systems are capable of depleting 4 to 6 logs of tumor cells from a graft. However, even at such high efficiencies, and given the limits of our ability to detect residual tumor cells, the clinical value of purging autologous grafts is debatable. There has also been a decline in interest in purging techniques because of the potential benefits of a graft-versus-tumor (GVT) effect detected in recipients of allogeneic grafts.

T-Cell Depletion of Allogeneic Products

T cells in HPC grafts have the potential to cause severe or lethal GVHD or exert a beneficial GVT effect.[12] Considerable work has been done to determine whether or not these opposing effects are produced by distinct subpopulations of T lymphocytes. This would allow ex vivo manipulation of allogeneic grafts to remove differentially the GVHD-producing T cells while sparing those that mediate GVT responses. Various subpopulations of T cells have been identified as candidate effector subpopulations; however, there is still no widespread consensus as to which subsets should be targeted.

Methods are available for Pan-T cell elimination from grafts using approaches similar to those used for purging tumor cells (see Table 103.3). Early techniques included the use of soybean agglutinin to aggregate the majority of non-progenitor cells and the rosetting of sheep erythrocytes with T cells to facilitate their removal. Although successful, these techniques are not “FDA friendly” and do not offer the specificity that likely is required to engineer T-cell subpopulations in allogeneic grafts.[13–15] This is possible by using MAb directed toward the antigens that currently are used to identify T-lymphocyte subpopulations. The target population then can be removed with high efficiency using immunomagnetic separation, as described previously for purging autologous grafts. The challenge remains to identify the appropriate target T cell populations and to source clinical-grade MAb for these procedures. Several potential target antigens have been identified and separation techniques implemented. They range from pan–T-cell depletions using MAb to CD3 and CD2, depletions of helper and cytotoxic T cells using MAb against CD4 and CD8, to stimulation and removal of alloreactive populations by targeting activation antigens. In addition, methods have been developed for the selective manipulation of natural killer (NK)[16] cells, natural killer T (NKT) cells,[17] myeloid suppressor populations, and MSCs for administration to stem cell transplant recipients to prevent or treat GVHD.

Efforts have recently focused on the depletion of αβ-positive T cells from the graft. This spares the donor-derived alloreactive NK and γδ T cells, which may provide a tumor-directed response. Promising early clinical results have been obtained using this approach. Twelve patients received marrow or PBPC grafts depleted of ~4 logs of αβ T cells, and all were alive at 3 months, and after 1 year, two had died. There was no severe GVHD (grade III to IV) post-infusion. A systematic review of the impact of γδ cells on the outcome of transplants concluded from 11 studies (919 patients), with a median follow-up of 30 months, that high γδ numbers were associated with lower relapse, fewer viral infections, and higher overall and disease-free survivals; however, no association was found between high γδ numbers and GVHD incidence.

Regulatory T Cells

A different approach for GVHD is to administer T_{reg} cells post-transplant to prevent and treat GVHD. A first-in-man clinical study was performed using in vitro expanded T_{reg} from partially HLA-matched third-party umbilical cord blood units that were administered to 23 patients receiving double cord blood transplants. Results were compared to 108 historical controls. No T_{reg} acute toxicities were seen, and there was a reduced incidence of grades II-IV acute GVHD but since this was a Phase 1 study, it was not designed to demonstrate efficacy. Later studies support the finding that donor T_{reg} infusions early after transplant can prevent GVHD after allogeneic transplantation. There are several questions still to be addressed. These include the source of T_{reg} cells (cord versus peripheral blood—cord blood generally yields cells of a higher purity but lower numbers); whether the cells should be expanded in vitro before use; the best method for their purification; and the best conditions for expansion.

T_{reg} represents a rare cell population whose phenotype cannot be categorically defined. They are generally enriched using their expression of CD25, sometimes incorporating prior depletion of C8 and CD19-positive cells. This leads to an enrichment of $CD4^+CD25^+FOXP3^+$ cells which can be further increased to 87% purity by further in vitro expansion. The optimal method for enrichment appears to be the separation of $CD4^+CD25^{hi}CD127^{lo}$ cells. Peripheral blood T_{reg} cells can be effectively expanded in vitro by stimulation with CD3 and CD28 MAb and high concentrations of IL-2. Alternatively, artificial antigen-presenting cells (APCs) primed with CD3 (and CD28) MAb have been shown to preserve FOXP3 expression while achieving cell expansion. Expansion of cord blood T_{re} can be further improved by using APC additionally primed with OX40L or 4-IBBL. Rapamycin can also be used to enhance TGF-β-dependent FOXP3 expression and to limit the activation and expression of effector T cells. It should be noted that cryopreservation of T_{reg} may impair their function.

Positive Selection of Progenitor Cells

Methods that eliminate or physically remove either T cells or tumor cells from grafts are referred to as *negative selection techniques*. They are affected by variables such as target antigen expression, the sensitivity of detection technologies for quantitating separation efficiency, and other technical hurdles. These may be difficult to control in order to achieve the ideal composition of the graft, and the target level of residual T cells, or T cell subsets, remains to be established. A dose of 10^5T cells/kg is generally regarded as the goal to minimize the risk of GVHD while facilitating engraftment. There are also no approved devices for negative selection, so these types of procedures must be performed under an IND.

For many years, the goal was to replace the negative selection with a procedure in which HPC populations could be enriched by positive selection. This would effectively deplete T cells, or tumor cells, from allogeneic and autologous grafts, respectively. The problem was the lack of a method for identifying the target HSC until the CD34 antigen was identified on a small population of progenitor cells, including the pluripotent cells required for hematopoietic transplantation. The subsequent availability of MAb directed against this antigen made possible the development of techniques for the enrichment of these cells. Immobilization of the antibodies on a matrix (e.g., plastic sheets),cellulose, and magnetic particles were used as the primary approach, and several devices were commercially developed (see Table 103.3). The first to achieve FDA approval for use with apheresis products were the Baxter Isolex 300i, which used Dynal 5 μm magnetic beads as the separation modality and released CD34$^+$ cells from the beads using a competitive binding peptide. This device was taken over by Nexell, which ultimately went out of business in 2002.

The alternative is the CliniMACS system (Miltenyi Biotec, Fig. 103.1C), which currently has regulatory approval in Europe, but still requires an IND for use in the United States for other than one specific indication (the depletion of T cells by CD34$^+$ cells selection for the treatment of acute myeloid leukemia). This uses anti-CD34 or anti-CD133 nanoparticles to effect separation. The labeling with and removal of unbound MAb reagent is performed manually. The CD34 reagent-treated cells are then processed on the device, where they are retained on a column located in a high-gradient magnetic field. Nonlabeled cells flow through the column and are collected in the negative fraction. The labeled cells are recovered from the column after several automated separations and washing cycles by removing the magnetic field. The nanoparticles remain on the CD34/133-positive cells; however, they are biocompatible and may be infused into the recipient. Recently the company has come up with REAlease nanoparticles that can be removed from the targeted cells, however, these are not yet clinically approved. The CliniMACS device normally achieves purities over 90% with yields of approximately 60%. This results in the passive depletion of 4 to 6 logs of allogeneic T cells or autologous tumor cells. The device may be used with a variety of MAb directed against antigens expressed by various types (T, B, and NK cells), potentially allowing it to be used as a platform for multiple types of graft manipulation.

Miltenyi has also introduced the Prodigy device (Fig. 103.1B), which can automatically perform many of the steps requiring manual intervention on the CliniMACS. Additional features provide the potential ability to fully automate the preparation of a variety of cellular therapy products (e.g., CAR-T cells) in a functionally closed system. The regulatory status of these devices should be discussed with the appropriate regulatory agencies before clinical use.

Positive selection techniques may, however, passively deplete from the graft certain cells that could be of potential benefit to the recipient. These include some stromal elements, GVT-mediating T cells, and other populations that may facilitate engraftment. As our understanding of the identity of these populations improves, it may be possible to recover them from the normally discarded negative fraction and add them back to the CD34$^+$ cells or to administer them in the post-transplant period as donor leukocyte infusions).

Table 103.3 lists various methods for manipulation of HSC grafts to achieve tumor removal from autologous products and T cells depletion by HPC enrichment.

EVALUATION OF MANIPULATED ALLOGENEIC GRAFTS

Most allograft engineering has focused on T-lymphocyte depletion and has emphasized quantitative versus qualitative removal. The majority of allografts are infused immediately after preparation rather than after cryopreservation and storage. This restricts the types of assays that can be used to evaluate graft composition to those that have a fast turnaround since the implications of infusing large numbers of T lymphocytes can be severe or lethal. Therefore, it is important to have available methods that can rapidly enumerate the numbers of T lymphocytes within the graft. Early methods used detection of E-rosette–forming cells or manual immunofluorescence after staining with pan-T-lymphocyte–directed monoclonal antibody, however, most laboratories now rely on flow cytometry. This technology is widely used in routine clinical laboratories; however, some precautions must be taken when it is used for T-depleted allografts.

Flow Cytometry

Accurate enumeration of small numbers of target cells by cytometry requires rare event analysis. In this technique, large numbers of events must be accumulated and carefully analyzed if reliable data are to be obtained. This approach has been widely adopted for counting CD34$^+$ cells in unfractionated grafts but is often neglected when enumerating T lymphocytes in depleted grafts. The choice of MAb for detection of the T-lymphocyte population also is critical when using MAb-mediated depletion technology. The same MAb should not be used for both depletion and analysis because cells that became coated with the antibody during the depletion phase but were not effectively removed will be blocked from detection. However, they can be detected by adding an anti-immunoglobulin antibody conjugated to a fluorochrome different from the one conjugated to the T-lymphocyte–directed MAb. The most sensitive detection is achieved by using panels of non–cross-blocking anti–T-lymphocyte antibodies directed against a variety of epitopes.

It is important to include a viability stain in the analysis panel, especially when in situ elimination methods are used. In these cases, the depleted allograft may contain dead or dying T lymphocytes that will be detected by flow but may not contribute to post-infusion events. Suitable viability stains include propidium iodide and 7-aminoactinomycin (7-AAD). Analysis of cell viability after ex vivo depletion is not straightforward. Cell death may not be expressed immediately but develops in the hours or days after processing and infusion. Under such circumstances, the analysis of apoptotic cells by a combination of Annexin-V staining with 7-AAD may provide a more accurate estimate of cell damage. This approach can be used in combination with simultaneous staining for T-cell surface markers to provide additional information. In some cases, incubating the cells for a period before analysis is advisable (e.g., in the case of depletion by immunotoxins, cells require time to divide to manifest the toxic effects).

Functional Assays

Flow cytometry detects residual cells by their ability to bind MAb. Routine flow does not provide information on the functional capacity of these cells, which may be important when assessing the graft for its potential to mediate GVHD or GVT. Stimulation of cells with interleukin-2 (IL-2) and ex vivo culture also has been used to detect functional residual T lymphocytes. In some cases, this method has shown a correlation between the numbers of T lymphocytes in the cultured sample and the development of clinical GVHD in the graft recipient. Limiting dilution analysis also provides a sensitive method for detecting residual T cells. However, because of the complexity of the results obtained, its use as a routine procedure to guide immunosuppression is not recommended.

Release of Manipulated Grafts for Clinical Administration

A selection procedure that does not alter the biological status of the progenitor cells is not generally regarded as more-than-minimal manipulation by the FDA. This usually means that the procedure can be performed according to GTP regulations, and product release does not require a formal CofA. If, however, a commercial device is to be used in the procedure, this complicates the situation. For example, the Miltenyi CliniMACS device is approved as a Humanitarian Use Device for CD34 selection in certain patients in their first complete remission. The FDA recognizes that some investigators may use it in an off-label indication but has indicated that it will monitor sales to determine whether this is happening. Miltenyi also sells nanoparticles for the depletion of CD3/CD19 and TCRαβ T cells for research use only. If the system is to be used for clinical T cell depletion, it is advisable to contact the company and the FDA to determine whether an IND application is required.

If it is determined that an IND is necessary, the cells must be prepared under GMP regulations. These require that manufacturing be performed by trained staff following formal standard operating procedures. The chemistry manufacturing and control (CMC) section of the IND will specify how the product is prepared, the reagents and materials that will be used, and the criteria for the release of the product for clinical use. The FDA has published Guidances for preparing the CMC section of the IND application[18] and for the design of clinical trials of cell and gene therapy products.[19]

Release criteria are test specifications that are designed to ensure that the product is sterile and pure, and they may include assays for functionality. The specific tests for sterility and purity that have been approved by the FDA are described in 21CFR 610.12. In the normal release mechanism for a cellular therapy product, the quality unit reviews the production records and issues a certificate of analysis (CofA). This document details the testing that was performed together with the test method, the testing laboratory, the specification required by the FDA, and the actual results obtained. Routine testing required for most cellular therapy products consists of aerobic, anaerobic, and fungal sterility; endotoxin levels (by Limulus amebocyte method [e.g., Endosafe from Charles River]); mycoplasma for ex vivo expanded cells (assayed by the culture method, by a validated polymerase chain reaction-based method [e.g., MycoTOOL from Roche Applied Science or by MycoAlert with FDA approval]); identity and purity (by flow cytometry and, in some cases, HLA typing); and, for products in later stages of evaluation, functionality (e.g., cytotoxic activity toward target cells or secretion of specific bioactive products). Products that have been transduced with a retroviral vector will also require testing for replication-competent viruses. The use of non-FDA-approved test methods should be cleared by the FDA at the IND application stage. Regulatory agencies also like to see some form of stability testing program that evaluates the stability of the cellular product over time in storage in the frozen state and when thawed for administration.

GTP products are not released using a CofA procedure. Fresh cells are usually administered at risk unless it is decided to perform a Gram stain for sterility. Normally a 14-day sterility test is still performed, and there must be an SOP in place to inform the recipient's physician if a positive result is obtained, identify the organism involved, and obtain antibiotic sensitivities. Other tests may also be performed, for example, endotoxin, immunophenotyping. In most cases, cryopreserved products should be able to undergo full release testing before administration.

CELLULAR THERAPY PRODUCTS

Many therapy products, including some that have been genetically modified, are being evaluated in clinical trials to determine their value in preventing or treating GVHD and for potential anti-tumor responses (Table 103.4 and Fig. 103.2). These include unmanipulated leukoinfusions from HPC donors,[13] cytokine-induced T cells, lymphokine-activated killer (LAK) cells, tumor-infiltrating lymphocytes (TILs,[20] T-regulatory cells, antigen-specific T cells,[21] mesenchymal cells,[2] dendritic cells [DCs], and NK cells[16]). Detailed accounts of the scientific basis for such studies as well as results of clinical trials are given in other chapters in this volume. This section focuses on processing and product evaluation issues.

TABLE 103.4 Immunotherapeutic Cells Frequently Used in Hematopoietic Stem Cell Transplant Recipients

Cell Type	Clinical Use
T regulatory cells	Prevention/treatment of GVHD
Mesenchymal stromal cells	Immunosuppressive effect (GVHD) Regenerative applications
NK cells, TIL cells, LAK cells, NKT cells	Anti-cancer response (Some for prevention/treatment GVHD)
CAR-T cells	Anti-cancer response
Antigen-specific T cells	Anti-cancer response Anti-virus response
Dendritic cells	Anti-cancer vaccines Antigen-presenting cells (unprimed)

Given that the frequency of tumor or viral antigen-specific T cells in most cases is considerably lower than that of alloreactive T cells, it is necessary to expand antigen-specific T cells ex vivo not only to enhance antitumor activity but also to try to separate GVHD from GVT effect.
CAR, Chimeric antigen receptor; *GVHD*, graft-versus-host disease; *LAK*, lymphokine activated; *TIL*, tumor infiltrating lymphocytes; *NK*, natural killer cells; *NKT*, natural killer T cells.

Donor Leukocyte Infusions

The ability of donor leukocyte infusions (DLI) to mediate antitumor responses was originally described in patients with chronic myeloid leukemia (CML) in hematologic relapse after allogeneic stem cell transplantation (SCT), but lymphomas and Hodgkin disease also are sensitive to the effects of DLI.[13] Remission rates of up to 80% have been reported in CML patients who relapse after transplant. DLI have also been used successfully in patients with myelodysplastic syndrome, and up to 90% of responses have been described in patients with Epstein-Barr virus (EBV)—associated lymphoproliferative disease. Moderate success has been achieved using DLI after relapse in other malignancies, such as acute myeloid leukemia (15% to 40%), low-grade lymphomas (≤60%), and metastatic multiple myeloma (40% to 60%). Fewer than 5% of patients with relapsed acute lymphoblastic leukemia respond to DLI alone. Although the etiology is unclear, the reason could be a lack of antigenic expression, downregulation of T-cell recognition molecules, or tumor burden at the time of treatment.

In practice, DLI are usually quantitated by their T cell (e.g., CD3, concentration, and frozen down in aliquots that provide a range of doses for the intended recipient). Initially, doses of 1×10^5 to 1×10^6kg are used, followed at intervals of 4 to 12 weeks with escalated doses, which may increase by 5- to 10-fold on each occasion. The timing of administration can be assisted by tracking the status of minimal residual disease or by mixed chimerism.

The regulatory situation for DLI is complicated and in transition. At present, non-manipulated DLI are classified as Type 361 products and fall under Part 1271 of 21CFR. It is possible that they will be reclassified as Type 351 products requiring an IND application. Currently, formal release criteria are not required; however, the normal practice is to evaluate T-cell content by flow cytometry and to test for sterility. This may be done by a Gram stain (used for immediate release) accompanied by 14-day culture-based methods. These provide results after the product has been infused. Formal procedures should be in place to inform the recipient's physician if a positive test result is subsequently received. The contaminant should be speciated and antibiotic sensitivities obtained and communicated to the physician. DLI that have been manipulated ex vivo in any way (e.g., by targeting them to specific antigens or by vector transduction, as described later) will be classified as Type 351 products requiring an IND. These products will require formal release testing as described previously for other cellular therapy products.

Allodepleted Cells

The major drawback of DLI is the significant risk of GVHD, which is the most important source of treatment-related mortality.

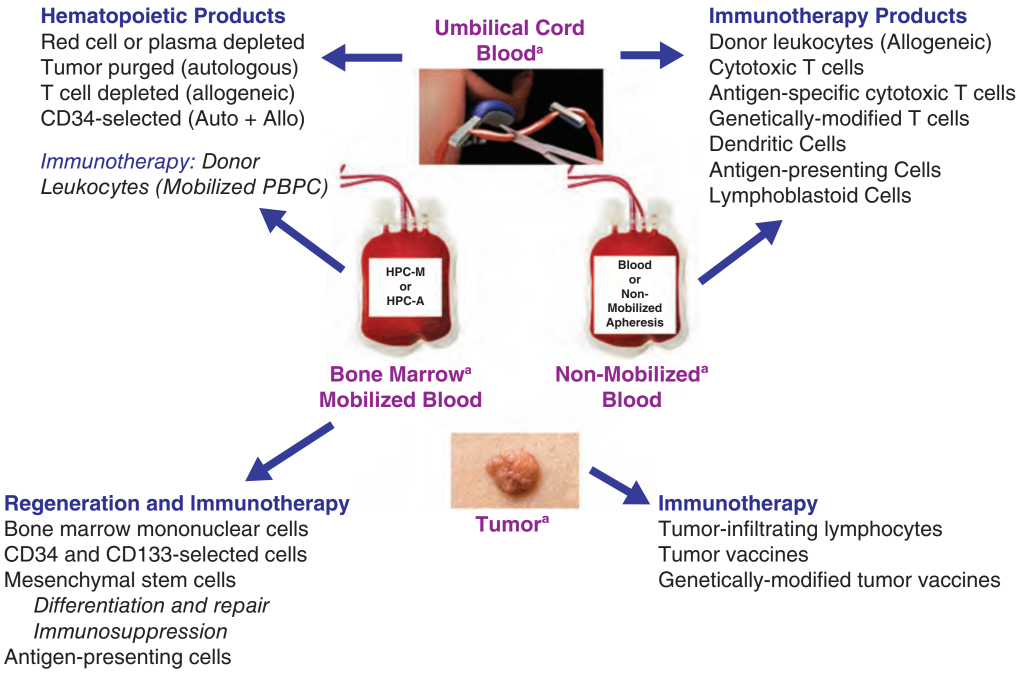

Hematopoietic Products
Red cell or plasma depleted
Tumor purged (autologous)
T cell depleted (allogeneic)
CD34-selected (Auto + Allo)
Immunotherapy: Donor Leukocytes (Mobilized PBPC)
Umbilical Cord Blood[a]
Immunotherapy Products
Donor leukocytes (Allogeneic)
Cytotoxic T cells
Antigen-specific cytotoxic T cells
Genetically-modified T cells
Dendritic Cells
Antigen-presenting Cells
Lymphoblastoid Cells
HPC-M or HPC-A
Blood or Non-Mobilized Apheresis
Bone Marrow[a] Mobilized Blood
Non-Mobilized[a] Blood
Regeneration and Immunotherapy
Bone marrow mononuclear cells
CD34 and CD133-selected cells
Mesenchymal stem cells
Differentiation and repair
Immunosuppression
Antigen-presenting cells
Tumor[a]
Immunotherapy
Tumor-infiltrating lymphocytes
Tumor vaccines
Genetically-modified tumor vaccines

Figure 103.2 CELL SOURCES FOR HEMATOPOIETIC TRANSPLANT AND POST-TRANSPLANT IMMUNOTHERAPY. *([a]From iStock Getty images.)*

A method for separating GVHD from the GVT effect is to selectively deplete alloreactive donor T cells ex vivo.[14,15] Selective allodepletion is performed by removing donor T cells that express activation markers after co-culture with non-leukemic recipient cells. Activation markers investigated include CD25 and CD69 and the increased sensitivity of activated T cells to photosensitizing dyes. Many phase I/II studies using immunotoxin directed against CD25 have demonstrated the feasibility of this approach, with accelerated reconstitution of virus-specific and total T-cell numbers and low rates of severe GVHD. Relapse rates remained high in this study, which probably reflects the high-risk nature of the patient population.

Potential recipients of allodepleted cells often receive a CD34-selected HPC graft. As a part of the CD34 selection process, a negatively selected population of cells that includes T cells becomes available. However, this fraction usually is not used as the source of cells for allodepletion. These cells usually have been obtained from donors who received granulocyte-macrophage colony-stimulating factor (GM-CSF) for mobilization of PBPC, and during processing for CD34 selection, they were exposed to an anti-CD34 monoclonal antibody. Therefore, it is preferable to obtain cells for allodepletion from a peripheral blood draw of the donor before administration of growth factor for mobilization. The T cell-enriched fraction usually is obtained by centrifugation on a Ficoll-Hypaque density cushion. The cells are washed and co-incubated with irradiated recipient mononuclear cells, which act as stimulators. A convenient source for stimulator cells is obtained by generating a cell line from the donor by infection of their peripheral blood mononuclear cells with a laboratory strain of EBV. The co-incubation step produces stimulation of alloreactive donor T cells with the resulting expression of activation markers, which then can be targeted to remove the alloreactive population. Methods for elimination include cytotoxic drugs (e.g., methotrexate), anti-CD25 monoclonal antibody conjugated to the toxin ricin, and immunomagnetic selection. All of the regulatory issues associated with the manufacturing and release of Type 351 products apply to allodepleted T-cell products. Release criteria include routine assays for sterility and purity. HLA typing may be included as a confirmation that the cells in the product are of donor origin. Functional assays may include a demonstration that the allodepleted cells fail to proliferate when cultured in a primary mixed lymphocyte reaction (MLR). If a suicide gene has been introduced (see below), testing will include demonstration that it can be efficiently activated.

Tumor-Infiltrating Lymphocytes

TIL are cells harvested from tumor sites (see Fig. 103.2),[20] which are expanded ex vivo with IL-2. As the cells are being expanded, increased tumor specificity can be achieved by pulsing the cells with tumor-specific peptides or transducing them with a retrovirus encoding a tumor-specific TCR. A major limitation is that patients must have pre-existing lymphocytes that can both respond to the tumor and be expanded ex vivo. Transferring these cells has led to tumor regression in 50% of lymphodepleted patients with metastatic melanoma.

Many methods have been developed for the isolation of TIL from tumors. They include mechanical disruption, enzymatic treatment, differential centrifugation approaches, and positive immunomagnetic selection. The method of choice depends on the type of tissue from which the cells are to be extracted and the availability of reagents, such as MAb, to enrich the T-cell population of interest. As with T-cell depletion of allogeneic grafts, the predominant effector TIL cell population is not fully characterized, and the availability of this information should facilitate the design of more effective separation techniques. A number of approved enzymes and centrifugation media are available, and several companies have suitable MAb that have been prepared under GMP conditions but have not been submitted for FDA approval.

The extracted cells are expanded ex vivo, usually starting in semi-open systems such as cluster plates. The initial populations may be tested in a cytotoxicity assay in an attempt to identify the effector population, which can then be selected for expansion. The expansion phase usually progresses through a number of types of cultures as cell numbers increase. These may involve growth in plates initially, progressing to T flasks and gas-permeable bags and on to hollow fiber and culture bag bioreactors. The culture medium contains IL-2 as the primary cytokine, although other agents alone and in combination may be used to promote the outgrowth of specific cell subpopulations.

Some investigators have used irradiated tumor cells to restimulate TIL during culture; others have performed selective separations to enrich the effector cells during expansion. Highly characterized tissue culture media, such as the serum-free lymphocyte medium AIM V, have been used for expansion. The goal is to use the simplest medium with the fewest additives that will support the growth of functional cells. When possible, the media should be free of animal serum and proteins to simplify the regulatory issues. However, the FDA is aware that complex media and serum combinations may be required to support the growth of some cell types and is willing to consider them, particularly if the cells can be washed into an approved excipient for administration.

Initiation of a phase I study using TIL or TIL subpopulations will not require complete characterization of the effector cell population, but some preliminary information should be available that allows quantification of the putative effectors for dosing purposes. In most cases, flow cytometric analysis will be used, and the target antigens may be pan–T-cell markers or specific combinations of T subset markers. When other constituents of the product may adversely affect the activity of effector cells, it may be necessary to set an upper limit for contamination by these cells in the clinical product. Most facilities perform some type of functional assay as a part of the release process, which in the case of TIL may be cytokine release or cytotoxic activity toward tumor cells of the appropriate histologic type.

Suicide Gene-Transduced Lymphocytes

Although treatment with DLI has led to remission in patients with the disease after HSC transplantation, unmanipulated cells also contain alloreactive T cells and can induce GVHD. The incidence of GVHD ranges from 55% to 90% and is associated with a treatment-related mortality rate of about 20%. Approaches that maintain the GVT effect while decreasing the incidence of GVHD have been evaluated and include transduction of donor T cells with a “suicide gene”.[22] Genes can be introduced into DLI or alloreactive-depleted cells to express the herpes simplex virus-1 thymidine kinase (HSV-tk). If GVHD develops, Ganciclovir is administered to the recipient, resulting in the suicide of the transduced donor leukocytes.

Alternative suicide genes based on the dimerization of Fas or Caspase-9 in the apoptotic pathway have been developed to circumvent the problem of immunogenicity of HSV-tk. The inducible Caspase construct also encodes a selection marker, such as CD19, that can be used to enrich the transduced population. If GVHD develops after administration of the Caspase-transduced cells, the suicide activation drug can be administered to the recipient to eradicate the transduced T cells by dimerization of Caspase 9 and activation of the apoptosis pathway. This approach is now also being used to control CAR-T cells administered post-transplant.

Antigen-Specific Cytotoxic T Lymphocytes

Two major prerequisites for generating antigen-specific cytotoxic T lymphocytes (CTL) are the identification of appropriate viral (in the case of EBV-associated lymphomas)[21] or tumor target antigens and the availability of suitable APCs. After being identified, CTL lines can be generated by co-culturing T cells with APC that express the target antigen. The lines are then expanded by re-stimulation with the antigen of choice and the addition of cytokines such as IL-2.

To generate antigen-specific CTLs, it is necessary to have a good source of APCs and a source of antigen to present to T cells. A variety of APCs have been evaluated, including fibroblasts, monocytes, and DCs, using different sources of antigen, including virus lysate or lysate of antigen-positive cells, peptides, or transduction of the APC, such as lymphoblastoid cells (LCLs) and DC with an immunodominant antigen. The use of lysates as the antigen source can be problematic because they are likely to be variable and difficult to standardize. Alternatively, many processing laboratories may not be experienced in handling virus and virus-infected cells.

Lymphoblastoid cells prepared using laboratory strains of EBV make excellent APCs for use in manufacturing EBV-specific T cells. They present EBV antigens efficiently, and they express high levels of co-stimulatory molecules. The typical procedure for generating the LCL is co-incubation with EBV derived from the tamarin B95-8 cell line. This line has undergone extensive testing and has been approved for use as a source of “clinical” EBV. The resulting LCLs are cultured in media containing acyclovir to eliminate any residual EBV. The LCLs are used to repeatedly stimulate T cells (as a mononuclear cell fraction of peripheral blood leukocytes), resulting in a population that is directed toward the immunodominant EBV antigens. These CTLs can be frozen while release testing is performed. They are delivered frozen to the bedside, where they are thawed and administered intravenously. Release testing consists of the routine tests described earlier for Type 351 products. For allogeneic CTL, functionality usually is assessed by cytotoxicity assays, which must demonstrate less than 10% killing of autologous blasts.

One limitation of this approach is that expanding of virus-specific T cells to achieve adequate dose levels is extremely time-consuming. All of these procedures require several weeks to generate APCs. More recently, accelerated methods of production have been developed. These include using direct T cell stimulation using mixtures of peptides for overlapping regions of the target antigen(s) (Pepmixes) and using disposable bioreactors that provide improved gas exchange, thereby markedly improving rates of T cell expansion.

An alternative strategy is to manufacture and bank third-party allogeneic antigen-specific CTLs with a range of HLA types so that an “off-the-shelf” product is available.[21] Anti-viral clinical responses have been described for patients who received partially matched allogeneic CTL. The generation of banks of CTL requires careful donor screening and HLA typing to allow at least partial matching with the intended recipient.

Chimeric Antigen Receptor T Cells

The use of T cells transduced to express CARs directed against tumor antigens has produced very promising results in clinical trials in CD19-positive leukemias and is now being evaluated for the treatment of solid tumors. The CAR often incorporates other moieties that are designed to dampen inhibitory signals from cancer or to amplify a beneficial immune response. CAR design is continuously evolving (Fig. 103.3). Initially, first-generation CARs were used, which included activating receptors such as CD8/CD3ζ. Second-generation CARs provided dual signaling combining activating and costimulatory signals. Third-generation CARs comprise more complex structures with at least three signaling domains, and fourth-generation receptors include a cytokine transgene.[23] Manufacture of these cells requires the preparation of an appropriate construct and delivery system (viral or non-viral vector) that is used to modify the T-cell population.[24,25] Traditionally, gamma retroviral or lentiviral vectors have been used for transduction. Transposases and RNA electroporation or cell surface conjugation are also being used to achieve transient CAR expression with the resulting CAR-T cells persisting for about two weeks.

There are numerous methods for preparing CAR-T cells involving the selection of different T cell subsets ($CD4^+$, $CD8^+$, γδ and αβ, naive, central memory, effector memory, virus-specific T cells, and a stem-like memory T cell). Magnetic separation methods are generally used for their enrichment, and expansion is achieved using a variety of cultural conditions. Several other cell types (e.g., NK-T) T_{reg}, and NK cells are also now being transduced to express CARs. Release testing is similar to that required for other gene-modified cellular therapeutics.

Analysis of Antigen-Specific T cells

Tetramer Analysis Enumeration of T cells bearing receptors for specific antigens can be achieved using tetramer analysis. Soluble versions of the heavy chain of major histocompatibility complex (MHC) molecules are first synthesized and adopt the appropriate conformation when a synthetic peptide representing the epitope is recognized by the T-cell receptor (TCR), and β2-microglobulin is added. The

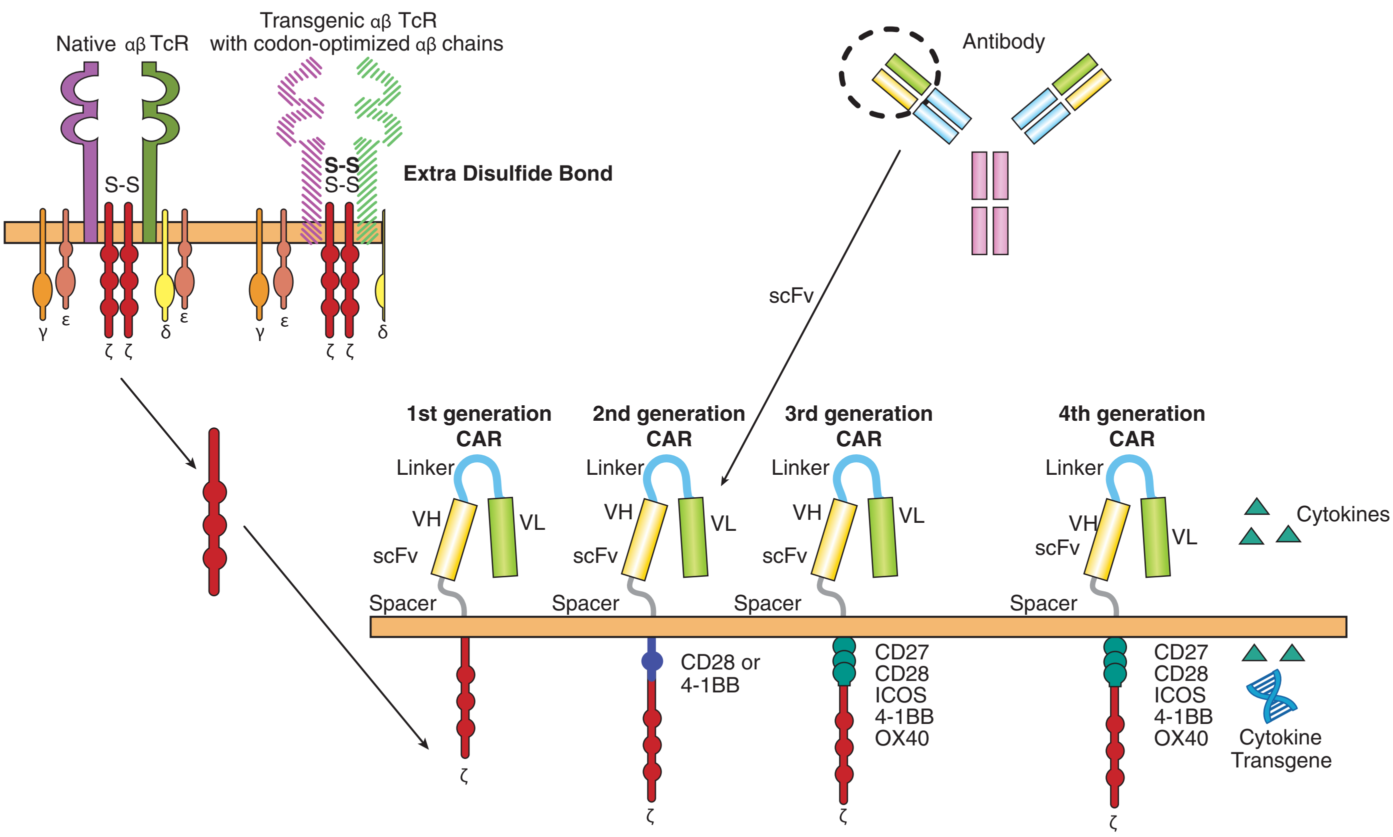


Figure 103.3 EVOLUTION OF CAR ANTIGEN RECEPTORS.

carboxyl-terminus of the MHC molecule is biotinylated, and four of these peptide/MHC—biotin complexes assemble into a tetramer when streptavidin is added. The streptavidin is tagged with a fluorochrome, therefore, T cells reactive with the chosen peptide—MHC complex become fluorescently stained and can be detected by flow cytometry.

Enzyme-Linked Immunosorbent Spot

Another approach is the use of an enzyme-linked immunosorbent spot (ELISpot) assay in which cells are stimulated to produce an analyte that is characteristic of their normal function. The cells are plated onto a surface that has been coated with an antibody directed against that analyte and incubated for a fixed period. The secreted analyte binds to this antibody, and the cells and any other unbound material are washed away. The surface is incubated with a biotinylated antibody directed against the analyte, washed, and incubated with alkaline phosphatase linked to streptavidin. After washing, the plate is incubated with a substrate solution. A blue-black precipitate will appear at sites where the analyte was produced, with each spot representing an analyte-secreting cell. The spots can be enumerated manually or by using an ELISpot reader.

These assays are not suitable for use as release tests because of their turnaround time, but they can provide retrospective information that may correlate with clinical outcomes.

Natural Killer Cells

NK cells are effectors from the innate immune system that also mediate antiviral and antitumor immunity. Studies have shown that haploidentical NK cells infused after lymphodepleting chemotherapy can have antitumor effects.[16] There are more than one thousand clinical NK cell trials completed or in progress. The majority are in the United States, followed by China, and are in use for the treatment of both leukemias and solid tumors.

NK cells can be prepared from Ficoll-Hypaque-enriched PBPC collections seeded into G-Rex bioreactors (Wilson-Wolfe Manufacturing) at 2 to 8 × 10^4 $CD56^+$ $CD3^-$ cells/cm^2. These are stimulated with K562 APC expressing mb15–41BBL at a 10:1 ratio of APC to NK cells. A simple positive selection with clinical scale immunomagnetic methods involving CD56 selection and CD3 depletion can be used to enrich NK cells from the expanded product.[26]

These procedures must be done under an IND because of the lack of commercially available devices and reagents for this indication. Recent evidence has suggested that cryopreserved NK cells have impaired functionality on recipients, in contrast to cells that are infused fresh. This poses an additional challenge to the manufacturer, who must coordinate testing and possible shipment of the fresh product. It is not clear whether the same findings may apply to other cell therapy products.

When preparing an IND application for NK cell enrichment, care should be taken to ensure that the MAb used for the selection, or depletion, are of the highest quality available. CofA for these reagents should be submitted with the IND application, and the agency probably will require detailed information on their manufacturing (i.e., to ensure that the process includes robust procedures for virus inactivation or removal). The same type of information may be required for any ancillary reagents (e.g., buffers and protein sources used to supplement buffers). Preclinical data demonstrating that the enrichment technique is effective should be provided and accompanied by data from clinical scale validation enrichments, providing evidence that the NK cells can be separated with acceptable viability, purity, and yield for the proposed study. The CMC section of the IND application should include information on the methods used to store, label, and administer the cells. Engineered APCs, such as the K562-mb15–41BBL cell line, if used to stimulate NK cells, should initially have been grown and tested as a Master Cell Bank, from which a Working Cell Bank is generated and tested for routine use.

Natural Killer T Cells

NK-T cells are innate-like CD1d-restricted T cells that recognize lipid antigens in a non-HLA-dependent manner and can rapidly respond to antigenic stimulation to produce large amounts of chemokines and cytokines, which can modulate both innate and adaptive immune responses.[17] Type I NK-T cells recognize α-galactosylceramide (α-GalCer), whereas Type II NK-T cells do not. Type II NK-T occurs with a higher frequency than Type I in humans. NK-T cells can kill CD1d-expressing tumor cells by direct cytolysis using perforin, granzyme B, Fas ligand, or TNF-α-mediated cytotoxic pathways. CD1d-bearing tumor cells are mainly myelomonocytic or of the B cell lineage. Type I NK-T murine cells have been generated ex vivo from CD34$^+$ cord blood cells using IL-15 and stem cell factor (flt-3 ligand).

Clinical trials using autologous NK-T cells transduced with a GD2-specific CAR (14 G.28.IL-15) vector and allogeneic third-party NK-T cells using a CD19 CAR vector construct that includes IL-15 and shRNA to reduce HLA expression are ongoing at Baylor College of Medicine. The cells are selected using the CliniMACS device and anti-NKT beads (Miltenyi Biotec). The selected cells are cultured with the irradiated negative fraction in the presence of α-Gal-Cer, IL-2, and IL-21 in G-Rex bioreactors (Wilson Wolfe), after which they are transduced with the retroviral vector and further expanded. Additional clinical trials are underway in China and Korea.

Dendritic Cells

DCs are powerful APCs that can be used in vitro or in vivo to elicit an immune response to the antigen they are presenting. A number of studies have evaluated tumor antigen-primed DCs for the treatment of hematologic malignancies and solid tumors.[27] Antigen-primed DCs are used to target T cells in vitro toward specific antigens. These cells then can be used therapeutically to eliminate viruses or tumor cells bearing that antigen. DC can be derived from BM or cord blood and mobilized and resting peripheral blood obtained from normal donors and patients. The typical procedure consists of enriching monocytes from the source material, which has been accomplished by plastic adherence or CD14-based immunomagnetic selection (or by depletion of CD19$^+$ B cells and CD2$^+$ T cells). An alternative approach is the use of elutriation to collect an enriched monocyte fraction from the donor. A purpose-built elutriation system for the collection of monocytes is available (the Elutra). This method also has been used successfully to enrich monocytes from cryopreserved mobilized apheresis collections.

The monocyte fraction can be cultured in polystyrene tissue culture flasks or gas-permeable bags in a culture medium containing IL-4 and GM-CSF to induce DC differentiation. This is followed by culture in a medium containing pro-inflammatory mediators to accelerate maturation. A number of supplements have been used during this phase, including CD40 ligand or poly(I:C), interferon-α, tumor necrosis factor-α, IL-6, IL-1α, and prostaglandin E_2. Some protocols add pro-inflammatory mediators at the initiation of the cultures rather than after induction of differentiation. One issue for manufacturers is finding clinical or GMP-grade cytokines and growth factors. Some sources are now available, and many manufacturers assist investigators by providing information on test procedures and stability information. Attempts have been made to expand DC in serum-free medium with varying degrees of success; improved growth and maturation have generally been obtained in the presence of human AB or autologous serum. Of note, minor changes in the composition of the culture medium and even in the type of vessel used for culture have been reported to affect the yield, phenotype, and functional activity of the resulting DCs. Cultured DCs have been effectively transfected using native tumor DNA or lentiviral or adenoviral vectors. The cells can be cryopreserved for later administration or use.

Assessment of DC product usually involves immunophenotyping (CD1a$^+$, CD80$^+$, CD83$^+$) and some type of functional assay (ability to generate an allogeneic T-cell response or response to a recall antigen as measured by proliferation assay) in addition to the standard release assays.

Mesenchymal Stromal Cells

MSCs are cells with multilineage potential that have shown efficacy in promoting engraftment and suppressing GVHD.[2] They are also being widely used in regenerative medicine and cancer immunotherapy applications,[2] where their mode of action is not well understood.[28] In vitro MSCs are capable of differentiating into bone, cartilage, cardiac and skeletal muscle, neuronal cells, adipose and connective tissue, and tendons. The cells are not inherently immunogenic and do not appear to be recognized by allogeneic T cells or NK cells. They express very low levels of MHC class II and intermediate levels of class I antigens. They appear to be able to suppress in vitro T-cell proliferation and function of both memory T and naive T cells and inhibit the development of monocyte-derived DCs in vitro.

A number of technical variables affect MSC culture and expansion ex vivo. They include culture medium, passaging density, serum type and concentration, population selection, culture vessel, and use of growth factors. To reduce some of this variability and decrease the cost of preclinical testing, the FDA often encourages investigators to adopt a manufacturing process that is in current clinical trials. The following is a basic procedure. MSCs usually are isolated from BM collected from the iliac crest. The cells are diluted, and the mononuclear fraction is isolated using a Ficoll-Hypaque density cushion. This fraction is plated into culture flasks to enrich adherent cells. The non-adherent population is removed after approximately 7 days of incubation. The adherent cells are washed and detached by incubation with trypsin/ethylenediaminetetraacetic acid (EDTA) (alternatively, Trypzean, a recombinant form of trypsin, can be substituted) and then cultured and passaged at weekly intervals. In a study designed to optimize culture conditions, the best results were obtained when cells were cultured in low-glucose Dulbecco modified Eagle medium (DMEM)based media containing 10% fetal bovine serum (human platelet lysate is now frequently substituted) and GlutaMAX instead of l-glutamine. The cells also proliferated better when plated at low cell densities (5000 to 10,000 cells/cm^2) in Falcon flasks. The use of basic fibroblast growth factor as a growth supplement was found to be effective; however, it caused HLA-DR induction and upregulated HLA class I expression. This did not affect the immunosuppressive capabilities of the cells. An increase in osteogenic and adipogenic potential was noted, but neurogenesis and engraftment support for CD34-positive cells were slightly suppressed. A number of media specifically designed for the culture of MSC are commercially available (e.g., PRIME XV from Irvine Scientific, STEMPRO MSC-SFM, a serum-free formulation from Invitrogen, and Mesencult, a basal culture medium from StemCell Technologies). Various investigators have reported success growing MSCs in serum-free media or using platelet lysate, autologous serum, or human AB serum as alternatives to fetal bovine serum. The traditional method for producing MSC is tissue culture flasks is laborious and involves many open procedures that could result in product contamination. An alternative method is to use a functionally closed, hollow fiber bioreactor, such as the Quantum from Terumo. In this system whole marrow is loaded into the reactor and the adherent cells attach to the fibers. Non-adherent cells are flushed from the fibers, which are perfused with the culture medium. Growth is monitored by glucose consumption and lactate production, and the flow rate of the medium is increased based on these readings. The cells are removed from the fibers by routine enzymatic treatment.

MSCs can be characterized by their expression of CD105, CD73, and CD90, and their lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR. They must be plastic adherent under standard culture conditions and must be capable of differentiating into osteoblasts, adipocytes, and chondroblasts in vitro. Differentiation assays involve growing MSCs under conditions that promote differentiation along the specific pathway. For neurogenic differentiation, the medium contains linoleic acid, platelet-derived growth factor, and epidermal growth factor. Neural cells are identified by immunostaining with antibodies against tubulin BIII, synaptophysin, galactocerebroside, neurofilament M, and neuronal nuclei (NeuN). To assess adipogenic potential, the medium contains dexamethasone, isobutylmethylxanthine, and indomethacin. The lipid droplets in the generated adipocytes are visualized by staining with Sudan Black IV. Osteogenic differentiation is measured by culturing the cells in a medium containing dexamethasone, β-glycerophosphate, and l-ascorbic acid 2-phosphate. Calcium accumulation and alkaline phosphatase activity in the resulting cells are visualized by alkaline phosphatase/Von Kossa staining, and the osteogenic differentiation is measured as the percentage of mineralized area in the total cultured area.

The ability of MSC to suppress an MLR can be used as an indication of their immunosuppressive activity. A traditional MLR assay is used; however, in the test wells, the MLR is performed on a layer of MSC seeded the day before. The response, traditionally, is measured by uptake of tritiated thymidine. Numerous animal assays for MSC are available but are predominantly used in preclinical studies and are not useful as release assays. Release criteria consist of the usual assays for sterility, endotoxin, and mycoplasma, with immunophenotyping for the MSC population. Depending on the intended use of the MSC, the release process may require an assay showing potential for differentiation into a specific lineage or ability to suppress the immune response, such as in the MLR assay.

Cancer Vaccines

Tumor vaccines as an approach to inducing or stimulating immunity have been evaluated.[29,30] Various techniques have been tested, including the use of DCs pulsed with a tumor-associated antigen preparation and the use of the entire tumor cell as an immunogen, either unmodified or transduced with immunostimulatory genes, aimed at eliciting a broad-based, robust immune response.

From the manufacturing perspective, the autologous product is much more of a challenge because a stable tumor cell line must be isolated from each patient. This can be an extremely difficult process. Many isolated lines do not expand well in culture, or they change phenotype over time. In some patients, the line cannot be generated; other patients progress clinically before the vaccine is available and are excluded from the study. As a result, the autologous approach has a relatively high “failure rate.” The allogeneic cell line approach is technically easier because a line is selected before the study and can be used as the generic immunogen. This line must be tested extensively for infectious agents and should pass the FDA testing criteria for Master and Working Cell Banks. Scientifically, the drawback is that the selected line may not adequately express the antigen or range of antigens present on each patient’s tumor. In addition, the choice of immunostimulatory molecules that are expressed or enhanced by transduction of the cell line may not be those that would most effectively evoke an immune response. These products are relatively expensive to produce, involving not only the costs of testing the tumor cell line but also the expense of manufacturing and testing the vectors used for transduction. However, these are one-time costs because the same vaccine is used for all patients in the study.

Release testing of cellular vaccines involves testing for sterility, endotoxin, and mycoplasma, with immunophenotyping of the cells and some form of test demonstrating that the line has been effectively transduced (e.g., flow cytometry for CD40 ligand and IL-2 production in the examples described earlier). Because the product will be stored cryopreserved throughout the study and thawed for administration to each patient, the requirement for an ongoing stability study should be anticipated.

An alternative strategy is the administration of peptides identified from tumor-associated or tumor-specific antigens used in combination with a variety of cytokines (e.g. IL-2, Il-7), radiotherapy, checkpoint inhibitors, chemotherapy, or small molecules (e.g. tyrosine kinase inhibitors) to stimulate an immune response in the recipients.

FUTURE DIRECTIONS

The practice of hematopoietic transplantation has changed dramatically over the past 10 years as a result of an improved understanding of the immunological interplay between GVHD, GVT, and anti-tumor responses mediated by post-transplant cellular therapies. Access to transplants has been improved by using matched unrelated and haploidentical grafts and by the availability of cord blood banks. GVHD has been reduced by the administration of T_{reg} and MSC post-transplant, and efforts are underway to exploit GVT responses. A major development has been the successful use of CAR T cells to destroy leukemic cells, and work is in progress to target solid tumors. We have seen some of these cells approved as licensed therapeutics, marking a major step forward for cellular therapies. At the same time the regulatory environment has become more stable and has recognized the issues associated with the rapid development pathway associated with these new types of treatments.

For the research community and cell manufacturing facility, life has been made easier by the availability of GMP-grade reagents and new cell culture and separation devices. There are also rapid testing methods available for endotoxin and mycoplasma, simplifying the release of products for clinical use.

As our understanding of the immunology of transplantation and immunotherapy increases, it is likely that we will continue to see the development of even more effective therapies.

REFERENCES

1. Frigault MJ, Maus MV. State of the art in CAR T cells therapy for CD19+ B cell malignancies. *J Clin Invest*. 2020;130:1586.
2. Fisher SA, Cutler A, Doree C, Brunskill SJ, et al. Mesenchymal stem cells as treatment or prophylaxis of acute or chronic graft-versus-host disease in hematopoietic stem cell transplant (HSCT) in recipients with a haematological condition. *Cochrane Database Syst Rev* 10.1002/14651858.CD009768.
3. cGMP in Manufacturing, Processing, Packing, or Holding of Drugs and Finished Pharmaceuticals. Code of Federal Regulations Title 21, Parts 210 and 211.
4. Guidance for Industry: CGMP for Phase 1 Investigational Drugs. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research, July 2008.
5. Human Cells, Tissues, and Cellular and Tissue Based Products. Code of Federal Regulations Title 21, Part 1271.
6. FACT-JACIE International Standards for Cellular Therapy Product Collection: Processing and Administration. Foundation for Accreditation of Cellular Therapy, Omaha, NE.
7. FACT Common Standards for Cellular Therapy. Foundation for Accreditation of Cellular Therapy, Omaha, NE.
8. AABB Standards for Cellular Therapy Services, AABB, Bethesda, MD.
9. NetCord-FACT International Standards for Cord Blood Collection, Processing, and Release for Administration, Omaha, NE.
10. Gee AP. *Cell Therapy—cGMP Facilities and Manufacturing*. New York, NY: Springer; 2009.
11. Shaz BH, Schwartz J. *Best Practices in Processing and Storage for Hematopoietic Cell Transplantation*. New York, NY: Springer; 2018.
12. Bertaina A, Roncarolo MG,. Graft engineering and adoptive immunotherapy: new approaches to promote immune tolerance after hematopoietic stem cells transplantation. *Front Immunol*. 2019;10:1342.
13. Greiner J, Gotz M, Bunjes D, Hofmann S, Wais V. Immunological and clinical impact of manipulated and unmanipulated DLI after allogeneic stem cell transplantation of AML patients. *J Clin Med*. 2020;9:13.
14. Li Pira G, Di Cecca S, Montanarti M, Moretta L, Manca F. Specific removal of alloreactive T cells to prevent GvHD in hemopoietic stem cell transplantation: rationale, strategies and perspectives. *Blood Rev*. 2016;30:297.
15. Amrolia PJ, Muccioli-Casadei G, Yvon E, Huls H, et al. Selective depletion of donor alloreactive T cells without loss of antiviral or antileukemic responses. *Blood*. 2003;102:2292.
16. Hu W, Wang G, Huang D, Sui M, et al. Cancer immunotherapy based on natural killer cells: Current progress and new opportunities. *Front Immunol*. 2019;10:1205.
17. Nair S, Dhodapkar MV. Natural killer T cells in cancer immunotherapy. *Front Immunol*. 2017;8:1178.

18. Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs). U.S. Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research; April 2008.
19. Guidance for Industry: Considerations for the Design of Early-Phase Clinical Trials of Cellular and Gene Therapy Products. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research, June 2015.
20. Nguyen LT, Sabil SD, Sotov V, Le MX, et al. Phase II clinical trial of adoptive cell therapy for patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and low-dose interleukin-2. *Cancer Immunol Immunother*. 2019;68:773.
21. Tzannou I, Papadopolou A, Naik S, Leung K, et al. Off-the-shelf virus-specific T cells to treat BK virus, human herpesvirus 6, cytomegalovirus, Epstein-Barr virus, and adenoviral infections after allogeneic hematopoietic stem-cell transplantation. *J Clin Oncol*. 2017;35:3547.
22. Ren YB, Sun SJ, Han SY. Safety strategies of genetically engineered T cells in cancer immunotherapy. *Curr Pharm Des*. 2018;24:78.
23. Stoiber S, Cadilha BL, Benmebarek M-R, Lesch S, et al. Limitations in the design of chimeric antigen receptors for cancer therapy. *Cells*. 2019;8:472.
24. Vormittag P, Gunn R, Gorashian S, Veraitch FS. A guide to manufacturing CAR T cell therapies. *Curr Opin Biotechnol*. 2018;53:164.
25. Stock S, Schmitt M, Sellner L. Optimizing manufacturing protocols of chimeric antigen receptor T cells for improved anticancer immunotherapy. *Int J Molec Sci*. 2019;20:6223.
26. Oberschmidt O, Morgan Huppert V, Kessler J, et al. Development of automated separation, expansion and quality control protocols for clinical-scale manufacturing of primary human NK cells and alpharetroviral chimeric antigen receptor engineering. *Hum Gene Ther Methods*. 2019;3:102.
27. Baldin AV, Savvateeva LV, Bazhin AV, Zamyatnin AA. Dendritic cells in anticancer vaccination: rationale for ex vivo loading or in vivo targeting. *Cancers*. 2020;12:590.
28. Christodoulo J, Goulielmaki M, Devetzi M, Panagiotidis M, et al. Mesenchymal stem cells in preclinical cancer cytotherapy: a systematic review. *Stem Cell Res Ther*. 2018;9:336.
29. Banchereau J, Palucka K. Immunotherapy: cancer vaccines on the move. *Nat Rev Clin Oncol*. 2018;15:9.
30. Gatti-Mays ME, Redman JN, Collins JM, Bilusic M. Cancer vaccines: enhances immunogenic modulation through therapeutic combinations. *Hum Vaccin Immunother*. 2017;13:2561.

CHAPTER 104

PRINCIPLES OF CELL-BASED GENETIC THERAPIES

David A. Williams

The use of gene transfer to treat human diseases has now been demonstrated to be efficacious in a growing number of indications. Proof-of-principle successes in several monogenic diseases—both hematologic and non-hematologic—have been published and widely publicized in the past decade. The previous occurrence of serious adverse events in some trials related to insertional mutagenesis has stimulated the development of safer vector systems and the improved efficiency of gene editing methods at a clinical scale, particularly those using clustered regularly interspaced short palindromic repeat (CRISPR) technology, holds great promise in the near term. This chapter discusses the basic biology of vector systems applicable to blood diseases, the details of the application of gene therapy to blood diseases using specific trials as examples of this technology, and the modifications in vector systems driven by clinical experience that predict future trials. The chapter also discusses the evolving field of genome editing that is already being tested in clinical trials.

HEMATOLOGIC DISEASES, CELLULAR TARGETS, AND THE BASIS FOR GENETIC THERAPIES

Gene therapy is defined as the introduction of new genetic material into the cells of an organism for therapeutic purposes. Broadly speaking, two types of gene therapy can be envisioned. The introduction of genetic material into germ cells such that the new DNA can be expected to be passed into the gene pool. This is termed *germline gene therapy* and is currently banned in the United States and around the world. The potential for newer methods of genome engineering to be utilized in clinical applications (discussed later) has led to more recent calls for extending this restriction. In contrast, the introduction of new genetic material into specialized cells of the body with no risk of the new genetic material being passed onto subsequent generations is termed *somatic gene therapy*. The ultimate goal of gene therapy is to correct the phenotypic abnormalities of the targeted genetic disease by *replacement* of the defective gene in situ. Such gene replacement could be envisioned via a process termed *homologous recombination*. Homologous recombination in mammalian cells is widely practiced in laboratories, but up to now, has been relatively inefficient, although newer technology appears to overcome the previous limitations of efficient genome editing. Advantages of this approach would include a reduction in the risk of inadvertent disruption or dysregulation of expression of a critical gene sequence and regulated (appropriate level and distribution) expression of the normal (replaced) gene. However, the frequency of homologous recombination (in contrast to random chromosomal integration or even editing using non-homologous methods) in mammalian cells and primary tissues at a clinical scale makes therapeutic use of homologous recombination still somewhat impractical at this point. Methods to effect homologous recombination efficiency have improved in the past 5 years and may make this goal attainable in the future.

The requirements for successful application of our current gene transfer technology for the treatment of human diseases include knowledge of the abnormal gene sequence responsible for the disease phenotype and the availability of the corresponding normal gene sequence that can be packaged into current vector backbones for efficient recombinant virus production. In addition, the cells responsible for the disease phenotype must be identified and accessible for genetic manipulation. Finally, a means of introducing and expressing the correct gene sequence in cells such that the disease phenotype can be reversed is needed. Although effectively accomplished in the early 1980s in murine studies, this latter requirement encompassing both in vivo administration of DNA sequences and ex vivo cell manipulation has been more difficult to consistently meet in human applications using current gene transfer technology.

Since the early development of virus vectors, blood-forming cells have been used as one optimal target for ex vivo gene transfer studies. For this purpose, hematopoietic stem and progenitor cells (HPSCs) are isolated, manipulated in the laboratory and administered back to the patient. The advantages of these cells as targets of gene transfer are multiple. First, all blood cells are derived from a common progenitor cell, the hematopoietic stem cell (HSC), which is both long-lived in vivo and capable of significant self-renewal. The latter capacity and the pluripotency of HSC is exploited to amplify the genetically manipulated cells into large numbers of progeny cell numbers of multiple blood lineages derived from the genetically altered cells in vivo. There is a long and successful experience in obtaining these stem cells from the bone marrow (BM) and peripheral and umbilical cord blood. There is extensive experience in the use of HSCs in the clinical setting for autologous and allogeneic transplantation, and there is the experience in the purification of these cells and limited knowledge of the requirements for ex vivo manipulation of the cells. In addition, the experience of HSC transplantation (HSCT) has defined a variety of genetic diseases in which the phenotype can be altered by the successful engraftment of normal allogeneic donor cells. Finally, the blood system is involved as a major dose-limiting organ in cancer therapies and both a target and an effector organ in immune reactions provide a large group of diseases that could theoretically be approached using gene transfer technology. As noted earlier, there are already many monogenic diseases of the blood extensively characterized with more being defined at the molecular level on a regular basis as whole-exome and whole-genome sequencing is being applied to rare disease phenotypes. In addition to HSC targets, another application of gene transfer technology exploits the experience in adoptive T-cell immunotherapy. In this application, T cells (and less well-developed to this point, other immune effector cells) are modified ex vivo to enhance potency and specificity. This application of gene transfer technology will not be reviewed here.

The field of gene therapy is rapidly evolving. Successes of "proof-of-principle" small trials have demonstrated the utility of the gene transfer approach in a growing number of patients but in a limited number of diseases leading to the first genetic therapies to be approved by regulatory agencies worldwide as therapeutic medicines. The technology itself is quickly evolving in response to new understandings of viruses, the regulation of gene expression, and improved gene-editing technologies. The application of gene transfer technology to HSC gene therapy has been made possible by the exploitation of viruses that have evolved the capacity to insert viral genomes efficiently and precisely into cellular chromosomes of infected cells. The field has taken >30 years to evolve to its current state of clinical application. Although this might be viewed as a slow pace, this time frame parallels the development of many other novel therapies. This developmental phase also reflects the complexities of the biologic systems involved and the caution required in moving forward in the face of serious adverse events seen in early safety trials. It is indeed an exciting time with respect to the clinical application of gene transfer technology in human diseases.

VECTOR SYSTEMS

The initial impetus to develop gene transfer for human studies derived from the exploitation of oncoretroviruses, mainly murine gamma (γ)-retroviruses, as vectors for gene delivery in the early 1980s. However, since these early studies, a multitude of virus vectors have been developed. All vector systems exploit the virus life cycle to increase the frequency and fidelity of the gene transfer. Although many vector systems have been developed, retrovirus and lentivirus vectors have become the most used platforms for human gene therapy trials involving HSC and T cell targets and this review will focus primarily on these vector systems (reviewed by Touw and Erkeland).[1] As noted, the majority of trials registered with the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health use retroviruses (both γ-retroviruses and lentiviruses) with non-integrating adenovirus vectors, adeno-associated virus, and nonvirus (liposomes and plasmids) systems making up the second and third largest groups. The latter is primarily focused on immune stimulation trials in cancer and have limited relevance to the use of HPSCs for the treatment of genetic blood diseases.

Retrovirus Vectors

The use of γ-retroviruses as gene transfer vectors takes advantage of the normal virus life cycle. In general, lentivirus vectors exploit a similar lifecycle as a vector system. The virus, a membrane-bound particle enclosing a dimer of genomic RNA, gag, and reverse transcriptase proteins, interacts with specific cell surface receptors on the target cell. After entry into the cytoplasm, the virus is uncoated, and the genomic messenger (m)RNA is reverse transcribed into DNA. Subsequent polymerase activity yields a double-stranded (DS) DNA provirus molecule. For γ-retroviruses, transport into the nucleus depends on the loss of the nuclear membrane, which accompanies cell division (see later discussion). Integration of the DS provirus in the chromosome is semi-random. The occurrence of insertional activation of oncogenes in several human trials and the subsequent scrutiny of insertion sites in HSC-derived progeny in both murine and human cells using deep sequencing methods have provided a more detailed understanding of subtle but biologically relevant preferences for insertions of these vectors (see later discussion). After being integrated, the provirus can give rise to mRNA leading to encoded protein products. Full-length (genomic) mRNA can also be used as the genomic nucleic acid in newly formed virus particles, which are budded nonlytically from the cell surface after assembly in the cytoplasm of the infected cell. The use of retroviruses for gene delivery depends on the capacity to replace viral genes with other heterologous gene sequences and to provide necessary viral proteins *in trans* in specialized cell lines, called *packaging cells*. The advanced generation of γ-retroviruses packaging cells appears to be capable of generating pure stocks of the recombinant virus without contaminating wild-type helper virus, an important safety consideration. Indeed, to date in human trials, there have been no reports of inadvertent generation of infectious viruses. Thus, the infection with replication-incompetent (i.e., helper-free) retrovirus vectors would be predicted to yield integration into the targeted cell population but no further spread of the virus in the body of the treated patient. The proteins provided *in trans* for γ-retroviruses are generally gag, reverse transcriptase, and envelope proteins, the latter defining the host range of infection. In summary, the advantages of retrovirus vectors include the high efficiency of stable transfer of intact DNA sequences, the broad range of host cells susceptible to infection by retroviruses, and for γ-retroviruses the ability to generate helper-free recombinant virus via stable packaging cell lines.

Despite these advantages, the application of retrovirus vectors for the treatment of human blood diseases in early trials was disappointing. In multiple studies, transduction of long-lived and transplantable HSCs was extremely low. In most studies, the frequency of circulating marked blood cells was too low to effect phenotypic correction of any disease, usually less than 0.1%. The biologic parameters contributing to the poor results in human trials are varied. The major impediments appear to include the low levels of viral receptors on the surface of human HSCs, reducing the efficiency of interaction of virus particles with these target cells, and the quiescent nature of the majority of HSC, which hinders the transport of the provirus into the nucleus and thus reduces the integration frequency. Practical issues, including the difficulty in obtaining high-titer viruses in large-scale preparations required for human trials, have also been noted.

These difficulties have led to various strategies and the development of entirely new vector systems, which seek to improve gene transfer methods in human HSCs. These strategies include attempts to increase virus–cell interactions or methods to enhance the chances of successful DNA integration. Different viral envelopes were used to pseudotype recombinant particles to more efficiently target CD34 cells. The use of various cell surface markers, such as CD34, to purify the target cell population can also increase the multiplicity of infection at a given virus titer and has been used in clinical transplantation protocols. Thus, the development of antibody-based enrichment of the $CD34^+$ HPSC compartment from human hematopoietic tissues using magnetic column purification provides a rapid, clinically applicable method to further enhance retroviral transduction by increasing the vector to target cell ratio. Methods to increase physical interactions between vector particles and target cells include co-localization on fibronectin and centrifugation methods. Where polycations such as polybrene had previously been used to enhance transduction frequencies by negating electrostatic charge repulsion between target cells and viral particles, the characterization of the recombinant CH296 fibronectin fragment (Retronectin™) as a matrix upon which one could co-localize HSCs and viral particles was a significant advance in the quest to improve $CD34^+$ transduction frequencies. More recently effective combinations of agents called "transduction enhancers" have been increasingly utilized in clinical trials. Other efforts to increase the chances of DNA integration have focused on attempts to increase the number of HSC that are undergoing cell division (primarily the use of cytokines that affect stem cell proliferation). The development of improved in vitro growth media formulations incorporating novel cytokine cocktails achieved the dual aim of promoting HSC division, which is required for transduction with γ-retroviral vectors and "activation" required for lentivirus vectors while minimizing stem cell loss via apoptosis or differentiation. Finally, the use of new virus systems that do not require nuclear membrane disruption (and, therefore, cell division) for entry of the provirus DNA into the nucleus, including primarily lentivirus vectors, but potentially also vectors based on foamy viruses, appears to be the most significant development in the field in the past decade. Lentivirus vectors will be discussed in more detail later.

In addition to advancing stem cell transduction methodology, additional work has focused on developing retroviral vectors that would express transgene cassettes at levels that would be high enough to elicit a therapeutic benefit and be resistant to gene silencing. Advances in vector design such as the optimization of long-terminal repeat (LTR) enhancer and promoter elements and viral leader sequences resulted in recombinant vectors that were able to mediate high-level transgene expression in both primitive and mature hematopoietic cells. As discussed in detail later, although these powerful promoter and enhancer elements provided a robust expression of transgenes, they appear also to be capable of long-range activation of endogenous regulatory sequences as a form of insertional mutagenesis that can have significant deleterious effects. Taken together, these technological advances served as the platform for the first successful gene therapy trial in humans.

The use of pharmacologic in vivo selection in combination with gene transfer, both in the setting of cancer trials and in genetic diseases, remains a potentially important method to enhance the reconstitution of human recipients with gene-modified blood cells but has not yet gained widespread usage. General considerations include the need for a particular drug to effect damage to BM stem or progenitor cells. A gene's or genes' encoding resistance to this agent would need to be identified and resistance in vivo to the agent would need to be demonstrated after overexpression of this gene in BM cells.

For applications in cancer therapies, dose intensification of drugs used within chemotherapeutic regimens should improve antitumor efficacy. Work is ongoing to use the transgenic expression of O^6-methylguanine methyltransferase (MGMT) which generates resistance to bis-chloroethylnitrosourea (BCNU), temazolamide (TMZ) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and cytidine deaminase which generates resistance to cytosine arabinoside, gemcitabine, decitobine, and azacytodine for applications in cancer. For non-cancer applications (for the co-selection of a non-selectable therapeutic gene in a genetic disease application), the mutagenic potential of the chemotherapy agent must be considered as a risk in relation to the overall benefit of the gene therapy procedure. Several chemoresistance genes and chemotherapy drug combinations are currently under investigation for this application and encouraging preclinical studies in model systems, including murine and canine studies, have led to a limited number of early-phase human studies in cancer patients.

Lentivirus Vectors

One of the key advances in the gene therapy field in the past decade has been the development of recombinant vectors based on lentiviruses, including the human immunodeficiency virus (HIV). These vectors were originally developed after the observation that targets of HIV included more differentiated cells, such as macrophages, that are often also post-mitotic, suggesting this group of retroviruses has evolved a method of circumventing the block in infection of γ-retroviruses seen in non-dividing cells. Investigators demonstrated that lentivirus vectors derived from HIV were capable of infecting non-dividing neurons after direct injection into the brain. Subsequently, Naldini and colleagues showed efficient infection of growth-arrested cells. As with γ-retroviruses, lentivirus vectors use key viral gene products *in trans* to generate replication-defective infectious particles carrying the transgene of interest. To date, most lentivirus vectors use vesicular stomatitis virus as an envelope sequence, which provides a very broad range of target cells susceptible to lentivirus vector transduction. In the case of lentivirus vectors, viral gag and pol, as well as tat and rev proteins, expression are required *in trans* for efficient virus production along with the envelope proteins. These proteins are usually supplied from separate plasmids, and recombinant lentivirus production is today generally created using the "four plasmid" systems encompassing all the necessary viral proteins on three plasmids and the transfer vector sequences of the fourth plasmid. In addition to *gag* and *pol*, the transfer vector contains all the virus regulatory sequences required *in cis* for packaging an infectious particle, including the *psi* packaging sequence, integrase, and reverse transcriptase. In almost all clinical applications to date, the recombinant lentivirus vector is generated as a transient supernatant from a population of transfected producer cells rather than a clone and each large-scale batch of viruses has variable yields. Thus, the generation of high-titer recombinant virus is more complicated than γ-retroviruses and remains a significant issue for large-scale, clinical-grade production for use in human trials.

To reduce the chances of the generation of replication-competent retroviruses (RCRs), a major safety concern with HIV, most of the nonessential viral sequences have been removed from currently utilized vectors. This included *viv, vpu, vpr*, and *nef* genes and subsequently *tat*, an important regulator of viral transcription. Recombinant vectors generated without these sequences were demonstrated to efficiently infect a variety of cells. RCR testing is based on sensitive assays to detect a gag protein by p24 immunoassays or polymerase chain reaction. In addition, lentivirus vectors have traditionally been produced using the "self-inactivating design" (SIN) for added safety because of the reduced risk of recombination with and subsequent mobilization of endogenous HIV viruses. The transfer vector thus contains a deletion of the 5′ LTR U3 region and are devoid of viral enhancer and promoter sequences. During reverse transcription, the 5′ LTR is replicated, and the integrated provirus is thus devoid of both 5′ and 3′ enhancers. In SIN constructs, the transgene of interest is thus expressed from an internal promoter that can be chosen with varying strengths or lineage specificities. This added safety feature ultimately has proven important in reducing the risk of insertional mutagenesis (see later) by which integration near cellular genes are inadvertently activated by the LTR enhancer sequences of vectors.

Lentivirus vectors were originally developed for use in a wide range of tissues in which cells are largely non-dividing. Early work focused on the brain, retinal cells, liver, pancreatic islets, airway epithelium, and muscle. However, it was subsequently appreciated that the requirement of stimulating HSCs into division with various cytokines to effect efficient transduction with γ-retrovirus vectors may have negative effects on engraftment or that a large fraction of HSCs remain quiescent and, therefore, resistant to transduction during clinical transduction protocols. After several groups reported successful transduction of primitive HSC populations in protocols in which these cells remained resistant to transduction by γ-retroviruses, the adoption of lentivirus vectors has now included multiple human trials with encouraging results. One of the earliest trials to utilize lentivirus vectors was in childhood cerebral adrenoleukodystrophy (CCALD)[2] in which long-term "marking" in the myeloid compartment appears to be 10% to 20%, a level that is about 100-fold higher than the marking in the myeloid compartment seen in previous trials in immunodeficiency conditions that used γ-retrovirus vectors. In addition to the safety advantage of SIN vector design used in all lentivirus vectors, there is an additional theoretical advantage of the preference of lentiviruses for integration away from transcription start sites (TSS) of genes in contrast to γ-retroviruses. However, the recent experience with a lentivirus vector used in a single patient with thalassemia[3] in which abnormal splicing resulted in clonal expansion in the erythroid compartment, suggests that insertion within genes including intronic and non-coding regions of genes may also have potential adverse effects on endogenous sequences. These trials are described in more detail later. Thus, the long-term safety of lentiviruses in human trials remains to be determined, and important aspects of insertional mutagenesis are described in more detail later.

EXPERIENCE IN HEMATOLOGIC CLINICAL TRIALS TO DATE

X-Linked Severe Combined Immunodeficiency

Severe combined immunodeficiency (SCID) comprises a number of rare monogenic diseases with the common feature of a block in T-cell differentiation and impaired B-cell and natural killer (NK) cell immunity. Studies of the pattern of inheritance, immune function, and genotypes have led to the identification of at least 18 distinct SCID conditions with the number increasing as genomic sequencing identifies mutations in additional immunodeficiency phenotypes. The most common variant of SCID results from the deficiency in the expression or function of the common cytokine receptor γ chain, which is shared by the receptors for interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15, and IL-21. This condition is inherited in a sex-linked fashion (X-linked SCID or SCID-X1) and accounts for ~30% of all SCID cases. SCID-X1 is characterized by abnormal development or function of T, B, and NK cells, although B cells are usually present in humans (so-called T-minus, B-plus SCID). Survival depends on the reconstitution of T-cell development and function by allogeneic BM transplantation (BMT). If a genotypically matched family donor is available, HSCT confers a greater than 80% chance of long-term survival. The absence of T and NK cells in the patient allows for the engraftment of donor cells without preparative chemotherapy conditioning; thus, this is the treatment of choice with minimal toxicity. When a genotypically matched family member is not available, haploidentical donors (e.g., a parent) or closely matched unrelated donors are used, with varying preference from center to center, and a survival rate of 64% to 78% has been reported. These inferior outcomes may be attributed to the increased risk of graft rejection or graft-versus-host disease (GVHD), as well as the effects of T-cell depletion, immune suppression causing slower immune reconstitution, or conditioning with an increased risk of infection. Haploidentical transplants rigorously depleted of T cells, similar to genotypically related transplants, are performed in some institutions without preparative chemotherapy conditioning; however, B-cell reconstitution is poor, and the majority of patients require intravenous immunoglobulin (IVIG) replacement for life. Interestingly, spontaneous partial correction of severe T-cell immunodeficiencies, including SCID, has previously been reported, suggesting a selective advantage of wild-type T cells over defective T cells.

Multiple independent gene therapy trials aimed at correcting the immunologic defect of SCID-X1 patients who lack a genotypically matched BM donor have been reported.[4] In the initially reported trials, done in Paris and London, a total of 20 patients have been treated.[5,6] Despite minor technical differences in the two protocols, the basic design of both gene therapy trials is quite similar: The complete coding region of the human γ chain was cloned into a "first-generation" γ-retroviral vector regulated by the murine leukemia virus (MLV) LTR sequences, which was used to infect BM-derived CD34$^+$ cells in vitro. Results in both trials have been extremely encouraging.

In the French trial, 10 children younger than the age of 1 year were enrolled between 1999 and 2002. Nine of 10 infants developed normal numbers of T and NK cells, with good immune function. In seven of the nine patients who developed T cells, T-cell counts reached normal levels within three months and have remained normal at the time of the last published follow-up. Protective levels of antibodies, including antibody production after immunization, were achieved, and the prophylactic administration of IVIG was discontinued. At almost >10 years after gene therapy, these patients continued to retain a functional immune system, enabling them to live normally.

However, serious adverse events related to gene therapy have been reported in five patients in the French trial, occurring up to 15 years after gene therapy and in one patient in the British trial. In these patients, untoward effects of viral integration into the genome resulted in T-cell leukemia, leading to the death of one of the affected patients. Much research has subsequently been directed at elucidating the mechanism responsible for these adverse events. Retroviral integration in the proximity of proto-oncogenes, particularly the *LIM domain only 2* (*LMO2*) promoter, was involved in leukemogenesis in three French patients and one British patient. Integration of the unaltered γ chain–encoding viral vector on chromosome 11q13, near the first exon of the *LMO2* gene, led to the unregulated transcription of LMO2, giving rise to a T-cell acute lymphoblastic leukemia (T-ALL)–like lymphoproliferation in the initial two patients. In the patient in the British trial, the integration of the vector 35 kb upstream of the *LMO2* locus cooperated with secondary genetic aberrations, including a gain of function mutation of NOTCH1, a deletion at the CDKN2A tumor suppressor gene locus, and translocation of the T-cell receptor β region, to give rise to T-ALL. LMO2 is a master regulator of human hematopoiesis that is involved in stem cell growth and is not normally expressed in T cells. However, LMO2 activation has been implicated in some cases of human T-cell leukemia. In addition, LMO2 transgenic mice have been shown to develop T-ALL within 10 months. It is increasingly clear that retroviral vectors may "turn on" cellular proto-oncogenes adjacent to their integration site in the genome. The strong promoter or enhancer activity of the retroviral LTR element shows a propensity to the upregulation of genes neighboring the integration site. Multiple studies now indicate that γ-retroviral vectors, such as the vectors used in the two SCID-X1 trials, preferentially integrate into the 5′ end of genes near the TSS. In addition, γ-retroviral vectors have been shown to integrate into or near a number of proto-oncogenes that are actively expressed in human CD34$^+$ cells. When human CD34$^+$ cells were transduced with retroviral vectors ex vivo, 21% of retroviral integrations occurred at recurrent insertion sites ("hot spots"), which were highly enriched for proto-oncogenes and growth-controlling genes. A series of papers investigating the vector integration sites in both SCID-X1 trials and a trial treating Adenosine Deaminase (ADA) Deficiency SCID trial (see below) observed a greater-than-random frequency of vector integrations near the TSS of genes that are active in HSCs. Interestingly, in the SCID-X1 trial, a skewing of vector integration site distribution in vivo was noted. Compared with retroviral integration sites (RIS) recovered from transduced CD34$^+$ cells, RIS recovered from T cells in vivo 9 to 30 months after transplantation showed an overrepresentation of RIS within or near genes encoding proteins with kinase activity, transferase activity, or proteins involved in phosphorous metabolism. This skewing of RIS in vivo suggests a selection of T cells as a result of viral integration in certain growth- and survival-promoting genes. A subsequent trial in X-SCID utilizing a SIN γ-retrovirus design (NCT01129544) reports follow-up on nine patients, although the trial has now closed for accruing patients.[4] The virus design is noteworthy in that it is more directly comparable to a lentivirus since the viral enhancer elements are deleted and the expression of the IL-2 γ chain is from an "internally positioned" cellular promoter. The initial results reported efficacy similar to the previous SCID-X1 trials, no leukemia and integration analysis that appears safer than those seen in the previous two trials. In the eight evaluable patients treated in this trial, six demonstrated correction of T-cell reconstitution. One patient died prior to full engraftment of gene-modified cells of pre-existing viral infection that did not resolve after gene therapy. The two failures correlated with lower VCN in the transduced product as a result of inadequate gene transfer. This study is particularly noteworthy as the only human study in which a γ-retrovirus vector deleted of enhancer elements has been utilized. It thus provides a platform for more direct comparison with previous γ-retrovirus and lentivirus trials with respect to safety and efficacy. More recently, a trial using similar cis-regulatory elements driving the gamma chain cDNA in a lentivirus vector has been reported, which includes the successful reconstitution of the B-cell compartment after including conditioning with busulfan.[7]

In summary, thus far, six of 20 patients treated with gene therapy for SCID-X1 have encountered a life-threatening severe adverse event, thought to be triggered by retroviral activation of LMO2 in four patients. Four patients were salvaged with chemotherapy, and one patient succumbed to the disease after an unsuccessful allogeneic BMT. At this point, the use of MLV-based retroviral vectors with LTR promoter enhancer elements intact is viewed as contraindicated in this disease by most investigators in the field. The continued development of safety-enhanced vectors and the validation of these vectors in clinically relevant systems have emerged as a major priority in the field, and an international trial has recently been reported using a γ-retrovirus that is deleted of LTR enhancer elements. Transgene expression is mediated by a weak cellular promoter in this vector. Data suggest that early efficacy is maintained despite lower expression of the cγ chain and that the deletion of the γ-retrovirus enhancer is a key to improved safety.[4]

Adenosine Deaminase Deficiency

ADA is a housekeeping enzyme of the purine metabolic pathway that is expressed in all tissues of the body. Deficiency of this enzyme leads to a buildup of toxic metabolites with detrimental systemic effects, including neurodevelopmental deficiencies, sensorineuronal deafness, and skeletal abnormalities. Importantly, ADA deficiency causes abnormal T-, B-, and NK-cell development, resulting in the SCID phenotype. As is the case with the more common SCID-X1, untreated patients generally succumb to severe opportunistic infections in the first year of life. Treatment strategies used to manage affected patients include allogeneic HSCT; enzyme replacement therapy; and more recently, gene therapy. Allogeneic HSCT from a human leukocyte antigen (HLA)–matched family donor offers good immunologic and biochemical correction with reasonable survival. However, outcomes after mismatched and haploidentical transplants are less impressive. Likewise, the exogenous replacement of ADA, administered in a polyethylene glycol (PEG) conjugate by intramuscular injection on a weekly or twice-weekly schedule, results in systemic detoxification and immune reconstitution. In the long term, however, about half of the patients receiving PEG-ADA replacement continue to require IVIG infusions, and some patients show a decline in T-cell numbers over time.

A number of gene therapy trials for ADA deficiency were initiated in the early 1990s, targeting retroviral gene transfer into various cell types, including peripheral blood lymphocytes, umbilical cord blood, BM, and CD34$^+$ selected stem cells. These early studies failed to produce clear efficacy. By contrast, later studies introduced key modifications to the gene therapy protocol, including the use of a reduced-intensity myelosuppressive conditioning regimen and the withdrawal of concurrent PEG-ADA replacement. The Milan-based group of Aiuti and colleagues reported on their initial experience with 10 children. Patients were conditioned with 4 mg/kg of busulfan before the infusion of transduced cells. The mean age at the time of gene therapy was 2.2 years. All children on this trial were reported as healthy and thriving, with the longest published follow-up time more than 64 months. Gene therapy resulted in a substantial increase in lymphocyte counts and normalization of T-cell function. An update on this trial,[8] published in abstract form, reports that 18 patients have been treated and are alive and, of these, 15 do not require replacement enzyme treatment (reviewed in 2017 by Ferrua and Auiti).[9]

Similarly, four patients have been treated in London.[10] One patient that has been reported in detail had been treated with PEG-ADA for 3 years but showed a gradual decline in T-cell numbers despite effective metabolic correction. Because a matched BM donor was not available, the patient was enrolled on the ADA-SCID gene therapy trial. The PEG-ADA replacement was stopped one month before gene therapy, and the patient was conditioned with a single dose of 140 mg/m^2 of melphalan before the infusion of the transduced BM CD34$^+$ cells. At the time of the last published follow-up, the patient was 2 years from gene therapy, clinically well, and off prophylactic antibiotic therapy. An increase in T-cell numbers and normalization of the proliferative response have been noted. Importantly, no adverse events have occurred thus far in the patients treated for ADA-SCID at these two centers.

Aiuti and colleagues recently published a comprehensive genome-wide analysis of RIS of five patients treated in Milan. This paper analyzed the RIS patterns in CD34$^+$ cells before infusion as well as RIS in vivo, up to 47 months after gene therapy. As anticipated, a nonrandom proviral integration pattern, favoring TSS and gene dense regions, was observed in the pretransplant cells. RIS observed in vivo in T cells were additionally enriched for TSS, suggesting the occurrence of in vivo selection. More recently, Aiuti and colleagues[11] have demonstrated that cellular genes in the proximity of the proviral integration site are subject to moderate dysregulation in gene-modified T-cell clones isolated from patients. However, in contrast to the SCID-X1 trial, no in vivo skewing toward RIS in genes affecting survival, cell cycling, signal transduction, or proliferation were observed, making a clonal dominance effect appear less likely. Interestingly, only one *RIS* was detected at the myelodysplastic syndrome (MDS)-EVI1 locus and became undetectable at later time points. This is in contrast to the clonal dominance of *MDS-EVI1* integration sites observed in the X-linked Chronic Granulomatous Disease (CGD) trial (see below). Additionally, an overrepresentation of RIS was noted in the proximity of the *CCND2* and *LMO2* gene with a total of five of 523 RIS recovered in vivo. Notably, the *CCND2* insertions were detected only in the first 2 years of follow-up and not subsequently. *LMO2* insertions were also overrepresented in the pretransplant CD34$^+$ samples, highlighting the fact that the *LMO2* gene is a hot spot for retroviral integration in human CD34$^+$ cells. The lack of in vivo expansion of clones carrying LMO2 RIS indicates that this integration site may not be sufficient to mediate clonal dominance and leukemic transformation. Rather, additional cooperating mutations or insertions are required for malignant transformation. The γ-retrovirus vector (Strimvelis™) has been approved by the European Medicines Agency (EMA). However, recent leukemia has been reported in a patient treated with this vector attributable to insertional mutagenesis. A multi-center trial utilizing a lentivirus platform that has completed accrual (NCT01279720) with encouraging results is currently being reported.[12]

CHRONIC GRANULOMATOUS DISEASE

CGD is an inherited disorder of phagocyte dysfunction characterized by often life-threatening invasive fungal and bacterial infections and by granuloma formation in vital organs. CGD results from a mutation in one of four subunits of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase of phagocytes. The inability to form microbiocidal oxygen species renders the phagocytes unable to fight invasive infections. Almost 70% of CGD cases result from defects in the X-linked gene encoding gp91^{phox} (X-CGD). With conventional therapy, including lifelong antimicrobial prophylaxis and interferon-γ therapy, the yearly mortality rate of X-CGD remains at 5%. HSC transplantation is curative for patients with a perfectly matched sibling donor but remains risky in patients with active infections. Unrelated donor transplantations are not routinely recommended. Thus, the development of a gene therapy approach that uses autologous HSC provides an important therapeutic advance for this patient group. In previous clinical gene therapy trials conducted without myeloreductive conditioning, the engraftment level of gene-modified cells remained low.

In 2002, the German group of Grez and colleagues in Frankfurt, Germany, initiated a gene therapy trial of X-CGD. The initial patients received a mild immunosuppressive preparative regimen and failed to engraft significant numbers of gene-modified cells. However, 2 years later, low-dose busulfan—modeled on the successful gene therapy trial for ADA-SCID—was incorporated into the preparative regimen, and additional patients were treated. This group of patients has been followed with unprecedented sophistication by the prospective monitoring of integration sites that mark each hematopoietic cell before transplantation and then allows the tracking of these cells in vivo. The initial two patients treated were 26 and 25 years old. Both subjects carried the diagnosis of X-CGD and had failed to clear invasive infections, including a *Staphylococcus aureus* liver abscess and pulmonary aspergillosis with medical treatment. Thus, autologous peripheral blood CD34$^+$ cells were mobilized with the granulocyte colony-stimulating factor (G-CSF) and collected. Gene transfer was performed using a gammaretroviral vector SF71gp91^{phox}. This vector, containing the spleen focus-forming virus LTR elements (in contrast to the Moloney MLV LTR in the two SCID trials) was chosen for its ability to achieve high expression levels in transduced HSCs. The in vitro transduction rates in the two patients were 45% and 39.5%, respectively, with a proviral copy number of 2.6 and 1.5 per transduced cell. Proviral integration occurred preferentially in gene-coding regions (47% to 52%) and was highly skewed toward the 5-kb sequence surrounding TSS. Moreover, the clonal distribution pattern was not stable over time. Rather, starting five months after therapy, a less diverse integration pattern emerged, indicating the appearance of dominant clones.

Clinically, following a period of cytopenia after the conditioning and cell infusion, the initial engraftment rates detected in the peripheral blood were 12% to 13%. Significant improvement in the previously refractory infections was noted 50 to 60 days after therapy. A gradual increase in the number of gene-corrected cells up to 50% to 60% of all peripheral blood cells was observed, starting around day 150 after transplant. This coincided with increased oxidase activity and occurred in the absence of altered blood counts. These events were accompanied by a selective outgrowth of progenitors carrying vector insertions that activated one of three oncogenes, PR domain containing 16 (*PRDM16), SETBP1*, and most notably *MDS-EVI1*. Although all three genes are well-known cancer-associated genes, most clonal outgrowths were exhausted after a few months with the exception of *MDS-EVI1*, which increased to 67% to 90% in both patients approximately 1 year after cell infusion. Of note, the dominant *MDS-EVI1* clones initially did not transgress the boundaries of the normal myeloid pool because these cells remained cytokine-dependent in vitro and failed to engraft in immunocompromised mice, suggesting their benign nature. Thus, the expansion of gp91^{phox+} cells, although clearly providing therapeutic benefit during the initial phase, was viewed with mixed feelings by the investigators and the general gene therapy community.

A follow-up on these two study patients has been provided.[13,14] Indeed, although gene-marking remained high in both patients, downregulation of gene expression was noted as a result of CpG methylation in the viral LTR promoter. As a consequence, gp91^{phox}

expression was suppressed, but the capacity of the LTR encoded enhancer to transactivate nearby genes remained intact. One patient died 2.5 years after therapy of severe sepsis. The second patient developed monosomy 7 and MDS and died after an unsuccessful unrelated donor BMT.[13] Of note, the *EVI1* locus has previously been identified as a common target of retroviral oncogenesis. EVI1, which is not detected in normal hematopoietic cells, has been associated with myeloid leukemia and MDS. The constitutive overexpression of Evi1 in mouse BM cells has been shown to induce MDS in mice and data from the CGD trial suggest that dysregulated expression is associated with genomic instability, presumably contributing to the acquisition of additional somatic mutations. Despite these molecular events, the infusion of gene-corrected CD34$^+$ cells was highly effective with regard to clearing refractory pyogenic infections, raising the possibility of using gene therapy to bridge patients with refractory pyogenic infections into eligibility for allogeneic HSCT. A multi-institutional international trial has recently opened in which the GP91^{phox} gene is expressed in a lentivirus backbone via a chimeric myeloid internal promoter (NCT01855685). The initial report of results of this trial demonstrated 6 of 9 treated patients has 16% to 46% oxidase-positive neutrophils at 12 months post-treatment. No CGD-related infections were seen in the surviving patients.[15]

Wiskott-Aldrich Syndrome

Wiskott-Aldrich syndrome (WAS) is an X-linked immunodeficiency caused by inactivating mutations in the WAS protein (WASP). WASP plays a regulatory role in cell signaling and cytoskeletal reorganization in hematopoietic cells. The disease is fatal and characterized by SCID, thrombocytopenia, elevated frequency of tumor formation, eczema, and other autoimmune manifestations. The only currently available curative therapy for WAS is a BM transplant, but as with the other primary immunodeficiencies, the availability of suitably matched donors is limiting. A clinical trial for the genetic correction of WAS via retroviral delivery of the WASP cDNA into autologous CD34$^+$ cells was recently reported.[16] A combination of a relatively high cell dose (7 to 8 × 10^6 CD34$^+$/kg body weight) and good transduction efficiency led to gene marking across both myeloid and lymphoid lineages. A marked clinical benefit from gene therapy has been reported in one of the patients.[16] Of particular note is the fact that the architecture of the vector backbone used in this trial is similar to that used in both the CGD and the SCID trials described earlier. That is, the gammaretroviral vector has an intact LTR that contains the enhancer and promoter from the spleen focus forming virus.[17] Leukemias from insertional mutagenesis in seven of the ten patients in this trial have been described.[18] Thus, a common molecular etiology in this trial strongly suggests that the initial vector design (intact strong viral LTRs containing enhancer elements that can transactivate promoters over long distances) are unsafe. In addition, the occurrence of T-cell leukemia in a disease other than SCID weakens the argument that the leukemias in X-SCID was related either to the transgene or to some unknown disease-specific characteristic. In any regard, use of the "first–generation" MLV backbone in human trials may now be ended. Several more recent clinical trials have utilized a SIN lentivirus vector for the treatment of WAS (NCT02333760, NCT01410825, NCT01347346). These trials utilize the same vector backbone. In a total of 10 patients studied, nine were reported alive and showed clinical benefit with reduced autoimmunity, including improvement in eczema and reduced platelet transfusion dependence with no serious bleeding. One subject died from a pre-existing viral infection that did not resolve after gene therapy. One study noted that the degree of myeloid engraftment and platelet reconstitution in this disease treatment correlated with the dosage of gene-corrected cells.

Childhood Cerebral X-Linked Adrenoleukodystrophy

Childhood cerebral X-linked CCALD is a fatal neurodegenerative disease that results from progressive neural demyelination within the brain. The defective gene that is responsible for the phenotype, adenosine triphosphate–binding cassette D1 (*ABCD1*), encodes a transmembrane transport protein that is responsible for the shuttling of fatty acids into peroxisomes, where they are subsequently degraded. ALD is characterized by an accumulation of very long chain fatty acids, although the exact pathophysiology of the disease is unknown. Cerebral demyelination is associated with inflammation evident on gadolinium magnetic resonance imaging studies. Most patients progress from no symptoms to a vegetative state and death within 5 years of diagnosis. Allogeneic BM transplant has been found to be therapeutic for cerebral demyelination, presumably because of the infiltration of donor-derived microglia cells into the brain. However, because of the very rapidly progressive nature of the demyelination and the time required to generate mature microglia from transplanted HSCs, BM transplant is most effective when administered as soon as possible after the development of demyelination evident by imaging methods. In cases in which CCALD is successfully treated with HSCT, continued progression of the disease occurs for 18 months to 2 years before disease arrest. Myelin inflammation resolves, but lost neurologic function is not regained in successfully treated patients. Because of the time constraints imposed upon finding a suitable HLA-matched donor and the occurrence of GVHD, the genetic correction of autologous BM CD34$^+$ cells is an attractive experimental therapeutic option.

A phase 1 gene therapy trial using a recombinant HIV-1 based lentiviral vector to deliver the ABCD1 cDNA into CD34$^+$ cells has been reported from Paris.[2] This trial is particularly noteworthy in that it is the first trial to report reconstitution of BM cells that have been transduced with a recombinant lentiviral vector. Molecular characterization of lentiviral integration sites in engrafting cells indicated that the reconstitution was polyclonal, but detailed analysis of genomic loci targeted by this vector has yet to be reported. A multisite, biotechnology-sponsored phase 2/3 registration trial accruing patients in several countries using a similar lentivirus vector in this disease has been completed and initial results reported showing that 15 of 17 boys treated were alive and free from major functional disabilities with minimal clinical symptoms.[19] This study has accrued 32 patients and recently closed for additional enrollment, but final results have not been reported. One ongoing trial is accruing additional patients utilizing the same vector but with a modified conditioning regimen (NCT03852498).

β-Thalassemia

Thalassemia results from mutations that attenuate the expression of either the α or β globin chains that compromise hemoglobin synthesis and thus causes ineffective erythropoiesis. Because adult hemoglobin consists of a tetramer of two α and two β chains, inherited mutations at the β globin locus cause a mismatch in the ratio of these two chains, thus preventing the correct assembly of the hemoglobin molecule. Clinically, this may result in transfusion-dependent anemia, which in turn can promote the serious side effect of iron overload. In general, disease severity correlates with the degree to which inactivation mutations inhibit β-globin expression. However, other genetic loci can modulate the disease phenotype, for example, by inducing adult expression of the fetal γ-globin gene, which can be efficiently incorporated into functional hemoglobin in place of the β-globin chain. For instance, several groups have recently demonstrated that the transcription factor BCL11A plays a major role in regulating the expression of fetal hemoglobin and a key to switching γ globin off and adult β-globin expression on during the transition from fetal to adult life and the BCL11A gene has become a therapeutic target for the treatment of β-thalassemia.[20] Gene therapy of β-thalassemia is complicated by the requirement for an exact stoichiometry of α- and β-globin chains to facilitate efficient assembly of hemoglobin. Thus, an effective gene therapy vector must be able to facilitate high-level expression of β-globin in the range of that mediated by the normal endogenous gene in an erythroid-specific context.

Patients treated in an initial clinical trial that used a lentiviral vector for the correction of β-thalassemia have been reported.[3] The lentiviral vector used in the trials mentioned above comprises a SIN configuration with elements from the β-globin LCR driving expression of the β-globin cDNA that has been mutated to enhance β-globin chain stability. An interim report on the findings of the clinical trial describes that, in 2007, a single patient with severe transfusion-dependent β-thalassemia major received $CD34^+$ cells transduced with the lentiviral vector described earlier.[3] There was a clear demonstration of clinical efficacy because the patient has been transfusion independent for 15 months. Of note, posttransplant molecular analysis revealed a mild clonal skewing comprising less than 5% of peripheral blood cells. The clone, which harbors a proviral integration within the high-mobility group A2 proteins (*HMGA2*) gene locus, is reported to be stable. Insertion of the provirus into this locus resulted in the production of a 3′ truncated mRNA resulting from the introduction of a cryptic splice acceptor, present in the vector insulator element, into intron 3 of the gene. This truncated mRNA, comprising exons 1 through 3, was expressed at elevated levels because of a loss of negative posttranscriptional regulation by Let-7 miRNAs because the miRNA target sequence in exon 5 was lost. *HMGA2* has been found to be mutated in chromosomal translocations primarily in benign tumors and less often in malignant tumors. Although the clinical implications of this clonal outgrowth are unclear, this event clearly demonstrates that lentiviral vectors can contribute to insertional mutagenesis albeit, in this case, via modulation of posttranscriptional regulation of gene expression. Indeed, the propensity to insert in coding sequences may lead to an abundance of abnormal mRNA splicing variants.

More recently, data from a multicenter gene therapy trial using a lentivirus vector to treat β-thalassemia has been reported.[21] In 12 of 13 non β^0/β^0 (less severe phenotype) patients treated, red cell transfusions were able to be discontinued with hemoglobin levels increasing to 8.2 to 13.7 gm/dL. In 9 β^0/β^0 (severe phenotype) patients treated the annualized red cell transfusion burden decreased 73% and in 3/9 patients, it was reported that red cell transfusions were discontinued altogether.

Sickle Cell Disease

The treatment of sickle cell disease by gene therapy approaches has utilized multiple approaches. Several trials use lentivirus vectors to express either sickling-resistant β-globin protein, γ-globin, or to increase expression of protective fetal hemoglobin by reversing the so-called fetal to adult globin switch based on recent data showing the "cure" of humanized sickle mice[22] by genetic deletion of BCL11A. The largest study has reported 19 patients in an ongoing phase 1/2 trial (NCT 02140554) treated with the $\beta^{A\text{-}T87Q}$ Lentiglobin™ vector using mobilized peripheral blood CD34 cells.[23] In this study, 13 patients stopped all red cell transfusions with a median sickle hemoglobin (HbS) reduced to <50% and correction of anemia with median Hb levels of 11.5 gm/dL. Eight patients who had a median annualized incidence of 5.3 vasoocclusive or acute syndrome episodes prior to treatment had no episodes after genetic therapy. While these results are encouraging, two patients treated in an earlier version of this trial have developed acute myeloid leukemia (AML). The etiology of these leukemias is unclear, but at least in one case it was not associated with insertional mutagenesis.[24]

An alternative approach to treating SCD is to silence the BCL11A gene. As noted above, BCL11A is a major repressor of fetal globin expression. The silencing of BCL11A has been accomplished either by expressing an shRNA against this transcription factor embedded in a microRNA or by gene-edited deletion of the *BCL11A* enhancer gene in HSCs and preliminary results of both approaches have been reported and show promising results.[25,26] In both reported trials, significant induction of fetal hemoglobin was accompanied by mitigation of anemia and vaso-occlusive events with good safety profiles, although follow-up is relatively short. These trials represent important extensions of the technology from very rare diseases to a disease with a much larger patient population.

INSERTIONAL MUTAGENESIS

From an early stage in the development of retroviral vectors for gene therapy applications, there has been a concern that recombinant vectors could elicit cellular transformation by altering the expression of either cellular proto-oncogenes or tumor suppressor genes that are proximal to the genomic integration site. This phenomenon, referred to as insertional mutagenesis, was characterized as a property of wild-type γ-retroviruses. Having greatly reduced the likelihood that retroviral gene therapy vectors could generate a replication-competent virus, the risk of a recombinant vector being able to transform a cell via insertional mutagenesis was initially perceived by many investigators to be very low. The lack of efficacy in preclinical models using human hematopoietic stem and progenitor ($CD34^+$) cells also shifted emphasis away from the risks of insertional mutagenesis. However, soon after the publication detailing the efficacy of the Paris-based SCID-X1 trial, work emerged from the group of Baum and colleagues that would reestablish the importance of insertional mutagenesis as a significant risk factor in the retroviral-mediated genetic correction of hematopoietic cells and that ultimately predicted the appearance of leukemia in the SCID (both SCID-X1 and ADA-SCID), CGD, and WAS gene therapy trials described earlier. Baum demonstrated for the first time that a replication-incompetent retroviral vector backbone designed for gene therapy applications could cause cellular transformation via insertional mutagenesis in the context of a transplant model of transduced murine HSCs. In this, and a subsequent study, it was found that a single retroviral insertion in the vicinity of the ecotropic viral integration site 1 (*Evi1*) gene or the related *PRDM16* gene resulted in their overexpression likely because of the influence of the LTR viral enhancer element and was sufficient to initiate a cascade of events resulting in a leukemic transformation in vivo. Furthermore, a high copy number infection of murine BM with recombinant retroviral vectors was able to facilitate combinatorial hits which caused leukemogenesis. The pattern of cellular genes that combine to promote cellular transformation demonstrated a significant overlap with those that are deregulated in experiments that used replication-competent retrovirus vectors to provoke the development of leukemia. Although murine HSCs likely represent a more readily transformed target than their human counterparts, these studies formally established the mutagenic potential of recombinant retroviral vectors intended for gene therapy applications.[27]

Thus far, leukemias associated with replication-incompetent retrovirus vectors are associated with insertional activation of known proto-oncogenes by a viral promoter and enhancer sequences (reviewed by Kohn et al.[28]).

Wild-type and recombinant retroviral vectors (including α, γ, spuma, and lenti) integrate into the host genome in a semi-random manner and demonstrate insertion site biases that are dependent on the accessibility of the insertion site in the target cell and variations in the viral integrase enzyme that depend on the retroviral genus. γ-retroviruses such as MLV have been shown to exert a clear preference for integration in the region immediately surrounding the TSS of actively transcribed genes. Although lentiviral vectors also demonstrate a preference to integrate within the loci of actively transcribed genes, their integration profile favors sites that are downstream of the TSS within the body of the primary transcript.

As noted earlier, other related retroviral vector systems have also been shown to have an integration pattern that is distinct from gammaretroviral vectors and, as such, may represent a safer vector configuration. Recombinant foamy virus vectors do not preferentially integrate within genes, and their integration pattern does not significantly correlate with actively transcribed genes. Likewise, avian sarcoma leukosis (alpharetrovirus) vectors do not favor gene-rich regions or TSS as preferred integration sites. However, these novel vectors systems have not been as well characterized as gammaretroviral or lentiviral vectors with regards to safety and efficacy and have not yet been translated to clinical use in the near future.

RECENT MODIFICATIONS OF VECTOR SYSTEMS BASED ON CLINICAL EXPERIENCE

New Cell Targets in Genetic Engineering

Despite recent advances in improving ex vivo manipulation of HSC for the purpose of gene transfer, an ongoing limitation of this technology is the loss of engraftment potential of manipulated cells. This is dramatically emphasized in genetic diseases such as Fanconi anemia, in which the disease process itself leads to a marked reduction in HSC targets and, in addition, HSCs, which appear to have increased susceptibility to in vitro stress. An exciting area of recent research advances that may in the future address this issue and also allow prescreening at the molecular level of insertions to determine safety is the use of embryonic stem (ES) or *induced pluripotent stem* (iPS, see next paragraph) cells. These cells offer exciting possibilities for studying mechanisms of pluripotency; establishing models for disease-specific investigations; and enabling future applications in genetic and cellular therapies, including tissue engineering for regenerative medicine. ES cells can be propagated and expanded in vitro, making them amenable to manipulations, including the genetic correction of molecular defects. ES cells can be differentiated into a number of cells resembling mature cell types in vitro. In theory, cell therapy using donor cells of the same genetic constitution as the recipient may avoid the issues related to the immune barrier of allogeneic transplantation. One of the key ethical and practical barriers to applying ES cell research to human diseases is the need for obtaining human oocytes or embryos for the in vitro generation of ES cell lines.

A key finding with potential applications to cell and gene therapy has been the observation that many somatic tissues can be "reprogrammed" into pluripotent stem cells with characteristics of ES cells, termed iPS cells, and direct reprogramming of differentiated somatic cells by gene transfer of a small number of defined transcription factors has been shown to yield cells that are indistinguishable from inner cell mass-derived ES cells. Takahashi et al.[29] reasoned that forcing the expression of ES cell-specific genes, particularly transcription factors in somatic cells might induce (reprogram) somatic cells to take on the properties of ES cells, much like factors present in oocytes can reprogram somatic nuclei in mammals. By systematic screening experiments, four factors, including some known to be involved in the process of self-renewal (Oct3/4, Sox2), and others associated with transformation and maintenance of ES cell pluripotency (c-Myc, Klf 4), were identified as sufficient to achieve reprogramming. The resultant cells, termed iPS cells, exhibited key features of ES cells, including the fulfillment of stringent pluripotency requirements. This work was subsequently confirmed and expanded upon by other groups describing the application of this technology to human cells. Proof-of-concept for the utility of iPS cells in regenerative medicine of inherited blood disorders was recently provided when iPS cells were generated from tail tip fibroblast in a humanized sickle cell anemia mouse model. The genetic sickle hemoglobin defect of the iPS cells was corrected in vitro via homologous recombination. Using established protocols for the differentiation of hematopoietic progenitors from ES cells, hematopoietic cells capable of reconstituting lethally irradiated recipient mice were generated.

Transplantation of these cells into irradiated recipients resulted in robust engraftment and amelioration of the sickle cell phenotype in transplant recipients. Reprogramming of a panel of disease-specific human iPS cells, including patients with blood diseases, has been demonstrated. Human iPS cell lines shared defining features with human ES cells, including morphology, proliferation, feeder dependence, surface markers, gene expression, promoter and telomerase activities, in vivo differentiation, and teratoma formation. In analogy to the murine system, the reprogramming viruses are strongly silenced in human iPS cells, indicating that the maintenance of pluripotency does not depend on continuous transgene expression. Enforced transgene expression appears to initiate a sequence of stochastic events over several days that eventually induces a small fraction of cells (0.001% to 0.5% of cells) to acquire a stable pluripotent state. During direct reprogramming, gradual changes lead to a stable epigenetic state that is indistinguishable from inner cell mass-derived ES cells. For example, the Dnmt3a and Dnmt3b methyltransferases become activated and silence the viral transgenes as endogenous pluripotency factors are transcriptionally reactivated. Human iPS cell lines thus represent a novel stem cell population that can be studied with regard to normal and pathologic tissue formation in vitro, enabling disease investigation, drug development, and a platform for producing autologous cell therapies that avoid immune rejection. Moreover, the creation of iPS cells allows the correction of genetic defects and extensive molecular characterization at the clonal level before tissue reconstruction. While the genetic transduction with exogenous genes, particularly oncogenes such as *c-MYC* and *KLF4*, and the use of integrating retroviral delivery systems are clear handicaps of this technology with regard to future clinical translation, newer methods have utilized non-integrating vectors and even mRNA transduction to generate iPS. However, to date, robust reconstitution of murine hematopoiesis has not been consistently demonstrated, and there are no successful reports of in vivo reconstitution of human hematopoiesis in model systems. These all represent significant barriers to the translation of this powerful technology into human therapies.

One utility of targeting iPS cells for genetic therapies is that these cells can be cloned and expanded, allowing analysis of vector insertion sites before clinical use. In addition, reprogramming has now been accomplished, albeit at lower efficiencies using both non-integrating vectors and protein transduction. Reprogramming has also now been accomplished with the expression of fewer transcription factors, most notably without c-Myc. Several laboratories have demonstrated that targeting specific loci is associated with limited or no adverse effects on the expression of neighboring genes (reviewed by Sadelain et al.[30]). For example, targeting of the *AAVS1* locus located on chromosome 19 has been well characterized. This locus encodes the *PPP1R1C* gene that is ubiquitously expressed. Insertion at this site appears to provide a "safe harbor" with respect to genotoxicity and allows stable and long-term expression of transgenes in human ES cells (hHSCs). Finally, Naldini and colleagues have demonstrated that targeted insertion into two genomic sites (*IL2RG* and *CCR5*) using zinc-finger technology leads to no detectable alteration in the expression of nearby genes and sustained expression of the transgene cargo. The frequency of directed site-specific integration in primary fibroblasts was as high as 10%.[31] In addition, elegant work by Sadelain and colleagues[32] has demonstrated the feasibility of screening large numbers of iPS-derived clones for both integration in operationally defined safe harbors and for clinically relevant transgene expression using vectors that express globin genes.[32] Thus, this approach offers exciting future applications in treating a variety of human diseases.

Site-Directed Homologous Recombination to Correct Gene Mutations

A long-standing but unrealized goal of genetic therapy has been homologous recombination to affect the *replacement* of abnormal disease-causing gene mutations. Although human ES cells theoretically provide an ideal target for such correction, gene targeting in these cells has proven difficult. Zinc-finger nuclease (ZFN)-mediated DS breaks allow high-efficiency site-specific homologous recombination and has been used to target a number of genes in human cells. ZFNs are generated by fusing the FokI nuclease domain to a DNA recognition domain composed of engineered C2H2 zinc finger motifs that specify genomic DNA binding sites for the chimeric protein. Compared with standard homologous recombination vectors, one advantage of ZFN is the relatively short stretch of homology needed (500 bp vs. 10 to 12 kb) to mediate genomic targeting. The binding of two fusion proteins to cognate DNA allows dimerization of the nuclease, leading to the generation of a DS DNA break. When donor DNA with homology to sequence flanking the DS break is present, repair occurs with the incorporation of the incoming DNA sequence. This system has been successfully used to target genes in multiple species and has recently been demonstrated in both human ES cells and iPS cells to effectively target both expressed and nonexpressed genes with a frequency of 1% to 20% and has been termed *genome editing*. Because the development of a DNA DS break is a prerequisite to ZFN-mediated gene editing and such breaks induce both p53 and HDR, this approach, although attractive, could also have significant "off-target" effects. In addition, as efficiency improves, direct targeting in HSC is possible and a trial is currently under development to utilize ZFN-mediated deletion of *BCL11A*, which represses fetal hemoglobin expression in HSC, for the treatment of β-thalassemia.[33]

Other systems that seek to target specific genetic loci have also been described and are at different levels of development. As with ZFN nucleases, these approaches use cellular DNA repair mechanisms to introduce exogenous DNA sequences into the chromosome. Thus, methods to increase the efficiency of targeting by introducing DNA DS breaks, and several methods have exploited endonucleases that target rare DNA sequences to establish these DS breaks. Alternative methods in development include meganucleases and transcription activator-like effector nucleases.[34] Even more recently, bacterial immunity-related CRISPRs have shown higher efficiencies of genome editing by expressing the Cas9 protein and guide RNAs to target mammalian genomes at specific locations. Challenges of all of these approaches include efficiency of targeting in primary cells and potential off-target effects on the genome and will likely also depend on technological advances that allow ex vivo HSC cloning and large-scale expansion. These technologies have now developed at a scale and efficiency that has led to the first in human trials in thalassemia and SCD, with particularly noteworthy recent studies using CRISPR technology published in a very limited number of patients.[26]

New genome editing tools called base editors, which induce the targeted conversion of individual nucleotides, have been generated by fusing inactive Cas9 to deaminases which catalyze the enzymatic conversion of C to T (G-to-A on the opposing strand) or A to G (T-to-C on the opposing strand). This method does not induce DNA double-strand breaks and could thus be considered safer for use in therapeutic applications. The latest addition to the base editing repertoire, "prime" editing, has the ability to catalyze arbitrary nucleotide exchanges including correction of the sickle mutation. It consists of a fusion between a catalytically impaired Cas9 and an engineered reverse transcriptase, which directly copies the corrected sequence from an extended guide RNA into the target genomic locus. The proof of principle for the efficient correction of the sickling mutation has been shown in cell lines with up to 52% of gene correction. This technology will certainly undergo refinement, further optimization and characterization for use in patient HSCs in the near future.

FUTURE DIRECTIONS

The use of gene transfers to treat human diseases has now been successful in several diseases. Compared with allogeneic BMT, ex vivo gene therapy using autologous cells obviates the need to search for an appropriate donor, eliminates the risk of GVHD and in some cases reduces the intensity of the preparative regimen required before transplant, which also reduces toxicity. Thus, in some diseases, this therapeutic approach can now be considered an alternative to standard therapy. Insertional mutagenesis, which has resulted in serious adverse events in several trials, has stimulated the rapid development of putative safer vector systems that are being tested in human trials, but remains a challenge. Rapid progress in molecular technology, such as high-throughput sequencing, high-efficiency gene editing and the development of new sources of expandable stem cell sources, offers significant potential for ongoing development of gene transfer in regenerative biology for a wide range of human conditions.

ACKNOWLEDGMENTS

Funding from NIH HL137848; OT2 HL154815; UO1 A1125051; UO1 TR001814; CIRM CLIN2 SCD-12031; Gates INV-021791. I would like to thank members of my laboratory and members of the Transatlantic Gene Therapy Consortium for productive collaborations.

SUGGESTED READINGS

Aiuti A, Cassani B, Andolfi G, et al. Multilineage hematopoietic reconstitution without clonal selection in ADA-SCID patients treated with stem cell gene therapy. *J Clin Invest*. 2007;117:2233.

Aiuti A, Slavin S, Aker M, et al. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science*. 2002;296:2410.

Anzalone AV, Randolph PB, Davis JR, et al. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature*. 2019;576(7785):149–157.

Bauer Jr. TR, Allen JM, Hai M, et al. Successful treatment of canine leukocyte adhesion deficiency by foamy virus vectors. *Nat Med*. 2008;14:93.

Cartier N, Hacein-Bey-Abina S, Bartholomae CC, et al. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science*. 2009;326:818.

Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease [see comments]. *Science*. 2000;288:669.

Cavazzana-Calvo M, Payen E, Negre O, et al. Transfusion independence and HMGA2 activation after gene therapy of human β-thalassaemia. *Nature*. 2010;467:318.

Gaspar HB, Parsley KL, Howe S, et al. Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet*. 2004;364:2181.

Hacein-Bey-Abina S, Le Deist F, Carlier F, et al. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N Engl J Med*. 2002;346:1185.

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science*. 2003;302:415.

Hanna J, Wernig M, Markoulaki S, et al. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science*. 2007;318:1920.

Hirschhorn R, Yang DR, Puck JM, et al. Spontaneous in vivo reversion to normal of an inherited mutation in a patient with adenosine deaminase deficiency. *Nat Genet*. 1996;13:290.

Howe SJ, Mansour MR, Schwarzwaelder K, et al. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest*. 2008;118:3143.

Komor AC, Kim YB, Packer MS, et al. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature*. 2016;533(7603):420–424.

Laufs S, Nagy KZ, Giordano FA, et al. Insertion of retroviral vectors in NOD/SCID repopulating human peripheral blood progenitor cells occurs preferentially in the vicinity of transcription start regions and in introns. *Mol Ther*. 2004;10:874.

Li Z, Düllmann J, Schiedlmeier B, et al. Murine leukemia induced by retroviral gene marking. *Science*. 2002;296:497.

Milsom M, Schambach A, Williams DA, et al. Chemoprotective gene delivery. *Viral Ther Cancer*. 2008;376

Miyoshi H, Smith KA, Mosier DE, et al. Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science*. 1999;283:682.

Moritz T, Patel VP, Williams DA. Bone marrow extracellular matrix molecules improve gene transfer into human hematopoietic cells via retroviral vectors. *J Clin Invest*. 1994;93:1451.

Naldini L, Blömer U, Gallay P, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector [see comments]. *Science*. 1996;272:263.

Ott MG, Stein S, Schultze-Strasser S, et al. Phase I/II gene therapy study for chronic granulomatous disease: results, lessons and perspectives. *Nat Med*. 2007;503

Pai SNL, Harris C, Cattaneo F, et al. Somatic gene therapy for x-linked severe combined immunodeficiency using a self-inactivating modified gammaretroviral vector results in an improved preclinical safety profile and early clinical efficacy in a human patient. *Blood (ASH Annual Meeting Abstracts)*. 2011;118:164.

Park IH, Zhao R, West JA, et al. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature*. 2008;451:141.

Porteus MH, Carroll D. Gene targeting using zinc finger nucleases. *Nat Biotechnol*. 2005;23:967.

Sadelain M, Papapetrou EP, Bushman FD. Safe harbours for the integration of new DNA in the human genome. *Nat Rev Cancer*. 2012;12:51.

Sankaran VG, Menne TF, Xu J, et al. Human fetal hemoglobin expression is regulated by the developmental stage-specific repressor BCL11A. *Science*. 2008;322:1839.

Stein S, Ott MG, Schultze-Strasser S, et al. Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. *Nat Med*. 2010;16:198.
Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007;131:861.
Thrasher AJ, Gaspar HB, Baum C, et al. Gene therapy: X-SCID transgene leukaemogenicity. *Nature*. 2006;443:E6–E7. E5, discussion.
Urnov FD, Miller JC, Lee Y-L, et al. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature*. 2005;435:646.
Williams DA, Thrasher AJ. Concise review: lessons learned from clinical trials of gene therapy in monogenic immunodeficiency diseases. *Stem Cells Transl Med*. 2014;3(5):636–642.
Xu J, Peng C, Sankaran VG, et al. Correction of sickle cell disease in adult mice by interference with fetal hemoglobin silencing. *Science*. 2011;334:993.
Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science*. 2007;318:1917.

REFERENCES

1. Touw IP, Erkeland SJ. Retroviral insertion mutagenesis in mice as a comparative oncogenomics tool to identify disease genes in human leukemia. *Mol Ther*. 2007;15(1):13–19.
2. Cartier N, Hacein-Bey-Abina S, Bartholomae CC, et al. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science*. 2009;326(5954):818–823.
3. Cavazzana-Calvo M, Payen E, Negre O, et al. Transfusion independence and HMGA2 activation after gene therapy of human β-thalassaemia. *Nature*. 2010;467(7313):318–322.
4. Hacein-Bey-Abina S, Pai S-Y, Gaspar HB, et al. A modified γ-retrovirus vector for X-linked severe combined immunodeficiency. *N Engl J Med*. 2014;371(15):1407–1417.
5. Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease [see comments]. *Science*. 2000;288(5466):669–672.
6. Gaspar HB, Parsley KL, Howe S, et al. Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet*. 2004;364(9452):2181–2187.
7. Mamcarz E, Zhou S, Lockey T, et al. Lentiviral gene therapy combined with low-dose busulfan in infants with SCID-X1. *N Engl J Med*. 2019;380(16):1525–1534.
8. Sauer AV, Di Lorenzo B, Carriglio N, Aiuti A. Progress in gene therapy for primary immunodeficiencies using lentiviral vectors. *Curr Opin Allergy Clin Immunol*. 2014;14(6):527–534.
9. Ferrua F, Aiuti A. Twenty-five years of gene therapy for ADA-SCID: from bubble babies to an approved drug. *Hum Gene Ther*. 2017;28(11):972–981.
10. Thrasher AJ, Gaspar B. Successful bone marrow gene therapy for SCID. *Blood Cells Mol Dis*. 2008;40(2):287–288.
11. Aiuti A, Cattaneo F, Galimberti S, et al. Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *N Engl J Med*. 2009;360(5):447–458.
12. Kohn DB, Booth C, Shaw KL, et al. Autologous ex vivo lentiviral gene therapy for adenosine deaminase deficiency. *N Engl J Med*. 2021;384(21):2002–2013.
13. Stein S, Ott MG, Schultze-Strasser S, et al. Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. *Nat Med*. 2010;16(2):198–204.
14. Kang EM, Choi U, Theobald N, et al. Retrovirus gene therapy for X-linked chronic granulomatous disease can achieve stable long-term correction of oxidase activity in peripheral blood neutrophils. *Blood*. 2010;115(4):783–791.
15. Kohn DB, Booth C, Kang EM, et al. Lentiviral gene therapy for X-linked chronic granulomatous disease. *Nat Med*. 2020;26(2):200–206.
16. Boztug K, Schmidt M, Schwarzer A, et al. Stem-cell gene therapy for the Wiskott-Aldrich syndrome. *N Engl J Med*. 2010;363(20):1918–1927.
17. Avedillo Díez I, Zychlinski D, Coci EG, et al. Development of novel efficient SIN vectors with improved safety features for Wiskott-Aldrich syndrome stem cell based gene therapy. *Mol Pharm*. 2011;8(5):1525–1537.
18. Braun CJ, Boztug K, Paruzynski A, et al. Gene therapy for Wiskott-Aldrich syndrome—long-term efficacy and genotoxicity. *Sci Transl Med*. 2014;6(227). 227ra33.
19. Eichler F, Duncan C, Musolino PL, Orchard PJ, De Oliveira S, Thrasher AJ, et al. Hematopoietic stem-cell gene therapy for cerebral adrenoleukodystrophy. *N Engl J Med*. 2017;377(17):1630–1638.
20. Sankaran VG, Menne TF, Xu J, et al. Human fetal hemoglobin expression is regulated by the developmental stage-specific repressor BCL11A. *Science*. 2008;322(5909):1839–1842.
21. Thompson AA, Walters MC, Kwiatkowski J, et al. Gene therapy in patients with transfusion-dependent β-thalassemia. *N Engl J Med*. 2018;378(16):1479–1493.
22. Xu J, Peng C, Sankaran VG, et al. Correction of sickle cell disease in adult mice by interference with fetal hemoglobin silencing. *Science*. 2011;334(6058):993–996.
23. Kanter J, Walters MC, Hsieh M, et al. Interim results from a phase 1/2 clinical study of lentiglobin gene therapy for severe sickle cell disease. *Blood*. 2017;130(suppl 1):527.
24. Hsieh MM, Bonner M, Pierciey FJ, et al. Myelodysplastic syndrome unrelated to lentiviral vector in a patient treated with gene therapy for sickle cell disease. *Blood Adv*. 2020;4(9):2058–2063.
25. Esrick EB, Lehmann LE, Biffi A, et al. Post-transcriptional genetic silencing of BCL11A to treat sickle cell disease. *N Engl J Med*. 2021;384(3):205–215.
26. Frangoul H, Altshuler D, Cappellini MD, et al. CRISPR-Cas9 gene editing for sickle cell disease and β-thalassemia. *N Engl J Med*. 2021;384(3):252–260.
27. Li Z, Düllmann J, Schiedlmeier B, … Baum C. Murine leukemia induced by retroviral gene marking. *Science*. 2002;296:497.
28. Kohn DB, Sadelain M, Glorioso JC. Occurrence of leukaemia following gene therapy of X-linked SCID. *Nat Rev Cancer*. 2003;3(7):477–488.
29. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007;131:861..
30. Sadelain M, Papapetrou EP, Bushman FD. Safe harbours for the integration of new DNA in the human genome. *Nat Rev Cancer*. 2012;12(1):51–58.
31. Lombardo A, Genovese P, Beausejour CM, et al. Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nat Biotechnol*. 2007;25(11):1298–1306.
32. Papapetrou EP, Lee G, Malani N, … Sadelain M. Genomic safe harbors permit high β-globin transgene expression in thalassemia induced pluripotent stem cells. *Nat Biotechnol*. 2011;29(1):73–78.
33. Breda L, Rupon JW, Motta I, et al. Comparing strategies to reactivate fetal globin expression for the treatment of beta-globinopathies. *Blood (ASH Annual Meeting Abstracts)*. 2014;124(21):333.
34. Boch J. TALEs of genome targeting. *Nat Biotechnol*. 2011;29(2):135–136.

CHAPTER 105

INDICATIONS, OUTCOMES, AND DONOR SELECTION FOR ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION FOR HEMATOLOGIC MALIGNANCIES IN ADULTS

Saurabh Chhabra, Mehdi Hamadani, and Parameswaran N. Hari

BACKGROUND

Early reports of leukemia-free survival, following human leukocyte antigen (HLA)-identical sibling hematopoietic cell transplantation (HCT) for refractory acute leukemia, was reported in the 1970s. Since then, allogeneic HCT has evolved into an effective and increasingly safe, curative, and intent therapeutic modality for hematologic malignancies.[1] The indications and applicability of HCT for specific diseases change over time, especially as non-transplant treatment options and our understanding of disease biology improve. For example, in chronic myelogenous leukemia (CML), molecularly targeted therapy has almost completely replaced HCT since the early 2000s. At the same time, developments in HCT, such as the advent of reduced intensity conditioning (RIC), the use of mismatched, haploidentical-related donor and cord blood grafts, and improvements in supportive care, have increased the scope and applicability of allogeneic HCT. Allogeneic HCT is increasingly used in older patients, ethnic minorities, those for whom fully HLA-matched donors are not available, and those in whom fully HLA-matched donors are used as a salvage strategy after relapse. Transplant-related mortality (also referred to as non-relapse mortality [NRM]), despite declining over the years, remains an important challenge; posttransplant relapse has emerged as a major therapeutic focus. General principles, indications, donor selection, and optimal timing of allogeneic HCT for hematologic malignancies in adults and post-HCT outcomes are discussed in this chapter.

Allogeneic HCT involves administration of a conditioning (or preparative) regimen of chemotherapy (with or without radiation) and immunosuppressive medications, followed by infusion of donor hematopoietic progenitor cells. Most patients then receive several months of immune suppression therapy to prevent or treat graft-versus-host disease (GVHD). The purpose of the conditioning regimen is twofold: to eradicate malignant cells and to eliminate host immune cells (capable of rejecting even HLA-identical sibling donor cells). The ability to restore hematopoiesis with donor hematopoietic progenitor cells permits the administration of substantially higher (myeloablative) doses of cytotoxic therapy, than is otherwise possible. Additionally graft-versus-malignancy (GVM) effects, conferred by alloreactive donor immune cells, contribute substantially to cancer eradication and relapse prevention.[2]

PATIENT POPULATION

The Center for International Blood and Marrow Transplant Research (CIBMTR) collects data on HCT outcomes from over 500 worldwide participating centers and on all allogeneic transplants in the United States.[3] Approximately 83,700 allogeneic HCT were performed in the United States between 2010 and 2018.[4] In 2018, approximately 2371 matched sibling donors, 4309 unrelated adult donors, 1503 haploidentical donors (defined as a related donor with two or more antigen mismatches), and 466 cord blood transplants were performed in the United States,[4] including approximately 7500 in adult patients. Hematologic malignancies are the most common indications for allogeneic HCT. Acute myeloid leukemia (AML), myelodysplastic syndrome/myeloproliferative neoplasms (MDS/MPN), and acute lymphoblastic leukemia (ALL) currently constitute the three most common indications of allogeneic HCT (Fig. 105.1).

In recent years, improved supportive care and the use of RIC have led to an increase in allogeneic HCT for older adults. Only 4% of allogeneic HCT-recipients, from 1987 to 1992, were older than 50 years. In 2018, 45% were older than 55 years and 20% were 65 years or older. The use of allogeneic HCT in patients 70 years and older in the United States increased substantially in the past decade, from 5 (0.1%) in 2000, to 283 (3.85%) in 2013.[5] Allogeneic transplantation, in patients without HLA-identical siblings, was facilitated by establishment of large unrelated donor registries around the globe. From 1987 to 1992, less than 10% of HCTs, for hematologic malignancies, used unrelated donors; in 2018, this figure was approximately 80%. Increased utilization of HCT among older adults, increasing use of unrelated donors, and more recently, haploidentical-related donors have been the main reasons for the steady growth in allogeneic HCT over the last decade. In 2011, approximately 300 haploidentical-related donor transplants were reported to the CIBMTR, compared with nearly 1500 transplants in 2018. The number of cord blood transplants declined concomitantly from a high of 816 in 2011, to approximately 500 in 2018. Over the years in the United States, the increase in the number of allogeneic HCT has been most striking in African Americans, while the number of allogeneic HCT for all indications increased by 7% from 2010 to 2018 for White people, the growth was 63% for African Americans, and 21% for Hispanics.[4] A threefold increase occurred between 2010 and 2018 in haploidentical HCT; in 2018, approximately 20% of haploidentical HCT were performed in African Americans.

CONDITIONING REGIMENS

Historically, conditioning regimens included myeloablative doses of cytotoxic drugs—with or without radiation—intended to provide disease control and induce host immunosuppression (to prevent graft rejection). Myeloablative conditioning (MAC) regimens for hematologic malignancies often involve a combination of cyclophosphamide (commonly 60 mg/kg/day for 2 days) and either busulfan (typically 3.2 mg/kg/day intravenously for 4 days or a similar pharmacokinetic guided dose) or, historically, conditioning regimens included total body irradiation (TBI) (5 to 15 Gy, single or fractionated doses). Posttransplant survival rates are broadly similar with either of the above approaches, although an advantage for pharmacokinetic-guided busulfan regimens has been shown for myeloid malignancies.[6] In a variety of regimens, other drugs such as fludarabine, etoposide, melphalan, and thiotepa are sometimes added to or substituted for cyclophosphamide and/or busulfan in efforts to provide better, generally disease-specific, antineoplastic activity. An Italian phase III randomized study in AML patients compared the two common MAC regimens (intravenous busulfan plus cyclophosphamide [BU CY],

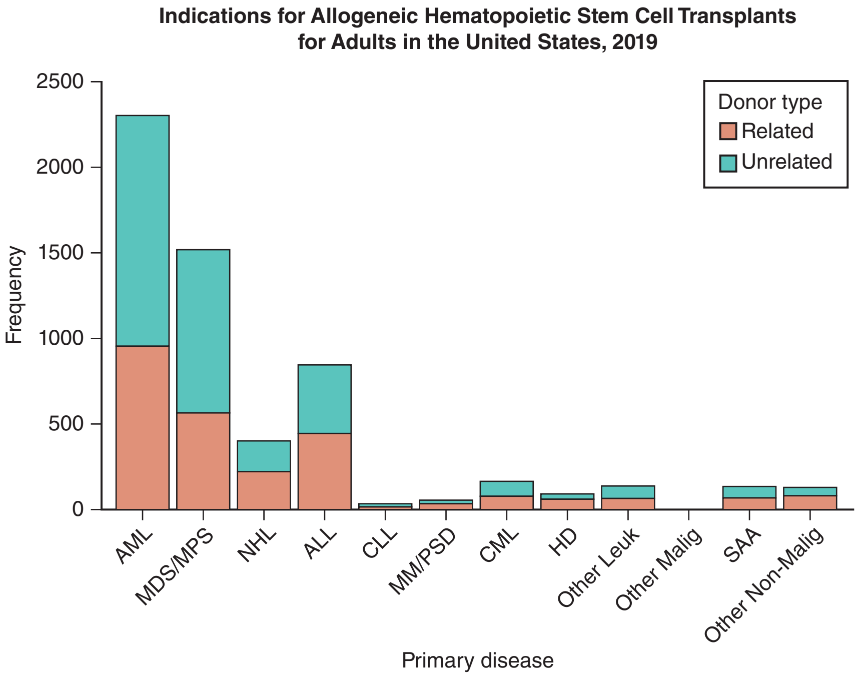


Figure 105.1 INDICATIONS FOR ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION IN ADULTS (US DATA), 2019. *ALL, Acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; HD, Hodgkin disease; Leuk, leukemia; Malig, malignancy; MDS, myelodysplastic syndrome; MM, multiple myeloma; MPS, myeloproliferative syndrome; NHL, non-Hodgkin lymphoma; PCD, plasma cell dyscrasia; SAA, severe aplastic anemia.*

with busulfan plus fludarabine [Flu BU]) and demonstrated lower NRM (with busulfan plus fludarabine), without affecting relapse risk.[7] In a study randomizing AML patients to high-dose cyclophosphamide and either 12 Gy or 15.75 Gy TBI as conditioning, a lower relapse risk in the 15.75 Gy TBI group was offset by higher NRM risk leading to similar OS.[8] A more recent CIBMTR study also reported that higher doses of TBI over the standard dose (12 Gy) were associated with worse NRM and offered no survival benefit.[9]

Toxicity associated with MAC regimens can be minimized and efficacy can be improved with careful pharmacokinetic monitoring of certain drugs, for example, busulfan. Another strategy for lowering NRM is reducing the dose-intensity of the conditioning regimen. This approach uses lower doses of cytotoxic drugs and/or radiation to facilitate donor cell engraftment and relies more on GVM effects to eradicate malignant cells. The lower doses of cytotoxic agents produce less host tissue damage and inflammatory cytokine secretion, resulting in lower rates of regimen-related morbidity and mortality. In patients ineligible for traditional MAC regimens, the use of RIC regimens has greatly increased the applicability of allogeneic HCT because of age or comorbidities. The development of RIC regimens has led to a substantial increase in the utilization of HCT in older patients (Fig. 105.2).

Consensus criteria developed by the CIBMTR and the European Society for Blood and Marrow Transplantation (EBMT) distinguish between myeloablative, reduced intensity and nonmyeloablative regimens based on the likelihood of the regimen to produce toxicity in the recipient marrow (Table 105.1).[10] MAC regimens produce profound pancytopenia and are usually fatal in the absence of stem cell rescue. Nonmyeloablative regimens cause brief and often less severe pancytopenia, and in the absence of donor stem cell rescue, autologous hematopoietic recovery is likely to occur. RIC regimens are an intermediate category that does not fit well in either of the above, since they produce pancytopenia that may recover without stem cell rescue. RIC regimens induce less immune compromise in the immediate post-HCT setting as the duration and depth of neutropenia are reduced and host-derived immunocompetent cells are not immediately eliminated.[3,11]

It is generally accepted that RIC regimens allow HCT in some patients who are ineligible or not offered HCT with MAC. For example, in the United States, the proportion of transplants performed in patients 60 years and older increased from less than 5% before 2000, to ~34% in 2018. Two-thirds of the latter patients received RIC regimens. However, RIC regimens are increasingly used in patients of all ages who receive both related and unrelated donor transplants, as is shown in Fig. 105.2. Despite the growing popularity of RIC regimens, large retrospective database studies from the EBMT and CIBMTR (summarized in Table 105.2) have generally failed to show a definite survival advantage resulting from the use of such conditioning regimens. Although RIC regimens allow a potent graft-versus-leukemia response to occur with lower toxicity, there are concerns about a higher risk of disease relapse following RIC regimens. Since the distribution of conditioning regimens in registry studies reflects physician choice or patient selection bias, retrospective data are of limited value and randomized trials have addressed this question. The prospective US Bone Marrow Transplant Clinical Trial Network (BMT CTN) 0901 trial randomized patients with myeloid malignancies eligible for myeloablative conditioning to RIC versus MAC regimens. In this trial, a superior relapse-free survival was evident for MAC after enrolling 272 out of the planned 356 patients, leading to early closure of the study. Notably, an overall survival (OS) advantage did not emerge.[12] A prospective randomized European trial (RICMAC) showed similar 2-year, relapse-free, and overall survival for busulfan-based RIC versus MAC in patients with MDS and secondary AML.[13]

Donor Factors

NRM rates are generally lower after HLA-identical sibling than after unrelated donor or alternative donor transplants because of less graft failure, faster immune reconstitution, and less GVHD. However, considerable progress has been made in reducing NRM rates after both HLA-identical sibling and alternative donor HCT. For matched sibling donor HCT (for AML in CR1), the incidence of NRM at 1 year decreased from 23% from 1985 to 1989, to about 11% from

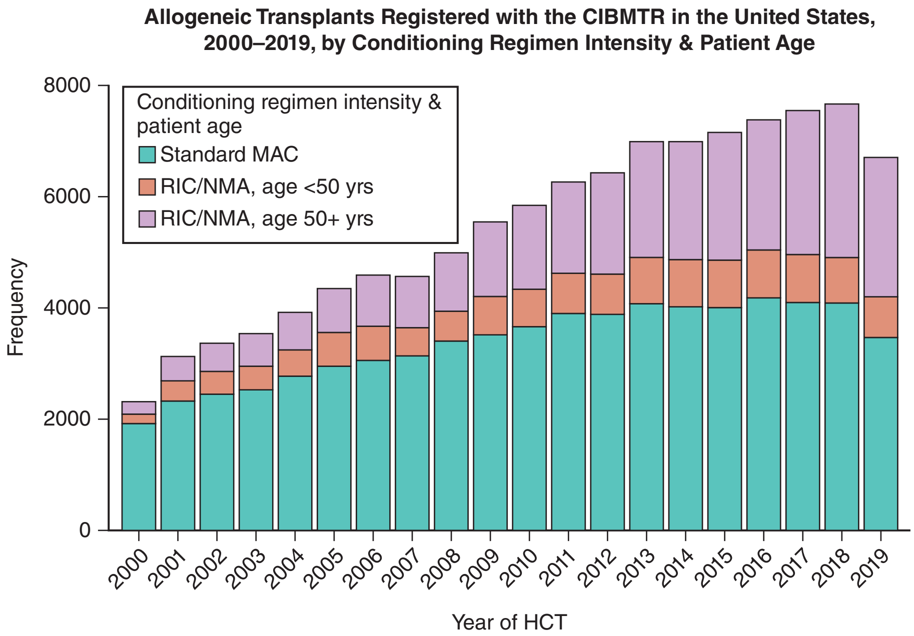


Figure 105.2 CONDITIONING REGIMEN INTENSITY AND PATIENT AGE: CHANGING TREND OVER TIME (2000–2019). *CIBMTR, Center for International Blood and Marrow Transplant Research; HCT, hematopoietic cell transplantation; MAC, myeloablative conditioning; NMA, nonmyeloablative; RIC, reduced intensity conditioning.*

TABLE 105.1 **Consensus Definition of Conditioning Regimen Intensity**

Myeloablative Conditioning
Profound Cytopenia, Not Likely to Recover Without Hematopoietic Cell Rescue
Total body irradiation >5 Gy single dose or >8 Gy fractionated
Busulfan >8 mg/kg orally or intravenous equivalent
Nonmyeloablative Conditioning
Minimal Cytopenia, Autologous Recovery of Hematopoiesis Likely Even Without Transplant
Total body irradiation <2 Gy + purine nucleoside analogue
Fludarabine + cyclophosphamide + antithymocyte globulin
Fludarabine + cytarabine + idarubicin
Cladribine + cytarabine
Total lymphoid irradiation + antithymocyte globulin
Reduced Intensity Conditioning
Regimens that are intermediate between the above categories

2000 to 2004, and down to 6.3% in 2018. The 1-year incidence of NRM for unrelated donor HCT (for AML in CR1) from 1990 to 1994 was 34%, which decreased to 26% from 2000 to 2004, and down to 9.5% in 2018.[14] Among alternative donor transplants, those from more closely HLA-matched donors tend to have lower risks of GVHD and NRM. Donor-recipient HLA mismatching is associated with increased risk of posttransplant complications, including graft rejection, acute and chronic GVHD, and mortality; risks increase progressively with multiple HLA mismatches. With modern molecular HLA typing techniques (allowing selection of more closely HLA-matched donors) and current GVHD prevention strategies, the difference in outcomes between HLA-matched sibling and unrelated donor transplantation has narrowed (Fig. 105.3). Adoption of molecularly defined, HLA matching techniques, calcineurin inhibitor–based GVHD prophylaxis, fungal prophylaxis with azoles, leukocyte reduction of blood products, newer assays for viral reactivation, pharmacokinetic testing of conditioning agents, and posttransplant cyclophosphamide for induction of allogeneic tolerance are some of the major innovations that have impacted NRM.

GRAFT SOURCES

The sources of hematopoietic cells for transplantation, historically bone marrow from donors, now also include peripheral blood hematopoietic cells, collected by leukapheresis and umbilical cord blood. The composition of grafts from bone marrow, peripheral blood, and cord blood varies in terms of the proportion of pluripotent stem cells, the proportion of lineage-committed late progenitor cells, and in the proportion and characteristics of immune-reactive cells. Bone marrow is the primary source of allogeneic donor cells for transplantation in children, and peripheral blood is the primary source in adults (Fig. 105.4).

Several randomized trials have compared matched-sibling bone marrow versus peripheral blood grafts in patients with hematologic malignancy, following MAC regimens. In general, these studies showed no difference in the rates of acute GVHD between the two graft sources and showed no difference between higher risk of chronic GVHD with peripheral blood grafts. While survival outcomes were similar in most studies, the US and Canadian randomized studies reported a disease-free and overall survival advantage, respectively, favoring peripheral blood grafts.[15,16] A meta-analysis, using individual patient-level data from nine randomized trials of related donor peripheral blood versus bone marrow transplants suggested that patients receiving peripheral blood grafts had faster hematopoietic recovery,[15] a lower relapse rate if transplanted for hematologic malignancy, and higher overall and disease-free survival if transplanted for advanced-stage disease. However, peripheral blood was also associated with a significantly increased risk of extensive chronic GVHD. Among unrelated donor transplant recipients, the prospective randomized BMT-CTN 0201 trial showed a higher risk of chronic GVHD in patients receiving peripheral blood grafts (53% versus 41% for marrow grafts).[17] Notably, survival and the incidence of relapse and acute

TABLE 105.2 Retrospective Registry-Based Comparisons of Nonmyeloablative/Reduced Intensity Conditioning Versus Myeloablative Allogeneic Hematopoietic Cell Transplantation

Group	Disease	Donor	RIC vs. MAC (*N*)	NRM RIC vs. MAC	Relapse	Comments
EBMT[161]	MDS or sAML, >50 years	UD 39%	833 vs. 500	32% vs. 44% at 4 years	41% vs. 33%	Survival 31% at 4 years. RIC predicted for greater relapse but lower NRM.
EBMT[162]	AML, >50 years	Sibling	315 vs. 407	18% vs. 32% at 2 years	41% vs. 34%	No difference in leukemia-free and overall survival between RIC and. MAC.
EBMT[163]	MM	MUD 12%	320 vs. 196	24% vs. 37% at 2 years	27% vs. 54%	NRM was lower after RIC but relapse risk was double.
EBMT[164]	AML	Sibling	215 vs. 621	22% vs. 32% at 3 years	45% vs. 27%	Relapse rate higher and NRM lower in RIC but OS similar (41% vs. 45%) in both groups.
EBMT[165]	CLL	MUD 22%	73 vs. 82	19% vs. 26%	28% vs. 11%	Similar NRM but higher relapse risk observed after RIC.
EBMT[166]	HL	MUD 13%	89 vs. 79	23% vs. 46% at 1 year	57% vs. 30%	Relapse rate was higher and NRM lower in RIC but OS was similar.
CIBMTR[167]	Follicular NHL	Sibling	88 vs. 120	23% in both at 1 year	17% vs. 8%	RIC associated with higher risk of relapse but similar NRM while lower KPS impacted on NRM. OS was similar.
EBMT[168]	ALL	Sibling	127 vs. 449	21% vs. 29%	32% vs. 38%	NRM lower after RIC but higher relapse rate. Leukemia-free survival similar to MAC.
EBMT[169]	AML	MUD	401 vs. 1154	<50 years: 25% vs. 27%;≥50 years: 25% vs. 39% at 2 years	<50 years: 38% vs. 30%;≥50 years: 42% vs. 29%	RIC (vs. MAC) was associated with higher relapse <50 years and decreased NRM ≥50 years. Leukemia-free survival was similar after both RIC and MAC, regardless of age.
CIBMTR[170]	AML/MDS	MUD/ Sibling	1448 vs. 3731	3-year NRM similar	Lower risk of relapse in myeloablative	Overall and disease-free survival was highest for myeloablative group.
CIBMTR[119]	CML, 18–60 years	MUD/ Sibling	191 vs. 1204	29% vs. 32% at 5 years	25% vs. 26%	Similar leukemia-free and overall survival and lower chronic GVHD risk with RIC vs. MAC in TKI era.

ALL, Acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *CIBMTR*, Center for International Blood and Marrow Transplant Research; *CLL*, chronic lymphocytic leukemia; *EBMT*, European Society for Blood and Marrow Transplantation; *GVHD*, graft-versus-host disease; *HCT*, hematopoietic cell transplantation; *HL*, Hodgkin lymphoma; *KPS*, Karnofsky performance score; *MAC*, myeloablative conditioning; *MDS*, myelodysplastic syndrome; *MM*, multiple myeloma; *MUD*, matched unrelated donor; *NHL*, non-Hodgkin lymphoma; *NMA*, nonmyeloablative; *NRM*, non-relapse mortality; *OS*, overall survival; *RIC*, reduced intensity conditioning; *sAML*, secondary acute myeloid leukemia; *TKI*, tyrosine kinase inhibitors.

GVHD were similar, while graft failure was more common among recipients of marrow grafts.[17] The relative importance of graft sources in patients receiving lower-intensity conditioning is not known, but a CIBMTR study comparing unrelated bone marrow versus peripheral blood grafts, following RIC, showed no difference between the two sources in terms of rates of acute or chronic GVHD, relapse risk, and survival outcomes.[18]

Donor Selection

While donor-derived alloimmunity in the form of a graft versus tumor effect leads to tumor control, the same phenomenon directed against normal host tissues leads to GVHD, which is a major determinant of NRM after allogeneic transplantation. The major criterion for selecting an allogeneic donor is the degree of histocompatibility between the donor and recipient because the risks of both graft rejection and GVHD increase with the degree of genetic disparity.[19–21] Alloreactivity is reduced by matching recipients and donors at loci in the major histocompatibility complex (MHC), including HLA that is encoded by class I (*HLA-A*, *HLA-B*, and *HLA-C*) and class II (*HLA-DR*, *HLA-DQ*, and *HLA-DP*) genes. HLA molecules were originally defined by serology, but DNA-based, high-resolution testing is now routine.[22] Even in the setting of MHC-matched, donor-recipient pairs, minor histocompatibility antigen (MiHA) (cell surface peptides found in association with class I or II MHC molecules) differences that are detectable by T cells can stimulate an MHC-restricted, alloreactive response, resulting in GVHD. The optimal donor is a sibling sharing HLA class I and HLA class II alleles, but matched sibling donors are available for only about 30% of patients.

For unrelated donor HCT, the primary driver for successful outcome is the degree of donor-recipient HLA matching. In 1986, the National Marrow Donor Program (NMDP; "Be the Match Registry") was established as a repository for HLA typing information so that unrelated donors and recipients could be adequately matched.[23,24] The NMDP HapLogic donor search considers over 20 million donors (87% typed for HLA-A, HLA-B, and HLA-DR) and additionally provides a match report of an additional 13 million donors listed in World Marrow Donor Association Search and Match.[25] Such a large number of donors is necessary because great diversity exists in the HLA system, with over 5500 class I alleles (*HLA-A*, *-B*, and *-C*) and over 1600 class II alleles (*HLA-DRB1* and *-DQB1*), resulting in several million potential HLA combinations.[26] The likelihood of finding an optimal adult unrelated donor (i.e., a donor matched at high resolution at *HLA-A, HLA-B, HLA-C*, and *-DRB1* alleles or a "8/8 match") varies among racial and ethnic

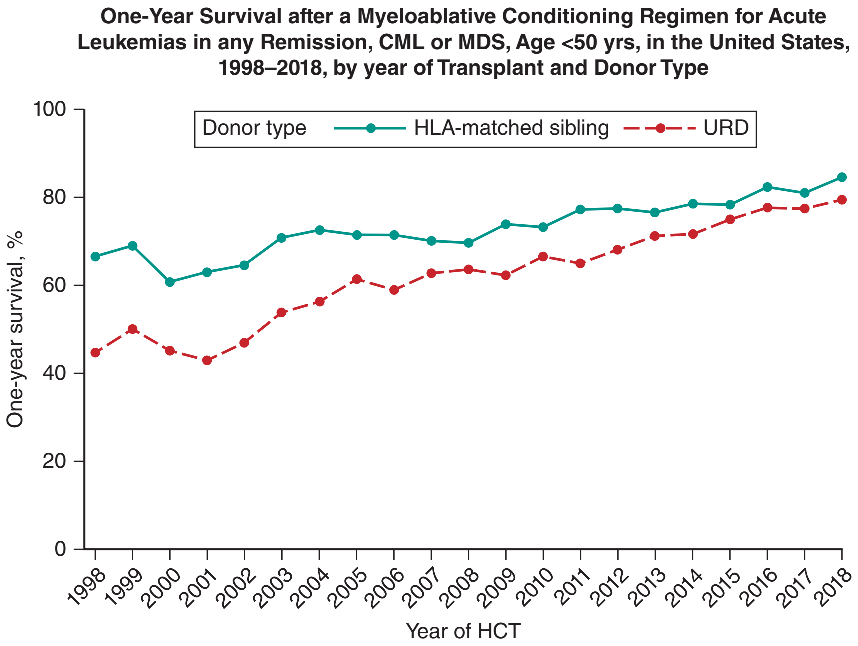


Figure 105.3 ONE-YEAR SURVIVAL AFTER MYELOABLATIVE HCT IN YOUNGER PATIENTS (RELATED VERSUS UNRELATED DONOR). *CML, Chronic myeloid leukemia; HCT, hematopoietic cell transplantation; HLA, human leukocyte antigen; MDS, myelodysplastic syndrome; URD, unrelated donors.*

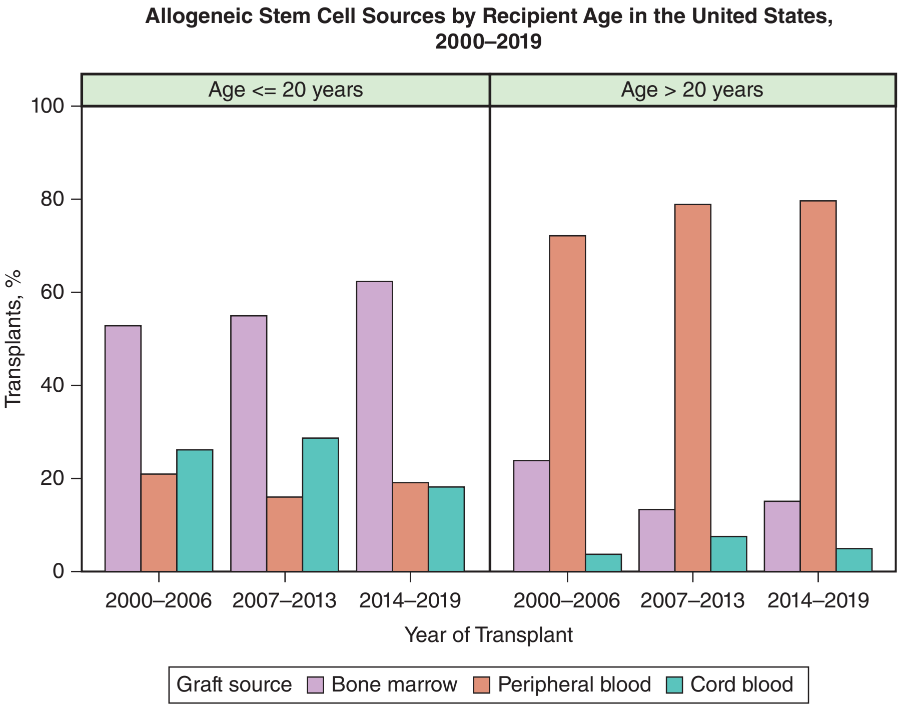


Figure 105.4 ALLOGENEIC STEM CELL SOURCES, BY DONOR TYPE VERSUS DONOR AGE, 2000–2019.

groups, depending on the size and composition of unrelated donor registries. Within the US population, the highest likelihood for finding an optimal donor is 75% for White people of European descent, and the lowest is 16% among Black people of South or Central American descent.[27] The development of large donor registries has expanded access to transplantation, as well as increasing the likelihood that a recipient will find a well-matched donor.[28] However, this also means that patients who do not find a suitably matched donor in the large registry pool have rare HLA genotypes for whom waiting for a fully matched donor from among new additions recruited to the donor pool is not recommended. Such patients should proceed to alternative donor transplantation with partially matched sources; haploidentical or cord blood donor grafts.

The adoption of high-resolution HLA typing for matching and selection of unrelated donors, have been associated with improved outcomes.[20,29–32] A study examining unrelated donor characteristics predictive of outcomes reported that after adjusting for the disease and comorbidities, when the donor was a 10/10 match (matched for *HLA-A, -B, -C, -DRB1*, and *-DQB1*). Age is the most important factor, after donor-recipient HLA match: survival was significantly improved if the donor was young (aged 18 to 32 years).[33] A 5.5% increase occurred in the hazard for overall mortality for every 10-year increment in donor age. If multiple 8/8 matched unrelated donors are available, other factors could be considered, such as donor age, matching at DPB1 or permissive DPB1 mismatching, avoidance of donor allotypes targeted by donor-specific antibodies in the recipient, and blood group or CMV-status matching. With current high-resolution HLA typing and donor selection, unrelated donor transplants have survival comparable to that of matched sibling donor setting.[34–37]

For patients lacking a matched sibling or 8/8 matched unrelated donors, the options are a mismatched and unrelated donor, a haploidentical-related donor, or a cord-blood unit (collectively denoted as "alternative donors"). Each choice has benefits and disadvantages (Table 105.3). As a source of donor hematopoietic cells, umbilical cord blood offers several advantages. It is readily available as cryopreserved units, entails no risk to the donor and the HLA-matching criteria for cord blood transplantation need not be as stringent as for adult donor transplantation. Hence, mismatched cord blood units, at one or two HLA loci, are available for almost all patients less than 20 years of age and for more than 80% of patients ≥20 years of age, regardless of racial and ethnic background.[9,27] Drawbacks of cord blood transplantation include procurement costs, such as the fixed and often limited dose of hematopoietic cells (based on recipient body weight) in each cord blood unit and relatively high rates of NRM. However, double cord blood transplantation, with each unit sharing at least 4/6 HLA antigens with the recipient, has been shown to overcome the problem of dose and extend the use of cord blood transplant to adult patients.[38] Other approaches to facilitate engraftment with limited cord blood cell doses, including ex vivo cell expansion techniques, are in clinical trials.[39]

HLA haploidentical-related donor HCT is another option for patients without matched siblings or unrelated donors. Haploidentical donors are usually parents, siblings, or children of patients who are mismatched at ≥2 loci *HLA-A, HLA-B, HLA-C*, and *HLA-DRB1*. Historically, T-cell-replete haploidentical HCT was prohibited by severe GVHD and NRM, approaching 50%.[40] Haploidentical HCT have been made significantly safer by the administration of high-dose cyclophosphamide after transplantation (posttransplant CY), which promotes tolerance by elimination of alloreactive host and donor T cells, leading to suppression of graft rejection and GVHD, without toxicity to donor hematopoietic cells.[41,42] Immediate and near-universal donor availability and theoretically lower costs are advantages of haploidentical grafts. Several retrospective registry analyses suggest haploidentical HCT, performed using T-cell-replete grafts and posttransplant cyclophosphamide, achieves outcomes comparable with those of transplantation performed using matched related or unrelated donors.[44–50] An increased risk of NRM has been suggested in patients receiving haploidentical HCT, using posttransplant CY compared with matched sibling donor HCT and calcineurin-inhibitor-based[51] or posttransplant cyclophosphamide-based GVHD prophylaxis.[52] These data caution against using haploidentical-related donors in lieu of adult, matched-related donors outside of a clinical trial.

TABLE 105.3 Donor Choice

	Mismatched Unrelated Donor	Haploidentical Donor	Cord
Availability	Over 80%	Over 95%	Over 80%
Time to procure	1–4 months	Immediate	2–5 days
Engraftment	Over 95%	Over 95%	Over 90% with slower engraftment
GVHD	Moderate	More genetic disparity but moderate with most modern regimens	Moderate—may be less acute but chronic similar
Availability donor for posttransplant immune therapy	Usually, but takes 2–4 weeks	Yes	No
Relative cost	Moderate	Low if unmanipulated product. Moderate if ex vivo selection	Moderate (1 unit) or high (2 units)
Risk of relapse	Moderate	Higher with regimens that are effective in preventing GVHD	Moderate

GVHD, Graft-versus-host disease.

In patients lacking adult, 8/8-matched (sibling or unrelated) donors, the choice between cord blood versus haploidentical HCT has been a matter of much debate. The phase III randomized BMT-CTN 1101 study compared double umbilical cord blood versus haploidentical marrow grafts and reported that NRM and overall survival at 2 years were superior in patients who received haploidentical marrow transplantation, while there was no significant difference in the primary endpoint of progression-free survival between the two types of transplant.[53] Other techniques for facilitating haploidentical transplants have been developed around the world that involve combinations of selective T-cell depletion ex vivo, mega dose infusion of CD34 progenitors, or induction of immune tolerance with G-CSF and anti-thymocyte globulin (ATG).[54,55]

In a minority of patients, especially those with donor-specific, anti-HLA antibodies (DSAs), appropriate cord and haploidentical donors may be unavailable. Unrelated donor grafts, mismatched at more than one allele, have now emerged as an alternative source in the context of post-transplant CY-based GVHD prophylaxis, similar to haploidentical-related donor HCT.[56,57] A single-center study among recipients of nonmyeloablative conditioning for HCT from mismatched unrelated donors (majority at two or more loci), using posttransplant CY/sirolimus and mycophenolate as GVHD prophylaxis, demonstrated a promising 2-year NRM and overall survival of 6% and 67%, respectively.[58]

Graft-Versus-Host Disease Prophylaxis

The combination of a calcineurin inhibitor, such as cyclosporine, tacrolimus, or methotrexate, has been the mainstay of GVHD prevention, after allogeneic HCT.[59–63] For RIC regimens, an acceptable alternative is a combination of a calcineurin inhibitor and mycophenolate mofetil.[64–66] Despite GVHD prophylaxis, over 50% of allogeneic HCT recipients will develop acute and/or chronic GVHD. Studies have demonstrated that in vivo T-cell depletion with anti-thymocyte ATG can reduce chronic GVHD,[67] though the effect on survival has been questioned. Prospective phase III randomized trials have compared the use of ATG as part of GVHD prophylaxis in unrelated and related donor HCT and generally suggest lower rates of acute and/or chronic GVHD, without an impact on relapse, survival, or NRM.[68–71] Comparisons are difficult due to differences in clinical trial designs, potency, dosage of ATG preparations due to different production methods, and absolute lymphocyte count at the time of ATG administration.[72,73]

Administration of cyclophosphamide after HCT has moved from early use in the haploidentical HCT setting to the HLA-matched, related and unrelated donor HCT settings.[74–76] A prospective phase III randomized HOVON-96 trial done with recipients of matched-donor grafts in the RIC setting reported a significant reduction in both acute and chronic GVHD and 1-year GVHD-free and relapse-free survival with the use of post-transplant cyclophosphamide, without any difference in progression-free and overall survival.[77] The control arm in this study utilized cyclosporine and mycophenolate, which precludes comparison with the more common methotrexate-based

GVHD prophylaxis approach, after RIC transplantation.[64] A phase II randomized BMT CTN 1203 trial also suggested improved GVHD-free and relapse-free survival, along with lower risk of severe acute and chronic GVHD for post-transplant, CY-based GVHD prophylaxis regimen in RIC HCT, compared with non-randomized contemporaneous controls.[78] A large randomized trial of post-transplant cyclophosphamide versus the current standard of tacrolimus and methotrexate is now enrolling patients (BMT CTN 1703).

IMPACT OF MEASURABLE RESIDUAL DISEASE

At the time of transplant, disease remission status is a strong predictor of survival post transplantation for hematologic malignancies. Evaluation of measurable or minimal residual disease (MRD) status, using sensitive techniques (multiparameter flow cytometry, sequencing), is one of the most important established prognostic tools in ALL and is now emerging to be an important predictive and prognostic marker in other hematologic malignancies, such as AML, myeloma, and B-cell lymphomas. In ALL, MRD is present in up to 30% to 50% patients in a traditional CR.[79,80] Consideration of MRD impacts several clinical decisions in HCT. Among these are: assessing the suitability of proceeding to HCT versus additional consolidative therapy in patients who achieve a traditional CR but with residual MRD, the intensity of conditioning regimen used for HCT (RIC versus MAC), the role of pre-emptive posttransplant therapy to address relapse risk, and the need for HCT in those with an MRD-negative remission.

PREVENTION OF RELAPSE AFTER ALLOGENEIC TRANSPLANTATION

Primary causes of death after allogeneic HCT for hematologic malignancies are shown in Fig. 105.5. With improvements in prophylaxis and treatment of GVHD and supportive care advancements, relapse is now the leading cause of transplant failure. Maintenance therapy administered to patients in remission and after HCT attempts to reduce the risk of relapse. The ideal maintenance agent should have minimal interference with GVM effects, should not incite GVHD, nor interact with routine posttransplant drugs and should be tolerable with an acceptable toxicity profile. Targeted agents with minimal off-target toxicities, the list of potential maintenance options is growing. Maintenance therapy in Philadelphia chromosome-positive (Ph+) leukemias, including chronic myeloid leukemia and Ph+ ALL, comprises Abelson tyrosine kinase inhibitors (TKIs), based on single-arm studies and retrospective evidence supporting their adoption as the current standard.[81–84] In AML, the presence of an FMS-like tyrosine kinase internal tandem duplication (FLT3-ITD) mutation predicts for higher relapse risk, even after HCT in first complete remission (CR1). Gilteritinib, a selective FLT3 TKI approved for relapsed/refractory AML treatment, is being evaluated as post-HCT maintenance in a phase III randomized, double-blind, placebo-controlled trial (BMT CTN 1506). A non-targeted maintenance approach for AML and MDS with hypomethylating agents has been demonstrated to be feasible in several single-arm prospective trials and retrospective case series, but the efficacy of this strategy still needs to be proven in a phase III randomized trial.[85–88] Maintenance using novel targeted agents, such as IDH inhibitors, are also expected to be tested in specific molecular subtypes in the future.

Timing of Transplantation

In general, HCT outcomes are better when transplantation is done earlier in the disease course. Transplantation for advanced disease is associated with higher risks of both relapse and NRM. High NRM, in the setting of advanced disease, likely reflects patients' poorer clinical status and the cumulative effects of more extensive prior treatment. Since some HMs have excellent prognosis with non-transplant therapy (e.g., children with standard-risk ALL or adults with acute promyelocytic leukemia), appropriate timing of transplantation requires consideration of likely outcomes with transplant and non-transplant therapies. However, even when not used early, transplantation should not be

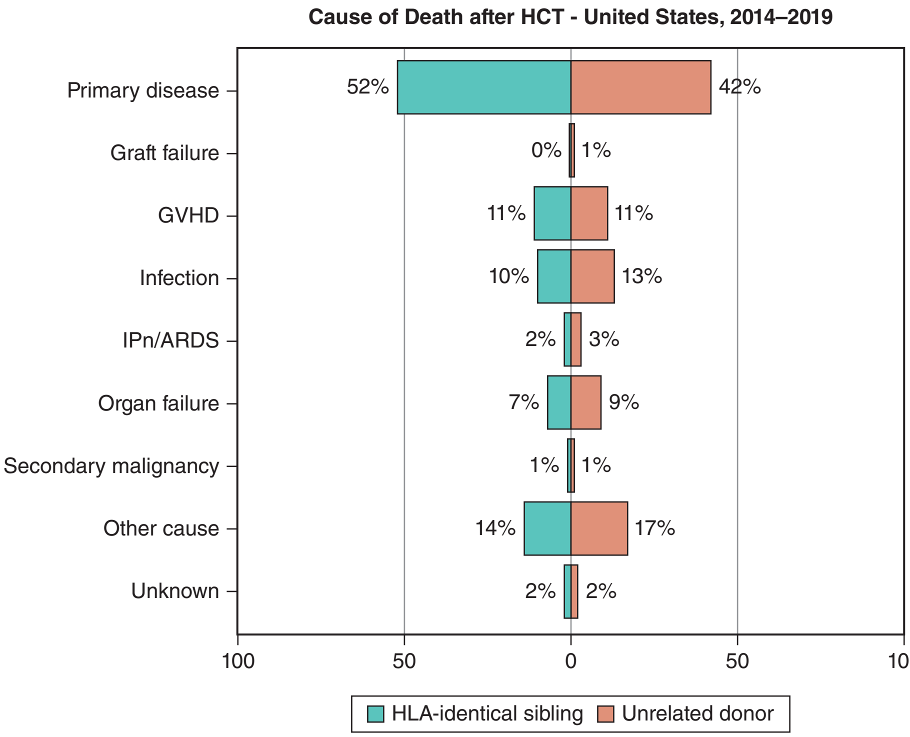


Figure 105.5 CAUSES OF DEATH AFTER TRANSPLANTATION, 2014–2019. *ARDS, Acute respiratory distress syndrome; GVHD, graft-versus-host disease; HLA, human leukocyte antigen; IPn, idiopathic pneumonia syndrome.*

inordinately delayed, since patients with refractory disease or severe complications from extensive prior therapy are unlikely to benefit. For patients with diseases potentially curable by allografting, appropriate timing of transplantation should be considered early in the overall management strategy. This includes early determination of donor availability. The American Society for Transplantation and Cellular Therapy (ASTCT) has developed evidence-based guidelines (Box 105.1), based upon current clinical practice and available literature (available online at https://www.astct.org/practice/practice-guidelines).[89]

Patient- and Disease-Related Factors

NRM is lower and, consequently, survival higher, in patients who are young, cytomegalovirus antibody screen-negative, have good performance scores, and have no active infection. While age is still a significant barrier to successful allogeneic HCT, the presence and extent of medical comorbid conditions rather than an arbitrary chronologic age cutoff, is increasingly recognized as a major determinant of NRM. Box 105.2 depicts the commonly used HCT-Comorbidity Index (HCT-CI), used for estimating risk of NRM.[90] Although many factors influence the outcome of allogeneic HCT, disease type and disease status at the time of transplantation are the strongest disease-related determinants of post-transplant survival. The CIBMTR Refined Disease Risk Index (R-DRI) (Box 105.3) is a validated prognostic tool that predicts overall survival after allogeneic HCT, based on patient's disease type and remission status.[91] Both HCT-CI and R-DRI are increasingly used in clinical decision making to determine patient eligibility and optimal conditioning intensity pre-HCT.

BOX 105.1 American Society for Transplantation and Cellular Therapy Indications for Allogeneic Transplantation in Adults (see full recommendations at https://www.astct.org/learn/practice-guidelines)

The ASTCT committee on practice classified the proposed indications for transplant as "standard of care" (S) or "clinical evidence available" (C), or "rare indication" (R). Emerging indications still in experimental stages were noted as "developmental" (D).

Disease State	Policy Regarding: Allogeneic Transplantation
AML in adults	• Standard-of-care for patients with AML with intermediate and high risk who are in CR1, for treatment of AML in ≥CR2 and for AML patients not in remission (all S) • Recommended for patients with therapy-related AML in CR1 (S) • Allogeneic HCT was not recommended for AML patients with low-risk disease and CR1
Acute promyelocytic leukemia	• Recommended for patients with APL in CR2 and with lack of molecular remission (S) • Clinical evidence supports allogeneic HCT for APL in CR2 with molecular remission, CR≥3, patients not in remission and patients relapsing after prior autologous HCT (all C) • Allogeneic HCT not recommended for APL patients in CR1
ALL	• Recommended as standard-of-care for treatment of ALL patients with standard-risk and high-risk in CR1, and patients with CR≥2 (all S) • For patients with ALL who are not in remission, allogeneic HCT can be considered (S)
CML	• Recommended as standard-of-care for patients with CML in CP2+, accelerated and blast phase (all S) • Standard-of-care with clinical evidence for CML patients in CP1, if refractory to or intolerant to multiple TKIs (C)
Myelofibrosis and myeloproliferative diseases	• Evidence supporting allogeneic HCT for treatment of patients with low-, intermediate- and high-risk primary disease and for secondary myelofibrosis (all C). Hypereosinophilic syndrome is a rare indication (R)
Myelodysplastic syndrome	• Recommended in highintermediate 2 risk disease (S) and recommended with clinical evidence in low/intermediate-1 risk (C)
Plasma cell disorders	• Considered standard-of-care for treatment of chemosensitive relapsed myeloma, including relapse after autologous HCT and for plasma cell leukemia (S) • There is developmental evidence to support allogeneic HCT for refractory myeloma (D)
Hodgkin lymphoma	• Allogeneic HCT is standard-of-care for chemosensitive first relapse, second or greater relapse and relapse after autologous HCT (all S) It can be considered for chemotherapy resistant first relapse, primary refractory disease; (all C) • Allogeneic HCT not recommended for Hodgkin lymphoma in CR1
DLBCL	• Allogeneic HCT is standard-of-care for primary refractory DLBCL (sensitive- or resistant-), first relapse (sensitive- or resistant-), second or greater relapse, and relapse after autologous transplant (all S). • There is insufficient evidence to recommend allogeneic HCT for high-grade B cell lymphoma, with MYC and BCL2 and/or BCL6 rearrangements, in CR1 and for primary central nervous system lymphoma in CR1 or in relapse
Follicular lymphoma (FL)	• Recommended as standard-of-care for primary refractory (resistant) FL, first relapse (resistant), and second or greater relapse, including relapse after autologous transplant (all S) • There is sufficient evidence to recommend allogeneic HCT for FL transforming to high-grade lymphoma. • Allogeneic HCT is not recommended for FL in CR1, first relapse (sensitive), including progression within 2 years
Mantle cell lymphoma (MCL)	• Recommended as standard of care for primary-refractory (sensitive) MCL, sensitive first relapse, second or greater relapse, and relapse after autologous transplant (all S) • There is evidence to support use of allogeneic HCT CR1/first partial remission, primary refractory (resistant) and first relapse (resistant) MCL (all C)
T-cell lymphoma	• Recommended as standard-of-care for CR1/first partial remission, primary refractory (sensitive), chemosensitive first relapse, second or greater relapse, and relapse after autologous HCT (all S) • Allogeneic HCT may also be considered for primary refractory (resistant) and chemoresistant first relapse (all C) • Allogeneic HCT is considered standard-of-care for relapsed cutaneous T-cell lymphoma (S)
Chronic lymphocytic lymphoma (CLL)	• Recommended as standard-of-care for high-risk CLL, first or greater remission and T-prolymphocytic leukemia (all S) • There is sufficient evidence to recommend allogeneic HCT for transformation to high-grade lymphoma (C)

ALL, Acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *CR*, complete remission; *DLBCL*; diffuse large B-cell lymphoma; *HCT*, hematopoietic cell transplantation.

BOX 105.2 Hematopoietic Cell Transplantation-Specific Comorbidity Index

Comorbidity	Explanation	HCT-CI score
Arrhythmia	Atrial fibrillation or flutter, sick sinus syndrome, or ventricular arrhythmias	1
Cardiac	Coronary artery disease, congestive heart failure, myocardial infarction, or ejection fraction ≤50%	1
Inflammatory bowel disease	Crohn disease or ulcerative colitis	1
Diabetes	Requiring treatment with insulin or oral hypoglycemics	1
Cerebrovascular disease	Transient ischemic attack or cerebrovascular accident	1
Psychiatric disturbance	Depression or anxiety requiring psychiatric consult or treatment	1
Hepatic, mild	Chronic hepatitis, bilirubin > ULN to 1.5 × ULN, or AST/ALT > ULN to 2.5 × ULN	1
Obesity	Patients with a body mass index >35 kg/m$^+$	1
Infection	Requiring treatment after day 0	1
Rheumatologic	SLE, rheumatoid arthritis, polymyositis, mixed connective tissue disease, or polymyalgia rheumatica	2
Peptic ulcer	Requiring treatment	2
Moderate/severe renal	Serum creatinine >2 mg/dL, on dialysis, or prior renal transplantation	2
Moderate pulmonary	DLCO and/or FEV$_1$ 66% to 80% or dyspnea on slight activity	2
Prior solid tumor	Treated at any time point in the patient's past history, excluding nonmelanoma skin cancer	3
Heart valve disease	Except mitral valve prolapse	3
Severe pulmonary	DLCO and/or FEV$_1$ ≤65% or dyspnea at rest or requiring oxygen	3
Moderate/severe hepatic	Liver cirrhosis, bilirubin > 1.5 × ULN, or AST/ALT > 2.5 × ULN	3

HCT-CI of 0 predicts for 2-year NRM of 14%
HCT-CI of 1–2 predicts for 2-year NRM of 21%
HCT-CI of ≥3 predicts for 2-year NRM of 41%

ALT, Alanine aminotransferase; *AST*, aspartate aminotransferase; *CI*, confidence interval; *DLCO*, diffusing capacity of the lungs for carbon monoxide; *FEV1*, forced expiratory volume in the first second of expiration; *HCT*, hematopoietic cell transplantation; *SLE*, systemic lupus erythematosus; *ULN*, upper limit of normal.

BOX 105.3 Refined Disease Risk Index

Refined DRI Category	Definition of Refined DRI Category	2-Year Overall Survival
Low	• Hodgkin lymphoma, mantle cell lymphoma, indolent non-Hodgkin lymphoma (NHL), chronic lymphocytic leukemia (CLL) or favorable-cytogenetics acute myeloid leukemia (AML) in complete remission (CR) • CLL or indolent NHL in partial remission (PR) • First or second chronic phase chronic myelogenous leukemia (CML)	66%
Intermediate	• CML advance phase • Mantle cell lymphoma, T-cell NHL, Hodgkin lymphoma, or aggressive NHL in PR • CLL or indolent NHL with stable or progressive disease • Acute lymphoblastic leukemia (ALL) in first CR • Intermediate-cytogenetics AML or aggressive NHL in CR • Myeloproliferative neoplasm • Low-risk myelodysplastic syndrome (MDS) with adverse-cytogenetic or high-risk MDS with intermediate-cytogenetics • Multiple myeloma in CR/very good PR/PR	51%
High	• T-cell NHL, Hodgkin lymphoma, mantle cell lymphoma, myeloma with stable or progressive disease • Burkitt lymphoma in CR • ALL in second or third CR • Adverse-cytogenetics AML in CR • Intermediate-cytogenetic AML with induction failure or active relapse • High-risk MDS with intermediate cytogenetics and advanced disease • Any-risk MDS with adverse cytogenetics and advanced disease	33%
Very High	• CML blast phase • Burkitt lymphoma in PR • Adverse-cytogenetic AML or ALL with induction failure or active relapse • Aggressive NHL with stable or progressive disease	23%

LONG-TERM SURVIVAL AFTER ALLOGENEIC TRANSPLANTATION

Most HCT-related deaths occur within the first 2 years. However, an analysis of 2 year survivors found that, compared with age- and nationality-matched death rates among the general population, subsequent survival was still inferior for HCT recipients.[92] Overall, 85% of 2-year survivors were still alive at 10 years post-HCT. Late relapse was the major cause of late deaths and was associated with advanced disease at HCT. Older age and GVHD were the other major predictors of late mortality.

The number of long-term HCT survivors is growing. Most 5-year survivors are well, off all immune suppression and leading normal lives. However, transplant recipients remain at risk for a variety of late complications, including late infections, cataracts, abnormalities of growth and development, thyroid disorders, chronic lung disease, osteoporosis, and avascular necrosis. There is also an increased incidence of leukemias, myelodysplasia, and solid tumors in transplant recipients, compared with the general population. Lifelong surveillance is necessary, as is increased awareness of late complications among the many non-transplant physicians who will care for these patients. Recommended long-term follow-up (LTFU) of HCT recipients is summarized in Box 105.4.[93] HCT-specific LTFU or survivorship clinics, though formally operational in less than half the North American transplant centers, are helpful in delivering individualized longitudinal survivorship care and transition of care from pediatric to adult setting. They also assist local physicians remote from the transplant center.[94]

DISEASE-SPECIFIC INDICATIONS FOR ALLOGENEIC TRANSPLANTATION

Acute Myeloid Leukemia

There is general agreement that most patients under the age of about 60 years who fail conventional therapy for AML and who possess good performance status are best treated with allogeneic transplantation if an appropriate related or unrelated donor is available. HCT is not recommended for those with favorable risk cytogenetics in CR1. The National Comprehensive Cancer Network (NCCN) guidelines, widely used to guide cancer therapy in the United States, distinguishes between AML patients ≤60 versus those greater than 60 years of age. Among younger patients, the panel recommended a post-remission strategy, based on factors such as the expected relapse rates with non-transplant therapy alone, salvage treatment options at relapse and patient-specific comorbidities that predict for NRM. The guidelines uniformly endorsed allogeneic HCT from related or unrelated donors, including cord blood HCT, in first CR for patients with unfavorable cytogenetic or molecular abnormalities, therapy-related AML, or prior myelodysplasia. Among those older than 60 years, the recommendation was to consider HCT as an early option in those achieving CR and as treatment for induction failure only in patients with low-volume residual disease. For relapsed AML, HCT was recommended irrespective of age but only after a second CR was achieved or in the context of a clinical trial. For patients with acute promyelocytic leukemia, the recommendation was to reserve allogeneic HCT for relapsed patients with persistent disease, despite salvage therapy.

The ASTCT guidelines for allogeneic HCT recently recommended HCT for AML patients in CR1, with intermediate-risk and high-risk disease and in the relapse setting (preferably after achievement of CR).[95] The European LeukemiaNet guidelines are in line with the ASTCT evidence-based policy and recommend allogeneic HCT for patients with intermediate-risk and adverse-risk cytogenetics in CR1, for patients with primary refractory disease, and for patients in CR2.[96] Patients in CR, but with persistent MRD or recurrent MRD, may proceed directly to HCT using MAC or may receive salvage therapy and proceed to HCT before relapse, depending on the likely efficacy of salvage therapy.

A systematic review of various national guidelines found the following:[97]

a. Consistent recommendations that patients with relapsed or refractory disease or high-risk cytogenetics be considered for HCT early;
b. Consistent recommendations that patients with good prognosis cytogenetics receive HCT only after failure of non-transplant therapy;
c. Inconsistent recommendations for patients with intermediate risk disease or without a family donor regarding transplantation in first remission.

Most national guidelines recommend a risk-based approach to guide selection of chemotherapy versus transplant approaches, using factors such as patient age, comorbid conditions, disease phenotype at diagnosis, and the number of cycles of therapy before achieving remission. MRD identified by multicolor flow cytometry,[98,99] real-time quantitative polymerase chain reaction,[100] and next-generation sequencing (NGS)[101] have been found to be an independent prognostic indicator in AML that is predictive of post-transplant outcomes in AML patients, and that pre-transplant MRD assessment by multi-color flow cytometry or molecular techniques can be used for risk stratification at the time of HCT.[102–106] A retrospective analysis of MRD and conditioning intensity was performed using pre-transplant samples from the phase III MAC versus RIC trial (BMT CTN 0901). Ultra-deep, DNA-based sequencing for 13 commonly mutated genes in AML was used for MRD detection.[107] Patients with no MRD had similar survival, regardless of conditioning intensity. In patients with detectable mutation, RIC (compared with MAC) was associated with higher relapse and lower relapse-free and overall survival, indicating that MAC can improve survival in MRD-positive AML patients in CR.

Acute Lymphoblastic Leukemia

Allogeneic HCT in first remission, from related or unrelated donors, is generally accepted as the most effective therapy for patients with Ph+ ALL. For adults with Ph-negative ALL (Ph− ALL), the appropriate timing of HCT is more controversial, despite the largest prospective study (Medical Research Council/Eastern Cooperative Oncology Group [MRC/ECOG]) and a meta-analysis suggesting a survival advantage for those assigned to transplant in CR1.[108,109] Another meta-analysis, limited to non-Ph+ ALL patients in CR, showed an overall survival benefit with allogeneic HCT, using a matched sibling donor for patients less than 35 years of age but not for older patients.[110] Allogeneic HCT is recommended in ASTCT guidelines for adult patients in CR2 and beyond and with high-risk ALL (including Ph+ ALL, t(4;11)) in CR1.[89] An evidence-based review and the most recent consensus recommendations from the ASTCT recommend allogeneic HCT for patients with standard-risk ALL in CR1, even while acknowledging the similar or superior outcomes with treatment on intensified pediatric-inspired protocols and without upfront allogeneic HCT in patients with no MRD.[111] Consolidation with allogeneic HCT should be considered after induction for patients with MRD, albeit after attempting to eliminate MRD with additional therapy. For example, treatment with blinatumomab to MRD negativity, before HCT, should be considered in B-ALL.[112] Among Ph+ ALL patients receiving HCT in CR1, conditioning intensity (RIC vs. MAC) did not influence outcomes before HCT for those without MRD, whereas those with residual MRD had inferior outcomes following RIC.[111] In the absence of randomized data against HCT, patients with high-risk ALL should still be considered for HCT even if they achieve MRD negativity.[111] For standard-risk adolescents and young adults (AYA) ALL patients with early MRD-negative CR1 status and pediatric-inspired regimens, continued chemotherapy consolidation and maintenance is recommended, while reserving allogeneic HCT for patients in CR2. For AYA patients with high-risk ALL and those with persistent MRD after induction, upfront allogeneic HCT consolidation should be

BOX 105.4 Recommended Practices for Allogeneic Transplant Recipients in Long-Term Follow-Up

	6 Months	12 Months	Annual	Comments (a)
Liver:				
Liver function tests	√	√	[a]	Annual check only if previously abnormal or new signs/ symptoms
Serum ferritin		√	[a]	
Respiratory:				
Clinical assessment	√	√	√	
Smoking/tobacco avoidance	√	√	√	
Formal pulmonary function tests		√	[a]	Annual study/imaging as needed in follow up of prior/new abnormalities
Chest X-ray	[a]	[a]	[a]	
Bone Density Screening		√	[a]	Consider repeat testing in ones with recognized defects, ongoing risk factors or for response assessment
Kidney:				
Blood pressure screen	√	√	√	
Proteinuria screen	√	√	[a]	Annually as needed
BUN/creatinine level	√	√	√	
Nervous System:				
Clinical assessment		√	[a]	Annually as needed
Endocrine:				
Thyroid function		√	[a]	Annually as needed
Gonadal function in postpubertal women		√	√	
Cardiac/Vascular:				
Cardiovascular risk factor screen		√	√	
Immune System/Infection Risk:				
Encapsulated organism prophylaxis	[a]	[a]	[a]	If on immune suppression or with ongoing GVHD
Pneumocystis prophylaxis	√	[a]	[a]	
CMV test	√	√		
Immunizations		√	√	
Endocarditis prophylaxis		[a]	[a]	Follow AHA guidelines
Antifungal/antiherpes viral prophylaxis	[a]	[a]	[a]	If on immune suppression or with GVHD; if not, no consensus
Second Cancer Risk:				
Cancer risk assessment and education		√	√	
Pap smear		√	√	
Mammogram (women over 40 years)		√	√	
Breast/Skin/Testes self-examination		√	√	
Clinical screen for second cancers		√	√	
Psychosocial:				
Clinical psychosocial and QOL screen	√	√	√	
Sexual function screen	√	√	√	
Dental Assessment	√	√	√	
Ocular:				
Clinical evaluation	√	√	√	
Fundus exam		√	[a]	Annually as needed
Schirmer test		[a]	[a]	If with ongoing GVHD or on immune suppression

AHA, American Heart Association; *BUN*, blood urea nitrogen; *CMV*, cytomegalovirus; *GVHD*, graft-versus-host disease; *QOL*, quality of life.

considered. Post-transplant TKI for maintenance or as preemptive MRD-guided therapy should be considered in Ph+ ALL, despite the lack of high-quality prospective randomized data. A position statement from the Acute Leukemia Working Party of the EBMT and European Working Group for Adult Acute ALL also recommended allogeneic HCT for Ph- ALL with high-risk features, including MRD persistence ($<10^{-3}$ in marrow).[113] However, in Ph- ALL patients in CR1 with absence of MRD, continued intensified pediatric-inspired treatment, rather than upfront allogeneic HCT, is increasingly considered an acceptable strategy.[113] ALL patients who do not achieve MRD negativity after induction chemotherapy should be considered for allogeneic HCT,[79] acknowledging that their outcomes are likely inferior compared with patients undergoing HCT without evidence of MRD. Anti-CD19 CAR-T cell therapy has proven efficacy and shown impressive outcomes in patients with relapsed and refractory B-ALL: tisagenlecleucel (Kymriah, Novartis Pharmaceuticals Corp., Basel, Switzerland) and is approved by the US FDA for treatment of children and young adults up to 25 years of age, with second or later relapse and with refractory B-ALL.[114] Additional clinical data are required to determine the optimal utilization and sequencing of CAR-T-cell therapies in relation to allogeneic HCT, for instance, as a bridge to HCT, as definitive MRD-dictated intensification versus as treatment of ALL relapse post-HCT—current guidelines lack recommendations on these open questions.

Chronic Myelogenous Leukemia

In general, allogeneic HCT is indicated for patients whose initial presentation is in blast phase (BP) and should be considered for those with a suboptimal response or relapse while on TKIs.[115] HCT is also a consideration for patients who have *BCR-ABL* mutations that predict for TKI non-response (e.g., *T315I* mutation). It is important to establish a monitoring plan for early signs of progression and for mutation screening, since outcomes are significantly better if HCT is performed before transformation to an advanced disease stage. Definitions of suboptimal response and failure with TKIs and guidelines for monitoring have been developed.[116,117] European LeukemiaNet guidelines have addressed timing of allogeneic HCT in CML.[117] Allogeneic HCT is recommended in CML patients presenting in or progressing to BP, for patients progressing to accelerated phase (AP) during treatment, and patients presenting with AP who have suboptimal response to TKIs. For patients in AP and BP, allogeneic HCT should be offered after achieving disease control, since patients in second chronic phase (CP) before HCT have improved outcomes.[118] For patients in CP, a CIBMTR registry study showed a 5-year OS of 75% in early chronic phase CML, making it a potential curative clinical option in this subset of patients under special circumstances. We consider allogeneic HCT for those who are refractory or intolerant to at least one second-generation TKI in first- or second-line therapy, and in patients with T315I mutation, especially in those with suboptimal response to ponatinib.[89] A registry-based, retrospective comparison of myeloablative versus RIC and non-myeloablative conditioning in CML patients up to 60 years of age revealed similar survival, despite higher relapse risk with non-MAC regimens.[119]

Chronic Lymphocytic Leukemia

The introduction of signaling pathway inhibitors, such as Bruton tyrosine kinase inhibitor (BTKi; ibrutinib), phosphatidylinositol-3-kinase inhibitor (PI3Ki; idelalisib), and BCL2-inhibitor (BCL2i; venetoclax) has altered the position of HCT in the chronic lymphocytic leukemia (CLL) treatment algorithm. Recent EBMT consensus is that patients with chemoimmunotherapy-resistant CLL, with *TP53* abnormalities, but responsive to a first pathway inhibitor (BTKi or BCL2i), could be offered allogeneic HCT if deemed low risk for NRM, even though most of these patients could continue to be treated with pathway inhibitors.[115,120] CLL patients with resistance to both chemoimmunotherapy and at least one pathway inhibitor should be considered for allogeneic HCT, even if responding to an alternate pathway inhibitor. These recommendations supplant ASTCT Practice Guidelines that favor allogeneic HCT for relapsed/refractory CLL patients, with del(17p) and/or *TP53* mutations, with or without complex karyotype, regardless of sensitivity to pathway inhibitors (responsive or refractory to BTKi and/or BCL-2i).[121] In addition, allogeneic HCT is a strong consideration for CLL patients developing Richter transformation, after achieving a response to anthracycline-based chemotherapy.[121] HCT and pathway inhibitor drugs are not mutually exclusive in the CLL landscape: both have a role in the management of CLL, which continues to evolve.[120] With the potentially complementary effects of pathway inhibitors and allogeneic HCT (graft-versus-leukemia effect), strategic and non-competitive employment of pathway inhibitors before and/or after HCT will likely synergistically improve outcomes in patients with high-risk CLL. CAR-T-cell therapy, targeted at CD 19, has been in clinical trials for CLL and once mature data are available, the position and timing of allogeneic HCT would require a reappraisal.

Diffuse Large B-Cell Lymphoma

Allogeneic HCT role in diffuse large B-cell lymphoma (DLBCL) has undergone recent evolution with the availability of CAR-T-cell therapy. A decline in the number of autologous HCT for DLBCL was noted in 2018 compared with previous years,[4] following the approval of CD19-directed CAR-T cells in 2017 (axicabtagene ciloleucel, Yescarta) (Kite Pharma, A Gilead Company, Los Angeles, CA); tisagenlecleucel, Kymriah (Novartis Pharmaceuticals Corp., Basel, Switzerland) is used for treatment of DLBCL patients failing at least two lines of therapy, with or without autologous HCT.[122–124] Notably, an EBMT report recommended allogeneic HCT as the established standard for chemosensitive relapse, after failure of a prior autologous HCT.[115] Understandably, in the absence of comparative studies there is growing uncertainty regarding the role of allogeneic HCT in DLBCL management. In the United States, CAR-T cell therapy has rapidly become the preferred option for relapsed/refractory DLBCL as a result of its safety and efficacy in refractory disease, while allogeneic HCT appears to be positioned as treatment of post-CAR-T relapses.[125,126] A few exceptions include relapsed DLBCL patients with refractory cytopenias and therapy-related myelodysplastic syndrome, which are indications to be considered for allogeneic HCT rather than CAR-T therapy.[125] A collaborative registry-based (EBMT and CIBMTR) analysis compared outcomes of RIC allogeneic HCT for relapsed DLBCL, using the three major donor types: haploidentical donor grafts using post-transplant CY, matched sibling donor, or matched unrelated donor.[49] No differences in NRM, relapse, or overall survival between haploidentical and matched donor HCT were observed. A 3-year OS of 46 and PFS of 38% underscored the promise of allogeneic HCT to rescue DLBCL patients.

Follicular Lymphoma

The vast majority of relapsed, chemotherapy-sensitive FL patients can experience durable disease control with autologous HCT. Allogeneic HCT can be considered after autologous HCT failure, and in those who have heavily pretreated disease, persistent marrow involvement, or chemo-unresponsive (but relatively low bulk disease). In the allogeneic setting, RIC is the standard of care, with limited role for MAC regimens.[127] The recent ASTCT guidelines added a category of patients with follicular lymphoma with early treatment failure, defined as a lack of response or relapse/progression within 2 years of frontline chemoimmunotherapy.[89] Registry-based analyses suggest efficacy of autologous HCT (with low NRM) and allogeneic HCT from a matched sibling donor (with low relapse rate), resulting in improved overall survival in this high-risk subset of follicular lymphoma patients.[128,129] CAR-T-cell therapies (axicabtagene ciloleucel or tisagenlecleucel) have emerged as an effective salvage option for patients who have failed ≥2 prior chemoimmunotherapy regimens for transformed follicular lymphoma, who may or may not be eligible for HCT.[124] CAR-T-cell therapies for B-cell malignancies are expected to impact the role of allogeneic HCT for relapsed FL in the near future.

Mantle Cell Lymphoma

NCCN guidelines recommend autologous transplantation as an adjunct to initial therapy in eligible patients.[124,130] Expert consensus opinion is that allogeneic HCT has a limited role as upfront consolidation in chemotherapy-sensitive disease.[89,115] Given the poor prognosis of relapsed mantle cell lymphoma and the curative potential of allogeneic HCT, it is a reasonable option for patients relapsing after upfront autologous transplantation or those with BTKi-refractory disease. KTE-X19, an anti-CD19 CAR T-cell therapy, was evaluated in a single-arm phase II trial for relapsed/refractory mantle cell lymphoma patients, progressing on or after BTKi therapy and resulted in durable remissions in a majority of patients.[131] The recent approval of KTE-X19, now known as brexucabtagene autoleucel, may augur changes in the utilization and timing of allogeneic HCT in mantle cell lymphoma.

T-Cell Lymphoma

ASTCT and NCCN guidelines recommend consideration of allogeneic HCT in the relapsed or refractory setting in T-cell lymphoma.[89,124,132] Autologous HCT is recommended as front-line consolidation in and for relapsed-sensitive T-cell lymphoma subtypes, including peripheral T-cell lymphoma-not otherwise specified (PTCL-NOS), angioimmunoblastic T-cell lymphoma (AITL), anaplastic large-cell lymphoma, anaplastic lymphoma kinase (ALCL-ALK)-negative, natural killer (NK)/T-cell (disseminated), and hepatosplenic lymphoma, despite the lack of randomized evidence.[133–135]. Autologous HCT is recommended for relapsed-sensitive ALCL-ALK-positive, where it is not offered in front-line consolidation.[133] Allogeneic HCT is strongly recommended (based on retrospective and registry data) for high-risk histologies and in settings where chemotherapy-based approaches are non-curative (e.g., front line in adult T-cell leukemia/lymphoma) (ATLL; acute and lymphoma type), hepatosplenic lymphoma, NK cell leukemia, and for chemotherapy sensitive relapse of NK/T-cell (disseminated), mycosis fungoides/Sezary syndrome (advanced stage); and for relapse post autologous transplant in subtypes that are typically offered autologous HCT in the first-line (PTCL-NOS, AITL, ALCL-ALK-negative).[136–144]

Hodgkin Lymphoma

With the recent approval of three novel agents (brentuximab vedotin,[145] and checkpoint inhibitors, nivolumab[146] and pembrolizumab[147]), allogeneic HCT is currently recommended only for Hodgkin lymphoma patients, relapsing after a prior autologous HCT and after brentuximab vedotin failure.[89,148,149] In high-risk Hodgkin lymphoma (with a median age in the early 30 s in most published data), patients who have progressed after autologous HCT and brentuximab vedotin, checkpoint inhibitors result in modest response with suboptimal durability.[146,147] Such patients have improved survival outcomes with allogeneic HCT, and a CIBMTR analysis suggested that the haploidentical-related donor HCT with post-transplant CY (vs. matched sibling donor HCT) resulted in lower relapse and comparable survival in relapsed Hodgkin lymphoma patients.[50] NCCN guidelines currently consider allogeneic HCT in progressive or relapsed Hodgkin lymphoma as a category 3 recommendation (major disagreement).[150]

Allogeneic HCT is a frequently considered treatment option in heavily pretreated HL patients, including those relapsing.

Multiple Myeloma

First-line autologous HCT continues to be the standard of care for newly diagnosed myeloma patients, which in turn remains the most common indication for autologous HCT.[4,151,152] Allogeneic HCT with RIC has been studied in randomized studies, comparing it in the tandem auto-allo HCT fashion versus tandem autologous transplantation.[153–155] Results have been disparate with the large US-based BMT CTN 0102 study failing to show a PFS or OS advantage for allogeneic HCT, while a similar EBMT study did show a long-term survival and PFS advantage. A long-term follow-up study, using a large pooled dataset of four prospective trials with biological randomization between tandem autologous HCT and autologous-allogeneic HCT, showed significantly improved OS, as well as PFS and post-relapse survival in favor of allogeneic HCT.[156] Given the significant expansion in myeloma therapeutics, the use of upfront allogeneic HCT has declined.[4] Allogeneic HCT has been considered for ultra-high-risk myeloma in clinical trials (BMT CTN 1302), but an optimal patient population is still not uniformly agreed upon. ASTCT guidelines do not recommend routine upfront myeloablative allogeneic HCT, nor planned tandem autologous-RIC allogeneic HCT.[157] However these guidelines support allogeneic HCT in myeloma in the context of a clinical trials or in clear high-risk disease, such as plasma cell leukemia. Prospective trial data on allogeneic HCT for relapsed myeloma are sparse; a CIBMTR analysis suggested no survival advantage in favor of salvage allogeneic HCT, over autologous HCT in relapse setting.[158] Several post-relapse survival comparisons of patients relapsing after allogeneic HCT versus single or tandem autologous HCT have shown post-relapse survival advantage in allogeneic HCT recipients.[155,159,160]

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Anasetti C, Logan BR, Lee SJ, et al. Peripheral-blood stem cells versus bone marrow from unrelated donors. *N Engl J Med*. 2012;367:1487–1496.

Armand P, Kim HT, Logan BR, et al. Validation and refinement of the Disease Risk Index for allogeneic stem cell transplantation. *Blood*. 2014;123: 3664–3671.

Bacigalupo A, Ballen K, Rizzo D, et al. Defining the intensity of conditioning regimens: working definitions. *Biol Blood Marrow Transplant*. 2009;15:1628–1633.

Bashey A, Zhang X, Sizemore CA, et al. T-cell-replete HLA-haploidentical hematopoietic transplantation for hematologic malignancies using post-transplantation cyclophosphamide results in outcomes equivalent to those of contemporaneous HLA-matched related and unrelated donor transplantation. *J Clin Oncol*. 2013;31:1310–1316.

Bolaños-Meade J, Reshef R, Fraser R, et al. Three prophylaxis regimens (tacrolimus, mycophenolate mofetil, and cyclophosphamide; tacrolimus, methotrexate, and bortezomib; or tacrolimus, methotrexate, and maraviroc) versus tacrolimus and methotrexate for prevention of graft-versus-host disease with haemopoietic cell transplantation with reduced-intensity conditioning: a randomised phase 2 trial with a non-randomised contemporaneous control group (BMT CTN 1203). *Lancet Haematol*. 2019;6:e132–e143.

Brissot E, Labopin M, Beckers MM, et al. Tyrosine kinase inhibitors improve long-term outcome of allogeneic hematopoietic stem cell transplantation for adult patients with Philadelphia chromosome positive acute lymphoblastic leukemia. *Haematologica*. 2015;100:392–399.

Brunstein CG, et al. Results of blood and marrow transplant clinical trials Network protocol 1101: a multicenter phase III randomized trial of transplantation of double umbilical cord blood vs. HLA-haploidentical related bone marrow for hematologic malignancy. *Biol Blood Marrow Transplant Abstract LBA*. 2020;1

Dehn J, Spellman S, Hurley CK, et al. Selection of unrelated donors and cord blood units for hematopoietic cell transplantation: guidelines from NMDP/CIBMTR. *Blood*. 2019;134(12):924–934.

Gahrton G, Iacobelli S, Bjorkstrand B, et al. Autologous/reduced-intensity allogeneic stem cell transplantation vs autologous transplantation in multiple myeloma: long-term results of the EBMT-NMAM2000 study. *Blood*. 2013;121:5055–5063.

Gökbuget N, Dombret H, Bonifacio M, et al. Blinatumomab for minimal residual disease in adults with B-cell precursor acute lymphoblastic leukemia. *Blood*. 2018;131:1522–1531.

Goldstone AH, Richards SM, Lazarus HM, et al. In adults with standard-risk acute lymphoblastic leukemia, the greatest benefit is achieved from a matched sibling allogeneic transplantation in first complete remission, and an autologous transplantation is less effective than conventional consolidation/maintenance chemotherapy in all patients: final results of the International ALL Trial (MRC UKALL XII/ECOG E2993). *Blood*. 2008;111:1827–1833.

Gragert L, Eapen M, Williams E, et al. HLA match likelihoods for hematopoietic stem-cell grafts in the U.S. registry. *N Engl J Med*. 2014;371:339–348.

Hochhaus A, Baccarani M, Silver RT, et al. European LeukemiaNet 2020 recommendations for treating chronic myeloid leukemia. *Leukemia*. 2020;34:966–984.

Hourigan CS, Dillon LW, Gui G, et al. Impact of conditioning intensity of allogeneic transplantation for acute myeloid leukemia with genomic evidence of residual disease. *J Clin Oncol*. 2020;38:1273–1283.

Kanate AS, Majhail NS, Savani BN, et al. Indications for hematopoietic cell transplantation and immune effector cell therapy: guidelines from the American society for transplantation and cellular therapy. *Biol Blood Marrow Transplant*. 2020;26:1247–1256.

Kharfan-Dabaja MA, Kumar A, Ayala E, et al. Clinical practice recommendations on indication and timing of hematopoietic cell transplantation in mature T cell and NK/T cell lymphomas: an International collaborative effort on behalf of the guidelines committee of the American society for blood and marrow transplantation. *Biol Blood Marrow Transplant*. 2017;23:1826–1838.

Kröger N, Solano C, Wolschke C, et al. Antilymphocyte globulin for prevention of chronic graft-versus-host disease. *N Engl J Med*. 2016;374:43–53.

Lee SJ, Klein J, Haagenson M, et al. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. *Blood*. 2007;110:4576–4583.

Luznik L, O'Donnell PV, Symons HJ, et al. HLA-Haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose, posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant*. 2008;14:641–650.

Majhail NS, Farnia SH, Carpenter PA, et al. Indications for autologous and allogeneic hematopoietic cell transplantation: guidelines from the American Society for Blood and Marrow Transplantation. *Biol Blood Marrow Transplant*. 2015;21:1863–1869.

Majhail NS, Rizzo JD, Lee SJ, et al. Recommended screening and preventive practices for long-term survivors after hematopoietic cell transplantation. *Bone Marrow Transplant*. 2012;47:337–341.

Muffly L, Pasquini MC, Martens M, et al. Increasing use of allogeneic hematopoietic cell transplantation in patients aged 70 years and older in the United States. *Blood*. 2017;130:1156–1164.

Nash RA, Antin JH, Karanes C, et al. Phase 3 study comparing methotrexate and tacrolimus with methotrexate and cyclosporine for prophylaxis of acute graft-versus-host disease after marrow transplantation from unrelated donors. *Blood*. 2000;96:2062–2068.

Rambaldi A, Grassi A, Masciulli A, et al. Busulfan plus cyclophosphamide versus busulfan plus fludarabine as a preparative regimen for allogeneic haemopoietic stem-cell transplantation in patients with acute myeloid leukaemia: an open-label, multicentre, randomised, phase 3 trial. *Lancet Oncol*. 2015;16:1525–1536.

Scott BL, Pasquini MC, Logan BR, et al. Myeloablative versus reduced-intensity hematopoietic cell transplantation for acute myeloid leukemia and myelodysplastic syndromes. *J Clin Oncol*. 2017;35:1154–1161.

Soiffer RJ, Kim HT, McGuirk J, et al. Prospective, randomized, double-blind, phase III clinical trial of anti–T-lymphocyte globulin to assess impact on chronic graft-versus-host disease–free survival in patients undergoing HLA-matched unrelated myeloablative hematopoietic cell transplantation. *J Clin Oncol*. 2017;35:4003–4011.

Smith SM, Burns LJ, van Besien K, et al. Hematopoietic cell transplantation for systemic mature T-cell non-Hodgkin lymphoma. *J Clin Oncol*. 2013;31:3100–3109.

Smith SM, Godfrey J, Ahn KW, et al. Autologous transplantation versus allogeneic transplantation in patients with follicular lymphoma experiencing early treatment failure. *Cancer*. 2018;124:2541–2551.

Sorror ML, Maris MB, Storb R, et al. Hematopoietic cell transplantation (HCT)-specific comorbidity index: a new tool for risk assessment before allogeneic HCT. *Blood*. 2005;106:2912–2919.

Storb R, Deeg HJ, Whitehead J, et al. Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. *N Engl J Med*. 1986;314:729–735.

REFERENCES

1. Thomas E, Storb R, Clift RA, et al. Bone-marrow transplantation (first of two parts). *N Engl J Med*. 1975;292:832–843.
2. Mavroudis D, Barrett J. The graft-versus-leukemia effect. *Curr Opin Hematol*. 1996;3:423–429.
3. Center for International Blood and Marrow Transplant Research (CIBMTR). Annual Report 2019. https://www.cibmtr.org/About/AdminReports/Documents/2019CIBMTRAnnualReport.pdf. Accessed June 9, 2020.
4. D'Souza A, Fretham C, Lee SJ, et al. Current use of and trends in hematopoietic cell transplantation in the United States. *Biol Blood Marrow Transplant*. 2020;26(8):e177–e182.
5. Muffly L, Pasquini MC, Martens M, et al. Increasing use of allogeneic hematopoietic cell transplantation in patients aged 70 years and older in the United States. *Blood*. 2017;130:1156–1164.
6. Bredeson C, LeRademacher J, Kato K, et al. Prospective cohort study comparing intravenous busulfan to total body irradiation in hematopoietic cell transplantation. *Blood*. 2013;122:3871–3878.
7. Rambaldi A, Grassi A, Masciulli A, et al. Busulfan plus cyclophosphamide versus busulfan plus fludarabine as a preparative regimen for allogeneic haemopoietic stem-cell transplantation in patients with acute myeloid leukaemia: an open-label, multicentre, randomised, phase 3 trial. *Lancet Oncol*. 2015;16:1525–1536.
8. Clift RA, Buckner CD, Appelbaum FR, et al. Allogeneic marrow transplantation in patients with acute myeloid leukemia in first remission: a randomized trial of two irradiation regimens. *Blood*. 1990;76:1867–1871.
9. Sabloff M, Chhabra S, Wang T, et al. Comparison of high doses of total body irradiation in myeloablative conditioning before hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2019;25:2398–2407.
10. Bacigalupo A, Ballen K, Rizzo D, et al. Defining the intensity of conditioning regimens: working definitions. *Biol Blood Marrow Transplant*. 2009;15:1628–1633.
11. Sorror ML, Sandmaier BM, Storer BE, et al. Long-term outcomes among older patients following nonmyeloablative conditioning and allogeneic hematopoietic cell transplantation for advanced hematologic malignancies. *JAMA*. 2011;306:1874–1883.
12. Scott BL, Pasquini MC, Logan BR, et al. Myeloablative versus reduced-intensity hematopoietic cell transplantation for acute myeloid leukemia and myelodysplastic syndromes. *J Clin Oncol*. 2017;35:1154–1161.
13. Kröger N, Iacobelli S, Franke GN, et al. Dose-reduced versus standard conditioning followed by allogeneic stem-cell transplantation for patients with myelodysplastic syndrome: a prospective randomized phase III study of the EBMT (RICMAC Trial). *J Clin Oncol*. 2017;35:2157–2164.
14. Horan JT, Logan BR, Agovi-Johnson MA, et al. Reducing the risk for transplantation-related mortality after allogeneic hematopoietic cell transplantation: how much progress has been made? *J Clin Oncol*. 2011;29:805–813.
15. Couban S, Simpson DR, Barnett MJ, et al. A randomized multicenter comparison of bone marrow and peripheral blood in recipients of matched sibling allogeneic transplants for myeloid malignancies. *Blood*. 2002;100:1525–1531.
16. Mielcarek M, Storer B, Martin PJ, et al. Long-term outcomes after transplantation of HLA-identical related G-CSF-mobilized peripheral blood mononuclear cells versus bone marrow. *Blood*. 2012;119:2675–2678.
17. Anasetti C, Logan BR, Lee SJ, et al. Peripheral-blood stem cells versus bone marrow from unrelated donors. *N Engl J Med*. 2012;367:1487–1496.
18. Eapen M, Logan BR, Horowitz MM, et al. Bone marrow or peripheral blood for reduced-intensity conditioning unrelated donor transplantation. *J Clin Oncol*. 2015;33:364–369.
19. Fürst D, Müller C, Vucinic V, et al. High-resolution HLA matching in hematopoietic stem cell transplantation: a retrospective collaborative analysis. *Blood*. 2013;122:3220–3229.
20. Lee SJ, Klein J, Haagenson M, et al. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. *Blood*. 2007;110:4576–4583.
21. Loiseau P, Busson M, Balere M-L, et al. HLA Association with hematopoietic stem cell transplantation outcome: the number of mismatches at HLA-A, -B, -C, -DRB1, or -DQB1 is strongly associated with overall survival. *Biol Blood and Marrow Transplant*. 2007;13:965–974.
22. Nunes E, Heslop H, Fernandez-Vina M, et al. Definitions of histocompatibility typing terms. *Blood*. 2011;118:e180–e183.
23. Horowitz MM. Uses and growth of hematopoietic cell transplantation. In: Forman SJ, Negrin RS, Antin JH (eds.) *Thomas' Hematopoietic Cell Transplantation*, vol. 1. 5th ed. Chichester: Wiley Blackwell; 2015:8–17.
24. Stroncek D, Bartsch G, Perkins HA, et al. The National Marrow Donor Program. *Transfusion*. 1993;33:567–577.
25. Dehn J, Spellman S, Hurley CK, et al. Selection of unrelated donors and cord blood units for hematopoietic cell transplantation: guidelines from NMDP/CIBMTR. *Blood*. 2019;134(12):924–934.
26. Lown RN, Shaw BE. Beating the odds: factors implicated in the speed and availability of unrelated haematopoietic cell donor provision. *Bone Marrow Transplant*. 2013;48:210–319.
27. Gragert L, Eapen M, Williams E, et al. HLA match likelihoods for hematopoietic stem-cell grafts in the U.S. Registry. *N Engl J Med*. 2014;371:339–348.
28. Anasetti C. Use of alternative donors for allogeneic stem cell transplantation. *Hematology*. 2015;2015:220–224.
29. Morishima Y, Sasazuki T, Inoko H, et al. The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients receiving a marrow transplant from serologically HLA-A, HLA-B, and HLA-DR matched unrelated donors. *Blood*. 2002;99:4200–4206.
30. Petersdorf EW. HLA matching in allogeneic stem cell transplantation. *Curr Opin in Hematol*. 2004;11:386–391.
31. Flomenberg N, Baxter-Lowe LA, Confer D, et al. Impact of HLA class I and class II high-resolution matching on outcomes of unrelated donor bone marrow transplantation: HLA-C mismatching is associated with a strong adverse effect on transplantation outcome. *Blood*. 2004;104:1923–1930.
32. Gratwohl A, Pasquini MC, Aljurf M, et al. One million haemopoietic stem-cell transplants: a retrospective observational study. *Lancet Haematol*. 2015;2:e91–e100.
33. Kollman C, Howe CWS, Anasetti C, et al. Donor characteristics as risk factors in recipients after transplantation of bone marrow from unrelated donors: the effect of donor age. *Blood*. 2001;98:2043–2051.
34. Gupta V, Tallman MS, He W, et al. Comparable survival after HLA-well-matched unrelated or matched sibling donor transplantation for acute myeloid leukemia in first remission with unfavorable cytogenetics at diagnosis. *Blood*. 2010;116:1839–1848.
35. Moore J, Nivison-Smith I, Goh K, et al. Equivalent survival for sibling and unrelated donor allogeneic stem cell transplantation for acute myelogenous leukemia. *Biol Blood Marrow Transplant*. 2007;13:601–607.
36. Saber W, Opie S, Rizzo JD, et al. Outcomes after matched unrelated donor versus identical sibling hematopoietic cell transplantation in adults with acute myelogenous leukemia. *Blood*. 2012;119:3908–3916.
37. Walter RB, Pagel JM, Gooley TA, et al. Comparison of matched unrelated and matched related donor myeloablative hematopoietic cell transplantation for adults with acute myeloid leukemia in first remission. *Leukemia*. 2010;24:1276.
38. Wagner JE, Eapen M, Carter S, et al. One-unit versus two-unit cord-blood transplantation for hematologic cancers. *N Engl J Med*. 2014;371:1685–1694.
39. Horwitz ME, Wease S, Blackwell B, et al. Phase I/II study of stem-cell transplantation using a single cord blood unit expanded ex vivo with nicotinamide. *J Clin Oncol*. 2019;37:367–374.
40. Beatty PG, Clift RA, Mickelson EM, et al. Marrow transplantation from related donors other than HLA-identical siblings. *N Engl J Med*. 1985;313:765–771.
41. Luznik L, O'Donnell PV, Symons HJ, et al. HLA-haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose, posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant*. 2008;14:641–650.
42. Luznik L, Jalla S, Engstrom LW, et al. Durable engraftment of major histocompatibility complex-incompatible cells after nonmyeloablative conditioning with fludarabine, low-dose total body irradiation, and posttransplantation cyclophosphamide. *Blood*. 2001;98:3456–3464.
43. Kanate AS, Szabo A, Raj RV, et al. Comparison of graft acquisition and early direct charges of haploidentical related donor transplantation versus umbilical cord blood transplantation. *Biol Blood Marrow Transplant*. 2019;25:1456–1464.
44. Kanate AS, Mussetti A, Kharfan-Dabaja MA, et al. Reduced-intensity transplantation for lymphomas using haploidentical related donors vs HLA-matched unrelated donors. *Blood*. 2016;127:938–947.
45. Ghosh N, Karmali R, Rocha V, et al. Reduced-intensity transplantation for lymphomas using haploidentical related donors versus HLA-matched sibling donors: a center for international blood and marrow transplant research analysis. *J Clin Oncol*. 2016;34:3141–3149.
46. Ciurea SO, Zhang MJ, Bacigalupo AA, et al. Haploidentical transplant with posttransplant cyclophosphamide vs matched unrelated donor transplant for acute myeloid leukemia. *Blood*. 2015;126:1033–1040.
47. Rashidi A, Hamadani M, Zhang MJ, et al. Outcomes of haploidentical vs matched sibling transplantation for acute myeloid leukemia in first complete remission. *Blood Adv*. 2019;3:1826–1836.
48. Bashey A, Zhang X, Sizemore CA, et al. T-cell-replete HLA-haploidentical hematopoietic transplantation for hematologic malignancies using post-transplantation cyclophosphamide results in outcomes equivalent to

those of contemporaneous HLA-matched related and unrelated donor transplantation. *J Clin Oncol*. 2013;31:1310–1316.

49. Dreger P, Sureda A, Ahn KW, et al. PTCy-based haploidentical vs matched related or unrelated donor reduced-intensity conditioning transplant for DLBCL. *Blood Advances*. 2019;3:360–369.
50. Ahmed S, Kanakry JA, Ahn KW, et al. Lower graft-versus-host disease and relapse risk in post-transplant cyclophosphamide-based haploidentical versus matched sibling donor reduced-intensity conditioning transplant for Hodgkin lymphoma. *Biol Blood Marrow Transplant*. 2019;25:1859–1868.
51. Battipaglia G, Boumendil A, Labopin M, et al. Unmanipulated haploidentical versus HLA-matched sibling allogeneic hematopoietic stem cell transplantation in relapsed/refractory acute myeloid leukemia: a retrospective study on behalf of the ALWP of the EBMT. *Bone Marrow Transplant*. 2019;54:1499–1510.
52. Sanz J, Galimard JE, Labopin M, et al. Post-transplant cyclophosphamide after matched sibling, unrelated and haploidentical donor transplants in patients with acute myeloid leukemia: a comparative study of the ALWP EBMT. *J Hematol Oncol*. 2020;13:46.
53. Fuchs EJ, O'Donnell PV, Eapen M, et al. Double unrelated umbilical cord blood vs HLA-haploidentical bone marrow transplantation: the BMT CTN 1101 trial. *Blood*. 2021;137(3):420–428.
54. Aversa F, Terenzi A, Tabilio A, et al. Full haplotype-mismatched hematopoietic stem-cell transplantation: a phase II study in patients with acute leukemia at high risk of relapse. *J Clin Oncol*. 2005;23
55. Huang XJ, Liu DH, Liu KY, et al. Haploidentical hematopoietic stem cell transplantation without in vitro T-cell depletion for the treatment of hematological malignancies. *Bone Marrow Transplant*. 2006;38:291–297.
56. McCurdy SR, Kanakry JA, Showel MM, et al. Risk-stratified outcomes of nonmyeloablative HLA-haploidentical BMT with high-dose posttransplantation cyclophosphamide. *Blood*. 2015;125:3024–3031.
57. Bacigalupo A, Dominietto A, Ghiso A, et al. Unmanipulated haploidentical bone marrow transplantation and post-transplant cyclophosphamide for hematologic malignancies following a myeloablative conditioning: an update. *Bone Marrow Transplant*. 2015;50:S37–S39.
58. Kasamon YL, Ambinder RF, Fuchs EJ, et al. Prospective study of nonmyeloablative, HLA-mismatched unrelated BMT with high-dose posttransplantation cyclophosphamide. *Blood Advances*. 2017;1:288–292.
59. Storb R, Deeg HJ, Whitehead J, et al. Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. *N Engl J Med*. 1986;314:729–735.
60. Ratanatharathorn V, Nash RA, Przepiorka D, et al. Phase III study comparing methotrexate and tacrolimus (prograf, FK506) with methotrexate and cyclosporine for graft-versus-host disease prophylaxis after HLA-identical sibling bone marrow transplantation. *Blood*. 1998;92:2303–2314.
61. Nash RA, Antin JH, Karanes C, et al. Phase 3 study comparing methotrexate and tacrolimus with methotrexate and cyclosporine for prophylaxis of acute graft-versus-host disease after marrow transplantation from unrelated donors. *Blood*. 2000;96:2062–2068.
62. Hiraoka A, Ohashi Y, Okamoto S, et al. Phase III study comparing tacrolimus (FK506) with cyclosporine for graft-versus-host disease prophylaxis after allogeneic bone marrow transplantation. *Bone Marrow Transplant*. 2001;28:181–185.
63. D'Souza A, Lee S, Zhu X, et al. Current use and trends in hematopoietic cell transplantation in the United States. *Biol Blood Marrow Transplant*. 2017;23:1417–1421.
64. Chhabra S, Liu Y, Hemmer MT, et al. Comparative analysis of calcineurin inhibitor-based methotrexate and mycophenolate mofetil-containing regimens for prevention of graft-versus-host disease after reduced-intensity conditioning allogeneic transplantation. *Biol Blood Marrow Transplant*. 2019;25:73–85.
65. Kharfan-Dabaja M, Mhaskar R, Reljic T, et al. Mycophenolate mofetil versus methotrexate for prevention of graft-versus-host disease in people receiving allogeneic hematopoietic stem cell transplantation. *Cochrane Database Syst Rev*. 2014;7:CD010280.
66. Penack O, Marchetti M, Ruutu T, et al. Prophylaxis and management of graft versus host disease after stem-cell transplantation for haematological malignancies: updated consensus recommendations of the European Society for Blood and Marrow Transplantation. *Lancet Haematol*. 2020;7:e157–e167.
67. Bacigalupo A, Lamparelli T, Barisione G, et al. Thymoglobulin prevents chronic graft-versus-host disease, chronic lung dysfunction, and late transplant-related mortality: long-term follow-up of a randomized trial in patients undergoing unrelated donor transplantation. *Biol Blood Marrow Transplant*. 2006;12:560–565.
68. Finke J, Bethge WA, Schmoor C, et al. Standard graft-versus-host disease prophylaxis with or without anti-T-cell globulin in haematopoietic cell transplantation from matched unrelated donors: a randomised, open-label, multicentre phase 3 trial. *Lancet Oncol*. 2009;10:855–864.
69. Walker I, Panzarella T, Couban S, et al. Pretreatment with anti-thymocyte globulin versus no anti-thymocyte globulin in patients with haematological malignancies undergoing haemopoietic cell transplantation from unrelated donors: a randomised, controlled, open-label, phase 3, multicentre trial. *Lancet Oncol*. 2016;17:164–173.
70. Soiffer RJ, Kim HT, McGuirk J, et al. Prospective, randomized, double-blind, phase III clinical trial of anti–T-lymphocyte globulin to assess impact on chronic graft-versus-host disease–free survival in patients undergoing HLA-matched unrelated myeloablative hematopoietic cell transplantation. *J Clin Oncol*. 2017;35:4003–4011.
71. Kröger N, Solano C, Wolschke C, et al. Antilymphocyte globulin for prevention of chronic graft-versus-host disease. *N Engl J Med*. 2016; 374:43–53.
72. Admiraal R, Nierkens S, de Witte MA, et al. Association between anti-thymocyte globulin exposure and survival outcomes in adult unrelated haemopoietic cell transplantation: a retrospective, pharmacodynamic cohort analysis. *Lancet Haematol*. 2017;4:e183–e191.
73. Kennedy VE, Chen H, Savani BN, et al. Optimizing anti-thymocyte globulin dosing based on recipient absolute lymphocyte count after unrelated allogeneic hematopoietic cell transplant. *Blood*. 2015;126 4410-4410.
74. Ruggeri A, Labopin M, Bacigalupo A, et al. Post-transplant cyclophosphamide for graft-versus-host disease prophylaxis in HLA matched sibling or matched unrelated donor transplant for patients with acute leukemia, on behalf of ALWP-EBMT. *Hematol Oncol*. 2018;11:40.
75. Robinson TM, O'Donnell PV, Fuchs EJ, et al. Haploidentical bone marrow and stem cell transplantation: experience with post-transplantation cyclophosphamide. *Semin Hematol*. 2016;53:90–97.
76. Kanakry CG, Tsai H-L, Bolaños-Meade J, et al. Single-agent GVHD prophylaxis with posttransplantation cyclophosphamide after myeloablative, HLA-matched BMT for AML, ALL, and MDS. *Blood*. 2014;124:3817–3827.
77. De Jong CN, Meijer E, Bakunina K, et al. Post-transplantation cyclophosphamide after allogeneic hematopoietic stem cell transplantation: results of the prospective randomized HOVON-96 Trial in recipients of matched related and unrelated donors. *Blood*. 2019;134 1-1.
78. Bolaños-Meade J, Reshef R, Fraser R, et al. Three prophylaxis regimens (tacrolimus, mycophenolate mofetil, and cyclophosphamide; tacrolimus, methotrexate, and bortezomib; or tacrolimus, methotrexate, and maraviroc) versus tacrolimus and methotrexate for prevention of graft-versus-host disease with haemopoietic cell transplantation with reduced-intensity conditioning: a randomised phase 2 trial with a non-randomised contemporaneous control group (BMT CTN 1203). *Lancet Haematol*. 2019;6:e132–e143.
79. Gökbuget N, Kneba M, Raff T, et al. Adult patients with acute lymphoblastic leukemia and molecular failure display a poor prognosis and are candidates for stem cell transplantation and targeted therapies. *Blood*. 2012;120:1868–1876.
80. Bassan R, Spinelli O, Oldani E, et al. Improved risk classification for risk-specific therapy based on the molecular study of minimal residual disease (MRD) in adult acute lymphoblastic leukemia (ALL). *Blood*. 2009;113:4153–4162.
81. Olavarria E, Siddique S, Griffiths MJ, et al. Posttransplantation imatinib as a strategy to postpone the requirement for immunotherapy in patients undergoing reduced-intensity allografts for chronic myeloid leukemia. *Blood*. 2007;110:4614–4617.
82. Shimoni A, Volchek Y, Koren-Michowitz M, et al. Phase 1/2 study of nilotinib prophylaxis after allogeneic stem cell transplantation in patients with advanced chronic myeloid leukemia or Philadelphia chromosome–positive acute lymphoblastic leukemia. *Cancer*. 2015;121:863–871.
83. Carpenter PA, Johnston L, Fernandez H, et al. A multicenter Phase I/II study of relapse prophylaxis with nilotinib after hematopoietic cell transplantation (HCT) for high-risk philadelphia chromosome-positive (Ph+) leukemias. *Biol Blood Marrow Transplant*. 2015;21:S274–S276.
84. Brissot E, Labopin M, Beckers MM, et al. Tyrosine kinase inhibitors improve long-term outcome of allogeneic hematopoietic stem cell transplantation for adult patients with Philadelphia chromosome positive acute lymphoblastic leukemia. *Haematologica*. 2015;100:392–399.
85. de Lima M, Oran B, Champlin RE, et al. CC-486 Maintenance after stem cell transplantation in patients with acute myeloid leukemia or myelodysplastic syndromes. *Biol Blood Marrow Transplant*. 2018;24: 2017–2024.

86. Pusic I, Choi J, Fiala MA, et al. Maintenance therapy with decitabine after allogeneic stem cell transplantation for acute myelogenous leukemia and myelodysplastic syndrome. *Biol Blood Marrow Transplant*. 2015;21:1761–1769.
87. Goodyear OC, Dennis M, Jilani NY, et al. Azacitidine augments expansion of regulatory T cells after allogeneic stem cell transplantation in patients with acute myeloid leukemia (AML). *Blood*. 2012;119:3361–3369.
88. Vij R, Le-Rademacher J, Laumann K, et al. A Phase II multicenter study of the addition of azacitidine to reduced-intensity conditioning allogeneic transplant for high-risk myelodysplasia (and older patients with acute myeloid leukemia): results of CALGB 100801 (alliance). *Biol Blood Marrow Transplant*. 2019;25:1984–1992.
89. Kanate AS, Majhail NS, Savani BN, et al. Indications for hematopoietic cell transplantation and immune effector cell therapy: guidelines from the American Society for Transplantation and Cellular Therapy. *Biol Blood Marrow Transplant*. 2020;26:1247–1256.
90. Sorror ML, Maris MB, Storb R, et al. Hematopoietic cell transplantation (HCT)-specific comorbidity index: a new tool for risk assessment before allogeneic HCT. *Blood*. 2005;106:2912–2919.
91. Armand P, Kim HT, Logan BR, et al. Validation and refinement of the Disease Risk index for allogeneic stem cell transplantation. *Blood*. 2014;123:3664–3671.
92. Socié G, Stone JV, Wingard JR, et al. Long-term survival and late deaths after allogeneic bone marrow transplantation. Late effects working committee of the international bone marrow transplant registry. *N Engl J Med*. 1999;341:14–21.
93. Majhail NS, Rizzo JD, Lee SJ, et al. Recommended screening and preventive practices for long-term survivors after hematopoietic cell transplantation. *Bone Marrow Transplantat*. 2012;47:337–341.
94. Hashmi SK, Lee SJ, Savani BN, et al. ASBMT Practice Guidelines Committee survey on long-term follow-up clinics for hematopoietic cell transplant survivors. *Biol Blood Marrow Transplant*. 2018;24:1119–1124.
95. Majhail NS, Farnia SH, Carpenter PA, et al. Indications for autologous and allogeneic hematopoietic cell transplantation: Guidelines from the American Society for Blood and Marrow Transplantation. *Biol Blood Marrow Transplant*. 2015;21:1863–1869.
96. Döhner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*. 2017;129:424–447.
97. Hübel K, Weingart O, Naumann F, et al. Allogeneic stem cell transplant in adult patients with acute myelogenous leukemia: a systematic analysis of international guidelines and recommendations. *Leuk Lymphoma*. 2011;52:444–457.
98. Freeman SD, Virgo P, Couzens S, et al. Prognostic relevance of treatment response measured by flow cytometric residual disease detection in older patients with acute myeloid leukemia. *J Clin Oncol*. 2013;31:4123–4131.
99. Terwijn M, van Putten WL, Kelder A, et al. High prognostic impact of flow cytometric minimal residual disease detection in acute myeloid leukemia: data from the HOVON/SAKK AML 42A study. *J Clin Oncol*. 2013;31:3889–3897.
100. Ivey A, Hills RK, Simpson MA, et al. Assessment of minimal residual disease in standard-risk AML. *N Engl J Med*. 2016;374:422–433.
101. Jongen-Lavrencic M, Grob T, Hanekamp D, et al. Molecular minimal residual disease in acute myeloid leukemia. *N Engl J Med*. 2018;378:1189–1199.
102. Araki D, Wood BL, Othus M, et al. Allogeneic hematopoietic cell transplantation for acute myeloid leukemia: time to move toward a minimal residual disease-based definition of complete remission? *J Clin Oncol*. 2016;34:329–336.
103. Thol F, Gabdoulline R, Liebich A, et al. Measurable residual disease monitoring by NGS before allogeneic hematopoietic cell transplantation in AML. *Blood*. 2018;132:1703–1713.
104. Walter RB, Gooley TA, Wood BL, et al. Impact of pretransplantation minimal residual disease, as detected by multiparametric flow cytometry, on outcome of myeloablative hematopoietic cell transplantation for acute myeloid leukemia. *J Clin Oncol*. 2011;29:1190–1197.
105. Zhou Y, Othus M, Araki D, et al. Pre- and post-transplant quantification of measurable ("minimal") residual disease via multiparameter flow cytometry in adult acute myeloid leukemia. *Leukemia*. 2016;30:1456–1464.
106. Buckley SA, Wood BL, Othus M, et al. Minimal residual disease prior to allogeneic hematopoietic cell transplantation in acute myeloid leukemia: a meta-analysis. *Haematologica*. 2017;102:865–873.
107. Hourigan CS, Dillon LW, Gui G, et al. Impact of conditioning intensity of allogeneic transplantation for acute myeloid leukemia with genomic evidence of residual disease. *J Clin Oncol*. 2020;38:1273–1283.
108. Goldstone AH, Richards SM, Lazarus HM, et al. In adults with standard-risk acute lymphoblastic leukemia, the greatest benefit is achieved from a matched sibling allogeneic transplantation in first complete remission, and an autologous transplantation is less effective than conventional consolidation/maintenance chemotherapy in all patients: final results of the International ALL Trial (MRC UKALL XII/ECOG E2993). *Blood*. 2008;111:1827–1833.
109. Pidala J, Djulbegovic B, Anasetti C, et al. Allogeneic hematopoietic cell transplantation for adult acute lymphoblastic leukemia (ALL) in first complete remission. *Cochrane Database Syst Rev*. 2011;2011(10):CD008818 Oct 5.
110. Gupta V, Richards S, Rowe J. Allogeneic, but not autologous, hematopoietic cell transplantation improves survival only among younger adults with acute lymphoblastic leukemia in first remission: an individual patient data meta-analysis. *Blood*. 2013;121:339–350.
111. DeFilipp Z, Advani AS, Bachanova V, et al. Hematopoietic cell transplantation in the treatment of adult acute lymphoblastic leukemia: updated 2019 evidence-based review from the American society for transplantation and cellular therapy. *Biol Blood Marrow Transplant*. 2019;25:2113–2123.
112. Gökbuget N, Dombret H, Bonifacio M, et al. Blinatumomab for minimal residual disease in adults with B-cell precursor acute lymphoblastic leukemia. *Blood*. 2018;131:1522–1531.
113. Giebel S, Marks DI, Boissel N, et al. Hematopoietic stem cell transplantation for adults with Philadelphia chromosome-negative acute lymphoblastic leukemia in first remission: a position statement of the European Working Group for Adult Acute Lymphoblastic Leukemia (EWALL) and the Acute Leukemia Working Party of the European Society for Blood and Marrow Transplantation (EBMT). *Bone Marrow Transplant*. 2019;54:798–809.
114. Maude SL, Laetsch TW, Buechner J, et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *N Engl J Med*. 2018;378:439–448.
115. Duarte RF, Labopin M, Bader P, et al. Indications for haematopoietic stem cell transplantation for haematological diseases, solid tumours and immune disorders: current practice in Europe, 2019. *Bone Marrow Transplant*. 2019;54:1525–1552.
116. Radich JP, Deininger M, Abboud CN, et al. Chronic Myeloid Leukemia, Version 1.2019, NCCN clinical practice guidelines in oncology. *J Natl Compr Canc Netw*. 2018;16:1108–1135.
117. Hochhaus A, Baccarani M, Silver RT, et al. European LeukemiaNet 2020 recommendations for treating chronic myeloid leukemia. *Leukemia*. 2020;34:966–984.
118. Lübking A, Dreimane A, Sandin F, et al. Allogeneic stem cell transplantation for chronic myeloid leukemia in the TKI era: population-based data from the Swedish CML registry. *Bone Marrow Transplant*. 2019; 54:1764–1774.
119. Chhabra S, Ahn KW, Hu ZH, et al. Myeloablative vs reduced-intensity conditioning allogeneic hematopoietic cell transplantation for chronic myeloid leukemia. *Blood Adv*. 2018;2:2922–2936.
120. Dreger P, Corradini P, Kimby E, et al. Indications for allogeneic stem cell transplantation in chronic lymphocytic leukemia: the EBMT transplant consensus. *Leukemia*. 2007;21:12–17.
121. Kharfan-Dabaja MA, Kumar A, Hamadani M, et al. Clinical practice recommendations for use of allogeneic hematopoietic cell transplantation in chronic lymphocytic leukemia on behalf of the guidelines committee of the American society for blood and marrow transplantation. *Biol Blood Marrow Transplant*. 2016;22:2117–2125.
122. Neelapu SS, Locke FL, Bartlett NL, et al. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. *N Engl J Med*. 2017; 377:2531–2544.
123. Schuster SJ, Svoboda J, Chong EA, et al. Chimeric antigen receptor T cells in refractory B-cell lymphomas. *N Engl J Med*. 2017;377:2545–2554.
124. Zelenetz AD, Wierda WG, Abramson JS, et al. Non-Hodgkin's lymphomas, version 1.2013. *J Natl Compr Canc Netw*. 2013;11:257–272. quiz 273.
125. Dreger P, Fenske TS, Montoto S, et al. Cellular immunotherapy for refractory diffuse large B cell lymphoma in the chimeric antigen receptor-engineered T cell era: still a role for allogeneic transplantation? *Biol Blood Marrow Transplant*. 2020;26:e77–e85.
126. Jain T, Bar M, Kansagra AJ, et al. Use of chimeric antigen receptor T cell therapy in clinical practice for relapsed/refractory aggressive B cell non-Hodgkin lymphoma: an expert panel opinion from the American Society for Transplantation and Cellular Therapy. *Biol Blood Marrow Transplant*. 2019;25:2305–2321.
127. Ghosh N, Ahmed S, Ahn KW, et al. Association of reduced-intensity conditioning regimens with overall survival among patients with non-Hodgkin lymphoma undergoing allogeneic transplant. *JAMA Oncol*. 2020;6(7):1011–1018.
128. Smith SM, Godfrey J, Ahn KW, et al. Autologous transplantation versus allogeneic transplantation in patients with follicular lymphoma experiencing early treatment failure. *Cancer*. 2018;124:2541–2551.

129. Casulo C, Friedberg JW, Ahn KW, et al. Autologous transplantation in follicular lymphoma with early therapy failure: a National LymphoCare Study and Center for International Blood and Marrow Transplant Research analysis. *Biol Blood Marrow Transplant*. 2018;24:1163–1171.
130. Dreyling M, Lenz G, Hoster E, et al. Early consolidation by myeloablative radiochemotherapy followed by autologous stem cell transplantation in first remission significantly prolongs progression-free survival in mantle-cell lymphoma: results of a prospective randomized trial of the European MCL Network. *Blood*. 2005;105:2677–2684.
131. Wang M, Munoz J, Goy A, et al. KTE-X19 CAR T-cell therapy in relapsed or refractory mantle-cell lymphoma. *N Engl J Med*. 2020;382:1331–1342.
132. Zelenetz AD, Gordon LI, Abramson JS, et al. NCCN Guidelines Insights: B-cell lymphomas, Version 3.2019. *J Natl Compr Canc Netw*. 2019;17:650.
133. Kharfan-Dabaja MA, Kumar A, Ayala E, et al. Clinical practice recommendations on indication and timing of hematopoietic cell transplantation in mature T cell and NK/T cell lymphomas: an international collaborative effort on behalf of the guidelines committee of the American Society for Blood and Marrow Transplantation. *Biol Blood Marrow Transplant*. 2017;23:1826–1838.
134. Yhim H-Y, Kim JS, Mun Y-C, et al. Clinical outcomes and prognostic factors of up-front Autologous stem cell transplantation in patients with extranodal natural killer/T cell lymphoma. *Biol Blood Marrow Transplant*. 2015;21:1597–1604.
135. d'Amore F, Relander T, Lauritzsen GF, et al. Up-front autologous stem-cell transplantation in peripheral T-cell lymphoma: NLG-T-01. *J Clin Oncol*. 2012;30:3093–3099.
136. Kanate AS, DiGilio A, Ahn KW, et al. Allogeneic haematopoietic cell transplantation for extranodal natural killer/T-cell lymphoma, nasal type: a CIBMTR analysis. *Br J Haematol*. 2018;182:916–920.
137. Hamadani M, Kanate AS, DiGilio A, et al. Allogeneic hematopoietic cell transplantation for aggressive NK cell leukemia. A Center for International Blood and Marrow Transplant Research Analysis. *Biol Blood Marrow Transplant*. 2017;23:853–856.
138. Epperla N, Ahn KW, Litovich C, et al. Allogeneic hematopoietic cell transplantation provides effective salvage despite refractory disease or failed prior autologous transplant in angioimmunoblastic T-cell lymphoma: a CIBMTR analysis. *J Hematol Oncol*. 2019;12:6.
139. Tanase A, Schmitz N, Stein H, et al. Allogeneic and autologous stem cell transplantation for hepatosplenic T-cell lymphoma: a retrospective study of the EBMT Lymphoma Working Party. *Leukemia*. 2015;29:686–688.
140. Utsunomiya A, Miyazaki Y, Takatsuka Y, et al. Improved outcome of adult T cell leukemia/lymphoma with allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant*. 2001;27:15–20.
141. Hishizawa M, Kanda J, Utsunomiya A, et al. Transplantation of allogeneic hematopoietic stem cells for adult T-cell leukemia: a nationwide retrospective study. *Blood*. 2010;116:1369–1376.
142. Bazarbachi A, Cwynarski K, Boumendil A, et al. Outcome of patients with HTLV-1-associated adult T-cell leukemia/lymphoma after SCT: a retrospective study by the EBMT LWP. *Bone Marrow Transplant*. 2014;49:1266–1268.
143. Duarte RF, Boumendil A, Onida F, et al. Long-term outcome of allogeneic hematopoietic cell transplantation for patients with mycosis fungoides and Sézary syndrome: a European Society for Blood and Marrow Transplantation Lymphoma Working Party extended analysis. *J Clin Oncol*. 2014;32:3347–3348.
144. Smith SM, Burns LJ, van Besien K, et al. Hematopoietic cell transplantation for systemic mature T-cell non-Hodgkin lymphoma. *J Clin Oncol*. 2013; 31:3100–3109.
145. Younes A, Gopal AK, Smith SE, et al. Results of a pivotal phase II study of brentuximab vedotin for patients with relapsed or refractory Hodgkin's lymphoma. *J Clin Oncol*. 2012;30:2183–2189.
146. Armand P, Engert A, Younes A, et al. Nivolumab for relapsed/refractory classic Hodgkin lymphoma after failure of autologous hematopoietic cell transplantation: extended follow-up of the multicohort single-arm phase II checkmate 205 trial. *J Clin Oncol*. 2018;36:1428–1439.
147. Chen R, Zinzani PL, Lee HJ, et al. Pembrolizumab in relapsed or refractory Hodgkin lymphoma: 2-year follow-up of KEYNOTE-087. *Blood*. 2019;134:1144–1153.
148. Sarina B, Castagna L, Farina L, et al. Allogeneic transplantation improves the overall and progression-free survival of Hodgkin lymphoma patients relapsing after autologous transplantation: a retrospective study based on the time of HLA typing and donor availability. *Blood*. 2010;115:3671–3677.
149. Ahmed S, Ghosh N, Ahn KW, et al. Impact of type of reduced-intensity conditioning regimen on the outcomes of allogeneic haematopoietic cell transplantation in classical Hodgkin lymphoma. *Br J Haematol*. 2020;190(4):573–582.
150. Richard TH, Ranjana HA, Weiyun ZA, et al. NCCN Guidelines Insights: Hodgkin lymphoma, Version 1.2018. *J Natl Compr Canc Netw*. 2018;16:245–254.
151. Dhakal B, Szabo A, Chhabra S, et al. Autologous transplantation for newly diagnosed multiple myeloma in the era of novel agent induction: a systematic review and meta-analysis. *JAMA Oncol*. 2018;4:343–350.
152. Kumar SK, Callander NS, Alsina M, et al. NCCN Guidelines Insights: Multiple myeloma, Version 3.2018. *J Natl Compr Canc Netw*. 2018;16:11.
153. Bruno B, Rotta M, Patriarca F, et al. A comparison of allografting with autografting for newly diagnosed myeloma. *N Engl J Med*. 2007;356: 1110–1120.
154. Krishnan A, Pasquini MC, Logan B, et al. Autologous haemopoietic stem-cell transplantation followed by allogeneic or autologous haemopoietic stem-cell transplantation in patients with multiple myeloma (BMT CTN 0102): a phase 3 biological assignment trial. *Lancet Oncol*. 2011;12:1195–1203.
155. Gahrton G, Iacobelli S, Bjorkstrand B, et al. Autologous/reduced-intensity allogeneic stem cell transplantation vs autologous transplantation in multiple myeloma: long-term results of the EBMT-NMAM2000 study. *Blood*. 2013;121:5055–5063.
156. Costa LJ, Iacobelli S, Pasquini MC, et al. Long-term survival of 1338 MM patients treated with tandem autologous vs. autologous-allogeneic transplantation. *Bone Marrow Transplant*. 2020;55(9):1810–1816.
157. Shah N, Callander N, Ganguly S, et al. Hematopoietic stem cell transplantation for multiple myeloma: guidelines from the American society for blood and marrow transplantation. *Biol Blood Marrow Transplant*. 2015;21:1155–1166.
158. Freytes CO, Vesole DH, LeRademacher J, et al. Second transplants for multiple myeloma relapsing after a previous autotransplant-reduced-intensity allogeneic vs autologous transplantation. *Bone Marrow Transplant*. 2014;49:416–421.
159. Chhabra S, Szabo A, Glisch C, et al. Relapse after allogeneic hematopoietic cell transplantation for multiple myeloma: survival outcomes and factors influencing them. *Biol Blood Marrow Transplant*. 2020;26(7):1288–1297.
160. Htut M, D'Souza A, Krishnan A, et al. Autologous/allogeneic hematopoietic cell transplantation versus tandem autologous transplantation for multiple myeloma: comparison of long-term postrelapse Survival. *Biol Blood Marrow Transplant*. 2018;24:478–485.
161. Lim Z, Brand R, Martino R, et al. Allogeneic hematopoietic stem-cell transplantation for patients 50 years or older with myelodysplastic syndromes or secondary acute myeloid leukemia. *J Clin Oncol*. 2010;28:405–411.
162. Aoudjhane M, Labopin M, Gorin NC, et al. Comparative outcome of reduced intensity and myeloablative conditioning regimen in HLA identical sibling allogeneic haematopoietic stem cell transplantation for patients older than 50 years of age with acute myeloblastic leukaemia: a retrospective survey from the Acute Leukemia Working Party (ALWP) of the European Group for Blood and Marrow Transplantation (EBMT). *Leukemia*. 2005;19:2304–2312.
163. Crawley C, Iacobelli S, Björkstrand B, et al. Reduced-intensity conditioning for myeloma: lower nonrelapse mortality but higher relapse rates compared with myeloablative conditioning. *Blood*. 2007;109:3588–3594.
164. Martino R, Iacobelli S, Brand R, et al. Retrospective comparison of reduced-intensity conditioning and conventional high-dose conditioning for allogeneic hematopoietic stem cell transplantation using HLA-identical sibling donors in myelodysplastic syndromes. *Blood*. 2006;108:836–846.
165. Dreger P, Brand R, Milligan D, et al. Reduced-intensity conditioning lowers treatment-related mortality of allogeneic stem cell transplantation for chronic lymphocytic leukemia: a population-matched analysis. *Leukemia*. 2005;19:1029–1033.
166. Sureda A, Robinson S, Canals C, et al. Reduced-intensity conditioning compared with conventional allogeneic stem-cell transplantation in relapsed or refractory Hodgkin's lymphoma: an analysis from the Lymphoma Working Party of the European Group for Blood and Marrow Transplantation. *J Clin Oncol*. 2008;26:455–462.
167. Hari P, Carreras J, Zhang MJ, et al. Allogeneic transplants in follicular lymphoma: higher risk of disease progression after reduced-intensity compared to myeloablative conditioning. *Biol Blood Marrow Transplant*. 2008;14:236–245.
168. Mohty M, Labopin M, Volin L, et al. Reduced-intensity versus conventional myeloablative conditioning allogeneic stem cell transplantation for patients with acute lymphoblastic leukemia: a retrospective study from the European Group for Blood and Marrow Transplantation. *Blood*. 2010;116:4439–4443.
169. Ringdén O, Labopin M, Ehninger G, et al. Reduced intensity conditioning compared with myeloablative conditioning using unrelated donor transplants in patients with acute myeloid leukemia. *J Clin Oncol*. 2009;27:4570–4577.
170. Luger SM, Ringdén O, Zhang MJ, et al. Similar outcomes using myeloablative vs reduced-intensity allogeneic transplant preparative regimens for AML or MDS. *Bone Marrow Transplant*. 2012;47:203–211.

CHAPTER 106

UNRELATED DONOR HEMATOPOIETIC CELL TRANSPLANTATION

Effie Wang Petersdorf and Katharine Hsu

The outcomes of unrelated donor hematopoietic cell transplantation (HCT) have greatly improved as a result of a better understanding of the diversity of human leukocyte antigen (HLA) and killer-cell immunoglobulin-like receptor (KIR) genes. The HLA and KIR genetic systems regulate the transplantation barrier. Clinical outcomes after unrelated donor transplantation can be achieved with donor matching for the highly polymorphic HLA loci and through consideration of donor KIR genes. When HLA disparity cannot be avoided, judicious selection of a donor with the fewest HLA mismatches and avoidance of certain loci may provide patients with the opportunity for life-saving transplantation. The disease stage remains a strong predictor of overall transplant outcome, and expediency in timing of transplantation for patients with high-risk disease is paramount. New research avenues include the identification of major histocompatibility complex (MHC) and KIR genetic variation that may contribute to risks of graft-versus-host disease (GVHD) and relapse. This chapter chronicles the role of HLA and KIR genes in unrelated donor HCT.

THE HUMAN LEUKOCYTE ANTIGEN AND KILLER-CELL IMMUNOGLOBULIN-LIKE RECEPTOR GENETIC SYSTEMS

Genetics of the Human Leukocyte Antigen Complex

The advent of molecular techniques has made possible the definition of unique sequence variants (alleles) of HLA molecules that are recognized by an antibody (Table 106.1). Polymorphism ensures that a large array of foreign peptides can be presented to the immune system by HLA molecules. As of May 2020, a total of 26,887 alleles are recognized.[1] HLA nomenclature accommodates the steady discovery of new human variants and has no limits on the number of novel variants. The HLA prefix is followed by a hyphen and the gene name (e.g., HLA-A). An asterisk separates the gene from the unique sequence (HLA-A*). The unique sequence name embodies up to four kinds of information, each delimited by a colon. The first set of numbers after the asterisk and before the first colon correspond to the serologic antigen equivalent (e.g., HLA-A*02 refers to sequences of the HLA-A2 antigen family). The second set of numbers provides the unique protein that corresponds to the subtype (HLA-A*02:101). The third set of numbers indicates synonymous substitutions (HLA-A*02:101:01). The last series of numbers give information on noncoding variation often denoting expression (HLA-A*02:101:01:02 N). Letter suffixes are used to denote alleles that are not expressed (also known as "null," N), low cell surface expression (L), a soluble secreted molecule not present on the surface of the cell (S), a cytoplasmic product not expressed on the cell surface (C), a protein with aberrant expression (A), and a sequence of questionable expression (Q).

If HLA alleles can be expressed in any combination and if the inheritance of alleles were random, then the total estimated number of possible five-locus HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1 genotypes would be exceedingly high, and the chances of identifying a fully matched donor would be very low. Clinical experience demonstrates that donor identification is successful and is due to the underlying genetic hallmark of the MHC, linkage disequilibrium (LD). LD refers to the observation that HLA alleles are found in association with each other at an observed frequency that exceeds their expected frequency. The probability of identifying a matched donor for a given patient is higher when the patient and donor share a similar ethnic background. HLA gene and haplotype frequencies provide important data for estimating optimal registry size and composition.[2] Given the polymorphism of allele sequences that encompass variants of a single serologically defined antigen, it is not surprising that antigen-matched donor and patient pairs may differ by their alleles.

HUMAN LEUKOCYTE ANTIGEN TYPING METHODS

The development of standardized typing methods and nomenclature for HLA genes have been facilitated by a series of 17 international histocompatibility workshops (Table 106.2). The terms "low-resolution," "intermediate-resolution", and "high-resolution" are a reflection of the evolution of DNA methods and their equivalency to serologically defined specificities. The term "6/6" match refers to recipients and donors who share the same low-resolution-defined HLA-A, HLA-B, and HLA-DR genes. The term "8/8" refers to high-resolution matching at the four loci HLA-A, HLA-B, HLA-C, and HLA-DRB1; "10/10" to the addition of HLA-DQB1, and "12/12" to the addition of HLA-DPB1.

Next-generation sequencing (NGS) platforms are the most current sequencing methodology which provide not only "ultra-high resolution" of HLA alleles (typing of the gene beyond its coding regions) but also short-range phasing of exons 2, 3, and 4 of class I genes, and of exons 2 and 3 of class II genes.[3,4] NGS has the capability of linking sequences across exons that substantially reduces ambiguities of allele combinations.[5,6] NGS approaches for HLA typing are supported by software for automated allele assignment. Because many samples may be tested simultaneously, NGS is a cost-effective typing method for typing donors recruited into registries.[4]

GENETICS OF THE KILLER-CELL IMMUNOGLOBULIN-LIKE RECEPTORS SYSTEM

Recognition that natural killer (NK) cells engage HLA, leading to the capacity to discern self from non-self, has generated interest in the NK receptors responsible for HLA binding (Table 106.3). Located on chromosome 19, the KIR gene region is similar to the HLA gene complex in that it is also polygenic and polymorphic; however, unlike the HLA genes, whose loci are conserved across all individuals, KIR genes frequently differ from individual to individual. As few as eight and as many as 14 different KIR genes and pseudogenes may be found on one haplotype, with some genes existing more than once.[7] KIR genes encode two broad categories of receptors: mmunoreceptor tyrosine-based inhibition motif (ITIM)-containing inhibitory receptors and mmunoreceptor tyrosine-based activation motif (ITAM)-binding activating receptors. Nomenclature for the KIR genes is structurally inspired: the first two characters (2D or 3D) refer to the number of extracellular Ig-like domains characterizing the receptor; the third character (L or S) refers to the "long" inhibitory or "short" activating cytoplasmic tail of the receptor, and the last character (numbering 1 through 5) differentiates receptors with similar numbers of extracellular domains and cytoplasmic tail length. The inhibitory receptors

TABLE 106.1 Common Definitions in Human Leukocyte Antigen and Killer-Cell Immunoglobulin-Like Receptor Genetics

Term	Definition	Example
Allele	Unique sequence of an HLA gene defined by molecular methods	DRB1*04:01 allele is a unique sequence defined as DR4 by serologic methods
Antigen	Antibody-defined protein	DR4 antigen is a serologically defined protein product of an HLA gene
Antigen matched	HLA-B*44	HLA-B*44
Antigen mismatched	HLA-B*44	HLA-B*27
Antigen matched and allele matched	HLA-B*44:02	HLA-B*44:02
Antigen matched but allele mismatched	HLA-B*44:03	HLA-B*44:02
HLA haplotype	HLA genes inherited as a chromosomal unit	HLA-A1, HLA-B8, HLA-DR3 are common haplotypes among White populations
HLA genotype	Molecularly defined HLA allele or sequence	Genotypically matched donor and recipient are identical for the HLA alleles at a given HLA gene (e.g., HLA-DRB1*04:01)
HLA phenotype	Serologically defined HLA protein or antigen	Phenotypically matched donor and recipient share the same HLA antigen (e.g., HLA-DR4)
KIR haplotype	KIR genes inherited as a chromosomal unit	KIR2DL3, KIR2DL1, KIR3DL1, and KIR2DS4 are present together on a common haplotype
KIR genotype	Repertoire of KIR genes present in an individual	
KIR phenotype	Cell surface expression of KIR receptors within the NK population, both at the level of frequency of expression and density of expression	KIR2DL1 alleles are expressed at different frequencies within the NK population and at different cell surface densities on an individual NK cell
KIR ligand	Molecule binding the KIR receptor	HLA alleles belonging to the HLA-Bw4, HLA-C1, and HLA-C2 groups
KIR ligand incompatibility	Dissimilar KIR ligands in the HCT recipient and donor	Donor has HLA-Bw4, C1, and C2 KIR ligands, but the recipient has HLA-C1 only
Missing self	HCT recipient lacking KIR ligand present in the donor.	Donor has HLA-Bw4, C1, and C2 KIR ligands, but recipient lacks HLA-C2
Missing KIR ligand	Individual having the inhibitory KIR receptor without the ligand	Individual is positive for KIR2DL1 but negative for HLA-C2
NK education	Capacity for effector response against missing self. Dictated by interaction between inhibitory KIR and its ligand.	NK cells expressing KIR3DL1 in an HLA-Bw4+ individual are educated for response.

HLA, Human leukocyte antigen; *KIR*, killer-cell immunoglobulin-like receptor; *NK*, natural killer.

recognize HLA class I ligands, and interaction between the receptor and its cognate HLA ligand is responsible for establishing the response threshold upon encountering a putative target. It is in this way that a KIR-expressing NK cell becomes educated to behave indifferently toward cells bearing self-HLA ligands and aggressively toward cells that have lost or altered HLA expression.

The KIR genes are arranged neatly in a head-to-tail manner within a highly organized cluster with prototypic gene order, leading to LD between pairs and clusters of KIR genes and alleles.[7–9] The so-called anchor genes and pseudogenes, recognized for their distinctive positions on nearly all identified KIR haplotypes, demarcate the beginning, middle and end of the KIR gene cluster. KIR3DL3 and KIR3DL2 flank the KIR genetic region, while KIR3DP marks the end of the centromeric portion and KIR2DL4 marks the beginning of the telomeric portion of the haplotype.[7,10,11] The centromeric and telomeric portions appear to have evolved and diversified separately due to reproductive and pathogenic pressures,[12] and the gene content distinctive to each region may have clinical relevance in HCT.[13]

Population studies of KIR genotypes identified the presence of two major KIR haplotype groups, the A-haplotype and the B-haplotype,[10,11] where, apart from the anchor genes, the canonical A-haplotype is comprised of the genes KIR2DL3, -2DL1, -3DL1, and -2DS4. By contrast, B haplotypes are more variable in gene content, exhibiting more than one activating KIR gene—KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, and KIR3DS1—in the centromeric and/or telomeric regions.[14]

Allelic polymorphism of each KIR locus lends additional diversity to the genetic region, with new alleles reported regularly.[15] The loci encoding inhibitory KIR receptors are the most diverse, with 183 KIR3DL1, 165 KIR3DL3, 164 KIR3DL2, 111 KIR2DL1, 64 KIR2DL3, and 34 KIR2DL2 alleles reported as of March 2020. KIR allele sequences are named in an analogous fashion to HLA.[16] An asterisk separates the KIR gene name (e.g., KIR2DL1) from the numeric allele sequence (KIR2DL1*). The first three digits following the asterisk indicate alleles that encode different protein sequences (KIR2DL1*003), followed by two digits that distinguish alleles whose DNA differences within the coding sequence are synonymous (KIR2DL1*00302). The last 2-digit field identifies alleles that differ by DNA substitutions in non-coding regions (KIR2DL1*0030202). For some KIR loci (e.g., KIR3DL1 and KIR2DL1), alleles can be grouped into subtypes based on shared sequences that distinguish them based on cell surface expression or function.[17–19]

TABLE 106.2 International Histocompatibility Workshops and Conferences

Workshop	Year	Chairman	Venue	Advances	
1st	1964	D.B. Amos	Durham, NC	Definition of “Hu-L,” “LA,” and “Four” antigen specificities	
2nd	1965	J.J. Van Rood	Leiden, The Netherlands	MLC testing	
3rd	1967	R. Ceppellini	Turin, Italy	Family studies HLA in renal transplantation	
4th	1970	P. Terasaki	Los Angeles, CA	Definition of 27 HLA-A, HLA-B, and HLA-C specificities	
5th	1972	J. Dausset	Evian, France	Worldwide typing of 49 populations	
6th	1975	F. Kissmeyer	Aarhus, Denmark	Description of Dw specificities, Nielsen	
7th	1977	W. Bodmer	Oxford, England	Definition of DR1-7 specificitiesHTC testing	
8th	1980	P. Terasaki	Los Angeles, CA	Definition of HLA-MB (DQ) MT (DR52/53) HLA in renal transplantation and disease association	
9th	1984	E. Albert W. Mayr	Munich, Germany Vienna, Austria	New class I and II specificities HLA class II in renal transplantation	
10th	1987	B. Dupont	Scanticon, NJ New York, NY	Establishment of RFLP/T cell clones and HTC methods Creation of panel of homozygous cell lines	
11th	1991	T. Sasazuki K. Tsuji	Yokohama, Japan	HLA class I PCR typing anthropology	
12th	1996	D. Charron	Saint-Malo, France Paris, France	Sequencing Class I DNA typing/HLA in medicine	
13th	2002	J. Hansen	Seattle, WA Victoria, British Columbia, Canada	Virtual DNA analysis Identification of SNP markers HLA in anthropology, disease association, HCT	http://www.ihwg.org
14th	2005	J. McCluskey	Melbourne, Australia	MHC and anthropology, disease, infection, HCT, cancer nonclassic genes, NK-KIR, cytokine genes	http://www.ihwg.org
15th	2008	M. Gerbase and M.-E. Moraes	Buzios, Brazil	Brazil population studies, bioinformatics tools	
16th	2012	S.G.E. Marsh and D. Middleton	Liverpool, UK	Population-based alleles and haplotypes; next-generation sequencing tools	
17th	2017	M. Fernandez-Vina	Stanford, CA	Next-generation sequencing	
18th	2022	E. Speirings and S. Heidt	Amsterdam, NL	Next-generation sequencing; population studies	

HCT, Hematopoietic cell transplantation; *HLA*, human leukocyte antigen; *HTC*, homozygous typing cells; *KIR*, killer immunoglobulin-like receptor; *MHC*, major histocompatibility complex; *MLC*, mixed lymphocyte culture; *NK*, natural killer; *PCR*, polymerase chain reaction; *RFLP*, restriction fragment length polymorphism; *SNP*, single nucleotide polymorphism.

TABLE 106.3 Donor Killer-Cell Immunoglobulin-Like Receptor Models

		Examples	
Definition	**Donor-Recipient HLA Match**	**Donor**	**Recipient**
Missing self	Mismatched	KIR3DL1 + HLA-Bw4	Lacking HLA-Bw4
Missing ligand	Matched or mismatched	KIR2DL1 + HLA-C1/C1	HLA-C1/C1(missing HLA-C2)
KIR haplotype-B	N/A	KIR haplotype-B members (activating KIR-rich)	N/A
KIR2DS1 + HLA-C1	Matched	KIR2DS1 + HLA-C1	HLA-C1
KIR3DL1 inhibition	Matched	KIR3DL1-H + HLA-Bw4-80T	HLA-Bw4-80T

HLA, Human leukocyte antigen; *KIR*, killer-cell immunoglobulin-like receptor.

KILLER-CELL IMMUNOGLOBULIN-LIKE RECEPTORS TYPING METHODS

Identification of KIR genotypes can be performed at the resolution of the KIR gene, subtype, and allele by a number of different molecular approaches. Currently, polymerase chain reaction (PCR) sequence-specific primers (SSP) and sequence-specific oligonucleotide probes (SSOP) are employed by clinical laboratories for KIR gene content. For research purposes, PCR-SSP is used to identify subtypes and some alleles.[8,20,21] Whole-genome sequencing and RNA-seq databases are rich resources for algorithmic extraction of KIR gene content and copy number.[22,23] More recently, NGS has emerged as a powerful research tool for KIR gene and allele discrimination, with the anticipation of more widespread use as evidence for the clinical importance of KIR genotyping increases.[24]

DONOR IDENTIFICATION AND LIKELIHOOD OF TRANSPLANTATION

The field of unrelated donor HCT has witnessed rapid growth over the past 30 years. Among all allogeneic transplantations performed in the US, the number of transplants from unrelated donors exceeds that from related donors since 2006.[25] The primary indications for unrelated donor HCT are acute myeloid leukemia (AML), myelodysplastic syndrome/myeloproliferative disorders (MDS/MPD), acute lymphoblastic leukemia (ALL), non-Hodgkin lymphoma (NHL), and other nonmalignant disorders. Starting in 2005, patients between 60 and 69 years of age comprised the fastest-growing group of transplant recipients as a result of the introduction of non-myeloablative and reduced-intensity conditioning regimens. Since 2011, patients at the age of 70 or older are the fastest-growing group of allogeneic transplant recipients.

Unrelated HCT has been made feasible by the establishment of registries of volunteer donors worldwide. The Anthony Nolan Appeal was the first effort to demonstrate the feasibility of donor recruitment. Now known as the Anthony Nolan Research Institute, this registry was the first to promote access to HLA-matched bone marrow (BM) donors for patients around the world. In the United States, early efforts for donor recruitment were spearheaded by individual centers. The growing interest in unrelated donor HCT led the US Congress to authorize the creation of a national registry comprising a network of donor centers, transplant centers, and a national coordinating center through the Transplant Act of 1984. Two years later, a federal contract to establish a national registry was awarded to the National Marrow Donor Program (NMDP). In the Netherlands, Professor Jon J. van Rood led the Europdonor Foundation in the collection of HLA data from donor registries around the world and the formation of a database of HLA phenotypes known as Bone Marrow Donors Worldwide (BMDW).[25] Continued growth in registry size worldwide has increased the chances that well-matched donors can be identified. Today, more than 37 million donors are available through international cooperative agreements with registries around the world.[25] The likelihood of identifying an HLA-A, -B, -C, -DRB1-matched (HLA-8/8) unrelated donor depends on the patient's race and ethnicity, with higher rates in patients of White European descent than other ethnicities. In the Japanese population, the probability of identifying an HLA-8/8 match increases as registry size increases but is predicted to reach a limit due to population-specific allele and haplotype frequencies.[26] Strategies to estimate the likelihood of any given patient's success in identifying HLA-matched donors take into account the patient's race and ethnicity. When criteria are relaxed to permit mismatching, over 98% of patients of European descent and over 80% of patients of non-European origin have HLA-7/8 donors.[27] Use of cord blood can further extend access to transplantation.[28]

The identification of HLA-matched donors is only one potential barrier to transplantation for patients. Donor availability, donor health issues, donor race, delays due to coordination, and deterioration of patient health may also lower the chances that a patient can undergo transplantation (Box 106.1).[29] Identification of primary and backup donors allows patients to reach transplantation and improves the overall survival of patients with good performance status, compared to not having a suitable donor.[30]

HUMAN LEUKOCYTE ANTIGEN CRITERIA

Selection of unrelated donors includes consideration for the degree of HLA matching and the presence of recipient anti-donor HLA antibodies, which place the patient at high risk for non-engraftment in the setting of HLA mismatching.[31] Evaluation of unrelated donors includes a health screening history, physical examination, and laboratory testing for risks associated with transmissible elements with a special focus on hepatitis B, hepatitis C, human immunodeficiency virus (HIV 1 and 2), human T-cell lymphotrophic virus I and II, malaria, West Nile virus, transmissible spongiform encephalitis (Creutzfeldt-Jacob disease), Chagas disease, *Treponema pallidum*, and cytomegalovirus (CMV).[32] This core screening is supplemented with an infectious disease health questionnaire screening with novel epidemics including Zika risk areas and COVID-19 exposure.[25] Increased awareness for the health and safety of the unrelated donor has led to the establishment of standards for donation.[25,33]

A large-scale registry study demonstrates that donor HLA match status and donor age are the two most important determinants of transplant survivorship.[34] Two-year survival was 3% better when a donor 10 years or younger is selected. These data demonstrate that transplant centers have the flexibility in donor selection, with the prioritization of HLA-matched young donors where possible (see Box 106.1).

HUMAN LEUKOCYTE ANTIGEN VECTOR OF MISMATCHING

The "vector" or "direction" of HLA compatibility between a donor and a recipient has biologic relevance in risks of graft failure and GVHD and applies to all classical HLA loci. The presence of donor

BOX 106.1 Clinical Relevance to Reaching Transplantation from the Initiation of an Unrelated Donor Search: Patient and Donor Factors[a]

Outcome	Patient Factors	Donor Factors
Probability of the patient reaching transplantation	Increased age[a]	HLA-A, B, C, DRB1, DQB1, DPB1 match status[b]
	Advanced disease risk/urgency of HCT[c]	Availability of high-resolution typing at search
Probability of patient survival	Karnofsky Performance Status (KPS)[a]	Availability/attrition[d]
		Increased age

[a] Pidala J, Kim J, Schell M, et al. Race/ethnicity affects the probability of finding an HLA-A, -B, -C and -DRB1 allele-matched unrelated donor and likelihood of subsequent transplant utilization. *Bone Marrow Transplant.* 2013;48:346–350.

[b] Donor HLA matching remains the most significant risk factor for transplant outcomes.

[c] Disease risk as categorized by the Center for International Blood and Marrow Transplantation Research (CIBMTR).

[d] Identifying the primary donor with a back-up will give flexibility in the scheduling of a transplant.

After the initiation of a search for an unrelated donor, older patients with more advance disease and no HLA-matched donor are associated with lower odds of reaching transplantation. Patients whose Karnofsky Performance Status is 90% to 100% and who have an available donor have significantly reduced hazard of death.[a]

alleles not shared by the recipient determines HVG allorecognition; the presence of recipient alleles not shared by the donor provides the immunologic basis for GVH allorecognition (Table 106.4). "Bidirectional" mismatching occurs when both HVG and GVH vectors are present at a given HLA locus. A recent analysis of the vector of mismatching confirms that outcome is associated with a GVH vector mismatch, and not when a GVH mismatch is absent.[35]

HUMAN LEUKOCYTE ANTIGEN HAPLOTYPES

Patients undergo a family study to determine the availability of potential HLA genotypically identical siblings who could serve as a donor (Box 106.2). HLA genes segregate in classical Mendelian fashion and there is a 25% probability that a sibling inherits the same parental haplotypes (genotypically identical), a 50% probability that a sibling shares one parental haplotype (haploidentical), and a 25% chance that the sibling is completely mismatched.

When no related donor is available or suitable, a search for an unrelated donor is initiated. A search of all available international registries today includes consideration of more than 37 million donors worldwide.[25,32] Unrelated donors are assessed for allele matching at each HLA locus. However, gene-by-gene identity for HLA-A, HLA-B, HLA-C, HLA-DR, and HLA-DQ between two unrelated individuals does not necessarily guarantee the HLA alleles are linked on the same chromosomal haplotype. HLA-matched transplants whose alleles are physically linked on the same extended haplotypes have lower GVHD than HLA-matched transplants whose alleles are encoded on different haplotypes.[36] In an independent study, the inferred phases of HLA-10/10-matched unrelated transplants based on haplotype frequencies did not correlate with the outcome.[37] Sequence-defined haplotypes in Japanese patients are themselves associated with the outcome.[38]

DONOR KILLER-CELL IMMUNOGLOBULIN-LIKE RECEPTOR CRITERIA

Consideration of donor KIR genotype differs significantly from HLA in that there is no conclusive evidence that matching for KIR genotype between donors and recipients has significant relevance to the clinical outcome of overall survival.[39–41] This is not surprising, given their differing contributions to immunity, where HLA is present on the target cell, while KIR resides on the effector cell. Unlike HLA, therefore, there is no negative consequence to the KIR gene or allele mismatching between HCT donor and recipient, as the KIR protein itself does not appear to be immunogenic. Nevertheless, consideration of donor KIR has a role in allogeneic HCT, but an understanding of how the interaction between KIR and HLA impacts NK biology is vital to the successful implementation of KIR assessment for donor selection in HCT.

NK cell response, its likelihood as well as its amplitude, is governed by the interaction between inhibitory receptors and environmental HLA ligands via an incompletely understood process referred to as "NK education."[42] Inhibitory KIR contributing to NK education include KIR3DL1, which recognizes the HLA-Bw4 epitope present on some HLA-A and -B allotypes; KIR2DL2 and KIR2DL3, which recognize HLA-C molecules characterized by Ser77,Asn80 (collectively referred to as HLA-C group 1); and KIR2DL1, which recognizes HLA-C molecules characterized Asn77,Lys80 (collectively referred to as HLA-C group 2). Although KIR3DL2 binds HLA-A*03 and -A*11, the binding appears to be peptide-dependent, and the receptor-ligand relationship does not appear to contribute to NK

BOX 106.2 PRINCIPLES OF HUMAN LEUKOCYTE ANTIGEN MATCHING AND DONOR SELECTION

Establish the Patient's Haplotypes

When an allogeneic transplant is being considered a part of the treatment regime for a patient, human leukocyte antigen (HLA) typing of the patient and first-degree relatives is performed early in the planning process to identify suitable related donors. Typing of family members provides 2 key pieces of information: (1) the availability of an HLA genotypically matched sibling or a suitable haploidentical related donor, and (2) confirmation of the patient's HLA tissue type. When both parents of the patient are available for tissue typing, the family study allows confirmation of the paternal and maternal HLA haplotypes, and this information is invaluable for predicting the probability of finding unrelated donors. In the absence of parents, tissue typing of available siblings might yield sufficient information for the four parental haplotypes.

Characterize Human Leukocyte Antigens at High Resolution

DNA-based methods are the mainstay for tissue typing. Molecular methods provide information of allelic variants at HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 that have been shown to have biological implications in graft-versus-host and host-versus-graft allorecognition. When a search for an unrelated donor yields potential registry donors that lack high-resolution typing, often knowledge of the patient's haplotypes may help to direct typing of donors that have the highest probability of matching the patient's alleles.

Determine the Presence of Anti-Donor Antibodies Against Mismatched HLA Proteins

The risk of graft failure is significantly increased when the patient has mounted an anti-HLA response against donor mismatched antigens. Screening donors with patient sera especially when the donor has known HLA mismatches, is an essential step of donor selection. When no HLA-matched donors are available, avoiding the use of donors whose HLA mismatches are the same specificity as the anti-HLA antibodies in the patient, may reduce the risk of graft failure.

Identify Back-up Donors

The efficiency of the unrelated donor search process is highly dependent on the racial and ethnic background of the recipient and the composition of the donor registries. The availability of unrelated donors must also factor into the planning of the transplant, including the identification of a primary donor and backup donors.

TABLE 106.4 Vector of Human Leukocyte Antigen Mismatch

Vector	Definition	Examples	
		Donor	Recipient
HVG	Presence of donor alleles not present in the recipient	DRB1*01:01,04:01[a]	DRB1*01:01,04:10
		DRB1*01:01,04:01[b]	DRB1*01:01,01:01
GVH	Presence of recipient alleles not present in the donor	DRB1*01:01,04:01[a]	DRB1*01:01,04:10
		DRB1*01:01,01:01[b]	DRB1*01:01,04:10

[a] These combinations contain bidirectional (both HVG and GVHD) mismatch vectors.
[b] Unidirectional mismatches.
GVH, Graft versus host; *HVG*, host versus graft.

education.[43,44] Because the HLA and KIR regions exist on separate chromosomes and are inherited in classic Mendelian fashion, it is common that an individual inherits KIR genes for which it lacks the encoded HLA ligand and vice versa. Expression of the KIR receptors is largely stochastic, leading to a repertoire containing NK cells that express inhibitory KIR for self-HLA and NK cells that express inhibitory KIR for which the HLA ligand may not be present. Tolerance to self is achieved via inhibitory signaling that occurs upon engagement of the KIR-bearing NK cell with cognate (self) HLA ligand on autologous neighboring cells. The same interaction leads to the differential acquisition of responsiveness, such that only NK cells exhibiting inhibitory receptors for self-HLA ligands have high response capacity and are "educated" for a response when confronted with a target cell lacking self-HLA ligand ("missing self").[45] In contrast, NK cells lacking inhibitory receptors for self-HLA, including NK cells expressing inhibitory receptors for non-self HLA (e.g., "missing ligand"), are "uneducated" and hyporesponsive. Both educated and uneducated NK cells play a role in allogeneic HCT.

The majority of activating KIR receptors do not recognize HLA molecules as a ligand themselves and, instead, bind a variety of viral epitopes.[46,47] The one exception is KIR2DS1, which signals NK activation upon engagement of the HLA-C2 ligand. Interestingly, KIR2DS1 also participates in NK education, tolerizing the NK cell based on the environmental "dosage" of HLA-C2, leading to hyporesponsive KIR2DS1$^+$ cells in individuals homozygous for HLA-C2 ligands.[19,48,49]

CLINICAL SIGNIFICANCE OF HUMAN LEUKOCYTE ANTIGEN AND KILLER-CELL IMMUNOGLOBULIN-LIKE RECEPTOR IN TRANSPLANTATION

Human Leukocyte Antigen Matched Transplantation

The impact of more complete and precise donor HLA matching is dramatic. Overall survival after transplantation for the treatment of AML, MDS, ALL, and CML from an HLA-8/8-matched unrelated donor can approach the results observed after HLA-identical sibling transplantation.[32] For all modalities, the underlying disease diagnosis and stage of disease at transplantation remain the most important prognostic features that affect disease-free survival (DFS). The general rules for donor HLA matching are applicable to different graft sources (BM or peripheral blood stem cells) and to different intensities of the conditioning regimen (ablative, reduced intensity, non-myeloablative) in which HLA matching is globally associated with a better outcome than HLA mismatching.[35] Finally, among HLA-10/10-matched unrelated donor transplants, the race and ethnicity of the patient influence outcome.[50] Risks of both acute GVHD and relapse are lower in patients of Asian ancestry than other races and demonstrate the importance of non-HLA factors in influencing the clinical outcome.

Single-Locus Mismatched Transplantation

When an HLA-matched donor is not available, transplantation from a donor with one HLA-A, -B, -C, -DRB1, or -DQB1 mismatch may be considered; however, the risks depend on the mismatched locus (Table 106.5). In the largest analysis of HLA in unrelated transplantation to date, the impact of HLA was studied in over 33,000 transplants performed by the international community.[35] The risks of mortality, DFS, non-relapse mortality and acute and chronic GVHD each increased with each additional HLA-A, -B, -C, -DRB1 or -DQB1 mismatched locus. Mortality increased by ≥20% with each additional mismatch. Among single HLA mismatches, HLA-B mismatches were associated with the lowest DFS and highest acute GVHD risk compared to single HLA-DQB1 mismatches, such that a hierarchy of risks can be discerned: HLA-DQB1, HLA-DRB1, HLA-C, HLA-A, HLA-B.

Among HLA-9/10 unrelated donor transplants, the presence of HLA-DRB1 alleles with certain amino acid motifs is associated with higher mortality.[51] In an MHC-wide study of single nucleotide polymorphism (SNP) associations in HLA 9/10 unrelated donor transplants, rs429916 was validated as a marker for mortality. Fine-mapping over 400,000 base pairs of the class II region identified residues 26, 28, and 30 of HLA-DRβ to be the susceptibility feature. Patients whose HLA-DRB1 alleles encode 26F-28E-30Y have a significantly higher risk of mortality than patients whose HLA-DRB1 alleles encode other residues at these three positions. The frequency of 26F-28E-30Y-positive HLA-DRB1 alleles is higher among individuals of African and Hispanic ancestry than those of White European or Asian backgrounds. The 26F-28E-30Y motif, when present, increases mortality after transplantation regardless of the patient's ethnicity. These results demonstrate that the patient's germline HLA features influence transplant outcome and may aid in pre-transplant risk assessment.

Locus-specific features. Intense efforts directed at identifying HLA features that predict better tolerated ("permissive") mismatches than others ("non-permissive") have identified key concepts: (1) allele compared to antigen mismatches; (2) mismatching for specific amino acid residues in the mature HLA molecule; (3) the level of expression of the mismatched patient allele, and (4) variation residing outside of the peptide-binding region (Box 106.3).

Alleles and antigens. The concept that allele mismatches are associated with lower risks than antigen mismatches was borne out of differences that might provoke different T-cell responses: the number of nonsynonymous substitutions (a change in amino acids) and in the location of the mismatch in the α_1 and α_2 domains of the class I molecule, features affect peptide binding (see Table 106.5). Among HLA-C allele mismatches, the high-frequency C*03:03/C*03:04 allele mismatch is associated with risks comparable to HLA-C-matching and attributed to low cell-surface expression levels.[52] These data are consistent with the early graft failure experience and imply that when faced with a choice between donors with allele or antigen mismatches, selection of an allele mismatch may help to lower risks.

Mismatching at specific HLA residues. The hypothesis that donor-recipient mismatching at certain amino acid substitutions in the class I HLA molecule may be associated with higher post-transplant risks compared with mismatching at other residues has been evaluated in several studies and has particular clinical significance in HLA-DP.[53] HLA-DPB1 T-cell epitope groups correlate with alloreactive T-cell cross-reactivity patterns and predict GVHD risk.

Level of HLA expression. HLA class I proteins are expressed at different levels and this differential expression is a key feature of alloimmunity in infections and autoimmune diseases including HIV-AIDs and Crohn's disease.[54] The level of expression of HLA-C and HLA-DP allotypes is an important feature of the permissivity of mismatches at these two loci.[55,56] The concept behind HLA expression in diseases pertains to the robust presentation of foreign antigens when HLA molecules are expressed at high levels on the cell surface. This contributes to improved immune surveillance. In transplantation, high-expression HLA molecules may provoke a strong attack on host tissues leading to autoimmunity or GVHD in transplantation.

HLA-C. HLA-C expression is regulated in part by rs9264942 ("−35" SNP), which maps equidistant from HLA-B and -C[57] and ablates the miR-148a binding site.[58] In addition to rs9264942, variation in an OCT1 binding site in the promoter provides further control of HLA-C expression.[59] Although each marker is associated with differential HLA-C expression, they do not completely explain its regulation. In HLA-C-mismatched transplantation, as the level of expression of the patient's mismatched HLA-C allotype increases, the risks of acute GVHD and non-relapse mortality increase.[55] Compared to low-expression C*03 and 07 mismatches, high-expression C*01 and 14 mismatches are associated with increased risk when the HLA-C mismatch was also mismatched at residue 116 and for natural KIR ligands. Residue 116 and KIR ligand mismatches that were low-expression had similar risks as HLA-C matches. These data demonstrate that both expression and qualitative features define permissivity.

HLA-DP. The 3′ UTR of HLA-DPB1 is resident to rs9277534 which affects the level of expression of HLA-DP. Haplotypes defined

TABLE 106.5 Impact of Human Leukocyte Antigen Mismatching and Donor Killer-Cell Immunoglobulin-Like Receptor on Outcome of Unrelated Donor Transplantation

Mismatched Locus	Graft Failure	GVHD	Relapse	Survival	Notes
HLA-A	↑[1,2]	↑[4–6]		↓[4,6,7]	Allele and antigen mismatches are similarly risky; in some reports, antigen mismatches are riskier than allele mismatches
HLA-B	↑[2]	↑[4–6]		↓ [6,7]	Insufficient data on allele vs. antigen mismatches
HLA-C	↑[2,3]	↑[4–6,8,9]	Yes[7]	↓[6–8]	Antigen mismatches are much riskier than allele mismatches. C*03:03/03:04 mismatch is low-risk. GVL effect present
HLA-DRB1		↑↓[4–6]		↓[6,7]	Insufficient data on allele vs. antigen mismatches. global trend for lower survival
HLA-DQB1		↓[4]			Only when DQB1 is the only mismatched locus (HLA 9/10)
HLA-DPB1		↑[6,10,13]	Yes[7]	↓[6,7]	GVL effect present
HLA-C Expression		↑[9]			High-expression patient mismatch
HLA-DPB1 T-cell epitope		↑[11]		↓[11,14,15]	TCE non-permissive
HLA-DP expression		↑[16]			High-expression patient mismatch
HLA-B leader		↑[6]			Leader-mismatching
HLA-E				↓[6]	E*01:03 lowers disease-free survival
MICA		↑[17–19]			Heterogeneous data
Missing self			↓[20,21]	↑[20,21]	Protective from AML relapse (when PT-Cy is not used)
Missing ligand			↓[22–26]	↑[22–26]	Lower AML relapse
KIR3DS1		↓[27]		↑[27]	Lower GVHD
KIR haplotype-B			↓[28–31]	↑[28–30]	Lower relapse, heterogeneous data
KIR2DS1+HLA-C1			↓[32–34]	↑[32–34]	Lower AML relapse, heterogeneous data
KIR3DL1 inhibition			↓[34,35]	↑[34,35]	Lower AML relapse, heterogeneous data

AML, Acute myeloid leukemia; *GVL*, graft-versus-leukemia; *GVHD*, graft-versus-host disease; *KIR*, killer cell immunoglobulin-like receptor; *MICA*, major histocompatibility complex class I chain-related A; *TCE*, T-cell epitope; *T-Cy*, post-transplantation cyclophosphamide.

[1] Anasetti C, et al. Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. *N Engl J Med*. 1989;320:197–204.

[2] Anasetti C, Hansen JA. Effect of HLA incompatibility in marrow transplantation from unrelated and HLA-mismatched related donors. *Transfus Sci*. 1994;15:221–230.

[3] Petersdorf EW, et al. Major-histocompatibility-complex class I alleles and antigens in hematopoietic-cell transplantation. *N Engl J Med*. 2001;345:1794–1800.

[4] Lee SJ, et al. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. *Blood*. 2007;110:4576–4583.

[5] Hurley CK, et al. The impact of HLA unidirectional mismatches on the outcome of myeloablative hematopoietic stem cell transplantation with unrelated donors. *Blood*. 2013;121:4800–4806. [6] Petersdorf EW, et al. Role of HLA-B exon 1 in graft-versus-host disease after unrelated haemopoietic cell transplantation: a retrospective cohort study. *Lancet Haematol*. 2020;7(1):e50–e60. doi:10.1016/S2352-3026(19)30208-X. [7] Morishima Y, et al. Biological significance of HLA locus matching in unrelated donor bone marrow transplantation. *Blood*. 2015;125:1189–1197. [8] Woolfrey A, et al. HLA-C antigen mismatch is associated with worse outcome in unrelated donor peripheral blood stem cell transplantation. *Biol Blood Marrow Transplant*. 2011;17:885–892. [9] Petersdorf EW, et al. HLA-C expression levels define permissible mismatches in hematopoietic cell transplantation. *Blood*. 2014;124:3996–4003. [10] Fernandez-Vina MA, et al. Multiple mismatches at the low expression HLA loci DP, DQ, and DRB3/4/5 associate with adverse outcomes in hematopoietic stem cell transplantation. *Blood*. 2013;121:4603–4610. [11] Fleischhauer K, et al. Effect of T-cell-epitope matching at HLA-DPB1 in recipients of unrelated-donor haemopoietic-cell transplantation: a retrospective study. [Erratum appears in *Lancet Oncol*. 2012;13(4):e134–e135.] *Lancet Oncol*. 2012;13:366–374. [12] Zino E, et al. A T-cell epitope encoded by a subset of HLA-DPB1 alleles determines nonpermissive mismatches for hematologic stem cell transplantation. *Blood*. 2004;103:1417–1424. [13] Pidala J, et al. Nonpermissive HLA-DPB1 mismatch increases mortality after myeloablative unrelated allogeneic hematopoietic cell transplantation. *Blood*. 2014;124:2596-2606. [14] Meurer T, et al. Dissecting genetic control of HLA-DPB1 expression and its relation to structural mismatch models in hematopoietic stem cell transplantation. *Front Immunol*. 2018;9:2236. [15] Lorentino F, et al. Comparative evaluation of biological HLA-DPB1 mismatch models for survival and graft versus host disease prediction after unrelated donor hematopoietic cell transplantation. *Haematologica*. 2019. doi:10.3324/haematol.2019.225177. [16] Petersdorf EW, et al. High HLA-DP expression and graft-versus-host disease. *N Engl J Med*. 2015;373:599–609. [17] Carapito R, et al. Matching for the nonconventional MHC-I MICA gene significantly reduces the incidence of acute and chronic GVHD. *Blood*. 2016;128(15):1979–1986. [18] Fuerst D, et al. Matching for the MICA-129 polymorphism is beneficial in unrelated hematopoietic stem cell transplantation. *Blood*. 2016;128(26):3169–3176. [19] Askar M, et al. MHC class I chain-related gene A (MICA) donor-recipient mismatches and MICA-129 polymorphism in unrelated donor hematopoietic cell transplantations has no impact on outcomes in acute lymphoblastic leukemia, acute myeloid leukemia, or myelodysplastic syndrome: a center for international blood and marrow transplant research study. *Biol Blood Marrow Transplant*. 2017;23(3):436–444. [20] Ruggeri L, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood*. 1999;94:333–339. [21] Russo A, et al. NK cell recovery after haploidentical HSCT with posttransplant cyclophosphamide: dynamics and clinical implications. *Blood*. 2018;131:247–262. [22] Hsu KC, et al. Improved outcome in HLA-identical sibling hematopoietic stem-cell transplantation for acute myelogenous leukemia predicted by KIR and HLA genotypes. *Blood*. 2005;105(12):4878–4884. [23] Hsu KC, et al. KIR ligands and prediction of relapse after unrelated donor hematopoietic cell transplantation for hematologic malignancy. *Biol Blood Marrow Transplant*. 2006;12:828–836. [24] Shimoni A, et al. Missing HLA C group 1 ligand in patients with AML and MDS is associated with reduced risk of relapse and better survival after allogeneic stem cell transplantation with fludarabine and treosulfan reduced toxicity conditioning. *Am J Hematol*. 2017;92(10):1011-1019. [25] Arima N, et al. Increased relapse risk of acute lymphoid leukemia in homozygous HLA-C1 patients after HLA-matched allogeneic transplantation: a Japanese national registry study. *Biol Blood Marrow Transplant*. 2020; 26(3):431–437. [26] Martínez-Losada C, et al. Patients lacking a KIR-ligand of HLA group C1 or C2 have a better outcome after umbilical cord blood transplantation. *Front Immunol*. 2017;8:810. [27] Venstrom JM, et al. Donor activating KIR3DS1 is associated with decreased acute GVHD in unrelated allogeneic hematopoietic stem cell transplantation. *Blood*. 2010;115(15):3162–3165. [28] Cooley S, et al. Donors with group B haplotypes improve relapse-free survival after unrelated hematopoietic transplantation for acute myeloid leukemia. *Blood*. 2009;113:726–732. [29] Sobecks RM, et al. Impact of KIR and HLA genotypes on outcomes after reduced-intensity conditioning hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2015;21(9):1589–1596. [30] Weisdorf D, et al. KIR B donors improve the outcome for AML patients given reduced intensity conditioning and unrelated donor transplantation. *Blood Adv*. 2020;4(4):740–754. [31] Babor F, et al. Presence of centromeric but absence of telomeric group B KIR haplotypes in stem cell donors improve leukaemia control after HSCT for childhood ALL. *Bone Marrow Transplant*. 2019; 54:1847–1858. [32] Venstrom JM, et al. HLA-C-dependent prevention of leukemia relapse by donor activating KIR2DS1. *N Engl J Med*. 2012;367(9):805–816. [33] Cooley S, et al. Donor killer cell Ig-like receptor B haplotypes, recipient HLA-C1, and HLA-C mismatch enhance the clinical benefit of unrelated transplantation for acute myelogenous leukemia. *J Immunol*. 2014;192(10):4592–4600. [34] Schetelig J, et al. External validation of models for KIR2DS1/KIR3DL1-informed selection of hematopoietic cell donors fails. *Blood*. 2020;135(16):1386–1395. [35] Boudreau JE, et al. KIR3DL1/HLA-B subtypes govern acute myelogenous leukemia relapse after hematopoietic cell transplantation. *J Clin Oncol*. 2017;35(20):2268–2278.

BOX 106.3 RESEARCH IN PROGRESS: NEW IMMUNOGENETIC FACTORS FOR CONSIDERATION IN TRANSPLANTATION

Human Leukocyte Antigen

When a human leukocyte antigen (HLA)-matched donor is not available, criteria for the selection of mismatched donors is needed. Beyond prioritizing an allele mismatch over an antigen mismatch, research on the immunogenicity of residues of class I and II molecules and their amino acid substitutions remain important concepts. Hypervariable positions having a direct influence on the peptide-binding region of the molecule have been most commonly studied, and show that some amino acid substitutions at certain positions are more detrimental than others; these include residues 99 and 116 of HLA-C, residue 9 of HLA-B, and residues that define hypervariable regions of HLA-DP. Further elucidation of the features of residue mismatching will provide more specific information on the combinations of mismatches that are permissive.

The level of HLA expression of patient mismatches influences GVHD. A minority of the putative variants that control HLA expression are known, and more information is needed to understand the mechanisms. The role of non-coding variation such as the HLA-B leader dimorphism will advance understanding of the mechanisms of T- and natural killer (NK)-cell recognition and provide new avenues for donor selection.

A role for highly conserved HLA haplotypes and transplant outcomes is emerging in selected populations. Haplotype content and organization in ethnically diverse populations will continue to provide invaluable information.

Killer-Cell Immunoglobulin-Like Receptor

Donor NK alloreactivity in hematopoietic cell transplantation (HCT) is manifested as improved control of myeloid malignancy, leading to a reduced risk of leukemia relapse and increased survival following transplantation. It has become increasingly evident that killer-cell immunoglobulin-like receptor (KIR) driven donor NK effects can occur in the HLA-matched setting. Several models of NK activation in transplantation have been tested in retrospective studies in efforts to use KIR and HLA genotypes to select advantageous donors and improve transplant outcomes.

Donors with KIR genotypes encoding activating KIR provide protection from relapse of myeloid malignancy following transplantation, but additional information is needed to understand how HLA moderates this benefit. Donors with KIR3DL1 subtypes that signal less NK inhibition also provide protection from disease relapse. KIR-based donor selection that promotes NK activation while minimizing NK inhibition will therefore provide the greatest benefit to patients with myeloid malignancies. Novel findings in NK education will continue to guide future studies of KIR and HLA in transplantation.

Novel Markers

HLA haplotype encodes additional genes that have clinical relevance. Future research into the identification of the causative genes will shed light on the pathways involved in graft-versus-host and host-versus-graft allorecognition. The use of next-generation sequencing (NGS) methods for both clinical and research applications will accelerate the identification of novel markers, particularly in non-coding regions of HLA genes.

by HLA-DPB1 exon 2 and rs9277534 permit two families of alleles to be defined: low-expression rs9177534A and high-expression rs9277534G. Haplotypes have been confirmed in a large study of registry donors.[60] In HLA-11/12 transplants with one HLA-DPB1 mismatch, mismatching against a high-expression patient allele was associated with significantly higher acute GVHD than mismatching against a low-expression allele.[56] The role of HLA-DP expression in permissive mismatches was recently validated in Japanese transplants[61] and in solid organ transplantation in which high-expression rs9277534G HLA-DP antigens are associated with a stronger B-cell flow crossmatch compared to low-expression rs9277534A antigens.[62] Both T-cell epitope (TCE) and HLA-DP expression inform GVHD.[63]

HLA-DRB3, DRB4, DRB5. The low-expression loci HLA-DRB3, -DRB4, and -DRB5 contribute to clinical outcome.[52] The cumulative impact of multi-loci mismatching inclusive of DRB3/4/5 on mortality is significant, particularly when three or more mismatches are present. These data suggest that when an HLA-DRB1-mismatched donor is identified, additional characterization of HLA-DRB3/4/5 on relevant HLA-DR haplotypes is warranted.

Variation outside of the HLA peptide-binding groove. HLA research has focused on the peptide-binding groove because of the role of antigen presentation in immunity and GVHD. Variation encoded outside of the peptide-binding region may also affect the outcome, particularly when it defines haplotypes across an HLA genetic region. The HLA-B leader is an example of a functional polymorphism that is encoded within the HLA-B locus, but outside of the peptide-binding region.[35]

The HLA-B leader is encoded by exon 1. A C/T dimorphism at rs1050458 (position -21) gives rise to leader peptides with either methionine (M) or threonine (T) at the second residue of the leader peptide. HLA-B alleles that encode M-leaders are a family distinct from alleles that encode T-leaders. M- and T-leaders differentially influence HLA-E expression and the strength of inhibitory and activating NK and T-cell responses.[64]

In HLA-9/10 unrelated donor transplants with one HLA-B mismatch (and one HLA-B match), the leaders of the mismatched patient and donor alleles as well as the leader of the matched allele both influence GVHD risk.[35] When the patient/donor mismatched alleles are both from the same leader family (both M or both T), acute GVHD is significantly lower than when the mismatched alleles are derived from different leader families (an M-leader allele mismatched with a T-leader allele). When the matched allele encodes an M-leader, GVHD is higher than when the matched allele has a T-leader. These data implicate a polymorphism outside of the peptide-binding groove in transplant outcome. Both HLA-B haplotypes contribute to the immune response. Use of the HLA-B leader to select permissive HLA-B mismatches may provide patients with options.

Importance of Killer-Cell Immunoglobulin-Like Receptor in Relapse After Transplantation

KIR ligand incompatibility and missing self in HLA-mismatched HCT. The clinical significance of donor KIR genotype in allogeneic HCT first came to light in the setting of HLA-haploidentical HCT using $CD34^+$-selected allografts, where the high degree of HLA-mismatching led to the frequent circumstance in which a KIR ligand present in the donor was lacking in the recipient, thereby fulfilling the criteria for the "missing self" from the standpoint of the donor NK cell (see Table 106.5). Citing missing self due to KIR ligand incompatibility in the graft-versus-host direction, investigators reported a lower risk of relapse of myeloid malignancy following HCT.[65] The observation that favorable HCT outcomes were associated with the absence of donor KIR ligands in the recipient suggested that donor-derived NK alloreactivity was the source of leukemia control and that KIR receptors were the mediators of the disease control. The successful isolation of educated, KIR^+ donor NK clones reacting according to missing self in vitro provided further accuracy in predicting leukemic control in patients lacking donor KIR ligands.[66] That myeloid malignancies were more sensitive to NK control compared to lymphoid malignancies provided additional clues to the biology of NK cells, which not only require a lack of inhibitory signals from the target cell to unleash cytotoxicity but also require simultaneous activating signals. Indeed, myeloid malignancies express abundant activating ligands, such as MIC-A, a protein recognized by the NKG2D activating receptor on NK cells.[67] Interestingly, a recent study of transplantation using unmanipulated haploidentical T-cell-replete allografts with the administration of post-transplant cyclophosphamide for prevention of graft-versus-host disease did not replicate the original findings of protection from disease relapse, possibly due to the elimination of mature donor KIR^+ anti-leukemic NK cells in the allograft.[68]

Missing ligand effect in HLA-matched HCT. Although HLA-mismatched HCT is becoming more common, the prevailing preference is for a stem cell donor that is matched for as many HLA alleles as possible, with possible exceptions for permissive mismatching at HLA-DP.[56] By definition, matching donor and recipient for HLA alleles results in KIR ligand matching, which eliminates the possibility for educated NK alloreactivity due to missing self. It was therefore surprising that a lack of KIR ligands was found to be predictive of AML relapse following HLA-matched sibling transplantation, where homozygosity for HLA-C1, HLA-C2, or HLA-Bw6 was predictive of lower relapse and higher survival.[69] The lack of KIR ligands (e.g. lack of HLA-C2 ligand in HLA-C1 homozygous patients, lack of HLA-Bw4 in HLA-Bw6 homozygous patients) invoked the possibility that uneducated NK cells expressing the KIR receptors for the "missing ligands" could be activated by the post-HCT inflammatory environment for cytotoxicity.[70] Findings of improved outcomes with lack of KIR ligands have since been replicated with HLA-matched sibling and unrelated donor allografts,[71–73] and HLA-mismatched umbilical cord allografts.[74]

KIR B-haplotypes. Capturing donor NK alloreactivity according to missing self and missing ligand mechanisms of NK activation, however, ultimately depends on the recipient's HLA genotype, which encodes the patient's KIR ligands and cannot be altered. Efforts to identify benefits associated with donor KIR gene content irrespective of KIR ligand has yielded positive findings, again compellingly confined to the AML patient population. KIR3DS1 is an activating variant of the inhibitory KIR3DL1 gene and characterizes KIR B-haplotypes with telomeric activating KIR.[7] A large retrospective study of patients undergoing unrelated donor HCT identified the association of donor KIR3DS1 with lower GVHD and higher overall survival, with increased benefit seen in donors exhibiting more than one KIR3DS1 copy.[75] A separate study revealed that both centromeric B-haplotypes (e.g. those containing KIR2DS2) and telomeric B-haplotypes confer a protective effect from AML relapse with superior survival, but that centromeric B-haplotypes (cenB) were the more protective of the two.[13] Accordingly, homozygosity for cenB was associated with the greatest protection from AML relapse. Together, these data support the selection of a donor with KIR B-haplotype to reduce relapse and GVHD in patients with myeloid malignancies.

KIR2DS1 and HLA-C1. The telomeric KIR2DS1 is the only activating KIR that contributes to NK education, as evidenced in vitro by progressive hyporesponsiveness among KIR2DS1$^+$ NK cells from donors with increasing HLA-C2 allele content.[19,48,49] Correspondingly, the protective effect of donor KIR2DS1 against AML relapse following HLA-compatible HCT is lost in patients whose donors are HLA-C2 homozygous.[76,77] Interestingly, donor HLA-C2 homozygosity silences the benefits seen with all KIR haplotype-B donors and not just those with KIR2DS1.[76,78] Thus, to gain the protective effects of donor KIR2DS1$^+$ and other activating KIR, an HLA-C1 donor is required.

KIR3DL1 and HLA-Bw4 subtypes. NK cells expressing the same KIR receptor and derived from individuals with similar KIR ligands can still display a wide variation of response capacity, due to allele-encoded differences in receptor expression, ligand expression, and affinity between receptor-ligand pairs.[18,79] The KIR-HLA relationship most illustrative of how polymorphism impacts NK function, is the KIR3DL1/HLA-Bw4 combination. KIR3DL1 alleles differ significantly in surface expression, with variants expressed at high or low density on the surface and with one common variant retained intracellularly, leading to the subtype designations of KIR3DL1-h, KIR3DL1-l, and KIR3DL1-n.[80–82] HLA-B Bw4 alleles can be segregated based on dimorphism at amino acid position 80 into subtype groups characterized by an isoleucine (Bw4-80I) or threonine (Bw4-80T) residue. Combinations of high vs low expression KIR3DL1 subtypes with Bw4-80I or Bw4-80T subtypes generate distinct differences in response capacity and inhibitory strengths, partially enhanced by differences in binding affinities.[18,79,83] In individuals with two KIR3DL1 alleles, the low-expression allele dominates, leading to a compound subtype designation of KIR3DL1-L, and in individuals with two HLA-Bw4 alleles, the Bw4-80I allele dominates. High inhibition combinations include KIR3DL1-H + Bw4-80I and KIR3DL1-L + Bw4-80T, while low inhibition combinations include KIR3DL1-H + Bw4-80T and KIR3DL1-L + Bw4-80I.

Among AML patients receiving a 9/10 or 10/10 HLA allele-matched unrelated donor HCT, those whose donor KIR3DL1 subtype is paired with a low inhibitory HLA-B subtype experience lower relapse following HCT, compared to patients whose donor KIR3DL1 subtype is paired with a high inhibitory HLA-B subtype.[83] Patients whose donors express a non-inhibitable KIR3DL1 allele (e.g., KIR3DL1-n or KIR3DS1) or who lack the HLA-Bw4 epitope (e.g., HLA-Bw6 homozygous) experienced relapse rates similar to patients with low inhibitory donor KIR3DL1/HLA-Bw4 relationships. Because KIR3DL1 is essentially ubiquitous within the population, and its expression subtypes are fairly equally distributed, identifying an unrelated donor with a low/no KIR3DL1 inhibition subtype for an HLA-Bw4$^+$ patient is an achievable goal.

Mixed results for KIR and HCT outcomes. The prospect that donors can be selected based on KIR and HLA genotypes to harness NK alloreactivity is bolstered by compelling results from numerous retrospective trials, frequently involving cohorts of more than 1000 patients. Validation of the beneficial impacts of each of these KIR-HLA models has, however, provided mixed results. A retrospective study of donor KIR B-haplotypes in recent transplants found lower AML relapse and higher survival following HCT but only in patients who received reduced-intensity, and not myeloablative, conditioning;[78] another study, however, found no benefit of KIR B-haplotypes in reduced intensity HCT.[84] While one retrospective analysis found no apparent benefit from donor KIR B-haplotypes in pediatric HCT patients with either myeloid or lymphoid malignancies,[85] another showed a benefit of donor cenB (but not telomeric-B haplotypes) in pediatric ALL patients.[86] Similarly, a large retrospective study was not able to validate the protective effects of the donor KIR2DS1 + HLA-C1 combination and low inhibition donor KIR3DL1 + HLA-Bw4 combinations.[87] A greater understanding of NK alloreactivity, its predictive genetics, and the transplant conditions that favor its effects is still required. Well-designed prospective clinical trials of donor selection based on biologically supported KIR and HLA models will undoubtedly help to resolve some of these discrepancies.

SUMMARY

Based on a large-scale registry and consortium studies, recommendations for donor selection are as follows: (1) If the patient has many potential HLA-8/8 donors, additional matching for HLA-DQB1 (HLA-10/10) and HLA-DPB1 (HLA-12/12) may further enhance patient outcomes. (2) When an HLA-10/10 matched donor cannot be identified, the use of a donor mismatched for a single HLA-DQB1 allele can be considered. (3) When HLA-10/10 donors are available, selection of a 12/12 donor matched at HLA-DPB1 may further lower GVHD risks. (4) When an HLA-12/12 donor is not available, consideration of HLA-11/12 donors with a single HLA-DPB1 mismatch using either the TCE or expression algorithm, will help to lower GVHD risk. (5) Multiple mismatches are less well tolerated and should be limited. (6) When HLA-DRB1-mismatched donors (7/8 or 9/10) are identified, assessment of HLA-DRB3, DRB4 or DRB5 may help to uncover coincident mismatching at these loci; cumulative mismatching at multiple HLA-DRB genes increases risks after transplantation and should be avoided. (7) Polymorphisms outside of the classical HLA loci may be clinically significant. (8) If an HLA-mismatched donor cannot be avoided, selection of a donor whose KIR ligands are missing in the patient may protect the patient from relapse of myeloid malignancy. This protection may be lost, however, with the use of post-transplant cyclophosphamide. (9) In HLA-matched HCT, the selection of an HLA-C1$^+$ KIR2DS1$^+$ donor may reduce the risk for myeloid malignancy relapse. (10) Selection of a donor with a KIR B-haplotype (e.g., centromeric KIR2DS2 or telomeric KIR3DS1) may provide protection against relapse of myeloid malignancy. (11) For patients who are HLA-Bw4$^+$ and for whom multiple donors are available, consideration should be made to select a donor with a KIR3DL1 subtype predicted to have low inhibitory interaction with the patient's HLA-Bw4 subtype to decrease the risk of post-HCT myeloid leukemia relapse and increase survival (see Table 106.5).

Beyond Classical Human Leukocyte Antigen

The MHC is the most diverse region in the human genome known to date, encoding more than 300 loci involved in immune function within the extended 7.6-megabase (Mb) region.[88] Hence, current donor matching is performed for less than 5% of the total gene content of the MHC. In addition to the classical HLA loci, the MHC is residence to the nonclassical HLA-E, HLA-F, HLA-G, MICA, and MICB genes. The recent analysis of HLA-9/10 transplants identified donor HLA-E*01:03 to be associated with lower DFS.[35] The association of HLA-E*01:03 with outcome provides new insight into dual pathways: potential T-cell as well as CD94/NKG2 NK recognition of the ligand.

Information on MICA in transplantation has become available.[89–91] In some series, HLA-10/10 transplants had higher severe acute GVHD when the patient and donor were MICA-mismatched or when the mismatch involved residue 129.[91] However, in other series, no association of MICA alleles or mismatching can be demonstrated.[89,92] The clinical significance of MICA alleles and mismatching remains an important research question.

The Major Histocompatibility Complex in the Next-Generation Sequencing Era

The availability of NGS methods has uncovered unprecedented levels of haplotype-associated variation of the MHC. The routine use of NGS-based HLA typing is redefining the landscape of the MHC. Emerging data indicate that HLA-10/10 unrelated donor pairs harbor substantial degrees of undetected variation; furthermore, the clinical outcome is superior when patients and donors are matched across class I genes by NGS criteria.[93] As more NGS information becomes available, it is anticipated that both coding and non-coding variation with biological significance will be discovered. In this way, the validation and cataloguing of human genetic variation remain a vital resource for future research (see Box 106.3).

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Anasetti C, Logan BR, Lee SL, et al. Peripheral-blood stem cells versus bone marrow from unrelated donors. *N Engl J Med*. 2012;367:1487–1496.

Boudreau JE, Giglio F, Gooley TA, et al. KIR3DL1/HLA-B subtypes govern acute myelogenous leukemia relapse after hematopoietic cell transplantation. *J Clin Oncol*. 2017;35(20):2268–2278.

Boudreau JE, Hsu KC. Natural killer cell education and the response to infection and cancer therapy: stay tuned. *Trends Immunol*. 2018;39(3):222–239.

Cooley S, Trachtenberg E, Bergemann T, et al. B haplotypes improve relapse-free survival after unrelated hemaotpoietic transplantation for acute myeloid leukemia Donors with group. *Blood*. 2009;113:726–732.

Fauriat C, Ivarsson MA, Ljunggren H-G, et al. Education of human natural killer cells by activating killer cell immunoglobulin-like receptors. *Blood*. 2010;115(6):1166–1174.

Fleischhauer K, Shaw BE, Gooley T, et al. Effect of T-cell-epitope matching at HLA-DPB1 in recipients of unrelated-donor haemopoietic-cell transplantation: a retrospective study [Erratum appears in *Lancet Oncol*. 2012 Apr;13(4):e134-e135]. *Lancet Oncol*. 2012;13:366–374.

Gragert L, Eapen M, Williams E, et al. HLA match likelihoods for hematopoietic stem-cell grafts in the US registry. *N Engl J Med*. 2014;371:339–348.

Hsu KC, Chida S, Geraghty DE, Dupont B. The killer cell immunoglobulin-like receptor (KIR) genomic region: gene-order, haplotypes and allelic polymorphism. *Immunol Rev*. 2002;190:40–52.

Hsu KC, Keever-Taylor CA, Wilton A, et al. Improved outcome in HLA-identical sibling hematopoietic stem-cell transplantation for acute myelogenous leukemia predicted by KIR and HLA genotypes. *Blood*. 2005;105(12):4878–4884.

Morishima Y, Kawase T, Malkki M, et al. Significance of ethnicity in the risk of acute graft-versus-host disease and leukemia relapse after unrelated donor hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2013;19(8):1197–1203.

Petersdorf EW, Malkki M, Gooley TA, et al. MHC haplotype matching for unrelated hematopoietic cell transplantation. *PLoS Med*. 2007;4:e8.

Petersdorf EW, Malkki M, O'hUigin C, et al. High HLA-DP expression and graft-versus-host disease. *N Engl J Med*. 2015;373:599–609.

Petersdorf EW, Carrington M, O'hUigin C, et al. Role of HLA-B exon 1 in graft-versus-host disease after unrelated haemopoietic cell transplantation: a retrospective cohort study. *Lancet Haematol*. 2020;7(1):e50–e60.

Ruggeri L, Capanni M, Casucci M, Velardi A, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood*. 1999;94:333–3339.

Shaw BE, Logan BR, Spellman SR, et al. Development of an unrelated donor selection score predictive of survival after HCT: donor age matters most. *Biol Blood Marrow Transplant*. 2018;24:1049–1056.

Valiante NM, Uhrberg M, Shilling HG, et al. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity*. 1997;7:739–751.

Venstrom JM, Pittari G, Gooley TA, et al. HLA-C-dependent prevention of leukemia relapse by donor activating KIR2DS1. *N Engl J Med*. 2012;367(9):805–816.

REFERENCES

1. European Bioinformatics Institute. Statistics. Retrieved from https://www.ebi.ac.uk/ipd/imgt/hl.
2. Allele Frequency Net Database. Allele Frequency Net Database. Retrieved from http://www.allelefrequencies.net/.
3. Klasberg S, Surendranath V, Lange V, Schöfl G. Bioinformatics strategies, challenges, and opportunities for next generation sequencing-based HLA genotyping. *Transfusion Medicine and Hemotherapy*. 2019;46(5):312–325. https://doi.org/10.1159/000502487.
4. Mayor NP, Robinson J, McWhinnie AJ, et al. HLA typing for the Next Generation. *PLoS One*. 2015;10(5):e0127153. https://doi.org/10.1371/journal.pone.0127153.
5. Allen ES, Yang B, Garrett J, et al. Improved accuracy of clinical HLA genotyping by next-generation DNA sequencing affects unrelated donor search results for hematopoietic stem cell transplantation. *Hum Immunol*. 2018;79(12):848–854. https://doi.org/10.1016/j.humimm.2018.10.008.
6. Balgansuren G, Regen L, Sprague M, et al. Identification of the rs9277534 HLA-DP expression marker by next generation sequencing for the selection of unrelated donors for hematopoietic cell transplantation. *Hum Immunol*. 2019;80(10):828–833. https://doi.org/10.1016/j.humimm.2019.05.010.
7. Hsu KC, Chida S, Geraghty DE, Dupont B. The killer cell immunoglobulin-like receptor (KIR) genomic region: gene-order, haplotypes and allelic polymorphism. *Immunol Rev*. 2002;190:40–52. https://doi.org/10.1034/j.1600-065x.2002.19004.x.
8. Le Luduec JB, Kudva A, Boudreau JE, Hsu KC. Novel multiplex PCR-SSP method for centromeric KIR allele discrimination. *Sci Rep*. 2018;8(1):14853. https://doi.org/10.1038/s41598-018-33135-1.
9. Vierra-Green C, Roe D, Hou L, et al. Allele-level haplotype frequencies and pairwise linkage disequilibrium for 14 KIR loci in 506 European-American individuals. *PLoS One*. 2012;7(11):e47491. https://doi.org/10.1371/journal.pone.0047491.
10. Uhrberg M, Valiante NM, Shum BP, et al. Human diversity in killer cell inhibitory receptor genes. *Immunity*. 1997;7(6):753–763. https://doi.org/10.1016/s1074-7613(00)80394-5.
11. Valiante NM, Uhrberg M, Shilling HG, et al. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity*. 1997;7(6):739–751. https://doi.org/10.1016/s1074-7613(00)80393-3.
12. Norman PJ, Hollenbach JA, Nemat-Gorgani N, et al. Co-evolution of human leukocyte antigen (HLA) class I ligands with killer-cell immunoglobulin-like receptors (KIR) in a genetically diverse population of sub-Saharan Africans. *PLoS Genet*. 2013;9(10):e1003938. https://doi.org/10.1371/journal.pgen.1003938.
13. Cooley S, Trachtenberg E, Bergemann TL, et al. Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia. *Blood*. 2009;113(3):726–732. https://doi.org/10.1182/blood-2008-07-171926.
14. Hsu KC, Liu XR, Selvakumar A, et al. Killer Ig-like receptor haplotype analysis by gene content: evidence for genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets. *J Immunol*. 2002;169(9):5118–5129. https://doi.org/10.4049/jimmunol.169.9.5118.
15. EMBL-EBI. (2020). IPD-KIR. Retrieved from https://www.ebi.ac.uk/ipd/kir
16. Marsh SGE, Parham P, Dupont B, et al. Killer-cell immunoglobulin-like receptor (KIR) nomenclature report, 2002. *Immunogenetics*. 2003;55(4):220–226. https://doi.org/10.1007/s00251-003-0571-z.
17. Bari R, Bell T, Leung WH, et al. Significant functional heterogeneity among KIR2DL1 alleles and a pivotal role of arginine 245. *Blood*. 2009;114(25):5182–5190. https://doi.org/10.1182/blood-2009-07-231977.
18. Boudreau JE, Mulrooney TJ, Le Luduec JB, et al. KIR3DL1 and HLA-B density and binding calibrate NK education and response to HIV. *J Immunol*. 2016;196(8):3398–3410. https://doi.org/10.4049/jimmunol.1502469.
19. Le Luduec JB, Boudreau JE, Freiberg JC, Hsu KC. novel approach to cell surface discrimination between KIR2DL1 subtypes and KIR2DS1 identifies hierarchies in NK repertoire, education, and tolerance. *Front Immunol*. 2019;10:734. https://doi.org/10.3389/fimmu.2019.00734.
20. Bari R, Schell S, Tuggle M, Leung W. An improved method with high specificity for KIR2DL1 functional allele typing. *Laboratory Medicine*. 2015;46(3):207–213. https://doi.org/10.1309/LMMN1K6SBRJCGD9J.
21. Boudreau JE, Le Luduec JB, Hsu KC. Development of a novel multiplex PCR assay to detect functional subtypes of KIR3DL1 alleles. *PLoS One*. 2014;9(6):e99543. https://doi.org/10.1371/journal.pone.0099543.
22. Norman PJ, Hollenbach JA, Nemat-Gorgani N, et al. Defining KIR and HLA class I genotypes at highest resolution via high-throughput sequencing. *Am J Hum Genet*. 2016;99(2):375–391. https://doi.org/10.1016/j.ajhg.2016.06.023.
23. Trefny MP, Rothschild SI, Uhlenbrock F, et al. A variant of a killer cell immunoglobulin-like receptor is associated with resistance to PD-1 blockade in lung cancer. *Clin Cancer Res*. 2019;25(10):3026–3034. https://doi.org/10.1158/1078-0432.Ccr-18-3041.
24. Maniangou, B., Legrand, N., Alizadeh, M., et al. Killer immunoglobulin-like receptor allele determination using next-generation sequencing technology. *Front Immunol*, 8, 547. doi: 10.3389/fimmu.2017.00547
25. World Marrow Donor Association. www.wmda.info. Retrieved from https://wmda.info/
26. Nishiwaki S, Tanaka H, Kojima H, Okamoto S. Availability of HLA-allele-matched unrelated donors: estimation from haplotype frequency in the Japanese population. *Bone Marrow Transplant*. 2019;54(2):300–303. https://doi.org/10.1038/s41409-018-0263-9.
27. Buck K, Wadsworth K, Setterholm M, et al. High-resolution match rate of 7/8 and 9/10 or better for the be the match unrelated donor registry. *Biol Blood Marrow Transplant*. 2016;22(4):759–763. https://doi.org/10.1016/j.bbmt.2015.12.012.
28. Barker JN, Boughan K, Dahi PB, et al. Racial disparities in access to HLA-matched unrelated donor transplants: a prospective 1312-patient analysis. *Blood Adv*. 2019;3(7):939–944. https://doi.org/10.1182/bloodadvances.2018028662.
29. Anthias C, Shaw BE, Bruce JG, et al. Role of race/ethnicity in donor decisions about unrelated hematopoietic progenitor cell donation: exploring reasons for higher attrition among racial/ethnic minorities. *Biol Blood Marrow Transplant*. 2020;26(3):593–599. https://doi.org/10.1016/j.bbmt.2019.10.012.
30. Pidala J, Kim J, Schell M, et al. Race/ethnicity affects the probability of finding an HLA-A, -B, -C and -DRB1 allele-matched unrelated donor and likelihood of subsequent transplant utilization. *Bone Marrow Transplant*. 2013;48(3):346–350. https://doi.org/10.1038/bmt.2012.150.
31. Dehn J, Spellman S, Hurley Selection of unrelated donors and cord blood units for hematopoietic cell transplantation: guidelines from the NMDP/CIBMTR. *Blood*. 2019;134(12):924–934. https://doi.org/10.1182/blood.2019001212.
32. National Marrow Donor Program. (2020). https://bethematchclinical.org/. Retrieved from https://bethematchclinical.org/
33. Navarro WH, Switzer GE, Pulsipher M. National marrow donor program session: donor issues. *Biol Blood Marrow Transplant*. 2013;19(1 Suppl):S15–S19. https://doi.org/10.1016/j.bbmt.2012.10.019.
34. Shaw BE, Logan BR, Spellman SR, et al. Development of an unrelated donor selection score predictive of survival after HCT: donor age matters most. *Biol Blood Marrow Transplant*. 2018;24(5):1049–1056. https://doi.org/10.1016/j.bbmt.2018.02.006.
35. Petersdorf EW, Carrington M, O'HUigin C, et al. Role of HLA-B exon 1 in graft-versus-host disease after unrelated haemopoietic cell transplantation: a retrospective cohort study. *Lancet Haematol*. 2020;7(1):e50–e60. https://doi.org/10.1016/s2352-3026(19)30208-x.
36. Petersdorf EW, Malkki M, Gooley TA, et al. MHC haplotype matching for unrelated hematopoietic cell transplantation. *PLoS Med*. 2007;4(1):e8. https://doi.org/10.1371/journal.pmed.0040008.
37. Buhler S, Baldomero H, Ferrari-Lacraz S, et al. High-resolution HLA phased haplotype frequencies to predict the success of unrelated donor searches and clinical outcome following hematopoietic stem cell transplantation. *Bone Marrow Transplant*. 2019;54(10):1701–1709. https://doi.org/10.1038/s41409-019-0520-6.
38. Morishima S, Ogawa S, Matsubara A, et al. Impact of highly conserved HLA haplotype on acute graft-versus-host disease. *Blood*. 2010;115(23):4664–4670. https://doi.org/10.1182/blood-2009-10-251157.
39. Faridi RM, Kemp TJ, Dharmani-Khan P, et al. Donor-recipient matching for KIR genotypes reduces chronic GVHD and missing inhibitory KIR Ligands protect against relapse after myeloablative, HLA matched hematopoietic cell Transplantation. *PLoS One*. 2016;11(6):e0158242. https://doi.org/10.1371/journal.pone.0158242.
40. Gagne K, Busson M, Balere-Appert ML, et al. Relevance of KIR gene matching in unrelated hematopoietic stem cell transplantations. *Tissue Antigens*. 2007;69(Suppl 1):118–122. https://doi.org/10.1111/j.1399-0039.2006.76210.x.
41. Sahin U, Dalva K, Gungor F, et al. Donor-recipient killer immunoglobulin like receptor (KIR) genotype matching has a protective effect on chronic graft versus host disease and relapse incidence following HLA-identical sibling hematopoietic stem cell transplantation. *Ann Hematol*. 2018;97(6):1027–1039. https://doi.org/10.1007/s00277-018-3274-0.
42. Boudreau JE, Hsu KC. Natural killer cell education and the response to infection and cancer therapy: stay tuned. *Trends Immunol*. 2018;39(3):222–239. https://doi.org/10.1016/j.it.2017.12.001.

43. Hansasuta P, Dong T, Thananchai H, et al. Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific. *Eur J Immunol*. 2004;34(6):1673–1679. https://doi.org/10.1002/eji.200425089.
44. Yawata M, Yawata N, Draghi M, et al. MHC class I-specific inhibitory receptors and their ligands structure diverse human NK-cell repertoires toward a balance of missing self-response. *Blood*. 2008;112(6):2369–2380. https://doi.org/10.1182/blood-2008-03-143727.
45. Anfossi N, Andre P, Guia S, et al. Human NK cell education by inhibitory receptors for MHC class I. *Immunity*. 2006;25(2):331–342. https://doi.org/10.1016/j.immuni.2006.06.013.
46. Naiyer MM, Cassidy SA, Magri A, et al. KIR2DS2 recognizes conserved peptides derived from viral helicases in the context of HLA-C. *Sci Immunol*. 2017;2(15). https://doi.org/10.1126/sciimmunol.aal5296.
47. van der Ploeg K, Chang C, Ivarsson MA, et al. Modulation of human leukocyte antigen-C by human cytomegalovirus stimulates KIR2DS1 recognition by natural killer cells. *Front Immunol*. 2017;8:298. https://doi.org/10.3389/fimmu.2017.00298.
48. Chewning JH, Gudme CN, Hsu KC, et al. KIR2DS1-positive NK cells mediate alloresponse against the C2 HLA-KIR ligand group in vitro. *J Immunol*. 2007;179(2):854–868. https://doi.org/10.4049/jimmunol.179.2.854.
49. Fauriat C, Ivarsson MA, Ljunggren HG, et al. Education of human natural killer cells by activating killer cell immunoglobulin-like receptors. *Blood*. 2010;115(6):1166–1174. https://doi.org/10.1182/blood-2009-09-245746.
50. Morishima Y, Kawase T, Malkki M, et al. Significance of ethnicity in the risk of acute graft-versus-host disease and leukemia relapse after unrelated donor hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2013;19(8):1197–1203. https://doi.org/10.1016/j.bbmt.2013.05.020.
51. Petersdorf EW, Stevenson P, Malkki M, et al. Patient HLA germline variation and transplant survivorship. *J Clin Oncol*. 2018;36(24):2524–2531. https://doi.org/10.1200/jco.2017.77.6534.
52. Fernandez-Vina MA, Klein JP, Haagenson M, et al. Multiple mismatches at the low expression HLA loci DP, DQ, and DRB3/4/5 associate with adverse outcomes in hematopoietic stem cell transplantation. *Blood*. 2013;121(22):4603–4610. https://doi.org/10.1182/blood-2013-02-481945.
53. Fleischhauer K, Shaw BE, Gooley T, et al. Effect of T-cell-epitope matching at HLA-DPB1 in recipients of unrelated-donor haemopoietic-cell transplantation: a retrospective study. *Lancet Oncol*. 2012;13(4):366–374. https://doi.org/10.1016/s1470-2045(12)70004-9.
54. Apps R, Qi Y, Carlson JM, et al. Influence of HLA-C expression level on HIV control. *Science*. 2013;340(6128):87–91. https://doi.org/10.1126/science.1232685.
55. Petersdorf EW, Gooley TA, Malkki M, et al. HLA-C expression levels define permissible mismatches in hematopoietic cell transplantation. *Blood*. 2014;124(26):3996–4003. https://doi.org/10.1182/blood-2014-09-599969.
56. Petersdorf EW, Malkki M, O'HUigin C, et al. High HLA-DP expression and graft-versus-host disease. *N Engl J Med*. 2015;373(7):599–609. https://doi.org/10.1056/NEJMoa1500140.
57. Thomas R, Apps R, Qi Y, et al. HLA-C cell surface expression and control of HIV/AIDS correlate with a variant upstream of HLA-C. *Nat Genet*. 2009;41(12):1290–1294. https://doi.org/10.1038/ng.486.
58. Kulkarni S, Savan R, Qi Y, et al. Differential microRNA regulation of HLA-C expression and its association with HIV control. *Nature*. 2011;472(7344):495–498. https://doi.org/10.1038/nature09914.
59. Vince N, Li H, Ramsuran V, Naranbhai V, Duh FM, Fairfax BP, Carrington M. HLA-C Level is regulated by a polymorphic oct1 binding site in the HLA-C promoter region. *Am J Hum Genet*. 2016;99(6):1353–1358. https://doi.org/10.1016/j.ajhg.2016.09.023.
60. Schone B, Bergmann S, Lang K, Wagner I, Schmidt AH, Petersdorf EW, Lange V. Predicting an HLA-DPB1 expression marker based on standard DPB1 genotyping: linkage analysis of over 32,000 samples. *Hum Immunol*. 2018;79(1):20–27. https://doi.org/10.1016/j.humimm.2017.11.001.
61. Morishima S, Shiina T, Suzuki S, Ogawa S, Sato-Otsubo A, Kashiwase K, Morishima Y. Evolutionary basis of HLA-DPB1 alleles affects acute GVHD in unrelated donor stem cell transplantation. *Blood*. 2018;131(7):808–817. https://doi.org/10.1182/blood-2017-08-801449.
62. Soe NN, Yin Y, Valenzuela NM, Jin Y-P, Rossetti M, Reed EF, Zhang QJ. OR3 HLA-DPB1 single nucleotide polymorphism determines DP molecule expression and B lymphocyte crossmatch results. *Hum. Immunol*. 2017;78:3. https://doi.org/10.1016/j.humimm.2017.06.009.
63. Lorentino F, Sacchi N, Oldani E, Miotti V, Picardi A, Gallina AM, Bonifazi F. Comparative evaluation of biological human leukocyte antigen DPB1 mismatch models for survival and graft-versus-host disease prediction after unrelated donor hematopoietic cell transplantation. *Haematologica*. 2020;105(4):e186–e189. https://doi.org/10.3324/haematol.2019.225177.
64. Braud V, Jones EY, McMichael A. The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. *Eur J Immunol*. 1997;27(5):1164–1169. https://doi.org/10.1002/eji.1830270517.
65. Ruggeri L, Capanni M, Casucci M, Volpi I, Tosti A, Perruccio K, Velardi A. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood*. 1999;94(1):333–339.
66. Ruggeri L, Mancusi A, Capanni M, Urbani E, Carotti A, Aloisi T, Velardi A. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. *Blood*. 2007;110(1):433–440. https://doi.org/10.1182/blood-2006-07-038687.
67. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature*. 2002;419(6908):734–738. https://doi.org/10.1038/nature01112.
68. Russo A, Oliveira G, Berglund S, Greco R, Gambacorta V, Cieri N, Vago L. NK cell recovery after haploidentical HSCT with posttransplant cyclophosphamide: dynamics and clinical implications. *Blood*. 2018;131(2):247–262. https://doi.org/10.1182/blood-2017-05-780668.
69. Hsu KC, Keever-Taylor CA, Wilton A, Pinto C, Heller G, Arkun K, Dupont B. Improved outcome in HLA-identical sibling hematopoietic stem-cell transplantation for acute myelogenous leukemia predicted by KIR and HLA genotypes. *Blood*. 2005;105(12):4878–4884. http://doi.org/10.1182/blood-2004-12-4825.
70. Yu J, Venstrom JM, Liu XR, Pring J, Hasan RS, O'Reilly RJ, Hsu KC. Breaking tolerance to self, circulating natural killer cells expressing inhibitory KIR for non-self HLA exhibit effector function after T cell-depleted allogeneic hematopoietic cell transplantation. *Blood*. 2009;113(16):3875–3884. http://doi.org/10.1182/blood-2008-09-177055.
71. Arima N, Kanda J, Yabe T, Morishima Y, Tanaka J, Kako S, Kanda Y. Increased relapse risk of acute lymphoid leukemia in homozygous HLA-C1 patients after HLA-matched allogeneic transplantation: a Japanese National Registry Study. *Biol Blood Marrow Transplant*. 2020;26(3):431–437. https://doi.org/10.1016/j.bbmt.2019.10.032.
72. Hsu KC, Gooley T, Malkki M, Pinto-Agnello C, Dupont B, Bignon JD, Petersdorf E. KIR ligands and prediction of relapse after unrelated donor hematopoietic cell transplantation for hematologic malignancy. *Biol Blood Marrow Transplant*. 2006;12(8):828–836. https://doi.org/10.1016/j.bbmt.2006.04.008.
73. Shimoni A, Vago L, Bernardi M, Yerushalmi R, Peccatori J, Greco R, Ciceri F. Missing HLA C group 1 ligand in patients with AML and MDS is associated with reduced risk of relapse and better survival after allogeneic stem cell transplantation with fludarabine and treosulfan reduced toxicity conditioning. *Am J Hematol*. 2017;92(10):1011–1019. http://doi.org/10.1002/ajh.24827.
74. Martinez-Losada C, Martin C, Gonzalez R, Manzanares B, Garcia-Torres E, Herrera C. Patients lacking a KIR-ligand of HLA group C1 or C2 have a better outcome after umbilical cord blood transplantation. *Front Immunol*. 2017;8:810. https://doi.org/10.3389/fimmu.2017.00810.
75. Venstrom JM, Gooley TA, Spellman S, Pring J, Malkki M, Dupont B, Hsu KC. Donor activating KIR3DS1 is associated with decreased acute GVHD in unrelated allogeneic hematopoietic stem cell transplantation. *Blood*. 2010;115(15):3162–3165. https://doi.org/10.1182/blood-2009-08-236943.
76. Cooley S, Weisdorf DJ, Guethlein LA, Klein JP, Wang T, Marsh SG, Miller JS. Donor killer cell Ig-like receptor B haplotypes, recipient HLA-C1, and HLA-C mismatch enhance the clinical benefit of unrelated transplantation for acute myelogenous leukemia. *J Immunol*. 2014;192(10):4592–4600. https://doi.org/10.4049/jimmunol.1302517.
77. Venstrom JM, Pittari G, Gooley TA, Chewning JH, Spellman S, Haagenson M, Hsu KC. HLA-C-dependent prevention of leukemia relapse by donor activating KIR2DS1. *N Engl J Med*. 2012;367(9):805–816. https://doi.org/10.1056/NEJMoa1200503.
78. Weisdorf D, Cooley S, Wang T, Trachtenberg E, Vierra-Green C, Spellman S, Miller JS. KIR B donors improve the outcome for AML patients given reduced intensity conditioning and unrelated donor transplantation. *Blood Advances*. 2020;4(4):740–754. https://doi.org/10.1182/bloodadvances.2019001053.
79. Saunders PM, Pymm P, Pietra G, Hughes VA, Hitchen C, O'Connor GM, Vivian JP. Killer cell immunoglobulin-like receptor 3DL1 polymorphism defines distinct hierarchies of HLA class I recognition. *J Exp Med*. 2016;213(5):791–807. https://doi.org/10.1084/jem.20152023.
80. Carr WH, Pando MJ, Parham P. KIR3DL1 polymorphisms that affect NK cell inhibition by HLA-Bw4 ligand. *J Immunol*. 2005;175(8):5222–5229. https://doi.org/10.4049/jimmunol.175.8.5222.
81. Gardiner CM, Guethlein LA, Shilling HG, Pando M, Carr WH, Rajalingam R, Parham P. Different NK cell surface phenotypes defined by

the DX9 antibody are due to KIR3DL1 gene polymorphism. *J Immunol*. 2001;166(5):2992–3001. https://doi.org/10.4049/jimmunol.166.5.2992.

82. Pando MJ, Gardiner CM, Gleimer M, McQueen KL, Parham P. The protein made from a common allele of KIR3DL1 (3DL1*004) is poorly expressed at cell surfaces due to substitution at positions 86 in Ig domain 0 and 182 in Ig domain 1. *J Immunol*. 2003;171(12):6640–6649. https://doi.org/10.4049/jimmunol.171.12.6640.
83. Boudreau JE, Giglio F, Gooley TA, et al. KIR3DL1/HLA-B subtypes govern acute myelogenous leukemia relapse after hematopoietic cell transplantation. *J Clin Oncol*. 2017;35(20):2268–2278. https://doi.org/10.1200/jco.2016.70.7059.
84. Sobecks RM, Wang T, Askar M, et al. Impact of KIR and HLA genotypes on outcomes after reduced-intensity conditioning hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2015;21(9):1589–1596. https://doi.org/10.1016/j.bbmt.2015.05.002.
85. Verneris MR, Miller JS, Hsu KC, Wang T, Sees JA, Paczesny S, Lee SJ. Investigation of donor KIR content and matching in children undergoing hematopoietic cell transplantation for acute leukemia. *Blood Adv*. 2020;4(7):1350–1356. https://doi.org/10.1182/bloodadvances.2019001284.
86. Babor F, Peters C, Manser AR, Glogova E, Sauer M, Potschger U, Meisel R. Presence of centromeric but absence of telomeric group B KIR haplotypes in stem cell donors improve leukaemia control after HSCT for childhood ALL. *Bone Marrow Transplant*. 2019;54(11):1847–1858. https://doi.org/10.1038/s41409-019-0543-z.
87. Schetelig J, Baldauf H, Heidenreich F, Massalski C, Frank S, Sauter J. External validation of models for KIR2DS1/KIR3DL1-informed selection of hematopoietic cell donors fails. *Blood*. 2020;135(16):1386–1395. https://doi.org/10.1182/blood.2019002887.
88. Shiina T, Hosomichi K, Inoko H, Kulski JK. The HLA genomic loci map: expression, interaction, diversity and disease. *J Hum Genet*. 2009;54(1):15–39. https://doi.org/10.1038/jhg.2008.5.
89. Askar M, Sobecks R, Wang T, et al. MHC Class I Chain-Related Gene A (MICA) Donor-Recipient Mismatches and MICA-129 Polymorphism in unrelated donor hematopoietic cell transplantations has no impact on outcomes in acute lymphoblastic leukemia, acute myeloid leukemia, or myelodysplastic syndrome: a center for international blood and marrow transplant research study. *Biol Blood Marrow Transplant*. 2017;23(3):436–444. https://doi.org/10.1016/j.bbmt.2016.11.021.
90. Carapito R, Jung N, Kwemou M, et al. Matching for the nonconventional MHC-I MICA gene significantly reduces the incidence of acute and chronic GVHD. *Blood*. 2016;128(15):1979–1986. https://doi.org/10.1182/blood-2016-05-719070.
91. Fuerst D, Neuchel C, Niederwieser D, et al. Matching for the MICA-129 polymorphism is beneficial in unrelated hematopoietic stem cell transplantation. *Blood*. 2016;128(26):3169–3176. https://doi.org/10.1182/blood-2016-05-716357.
92. Anderson E, Grzywacz B, Wang H, et al. Limited role of MHC class I chain-related gene A (MICA) typing in assessing graft-versus-host disease risk after fully human leukocyte antigen-matched unrelated donor transplantation. *Blood*. 2009;114(21):4753–4754. author reply 4754-4755. doi:10.1182/blood-2009-08-239301.
93. Mayor NP, Hayhurst JD, Turner TR, et al. Recipients receiving better HLA-matched hematopoietic cell transplantation grafts, uncovered by a novel HLA typing method, have superior survival: a retrospective study. *Biol Blood Marrow Transplant*. 2019;25(3):443–450. https://doi.org/10.1016/j.bbmt.2018.12.768.

CHAPTER 107

HAPLOIDENTICAL HEMATOPOIETIC STEM CELL TRANSPLANTATION

Stefan O. Ciurea

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (alloHSCT) is an effective treatment for a wide range of hematologic malignancies, nonmalignant hematologic diseases, and immunologic disorders.[1] Sources of donor stem cells for alloHSCT include human leukocyte antigen (HLA)-matched siblings (MSD), HLA-matched unrelated adult donors (MUD) matched at least at HLA-A, -B, -C, -DRB1, HLA-mismatched unrelated donors, unrelated umbilical cord blood, and HLA half-matched related donors, mismatched at 2 or more HLA antigens (haploidentical donors, HRD). Haploidentical donors are matched at one full haplotype (see later) with various degree of matching in the other haplotype (6 to 10 out of 12 allele match). Historically, the most important consideration in choosing between graft sources has been the degree of match between donor and recipient. A fully HLA-matched sibling has been traditionally the preferred donor because transplants using such donors have been associated with the lowest incidence of treatment-related complications and best overall survival (OS). Unfortunately, only 25% to 30% of patients referred for transplantation have an MSD available. For other patients, a MUD has been the preferred alternative; however, availability of a MUD varies widely based on patient race/ethnicity, from approximately 70% for individuals of Northern European descent to less than 20% for African Americans or Asians.[2] In addition, a longer time to obtain the unrelated donor cells, on average of 3 to 4 months, may expose the patients in urgent need for a transplant to risk of disease progression.[3] Increased HLA mismatching has been associated with increased alloreactivity between donor and recipient T cells, leading, in general, to worse outcomes including higher risks of graft rejection, graft-versus-host disease (GVHD), and nonrelapse mortality (NRM). Over the past two decades, significant progress has been made in mitigating alloreactive reactions between the donor and recipient, which has enabled an increase in use of haploidentical donors for transplantation, now the fastest growing source of hematopoietic stem cells,[4] with improved transplant outcomes, similar to HLA-matched donor transplants, and potential to become the alternative donor of choice for patients without a MSD, at least for those in need of an urgent transplant.

There are several advantages of using haploidentical donors for transplantation (see box on The Increasing Popularity of Human Leukocyte Antigen–Haploidentical Donors):

1. *Near-universal availability of highly motivated donors:* Patients have an average of 2.7 potential HLA-haploidentical donors among first-degree relatives;
2. *Rapid availability:* As compared with MUDs, a haploidentical donor can be identified, screened, and collected within 2 weeks, and transplant can be performed in 3 weeks;
3. *Low cost of graft acquisition:* The costs of graft acquisition from MUD and especially from umbilical cord blood banks can be substantially higher than from related donors, including haploidentical donors[5];
4. *Adequate doses of HSCs:* An adequate number of HSCs can be obtained from HLA-haploidentical donors for adult recipients, as compared with umbilical cord blood grafts. The number of total nucleated calls infused has been correlated with all transplant outcomes[6,7];
5. *Availability of the donor for repeated donations of HSCs (stem cell boost) in case of poor graft function or therapeutic cells (lymphocytes) to prevent/treat relapse or viral infections;*

GLOBAL EXPANSION OF HUMAN LEUKOCYTE ANTIGEN–HAPLOIDENTICAL DONORS

The number of human leukocyte antigen (HLA)-haploidentical stem cell transplant procedures performed is increasing on a global scale. China is the leader in HLA-haploidentical ("haplo") stem cell transplants (SCTs) on a per-country basis, perhaps because of the former one-child policy that has nullified the availability of an HLA-matched sibling for many patients. In the past 5 years, the number of haplo transplants performed in Europe and the United States has more than doubled. Reports of successful haplo SCTs are appearing in the published literature from countries with more limited economic resources, such as Brazil, India, and Romania.

There are clinical, practical, and economic reasons for the increasing popularity of HLA-haploidentical SCT. Clinically, results of the procedure have improved dramatically over the past 10 to 15 years, to the point that the outcomes of haplo SCT approach or equal those of HLA-matched sibling or unrelated donor transplantation (see Comparative Outcomes Between Haploidentical and Other Graft Sources). Another major practical advantage of the haplo option is donor availability. A haplo donor can be found for nearly every patient referred for allogeneic SCT, because every biologic child or parent of a patient is HLA haploidentical, and each sibling or half-sibling has a 50% chance of being HLA haploidentical. The likelihood of finding a haplo donor increases further if one is willing to consider second-degree relatives, such as aunts, uncles, nieces, nephews, or cousins, as donors. The wide availability of haplo donors is especially important for members of ethnic minority groups that are underrepresented in registries of volunteer unrelated donors. Relatives, especially parents and children, tend to be highly motivated to donate and can do so more than once if needed to treat graft failure or relapse. The treating center has greater control over the timing of transplants when haplo donors rather than unrelated donors are used. Clinical trials and advances in adoptive cellular therapy of cancer may be easier using related donors because unrelated donor lymphocytes for infusion are regulated by the US Food and Drug Administration as a biologic. Thus an investigational new drug application must be filed for any clinical trial employing the infusion of lymphocytes from unrelated donors, but not for minimally manipulated lymphocytes from related donors.

Finally, the cost of obtaining the graft from haploidentical donors is cheaper compared with stem cells from unrelated adult volunteers or from umbilical cord blood. This, combined with the growing availability of inexpensive methods of graft-versus-host disease prophylaxis for haplo SCT (especially posttransplant cyclophosphamide), makes haploidentical transplants increasingly attractive in countries with limited economic resources and a fixed budget for allogeneic stem cell transplantation. Thus, haploidentical donor transplant as related donor transplants, largely eliminate cost differences compared with HLA-matched related donor transplants.

6. *Potential for a stronger graft-versus-leukemia (GVL) effect:* Increase alloreactive reactions generated by higher degree of HLA mismatch may be associated with a stronger GVL effect compared with MSD transplants, with some studies suggesting a lower cumulative incidence of relapse[8] and an improved survival[9–11]; however, the full potential of this stronger GVL has not yet been fully realized.

HISTORICAL PERSPECTIVE

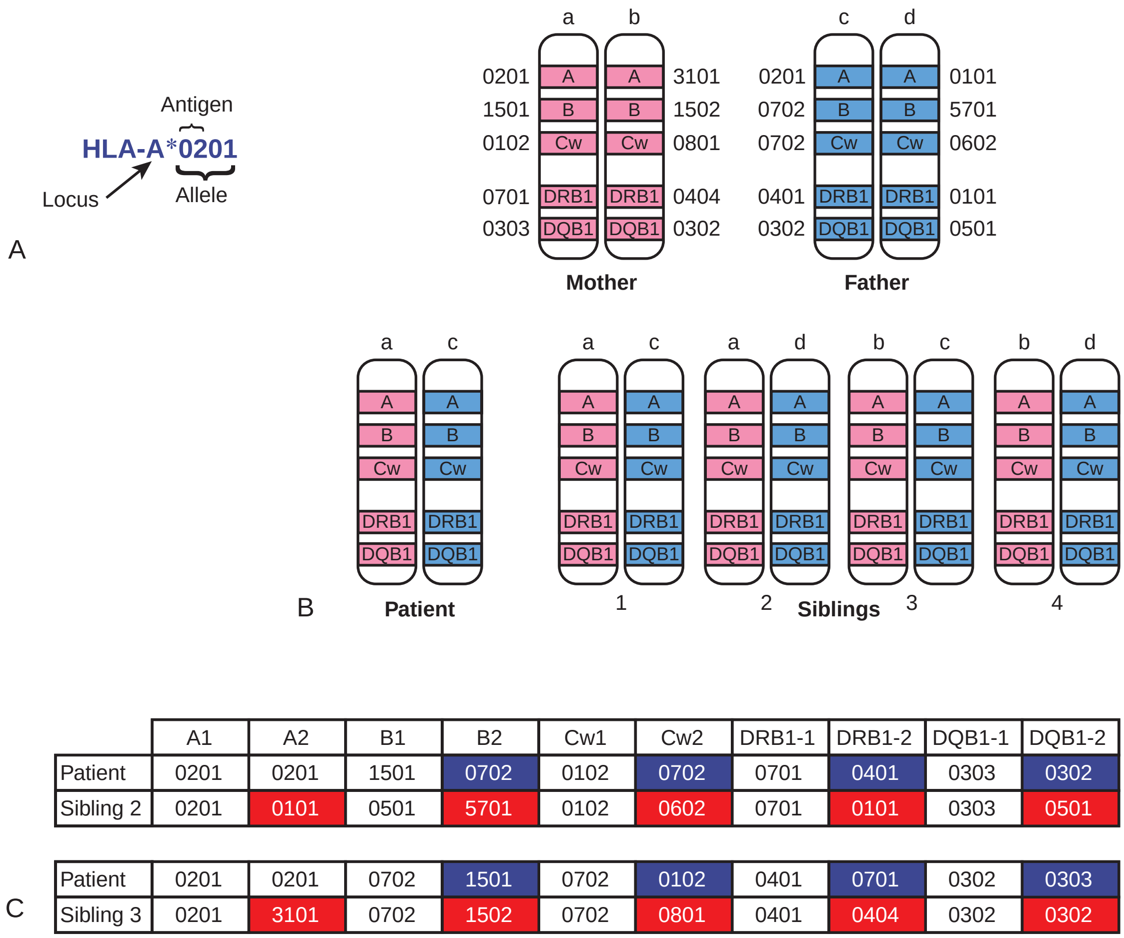
The major challenge in performing haploidentical HSCT (haploSCT) with conventional GVHD prophylaxis has been overcoming the intense bidirectional alloreactive reactions in the host-versus-graft (HVG) and graft-versus-host (GVH) direction, primarily mediated by donor T cells against recipient's HLA antigens,[12] resulting in high incidence of graft rejection, hyperacute GVHD, and treatment-related mortality.[13] In an analysis of more than 2000 allogeneic transplants performed between 1985 and 1991 and reported to the International Bone Marrow Transplant Registry,[14] compared with MSD transplants and using conventional GVHD prophylaxis, two HLA antigen-mismatched related donor transplants resulted in higher incidence of NRM (55% vs. 21% at 3 years post-transplant), graft failure (16% vs. 1%), moderate-severe acute GVHD (56% vs. 29%), and chronic GVHD (60% vs. 42%). Complete T-cell depletion (TCD) using CD34+ selected grafts has been subsequently used to reduce the incidence of acute GVHD; however, an increased risk of graft rejection and treatment-related mortality secondary to infectious complications emerged.[15–17] Infusion of "mega-dose" CD34+ cells[18] decreased the incidence of graft failure; however, it could not compensate for the almost complete lack of donor T cells (especially memory T cells) in the graft, and a higher incidence of infectious complications, especially viral infections, persisted, which was the main cause of death in these patients.[19] Consequently, it became apparent that complete T-cell depletion cannot provide better outcomes, and various attempts to maintain different lymphocyte subsets in the graft have been subsequently studied.[20,21] The Tübingen and Memphis groups introduced $CD3^+/CD19^+$ depletion rather than positive selection of $CD34^+$ cells to produce a graft that retains other cell subsets in the graft (such as NK cells, monocytes, dendritic cells, and other myeloid cells) that might facilitate a faster immune recovery without excessive GVHD, [22] and generate a lower treatment-related mortality.[20] In one study in adults, all but 1 of 29 patients engrafted; however, grades II–IV acute GVHD occurred in 48% of patients, 8 patients (28%) had NRM, 7 died as a result of infection. The 2-year survival was only approximately 20%. However, this approach appeared to be associated with better outcomes in children. In a study of 46 children receiving $CD3^+/CD19^+$-depleted grafts after myeloablative conditioning, successful engraftment was shown in 88% of patients, and grades II–IV acute GVHD occurred in 20%, grades III–IV acute GVHD in 7%, chronic GVHD in 21%, and NRM in 20%.[22] In this latter study, relapse occurred in 63% of patients after 2 years of follow-up; however, 43% of patients undergoing transplant had active disease at the time of treatment. The increased risk of infection seen after TCD haploSCT may be due to the depletion from the adult donor graft of pathogen-specific memory T cells that provide protection from infections until new donor T cells differentiate from hematopoietic precursors in the recipient thymus. Subsequent research has been focused on infusing grafts containing selected populations of T cells to enhance immune reconstitution without substantially increasing the risk of GVHD. Adding back a fixed number of T cells that have been depleted of alloreactive cells or that have been anergized was proposed, with various degrees of success in improving immune reconstitution post-transplant.[23,24] Several major advances emerged after year 2000, when graft manipulation focused on partial T-cell depletion of alloreactive T cells[25,26] and pharmacologic modulation applied early post-transplant,[27] have made major progress in reducing the incidence of infectious complications post-transplant, without increasing the incidence of GVHD and NRM. These maneuvers have significantly improved survival of patients receiving haploidentical transplantation,[19,28–30] making this graft source an equal alternative to unrelated donor grafts, with similar outcomes and pattern of immunologic reconstitution post-transplant (see Modern Approaches to Haploidentical Transplantatiom).[31–33]

IMMUNOLOGICAL CONSIDERATIONS OF MAJOR HUMAN LEUKOCYTE ANTIGEN MISMATCHED GRAFTS

A haplotype is a set of genes closely grouped together on a chromosome, inherited as a biologic unit. The HLA locus on chromosome 6p13.2 comprises a set of tightly linked genes encoding molecules that present peptide antigens to T cells. The HLA locus contains three regions: (1) Class I region—encodes the "classical" class I HLA genes HLA-A, HLA-B, and HLA-C, which present antigens to $CD8^+$ T cells, as well as nonclassical HLA-E, HLA-F, and HLA-G molecules; (2) Class II region—encodes HLA-DRB1, HLA-DQB1, and HLA-DPB1, which present antigens to $CD4^+$ T cells, as well as nonclassical class II molecules HLA-DM and HLA-DO; (3) Class III region—encodes molecules not known to be involved in histocompatibility reactions. An HLA haplotype is defined as the set of histocompatibility genes present on chromosome 6 which are transmitted together. Each individual has two HLA haplotypes, one inherited from each parent. An HLA-haploidentical donor is a related donor who shares one full haplotype with the transplant recipient, while the degree of HLA matching on the mismatched haplotype may vary between 0 (full haplotype mismatch, 5/10 HLA matched) to 3 (8/10) (of note a 9/10 HLA related donor is not considered a haploidentical donor). Recently, high-resolution HLA typing is usually performed for three HLA class I genes, HLA-A, HLA-B, and HLA-C, and three class II genes, HLA-DRB1, HLA-DQB1, and HLA-DPB1, to confirm that the donor and recipient share at least at one full haplotype as, rarely, mismatches can occur during meiosis (crossover). By definition, a parent and a child are HLA haploidentical to each other, and each biological sibling or half-sibling of a patient has a 50% chance of being HLA haploidentical to each other. Other potential haploidentical donors include second- or third-degree related donors—aunts, uncles, nieces, and nephews, who each have a 50% chance of being HLA haploidentical, and cousins, who have a 25% chance of being HLA haploidentical with the recipient.

Mismatching of HLA alleles or antigens can occur in the GVH direction only, the HVG direction only, or bidirectionally. When the donor is homozygous for an HLA allele but the recipient is heterozygous at the same genetic locus, there is a mismatch in the GVH direction only. Conversely, when the recipient is homozygous for an HLA allele but the donor is heterozygous, there is a mismatch in the HVG direction only. In general, HLA mismatches in the GVH direction favor GVHD, whereas HLA mismatches in the HVG direction predispose to rejection of the hematopoietic stem cell graft mostly by recipient's T cells. The number of HLA mismatches between a haploidentical donor and recipient should be expressed as the number of mismatches in the GVH direction, as well as the number of mismatches in the HVG direction. For example, the patient in Fig. 107.1 differs from sibling 2 by four antigens (and alleles) in the GVH direction and by five antigens (and alleles) in the HVG direction, and from sibling 3 by three antigens (vs. four alleles) in the GVH direction and by three antigens (versus five alleles) in the HVG direction. More recently, molecular mismatches evaluating distinct amino acid configurations on the surface of the HLA molecule that could elicit immune responses (eplets) in both HVG and GVH directions are being evaluated, as early data suggest that could impact transplant outcomes.[34,35]

The biologic mechanisms underlying the high incidence of graft rejection and severe GVHD when crossing major HLA barriers remain to be elucidated; however, two fundamental characteristics of T-cell alloreactivity are likely responsible. The first is the high frequency of



	A1	A2	B1	B2	Cw1	Cw2	DRB1-1	DRB1-2	DQB1-1	DQB1-2
Patient	0201	0201	1501	0702	0102	0702	0701	0401	0303	0302
Sibling 2	0201	0101	0501	5701	0102	0602	0701	0101	0303	0501

Patient	0201	0201	0702	1501	0702	0102	0401	0701	0302	0303
Sibling 3	0201	3101	0702	1502	0702	0801	0401	0404	0302	0302

Figure 107.1 HIGH-RESOLUTION HUMAN LEUKOCYTE ANTIGEN TYPING IN A REPRESENTATIVE FAMILY. (A) Nomenclature employed for high-resolution typing. The human leukocyte antigen (HLA) genetic locus is identified by the capital letter after the hyphen. The first two digits after the asterisk are the antigen designation, which facilitates comparisons of results of low- or intermediate-resolution versus high-resolution HLA typing. The four-digit code designates allele level type by high-resolution methods, such as sequence-based typing or a combination of sequence-specific priming and sequence-specific oligonucleotide probes. (B) Family typing. Results of high-resolution HLA typing are shown for the parents. Based upon HLA typing of the children, membership of individual HLA alleles in haplotypes, designated "a," "b," "c," and "d," are assigned. The allele composition of the haplotypes is then used to assign haplotype inheritance in the children. The patient is genotypically HLA matched to sibling 1 and is completely mismatched for both HLA haplotypes with sibling 4. Siblings 2 and 3 are partially HLA mismatched, or HLA haploidentical, to the patient, and would be considered potential donors for allogeneic stem cell transplant only if sibling 1 is not a suitable or willing donor. (C) High-resolution HLA typing of the patient is aligned with typing of the HLA-haploidentical siblings to facilitate analysis of the degree of mismatching in both the rejection (host-versus-graft) and graft-versus-host directions. Alleles of the shared haplotype are aligned in columns A1, B1, Cw1, DRB1-1, and DQB1-1. Alleles that are mismatched in the graft-versus-host direction are in *blue boxes*, whereas alleles that are mismatched in the host-versus-graft direction are outlined in *red boxes*.

T cells reactive against allogeneic HLA molecules. T cells recognize a complex determinant comprising specific amino acid residues of the HLA molecule as well as amino acid residues of the bound peptide. It has been estimated that there are 50,000 to 100,000 copies of each HLA molecule on a cell surface, with as many as 5000 different peptides being presented. Each allogeneic HLA molecule provides as many as 5000 distinct recognition units corresponding to 5000 distinct alloreactive T cells. In contrast, a non-HLA or "minor" histocompatibility antigen consists of a single allelic peptide presented by a single species of HLA molecule. The frequency of T cells reactive to a single minor histocompatibility antigen is on the order of 1 in 50,000 the frequency of anti-HLA alloreactive T cells.[12] The higher frequency of T cells reactive to allogeneic HLA molecules compared to minor histocompatibility antigens corresponds to a higher incidence of graft rejection and GVHD after HLA-haploidentical SCT than after HLA-matched SCT. The second property of alloreactive T cells that contributes to a high incidence of graft failure and GVHD after haploSCT is a significant proportion of HLA-alloreactive memory T cells,[36] even in donors and recipients who have not been exposed to allogeneic HLA molecules through pregnancy or blood transfusions. T cells that are immunized against environmental antigens, especially viruses, can cross-react against allogeneic major histocompatibility complex (MHC) molecules.[37,38] The phenomenon, in which viral infection triggers cross-reactive memory against allogeneic HLA molecules, is termed *heterologous immunity* and may be a powerful barrier to the establishment of donor hematopoietic cell chimerism[39] or the induction of tolerance to transplanted organs.[40] Unlike naïve T cells, which are sensitive to chemotherapy-induced apoptosis and to immunologic tolerance induction by antigen without costimulation,[41] memory T cells are more resistant to chemotherapy-induced death, can induce costimulatory signals on antigen-presenting cells that they encounter,[42] and are resistant to tolerance induction by regulatory T cells (Tregs)[43,44] or to T-cell–depleting antibodies.[45–47] The presence and substantial number of memory T cells reactive to allogeneic HLA molecules make control of graft rejection and GVHD more challenging after haploidentical versus HLA-matched transplantation.

B cells and NK cells also participate in the immune response to HLA-mismatched tissues. Allogeneic HLA molecules can elicit the formation of alloantibodies. Preexisting donor-specific antibodies

(DSA) against HLA molecules are a major risk factor for development of primary graft failure after HLA-haploidentical SCT.[48,49] Pregnancy and blood transfusions are sensitizing events that can lead to the formation of antibodies against HLA molecules. Pregnancy is the main mechanism of allosensitization, evident by the fact that the highest DSA levels were found in parous women.[50] The prevalence of DSA in parous women has been reported to be as high as 42%[51]; such sensitization is directed against unshared HLA molecules expressed by their children (father's HLA antigens).

DSA can be detected by flow cytometry using a Luminex platform using beads coated with single HLA molecules or by complement-dependent cytotoxicity (CDC) testing, in which the patient's serum is mixed with donor lymphocytes in the presence of complement.[50] In one study, a positive crossmatch for anti-donor lymphocytotoxic antibodies was associated with a 2.3-fold increased risk of graft failure.[52] Donors with high DSA levels, especially with complement-binding DSAs and/or a positive CDC crossmatch, are, in general, avoided for transplantation, unless a significant response to desensitization treatment is accomplished, as discussed later in this chapter.

Natural Killer Cell Alloreactivity After Allogeneic Transplantation

NK cells are a group of lymphoid cells part of the innate immune system capable of recognizing and eliminating malignantly transformed or virally infected cells. NK cells may play a significant role in inducing GVL effects after haploSCT in humans. Unlike T and B cells, they do not express rearranging receptors for antigen, but like $CD8^+$ T cells, they express receptors for HLA class I molecules, including HLA-B and HLA-C, secrete interferon-γ, and kill target cells via granzyme- and perforin-mediated cytotoxicity. Karre and Kiessling initially proposed in the "missing self" hypothesis as mechanism of action for NK cells.[53,54] It was subsequently found that NK cells are able to detect and rapidly eliminate virally infected or tumor cells that have downregulated cell surface expression of MHC class I molecules to evade the $CD8^+$ T-cell immune response. The molecular basis of NK-cell alloreactivity is incompletely understood; however, it involves a dynamic balance of signals through activating as well as inhibitory receptors on the surface of the NK cells. The killer immunoglobulin-like receptors (KIRs) are encoded by a set of linked genes called the leukocyte receptor complex (LRC) on human chromosome 19q13.4.[55] KIRs contain two or three extracellular Ig-like domains and either a short (S) or a long (L) cytoplasmic tail, which mediate activating or inhibitory signals, respectively. The LRC is marked by significant interindividual variation in KIR gene content as well as significant allelic variation in individual KIR genes.[56] As a consequence, the KIR locus is second only to the HLA locus in the number of polymorphisms, and unrelated individuals are unlikely to share KIR genotypes.

Distinct HLA class I molecules comprise the ligands for specific inhibitory KIRs (iKIRs). An organizing principle of NK-cell biology is that NK-cell self-tolerance is mediated by inhibitory signals delivered by self HLA class I molecules through iKIRs. The ontogeny of receptor expression on individual NK cells is poorly understood, but it is currently thought that each NK cell expresses at least one inhibitory receptor for a self HLA class I molecule.[57] There are four distinct categories of HLA ligands for iKIRs (Fig. 107.2). The C1 group of HLA ligands is characterized by the presence of an asparagine residue at position 80 of the HLA-C molecule and is recognized by either KIR2DL2 or KIR2DL3. The complementary C2 group of HLA ligands is distinguished by a lysine residue at position 80 of the HLA-C molecule and is recognized by KIR2DL1. The Bw4 serologic group is recognized by KIR3DL1. Finally, KIR3DL2 recognizes HLA-3 and HLA-11 molecules. Developing NK cells undergo a host MHC class I–dependent functional maturation process, termed *licensing*[58,59] or, more recently, *education*.[60] Education endows an NK cell with the ability to kill MHC class I–deficient targets but also provides a mechanism for self-tolerance because the same MHC class I ligand that licenses the developing NK cell also inhibits the activity of the mature NK cell. Licensing is not an all-or-none phenomenon, and there are degrees of licensing depending upon the affinity of an iKIR for its HLA ligand. Furthermore, there is evidence that transplanted NK cells can attune themselves to the new HLA environment, indicating that NK-cell alloreactivity is a complex and dynamic phenomenon.[61] Because the genes comprising the LRC and the HLA locus are on chromosomes 19 and 6, respectively, KIR and HLA molecules are inherited independently, so individuals can inherit an iKIR but not its ligand, resulting in an NK cell that cannot be licensed during development. Unlicensed NK cells would be predicted to be poorly functional; unexpectedly, however, they dominate the early response to cytomegalovirus (CMV) infection in mice,[62] and they also may be potent mediators of antibody-dependent cellular cytotoxicity.[63] DNA damage resulting from transplant conditioning or inflammatory conditions such as viral infection may be sufficient to induce stress ligands of NK-cell activation receptors,[64] break tolerance in unlicensed NK cells, and generate autoreactivity,[65–67] including tumor regression. In contrast to HLA-matched transplants, where donors and recipients express the same ligands for iKIRs, in HLA-haploidentical SCTs, the recipient may lack the HLA ligand for the iKIR on a licensed donor NK cell, releasing the cell from inhibition, and resulting in donor NK-cell alloreactivity. Unlicensed donor NK cells may also be activated by a dominance of stimulatory NK-cell ligands in the inflammatory milieu of a conditioned recipient, but this scenario is not unique to HLA-haploidentical SCT and may also occur after HLA-matched SCT as long as the recipient lacks HLA ligands for iKIR molecules. Although the mechanisms of NK-cell alloreactivity remain to be fully elucidated, there is great interest in harnessing their activity for preventing or controlling relapse of hematologic malignancy after HLA-haploidentical SCT.[68]

Models of Natural Killer Cell Alloreactivity After Transplant

The KIR ligand incompatibility model was first formulated by Ruggeri et al.[69–71] to account for NK alloreactivity after rigorously T-cell–depleted, stem-cell–enriched, HLA-haploidentical SCT.[18,72] The model predicts NK cell alloreactivity when the donor expresses, but the recipient lacks, an HLA ligand (HLA group C1, C2, or Bw4) for an iKIR. In this situation, the donor is predicted to contain NK cells that are activated by recipient cells lacking expression of that HLA molecule. Support for the ligand incompatibility model was provided by the ability to generate donor alloreactive NK clones in all 51 ligand incompatible donor—recipient pairs but in none of the 61 donors who were KIR ligand matched with their recipients.[71] NK alloreactivity in the GVH direction was predicted to have three functional consequences[70]: (1) a GVL effect arising from donor NK cytotoxicity against leukemia cells; (2) a decreased rate of graft rejection arising from donor NK-cell killing of host T cells; and (3) a decreased rate of GVHD arising from donor NK-cell elimination of host antigen-presenting cells such as dendritic cells, which are required to initiate GVH reactions.[73] This group (hereinafter the Perugia group) has demonstrated a strong antitumor effect of KIR ligand incompatibility in acute myeloid leukemia (AML) but not in acute lymphoblastic leukemia (ALL) patients undergoing T-cell depleted haploidentical transplantation.

The missing ligand model predicts NK-mediated GVH reactions when the transplant recipient is missing at least one of the three major classes of HLA ligands for iKIR. The missing ligand model differs from the ligand incompatibility model only in that it does not require the presence on donor cells of the HLA ligand that is missing in the recipient. Consequently, donors who are predicted by the ligand incompatibility model to contain alloreactive NK cells against their recipients are a subset of the donors who are predicted by the missing ligand model to contain anti-recipient alloreactive NK cells. The KIR

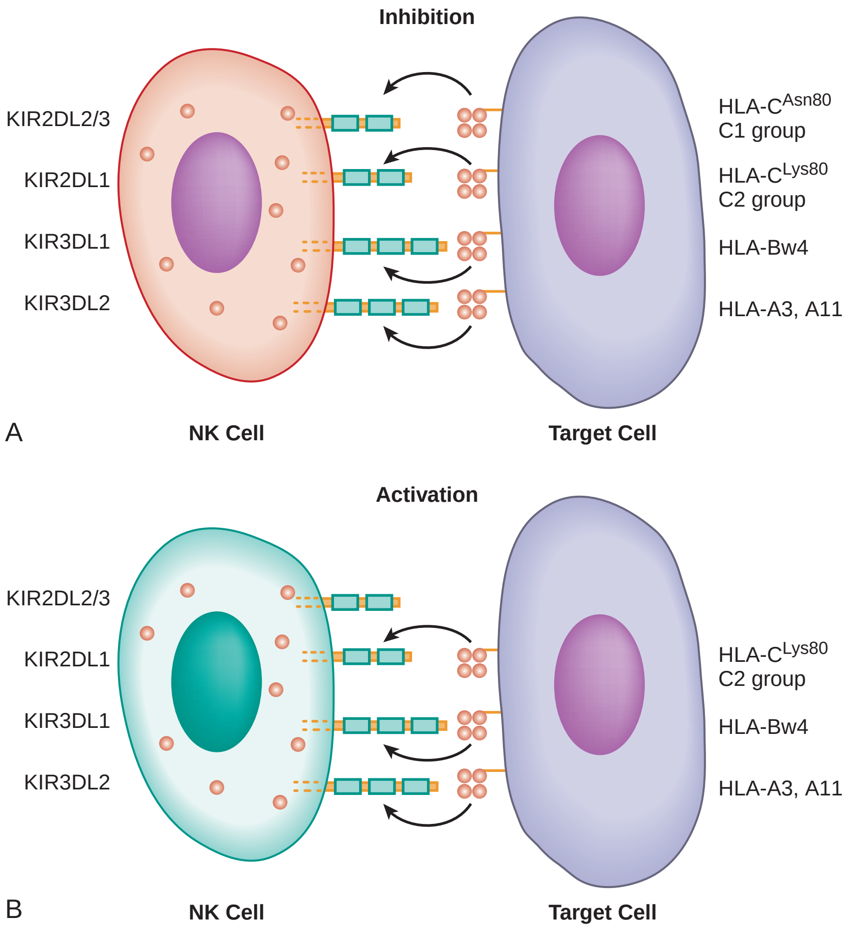


Figure 107.2 INTERACTIONS BETWEEN INHIBITORY KILLER IMMUNOGLOBULIN-LIKE RECEPTORS AND THEIR HUMAN LEUKOCYTE ANTIGEN LIGANDS OF RELEVANCE TO NATURAL KILLER CELL ALLOREACTIVITY AFTER ALLOGENEIC STEM CELL TRANSPLANT. For convenience, a single natural killer (NK) cell expressing four distinct inhibitory killer immunoglobulin-like receptors (iKIRs) is shown. Each NK cell need express only one molecular species of iKIR for functional maturation to occur. Individual members of the human leukocyte antigen (HLA)-C1, HLA-C2, and HLA-Bw4 groups are listed in Table 107.1. (A) High-resolution HLA typing is required to determine whether specific alleles of HLA-B and HLA-Cw are ligands of specific iKIRs. HLA typing of the recipient and KIR genotyping of donor NK cells are incorporated into the ligand incompatibility, receptor ligand, and missing ligand models of NK cell alloreactivity. Interactions between KIR3DL2 and HLA-A3 or HLA-A11 are generally not considered in these models. (B) Schematic view of missing ligand model. When the recipient cell lacks one or more HLA ligands (C1 is missing) for the licensed donor inhibitory KIR, the NK cell alloreactivity occurs in the graft-versus-host direction, possibly leading to the graft-versus-tumor effect.

ligand incompatibility and missing KIR ligand models were compared for their ability to predict relapse after T-cell–replete (TCR), unrelated SCT for hematologic malignancies.[74] Among recipients of HLA-mismatched transplants, recipient homozygosity for HLA-B or HLA-C KIR epitopes was used to define “missing” KIR ligands and was associated with a decreased hazard of relapse (hazard ratio [HR], 0.61; 95% confidence interval [CI], 0.43 to 0.85; $P = .004$). The effect was observed in patients with AML, chronic myeloid leukemia, or ALL. The same effect was not observed in HLA-identical transplants. KIR ligand incompatibility was not associated with a decreased risk of relapse in recipients of either HLA-mismatched or HLA-matched grafts.

With novel approaches to haploidentical transplantation, especially using post-transplantation cyclophosphamide, there has been a renewed interest in harnessing antitumor effect of NK cells. Although several large retrospective studies failed to show a survival benefit of a KIR ligand mismatched donor as described by the Perugia group[35,75] and cannot be recommended now for donor selection, at least for T-cell replete haploidentical transplants (see Selection of Donors for Haploidentical Transplantation),[76] recent data outlined the role of NK cells infused with the graft in prevention of GVHD, relapse, and survival post-transplant.[7]

MODERN APPROACHES TO HAPLOIDENTICAL TRANSPLANTATION (FIG. 107.3)

Refinements to the T-Cell Depletion Platform to Improve Immune Reconstitution

Early studies of TCD HLA-haploidentical SCT were characterized by lower incidence of GVHD; however, patients experienced a higher incidence of engraftment failure and high NRM, mostly due to infectious complications related to a significant delay in immunologic reconstitution.[19,33,77] These outcomes spurred efforts to improve engraftment rate and immune reconstitution after TCD haploSCT.

In order to improve immunologic reconstitution after transplant, the Perugia group proposed administration of a fixed dose of conventional T (Tcons) cells after TCD haploidentical transplantation, and, to prevent development of severe GVHD, infusion of immunomagnetically selected Tregs 4 days before transplant followed by Tcons cells on the same day as the TCD allograft.[78,79] In the first study of 28 patients only two developed grades II–IV acute GVHD, and both received the highest T-cell doses in the study cohort, while no chronic GVHD was observed.[78] T-cell reconstitution was improved with

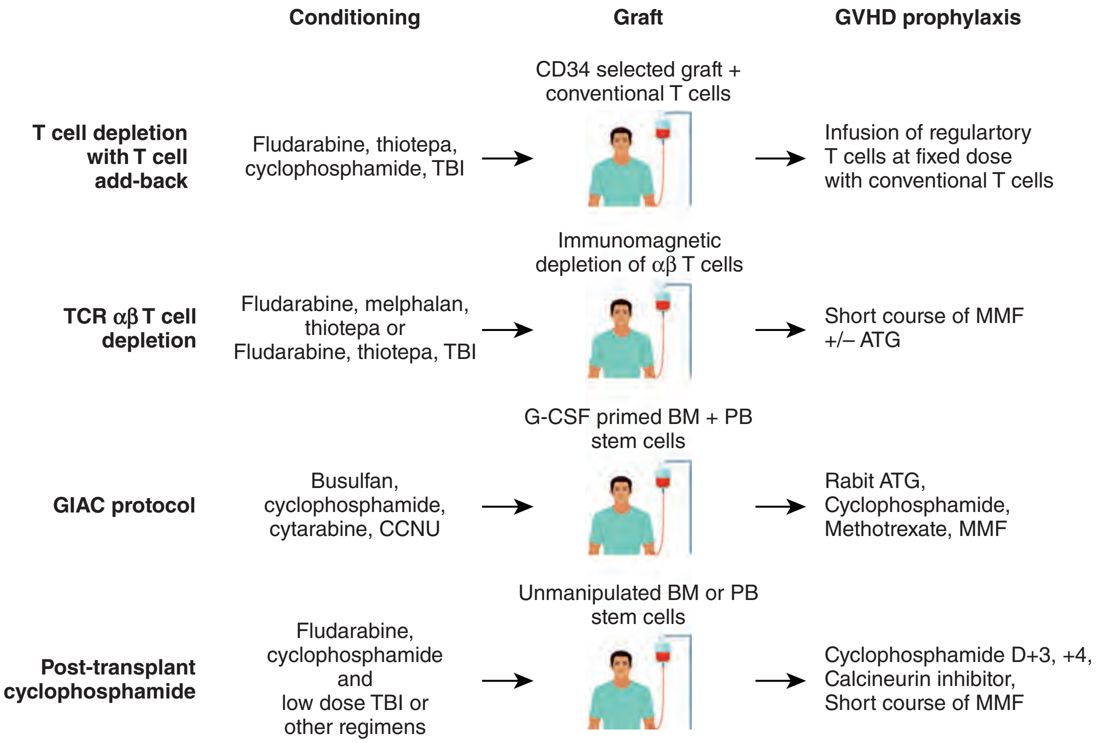

Conditioning
Graft
GVHD prophylaxis
T cell depletion with T cell add-back
Fludarabine, thiotepa, cyclophosphamide, TBI
CD34 selected graft + conventional T cells
Infusion of regulartory T cells at fixed dose with conventional T cells
TCR αβ T cell depletion
Fludarabine, melphalan, thiotepa or Fludarabine, thiotepa, TBI
Immunomagnetic depletion of αβ T cells
Short course of MMF +/– ATG
GIAC protocol
Busulfan, cyclophosphamide, cytarabine, CCNU
G-CSF primed BM + PB stem cells
Rabit ATG, Cyclophosphamide, Methotrexate, MMF
Post-transplant cyclophosphamide
Fludarabine, cyclophosphamide and low dose TBI or other regimens
Unmanipulated BM or PB stem cells
Cyclophosphamide D+3, +4, Calcineurin inhibitor, Short course of MMF

Figure 107.3 COMPONENTS OF EACH TRANSPLANTATION PLATFORM. Interventions on the donor or the recipient for each transplantation platform are shown at each stage of the transplant procedure. *ATG*, Antithymocyte globulin; *BM*, bone marrow; *BMT*, blood or bone marrow transplant; *GCSF*, granulocyte colony-stimulating factor; *GIAC*, granulocyte colony-stimulating factor stimulation of the donor; intensified immunosuppression including cyclosporine A, mycophenolate mofetil, and methotrexate; antithymocyte globulin; and combination of peripheral blood and bone marrow allografts; *G-PBSCs*, granulocyte colony-stimulating factor-mobilized peripheral blood stem cells; *MMF*, mycophenolate mofetil; *PBSCs*, peripheral blood stem cells; *PTCy*, post-transplant cyclophosphamide; *TBI*, total-body irradiation; *TCD*, T-cell depletion; *TCR*, T-cell receptor.

early expansion of T-cell repertoires and improved pathogen-specific responses were observed. Although none of the patients developed CMV-associated disease, NRM still occurred in 13 patients (50%), 8 of whom died of infectious complications. In an updated report on an expanded cohort of patients the authors showed similar results and suggested that, in both mouse models and human studies, the infusion of Tregs appears to control GVHD without affecting graft-versus-tumor immunity.[79] While relapse rates for patients treated in this study appeared better than in historical controls, NRM remained high, particularly death as a result of infection, despite laboratory evidence of improved immune reconstitution post-transplant.

Viral reactivation is a major cause of morbidity and mortality early, especially after TCD haploidentical transplantation, and, in general, a higher number of viral reactivations has been associated with higher NRM.[80] Hence, prevention and early treatment of viral infections post-transplant is crucial for the success of transplantation. Several groups explored infusion of viral-specific cytotoxic T lymphocytes (CTLs) for the prevention or treatment of viral infections early post-transplant.[81] These T cells expand in vivo following infusion and exert antiviral effects without causing GVHD.[81,82] Leen and colleagues showed that CTLs against single or multiple viruses that commonly reactivate post-transplant can be safely infused with evidence of antiviral activity in these patients.[81]

Due to the fact that administration of unmodified CD3+ selected donor lymphocytes early post-transplant carries a major risk of development of acute GVHD, infusion of genetically modified donor lymphocytes expressing a suicide gene that could be activated by administration of ganciclovir if GVHD develops has been explored.[83,84] In a study of 50 patients, the use of this treatment strategy markedly accelerated immune reconstitution.[83] When GVHD did occur, it was promptly and effectively abated by induction of the suicide gene. Although this appeared to be a promising approach, NRM in this study was still high (approximately 40% of the patients). In addition, due to the fact that ganciclovir is a commonly used drug in transplantation for treatment of CMV reactivation, the Baylor group developed an alternative approach using an inducible caspase 9 suicide gene incorporated in donor lymphocytes, which could be eliminated using an inert molecule as dimerizing drug.[84] Administration of this drug resulted in successful elimination of greater than 90% of lymphocytes and promptly stopped the development of GVHD.[84] In a follow-up study, the same group treated 10 haploidentical transplant patients with iCasp9-modified donor lymphocyte infusion (DLI) and showed a favorable effect on immune responses against viral and fungal pathogens.[85] This approach was also studied in conjunction with alpha/beta TCD haploidentical transplantation (see following section), with excellent results.

Alpha/Beta T-Cell Depleted Haploidentical Transplantation

Acute and chronic GVHD reactions associated with a major HLA disparity between the donor and the recipient have been a main concern since the first introduction of haploidentical donor transplantation.[86,87] As previously mentioned, while a full HLA haploidentical graft with conventional GVHD prophylaxis has been associated with unacceptable incidence of GVHD, the use of complete T-cell depletion methods resulted in very high treatment-related mortality.[18,20,21,88] These results have emphasized the beneficial effect of donor T lymphocytes and other immune cells in the graft, and led to the development of novel techniques of graft manipulation with the goal to selectively deplete T-cell subgroups most likely responsible for development of GVHD.

The majority of peripheral blood T cells express αβ T-cell receptor (TCR), while approximately 5% express γδ TCR. It has been shown that a donor-derived αβ T-cell subset plays a major role in the pathogenesis of GVHD, unlike innate-like γδ T cells, which are capable of directly recognizing targets in an MHC-independent manner, enabling them to exert anti-tumor activity by directly identifying stress-induced antigens expressed on malignant cells, without recognition of classical MHC-associated alloantigens.[89–91] Several studies have demonstrated that an increased number of donor-derived γδ T cells following HLA mismatched related allogeneic hematopoietic stem cell transplants is significantly associated with better disease-free survival.[92,93]

Based on these findings, the immunomagnetic method of ex vivo selective depletion of αβ T cells from mobilized peripheral blood grafts has been pioneered by Chaleff and Handgretinger using a biotinylated anti-TCRαβ antibody, followed by an anti-biotin antibody conjugated to magnetic microbeads, which causes approximately 4 log reduction of αβ T cells, while maintaining in the graft the majority of CD34+ cells, as well as the NK cells and γδ T cells.[26,94] Several groups have demonstrated that the efficacy in T-cell reduction using this method was comparable to CD34+ positive selection and more effective than CD3+/CD19+ T-cell depletion, whereas recovery of CD34+ cells after depletion is comparable in all three procedures.[26] In addition, results from animal models of transplantation of αβ+ T-cell depleted graft showed rapid engraftment.[94] This method was subsequently combined with depletion of CD19+ B lymphocytes in order to prevent post-transplant EBV-associated lymphoproliferative disease. In the first clinical experience, 23 pediatric patients with advanced hematologic malignancies treated in Tübingen and Rome received TCRαβ+/CD19+ depleted haploidentical peripheral blood grafts, with either reduced-intensity (melphalan, thiotepa, fludarabine, or clofarabine and OKT-3 or ATG) or myeloablative (fractionated TBI, thiotepa, fludarabine, and ATG) conditioning regimen. All patients achieved sustained engraftment, rapid immune reconstitution, and had low incidence of GVHD, despite that no post-transplant pharmacologic GVHD prophylaxis was administered.[95]

Lang et al. reported in abstract format long-term follow-up results of 60 patients (30 adults, 30 pediatric patients) with malignant and nonmalignant diseases who received TCRαβ+/CD19+ depleted peripheral blood stem cell grafts from haploidentical donors as part of a prospective, multi-center, single-arm, phase I/II clinical trial. All patients received reduced-intensity conditioning (RIC) with fludarabine, melphalan, thiotepa, and either ATG or total nodal irradiation, and a short course of mycophenolate mofetil for GVHD prophylaxis. Rapid engraftment was observed with median time to neutrophil and platelet engraftment of 13 and 15 days, respectively. Eight of nine patients who had graft rejection were successfully re-transplanted. None of the patients developed severe acute GVHD, while severe chronic GVHD occurred in only 9%. However, a higher rate of viral reactivation was observed, with three patients dying of adenovirus disease. At 2-year follow-up, OS and DFS were 62% and 53%, respectively, while cumulative incidences of relapse and NRM were 34% and 20%, respectively.[96] Similarly, successful engraftment and lower rate of GVHD have been confirmed in several studies, both in pediatric and adult patients.[97–101]

Post-transplant immunologic recovery has also been investigated after TCRαβ+/CD19+ depleted haploidentical transplantation. Results from an immunologic reconstitution study of 41 recipients of TCRαβ+/CD19+ depleted haploidentical transplantation showed a significantly higher number of CD3+, CD3+CD4+, and CD56+ cells early post-transplant compared to a historical control group of 36 pediatric patients receiving positive CD34+ selected haploidentical stem cells. In this study, recovery of γδ T cells started at day +7 post-transplant and preceded αβ T-cell reconstitution.[99] Airoldi and colleagues found that γδ T cells are the predominant T-cell population in patients during the first weeks after transplantation, derived not only from cells infused with the graft but also γδ T cells differentiated from donor's hematopoietic stem cells.[32]

Not only has a rapid increase in number of donor-derived immunologic cell subsets post-transplant been observed, but these cells also might provide a benefit in graft-versus-tumor effect, as evidenced in several studies. Focusing on pediatric patients with acute leukemia, Locatelli et al. reported outcomes of 80 patients of TCRαβ+/CD19+ depleted haploidentical transplant using myeloablative conditioning without post-transplant GVHD prophylaxis, and showed a high GVHD-free, relapse-free survival (GRFS) of 71% at 5 years, comparable to those of children given transplantation from an HLA-identical sibling or a 10/10 MUD treated during the same period of time.[100] Similar results were also reported in other studies, in both pediatric and adult patients with high-risk leukemias.[101–103]

The benefit of lower incidence of GVHD in TCRαβ+/CD19+ depleted haploidentical transplants may be more important for patients with non-malignant diseases. A prospective study conducted in 23 pediatric patients with non-malignant diseases treated with TCRαβ+/CD19+ depleted haploidentical transplants showed promising outcomes with high engraftment rate and very low incidence of both acute and chronic GVHD. In this study, the cumulative incidence of grade I–II aGVHD was only 13.1%, no chronic GVHD was observed, and transplant-related mortality was only 9%. With a median follow-up of 18 months, 21 of 23 children were alive and disease-free with the 2-year probability of disease-free survival of 91.1%. Considering both primary and secondary graft failure as events, the probability of EFS was 74%.[98] Another study of 14 pediatric patients with thalassemia also reported a high rate of engraftment. In this study the cumulative incidence of engraftment failure was 14%, significantly lower than a historical group of 40 patients with hemoglobinopathies who received CD34+ selected haploidentical grafts (45%). Overall survival and disease-free survival were 78% and 69%, respectively. However, a higher incidence of GVHD was observed in this study despite receiving post-transplant pharmacological prophylaxis up to 60 days (with 28% incidence of grade II–III acute GVHD and 21% chronic extensive GVHD), and viral reactivations, such as CMV, ADV, and BKV, were also common.[104] Although the number of patients treated so far is relatively small and no definitive conclusions can be drawn, it is important to notice that a higher incidence of GVHD has also been seen in adults receiving TCRαβ+/CD19+ depleted haploidentical transplantation in some studies.[105]

Taken together, current available data indicate that TCRαβ+/CD19+ depleted haploidentical transplantation can provide an alternative option for patients without an HLA-matched donor with several studies that reported outcomes comparable to HLA-matched related or unrelated donor transplants.[99,100,102,106] Moreover, the low risk of GVHD without the need of long-term post-transplant immunosuppression may provide a better opportunity to apply cellular therapy post-transplant and help improve quality of life, which could make this type of haploidentical transplant more appealing, especially for pediatric patients. Novel pharmacologic and adoptive cellular therapy approaches to enhance anti-tumor effect and immune reconstitution and improve outcomes after TCRαβ+/CD19+ depleted haploidentical transplantation are being investigated.

G-CSF Stimulation of the Donor, Intensified Immunosuppression (CsA, MMF, and MTX), Anti-Thymocyte Globulin; and Combination of Peripheral Blood and Bone Marrow Allografts ("GIAC" Protocol)

Treatment of bone marrow donors with G-CSF before donation increases marrow CD34$^+$ cells and granulocyte-macrophage colony-forming units, reduces total lymphocytes, and reverses the CD4$^+$/CD8$^+$ T-cell ratio. To enhance engraftment by increasing the dose of transplanted HSCs, 15 patients with high-risk leukemia received myeloablative conditioning with cytarabine, cyclophosphamide, and 1000 cGy TBI, and G-CSF–primed bone marrow from haploidentical donors. GVHD prophylaxis consisted of rabbit ATG (5 mg/kg/day on days −4 through −1), cyclosporine (CsA), methotrexate, and mycophenolate mofetil (MMF).[107] At the time of reporting, all 15 patients had prompt trilineage hematopoietic engraftment; the cumulative incidence of GVHD was 33%; and 9 of 15 patients were alive at a median follow-up of 22 months (range, 13 to 35 months). Subsequently, Lu et al. compared outcomes of 293 patients with leukemia receiving HLA-matched sibling ($n = 158$) or

HLA-haploidentical related grafts ($n = 135$) from G-CSF–primed donors.[108] Patients undergoing haploidentical SCT were conditioned with cytarabine, oral busulfan, cyclophosphamide, and methyl-CCNU (1-[2-chloroethyl]-3-[4-methylcyclohexyl]-1-nitrosourea), received G-CSF primed bone marrow on day 0 ($n = 134$) and/or G-CSF–primed peripheral blood on day 1 ($n = 131$), and GVHD prophylaxis with ATG 2.5 mg/kg/day on days −4 through −1, CsA, MTX, and MMF. All but two haploidentical SCT patients treated with the GIAC protocol had sustained engraftment of donor neutrophils. The cumulative incidence of acute grades II–IV, grades III–IV, and chronic GVHD in recipients of matched versus mismatched SCT were 32% versus 40% ($P = .13$), 11% versus 16%, and 56% versus 55%. Mismatched patients had a higher incidence of CMV antigenemia (65% vs. 39%; $P < .001$) and hemorrhagic cystitis (35% vs. 13%; $P < .001$), but not of CMV disease. Two-year relapse rate was 13% versus 18% ($P = .40$), NRM 14% versus 22% ($P = .10$), LFS 71% versus 64%, and OS 72% versus 71% ($P = .72$), in the matched and mismatched cohorts, respectively. In a follow-up report of 157 recipients treated with this approach, recipients of CD3$^+$ T-cell doses higher than the median (1.77×10^8/kg) had a significantly lower NRM, better LFS, and better OS.[109] Two other Chinese groups reported similar results of retrospective studies comparing outcomes of patients who received haploSCT with those of patients who received HLA-matched sibling or HLA-matched unrelated transplants.[110,111] Moreover, in a prospective, multicenter study comparing haploSCT ($n = 231$) versus HLA-matched sibling transplants ($n = 219$) using biologic randomization based on donor availability, similar outcomes were noted between the two groups.[112]

Although outcomes are in general very good (especially because patients treated are up to age 60) a higher proportion of patients develop severe acute and mostly chronic GVHD. Several groups have attempted to reduce the incidence of GVHD seen with this protocol.[113–116] In a Korean study in which a modified version of this platform was used with RIC and only G-CSF mobilized PBSC allografts, authors showed a lower incidence of grades II–IV acute (20%) and chronic (34%) GVHD.[113] A different group modified this protocol to use TBI-based conditioning, only GCSF-primed bone marrow, and added basiliximab to enhance GVHD prophylaxis.[114,115] Although this approach resulted in lower incidence of grades II–IV acute GVHD of 11%, chronic GVHD was still seen in most patients, yet was mostly limited in severity. The Rome Transplant Network employed the same GVHD prophylaxis and reported in 97 patients encouraging results in terms of GVHD control (grades II–IV and grades III–IV acute GVHD of 31% and 9%, respectively, and extensive chronic GVHD of 12%); however, 1-year NRM was 32%.[116]

Survival outcomes of patients with acute leukemia receiving haploSCT with this protocol have been particularly encouraging, owing primarily to lower incidence of relapse in the haploidentical versus HLA matched group (26% vs. 49%; $P = .008$).[33] In 2009, the Peking University group reported the results of a study of 250 consecutively treated patients with acute leukemia, 108 of whom had AML and 142 of whom had ALL.[117] Survival of patients with AML were particularly favorable, with 3-year OS of 73% and 56% reported for standard-risk and high-risk groups, respectively.[112] However, outcomes of patients with high-risk ALL appeared poor because of higher incidence of NRM (51%) and relapse (49%) at 3 years, presumably due to intense myeloablative conditioning, usually less well tolerated in this group of patients.[117]. Patients with standard-risk ALL, however, had 3-year OS of 60%.[117] Two other studies have confirmed excellent survival of patients with ALL in CR1 treated with haploSCT using this protocol.[112,118] In another ALL study,[119] patients received consolidation with either 2 years of chemotherapy ($n = 104$) or haploSCT ($n = 79$) (as preferred by the patients); those who received haploSCT had markedly better 3-year disease-free survival (DFS) (64% vs. 21%), OS (72% vs. 27%), due to a much lower cumulative incidence of relapse (19% vs. 61%) compared with chemotherapy-treated patients.[119] In multivariate analyses, consolidation of remission with a haploSCT was the only factor associated with lower relapse and better OS in these patients.

In all these studies, the extent of HLA disparity did not affect survival post-transplant.[110,120,121] However, donor characteristics appeared to impact patient outcomes; lowest NRM and best survival were seen with younger, male donors, and lower incidence of acute GVHD occurred when the donor was the patient's child or an HLA-haploidentical relative who was mismatched for non-inherited maternal HLA antigens (NIMA), while the use of maternal donors was associated with higher incidence of acute and chronic GVHD, and worse survival.[121]

A few studies compared outcomes of unmanipulated haploSCT using the GIAC protocol with post-transplantation cyclophosphamide approach and will be discussed later in this chapter.

Haploidentical Transplantation With Post-Transplant Cyclophosphamide

The effects of cyclophosphamide (Cy), one of the oldest chemotherapeutic agents, have been studied since the early 1960s.[122] High-dose Cy was found to be effective in prolonging the survival of MHC-mismatched mouse skin allografts only when the drug was given up to the fourth day post-transplant, with the optimal effectiveness of this treatment being at 2 days post-transplant.[123] This work was continued by a number of groups into the mid-1990s, which investigated mechanisms of tolerance induction by Cy.[124,125] By administering donor spleen cells followed 48 to 72 hours later by administration of cyclophosphamide, long-lasting tolerance to MHC-compatible, but not MHC-incompatible, skin allografts was established. Three primary mechanisms of post-transplant cyclophosphamide (PTCy)-induced tolerance were identified in this model: (1) Direct elimination of host T cells responding to donor antigens in the periphery, (2) intra-thymic clonal deletion of donor-reactive host T cells, and (3) generation of tolerogen-specific host suppressor T cells.[126] Suppressor T cells were found to inhibit responses to both major and minor histocompatibility antigens through active suppression but not clonal deletion of alloreactive T cells.[127] Induction of tolerance by PTCy was disrupted by the administration of CsA or corticosteroids before adoptive cell transfer and cyclophosphamide treatment,[128,129] while it was not affected by the administration of G-CSF starting the day after PTCy treatment.[130]

PTCy found its greatest applicability in haploidentical transplantation. In the initial experiments in MHC-mismatched mouse models, treatment with PTCy reduced the dose of radiation required to induce reliable engraftment,[131] prevented GVHD, and prolonged survival.[27] Graft failure could be further reduced by the use of antilymphocyte globulin,[132] and radiation in the conditioning regimen could be replaced, at least in part by fludarabine, although high levels of donor chimerism required at least 100 cGy of TBI.[27] The resultant mixed chimeras were tolerant to both donor and host but maintained reactivity against third-party alloantigens in mixed lymphocyte culture.[27,131,132] This tolerogenic effect allowed skin and heart allografts from the MHC-mismatched donor strain to survive, whereas MHC-disparate third-party grafts were rejected.[131,133]

Parallel to the effects seen on host T cells in skin allograft models, in transplant mouse models, treatment with PTCy inhibited GVHD through elimination of alloreactive donor T cells.[134] Donor T cells exposed to antigen on day 0 were largely depleted by PTCy, whereas non-alloreactive donor T cells, which divided more slowly in a lymphopenic environment, were relatively spared (Fig. 107.4).[134] However, elimination of alloreactive donor T cells was necessary but not sufficient for PTCy-induced tolerance. The role of Tregs in inducing, as opposed to maintaining, tolerance is controversial. In mouse models of MHC-matched transplants in which donor CD4$^+$ T cells promote GVHD, donor Tregs were necessary to prevent lethal GVHD after PTCy treatment,[135,136] an effect consistent with the results from skin allograft models. In mouse models as well as human studies, donor Tregs were resistant to PTCy-induced cytotoxicity, owing to increased expression of aldehyde dehydrogenase, the enzyme primarily responsible for in vivo detoxification of cyclophosphamide.[135–137] In contrast, PTCy can induce tolerance in alloreactive CD8$^+$ T cells in the absence of CD4$^+$ T cells, including CD4$^+$ Tregs.[135] In

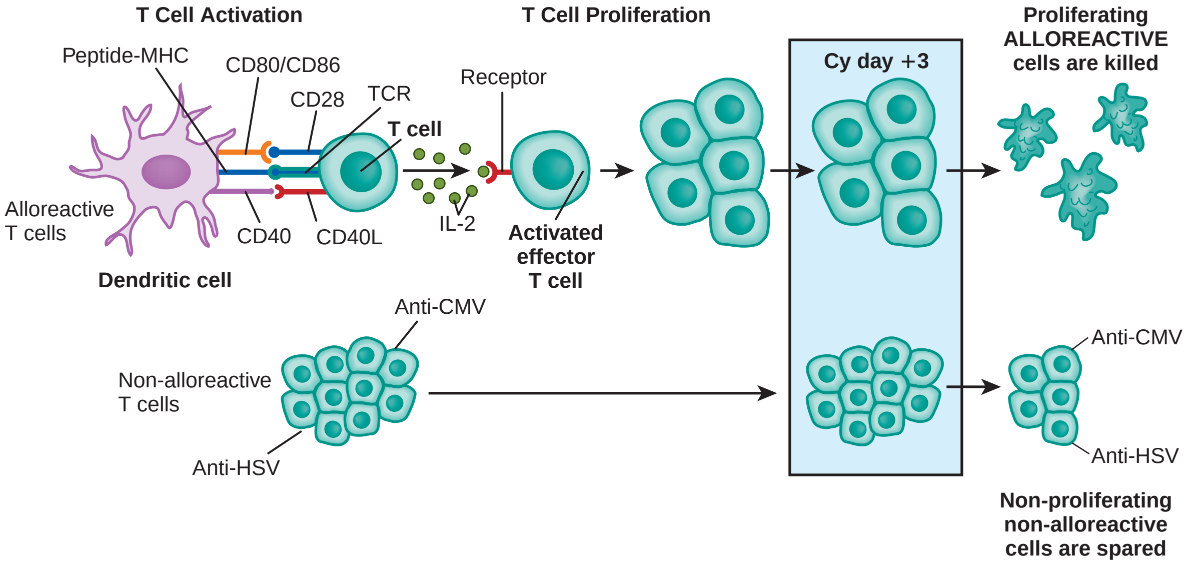


Figure 107.4 CYCLOPHOSPHAMIDE-INDUCED TOLERANCE TO HISTOCOMPATIBILITY ANTIGENS. On the day of transplant, alloreactive donor and host T cells encounter alloantigen on the surface of host and donor antigen-presenting cells, which also provide costimulatory ligands for T-cell activation *(top left)*. The alloreactive T cells secrete cytokines such as interleukin (IL)-2 and proliferate. Because cyclophosphamide is more toxic to proliferating than to resting cells, the drug induces substantial killing of the alloreactive population while relatively sparing the resting, nonalloreactive T cells, including cells responsible for immunity to pathogens *(bottom)*. Thus this protocol provides for selective allodepletion of alloreactive T cells while permitting a more rapid immune reconstitution compared with complete depletion of T cells from the graft. *CMV*, Cytomegalovirus; *HSV*, herpes simplex virus; *MHC*, major histocompatibility complex; *TCR*, T-cell receptor.

addition, other studies have shown that Tregs are not required for cyclophosphamide-induced tolerance of tumor-reactive T cells[138] or for transplant tolerance induced by a different alkylating agent, bendamustine.[139]

Clinical Studies Using Post-Transplant Cyclophosphamide-Based Graft-Versus-Host Disease Prophylaxis

In an initial phase I clinical trial of 13 patients done at John Hopkins, patients received a non-myeloablative (NMA) conditioning regimen with fludarabine ± cyclophosphamide and low-dose (200 cGy) TBI, a T-cell replete haploidentical bone marrow graft, and one dose of PTCy 50 mg/kg on day +3 post-transplant.[140] For GVHD prophylaxis, in addition to tacrolimus, MMF was also added starting the day after patients received PTCy and continued for at least 30 days. Approximately 80% of patients who had fludarabine, cyclophosphamide, and 2GyTBI successfully engrafted, and half the patients were alive and in remission after a median follow-up of 284 days. Grade II–IV acute GVHD developed in 6 patients. Application of two doses of cyclophosphamide was found to be more effective in facilitating engraftment and in preventing GVHD. Subsequently, a phase I/II study performed at two institutions enrolled 68 patients,[141] and the majority received two doses of PTCy on day +3 and +4 post-post-transplant. Eighty-seven percent of patients engrafted and achieved complete or near-complete donor chimerism by 1 to 2 months post-transplant. Grades II–IV and grades III–IV acute GVHD occurred in 34% and 6% of patients, respectively. The incidence of extensive chronic GVHD was very low, significantly lower in patients receiving two doses of PTCy (5%). Longer follow-up of an expanded cohort of 210 patients treated according to the JHH protocol confirmed very low incidence of NRM, acute GVHD, and chronic GVHD.[142] The extent of HLA disparity had no negative effects on acute GVHD or progression-free survival (PFS).[143] In addition, a relatively high rate of relapse (55%) was observed, in part reflected by the use of NMA conditioning in patients with acute leukemia and by the advanced disease state of patients who received transplants. Interestingly, a disease risk–stratified analysis of 372 patients performed by the Johns Hopkins group showed that survival outcomes were comparable with those of patients receiving HLA-matched allogeneic stem cell transplantation.[144]

Nevertheless, in an effort to reduce relapse rates post-transplant, several groups explored intensified conditioning with either a melphalan-, total body irradiation (TBI)-, thiotepa-, or busulfan-based regimen for haploidentical transplants in the adult and pediatric population.[28–30,145,146] These studies showed, in general, higher NRM (3% to 21%) lower incidence of relapse (19% to 35%), comparable incidence of grades II–IV aGVHD (12% to 30%), III–IV aGVHD (3% to 10%), and cGVHD (10% to 26%), and at least as good survival as NMA conditioning (1-year DFS 51% to 70%). It is now generally accepted that, as in HLA matched transplants,[147] younger, fit patients with acute leukemia should receive a myeloablative conditioning regimen, while older, less fit individuals and lymphoma patients should be treated with a RIC/NMA conditioning regimen.[148,149] A summary of commonly employed conditioning regimens for haploSCT with PTCy-based GVHD prophylaxis is presented in Table 107.1.

An alternative two-step approach to myeloablative PTCy haploSCT separates the induction of tolerance to donor cells (step 1) from hematopoietic rescue (step 2). Patients received a TBI-based conditioning and peripheral blood T cells at a fixed dose on pre-transplantation day −6, two doses of cyclophosphamide 60 mg/kg/day on days −3 and −2 for tolerance induction (step 1), followed by a CD34-selected PBSC graft from the same donor on day 0.[150,151] This procedure mimicked the timing of the standard PTCy platform, except that stem cells were spared exposure to cyclophosphamide. Although there were concerns initially with exposure of stem cells to cyclophosphamide, no detrimental effects have been observed long term, now after greater than 10 years of application of Cy in haploSCT. Results for patients who were in remission at the time of transplant showed a low incidence of grades III–IV acute (4%) and chronic (21%) GVHD, NRM of 3.6%, and relapse-related mortality of 19%, leading to a 2-year DFS of 74%.

Although initially a bone marrow graft was used almost exclusively for haploidentical transplantation, several groups have explored the use of peripheral blood stem cells (PBSCs) for haploidentical transplantation with PTCy,[28,152–154] and several studies compared these two donor sources for haploidentical transplantation.[154–157] As seen in a randomized study conducted by the Blood and Marrow Transplant Clinical Trials Network (BMTCTN) in unrelated donor

TABLE 107.1 **Commonly Used Conditioning Regimens Used in Unmanipulated Haploidentical Transplantation With Post-Transplantation Cyclophosphamide**

Conditioning Regimen	Study	Type	N	Age Median (Range)	aGVHD gr II-IV	NRM	Relapse rate	PFS	OS	Reference
Fludarabine 150 mg/m^2 Cyclophosphamide 29 mg/kg TBI 2Gy	Prospective, two centers	NMA	68	46 (1–71)	34%	15% at 1 year	51% at 1 year	26% at 2 years	36% at 2 years	141
Fludarabine 150 mg/m^2 Cyclophosphamide 29 mg/kg TBI 2Gy	Prospective, multicenter, randomized	NMA	182	60 (20–70)	28%	11% at 2 years	48% at 2 years	41% at 2 years	57% at 2 years	292
Fludarabine 160 mg/m^2 Melphalan 100–140 mg/m^2 TBI 2 Gy *or* Fludarabine 160 mg/m^2 Melphalan 100–140 mg/m^2 Thiotepa 5 mg/kg	Prospective, single center	RIC MAC	60	45 (20–63)	28%	21% at 1 year	19% at 1 year	60% at 1 year 53% at 2 years	70% at 1 year 55% at 2 years	30
Cyclophosphamide 100 mg/kg and Busulfan 3.2 mg/kg, 128 mg/m$^{2\,a}$ *or* Cyclophosphamide 100 mg/kg and TBI 12 Gy	Prospective,	MAC	96	42 (1–65)	11%	11% 19% for >55 years	35% at 1 year 43% at 3 years	57% at 1 year 49% at 3 years	73% at 1 year 54% at 3 years	145
Fludarabine 90 mg/m^2 TBI 12 Gy	Retrospective, single center	MAC	82	42 (19–61)	52%	13% at 4 years	27%	60% at 4 years	67% at 4 years	28
Thiotepa 10 mg/kg Busulfan 9.6 mg/kg Fludarabine 150 mg/kg	Retrospective, single center	MAC	148	47 (17–74)	24%	14%	NR	NR	77% CR1 49% CR2 38% advanced	293

MAC, Myeloablative conditioningn; *NMA*, non-myeloablative conditioning; *RIC*, reduced-intensity conditioning; *TBI*, total body irradiation.

transplantation,[158] the use of peripheral blood graft has been, in general, associated with a slightly higher engraftment rate yet a higher incidence of acute and chronic GVHD, with similar survival in most studies, including in a recent meta-analysis.[155–157,159] However, for patients with acute lymphoblastic leukemia, a bone marrow graft should be preferred as it was associated with lower incidence of GVHD and NRM and better DFS and GRFS survival in a European Blood and Marrow Transplant (EBMT) analysis.[160]

Post-Transplant Cyclophosphamide in Combined Solid Organ—Hematopoietic Stem Cell Transplantation

PTCy has shown promise as a tolerizing agent in facilitating mismatched hematopoietic stem cell grafts infused during or soon after solid-organ transplantation with the goal to achieve complete tolerance of the solid organ grafts. Leventhal et al. reported on combined kidney and hematopoietic transplants in which fludarabine/cyclophosphamide conditioning was administered before renal transplant and column-selected PBSCs were given the day after renal transplant.[161–163] Mycophenolate and tacrolimus were started 2 days before kidney transplant and one dose of PTCy (50 mg/kg) was given 3 days post-transplant. According to the latest report, 12 of 19 patients[163] achieved functional tolerance after full donor chimerism as demonstrated by successful cessation of all immunosuppression without graft rejection. This approach suggests that tolerance induction in solid organ transplantation appears to be dependent on achieving sustained donor chimerism.[162] Future studies will explore the use of haploSCT with PTCy after solid organ transplantation to achieve complete tolerance of the transplanted organ to the recipient's immune system.

COMPARATIVE OUTCOMES BETWEEN HAPLOIDENTICAL AND OTHER DONOR GRAFTS

With multiple platforms being developed for patients receiving haploidentical transplantation, the natural question is how outcomes of haploidentical transplants using different approaches compare, and how outcomes of haploidentical transplants compare with other donor types. Only a few studies to date have retrospectively compared outcomes using different haploidentical transplantation platforms. In an initial retrospective study of 65 adult patients receiving haploSCT using either PTCy or megadose T-cell–depleted stem cells for GVHD prophylaxis, survival was significantly better for patients receiving PTCy.[19] NRM was markedly lower after TCR graft and PTCy-based GVHD prophylaxis versus TCD haploidentical transplantation (16% vs. 42% at 1 year), with the difference largely a result of lower risk of viral (twofold lower) and fungal (fivefold lower) infections post-transplant. A more rapid immunologic reconstitution was noted in patients receiving PTCy, with similar incidence of aGVHD, lower incidence of cGVHD (7% vs. 18%), similar relapse rate post-transplant, and significantly better survival (64% vs. 30%, $P = .02$).[19]

Another study compared haploidentical transplants performed with PTCy and GIAC protocol (anti-thymocyte globulin [ATG]) for patients with AML.[164] Patients in the PTCy group ($n = 193$) had significantly less severe (grades III–IV) aGVHD than patients in the GIAC group ($n = 115$), 5% versus 12%, respectively ($P = .01$), with similar incidence of chronic GVHD and relapse incidence. However, recipients of PTCy had a significantly lower incidence of NRM (22% vs. 30%; $P = .02$) and improved LFS (HR, 1.48; 95% CI, 1.03 to 2.12; $P = .03$). Taken together, these results suggest that TCR haploSCT with PTCy may the best haploidentical transplant platform to date, yet additional studies are needed, including comparing PTCy with αβTCD.

As noted earlier in this chapter, traditional donor choices in patients receiving conventional GVHD prophylaxis relied on HLA matched donors, as outcomes with haploidentical donors were associated with unacceptable toxicity. HLA-matched sibling donors were preferred, as they were associated with the best outcomes. If an HLA-matched sibling donor was not available, the preferred donor was a well-matched (at least 8/8 allele match at HLA-A, HLA-B, HLA-C, HLA-DRB1) MUD and, in the absence of an HLA-matched sibling or MUD, the choices may be a partially HLA-matched unrelated donor, an unrelated umbilical cord blood, and an HLA-haploidentical first-degree relative. The introduction of PTCy for GVHD prophylaxis has significantly improved transplant outcomes with haploidentical donors, now similar at least with MUDs, and raised questions whether traditional donor choice should continue be used, or if other factors should be considered such as urgency to transplantation.[3] Multiple studies, mostly retrospective, compared outcomes of haploidentical transplants with PTCy with other donor sources; the most relevant are summarized below.

Haploidentical Versus Cord Blood Transplants

The Acute Leukemia Working Party of the EBMT group compared outcomes after UCBT and haploSCT in adults with de novo AML ($n = 918$, comprising 360 haploSCT, 558 UCBT) and ALL ($n = 528$, comprising 158 haploSCT, 370 UCBT).[165] Conditioning regimens were a mixture of myeloablative and RIC, and GVHD prophylaxis for recipients of haplo transplants included PTCy in 163, ATG in 244, and neither ATG nor PTCy in 111 patients. UCBT was associated with delayed engraftment and a higher incidence of graft failure, but a lower incidence of chronic GVHD, for both AML and ALL patients. There were no significant differences between graft sources in the incidence of NRM or relapse or in LFS. The authors concluded that both haplo and UCBT grafts are acceptable approaches for patients with acute leukemia lacking an HLA-matched donor.[165] However, the main limitation of this study was that it included haploidentical transplants performed with methods other than PTCy, which have been associated with worse outcomes.[166,167]

The Blood and Marrow Transplant Clinical Trials Network (BMT CTN) in the United States conducted two prospective parallel trials of RIC followed by either double-UCBT (dUCBT) (BMT CTN 0604) or haploSCT with PTCy for patients with acute leukemia or lymphoma.[168,169] The 100-day cumulative incidence of grades II–IV acute GVHD was 40% after dUCBT and 32% after haploSCT. The 1-year cumulative incidence rates of NRM and relapse after dUCBT transplant were 24% and 31%, respectively, with corresponding results of 7% and 45% after haploSCT. OS and PFS were 39% (95% CI, 26% to 53%) and 36% (95% CI, 23% to 49%), respectively, after dUCBT and 54% (95% CI, 39% to 67%) and 35% (95% CI, 21% to 48%), respectively, after haploSCT. The similar PFS seen in these two trials provided equipoise for the multicenter randomized phase III BMT CTN trial (ClinicalTrials.gov identifier NCT01597778) comparing these two different graft sources after RIC. Outcomes from this study were recently reported.[170] Three hundred sixty-eight patients (18 to 70 years old) with leukemia and lymphoma were randomized to receive either a cord blood transplant ($n = 186$) or a haploidentical donor transplant ($n = 182$) with a similar conditioning regimen including fludarabine, cyclophosphamide, and 2Gy TBI. The primary endpoint was PFS, and all outcomes reported are at 2 years. The PFS was 35% versus 41% ($P = .41$) in favor of haploSCT, NRM was higher in the CBT, 18% versus 11% ($P = .04$) and OS was 46% versus 57% ($P = .04$), respectively. This study confirmed lower NRM and better survival with haploidentical donor transplants, and suggested that a haploidentical donor, when available, should be preferred to a double umbilical cord blood transplant.[170]

Several other large retrospective studies compared outcomes between haploidentical donor transplants and UCBT for different diseases and showed, in general, better outcomes with haploSCT.[171–174]

Haploidentical Versus Human Leukocyte Antigen Matched and Mismatched Unrelated Donor Transplants

GIAC Protocol

The Beijing group and the EBMT conducted a retrospective comparison of patients with AML and intermediate-risk cytogenetics receiving haploidentical transplants using the GIAC protocol ($n = 87$) versus control subjects receiving fully matched (10/10) URD transplants ($n = 87$).[175] Cases and control subjects were matched for age ±5 years, interval from CR1 to transplant, and the number of induction courses required to achieve CR1. Outcomes were similar in both groups: the 5-year LFS was 60.3% in the URD group versus 73.5% in the haplo group ($P = .15$), OS was 63.6% versus 78.2% ($P = .15$), relapse incidence was 24% versus 12.7% ($P = .08$), and NRM was 15.7% versus 13.8% ($P = .96$).[175]

α/β T-Cell Depleted Haploidentical Transplantation

α/β T-cell/B-cell depleted haploSCT in children has been compared with HLA matched and mismatched unrelated donor transplantation. Outcomes of 98 patients receiving haploSCT, 127 MUD, and 118 mismatched MUD (MMUD) were compared in a multicenter retrospective study.[106] Recipients of haploSCT had significantly lower incidence of grade II–IV and III–IV aGVHD (16% and 0% vs. 35% and 47% for MUD, $P < .001$) and chronic GVHD, and 5-year LFS of 62% versus 67% and 55% respectively ($P < .001$) with no significant differences in LFS and GRFS between MUD and α/β T-cell/B-cell depleted haploSCT.[106]

Post-Transplant Cyclophosphamide

Multiple retrospective studies compared outcomes of haploSCT with PTCy with MUD transplants. Two CIBMTR studies compared outcomes of MUD transplants with those of haploSCT with PTCy for patients with AML[176] as well as for patients with lymphoma.[177] For patients with AML receiving MAC, haploidentical transplants had a lower incidence of grade II–IV aGVHD (16 vs. 33%; $P < .0001$) and 3-year chronic GVHD (30% vs. 53%; $P < .0001$). Similar differences in GVHD were also seen after RIC/NMA conditioning. Three-year probability of OS after myeloablative conditioning was 45% after haploSCT versus 50% after MUD transplants ($P = .38$), while the corresponding rates after RIC were 46% for haplo and 44% for MUD transplants ($P = .71$).[176]

A similar study evaluated outcomes of patients with lymphoma receiving RIC and compared three groups: haploidentical transplants with PTCy versus MUD transplants, without and with ATG. The 1-year cumulative incidence rates of chronic GVHD were 13%, 51%, and 33%, respectively ($P < .001$). In multivariate analysis, incidence of grade III–IV aGVHD was higher in MUD without ATG ($P = .001$), as well as MUD with ATG ($P = .01$), relative to haploidentical transplants. Cumulative incidence rates of relapse at 3 years were 36%, 28%, and 36% in the haploidentical, MUD without ATG, and MUD with ATG groups, respectively ($P = .07$), and the corresponding 3-year OS rates were 60%, 62%, and 50%, respectively, in the three groups, with multivariate analysis showing no

TABLE 107.2 Selected Retrospective Registry Studies Comparing Outcomes of Haploidentical Transplants With Post-Transplant Cyclophosphamide With Matched Unrelated Donor Transplants

Disease Subtype	N	aGVHD	cGVHD	TRM	Relapse	3-Year PFS	GRFS
AML[176]							
MAC	104	16%	30%	14%	44%	45%	——
Haplo	1245	33%	53%	20%	39%	50%	
MUD	88	19%	34%	9%	58%	46%	
RIC	737	28%	52%	23%	42%	44%	
Lymphomas[294]							
Haplo	185	27%	13%	11%	36%	47%	—
MUD with ATG	241	40%	33%	13%	36%	38%	—
MUD without ATG	491	49%	51%	20%	28%	49%	—
Hodgkin's Lymphoma[179]							
Haplo	98	33%	26%	17%	39%	43%[a]	—
MUD	273	30%	41%	21%	32%	45%[a]	
DLBCL[180]							
Haplo	132	34%	18%	22%	41%	38%	36%
MUD TCD+	403	32%	27%	17%	38%	36%	33%
MUD TCD–	378	42%	57%	26%	34%	37%	19%
ALL[181]							
Haplo	136	28%	44%	23%	28%	49%	44%
MUD	809	32%	39%	19%	28%	53%	39%
ALL[182 a,b]							
Haplo	487	33%	30%	24%	37%	39%	31%
MUD	974	32%	28%	24%	34%	41%	32%

[a] Two-year survival is reported.
[b] Haploidentical recipients were matched (1:2) with MUD controls.
aGVHD, Acute graft-versus-host disease; *ALL*, acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; cGVHD, chronic graft-versus-host disease; *DLBCL*, diffuse large B-cell lymphoma; *GRFS*, GVHD-free, relapse-free survival; *HaploSCT*, allogeneic hematopoietic stem cell transplantation; *MAC*, myeloablative conditioning; *MUD*, matched unrelated donors; *NRM*, non-relapse mortality; *PFS*, progression-free survival; *RIC*, reduced intensity conditioning; *TCD*, T-cell depletion.

survival difference between MUD without ATG ($P = .21$) relative to haploidentical transplants and significantly worse compared with MUD with ATG. These data demonstrated that, for patients with lymphoma, haploidentical transplants with PTCy produce survival outcomes as good as those seen after transplants using MUD transplants without ATG, with much less cGVHD, which could translate into a long-term survival benefit. A follow-up study is thus needed to appreciate long-term survival between these groups.

Other important registry studies compared outcomes between haploidentical, MUD ± MSD transplants and yielded similar results between haploSCT and matched transplants across many diseases including lymphoma[178–180] and acute lymphoblastic leukemia (Table 107.2).[181,182]

Haploidentical Versus Human Leukocyte Antigen-Matched Sibling Donor Transplants

Encouraging outcomes between haploidentical and unrelated donor transplants prompted comparisons between haploidentical donor transplant and MSD transplants for patients with hematological malignancies and different diseases. Part of these studies have been detailed above.

GIAC Protocol

Using the GIAC protocol, the Beijing group performed several single-center, nonrandomized studies which suggested that survival after haploidentical transplants using the GIAC protocol was similar with HLA-matched sibling donor transplants.[108,111] These studies motivated a prospective, multicenter trial with biologic assignment, in which patients with AML in CR1 were assigned to receive a transplant after myeloablative conditioning from an HLA-matched sibling ($n = 219$) or, if a matched sibling was not available, an HLA-haploidentical donor ($n = 231$).[183] Although patients receiving haplo grafts had significantly higher incidence of grades II–IV acute GVHD (36% vs. 13%; $P < .001$), grades III–IV acute GVHD (10% vs. 3%; $P = .004$), and chronic GVHD (42% vs. 15%; $P < .001$) than patients receiving grafts from matched siblings, there were no significant differences between the two groups in 3-year incidence of relapse (15% vs. 15%; $P = .98$), NRM (13% vs. 8%; $P = .13$), DFS (74% vs. 78%; $P = .34$), or OS (79% vs. 82%; $P = .36$) between the two groups.[183]

Post-transplant Cyclophosphamide

A variety of single center reports have retrospectively analyzed the outcomes of transplants from either matched siblings or haploidentical first-degree relatives.[31,184–186] Bashey et al.[185] reported that patients treated with haploSCT with PTCy had very similar outcomes with MUD and MSD transplants except higher 2-year overall survival (57% vs. 59% vs. 72%, $P = .004$) with similar PFS between all 3 groups (54% vs. 50% vs. 56%, $P =$ NS) and lower incidence of moderate to severe cGVHD in favor of haploidentical transplants (31% vs. 47% vs. 44%, $P = .004$). Several other studies showed similar survival after haploSCT, and two studies[185,186] showed a significantly lower incidence of chronic GVHD.

The CIBMTR recently compared outcomes of patients with lymphoma undergoing RIC followed by either HLA-matched sibling SCT ($n = 807$) or haploSCT with PTCy as part of GVHD prophylaxis ($n = 180$).[187] Although platelet recovery was delayed among those treated with haploSCT plus PTCy, the cumulative incidence of chronic GVHD was again significantly lower after haploSCT (12% vs. 45%; $P < .001$), and there were no significant differences between haploSCT and MSD SCT in 3-year NRM (15% vs. 13%; $P = .41$), relapse (37% vs. 40%; $P = .51$), PFS (48% vs. 48%; $P = .96$), or OS (61% vs. 62%; $P = .82$). Another retrospective study performed by CIBMTR evaluated outcomes of donor-recipient pairs in younger (<55 years) or older (±55 years) recipients with acute leukemia receiving an MSD or haploidentical donor transplant and found that, in younger individuals, the 2-year DFS was identical (61% vs. 61%) for patients receiving a haploidentical versus a MSD transplant, while for older individuals, 2-year DFS was better using a MSD versus haploidentical donor (51% vs. 44%) due to a higher NRM in the haplo group, yet lower cGVHD was again observed in the haploidentical versus MSD groups.[188]

These remarkable results in patients receiving a haploSCT with PTCy-based GVHD prophylaxis with similar survival compared with HLA matched transplants yet lower incidence of acute and mostly cGVHD prompted a shift toward adoption of this method of GVHD prophylaxis for HLA matched transplants. A recent study from EBMT compared outcomes between haploidentical with bone marrow graft and MUD transplants with peripheral blood graft for patients with acute leukemia using the same PTCy-based GVHD prophylaxis, and found that, despite the fact that the haploidentical group had lower incidence of acute GVHD (grade II–IV 19.4% vs. 29.7%, $P = .01$; grade III–IV 6.5% vs. 9.2%, $P = .27$) and chronic GVHD (overall 28.9% vs. 39.1%, $P = .058$, and extensive 8.6% vs. 21.1%, $P = .001$), there were no survival differences between the haploidentical and MUD groups at 2-year for DFS (58% vs 61.9 %, $P = .18$), OS (65.4% vs. 64.2%, $P = .67$) and GRFS (54% vs. 44.1%, $P = .45$), respectively.[189]

POTENTIAL COMPLICATIONS AFTER HAPLOIDENTICAL TRANSPLANTATION

HaploSCT with PTCy has generally been well tolerated, yet several potential complications can occur. Some of these complications are characteristic of allogeneic stem cell transplantation in general, such as toxicity from chemotherapy or the development of GVHD, while others are more particular or have a higher incidence with this type of transplant, due to higher HLA disparity between the donor and recipient, such as higher risk of engraftment failure, in part due to risk of development of donor-specific anti-HLA antibodies (DSA), development of CRS in the first few days after haploidentical transplant, especially with a peripheral blood stem cell graft, a higher risk of viral reactivation early post-transplant (CMV, HHV6, BK virus), development of hemorrhagic cystitis (most commonly related to BK virus, more rarely related to cyclophosphamide), and relapse post-transplant. Notably, Epstein-Barr virus–related post-transplant lymphoproliferative disease within the first-year post-transplant is very rare (not seen in a study of 785 patients treated with PTCy),[190] and no increase in donor-derived malignancies was detected after large number of patients received PTCy to date.[191]

The following section details several potential complications after haploidentical transplantation and mitigating strategies.

Graft Failure

Successful donor hematopoietic cell engraftment is the first important step toward the ultimate goal of allogeneic hematopoietic stem cell transplantation to cure the malignant disease, restore normal hematopoiesis and donor immune system to provide beneficial graft-versus-tumor effect. Patients with engraftment failure, either primary, defined as absence of donor hematopoiesis within 28 days post-transplant (or 42 days after UCBT), or secondary, defined as loss of donor hematopoiesis after initial engraftment, can suffer from protracted pancytopenia, risk of fatal infections, and major bleeding in the absence of a second transplant, particularly with more intense conditioning (myeloablative) where autologous hematopoietic reconstitution rarely occurs.

The incidence of graft failure varies significantly in different transplant settings. Several factors are known to be associated with the risk of graft failure, such as older recipient age, highly alloimmunized recipients, greater degree of donor-recipient HLA disparity, major ABO incompatibility, use of nonmyeloablative conditioning, bone marrow stem cell graft, or T-cell depleted graft.[192,193] In contemporary haploidentical stem cell transplantation, the reported incidence of graft failure is up to 10%; however, it can be up to 20% in recipients of T-cell depleted haploidentical transplants.[19,99,106,194] The incidence of engraftment failure has been improved with the use of high-dose post-transplant cyclophosphamide, in part because of more effective elimination of residual host T cells. In addition, the recognition of donor-specific anti-HLA antibodies (DSA) as a major cause of engraftment failure, especially in haploidentical stem cell transplantation, has contributed to lower incidence of engraftment failure in this type of transplant.[48,49,195,196]

The main mechanism of primary graft failure is recipient immunologic reaction against donor cells,[197] mediated primarily by residual host T and NK cells that survived the preparative chemotherapy.[198,199] This mechanism of graft rejection is more important in major HLA mismatched transplants, making haploidentical stem cell transplant recipients more susceptible to develop graft rejection compared to HLA matched donor transplants. Nonetheless, recipients of HLA matched transplants are also at risk of developing T-cell-mediated graft rejection as a result of responses directed against minor histocompatibility antigens.[198]

While the use of myeloablative preparative chemotherapy as well as high-dose post-transplant cyclophosphamide in haploidentical transplantation can decrease the risk of cellular mediated graft rejection,[152] the adaptive immune response caused by recipient preformed alloantibodies caused by recipient's B cells cannot be easily mitigated by the escalation of chemotherapy intensity or post-transplant immunosuppression because of persistence of such antibodies in recipient's serum and/or tissues.

Although many anti-HLA antibodies can be detected in alloimmunized hematopoietic stem cell transplant recipients, only antibodies against donor HLA antigens (DSA) have been shown to be associated with transplant outcomes, and have become an important cause of graft rejection in HLA mismatched hematopoietic stem cell transplantation during the past decade.[48,49,52,195,200,201] This form of graft rejection is particularly important and more common in haploidentical transplants than in other types of HLA mismatched transplants due to the allosensitization of the female recipient through pregnancy against non-shared HLA antigens in a child donor.[48,49]

Anassetti and colleagues analyzed graft outcome of patients receiving allogeneic stem cell transplant from an HLA matched and mismatched relative donor and found that a positive crossmatch for anti-donor lymphocytotoxic antibody was an independent risk factor for engraftment failure, raising concerns about the role of HLA antibodies in graft rejection in allogeneic hematopoietic stem cell transplants with HLA mismatched donors.[52] Whether anti-HLA antibodies are causing engraftment failure has remained a matter of debate; however, several preclinical studies have showed that preformed anti-HLA antibodies present at the time of marrow infusion in multiply transfused mice, rather than primed T cells, are a major barrier against marrow engraftment.[202] Moreover, antibody-mediated rejection was extremely rapid in primed mice, and a higher bone marrow cell dose could partly overcome the predisposition to graft failure in sensitized mice,[202] presumably a higher antigen load would block more effectively circulating antibodies and allow engraftment. With evolving technology in recent years, novel solid phase immunoassays (SPI) that utilize purified preparations of molecules corresponding to single HLA antigens allow accurate identification of

HLA-antibody specificities.[203] Using modern technology of antibody detection, Ciurea et al. have demonstrated initially in haploidentical transplant recipients the clear association between DSAs and primary graft failure in recipients of T-cell depleted haploidentical stem cell transplantation with mega-doses of CD34+ stem cells. In this study, all patients with DSA were multiparous females and the most common reactivity was detected against HLA-DR and class I HLA antigens.[48] However, primary graft failure related to the development of antibodies against donor low expression HLA loci such as HLA-DPB1 and HLA-DQB1 has also been reported.[201] The significance of DSA in influencing the risk of graft failure has been confirmed in several subsequent studies using different platforms of haploidentical transplantation,[195,200] as well as in hematopoietic stem cell transplants with other mismatched grafts.[201,204,205] DSA have been shown not only to have a negative impact on engraftment but also have been shown to be associated with delayed engraftment, primary poor graft function,[200] and lower survival of haploidentical transplant recipients with DSA.[201]

The identification and specification of anti-HLA antibodies in transplant recipients can be performed using either cell-based crossmatch assays or SPIs. In cell-based crossmatch assays such as CDC or flow cytometry crossmatch test, donor T or B leukocytes are incubated with recipient serum to assess antibody reactivity with the donor's cell surface antigens via both complement dependent and non-complement dependent pathways. Even though it has been reported that the positive crossmatch test is strongly associated with the occurrence of primary graft failure,[52] this method has low sensitivity and specificity due to the possibility of detecting non-HLA antibodies, which might not be clinically relevant. Moreover, the cell-based crossmatch assays cannot identify antibody specificities in patients with complex antibody profiles.

In SPIs, the patient's serum is incubated with soluble HLA antigens bound to a solid matrix either by a microtiter plate (enzyme-linked immunosorbent assay; ELISA) or polystyrene beads (multiplexed multianalyte bead arrays) performed on a conventional flow cytometer or a fluoroanalyzer (Luminex). These tests are more sensitive than the conventional cell-based crossmatch assays and can be used for both antibody identification and semi-quantitively assess strength of HLA alloantigen-specific antibodies. Nonetheless, the assessment of DSA level using SPIs has not been well inter-laboratory standardized. Although cutoff values have been evaluated for different types of transplants, it is increasingly clear that there is a progressive increase in risk of rejection (in the absence of any treatment) with higher DSA levels, and, in TCR haploidentical transplants a higher risk was appreciated for patients with DSA > 10,000 MFI.[200]

Even though the exact mechanism by which DSA cause graft rejection in hematopoietic stem cell transplantation remains unclear, ample evidence in solid organ transplants has pointed toward complement-mediated cytotoxicity,[206–209] which has led to the assessment of functionality and ability of anti-HLA antibodies in complement fixation such as C4d and C1q assays. In haploidentical stem cell transplantation, our group has shown an association between high level of DSA (>5000 MFI) and their ability to activate complement pathway assessed by C1q test, and showed that patients who remained C1q positive at time of transplant experienced engraftment failure, while those who became C1q negative pre-transplant engrafted the donor cells.[49] Results from this study have prompted the EBMT recommendations on pre-transplant DSA and C1q assessment in all haploidentical transplant recipients when possible.[50] A follow-up study in patients who received treatment for DSAs confirmed these findings.[210]

Selecting a donor without the corresponding HLA antigens is the ideal option for recipients with DSA; however, when it is not possible pre-transplant desensitization treatment to decrease DSA levels and ensure a successful engraftment is required. To date several desensitization approaches have been studied, mostly in small numbers of patients with different graft outcomes. Most of these are based on a combination of different methods of immunosuppression, antibody removal, neutralization as well as antibody absorption.[49,195,211–215]

The investigators from MD Anderson Cancer Center (MDACC) initially reported outcomes of 12 haploidentical transplant patients with DSA receiving multimodality DSA desensitization with 3 sessions of alternate day plasma exchange with 1 to 1.5 × plasma volume (replaced with either fresh frozen plasma or 4% to 5% human plasma albumin), starting 1 week prior to admission for transplantation, followed by a single dose of rituximab 375 mg/m^2 the next day after completion of plasma exchange, and one day later by one dose of IvIg 1 g/kg. In addition, 5 patients received a buffy coat infused on day −1, prepared from 1 unit of blood from the same haploidentical donor to neutralize the remaining DSA prior to administration of hematopoietic stem cells. Even though the significant change of DSA level was not immediately observed, all patients who became C1q negative after treatment/before transplant successfully engrafted with the donor cells, suggesting that C1q negativity should be the primary goal of treatment rather than clearing of the non-complement binding DSA.[49] This protocol was later adopted by other institutions. In the updated report of 37 haploidentical recipients with DSA treated with this regimen at MDACC and City of Hope National Medical Center, reduction of DSA levels was observed after treatment and graft outcome and post-transplant survival of patients with initial DSA < 20,000 MFI and those with negative C1q after desensitization were comparable with control patients without DSA. In multivariable analysis, patients with initial DSA > 20,000 MFI and with persistent C1q+ after desensitization had a significantly lower engraftment rate, resulting in a significantly higher non-relapse mortality and worse overall survival than control group of patients without DSA.[210]

Treatment of Patients With Graft Failure After Transplantation

One of the main goals of transplantation is to prevent engraftment failure, which is associated with very high NRM and exposes patients to relapse due to lack of a functioning graft. As mentioned above, there are two main causes of engraftment failure—T-cell-mediated rejection and humoral rejection caused by DSAs. Knowing the cause of graft failure will help guide the treatment, which will need to include a second transplant performed as soon as possible after day 28 to avoid prolonged neutropenia and high mortality associated with this event.[216]

Only a few studies have explored salvage transplants after engraftment failure after transplantation.[217–219] In a recent study, Kongtim et al. showed that an urgent second transplant with a haploidentical donor and non-myeloablative conditioning (using the flurabine, cyclophosphamide and 2 Gray TBI regimen [Flu/Cy/TBI]) was associated with better long-term survival compared with any other salvage approach, and that changing the haploidentical donor to a donor with a different mismatched haplotype hopefully without DSA for those who received a prior haplo transplant was associated with better results. In addition, patients with AML/MDS and those who had a lower HCT-CI did better after salvage transplantation. Patients with AML/MDS who received an urgent haploidentical transplant from a different mismatched haplotype and with Flu/Cy/TBI conditioning had 3-year OS of 64.3%.[216]

In conclusion, growing evidence in the past decade has supported the significant role of DSA in mediating engraftment failure in haploidentical stem cell transplantation. Although several desensitization approaches have been explored, the number of patients treated is very small and it is unclear how effective these regimens are in reducing the risk of graft failure especially in patients with high DSA levels. The best available data is on the desensitization protocol developed at MDACC, described above. Until the more effective desensitization treatments are developed, this approach should be considered standard of care for patients with DSA levels up to 20,000 MFI. Patients with DSA should undergo desensitization treatment over 1 week with repeat DSA and C1q levels after 3 to 4 weeks and, if DSA remain greater than 10,000 or and/or C1q positive, transplantation should be discouraged. If levels decrease less than 10,000 MFI and C1q becomes negative, another course of treatment over 1 week followed by admission for transplant is recommended. If engraftment failure occurs an urgent rescue transplant with a haploidentical donor and non-myeloablative conditioning should be considered.

Toxicity From Chemotherapy

Complications Related to High-Dose Post-Transplant Cyclophosphamide

Hemorrhagic cystitis occurs in approximately a third of haploSCT patients receiving PTCy-based GVHD prophylaxis, is generally of limited severity, and most often attributed to polyomavirus (BK virus) infection.[220–223] Most patients tolerate very well PTCy, yet hemorrhagic cystitis can occur a few days following cyclophosphamide administration and is usually successfully prevented by adequate hydration and use of mesna during its administration.[224] As compared with hemorrhagic cystitis occurring within days after transplant, which is usually related to cyclophosphamide administration, cystitis that occurs a few weeks to a few months after transplant is usually related to BK virus reactivation (see Delayed Immunologic Reconstitution and Infectious Complications).

Another potential complication with the use of PTCy is development of cardiomyopathy (CMP), usually transient, which occurs in approximately 20% of patients, usually within the first month post-transplant. A retrospective analysis of 176 patients treated at a single institution found that impaired pre-transplant heart function did not influence the development of post-transplant CMP, rather other factors were more likely to be associated with development of CMP post-transplant, such as older age (>60) and a higher HCT-CI score ≥4.[225] Although in a case matched analysis, PTCy did not appear to be significantly associated with the development of CMP (21.9% vs. 14.8%, $P = .33$), a higher NRM and worse survival were observed in those who developed CMP post-transplant.[225,226] In addition to chemotherapy, excessive administration of IV fluids during chemotherapy may contribute to transient worsening in heart function and negatively impact transplant outcomes in these patients.[227,228]

Cytokine Release Syndrome

CRS is a syndrome characterized by the release of proinflammatory cytokines by the activated T cells, initially described as a complication of CAR-T-cell therapy. It manifests as fever (almost always present), which can progress to hypotension, hypoxia, and multiorgan failure.[228] A similar syndrome has been described in patients receiving haploidentical transplants.[229–231] CRS occurs typically within the first few days posttransplant, particularly when a peripheral blood stem cell graft is used. Patients develop high fever usually 24 to 48 hours after infusion of the graft, with negative blood cultures, which resolves after the first or more frequently after the second dose of cyclophosphamide administration. In several studies, approximately 90% of recipients of haploidentical transplants develop CRS,[230,231,233] usually mild (grade I–II). However, 12% to 17% can develop severe (grade III–V) CRS, which has been associated, in about half the patients, with multi-organ toxicity, higher NRM, and worse survival.[230,231,233] In the largest retrospective multicenter study to date, 451 patients receiving haploSCT were evaluated and authors found that severe CRS was significantly higher with peripheral blood versus bone marrow grafts (19% vs. 4.9%), and was associated with significantly higher NRM and worse survival.[233] In another study of 146 haploidentical transplant patients receiving PBSC grafts the cumulative incidence of NRM at 6 months for patients with severe CRS was 38% versus 8% for those who did not develop severe CRS.[231] Some reports suggested that patients with severe CRS may have delay in engraftment[230] and are more likely to develop acute GVHD post-transplant,[232] while others showed that mild CRS may be associated with a lower relapse and better survival post-transplant.[233]

Similar to CRS occurring in the setting of CAR-T-cell therapy, patients developing CRS after haploidentical transplantation were found to have higher IL-6 levels, and treatment with tocilizumab appears to be very effective for these patients.[230]

Delayed Immunologic Reconstitution and Infectious Complications

Rapid reconstitution of innate and adaptive immunity is critical for resistance to opportunistic infections after allogeneic SCT. Patients who undergo HLA-haploidentical SCT have had significantly impaired immune reconstitution as measured by delayed recovery of $CD4^+$ T cells compared with recipients of HLA-matched sibling grafts.[234] Similar findings have been reported in a pediatric group of patients.[235] This group showed that early reconstitution of $CD4^+$ T cells was associated with lower NRM and better overall survival. A number of factors contribute to impaired immune reconstitution after HLA-haploidentical SCT. Damage to lymphoid tissues resulting from conditioning may interfere with T-cell homing and the generation of immunologic memory. The rigorous TCD necessary to prevent GVHD in the haploidentical setting results in profound post-transplant immunodeficiency.[236,237] Finally, acute and chronic GVHD interfere with immune reconstitution,[238] in part through inhibition of thymopoiesis.[239] The delayed immune reconstitution after T-cell–depleted haploidentical SCT resulted in increased incidence of morbidity and NRM secondary to opportunistic infections. TCD haploidentical transplantation has been associated with delay in absolute lymphocyte count recovery[240] and a higher incidence of infectious complications compared with unmanipulated graft and PTCy-based GVHD prophylaxis.[19] We have also found that graft composition was independently associated with transplant outcomes. A balanced $CD4^+/CD8^+$ ratio (close to 1) was the most important determinant of transplant outcomes in haploidentical bone marrow transplantation; a low $CD4^+/CD8^+$ ratio (<0.85) and low $CD19^+$ numbers in the graft have been associated with higher NRM almost exclusively related to infectious complications,[7] findings that align prior reports which found a low $CD4^+$ count in the peripheral blood early post-transplant to be associated with higher NRM and worse survival.

Infectious Complications After Haploidentical Stem Cell Transplantation

Viral reactivation is common after haploidentical stem cell transplantation and occurs in approximately 70% of the patients by day 100 post-transplant.[221] Although less frequently encountered in haploidentical transplantation with PTCy-based GVHD prophylaxis,[19] viral reactivation remains a common complication, especially for CMV, HHV6, and BK virus (BKV), with very low incidence of EBV and adenovirus in adults.[221,241]

CMV reactivation occurs in 42% to 70% of patients receiving a haploidentical transplant with PTCy[220,221,241,242] after a median of approximately 40 days post-transplant. Although believed to be related mostly to haploidentical grafts, in a recent study performed by CIBMTR which compared incidence of CMV reactivation in three cohorts of patients: MSD with conventional GVHD prophylaxis ($n = 1605$), MSD with PTCy-based GVHD prophylaxis ($n = 403$), and haploidentical transplants with PTCy-based GVHD prophylaxis ($n = 757$) - it was found that the use of PTCy rather than a haploidentical donor was mainly responsible for a higher incidence of CMV reactivation (23%, 37%, and 42%, respectively; $P < .001$).[243] A major recent advance in prevention of CMV reactivation and development of CMV disease has been the use of letermovir in the first 100 days post-transplant,[244] which has also been shown to decrease significantly the incidence of CMV reactivation in haploidentical transplant patients receiving PTCy-based GVHD prophylaxis (68.8% to 21.9%, $P < .001$).[245]

BK virus hemorrhagic cystitis is seen with increased frequency in alternative donor transplants (MUD, cord blood, and haploidentical transplants)[246–248] and is more common in younger individuals[246] and patients receiving myeloablative conditioning.[222,247,249]

Although the great majority of patients receiving a haploidentical transplant with PTCy may have asymptomatic BK viruria (80% to 90%) only approximately half will develop symptomatic cystitis, mostly between 1 and 2 months post-transplant (median 38 days). The incidence of BKV cystitis appears to vary based on intensity and type of conditioning, with lowest incidence with NMA conditioning (23%),[220] 30% to 40% with melphalan-based conditioning and TBI-based conditioning,[241] and up to 75% with busulfan-based conditioning.[242] Other factors associated with BKV cystitis were CMV reactivation, development of GVHD, and/or treatment with steroids.[222,249] In a study from MDACC, significant (grade II–IV) BKV cystitis occurred in 24% of the patients receiving melphalan-based conditioning, while 8% experienced severe cystitis requiring bladder irrigation or nephrostomy tube placement.[241,250] Although development of BKV cystitis has been associated with increased morbidity and prolonged hospitalization, no impact on survival has been observed in several studies as 90% of the cases resolve after 4 to 6 weeks.[222,241,249] Treatment of BKV hemorrhagic cystitis remains mostly symptomatic with adequate hydration to prevent significant clot formation and ureteral obstruction, bladder irrigation, and pain control, as no effective drug treatment exists. However, recent success has been seen with viral CTLs, either targeting only BKV or more commonly with multi-virus CTLs with resolution of symptoms within 4 weeks of treatment in most patients.[251,252]

Relapse Post-Transplant

Mechanisms of Disease Relapse Post Haplo-Transplant

As treatment-related mortality has progressively improved post-transplant,[253] relapse has emerged as the most common cause of treatment failure, including after HLA-haploidentical SCT. There is substantial controversy regarding whether HLA disparity between donor and recipient reduces relapse through a more intense GVH reaction. Most studies have not shown decreased relapse rates after HLA-haploidentical as compared with HLA-matched SCT,[177,187,254] although such studies may be confounded by significant differences between donor types in the GVHD prophylaxis regimens employed. Other studies have shown a decreased risk of relapse associated with HLA mismatching,[255] especially for patients with poor-risk hematologic malignancies.[8] More recent data suggests that it is not the number of mismatched HLA loci associated with lower relapse rate but rather a higher number of molecular mismatches quantified by mismatched eplet load at each HLA locus.[35]

Approximately one-third of leukemic relapses after TCD HLA-haploidentical SCT are associated with loss of the mismatched HLA locus due to acquired uniparental disomy, a process in which the unshared HLA haplotype on chromosome 6 is replaced by the shared HLA haplotype.[256,257] HLA-alloreactive T cells may be providing the selective immunological pressure that results in the emergence of these variants, because relapse is never associated with loss of the shared HLA haplotype, and HLA loss by uniparental disomy suggesting that this could be a mechanism specific to haploSCT. However, recent data found this mechanism of relapse in transplants with all donor types and seem to correlate with the number of HLA mismatches.[258] In addition, there is growing evidence of another immune escape mechanism as cause of relapse after allogeneic transplantation, in general, with downregulation of HLA class II molecules or upregulation of inhibitory molecules by the tumor cells.[259–261] Clinically, relapses of AML associated with loss of mismatched HLA tend to occur later than relapses with preserved HLA expression (307 days vs. 88 days; $P < .001$),[262] suggesting that they originate in a smaller clone, resistant to the immunologic effect of the graft,[258] and tend to carry a similarly poor prognosis (OS at 6 months after relapse was 28.5% for patients with HLA loss and 27.4% for patients with "classic" relapse). These considerations may have important therapeutic implications as donor lymphocyte infusion (DLI) may not be effective in light of the loss of the major targets of alloreactive T cells, while upregulation of inhibitory molecules may potentially respond to checkpoint inhibition.[258] Survival may be improved by performing a second transplant for patients with HLA loss, especially from a donor with a different mismatched haplotype.[263]

Treatment of Relapsed Hematologic Malignancy After Human Leukocyte Antigen-Haploidentical Stem Cell Transplants

Unmanipulated DLIs have been administered to patients with relapsed hematologic malignancy after HLA-haploidentical SCT with PTCy. In one study, DLI was administered as a series of escalating infusions (ranging from 10^3 to 10^7 T cells/kg), with or without preceding chemotherapy, to patients with acute leukemia, in molecular ($n = 20$) or hematologic ($n = 12$) relapse, or to patients with Hodgkin lymphoma ($n = 10$).[264] The incidence of acute GVHD after haploidentical DLI in these groups was 15%, 17%, and 10%, respectively, and the response rates were 45%, 33%, and 70%. Two-year actuarial survival rates were 43%, 19%, and 80%, respectively. In a second report, 40 patients, including 16 with AML and 11 with lymphoma, received DLI starting at a dose of 10^5 $CD3^+$ T cells/kg with subsequent dose escalation.[265] The most commonly used first dose was 10^6 $CD3^+$ T cells/kg. Acute GVHD developed in 10 patients, 6 patients had grades III–IV, and 3 developed chronic GVHD. Twelve patients (30%) achieved a complete response with a median duration of 11.8 months. Eight patients were alive in complete response at the time of reporting, six for more than 1 year.[265]

The Beijing group developed a modified DLI approach in which patients with relapsed leukemia after haploSCT received chemotherapy and peripheral blood leukocytes from filgrastim-treated donors followed by an immunosuppressive drug (CsA or methotrexate) for 2 to 8 weeks after DLI.[266] One hundred twenty-four patients in relapse after haploSCT received chemotherapy followed by the modified DLI containing a median of 4×10^7 $CD3^+$ T cells/kg of recipient weight. The cumulative incidence of DLI-associated acute GVHD was 53.2% for grades II–IV and 28.4% for grades III–IV. The duration of GVHD prophylaxis after DLI was the only risk factor for DLI-associated grades III–IV acute GVHD ($P < .05$). The 2-year OS, NRM, and cumulative incidence of relapse after DLI were 47.2%, 34.1%, and 34.6%, respectively. A subsequent study compared the antileukemic effects of chemotherapy alone ($n = 32$) versus chemotherapy followed by modified DLI ($n = 50$), with immunosuppression given for 4 to 8 weeks after DLI, in patients with relapsed acute leukemia after haploSCT.[265] In patients receiving chemotherapy followed by modified DLI, the CR rate was significantly higher (64.0% vs. 12.5%; $P = .000$), the incidence of relapse was significantly lower (50.0% vs. 100.0%; $P = .000$), and DFS was significantly improved (36.0% vs. 0.0%; $P = .000$) compared with patients receiving chemotherapy alone. Multivariate analysis demonstrated that patients with chronic GVHD after intervention ($P = .000$) and patients receiving chemotherapy followed by modified DLI ($P = .037$) had a lower relapse rate.

In summary, hematologic malignancies in relapse after HLA-haploidentical SCT can be treated with chemotherapy + DLI with the goal to achieve remission. Patients with relapse of acute leukemia within the first 6 months after transplant generally have a poor prognosis. Only a minority of patients will achieve remission to salvage therapy ± DLI and, if they do, are younger, greater than 6 months from the first transplant, no infection, good performance status, and low comorbidity index and are taken urgently to a second transplant, preferably with a donor with a different mismatched haplotype and conditioning regimen.[263] For patients with relapse of leukemia after 6 months, HLA typing of the leukemic blasts is recommended. If mismatched HLA expression is preserved, chemotherapy ± DLI may be used. For patients in overt hematologic relapse treated with PTCy-based GVHD prophylaxis, a starting dose of 10^6 $CD3^+$ T cells/kg is usually recommended. If mismatched HLA alleles have been lost, salvage chemotherapy followed by an urgent second transplant procedure from a different donor may provide the best outcome.[263,268]

Prevention of Relapse After Haploidentical Stem Cell Transplantation

Outcomes of patients with disease relapse after transplant are very poor, with only approximately 20% of patients responding to salvage treatment, and only approximately 20% of these patients will achieve disease control and meet transplant eligibility, and are able to proceed to a second transplant. Hence preventing post-transplant relapse, especially in patients with high-risk leukemia, becomes of paramount importance to improve survival after allogeneic transplantation.

Several cell-based approaches have been used to prevent disease relapse post-transplant (Table 107.3), initially with unmodified DLI, more recently with genetically modified T cells and ex vivo expanded NK cells. These reports suggest that cellular therapy with lymphocytes obtained from the same haploidentical donor can be safely and successfully applied post-transplant with encouraging results.[267]

Unmodified Donor Lymphocyte Infusion

One of the major advantages of using haploidentical donors for transplantation is the opportunity to collect donor T cells or NK cells to treat or prevent relapse post-transplant. This approach has been used primarily in patients with persistent MRD or with decrease in chimerism after transplant.[270–275] Although there is concern with increase in the potential to induce severe aGVHD after haploidentical DLI, the incidence of GVHD was not shown, in general, to be higher than in HLA matched transplants,[264,265,276] possibly in part due to the tolerizing effect of PTCy. In one of the earliest reports, 40 patients with hematological malignancies received an unmodified haploidentical DLI for relapsed disease after haploSCT with PTCy-based GVHD prophylaxis).[265] Most patients received 1×10^6/kg CD3$^+$ T cells. Acute GVHD developed in 25% of patients, and 15% of patients had grade III–IV aGVHD. Efficacy of DLI appears to be improved with the use of cytoreductive therapy prior to the DLI infusion, with higher likelihood of achieving CR and improved survival in some studies.[264,276] DLI is expected to be more effective in patients with low tumor burden, such as patients with minimal residual disease,[277] in patients with lymphoid malignancies,[264,278] and when administered in conjunction with cytoreductive chemotherapy.[265,272]

Modified Donor Lymphocyte Infusion With a Safety Switch

The most important risk of administering donor lymphocytes early post-transplant is the development of severe acute GVHD, which could be potentially controlled by inserting a suicide gene in the donor T cells. If significant aGVHD develops, T cells can be turned off by activating this switch with a drug to avoid excessive aGVHD. This approach has so far been investigated to boost post-transplant immune recovery after T-cell-depleted haploidentical grafts. Ciceri et al. infused DLI engineered T cells to express herpes simplex virus-thymidine kinase suicide gene that can be targeted by ganciclovir to induce apoptosis.[83] Grade I–IV aGVHD developed in 20% of the patients and GVHD was successfully controlled by inducing the suicide gene with ganciclovir. However, ganciclovir is commonly used post-transplant to control CMV reactivation or disease thus may not be the ideal drug in this setting. Another approach developed by the Baylor group used engineered DLI to express an inducible caspase-9 transgene.[84] This gene can be induced by an inert dimerizing drug leading to rapid cell death. In an initial report, GVHD which occurred in 4 of the 5 haploSCT patients after infusing the safety switch-modified T cells, was rapidly stopped by administration of the dimerizing drug.[84] Long-term outcomes were reported for 10 haploSCT pediatric patients infused with the same product, showing persistence of these modified cells for over 2 years and potentially contributing to control of opportunistic infections post-transplant and promoting endogenous T-cell recovery.[85] This approach has been explored after TCD haploidentical transplantation with encouraging results in pediatric patients with acute leukemia.[279]

TABLE 107.3 **Post-Transplant Cellular Therapies to Prevent Relapse After Haploidentical Transplantation (Selected Studies)**

Approach	Advantages	Limitations	References
Unmodified DLI	Increase graft-versus-tumor effect	Not targeted to specific antigen(s) Potential for development of severe GVHD especially with higher doses Efficacy possibly higher with chemotherapy	265,274,276,297
Modified DLI with a "safety switch"	Increase graft-versus-tumor effect "Safety switch" allows elimination of T cells in case of GVHD development	Not targeted to specific antigen(s) Clinical efficacy not yet demonstrated in large number of patients	83,84,298
Chimeric antigen receptor modified T cells (CAR-T)	Genetically modified T cells to express a CAR (e.g., CD19) Could provide graft-versus-tumor effect Low risk of GVHD	A small number of patients treated to date Efficacy unproven	284,299
Ex vivo expanded NK cells	Potential graft-versus-leukemia effect without GVHD	Clinical efficacy demonstrated in a phase II study	68,289
Photodepleted T cells	Depletion of alloreactive T cells by photodepletion	Efficacy in phase II study Randomized phase III study stopped early	300,301
δγ DLI	Selected δγ T cells Potential to increase anti-tumor effect especially after αββTCD haploSCT ? Limited/no GVHD potential	Efficacy unproven	302

DLI, Donor lymphocyte infusion; *GVHD*, graft-versus-host disease; *NK*, natural killer; *TCD*, T-cell depleted.

Chimeric Antigen Receptor Modified T Cells

While DLI offers a non-specific anti-tumor activity, its effect does not target directly the malignant cells. The recent success of CAR-T-cell therapy in patients with CD19 B-cell lymphoid malignancies[280–282] prompted investigation of this form of therapy for patients with advanced B-cell ALL after transplant to improve relapse rate.[283,284] Several studies reported experience with donor-derived CAR-T-cell therapy administered for relapsed disease after allogeneic stem cell transplantation.[283,285,286] Kochenderfer and colleagues used donor-derived allogeneic CAR-T cells for patients with B-cell lymphoid malignancies relapsed after allogeneic stem cell transplant without lymphodepleting chemotherapy and obtained response in 30% of the patients.[283] The MDACC group explored the use of CAR-T cells administered in the peri-transplant setting to prevent disease relapse for patients with B-cell lymphoid malignancies and improve outcomes after autologous and allogeneic transplantation. Results from the phase I clinical trial for patients with B-cell ALL receiving allogeneic transplant showed safety of this approach and very low relapse rate. Eight patients received a haploSCT followed by donor-derived CAR-T-cell infusion with 1-year PFS and OS of 75% and 100%, respectively, and with no increased risk of GVHD.[282] These preliminary studies suggested that allogeneic CAR-T cells can be safely administered post-transplant to treat or prevent disease relapse without significant GVHD.

Infusion of Ex Vivo Expanded Natural Killer Cells

NK cells are part of the innate immune system capable of recognizing and killing malignant or virally infected cells. NK cells recognize and target "foreign" cells that lack one or more HLA class I alleles specific to the inhibitory receptors (killer immunoglobulin-like receptors-KIRs) or by increasing activator signals on malignant cells.[287] High NK cell numbers early post-transplant,[288] as well as high NK cell numbers in the haploidentical bone marrow graft,[7] have been associated with lower incidence of GVHD. Although prior studies in T-cell depleted haploidentical transplants showed lower incidence of relapse when patients had a KIR-ligand mismatched (KIR-LM) donor,[70] several studies in T-cell-replete (TCR) haploSCT (both ATG-based or PTCy-based) suggest no role of KIR-LM in lowering relapse risk, at least after TCR haploidentical transplantation.[76] Although there is no convincing role of KIR-LM in decreasing relapse post-transplant, our group explored the anti-tumor effects of NK cells in the post-transplant setting and hypothesized that high doses of ex vivo expanded NK cells would decrease the relapse rate after transplant with very encouraging results. In a phase I dose-escalation study, using mb-IL21 expanded NK cells, 13 patients with high-risk myeloid malignancies received escalating doses of donor-derived NK cells on days −2, +7, and +28 of haploSCT.[68] The study demonstrated feasibility of infusing high doses of ex vivo expanded NK cells after transplant without toxicities or GVHD. A remarkable low post-transplant relapse was observed.[68] Preliminary results from the phase II study confirmed these findings, and, in an independent matched-pair analysis performed by CIBMTR, NK-cell-treated patients showed a significantly lower 2-year relapse rate of 4% vs. 38% ($P = .014$) and improved PFS (66% vs. 44%) ($P = .1$) compared with patients who did not receive NK cells.[289] Based on these promising results, phase II and a phase III randomized study with and without NK-cell administration early post-transplant are planned.

DONOR SELECTION FOR HAPLOIDENTICAL TRANSPLANTATION (TABLE 107.4)

The great majority of patients have more than one potential haploidentical donor available for donation and include first-degree relatives (parents, children, siblings, half-siblings) and second-degree relatives (cousins, nieces, nephews, and grandchildren). Several patient and donor characteristics have been shown to influence the choice of donor, including medical and psychologic suitability of the donor, donor age, sex, the presence of DSA in the recipient's serum, degree of donor-recipient HLA mismatch, red blood cell ABO group compatibility, and donor and recipient CMV serology. Regardless of transplant platform, there are two absolute contraindications to donation: (1) the donor is medically or psychologically unfit to donate or (2) the recipient has high levels of DSA against donor HLA antigens (>20,000 MFI or C1q remains positive after desensitization therapy)[210] (see also Graft Failure).

TABLE 107.4 Algorithm for Donor Selection in Haploidentical Stem Cell Transplantation With Post-Transplant Cyclophosphamide

Priority	TCR Haploidentical SCT
1	No DSA against donor[a]
2	Younger preferred to older donor
3	Male donor for male recipient[b]
4	Sibling or offspring over parent donor
5	Between parents, father is preferred over mother
6	ABO matched preferred to minor ABO mismatch to major ABO mismatch
7	First-degree relative preferred over second-degree relative[c]
8	KIR ligand match preferred to KIR ligand mismatch[c]
9	NIMA mismatch preferred over NIPA mismatch[c]

[a] If a donor without DSA is not identified desensitization treatment may be performed for patients with DSA up to 20,000 MFI (MDACC desensitization protocol only).
[b] Avoid older female donors.
[c] Data available at present only for the GIAC protocol.
KIR, Killer immunoglobulin ligand; *NIMA*, non-inherited maternal antigens; *NIPA*, non-inherited paternal antigens; *TCR*, T-cell–replete.
Adapted from Ciurea SO, Al Malki MM, Kongtim P, et al. The European Society for Blood and Marrow Transplantation (EBMT) consensus recommendations for donor selection in haploidentical hematopoietic cell transplantation. *Bone Marrow Transplant*. 2020;55(1):12–24.

First, it is important to mention that the transplant platform (i.e., T-cell-depleted versus T-cell-replete haploidentical transplantation) may influence donor choice. For example, in a study of 118 patients receiving mega-dose TCD transplants, 5-year event-free survival was better in patients who received transplants from the mother than from the father (50.6% ± 7.6% vs. 11.1% ± 4.2%; $P < .001$), with better survival resulting from both a reduced incidence of relapse and transplant-related mortality. The 5-year event-free survival was 27.0% ± 6% and 25.5% ± 9% for patients who received transplants from an HLA haploidentical sister ($n = 30$) or brother ($n = 49$), respectively ($P = .63$). On the basis of these results, the authors suggested that the mother of the patient is the preferred HLA-haploidentical donor for TCD haploSCT. The opposite is true for TCR haploidentical transplants where older female donors have been found to be associated with higher incidence of severe acute GVHD, higher NRM, and worse survival.[7,76] Incidence of severe grade III–IV aGVHD was found to be as high as 54% in older female donors owing to a higher CD4/CD8 ratio in the bone marrow graft of these donors, combined with lower % CD56 (NK cells), lower CC34+ (stem cells), and CD19+ (B-cells) in the graft found in older donors caused increased NRM in these patients.[7] Similar results were reported by Wang et al. who studied outcomes of 1210 patients treated with the GIAC protocol. With this platform, younger donors and male donors were found to be associated with less NRM and better survival. By contrast, use of maternal donors was associated with higher incidence of acute and chronic GVHD, and worse survival.[121] Similar results have been reported in haploSCT with PTCy.[290,291] Other important factors in selecting donors for haploidentical stem cell transplantation include children rather than siblings for older recipients,[290,291] ABO matched vs. minor ABO mismatch vs. major ABO mismatch,[292] first-degree versus second-degree haploidentical donor, KIR ligand match versus KIR ligand mismatch donor, and NIMA versus NIPA mismatch donor for TCR haploSCT patients treated with the GIAC protocol.[121] The use of second-degree family donors has been reported by the Johns Hopkins group to be feasible and associated with similar outcomes as use of first-degree haploidentical donors[293]; however, to date, no study has evaluated outcomes with second- versus first-degree haploidentical donors in patients receiving PTCy-based GVHD prophylaxis (Table 107.4). The last important issue is the role of NK-cell alloreactivity appreciated by the presence of KIR ligand mismatch (KIR-LM) between the donor and recipient in haploidentical transplant recipients receiving a TCR graft, previously shown to be associated with lower relapse and improved survival in patients receiving TCD haploSCT.[70] Recent data on a large number of patients receiving PTCy-based GVHD prophylaxis showed that patients with a KIR-LM had higher NRM and decreased survival compared with KIR ligand match recipients,[75] findings concordant with reported data by the Beijing group with the GIAC protocol.[121] Consequently, the use of a KIR-LM haploidentical donor cannot be recommended at the present time in patients receiving a TCR haploidentical transplantation.

CONCLUSIONS AND FUTURE DIRECTIONS

HLA-haploidentical donors are a rapid and near universally available source of stem cells for transplantation. Major HLA mismatch is associated with intense bidirectional reactions resulting in higher incidences of graft failure and GVHD, thus effective control of alloreactive reactions is needed in this setting. Due to higher NRM, haploidentical transplants have evolved from complete depletion of T cells from the graft to a partial T-cell depletion or infusion of full graft with effective GVHD prevention. Three modern approaches to haploSCT have emerged in the recent years: (1) High-dose post-transplant cyclophosphamide-based GVHD prophylaxis, (2) the GIAC protocol involving administration of ATG, filgrastim-mobilized marrow and PBSCs, and intensive posttransplant immunosuppression, and (3) alpha-beta T-cell depleted haploSCT. While multiple retrospective studies comparing outcomes with different donor sources have been performed, only one randomized study to date has been done comparing outcomes of haploidentical transplants with PTCy and cord blood transplants. Prospective randomized trials comparing different approaches to haploidentical transplantation as well as different donor sources with intent-to-treat will be needed to determine the donor priority for patients referred for allogeneic transplantation.

Priorities for future research include prevention of disease relapse, improvement of immunologic reconstitution post-transplant, as well as exploration of graft manipulation to improve outcomes after haploidentical stem cell transplantation.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Al Malki MM, Jones R, Ma Q, et al. Proceedings from the fourth haploidentical stem cell transplantation symposium (HAPLO2016), San Diego, California, December 1, 2016. *Biol Blood Marrow Transplant*. 2018;24:895–908.

Bachegowda LS, Saliba RM, Ramlal R, et al. Predictive model for survival in patients with AML/MDS receiving haploidentical stem cell transplantation. *Blood*. 2017;129:3031–3033.

Bittencourt MCB, Ciurea SO. Recent advances in allogeneic hematopoietic stem cell transplantation for acute myeloid leukemia. *Biol Blood Marrow Transplant*. 2020;26:e215–e221.

Canaani J, Labopin M, Huang XJ, et al. Minimal residual disease status predicts outcome of acute myeloid leukaemia patients undergoing T-cell replete haploidentical transplantation. An analysis from the Acute Leukaemia Working Party (ALWP) of the European Society for Blood and Marrow Transplantation (EBMT). *Br J Haematol*. 2018;183:411–420.

Im A, Rashidi A, Wang T, et al. Risk factors for graft-versus-host disease in haploidentical hematopoietic cell transplantation using post-transplant cyclophosphamide. *Biol Blood Marrow Transplant*. 2020;26:1459–1468.

Imus PH, Tsai HL, DeZern AE, et al. Thrombotic microangiopathy after post-transplantation cyclophosphamide-based graft-versus-host disease prophylaxis. *Biol Blood Marrow Transplant*. 2020;26:2306–2310.

Kanakry CG, Fuchs EJ, Luznik L. Modern approaches to HLA-haploidentical blood or marrow transplantation. *Nat Rev Clin Oncol*. 2016;13:132.

Nagler A, Labopin M, Dholaria B, et al. Comparison of haploidentical bone marrow versus matched unrelated donor peripheral blood stem cell transplantation with posttransplant cyclophosphamide in patients with acute leukemia. *Clin Cancer Res*. 2021;27:843–851.

Piemontese S, Boumendil A, Labopin M, et al. Leukemia relapse following unmanipulated haploidentical transplantation: a risk factor analysis on behalf of the ALWP of the EBMT. *J Hematol Oncol*. 2019;12:68.

Robin M, Porcher R, Ruggeri A, et al. HLA-mismatched donors in patients with myelodysplastic syndrome: an EBMT registry analysis. *Biol Blood Marrow Transplant*. 2019;25:114–120.

Sahebi F, Garderet L, Kanate AS, et al. Outcomes of haploidentical transplantation in patients with relapsed multiple myeloma: an EBMT/CIBMTR report. *Biol Blood Marrow Transplant*. 2019;25:335–342.

Santoro N, Labopin M, Ciceri F, et al. Impact of conditioning intensity on outcomes of haploidentical stem cell transplantation for patients with acute myeloid leukemia 45 years of age and over. *Cancer*. 2019;125:1499–1506.

Sanz J, Galimard JE, Labopin M, et al. Post-transplant cyclophosphamide after matched sibling, unrelated and haploidentical donor transplants in patients with acute myeloid leukemia: a comparative study of the ALWP EBMT. *J Hematol Oncol*. 2020;13:46.

Shah NN, Ahn KW, Litovich C, et al. Outcomes of Medicare-age eligible NHL patients receiving RIC allogeneic transplantation: a CIBMTR analysis. *Blood Adv*. 2018;2:933–940.

Solomon St SR, Martin A, Shah NN, et al. Myeloablative vs reduced intensity T-cell-replete haploidentical transplantation for hematologic malignancy. *Blood Adv*. 2019;3:2836–2844.

Stabile H, Nisti P, Fionda C, et al. NK cell reconstitution in paediatric leukemic patients after T-cell-depleted HLA-haploidentical haematopoietic stem cell transplantation followed by the reinfusion of iCasp9-modified donor T cells. *J Clin Med*. 2019:8.

Van Elssen C, Ciurea SO. NK cell alloreactivity in acute myeloid leukemia in the post-transplant cyclophosphamide era. *Am J Hematol*. 2020;95: 1590–1598.

REFERENCES

1. Copelan EA. Hematopoietic stem-cell transplantation. *N Engl J Med*. 2006;354:1813–1826.
2. Gragert L, Eapen M, Williams E, et al. HLA match likelihoods for hematopoietic stem-cell grafts in the U.S. registry. *N Engl J Med*. 2014; 371:339–348.
3. Ciurea SO, Bittencourt MCB, Milton DR, et al. Is a matched unrelated donor search needed for all allogeneic transplant candidates? *Blood Adv*. 2018;2:2254–2261.
4. Passweg JR, Baldomero H, Chabannon C, et al. The EBMT activity survey on hematopoietic-cell transplantation and cellular therapy 2018: CAR-T's come into focus. Bone Marrow Transplant 2020;55:1604-13.
5. Debals-Gonthier M, Siani C, Faucher C, et al. Cost-effectiveness analysis of haploidentical vs matched unrelated allogeneic hematopoietic stem cells transplantation in patients older than 55 years. *Bone Marrow Transplant*. 2018;53:1096–1104.
6. Rocha V, Labopin M, Gluckman E, et al. Relevance of bone marrow cell dose on allogeneic transplantation outcomes for patients with acute myeloid leukemia in first complete remission: results of a European survey. *J Clin Oncol*. 2002;20:4324–4330.
7. Saliba RM, Veltri L, Rondon G, et al. Impact of graft composition on outcomes of haploidentical bone marrow stem cell transplantation. *Haematologica*. 2020;106:269–274.
8. Kanda Y, Chiba S, Hirai H, et al. Allogeneic hematopoietic stem cell transplantation from family members other than HLA-identical siblings over the last decade (1991–2000). *Blood*. 2003;102:1541–1547.
9. Wang Y, Liu DH, Xu LP, et al. Superior graft-versus-leukemia effect associated with transplantation of haploidentical compared with HLA-identical sibling donor grafts for high-risk acute leukemia: an historic comparison. *Biol Blood Marrow Transplant*. 2011;17:821–830.
10. Burroughs LM, O'Donnell PV, Sandmaier BM, et al. Comparison of outcomes of HLA-matched related, unrelated, or HLA-haploidentical related hematopoietic cell transplantation following nonmyeloablative conditioning for relapsed or refractory Hodgkin lymphoma. *Biol Blood Marrow Transplant*. 2008;14:1279–1287.
11. Saini NY, Saliba RM, Rondon G, et al. Impact of donor type and melphalan dose on allogeneic transplantation outcomes for patients with lymphoma. *Biol Blood Marrow Transplant*. 2019;25:1340–1346.
12. Suchin EJ, Langmuir PB, Palmer E, Sayegh MH, Wells AD, Turka LA. Quantifying the frequency of alloreactive T cells in vivo: new answers to an old question. *J Immunol*. 2001;166:973–981.
13. Powles RL, Morgenstern GR, Kay HE, et al. Mismatched family donors for bone-marrow transplantation as treatment for acute leukaemia. *Lancet*. 1983;1:612–615.
14. Szydlo R, Goldman JM, Klein JP, et al. Results of allogeneic bone marrow transplants for leukemia using donors other than HLA-identical siblings. *J Clin Oncol*. 1997;15:1767–1777.
15. Ash RC, Horowitz MM, Gale RP, et al. Bone marrow transplantation from related donors other than HLA-identical siblings: effect of T cell depletion. *Bone Marrow Transplant*. 1991;7:443–452.
16. Mulanovich VE, Jiang Y, de Lima M, Shpall EJ, Champlin RE, Ciurea SO. Infectious complications in cord blood and T-cell depleted haploidentical stem cell transplantation. *Am J Blood Res*. 2011;1:98–105.
17. Bayraktar UD, Champlin RE, Ciurea SO. Progress in haploidentical stem cell transplantation. *Biol Blood Marrow Transplant*. 2012;18:372–380.
18. Aversa F, Terenzi A, Tabilio A, et al. Full haplotype-mismatched hematopoietic stem-cell transplantation: a phase II study in patients with acute leukemia at high risk of relapse. *J Clin Oncol*. 2005;23:3447–3454.
19. Ciurea SO, Mulanovich V, Saliba RM, et al. Improved early outcomes using a T cell replete graft compared with T cell depleted haploidentical hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2012;18:1835–1844.
20. Bethge WA, Faul C, Bornhäuser M, et al. Haploidentical allogeneic hematopoietic cell transplantation in adults using CD3/CD19 depletion and reduced intensity conditioning: an update. *Blood Cells Mol Dis*. 2008; 40:13–19.
21. Federmann B, Bornhauser M, Meisner C, et al. Haploidentical allogeneic hematopoietic cell transplantation in adults using CD3/CD19 depletion and reduced intensity conditioning: a phase II study. *Haematologica*. 2012;97:1523–1531.
22. Lang P, Teltschik HM, Feuchtinger T, et al. Transplantation of CD3/CD19 depleted allografts from haploidentical family donors in paediatric leukaemia. *Br J Haematol*. 2014;165:688–698.
23. Amrolia PJ, Muccioli-Casadei G, Huls H, et al. Adoptive immunotherapy with allodepleted donor T-cells improves immune reconstitution after haploidentical stem cell transplantation. *Blood*. 2006;108:1797–1808.
24. Bacchetta R, Lucarelli B, Sartirana C, et al. Immunological outcome in haploidentical-HSC transplanted patients treated with IL-10-anergized donor T cells. *Front Immunol*. 2014;5:16.
25. Bastien JP, Krosl G, Therien C, et al. Photodepletion differentially affects CD4+ Tregs versus CD4+ effector T cells from patients with chronic graft-versus-host disease. *Blood*. 2010;116:4859–4869.
26. Schumm M, Lang P, Bethge W, et al. Depletion of T-cell receptor alpha/beta and CD19 positive cells from apheresis products with the CliniMACS device. *Cytotherapy*. 2013;15:1253–1258.
27. Luznik L, Jalla S, Engstrom LW, Iannone R, Fuchs EJ. Durable engraftment of major histocompatibility complex-incompatible cells after nonmyeloablative conditioning with fludarabine, low-dose total body irradiation, and posttransplantation cyclophosphamide. *Blood*. 2001;98:3456–3464.
28. Solomon SR, Sizemore CA, Sanacore M, et al. Haploidentical transplantation using T cell replete peripheral blood stem cells and myeloablative conditioning in patients with high-risk hematologic malignancies who lack conventional donors is well tolerated and produces excellent relapse-free survival: results of a prospective phase II trial. *Biol Blood Marrow Transplant*. 2012;18:1859–1866.
29. Raiola AM, Dominietto A, Ghiso A, et al. Unmanipulated haploidentical bone marrow transplantation and posttransplantation cyclophosphamide for hematologic malignancies after myeloablative conditioning. *Biol Blood Marrow Transplant*. 2013;19:117–122.
30. Gaballa S, Ge I, El Fakih R, et al. Results of a 2-arm, phase 2 clinical trial using post-transplantation cyclophosphamide for the prevention of graft-versus-host disease in haploidentical donor and mismatched unrelated donor hematopoietic stem cell transplantation. *Cancer*. 2016;122:3316–3326.
31. Di Stasi A, Milton DR, Poon LM, et al. Similar transplantation outcomes for acute myeloid leukemia and myelodysplastic syndrome patients with haploidentical versus 10/10 human leukocyte antigen-matched unrelated and related donors. *Biol Blood Marrow Transplant*. 2014;20:1975–1981.
32. Airoldi I, Bertaina A, Prigione I, et al. γδ T-cell reconstitution after HLA-haploidentical hematopoietic transplantation depleted of TCR-αβ+/CD19 + lymphocytes. *Blood*. 2015;125:2349–2358.
33. Ciurea SO, Bayraktar UD. "No donor"? Consider a haploidentical transplant. *Blood Rev*. 2015;29:63–70.
34. Rimando J, Slade M, DiPersio JF, et al. HLA epitope mismatch in haploidentical transplantation is associated with decreased relapse and delayed engraftment. *Blood Adv*. 2018;2:3590–3601.
35. Zou J, Ciurea SO, Kongtim P, et al. Molecular disparity in human leukocyte antigens is associated with outcomes in haploidentical stem cell transplantation. *Blood Adv*. 2020;4:3474–3485.
36. Melenhorst JJ, Scheinberg P, Williams A, et al. Alloreactivity across HLA barriers is mediated by both naïve and antigen-experienced T cells. *Biol Blood Marrow Transplant*. 2011;17:800–809.
37. Finberg R, Burakoff SJ, Cantor H, Benacerraf B. Biological significance of alloreactivity: T cells stimulated by Sendai virus-coated syngeneic cells specifically lyse allogeneic target cells. *Proc Natl Acad Sci U S A*. 1978; 75:5145–5149.
38. Amir AL, D'Orsogna LJ, Roelen DL, et al. Allo-HLA reactivity of virus-specific memory T cells is common. *Blood*. 2010;115:3146–3157.
39. Westerhuis G, de Witte M, Schumacher TN, Toes RE, Fibbe WE. Barriers to chimerism after major histocompatibility complex-mismatched stem cell transplantation: a potential role for heterologous immunity. *Exp Hematol*. 2014;42:753–760.
40. Nadazdin O, Boskovic S, Murakami T, et al. Host alloreactive memory T cells influence tolerance to kidney allografts in nonhuman primates. *Sci Transl Med*. 2011;3:86ra51.
41. Fuchs EJ, Matzinger P. B cells turn off virgin but not memory T cells. *Science*. 1992;258:1156–1159.
42. Ashwell JD, DeFranco AL, Paul WE, Schwartz RH. Antigen presentation by resting B cells. Radiosensitivity of the antigen-presentation function and two distinct pathways of T cell activation. *J Exp Med*. 1984;159:881–905.
43. Yang J, Brook MO, Carvalho-Gaspar M, et al. Allograft rejection mediated by memory T cells is resistant to regulation. *Proc Natl Acad Sci U S A*. 2007;104:19954–19959.
44. Afzali B, Mitchell PJ, Scottà C, et al. Relative resistance of human CD4(+) memory T cells to suppression by CD4(+) CD25(+) regulatory T cells. *Am J Transplant*. 2011;11:1734–1742.
45. Ayasoufi K, Yu H, Fan R, Wang X, Williams J, Valujskikh A. Pretransplant antithymocyte globulin has increased efficacy in controlling donor-reactive memory T cells in mice. *Am J Transplant*. 2013;13:589–599.
46. Gurkan S, Luan Y, Dhillon N, et al. Immune reconstitution following rabbit antithymocyte globulin. *Am J Transplant*. 2010;10:2132–2141.
47. Pearl JP, Parris J, Hale DA, et al. Immunocompetent T-cells with a memory-like phenotype are the dominant cell type following antibody-mediated T-cell depletion. *Am J Transplant*. 2005;5:465–474.

48. Ciurea SO, de Lima M, Cano P, et al. High risk of graft failure in patients with anti-HLA antibodies undergoing haploidentical stem-cell transplantation. *Transplantation*. 2009;88:1019–1024.
49. Ciurea SO, Thall PF, Milton DR, et al. Complement-binding donor-specific anti-HLA antibodies and risk of primary graft failure in hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2015;21:1392–1398.
50. Ciurea SO, Cao K, Fernandez-Vina M, et al. The European Society for Blood and Marrow Transplantation (EBMT) consensus guidelines for the detection and treatment of donor-specific anti-HLA antibodies (DSA) in haploidentical hematopoietic cell transplantation. *Bone Marrow Transplant*. 2018;53:521–534.
51. Gladstone DE, Zachary AA, Fuchs EJ, et al. Partially mismatched transplantation and human leukocyte antigen donor-specific antibodies. *Biol Blood Marrow Transplant*. 2013;19:647–652.
52. Anasetti C, Amos D, Beatty PG, et al. Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. *N Engl J Med*. 1989;320:197–204.
53. Kärre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature*. 1986;319:675–678.
54. Ljunggren HG, Kärre K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today*. 1990;11:237–244.
55. Martin AM, Kulski JK, Witt C, Pontarotti P, Christiansen FT. Leukocyte Ig-like receptor complex (LRC) in mice and men. *Trends Immunol*. 2002; 23:81–88.
56. Shilling HG, Guethlein LA, Cheng NW, et al. Allelic polymorphism synergizes with variable gene content to individualize human KIR genotype. *J Immunol*. 2002;168:2307–2315.
57. Valiante NM, Uhrberg M, Shilling HG, et al. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity*. 1997;7:739–751.
58. Kim S, Poursine-Laurent J, Truscott SM, et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature*. 2005;436:709–713.
59. Yokoyama WM, Kim S. Licensing of natural killer cells by self-major histocompatibility complex class I. *Immunol Rev*. 2006;214:143–154.
60. Shifrin N, Raulet DH, Ardolino M. NK cell self tolerance, responsiveness and missing self recognition. *Semin Immunol*. 2014;26:138–144.
61. Boudreau JE, Liu XR, Zhao Z, et al. Cell-extrinsic MHC class I molecule engagement augments human NK cell education programmed by cell-intrinsic MHC class I. *Immunity*. 2016;45:280–291.
62. Orr MT, Murphy WJ, Lanier LL. 'Unlicensed' natural killer cells dominate the response to cytomegalovirus infection. *Nat Immunol*. 2010;11:321–327.
63. Du J, Lopez-Verges S, Pitcher BN, et al. CALGB 150905 (Alliance): rituximab broadens the antilymphoma response by activating unlicensed NK cells. *Cancer Immunol Res*. 2014;2:878–889.
64. Gleimer M, Parham P. Stress management: MHC class I and class I-like molecules as reporters of cellular stress. *Immunity*. 2003;19:469–477.
65. Gasser S, Orsulic S, Brown EJ, Raulet DH. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature*. 2005;436:1186–1190.
66. Dokun AO, Kim S, Smith HR, Kang HS, Chu DT, Yokoyama WM. Specific and nonspecific NK cell activation during virus infection. *Nat Immunol*. 2001;2:951–956.
67. Wang LL, Chu DT, Dokun AO, Yokoyama WM. Inducible expression of the gp49B inhibitory receptor on NK cells. *J Immunol*. 2000;164:5215–5220.
68. Ciurea SO, Schafer JR, Bassett R, et al. Phase 1 clinical trial using mbIL21 ex vivo-expanded donor-derived NK cells after haploidentical transplantation. *Blood*. 2017;130:1857–1868.
69. Ruggeri L, Capanni M, Casucci M, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood*. 1999;94:333–339.
70. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. 2002;295:2097–2100.
71. Ruggeri L, Mancusi A, Capanni M, et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. *Blood*. 2007;110:433–440.
72. Aversa F, Tabilio A, Velardi A, et al. Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. *N Engl J Med*. 1998;339:1186–1193.
73. Shlomchik WD, Couzens MS, Tang CB, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science*. 1999;285:412–415.
74. Hsu KC, Gooley T, Malkki M, et al. KIR ligands and prediction of relapse after unrelated donor hematopoietic cell transplantation for hematologic malignancy. *Biol Blood Marrow Transplant*. 2006;12:828–836.
75. Shimoni A, Labopin M, Lorentino F, et al. Killer cell immunoglobulin-like receptor ligand mismatching and outcome after haploidentical transplantation with post-transplant cyclophosphamide. *Leukemia*. 2019;33:230–239.
76. Ciurea SO, Al Malki MM, Kongtim P, et al. The European Society for Blood and Marrow Transplantation (EBMT) consensus recommendations for donor selection in haploidentical hematopoietic cell transplantation. *Bone Marrow Transplant*. 2020;55:12–24.
77. Ciurea SO, Champlin RE. Outcomes of patients treated and one human leukocyte antigen-mismatched related compared with matched unrelated donors. *Biol Blood Marrow Transplant*. 2011;17:1261.
78. Di Ianni M, Falzetti F, Carotti A, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood*. 2011;117:3921–3928.
79. Martelli MF, Di Ianni M, Ruggeri L, et al. HLA-haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents acute leukemia relapse. *Blood*. 2014;124:638–644.
80. Guenounou S, Borel C, Bérard E, et al. Prognostic impact of viral reactivations in acute myeloid leukemia patients undergoing allogeneic stem cell transplantation in first complete response. *Medicine (Baltimore)*. 2016;95:e5356.
81. Leen AM, Christin A, Myers GD, et al. Cytotoxic T lymphocyte therapy with donor T cells prevents and treats adenovirus and Epstein-Barr virus infections after haploidentical and matched unrelated stem cell transplantation. *Blood*. 2009;114:4283–4292.
82. Melenhorst JJ, Castillo P, Hanley PJ, et al. Graft versus leukemia response without graft-versus-host disease elicited by adoptively transferred multivirus-specific T-cells. *Mol Ther*. 2015;23:179–183.
83. Ciceri F, Bonini C, Stanghellini MT, et al. Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I–II study. *Lancet Oncol*. 2009;10:489–500.
84. Di Stasi A, Tey SK, Dotti G, et al. Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med*. 2011;365:1673–1683.
85. Zhou X, Di Stasi A, Tey S-K, et al. Long-term outcome after haploidentical stem cell transplant and infusion of T cells expressing the inducible caspase 9 safety transgene. *Blood*. 2014;123:3895–3905.
86. Falk PM, Herzog P, Lubens R, et al. Bone marrow transplantation between a histocompatible parent and child for acute leukemia. *Transplantation*. 1978;25:88–90.
87. Dupont B, O'Reilly RJ, Pollack MS, Good RA. Use of HLA genotypically different donors in bone marrow transplantation. *Transplant Proc*. 1979;11 :219–224.
88. Aversa F, Tabilio A, Terenzi A, et al. Successful engraftment of T-cell-depleted haploidentical "three-loci" incompatible transplants in leukemia patients by addition of recombinant human granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells to bone marrow inoculum. *Blood*. 1994;84:3948–3955.
89. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol*. 2012;12:443–458.
90. Kawanishi Y, Passweg J, Drobyski WR, et al. Effect of T cell subset dose on outcome of T cell-depleted bone marrow transplantation. *Bone Marrow Transplant*. 1997;19:1069–1077.
91. Song Y, Zhu Y, Hu B, et al. Donor γδT cells promote GVL effect and mitigate aGVHD in allogeneic hematopoietic stem cell transplantation. *Front Immunol*. 2020;11:558143.
92. Godder KT, Henslee-Downey PJ, Mehta J, et al. Long term disease-free survival in acute leukemia patients recovering with increased gammadelta T cells after partially mismatched related donor bone marrow transplantation. *Bone Marrow Transplant*. 2007;39:751–757.
93. Lamb Jr. LS, Henslee-Downey PJ, Parrish RS, et al. Increased frequency of TCR gamma delta + T cells in disease-free survivors following T cell-depleted, partially mismatched, related donor bone marrow transplantation for leukemia. *J Hematother*. 1996;5:503–509.
94. Chaleff S, Otto M, Barfield RC, et al. A large-scale method for the selective depletion of alphabeta T lymphocytes from PBSC for allogeneic transplantation. *Cytotherapy*. 2007;9:746–754.
95. Handgretinger R, Lang P, Feuchtinger TF, et al. Transplantation of TcRαβ/CD19 depleted stem cells from haploidentical donors: robust engraftment and rapid immune reconstitution in children with high risk leukemia. *Blood*. 2011;118 1005-.
96. Lang P, Handgretinger R, Meisel R, et al. Results of a prospective, multicenter, phase I/II clinical study in pediatric and adult patients using TCRαβ and CD19 depleted haploidentical hematopoietic stem cell grafts following reduced-intensity conditioning. *Blood*. 2018;132 604-.

97. Bhat S, Ngangbam S, Iqbal W, et al. Outcomes of myeloablative haploidentical hematopoietic stem cell transplant in pediatric patients with TCR α/β and CD 19 depletion. *Biol Blood Marrow Transplant*. 2017;23:S191–S192.
98. Bertaina A, Merli P, Rutella S, et al. HLA-haploidentical stem cell transplantation after removal of αβ+ T and B cells in children with nonmalignant disorders. *Blood*. 2014;124:822–826.
99. Lang P, Feuchtinger T, Teltschik HM, et al. Improved immune recovery after transplantation of TCRαβ/CD19-depleted allografts from haploidentical donors in pediatric patients. *Bone Marrow Transplant*. 2015;50(suppl 2):S6–S10.
100. Locatelli F, Merli P, Pagliara D, et al. Outcome of children with acute leukemia given HLA-haploidentical HSCT after αβ T-cell and B-cell depletion. *Blood*. 2017;130:677–685.
101. Kaynar L, Demir K, Turak EE, et al. TcRαβ-depleted haploidentical transplantation results in adult acute leukemia patients. *Hematology*. 2017;22:136–144.
102. Maschan M, Shelikhova L, Ilushina M, et al. TCR-alpha/beta and CD19 depletion and treosulfan-based conditioning regimen in unrelated and haploidentical transplantation in children with acute myeloid leukemia. *Bone Marrow Transplant*. 2016;51:668–674.
103. Shelikhova L, Ilushina M, Shekhovtsova Z, et al. αβ T cell-depleted haploidentical hematopoietic stem cell transplantation without antithymocyte globulin in children with chemorefractory acute myelogenous leukemia. *Biol Blood Marrow Transplant*. 2019;25:e179–e182.
104. Gaziev J, Isgrò A, Sodani P, et al. Haploidentical HSCT for hemoglobinopathies: improved outcomes with TCRαβ(+)/CD19(+)-depleted grafts. *Blood Adv*. 2018;2:263–270.
105. Prezioso L, Manfra I, Bonomini S, et al. Haploidentical hematopoietic stem cell transplantation in adults using the αβTCR/CD19-based depletion of G-CSF-mobilized peripheral blood progenitor cells. *Bone Marrow Transplant*. 2019;54:698–702.
106. Bertaina A, Zecca M, Buldini B, et al. Unrelated donor vs HLA-haploidentical α/β T-cell- and B-cell-depleted HSCT in children with acute leukemia. *Blood*. 2018;132:2594–2607.
107. Ji SQ, Chen HR, Wang HX, et al. G-CSF-primed haploidentical marrow transplantation without ex vivo T cell depletion: an excellent alternative for high-risk leukemia. *Bone Marrow Transplant*. 2002;30:861–866.
108. Lu DP, Dong L, Wu T, et al. Conditioning including antithymocyte globulin followed by unmanipulated HLA-mismatched/haploidentical blood and marrow transplantation can achieve comparable outcomes with HLA-identical sibling transplantation. *Blood*. 2006;107:3065–3073.
109. Dong L, Wu T, Zhang MJ, Gao ZY, Lu DP. CD3+ cell dose and disease status are important factors determining clinical outcomes in patients undergoing unmanipulated haploidentical blood and marrow transplantation after conditioning including antithymocyte globulin. *Biol Blood Marrow Transplant*. 2007;13:1515–1524.
110. Chen XH, Gao L, Zhang X, et al. HLA-haploidentical blood and bone marrow transplantation with anti-thymocyte globulin: long-term comparison with HLA-identical sibling transplantation. *Blood Cells Mol Dis*. 2009;43:98–104.
111. Luo Y, Xiao H, Lai X, et al. T-cell-replete haploidentical HSCT with low-dose anti-T-lymphocyte globulin compared with matched sibling HSCT and unrelated HSCT. *Blood*. 2014;124:2735–2743.
112. Chen H, Liu KY, Xu LP, et al. Haploidentical hematopoietic stem cell transplantation without in vitro T cell depletion for the treatment of Philadelphia chromosome-positive acute lymphoblastic leukemia. *Biol Blood Marrow Transplant*. 2015;21:1110–1116.
113. Lee KH, Lee JH, Lee JH, et al. Reduced-intensity conditioning therapy with busulfan, fludarabine, and antithymocyte globulin for HLA-haploidentical hematopoietic cell transplantation in acute leukemia and myelodysplastic syndrome. *Blood*. 2011;118:2609–2617.
114. Chen HR, Ji SQ, Wang HX, et al. Humanized anti-CD25 monoclonal antibody for prophylaxis of graft-vs-host disease (GVHD) in haploidentical bone marrow transplantation without ex vivo T-cell depletion. *Exp Hematol*. 2003;31:1019–1025.
115. Ji SQ, Chen HR, Yan HM, et al. Anti-CD25 monoclonal antibody (basiliximab) for prevention of graft-versus-host disease after haploidentical bone marrow transplantation for hematological malignancies. *Bone Marrow Transplant*. 2005;36:349–354.
116. Arcese W, Picardi A, Santarone S, et al. Haploidentical, G-CSF-primed, unmanipulated bone marrow transplantation for patients with high-risk hematological malignancies: an update. *Bone Marrow Transplant*. 2015; 50(Suppl 2):S24–S30.
117. Huang XJ, Liu DH, Liu KY, et al. Treatment of acute leukemia with unmanipulated HLA-mismatched/haploidentical blood and bone marrow transplantation. *Biol Blood Marrow Transplant*. 2009;15:257–265.
118. Al-Homsi AS, Cole K, Bogema M, Duffner U, Williams S, Mageed A. Short course of post-transplantation cyclophosphamide and bortezomib for graft-versus-host disease prevention after allogeneic peripheral blood stem cell transplantation is feasible and yields favorable results: a phase I study. *Biol Blood Marrow Transplant*. 2015;21:1315–1320.
119. Sun YQ, Wang J, Jiang Q, et al. Haploidentical hematopoietic SCT may be superior to conventional consolidation/maintenance chemotherapy as post-remission therapy for high-risk adult ALL. *Bone Marrow Transplant*. 2015;50:20–25.
120. Huang XJ, Liu DH, Liu KY, et al. Haploidentical hematopoietic stem cell transplantation without in vitro T-cell depletion for the treatment of hematological malignancies. *Bone Marrow Transplant*. 2006;38:291–297.
121. Wang Y, Chang YJ, Xu LP, et al. Who is the best donor for a related HLA haplotype-mismatched transplant? *Blood*. 2014;124:843–850.
122. Maguire Jr. HC, Maibach HI, Minisce Jr. LW. Inhibition of guinea pig anaphylactic sensitization with cyclophosphoramide. *J Invest Dermatol*. 1961;36:235–236.
123. Berenbaum MC, Brown IN. Prolongation of homograft survival in mice with single doses of cyclophosphamide. *Nature*. 1963;200:84.
124. Mayumi H, Tokunaga K. Cyclophosphamide-induced chimera-type tolerance to allografts: an overview of drug-induced immunological tolerance. *Fukuoka Igaku Zasshi*. 1990;81:20–39.
125. Mayumi H, Umesue M, Nomoto K. Cyclophosphamide-induced immunological tolerance: an overview. *Immunobiology*. 1996;195: 129–139.
126. Eto M, Mayumi H, Tomita Y, Yoshikai Y, Nishimura Y, Nomoto K. Sequential mechanisms of cyclophosphamide-induced skin allograft tolerance including the intrathymic clonal deletion followed by late breakdown of the clonal deletion. *J Immunol*. 1990;145:1303–1310.
127. Kong YY, Eto M, Omoto K, Umesue M, Hashimoto A, Nomoto K. Regulatory T cells in maintenance and reversal of peripheral tolerance in vivo. *J Immunol*. 1996;157:5284–5289.
128. Nomoto K, Eto M, Yanaga K, Nishimura Y, Maeda T, Nomoto K. Interference with cyclophosphamide-induced skin allograft tolerance by cyclosporin A. *J Immunol*. 1992;149:2668–2674.
129. Dukor P, Dietrich FM. Prevention of cyclophosphamide-induced tolerance to erythrocytes by pretreatment with cortisone. *Proc Soc Exp Biol Med*. 1970;133:280–285.
130. Nishimura Y, Mayumi H, Tomita Y, Eto M, Maeda T, Nomoto K. Recombinant human granulocyte colony-stimulating factor improves the compromised state of recipient mice without affecting the induction of specific tolerance in the cyclophosphamide-induced tolerance system. *J Immunol*. 1991;146:2639–2647.
131. Colson YL, Wren SM, Schuchert MJ, et al. A nonlethal conditioning approach to achieve durable multilineage mixed chimerism and tolerance across major, minor, and hematopoietic histocompatibility barriers. *J Immunol*. 1995;155:4179–4188.
132. Colson YL, Li H, Boggs SS, Patrene KD, Johnson PC, Ildstad ST. Durable mixed allogeneic chimerism and tolerance by a nonlethal radiation-based cytoreductive approach. *J Immunol*. 1996;157:2820–2829.
133. Luznik L, Engstrom LW, Iannone R, Fuchs EJ. Posttransplantation cyclophosphamide facilitates engraftment of major histocompatibility complex-identical allogeneic marrow in mice conditioned with low-dose total body irradiation. *Biol Blood Marrow Transplant*. 2002;8:131–138.
134. Ross D, Jones M, Komanduri K, Levy RB. Antigen and lymphopenia-driven donor T cells are differentially diminished by post-transplantation administration of cyclophosphamide after hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2013;19:1430–1438.
135. Ganguly S, Ross DB, Panoskaltsis-Mortari A, et al. Donor CD4+ Foxp3+ regulatory T cells are necessary for posttransplantation cyclophosphamide-mediated protection against GVHD in mice. *Blood*. 2014;124:2131–2141.
136. Kanakry CG, Ganguly S, Zahurak M, et al. Aldehyde dehydrogenase expression drives human regulatory T cell resistance to posttransplantation cyclophosphamide. *Sci Transl Med*. 2013;5 211ra157.
137. Emadi A, Jones RJ, Brodsky RA. Cyclophosphamide and cancer: golden anniversary. *Nat Rev Clin Oncol*. 2009;6:638–647.
138. Darrasse-Jèze G, Bergot AS, Durgeau A, et al. Tumor emergence is sensed by self-specific CD44hi memory Tregs that create a dominant tolerogenic environment for tumors in mice. *J Clin Invest*. 2009;119:2648–2662.
139. Stokes J, Hoffman EA, Zeng Y, Larmonier N, Katsanis E. Post-transplant bendamustine reduces GvHD while preserving GvL in experimental haploidentical bone marrow transplantation. *Br J Haematol*. 2016;174: 102–116.
140. O'Donnell PV, Luznik L, Jones RJ, et al. Nonmyeloablative bone marrow transplantation from partially HLA-mismatched related donors using posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant*. 2002;8:377–386.

141. Luznik L, O'Donnell PV, Symons HJ, et al. HLA-haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose, posttransplantation cyclophosphamide. *Biol Blood Marrow Transplant*. 2008;14:641–650.
142. Munchel A, Kesserwan C, Symons HJ, et al. Nonmyeloablative, HLA-haploidentical bone marrow transplantation with high dose, post-transplantation cyclophosphamide. *Pediatr Rep*. 2011;3(Suppl 2):e15.
143. Kasamon YL, Luznik L, Leffell MS, et al. Nonmyeloablative HLA-haploidentical bone marrow transplantation with high-dose posttransplantation cyclophosphamide: effect of HLA disparity on outcome. *Biol Blood Marrow Transplant*. 2010;16:482–489.
144. McCurdy SR, Kanakry JA, Showel MM, et al. Risk-stratified outcomes of nonmyeloablative HLA-haploidentical BMT with high-dose posttransplantation cyclophosphamide. *Blood*. 2015;125:3024–3031.
145. Symons HJ, Zahurak M, Cao Y, et al. Myeloablative haploidentical BMT with posttransplant cyclophosphamide for hematologic malignancies in children and adults. *Blood Adv*. 2020;4:3913–3925.
146. Shabbir-Moosajee M, Lombardi L, Ciurea SO. An overview of conditioning regimens for haploidentical stem cell transplantation with post-transplantation cyclophosphamide. *Am J Hematol*. 2015;90:541–548.
147. Scott BL, Pasquini MC, Logan BR, et al. Myeloablative versus reduced-intensity hematopoietic cell transplantation for acute myeloid leukemia and myelodysplastic syndromes. *J Clin Oncol*. 2017;35:1154–1161.
148. Ciurea SO, Kongtim P, Varma A, et al. Is there an optimal conditioning for older patients with AML receiving allogeneic hematopoietic cell transplantation? *Blood*. 2020;135:449–452.
149. Kharfan-Dabaja MA, El-Jurdi N, Ayala E, Kanate AS, Savani BN, Hamadani M. Is myeloablative dose intensity necessary in allogeneic hematopoietic cell transplantation for lymphomas? *Bone Marrow Transplant*. 2017;52:1487–1494.
150. Grosso D, Carabasi M, Filicko-O'Hara J, et al. A 2-step approach to myeloablative haploidentical stem cell transplantation: a phase 1/2 trial performed with optimized T-cell dosing. *Blood*. 2011;118:4732–4739.
151. Grosso D, Gaballa S, Alpdogan O, et al. A two-step approach to myeloablative haploidentical transplantation: low nonrelapse mortality and high survival confirmed in patients with earlier stage disease. *Biol Blood Marrow Transplant*. 2015;21:646–652.
152. Solomon SR, Sizemore CA, Sanacore M, et al. Total body irradiation-based myeloablative haploidentical stem cell transplantation is a safe and effective alternative to unrelated donor transplantation in patients without matched sibling donors. *Biol Blood Marrow Transplant*. 2015;21:1299–1307.
153. Raj K, Pagliuca A, Bradstock K, et al. Peripheral blood hematopoietic stem cells for transplantation of hematological diseases from related, haploidentical donors after reduced-intensity conditioning. *Biol Blood Marrow Transplant*. 2014;20:890–895.
154. Castagna L, Crocchiolo R, Furst S, et al. Bone marrow compared with peripheral blood stem cells for haploidentical transplantation with a nonmyeloablative conditioning regimen and post-transplantation cyclophosphamide. *Biol Blood Marrow Transplant*. 2014;20:724–729.
155. Bashey A, Zhang MJ, McCurdy SR, et al. Mobilized peripheral blood stem cells versus unstimulated bone marrow as a graft source for T-cell-replete haploidentical donor transplantation using post-transplant cyclophosphamide. *J Clin Oncol*. 2017;35:3002–3009.
156. Ruggeri A, Labopin M, Bacigalupo A, et al. Bone marrow versus mobilized peripheral blood stem cells in haploidentical transplants using posttransplantation cyclophosphamide. *Cancer*. 2018;124:1428–1437.
157. Yu X, Liu L, Xie Z, et al. Bone marrow versus peripheral blood as a graft source for haploidentical donor transplantation in adults using post-transplant cyclophosphamide-A systematic review and meta-analysis. *Crit Rev Oncol Hematol*. 2019;133:120–128.
158. Anasetti C, Logan BR, Lee SJ, et al. Peripheral-blood stem cells versus bone marrow from unrelated donors. *N Engl J Med*. 2012;367:1487–1496.
159. O'Donnell PV, Eapen M, Horowitz MM, et al. Comparable outcomes with marrow or peripheral blood as stem cell sources for hematopoietic cell transplantation from haploidentical donors after non-ablative conditioning: a matched-pair analysis. *Bone Marrow Transplant*. 2016;51:1599–1601.
160. Nagler A, Dholaria B, Labopin M, et al. Bone marrow versus mobilized peripheral blood stem cell graft in T-cell-replete haploidentical transplantation in acute lymphoblastic leukemia. *Leukemia*. 2020;34: 2766–2775.
161. Leventhal J, Abecassis M, Miller J, et al. Chimerism and tolerance without GVHD or engraftment syndrome in HLA-mismatched combined kidney and hematopoietic stem cell transplantation. *Sci Transl Med*. 2012;4 :124ra28.
162. Leventhal J, Abecassis M, Miller J, et al. Tolerance induction in HLA disparate living donor kidney transplantation by donor stem cell infusion: durable chimerism predicts outcome. *Transplantation*. 2013;95:169–176.
163. Leventhal JR, Elliott MJ, Yolcu ES, et al. Immune reconstitution/ immunocompetence in recipients of kidney plus hematopoietic stem/ facilitating cell transplants. *Transplantation*. 2015;99:288–298.
164. Ruggeri A, Sun Y, Labopin M, et al. Post-transplant cyclophosphamide versus anti-thymocyte globulin as graft-versus-host disease prophylaxis in haploidentical transplant. *Haematologica*. 2017;102:401–410.
165. Ruggeri A, Labopin M, Sanz G, et al. Comparison of outcomes after unrelated cord blood and unmanipulated haploidentical stem cell transplantation in adults with acute leukemia. *Leukemia*. 2015;29:1891 –1900.
166. Robin M, Porcher R, Ciceri F, et al. Haploidentical transplant in patients with myelodysplastic syndrome. *Blood Adv*. 2017;1:1876–1883.
167. Nagler A, Kanate AS, Labopin M, et al. Post-transplant cyclophosphamide versus anti-thymocyte globulin for graft-versus-host disease prevention in haploidentical transplantation for adult acute lymphoblastic leukemia. *Haematologica*. 2020
168. Brunstein CG, Fuchs EJ, Carter SL, et al. Alternative donor transplantation after reduced intensity conditioning: results of parallel phase 2 trials using partially HLA-mismatched related bone marrow or unrelated double umbilical cord blood grafts. *Blood*. 2011;118:282–288.
169. Eapen M, O'Donnell P, Brunstein CG, et al. Mismatched related and unrelated donors for allogeneic hematopoietic cell transplantation for adults with hematologic malignancies. *Biol Blood Marrow Transplant*. 2014;20 :1485–1492.
170. Fuchs EJ, O'Donnell PV, Eapen M, et al. Double unrelated umbilical cord blood versus HLA-haploidentical bone marrow transplantation (BMT CTN 1101). *Blood*. 2020
171. Robin M, Porcher R, Ruggeri A, et al. HLA-Mismatched donors in patients with myelodysplastic syndrome: an EBMT registry analysis. *Biol Blood Marrow Transplant*. 2019;25:114–120.
172. Giannotti F, Labopin M, Shouval R, et al. Haploidentical transplantation is associated with better overall survival when compared to single cord blood transplantation: an EBMT-Eurocord study of acute leukemia patients conditioned with thiotepa, busulfan, and fludarabine. *J Hematol Oncol*. 2018;11:110.
173. Fatobene G, Rocha St V, Martin A, et al. Nonmyeloablative alternative donor transplantation for Hodgkin and non-Hodgkin lymphoma: from the LWP-EBMT, Eurocord, and CIBMTR. *J Clin Oncol*. 2020;38:1518–1526.
174. Wieduwilt MJ, Metheny III L, Zhang M-J, et al. Comparison of haploidentical donor hematopoietic cell transplantation using post-transplant cyclophosphamide to matched-sibling, matched-unrelated, mismatched-unrelated, and umbilical cord blood donor transplantation in adults with acute lymphoblastic leukemia: a CIBMTR study. *Blood*. 2020;136:50–51.
175. Sun Y, Beohou E, Labopin M, et al. Unmanipulated haploidentical versus matched unrelated donor allogeneic stem cell transplantation in adult patients with acute myelogenous leukemia in first remission: a retrospective pair-matched comparative study of the Beijing approach with the EBMT database. *Haematologica*. 2016;101:e352–e354.
176. Ciurea SO, Zhang MJ, Bacigalupo AA, et al. Haploidentical transplant with posttransplant cyclophosphamide vs matched unrelated donor transplant for acute myeloid leukemia. *Blood*. 2015;126:1033–1040.
177. Kanate AS, Mussetti A, Kharfan-Dabaja MA, et al. Reduced-intensity transplantation for lymphomas using haploidentical related donors vs HLA-matched unrelated donors. *Blood*. 2016;127:938–947.
178. Dietrich S, Finel H, Martinez C, et al. Post-transplant cyclophosphamide-based haplo-identical transplantation as alternative to matched sibling or unrelated donor transplantation for non-Hodgkin lymphoma: a registry study by the European society for blood and marrow transplantation. *Leukemia*. 2016;30:2086–2089.
179. Martínez C, Gayoso J, Canals C, et al. Post-transplantation cyclophosphamide-based haploidentical transplantation as alternative to matched sibling or unrelated donor transplantation for Hodgkin lymphoma: a registry study of the lymphoma working party of the European Society for Blood and Marrow Transplantation. *J Clin Oncol*. 2017;35:3425–3432.
180. Dreger P, Sureda A, Ahn KW, et al. PTCy-based haploidentical vs matched related or unrelated donor reduced-intensity conditioning transplant for DLBCL. *Blood Adv*. 2019;3:360–369.
181. Shem-Tov N, Peczynski C, Labopin M, et al. Haploidentical vs. unrelated allogeneic stem cell transplantation for acute lymphoblastic leukemia in first complete remission: on behalf of the ALWP of the EBMT. *Leukemia*. 2020;34:283–292.
182. Al Malki MM, Yang D, Labopin M, et al. Comparing transplant outcomes in ALL patients after haploidentical with PTCy or matched unrelated donor transplantation. *Blood Adv*. 2020;4:2073–2083.
183. Wang Y, Liu QF, Xu LP, et al. Haploidentical vs identical-sibling transplant for AML in remission: a multicenter, prospective study. *Blood*. 2015;125 :3956–3962.

184. McCurdy SR, Kanakry CG, Kasamon YL, et al. Graft-versus-host disease (GVHD) and survival outcomes after HLA-haploidentical (Haplo) bone marrow transplant (BMT) compare favorably with matched related donor (MRD), and matched unrelated donor (MUD) BMT utilizing high-dose posttransplantation cyclophosphamide (PTCy). *Blood*. 2014;124:730.
185. Bashey A, Zhang X, Jackson K, et al. Comparison of outcomes of hematopoietic cell transplants from t-replete haploidentical donors using post-transplantation cyclophosphamide with 10 of 10 HLA-A, -B, -C, -DRB1, and -DQB1 allele-matched unrelated donors and HLA-identical sibling donors: a multivariable analysis including disease risk index. *Biol Blood Marrow Transplant*. 2016;22:125–133.
186. Blaise D, Fürst S, Crocchiolo R, et al. Haploidentical T cell-replete transplantation with post-transplantation cyclophosphamide for patients in or above the sixth decade of age compared with allogeneic hematopoietic stem cell transplantation from an human leukocyte antigen-matched related or unrelated donor. *Biol Blood Marrow Transplant*. 2016;22:119–124.
187. Ghosh N, Karmali R, Rocha V, et al. Reduced-intensity transplantation for lymphomas using haploidentical related donors versus HLA-matched sibling donors: a center for international blood and marrow transplant research analysis. *J Clin Oncol*. 2016;34:3141–3149.
188. Robinson TM, Fuchs EJ, Zhang MJ, et al. Related donor transplants: has posttransplantation cyclophosphamide nullified the detrimental effect of HLA mismatch? *Blood Adv*. 2018;2:1180–1186.
189. Nagler A, Labopin M, Dholaria B, et al. Comparison of haploidentical bone marrow versus matched unrelated donor peripheral blood stem cell transplantation with posttransplant cyclophosphamide in patients with acute leukemia. *Clin Cancer Res*. 2021;27(3):843–851.
190. Kanakry JA, Kasamon YL, Bolaños-Meade J, et al. Absence of post-transplantation lymphoproliferative disorder after allogeneic blood or marrow transplantation using post-transplantation cyclophosphamide as graft-versus-host disease prophylaxis. *Biol Blood Marrow Transplant*. 2013; 19:1514–1517.
191. Symons HJ, Mogri H, Kanakry JA, et al. Rarity of donor-derived malignancy after allogeneic BMT with high-dose post-transplantation cyclophosphamide. *Biol Blood Marrow Transplant*. 2014; 20:S252.
192. Olsson RF, Logan BR, Chaudhury S, et al. Primary graft failure after myeloablative allogeneic hematopoietic cell transplantation for hematologic malignancies. *Leukemia*. 2015;29:1754–1762.
193. Cluzeau T, Lambert J, Raus N, et al. Risk factors and outcome of graft failure after HLA matched and mismatched unrelated donor hematopoietic stem cell transplantation: a study on behalf of SFGM-TC and SFHI. *Bone Marrow Transplant*. 2016;51:687–691.
194. Balashov D, Shcherbina A, Maschan M, et al. Single-center experience of unrelated and haploidentical stem cell transplantation with TCRαβ and CD19 depletion in children with primary immunodeficiency syndromes. *Biol Blood Marrow Transplant*. 2015;21:1955–1962.
195. Yoshihara S, Maruya E, Taniguchi K, et al. Risk and prevention of graft failure in patients with preexisting donor-specific HLA antibodies undergoing unmanipulated haploidentical SCT. *Bone Marrow Transplant*. 2012;47:508–515.
196. Yoshihara S, Taniguchi K, Ogawa H, Saji H. The role of HLA antibodies in allogeneic SCT: is the 'type-and-screen' strategy necessary not only for blood type but also for HLA? *Bone Marrow Transplant*. 2012;47:1499–1506.
197. Rondon G, Saliba RM, Khouri I, et al. Long-term follow-up of patients who experienced graft failure postallogeneic progenitor cell transplantation. Results of a single institution analysis. *Biol Blood Marrow Transplant*. 2008;14:859–866.
198. Voogt PJ, Fibbe WE, Marijt WA, et al. Rejection of bone-marrow graft by recipient-derived cytotoxic T lymphocytes against minor histocompatibility antigens. *Lancet*. 1990;335:131–134.
199. Huang Y, Rezzoug F, Chilton PM, Grimes HL, Cramer DE, Ildstad ST. Matching at the MHC class I K locus is essential for long-term engraftment of purified hematopoietic stem cells: a role for host NK cells in regulating HSC engraftment. *Blood*. 2004;104:873–880.
200. Chang YJ, Zhao XY, Xu LP, et al. Donor-specific anti-human leukocyte antigen antibodies were associated with primary graft failure after unmanipulated haploidentical blood and marrow transplantation: a prospective study with randomly assigned training and validation sets. *J Hematol Oncol*. 2015;8:84.
201. Ciurea SO, Thall PF, Wang X, et al. Donor-specific anti-HLA Abs and graft failure in matched unrelated donor hematopoietic stem cell transplantation. *Blood*. 2011;118:5957–5964.
202. Taylor PA, Ehrhardt MJ, Roforth MM, et al. Preformed antibody, not primed T cells, is the initial and major barrier to bone marrow engraftment in allosensitized recipients. *Blood*. 2007;109:1307–1315.
203. Pei R, Lee JH, Shih NJ, Chen M, Terasaki PI. Single human leukocyte antigen flow cytometry beads for accurate identification of human leukocyte antigen antibody specificities. *Transplantation*. 2003;75:43–49.
204. Spellman S, Bray R, Rosen-Bronson S, et al. The detection of donor-directed, HLA-specific alloantibodies in recipients of unrelated hematopoietic cell transplantation is predictive of graft failure. *Blood*. 2010;115:2704–2708.
205. Ruggeri A, Rocha V, Masson E, et al. Impact of donor-specific anti-HLA antibodies on graft failure and survival after reduced intensity conditioning-unrelated cord blood transplantation: a Eurocord, Société Francophone d'Histocompatibilité et d'Immunogénétique (SFHI) and Société Française de Greffe de Moelle et de Thérapie Cellulaire (SFGM-TC) analysis. *Haematologica*. 2013;98:1154–1160.
206. Damman J, Seelen MA, Moers C, et al. Systemic complement activation in deceased donors is associated with acute rejection after renal transplantation in the recipient. *Transplantation*. 2011;92:163–169.
207. Muller TF, Kraus M, Neumann C, Lange H. Detection of renal allograft rejection by complement components C5A and TCC in plasma and urine. *J Lab Clin Med*. 1997;129:62–71.
208. Welch TR, Beischel LS, Witte DP. Differential expression of complement C3 and C4 in the human kidney. *J Clin Invest*. 1993;92:1451–1458.
209. Keslar K, Rodriguez ER, Tan CD, Starling RC, Heeger PS. Complement gene expression in human cardiac allograft biopsies as a correlate of histologic grade of injury. *Transplantation*. 2008;86:1319–1321.
210. Ciurea SO, Al Malki MM, Kongtim P, et al. Treatment of allosensitized patients receiving allogeneic transplantation. *Blood Adv*. 2021;5(20): 4031–4043.
211. Barge AJ, Johnson G, Witherspoon R, Torok-Storb B. Antibody-mediated marrow failure after allogeneic bone marrow transplantation. *Blood*. 1989;74:1477–1480.
212. Maruta A, Fukawa H, Kanamori H, et al. Donor-HLA-incompatible marrow transplantation with an anti-donor cytotoxic antibody in the serum of the patient. *Bone Marrow Transplant*. 1991;7:397–400.
213. Braun N, Faul C, Wernet D, et al. Successful transplantation of highly selected CD34+ peripheral blood stem cells in a HLA-sensitized patient treated with immunoadsorption onto protein A. *Transplantation*. 2000; 69:1742–1744.
214. Pollack MS, Ririe D. Clinical significance of recipient antibodies to stem cell donor mismatched class I HLA antigens. *Hum Immunol*. 2004;65:245–247.
215. Leffell MS, Jones RJ, Gladstone DE. Donor HLA-specific Abs: to BMT or not to BMT? *Bone Marrow Transplant*. 2015;50:751–758.
216. Kongtim P, Bittencourt M, Srour SA, et al. Haploidentical transplants for patients with graft failure after the first allograft. *Am J Hematol*. 2020 Jun 29. doi:10.1002/ajh.25917.
217. Lund TC, Liegel J, Bejanyan N, et al. Second allogeneic hematopoietic cell transplantation for graft failure: poor outcomes for neutropenic graft failure. *Am J Hematol*. 2015;90:892–896.
218. Jabbour E, Rondon G, Anderlini P, et al. Treatment of donor graft failure with nonmyeloablative conditioning of fludarabine, antithymocyte globulin and a second allogeneic hematopoietic transplantation. *Bone Marrow Transplant*. 2007;40:431–435.
219. Byrne BJ, Horwitz M, Long GD, et al. Outcomes of a second non-myeloablative allogeneic stem cell transplantation following graft rejection. *Bone Marrow Transplant*. 2008;41:39–43.
220. Raiola A, Dominietto A, Varaldo R, et al. Unmanipulated haploidentical BMT following non-myeloablative conditioning and post-transplantation CY for advanced Hodgkin's lymphoma. *Bone Marrow Transplant*. 2014; 49:190–194.
221. Crocchiolo R, Bramanti S, Vai A, et al. Infections after T-replete haploidentical transplantation and high-dose cyclophosphamide as graft-versus-host disease prophylaxis. *Transpl Infect Dis*. 2015;17:242–249.
222. Ruggeri A, Roth-Guepin G, Battipaglia G, et al. Incidence and risk factors for hemorrhagic cystitis in unmanipulated haploidentical transplant recipients. *Transpl Infect Dis*. 2015;17:822–830.
223. Rimondo A, Crocchiolo R, El-Cheikh J, et al. The calcineurin inhibitor and the intensity of the conditioning regimen may affect the occurrence of polyomavirus-associated hemorrhagic cystitis after haploidentical hematopoietic stem cell transplantation with post-transplant cyclophosphamide. *Bone Marrow Transplant*. 2017;52:135–137.
224. Vose JM, Reed EC, Pippert GC, et al. Mesna compared with continuous bladder irrigation as uroprotection during high-dose chemotherapy and transplantation: a randomized trial. *J Clin Oncol*. 1993;11:1306–1310.
225. Lin CJ, Vader JM, Slade M, DiPersio JF, Westervelt P, Romee R. Cardiomyopathy in patients after posttransplant cyclophosphamide-based hematopoietic cell transplantation. *Cancer*. 2017;123:1800–1809.
226. Modi D, Albanyan O, Kim S, et al. Toxicities after high dose post-transplant cyclophosphamide in haploidentical donor transplants: risk factors and impact on survival. *J Clin Oncol*. 2020;38 7545-.

227. Rondón G, Saliba RM, Chen J, et al. Impact of fluid overload as new toxicity category on hematopoietic stem cell transplantation outcomes. *Biol Blood Marrow Transplant*. 2017;23:2166–2171.
228. Varma A, Rondon G, Srour SA, et al. Endothelial activation and stress index (EASIX) at admission predicts fluid overload in recipients of allogeneic stem cell transplantation. *Biol Blood Marrow Transplant*. 2020;26:1013–1020.
229. Lee DW, Santomasso BD, Locke FL, et al. ASTCT consensus grading for cytokine release syndrome and neurologic toxicity associated with immune effector cells. *Biol Blood Marrow Transplant*. 2019;25:625–638.
230. Abboud R, Keller J, Slade M, et al. Severe cytokine-release syndrome after T cell-replete peripheral blood haploidentical donor transplantation is associated with poor survival and anti-IL-6 therapy is safe and well tolerated. *Biol Blood Marrow Transplant*. 2016;22:1851–1860.
231. Imus PH, Blackford AL, Bettinotti M, et al. Severe cytokine release syndrome after haploidentical peripheral blood stem cell transplantation. *Biol Blood Marrow Transplant*. 2019;25:2431–2437.
232. Solán L, Landete E, Bailén R, et al. Cytokine release syndrome after allogeneic stem cell transplantation with posttransplant cyclophosphamide. *Hematol Oncol*. 2020;38:597–603.
233. Abboud R, Wan F, Mariotti J, et al. Cytokine release syndrome after haploidentical hematopoietic cell transplantation: an international multicenter collaboration. *Biol Blood Marrow Transplant*. 2020;26:S55–S56.
234. Berger M, Figari O, Bruno B, et al. Lymphocyte subsets recovery following allogeneic bone marrow transplantation (BMT): CD4+ cell count and transplant-related mortality. *Bone Marrow Transplant*. 2008;41:55–62.
235. van Roessel I, Prockop S, Klein E, et al. Early CD4+ T cell reconstitution as predictor of outcomes after allogeneic hematopoietic cell transplantation. *Cytotherapy*. 2020;22:503–510.
236. Eyrich M, Lang P, Lal S, et al. A prospective analysis of the pattern of immune reconstitution in a paediatric cohort following transplantation of positively selected human leucocyte antigen-disparate haematopoietic stem cells from parental donors. *Br J Haematol*. 2001;114:422–432.
237. Haddad E, Landais P, Friedrich W, et al. Long-term immune reconstitution and outcome after HLA-nonidentical T-cell-depleted bone marrow transplantation for severe combined immunodeficiency: a European retrospective study of 116 patients. *Blood*. 1998;91:3646–3653.
238. Noel DR, Witherspoon RP, Storb R, et al. Does graft-versus-host disease influence the tempo of immunologic recovery after allogeneic human marrow transplantation? An observation on 56 long-term survivors. *Blood*. 1978;51:1087–1105.
239. Weinberg K, Blazar BR, Wagner JE, et al. Factors affecting thymic function after allogeneic hematopoietic stem cell transplantation. *Blood*. 2001; 97:1458–1466.
240. Ciurea SO, Mulanovich V, Jiang Y, et al. Lymphocyte recovery predicts outcomes in cord blood and T cell-depleted haploidentical stem cell transplantation. *Biol Blood Marrow Transplant*. 2011;17:1169–1175.
241. Ramlal R, Sasaki K, Cerrada SL, et al. Viral reactivation in haploidentical transplants using post-transplantation cyclophosphamide—a single institution experience. *Biol Blood Marrow Transplant*. 2016;22:S375–S376.
242. Solomon SR, Solh M, Morris LE, Holland HK, Bashey A. Myeloablative conditioning with PBSC grafts for T cell-replete haploidentical donor transplantation using posttransplant cyclophosphamide. *Adv Hematol*. 2016;2016:9736564.
243. Goldsmith SR, Abid MB, Auletta JJ, et al. Posttransplant cyclophosphamide is associated with increase in cytomegalovirus infection: a CIBMTR analysis. *Blood*. 2021;137(20):3291–3305.
244. Marty FM, Ljungman P, Chemaly RF, et al. Letermovir prophylaxis for cytomegalovirus in hematopoietic-cell transplantation. *N Engl J Med*. 2017;377:2433–2444.
245. Lin A, Flynn J, DeRespiris L, et al. Letermovir for prevention of cytomegalovirus reactivation in haploidentical and mismatched adult donor allogeneic hematopoietic cell transplantation with post-transplantation cyclophosphamide for graft-versus-host disease prophylaxis. *Biol Blood Marrow Transplant*. 2020
246. El-Zimaity M, Saliba R, Chan K, et al. Hemorrhagic cystitis after allogeneic hematopoietic stem cell transplantation: donor type matters. *Blood*. 2004;103:4674–4680.
247. Silva Lde P, Patah PA, Saliba RM, et al. Hemorrhagic cystitis after allogeneic hematopoietic stem cell transplants is the complex result of BK virus infection, preparative regimen intensity and donor type. *Haematologica*. 2010;95:1183–1190.
248. Copelan OR, Sanikommu SR, Trivedi JS, et al. Higher incidence of hemorrhagic cystitis following haploidentical related donor transplantation compared with matched related donor transplantation. *Biol Blood Marrow Transplant*. 2019;25:785–790.
249. Mukherjee A, Milton D, Ledesma C, et al. Influence of the intensity of the allogeneic conditioning regimen on the risk of hemorrhagic cystitis (HC) in patients receiving post- transplant cyclophosphamide (PT-Cy) as GVHD prophylaxis. *Biol Blood Marrow Transplant*. 2019;25:S147–S148.
250. Khan H, Oberoi S, Mahvash A, et al. Reversible ureteral obstruction due to polyomavirus infection after percutaneous nephrostomy catheter placement. *Biol Blood Marrow Transplant*. 2011;17:1551–1555.
251. Leen AM, Bollard CM, Mendizabal AM, et al. Multicenter study of banked third-party virus-specific T cells to treat severe viral infections after hematopoietic stem cell transplantation. *Blood*. 2013;121:5113–5123.
252. Tzannou I, Papadopoulou A, Naik S, et al. Off-the-shelf virus-specific T cells to treat BK virus, human herpesvirus 6, cytomegalovirus, Epstein-Barr virus, and adenovirus infections after allogeneic hematopoietic stem-cell transplantation. *J Clin Oncol*. 2017;35:3547–3557.
253. Gooley TA, Chien JW, Pergam SA, et al. Reduced mortality after allogeneic hematopoietic-cell transplantation. *N Engl J Med*. 2010;363:2091–2101.
254. Ringdén O, Labopin M, Ciceri F, et al. Is there a stronger graft-versus-leukemia effect using HLA-haploidentical donors compared with HLA-identical siblings? *Leukemia*. 2016;30:447–455.
255. Shaw BE, Marsh SG, Mayor NP, Russell NH, Madrigal JA. HLA-DPB1 matching status has significant implications for recipients of unrelated donor stem cell transplants. *Blood*. 2006;107:1220–1226.
256. Vago L, Perna SK, Zanussi M, et al. Loss of mismatched HLA in leukemia after stem-cell transplantation. *N Engl J Med*. 2009;361:478–488.
257. Villalobos IB, Takahashi Y, Akatsuka Y, et al. Relapse of leukemia with loss of mismatched HLA resulting from uniparental disomy after haploidentical hematopoietic stem cell transplantation. *Blood*. 2010;115:3158–3161.
258. Vago L. Clonal evolution and immune evasion in posttransplantation relapses. *Hematology*. 2019;2019:610–616.
259. Christopher MJ, Petti AA, Rettig MP, et al. Immune escape of relapsed AML cells after allogeneic transplantation. *N Engl J Med*. 2018;379: 2330–2341.
260. Toffalori C, Zito L, Gambacorta V, et al. Immune signature drives leukemia escape and relapse after hematopoietic cell transplantation. *Nat Med*. 2019;25:603–611.
261. Zeiser R, Vago L. Mechanisms of immune escape after allogeneic hematopoietic cell transplantation. *Blood*. 2019;133:1290–1297.
262. Crucitti L, Crocchiolo R, Toffalori C, et al. Incidence, risk factors and clinical outcome of leukemia relapses with loss of the mismatched HLA after partially incompatible hematopoietic stem cell transplantation. *Leukemia*. 2015;29:1143–1152.
263. Srour SA, Kongtim P, Rondon G, et al. Haploidentical transplants for patients with relapse after the first allograft. *Am J Hematol*. 2020
264. Ghiso A, Raiola AM, Gualandi F, et al. DLI after haploidentical BMT with post-transplant CY. *Bone Marrow Transplant*. 2015;50:56–61.
265. Zeidan AM, Forde PM, Symons H, et al. HLA-haploidentical donor lymphocyte infusions for patients with relapsed hematologic malignancies after related HLA-haploidentical bone marrow transplantation. *Biol Blood Marrow Transplant*. 2014;20:314–318.
266. Yan CH, Liu DH, Xu LP, et al. Modified donor lymphocyte infusion-associated acute graft-versus-host disease after haploidentical T-cell-replete hematopoietic stem cell transplantation: incidence and risk factors. *Clin Transplant*. 2012;26:868–876.
267. Yan CH, Wang JZ, Liu DH, et al. Chemotherapy followed by modified donor lymphocyte infusion as a treatment for relapsed acute leukemia after haploidentical hematopoietic stem cell transplantation without in vitro T-cell depletion: superior outcomes compared with chemotherapy alone and an analysis of prognostic factors. *Eur J Haematol*. 2013;91:304–314.
268. Srour SA, Saliba RM, Bittencourt MCB, et al. Haploidentical transplantation for acute myeloid leukemia patients with minimal/measurable residual disease at transplantation. *Am J Hematol*. 2019;94:1382–1387.
269. Kongtim P, Lee DA, Cooper LJ, Kebriaei P, Champlin RE, Ciurea SO. Haploidentical hematopoietic stem cell transplantation as a platform for post-transplantation cellular therapy. *Biol Blood Marrow Transplant*. 2015; 21:1714–1720.
270. Schmid C, Schleuning M, Ledderose G, Tischer J, Kolb HJ. Sequential regimen of chemotherapy, reduced-intensity conditioning for allogeneic stem-cell transplantation, and prophylactic donor lymphocyte transfusion in high-risk acute myeloid leukemia and myelodysplastic syndrome. *J Clin Oncol*. 2005;23:5675–5687.
271. Solomon SR, Sizemore CA, Zhang X, et al. Preemptive DLI without withdrawal of immunosuppression to promote complete donor T-cell chimerism results in favorable outcomes for high-risk older recipients of alemtuzumab-containing reduced-intensity unrelated donor allogeneic transplant: a prospective phase II trial. *Bone Marrow Transplantation*. 2014;49:616–621.
272. Mo XD, Zhang XH, Xu LP, et al. Salvage chemotherapy followed by granulocyte colony-stimulating factor-primed donor leukocyte infusion with graft-vs.-host disease control for minimal residual disease in acute leukemia/myelodysplastic syndrome after allogeneic hematopoietic stem cell

transplantation: prognostic factors and clinical outcomes. *Eur J Haematol*. 2016;96:297–308.

273. Di Grazia C, Pozzi S, Geroldi S, et al. Wilms tumor 1 expression and pre-emptive immunotherapy in patients with acute myeloid leukemia undergoing an allogeneic hemopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2016;22:1242–1246.
274. Yan CH, Liu QF, Wu DP, et al. Prophylactic donor lymphocyte infusion (DLI) followed by minimal residual disease and graft-versus-host disease-guided multiple DLIs could improve outcomes after allogeneic hematopoietic stem cell transplantation in patients with refractory/relapsed acute leukemia. *Biol Blood Marrow Transplant*. 2017;23:1311–1319.
275. Schmid C, Labopin M, Schaap N, et al. Prophylactic donor lymphocyte infusion after allogeneic stem cell transplantation in acute leukaemia—a matched pair analysis by the Acute Leukaemia Working Party of EBMT. *Br J Haematol*. 2019;184:782–787.
276. Goldsmith SR, Slade M, DiPersio JF, et al. Donor-lymphocyte infusion following haploidentical hematopoietic cell transplantation with peripheral blood stem cell grafts and PTCy. *Bone Marrow Transplant*. 2017;52:1623–1628.
277. Tan Y, Du K, Luo Y, et al. Superiority of preemptive donor lymphocyte infusion based on minimal residual disease in acute leukemia patients after allogeneic hematopoietic stem cell transplantation. *Transfusion*. 2014; 54:1493–1500.
278. Dholaria B, Savani BN, Labopin M, et al. Clinical applications of donor lymphocyte infusion from an HLA-haploidentical donor: consensus recommendations from the acute leukemia working party of the EBMT. *Haematologica*. 2020;105:47–58.
279. Locatelli F, Ruggeri A, Merli P, et al. Administration of BPX-501 cells following αβ T and B-cell-depleted HLA haploidentical HSCT (haplo-HSCT) in children with acute leukemias. *Blood*. 2018;132 307-.
280. Neelapu SS, Locke FL, Bartlett NL, et al. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. *N Engl J Med*. 2017; 377:2531–2544.
281. Maude SL, Laetsch TW, Buechner J, et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *N Engl J Med*. 2018;378 :439–448.
282. Schuster SJ, Bishop MR, Tam CS, et al. Tisagenlecleucel in adult relapsed or refractory diffuse large B-cell lymphoma. *N Engl J Med*. 2019;380:45–56.
283. Kochenderfer JN, Dudley ME, Carpenter RO, et al. Donor-derived CD19-targeted T cells cause regression of malignancy persisting after allogeneic hematopoietic stem cell transplantation. *Blood*. 2013;122:4129–4139.
284. Kebriaei P, Singh H, Huls MH, et al. Phase I trials using sleeping beauty to generate CD19-specific CAR T cells. *J Clin Invest*. 2016;126:3363–3376.
285. Cruz CR, Micklethwaite KP, Savoldo B, et al. Infusion of donor-derived CD19-redirected virus-specific T cells for B-cell malignancies relapsed after allogeneic stem cell transplant: a phase 1 study. *Blood*. 2013;122:2965–2973.
286. Brudno JN, Somerville RP, Shi V, et al. Allogeneic T cells that express an anti-CD19 chimeric antigen receptor induce remissions of B-cell malignancies that progress after allogeneic hematopoietic stem-cell transplantation without causing graft-versus-host disease. *J Clin Oncol*. 2016;34:1112–1121.
287. Morvan MG, Lanier LL. NK cells and cancer: you can teach innate cells new tricks. *Nat Rev Cancer*. 2016;16:7–19.
288. Savani BN, Mielke S, Adams S, et al. Rapid natural killer cell recovery determines outcome after T-cell-depleted HLA-identical stem cell transplantation in patients with myeloid leukemias but not with acute lymphoblastic leukemia. *Leukemia*. 2007;21:2145–2152.
289. Ciurea SO, Bassett R, Soebbing D, et al. Improved outcomes for patients receiving high-doses of IL-21 ex vivo expanded NK cells after haploidentical transplantation (haploSCT): long-term follow-up of a phase 1/2 clinical trial with comparison to CIBMTR controls. *Blood*. 2019;134:700.
290. Ciurea SO, Shah MV, Saliba RM, et al. Haploidentical transplantation for older patients with acute myeloid leukemia and myelodysplastic syndrome. *Biol Blood Marrow Transplant*. 2018;24:1232–1236.
291. Canaani J, Savani BN, Labopin M, et al. Donor age determines outcome in acute leukemia patients over 40 undergoing haploidentical hematopoietic cell transplantation. *Am J Hematol*. 2018;93:246–253.
292. Canaani J, Savani BN, Labopin M, et al. Impact of ABO incompatibility on patients' outcome after haploidentical hematopoietic stem cell transplantation for acute myeloid leukemia—a report from the acute leukemia working party of the EBMT. *Haematologica*. 2017;102:1066–1074.
293. Elmariah H, Kasamon YL, Zahurak M, et al. Haploidentical bone marrow transplantation with post-transplant cyclophosphamide using non-first-degree related donors. *Biol Blood Marrow Transplant*. 2018; 24:1099–1102.
294. Fuchs EJ, O'Donnell PV, Eapen M, et al. Double unrelated umbilical cord blood vs HLA-haploidentical bone marrow transplantation: the BMT CTN 1101 trial. *Blood*. 2021;137:420–428.
295. Bacigalupo A, Dominietto A, Ghiso A, et al. Unmanipulated haploidentical bone marrow transplantation and post-transplant cyclophosphamide for hematologic malignancies following a myeloablative conditioning: an update. *Bone Marrow Transplant*. 2015;50(Suppl 2). S37-9.
296. Kanate AS, Mussetti A, Kharfan-Dabaja MA, et al. Reduced-intensity transplantation for lymphomas using haploidentical related donors vs HLA-matched unrelated donors. *Blood*. 2016;127:938–947.
297. Cauchois R, Castagna L, Pagliardini T, et al. Prophylactic donor lymphocyte infusions after haploidentical haematopoietic stem cell transplantation for high risk haematological malignancies: a retrospective bicentric analysis of serial infusions of increasing doses of CD3(+) cells. *Br J Haematol*. 2019;185:570–573.
298. Zhou X, Di Stasi A, Tey SK, et al. Long-term outcome after haploidentical stem cell transplant and infusion of T cells expressing the inducible caspase 9 safety transgene. *Blood*. 2014;123:3895–3905.
299. Liu J, Zhong JF, Zhang X, Zhang C. Allogeneic CD19-CAR-T cell infusion after allogeneic hematopoietic stem cell transplantation in B cell malignancies. *J Hematol Oncol*. 2017;10:35.
300. Roy DC, Lachance S, Cohen S, et al. Allodepleted T-cell immunotherapy after haploidentical haematopoietic stem cell transplantation without severe acute graft-versus-host disease (GVHD) in the absence of GVHD prophylaxis. *Br J Haematol*. 2019;186:754–766.
301. Roy D-C, Walker I, Maertens J, et al. Efficacy and safety of a single dose of donor lymphocytes depleted of alloreactive T-cells (ATIR101) following T-cell-depleted haploidentical HSCT: a pooled analysis of two phase II studies. *Blood*. 2018;132 120-.
302. Rådestad E, Wikell H, Engström M, et al. α/β T-cell depleted grafts as an immunological booster to treat graft failure after hematopoietic stem cell transplantation with HLA-matched related and unrelated donors. *J Immunol Res*. 2014;2014:578741.

CHAPTER 108

CORD BLOOD TRANSPLANTATION

Joseph E. Maakaron, Najla El-Jurdi, and Claudio G. Brunstein

CORD BLOOD AS A STEM CELL SOURCE

It is well established that ethnic minorities have a low likelihood of matching with an unrelated hematopoietic stem cell donor.[1] In recent years, there has been increasing interest in alternative stem cell donors given the genetic heterogeneity of our population coupled with diminishing family size. Cord blood (CB) represents an alternative donor type for hematopoietic cell transplantation (HCT) and yields outcomes similar to matched donors. In addition, CB has several unique characteristics. CB units are readily available for procurement from CB banks when urgent transplants are needed. Properties of the unit are also known ahead of time including any evidence of microbial contamination, total nucleated cell (TNC) dose, and in many instances, the CD34+ cell dose and viability. Given the immaturity of stem cells and accompanying T cells, the risks of acute and chronic graft-versus-host-disease are relatively low, requiring less stringent HLA (human leukocyte antigen) matching.[2] Cord blood transplantation (CBT) has also been associated with a decreased risk of relapse, especially in the setting of minimal residual disease.[3]

CORD BLOOD UNIT SELECTION

Just as optimal donor selection is key to the success of a stem cell transplant (SCT), a successful CBT depends on ideal unit selection. Experience in CB unit selection requires time and understanding of multiple variables that represent CB unit quality and affect outcomes. Both the American Society of Transplant and Cellular Therapy and the National Marrow Donor Program have resources to support graft selection (https://network.bethematchclinical.org/education/education-catalog/cord-blood-unit-selection/). Consultation with a transplant center experienced with CB transplantation may also be considered. There are several characteristics that are typically considered in the selection process, and transplant centers may have different CB unit selection algorithms where they may give priority to one CB characteristic over another, as recently outlined.[2] The main CB unit characteristics to be considered in graft selection are discussed in the following sections.

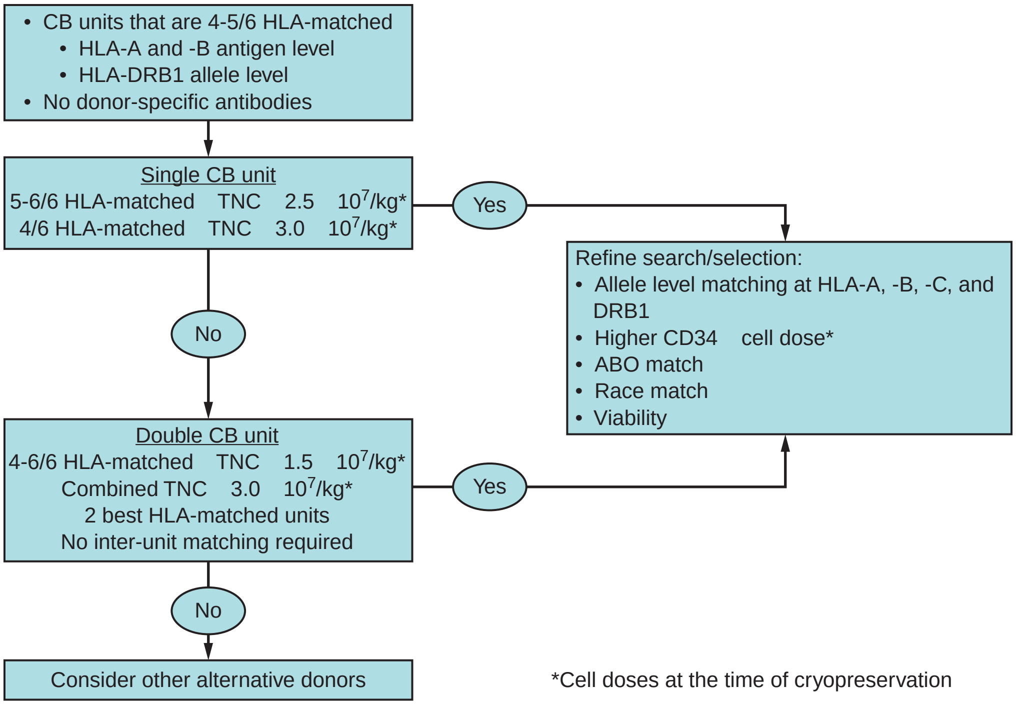
Cell Dose

One of the main determinants of transplant-related mortality (TRM) in CBT is the infused TNC and CD34+ cell doses as they correlate with incidence and time to hematopoietic recovery.[4] Historically, TNC was the only cell dose variable considered at the time of CB unit selection. The TNC dose is still relevant today, and most centers consider a TNC of 2.5 to 3.0 × 10^7/kg suitable as a single CB unit graft. However, the underlying diagnosis must be taken into consideration as those with nonmalignant disease require higher cell doses to achieve reproducible engraftment, estimated between 4 to 5 × 10^7/kg.[5–7] Cell doses below this threshold result in delayed hematopoietic recovery, higher risk of graft failure, and increased treatment-related mortality (TRM). The limited cell dose in each individual unit can be in part overcome by the utilization of double CB unit grafts, as outlined in the following sections. More recently, greater emphasis has been given to the importance of considering the CD34+ cell dose as a measure of CB unit quality and potency. A minimum pre-cryopreservation CD34+ cell dose of 0.9 to 1.1 × 10^7/kg is typically considered. Recently stored units will have both pre-cryopreservation TNC and CD34+ cell counts available for graft selection, especially with red cell depletion and modern banking procedures. While TNC and CD34+ cell counts were expected to be correlated, a recent study found that the correlation between TNC and CD34+ was in fact poor ($r = .024$).[8] The ratio of CD34+ to TNC was 0.34% with an interquartile range of 0.23% to 0.48%.[4,8] Note that when the TNC/CD34+ ratio is significantly outside this range, other unit characteristics, delivery conditions, and experience with the CB bank should be considered in unit selection. In summary, current recommendations are to take both the TNC and CD34+ cell counts into consideration in CB unit selection (Fig. 108.1).[2]

The use of CB for allogeneic HCT started in a pediatric setting, where younger and smaller patients may receive a TNC dose of 5 to 10 × 10^7/kg. In part due to TNC dose, CB grafts in children achieve consistent engraftment, with outcomes similar to unrelated adult donors.[9,10] As investigators attempted to extend CB to adult patients, it became evident that cell dose was a barrier to the efficacy of this donor type for larger and adult patients. Initially developed at the University of Minnesota as a platform to study novel graft manipulations, the use of two CB units as a graft (double CB graft) extended this donor type to a physically larger patient population.[11] Double CB transplantation rapidly became an approach commonly used to overcome the cell dose limitation of a single unit. While the principles of quality and HLA-matching apply to the selection of each unit composing the double CB graft, the most commonly used TNC cell dose threshold for each of the two units is a minimum of 1.5 × 10^7 TNC/kg. There is no widely defined CD34+ cell dose for each individual unit in double CB transplantation. Prospective randomized data in children[9] and retrospective registry-based data in adults[12,13] have shown that the outcomes of transplantation with single or double units result in similar outcomes once the pre-cryopreservation TNC threshold ≥2.5 × 10^7 TNC/kg is met. However, children are still more likely to find suitable single units than adults. Thus, the use of double CB graft is still important in extending this donor type to older and larger patients.

The growth of the inventory of CB over the years has allowed for more frequent identification of single unit grafts with the adequate cell dose for children and certain adult patient populations. Once these larger units become available in the national and international donor search platforms, they tend to be in the CB banks inventory for a short period of time. Moreover, to meet HLA-matching requirements, minorities continue to find only smaller units and require double CB drafts.[14] Current efforts in overcoming the cell dose limitation and making a larger proportion of the existing inventory available to more patients are focused on ex vivo expansion, as discussed later in this chapter.

While methods used to collect and cryopreserve a CB unit may impact its quality, conditions of storage and thaw of the CB unit may also have an impact on the final quality of the CB product. Thus, beyond using proper established and validated thaw methodology, it is necessary to monitor post-thaw cell counts as these counts also play a crucial part in optimizing outcomes. A recent report in the double CB setting described the effect of post-thaw cell count and viability on outcomes and found that a higher CD34+ cell dose and a CD34+ cell dose viability >75% resulted in a higher likelihood of engraftment and CB unit predominance.[15] While this study was in the


• CB units that are 4-5/6 HLA-matched
• HLA-A and -B antigen level
• HLA-DRB1 allele level
• No donor-specific antibodies
Single CB unit
5-6/6 HLA-matched TNC 2.5 10^7/kg*
4/6 HLA-matched TNC 3.0 10^7/kg*
Yes
Refine search/selection:
• Allele level matching at HLA-A, -B, -C, and DRB1
• Higher CD34 cell dose*
• ABO match
• Race match
• Viability
No
Double CB unit
4-6/6 HLA-matched TNC 1.5 10^7/kg*
Combined TNC 3.0 10^7/kg*
2 best HLA-matched units
No inter-unit matching required
Yes
No
Consider other alternative donors

*Cell doses at the time of cryopreservation

Figure 108.1 SUGGESTED CONSIDERATIONS DURING CORD BLOOD GRAFT SELECTION.
CB, Cord blood; *TNC*, total nucleated cell

double CB transplant setting, these findings must also be considered in the context of single CB transplantation; if a single CB unit graft has poor TNC or CD34+ recovery and/or viability, close monitoring and early intervention for graft failure should be considered.

HLA-Matching

The HLA-matching of CB units to the patient historically required 4–6/6 matching at HLA-A and HLA-B at the antigen level and HLA-DRB1 at the allele level. Despite this low level of HLA-matching in most cases, the incidence of graft-versus-host disease (GVHD) with CBT has been relatively low compared to bone marrow (BM) transplants using other stem cell sources. There is growing consensus in the literature regarding the importance of better HLA-matching in CB transplantation. The data in single CB in children suggest that 6/6 allele level HLA-matched CB results in the best outcomes.[16] In contrast, in adult patients closer HLA-matching considering 6 to 10 loci does not seem to affect survival in either single or double CB settings.[17–19] However, in the double CB setting when the dominant unit is better HLA-matched to the recipient, it seems to lead to improved outcomes.[20] In the adult population, in particular in ethnic minorities, selected CB units tend to be more HLA-mismatched in order to meet minimum TNC criteria. A lower level of HLA-match may result in a lower risk of relapse, but this is offset by an increased risk of TRM, resulting in similar survival. The novel ex vivo expansion platforms have the potential to shift this balance by allowing the selection of initially smaller but better HLA-matched CB units that can then be expanded to suitable cell doses for most patients. When widely available, this type of innovation will make a larger proportion of the CB inventory available for transplantation of larger and adult patients. Currently, HLA-matching in CB graft selection still starts with the historical standards described. Once this initial search identifies numerous potential CB units, experienced CB transplant centers will further refine the selection by selecting units with the closest allele level HLA-matching considering HLA-A, -B, -C, and -DRB1. In the setting of double CB transplantation, the two units should be selected based on each individual unit's characteristics aiming for the best conventional and, if possible, allele HLA-matching to the recipient, with no need to match the units to each other.[21]

Cord Blood Bank

CB banks are a key element for successful CB transplantation. Under appropriate CB unit selection for banking, quality control, cryopreservation, and storage standards, CB units can be stored and available for treatment for many years. For example, the storage of CB units that have had their volume reduced to around 25 mL have better cell recovery as compared to those that have larger volumes.[15] It is also important that units are red cell depleted as units not depleted of red cells have been associated with a high risk of serious infusion reactions. The familiarity of the transplant center with the CB banks and their practices is another factor to be considered in CB graft selection. The CB bank community is self-regulated with most banks seeking accreditation by NetCord-Foundation for the Accreditation for Cellular Therapy (FACT), which has helped make CB Bank practice more consistent. Notably, CB banks that were NetCord-FACT accredited had higher and more consistent post-thaw cell viability in one study.[15] In addition, CB banks may also undergo licensure by the US Federal Drug Administration (FDA). CB units collected by licensed CB banks are labeled, licensed, and dispensed as a drug, whereas units collected prior to licensure or in unlicensed CB banks and labeled as unlicensed have to be dispensed and used for transplantation under an Investigational New Drug (IND) application. Engraftment and survival are similar whether using a licensed or unlicensed CB unit.[22] Please refer to papers on the topic of CB banking for a more complete discussion of this topic.

DONOR-SPECIFIC ANTIBODIES

The testing for donor-specific HLA-antibodies (DSA) is an important selection criterion in unrelated and mismatched HCT. The data in single CBT have consistently shown that the presence of DSA with mean fluorescence intensity (MFI) of 1000 is associated with a significantly higher incidence of graft failure that ranges from 20% to 68%.[23–26] The presence of HLA-antibodies, even if not directed against the graft, may be associated with a higher risk of graft failure.[23] This is further suggested by the presence of higher titer antibodies resulting in non-engraftment with either CB unit in double CB transplantation.[26] When comparing patients with DSA who were engrafted to those who had graft failure, the latter had a higher median MFI. There are also some data suggesting that DSA against HLA-C, DP, DQ, and DRB3/4/5 may result in a higher risk of graft failure.[25] The data in double CB transplantation were less clear in part because of the varying MFI thresholds used in different reports.[26–29] Nonetheless, owing to the strong impact seen in single CB transplantation, testing for DSA has also been incorporated in double CB graft selection by most centers. In summary, testing for DSAs against high expression HLA loci and using virtual cross-matching to assist with the selection of CB units is highly recommended. While HLA A-, B-, C-, -DQB1, and -DRB1 are typically available in current confirmatory typing reports, some cases may require additional HLA typing for determinating HLA-DP and -DRB3/4/5, adding time and complexity to the selection process. While most centers will use an MFI greater than 1000 as positive, higher-risk DSAs are those with MFI greater than 3000. Consultation with the immunology laboratory to determine the relevance of DSAs is also valuable. Administration of rituximab, intravenous immunoglobulin, and bortezomib with or without plasma exchange has been used immediately prior to conditioning to reduce the DSA titer and proceed with the transplant with a specific CB unit.[30,31] DSA debulking requires further evaluation before it can be widely applied in CB transplantation care, and selecting an alternative CB unit with no DSA may be the best course of action, if possible.

CONDITIONING REGIMENS IN CORD BLOOD TRANSPLANTATION

Initial CBT conditioning regimens were designed to provide additional immunosuppression to facilitate engraftment in the context of HLA-mismatching. Early regimens included anti-thymocyte globulin (ATG) and a short course of steroids, which resulted in successful engraftment in children but had limited success in adult patients.[32] The introduction of fludarabine (FLU) to the conditioning regimens

TABLE 108.1 **Selected Conditioning Regimens Used in Cord Blood Transplantation**

Reference	Center	Conditioning Regimen
Myeloablative Conditioning Regimens		
Barker et al. Blood 2005[11]	University of Minnesota, USA	• Cyclophosphamide 60 mg/kg/day days –7 and –6 • Fludarabine 25 mg/m^2/day days –8 to –6 • Total body irradiation 1320 cGy (180 cGy twice daily) days –4 to –1
Anand et al. BBMT 2017[38]	Duke University, USA	• Thiotepa 5 mg/kg/day days –11 to –10 • Fudarabine 40 mg/m^2/day per day i.v. on days –5 to –2 • Total body irradiation 1350 cGy (150 cGy twice daily) on days –9 to –5
Sanz et al. BMT 2012[36]	University of Valencia, Spain	• Thiotepa 5 mg/kg/day on days –7 and –6 • Busulfan 3.2 mg/kg/day as a single daily dose on days –5 to –3, adjusted in patients weighing <34 kg • Fudarabine 50 mg/m^2/day on days –5 to –3 • Rabbit ATG 2 mg/kg/day on days –5 to –2.
Atsuta et al. Blood 2009[161]	University of Tokyo, Japan	• Cyclophosphamide 60 mg/kg/day days –4 and –3 • Total body irradiation 1200 cGy day –9 to –7 • Cytarabine 3 g/m^2 twice daily days –6 and –5 • Granulocyte colony-stimulating factor 5 μg/kg continuous intravenous infusion, from 12 h prior to start cytarabine stopping after the last dose
Reduced Intensity and Non-Myeloablative Regimens		
Brunstein et al. Blood 2007[33]	University of Minnesota, USA[a]	• Cyclophosphamide 50 mg/kg/day day –6 • Fludarabine 30–50 mg/m^2/day days –6 to –2 • Total body irradiation 200 cGy day –1
Ponce et al. Blood 2013[37]	Memorial Sloan Kettering Cancer Center, USA	• Cyclophosphamide 50 mg/kg/day day –6 • Fludarabine 30 mg/m^2/day days –6 to –2 • Thiotepa 5 mg/kg/day days –5 and –4 • Total body irradiation 400 cGy (200 cGy daily) day –2 and –1
DeFilipp et al. BMT 2020[47]	Dana Farber Cancer Institute and Massachusetts General Hospital, USA	• Fudarabine 30 mg/m^2/day days –7 to –2 • Melphalan 100 mg/m^2/day day –1 • Total body radiation 200 cGy day 0
Ballen et al. BBMT 2007[39]	Dana Farber Cancer Institute and Massachusetts General Hospital, USA	• Fudarabine 30 mg/m^2/day days –8 to –3 • Melphalan 100 mg/m^2/day day –2 • Rabbit ATG 1.5 mg/kg/day days –7, –5, –3, and –1
Ciurea et al. Leuk Lymphoma 2012[40]	MD Anderson Cancer Center, USA	• Melphalan 140 mg/m^2 on day –8 • Thiotepa 10 mg/kg (day –7), fludarabine 160 mg/m^2 over 4 days (days –6 to –3) and rabbit antithymocyte globulin (ATG) 1.25 mg/kg (day –4) and 1.75 mg/kg (day –3)
Yamamoto et al. BBMT 2016[46]	Toranomon Hospital, Japan	• Fudarabine 30 mg/m^2/day days –7 to –2 • Melphalan 40 mg/m^2/day days –3 and –2 • Busulfan 3.2 mg/kg 4 times daily, days –7 to –4
Deeg et al. BBMT 2018[45]	Fred Hutchinson Cancer Research Center, USA	• Treosulfan, 14 g/m^2/day on days –6 to –4 • Fludarabine, 30 mg/m^2/day on days –6 to –2 • Total body radiation 200 cGy day 0

[a] Horse ATG 15 mg/kg twice daily days –6 to –4, if no recent immunosuppressive chemotherapy or previous autologous transplant,
ATG, Anti-thymocyte globulin.

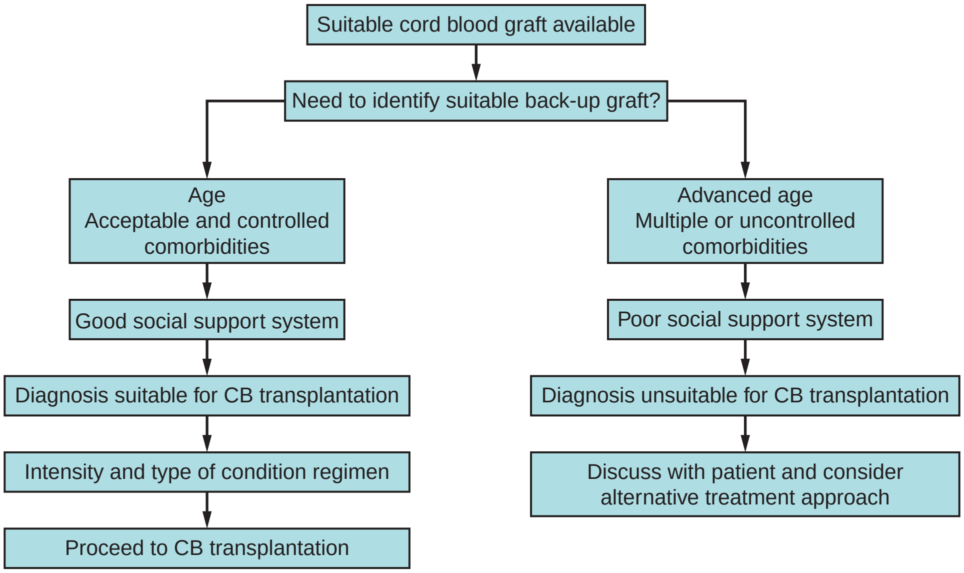

Suitable cord blood graft available
Need to identify suitable back-up graft?
Age
Acceptable and controlled comorbidities
Good social support system
Diagnosis suitable for CB transplantation
Intensity and type of condition regimen
Proceed to CB transplantation
Advanced age
Multiple or uncontrolled comorbidities
Poor social support system
Diagnosis unsuitable for CB transplantation
Discuss with patient and consider alternative treatment approach

Figure 108.2 CONSIDERING PATIENT SUITABILITY FOR CORD BLOOD TRANSPLANTATION. *CB*, Cord blood.

for CBT significantly improved engraftment in general and was key in extending this donor type to adults in the myeloablative and reduced intensity settings.[11,33] Table 108.1 summarizes some of the conditioning regimens used in CBT. It is important to keep in mind that in CBT, the conditioning regimen is part of the platform and that success using this donor type is a full package that includes graft selection, patient evaluation, and supportive care, which depends heavily on center experience (Fig. 108.2).

Myeloablative Conditioning

In children, the myeloablative regimen with cyclophosphamide (CY), FLU, and 1200 to 1320 Gy total body irradiation (TBI; TCF regimen) developed by the University of Minnesota has been widely used and has resulted in high rates of engraftment.[11] In a recent multicenter study, this conditioning regimen improved outcomes among children and young adults with acute leukemia,[9] as evidenced by a comparison with chemotherapy only and non-FLU-containing TBI regimens.[10] Taken together, these data support the use of TCF as a preferred regimen for patients with leukemia who are candidates for MA.

Given that this is the main indication for allogeneic transplantation in this age group, it was extrapolated for other conditions. In adults, the TCF regimen has also been shown to result in high rates of engraftment and progression-free survival compared to transplantation using other donor types.[34] At least in part, the long-term disease control seems to be driven by a potent graft versus leukemia effect.[3] The use of this myeloablative regimen in centers with less experience in CBT has proved challenging.[35] This led to increased interest in reduced toxicity FLU-based regimens with thiotepa. These regimens have been shown to produce very promising outcomes in single[36] and double[37,38] CBT and potentially better applicability (see Table 108.1). An advantage of these regimens is the ability to extend intensive conditioning regimens to older fit patients, potentially improving outcomes.

Reduced-Intensity and Non-Myeloablative Conditioning

Reduced-intensity and non-myeloablative conditioning regimens are mostly used for the treatment of adult patients who are older or those who have comorbid conditions that would otherwise increase their risk of TRM. Similar to the myeloablative setting, FLU is a critical component of these conditioning regimens. Non-myeloablative conditioning consisting of CY 50 mg/kg, FLU 150 to 200 mg/m^2, and TBI 200 cGy (Cy-Flu-TBI-200) described by the University of Minnesota is the most commonly used regimen.[33] Other reduced-intensity nonmyeloablative conditioning regimens have been described,[39–41] but a retrospective registry-based comparison suggested that Cy-Flu-TBI-200 resulted in superior long-term outcomes.[42] However, the reported outcomes from the centers that developed each conditioning regimen are very similar. Thus, center experience with a treatment platform that includes conditioning regimen, immune suppression, and supportive care impacts outcomes.

In contrast to myeloablative conditioning regimens, indications for CBT transplantation using a reduced-intensity or a non-myeloablative conditioning regimen include not only acute leukemia, but also chronic leukemia, myelodysplasia, and lymphomas. To facilitate engraftment in these older patients whose disease was not treated with potent immunosuppressive chemotherapy, ATG was added to the conditioning regimens to improve engraftment. However, ATG was associated with a higher risk of viral reactivations, post-transplant lymphoproliferative disorders, and slower immune reconstitution.[43,44] Thus, investigators have developed ATG-free regimens by replacing it with thiotepa, treosulfan, busulfan, or TBI.[36,45–47] A new strategy dosing the ATG based on the absolute lymphocyte count may allow for a better therapeutic window of this drug as part of the conditioning regimens while allowing adequate immune reconstitution after UCB transplantation.[48,49]

Conditioning Regimens for Non-Malignant Disease in Children

The indications of UCB transplantation in children extend to nonmalignant diseases such as hemoglobinopathies, immune deficiencies, marrow failure syndromes, and metabolic diseases. In these diseases, there is a need to balance the intensity of the immunosuppression with specific disease manifestations that could impact the recipient's tolerance of toxic drugs commonly used in conditioning regimens and TBI. Examples of such diseases include Fanconi Anemia and Dyskeratosis Congenita. In these situations, there is increased sensitivity to adverse events of chemotherapy and radiation.[50] To be immune ablative and reduce the risk of graft rejection, these regimens often use in vivo T cell depletion with ATG or alemtuzumab.[51] Thus, in this setting, the conditioning regimen will be somewhat disease-specific and is determined by the center's expertise. It is challenging to present specific outcomes about conditioning regimens because, as mentioned before, the outcomes depend on the whole transplant "package" adopted by the transplant center.[51]

GRAFT-VERSUS-HOST DISEASE IN CORD BLOOD TRANSPLANTATION

The growth of the CB inventory has allowed for the identification of larger and better HLA-matched CB units, in particular in children. Nonetheless, most CB grafts remain HLA-mismatched to the patients, and many adults continue to require double CB grafts. Despite the presence of HLA-mismatches, there is a relatively low incidence of acute and chronic GVHD (cGVHD) after CB transplantation, as compared to HLA-mismatched grafts in other donor types.[34,52–54] The distribution of the severity of cGVHD manifestations after CBT does not differ from other donor types, with approximately two-thirds having mild to moderate symptoms.[54] There seems to be a lower risk of development of cutaneous sclerosis after CBT as compared to other donor types,[54,55] with late acute GVHD (aGVHD) being a frequent clinical presentation of GVHD after CBT.[55] Recipients of CBT also seem to have superior GVHD-free, relapse-free survival as compared to other donor types.[54] The risk of aGVHD in single CBT ranges from 10% to 50% with higher incidences observed in adults receiving CB units that are more HLA-mismatched, as discussed in this section. Studies found no impact of the degree of HLA-mismatch when conventional HLA-matching criteria are considered in studying the risk of GVHD after CBT.[56] More recently, studies in single CBT evaluated the impact of allele level HLA-matching and found an approximately 1.5-fold relative increase in the risk of aGVHD with ≥2 allele level mismatches, but no effect on cGVHD.[18] As compared to single CBT, double CBT has overcome the barrier of low cell dose in single CBT and expanded its use to more adult recipients, but it resulted in a higher incidence of aGVHD and cGVHD, as compared to single CBT.[9,12,37] After double CBT, the incidence of acute grades II to IV GVHD ranges from 25% to 75% and cGVHD from 20% to 50%. When considering the historical HLA-matching practice (HLA-A and -B at the antigen level and -DRB1 at the allele level), there is no obvious effect on the risk of GVHD when there are up to 2 HLA-mismatches. In contrast to single CBT, the effect of allele level matching at 8 to 10 loci is less clear.[17,57] This discrepancy may result from differences in patient population and numbers, institutional supportive care, and immunosuppression practice but is not limiting to the efficacy of double CBT. Maintaining higher serum levels of calcineurin inhibitors (CNI) and administration of higher doses of MMF seems to provide superior GVHD prophylaxis in double CBT, as discussed in the following section.

Graft-Versus-Host Disease Prophylaxis in Cord Blood Transplantation

GVHD prophylactic regimens have changed, and currently, the most commonly used regimen used in the United States is the combination of a CNI and MMF, which has largely replaced the short course of corticosteroids. This combination is currently used both in adults and children, with myeloablative and reduced-intensity conditioning regimens and with single and double CB transplantation. The choice of the CNI, whether tacrolimus or cyclosporine-A, is driven by institutional preference. However, data show that keeping the cyclosporine-A level in the upper range of the target serum level of 200 to 400 ng/mL in the first 30 days after CB transplantation results in a lower risk of GVHD.[58] Based on pharmacokinetic studies suggesting suboptimal serum levels of mycophenolic acid (MPA), some centers adopted either a higher dose of MMF (from 2 g/day to 3 g/day) or monitored MPA levels resulting in higher exposure to MPA and lower risk of aGVHD.[59,60] The combination CNI with methotrexate (MTX), commonly used in sibling and unrelated donor (URD) transplantation, is also used in CB transplantation in Japan and to a lesser extent in Europe.[61] The CNI/MTX combination was associated with delayed engraftment after CB transplantation, and further studies demonstrated that reduced dose and frequency of MTX (mini MTX) given at 5 to 10 mg/m^2 on days +1 and 5 to 7 mg/m^2 on days +3 and +6 improved engraftment, effectively suppressed GVHD, and reduced TRM.[62–64] Further reduction in the MTX to only days +1 and +3 resulted in an increased risk of aGVHD after CB transplantation.[63] Retrospective data suggest that in patients with acute leukemia receiving a myeloablative or reduced-intensity conditioning single-unit CBT, CNI plus MTX results in a lower incidence of grade III to IV aGVHD compared to CNI plus MMF.[65,66] ATG is still being used by some centers as part of the conditioning regimen to further ablate the recipient's immune system and improve engraftment rather than GVHD prophylaxis.[33] Registry data demonstrate that ATG reduces the incidence of aGVHD in single and double CB transplantation.[56] The effect of ATG in reducing the risk of GVHD is variable depending on dose, ATG source (rabbit or equine), and timing of administration in relation to the CB graft infusion. After being originally tested in matched related donor peripheral blood stem cell (PBSC) transplantation, the mammalian target of rapamycin (mTOR) inhibitor sirolimus in combination with tacrolimus was studied in reduced-intensity double CB transplantation.[67] In this study, sirolimus/tacrolimus immunosuppression resulted in remarkably low incidences of grade II–IV aGVHD (3 of 32 patients; 9.4%) and cGVHD (13%). Another group studied the combination of sirolimus and MMF in the non-myeloablative setting.[68] This combination was based on studies that involved the adoptive transfer of CB-derived regulatory T cells.[69,70] However, sirolimus/MMF alone resulted in similar rates of GVHD but was better tolerated with a lower overall incidence of infections and less renal toxicity, as compared to cyclosporine and MMF.[68] There was, however, a higher risk of HHV-6 reactivation and delayed engraftment, which may require foscarnet suppression. Neither sirolimus with tacrolimus nor MMF results have yet been reproduced by other groups.

Graft-Versus-Host Disease Outcomes in Cord Blood Transplantation

All-grade aGVHD occurs less frequently in mismatched CBT relative to URD transplantation.[34,71–73] Severe aGVHD after CBT is overall similar to that of matched URD transplantation.[74–76] The most commonly involved organs in all-grade aGVHD are skin and the gastrointestinal tract.[9,57,77] As compared to other donor sources, aGVHD is reported to be overall more responsive to first-line immunosuppressive therapies, with a less detrimental impact on overall survival.[53,57,78] The incidence of overall and severe cGVHD is lower in CBT than in URD and matched sibling transplants.[34,52,53,57] Additionally, the median time to discontinuation of immunosuppression and immune suppression burden including late infections are reported to be lower in CBT than URD peripheral blood transplants with increased corticosteroid responsiveness.[52,79]

OUTCOMES OF CORD BLOOD TRANSPLANTATION

Acute Leukemia and Myelodysplastic Syndrome

The use of CB transplantation has been successfully used worldwide for the treatment of patients with acute leukemia and myelodysplastic syndromes (MDS), both in adult and pediatric patients. In children, most CB transplantation has been after myeloablative conditioning regimens, while in the adult population, a large proportion of patients have received reduced-intensity or non-myeloablative conditioning regimens.

Prospective and retrospective studies in children undergoing CB transplantation for acute leukemia and MDS have found that the probabilities of overall survival and disease-free survival range between 65% to 73% and 47% to 70%, respectively.[9,10,80] In a prospective randomized trial that compared single and double CB transplantation in children, the better outcomes were in children with

AML, self-reported white race[9] and confirmed the positive effect of HLA matching on leukemia-free survival.[9,81] This prospective study observed no difference in the outcomes between recipients of single or double CB grafts as previously reported,[82] but data suggest better disease control among children with minimal residual disease receiving a double CB graft.[83] As children who receive less well HLA-matched CB grafts seem to have a lower risk of relapse,[9] it is possible that better disease control was driven by the lower degree of HLA-matching observed in those patients who require a double CB graft. Recent data demonstrate that the TCF conditioning regimen (see the Conditioning Regimens section) was, at least in part, the main reason for the improved outcomes of children receiving CBT transplants over time.[10,84] The use of a novel treosulfan-containing regimen in children with AML and MDS seems to also result in excellent disease control and survival.[85] Taken together, these data support CB as an effective and valuable graft source in children with leukemia and MDS.

In adults, CB transplantation has also been extensively used for the treatment of leukemia and MDS, with outcomes that are similar to those of matched related and URD transplantations.[3,34,37,42,86–88] However, the long-term outcomes vary depending on the intensity of the conditioning regimen and patient population. In centers with extensive experience in CB transplantation, outcomes after myeloablative regimens and the more recently described myeloablative-reduced-toxicity (thiotepa- or melphalan-containing) regimens may result in overall survival of 50% to 73% and DFS of 45% to 68%.[3,34,37,38,47,89] In the myeloablative setting, there was also a suggestion of better disease control in patients who received CB grafts taken to transplantation with MRD+ disease.[3] It seems that a TBI-containing myeloablative regimen provided better disease control for patients with acute lymphoblastic leukemia, whereas TBI-containing and chemotherapy-only regimens were similarly effective in acute myeloid leukemia.[90] After non-myeloablative CB transplantation, the overall survival was 40% to 54% and PFS was 30% to 45%.[42,91,92] These outcomes are driven by a higher risk of relapse that ranges from 35% to 47%. Notably, in both myeloablative and non-myeloablative settings, the TRM after CB transplantation tended to be higher as compared to other donor types, at least in part, due to the longer time to neutrophil recovery and risk of infections.

Mature Lymphoid Malignancies

There is abundant evidence of the efficacy of CB transplantation for the treatment of mature lymphoid malignancies in the literature. However, a significant amount of these data are combined in reports of CB for the treatment of hematological malignancies and numerous case reports. Most of the data on these diseases are in adult patients. Registry-based studies of CB transplantation for mature lymphoid malignancies found an overall survival of 41% to 56%, progression-free survival of 31% to 41%, incidence of relapse of 28% to 30%, and non-relapse mortality of 29% to 37%.[93,94] In these reports, survival and mortality were similar to that of matched URD transplantation even after adjusting for relevant variables. In another report limited to diffuse large cell B-cell lymphoma, 4-year overall survival was 27%, relapse-free survival was 27%, incidence of relapse was 59%, and non-relapse mortality was 17%.[95] Outcomes were better in patients in partial or complete remission and in those with a longer time from diagnosis to transplantation. Although this study had a relatively small number of patients, the results were similar to those of BM transplantation, and the outcomes reflect the high-risk nature of treated patients. Data on Hodgkin lymphoma showed that overall survival was 46% to 80%, progression-free survival was 26% to 53%, the incidence of relapse was 36% to 44%, and non-relapse mortality was 11% to 30%.[96–98] In multivariate analysis, patients in complete or partial remission and those who received a TCF non-myeloablative conditioning regimen had better survival.[97] Although chronic lymphocytic leukemia is less often an indication of allogeneic transplantation, CB has also been shown to provide long-term survival for those with advanced or high-risk disease (i.e., mutated *p53*). In a series on chronic lymphocytic leukemia from EBMT, the overall survival, progression-free survival, relapse, non-relapse mortality rates were 16%, 39%, 54%, and 45%, respectively.[99] Patients who were CMV seronegative, had FLU-sensitive disease, received less than three previous lines of chemotherapy, and received a non-myeloablative regimen with low-dose TBI regimens had better overall survival. In a report on adult T-cell leukemia/lymphoma (ATLL), CB transplantation resulted in an overall lower probability of overall survival as compared to unrelated BM transplantation.[100] The difference was driven by higher non-relapse mortality in the low-risk disease group (27% vs. 39%). However, in ATLL patients with intermediate and high-risk disease, the efficacy of CB transplantation was similar to unrelated BM transplantation. Recent registry-based studies have found that patients with lymphoma undergoing CB transplantation had similar relapse risk, but somewhat lower progression-free and overall survival, as compared to partially matched related donor transplantation.[94,96] The difference in outcomes resulted from the remarkably low non-relapse mortality observed among recipients of partially matched related donor transplantation. Nonetheless, the data above support the use of CB for patients with lymphoid malignancies who lack other donors.

Non-Malignant Diseases in Pediatrics

CB has been shown to be a valuable alternative donor type for children with non-malignant diseases who lack a matched related or URD.[51] In diseases that require urgent transplantation to avoid organ damage and complications or death from the underlying disease (e.g., severe combined immunodeficiency, Hurler's syndrome, adrenoleukodystrophy, and Krabbe's disease), CB offers a readily available source of stem cells.[7,101–103] While it may not always reverse the disease manifestations, similar to other donor types, CB may provide stabilization of symptoms and avoid further disease progression. Patients with Wiskott–Aldrich syndrome transplanted before 2 years of age have an OS of 75%,[104] and those with Fanconi anemia with no history of opportunistic infections may have survival over 90%.[50] While not as successful in about half of children with hereditary hemophagocytic syndrome and osteopetrosis, CB transplantation may achieve long-term survival.[105,106] Advances in the immunosuppressive properties of conditioning regimens have allowed for better outcomes of patients receiving CB grafts for the treatment of severe acquired aplastic anemia (SAA). One approach that has resulted in a 2-year survival of 84% in patients with SAA involved the selection of a single or double CB graft with a minimum TNC dose of 4.0×10^7/kg and a conditioning regimen with CY 30 mg/kg and FLU 30 mg/m^2 daily for 4 days, rabbit total ATG 5.0 mg/kg, and TBI 200 cGy.[107] Thus, such a strategy could be considered for adults with SAA but is most appropriate if done in the context of a clinical trial. However, the best outcomes with CB transplantation in these complex and very different diseases are achieved with adequate CB graft selection, tailored conditioning, supportive care, and a transplant center with both CB transplantation and target disease experience.

OTHER DISEASES

Multiple myeloma has been a progressively less frequent indication of allogeneic transplantation, and the small number of patients treated in individual centers have been reported in series with other hematological malignancies. In registry studies, the progression-free survival and overall survival were 13% to 24% and 15% to 40%, respectively.[108,109] Interestingly, patients with multiple myeloma undergoing planned tandem autologous transplantation followed by CB transplantation had an overall survival of 46%.[109] The use of ATG, high-risk cytogenetics, and chemotherapy-refractory multiple myeloma were predictors of worse survival.[108,109] CB has been used as the graft source for chronic myeloid leukemia and other myeloproliferative diseases. In these less frequent indications of allogeneic transplantation, if CB transplantation is considered the best option for the patient, it should be considered in a clinical trial and/or in consultation with a center experienced with this donor type.

OUTCOMES OF CORD BLOOD RELATIVE TO OTHER DONOR TYPES

Numerous studies have compared the outcomes of CB to other donor types in the myeloablative and non-myeloablative settings, pediatric and adult patients, and leukemia and lymphoid malignancies. Selected studies on this topic published in the last decade (2010 to 2020) are summarized in Tables 108.2, 108.3, and 108.4. Overall, earlier in the last decade, these studies showed that CB leads to outcomes similar to other donor types that at the time were mostly matched siblings and matched and mismatched URDs. As advances in supportive care, HLA typing, donor selection, and GVHD prophylaxis were made, there has been a shift in practices with renewed interest in mismatched unrelated and partially matched related donors (haploidentical donors) and a reduction in the utilization of CB as a graft source in HCT. While recent studies in adults showed that CB may have similar progression-free and disease-free survival after HCT as compared to haploidentical donor transplantation, the latter seems to provide better overall survival, at least in part, because of very low non-relapse mortality.[92,94,110–112] Many factors influence the differences observed in studies comparing CB to other donor types including, but not limited to, patient population and source of the report. In children, for example, CB transplantation is a well-established practice and results in outcomes similar to other donor types (see Table 108.4).[16,80,87,113–116] In contrast, in older adults, CB transplantation has to be considered with caution as this patient population was found to have higher overall mortality, possibly related to a combination of longer engraftment time, more frequent presence of co-morbidities, and disease characteristics (see Table 108.3).[117–119] Notably, reports from a center with a focus on CB as an alternative donor type for HCT consistently showed outcomes similar to other donor types, demonstrating the importance of transplant center expertise in the outcomes of CB transplantation (see Table 108.2).[3,34,86,120] In summary, in the context of smaller families, issues with donor availability, and the presence of donor-specific antibodies, CB remains a valuable donor option for HCT for those patients who lack a matched donor.

STRATEGIES TO IMPROVE CORD BLOOD TRANSPLANTATION OUTCOMES

To reduce the longer time to neutrophil recovery after CB transplantation as compared to BM or PBSC transplantation, numerous strategies have been studied. The reader may refer to a review on this topic for more details.[121,122] We limited this section to more contemporaneous strategies.

Ex Vivo Cord Blood Expansion

The demonstration of the safety of the double CB graft platform set the stage for most of the recent studies in ex vivo CB expansion. In phase 1/2 studies, these strategies used myeloablative or myeloablative-reduced-toxicity conditioning regimens on the double CB platform, where one unit was ex vivo expanded and the other unit was infused unmanipulated as a safety measure. Notably, in contrast with previous strategies in which the ex vivo expanded product was CD3+ depleted, more recent strategies saved and co-infused CD3+ cells derived from the ex vivo expanded unit at the time of transplantation in order to establish long-term chimerism of the ex vivo expanded CB unit; these studies are summarized in Table 108.5. The strategy using nicotinamide (omidubicel) in combination with cytokines is further along in development, and investigators recently completed a randomized phase 3 study comparing the best CB graft available.[123,124] The primary endpoint of this study was time to neutrophil recovery, and while it took 21 days for production, it seems to provide reproducible expansion. The strategy using an SR-1 inhibitor completed phase 1/2 and is currently being evaluated in a phase 2 expansion cohort study[125] testing a cryopreserved product; this strategy seems to provide robust ex vivo expansion. The UM171-based strategy that recently completed phase 1/2 and a small phase 2 expansion phase is the platform with the shortest ex vivo culture time, potentially allowing the culture to occur while the patient is receiving the conditioning regimen.[126] The copper chelator tetraethylenepentamine (carlecortemcel-L) also provided robust expansion,[127] but the time to neutrophil recovery was similar to the 24 to 26 days of conventional single or double CB transplantation. However, in this platform, they only ex vivo expanded a portion of a single CB unit, and it could be speculated that if the investigators expanded the whole unit, time to neutrophil recovery could have been shorter. In summary, there are very promising ex vivo CB expansion platforms in development, and each has different strengths. It is possible that we will have an FDA-approved product in the next 1 to 2 years.

Improving Homing of Cord Blood

One of the strategies pursued in improving engraftment after CB was to improve the homing efficiency of the reduced number of progenitor and stem cells contained in CB units, as compared to BM and PBSC grafts. In a phase 1 study, investigators tested dimethyl prostaglandin E2 (dmPGE2), which had been shown to modulate homeostasis and engraftment in in vitro and in vivo models.[128] The study in the double CB setting in which only one of the CB units was primed with dmPGE2 demonstrated the safety of the strategy, observed a shorter time to neutrophil recovery as compared to historical data (17 vs. 21 days), and found skewed single-unit predominance toward the primed unit. These promising results set the stage for a multicenter phase 2 study that has been completed and is pending reporting. Another group hypothesized that treating one of two CB units composing the graft with fucosyltransferase-VI and guanosine diphosphate fucose would result in higher fucosylation of selectins, resulting in improved homing and hematopoietic recovery after CB transplantation.[129] This study also observed a shorter time to neutrophil and platelet recovery after CB transplantation. This group is also studying fucosylation to improve homing of other cell populations, such as regulatory T cells.[130] In contrast to other strategies that primed or treated the CB ex vivo, one group of investigators administered sitagliptin to the patient; sitagliptin is a dipeptidyl peptidase-4 (DPP-4) inhibitor that prevents the cleavage of SDF1α, allowing for interaction with CXCR4, which is critical for stem cell homing.[131,132] This approach was studied in the single CB graft setting. After a dose-finding study,[131] a phase 2 study demonstrated inhibition of DPP-4 in the plasma and observed time to neutrophil recovery of 19 days. While alone these strategies seem to have only a modest effect, it is possible that they could be combined with ex vivo CB expansion for additive or synergistic effects that could result in better neutrophil recovery and reduce the risk of graft rejection.

Combination of Cord Blood and Haploidentical Donor

The combination of a single CB graft with a CD34+ selected haploidentical PBSC was studied initially in the myeloablative setting[133] and more recently in the non-myeloablative setting.[134] The short-term goal is to temporarily bridge the period of neutropenia with neutrophils derived from the haploidentical donor myeloid progenitors. The ultimate goal is for the haploidentical graft that is lymphocyte depleted to be rejected by the CB, which will establish chimerism and long-term hematopoiesis. Studies in this platform used CD34+ immunomagnetic selection of granulocyte colony-stimulating factor mobilized PBSCs with the goal of obtaining a graft containing 2.5 to 3×10^6 CD34+ cells/kg and less than 1×10^4 CD3+ cells/kg.[133]

Downloaded for roney wuhan (xpsyngd@telegmail.com) at University of Helsinki from ClinicalKey.com by Elsevier on April 14, 2023. For personal use only. No other uses without permission. Copyright ©2023. Elsevier Inc. All rights reserved.

TABLE 108.2 Studies Comparing the Outcomes of Transplantation With Cord Blood and Other Donor Types in Adult Patients

Reference	Diseases	Conditioning Intensity	Donor Type	OS	DFS/PFS	GRFS	TRM/NRM	Relapse	Outcome Years/ Notes
Atsuta et al. Blood 2009[161]	16 y.o. AML ALL	MAC	8/8 BM ($n = 533$) Single CB ($n = 287$)	60% RR = 1.0 (p-value) 43% AML 1.45 (.28) ALL 1.06 (.78)	51% RR = 1.0 (p-value) 45% AML 1.48 (0.12) ALL 1.22 (.28)	NA NA	33% RR = 1.0 (p-value) 22% AML 1.47 (.85) ALL 1.01 (.98)	24% RR = 1.0 (p-value) 31% AML 1.21 (.38) ALL 1.42 (.19)	2 years
Brunstein et al. Blood 2010[34]	AML ALL CML MDS	MAC	dUCB ($n = 128$) MRD ($n = 204$) URD 8/8 ($n = 152$) URD 7/8 ($n = 52$)	NA NA NA NA	51% RR 1.0 (p-value) 33% 1.09 (.59) 48% 0.85 (.36) 38% 1.12 (.60)	NA NA NA NA	34% RR 1.0 (p-value) 24% 0.31 (<.01) 14% 0.61 (.13) 27% 0.38 (<.01)	15 RR 1.0 (p-value) 43% 3.67 (<.01) 37% 3.05 (<.01) 35% 2.50 (<.01)	5 years
Brunstein et al. Blood 2011[91]	Hematologic malignancies	RIC–NMA	Double CB ($n = 50$) Haplo BM ($n = 50$)	54% 62%	46% 48%	NA NA	24% 7%	31% 45%	BMT CTN 0604; 1 year BMT CTN 0603; 1 year
Brunstein et al. Blood 2012[42]	AML ALL	RIC-NMA	URD 8/8 PBSC ($n = 313$) URD 7/8 PBSC ($n = 111$) Double CB TCF ($n = 121$) Double CB Other ($n = 40$)	44% RR = 1.0 (p-value) 37% 1.24 (.117) 37% 1.08 (.602) 19% 1.47 (.046)	35% RR = 1.0 (p-value) 29% 1.28 (.057) 31% 1.13 (.368) 15% 1.13 (.046)	NA NA NA NA	21% RR = 1.0 (p-value) 28% 1.62 (.020) 19% 0.92 (.719) 52% 2.72 (<.001)	44% RR = 1.0 (p-value) 44% 1.10 (.578) 49% 1.26 (.115) 44% 0.92 (.756)	2 years
Atsuta et al. BBMT 2012[162]	16 y.o. AML ALL MDS	MAC	URD BM class II mismatch ($n = 248$) URD BM class I mismatch ($n = 424$) URD BM 6/8 ($n = 356$) Single UCB ($n = 351$)	41 % RR = 1.0 (p-value) 47% A or B 0.84 (.21) C 0.89 (.32) 38% C + DR 0.97 (.83) A/B +C 1.22 (.14) Other 1.25 (.15) 47% 0.85 (.15)	36% RR = 1.0 (p-value) 44% A or B 0.82 (.16) C 0.86 (.17) 36% C + DR 0.95 (.72) A/B +C 1.15 (.30) Other 1.13 (.41) 42% 0.97 (.75)	NA NA NA NA	RR = 1.0 (p-value) A or B 1.07 (.69) C 1.13 (.39) C + DR 1.10 (.60) A/B +C 1.42 (.032) Other 1.48 (0.35) 0.68 (.011)	RR = 1.0 (p-value) A or B 0.65(.06) C 0.60 (.007) C + DR 0.76 (.20) A/B +C 0.70 (.12) Other 0.60 (.06) 1.28 (.125)	3 year CB outcome similar to 7/8 BM class I or II mismatch

Continued

Downloaded for roney wuhan (xpsyngd@telegmail.com) at University of Helsinki from ClinicalKey.com by Elsevier on April 14, 2023. For personal use only. No other uses without permission. Copyright ©2023. Elsevier Inc. All rights reserved.

TABLE 108.2 **Studies Comparing the Outcomes of Transplantation With Cord Blood and Other Donor Types in Adult Patients—cont'd**

Reference	Diseases	Conditioning Intensity	Donor Type	OS	DFS/PFS	GRFS	TRM/NRM	Relapse	Outcome Years/ Notes
Chen et al. BBMT 2012[86]	Hematologic malignancies	RIC	URD 7–8/8 ($n = 221$) Double CB ($n = 64$)	50% HR 1.0 (p-value) 46% 1.34 (.16)	40% HR 1.0 (p-value) 30% 1.19 (.35)	NA NA	10% HR 1.0 (p-value) 27% 3.55 (.0002)	50% HR 1.0 (p-value) 43% 0.68 (.08)	3 years
Raiola et al. BBMT 2014[110]	Hematologic malignancies	MAC RIC	MSD ($n = 176$) URD 8/8 ($n = 43$) Urd 7/8 ($n = 43$) Single CB ($n = 105$) Haplo BM ($n = 92$)	45% HR 1.0 (p-value) 43% (.96) 40% 1.21 (.40) 34% 1.40 (.03) 52% 0.95 (.80)	32% 36% 34% 33% 43%	NA NA NA NA NA	24% 33% 35% 35% 18%	40% 23% 30% 30% 35%	4 years
Marks et al. Haematologica 2014[88]	ALL	MAC	Single and double CB ($n = 116$) Urd 8/8 PBSC and BM ($n = 546$) Urd 7/8 PBSC and BM ($n = 140$)	44% HR 1.0 (p-value) 44% 0.89 (.38) 43% 0.96 (.81)	NA HR 1.0 (p-value) .97 (.82) 1.03 (.85)	NA NA NA	42% HR 1.0 (p-value) 31% 0.74 (.07) 39% 0.83 (.35)	22% HR 1.0 (p-value) 25% 1.35 (.17) 28% 1.37 (.24)	3 years
Ponce et al. BBMT 2015[120]	AML ALL CML	MAC RIC	dUCB ($n = 55$) Urd 8/8 ($n = 66$) Urd 7/8 ($n = 45$)	73% 57% 44%	68% HR 1.0 (p-value) 57% 1.43 (.257) 41% 1.91 (.037)	NA NA NA	24% 18% 39%	9% 23% 20%	3 years
El-Cheikh et al. Cancer 2015[163]	Adults Hematologic malignancies	RIC-NMA	Haplo BM and PBSC ($n = 69$) Single and double CB ($n = 81$)	69% 45%	65% 36%	NA NA	18% 23%	NA NA	2 years -UCB 63% acute leukemia -Haplo 85% LPD
Robin et al. BBMT 2015[164]	Adults MDS	RIC-NMA	Single and double CB ($n = 129$) Urd 10/10 PBSC ($n = 379$) Urd 9/10 PBSC ($n = 107$)	30% HR 1.0 (p-value) 50% 0.56 (.0001) 43% .73 (.10)	28% HR 1.0 (p-value) 45% .57 (.0002) 36% 0.70 (.06)	NA NA NA	42% HR 1.0 (p-value) 32% 0.57 (.003) 36% 0.71 (.16)	30% HR 1.0 (p-value) 23% .57 (.02) 28% .70 (.21)	2 years

Downloaded for roney wuhan (xpsyngd@telegmail.com) at University of Helsinki from ClinicalKey.com by Elsevier on April 14, 2023. For personal use only. No other uses without permission. Copyright ©2023. Elsevier Inc. All rights reserved.

TABLE 108.2 Studies Comparing the Outcomes of Transplantation With Cord Blood and Other Donor Types in Adult Patients—cont'd

Reference	Diseases	Conditioning Intensity	Donor Type		OS	DFS/PFS	GRFS	TRM/NRM	Relapse	Outcome Years/ Notes
Ruggeri et al. Leukemia 2015[165]	Adults AML ALL	MAC RIC RIC-NMA	AML ALL	Haplo PBSC and BM ($n = 360$) CB single and double ($n = 558$) Haplo PBSC and BM ($n = 158$) CB single and double ($n = 370$)	NA NA NA	32% HR 1.0 (*p*-value) 38% 0.78 (.078) 28% HR 1.0 (*p*-value) 34% 1.002 (.84)	NA NA NA NA	HR 1.0 (*p*-value)1.16 (.76) HR 1.0 (*p*-value)1.23 (.23)	HR 1.0 (*p*-value) 0.95 (.47) HR 1.0 (*p*-value) 0.82 (.31)	2 years
Tanaka et al. BBMT 2015[118]	Adult ≥50 y.o. AML ALL MDS	MAC RIC RIC-NMA	Urd Urd	8/8 BM ($n = 516$) 7/8 BM ($n = 295$) Single CB ($n = 566$)	51% 39% 35% vs. 8/8 HR 1.47 (<.001) vs. 7/8 HR 1.03 (.75)	NA NA NA	NA NA NA	32% HR 1.0 (*p*-value) 40% 38% vs. 8/8 HR 1.32 (<.013) vs. 7/8 HR 0.98 (.88)	18% HR 1.0 (*p*-value) 21% 26% vs. 8/8 HR 1.35 (<.02) vs. 7/8 HR 1.18 (.26)	2 years
Milano et al. NEJM 2016[3]	AML ALL MDS	MAC RIC	CB single and double ($n = 140$) Urd 8/8 BM and PBSC ($n = 344$) Urd 7/8 BM and PBSC ($n = 98$)		71% HR 1.0 (*p*-value) 63% 0.78 (.33) MDR+ 1.69 (.08) 49% 1.36 (.30) MRD+ 2.92 (.001)	NA NA NA	NA NA NA	18% 17% 28%	15% HR 1.0 (*p*-value) 24% 1.30 (.46) MRD+ 2.92 (.007) 25% 1.28 (.60) MRD+ (3.01 (.02)	4 years Superior outcomes for CB when MRD+ at HCT
Giannotti et al. J Hematol Oncol 2018[166]	Adults AML	MAC	Haplo BM and PBSC ($n = 186$) Single CB ($n = 147$)		69% HR 1.0 (*p*-value) 42% 2.39 (.003)	63% HR 1.0 (*p*-value) 40% 1.95 (.007)	56% HR 1.0 (*p*-value) 30% 2.39 (<.001)	21% HR 1.0 (*p*-value) 48% 2.63 (.002)	17% HR 1.0 (*p*-value) 12% 0.81 (.65)	2 years

Continued

Downloaded for roney wuhan (xpsyngd@telegmail.com) at University of Helsinki from ClinicalKey.com by Elsevier on April 14, 2023. For personal use only. No other uses without permission. Copyright ©2023. Elsevier Inc. All rights reserved.

TABLE 108.2 Studies Comparing the Outcomes of Transplantation With Cord Blood and Other Donor Types in Adult Patients—cont'd

Reference	Diseases	Conditioning Intensity	Donor Type	OS	DFS/PFS	GRFS	TRM/NRM	Relapse	Outcome Years/ Notes
Baron et al. Blood Cancer J 2019[167]	Refractory and relapsed AML	MAC RIC	Single and double CB ($n = 285$) Urd 10/10 PBSC + BM ($n = 2001$) Urd 9/10 PBSC + BM ($n = 677$)	18% HR = 1.0 (p-value) 37% 0.60 (<.001) 27% 0.80 (.004)	16% HR = 1.0 (p-value) 21% 0.60 (<.001) 23% 0.70 (.002)	11% HR = 1.0 (p-value) 20% 0.7 (<.001) 16% 0.80 (.008)	38% HR = 1.0 (p-value) 26% 0.60 (<.001) 33% 0.80 (0.1)	47% HR = 1.0 (p-value) 43% 0.70 (<.001) 44% 0.87 (.005)	2 years Achieved CR CB 48%, 10/10 69%, 9/10 70%
Sanz et al. BBMT 2020[111]	AML ALL CML MDS	MAC	Single CB ($n = 19$) Haplo ($n = 26$)	35% 59%	30% 54%	NA NA	52% 23%	17% 23%	2 years Prospective randomized study
Fatobene et al. J Clin Oncol 2020[94]	Lymphoma	RIC- NMA	Single and double UCB ($n = 214$) Haplo PBSC ($n = 169$) Haplo BM ($n = 357$)	49% vs. PBSC HR 1.59 (.005) vs. BM HR 1.55 (.001) 59% HR 1.0 (p-value) 58% HR 1.0 (p-value)	36% vs. PBSC HR 1.86 (<.001) vs. BM HR 1.44 (.002) 52% HR 1.0 (p-value) 46% HR 1.0 (p-value)	NA NA NA	33% vs. PBSC HR 2.27 (.0002) vs. BM HR 1.91 (.0001) 18% HR 1.0 (p-value) 20% HR 1.0 (p-value)	31% vs. PBSC 1.52 (.04) vs. BM HR 1.11 (.54) 30% HR 1.0 (p-value) 34% HR 1.0 (p-value)	4 years
Mehta et al. J Clin Oncol 2020[168]	AML ALL CML MDS	MAC RIC	Haplo MAC/RIC, BM/PBSC ($n = 159$) CB MAC ($n = 394$) RIC ($n = 444$) Urd 7/8 BM ($n = 241$)	HR 1.0 (p-value 1.73 (.0003) 1.69 (.00008) 1.65 (.0010)	HR 1.0 (p-value 1.28 1.64 1.28	HR 1.0 (p-value) 1.90 (<.0001) 1.71 (<.0001) 1.58 (.001)	NA NA NA NA	HR 1.0 (p-value 0.44 (.0002) 1.47 (.04) 0.57 (.004)	Report also on CRFS

Downloaded for roney wuhan (xpsyngd@telegmail.com) at University of Helsinki from ClinicalKey.com by Elsevier on April 14, 2023. For personal use only. No other uses without permission. Copyright ©2023. Elsevier Inc. All rights reserved.

TABLE 108.2 Studies Comparing the Outcomes of Transplantation With Cord Blood and Other Donor Types in Adult Patients—cont'd

Reference	Diseases	Conditioning Intensity	Donor Type		OS	DFS/PFS	GRFS	TRM/NRM	Relapse	Outcome Years/ Notes
			URD 7/8 PBSC	MAC sero+ ($n = 256$)	1.52 (.005)	1.25	1.44 (.005)	NA	0.73 (.13)	
				MAC, no sero ($n = 368$)	1.81 (<.0001)	1.41	2.37 (<.0001)	NA	0.68 (.04)	
				RIC sero+ ($n = 157$)	1.58 (.003)	1.42	1.64 (.0007)	NA	0.91 (.69)	
				RIC, no sero ($n = 179$)	1.55 (.0001)	1.36	1.97 (<.0001)	NA	0.71 (.14)	
Brunstein et al. BBMT 2020[112]	Adults Hematologic malignancies	RIC- NMA	Haplo BM ($n = 182$)		59%	43% HR 1.0 (p-value)	NA	19%	44%	2 years Intent to treat analysis
			double UCB ($n = 186$)		47%	38% 1.30 (0.060)	NA	11%	46%	

ALL, Acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *BM*, bone marrow; *CB*, cord blood; *DFS*, disease-free survival; *dUCB*, double umbilical cord blood; *GRFS*, graft-vs-host and relapse-free survival; *HR*, hazard ratio; *MAC*, myeloablative conditioning; *MDS*, myelodysplastic syndrome; *MRD*, minimal residual disease; *NA*, not available; *NMA*, nonmyeloablative; *NRM*, non-relapse mortality; *OS*, overall survival; *PBSC*, peripheral blood stem cells; *PFS*, progression-free survival; *RIC*, reduced-intensity conditioning; *RR*, relative risk; *TRM*, treatment-related mortality; *URD*, unrelated donor.

TABLE 108.3 **Studies Comparing the Outcomes of Transplantation With Cord Blood and Other Donor Types in Adult Patient ≥50 years Old**

Reference	Population	Conditioning Intensity	Donor Type		OS	DFS/ PFS	GRFS	TRM/NRM	Relapse	Outcome Years
Peffault de Latour et al. BBMT 2013[119]	AML	RIC-NMA	MSD PBSC ($n = 82$)		51% HR 1.0 (p-value)	48%	NA	18%	33%	3 years
			Urd PBSC ($n = 35$)		53% 1.16 (.73)	57% 1.26 (.56)	NA	14% 1.55 (.53)	29% 1.34 (.54)	
			Single and double CB ($n = 80$)		45% 1.23 (.45)	33% 1.23 (.45)	NA	24% 0.95 (.90)	43% 1.51 (.24)	
Tanaka et al. BBMT 2015[118]	AML ALL MDS	MAC RIC RIC-NMA	Urd	8/8 BM ($n = 516$)	51%	NA	NA	32% HR 1.0 (p-value)	18% HR 1.0 (p-value)	2 years
			Urd	7/8 BM ($n = 295$)	39%	NA	NA	40%	21%	
			Single CB ($n = 566$)		35% vs. 8/8 1.47 (<.001) vs. 7/8 1.03 (.75)	NA	NA	38% vs. 8/8 1.32 (<.013) vs. 7/8 0.98 (.88)	26% vs. 8/8 1.35 (<.02) vs. 7/8 1.18 (.26)	
Konuma et al. Am J Hematol 2016[117]	AML ALL MDS	MAC RIC	Single CB ($n = 1310$)		36% HR 1.0 (p-value)	NA	25% HR 1.0 (p-value)	41%% HR 1.0 (p-value)	27% HR 1.0 (p-value)	2 years
			MSD PB ($n = 462$)		47% 0.75 (.002)	NA	17% 1.12 (.14)	26% 0.63 (<.001)	31% 1.13 (.35)	
			MSD BM ($n = 319$)		54% 0.67 (<.001)	NA	25% 0.94 (.53)	26% 0.61 (<.001)	30% 1.1 (.47)	
			URD 7/8 PBSC	MAC sero+ ($n = 256$)	1.52 (.005)	1.25	1.44 (.005)	NA	0.73 (.13)	
				MAC, no sero ($n = 368$)	1.81 (<.0001)	1.41	2.37 (<.0001)	NA	0.68 (.04)	
				RIC sero+ ($n = 157$)	1.58 (.003)	1.42	1.64 (.0007)	NA	0.91 (.69)	
				RIC, no sero ($n = 179$)	1.55 (.0001)	1.36	1.97 (<.0001)	NA	0.71 (.14)	

ALL, Acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *BM*, bone marrow; *CB*, cord blood; *DFS*, disease-free survival; *dUCB*, double umbilical cord blood; *GRFS*, graft-vs-host and relapse-free survival; *HR*, hazard ratio; *MAC*, myeloablative conditioning; *MDS*, myelodysplastic syndrome; *MRD*, minimal residual disease; *NA*, not available; *NMA*, nonmyeloablative; *NRM*, non-relapse mortality; *OS*, overall survival; *PBSC*, peripheral blood stem cells; *PFS*, progression-free survival; *RIC*, reduced-intensity conditioning; *RR*, relative risk; *TRM*, treatment-related mortality; *URD*, unrelated donor.

The CD34+ selected cell dose was capped to reduce the risk of long-term engraftment of the haploidentical graft. Using this strategy in the myeloablative setting, the median time to neutrophil engraftment was 12 days with 95% of patients engrafted with a CB graft by 40 days after transplantation. Platelet engraftment occurred at a median of 36 days with a 78% cumulative incidence of engraftment by day 100. Cumulative incidence of grade II to IV aGVHD and cGVHD were both 21%. Overall survival with 60 months of median follow-up was estimated at 44%.[133] In the non-myeloablative setting, the times to neutrophil and platelet recovery were 11 days and 19 days, respectively. Acute and chronic GVHD occurred at rates of 25% and 5%, respectively, and estimated survival at 1 year was 55%.[134] Notably, in the non-myeloablative setting, a small number of patients had haploidentical donor-derived long-term hematopoiesis instead of engraftment of the CB graft. This combination is valuable and promising and seems to achieve results similar to matched URD transplantation but requires an institution able to perform the immunomagnetic selection.[135]

TABLE 108.4 Studies Comparing the Outcomes of Transplantation With Cord Blood and Other Donor Types in Pediatric Patients

Reference	Diseases	Conditioning Intensity	Donor Type		OS	DFS/PFS	GRFS	TRM/NRM	Relapse	Outcome Years
Eapen et. al. Lancet 2007[16]	AML ALL	MAC	Urd BM	8/8 (I = 116)	NA	38% RR 1.0 (p-value)	NA	RR 1.0 (p-value)	RR 1.0 (p-value)	5 years
				6–7/8 (n = 166)	NA	37% 0.99 (.973)	NA	1.42 (.157)	0.77 (.217)	
			Single CB	6/6 (n = 35)	NA	60% 0.54 (.041)	NA	0.26 (.066)	0.68 (.252)	
				5/6 HD (n = 157)	NA	45% 0.94 (.730)	NA	1.48 (.133)	0.67 (.059)	
				5/6 LD (n = 44)	NA	37% 1.12 (.634)	NA	1.88 (.046)	0.72 (.389)	
				4/6 (n = 267)	NA	33% 1.17 (.297)	NA	2.31 (<.001)	0.54 (.005)	
González-Vicent et al. Eur J Haematol 2011[113]	AML ALL	MAC	Haplo PBSC CD34+ selected (n = 29)		NA	44% AML 51% ALL 41%	NA	25%	48%	
			Single CB (n = 38)		NA	32% AML 46% ALL 26%	NA	47%	25%	
Zheng et al. Ann Hematol 2015[114]	AML ALL	MAC	MSD PBSC and BM (n = 25)		73%	63%	NA	13%	45%	5 years
			Single CB (n = 90)		61%	59%	NA	33%	13%	
Sakaguchi et al. BBMT 2016[115]	AML ALL	MAC	MSD BM (n = 159)		HR 1.0 (p-value)	HR 1.0 (p-value)	NA	HR 1.0 (p-value)	HR 1.0 (p-value)	
			Urd 7-8/8 BM (n = 218)		0.90 (.67)	0.95 (.80)	NA	1.69 (.15)	0.63 (.04)	
			Single CB (n = 200)		1.15 (.57)	1.23 (.30)	NA	2.20 (.03)	0.95 (.81)	
Mo et al. Int J Cancer 2016[116]	ALL	MAC	Haplo (n = 65)		82% HR 1.0 (p-value)	71% HR 1.0 (p-value)	NA	12% HR 1.0 (p-value)	16% HR 1.0 (p-value)	2 years
			Single CB (n = 64)		70% 0.90 (.836)	57% 0.77 (0.533)	NA	19% 0.77 (.686)	24% 0.62 (.397)	
Mehta et al. Blood Adv 2019[87]	AML ALL	MAC	CB (n = 1441)		59% HR 1.0 (p-value)	52% HR 1.0 (p-value)	33% HR 1.0 (p-value)	NA	30% HR 1.0 (p-value)	2 years
			Urd 7/8 BM (n = 172)		59% 1.07 (N.S.)	49% 2.14 (.007)	22% 1.12 (N.S.)	NA	24% 1.44 (.03)	
Keating et al. Blood Adv 2019[80]	AML	MAC	UCB (n = 122) vs. 7-8/8 MUD (n = 73)		HR (p-value) 1.0 (1.0)	HR (p-value) 1.0 (1.0)	HR (p-value) 0.56 (.033)	HR (p-value) 1.6 (1.0)	HR (p-value) 0.59 (.84)	2 years

Continued

TABLE 108.4 Studies Comparing the Outcomes of Transplantation With Cord Blood and Other Donor Types in Pediatric Patients—cont'd

Reference	Diseases	Conditioning Intensity	Donor Type	OS	DFS/PFS	GRFS	TRM/NRM	Relapse	Outcome Years
			MSD ($n = 61$) vs. Urd 7-8/8 BM ($n = 73$)	0.67 (1.0)	0.83 (1.0)	0.67 (.73)	1.1 (1.0)	0.71 (1.0)	
			MSD ($n = 61$) vs. Single CB ($n = 122$)	0.67 (1.0)	0.77 (1.0)	1.1 (.65)	0.67 (1.0)	1.2 (1.0)	
			MSD ($n = 61$) vs. Double CB ($n = 61$)	0.43 (.091)	0.56 (0.406)	1 (.95)	0.42 (.497)	0.91 (1.0)	

ALL, Acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *BM*, bone marrow; *CB*, cord blood; *DFS*, disease-free survival; *dUCB*, double umbilical cord blood; GRFS, graft-vs-host and relapse-free survival; *HR*, hazard ratio; *MAC*, myeloablative conditioning; *MDS*, myelodysplastic syndrome; *MRD*, minimal residual disease; *NA*, not available; *NMA*, nonmyeloablative; *NRM*, non-relapse mortality; *OS*, overall survival; *PBSC*, peripheral blood stem cells; *PFS*, progression-free survival; *RIC*, reduced-intensity conditioning; *RR*, relative risk; *TRM*, treatment-related mortality; *URD*, unrelated donor.

TABLE 108.5 Summary of Selected Ex Vivo Expansion Platforms

Reference	Strategy Type	Reagent Class	Research Phases Completed	Time to Complete Ex Vivo Expansion	Median CD34+ Expansion	Minimum Required TNC for Expanded CB Unit	Median Time to ANC Engraftment	Median Time to PLT Engraftment
Horwitz et al. JCO 2019[123]	Nicotinamide (Omidubicel)	Amide form of vitamin B_3 (niacin)	Phase 1 Phase 2 Phase 3 (recently completed)	21 days	33-fold	1.5 × 10e7/kg	12 days	34 days
Wagner et al. Cell Stem Cell 2016[125]	SR1-inhibitor MGTA-456 (former HSC835)	Aryl hydrocarbon receptor inhibitor	Phase 1/2 Phase 2 (ongoing extension)	15 days	330-fold	1.5 × 10e7/kg	15 days	49 days
Cohen et al. Lancet Haematol 2020[126]	UM171	Pyrimido-indole derivative	Phase 1/2	7 days	28-fold	Phase 1: 2.0 ×10e7/kg Phase 2: 1.5 ×10e7/kg	18 days	42 days
Stiff et al. BBMT 2018[127]	Carlecortemcel-L	Copper chelator tetraethylenepentamine	Phase 1/2	22 days	90-fold	×10e7/kg In dual cryo bags	21 days	54 days

ALL, Acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *BM*, bone marrow; *CB*, cord blood; *DFS*, disease-free survival; *dUCB*, double umbilical cord blood; *GRFS*, graft-vs-host and relapse-free survival; *HR*, hazard ratio; *MAC*, myeloablative conditioning; *MDS*, myelodysplastic syndrome; *MRD*, minimal residual disease; *NA*, not available; *NMA*, nonmyeloablative; *NRM*, non-relapse mortality; *OS*, overall survival; *PBSC*, peripheral blood stem cells; *PFS*, progression-free survival; *RIC*, reduced-intensity conditioning; *RR*, relative risk; *TRM*, treatment-related mortality; *URD*, unrelated donor.

SUPPORTIVE CARE IN CORD BLOOD TRANSPLANTATION

While most supportive care measures used after hematopoietic SCT are similar to other donor types, infection prophylaxis and growth factor support require special attention after CB transplantation.

Infection Prophylaxis

Cytomegalovirus (CMV) reactivation is a frequent event after CB transplantation, and weekly monitoring and preemptive therapy is a mandatory practice to avoid CMV organ disease. The incidence of reactivation in CMV-seropositive patients using PCR ranges from 50% to 100% and typically occurs in the first 3 months after CB transplantation. The sensitivity of the PCR and viral copy number threshold to start preemptive therapy varies depending on the institution. While there has been a suggestion that patients who have CMV reactivation may be protected from relapse,[136,137] the treatment of CMV reactivation early after CB transplantation is challenging because of the potential morbidity and need for hospitalization caused by anti-CMV therapy complications, namely myelosuppression induced by ganciclovir and electrolyte disturbances and kidney injury caused by foscarnet. Thus, centers with expertise in CB transplantation pursue some form of CMV suppressive therapy,[138–141] and many centers have now adopted letermovir CMV prophylaxis for CMV-positive patients undergoing CB transplantation.[142]

Epstein-Barr virus (EBV) reactivation after allogeneic transplantation is most frequent in those receiving HLA mismatched grafts, when the donor is unrelated, and with the use of T cell-depleting agents or techniques (e.g., ATG, CD34+ selection). Not surprisingly, EBV reactivation may be seen in approximately 25% of patients after CB transplantation, with progression to post-transplant lymphoproliferative disease (PTLD) in 10% to 20% of patients.[43,44,143] Once PTLD is established, the prognosis is poor.[43,143] Especially when using ATG as part of the CB transplantation conditioning regimen, monitoring for EBV viremia and administering preemptive therapy with rituximab has been shown to reduce the risk of progression to PTLD.[144] More recently, administration of rituximab pre-CB transplantation has also been shown to be a potential approach in reducing the risk of EBV PTLD with this donor type.[145]

Human herpesvirus-6 (HHV-6) reactivation seems to be a frequent event typically happening 4 to 6 weeks after CB transplantation.[146–148] The more significant clinical manifestations of HHV-6 reactivation after CB transplantation include a maculopapular rash, fevers, delayed hematopoietic count recovery/graft failure, and limbic encephalitis.[149–151] Notably, the frequency of limbic encephalitis varies from center to center, and the reasons for this variation are not completely understood.[152] It is possible that those receiving sirolimus-based GVHD prophylaxis are at higher risk of HHV-6 reactivation.[68] The potentially devastating impact of graft failure and encephalitis led to attempts at prophylactic therapy or monitoring HHV-6 reactivation and instituting preemptive therapy with either foscarnet or valganciclovir.[153–155] The toxicity of these two antivirals has been challenging, and these prophylactic strategies have not been shown to prevent limbic encephalitis.[154] The impact of HHV-6 therapy in preventing delayed engraftment and graft failure is yet to be determined. It is also unclear what viral load should be considered before beginning any therapy. Thus, patients undergoing CB transplantation should be monitored for HHV-6 reactivation every 1 to 2 weeks. The detection of a positive or rising viral load needs to be considered in the context of PCR test sensitivity, sample (whole blood vs. plasma), and methodology of HHV-6 testing as very high viral load may represent DNA viral integration rather than HHV-6 reactivation. Therapy with foscarnet or valganciclovir should be considered in those patients with rising HHV-6 viral load and symptoms (e.g., rash, fevers, slow hematopoietic recovery, and limbic encephalitis) and take into account the clinical context (e.g., pancytopenia and kidney function). Virus-specific T cells are currently under investigation for this indication (NCT02108522).

Antifungal and antibacterial prophylaxis is recommended to all patients undergoing CB transplantation and follows the clinical practice applied to other donor types. Antibacterial prophylaxis is typically delivered until neutrophil recovery, and fluoroquinolones have been primarily used. While fluconazole is an acceptable antifungal prophylaxis, patients who have a history of fungal infection or chest CT scan changes suggestive of fungal infection should be considered for anti-mold prophylaxis with micafungin[156] or micafungin followed by voriconazole.[157] In patients who initially receive fluconazole prophylaxis and have slow count recovery and fevers after CB transplantation, advancing anti-fungal coverage to target molds should be considered. Bacterial and *Pneumocystis jirovecii* prophylaxis should follow the same principles of other donor types.

Growth Factor Support

Most patients will receive GCSF support after undergoing CB transplantation. Dosing and route of administration follow the same standard of care of other donor types. The timing of initiation of GCSF varies based on institutional practice, clinical protocols, and availability, which may be limited or restricted in some countries. Most commonly, GCSF is started between days +1 and +7 after transplantation, but some centers will not start until at least 21 days with no evidence of white cell counts and/or neutrophil recovery. Considering the delay in hematologic recovery after CB as compared to other donor types, centers with CB expertise in the United States will start GCSF in the first-week post-transplantation and continue until neutrophil engraftment. As the time to platelet recovery is also longer after CB transplantation, leading to increased blood product utilization,[158] emerging data suggest that thrombopoietin receptor mimetics or agonists may be of value but should be used in the context of a clinical trial.[159,160] Careful attention to drug toxicity and viral infections are critical in reducing the risk of delayed hematopoietic recovery.

SUMMARY

There is a significant body of evidence to support CB as a valuable alternative source of hematopoietic cells for transplantation. Successful CB transplantation requires appropriate unit selection, adequate immunosuppressive conditioning regimen, effective GVHD prophylaxis, and adequate infection monitoring and management. Moreover, as experience also plays a role in the success of CB transplantation, less experienced centers are encouraged to access unit selection resources and reach out to a center with CB expertise for advice and guidance. In addition to better tolerated more intensive conditioning regimens, new CB expansion and homing enhancement methodologies are likely to improve the efficacy of this donor type.

SUGGESTED READINGS

Admiraal R, Nierkens S, de Witte MA, et al. Association between anti-thymocyte globulin exposure and survival outcomes in adult unrelated haemopoietic cell transplantation: a multicentre, retrospective, pharmacodynamic cohort analysis. *Lancet Haematol*. 2017;4(4):e183–e191.

Anand S, Thomas S, Corbet K, et al. Adult umbilical cord blood transplantation using myeloablative thiotepa, total body irradiation, and fludarabine conditioning. *Biol Blood Marrow Transplant*. 2017;23(11):1949–1954.

Arora M, Cutler CS, Jagasia MH, et al. Late acute and chronic graft-versus-host disease after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2016;22(3):449–455.

Barker JN, Kempenich J, Kurtzberg J, et al. CD34(+) cell content of 126 341 cord blood units in the US inventory: implications for transplantation and banking. *Blood Adv*. 2019;3(8):1267–1271.

Barker JN, Kurtzberg J, Ballen K, et al. Optimal practices in unrelated donor cord blood transplantation for hematologic malignancies. *Biol Blood Marrow Transplant*. 2017;23(6):882–896.

Brunstein CG, Barker JN, Weisdorf DJ, et al. Umbilical cord blood transplantation after nonmyeloablative conditioning: impact on transplantation outcomes in 110 adults with hematologic disease. *Blood*. 2007;110(8):3064–3070.

Brunstein CG, Gutman JA, Weisdorf DJ, et al. Allogeneic hematopoietic cell transplantation for hematological malignancy: relative risks and benefits of double umbilical cord blood. *Blood*. 2010;116(22):4693–4699.

Cohen S, Roy J, Lachance S, et al. Hematopoietic stem cell transplantation using single UM171-expanded cord blood: a single-arm, phase 1–2 safety and feasibility study. *Lancet Haematol*. 2020;7(2):e134–e145.

Eapen M, Rubinstein P, Zhang MJ, et al. Outcomes of transplantation of unrelated donor umbilical cord blood and bone marrow in children with acute leukaemia: a comparison study. *Lancet*. 2007;369(9577):1947–1954.

Eapen M, Wang T, Veys PA, et al. Allele-level HLA matching for umbilical cord blood transplantation for non-malignant diseases in children: a retrospective analysis. *Lancet Haematol*. 2017;4(7):e325–e333.

Fuchs EJ, O'Donnell PV, Eapen M, et al. Double unrelated umbilical cord blood versus HLA-haploidentical bone marrow transplantation: the BMT CTN 1101 trial. *Blood*. 2021;137(3):420–428.

Horwitz ME, Wease S, Blackwell B, et al. Phase I/II study of stem-cell transplantation using a single cord blood unit expanded ex vivo with nicotinamide. *J Clin Oncol*. 2019;37(5):367–374.

Keating AK, Langenhorst J, Wagner JE, et al. The influence of stem cell source on transplant outcomes for pediatric patients with acute myeloid leukemia. *Blood Adv*. 2019;3(7):1118–1128.

Konuma T, Tsukada N, Kanda J, et al. Comparison of transplant outcomes from matched sibling bone marrow or peripheral blood stem cell and unrelated cord blood in patients 50 years or older. *Am J Hematol*. 2016;91(5):E284–E292.

MacMillan ML, Weisdorf DJ, Brunstein CG, et al. Acute graft-versus-host disease after unrelated donor umbilical cord blood transplantation: analysis of risk factors. *Blood*. 2009;113(11):2410–2415.

Mehta RS, Holtan SG, Wang T, et al. Composite GRFS and CRFS outcomes after adult alternative donor HCT. *J Clin Oncol*. 2020;38(18): 2062–2076.

Mehta RS, Holtan SG, Wang T, et al. GRFS and CRFS in alternative donor hematopoietic cell transplantation for pediatric patients with acute leukemia. *Blood Adv*. 2019;3(9):1441–1449.

Milano F, Gooley T, Wood B, et al. Cord-blood transplantation in patients with minimal residual disease. *N Engl J Med*. 2016;375(10):944–953.

Ponce DM, Hilden P, Devlin SM, et al. High disease-free survival with enhanced protection against relapse after double-unit cord blood transplantation when compared with T cell-depleted unrelated donor transplantation in patients with acute leukemia and chronic myelogenous leukemia. *Biol Blood Marrow Transplant*. 2015;21(11):1985–1993.

Ponce DM, Sauter C, Devlin S, et al. A novel reduced-intensity conditioning regimen induces a high incidence of sustained donor-derived neutrophil and platelet engraftment after double-unit cord blood transplantation. *Biol Blood Marrow Transplant*. 2013;19(5):799–803.

Robin M, Ruggeri A, Labopin M, et al. Comparison of unrelated cord blood and peripheral blood stem cell transplantation in adults with myelodysplastic syndrome after reduced-intensity conditioning regimen: a collaborative study from Eurocord (Cord Blood Committee of Cellular Therapy & Immunobiology Working Party of EBMT) and Chronic Malignancies Working Party. *Biol Blood Marrow Transplant*. 2015;21(3):489–495.

Ruggeri A, Rocha V, Masson E, et al. Impact of donor-specific anti-HLA antibodies on graft failure and survival after reduced intensity conditioning-unrelated cord blood transplantation: a Eurocord, Sociéte Francophone d'Histocompatibilite et d'Immunogenetique (SFHI) and Sociéte Française de Greffe de Moelle et de Therapie Cellulaire (SFGM-TC) analysis. *Haematologica*. 2013;98(7):1154–1160.

Takanashi M, Atsuta Y, Fujiwara K, et al. The impact of anti-HLA antibodies on unrelated cord blood transplantations. *Blood*. 2010;116(15):2839–2846.

Wagner Jr. JE, Brunstein CG, Boitano AE, et al. Phase I/II trial of StemRegenin-1 expanded umbilical cord blood hematopoietic stem cells supports testing as a stand-alone graft. *Cell Stem Cell*. 2016;18(1): 144–155.

Wagner JE, Barker JN, DeFor TE, et al. Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. *Blood*. 2002;100(5):1611–1618.

REFERENCES

1. Gragert L, Eapen M, Williams E, Freeman J, Spellman S, Baitty R, et al. HLA match likelihoods for hematopoietic stem-cell grafts in the U.S. registry. *N Engl J Med*. 2014;371(4):339–348.
2. Barker JN, Kurtzberg J, Ballen K, Boo M, Brunstein C, Cutler C, et al. Optimal practices in unrelated donor cord blood transplantation for hematologic malignancies. *Biol Blood Marrow Transpl*. 2017;23(6):882–896.
3. Milano F, Gooley T, Wood B, Woolfrey A, Flowers ME, Doney K, et al. Cord-blood transplantation in patients with minimal residual disease. *N Engl J Med*. 2016;375(10):944–953.
4. Wagner JE, Barker JN, DeFor TE, Baker KS, Blazar BR, Eide C, et al. Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. *Blood*. 2002;100(5):1611–1618.
5. Gluckman E, Rocha V, Ionescu I, Bierings M, Harris RE, Wagner J, et al. Results of unrelated cord blood transplant in fanconi anemia patients: risk factor analysis for engraftment and survival. *Biol Blood Marrow Transpl*. 2007;13(9):1073–1082.
6. Peffault de Latour R, Purtill D, Ruggeri A, Sanz G, Michel G, Gandemer V, et al. Influence of nucleated cell dose on overall survival of unrelated cord blood transplantation for patients with severe acquired aplastic anemia: a study by eurocord and the aplastic anemia working party of the European Group for Blood and Marrow Transplantation. *Biol Blood Marrow Transpl*. 2011;17(1):78–85.
7. Fernandes JF, Rocha V, Labopin M, Neven B, Moshous D, Gennery AR, et al. Transplantation in patients with SCID: mismatched related stem cells or unrelated cord blood? *Blood*. 2012;119(12):2949–2955.
8. Barker JN, Kempenich J, Kurtzberg J, Brunstein CG, Delaney C, Milano F, et al. CD34(+) cell content of 126 341 cord blood units in the US inventory: implications for transplantation and banking. *Blood Adv*. 2019;3(8):1267–1271.
9. Wagner Jr. JE, Eapen M, Carter S, Wang Y, Schultz KR, Wall DA, et al. One-unit versus two-unit cord-blood transplantation for hematologic cancers. *N Engl J Med*. 2014;371(18):1685–1694.
10. Eapen M, Kurtzberg J, Zhang MJ, Hattersely G, Fei M, Mendizabal A, et al. Umbilical cord blood transplantation in children with acute leukemia: impact of conditioning on transplantation outcomes. *Biol Blood Marrow Transpl*. 2017;23(10):1714–1721.
11. Barker JN, Weisdorf DJ, DeFor TE, Blazar BR, McGlave PB, Miller JS, et al. Transplantation of 2 partially HLA-matched umbilical cord blood units to enhance engraftment in adults with hematologic malignancy. *Blood*. 2005;105(3):1343–1347.
12. Scaradavou A, Brunstein CG, Eapen M, Le-Rademacher J, Barker JN, Chao N, et al. Double unit grafts successfully extend the application of umbilical cord blood transplantation in adults with acute leukemia. *Blood*. 2013;121(5):752–758.
13. Baron F, Ruggeri A, Beohou E, Labopin M, Mohty M, Blaise D, et al. Single- or double-unit UCBT following RIC in adults with AL: a report from Eurocord, the ALWP and the CTIWP of the EBMT. *J Hematol Oncol*. 2017;10(1):128.
14. Barker JN, Boughan K, Dahi PB, Devlin SM, Maloy MA, Naputo K, et al. Racial disparities in access to HLA-matched unrelated donor transplants: a prospective 1312-patient analysis. *Blood Adv*. 2019;3(7):939–944.
15. Purtill D, Smith K, Devlin S, Meagher R, Tonon J, Lubin M, et al. Dominant unit CD34+ cell dose predicts engraftment after double-unit cord blood transplantation and is influenced by bank practice. *Blood*. 2014;124(19):2905–2912.
16. Eapen M, Rubinstein P, Zhang MJ, Stevens C, Kurtzberg J, Scaradavou A, et al. Outcomes of transplantation of unrelated donor umbilical cord blood and bone marrow in children with acute leukaemia: a comparison study. *Lancet*. 2007;369(9577):1947–1954.
17. Brunstein CG, Petersdorf EW, DeFor TE, Noreen H, Maurer D, MacMillan ML, et al. Impact of Allele-level HLA mismatch on outcomes in recipients of double umbilical cord blood transplantation. *Biol Blood Marrow Transpl*. 2016;22(3):487–492.
18. Eapen M, Klein JP, Ruggeri A, Spellman S, Lee SJ, Anasetti C, et al. Impact of allele-level HLA matching on outcomes after myeloablative single unit umbilical cord blood transplantation for hematologic malignancy. *Blood*. 2014;123(1):133–140.
19. Sanz J, Jaramillo FJ, Planelles D, Montesinos P, Lorenzo I, Moscardo F, et al. Impact on outcomes of human leukocyte antigen matching by allele-level typing in adults with acute myeloid leukemia undergoing umbilical cord blood transplantation. *Biol Blood Marrow Transpl*. 2014;20(1):106–110.
20. Tozatto-Maio K, Giannotti F, Labopin M, Ruggeri A, Volt F, Paviglianiti A, et al. Cord blood unit dominance analysis and effect of the winning unit on outcomes after double-unit umbilical cord blood transplantation in adults with acute leukemia: a retrospective study on Behalf of Eurocord, the Cord Blood Committee of Cellular Therapy, Immunobiology Working Party, and the Acute Leukemia Working Party of the European Group for Blood and Marrow Transplantation. *Biol Blood Marrow Transpl*. 2018;24(8):1657–1663.
21. Brunstein C, Zhang MJ, Barker St J, Martin A, Bashey A, de Lima M, et al. The effect of inter-unit HLA matching in double umbilical cord blood transplantation for acute leukemia. *Haematologica*. 2017; 102(5):941–947.
22. Ballen K, Logan BR, Chitphakdithai P, Kuxhausen M, Spellman SR, Adams A, et al. Unlicensed umbilical cord blood units provide a safe and effective graft source for a diverse population: a study of 2456 umbilical cord blood recipients. *Biol Blood Marrow Transpl*. 2020;26(4):745–757.
23. Takanashi M, Atsuta Y, Fujiwara K, Kodo H, Kai S, Sato H, et al. The impact of anti-HLA antibodies on unrelated cord blood transplantations. *Blood*. 2010;116(15):2839–2846.
24. Fuji S, Oshima K, Ohashi K, Sawa M, Saito T, Eto T, et al. Impact of pretransplant donor-specific anti-HLA antibodies on cord blood transplantation on behalf of the Transplant Complications Working Group of Japan Society for Hematopoietic Cell Transplantation. *Bone Marrow Transpl*. 2020;55(4):722–728.
25. Yamamoto H, Uchida N, Matsuno N, Ota H, Kageyama K, Wada S, et al. Anti-HLA antibodies other than against HLA-A, -B, -DRB1 adversely affect engraftment and nonrelapse mortality in HLA-mismatched single cord blood transplantation: possible implications of unrecognized donor-specific antibodies. *Biol Blood Marrow Transpl*. 2014;20(10):1634–1640.
26. Ruggeri A, Rocha V, Masson E, Labopin M, Cunha R, Absi L, et al. Impact of donor-specific anti-HLA antibodies on graft failure and survival after reduced intensity conditioning-unrelated cord blood transplantation: a Eurocord, Societé Francophone d'Histocompatibilite et d'Immunogenetique (SFHI) and Societé Française de Greffe de Moelle et de Therapie Cellulaire (SFGM-TC) analysis. *Haematologica*. 2013;98(7):1154–1160.
27. Cutler C, Kim HT, Sun L, Sese D, Glotzbecker B, Armand P, et al. Donor-specific anti-HLA antibodies predict outcome in double umbilical cord blood transplantation. *Blood*. 2011;118(25):6691–6697.
28. Brunstein CG, Noreen H, DeFor TE, Maurer D, Miller JS, Wagner JE. Anti-HLA antibodies in double umbilical cord blood transplantation. *Biol Blood Marrow Transpl*. 2011;17(11):1704–1708.
29. Dahi PB, Ponce DM, Devlin S, Evans KL, Lubin M, Gonzales AM, et al. Donor-recipient allele-level HLA matching of unrelated cord blood units reveals high degrees of mismatch and alters graft selection. *Bone Marrow Transpl*. 2014;49(9):1184–1186.
30. Yamashita T, Ikegame K, Kojima H, Tanaka H, Kaida K, Inoue T, et al. Effective desensitization of donor-specific HLA antibodies using platelet transfusion bearing targeted HLA in a case of HLA-mismatched allogeneic stem cell transplantation. *Bone Marrow Transpl*. 2017;52(5):794–796.
31. Choe H, Gergis U, Hsu J, Phillips A, Shore T, Christos P, et al. Bortezomib and immune globulin have limited effects on donor-specific HLA antibodies in haploidentical cord blood stem cell transplantation: detrimental effect of persistent haploidentical donor-specific HLA antibodies. *Biol Blood Marrow Transpl*. 2019;25(2):e60–e64.
32. Cornetta K, Laughlin M, Carter S, Wall D, Weinthal J, Delaney C, et al. Umbilical cord blood transplantation in adults: results of the prospective cord blood transplantation (COBLT). *Biol Blood Marrow Transpl*. 2005;11(2):149–160.
33. Brunstein CG, Barker JN, Weisdorf DJ, DeFor TE, Miller JS, Blazar BR, et al. Umbilical cord blood transplantation after nonmyeloablative conditioning: impact on transplantation outcomes in 110 adults with hematologic disease. *Blood*. 2007;110(8):3064–3070.
34. Brunstein CG, Gutman JA, Weisdorf DJ, Woolfrey AE, Defor TE, Gooley TA, et al. Allogeneic hematopoietic cell transplantation for hematological malignancy: relative risks and benefits of double umbilical cord blood. *Blood*. 2010;116(22):4693–4699.
35. Barker JN, Fei M, Karanes C, Horwitz M, Devine S, Kindwall-Keller TL, et al. Results of a prospective multicentre myeloablative double-unit cord blood transplantation trial in adult patients with acute leukaemia and myelodysplasia. *Br J Haematol*. 2015;168(3):405–412.
36. Sanz J, Boluda JC, Martin C, Gonzalez M, Ferra C, Serrano D, et al. Single-unit umbilical cord blood transplantation from unrelated donors in patients with hematological malignancy using busulfan, thiotepa, fludarabine and ATG as myeloablative conditioning regimen. *Bone Marrow Transpl*. 2012;47(10):1287–1293.
37. Ponce DM, Sauter C, Devlin S, Lubin M, Gonzales AM, Kernan NA, et al. A novel reduced-intensity conditioning regimen induces a high incidence of sustained donor-derived neutrophil and platelet engraftment after double-unit cord blood transplantation. *Biol Blood Marrow Transpl*. 2013;19(5):799–803.

38. Anand S, Thomas S, Corbet K, Gasparetto C, Long GD, Lopez R, et al. Adult umbilical cord blood transplantation using myeloablative thiotepa, total body irradiation, and fludarabine conditioning. *Biol Blood Marrow Transpl*. 2017;23(11):1949–1954.
39. Ballen KK, Spitzer TR, Yeap BY, McAfee S, Dey BR, Attar E, et al. Double unrelated reduced-intensity umbilical cord blood transplantation in adults. *Biol Blood Marrow Transpl*. 2007;13(1):82–89.
40. Ciurea SO, Saliba RM, Hamerschlak N, Karduss Aurueta AJ, Bassett R, Fernandez-Vina M, et al. Fludarabine, melphalan, thiotepa and anti-thymocyte globulin conditioning for unrelated cord blood transplant. *Leuk Lymphoma*. 2012;53(5):901–906.
41. Uchida N, Wake A, Takagi S, Yamamoto H, Kato D, Matsuhashi Y, et al. Umbilical cord blood transplantation after reduced-intensity conditioning for elderly patients with hematologic diseases. *Biol Blood Marrow Transpl*. 2008;14(5):583–590.
42. Brunstein CG, Eapen M, Ahn KW, Appelbaum FR, Ballen KK, Champlin RE, et al. Reduced-intensity conditioning transplantation in acute leukemia: the effect of source of unrelated donor stem cells on outcomes. *Blood*. 2012;119(23):5591–5598.
43. Brunstein CG, Weisdorf DJ, Defor T, Barker JN, Tolar J, van Burik JA, et al. Marked increased risk of Epstein-Barr virus-related complications with the addition of antithymocyte globulin to a nonmyeloablative conditioning prior to unrelated umbilical cord blood transplantation. *Blood*. 2006;108(8):2874–2880.
44. Ballen KK, Cutler C, Yeap BY, McAfee SL, Dey BR, Attar EC, et al. Donor-derived second hematologic malignancies after cord blood transplantation. *Biol Blood Marrow Transpl*. 2010;16(7):1025–1031.
45. Deeg HJ, Stevens EA, Salit RB, Ermoian RP, Fang M, Gyurkocza B, et al. Transplant conditioning with treosulfan/fludarabine with or without total body irradiation: a randomized phase II trial in patients with myelodysplastic syndrome and acute myeloid leukemia. *Biol Blood Marrow Transpl*. 2018;24(5):956–963.
46. Yamamoto H, Uchida N, Yuasa M, Kageyama K, Ota H, Kaji D, et al. A novel reduced-toxicity myeloablative conditioning regimen using full-dose busulfan, fludarabine, and melphalan for single cord blood transplantation provides durable engraftment and remission in nonremission myeloid malignancies. *Biol Blood Marrow Transpl*. 2016;22(10):1844–1850.
47. DeFilipp Z, Li S, Avigan D, Armand P, Ho VT, Koreth J, et al. A phase II study of reduced intensity double umbilical cord blood transplantation using fludarabine, melphalan, and low dose total body irradiation. *Bone Marrow Transpl*. 2020;55(4):804–810.
48. Admiraal R, Nierkens S, de Witte MA, Petersen EJ, Fleurke GJ, Verrest L, et al. Association between anti-thymocyte globulin exposure and survival outcomes in adult unrelated haemopoietic cell transplantation: a multicentre, retrospective, pharmacodynamic cohort analysis. *Lancet Haematol*. 2017;4(4):e183–e191.
49. Admiraal R, Lindemans CA, van Kesteren C, Bierings MB, Versluijs AB, Nierkens S, et al. Excellent T-cell reconstitution and survival depend on low ATG exposure after pediatric cord blood transplantation. *Blood*. 2016;128(23):2734–2741.
50. MacMillan ML, DeFor TE, Young JAH, Dusenbery KE, Blazar BR, Slungaard A, et al. Alternative donor hematopoietic cell transplantation for Fanconi anemia. *Blood*. 2015;125(24):3798–3804.
51. Eapen M, Wang T, Veys PA, Boelens St JJ, Martin A, Spellman S, et al. Allele-level HLA matching for umbilical cord blood transplantation for non-malignant diseases in children: a retrospective analysis. *Lancet Haematol*. 2017;4(7):e325–e333.
52. Gutman JA, Ross K, Smith C, Myint H, Lee CK, Salit R, et al. Chronic graft versus host disease burden and late transplant complications are lower following adult double cord blood versus matched unrelated donor peripheral blood transplantation. *Bone Marrow Transpl*. 2016;51(12):1588–1593.
53. Lazaryan A, Weisdorf DJ, DeFor T, Brunstein CG, MacMillan ML, Bejanyan N, et al. Risk factors for acute and chronic graft-versus-host disease after allogeneic hematopoietic cell transplantation with umbilical cord blood and matched sibling donors. *Biol Blood Marrow Transpl*. 2016;22(1):134–140.
54. Arora M, Cutler CS, Jagasia MH, Pidala J, Chai X, Martin PJ, et al. Late acute and chronic graft-versus-host disease after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transpl*. 2016;22(3):449–455.
55. Newell LF, Flowers MED, Gooley TA, Milano F, Carpenter PA, Martin PJ, et al. Characteristics of chronic GVHD after cord blood transplantation. *Bone Marrow Transpl*. 2013;48(10):1285–1290.
56. Chen YB, Wang T, Hemmer MT, Brady C, Couriel DR, Alousi A, et al. GvHD after umbilical cord blood transplantation for acute leukemia: an analysis of risk factors and effect on outcomes. *Bone Marrow Transpl*. 2017;52(3):400–408.
57. Ponce DM, Gonzales A, Lubin M, Castro-Malaspina H, Giralt S, Goldberg JD, et al. Graft-versus-host disease after double-unit cord blood transplantation has unique features and an association with engrafting unit-to-recipient HLA match. *Biol Blood Marrow Transpl*. 2013;19(6):904–911.
58. Rogosheske JR, Fargen AD, DeFor TE, Warlick E, Arora M, Blazar BR, et al. Higher therapeutic CsA levels early post transplantation reduce risk of acute GVHD and improves survival. *Bone Marrow Transpl*. 2014;49(1):122–125.
59. Bejanyan N, Rogosheske J, DeFor T, Lazaryan A, Esbaum K, Holtan S, et al. Higher dose of mycophenolate mofetil reduces acute graft-versus-host disease in reduced-intensity conditioning double umbilical cord blood transplantation. *Biol Blood Marrow Transpl*. 2015;21(5):926–933.
60. Harnicar S, Ponce DM, Hilden P, Zheng J, Devlin SM, Lubin M, et al. Intensified mycophenolate mofetil dosing and higher mycophenolic acid trough levels reduce severe acute graft-versus-host disease after double-unit cord blood transplantation. *Biol Blood Marrow Transpl*. 2015;21(5):920–925.
61. Kanda J, Hayashi H, Ruggeri A, Kimura F, Volt F, Takahashi S, et al. Prognostic factors for adult single cord blood transplantation among European and Japanese populations: the Eurocord/ALWP-EBMT and JSHCT/JDCHCT collaborative study. *Leukemia*. 2020;34(1):128–137.
62. Fuji S, Tada Y, Nakata R, Nakata K, Koike M, Kida S, et al. GVHD prophylaxis by tacrolimus and mini-MTX in single-unit CBT: a single institute experience. *Int J Hematol*. 2020;111(2):278–283.
63. Hattori N, Saito B, Matsui T, Nakata A, Sasaki Y, Shimada S, et al. Comparative study of tacrolimus and short-term methotrexate: 2-day versus 3-day methotrexate as graft-versus-host-disease prophylaxis after umbilical cord blood transplantation in adults. *Biol Blood Marrow Transpl*. 2020;26(2):367–372.
64. Shiratori S, Ohigashi H, Takahashi S, Ara T, Goto H, Nakagawa M, et al. Reduced dose of MTX for GVHD prophylaxis promotes engraftment and decreases non-relapse mortality in umbilical cord blood transplantation. *Ann Hematol*. 2020;99(3):591–598.
65. Terakura S, Wake A, Inamoto Y, Murata M, Sakai R, Yamaguchi T, et al. Exploratory research for optimal GvHD prophylaxis after single unit CBT in adults: short-term methotrexate reduced the incidence of severe GvHD more than mycophenolate mofetil. *Bone Marrow Transpl*. 2017;52(3):423–430.
66. Terakura S, Kuwatsuka Y, Yamasaki S, Wake A, Kanda J, Inamoto Y, et al. GvHD prophylaxis after single-unit reduced intensity conditioning cord blood transplantation in adults with acute leukemia. *Bone Marrow Transpl*. 2017;52(9):1261–1267.
67. Cutler C, Stevenson K, Kim HT, Brown J, McDonough S, Herrera M, et al. Double umbilical cord blood transplantation with reduced intensity conditioning and sirolimus-based GVHD prophylaxis. *Bone Marrow Transpl*. 2011;46(5):659–667.
68. Bejanyan N, Rogosheske J, DeFor TE, Lazaryan A, Arora M, Holtan SG, et al. Sirolimus and mycophenolate mofetil as calcineurin inhibitor-free graft-versus-host disease prophylaxis for reduced-intensity conditioning umbilical cord blood transplantation. *Biol Blood Marrow Transpl*. 2016;22(11):2025–2030.
69. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood*. 2011;117(3):1061–1070.
70. Brunstein CG, Miller JS, McKenna DH, Hippen KL, DeFor TE, Sumstad D, et al. Umbilical cord blood-derived T regulatory cells to prevent GVHD: kinetics, toxicity profile, and clinical effect. *Blood*. 2016;127(8):1044–1051.
71. Rocha V, Cornish J, Sievers EL, Filipovich A, Locatelli F, Peters C, et al. Comparison of outcomes of unrelated bone marrow and umbilical cord blood transplants in children with acute leukemia. *Blood*. 2001;97(10):2962–2971.
72. Rocha V, Labopin M, Sanz G, Arcese W, Schwerdtfeger R, Bosi A, et al. Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. *N Engl J Med*. 2004;351(22):2276–2285.
73. Grewal SS, Barker JN, Davies SM, Wagner JE. Unrelated donor hematopoietic cell transplantation: marrow or umbilical cord blood? *Blood*. 2003;101(11):4233–4244.
74. Barker JN, Davies SM, DeFor T, Ramsay NK, Weisdorf DJ, Wagner JE. Survival after transplantation of unrelated donor umbilical cord blood is comparable to that of human leukocyte antigen-matched unrelated donor bone marrow: results of a matched-pair analysis. *Blood*. 2001;97(10):2957–2961.
75. Terakura S, Atsuta Y, Tsukada N, Kobayashi T, Tanaka M, Kanda J, et al. Comparison of outcomes of 8/8 and 7/8 Allele-matched unrelated bone marrow transplantation and single-unit cord blood transplantation in adults with acute leukemia. *Biol Blood Marrow Transpl*. 2016;22(2):330–338.

76. Sakai R, Taguri M, Oshima K, Mori T, Ago H, Adachi S, et al. A comparison of tacrolimus and cyclosporine combined with methotrexate for graft-versus-host disease prophylaxis, stratified by stem cell source: a retrospective nationwide survey. *Int J Hematol*. 2016;103(3):322–333.
77. MacMillan ML, Weisdorf DJ, Brunstein CG, Cao Q, DeFor TE, Verneris MR, et al. Acute graft-versus-host disease after unrelated donor umbilical cord blood transplantation: analysis of risk factors. *Blood*. 2009;113(11):2410–2415.
78. Kanda J, Hishizawa M, Utsunomiya A, Taniguchi S, Eto T, Moriuchi Y, et al. Impact of graft-versus-host disease on outcomes after allogeneic hematopoietic cell transplantation for adult T-cell leukemia: a retrospective cohort study. *Blood*. 2012;119(9):2141–2148.
79. Arora M, Nagaraj S, Wagner JE, Barker JN, Brunstein CG, Burns LJ, et al. Chronic graft-versus-host disease (cGVHD) following unrelated donor hematopoietic stem cell transplantation (HSCT): higher response rate in recipients of unrelated donor (URD) umbilical cord blood (UCB). *Biol Blood Marrow Transpl*. 2007;13(10):1145–1152.
80. Keating AK, Langenhorst J, Wagner JE, Page KM, Veys P, Wynn RF, et al. The influence of stem cell source on transplant outcomes for pediatric patients with acute myeloid leukemia. *Blood Adv*. 2019;3(7):1118–1128.
81. Eapen M, Logan BR, Confer DL, Haagenson M, Wagner JE, Weisdorf DJ, et al. Peripheral blood grafts from unrelated donors are associated with increased acute and chronic graft-versus-host disease without improved survival. *Biol Blood Marrow Transpl*. 2007;13(12):1461–1468.
82. Verneris MR, Brunstein CG, Barker J, MacMillan ML, DeFor T, McKenna DH, et al. Relapse risk after umbilical cord blood transplantation: enhanced graft-versus-leukemia effect in recipients of 2 units. *Blood*. 2009;114(19):4293–4299.
83. Balligand L, Galambrun C, Sirvent A, Roux C, Pochon C, Bruno B, et al. Single-unit versus double-unit umbilical cord blood transplantation in children and young adults with residual leukemic disease. *Biol Blood Marrow Transpl*. 2019;25(4):734–742.
84. Spees LP, Martin PL, Kurtzberg J, Stokhuyzen A, McGill L, Prasad VK, et al. Reduction in mortality after umbilical cord blood transplantation in children over a 20-year period (1995–2014). *Biol Blood Marrow Transpl*. 2019;25(4):756–763.
85. Nemecek ER, Hilger RA, Adams A, Shaw BE, Kiefer D, Le-Rademacher J, et al. Treosulfan, fludarabine, and low-dose total body irradiation for children and young adults with acute myeloid leukemia or myelodysplastic syndrome undergoing allogeneic hematopoietic cell transplantation: prospective phase II trial of the pediatric blood and marrow transplant consortium. *Biol Blood Marrow Transpl*. 2018;24(8):1651–1656.
86. Chen YB, Aldridge J, Kim HT, Ballen KK, Cutler C, Kao G, et al. Reduced-intensity conditioning stem cell transplantation: comparison of double umbilical cord blood and unrelated donor grafts. *Biol Blood Marrow Transpl*. 2012;18(5):805–812.
87. Mehta RS, Holtan SG, Wang T, Hemmer MT, Spellman SR, Arora M, et al. GRFS and CRFS in alternative donor hematopoietic cell transplantation for pediatric patients with acute leukemia. *Blood Adv*. 2019;3(9):1441–1449.
88. Marks DI, Woo KA, Zhong X, Appelbaum FR, Bachanova V, Barker JN, et al. Unrelated umbilical cord blood transplant for adult acute lymphoblastic leukemia in first and second complete remission: a comparison with allografts from adult unrelated donors. *Haematologica*. 2014;99(2):322–328.
89. Sharma P, Pollyea DA, Smith CA, Purev E, Kamdar M, Haverkos B, et al. Thiotepa-based intensified reduced-intensity conditioning adult double-unit cord blood hematopoietic stem cell transplantation results in decreased relapse rate and improved survival compared with transplantation following standard reduced-intensity conditioning: a retrospective cohort comparison. *Biol Blood Marrow Transpl*. 2018;24(8):1671–1677.
90. Sanz J, Wagner JE, Sanz MA, DeFor T, Montesinos P, Bachanova V, et al. Myeloablative cord blood transplantation in adults with acute leukemia: comparison of two different transplant platforms. *Biol Blood Marrow Transpl*. 2013;19(12):1725–1730.
91. Brunstein CG, Fuchs EJ, Carter SL, Karanes C, Costa LJ, Wu J, et al. Alternative donor transplantation after reduced intensity conditioning: results of parallel phase 2 trials using partially HLA-mismatched related bone marrow or unrelated double umbilical cord blood grafts. *Blood*. 2011;118(2):282–288.
92. Fuchs EJ, O'Donnell PV, Eapen M, Logan B, Antin JH, P. D, et al. Double unrelated umbilical cord blood versus HLA-haploidentical bone marrow transplantation: the BMT CTN 1101 trial. *Blood*. 2021;137(3):420–428.
93. Bachanova V, Burns LJ, Ahn KW, Laport GG, Akpek G, Kharfan-Dabaja MA, et al. Impact of pretransplantation (18)f-fluorodeoxy glucose–positron emission tomography status on outcomes after allogeneic hematopoietic cell transplantation for non-Hodgkin lymphoma. *Biol Blood Marrow Transpl*. 2015;21(9):1605–1611.
94. Fatobene G, Rocha St V, Martin A, Hamadani M, Robinson S, Bashey A, et al. Nonmyeloablative alternative donor transplantation for Hodgkin and non-Hodgkin lymphoma: from the LWP-EBMT, Eurocord, and CIBMTR. *J Clin Oncol: Off J Am Soc Clin Oncology*. 2020;38(14):1518–1526.
95. Izumi K, Kanda J, Nishikori M, Arai Y, Ishikawa T, Yoshioka S, et al. Outcomes of allogeneic stem cell transplantation for DLBCL: a multi-center study from the Kyoto Stem Cell Transplantation Group. *Ann Hematol*. 2019;98(12):2815–2823.
96. Gauthier J, Castagna L, Garnier F, Guillaume T, Socié G, Maury S, et al. Reduced-intensity and non-myeloablative allogeneic stem cell transplantation from alternative HLA-mismatched donors for Hodgkin lymphoma: a study by the French Society of Bone Marrow Transplantation and Cellular Therapy. *Bone Marrow Transpl*. 2017;52(5):689–696.
97. Paviglianiti A, Tozatto Maio K, Rocha V, Gehlkopf E, Milpied N, Esquirol A, et al. Outcomes of advanced Hodgkin lymphoma after umbilical cord blood transplantation: a Eurocord and EBMT Lymphoma and Cellular Therapy & Immunobiology Working Party Study. *Biol Blood Marrow Transpl*. 2018;24(11):2265–2270.
98. Thompson PA, Perera T, Marin D, Oran B, Popat U, Qazilbash M, et al. Double umbilical cord blood transplant is effective therapy for relapsed or refractory Hodgkin lymphoma. *Leukemia & Lymphoma*. 2016;57(7):1607–1615.
99. Xavier E, Cornillon J, Ruggeri A, Chevallier P, Cornelissen JJ, Andersen NS, et al. Outcomes of cord blood transplantation using reduced-intensity conditioning for chronic lymphocytic leukemia: a study on Behalf of Eurocord and Cord Blood Committee of Cellular Therapy and Immunobiology Working Party, Chronic Malignancies Working Party of the European Society for Blood and Marrow Transplantation, and the Societé Française de Greffe de Moelle et Therapie Cellulaire. *Biol Blood Marrow Transpl*. 2015;21(8):1515–1523.
100. Fuji S, Kurosawa S, Inamoto Y, Murata T, Utsunomiya A, Uchimaru K, et al. A decision analysis comparing unrelated bone marrow transplantation and cord blood transplantation in patients with aggressive adult T-cell leukemia-lymphoma. *Int J Hematol*. 2020;111(3):427–433.
101. Staba SL, Escolar ML, Poe M, Kim Y, Martin PL, Szabolcs P, et al. Cord-blood transplants from unrelated donors in patients with Hurler's syndrome. *N Engl J Med*. 2004;350(19):1960–1969.
102. van den Broek BTA, Page K, Paviglianiti A, Hol J, Allewelt H, Volt F, et al. Early and late outcomes after cord blood transplantation for pediatric patients with inherited leukodystrophies. *Blood Adv*. 2018;2(1):49–60.
103. Escolar ML, Poe MD, Provenzale JM, Richards KC, Allison J, Wood S, et al. Transplantation of umbilical-cord blood in babies with infantile Krabbe's disease. *N Engl J Med*. 2005;352(20):2069–2081.
104. Shekhovtsova Z, Bonfim C, Ruggeri A, Nichele S, Page K, AlSeraihy A, et al. A risk factor analysis of outcomes after unrelated cord blood transplantation for children with Wiskott–Aldrich syndrome. *Haematologica*. 2017;102(6):1112–1119.
105. Furtado-Silva JM, Paviglianiti A, Ruggeri A, Boelens JJ, Veys P, Ahmari AA, et al. Risk factors affecting outcome of unrelated cord blood transplantation for children with familial haemophagocytic lymphohistiocytosis. *Br J Haematol*. 2019;184(3):397–404.
106. Chiesa R, Ruggeri A, Paviglianiti A, Zecca M, Gónzalez-Vicent M, Bordon V, et al. Outcomes after unrelated umbilical cord blood transplantation for children with osteopetrosis. *Biol Blood Marrow Transpl*. 2016;22(11):1997–2002.
107. Peffault de Latour R, Chevret S, Jubert C, Sirvent A, Galambrun C, Ruggeri A, et al. Unrelated cord blood transplantation in patients with idiopathic refractory severe aplastic anemia: a nationwide phase 2 study. *Blood*. 2018;132(7):750–754.
108. Kawamura K, Takamatsu H, Ikeda T, Komatsu T, Aotsuka N, Amano I, et al. Cord blood transplantation for multiple myeloma: a study from the Multiple Myeloma Working Group of the Japan Society for Hematopoietic Cell Transplantation. *Biol Blood Marrow Transpl*. 2015;21(7):1291–1298.
109. Paviglianiti A, Xavier E, Ruggeri A, Ceballos P, Deconinck E, Cornelissen JJ, et al. Outcomes of unrelated cord blood transplantation in patients with multiple myeloma: a survey on behalf of Eurocord, the Cord Blood Committee of Cellular Therapy and Immunobiology Working Party, and the Chronic Leukemia Working Party of the EBMT. *Haematologica*. 2016;101(9):1120–1127.
110. Raiola AM, Dominietto A, di Grazia C, Lamparelli T, Gualandi F, Ibatici A, et al. Unmanipulated haploidentical transplants compared with other alternative donors and matched sibling grafts. *Biol Blood Marrow Transpl*. 2014;20(10):1573–1579.
111. Sanz J, Montoro J, Solano C, Valcárcel D, Sampol A, Ferrá C, et al. Prospective randomized study comparing myeloablative unrelated umbilical cord blood transplantation versus HLA-haploidentical related stem cell transplantation for adults with hematologic malignancies. *Biol Blood Marrow Transpl*. 2020;26(2):358–366.

112. Fuchs EJ, O'Donnell PV, Eapen M, et al. Double unrelated umbilical cord blood vs HLA-haploidentical bone marrow transplantation: the BMT CTN 1101 trial. *Blood*. 2021;137(3):420–428.
113. González-Vicent M, Molina B, Andión M, Sevilla J, Ramirez M, Pérez A, et al. Allogeneic hematopoietic transplantation using haploidentical donor vs. unrelated cord blood donor in pediatric patients: a single-center retrospective study. *Eur J Haematol*. 2011;87(1):46–53.
114. Zheng C, Zhu X, Tang B, Yao W, Song K, Tong J, et al. Comparative analysis of unrelated cord blood transplantation and HLA-matched sibling hematopoietic stem cell transplantation in children with high-risk or advanced acute leukemia. *Ann Hematol*. 2015;94(3):473–480.
115. Sakaguchi H, Watanabe N, Matsumoto K, Yabe H, Kato S, Ogawa A, et al. Comparison of donor sources in hematopoietic stem cell transplantation for childhood acute leukemia: a nationwide retrospective study. *Biol Blood Marrow Transpl*. 2016;22(12):2226–2234.
116. Mo XD, Tang BL, Zhang XH, Zheng CC, Xu LP, Zhu XY, et al. Comparison of outcomes after umbilical cord blood and unmanipulated haploidentical hematopoietic stem cell transplantation in children with high-risk acute lymphoblastic leukemia. *Int J Cancer*. 2016;139(9):2106–2115.
117. Konuma T, Tsukada N, Kanda J, Uchida N, Ohno Y, Miyakoshi S, et al. Comparison of transplant outcomes from matched sibling bone marrow or peripheral blood stem cell and unrelated cord blood in patients 50 years or older. *Am J Hematol*. 2016;91(5):E284–E292.
118. Tanaka M, Miyamura K, Terakura S, Imai K, Uchida N, Ago H, et al. Comparison of cord blood transplantation with unrelated bone marrow transplantation in patients older than fifty years. *Biol Blood Marrow Transpl*. 2015;21(3):517–525.
119. Peffault de Latour R, Brunstein CG, Porcher R, Chevallier P, Robin M, Warlick E, et al. Similar overall survival using sibling, unrelated donor, and cord blood grafts after reduced-intensity conditioning for older patients with acute myelogenous leukemia. *Biol Blood Marrow Transpl*. 2013;19(9):1355–1360.
120. Ponce DM, Hilden P, Devlin SM, Maloy M, Lubin M, Castro-Malaspina H, et al. High disease-free survival with enhanced protection against relapse after double-unit cord blood transplantation when compared with T cell-depleted unrelated donor transplantation in patients with acute leukemia and chronic myelogenous leukemia. *Biol Blood Marrow Transpl*. 2015;21(11):1985–1993.
121. Mehta RS, Dave H, Bollard CM, Shpall EJ. Engineering cord blood to improve engraftment after cord blood transplant. *Stem Cell Investig*. 2017;4:41.
122. Thompson PA, Rezvani K, Hosing CM, Oran B, Olson AL, Popat UR, et al. Umbilical cord blood graft engineering: challenges and opportunities. *Bone Marrow Transpl*. 2015;50(Suppl 2):S55–S62.
123. Horwitz ME, Wease S, Blackwell B, Valcarcel D, Frassoni F, Boelens JJ, et al. Phase I/II study of stem-cell transplantation using a single cord blood unit expanded ex vivo with nicotinamide. *J Clin Oncol.:* 2019;37(5):367–374.
124. Anand S, Thomas S, Hyslop T, Adcock J, Corbet K, Gasparetto C, et al. Transplantation of ex vivo expanded umbilical cord blood (NiCord) decreases early infection and hospitalization. *Biol Blood Marrow Transpl*. 2017;23(7):1151–1157.
125. Wagner Jr. JE, Brunstein CG, Boitano AE, DeFor TE, McKenna D, Sumstad D, et al. Phase I/II trial of StemRegenin-1 expanded umbilical cord blood hematopoietic stem cells supports testing as a stand-alone graft. *Cell Stem Cell*. 2016;18(1):144–155.
126. Cohen S, Roy J, Lachance S, Delisle JS, Marinier A, Busque L, et al. Hematopoietic stem cell transplantation using single UM171-expanded cord blood: a single-arm, phase 1–2 safety and feasibility study. *Lancet Haematol*. 2020;7(2):e134–e145.
127. Stiff PJ, Montesinos P, Peled T, Landau E, Goudsmid NR, Mandel J, et al. Cohort-controlled comparison of umbilical cord blood transplantation using carlecortemcel-L, a single progenitor-enriched cord blood, to double cord blood unit transplantation. *Biol Blood Marrow Transpl*. 2018;24(7):1463–1470.
128. Cutler C, Multani P, Robbins D, Kim HT, Le T, Hoggatt J, et al. Prostaglandin-modulated umbilical cord blood hematopoietic stem cell transplantation. *Blood*. 2013;122(17):3074–3081.
129. Popat U, Mehta RS, Rezvani K, Fox P, Kondo K, Marin D, et al. Enforced fucosylation of cord blood hematopoietic cells accelerates neutrophil and platelet engraftment after transplantation. *Blood*. 2015;125(19):2885–2892.
130. Parmar S, Liu X, Najjar A, Shah N, Yang H, Yvon E, et al. Ex vivo fucosylation of third-party human regulatory T cells enhances anti-graft-versus-host disease potency in vivo. *Blood*. 2015;125(9):1502–1506.
131. Farag SS, Srivastava S, Messina-Graham S, Schwartz J, Robertson MJ, Abonour R, et al. In vivo DPP-4 inhibition to enhance engraftment of single-unit cord blood transplants in adults with hematological malignancies. *Stem Cell Dev*. 2013;22(7):1007–1015.
132. Farag SS, Nelson R, Cairo MS, O'Leary HA, Zhang S, Huntley C, et al. High-dose sitagliptin for systemic inhibition of dipeptidylpeptidase-4 to enhance engraftment of single cord umbilical cord blood transplantation. *Oncotarget*. 2017;8(66):110350–110357.
133. Kwon M, Bautista G, Balsalobre P, Sanchez-Ortega I, Serrano D, Anguita J, et al. Haplo-cord transplantation using CD34+ cells from a third-party donor to speed engraftment in high-risk patients with hematologic disorders. *Biol Blood Marrow Transpl*. 2014;20(12):2015–2022.
134. Liu H, Rich ES, Godley L, Odenike O, Joseph L, Marino S, et al. Reduced-intensity conditioning with combined haploidentical and cord blood transplantation results in rapid engraftment, low GVHD, and durable remissions. *Blood*. 2011;118(24):6438–6445.
135. Tsai SB, Rhodes J, Liu H, Shore T, Bishop M, Cushing MM, et al. Reduced-intensity allogeneic transplant for acute myeloid leukemia and myelodysplastic syndrome using combined CD34-selected haploidentical graft and a single umbilical cord unit compared with matched unrelated donor stem cells in older adults. *Biol Blood Marrow Transpl*. 2018;24(5):997–1004.
136. Davis ZB, Cooley SA, Cichocki F, Felices M, Wangen R, Luo X, et al. Adaptive natural killer cell and killer cell immunoglobulin-like receptor-expressing T cell responses are induced by cytomegalovirus and are associated with protection against cytomegalovirus reactivation after allogeneic donor hematopoietic cell transplantation. *Biol Blood Marrow Transpl*. 2015;21(9):1653–1662.
137. Cichocki F, Taras E, Chiuppesi F, Wagner JE, Blazar BR, Brunstein C, et al. Adaptive NK cell reconstitution is associated with better clinical outcomes. *JCI Insight*. 2019;4:e125553 in press.
138. Montesinos P, Sanz J, Cantero S, Lorenzo I, Martin G, Saavedra S, et al. Incidence, risk factors, and outcome of cytomegalovirus infection and disease in patients receiving prophylaxis with oral valganciclovir or intravenous ganciclovir after umbilical cord blood transplantation. *Biol Blood Marrow Transpl*. 2009;15(6):730–740.
139. Milano F, Pergam SA, Xie H, Leisenring WM, Gutman JA, Riffkin I, et al. Intensive strategy to prevent CMV disease in seropositive umbilical cord blood transplant recipients. *Blood*. 2011;118(20):5689–5696.
140. Green JS, Shanley RM, Brunstein CG, Young JAH, Verneris MR. Mixed vs full donor engraftment early after hematopoietic cell transplant: Impact on incidence and control of cytomegalovirus infection. *Transpl Infect Dis*. 2019;21(3):e13070.
141. Dahi PB, Perales MA, Devlin SM, Olson A, Lubin M, Gonzales AM, et al. Incidence, nature and mortality of cytomegalovirus infection after double-unit cord blood transplant. *Leukemia & Lymphoma*. 2015;56(6):1799–1805.
142. Sharma P, Gakhar N, MacDonald J, Abidi MZ, Benamu E, Bajrovic V, et al. Letermovir prophylaxis through day 100 post transplant is safe and effective compared with alternative CMV prophylaxis strategies following adult cord blood and haploidentical cord blood transplantation. *Bone Marrow Transpl*. 2020;55(4):780–786.
143. Sanz J, Arango M, Senent L, Jarque I, Montesinos P, Sempere A, et al. EBV-associated post-transplant lymphoproliferative disorder after umbilical cord blood transplantation in adults with hematological diseases. *Bone Marrow Transpl*. 2014;49(3):397–402.
144. Blaes AH, Cao Q, Wagner JE, Young JAH, Weisdorf DJ, Brunstein CG. Monitoring and preemptive rituximab therapy for Epstein-Barr virus reactivation after antithymocyte globulin containing nonmyeloablative conditioning for umbilical cord blood transplantation. *Biol Blood Marrow Transpl*. 2010;16(2):287–291.
145. Van Besien K, Bachier-Rodriguez L, Satlin M, Brown MA, Gergis U, Guarneri D, et al. Prophylactic rituximab prevents EBV PTLD in haplo-cord transplant recipients at high risk. *Leuk Lymphoma*. 2019;60(7):1693–1696.
146. Ogata M, Oshima K, Ikebe T, Takano K, Kanamori H, Kondo T, et al. Clinical characteristics and outcome of human herpesvirus-6 encephalitis after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transpl*. 2017;52(11):1563–1570.
147. Betts BC, Young JA, Ustun C, Cao Q, Weisdorf DJ. Human herpesvirus 6 infection after hematopoietic cell transplantation: is routine surveillance necessary? *Biol Blood Marrow Transpl*. 2011;17(10):1562–1568.
148. Inui Y, Yakushijin K, Okamura A, Tanaka Y, Shinzato I, Nomura T, et al. Human herpesvirus 6 encephalitis in patients administered mycophenolate mofetil as prophylaxis for graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Transpl Infect Dis*. 2019;21(1):e13024.
149. Zerr DM, Corey L, Kim HW, Huang ML, Nguy L, Boeckh M. Clinical outcomes of human herpesvirus 6 reactivation after hematopoietic stem cell transplantation. *Clin Infect Dis*. 2005;40(7):932–940.

150. Isomura H, Yamada M, Yoshida M, Tanaka H, Kitamura T, Oda M, et al. Suppressive effects of human herpesvirus 6 on in vitro colony formation of hematopoietic progenitor cells. *J Med Virol*. 1997;52(4):406–412.
151. Lagadinou ED, Marangos M, Liga M, Panos G, Tzouvara E, Dimitroulia E, et al. Human herpesvirus 6-related pure red cell aplasia, secondary graft failure, and clinical severe immune suppression after allogeneic hematopoietic cell transplantation successfully treated with foscarnet. *Transpl Infect Dis*. 2010;12(5):437–440.
152. Ogata M, Kikuchi H, Satou T, Kawano R, Ikewaki J, Kohno K, et al. Human herpesvirus 6 DNA in plasma after allogeneic stem cell transplantation: incidence and clinical significance. *J Infect Dis*. 2006;193(1):68–79.
153. Ogata M, Satou T, Inoue Y, Takano K, Ikebe T, Ando T, et al. Foscarnet against human herpesvirus (HHV)-6 reactivation after allo-SCT: breakthrough HHV-6 encephalitis following antiviral prophylaxis. *Bone Marrow Transpl*. 2013;48(2):257–264.
154. Ishiyama K, Katagiri T, Hoshino T, Yoshida T, Yamaguchi M, Nakao S. Preemptive therapy of human herpesvirus-6 encephalitis with foscarnet sodium for high-risk patients after hematopoietic SCT. *Bone Marrow Transpl*. 2011;46(6):863–869.
155. Ogata M, Takano K, Moriuchi Y, Kondo T, Ueki T, Nakano N, et al. Effects of prophylactic foscarnet on human herpesvirus-6 reactivation and encephalitis in cord blood transplant recipients: a prospective, multicenter trial with an historical control group. *Biol Blood Marrow Transpl*. 2018;24(6):1264–1273.
156. Yasu T, Konuma T, Oiwa-Monna M, Mizusawa M, Isobe M, Kato S, et al. Efficacy and safety of micafungin in unrelated cord blood transplant recipients. *Ann Hematol*. 2019;98(11):2593–2600.
157. Rosillo C, Avila AM, Huang YT, Devlin S, Cho C, Montoro J, et al. Sequential systematic anti-mold prophylaxis with micafungin and voriconazole results in very low incidence of invasive mold infections in patients undergoing allogeneic hematopoietic stem cell transplantation. *Transpl Infect Dis*. 2018;20(4):e12897.
158. Solh M, Brunstein C, Morgan S, Weisdorf D. Platelet and red blood cell utilization and transfusion independence in umbilical cord blood and allogeneic peripheral blood hematopoietic cell transplants. *Biol Blood Marrow Transpl*. 2011;17(5):710–716.
159. Christakopoulos GE, DeFor TE, Hage S, M, et al. Romiplostim improves platelet recovery after UCB transplant. *Blood*. 2019;134(Supplement_1):1979.
160. Mahat U, Rotz SJ, Hanna R. Use of thrombopoietin receptor agonists in prolonged thrombocytopenia after hematopoietic stem cell transplantation. *Biol Blood Marrow Transpl*. 2020;26(3):e65–e73.
161. Atsuta Y, Suzuki R, Nagamura-Inoue T, Taniguchi S, Takahashi S, Kai S, et al. Disease-specific analyses of unrelated cord blood transplantation compared with unrelated bone marrow transplantation in adult patients with acute leukemia. *Blood*. 2009;113(8):1631–1638.
162. Atsuta Y, Morishima Y, Suzuki R, Nagamura-Inoue T, Taniguchi S, Takahashi S, et al. Comparison of unrelated cord blood transplantation and HLA-mismatched unrelated bone marrow transplantation for adults with leukemia. *Biol Blood Marrow Transpl*. 2012;18(5):780–787.
163. El-Cheikh J, Crocchiolo R, Furst S, Bramanti S, Sarina B, Granata A, et al. Unrelated cord blood compared with haploidentical grafts in patients with hematological malignancies. *Cancer*. 2015;121(11):1809–1816.
164. Robin M, Ruggeri A, Labopin M, Niederwieser D, Tabrizi R, Sanz G, et al. Comparison of unrelated cord blood and peripheral blood stem cell transplantation in adults with myelodysplastic syndrome after reduced-intensity conditioning regimen: a collaborative study from Eurocord (Cord blood Committee of Cellular Therapy & Immunobiology Working Party of EBMT) and Chronic Malignancies Working Party. *Biol Blood Marrow Transpl*. 2015;21(3):489–495.
165. Ruggeri A, Labopin M, Sanz G, Piemontese S, Arcese W, Bacigalupo A, et al. Comparison of outcomes after unrelated cord blood and unmanipulated haploidentical stem cell transplantation in adults with acute leukemia. *Leukemia*. 2015;29(9):1891–1900.
166. Giannotti F, Labopin M, Shouval R, Sanz J, Arcese W, Angelucci E, et al. Haploidentical transplantation is associated with better overall survival when compared to single cord blood transplantation: an EBMT-Eurocord study of acute leukemia patients conditioned with thiotepa, busulfan, and fludarabine. *J Hematol Oncol*. 2018;11(1):110.
167. Baron F, Labopin M, Ruggeri A, et al. Umbilical cord blood versus unrelated donor transplantation in adults with primary refractory or relapsed acute myeloid leukemia: a report from Eurocord, the Acute Leukemia Working Party and the Cord Blood Committee of the Cellular Therapy and Immunobiology Working Party of the EBMT. Blood. *Cancer J*. 2019;9(4):46.
168. Mehta RS, Holtan SG, Wang T, et al. Composite GRFS and CRFS outcomes after adult alternative donor HCT. *J Clin Oncol: Off J Am Soc Clin Oncology*. 2020;38(18):2062–2076.

CHAPTER 109

GRAFT-VERSUS-HOST DISEASE AND GRAFT-VERSUS-LEUKEMIA RESPONSES

Mary Riwes, James L. Ferrara, Pavan Reddy, and John M. Magenau

The ability of allogeneic hematopoietic cell transplantation (HCT) to cure certain hematologic malignancies is widely recognized. An important therapeutic aspect of HCT in eradicating malignant cells is the graft-versus-leukemia (GVL) effect. The importance of the GVL effect in allogeneic HCT has been recognized since the earliest experiments in stem cell transplantation. Forty years ago, Barnes and colleagues noted that leukemic mice treated with a subtherapeutic dose of radiation and a syngeneic (identical twin) graft transplant were more likely to relapse than mice given an allogeneic stem cell transplant. They hypothesized that the allogeneic graft contained cells with immune reactivity necessary for eradicating residual leukemia cells. They also noted that recipients of allogeneic grafts, although less likely to relapse, died of a “wasting syndrome” now recognized as graft-versus-host disease (GVHD). Thus, in addition to describing GVL, these experiments highlighted for the first time the intricate relationship between GVL and GVHD. Since these early experiments, both GVHD and the GVL effect have been studied extensively. This chapter reviews the pathophysiology, clinical features, and treatment of GVHD and summarizes current understanding of the relationships between GVHD and the GVL effect.

GRAFT-VERSUS-HOST DISEASE: CLINICAL AND PATHOLOGIC ASPECTS

Ten years after the work of Barnes and Loutit, Billingham formulated the requirements for the development of GVHD: the graft must contain immunologically competent cells, the recipient must express tissue antigens that are not present in the transplant donor, and the recipient must be incapable of mounting an effective response to destroy the transplanted cells. According to these criteria, GVHD can develop in various clinical settings when tissues containing immunocompetent cells (blood products, bone marrow, and some solid organs) are transferred between persons. The most common setting for the development of GVHD is following allogeneic HCT; without prophylactic immunosuppression, most allogeneic HCTs will be complicated by GVHD. GVHD is induced by mismatches between histocompatibility antigens between the donor and recipient. Matching of the major histocompatibility complex (MHC) antigens hastens engraftment and reduces the severity of GVHD. In humans, the MHC region lies on the short arm of chromosome 6 and is called the human leukocyte antigen (HLA) region. The HLA region is divided into two classes, class I and class II, each containing numerous gene loci that encode a large number of polymorphic alleles. MHC class I molecules are involved in the presentation of peptides to $CD8^+$ T cells, and class II molecules present peptides to $CD4^+$ T cells.

The determination of HLA types has become much more accurate with molecular techniques that replace earlier serologic or cellular methods. The average patient has a 25% chance of having an HLA match within his or her immediate family. Patients who lack an HLA-identical family member donor must seek an unrelated haploidentical or cord blood donor. In patients whose ancestry involves extensive interracial mixing, the chances of identifying an HLA identical donor are diminished. Using a population-based genetic model, it is estimated that 75% of Whites of European decent will have a high-resolution matched unrelated donor at the key major class 1 and class II loci; however, patients of African or South Asian ancestry have a less than 30% chance of identifying a similar pairing. Advances in the clinical application of haploidentical (partial HLA match) HCT have now expanded alternate options for ethnic groups disproportionately affected by limited access to unrelated donors.[1]

Despite HLA identity between a patient and donor, substantial numbers of patients still develop GVHD because of differences in minor histocompatibility antigens (MiHAs) that lie outside the HLA loci. Genome-wide arrays demonstrate that single nucleotide polymorphisms (SNPs), reflecting amino-acid coding differences between donor-recipient pairs, are themselves sufficient for producing clinical GVHD. For example, each 1% difference in SNPs resulted in 20% relative risk of developing grade III to IV GVHD.[2] Nonetheless, the identity of the relevant minor antigens that comprise differences in peptides that are expressed on the cell surface bound to specific HLA molecules has yet to be precisely elucidated.

Acute Graft-Versus-Host Disease

Acute GVHD typically occurs within 30 days of transplantation (in HLA-matched recipients receiving standard GVHD prophylaxis) but occurs in diminishing frequencies for up to 6 months.[3,4] The incidence ranges from less than 10% to more than 80%, depending on the degree of histoincompatibility between donor and recipient, the number of T cells in the graft, the patient’s age, and the GVHD prophylactic regimen. The principal target organs include the immune system, skin, liver, and intestine. GVHD occurs first and most commonly in the skin as a pruritic maculopapular rash, often involving the palms, soles, and ears; it can progress to total-body erythroderma, with bullae formation, rupture along the epidermal-dermal border, and desquamation in severe cases. Gastrointestinal (GI) and liver manifestations often appear later and rarely represent the first and only findings. Intestinal symptoms include anorexia, nausea, diarrhea (sometimes bloody), abdominal pain, and paralytic ileus. Liver dysfunction includes hyperbilirubinemia and increased serum alkaline phosphatase and aminotransferase values. Coagulation studies may become abnormal, and hepatic failure with ascites and encephalopathy may develop in severe cases. Hepatic GVHD can be distinguished from hepatic venoocclusive disease (VOD) by weight gain or pain in the right upper quadrant in the latter. Acute GVHD also results in the delayed recovery of immunocompetence. The clinical result is profound immunodeficiency and susceptibility to infections, often further accentuated by the immunosuppressive agents used to treat GVHD.

Pathologically, the sine qua non of acute GVHD is selective epithelial damage of target organs. The epidermis and hair follicles are damaged and sometimes destroyed. Small bile ducts are profoundly affected, with segmental disruption. The destruction of intestinal crypts results in mucosal ulcerations that may be either patchy or diffuse. Other epithelial surfaces, such as the conjunctivae, vagina, and esophagus, are less commonly involved. A peculiarity of GVHD histology is the early paucity of mononuclear cell infiltrates; however, as the disease progresses, the inflammatory component may be

sevier on April
ıghts reserved.

substantial. Studies that identified inflammatory cytokines as soluble mediators of GVHD have suggested that direct contact between target cells and lymphocytes is not always required (see following sections). GVHD lesions are not evenly distributed: in the skin, damage is prominent at the tip of rete ridges; in the intestine, at the base of the crypts; and in the liver, in the periductular epithelium. These areas all contain a high proportion of stem cells, giving rise to the idea that GVHD targets may be undifferentiated epithelial cells with primitive surface antigens.

The histologic severity of GVHD is at best semiquantitative in part due to inability to sample tissues in the small intestine, and consequently pathologic scores are not used to grade GVHD. Because it is often difficult to obtain an adequate tissue biopsy, and because it can be very difficult to distinguish GVHD from other post-HCT complications such as drug eruptions or infectious complications, the physician is left to use clinical judgment.

Standard grading systems generally include clinical changes in the skin, GI tract, liver, and performance status (Table 109.1). Although the severity of GVHD is often difficult to quantify, the overall maximal grade correlates with disease outcome: mild GVHD (grade I or II) is associated with little mortality, whereas higher grades are associated with significantly decreased survival. Refined staging methods that standardize diagnosis and clinical grading (Mount Sinai Acute GVHD International Consortium [MAGIC]) are becoming more widely adopted in clinical practice and research.[5] However, consistent across all clinical staging systems is the association between high-grade organ involvement (grade III to IV) or disease affecting multiple organs and increased mortality. Once severe GVHD is established, the likelihood of responding to treatment is less than 50% and patients are at twice the risk of succumbing to GVHD or its sequela than mild to moderate forms of disease.[6] Although GVHD remains a clinical diagnosis, recent advances in the use of biomarkers may improve diagnostic sensitivity or provide early prognostic information to inform primary therapy.

Clinical Features of Acute Graft-Versus-Host Disease

The clinical features, staging, and grading of acute GVHD are summarized in Tables 109.1 and 109.2. In a comprehensive review of patients receiving therapy for acute GVHD, Martin and colleagues found that 81% had skin involvement, 54% had GI involvement, and 50% had liver involvement at the initiation of therapy. After high-intensity (myeloablative) conditioning, acute GVHD generally occurs within 14 to 35 days of stem cell infusion. A rapid and severe form of GVHD may occur in patients with severe HLA mismatches and in patients who receive T cell–replete transplants without or with inadequate in vivo GVHD prophylaxis. Although such GVHD is sometimes called “hyperacute,” this term is misleading because it is pathophysiologically distinct from hyperacute rejection after solid organ allografting, which is due to preformed antibodies. This form of GVHD, which is manifested by fever, generalized erythroderma and desquamation, and often edema, typically occurs about 1 week after stem cell infusion and may be rapidly fatal. In patients receiving standard (in vivo) GVHD prophylaxis such as a combination of calcineurin inhibitors, such as cyclosporine (CSP) or tacrolimus, and methotrexate, the median onset of GVHD is typically 21 to 25 days after transplantation; however, after in vitro T-cell depletion of the graft, the onset of GVHD symptoms may be much later. Thus the findings of rash and diarrhea by 1 week after transplantation would very likely be due to ineffective prophylaxis and would be very unlikely with the use of calcineurin inhibitors or in vitro T-cell depletion of the stem cell inoculum. A less ominous syndrome of fever, rash, and fluid retention occurring in the first 1 to 2 weeks after stem cell infusion is the “engraftment syndrome.” These manifestations may be seen with either allogeneic or autologous transplantation. Although this syndrome’s pathophysiology is poorly understood, it is thought to be due to a wave of cytokine production as the graft starts to recover. These symptoms are related to, but distinct from, the “cytokine storm” of acute GVHD because there is no concomitant T cell–mediated attack. This syndrome responds immediately to steroids in most patients, and it typically presents earlier than acute GVHD.

TABLE 109.1 Clinical Manifestations and Staging of Acute Graft-Versus-Host Disease

Organ	Clinical Manifestations	Staging
Skin	Erythematous, maculopapular rash involving palms and soles; may become confluent Severe disease: bullae	Stage 1: <25% rash Stage 2: 25%–50% rash Stage 3: generalized erythroderma Stage 4: bullae
Liver	Painless jaundice with conjugated hyperbilirubinemia and increased alkaline phosphatase	Stage 1: bili 2–3 mg/dL Stage 2: bili 3.1–6 mg/dL Stage 3: bili 6.1–15 mg/dL Stage 4: bili >15 mg/dL
Gastrointestinal tract	Upper: nausea, vomiting, anorexia Lower: diarrhea, abdominal cramps, distension, ileus, bleeding	Stage 1: diarrhea > 500 mL/day Stage 2: diarrhea > 1000 mL/day Stage 3: diarrhea > 1500 mL/day Stage 4: ileus, bleeding

Skin is the most commonly affected organ (Fig. 109.1). In patients receiving transplants after myeloablative conditioning, the skin is usually the first organ involved, and GVHD often coincides with engraftment. However, the presentation of GVHD is more varied following nonmyeloablative transplants or donor lymphocyte infusions. The characteristic maculopapular rash can spread throughout the rest of the body but usually spares the scalp; it is often described as feeling like a sunburn, tight or pruritic. In severe cases the skin may blister and ulcerate. Histologic confirmation is critical to rule out drug reactions, viral infections, etc. but is optimally performed 1 to 2 days after the onset of rash in cases of patchy involvement. Pathologic findings that describe apoptosis at the base of dermal crypts is characteristic. Other features include dyskeratosis, exocytosis of lymphocytes, satellite lymphocytes adjacent to dyskeratotic epidermal keratinocytes, and dermal perivascular lymphocytic infiltration.

GI tract involvement of GVHD may present as nausea, vomiting, anorexia, diarrhea, and/or abdominal pain. It is a panintestinal process, often with differences in severity between the upper and lower GI tracts. Although gastroparesis is seen after bone marrow transplant, it is usually not associated with GVHD. The diarrhea of GVHD is secretory; in extreme cases significant GI blood loss may occur as a result of mucosal ulceration and is associated with a poor

TABLE 109.2 Glucksberg Criteria for Staging of Acute Graft-Versus-Host Disease[a]

Overall Grade	Skin	Liver		Gut
I	1–2	0		0
II	1–3	1	and/or	1
III	2–3	2–4	and/or	2–3
IV	2–4	2–4	and/or	2–4

[a] See Table 109.1 for individual organ staging. Traditionally, individual organs are staged without regard to attribution. However, the overall grade of graft-versus-host disease (GVHD) reflects the actual extent of GVHD. To achieve each overall grade, skin disease plus liver and/or gut involvement are required.

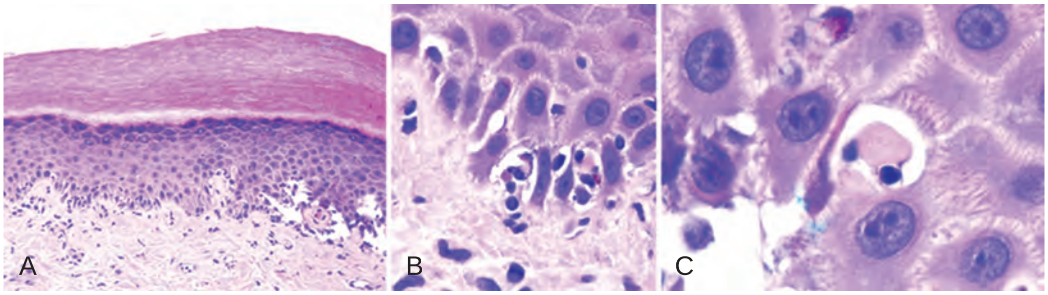


Figure 109.1 GRAFT-VERSUS-HOST DISEASE, SKIN BIOPSY. This 40-year-old man with a history of relapsed Hodgkin lymphoma was status postallogeneic stem cell transplant with donor lymphocyte infusion. He developed painful oral ulcers and a macular-papular rash on the arms, hand, and chest. The skin biopsy is from the palmar surface of the hand (A). It shows a scant lymphoid infiltrate in the dermis with a developing subepithelial blister *(right)*. There is basal vacuolar change (B) with single lymphocytes in the epithelium, as well as apoptotic keratinocyte accompanied by lymphocytes (B, and detail, C). *(Courtesy Vesna Petronic-Rosic and Mark Racz, University of Chicago.)*

prognosis. In advanced disease, diffuse, severe abdominal pain and distension is accompanied by voluminous diarrhea (>2 L/day).

Radiologic findings of the GI tract include luminal dilatation with thickening of the wall of the small bowel and air/fluid levels suggestive of an ileus on abdominal flat plates or small bowel series. Abdominal computed tomography may show the "ribbon" sign of diffuse thickening of the small bowel wall. Little correlation exists between the extent of disease and the appearance of mucosa on endoscopy, but mucosal sloughing is pathognomonic for severe disease. Nevertheless, some studies have shown that antral biopsies correlate well with the severity of GVHD in the duodenum and in the colon even when the presenting symptom is diarrhea. Histologic analysis of tissue is imperative to establish the diagnosis. The histologic features of GI GVHD are the presence of apoptotic bodies in the base of crypts, crypt abscesses, crypt loss, and flattening of the surface epithelium.

Liver function test abnormalities are common after bone marrow transplant and occur secondary to VOD, drug toxicity, viral infection, sepsis, iron overload, and other causes of extrahepatic biliary obstruction. The exact incidence of hepatic GVHD is unknown because many patients do not undergo liver biopsies. The development of jaundice or an increase in the alkaline phosphatase and bilirubin may be the initial features of acute GVHD of the liver. The histologic features of hepatic GVHD are endothelialitis, lymphocytic infiltration of the portal areas, pericholangitis, and bile duct destruction and loss.

Other Organs

Whether GVHD affects organs other than the classic triad of skin, liver, and gut has remained a matter of debate, although numerous reports suggest additional organ manifestations. The most likely candidate is the lung. Lung toxicity, including interstitial pneumonitis and diffuse alveolar hemorrhage, may occur in 20% to 60% of allogeneic transplant recipients but in fewer autologous transplant recipients. Causes of pulmonary damage other than GVHD include engraftment syndrome (see earlier), infection, radiation pneumonitis, and chemotherapy-related toxicity (e.g., methotrexate, busulfan). One retrospective analysis failed to link severe pulmonary complications to clinical acute GVHD per se. The mortality due to pneumonia increases with the severity of GVHD, but this association may be related to increased immunosuppressive therapy.

Despite the ability of kidneys and hearts to serve as targets of transplant rejection, there is no convincing evidence for direct renal or cardiac damage from acute GVHD that is not secondary to drugs or infection. Similarly, neurologic complications are also common after transplantation but most can be attributed to drug toxicity, infection, or vascular insults.

Differential Diagnosis

Acute GVHD ought to be distinguished from any process that causes a constellation of fever, erythematous skin rash, and pulmonary edema that may occur during neutrophil recovery and has been termed engraftment or capillary leak syndrome. In allogeneic transplant recipients, distinction from acute GVHD is difficult. Engraftment syndrome is thought to reflect cellular and cytokine activities during early recovery of (donor-derived) blood cell counts and/or homeostatic proliferation of lymphocytes, but a precise delineation of the activated cells and mechanisms has not been demonstrated. Engraftment syndrome may be associated with increased mortality, primarily but not exclusively from pulmonary failure. Corticosteroid therapy may be effective particularly for the treatment of pulmonary manifestations. Skin rashes may reflect delayed reactions to the conditioning regimen, antibiotics, or infections; furthermore, histopathologic skin changes consistent with acute GVHD can be mimicked by chemoradiotherapy and drug reactions. Diarrhea can be a consequence of total body irradiation (TBI), viral infection (especially with cytomegalovirus [CMV] and other herpes viruses), parasitic infection, *Clostridioides difficile* infection, nonspecific gastritis, narcotic withdrawal, and drug reactions—all of which mimic GVHD of the gut. Liver dysfunction can be due to parenteral nutrition, VOD, and viral- or drug-induced hepatitis. Thus establishing a diagnosis of GVHD requires consideration of the timing of events in relation to HCT conditioning as well as exclusion of relevant infectious etiologies in HCT patients.

Genetic Basis of Graft-Versus-Host Disease

The graft-versus-host (GVH) reaction was first noted when irradiated mice were infused with allogeneic marrow and spleen cells. Although mice recovered from radiation-induced injury and marrow aplasia, they subsequently died with "secondary disease," a phenomenon subsequently recognized as acute GVHD. Three requirements for the development of GVHD were formulated by Billingham. First, the graft must contain immunologically competent cells, now recognized as mature T cells. In both experimental and clinical allogeneic HCT, the severity of GVHD correlates with the number of donor T cells transfused. The precise nature of these cells and the mechanisms they use are now understood in greater detail (see later). Second, the recipient must be incapable of rejecting the transplanted cells (i.e., immunocompromised). After allogeneic HCT, the recipient is typically immunosuppressed by chemotherapy and/or radiotherapy before the hematopoietic cell infusion. Third, the recipient must express tissue antigens that are not present in the transplant donor. Thus Billingham's third postulate stipulates that the GVH reaction occurs when donor immune cells recognize disparate host antigens. These differences are governed by the genetic polymorphisms.

Human Leukocyte Antigen Matching

Recognition of alloantigens depends on the match with the presenting major histocompatibility molecule. In humans, the MHC is governed by the HLA antigens that are encoded by the MHC gene complex on the short arm of chromosome 6 and can be categorized as class I, II, and III. Class I antigens (HLA-A, B, and C) are expressed on almost all cells of the body. Class II antigens (DR, DQ, and DP) are primarily expressed on hematopoietic cells, although their expression can also be induced on other cell types following inflammation. The incidence of acute GVHD is directly related to the degree of MHC mismatch. For example, recipients of single loci mismatched unrelated donors have higher rates of grade II to IV GVHD, higher transplant-related mortality, and inferior survival compared with 8/8 HLA-matched unrelated donors.[7,8] In the context of 8/8 HLA matching, mismatching that was nonpermissive (based on T-cell epitope grouping) was associated with increased mortality compared with permissive mismatches.[9] In alternative donor HCT the degree of HLA disparities also correlates with GVHD risk; however, there appears to be greater permissiveness as compared with unrelated donor transplant. However, it should be acknowledged that direct comparisons between various donor source are challenging because conditioning regimens and GVHD prevention platforms may differ significantly.

Minor Histocompatibility Antigens

In most clinical allogeneic transplants where the MHC of donor and recipient are matched, donor T cells recognize MHC-bound peptides derived from the protein products of polymorphic genes (MiHAs) that are present in the host but not in the donor. Substantial numbers (50%) of patients will develop acute GVHD despite receiving HLA-identical grafts as well as optimal postgrafting immune suppression. MiHAs are widely but variably expressed in different tissue, which is one possible explanation for the unique target organ distribution in GVHD. Many MiHAs such as HA-1 and HA-2 are expressed on hematopoietic cells, which may be one reason for the host immune system to be a primary target for the GVH response, and help to explain the critical role of direct presentation by professional recipient antigen-presenting cells (APCs) in the GVH response. By contrast, other MiHAs such as H-Y and HA-3 are expressed ubiquitously. MiHAs do not all equally induce lethal GVHD but show hierarchic immunodominance. Furthermore, the difference in a single immunodominant MiHA is insufficient to elicit GVHD in murine models, even though a single MiHA can elicit T cell–mediated damage in a skin explant model. However, the role of specific MiHAs that are able to induce clinical GVHD has not been systematically evaluated in large groups of patients.

Other Non-HLA Genes

Genetic polymorphisms in several non-HLA genes such as in killer-cell immunoglobulin (Ig)-like receptors (KIRs), cytokines, and nucleotide-binding oligomerization domain containing 2 *(NOD2)* genes have been shown to modulate the severity and incidence of GVHD.

KIRs on natural killer (NK) cells that bind to the HLA class I gene products are encoded on chromosome 19. Polymorphisms in the transmembrane and cytoplasmic domains of KIRs govern whether the receptor has inhibitory (e.g., KIR2DL1, -2DL2, -2DL3, and -3DL1) or activating potential. Two competing models have been proposed for HLA-KIR allorecognition by donor NK cells following allogeneic HCT: the “mismatched ligand” and the “missing ligand” models. Both models are supported by several clinical observations, albeit in patients receiving very different transplant and immunosuppressive regimens (see Chapters 21 and 107).

Proinflammatory cytokines involved in the classic cytokine storm of GVHD cause pathologic damage to target organs, such as the skin, liver, and GI tract (see later). Several cytokine gene polymorphisms in both recipients and donors have been implicated. Specifically, tumor necrosis factor (TNF) polymorphisms (TNFd3/d3 in the recipient, TNF863 and TNF857 in donors and/or recipients, and TNFd4, TNF-α-1031C, and TNFRII-196R in the donors) have been associated with an increased risk for acute GVHD and transplant-related mortality (TRM). The three common haplotypes of the interleukin (IL)-10 gene promoter region in recipients, representing high, intermediate, and low production of IL-10, have been associated with severity of acute GVHD following HLA-matched sibling donor allogeneic HCT. By contrast, smaller studies have found neither IL-10 nor TNF-α polymorphisms to be associated with GVHD following HLA-mismatched cord blood transplant (CBT). Interferon-γ (IFN-γ) polymorphisms of the 2/2 genotype (high IFN-γ production) and 3/3 genotype (low IFN-γ) have been associated with decreased or increased acute GVHD, respectively.

NOD2/caspase-activating recruitment domain 15 *(CARD15)* gene polymorphisms in both the donors and recipients were recently shown to have a striking association between GI GVHD and overall mortality following related and unrelated donor allogeneic HCT. It is likely that the importance of non-HLA gene polymorphisms in GVHD will differ depending on the donor source (related vs. unrelated), HLA disparity (matched vs. mismatched), graft source (cord blood vs. bone marrow vs. peripheral blood stem cells), and the intensity of the conditioning.

PATHOPHYSIOLOGY OF ACUTE GRAFT-VERSUS-HOST DISEASE

It is helpful to remember two important principles when considering the pathophysiology of acute GVHD. First, acute GVHD represents exaggerated but normal inflammatory responses against foreign antigens (alloantigens) that are ubiquitously expressed in a setting where they are undesirable. The donor lymphocytes that have been infused into the recipient function appropriately, given the foreign environment they encounter. Second, donor lymphocytes encounter tissues in the recipient that have been often profoundly damaged. The effects of the underlying disease, prior infections, and the intensity of conditioning regimen all result in substantial changes not only in the immune cells but also in the endothelial and epithelial cells. Thus the allogeneic donor cells rapidly encounter not simply a foreign environment, but one that has been altered to promote the activation and proliferation of inflammatory cells. Therefore the pathophysiology of acute GVHD may be considered a distortion of the normal inflammatory cellular responses that, in addition to the absolute requirement of donor T cells, involves multiple other innate and adaptive cells and mediators. The development and evolution of acute GVHD can be conceptualized in three sequential phases (Fig. 109.2) to provide a unified perspective on the complex cellular interactions and inflammatory cascades that lead to acute GVHD: (1) activation of the APCs; (2) donor T-cell activation, differentiation, and migration; and (3) effector phase. It is important to note that this three-phase description permits a unified perspective on GVHD biology, but it is not meant to suggest that all three phases are of equal importance or that GVHD occurs in a stepwise and sequential manner. The spatiotemporal relationships among these biologic processes, depending on the context, are likely to of vary, and their relevance to the induction, severity, and maintenance of GVHD may depend on the factors cited earlier.

Phase 1: Host Tissue Injury and Activation of Antigen-Presenting Cells

The earliest phase of acute GVHD is initiated by the profound damage caused by the underlying disease and infections and further exacerbated by BMT conditioning regimens (which include TBI and chemotherapy) that are administered even before the infusion of donor cells. This first step results in activation of the APCs. Specifically, secretion of proinflammatory cytokines from APCs, such as TNF-α and IL-1, described as the “cytokine storm.” More recently, HCT models have revealed an emerging role for damage-associated molecular patterns (DAMPs), released from injured host tissues, and pathogen-associated molecular patterns (PAMPs), from microbes, in amplifying inflammation and GVHD. These “danger signals” engage TLRs and other pattern recognition receptors to activate host APCs. Damage to the GI tract from the conditioning is particularly important in this process because it allows for systemic translocation of immunostimulatory PAMPs such as lipopolysaccharide (LPS) that further enhance the activation of host APCs, and the secondary lymphoid tissue in the GI tract is likely the initial site of interaction between activated APCs and donor T cells. This scenario accords with the observation that an increased risk for GVHD is

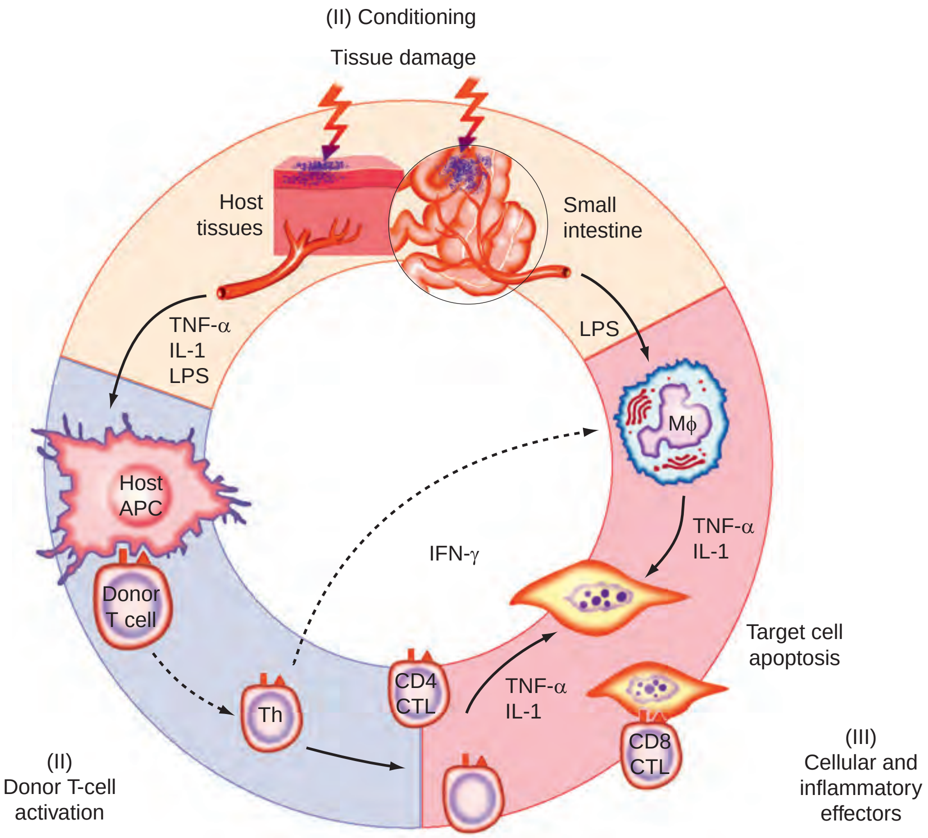


Figure 109.2 PATHOPHYSIOLOGY OF GRAFT-VERSUS-HOST DISEASE. During step 1, irradiation and chemotherapy both damage and activate host tissues, including intestinal mucosa, liver, and the skin. Activated cell hosts then secrete inflammatory cytokines (e.g., tumor necrosis factor [TNF]-α and interleukin [IL]-1), which can be measured in the systemic circulation. The cytokine release has important effects on antigen-presenting cells (APCs) of the host, including increased expression of adhesion molecules (e.g., intracellular adhesion molecule-1, vascular cell adhesion molecule-1) and of major histocompatibility (MHC) class II antigens. These changes in the APCs enhance the recognition of host MHC and/or minor H antigens by mature donor T cells. During step 2, donor T-cell activation is characterized by proliferation of GVHD T cells and secretion of the Th1 cytokines IL-2 and interferon (IFN)-γ. Both of these cytokines play central roles in clonal T-cell expansion, induction of cytotoxic T lymphocyte (CTL) and natural killer (NK) cell responses, and the priming of mononuclear phagocytes. In step 3, mononuclear phagocytes primed by IFN-γ are triggered by a second signal such as endotoxin (lipopolysaccharide [LPS]) to secrete cytopathic amounts of IL-I and TNF-α. LPS can leak through the intestinal mucosa damaged by the conditioning regimen to stimulate gut-associated lymphoid tissue or Kupffer cells in the liver; LPS that penetrates the epidermis may stimulate keratinocytes, dermal fibroblasts, and macrophages to produce similar cytokines in the skin. This mechanism results in the amplification of local tissue injury and further production of inflammatory effectors such as nitric oxide, which, together with CTL and NK effectors, leads to the observed target tissue destruction in the stem cell transplant host. CTL effectors use Fas/Fas ligand, perforin/granzyme B, and membrane-bound cytokines to lyse target cells.

associated with intensive conditioning regimens that cause extensive injury to epithelial and endothelial surfaces with a subsequent release of inflammatory cytokines and increases in expression of cell surface adhesion molecules. The relationship among conditioning intensity, inflammatory cytokine, and GVHD severity has been supported by elegant murine studies. Furthermore, the observations from these experimental studies have led to two recent clinical innovations to reduce clinical acute GVHD: (1) reduced intensity conditioning to decrease the damage to host tissues and thus limit activation of host APC and (2) KIR mismatches between donor and recipients to eliminate the host APCs by the alloreactive NK cells.

Host-type APCs that are present and have been primed by conditioning are critical for the induction of this phase; recent evidence suggests that donor-type APCs exacerbate GVHD, but in certain experimental models, donor-type APC chimeras also induce GVHD. In clinical situations, if donor-type APCs are present in sufficient quantity and have been appropriately primed, they too might play a role in the initiation and exacerbation of GVHD. Among the cells with antigen-presenting capability, dendritic cells (DCs) are the most potent and play an important role in the induction of GVHD. Experimental data suggest that GVHD can be regulated by qualitatively or quantitatively modulating distinct DC subsets. Langerhans cells were also shown to be sufficient for the induction of GVHD when all other APCs were unable to prime donor T cells, although the role for Langerhans cells when all APCs are intact is dispensable. Studies have yet to define roles for other DC subsets. In one clinical study, persistence of host DC after day 100 correlated with the severity of acute GVHD, whereas elimination of host DCs was associated with reduced severity of acute GVHD. The allostimulatory capacity of mature monocyte-derived DCs (mDCs) after reduced intensity transplants was lower for up to 6 months compared with the mDCs from myeloablative transplant recipients, thus suggesting a role for host DCs and the reduction in danger signals secondary to less intense conditioning in acute GVHD. Nonetheless this concept of enhanced host APC activation explains a number of clinical observations such as increased risks for acute GVHD associated with advanced-stage malignancy, conditioning intensity, and histories of viral infections.

Other professional APCs such as monocytes/macrophages or semiprofessional APCs might also play a role in this phase. For example, recent data suggest that host-type B cells might play a regulatory role under certain contexts, whereas in other contexts, they were dispensable for GVHD induction. Similarly, host basophils have been shown not to affect the induction or severity of acute GVHD. Recent data suggest that radiosensitive hematopoietic-derived APCs may not be obligatory for induction of APCs. In addition, host- or donor-type nonhematopoietic stem cells (e.g., mesenchymal stem cells, endothelial cells, epithelial cells, or stromal cells) can function as APCs in the context of inflammation. The role of these cells in the presence and absence of professional hematopoietic-derived APCs remains to be elucidated.

Host Tissue Injury and the Microbiome

GI tract cells such as intestinal epithelial cells (IECs), intestinal stem cells (ISCs), and Paneth cells are targeted in GI GVHD. GI tract damage from conditioning along with subsequent disruptions in intestinal homeostasis, which are further compounded by the use of broad-spectrum antibiotics and alterations in nutrition that occur during the process of allogeneic HCT, are now understood to play important roles in augmenting GVHD.

Maintenance of intestinal homeostasis is in part regulated by the intestinal microbiota and its interactions with the host immune system.[10] The host immune system has been shown to modulate the intestinal microbiota and its disturbances result in intestinal microbiota dysbiosis. For example, retinoic acid receptor–related orphan receptor-γt (RORγt)$^+$ group 3 innate lymphoid cells (ILC3s) regulate intestinal bacteria by limiting local and systemic inflammation, and the absence of intestinal immune cells, particularly isolated lymphoid follicles (ILFs), has been shown to result in alteration of the intestinal microbiota of mice by over representation of gram-negative bacteria. The intestinal microbiota also modulates host immune responses. Germ-free mice developed an increase in IFN-γ after colonization with *Escherichia coli*. In another study, germ-free mice colonized with conventional intestinal microbiota developed structural changes in the depth of the intestinal epithelial crypts as well as expansion in the lamina propria with an increase in immune cells after exposure to intestinal bacteria. Multiple studies have linked the intestinal microbiota to regulation of T cells. In one study, it was shown that colonization of mice with segmented filamentous bacteria (SFB) induces CD4$^+$ T helper cells in the lamina propria. In other studies, several intestinal microbes have been shown to induce forkhead box protein (Foxp3)-expressing regulatory T cells (Tregs). Furthermore, interactions between the intestinal microbiota and the host promote the functions of nonimmune cells such as IECs and goblet cells which play important roles in regulating the barrier function of the GI tract. Thus emerging data now strongly point to intestinal microbiota as playing an important role in intestinal homeostasis and augmenting GVHD.

Microbial Changes in Acute Graft-Versus-Host Disease

After allogeneic HCT there is loss of microbial diversity with a shift towards *Enterococci* and fewer obligate anaerobic bacteria, such as *Frimicutes*. This change can be pronounced with routine clinical use of antibiotics and the development of GVHD. Acute GVHD itself has been associated with major fluctuations in the intestinal microbiota, including a general loss of diversity and expansion of *Enterobacteriales* (including *Escherichia*, *Klebsiella*, and *Enterobacter*), *Lactobacillales* (including *Lactobacillus*, *Enterococcus*, and *Streptococcus*), *Proteobacteria*, and *Akkermansia*. This expansion is accompanied with loss of beneficial microbes including *Firmicutes* (including *Clostridia* and *Blautia*).[11] Accompanying these shifts is Paneth cell loss in experimental GVHD that results in decreased secretion of α-defensins, leading to further intestinal dysbiosis, and that loss of Paneth cells and dysbiosis were associated with nonrelapse mortality (NRM) in clinical GVHD.[12] Several studies have noted that expansion of specific pathogenic bacteria (VRE, *Enterococcus*) often preceded bloodstream infections by the same organisms and identified treatment with certain antibiotics with anaerobic coverage such as metronidazole as a risk factors for bacterial expansion. The intestinal microbiota can also modulate acute GVHD severity. In the 1970s, investigators paradoxically found less GVHD in mice transplanted in germ-free conditions or receiving gut decontamination antibiotics. These studies have not been confirmed in modern day germ-free mice facilities. However, early clinical studies also showed similar beneficial effects of bacterial decontamination in allogeneic HCT patients, although subsequent studies did not replicate these benefits. More recent research made major strides in investigating the specific alterations in the composition of the intestinal microbiota and their association with acute GVHD. In one study, high intestinal abundance of the anaerobic bacteria *Blautia* was correlated with lower rates of GVHD and improved survival. Loss of diversity of the intestinal microbiota was associated with increased mortality in allogeneic HCT patients which was attributed to increased death due to GVHD or infection rather than relapse. Another study showed associations between the expansion of certain bacteria *Lactobacillales* as well as general loss of diversity and acute GVHD severity. This study also showed that expansion of Lactobacillales was not the cause for the development of GVHD because eliminating Lactobacillales from the intestinal flora in mice before allogeneic HCT caused more severe GVHD and reintroducing Lactobacillales alleviated this effect. However, another study found that that modifying the intestinal microbiota using the probiotic microorganism *Lactobacillus rhamnosus GG* reduced experimental GVHD. Similarly, administration of a cocktail of 17 species of *Clostridia* reduced experimental GVHD and improved survival.[13] These data suggest that probiotic therapy might become a rational strategy for ameliorating GVHD. As such, limited published early clinical experiences explored the use of probiotics, including via fecal microbiota transplantation (FMT) in allogeneic HCT patients. However, such approaches require rigorous assessments of safety in immunocompromised populations, as well as challenges related to scalability for widespread application in large prospective trials.

Use of Antibiotics

A recent retrospective study showed that broad-spectrum antibiotic treatment with imipenem-cilastatin and piperacillin-tazobactam was associated with increased GVHD-related mortality in patients and that treatment with imipenem-cilastatin was associated with loss of the protective mucus lining of the colon and a shift toward *Akkermansia* in mice. Antibiotic agents with more limited spectra of activity such as rifaximin, cefepime, and aztreonam were associated with reduced GVHD severity. The use of rifaximin correlated with a lower enterococcal load after allogeneic HCT and the use of cefepime and aztreonam correlated with less alterations to the composition of the intestinal microbiota post allogeneic HCT when compared with broader-spectrum antibiotics with increased anaerobic activity.

Microbial Metabolites in Acute Graft-Versus-Host Disease

The intestinal microbiota metabolize nutrients ingested by the host and produce microbial metabolites which play critical roles in the microbiota's interactions with the host and in maintaining intestinal homeostasis. Microbial metabolites include essential fatty acids and amino acids for the host. Short-chain fatty acids (SCFAs) are the most studied microbial metabolites. Examples of other microbial metabolites are bile acids, polyamines, and aryl hydrocarbon receptor (AhR) ligands. These metabolites impact both nonimmune and immune intestinal cells as well as the microbiome and have varying functions in maintaining the barrier surface of the GI tract, as well as modulating the innate and adaptive host immune responses.

Short-Chain Fatty Acids

SCFAs (e.g., butyrate, proprionate, and acetate) are produced from fermentation of indigestible carbohydrates by intestinal anaerobic commensal bacteria such as *Clostridia* species and impact both nonimmune and immune intestinal cells. SCFAs serve as an energy source for the intestinal microbiota and IECs and assist in maintaining the integrity of the mucosal barrier. For example, goblet cells upregulated their expression of mucin genes in response to SCFAs and inoculating germ-free rats with SCFA-producing *Bacteroides thetaiotaomicron* or *Faecalibacterium prausnitzii* induced goblet cell differentiation and mucus production. Furthermore, colonization with *Bifidobacterium longum* protected mice against death induced by a lethal infection by increasing production of SCFA acetate, which maintained the barrier integrity of IECs and inhibited translocation of the *E. coli* O157:H7 Shiga toxin from the gut lumen into the blood. Finally, SCFAs play an important part in innate immunity because they are histone deacetylase (HDAC) inhibitors with antiinflammatory effects. These observations reveal a crucial protective function of the microbiome in regulating the response of host tissues to immunologic and/or cytotoxic insults that occur after HCT.

Dietary Resistant Starch and Short-Chain Fatty Acids

One potential approach for stimulating SCFA production could be via dietary supplementation with carbohydrates that are resistant to degradation by human enzymes but can be metabolized by select microbes in the gut. Multiple studies have reported increased fecal SCFAs, particularly butyrate, by dietary supplementation with different formulations of resistant starch (RS). Another study showed that one such RS prepared from potatoes (RPS) is more potent at increasing average fecal butyrate levels compared with other RS formulations. Butyrate production from RS is a complex multistep process, and understanding all the gut microbes involved in this process is an ongoing challenge. Only a limited number of gut bacteria can degrade RS; however, most primary RS degraders are not among the known butyrate producers. Thus, for dietary supplementation with RS to stimulate butyrate production, the activities of secondary fermenters are required. These secondary fermenters capture degradation and fermentation products from primary degraders and metabolize them into new molecules, including butyrate. Known primary RS degraders include *Ruminococcus bromii*, *Ruminococcus lactaris*, *Ruminococcus gnavus*, and *Bifidobacterium* spp., and known butyrate producers (secondary fermenters) include *Roseburia* spp., *F. prausnitzii*, *Eubacterium rectale*, and *Anaerostipes* spp.

Short-Chain Fatty Acids and Acute Graft-Versus-Host Disease

A recent murine study explored the role of microbial metabolites, specifically SCFAs, in acute GVHD severity and found that the SCFA butyrate was significantly decreased in the IECs of mice experiencing acute GVHD.[13] Restoring butyrate levels, either by direct administration of butyrate or by increasing intestinal butyrate-producing bacteria, reduced acute GVHD. Two SCFAs, butyrate and propionate, were shown to directly protect IECs and reduce acute GVHD severity in mice. A small clinical study found that fecal butyrate and propionate levels are decreased in patients after allogeneic HCT and are lower with antianaerobic antibiotic exposure. Another recent clinical study examined fecal SCFA concentrations after allogeneic HCT and found that higher levels of butyrate and other SCFAs correlated with abundance of butyrate-producing organisms in the intestinal microbiota and this abundance was associated with resistance against lower tract respiratory infections. However, the impact of SCFA levels on acute GVHD has yet to be examined in patients post allogeneic HCT.

Dietary Manipulation of the Microbiome-Metabolome Axis and Acute Graft-Versus-Host Disease

Diet affects the composition of the intestinal microbiome and the associated metabolome is correspondingly altered. In one study, high-protein and low-carbohydrate diet was associated with loss of members of *Clostridiales*, including *Roseburia*, *Faecalibacterium*, *Ruminococcus*, and *Blautia*. Similar patterns were seen with diets derived entirely from animal products. Turnbaugh et al. showed that changes in diet from a low-fat, plant polysaccharide–rich diet to a high-fat, high-sugar diet in mice that were colonized with human feces altered the intestinal microbiota composition and this was accompanied by changes in the metabolic milieu. In two other studies, researchers showed that total parenteral nutrition resulted in a proinflammatory pattern of immune responses in mice and humans. These data suggest that diet can alter the intestinal microbiota and the metabolome and thereby regulate intestinal homeostasis.

Specific elements of the diet called prebiotics, which usually refer to indigestible carbohydrates that are metabolized by the intestinal microbiota to produce SCFAs, are particularly influential in modulating the structure and function of the intestinal microbiota. Despite their wide commercial availability, here is lack of published clinical data on the effect of prebiotics in acute GVHD. However, several studies showed that the prebiotics inulin and fructo-oligosaccharides increased intestinal microbiota diversity and decreased disease activity in inflammatory bowel disease patients. These studies, along with the evidence that SCFAs protect IECs and reduce experimental acute GVHD severity, provide a compelling rationale for now ongoing studies of dietary manipulation of the microbiome-metabolome axis using prebiotics to mitigate acute GVHD in patients receiving allogeneic HCT.

Phase 2: Donor T-Cell Activation, Differentiation, and Migration

The infused donor T cells interact with the primed APCs, leading to the initiation of the second phase of acute GVHD. This phase includes antigen presentation by primed APCs and the subsequent activation, proliferation, differentiation, and migration of alloreactive donor T cells. After allogeneic HSC transplants, both host- and donor-derived APCs are present in secondary lymphoid organs. The T-cell receptor (TCR) of the donor T cells can recognize alloantigens either on host APCs (direct presentation) or donor APCs (indirect presentation). In direct presentation, donor T cells recognize either the peptide bound to allogeneic MHC molecules or allogeneic MHC molecules without peptide. During indirect presentation, T cells respond to the peptide generated by degradation of the allogeneic MHC molecules presented on self-MHC. Experimental studies demonstrated that APCs derived from the host, rather than from the donor, are critical in inducing GVHD across MiHA mismatch. Recent data suggest that presentation of distinct target antigens by the host- and donor-type APCs might play a differential role in mediating target organ damage. In humans, most cases of acute GVHD developed when both host DCs and donor DCs were present in peripheral blood after BMT.

Costimulation

The interaction of donor lymphocyte TCR with the host allopeptide presented on the MHC of APCs alone is insufficient to induce T-cell activation. Both TCR ligation and costimulation via a “second” signal through interaction between the T-cell costimulatory molecules and their ligands on APCs are required to achieve T-cell proliferation, differentiation, and survival. The danger signals generated in phase 1 augment these interactions, and significant progress has been made on the nature and impact of these second signals. Costimulatory pathways are now known to deliver both positive and negative signals, and molecules from two major families, the B7 family and the TNF receptor (TNFR) family, play pivotal roles in GVHD. Interruption of the second signal by blockade of various positive costimulatory molecules (CD28, ICOS, CD40, CD30, 4-1BB, and OX40) reduces acute GVHD in several murine models, whereas antagonism of the inhibitory signals (PD-1 and CTLA-4) exacerbates the severity of acute GVHD. Blockade of activating costimulatory signals with the CTLA-4 fusion protein Abatacept has shown initial evidence of efficacy for prevention of GVHD in phase II clinical trials, particularly in settings of mismatched HCT.[14] Various T-cell and APC costimulatory molecules and the impact on acute GVHD are summarized in Table 109.3. The specific context and the hierarchy in which each of these signals plays a dominant role in the modulation of GVHD remain to be determined.

T-Cell Subsets

T cells consist of several subsets whose responses differ based on antigenic stimuli, activation thresholds, and effector functions. The alloantigen composition of the host determines which donor T-cell subsets proliferate and differentiate.

TABLE 109.3 **T Cell–Antigen-Presenting Cell Interactions**

T Cell	APC
Adhesion	
ICAMs	LFA-1
LFA-1	ICAMs
CD2 (LFA-2)	LFA-3
Recognition	
TCR/CD4	MHC II
TCR/CD8	MHC I
Costimulation	
CD28	CD80/86
CD152 (CTLA-4)	CD80/86
ICOS	B7H/B7RP-1
PD-1	PD-L1, PD-L2
Unknown	B7-H3
CD154 (CD40L)	CD40
CD134 (OX 40)	CD134L (OX40L)
CD137 (4-1BB)	CD137L (4-1BBL)
HVEM	LIGHT

APC, Antigen-presenting cell; *HVEM*, HSV glycoprotein D for herpesvirus entry mediator; *LIGHT*, homologous to lymphotoxins, shows inducible expression, and competes with HVEM, a receptor expressed by T lymphocytes; *MHC*, major histocompatibility complex; *TCR*, T-cell receptor.

CD4$^+$ and CD8$^+$ *Cells*

CD4 and CD8 proteins are coreceptors for constant portions of MHC class II and class I molecules, respectively. Therefore MHC class I (HLA-A, -B, -C) differences stimulate CD8$^+$ T cells, and MHC class II (HLA-DR -DP, -DQ) differences stimulate CD4$^+$ T cells. However, clinical trials of CD4$^+$ or CD8$^+$ depletion have been inconclusive. Perhaps this is not surprising, because in the majority of HLA-identical BMT, GVHD is induced by MiHAs, which are peptides derived from polymorphic cellular proteins that are presented by MHC molecules. Because the manner of protein processing depends on genes of the MHC, two siblings will have many different peptides in the MHC groove. Thus, in the majority of HLA-identical BMT, acute GVHD can be induced by either or both CD4$^+$ and CD8$^+$ subsets in response to MiHAs. The peptide repertoire for class I or class II MHC remains unknown and might even be unique in different individuals. However, it is plausible that only a few of the many of these peptides might behave as immunodominant “major minor” antigens that can potentially induce GVHD. In any event, such antigens remain to be identified and validated in large patient population.

Naïve and Memory Subsets

Intriguingly, several independent groups have found that, although the naïve (CD62L$^+$) T cells were alloreactive and caused acute GVHD, this was not the case for the memory (CD62L$^-$) T cells across different donor/recipient strain combinations. Furthermore, expression of naïve T-cell marker CD62L was also found to be critical for regulation of GVHD by donor natural Tregs. By contrast, another recent study demonstrated that alloreactive memory T cells and their precursor cells (memory stem cells) caused robust GVHD. It remains as yet unknown whether the reduced GVHD potential of memory-type T cells from a naïve murine donor, in contrast to their ability to cause greater solid organ allorejection, is a consequence of the intense conditioning regimen and/or altered trafficking or from a restricted repertoire and/or T-cell intrinsic defect.

Regulatory T Cells

Recent advances indicate that distinct subsets of regulatory CD4$^+$CD25$^+$, CD4$^+$CD25$^-$IL10$^+$ T responder (Tr) cells, γδT cells, double negative (DN$^-$) T cells, NK T cells, and regulatory DCs control immune responses by induction of anergy or active suppression of alloreactive T cells. Several studies have demonstrated a critical role for the natural donor CD4$^+$CD25$^+$ Foxp3$^+$ Tregs, obtained from naïve animals or generated ex vivo, in the outcome of acute GVHD. Donor CD4$^+$CD25$^+$T cells suppressed the early expansion of alloreactive donor T cells and their capacity to induce acute GVHD without abrogating GVL effector function against these tumors. CD4$^+$CD25$^+$ T cells induced and/or generated by of immature or regulatory host-type DCs and by regulatory donor-type myeloid APCs were also able to suppress acute GVHD. One of the clinical studies that evaluated the relationship between donor CD4$^+$CD25$^+$ cells and acute GVHD in humans after matched sibling donor grafts found that in contrast to the murine studies, donor grafts containing larger numbers of CD4$^+$CD25$^+$T cells developed more severe acute GVHD. These data suggest that coexpression of CD4$^+$ and CD25$^+$ is insufficient because an increase in CD25$^+$ T cells in donor grafts is associated with greater risks for acute GVHD after clinical HCT. Another recent study found that Foxp3 mRNA expression (considered a specific marker for naturally occurring CD4$^+$CD25$^+$Tregs) was significantly decreased in peripheral blood mononuclear cells from patients with acute GVHD, but Foxp3 expression in humans, unlike mice, may not be specific for T cells with a regulatory phenotype. It is likely that the precise role of Tregs in clinical acute GVHD will therefore depend not only on identification of specific molecular markers in addition to Foxp3 but also on the ability for ex vivo expansion of these cells in sufficient numbers. The presence of STAT1 signaling has been shown to enhance Treg-mediated suppression of GVHD. Recent observations have demonstrated key roles for host APCs in the induction and for donor APCs in the sustenance of infused mature Tregs. Several early-phase clinical trials have been conducted in the United States and Europe using various methods to expand these cells ex vivo and use for prevention of GVHD. Emerging data from these trials suggest that donor Tregs can reduce incidence of clinical GVHD and do not prevent engraftment.[15]

Host NK1.1$^+$ T cells are another T-cell subset that has been shown to suppress acute GVHD in an IL-4 dependent manner. By contrast, donor NKT cells were found to reduce GVHD and enhance perforin-mediated GVL in an IFN-γ–dependent manner. Clinical data suggest that enhancing recipient NKT cells by repeated total lymphoid irradiation (TLI) conditioning promoted T-helper 2 (Th2) polarization and dramatically reduced GVHD. Experimental data also show that activated donor NK cells can reduce GVHD through the elimination of host APCs or by secretion of transforming growth factor-β (TGF-β) secretion. A BMT study using mice lacking SH2-containing inositol phosphatase (SHIP), in which the NK compartment is dominated by cells that express two inhibitory receptors capable of binding either self or allogeneic MHC ligands, suggests that host NK cells may play a role in the initiation of GVHD.

T-Cell Apoptosis

Deletional mechanisms of tolerance fall into two categories: (1) central (thymic) deletion and (2) peripheral deletion. Central deletion is an effective way to eliminate continued thymic production of alloreactive T cells. To this end, lymphoablative treatments have been used as a condition to create a mixed hematopoietic chimeric state in murine BMT models. In this strategy, donor cells seed the thymus and maturing donor-reactive T-cell clones are deleted through intrathymic apoptosis. The proportion of the peripheral T-cell repertoire that can respond to allogeneic MHC antigens may play a critical role in the development of tolerance. In the case of MHC-mismatched transplantation, the frequency of alloreactive T cells is at least five orders of magnitude greater than the frequency of peptide-specific T cells responding to a nominal antigen. The pathways of T-cell apoptosis by which peripheral deletion occurs can be broadly categorized into activation-induced cell death (AICD) and passive cell death (PCD). An important mediator of AICD in T cells is the Fas receptor. Activated T cells expressing the Fas molecule undergo apoptotic cell death when brought into contact with cells expressing Fas ligand (FasL). A critical role for Fas-mediated AICD has been clearly demonstrated in attenuation of acute GVHD by several Th1 cytokines.

PCD, or "death by neglect," illustrates the exquisite dependence of activated T cells on growth factors (e.g., IL-2, IL-4, IL-7, and/or IL-15) for survival; apoptotic cell death in this instance is largely due to rapid downregulation of BCL2. Transplantation of BCL-XL T cells into nonirradiated recipients significantly exacerbates GVHD; however, no difference in GVHD mortality is observed in animals that have been lethally irradiated. Selective elimination of donor T cells in vivo after BMT using transgenic T cells in which a thymidine kinase (TK) suicide gene is targeted to T cells has also been shown to attenuate the severity of acute GVHD. Another recent approach to prevent GVHD is the selective depletion of alloantigen-specific donor T cells by photodynamic cell purging process, wherein donor T cells are treated with photoactive 4,5-dibromorhodamine 123 and subsequently exposed to visible light. Thus several deletional mechanisms have been shown to reduce acute GVHD, but the conditions under which one or another of these deletional mechanisms predominates role remain to be determined.

Cytokines and T-Cell Differentiation

APC and T-cell activation results in rapid intracellular biochemical cascades that induce the transcription of many genes, including cytokines and their receptors. The Th1 cytokines (IFN-γ, IL-2, and TNF-α) have been implicated in the pathophysiology of acute GVHD. IL-2 production by donor T cells remains the main target of many current clinical therapeutic and prophylactic approaches, such as CSP, tacrolimus, and monoclonal antibodies (mAbs) against the IL-2 and its receptor to control acute GVHD. However, emerging data indicate an important role for IL-2 in the generation and maintenance of CD4$^+$CD25$^+$ Foxp3$^+$ Tregs, suggesting that prolonged interference with IL-2 may have an unintended consequence in the prevention of the development of long-term tolerance after allogeneic HCT. Similarly, the role of other Th1 cytokines, IFN-γ, or their inducers as regulators or inducers of GVHD severity depends on the degree of allomismatch, the intensity of conditioning, and the T-cell subsets that are involved after BMT. Thus, although the cytokine storm initiated in phase 1 and amplified by the Th1 cytokines correlates with the development of acute GVHD, early Th1 polarization of donor T cells to HCT recipients can attenuate acute GVHD, suggesting that physiologic and adequate amounts of Th1 cytokines are critical for GVHD induction, whereas inadequate production (extremely low or high) could modulate acute GVHD through a breakdown of negative feedback mechanisms for activated donor T cells. Several different cytokines that polarize donor T cells to Th2 such as IL-4, granulocyte colony-stimulating factor (G-CSF), IL-18, IL-11, rapamycin, and the secretion of IL-4 by NK1.1$^+$ T cells can reduce acute GVHD. However, Th1 and Th2 subsets cause injury of distinct acute GVHD target tissues, and some studies failed to show a beneficial effect of Th2 polarization on acute GVHD. Thus the Th1/Th2 paradigm of donor T cells in the immunopathogenesis of acute GVHD has evolved over the past few years, and its causal role in acute GVHD is complex and incompletely understood.

IL-10 plays a key role in suppression of immune responses, and its role in regulating experimental acute GVHD is unclear. Recent clinical data demonstrate an unequivocal association of IL-10 polymorphisms with the severity of acute GVHD. TGF-β, another suppressive cytokine, was shown to suppress acute GVHD but to exacerbate chronic GVHD. The roles of some other cytokines, such as IL-7 (which promotes immune reconstitution) and IL-13 remain unclear. The role of IL-17–producing CD4$^+$ T cells (Th17) in GVHD is also unclear at present. Initial studies showed that donor T cells deficient in IL-17A augmented Th1 differentiation and exacerbated acute GVHD. In contrast, another study using a similar model showed that IL-17 contributed to CD4$^+$-mediated GVHD without affecting overall survival rates. In addition, the differentiation of Th17 cells in vitro appeared to cause lethal acute GVHD with severe cutaneous and pulmonary damage. More recently, IL-21, which is known to enhance Th17 cell differentiation while inhibiting the conversion of inducible Tregs from naïve T cells, has been shown to increase GVHD-induced injury. Consistent with this finding, the abrogation of IL-21 signaling in donor T cells has led to reduced GVHD mortality, which is associated with donor Treg expansion and decreased TNF-α. These conflicting results may be due to variations among the model systems, and further analyses are required to define the role of Th17 cells in GVHD.

CD4$^+$ and CD8$^+$ *T Cells*

As mentioned previously, in the majority of HLA-matched allogeneic HCTs, acute GVHD may be induced by either or both CD4$^+$ and CD8$^+$ subsets responses to MiHAs. The repertoire and immunodominance of the GVHD-associated peptides presented by MHC class I and class II molecules have not been clearly defined. One approach to retaining the beneficial GVL effects while eliminating the negative effects of GVHD is to selectively deplete subsets of donor alloreactive T cells in the hematopoietic cell inoculums using a TCR Vβ repertoire analysis with CDR3-size spectratyping.

Naïve and Memory T Cells

T cells in murine models can be categorized into naïve (CD62L$^+$ CD44$^-$), central memory (CD62L$^+$ CD44$^+$), and terminally differentiated effector/effector memory (CD62L$^-$ CD44$^-$) subsets. Donor naïve CD62L$^+$ T cells are the primary alloreactive T cells that drive the GVHD reaction, whereas the donor effector memory CD62L$^-$ T cells do not. Shedding of CD62L by antigen encountered T cells facilitates their egress from peripheral lymph tissues to the circulation, where they participate in helper and effector functions. Of interest, donor Treg cells expressing CD62L are critical to the regulation of GVHD. Specifically, it has been shown that CD62L^{hi} Tregs attenuate GVHD and promote allogeneic BM engraftment. The expression of CD62L is important for the migration of Tregs to secondary lymphoid organs, thereby inhibiting the initial expansion of donor T cells. We now know that it is possible to modulate the alloreactivity of naïve T cells by inducing anergy with costimulation blockade, deletion via cytokine modulation, or mixed chimerism. Donor effector memory T cells that are nonalloreactive do not induce GVHD yet are able to transfer functional memory and mediate GVL. In addition, lymphopenia-induced proliferation gives rise to cells that are like memory T cells and enhance the GVL effect after donor leukocyte infusion (DLI). As proof-of-concept, clinical studies of donor grafts depleted of CD34$^+$ cells and CD45RA$^+$ naïve T cells, with add-back of memory T cells have reported low rates of severe (corticosteroid-resistant) GVHD and chronic GVHD.[16] In contrast, memory T cells that are alloreactive can cause severe GVHD. However, more recently, Anderson and colleagues demonstrated that enhancing the alloreactivity of effector memory T cells can cause GVHD, although a less severe and possibly more transient GVHD in nature. These findings may be better understood with further analyses that would enable tracking of alloantigen-specific T cells (naïve, central memory, and effector memory subsets) in experimental GVHD and GVL models with defined TCR repertoires.

Leukocyte Migration

Donor T cells migrate to lymphoid tissues, recognize alloantigens on either host or donor APCs, and become activated. They then exit the lymphoid tissues, traffic to the target organs, and cause tissue damage. The molecular interactions necessary for T-cell migration and the role of lymphoid organs during acute GVHD have recently become the focus of a growing body of research. Chemokines play a critical role in the migration of immune cells to secondary lymphoid organs and target tissues. T-lymphocyte production of macrophage inflammatory protein-1α (MIP-1α) is critical to the recruitment of CD8$^+$ but not CD4$^+$ T cells to the liver, lung, and spleen during acute GVHD. Several chemokines such as CCL2–5, CXCL2, CXCL9–11, CCL17, and CCL27 are overexpressed and might play a critical role in the migration of leukocyte subsets to target organs liver, spleen, skin, and lungs during acute GVHD. CXCR3$^+$ and CCR5$^+$ T cells cause acute GVHD in the liver and intestine. CCR5 expression has also been found to be critical for Treg migration in GVHD. In addition to chemokines and their receptors, expression of selectins and integrins and their ligands also regulate the migration of inflammatory cells to target organs. For example, interaction between α4β7 integrin and its ligand MadCAM-1 are important for homing of donor T cells to Peyer patches and in the initiation of intestinal GVHD. αLβ2 intracellular adhesion molecule-1,2,3 (ICAM1,2,3) and α4β1/vascular cell adhesion molecule-2 (VCAM-2) interactions are important for homing to the lung and liver after experimental HCT. The expression of CD62L on donor Tregs is critical for their regulation of acute GVHD, suggesting that their migration in secondary tissues is critical for their regulatory effects. The migratory requirement of donor T cells to specific lymph nodes (e.g., Peyer patches) for the induction of GVHD might depend on other factors such as the conditioning regimen, inflammatory milieu, etc. Furthermore, FTY720, a pharmacologic sphingosine-1-phosphate receptor agonist, inhibited GVHD in murine but not in canine models of HCT. Thus significant species differences may also factor in the ability of these molecules to regulate GVHD.

Phase 3: Effector Phase

The effector phase that leads to the GVHD target organ damage is a complex cascade of multiple cellular and inflammatory effectors that further modulate each other's responses either simultaneously or successively. Effector mechanisms of acute GVHD can be grouped into cellular and inflammatory effectors. Inflammatory chemokines expressed in inflamed tissues upon stimulation by proinflammatory effectors, such as cytokines, are specialized for the recruitment of effector cells, such as cytotoxic T lymphocytes (CTLs). Furthermore, the spatiotemporal expression of the cytochemokine gradients might determine not only the severity but also the unusual cluster of GVHD target organs (skin, gut, and liver).

Cellular Effectors

CTLs are the major cellular effectors of GVHD. The principal CTL effector pathways that have been evaluated after allogeneic BMT are the Fas-FasL, the perforin-granzyme (or granule exocytosis), and the TNFR-like death receptors (DRs), such as TNF-related apoptosis-inducing ligand (TRAIL: DR4, 5 ligand), and TNF-like weak inducers of apoptosis (TWEAK: DR3 ligand). The involvement of each of these molecules in GVHD has been tested by using donor cells that are unable to mediate each pathway. Perforin is stored in cytotoxic granules of CTLs and NK cells, together with granzymes and other proteins. Although the exact mechanisms remain unclear, following the recognition of a target cell through the TCR-MHC interaction, perforin is secreted and inserted into the cell membrane, forming "perforin pores" that allow granzymes to enter the target cells and induce apoptosis through various downstream effector pathways such as caspases. Ligation of Fas results in the formation of the death-inducing signaling complex (DISC) and also activates caspases.

Transplantation of perforin-deficient T cells results in a marked delay in the onset of GVHD in transplants across MiHA disparities only, both MHC and MiHA disparities, and across isolated MHC I or II disparities. However, mortality and clinical and histologic signs of GVHD were still induced even in the absence of perforin-dependent killing in these studies, demonstrating that the perforin-granzyme pathway plays little role in target organ damage. A role for the perforin-granzyme pathway for GVHD induction is also evident in studies using donor T-cell subsets. Perforin- or granzyme B-deficient CD8$^+$ T cells caused less mortality than wild-type T cells in experimental transplants across a single MHC class I mismatch. However, this pathway seems to be less important compared with Fas/FasL pathway in CD4-mediated GVHD. Thus it seems that CD4$^+$ CTLs preferentially use the Fas-FasL pathway, whereas CD8$^+$ CTLs primarily use the perforin-granzyme pathway.

Fas, a TNF-receptor family member, is expressed by many tissues, including GVHD target organs. Its expression can be upregulated by inflammatory cytokines such as IFN-γ and TNF-α during GVHD, and the expression of FasL is also increased on donor T cells, indicating that FasL-mediated cytotoxicity may be a particularly important effector pathway in GVHD. FasL-defective T cells cause less GVHD in the liver, skin, and lymphoid organs. The Fas-FasL pathway is particularly important in hepatic GVHD, consistent with the keen sensitivity of hepatocytes to Fas-mediated cytotoxicity in experimental models of murine hepatitis. Fas-deficient recipients are protected from hepatic GVHD but not from other organ GVHD, and administration of anti-FasL (but not anti-TNF) mAbs significantly blocked hepatic GVHD damage occurring in murine models. Although the use of FasL-deficient donor T cells or the administration of neutralizing FasL MAbs had no effect on the development of intestinal GVHD in several studies, the Fas-FasL pathway may play a role in this target organ, because intestinal epithelial lymphocytes exhibit increased FasL-mediated killing potential. Elevated serum levels of soluble FasL and Fas have also been observed in at least some patients with acute GVHD.

The use of a perforin-granzyme and FasL cytotoxic double-deficient (cdd) mouse provides an opportunity to address whether other effector pathways are capable of inducing GVHD target organ pathology. An initial study demonstrated that cdd T cells were unable to induce lethal GVHD across MHC class I and class II disparities after sublethal irradiation. However, subsequent studies demonstrated that cytotoxic effector mechanisms of donor T cells are critical in preventing host resistance to GVHD. Thus, when recipients were conditioned with lethal dose of irradiation, cdd CD4$^+$ T cells produced similar mortality to wild-type CD4$^+$ T cells. These results were confirmed by a recent study demonstrating that GVHD target damage can occur in mice that lack alloantigen expression on the epithelium, preventing direct interaction between CTLs and target cells.

The participation of another death ligand receptor signaling pathway, TNF/TNFRs, has also been evaluated. Experimental data suggest that this pathway is crucial for GI GVHD (discussed in more detail later). Recently, several additional TNF family apoptosis-inducing receptors/ligands have been identified, including TWEAK, TRAIL, and LTβ/LIGHT, all of which have been proposed to play a role in GVHD and GVL responses. However, whether these distinct pathways play a more specific role for GVHD mediated by distinct T-cell subsets in certain situations remains unknown. Intriguingly, recent data suggest that none of these pathways might be critical for mediating the rejection of donor grafts. Thus it is likely that their role in GVHD might be modulated by the intensity of conditioning and by the recipient T-cell subsets. Existing experimental data suggest that perforin and TRAIL cytotoxic pathways are associated with CD8$^+$ T cell–mediated GVL. The available experimental data are strongly skewed toward CD8$^+$ T cell–mediated GVL, based on the dominant role of this effector population in most murine GVT models; however, CD4$^+$ T cells can mediate GVL and might be crucial in clinical BMT, depending on the type of malignancy and the expression of immunodominant antigens.

Taken together, experimental data suggest some distinction between the use of different lytic pathways for the specific GVHD target organs and GVL, but the clinical applicability of these observations is as yet largely unknown.

Inflammatory Effectors

Inflammatory cytokines synergize with CTLs, resulting in the amplification of local tissue injury and further promotion of an inflammation, which ultimately leads to the observed target tissue destruction in the transplant recipient. Macrophages, which had been primed with IFN-γ during step 2, produce inflammatory cytokines TNF-α and IL-1 when stimulated by a secondary triggering signal. This stimulus may be provided through Toll-like receptors (TLRs) by microbial products such as LPS and other microbial particles, which can leak through the intestinal mucosa damaged by the conditioning regimen and gut GVHD. It is now apparent that immune recognition through both TLR and non-TLRs (e.g., NOD) by the innate immune system also controls activation of adaptive immune responses. Recent clinical studies of GVHD suggested the possible association with TLR/NOD polymorphisms and severity of GVHD. LPS and other immunogenic stimuli such as DAMPs may stimulate gut-associated lymphocytes, keratinocytes, dermal fibroblasts, and macrophages to produce proinflammatory effectors that play a direct role in causing target organ damage. Indeed, experimental data with MHC mismatched BMT suggest that under certain circumstances these inflammatory mediators are sufficient in causing GVHD damage even in the absence of direct CTL-induced damage. The severity of GVHD appears to be directly related to the level of innate and adaptive immune cell priming and release of proinflammatory cytokines such as TNF-α, IL-1, and nitric oxide (NO).

The cytokines TNF-α and IL-1 are produced by an abundance of cell types during processes of both innate and adaptive immunity; they often have synergistic, pleiotropic, and redundant effects on both activation and effector phases of GVHD. A critical role for TNF-α in the pathophysiology of acute GVHD was first suggested more than 20 years ago because mice transplanted with mixtures of allogeneic BM and T cells developed severe skin, gut, and lung lesions that were associated with high levels of TNF-α messenger RNA (mRNA) in these tissues. Target organ damage could be inhibited by infusion of anti–TNF-α mAbs, and mortality could be reduced from 100% to 50% by the administration of the soluble form of the TNF-α receptor (sTNFR), an antagonist of TNF-α. Accumulating experimental data further suggest that TNF-α is involved in a multistep process of GVHD pathophysiology. TNF-α can (1) cause cachexia, a characteristic feature of GVHD; (2) induce maturation of DCs, thus enhancing alloantigen presentation; (3) recruit effector T cells, neutrophils, and monocytes into target organs through the induction of inflammatory chemokines; and (4) cause direct tissue damage by inducing apoptosis and necrosis. TNF-α also involves in donor T-cell activation directly through its signaling via TNFR1 and TNFR2 on T cells. TNF-TNF1 interactions on donor T cells promote alloreactive T-cell responses and TNF-TNFR2 interactions are critical for intestinal GVHD. TNF-α also seems to be an important effector molecule in GVHD in skin and lymphoid tissue. In addition, TNF-α might also be involved in hepatic GVHD, probably by enhancing effector cell migration to the liver via the induction of inflammatory chemokines. An important role for TNF-α in clinical acute GVHD has been suggested by studies demonstrating elevated serum levels or TNF-α or elevated TNF-α mRNA expression in peripheral blood mononuclear cells in patients with acute GVHD and other endothelial complications, such as hepatic VOD.

The second major proinflammatory cytokine that appears to play an important role in the effector phase of acute GVHD is IL-1. Secretion of IL-1 appears to occur predominantly during the effector phase of GVHD of the spleen and skin, two major GVHD target organs. A similar increase in mononuclear cell IL-1 mRNA has been shown during clinical acute GVHD. Indirect evidence of a role for IL-1 in GVHD was obtained with administration of this cytokine to recipients in an allogeneic murine BMT model. Mice receiving IL-1 displayed a wasting syndrome and increased mortality that appeared to be an accelerated form of disease. By contrast, intraperitoneal administration of IL-1 receptor antagonist (IL-1RA) starting on day 10 posttransplant was able to reverse the development of GVHD in the majority of animals, providing a significant survival advantage to treated animals. However, the attempt to use IL-1RA to prevent acute GVHD in a randomized trial was not successful.

As a result of activation during GVHD, macrophages also produce NO, which contributes to the deleterious effects on GVHD target tissues, particularly immunosuppression. NO also inhibits the repair mechanisms of target tissue destruction by inhibiting proliferation of epithelial stem cells in the gut and skin. In humans and rats, the development of GVHD is preceded by an increase in serum levels of NO oxidation products.

IL-6 has also been identified as a critical cytokine that promotes a proinflammatory response during GVHD and inhibits the reconstitution of Tregs. Although Tawara and colleagues confirmed an important role for IL-6 in GVHD, their findings were independent of T effector cell expansion or donor Treg responses. It is possible that reduction in GVHD is a consequence of direct reduction in IL-6–induced inflammation and cytopathic damage of the target tissues. Existing data demonstrate important roles for various inflammatory effectors in GVHD. However, the relevance of currently studied or as yet unknown specific effectors might be determined by other factors, including the intensity of preparatory regimens, the type of allograft, the T-cell subsets and the duration of BMT. In any event, both experimental and clinical data suggest an important role for both the cellular and inflammatory mediators in GVHD-induced target organ damage.

BIOMARKERS OF GRAFT-VERSUS-HOST DISEASE

Large datasets have identified and validated plasma biomarkers with important prognostic value at the onset of symptoms and treatment of acute GVHD. Several initial reports based on proteomic discovery of plasma samples taken from patients with systemic GVHD initially described markers including IL-2Rα, TNFR1, IL-8, and hepatocyte growth factor (HGF). Elafin has been identified as a biomarker specific for skin GVHD, and regenerating islet-derived 3α (REG3α) as a biomarker of GI GVHD, and ST2 or the soluble IL-33 receptor as a maker of resistance to therapy. Subsequently, in an effort to improve diagnostic precision panels that combine three plasma biomarkers (TNFR1, ST2, and Reg3α) were used in the creation of an algorithm that computed the probability of nonrelapse mortality for individual patients.[17] The algorithm was developed in a multicenter training set and two separate multicenter validation sets, respectively. The investigators identified thresholds that created three distinct Ann Arbor GVHD scores. In all three datasets (training, test, and validation), the cumulative incidence of 6-month nonrelapse mortality significantly increased as the Ann Arbor GVHD score increased: 8% for score 1, 27% for score 2, and 46% for score 3 ($P < .0001$). Conversely, the response to primary GVHD treatment decreased as the GVHD score increased: 86% for score 1, 67% for score 2, and 46% for score 3 ($P < .0001$). Two-biomarker algorithms involving only ST2 and REG3α have demonstrated similar value to the aforementioned three marker panels and were predictive of corticosteroid resistance associated with elevated TRM.[18]

These biomarkers may also provide new insights into the biology of GVHD. For example, the IL-22–Reg3 axis protects the epithelial barrier function of the intestinal mucosa. ISCs are principal cellular targets of GVHD in the GI tract, whereas discussed previously certain strains of intestinal flora are critical for amplification of GVHD damage. ISCs are protected by antibacterial proteins such as REG3 α secreted by neighboring Paneth cells. Mucosal barrier disruption caused by stem cell dropout and subsequent lack of mucosal regeneration may preferentially allow Paneth cell proteins, including REG3 α, to traverse into the bloodstream. Other groups have extended biologic links between intestinal immunologic injury and biomarkers. For instance, the number of Paneth cells themselves in duodenal biopsies for GVHD, which can be eliminated during intestinal crypt damage during GVHD, has been shown to correlate with response to treatment and survival.[9] Similarly, high plasma levels of amphiregulin, a plasma EGFR ligand involved in GI mucosal repair, has been shown to be elevated at GVHD onset in patients with poor subsequent outcomes.[19] Finally, as a consequence of early mucosal barrier permeability the concentration of certain stool analytes such as α_1-antitrypsin and calprotectin are elevated in the setting of intestinal injur,[20] although to date the diagnostic utility of these markers is less developed in clinical settings.

These findings suggest that biomarker-based scores might one day be used to guide risk-adapted therapy strategies, together with clinical staging, when acquired at the onset of acute GVHD. For example, high-risk patients are candidates for intensive primary therapy, whereas low-risk patients are candidates for rapid tapers of systemic steroid therapy. Whether any such markers can be successfully integrated into prospective trials in real time and the degree to which modulating the intensity of frontline therapy can successfully ameliorate the natural history of high risk GVHD remain to be determined.

Determining patients at risk of significant morbidity and mortality in chronic GVHD remains a significant unmet need. However, at this time no biomarkers of chronic GVHD have been reliably validated in clinical studies. This likely reflects the relative complexity of accurately characterizing the various subforms of chronic GVHD in addition to the understanding of underlying chronic GVHD biology. Nonetheless, some promising candidates are beginning to emerge. CXCL9 has been identified and validated in several hundred patients from at least two HCT centers. Other biomarkers with potential utility include soluble B-cell activation factor (sBAFF), anti-double strand DNA antibody, soluble IL-2 receptor-α (sIL-2Rα), and soluble CD13 (sCD13); sBAFF and anti-dsDNA also may be elevated in patients with late-onset chronic GVHD, but these biomarkers need to be validated in much larger cohorts before definitive conclusions can be drawn.

Prevention of Acute Graft-Versus-Host Disease

Elimination of T cells with mAbs, immunotoxins, lectins, CD34 columns, or physical techniques are effective at reducing GVHD. A typical unmanipulated marrow transplant entails the infusion of approximately 10^7 T cells per kg of recipient weight. A T-cell dose 10^5 or less per kg has been associated with complete control of GVHD. More recently, the combination of very high stem cell numbers and less than 3×10^4 CD3 cells/kg allowed haploidentical transplantation without GVHD. Presumably, host immune cells that survive the initial conditioning are responsible for graft rejection. When the stem cell source contains a large numbers of T cells, the GVH reaction further reduces the residual population capable of host versus graft alloreactivity, thus decreasing graft rejection. To some degree, the higher graft failure rates may be controlled by increasing the intensity of the immunosuppression of the conditioning regimen or by adding back T cells. Overall there has been no improvement in survival that can be definitively attributed to T-cell depletion.

Evolution of Modern Clinical Graft-Versus-Host Disease Prevention Strategies

Treatment of established GVHD with specific T-cell antibodies has produced mixed results. Although antithymocyte globulin has definite activity in established GVHD and prevention of GVHD, the nonspecific clearance of T cells may result in increased opportunistic infections and no improvement in survival. More specific therapies targeting single cytokines or immune cell subsets with humanized anti–IL-2 receptor antibody daclizumab or the humanized anti-CD3 antibody visilizumab were initially promising because they offer the potential of selectively removing the activated T cells. However, an increased risk for infection or relapse may still be observed; thus improvement in overall survival has been less clear.

The first generally prescribed GVHD preventive regimen was the administration of intermittent low-dose methotrexate as developed in a dog model by Thomas and Storb. The principle of this approach was to administer low dosages a cell cycle–specific chemotherapeutic agent immediately after the transplant, when the T cells have started to divide after exposure to allogeneic antigens. Subsequently, the addition of antithymocyte globulin, prednisone, or both resulted in incremental improvement in the GVHD rate but no improvement in survival. Ultimately, the course of methotrexate was abbreviated and combined with a T-cell activation inhibitor, such as CSP or tacrolimus. The introduction of CSP in the late 1970s was a significant advance in GVHD prevention. A similar agent, tacrolimus, has been shown to provide similar control of GVHD. As a single agent, CSP was about as effective as methotrexate. However, in combination with methotrexate, there was a significant reduction in the incidence of GVHD and an improvement in survival. Subsequent trials of tacrolimus and methotrexate compared with CSP and methotrexate showed no clear advantage for either combination. The addition of prednisone to the conventional two-drug regimen resulted in similar rates of GVHD and no improvement in survival.

Sirolimus (rapamycin) is a macrocyclic lactone immunosuppressant that is similar in structure to tacrolimus and CSP. All three drugs bind to immunophilins; however, sirolimus complexed with FKBP12 inhibits T-cell proliferation by interfering with signal transduction and cell cycle progression and can prevent GVHD. Because sirolimus acts through a separate mechanism from the tacrolimus-FKBP complex (and CSP -cyclophilin complex), it is synergistic with both tacrolimus and CSP. In addition, mycophenolate mofetil (MMF) has been studied as a substitute for methotrexate particularly as a component of reduced intensity regimens based on the premise that this may limit cytotoxic effects on mucosal tissues thereby limiting early mucositis. MMF is the prodrug of mycophenolic acid (MPA), a selective inhibitor of inosine monophosphate dehydrogenase, an enzyme critical to the de novo synthesis of guanosine nucleotide. Because T lymphocytes are more dependent on such synthesis than myeloid or mucosal cells, MPA preferentially inhibits proliferative responses of T cells. To date, prospective comparisons between either MMF and MTX combined with calcineurin inhibitors are lacking, but meta-analysis have not clearly suggested superiority of either approach. HLA barriers have historically restricted transplantation of haploidentical donors using conventional calcineurin inhibitor–based GVHD prophylaxis due to high rates of graft failure and grade III to IV GVHD. However, the addition of posttransplant cyclophosphamide (PtCy) has recently emerged as a standard component of acute GVHD prophylaxis regimens in haploidentical HCT, likely due to deletion of alloreactive T cells without eliminating $CD34^+$ stem cells that are thought to be protected by expressing high levels of aldehyde dyhyrogenase.[21] In these HLA-mismatched situations, PtCy together with tacrolimus and MMF results in stable engraftment and low rates of grade III to IV acute GVHD and chronic GVHD. Current prospective studies are now comparing PtCy to standard tacrolimus/MTX-based prevention regimens in HLA-matched HCT.[22] A major consequence of this advance is the improved availability of donor options in critically underrepresented ethnic groups.

Therapy for Acute Graft-Versus-Host Disease

Glucocorticoid steroids are the initial therapy for acute GVHD. The mechanisms by which steroids work are multifactorial; they act as lympholytic agents and inhibit the release of inflammatory cytokines such as IL-1, IL-2, IL-6, IFN-γ, and TNF-α. Because of its intravenous (IV) availability, methylprednisolone is the steroid most commonly given for acute GVHD. Various dosing regimens have been used, none of which is clearly superior. High-bolus doses (10 to 20 mg/kg or 500 mg/m^2) may have higher initial response rates, but flares on tapering and opportunistic infections are common. Both the Seattle and Minnesota transplant groups have found that treatment with steroids was as effective as, or more effective than, other therapies or combination of therapies, with 20% to 40% of patients having durable long-term responses. Long-term salvage rates for patients who did not respond to steroids were 20% or less; most patients eventually died from infection, acute GVHD, and/or chronic GVHD. Clinically, two types of failure of corticosteroid treatment of acute GVHD can be distinguished: true steroid resistance (i.e., progression of GVHD symptoms and manifestations while patients are receiving full-dose corticosteroid treatment) and steroid dependence (i.e., reoccurrence [or flare] of GVHD during or after tapering of steroid treatment). In general, the prognosis with true steroid-resistant GVHD is worse than the prognosis of steroid-dependent patients. A comparison of trials dealing with steroid-resistant GVHD is hampered by variable inclusion of both patient groups in many of these trials. A number of agents have been tested, including chemical immunosuppressants such as MMF, antithymocyte globulin (ATG), anti-CD3 anti–T-cell antibodies, and more specific agents directed against activation or adhesion molecules anti-CD25, anti-CD147, or cytokines or extracorporeal photopheresis. Similarly, a recent head-to-head prospective phase III trial assessing the IL-2 receptor antibody inolimumab, when compared with ATG, did not show superiority for either agent with respect to survival.[23] A number of other promising phase II trials have also suggested a role for more selective approaches such as TNF inhibition when added to steroids in treating GVHD; however, a randomized trail failed to demonstrate any difference when compared with the addition of pentostatin or anti–IL-2 to steroids and was inferior to the addition of MMF. It should be noted that a follow-up trial for frontline therapy comparing MMF with corticosteroids versus corticosteroids alone was ended prematurely due to lack of efficacy with combination therapy. More recently, based on preliminary studies in the setting of steroid-refractory acute GVHD, IL-6 antagonism has been adopted at some centers as a treatment for steroid-refractory acute GVHD but controlled studies for this agent are presently lacking.[24]

Considering the overlapping roles that individual cytokines play in the complex immune biology, such approaches may be limited in their capacity to treat aggressive forms of GVHD. These trials also underscore the challenges in improving outcomes in treatment of established GVHD whereby potentially irreversible underlying damage to tissues that can complicate a full recovery.

A recent advance in GVHD treatment has been licensing of the JAK1/2 inhibitor ruxolitinib as the first US Food and Drug Administration (FDA)-approved therapy for SR-GVHD. This agent targets multiple pathways distal to cytokine-regulated signaling via small molecule tyrosine kinase inhibition of JAKs and STATs that has demonstrated some success in treating SR-GVHD. After high response rates were observed in a retrospective survey, a prospective, open label, multicenter trial of the JAK1/2 inhibitor ruxolitinib reported more than half of SR-GVHD patients met the primary end point of day 28 overall response, including a median duration of response of approximately 1 year.[25] As alluded to previously, given the lack of randomized data, this approach will require careful monitoring, especially considering blood cytopenias and infections can frequently complicate this treatment in severe GVHD.

CHRONIC GRAFT-VERSUS-HOST DISEASE

Chronic GVHD was initially defined as a GVHD syndrome presenting more than 100 days after transplant; its onset occurred either as an extension of acute GVHD (overlap), after a disease-free interval (quiescent), or with no precedent (de novo). Chronic GVHD may be limited or extensive (see Table 109.3). Any grade of acute GVHD increases the probability of chronic GVHD, although no singular pathologic feature of the former predicts the development of the latter. Its incidence ranges from 30% to 60% after transplantation with the bone marrow, and with increased prevalence after peripheral blood progenitor transplants.

As with acute GVHD, the immune system appears to be affected in all patients, who are highly susceptible to bacterial, viral, fungal, and opportunistic infections. Specific abnormalities of cellular immunity include decreases in the production of antibodies against specific antigens, defects in the number and function of CD4$^+$ T cells, and increases in the number of nonspecific suppressor cells, which further diminish lymphocyte responses. Skin changes resembling widespread lichen planus with papulosquamous dermatitis, plaques, desquamation, dyspigmentation, and vitiligo occur in 80% of patients. Destruction of dermal appendages leads to alopecia and onychodysplasia. Severe chronic GVHD of the skin can resemble scleroderma, with induration, joint contractures, atrophy, and chronic skin ulcers. Chronic cholestatic liver disease occurs in 80% of patients and often resembles acute GVHD; it rarely progresses to cirrhosis. Severe mucositis of the mouth and esophagus can result in weight loss and malnutrition. However, intestinal involvement is infrequent and, when involved, typically affects the upper GI tract with stricturing. Chronic GVHD also produces a sicca syndrome, with atrophy and dryness of mucosal surfaces caused by lymphocytic destruction of exocrine glands, usually affecting the eyes, mouth, airways, skin, and esophagus. The hematopoietic system may also be affected, and thrombocytopenia is an unfavorable prognostic factor in patients with chronic GVHD. Important predictors of unfavorable outcome are progressive onset, lichenoid skin changes, elevated serum bilirubin level, continued thrombocytopenia, and failure to respond to 9 months of therapy. Among patients with none of these risk factors, 70% are expected to survive, compared with less than 20% with two or more of these risk factors.

Histologic examination of the immune system reveals involution of thymic epithelium, disappearance of Hassall corpuscles, depletion of lymphocytes, and absence of secondary germinal centers in lymph nodes. Pathologic skin findings include epidermal atrophy with changes characteristic of lichen planus and striking inflammation around eccrine units. Sclerosis of the dermis and fibrosis of the hypodermis subsequently develop. GI lesions include localized inflammation of the mucosa and stricture formation in the esophagus and small intestine. Histologic findings in the liver are often similar to those that occur in acute GVHD but are more intense, with chronic changes such as fibrosis and hyalinization of portal triads, obliteration of bile ducts, and hepatocellular cholestasis. The endocrine glands of the eyes, mouth, esophagus, and bronchi show destruction focused on centrally draining ducts, with secondary involvement of alveolar components. Findings of bronchiolitis obliterans, similar to those that occur in rejection of lung transplants, are now generally considered a pulmonary manifestation of chronic GVHD, although the pathogenesis of this process remains unclear.

Clinical Manifestations of Chronic Graft-Versus-Host Disease

Chronic GVHD can present with a plethora of clinical manifestations. Because of its unpredictable pattern and the late onset, when patients are no longer receiving care at their transplant center, the diagnosis is often delayed or not recognized. However, multiorgan diagnostic criteria developed by the National Institutes of Health (NIH) is now the accepted as the standard for diagnosing and evaluating responses in most clinical trials and research settings for chronic GVHD.

Dermatologic

Skin involvement in chronic GVHD presents with varied features. Lichenoid chronic GVHD presents as an erythematous, papular rash that resembles lichen planus with no typical distribution pattern. Sclerodermatous GVHD may involve the dermis and/or the muscular fascia and clinically resembles systemic sclerosis. The skin is thickened, tight, and fragile, with very poor wound healing. Either hypopigmentation or hyperpigmentation may occur. In severe cases the skin may become blistered and ulcerate. Hair changes can include increased brittleness, premature graying, and alopecia. Fingernails and toenails may also be affected by chronic GVHD. Destruction of sweat glands can cause hyperthermia.

Ocular

Ocular GVHD usually presents with xerophthalmia or dry eyes. Irreversible destruction of the lacrimal glands results in dryness, photophobia, and burning. Local therapy with preservative-free tears and ointment or the placement of punctal plugs by an ophthalmologist might be required. Conjunctival GVHD, a rare manifestation of severe chronic GVHD, has a poor prognosis.

Oral

Oral GVHD causes xerostomia and/or food sensitivity. More advanced disease may cause odynophagia due to esophageal damage and strictures, although esophageal involvement occurs rarely without oral disease. Physical examination may reveal only erythema with a few white plaques, prompting a misdiagnosis of thrush or herpetic infections. Lichenoid changes in advanced disease can cause extensive plaque formation.

Gastrointestinal

Patients with chronic GVHD have GI complaints that mimic other disease states, including acute GVHD, infection, dysmotility, lactose intolerance, pancreatic insufficiency, and drug-related side effects. In one retrospective review of the intestinal biopsies of patients with chronic GVHD and persistent GI symptoms, a majority of patients had evidence of both acute and chronic GVHD, and only 7% of the patients had isolated chronic GVHD. Thus, although chronic GVHD may involve the GI tract alone, it may be difficult to diagnose in those circumstances without concurrent acute GVHD.

Hepatic

Hepatic disease typically presents as cholestasis with elevated serum levels of alkaline phosphatase and bilirubin. Isolated hepatic chronic GVHD has become more common with the increasing use of donor lymphocyte infusions. Liver biopsy is required to confirm the diagnosis of chronic hepatic GVHD in patients with no other target organ involvement.

Pulmonary

Bronchiolitis obliterans is a late and serious manifestation of chronic GVHD. Patients typically present with a cough or dyspnea. Severe sclerotic disease of the chest wall may also give rise to similar symptoms with no intrinsic pulmonary disease. Pulmonary function tests demonstrate obstructive physiology and a reduction in diffusion capacity (DLCO). Chest computed tomography results may be normal or may show hyperinflation with a ground-glass appearance. Overall, patients with bronchiolitis obliterans have minimal response to therapy and a very poor prognosis. Patients with chronic GVHD are also at risk for chronic sinopulmonary infections, but symptoms may be minimal.

Hematopoietic

Cytopenias in chronic GVHD are common. This may be a result of stromal damage, but autoimmune neutropenia, anemia, and/or thrombocytopenia are also seen. Thrombocytopenia at the time of chronic GVHD diagnosis is associated with poor prognosis. However, thrombocytopenia post transplant is a poor prognostic factor regardless of GVHD, and eosinophilia is occasionally seen with chronic GVHD.

Immunologic

Chronic GVHD is inherently immunosuppressive. Functional asplenia with an increased susceptibility to encapsulated bacteria is common and circulating Howell-Jolly bodies can be seen on peripheral blood smear. Patients are also at risk for invasive fungal infections and *Pneumocystis carinii* pneumonia (PCP). Hypoglobulinemia is common, and patients with levels less than 500 mg/dL should be supplemented with IV Ig.

Musculoskeletal

Fascial involvement in sclerodermatous GVHD is usually associated with skin changes. Fasciitis in joint areas can cause severe restriction of range of motion. Muscle cramps are a common complaint in patients with chronic GVHD, but myositis with elevated muscle enzymes is rare. Many patients with chronic GVHD are on steroid therapy and have low levels of sex hormone posttransplant. Thus avascular necrosis, osteopenia, and osteoporosis are frequent complications.

Although several cases have been described, it is yet to be determined in large studies whether kidney involvement and associated nephrotic syndrome, which are primary targets in some animal models of chronic GVHD, are also involved. Among the myriad clinical features of chronic GVHD, three definitive signs appear to be risk factors for increased mortality: (1) extensive skin GVHD involving greater than 50% of the body surface area, (2) platelet count of less than 100,000/μL, and (3) progressive onset and acute GVHD that continues uninterrupted beyond day 100. However, chronic GVHD remains, except in cases with obvious features, a difficult diagnosis; response to therapy is even more difficult to assess. Recent criteria established by an NIH consensus conference might prove to be beneficial in establishing uniform guidelines for diagnosis, treatment, and response.

Differential Diagnosis

The distinction between chronic and acute GVHD has been traditionally based on the time of onset. However, with the advent of low-intensity HCT, that distinction has become less relevant. The NIH Working Group has, in addition to the two main categories of GVHD, added two subcategories. The broad category of acute GVHD includes (1) classic acute GVHD (maculopapular rash, nausea, vomiting, anorexia, profuse diarrhea, ileus, or cholestatic hepatitis), occurring within 100 days after transplantation or DLI (without diagnostic or distinctive signs of chronic GVHD), and (2) persistent, recurrent, or late acute GVHD: features of classic acute GVHD without diagnostic or distinctive manifestations of chronic GVHD occurring beyond 100 days of transplantation or DLI (often seen after withdrawal of immune suppression). The broad category of chronic GVHD includes (1) classic chronic GVHD without features characteristic of acute GVHD and (2) an overlap syndrome in which features of chronic and acute GVHD appear together. In the absence of histologic or clinical signs or symptoms of chronic GVHD, the persistence, recurrence, or new onset of characteristic skin, GI tract, or liver abnormalities should be classified as acute GVHD regardless of the time after transplantation. With appropriate stratification, patients with persistent, recurrent, or late acute GVHD or overlap syndrome can be included in clinical trials with patients who have chronic GVHD.

CHRONIC GRAFT-VERSUS-HOST DISEASE: PATHOPHYSIOLOGY

The pathophysiology of chronic GVHD is generally much less well understood than that of acute GVHD and has undergone less intensive experimental modeling. It is important to recognize that chronic GVHD was originally defined as a temporal rather than a clinical or pathophysiologic entity. The initial clinical reports of chronic GVHD described abnormalities that occurred at least 150 days after stem cell infusion. By convention, day 100 after stem cell infusion is used as an arbitrary divider between acute and chronic GVHD. However, some manifestations of acute GVHD occur after day 100, and some manifestations of chronic GVHD may occur before day 100. Thus it is preferable to consider the clinical symptoms and signs per se rather than their timing of onset.

Relatively little is known about the pathophysiology of chronic GVHD. This is due in part to the absence of appropriate animal models that can capture the kinetics and the protean manifestation of chronic GVHD. Based on certain clinical features, chronic GVHD has been considered to be an autoimmune disease, with some experimental data suggesting that chronic GVHD results from defective central negative selection, which leads to the generation of autoreactive clones that escape tolerogenic mechanisms operating in the periphery.

This would indicate that the nonpolymorphic antigens expressed in both the donor and recipient rather than MiHA antigens are the likely targets. In contrast to T cells from animals with acute GVHD that are specific for host alloantigens, T cells from animals with chronic GVHD are specific for a public (common) determinant of MHC class II molecules. These T cells are considered autoreactive because they recognize public MHC class II determinants that are common to both donor and recipient rather than polymorphic histocompatibility antigens that are specific for the host. The autoreactive cells of chronic GVHD are associated with a damaged thymus, which can be injured by several mechanisms, including acute GVHD, the conditioning regimen, or age-related involution and atrophy. In chronic GVHD the ability of the thymus to delete autoreactive T cells (negative selection) and to induce tolerance is impaired. However, no clear data exist on the isolation of “autoreactive” donor-derived T-cell clones that equally recognize nonpolymorphic antigens from the donor and recipient cells. However, this does not directly preclude the existence of a causative role for autoreactive T cells. Chronic GVHD could also be a product of T cells that have undergone relatively chronic antigen stimulation as a result of the presence of inexhaustible and ubiquitous MiHA antigens. Allo-T cells under circumstances of chronic MiHA antigen stimulation can induce syndromes resembling those induced by the chronic antigen stimulation in autoimmune diseases. This concept is also consistent with the proposal of acute GVHD as a risk factor for chronic GVHD. The antigens targeted in chronic GVHD could be the same dominant ones targeted in acute GVHD, but the reactive T cells could be different; for example, they may secrete TGF-β. However, antigens other than those that were initially immunodominant (even those not initially targeted but introduced through epitope spread of either the nonpolymorphic “autoantigens” or the now-distinct MiHAs) could be important. It is also conceivable that regulatory mechanisms could frequently fail in allo-HCT, resulting in activation and expansion of T cells that recognize both nonpolymorphic and MiHA epitopes. One recent murine study suggested that development of chronic GVHD-like syndrome is target antigen-dependent. Furthermore, chronic GVHD pathogenesis could, in part, be a consequence of T cell priming by donor-derived APCs. In some patient subsets, responses to rituximab, presence of MiHA-specific antibodies, and the presence of chronic GVHD after T cell–depleted (TCD) allo-BMT would indicate that, in addition to donor T cells, donor B cells might be a direct effector or might have a role in priming T cells as APCs. Given the myriad clinical presentations of chronic GVHD that tend to occur at variable times after HCT, it is possible that separate pathogenic mechanisms are involved in causing distinct manifestations and that no single putative mechanism is sufficient to cause chronic GVHD. Nonetheless, a murine model demonstrated a pathogenic role for donor B cells and alloantibody production in causing experimental chronic GVHD.

THERAPY FOR CHRONIC GRAFT-VERSUS-HOST DISEASE

Chronic GVHD has a major impact on both quality of life and survival, frequently involves multiple organs, and necessitates prolonged immunosuppressive therapy. One report noted that 15% of cancer-free patients were still receiving immunosuppressive therapy after 7 years. The more severe forms of chronic GVHD are clearly associated with a lower disease-free survival. Thus the potential benefit of a GVL effect is shadowed by significant treatment-related mortality.

Current therapies for chronic GVHD have historically been of limited efficacy, particularly for patients who do not respond to frontline steroid-based therapy. A recent development was the FDA approval of the BTK inhibitor ibrutinib for the treatment of chronic GVHD following failure of one or more prior lines of therapy. This was based on an open label prospective design that reported an overall best response rate of 67% by NIH criteria, whereby a majority of responders experienced disease remission for greater than 5 months and cumulative reductions in their dosage of corticosteroids.[26] Another frequently used approach in some centers is ECP, which is thought to modulate alloreactivity of circulating T cells and DCs. Based on systemic review of prospective trials, extracorporeal photopheresis (ECP) demonstrated the greatest clinical effects in patients with cutaneous and GI tract involvement, as well as promoting steroid-sparing effects.[27] Nonetheless, the overall lack of standardized response criteria to measure therapeutic efficacy and the protean nature of this disease pose major obstacles to pursuing therapeutic trials in chronic GVHD. The NIH-sponsored consensus project has provided, for the first time, a set of standardized measures and definitions to use as response criteria in chronic GVHD. The NIH consensus conference has defined response measures classified in two main groups: clinician assessed and patient reported (Table 109.4).

The prevention of acute GVHD has not consistently resulted in a lower incidence of chronic GVHD. A clear example is the use of reduced-intensity transplants, consistently associated with a lower incidence of acute GVHD but with no major impact on chronic GVHD. The extended use of GVHD prophylaxis with CSP, or variations in the CSP dosage used, showed no beneficial effects on the incidence of chronic GVHD. The addition of thalidomide to CSP and methotrexate prophylaxis, the administration of IV Ig, and early treatment based on biopsy findings of subclinical GVHD in an attempt to preemptively treat chronic GVHD were unsuccessful. Currently, only the most-established practices for limiting chronic GVHD have involved use of bone marrow grafts and various methods that significantly deplete T cells in the graft inoculum (ATG, PtCy, CD34$^+$ selection), although as alluded to previously, none of these approaches have clearly established a survival advantage after HCT. The most commonly used therapy to treat chronic GVHD is prednisone. Sullivan and colleagues reported that prednisone alone is superior to prednisone plus azathioprine for primary treatment of patients with chronic GVHD. However, in patients classified as high risk on the basis of platelet counts less than 100,000/μL, treatment with prednisone alone resulted in only 26% 5-year survival. When a similar group of patients was treated with alternating-day CSA and prednisone, 5-year survival exceeded 50%. A recent randomized study of 287 patients with extensive GVHD found no statistically significant difference in nonrelapse death at 5 years or in cumulative incidence of secondary therapy at 5 years when prednisone alone was compared with prednisone plus CSA. For chronic GVHD that recurs or fails to respond to initial therapy, there is no standard treatment. Experimental therapies currently under evaluation include psoralen plus ultraviolet light A, MMF, thalidomide, total lymphoid

TABLE 109.4 National Institutes of Health Chronic GVHD Measures

Measure	Clinician-Assessed	Patient-Reported
Chronic GVHD-Specific Core Measures		
Signs	Organ-specific measures	Not applicable
Symptoms	Clinician-assessed symptoms	Patient-reported symptoms
Global rating	Mild, moderate, or severe	Mild, moderate, or severe
	0–10 severity scale	0–10 severity scale
	7-point change scale	7-point change scale
Chronic GVHD Nonspecific Ancillary Measures		
Function	Grip strength 2-min walk time	Patient-reported function
Quality of life	—	Patient-reported health-related quality of life

GVHD, Graft-versus-host disease.

irradiation, extracorporeal photopheresis, pentostatin, and acitretin. (Table 109.5 lists the commonly used GVHD drugs and their side effects.)

SYNGENEIC GRAFT-VERSUS-HOST DISEASE

A GVHD-like syndrome that is usually self-limited and predominantly affects the skin can occur in recipients of syngeneic or autologous transplants. It is also possible that some of the reported cases of syngeneic GVHD have reflected a mistaken assumption that the donor was syngeneic without extensive molecular confirmation. The condition manifests primarily as a rash that usually responds promptly to corticosteroid therapy. Although the level of severity may be grade II or III, the disease generally resolves promptly with the administration of glucocorticoids and is not life-threatening. Virtually all patients in whom a GVHD-like syndrome develops after syngeneic transplantation have been prepared with intensive conditioning regimens, usually involving irradiation. Experimental studies suggest that such conditioning is essential for the induction of thymic dysfunction, which is necessary for the development of the disease. Generation of autoreactive cells (a defect in thymic negative selection) and elimination of regulatory cells appear to be the requirements for the development of this disease. An additional hypothesis is that in some individuals maternal cells transmitted to them during their fetal development remain present throughout adult life. These observations suggest the possibility that, in some instances, small numbers of HLA-incompatible cells (derived from the donor's mother) may be transmitted with HLA-identical transplants. Transplacentally transferred maternal cells may also play a role in the development of neonatal GVHD.

TABLE 109.5 Commonly Administered Drugs for Graft-Versus-Host Disease Prophylaxis and Treatment

Drug	Mechanism	Adverse Effects
Corticosteroids	Direct lymphocyte toxicity; suppress proinflammatory cytokines such as tumor necrosis factor (TNF)-α	Hyperglycemia; acute psychosis; severe myopathy; neuropathy; osteoporosis; cataract development
Methotrexate (MTX)	Antimetabolite: inhibit T-cell proliferation	Significant renal, hepatic, and gastrointestinal toxicities
Cyclosporine A (CSA)	Interleukin (IL)-2 suppressor; blocks Ca^{2+}-dependent signal transduction distal to T-cell receptor (TCR) engagement	Renal and hepatic insufficiency; hypertension; hyperglycemia; headache; nausea and vomiting; hirsutism; gum hypertrophy; seizure with severe toxicity
Tacrolimus (FK506)	IL-2 receptor; blocks Ca^{2+}-dependent signal transduction distal to TCR engagement	Similar to CSA
Mycophenolate mofetil (MMF)	Inhibits de novo purine synthesis	Body aches; abdominal pain; nausea and vomiting; diarrhea; neutropenia
Sirolimus	Mammalian target of rapamycin (mTOR) inhibitor	Thrombocytopenia; hyperlipidemia, TTP
Antithymocyte globulin (ATG)	Polyclonal immunoglobulin	Anaphylaxis; serum sickness
JAK inhibitors	Targets multiple pathways distal to cytokine-regulated signaling via small molecule tyrosine kinase inhibition of JAKs and STATs	Blood cytopenias and infections
BTK inhibitors	Irreversible inhibition of both BTK and IL-2–inducible T-cell kinase (ITK) that impairs B-cell and T-cell function, respectively	Blood cytopenias and infections

Transfusion-Associated Graft-Versus-Host Disease

Most blood products administered to immunocompromised patients are now irradiated or at least leukocyte-depleted to avoid the transfusion of viable alloreactive T cells. With most homologous blood products, the MHC incompatibility between donor and recipient results in rapid clearance of transfused T cells by the recipient's immune system. However, occasionally, transfusions from donors who are homozygous for one of the recipient's MHC haplotypes are not recognized as foreign by the recipient. These cells can survive, "engraft," and mount an immunologic attack against the unshared haplotype in the patient, resulting in transfusion-induced GVHD. Transfusion-associated GVHD differs from GVHD occurring after transplantation in terms of kinetics and manifestations (i.e., with transfusion-associated GVHD, the recipient marrow is a major target). Because the number of stem cells in the offending blood product is inadequate, there is no hemopoietic recovery from donor cells. This syndrome is generally fatal as a result of refractory pancytopenia and/or other organ involvement.

GRAFT-VERSUS-LEUKEMIA RESPONSES

The GVL response after allogeneic HCT results from the immunologic attack of the host tissue and, by extension, the leukemia (i.e., the tumor). This response represents a potent form of immunotherapy that circumvents some of the "immunoediting" mechanisms used by tumor cells to develop in the hosts. The power of the alloimmune response to eliminate malignancy was first reported more than 50 years ago in experimental models by Barnes et al. However, GVL as its own entity and its close association with GVHD were not established until another 15 years later.

Clinical Features

Clinical evidence that the donor graft mediates important antileukemic effect comes from higher relapse rates for recipients of syngeneic stem cells than for recipients of HLA-matched sibling grafts. These findings have also been confirmed in a multicenter analysis of HCT recipients with acute myelogenous leukemia (AML) in first remission and subsequent retrospective analyses by the International Bone Marrow Transplant Registry (IBMTR). The second IBMTR analysis also showed that the magnitude of this GVL effect is greater for patients with chronic myelogenous leukemia (CML) and AML and not statistically significant for patients with acute lymphoblastic leukemia (ALL) in first remission.

Several case reports of patients with relapse of leukemia after allogeneic HCT noted remissions of the malignancy either after abrupt withdrawal of immunosuppression or during a flare of acute GVHD. Patients who develop GVHD after allogeneic HCT experience relapse less frequently than similar patients who do not develop clinical disease. GVHD is protective against relapse both for HCT recipients with advanced leukemia and for patients who receive transplants in earlier stages of malignancy. Additional analyses also suggest that the magnitude of the GVL effect appears to be disease and stage specific. Initial reports suggested that chronic GVHD was most protective against relapse, but other analyses demonstrate that acute GVHD is also protective. Based on these reports, newer trials of immunotherapy are designed to include cessation of immunosuppressive therapy (without taper). Withdrawal of immunosuppression can induce a GVL reaction for patients whose malignancy has relapsed after HCT, although such responses tend to occur in a minority of patients, coincide with GVHD, and may be transient in nature without additional consolidation (second HCT or DLI).

Another line of clinical evidence regarding the GVL effect of allogeneic HCT and its tight linkage to GVHD comes from the studies using T-cell depletion of the donor graft. Donor T cells included in the stem cell graft are critical for acute GVHD, and T-cell depletion by various strategies is one of the most successful means of reducing the incidence and severity of GVHD after allogeneic HCT. Unfortunately, although T-cell depletion results in less treatment-related morbidity and mortality, improved overall survival rates have not been reliably demonstrated. This failure is due in large part to a reciprocal increase in the subsequent relapse rate after T-cell depletion, as well as to graft failure and other complications. T-cell depletion increases relapse rates particularly in CML. This observation provides further strong, albeit indirect, evidence that allogeneic donor T cells are important mediators not only of GVHD but also of the GVL properties of the allogeneic stem cell graft. Finally, the most compelling evidence of donor T cells in mediating GVL comes from the observations made from donor lymphocyte infusions (discussed later). The induction of GVL is a complex process.

Genetic Basis

The immunotherapeutic effect that occurs in allo-BMT setting is primarily mediated by allogeneic donor T or NK cells directed against the alloantigens shared by the recipient tumor and target tissues and/or tumor-specific antigens that have the advantage of not being subjected to tolerance mechanisms by the host tumor. Understanding of the exquisite specificity of T-cell responses led to attempts to identify specific antigens that are responsible for the GVL effect. Much of the focus has been on the identification of (1) certain oncogenic viral proteins (because these are absent in normal cells but expressed by transformed tumor cells [certain Epstein-Barr virus (EBV) peptides such as EBNA-1, LMP-1, LMP, LCL]), (2) antigens that are expressed in a tissue-specific fashion (melanoma-specific proteins), and (3) proteins that are overexpressed in tumors (WT1, proteinase 3, survivin, telomerase reverse transcriptase, CYPB1, and HER2/neu). Although these antigens are specific, most T-cell responses to these antigens are limited because of the poor immunogenicity of these proteins, expression on normal cells, defects in the processing or presentation of tumor antigens, or production of factors that disable T-cell responses. Thus clinical attempts to obtain high specificity of T-cell responses have been offset with difficulties in obtaining enough sensitivity and vice versa. Furthermore, given the current concepts of stem cell origins of leukemia and cancers, identification of the immunogenic proteins that are specifically expressed in the malignant stem cells and harnessing T-cell responses to those antigens will be needed for the optimal GVL effect to cure malignancy.

In contrast to the tumor-specific or tumor-associated antigens discussed earlier, alloantigens are not subjected to tolerance mechanisms. Vaccination strategies with autologous T cells using tumor-associated or tumor-specific antigens have yielded disappointing clinical antitumor responses. By contrast, allogeneic HCT has met with remarkable GVL responses, perhaps owing to recognition of minor alloantigens in addition to the tumor-associated antigens. This concept has been demonstrated by recent murine studies, which showed that alloantigen on the tumor cells is required for GVL responses and that the principal targets of GVL are the immunodominant allogeneic MiHAs rather than the tumor-associated antigens. Thus T cells specific for MiHA antigens could provide for a potent GVL effect. Significant progress has been made in the identification of MiHAs that are specifically expressed in the host hematopoietic tissues and therefore might allow for a GVL response without causing GVHD. Together, these results suggest that, in addition to tumor-specific proteins, expression of alloantigens and cognate interactions between donor T cells and the tumor tissues are required for the effective induction of the majority of GVL responses. However, T cells specific for some MiHAs are also responsible for GVHD, and a means of consistently separating the beneficial GVL effect from GVHD has not yet been clinically achieved.

KIR Polymorphisms

The two competing models described earlier, the "mismatched ligand" and the "missing ligand" models for HLA-KIR allorecognition, have been supported by clinical observations of GVL responses in different patient and transplant populations. The former model has been shown to separate GVL and GVHD responses in the context of TCD haploidentical HCT for AML. Even though this model is supported by elegant laboratory studies, it was found to be invalid for ALL and also for AML after unrelated donor HCT with immunosuppression. Recent retrospective clinical data suggest that GVHD and GVL can be separated by the "missing ligand" model in CML/AML and myelodysplastic syndrome patients after TCD HLA-identical sibling HCT. Further validation of either models by clinical prospective studies and a better understanding of the balance between the inhibitory and activating receptor-ligand interaction of the NK cells are needed to adequately exploit the interface between HLA-KIR genetics to separate GVHD from GVL.

Immunobiology of Graft-Versus-Leukemia Responses

Given the tight association of clinical GVHD and GVL, as well as the common biologic principles governing these responses after allogeneic HCT, it is important to discuss the similarities and distinctions between them in the context of the three cellular phases of GVHD.[28]

Phase 1: Activation of Antigen-Presenting Cells

The concept that tumor eradication after allogeneic HCT might not require toxic chemoradiotherapy and could be achieved primarily by the immunotherapeutic effect from the GVL responses has led to the clinical development of nonmyeloablative HCT for hematologic and nonhematologic malignancies. Phase 1 is characterized by the development of cytokine storm–generated danger signals from the conditioning regimen and the subsequent activation of APCs. Experimental data suggested that the reduction in conditioning would attenuate the cytokine storm, lead to the development of mixed donor-host chimerism, and confine the GVH response primarily to secondary lymphoid organs, thus causing less severe GVHD without impairing GVL responses. However, nonmyeloablative HCT has delayed the kinetics but did not reduce the overall incidence of GVHD, and a significant number of patients either failed to respond or relapsed. Furthermore, recent murine and human studies have suggested that homeostatic expansion of T cells in a lymphopenic environment induced by conditioning (as opposed to mere immunosuppression) improves the antitumor efficacy of adoptively transferred syngeneic or autologous T cells by increasing the availability of space, enhancing the memory responses, and reducing the competition for homeostatic cytokines (e.g., IL-7 and IL-15) for transferred T cells while eliminating Tregs. Thus low-intensity HCT clearly demonstrates the principle of GVL effect, but the roles of cytokine storm and homeostatic expansion of allogeneic T cells in shaping the intensity of GVL responses are not known.

Host and donor APCs are critical for the induction and severity of GVHD.[8] Activation of APCs is the key step in phase 1 of GVHD. Significant progress has been made in understanding the role of APCs in GVL. Recent experimental evidence has demonstrated a crucial role for professional host APCs in the induction of GVL responses mediated by donor T cells, even when the tumor cells showed some features of APCs. Tumors that merely express costimulatory molecules may still be unable to stimulate an effective immune response because of their various "immunoediting" processes that cause ineffective antigen presentation. However, when the tumor cell itself functions as a professional APC, as with CML, it can generate an effective GVL response. By contrast, cancers such as acute leukemias that seldom differentiate into APCs generate poor GVL responses. Data also demonstrated that given sufficient time and a low tumor burden, cross-presentation of tumor-associated antigens and/or alloantigens by professional donor APCs can occur and may promote or sustain GVL responses by maintaining or expanding alloreactive T cells after initial priming on host APCs. This concept is consistent with clinical GVL responses in CML in which the final stage of a GVL response to CML may be the result of donor T cells responding not directly to the small number of CML stem cells or progenitors (which would be undifferentiated and therefore poor APCs) but to tumor antigens cross-presented on professional donor APCs. Emerging data suggest that enhancing such cross-presentation is sufficient to elicit effective GVL responses against acute or advanced leukemia. However, these data suggest that GVL responses generated after low-intensity conditioning may not be as robust as those after full intensity HCT and highlight the need for a clearer understanding of the effects of cytokine storm and lymphopenia generated danger signals on the activation of APCs in mediating GVL.

Phase 2: Donor T Cell Activation

The core of GVL responses, as with GVHD, is also dependent on the activation of appropriate numbers of T cells. The "second" signals from professional APCs (or certain tumor cells that function as effective APCs) are critical for generating an effective GVL response. Several of the costimulatory pathways that modulate GVHD have also been evaluated in mediating GVL responses. Blockade of CD28 costimulation preserved GVL responses but reduced GVHD in murine studies. However, when the tumor cells also expressed B7 molecules, such blockade reduced the GVL responses. Ex vivo blockade of CD40-CD40L interaction has been shown to reduce GVHD by generating Tregs but still preserve GVL. By contrast, blockade of the 4-1BB pathway reduced both GVHD and GVL. The other costimulatory molecules (OX40 and ICOS) and the inhibitory molecules (CTLA-4 and PD-1) also modulate antitumor responses. A better understanding of the context (i.e., low intensity or DLI) and the hierarchy of timing, duration, and extent of costimulatory requirements of donor T-cell subsets might allow for balancing the intensity of GVL and GVHD responses. Clinical and experimental evidence suggests that donor T cell numbers correlate with the severity of GVHD and GVL responses. TCD grafts had reduced GVHD but increased disease relapse, suggesting a role for T-cell numbers in GVL responses as well. Clinical attempts to separate GVHD and GVL by regulating allogeneic T cell dose have met with limited success. For example, administration of 1×10^5 T cells/kg after HLA-matched sibling transplantation did not mediate GVL effects and yet was associated with a measurable incidence of GVHD. Thus infusion of right numbers of donor T cell effectors is crucial for GVL responses. This has been demonstrated by durable responses that are observed in CML and other malignancies after DLI (see later), despite the experimental evidence that host APCs stimulate a stronger GVL response than do donor APCs. This could be because, clinically, DLI is almost always given without immunosuppression to an individual who has not developed GVHD either from the chemical immunosuppression or physical removal of donor T cells from the allograft. This lack of immunosuppression after DLI increases the likelihood of a GVH response, and DLI is almost always associated with clinical GVHD. The delivery of additional allogeneic effector cells in DLI also increases the effector:target ratio compared with the time of initial HCT. The latter is also clinically demonstrated by a more effective GVL response to DLI against minimal residual disease (BCR-ABL positivity by polymerase chain reaction [PCR]) compared with the response against high leukemic burden (e.g., blast crisis) in CML patients. Thus DLI provides the proof in principle for the concepts that sufficient T-cell numbers and appropriate antigen presentation are required for both GVHD and an effective GVL response.

T-Cell Subsets

Most experimental studies have implicated donor $CD8^+$ T cells as the primary mediators of GVL, but there are no clinical data for $CD8^+$-mediated GVL responses in the absence of $CD4^+$ T cells. Moreover, some clinical data suggest a role for greater CD4-mediated GVL responses without an increase in GVHD after allogeneic HCT and DLI, but it is unclear whether $CD4^+$ T cell–initiated GVL responses occur in the absence of generation of MiHA-specific $CD8^+$ T cells. Given the critical requirement of alloantigens for most GVL responses, the specific requirement of CD4 and/or CD8 T cells for GVL and GVHD is likely to be determined by the expression of the relevant immunodominant MiHAs and/or tumor-associated proteins. Therefore it is unlikely that GVHD and GVL responses can be separated under all circumstances merely by depletion of either subset of alloreactive T cells. However, experimental data suggest it might be possible to separate GVHD and GVL when certain donor T-cell subsets are either depleted or infused (DLI) at appropriate interval after transplant. But the optimal time interval, if any, after clinical HCT is yet to be determined.

Because of recent identification and understanding of the role of various T-cell subsets in mediating immune responses, depletion of specific T-cell subsets to separate GVHD and GVL remains an area of active investigation. For example, recent experimental data suggest that CD62L-expressing naïve T cells home to secondary lymph nodes and are critical for initiating GVHD. In contrast, CD62L-negative effector memory T cells with enhanced reactivity to recall antigens mediated GVL responses with minimal GVHD. An important caveat to these data is the fact that the lack of a priori knowledge of the repertoire of human memory T cells would make it difficult to predict whether these cells might cross-react only with tumor-associated antigens or with the recipient's alloantigens. Using CD62L status alone as determinant of GVHD potential can also have other unintended consequences; recent studies have demonstrated that its expression is critical for the regulation of GVHD by Tregs (see later). Moreover, it is not known whether the behavior of human memory T cells parallels that of murine memory T cells in their migratory, functional, and cytolytic capabilities. Although Tregs reduce antitumor immunity in murine models and in human subjects, experimental data suggest that administration of donor-type Tregs either at the time of HCT or when delayed reduced GVHD but preserved $CD8^+$-mediated perforin-dependent GVL responses. Similar preservation of experimental GVL was also observed by harnessing donor NKT function with G-CSF analogs. However, it remains unclear whether these observations are valid after clinical HCT when the GVL responses might not be entirely dependent on $CD8^+$ T cells.

T-Cell Migration

It is conceivable that manipulation of these interactions to focus the alloimmune response to lymphohematopoietic tissues would enhance GVL responses but not GVHD. For example, blockade of CCR9 ligand TECK or CCR5 and CCL17 may prevent the migration of donor T cells to GI tract and skin, respectively, but preserve GVL. Pharmacologic manipulation with the immunosuppressive agent FTY720 has recently provided the proof in principle for this approach. Given the redundancy, strategies to modulate the chemokine biology for separation of GVHD and GVL will require greater understanding of these networks in modulating the migration not only of specific T-cell subsets but also of the other immune cells in the context of different conditioning regimens.

Phase 3: Effector Phase of Graft-Versus-Leukemia

The effector arm of GVL is also characterized primarily by the antigen-specific cellular components and less by the inflammatory components of alloresponse. Experimental data demonstrate that neutralization of IL-1α reduced GVHD but preserved GVL. By contrast, donor TNF-α secretion contributes to both GVHD and GVL effects, and in some cases, antagonism of TNF-α reduced GVHD and GVL responses. Nonetheless, antagonism of nonspecific inflammatory effectors (e.g., either IL-1 or TNF-α) appears to regulate GVHD to a greater extent than GVL responses after experimental allogeneic HCT.

Several lines of experimental and clinical data demonstrate that antigen-specific donor T-cell subsets and NK cells are the key effectors of GVL. The cytotoxic pathways that are operative in the NK and T cell–mediated antitumor responses have been well characterized. Fas-mediated CTL of tumor targets is used by both NK and T (mostly Th1) cells, but most murine experiments with FasL-deficient donor T cells suggested that FasL is a key effector molecule for causing GVHD but not GVL. However, one study found that FasL is required for $CD4^+$–mediated GVL against myeloid leukemia. By contrast, even though perforin-mediated CTL pathways are also used by T (mostly Th2) and NK cells, experimental data with perforin-deficient donor T cells demonstrated a loss of GVL with a diminution in the severity of GVHD. In some other experimental models, perforin was required only for GVL but not for GVHD. Recent data showed that TRAIL-mediated CTL had no effect on GVHD severity but was required for optimal GVL. Therefore strategies that increase donor T-cell TRAIL expression or enhance the susceptibility of tumors to TRAIL-mediated CTL (e.g., HDAC inhibitors) may promote a robust GVL effect without exacerbating GVHD. Thus a significant progress has been made in recent understanding of the CTL pathways used by donor T cells for GVL responses, but the role and context of use of these pathways by donor NK and NKT cells after allogeneic HCT are not known.

DONOR LEUKOCYTE INFUSIONS

Until recently, the evidence for an important GVL effect in clinical transplantation was strong but largely circumstantial. The use of DLI to treat relapses after allogeneic HCT has now provided direct evidence of the GVL effect. Kolb and colleagues first reported three patients with relapsed CML who achieved complete cytogenetic remission after treatment with IFN-α and DLI from the original donors. Subsequently, these findings have been confirmed in several reports. Two large retrospective studies of DLI have been reported from Europe and North America. Although the treatment protocols varied slightly among institutions, the results have been remarkably consistent. When results from these trials are combined, the complete remission rate for patients treated for relapsed CML is consistently 60% to 80%. In many patients with CML, the response to DLI is not immediate. The average time to obtain a molecular remission is between 4 and 6 months, but disease-free survival after DLI depends on the stage of CML. Complete cytogenetic and molecular responses are achieved in almost 80% of patients treated with either early relapse (cytogenetic or molecular) or hematologic relapse of chronic phase. Patients with more advanced CML (accelerated phase or blast crisis) are less likely to respond. Two recent analyses suggest that the outcome after unrelated DLI is similar to matched sibling DLI in CML patients.

DLI has also produced remissions in acute leukemias. Several retrospective studies have reported response rates in AML ranging from 20% to 65%. A prospective study of 65 patients with advanced myeloid leukemia who received cytarabine and G-CSF–primed DLI showed that 47% of the patients achieved complete remission, with an overall survival of 19% at 2 years. A recent European Blood and Marrow Transplantation (EBMT) analysis of DLI in patients with relapsed AML showed a response rate of 41%, which did not change if chemotherapy was used before DLI. In general, DLI produces lower response rates and higher relapse rates in patients with AML than in those with CML. Extramedullary relapses at multiple regions that are usually not considered sanctuary sites for leukemia have been observed after DLI.

Although GVL responses to ALL after allogeneic HCT have been noted, DLI for ALL has generally been ineffective. Reports both from Europe and the United States found little benefit for DLI in ALL patients. Furthermore, Ruggeri and others found that, following TCD haploidentical HCT where the donors had antirecipient NK cells, the probability of relapse was 85% for ALL in contrast with 0% for AML.

The experience with DLI for other hematologic malignancies such as non-Hodgkin lymphoma and multiple myeloma (MM) is much more limited. Case reports and small series demonstrate responses to DLI in patients with posttransplant relapses of low-grade lymphoma and CLL and also after low-intensity HCT. In MM, complete responses to DLI were observed in 25% of the patients in two small series. DLI has also been shown to induce complete remission in a majority of patients with posttransplant lymphoproliferative disorders (PTLDs) after allogeneic HCT. Viral infections may be treatable with DLI, and adoptive immune therapy with T cells specific for EBV and CMV have been shown to both treat and prevent these complications.

Complications of Donor Leukocyte Infusions

Adoptive immunotherapy with DLI causes significant morbidity. The major complications are myelosuppression and GVHD. Myelosuppression with anemia and/or thrombocytopenia and/or leucopenia and/or pancytopenia occurs in 34% of the patients. Marrow aplasia presumably results from the destruction of host leukemia cells before recovery of normal donor hematopoiesis. This idea is supported by the observation that patients treated with donor mononuclear cell infusions for cytogenetic or molecular relapse rarely experience pancytopenia. Occasionally, marrow aplasia has been persistent, although this toxicity has been successfully reversed with the infusion of additional donor stem cells in some patients. If pancytopenia is associated with chronic GVHD, then immunosuppression might be the most appropriate therapy. Acute GVHD and chronic GVHD have been the major direct complications from DLI. In retrospective and prospective studies, acute or chronic GVHD has been reported in 40% to 60% of evaluable patients. In most studies, GVHD correlates with GVL activity and response. In the North American analysis, more than 90% of complete responders developed acute GVHD, and 88% of responders developed chronic GVHD. Of 23 patients who did not experience GVHD, only 3 achieved a complete remission. In 92 patients who had no response, only 35% had acute GHVD and only 13% had chronic GVHD. In the EBMT analysis, 41% of DLI recipients developed grade II to IV acute GVHD. However, it should be noted that many patients who fail to respond to GVL induction will die shortly of progressive disease and may not survive long enough for GVHD to develop. This is particularly important for patients with acute leukemia. It is also important to emphasize that a number of complete responses were seen in patients without any sign of GVHD. The GVL effect in the absence of clinical GVHD provides important evidence for GVL activity separate from GVHD.

In general, GVHD that occurs after DLI has been mild to moderate and has been responsive to immunosuppressive therapy. This observation is important because the dose of T cells often administered with DLI may be 10- to 100-fold higher than the T-cell dose administered at the time of transplant, and DLI is given without additional immunosuppression. At the time of transplant, toxicity is unacceptable if immunosuppression is withheld or if a dose of donor T cells similar to that used for DLI is given (to augment GVL). This may be because the effects of GVHD are more tolerable when separated from the acute transplant toxicity. GVHD can be stimulated and exacerbated by the cytokine storm that may accompany transplantation. Tissue damage from the intensive conditioning regimen, infections, and other physiologic insults results in a cascade of events that ultimately augments the GVHD reaction. When GVHD is induced independently of other transplant related toxicity, it may be more manageable with appropriate immunosuppression. Nevertheless, in some cases DLI-induced GVHD may be quite severe; as shown in Table 109.2 , 20% to 35% of DLI recipients can be anticipated to develop grade III or IV acute GVHD. Furthermore, acute GVHD has contributed to death in almost 10% of patients. Chronic GVHD occurs in 30% to 60% of recipients of unmanipulated DLI.

It is notable that the clinical presentation of GVHD after DLI is somewhat different from that after myeloablative allogeneic HCT. For instance, the onset of GVHD may be later after DLI. The median time to onset of acute GVHD is approximately 32 to 42 days compared with a median time to onset of 16 to 20 days following myeloablative T cell–replete transplantation. Therefore, not only can the severity of GVHD be influenced by the use of intensive conditioning, but the tissue damage and inflammatory cytokine release may influence the pace of GVHD development as well. This possibility is supported by the finding that time to onset of acute GVHD is also delayed following reduced intensity transplantation. The target organs of acute GVHD following DLI are the same as those seen following HCT, but the clinical manifestations may differ. A hepatic variant of liver GVHD characterized by marked elevations of serum aminotransferase levels more than 10 times the upper limit of normal was observed in 11 of 73 (15%) patients who received DLI at Johns Hopkins University. Characteristic skin, liver, and intestinal acute GVHD manifestations can be seen following DLI, but their frequency and severity has not been well described. In a study of 81 patients who received DLI for relapse, mixed chimerism, or prophylaxis following reduced-intensity HCT, skin, liver, and intestinal GVHD developed in 26%, 8%, and 14%, respectively. Likewise, characteristic findings of chronic GVHD can be seen following DLI, and on occasion both acute and chronic GVHD can develop simultaneously following DLI.

In recent years, DLI has been used in the context of reduced-intensity allogeneic HCT. When conditioning is minimal and GVHD has not already occurred, DLI is often required to either reverse mixed chimerism or treat persistent disease. GVHD after DLI following nonmyeloablative setting develops 19% to 45% of the time, whereas chronic GVHD develops 28% to 34% of the time. In two studies of a combined 134 subjects who received DLI after reduced-intensity transplantation, 37 (28%) patients developed acute GVHD, 15 (11%) developed grade III or IV GVHD, and 8 (6%) died from GVHD. There was no statistically significant relationship between DLI dose and GVHD in either study. The clinical features of GVHD after DLI following reduced-intensity HCT are similar to the GVHD that occurs after DLI is used to treat relapse following myeloablative HCT. The similarity between GVHD after DLI in either setting suggests that the reduction of tissue damage associated with either no chemotherapy (as in DLI for relapse following prior myeloablative HCT) or reduced-dose chemotherapy (as in DLI after reduced-intensity HCT) may be an important common theme.

It is likely that effector cells responsible for the GVL and GVHD effects of HCT will similarly be responsible for the GVL effect associated with DLI, although this assumption has not been formally proven. The administration of select subsets of donor mononuclear cell fractions is the ideal setting in which to dissect the cellular mechanisms responsible for GVL induction, and strategies that delay the infusion of these various cellular subsets will help to define the mechanisms and enhance the efficacy of DLI.

FUTURE DIRECTIONS

Complications of HCT, particularly GVHD, remain major barriers to the wider application of allogeneic HCT for a variety of diseases. Recent advances in the biology of genetic polymorphisms, host microbiome, chemocytokine networks, several novel cellular subsets including Tregs, and the direct mediators of cellular cytotoxicity have led to improved understanding of this complex disease process. Animal studies show that modulation of several mediators of the complex GVHD cascade may be able to reduce the undesirable inflammatory aspects of GVHD while preserving the benefits of GVL. However, most of the laboratory observations remain to be studied in well-controlled clinical trials. Multiple cellular effectors may be involved in GVL, although donor T-cell recognition of host antigens is an important element of this process. Cellular immunotherapy such as DLI offers a strategy for separating GVHD and the GVL effect. Both experimental and clinical data suggest that posttransplantation cellular immunotherapy can be performed relatively safely and effectively, and optimization of patient selection, cell dose, and timing of administration may all serve to limit toxicity and enhance the potential GVL effects.

REFERENCES

1. Gragert L, Eapen M, Williams E, et al. HLA match likelihoods for hematopoietic stem-cell grafts in the U.S. registry. *N Engl J Med*. 2014; 371:339.
2. Martin PJ, Levine DM, Storer BE. Genome-wide minor histocompatibility matching as related to the risk of graft-versus-host disease. *Blood*. 2017; 129(6):791.
3. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet*. 2009;373(9674):1550–1561.
4. Hill GR, Betts BC, Tkachev V, Kean LS, Blazar BR. Current concepts and advances in graft-versus-host disease immunology. *Annu Rev Immunol*. 2021;39:19–49. Epub 2021/01/12; PMCID: PMC8085043.
5. Harris AC, Young R, Devine S. International, multi-center standardization of acute graft-versus-host disease clinical data collection: a report from the MAGIC consortium. *Biol Blood Marrow Transplant*. 2016;22(1):4.
6. MacMillan ML, Robin M, Harris AC. A refined risk score for acute graft-versus-host disease that predicts response to initial therapy, survival, and transplant-related mortality. *Biol Blood Marrow Transplant*. 2015; 21:761.
7. Verneris MR, Lee SJ, Ahn KW. HLA mismatch is associated with worse outcomes after unrelated donor reduced-intensity conditioning hematopoietic cell transplantation: an analysis from the center for international blood and marrow transplant research. *Biol Blood Marrow Transplant*. 2015;21:1783.
8. Lee SJ, Klein J, Haagenson M. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. *Blood*. 2007;110:4576.
9. Pidala Lee SJ, Ahn KW. Nonpermissive HLA-DPB1 mismatch increases mortality after myeloablative unrelated allogeneic hematopoietic cell transplantation. *Blood*. 2014;124:2596.
10. Shono Y, van den Brink MRM. Gut microbiota injury in allogeneic haematopoietic stem cell transplantation. *Nat Rev Cancer*. 2018;18(5): 283–295.
11. Jenq RR, Ubeda C, Taur Y, et al. Regulation of intestinal inflammation by microbiota following allogeneic bone marrow transplantation. *J Exp Med*. 2012;209:5.
12. Levine JE, Huber E, Hammer STG, et al. Low Paneth cell numbers at onset of gastrointestinal graft-versus-host disease identify patients at high risk for nonrelapse mortality. *Blood*. 2013;122:8.
13. Mathewson ND, Jenq R, Mathew AV, et al. Gut microbiome-derived metabolites modulate intestinal epithelial cell damage and mitigate graft-versus-host disease. *Nat Immunol*. 2016;17:5.
14. Watkins B, Qayed M, McCracken C. Phase II trial of costimulation blockade with abatacept for prevention of acute GVHD. *J Clin Oncol*. 2021;39(17):1865–1877.
15. Brunstein CG, Miller JS, Cao Q. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood*. 2011;117:1061.
16. Bleakley M, Heimfeld S, Loeb KR. Outcomes of acute leukemia patients transplanted with naive T cell-depleted stem cell grafts. *J Clin Invest*. 2015;125:2677.
17. Levine JE, Braun TM, Harris AC. A prognostic score for acute graft-versus-host disease based on biomarkers: a multicentre study. *Lancet Haematol*. 2015;2(1):e21–e29.
18. Major-Monfried H, Renteria AS, Pawarode A. MAGIC biomarkers predict long-term outcomes for steroid-resistant acute GVHD. *Blood*. 2018; 131(25):2846.

19. Holtan SG, Versluis J, Weisdorf DJ, Cornelissen JJ. Optimizing donor choice and GVHD prophylaxis in allogeneic hematopoietic cell transplantation. *J Clin Oncol*. 2021;39(5):373–385.
20. O'Meara A, Kapel N, Xhaard A, et al. Fecal calprotectin and alpha1-antitrypsin dynamics in gastrointestinal GvHD. *Bone Marrow Transplant*. 2015;50(8):1105.
21. Robinson T, O'Donnell PV, Fuchs EJ. Haploidentical bone marrow and stem cell transplantation: experience with post-transplantation cyclophosphamide. *Semin Hematol*. 2016;53(2):90.
22. Bolaños-Meade J, Reshef R, Fraser R. Prevention of graft versus host disease with hematopoietic cell transplantation with reduced intensity conditioning—a comparison of three prophylaxis regimens (tacrolimus/mycophenolate mofetil/cyclophosphamide, tacrolimus/methotrexate/bortezomib or tacrolimus/methotrexate/maraviroc) versus tacrolimus/methotrexate: a randomised phase 2 trial with a non-randomised contemporaneous control group (BMT CTN 1203). *Lancet Haematol*. 2019;6(3):e132.
23. Socié G, Vigouroux S, Yakoub-Agha I. A phase 3 randomized trial comparing inolimomab vs usual care in steroid-resistant acute GVHD. *Blood*. 2017;129(5):643.
24. Drobyski WR, Pasquini M, Kovatovic K. Tocilizumab for the treatment of steroid refractory graft-versus-host disease. *Biol Blood Marrow Transplant*. 2011;17(12):1862.
25. Jagasia M, Perales M-A, Schroeder MA. Ruxolitinib for the treatment of steroid-refractory acute GVHD (REACH1): a multicenter, open-label phase 2 trial. *Blood*. 2020;135(20):1739.
26. Miklos D, Cutler CS, Arora M, et al. Ibrutinib for chronic graft-versus-host disease after failure of prior therapy. *Blood*. 2017;130(21): 2243.
27. Abu-Dalle I, Reljic T, Nishihori T, et al. Extracorporeal photopheresis in steroid-refractory acute or chronic graft-versus-host disease: results of a systematic review of prospective studies. *Biol Blood Marrow Transplant*. 2014;20:1677.
28. Blazar BR, Hill GR, Murphy WJ. Dissecting the biology of allogeneic HSCT to enhance the GvT effect whilst minimizing GvHD. *Nat Rev Clin Oncol*. 2020;17(8):475–492.

CHAPTER 110

SUPPORTIVE CARE FOR THE TRANSPLANT PATIENT

Abraham S. Kanate and Navneet S. Majhail

Although hematopoietic cell transplantation (HCT) is a therapeutic option for various hematological and non-hematological disorders, it can result in toxicities and complications, leading to significant treatment related morbidity and non-relapse mortality (NRM). Based on the temporal association from the date of HCT, these may be divided into early complications (within the first 3 months of HCT) or late complications (beyond 3 months of HCT), but can vary between patients based on comorbidities, type of HCT (autologous versus allogeneic), conditioning regimens and intensity, type and duration of immunosuppressant use, antimicrobial prophylaxis, and long-term post-transplant survivorship care (Fig. 110.1). By etiology post-HCT complications may also be classified as infectious, early non-infectious complications, late non-infectious complications, and graft-versus-host disease (GVHD) (Table 110.1). Identification of risk factors for complications allows the design of risk-specific treatment and supportive care plans that may reduce treatment-related morbidity and mortality.

INFECTIONS

Infections are common causes of NRM in HCT recipients and cause significant morbidity, in both the early and late transplant period (Table 110.2). Immune defects occurring in the post-transplant period can be divided into predictable phases based on time from transplant, with characteristic infections in each phase (see Fig. 110.1). Antimicrobial prophylaxis tailored to address the risk of specific infections are effective in decreasing the incidence of post-transplant infections (Table 110.3). Evidence-based guidelines for preventive strategies have been published and can be used as a reference for determining infection risk and assigning antimicrobial prophylaxis for an individual patient.[1,2]

Neutrophil engraftment (sustained absolute neutrophil count >500/μL) generally occurs within 10 to 14 days in autologous and 14 to 28 days in allogeneic HCT recipients. Allografts utilizing bone marrow or umbilical cord (UCB) as the graft source tend to engraft later compared to peripheral blood allografts. Importantly, 5% to 10% of allo-HCT recipients may have primary graft failure, causing prolonged neutropenia and transfusion dependence. Risk factors for infection during the neutropenic phase are disruption of mucocutaneous barriers and indwelling venous catheters. Bacterial infections can occur in ~30% of transplant recipients during this initial period and usually arise from normal flora of the skin (coagulase-negative *Staphylococcus*), oropharynx, and gastrointestinal tract (*Streptococcus viridans*, *Enterococcus* spp. and enteric gram-negative bacilli). Colonizing yeasts or molds may also cause systemic mycotic infections (usually *Candida* or *Aspergillus* spp.) in ~10% to 15% of patients due to neutropenia and disruption of normal host flora. Reactivation of herpes virus can occur in the absence of prophylaxis.

The predominant defect seen in the early to late post-engraftment period is impaired cellular and humoral immunity. This state of underlying severe immune dysfunction is enhanced and prolonged by acute and chronic GVHD needing corticosteroids and other immunosuppressive agents, sometimes lasting beyond 2 years of HCT. The incidence of late opportunistic infections is lower in autologous HCT recipients because of relatively faster immune reconstitution. Patients with chronic GVHD can be functionally asplenic and at risk of infections by encapsulated bacteria. In addition, chronic GVHD patients on long-term immunosuppression are susceptible to fungi (*Aspergillus* spp., *Candida* spp., and *Pneumocystis jiroveci*) and viruses (cytomegalovirus [CMV]) and varicella zoster virus [VZV]). Additional factors that can delay immune reconstitution include donor-recipient human-leukocyte antigen (HLA) disparity, T-cell depletion (either by graft manipulation or in vivo by use of antithymocyte globulin or post-transplant cyclophosphamide) and donor type (unrelated donor, haploidentical donor and UCB transplantation).[3] Recommended antimicrobial prophylaxis should continue beyond the initial post-transplant period, typically for at least 3 to 6 months after cessation of all immunosuppression, especially in those with chronic GVHD. Some centers use total T-cell (CD3+) and particularly CD4 cell levels as surrogate markers of T-cell immunity and to guide duration of antimicrobial prophylaxis. Supplemental intravenous immunoglobulin (IVIG) has been considered for patients with persistent hypogammaglobulinemia (IgG levels <400 mg/dL), but its prophylactic use is costly, does not prolong survival or prevent late infections and may impair humoral immune reconstitution. Patients with GVHD and those with indwelling venous access who undergo dental procedures should receive antibiotics for endocarditis prophylaxis. Published guidelines are available for recommendations on immunization of HCT recipients (Table 110.4).[1,4,5]

Owing to the shorter duration of neutropenia in reduced intensity/non-myeloablative allogeneic (RIC/NMA) HCT, the incidence of bacterial infections is usually lower. However, the degree and duration of lymphodepletion are comparable to myeloablative regimens and the risks of opportunistic infections such as pneumocystis pneumonia, invasive aspergillosis and CMV reactivation remain unchanged. Among UCB and haploidentical donor allo-HCT recipients, neutrophil engraftment and immune reconstitution can be delayed and a higher incidence of bacterial and viral infections in the early post-transplant period has been reported,[6,7] but does not affect overall mortality and NRM. Infections that are unique to the HCT recipients are discussed in further detail here.

Febrile Neutropenia

Fever is a common occurrence in the early post-transplantation neutropenic phase, although an infectious pathogen is identified in only ~50% of the patients. Fever may also be due to tissue inflammation (oropharyngeal or enteric mucositis), transfusions, or drug reactions. Bacterial infections due to aerobic bacteria such as *viridans* streptococci and enteric gram-negative bacilli are the primary concern during this period, although yeast infections are also a possible cause of febrile neutropenia. Prophylactic strategies including suppressive antimicrobials such as fluoroquinolones, and azoles are considered during this period.

At the onset of febrile neutropenia, empiric therapy with broad spectrum antibiotics is promptly started along with appropriate clinical and microbiologic evaluation. The choice of antibiotics depends on prior and current antibiotic usage, accounting for local resistance patterns. These recommendations are based on the available guidelines for the treatment of febrile neutropenia in cancer patients.[8] Among patients with persistent febrile neutropenia, that is fever without an identified focus that continues despite 3 to 5 days of appropriate broad-spectrum antibiotics, invasive fungal infections

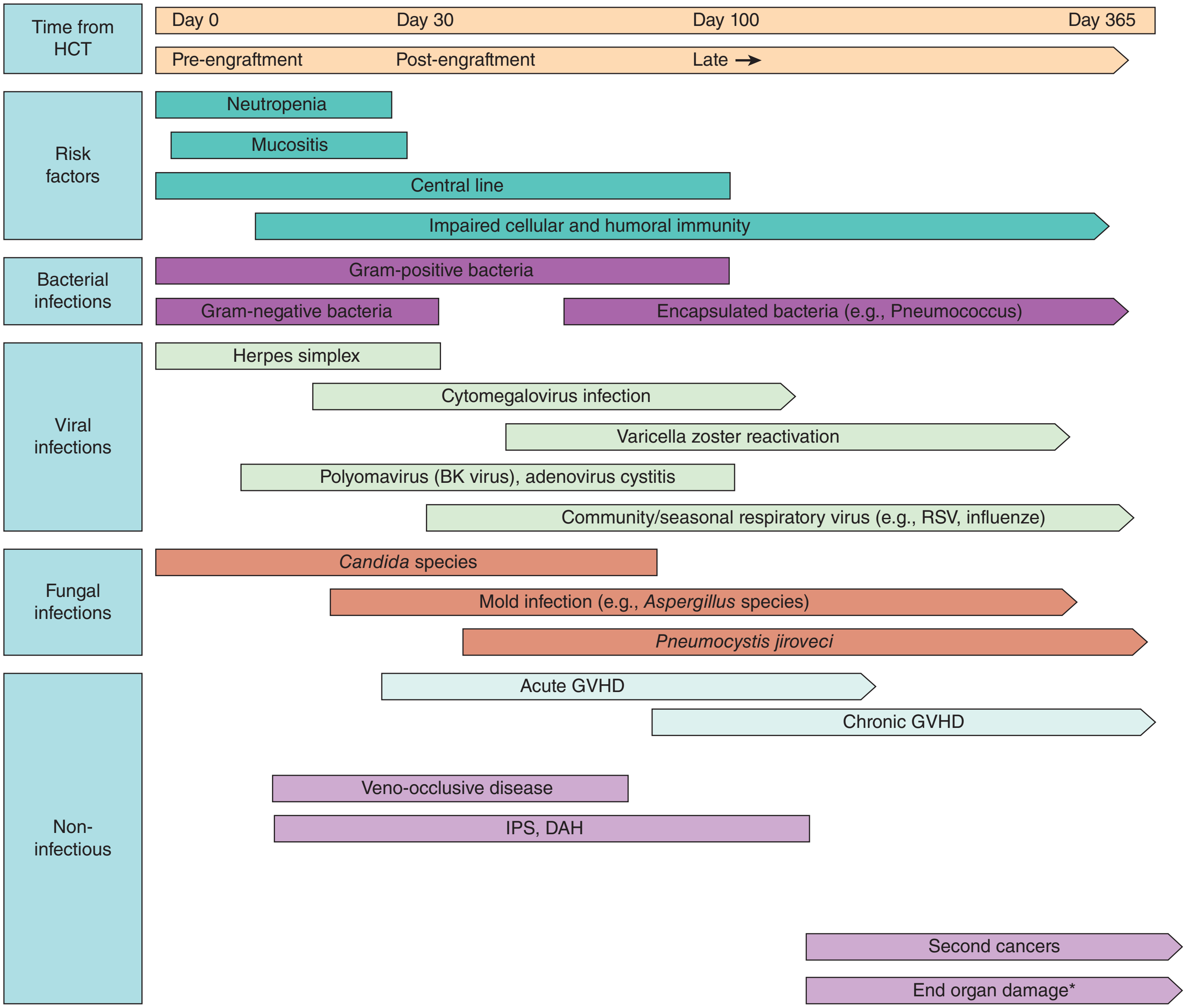

Time from HCT
Day 0
Day 30
Day 100
Day 365
Pre-engraftment
Post-engraftment
Late →
Risk factors
Neutropenia
Mucositis
Central line
Impaired cellular and humoral immunity
Bacterial infections
Gram-positive bacteria
Gram-negative bacteria
Encapsulated bacteria (e.g., Pneumococcus)
Viral infections
Herpes simplex
Cytomegalovirus infection
Varicella zoster reactivation
Polyomavirus (BK virus), adenovirus cystitis
Community/seasonal respiratory virus (e.g., RSV, influenze)
Fungal infections
Candida species
Mold infection (e.g., *Aspergillus* species)
Pneumocystis jiroveci
Non-infectious
Acute GVHD
Chronic GVHD
Veno-occlusive disease
IPS, DAH
Second cancers
End organ damage*

Figure 110.1 TIMELINE OF COMMON INFECTIOUS AND NON-INFECTIOUS POST-TRANSPLANT COMPLICATIONS. *Late end-organ complications include cardiovascular, pulmonary, endocrinopathy, ophthalmologic, renal, iron metabolism and bone health. *DAH*, Diffuse alveolar hemorrhage; *EBV*, Epstein-Barr virus; *GVHD*, graft-versus-host disease; *HCT*, hematopoietic cell transplantation; *IPS*, idiopathic pulmonary syndrome; *RSV*, respiratory syncytial virus.

should be considered. Initiating empiric antifungal therapy with mold-active agents such as posaconazole, voriconazole or amphotericin is appropriate at this stage. The choice of agent is dependent on prior exposure to anti-mold agents for prophylaxis where resistant species (e.g., zygomycetes) may emerge. Empiric mold specific therapy can be started earlier in patients who have experienced prolonged periods of neutropenia pre-HCT (e.g., patients with myelodysplastic syndromes [MDSs]). Repeated vigorous investigation to identify sources of infection (e.g., computed tomography [CT] scans of the chest and sinuses), is essential. Although administration of myeloid growth factors (granulocyte colony-stimulating factor [G-CSF] or granulocyte-macrophage colony-stimulating factor [GM-CSF]) reduces the duration of neutropenia and accelerates neutrophil engraftment, they have not demonstrated reductions in mortality from early post-transplant infections. Granulocyte infusions have rarely been used for life-threatening infections during the pre-engraftment phase. However, questionable efficacy in "real-world" scenarios and logistical challenges have resulted in this strategy falling out of favor.

Cytomegalovirus Infection

Epidemiology and Risk Factors

Despite the introduction of effective antiviral therapies, CMV reactivation and infection can be a major cause of morbidity and mortality in allogeneic HCT recipients.[9] The risk of CMV reactivation spans both the early and late transplant period, especially in patients with active GVHD requiring prolonged immunosuppression. Although the incidence of early CMV disease with organ involvement has declined to 3% to 6% with the use of empiric antiviral drug therapy directed by routine surveillance with CMV DNA polymerase chain reaction (PCR) or antigenemia testing (pre-emptive strategy), late onset CMV infection and organ disease remain significant clinical concerns.[9]

Seropositivity of the recipient is the most important risk factor for CMV infection in HCT recipients, and reactivation of latent virus is the most important mechanism resulting in CMV disease. CMV infections (<5%) in seronegative recipients are extremely rare

TABLE 110.1 Major Complications of Hematopoietic Cell Transplantation

Complication	Incidence
Infections	
Bacterial Infections	
Gram-positive bacteremia	20%–30%
Gram-negative bacteremia	5%–10%
Viral Infections	
Cytomegalovirus	5%–40% in high-risk patients[a]
Herpes simplex virus	5%–10% in seropositive patients
Varicella-zoster virus	10%–50% in seropositive patients
Respiratory viruses	10%–20%
Fungal Infections	
Candida	5%–10%
Aspergillus and other molds	5%–15%
Pneumocystis jirovecii	<1%
Other Infections	
Toxoplasma gondii	2%–7% in seropositive patients
Early Non-infectious Complications (0–3 months)	
Regimen-Related Toxicity	
Mucositis	60%–75%
Hemorrhagic cystitis	5%–10%
Veno-occlusive disease	5%–40%
Pneumonitis	10%–20%
Alveolar hemorrhage	5%–10%
Graft failure	2%–10%
Adverse drug reactions	Common
Late Non-infectious Complications (>3 months)	
Organ-Specific Late Effects	
Cataracts	25%–40%
Hypothyroidism	30%–50%
Sterility/hypogonadism	50%–90%
Growth disturbances	30%–50% in prepubertal children
Osteoporosis/avascular necrosis	5%–20%
Malignant relapse	Variable
Second cancers	2%–12%
Graft-Versus-Host Disease	
Acute	20%–50% with related, 40%–90% with unrelated donors, 20%–50% with UCB, 10%–20% with haplo
Chronic	20%–40% with related, 40%–70% with unrelated donors, 20%–40% with UCB, 10%–20% with haplo

[a] CMV-seropositive HCT recipients.

CMV, Cytomegalovirus; *HCT*, hematopoietic cell transplantation; *UCB*, umbilical cord.

and likely result from exogenous exposure (primary CMV infection). The use of a CMV-seronegative donor in a seropositive recipient carries the worst risk for reactivation and is associated with repeated infections and decreased survival. Other risk factors for CMV reactivation include use of an unrelated donor, UCB or haploidentical donor HCT, in vivo T-cell depletion, older recipient age, increasing donor-recipient HLA mismatch, acute and chronic GVHD, and the need for prolonged immunosuppression, especially with high-dose corticosteroids. CMV reactivation can occur in autologous HCT patients as well, especially in those with extensive lymphodepleting prior therapies (e.g., fludarabine) but the risk of CMV end-organ disease is rare. Significant debate and research have gone into the evaluation of the potential role of CMV reactivation in decreasing post-HCT risk of disease relapse. The largest published study to date, including more than 9000 patients, showed no benefit in relapse risk but a higher a risk of NRM with CMV reactivation.[10]

Clinical Presentation and Diagnosis

CMV infection is often identified as asymptomatic reactivation noted by screening antigenemia or DNA PCR testing. The organs that are usually affected by CMV disease include the lungs (pneumonitis) and the gut (enteritis). CMV retinitis, hepatitis and encephalitis are less common and are usually seen in late-onset, recurrent CMV infection. Indirect effects of CMV infection may include increased risks of secondary graft failure and bacterial and fungal superinfection. Preceding CMV viremia is a strong predictor of subsequent clinical disease. CMV pneumonitis develops in 60% of patients with untreated asymptomatic viremia, and conversely effective treatment of CMV viremia reduces the incidence of CMV pneumonitis.

The diagnosis of CMV can be made either by demonstration of characteristic cytopathic effects in tissue or using more sensitive molecular methods that detect viral protein or DNA. Commonly used molecular assays include CMV DNA detection methods, and the pp65 antigenemia assay. Detection of the CMV pp65 antigen in leukocytes has been a commonly used method for CMV surveillance after HCT but is ineffective during early post-HCT leukopenia. Direct detection of CMV DNA either by PCR or DNA hybrid capture assay is the most sensitive method to detect CMV viremia. Furthermore, plasma CMV DNA PCR can be a valuable tool to monitor CMV during periods of neutropenia when CMV antigenemia testing is unreliable. In addition, quantitative real-time PCR assays allow estimation of viral load which can assist in determining the need for therapy, risk of disease progression and in monitoring response to anti-CMV treatment. Viral cultures of urine, saliva, blood or bronchioalveolar lavage (BAL), using either rapid shell-vial or routine culture techniques, have limited clinical utility since they are less sensitive than antigen or DNA detection techniques and take much longer to report. Since asymptomatic viral shedding in the airways is common, CMV DNA detected in BAL should be carefully interpreted. Higher viral loads and pre-test probability of CMV pneumonitis in these patients increase the positive predictive value of these tests.[9]

Prevention and Treatment

For seronegative recipients, the use of seronegative donors and CMV-safe blood products is the mainstay of prevention of CMV disease (see box on Approach to Prevention and Treatment of CMV Infection). For high-risk patients (seropositive recipients), two general strategies can be utilized, both of which have been effective in reducing early CMV infection rates to less than 10%.[9] In the "pre-emptive therapy" strategy, patients are monitored periodically (usually weekly) by quantitative PCR with prompt treatment of early CMV viremia with ganciclovir, valganciclovir, or foscarnet before it can lead to clinical disease. In "prophylactic therapy" at-risk patients are treated with anti-viral prophylaxis. Earlier studies evaluating the use of prophylactic ganciclovir and foscarnet did reduce rates of CMV infection but did not result in improved survival and was offset by drug related toxicities such as myelosuppression and renal dysfunction.[9] The antiviral, letermovir was

TABLE 110.2 Common Infections in Hematopoietic Cell Transplant Recipients

Pathogen	Risk Period After HCT (Weeks)	Risk Factors	Common Clinical Syndromes	Treatment
Gram-positive cocci	1–4	Neutropenia Mucositis Central venous catheters Skin breakdown	Bacteremia	Antibiotics based on susceptibility testing
Enterobacteriaceae spp.	1–4	Neutropenia Skin breakdown GI mucosal breakdown	Bacteremia	Antibiotics based on susceptibility testing
Clostridoides difficile	1–8	Antibiotics	Colitis	Metronidazole Oral vancomycin
Encapsulated bacteria[a]	>12	Chronic GVHD Chronic immunosuppression	Sinusitis Pneumonia	Antibiotics based on susceptibility testing
Candida spp.	1–4	Neutropenia Skin breakdown GI mucosal breakdown	Candidemia Muco-cutaneous Hepato-splenic	Azoles Echinocandins Amphotericin
Aspergillus spp.	1–4>8	HLA-disparity CMV infection Acute or chronic GVHD Chronic immunosuppression High-dose corticosteroids	Sinusitis Pulmonary nodules or infiltrates	Mold specific azoles Echinocandins Amphotericin
Pneumocystis jirovecii	>4	Chronic GVHD Chronic immunosuppression	Pneumonia	TMP-SMX Dapsone Pentamidine
CMV	>4	Recipient or donor seropositivity HLA-disparity Acute or chronic GVHD Chronic immunosuppression	Viremia Enteritis Interstitial pneumonitis	Ganciclovir Foscarnet Valganciclovir
HSV	1–4	Recipient seropositivity	Oro-pharyngeal Esophagitis	Acyclovir Valacyclovir Foscarnet
VZV	>4	Recipient seropositivity History of chickenpox HLA disparity Acute or chronic GVHD Chronic immunosuppression	Cutaneous Interstitial pneumonitis Hepatitis	Acyclovir Valacyclovir Foscarnet
EBV	>4	HLA disparity T-cell depletion	Viremia PTLD	Rituximab Reduce immunosuppression Virus-specific T cells Cytotoxic chemotherapy

[a] Includes *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis*.
CMV, Cytomegalovirus; *EBV*, Epstein-Barr virus; *GI*, gastrointestinal tract; *GVHD*, graft-versus-host disease; *HCT*, hematopoietic cell transplantation; *HLA*, human leukocyte antigen; *HSV*, herpes simplex virus; *PTLD*, post-transplant lymphoproliferative disorder; *TMP-SMX*, trimethoprim-sulfamethoxazole.

studied in a phase III randomized trial versus placebo in CMV-seropositive allogeneic HCT recipients for 12 weeks post-transplant and resulted in reduced CMV infections and improved all-cause mortality at 24 weeks.[11] Letermovir should be considered in these patients. However, they do need additional acyclovir for prophylaxis against herpes simplex and varicella. Both approaches require aggressive surveillance to allow prompt detection of infection. Unlike ganciclovir, which is administered intravenously, its prodrug valganciclovir, has excellent oral bioavailability and is often used for prophylaxis and pre-emptive therapy of CMV infection among HCT recipients. Surveillance for CMV is continued weekly until at least day 100 post-transplant for high-risk patients and is continued longer in patients with chronic GVHD on high-dose immunosuppression. In patients receiving prophylactic letermovir,

TABLE 110.3 Recommended Antimicrobial Prophylaxis Against Common Infections

Pathogen	Preventing Early Disease (0–100 Days After HCT)	Preventing Late Disease (>100 Days After HCT)
Bacterial infections	Quinolone prophylaxis during period of neutropenia	Antibiotics against encapsulated bacteria (e.g., *Streptococcus pneumoniae*) in patients on chronic immunosuppression
CMV	Letermovir prophylaxis recommended[a] or pre-emptive treatment with ganciclovir or valganciclovir	Pre-emptive treatment with ganciclovir or valganciclovir
HSV	Acyclovir/valacyclovir in seropositive patients	Acyclovir/valacyclovir in patients with recurrent HSV infections
VZV	Acyclovir/valacyclovir	Acyclovir/valacyclovir
Yeast infections	Fluconazole	Fluconazole in patients on chronic immunosuppression
Mold infections	Anti-mold triazole (e.g., posaconazole) prophylaxis in patients with GVHD[b]	Anti-mold triazole (e.g., posaconazole) prophylaxis in patients with GVHD[b]
Pneumocystis jirovecii	Trimethoprim-sulfamethoxazole (preferred) or dapsone or pentamidine or atovaquone	Trimethoprim-sulfamethoxazole (preferred) or dapsone or pentamidine or atovaquone in patients on chronic immunosuppression

[a] CMV-seropositive HCT recipients.
[b] Limited data available. Prospective testing of voriconazole and posaconazole suggests possible benefit as prophylaxis. No impact on mold-related mortality.
CMV, Cytomegalovirus; *GVHD*, graft-versus-host disease; *HCT*, hematopoietic cell transplantation; *HSV*, herpes simplex virus.

continued monitoring after cessation of drug is recommended to detect rebound infections.

CMV disease, especially pneumonia, must be diagnosed and treated promptly as it is associated with high rates of mortality. The combined use of ganciclovir and IVIG has been the most successful treatment for CMV pneumonia, with resolution in 50% to 75% of non-ventilator dependent patients. Prolonged therapy (>2 months) with the combination is indicated because shorter treatment regimens have been associated with a recurrence of CMV pneumonia, and granulocyte colony stimulating factor (GCSF) often necessary to manage ganciclovir-induced myelosuppression. Foscarnet, cidofovir or the combination of intravenous ganciclovir with foscarnet can be considered as second line therapy and early studies have shown promising data with maribavir for resistant or refractory cytomegalovirus disease.[9] The antiviral management of CMV enteritis, hepatitis and retinitis is similar, although the use of IVIG in these settings is more controversial.

Although rare, rising CMV viral loads and worsening end-organ dysfunction despite optimal therapy, should raise the concern of CMV antiviral resistance. Ganciclovir resistance occurs due to mutations to human cytomegalovirus gene UL97 and ganciclovir, foscarnet and cidofovir resistance through UL54 mutations.[9] Adoptive cellular therapies, using approaches to enhance natural killer (NK) or more commonly infusing donor or third-party CMV-specific cytotoxic T-cells (CTLs) have been studied. CMV-specific CTLs have shown efficacy although third party cells may be hampered by low proliferative capacity and persistence.

TABLE 110.4 Recommended Vaccinations for Hematopoietic Cell Transplant Recipients

Vaccine[a]	Time Post-HCT to Initiate Vaccine (Months)	No. of Doses[b]
PCV	3–6	2–3[c]
Tetanus, diphtheria, acellular pertussis[d]	6–12	3
Haemophilus influenzae conjugate	6–12	3
Inactivated polio	6–12	3
Recombinant hepatitis B	6–12	3
Inactivated herpes zoster	6–12	2
Inactivated influenza	4–6	1–2 yearly[e]
Measles-mumps-rubella (live)	24	1–2[f]
Varicella zoster (live)[g]	24	1[f]

[a] Vaccinations are deferred in patients with chronic GVHD until discontinuation of immunosuppression.
[b] A minimum of 1-month interval between doses is suggested.
[c] Following the primary series of three PCV doses, a dose of the PPSV23 to broaden the immune response might be given. For patients with chronic GVHD who are likely to respond poorly to PPSV23, a fourth dose of the PCV should be considered instead of PPSV23.
[d] DTaP (diphtheria tetanus pertussis vaccine) is preferred; however, Tdap can be used if DTaP is not available.
[e] For children <9 years of age, two doses are recommended yearly between transplant and 9 years of age.
[f] Not recommended <24 months post-HCT, in patients with active GVHD and in patients on immune suppression. In children, two doses of MMR (mumps, measles, and rubella) are favored. Lower viral dose vaccines (varicella vaccine, live [varivax], not zoster vaccine, live [zostavax]) may be preferred as potentially safer.
[g] Inactivated herpes zoster preferred and administered earlier in the course.
GVHD, Graft-versus-host disease; *HCT*, hematopoietic cell transplant; *PCV*, pneumococcal conjugate; *PPSV23*, 23-valent polysaccharide pneumococcal vaccine.

APPROACH TO PREVENTION AND TREATMENT OF CYTOMEGALOVIRUS INFECTION

Prevention

1. Seronegative recipient: Choose seronegative donor when possible. Consider chemoprophylaxis is seropositive donor is considered. Transfuse only cytomegalovirus (CMV) safe blood products. Leukocyte depletion by filtration and blood from CMV-seronegative donors are clinically equivalent alternatives.
2. Seropositive recipients: Avoid seronegative donor when possible. Letermovir prophylaxis is recommended in this setting, considering improvement in 24-month all-cause mortality. Ganciclovir (or valganciclovir) is also effective but is myelosuppressive. Intensive surveillance and early pre-emptive therapy required if letermovir prophylaxis not possible.
3. All patients need periodic (weekly) monitoring for CMV DNA PCR for at least 12 weeks post-transplantation. Longer duration (beyond 12 weeks) surveillance is appropriate for allograft recipients with graft-versus-host disease (GVHD) and those previously on chemoprophylaxis to detect rebound.

Treatment

1. Asymptomatic infections: Ganciclovir or valganciclovir treatment of asymptomatic infection detected in blood or bronchioalveolar lavage (BAL), by either molecular detection or antigenic methods, is recommended to prevent the development of CMV pneumonia. Intensive induction treatment (2 weeks) followed by a maintenance phase of 5 days/week therapy for an additional 4–8 weeks is necessary.
2. CMV pneumonia: Ganciclovir in combination with immunoglobulin is recommended. This should be instituted promptly. Once the disease has progressed to cause respiratory failure and ventilator dependence, outcomes are bleak.
3. Foscarnet and cidofovir are alternatives to CMV disease that is resistant or refractory to ganciclovir.

Other Latent Viral Infections

Primary and reactivation of other herpes viruses can occur after transplantation. Herpes simplex virus (HSV) infection is uncommon with the routine use of acyclovir or valacyclovir prophylaxis in serologically positive patients. Albeit uncommon, acyclovir-resistant HSV infection may occur in patients given a low-dose or intermittent prophylaxis and recipients of T-cell depleted grafts. Foscarnet is the drug of choice for resistant disease with cidofovir reserved as an alternative agent. Varicella zoster reactivation can occur in 30% to 50% of HCT recipients with previous exposure to VZV and can be effectively prevented by acyclovir prophylaxis. Acyclovir prophylaxis is recommended for the first year after transplantation for VZV seropositive autologous and allogeneic HCT recipients, although patients with chronic GVHD on immunosuppression and patients on maintenance proteasome inhibitors will need extended prophylaxis.

Human polyomavirus type I, also known as BK virus, can cause hemorrhagic cystitis in the early post-transplant period. Urine PCR can identify BK virus and distinguish it from hemorrhagic cystitis caused by other infections (e.g., adenovirus) and urotoxic agents (e.g., cyclophosphamide). Lowering the immunosuppressive therapy, if possible, should be the first step. Quinolone antibiotics suppress BK virus replication in vivo and in vitro and may have a role as prophylaxis in patients at high risk for hemorrhagic cystitis. Intravesical or intravenous cidofovir has been used for the treatment of BK virus hemorrhagic cystitis.[1] BK-specific CTLs have been used in severe, progressive hemorrhagic cystitis. Rarely, BK virus encephalitis has been described post-allogeneic HCT. Another polyoma virus, John Cunningham (JC) virus, can reactivate in immunocompromised HCT recipients and cause progressive multifocal leukoencephalopathy, a fatal demyelinating disease of the central nervous system.

Human herpes virus 6 (HHV-6) can also reactivate from latency post-HCT, with incidence ranging from 20% to 60%, depending on donor type and graft source. Although reactivation is a common, routine surveillance is not recommended. Treatment with ganciclovir or foscarnet is recommended in symptomatic patients (headache, mental status changes, unexplained fever and/or rash, bone marrow suppression) with accompanying high viral load (>25,000 copies). Asymptomatic reactivation of HHV6 is common and is not associated with adverse post-HCT outcomes.[12]

Acquired Viral Infections

HCT recipients are prone to seasonal and community-acquired respiratory viral (CRV) infections including influenza, parainfluenza, and respiratory syncytial virus (RSV). Upper respiratory infections can rapidly progress to more serious lower respiratory infections in immunocompromised HCT recipients. It is of vital importance to promptly initiate appropriate diagnostic testing (e.g., nasopharyngeal swabs) to identify the virus if possible. Zanamivir or oseltamivir can be used as chemoprophylaxis for HCT recipients with known contact to a case, as well as for treatment of influenza. Aerosolized ribavirin may be considered in patients with RSV lower respiratory tract infection. Novel antivirals are needed to treat respiratory viral infections in HCT recipients. A novel inhaled antiviral, DAS181 (Fludase), cleaves sialic acid-containing receptors on the surface of host respiratory epithelial cells, thus preventing parainfluenza virus attachment to and infection of respiratory cells has shown some activity.

In 2020, a novel coronavirus (SARS-CoV-2) caused a global pandemic of COVID-19 infection characterized by a viral syndrome with a predominance of respiratory symptoms. At the time of writing this chapter, the epidemiology, risk factors, clinical presentation, and outcomes in HCT recipients were not well described. However, HCT recipients were anticipated to be at high-risk for getting infection and for developing more severe manifestations and adverse outcomes.[13] It is expected that this virus will continue in circulation for some time, and its impact and management in the transplant population will become clearer with increasing experience.

Fungal Infections

Invasive fungal infections are among the leading causes of morbidity and mortality in HCT recipients. Most fungal infections in this setting are caused by yeasts (*Candida* spp.) or molds (*Aspergillus* spp.).

Candida infections

Candida albicans has been the leading cause of yeast infections in HCT recipients, but the current widespread use of azole (e.g., fluconazole) prophylaxis in the early transplant period has led to the emergence of a variety of non-*albicans* species, such as *Candida tropicalis, Candida krusei*, and *Candida glabrata*, as important pathogens.[14] Yeasts are normal inhabitants of the skin, oral and gastrointestinal mucosa. Breakdown of mucosal surfaces due to radiation and chemotherapy, compounded by neutropenia in the pre-engraftment period, can greatly increase the risk of invasion and systemic infections. The presence of indwelling central venous catheters, parenteral nutrition and alteration of normal surface flora due to antibiotics are additional risk factors for *Candida* infections.[1]

Clinical manifestations can range from localized mucocutaneous to disseminated deep-tissue infection. A high index of suspicion is needed for the diagnosis of *Candida* infections, especially in patients with persistent febrile neutropenia since blood cultures are usually not very sensitive for isolation and identification of *Candida* spp. Oral and esophageal candidiasis frequently occurs in the early post-transplant period and should be treated aggressively as these can serve as portals for subsequent systemic infection. Venous catheter infections can be difficult to eradicate with antifungal agents alone and necessitate removal of the central line. Patients with candidemia are also at risk for endovascular infections such as endocarditis and thrombophlebitis. Hepatosplenic candidiasis is the most common manifestation of disseminated candidiasis although it is increasingly rare with widespread use of effective anti-candida azoles and echinocandins. Specific signs or symptoms related to organ involvement may be absent and the diagnosis frequently must be made by abdominal CT scan imaging.

Prophylaxis with fluconazole is recommended in the pre-engraftment and early post-engraftment period, especially among allogeneic HCT recipients.[1] In patients who are at high-risk for mold infections (e.g., severe GVHD), triazole antifungals such as posaconazole or voriconazole should be considered. Alternatively, caspofungin or micafungin can also be considered. In patients with severe GVHD, posaconazole prophylaxis compared to fluconazole was noted to be superior in preventing invasive aspergillosis and deaths due to fungal infections in a large phase III trial.[15] *C. krusei* and *C. glabrata* are intrinsically resistant to fluconazole and other antifungal agents (e.g., posaconazole, micafungin) should be preferred for prophylaxis in those colonized with fluconazole-resistant *Candida* species. Itraconazole is another active agent, but its use is limited by its tolerability and absorption. Cross-resistance to azoles can occur among *Candida* species. Anti-fungal agents for treatment of suspected or known invasive candidiasis include voriconazole or posaconazole, echinocandins or an amphotericin formulation, especially when infection occurs in the setting of ongoing fluconazole prophylaxis.

Aspergillus Infections

Most mold infections in HCT recipients are due to *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger*, which gain entry through breakdown of mucosal surfaces or through the nasal passages and respiratory tract. Examples of non-Aspergillus molds causing infections in this setting include *Fusarium* species, zygomycetes and rarely *Scedosporium*. *Aspergillus* infections can occur early after HCT (during the neutropenic phase) or later, especially with prolonged immunosuppression associated with acute or chronic GVHD. Risk factors for *Aspergillus* infections include allogeneic HCT, prolonged neutropenia, concomitant CMV infection, and severe GVHD and high-dose steroids is a risk factor for invasive aspergillosis-associated mortality. Underlying primary hematologic diagnosis such as chronic granulomatous disease, aplastic anemia and MDS, which are associated with prolonged pre-transplant neutropenia also increase the risk. A prior history of *Aspergillus* infection has also been observed to be a risk factor for reactivation after HCT. As pointed out earlier, mold-active triazole prophylaxis should be considered in high-risk patients.

Aspergillus infection is intrinsically difficult to diagnose premortem. A high index of suspicion and an aggressive approach are required to establish diagnosis and initiate therapy. Since the nasal passages and tracheo-bronchial tree are the most common portals of entry for *Aspergillus* spp., these two sites are also the most common sites of infection. Sinusitis is frequently symptomatic and more advanced disease can be associated with erosion and necrosis of surrounding structures. Pulmonary manifestations typically include nodular infiltrates, usually distributed along the lung periphery, with pleuritic pain or cough as an initial symptom. Frank consolidation (with a halo sign on CT scan) or cavitary lesions can be seen in more advanced stages of pulmonary involvement. *Aspergillus* has angioinvasive properties and can present with hemoptysis or with intravascular dissemination to the skin or brain.

Blood cultures have a very low sensitivity in detecting *Aspergillus* and diagnosis relies on demonstration of typical fungal morphology on histopathology or on tests to detect fungal components or nucleic acids. Nasal and bronchial washings for *Aspergillus* may also not be sensitive and sinus or lung biopsy are often required to confirm diagnosis. The galactomannan assay is an enzyme-linked immunosorbent assay to detect the *Aspergillus* cell wall glycoprotein; it has a high specificity, but low sensitivity for diagnosing invasive aspergillosis and has not been reproducible. Molecular methods for diagnosis, including PCR for *Aspergillus* DNA, are also undergoing development. Assays to detect fungal cell wall component (1–3)-β-ᴅ-glucan are sensitive to detect most invasive fungal infections (except *Cryptococcus* and zygomycetes) but are not specific to *Aspergillus*. Often, tissue biopsy is needed to confirm the presence of *Aspergillus* in suspicious lesions. When biopsy is not possible, empiric therapy is recommended if clinical suspicion for mold infection is high.

The use of high-efficiency air filters has reduced the nosocomial acquisition of *Aspergillus*, at least during the early neutropenic phase. The optimal pharmacologic strategy for primary prophylaxis against aspergillosis is not well defined. Posaconazole is effective and recommended as prophylaxis in patients with severe GVHD. A large, randomized trial showed no difference in fungal-free survival between voriconazole and fluconazole prophylaxis in HCT recipients at low risk for disease progression or early HCT mortality, although there was a trend towards fewer *Aspergillus* infections and less empiric antifungal use in the voriconazole arm.[16] In patients with a previous history of invasive aspergillosis, secondary prophylaxis with a mold-specific azole (e.g., oral voriconazole or posaconazole), parenteral echinocandins, or an amphotericin preparation is recommended during the peri-transplant period and possibly for the duration of intensive immunosuppression. Similarly, in high-risk patients with persistent febrile neutropenia not responding to antibiotics, mold-directed therapy should be promptly initiated. Azoles and echinocandins have a more favorable side effect profile compared to amphotericin formulations. Patients with disease progressing on a single anti-mold drug might need combination therapy. The role of adjunctive measures such as cytokine growth factors, immunoglobulin infusion, or granulocyte transfusion remains undefined.

Pneumocystis jirovecii Pneumonia

Pneumonitis caused by *Pneumocystis jirovecii* (PJP) has a typical bilateral distribution with a "butterfly" pattern on chest radiograph and is clinically associated with dyspnea and significant hypoxemia. The risk of *Pneumocystis* pneumonia is effectively eliminated with routine use of prophylaxis with TMP-SMX (first choice), dapsone, atovaquone, or aerosolized pentamidine. For those prescribed PJP prophylaxis, compliance must be ensured before ruling it out as a possible etiology. Diagnostic confirmation requires cytologic evaluation of silver-stained preparations of BAL cells or sputum, although transbronchial lung biopsy may slightly increase the diagnostic yield of a bronchoscopy examination. Although not confirmatory, positive (1–3)-β-ᴅ-glucan assay can be a useful marker for PJP besides invasive fungal infections. *Pneumocystis* pneumonia is effectively treated with high dose TMP-SMX or parenteral pentamidine. PJP prophylaxis is recommended through the period of immunosuppression (6 to 12 months) or for the duration of any chronic GVHD therapy in allogeneic HCT recipients and up to 3 months for autologous HCT recipients.

EARLY NON-INFECTIOUS COMPLICATIONS

Chemotherapy and radiation are used as conditioning agents before transplantation for their antineoplastic and immunosuppressive effects. However, these regimens, especially myeloablative conditioning (MAC) can cause recipient tissue damage, resulting in significant morbidities. Early HCT-associated complications can frequently simulate infections or be compounded by concurrent infections. Additionally, delays in hematopoietic recovery can potentially hinder epithelial tissue repair and cause local microinvasive infections, thus exacerbating and prolonging the toxicities.

Graft Failure

Failure to establish hematologic engraftment (primary graft failure) and loss of an established graft (secondary graft failure) are serious complications of both autologous and allogeneic HCT. Delayed or poor graft function can exaggerate and prolong the risks of infection and increase the risk of peri-transplant mortality. Failure to engraft can occur if insufficient hematopoietic progenitors are infused. If bone marrow is used as the graft source, a minimum of ~2×10^4 colony-forming cells from marrow per kilogram (kg) recipient weight are needed to establish autologous engraftment. This is accomplished by infusing approximately 1×10^8 autologous marrow mononuclear cells/kg. Bone marrow is rarely used for autologous HCT. For bone marrow allografts, a minimum of 2×10^8 mononuclear cells/kg cell dose is recommended to ensure adequate hematopoietic recovery. In UCB transplants, a lower cell dose is usually sufficient to ensure hematopoietic recovery. It is recommended that a single UCB unit has a cell dose of greater than 2.5 to 3×10^7/kg mononuclear cells and when double UCB units are used, each unit should have greater than 1.5×10^7/kg mononuclear cells.[17]

Obtaining hematopoietic stem cells and progenitor cells from peripheral blood by apheresis instead of bone marrow harvest is widely prevalent, both for autologous and allogeneic HCT. For autologous transplantation, peripheral blood hematopoietic cell grafts are collected after mobilization of marrow-derived progenitors into the blood by cytokine (G-CSF or GM-CSF) therapy or during recovery from myelosuppressive chemotherapy, often in combination with growth factors. Hematopoietic cell content of blood or marrow grafts is assayed by quantitation of mononuclear cells expressing the hematopoietic progenitor-associated surface marker CD34. Peripheral graft mobilization can be further enriched using plerixafor, an inhibitor of CXCR4 that releases cells from the marrow. Allogeneic grafts are mobilized from healthy donors using growth factors alone, nearly exclusively using G-CSF, though clinical trials with plerixafor have been completed.

Graft failure is uncommon if 2×10^6 CD34+ cells/kg or more are utilized in autologous and allogeneic HCT. Mobilized peripheral blood derived hematopoietic cells yield satisfactory and more rapid trilineage hematopoietic recovery than marrow-derived grafts. Secondary graft failure is unlikely (<5%) after transplantation using peripheral blood hematopoietic cells. Recipient factors such as myelofibrosis and splenomegaly can impair hematopoietic recovery due to faulty homing of progenitor cells in the marrow microenvironment or sequestration of cells in the spleen, respectively. Graft failure or poor graft function can also be associated with the use of methotrexate, anti-thymocyte globulin (ATG), acyclovir, ganciclovir, trimethoprim-sulfamethoxazole (TMP-SMX), and mycophenolate mofetil (MMF) in the peri-transplant period. Post-transplant complications such as CMV, HHV-6 or fungal infections, and acute and chronic GVHD can also compromise successful hematopoietic function.

Allogeneic transplantation from a matched sibling donor at HLA-A, -B, and -DR loci are rarely (1% to 3%) associated with graft failure; however, the probability of graft failure increases with greater degrees of HLA incompatibility. In a matched unrelated donor (at HLA-A, -B, -C, and -DRB1) allo-HCT the rate of graft failure is less than 5% but is higher in mismatched donor HCT. Thus, the degree of HLA-match is a significant determinant to decrease the risk of graft failure. The presence of donor-specific antibodies (DSA) in the recipient against the donor HLA molecules is associated with increased risk of graft failure in the setting of a matched/mismatched unrelated donor, haploidentical donor and UCB transplantation. Major ABO incompatibility, inadequate immunosuppression, and reduced intensity conditioning are other risk factors for graft failure in allogeneic HCT. Evaluating donor chimerism by PCR amplification can distinguish between donor and recipient hematopoietic elements. Increasing recipient T-cell repertoire in the post-HCT setting is a risk factor for subsequent graft failure, especially after RIC/NMA conditioning. Early failure of an allogeneic graft can be due to the emergence of cytotoxic T-lymphocytes of host origin, presumably representing immune-mediated graft rejection. T-lymphocyte depletion (ex vivo or in vivo) of allografts for GVHD prophylaxis can potentially affect hematopoietic engraftment.

Neutrophil recovery time is usually delayed in patients receiving UCB grafts, compared to other sibling/unrelated allografts. Additionally, higher rates of graft rejection are noted with UCB grafts (5% to 15%). The risk of graft rejection can be minimized by improving the degree of HLA matching, ensuring adequate cell dose (>2.5 to 3×10^7/kg mononuclear cells for single UCB and greater than 1.5×10^7/kg mononuclear cells per unit for double UCB transplants) and avoiding units for which the recipient exhibits DSA. Ex vivo expansion is a potentially useful strategy to improve the infused cell dose in this setting. In recipients with anti-HLA antibodies, IVIG and anti-CD20 monoclonal antibodies have been used successfully to decrease antibody titer, although its clinical utility in this setting has not been well established. Growth factors such as GCSF and GM-CSF stimulate myelopoiesis and may be utilized prophylactically to ensure prompt neutrophil recovery in UCB and haploidentical donor transplantation. They are successful in improving myelopoiesis and neutrophil recovery in most cases of secondary graft failure and associated neutropenia. The use of recombinant human thrombopoietin and erythropoiesis stimulating agents to aid platelet and red cell recovery in this setting is controversial.

In the case of a failed autograft, infusion of previously harvested and frozen marrow or blood cells frequently reestablishes functional hematopoiesis. In allograft recipients with poor hematopoietic recovery, assessment of sorted T-cell chimerism can assist in distinguishing between immune mediated graft rejection (usually <5% donor derived T-cells detected) versus poor graft function in the setting of mixed chimerism.[18] It is vital to rule out other causes of secondary graft failure, such as infections and drug reactions. In the case of graft failure associated with low T-cell chimerism, a second infusion of donor marrow or cytokine-mobilized peripheral blood stem cells (stem cell boost) may allow successful engraftment. Due to the likelihood of immune-mediated rejection, immune/lymphodepleting reconditioning regimens are necessary prior to the stem cell boost to ensure adequate host immunosuppression. In recipients with mixed chimerism (5% to 95% donor T-cell) and poor graft function not responding to growth factors and tapering of immunosuppressive agents; donor lymphocyte infusions (DLI) may be considered to improve the donor chimerism to avoid impeding graft failure.[19] Stem cell boosts are readily available in the setting of related donors but can be more problematic in unrelated and UCB transplants. In case of graft rejection after UCB transplantation, a second transplant using cells from a different UCB or volunteer adult donor (related or unrelated) are possible options. Unfortunately, there is very little data on the outcomes of second allografts in these situations.

Veno-occlusive Disease

Hepatic veno-occlusive disease, also called sinusoidal obstruction syndrome (VOD/SOS), is a serious liver disorder characterized by jaundice, ascites, fluid retention and tender hepatomegaly, which complicates up to 5% to 50% of HCT.[20] The varying incidence is dependent on patient and transplant-related factors as well as stringency of diagnostic criteria used (see box on Veno-occlusive Disease). The primary initiating event is thought to be the conditioning agents (chemotherapy or radiation) causing sinusoidal endothelial injury, which leads to the release of fibronectin and factor VIII/von Willebrand factor, thus activating the coagulation cascade and formation of sinusoidal microthrombi and obstruction. This results in portal hypertension and centrilobular hepatocyte damage. Decreased plasma protein C levels, lower antithrombin III levels and elevated factor VIII and fibrinogen levels are usually noted. Cytokines such as tumor necrosis factor (TNF)-α and alterations in the levels of nitric oxide and matrix metalloproteinases may also have a role in its pathogenesis.

Risk factors associated with the development of VOD/SOS include patient-related factors such as age, performance score, underlying diagnosis, history of pre-transplant hepatitis or liver injury/radiation,

VENO-OCCLUSIVE DISEASE

Diagnostic Criteria for Veno-Occlusive Disease/Sinusoidal Obstruction Syndrome (VOD/SOS)

- Bilirubin ≥ 2 mg/dL before day 21 post-HCT and at least 2 of the following: (1) hepatomegaly or right upper quadrant pain, (2) ascites, or (3) weight gain >5% over baseline [Baltimore criteria].
- VOD/SOS is a clinical diagnosis; liver ultrasound with Doppler studies or liver biopsy can support or confirm the diagnosis.

Risk Factors

Pre-transplant Factors

- Prior hepatic inflammatory disease (e.g., hepatitis B/C, non-alcoholic steatohepatitis, alcoholic hepatitis)
- Prior hepatic fibrotic disease (e.g., cirrhosis)
- Extensive pre-HCT chemotherapy
- Prior exposure to gemtuzumab ozogamicin (AML) or inotuzumab ozogamicin (ALL)
- Prior liver irradiation

Transplant-Related Factors

- Conditioning regimen (e.g., myeloablative total body irradiation or alkylator (e.g., busulfan) conditioning
- Concomitant medications (e.g., sirolimus, itraconazole)

Prognostic Factors

Adverse prognostic factors include development of multi-organ failure (pulmonary, renal, encephalopathy), rapid increase in weight, rapid increase in bilirubin.

Prevention

- Ursodeoxycholic acid (prophylaxis)
- Defibrotide (investigational, phase III study was terminated early for lack of efficacy)
- Low-dose heparin and low molecular weight heparin (prophylaxis, not effective)

Treatment

Mild to moderate VOD/SOS: supportive care, aggressive fluid and weight management, avoid hepatotoxic agents. More 70% will have complete recovery.

Severe VOD/SOS: prompt therapy vital, mortality historically >80%. Initiate Defibrotide as soon as possible (only FDA approved therapy). Other measures such as tissue plasminogen activators and antithrombin III are investigational. Non-pharmacologic measures include supportive care, transjugular intrahepatic porto-systemic shunt (TIPS), and liver transplantation.

prior exposure to oxaliplatin, gemtuzumab ozogomicin or inotuzumab ozogomicin; transplant-related factors such as MAC regimens, higher dosage of total body irradiation (TBI) (>10 to 12 Gy), dose rate and increased busulfan dose, and concomitant use of other alkylators such as cyclophosphamide or melphalan. VOD may also be more frequent after mismatched related or unrelated donor transplantation. The use of sirolimus as GVHD prophylaxis in the context of busulfan containing MAC regimen can increase the risk. An online risk calculator is available to calculate the risk of developing VOD/SOS and may direct careful monitoring and early initiation of therapy (https://www.cibmtr.org/ReferenceCenter/Statistical/Tools/Pages/VOD.aspx).

The classical signs and symptoms of VOD are noted within 3 weeks after allo-HCT and two diagnostic systems have traditionally been used (1) modified Seattle criteria: bilirubin greater than 2 mg/dL, hepatomegaly, and greater than 2% to 5% weight gain (>2 parameters required) or (2) Baltimore criteria: bilirubin ≥2 mg/dL plus at least 2 of: hepatomegaly, ascites, ≥5% weight gain.[20,21] However, late-onset VOD/SOS and differences in clinical presentation among children are now recognized and are taken into account in revised criteria.[22] Progressive and severe VOD can be devastating, presenting renal and pulmonary complications, multi-organ failure, encephalopathy, and death. Early recognition and diagnosis of VOD/SOS has important clinical ramifications and cannot be over emphasized. It is imperative that the patient be evaluated carefully to rule out other causes of hyperbilirubinemia and weight gain (e.g., drugs, hepatitis, capillary leak, cardiac failure, volume overload). Although the gold standard for confirming the diagnosis is a hepatic biopsy, it may be challenging and associated with high risk of hemorrhage. Percutaneous or transabdominal needle biopsy in patients with thrombocytopenia and coagulopathy is not recommended. Transvenous biopsies may provide histologic material for diagnosis and may allow determination of hepatic wedge pressure product (>10 mm is associated with VOD), but the risk of bleeding should be considered. Ultrasonographic Doppler flow studies demonstrating reversal of portal flow or a higher portal vein resistive index may aid in the diagnosis. The diagnosis is almost always based on clinical parameters and hence a high index of suspicion must be maintained in at-risk patients for the early diagnosis and prompt initiation of therapy. Based on clinical parameters such as degree of hyperbilirubinemia, weight gain, other-organ dysfunction, VOD is graded as mild, moderate, and severe. Severe VOD is associated with very high mortality (>80%).

The most important factor in preventing VOD/SOS is probably calculating the risk score, which allows for careful patient selection and potentially modifying transplant-related factors to lessen the risk of subsequent VOD/SOS (e.g., RIC/NMA conditioning may be considered for a younger patient with prior hepatitis and exposure to gemtuzumab). Limited understanding of the cellular and microvascular pathophysiology of VOD/SOS has historically confounded development of more rational approaches to its prevention. Preventive strategies such as low-dose heparin, prostaglandin E, pentoxifylline (a TNF-α blocking agent), or ursodiol, have been studied although none has proved effective in prospective trials. Nevertheless, ursodiol prophylaxis is common in many centers due to its favorable safety profile and potential impact on liver toxicities and possibly survival.[23] Defibrotide (see below) has been evaluated as preventive strategy in prospective studies with promising results, especially in pediatric population.[24] However, a phase III trial stopped enrollment early based on interim analysis concluding that it was unlikely to meet primary objectives. For the treatment of VOD/SOS several pharmacologic and invasive measures have been studied with mixed results; recombinant tissue plasminogen factor (variable efficacy, risk of bleeding), diuretics (for fluid management), prostaglandins, transjugular intrahepatic portosystemic shunts (TIPS—limited and transient efficacy). Defibrotide, a single-stranded polyribonucleotide of porcine origin with probable anti-inflammatory, anti-thrombotic and anti-ischemic properties (exact mechanism of action remains unknown), showed favorable responses with 30% to 70% of patients with severe VOD/SOS achieving complete remission and noted improvement in survival compared to historical controls.[24] Defibrotide received Food and Drug Administration (FDA) approval based on the open label, phase III trial in patients with severe VOD/SOS defined by the Baltimore criteria plus renal/pulmonary dysfunction by day +28 and noted improved remission of VOD/SOS and day +100 survival rates, compared to historical controls.[25] It is recommended that defibrotide (6.25 mg/kg intravenously every 6 hours for at least 21 days) be promptly initiated in patients meeting diagnostic criteria, as delays in initiation of therapy can be potentially fatal.

Interstitial Pneumonitis

Pulmonary complications in the aftermath of HCT is common and ranges from atelectasis, volume overload, transfusion related pulmonary complications, engraftment reaction, bacterial pneumonia, interstitial pneumonitis (IP) and GVHD, spanning the early and late transplant period. IP is a frequent and potentially fatal complication that can affect up to 35% of recipients, especially allogeneic HCT. It is notably less common after autologous HCT. Although advances in supportive care and RIC/NMA have improved outcomes, it remains a cause for significant post-transplant morbidity. It is characterized by diffuse, interstitial inflammation of non-bacterial etiology accompanied by hypoxemia, dyspnea, and non-productive cough, and is associated with fever. Risk factors associated with the development of IP include the use of methotrexate for GVHD prophylaxis rather than cyclosporine, older age, severe early onset GVHD, time from diagnosis to HCT of >6 months, poor performance status and higher dose rate of irradiation during conditioning (>4 cGy/min). In one study, the risk of IP was 8% when none of these risk factors were present, compared to 94% when all six factors were present.[26] The clinical course of IP can be catastrophic, manifesting with rapidly progressive tachypnea, hypoxemia and hemodynamic compromise. Therefore, therapeutic interventions, often empiric based on clinical features, must occur before diagnostic tests can definitively confirm the diagnosis.

Infectious Causes of Interstitial Pneumonitis

Infections are the most common cause of IP in HCT recipients. Cytomegalovirus and *Aspergillus* are common infectious etiologies of IP and have been discussed in detail earlier in this chapter. Other important, uncommon infections to consider include *P. jirovecii* and RSV and similar, seasonal respiratory viruses. Clinical presentation and management of respiratory infections has been discussed in the infectious complications section above.

Non-infectious Causes of Interstitial Pneumonitis

Idiopathic Pulmonary Syndrome

Idiopathic pulmonary syndrome (IPS) is a serious complication characterized by IP that occurs in about 10% of HCT recipients, usually within the early post-transplant period (median time 2 to 4 weeks) and is indeed a diagnosis of exclusion after ruling out other known etiologies. Risk factors for IPS include older age, extensive prior chemotherapy, MAC regimens with high-dose cyclophosphamide and TBI (higher total dose and dose rate), blood transfusions, methotrexate for GVHD prophylaxis and active GVHD. It is thought that conditioning regimen and intensity plays a vital role in the pathophysiology of IPS by inciting pulmonary epithelial damage and subsequent macrophage and lymphocyte mediated immune response. Alveolar damage presenting as multilobar opacities on imaging, increased alveolar-arterial oxygen gradient, hypoxemia and exclusion of other causative factors are necessary to establish diagnosis. The management of IPS largely revolves around supportive care. High-dose glucocorticoids (>1 mg/kg) are often administered but their efficacy is not well established and progressive IPS carries a mortality risk of nearly 90%. Given the observation that inflammatory cytokines such as interleukin-1, -6, -8, and TNF-α, have been implicated in IPS, etanercept, a TNF-α binding protein has been evaluated in this setting. In a small series, etanercept with glucocorticoids resulted in clinical response but did not seem to improve survival outcomes. A phase III randomized trial comparing etanercept/placebo with glucocorticoids closed early due to poor accrual and did not show any clear benefit with etanercept over placebo.[27]

Diffuse Alveolar Hemorrhage

Diffuse alveolar hemorrhage (DAH) is a clinical syndrome of acute onset characterized by diffuse pulmonary infiltrates on imaging and the diagnosis is usually established by bronchoscopy showing progressively bloody fluid on sequential lavage.[28] Onset typically occurs within 3 months of HCT and presenting symptoms include dyspnea, hypoxia and cough, although late-onset alveolar hemorrhage has also been described. Alveolar hemorrhage may occur in the setting of lung infections or accelerated GVHD where, apart from supportive measures, treatment should be targeted at the underlying etiology. When no discernible etiology is identified, DAH represents a spectrum of non-infectious pulmonary complications which include IPS. Interestingly, DAH can occur after both autologous and allogeneic HCT, older age, and underlying GVHD are risk factors. Its pathogenesis remains poorly described, but likely is a result of a complex interactions, including alveolar injury from radiation and chemotherapy, inflammatory damage due to neutrophils and cytokines, and underlying infections. DAH can rapidly evolve into severe respiratory failure with mortality rates of greater than 70%. The mainstay of therapy includes prompt management of underlying etiology (e.g., infection or GVHD), correction of any coagulopathy, and aggressive ventilatory support. For DAH, high-dose corticosteroids remain the mainstay of therapy in most centers, although efficacy data are scant. Small case series have reported the successful use of recombinant factor VIIa and aminocaproic acid, but their efficacy needs to be confirmed in clinical trials and prothrombotic risks should carefully be counter balanced.

Other Complications

The early post-transplant period is invariably characterized by some degree of cytopenias needing platelet and red cell transfusion support, although this may be less pronounced after NMA/RIC conditioning. Judicious use of blood products is suggested for carefully balancing the benefits versus risks. Bleeding diathesis due to severe thrombocytopenia and coagulopathy resulting from vitamin K deficiency or infectious complications should be monitored. Transplant-related thrombotic microangiopathy (TMA) is a unique disorder typically seen in MAC allogeneic-HCT recipients taking tacrolimus and/or sirolimus for GVHD prophylaxis and is characterized by complement mediated injury causing microangiopathy, intravascular hemolysis, renal insufficiency, and neurological dysfunction. Management is supportive and using alternative therapy for GVHD prevention should be considered. Observational studies suggest that complement blockade with eculizumab may be an effective strategy in patients with end organ dysfunction and may lead to improved survival compared to historical controls. Gastrointestinal toxicities including oral mucositis, nausea/vomiting, diarrhea, and enterocolitis are commonly seen in the pre-engraftment phase and are usually related to conditioning toxicity and cytopenias. Treatment strategies mostly rely on supportive care, pain and symptom management and antimicrobials. For oral mucositis cryotherapy during MAC (e.g., radiation, melphalan) is commonly employed. Prophylaxis with palifermin (recombinant human keratinocyte growth factor) in patients undergoing high-dose conditioning has shown modest benefits. Deconditioning, functional incapacitation, nutritional deficiencies, and psychosocial turmoil are common during the transplant period and require the treatment team to be cognizant and responsive to the patient's individual needs in a multidisciplinary fashion.

LATE NON-INFECTIOUS COMPLICATIONS

Transplantation techniques and supportive care improvements have not only resulted in increased use of transplantation as a therapeutic strategy but have led to improvements in outcomes and long-term survival. The biggest challenge to long-term survival after HCT is relapse of the primary disease, and for recipients who remain disease-free through 2 to 5 years post-transplantation, the probability of survival over the next 10 to 20 years is greater than 80%.[23,29] However, survivors remain at considerable risk for late transplant-associated complications, which can include organ specific dysfunction, second cancers, infections due to ongoing immunodeficiency, and functional and psycho-social impairments and changes in quality of life (QOL). Among 5-year post-HCT survivors, 45% and 79% of autologous and allogeneic HCT recipients develop at least one late complication, respectively. Over a quarter of the allogeneic HCT patients may have >3 late effects. Unfortunately, transplant survivors appear to have higher risk of pulmonary complications, infections, hospitalization and all-cause mortality compared to their age-matched peers in the general population.[30] It is of paramount importance that physicians and other healthcare providers caring for transplant recipients be familiar with published guidelines for screening and prevention of late effects in HCT survivors.[4] Additionally, it is also important to follow general population guidelines for the screening and prevention of cancers, as well as chronic diseases and health maintenance.

The development of late effects is dependent on a myriad of patient-related (age, pre-HCT comorbidities and performance scores, existing lifestyle, underlying disease and therapies received), transplant-related (allogeneic HCT, conditioning intensity, radiation) and the presence of early post-transplant complications. Allogeneic HCT recipients who develop GVHD require long-term, and sometimes life-long immunosuppression. This can significantly affect their overall functional status and QoL, as well as increase susceptibility to adverse effects to drugs, infections, and second malignancies.

Organ-Specific Late Effects

Although any organ system can be involved, certain organs have a greater predilection for late-onset post-transplant complications (Table 110.5).[31] Dedicated long-term follow-up clinics and survivorship plans that monitor and screen patients for late complications per established guidelines are ideal (see Table 110.5). Early identification and management of late toxicities can potentially circumvent debilitating morbidities and mortality related to end-organ dysfunction. Late pulmonary complications (besides infections) including bronchiolitis obliterans and pulmonary hypertension negatively impact mortality. Cataracts develop in more than one-third of patients by 5-years post transplantation and are worsened by being on glucocorticoids, often requiring surgical therapy. Hypothyroidism can be seen in ~50% and hypogonadism in ~90% of HCT survivors. The majority of HCT survivors become permanently sterile, although HCT without TBI and use of RIC/NMA can be fertility-sparing. Prepubertal children may retain fertility, although secondary sexual development is often delayed. Up to 50% of children undergoing HCT also develop growth retardation. Musculoskeletal complications including osteoporosis and avascular necrosis can be particularly debilitating. An increased incidence of cardiovascular events and diabetes has also been reported. The risk for most organ-specific late complications continues to increase with time and continued surveillance for these problems are indicated in all HCT survivors.

Second Cancers

Secondary malignancies are rare but devastating complications of HCT. They account for 5% to 10% of deaths among survivors beyond 2 years after HCT. These include (1) post-transplant lymphoproliferative disorders (PTLD), (2) myelodysplasia and other hematologic malignancies, and (3) solid malignancies.[32]

PTLD comprises of a heterogeneous group of lymphoid proliferations, primarily involving B-lymphocytes that develop as a result of uncontrolled Epstein-Barr virus (EBV) infection and is usually donor-derived. They occur almost exclusively in allogeneic HCT recipients,

TABLE 110.5 Selected Late Complications of Hematopoietic Cell Transplantation

Complication	Risk Factors	Monitoring and Prevention
Endocrine		
Hypothyroidism	TBI/radiation	Assessment of thyroid and gonadal function at 6-months and annually thereafter
Hypogonadism	Chronic GVHD	
Growth retardation	Chemotherapy	
Ocular		
Cataracts	TBI/radiation	Ophthalmology evaluation at 6-months and annually thereafter
Keratoconjunctivitis sicca	Corticosteroids	
	Chronic GVHD	
Oral		
Dental caries	TBI/radiation	Dental evaluation, at 6-months and annually Thereafter
Dry mouth	Chronic GVHD	
Cardiovascular		
Coronary artery disease	TBI/radiation	Risk factor modification (monitor and manage hypertension, lipid panel, diabetes)
Cerebrovascular disease	Chemotherapy	Periodic clinical evaluation
Respiratory		
Bronchiolitis obliterans	TBI/radiation	Smoking cessation, clinical evaluation
Interstitial pneumonitis	Chronic GVHD	Pulmonary function tests, at 6-months and annually thereafter
	Infections	
Hepatic		
Cirrhosis	Hepatitis B or C	Periodic liver function tests
Iron overload	Transfusions	Serum ferritin level (at 1-year and as indicated)
Renal		
Nephropathy	TBI/radiation	Monitor renal parameters
	Chemotherapy	Monitor urine protein (annually)
	Cyclosporine	Control hypertension
Skeletal		
Osteoporosis	TBI/radiation	Periodic bone densitometry
Avascular necrosis	Corticosteroids	Imaging as indicated
Second cancers	TBI/radiation	Cancer screening as recommended
	Chemotherapy	
	Chronic GVHD	

GVHD, Graft-versus-host disease; *HLA*, human leukocyte antigen; *PCP*, *Pneumocystis carinii* pneumonia.

with an overall incidence rate of 1% to 2%. More than 80% of PTLD are diagnosed in the first year, although late occurrences have been described. Since T-lymphocytes play an important role in preventing proliferation of EBV-infected B-lymphocytes, removal of T-cells from the hematopoietic graft source (ex vivo or in vivo) is a major risk factor for PTLD. A greater degree of immunosuppression, usually noted in unrelated or mismatched allografts, the use of antithymocyte globulin or post-transplant cyclophosphamide, GVHD, all increase the risk for PTLD. Risk of PTLD is higher when EBV-seronegative patients receive EBV-seropositive allograft.

Clinical presentation of PTLD can be variable with patients presenting with constitutional symptoms, lymphadenopathy and end-organ dysfunction. Diagnosis usually requires histopathological confirmation. PTLD is classified as (1) early lesions (polyclonal), (2) polymorphic (polyclonal and monoclonal not meeting criteria for B-cell or NK/T-cell lymphoma), (3) monomorphic (monoclonal, meeting criteria for B-cell or NK/T-cell lymphoma), and (4) Hodgkin lymphoma-like. Treatment for early lesions and polymorphic-PTLD usually constitutes withdrawal of immunosuppression as tolerated and anti-CD20 monoclonal antibody such as rituximab. For monomorphic and Hodgkin lymphoma-like PTLD, combination chemotherapy ± rituximab (based on CD20 status) is necessary. Most transplant centers monitor for EBV viremia by quantitative PCR and consider pre-emptive therapy with rituximab (thus depleting the B-cells where EBV resides and replicates). Since PTLD can be associated with considerable morbidity and mortality, active surveillance and pre-emptive therapy is recommended for high-risk patients. EBV-specific cytotoxic lymphocytes have been shown to be efficacious in the treatment of refractory PTLD and clinical trials testing this approach in a multi-center setting are ongoing.

Secondary MDS and acute myeloid leukemia (AML) is seen in 5% to 5% of autologous HCT recipients, but is rare among allogeneic HCT patients, and has a latency period ranging between 3 and 5 years post-transplant.[32] Cytogenetic evaluation by fluorescent in-situ hybridization/karyotype and mutation analysis by next generation sequencing (NGS) may reveal characteristic chromosomal aberrations of therapy-related MDS/AML. Risk factors include older age, prior therapies (especially alkylators, topoisomerase inhibitors etc.) and conditioning regimens (e.g., irradiation). Overall outcomes are poor and long-term survival is less than 20% even with an allogeneic HCT.

Secondary solid cancers have a latency period of 3 to 5 years following HCT and the risk of incidence continues to rise with time and is higher than their age- and gender-matched peers in the general population. The cumulative incidence ranges from 1% to 2% at 5 years, 2% to 6% at 10 years, and 4% to 15% at 15 years post-transplantation.[32] Younger age at transplantation, prior therapy including immunomodulator therapy for myeloma, use of TBI and chronic GVHD are important risk factors for solid cancers. Solid cancers at a variety of sites have been reported, including cancers of the head and neck, breast, skin, liver, brain and nervous system, thyroid, and bone and connective tissue. Counseling regarding maintaining a healthy life style, smoking cessation, skin protection, and lifelong cancer screening is recommended for all HCT survivors according to established guidelines specific to transplant survivors and general population (Table 110.6).[4,33]

Quality of Life After Transplantation

Despite the significant early morbidities associated with HCT, most survivors attain high levels of physical and psychological QOL and return to full-time employment by 3 to 5 years post-transplant. Unfortunately, about 20% to 40% of long-term survivors carry the burden of therapy received and endure physical, functional, psychological, cognitive, sexual, and social impairments years after transplantation. The major risk factors for poor QOL are older age and advanced disease at transplantation, chronic GVHD, late complications of HCT described above and other medical problems. The patient's own personal and cultural background and expectations affect perceptions of QOL. Although chronic GVHD is a strong predictor of poor QOL, the overall health and functional status improves with resolution of GVHD and eventually reaches a level comparable to that seen in patients with no history of chronic GVHD. Gender-specific differences in QOL have also been observed, with females more likely to report impairments in psychological and sexual domains. Cognitive deficits, particularly involving executive function, memory, and motor skills, have been reported in 30% to 60%

TABLE 110.6 Screening Guidelines for Common Cancers After Hematopoietic Cell Transplantation

Site	Screening Recommendations[a]
Breast	Annual mammogram from age 40–45; for women receiving >800 cGy chest radiation screening to begin at age 25 or 8 years after radiation, whichever is later
Cervix	Start at age 21; Pap test every 3 years for ages 21–29; co-testing (Pap + HPV test) for ages 30–65
Colorectal	Start at age 45; stool-based tests (immunohistochemical, DNA) annually or direct visualization (e.g., colonoscopy every 10 years); certain high-risk groups (e.g., patients with inflammatory bowel disease) may need earlier initiation and more frequent screening
Lung	Imaging as clinically indicated; for high-risk patients between the ages 55 and 75 low-dose CT scan as per guidelines
Oral	Routine oral cavity exam
Thyroid	Annual TSH and thyroid exam; imaging as indicated
Skin	Annual skin checks

[a] Recommendations are based on published guidelines specific to HCT recipients and American Cancer Society recommendations for the general public.
HCT, Hematopoietic cell transplantation; *HPV*, human papilloma virus..

of HCT survivors. The risk of developing these neuropsychological sequelae is increased in patients receiving transplantation at an older age, and with the use of irradiation based conditioning regimens and cyclosporine. Survivorship plans that incorporate periodic QOL-questionnaires are important for early identification and mitigation of QOL issues. A structured survivorship clinic and multi-disciplinary approach involving supportive care services, onco-psychiatry, and physical and mental rehabilitation are increasingly being employed and are recommended. Frailty and geriatric assessments prior to HCT and their impact on transplant outcomes, including QOL measures, are areas of active research.

GRAFT-VERSUS-HOST DISEASE

GVHD is a multisystem, clinicopathologic syndrome of donor-derived lymphocyte-mediated alloreactivity that commonly occurs as a complication of allogeneic HCT and leads to significant morbidity and mortality. It is usually classified as acute or chronic GVHD. Acute GVHD is further classified as classic acute GVHD (onset ≤100 days) and persistent, recurrent or late-onset acute GVHD (onset >100 days). In the absence of clinical features characteristic of acute GVHD, chronic GVHD is called classic chronic GVHD. When features of both acute and chronic GVHD appear together, it is classified as overlap syndrome, based upon a consensus conference on proposed definitions.[34,35]

Current understanding of the pathogenesis of GVHD suggests that alloreactive donor T-lymphocytes recognize histocompatibility antigens on host cells and initiate secondary inflammatory injury, leading to the clinical symptoms of GVHD. Acute GVHD is indeed driven by donor T-lymphocytes and inflammatory cytokines resulting in tissue destruction, whereas chronic GVHD is more complex and involves interactions between host immune system, autoreactivity, loss of immune tolerance and donor-derived dysregulated T- and B-lymphocytes.[36] The alloreactive response can be initiated or accelerated by conditioning regimen–induced tissue injury with release of proinflammatory cytokines, primarily interleukin (IL)-1 and TNF-α. GVHD is more frequent and more severe in recipients of partially matched or HLA-incompatible transplants, suggesting that major histocompatibility complex encoded molecules may be the prime antigenic targets initiating the alloreactive T-cell response. Minor histocompatibility antigens also play an important role in its pathogenesis. Activated donor derived T-cells produce IL-2 and interferon-γ, expand, and differentiate into effector cells and recruit mononuclear phagocytes and neutrophils that ultimately yield host tissue destruction, primarily through apoptosis. T-cell depletion of the graft (ex vivo or in vivo) reduces the risk of acute and chronic GVHD, albeit at the cost of increasing risks of disease relapse due to blunting of the graft-versus-tumor response.

Acute Graft-Versus-Host Disease

Risk Factors and Clinical Features

Published reports suggest that 10% to 50% of patients receiving HLA-identical sibling-donor develop acute GVHD. It is more common after unrelated donor and mismatched allografts. Donor-recipient HLA disparity is the most important risk factor for acute GVHD. Additional risk factors include increasing recipient and donor age, use of alloimmunized donors such as parous women, and HCT from unrelated instead of sibling donors. Among recipients of UCB, acute GVHD occurs in 20% to 60% of patients. Haploidentical donor transplantation is also a risk factor for acute GVHD, although it is partially reversed with the current strategy of post-transplant cyclophosphamide for GVHD prevention.

The skin, liver, and gastrointestinal tract are the most common sites of acute GVHD. Skin involvement is characterized by a maculopapular rash that, when severe, can lead to bullae formation and resemble toxic epidermal necrolysis. Hepatic involvement manifests as cholestatic jaundice with marked elevation of serum bilirubin and alkaline phosphatase, but usually only mild transaminase alterations. In the intestine, upper gastrointestinal tract GVHD can produce nausea, vomiting and anorexia, whereas small bowel and colon GVHD produces large volume secretory diarrhea. Acute GVHD is graded according to organs involved (skin, liver or gastrointestinal tract) and the extent (Stage) of each organ involvement (Table 110.7).[34] The diagnosis is clinical but frequently requires histologic confirmation to distinguish it from other frequent toxicities in the early post-transplantation period (e.g., hypersensitivity drug rash, drug-induced cholestasis, VOD/SOS, or infectious enteritis). The histologic hallmark of acute GVHD is apoptosis of the proliferative and regenerative cell layer of the epidermis, intestinal or biliary epithelium. Plasma biomarkers (ST2, REG3alpha, TNFR1) in patients with acute GVHD have been used to establish a prognostic algorithm that can predict response to therapy and NRM at 6 months.[37] The utility of implementing these biomarkers in routine clinical practice is an area of ongoing research.

Prophylaxis of Graft-Versus-Host Disease

The most effective techniques for GVHD prevention have involved ex vivo depletion of donor T-lymphocytes, most often coupling immunologic recognition (monoclonal anti-T cell antibodies) with depletion techniques (immunomagnetic beads, complement cytotoxicity or toxin immunoconjugates). Although vigorous T-cell depletion prevents acute GVHD, it also increases the risk of graft failure and relapse of primary malignancy after transplantation.

Pharmacologic immunosuppression and in vivo T-cell depletion administered in the early peri-transplant period prevent or blunt the initiating T-cell recognition and proliferative response that triggers GVHD and can allow development of immune system tolerance without impeding lymphohematopoietic recovery and donor chimerism. Methotrexate, corticosteroids, antithymocyte globulin, cyclosporine, tacrolimus, mycophenolate, and sirolimus have been used for prophylaxis of GVHD and have successfully reduced both the frequency and the severity of clinical GVHD. Despite the potential role of inflammatory cytokines in initiation and amplification of GVHD, clinical blockade of IL-1, IL-2, or TNF-α has not been

TABLE 110.7 Acute Graft-Versus-Host Disease Staging by Consensus Criteria[34]

Stage	Skin	Liver (Bilirubin)	Gastrointestinal
0	No skin rash	<2 mg/dL	<50 mL/day or persistent nausea alone
1	Maculopapular rash <25% BSA	2.1–3 mg/dL	500–1000 mL/day, or persistent nausea, vomiting, anorexia, or positive upper GI biopsy
2	Maculopapular rash 25%–50% BSA	3.1–6 mg/dL	1000–1500 mL/day
3	Maculopapular rash >50% BSA	6.1–15 mg/dL	>1500 mL/day
4	Generalized erythroderma, plus bullae or desquamation	>15 mg/dL	> 2000 mL/day, severe abdominal pain ±ileus

Clinical Grade	Skin	Liver	Gastrointestinal
I	Stage 1–2	None	None
II	Stage 3 or	Stage 1 or	Stage 1
III	—	Stage 2–3 or	Stages 2–4
IV	Stage 4 or	Stage 4	—

[a] Milliliter/day of liquid stool.
BSA, Body surface area; *GI*, gastrointestinal.

effective in GVHD prophylaxis. The most commonly used backbone of most GVHD prevention strategies includes calcineurin inhibitors with methotrexate or mycophenolate with or without antithymocyte globulin. In the last decade, high-dose post-transplant cyclophosphamide has emerged as a valuable GVHD prevention strategy in the context of haploidentical donor HCT. Several randomized trials are evaluating this strategy in transplant strategies using donors other than haploidentical related donors. In a phase III trial post-transplant cyclophosphamide was found to reduce the incidence of severe acute GVHD and chronic GVHD compared cyclosporine-based prophylaxis in matched related and unrelated donor RIC/NMA allo-HCT.[38]

Treatment of Acute Graft-Versus-Host Disease

Therapy for acute GVHD requires both immunosuppression to negate the T-cell mediated immune response that causes tissue injury and appropriate supportive care. Corticosteroids are the mainstay of initial therapy. GVHD involving limited areas of the skin (stage 1 to 2, overall grade I) can be treated with topical corticosteroids alone. Oral beclomethasone can be used to treat isolated GVHD of the upper gastrointestinal tract. Systemic corticosteroids (2 mg/kg/day prednisone or equivalent) are the standard initial therapy of more advanced GVHD with an expected response rate of ~50%. No regimen has shown a consistent increase in response rates or improvement in survival compared to corticosteroids alone. In a phase II randomized trial comparing corticosteroids with mycophenolate, pentostatin, etanercept or denileukin diftitox, efficacy and toxicity data suggested the combination of mycophenolate plus corticosteroids to be most promising in the initial therapy of acute GVHD.[39] However, this combination did not yield any improvements over corticosteroids alone in a randomized phase III trial.[40] For steroid refractory acute GVHD outcomes are generally poor. Salvage therapy with antithymocyte globulin or other agents like sirolimus, tacrolimus, MMF, pentostatin or cyclosporine yields a response rate of about 10% to 40%. The selective Janus kinase inhibitor ruxolitinib was compared to the investigator's choice second-line therapy for steroid-refractory acute GVHD in a large phase III trial and showed significant improvement in response rates at day +28 (62% vs. 39%).[41] Ruxolitinib is now FDA approved for this indication. Monoclonal antibodies and immunotoxins directed against T-cells or inflammatory cytokines have been investigated, although their effectiveness has not been demonstrated outside of small case series. Specific agents with reported activity in steroid-refractory acute GVHD include pentostatin, etanercept and infliximab (TNF-α receptor blockers), dacluzimab and denileukin diftitox (IL-2 receptor inhibitors), visilizumab (anti-CD3 antibody), and α-1 antitrypsin (protease inhibitor). Extra-corporeal photopheresis (ECP) has been reported to have some efficacy in steroid refractory acute GVHD. In addition to effective immunosuppression, successful management of acute GVHD involves attention to supportive care, particularly skin care, nutrition, and limitation of polypharmacy-associated drug interactions. Infections are a leading cause of death in patients with GVHD and attention should be paid to infection surveillance and prophylaxis.

TABLE 110.8 Common Clinical Manifestations of Chronic Graft-Versus-Host Disease[35]

Organ System	Clinical Manifestations
Cutaneous	Poikiloderma, lichen planus, dermal sclerosis, morphea-like features, hypo- or hyper-pigmentation, ichthyosis, nail dystrophy, onycholysis
Ocular	Keratoconjunctivitis sicca, conjunctivitis, corneal ulcerations
Oral	Lichen planus, hyperkeratotic plaques, xerostomia, mucosal atrophy, ulcers, restriction of mouth opening from sclerosis
Pulmonary	Bronchiolitis obliterans, bronchiolitis obliterans-organizing pneumonia (BOOP)
Gastrointestinal	Esophageal web and strictures, malabsorption syndrome, exocrine pancreatic insufficiency
Hepatic	Cholestasis
Genitourinary	Vaginal stenosis or scarring, lichen planus
Musculoskeletal	Fasciitis, joint contractures from sclerosis, myositis or polymyositis, arthritis
Hematopoietic	Thrombocytopenia, eosinophilia, lymphopenia, hemolytic anemia, hypogammaglobulinemia

Chronic Graft-Versus-Host Disease

Risk Factors and Clinical Features

Chronic GVHD is a complex syndrome that affects allogeneic HCT patients to varying degrees. Usually presenting beyond day +100, the diagnosis is based on clinical presentation rather than timing alone. Preceding acute GVHD, unrelated donor HCT and peripheral blood as the graft source are the main risk factors for subsequent chronic GVHD. Like acute GVHD, ex vivo or in vivo T-cell depletion of the graft can reduce the incidence of chronic GVHD.

Chronic GVHD can affect any organ system; however, certain pathognomonic clinical signs and symptoms aid in establishing the diagnosis (Table 110.8).[35] Histological confirmation, though not always required, may help confirm the diagnosis and rule out other etiologies, especially when patients exhibit unusual presentation. The skin, mouth, eyes, and liver are the most commonly involved sites of chronic GHVD. The cutaneous manifestations resemble autoimmune disease and can include poikiloderma, lichen planus-like eruptions or scleroderma. An inflammatory dermatitis can progress to severe dermal and periarticular fibrosis with loss of skin appendages (hair and sweat glands) as well as significant skin tightness, fasciitis, and loss of joint flexibility. Additional manifestations include dry eyes and mouth, which can resemble Sjögren's syndrome clinically and histologically; enteritis with anorexia, early satiety, malabsorption, weight loss and failure to thrive, or esophageal dysmotility or stricture; and cholestatic (rarely hepatitic) jaundice. Pulmonary involvement in the form of bronchiolitis obliterans is an uncommon manifestation but can be particularly debilitating and lethal. In addition, the profound immune dysfunction associated with chronic GVHD, due to hypogammaglobulinemia, impaired cellular immunity and functional asplenia, greatly increases the risk of secondary infections from bacteria, viruses, and fungi. After the onset of chronic GVHD, 25% to 40% of patients die within 2 years, with infections often being the immediate cause. Chronic GVHD can be classified as mild, moderate, or severe depending on the number of organs involved and the degree of individual organ involvement. In general, factors associated with an adverse prognosis include thrombocytopenia, progressive onset from acute GVHD, poor performance status, lack of response to initial therapy and extensive skin or lung involvement.

Treatment of Chronic Graft-Versus-Host Disease

Although acute GVHD is one of the strongest predictors for chronic GVHD, strategies to limit acute GVHD, such as prolonging or intensifying initial immunosuppression, have not been consistently effective in preventing subsequent chronic GVHD. As with acute GVHD, the use of corticosteroids, usually in combination with calcineurin inhibitor, is the mainstay of therapy. The protracted nature of chronic GVHD syndrome warrants long-term therapy. Lowest possible doses and alternate day steroid therapy are useful strategies to potentially minimize the chronic complications of prolonged corticosteroid therapy. Typical corticosteroid regimens start with daily prednisone 1 mg/kg/day and responding patients are tapered as tolerated. Chronic immunosuppression is usually continued long-term even for quiescent chronic GVHD to avoid exacerbations. Local treatment strategies targeting affected organ systems (e.g., cyclosporine eye drops, dexamethasone mouth rinses) are useful in limiting systemic immunosuppression, especially for limited stage chronic GVHD. For chronic GVHD that is steroid-dependent or refractory, salvage therapies are needed. If not already on it, a calcineurin inhibitor may be added. Other therapeutic options include sirolimus, mycophenolate, ruxolitinib, ibrutinib (FDA approved), rituximab, and imatinib. Most salvage strategies work by modulation of T-cell function, B-cell depletion, induction of immune tolerance, and cytokine blockade. T-cell immunomodulation using extracorporeal photopheresis has been shown to have some activity, especially in sclerotic cutaneous chronic GVHD. The treatment of chronic GVHD demands attention to aggressive antimicrobial prophylaxis and immunizations to prevent opportunistic infections. Most successful strategies for treating chronic GVHD incorporate long-term reduced-dose immunosuppressive therapy, aggressive antimicrobial prophylaxis, and supportive care. Strategies favoring development of donor-derived regulatory T cells (Tregs) which may facilitate the development of immunologic tolerance are being studied.

FUTURE DIRECTIONS

Complications of HCT are one of the major barriers to the wider application of transplantation for a variety of diseases. Currently, only one in four adult HCT recipients survives to 1 year post-HCT without experiencing a major complication. The impact of newer transplantation modalities, including the use of NMA/RIC regimens, alternative donor transplantation with haploidentical donor and UCB, and incorporation of immune-based therapies within transplantation regimens, on early and late complications continue to evolve. Better tools to predict the risk of post-HCT complications and NRM are needed as well. Genomic and proteomic approaches to HCT complications are being investigated to monitor and predict complications. Although they are some years from use in clinical practice, these approaches could have many potential applications in transplantation, including prediction of risk for complications and GVHD, refining donor selection, and utilization of pharmacogenomic data to individualize conditioning regimens and immunosuppression. Each of these facets represent areas of ongoing vigorous research to improve the safety and effectiveness of HCT.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Bolaños-Meade J, Logan BR, Alousi AM, et al. Phase 3 clinical trial of steroids/mycophenolate mofetil vs steroids/placebo as therapy for acute GVHD: BMT CTN 0802. *Blood*. 2014;124(22):3221–3335.

Carreras E. How I manage sinusoidal obstruction syndrome after haematopoietic cell transplantation. *Br J Haematol*. 2015;168(4):481–491.

Chow EJ, Cushing-Haugen KL, Cheng G-S, et al. Morbidity and mortality differences between hematopoietic cell transplantation survivors and other cancer survivors. *J Clin Oncol*. 2016;35(3):306–313.

Cordonnier C, Einarsdottir S, Cesaro S, et al. Vaccination of haemopoietic stem cell transplant recipients: guidelines of the 2017 European Conference on Infections in Leukaemia (ECIL 7). *Lancet Infect Dis*. 2019;19(6): e200–e212.

Crocchiolo R, Bramanti S, Vai A, et al. Infections after T-replete haploidentical transplantation and high-dose cyclophosphamide as graft-versus-host disease prophylaxis. *Transpl Infect Dis*. 2015;17(2):242–249.

Dehn J, Spellman S, Hurley CK, et al. Selection of unrelated donors and cord blood units for hematopoietic cell transplantation: guidelines from the NMDP/CIBMTR. *Blood*. 2019;134(12):924–934.

De Jong CN, Meijer E, Bakunina K, et al. Post-transplantation cyclophosphamide after allogeneic hematopoietic stem cell transplantation: results of the prospective randomized HOVON-96 trial in recipients of matched related and unrelated donors. *Blood*. 2019;134(suppl_1). 1-1.

Doede T, Viddam A, Villa KF, et al. Diagnosis of veno-occlusive disease/sinusoidal obstruction syndrome (VOD/SOS) with or without multi-organ dysfunction (MOD) after hematopoietic stem cell transplantation (HSCT): analysis of a multicenter chart review. *Blood*. 2016;128(22). 5756-5756.

Gooley TA, Chien JW, Pergam SA, et al. Reduced mortality after allogeneic hematopoietic-cell transplantation. *N Engl J Med*. 2010;363(22): 2091–2101.

Jagasia MH, Greinix HT, Arora M, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. The 2014 Diagnosis and Staging Working Group report. *Biol Blood Marrow Transplant*. 2015;21(3):389–401.e1.

Khera N, Storer B, Flowers MED, et al. Nonmalignant late effects and compromised functional status in survivors of hematopoietic cell transplantation. *J Clin Oncol*. 2011;30(1):71–77.

Levine JE, Braun TM, Harris AC, et al. A prognostic score for acute graft-versus-host disease based on biomarkers: a multicentre study. *Lancet Haematol*. 2015;2(1):e21–e29.

Ljungman P, de la Camara R, Robin C, et al. Guidelines for the management of cytomegalovirus infection in patients with haematological malignancies and after stem cell transplantation from the 2017 European Conference on Infections in Leukaemia (ECIL 7). *Lancet Infect Dis*. 2019;19(8): e260–e272.

Ljungman P, Mikulska M, de la Camara R, et al. The challenge of COVID-19 and hematopoietic cell transplantation; EBMT recommendations for management of hematopoietic cell transplant recipients, their donors, and patients undergoing CAR T-cell therapy *Bone Marrow Transpl*. 2020;55(11):2017–2076.

Majhail NS. Secondary cancers following allogeneic haematopoietic cell transplantation in adults. *Br J Haematol*. 2011;154(3):301–310.

Majhail NS, Parks K, Defor TE, Weisdorf DJ. Diffuse alveolar hemorrhage and infection-associated alveolar hemorrhage following hematopoietic stem cell transplantation: related and high-risk clinical syndromes. *Biol Blood Marrow Transplant*. 2006;12(10):1038–1046.

Majhail NS, Rizzo JD, Lee SJ, et al. Recommended screening and preventive practices for long-term survivors after hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2012;18(3):348–371.

Marty FM, Ljungman P, Chemaly RF, et al. Letermovir prophylaxis for cytomegalovirus in hematopoietic-cell transplantation. *N Engl J Med*. 2017;377(25):2433–2444.

Mattsson J, Ringdén O, Storb R. Graft failure after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2008;14(1, Suppl):165–170.

McDonald GB. Hepatobiliary complications of hematopoietic cell transplantation, 40 years on. *Hepatology*. 2010;51(4):1450–1460.

McDonald GB, Sandmaier BM, Mielcarek M, et al. Survival, nonrelapse mortality, and relapse-related mortality after allogeneic hematopoietic cell transplantation: comparing 2003–2007 versus 2013–2017 cohorts. *Ann Intern Med*. 2020;172(4):229–239.

Mohty M, Malard F, Abecassis M, et al. Revised diagnosis and severity criteria for sinusoidal obstruction syndrome/veno-occlusive disease in adult patients: a new classification from the European Society for Blood and Marrow Transplantation. *Bone Marrow Transplant*. 2016;51(7):906–912.

Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant*. 1995;15(6):825–828.

Richardson PG, Riches ML, Kernan NA, et al. Phase 3 trial of defibrotide for the treatment of severe veno-occlusive disease and multi-organ failure. *Blood*. 2016;127(13):1656–1665.

Taplitz RA, Kennedy EB, Bow EJ, et al. Antimicrobial prophylaxis for adult patients with cancer-related immunosuppression: ASCO and IDSA clinical practice guideline update. *J Clin Oncol*. 2018;36(30):3043–3054.

Teira P, Battiwalla M, Ramanathan M, et al. Early cytomegalovirus reactivation remains associated with increased transplant-related mortality in the current era: a CIBMTR analysis. *Blood*. 2016;127(20):2427–2438.

Tomblyn M, Chiller T, Einsele H, et al. Guidelines for preventing infectious complications among hematopoietic cell transplantation recipients: a global perspective. *Biol Blood Marrow Transplant*. 2009;15(10):1143–1238.

Wingard JR, Carter SL, Walsh TJ, et al. Randomized, double-blind trial of fluconazole versus voriconazole for prevention of invasive fungal infection after allogeneic hematopoietic cell transplantation. *Blood*. 2010;116(24):5111–5118.

Zeiser R, von Bubnoff N, Butler J, et al. Ruxolitinib for glucocorticoid-refractory acute graft-versus-host disease. *N Engl J Med*. 2020;382(19):1800–1810.

REFERENCES

1. Tomblyn M, Chiller T, Einsele H, et al. Guidelines for preventing infectious complications among hematopoietic cell transplantation recipients: a global perspective. *Biol Blood Marrow Transpl*. 2009;15(10):1143–1238. https://doi.org/10.1016/j.bbmt.2009.06.019.
2. Taplitz RA, Kennedy EB, Bow EJ, et al. Antimicrobial prophylaxis for adult patients with cancer-related immunosuppression: ASCO and IDSA clinical practice guideline update. *J Clin Oncol*. 2018;36(30):3043–3054. https://doi.org/10.1200/JCO.18.00374.
3. Wingard JR, Hsu J, Hiemenz JW. Hematopoietic stem cell transplantation: an overview of infection risks and epidemiology. *Infect Dis Clin North Am*. 2010;24(2):257–272. https://doi.org/10.1016/j.idc.2010.01.010.
4. Majhail NS, Rizzo JD, Lee SJ, et al. Recommended screening and preventive practices for long-term survivors after hematopoietic cell transplantation. *Biol Blood Marrow Transpl*. 2012;18(3):348–371. https://doi.org/10.1016/j.bbmt.2011.12.519.
5. Cordonnier C, Einarsdottir S, Cesaro S, et al. Vaccination of haemopoietic stem cell transplant recipients: guidelines of the 2017 European Conference on Infections in Leukaemia (ECIL 7). *Lancet Infect Dis*. 2019;19(6): e200–e212. https://doi.org/10.1016/S1473-3099(18)30600-5.
6. Van Burik J-A, Brunstein CG. Infectious complications following unrelated cord blood transplantation. *Vox Sanguinis*. 2007;92(4):289–296. https://doi.org/10.1111/j.1423-0410.2007.00900.x.
7. Crocchiolo R, Bramanti S, Vai A, et al. Infections after T-replete haploidentical transplantation and high-dose cyclophosphamide as graft-versus-host disease prophylaxis. *Transpl Infect Dis*. 2015;17(2):242–249. https://doi.org/10.1111/tid.12365.
8. Taplitz RA, Kennedy EB, Bow EJ, et al. Outpatient management of fever and neutropenia in adults treated for malignancy: American Society of Clinical Oncology and Infectious Diseases Society of America Clinical Practice Guideline Update. *JCO*. 2018;36(14):1443–1453. https://doi.org/10.1200/JCO.2017.77.6211.
9. Ljungman P, de la Camara R, Robin C, et al. Guidelines for the management of cytomegalovirus infection in patients with haematological malignancies and after stem cell transplantation from the 2017 European Conference on Infections in Leukaemia (ECIL 7). *Lancet Infect Dis*. 2019;19(8):e260–e272. https://doi.org/10.1016/S1473-3099(19)30107-0.
10. Teira P, Battiwalla M, Ramanathan M, et al. Early cytomegalovirus reactivation remains associated with increased transplant-related mortality in the current era: a CIBMTR analysis. *Blood*. 2016;127(20):2427–2438. https://doi.org/10.1182/blood-2015-11-679639.
11. Marty FM, Ljungman P, Chemaly RF, et al. Letermovir prophylaxis for cytomegalovirus in hematopoietic-cell transplantation. *N Engl J Med*. 2017;377(25):2433–2444. https://doi.org/10.1056/NEJMoa1706640.
12. Betts BC, Young J-AH, Ustun C, et al. Human herpesvirus 6 infection after hematopoietic cell transplantation: is routine surveillance necessary? *Biol Blood Marrow Transpl*. 2011;17(10):1562–1568. https://doi.org/10.1016/j.bbmt.2011.04.004.
13. Ljungman P, Mikulska M, de la Camara R, et al. The challenge of COVID-19 and hematopoietic cell transplantation; EBMT recommendations for management of hematopoietic cell transplant recipients, their donors, and patients undergoing CAR T-cell therapy *Bone Marrow Transpl*. 2020;55(11):2017–2076. https://doi.org/10.1038/s41409-020-0919-0. Published online May 13.
14. Wingard JR. The changing face of invasive fungal infections in hematopoietic cell transplant recipients. *Curr OpOncol*. 2005;17(2):89–92. https://journals.lww.com/co-oncology/Fulltext/2005/03000/The_changing_face_of_invasive_fungal_infections_in.3.aspx.
15. Ullmann AJ, Lipton JH, Vesole DH, et al. Posaconazole or fluconazole for prophylaxis in severe graft-versus-host disease. *N Engl J Med*. 2007;356(4): 335–347. https://doi.org/10.1056/NEJMoa061098.
16. Wingard JR, Carter SL, Walsh TJ, et al. Randomized, double-blind trial of fluconazole versus voriconazole for prevention of invasive fungal infection after allogeneic hematopoietic cell transplantation. *Blood*. 2010;116(24): 5111–5118. https://doi.org/10.1182/blood-2010-02-268151.
17. Dehn J, Spellman S, Hurley CK, et al. Selection of unrelated donors and cord blood units for hematopoietic cell transplantation: guidelines from the NMDP/CIBMTR. *Blood*. 2019;134(12):924–934. https://doi.org/10.1182/blood.2019001212.
18. Baron F, Sandmaier BM. Chimerism and outcomes after allogeneic hematopoietic cell transplantation following nonmyeloablative conditioning. *Leukemia*. 2006;20(10):1690–1700.
19. Mattsson J, Ringdén O, Storb R. Graft failure after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2008;14(1, Supplement): 165–170. https://doi.org/10.1016/j.bbmt.2007.10.025.
20. McDonald GB. Hepatobiliary complications of hematopoietic cell transplantation, 40 years on. *Hepatology*. 2010;51(4):1450–1460. https://doi.org/10.1002/hep.23533.
21. Doede T, Viddam A, Villa KF, et al. Diagnosis of veno-occlusive disease/sinusoidal obstruction syndrome (VOD/SOS) with or without multi-organ dysfunction (MOD) after hematopoietic stem cell transplantation (HSCT): analysis of a multicenter chart review. *Blood*. 2016;128(22). https://doi.org/10.1182/blood.V128.22.5756.5756. 5756-5756.
22. Mohty M, Malard F, Abecassis M, et al. Revised diagnosis and severity criteria for sinusoidal obstruction syndrome/veno-occlusive disease in adult patients: a new classification from the European Society for Blood and Marrow Transplantation. *Bone Marrow Transpl*. 2016;51(7):906–912. https://doi.org/10.1038/bmt.2016.130.
23. Gooley TA, Chien JW, Pergam SA, et al. Reduced mortality after allogeneic hematopoietic-cell transplantation. *N Engl J Med*. 2010;363(22): 2091–2101. https://doi.org/10.1056/NEJMoa1004383.
24. Carreras E. How I manage sinusoidal obstruction syndrome after haematopoietic cell transplantation. *Br J Haematology*. 2015;168(4): 481–491. https://doi.org/10.1111/bjh.13215.
25. Richardson PG, Riches ML, Kernan NA, et al. Phase 3 trial of defibrotide for the treatment of severe veno-occlusive disease and multi-organ failure. *Blood*. 2016;127(13):1656–1665. https://doi.org/10.1182/blood-2015-10-676924.
26. Weiner RS, Bortin MM, Gale RP, et al. Interstitial pneumonitis after bone marrow transplantation: assessment of risk factors. *Ann Intern Med*. 1986;104(2):168–175. https://doi.org/10.7326/0003-4819-104-2-168.
27. Yanik GA, Horowitz MM, Weisdorf DJ, et al. Randomized, double-blind, placebo-controlled trial of soluble tumor necrosis factor receptor: enbrel (etanercept) for the treatment of idiopathic pneumonia syndrome after allogeneic stem cell transplantation: blood and marrow transplant clinical trials network protocol. *Biol Blood Marrow Transpl*. 2014;20(6):858–864. https://doi.org/10.1016/j.bbmt.2014.02.026.
28. Majhail NS, Parks K, Defor TE, et al. Diffuse alveolar hemorrhage and infection-associated alveolar hemorrhage following hematopoietic stem cell transplantation: related and high-risk clinical syndromes. *Biol Blood Marrow Transplant*. 2006;12(10):1038–1046. https://doi.org/10.1016/j.bbmt.2006.06.002.
29. McDonald GB, Sandmaier BM, Mielcarek M, et al. Survival, nonrelapse mortality, and relapse-related mortality after allogeneic hematopoietic cell transplantation: comparing 2003–2007 versus 2013–2017 cohorts. *Ann Intern Med*. 2020;172(4):229–239. https://doi.org/10.7326/M19-2936.
30. Chow EJ, Cushing-Haugen KL, Cheng G-S, et al. Morbidity and mortality differences between hematopoietic cell transplantation survivors and other cancer survivors. *JCO*. 2016;35(3):306–313. https://doi.org/10.1200/JCO.2016.68.8457.
31. Khera N, Storer B, Flowers MED, et al. Nonmalignant late effects and compromised functional status in survivors of hematopoietic cell transplantation. *JCO*. 2011;30(1):71–77. https://doi.org/10.1200/JCO.2011.38.4594.
32. Majhail NS. Secondary cancers following allogeneic haematopoietic cell transplantation in adults. *Br J Haematology*. 2011;154(3):301–310. https://doi.org/10.1111/j.1365-2141.2011.08756.x.
33. Croke L. Cancer screening: ACS releases annual summary of recommendations. *Am Fam Physician*. 2019;99(11):719–722.
34. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus conference on acute GVHD grading. *Bone Marrow Transpl*. 1995;15(6): 825–828.
35. Jagasia MH, Greinix HT, Arora M, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. The 2014 diagnosis and staging working group report. *Biol Blood Marrow Transpl*. 2015;21(3):389–401.e1. https://doi.org/10.1016/j.bbmt.2014.12.001.
36. Ferrara JLM, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet*. 2009;373(9674):1550–1561. https://doi.org/10.1016/S0140-6736(09)60237-3.
37. Levine JE, Braun TM, Harris AC, et al. A prognostic score for acute graft-versus-host disease based on biomarkers: a multicentre study. *Lancet Haematology*. 2015;2(1):e21–e29. https://doi.org/10.1016/S2352-3026(14)00035-0.
38. De Jong CN, Meijer E, Bakunina K, et al. Post-transplantation cyclophosphamide after allogeneic hematopoietic stem cell transplantation: results of the prospective randomized HOVON-96 trial in recipients of matched related and unrelated donors. *Blood*. 2019;134(suppl_1). https://doi.org/10.1182/blood-2019-124659. 1-1.

39. Alousi AM, Weisdorf DJ, Logan BR, et al. Etanercept, mycophenolate, denileukin, or pentostatin plus corticosteroids for acute graft-versus-host disease: a randomized phase 2 trial from the Blood and Marrow Transplant Clinical Trials Network. *Blood*. 2009;114(3):511–517. https://doi.org/10.1182/blood-2009-03-212290.
40. Bolaños-Meade J, Logan BR, Alousi AM, et al. Phase 3 clinical trial of steroids/mycophenolate mofetil vs steroids/placebo as therapy for acute GVHD: BMT CTN 0802. *Blood*. 2014;124(22):3221–3335. https://doi.org/10.1182/blood-2014-06-577023.
41. Zeiser R, von Bubnoff N, Butler J, et al. Ruxolitinib for glucocorticoid-refractory acute graft-versus-host disease. *N Engl J Med*. 2020;382(19):1800–1810. https://doi.org/10.1056/NEJMoa1917635.

PART X TRANSFUSION MEDICINE

CHAPTER 111

HUMAN BLOOD GROUP ANTIGENS AND ANTIBODIES

William J. Lane, Connie M. Westhoff, Jill R. Storry, and Beth H. Shaz

Pretransfusion testing includes ABO and Rhesus (Rh) type, and antibody screening to determine whether a patient has an unexpected red blood cell (RBC) antibody. If the antibody screen is positive, an identification panel is performed to identify the specificity. Unexpected antibodies can be clinically significant causing hemolysis (i.e., acute or delayed hemolytic reaction) after transfusion of RBCs carrying the reciprocal antigen, or can be insignificant. The clinical significance of an antibody is assessed by correlating the serologic information with clinical experiences reported in the literature and with the patient's medical history. Notably, the majority of clinically significant antibodies (outside the ABO system) are in response to RBC antigen exposure either through transfusion or pregnancy. Other antibody characteristics that are used to predict clinical significance include immunoglobulin (Ig) class and in vitro characteristics such as strength of reactivity and titer; however, no foolproof method exists to predict the clinical significance. For antibodies with well-known clinical significance, antigen-negative blood is selected for transfusion. Predicting clinical significance is more difficult when a patient has an antibody to a novel or rare high-prevalence antigen, and requires a transfusion but antigen-negative blood is not available.

ERYTHROCYTE BLOOD GROUP ANTIGENS

Erythrocyte blood group antigens are polymorphic, inherited, carbohydrate, or protein structures located on the surface of the RBC membrane. There are more than 300 blood group antigens, most of which are included in 38 different blood group systems (Table 111.1). The protein antigens are primarily located on integral transmembrane proteins, but a few are on glycosylphosphatidylinositol (GPI)–linked proteins (Fig. 111.1). Some antigens are carbohydrates attached to proteins or lipids, some require a combination of a specific portion of protein and carbohydrate, and a few antigens are carried on proteins that are adsorbed from the plasma. Many of the proteins carrying blood group antigens reside in the erythrocyte membrane as complexes with other proteins.

Recognition of a new blood group antigen begins with discovery of an antibody. When an individual whose RBCs lack an antigen is exposed to RBCs that possess the antigen, they may mount an immune response and produce antibodies that react with the antigen. Depending on the characteristics of the antibody and the number and topology of antigens in the RBC membrane, the interaction in vivo between antibody and antigen may result in removal of antibody-coated RBCs by the reticuloendothelial system or in hemolysis if complement is activated.

In blood group testing, most assays are designed to detect antibody-antigen binding with clumping of the RBCs ("agglutination") as the detectable endpoint. The ability to detect and identify blood group antigens and antibodies has contributed significantly to current safe supportive blood transfusion practice, to the appropriate management of pregnancies at risk for hemolytic disease of the fetus and newborn (HDFN), and to management of hematopoietic progenitor cell and solid organ transplantation.

Terminology

Some blood group systems bear the family surname in which the antibody was first discovered (Kell, Kidd, Duffy, etc.), with abbreviations to indicate antigens (K/k, Jk^a/Jk^b, Fy^a/Fy^b, etc.). Others have been given letter designations (A, B, D, M, N, etc.). A committee for terminology of RBC surface antigens and alleles, organized by the International Society of Blood Transfusion (ISBT), works to standardize terminology of new blood group antigens and the coding alleles.

DNA-Based Typing for Blood Group Antigens

The majority of genes encoding blood group antigens have been identified and cloned, and the molecular basis of most blood group antigens has been determined.[1,2] Details concerning the alleles associated with blood group antigens are found on the ISBT Red Cell Immunogenetics and Blood Group Terminology working party webpage, and the Blood Antigens and Erythrogene websites (www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/; bloodantigens.com; erythrogene.com). Knowledge of the genes has advanced understanding of the structure and function of the components carrying antigens, and resulted in an appreciation of diseases associated with loss of expression of some blood groups, for example, null phenotypes (see Table 111.1). Of importance, knowledge of the gene has made it possible to perform DNA analyses to predict the serologic phenotype, to determine gene dosage (zygosity), to perform noninvasive fetal typing, and to type for numerous blood group antigens in a single assay.

Although the simple hemagglutination test remains the principal assay for RBC antigen typing for ABO and Rh, antibody screen, and compatibility testing; DNA-based typing (genotyping), especially for the minor blood group antigens, has become commonplace in some clinical situations (Table 111.2).[3] These include determination of the extended blood group phenotype in patients who are multiply transfused, which avoids false typing because of contaminating donor RBCs and aids determination of antibody specificity. This approach is also preferred in patients with strongly direct antiglobulin test (DAT)-positive RBCs, as well as for typing for antigens when no serologic reagents are available and for fetal typing from amniocytes or from free DNA present in the maternal plasma. In these instances[4] and others (see Table 111.2), hemagglutination is not helpful, and genomic analysis is a useful adjunct to routine testing. More recently, genotyping is useful in patients treated with daratumumab (anti-CD38) because treatment results in panreactivity on antibody

TABLE 111.1 Blood Group Systems, Antigens, Expression, and Disease Associations

ISBT System Name (Number)	Gene Name	Predicted Topology (Number of Amino Acids [AA])	Component Name	Principal Associated Blood Group Antigens (Null Phenotype)	Present in Other Tissue	Disease Association	Function
ABO (001)	*ABO*	Glycosyl-transferases Type II^a (354 AA)	Carbohydrate	A, B, AB, A1 (group O)	Secretions, platelets, broad tissue distribution	Altered in some hematologic disorders, leukemia	Glycosylation
MNS (002)	*GYPA (MN)* *GYPB (Ss)*	Type I (131 AA) Type I^a (72 AA)	GPA GPB	M, N, S, s, U, Vw; M^k M^k lack GPA and GPB; En(a–) lack GPA; S–s–U– lack GPB	Renal endothelium and epithelium	Decreased *Plasmodium falciparum* invasion May be receptor for *Escherichia coli*	Negative charge on sialic acid; receptor for microbes
P1PK (003)	*A4GALT*	Galactosyl-transferase Type II (353 AA)	Carbohydrate	P1, Pk (P1–, PP1Pk–)	Lymphocytes, granulocytes, monocytes, platelets	Receptor *E. coli* and *Parvovirus-B19* Miscarriage	Glycosylation
Rh (004)	*RHD* *RHCE*	Multipass—12 spans (417 AA)	RhD RhCE	D, C, E, c, e, G, V/VS (Rh$_{null}$ syndrome)	RBC-specific	Hemolytic anemia; stomatocytosis Reduced expression and mosaicism in hematologic malignancies	Structural link to underlying cytoskeleton
Lutheran (005)	*BCAM* *(LU)*	Type I IgSF (597 AA) (557 AA)	Lutheran glycoprotein B-CAM	Lu^a, Lu^b, Lu3, Au^a, Au^b (recessive Lu(a–b–))	Broad tissue distribution Not on lymphocytes, granulocytes, monocytes or platelets	Increased expression possibly involved in vasoocclusion in sickle cell disease	Possibly adhesion; may mediate intracellular signalling Binds to laminin
Kell (006)	*KEL*	Type II (732 AA)	Kell glycoprotein	K, k, Kp^a, Kp^b, Ku, Js^a, Js^b (K$_0$ or K$_{null}$)	Broad tissue distribution Bone marrow, fetal liver, testes, brain, heart, skeletal muscle	Depressed in McLeod syndrome (see XK)	Cleaves big endothelin 3 to ET-3 (a potent vasoconstrictor)
Lewis (007)	*FUT3 (LE)*	Not endogenous to RBCs Type II (361 AA)	Carbohydrate Adsorbed from plasma	Le^a, Le^b, Le^{ab}, Le^{bh}, ALe^b, BLe^b (Le(a–b–))	Saliva and body fluids, Blood cells, GI, skeletal muscle, kidney, adrenal	Increased expression in fucosidosis	Fucosyl transferase
Duffy (008)	*ACKR1 (DARC, FY)*	Multipass—7 spans (338 AA)	Fy glycoprotein	Fy^a, Fy^b, Fy3, Fy6 (Fy(a–b–))	Broad tissue distribution Endothelial and epithelial cells, Purkinje cells, colon, lung, spleen, thyroid, thymus, kidney	*Plasmodium vivax* receptor RBC null-resistant to *P. vivax*	Chemokine receptor
Kidd (009)	*SLC14A1 (JK)*	Multipass—10 spans (389 AA)	Kidd glycoprotein	Jk^a, Jk^b, Jk3 (Jk(a–b–) or Jk$_{null}$)	Kidney: vasa recta endothelium Renal medulla	Urine concentrating defect	Urea transport
Diego (010)	*SLC4A1 (DI)*	Multipass—14 spans (911 AA)	Band 3, AE1	Di^a, Di^b, Wr^a, Wr^b, (1 reported—transfusion dependent; predicted to be incompatible with life)	Kidney: intercalated cells of distal and collecting tubules	Southeast Asian ovalocytosis, hereditary spherocytosis, renal tubular acidosis	Anion transport CO_2/HCO_3^- exchange

Continued

TABLE 111.1 Blood Group Systems, Antigens, Expression, and Disease Associations—cont'd

ISBT System Name (Number)	Gene Name	Predicted Topology (Number of Amino Acids [AA])	Component Name	Principal Associated Blood Group Antigens (Null Phenotype)	Present in Other Tissue	Disease Association	Function
Yt (011)	*ACHE (YT)*	GPI-linked (557 AA)	Acetyl-cholinesterase	Yt^a, Yt^b	Brain, muscle, nerves	Absent from PNH III RBCs	Enzymatic
Xg (012)	*XG*	Type I (180 AA) (163 AA)	Xg^a glycoprotein	Xg^a	The antigen may be restricted to RBC, but CD99 has broad tissue distribution		Adhesion molecule
Scianna (013)	*ERMAP (SC)*	Type I (475 AA)	ERMAP	Sc1, Sc2, Sc3, Rd (Sc −1, −2, −3)			Possible adhesion
Dombrock (014)	*ART4 (DO)*	GPI-linked (314 AA)	Do glycoprotein; ART 4	Do^a, Do^b, Gy^a, Hy, Jo^a (Gy(a−))	Lymphocytes, spleen, lymph nodes, GI, ovary, testes, heart, liver	Absent from PNH III RBCs	Enzymatic
Colton (015)	*AQP1 (CO)*	Multipass—6 spans (269 AA)	Aquaporin	Co^a, Co^b, Co3 (Co(a−b−))	Broad tissue distribution Kidney, liver, gallbladder, eye, capillary endothelium	Monosomy 7, congenital dyserythropoietic anemia	Water transport
Landsteiner-Wiener (016)	*ICAM4 (LW)*	Type I IgSF (241 AA)	LW glycoproteinICAM-4	LW^a, LW^b, LW^{ab}(LW(a−b−))		Depressed in some malignant diseases; decreased in Rh$_{null}$ syndrome	Ligand for integrins
Chido/ Rodgers (017)	*C4A, C4B (CH/RG)*	Not endogenous to RBC (1191 AA)	C4A; C4B	Ch1, Ch2, Rg1	Adsorbed from plasma	Certain phenotypes increased susceptibility to autoimmune conditions and infections C4-deficient Predisposes for SLE	Part of the complement cascade
H (018)	*FUT1(H)*	Fucosyl-transferase Type II (365 AA)	Carbohydrate	H (Bombay O$_h$)	Broad distribution Soluble—all fluids except CSF in secretors	Decreased in some tumor cells Increased in hematopoietic stress	Glycosylation
Kx (019)	*XK (XK)*	Multipass—10 spans (444 AA)	XK glycoprotein	Kx (McLeod)	Fetal liver, adult skeletal muscle, brain, pancreas, heart	X-linked midlife onset neuropathy, elevated CPK, muscular dystrophy, acanthocytosis; sometimes associated with CGD	Transport; possible neurotransmitter
Gerbich (020)	*GYPC (GE)*	Type I (128 AA) (107 AA)	GPC GPD	Ge2, Ge3, Ge4 (Leach phenotype)	Fetal liver, renal endothelium	Hereditary elliptocytosis, hemolytic anemia, receptor *P. falciparum*	Structural Interacts with protein 4.1 and p55

Continued

TABLE 111.1 Blood Group Systems, Antigens, Expression, and Disease Associations—cont'd

ISBT System Name (Number)	Gene Name	Predicted Topology (Number of Amino Acids [AA])	Component Name	Principal Associated Blood Group Antigens (Null Phenotype)	Present in Other Tissue	Disease Association	Function
Cromer (021)	*CD55 (CROM)*	GPI-linked (347 AA)	DAF	Cr^a, Tc^a, Tc^b, Tc^c, Dr^a, Es^a, IFC (Inab)	Vascular endothelium Epithelial GI, GU, CNS Soluble form in plasma and urine	Absent from PNH III RBCs Dr^a is receptor for uropathogenic *E. coli*	Complement regulation; binds C3b; disassembles C3/C5 convertase
Knops (022)	*CR1 (KN)*	Type I (1998 AA)	CR1	Kn^a, Kn^b, McC^a, Sl^a, Yk^a, KCAM (no nulls reported)	Blood cells, glomerular podocytes, follicular dendritic cells	Antigens depressed in certain autoimmune and malignant conditions	Complement regulation; binds C3b and C4b; mediates phagocytosis
Indian (023)	*CD44 (IN)*	Type I (341 AA)	Hermes antigen	In^a, In^b	Wide tissue distribution	1 case—congenital dyserythropoietic anemia	Binds hyaluronic acid; mediates adhesion of leukocytes
Ok (024)	*BSG (OK)*	Type I IgSF (248 AA)	Basigin	Ok^a	All cells tested	Receptor *P. falciparum*	Possible adhesion
RAPH (025)	*MER2*	Multipass—4 spans (253 AA)	CD151	MER2 (Raph–)	Fibroblasts	Absence associated with renal disease and kidney failure	
JMH (026)	*SEMA7A(JMH)*	GPI-linked (656 AA)	Semaphorin 7A	JMH		Absent from PNHIII RBCs	Adhesion molecule
I (027)	*GCNT2*	*N*-acetyl-glucosaminyl-transferase type II (400 AA)	Carbohydrate	I (I– or i adult)	Broad tissue distribution	Cataracts in Asians	Glycosylation
Globoside (028)	*B3GALT1*	*N*-acetyl-galactosaminyl-transferase type II (331 AA)	Carbohydrate (Gb$_4$, globoside)	P (P–) P^k and p	Broad tissue distribution Placenta (trophoblasts and interstitial cells)	Receptor *E. coli* and *Parvovirus-B19* Spontaneous abortion	Glycosylation
GIL (029)	*AQP3 (GIL)*	Multipass—6 spans (292 AA)	AQP3	GIL (GIL–)	Board tissue distribution Kidney, prostate, GI tract, spleen, skin, eye		Glycerol/ water/ urea transport
RHAG (030)	*RHAG*	Multipass—12 spans (409 AA)	Rh-associated glycoprotein	RHAG1 or Duclos, RHAG2 or Ol^a, RHAG3 or DL, RHAG4 (Rh$_{null}$ regulator)	RBC-specific but homologs RhBG, RhCG in kidney, liver, skin, GI tract	Hereditary overhydrated stomatocytosis	Ammonia transport
FORS (031)	*GBGT1*	Type II (347 AA)	Carbohydrate	FORS1	Broad tissue distribution		Glycosyltransferase
JR (032)	*ABCG2*	Type III—6 spans (655 AA)	ATP-binding cassette sub-family G member 2	Jr^a (Jr(a–))	Broad tissue distribution High in placenta, seminal vesicles	Gout; multidrug resistance in cancer	Urate exporter; porphyrin hemostasis
LAN (033)	*ABCB6*	Type III—6 spans (842 AA)	ATP-binding cassette sub-family B member 6, mitochondrial	Lan (Lan–)	Broad tissue distribution	Dyschromatosis universalis hereditaria; Microphthalmia	Binds heme and porphyrins. Important in heme synthesis

Continued

TABLE 111.1 Blood Group Systems, Antigens, Expression, and Disease Associations—cont'd

ISBT System Name (Number)	Gene Name	Predicted Topology (Number of Amino Acids [AA])	Component Name	Principal Associated Blood Group Antigens (Null Phenotype)	Present in Other Tissue	Disease Association	Function
VEL (034)	*SMIM1*	Type 1 (78 AA)	SMIM1	Vel (Vel–)	RBCs, salivary glands, testis		Not known
CD59 (035)	*CD59*	GPI-linked (128 AA)	CD59 glycoprotein	CD59.1 (CD59.1–)	Broad tissue distribution; soluble form in plasma	Hemolytic anemia, with or without polyneuropathy	Complement regulation; inhibits MAC complex
AUG (036)	*SLC29A1*	Type III—11 spans (456 AA)	Equilibrative nucleoside transporter 1	At^a (At(a–)	Broad tissue distribution	Pseudo gout in the null phenotype	Nucleoside transmembrane transporter
KANNO (037)	*PRNP*	(253 AA)	Prion	KANNO	Broad tissue distribution	Absence of KANNO possibly protective for Creutzfeldt-Jakob disease	Possible role in neuronal development and synaptic plasticity
Sid (038)	*B4GALNT2*	Beta-1,4 N-acetylgalactosaminyltransferase 2 (566 AA)	Carbohydrate	Sd^a ((Sd(a–))	Kidney, colon, stomach	Extra strong expression of Sd^a Sd(a++) makes cells resistant to malarial parasite *P. falciparum*	Glycosylation

Type I, protein with a single pass through the RBC lipid bilayer with its amino terminus to the outside of the cell; type II, protein with a single pass through the RBC lipid bilayer with its amino terminus to the inside of the cell. *AE1*, Anion exchanger 1; *AQP*, aquaporin; *C4*, fourth component of complement; *CGD*, chronic granulomatous disease; *CR1*, complement receptor 1; *CSF*, colony-stimulating factor; *CNS*, central nervous system; *DAF*, decay-accelerating factor; *ERMAP*, erythrocyte membrane-associated protein; *GPA*, glycophorin A; *GPB*, glycophorin B; *GPC*, glycophorin C; *GPD*, glycophorin D; *GI*, gastrointestinal; *GPI*, glycosylphosphatidylinositol; *GU*, genitourinary; *ICAM4*, intercellular adhesion molecule 4; *IgSF*, immunoglobulin super family; *ISBT*, International Society of Blood Transfusion; *PNH*, paroxysmal nocturnal hemoglobinuria; *RBC*, red blood cell; *SLE*, systemic lupus erythematosus.

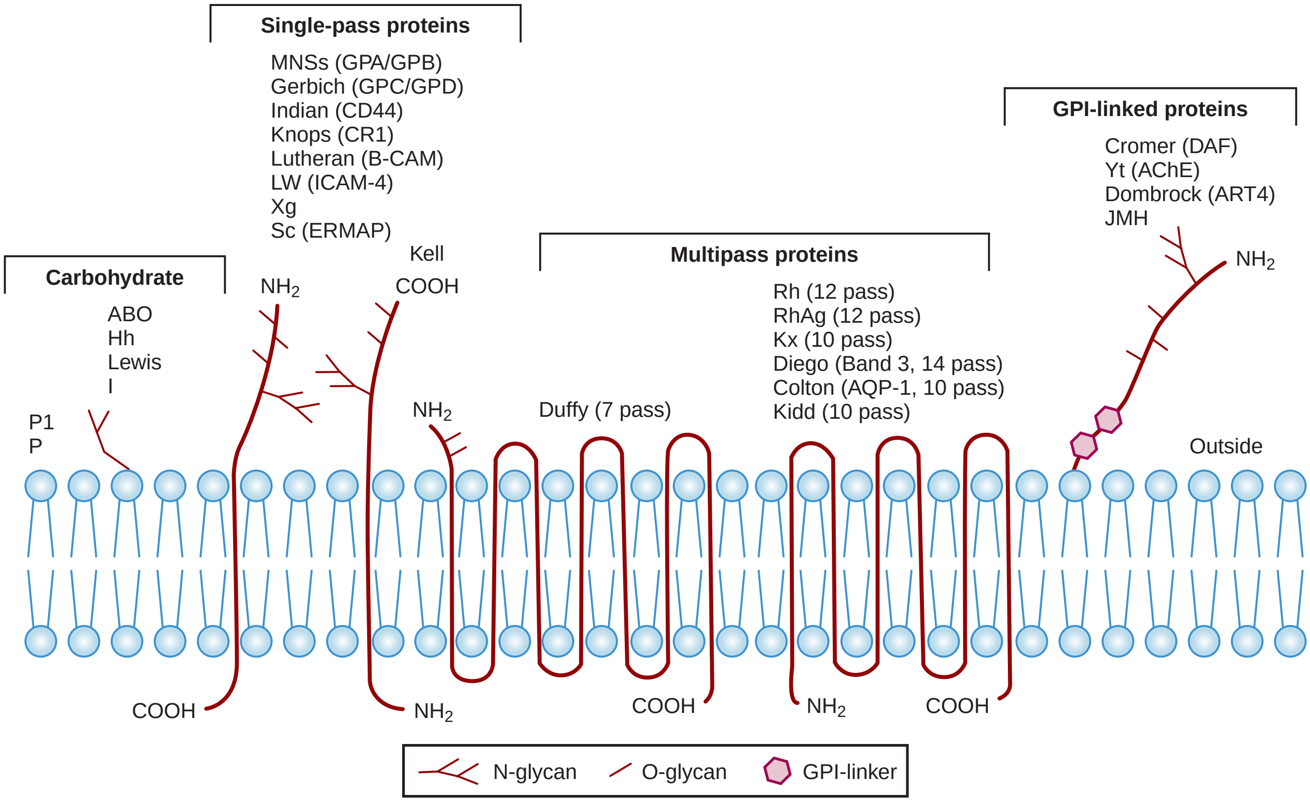


Figure 111.1 MODEL OF BLOOD GROUP PROTEINS IN THE RED BLOOD CELL MEMBRANE. Schematic representation of the red blood cell (RBC) molecules that carry blood group antigens and the predicted type of structure. These include carbohydrates, single and multipass proteins, and GPI-linked proteins in the RBC membrane (represented by the blue molecules arranged as a bilayer). Note that not all blood group–bearing structures are listed but those that are more commonly encountered. NH_2 indicates orientation of the N-terminus of the protein; COOH indicates the orientation of the C-terminus. Glycosylation of proteins is shown as indicated, and may be small O-linked carbohydrate chains or more complex N-linked sugar structures. The extracellular surface of the lipid bilayer is also heavily glycosylated with blood group–bearing carbohydrates.

TABLE 111.2 **Uses of DNA-Based Genotyping Assays for Transfusion Medicine**

Type patients who have been recently transfused
Type patients who are being treated with monoclonal antibody therapy, for example anti-CD38
Type RBCs coated with immunoglobulin
Type patients with AIHA (to select antigen-negative RBCs for transfusion and absorption of autoantibodies when searching for underlying alloantibodies)
Type RBCs when commercial antisera are not available
Type for numerous blood group antigens in a single assay
Identify weak D and partial D (to determine candidate for Rh immune globulin or avoid use of limited Rh-negative donor supply)
Resolve blood group typing discrepancies
Determine paternal zygosity for *RHD* and HPA
Type fetus to determine risk for HDFN or NAIT
ABO genotyping of buccal swabs for screening of stem cell transplant donors

AIHA, Autoimmune hemolytic anemia; *HDFN*, hemolytic disease of the fetus and newborn; *HPA*, human platelet antigen; *NAIT*, neonatal alloimmune thrombocytopenia; *RBC*, red blood cell.

screening (all cells being reactive). High-throughput genotyping systems have enabled blood centers to screen donors for a large number of antigens in a single assay. *RHD* genotyping is also well suited for the precise determination of known weak D and partial D variations.

Polymerase chain reaction (PCR) -based genotyping assays currently dominate the market. However, next generation sequencing (NGS) has proven to be a naive, but promising approach.[5,6] NGS allows for the sequencing of large genomic regions, making it ideal for novel antigen discovery, and has been used to determine the genetic basis of several recent blood group systems. Interpretation of NGS based whole genome and whole exome data can be used to genotype for essentially all antigens with a known genetic basis. Furthermore, the development of automated interpretive software has allowed for genotyping from large scale genomic sequencing projects. In addition, targeted NGS of either one or multiple blood group genes is possible, including the ABO and Rh blood group systems. Targeted NGS based *ABO* genotyping particularly has value alongside NGS based human leukocyte antigen (HLA) typing of buccal swabs when screening stem cell transplant donors. In addition, NGS, especially whole genome sequencing (WGS), is well suited for the evaluation of structural variations including large deletions (e.g. MNS U– *GYPB* deletion) and gene recombinations (e.g., Rh partial D DIIIa-CE(4-7)-D).

Blood Group Antibodies

The common causes of immunization against blood group antigens are transfusion, pregnancy, transplantation, or occasionally, practices such as sharing needles. “Naturally occurring” antibodies are not a result of RBC exposure; rather, a response to microbes encountered by way of the digestive tract and other mucosal surfaces regularly (e.g., anti-A, anti-B,) or sometimes (e.g., anti-M, -P, -P^k, -P1, -Le^a, -Le^b, -I, -IH) which result in production of antibodies with these specificities. These are the most common antibodies present in children and nontransfused male patients, and are primarily

TABLE 111.3 **Characteristics of Some Blood Group Alloantibodies (Listed in Approximate Order of Clinical Significance)**

Antibody Specificity	IgM	IgG	Clinical	
			Transfusion Reaction	**HDFN**
ABO	Most	Some	Immediate; mild to severe	Common; mild to moderate
H in Bombay	Most	Some	Immediate; mild to severe	Rare; mild
Rh	Some	Most	Immediate/delayed; mild to severe	Common; mild to severe
RhAG	Rare	Most	Immediate/delayed; mild to severe	Mild to severe
Kell	Some	Most	Immediate/delayed; mild to severe	Mild to severe
Kidd	Few	Most	Immediate/delayed; mild to severe	Rare; mild
Duffy	Rare	Most	Immediate/delayed; mild to severe	Rare; mild
S	Some	Most	Delayed/mild	Rare; mild to severe
s	Rare	Most	Delayed/mild	Rare; mild to severe
U	Rare	Most	Immediate/delayed; mild to severe	Rare; severe
PP1P^k	Most[a]	Most[a]	Immediate; mild to severe	Mild to severe[b]
Vel	Most[a]	Most[a]	Immediate/delayed; mild to severe	Mild to severe
Diego	Some	Most	Delayed; none to severe	Mild to severe
Colton	Rare	Most	Delayed; mild	Rare; mild to severe
Lutheran	Some	Most	Delayed	Rare; mild
Dombrock	Rare	Most	Immediate/delayed; mild to severe	Rare; mild
M	Some	Most	Delayed (rare)	Rare; mild to severe
N	Most	Rare	None	None
LW^a	Rare	Most	Delayed; none to mild	Rare; mild
Yt^a	Rare	Most	Delayed (rare); none to mild	None
Ch/Rg	Rare	Most	Anaphylactic (rare)	None
JMH	Rare	Most	Delayed (rare in genetic variants); none to mild	None
P1	Most	Rare	None (rare)	None
Le^a	Most	Few	Immediate (rare)	None
Le^b	Most	Few	None	None
I	Most	Rare	None to mild in I adults	None
Knops	Rare	Most	None	None
Xg^a	Rare	Most	None	None

[a] Most examples of these antibodies are both IgM and IgG.

[b] Seldom hemolysis of fetal cells but high incidence of recurrent spontaneous abortions.

IgG, Immunoglobulin G; *IgM*, immunoglobulin M; *HDFN*, Hemolytic disease of the fetus and newborn; *Rh*, rhesus; *RhAG*, Rh-associated glycoprotein.

IgM. These multivalent IgM antibodies directed to carbohydrate antigens optimally bind and directly agglutinate to RBCs at temperatures below 37°C. Most are not clinically significant (outside of ABO). Exceptions occur if the antibody is reactive at 37°C and/or has an IgG component. Antibodies that are considered not to be clinically significant unless the antibody reacts in tests performed at 37°C include those to A1, P1, M, N, Lu^a, Le^a, Le^b, I, IH, and Sd^a antigens.

In contrast, antibodies occurring following immunization to protein antigens such as those in the Rh, Kell, Kidd, and Duffy blood group systems are primarily of the IgG isotype that react at 37°C and are detected by the indirect antiglobulin test (IAT). These bivalent antibodies optimally bind to, but do not directly agglutinate, RBCs at 37°C. The addition of an antiglobulin reagent (i.e., antihuman globulin [AHG], also known as Coombs serum) is required to induce RBC agglutination. Most of these are clinically significant antibodies, with the exception of antibodies to Knops (Kn), Chido/Rodgers (Ch/Rg) and John Milton Hagen (JMH) systems (Table 111.3 summarizes the Ig class and clinical significance associated with alloantibodies; see also box on Indirect Antiglobulin Test and Direct Antiglobulin Test).

Antibodies recognizing antigens in the ABO system are by far the most clinically significant and are present in nearly all individuals who lack the antigen (they typically appear by 4 months of age). Other clinically significant antibodies occur in the following approximate order, from the most to the least commonly encountered in transfusion practice: anti-D, anti-K, anti-E, anti-c, anti-Fy^a, anti-C, anti-Jk^a, anti-S, and anti-Jk^b. Clinically significant antibodies occur in approximately 3% of transfused patients[7] but have a higher incidence of 35% to 55% in patients undergoing chronic transfusion.[8–12] The frequency of antibody production depends on the antigen immunogenicity and prevalence of the antigen in a population.

Compatibility Procedures and Location of Antigen-Negative Blood

Manual tube, solid phase, or gel-column methods based on agglutination of RBCs are the most common serologic assays performed in transfusion medicine laboratories.

INDIRECT ANTIGLOBULIN TEST AND DIRECT ANTIGLOBULIN TEST

The indirect antiglobulin test (IAT) is used to detect alloantibodies in patient sera in vitro following incubation at 37°C and includes antibody screening and identification and crossmatching with donor red blood cells (RBCs). IAT is also sometimes used for antigen typing to detect RBCs coated with antibody following incubation with reagent antisera. After incubation, unbound antibodies are removed by washing with saline and an antiglobulin reagent containing either antihuman IgG (AHG) or a mixture of AHG and monoclonal antihuman complement is added. Differential agglutination suggests the presence of antibodies to one or more specific RBC antigens while agglutination of all cells suggests the presence of an antibody to a high-prevalence antigen or the presence of an autoantibody.

The direct antiglobulin test (DAT) is used to detect the presence of antibody or complement (or both) on the surface of RBCs in vivo such as autoantibodies coating the patient's cells in warm autoimmune hemolytic anemia, cold hemagglutinin disease, or alloantibodies coating the patient's cells in immediate or delayed transfusion reactions, or hemolytic disease of the fetus and newborn. Patient RBCs obtained in ethylenediaminetetraacetic acid, to prevent in vitro complement deposition on the RBCs, are washed with saline and then incubated with a commercial antiglobulin reagent containing AHG or antihuman complement or a mixture of the two. Antiglobulin reagents containing anti-IgM or anti-IgA are available in specialized centers to detect coating of RBCs by antibodies of these isotypes.

Rh IMMUNE GLOBULIN

Rh immune globulin (RhIG) is a human plasma-derived hyperimmunoglobulin product consisting of IgG antibodies to D antigen that is administered to D-negative pregnant women who are at risk for D sensitization. RhIG is administered (1) at 28 weeks gestational age, (2) when there is a risk for fetal maternal hemorrhage through amniocentesis, trauma, or other procedures, and (3) postpartum in the case of a known or potential D-positive newborn or fetus. If the Rh(D) typing of a pregnant woman is discrepant with prior results, or typing reactions are weaker than expected, or if variable reactivity is seen, RHD genotyping should be considered to guide RhIG prophylaxis and selection of blood for transfusion.

RhIG is sometimes administered outside of pregnancy to D-negative patients who receive D-positive blood products, most commonly whole blood derived platelet products. This is primarily considered for females of childbearing potential when the formation of anti-D has serious consequences. Because the risk for D alloimmunization from whole blood derived platelet transfusion is less than 4%, and even less for apheresis platelets, the majority of D-incompatible platelets are given without RhIG administration. RhIG in significantly higher doses is used to treat immune thrombocytopenic purpura (ITP) in patients who are D-positive and have not been splenectomized.

For prevention of D-sensitization in the United States, 300 μg are routinely administered, but dosing is increased if there is evidence of large fetal-maternal hemorrhage (300 μg for every 15 mL of RBC exposure or 30 mL of whole blood exposure). The dose is calculated based on the estimated volume of D-positive fetal RBCs from Kleihauer-Betke or flow cytometry, which is more precise. RhIG dosing calculators are available. RhIG should be given within 72 hours, which was the time period for the original studies, but should not be withheld if not administered within this time period. Adverse events to low doses used to prevent D immunization include fever, chills, and pain at the injection site. Rarely, hypersensitivity reactions are noted. RhIG doses used to treat ITP are substantial: 50 μg/kg for hemoglobin values ≥10 g/dL and 25–40 μg/kg when hemoglobin is 8–10 g/dL. Adverse events include possible anemia, hemolysis, disseminated intravascular coagulopathy, and rarely, death (Chapter 131).

ABO

Commercially available mouse monoclonal anti-A and anti-B are used to determine ABO type, and these reagents directly agglutinate RBCs at room temperature. To confirm the RBC ABO reactivity, the plasma is tested for the presence of the corresponding agglutinins by testing with commercially available group A1 and group B RBCs. In both tests, agglutination is macroscopically visible.

Rhesus

Patient and donor RBCs are routinely tested for the presence of the D antigen in the Rh system. Reagents containing monoclonal anti-D that directly agglutinate D-positive (Rh-positive) RBCs suspended in saline at room temperature are commonly used for testing. Testing by a method that detects expression of a weak D antigen on RBCs is required for donors, but additional testing for weak D is optional when testing patient samples. Exceptions include typing the RBCs of a newborn when the mother is D-negative to determine whether she is a candidate for Rh immune globulin (RhIG) (see box on Rh Immune Globulin).

Antibody Screening

Patient plasma is incubated at 37°C with commercially available reagent RBCs of known antigen type. After incubation, unbound antibodies are removed by washing with saline, and an antiglobulin reagent containing either AHG, or a mixture of AHG and antihuman complement is added. Column agglutination technology is now widely used and eliminates the requirement to wash unbound IgG. If the antibody screen is positive, the specificity of the antibody is determined by testing the plasma against a panel of different group O reagent RBCs (usually 10) varying in antigen phenotype (termed antibody identification).

Compatibility Testing

Once a patient is actively immunized to an RBC antigen and produces a clinically significant alloantibody, the patient is considered immunized for life and should be transfused with antigen-negative RBCs, even if the antibody is no longer detectable. Patients with passively acquired antibody (e.g., neonates with maternal antibody; recipients of plasma and platelet products or RhIG) need to be transfused with antigen-negative RBCs only while the passive antibody is present. Selection of blood for transfusion to patients with alloantibodies requires typing of donor units for the corresponding antigen to identify antigen-negative units and crossmatch of the selected units with the patient's plasma. Antigen-negative units are provided by, or can be located by, most donor centers. Provision of antigen-negative blood will to some extent depend on the prevalence of the target antigen(s) in the donor population. Transfusion service staff are vital for communication between the patient's physician and/or consultant transfusion medicine specialist to determine the immediate and ongoing transfusion needs of the patient and to ensure that antigen-negative blood is available. Understanding the risks and benefits of transfusion are important, as well as understanding the potential clinical significance of the antibody and the urgency of transfusion. When a patient's antibody is directed at a high-prevalence antigen, it is important to test siblings in the quest for compatible blood and to urge the patient to donate blood for long-term storage when clinical status permits. In hemolytic anemia because of warm-reactive autoantibodies, compatibility may be difficult to demonstrate. In this scenario, the important issue is to be sure that there are no clinically significant alloantibodies underlying the warm reactive autoantibodies. Donor RBCs antigen-matched with the patient for clinically significant blood group antigens should be considered in lieu of transfusion with "least incompatible" blood to minimize alloimmunization.

Prevention of Alloimmunization

Transfusion management of patients who require chronic transfusion therapy, in particular patients with sickle cell disease can be challenging.[13–15] Many programs attempt to reduce or prevent alloimmunization by transfusion of RBCs that are prophylactically antigen-matched, typically for C, E, and K,[16] and some match for additional antigens. Some centers match for extended antigens once the patient makes an antibody (Table 111.4). The goal is to prevent

TABLE 111.4 Approaches to Supplying Red Blood Cell Products to Prevent Alloimmunization in Patients With Sickle Cell Disease or Other Transfusion-Dependent Anemia

Phenotype or genotype for clinically significant antigens before transfusion
Provide antigen-matched blood for C, E, and K prophylactically
Provide blood negative for the major antigens that the patient lacks after the patient makes an antibody

hemolytic or delayed transfusion reactions, which are known to be underreported because they can manifest as a sickle cell crisis and may result in decreasing the transfusion interval.

Blood Group Disease Association

The absence of some blood group antigens and their carrier molecules can result in disease. For example, an absence of the Rh and Rh-associated glycoprotein (RhAG) proteins causes stomatocytosis[17] and anemia, termed *Rh*$_{null}$ *syndrome*. The absence of Xk protein is associated with the *McLeod syndrome*, which is associated with myopathy and neurodegeneration. RBCs and white blood cells, such as T cells, from patients with leukocyte adhesion deficiency II (also known as *congenital disorder of glycosylation type II*) lack antigens that are dependent on fucose. The RBCs have the Le(a−b−) Bombay phenotype and the white blood cells lack sialyl-Le^x, which explains the high white blood cell count and infections in these patients. Hemagglutination is a simple test that can be used to diagnose these syndromes. In patients with paroxysmal nocturnal hemoglobinuria, a proportion of the RBCs will lack antigens carried on GPI-linked proteins. Other associations between blood group antigens and diseases are summarized in Table 111.1.

Diseases associated with antibodies to blood group antigens include hemolytic disease of the newborn, warm autoimmune hemolytic anemia, cold hemagglutinin disease, and paroxysmal cold hemoglobinuria. Hemagglutination is a valuable aid in diagnosis of these conditions.

Blood Group Systems

Presented here is a brief description of the most clinically relevant blood group systems in approximate order of clinical significance. For further information and prevalence of blood group antigens in different populations, refer to specialized texts such as *Human Blood Groups*, *The Blood Group Antigen Facts Book*, and the AABB *Technical Manual*.[2,3,18]

Carbohydrate Blood Groups

ABO and H

The ABO blood group system is by far the most clinically significant, because of the presence of naturally occurring IgM antibodies (and sometimes IgG). The original observation by Landsteiner that certain human erythrocyte suspensions were agglutinated by other human sera led to the recognition of ABO polymorphism.[19] This initial observation is still the cornerstone of modern transfusion practice more than a century later.

Antigens and Their Synthesis

H antigens (ABH) occur on glycoproteins and glycolipids and are synthesized in a stepwise fashion by glycosyltransferases that sequentially add specific monosaccharides in specific linkages to a growing oligosaccharide precursor chain (reviewed in Clausen and Hakomori[20]). The terminal sugar determines antigen specificity (Fig. 111.2). Group O individuals have H antigen only, the terminal sugar of which is fucose, and this is the precursor substrate for A and B antigens. Group O individuals have defective A or B transferases. The A and B transferase enzymes differ only by the nature of the monosaccharide added to the chain. *N*-acetyl-D-galactosamine is added by A-transferase, and D-galactose is added by B-transferase. In clinical practice, four ABO phenotypes (A, B, O, and AB) are discriminated. In addition, two common variations of group A (A$_1$ and A$_2$) can be distinguished. The differences between A$_1$ and A$_2$ phenotypes are quantitative and qualitative. Not only is the A$_1$ transferase more efficient in converting H to A antigen (approximately five times more A sites per RBC on A$_1$ RBCs than on A$_2$ RBCs), it also has the capacity to make A$_1$ antigen on the repetitive A epitope. Quantitatively normal ABH expression also requires the branching of carbohydrate chains, which is performed by the blood group I enzyme. Some H antigen precursor remains on A and B RBCs in this order: $A_2 > B > A_2B > A_1 > A_1B$.

Inherited and Acquired ABH Variation. In addition to the main ABO types, there are many other inherited phenotypes with a weaker expression of the specified antigen, for example, A$_3$, A$_x$, A$_{el}$, B$_3$, B(A), and cis-AB. This can cause problems in determining the ABO blood group, but for patients needing immediate transfusion, the selection of group O red cells and AB plasma products is an option. In blood donors, if a very weak expression of A or B antigens on the RBCs is not detected, the major risk is that they may be transfused to a patient whose antibodies may cause accelerated destruction of the transfused cells.

Rare Bombay (O$_h$) phenotype RBCs, first reported in Bombay (Mumbai), India, lack H antigen and, consequently, A and B antigens. Other variants with weak H expression on RBCs, with or without H in secretions, also occur (para-Bombay) and have been reviewed.[21] Of clinical relevance, potent anti-H with the same hemolytic potential as anti-A and anti-B can be produced by Bombay individuals. Anti-H is often found in para-Bombay individuals but is generally not a potent antibody. HDFN caused by anti-H has not been reported.

Acquired B antigen is a rare phenomenon that results from the action of bacterial deacetylase, an enzyme that can remove an acetyl group from the A-terminal sugar, *N*-acetylgalactosamine. Galactosamine is similar to galactose, the B-specific terminal residue, and anti-B reagents can cross-react with the deacetylated structure. Acquired B can occur in individuals suffering from gram-negative infections of gastrointestinal origin or carcinoma and can be clinically significant if a patient's blood group is misinterpreted and group AB blood is transfused. Other polyagglutinable states (e.g., T, Tn, Tk) are detected by naturally occurring antibodies found in the serum of most people; these can be identified by a panel of lectins.

A or B antigen expression can weaken in patients with acute leukemia or stress hematopoiesis or, occasionally, during pregnancy. Chromosomal deletions or lesions that involve the *ABO* locus can result in the loss of transferase expression in the leukemic cell population. A decrease in A or B antigen expression, when found without a hematologic disorder, can be prognostic of a preleukemic state.

Genes and Enzymes. The ABO gene was cloned in 1990 following purification of A transferase; since then, over 200 different alleles have been described.[22] There are only four amino acid differences between A and B transferases in the catalytic domain, two of which (Leu266Met and Gly268Ala) are primarily responsible for the substrate specificity. The group O phenotype results from mutations in ABO that cause a loss of glycosyltransferase activity. The most common group O allele (*ABO*O1*) results from a single nucleotide deletion near the 5' end of the gene that causes a frameshift and early termination with no active enzyme production. The rare B(A) and cis-AB phenotypes have both A and B enzyme activity from a single allele caused by variant glycosyltransferases that have a combination of A-specific and B-specific residues.

The fucosyltransferases required for H synthesis are encoded by two closely linked genes on chromosome 19, *FUT1* (or H) and *FUT2* (or *Se* for secretor), which have different substrate specificity and expression in tissues. Homozygosity for defective *FUT2* alleles is responsible for the common nonsecretor phenotype in which A, B, and/or H antigen are not present in secretions. Individuals homozygous for null alleles at both the *FUT1* and *FUT2* loci have the Bombay phenotype (see earlier section).

ABO and Transplantation. As tissue antigens, ABO antigens are important in solid organ transplantation. Recipient antibodies will

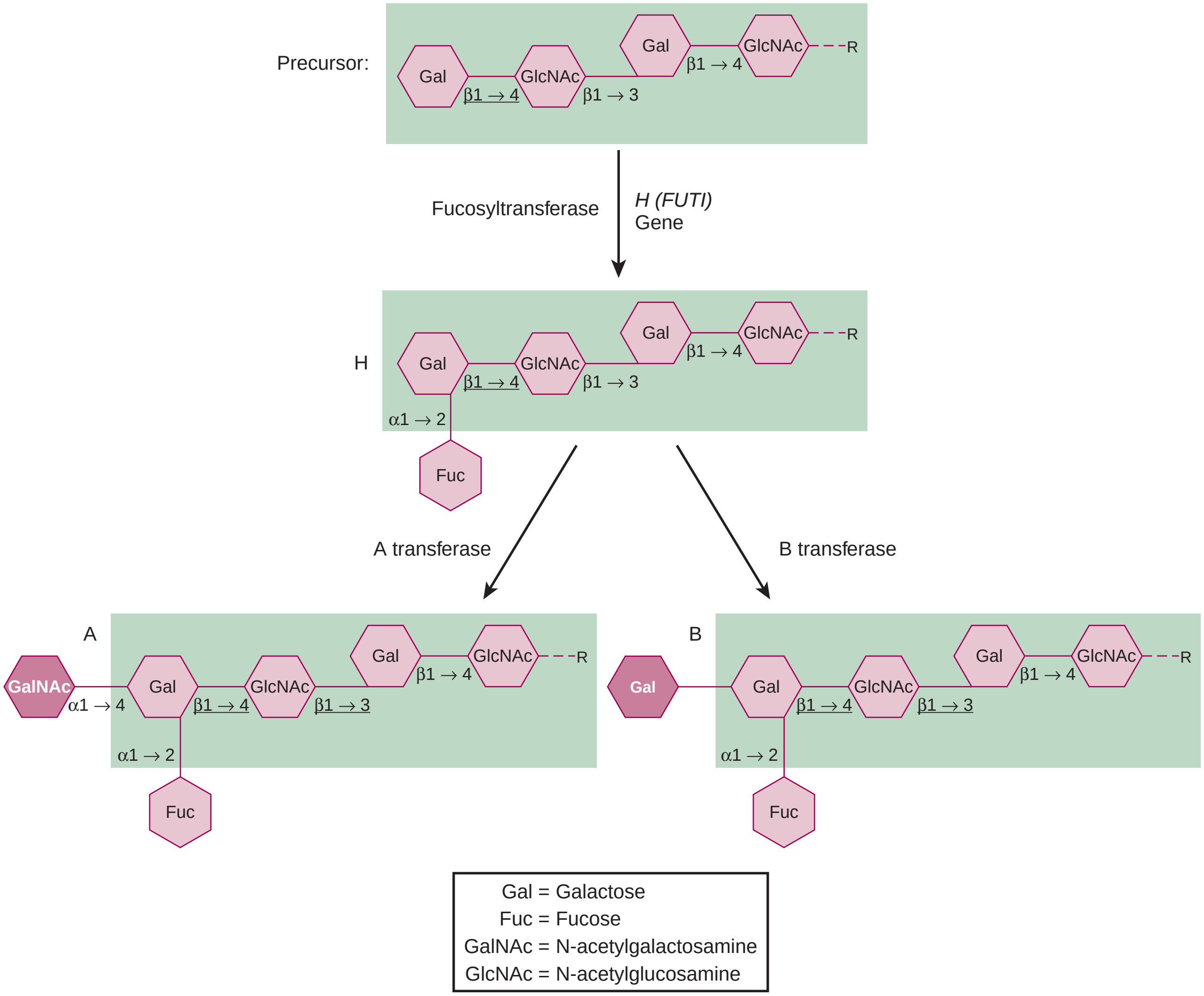


Figure 111.2 BIOCHEMICAL STRUCTURES OF ABH ANTIGENS. Schematic representation of the terminal portions of the carbohydrate structures carrying the H, A, and B antigens on red blood cells.

react with antigens on the transplanted organ and complement activation at the surface of endothelial cells can result in rapid destruction and hyperacute rejection. However, successful transplantation across ABO barriers is possible, particularly with blood group A_2 to O and with current immunosuppressive and pretreatment regiments including removal of ABO antibodies.[23] Allogeneic hematopoietic stem cell transplantations are routinely performed regardless of ABO compatibility, but occasionally initial hemolysis or pure red cell anemia because of persisting anti-A or anti-B titers in the recipient can result.

Antibodies. Anti-A and anti-B are found in the sera of individuals who lack the corresponding antigens. They are produced in response to environmental stimulants, such as bacteria. These antibodies are produced after birth, reaching a peak at 5 to 10 years of age, and declining with increasing age. The antibodies are mostly IgM and can activate complement, which in conjunction with the high density of ABO antigen sites on RBCs, are responsible for the severe, life-threatening transfusion reactions that may result following ABO-incompatible transfusions. In contrast, HDFN caused by ABO antibodies is usually mild because (1) placental transfer is limited to the fraction of IgG anti-A and anti-B found in maternal serum, (2) ABH antigens are not fully developed on fetal RBCs because of a lack of fully branched carbohydrate chains, and (3) tissue ABH antigens provide additional targets for the antibodies.

Platelets have intrinsic A, B, and H antigens; thus, ABO incompatibility can decrease the posttransfusion platelet increment, but this is often not of clinical significance.[24] However, platelets from donors with an A_2 phenotype lack both A and H antigens. Approximately 20% of group A platelets would be from A_2 donors and would be appropriate for "universal" use. Platelets from A_2 donors may also be a superior product for patients undergoing A/O major mismatch allogeneic progenitor cell transplantation.[25]

Potent anti-H (along with anti-A and anti-B) found in O_h (Bombay) individuals will destroy transfused RBCs of any ABO group, so these individuals must be transfused only with H−RBCs. In contrast, anti-H identified in individuals with low expression of H antigen, notably A_1B and A_1, is usually IgM, reacts only at lower temperatures, and is thus clinically insignificant.

Other Carbohydrate Blood Group Systems

As for all glycoconjugate structures, sequential enzymatic action is required to build other carbohydrate antigenic epitopes, and the genetic background of all these involves different glycosyltransferase loci.

The null p phenotype, P_2^k and P_1^k, are of clinical interest because of potent naturally occurring antibodies that are present in plasma of individuals whose RBCs lack the glycolipid-based antigens P1/P/P^k, P1/P/PX2, or P/PX2, respectively. In analogy with the ABO blood group system, antibodies of IgM and IgG class (anti-PP1P^k, anti-P1PPX2, or anti-PPX2) are made against the missing antigens. Although the incidence of the null phenotypes is only 5 to 10 per million, they have attracted considerable interest because of their relationship to disease and as receptors for pathogens. Women with p and P^k phenotypes suffer a high incidence of spontaneous abortion, a phenomenon most likely caused by destruction of the placenta by anti-P. In addition, anti-P and anti-P^k cause hemolytic transfusion reactions if antigen-positive RBCs are transfused. Transient autoanti-P, produced following a viral infection, causes paroxysmal cold hemoglobinuria and lysis of autologous P-positive RBC. P antigen (also known as *globoside*) is the cellular receptor for the parvo-B19 virus that causes erythema infectiosum (fifth disease) in children, sometimes complicated by severe aplastic anemia because of lysis of early erythroid precursors. P-fimbriated *Escherichia coli* expresses both P-binding and P^k-binding molecules at the tips of their pili, a finding with implications for uropathogenicity. Individuals lacking P, or P^k and P, appear to be naturally resistant to these bacterial and viral infections. In contrast to anti-P and anti-P^k, it should be noted that anti-P1 is a cold-reactive agglutinin that seldom has clinical importance. The clinical importance of anti-PX2 made by P^k individuals is unclear.

Lewis antigens are fucosylated glycolipids that are synthesized by nonerythroid cells, circulate in plasma, and are passively adsorbed onto RBC. Antibodies to Lewis can be made by individuals with the Le(a−b−) phenotype. These antibodies are of IgM class and seldom cause any clinical problems. Lewis antibodies are commonly found in pregnant women.

The i and I antigens are nonterminal epitopes on linear and branched carbohydrate structures, respectively, carrying ABH antigens at their terminal ends. During the first years of life, linear chains are modified into branched chains, resulting in the appearance of I antigens. The i phenotype is very rare among adults, but it is the normal state on RBCs from fetuses and infants. The gene encoding the I-branching β-1,6-N-acetylglucosaminyltransferase (GCNT2) has three alternative forms of exon 1 with common exons 2 and 3. Mutations in exon 2 or exon 3 silence GCNT2 and give rise to the form of the i phenotype that is associated with cataracts in Asians. Mutations in exon 1C silence the gene in erythrocytes (but not in other tissues) and lead to the i phenotype without cataracts.

Alloanti-I made by a person with the rare i adult phenotype can be clinically significant and cause destruction of transfused I-positive RBCs. However, the sera of all I-positive individuals contain autoanti-I that is clinically benign and reactive only at or below room temperature. In contrast, cold hemagglutinin disease is characterized by a high titer of complement-fixing monoclonal anti-I, which causes in vivo hemolysis and hemolytic anemia. The titer and thermal range of autoanti-I is often increased following infection with *Mycoplasma pneumoniae*. If transfusion cannot be avoided, donor RBCs should be transfused through a blood warmer.

The FORS1 blood group antigen is a rare low prevalence antigen that is A-like in that it is defined by a terminal *N*-acetylgalactosamine and was first recognized as a weak A subgroup. The antigen has been defined as Forssman antigen, commonly found on the RBCs of nonprimate mammals, and arises from a gain-of-function mutation in the *GBGT1* pseudogene. The clinical relevance of anti-FORS1 found naturally occurring in the plasma of most individuals is not known.

The blood group system Sid (symbol SID; number 038) was recently assigned to the β-1,4-N-acetylgalactosaminyltransferase (β4GalNAc-T2) encoded by the *B4GALNT2* gene. Through a combination of experimental and bioinformatics approaches, it was shown that that Sd(a−) was due to a c.1396T>C variation in the *B4GALNT2* gene leading to a likely deleterious amino acid polymorphism (p.Cys466Arg).[26]

Protein Blood Groups

Rhe, Rhe-associated Glycoprotein, and LW Blood Group Systems

The Rh system is second only to the ABO system in importance in transfusion medicine. Rh antigens, especially D, are highly immunogenic; thus in most countries, blood for transfusion is tested and labeled with the D antigen type (Rh-positive or Rh-negative) and D− recipients are transfused with D−RBC products.

TABLE 111.5 Prevalence of the Principal Rhesus Haplotypes

		Incidence (%)		
Fisher-Race Haplotype	Modified Weiner Haplotype	White	African Black	Asian
Rh-Positive				
DCe	R_1	42	17	70
DcE	R_2	14	11	21
Dce	R_0	4	44	3
DCE	R_Z	<0.01	<0.01	1
Rh-Negative				
ce	r	37	26	3
Ce	r′	2	2	2
cE	r″	1	<0.01	<0.01
CE	r^y	<0.01	<0.01	<0.01

Three systems for naming Rh antigens have been used. Two are shown in Table 111.5, which indicates the incidence of the common Rh haplotypes present in different ethnic groups. The Fisher-Race nomenclature was based on the premise that there were three closely linked genes (D, C/c, and E/e), whereas the Wiener nomenclature (Rh-Hr) was based on the belief that a single gene encoded multiple factors (antigens). Although it is now well established that two genes, the *RHD* and *RHCE*, encode the Rh proteins, the Fisher-Race (D, C/c, and E/e) terminology is often preferred for written communication; for spoken communication, a modified version of the Wiener nomenclature is preferred. Uppercase *R* indicates that D antigen is present and use of a lowercase *r* (or "little r") indicates that it is absent. The C or c and E or e antigens carried with D are represented by subscripts: 1 for Ce (R_1), 2 for cE (R_2), 0 for ce (R_0), and Z for CE (R_z). The presence of these antigens without D is represented by a superscript: prime for Ce (r′), double-prime for cE (r″), and y for CE (r^y). This terminology allows one to convey the common Rh antigens (the phenotype) with a single term. The third system of numeric designations is not widely used in the laboratory, with a few exceptions (Rh17, Rh32, Rh33).

Genes, Proteins, Antigens, and Phenotypes. The Rh proteins are designated RhD (encoded by RHD), which carries the D antigen, and RhCE (encoded by RHCE), which carries the CE antigens (either ce, cE, Ce, or CE). RhD differs from the various forms of RhCE by 32 to 35 amino acids. RhD and RhCE are not glycosylated but form a complex in the RBC membrane with RhAG (Rh-associated glycoprotein). Other proteins present in the Rh-complex are CD47 (an integrin-associated protein), Lansteiner-Wiener (LW) proteins, and glycophorin B. The Rh-complex also associates with band 3 (the anion exchanger) as a macrocomplex in the membrane.

The D-negative (Rh-negative) phenotype is prevalent in Whites (15% to 17%), less common in African Blacks (3% to 5%), and rare in Asians (<0.1%). The absence of D in Europeans is primarily caused by a deletion of the *RHD* gene. African Blacks, and rare D-negative Whites and Asians carry a *RHD* gene that is silenced by a variety of molecular events.[7]

RBCs with weak D have D antigen but at lower levels than normal because of one or more amino acid changes that are often predicted to be in the intracellular or transmembrane regions of RhD. The RBCs do not lack, or have altered, epitopes of D. Many individuals with a serologic weak D phenotype have weak D types 1, 2, and 3 by *RHD* genotyping, and individuals with these genotypes can safely receive D-positive blood and do not make clinically significant anti-D.[27,28]

Partial D antigens (previously called *D categories* or *D mosaics*) are caused either by point mutations in *RHD* that encode amino acid changes that alter D epitopes, or by replacement of *RHD* nucleotides or exons by the equivalent part of *RHCE* that result in loss of D

WEAK OR VARIABLE D-TYPING

Clinically significant D-sensitization potentially results in a pregnancy with a fetus at risk for hemolytic disease of the fetus and newborn and hemolytic transfusion reactions if transfused with D-positive red blood cells (RBCs). Individuals with RBCs that express a partial D antigen are at risk for D-sensitization, whereas those with weak D antigen are usually not at risk. These cannot be distinguished by serologic reactivity, because either may present as weak or moderately positive or give variable results with anti-D reagents. Particularly in the prenatal setting, *RHD* genotyping should be done to distinguish weak D from partial D. Women with weak D expression, particularly weak D types 1, 2, and 3, are not at risk for clinically significant D-sensitization and therefore are not candidates for RhIG prophylaxis. In contrast, individuals with partial D, lack D epitopes and have produced clinically significant anti-D and should receive RhIG prophylaxis. *RHD* genotyping avoids unnecessary treatment with RhIG and excess use of Rh-negative blood in patients with weak D antigen.

TRANSFUSION MANAGEMENT OF PATIENTS WITH WARM AUTOIMMUNE HEMOLYTIC ANEMIA

Patients with warm autoimmune hemolytic anemia may present with jaundice, fatigue, and anemia, or they may show no overt clinical manifestations. The antibody screen and antibody identification panel will show all red blood cells (RBCs) positive (panagglutinin) with anti-IgG in the indirect antiglobulin test. The autocontrol (patient's own plasma and RBCs) will also be positive.

History: A transfusion history should be obtained to differentiate these results from a hemolytic transfusion reaction or hemolysis because of an alloantibody.

DAT: A direct antiglobulin test should be performed with anti-IgG and anti-C3. In clinically significant hemolysis, the DAT is usually strongly positive.

Eluate: If patient has been recently transfused (3–4 months), an eluate should be prepared from the patient cells to remove the antibody(ies); the eluate should be tested to determine specificity. The eluate is usually reactive with all cells when tested by the IAT with anti-IgG.

Phenotype or genotype: Type the patient's RBCs for minor blood group antigens (Cc, Ee, K, Jka/b, Fya/b, Ss) if the patient has not been recently transfused. When possible, IgM typing reagents are used because the patient's own antibody-coated RBCs may result in false-positive typing. Some laboratories are able to remove the IgG from the RBCs and perform a phenotype, but alternatively, genotyping for minor blood group antigens including $\text{Do}^{a/b}$ antigens (there is no serologic reagent) can be performed.

Adsorption: Adsorb the serum autoantibody onto the patient's own RBCs to test for underlying alloantibody if the patient has not been recently transfused (3–4 months). If the patient has been recently transfused or if the low hematocrit results in insufficient autologous RBCs, perform alloadsorption with well-characterized RBCs (usually three with known antigen profiles). Test the adsorbed serum for underlying alloantibodies.

Crossmatch: Perform with neat and with adsorbed plasma. Crossmatch performed with neat plasma will usually be incompatible.

Communication: Inform ordering physician of reactivity and of delay in receiving crossmatched RBCs. Provide emergency-release RBCs if patient's clinical situation warrants. Inform the physician that the patient may hemolyze transfused RBCs similar to hemolysis of his or her own RBCs.

Transfusion: Consider providing RBC units negative for minor antigens that the patient also lacks. Consider matching for Cc, Ee, K, Jka/b, Fya/b, Ss (and Doa/b if possible). This potentially enables RBC units to be available before completion of the antibody identification testing. Transfusion with antigen-matched units potentially allows continued transfusion without need for autoadsorption or alloadsorption unless signs and symptoms of RBC destruction occur or there is a change in reactivity in antibody screening or the DAT.

epitopes. RBCs with a partial D antigen may have strong or weak reactivity with anti-D. Because patients with partial D antigens can make anti-D directed to the D epitopes that are altered or absent, they ideally should receive D-negative blood and women of childbearing potential are candidates for Rh immune globulin. In practice, many type as D-positive and are recognized only after they make anti-D. However, in the United States monoclonal D typing reagents licensed by the Food and Drugs Administration for patient testing classify partial Rhesus antigen D category VI (DVI) phenotypes as D-negative in direct testing, and as D-positive by IAT. *RHD* genotyping is very useful to distinguish weak D phenotypes from partial D to guide selection of blood for transfusion and prevent D alloimmunization and to avoid unnecessary Rh immune globulin injection (see box on Weak or Variable D Typing). Several phenotypes, including D− −, Dc−, and $DC^{w}-$, have an enhanced expression of D antigen and no, or variant, CE antigens. They are caused by replacement of portions of *RHCE* by *RHD*. The RhD sequences in RhCE, along with a normal RhD, explain the enhanced D and account for the lack, or reduced expression, of CE antigens. Immunized individuals with these CE-depleted phenotypes can make antibodies to high-prevalence Rh antigens.

C and c antigens differ by four amino acids, but only residue Ser103Pro is predicted to be extracellular. E and e differ by one amino acid, Pro226Ala. The RhD and various combinations of RhCE proteins (ce, Ce, cE, and CE) are typical for the majority of White transfusion recipients. However, Rh proteins in other ethnic groups often carry additional polymorphisms, particularly in individuals of African descent, and this fact often complicates transfusion in patients with sickle cell disease. For example, the RBCs of more than 30% of Blacks are VS+ because of a Leu245Val substitution in Rhce, and expression of this antigen is associated with variant expression of e antigen. Many other amino acid changes in Rhce, as well as in RhD, are associated with production of Rh antibodies in patients with sickle cell disease. RH genotyping by DNA methods allows enhanced Rh antigen matching of patients and donors and is particularly important in patients who present with Rh antibodies reacting with all, or the majority, of cells tested.

The Rh_{null} phenotype is very rare and occurs on two genetic backgrounds: the "regulator" type, caused by mutations in *RHAG*, which encodes an Rh-associated glycoprotein, and the "amorph" type, caused by mutations in *RHCE* on a D− (deleted *RHD*) background. Rh_{null} RBCs are stomatocytic, fragile, and associated with anemia. RhAG is involved in maintenance of cation balance in RBCs.[17]

Antibodies. Most Rh antibodies are IgG and do not activate complement. As a result, primarily extravascular hemolysis, rather than intravascular hemolysis, occurs in transfusion reactions involving Rh antibodies. The antibodies are almost always caused by RBC immunization from pregnancy or transfusion and usually persist for years. Anti-D can cause severe hemolytic transfusion reactions and HDFN, but the incidence of anti-D has decreased with the prophylactic use of Rh immune globulin. Most Rh antibodies should be considered as having the potential to be clinically significant for HDFN and hemolytic transfusion reactions. If serum antibody levels fall below detectable levels, subsequent exposure to the antigen characteristically produces a rapid secondary immune response. Autoantibodies in the sera of patients with warm autoimmune hemolytic anemia, as well as in some cases of drug-induced autoimmune hemolytic anemia, appear to demonstrate relative specificity to high-prevalence Rh antigens, although specificity for other members of the Rh complex have not been ruled out (see box on Transfusion Management of Patients With Warm Autoimmune Hemolytic Anemia).

RHAG Blood Group System

RhAG glycoprotein, encoded by *RHAG*, is highly similar to the RhD and RhCE proteins. It carries four blood group antigens: two of high prevalence (RHAG1 and 3) and two of low prevalence (RHAG 2 and 4). Antibodies to RHAG4 cause HDFN. RhAG is important for erythrocyte ion balance in RBCs and is required for the expression of RhD and RhCE proteins forming the core of the Rh-complex.

Lansteiner-Wiener Blood Group System

Rh and LW are independent blood group systems but have a phenotypic relationship. In adults, D-positive RBCs have a stronger expression of LW antigen than D-negative RBCs, and anti-LW can be confused with anti-D. Transient loss of LW antigens from RBCs has been described in pregnancy and in patients with diseases, particularly Hodgkin disease, lymphoma, leukemia, sarcoma, and other forms of malignancy. Loss of LW antigens is usually associated with the production of antibodies that appear to be alloanti-LW.

Kell and Kx Systems

The Kell glycoprotein is highly folded through multiple intrachain disulfide bonds and is covalently linked to the XK protein in the RBC membrane. Kell is highly polymorphic because of single amino acid substitutions in the glycoprotein that account for 35 of 36 antigens described to date. The K antigen is remarkably immunogenic even though it differs from wild-type (k, small k) by only one amino acid, and it appears that loss of an *N*-glycan exposes the peptide, thereby rendering it immunogenic.

Inherited weak expression of Kell antigens, termed K_{mod} phenotype, occurs with amino acid changes in the protein, with Kp^a in cis, and in the McLeod phenotype. Transient depression of Kell system antigens may also occur in autoimmune hemolytic anemia, in microbial infections, and was reported in two cases of idiopathic thrombocytopenia purpura. The lack of Kell antigens (the K_0 or K_{null} phenotype) is caused by multiple different gene defects.

HDFN caused by anti-K can result in severe neonatal anemia, and unlike anti-D, maternal antibody titers and amniotic bilirubin levels are not good predictors of the severity of the disease. Kell antigens are expressed very early during erythropoiesis, and anti-K has been shown to suppress erythropoiesis in vitro. This may explain the low level of bilirubin observed in cases of neonatal anemia; thus Doppler screening of the fetal middle cerebral artery peak systolic velocity is used to monitor anemia. Other unusual consequences of Kell antibodies include risk for fetal thrombocytopenia and neutropenia.

McLeod Syndrome. This uncommon syndrome is associated with the loss of expression of Kx protein caused by mutations and deletions in the *XK* gene. The syndrome, which is X-linked and manifests only in males, may be underdiagnosed. The physical characteristics, which often develop only after the fourth decade of life, include muscular and neurologic problems. A minority of patients with chronic granulomatous disease also have the McLeod phenotype as a result of X-chromosome deletions encompassing both genes. Carrier females have two populations of RBCs (one of the McLeod phenotype and one of normal phenotype).

Duffy Blood Group System

The Duffy blood group protein, Atypical chemokine receptor 1, is a promiscuous chemokine receptor found on RBCs and on endothelial cells in the kidney and brain that binds a family of chemotactic and proinflammatory peptides from the CXC (IL-8, MGSA) and the CC (RANTES, MCP-1, MIP-1) classes. The physiologic role of FY is clear, but on RBCs the receptor may allow RBCs to act as scavengers for excess chemokines. FY is also a receptor to which *Plasmodium vivax* merozoites can bind to invade RBC and cause malaria.

Antigens. The Fy^a and Fy^b antigens differ by a single amino acid (Gly42Asp) located on the N-terminal extracellular domain of the FY glycoprotein and is responsible for the common Fy(a+b−), Fy(a−b+), and Fy(a+b+) phenotypes. The null Fy(a−b−) phenotype is rare in most ethnic groups, but it is common in people of African and Arabian origins. The null phenotype most often results from a mutation in the promoter region of FY that disrupts a binding site for the erythroid transcription factor GATA-1 and results in loss of FY on RBCs. Because the erythroid promoter controls expression only in erythroid cells, FY expression in other tissues is unaffected. All individuals of African origin with a mutated GATA box to date have been shown to carry FYB and therefore Fy^b is expressed on nonerythroid tissues. This explains why Fy(a−b−) individuals make anti-Fy^a but not anti-Fy^b. Fy(a−b−) caused by a mutated GATA box on an FYA allele has been found in Papua New Guinea, another malaria-endemic region.

Antibodies. FY antigens are much less immunogenic than Rh and K. Anti-Fy^b is less common than anti-Fy^a, and both antibodies can cause delayed hemolytic transfusion reaction (DHTR) and rarely HDFN. Anti-Fy3 is made by Fy(a−b−) individuals who are exceptions to above, and it is speculated they may lack FY protein on all cells.

Kidd Blood Group System

The Kidd (JK) blood group protein was implicated in urea transport when RBCs lacking the antigens were shown to resist lysis in 2 M urea. The protein is present in RBCs and kidney medulla and is a constitutive urea transporter, but failure to express Kidd does not result in an overt clinical syndrome; the only observed manifestation is a reduced capacity to concentrate urine.

Antigens. The Jk^a and Jk^b antigens differ by a single amino acid (Asp280Asn) and are responsible for the common Jk(a+b−), Jk(a−b+), and Jk(a+b+) phenotypes. The Jk(a−b−) or Jk_{null} phenotype is uncommon and occurs with greater incidence in Polynesians, Asians, and Finns. Many different molecular changes in both JK antigen A (JKA) and JK antigen B (JKB) alleles have been shown to abolish expression of the Kidd blood group protein. Weakly expressed variants of Jk^a and Jk^b antigens are also not uncommon.

Antibodies. JK antibodies are responsible for at least one-third of cases of DHTR. The antibodies often drop to undetectable levels or react only with cells that are homozygous for the antigen and escape detection in the sensitized patient's serum before transfusion. JK antibodies only rarely cause HDFN, and if they do, it is typically not severe. Anti-Jk3, sometimes referred to as anti-Jk^{ab}, is produced by Jk(a−b−) individuals, and rare donors must be located for transfusion.

MNS System

M and N antigens from the MNS antigen system (MNS) are carried on alternative forms of glycophorin A (GPA/GYPA), whereas S and s antigens are carried on alternative forms of glycophorin B (GPB/GYPB). M/N and S/s are homologous proteins encoded by adjacent genes and consequently show linkage disequilibrium in inheritance of the antigens. The MNS system is highly polymorphic and most of the antigens are the result of amino acid substitutions or rearrangements between *GYPA* and *GYPB*. Persons who are S−s− are usually of African origin. They either also lack the high-prevalence U antigen arising from a deletion of *GYPB* or express variant weak U antigen as a result of an altered form of *GYPB*.

Antibodies. Anti-S, anti-s, and anti-U are usually IgG and can be clinically significant antibodies. Anti-M and anti-N can be naturally occurring, may be reactive at room temperature or below, and are often clinically insignificant.

Other Protein Antigens

Antibodies to antigens in the following systems are less common than those described earlier in this chapter, and information regarding their general clinical significance is summarized in Table 111.3.

Lutheran System

Lutheran (Lu), along with Secretor, provided the first example of autosomal linkage in humans, the first example of autosomal crossing over, and the first indication that crossing over in humans is more common in females than in males. The Lu system consists of four antithetical pairs of antigens and 16 independent high-prevalence antigens. The Lu(a−b−) phenotype is rare, but in the majority of individuals, it is caused by heterozygosity for silencing mutations in the *EKLF/KLF1* gene. KLF1 is a transcription factor that regulates many erythroid-specific genes, and the expression of antigens in other blood group systems (e.g., Kn, In) is also affected. In one family, heterozygosity for a mutation in the *GATA1* gene was shown to be responsible for the Lu(a−b−) phenotype.

Antibodies. Antibodies in this system are rarely encountered because the antigens are not highly immunogenic. They are usually IgG and give characteristic agglutinates surrounded by unagglutinated RBCs. They can cause mild transfusion reactions, but do not typically cause HDFN. Anti-Lu3 is found in the serum of immunized people of the rare recessive Lu(a−b−) phenotype, and the antibody is usually IgG and may cause DHTR or HDFN. Blood with the Lu(a−b−) phenotype should be used for transfusion of patients with these antibodies.

Diego System

The Diego (Di) blood group antigens are on Band 3 anion transport protein (AE1), one of the most abundant erythrocyte glycoproteins. AE1 forms complexes with many other proteins in the cell membrane and is important for RBC stability. The Diego blood group system contains three antithetical pairs of antigens and 16 low-prevalence antigens. Di^b antigen has a prevalence of greater than 99.9%, but Di^a is rare in most populations. Exceptions include Indigenous South Americans (Di^a occurs in 54% of this population) and Native Americans, approximately 12% of whom are Di(a+).

Antibodies. Di antibodies are usually IgG and do not bind complement. These antibodies have caused DHTR (usually delayed) and HDFN. Autoantibodies to band 3 are common in patients with warm autoimmune hemolytic anemia.

Yt Blood Group System

The Yt system was named in 1956 when an antibody was found in the serum of patient whose last name was Cartwright. Yt^a occurs with a prevalence of more than 99% in random blood samples, and Yt^b is found with a prevalence of approximately 8%, except in Israelis, in whom it has a prevalence of 20% or higher.

Antibodies. Yt antibodies usually are IgG and do not bind complement. These antibodies have caused DHTR but not HDFN.

Scianna Blood Group System

The Scianna (Sc) antigens are expressed by the RBC adhesion protein, erythrocyte membrane-associated protein. Sc1 is a high-prevalence antigen (prevalence ≈99.9%), and Sc2 is a low-prevalence antigen (1%); there are five other Scianna antigens.

Antibodies. Sc antibodies are usually IgG, and some bind complement. These antibodies have not caused DHTR, and although they have caused a positive DAT in cord RBCs, they have not caused HDFN. Several examples of autoanti-Sc1 have been reported, some reactive in tests using patient serum but not plasma. Autoanti-Sc3–like antibodies have been described in one patient with lymphoma and in one patient with Hodgkin disease whose RBCs had suppressed Sc antigens.[2]

Dombrock Blood Group System

Dombrock (Do) antigens are carried on a GPI-linked glycoprotein that is a member of the mono-ADP-ribosyltransferase family, although Do has no demonstrable enzyme activity on the RBC. The Do blood group system consists of two antithetical antigens, Do^a and Do^b, and eight other antigens of high prevalence. The null phenotype is Gy(a−).

Antibodies. Do^a and Do^b antigens are poor immunogens, and anti-Do^a and anti-Do^b are rarely found as single specificities. Antibodies in the Do system are usually IgG and do not bind complement. These antibodies have caused DHTR and a positive DAT but no clinical HDFN.

Colton Blood Group System

The Colton (Co) antigens are carried on aquaporin-1 (AQP-1), the first water channel protein characterized in mammals, and are also found in the kidney. The function of AQP-1 in RBCs may be to rehydrate rapidly after shrinking in the hypertonic environment of the renal medulla. Co^a has a prevalence of 99.9%, its antithetical antigen Co^b has a prevalence of 10%, and Co3 and Co4 are present on all RBCs except those of the very rare Co(a−b−) null phenotype. Apparently healthy propositi with the Co(a−b−) phenotype and AQP-1 deficiency have RBCs with an 80% reduction in the ability to transport water. The residual water transport in these RBCs may be through another member of the water channel protein family, AQP-3, which transports water, glycerol, and urea, and carries the blood group antigen aquaporin 3 (AQP3/GIL).

Antibodies. Antibodies in the Co system are usually IgG and some bind complement. The antibodies have caused DHTR and HDFN.

Gerbich Blood Group System

The Gerbich system antigens are carried on glycophorin C (GPC) and glycophorin D (GPD). There are six high-prevalence antigens and five low-prevalence antigens. The two glycoproteins are products of the *GYPC* gene. The gene consists of four exons, and the smaller GPD is generated by the use of an alternative translation initiation site.

Antibodies. The antibodies may be immune or naturally occurring. Most are IgG and some of these bind complement. Some antibodies may be IgM. Although some antibodies have caused DHTR, others have been benign. Anti-Ge3 has caused HDFN, and similar to Kell antibodies, the disease is associated with severe anemia. Clinical HDFN associated with anti-Ge2 has not been reported, but the antibodies have been eluted from DAT-positive cord RBCs.

Cromer Blood Group System

The Cromer (Cr) antigens are carried on decay-accelerating factor (DAF, CD55), a complement control protein attached to the RBC membrane through GPI-linkage. Cr is a system of two sets of antithetical antigens (Tc^a/Tc^b/Tc^c and WES^a/WES^b), 16 high-prevalence antigens, and three low-prevalence antigens. The Cr(a−) phenotype is the least rare of the negative phenotypes, and with the exception of one Spanish-American woman, all people with Cr(a−) RBCs are Black. Most of the other phenotypes are exceedingly rare.

Antibodies. Antibodies in the Cr system are usually IgG and do not bind complement. The antibodies have caused mild DHTR but not HDFN.

Knops Blood Group System

The Kn blood group antigens are carried on complement receptor 1 (CR1). Kn^a, Sl^a, and McC^a antigens are fairly common and have a similar prevalence (>90%) in different populations; however, Sl^a is present on RBCs of 98% of Whites, but on only 60% of African Americans. Typing for Kn system antigens can be challenging because the low level of expression on the RBCs in some disease processes gives false-negative results. RBC CR1 is important in the processing of immune complexes, binding them for transport to the liver and spleen for removal from the circulation. The CR1 copy number per RBC (and thus antigen strength) is reduced in SLE, cold agglutinin disease, paroxysmal nocturnal hemoglobinuria (PNH), hemolytic anemia, insulin-dependent diabetes mellitus, acquired immunodeficiency syndrome, some malignant tumors, and any condition associated with increased clearance of immune complexes. CR1 (the Sl^a antigen in particular) may act as a receptor for the malarial parasite *Plasmodium falciparum*; thus the Sl(a−) phenotype may provide selective advantage.

Antibodies. Antibodies in the Kn system are usually IgG, and they do not bind complement. The antibodies do not cause DHTR or HDFN, and once identified, they can usually be ignored for clinical purposes. Identification may be complicated by the fluctuation of antigen expression on RBCs. In the Kn system, anti-Kn^a is the most common antibody in Whites, and anti-Sl^a is the most common in African Americans.

Indian Blood Group System

The antigens of the In system are carried on CD44. CD44 has a diverse range of biologic functions involving cell-cell and cell-matrix interactions in cells other than RBCs. It is an adhesion molecule in lymphocytes, monocytes, and some tumor cells. CD44 binds to hyaluronate and other components of the extracellular matrix and is also involved in immune stimulation, as well as signaling between cells. In^b is a common antigen, and In^a is rare in White persons but has a prevalence of 4% in the population of India, 10% in Iranians, and nearly 12% in Arabs.

Antibodies. Antibodies in the Indian system are usually IgG and do not bind complement. Some antibodies may directly agglutinate RBCs, but the reactivity is greatly enhanced by the IAT. These antibodies have caused decreased RBC survival and a positive DAT in the neonate, but not HDFN. A severe DHTR caused by anti-In^b has been reported.

Chido/Rodgers Blood Group System

Although the Ch and Rg antigens are readily detected on RBCs, they are located on the fourth component of complement (C4), which becomes bound to RBCs from the plasma. In complement activation through the classic pathway, C4 becomes bound to the RBC membrane and undergoes further cleavage; ultimately, a tryptic fragment, C4d, remains on the RBC. This C4d glycoprotein carries the Ch/Rg blood group antigens. The antigens are stable in stored serum or plasma, and the phenotypes of this system are most accurately defined in plasma by agglutination inhibition tests.

Antibodies. Antibodies in the Ch/Rg system are usually IgG, do not activate complement, and are considered benign. Considerable variation may be common in the reaction strength obtained with different RBC samples. Although these antibodies do not generally cause DHTR, they have caused anaphylactic reactions. The antibodies have not caused HDFN.

Jr Blood Group System

The Jr^a antigen is carried on ABCG2, a member of the ATP-binding cassette family of multipass membrane proteins. The protein is broadly distributed and is a high-affinity urate transporter. It is also involved in porphyrin transport and in multidrug resistance in some cancers. The Jr(a−) phenotype is the null phenotype and Jr(a−) individuals lack the protein. Many different mutations have been identified for this phenotype although it is more common among Japanese people (where it is associated with gout) and the Roma population in Europe.

Antibodies. Anti-Jr^a are usually IgG, do not activate complement and do not generally cause DHTR or HDFN, however two cases of severe HDFN have been reported.

Lan Blood Group System

Lan is carried by an ATP-binding cassette protein, ABCB6, a protein that is widely expressed. It is highly expressed in fetal liver and upregulated during erythropoiesis. ABCB6 is important in heme synthesis and transports heme, and porphyrins into the mitochondria. The Lan− phenotype is the null phenotype and it arises from many different molecular backgrounds, but is not associated with a disease phenotype.

Antibodies. Anti-Lan are generally IgG and do not bind complement but have caused mild to severe DHTR and mild HDFN.

Vel Blood Group System

The Vel blood group antigen is dependent on the SMIM1 protein for expression and a 17-bp mutation in the *SMIM1* gene accounts for the vast majority of Vel− individuals and absence of SMIM1. The protein is widely expressed but more highly expressed on erythrocytes, salivary glands, and testes, although its function is unknown. Vel antigen expression is variable on RBCs.

Antibodies. Anti-Vel are often a mixture of IgM and IgG and readily bind complement. While HDFN caused by anti-Vel is rare, the antibodies can cause severe hemolytic transfusion reactions.

CD59 Blood Group System

An antibody in the plasma of a young patient with CD59 deficiency qualified CD59 as a blood group antigen. CD59 is a GPI-linked protein on the erythrocyte and is important in complement regulation. It inhibits the formation of the membrane attack complex (MAC) by specifically binding to C8 and C9. Like other GPI-linked proteins, it is absent from the erythrocytes of PNH patients and its absence is the underlying cause of hemolysis.

Antibodies. Only one example of the antibody has been reported. It was clinically benign although a positive DAT was observed following one of the transfusions.

Augustine Blood Group System

The At(a−) phenotype in individuals of African origin is defined by an amino acid polymorphism on SLC29A1 (p.Glu391Lys), while the At(a−) members of a rare family affected by bone malformation lacked the protein due to an inactivating mutation in the *SLC29A1* gene: c.589+1 G>C. The antigen defined by the antibody produced by the null phenotype was named AUG1, and the antigen defined by the amino acid Glu391 (At^a) was named AUG2.

Antibodies. Anti-At^a are mostly IgG although may contain IgM. While HDFN due to anti-At^a is rare, the antibodies can cause severe hemolytic transfusion reactions.

KANNO Blood Group System

The blood group system KANNO (symbol KANNO; number 037) was recently assigned to the prion protein (PrP) encoded by the *PRNP* gene. Through a combination of genome-wide association studies and whole exome sequencing which showed that KANNO− was due to a c.655 G>A variation leading to the amino acid polymorphism (p.Glu219Lys).[29]

Antibodies. Anti-KANNO are mostly IgG. Anti-KANNO was first identified in a Japanese woman who was never transfused, but had a history of two deliveries.[30] Although anti-KANNO appears to be stimulated by pregnancy, it has not been reported to cause hemolytic disease of the fetus and newborn or hemolytic transfusion reactions.

REFERENCES

1. Reid ME, Lomas-Francis C, Olsson ML. *Blood group antigen facts book*. ed 3 San Diego: Academic Press; 2012.
2. Daniels G. *Human blood groups*. ed 3 Oxford: Wiley-Blackwell; 2013.
3. Association Bulletin #16-02 *Mitigating the anti-CD38 interference with serologic testing*. AABB; 2016. <https://www.aabb.org/programs/publications/bulletins/Documents/ab16->.
4. Westhoff CM. Blood group genotyping. *Blood*. 2019 Apr 25;133(17):1814–1820.
5. Hyland CA, Roulis EV, Schoeman EM. Developments beyond blood group serology in the genomics era. *Br J Haematol*. 2019;184(6):897–911.
6. Orzinska A, Guz K, Brojer E. Potential of next-generation sequencing to match blood group antigens for transfusion. *Int J Clin Transfus Med*. 2019;7:11–22.
7. Heddle NM, Soutar RL, O'Hoski PL, et al. A prospective study to determine the frequency and clinical significance of alloimmunization post-transfusion. *Br J Haematol*. 1995;91:1000.
8. Rosse WF, Gallagher D, Kinney TR, et al. Transfusion and alloimmunization in sickle cell disease. *Blood*. 1990;76:1431.
9. Aygun B, Padmanabhan S, Paley C, et al. Clinical significance of RBC alloantibodies and autoantibodies in sickle cell patients who received transfusions. *Transfusion*. 2002;42:37.
10. Vichinsky EP, Earles A, Johnson RA, et al. Alloimmunization in sickle cell anemia and transfusion of racially unmatched blood. *N Engl J Med*. 1990;322:1617.

11. Cox JV, Steane E, Cunningham G, et al. Risk of alloimmunization and delayed hemolytic transfusion reactions in patients with sickle cell disease. *Arch Intern Med*. 1988;148:2485.
12. Chou ST, Jackson T, Vege S, et al. High prevalence of red blood cell alloimmunization in sickle cell disease despite transfusion from Rh-matched minority donors. *Blood*. 2013;122:1062.
13. Chou ST, Westhoff CM. The role of molecular immunohematology in sickle cell disease. *Transfus Apher Sci*. 2011;44:73.
14. Yazdanbakhsh K, Ware RE, Noizat-Pirenne F. Red blood cell alloimmunization in sickle cell disease: pathophysiology, risk factors, and transfusion management. *Blood*. 2012;120:528.
15. Matteocci A, Pierelli L. Red blood cell alloimmunization in sickle cell disease and in thalassaemia: current status, future perspectives and potential role of molecular typing. *Vox Sang*. 2014;106:197.
16. Osby M, Shulman IA. Phenotype matching of donor red blood cell units for nonalloimmunized sickle cell disease patients: a survey of 1182 North American laboratories. *Arch Path Lab Med*. 2005;129:190.
17. Bruce LJ, Guizouarn H, Burton NM, et al. The monovalent cation leak in overhydrated stomatocytic red blood cells results from amino acid substitutions in the Rh-associated glycoprotein. *Blood*. 2009;113:1350.
18. Fung MK, Grossman BJ, Westhoff CM, eds. *Technical manual*. ed 18 Bethesda, MD: American Association of Blood Banks; 2014.
19. Schwarz HP, Dorner F. Karl Landsteiner and his major contributions to haematology. *Br J Haematol*. 2003;121:556–565.
20. Clausen H, Hakomori S. ABH and related histo-blood group antigens; immunochemical differences in carrier isotypes and their distribution. *Vox Sang*. 1989;56:1.
21. Oriol R, Candelier JJ, Mollicone R. Molecular genetics of H. *Vox Sang*. 2000;78:105.
22. Storry JR, Olsson ML. The ABO blood group system revisited: A review and update. *Immunohematol*. 2009;25:48.
23. Rydberg L. ABO-incompatibility in solid organ transplantation. *Transfus Med*. 2001;11:325.
24. Curtis BR, Edwards JT, Hessner MJ, et al. Blood group A and B antigens are strongly expressed on platelets of some individuals. *Blood*. 2000;96:1574.
25. Cooling LL, Kelly K, Barton J, et al. Determinants of ABH expression on human blood platelets. *Blood*. 2005;105:3356.
26. Stenfelt L, Hellberg Å, Möller M, et al. Missense mutations in the C-terminal portion of the B4GALNT2-encoded glycosyltransferase underlying the Sd(a-) phenotype. *Biochem Biophys Rep*. 2019;19:100659.
27. Flegel WA. Homing in on D antigen immunogenicity. *Transfusion*. 2005;45:466.
28. Sandler SG, Flegel WA, Westhoff CM, et al. It's time to phase in RHD genotyping for patients with a serologic weak D phenotype. *Transfusion*. 2015;55:680.
29. Omae Y, Ito S, Takeuchi M, et al. Integrative genome analysis identified the KANNO blood group antigen as prion protein. *Transfusion*. 2019;59(7):2429–2435.
30. Kawabata K, Uchikawa M, Ohto H, et al. Anti-KANNO: a novel alloantibody against a red cell antigen of high frequency. *Transfus Med Rev*. 2014;28(1):23–28.

CHAPTER 112

PRINCIPLES OF RED BLOOD CELL TRANSFUSION

Robert A. DeSimone, Paul M. Ness, and Melissa M. Cushing

The clinical practice of transfusion medicine has evolved substantially since the discovery of the ABO system around 1900. Two technological advances set the stage for clinical practice through blood component therapy. First, the introduction of a safe and effective anticoagulant-preservative solution (suggested by Loutit and Mollison) allowed for the preservation of blood products. Second, in the mid-1960s, the introduction of plastic blood bags by Walter and Murphy, combined with the ability to store blood for extended periods, created opportunities to use transfusions in varied clinical settings. With these discoveries, the era of modern component therapy began. In 2017, approximately 12.2 million units of whole blood/red blood cells (RBCs) were transfused in the United States. This chapter reviews appropriate RBC transfusion practice in a variety of clinical settings, the clinical implications of RBC storage, the pathogenesis of red cell alloimmunization, and existing and emerging alternatives to allogeneic RBC transfusions.

RED BLOOD CELL COMPONENTS

Modern transfusion medicine practice aims to provide the specific component of blood required, rather than whole blood: red cells for oxygen-carrying capacity, plasma for coagulation proteins, and platelets for microvascular bleeding. The component therapy approach allows for optimal use of a limited community resource (Table 112.1). Today, the clinician wishing to increase the patient's oxygen-carrying capacity is more likely to use an RBC concentrate than whole blood, although there may still be situations in which whole blood, if available, is appropriate. For particular clinical applications, several modifications can be made to RBC products to render them leukocyte-or plasma-depleted. RBCs can also be frozen for long-term storage.

Whole Blood

A unit of whole blood is collected in citrate phosphate dextrose adenine (CPDA)-1 anticoagulant, giving it a shelf-life of 35 days and a volume of approximately 510 mL (450 mL of blood plus 63 mL of CPDA-1). Within 24 hours of collection, the granulocytes are dysfunctional, and several plasma coagulation factor levels including factors V and VIII have fallen. Although clinicians have been taught that refrigerated whole blood stored has no functional platelets, evolving studies have demonstrated in vitro that platelet quantities and function are maintained for 10 to 14 days at 4°C after collection, leading to renewed interest in the use of whole blood for resuscitation.

Whole blood has the advantage of correcting simultaneous deficits in oxygen-carrying capacity, coagulation factor deficiencies, and blood volume. Therefore, whole blood is useful in the management of trauma or in surgical cases involving extensive blood loss. In this setting, whole blood has two distinct advantages: (a) it provides colloid osmotic pressure and coagulation factors not supplied by crystalloid solutions and (b) it does not expose the recipient to RBCs and plasma from different donors.

There has been recent renewed interest in fresh whole blood for patients with severe coagulopathy and shock. Few prospective trials have compared fresh whole blood with component therapy. The potential advantages for fresh whole blood are a relative increase in hemoglobin (Hb) concentration, coagulation factors, and platelets compared with component therapy. In addition, fresh products avoid some of the negative effects of storage and processing. A Food and Drug Administration (FDA) approved platelet-sparing leukocyte reduction filter is available for fresh whole blood. However, the use of fresh whole blood is limited due to the lack of published randomized controlled studies on specific indications and demonstration of significant clinical benefits, as well as logistical considerations such as wastage. The methods to achieve adequate pathogen removal and the determination of optimal storage conditions for whole blood products are the subjects of active investigations.

There has also been recent interest in the use of stored, low-titer group O whole blood (LTOWB) for civilian trauma building on successful military experience. "Low-titer" refers to low titers of anti-A (and sometimes anti-B as well) which are found in the plasma component of whole blood and may cause hemolysis of the recipient's RBCs, but a universal testing process and titer threshold to define low-titer has not been established. LTOWB can be used if a recipient's ABO group is unknown at the time of transfusion, but every local institution must adopt policies to define specific patient populations eligible to receive LTOWB and establish monitoring for potential hemolysis. Monitoring may involve regular post-transfusion hemolysis labs as well as clinical assessment of hemolysis symptoms, which are often non-specific (i.e., fever, back pain).

Increasingly studies in trauma patients have demonstrated a benefit to the early use of increased ratios of plasma and platelets to RBCs. The PROPPR study found there was a significant reduction in the time to hemostasis and a reduction in death by exsanguination within 24 hours for patients receiving a 1:1:1 ratio of RBC:plasma:platelets versus a 2:1:1 ratio. In addition, the PAMPer trial demonstrated a significant 30-day survival benefit in patients administered pre-hospital thawed plasma relative to standard-care resuscitation.

Red Blood Cells

RBCs (also referred to as *packed RBCs* or *RBC concentrates*) are obtained from anticoagulated whole blood after removal of most of the platelet-rich plasma for the production of frozen plasma or platelets, or both. At most blood centers, the RBCs are then mixed with 100 mL of an additive nutrient solution that extends the storage period to 42 days and results in flow properties similar to those of whole blood.

RBCs are the product of choice for the correction of an isolated defect in oxygen-carrying capacity, as in cases of chronic anemia. In addition, RBCs rather than whole blood are most commonly used for the emergent transfusion of patients of unknown ABO type. The use of concentrated group O RBCs without plasma containing anti-A and anti-B can minimize potential hemolysis of the recipient's red cells.

Leukocyte-Reduced Red Blood Cells

Leukocyte-reduced RBCs (LRRCs) can be prepared by a variety of methods, resulting in differing degrees of white blood cell (WBC) removal. Currently, the most widely used method of leukoreduction is pre-storage filtration that is performed at blood collection facilities after collection using blood bags with in-line filters. The various filters on the market result in greater than 99% leukocyte reduction while depleting less than 10% of the red cells.

TABLE 112.1 Red Blood Cell Components: Characteristics and Indications

Component	Characteristics	Indications
Whole blood	High volume; good flow	Combined red cell/volume deficit (massive hemorrhage; exchange transfusion)
RBCs	Lower volume Higher hematocrit	Red cell deficit
Leukocyte-reduced RBCs	Good flow in AS-1	Prevention of febrile reactions Reduction of alloimmunization Reduction of immunomodulatory effects
Washed RBCs	Plasma depletion Must use within 24 h	Prevention of severe allergic reactions Prevention of anaphylaxis in IgA deficiency
Frozen RBCs	Long-term storage Plasma and leukocyte depletion Must use within 24 h of thawing	Rare donor unit storage Autologous storage for postponed surgery

Ig, Immunoglobulin; *RBC*, red blood cell.

The major indication for the use of LRRCs is the prevention of the febrile nonhemolytic transfusion reaction, a common adverse effect of transfusion, particularly in multiply transfused patients or multiparous females. These reactions are believed to be mediated by antibodies directed against leukocyte antigens (human leukocyte antigen, HLA, or human neutrophil antigen, HNA). Depletion of leukocytes to less than 5×10^6 has been shown to prevent, or at least ameliorate, such reactions in most patients. Increasing evidence suggests that cytokines play a role in causing these reactions. Because cytokines may be released from leukocytes during storage, pre-storage leukoreduction is preferred.

A second important indication for LRRCs is the mitigation of alloimmunization to HLA antigens that can adversely affect post-transfusion platelet increments and the search for HLA-compatible donors for stem cell and solid organ transplantations. This approach will be effective only if leukoreduced platelets are also used. According to current AABB standards, the total leukocyte number must be less than 5×10^6 when intended for this purpose, an achievable goal with third-generation leukoreduction filters. A multicenter study known as TRAP (Trial to Reduce Alloimmunization to Platelets) showed that the use of leukoreduction filters for platelet products significantly decreased the rate of alloimmunization but did not completely eliminate the problem.

A third indication for the use of LRRCs is to prevent transfusion-transmitted cytomegalovirus (CMV). CMV is found in low copy numbers outside of cells, and leukoreduced blood components are considered CMV safe. RBCs that are either leukoreduced or from CMV-seronegative donors are associated with only 1% to 1.5% CMV-transmission rates; thus, many institutions provide leukoreduced RBCs to mitigate CMV transmission.

A final indication for the use of LRRCs is to prevent transfusion-related immunomodulation (TRIM). A large meta-analysis previously demonstrated that patients who receive a blood transfusion are more likely to experience a postoperative infection than patients not transfused. This effect has been found to be dose-dependent and is thought to be mediated by suppressing the patient's immune function. In addition, some studies have suggested that TRIM may be associated with cancer recurrence, but other studies have refuted this notion. The mechanism of TRIM has yet to be defined, but multiple theories have been proposed. It has been suggested that immunologically active WBCs or soluble biologic response modifiers released from WBCs during storage downregulate the recipient's immune function. Alternative theories suggest that soluble mediators circulating in allogeneic plasma may have an immune-modulatory effect. Universal leukoreduction has been found by some studies, but not by others, to mitigate the immunomodulatory effect of allogeneic transfusions.

Washed Red Blood Cells

RBCs are washed using isotonic saline solutions mainly by automated techniques. There is always some degree of RBC loss with each wash cycle. Because most automated methods utilize open systems, the resulting product must be transfused within 24 hours to prevent bacterial growth.

The primary aim of washing is to remove plasma proteins, although some leukocytes and platelets are removed simultaneously. The major indication for washed RBCs is the prevention of severe allergic transfusion reactions, most likely mediated by recipient IgE antibodies against donor plasma proteins. Washing is recommended when reactions are severe and refractory to pre-transfusion steroid and antihistamine administration. In IgA-deficient patients who have preformed antibodies to IgA, the exposure to IgA-containing plasma can cause anaphylaxis. Therefore, high volume cell washes may be required to prepare cellular components for transfusion in IgA-deficient patients.

Irradiated Red Blood Cells

RBCs are irradiated with either gamma-ray or X-ray technology with a minimum dose of 25 Gy not exceeding 50 Gy. RBCs expire 28 days after irradiation or on the original expiration date, whichever is sooner. A method is used to ensure that irradiation has occurred with each batch. The primary aim of irradiation is to prevent the rare, but often fatal risk of transfusion-associated graft-versus-host disease (TA-GVHD) by the abrogation of the proliferative potential of donor T lymphocytes. GVHD can occur after the transfusion of immunologically competent donor lymphocytes, usually to an immuno-incompetent recipient. Some patient populations with indications for irradiated products include neonates, patients with hematologic malignancies, stem cell transplant recipients, and patients with congenital immune deficiencies. There is still much debate among experts regarding which additional patient populations may be at risk for TA-GVHD. It has been suggested that a policy of universal blood component irradiation could prevent TA-GVHD in patients with currently unsuspected risks, including advanced age, unrecognized immune deficiencies, or unsuspected donor-recipient immune similarities. If efficacious systems to apply pathogen reduction to red cell components by induction of DNA/RNA cross-linking become approved, another advantage would be the inactivation of white cells, which would eliminate the need for gamma irradiation to prevent TA-GVHD.

Frozen Red Blood Cells

RBCs can be frozen (with glycerol used as a cryoprotective agent) and stored at −65°C or colder. The required concentration of glycerol depends on the rate and the temperature of freezing. The freezing process destroys other blood constituents, except for a small percentage of immunocompetent lymphocytes. RBCs are prepared for transfusion by thawing and washing away the glycerol using a series of progressively less hypertonic crystalloid solutions, allowing glycerol to diffuse gradually from the cells to prevent hemolysis. The cells are resuspended in an isotonic saline solution containing glucose. The extensive washing removes approximately 99.9% of the plasma as well as cellular debris.

RBCs can be stored in the frozen state for at least 10 years with good viability. After thawing and washing, storage is typically limited to 24 hours because of the open system. Frozen cells have been shown to maintain prefreezing adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (DPG; also known as 2,3-bisphosphoglycerate or 2,3-BPG) levels. To maintain these factors at high levels, the standard is to freeze within 6 days of collection. When it is necessary to freeze older units, rejuvenation with a solution containing pyruvate, glucose, phosphate, and adenine has provided excellent results. The major indication for frozen RBCs is the stockpiling of rare donor units for patients who have developed alloantibodies. Some patients with rare phenotypes can make autologous donations that can be frozen for later use. Cells from autologous donors can be frozen if more units are required than can be collected in the 42-day liquid storage period or if surgery is postponed. Because of the high cost, lower product quality, and cumbersome nature of freeze-thaw procedures, other uses of frozen RBCs are somewhat difficult to justify.

APPROPRIATE TRANSFUSION PRACTICE IN VARIOUS CLINICAL SETTINGS

The response to RBC transfusion varies from patient to patient. In the absence of increased red cell destruction or sequestration, one unit of RBCs can be expected to increase the Hb level by 1 g/dL or the hematocrit level by approximately 3%. This rise is usually not fully realized until approximately 24 hours after transfusion, when the plasma volume has had time to return to normal. On the basis of a half-life of approximately 57.7 days for donor red cells, Mollison and associates calculated that an average-sized adult requires 24 mL of RBCs per day to maintain a given hematocrit level, assuming no red cell production. Patients with red cell aplasia require approximately 2 units of RBCs every 2 weeks.

Several factors can adversely affect the survival of transfused red cells. Hemolysis, caused by either immune-mediated red cell damage or mechanical trauma, shortens the survival of transfused cells, much as it shortens the survival of the patient's own cells. Hypersplenism can lead to initial sequestration as well as increased destruction of red cells. Continued blood loss is another obvious cause of suboptimal response to transfusion. It should also be emphasized that transfusion suppresses erythropoiesis, so that the net result of transfusion may be less than expected if transfusions are administered on a chronic basis.

Chronic Anemia

As a rule, signs and symptoms attributable to anemia are unlikely to develop at a Hb level of greater than 7 g/dL. When the anemia is of gradual onset, the body's compensatory mechanisms for maintaining oxygen delivery to the tissues come into play. Both cardiac output and intracellular 2,3-DPG increase, and thus, oxygen unloads at a lower oxygen saturation of Hb. When chronic anemia is due to red cell destruction, the healthy bone marrow responds by increasing erythropoiesis up to sixfold.

RBC transfusion provides symptomatic support rather than definitive therapy for anemia. Transfusion should be used only when there is no definitive treatment for the underlying cause (i.e., iron, vitamin B12, or folate supplementation) or when the severity of the anemia and the clinical manifestations in the patient make it impossible to wait for the effects of treatment to be realized.

Generalizations about RBC transfusion indications and practices are difficult to make and are usually inappropriate. The clinical impact of anemia varies depending on its pathogenesis, rate of onset, the presence or absence of accompanying hypovolemia, and, most importantly, the individual patient. The Hb level at which a given individual manifests the signs and symptoms of anemia relates, in part, to underlying health status, cardiorespiratory reserve, and tissue oxygen demand. Studies have shown that the risk of death increases with the severity of chronic anemia; it is prudent to maintain the Hb level above four for otherwise healthy patients and six for patients with cardiac disease to avoid sudden cardiac arrhythmias that are often fatal.

Perioperative Period

Many generalizations have been made about the appropriate transfusion management of acute blood loss, often with little evidence to support the arguments. One rule of thumb is that blood loss of 10% or less of total blood volume requires no replacement therapy at all; loss of up to 20% can be replaced exclusively with crystalloid solutions; and loss of greater than 25% generally requires RBC transfusion to restore oxygen-carrying capacity, along with crystalloid and sometimes colloid solutions to restore intravascular oncotic pressure to achieve adequate perfusion. A RBC transfusion trigger of 7 g/dL of Hb is now commonly used during the perioperative period. Each case must be evaluated individually on the basis of clinical signs and symptoms, rather than laboratory values alone. If the cardiovascular system is healthy and the degree of hypoperfusion is not significant, good tissue oxygenation can be maintained at much lower Hb levels. Many anesthesiology and surgery clinical guidelines suggest that most surgical patients do not need transfusion unless the Hb level falls to less than 7 g/dL. Given that RBC transfusion should be tailored to individual needs, the question arises as to whether there is any readily available, objective measurement that can be used to determine how low the Hb level can safely be allowed to fall before RBC transfusion is initiated.

Global hemodynamic parameters do not always correlate with microvascular perfusion. Assessment of tissue oxygenation at the microvascular level would help evaluate the effectiveness of a red cell transfusion, evaluate the effect of red cell storage on end-organ perfusion, and provide data about when to transfuse. Several general methods are available to evaluate the microcirculation and include direct assessment using image techniques and indirect methods of assessment, such as measures of microvascular oxygen availability and function. Direct assessment can be performed using laser Doppler flowmetry, imaging of the microcirculation, intravital microscopy, orthogonal polarization spectral imaging, and sidestream dark-field imaging. Assessments of oxygen availability include oxygen electrodes, reflectance spectrophotometry, and near-infrared spectroscopy. The techniques described are currently considered research tools and are not available in routine clinical practice. None has yet to prove reliable and reproducible in the clinical setting. Further, some are only useful in specific organ systems and do not reflect the global oxygenation of the patient. To be useful at the bedside, a technique must be technically simple, rapid, and noninvasive without large interoperator variation. Such a device has yet to become available.

In 2019, the Society of Cardiovascular Anesthesiologists published clinical practice guidelines focusing on blood conservation interventions, such as erythropoietin or antifibrinolytic administration, intraoperative blood salvage, or normovolemic hemodilution, and provided practical intraoperative transfusion algorithms guided by point-of-care viscoelastic methods and standard coagulation laboratory values.

Randomized clinical trials have evaluated the effects of different transfusion thresholds in distinct clinical settings; however, the thresholds used in the studies differ widely. Many of the studies found no difference in outcome. Most studies were not powered to adequately evaluate clinically important outcomes. Few studies included more than 100 patients. The TRICC trial included 838 intensive care unit (ICU) patients who were randomized to a restrictive transfusion strategy (transfused at Hb 7 g/dL) or liberal strategy (transfused at 10 g/dL). The 30-day mortality was slightly lower in the restrictive group (18.7% vs. 23.3%), but not significantly lower. The FOCUS trial, a 2600 patient, multicenter randomized trial designed to determine whether patients with cardiovascular disease or risk factors undergoing surgical repair of the hip benefit from a lower (<8 g/dL) or liberal transfusion trigger (transfused at 10 g/dL) showed that liberal transfusion did not reduce mortality or in-hospital morbidity in this patient cohort. Other studies have now been published in sepsis (TRISS), upper gastrointestinal (GI) bleeding (Villanueva et al.), pediatric intensive care (TRIPICU), and cardiac surgery (TRACS, TITRe2) that support the use of conservative transfusion triggers.

Red Blood Cell Transfusion in Neonates

In neonates, it is convenient to consider periodic, small-volume transfusion separately from massive transfusion situations. The trigger for transfusion and the optimal type of component are very different in these two settings. The potential adverse effects may be quite distinct.

Low-volume RBC transfusion is rarely indicated in full-term infants unless acute blood loss has occurred at birth or an intrauterine situation has led to prenatal anemia. In contrast, premature infants are frequent recipients of transfusions. In the intensive care setting, the premature infant is subjected to frequent blood sampling, and iatrogenic anemia may necessitate transfusion. Anemia of prematurity is also a well-recognized entity; premature infants have a slightly lower Hb value at birth. In addition, the postnatal decline in Hb occurs earlier and is more pronounced in premature infants. The mechanism for anemia of prematurity appears to involve a relatively lower output of erythropoietin in response to a given degree of anemia. This phenomenon is attributed in part to the fact that the liver, rather than the kidney, is the major site of erythropoietin production in these infants. Although some practitioners have considered this degree of anemia to be physiologic, the benign nature of this condition remains controversial.

Another debate among neonatologists concerns the triggers for RBC transfusion, as in what clinical signs and symptoms are valid reflections of poor tissue oxygenation. Congestive heart failure and severe pulmonary disease are generally accepted indications for transfusion, but recurrent apnea, tachypnea, tachycardia, and failure to thrive are also used as transfusion triggers. In recent times, the rate of transfusion and the donor exposure rate of premature infants have consistently declined. These changes, however, reflect improvements in patient care (e.g., microtesting methods resulting in less iatrogenic blood loss; the use of surfactant resulting in decreased respiratory distress and the use of a single unit to supply one infant over a longer period) rather than being attributable to changes in the transfusion trigger. The prolonged use of single units has become possible with the advent of the sterile docking technology that preserves the full shelf-life of the unit of RBCs, as well as with the accumulating evidence that fresh blood is not necessary for low-volume transfusions in neonates because supernatant potassium and decreased pH are not of concern in this setting. Recent studies have provided additional information about the relative risks and benefits of using restrictive rather than more liberal criteria for very low birth weight infants. Two studies showed that a liberal transfusion practice resulted in more infants receiving transfusion but conferred little evidence of clinical benefit. One study showed a lower risk of apnea and major brain injury for the liberal transfusion arm of the study. Therefore, the safest transfusion trigger in the preterm infant still remains unclear, and further studies are indicated. Most institutions assess the clinical situation and consider the postnatal age and whether a neonate has oxygen requirements when determining the need for a RBC transfusion.

The dose of a RBC transfusion in a neonate can vary by institution between 10 and 20 mL/kg. Few studies have assessed the optimal dose in this patient population, and further studies are needed. Paul et al. compared 10 and 20 mL/kg and found that the larger volume did not cause impaired pulmonary function. Wong et al. demonstrated extra transfusion episodes could be avoided with 20 mL/kg versus 15 mL/kg, without any additional risk to the patient. Many transfusion services now routinely use RBCs stored in additive solutions for low-volume RBC transfusion and thus prefer a dose of 20 mL/kg to account for the lower hematocrit of an additive unit.

Finally, several trials of erythropoietin therapy in premature infants have been undertaken. The administration of relatively high-dose erythropoietin has been shown to raise Hb levels and reticulocyte counts in healthy premature infants, but the effect in sicker neonates is unclear. Although transfusion exposure was decreased, the significance of this observation is diminished, given the promise of new strategies for limiting transfusions and donor exposure. The high cost and the increased risk of retinopathy associated with erythropoietin treatment does not justify its use in this patient population.

In the case of massive transfusion, the situation differs. There have been marked increases in massive transfusion in recent years in full-term as well as premature infants. Hemolytic disease of the newborn remains a prominent indication for exchange transfusion; however, the recent use of intravenous immune globulin to decrease red cell antibody levels in newborns has decreased the necessity of this procedure. The two triggers for exchange are (a) rapidly rising levels of unconjugated bilirubin that may lead to kernicterus and permanent central nervous system damage and (b) congestive heart failure secondary to severe anemia. Whole blood exchange transfusion is especially beneficial in cases of hemolytic disease of the newborn because it clears the bilirubin, the offending antibody, and the antibody-coated red cells before lysis while providing a source of red cells lacking the offending antigen. A two-blood-volume exchange is commonly performed by using a fresh unit of blood concentrated to a final hematocrit level of approximately 50%. In cases of hyperbilirubinemia resulting from other causes (e.g., liver immaturity in premature infants), phototherapy is the treatment of choice because its effects are usually more sustained, and exchange transfusion is used only for cases of marked elevations. Extracorporeal membrane oxygenation and open-heart surgery are two other situations when the neonate may be exposed to large volumes of allogeneic RBCs. The extracorporeal membrane oxygenation circuit requires a prime with RBCs, as do many of the types of extracorporeal circuits used for cardiopulmonary bypass.

Although accumulating evidence supports the safety of using RBC units of any age and with any preservative solution for low-volume transfusions in neonates, the same transfusion policies may not apply to massive transfusion. Newborn physiology is unique in several ways that may have implications for massive transfusion therapy. The newborn does not handle metabolites in a mature fashion. Renal immaturity may lead to problems in clearing potassium or acid from stored RBCs, and the immature liver may not catabolize citrate efficiently. These problems are accentuated and protracted in the premature infant. To address the concern about potassium load, fresh (<7 days old) or washed RBCs are often used, although the necessity of this practice is actively debated. Fresh blood may also be preferred because of its higher 2,3-DPG levels and better red cell integrity. The citrate problem is probably best handled by using slow infusion rates because the use of bicarbonate or calcium replacement to counteract the acid load or calcium-chelating effects of citrate is controversial. AS-3 may be preferentially chosen over AS-1 because of the theoretical concern over mannitol content that is absent in AS-3 but present in AS-1. However, in the context of multiple, small-volume transfusions, either AS is considered safe.

The humoral and cellular immune systems of the neonate are immature, especially in the premature infant. There is a small but real risk of transfusion-induced GVHD in premature infants receiving RBC transfusions and in the fetus undergoing intrauterine transfusion. Irradiation of RBCs should be performed in both settings. Another risk of transfusion in low birth weight (<1500 g) premature infants is the development of clinical CMV infection in infants of CMV-seronegative mothers. CMV-safe blood, either CMV seronegative or leukoreduced, should be provided to these infants. Some retrospective observational studies have suggested that other rare RBC transfusion-associated complications in this particularly vulnerable patient group may include necrotizing enterocolitis, intraventricular hemorrhage, bronchopulmonary dysplasia, and retinopathy of prematurity. However, a causal relationship has yet to be established through clinical studies. In multivariable analyses, some studies have suggested that severe anemia, and not transfusion itself, may be associated with these outcomes.

Ideas to prevent anemia and decrease donor exposure in premature infants and other neonates include delayed cord clamping, cord milking, umbilical vein blood sampling from the delivered placenta, and autologous cord blood transfusion. One review of 10 delayed cord clamping studies demonstrated lower transfusion requirements in the delayed versus early clamped group. However, a randomized controlled trial by Strauss et al. found no difference in transfusion needs between delayed and early clamped groups. A few studies have looked at autologous cord blood transfusions in neonates. One study found that the amount of blood harvested was insufficient to cover all transfusions in low-birth-weight infants. In addition, studies have demonstrated that blood processing problems, bacterial contamination, and costs are all barriers to the routine collection and autotransfusion of cord blood.

Red Blood Cell Transfusion in the Allogeneic Hematopoietic Stem Cell Transplantation Recipient

Red cell engraftment is usually the last phase of hematopoietic recovery after stem cell transplantation; therefore, RBC transfusion is common during the posttransplantation period. As hematopoietic stem cells and progenitor cells lack ABO antigens, the transplantation outcome is not significantly affected by the red cell antigen/antibody incompatibility between the donor and the recipient. However, RBC transfusion requirements and blood product selection can vary significantly depending on the type of ABO incompatibility. The phenotyping of non-ABO/Rh red cell antigens from the donor and recipient is not required in the absence of a positive red cell antibody screen in the recipient. Patients with an autologous stem cell transplant have fewer RBC transfusion requirements when compared with patients receiving allogeneic stem cell transplantation.

In the setting of allogeneic stem cell transplantation, there are four major categories of ABO antigen matching: full compatibility, minor incompatibility, major incompatibility, and bidirectional incompatibility. In addition, Rh type must be taken into consideration. Rh-positive recipients with Rh-negative donors should receive Rh-negative RBCs, but Rh-negative recipients with Rh-positive donors may receive Rh-positive RBCs. Apheresis platelets and plasma may be given without regard to Rh. The blood product selection algorithm is shown in (Table 112.2). Minor incompatibility is defined as the presence of blood group antibodies in donor plasma (e.g., group O donor to group A recipient). In minor incompatible stem cell transplantation, the incompatible plasma in the donor stem cell product may result in some hemolysis of the recipient's endogenous RBCs during the early phase of the posttransplantation period. Minor incompatibility is occasionally complicated by passenger lymphocyte syndrome, where transient hemolysis may occur if donor-derived lymphocytes in the stem cell graft remain viable and form blood group–specific antibodies that are incompatible with the recipient's red cells. Typically, passenger lymphocyte syndrome involves ABO incompatibility, but hemolysis resulting from serologic incompatibility in other blood group systems has been reported. If hemolysis increases a few days after transplantation, passenger lymphocyte syndrome should be considered. Once the donor is engrafted and incompatible recipient red cells are removed, donor red cells will have normal survival in the recipient. Major incompatibility is defined by the presence of blood group antibodies in recipient plasma (e.g., group A donor to a group O recipient). Major ABO-incompatible transplantation may result in pure red cell aplasia (PRCA) due to persisting incompatible ABO antibodies targeting the donor's engrafting erythropoietic precursors expressing ABO antigens. Although there may be no evident hemolysis, the donor red cell engraftment could be further delayed and result in prolonged RBC transfusion support. Finally, bidirectional incompatibility is defined as the presence of incompatible ABO antigens and antibodies contributed by both donor and recipient (e.g., group A donor to group B recipient). Bidirectional incompatible transplants may cause the problems associated with both minor and major ABO-incompatible transplants. To predict the severity of these complications and provide management, an ABO antibody titer can be performed on either the stem cell product or the recipient. However, titers do not correlate perfectly with the clinical outcomes. When a high titer incompatible ABO antibody is discovered in a minor incompatibility, the stem cell product can be plasma-reduced to avoid an immediate hemolytic reaction. Occasionally, in major incompatibilities, plasma exchange can be considered if the recipient has a high level of incompatible ABO antibodies to prevent hemolysis at the time of transplant or PRCA.

TABLE 112.2 Transfusion After ABO-Incompatible Hematopoietic Stem Cell Transplantation

ABO Group			Product Selection	
Donor	Recipient	Type of Mismatch	RBC	Plasma/ Platelets
A	O	Major	O	A
A	B	Bidirectional	O	AB
A	AB	Minor	A	AB
B	O	Major	O	B
B	A	Bidirectional	O	AB
B	AB	Minor	B	AB
O	A	Minor	O	A
O	B	Minor	O	B
O	AB	Minor	O	AB
AB	A	Major	A	AB
AB	B	Major	B	AB
AB	O	Major	O	AB

RBC, Red blood cell.

RED BLOOD CELL PRESERVATION AND STORAGE

The first key to the storage of blood is a stable, minimally toxic anticoagulant with preservative properties. During the early 1900s, it was recognized that citrate met these criteria. Citrate is slightly more toxic than heparin, especially when given rapidly and in large amounts, but citrate has preservative action that heparin lacks. Citrate has the added advantage of not causing systemic anticoagulation in the recipient.

The other factor essential for long-term storage is a mechanism to maintain cell viability and function. Freshly transfused RBCs have a good survival rate in the recipient's circulation, with a destruction rate approximately equal to that of the recipient's own cells: 1% per day.

However, changes within the RBC and its supernatant during RBC storage have been associated with reduced tissue oxygenation and other adverse effects in patients receiving RBC components stored for extended periods. The biochemical, structural, and functional changes are collectively termed the red cell storage lesion. RBC donor characteristics such as social behaviors and inherited genetic variants as well as RBC component processing techniques may also affect the storage lesion. The alterations found with the storage lesion along with the clinical implications are discussed in this section.

BIOCHEMICAL CHANGES ASSOCIATED WITH RED BLOOD CELL STORAGE

Adenosine Triphosphate Levels

ATP levels appear to be a major determinant of red cell viability. The drop in cellular ATP levels during storage has been correlated with increased cell rigidity and with loss of membrane lipid, leading to decreased red cell life span. For this reason, most efforts to extend RBC storage have focused on ways to maintain intracellular ATP levels. First, dextrose was introduced into the citrate solution (citrate-phosphate-dextrose [CPD]: 21-day shelf-life), and then adenine was added (CPDA-1: 35-day shelf-life). Three additive solutions containing additional dextrose and adenine (Nutricel, or AS-3) or dextrose and adenine plus mannitol (Adsol, or AS-1) and (Optisol, or AS-5) allow extension of the maximum storage time to 42 days (Table 112.3). Most of today's RBC supply is stored in an additive solution.

TABLE 112.3 **Biochemical Changes in Stored Red Blood Cells**

Variable	CPDA-1	CPDA-1	Adsol
In vivo survival (at 24 h) (%)	100	<71.0	<88.0
pH	<7.5	<6.7	<6.7
ATP (% initial)	100	<45.0	<76.0
2,3-DPG (% initial)	100	<10.0	<10.0
Plasma K^+ (mEq/L)	5.1	<78.5	<49.0

ATP, adenosine triphosphate; *CPDA*-1, citrate phosphate dextrose adenine-1; *DPG*, diphosphoglycerate.
Data from Zuck TF, Bensinger TA, Peck CC, et al. The in vivo survival of red blood cells stored in modified CPD with adenine: report of a multi-institutional cooperative effort. *Transfusion*. 1977;17:34; and Moore GL, Peck CC, Sohmer PR, et al. Some properties of blood stored in anticoagulant CPDA-1 solution. a brief summary. *Transfusion*. 1981;21:135.

2,3-Diphosphoglycerate Levels

Stored RBCs must also maintain their capacity to deliver oxygen. It was not until 1967 that the central role of 2,3-DPG in releasing oxygen from oxyhemoglobin was recognized. Attention was then focused on ways to maintain high levels of 2,3-DPG in stored RBCs. The first anticoagulant introduced on a large scale, acid citrate-dextrose, was ineffective because of its low initial pH; however, the subsequently developed CPD, with its higher initial pH and slower fall in pH, was superior. CPDA-1 and additive solutions have not further improved 2,3-DPG maintenance. Although 2,3-DPG depletion of stored RBCs is known to decrease oxygen delivery, the clinical significance of this finding is unclear. 2,3-DPG levels in stored RBCs are rapidly regenerated in vivo, rising to greater than 50% of normal within several hours and to normal within 24 hours of transfusion. Although a patient with normal cardiac status should be able to compensate by increasing cardiac output to maintain normal oxygen delivery until 2,3-DPG levels are regenerated, an improvement in 2,3-DPG preservation in stored RBCs is still desirable.

Citrate

Infusion of large volumes of blood with citrate anticoagulant over a short period may cause plasma citrate levels to reach the toxic range. The primary concern is the cardiovascular effects of hypocalcemia caused by the chelation of calcium by citrate. The risk of citrate toxicity is exacerbated by liver dysfunction or liver immaturity. Despite these theoretical considerations, there is little documented evidence of clinical citrate toxicity, which can usually be prevented by slower infusion. If large amounts of blood have to be infused over a very short period, administration of calcium gluconate can be considered, but whether the benefits justify the risk is controversial.

Potassium

Another issue with prolonged storage is the excess potassium in the RBC supernatant that could potentially cause cardiac arrhythmias. At a storage temperature of 4°C, the red cell sodium-potassium pump is essentially nonfunctional, and intracellular and extracellular levels gradually equilibrate. In addition, hemolysis results in increased potassium in the supernatant. However, because the total volume of plasma in RBC concentrates is low (approximately 70 mL), the total potassium burden is only approximately 5.5 mEq at product expiration. Practically speaking, the potassium load is rarely a clinical problem except in the setting of preexisting hyperkalemia and renal failure in adults or in some recipients of massive amounts of irradiated red cells In children with rapid or massive transfusions, hyperkalemic cardiac arrest is more commonly recognized. In these situations, fresher units of RBCs or washed RBCs can be used.

Di(2-ethylhexyl)phthalate

Since their introduction in the 1960s, plastic blood bags used for storing RBCs have been made from polyvinylchloride containing the lipophilic plasticizer di(2-ethylhexyl)phthalate (DEHP), which confers pliability. The safety of DEHP has been questioned for years owing to its tendency to leach from the bag and to be present at levels of 50 to 70 mg/L in stored RBCs. The storage of RBCs in bags made of polyvinyl chloride plasticized with DEHP has caused more recent concern because of the reported association between DEHP exposure and impaired development of the male genital tract. Due to these concerns, there has been increased discussion in Europe surrounding eliminating DEHP from blood bags. One of the benefits of using DEHP for RBC storage is the prevention of hemolysis. DEHP leaches out from the plastic bag and intercalates and stabilizes the red cell membrane. Shorter storage lessens the load of DEHP delivered to the recipient. Although there are potential replacement plasticizers, DEHP is most commonly used since the exposure to DEHP through transfusion is generally felt to be less than other environmental exposures.

Storage Length of Red Blood Cells

Current Status

The current expiration time of an RBC unit stored in an additive solution is 42 days. The allowable storage time is regulated by the FDA and requires: (1) the recovery of at least 75% of red cells transfused 24 hours after infusion, and (2) less than 1% hemolysis, both at the end of the storage limit. There is no criterion based on the clinical ability of transfused red cells to oxygenate tissue. The 2017 National Blood Collection and Utilization Survey reported that 18% of RBCs are transfused during their last week of life, between days 35 to 42.

Many variables affect the age of a specific RBC unit at transfusion. The blood group of the unit will impact the length of storage. Group O units tend to be issued quickly due to their universal compatibility; as a result, Group O units are often issued with a shorter age. Group B and AB tend to be stored the longest. Transfusion service policies will also affect the overall age of RBC units at the time of transfusion. Busy tertiary care hospitals tend to transfuse some of the oldest units since they may receive units returned from smaller community centers that did not expect to use them before their outdate. Hospitals that have blood refrigerators outside the blood bank tend to age units in the refrigerators because it is cumbersome to rotate the units out frequently. Hospitals with high crossmatched to transfused ratios also tend to have older units on their shelves.

Red Blood Cell Storage Lesion

The RBC storage lesion includes all the changes that occur to blood components during blood bank storage. The lesion includes biochemical and structural changes to the red cell, as well as changes that occur in the storage supernatant. The structural changes include red cell membrane loss that leads to the reversible evolution of the shape of the red cell from a biconcave disc to a spheroechinocyte. After this stage, further red cell membrane loss becomes irreversible, and microvesicles are produced. Red cell vesicles are quickly cleared by macrophages due to exposed negatively charged lipids. The infusion of a large amount of red cell vesicles during a RBC transfusion may overwhelm the reticuloendothelial system and cause a proinflammatory and prothrombotic response. The shape changes are also associated with a rheologic effect, including increased viscosity and reduced flow within the capillaries, leading to decreased tissue perfusion. Many of the biochemical and structural changes, aside from vesiculation, are reversible when the RBCs enter human circulation where pH, ATP levels, and 2,3-DPG levels are normal.

Older red cells become more susceptible to oxidative damage, although this change generally occurs at a lower rate during in vitro conditions than in vivo due to the lower storage temperature. However, during a transfusion, the human circulation is confronted by a bolus of equally damaged RBCs that may overwhelm the reticuloendothelial system. The work of Hod and Spitalnik emphasized that up to 25% of transfused RBCs with prolonged storage are cleared within 24 hours. The rate of delivery of heme-iron to the reticuloendothelial system may abruptly increase as much as 60-fold after transfusion of even a single unit of RBCs. This can surpass the rate of uptake by transferrin and produce circulating nontransferrinbound iron that may in turn lead to the myriad of problems associated with iron overload.

Irradiated cells are exposed to additional oxidative stress that can damage red cell protein and lipid. WBCs in the component also break down during storage and release proteases and lipases. Lysophospholipids and glycosidases are produced. Glycosidases may remove sialic acid and other sugars from the red cell membrane and can cause increased binding of stored red cells to endothelial cells and potentially contribute to endothelial inflammation. Increased lysophospholipids, such as platelet activating factor, have been found in units that have caused TRALI.

Certain blood donor characteristics may also enhance the red cell storage lesion. For example, glucose-6-phosphate dehydrogenase (G6PD) deficiency decreases the ability of RBCs to withstand oxidative stress, and RBCs from G6PD-deficient donors have been associated with reduced 24-hour post-transfusion recovery (mean 78.5% vs. 85.3%, $P = .0009$). In addition, blood donor smoking has been associated with increased RBC osmotic fragility and oxidative stress. RBCs donated by smokers that undergo irradiation have been associated with reduced post-transfusion Hb increments compared to RBCs irradiated from non-smokers (mean 0.74 g/dL vs. 0.94 g/dL, $P = .045$), suggesting a potential two-hit mechanism on RBC quality.

Clinical Relevance of the Red Cell Storage Lesion

Retrospective or prospective observational studies in many diverse patient populations have suggested numerous adverse events that may be associated with prolonged RBC storage, including increased risk for mortality, postoperative infection, multiorgan failure, deep venous thrombosis, or increased length of stay in the ICU or hospital. The observational studies on this topic have a number of significant limitations. First, larger volumes of RBC transfusion predict worse outcomes. Patients who are transfused larger volumes are statistically more likely to receive older RBCs. Second, confounding factors may not be recognized and, as a result, are discounted in nonrandomized studies. Third, individual methodologies in the presently available studies have varied markedly. Some studies have looked at the effect of mean storage age of all units transfused on outcome, and some have broken storage time into categorical groups (i.e., is storage less than 14 days safer than storage beyond 14 days). In addition, studies have not used a single definition for “older” units; some have defined older units as greater than 14 days, greater than 21 days, or greater than 28 days. The definitions of age have not been based on either clinical or microcirculatory relevance or related to the feasibility and practicality of blood collection. Most studies have been designed for convenience and feasibility; a red cell storage age study with a definition of an older RBC unit as greater than 14 days of storage is achievable given current hospital inventories. Fourteen days is close to the average age of RBCs stored in a blood bank, but from a blood collection and inventory management perspective, an expiration time of 14 days would be disastrous for both hospitals and blood collection facilities. Authorities have argued that studies should be designed to measure storage age differences between lengths of storage that would actually be achievable given current inventory levels and difficulties in donor recruiting.

Published studies have focused on three patient groups that consume large numbers of RBC components: cardiac surgery, trauma, and critical care patients. A publication on the effect of RBC storage age on hematopoietic transplant recipients has also been recently published.

The Age of RBCs in Premature Infants study, a double-blinded study evaluating the effectiveness of RBCs stored no longer than 7 days versus standard-issue RBCs in 377 neonates requiring transfusions has been published. The primary outcome revealed that using fresh blood of less than 7 days-old did not improve the clinical outcome of these vulnerable premature infants. The Age of Blood in Children in Pediatric ICUs trial (ABC PICU) examined the effect of transfusing less than 7-day old blood (versus standard-issue age) in 1538 pediatric ICU patients (age range 3 days to 16 years, median age 1.8 years). The randomized study found that fresh versus standard-issue red cells did not decrease new or progressive multiple organ dysfunction syndrome or ICU mortality. The two groups also had similar rates of sepsis and acute respiratory distress syndrome.

The Red Cell Storage Duration Study evaluated 1098 patients undergoing complex cardiac surgical procedures who were likely to require RBC transfusion. Patients were randomized to receive RBC units stored for either 10 or fewer days or 21 or more days. The median storage times of the transfused RBCs were 7 days versus 28 days for the two groups. RBC storage duration was not associated with a change in the Multiple Organ Dysfunction Score (MODS) or the 28-day mortality. Adverse events did not differ between the groups, except that hyperbilirubinemia was more common in the older storage age group.

The Age of Blood Evaluation study investigated the effect of leukoreduced RBCs stored 7 days or less versus leukoreduced standard-issue RBCs on 90-day all-cause mortality. Investigators enrolled 2430 adult participants receiving their first RBC unit in an ICU. The study found that fresh versus standard-issue RBCs did not decrease 90-day mortality among critically ill adults.

The current RBC inventory almost always meets hospital needs with a 42-day RBC expiration period but is not currently equipped to meet the needs of a less than 7-day inventory, or even a less than 21-day inventory. Although it is logical to expect that there is a limit to RBC storage, beyond which the risks of transfusion outweigh the benefits of transfusion, none of the randomized studies described above is designed to determine this limit. In a study of autologously transfused RBCs, those transfused at later times (days 40 to 42 of storage) were associated with increased post-transfusion bilirubin and non-transferrin-bound iron, as well as increase in transferrin saturation, compared to transfusion of fresh units (days 3 to 7 days of storage). This finding suggests that units transfused in the last week of storage (days 35 to 42) may demonstrate decreased quality, and raises potential safety concerns and implications for blood bank inventory management.

RED CELL ALLOIMMUNIZATION

Overview

RBCs have over 300 blood group antigens that are capable of mounting humoral responses in antigen-naïve transfused recipients. Direct exposure to nonself red cells from allogeneic blood transfusion, circulating fetal red cells in pregnant females, or even occasionally stem cell/organ transplantation can cause red cell alloimmunization. In addition, recent studies have shown that indirect immune modulation mediated via proinflammatory factors in stored RBCs, a proinflammatory state in the host (e.g., endothelial damage or higher turnover rate of red cells in patients with chronic transfusion), and passive transfer of WBC antibodies (anti-HLA and anti-HNA) are associated with a higher risk of RBC alloimmunization. Matzinger’s

Danger Theory/Model further proposes that nonself antigen exposure with local danger signals from injured cells can synergize immune responses. Therefore, red cell alloimmunization represents a complex interplay between transfused allogeneic RBCs and host immunity.

The humoral response can be categorized into two phases. The primary response occurs immediately when foreign antigen activates B lymphocytes leading to the high-affinity mutation-selection process. This process allows the activated B cells to proliferate and differentiate into memory/plasma B cells that produce a specific antibody against the antigen, although the initially generated antibody may not lead to significant hemolysis of sensitized red cells. Upon re-exposure to the same antigen (e.g., transfusing an individual who had preformed antibody but at an undetectable level), the second phase of the immune response (anamnestic response) can lead to rapid and robust antibody production from the pre-generated antigen-specific memory/plasma B cells. This response often causes clinically significant hemolysis that may even lead to a fatality in these transfused individuals. There are two isotypes of red cell antibodies that are routinely evaluated in the blood bank. Immunoglobulin M (IgM) is a pentavalent antibody that appears during the early phase of antibody generation. It is primarily restricted to the intravascular compartment, can fix complement on the transfused red cells with cognate antigens, and could result in intravascular hemolysis. On the contrary, IgG antibody is produced mostly by memory/plasma B cells that can migrate to the extravascular compartment (e.g., amniotic fluid and tissues). Due to its capacity to move between compartments, IgG antibodies are commonly associated with extravascular hemolysis and fetal hemolysis.

Individuals at Risk

Both the immunogenicity and quantity of foreign red cell antigens are critical determinants of alloimmunization. Therefore, frequent transfusion exposure and transfusion across different ethnic groups are the most common factors associated with alloimmunization. This issue is of great concern in patients with transfusion-dependent hemoglobinopathies (e.g., sickle cell disease [SCD] and thalassemia) and PRCA (e.g., Diamond-Blackfan anemia). In particular, SCD is associated with rapid sequestration of defective red cells and endothelial damage. The racial discrepancy in the prevalence of certain red cell antigens, such as in the Rh system (DCcEe) and Kell (K1), between blood donors (mostly European descent) and SCD patients (mostly African descent) significantly contributes to the increase in red cell alloimmunization rate compared with the general population. Interestingly, by molecular genotyping, it was found that SCD patients have a high frequency of polymorphisms that can result in red cell alloimmunization even when serologically matched red cells are transfused. In addition, the endothelial damage and chronic inflammatory state associated with SCD have been hypothesized to cause synergy in red cell alloimmunization. Genetic factors predisposing SCD patients to red cell alloimmunization have been studied, and patients with HLA-B35 have a sixfold higher likelihood of making red cell alloantibodies compared with SCD patients lacking HLA-B35. The Genome-Wide Association Studies (GWAS) currently being pursued may clarify the role of genetic factors. Interestingly, the rate of alloimmunization was not proportionally observed in patients receiving massive transfusion with un-crossmatched RBCs. This observation may be due to transient immunosuppression in the setting of severe physiologic stress or by immune modulation due to the overwhelming number of foreign RBCs transfused. This phenomenon was also shown in pediatric SCD patients receiving RBC exchange. On rare occasions, solid organ transplant can also be a source of red cell alloimmunization as it may expose the recipient to residual donor red cells with foreign antigens, despite immediate saline perfusion to rinse blood from the organ. Clinical hemolysis may be observed, but severe hemolytic complications are more likely a result of passenger lymphocytes that are capable of mounting an anamnestic response to antigen-positive recipient red cells (passenger lymphocyte syndrome). From the knowledge gained through observational studies, the risk of red cell alloimmunization seems to be associated with both red cell exposure and host immune responses. However, the complete pathophysiology still remains to be determined.

Molecular Pathophysiology

The exact cause of human RBC alloimmunization under myriad clinical circumstances is multifactorial and difficult to accurately identify. Thus, reductionist animal model–based studies will be needed to investigate the mechanisms and identify the specific etiologies of this clinical entity. Given the well-defined genetic background, ready availability of a large number of participants, ability to adapt to many genetic manipulations, and largely conserved lineage-specific genes in the mouse immune system, mouse models are ideal to investigate this complex clinical entity. For example, transgenic human Rh, Kell, and Duffy antigen-expressing mice have been generated to study the interaction with their cognate alloantibodies. In addition, human hemolytic disease of the fetus and newborn (HDFN) associated with alloantibody against the Kell antigen has been closely recapitulated in pregnant transgenic Kell mice. Unlike the HDFN associated with alloantibody against the RhD antigen that can be prevented with the administration of Rh immune globulin, HDFN associated with alloantibody against the Kell antigen still lacks effective prevention. Therefore, this anti-Kell mouse model will be useful in revealing the mechanism of alloimmunization against the Kell antigen.

The transgenic murine model of SCD revealed that the sickle cell genetic mutation alone was not sufficient to independently cause RBC alloimmunization. One study further suggests the critical roles of environmental factors involved in this immune response. Indeed, other mouse model studies have found that viral analog-induced inflammation (e.g., polyinosinic polycytidylic acid, or poly:IC) can independently enhance RBC alloimmunization. Furthermore, experimental mice transfused in the absence of inflammation developed immune tolerance against the transfused foreign red cell antigens as compared with control mice in the presence of poly:IC. This observation is supported by the fact that most transfused individuals without infections do not make alloantibodies to nonself red cell antigens. Also, exogenous inflammatory factors accumulated during RBC storage (storage lesion) were found to enhance sensitization to foreign RBCs leading to a more than 10-fold increase in alloantibody production. This concept has not yet been tested in prospective controlled clinical trials.

While animal models are able to provide the reductionist view of red cell alloimmunization and can serve as a platform to study the mechanism of red cell alloimmunization, the study conclusions may have limited general applicability. For example, the transgenic SCD mouse model has inherent differences in disease manifestations (e.g., massive splenomegaly and large vessel vasculopathy) that are not observed in human SCD patients. In addition to the interspecies genetic variation, the homogeneous nature of the experimental mouse's genetic background may not fully capture the true pathophysiology of red cell alloimmunization in the human population. Nevertheless, mouse models provide a molecular view of the disease pathogenesis and generate many new hypotheses to further elucidate the mechanism of red cell alloimmunization.

Red Blood Cell Alloimmunization Mitigation Strategies

Blood transfusion is one of the most common therapies provided in a hospital, and millions of blood products are transfused every year worldwide. Therefore, a carefully planned strategy to prevent red cell alloimmunization is urgently needed. Reducing red cell alloimmunization can occur in three areas: blood collection/manufacturing, hospital transfusion services, and through patient care. At the blood center, prestorage leukoreduction can effectively remove more than 99.99% of the original WBCs. This process minimizes the inflammatory or "danger" signals infused into recipients at the time of foreign red cell antigen exposure and thus may reduce subsequent alloimmunization. Some blood centers have also begun to provide non-ABO red cell antigen typing on RBC units to minimize transfusing RBCs with mismatched red cell antigens. Clinical interventions have also been proposed to reduce red cell alloimmunization risk. Surgical splenectomy was shown in mouse models and observed in transfusion-dependent patients (e.g., thalassemia) to reduce the alloimmunization rate; however, this procedure is not as effective if the initial antigen exposure occurred prior to spleen removal. Since sickle cell patients develop asplenia at early ages, this finding may also not be relevant to the high incidence of alloimmunization in sickle cell anemia. Immunosuppression can also theoretically retard the red cell alloimmunization rate. However, one must consider the adverse clinical impact of these drugs. More studies on drug types and dosages are needed to refine this strategy. Finally, blood bank practice plays a daily pivotal role in preventing red cell alloimmunization in hospital patients. Molecular/genetic typing can permit red cell antigen matching at a higher resolution and precision than traditional serologic red cell antigen typing. This additional testing is particularly helpful in patients with hemoglobinopathies or high titer warm autoantibodies, who are especially vulnerable to red cell alloantibody formation. Expert hematology guidelines now strongly recommend extended molecular RBC genotypes in all SCD patients with subsequent prophylactic RBC matching for Rh and K1 antigens to prevent alloimmunization from transfusion.

ALTERNATIVES TO ALLOGENEIC RED CELL TRANSFUSIONS

There are many alternatives to standard allogeneic red cell transfusions for a patient requiring elective surgery (Table 112.4). Potential alternatives include banking autologous units before the surgery, acute normovolemic hemodilution, pharmacologic therapies (i.e., erythropoietin or fibrinolysis inhibitors), perioperative salvage, virally inactivated donor red cells, or blood substitutes. All current options have their own unique benefits and drawbacks, and some of these alternatives are not yet available. In cases where an emergency transfusion is needed, the first three options would not be possible as they all require significant planning. Only virally inactivated components and red cell substitutes (both of which are still works in progress) would be available for unanticipated transfusion needs.

Autologous Blood Transfusion

Advantages of Autologous Blood Transfusion

The substitution of autologous blood components for those collected from other (allogeneic) donors eliminates transfusion-transmitted diseases such as viral hepatitis and acquired immunodeficiency syndrome. Immunologic complications related to the transfusion of foreign cells, including hemolysis and febrile reactions to WBCs, are also prevented. Other advantages, though possible, are less clearly established. For example, erythropoiesis may be sufficiently stimulated in the repeatedly bled autologous donor to hasten recovery from postoperative anemia. Intraoperatively salvaged red cells are spared the acquired membrane defects ("storage lesion") and 2,3-DPG deficiencies of refrigerated red cells.

TABLE 112.4 Alternatives to Standard Allogeneic Transfusions

Hemodilution
Intraoperative autologous transfusion
Perioperative blood salvage
Lower transfusion trigger
Pharmacologic therapies
Pathogen inactivation
Red cell substitutes
Stem cell derived red blood cells

An important drawback to these techniques is their increased expense in contrast to the simpler allogeneic transfusions they replace. In addition, the availability of autologous components may result in their use in situations where transfusion might not have otherwise been considered. For orthopedic and urologic elective surgeries, the recent advent of less invasive intraoperative procedures has led to reduced need for transfusion. Patients with suboptimal compensatory erythropoiesis and donation-induced anemia at the time of surgery are also more likely to be given transfusions. Based upon the current level of viral safety in blood components in the developed world, the use of autologous blood has dropped significantly from times when viral testing was not available or reliable.

Preoperative Autologous Blood Collection

The typical volunteer allogeneic blood donor is allowed to give one unit of blood no more than once every 8 weeks to prevent iron deficiency. However, provided that bone marrow erythropoiesis can be stimulated and satisfactory iron supplies maintained, blood can be collected as frequently as once a week from an autologous donor. Although the shelf-life of refrigerated RBCs is limited to 42 days, frozen storage for up to 10 years is possible at below −65°C using glycerol as a cryopreservative.

From a cardiovascular standpoint, phlebotomy is well tolerated by a variety of seemingly high-risk donors, including the elderly, children, pregnant women, and patients with coronary artery disease. By contrast, anemia frequently develops during the donation interval and limits the number of autologous units that can be collected. In addition to marginal iron stores, erythropoietin levels often do not increase during the donation interval, probably because the hematocrit level of most donors is not allowed to fall to less than 30%. This situation may be improved by the administration of the recombinant erythropoietin to autologous donors. Since many procedures are now performed using laparoscopy or other techniques to limit blood loss, the demand for predeposit autologous transfusion support has fallen in recent years.

Autologous blood has been safely collected from women during pregnancy for use during childbirth. Nevertheless, the transfusion rate at delivery is quite low (<2.5% in many institutions) and most autologous donations are unused. Long-term (frozen) storage of autologous RBCs in the absence of a planned transfusion episode is largely ineffective and expensive.

Intraoperative Blood Salvage

Cell salvage occurs in three phases: collection, washing, and reinfusion. RBCs are collected from the operative field using a dedicated double lumen suction device. One lumen suctions blood from the operative field and the other lumen adds heparinized saline to the salvaged blood. The anticoagulated blood then passes through a filter and is collected in a reservoir. If less than 1 L of blood is collected, further processing is foregone and the collected blood is discarded.

In most circumstances, the contents in the bag can be washed to remove free Hb, surgical irrigation solutions, and other debris. Instruments are available that include both a reservoir for collecting salvaged blood and a centrifugal washer. Large aliquots (>500 mL) can be fully washed in as little as 3 minutes. As a result of this speed, autologous blood salvage has become practical in situations in which blood loss may be extremely rapid, such as trauma or liver transplantation.

The hematocrit level of unwashed blood is typically low because of dilution from irrigating surgical fluids and some degree of mechanical hemolysis. Free Hb levels are sometimes greater than 1000 mg in unwashed blood, and hemoglobinemia and hemoglobinuria may occur after the transfusion, although renal sequelae are surprisingly

TABLE 112.5 **Autologous Blood Salvage Systems[a]: Characteristics of Collected Blood**

System	Hardware	Software	Hematocrit	Free Hemoglobin	Platelet Count	Coagulation Factors	Fibrin Degradation Products
Collection without washing	Rigid plastic container	Plastic bag	Low (25%)	Very high (200 mg%)	Low (100,000/mm^3)	Low (35%–75%)	High (300 mg%)
Collection followed by washing	Integral or separate blood cell processor	Disposable plastic bowl and tubing	High (60%)	Low (<50 mg%)	Very low (10,000/mm^3)	Absent (0%)	Absent (0%)

[a]Typical results of laboratory tests are shown. Transfusion of large volumes of salvaged blood results in similar alterations in these tests in the recipient.
Data from Noon GP. Intraoperative autotransfusion. *Surgery*. 1978;84:719; and Silva R, Moore EE, Bar-Or D, et al. The risk: benefit ratio of autotransfusion–comparison to banked blood in a canine model. *J Trauma*. 1984;24:557.

low. Despite this evidence of red cell injury, the survival rate of ^{51}Cr-labeled salvaged cells is normal in most patients studied.

There are many potential complications associated with cell salvage, such as nonimmune hemolysis, air embolus, febrile nonhemolytic transfusion reactions, mistransfusion, coagulopathy, and contamination with drugs. Transfusion of salvaged blood has resulted in coagulation abnormalities, including hypofibrinogenemia, prolonged prothrombin time and partial thromboplastin time, elevated fibrin degradation products, and thrombocytopenia. These coagulation abnormalities most likely reflect the characteristics of the salvaged blood itself, which, after exposure to serosal surfaces, becomes deficient in coagulation factors and platelets and, in the case of unwashed blood, has high levels of fibrin degradation products (Table 112.5). Coagulation abnormalities can also occur when extensive red cell salvage has been given without adequate plasma or platelet support.

Fat, fibrin, bone fragments, and microaggregates often contaminate salvaged autologous blood. However, infusion of unwashed blood has not been proved harmful in either animals or humans, possibly because routine blood filters remove most particulate material. Other contaminants, such as heparin, topical antibiotics, hemostatic agents, and biologic substances such as tissue enzymes, can be at least partially removed by washing. Complete removal of bacteria is also not possible, even when the salvaged blood is washed with antibiotics. Thus, collection of blood from a contaminated site (e.g., with intestinal contents) is usually considered to be contraindicated; in fact, manufacturers contraindicate the use of cell salvage in cases where there is potential contamination of salvaged blood with enteric contents. However, in recent years, this viewpoint has been reconsidered as studies have found that the autotransfusion of microbiologically contaminated salvaged blood has demonstrated no adverse outcomes or increase in postoperative infectious complications. Tumor cells have been found in blood salvaged during cancer operations and thus many practitioners consider cancer another contraindication. Others believe that filtration would remove salvaged tumor cells.

Approximately one-half of the blood lost during surgery can be salvaged. The rest is usually irretrievably absorbed in drapes and sponges or damaged during collection. The use of salvaged autologous blood has been associated with a 50% reduction in allogeneic blood use in orthopedic procedures such as spinal surgery and hip replacement and is also effective in vascular surgical procedures such as aortic reconstruction. Autologous salvage has been a useful adjunct in the treatment of some Jehovah's Witnesses whose literal acceptance of the Bible includes abstention from routine allogeneic blood transfusions.

Both the canister systems and RBC processors used to collect intraoperative autologous blood can also be used to collect postoperative blood drainage, such as that from the mediastinum after open-heart surgery, from the knee or hip after orthopedic procedures, or from the peritoneal cavity after hepatic injury. Because blood salvaged from a serosal cavity has little residual fibrinogen and platelets, clotting is not a problem, and the addition of anticoagulants is usually unnecessary. Shed mediastinal blood after open-heart surgery contains high levels of cardiac muscle enzymes, especially creatine kinase, as well as lactate dehydrogenase from hemolyzed RBCs. Therefore, reinfusion of shed blood results in elevated levels of both enzymes that can confound the diagnosis of myocardial infarction (MI) in the postoperative period. Reinfusion of shed mediastinal blood has been shown to reduce the need for allogeneic transfusions.

Hemodilution

The collection of autologous blood at the onset of surgery for later reinfusion at the end of the procedure was first suggested in open-heart operations, in which it was hoped that a supply of platelets undamaged by exposure to the membrane oxygenator might reduce the incidence of coagulopathies. Hemodilution itself reduces RBC loss: a patient with a hematocrit level of 45% and 2 L blood loss during surgery loses roughly 900 mL of RBCs, but one with a hematocrit level of 20% from hemodilution loses only 400 mL of RBCs. Hemodilution is less expensive to accomplish than preoperative autologous blood donation and may be the only option available when surgery is performed in other than elective settings. Proponents claim that the induced anemia may even be beneficial to the patient, in that oxygen delivery at a hematocrit level of 30% is enhanced by an increased cardiac output resulting from the decreased blood viscosity. Other advantages of hemodilution over predeposit autologous transfusion are the provision of fresh red cells along with plasma and platelets that may be important in maintaining hemostasis.

Reductions in allogeneic blood needs have been reported after marked intraoperative hemodilution (after the hematocrit is lowered by 50%). More modest hemodilution (e.g., removal of 2 units of blood at the beginning of surgery) is also beneficial according to some investigators, but the amount of red cells saved is small.

BLOOD SUBSTITUTES

The search for blood substitutes began when our early scientific ancestors tested alternatives to human blood, including animal blood and Hb solutions, milk, and wine. Further work revealed that human and bovine hemosylates caused renal dysfunction in human recipients. The suspected cause of this nephrotoxicity was the stromal lipid component of the RBC membrane. The logical next step in the search for the ideal substitute was the development of stroma-free Hb (Hb tetramer). Unfortunately, in 1978, Savitsky et al. demonstrated renal dysfunction, hypertension, and abdominal pain using stroma-free Hb in healthy volunteers. It was hypothesized that these adverse events were due to the instability of the Hb tetramer. Since then, efforts have been made to produce stabilized products with desirable oxygen off-loading characteristics and extended intravascular retention times.

Today, the United States blood supply is increasingly safe and has sufficient capacity to meet most patient needs. There is room for considerable improvement, however, in supply levels and risk reduction. The shrinking donor pool (owing to lack of willingness and/or ability) and the increasing transfusion requirements of an aging population may lead to a shortage of blood products. The continuous battle against emerging infectious diseases underscore the risk of a tainted

TABLE 112.6 The Ideal Red Cell Substitute

Delivers oxygen (and maybe enhances delivery)
Does not transmit disease
Does not have immunosuppressive effects
Available in abundant supply
Universally compatible (no need to type and crossmatch)
Prolonged shelf-life and stable at a range of temperatures
Similar in vivo half-life to the RBC
Available at a reasonable cost
Easy to administer
Able to access all areas of the human body (including ischemic tissue)
Effective on room air or ambient conditions

RBC, Red blood cell.

blood supply and depletion of transfusion resources. Theoretically, the ideal red cell substitute would solve both of these issues (Table 112.6). There have been many attempts to develop red cell substitutes in the past, but no product has been able to fulfill all of the presented criteria or meet the FDA requirements of purity, potency, and safety.

RED BLOOD CELL SUBSTITUTES

It is important to differentiate between "blood substitutes" and red cell substitutes. Red cell substitutes are oxygen carriers and do not replace all components and functions of blood, for example, coagulation factors and WBCs. Blood substitutes also have a short intravascular survival limiting their potential efficacy as a replacement for red cell transfusions. There remains one category of oxygen carriers that still shows promise as RBC substitutes: Hb-based oxygen carriers (HBOCs).

Hemoglobin-Based Oxygen Carriers

As RBCs age and degrade, the stroma and unmodified Hb released can lead to various adverse effects in the transfused recipients. Therefore, the HBOCs that were most recently in clinical development were stroma-free and engineered to produce desirable oxygen dissociation characteristics as well as an adequate in vivo half-life with minimal toxicities. Stroma-free Hb has a very high oxygen affinity compared with native Hb in a RBC because of a lack of 2,3-DPG. Furthermore, the Hb tetramer is such a small molecule that the kidney rapidly removes it.

Four different methods have been suggested to avoid toxicities: stabilization, polymerization, conjugation, and Hb vesicles. HBOCs can be prepared from different Hb sources (e.g., bovine RBCs or recombinant Hb harvested from bacteria/yeasts). However, the safety concerns of using Hb from nonhuman sources, such as the potential for transmitting diseases, immunogenicity, and toxicities have not been fully addressed. While one of the commercially available HBOCs (e.g., bovine-derived oxyglobin, HBOC-301) is approved for veterinary use, there is no HBOC product approved for clinical use although several products are in clinical development phases. A large meta-analysis by Natanson et al. in 2008 identified 16 randomized controlled trials in which adult patients received HBOCs therapeutically. The analysis reviewed the association between HBOCs and the risk of MI and mortality in clinical trials. The study included five different HBOCs in the analysis and reported a 30% increase in the risk of death and a threefold increase in the risk of MI when all HBOC trials were pooled. The meta-analysis has been criticized for including trials of varying HBOC products performed on heterogeneous patient populations in different settings with different controls. The authors of the meta-analysis criticized the United States government oversight, as well as the transparency and timeliness in reporting the results of the HBOC clinical trials. The editorial that accompanied the publication in the *Journal of the American Medical Association* made a recommendation that further phase III trials of HBOCs should not be conducted until the mechanisms and potential toxicities are better understood. The effects may be due to the interaction of Hb and nitric oxide (NO), a concern not fully appreciated in the early days of blood substitute research, but potentially addressed by the expansion of knowledge about NO and mitigating its effects. Although some HBOCs are still being investigated, there are no licensed products in the United States.

Potential Clinical Applications

RBC substitutes have many potential applications. One of the most compelling needs would be the use for rapid resuscitation during military and civilian traumas. In developing a specific plan to avoid the consequences of blood unavailability after national disasters involving large-scale injuries, blood substitutes could play a key role as an immediate treatment option. In addition to these acute emergency situations, there is a wide range of less urgent applications where oxygen carriers might provide a viable solution to difficult situations, including supporting solid organ perfusion during transport/storage, augmenting perioperative hemodilution, transfusion of patients who refuse human blood components because of religious beliefs (e.g., Jehovah's Witnesses), transfusion of patients with rare blood types or antibodies to high incidence antigens, transfusion in patients with autoimmune hemolytic anemia in whom it is difficult to detect underlying antibodies, and reducing alloimmunization risk in patients receiving frequent transfusions. Since blood transfusion therapy is not available to handle some of these indications, there remains an unmet medical need for a safe and efficacious RBC substitute.

Red Blood Cells Derived From Stem Cells

Due to the threat of future blood shortages, the possible shortening of RBC storage, and unlikely availability of RBC substitutes in clinical use, a new source of RBCs is needed. Blood substitutes will not answer this need in the near future. The generation of RBCs derived from stem cells could meet this need, and this method is currently under development by researchers worldwide.

Umbilical cord blood is an accessible source of stem cells with proliferative capacity; however, the proliferation is not infinite, requiring a system of production in batches. Neildez-Nguyen et al. first reported the possibility of converting umbilical cord stem cells into erythroid progenitors that can continue to mature into terminally differentiated RBCs in vivo. Giarratana et al. subsequently demonstrated that adult mobilized hematopoietic stem progenitor cells can also be differentiated into RBCs and found that the survival of the cultured RBCs was similar to that of native RBCs. Under optimized expansion and differentiation conditions, Giarratana et al. reported that they were able to generate 4 to 30 million RBCs from one hematopoietic stem progenitor cell obtained from apheresis donors. While this expansion scheme could be adequate to generate clinically useful transfusable RBCs, it may not justify the donor risks (e.g., G-CSF–mobilized apheresis collection) for the sole purpose of RBC production. Stem cells cultured from umbilical cords would necessitate the use of cord blood banks and would still be dependent on human donation. In addition, cord cells produce fetal Hb with a higher tendency to denature and to cause membrane damage compared with adult Hb. Alternatively, embryonic stem cells and induced pluripotent stem cells (iPSCs) can be obtained, generated, then maintained indefinitely in culture, thus providing an unlimited source of cells. Like embryonic stem cells, iPSCs are capable of in vitro self-renewal and differentiation into cell types from all germ layers. However, they possess an advantage over embryonic cells in that they pose no ethical dilemma and can be selected based on known phenotypes. Researchers have been able to demonstrate that iPSCs can differentiate into terminally mature and fully functional red cells. More recently, Heshusius et al. have optimized large-scale cultures of RBCs from peripheral blood mononuclear cells using a good manufacturing practice (GMP) grade culture medium. The cultures yield a 10^7-fold increase in erythroblasts in 25 days and can be differentiated to RBCs in 12 days with greater than 90% enucleation. Furthermore, the cultured RBCs synthesize adult Hb and the expected blood group antigens.

A number of challenges must be overcome before we have an unlimited supply of clinical grade RBCs. The ideal choice for the initial cell type (e.g., hematopoietic stem cell and erythroblast) remains to be determined. The method of reprogramming the cells must be established to avoid the risk of potential mutation and tumor development (although this is less of an issue for red cells as they are enucleate and can be safely irradiated) while still ensuring the functionality of the end product. Finally, the use of GMP conditions for mass production within a reasonable amount of time and at a reasonable cost seems to be the greatest hurdle to date. The current challenges in utilizing iPSCs to generate RBCs are the inefficiency in erythroid commitment and the suboptimal cellular amplification. A number of strategies are under development to solve this crucial problem. Until mass amplification is possible, the iPSCs could be developed, not as an unlimited supply, but as a source of cells expressing a rare blood group used to treat allo-immunized patients with rare blood types. Currently, the available methods for ex vivo manufacturing of RBCs for clinical use are expensive and unrealistic.

SUGGESTED READING

Ashworth A, Klein AA. Cell salvage as part of a blood conservation strategy in anaesthesia. *Br J Anaeth*. 2010;105(4):401–416.

Bell EF, Strauss RG, Widness JA, et al. Randomized trial of liberal versus restrictive guidelines for red blood cell transfusion in preterm infants. *Pediatrics*. 2005;115(6):1685–1691.

Chou ST, Jackson T, Vege S, et al. High prevalence of red blood cell alloimmunization in sickle cell disease despite transfusion from Rh-matched minority donors. *Blood*. 2013;122(6):1062–1071.

Chou ST, Alsawas M, Fasano RM, et al. American Society of Hematology 2020 guidelines for sickle cell disease: transfusion support. *Blood. Adv*. 2020;4(2):327–355.

D'Alessandro A, Culp-Hill R, Reisz JA, et al. Heterogeneity of blood processing and storage additives in different centers impacts stored red blood cell metabolism as much as storage time: lessons from REDS-III-Omics. *Transfusion*. 2019;59(1):89–100.

DeSimone RA, Hayden JA, Mazur CA, et al. Red blood cells donated by smokers: a pilot investigation of recipient transfusion outcomes. *Transfusion*. 2019;59(8):2537–2543.

DeSimone RA, Plimier C, Lee C, et al. Additive effects of blood donor smoking and gamma irradiation on outcome measures of red blood cell transfusion. *Transfusion*. 2020;60(6):1175–1182.

Dzik WH, Anderson JK, O'Neill EM, et al. A prospective, randomized clinical trial of universal WBC reduction. *Transfusion*. 2002;42(9): 1114–1122.

Fergusson DA, Hébert P, Hogan DL, et al. Effect of fresh red blood cell transfusions on clinical outcomes in premature, very low-birth-weight infants: the ARIPI randomized trial. *JAMA*. 2012;308(14):1443–1451.

Francis RO, D'Alessandro A, Eisenberger A, et al. Donor glucose-6-phosphate dehydrogenase deficiency decreases blood quality for transfusion. *J Clin Invest*. 2020;130(5):2270–2285.

Giarratana MC, Rouard H, Dumont A, et al. Proof of principle for transfusion of in vitro-generated red blood cells. *Blood*. 2011;118(19):5071–5079.

Heeger LE, Counsilman CE, Bekker V, et al. Restrictive guideline for red blood cell transfusions in preterm neonates: effect of a protocol change. *Vox Sang*. 2019;114(1):57–62.

Hebert PC, Wells G, Blajchman MA, et al. A multicenter, randomized, controlled clinical trial of transfusion requirements in critical care. Transfusion Requirements in Critical Care Investigators, Canadian Critical Care Trials Group. *N Engl J Med*. 1999;340(6):409–417.

Hendrickson JE, Tormey CA, Shaz BH. Red blood cell alloimmunization mitigation strategies. *Transfus Med Rev*. 2014;28(3):137–144.

Heshusius S, Heideveld E, Burter P, et al. Large-scale in vitro production of red blood cells from human peripheral blood mononuclear cells. *Blood Adv*. 2019;3(21):3337–3350.

Hod EA, Brittenham GM, Billote GB, et al. Transfusion of human volunteers with older, stored red blood cells produces extravascular hemolysis and circulating non-transferrin-bound iron. *Blood*. 2011;118(25):6675–6682.

Hod EA, Zhang N, Sokol SA, et al. Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation. *Blood*. 2010;115(21):4284–4292.

Holcomb JB, Tilley BC, Baraniuk S, et al. Transfusion of plasma, platelets, and red blood cells in a 1:1:1 vs a 1:1:2 ratio and mortality in patients with severe trauma: the PROPPR randomized clinical trial. *JAMA*. 2015;313(5):471–482.

Howarth C, Banerjee J, Aladangady N. Red blood cell transfusion in preterm infants: current evidence and controversies. *Neonataology*. 2018;114(1):7–16.

Jackman RP, Deng X, Bolgiano D, et al. Leukoreduction and UV treatment reduce both the magnitude and duration of the anti-HLA antibody response. *Transfusion*. 2015;54(3):672–680.

King KE, Ness PM. Treatment of autoimmune hemolytic anemia. *Semin Hematol*. 2005;42(3):131–136.

Kirpalasni H, Whyte RK, Andersen C, et al. The Premature Infants in Need of Transfusion (PINT) study: a randomized, controlled trial of a restrictive (low) versus liberal (high) transfusion threshold for extremely low birth weight infants. *J Pediatr*. 2006;149(3):301–307.

Klein HG. Transfusion-associated graft-versus-host disease: less fresh blood and more gray (Gy) for an aging population. *Transfusion*. 2006;46(6):878–880.

Klein HG, Anstee DJ. Mollison's blood transfusion. In: *Clinical Medicine*. 11th ed. Malden, MA: Blackwell Publishing; 2005:389.

Lee AC, Reduque LL, Luban NLC, et al. Transfusion-associated hyperkalemic cardiac arrest in pediatric patients receiving massive transfusion. *Transfusion*. 2014;54(1):244–254.

Mazurier C, Douay L, Lapillonne H. Red blood cells from induced pluripotent stem cells: hurdles and developments. *Curr Opin Hematol*. 2011;18(4): 249–253.

Murphy GJ, Pike A, Rogers CA, et al. Liberal or restrictive transfusion after cardiac surgery. *N Engl J Med*. 2015;372(11):997–1008.

Natanson C, Kern SJ, Lurie P, et al. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. *JAMA*. 2008;299(19):2304–2312.

Neildez-Nguyen TM, Wajcman H, Marden MC, et al. Human erythroid cells produced ex vivo at large scale differentiate into red blood cells in vivo. *Nat Biotechnol*. 2002;20(5):467–472.

Ness PM, Bourke DL, Walsh PC. A randomized trial of perioperative hemodilution versus transfusion of preoperatively deposited autologous blood in elective surgery. *Transfusion*. 1992;32(3):226–230.

Ness PM, Cushing MM. Oxygen therapeutics: pursuit of an alternative to the donor red blood cell. *Arch Pathol Lab Med*. 2007;131(5):734–741.

Paglino JC, Pomper GJ, Fisch GS, et al. Reduction of febrile but not allergic reactions to RBCs and platelets after conversion to universal prestorage leukoreduction. *Transfusion*. 2004;44(1):16–24.

Patel RM, Knezevic A, Shenvi N, et al. Association of red blood cell transfusion, anemia, and necrotizing enterocolitis in very low-birth-weight infants. *JAMA*. 2016;315(9):889–897.

Paul DA, Leef KH, Locke RG, et al. Transfusion volume in infants with very low birth weight: a randomized trial of 10 versus 20 mL/kg. *J Pediatr Hematol Oncol*. 2002;24(1):43–46.

Peyrard T, Bardiaux L, Krause C, et al. Banking of pluripotent adult stem cells as an unlimited source for red blood cell production: potential applications for alloimmunized patients and rare blood challenges. *Transfus Med Rev*. 2011;25(3):206–216.

Raphael J, Mazer CD, Subramani S, et al. Society of Cardiovascular Anesthesiologists Clinical Practice Improvement Advisory for management of perioperative bleeding and hemostasis in cardiac surgery patients. *Anesth Analg*. 2019;129(5):1209–1221.

Ryder AB, Zimring JC, Hendrickson JE. Factors influencing RBC alloimmunization: lessons learned from murine models. *Transfus Med Hemother*. 2014;41(6):406–419.

Sapiano MRP, Jones JM, Savinkina AA, et al. Supplemental findings of the 2017 national blood collection and utilization survey. *Transfusion*. 2020;60(Suppl 2):S17–S37.

Sehgal LR, Sebala LP, Takagi I, et al. Evaluation of oxygen extraction ratio as a physiologic transfusion trigger in coronary artery bypass graft surgery patients. *Transfusion*. 2001;41(5):591–595.

Sperry JL, Guyette FX, Brown JB, et al. Prehospital plasma during air medical transport in trauma patients at risk for hemorrhagic shock. *N Engl J Med*. 2018;379(4):315–326.

Steiner ME, Ness PM, Assmann SF, et al. Effects of red-cell storage duration on patients undergoing cardiac surgery. *N Engl J Med*. 2015;372(15):1419–1429.

Spinella PC, Tucci M, Fergusson DA, et al. Effect of fresh vs standard-issue red blood cell transfusions on multiple organ dysfunction syndrome in critically ill pediatric patients: a randomized clinical trial. *JAMA*. 2019;322(22):2179–2190.

Strauss RG, Mock DM, Johnson KJ, et al. A randomized clinical trial comparing immediate versus delayed clamping of the umbilical cord in preterm infants: short-term clinical and laboratory endpoints. *Transfusion*. 2008;48(4):658–665.

Tinmouth A, Fergusson D, Yee IC, et al. Clinical consequences of red cell storage in the critically ill. *Transfusion*. 2006;46(11):2014–2024.

TRAP Study Group. Leukocyte reduction and ultraviolet B irradiation of platelets to prevent alloimmunization and refractoriness to platelet transfusions. *N Engl J Med*. 1997;337(26):1861–1869.

Venkatesh V, Khan R, Curley A, et al. The safety and efficacy of red cell transfusions in neonates: a systematic review of randomized controlled trials. *Br J Haematol*. 2012;158(3):370–385.

Villanueva C, Colomo A, Bosch A, et al. Transfusion strategies for acute upper gastrointestinal bleeding. *N Engl J Med*. 2013;368(1):11–21.

Von Lindern JS, Brand A. The use of blood products in perinatal medicine. *Semin Fetal Neonatal Med*. 2008;13(4):272–281.

Wang D, Cortés-Puch I, Sun J, et al. Transfusion of older stored blood worsens outcomes in canines depending on the presence and severity of pneumonia. *Transfusion*. 2014;54(7):1712–1724.

Wong H, Connelly R, Day A, et al. A comparison of high and standard blood transfusion volumes in premature infants. *Acta Paediatr*. 2005;94(5):624–625.

Yazer MH, Cap AP, Spinella PC, et al. How do I implement a whole blood program for massively bleeding patients? *Transfusion*. 2018;58(3):622–628.

CHAPTER 113

CLINICAL CONSIDERATIONS IN PLATELET TRANSFUSION THERAPY

Richard M. Kaufman

PLATELET COLLECTION AND MANUFACTURING

Platelet components are either prepared from whole blood donations (platelet concentrates) or are collected by apheresis (single donor platelets). In the United States, whole blood–derived platelet concentrates are produced using the platelet-rich plasma (PRP) method. First, a whole blood unit is separated by gentle centrifugation (slow spin) into red blood cells (RBCs) and PRP. The PRP is then centrifuged a second time (hard spin) to isolate one platelet concentrate plus one unit of plasma. Each platelet concentrate contains approximately 5.5×10^{10} platelets suspended in a plasma volume of approximately 50 mL. In Europe and Canada, the alternative buffy coat method is used to produce platelet concentrates. To prepare an adult dose of platelets for transfusion, four to six platelet concentrates are pooled together.

Apheresis platelet units are collected from a single platelet donor by continuous flow centrifugation using an automated device. A high volume of whole blood is processed through the machine, and the platelets are retained in a sterile collection bag. According to AABB (formerly, the American Association of Blood Banks) standards, an apheresis platelet unit should contain a minimum of 3×10^{11} platelets, a dose that is approximately equivalent to five pooled platelet concentrates. Current devices allow many different combinations of blood products to be collected during a single apheresis donation, such as 1 unit of platelets plus 1 unit of RBCs. Apheresis platelet units generally contain less than 1×10^6 white blood cells (WBCs) per unit; thus they meet the current AABB definition for leukoreduced blood products ($<5 \times 10^6$ WBCs/unit). Although apheresis platelets cost more to produce than whole blood–derived platelet concentrates, they have become increasingly popular. In the United States in 2015, approximately 2 million therapeutic doses of platelets were provided as apheresis platelets; in contrast, only approximately 200,000 equivalent doses of whole blood–derived platelet concentrates were distributed.

Apheresis platelets provide limited advantages over whole blood–derived platelet concentrates. Radiolabeling studies indicate that apheresis platelets circulate longer in vivo than pooled concentrates, most likely reflecting gentler handling and less platelet activation during collection. Recipients of apheresis platelets are exposed to fewer donors per transfusion (one donor versus four to six as with a pool of platelet concentrates), so in principle, apheresis platelets should pose a lower risk of viral transmission than whole blood–derived platelets. However, given that the per-unit transfusion-transmission risks for human immunodeficiency virus (HIV) and hepatitis C virus have been reduced to less than 1 per 1,000,000, the viral safety advantage of apheresis platelets over whole blood–derived platelets is marginal. Data from surveillance culture studies suggest that apheresis platelets may be less likely than platelet concentrates to become contaminated with bacteria. At one time, it was predicted that apheresis platelets would be less likely than pooled concentrates to provoke platelet alloimmunization by virtue of exposing recipients to fewer unique donor human leukocyte antigens (HLAs). However, this hypothesis was not confirmed empirically. Although they express surface HLA class I antigens, platelets appear to be rather poor immunogens. HLA alloimmunization to platelets primarily appears to be triggered by contaminating WBCs within a platelet unit. Thus alloimmunization is not platelet dose dependent, and simply providing leukoreduced platelet units prevents most cases of immune-mediated platelet refractoriness. When platelet refractoriness does occur, it is often in multiparous women, who were initially sensitized to foreign (paternal) HLA antigens in previous pregnancies.

PROPHYLACTIC PLATELET TRANSFUSION

Most platelet units are transfused to prevent bleeding in nonbleeding patients, rather than to treat active bleeding. Before 1960, platelet transfusions were not widely available, and death from hemorrhage was common among patients with leukemia who received chemotherapy. In 1962, Gaydos and colleagues[1] published a seminal study demonstrating an inverse relationship between days with bleeding and platelet count. After this study was published, prophylactic platelet transfusion rapidly became standard practice. Notably, based on their data, no specific platelet transfusion trigger was suggested by the authors. Regardless, a platelet count of 20,000/μL was widely adopted at the time as the standard prophylactic platelet transfusion trigger.

Later studies suggested that a lower transfusion trigger would be as effective as a trigger of 20,000/L. For instance, Slichter and Harker[2] performed RBC radiolabeling studies in thrombocytopenic patients who were not receiving platelet transfusions. They demonstrated that only patients with platelet counts less than 5000/L had significantly elevated fecal RBC loss. Years later, several clinical studies directly challenged the 20,000/L trigger. Platelet transfusion triggers of 10,000/L versus 20,000/L were compared directly in three randomized prospective studies of patients with acute leukemia.[3–5] These studies, as well as a nonrandomized prospective trial,[6] did not show an increased incidence of bleeding when a trigger of 10,000/L is used. More recently, the Platelet Dosing (PLADO) trial demonstrated that the risk of bleeding among patients with hypoproliferative thrombocytopenia increased only at platelet counts less than 6000/μL.[7] As shown in Fig. 113.1, the risk of spontaneous bleeding was equivalent between platelet counts of 6000/μL and 80,000/μL.

Severe hemorrhage in the setting of therapy-related hypoproliferative thrombocytopenia is now quite rare. Given the substantial changes in both chemotherapy and the supportive care of patients with hematologic malignancy that have occurred over the past several decades, two randomized controlled trials examined whether routine platelet prophylaxis still provides a clinical benefit. In the study by Wandt and colleagues,[8] patients receiving chemotherapy or undergoing autologous hematopoietic stem cell transplantation (HSCT) were randomly assigned to receive platelet transfusions only when bleeding occurred, or standard prophylactic platelet transfusions for a morning platelet count at or less than 10,000/μL. Grade 2 or higher bleeding occurred in 42% of patients receiving therapeutic platelet transfusions only, versus 19% in patients receiving platelet prophylaxis ($P < .001$). There were significantly more intracerebral hemorrhages in the no-prophylaxis group (7% vs. 2%, $P = .01$), and there were two deaths due to bleeding in the no-prophylaxis group compared with zero in the prophylaxis group. In the Trial of Prophylactic Platelets,[9] a similar population of patients was randomized to no prophylactic platelet transfusions or routine prophylaxis. Grade 2 or higher bleeding occurred in 50% of patients in the no-prophylaxis group versus 43% in the prophylaxis group ($P = .06$ for noninferiority). In this study, there were no deaths due to bleeding. In both trials, bleeding occurred more frequently among patients being treated for acute leukemia versus autologous stem cell transplant recipients. Overall,

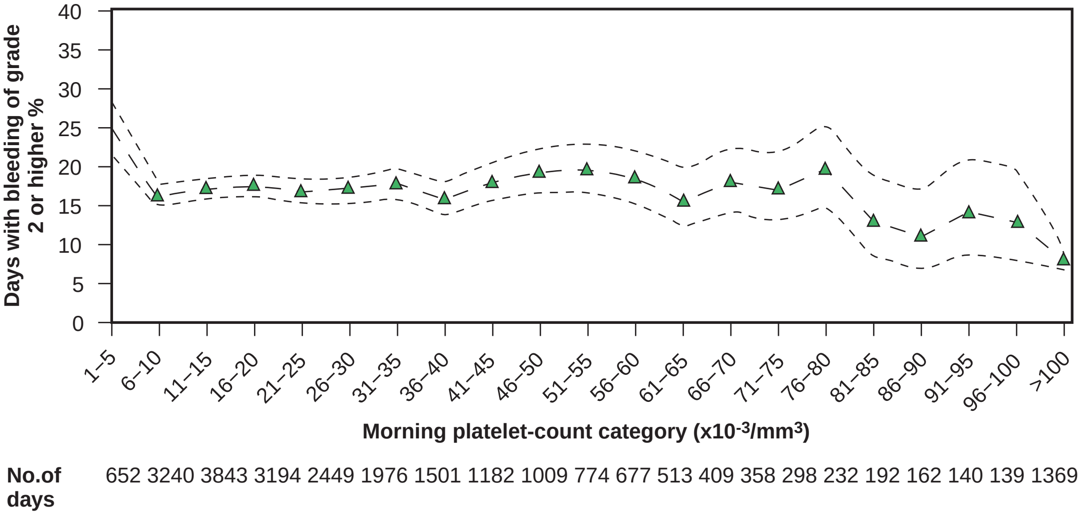


Figure 113.1 RELATIONSHIP BETWEEN PLATELET COUNT AND SPONTANEOUS BLEEDING AMONG PATIENTS WITH THERAPY-INDUCED HYPOPROLIFERATIVE THROMBOCYTOPENIA. The percentage of days that adult or pediatric patients with hematologic malignancy enrolled in the Platelet Dosing (PLADO) trial had grade 2 or higher bleeding is shown as a function of morning platelet count. The 95% confidence intervals are shown as dashed lines. The bleeding risk was equivalent for platelet counts between 6000 and 80,000/μL.[12]

the results of these trials support the continued use of prophylactic platelet transfusions for patients with hematologic malignancy and therapy-induced hypoproliferative thrombocytopenia.[10] However, using a no-prophylaxis strategy specifically for adult autologous stem cell transplant recipients has been endorsed by the American Society of Clinical Oncology (ASCO).[11]

Prophylactic platelet transfusions are mainly given in the setting of oncology, but they are also used to prevent bleeding in thrombocytopenic neonates. Notably, a multicenter randomized controlled trial of 660 preterm infants found that using a high platelet count threshold for administering prophylactic platelet transfusions (50,000/μL) was associated with significantly higher mortality or major bleeding events than using a low threshold (25,000/μL).[12]

Prophylactic Platelet Dosing

There are currently thought to be two distinct clearance mechanisms for platelets. Most platelets undergo senescence after circulating in the peripheral blood for 8 to 10 days. But there is also evidence for a second clearance route in which there is a fixed daily loss of platelets that occurs independent of platelet age. Platelets exiting the circulation via this second route are postulated to function in maintaining vascular integrity. In principle, a low dose of platelets could be used to meet this daily requirement. This hypothesis was tested by the 2010 PLADO trial. A total of 1272 patients receiving HSC transplantation or chemotherapy were randomly assigned to receive low-, medium-, or high-dose platelets for a morning count of 10,000/μL or lower. The risk of spontaneous bleeding was not increased until patient platelet counts decreased to 5000/μL or lower. No differences were observed in bleeding rates among the three treatment groups, supporting the concept that few platelets are required to maintain hemostasis. Although significantly fewer total platelets were transfused to patients in the low-dose group, platelet transfusions were required more frequently.[7]

Prophylactic Platelets for Invasive Bedside Procedures

To date, there have been no large randomized controlled trials evaluating the need for platelet prophylaxis before invasive bedside procedures such as lumbar puncture. However, retrospective data provide reassurance that moderate thrombocytopenia does not pose a serious risk for performing such procedures. Howard et al.[13] reviewed the records of 956 consecutive pediatric patients with newly diagnosed acute lymphoblastic leukemia who underwent lumbar puncture. No serious hemorrhagic complications were observed after 5223 lumbar punctures, including 170 procedures that were done when the platelet count was 11,000 to 20,000/μL. The authors concluded that prophylactic platelet transfusion was unnecessary in patients with platelet counts greater than 10,000/μL. Lumbar punctures were performed in only 29 patients with platelet counts of 10,000/μL or less, making it difficult to assess the risk of bleeding in patients with very low platelet counts. A similar, albeit much smaller, retrospective study examined the same issue in adult patients with acute leukemia.[14] No hemorrhagic complications were observed after 195 lumbar punctures, including 35 that were done with platelet counts of 20,000 to 30,000/μL. With very limited published data available on severely thrombocytopenic adults, AABB has suggested 50,000/μL as a minimum safe platelet count for lumbar puncture in adult patients. For central venous catheter placements, AABB has suggested 20,000/μL as a minimum safe platelet count, based on multiple observational studies.[10] Relatively few studies have been published to date addressing the question of a safety minimum platelet count for other types of bedside procedures (thoracentesis, paracentesis, etc.)

Prophylactic Platelets for Major Surgical Procedures

There are currently no data from randomized trials addressing the question of what constitutes an adequate platelet count before surgery. However, retrospective studies suggest that patients with platelet counts of 50,000/μL or higher are not at excess bleeding risk during surgery. Bishop and colleagues[15] reported a series of 95 patients with acute leukemia who underwent 130 surgical procedures with platelet counts of less than 50,000/μL. Intraoperative blood loss exceeded 500 mL in only 7% of cases. No relationship was seen between the preoperative platelet count and surgical blood loss. These data suggest that prophylactic platelet transfusions need not be administered before surgery when the preoperative platelet count is at least 50,000/μL. This rule of thumb is thought to apply to most types of surgery (cardiac, orthopedic, and so on). However, for a few types of surgeries, requiring a higher platelet count (70,000 to 100,000/μL) is traditional, although no published data currently exist either to support or refute this approach. These settings include neurosurgery, retinal surgeries, and other procedures in which the risk is not that the patient may exsanguinate but rather that even a minor bleed might cause clinically significant damage in a vulnerable vital structure such as the brain.

Platelets to Treat Bleeding

High-quality data to guide platelet use in the setting of active bleeding are lacking. A small randomized controlled trial in patients on antiplatelet medication who suffered intracerebral hemorrhage found worse outcomes among patients transfused with platelets compared with patients receiving standard care only.[16]

ADVERSE EFFECTS OF PLATELET TRANSFUSION

Infectious Risks

Platelets are associated with essentially the same range of infectious pathogens as other blood components, but septic transfusion reactions caused by bacterially contaminated units comprise a unique risk of platelet transfusion. Over time, improvements in donor screening virtually eliminated the risk of transfusion transmission of hepatitis B virus, hepatitis C virus, and HIV. The risk of septic transfusion reactions remained fairly constant over this same period, so eventually, platelet bacterial contamination became, by default, the most frequent infectious risk of transfusion. Unlike other blood components, which are stored either refrigerated or frozen, platelets are stored at room temperature. The reason is that if platelets are refrigerated before transfusion, they are cleared rapidly from the recipient's circulation.[17] Although room temperature storage allows transfused platelets to circulate in vivo, it has the downside of promoting bacterial growth. Because of this risk, platelet storage is limited to only 5 to 7 days, making platelet inventory management extremely challenging. It has been observed that platelets stored under refrigeration (4°C) demonstrate better hemostatic function than room temperature platelets in most in vitro assays. Future clinical trials will determine whether administering refrigerated platelets leads to improved outcomes among patients with acute bleeding. Other alternatives to standard platelets (e.g., frozen platelets, artificial platelets) are also being explored.

In the 1990s, numerous studies demonstrated that contaminating bacteria, usually representing skin flora from the donor, could be cultured out of approximately 1 of 3000 platelet units. Clinically apparent septic transfusion reactions were thought to occur after approximately 1 of 25,000 platelet transfusions, although there is considerable uncertainty around this point estimate. In response to the issue, the US Food and Drug Administration (FDA) in 2019 issued a final guidance requiring enhanced bacterial detection/prevention for platelet units. A common approach is to use automated culture systems (BacT/ALERT, Biomerieux) to detect bacterial contamination before platelets are released for clinical use. Rapid, point-of-issue tests that can be used to test the sterility of a platelet unit just before issue have also been developed but are not widely used due to issues of logistics and cost. Pathogen reduction systems represent the strongest approach to the problem of platelet bacterial contamination. These systems use photoactivated reagents such as amotosalen or riboflavin plus ultraviolet (UV) light to provide up to 6 logs of killing of spiked virus or bacteria within 1 unit of platelets. Such systems have been used in Europe and elsewhere, and in 2015, a pathogen reduction system for platelets and plasma was licensed for use in the United States. These technologies are highly effective for ensuring the sterility of platelet units. Platelet increments are a bit lower when pathogen-reduced platelets are administered, but clinical outcomes in patients receiving platelet prophylaxis are similar.

Allergic and Febrile Nonhemolytic Transfusion Reactions

The typical RBC unit contains approximately 20 mL of plasma. Platelet units contain far more plasma, approximately 200 mL on average. There are multiple potential adverse effects associated with this large plasma content. Indeed, in the PLADO trial, higher platelet doses were associated with significantly increased risk of transfusion-related adverse events.[18] Febrile nonhemolytic reactions, defined as an increase in temperature of more than 1°C, are the most common reactions seen after platelet transfusion. In a classic study, Heddle and colleagues[19] demonstrated that the plasma component of platelet units, rather than the cellular component, causes most reactions. Cytokines that accumulate during product storage have been implicated. Allergic transfusion reactions are the second most-common type of adverse event associated with platelet transfusion.[18] Allergic reactions to blood products occur when the recipient has a preexisting allergy to a plasma protein component. Allergic reactions range from mild, uncomplicated urticarial reactions (most common) to full-blown anaphylaxis. Platelet additive solution (PAS) platelet units are those in which a preservative solution replaces most of the residual plasma. Using PAS platelets has been shown to reduce the risk of allergic reactions.[20]

ABO and Hemolytic Reactions to Platelets

Whenever possible, platelet units are assigned so as to match the donor plasma with recipient RBC type. For example, a type A patient would ordinarily receive type A or AB platelets, which do not contain anti-A antibody. However, when platelet inventories are constrained, it is common for blood banks to issue ABO-incompatible platelets. For example, a type A patient might receive type O platelets, containing anti-A. The passive transfusion of donor anti-A or anti-B to a patient usually does not cause adverse sequelae. Rarely, however, hemolysis may be observed. Most often, this occurs with units from type O donors, who occasionally have high titer anti-A, anti-B, or anti-A,B antibody. In a typical type O adult, the titer of circulating anti-A is on the order of 128 to 256. However, some donors have anti-A titers of 10,000 or higher. Recipients of products from donors with high-titer anti-A (and less frequently, anti-B) rarely do have clinically apparent hemolysis, and a small number of fatalities have been reported. This is considered to be a low-risk event, but it may be on the rise in the United States because of the increased number of single-donor apheresis platelet units used. One strategy to deal with this issue is to measure the anti-A/B titer on all platelet donors; components exceeding a threshold titer are assigned to type-specific recipients. An alternative strategy is to reduce the load of plasma in a platelet unit before transfusing the unit to a non–ABO-identical recipient, either by washing or concentrating the unit, or by using a PAS unit (earlier.)

Transfusion-Related Acute Lung Injury

Transfusion-related acute lung injury (TRALI) is an acute respiratory distress syndrome associated with the transfusion of any plasma-containing blood component, including platelets.[21] Most if not all cases of TRALI appear to be precipitated by the passive transfusion of donor anti-HLA or (less commonly) antineutrophil antibody. It is believed that products containing higher volumes of plasma such as fresh-frozen plasma (FFP) and apheresis platelets carry a higher risk of TRALI. Approximately one-third of female blood donors have circulating anti-HLA antibody because of prior sensitization during pregnancy. To help mitigate against the risk of TRALI, many countries, including the United States, now produce FFP from the blood of male donors only. Although this strategy has been relatively easy to apply to FFP production, female platelet donors are still needed to ensure an adequate platelet supply. A variety of strategies are being implemented to screen female platelet donors for anti-HLA antibody and to defer women who are antibody-positive.

D Sensitization

The Rh(D) antigen is the most immunogenic RBC protein antigen. More than 80% of individuals who are D-negative will form anti-D after a single D-positive RBC transfusion. Anti-D is associated with both hemolytic transfusion reactions and hemolytic disease of the fetus and newborn. For this reason, it is standard practice to provide D-negative individuals exclusively with D-negative RBC units. Platelet units contain a small number of contaminating RBCs. In the case of whole blood-derived platelet concentrates, approximately 0.3 to 0.5 mL RBCs may be present. In contrast, current apheresis platelets contain far fewer RBCs (≈0.0002 to 0.0007 mL per unit). When possible, D-negative platelet units are given to D-negative recipients. However, inventory constraints often force blood banks to issue D-positive platelet products to D-negative recipients. Transfusion of such units is associated with a low but nonzero risk of sensitization and formation of anti-D. D sensitization can be prevented by administering Rh immune globulin (RhIG), as is done to prevent fetomaternal Rh sensitization in D-negative mothers of D-positive children. The risk of sensitization is very low among D-negative immunocompromised patients transfused with D-positive platelets (e.g., HSCT patients).[22] In this setting, the value of RhIG prophylaxis appears to be low.

PLATELET REFRACTORINESS

Causes of Refractoriness to Platelet Transfusion

Platelet refractoriness is defined as an inappropriately low platelet increment after repeated platelet transfusions. It can be caused by nonimmune or immune factors (Table 113.1). The most commonly reported nonimmune causes of platelet refractoriness include fever, sepsis, bleeding, splenomegaly, and disseminated intravascular coagulation. In a small subset of cases, platelet refractoriness is immune-mediated. Platelets express HLA class I antigens, ABO antigens, and several platelet-specific antigens. Any of these molecules may potentially serve as an immune stimulus in a transfusion recipient. Antibodies directed against HLA molecules are responsible for most cases of immune-mediated platelet refractoriness, whereas antibodies to the human platelet antigens (anti-HPA) are less frequently implicated.

Diagnosis of Platelet Refractoriness

Because fewer than half of all platelet-refractory patients have demonstrable anti-HLA or antiplatelet antibodies, evaluation of both an immediate response to platelet transfusion and an 18- to 24-hour posttransfusion platelet survival is needed to help establish the cause of platelet refractoriness. Platelet counts obtained from 10 minutes to 1 hour after transfusion that repeatedly fail to demonstrate a corrected count increment of more than 5000/μL usually indicate immune-mediated platelet refractoriness. If the 10-minute to 1-hour posttransfusion platelet count shows a reasonable increment but the platelet count decreases to baseline by 18 to 24 hours, a nonimmune mechanism of refractoriness may be presumed (see Table 113.1). In cases of suspected immune-mediated refractoriness, HLA antibody screening (panel reactive antibody [PRA]) provides valuable supporting evidence that allosensitization has occurred. A patient with a PRA greater than 70% may be considered to be “severely immunized” and a good candidate for HLA-matched platelets (later).

TABLE 113.1 Causes of Refractoriness to Platelet Transfusion

Nonimmune	
Fever	
Sepsis	
Drug-associated	
Active bleeding	
Splenomegaly	
Disseminated intravascular coagulation	
Venoocclusive disease	
Immune	
Anti-HLA antibodies	
Anti-HPA antibodies	
ABO mismatch	
Drug-dependent antibodies	

HLA, Human leukocyte antigen; *HPA*, human platelet antigen.

Detection of Anti-Human Leukocyte Antigen Antibodies

Most commonly, HLA antibodies are identified using flow cytometric detection of antibody binding to beads coated with purified HLA antigens. The flow cytometry-based methodology has significantly improved sensitivity over lymphocytotoxicity assays (LCA), and, similar to solid-phase assays, it can detect both complement-fixing and non–complement-fixing antibodies.

Detection of Antiplatelet Antibodies

The most commonly used methods for detection of anti-HPA antibodies are solid-phase assays using purified platelet antigens for detection of antibody specificity. However, testing for anti-HPA antibodies is not typically performed in the work-up of platelet-refractory patients, mainly because the importance of these antibodies in causing clinical refractoriness is not well established.

Prevention of Alloimmunization

Although they express HLA class I antigens, platelets themselves are fairly weak immunogens. It has been shown that contaminating leukocytes in platelet products are primarily responsible for stimulating HLA antibody formation in platelet transfusion recipients. Thus removing WBCs from blood products (leukoreduction) is an essential means of preventing alloimmunization and subsequent platelet refractoriness. The definitive study showing this was the Trial to Reduce Alloimmunization to Platelets (TRAP)[23] which compared alloimmunization rates in 530 newly diagnosed patients with acute myeloid leukemia randomized to receive unmodified, pooled platelet concentrates (control); filtered, pooled platelet concentrates (F-PCs); filtered, single-donor apheresis platelets (F-APs); or UV-B-irradiated pooled platelet concentrates (UVB-PCs). Anti-HLA antibodies were detected in 45% of control participants compared with 17% to 21% of patients receiving modified platelets. A total of 13% of control group patients became platelet refractory versus only 3% in the F-PC group, 4% in the F-AP group, and 5% in the UVB-PC group.

Management of Platelet-Refractory Patients

When platelet refractoriness has been demonstrated, several strategies may facilitate achieving therapeutic platelet increments in vivo (Table 113.2). A trial of ABO-matched, fresh (1 to 2 days old) platelets may be helpful. In cases of immune-mediated refractoriness, a trial of HLA-matched platelets, antigen-negative platelets, or crossmatched platelets should be considered. The basic principles for selection of HLA-matched platelets are outlined in Table 113.3. In most cases, alloimmune refractory patients will show some degree of response to HLA-matched platelets. Because of the high degree of polymorphism of the HLA loci, it is often not possible to find perfect HLA-A and HLA-B locus matches, leading to the use of platelets mismatched at one or more loci (Table 113.4). In general, transfusion of grade A- or BU-matched platelets can result in an increase in platelet count that is superior to platelet increment obtained using either crossmatched

TABLE 113.2 Management of the Platelet-Refractory Patient

Obtain 10-min to 1-h posttransfusion platelet count on two occasions

If there is an appropriate increment in platelet count after a single platelet transfusion, continue to transfuse random platelets and treat nonimmune factors associated with decreased platelet survival such as sepsis and DIC, discontinue offending drugs, and so on

If the 10-min to 1-h posttransfusion platelet counts demonstrate no platelet increment (or only a marginal increment), obtain the patient's HLA type and screen the patient for the presence of anti-HLA antibodies (panel reactive antibody test)

While awaiting HLA antigen typing and antibody screening results, consider transfusion of "fresh" (<3 days old) ABO-matched platelets

If PRA shows less than 20% reactivity, continue with "fresh" ABO-matched random platelets

If PRA shows more than 20% reactivity, obtain anti-HLA antibody specificity. Use the recipient's HLA typing information to locate grade A– or grade B–matched platelet units for transfusion. Avoid transfusion of platelets containing antigens against which the recipient has antibodies. Consider recruiting recipient's family for platelet donation

Transfusion of crossmatch-compatible platelets is an option if there is a poor response to grade A– or grade B–matched platelets or if the recipient has antibodies to platelet-specific antigens

All HLA-matched or crossmatched platelet units must be irradiated before transfusion to prevent transfusion-associated GVHD

DIC, Disseminated intravascular coagulation; *GVHD*, graft-versus-host disease; HLA, human leukocyte antigen; *PRA*, panel reactive antibody.

TABLE 113.3 Traditional Platelet Selection Guidelines for Refractoriness Caused by Alloimmunization

ABO antigens are expressed on platelets. Consider transfusion of ABO-matched platelets while awaiting the recipient's HLA type and antibody specificity

Platelet matching for the recipient's HLA-A and HLA-B antigens is important

Platelet matching for the recipient's HLA-C antigens is not essential

Determine the antigen specificity of recipient's antiplatelet antibodies and try to transfuse antigen negative platelets

Some HLA antigens may be weakly expressed on platelets. Consider giving platelets mismatched for those antigens (e.g., HLA-B12 and its splits B44 and B45)

If HLA-matched platelets are unavailable, consider transfusion of platelets mismatched for HLA antigens that are serologically cross-reactive with the recipient's HLA antigens

If platelets mismatched for serologically cross-reactive antigens are not effective, matching for HLA-associated antigen systems such as Bw4/Bw6 may be helpful

HLA, Human leukocyte antigen.

platelets or platelets with different degrees of HLA mismatching (BX, C, or D). An additional step that may help in finding compatible platelets is flow cytometric detection of anti-HLA antibody specificity using single HLA antigen-coated beads. This information can be used to find donors who may be HLA-mismatched with the recipient but whose platelets lack the antigens to which the patient has specific antibodies. Furthermore, family members may be considered as platelet donors in addition to the available pool of HLA-matched volunteer donors. As an alternative to HLA antigen matching, patients may benefit from receiving donor platelets that are crossmatch-compatible with the patient's serum. The major benefit of crossmatching is a potentially larger pool of donors who would have been excluded by strict HLA antigen matching. In addition, platelet crossmatching may be helpful in cases of refractoriness caused by antibodies directed against platelet-specific antigens.[24]

The most challenging cases are the rare instances involving immune-refractory patients who are actively bleeding and HLA-matched platelets or crossmatched platelets are either ineffective or unavailable. In such cases, patients are typically transfused with repeated doses of random HLA-incompatible platelets during hemorrhagic episodes. The efficacy of this practice is unclear.

TABLE 113.4 Grades of Human Leukocyte Antigen–Matched Platelets

Match Grade	Description
A	4-antigen match (donor and recipient match at both HLA-A and HLA-B loci)
B1U	1 antigen unknown or blank (e.g., donor is: A2, –; B5, 27)
B1X	1 cross-reactive group[a]
B2UX	1 antigen blank and 1 cross-reactive[a]
C	1 mismatched antigen present
D	≥2 mismatched antigens present
R	Random

[a] The clusters of human leukocyte antigen (HLA) that share antigenic epitopes can be classified into cross-reactive antigen groups. Antibodies recognizing one HLA molecule within the group cross-react with other members of the same group.

Adapted from Brecher ME, ed. *Technical Manual*. 15th ed. Bethesda, MD: American Association of Blood Banks; 2005.

REFERENCES

1. Gaydos LA, Freireich EJ, Mantel N. The quantitative relation between platelet count and hemorrhage in patients with acute leukemia. *N Engl J Med*. 1962;266(18):905–909.
2. Slichter SJ, Harker L. Thrombocytopenia: mechanisms and management of defects in platelet production. *Clin Haematol*. 1978;7:523.
3. Heckman K, Weiner G, Davis C, et al. Randomized study of prophylactic platelet transfusion threshold during induction therapy for adult acute leukemia: 10,000/microL versus 20,000/microL. *J Clin Oncol*. 1997;15(3):1143–1149.
4. Rebulla P, Finazzi G, Marangoni F, et al. The threshold for prophylactic platelet transfusions in adults with acute myeloid leukemia. Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto. *N Engl J Med*. 1997;337(26):1870–1875.
5. Zumberg MS, del Rosario MU, Nejame CF, et al. A prospective randomized trial of prophylactic platelet transfusion and bleeding incidence in hematopoietic stem cell transplant recipients: 10,000/L versus 20,000/microLtrigger. *Biol Blood Marrow Transplant*. 2002;8(10):569–576.
6. Gmur J, Burger J, Schanz U, et al. Safety of stringent prophylactic platelet transfusion policy for patients with acute leukaemia. *Lancet*. 1991;338(8777):1223–1226.
7. Slichter SJ, Kaufman RM, Assmann SF, et al. Dose of prophylactic platelet transfusions and prevention of hemorrhage. *N Engl J Med*. 2010;362(7):600–613.
8. Wandt H, Schaefer-Eckart K, Wendelin K, et al. Therapeutic platelet transfusion versus routine prophylactic transfusion in patients with haematological malignancies: an open-label, multicentre, randomised study. *Lancet*. 2012;380(9850):1309–1316.
9. Stanworth SJ, Estcourt LJ, Powter G, et al. A no-prophylaxis platelet-transfusion strategy for hematologic cancers. *N Engl J Med*. 2013;368(19):1771–1780.
10. Kaufman RM, Djulbegovic B, Gernsheimer T, et al. Platelet Transfusion: a clinical practice guideline from the AABB. *Ann Intern Med*. 2015;162(3):205–213.
11. Schiffer CA, Bohlke K, Delaney M, et al. Platelet transfusion for patients with cancer: clinical practice guidelines of the American Society of Clinical Oncology. *J Clin Oncol*. 2018;36(3):283–301.

12. Curley A, Stanworth SJ, Willoughby K, et al. Randomized trial of platelet-transfusion thresholds in neonates. *N Engl J Med*. 2019;380(3):242–251.
13. Howard S, Gajjar A, Ribeiro R, et al. Safety of lumbar puncture for children with acute lymphoblastic leukemia and thrombocytopenia. *JAMA*. 2000;284(17):2222–2224.
14. Vavricka SR, Walter RB, Irani S, et al. Safety of lumbar puncture for adults with acute leukemia and restrictive prophylactic platelet transfusion. *Ann Hematol*. 2003;82(9):570–573.
15. Bishop J, Schiffer C, Aisner J, et al. Surgery in acute leukemia: a review of 167 operations in thrombocytopenic patients. *Am J Hematol*. 1987;26(2):147–155.
16. Baharoglu MI, Cordonnier C, Al-Shahi Salman R, et al. Platelet transfusion versus standard care after acute stroke due to spontaneous cerebral haemorrhage associated with antiplatelet therapy (PATCH): a randomised, open-label, phase 3 trial. *Lancet*. 2016;387(10038):1–9.
17. Murphy S, Gardner F. Effect of storage temperature on maintenance of platelet viability--deleterious effect of refrigerated storage. *N Engl J Med*. 1969;280(20):1094–1098.
18. Kaufman RM, Assmann SF, Triulzi DJ, et al. Transfusion-related adverse events in the Platelet Dose study. *Transfusion*. 2014;55(1):144–153.
19. Heddle N, Klama L, Singer J, et al. The role of the plasma from platelet concentrates in transfusion reactions. *N Engl J Med*. 1994;331(10):625–628.
20. Tobian AA, Fuller AK, Uglik K, et al. The impact of platelet additive solution apheresis platelets on allergic transfusion reactions and corrected count increment (CME). *Transfusion*. 2013;54(6):1523–1529.
21. Toy P, Gajic O, Bacchetti P, et al. Transfusion-related acute lung injury: incidence and risk factors. *Blood*. 2012;119(7):1757–1767.
22. Cid J, Lozano M, Fernández-Avilés F, et al. Anti-D alloimmunization after D-mismatched allogeneic hematopoietic stem cell transplantation in patients with hematologic diseases. *Transfusion*. 2006;46(2):169–173.
23. Slichter SJ. Leukocyte reduction and ultraviolet B irradiation of platelets to prevent alloimmunization and refractoriness to platelet transfusions. The Trial to Reduce Alloimmunization to Platelets Study Group. *N Engl J Med*. 1997;337(26):1861–1869.
24. Vassallo RR, Fung M, Rebulla P, et al. Utility of cross-matched platelet transfusions in patients with hypoproliferative thrombocytopenia: a systematic review. *Transfusion*. 2013;54(4):1180–1191.

CHAPTER 114

HUMAN LEUKOCYTE ANTIGEN AND HUMAN NEUTROPHIL ANTIGEN SYSTEMS

Ena Wang, Sharon Adams, David F. Stroncek, and Francesco M. Marincola

This chapter reviews human leukocyte antigen (HLA) and human neutrophil antigen (HNA) systems. A general background of the structure, function, and nomenclature of both systems and their relevance in clinical hematology is presented. Analysis of HLA gene products is applied in clinical settings (1) to select compatible donor-recipient pairs for transplantation, (2) to select HLA-compatible single-donor platelet products for thrombocytopenic patients refractory to standard transfusion of random pooled platelets, (3) to screen for genetic factors that may contribute to the prevalence of diseases, and (4) for forensic purposes in which the identity of individuals may contribute to solving legal disputes or criminal investigations. In addition, we discuss new applications that have broadened the relevance of HLA in the area of immune pathology. HLA phenotypes determine the suitability of patients for epitope-specific immunization. Tetrameric HLA/epitope complexes (tHLA) allow enumeration of antigen-specific T-cell responses. Furthermore, molecular identification of T-cell epitopes associated with distinct diseases and characterization of the communication between immune effector cells through HLA-HLA ligand interactions extend the relevance of HLA to biologic fields. These biologic fields encompass natural killer (NK) and cytotoxic T-cell function, antigen recognition in the context of infection, autoimmunity, the graft-versus-neoplasia (GVN) effect, and autologous cancer rejection. Finally, the recognition that polymorphism extends to other protein families relevant to immune pathology including cytokines, their receptors, and killer cell–inhibitory receptors has broadened the significance of immunogenetics beyond HLA. Thus, this chapter emphasizes the importance of viewing human pathologic conditions through the kaleidoscopic complexity of human polymorphism.

THE GENETICS, STRUCTURE, AND FUNCTION OF HUMAN LEUKOCYTE ANTIGEN MOLECULES

HLAs comprise a family of genes clustered in the short arm of chromosome 6 as the human version of the major histocompatibility complex (MHC), initially identified in mice as responsible for graft rejection between genetically unrelated strains (transplantation antigens).[1] Credit for the description of the human MHC goes to three individuals. In 1952, Jean Dausset observed that the serum of individuals who had received several transfusions contained hemagglutinins specific for donors' leukocytes. In 1958, Rose Payne noted that the only requirement for the development of hemagglutinins against leukocytes was a history of previous transfusion or pregnancy and concluded that these antibodies were directed against antigens on the surface of circulating leukocytes. This conclusion was concomitantly and independently confirmed by Jon van Rood, who observed that multiple pregnancies immunize mothers against leukocytes leaked from the fetus into the mother's circulation. Based on these discoveries, the term *human leukocyte antigen* was subsequently adopted.[2] It should be clarified, however, that this historical name is misleading. HLA molecule expression is not limited to leukocytes nor do they display, in natural conditions, antigenic behavior. In fact, several are expressed by most somatic cells, and, rather than being antigens, they chaperone protein bioproducts to the cell surface for recognition by T cells. There is, however, some substance to the name, because HLAs, by virtue of being densely packed on the cell surface, are exposed to recognition in a foreign environment, such as allotransplantation or xenoinfusion performed to induce anti-HLA antibodies as diagnostic reagents.

ORGANIZATION OF THE HUMAN LEUKOCYTE ANTIGEN GENES

HLA genes constitute a string of coding sequences that regulate the expression of molecules with similar but not identical function. Residing in a region that spans approximately 4000 kilobases of the short arm of chromosome 6 (Fig. 114.1),[3] HLA contains several genes and pseudogenes characterized by sequence homology and functional similarity. Of them, 47 are officially recognized by the World Health Organization (WHO) Nomenclature Committee[4] and include classic HLA class I and class II genes associated with antigen processing, such as PSMB8 and PSMB9 (proteasomal units) or TAP1 and TAP2 associated with peptide transport. Both HLA and HLA-associated genes can be physically grouped into three subregions according to chromosomal location. In the centromeric to telomeric direction, the first is HLA class II region comprising the α-and β-chains of HLA-DR, -DQ, -DP, -DM, and -DO as well as TAP and PSMB. Sandwiched between the class II and class I region, the class III region encodes for functionally unrelated genes such as complement components, heat shock proteins, and the tumor necrosis factor. The reason for their genetic link to the HLA complex is unknown, but their immunologic function seems more than coincidental. The class I region is most telomeric and includes HLA-A, -B, and -C loci; the nonclassic HLA-E, -F, and -G loci; and several pseudogenes.

General terminology separates HLA genes into classic and nonclassic. Classic HLA genes have been well characterized and are clearly associated with the presentation of antigens to immune cells. They are further subdivided into class I (HLA-A, -B, -C) and class II (HLA-DR, -DQ, and -DP). In general, HLA class I and II genes have very similar structure and function.[5,6] They contain six to eight exons coding for functionally distinct domains (Fig. 114.2). The first exon encodes a leader sequence; the following exons (exons 2 to 4) are highly polymorphic and encode extracellular domains responsible for peptide binding and T cell–antigen receptor (TCR) engagement. Because they are exposed on the cell surface, these domains are also responsible for alloreactivity. The last exons encode a conserved transmembrane and small intracellular domains whose function is unclear.

Only the heavy chain of HLA class I is encoded in the MHC region. Genes encoding HLA-A, -B, and-C contain three exons coding for α_1, α_2, and α_3 extracytoplasmic domains, one transmembrane, and three cytoplasmic domains (Fig. 114.3). The associated class I light chain, β_2-microglobulin, is encoded on chromosome 15.[7] By contrast, the HLA class II molecule is composed of a heterodimer of an α-chain and a β-chain encoded in the MHC region. Although the genetics are different, the protein product is structurally similar to HLA class I, with two helices resulting in the antigen-presenting part of the molecule (Fig. 114.4).

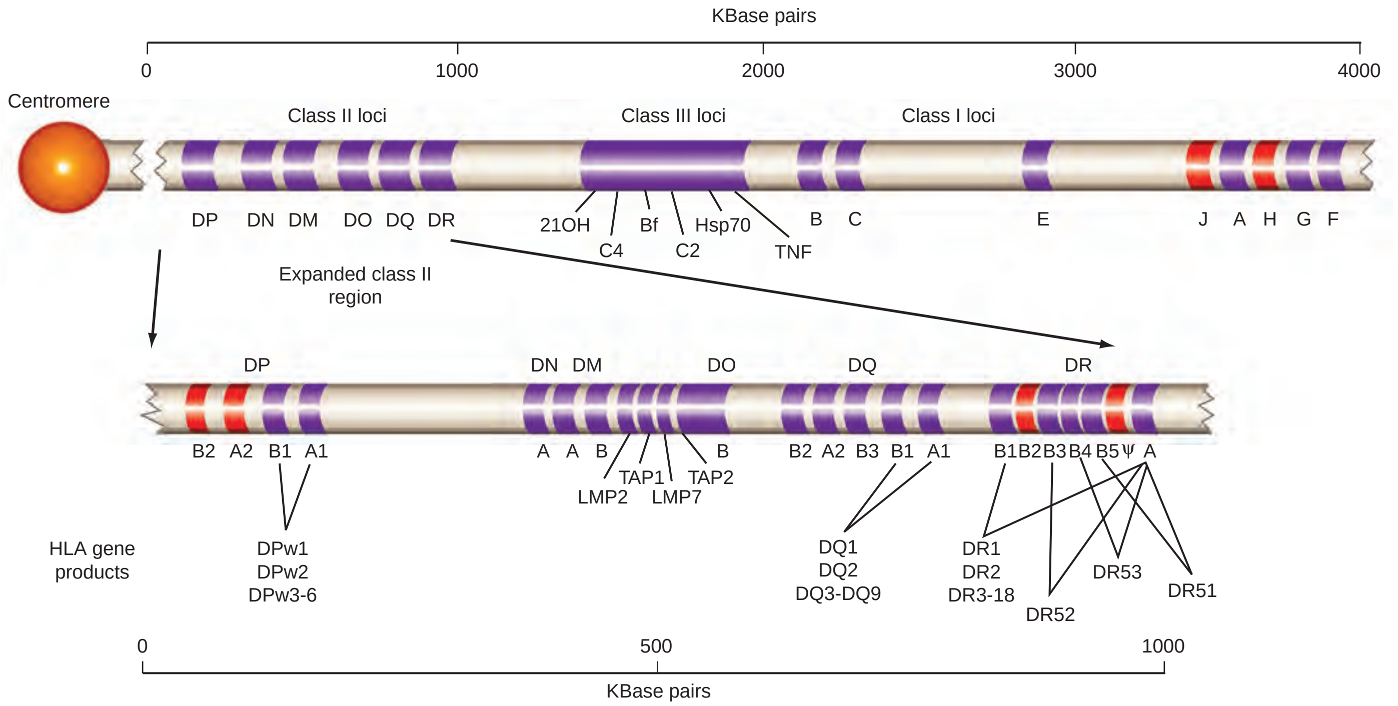

KBase pairs
0
1000
2000
3000
4000
Centromere
Class II loci
Class III loci
Class I loci
DP
DN
DM
DO
DQ
DR
21OH
C4
Bf
C2
Hsp70
TNF
B
C
E
J
A
H
G
F
Expanded class II region
DP
DN
DM
DO
DQ
DR
B2
A2
B1
A1
A
A
B
TAP1
TAP2
LMP2
LMP7
B
B2
A2
B3
B1
A1
B1
B2
B3
B4
B5
ψ
A
HLA gene products
DPw1
DPw2
DPw3-6
DQ1
DQ2
DQ3-DQ9
DR1
DR2
DR3-18
DR52
DR53
DR51
0
500
1000
KBase pairs

Figure 114.1 PHYSICAL MAP OF THE HUMAN LEUKOCYTE ANTIGEN (HLA) GENETIC COMPLEX, ILLUSTRATING THE CLUSTERS OF GENES ACCORDING TO THE CLASS OF ENCODED GENE PRODUCTS. The symbol ψ represents four DRB pseudogenes, designated 7, 8, and 9. Other pseudogenes are shown in *gray*.

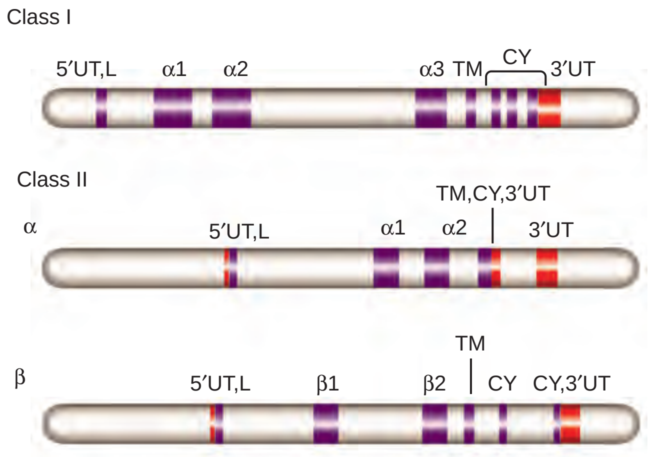

Class I
5′UT,L
α1
α2
α3
TM
CY
3′UT
Class II
α
5′UT,L
α1
α2
TM,CY,3′UT
3′UT
β
5′UT,L
β1
β2
TM
CY
CY,3′UT

Figure 114.2 ORGANIZATION OF CLASS I AND II MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) GENES. *5′ UT* and *3′ UT*, Untranslated regions in the 5′ and 3′ ends of the gene; *α, β*, exons encoding extracellular domains; *CY*, exon encoding cytoplasmic tail; *L*, leader sequence; *TM*, transmembrane exon. *(From Germain RN, Malissen B. Analysis of the expression and function of class-II major histocompatibility complex-encoded molecules by DNA-mediated gene transfer. Annu Rev Immunol. 4:281, 1986.)*

All DR molecules use DRA for α-chains but can use alleles coded by DRB1, DRB3, DRB4, or DRB5 for β-chains. DP, DQ, DM, and DO molecules are the product of DPA1 and DPB1, DQA1 and DQB1, DMA and DMB, and DOA and DOB genes, respectively. The HLA DRB1 locus is expressed in all HLA haplotypes (the set of HLA alleles derived from the same parental chromosome and therefore genetically linked). However, only one other HLA DR locus is present in each individual chromosome. Thus, each haplotype can have either DRB5 (DR1 haplotype); DRB3, -B4, or -B5 loci (DR2 haplotype); DRB4 locus (DR4 and DR7 haplotype); or none (DR8 and DR10 haplotype).[3]

INHERITANCE AND LINKAGE DISEQUILIBRIUM

Because of their proximity within a short chromosomal distance, HLA genes are inherited en bloc from each parent unless a recombinant event occurs. Thus each HLA haplotype behaves as a unit and is transmitted through generations according to mendelian principles. Because there are four possible genotypes (two from each parent), the probability of genotypic identity between two siblings is 25%. Most HLA phenotypically identical siblings are also HLA genotypically identical, because the genetic pool of derivation is restricted to the parents. In 2% of cases, recombinant HLA haplotypes (a set of genes derived partially from two chromosomes through recombination) deviate from this rule.

The occurrence of HLA haplotypes within a population with a frequency higher than expected from the prevalence of individual alleles is called *linkage disequilibrium*. In large populations, gene frequencies achieve equilibrium within a few generations unless selective pressure influences individuals' survival and mating capacity (Hardy-Weinberg principle). In equilibrium, gene prevalence is maintained based solely on its frequency. Thus, assuming that there were 18, 36, and 8 alleles for the HLA-A, -B, and -C loci, respectively (number of alleles known when this example was described[2]), theoretically, 18 × 36 × 8=5184 HLA class I allelic combinations or haplotypes would be possible. Adding HLA class II genes to the calculation yields an astronomical number, making the identification of two HLA-matched individuals unlikely. However, individual alleles occur with different frequency, and an allele that occurs with high frequency is predominant in a given population, such as HLA-A2 in persons of European ancestry and A24 or A11 in Asians.[8] Because predominant alleles come with the related haplotype, most theoretical permutations never occur, and the chances of identifying matched individuals are much higher than theoretically possible.

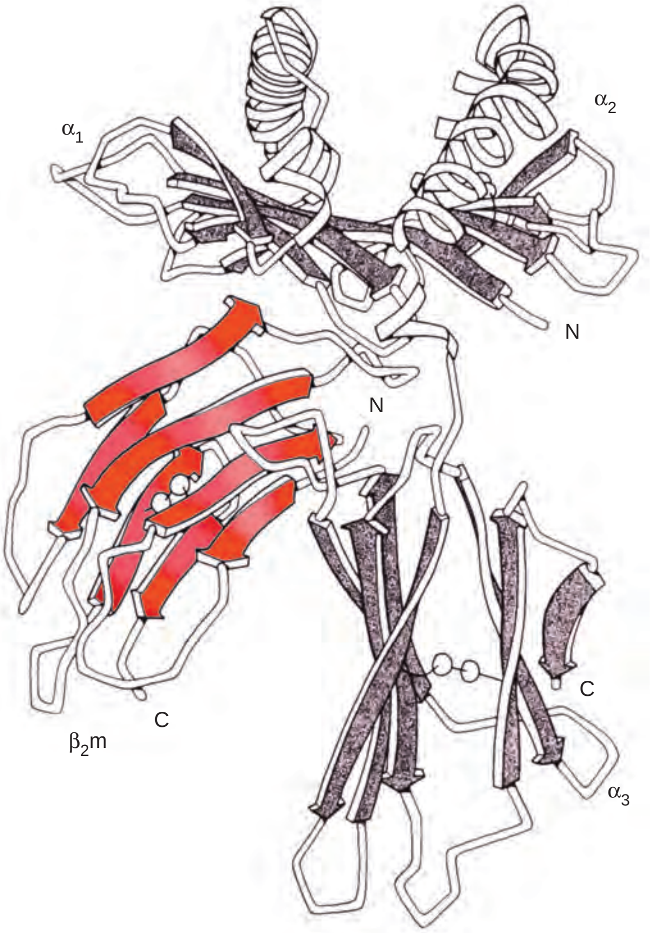


Figure 114.3 THREE-DIMENSIONAL CONFIGURATION OF HLA-A2, MODELED FROM X-RAY CRYSTALLOGRAPHIC STUDIES. *HLA*, Human leukocyte antigen. *(From Bjorkman PJ, Saper MA, Samraoui B, et al. Structure of the human class I histocompatibility antigen, HLA-A2. Nature. 329:506, 1987.)*

STRUCTURE OF THE HUMAN LEUKOCYTE ANTIGEN CLASS I AND II

The structure of HLA molecules and their relationship with their natural ligand, the TCR, has been well characterized by crystallography.[9–11] HLA molecules are heterodimer glycoproteins belonging to the immunoglobulin superfamily with common features (see Fig. 114.4). This includes two α-helical domains protruding toward the extracellular milieu. Between them lies a flat surface formed by β-sheet structures that contributes to the formation of a groove accommodating peptides generated from intracellular (HLA class I) or extracellular proteins (HLA class II) (Fig. 114.5). The helices/peptide complex is exposed for TCR recognition. Because HLA polymorphism is clustered within the α-helixes and β-sheets domains, peptides display variable affinity for distinct HLA alleles.[12,13] It has been proposed that a given peptide can bind to closely related alleles, and HLA superfamilies with similar binding characteristics have been described.[14–16] However, peptide binding to related but distinct HLA alleles is associated with conformational dissimilarity caused by differential interaction with variant residues in the binding groove.[17] The TCR interaction with HLA required for productive engagement spans a surface including the peptide and portions of the α- and β-helix.[11,18–20] This double requirement of interaction between the TCR and the HLA/peptide complex represents the structural basis for HLA restriction. Degenerate and promiscuous TCR recognition of peptides presented by distinct HLA alleles within the same superfamily has also been described.[21] Although this concept holds in general, several exceptions can be expected because single amino acid variants in the HLA molecule may disallow binding of a peptide[22] or may not be permissive to TCR engagement.[23,24]

The binding affinity of a given peptide for an HLA allele can be predicted through algorithms that compile available information to identify amino acid residues that fit distinct pockets of the HLA groove.[13,25] Several algorithms implement this information with experimental testing based on the refolding capability of HLA heavy chains exposed to known peptide sequences in the presence of β_2-microglobulin and/or their dissociation rates (see www.bimas.dcrt.nih.gov or www.uni-tuebingen.de/uni/kxi). This is based on the principle that the affinity of a peptide for a given HLA promotes the stability of the noncovalent assembly of heavy chain with β_2-microglobulin.[13,26] Finally, peptide binding can be shown by direct elutriation from purified HLA heavy chains.[12,27]

Beyond the interactive component of the HLA molecule with the TCR, other domains act as coreceptors for the TCR. CD8$^+$ cytotoxic T cells bind to the α_3-domain of HLA class I through CD8, whereas CD4$^+$ T cells interact with HLA class II–specific domains. Although the coreceptor-TCR interaction is not an absolute requirement in most cases, it determines HLA class I or II restriction of individual T cells.[28] Specific to HLA class I is the assembly with β_2-microglobulin on stabilization in the presence of high-affinity peptides derived from the cleavage of intracellular proteins (endogenous pathway of antigen presentation).[29] Most peptides derive from degradation of self-protein in the cytosol by proteasomes (PSMB) and other proteases.[30] Soluble peptides (9 to 10 amino acids long) are then chaperoned into the endosomal compartment by transporter molecules associated with antigen processing (TAP1 and TAP2). Within this compartment, they bind to heavy chains according to each individual's HLA phenotype. The binding and stability of the HLA-peptide complex depends on the affinity of each peptide for a particular allele. If the stability is sufficient, the peptide-loaded HLA migrates to the cell surface. Intracellular pathogens produce proteins that are also degraded by proteasomes into peptides that compete with self-peptides for binding to HLA class I molecules. Thus, the function of the endogenous pathway of antigen presentation is to provide information to the extracellular compartment of intracellular events. In physiologic conditions, only self-peptides are presented, and therefore minimal interactions occur with circulating T cells. During infection, pathogen-derived peptides signal cellular infection to T cells, whereas antibodies that cannot cross the cell membrane remain insensitive to intracellular pathogens. Several pathogens, such as cytomegalovirus, can interfere with this process as well as with reduction of HLA/peptide density on the cell surface and, consequently, diminished T-cell recognition.[31,32] This escape mechanism can be counteracted by the host through increased susceptibility of virally infected cells to NK cell-mediated cytolysis.[33] Reciprocally, viruses can evade NK cells and escape recognition by modulating HLA expression.[34–36]

HLA class II molecules bind to longer peptides derived from the metabolism of molecules endocytosed from the extracellular compartment (exogenous pathway of antigen presentation).[29] This is a specialized process used by professional antigen-presenting cells, such as macrophages and dendritic cells. HLA class II molecules are assembled within the endosomal compartment where they are composed of a heterotrimer. This includes the α- and β-chains plus a short invariant chain that stabilizes the molecule by occupying its groove while chaperoning its migration to the endosomal compartment where exogenous antigen is processed. Upon uptake, the exogenous antigen undergoes limited proteolysis in the membrane-bound acidic endosomal compartment (MHC class II peptide-loading compartment, MIIC). Upon entering the MIIC, the pre-HLA class II complex is degraded, and antigenic peptides are loaded with the help of the nonclassic HLA-DM molecule.[37]

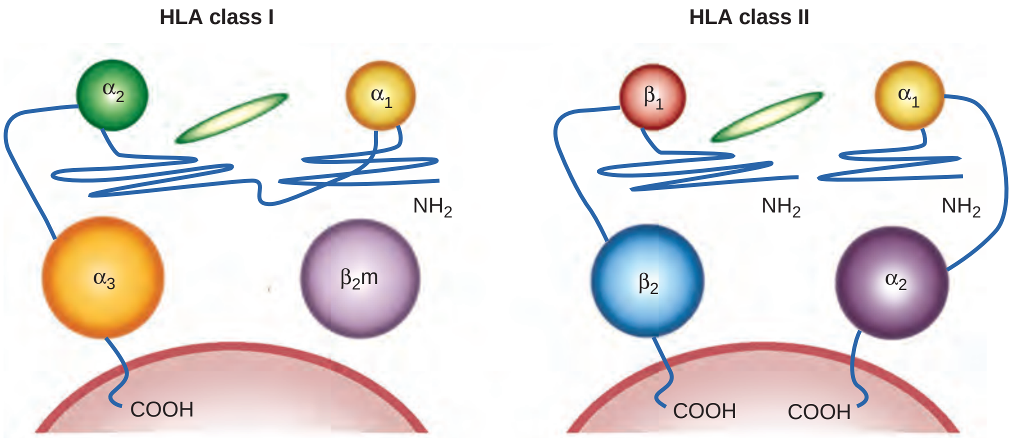


Figure 114.4 SCHEMATIC DIAGRAM OF HUMAN LEUKOCYTE ANTIGEN (HLA) CLASS I AND CLASS II MOLECULES POINTING TO THEIR STRUCTURAL SIMILARITY. In both HLA classes, two immunoglobulin-type domains reside close to the cell membrane (α_3 and β_2m for class I and α_2 and β_2 for class II). The other two domains project toward the extracellular milieu with α-helices (α_1 and α_2 for class I and α_1 and β_1 for class II) and a platform of parallel β-sheets that form a peptide-binding groove.

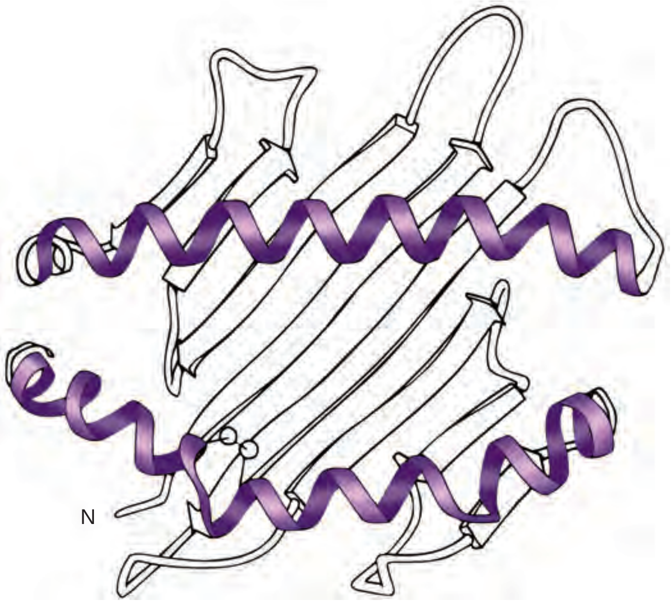


Figure 114.5 SCHEME OF THE PEPTIDE GROOVE OF A HUMAN LEUKOCYTE ANTIGEN (HLA) CLASS I MOLECULE, FORMED BY THE α1 AND α2 DOMAINS AT THE SIDE AND THE β-SHEETS AT THE BOTTOM. The view is looking down on the vertically oriented molecule: the T-cell receptor point of view. *(From Bjorkman PJ, Saper MA, Samraoui B, et al. Structure of the human class I histocompatibility antigen, HLA-A2. Nature. 1987;329:506.)*

EXPRESSION OF HUMAN LEUKOCYTE ANTIGEN MOLECULES

There are genetic and structural differences between the two classes of HLA alleles. The most striking difference is functional, because HLA class I molecules are expressed by most nucleated cells with the exception of germinal cells, whereas HLA class II molecules are expressed mainly by specialized antigen-presenting cells.[38,39] HLA class I molecules are also expressed by platelets.[40] They are responsible for refractoriness after multiple transfusions[41–44] and sufficiently, though minimally, expressed by reticulocytes to be targets of alloantibodies during hemolytic transfusion reactions.[45] HLA class II proteins are expressed constitutively by cells associated with the initiation of the immune response, such as monocytes, macrophages, and B cells,[46] or by immune cells activated by cytokines during inflammation. Thus HLA class I molecules instruct the host about the condition of individual cells as potential targets for CD8$^+$ cytotoxic T cells responsible for clearing the organisms of altered cells. HLA class II molecules initiate immune responses by taking up pathogen components and presenting them to CD4$^+$ helper T cells, which can in turn initiate humoral and facilitate cellular immune responses.

Expression of HLA alleles is sensitive to environmental conditions and can be modulated by cytokines among which interferons play a major role.[47] This is particularly important for HLA-B and HLA-C normally expressed at a lower density than HLA-A but more sensitive to cytokine induction.[48,49] In addition, HLA class II molecules can be expressed by most cells following cytokine stimulation.[50,51] Thus the ability of cells to present antigens in association with HLA is strongly influenced by the surrounding environment. This might explain why chronic inflammation induced by alloreactions during graft-versus-host disease (GVHD) may facilitate the presentation of tumor-specific antigens by tumor cells with consequent development of the GVN effect. In addition, it might explain how systemic administration of proinflammatory cytokines such as interleukin-2 may stimulate immune responses by increasing the antigen-presenting ability of cells within the tumor microenvironment.[52]

HUMAN LEUKOCYTE ANTIGEN POLYMORPHISM AND ITS CLINICAL SIGNIFICANCE

A striking characteristic of the HLA system is its extreme polymorphism.[53] By chance, most individuals are heterozygote and therefore carry two different alleles for each HLA gene that, being codominant, are equally expressed on the cell surface. Because everybody carries three HLA class I (-A,-B, and -C) and three HLA class II (-DR, -DQ, and -DP) genes, most individuals (with the exception of homozygotes) express six different HLA class I and six different HLA class II molecules on the surface of their cells. This has important functional implications, because HLA polymorphism is clustered in domains of the HLA molecule associated peptide binding and interaction with the TCR. Thus, most individuals have a broad repertoire of molecules capable of presenting different pathogen components to immune cells. Therefore, it is believed that HLA polymorphism improves the chances of a given species of surviving infection.[54] This paradigm is difficult to demonstrate in human pathologic conditions in which the natural history of infectious diseases rarely correlates with HLA phenotype. An exception is the strong association between

HLA-B*5701 and lack of progression to acquired immunodeficiency syndrome of individuals infected with human immunodeficiency virus (HIV).[55] Associations have been observed between HLA phenotype and predisposition for nasopharyngeal carcinoma.[56,57] This is of particular interest because nasopharyngeal carcinoma is a virally induced cancer against which T cells can mediate immune surveillance. HLA associations have also been described with less consistency for other virally driven tumors, such as cervical carcinoma,[58] or immunogenic tumors, such as melanoma.[59,60]

The difficulty in demonstrating conclusive associations between infectious disease and HLA in humans could be caused by the successful implementation of antigen presentation through a broad repertoire of HLA genes, which could compensate for each other in limitations in antigen presentation. Chickens carry a single MHC locus, and their susceptibility to infection appears to be clearly related to MHC. For instance, Kaufman et al.[54] have shown that chickens carrying a particular Bf (the only MHC class I gene in chickens) allele are fully protected from Rous virus-induced sarcomas because this allele (B-f12) can bind several antigenic peptides from its proteins. On the contrary, chickens homozygous for B-f4 are killed by the same infection because this allele cannot bind peptides from sarcoma virus proteins.[54]

HLA polymorphism is the basis of alloimmunization because most individuals are likely to have different HLA molecules on the surface of their cells. Hundreds of HLA alleles have been identified through high-resolution typing, making the chances of two individuals having identical HLA phenotypes extremely low. Thus partial mismatches are commonly accepted in transplantation cases, which are at the root of hyperacute, acute, and chronic rejections. Hyperacute rejection is caused by preformed antibodies against donor HLA alleles in patients presensitized by multiple transfusions.[61] Acute and chronic rejection result from a combination of humoral and cellular immune reactivity toward HLA alleles of the donor.[62,63] In addition to allosensitization, HLA alleles can mediate GVHD, whereby hematopoietic cells derived from the grafted tissue recognize and reject the host tissues. They do this by identifying polymorphisms of intracellular proteins of the host (minor histocompatibility antigens [mHags]) presented in association with donor-recipient matched HLA alleles.

NONCLASSIC MAJOR HISTOCOMPATIBILITY COMPLEX AND MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I CHAIN-RELATED MOLECULES

Besides the three ubiquitously expressed highly polymorphic classic HLA class I molecules, humans encode three relatively conserved nonclassic, selectively expressed (HLA-E, -F, and -G) MHC class I genes (also known as MHC-Ib). These evolved at different rates in primates reflecting differential involvement in the modulation of immune responses.[4,64,65] In addition, these molecules are characterized by unique patterns of transcription, protein structure, and immunologic function.[66]

The MHC class I–related chain genes (MICA and MICB) are located within the MHC region and are characterized by high polymorphism (more than 50 alleles so far identified).[67] The molecules encoded by these genes do not appear to bind peptides or associate with β_2-microglobulin. Their polymorphic variants are not concentrated around the peptide-binding groove, yet they seem to have functional significance, because most mutations are nonsynonymous, suggesting selective pressure as a driving force. Their tissue distribution is restricted to epithelial and endothelial cells and fibroblasts. It appears that MIC genes modulate the function of NK and CD8$^+$ T cells by binding the NKG2D stimulating receptor.[68] Also, MIC genes have been implicated in transplant rejection because alloantibodies against them are often found in transplant recipients that may exert complement-mediated cytotoxicity against endothelial cells from the graft.

Other unusual MHC-like molecules are present in the genome and have disparate functions, including presentation of lipid antigens (CD1), transport of immunoglobulins (Fc receptor), and regulation of iron metabolism (hemochromatosis gene product).[69] Contrary to classic MHC class I genes that are constitutively expressed, nonclassic MHC and MIC gene expression depends on stimulation by proinflammatory cytokines.[70] In addition, two nonclassic MHC class II proteins (HLA-DM and HLA-DO) have been described that function as mediators of peptide exchange by stabilizing empty MHC class II molecules.[37] Finally, it is possible that several nonclassic MHC molecules whose function is to present peptides to lymphocytes may be present throughout the genome. Because of their limited polymorphism, however, these genes may have evolved to serve specialized presentation functions.[71]

Characterized by low polymorphism, the regulation of HLA-G expression follows a nonclassic behavior. Aberrant cytokine-responsive regulatory sequences[72–74] may be responsible for its predominant expression by trophoblasts that do not express other HLA proteins.[75] It may also account for its low levels in a variety of human tissues.[76] Lack of responsiveness to common immunostimulatory pathways (NF-κB, interferon-γ, or CIITA) is most pronounced in HLA-G cells and is shared by other nonclassic MHC such as HLA-E.[77] Also, HLA-G is expressed in a variety of cancers.[72] It is hard to know what the relevance of HLA-G expression is because it can occur in various membrane-bound or soluble isoforms with distinct functional characteristics.[72] Functional isoforms that include the α-1 and α-2 domains bind and present peptides from cytoplasmic proteins.[78] Because of the minimal polymorphism, however, the repertoire of peptides presented is likely to be limited, suggesting that peptide binding is necessary to stabilize the molecule rather than being involved in antigen presentation.

Functionally, HLA-G is thought to modulate the function of NK cells through interactions with their inhibitory receptors.[79] In addition, the HLA-G leader sequence contains a peptide that can bind and stabilize the expression of HLA-E, which in turn inhibits NK cells.[80] Because the HLA-G–derived leader peptide has the strongest affinity for HLA-E (among all HLA class I molecules), it is likely that HLA-G is a powerful direct and indirect inhibitor of NK cells, reducing the risk for cardiac rejection[81] or inducing immune escape of cancer cells.[72] Although much has been published about the immunoregulatory role of HLA-G, its true function remains mysterious, principally because of discordant findings reported by various groups.[74] With the goal of achieving consensus, a workshop was recently organized to standardize methods of analysis of HLA-G.[66]

HLA-E is minimally polymorphic,[4] binds hydrophobic peptides from other HLA class I leader sequences, and interacts with CD94/NKG2 lectin-like receptors present predominately on NK and partially on CD8$^+$ T cells.[82–85] The peptide binding is highly specific and stabilizes the HLA-E protein, allowing its migration to the cell surface. Thus surface density of HLA-E is an indirect reflection of the number of HLA class I alleles expressed by a cell.[86] The interaction of HLA-E with CD94/NKG2 protects HLA-E–expressing cells from killing. Cells damaged by viral infection or neoplastic degeneration may lose HLA class I expression. As a backup mechanism of protection, reduced HLA class I expression results in decreased expression of HLA-E, leading to vulnerability to NK cells.[87] Some viruses express mimic peptides that bind and stabilize HLA-E so that, although classic MHC molecules are downregulated, HLA-E expression is maintained, allowing the pathogen to simultaneously escape CD8$^+$ T- and NK-cell killing.[88]

The function of HLA-F remains enigmatic. Its transcriptional regulation is closest to classic HLA molecules in that it can be induced by NF-κB, interferon regulatory factor-1, and class II trans-activator.[89] However, contrary to classic HLA molecules, HLA-F is predominantly empty, mostly intracellular, with a restricted pattern of expression.[90] Its tissue distribution appears to be limited to B cells, and therefore it is mostly found in lymphatic organs.[90] Structural studies suggest that HLA-F is a peptide-binding molecule and may reach the cell surface under favorable conditions when a suitable peptide is present.[91] Once on the cell surface, HLA-F may interact with the effector-cell receptors IL-T2 and IL-T4, as suggested by HLA-F tetrameric complexes–binding studies.[91] Thus it is possible that in specific yet unknown conditions, HLA-F may modulate the function of immune effector cells similarly to HLA-E and HLA-G.

NON-HUMAN LEUKOCYTE ANTIGEN POLYMORPHISM AND ITS CLINICAL SIGNIFICANCE

Although this chapter is dedicated to HLA, it would be incomplete without mentioning the increasingly recognized polymorphisms of other immune modulators. The significance of non-HLA polymorphism is evidenced by the development of GVHD in the presence of HLA identical matching among relatives. As recently summarized,[92] three general areas of polymorphism are being investigated: NK cell–receptor genes, minor histocompatibility antigens, and cytokines. Over the last decade, much progress has been made in identifying the mechanisms of action of NK cells. A major breakthrough was made in the discovery of HLA class I-specific inhibitory receptors and in the role that they play in the regulation of NK function with consequent effects on the eradication of hematologic malignancies, prevention of graft rejection, or induction of GVHD.[93] NK cells recognize HLA molecules via killer immunoglobulin-like receptors (KIRs). The regulation of NK cell function by KIRs is further discussed in Chapter 12. KIRs are glycoproteins encoded by at least 17 different genes located on chromosome 19q13.4.[94] All human KIR genes derive from a gene encoding three immunoglobulin (Ig)-like domains (D0, D1, and D2) and a long cytoplasmic tail. However, the KIRs genes are diverse and may encode either two or three Ig-like domains and either a long or short cytoplasmic tail (Table 114.1). The long cytoplasmic tails contain one or two immunoreceptor tyrosine-based inhibition motifs.[95] Although KIR molecules with long cytoplasmic tails inhibit NK cytotoxicity, those with short tails do not. The names given to KIR genes are based on the molecule that they encode. The first digit corresponds to the number of Ig-like domains in the molecule and a *D* denotes domain. The D is followed by either an *L* for long cytoplasmic tail or *S* for short cytoplasmic tail or *p* for a pseudogene. The last digit indicates the number of the KIR gene.[96]

Expression of KIR in individual NK cells is complex because NK cells may express several members of the KIR family. The number of KIR genes in each haplotype varies among individuals. The most common haplotype is known as group A, which is made up of six genes (2DL1, 2DL2 or 2DL3, 3DL1, 3DL2, 2DS4, and 2DL4). Various KIR genes can recognize different HLA-A, HLA-B, and HLA-C molecules. HLA-C antigens can be divided into two groups based on polymorphisms at amino acid positions 77 and 80 of their class I heavy chains. One group has asparagine (Asn) at position 77 and lysine (Lys) at 80, and the other has serine (Ser) at 77 and Asn at 80. Some KIRs recognize HLA-C antigens with Asn 77 and Lys 80, whereas other KIRs recognize HLA-C antigens with Ser 77 and Lys 80. The polymorphism at position 80 is most important. Another group of KIR reacts with HLA-B antigens that carry specific combinations of amino acids at positions 77 and 83 of the heavy chain that forms HLA-Bw4. Because a single KIR can interact with multiple HLA class I alleles, KIR recognition of HLA class I molecules is degenerate. Another NK inhibitory receptor (CD94-NKG2A) recognizes the nonclassical HLA-E molecule. In addition, each of the KIR genes is extensively polymorphic.

Because the genes for KIR, HLA, and CD94-NKG2A are located in separate chromosomes, they segregate independently, and consequently individuals can carry genes for KIR for which there is no correspondent HLA ligand.[97] Because HLA-E is expressed in all individuals, NK cells that bear the CD94-NKG2A receptor are not alloreactive. Because the specificity of KIR for their ligands is broad and each individual carries several KIRs, it is likely that in most cases all NK cells of a given person express at least one KIR that is specific for a self-HLA class I allele. Thus in autologous settings, NK cells kill only aberrant cells that have lost HLA class I expression. By contrast, NK cells can kill allogeneic cells that do not express HLA class I alleles recognized by their KIR. Thus, by knowing the KIR repertoire of a given transplant recipient and the HLA type of the donor, it would theoretically be possible to predict the likelihood of an NK-mediated alloreaction. Importantly, it appears that alloreactive NK cells undergo proliferation on exposure to the stimulatory cells and therefore can preferentially expand in the presence of allogeneic tissue. NK cells also express activating receptors that are responsible for their lytic activity. Although the identity of the ligands for these receptors has not been identified, it is possible that they are expressed primarily by activated or proliferating cells. It is therefore possible that during the inflammatory process induced in allogeneic conditions, normal cells can become activated by cytokines and express ligands, which are responsible for NK activation in the absence of HLA class I molecules reactive with the inhibitory receptors.[93] The relevance of KIR in transplantation has been well studied in the context of haploidentical hematopoietic transplantation. In this case, several combinations are possible: NK cells from the graft express KIRs that do not interact with the donor's HLA (graft-versus-host alloreactivity). It seems that the presence of graft-versus-host reactive NK cells associated with incompatibilities between donor and recipient (especially HLA-C families) has favorable effects on the outcome of acute myeloid leukemia.[98] Alternatively, a good match may be present between graft NK and host HLA as well as between host NK and graft HLA. In such a case, no alloreactivity occurs. Finally, the graft's HLA type may be unsuitable for the host's NK repertoire, and the host's reactivity may lead to graft rejection. Alloreactive grafted NK cells seem to prevent GVHD while inducing GVN.[93]

TABLE 114.1 **HLA Class I Alleles Recognized by Different Killer Immunoglobulin-Like Receptors**

KIR	HLA Class I Allele	Amino Acid Sequence Motif
P58.1 (KIR2DL1)	HLA-C2, -C4, -C5, -C6	Asn 77, Lys 80
P58.2 (KIR2DL2/3)	HLA-C1, -C3, -C7, -C8	Ser 77, Asn 80
P70 (KIR3DL1)	HLA-Bw4 public specificity	Aa 77–83
P140 (KIR3DL2)	HLA-A3, -A11	
(KIR2DL4)	HLA-G	

Aa, Amino acid; *Asn*, asparagine; *HLA*, human leukocyte antigen; *KIR*, killer immunoglobulin-like receptor; *Lys*, lysine; *Ser*, serine.

Like HLA, KIR genes are polymorphic, and their variability is clustered in positions likely to affect the overall structure of the molecule. The relevance of KIR gene polymorphism in the outcome of bone marrow transplantation (BMT) is unclear. It appears that the risk for GVHD is highest in the context of unrelated BMT when the recipient KIR genotype is included in the donor KIR genotype.[99] These results show that compatibility between KIR genotypes themselves may influence the outcome of BMT.

The minor histocompatibility antigens (mHags) are represented by polymorphic molecules whose peptides containing variant sequences are presented by HLA alleles. They have been shown to be targets of cytotoxic T cells, which can lyse leukemia cells.[98,100] In addition, some mHags are selectively expressed by neoplastic cells.[101] At present, little is known about the identity of mHag epitopes in the context of various HLA types and their significance in the development of GVHD and GVN.

Cytokines are another large family of molecules associated with antigen recognition, graft rejection, and GVHD. Their polymorphism is becoming an important area of investigation in the context of transplantation, autoimmunity, and cancer.[102] Polymorphic sites reside in regulatory regions so that genetic variants are associated with high or low production of a given cytokine rather than differences in its function. A website compiles information about cytokine polymorphism (www.bris.ac.uk/Depts/PathAndMicro/services/GAI/cytokine4.htm). Although no consensus has been achieved yet, several studies have shown associations between various cytokine genotypes, the propensity for disease, and transplant outcome. These studies have been summarized elsewhere.[92,103] A strong association was recently noted between a low interleukin-10 producer genotype and a tendency to develop melanoma and prostate cancer.[104,105]

HUMAN LEUKOCYTE ANTIGEN NOMENCLATURE

The history of the HLA system nomenclature was summarized by Sir Walter Bodmer who, together with Ruggero Ceppellini, was primarily involved in its development.[106] It began as HL-A, for *human leukocyte locus A*. With the recognition that HLA molecules are encoded by more than one locus, the A came to stand for *antigen*, and a locus designation was added after HLA (i.e., HLA-A, -B, -C, -D, etc.)[2]. From then on, the WHO has updated nomenclature on a quarterly basis. The most recent update was assigned in January 2015, as seen in Table 114.2.[4] At present, two systems are used. An immunologically defined nomenclature is based on the identification of HLA antigens on the surface of leukocytes. Therefore, HLA phenotypes described by immunologic methods are conventionally called *HLA antigens*.[107] The second system is based on the molecular identification of nucleotide sequences in genomic deoxyribonucleic acid (DNA), and results are conventionally referred to as *HLA alleles*. Because molecular typing has a higher resolution, it has gained increasing popularity.

IMMUNOLOGICALLY DEFINED HUMAN LEUKOCYTE ANTIGEN NOMENCLATURE

Immunologically defined nomenclature follows this rule: HLA separated by a hyphen from a capital letter identifying the locus encoding distinct HLA class I (-A, -B, -C) or class II (-DR, -DQ, -DP) antigens. The letter is followed by a number that identifies a serologic family of alleles sharing epitopes recognized by alloantibodies or alloreactive cytotoxic T cells. With improved understanding of the molecular genetics of the HLA region, various appendages have been removed from the HLA nomenclature. For instance, the letter *w* was used to indicate a provisional assignment, and this has been discontinued, but it is occasionally added to HLA-C antigen nomenclature to distinguish it from complement genes; DP and DW also maintained this letter to reinforce the dependency of their immune identification predominantly through cellular techniques. Finally, HLA-Bw4 and -Bw6 retain the *w* to emphasize that the public epitopes are shared by several HLA-B and some HLA-antigens. More recently, a bridge between immunologic and molecular nomenclature has been proposed whereby HLA antigens that encompass a single gene product can be assigned a two-digit numeric extension corresponding to the molecular nomenclature for that allele.[108]

SEQUENCE-DEFINED ALLELIC NOMENCLATURE

In 1987, the 10th International Histocompatibility Workshop recommended a sequence-based nomenclature[108] to describe alleles not distinguishable by immunologic methods. Since then, the number of HLA alleles has rapidly increased. As of January 2015, a total of 13,023 alleles for HLA exist. This is a drastic increase from the original numbers established in 2003. Other designations are summarized in Table 114.2. HLA designates molecules belonging to the human MHC followed by the locus (-A, -B, etc). Alleles are then identified after an asterisk (*). Each HLA allele name has a unique number corresponding to up to four sets of digits separated by colons. The length of the allele designation is dependent on the sequence of the allele and that of its nearest relative. All alleles receive at least a four-digit name, which corresponds to the first two sets of digits; longer names are only assigned when necessary. The digits before the first colon describe the type, which often corresponds to the serological antigen carried by an allotype. The next set of digits is used to list the subtypes, numbers being assigned in the order in which DNA sequences have been determined. Alleles whose numbers differ in the two sets of digits must differ in one or more nucleotide substitutions that change the amino acid sequence of the encoded protein. Alleles that differ only by synonymous nucleotide substitutions (also called silent or non-coding substitutions) within the coding sequence are distinguished by the use of the third set of digits. Alleles that only differ by sequence polymorphisms in the introns or in the 5′ or 3′ untranslated regions that flank the exons and introns are distinguished by the use of the fourth set of digits.[109] A four-digit number is used in which the first two digits refer to the original serologic family (e.g., HLA-A2 serologically would be HLA-A*02). Often numbers are missing because an original assignment was revoked (e.g., this is why there is no HLA-A*24:01). When silent mutations are identified (variation in nucleotide sequence that does not translate into changes in amino acid sequence), the name of the allele remains identical, but another two digits are added to designate a variant that has no functional significance. New sequences are submitted to European Molecular Biology Laboratory (EMBL; www.ebi.ac.uk/Submissions/index.html), GenBank (www.ncbi.nlm.nih.gov/Genbank/index.html), or DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/submission-e.html).

Requirements for new allele naming were described by Marsh et al.[4] The WHO Nomenclature Committee also made recommendations about naming alleles with aberrant expression, such as HLA-G isoforms and KIR. Some alleles are identifiable at the genomic level but are not translated into protein (pseudogenes). These are indicated by the addition of an *N* (for null) following the numerical designation of the allele. Mutations inducing a reduction of expression are marked by *L* (low expression). An *S* denotes alleles expressed as soluble secreted molecules, such as HLA-B*44:02:01:02 S characterized by an intronic variant that disallows the expression of the transmembrane domain of the HLA molecule and is therefore only in soluble form. Differential splicing of HLA-G leads to production of membrane-bound and soluble forms, which are respectively denoted by a lowercase *m* or *s* before HLA. Limited cytoplasmic expression is denoted by *C* and aberrant expression by *A*. Finally, KIR polymorphism will be classified by a new system that is in preparation.[4,97,110] A nomenclature system for cytokine polymorphism has not yet been developed.[111]

HUMAN LEUKOCYTE ANTIGEN TYPING IN CLINICAL HEMATOLOGY AND DETERMINATION OF COMPATIBILITY HUMAN LEUKOCYTE ANTIGEN TYPING

Originally HLA typing was done primarily in support of transplantation or transfusion needs with the purpose of identifying histocompatibility through the best match between donor and recipient. Allosensitization of recipients previously exposed to heterologous cell products is tested by identifying alloreactive antibodies in serum, and crossmatch procedures are performed to grade compatibility of candidate donor-recipient pairs. In addition, HLA testing has been applied to identify links between a given disease and the genetic makeup of its carriers.[112] Strong associations are exemplified by birdshot uveitis (a disease occurring exclusively in HLA-A29 individuals),[113] type 1 diabetes and other autoimmune diseases,[114–116] or long-term survival of HIV-infected individuals.[55] These studies evaluated the role that genetic background may have played over environmental factors.[112,117] HLA associations are thought to be caused by the differential ability of distinct alleles to present immunogenic epitopes[113,115] or by the close linkage to the HLA class III region where potent immunomodulators such as tumor necrosis factor-α are located.[118] It has also been suggested that HLA associations may be predictors of immune responsiveness of cancer to immune therapy.[60] However, such associations have remained quite difficult to reproduce.[119] HLA typing is requested for enrollment of patients into and interpretation of immunization protocols,[24] because specific HLA-epitope combinations require high-resolution typing.[22,120]

Serologic testing takes advantage of fetomaternal sensitization. In mammals, the progeny carries a full haplotype of paternal origin, and pregnant women may develop antibodies against the paternal haplotype. Maternal serum samples are collected at term and characterized by testing their ability to kill HLA-bearing repository cell lines of known phenotype in the presence of complement (complement-dependent

TABLE 114.2 Name of Genes in the HLA Region Considered by the WHO Nomenclature Committee

Name	Previous Equivalents	Molecular Characteristics	No. of Alleles January (2015)
HLA-A	–	Class I α-chain	3107
HLA-B	–	Class I α-chain	3887
HLA-C	–	Class I α-chain	2623
HLA-E	E, “6.2”	Associated with class I 6.2-kBa Hind III fragment	17
HLA-F	F, “5.4”	Associated with class I 5.4-kBa Hind III fragment	22
HLA-G	G, “6.0”	Associated with class I 6.0-kBa Hind III fragment	50
HLA-H	H, AR, “12.4”, HLA-54	Pseudogene association with class I 5.4-kBa Hind III fragment	12
HLA-J	cda 12, HLA-59	Pseudogene association with class I 5.9-kBa Hind III fragment	9
HLA-K	HLA-70	Pseudogene association with class I 7.0-kBa Hind III fragment	6
HLA-L	HLA-92	Pseudogene association with class I 9.2-kBa Hind III fragment	5
HLA-X	–	Class I gene fragment	0
HLA-DRA	DRα	DR α-chain	7
HLA-DRB1	DRβI, DR1B	DR β_1 determining specificity for DR1, DR2, DR3, etc.	1726
HLA-DRB2	DRβII2	Pseudogene with DRβ-like sequence	1
HLA-DRB3	DRβIII, DR3B	DR β3 determining DR52, Dw24, w25, -26 specificity	59
HLA-DRB4	DRβIV, DR4B	DR β4 determining DR53 specificity	15
HLA-DRB5	DRβV, DR5B	DR β5 determining DR52, Dw24, w25, -26 specificity	21
HLA-DRB6	DRBX, DRBσ	Pseudogene found in DR1, DR2, and DR10 haplotypes	3
HLA-DRB7	DRBψ1	Pseudogene found in DR4, DR7, and DR9 haplotypes	2
HLA-DRB8	DRBψ2	Pseudogene found in DR4, DR7, and DR9 haplotypes	1
HLA-DRB9	M 4.2 β exon	Pseudogene isolated fragment	1
HLA-DQA1	DQα1, DQ1A	DQ α-chain as expressed	54
HLA-DQB1	DQβ1, DQ1B	DQ β-chain as expressed	780
HLA-DOA	DNA,DZα, DOα	DO α-chain	12
HLA-DOB	DOβ	DO β-chain	13
HLA-DMA	RING 6	DM α-chain	7
HLA-DMB	RING 7	DM β-chain	13
HLA-DPA1	DPα1, DP1A	DP α-chain as expressed	33
HLA-DPB1	DPβ1, DP1B	DP β-chain as expressed	520
TAP1	ABCB2, RING4	ABC (ATP-binding cassette) transporter	12
TAP2	ABCB3, RING 11	ABC (ATP-binding cassette) transporter	12
MICA	PERB 11.1	Class I chain–related gene	101
MICB	PERB 11.2	Class I chain–related gene 18	41

ATP, Adenosine triphosphate; *HLA*, human leukocyte antigen; *WHO*, World Health Organization.
Data from Marsh SGE, Albert AD, Bodmer WF, et al. Nomenclature for factors of the HLA system, 2010. *Tissue Antigens*. 75:291, 2010.

cytotoxicity [CDC]).[121] CDC is used for HLA typing by exposing circulating cells expressing HLA class I (most cells) and class II (predominantly B cells) antigens from the individual (to be typed to previously characterized sera or monoclonal antibodies).[122] Conversely, already typed repository cell lines are used in CDC to identify alloantibodies in sera of sensitized individuals. The fraction of cell lines killed by the sera roughly grades the intensity of allosensitization or panel reactive antibody (PRA) reactivity. Some antibodies activate complement and kill with poor efficiency, known as the *cytotoxicity-negative absorption-positive (CYNAP) phenomenon*.[123] As judged by cytotoxicity testing, CYNAP may underestimate allosensitization. By modifying CDC with the addition of antihuman antibodies suitable for complement activation, CYNAP can be circumvented (augmented CDC). In this case, however, relatively innocuous antibodies can cause overestimation of clinically relevant allosensitization.[124]

Other methods identify alloantibodies, including immobilization of HLA molecules on solid surfaces to capture soluble antibodies[125–127] and flow cytometry using a spectrum of microbeads loaded with known HLA alleles.[128,129] An interlaboratory comparison of serum screening for HLA-antibody determination suggested that enzyme-linked immunosorbent assay and flow cytometry yield higher PRA activity values compared with CDC or augmented CDC.[125,127] However, the study suggested a lack of consistency among participant laboratories, leaving unsolved the question of which method most accurately defines clinically relevant allosensitization, and a panel of various methods may be most informative.[72]

CDC is declining in interest in the United States because most laboratories are switching to easier-to-handle and higher-resolution molecular methods. However, immunologic methods remain valuable to characterize functional aspects of HLA because molecular methods cannot define whether an HLA allele is expressed nor, at least until recently,[130] grade allosensitization. Thus it is likely that immunologic methods will continue to complement molecular methods in the future.[130]

The usefulness of conventional serologic typing of HLA antigens has been limited by the availability of allele-specific sera. Most importantly, because antibodies identify structural differences on the surface of HLA molecules, variants caused by nucleotide polymorphism in nonexposed areas such as the peptide-binding groove of the HLA heavy chain are not detectable. However, these differences are of functional significance because they determine the specificity and affinity of peptide binding[131,132] and T-cell recognition of self and allogeneic target cells.[23,133] DNA-based typing directly determines the sequence[134] and provides higher resolution typing.

DNA-based typing can be performed with polymerase chain reaction (PCR)-based methods, among which are sequence-specific primer,[134,135] sequence-specific oligonucleotide probe,[136,137] and DNA sequencing.[138] Molecular typing using sequence-specific oligonucleotide primers or probes provides higher level typing than serological typing, and DNA sequencing provides allele-level typing.[138] The rich nature of HLA has led to proportionally increasing complexity of the assays used to cover all possible alleles. As a consequence, accurate HLA typing for donor and recipient matching in transplantation has become increasingly complex and burdensome. In addition, because of the important role that HLA molecules play in antigen presentation and the stringency of the relationship between epitope and associated HLA allele, high-resolution typing is increasingly requested for appropriate enrollment of patients into immunization protocols aimed at the enhancement of T-cell responses.[139] Therefore high-resolution HLA typing is increasingly in demand in clinical and experimental settings.

Although oligonucleotide-based methods could theoretically discriminate any known polymorphic site, they have two major limitations. First, they require a specific PCR reaction for each allele investigated. Because each individual has only two alleles for each locus, a disproportionately large number of PCR reactions must be performed to cover all possible polymorphisms to identify the two borne by the individual tested. Because both methods are based on specific interactions with known oligonucleotide sequences unique to a particular allele, they cannot identify unknown polymorphisms unless the variation occurs within the region spanned by one of the oligonucleotides used in the assay. Because of these limitations, definitive typing methods that yield conclusive information about the identity of the alleles typed using DNA sequence-based typing have been developed.

Sequence-based typing is being performed using Sanger sequencing and next generation sequencing.[138,140] Most methods begin with PCR amplification of the HLA locus of interest. Sanger sequencing involves chain termination sequencing techniques and does not allow the separation of sequences of maternally and paternally derived alleles. Sanger sequencing cannot distinguish *cis*/*trans* polymorphisms since both alleles are sequenced in one reaction and it is difficult to determine if two variants are from the same chromosome (*cis*) or from the opposite chromosome (*trans*).[140a] Therefore, some combinations of HLA class I and class II alleles result in ambiguous allele combinations that require additional testing for resolution.[141] Sanger sequencing instruments analyze only one sample at a time, which limits sample throughput and further limits the usefulness of this technology.

Next-generation sequencing allows for the parallel sequencing of multiple single strands of DNA. This allows for the determination of the sequence of both maternal and paternal HLA sequences, which reduces ambiguities. It also provides information on HLA genes not previously sequenced, allowing for the identification of new alleles.[142] Next-generation sequencing instruments permit the analysis of many samples at one time, allowing for high throughput analysis and reduces costs.[138,140] A number of next-generation sequencing technologies and instruments are available.

High-resolution methods yield high-resolution information of an individual's HLA type. However, the wealth of information is counterbalanced by increased difficulty in identifying suitable HLA alleles during donor-recipient pairing or accrual into immunization protocols restricted to specific HLA-epitope combinations. Thus, at present, clinicians are faced with the daunting task of applying high-resolution typing results of unclear relevance to clinical settings.[143]

TESTING FOR ALLOSENSITIZATION AND DETERMINATION OF COMPATIBLE DONOR-RECIPIENT PAIRS

Any cell-containing product transfused or transplanted between different individuals should be compatible in an ideal situation. Yet, in most cases, histocompatibility is not prospectively sought. Thus patients with multiple exposures to blood products often become reactive to various antigens, including HLA. Transplant candidates often develop prior alloreactivity following transfusion of platelet concentrates contaminated with leukocytes, even though the incidence of allosensitization is much less because of leukodepletion of blood products. Alloreactivity must be documented before transplantation because alloreactive patients can still undergo transplantation, provided that the donor has no mismatched HLA antigens reacting with the patient's antibodies. Patients who have received repeated platelet transfusions may become allosensitized and consequently refractory to further transfusions unless HLA-compatible platelets are used. Obviously, the best compatibility consists of identical matching. It is often impossible to identify a perfectly matched unrelated donor, particularly in the case of rare HLA types. Thus other strategies are adopted to identify the best possible match or compatible mismatch. Selection of unrelated donor-recipient pairs is carried out through typing with serologic, cellular, and molecular methods.[144] The chances of identifying compatible donors based on full or partial HLA matching have become increasingly low with the increasing resolution of the typing methods.[143] To broaden compatibility, matching criteria of donor-recipient pairs are based on shared public epitopes assigned to cross-reactive groups (CREGs)[145] or shared amino acid polymorphisms defined through sequence information (Table 114.3).[146] The preexistence of alloantibodies restricts the identification of compatible donors even further. Highly sensitized recipients with PRA activity exceeding 85% of tested specificities (generally between 30 and 60) represent a particularly challenging group.[147] An alternative approach to the exclusion of alloreactive determinants is the inclusion of acceptable antigen mismatches expressed in a panel of cells that give negative reactions with the recipient sera,[148] which extends the repertoire of possible donors. All of these are fundamental tools for the identification of nonrelated, not fully matched donor-recipient pairs. Unfortunately, even these compromises often fail to identify a suitable match.

Duquesnoy[147] described a molecularly based algorithm to identify histocompatible pairs called HLAMatchmaker. This method focuses on the structural basis of HLA class I polymorphism so that compatible HLA mismatches can be identified without extensive serum screening. This algorithm is based on the principle that short amino acid sequences (triplets) characterizing polymorphic sites of the HLA molecules are critical components of allosensitizing epitopes. Such amino acids reside in the α-helices and β-loops of the heavy chain. Because each HLA molecule expresses a characteristic string of these determinants, it is possible to characterize each molecule according to the linear sequence of amino acid triplets present on its surface. Based on the reasonable assumption that none of the triplets present in the HLA repertoire of the recipient is self-immunogenic, it is possible through a process of electronic recombination to identify donors with HLA alleles different from the recipient's but containing exclusively shared triplets. These HLA alleles will be compatible because they do not contain any epitope that is absent in the recipient.

In theory, a large number of triplets could occur if polymorphisms were randomly distributed. However, most HLA molecules span conserved domains, and only a total of 142 different polymorphic triplets designate serologically defined HLA-A, -B, and -C antigens.[147] Triplet polymorphism can occur in 30 locations on HLA-A, 27

TABLE 114.3 **Population Frequencies of Major Cross Reactive or Determinants Present on HLA-A and -B Gene Products**

Major Cross-Reactive Group	Public Epitope	Associated Private Epitopes	Approximate Epitope Frequency (%)[a]
1C	1p	A1, 3, 9 (23, 24), 11, 29, 30, 31, 36, 80	79
	10p	A10 (25, 26, 34, 43, 66), 11, 28 (68, 69), 32, 33, 74	
2C	28p	A2, 28 (68, 69), 9, 17	70
	9p	A2, 28 (68, 69), 9 (23, 24)	
	17p	A2, B17 (57, 58)	
5C	5p	B5 (51, 52), 18, 35, 53, 78	50
	21p	B5 (51, 52), 15 (62, 63, 75, 76, 77), 17 (57, 58), 21 (49, 50), 35, 53, 70 (71, 72), 73, 74, 78	
7C	7p	B7, 8, 41, 42, 48, 81	54
	22p	B7, 22 (54, 55, 56), 27, 42, 46	
	27p	B7, 13, 27, 40 (60, 61), 47	
8C	8p	B8, 14 (64, 65), 16 (38, 39), 18	38
12C	12p	B12 (44, 45), 13, 21 (49, 50), 40 (60, 61), 41	44
Bw4	Bw4	B13, 27, 37, 38, 47, 49, 51, 52, 53, 57, 58, 59, 63, 77, A24, 25, 32	79
Bw6	Bw6	B7, 8, 18, 35, 39, 41, 42, 45, 46, 48, 50, 54, 55, 56, 60, 61, 62, 64, 65, 67, 71, 72, 73, 75, 76, 78, 81	87

[a] North American White populations of European origin.

in HLA-B, and 19 in HLA-C chains. Because the HLAMatchmaker algorithm includes interlocus comparison, it is possible to accumulate the information into a single database. Among the 142 polymorphic triplets, 29 are polymorphic for one class I locus but monomorphic for another class I locus. Such polymorphic triplets cannot be immunogenic because they are always present on the patient's own HLA antigens, whereas the remaining 113 triplets have immunogenic potential. With this algorithm, it is possible to significantly broaden the number of molecularly matched HLA alleles and significantly increase the chances of identifying a compatible donor, particularly in those cases in which the recipient has a rare HLA phenotype. In addition, HLAMatchmaker considers triplets that are present in the panel cells that give negative reactions with the recipient's serum. These negative panel cells can be expected to share antigens with the patient's, but other HLA antigens may be present and contain mismatched triplets apparently not immunogenic for that patient. Such triplets are therefore acceptable and can be added to the algorithm for the identification of possible donors. Thus HLAMatchmaker assesses HLA compatibility at a molecular level by determining whether or not a triplet in a given position of a mismatched HLA antigen is also found in the same position in any of the recipient's own HLA-A, HLA-B, and HLA-C molecules. A shared triplet in the same position on a mismatched HLA antigen cannot elicit a specific antibody response in that patient. Preliminary verification of the algorithm in a series of high-PRA renal patients suggested that this is a proper strategy, at least in highly sensitized renal transplantation candidates waiting for unrelated donors.[149,150] HLAMatchmaker is also effective at selecting an optimal HLA-typed platelet component for alloimmunized thrombocytopenic patients.[150]

A new version of HLAMatchmaker considers so-called eplets and is based on the structural definitions of functional epitopes on well-characterized protein antigens that have been complexed with antibody.[151] Eplets represent amino acid residue configurations within a 3- to 3.5-Å radius of each polymorphic residue on the HLA molecular surface. Many eplets correspond to triplets, but eplets represents a more complete repertoire of structurally defined epitopes.

THE HUMAN LEUKOCYTE ANTIGEN MOLECULES AS ANTIGENS AND HUMAN LEUKOCYTE ANTIGEN ALLOIMMUNIZATION

By no means are HLA molecules antigenic in physiologic condition (with the exception of maternofetal alloimmunization). However, because of their high density on the surface of cells, they can become highly immunogenic in the nonphysiologic event in which cells from different individuals are exposed to another person's immune system. The mechanism or mechanisms leading to allosensitization are believed to follow two pathways. The first pathway mimics the one followed during most immune reactions in which antigen is uptaken by antigen-presenting cells and presented to autologous lymphocytes (indirect pathway). In this case, the donor's HLA molecules are processed into peptides through the exogenous pathway of antigen presentation and presented to autologous T cells as linear peptides.[152,153] This pathway is believed to be responsible for the development of alloantibodies as well as T-helper cell responses, but its role in the development of cytotoxic T-cell responses remains unclear. Because this pathway depends on the presentation of donor HLA molecules by recipient HLA alleles, it may explain why the humoral response to HLA class I allodeterminants correlates with the HLA phenotype of the responder.[154] The indirect pathway of HLA allorecognition has been associated with allograft rejection.[155] Because the function of HLA molecules is to present antigenic determinants to T cells, it could be easily envisioned how minor changes in their structure could be misinterpreted as antigenic epitopes. Intact HLA molecules residing on the surface of donor cells are a perfect target for T-cell-mediated allorecognition (direct pathway) either through the direct cytotoxic effect of T cells against target cells or by the activation of helper T cells through HLA class II engagement, which leads to stimulation of antibody-mediated immune responses.[156]

Humoral responses mediated most frequently by IgM are predominant in the sensitization to infrequent allogeneic exposure because they require smaller amounts of antigenic material. T-cell responses become more manifest in the context of transplantation in which the persistence of the allogeneic stimulus allows the expansion and sustenance of alloreactive cytotoxic T cells. Antibodies and TCR have different requirements for their engagement, which means epitopes recognized by T cells and antibodies are different. Antibodies require interaction with a small structure, including a limited number of amino acids; thus, any sequence combination on the surface of an HLA allele not present in the individual exposed to the alloreaction may represent an epitope. T cells have much lower binding affinity for their ligand and require a complete interaction with the peptide as well as the α and β helices of the HLA class I heavy chain.[11] Although several B-cell epitopes recognized by antibodies can be identified in a given HLA molecule, generally the whole HLA molecule is necessary for T cell–dependent allorecognition. Definition and topographic mapping of epitopes defined by serologic or cellular methods has revealed distinct regions of hypervariability in the α-1 and α-2 domains of the class I heavy chains and in the α-1 and β-1 domains of class II molecules.[157] Two types of antibody-defined epitopes can be identified according to their frequency among HLA alleles. Private epitopes are almost but not totally unique for a single serologically defined HLA antigen and are used for typing. These epitopes are generally shared by all molecular alleles present in that given family, and fine differences among alleles within a general family cannot be distinguished by antibodies. Public epitopes are more widely distributed and cluster distinct serologic families into groups. These epitopes bear an immunodominant character. Immune sera that identify public epitopes have been considered predictive of major CREGs with the idea that alloreactivity among patients belonging to the same CREG may be less likely (see Table 114.3). The predictive value of CREGs in transplant outcome or platelet transfusion results, however, remains to be demonstrated.

Not all subjects who have been exposed to alloantigens develop alloantibodies, and in fact exposure to low doses of donor-specific HLA antigens through donor transfusion may have a beneficial effect on graft survival.[158] Obviously, the degree of compatibility in the context of allosensitization may vary according to the degree of mismatch between donor and recipient. In addition, alloantibodies are one aspect of alloreaction that does not take into account cellular responses. These have been more difficult to document, although they are likely to play an important role in the context of acute transplant rejection. Several hypotheses have been discussed about the reason or reasons for the capriciousness of allosensitization, including the presence of regulatory immune responses or cytokine-mediated immunosuppression. Currently the mechanism modulating the quality and quantity of alloimmunity remains elusive, and different aspects of this algorithm are discussed ad hoc in this chapter, with particular attention to molecularly defined algorithms for the prediction of histocompatibility.[130,147,159,160]

Clearly, HLA matching is beneficial in patients undergoing renal transplantation. An analysis of more than 150,000 recipients receiving transplants in different centers participating in the Collaborative Transplant Study showed that a complete mismatch (6 HLA-A+B+DR) had a 17% lower survival expectation than no mismatch ($P < .0001$).[161] Matching was particularly beneficial in patients with highly reactive preformed alloantibodies. The same study suggested that high-resolution matching based on molecular typing improved graft survival. Similar results were observed in cases of cardiac transplantation in which HLA matching yielded significantly better results ($P < .0001$). This is particularly important because donor hearts are currently not allocated according to HLA matches in most centers. In cases of liver transplantation, HLA matching was not beneficial.[162]

Donor-specific hyporesponsiveness has been particularly well documented in the context of renal allotransplantation and may limit the need for immunosuppression. A recent randomized study suggested that pretransplant donor transfusions improved the survival of cadaver kidney grafts in patients receiving modern immunosuppressive regimens, but the mechanism remains unclear.[163,164] Although most centers currently do not implement deliberate blood transfusions, the usefulness of this approach needs to be investigated further.

Approximately 5% to 10% of platelet transfusions are given to patients who have been previously exposed to HLA class I–expressing heterologous cells and are reactive to HLA antigens. Such patients are refractory to random-donor platelets and must be given HLA-matched or semi-matched platelet pheresis components.[41–44] However, a provision of HLA-matched platelets does not always improve platelet recovery and survival. Possibly, the ineffective platelet transfusion is in part caused by unrecognized HLA mismatches between the donor and recipient resulting from the low-resolution methods used for typing, and higher resolution methods have been advocated. It is currently controversial whether or not molecularly based HLA typing confers an advantage over serologic typing, and this principle was recently questioned in the context of hematopoietic cell transplantation.[165,166]

HUMAN LEUKOCYTE ANTIGEN AS A FUNCTIONAL MEDIATOR OF GRAFT-VERSUS-HOST DISEASE AND/OR GRAFT-VERSUS-NEOPLASIA EFFECT

Allogeneic or syngeneic BMT is used predominately for the treatment of hematologic malignancies[167–169] and other hematologic disorders such as aplastic anemia,[170] thalassemia,[171] or myelodysplastic syndrome.[172] This strategy can induce long-term disease-free survival in chronic myelogenous leukemia patients.[167,169] The objective of BMT in cases of malignancy is to cure the patient by eradication of the neoplastic cells with myeloablative chemotherapy followed by restoration of hematopoiesis through the transplantation of normal hematopoietic stem cells derived from HLA-compatible normal donors. This strategy is characterized by the insurgence of an immune reaction toward the host's normal cells (GVHD),[173–175] which is often associated and preferentially targets neoplastic cells (GVN effect).[176–183] Both the GVHD and the GVN effect occur in the presence of a full HLA match; thus the HLA molecules are not targets of allosensitization themselves but present polymorphic molecules expressed by the recipient's cells recognized by the grafted immune cells.

GRAFT-VERSUS-HOST DISEASE

GVHD represents the alloimmune reaction of donor lymphocytes against normal cells of the recipient. GVHD occurs predominantly in association with hematopoietic progenitor cell transplantation (HPCT) compared to other types of transplants because the hematopoietic transplant is enriched with immune cells. Myeloablative therapy is generally administered before transplantation and, as a consequence, prevents graft rejection in most cases. GVHD is a major complication of HPCT, and a fine balance between GVHD and graft rejection is maintained by modulating the level of post-transplantation immunosuppression.[177] In addition, other major disturbances associated with HPCT, such as overwhelming infection, leukemia, tumor relapse, and other regimen-related morbidities are strongly influenced by the treatment of GVHD. With the advent of nonmyeloablative HPCT for the treatment of nonhematologic diseases such as solid tumors, GVHD has reached a predominant role because of its close association with the GVN effect.

T-cell depletion has been advocated for the prevention of GVHD by decreasing the probability of cellular and humoral alloresponses.[184,185] This strategy decreases the occurrence of GVHD but is associated with an increased risk for graft rejection and tumor or leukemia relapse.[177] In fact, Weiden et al.[186] observed that survivors of severe acute GVHD had a significantly lower incidence of tumor relapse compared with patients who did not experience GVHD. This association appeared mandatory, and it was believed that the beneficial GVN reaction was inseparable from GVHD.

The risk for GVHD increases with genetic distancing between donor and recipient. Thus recipients of transplants from HLA-identical twins have a lesser chance of developing GVHD than recipients of transplants from HLA-unrelated donors and from donors with only a partial HLA match.[187,188] Interestingly, however, although the genetic closeness between donor and recipient appears to decrease the risk for GVHD, it also decreases the therapeutic benefit, with increased chances of tumor relapse.

GRAFT-VERSUS-NEOPLASIA EFFECT

It was originally observed that a beneficial collateral effect of GVHD was the rejection of neoplastic cells by the donor immune system in the context of hematologic malignancies (graft-versus-leukemia effect).[181] It was rapidly recognized that the graft-versus-leukemia effect could play a powerful therapeutic role in the treatment of refractory malignant disorders, including some solid tumors (graft-versus-tumor effect).[178] Because the biology and clinical principles underlining the two effects are likely similar, for simplicity, in this chapter we coin a unifying term: *graft-versus-neoplasia effect*. Indeed, there is a perception that the GVN reaction is the most potent form of tumor immunotherapy currently in clinical use. Its mechanism of action is still poorly understood. T cells definitely play a fundamental role in the initiation and maintenance of the alloreaction toward neoplastic cells.[189] A sevenfold increase in the chance of relapse was noted in patients with chronic myelogenous leukemia who received a T-cell-depleted BMT as compared with a subset of patients who had received a T-cell-repleted BMT but did not develop GVHD.[184,185] This result suggested that GVHD is a biologic entity different from the GVN effect. In addition, on leukemia relapse, administration of donor lymphocyte infusion could reinduce clinical remission.[190] Finally, leukemia-specific CD8$^+$ T cells have been identified in circulating lymphocytes at the time of leukemia regression.[191] NK cells play an additional role in mediating this phenomenon, and clinical data suggest that mismatch of NK receptor and ligands during allogeneic BMT can be used to enhance the GVN effect.[93,192] Appreciation of the GVN effect has led to the development of nonmyeloablative stem cell transplants designed to immunosuppress the host to a level sufficient to permit engraftment of the donor immune cells to generate GVN without inducing the serious complications associated with myeloablation.[177] The GVN effect has gained popularity in the last decade to the point that allogeneic-based immunotherapeutic approaches have been advocated for several nonhematologic malignancies.[177] The rationale is largely based on the assumption that the immune cell repertoire capable of recognizing cancer cells in the allogeneic context is broader than in the autologous system. Donor T cells can target not only tumor-specific antigens but also allelic variants of these antigens, mHag, and, in the case of HLA-mismatched transplants, HLA antigens disparate from the donor expressed by the tumor cells.[193–195] Although there are several theories about how the GVN effect occurs, it remains unclear why allo-T cells have a better chance of targeting tumor cells in an allogeneic context as compared with the natural insurgence of antitumor immunity described for several solid tumors.

HUMAN LEUKOCYTE ANTIGEN AND T-CELL-DIRECTED IMMUNIZATION

The last decade has witnessed remarkable progress in the identification and mapping of T-cell epitopes for various infectious diseases and cancer. In particular, progress was made in mapping HLA-associated epitopes for HIV, cytomegalovirus, and Epstein-Barr virus.[196–199] In addition, the molecular identification of tumor-associated antigens has yielded a large number of epitopes that could be used to immunize against neoplasia.[200] A comprehensive discussion of these topics is beyond the scope of this chapter, but we address a few points framing the relevance of HLA in the context of T-cell-directed immunization (viral specific T cells).

The identification of T-cell epitopes led to two major areas of clinical investigation. The first is active-specific immunization to prevent or treat ongoing infections or cancer. The second is the harvest and in vitro expansion of immunization-induced T cells for adoptive transfer. In general, active immunization has proved successful in inducing epitope-specific T cells easily detectable among circulating lymphocytes.[120,201–203] However, often the immunization-induced enhancement of T-cell function is not associated with clinical improvement. Although the reason for the clinical ineffectiveness of immunization-induced T cells is unclear, it has been postulated that they may be quantitatively[201] or qualitatively[204] inadequate for eradicating disease. Therefore a second strategy is being pursued whereby the number of antigen-specific T cells is amplified in vitro for autologous or donor-derived adoptive transfer. This second strategy has met some promising success in the context of ganciclovir-resistant cytomegalovirus infection,[205] Epstein-Barr virus–induced posttransplantation lymphoproliferative disorders,[206] and metastatic melanoma.[207]

Whether delivered as a primary form of therapy or to prime in vivo T cells for further ex vivo expansion, epitope-specific vaccination encounters the major limitation of a stringent requirement for HLA allelic association. Although super families of HLA alleles may share epitopes,[14,16] in practical terms, clinically relevant HLA-epitope associations are restricted to few peptide-allele combinations for a given protein.[22] Patients considered for enrollment in immunization protocols are best served by high-resolution HLA typing to exclude subtypes with unproven immunogenic potential for a given epitope. To bypass the stringent HLA requirements demanded by epitope-specific vaccination, whole-antigen delivery is suggested. This is based on the assumption that the epitope repertoire of a protein can be adjusted to distinct HLA phenotypes by a cellular process of self-selection naturally coupling peptides to HLA according to binding affinity. Although theoretically indisputable, in practice, the truth of this assumption depends on the efficiency with which individual molecules are processed and presented in association with distinct HLA alleles. Even in these settings, high-resolution typing is desirable because it allows accurate interpretation of immunization results by allowing a comparison between the detailed genetic makeup of the individual receiving the vaccine and his or her antigen-specific immune response. It is likely that in the future HLA laboratories will receive increasing demands for high-resolution, definitive typing for appropriate patient enrollment and for subsequent interpretation of immune responses.

MONITORING IMMUNE RESPONSES WITH TETRAMERIC HUMAN LEUKOCYTE ANTIGEN-PEPTIDE COMPLEXES

A growing understanding of the molecular immunology of T-cell interactions with HLA/epitope complexes in the context of infectious disease, virally induced malignancies, and spontaneous tumors as well as interest in their treatment with T-cell-directed vaccines has generated more attention to the issue of using accurate methods to quantify ex vivo the extent of antigen-specific immune responses.[208] The most commonly used assays available for the enumeration of antigen-specific T cells include tHLA; intracellular fluorescence-activated cell sorter staining for cytokine expression on cognate stimulation; detection of cytokine release by enzyme-linked immunospot assay; and quantitative real-time PCR, recently reviewed by Keilholz et al.[209] tHLAs are complexes of four HLA molecules combined with a specific peptide and bound to a fluorochrome (Fig. 114.6). These complexes bind to complementary TCR and identify antigen-specific T cells,[210] measuring cellular responses against specific epitopes with sensitivity as low as 1 in 5000 CD8$^+$ T cells. To synthesize tHLA molecules, soluble HLA heavy chains containing a biotinylation site and recombinant β_2-microglobulin are synthesized and purified. They are then refolded in the presence of the specific epitope, and the monomer is isolated by gel filtration and is biotinylated. Fluorescent streptavidin is added to induce tetramerization. An aliquot of tetramers is added to the peripheral blood mononuclear cells together with other antibodies for a more detailed characterization of antigen-specific T cells.[204] Analysis is performed using a flow cytometer. Because of the specificity of vaccines, the patient's HLA type and specific peptide must be identified and synthesized to provide the adequate tetramer. Pentameric HLA/peptide complexes have recently become available and in some laboratories have replaced the use of tHLA.

Analysis of tHLA offers many potential advantages over other T-cell assays. This method is quantitative and enables an estimation of the avidity between TCR and peptide-loaded HLA class I molecules. tHLA staining does not kill the labeled cells, allowing sorting of subpopulations by flow cytometry for additional analysis or expansion for adoptive transfer. With tHLA specific T cells can be analyzed from blood samples without the prerequisite of in vitro culture, and all specific cytotoxic T lymphocytes are detected, regardless of their functional status.[201,204,211]

HUMAN LEUKOCYTE ANTIGEN SUMMARY

The relevance of HLA in clinical pathology has broadened from its role as a predictor of allosensitization to a mediator of GVHD and

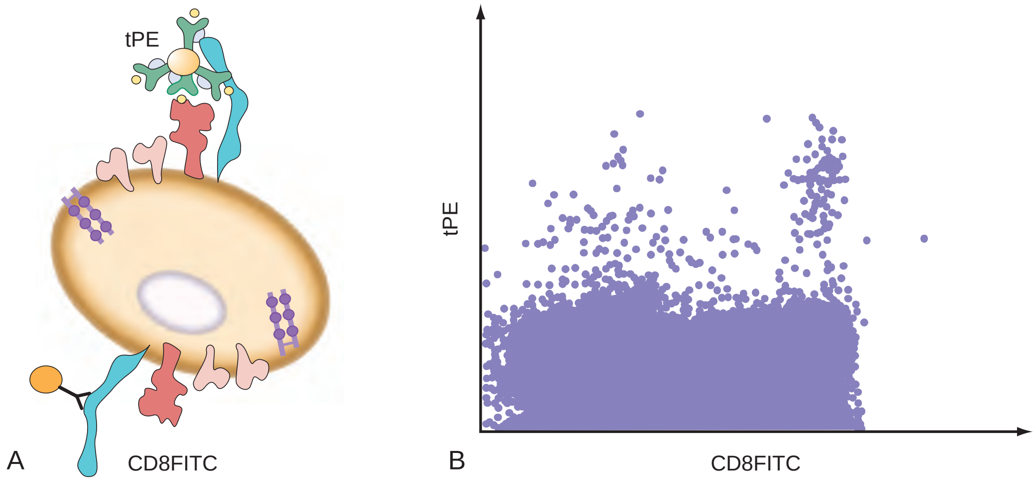


Figure 114.6 SCHEMATIC REPRESENTATION OF THE MECHANISM OF BINDING OF TETRAMERIC PEPTIDE/HUMAN LEUKOCYTE ANTIGEN COMPLEXES (THLA) TO ANTIGEN-SPECIFIC T CELLS. (A) Binding of tHLA to T-cell receptor. The tHLA consists of four HLA/peptide complexes identical to the ones recognized by the T cell on the surface of live cells. Each HLA molecule is modified to contain one biotin molecule that serves as a bridge for binding to tetravalent streptavidin molecules fluorescently labeled. (B) Actual fluorescence-activated cell sorter (FACS) analysis result of CD8$^+$, tHLA-positive T cells. *(Modified from Monsurro V, Nargosen D. Immunotracking of specific cancer vaccine CD8$^+$ lymphocytes, ASHI Q. 2003;26:100.)*

GVN. The understanding of action mechanisms in various nonclassic HLA genes as well as KIR has opened a new field. The study examining the function of the innate immune response in the context of transplantation and other immunopathologies is a result of this. Together with HLA, immunogenetics is rapidly growing, driven by the realization that polymorphism is the hallmark of human immunopathology as its spans mHag, KIR, cytokines, and their receptors. Each of these is interwoven in an intricate array of interdependent functions that cannot be discounted. Modern methods should be able to adopt high-throughput systems for the parallel assessment of all of these variables when addressing the genetic makeup of an individual in correlation with the natural or therapeutic history of his or her disease. The comparison of donor and recipient protein profiling and cytokine polymorphism may offer insights into the rejection mechanism when matched donor grafts are used. Finally, the increased use of T-cell-directed immunization is driving renewed interest in high-resolution typing of HLA molecules to allow a more accurate interpretation of clinical and immunologic results. In the coming years, there will be an abundance of data generated from various methods for next generation sequencing. Utilization of this data will have tremendous impact on understanding the clinical and immunological implications in regard to HLA. This data will require an enormous effort with bioinformatics at the forefront.

HUMAN NEUTROPHIL ANTIGENS AND THEIR CLINICAL SIGNIFICANCE

Lalezari et al. described the first granulocyte antigens. These antigens were designated *N* for neutrophil. Each antigen system was described alphabetically, and each allele was described numerically in order of discovery. They identified NA1 in 1966 and its allele, NA2, in 1972.[212,213] In 1971, Lalezari et al.[213] described another granulocyte antigen, NB1. Reports of several other granulocyte antigens followed.

A new nomenclature was established in 1998 by the International Society of Blood Transfusion Working Party (Table 114.4).[214] In this nomenclature, antigen systems are referred to as *human neutrophil antigens*, or HNA. The antigen systems—that is, the polymorphic forms of the immunogenic proteins—are indicated by integers, and specific antigens within each system are designated alphabetically by date of publication. Alleles of the coding genes are named according to the Guidelines for Human Gene Nomenclature. Neutrophil antigens NA1 and NA2 became HNA-1a and HNA-1b, and NB1 became HNA-2. The serology, biochemistry, molecular biology, and clinical significance of these antigens are reviewed here.

THE HNA-1 ANTIGEN SYSTEM

Expression of HNA-1 Antigens

HNA-1 antigens are expressed only on neutrophils. HNA-1 antigens are located on the low-affinity Fc-γ receptor IIIb (FcγRIIIb), CD16.[215–218] FcγRIIIb, HNA-1a, HNA-1b, HNA-1c, and HNA-1d antigens are expressed on all segmented neutrophils, approximately one-half of neutrophilic metamyelocytes, and approximately 10% of neutrophilic myelocytes.[219] Neutrophil expression of FcγRIIIb and HNA-1 antigens are diminished by the treatment of neutrophils with stimulants such as complement component C5a and chemotaxin F-met-leu-phe or granulocyte colony-stimulating factor (G-CSF). Soluble Fc γ RIIIb is present in plasma, and it has the same HNA-1 polymorphisms found on neutrophils. Fc γ RIIIb released from granulocytes is the source of the soluble Fc γ RIIIb and HNA-1 antigens.[220]

Biochemistry

Fc γ RIIIb is a glycoprotein with 233 amino acids and is glycosylphosphatidylinositol (GPI) anchored.[215–218] Its molecular weight is 50 to 80 kDa, and the glycoprotein has *N*-linked carbohydrate side chains. HNA-1a form of Fc γ RIIIb is 50 to 65 kDa, the HNA-1b form of Fc γ RIIIb is 65 to 80 kDa, and the heterozygous form is 50 to 80 kDa. Differences in *N*-glycosylation account for the differences in molecular mass.

Molecular Biology

Fc γ RIIIb and the HNA-1 antigens are encoded by the FCGR3B gene located on chromosome 1q23–24 within a cluster of two families of Fc γ R genes, FCGR2 and FCGR3. The FCGR3 family is made up of FCGR3A and FCGR3B. FCGR3B is highly homologous to FCGR3A, which encodes Fc γ RIIIa. Only four nucleotides differ between FCGR3B and FCGR3A (Table 114.5). The most important difference between the two genes is a C to T change at 733 in FCGR3B that creates a stop codon. As a result, FCGR3A has 21 more amino acids than FCGR3B, and FCGR3A is a transmembrane rather than GPI-anchored glycoprotein. Fc γ RIIIa is not recognized by antibodies specific to HNA-1 antigens, but the similarities between FCGR3A and FCGR3B complicate genotyping of HNA-1 alleles.

HNA-1a, -1b, -1c and -1d Polymorphisms

The neutrophil-specific HNA-1 antigen system is made up of four alleles: HNA-1a, -1b, -1c and -1d (see Table 114.4).[221] The gene frequencies of the alleles vary widely among different racial groups. Among Whites of European descent, the frequency of the gene encoding HNA-1a, FCGR3B*01, is between 0.30 and 0.37, and the frequency of the gene encoding HNA-1b, FCGR3B*02, is between 0.63 and 0.70.[222–226] In Japanese and Chinese populations, the FCGR3B*01 gene frequency is between 0.60 and 0.66, and the FCGR3B*02 gene frequency is between 0.30 and 0.33.[215,216,219,223–226] The gene frequency of the gene encoding HNA-1c, FCGR3B*03, also varies among racial groups. FCGR3B*03 is expressed by neutrophils in 4% to 5% of Whites and 25% to 38% of African Americans.[227]

The FCGR3B*01 gene differs from the FCGR3B*02 gene by only five nucleotides in the coding region, at positions 141, 147, 227, 277, and 349 (see Table 114.5).[215–218] Four of the nucleotide changes result in changes in the amino acid sequence between the HNA-1a and HNA-1b forms of the glycoprotein. The fifth polymorphism at 147 is silent. The glycosylation pattern of the protein differs between the two antigens because of two nucleotide changes at bases 227 and 277. The HNA-1b form has six *N*-linked glycosylation sites, and the HNA-1a form has four glycosylation sites.

TABLE 114.4 International Society of Blood Transfusion Nomenclature

Antigen System	Antigens	Location	Former Name	Alleles
HNA-1	HNA-1a	FcγRIIIb, CD16	NA1	FCGR3B*01
	HNA-1b	FcγRIIIb, CD16	NA2	FCGR3B*02
	HNA-1c	FcγRIIIb, CD16	SH	FCGR3B*03
	HNA-1d	FcγRIIIb, CD16		FCGR3B*02
HNA-2	HNA-2	CD177 (NB1 gp)	NB1	CD177
HNA-3	HNA-3a	CTL-2	5b	SLC44A2*01
HNA-4	HNA-4a	CD11b (CR3)	Mart(a)	ITGAM*01
HNA-5	HNA-5a	CD11a (LFA-1)	Ond(a)	ITGAL*01

CR3, C3bi receptor; *gp*, glycoprotein; *HNA*, human neutrophil antigen; *LFA-1*, leukocyte function–associated antigen-1.

The gene encoding the HNA-1c form of Fc γ RIIIb, FCGR3B*03, is identical to FCGR3B*02 except for a C to A substitution at nucleotide 266, resulting in an alanine to aspartate change at amino acid 78 of Fc γ RIIIb (see Table 114.5).[221] In many cases, FCGR3B*03 exists on the same chromosome with a second or duplicate FCGR3B gene.[221,228]

Antibodies to HNA-1d have recently been described in two cases of neonatal immune neutropenia. The antigen HNA-1d is also encoded by FCGRB*02.[229]

FC γ RIIIB Deficiency

Blood cells from patients with paroxysmal nocturnal hemoglobinuria lack the GPI-linked glycoproteins, and their granulocytes express reduced amounts of Fc γ RIIIb and the HNA-1 antigens.[218] Genetic deficiencies of granulocyte Fc γ RIIIb and HNA-1 antigens have also been reported. With inherited deficiency of Fc γ RIIIb, the FCGR3B gene is deleted along with an adjacent gene, FCGR2C.[222,230] Among White subjects, the incidence of individuals homozygous for FCGR3B deletion is about 0.1%.[231,232] However, among Africans and African Americans, the incidence is much higher; in one study, 3 of 126 Africans were found to be FCGR3B-deficient,[227] and in another, 1 of 53 was found to be FCGR3B-deficient.[225]

Function of HNA-1 Antigens

The low-affinity Fc-γ receptors link humoral immunity to cellular immune function; specifically, Fc γ Rs on effector cells recognize cytotoxic IgG molecules and immune complexes containing IgG molecules. Polymorphisms in Fc γ RIIIb affect neutrophil function. Neutrophils that are homozygous for HNA-1b have a lower affinity for IgG3 than granulocytes homozygous for HNA-1a.[233] Neutrophils from people who are homozygous for HNA-1b phagocytize erythrocytes sensitized with IgG1 and IgG3 anti-Rh monoclonal antibodies and bacteria opsonized with IgG1 at a lower level than granulocytes homozygous for HNA-1a.[234,235]

The Clinical Relevance of FC γ RIIIB Deficiency

Despite the important role of Fc γ RIIIb in neutrophil function, deletion of the entire Fc γ RIIIB gene does not cause major clinical problems, and most people with Fc γ RIIIb deficiency are healthy. However, too few patients have been studied to identify a slight increase in susceptibility to infection or autoimmune disease due to Fc γ RIIIb deficiency.

THE HNA-2 ANTIGEN SYSTEM

HNA-2 is an isoantigen without allelic variation. It was previously known as NB1.[236] Monoclonal antibodies specific to HNA-2 have been clustered as CD177.

TABLE 114.5 Nucleotide Differences Among the Genes Encoding the HNA-1a, HNA-1b, and HNA-1c Antigens of Fcγ RIIIb Base Pair Position

Gene	141	147	227	266	277	349	473	505	559	641	733
FCGR3B*01	AGG	CTC	AAC	GCT	GAC	GTC	GAC	CAC	GTT	TCT	TGA[a]
FCGR3B*02	AGC	CTT	AGC	GCT	AAC	ATC	GAC	CAC	GTT	TCT	TGA[a]
FCGR3B*03	AGC	CTT	AGC	GAT	AAC	ATC	GAC	CAC	GTT	TCT	TGA[a]
FCGR3A	AGG	CTC	AGC	GCT	GAC	ATC	GGC	TAC	TTT	TTT	CGA

Note: Differences among genes are in bold.
HNA, Human neutrophil antigen.
*N-Glycosylation site.
[a] Stop codon.

Expression of HNA-2

HNA-2 is is expressed only on neutrophils, neutrophilic metamyelocytes, and myelocytes.[219,237] HNA-2 is unique in that it is expressed on subpopulations of neutrophils. The mean size of the HNA-2–positive subpopulation of neutrophils is 45% to 65%.[238–240] The expression of HNA-2 is greater on neutrophils in women.[239,241] The size of the HNA-2–positive subpopulation of neutrophils in women is approximately 60%, compared with approximately 50% for men. Neutrophil expression of HNA-2 is greater in pregnant women than in healthy female blood donors.[241] The surface expression of HNA-2 is slightly upregulated by treatment with the chemotactic peptide F-met-leu-phe.[238] The administration of G-CSF to healthy subjects for several days increases the proportion of neutrophils expressing HNA-2 to near 90%.[242]

CD177 Glycoprotein Biochemistry

The glycoprotein carrying HNA-2a, CD177, was previously known as the NB1 glycoprotein. It is located on neutrophil plasma membranes and secondary granules[238,243] and is linked to the plasma membrane via a GPI anchor.[238] Although some GPI-anchored proteins are shed by F-met-leu-phe–treated neutrophils, CD177 is not, nor is soluble CD177 glycoprotein present in plasma.[238] The molecular weight of CD177 is 58 to 64 kDa, and it contains *N*-linked carbohydrate side chains.[238,243]

Molecular Biology

The gene encoding CD177 is located on chromosome 19q13.31, and its coding region consists of 1311 bp that code for a protein of 416 and a signal peptide of 21 amino acids.[244,245] The predicted protein has two cysteine-rich domains, three potential *N*-linked glycosylation sites, and a potential ω-site for attachment of the GPI anchor.[245] This gene belongs to the Ly-6 snake toxin superfamily. Other genes in this family include urokinase-type plasminogen activator receptor (uPAR) (CD87) and decay-accelerating factor (CD59).

Polymorphisms

HNA-2 is expressed on neutrophils by approximately 97% of persons of European origin, 95% of African Americans, and 89% to 99% of Japanese.[239,246–248] A HNA-2 negative neutrophil phenotype is caused by a CD177 transcription defect that is not completely understood.[249,250] The most common cause is an A to T change at position 787 that results in a premature stop codon within exon 7. Heterozygosity for 787 A and 787 T has been associated with reduced expression of HNA-2.[250]

Clinical Relevance of HNA-2 Antigens

The rare women who produce HNA-2–specific alloantibodies and who lack NB1 glycoprotein are healthy. The expression of HNA-2 is reduced on neutrophils from people with paroxysmal nocturnal hemoglobinuria and chronic myelogenous leukemia,[237] but it is unknown whether this has any clinical significance. CD177 mRNA is overexpressed by neutrophils from patients with polycythemia rubra vera and essential thrombocytosis.[251,252] The role of CD177 in neutrophil function is unknown. In some studies, HNA-2 may have a role in the adhesion of neutrophils to endothelial cells.[253]

THE HNA-3 ANTIGEN SYSTEM

The HNA-3 antigen system has one antigen, HNA-3a, which was previously known as 5b. HNA-3a is expressed by neutrophils, lymphocytes, platelets, endothelial cells, and kidney, spleen, and placental cells. HNA-3a has a gene frequency of 0.66.[254] HNA-3a is located on the choline transport-like protein-2 (CTL-2) and is encoded by SLC44A2. The HNA-3a phenotype is due to a nucleotide polymorphism that results in an arginine rather than a glutamine at position 152 of CTL-2.[250,255,256] Several cases of transfusion-related acute lung injury (TRALI) have been associated with transfusion of plasma containing anti-HNA-3a.[257,258]

HNA-4 AND HNA-5 ANTIGEN SYSTEMS

HNA-4 and HNA-5 antigens are located in the β_2-integrins. Each antigen system contains only a single antigen, HNA-4a and HNA-5a, respectively. The HNA-4a antigen was previously known as Mart(a). HNA-4a has a phenotype frequency of 99.1% in White individuals. HNA-4a has been located on the αM chain (CD11b) of the C3bi receptor (CR3) and is caused by a single nucleotide substitution of G to A at position 230 of the gene ITGAM.[250,259] The substitution would be predicted to result in an arginine-to-histidine polymorphism at amino acid 61.

A second polymorphism of the β_2-integrins, HNA-5a, was first described as Ond(a). HNA-5a was found to be expressed on the αL integrin unit, leukocyte function–associated antigen-1 (LFA-1) (CD11a) and is caused by a G to C single nucleotide substitution at position 2372 of the gene ITGAL. This change predicts an amino acid change of arginine to threonine at amino acid 766.[250,259]

CLINICAL SIGNIFICANCE OF ANTIBODIES TO NEUTROPHIL ANTIGENS

Alloimmune Neonatal Neutropenia

During pregnancy, mothers can become alloimmunized to neutrophil antigens. Maternal IgG directed to neutrophils can cross the placenta and destroy the neonate's neutrophils. Maternal alloimmunization to neutrophil antigens can occur in utero and affect the first child. Most neonates experience isolated neutropenia, but the cytopenias are self-limiting and resolve as the antibody is cleared. The alloimmunized mothers produce and carry the antibodies, but the antibodies do not react with the mother's blood cells or tissues, and they have normal neutrophil counts. Antibodies to neutrophil-specific antigens HNA-1a, HNA-1b, and HNA-2 most commonly cause neonatal alloimmune neutropenia (Table 114.6).[254,260] Antibodies to HNA-1c, HNA-3a, and HNA-3b rarely cause alloimmune neonatal neutropenia.[261] Mothers with FcγRIIIb deficiency have produced FcγRIIIb-specific antibodies that caused neonatal neutropenia.[231,254,260]

Newborns with alloimmune neutropenia are usually asymptomatic. Most often, the neutropenia is detected in the first week of life when the neonate becomes febrile or develops an infection and a neutrophil count is done. Typically, the counts are 0.100 to 0.200 × 10^9/L. Some neonates have normal neutrophil counts the first day of life, but they become neutropenic on their second day.[260] White blood cell count, platelet count, and hemoglobin level are usually normal, but eosinophilia or monocytosis may be present. If a bone marrow biopsy is performed, it often shows normal numbers of erythroid progenitors and megakaryocytes with hyperplasia of myeloid progenitors.

The clinical course is quite variable. An occasional infant is asymptomatic, but almost all affected children have an infection. The most common infections are umbilicus infections, skin infections, abscesses, and respiratory tract infections. Less commonly, infants experience otitis media, urinary tract infections, and gastroenteritis. Serious infections such as sepsis, pneumonia, and meningitis can occur. The duration of the neutropenia may be as short as a few days or as long as 28 weeks.[260] The mean duration of neutropenia is about 11 weeks.[260]

For an asymptomatic child, no immediate treatment may be required. Prompt and aggressive antibiotic treatment of children with fevers or other signs of infections is indicated. Intravenous

TABLE 114.6 Specificities of Antibodies in Alloimmune Neonatal Neutropenia

Antigen	$N = 18$[211]	$N = 48$[253]
HNA-1a	28%	10%
HNA-1b	22%	8%
FcγRIIIb	0%	8%
HNA-2a	11%	2%
HNA-3a	0%	2%
Unknown	0%	15%
HLA class I[251]	11%	54%
Negative	28%	38%

HLA, Human leukocyte antigen; *HNA*, human neutrophil antigen.

immunoglobulin has a limited role in the treatment of neonatal alloimmune neutropenia. Approximately half the patients treated have a transient increase in count lasting only a few days. The use of G-CSF to treat alloimmune neutropenia has also had mixed results. The administration of G-CSF elevates the neutrophil count in some, but not all, neonates.[260]

AUTOIMMUNE NEUTROPENIA OF CHILDHOOD

Autoimmune neutropenia has been well described in children.[262–265] Typically the onset of the autoimmune neutropenia of children begins at 8 months of age, but children between 1 and 36 months of age can be affected. Most studies found that neutrophil counts recover spontaneously by the age of 5 years, with a median of 13 to 20 months of neutropenia.[262–265]

In most cases, children presented with severe neutropenia having neutrophil counts of less than 0.5×10^9/L. Monocytosis has been reported to occur in up to 38% of patients. Results of bone marrow biopsies in affected patients usually show normal to hypercellular marrow with a decreased number of mature granulocytes. Febrile episodes and infections, including bacterial skin infections, otitis media, respiratory tract infections, and urinary tract infections, are common. Life-threatening complications are rare.

Antibodies to neutrophils can be detected in up to 98% of affected patients. If an antibody specificity is identified, the antibodies are almost always specific to epitopes located on FcγRIIIb. The antibodies are directed to HNA-1a in 10% to 46% of patients, to HNA-1b in 2% to 3% of patients, and rarely to FcγRIIIb epitopes expressed by granulocytes from all donors.[262,265]

Autoimmune neutropenia has been treated with corticosteroids, intravenous immunoglobulin, and G-CSF. Approximately half the patients responded to intravenous immunoglobulin, but neutrophil counts remained elevated for only 1 week.[262] Almost all the patients responded to G-CSF and 75% to corticosteroids, and neutrophil counts remained elevated as long as the drugs were given.

TRANSFUSION REACTIONS

Antibodies to neutrophil and HLA antigens can cause febrile nonhemolytic transfusion reactions and TRALI. Before the widespread transfusion of leukocyte-reduced blood components, approximately 0.5% of transfusions were associated with febrile nonhemolytic transfusion reactions, and leukocyte antibodies are a common cause of these reactions. These febrile reactions are caused by the interaction of leukocyte antibodies in the transfusion recipient with leukocytes contained in the transfused blood components. These reactions can be prevented by the use of components that have been filtered to remove leukocytes.

A more serious type of transfusion reaction associated with leukocyte antibodies is the acute noncardiac pulmonary edema, or TRALI. This entity is characterized by acute respiratory distress that usually occurs within 4 hours after a transfusion. These reactions are characterized by dyspnea, hypoxia, and bilateral pulmonary infiltrates on chest radiograph without cardiomegaly or pulmonary vascular congestion. The mortality rate associated with TRALI is approximately 5%.[266] Of patients with TRALI, 80% have rapid resolution of pulmonary infiltrates and return of arterial blood gas values to normal within 96 hours after the initial respiratory insult. However, pulmonary infiltrates have persisted for at least 7 days after the transfusion reaction in 17% of TRALI patients.

TRALI has been associated with both neutrophil and HLA antibodies. Antibodies reported in these reactions include HNA-1a, HNA-1b, HNA-2, HNA-3a, and HLA class I and II antibodies. Most of these cases involve the passive transfusion of the offending antibody in donor plasma, as contrasted with the reactivity of the recipient's antibody with donor leukocytes to cause febrile nonhemolytic reactions. Retrospective studies involving antibodies to HNA-3a have implicated blood components from single donors with anti-HNA-3a in several TRALI cases.[257]

NEUTROPHIL ANTIGENS SUMMARY

Five neutrophil antigen systems, HNA-1,HNA-2, HNA-3, HNA-4 and HNA-5, have been well described. HNA-1 antigens are located on FcγRIIIb, and antibodies to these antigens are frequently implicated in autoimmune and alloimmune neutropenia. HNA-2 is located on CD177 glycoprotein, and antibodies to HNA-2 are found in patients with alloimmune and autoimmune neutropenia. Antibodies to HNA-3a are rare, but, relative to other neutrophil antibodies, may be more frequently associated with cases of TRALI. The significance, if any, of HNA-4a and HNA-5a antibodies is uncertain.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Braun WE. Update in kidney transplantation: Increasing clinical success, expanding waiting lists. *Cleve Clin J Med*. 2002;69:501.

Bux J, Behrens G, Jaeger G, et al. Diagnosis and clinical course of autoimmune neutropenia in infancy: analysis of 240 cases. *Blood*. 1998;91:181.

Bux J, Jung KD, Kauth T, et al. Serological and clinical aspects of granulocyte antibodies leading to alloimmune neonatal neutropenia. *Transfus Med*. 1992;2:143.

Childs R, Srinivasan R. Advances in allogeneic stem cell transplantation: directing graft-versus-leukemia at solid tumors. *Cancer J Sci Am*. 2002;8:2.

Daser A, Michinson H, Michinson A, et al. Non-classical-MHC genetics of immunological disease in man and mouse: the key role of proinflammatory cytokine genes. *Cytokine*. 1996;8:593.

Dawkins RL, Degli-Esposti MP, Abraham LJ, et al. Conservation versus polymorphism of the MHC in relation to transplantation, immune responses and autoimmune disease. In: Klein J, Klein D, eds. *Molecular Evolution of the Major Histocompatibility Complex*. Berlin: Springer-Verlag; 1991:391.

De Haas M, Kleijer M, van Zwieten R, et al. Neutrophil FcγRIIIb deficiency, nature, and clinical consequences: a study of 21 individuals from 14 families. *Blood*. 1995;86:2403.

Duquesnoy RJ, Marrari M. HLAMatchmaker: a molecularly based algorithm for histocompatibility determination. II. Verification of the algorithm and determination of the relative immunogenicity of amino acid triplet-defined epitopes. *Hum Immunol*. 2002;63:353.

Hennecke J, Wiley DC. T cell-receptor-MHC interactions up close. *Cell*. 2001;104:1.

Kim CJ, Parkinson DR, Marincola FM. Immunodominance across the HLA polymorphism: implications for cancer immunotherapy. *J Immunother*. 1997;21:1.

Kissel K, Santoso S, Hofmann C, et al. Molecular basis of the neutrophil glycoprotein NB1 (CD177) involved in the pathogenesis of immune neutropenias and transfusion reactions. *Eur J Immunol*. 2001;31:1301.

Matzinger P. An innate sense of danger. *Semin Immunol*. 1998;10:399.

Opelz G, Vanrenterghem Y, Kirste G, et al. Prospective evaluation of pretransplant blood transfusion in cadaver kidney recipients. *Transplantation*. 1997;63:964.

Opelz G, Wujciak T, Dohler B, et al. HLA compatibility and organ transplant survival: collaborative transplant study. *Rev Immunogenet*. 1999;1:334.

Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J Immunol*. 1994;152:163.

Petersdorf EW, Hansen JA, Martin PJ, et al. Major-histocompatibility-complex class I alleles and antigens in hematopoietic-cell transplantation. *N Engl J Med*. 2001;345:1794.

Petz LD, Garratty G, Calhoun L, et al. Selecting donors of platelets for refractory patients on the basis of HLA antibody specificity. *Transfusion*. 2000;40:1446.

Stroncek DF, Skubitz KM, McCullough J. Biochemical nature of the neutrophil-specific antigen NB1. *Blood*. 1990;75:744.

Velardi A, Ruggeri L, Moretta A, et al. NK cells: A lesson from mismatched hematopoietic transplantation. *Trends Immunol*. 2002;23:438.

REFERENCES

1. Gorer PA. The detection of antigenic differences in mouse erythrocytes by employment of immune sera. *Br J Exp Pathol*. 1936;17(1):42–50.
2. Edidin M. Book review: The Natural History of the Major Histocompatibility Complex (Klein J. Wiley; 1986). *Immunol Today*. 1987; 8(5): 159–160.
3. Trowsdale J, Ragoussis J, Campbell RD. Map of the human MHC. *Immunol Today*. 1991;12:443–446.
4. Marsh SG, Albert ED, Bodmer WF, et al. Nomenclature for Factors of the HLA System, 2004. *Hum Immunol*. 2005;66:571–636.
5. Germain RN, Malissen B. Analysis of the expression and function of class-II major histocompatibility complex-encoded molecules by DNA-mediated gene transfer. *Annu Rev Immunol*. 1986;4:281–315.
6. Malissen M, Malissen B, Jordan BR. Exon/intron organization and complete nucleotide sequence of an HLA gene. *Proc Natl Acad Sci U S A*. 1982;79:893–897.
7. Goodfellow PN, Jones EA, Van Heyningen V, et al. The beta2-microglobulin gene is on chromosome 15 and not in the HL-A region. *Nature*. 1975;254:267–269.
8. Terasaki IP GD. HLA. UCLA Tissue Typing Laboratory 1997.
9. Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature*. 1987;329:506–512.
10. Brown JH, Jardetzky TS, Gorga JC, et al. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature*. 1993; 364:33–39.
11. Garboczi DN, Ghosh P, Utz U, Fan QR, Biddison WE, Wiley DC. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature*. 1996;384:134–141.
12. Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee HG. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature*. 1991;351:290–296.
13. Ruppert J, Sidney J, Celis E, Kubo RT, Grey HM, Sette A. Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules. *Cell*. 1993;74:929–937.
14. del Guercio MF, Sidney J, Hermanson G, et al. Binding of a peptide antigen to multiple HLA alleles allows definition of an A2-like supertype. *J Immunol*. 1995;154:685–693.
15. Sidney J, del Guercio MF, Southwood S, Sette A. The HLA molecules DQA1*0501/B1*0201 and DQA1*0301/B1*0302 share an extensive overlap in peptide binding specificity. *J Immunol*. 2002;169:5098–5108.
16. Sidney J, Grey HM, Southwood S, et al. Definition of an HLA-A3-like supermotif demonstrates the overlapping peptide-binding repertoires of common HLA molecules. *Hum Immunol*. 1996;45:79–93.
17. Madden DR, Garboczi DN, Wiley DC. The antigenic identity of peptide-MHC complexes: a comparison of the conformations of five viral peptides presented by HLA-A2. *Cell*. 1993;75:693–708.
18. Hennecke J, Carfi A, Wiley DC. Structure of a covalently stabilized complex of a human alphabeta T-cell receptor, influenza HA peptide and MHC class II molecule, HLA-DR1. *EMBO J*. 2000;19:5611–5624.
19. Hennecke J, Wiley DC. T cell receptor-MHC interactions up close. *Cell*. 2001;104:1–4.
20. Hennecke J, Wiley DC. Structure of a complex of the human alpha/beta T cell receptor (TCR) HA1.7, influenza hemagglutinin peptide, and major histocompatibility complex class II molecule, HLA-DR4 (DRA*0101 and DRB1*0401): insight into TCR cross-restriction and alloreactivity. *J Exp Med*. 2002;195:571–581.
21. Threlkeld SC, Wentworth PA, Kalams SA, et al. Degenerate and promiscuous recognition by CTL of peptides presented by the MHC class I A3-like superfamily: implications for vaccine development. *J Immunol*. 1997;159:1648–1657.
22. Bettinotti MP, Kim CJ, Lee KH, et al. Stringent allele/epitope requirements for MART-1/Melan A immunodominance: implications for peptide-based immunotherapy. *J Immunol*. 1998;161:877–889.
23. Rivoltini L, Loftus DJ, Barracchini K, et al. Binding and presentation of peptides derived from melanoma antigens MART-1 and glycoprotein-100 by HLA-A2 subtypes. Implications for peptide-based immunotherapy. *J Immunol*. 1996;156:3882–3891.
24. Kim CJ, Parkinson DR, Marincola F. Immunodominance across HLA polymorphism: implications for cancer immunotherapy. *J Immunother*. 1998;21:1–16.
25. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J Immunol*. 1994;152:163–175.
26. Parker KC, DiBrino M, Hull L, Coligan JE. The beta 2-microglobulin dissociation rate is an accurate measure of the stability of MHC class I heterotrimers and depends on which peptide is bound. *J Immunol*. 1992;149:1896–1904.
27. Hunt DF, Henderson RA, Shabanowitz J, et al. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science*. 1992;255:1261–1263.
28. Lanzavecchia A, Iezzi G, Viola A. From TCR engagement to T cell activation: a kinetic view of T cell behavior. *Cell*. 1999;96:1–4.
29. Yewdell JW, Bennink JR. The binary logic of antigen processing and presentation to T cells. *Cell*. 1990;62:203–206.
30. Pamer E, Cresswell P. Mechanisms of MHC class I-restricted antigen processing. *Annu Rev Immunol*. 1998;16:323–358.
31. Wiertz EJ, Jones TR, Sun L, Bogyo M, Geuze HJ, Ploegh HL. The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell*. 1996;84: 769–779.
32. Wiertz EJ, Mukherjee S, Ploegh HL. Viruses use stealth technology to escape from the host immune system. *Mol Med Today*. 1997;3: 116–123.
33. Falk CS, Mach M, Schendel DJ, Weiss EH, Hilgert I, Hahn G. NK cell activity during human cytomegalovirus infection is dominated by US2-11-mediated HLA class I down-regulation. *J Immunol*. 2002;169: 3257–3266.
34. Chen Y, Rocha V, Bittencourt H, et al. Relationship between HLA alleles and cytomegalovirus infection after allogenic hematopoietic stem cell transplant. *Blood*. 2001;98:500–501.
35. Lopez-Botet M, Llano M, Ortega M. Human cytomegalovirus and natural killer-mediated surveillance of HLA class I expression: a paradigm of host-pathogen adaptation. *Immunol Rev*. 2001;181:193–202.
36. Ulbrecht M, Martinozzi S, Grzeschik M, et al. Cutting edge: the human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis. *J Immunol*. 2000;164:5019–5022.
37. Kropshofer H, Hammerling GJ, Vogt AB. The impact of the non-classical MHC proteins HLA-DM and HLA-DO on loading of MHC class II molecules. *Immunol Rev*. 1999;172:267–278.
38. Daar AS, Fuggle SV, Fabre JW, Ting A, Morris PJ. The detailed distribution of HLA-A, B, C antigens in normal human organs. *Transplantation*. 1984;38:287–292.
39. Kowalik I, Kurpisz M, Jakubowiak A, Janecki A, Lukaszyk A, Szymczynski G. Evaluation of HLA expression on gametogenic cells isolated from human testis. *Andrologia*. 1989;21:237–243.
40. Datema G, Stein S, Eijsink C, Mulder A, Claas FH, Doxiadis II. HLA-C expression on platelets: studies with an HLA-Cw1-specific human monoclonal antibody. *Vox Sang*. 2000;79:108–111.
41. Petz LD, Garratty G, Calhoun L, et al. Selecting donors of platelets for refractory patients on the basis of HLA antibody specificity. *Transfusion*. 2000;40:1446–1456.
42. Ornstein DL, Mortara KL, Smith MB, et al. Treatment of severe thrombocytopenia in alloimmunized, transfusion-refractory patients. *Military Med*. 2001;166:269–274.
43. Kekomaki S, Volin L, Koistinen P, et al. Successful treatment of platelet transfusion refractoriness: the use of platelet transfusions matched for both human leucocyte antigens (HLA) and human platelet alloantigens (HPA) in alloimmunized patients with leukaemia. *Eur J Haematol*. 1998;60: 112–118.
44. Gelb AB, Leavitt AD. Crossmatch-compatible platelets improve corrected count increments in patients who are refractory to randomly selected platelets. *Transfusion*. 1997;37:624–630.
45. Panzer S, Puchler K, Mayr WR, Hocker P, Graninger W, Lechner K. Haemolytic transfusion reactions due to HLA antibodies. A prospective study combining red-cell serology with investigations of chromium-51-labelled red-cell kinetics. *Lancet*. 1987;1:474–478.
46. Daar AS, Fuggle SV, Fabre JW, Ting A, Morris PJ. The detailed distribution of MHC Class II antigens in normal human organs. *Transplantation*. 1984;38:293–298.
47. Skoskiewicz MJ, Colvin RB, Schneeberger EE, Russell PS. Widespread and selective induction of major histocompatibility complex-determined antigens in vivo by gamma interferon. *J Exp Med*. 1985;162:1645–1664.
48. Hakem R, Le Bouteiller P, Jezo-Bremond A, Harper K, Campese D, Lemonnier FA. Differential regulation of HLA-A3 and HLA-B7 MHC class I genes by IFN is due to two nucleotide differences in their IFN response sequences. *J Immunol*. 1991;147:2384–2390.
49. Hakem R, Jezo-Bremond A, Le Bouteiller P, Harper K, Lemonnier FA. Differential transcription inducibility by interferon of the HLA-A3 and HLA-B7 class-I genes. *Int J Cancer Suppl*. 1991;6:2–9.
50. Marincola FM, Shamamian P, Simonis TB, et al. Locus-specific analysis of human leukocyte antigen class I expression in melanoma cell lines. *J Immunother Emphasis Tumor Immunol*. 1994;16:13–23.
51. Marincola FM, Shamamian P, Alexander RB, et al. Loss of HLA haplotype and B locus down-regulation in melanoma cell lines. *J Immunol*. 1994;153:1225–1237.

52. Panelli MC, Wang E, Phan G, et al. Gene-expression profiling of the response of peripheral blood mononuclear cells and melanoma metastases to systemic IL-2 administration. *Genome Biol*. 2002;3 RESEARCH0035.
53. Dawkins RL, Degli-Esposti MP, Abraham LJ, et al. Conservation versus polymorphism of the MHC in relation to transplantation, immune responses and autoimmune disease. In: Klein J, Klein D, eds. *Molecular Evolution of the Major Histocompatibility Complex*. NATO ASI Series, Vol. 59. Heidelberg: Springer; 2019.
54. Kaufman J, Volk H, Wallny HJ. A "minimal essential Mhc" and an "unrecognized Mhc": two extremes in selection for polymorphism. *Immunol Rev*. 1995;143:63–88.
55. Migueles SA, Sabbaghian MS, Shupert WL, et al. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc Natl Acad Sci U S A*. 2000;97:2709–2714.
56. Hildesheim A, Apple RJ, Chen CJ, et al. Association of HLA class I and II alleles and extended haplotypes with nasopharyngeal carcinoma in Taiwan. *J Natl Cancer Inst*. 2002;94:1780–1789.
57. Goldsmith DB, West TM, Morton R. HLA associations with nasopharyngeal carcinoma in Southern Chinese: a meta-analysis. *Clin Otolaryngol Allied Sci*. 2002;27:61–67.
58. Wank R, Thomssen C. High risk of squamous cell carcinoma of the cervix for women with HLA-DQw3. *Nature*. 1991;352:723–725.
59. Lee JRJ, Platsoucas C, et al. Increased melanoma risk associated with the class II human leukocyte antigen DBQ1*301. *Int J Cancer*. 1994;59(4):510–513.
60. Marincola FM, Shamamian P, Rivoltini L, et al. HLA associations in the antitumor response against malignant melanoma. *J Immunother Emphasis Tumor Immunol*. 1995;18:242–252.
61. Braun WE. Update on kidney transplantation: increasing clinical success, expanding waiting lists. *Cleve Clin J Med*. 2002;69:501–504.
62. Shirwan H. Chronic allograft rejection. Do the Th2 cells preferentially induced by indirect alloantigen recognition play a dominant role? *Transplantation*. 1999;68:715–726.
63. Ponticelli C, Tarantino A, Vegeto A. Renal transplantation, past, present and future. *J Nephrol*. 1999;12(Suppl 2):S105–S110.
64. Adams EJ, Parham P. Species-specific evolution of MHC class I genes in the higher primates. *Immunol Rev*. 2001;183:41–64.
65. Natarajan K, Li H, Mariuzza RA, Margulies DH. MHC class I molecules, structure and function. *Rev Immunogenet*. 1999;1:32–46.
66. Paul P, Rouas-Freiss N, Moreau P, et al. HLA-G, -E, -F preworkshop: tools and protocols for analysis of non-classical class I genes transcription and protein expression. *Hum Immunol*. 2000;61:1177–1195.
67. Stastny P. Polymorphism and antigenicity of HLA-MICA. *ASHI Q*. 2002;26:64–69.
68. Cerwenka A, Lanier LL. Ligands for natural killer cell receptors: redundancy or specificity. *Immunol Rev*. 2001;181:158–169.
69. Wilson IA, Bjorkman PJ. Unusual MHC-like molecules: CD1, Fc receptor, the hemochromatosis gene product, and viral homologs. *Curr Opin Immunol*. 1998;10:67–73.
70. Daser A, Mitchison H, Mitchison A, Muller B. Non-classical-MHC genetics of immunological disease in man and mouse. The key role of pro-inflammatory cytokine genes. *Cytokine*. 1996;8:593–597.
71. Fischer Lindahl K. Peptide antigen presentation by non-classical MHC class I molecules. *Semin Immunol*. 1993;5:117–126.
72. Urosevic M, Dummer R. HLA-G: An ace up the sleeve? *ASHI Q*. 2002:106–109.
73. Gobin SJ, van den Elsen PJ. The regulation of HLA class I expression: is HLA-G the odd one out? *Semin Cancer Biol*. 1999;9:55–59.
74. Bainbridge D, Ellis S, Le Bouteiller P, Sargent I. HLA-G remains a mystery. *Trends Immunol*. 2001;22:548–552.
75. Ellis SA, Palmer MS, McMichael AJ. Human trophoblast and the choriocarcinoma cell line BeWo express a truncated HLA Class I molecule. *J Immunol*. 1990;144:731–735.
76. Onno M, Guillaudeux T, Amiot L, et al. The HLA-G gene is expressed at a low mRNA level in different human cells and tissues. *Hum Immunol*. 1994;41:79–86.
77. Gobin SJ, van den Elsen PJ. Transcriptional regulation of the MHC class Ib genes HLA-E, HLA-F, and HLA-G. *Hum Immunol*. 2000;61:1102–1107.
78. Lee N, Malacko AR, Ishitani A, et al. The membrane-bound and soluble forms of HLA-G bind identical sets of endogenous peptides but differ with respect to TAP association. *Immunity*. 1995;3:591–600.
79. Lopez-Botet M, Llano M, Navarro F, Bellon T. NK cell recognition of non-classical HLA class I molecules. *Semin Immunol*. 2000;12:109–119.
80. Lee N, Goodlett DR, Ishitani A, Marquardt H, Geraghty DE. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J Immunol*. 1998;160: 4951–4960.
81. Lila N, Amrein C, Guillemain R, et al. Human leukocyte antigen-G expression after heart transplantation is associated with a reduced incidence of rejection. *Circulation*. 2002;105:1949–1954.
82. Borrego F, Ulbrecht M, Weiss EH, Coligan JE, Brooks AG. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J Exp Med*. 1998;187:813–818.
83. Braud VM, Allan DS, O'Callaghan CA, et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature*. 1998;391: 795–799.
84. Garcia P, Llano M, de Heredia AB, et al. Human T cell receptor-mediated recognition of HLA-E. *Eur J Immunol*. 2002;32:936–944.
85. Lee N, Llano M, Carretero M, et al. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc Natl Acad Sci U S A*. 1998;95:5199–5204.
86. Maier S, Grzeschik M, Weiss EH, Ulbrecht M. Implications of HLA-E allele expression and different HLA-E ligand diversity for the regulation of NK cells. *Hum Immunol*. 2000;61:1059–1065.
87. O'Callaghan CA. Molecular basis of human natural killer cell recognition of HLA-E (human leucocyte antigen-E) and its relevance to clearance of pathogen-infected and tumour cells. *Clin Sci*. 2000;99:9–17.
88. Tomasec P, Braud VM, Rickards C, et al. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. *Science*. 2000;287:1031.
89. Carosella ED, Paul P, Moreau P, Rouas-Freiss N. HLA-G and HLA-E: fundamental and Pathophysiological aspects. *Immunol Today*. 2000;21: 532–534.
90. Wainwright SD, Biro PA, Holmes CH. HLA-F is a predominantly empty, intracellular, TAP-associated MHC class Ib protein with a restricted expression pattern. *J Immunol*. 2000;164:319–328.
91. Lepin EJ, Bastin JM, Allan DS, et al. Functional characterization of HLA-F and binding of HLA-F tetramers to ILT2 and ILT4 receptors. *Eur J Immunol*. 2000;30:3552–3561.
92. D T. The clinical relevance of non-HLA polymorphisms in transplantation. *ASHI Q*. 2002;6:64–69.
93. Velardi A, Ruggeri L, Alessandro M, Moretta L. NK cells: a lesson from mismatched hematopoietic transplantation. *Trends Immunol*. 2002;23: 438–444.
94. Trowsdale J, Barten R, Haude A, Stewart CA, Beck S, Wilson MJ. The genomic context of natural killer receptor extended gene families. *Immunol Rev*. 2001;181:20–38.
95. Moretta L, Moretta A. Killer immunoglobulin-like receptors. *Curr Opin Immunol*. 2004;16:626–633.
96. Middleton D, Williams F, Halfpenny IA. KIR genes. *Transpl Immunol*. 2005;14:135–142.
97. Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol*. 2002;20:217–251.
98. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. 2002;295:2097–2100.
99. Gagne K, Brizard G, Gueglio B, et al. Relevance of KIR gene polymorphisms in bone marrow transplantation outcome. *Hum Immunol*. 2002;63:271–280.
100. Falkenburg JH, Marijt WA, Heemskerk MH, Willemze R. Minor histocompatibility antigens as targets of graft-versus-leukemia reactions. *Curr Opin Hematol*. 2002;9:497–502.
101. Klein CA, Wilke M, Pool J, et al. The hematopoietic system-specific minor histocompatibility antigen HA-1 shows aberrant expression in epithelial cancer cells. *J Exp Med*. 2002;196:359–368.
102. Jin P, Wang E. Polymorphism in clinical immunology—From HLA typing to immunogenetic profiling. *J Transl Med*. 2003;1:8.
103. Keen LJ. The study of polymorphism in cytokine and cytokine receptor genes. *Trans Immunol*. 2002;2-3:143–146.
104. McCarron SL, Edwards S, Evans PR, et al. Influence of cytokine gene polymorphisms on the development of prostate cancer. *Cancer Res*. 2002; 62:3369–3372.
105. Howell WM, Turner SJ, Bateman AC, Theaker JM. IL-10 promoter polymorphisms influence tumour development in cutaneous malignant melanoma. *Genes Immun*. 2001;2:25–31.
106. Bodmer WF. HLA: what's in a name? A commentary on HLA nomenclature development over the years. *Tissue Antigens*. 1997;49:293–296.
107. Tiercy JM, Marsh SG, Schreuder GM, et al. Guidelines for nomenclature usage in HLA reports: ambiguities and conversion to serotypes. *Eur J Immunogenet*. 2002;29:273–274.
108. Dupont B. Nomenclature for factors of the HLA system, 1987. Decisions of the Nomenclature Committee on Leukocyte Antigens, which met in New York on November 21–23, 1987. *Hum Immunol*. 1989;26:3–14.

109. Robinson J, Mistry K, McWilliam H, Lopez R, Parham P, Marsh SG. The IMGT/HLA database. *Nucleic Acids Res*. 2011;39:D1171–D1176.
110. Shilling HG, Guethlein LA, Cheng NW, et al. Allelic polymorphism synergizes with variable gene content to individualize human KIR genotype. *J Immunol*. 2002;168:2307–2315.
111. Turner D, Choudhury F, Reynard M, Railton D, Navarrete C. Typing of multiple single nucleotide polymorphisms in cytokine and receptor genes using SNaPshot. *Hum Immunol*. 2002;63:508–513.
112. Svejgaard A, Ryder LP. HLA and disease associations: detecting the strongest association. *Tissue Antigens*. 1994;43:18–27.
113. Boisgerault F, Khalil I, Tieng V, et al. Definition of the HLA-A29 peptide ligand motif allows prediction of potential T-cell epitopes from the retinal soluble antigen, a candidate autoantigen in birdshot retinopathy. *Proc Natl Acad Sci U S A*. 1996;93:3466–3470.
114. Cope AP, Patel SD, Hall F, et al. T cell responses to a human cartilage autoantigen in the context of rheumatoid arthritis-associated and nonassociated HLA-DR4 alleles. *Arthritis Rheum*. 1999;42:1497–1507.
115. Friday RP, Trucco M, Pietropaolo M. Genetics of Type 1 diabetes mellitus. *Diabetes Nutr Metab*. 1999;12:3–26.
116. Luppi P, Alexander A, Bertera S, Noonan K, Trucco M. The same HLA-DQ alleles determine either susceptibility or resistance to different coxsackievirus-mediated autoimmune diseases. *J Biol Regul Homeost Agents*. 1999;13:14–26.
117. Luppi P, Rossiello MR, Faas S, Trucco M. Genetic background and environment contribute synergistically to the onset of autoimmune diseases. *J Mol Med*. 1995;73:381–393.
118. Jongeneel CV, Briant L, Udalova IA, Sevin A, Nedospasov SA, Cambon-Thomsen A. Extensive genetic polymorphism in the human tumor necrosis factor region and relation to extended HLA haplotypes. *Proc Natl Acad Sci U S A*. 1991;88:9717–9721.
119. Marincola FM. Possible biases in referral pattern to NIH and their effect on HLA association studies. *J Immunother Emphasis Tumor Immunol*. 1996;19:384.
120. Cormier JN, Salgaller ML, Prevette T, et al. Enhancement of cellular immunity in melanoma patients immunized with a peptide from MART-1/Melan A. *Cancer J Sci Am*. 1997;3:37–44.
121. Papassavas AC, Iniotaki-Theodoraki A, Stathakopoulou A, et al. Evaluation of HLA-class I alloantibodies using PRA-STAT and complement-dependent cytotoxicity techniques. *Transplant Proc*. 1998;30(3):724–726
122. Sakaguchi K, Ono R, Tsujisaki M, et al. Anti-HLA-B7,B27,Bw42,Bw54, Bw55,Bw56, Bw67,Bw73 monoclonal antibodies: specificity, idiotypes, and application for a double determinant immunoassay. *Hum Immunol*. 1988;21:193–207.
123. Lublin DM, Grumet FC. Mechanisms of the CYNAP phenomenon: evidence in the Bw49/Bw50 model for epitopes with different spatial orientation of antibody. *Hum Immunol*. 1982;4(2):137–145.
124. Scornik JC, Brunson ME, Schaub B, Howard RJ, Pfaff WW. The crossmatch in renal transplantation. Evaluation of flow cytometry as a replacement for standard cytotoxicity. *Transplantation*. 1994;57:621–625.
125. Buelow R, Chiang TR, Monteiro F, et al. Soluble HLA antigens and ELISA—a new technology for crossmatch testing. *Transplantation*. 1995; 60:1594–1599.
126. Christiaans MH, Nieman F, van Hooff JP, van den Berg-Loonen EM. Detection of HLA class I and II antibodies by ELISA and complement-dependent cytotoxicity before and after transplantation. *Transplantation*. 2000;69:917–927.
127. Marrari M, Duquesnoy RJ. Interlaboratory comparisons of serum screening for HLA antibody determination and crossmatching by ELISA and flow cytometry methods. *Hum Immunol*. 2002
128. Pei R, Lee J, Chen T, Rojo S, Terasaki PI. Flow cytometric detection of HLA antibodies using a spectrum of microbeads. *Hum Immunol*. 1999; 60:1293–1302.
129. Moses LA, Stroncek DF, Cipolone KM, Marincola FM. Detection of HLA antibodies by using flow cytometry and latex beads coated with HLA antigens. *Transfusion*. 2000;40:861–866.
130. Duquesnoy RJ, Marrari M. HLAMatchmaker: a molecularly based algorithm for histocompatibility determination. II. Verification of the algorithm and determination of the relative immunogenicity of amino acid triplet-defined epitopes. *Hum Immunol*. 2002;63:353–363.
131. Rotzschke O, Falk K, Stevanovic S, Jung G, Rammensee HG. Peptide motifs of closely related HLA class I molecules encompass substantial differences. *Eur J Immunol*. 1992;22:2453–2456.
132. Parker KC, Bednarek MA, Hull LK, et al. Sequence motifs important for peptide binding to the human MHC class I molecule, HLA-A2. *J Immunol*. 1992;149:3580–3587.
133. Sette A, Vitiello A, Reherman B, et al. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. *J Immunol*. 1994;153:5586–5592.
134. Bunce M, O'Neill CM, Barnardo MC, et al. Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). *Tissue Antigens*. 1995;46:355–367.
135. Krausa P, Browning MJ. A comprehensive PCR-SSP typing system for identification of HLA-A locus alleles. *Tissue Antigens*. 1996;47:237–244.
136. Itoh Y, Mizuki N, Shimada T, et al. High-throughput DNA typing of HLA-A, -B, -C, and -DRB1 loci by a PCR-SSOP-Luminex method in the Japanese population. *Immunogenetics*. 2005;57:717–729.
137. Ng J, Hurley CK, Carter C, et al. Large-scale DRB and DQB1 oligonucleotide typing for the NMDP registry: progress report from year 2. *Tissue Antigens*. 1996;47:21–26.
138. Madden K, Chabot-Richards D. HLA testing in the molecular diagnostic laboratory. *Virchows Arch*. 2019;474:139–147.
139. Lally KM, Mocellin S, Ohnmacht GA, et al. Unmasking cryptic epitopes after loss of immunodominant tumor antigen expression through epitope spreading. *Int J Cancer*. 2001;93(6):841–847.
140. Weimer ET. Clinical validation of NGS technology for HLA: An early adopter's perspective. *Hum Immunol*. 2016;77:820–823.
140a. Marsh SGE. WHO Nomenclature Committee for factors of the HLA system, update April, May, and June 2020. *HLA*. 2020;96(3):384–412.
141. Adams SD, Barracchini KC, Chen D, et al. Ambiguous allele combinations in HLA Class I and Class II sequence-based typing: when precise nucleotide sequencing leads to imprecise allele identification. *J Transl Med*. 2004;2:30.
142. Ingram KJ, Merkens H, O'Shields EF, Kiger D, Gautreaux MD. New HLA alleles discovered by next generation sequencing in routine histocompatibility lab work in a medium-volume laboratory. *Hum Immunol*. 2019;80:465–467.
143. Tiercy JM, Bujan-Lose M, Chapuis B, et al. Bone marrow transplantation with unrelated donors: what is the probability of identifying an HLA-A/B/Cw/DRB1/B3/B5/DQB1-matched donor? *Bone Marrow Transplant*. 2000;26:437–441.
144. Tiercy JM, Villard J, Roosnek E. Selection of unrelated bone marrow donors by serology, molecular typing and cellular assays. *Transpl Immunol*. 2002; 10:215–221.
145. Duquesnoy RJ, White LT, Fierst JW, et al. Multiscreen serum analysis of highly sensitized renal dialysis patients for antibodies toward public and private class I HLA determinants. Implications for computer-predicted acceptable and unacceptable donor mismatches in kidney transplantation. *Transplantation*. 1990;50:427–437.
146. Duquesnoy RJ, Marrari M. Determination of HLA-A,B residue mismatch acceptability for kidneys transplanted into highly sensitized patients: a report of a collaborative study conducted during the 12th International Histocompatibility Workshop. *Transplantation*. 1997;63:1743–1751.
147. Duquesnoy RJ. HLAMatchmaker: a molecularly based algorithm for histocompatibility determination. I. Description of the algorithm. *Hum Immunol*. 2002;63:339–352.
148. Claas FH, De Meester J, Witvliet MD, Smits JM, Persijn GG, Doxiadis II. Acceptable HLA mismatches for highly immunized patients. *Rev Immunogenet*. 1999;1:351–358.
149. Lobashevsky AL, Senkbeil RW, Shoaf JL, et al. The number of amino acid residues mismatches correlates with flow cytometry crossmatching results in high PRA renal patients. *Hum Immunol*. 2002;63:364–374.
150. Nambiar A, Duquesnoy RJ, Adams S, et al. HLAMatchmaker-driven analysis of responses to HLA-typed platelet transfusions in alloimmunized thrombocytopenic patients. *Blood*. 2006;107:1680–1687.
151. Duquesnoy RJ. A structurally based approach to determine HLA compatibility at the humoral immune level. *Hum Immunol*. 2006;67: 847–862.
152. Shoskes DA, Wood KJ. Indirect presentation of MHC antigens in transplantation. *Immunol Today*. 1994;15:32–38.
153. Semple JW, Freedman J. Recipient antigen-processing pathways of allogeneic platelet antigens: essential mediators of immunity. *Transfusion*. 2002;42:958–961.
154. Fuller TC, Fuller A. The humoral immune response against an HLA class I allodeterminant correlates with the HLA-DR phenotype of the responder. *Transplantation*. 1999;68:173–182.
155. Liu Z, Colovai AI, Tugulea S, et al. Indirect recognition of donor HLA-DR peptides in organ allograft rejection. *J Clin Invest*. 1996;98:1150–1157.
156. Benichou G, Valujskikh A, Heeger PS. Contributions of direct and indirect T cell alloreactivity during allograft rejection in mice. *J Immunol*. 1999;162:352–358.
157. Rodey GE, Fuller TC. Public epitopes and the antigenic structure of the HLA molecules. *Crit Rev Immunol*. 1987;7:229–267.
158. Salvatierra Jr. O, Melzer J, Potter D, et al. A seven-year experience with donor-specific blood transfusions. Results and considerations for maximum efficacy. *Transplantation*. 1985;40:654–659.

159. Clark BD, Geer LI, Park MS, Terasaki PI. Association of high sensitization to the structure of HLA class I alleles. *Clin Transpl*. 1991:347–362.
160. Hardy S, Lee SH, Terasaki PI. Sensitization 2001. *Clin Transpl*. 2001: 271–278.
161. Opelz G, Wujciak T, Dohler B, Scherer S, Mytilineos J. HLA compatibility and organ transplant survival. Collaborative Transplant Study. *Rev Immunogenet*. 1999;1:334–342.
162. Matzinger P. An innate sense of danger. *Semin Immunol*. 1998;10:399–415.
163. Opelz G, Vanrenterghem Y, Kirste G, et al. Prospective evaluation of pretransplant blood transfusions in cadaver kidney recipients. *Transplantation*. 1997;63:964–967.
164. Terasaki PI. The beneficial transfusion effect on kidney graft survival attributed to clonal deletion. *Transplantation*. 1984;37:119–125.
165. Rubinstein P. HLA matching for bone marrow transplantation—how much is enough? *N Engl J Med*. 2001;345:1842–1844.
166. Petersdorf EW, Hansen JA, Martin PJ, et al. Major-histocompatibility-complex class I alleles and antigens in hematopoietic-cell transplantation. *N Engl J Med*. 2001;345:1794–1800.
167. Champlin RE, Goldman JM, Gale RP. Bone marrow transplantation in chronic myelogenous leukemia. *Semin Hematol*. 1988;25:74–80.
168. Clift RA, Buckner CD. Marrow transplantation for acute myeloid leukemia. *Cancer Invest*. 1998;16:53–61.
169. Hansen JA, Gooley TA, Martin PJ, et al. Bone marrow transplants from unrelated donors for patients with chronic myeloid leukemia. *N Engl J Med*. 1998;338:962–968.
170. Storb R, Leisenring W, Anasetti C, et al. Long-term follow-up of allogeneic marrow transplants in patients with aplastic anemia conditioned by cyclophosphamide combined with antithymocyte globulin. *Blood*. 1997;89:3890–3891.
171. Lucarelli G, Clift RA, Galimberti M, et al. Marrow transplantation for patients with thalassemia: results in class 3 patients. *Blood*. 1996;87: 2082–2088.
172. Anderson JE, Appelbaum FR, Schoch G, et al. Allogeneic marrow transplantation for myelodysplastic syndrome with advanced disease morphology: a phase II study of busulfan, cyclophosphamide, and total-body irradiation and analysis of prognostic factors. *J Clin Oncol*. 1996; 14:220–226.
173. Teshima T, Ferrara JL. Understanding the alloresponse: new approaches to graft-versus-host disease prevention. *Semin Hematol*. 2002;39:15–22.
174. Ratanatharathorn V, Ayash L, Lazarus HM, Fu J, Uberti JP. Chronic graft-versus-host disease: clinical manifestation and therapy. *Bone Marrow Transplant*. 2001;28:121–129.
175. Flowers ME, Kansu E, Sullivan KM. Pathophysiology and treatment of graft-versus-host disease. *Hematol Oncol Clin North Am*. 1999;13: 1091–1112. viii-ix.
176. Barrett J, Childs R. The benefits of an alloresponse: graft-versus-tumor. *J Hematother Stem Cell Res*. 2000;9:347–354.
177. Barrett J, Childs R. New directions in allogeneic stem cell transplantation. *Semin Hematol*. 2002;39:1–2.
178. Childs R, Srinivasan R. Advances in allogeneic stem cell transplantation: directing graft-versus-leukemia at solid tumors. *Cancer J*. 2002;8:2–11.
179. Feinstein L, Sandmaier B, Maloney D, et al. Nonmyeloablative hematopoietic cell transplantation. Replacing high-dose cytotoxic therapy by the graft-versus-tumor effect. *Ann N Y Acad Sci*. 2001;938:328–337. discussion 37-9.
180. Mavroudis D, Barrett J. The graft-versus-leukemia effect. *Curr Opin Hematol*. 1996;3:423–429.
181. Michallet M. Graft-versus-host disease and graft-versus-leukemia. *Hematol Cell Ther*. 1996;38:459–460.
182. Porter DL, Antin JH. The graft-versus-leukemia effects of allogeneic cell therapy. *Annu Rev Med*. 1999;50:369–386.
183. Shimoni A, Giralt S, Khouri I, Champlin R. Allogeneic hematopoietic transplantation for acute and chronic myeloid leukemia: non-myeloablative preparative regimens and induction of the graft-versus-leukemia effect. *Curr Oncol Rep*. 2000;2:132–139.
184. Horowitz MM, Gale RP, Sondel PM, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*. 1990;75:555–562.
185. Marmont AM, Horowitz MM, Gale RP, et al. T-cell depletion of HLA-identical transplants in leukemia. *Blood*. 1991;78:2120–2130.
186. Weiden PL, Flournoy N, Thomas ED, et al. Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. *N Engl J Med*. 1979;300:1068–1073.
187. Sasazuki T, Juji T, Morishima Y, et al. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor. Japan Marrow Donor Program. *N Engl J Med*. 1998;339:1177–1185.
188. Hansen JA, Yamamoto K, Petersdorf E, Sasazuki T. The role of HLA matching in hematopoietic cell transplantation. *Rev Immunogenet*. 1999;1: 359–373.
189. Barrett AJ. Mechanisms of the graft-versus-leukemia reaction. *Stem Cells*. 1997;15:248–258.
190. Kolb HJ, Mittermuller J, Clemm C, et al. Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. *Blood*. 1990;76:2462–2465.
191. Barrett AJ, Malkovska V. Graft-versus-leukaemia: understanding and using the alloimmune response to treat haematological malignancies. *Br J Haematol*. 1996;93:754–761.
192. Farag SS, Fehniger TA, Ruggeri L, Velardi A, Caligiuri MA. Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. *Blood*. 2002;100:1935–1947.
193. de Bueger M, Bakker A, Van Rood JJ, Van der Woude F, Goulmy E. Tissue distribution of human minor histocompatibility antigens. Ubiquitous versus restricted tissue distribution indicates heterogeneity among human cytotoxic T lymphocyte-defined non-MHC antigens. *J Immunol*. 1992;149:1788–1794.
194. Goulmy E. Human minor histocompatibility antigens. *Curr Opin Immunol*. 1996;8:75–81.
195. Warren EH, Greenberg PD, Riddell SR. Cytotoxic T-lymphocyte-defined human minor histocompatibility antigens with a restricted tissue distribution. *Blood*. 1998;91:2197–2207.
196. Frahm N, Korber BT, Adams CM, et al. Consistent cytotoxic-T-lymphocyte targeting of immunodominant regions in human immunodeficiency virus across multiple ethnicities. *J Virol*. 2004;78:2187–2200.
197. Meij P, Leen A, Rickinson AB, et al. Identification and prevalence of CD8(+) T-cell responses directed against Epstein-Barr virus-encoded latent membrane protein 1 and latent membrane protein 2. *Int J Cancer*. 2002;99:93–99.
198. Provenzano M, Mocellin S, Bettinotti M, et al. Identification of immune dominant cytomegalovirus epitopes using quantitative real-time polymerase chain reactions to measure interferon-gamma production by peptide-stimulated peripheral blood mononuclear cells. *J Immunother*. 2002;25:342–351.
199. Solache A, Morgan CL, Dodi AI, et al. Identification of three HLA-A*0201-restricted cytotoxic T cell epitopes in the cytomegalovirus protein pp65 that are conserved between eight strains of the virus. *J Immunol*. 1999;163:5512–5518.
200. Rosenberg SA. Progress in human tumour immunology and immunotherapy. *Nature*. 2001;411:380–384.
201. Lee KH, Wang E, Nielsen MB, et al. Increased vaccine-specific T cell frequency after peptide-based vaccination correlates with increased susceptibility to in vitro stimulation but does not lead to tumor regression. *J Immunol*. 1999;163:6292–6300.
202. Lin CL, Lo WF, Lee TH, et al. Immunization with Epstein-Barr virus (EBV) peptide-pulsed dendritic cells induces functional CD8+ T-cell immunity and may lead to tumor regression in patients with EBV-positive nasopharyngeal carcinoma. *Cancer Res*. 2002;62:6952–6958.
203. Parmiani G, Castelli C, Dalerba P, et al. Cancer immunotherapy with peptide-based vaccines: what have we achieved? Where are we going? *J Natl Cancer Inst*. 2002;94:805–818.
204. Monsurro V, Nagorsen D, Wang E, et al. Functional heterogeneity of vaccine-induced CD8(+) T cells. *J Immunol*. 2002;168:5933–5942.
205. Einsele H, Roosnek E, Rufer N, et al. Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. *Blood*. 2002;99:3916–3922.
206. Green M. Management of Epstein-Barr virus-induced post-transplant lymphoproliferative disease in recipients of solid organ transplantation. *Am J Transplant*. 2001;1:103–108.
207. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science*. 2002;298:850–854.
208. Sznol M. Principles of immune monitoring in cancer vaccine trials MF. In: Rosenberg SA, ed. *Principles and Practice of the Biologic Therapy of Cancer*. Philadelphia: Lippincott Williams & Wilkins; 2000.
209. Keilholz U, Weber J, Finke JH, et al. Immunologic monitoring of cancer vaccine therapy: results of a workshop sponsored by the Society for Biological Therapy. *J Immunother*. 2002;25:97–138.
210. Altman JD, Moss PA, Goulder PJ, et al. Phenotypic analysis of antigen-specific T lymphocytes. *Science*. 1996;274:94–96.
211. Monsurro V, Nagorsen D. Immunotracking of specific cancer vaccine CD8$^+$ lymphocytes. *ASHI Q*. 2003;26:100–102.
212. Boxer LA, Yokoyama M, Lalezari P. Isoimmune neonatal neutropenia. *J Pediatr*. 1972;80:783–787.
213. Lalezari P, Bernard GE. An isologous antigen-antibody reaction with human neutrophiles, related to neonatal neutropenia. *J Clin Invest*. 1966; 45:1741–1750.
214. Bux J. Nomenclature of granulocyte alloantigens. ISBT Working Party on Platelet and Granulocyte Serology, Granulocyte Antigen Working Party. International Society of Blood Transfusion. *Transfusion*. 1999;39:662–663.

215. Trounstine ML, Peltz GA, Yssel H, et al. Reactivity of cloned, expressed human Fc gamma RIII isoforms with monoclonal antibodies which distinguish cell-type-specific and allelic forms of Fc gamma RIII. *Int Immunol*. 1990;2:303–310.
216. Ravetch JV, Perussia B. Alternative membrane forms of Fc gamma RIII(CD16) on human natural killer cells and neutrophils. Cell type-specific expression of two genes that differ in single nucleotide substitutions. *J Exp Med*. 1989;170:481–497.
217. Ory PA, Clark MR, Kwoh EE, Clarkson SB, Goldstein IM. Sequences of complementary DNAs that encode the NA1 and NA2 forms of Fc receptor III on human neutrophils. *J Clin Invest*. 1989;84:1688–1691.
218. Huizinga TW, Kleijer M, Tetteroo PA, Roos D, von dem Borne AE. Biallelic neutrophil Na-antigen system is associated with a polymorphism on the phospho-inositol-linked Fc gamma receptor III (CD16). *Blood*. 1990;75:213–217.
219. Stroncek DF, Shankar R, Litz C, Clement L. The expression of the NB1 antigen on myeloid precursors and neutrophils from children and umbilical cords. *Transfus Med*. 1998;8:119–123.
220. Huizinga TW, de Haas M, Kleijer M, Nuijens JH, Roos D, von dem Borne AE. Soluble Fc gamma receptor III in human plasma originates from release by neutrophils. *J Clin Invest*. 1990;86:416–423.
221. Bux J, Stein EL, Bierling P, et al. Characterization of a new alloantigen (SH) on the human neutrophil Fc gamma receptor IIIb. *Blood*. 1997;89:1027–1034.
222. Bux J, Stein EL, Santoso S, Mueller-Eckhardt C. NA gene frequencies in the German population, determined by polymerase chain reaction with sequence-specific primers. *Transfusion*. 1995;35:54–57.
223. Hessner MJ, Curtis BR, Endean DJ, Aster RH. Determination of neutrophil antigen gene frequencies in five ethnic groups by polymerase chain reaction with sequence-specific primers. *Transfusion*. 1996;36:895–899.
224. Lin M, Chen CC, Wang CL, Lee HL. Frequencies of neutrophil-specific antigens among Chinese in Taiwan. *Vox Sang*. 1994;66:247.
225. Matsuo K, Procter J, Stroncek D. Variations in genes encoding neutrophil antigens NA1 and NA2. *Transfusion*. 2000;40:645–653.
226. Ohto H, Matsuo Y. Neutrophil-specific antigens and gene frequencies in Japanese. *Transfusion*. 1989;29:654.
227. Kissel K, Hofmann C, Gittinger FS, Daniels G, Bux J. HNA-1a, HNA-1b, and HNA-1c (NA1, NA2, SH) frequencies in African and American Blacks and in Chinese. *Tissue Antigens*. 2000;56:143–148.
228. Koene HR, Kleijer M, Roos D, de Haas M, Von dem Borne AE. Fc gamma RIIIB gene duplication: evidence for presence and expression of three distinct Fc gamma RIIIB genes in NA(1+,2+)SH(+) individuals. *Blood*. 1998;91:673–679.
229. Reil A, Sachs UJ, Siahanidou T, Flesch BK, Bux J. HNA-1d: a new human neutrophil antigen located on Fcgamma receptor IIIb associated with neonatal immune neutropenia. *Transfusion*. 2013;53:2145–2151.
230. de Haas M, Kleijer M, van Zwieten R, Roos D, von dem Borne AE. Neutrophil Fc gamma RIIIb deficiency, nature, and clinical consequences: a study of 21 individuals from 14 families. *Blood*. 1995;86:2403–2413.
231. Fromont P, Bettaieb A, Skouri H, et al. Frequency of the polymorphonuclear neutrophil Fc gamma receptor III deficiency in the French population and its involvement in the development of neonatal alloimmune neutropenia. *Blood*. 1992;79:2131–2134.
232. Muniz-Diaz E, Madoz P, de la Calle Martin O, Puig L. The polymorphonuclear neutrophil Fc gamma RIIIb deficiency is more frequent than hitherto assumed. *Blood*. 1995;86:3999.
233. Nagarajan S, Chesla S, Cobern L, Anderson P, Zhu C, Selvaraj P. Ligand binding and phagocytosis by CD16 (Fc gamma receptor III) isoforms. Phagocytic signaling by associated zeta and gamma subunits in Chinese hamster ovary cells. *J Biol Chem*. 1995;270:25762–25770.
234. Salmon JE, Edberg JC, Kimberly RP. Fc gamma receptor III on human neutrophils. Allelic variants have functionally distinct capacities. *J Clin Invest*. 1990;85:1287–1295.
235. Bredius RG, Fijen CA, De Haas M, et al. Role of neutrophil Fc gamma RIIa (CD32) and Fc gamma RIIIb (CD16) polymorphic forms in phagocytosis of human IgG1- and IgG3-opsonized bacteria and erythrocytes. *Immunology*. 1994;83:624–630.
236. Lalezari P, Murphy GB, Allen Jr. FH. NB1, a new neutrophil-specific antigen involved in the pathogenesis of neonatal neutropenia. *J Clin Invest*. 1971;50:1108–1115.
237. Clement LT, Lehmeyer JE, Gartland GL. Identification of neutrophil subpopulations with monoclonal antibodies. *Blood*. 1983;61:326–332.
238. Goldschmeding R, van Dalen CM, Faber N, et al. Further characterization of the NB 1 antigen as a variably expressed 56-62 kD GPI-linked glycoprotein of plasma membranes and specific granules of neutrophils. *Br J Haematol*. 1992;81:336–345.
239. Matsuo K, Lin A, Procter JL, Clement L, Stroncek D. Variations in the expression of granulocyte antigen NB1. *Transfusion*. 2000;40:654–662.
240. Stroncek DF, Shankar RA, Noren PA, Herr GP, Clement LT. Analysis of the expression of NB1 antigen using two monoclonal antibodies. *Transfusion*. 1996;36:168–174.
241. Caruccio L, Bettinotti M, Matsuo K, Sharon V, Stroncek D. Expression of human neutrophil antigen-2a (NB1) is increased in pregnancy. *Transfusion*. 2003;43:357–363.
242. Stroncek DF, Jaszcz W, Herr GP, Clay ME, McCullough J. Expression of neutrophil antigens after 10 days of granulocyte-colony-stimulating factor. *Transfusion*. 1998;38:663–668.
243. Stroncek DF, Skubitz KM, McCullough JJ. Biochemical characterization of the neutrophil-specific antigen NB1. *Blood*. 1990;75:744–755.
244. Caruccio L, Bettinotti M, Director-Myska AE, Arthur DC, Stroncek D. The gene overexpressed in polycythemia rubra vera, PRV-1, and the gene encoding a neutrophil alloantigen, NB1, are alleles of a single gene, CD177, in chromosome band 19q13.31. *Transfusion*. 2006;46:441–447.
245. Kissel K, Santoso S, Hofmann C, Stroncek D, Bux J. Molecular basis of the neutrophil glycoprotein NB1 (CD177) involved in the pathogenesis of immune neutropenias and transfusion reactions. *Eur J Immunol*. 2001;31:1301–1309.
246. Bierling P, Poulet E, Fromont P, Seror T, Bracq C, Duedari N. Neutrophil-specific antigen and gene frequencies in the French population. *Transfusion*. 1990;30:848–849.
247. Taniguchi K, Kobayashi M, Harada H, et al. Human neutrophil antigen-2a expression on neutrophils from healthy adults in western Japan. *Transfusion*. 2002;42:651–657.
248. Stroncek DF, Shankar RA, Plachta LB, Clay ME, Dalmasso AP. Polyclonal antibodies against the NB1-bearing 58- to 64-kDa glycoprotein of human neutrophils do not identify an NB2-bearing molecule. *Transfusion*. 1993;33:399–404.
249. Kissel K, Scheffler S, Kerowgan M, Bux J. Molecular basis of NB1 (HNA-2a, CD177) deficiency. *Blood*. 2002;99:4231–4233.
250. Flesch BK, Reil A. Molecular genetics of the human neutrophil antigens. *Transfus Med Hemother*. 2018;45:300–309.
251. Temerinac S, Klippel S, Strunck E, et al. Cloning of PRV-1, a novel member of the uPAR receptor superfamily, which is overexpressed in polycythemia rubra vera. *Blood*. 2000;95:2569–2576.
252. Klippel S, Strunck E, Busse CE, Behringer D, Pahl HL. Biochemical characterization of PRV-1, a novel hematopoietic cell surface receptor, which is overexpressed in polycythemia rubra vera. *Blood*. 2002;100:2441–2448.
253. Stroncek DF, Herr GP, Plachta LB. Neutrophil-specific antigen NB1 inhibits neutrophil-endothelial cell interactions. *J Lab Clin Med*. 1994;123:247–255.
254. de Haas M, Muniz-Diaz E, Alonso LG, et al. Neutrophil antigen 5b is carried by a protein, migrating from 70 to 95 kDa, and may be involved in neonatal alloimmune neutropenia. *Transfusion*. 2000;40:222–227.
255. Curtis BR, Cox NJ, Sullivan MJ, et al. The neutrophil alloantigen HNA-3a (5b) is located on choline transporter-like protein 2 and appears to be encoded by an R>Q154 amino acid substitution. *Blood*. 2010;115:2073–2076.
256. Greinacher A, Wesche J, Hammer E, et al. Characterization of the human neutrophil alloantigen-3a. *Nature Med*. 2010;16:45–48.
257. Kopko PM, Marshall CS, MacKenzie MR, Holland PV, Popovsky MA. Transfusion-related acute lung injury: report of a clinical look-back investigation. *JAMA*. 2002;287:1968–1971.
258. Nordhagen R, Conradi M, Dromtorp SM. Pulmonary reaction associated with transfusion of plasma containing anti-5b. *Vox Sang*. 1986;51:102–107.
259. Simsek S, van der Schoot CE, Daams M, et al. Molecular characterization of antigenic polymorphisms (Ond(a) and Mart(a)) of the beta 2 family recognized by human leukocyte alloantisera. *Blood*. 1996;88:1350–1358.
260. Bux J, Jung KD, Kauth T, Mueller-Eckhardt C. Serological and clinical aspects of granulocyte antibodies leading to alloimmune neonatal neutropenia. *Transfus Med*. 1992;2:143–149.
261. Lopes LB, Abbas SA, Moritz E, et al. Antibodies to human neutrophil antigen HNA-3b implicated in cases of neonatal alloimmune neutropenia. *Transfusion*. 2018;58:1264–1270.
262. Bruin MC, von dem Borne AE, Tamminga RY, Kleijer M, Buddelmeijer L, de Haas M. Neutrophil antibody specificity in different types of childhood autoimmune neutropenia. *Blood*. 1999;94:1797–1802.
263. Bux J, Behrens G, Jaeger G, Welte K. Diagnosis and clinical course of autoimmune neutropenia in infancy: analysis of 240 cases. *Blood*. 1998;91:181–186.
264. Conway LT, Clay ME, Kline WE, Ramsay NK, Krivit W, McCullough J. Natural history of primary autoimmune neutropenia in infancy. *Pediatrics*. 1987;79:728–733.
265. Lalezari P, Khorshidi M, Petrosova M. Autoimmune neutropenia of infancy. *J Pediatr*. 1986;109:764–769.
266. Popovsky MA, Abel MD, Moore SB. Transfusion-related acute lung injury associated with passive transfer of antileukocyte antibodies. *Am Rev Respir Dis*. 1983;128:185–189.

CHAPTER 115

PRINCIPLES OF PLASMA AND PLASMA DERIVATIVES

Alexandra Jimenez, Christopher D. Hillyer, and Beth H. Shaz

Plasma and its derivatives are well-established clinical resources, but cost, risk of infectious disease transmission, although rare, and other adverse effects mandate their appropriate use. Even to this day, however, much still remains to be clarified regarding the appropriate clinical indications/protocols for plasma product use. Plasma can be separated from red blood cells (RBCs) through centrifugation of whole blood at the time of collection or can be collected by apheresis as a single product or as a by-product of platelet or RBC apheresis. Plasma can be processed into derivatives through cold ethanol fractionation (method of Cohn),[1] additional factor purification steps, and pathogen inactivation.[2] With the advent of genetic and cellular engineering technologies, the development of recombinant products with no human proteins is possible. In this chapter, the features and uses of plasma products, which include fresh frozen plasma (FFP), plasma frozen within 24 hours of phlebotomy (FP24), thawed plasma, liquid plasma, solvent detergent treated plasma (SD-plasma), pathogen-reduced plasma, cryoprecipitate, cryoprecipitate-reduced plasma (CRP), as well as plasma derivatives, including albumin, intravenous and intramuscular immunoglobulin, and purified and recombinant coagulation factor concentrates are discussed.

PLASMA

Plasma is the acellular, fluid compartment of blood and consists of 90% water, 7% protein and colloids, and 2% to 3% nutrients, crystalloids, hormones, and vitamins. The protein fraction contains the soluble clotting factors: fibrinogen, factor XIII, von Willebrand factor (vWF), factor VIII primarily bound to its carrier protein vWF, and the vitamin K-dependent coagulation factors II, VII, IX, and X. Primary indications for transfusion of plasma are either general clotting factor replacement or specific factor replacement when purified or recombinant products are not available.[3] Clotting proteins are the constituents for which transfusion of plasma is required. Plasma products include FFP, FP24, thawed plasma, SD-plasma, and pathogen-reduced plasma,[4] which are clinically equivalent.

Products

FFP and FP24

Plasma frozen at −18°C or colder within 8 hours of donation (or within 6 hours with the use of some storage bags after apheresis collection) can be labelled as FFP. A second type of frozen plasma, the most commonly used in the United States, is FP24 plasma. FP24 is frozen at −18°C or colder within 24 hours of collection. FFP and FP24 may be stored up to 1 year before use, at which time it is thawed at 37°C for 20 to 30 minutes. After thawing, plasma is stored at 1°C to 6°C and transfused within 24 hours, after which it becomes thawed plasma (described below). A direct comparison between FFP and FP24 mean factor activity immediately post-thaw indicates that either product can be used interchangeably such that they are considered clinically equivalent (Table 115.1).[5]

Thawed Plasma

Thawed FFP and FP24 stored at 1°C to 6°C for up to 5 days is termed thawed plasma and most coagulation factors remain relatively stable in it. Although there is evidence that activity levels for factors V, VII, and VIII decrease, this is not considered to be of clinical significance, as the mean factor activity levels for 5-day-old thawed plasma remain above the minimum activity required for surgical hemostasis (see Table 115.1).[6] Compared to frozen plasma, stored thawed plasma improves patient care as there is no preparation time required resulting in a decreased turnaround time, and because of longer shelf life, it reduces wastage rate.[7]

Cryoprecipitate-Reduced Plasma

CRP, also known as cryosupernatant or cryoreduced plasma, is the remaining supernatant after the removal of cryoprecipitate from FFP, which is subsequently refrozen. This product has significantly decreased levels of factor VIII, factor XIII, vWF, fibrinogen, and fibronectin. The primary indication of CRP is for transfusion or plasma exchange in patients with thrombotic thrombocytopenic purpura (TTP).[8] It can be thawed and stored for up to 5 days at 1°C to 6°C and is termed thawed plasma cryoprecipitate reduced.

Liquid Plasma

Liquid plasma is plasma that is separated from whole blood at any time during storage and up to 5 days after the whole blood expiration date. Liquid plasma is never frozen and is maintained at 1°C to 6°C and stored for 26 days or 40 days depending on the anticoagulant preservative used to collect the whole blood. Clotting factor levels progressively decrease over time, particularly labile clotting factors (i.e., factors V and VIII).[9] This product contains viable leukocytes, and thus would need product modifications (leukoreduction or irradiation) prior to transfusion in at-risk individuals (e.g., immunocompromised). It is used primarily for immediate treatment of patients undergoing massive transfusion due to massive hemorrhage.[10]

Lyophilized Plasma Also Known as Freeze-Dried Plasma

Lyophilized plasma is produced and pooled from 10 or fewer apheresis plasma donors. The plasma undergoes a cryodesiccation process to form a 215 g powder in a sterile bottle. The plasma is then rehydrated for use via 200 mL of sterile water with soft agitation. While not in use yet in the United States (but used in Europe and in the US military), clinical trials in the US are underway. Studies suggest that the product is safe and efficacious for treatment of traumatic hemorrhage.[11]

Solvent-Detergent Plasma

SD-plasma is US Food and Drug Administration (FDA)-approved (Octaplas, Octapharma USA Inc., Hoboken, NJ, USA; marketed as Octaplas LG in Europe and other markets) and is manufactured from ≤2500 pooled plasma products that have been treated with solvent (tributyl phosphate)/detergent (triton X-100) to inactivate lipid-enveloped viruses (HIV, hepatitis B, hepatitis C).[12,13] Additionally, the product is tested for hepatitis A and parvovirus B19. The product

TABLE 115.1 Average Coagulation Factor Activity in FFP and FP24 at the Time of Thaw and in Thawed Plasma After 5 Days of Storage at 1°C to 6°C

Factor	FFP	FP24	Thawed Plasma at Day 5	~Minimum Activity Required for Surgical Hemostasis
I (mg/dL)	280	309	303	100
II (IU/dL)	97	97	96	20–40
V (U/dL)	85	86	59	25
VII (IU/dL)	105	89	77	10–20
VIII (IU/dL)	81	66	48	30
IX (IU/dL)	82	88	84	25–30
X (IU/dL)	94	94	91	10–20

FFP, Fresh frozen plasma; *FP24*, 24 hours after phlebotomy.

is distributed in 200 mL containers, frozen at −18°C with a shelf life of 1 year. This pooled and treated plasma product has a lower rate of allergic reactions and has a substantially reduced risk of transfusion-related acute lung injury (TRALI), as the manufacturing process is considered to dilute neutrophil antibodies and soluble human leukocyte antigens (HLAs).[14]

Pathogen-Reduced Plasma

Other pathogen-reduction methods have been developed, including amotosalen photochemical treatment with ultraviolet (UVA) light (INTERCEPT plasma, Cerus Corp, Concord, CA), which is FDA approved. Riboflavin-treated plasma with UVA light (Mirasol Pathogen Reduction Technology, Terumo BCT, Denver, CO) and methylene blue-treated plasma (Theraflex System, MacoPharma, Mouvaux, France) are used outside of the United States, but these have not yet been FDA approved. Studies in Europe show that these methods are also quite effective at reducing the risk of pathogen contamination.[15]

Recovered and Source Plasma (Plasma for Manufacturing)

There are plasma products that are not used for transfusion but are used for further manufacturing into plasma derivatives. These products include recovered plasma that are derived from whole blood and are sent to a manufacturer from a collection facility through a "short supply agreement." Source plasma, an FDA licensed product, is collected by apheresis which is intended for further manufacturing. The Plasma Protein Therapeutics Association promotes safe collection and manufacturing practices of plasma derivatives. Source plasma can be collected more frequently under special donor programs; the donors can be compensated for their time.[15]

Convalescent Plasma

Convalescent plasma is plasma obtained from a patient who has recovered from or has been vaccinated against an infectious disease and has high titers of neutralizing antibodies (e.g., COVID-19). Convalescent plasma is transfused to infected patients to prevent or treat severe or life-threatening complications. COVID convalescent plasma potentially is beneficial if transfused early in the disease course or to patients who are immunocompromised.[16] Convalescent plasma can also be pooled and treated, as described below, to create a hyperimmune globulin.

Indications

Most guidelines consistently support plasma transfusions for the treatment of bleeding in patients with multiple acquired coagulation factor deficiencies, as seen in liver failure, disseminated intravascular coagulopathy (DIC), massive transfusion, and in certain indications as a factor replacement during therapeutic plasma exchange (TPE; Table 115.2).[17,18] However, the optimal transfusion strategy for these indications is not well established. Currently, there are no evidence-based laboratory value "triggers" for plasma administration, and any recommendation needs to be carefully weighed against the patient's presence, or risk, of bleeding.[9] Plasma transfusion is not indicated for use in clotting disorders where purified, concentrated, or recombinant clotting factors are available (e.g., factor VIII and IX deficiencies).

Plasma is typically indicated when prothrombin time (PT) and/or partial thromboplastin time (PTT) are greater than 1.5 to 1.7 times normal paired with the presence of bleeding or anticipated bleeding. These current recommendations are justified as there is compelling evidence that plasma transfusions are ineffective in correcting mild to moderate abnormalities of coagulation screening tests.[20] However, marked reductions in substantially elevated coagulation studies can occur with relatively modest plasma transfusion volumes. This response to plasma can be largely explained because of the nonlinear, exponential relationship between clotting factor activity levels and coagulation test results (Fig. 115.1).[21]

Audits of recent transfusion practices have consistently demonstrated that plasma product use is inappropriately high. Recent estimates suggest up to 83% (reported range: 10% to 83%) of plasma transfusions are not administered according to published guidelines.[22] The most commonly cited reason for plasma administration is a preprocedural elevation in coagulation studies.[22] This indication is not evidence-based, especially when the coagulation abnormality is mild-moderate.[24] Moreover, plasma should not be used as a volume expander or as a source of nutrients. Clinical situations where plasma transfusions may be beneficial are described below.

Liver Failure

Patients with liver failure may develop low levels of the vitamin K-dependent clotting factors (factors II, VII, IX, and X). These patients develop a prolonged PT/INR, PTT, and thrombin time.

TABLE 115.2 Indications for Plasma Product Transfusion

Indicated
Disseminated intravascular coagulation and bleeding or high risk of bleeding
Liver failure
Massive transfusion
Multiple acquired coagulation factor deficiency
Plasma infusion or exchange for thrombotic thrombocytopenic purpura and other thrombotic microangiopathies, diffuse alveolar hemorrhage, and catastrophic antiphospholipid syndrome
Rapid reversal of warfarin effect when prothrombin complex concentrate is not available
Replacement of an inherited single plasma factor deficiency for which no coagulation factor concentrate exists
Not Indicated
Burns
Immunodeficiency
Source of nutrients
Volume expansion
Wound healing

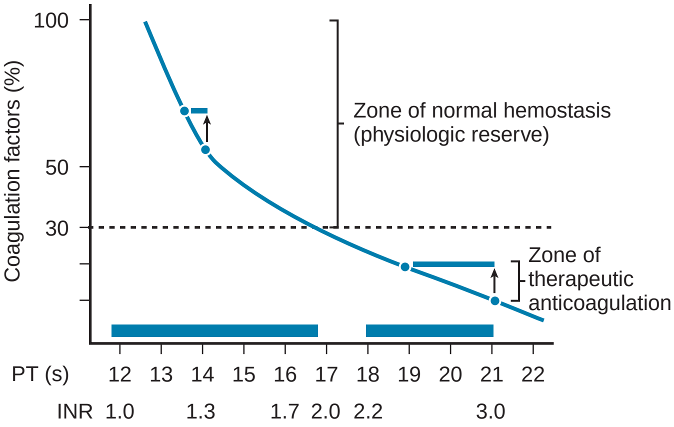


Figure 115.1 RELATIONSHIP BETWEEN FACTOR ACTIVITY LEVELS AND COAGULATION STUDIES. The general relationship between the concentration of coagulation factors and the result of PT and INR studies. The normalization of modest elevations in the INR required much larger volumes of plasma than would be expected and modest doses of plasma can result in marked changes in the INR when markedly elevated. The cause of this phenomenon can be explained by the nonlinear, exponential relationship between coagulation factor concentration and standard coagulation test results. Small increases of coagulation factors correlate with marked changes in coagulation studies when coagulation factors are depleted. The opposite is true when the coagulation factors are at higher concentrations. *INR*, International normalized ratio; *PT*, prothrombin time. *(Adapted from Kor DJ, Stubbs JR, Gajic O. Perioperative coagulation management: fresh frozen plasma. Best Pract Res Clin Anaesthesiol. 2010;24[1]:51–64.)*

Fibrin split products may also be elevated in these patients, and in later stages, the fibrinogen level may be decreased. Hemorrhage, most often secondary to an anatomic lesion, may be worsened by the coagulopathy. Patients undergoing orthotopic liver transplantation complicated by preexisting severe liver disease and liver disease with DIC are two examples that may require large plasma transfusion volumes.[25,26]

While elevations in coagulation tests are correlated with the incidence of bleeding in these patients, growing evidence now suggests that the PT and PTT are, in themselves, poor predictors of surgical bleeding.[27] The reason for this lack of association may be twofold. First, PT and PTT values do not correlate well with plasma factor activity levels. One study identified that up to 50% of patients with abnormal coagulation tests had coagulation activity levels considered sufficient for adequate thrombus formation. Moreover, studies demonstrate that mild abnormalities in these coagulation tests do not correct—even with infusion of large quantities of plasma—because of the mathematical difficulty of infusing normal levels of factors into mildly deficient blood to get enough plasma to decrease the PT/PTT (see Fig. 115.1).[28] Second, the lack of increased/excessive bleeding noted in some patients with liver disease and elevated coagulation tests may be caused by a parallel reduction in anticoagulant proteins, such as proteins C and S.[29,30] Therefore, patients with liver disease may not bleed as much as expected because they retain a homeostatic balance between coagulant and anticoagulant proteins. Evidence suggests that the use of plasma in the context of severe liver disease and perioperatively during liver transplant does not significantly improve outcome.[31] As a result, the use of plasma should be more limited in liver disease and hepatectomy patients. Plasma transfusion in these patients should be guided by a combination of clinical assessment, evidence and degree of bleeding, and coagulation test results. Plasma products are currently not recommended prophylactically before a surgical challenge or liver biopsy in these patients. However, as noted previously, plasma transfusions may be considered when the PT/PTT is greater than 1.5 to 1.7 times normal, or if the INR is 2.0 or greater when the risk of bleeding is considered high.

Massive Transfusion

Massive transfusion is generally defined as receiving 10 or more units of RBCs within 24 hours (or one blood volume). Trauma patients may arrive at the hospital with a prolonged PT (termed acute trauma-induced coagulopathy) due to protein C activation, fibrinogen depletion, and platelet dysfunction. Acute trauma-induced coagulopathy is associated with increased mortality and increased use of blood products.[32] Trauma patients can also develop a secondary coagulopathy, termed the lethal triad—dilutional coagulopathy, acidosis, and hypothermia.[33] The dilutional coagulopathy is secondary to the administration of crystalloid and RBCs without coagulation factor support.[34,35] Guidelines supported by randomized control trials recommend early use of tranexamic acid, an antifibrinolytic.[36]

Studies have shown that the early use of plasma and platelets in trauma patients undergoing massive transfusion appears to decrease the incidence of secondary coagulopathy (lethal triad) and improve survival in these patients. The Prospective Observational Multicenter Major Trauma Transfusion (PROMMTT) study[37] demonstrated that early transfusion of plasma (within minutes of arrival to a trauma center) was associated with improved 6-hour survival after admission. The Pragmatic, Randomized Optimal Platelet and Plasma Ratios (PROPPR) trial[38] compared the effectiveness and safety of a 1:1:1 (plasma:platelet:RBC) transfusion ratio with a 1:1:2 transfusion ratio in trauma patients who were predicted to receive a massive transfusion. This randomized clinical trial found no overall difference in survival based on transfusion ratio, but did find that those who were randomized to receive more plasma (1:1:1 ratio) achieved hemostasis more quickly and frequently.

Questions regarding best practice remain.[39] Currently, the use of cold stored whole blood is being evaluated and used in the prehospital and early hospital acute bleeding episodes. Studies support the use of whole blood early in trauma care prior to the need of specific blood components as a result of laboratory testing.[40] Other studies are investigating the early use of cryoprecipitate or fibrinogen concentrates as part of massive transfusion protocols.[41] Recently trials investigating the benefits of hospital plasma transfusion have been published. Prehospital plasma transfusion may benefit trauma victims who have longer transit time and suffered blunt trauma.

To improve the speed of identifying and treating coagulation abnormalities, some protocols incorporate thromboelastography (TEG) or other point-of-care tests.[44] TEG technology provides a dynamic and global assessment of the coagulation process and can provide rapid assessments of the patient's platelet function, coagulation cascade, and fibrinolysis, aiding in determining what medications or blood products the patient needs.[45]

Massive transfusion in other conditions, such as liver, cardiac, or orthopedic surgery and obstetric hemorrhage, likely have a different pathophysiology and thus transfusion management of these patients may be different than trauma patients.[46] Institutions should have policies in place for rapid availability of blood products and laboratory testing in these patient populations.

Disseminated Intravascular Coagulation

DIC may be secondary to sepsis, liver disease, hypotension, hypoperfusion, trauma, obstetric complications, leukemia (usually promyelocytic), or underlying malignancy. Successful treatment of the underlying cause is paramount. Recent guidelines suggest that plasma therapy should not be initiated based on abnormal laboratory results alone.[47] Rather, patients with DIC and bleeding, those requiring an invasive procedure, and those at risk for bleeding complications should be given plasma in amounts sufficient to correct or ameliorate the coagulopathy or hemorrhagic diathesis. Large volumes of plasma are often necessary to correct the coagulation defect in these patients (up to 30 mL/kg).[48] However, in patients with severe liver disease, bleeding, and DIC, plasma infusions often fail to normalize the PT and PTT.[49]

Rapid Reversal of Warfarin Effect

Warfarin inhibits the hepatic synthesis of vitamin K-dependent clotting factors (factors II, VII, IX, and X) by blocking the recovery of the form of vitamin K that is active in the carboxylation of these proteins. Warfarin therapy induces functional deficiencies of these factors, which correct within 48 hours after the discontinuation of warfarin if diet and vitamin K absorption are normal.

The use of plasma in the context of warfarin anticoagulation is well established but is no longer the preferred treatment. If available, four factor prothrombin complex concentrate (PCC) should be chosen as the first-line therapy for rapid reversal of life- or limb-threatening warfarin anticoagulation. In patients anticoagulated with warfarin who have active bleeding, require emergency surgery, or have serious trauma, the deficient clotting factors can be immediately provided by PCC. PCC can reverse a warfarin-induced coagulopathy faster with lower mortality and less volume overload than plasma or vitamin K alone.[50] Neither PCC nor plasma is indicated for warfarin reversal when the patient is not bleeding and has an INR less than 9, as vitamin K administration corrects the coagulopathy in 12 to 18 hours. INR levels need to be closely followed to ensure warfarin reversal is sustained. Plasma transfusion is only indicated for rapid reversal when no PCC is available.

Thrombotic Thrombocytopenic Purpura and Other Thrombotic Microangiopathies

In patients with TTP, TPE with plasma as the replacement fluid is lifesaving. Plasma infusion or exchange is also critical in the treatment of individuals who have congenital TTP. TPE has decreased the mortality of TTP from over 90% to less than 10% (see Chapter 132).[51] Six randomized control trials have demonstrated that TPE is most effective in patients who have an autoantibody to ADAMTS13 (ADAM metallopeptidase with thrombospondin type 1 motif 13).[52,53] This is caused by both the removal of a patient's plasma containing the inhibitor coupled with the addition of donor plasma containing the functional vWF-cleaving protease.[54] The FDA has also approved the use of CRP for TTP. Some authorities advocate CPR use as a first-line therapy for TTP, as these products have a lower level of vWF than FFP, comparable ADAMTS13 activity, and lower amounts of ADAMTS13–larger vWF multimer complexes. However, the most recent multicenter prospective randomized trial comparing TPE with plasma and CRP for the initial treatment of TTP demonstrated the two products were equivalent.[55]

The treatment of TTP has changed with the approval of caplacizumab, which inhibits the interaction between vWF multimers and platelets. Caplacizumab treatment is associated with faster platelet count recovery, lower incidence of TTP-related death or thromboembolism, and TTP recurrence.[56]

TPE is not indicated in the treatment of individuals with diarrhea-associated hemolytic uremic syndrome (HUS) (also termed thrombotic microangiopathy, Shiga toxin mediated) unless there are severe neurological symptoms. Seven randomized control trials evaluated the efficacy of TPE for typical cases of HUS. These trials found that the plasma use was not superior to supportive therapies alone.[53] TPE with plasma replacement fluid, however, is currently indicated in diarrhea-negative (atypical) HUS (aHUS), which is caused by several inherited and sporadic conditions that lead to the uncontrolled activation of the alternative complement system (i.e., deficiency or autoantibody to complement factor H). Typically, all patients diagnosed with aHUS are empirically treated with TPE or plasma transfusions until underlying disease or mutation is further defined, which then determines treatment.[57] TPE has been theoretically proposed to effectively remove the potentially causative autoantibody or mutated circulating complement regulator, while replacing absent or defective complement regulators. However, the clinical response varies depending on the underlying cause (see Chapter 132).

TPE with plasma replacement may also be indicated in other thrombotic microangiopathies including those due to medications, most notably ticlopidine and clopidogrel, and potentially cyclosporine or tacrolimus. Another example is in the treatment of thrombotic microangiopathy associated with stem cell transplantation.[51]

Plasma as replacement fluid, either partially or completely, for TPE is used in other diseases with risk of hemorrhage caused by the resulting coagulopathy, such as diffuse alveolar hemorrhage, liver failure, and perioperatively (see Chapter 116).

Prophylactic Use

Studies have shown that prophylactic administration of plasma to non-bleeding recipients with abnormal coagulation studies (i.e., PT, PTT, INR) is unlikely to produce a clinical benefit and unnecessarily exposes the patient to the risks of plasma transfusion. Moreover, systematic reviews of the predictive value of a prolonged PT or PTT found no significant difference in the risk of bleeding between patients with a prolonged PT or PTT and those with normal clotting parameters in the settings of bronchoscopy, central vein cannulation, angiography, liver biopsy, or lumbar puncture.[58] This is supported by randomized control trials and meta-analyses which have evaluated the efficacy of the prophylactic use of plasma products to reduce the risk of bleeding.[59,60] One trial, the Northern Neonatal Nursing Initiative Group Trial, randomized 776 neonates and evaluated whether plasma transfusion prophylaxis could prevent intraventricular hemorrhage in comparison with volume expanders (gelofusine or dextrose-saline).[61] In a second large randomized clinical trial, 275 patients were randomized to see whether plasma transfusions could prophylactically prevent bleeding in acute pancreatitis patients.[62] Neither large study showed clinical benefit of prophylactic plasma use. In one systematic review, 55 other randomized clinical trials were reviewed and evaluated. Only 17 of these 55 involved a control group that did not receive plasma.[63] Overall, like the two largest studies, the results of these randomized control trials failed to show evidence for the efficacy of prophylactic plasma use across multiple clinical and laboratory outcomes. Similarly, a second meta-analysis evaluated 25 independent studies of minor surgical procedures and found that there was no significant difference in bleeding risk between those who did and did not have a coagulopathy.[64] Despite this evidence, current recommendations still indicate that a pretransfusion INR of ≥ 1.5 to 1.7 be used as a transfusion trigger, as the prophylactic use of plasma is theoretically justified when the clinical risk of bleeding is greater than potential harms of using plasma.

Dosage

One unit of plasma derived from a unit of whole blood is 200 to 280 mL. When plasma is collected by apheresis, as much as 800 mL can be obtained from one individual ("jumbo" plasma units), but these are then divided into volume of 200 to 250 mL. On average, there is 0.7 to 1 unit/mL of activity of each coagulation factor per milliliter of plasma and 1 to 2 mg/mL of fibrinogen. The appropriate dose of plasma may be estimated from the plasma volume, the desired increment of factor activity, and the expected half-life of the factor being replaced (i.e., factor VII has a half-life of only 4 to 6 hours, and thus plasma doses should be repeated every few hours if replacing factor VII in a patient with factor VII deficiency). Alternatively, the plasma dosage may be estimated as 10 to 15 mL/kg, and with that dose all clotting factor activities will increase by ~30% in the absence of rapid ongoing consumption[65] Ideally, plasma should be ordered as the number of milliliters to be infused. The frequency of administration depends on the clinical response to the infusion and correction of laboratory parameters. Moreover, plasma infusions should be given as close to the time as it is needed to allow for its maximum hemostatic effect if given pre-procedure. Complete normalization of coagulation laboratory values will likely not be achieved with plasma infusion, and minimal elevations of PT/INR (e.g., INR <1.5) are usually not corrected with plasma administration.[66]

Compatibility

Plasma is screened for unexpected RBC antibodies during product testing and should be ABO-type compatible with the patient (see below).[18] However, following traumatic injury, the patient's blood type is often unknown. Emergency release plasma, traditionally group AB, is used until blood typing has been completed, and the plasma used can be switched to the patient's identified ABO type. The advantage in using group AB plasma is its lack of anti-A and anti-B antibodies, thus avoiding the risk of acute hemolytic transfusion reactions. However, only 4% of the population is group AB. In order to increase the availability of plasma for emergency use during massive transfusion, group A plasma, with or without assuring low titers of anti-B, is now being used.[67] Studies demonstrate no increased risk to the recipient and no adverse effect on clinical outcomes.[68] Since patients typically receive group O RBCs and about 80% of the population is group O or group A, the risk of hemolysis is low.

	Plasma Product ABO Type			
Patient ABO type	**O**	**A**	**B**	**AB**
O	Yes	Yes	Yes	Yes
A	No	Yes	No	Yes
B	No	No	Yes	Yes
AB	No	No	No	Yes

Yes = compatible blood types.
No = incompatible blood types.
Plasma units do not need to be matched for RhD, as the number of RBCs is negligible. Additionally, frozen plasma does not need to be leukoreduced or irradiated.

Adverse Events

Plasma transfusions, like all blood products, have the potential for infectious and noninfectious adverse events. Transfusion transmitted diseases, although rare, traditionally include HIV, hepatitis B, and hepatitis C (see Chapter 118). Noninfectious risks include allergic reactions, TRALI, transfusion-associated circulatory overload (TACO), and hemolytic reactions (see Chapter 117).[69]

CRYOPRECIPITATE

Cryoprecipitate is prepared from 1 unit of FFP thawed at 1°C to 6°C. The precipitate is then refrozen and stored at −18°C or colder for 1 year. Cryoprecipitate, volume of 10 to 15 mL, contains 80 to 100 units of factor VIII, 150 to 250 mg of fibrinogen, and 50 to 60 mg of fibronectin as well as vWF and factor XIII. Pathogen-reduced cryoprecipitated fibrinogen complex is now approved for use in acquired fibrinogen deficiency or for replacement of factor XIII or von Willebrand factor, but not for FVIII replacement, if no specific factor concentrates are available.

Cryoprecipitate takes 10 to 15 minutes to thaw at ~37°C, and then requires pooling before infusion. Prepooled (pooled before storage) cryoprecipitate products are available, easing the burden of preparation on the transfusion services. Once pooled and thawed, cryoprecipitate is maintained at 20°C to 24°C and outdates in 4 hours (6 hours if unpooled or pooled in a closed system).

Indications

Cryoprecipitate is used predominantly to treat bleeding associated with fibrinogen deficiency and in the manufacturing of fibrin sealants and glue (Table 115.3). Cryoprecipitate should not be used to treat factor XIII, vWF, and factor VIII deficiencies, as virally inactivated factor concentrates are available. Human fibrinogen concentrate is also available, and FDA approved, and is primarily used for congenital fibrinogen factor deficiency in the United States and broader indications in Europe.

Like plasma, recent studies also indicate that cryoprecipitate is not used appropriately in clinical practice. One large audit, for instance, demonstrated that across 25 Canadian hospitals and 4370 units of cryoprecipitate transfusion, only 24% of transfusions were considered clinically appropriate, and 34% of cryoprecipitate transfusions were deemed inappropriate according to published national guidelines (i.e., Transfusion Medicine Advisory Group of British Columbia, Canada Guidelines for cryoprecipitate transfusion).[70]

TABLE 115.3 Administration of Cryoprecipitate

Indicated
Congenital afibrinogenemia if fibrinogen concentrate unavailable
Dysfibrinogenemia if fibrinogen concentrate unavailable
Factor XIII deficiency if factor XIII concentrate unavailable
Fibrinogen deficiency if fibrinogen concentrate unavailable
Massive transfusion
Reversal of thrombolytic therapy
Possibly Indicated
Amniotic fluid embolism (used as last resort to replace depleted fibronectin)
Snake bites
Uremic bleeding

Fibrinogen Deficiency

Fibrinogen deficiency is the primary indication for cryoprecipitate transfusion. The deficiency may be caused by congenital afibrinogenemia or dysfibrinogenemia, severe liver disease, DIC, or massive transfusion.[48,71,72] Patients with the latter indications often have concomitant decreases in clotting factor levels and require the coadministration of plasma products. It is important to obtain fibrinogen measurements because levels less than 100 mg/dL cause prolongation of the PT and PTT, despite adequate clotting factor replacement. Very low levels of fibrinogen occur during liver transplantation (<100 mg/dL), where transfusion support with cryoprecipitate is vital.[17,28]

A specific purified human fibrinogen concentrate is an alternative for direct fibrinogen replacement in isolated fibrinogen deficiencies, such as inherited hypofibrinogenemia. Fibrinogen concentrates undergo viral inactivation and have a standardized fibrinogen content; these are used preferentially over cryoprecipitate in some countries, but studies have not demonstrated a clinical benefit over cryoprecipitate.[73–75]

Fibrin Glue/Sealant

Fibrin glue/sealant results from the mixture of a fibrinogen source (from plasma, platelet-rich plasma, or allogeneic/autologous cryoprecipitate) with a thrombin source (bovine, human, or recombinant). The enhanced local hemostasis achieved by the sealant product is through the action of thrombin on fibrinogen. "Fibrin glue" is a non–FDA-approved thrombin/preparation, and it has been widely used in Europe for many years. Fibrin sealants are FDA-approved alternatives to fibrin glue and are advantageous over locally made fibrin glues, because of standard dosing.[76] Fibrin glues/sealants can be used for multiple surgical purposes, including as a topical hemostat (creating a blood clot to halt bleeding), as a sealant (agents to prevent leakage of potentially nonclotting fluids [i.e., cerebrospinal fluid]), or as an adhesive (bonds different tissues together).

The safety profile of each product differs depending on the product components and source. Bovine thrombin has been reported to cause anaphylaxis (because of bovine allergies), coagulopathy through formation of antibodies to factor V or II, and rarely death caused by severe systemic hypotensive reactions.[77] Consequently, bovine products have an FDA mandated black-box warning on their package inserts. Pooled human plasma sources have the potential risk of viral or prion disease transmission. Reports indicate that hepatitis A and parvovirus B19 are particularly difficult to remove from these products despite current cleansing and filtration methods, and it is recommended that patients be counseled about this risk, although, given the current safety of the blood supply, the infectious risks are extremely low. Recombinant products, while eliminating the risk of infectious transmission or antibody formation, may also cause allergic reactions because of the hamster or snake proteins used to manufacture the product. Lastly, autologous fibrin glue preparations have been used, although the infectious risks (e.g., HIV and hepatitis) associated with the use of heterologous fibrin glue are eliminated by replacement with the autologous source, but are resource intensive.

Alternatively, albumin mixed with glutaraldehyde has been used to form both an effective sealant and adhesive.[78] The FDA has currently approved an albumin-based product to seal large blood vessel anastomoses and to reattach layers of the aorta in the context of an aortic dissection. Other successful reported uses include as a sealant in breast cancer surgery, and to reduce air leaks in lung volume reduction procedures. Side effects of this compound can be significant, however, and include nerve and muscle necrosis, sinoatrial node damage, calcium metabolism abnormalities, mucosal and skin irritation, adhesive emboli, limitation of aortic growth, and pseudoaneurysms.

Massive Transfusion

While most studies regarding massive transfusion evaluate the use of platelets and plasma, some studies suggest that regular doses of cryoprecipitate may also help improve survival. In general, the current use of cryoprecipitate in massive support is not standardized and the use is based on theoretic efficacy. According to the recently published PROMMTT study, there are wide differences (7% to 82%) in the use of cryoprecipitate at US level 1 trauma centers, and inclusion of cryoprecipitate in massive transfusion protocols vary significantly. Specifically, some PROMMTT sites used cryoprecipitate after a certain number of RBC units infused, while others used fibrinogen level triggers, such as 100 mg/dL.[76] While the PROMMTT study found no significant differences in mortality between those that did and did not receive cryoprecipitate, other studies have shown benefit. One study found that a high transfusion ratio involving cryoprecipitate in 214 massive transfusion patients resulted in improved 30-day survival (66% vs. 41%).[80] Another key study found that maintaining a ≥0.2 g fibrinogen/RBC (10 units of cryoprecipitate used for every 10 units of RBCs) unit ratio resulted in significantly higher survival rates (76% vs. 48%).[81] Lastly, military trauma patients in the MATTERs II study who received a combination of cryoprecipitate and tranexamic acid had the lowest observed mortality despite high injury severity scores (odds ratio, 0.34).[82] Consequently, while still under investigation, current data tentatively support the use of cryoprecipitate in the context of massive transfusion protocols, however cryoprecipitate remains indicated in the treatment of hypofibrinogenemia.[83] Outside of the United States and in clinical trials, fibrinogen concentrate is being used instead of cryoprecipitate in the treatment of massive hemorrhage.[84] See box on Severe Maternal Hemorrhage.

Uremic Bleeding

Abnormal bleeding is a common complication of uremia and is primarily caused by platelet dysfunction and defective interaction with endothelium. Use of cryoprecipitate as a source of vWF has been speculated to correct the platelet dysfunction. However, cryoprecipitate has been shown not to affect platelet aggregation in vitro, but it has been reported to shorten the bleeding time in some uremic patients.[90,91] Therefore, in addition to desmopressin, dialysis, erythropoietin, RBC transfusion, and conjugated estrogens, cryoprecipitate may have an adjunctive role.

SEVERE MATERNAL HEMORRHAGE

Major obstetric hemorrhage is a leading cause of maternal morbidity and mortality and is preventable and/or treatable. Significant obstetric hemorrhage is defined as active bleeding greater than 1000 mL within the 24 h following birth that continues despite the use of initial measures including first-line uterotonic agents and uterine massage. Early assessment and aggressive treatment of postpartum hemorrhage (PPH) are important for reducing morbidity and mortality rates. A critical first step in managing PPH is rapid recognition that clinically significant bleeding has occurred, with effective communication of the situation to the appropriate team members, both clinical and laboratory staff. Subsequent measures include immediate resuscitation with definitive action to arrest the bleeding (obstetric, surgical, and/or hematologic) and ongoing assessment and monitoring of the response to treatment. In these cases, blood ordering protocols specific to obstetric patients may be helpful. A massive transfusion protocol, like that seen in acute trauma patients, ensures sustained availability of blood products while the bleeding remains uncontrolled. Unique to maternal hemorrhage, hypofibrinogenemia is an important predictor for the later development of severe bleeding.[85] Consequently, point-of-care technologies, such as thromboelastography and rotational thromboelastometry, in addition to fibrinogen levels, can identify decreased fibrin clot quality during PPH, which correlate with low fibrinogen levels and can assist in transfusion management.[86,87] Early administration of 1–2 g tranexamic acid is also recommended, followed by an additional dose in cases of ongoing bleeding.[88,89] Early fibrinogen replacement using an appropriate dose of cryoprecipitate or fibrinogen concentrate may also be beneficial in these cases.

Dosage

The dosage of cryoprecipitate is calculated based on the amount of fibrinogen present in 1 unit of cryoprecipitate, the plasma volume, and the desired increment. The difficulty in determining the correct amount to administer is primarily caused by variability in the fibrinogen content of cryoprecipitate, and secondarily by variability in donors and component processing and preparation. The goal of therapy should be to maintain the measured fibrinogen at greater than 100 mL/dL, although some studies and consensus recommendations have revised target levels to at least 150 to 200 mg/dL, or a TEG maximum clot firmness (MCF) reading of 6 to 8 mm. It is estimated that each unit of cryoprecipitate raises the fibrinogen level of a 70-kg patient by at least 7 to 8 mg/dL; thus, a dose of 10 units of cryoprecipitate (equivalent of 1 blood volume's worth) will increase the fibrinogen in a 70 kg adult by 70 to 80 mg/dL, but how this dose affects a TEG is unclear. Dosing frequency should be determined based on clinical and laboratory responses; fibrinogen are very stable proteins with a half-life of 4 days.

Compatibility

Cryoprecipitate can contain minimal anti-A and/or anti-B antibodies and, as such, ABO and D compatibility is not necessary for most adult and pediatric patients. Cryoprecipitate does not need to be leukoreduced or irradiated.

Adverse Events

Cryoprecipitate has similar adverse event risk as other blood products, including transfusion-transmitted diseases and allergic reactions. Since it contains less plasma TACO and TRALI reactions are rare. Large volumes of ABO incompatible cryoprecipitate may cause a positive direct antiglobulin test.

PLASMA DERIVATIVES

Plasma derivatives, including albumin, human immune globulin (Ig), coagulation factor concentrates, are highly purified protein preparations that are obtained from large pools of whole blood or apheresis-derived plasma. The development of large-scale methods for the preparation of plasma derivatives began more than 70 years ago. There have been significant improvements in the preparation of these products since then, however the manufacturing is still based on modifications of the original method of cold ethanol fractionation, known as the Cohn fractionation process, developed by Dr. Edwin J. Cohn of Harvard Medical School and a team of scientists during the World War II era (Fig. 115.2).[1] Additional selective purification techniques involving ion exchange,[92] immune affinity chromatography, and other separation techniques are also used depending on the protein to be isolated and the individual manufacturer.[2]

Plasma contains thousands of different proteins and multiple products can be produced from a single raw material. Manufacturers begin by thawing frozen human plasma. During the Cohn's process, the ethanol concentration is serially increased and the precipitates and supernatants are separated by continuous-flow centrifugation or by large-scale filter presses. Fraction I, at 8% ethanol, yields the first precipitate in the fractionation process that contains fibrinogen and other poorly soluble proteins. Fractions II and III are then precipitated together and contain the immunoglobulins. Fraction IV-1 contains the vitamin K–dependent (VKD) clotting factors, antithrombin and α_1-proteinase inhibitor, whereas fraction IV-4 contains transferrin, haptoglobin, and some of the albumin. Fraction V is almost pure albumin (see Fig. 115.2).[1]

The extracted protein preparations are further subjected to additional steps (i.e. filtration, nanofiltration, solvent/detergent treatment, heat treatment, and pasteurization) to inactivate viruses and other infectious agents, making the risk of infectious disease transmission virtually zero.[93,94] The development of genetic engineering technology has allowed recombinant human plasma proteins to be produced in cell culture systems and transgenic animals.[95] This allows for greater control over the source of starting material, a reduction in the risk of infectious transmission from donors, and the easier production of a more uniform product with less variability. A description of select plasma derivatives is discussed below.

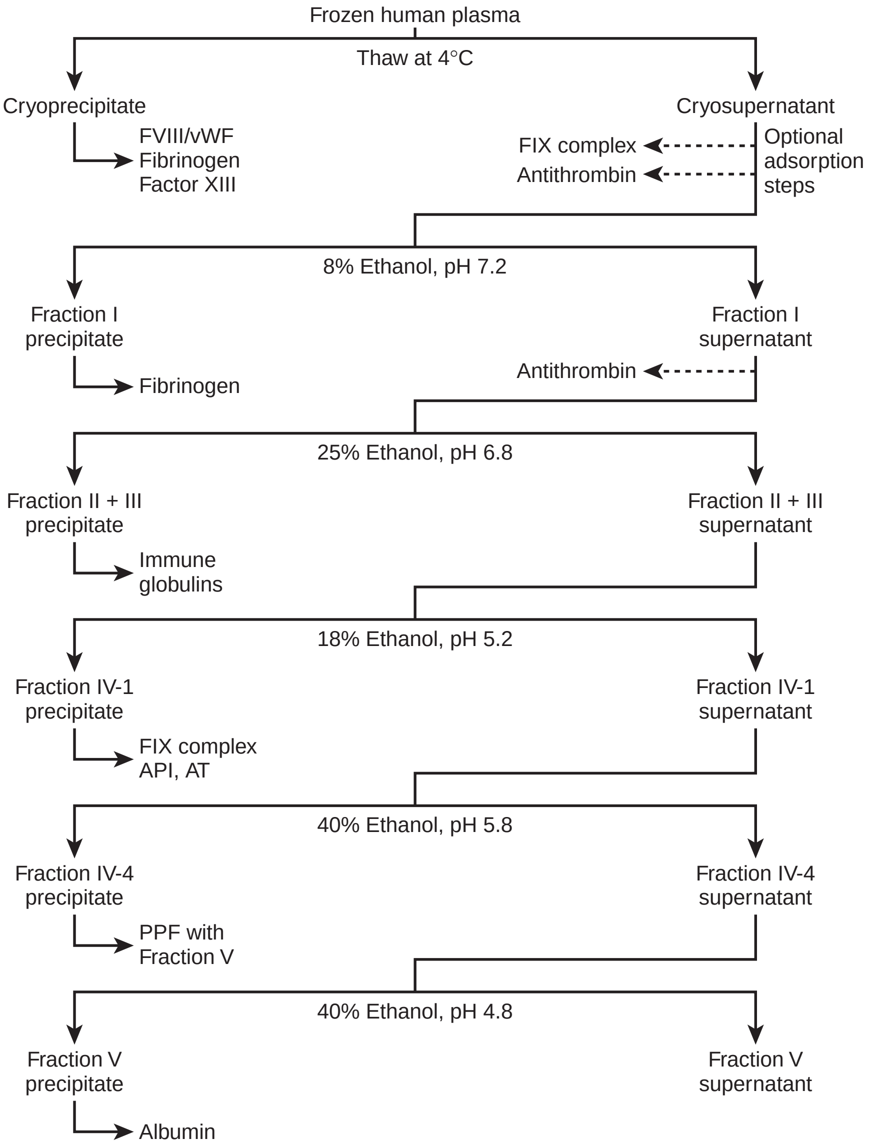

Frozen human plasma
Thaw at 4°C
Cryoprecipitate
FVIII/vWF
Fibrinogen
Factor XIII
Cryosupernatant
FIX complex
Antithrombin
Optional adsorption steps
8% Ethanol, pH 7.2
Fraction I precipitate
Fibrinogen
Fraction I supernatant
Antithrombin
25% Ethanol, pH 6.8
Fraction II + III precipitate
Immune globulins
Fraction II + III supernatant
18% Ethanol, pH 5.2
Fraction IV-1 precipitate
FIX complex
API, AT
Fraction IV-1 supernatant
40% Ethanol, pH 5.8
Fraction IV-4 precipitate
PPF with Fraction V
Fraction IV-4 supernatant
40% Ethanol, pH 4.8
Fraction V precipitate
Albumin
Fraction V supernatant

Figure 115.2 SCHEMATIC OF A TYPICAL PLASMA FRACTIONATION PROCESS. The *dashed lines* show optional steps. *API*, α_1-Proteinase inhibitor; *AT*, antithrombin; *FIX*, factor IX; *FVIII*, factor VIII; *PPF*, plasma protein fraction; *vWF*, von Willebrand factor.

ALBUMIN

Albumin, an important plasma protein, contributes primarily to the maintenance of plasma colloid oncotic pressure. Also, it is involved in the transport of numerous substances, such as unconjugated bilirubin, hormones, and drugs. Additionally, albumin has an established role in acid-base function, free radical scavenging, is antiapoptotic, antithrombotic, and has positive and negative effects on vascular integrity. The human body content of albumin is 4 to 5 g/kg and is responsible for 80% of the osmotic pressure of plasma.[96] Albumin is clinically available in four forms: 5% albumin solution; 25% albumin solution; albumin conjugated with polyethylene glycol; and purified protein fraction, which is 5% total protein (88% albumin and 12% globulins). These products are heat-treated, and albumin has not been documented to transmit infectious diseases (single outbreak occurred with albumin transfusion-associated hepatitis B with purified protein fraction in 1973 due to a failure in manufacturing process).[97]

Indications

A decrease in measured plasma albumin is found in many situations including chronic liver disease, chronic renal failure, sepsis, malignancy, burns, critical illness, severe head trauma, and hemorrhage, and is often not a clinically significant concern. Mild edema arising from hypoalbuminemia does not require albumin therapy. However, inadequate synthesis, as seen in severe liver disease and severe malnutrition, or excessive loss, as seen in nephrotic syndrome and protein-losing enteropathy, can lead to significant hypoalbuminemia with intravascular volume depletion, anasarca, ascites, and pleural effusions. Hypoalbuminemia is associated with poor clinical outcome in some studies, yet correction of low serum albumin levels in critically ill patients does not improve outcome measures such as mortality, duration of intensive care unit (ICU) and hospital stay, or mechanic ventilation. Historically, albumin had a broader use (i.e., nutritional support, correction of hypoalbuminemia, volume replacement); however recent studies support its benefit in fewer situations, including nephrotic syndrome resistant to potent diuretic therapy, after large-volume paracentesis, and in ovarian hyperstimulation syndrome (OHSS) (Table 115.4).[96]

TABLE 115.4 Administration of Albumin

Indicated
After large-volume paracentesis
Nephrotic syndrome resistant to potent diuretics
Ovarian hyperstimulation syndrome
Volume/fluid replacement in plasmapheresis
Possibly Indicated
Adult respiratory distress syndrome
Cardiopulmonary bypass pump priming
Fluid resuscitation in shock/sepsis/burns
Neonatal kernicterus/hyperbilirubinemia
To reduce enteral feeding intolerance
Not Indicated
Correction of measured hypoalbuminemia or hypoproteinemia
Nutritional deficiency, total parenteral nutrition
Preeclampsia
Red blood cell suspension
Simple volume expansion (surgery, burns)
Wound healing
Investigational
Cadaveric renal transplantation
Cerebral ischemia
Stroke
Common Usages
Cardiopulmonary bypass, pump priming
Extensive burns
Hypotension
Intraoperative fluid requirement exceeding 5–6 L in adults
Labile pulmonary, cardiovascular status
Liver disease, hypoalbuminemia, diuresis
Nephrotic syndrome, proteinuria, and hypoalbuminemia
Plasma exchange
Premature infant undergoing major surgery
Protein-losing enteropathy, hypoalbuminemia
Resuscitation
Serum albumin <20 g/dL

Intravascular Volume Expansion

As noted, albumin provides most of the plasma colloid oncotic pressure. Infused albumin provides colloid oncotic pressure; however, 50% of the infused protein is lost to the extravascular fluid compartment within 4 hours. Crystalloid may also provide volume expansion and is more quickly redistributed into total body fluids. Studies investigating the use of albumin in various situations including volume expansion during and after surgery, as priming solution in cardiopulmonary bypass, or in maintaining colloid oncotic pressure, found no clinical benefit compared with controls. In 1998, the Cochrane Injuries Group performed a systematic review of randomized control trials in albumin treatment of critically ill patients and concluded that there was no evidence that albumin use for volume expansion reduces patient mortality. They also suggested that albumin increases mortality, but this conclusion was not confirmed in later randomized control trials or subsequent meta-analyses.[98] The SAFE trial found, in 6997 patients across 16 ICUs, that there was no difference in survival between those ICU patients who received albumin versus normal saline.[99] In another randomized study, the SAFE trial investigators found that albumin use was associated with a trend toward better outcomes in patients with severe sepsis 28 days post-randomization.[100] A smaller prospective study further revealed that albumin was at least equivalent to plasma in clinical endpoints (perioperative/postoperative RBC transfusions, postoperative blood loss, duration of ICU stay, major complications) as a plasma expander in the context of pediatric craniofacial surgeries.[101] One conclusion from these prospective randomized trials is, contrary to previous reports, that the use of albumin is at least clinically equivalent to saline or plasma for intravascular volume resuscitation in some clinical settings. Moreover, the Italian Society of Transfusion Medicine and Immunohematology recently recommended that albumin may be useful for hypovolemia in some patients with hemorrhagic shock, patients undergoing major surgery, such as cardiac surgery, patients with severe burns, and patients post-liver transplant when crystalloids and other colloids did not provide adequate clinical benefit.[96]

Hypoalbuminemia

Low serum albumin is an independent predictor of morbidity and mortality in many clinical settings. However, correction of low serum albumin levels in ill patients does not improve outcome measures such as mortality.[102] However, two randomized controlled studies showed that correction of hypoalbuminemia did improve respiratory, cardiovascular, and central nervous system function. Current guidelines support the use of albumin to correct hypoalbuminemia for patients with ascites, large volume paracentesis, hepatorenal syndrome, and spontaneous bacterial peritonitis.[103] Recent studies with albumin infusions have also been done in end-stage liver disease patients for hypoalbuminemia. However, results are less encouraging, with studies indicating no additional benefits or reduction in morbidity.

Cirrhosis

The use of albumin in cirrhotic patients dates to before 1950. In this setting, albumin was recommended for temporary improvement in hyponatremia, spontaneous bacterial peritonitis, or prevention of the complications associated with paracentesis, including volume shifts and hyponatremia. A single randomized control trial of albumin use in cirrhotic patients with spontaneous bacterial peritonitis revealed that albumin administration with antibiotics resulted in reduced mortality and a reduced risk of renal failure in comparison with antibiotic use alone. Several studies demonstrated that after large-volume paracentesis (LVP) (>5 L), hyponatremia and renal insufficiency were improved with albumin infusion compared with other volume-expanding agents.[104,105] However, a recently published meta-analysis found insufficient evidence that albumin infusion after LVP lowered mortality in patients without hepatocellular carcinoma but with advanced liver disease.[106]

Nephrotic Syndrome

Albumin has been used to increase colloid oncotic pressure with the intention of increasing diuresis via increasing vascular pressure at the level of the glomerulus. Several studies have shown that albumin use in this context has resulted in no clinical benefit.[107] However, other studies have suggested that albumin use is associated with increased hypertension, respiratory distress, and electrolyte abnormalities. Consequently, the current recommended use of albumin for nephrotic syndrome patients is limited to patients in whom diuretic therapy is poorly tolerated or ineffective or in those with massive ascites or anasarca.

Ovarian Hyperstimulation Syndrome

OHSS is usually a result of iatrogenic administration of human chorionic gonadotrophin (hCG) to induce ovulation. OHSS is typified by enlarged ovaries which release vascular endothelial growth factor that can result in increased capillary permeability. This, in turn, leads to a fluid shift out of the intravascular compartment to the abdominal/pleural spaces resulting in ascites and hypovolemia. In the most severe form, the patient can develop tense ascites, oliguria, dyspnea, hemodynamic instability, and thromboembolism. Treatment includes fluid restriction, analgesics, and close monitoring; occasionally, hospitalization may be necessary.

Mild OHSS occurs in approximately one-third and moderate-severe in approximately 5% of women receiving exogenous hCG. Increased risk of OHSS includes young age, low body weight, polycystic ovarian syndrome, high-dose hCG, high or rapid rise in estradiol level, and previous history of OHSS. In addition, the risk is proportional to the number of developing follicles and number of oocytes retrieved.[118] Moderate-severe OHSS can be mitigated by closely monitoring women during treatment and subsequently withholding or reducing hCG administration when there is a large number of intermediate-size developing follicles present or when estradiol levels are elevated.[109,110]

In 2011, the Cochrane collaboration systematically reviewed eight randomized clinical trials of albumin administration in OHSS and concluded that there is only a borderline statistically significant decrease in the incidence and severity of OHSS when albumin was administered during oocyte retrieval in high-risk women. In contrast, the meta-analysis further revealed that the use of hydroxyethyl starch (HES) resulted in a markedly decreased incidence of severe OHSS.[111] In addition, Bellver et al. published a large, randomized trial that demonstrated no difference in moderate-severe OHSS when 40 g of albumin was administered after the retrieval of 20 or more oocytes.[112] Only one (nonrandomized) study to date has compared human albumin and 6% HES. This study concluded in 16 patients with severe OHSS that patients who received HES had a higher urine output, needed less abdominal paracentesis and drainage of pleural effusions, and had a shortened hospital stay than patients who received albumin.[113] Therefore, while still clinically used, albumin may be inferior to other therapies in the prevention of OHSS.

Therapeutic Apheresis

Albumin is the replacement fluid of choice for many apheresis indications (see Chapter 116).[51] Albumin reduces the risk of adverse events during apheresis procedures by reducing the risk of viral transmission, allergic reactions, and TRALI in comparison to plasma use (see plasma section). Albumin can also be used in combination with saline during apheresis procedures, but excessive use of saline results in hypotensive reactions. Albumin is also indicated if large (>15% of the total blood volume) blood volumes are removed to prevent hypotensive reactions in other therapeutic or collection apheresis procedures (leukapheresis, plateletpheresis).

While albumin is generally well tolerated in therapeutic apheresis patients, albumin use can result in significant hypotension, bradycardia, and flushing in patients receiving angiotensin converting enzyme inhibitor (ACE) therapy. ACE inhibitors prevent the patient's ability to metabolize bradykinins that are present in the albumin and activated during the apheresis procedure. In patients taking ACE inhibitors, symptoms can be prevented by using plasma, or halting ACE inhibitor use and delaying the start of apheresis therapy.

Fluid Resuscitation in Sepsis/Burns

Serum albumin has been shown to be a free radical scavenger. Because of this function, some guidelines recommend albumin infusion when patients with sepsis or acute burns require a substantial amount of crystalloids. However, meta-analyses have demonstrated no significant difference in mortality from albumin compared to other fluids in patients with these conditions.[17,114–118]

Dose

The volume and speed of administration should be determined by the patient's volume status, condition, and response to the product.[119] In an adult, the total daily dose should not exceed the theoretical amount present in normal plasma (2 g/kg/day), in the absence of acute hemorrhage. In the pediatric population, albumin dose again depends on the patient's condition, but 0.5 to 1.0 g/kg/dose with a maximum dose of 6 g/kg/day is used as a general guideline. Albumin 5% is oncotically equivalent to normal human plasma. Albumin 25% provides less infusion volume per amount of albumin and is usually administered to patients with fluid or sodium intake restriction. Albumin 25% expands the blood volume by 3.5 times by drawing fluid into the intravascular space.

Adverse Effects

Albumin is a plasma derivative used widely and associated with rare adverse reactions.[103] Allergic reactions, including urticaria, may be encountered.[119] Changes in vital signs (heart rate, blood pressure, respiration rate), nausea, emesis, and fever/chills have also been rarely reported. Volume overload and dilutional anemia as well as hypocalcemia may occur. In addition, hypotensive and allergic reactions have been associated more frequently with the use of plasma protein fraction than purified albumin. Albumin has not been associated with transfusion-transmitted diseases.

HUMAN IMMUNOGLOBULIN

Human immunoglobulins (Ig) are prepared by fractionation of large pools of human plasma.[96] There are numerous preparations available in the US and worldwide. Ig preparations can be made for intramuscular (IM), subcutaneous (SC), or intravenous (IV) administration. Each preparation is slightly different and has theoretic advantages and disadvantages and specific licensed indications. Ideally, Ig preparations should contain each IgG subclass; retain Fc receptor activity; have a normal half-life; demonstrate virus neutralization, opsonization, and intracellular killing; and have antibacterial capsular polysaccharide antibody.[120] Furthermore, vasoactive impurities should be absent, and no transmissible infectious agents should be present. The uses of Ig have been extensively reviewed elsewhere (see reference section) and the number of theoretical and accepted uses is rapidly expanding.

Indications

Human Ig is indicated for replacement of Igs or for its immunomodulatory effects.[121] Currently, there are six FDA approved indications for IVIg treatment: primary immunodeficiency, B-cell chronic lymphocytic leukemia (CLL) with infection, chronic inflammatory demyelinating polyradiculoneuropathy (CIPD), immune thrombocytopenia (ITP), Kawasaki disease, and multifocal motor neuropathy. IVIg is also considered first-line therapy for multiple other conditions, such as allogeneic stem cell transplantation, Guillain-Barré syndrome, neonatal alloimmune thrombocytopenia (NAIT), posttransfusion purpura (PTP), myasthenia gravis and stiff-person syndrome (Table 115.5). A brief summary of some of the more well-established uses of IVIg is presented in the following section.

Primary or Secondary Immune Deficiencies

Primary congenital immunodeficiency syndromes have been treated with intramuscular Ig for the past 30 years. The use of intramuscular Ig has certain disadvantages: delayed absorption, delivery of inadequate amounts because of small muscle mass, and pain at the

TABLE 115.5 **Administration of Intravenous Immunoglobulins**

Indicated
Ataxia-telangiectasia
Chronic inflammatory demyelinating polyneuropathy
Chronic lymphocytic leukemia
Common variable immunodeficiency
Cytomegalovirus-interstitial pneumonia after bone marrow transplantation
Guillain–Barré syndrome
Graft-versus-host disease after bone marrow transplantation
Hemolytic disease of the fetus and newborn
Idiopathic thrombocytopenic purpura
Immunoglobulin G subclass deficiency
Inflammatory myopathies (refractory dermatomyositis and polymyositis)
Myasthenia gravis
Mucocutaneous lymph node syndrome (Kawasaki disease)
Neonatal alloimmune thrombocytopenia
Parvovirus infection
Persistent deficit in antibody production after bone marrow transplantation
Posttransfusion purpura
Primary immunodeficiency syndromes
Secondary hypogammaglobulinemia
Severe combined immunodeficiency
Stiff-person syndrome
Wiskott-Aldrich syndrome
X-linked agammaglobulinemia
Possibly Indicated
Antiphospholipid syndrome in pregnancy
Autoimmune hemolytic anemia (warm type unresponsive to prednisone)
Factor VIII inhibitors (refractory)
Graves ophthalmopathy
Immune neutropenia
Multiple myeloma (stable disease, high risk for infections)
Pemphigus
Solid organ transplantation (kidney)
Systemic lupus erythematosus (refractory, severe, active)
Thrombocytopenia refractory to platelet transfusion
Vasculitis (refractory to standard therapy)
Investigational
Acquired von Willebrand disease
Amyotrophic lateral sclerosis
Burn patients
Chronic fatigue syndrome
Chronic human parvovirus B19 infection
Chronic idiopathic pericarditis
Chronic pain syndromes
Congestive heart failure
Dilated cardiomyopathy
Graft-versus-host disease
Human immunodeficiency virus infection
Immune-mediated aplastic anemia
Inflammatory bowel disease
Intractable childhood epilepsy
Multiple sclerosis
Myocarditis
Necrotizing fasciitis
Neonatal sepsis
Neonatal hemochromatosis
Postpartum cardiomyopathy
Prevention of nosocomial postoperative infections
Prophylaxis in transplant recipients against cytomegalovirus infection
Recurrent unexplained spontaneous abortions
Rheumatoid arthritis
Sepsis and septic shock
Toxic epidermal necrolysis/Stevens-Johnson syndrome
Toxic shock syndrome

injection site. IVIg overcomes these disadvantages and used prophylactically in patients with primary immunodeficiency has been demonstrated to reduce the number of febrile and infectious episodes as well as improve survival rate.[122] IVIg use in IgG subclass deficiencies is also beneficial. The typical maintenance dose for adults is 400 to 600 mg/kg every 3 to 4 weeks to maintain a trough IgG level of at least 500 mg/dL. SC Ig administration is now also FDA-approved for use in primary immunodeficiency treatment. Secondary/acquired immune deficiency may be caused by multiple etiologies including hematologic malignancy (e.g., CLL, multiple myeloma, protein-losing enteropathy, nephrotic syndrome, or other severe illnesses). Criteria for the use of IVIg in these conditions include hypogammaglobulinemia (IgG < 200 or total Ig < 400 mg/dL), absent or low natural antibodies, absent or low response to antigenic challenge (i.e., vaccines), and lack of antibody response to the infecting organism.

B-Cell Chronic Lymphocytic Leukemia With Infection

CLL may be associated with hypogammaglobulinemia and complications of repeated bacterial infections.[123] These infections are the most common cause of morbidity and mortality in CLL patients. The administration of Ig preparations may replace the missing immunoglobulins needed for humoral immunity. However, due to recent advances in management and treatment for CLL patients, IVIg should only be considered for those with recurrent serious bacterial infections and serum IgG levels less than 500 mg/dL despite immunization to diphtheria, tetanus, or pneumococcus. A typical dose of 300 to 500 mg/kg per month is needed to maintain a minimum IgG level of 500 mg/dL.

Chronic Inflammatory Demyelinating Polyradiculoneuropathy

CIDP is a chronic disorder resulting in demyelination of peripheral nerves that result in weakness and sensory changes. Equivalent outcomes have been observed after treatment with IVIg (reported dose 2 g/kg/day over 2 to 5 days, once each month), TPE, or corticosteroids.[124] The decision as to which treatment to use is made on an individual basis balancing the risks and benefits of each treatment modality.

Immune Thrombocytopenia

IVIg and Rh immune globulin (RhIg, described below) are routinely and effectively used in the treatment of acute and chronic ITP.[125] IVIg significantly raises the platelet count within 5 days in adults with chronic ITP and in children with acute ITP. The mechanism of action of IVIg in ITP is unknown; one postulation is Fc receptor blockade decreases the removal of antibody-coated platelets. Other proposed mechanisms include suppressed antiplatelet antibody synthesis, increased antiviral immunity, and blockage by anti-idiotypic antibodies. In general, IVIg induces responses in most patients within 1 to 2 days. Responses are of variable duration and are rarely sustained, although maintenance therapy may be of some value. IVIg may be effective in chronic ITP refractory to corticosteroids or splenectomy, and may show greater efficacy in conjunction with corticosteroids.

Although IVIg has shown equal efficacy with corticosteroids in pediatric acute ITP and in 75% of adults with chronic ITP, because of the transient responses and high cost, its use is justified only in clinical situations requiring rapid elevation of platelet count or if standard therapy has failed. IVIg is, therefore, indicated in acute bleeding episodes or before urgent surgery, including splenectomy; in patients at high risk of intracranial hemorrhage; and in those in whom corticosteroids are contraindicated or ineffective. IVIg has also been used to treat ITP during pregnancy, postinfectious thrombocytopenia, ITP associated with HIV infection, and neonatal thrombocytopenia (see Chapter 129).

Kawasaki Disease

Mucocutaneous lymph node syndrome (Kawasaki disease) has been treated with aspirin with or without concomitant IVIg administration. Coronary artery aneurysm, a serious complication of this disease, was significantly reduced in the IVIg-treated group. However, in a multicenter retrospective survey of all children treated with Kawasaki disease, persistent or recrudescent fever after their first course of IVIg was associated with a statistically significant risk of treatment failure. Furthermore, IVIg retreatment in those patients with persistent fever after IVIg treatment failure was approximately 60%.[126] Current randomized trials are now being done to compare the efficacy of newer, and perhaps better, therapies, such as infliximab (anti-tumor necrosis factor-α).[127] To date, these randomized control studies show that infliximab is at least as safe and as efficacious as IVIg in these refractory patients. One retrospective review has even suggested that infliximab might result in faster resolution of fever and fewer days of hospitalization in comparison with IVIg. While studies are still ongoing, IVIg may soon be replaced by alternative therapies in this refractory patient population.

Regarding the pathogenesis of Kawasaki disease, decreased peripheral blood lymphocyte apoptosis has been demonstrated.[128] Therefore, the effect of IVIg in Kawasaki disease has been postulated to partially reverse inhibited lymphocyte apoptosis.

An important related disorder is COVID-19-associated multi-inflammatory syndrome in children (MIS-C), which involves at least four organ systems and occurs after infection with COVID-19. IVIg is the most common treatment.[129]

Multifocal Motor Neuropathy

Multifocal motor neuropathy is a chronic progressive disorder resulting in primarily hand weakness. IVIg (SC administration under investigation) is now considered a first-line treatment for this condition,[130,131] and improved strength can be seen at a dose of 2.0 g/kg over 2 to 5 days.

Hematopoietic Stem Cell Transplantation

The use of prophylactic IVIg or cytomegalovirus (CMV)–IVIg in CMV-negative hematopoietic stem cell transplant (HSCT) recipients during the first 100 days posttransplant has been demonstrated to reduce the incidence of symptomatic CMV-associated disease, including CMV interstitial pneumonia, in some trials.[132] Because of the high cost of this treatment and the increasing use of prophylactic ganciclovir, IVIg is currently not indicated for the routine prophylaxis of CMV infections in HSCT recipients. IVIg should be given to allogeneic HSCT recipients with IgG less than 400 mg/dL and with recurrent infections.[133] In established CMV-interstitial pneumonia, IVIg in combination with ganciclovir has been shown to reduce the mortality rate and has become the recommended treatment modality. IVIg's role in preventing severe graft-versus-host disease is unclear.[134] Prolonged IVIg therapy during graft-versus-host disease prevention may suppress humoral immunity recovery.

Solid Organ Transplantation

The presence of high-titer reactive antibodies against incompatible graft HLA or ABO antigens increases the risk of early solid organ, antibody-mediated, graft rejection and mortality, especially in kidney and cardiac transplants. For some patients who have HLA antibodies to undergo transplantation, these antibodies must be removed or decreased. While morbidity and mortality can be reduced by selecting an adequately cross-matched donor, IVIg with or without plasma exchange and with or without rituximab has also been shown to decrease sensitization of incompatible antigens in patients awaiting renal and cardiac transplantation.[135] In addition, IVIg combined with rituximab and with or without TPE is used in the treatment of biopsy-proven antibody-mediated rejection. Consensus as to the appropriate treatment regimen, IVIg ± TPE ± rituximab, for incompatible solid organ transplants, is still under investigation.

Randomized trials have not yet been performed for ABO-incompatible heart, liver, or lung transplants. Moreover, there is a paucity of data for transplant outcomes, and current studies have small numbers of patients without data on donor-specific antibody levels.

Aplastic Anemia Secondary to Parvovirus

Parvovirus B19 infection can result in severe anemia and reticulocytopenia, especially in immunocompromised individuals[136] or individuals with sickle cell disease or thalassemia, and the use of IVIg is considered first-line therapy in the treatment of these patients (typical dose 0.5 g/kg weekly for 4 weeks).

Dermatomyositis

Dermatomyositis is a chronic inflammatory disorder that results in progressive weakness and rash. IVIg (typical dose 2.0 g/kg per month administered over 2 to 5 days) results in improved muscle strength and neuromuscular symptoms.[137]

Guillain-Barré Syndrome (Acute Inflammatory Demyelinating Polyneuropathy)

Guillain-Barré syndrome is an acute demyelinating peripheral neuropathy affecting both motor and sensory nerves. IVIg (typical dose 400 mg/kg/day for 5 days or 1.0 g/kg/day for 2 days or 2.0 g/kg as a single dose) is likely equivalent to TPE in improving disability and shortening the time to improvement.[138]

Hypogammaglobulinemia Associated With Multiple Myeloma

Multiple myeloma is a monoclonal B-cell (plasma cell) disorder with clinical symptoms arising as a result of plasma cell infiltration of the bone marrow, monoclonal Ig in the blood and urine, and immunosuppression. IVIg (SC administration under investigation) has shown to be beneficial in preventing serious infections in plateau-phase multiple myeloma or other hematologic malignancy, where the patients have hypogammaglobinemia, at doses of 0.4 g/kg every 4 weeks, with subsequent dosing adjusted based on trough levels.[138]

Lambert-Eaton Myasthenic Syndrome

Lambert-Eaton myasthenic syndrome results from antibodies to the neuromuscular junction, leading to autonomic dysfunction. One randomized control trial revealed that IVIg significantly improves generalized central and peripheral muscle strength and decreases serum calcium channel antibody titers. A total dose of 2.0 g/kg given over 2 to 5 days is a recommended initial treatment.[137]

Multiple Sclerosis

Multiple sclerosis is a chronic progressive or relapsing and remitting disorder characterized by brain white matter demyelination. There are two published meta-analyses,[140,141] and several randomized controlled clinical trials[142] in patients with relapsing-remitting multiple sclerosis using a wide range of IVIg doses that demonstrate the success of IVIg in reducing the number of exacerbations and disability in patients with relapsing-remitting multiple sclerosis in comparison with placebo. However, no studies to date have compared IVIg with standard therapies, and one clinical trial (PRIVIG trial)[143] has raised doubt that IVIg is effective as a routine treatment. Consequently, IVIg is considered a viable second-line option for those patients who fail, decline, or are unable to tolerate standard immunomodulatory therapies such as β-interferon and glatiramer acetate.

Myasthenia Gravis

Myasthenia gravis is a chronic neurologic autoimmune disorder characterized by weakness and fatigue upon repetitive skeletal muscle use, which improves with rest.[144] IVIg has been used successfully as a short-term measure for acute severe exacerbations of myasthenia gravis at a dose of 2.0 g/kg given over 2 to 5 days and outcome is comparable to treatment with TPE.[145]

Neonatal Alloimmune Thrombocytopenia

NAIT is a rare condition that results from maternal platelet alloantibodies against the fetal/neonatal platelets resulting in neonatal/fetal thrombocytopenia.[146] Studies evaluating the use of IVIg for NAIT are limited because of the rarity of the condition. The treatment of NAIT during pregnancy is maternal administration of 1.0 g/kg IVIg weekly as a first-line therapy beginning at 20 weeks of gestational age with the use of corticosteroids, or 2.0 g/kg weekly if steroids are not used.[147] Once at 32 weeks of gestation, the IVIg dose is increased to 2.0 g/kg weekly with corticosteroids. Moreover, the neonate may need to receive IVIg and platelet transfusions after delivery to increase fetal platelet counts to prevent intracerebral hemorrhage at a dose of 1.0 g/kg (see Chapter 129).

Hemolytic Disease of the Fetus and Newborn

Hemolytic disease of the fetus and newborn (HDFN) results from maternal RBC alloantibodies binding to fetal/neonatal RBCs and may result in hemolysis, leading to anemia or hydrops fetalis and death depending on the severity. Maternal IVIg infusion (with or without TPE) has been used in severe cases of HDFN where treatment must occur before the ability to perform intrauterine transfusions. After delivery, IVIg has been suggested to significantly reduce the need for exchange transfusions in neonates with HDFN, although there have been conflicting results in randomized controlled trials.[148] In addition, IVIg treatment of HDFN has been associated with increased risk of necrotizing enterocolitis in the neonate. IVIg is now consequently recommended at a dose of 0.5 g/kg to treat newborns with HDFN if there is established jaundice and a rising total serum bilirubin despite phototherapy.[149]

Posttransfusion Purpura

PTP is a rare complication of transfusion resulting in acute, profound thrombocytopenia, secondary to platelet antibodies that destroy both transfused and autologous platelets. While the evidence evaluating the effect of IVIg in these patients is limited to multiple case reports, the available evidence suggests that IVIg should be a first-line therapy in this condition. PTP treatment with IVIg at a dose of 2 g/kg over 2 days or 0.4 to 0.5 g/kg daily for 5 days with or without corticosteroids can result in a rapid increase in platelet count.[150]

Sepsis and Septic Shock in Adults

The use of IVIg in adult patients with bacterial sepsis or septic shock is potentially beneficial. One randomized control trial revealed that in ICU patients with sepsis and shock, IVIg with antibiotics was superior to antibiotics with albumin in improving patient survival.[151] Encouraging results have also been identified in patients receiving

IVIg for streptococcal toxic shock syndrome, but further studies are currently needed.

Stiff-Person Syndrome

Stiff-person syndrome is a neurologic disorder associated with truncal and limb rigidity and heightened sensitivity. One small, randomized control trial suggests that IVIg could play a positive role in improving stiffness and sensitivity symptoms. Currently, IVIg is considered a second-line therapy for those who fail or cannot tolerate GABA (glutamic acid decarboxylase)-ergic medications. A dose of 2.0 g/kg given over 2 to 5 days is the current recommended starting dose.[136]

Dosage

The FDA has approved IM, IV, and more recently SC Ig products. The dosage and frequency for each Ig product vary significantly depending on patient's age and clinical indication. Typical dosages were described in the preceding section. In general, patients require 200 to 800 mg/kg intravenously every 3 to 4 weeks to achieve adequate IgG levels if immunodeficient (usually 500 mg/dL) and needing protection against infection. Initially, serial IgG level determination may allow the physician to individualize the dose and schedule. These are affected by the recovery, half-life, redistribution, and catabolism of Ig preparation, which vary from product to product and patient to patient. Currently, direct comparisons between different IVIg formulations or brands are lacking, and so evidence-based recommendations cannot be made at this time.

Adverse Effects

Infusions of IVIg should be started slowly and patients should be closely monitored. If the initial rate (0.5 mL/kg/h) is well tolerated, the rate can be increased gradually, but not more than eightfold. That said, initial and maximum infusion rates vary by IVIg product, and product inserts should be consulted prior to selecting an appropriate infusion rate. Fever, headache, nausea, vomiting, fatigue, backache, leg cramps, urticaria, flushing, elevation of blood pressure, and thrombophlebitis may be seen.[152] Adverse events have been reported in 2% to 10% of infusions. Adverse reactions differ among different preparations, such that patients may tolerate one product better than another product. IgA-deficient patients may have IgG anti-IgA antibodies, which can cause anaphylactic reactions. This complication is rare and may be avoided by using products with a lower concentration of IgA. Aggregated IgG may produce chills, nausea, flushing, chest tightness, and wheezing. Rarely, IVIg preparations contain RBC antibodies that can produce hemolysis or interfere with serologic evaluations, including RBC compatibility testing. IVIg treatment will produce a false positive direct antiglobulin test, and sometimes positive hepatitis and CMV serologies. Serum sickness can also occur. Lastly, high-dose IVIg therapy has been associated with thrombosis, reversible acute renal failure, TRALI, and aseptic meningitis. Improved manufacturing processes currently in place render IVIg free of enveloped and nonenveloped viruses.

TABLE 115.6 **Hyperimmune and Intramuscular Immunoglobulins**

Antithymocyte globulin
Botulism immunoglobulin
Cytomegalovirus immunoglobulin
Hepatitis A immunoglobulin
Hepatitis B immunoglobulin
Rabies immunoglobulin
Respiratory syncytial virus immunoglobulin
Rh(D) immunoglobulin
Tetanus immunoglobulin
Vaccinia immunoglobulin
Varicella-zoster immunoglobulin
Western equine encephalitis immunoglobulin

HYPERIMMUNE IMMUNOGLOBULIN PRODUCTS

Hyperimmune globulins (Hyper-Ig) are concentrated Igs with specificity for an antigen or group of antigens.[153] These products are manufactured in a similar manner to that used for IVIg product production. However, donors for these specific products are unique in that they have high titers for the Ig specificity of interest. The donor high titers can be achieved via natural immunity, prophylactic immunization, or target immunization, depending on the antibody of interest. These products are generally used to provide passive immunity for a variety of conditions that are described in more detail in the following sections (Table 115.6).

Antithymocyte Globulin

Antithymocyte globulin is a purified concentrated globulin made from hyperimmune serum of horses immunized with human T lymphocytes. Antithymocyte globulin is used in renal transplant patients as an adjunct therapy in the treatment of graft rejection.[154] It is also used in patients with aplastic anemia who are not candidates for bone marrow transplantation.[155]

Infection-Associated Hyperimmune Immunoglobulin

Hyper-Ig is used to prevent the development of specific clinical disease or alter its symptomatology.[156] Hepatitis B Ig is used to provide passive immunity to hepatitis B virus associated with needle-stick exposure or sexual contact with hepatitis B surface antigen-positive individuals, post liver transplantation for prevention of recurrence, and prevention of hepatitis B vertical transmission. Other hyperimmunoglobulins include botulism, CMV,[157] hepatitis A, rabies,[158] respiratory syncytial virus, tetanus, vaccinia, and varicella-zoster virus Igs. Convalescent plasma can be made into hyperimmune Igs, which is being explored for treatment of COVID-19.[159]

WEAK, PARTIAL D, AND RhIg USE

Clinically significant D sensitization potentially results in a pregnancy with a fetus at risk for hemolytic disease of the fetus and newborn[161] and hemolytic transfusion reactions if transfused with D-positive red blood cells. In rare cases, as a result of a mutation in the *D* gene, a person may have amino acid substitutions affecting a part of the normal D protein that changes the antigen on the external aspect of the red blood cell. These patients are described as having "partial D." When someone with partial D is exposed to blood from someone with a normal form of D, they may make an antibody against the portions of the D antigen that they lack. Individuals with red blood cells that express a partial D antigen are thus at risk for D-sensitization and may benefit from RhIg administration to prevent D sensitization. Mutations which result in fewer "normal" D antigens on a red cell are known as having a "weak" D. Those with weak D antigens are usually not at risk for D sensitization because they have a normal D protein, and thus do not require RhIg administration. Partial and weak D antigens cannot be distinguished by serologic reactivity. However, reagents are manufactured that the most frequent partial D phenotypes give weak reactions. If a patient has weak or variable D typing, particularly in the prenatal setting, RhD genotyping can be done to distinguish weak D from partial D to help determine need for RhIg administration.[161,162]

Dosage

In general, formulations for the described hyper-Ig are generally prepared for IM administration but, in some instances, IV formulations are available. Refer to manufacturer's instructions for recommended dose and frequency of specific hyper-Igs as differences exist between formulations and for different conditions.

Rh Immunoglobulin

Rh immunoglobulin (RhIg) has two primary indications: prevention of D antigen alloimmunization and treatment of ITP.[160] It is given to D-negative mothers after potential exposure to fetal D-positive RBCs, such as after abortion or amniocentesis, as well as at 28 weeks gestational age, and postpartum if the child proves to be D positive.[161] The therapeutic effect is thought to be caused by antibody feedback with T-cell suppression of the B-cell clone responsible for the formation of anti-D antibody. (See box on Weak, Partial D, and Rh Ig Use.) RhIg can also be given to prevent immunization in D-negative individuals given D-positive blood products, such as platelets.[159,160] RhIg preparations are intended for either IM or IV administration.

Dosage

RhIg is dosed to adequately prevent D immunization. In the United States, 300 µg are administered after event resulting in maternal-fetal hemorrhage, 28 weeks and postpartum. Doses are increased for evidence of large maternal-fetal hemorrhage (300 µg for every 15 mL of RBC exposure). Factional calculated doses should be rounded to the next whole number. This dosing is also used in the prevention of D alloimmunization after receipt of RBC-containing blood product.[166] RhIg should be given within 72 hours after RBC exposure. Dosing can vary slightly by manufacturer. IV RhIg use in ITP is indicated in D-positive patients with intact spleen. It has been suggested that the mechanism of action may involve a shift in the immune-mediated destruction from platelets to the antibody-coated RBCs.[167] RhIg doses are substantially higher for the treatment of ITP: 50 µg/kg for hemoglobin ≥10 L/dL and 25 to 40 µg/kg when hemoglobin is 8 to 10 g/dL.

Adverse Effects

Adverse effects similar to those associated with IVIg can occur with use of hyper-Igs. Local adverse reactions such as tenderness, pain, soreness, or stiffness of the muscle may occur at an IM injection site. Individual hyper-Ig products have adverse effects specific to them. Animal derived hyper-Igs carry a risk of serum sickness. At low doses of RhIg, such as those used to prevent D immunization, fever, chills, and rarely hypersensitivity reactions are noted. Hemolysis, DIC, and rarely death have occurred with high doses of RhIg used to treat ITP.

COAGULATION FACTOR CONCENTRATES

Historically, the major source of coagulation factors for treatment of congenital deficiencies was plasma or cryoprecipitate. Plasma fractionation, along with improved testing and processing, has led to highly purified plasma-derived coagulation factor concentrates that have dramatically increased the quality of life and life expectancy for patients. In addition, recombinant products without human proteins can be produced in modified forms that may give them advantageous new properties. Each coagulation factor product differs in its composition, major indication, and dosage. The development of improved coagulation factor concentrates, as well as other therapies, continues to be a major focus of research.[168]

Recombinant Factor Concentrates

With the advent of genetic and cellular engineering technologies, it is possible to isolate the genes of specific coagulation factors, such as factor VIII, and to artificially produce properly functioning protein from cell culture. Almost all plasma proteins licensed for human use have been cloned and manufactured in biologically active form in the laboratory. The main advantages of recombinantly produced proteins include freedom from human viruses and a potentially unlimited supply, as reliance on blood donation is no longer needed.[169–171]

Recombinant proteins can also be produced in modified forms that may give them advantageous new properties such as increased potency, longer half-lives, or varied specificity. For example, some manufacturers are connecting factor VIII to another molecule that has a longer half-life such as albumin or an immunoglobulin fragment. This reduces the frequency of product infusions from every other day to once every 3 to 5 days.[172] Recombinant technology can design completely new proteins that do not occur in nature, such as B domain-deleted factor VIII. Research has shown that the B domain of factor VIII is not necessary for coagulant activity.[173] Eliminating the B domain increased the expression of the factor VIII molecule as much as 20-fold compared to the full-length factor. For patients with antibodies against human factor VIII recombinant porcine factor VIII is often indicated as antibodies do not often react to porcine factor, but the porcine molecule is active in the human coagulation process.

The use of plasma-derived clotting factor concentrates, and coagulation factor concentrates that are genetically engineered as therapy for specific clotting factor deficiencies, are discussed in detail in Chapter 136, Transfusion Therapy for Coagulation Factor Deficiencies.

Dosage

The appropriate factor concentrate is dictated by each patient's factor deficiency, but the selection of a specific product is often a function of product availability and clinician/patient preferences.[174] Before prescribing a coagulation factor concentrate, the specific details of its dosing, half-life, and other pharmacokinetic characteristics should be reviewed from the most current version of the drug product's package insert.

While factor concentrate manufacturers often suggest doses that are weight-based, these recommendations are often based on the in vivo recovery of circulating factor activity in normal nonbleeding test subjects. Thus, it is recommended that pre- and post-infusion samples be collected from the patient for the first few administrations to determine the in vivo circulating factor activity recovery, which can help guide optimal dosing of subsequent infusions in order to maintain the factor levels at or above a desired threshold. Replacement factor therapy must consider the patient's current and projected risk of bleeding.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Backholer L, GreenL Huish S, et al. A paired comparison of thawed and liquid plasma. *Transfusion*. 2017;57(4):881–889.

Bellver J, Munoz EA, Ballesteros A, et al. Intravenous albumin does not prevent moderate-severe ovarian hyperstimulation syndrome in high-risk IVF patients: a randomized controlled study. *Hum Reprod*. 2003;18(11): 2283–2288.

Burnouf T. Modern plasma fractionation. *Transfus Med Rev*. 2007;21(2):101–117.

Callum JL, Karkouti K, Lin Y. Cryoprecipitate: the current state of knowledge. *Transfus Med Rev*. 2009;23(3):177–188.

Chai-Adisaksopha C, Hillis C, Siegel DM, et al. Prothrombin complex concentrates versus fresh frozen plasma for warfarin reversal. A systematic review and meta-analysis. *Thromb Haemost*. 2016;116(5):879–890.

Dichtelmüller HO, et al. Contribution to safety of immunoglobulin and albumin from virus partitioning and inactivation by cold ethanol fractionation:

a data collection from Plasma Protein Therapeutics Association member companies. *Transfusion*. 2011;51(7):1412–1430.

Dunbar NM, Yazer MH. Confusion surrounding trauma resuscitation and opportunities for clarification. *Transfusion*. 2020;60(Suppl 3). S142-s149.

Eder AF, Sebok MA. Plasma components: FFP, FP24, and thawed plasma. *Immunohematology*. 2007;23(4):150–157.

Eljaiek R, Heylbroeck C, Dubois MJ. Albumin administration for fluid resuscitation in burn patients: a systematic review and meta-analysis. *Burns*. 2017;43(1):17–24.

Finfer S, Bellomo R, Boyce N, et al. A comparison of albumin and saline for fluid resuscitation in the intensive care unit. *N Engl J Med*. 2004;350(22): 2247–2256.

Holcomb JB, et al. The prospective, observational, multicenter, major trauma transfusion (PROMMTT) study: comparative effectiveness of a time-varying treatment with competing risks. *JAMA Surg*. 2013;148(2): 127–136.

Holcomb JB, et al. Transfusion of plasma, platelets, and red blood cells in a 1:1:1 vs a 1:1:2 ratio and mortality in patients with severe trauma: the PROPPR randomized clinical trial. *JAMA*. 2015;313(5):471–482.

Huber J, et al. Prophylactic plasma transfusion for patients without inherited bleeding disorders or anticoagulant use undergoing non-cardiac surgery or invasive procedures. *Cochrane Database Syst Rev*. 2019;11(11):Cd012745.

Key NS, Negrier C. Coagulation factor concentrates: past, present, and future. *Lancet*. 2007;370(9585):439–448.

Kor DJ, Stubbs JR, Gajic O. Perioperative coagulation management—fresh frozen plasma. *Best Pract Res Clin Anaesthesiol*. 2010;24(1):51–64.

Levi M, et al. Guidelines for the diagnosis and management of disseminated intravascular coagulation. British Committee for Standards in Haematology. *Br J Haematol*. 2009;145(1):24–33.

Levy JH, et al. Fibrinogen and hemostasis: a primary hemostatic target for the management of acquired bleeding. *Anesth Analg*. 2012;114(2):261–274.

Liumbruno GM, et al. Recommendations for the use of albumin and immunoglobulins. *Blood Transfus*. 2009;7(3):216–234.

McQuilten ZK, et al. Optimal dose, timing and ratio of blood products in massive transfusion: results from a systematic review. *Transfus Med Rev*. 2018;32(1):6–15.

Padmanabhan A, et al. Guidelines on the use of therapeutic apheresis in clinical practice—evidence-based approach from the Writing Committee of the American Society for Apheresis: The Eighth Special Issue. *J Clin Apher*. 2019;34(3):171–354.

Pandey S, Vyas GN. Adverse effects of plasma transfusion. *Transfusion*. 2012;52(Suppl 1):65S–79S. Suppl 1.

Patel A, et al. Randomised trials of human albumin for adults with sepsis: systematic review and meta-analysis with trial sequential analysis of all-cause mortality. *Bmj*. 2014;349:g4561.

Perez EE, Orange JS, Bonilla F, et al. Update on the use of immunoglobulin in human disease: a review of evidence. *J Allergy Clin Immunol*. 2017;139(3S):S1–S46.

Roback JD, et al. Evidence-based practice guidelines for plasma transfusion. *Transfusion*. 2010;50(6):1227–1239.

Sandler SG, et al. It's time to phase in RHD genotyping for patients with a serologic weak D phenotype. College of American Pathologists Transfusion Medicine Resource Committee Work Group. *Transfusion*. 2015;55(3):680–689.

Scott E, et al. Evaluation and comparison of coagulation factor activity in fresh-frozen plasma and 24-hour plasma at thaw and after 120 hours of 1 to 6°C storage. *Transfusion*. 2009;49(8):1584–1591.

Shaz BH, Hillyer CD, Reyes MR. *Transfusion Medicine and Hemostasis: Clinical and Laboratory Aspects*. 3rd ed. Elsevier; 2019.

Spotnitz WD, Burks S. State-of-the-art review: hemostats, sealants, and adhesives II: update as well as how and when to use the components of the surgical toolbox. *Clin Appl Thromb Hemost*. 2010;16(5):497–514.

Stanworth SJ. The evidence-based use of FFP and cryoprecipitate for abnormalities of coagulation tests and clinical coagulopathy. *Hematology Am Soc Hematol Educ Program*. 2007:179–186.

Triulzi D, Gottschall J, Murphy E, et al. A multicenter study of plasma use in the United States. *Transfusion*. 2015;55(6):1313–1319. quiz 1312.

REFERENCES

1. Cohn EJ, Strong LE, et al. Preparation and properties of serum and plasma proteins: a system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. *J Am Chem Soc*. 1946;68:459–475.
2. Burnouf T. Modern plasma fractionation. *Transfus Med Rev*. 2007;21(2):101–117.
3. Benjamin RJ, McLaughlin LS. Plasma components: properties, differences, and uses. *Transfusion*. 2012;52(Suppl 1):9s–19s.
4. AABB, ABC, ARC, ASBP. Circular of Information: For the use of human blood and blood components. 2017.
5. Scott E, et al. Evaluation and comparison of coagulation factor activity in fresh-frozen plasma and 24-hour plasma at thaw and after 120 hours of 1 to 6°C storage. *Transfusion*. 2009;49(8):1584–1591.
6. Eder AF, Sebok MA. Plasma components: FFP, FP24, and thawed plasma. *Immunohematology*. 2007;23(4):150–157.
7. Triulzi D, Gottschall J, Murphy E, et al. A multicenter study of plasma use in the United States. *Transfusion*. 2015;55(6):1313–1319. quiz 1312.
8. Yarranton H, et al. Coagulation factor levels in cryosupernatant prepared from plasma treated with amotosalen hydrochloride (S-59) and ultraviolet A light. *Transfusion*. 2005;45(9):1453–1458.
9. Cardigan R, Green L. Thawed and liquid plasma—what do we know? *Vox Sang*. 2015;109(1):1–10.
10. Backholer L, Green L, Huish S, et al. A paired comparison of thawed and liquid plasma. *Transfusion*. 2017;57(4):881–889.
11. Pusateri AE, et al. The need for dried plasma—a national issue. *Transfusion*. 2019;59(S2):1587–1592.
12. Horowitz B, et al. Solvent/detergent-treated plasma: a virus-inactivated substitute for fresh frozen plasma. *Blood*. 1992;79(3):826–831.
13. Liumbruno GM, et al. Solvent/detergent-treated plasma: a tale of 30 years of experience. *Expert Rev Hematol*. 2015;8(3):367–374.
14. Shaz BH, Stowell SR, Hillyer CD. Transfusion-related acute lung injury: from bedside to bench and back. *Blood*. 2011;117(5):1463–1471.
15. Domanović D, et al. Pathogen reduction of blood components during outbreaks of infectious diseases in the European Union: an expert opinion from the European Centre for Disease Prevention and Control Consultation Meeting. *Blood Transfus = Trasfus del sangue*. 2019;17(6):433–448.
16. Tobian A, Cohn CS, Shaz B. COVID-19 convalescent plasma. *Blood*. 2021 [epub]. doi: 10.1182/blood.2021012248.
17. Practice parameter for the use of fresh-frozen plasma cryoprecipitate platelets Fresh-Frozen Plasma, Cryoprecipitate, and Platelets Administration Practice Guidelines Development Task Force of the College of American Pathologists. *JAMA*. 1994;271(10):777–781.
18. Liumbruno G, et al. Recommendations for the transfusion of plasma and platelets. *Blood Transfus = Trasfus del sangue*. 2009;7(2):132–150.
19. Roback JD, et al. Evidence-based practice guidelines for plasma transfusion. *Transfusion*. 2010;50(6):1227–1239.
20. Abdel-Wahab OI, Healy B, Dzik WH. Effect of fresh-frozen plasma transfusion on prothrombin time and bleeding in patients with mild coagulation abnormalities. *Transfusion*. 2006;46(8):1279–1285.
21. Kor DJ, Stubbs JR, Gajic O. Perioperative coagulation management—fresh frozen plasma. *Best Pract Res Clin Anaesthesiol*. 2010;24(1):51–64.
22. Minuk L, et al. Identifying patterns of inappropriate plasma transfusion practices using a novel multicenter electronic data audit: over 80 percent of plasma transfused is inappropriate. *Blood*. 2019;134 3695-3695.
23. Dzik W, Rao A. Why do physicians request fresh frozen plasma? *Transfusion*. 2004;44(9):1393–1394.
24. Desborough M, Stanworth S. Plasma transfusion for bedside, radiologically guided, and operating room invasive procedures. *Transfusion*. 2012;52(Suppl 1):20s–29ss.
25. Devi AS. Transfusion practice in orthotopic liver transplantation. *Ind J Crit Care Med*. 2009;13(3):120–128.
26. Nusbacher J. Blood transfusion support in liver transplantation. *Transfus Med Rev*. 1991;5(3):207–213.
27. Chee YL Greaves M. Role of coagulation testing in predicting bleeding risk. *Hematol J*. 2003;4:373–378.
28. Youssef WI, et al. Role of fresh frozen plasma infusion in correction of coagulopathy of chronic liver disease: a dual phase study. *Am J Gastroenterol*. 2003;98(6):1391–1394.
29. Gajic O, Dzik WH, Toy P. Fresh frozen plasma and platelet transfusion for nonbleeding patients in the intensive care unit: benefit or harm? *Crit Care Med*. 2006;34(5 Suppl):S170–S173.
30. Tripodi A, Mannucci PM. The coagulopathy of chronic liver disease. *N Engl J Med*. 2011;365(2):147–156.
31. Donohue CI, Mallett SV. Reducing transfusion requirements in liver transplantation. *World J Transplant*. 2015;5(4):165–182.
32. Maegele M. Acute traumatic coagulopathy: incidence, risk stratification and therapeutic options. *World J Emerg Med*. 2010;1(1):12–21.
33. Gerecht R. The lethal triad. Hypothermia, acidosis and coagulopathy create a deadly cycle for trauma patients. *JEMS*. 2014;39(4):56–60.
34. Mikhail J. The trauma triad of death: hypothermia, acidosis, and coagulopathy. *AACN Clin Issues*. 1999;10(1):85–94.
35. Bolliger D, Görlinger K, Tanaka KA. Pathophysiology and treatment of coagulopathy in massive hemorrhage and hemodilution. *Anesthesiology*. 2010;113(5):1205–1219.
36. Napolitano LM, et al. Tranexamic acid in trauma: how should we use it? *J Trauma Acute Care Surg*. 2013;74(157U):1586.
37. Holcomb JB, et al. The prospective, observational, multicenter, major trauma transfusion (PROMMTT) study: comparative effectiveness of a time-varying treatment with competing risks. *JAMA Surg*. 2013;148(2):127–136.
38. Holcomb JB, et al. Transfusion of plasma, platelets, and red blood cells in a 1:1:1 vs a 1:1:2 ratio and mortality in patients with severe trauma: the PROPPR randomized clinical trial. *JAMA*. 2015;313(5):471–482.
39. Dunbar NM, Yazer MH. Confusion surrounding trauma resuscitation and opportunities for clarification. *Transfusion*. 2020;60(Suppl 3). S142-s149.
40. Yazer MH, Spinella PC. Review of low titre group O whole blood use for massively bleeding patients around the world in 2019. *ISBT Sci Ser*. 2019;14(3):276–281.
41. McQuilten ZK, et al. Optimal dose, timing and ratio of blood products in massive transfusion: results from a systematic review. *Transfus Med Rev*. 2018;32(1):6–15.
42. Sperry JL, et al. Prehospital plasma during air medical transport in trauma patients at risk for hemorrhagic shock. 2018;379(4):315–326.
43. Moore HB, et al. Plasma-first resuscitation to treat haemorrhagic shock during emergency ground transportation in urban area: a randomised trial. *Lancet* 2018;392(10144):283–291.
44. Hsu Y-M, Haas T, Cushing M. Massive transfusion protocols: current best practice. *Int J Clin Transfus Med*. 2016;2016:15.
45. Tapia NM, et al. TEG-guided resuscitation is superior to standardized MTP resuscitation in massively transfused penetrating trauma patients. *J Trauma Acute Care Surg*. 2013;74(2):378–385. discussion 385-6.
46. Patel EU, et al. Blood product utilization among trauma and nontrauma massive transfusion protocols at an urban academic medical center. *Anesth Analg*. 2017;125(3):967–974.
47. Di Nisio M, Thachil J, Squizzato A. Management of disseminated intravascular coagulation: a survey of the International Society on Thrombosis and Haemostasis. *Thromb Res*. 2015;136(2):239–242.
48. Levi M, et al. Guidelines for the diagnosis and management of disseminated intravascular coagulation. British Committee for Standards in Haematology. *Br J Haematol*. 2009;145(1):24–33.
49. Mueller MM, Bomke B, Seifried E. Fresh frozen plasma in patients with disseminated intravascular coagulation or in patients with liver diseases. *Thromb Res*. 2002;107(Suppl 1):S9–17.
50. Chai-Adisaksopha C, Hillis C, Siegel DM, et al. Prothrombin complex concentrates versus fresh frozen plasma for warfarin reversal. A systematic review and meta-analysis. *Thromb Haemost*. 2016;116(5):879–890.
51. Padmanabhan A, et al. Guidelines on the use of therapeutic apheresis in clinical practice—evidence-based approach from the Writing Committee of the American Society for Apheresis: The Eighth Special Issue. *J Clin Apher*. 2019;34(3):171–354.
52. Brunskill SJ, et al. A systematic review of randomized controlled trials for plasma exchange in the treatment of thrombotic thrombocytopenic purpura. *Transfus Med*. 2007;17(1):17–35.
53. Michael M, et al. Interventions for hemolytic uremic syndrome and thrombotic thrombocytopenic purpura: a systematic review of randomized controlled trials. *Am J Kidney Dis*. 2009;53(2):259–272.
54. Lankford KV, Hillyer CD. Thrombotic thrombocytopenic purpura: new insights in disease pathogenesis and therapy. *Transfus Med Rev*. 2000;14(3):244–257.
55. Zeigler ZR, et al. Cryoprecipitate poor plasma does not improve early response in primary adult thrombotic thrombocytopenic purpura (TTP). *J Clin Apher*. 2001;16(1):19–22.
56. Scully M, et al. Caplacizumab treatment for acquired thrombotic thrombocytopenic purpura. *N Engl J Med*. 2019;380(4):335–346.
57. Sridharan M, Go RS, Willrich MAV. Atypical hemolytic uremic syndrome: review of clinical presentation, diagnosis and management. *J Immunol Methods*. 2018;461:15–22.
58. Huber J, et al. Prophylactic plasma transfusion for patients without inherited bleeding disorders or anticoagulant use undergoing non-cardiac surgery or invasive procedures. *Cochrane Database Syst Rev*. 2019;11(11):Cd012745.
59. Yang L, et al. Is fresh-frozen plasma clinically effective? An update of a systematic review of randomized controlled trials. *Transfusion*. 2012;52(8):1673–1686. quiz 1673.

60. Stanworth SJ. The evidence-based use of FFP and cryoprecipitate for abnormalities of coagulation tests and clinical coagulopathy. *Hematol Am Soc Hematol Educ Program*. 2007:179–186.
61. Randomised trial of prophylactic early fresh-frozen plasma or gelatin or glucose in preterm babies: outcome at 2 years. *Lancet*. 1996;348(9022):229–232.
62. Leese T, et al. A multicentre controlled clinical trial of high-volume fresh frozen plasma therapy in prognostically severe acute pancreatitis. *Ann R Coll Surg Engl*. 1991;73(4):207–214.
63. Stanworth SJ, et al. Is fresh frozen plasma clinically effective? A systematic review of randomized controlled trials. *Br J Haematol*. 2004;126(1):139–152.
64. Segal JB, Dzik WH. Paucity of studies to support that abnormal coagulation test results predict bleeding in the setting of invasive procedures: an evidence-based review. *Transfusion*. 2005;45(9):1413–1425.
65. Green L, et al. British Society of Haematology guidelines on the spectrum of fresh frozen plasma and cryoprecipitate products: their handling and use in various patient groups in the absence of major bleeding. *Br J Haematol*. 2018;181(1):54–67.
66. Holland LL, Brooks JP. Toward rational fresh frozen plasma transfusion: the effect of plasma transfusion on coagulation test results. *Am J Clin Pathol*. 2006;126(1):133–139.
67. Zielinski MD, et al. Emergency use of prethawed Group A plasma in trauma patients. *J Trauma Acute Care Surg*. 2013;74(1):69–74. discussion 74-5.
68. Chhibber V, et al. Is group A thawed plasma suitable as the first option for emergency release transfusion? (CME). *Transfusion*. 2014;54(7):1751–1755. quiz 1750.
69. Pandey S, Vyas GN. Adverse effects of plasma transfusion. *Transfusion*. 2012;52(Suppl 1):65S–79S. Suppl 1.
70. Alport EC, et al. Cryoprecipitate use in 25 Canadian hospitals: commonly used outside of the published guidelines. *Transfusion*. 2008;48(10):2122–2127.
71. Levy JH, et al. Fibrinogen and hemostasis: a primary hemostatic target for the management of acquired bleeding. *Anesth Analg*. 2012;114(2):261–274.
72. Wong H, Curry N. Do we need cryoprecipitate in the era of fibrinogen concentrate and other specific factor replacement options? *ISBT Sci Ser*. 2018;13(1):23–28.
73. Callum J, et al. Effect of fibrinogen concentrate vs cryoprecipitate on blood component transfusion after cardiac surgery: the FIBRES randomized clinical trial. *JAMA*. 2019;322(20):1966–1976.
74. Theodoulou A, et al. Comparison of cryoprecipitate with fibrinogen concentrate for acquired hypofibrinogenaemia. *Transfus Apheresis Sci*. 2012;46(2):159–162.
75. Ahmed S, et al. The efficacy of fibrinogen concentrate compared with cryoprecipitate in major obstetric haemorrhage—an observational study. *Transfus Med*. 2012;22(5):344–349.
76. Spotnitz WD, Clinical WR. uses of fibrin sealant. In: Mintz PD, ed. *Transfusion Therapy: Clinical Principles and Practice*. Bethesda: AABB; 1999:199–222.
77. Berguer R, et al. Warning: fatal reaction to the use of fibrin glue in deep hepatic wounds. Case reports. *J Trauma*. 1991;31(3):408–411.
78. Spotnitz WD, Burks S. State-of-the-art review: hemostats, sealants, and adhesives II: update as well as how and when to use the components of the surgical toolbox. *Clin Appl Thromb Hemost*. 2010;16(5):497–514.
79. Holcomb JB, et al. Cryoprecipitate use in the PROMMTT study. *J Trauma Acute Care Surg*. 2013;75(1 Suppl 1):S31–S39.
80. Shaz BH, et al. Increased number of coagulation products in relationship to red blood cell products transfused improves mortality in trauma patients. *Transfusion*. 2010;50(2):493–500.
81. Stinger HK, et al. The ratio of fibrinogen to red cells transfused affects survival in casualties receiving massive transfusions at an army combat support hospital. *J Trauma*. 2008;64(2 Suppl):S79–S85. discussion S85.
82. Morrison JJ, et al. Association of cryoprecipitate and tranexamic acid with improved survival following wartime injury: findings from the MATTERs II Study. *JAMA Surg*. 2013;148(3):218–225.
83. Callum JL, Karkouti K, Lin Y. Cryoprecipitate: the current state of knowledge. *Transfus Med Rev*. 2009;23(3):177–188.
84. Callum J, et al. Effect of fibrinogen concentrate vs cryoprecipitate on blood component transfusion after cardiac surgery: the FIBRES randomized clinical trial. *JAMA*. 2019;322(20):1966–1976.
85. Charbit B, et al. The decrease of fibrinogen is an early predictor of the severity of postpartum hemorrhage. *J Thromb Haemost*. 2007;5(2):266–273.
86. de Lange NM, et al. Obstetric hemorrhage and coagulation: an update. thromboelastography, thromboelastometry, and conventional coagulation tests in the diagnosis and prediction of postpartum hemorrhage. *Obstet Gynecol Surv*. 2012;67(7):426–435.
87. Butwick AJ, Goodnough LT. Transfusion and coagulation management in major obstetric hemorrhage. *Curr Opin Anaesthesiol*. 2015;28(3):275–284.
88. Pabinger I, et al. Tranexamic acid for treatment and prophylaxis of bleeding and hyperfibrinolysis. *Wien Klin Wochenschr*. 2017;129(9-10):303–316.
89. WOMAN Trial Collaborators Effect of early tranexamic acid administration on mortality, hysterectomy, and other morbidities in women with post-partum haemorrhage (WOMAN): an international, randomised, double-blind, placebo-controlled trial. *Lancet*. 2017;389(10084):2105–2116.
90. Janson PA, et al. Treatment of the bleeding tendency in uremia with cryoprecipitate. *N Engl J Med*. 1980;303(23):1318–1322.
91. Triulzi DJ, Blumberg N. Variability in response to cryoprecipitate treatment for hemostatic defects in uremia. *Yale J Biol Med*. 1990;63(1):1–7.
92. Thyer J, et al. Prion-removal capacity of chromatographic and ethanol precipitation steps used in the production of albumin and immunoglobulins. *Vox Sang*. 2006;91(4):292–300.
93. Dichtelmüller HO, et al. Contribution to safety of immunoglobulin and albumin from virus partitioning and inactivation by cold ethanol fractionation: a data collection from plasma protein therapeutics association member companies. *Transfusion*. 2011;51(7):1412–1430.
94. Morgenthaler JJ, Omar A. Partitioning and inactivation of viruses during isolation of albumin and immunoglobulins by cold ethanol fractionation. *Dev Biol Stand*. 1993;81:185–190.
95. Burnouf T. Recombinant plasma proteins. *Vox Sang*. 2011;100(1):68–83.
96. Liumbruno GM, et al. Recommendations for the use of albumin and immunoglobulins. *Blood Transfus*. 2009;7(3):216–234.
97. Pattison CP, et al. An outbreak of type B hepatitis associated with transfusion of plasma protein fraction. *Am J Epidemiol*. 1976;103(4):399–407.
98. Cochrane Injuries Group Albumin Reviewers Human albumin administration in critically ill patients: systematic review of randomised controlled trials. *BMJ*. 1998;317(7153):235–240.
99. Finfer S, Bellomo R, Boyce N, et al. A comparison of albumin and saline for fluid resuscitation in the intensive care unit. *N Engl J Med*. 2004;350(22):2247–2256.
100. Finfer S, et al. Impact of albumin compared to saline on organ function and mortality of patients with severe sepsis. *Intensive Care Med*. 2011;37(1):86–96.
101. Kerner T, et al. A clinical pilot study of fresh frozen plasma versus human albumin in paediatric craniofacial repair. *J Int Med Res*. 2008;36:171–177.
102. SAFE Study Investigators, et al. Saline or albumin for fluid resuscitation in patients with traumatic brain injury. *N Engl J Med*. 2007;357(9):874–884.
103. Farrugia A. Concerning Chapter 5 - Human albumin; in Cross-Sectional Guidelines for Therapy with Blood Components and Plasma Derivatives, 4th ed. *Transfus Med Hemother*. 2009;36(6):399–407.
104. Bernardi M, et al. Albumin infusion in patients undergoing large-volume paracentesis: a meta-analysis of randomized trials. *Hepatology*. 2012;55(4):1172–1181.
105. Pericleous M, et al. The clinical management of abdominal ascites, spontaneous bacterial peritonitis and hepatorenal syndrome: a review of current guidelines and recommendations. *Eur J Gastroenterol Hepatol*. 2016;28(3):e10–e18.
106. Kütting F, et al. Insufficient evidence of benefit regarding mortality due to albumin substitution in HCC-free cirrhotic patients undergoing large volume paracentesis. *J Gastroenterol Hepatol*. 2017;32(2):327–338.
107. Caraceni P, Tufoni M, Bonavita ME. Clinical use of albumin. *Blood Transfus = Trasfus del sangue*. 2013;11(Suppl 4):s18–s25. Suppl 4.
108. Shaz BH, Hillyer CD, Reyes MR. *Transfusion Medicine and Hemostasis: Clinical and Laboratory Aspects*. 3rd ed. Elsevier; 2019.
109. Guo JL, et al. Pharmacologic interventions in preventing ovarian hyperstimulation syndrome: a systematic review and network meta-analysis. *Sci Rep*. 2016;6:19093.
110. Youssef MA, Mourad S. Volume expanders for the prevention of ovarian hyperstimulation syndrome. *Cochrane Database Syst Rev*. 2016(8):Cd001302.
111. Youssef MA, et al. Intra-venous fluids for the prevention of severe ovarian hyperstimulation syndrome. *Cochrane Database Syst Rev*. 2011(2):Cd001302.
112. Bellver J, Munoz EA, Ballesteros A, et al. Intravenous albumin does not prevent moderate-severe ovarian hyperstimulation syndrome in high-risk IVF patients: a randomized controlled study. *Hum Reprod*. 2003;18(11):2283–2288.
113. Abramov Y, et al. Hydroxyethylstarch versus human albumin for the treatment of severe ovarian hyperstimulation syndrome: a preliminary report. *Fertil Steril*. 2001;75(6):1228–1230.
114. Chang R, Holcmb JB. Choice of fluid therapy in the initial management of sepsis, severe sepsis, and septic shock. *Shock*. 2016;46(1):17–26.
115. Doig GS. Albumin may significantly increase mortality in burn patients: re-analysis of a systematic review. *Burns*. 2017;43(2):449–450.
116. Eljaiek R, Heylbroeck C, Dubois M-J. Albumin administration for fluid resuscitation in burn patients: a systematic review and meta-analysis. *Burns*. 2017;43(1):17–24.
117. Melinyshyn A, et al. Albumin supplementation for hypoalbuminemia following burns: unnecessary and costly! *J Burn Care Res*. 2013;34(1):8–17.

118. Patel A, et al. Randomised trials of human albumin for adults with sepsis: systematic review and meta-analysis with trial sequential analysis of all-cause mortality. *BMJ*. 2014;349:g4561.
119. Campos Munoz A, Jain NK, Gupta M. *AlbumColloid, StatPearls*. Treasure Island, FL: StatPearls Publishing Copyright ©; 2020. StatPearls Publishing LLC; 2020.
120. Radosevich M, Burnouf T. Intravenous immunoglobulin G: trends in production methods, quality control and quality assurance. *Vox Sang*. 2010;98(1):12–28.
121. Perez EE, Orange JS, Bonilla F, et al. Update on the use of immunoglobulin in human disease: a review of evidence. *J Allergy Clin Immunol*. 2017;139(3S):S1–S46.
122. Shehata N, et al. The use of immunoglobulin therapy for patients with primary immune deficiency: an evidence-based practice guideline. *Transfus Med Rev*. 2010;24(Suppl 1):S28–50.
123. Parikh SA, et al. Hypogammaglobulinemia in newly diagnosed chronic lymphocytic leukemia: natural history, clinical correlates, and outcomes. *Cancer*. 2015;121(17):2883–2891.
124. Eftimov F, et al. Intravenous immunoglobulin for chronic inflammatory demyelinating polyradiculoneuropathy. *Cochrane Database Syst Rev*. 2009(1):Cd001797.
125. De Mattia D, et al. Management of chronic childhood immune thrombocytopenic purpura: AIEOP consensus guidelines. *Acta Haematol*. 2010;123(2):96–109.
126. Burns JC, et al. Intravenous gamma-globulin treatment and retreatment in Kawasaki disease. US/Canadian Kawasaki syndrome study group. *Pediatr Infect Dis J*. 1998;17(12):1144–1148.
127. Hur G, et al. Infliximab treatment for intravenous immunoglobulin-resistant Kawasaki disease: a multicenter study in Korea. Korean. *Circ J*. 2019;49(2):183–191.
128. Matsubara T, Ichiyama T, Furukawa S. Immunological profile of peripheral blood lymphocytes and monocytes/macrophages in Kawasaki disease. *Clin Exp Immunol*. 2005;141(3):381–387.
129. Feldstein LR, et al. Multisystem inflammatory syndrome in US children and adolescents. *N Engl J Med*. 2020;383(4):334–346.
130. Kuwabara S, et al. Intravenous immunoglobulin for maintenance treatment of multifocal motor neuropathy: a multi-center, open-label, 52-week phase 3 trial. *J Peripher Nerv Syst*. 2018;23(2):115–119.
131. van Schaik IN, et al. Intravenous immunoglobulin for multifocal motor neuropathy. *Cochrane Database Syst Rev*. 2005(2):Cd004429.
132. Zaia JA. Prevention and treatment of cytomegalovirus pneumonia in transplant recipients. *Clin Infect Dis*. 1993;17(Suppl 2):S392–399.
133. Schmidt-Hieber M, et al. Prophylactic i.v. Igs in patients with a high risk for CMV after allo-SCT. *Bone marrow Transplant*. 2009;44(3):185–192.
134. Sokos DR, Berger M, Lazarus HM. Intravenous immunoglobulin: appropriate indications and uses in hematopoietic stem cell transplantation. *Biol Blood Marrow Transpl*. 2002;8(3):117–130.
135. Shehata N, et al. The use of immunoglobulin therapy for patients undergoing solid organ transplantation: an evidence-based practice guideline. *Transfus Med Rev*. 2010;24(Suppl 1):S7–s27.
136. Robinson P, et al. Evidence-based guidelines on the use of intravenous immune globulin for hematologic and neurologic conditions. *Transfus Med Rev*. 2007;21(2 Suppl 1):S3–S8.
137. Feasby T, et al. Guidelines on the use of intravenous immune globulin for neurologic conditions. *Transfus Med Rev*. 2007;21(2 Suppl 1):S57–S107.
138. Hughes RA, Swan AV, van Doorn PA. Intravenous immunoglobulin for Guillain-Barré syndrome. *Cochrane Database Syst Rev*. 2014;2014(9):Cd002063.
139. Chapel HM, et al. Randomised trial of intravenous immunoglobulin as prophylaxis against infection in plateau-phase multiple myeloma. The UK Group for Immunoglobulin Replacement Therapy in Multiple Myeloma. *Lancet*. 1994;343(8905):1059–1063.
140. Olyaeemanesh A, et al. Safety and effectiveness assessment of intravenous immunoglobulin in the treatment of relapsing-remitting multiple sclerosis: a meta-analysis. *Med J Islam Repub Iran*. 2016;30:336.
141. Sorensen PS, Fazekas F, Lee M. Intravenous immunoglobulin G for the treatment of relapsing-remitting multiple sclerosis: a meta-analysis. *Eur J Neurol*. 2002;9(6):557–563.
142. Fazekas F, et al. Randomised placebo-controlled trial of monthly intravenous immunoglobulin therapy in relapsing-remitting multiple sclerosis. Austrian Immunoglobulin in Multiple Sclerosis Study Group. *Lancet*. 1997;349(9052):589–593.
143. Fazekas F, et al. Intravenous immunoglobulin in relapsing-remitting multiple sclerosis: a dose-finding trial. *Neurology*. 2008;71(4):265–271.
144. Sanders DB, Wolfe GI, Narayanaswami P. Developing treatment guidelines for myasthenia gravis. *Ann N Y Acad Sci*. 2018;1412(1):95–101.
145. Gajdos P, Chevret S, Toyka KV. Intravenous immunoglobulin for myasthenia gravis. *Cochrane Database Syst Rev*. 2012;12(12):Cd002277.
146. Dunsmore KP. Intravenous immunoglobulin G therapy in fetal and neonatal alloimmune thrombocytopenia. *BioDrugs*. 1997;8(4):265–272.
147. Peterson JA, et al. Neonatal alloimmune thrombocytopenia: pathogenesis, diagnosis and management. *Br J Haematol*. 2013;161(1):3–14.
148. Louis D, et al. Intravenous immunoglobulin in isoimmune haemolytic disease of newborn: an updated systematic review and meta-analysis. *Arch Dis Child Fetal Neonatal Ed*. 2014;99(4):F325–F331.
149. Al-Lawama M, et al. Intravenous immunoglobulins as adjunct treatment to phototherapy in isoimmune hemolytic disease of the newborn: a retrospective case-control study. *J Clin Med Res*. 2019;11(11):760–763.
150. Hawkins J, Aster RH, Curtis BR. Post-transfusion purpura: current perspectives. *J Blood Med*. 2019;10:405–415.
151. Alejandria MM, et al. Intravenous immunoglobulin for treating sepsis, severe sepsis and septic shock. *Cochrane Database Syst Rev*. 2013;2013(9):Cd001090.
152. Cherin P, et al. Management of adverse events in the treatment of patients with immunoglobulin therapy: a review of evidence. *Autoimmun Rev*. 2016;15(1):71–81.
153. Späth PJ, Schneider C, von Gunten S. Clinical use and therapeutic potential of IVIG/SCIG, plasma-derived IgA or IgM, and other alternative immunoglobulin preparations. *Arch Immunol Ther Exp (Warsz)*. 2017;65(3):215–231.
154. Szczech LA, et al. Effect of anti-lymphocyte induction therapy on renal allograft survival: a meta-analysis. *J Am Soc Nephrol*. 1997;8(11):1771–1777.
155. Li X, et al. Outcomes of optimized over standard protocol of rabbit antithymocyte globulin for severe aplastic anemia: a single-center experience. *PLoS one*. 2013;8(3). e56648-e56648.
156. Stiehm ER. Appropriate therapeutic use of immunoglobulin. *Transfus Med Rev*. 1996;10(3):203–221.
157. Bonaros N, et al. CMV-hyperimmune globulin for preventing cytomegalovirus infection and disease in solid organ transplant recipients: a meta-analysis. *Clin Transpl*. 2008;22(1):89–97.
158. Manning SE, et al. Human rabies prevention—United States, 2008: recommendations of the Advisory Committee on Immunization Practices. *MMWR Recomm Rep*. 2008;57(Rr-3):1–28.
159. Nguyen AA, et al. Immunoglobulins in the treatment of COVID-19 infection: proceed with caution! *Clin Immunol*. 2020;216:108459.
160. Ayache S, Herman JH. Prevention of D sensitization after mismatched transfusion of blood components: toward optimal use of RhIG. *Transfusion*. 2008;48(9):1990–1999.
161. de Haas M, et al. Sensitivity of fetal RHD screening for safe guidance of targeted anti-D immunoglobulin prophylaxis: prospective cohort study of a nationwide programme in the Netherlands. *BMJ*. 2016;355:i5789.
162. Sandler SG, Chen LN, Flegel WA. Serological weak D phenotypes: a review and guidance for interpreting the RhD blood type using the RHD genotype. *Br J Haematol*. 2017;179(1):10–19.
163. Sandler SG, et al. It's time to phase in RHD genotyping for patients with a serologic weak D phenotype. College of American Pathologists Transfusion Medicine Resource Committee Work Group. *Transfusion*. 2015;55(3):680–689.
164 Cid J, et al. Low frequency of anti-D alloimmunization following D+ platelet transfusion: the Anti-D Alloimmunization after D-incompatible Platelet Transfusions (ADAPT) study. *Br J Haematol*. 2015;168(4):598–603.
165. Shaz BH, Hillyer CD. Residual risk of D alloimmunization: is it time to feel safe about platelets from D+ donors? *Transfusion*. 2011;51(6):1132–1135.
166. Sperling JD, et al. Prevention of RhD alloimmunization: a comparison of four national guidelines. *Am J Perinatol*. 2018;35(2):110–119.
167. Crow AR, Lazarus AH. The mechanisms of action of intravenous immunoglobulin and polyclonal anti-D immunoglobulin in the amelioration of immune thrombocytopenic purpura: what do we really know? *Transfus Med Rev*. 2008;22(2):103–116.
168. Escobar MA. Advances in the treatment of inherited coagulation disorders. *Haemophilia*. 2013;19(5):648–659.
169. Key NS, Negrier C. Coagulation factor concentrates: past, present, and future. *Lancet*. 2007;370(9585):439–448.
170. Pipe SW. Recombinant clotting factors. *Thromb Haemost*. 2008;99(5): 840–850.
171. Ragni MV. New-generation recombinant factor concentrates: bridge to gene therapy. *Haemophilia*. 2001;7(Suppl 1):28–35.
172. Peyvandi F, Garagiola I, Seregni S. Future of coagulation factor replacement therapy. *J Thromb Haemost*. 2013;11(Suppl 1):84–98.
173. Santagostino E. A new recombinant factor VIII: from genetics to clinical use. *Drug Design, Dev Ther*. 2014;8:2507–2515.
174. Shord SS, Lindley CM. Coagulation products and their uses. *Am J Health Syst Pharm*. 2000;57(15):1403–1417. quiz 1418–20.

CHAPTER 116

HEMAPHERESIS

Kamille A. West and Harvey G. Klein

Therapeutic bloodletting is an ancient therapy that dates back to the Galenic conceptualization of illness caused by the imbalance of the four humors. The practice remained fashionable, albeit unproved, well into the 19th century. About the time that scientific skepticism began to temper the widespread use of therapeutic phlebotomy, a new technique for blood removal, apheresis, appeared in the research laboratory.[1] The term *apheresis*, derived from a Greek verb meaning "to take away or withdraw," was coined to describe removal of one component of blood with return of the remaining components to the donor. Like phlebotomy, apheresis was used first to treat patients but later became more important for collecting blood components for transfusion. In 2017, about 1.8 million units (14.6%) of red blood cells (RBCs), 2.2 million units (91.3%) of platelets, 350,000 units (10.8%) of plasma, and 4000 granulocyte doses were collected by apheresis in the United States.[2] The Plasma Protein Therapeutics Association estimates that 40 million liters of source plasma are collected annually by plasmapheresis in approximately 30 million collections. Apheresis plasma is critical for preparing plasma-derived medicinal products such as intravenous immunoglobulin (IVIg) and albumin. Increasingly, apheresis techniques are also used to collect cells from the peripheral blood of healthy donors and patients for hematopoietic stem/progenitor cell (HSPC) transplantation and for manufacturing immune cell therapies such as chimeric antigen receptor (CAR) T cells. More than 50,000 HSPC and cellular therapy products are collected at hospital and blood center apheresis facilities annually.[3] In clinical settings, apheresis may also be employed in a therapeutic context to treat disease by removing pathologic cells or plasma-bound substances. Therapeutic applications of apheresis will constitute the focus of this chapter.

PRINCIPLES OF APHERESIS

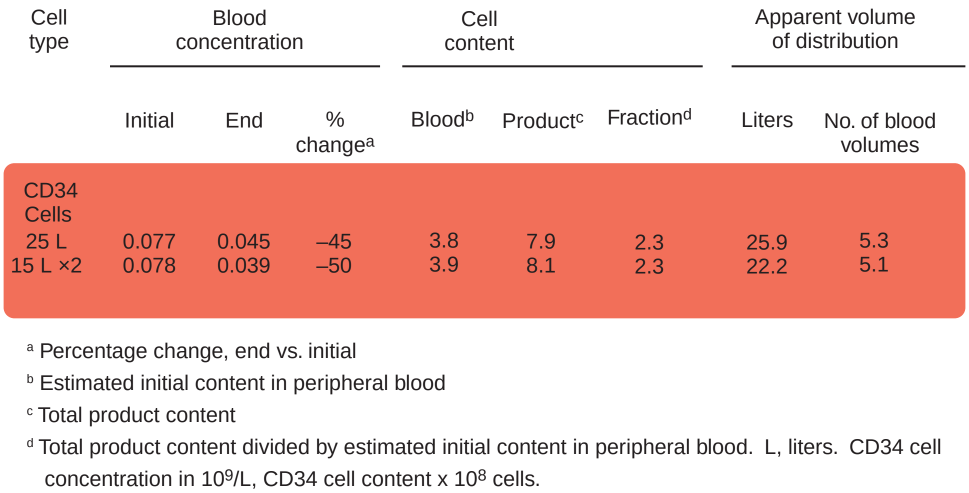
The principal objective of apheresis is efficient removal of some circulating blood component, either cells (cytapheresis) or some plasma solute (plasmapheresis). For most disorders, the treatment goal is to deplete the circulating cell or substance directly responsible for the disease process. Apheresis can also mobilize cells and plasma components from tissue depots. For example, lymphocytes may be mobilized from the spleen and lymph nodes of some patients with chronic lymphocytic leukemia (CLL), and low-density lipoproteins (LDLs) can be removed from tissue stores in patients with familial hypercholesterolemia. The apheresis procedure itself mobilizes $CD34^+$ cells from extravascular depots during peripheral blood HSPC donation, resulting in collection of more than twice as many $CD34^+$ cells than estimated based on preapheresis peripheral blood cell counts (Fig. 116.1). Apheresis may have other, less obvious effects. Lymphocyte depletion may modify immune responsiveness in some disease states, possibly by disturbing the control mechanisms of cellular immune regulation. Plasmapheresis enhances splenic clearance of immune complexes in certain autoimmune disorders. When therapeutic effect is judged by clinical improvement rather than by efficiency of solute removal, apheresis is more often a helpful adjunct than a form of first-line therapy.

Several mathematical models formulated for different clinical conditions describe the kinetics of apheresis. Removal of most blood constituents follows a logarithmic curve (Fig. 116.2). This model assumes that the substance removed is neither synthesized nor degraded substantially during the procedure, remains within the intravascular compartment, and mixes instantaneously and completely with any plasma replacement solution. When the goal of plasmapheresis is to supply a deficient substance, for example, the cleavase ADAM metallopeptidase with thrombospondin type 1 motif 13 (ADAMTS13) in the treatment of thrombotic thrombocytopenic purpura (TTP), replacement follows logarithmic kinetics similar to those developed for solute removal. Removal of 1.5 to 2.0 volumes will reduce an intravascular substance by approximately 80% (see Fig. 116.2); processing larger volumes results in little additional gain. Specific cell removal with centrifugal automated cell separators depend on the number of cells available, the volume of blood processed, the efficiency of the particular instrument, and the separation characteristics of the different cells. Most commercially available instruments remove platelets and lymphocytes extremely efficiently. Granulocytes and other mononuclear cells, including HSPCs from peripheral blood, cannot be cleanly separated from other cells by standard centrifugal apheresis equipment (Fig. 116.3). Optimal harvesting of these cells requires special techniques such as stimulating the donor with corticosteroids, cytokines or chemokine receptor antagonists, or adding sedimenting agents to enhance cell separation.

Whereas this model accurately estimates removal of cells and large proteins such as fibrinogen and immunoglobulin (Ig) M, removal of smaller solutes such as IgG and albumin-bound drugs are less efficient. Transfer of these moieties from the extravascular to the intravascular compartment depends both on diffusion along a concentration gradient and on active transport. The rate of clearance can be calculated using diffusion coefficients, sieving coefficients, and lymphatic flow rate, although in practice this degree of accuracy is rarely necessary.

TECHNOLOGY AND TECHNIQUES

Over 100 years ago, animal experiments formed the theoretical and practical basis for all modern extracorporeal blood purification methods. The plasmapheresis technique that originated in the animal laboratory required manual resuspension of RBCs and posed a substantial risk of microbial contamination of the components being reinfused. With the introduction of sterile, disposable, interconnected plastic blood bags, plasmapheresis became relatively safe and easy. However, manual apheresis proved too inefficient and labor intensive for collecting large component volumes, and raised concerns that the separated units of RBCs might be reinfused accidentally into the wrong donor or patient. The introduction of automated online blood cell separators solved these problems. Automated apheresis instruments use microprocessor technology to draw and anticoagulate blood, separate components either by centrifugation or by filtration, collect the desired component, recombine the remaining components for return to the patient or donor, and document procedure data. The equipment contains disposable plastic software in the blood path and uses anticoagulants containing citrate or combinations of citrate and heparin that do not result in clinical anticoagulation of the patient or donor. Most instruments function well at blood flow rates of 30 to 80 mL/min, and can operate from peripheral venous access or from a variety of multilumen central venous catheters.[4] Newer therapeutic apheresis devices are smaller and more automated, allowing for implementation of more safety functions and improved portability.



Cell type	Blood concentration			Cell content			Apparent volume of distribution	
	Initial	End	% change^a	Blood^b	Product^c	Fraction^d	Liters	No. of blood volumes
CD34 Cells								
25 L	0.077	0.045	–45	3.8	7.9	2.3	25.9	5.3
15 L ×2	0.078	0.039	–50	3.9	8.1	2.3	22.2	5.1

[a] Percentage change, end vs. initial

[b] Estimated initial content in peripheral blood

[c] Total product content

[d] Total product content divided by estimated initial content in peripheral blood. L, liters. CD34 cell concentration in 10^9/L, CD34 cell content x 10^8 cells.

Figure 116.1 Twenty healthy mobilized donors underwent either a single large volume leukapheresis procedure of 25 L or two consecutive-day smaller volume procedures of 15 L each. In both study groups, more than twice as many CD34 cells were collected than the estimated total content of these cells in the peripheral blood before apheresis, providing evidence for the existence of a large extravascular pool of peripheral blood stem cells, which becomes accessible to collection by leukapheresis. *(Modified from Bolan CD, Carter CS, Wesley RA, et al. Prospective evaluation of cell kinetics, yields and donor experiences during a single large-volume apheresis versus two smaller volume consecutive day collections of allogeneic peripheral blood stem cells. Br J Hematol. 2003;120:801, by permission of the authors.)*

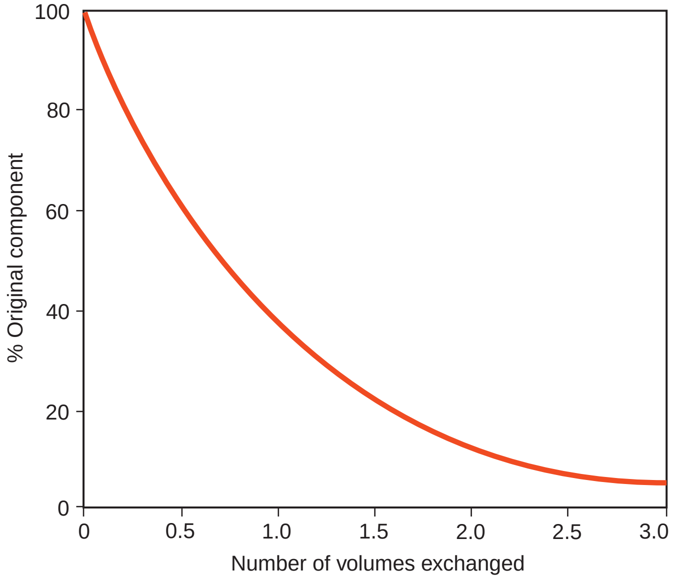


Figure 116.2 RELATIONSHIP BETWEEN VOLUMES REMOVED BY APHERESIS AND PERCENTAGE OF THE TARGET COMPONENT REMAINING. The relation is valid for blood volumes during red blood cell exchange or for plasma volumes during plasmapheresis if the target solute remains primarily within the intravascular compartment.

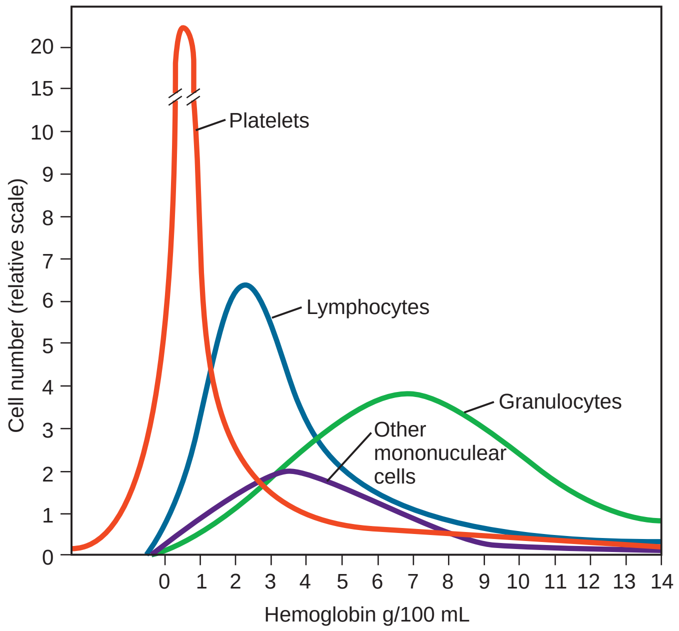


Figure 116.3 SCHEMATIC DISTRIBUTION OF CELLS AT THE COLLECTION PORT OF A CENTRIFUGAL CELL SEPARATOR. The number and percentage of each cell type collected can be varied by adjusting the site of collection along the interface or by changing centrifugal force, blood flow rate, or rate of cell removal.

In large-volume leukapheresis (LVL) procedures, prophylactic intravenous calcium supplementation minimizes the risk of hypocalcemia due to the citrate anticoagulant used.[5]

Because the ideal method for treating disorders mediated by abnormal plasma components is to remove the offending substance selectively, a variety of online filtration and column adsorption techniques have been introduced or proposed. Ligands bound to a column matrix may be relatively nonspecific chemical sorbents, such as charcoal or heparin, or specific ligands, such as monoclonal antibodies and recombinant protein antigens. Two such columns are commercially available: one using staphylococcal protein A, and the other using negatively charged dextran sulfate cellulose beads. Staphylococcal protein A has high affinity for the Fc portion of IgG1, IgG2, and IgG4 and for immune complexes containing these IgG subtypes. The dextran sulfate cellulose columns selectively remove LDL, very-low-density lipoprotein, and lipoprotein, and have proved effective in managing patients with homozygous hypercholesterolemia who have not responded to diet and cholesterol-lowering drug therapy (Fig. 116.4). A similar technique, heparin-induced extracorporeal lipoprotein precipitation, uses low pH and negatively charged heparin to precipitate lipoproteins and remove the precipitate by filtration online (H.E.L.P. Plasmat Futura, Braun Medical). Apheresis technology has also been adapted for extracorporeal phototherapy of patient leukocytes (photopheresis) to treat cutaneous T-cell lymphoma, and to modulate the pathologic immune response in graft-versus-host disease (GVHD), solid organ transplant.

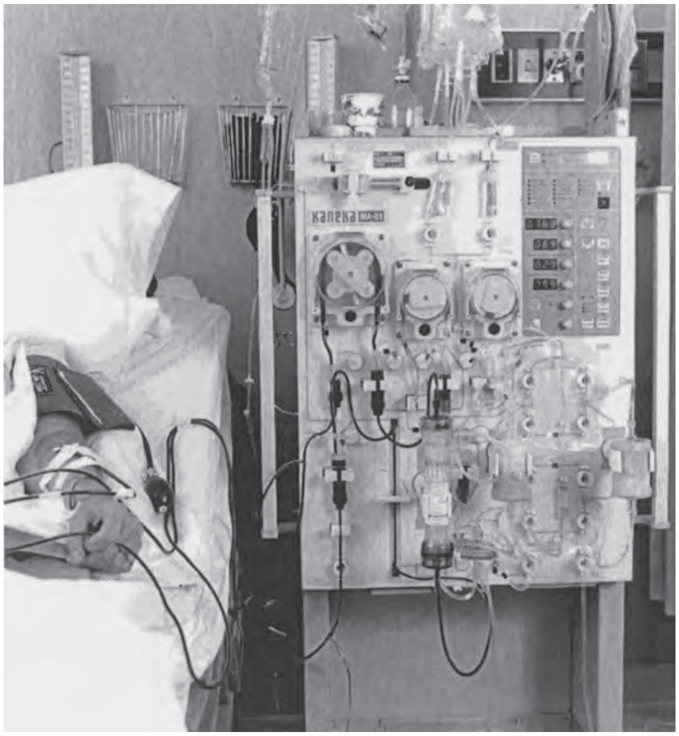


Figure 116.4 TWO-STAGE THERAPEUTIC PLASMAPHERESIS. Plasma is separated from cells by filtration and then passed through parallel adsorption columns to remove low-density lipoproteins from a patient with homozygous familial hypercholesterolemia.

Clinical Consultation With the Apheresis Service

Apheresis services are generally overseen by specialists in Hematology, Nephrology, or Transfusion Medicine; qualified nurses or technicians perform the apheresis procedures. When requesting a course of therapeutic apheresis, the team should specify the indication and urgency of the request as well as the therapeutic endpoint. Defining specific clinical or laboratory endpoints for success, a priori, is important to avoid continuing a fruitless course of apheresis with inherent risks but no clinical benefit. Successful therapeutic apheresis requires reliable vascular access, using either two large, durable peripheral veins or a central dual lumen catheter that is rigid enough to withstand significant flow pressures. The fluid volume removed by therapeutic apheresis must be replaced to prevent marked volume depletion. Some patients, such as small children or pregnant women, may require special technical considerations to appropriately manage volume shifts or minimize intraprocedural anemia. The apheresis schedule should be determined by the patient's condition, the pathologic substance targeted for removal, and the desired clinical and/or laboratory endpoint.

THERAPEUTIC PLASMA EXCHANGE (PLASMAPHERESIS)

Common clinical indications for therapeutic plasma exchange (TPE), frequently referred to as plasmapheresis, are outlined in Table 116.1. Most procedures are performed for treatment of immunologic and hematologic disorders. A course of plasmapheresis generally consists of five to seven exchanges of 1 to 1.5 plasma volumes each, either daily or with an interval of 1 to 2 days between procedures; the course of therapy varies depending on the specific disease indication and rate and duration of the response. Several expert committees have published practice guidelines for using plasmapheresis in a wide variety of disease states.[6] Some of the least controversial indications for plasmapheresis are supported by small series of uncontrolled cases that rely on some objective clinical or laboratory measurement of patient improvement. In recent years, effective biologic and pharmacologic agents such as IVIg have rendered TPE a backup therapy for some indications.

TABLE 116.1 Common Indications for Therapeutic Cytapheresis

Procedure	Indication (ASFA Category)
Red blood cells (RBC) exchange	Acute complications of sickle cell disease (SCD) (e.g., acute stroke [I], acute chest syndrome [II]) SCD, stroke prophylaxis (I) Recurrent vaso-occlusive crises (II) Severe parasitemia, e.g., babesiosis (II), malaria (III)
Erythrocytapheresis	Hemochromatosis, polycythemia vera
Leukapheresis	Symptomatic hyperleukocytosis (II)
Adsorptive cytapheresis	Behçet's disease (II)
Extracorporeal Photopheresis	Cutaneous T-cell lymphoma (I) Acute or chronic graft-versus-host-disease (II)
Cell Therapy Manufacture	Peripheral blood stem cell collection Chimeric antigen receptor T-cell therapy
Thrombocytapheresis	Symptomatic thrombocytosis

Adapted with permission from Schwartz J, Padmanabhan A, Aqui N, et al. Guidelines on the use of therapeutic apheresis in clinical practice—evidence-based approach from the Writing Committee of the American Society for Apheresis: the eighth special issue. *J Clin Apher.* 2019;34:171–354.

REPLACEMENT FLUIDS FOR PLASMA EXCHANGE

The success of therapeutic apheresis procedures seldom depends on the composition of the replacement solution; the major exception is TTP for which plasma is indicated to replace the depleted ADAMTS13 protein. With therapeutic plasmapheresis for most other disorders, the primary function of the replacement solution is to maintain intravascular volume. Additional requirements include restoration of important plasma proteins, maintenance of colloid osmotic pressure, maintenance of electrolyte balance, and preservation of trace elements lost during a prolonged course of plasmapheresis procedures. For most conditions, it is acceptable to perform 1 to 1.5 plasma volume exchanges per procedure. A single plasma volume exchange in an average-sized adult uses approximately 3 L of replacement fluid. In moderately well-nourished patients, homeostatic mechanisms normally obviate the need for precise plasma replacement, and 5% albumin in normal saline or combinations of albumin and crystalloid are usually sufficient. Patients with clinical conditions such as hypotension, hypoalbuminemia, or preexisting coagulopathies should receive solutions prepared specifically to meet their individual requirements. Problems of decreased availability and high cost of albumin have led some centers to develop protocols for alternatives to plasma-derived volume expanders, such as hydroxyethyl starch (HES), for full- or partial-volume replacement with plasma exchange. One center has used a combination of 3% HES and 5% albumin mixture successfully, but patients did experience mild adverse events more frequently than did historical control subjects.[7] Although such solutions are generally well tolerated, extensive replacement with HES in patients undergoing longer courses of plasmapheresis, especially those with impaired renal function, can result in diffuse tissue accumulation of the larger starch molecules (acquired lysosomal storage).

Hematologic Indications for Plasma Exchange

Two of the most common indications for plasmapheresis are treatment of TTP and treatment of clinical syndromes associated with paraproteinemias. Plasma exchange with fresh-frozen plasma (FFP) replacement has been estimated to improve survival rates of patients with TTP from 10% to more than 75%. Whereas plasma infusion is often initiated immediately, TPE is the superior treatment and should be started as soon as it can be arranged.[8] Comprehensive reviews of the clinical and laboratory evaluation and treatment of patients with suspected TTP, including management with plasma exchange therapy, have been published. Treatment usually involves daily single-volume plasma exchange with both frequency and duration of treatment guided by clinical response and continued until the platelet count is above 150,000/μL and lactate dehydrogenase is near normal for 2 to 3 consecutive days. The persistence of schistocytes on the peripheral blood smear does not preclude weaning or discontinuation of treatment. Typically, patients should respond within 2 or 3 days of beginning treatment. In desperately ill and deteriorating patients, escalating the intensity of plasma exchange to twice daily may be necessary.[9] Despite initial reports of improved response rates in certain patients, recent experience suggests that the use of cryoprecipitate-poor plasma may not be more effective than the use of standard FFP as a specific replacement fluid for plasma exchange in patients with TTP. The effectiveness of plasma exchange in this setting may derive from removal of antibody to or replacement of the von Willebrand factor–cleaving zinc metalloprotease, ADAMTS13. However, patients with clinical features of TTP and only moderate ADAMTS13 deficiency or even normal activity may respond to plasma exchange. Refractory TTP may become less common in the era of caplacizumab, a recent front-line therapeutic antibody directed at Von Willebrand Factor (anti-VWF), and novel agents such as recombinant ADAMTS13 which is being evaluated in clinical trials.[10]

TPE is generally not effective in the treatment of other thrombotic microangiopathies (TMA). Plasma exchange for hematopoietic progenitor cell transplant recipients exhibiting clinical features of TMA, now generally referred to as transplant-associated thrombotic microangiopathy (TA-TMA), has proved ineffective and potentially harmful. This syndrome likely differs in pathogenesis from classic TTP in many aspects, including the absence of severe ADAMTS13 deficiency, the spectrum of clinical symptoms, and the lack of evidence of systemic microthrombus formation. Atypical hemolytic uremic syndrome (aHUS), a rare form of thrombotic microangiopathy with high mortality, may be difficult to distinguish clinically from TTP in adults. aHUS results from uncontrolled complement activation, does not respond to plasma therapies, and is instead treated with eculizamab to block the terminal complement complex. Other causes of TMA, such as HUS due to Shiga toxin–producing *Escherichia coli* infection (STEC-HUS) or drug-induced TMA have not been shown to respond to plasma exchange.

Small, uncontrolled studies and extensive clinical experience support the use of plasmapheresis as an adjunctive therapy for patients with paraproteinemia and hyperviscosity syndrome, with some paraproteinemias in the absence of hyperviscosity. Waldenström macroglobulinemia manifests as a lymphoplasmacytic lymphoma with a monoclonal IgM protein in the plasma. Because IgM is a large molecule and resides predominantly in the intravascular space, as little as one apheresis procedure can result in dramatic improvement in symptoms. Recurrence of symptoms and rising plasma viscosity will determine the need and frequency of repeated exchanges. Definitive chemotherapy should be initiated concurrently. Comprehensive reviews describing the rationale and treatment schedules for plasmapheresis in patients with a variety of paraproteinemias, including cryoglobulinemia and Waldenström macroglobulinemia have been published.

In some immune disorders, such as immune thrombocytopenic purpura (ITP) and immune inhibitors to coagulation proteins, plasma exchange may be helpful during a catastrophic event, but in general, benefit of nonselective plasma exchange therapy is not established.

Neurological Disease Indications

Plasmapheresis is effective first- or second-line therapy in selected patients with certain neurologic disorders. Controlled clinical trials of plasmapheresis have demonstrated efficacy in at least two of the polyradiculoneuropathies. In Guillain-Barré syndrome, plasmapheresis should be considered when patients are unable to walk independently or require mechanical ventilation. However, IVIg alone may be equally effective and is more readily available. Periodic plasmapheresis may be necessary in patients with a chronic inflammatory demyelinating neuropathy. Because the long-term prognosis varies, plasmapheresis may be used in conjunction with steroids and IVIg. Rapid deterioration may occur upon discontinuation. Myasthenia gravis (MG) is one of the most common indications for TPE in many hospital settings, particularly in the context of myasthenic crises or refractory MG.[11] Other neurologic disorders characterized by circulating autoantibodies, such as neuromyelitis optica, N-methyl D-aspartate (NMDA)-encephalitis, and Eaton-Lambert syndrome, have also been treated successfully with TPE.

Multiple sclerosis (MS) is a relapsing and progressive disorder with demyelination of the central nervous system white matter. Patients who present with acute fulminant demyelination may benefit from early plasma exchange, particularly when they fail to respond to high-dose corticosteroids.[12] The majority of patients have a relapsing-remitting form of the disease, and plasmapheresis may be of benefit. Unfortunately, for chronic progressive forms of MS, plasma exchange has consistently been shown to be ineffective. Natalizumab (NTZ), an effective treatment for relapsing-remitting MS, may rarely be complicated by progressive multifocal leukoencephalopathy (PML); plasmapheresis has also been attempted to facilitate clearance of NTZ in this context.

Renal Disease Indications for Therapeutic Plasma Exchange

Filtration apheresis devices are commonly used by nephrology services since these devices can be used to perform plasma exchange as well as renal replacement therapy. Plasma exchange is considered first-line adjuvant therapy in patients with anti-glomerular basement membrane disease (Goodpasture syndrome), a disorder characterized by a specific pathogenic autoantibody directed against the renal glomerular and pulmonary alveolar basement membrane. Plasmapheresis has demonstrated similar success in antineutrophil cytoplasmic antibody (ANCA)-associated vasculitides, Plasma exchange has also been used to treat patients with focal segmental glomerulosclerosis, both for primary disease refractory to standard immunosuppressive therapy and for treatment of patients with recurrent disease after renal transplantation.

Plasma exchange appears to be a useful therapeutic option in renal transplant patients with refractory humoral rejection and is now widely used to overcome ABO and HLA incompatibilities between renal transplant patients and their only available donors. Several studies have shown successful reversal of acute humoral rejection mediated by HLA-specific donor antibody using a combination of plasmapheresis and IVIg, which is superior to high dose IVIg alone.[13] A conditioning regimen consisting of pretransplant plasmapheresis, immunosuppressive medications, and low-dose cytomegalovirus immune globulin effectively reduces donor-specific antibody and isoagglutinin titers with and without posttransplant splenectomy and anti-CD20 treatment. The strength of donor-specific antibodies is also important and can be determined by titration, but greater sensitivity and specificity may be obtained using Luminex flow-bead technology. Patients with potent HLA antibodies have increased mean bead fluorescence and were more likely to have acute rejection; however, the introduction of peri-transplantation apheresis reduced acute rejection from 66% to 7%.[14]

Immunoadsorption and Low-Density Lipoprotein Apheresis

Some of the conditions that can be treated with plasma exchange may alternatively be treated by selective adsorption of antibodies from plasma. The staphylococcal protein A–agarose immunoadsorption column (Immunosorba) has been approved by the US Food and Drug Administration (FDA) for removal of autoantibodies to factor VIII or factor IX. A phase III, multicenter, sham-controlled randomized study of staphylococcal protein A column immunoadsorption shows a significant increase in clinical response in adult patients with longstanding rheumatoid arthritis. The Prosorba staphylococcal protein A–silica column was approved in the US for the treatment of ITP but has not been manufactured since 2006.

Evidence that cutaneous lesions and vascular lesions regress in individuals with familial hypercholesterolemia as LDL levels are controlled by plasmapheresis has encouraged the use of apheresis column absorption procedures in patients with homozygous disease and in poorly controlled heterozygous patients. LDL apheresis removes apolipoprotein B–containing lipoproteins from the blood by a variety of techniques, including dextran sulfate cellulose adsorption, immunoadsorption, and heparin-induced extracorporeal precipitation. Short-term safety and efficacy have been demonstrated; however, additional experience will be required to prove long-term benefit for refractory hypercholesterolemia and coronary artery disease for heterozygotes in particular.[15] In 36 homozygous children in two studies, 20% to 22% developed new aortocoronary lesions, or showed progression of existing lesions while on apheresis despite impressive reductions in mean LDL cholesterol.[16,17] Side effects such as malaise, shivering, and pain at the phlebotomy site are common but mild, and the treatments are generally well tolerated. Patients with severe hypertriglyceridemia are at risk of developing acute pancreatitis. Plasma exchange appears to reduce recurrent episodes by an average 67% but requires continuation of medical therapy.

Simple plasma exchange may be used in patients with other inherited metabolic diseases, such as Refsum disease. The frequency of exchange depends primarily on total body burden, rate of synthesis, and plasma concentration of the solute to be removed (Fig. 116.5). Less evidence exists to support a role for repeated treatments in these diseases.

Therapeutic Cytapheresis

Common indications for therapeutic cell removal are listed in Table 116.2. Most of these procedures entail simple apheresis of the patient's peripheral blood with diversion for discarding the affected RBC, white blood cell (WBC), or platelet fraction. Special apheresis technologies have been approved, such as photopheresis devices, which divert a cell fraction that is photochemically treated in the extracorporeal circuit and returned to the patient's circulation, and adsorptive cytapheresis columns, which selectively remove leukocytes by binding to a solid matrix.

Erythrocytapheresis

RBC exchange (erythrocytapheresis with RBC replacement) is used most often to manage or prevent the acute vaso-occlusive complications of sickle cell disease (SCD). Compared to manual exchange transfusion, mechanical cell separators offer the advantages of speed and ease and reduce the risks of rapid blood volume alteration and increased blood viscosity that may occur with simple transfusion. Automated procedures can be performed with all centrifugal instruments, and built-in software programs allow accurate prediction of target hemoglobin (Hb) concentration and percent HbA at the conclusion of the procedure. A single volume exchange will remove about two-thirds of the circulating cells.

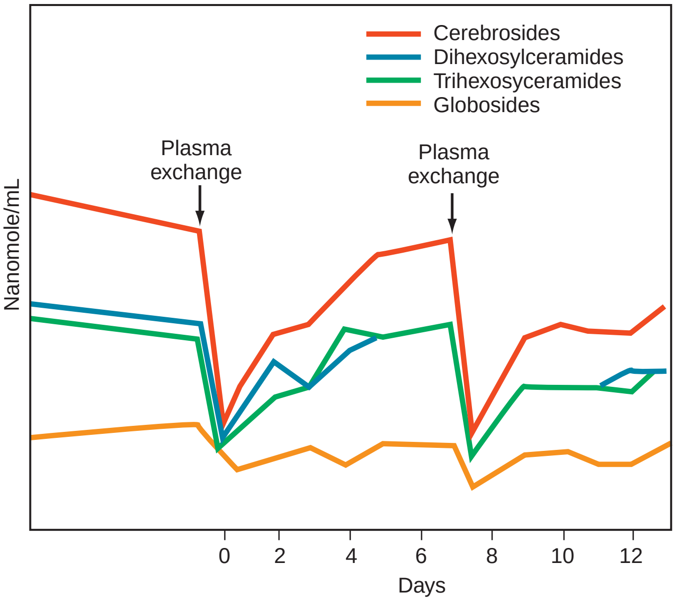


Figure 116.5 PLASMA EXCHANGE TO REMOVE PLASMA NEUTRAL GLYCOLIPIDS IN A PATIENT WITH FABRY DISEASE. The plasma lipid recovery curve appears to be biphasic, reflecting initial reequilibration from tissue stores and subsequent new synthesis of that glycolipid.

TABLE 116.2 **Common Indications for Therapeutic Plasma Exchange (Plasmapheresis)**

Indication (ASFA Category)
Hematologic diseases:
Thrombotic thrombocytopenic purpura (TTP) (I)
Hyperviscosity (I)
Severe cold agglutinin disease (II)
Transplantation:
ABOi liver transplant, desensitization (I)
ABO compatible renal transplant, desensitization (I)*
ABO compatible renal transplant, AMR (I)*
Neurologic diseases:
Myasthenia gravis (I)[a]
Guillain-Barré syndrome (I)[a]
Paraproteinemic demyelinating neuropathy (I)[a]
Renal diseases:
ANCA-associated vasculitis, RPGN (I)
Goodpasture syndrome, anti-GBM with DAH (I)
Recurrent FSGS in kidney transplant (I)[a]
Metabolic diseases:
Homozygous familial hypercholesterolemia (lipoprotein adsorption (I), TPE (II))

[a] Also an indication for immunoabsorption (IA).

Adapted with permission from Schwartz J, Padmanabhan A, Aqui N, et al. Guidelines on the use of therapeutic apheresis in clinical practice—evidence-based approach from the Writing Committee of the American Society for Apheresis: the eighth special Issue. *J Clin Apher*. 2019;34:171–354.

The pathophysiology of vaso-occlusive complications in SCD is complex, involving Hb polymerization, change in cell shape, adhesion to endothelial cells, dysregulated nitric oxide homeostasis, and release of free Hb and inflammatory cytokines. The rationale behind exchange transfusion involves improving tissue oxygenation, reducing hemolysis, and preventing microvascular sickling by diluting the patient's abnormal RBCs, simultaneously correcting anemia and favorably altering whole blood viscosity and rheology. No clinical data supports a single optimal level of HbA; however, as few as 30%

of transfused cells markedly decrease blood viscosity. At mixtures of 50% or greater, resistance to membrane filterability approaches normal. In nonemergency situations, such levels can often be achieved with a simple transfusion regimen. For simple and exchange transfusions, raising the level of HbA to between 60% and 70% while lowering the level of HbS to 30% is generally efficacious, although even higher levels of HbA are often used to treat an ongoing crisis. Clinical indications for exchange transfusion in patients with SCD remain controversial, with limited controlled study data available. Simple transfusion has been shown to improve renal concentrating ability and splenic function in young sickle cell patients; exchange transfusion improves exercise tolerance and reverses the periodic oscillations in cutaneous blood flow associated with this disease. Such observations have encouraged the use of exchange transfusion for acute complications of SCD such as acute chest syndrome, priapism, cerebrovascular accident, and hepatic and retinal infarction. Exchange transfusion for sickle cell patients has also been used for prophylaxis during pregnancy and before surgery, although prophylactic transfusion in these settings remains controversial. The only randomized trial of transfusion during pregnancy has shown that prophylactic transfusion sufficient to reduce the incidence of painful crises did not reduce other maternal morbidity or perinatal mortality. The risk of intrauterine growth restriction may be reduced by prophylactic exchange transfusion; however, the study is limited by the retrospective observational nature of the data. In a randomized study of patients with SCD undergoing surgery, a conservative simple transfusion regimen (to increase the Hb level to 10 g/dL) was as effective as an aggressive regimen (to lower the HbS level to <30%) with respect to perioperative complications not related to transfusion. The patients in the aggressive regimen group received twice as many units of blood, had a proportionally increased RBC alloimmunization rate, and had more hemolytic transfusion reactions. A subsequent trial has confirmed that preoperative transfusion is associated with decreased perioperative complications in patients with SCD who are scheduled to undergo low-risk and medium-risk surgeries.[18]

Transfusion prophylaxis is now clearly indicated for children at high risk for stroke. In the absence of primary stroke prevention, approximately 10% of children with sickle cell anemia will have overt stroke and an additional 20% to 35% will have silent cerebral infarction, which can cause cognitive decline and predispose them to additional silent infarcts and overt strokes. Acute stroke in SCD is an indication for RBC exchange, using HbS negative RBC units as the replacement fluid. Red cell units should ideally be antigen-matched for least C, E, and K.[6] A randomized controlled study demonstrated a risk reduction of 90% in the patients who were maintained at levels of 30% or less HbS by simple or exchange transfusion. A second trial addressed whether transfusion could be safely discontinued to avoid the cumulative long-term risks of iron overload and RBC alloimmunization. After 10 months of randomization, half of the patients who discontinued transfusion had developed central nervous system abnormalities, including reversion to abnormal transcranial Doppler findings and a small number of strokes, necessitating early termination of the trial. This result confirms earlier experience and indicates that in this group of children with sickle cell anemia, transfusion therapy should begin before the first event and continue indefinitely. For chronic management, repeated erythrocytapheresis may be preferable to simple transfusion for patients at high risk for stroke who have developed iron overload to levels associated with organ damage.

Exchange transfusion, although relatively safe and convenient, carries all the complications of RBC transfusion. Patients are exposed to a large number of donors and are at a small but increased risk of contracting hepatitis and other blood-borne infections. As many as 33% of all patients develop alloantibodies, and life-threatening delayed hemolytic transfusion reactions have been reported. In addition, an immunohematologic study of multiply transfused sickle cell patients shows that 85% of heavily transfused patients are alloimmunized to human leukocyte antigens (HLAs), platelet-specific antigens, or both. Most centers avoid inducing nonhemolytic transfusion reactions and HLA alloimmunization by using leukocyte-depleted RBCs. Extended RBC phenotyping at diagnosis and provision of phenotypically matched blood, when practical, can reduce the risk of RBC alloimmunization and associated hemolytic transfusion reactions. Isovolemic hemodilution (using saline) prior to standard red cell exchange may be safely employed in patients with SCD undergoing chronic RCE, and reduces the number of RBC units transfused.[19] Despite the removal of RBCs during exchange, most patients remain in positive iron balance, although iron accumulation is slow and chelation is rarely required to prevent transfusional hemosiderosis.

Other indications for RBC exchange are rare. The procedure has been used for patients with overwhelming RBC parasitic infections, such as severe and complicated malaria and babesiosis. Theoretically, automated exchange rapidly decreases the concentration of circulating parasites while improving the rheologic properties of the blood by replacing the infected RBCs. The exchange may also reduce levels of proinflammatory cytokines and may help sustain life until conventional therapy and natural immunity take effect. Although the efficacy of this therapy has not been evaluated by controlled trials, prospective studies and review of published cases suggest the use of erythrocytapheresis for parasitemia greater than 10% to 15% or even less in selected patients such as those with cerebral malaria or pulmonary edema. Case reports document RBC exchange in such diverse conditions as carbon monoxide poisoning and glucose-6-phosphate dehydrogenase (G6PD)–deficient hemolysis.

Automated RBC removal with saline replacement can be performed rapidly and safely in polycythemic subjects. This maneuver should be reserved for polycythemic patients with an urgent clinical indication to lower the hematocrit (e.g., evolving thrombotic stroke) for which standard single-unit manual phlebotomy might be inadvisably slow. Automated double RBC apheresis technology has more recently been used to treat individuals with hereditary hemochromatosis. This procedure removes excess iron more rapidly than manual phlebotomy, and is well received by patients because it lowers the frequency of clinic visits (Fig. 116.6).[20]

Leukapheresis

Therapeutic leukapheresis, also called leukocytapheresis or leukocytoreduction, has been used most successfully to help manage patients with acute leukemia and extremely high WBC numbers, so-called acute hyperleukocytic leukemia (AHL). When the fractional volume of leukocytes (leukocrit) exceeds 20%, blood viscosity increases and leukocytes can interfere with pulmonary and cerebral blood flow and compete with tissue for oxygen in the microcirculation. Investigations of the expression and function of adhesion receptors in leukemic cells, and the role of adhesion molecules in leukocyte-induced acute lung injury in sepsis, suggest that the pathophysiology of leukostasis in AHL may also be related to interactions between leukemic blasts, platelets, and endothelial cells mediated by locally released adhesion molecules. A single-volume leukapheresis procedure generally reduces the WBC count by 20% to 50%, depending on the differing sedimentation characteristics of the specific blast cell population. Hydroxyethyl starch may be used to enhance separation of red cells from blasts, increasing the efficiency of the procedure. Ordinarily, leukapheresis is initiated in a patient with acute myeloid leukemia (AML), or in the accelerated phases of chronic myeloid leukemia (CML) when the blast count exceeds 100,000/mm^3, or when rapidly rising blast counts are higher than 50,000/mm^3, especially when evidence of central nervous system or pulmonary symptoms appears. The threshold for initiation of leukapheresis in patients with acute lymphocytic leukemia (ALL) is generally higher (WBC count >200,000/mm^3). Leukostatic syndromes do not occur when concentrations of well-differentiated lymphocytes exceed even several million/mm^3.

Although leukapheresis is effective in reducing the number of circulating blasts, the evidence for clinical benefit is anecdotal. Case reports and small series describe dramatic improvement of patients with evolving strokes and respiratory insufficiency, but the absence of a randomized controlled trial necessitates reliance on retrospective

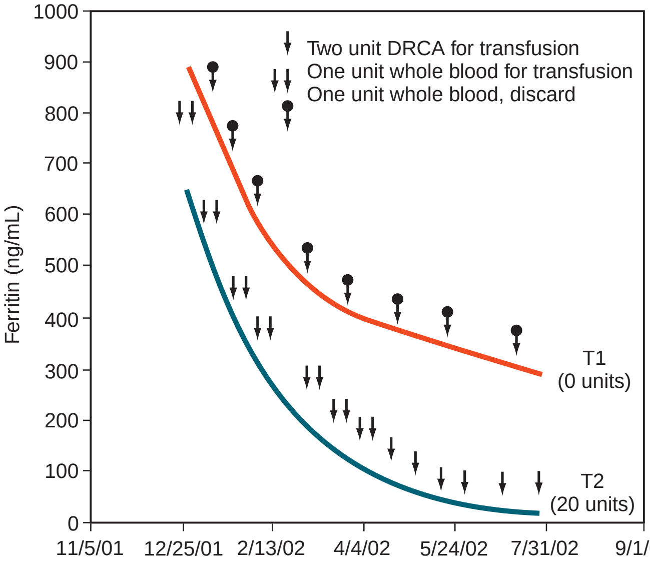


Figure 116.6 ONE TWIN WAS TREATED WITH MANUAL PHLEBOTOMY (T1) AND THE OTHER WITH DOUBLE RED BLOOD CELL APHERESIS (T2). In the same time period, ferritin levels declined more rapidly and to lower levels in the twin treated with double red blood cell apheresis. *DRCA*, Double red blood cell apheresis. *(Unpublished data from Bolan CD, Leitman SF, with permission of the authors.)*

studies. Leukapheresis does not improve the overall survival of patients with AML, and recent reports have not found evidence of a short-term mortality benefit.[21] Because one retrospective cohort study associated leukapheresis with a poorer outcome attributed to a delay in commencing chemotherapy,[22] it seems imprudent to delay other immediate measures for treatment of patients with AHL (infusion of intravenous [IV] fluids, administration of hydroxyurea, and uricosuric medication, urinary alkalization, correction of coagulopathy and thrombocytopenia) while awaiting leukapheresis.

Mechanical cytoreduction for managing other leukemic processes has limited value. Chronic leukapheresis can provide acceptable control of the peripheral WBC count in clinical situations such as pregnancy, when cytotoxic agents may best be avoided. In a small series of gravid patients, leukapheresis in combination with interferon has successfully controlled the disease until therapy with tyrosine kinase inhibitors could be initiated after delivery.[23]

Lymphocyte removal by apheresis has also been used to modify immune responsiveness in patients with autoimmune diseases, and to enhance solid organ allograft survival and reverse solid organ graft rejection. Evidence of clinical efficacy in these situations is sparse. Because leukocytes are a major source of inflammatory cytokines implicated in the pathogenesis of inflammatory bowel diseases (IBD), nonpharmacologic methods for selective leukoreduction have been developed to treat these chronic, relapsing, lifelong disorders. Although IBD respond to a variety of drug regimens, leukapheresis may have a role as a “steroid-sparing” intervention for patients who develop toxicity from long-term steroid use. Cellsorba removes leukocytes by extracorporeal filtration. Granulocyte-monocyte apheresis (Adacolumn) selectively adsorbs cells through columns filled with cellulose beads. Clinical trials using Adacolumn technology have promising results in the treatment of ulcerative colitis (UC), with remission rates in excess of 70% compared with conventional medical therapy, however in a randomized controlled trial the clinical remission rate at 12 months was comparable between the apheresis and control groups.[24] These studies should be interpreted with caution given their reliance on predominantly Japanese patients, which may limit how the results apply to populations with different genetic and environmental factors. The optimum course of therapy is yet to be defined. Recent trials have investigated the safety and efficacy of procedures varying from weekly to a daily basis.

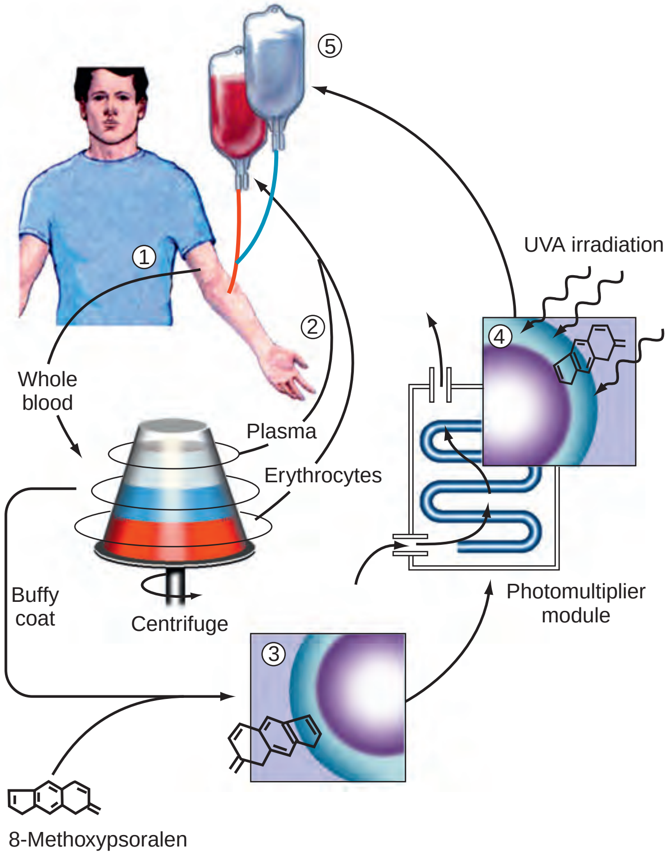


Figure 116.7 OVERVIEW OF A PHOTOPHERESIS PROCEDURE. (1) Vascular access is achieved by placement of a peripheral intravenous line or through a temporary central venous catheter. Whole blood is withdrawn from the patient, mixed with an anticoagulant solution, and pumped to a centrifuge, where it is separated into plasma, red blood cell, and mononuclear cell (buffy coat) fractions by elutriation. (2) After the collection of each mononuclear cell fraction, the uncollected red blood cells and plasma are pumped from the plasma/return bag through a blood filter and returned to the patient. (3) The mononuclear cell fractions are mixed with 8-methoxypsoralen and then (4) pumped through a sterile cassette surrounded by ultraviolet A *(UVA)* bulbs, resulting in a controlled rate and amount of UVA exposure. (5) The treated cell fraction is filtered and returned to the patient.

Extracorporeal Photopheresis

Photopheresis involves automated leukapheresis with extracorporeal photoactivation of WBC using 8-methoxypsoralen (8-MOP), a light-sensitizing agent, and ex vivo ultraviolet A irradiation. The treated leukocyte fraction is then returned to the patient (Fig. 116.7). Delivering the light-sensitizing agent directly to the extracorporeal leukocyte fraction avoids any potential toxicity from oral administration of 8-MOP. The mechanism of action of photopheresis is unproved, but may involve induction of apoptosis of pathogenic T lymphocytes and induction of a dendritic cell-mediated cytotoxic T-cell response. This therapy has minimal toxicity and is highly effective in the treatment of patients with advanced cutaneous T-cell lymphoma. Patients who present with the erythrodermic disease form and circulating malignant cells have the best clinical response. In this context, photopheresis is typically performed once or twice a month. Symptomatic patients with a higher circulating tumor burden benefit from a more intense regimen.[25] More recently, the major use of this technology involves modulation of immune rejection of transplanted organs and management of GVHD after stem cell transplantation in patients who do not respond to standard immunosuppressive therapy. In steroid-refractory acute GVHD, extracorporeal photopheresis (ECP) is thought to enhance regulatory T-cell activity, likely inhibiting the cytotoxic

T cells implicated in the pathogenesis of this condition. Response rates in steroid refractory acute GVHD in pediatric and adult patients range from 52% to 100%. Patients with chronic extensive GVHD have also been treated, with responses observed in approximately two-thirds of the steroid-dependent patients, with cutaneous, oral, and ocular forms of the disease. In the setting of GHVD, patients may require two treatments a week, every 1 to 2 weeks. A response may facilitate a reduction in immunosuppression ("steroid sparing") with continued photopheresis based on results from a randomized phase II trial.[26] In chronic visceral GVHD, where a clear benefit is yet to be established, consideration for treatment should include the logistic challenges of frequent hospital visits and the prolonged need for central venous catheter care and/or transfusion support.

Acute rejection in cardiac transplantation occurs in 25% of recipients in the first year. In 30% to 50% of cases, acute rejection is T-cell mediated. Prospective studies have shown benefit of photopheresis in prevention of rejection. In the case of lung transplant, chronic rejection is manifest as bronchiolitis obliterans syndrome (BOS), presenting as progressive dyspnea and airflow limitation. In a recent prospective nonrandomized study, the rate of decline in lung function stabilized within 6 months of starting ECP therapy compared with rates of FEV_1 (forced expiratory volume in the first second of expiration) which were worsening at baseline. Rates of retransplantation were lower and survival was higher among patients who received ECP treatment. Patients with cystic fibrosis and those with longer interval to BOS development since transplant were less likely to respond to therapy.[27] Of the substantial number of autoimmune disorders for which ECP therapy has been studied, data from small randomized trials have shown benefit in systemic sclerosis (improvement in skin and joint disease severity), and in Crohn's disease (steroid-sparing effect). No significant benefit was demonstrated in type I diabetes or in MS.[28] The acute adverse effects of photopheresis include photosensitivity, low-grade fever, and transient hypotension. Anemia and iron deficiency caused by incomplete reinfusion of RBCs may occur in patients undergoing repeated photopheresis. In patients with inadequate peripheral venous access requiring central venous catheter placement, infection, bleeding, and recurrent clotting are risk factors.

Thrombocytapheresis

Therapeutic plateletpheresis, or thrombocytapheresis, is generally reserved for patients with myeloproliferative disorders and hemorrhage or thrombosis associated with an increase in circulating platelets. Many centers consider using thrombocytapheresis when the patient's peripheral platelet count is greater than $10^6/mm^3$, although no consistent relationship between the level of platelet elevation and the occurrence of symptoms has been found, and no generally accepted assay of platelet dysfunction predicts which patients are at risk. A single cytapheresis procedure can lower the platelet count by 30% to 50%. Thrombocytapheresis can have dramatic effects for selected patients such as those with evolving digital gangrene. Attempts to maintain thrombocythemic patients at normal platelet counts by cytapheresis alone have not been successful; more practical long-term chemotherapy should be instituted concurrently. Because most patients with thrombocytosis do not develop symptoms, including patients with myeloproliferative disorders, prophylactic thrombocytapheresis seems unwarranted regardless of the platelet count. One possible exception involves pregnant patients with essential thrombocythemia who may be at increased risk of first trimester abortion; periodic thrombocytapheresis has been used in a limited series, with weekly procedures necessary to reduce the circulating platelet number until delivery.

Peripheral Blood Hematopoietic Stem and Progenitor Cell Collection

The most common application of apheresis technology for cell therapy indications is the collection of HSPCs from peripheral blood after administration of recombinant hematopoietic growth factor or chemotherapy, or both, to "mobilize" large numbers of HSPCs into the circulation. The collected mononuclear cell fraction contains a subset of progenitor cells that, on infusion, can home to and reconstitute the bone marrow in patients who receive ablative radiation or chemotherapy, or both. Pluripotent HSPCs, and quite possibly primordial hematopoietic stem cells capable of reconstituting the bone marrow and the immune system, have long been known to circulate in the peripheral blood. Numerous studies have confirmed the potential for rapid and durable engraftment of peripheral blood HSPC mobilized into the circulation by hematopoietic growth factors harvested by LVL procedures and subsequently administered to patients with marrow aplasia after high-dose chemotherapy. The concentration of HSPCs in the peripheral blood can be increased by the administration of cytokines, such as granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), and by the administration of chemotherapy. G-CSF for HSPC mobilization is generally administered at a dose of 10 to 16 μg/kg body weight by subcutaneous injection daily for 4 to 5 days prior to apheresis. $CD34^+$ hematopoietic cell counts in the peripheral blood generally rise 20- to 40-fold (from 1 to 3 cells/μL to 40 to 70 cells/μL) after administration of G-CSF alone, and they increase 100- to 1000-fold when chemotherapy rebound is enhanced by administration of G-CSF. Efforts to define optimal collection conditions have been limited by the lack of a standardized assay; however, time and level of increase of donor peripheral blood WBC and $CD34^+$ cell counts after administration of mobilizing agents have proved to be useful indicators. Despite ongoing challenges with interlaboratory standardization, flow cytometric analysis of cells labeled with a fluorochrome-conjugated CD34 antibody are used for "real-time" decision making about the timing and adequacy of PB HSPC collections. Several prestimulation donor variables are important. Increasing age, white ethnicity, and female gender are associated with significantly lower post–G-CSF $CD34^+$ cell counts, which would favor younger males as donors when high cell doses are required.[29]

Several clinical scale devices for $CD34^+$ cell enrichment of leukapheresis collections by immunoabsorption or immunomagnetic techniques are available. This "positive selection" of $CD34^+$ hematopoietic cells also results in reduced numbers of cells that do not express the CD34 antigen, such as T lymphocytes. T-cell–reduced products may confer a lower risk of GVHD, and the ability to produce apheresis products enriched for $CD34^+$ cells facilitates graft manipulations for experimental cell therapies such as ex vivo expansion of $CD34^+$ cells. However, some studies suggest a higher incidence of engraftment failure and possibly delayed immune reconstitution in certain patient populations when transplants are performed with highly purified, CD34-selected T-cell–depleted products.

Over the last decade, PB HSPCs have surpassed bone marrow HSPCs as the predominant graft source in the clinical setting for various hematopoietic stem cell transplants. PB HSPCs are the graft of choice in 99% of conditions where autologous transplants are indicated. For allogeneic transplants, especially those using stem cells from matched unrelated donors or in pediatric transplants, bone marrow stem cells still remain the popular graft source. PB HSPC transplants result in higher rates of clinically significant chronic GVHD in these situations. Table 116.3 provides a summary of comparisons of bone marrow versus PB HSPCs in different types of allogeneic stem cell transplants.

From a donor perspective, the discomfort and inconvenience of multiple-day cytokine administration may be a significant deterrent. Most donors experience some degree of malaise and bone pain, and some donors require hospitalization for more serious adverse reactions to G-CSF administration. However, a large prospective trial of the National Marrow Donor Program found intensity and duration of pain in the peri-collection period was similar for persons undergoing HSPC collection by apheresis (during G-CSF administration before apheresis) and following bone marrow harvest procedure in bone marrow HSPC donors). Furthermore, 3% of bone marrow donors reported ongoing low-grade site pain after 6 months of completing the procedure, whereas recovery was complete for PB HSPC donors by this time. Risk factors for worse toxicities, including older

TABLE 116.3 Bone Marrow Versus Cytokine Stimulated Peripheral Blood Stem Cells as Graft Source of Choice for Hematopoietic Stem Cell Transplantation

Disease Category	Recipient Age Group	Type of Donor	Preferred Graft Source (Unstimulated BM vs. Cytokine Stimulated PB HSPC)
Nonmalignant (e.g., severe aplastic anemia)	All ages	Sibling	BM HSPCs
Malignant	Pediatric	Sibling	BM HSPCs
Malignant	Adult	Sibling	PB or BM HSPCs (PB HSPCs preferred in high-risk leukemia)
Malignant	Adult	Matched unrelated donor	PB or BM HSPCs (PB HSPCs preferred if high graft failure risk; BM HSPCs if high cGVHD risk, prior immunosuppression)

BM, Bone marrow; *cGVHD*, chronic graft-versus-host disease; *HSPC*, hematopoietic stem and progenitor cell; *PB*, peripheral blood.

age, female gender, and obesity were similar among bone marrow and PB HSPC donors.[30] While concerns remain regarding the hypothetic long-term effects of exposure of healthy donors to growth factors, another recent prospective study with more than 20,000 donor-years of follow-up, showed no evidence of increased risk for cancer, autoimmune illness, and stroke in donors receiving G-CSF.[31] Splenic enlargement occurs commonly, and several instances of splenic rupture have been reported. Citrate toxicity is a common complication of HSPC collections; although usually mild and transient, it may be severe and even life-threatening in some individuals (see previous section). Venous access requires large-bore multilumen catheters, and these appear particularly susceptible to clotting, especially when patients receive recombinant cytokine stimulation. Hemorrhage, especially in thrombocytopenic patients, is another potentially severe complication of central venous catheter placement.

Some patients who have received multiple prior cycles of chemotherapy and a small proportion of normal healthy donors, referred to as "poor mobilizers," fail to respond adequately to mobilization regimens. In this circumstance, Plerixafor, a reversible CXCR4 antagonist that can block the adhesion of HSPCs to the bone marrow stroma can enhance collections by releasing these cells into the circulation. In a randomized controlled trial, the combination of G-CSF and a single dose of Plerixafor resulted in a significantly higher proportion of patients with non-Hodgkin lymphoma achieving their target cell dose in fewer apheresis days.[32] Similar success is achieved with multiple myeloma patients achieving a 4.8-fold increase peripheral blood CD34 cell count compared with 1.7-fold with G-CSF alone. Emerging evidence indicates that Plerixafor may be administered "just-in-time" to rescue collections for patients who mobilize poorly with G-CSF alone. While the optimum circumstances and timing for administration have not yet been determined, some guidelines recommend addition of Plerixafor for circulating $CD34^+$ cell counts less than 20×10^6/L on two consecutive days accompanied by increasing WBCs.

A novel indication for HSPC apheresis is to facilitate autologous gene therapy for patients with hemoglobinopathies. SCD and thalassemia are inherited hemoglobinopathies characterized by mutations the β-globin gene, causing production of abnormal hemoglobin (HbS) or inadequate globin chains and ineffective erythropoiesis. Allogeneic HSPC transplantation may be curative but identifying a related acceptable donor may be challenging. A novel approach is underway in clinical trials, utilizing transplantation of genetically modified autologous HSPC with either repair of the β-globin mutation or targeting genetic loci associated with reactivation of endogenous γ-globin expression. Bone marrow may be a source of HSPC for such approaches. However, recent trials in patients with SCD have shown that HSPC derived from mobilization and apheresis are superior to marrow due to higher HSPC yields and faster hematologic recovery compared to bone marrow, and a better cellular phenotype.[33] G-CSF mobilization with or without plerixafor results in safe and adequate $CD34^+$cell mobilization in patients with β-thalassemia; however, G-CSF is contraindicated in patients with SCD due to severe life-threatening complications in this population. Mobilization, using plerixafor as a single agent HSPC apheresis in participants with SCD, was impacted by known clinical factors that hinder stem cell mobilization in healthy adults such as age and bone marrow reserve as measured by pre-apheresis CD34/μL, white blood cell (WBC), and platelet counts. Disease severity as measured by hospitalization frequency in the preceding year and number of medications required to treat chronic pain, were also predictive of a lower total $CD34^+$ yield.

MONONUCLEAR CELL COLLECTION FOR CELLULAR IMMUNOTHERAPIES

Blood cell separators developed for hemapheresis are used to collect peripheral blood cells for cellular therapy. Donor leukocytes obtained from leukapheresis procedures may be used for donor lymphocyte infusion (DLI) or subject to further processing for immunotherapy. DLIs from allogeneic HSPC transplant donors are used to treat graft failure or disease relapse in recipients with hematologic malignancies. Collection of autologous lymphocytes from patients has been increasing with the expansion of the field of novel cellular therapies such as CAR-T cells. CAR-T-cell therapies are most commonly used to treat hematologic malignancies, primarily B-cell acute lymphoblastic leukemia or lymphoma; other indications include multiple myeloma and prostate cancer. Compared to healthy donors, patients who have previously undergone stem cell transplantation, or received lymphodepleting chemotherapy may be anemic and have significantly reduced total WBC and lymphocyte counts. This poses a challenge in collecting sufficient T cells to allow for CAR-T manufacture. Some CAR-T-cell manufacturers have recommended minimum lymphocyte counts in trials, such as an absolute lymphocyte count (ALC) >500/μL or $CD3^+$ >150/μL for the manufacture of tisagenlecleucel. In lymphopenic patients, the WBC, ALC or $CD3^+$ count prior to apheresis may be used to estimate the number of blood volumes needed to process to obtain the desired number of lymphocytes.

PEDIATRIC HEMAPHERESIS

Pediatric therapeutic apheresis accounts for about 10% of all apheresis procedures. The indications for apheresis in children are limited by a lack of clinical trial data. Therefore, the evidence for therapy in many diseases is extrapolated from trials in adults despite differing patient physiology and an age-dependent presentation and natural history of the disease. For example, hemolytic uremic syndrome is more common in children and responds to supportive care; at the other end of the spectrum of this disease, TTP is more common in adults and necessitates plasma exchange.

The majority of apheresis procedures in children are erythrocytapheresis for patients with SCD and autologous HSPC collections after chemotherapy rebound (e.g., for neuroblastoma). Increasingly, mononuclear cell collections for CAR-T cell manufacture to treat acute leukemia is becoming more frequent in the pediatric population with high-dose chemotherapy. ECP is used for GVHD; and both ECP and TPE have been used as adjunct treatment modalities for solid organ rejection, with sparse data thus far. In adults, TPE is shown to be efficacious in acute and chronic demyelinating neuropathies and in severe refractory MG. TPE may also benefit children with rare neurologic disorders; pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection (PANDAS) and Sydenham chorea (SC) are autoimmune neuropsychiatric disorders that occur rarely after infection with group A β-hemolytic streptococcal infections. Circulating immune factors may contribute to the pathogenesis of these diseases. These conditions respond to IVIg and plasmapheresis. In a small, randomized study, 50% of patients with SC also responded to plasmapheresis, and although superior to corticosteroids, treatment with IVIg had the best response, with 72% of patients having a reduction in symptoms of chorea. Small pediatric studies have demonstrated benefit of TPE specifically in recurrent focal segmental glomerulosclerosis, especially when it occurs post renal transplantation. It has been attempted with marginal success in severe refractory vasculitides such as Henoch-Schönlein purpura, Kawasaki disease, and ANCA-associated vasculitides.[34]

Technical Aspects

The mechanics of apheresis were developed for adults and therefore designed for their larger circulating blood volume. Therefore, depending on the size of the child, modifications to the apheresis procedure may be necessary. Central venous catheters are required in most circumstances because the caliber of peripheral venous access is too small to permit adequate blood flow. In pediatric apheresis, maintenance of isovolemia is essential to prevent circulatory compromise, particularly in an acutely ill child who may have some degree of cardiac or renal impairment. The beginning and end of the apheresis procedure involves negative and positive intravascular fluid shifts, respectively. Typically, volume shifts have negligible impact on an adult's circulation but can represent a substantial proportion of the total blood volume (TBV) of an infant. The volume of the patient's blood required to fill the apheresis circuit at the start of the procedure is the extracorporeal volume (ECV). Symptomatic hypovolemia becomes increasingly likely as ECV exceeds 15% of TBV. An ECV of 400 mL represents 8% of the TBV of a 70-kg patient but may be as much as 35% for a 15-kg infant. Modification of the apheresis procedure is necessary for safe management in infants and small children.

In adults, the apheresis circuit is primed with saline, which is then diverted to the collection or waste bag. One option permits the return of saline prime to the patient, which may be useful for larger children. For children who weigh less than 25 kg, priming the circuit with RBCs is often necessary to prevent intravascular volume depletion and severe anemia. Upon completion, fluids remaining in the apheresis circuit, typically returned to the adult patient, are not returned in pediatrics to avoid a positive fluid shift causing fluid overload.

COMPLICATIONS OF THERAPEUTIC APHERESIS

Automated apheresis is a minimal-risk procedure for normal healthy blood and plasma donors. The current generation of blood cell separators is remarkably reliable and equipped with sensitive detection and alarm systems to alert the operator to potential problems. Nevertheless, serious morbidity and rare deaths have been associated with therapeutic procedures. In most reports, deaths are related to either complications associated with the use of central venous access catheters, or to cardiac and respiratory problems in patients who were critically ill before apheresis; in the latter, the contributory role of the apheresis procedure is often questionable. The most common adverse effects of therapeutic apheresis are citrate-induced hypocalcemia, allergic reactions (usually to donor plasma or other blood components), vasovagal reactions, and hypovolemia. If transient paresthesia and mild vasovagal events are excluded, approximately 5% of all therapeutic apheresis procedures have medical complications. The frequency of adverse reactions is influenced by the experience of the operator and the nature of the patient population being treated. Expected and predictable effects of therapeutic apheresis include alterations in laboratory parameters caused by removal and dilution by replacement fluids. A 5% to 15% decrease in Hb and hematocrit, a 20% to 30% decrease in platelet count, and mild transient increase in leukocyte count are commonly observed. Significantly, but transiently abnormal coagulation test results are often observed (recovery generally occurs within 48 hours after a single-volume exchange procedure), as well as clinically insignificant decreased levels of other plasma proteins after serial plasma exchange procedures. The most common adverse effect of both donor and therapeutic apheresis procedures is symptomatic hypocalcemia caused by infusion of calcium-chelating citrate ions in the anticoagulant, and if used as replacement fluid, anticoagulated donor plasma. Hypocalcemia is usually manifested by mild perioral, acral paresthesia, or both, requiring no intervention other than slowing the reinfusion rate. The benefit of oral calcium supplements in this setting is questionable, although the practice is widespread. Hyperventilation may exacerbate the symptoms of hypocalcemia. More severe citrate toxicity is uncommon; signs may range from involuntary carpopedal spasm, nausea, and vomiting to frank tetany with spasm in other muscle groups, including life-threatening laryngospasm and grand mal seizure. Severe toxicity is most commonly experienced by small patients, particularly women, when the blood flow rate is rapid and the procedure is prolonged beyond a few hours. Symptoms correlate inversely with the level of ionized calcium. Extremely low concentrations of ionized calcium are encountered routinely in therapeutic procedures that process more than 15 L of blood. Acute severe hypocalcemia leading to fatal cardiac arrhythmia has been reported in patients who have undergone apheresis. Controlled infusions of 10% calcium gluconate or calcium chloride are effective in the prevention and management of these complications. Because metabolism of citrate occurs predominantly in the liver and kidney, patients with conditions affecting these organs are at increased risk for severe citrate reactions. Studies of TPE procedures and of HSPC donations performed with and without calcium gluconate or calcium chloride infusion show the effectiveness of continuous calcium infusion for the prevention of mild to moderate citrate toxicity (Fig. 116.8). A study of plateletpheresis donors demonstrated by

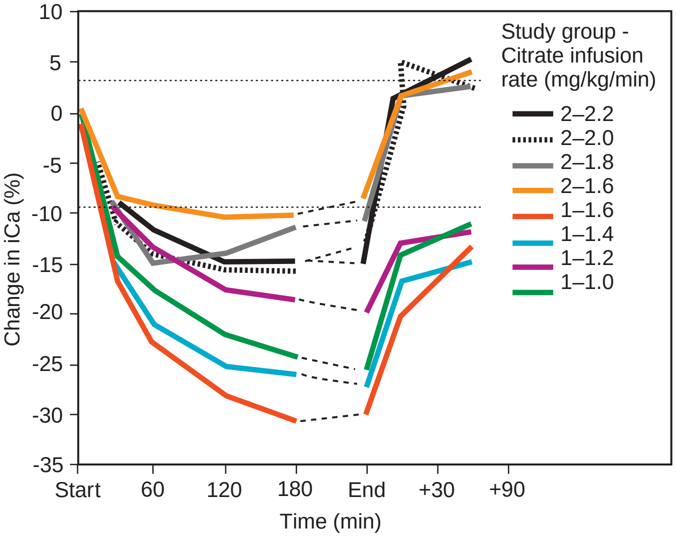


Figure 116.8 LEVELS OF IONIZED CALCIUM ARE MARKEDLY LOWER IN PROCEDURES PERFORMED WITHOUT PROPHYLACTIC CALCIUM INFUSION AND REMAIN BELOW THE REFERENCE RANGE 90 MINUTES AFTER COMPLETION OF APHERESIS. *Dotted lines* indicate upper and lower limits of the reference range. *Open symbols* represent procedures without prophylactic calcium; *solid symbols* represent those with calcium infusion. *Dashed lines* between 180 minutes and end-procedural values *(End)* reflect varying procedure duration. *iCa*, Ionized calcium. *(From Bolan CD, Cecco SA, Wesley RA, et al. Controlled study of citrate effects and response to i.v. calcium administration during allogeneic peripheral blood progenitor cell donation. Transfusion. 2002;42:935, used by permission.)*

PATIENT MANAGEMENT ISSUES FOR THERAPEUTIC APHERESIS

Important issues to consider when preparing a patient for therapeutic apheresis, for monitoring the patient during the procedure, and for managing the patient after removal from the apheresis device include:

Preparation

- Volume considerations: Calculate volume to be processed or exchanged; order replacement solutions; assess patient volume status. A blood prime may be necessary for children who weigh less than 25 kg.
- Vascular access: Assess need for apheresis line placement. Note that not all large catheters are suitable for apheresis procedures.
- Medications: Evaluate impact of medications (Coumadin, platelet inhibitory agents, angiotensin-converting enzyme inhibitors) and indications to suspend certain medications. Albumin-bound medications may be depleted by apheresis.
- Cell counts: Patient may require transfusion for preprocedure anemia or thrombocytopenia.
- Electrolytes: Assess risks for citrate toxicity; order calcium replacement solutions if indicated.

Procedure

- Volume considerations: Monitor replacement fluids (saline-to-albumin ratio). Rapid infusion of refrigerated solutions may cause hypothermia.
- Vascular access: Observe for impaired, intermittent, or obstructed flow. Kinked tubing can result in hemolysis.
- Medications: Limitations on blood or citrate flow rate caused by symptomatic citrate-induced hypocalcemia may necessitate addition of anticoagulant to the final apheresis product or inclusion of heparin in the anticoagulant regimen.
- Cell counts: Consider the impact of very high or low cell counts on device settings and procedure efficiency.
- Electrolytes: Continually monitor for adverse citrate effects; bolus or continuous intravenous calcium administration may be required.

Postprocedure Management

- Volume considerations: Review net volume balance and consider additional infusion or administration of diuretic if needed.
- Vascular access: The decision to remove the apheresis catheter should include the possibility of additional apheresis or procedures; monitor for complications associated with maintenance or removal of venous catheter.
- Medications: Determine when to restart medications; consider the impact of transiently reduced coagulation factor levels.
- Cell counts: The patient may require transfusion for postprocedure thrombocytopenia; monitor cumulative red blood cell and platelet loss after repeated procedures.
- Electrolytes: Assess for imbalance postprocedure with attention to ionized calcium and magnesium as indicated by symptoms.

changes in alkaline phosphatase, osteocalcin, parathyroid hormone, and 1,25-dihydroxyvitamin D levels, suggesting the potential for long-term effects on bone metabolism in these donors. Magnesium, another divalent cation, is also bound by citrate. Despite decreases in serum ionized magnesium levels to 39% below baseline, clinical effects are not typically observed even during LVL procedures. The most severe allergic complications occur when plasma is used as the replacement solution, and this risk increases with repeated exposure. Allergic reactions to ethylene oxide, an agent used in the sterilization of plastic disposable equipment, have been reported. IV diphenhydramine is usually effective in managing allergic reactions; premedication with steroids and precautions against anaphylaxis may be necessary for sensitized individuals. Atypical (hypotension and flushing) and anaphylactic reactions have been reported in patients receiving angiotensin-converting enzyme inhibitors undergoing different apheresis procedures, including immunoadsorption with staphylococcal protein A columns. These medications should be discontinued for at least 24 hours before LVL or plasma exchange. Unlike donors undergoing apheresis for donation of RBCs, platelets, or plasma, donors undergoing apheresis for collection of therapeutic granulocytes are stimulated with steroids and G-CSF before donation. The well-known association of long-term steroid treatment and posterior subcapsular cataracts is likely irrelevant in the context of short-term steroid stimulation for donor granulocyte mobilization. However, an increased number posterior subcapsular cataracts has been detected in three separate studies including an analysis of 100 granulocyte donors compared with age-matched plateletpheresis donors, suggesting that granulocyte donors may be at increased risk for cataract formation. Repeated steroid stimulation for granulocyte collection warrants regular ophthalmologic examination and close follow up.[35] Serious complications indirectly associated with therapeutic apheresis include adverse consequences of large-needle vascular access; namely, retroperitoneal or pericardial hemorrhage, phlebotomy site aneurysm formation, pneumo- or hemothorax, thrombosis, nerve damage, and infection. A retrospective review of 381 TPE procedures at one institution reported an approximately 1% incidence of severe complications, all of them related to central venous catheters (see box on Patient Management Issues for Therapeutic Apheresis).

CONCLUSIONS AND FUTURE DIRECTIONS

Apheresis is now most commonly performed to obtain blood components for transfusion or plasma for fractionation from healthy donors. As a therapeutic intervention, apheresis is used to treat neurologic, renal, and hematologic disorders where removal of a pathogenic antibody or cell population is considered helpful as a primary or temporizing measure. While the field of apheresis science is shifting to an evidence-based approach, therapeutic apheresis is still utilized in the absence of much supporting evidence of efficacy. Effective pharmacologic agents have supplanted therapeutic apheresis for many disease indications. Increasingly, apheresis is being used to collect starting material for hematopoietic stem cell transplantation, cellular immunotherapies, and potentially curative gene therapies. Medical oversight and optimization of these autologous donor procedures is likely to play a key role in the future of apheresis in the years to come.

REFERENCES

1. Abel JJ, Rowntree LG, Turner BB. Plasma removal with return of corpuscles (plasmaphaeresis). The Journal of Pharmacology and experimental therapeutics Vol. V. No. 6, July, 1914. *Transfus Sci*. 1990;11(166).
2. Jones JM, Sapiano MRP, Savinkina AA, et al. Slowing in decline in blood collection and transfusion in the United States. 2017. *Transfusion*. 2020;60(Suppl 2):S1–S9.
3. Whitaker BL, Henry RA. *The 2011 National Blood Collection and Utilization Survey Report*. Washington, DC: US Department of Health and Human Services; 2011.
4. Ipe TS, Marques MB. Vascular access for therapeutic plasma exchange. *Transfusion*. 2018;58(Suppl 1):580–589.
5. Bolan CD, Cecco SA, Wesley RA, et al. Controlled study of citrate effects and response to i.v. calcium administration during allogeneic peripheral blood progenitor cell donation. *Transfusion*. 2002;42(7):935–946.
6. Schwartz J, Padmanabhan A, Aqui N, et al. Guidelines on the use of therapeutic apheresis in clinical practice—evidence-based approach from the Writing Committee of the American Society for Apheresis: the eighth special issue. *J Clin Apher*. 2019;34(3):171–354.
7. Agreda-Vasquez GP, Espinosa-Poblano I, Sanchez-Guerrero SA, et al. Starch and albumin mixture as replacement fluid in therapeutic plasma exchange is safe and effective. *J Clin Apher*. 2008;23(5):163–167.
8. Rock GA, Shumak KH, Buskard NA, et al. Comparison of plasma exchange with plasma infusion in the treatment of thrombotic thrombocytopenic purpura. Canadian Apheresis Study Group. *N Engl J Med*. 1991;325(6):393–397.
9. Nguyen L, Li X, Duvall D, et al. Twice-daily plasma exchange for patients with refractory thrombotic thrombocytopenic purpura: the experience of the Oklahoma Registry, 1989 through 2006. *Transfusion*. 2008;48(2):349–357.
10. Joly BS, Coppo P, Veyradier A. Thrombotic thrombocytopenic purpura. *Blood*. 2017;129(21):2836–2846.
11. Farrugia ME, Goodfellow JA. A practical approach to managing patients with myasthenia gravis-opinions and a review of the literature. *Front Neurol*. 2020;11:604.

12. Llufriu S, Castillo J, Blanco Y, et al. Plasma exchange for acute attacks of CNS demyelination: predictors of improvement at 6 months. *Neurology*. 2009;73(12):949–953.
13. Lefaucheur C, Nochy D, Andrade J, et al. Comparison of combination plasmapheresis/IVIg/anti-CD20 versus high-dose IVIg in the treatment of antibody-mediated rejection. *Am J Transplant*. 2009;9(5):1099–1107.
14. Akalin E, Dinavahi R, Friedlander R, et al. Addition of plasmapheresis decreases the incidence of acute antibody-mediated rejection in sensitized patients with strong donor-specific antibodies. *Clin J Am Soc Nephrol*. 2008;3(4):1160–1167.
15. Bruckert E. Recommendations for the management of patients with homozygous familial hypercholesterolaemia: overview of a new European Atherosclerosis Society consensus statement. *Atheroscler Suppl*. 2014;15(2):26–32.
16. Hudgins LC, Kleinman B, Scheuer A, et al. Long-term safety and efficacy of low-density lipoprotein apheresis in childhood for homozygous familial hypercholesterolemia. *Am J Cardiol*. 2008;102(9):1199–1204.
17. Kolansky DM, Cuchel M, Clark BJ, et al. Longitudinal evaluation and assessment of cardiovascular disease in patients with homozygous familial hypercholesterolemia. *Am J Cardiol*. 2008;102(11):1438–1443.
18. Howard J, Malfroy M, Llewelyn C, et al. The transfusion alternatives preoperatively in sickle cell disease (TAPS) study: a randomised, controlled, multicentre clinical trial. *Lancet*. 2013;381(9870):930–938.
19. Ziemba Y, Xu C, Fomani KM, et al. Safety and benefits of automated red cell depletion-exchange compared to standard exchange in patients with sickle cell disease undergoing chronic transfusion. *Transfusion*. 2021;61(2):526–536.
20. Rombout-Sestrienkova E, Nieman FH, Essers BA, et al. Erythrocytapheresis versus phlebotomy in the initial treatment of HFE hemochromatosis patients: results from a randomized trial. *Transfusion*. 2012;52(3):470–477.
21. Bewersdorf JP, Giri S, Tallman MS, Zeidan AM, Stahl M. Leukapheresis for the management of hyperleukocytosis in acute myeloid leukemia-A systematic review and meta-analysis. *Transfusion*. 2020;60(10):2360–2369.
22. Chang MC, Chen TY, Tang JL, et al. Leukapheresis and cranial irradiation in patients with hyperleukocytic acute myeloid leukemia: no impact on early mortality and intracranial hemorrhage. *Am J Hematol*. 2007;82(11):976–980.
23. Klamova H, Markova M, Moravcova J, et al. Response to treatment in women with chronic myeloid leukemia during pregnancy and after delivery. *Leuk Res*. 2009;33(11):1567–1569.
24. Naganuma M, Yokoyama Y, Motoya S, et al. CAPTAIN study Group: Efficacy of apheresis as maintenance therapy for patients with ulcerative colitis in an open-label prospective multicenter randomised controlled trial. *J Gastroenterol*. 2020;55(4):390–400.
25. Knobler R, Berlin G, Calzavara-Pinton P, et al. Guidelines on the use of extracorporeal photopheresis. *J Eur Acad Dermatol Venereol*. 2014;28 (Suppl 1):1–37.
26. Flowers ME, Apperley JF, van Besien K, et al. A multicenter prospective phase 2 randomized study of extracorporeal photopheresis for treatment of chronic graft-versus-host disease. *Blood*. 2008;112(7):2667–2674.
27. Jaksch P, Knobler R. ECP and solid organ transplantation. *Transfus Apher Sci*. 2014;50(3):358–362.
28. Kuzmina Z, Stroncek D, Pavletic SZ. Extracorporeal photopheresis as a therapy for autoimmune diseases. *J Clin Apher*. 2015;30:224–237.
29. Vasu S, Leitman SF, Tisdale JF, et al. Donor demographic and laboratory predictors of allogeneic peripheral blood stem cell mobilization in an ethnically diverse population. *Blood*. 2008;112(5):2092–2100.
30. Pulsipher MA, Chitphakdithai P, Logan BR, et al. Acute toxicities of unrelated bone marrow versus peripheral blood stem cell donation: results of a prospective trial from the National Marrow Donor Program. *Blood*. 2013;121(1):197–206.
31. Pulsipher MA, Chitphakdithai P, Logan BR, et al. Lower risk for serious adverse events and no increased risk for cancer after PBSC vs BM donation. *Blood*. 2014;123(23):3655–3663.
32. DiPersio JF, Micallef IN, Stiff PJ, et al. Phase III prospective randomized double-blind placebo-controlled trial of plerixafor plus granulocyte colony-stimulating factor compared with placebo plus granulocyte colony-stimulating factor for autologous stem-cell mobilization and transplantation for patients with non-Hodgkin's lymphoma. *J Clin Oncol*. 2009;27(28):4767–4773.
33. Drysdale CM, Nassehi T, Gamer J, Yapundich M, Tisdale JF, Uchida N. Hematopoietic-stem-cell-targeted gene-addition and gene-editing strategies for β-hemoglobinopathies. *Cell Stem Cell*. 2021;28(2):191–208.
34. Kim YA, Sloan SR. Pediatric therapeutic apheresis: rationale and indications for plasmapheresis, cytapheresis, extracorporeal photopheresis, and LDL apheresis. *Pediatr Clin North Am*. 2013;60:1569–1580.
35. Clayton JA, Vitale S, Kim J, et al. Prevalence of posterior subcapsular cataracts in volunteer cytapheresis donors. *Transfusion*. 2011;51(5): 921–928.

CHAPTER 117

TRANSFUSION REACTIONS TO BLOOD AND HEMATOPOIETIC STEM CELL THERAPY PRODUCTS

Martin R. Schipperus and Johanna C. Wiersum-Osselton

A *transfusion reaction* can be defined broadly as any adverse event that occurs in association with the infusion of a blood or cell therapy product. Transfusion reactions are classified by how close to transfusion they occur (timing), how much morbidity is caused (severity), how strong the causal association of the event is with transfusion (imputability), as well as by its fit to a (standardized) definition of a transfusion reaction type. Every year, approximately 40 fatalities definitely, probably, or possibly attributable to transfusion are reported to the US Food and Drugs Administration (FDA). Similar rates of transfusion-associated fatalities are found in the International Haemovigilance Network's surveillance database ISTARE (0.26 cases per 100,000 units transfused; 2006–2016) and emerge from data on fatalities as reported to the European Commission annually by member states (0.26 per 100,000 units in 2017).

Stopping the transfusion at the first sign of a transfusion reaction, usually fever, is critical for preventing severe sequelae. Although the great majority of febrile reactions to blood transfusion are not caused by blood incompatibility, it is impossible to exclude this possibility at the bedside. All transfusion reactions need to be reported to the blood transfusion laboratory to exclude incompatibility.

It is important to recognize that many transfusion reactions can mimic pathology unrelated to transfusion. The differential diagnosis of any untoward clinical event should always consider adverse sequelae of transfusion, even when transfusion occurred weeks earlier. This chapter reviews the presentation, mechanisms, and management of transfusion reactions (Table 117.1). Approximate risks of selected transfusion reactions are shown in Fig. 117.1.

TABLE 117.1 Types of Acute Transfusion Reactions

Reaction Type	Presenting Signs and Symptoms
Acute hemolytic	Fever, chills, dyspnea, vomiting, hypotension, tachycardia, infusion site pain, back pain, hemoglobinuria, hemoglobinemia, indirect hyperbilirubinemia, renal failure, disseminated intravascular coagulation
Febrile reaction	Fever, chills, rigors
Allergic	Urticaria, pruritus, flushing, angioedema, dyspnea, bronchospasm, stridor, hypotension, tachycardia, abdominal cramping
Hypervolemic	Dyspnea, tachycardia, hypertension, headache, jugular venous distention
Septic	Fever, chills, hypotension, tachycardia, vomiting
Transfusion-related acute lung injury	Dyspnea, hypoxemia, fever, hypotension

HEMOLYTIC TRANSFUSION REACTIONS

Hemolytic transfusion reactions are caused by the immune-mediated clearance of transfused incompatible red blood cells (RBCs). Immune-mediated hemolysis can be classified clinically according to the timing of the reaction (acute or delayed), as caused by either ABO or non-ABO incompatibility, and mechanistically by the site of hemolysis: intravascular with terminal complement activation or extravascular with phagocytosis in liver and spleen (Table 117.2).

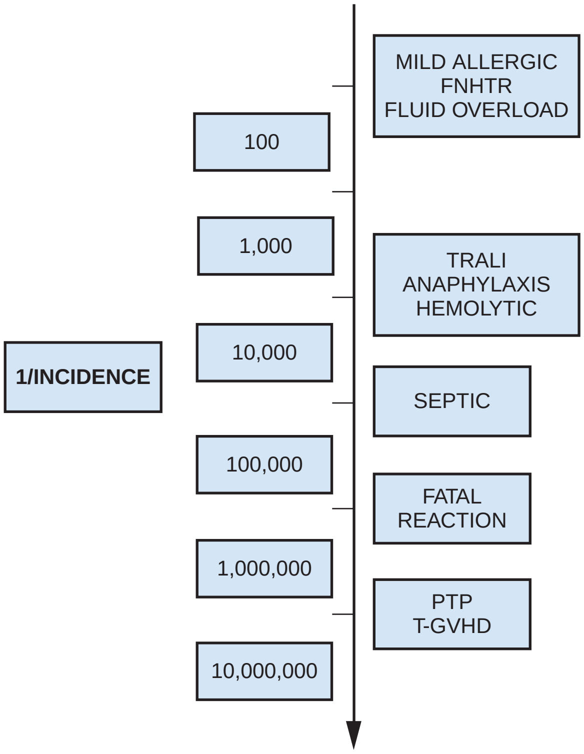


Figure 117.1 APPROXIMATE RISK OF VARIOUS TRANSFUSION COMPLICATIONS. *FNHTR*, Febrile nonhemolytic transfusion reaction; *PTP*, posttransfusion purpura; *t-GVHD*, transfusion-associated graft-versus-host disease; *TRALI*, transfusion-related acute lung injury.

TABLE 117.2 Hemolytic Transfusion Reactions: Serologic Presentation

Type	Antibody Detectable Initially	Primary Antibody Type	Degree of Complement Binding	Example
Acute intravascular	Yes	IgM	Full (C1–9)	ABO system
Acute extravascular	Yes	IgG	None/partial	Rh system
Delayed intravascular	No	IgG	Full (C1–9)	Kidd system
Delayed extravascular	No	IgG	None/partial	Duffy system

Although hemolytic transfusion reactions are mechanistically considered immune-mediated in most cases, thermal, osmotic, infectious, and mechanical destruction of RBCs also can lead to acute hemolysis.

ACUTE INTRAVASCULAR HEMOLYTIC TRANSFUSION REACTIONS

Pathophysiology

Acute hemolytic reactions are those that occur during or within 24 hours, typically after incompatible RBCs are transfused into a patient with preexisting antibodies. A reaction caused by a naturally occurring antibody against ABO-incompatible RBC is the prototypical example of an acute, intravascular hemolytic transfusion reaction. ABO antibodies are naturally occurring immunoglobulin IgM and less commonly IgG antibodies to foreign A and B blood group antigens. IgM antibodies efficiently fix and activate complement after binding to ABO-incompatible RBCs. The formation of excessive terminal membrane attack complexes consisting of complement components C5 through C9 creates multiple pores in the transfused RBC membranes, resulting in a clinically significant acute intravascular hemolytic transfusion reaction. Such a reaction could occur, for example, after transfusion of A RBCs into an O recipient who has significant amounts of circulating anti-A (see box on Acute Hemolytic Transfusion Reaction). The excess cell-free hemoglobin that is generated supersaturates the binding capacity of plasma albumin, haptoglobin, and hemopexin and manifests by hemoglobinemia and hemoglobinuria. Free heme induces renal vasoconstriction through nitric oxide scavenging. This may eventually result in acute tubular necrosis and renal failure. In IgM-mediated ABO-incompatible transfusion reactions, activation of the complement cascade generates anaphylatoxins C3a and C5a, leading to capillary leak, hypotension, and phagocyte and mast cell activation. Furthermore, the deposition of C3b on the RBC membrane increases extravascular hemolysis.

In addition to complement components, cytokines also play a role in the clinical syndrome of hemolysis and fever. For example, interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α have pyrogenic activity; IL-8 is a neutrophil chemotactic and activating factor. These four cytokines have been generated in various in vitro models of intravascular hemolysis and IgG-mediated RBC incompatibility. TNF-α induces tissue factor expression on endothelium while decreasing thrombomodulin, which contributes to disseminated intravascular coagulation (DIC). TNF-α also promotes endothelin production, promoting renal vasoconstriction. The clinical variability of hemolytic transfusion reactions is explained in part by the relative balance of cytokine production in the transfusion recipient. Factors that increase the circulating levels of proinflammatory cytokines and chemokines often result in more severe reactions. Factors that determine the severity of hemolysis include the antibody titer, antibody avidity, antibody subtype, antigen density on the RBC membrane, and volume and infusion rate of incompatible blood. Patient factors also play an important role. Transfusing as little as 30 mL of incompatible RBC concentrate can be fatal, and there is a direct relationship between increasing volumes of incompatible blood transfused and mortality. Conversely, there may be no reaction at all in an elderly or immune-compromised patient, or in the setting of massive transfusion.

Acute hemolytic reactions can also occur with incompatible plasma transfusion. Because of limited platelet inventories, platelet components with incompatible plasma are frequently transfused, for example, a group O platelet concentrate (with anti-A) transfused to a group A recipient. This plasma incompatibility (i.e., minor incompatibility) can occasionally result in acute ABO-incompatibility hemolytic reactions.

ACUTE HEMOLYTIC TRANSFUSION REACTION

A 15-year-old blood group O male with sickle cell disease presents for routine simple transfusion for primary prevention of stroke. He has no history of red blood cell (RBC) alloimmunization and his pretransfusion compatibility testing shows a negative screen for RBC alloantibodies. Seven minutes into the transfusion, the patient reports not feeling well. He quickly develops chills, abdominal pain, flank pain, and pain at the infusion site. The transfusion is stopped. Vital signs show a 15 mm Hg drop in systolic blood pressure from baseline value, pulse of 130, and a temperature increase from afebrile pretransfusion to 38.9°C. Gross hematuria is seen in a subsequent urine sample. Reinspection of the blood unit shows that it is group A and labeled with the name of the child receiving blood in the infusion chair next to him. The patient is transferred to the emergency room where he is evaluated for renal failure and diffuse intravascular coagulation (DIC).

Clinical Manifestations and Laboratory Testing

An acute intravascular hemolytic transfusion reaction is a medical emergency. Initial clinical symptoms can include fever and chills, shortness of breath, chest pain, dizziness, reddish urine, back or flank pain, hypotension, and oliguria. Some patients report feeling anxiety or pain or warmth ascending from the site of infusion. RBC transfusions must be stopped and the patient evaluated clinically and by laboratory testing if fever develops (≥2°C increase in temperature). The transfused incompatible RBCs undergo complement-mediated lysis, producing hemoglobinemia and hemoglobinuria. Cardinal signs of an acute intravascular hemolytic transfusion reaction are the presence of red plasma (hemoglobinemia) and red/dark urine (hemoglobinuria). Acute transfusion reactions can quickly progress to shock and acute renal failure. Many patients, curiously even anephric patients, often complain of lower back pain. It is speculated that this symptom is caused by ischemic muscle pain or vasospasm rather than by kidney pain from developing renal failure.

When an acute hemolytic transfusion is suspected, the transfusion should be stopped immediately and clerical checks performed to check ABO and Rh compatibility and verify that the correct unit is being transfused to the intended recipient. If it is confirmed that the transfusion was ABO-incompatible, it is important to investigate where an error occurred. If there has been a mix-up of samples or units, could a second patient be receiving an incorrect unit? There may be a systemic error that could put other patients at risk.

Laboratory findings confirming hemolysis include hemoglobinuria, hemoglobinemia, and a haptoglobin level that is low to undetectable. During the hemolytic episode, the bilirubin (especially indirect bilirubin) usually increases only modestly (2 to 3 mg/dL) if the patient has normal liver function. Because of the lysis of RBCs, levels of lactate dehydrogenase (LDH) may rise markedly. The direct antiglobulin test (DAT) becomes positive in an immune hemolytic reaction (if tested before all the incompatible RBCs are destroyed). Preparation of an antibody eluate is often necessary to identify the presence of an offending IgG antibody. Elution is a procedure that

WORKUP OF AN ACUTE INTRAVASCULAR HEMOLYTIC TRANSFUSION REACTION

If an acute transfusion reaction occurs:

1. Stop blood component infusion immediately.
2. Maintain intravenous access with a suitable crystalloid or colloid solution.
3. Maintain an adequate airway.
4. Monitor/maintain blood pressure and heart rate. Monitor renal status (blood urea nitrogen, creatinine, volume status).
5. Give a diuretic or institute fluid diuresis, or both.
6. Evidence is lacking for the use of corticosteroids and this is therefore not recommended.
7. Obtain blood and urine studies for the transfusion reaction workup.
8. Transfusion laboratory workup of suspected transfusion reaction:
    - Check paperwork and identification to ensure the correct blood component was transfused to the correct patient.
    - Observe plasma for hemoglobinemia.
    - Perform direct antiglobulin test.
    - Repeat compatibility testing (crossmatch).
    - Repeat other serologic testing as needed (ABO, Rh).
    - Analyze urine for hemoglobinuria.
9. Monitor coagulation status (prothrombin time, activated partial thromboplastin time, fibrinogen).
10. Monitor for signs of hemolysis (lactate dehydrogenase, bilirubin-total/direct, haptoglobin).

chemically separates the bound antibody from the RBCs and concentrates it so that it may be identified.

Initial therapy consists of immediately stopping the transfusion, administering intravenous fluids, cardiorespiratory support, and ensuring a brisk diuresis. Increasing renal blood flow is the best way to prevent acute oliguric renal failure. Usually, 0.9% NaCl is infused to maintain a urine output of 100 mL/h for approximately 24 hours. Diuresis can be achieved with loop diuretics or mannitol, but this is not evidence based. The mechanisms responsible for the beneficial effect of increased renal blood flow likely include increased clearance of free hemoglobin and a return of more physiologic control of renal vasodilation. Creatinine and blood urea nitrogen (BUN) should be monitored; dialysis may be necessary for the treatment of oliguric acute renal failure. Support of blood pressure and respiration may require the use of vasopressors, bronchodilators, or intubation. DIC can occur in severe cases. The prothrombin time, activated partial thromboplastin time, and fibrinogen level should be monitored (see box on Workup of an Acute Intravascular Hemolytic Transfusion Reaction). If the patient shows no signs of cardiovascular instability and if hemostatic and renal function are unchanged at least 24 hours after the incompatible transfusion, the episode can be considered to be over, with serious sequelae unlikely.

ACUTE EXTRAVASCULAR HEMOLYTIC TRANSFUSION REACTIONS

In an extravascular hemolytic transfusion reaction, complement is either not fixed at all or is fixed only to C3b. In either situation, because of the nature of the antigen-antibody reaction, complement activation with fixation of the C5b-9 complex does not occur. This presentation is commonly associated with Rh antibodies but can be seen with any number of non-ABO antigen-antibody complexes. The presence of IgG bound to the RBCs or C3b fixation results in an extravascular reaction because the antibody-coated cells are cleared by IgG receptors in the spleen or C3b receptors in the liver. In these circumstances, RBC lysis does not occur in the intravascular space. Because of the lack of generation of C3a or C5a, an extravascular hemolytic transfusion reaction does not usually present as a clinical emergency. It is characterized by a positive DAT caused by recipient RBC alloantibodies binding to the incompatible circulating donor RBCs. Moreover, an increase in indirect bilirubin, an increase in LDH, a decrease in hematocrit, a decrease in haptoglobin, and an increase in colorless urine urobilinogen can occur, but hemoglobinuria and hemoglobinemia are rarely present. The patient typically remains clinically stable. Renal failure, shock, and hemostatic abnormalities, such as DIC, are rarely seen unless the amount of incompatible blood infused is excessive. However, patients often have a low-grade fever.

When an extravascular hemolytic transfusion reaction is suspected, the diagnostic test of choice is a DAT with an eluate. The eluate is performed to identify the antibody coating the RBCs. The positive DAT result reflects the patient's antibody (or antibodies) coating the incompatible donor RBCs. Because this is not an autoantibody, the patient's own RBCs are not involved in the reaction.

Typically an acute extravascular hemolytic transfusion reaction requires no special therapeutic intervention if the volume of incompatible blood transfused is relatively low. The patient characteristically recovers in a few days as the incompatible donor RBCs are cleared from the circulation. If the volume of incompatible blood transfused was high, hemolysis can quickly lead to anemia through destruction of the transfused RBCs. Communication with the transfusion laboratory is key to identifying how many units of incompatible units were transfused.

Extravascular acute reactions may occur if the patient's preexisting alloantibody was missed by the blood transfusion laboratory during the antibody screening process. If a wrongly labeled sample was used, if the unit of blood was labeled for the wrong patient, or if the unit was transfused in the wrong patient, an extravascular acute reaction is a possible consequence.

Hyperhemolysis

Hyperhemolysis is a specific type of acute intravascular hemolysis of (autologous) bystander RBCs that do not express the antigen to which an immune-mediated hemolysis is directed.

Hyperhemolysis occurs primarily with transfusion in the setting of sickle cell disease and is also reported to occur in acute malarial infection, passenger lymphocyte syndrome, paroxysmal nocturnal hemoglobinuria, and select cases of autoimmune hemolytic anemia. Since hyperhemolysis is frequently associated with sickle cell disease, Petz and colleagues proposed the term *sickle cell hemolytic transfusion reaction syndrome* to describe the constellation of hemolysis, sickle cell pain crisis, reticulocytopenia, severe anemia, RBC transfusion leading to accelerated hemolysis, and lack of a clear serologic reason for hemolysis. Hyperhemolysis is frequently fatal because transfusion may exacerbate hemolysis and the primary treatment for severe anemia (RBC transfusion) makes anemia worse. Recognition of this syndrome is therefore critical because treatment should shift from transfusion to administering erythropoietin, glucocorticoids, intravenous immunoglobulin (IVIG), and rituximab, which have been used successfully in case series.

DELAYED HEMOLYTIC REACTIONS

The pathogenesis of delayed hemolytic transfusion reactions (DHTR) is similar to that described for acute hemolytic reactions. However, in DHTRs, the patient develops hemolysis 3 to 10 days after the transfusion as an anamnestic antibody response to a blood antigen previously known to the patient's immune system through transfusion, pregnancy, or hematopoietic stem cell transplantation (HSCT). Delayed hemolytic reactions are slower in onset than acute reactions and are less likely to present as a clinical emergency. Hemoglobinuria and hemoglobinemia can occur but are less pronounced than with an acute intravascular reaction. This is probably because of the gradual increase in antibodies, as well as the fact that most DHTRs are caused by antibodies not efficient at activating complement. The need for intervention is much less likely than with an acute hemolytic transfusion reaction, but hematologic and renal monitoring are prudent.

DHTRs are the most common presentation of transfusion-associated immune hemolysis. DHTRs often involve the Rh system. Patients present with a fever, a falling hemoglobin level, and the development of a positive DAT with an eluate demonstrating a new RBC alloantibody. Because these reactions are typically mild in nature, they are usually addressed with supportive care only. In patients with sickle cell disease, DHTRs can precipitate vaso-occlusive crises, autoantibody production, or hyperhemolysis. It is essential to take a transfusion history in people with sickle cell disease who present with new complications.

One final note regarding the serologic evaluation of a transfusion reaction: posttransfusion testing may be complicated and difficult to interpret because of the possibility of autoantibodies or the involvement of medications. In such circumstances, testing on the pretransfusion specimen is often helpful. In cases of more complex evaluations, consultation with an expert in immunohematology is recommended to detect and identify new alloantibodies in the patient's plasma, which may be responsible for a hemolytic transfusion reaction.

FEBRILE NONHEMOLYTIC TRANSFUSION REACTIONS

A febrile nonhemolytic transfusion reaction (FNHTR) is suspected if there is a rise in temperature of ≥1°C (with or without rigors/chills) during or within 4 hours following transfusion without any other cause such as hemolytic transfusion reaction, bacterial contamination, or underlying condition, with normalization within 24 hours after the transfusion. Cases with a rise in temperature ≥1°C (<2°C), without other relevant symptoms or signs are considered to be a mild (nonhemolytic) febrile reaction. The most serious FNHTR, with fever (≥39°C oral or equivalent and a change of ≥2°C from pretransfusion value) and chills/rigors, should always be adequately investigated. FNHTR could be present in the absence of fever (if chills or rigors without fever).

Evidence supports two mechanisms of FNHTR: antileukocyte antibodies and cytokines. Cytotoxic or agglutinating antibodies having human leukocyte antigen (HLA) specificity, neutrophil specificity, or platelet specificity may be present in the recipient's plasma and react against antigens present on transfused donor lymphocytes, granulocytes, or platelets. Conversely, donor plasma may contain the offending antibody that can react with the corresponding cellular antigens in the recipient's blood. Leukocyte-derived cytokines IL-8, IL-1β, and IL-6 accumulate in platelet products in particular and induce fever. IL-1, through prostaglandin PGE_2 synthesis, is thought to stimulate the thermoregulatory center of the hypothalamus to produce fever. Other mediators such as macrophage inflammatory proteins (e.g., MIP-1) may also participate in the febrile response, but this reaction is not mediated through prostaglandin synthesis. CD154 (CD40 ligand) derived from platelets is also involved in febrile reactions by inducing cyclooxygenase-2 and PGE_2. Leukoreduction should theoretically lead to a reduction of leukocyte-derived cytokines in the blood product but appears to be less effective at preventing FNHTRs to platelets than to red cell components because of platelet-derived biologic response modifiers. Nevertheless, studies show that the generation of cytokines during storage is directly proportional to the leukocyte count of the unit and the duration of storage. Implementation of prestorage leukoreduction has been shown to reduce the incidence of FNHTR.

The frequency of febrile reactions for a nonleukoreduced unit has been estimated to be 6.8 per 100 units RBCs transfused and 2.2 per 100 units platelet transfused. With the advent of prestorage leukoreduction, these risks have been decreased to about 0.09 to 1.1 per 100 units RBCs transfused and 0.04 to 1.56 per 100 platelet units transfused. Reactions are most commonly seen in recipients who have been exposed to multiple white cell or platelet antigens. Patients with bone marrow failure (primary or chemotherapy-induced) are at risk as a result of frequent transfusions, as are multiparous women who may have received multiple exposures during pregnancy and childbirth. These groups of patients can form multiple HLA-, granulocyte-, or platelet-specific antibodies that will react with white cells or platelets upon subsequent exposure.

The workup of a febrile reaction must be undertaken promptly because fever may also be the first sign of other, more severe reactions, including acute hemolysis or sepsis. A hemolytic transfusion reaction may be ruled out by reconfirming the ABO and Rh type of the patient and the donor unit, performing antiglobulin crossmatching to confirm patient-donor compatibility, evaluating the results of the pretransfusion and posttransfusion DATs, evaluating the serum for hemolysis, and rechecking the accuracy of paperwork. The posttransfusion DAT should yield negative findings because FNHTRs do not involve RBC alloantibodies.

As laboratory testing is being completed, the workup should include bedside patient evaluation. Fever and chills may be attributable to drugs or underlying diseases, or they may be associated with infection or inflammation. Neutropenic fever often complicates the clinical picture in patients undergoing intensive, myelosuppressive chemotherapy, a population of patients likely to receive repeated RBC or platelet transfusions. Blood cultures of the patient and the blood product should be considered, especially if the patient has high fever or shows signs of sepsis (see later text and box for a more in-depth discussion of suspected posttransfusion bacteremia/sepsis). Routinely, blood cultures and microbiological cultures of the blood product are ordered in order to differentiate an FNHTR from posttransfusion bacteremia that may be a reaction to a bacterially contaminated blood product or be associated with an infection in the patient. It is not routine to investigate for (or identify the specificities of) HLA, platelet, or granulocyte antibodies that could cause FNHTRs. Accordingly, the diagnosis of an FNHTR is usually made as a diagnosis of exclusion without isolating an identifiable antibody.

ALLERGIC TRANSFUSION REACTIONS

Allergic transfusion reactions complicate up to 3% of all transfusions. The allergic manifestations occur on a spectrum of severity. They can include flushing, urticaria, pruritus, angioedema, hypotension, bronchospasm, stridor, abdominal pain, and emesis. An anaphylactic reaction is a systemic immediate hypersensitivity reaction, which can be defined as allergic signs and symptoms in skin/mucosa and at least one other organ system (cardiovascular, respiratory, gastrointestinal). Shock is the most ominous manifestation of anaphylaxis, but bronchospasm and upper airway angioedema are more common manifestations (see box titled A Severe Allergic Transfusion Reaction).

Allergic transfusion reactions manifest similarly to other IgE-mediated, immediate hypersensitivity reactions. Mast cells are the primary allergic effector cells for immediate hypersensitivity reactions; basophils may play a secondary role. Mast cells and basophils can be activated by cross-linking cell surface high-affinity IgE receptors or via IgE-independent mechanisms, such as complement receptor binding by C5a. Upon activation, histamine is released immediately, as it is stored preformed in granules. Platelet-activating factor, a

A SEVERE ALLERGIC TRANSFUSION REACTION

A 34-year-old man with thrombotic thrombocytopenic purpura has been receiving daily 60 mg prednisone and daily plasmapheresis for 8 days without complication. Halfway through the ninth procedure, he rapidly develops severe angioedema of the face and tongue, stridor, wheezing, diffuse flushing, and urticaria. Tachycardia develops with stable blood pressure. The urgent intervention team is called and the patient is administered oxygen, epinephrine intramuscularly, albuterol nebulizer, diphenhydramine, ranitidine, and methylprednisolone. Symptoms resolve over the next 12 h without the need for escalated respiratory support. The patient receives another 3 weeks of plasma exchanges with two sporadic minor allergic reactions (focal urticaria and pruritus only).

potent arachidonic acid derivative that mediates anaphylactic shock, is produced rapidly, along with a variety of other eicosanoids, for example, leukotrienes.

Generally, allergic transfusion reactions are diagnosed clinically. The incidence is associated with the plasma content of the product, so it is thought that a plasma protein is responsible for many reactions.

Following a severe anaphylactic reaction, if relevant, an allergic mechanism can be confirmed by measuring a rise in serum tryptase. Further investigations may be performed to establish the trigger although most anaphylactic transfusion reactions remain idiopathic. Examples of IgG or IgE with specificity to IgA, haptoglobin, and C4 have been described. Case reports describe moderate or severe anaphylactic reactions in patients who are severely IgA deficient (<0.05 mg/dL) and have anti-IgA antibodies. However, the generalizability of this mechanism is low. Most cases of fatal anaphylaxis are not related to IgA deficiency, and most severely IgA-deficient people tolerate transfusions well. Thus, patients with incidental IgA deficiency may receive routine blood components, and IgA/anti-IgA testing should be reserved for patients with anaphylactic reactions. Quantitative haptoglobin can also be considered as a screening test particularly in Asian populations, as rare cases of haptoglobin deficiency are associated with anaphylactic reactions.

There are several reports of allergic transfusion reactions to autologous transfusion, suggesting that a storage lesion or conceivably a substance from the bag may be responsible for some reactions. Passively transferred IgE combined with allergen exposure in the recipient is a mechanism that has been described for food and antibiotic-mediated allergic transfusion reactions, but these are uncommon.

Over 90% of allergic transfusion reactions occur during infusion. A severe allergic (anaphylactic) transfusion reaction is a medical emergency and should be treated promptly as described in the box above. However, the majority of allergic transfusion reactions are milder. When allergic symptoms develop, transfusion should be stopped and the patient given an antihistamine like clemastine. The transfusion may resume, but only if the symptoms resolve and the patient feels well. A mild allergic reaction (urticaria and pruritus) during a blood transfusion usually does not progress to a more severe anaphylactic reaction after infusion of additional blood from the same unit. The severity of allergic transfusion reactions is not directly related to volume infused or infusion rate.

Even among the minority of patients with recurrent reactions, most transfusions are tolerated well. Patients who have had more than one mild allergic reaction may continue to receive routine units. Washed RBCs or plasma-reduced platelets can be used to prevent severe, recurrent reactions; however, washing cellular products compromises component quality. Washing RBCs leads to accelerated in vitro hemolysis, while washing platelets increases platelet activation and lowers posttransfusion platelet count increments. Platelets produced with platelet additive solution as storage medium and pooled, solvent detergent plasma are relatively new products that have been shown to be associated with a lower incidence of allergic transfusion reactions. Leukocyte depletion or the use of microaggregate filters does not reduce allergic transfusion reactions.

There is no evidence that antihistamine premedication prevents allergic transfusion reactions, although antihistamines do mitigate symptoms when they occur. Studies in healthy volunteers support a synergistic role for treating histamine-mediated reactions with both H1 and H2 receptor antagonists, for example, diphenhydramine and ranitidine, respectively. Corticosteroids, provided in advance of a transfusion, also may be useful in patients with serious recurrent reactions.

TRANSFUSION-RELATED ACUTE LUNG INJURY

Transfusion-related acute lung injury (TRALI) is an important pulmonary complication of blood transfusion in which inflammatory pulmonary edema develops within 6 hours of transfusion. Symptoms of TRALI range from mild dyspnea to severe noncardiogenic pulmonary edema, with features that include dyspnea, oxygen desaturation, respiratory failure, chills, fever, and hypotension. Most patients require oxygen support, and many require mechanical ventilation. TRALI is one of the leading causes of transfusion-related death reported to hemovigilance systems—the others being transfusion-associated circulatory overload (TACO), acute hemolytic transfusion reactions, or transfusion-transmitted bacterial infections (TTBIs), depending on the setting.

It is postulated that TRALI consists of a “two-hit” event, the first “hit” being an underlying clinical condition that leads to the sequestration and priming of neutrophils in the lung tissue. In some cases of TRALI, referred to as immune TRALI, the second hit consists of the transfusion of blood products containing anti-HLA or anti-HNA (human neutrophil antigen) antibodies to cognate antigens in the patient that activate the neutrophils in the lung parenchyma, leading to edema. Complement and monocyte activation with the aggregation of white blood cells also may occur when leukoagglutinins present in the recipient react with leukocytes contained in the infused donor blood. As a result of the leukocyte antigen-antibody reaction, the activated leukocytes express adhesive molecules on their surface (CD11/CD18), which then permit leukocytes to attach to pulmonary endothelial cells and migrate to the interstitial space between the pulmonary capillaries and the alveolar epithelium. Once in the interstitial space, neutrophils degranulate and through enzymatic digestion produce capillary dehiscence that results in fluid filling the alveolar sacs. Pulmonary leukostasis with pulmonary edema thus occurs as a result of microvascular occlusion and capillary leakage. Complement-activated granulocytes also produce oxygen radicals that damage pulmonary endothelial cells, resulting in a further increase in pulmonary vascular permeability and additional passage of fluid into alveolar spaces.

Other cases of TRALI are non-immune, and in these cases, the second hit is believed to arise from the presence in stored blood components of biological response modifiers such as bioactive lipids and soluble mediators, such as CD40L. Rodent model systems for TRALI have also been described, and some of these models suggest a role for platelets in TRALI. Further research is necessary to elucidate the different mechanisms leading to this syndrome, which may be a final common pathway from a variety of initiating insults.

In a typical case of TRALI, the patient becomes acutely dyspneic or shows decreased oxygen saturation during or up to 6 hours after transfusion. Physical examination reveals findings (crackles) of pulmonary oedema but without raised jugular venous pressure or elevation in cardiopulmonary pressures. The chest radiograph typically shows a pulmonary edema pattern with bilateral infiltrates but should not show vascular congestion. Copious amounts of fluid are produced in the lungs, and there may be frothy sputum. The alveolar oedema in TRALI is an inflammatory exudate as opposed to the transudate of cardiogenic oedema. TRALI usually follows infusion of relatively small volumes of blood, whereas fluid overload more often arises from larger volume transfusions. TRALI can be hard to distinguish from fluid overload without central cardiovascular pressure measurements. When a patient shows signs of increasing respiratory effort, the infusion should be immediately stopped, as it should be with all other reactions. Treatment of patients with TRALI is supportive (Table 117.3).

Because of the role of antileukocyte antibodies in triggering TRALI, these cases are reported to the blood establishment to allow for testing to identify donors with antileukocyte antibodies to the cognate antigens in the affected patient (the donor is then “implicated”) and their permanent deferral from blood donation. It should however be noted that anti-HLA antibodies found in such testing may match a frequent HLA type, and the finding of antibodies could have been coincidental. Prevention of TRALI has focused on providing blood components without anti-HLA. Antileukocyte antibodies are most likely present in blood donors who are (multi)parous women so many blood services now supply plasma for transfusion from male donors without a history of blood transfusion, and in some cases also from female donors who have tested negative for anti-HLA antibodies. Using male-only plasma started in the 2000s and led to a reduction in reported TRALI cases by about a third.

TABLE 117.3 Transfusion-Related Acute Lung Injury

Onset	Within 6h of the start of transfusion, usually within 2h
Frequency	1:10,000 transfusions
Signs and symptoms	Hypoxemia, fever, hypotension, tachypnea, dyspnea, diffuse pulmonary edema
Pathogenesis	HLA/granulocyte-specific antibodies (usually of donor origin) reacting with recipient leukocytes or directly with endothelium. Lysophosphatidylcholines that accumulate during component storage can activate neutrophils in an antibody-independent manner.
Diagnosis	Chest radiograph; blood gases; post/pretransfusion NT-BNP ratio <1.5; blood for HLA or antineutrophil antibodies
Differential diagnosis	Fluid overload; septic transfusion; anaphylaxis; unrelated acute lung injury
Treatment	Stop transfusion. Provide respiratory support (administer O_2, intubation may be needed), support blood pressure, no evidence supporting glucocorticoids or diuretics

HLA, Human leukocyte antigen; NT-*BNP*, N-terminal brain-natriuretic peptide.

The original TRALI case definition was developed in a consensus conference (Toronto, 2004). Subsequent research, both on this transfusion complication and on the acute respiratory distress syndrome (ARDS) has led a group of clinically based researchers to propose a redefinition incorporating newer insights on TRALI and ARDS (2019). Notably, there is an updated Berlin ARDS definition (2012) in which the former term of acute lung injury has been dropped outside the transfusion setting in favor of mild ARDS. For cases following transfusion, the group recommends classification as TRALI type I or type II based on the absence or presence of recognized risk factors for ARDS. In Type II TRALI, even if (mild) ARDS was already present, the patient can still be diagnosed with (Type II) TRALI if their respiratory status was stable for 12 hours before transfusion. Cases may also be classified as TRALI/TACO if it is impossible to distinguish between the two types of complication or both types of complication are present simultaneously. Validation of the revised TRALI definition for use in clinical and hemovigilance settings is awaited.

TRANSFUSION-ASSOCIATED CIRCULATORY OVERLOAD

TACO results from hydrostatic transudate accumulation in the lungs and should be considered in patients who, during or within 6 to 12 hours after a blood infusion, develop dyspnea, jugular venous distension, tachycardia, or other signs of congestive heart failure and fluid overload. An increase in blood pressure and widened pulse pressure may be observed.

Hemovigilance systems find that TACO is the leading cause of reported transfusion-related serious morbidity and mortality and particularly occurs in association with RBC transfusion. It is likely that symptoms of incipient circulatory overload in transfused patients often respond to extra diuretic therapy and go unreported.

TACO Risk Factors and TACO Prevention

Acute expansion of a (non-bleeding) patient's intravascular volume may exceed the capacity of the cardiovascular and renal systems to compensate, resulting in fluid overload. Risk factors for the development of TACO are compromised cardiopulmonary status, significant renal impairment, small body weight—infants, the elderly—or clinically significant preexisting positive fluid balance. If a patient is judged to be at increased risk, preventive measures should be considered (see box on TACO Preventive Measures).

TACO PREVENTIVE MEASURES

Transfuse a Single Unit and Review

State speed of infusion on the blood order (prescription), e.g., 1 mL/kg/h in patients at increased risk and otherwise 2–3 mL/kg/h in non-bleeding patients.

Consider prophylactic loop diuretic before start of transfusion e.g., 20–40 mg furosemide IV.

If TACO is suspected, the transfusion should be stopped and the patient's blood volume reduced by diuretics and oxygen or additional support given as indicated. Careful monitoring of a transfused patient's vital signs during transfusion may allow treatment to be instated before the patient progresses to full-blown TACO with respiratory compromise.

TACO, Transfusion-associated circulatory overload.

The initial stages of TACO may be difficult to distinguish from hemolytic transfusion reaction, FNHTR, allergic reaction, or TRALI. The absence of hemoglobinuria and hemoglobinemia and the absence of a positive posttransfusion DAT result distinguish the reaction from one caused by immune hemolysis. The presence of fever and chills is more typical in septic transfusion reactions, hemolysis, or febrile transfusion reactions; but new fever has been reported in up to a third of TACO cases. Radiographic imaging may support the diagnosis by showing a widened cardiac silhouette and vascular pedicle and changes of congestive pulmonary oedema; however, it can be challenging to distinguish between TACO and TRALI or infectious infiltrates. Blood levels of brain-type natriuretic peptide (BNP) or its precursor N-terminal pro-brain-natriuretic peptide (NT-proBNP), which are increased in left atrial strain, may aid in the diagnosis if NT-proBNP is at least 50% higher posttransfusion than pretransfusion.

POSTTRANSFUSION INFECTIONS AND BACTERIAL CONTAMINATION

In past decades, notably in the 1980s, public concern and scandal have focused on the occurrence of transfusion-transmitted infections (TTIs). Since that period, testing strategies, processing methods, and pathogen reduction technologies have contributed to large reductions of the infectious hazards. Nevertheless, it is important to remain aware of the possibility of bacterial, viral, or other TTI. A report can be classified as a TTI if:

- The recipient(s) had evidence of infection post transfusion with blood components, and there was no evidence of infection prior to transfusion, and no evidence of an alternative source of infection is found, and, either:
- At least one component received by the infected recipient(s) was donated by a donor who had evidence of the same transmissible infection or:
- At least one component received by the infected recipient was shown to contain the agent of infection

Posttransfusion Bacteremia/Sepsis

A posttransfusion bacteremia/sepsis is defined by clinical symptoms of bacteremia/sepsis arising during, directly after, or some time subsequent to a blood transfusion, with a relevant positive patient blood culture result; a causal link to a transfused blood component may or may not be confirmed through a finding of the same bacterial species in the component or other material from the donor.

When no signs or symptoms of a bacteremia or sepsis are present in the patient, but a positive culture of the blood product is found, this could be reported as bacterial contamination of a blood component as

SUSPECTED POSTTRANSFUSION BACTEREMIA/SEPSIS

A 29-year-old woman in week 38 of pregnancy received a unit of apheresis platelets prophylactically in a clinic for a chronic bone marrow failure syndrome of unclear etiology. During the infusion, she developed flank pain and coughing. The transfusion was stopped and the patient was admitted. She became febrile to 39.3°C 90 min after transfusion. Blood cultures from the patient and bag grew *Staphylococcus aureus* within 12 h.

Blood transfusion is common in people with bone marrow failure, either primary or secondary to intensive myelosuppressive chemotherapy. These patients are also often neutropenic and may have central venous catheters or take immunosuppressive medications. Despite these underlying risk factors, transfusion should always be considered a potential source of bacteremia. If there is a suspicion that blood components may be the source of infection it is critical to inform the blood establishment in order for other components manufactured from the same collection(s) to be blocked or recalled for investigation. Yomtovian and colleagues prospectively cultured all platelets issued from a large hospital blood bank and found contaminated units similar to other reported rates (~1:2000). When they relied on passive reporting of septic reactions from clinicians, the incidence fell to zero, only to increase back to baseline once active culturing of platelets resumed.

collected by some hemovigilance systems. Bacterial contamination is defined by the presence of relevant numbers of bacteria in a (remnant of) blood component or in the bacterial screen bottle of a platelet component, or in material from the same donation, demonstrated in the approved way with laboratory techniques, preferably including typing of the bacterial strain or strains.

Note: This commonly occurs in the setting of the routine bacterial screening of platelet concentrates, which are distributed "negative to date."

Posttransfusion bacteremia/sepsis resulting from a bacterially contaminated component—this can be termed transfusion-transmitted bacterial infection (TTBI)—is relatively rare. Only one red cell–related death per year has been reported from approximately 13,898,000 units of red cells or whole blood that are transfused in the United States. *Yersinia enterocolitica* is the most common bacterial contaminant of PRBC; other pathogens include *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Enterobacter* species. Incidence is directly related to the length of storage.

Platelet-related sepsis is more common than sepsis following RBC transfusion. The risk of symptomatic bacterial sepsis per pool or apheresis unit of platelets is in the order of 1:100.000. (See box on Suspected Posttransfusion Bacteremia/Sepsis.)

HYPOTENSIVE TRANSFUSION REACTION

While a drop in blood pressure may occur in patients with a range of transfusion reactions such as acute hemolysis, a severe allergic reaction or TRALI, in some cases an isolated, clinically relevant drop in systolic or diastolic blood pressure, arises during or up to 1 hour after administration of a blood component. These reactions are categorized as hypotensive transfusion reactions, with the ISBT definition and US Biovigilance protocols stating that in adults there should be a drop of 30 mmHg or more and a systolic pressure of 80 mmHg or lower. Typically, the patient responds to stopping the transfusion and supportive therapy with intravenous fluid (saline).

When hypotensive reactions were first described, an association was noted with an infusion of components leukoreduced using a particular type of bedside leukocyte filter. Moreover, reactions have been noted to occur in patients taking angiotensin-converting enzyme inhibitors. A role of bradykinin in the infused blood component has been postulated, but a definitive mechanism for hypotensive reactions in association with prestorage leukoreduced components has not been postulated. Hypotensive transfusion reaction remains a diagnosis of exclusion. If a patient receiving a blood transfusion shows an abrupt drop in blood pressure (even before the changes reach levels specified in the definitions), it is essential to respond promptly by stopping the transfusion, evaluating the patient clinically, and performing laboratory and any further investigations as clinically indicated.

POSTTRANSFUSION PURPURA

Posttransfusion purpura (PTP) is a rare and self-limiting thrombocytopenia occurring 5 to 10 days posttransfusion in patients lacking a specific platelet antigen, usually HPA-1a (also called PLA1, GPIIIa, CD61). These patients may have a history of prior transfusions or pregnancies. Indeed, about 85% of cases occur in women. After re-exposure to transfusion, patients can develop potent antibodies against the platelet-specific antigen that they are lacking but which is present on donor platelets. These platelet antibodies often have a high titer and can fix complement. As a result, the transfused platelets and the patient's own platelets are also destroyed through the adsorption of the antigen or immune complexes on their own platelets. A concurrent autoimmune process may also involve the destruction of the recipient's own platelets, as shown in one case report of a positive antibody against the patient's own platelets. The thrombocytopenia can be marked with a platelet count falling below 10,000/μL. The onset is sudden, although this complication is self-limiting, and usually resolves in 2 weeks. This severe thrombocytopenia can help distinguish PTP from heparin-induced thrombocytopenia, which can also be considered in the differential diagnosis when thrombocytopenia develops 5 to 10 days after combined heparin exposure and blood transfusion, for example, major surgery.

In patients who already have thrombocytopenia, diagnosing PTP can be difficult, especially when the patient already has immune-mediated platelet destruction. PTP can be considered if platelet refractoriness continues despite the transfusion of HLA-matched platelets. IVIG appears to be an effective treatment, although plasma exchange, corticosteroids, and splenectomy may also be useful. Patients with acute bleeding and needing platelet support should receive platelets without the platelet-specific antigen, if possible. If random donor platelets are given, patients can develop severe reactions, including allergic reactions. Recurrence of PTP is rare. The incidence of PTP has greatly declined in jurisdictions where universal leukodepletion is performed.

TRANSFUSION-ASSOCIATED GRAFT-VERSUS-HOST DISEASE

Transfusion-associated graft-versus-host disease (T-GVHD) occurs when immunologically competent lymphocytes are introduced into a host who cannot destroy the donor lymphocytes. The immunocompetent donor lymphocytes engraft, host HLA antigen is presented to donor lymphocytes, and the activated lymphocytes attack host tissues. T-GVHD occurs after transfusion of nonirradiated cellular blood components, either in immune-compromised patients or when the blood donor and recipient share HLA antigens. T-GVHD has a much higher fatality rate than HSCT-related GVHD because the donor lymphocytes produce recipient bone marrow aplasia in addition to typical liver, gut, and skin manifestations of acute GVHD. In GVHD after bone marrow transplantation, the bone marrow is of donor origin, and bone marrow aplasia does not occur.

T-GVHD is fatal in more than 90% of cases, primarily because of aplasia of the recipient's bone marrow. It often occurs 8 to 10 days after transfusion with marked pancytopenia, as well as gut, skin, and liver GVHD. The signs and symptoms include nausea, vomiting, anorexia, fever, diarrhea, liver dysfunction, and erythroderma. Patients often die of infection and hemorrhage within 3 to 4 weeks. There is no effective treatment, with the possible exception of bone marrow transplantation, if T-GVHD is recognized early and a suitable donor can be found in a short time.

Reports have shown that haploidentical directed donor units of blood may produce fatal T-GVHD even in immunocompetent recipients. The use of irradiated blood (2500 cGy) is thus recommended

RISK GROUPS FOR TRANSFUSION-ASSOCIATED GRAFT-VERSUS-HOST DISEASE

- Congenital T-cell defects (known or suspected)
- Immunologic immaturity (fetus or premature infant)
- Intrauterine transfusion
- Hematopoietic stem cell transplant
- Fludarabine treatment
- Haplotype sharing between donor and recipient
- Transfusions from blood relatives
- Human leukocyte antigen-matched platelets
- Granulocyte transfusions

in clinical situations in which T-GVHD is considered possible, such as when patients receive directed blood transfusions from their relatives. Leukocyte-reduction filters should not be used as prophylaxis against GVHD because the number of leukocytes needed to produce the disease is not known. Case reports of fatal T-GVHD in patients who received leukoreduced, but not irradiated, blood have been published. GVHD continues to be a rare, but extremely serious, complication of blood transfusion. From 2009 to 2013, two fatalities from T-GVHD were reported to the FDA. There is no clear evidence that prophylactic irradiation is indicated for patients with human immunodeficiency virus infection (see box on Risk Groups for Transfusion-Associated Graft-Versus-Host Disease). Of note, irradiation of RBCs causes membrane damage, permitting slow leakage of potassium and hemoglobin extracellularly. Nevertheless, rather than track which patients need irradiated blood, many centers provide universal irradiation of RBC components without adverse sequelae.

IRON OVERLOAD—HEMOSIDEROSIS

Transfusion-associated hemosiderosis is defined as a blood ferritin level of greater than 1000 mcg/L, with or without organ dysfunction, in the setting of repeated RBC transfusions. Iron loading from RBC transfusion is categorized by the ISBT as a transfusion reaction and is a common adverse consequence of chronic RBC transfusion. Further discussion of iron metabolism can be found in Chapter 36.

One milliliter of RBCs at a hematocrit of a typical RBC unit contains approximately 0.75 mg of iron. A unit of blood with 300 mL of RBCs thus contains approximately 225 mg of iron, and 4 units of blood contain ~1 g of iron, roughly the amount stored in the bone marrow. Men and nonmenstruating women lose only approximately 1 mg of iron each day. Continued use of transfusion therapy can thus result in the accumulation of excessive tissue stores of iron in individuals with hemolytic anemia, such as those with thalassemia or sickle cell disease (see Chapters 41, and Chapters 42 and 43, respectively), or ineffective hematopoiesis as in myelodysplastic syndromes (Chapter 61), in which iron is not lost from the body but is recycled. Over long periods, the iron that is stored in parenchymal cells results in oxidative injury and organ failure, particularly in the heart, liver, and endocrine organs. Iron chelation therapy is now widely used to mitigate positive iron balance. The availability of oral iron chelators such as deferasirox and deferiprone provides expanded access to iron chelation therapy.

HEMOVIGILANCE

Hemovigilance is defined by the International Haemovigilance Network as "A set of surveillance procedures covering the whole transfusion chain (from the collection of blood and its components to the follow-up of recipients), intended to collect and assess information on unexpected or undesirable effects resulting from the therapeutic use of labile blood products, and to prevent their occurrence or recurrence." Since the mid-1990s, in response to the "contaminated blood" scandals, many countries in the world have established national hemovigilance programs to which hospitals and blood establishments report untoward reactions and occurrences associated with blood transfusion and in some cases also plasma-derived products. Well-known examples are the professionally based SHOT (Serious Hazards of Transfusion, UK) and TRIP (Transfusion and Transplantation Related Incidents in Patients, The Netherlands), while Canada's Transfusion Transmitted Injuries Surveillance System, TTISS, is run by the public health agency and the French hemovigilance system by the regulator, the ANSM (Agence Nationale de Sécurité du Médicament et des Produits de Santé). In the United States, the National Healthcare Safety Network (NHSN) Hemovigilance Module was developed through a public-private partnership between the Centers for Disease Control and Prevention (CDC) and subject-matter experts convened by AABB (formerly the American Association of Blood Banks).

Importantly, these systems have legal safeguards to ensure that adverse reactions and events can be reported to them safely without risk of the institution or individual professionals being publicly identified or shamed. The collected data are reported publicly, provide transparency about the risks of transfusion, highlight risks, and make recommendations for improving safety.

Providing professionals in the health care facilities and blood establishments with standardized definitions and clear reporting instructions has been key in collecting data with the greatest possible potential for trend analysis, comparisons, and benchmarking. International definitions have been published by the International Society for Blood Transfusion in collaboration with the International Haemovigilance Network. The NHSN Hemovigilance Module provides standard criteria and definitions to participating facilities (http://www.cdc.gov/nhsn/PDFs/Biovigilance/BV-HV-protocol-current.pdf). Transfusion reactions are further classified by their severity and imputability—that is, the likelihood that the reaction is attributable to infusion of the blood product.

Healthcare facilities and blood establishments need to have protocols and provide training for staff in all relevant roles on how to handle blood products, correctly identify patients, recognize and respond to adverse reactions, and know what to report and how/to whom. Some establishments specifically qualify and revalidate nursing staff for performing blood transfusions. At best, a hemovigilance worker, also known as a transfusion practitioner, will be in post to support or run these activities (depending on institution size and local arrangements). Additional tasks are the development and overseeing of patient blood management activities and conducting audits of blood use.

Hemovigilance depends on clinicians and nursing staff considering the possibility of a transfusion reaction and reporting the signs and symptoms to the blood transfusion laboratory. The rate of such so-called spontaneous reports will generally be much lower than findings in clinical studies where active surveillance is in place, that is, where the outcome and associated symptoms are reported for every unit. When examining rates of reported transfusion reactions, it is essential to be aware of the method of data collection.

Traceability of a unit from donor to patient is essential, that is, ensuring that the blood establishment retains the record of donor identity for each unit and that transfusion facilities record (confirmed) transfusion of a unit to the patient or if a unit was not transfused, its final disposal. This allows for investigations in the (uncommon) event of a subsequent, potentially TTI in a donor or patient.

An early lesson from hemovigilance reporting was the data emerging from the SHOT scheme that showed that errors and notably "wrong blood" events (incorrect blood component transfused [IBCT]) more often caused more serious harm to patients than TTIs. Hemovigilance systems have also contributed to establishing the efficacy of safety interventions, for example, reduction in TRALI using plasma exclusively from male donors. They have the potential to identify emerging threats to transfusion safety, especially where events are uncommon, and are detectable only when large numbers of outcomes are evaluated in aggregate.

Although the earliest focus of hemovigilance was to monitor and improve the safety of clinical transfusion, this was followed by what has been called donor hemovigilance, “the systematic monitoring of adverse reactions and incidents in the whole chain of blood donor care, with a view to improving quality and safety for blood donors.” Alongside the centralizing and reporting of data on complications of blood donation, increasing research activity has demonstrated risk factors and shown the effectiveness of measures to reduce the occurrence of complications. The most frequent complication is dizziness or fainting (vasovagal reaction) during and following the donation of whole blood or blood components. By implementing best practices (e.g., an extra water drink shortly before donation, extra salt, social distraction), blood establishments can improve donors’ experience, which in turn increases the likelihood of future donations.

Both iron overload in chronically transfused patients and iron depletion blood donors, important longer-term complications of repeated transfusions and donations, remain challenging to capture and monitor in a meaningful way at the aggregate level. Chapter 37 discusses the management of iron overload of individual transfusion-dependent patients.

COMPLICATIONS ASSOCIATED WITH HEMATOPOIETIC STEM CELL INFUSION

Hematopoietic stem cells (HSCs), the most widely used cell therapy products, may be derived from bone marrow, peripheral blood, and umbilical cord blood. The infusion of HSCs carries the risk of the same types of transfusion reactions as with blood transfusions. However, additional risks are associated with HSC infusion, such as those related to cryopreservatives. In general, infusions of cryopreserved cells are more likely to cause reactions, as they contain higher concentrations of lysed RBCs and granulocytes than other blood products. For most reactions, it is difficult to identify the causative agent. Moreover, because of the irreplaceable nature of HSC products, higher risks may be tolerated. Only those reactions that are unique or more problematic for HSC infusions are discussed in this section. A summary of the reactions is given in Table 117.4.

Dimethyl Sulfoxide Toxicity

The most common reactions to HSC have been attributed to dimethyl sulfoxide (DMSO), the most widely used cryopreservative. A variety of symptoms are associated with DMSO infusion and are generally dose-dependent. A garlic odor commonly accompanies DMSO infusion, and nausea and vomiting are often reported. Additional DMSO-related symptoms include flushing, coughing, chest tightness, dyspnea, abdominal pain, hypotension, hypertension, cardiac toxicity (such as bradycardia and other arrhythmias), and rarely, neurologic toxicity (such as syncope and transient encephalopathy). Some cases of DMSO toxicity are thought to arise from the release of histamine. Most clinical services have protocols in place to limit the volume of DMSO that can be infused, such as setting an upper infusion limit of 1 g/kg/day, dividing HSC infusion doses, or washing HSC products to remove DMSO (this, however, may result in cellular loss).

Red Blood Cell Hemolysis

Although mismatched ABO and non-ABO blood groups do not prevent successful HSCT, these antigens (and their corresponding antibodies) can create problems during the transplant period. When considering incompatibilities within the ABO system, three scenarios are possible: (1) antibodies present in the recipient interact with incompatible cells present in the graft (major incompatibility), (2) antibodies present in the plasma portion of the graft mediate hemolysis of recipient RBCs (minor incompatibility), or (3) antibodies present in both the recipient and the donor interact with incompatible RBCs (bidirectional, or two-way, incompatibility). It has been shown that both major and bidirectional ABO incompatibility can delay stem cell engraftment. In such cases, anti-A and/or B antibodies suppress RBC precursors expressing these antigens, leading to reticulocytopenia and prolonged anemia. In the most severe form of this process, HSC recipients can develop pure RBC aplasia, a condition wherein bone marrow shows a virtual absence of immature erythroid elements more than 3 months posttransplantation. In cases of either delayed engraftment or pure RBC aplasia, patients are dependent on RBC transfusions for prolonged periods of time.

Another risk factor associated with HSC infusion is the possibility of RBC hemolysis. Both ABO and non-ABO antibodies are capable of mediating RBC lysis. In such conditions, preformed antibodies in either the recipient or HSC plasma can cause accelerated clearance of incompatible RBCs by either intra- or extravascular mechanisms. Presentation of hemolytic transfusion reactions immediately after HSC infusion is no different from that of typical transfusion reactions. Policies and protocols should be in place to prevent postinfusion HSC-related hemolysis—for example, plasma depletion of HSC products (in cases of minor mismatch and bidirectional ABO incompatibility), limiting the infusion of incompatible RBCs (in cases of major mismatch and bidirectional ABO), hydration of patients, and prospective monitoring for hemolysis. The reader is also referred to the chapters on stem cell graft harvesting and processing (Chapters 102 and 103).

TABLE 117.4 **Common Hematopoietic Stem Cell Infusion Reactions**

Types	Presentations	Treatment and Prevention
DMSO toxicity	Halitosis, nausea, vomiting, flushing, coughing, chest tightness, dyspnea, abdominal pain, hypotension, hypertension, cardiac toxicity (such as bradycardia and other arrhythmias), and rarely neurologic toxicity, such as syncope and transient encephalopathy	Antihistamine Antiemetics Limiting DMSO infusion volume
Minor ABO mismatch	Delayed hemolysis (1–2 weeks post HSC infusion)	Plasma depletion of HSC product RBC transfusion support compatible with donor and recipient Monitor for hemolysis RBC exchange with donor- and recipient-compatible blood if massive hemolysis
Major ABO mismatch	Hemolysis at the time of infusion Delayed RBC engraftment	Limiting the infusion of incompatible RBC Hydration Monitor for RBC engraftment Modify immunosuppression Plasmapheresis to remove isohemagglutinins if significant RBC engraftment delay

DMSO, Dimethyl sulfoxide; *HSC*, hematopoietic stem cell; *RBC* , red blood cell

REFERENCES

1. Alessandrino P, Bernasconi P, Caldera D, et al. Adverse events occurring during bone marrow or peripheral blood progenitor cell infusion: analysis of 126 cases. *Bone Marrow Transplant*. 1999;23:533.
2. Delaney M, Wendel S, Bercovitz RS, et al. Transfusion reactions: prevention, diagnosis, and treatment. *Lancet*. 2016;388(10061):2825–2836.
3. U.S. Food and Drug Administration. *Vaccines, Blood & Biologics: Fatalities reported to FDA following blood collection and transfusion*. Annual Summary for Fiscal Year 2018: https://www.fda.gov/media/136907/download.
4. Gilliss B, Looney M. Experimental models of transfusion-related acute lung injury. *Transfus Med Rev*. 2011;25:1 PMD 21134622.
5. Hardy J, de Moerloose P, Samama C. The coagulopathy of massive transfusion. *Vox Sang*. 2005;89:123 PMD 16146503.
6. Heddle NM, Blajchman MA, Meyer RM, et al. A randomized controlled trial comparing the frequency of acute reactions to plasma-removed platelets and prestorage WBC-reduced platelets. *Transfusion*. 2002;42:556 12084163.
7. Holcomb JB, Tilley BC, Baraniuk S, et al. Transfusion of plasma, platelets, and red blood cells in a 1:1:1 vs a 1:1:2 ratio and mortality in patients with severe trauma: the PROPPR randomized clinical trial. *JAMA*. 2015;313:471 25647203.
8. Kelly M, Roy DC, Labbe AC, et al. What is the clinical significance of infusing hematopoietic cell grafts contaminated with bacteria? *Bone Marrow Transplant*. 2006;38:183 PMD 16785868.
9. Parmar N, Pendergrast J, Lieberman L, Lin Y, Callum J, Cserti-Gazdewich C. The association of fever with transfusion-associated circulatory overload. *Vox Sang*. 2017;112:70–78.
10. Politis CP, Wiersum JC, Richardson C, et al. The International Haemovigilance Network database for the surveillance of adverse reactions and events in donors and recipients of blood components: technical issues and results. *Vox Sang*. 2016 Nov;111(4):409–417. https://doi.org/10.1111/vox.12447.
11. Sachs UJ, Wasel W, Bayat B, et al. Mechanism of transfusion-related acute lung injury induced by HLA class II antibodies. *Blood*. 2011;117:669 PMD 21030555.
12. Sandler SG, Eder AF, Goldman M, et al. The entity of immunoglobulin A-related anaphylactic transfusion reactions is not evidence based. *Transfusion*. 2015;55:199 PMD 25066014.
13. Savage WJ, Hamilton RG, Tobian AR, et al. Defining risk factors and presentations of allergic reactions to platelet transfusion. *J Allerg Clin Imm unol*. 2014;133:1772.
14. Shimada E, Tadokoro K, Watanabe Y, et al. Anaphylactic transfusion reactions in haptoglobin-deficient patients with IgE and IgG haptoglobin antibodies. *Transfusion*. 2002;42:766 PMD 12147031.
15. Sihler K, Napolitano L. Complications of massive transfusion. *Chest*. 2010;137:209 PMD 20051407.
16. Stowell SR, Winkler AM, Maier CL, et al. Initiation and regulation of complement during hemolytic transfusion reactions. *Clin Dev Immunol*. 2012;2012:307093 PMD 23118779.
17. Tobian A, Sokoll LJ, Tisch DJ, et al. N-terminal pro-brain natriuretic peptide is a useful diagnostic marker for transfusion-associated circulatory overload. *Transfusion*. 2008;48:1143 PMD 18298592.
18. Toy P, Gajic O, Bacchetti P, et al. Transfusion-related acute lung injury: incidence and risk factors. *Blood*. 2002;119:1757.
19. Uchida S, Tadokoro K, Takahashi M, et al. Analysis of 66 patients definitive with transfusion-associated graft-versus-host disease and the effect of universal irradiation of blood. *Transfusion Med*. 2013;23:416.
20. Vlaar A, Hofstra JJ, Determann RM, et al. The incidence, risk factors, and outcome of transfusion-related acute lung injury in a cohort of cardiac surgery patients: a prospective nested case-control study. *Blood*. 2011;117:4218 PMD 21325598.
21. Vlaar APJ, Toy P, Fung M, et al. A consensus redefinition of transfusion-related acute lung injury. *Transfusion*. 2019;59(7):2465–2476. https://doi.org/10.1111/trf.15311.
22. Wiersum-Osselton J, Middelburg RA, Beckers EA, et al. Male-only fresh-frozen plasma for transfusion-related acute lung injury prevention: before-and-after comparative cohort study. *Transfusion.*. 2011;51:1278. https://doi.org/10.1111/j.1537-2995.2010.02969.x.
23. Wiersum-Osselton JC, Whitaker B, Grey S, et al. Revised international surveillance case definition of transfusion-associated circulatory overload: a classification agreement validation study. *Lancet Haematol*. 2019;6(7):e350–e358. https://doi.org/10.1016/S2352-3026(19)30080-8.
24. Worel N, Greinix HT, Keil F, et al. Severe immune hemolysis after minor ABO-mismatched allogeneic peripheral blood progenitor cell transplantation occurs more frequently after nonmyeloablative than myeloablative conditioning. *Transfusion*. 2002;42 https://doi.org/10.1046/j.1537-2995.2002.00209.x. 1293-301.
25. Yomtovian RA, Palavecino EL, Dysktra AH, et al. Evolution of surveillance methods for detection of bacterial contamination of platelets in a university hospital, 1991 through 2004. *Transfusion*. 2006;46:719–730. PMD 16686839.

CHAPTER 118

TRANSFUSION-TRANSMITTED DISEASES

Lauren A. Crowder and Susan L. Stramer

Adverse reactions following blood transfusion reflect immunologic, pathophysiologic, and microbiologic events. This chapter presents information about transfusion-associated viral, bacterial, parasitic, and prion infections and discusses a number of emerging agents including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Transfusion-transmitted infection risk mitigation through blood donor screening and blood testing strategies are presented.[1] The boxed discussions provide insights into interventions aimed at reducing risk from known and emerging threats and new technologies for reducing or eliminating microbial contamination. Red cell, platelet, and plasma transfusions represent important therapeutic modalities for appropriately selected patients. Awareness of the hazards of transfusion and the rate at which these events occur should enable physicians to better determine the benefit/risk ratios when prescribing transfusions.

HEPATITIS VIRUSES

The hepatitis viruses can be classified according to their predominant modes of transmission, parenteral and enteric, with the parenterally transmitted agents, hepatitis B virus (HBV) and hepatitis C virus (HCV) dominating concerns about transfusion transmission because many individuals unknowingly infected with these agents become asymptomatic chronic carriers and may make blood donations.

Parenterally-Transmitted Hepatitides

Hepatitis B

HBV is a deoxyribonucleic acid (DNA) virus in the family *Hepadnaviridae*. The infectious virion is occasionally referred to as the Dane particle and has surface and core components, surface antigen (HBsAg) and core antigen (HBcAg), respectively. Epitopes on the viral surface provide a basis for epidemiologic studies and consist of the HBsAg "a," d/y, and w/r determinants. However, this approach is being replaced by genotyping and DNA sequencing methods. Recombinant vaccines containing the "a" determinant confer protective immunity to a high proportion of vaccinees and are dramatically altering the incidence and prevalence of HBV infection in the general population where they are in wide use and consequently also impact the frequency of HBV infection in the donor population.

The average incubation period (the time from infection to liver enzyme elevation and symptomatic hepatitis) is 59 days (range, 5 to 12 weeks) but may be as long as 6 months. Symptoms, which occur in 30% to 50% of infected persons aged 5 years and older, include fatigue, anorexia, nausea, vomiting, jaundice, dark urine, light stools, arthralgias, rashes, vasculitis, and glomerulonephritis. The risk for progression to chronic infection is inversely related to age at infection. HBV infection becomes chronic in more than 90% of infants, 25% to 50% of children 1 to 5 years of age, and fewer than 5% of older children and adults. Approximately 5% of the US population has serologic evidence of prior HBV infection (antibodies against HBcAg [anti-HBc] reactive). From the mid-1980s with increasing immunization in early childhood, the incidence of acute HBV infection has decreased. In 2017, 46 states reported over 3400 cases of acute HBV to the US Centers for Disease Control and Prevention (CDC), or 1.1 case per 100,000 population. Due to underreporting and asymptomatic cases that are not diagnosed and reported, estimates of the actual number of acute cases in 2017 is over 22,000. An additional 13,300 cases of chronic HBV cases were reported and approximately 862,000 people are estimated to be living with chronic HBV infection.[2] Approximately 1700 deaths occurred in 2017 resulting from hepatitis B and its complications.

Based largely on data from parenteral exposures of health care workers, HBV is 100 times more infectious than human immunodeficiency virus (HIV) and 10 times more infectious than HCV. The predominant mode of transmission to adults and adolescents is through sexual contact. Forty percent have infected partners, 15% are males having sex with other males, injecting drug users account for 14% of cases, and one-third have no identifiable risk. A recent study of risk factors in blood donors found to be infected with HBV found that of 292 infected donors surveyed, the characteristics most frequently associated with infection were living abroad or having immigrated to the United States (51% vs. controls at 6%), a family member infected with hepatitis (15% vs. 2%), having been in a jail or detention for 3 nights or more (19% vs. 5%), and having taken illegal drugs (20% vs. 12%).[3]

HBsAg is detectable in blood approximately 4 weeks (30 to 60 days) after infection. Subsequently, immunoglobulin M (IgM) anti-HBc antibodies appear coincident with symptom onset. High viral titers present at that time decline subsequently. HBsAg persists transiently in acute infections for up to 4 months (average 63 days). More recent data using extremely sensitive HBsAg assays for blood donation screening show the mean period of HBsAg duration to be 43 days.[4] Antibodies against HBsAg (anti-HBs) develop subsequently and are generally thought to protect against reinfection although recent studies on blood donors have identified a few breakthrough cases of infection. These cases were mainly caused by HBV genotypes differing from that of the vaccine strain and were mild and self-limited.[5] Such donations were not detected by HBsAg blood donation screening but required more sensitive HBV DNA screening.

Some anti-HBc–positive and HBsAg-negative individuals have circulating HBV DNA, and this pattern defines so-called occult HBV infection (OBI). In rare cases, OBI may be accompanied by anti-HBs, usually at levels below 200 mIU per mL. Donors with OBI may transmit HBV via blood transfusion, but the frequency of such infection is low, and seems to be absent if anti-HBs are present.[6] Although anti-HBs usually confers immunity to reinfection, sufficient virus remains in the liver to transmit HBV following liver transplant from anti-HBs–positive donors through reactivation under intense immunosuppression.

It should be noted that the FDA has eliminated the requirement to ask blood donors about a history of viral hepatitis, effective May 2016.[7] The detection of HBV DNA is performed by nucleic acid testing (NAT) in minipools of 6 to 16 donations each as now required per Food and Drug Administration (FDA) Guidance, Use of Nucleic Acid Tests on Pooled and Individual Samples from Donors of Whole Blood and Blood Components, including Source Plasma, to Reduce the Risk of Transmission of Hepatitis B Virus (www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm, October 2013) and made final in updates to the Code of Federal Regulations.[7] The risk for HBV transmission per unit in the United States has recently decreased substantially as a result of very sensitive HBsAg testing, the implementation of NAT, and a recent

TABLE 118.1 **Risk for Transfusion-Transmitted Diseases in the United States**

Pathogenic Agent or Disease	Average Estimated Risk Per Unit
Hepatitis A	Rare
Hepatitis B	1 per million
Hepatitis C	1 per 2 million
Human immunodeficiency virus	1 per 1.6 million
Human T-lymphotropic viruses 1, 2	1 per 3.3 million
Cytomegalovirus	Infrequent in screened products; 1% in unscreened products
Parvovirus B19	Rare
West Nile virus	Rare
Malaria	0–3 cases per year
Babesiosis	1 per 18,000 in highly endemic regions; 1 per 100,000 endemic regions overall
Chagas disease	20 transfusion-associated cases reported
Creutzfeldt-Jakob disease	4 vCJD transfusion-associated cases reported Transfusion transmission of non-variant CJD has not been shown
Bacterial Contamination:	
Red cells	1 per 30,000
Septic reactions	1 per 500,000–1 per 10 million
Platelets	1 per 3000–1 per 8000
Septic reaction	1 per 107,000
Emerging Infections:	Risk unknown
Arboviruses including dengue viruses, chikungunya and Zika viruses	
Pandemic influenza viruses (H5N1, H1N1)	

vCJD, Variant Creutzfeldt-Jakob disease.

decline in the incidence of HBV infections among donors undoubtedly because of universal vaccination (Table 118.1).[4,5,8,9] Before NAT implementation, most contemporary HBV transfusion-transmitted infections were attributable to blood donations from asymptomatic donors during acute infection preceding the development of detectable HBsAg. Early in infection, HBV replicates relatively slowly with a doubling time of approximately 2.6 days. The current window period between infection and the detection of HBV DNA by NAT ranges from 30 to 38 days depending on the infectious dose, which, early in infection, is estimated to be between 1 and 10 copies per mL.[10] Since the implementation of HBV NAT in the United States, there have been no documented cases of breakthrough HBV transfusion transmissions. The per unit residual risk for donations to the American Red Cross was estimated to be 1 per 843,000 to 1 per 1,208,000 in 2013 and 1 per 1,529,000 in 2015 to 2016 using an 18.5 day window period and weighted for all donors (first-time and repeat).[11] Residual risks of 1 per million using the same methods are documented from large multicenter national studies for 2018 to 2019.[12,13]

There is evidence that some HBsAg assays will not detect all HBsAg and HBc genetic variants of HBV. Because all blood donations are screened by HBV NAT, HBsAg, and anti-HBc, it is unlikely that any mutant strain of HBV would go undetected in the United States. Among 12.8 million blood donors to the American Red Cross during 2009 to 2011, 1 per 9337 were infected with HBV. Of these, 1090 were positive for HBsAg, with or without HBV DNA (1 per 11,736), and only 5 were identified solely by NAT in minipools (1 per 2.55 million).[8] However, more recent data, also from the American Red Cross, demonstrate the HBV DNA detection rate of seronegative donations by NAT to be 1 per 770,000 donations.[11] NAT on individual donation aliquots has the potential to reduce the risk for transfusion-transmitted HBV further by detecting newly infected donors slightly earlier than either minipool-NAT or HBsAg tests licensed for blood donor screening, but the additional cost of individual donation-NAT in the United States would be high with very little to no demonstrable clinical benefit.

Hepatitis D

Hepatitis D virus (HDV) was originally called the delta agent. It is a defective ribonucleic acid (RNA)–containing passenger virus that requires active synthesis of HBsAg to act as a "helper" for assembly of HDV virions. As many as 10% of HBV infections are accompanied by HDV worldwide. Its prevalence is very low in the United States but higher in intravenous drug users. HDV superinfection of chronic HBV carriers is associated with worsened chronic sequelae and with fulminant hepatitis. Screening for HBV acts synergistically to prevent transfusion-associated HDV cases by identifying donors that are co-infected with HBV and HDV. There has never been an HBV-HDV–related transfusion transmission reported.

Hepatitis C

HCV is an RNA virus in the family *Flaviviridae*, genus *Hepacivirus*. There are six genotypes that share similar epidemiology, pathogenesis, and natural histories. In the United States, genotype 1 causes 75% of infections, genotypes 2 or 3 causing 15% to 20% of infections, and genotypes 4, 5, and 6 causing less than 5% of infections. Genotype 1 responds relatively poorly to traditional treatment regimens using pegylated interferon combined with oral doses of ribavirin compared with genotypes 2 and 3; the overall cure rate for all genotypes using this regimen was no better than 40% to 50%. HCV protease inhibitors (direct acting antivirals) are materially improving these rates. Two oral regimens of Harvoni and Viekira Pak are now the standard of care in the United States, and result in a sustained virologic response of greater than 90% (https://www.cdc.gov/hepatitis/statistics/2017surveillance/index.htm#hepatitisB, Viral Hepatitis–2017 Surveillance).

HCV is distinguished by a low rate of recognized acute infection and by a high rate of chronic infection, with substantial morbidity and mortality over long periods of observation as a result. The CDC estimates that there were 2.4 million people living with chronic HCV infections in 2017.

The most common source of HCV acquisition is intravenous drug use. The prevalence of HCV in US adults (20 to 59 years old) with any history of illegal intravenous drug use is greater than 45%. Other risks include blood transfusion before donor serologic screening began in 1990, exposure in health care settings, including through dialysis, among infants born to HCV-infected mothers, and tattoos in unregulated settings. In a large series, 15% to 30% of patients report no risk factors. Vertical transmission occurs in 3% to 7% of infants of mothers with active infections. In contrast to HBV, sexual transmission is an inefficient route of infection and much less often reported but was found to be the most likely mode of transmission of HCV among HIV-infected men who have sex with men (MSM) in New York City. In 2012 the CDC recommended that individuals born during 1945 to 1965 have one-time HCV screening since among all persons living with HCV infection, about 75% were born during this time. Such individuals have a 3% prevalence, which is five times higher than the prevalence seen in adults born in other years. Among blood donors, 0.03% have confirmed-positive HCV test results. There have been studies on risk factors among HCV-infected blood donors, and despite policies requiring deferral of injection drug users, such use is the most common risk factor.[3,14] Of 316 blood donors followed who were HCV-confirmed positive, risks identified via a retrospective questionnaire revealed risks versus control donors of illegal drug use (37% vs. <1% for controls), followed by jail or detention (57% vs. 5%), history of a blood transfusion before screening (18% vs. 7%), and living in a household with someone having hepatitis (15% vs. 2%).

At most 20% to 30% of newly infected persons develop recognizable symptoms during acute HCV infection. Some 20% of infected individuals clear their infection over a relatively short initial period, remaining antibody-positive but RNA-negative. Chronic infection develops in 75% to 85% of persons infected after 45 years of age and in 50% to 60% of those infected as children or young adults. Chronic HCV infection progresses to cirrhosis in 15% to 30% over 30 years of observation. Hepatocellular carcinoma occurs in 1% to 4% per year in those with cirrhosis. HCV is among the most prevalent causes of chronic hepatitis, cirrhosis, and primary liver cancer in the developed world and is the most common indication for liver transplantation in the United States, resulting in around 2400 procedures annually.

A single positive anti-HCV result cannot distinguish between acute and chronic HCV infection or between current or cleared infection. In 2012 laboratory criteria for the confirmation of anti-HCV reactivity were modified to add one specific assay including RNA detection, using a supplemental anti-HCV assay (as available) or a single value above a specific threshold on the screening test.[15] The risk for posttransfusion HCV infection declined progressively with the introduction of surrogate markers for non-A, non-B hepatitis in the 1980s (alanine aminotransferase [ALT] and anti-HBc) and of serologic testing for HCV antibodies in May 1990, followed sequentially by improved serologic testing and NAT. The seronegative-window period for the first-generation HCV antibody test extended to 6 months from infection but was reduced to 82 and 70 days with second- and third-generation assays, respectively. NAT further reduced the window period to approximately 7 days. The risk per unit declined from an estimated 1 per 276,000 units to 1 per 2,600,000 units from 2007 to 2016 and in 2018 to 2019 in larger multicenter national studies to 1 per 2 million weighted for all donations from both first-time and repeat donors.[11,12]

Like HBV, in the United States, testing for HCV RNA has used NAT in small minipools combining aliquots from 16 to 24 donations (currently 6 to 16). Loss of test sensitivity because of sample pooling was tolerable given the rapid increase or burst of HCV viremia before antibody seroconversion (estimated doubling time of 10.8 hours in that period during the 40- to 50-day window period) and the high titer of viremia that remains preceding antibody seroconversion. However, the rest of the world (except the United States, Canada, the United Kingdom, and most of Germany) has implemented NAT for screening individual donation samples intended for transfusion. The HCV RNA yield by NAT in seronegative donations using data from the American Red Cross is 1 per 270,000 donations.[11]

As an alternative to NAT assays, enzyme immunoassays (EIAs) have been developed that detect HCV HBcAg in serum or plasma, either as an individual analyte in parallel with antibody assays or as HCV antigen-antibody combination assays. These tests reduce the pre-seroconversion window period and were adopted in some developing countries. They are less sensitive than HCV NAT, are not approved for blood donor screening in the United States, and use has decreased significantly.[16] Since the implementation of NAT, reports of transfusion-transmitted HCV are very infrequent.

The high seroprevalence of HCV at the onset of serology screening in the early 1990s, the prolonged interval between infection and clinical manifestations, and the relatively high rate of HCV clinical sequelae prompted blood collection facilities and hospitals to conduct "look-back" notification of previous recipients of blood given by donors found on subsequent donations to be HCV infected. Look-back was subsequently made mandatory in companion rules from FDA and the Centers for Medicare and Medicaid Services. In general, HCV look-back programs found half or fewer of targeted transfused individuals alive but were able to find both seropositive and RNA-positive recipients who were unaware of their status. This population has benefited significantly from identification and subsequent treatment with the advent of curative direct-acting antivirals.

Enterically Transmitted Hepatitides

Hepatitis A

The hepatitis A virus (HAV), a nonenveloped picornavirus, genus *Hepatovirus*, is transmitted predominantly by the fecal-oral route, with an average incubation period of 28 days (range, 15 to 50 days) with signs or symptoms persisting for less than 2 months. The incidence of HAV infection in the United States fell by 76% between 1997 and 2003 after the recommendation for targeted immunization of members in high-risk communities. Populations at risk include those in areas where extended community outbreaks occur and children living in states that have high and intermediate rates of disease, staff and residents of closed communities, close personal contacts of cases, the staff and parents of children in daycare centers, and those with common-source exposure to infected food or water. For many sporadic cases there is no recognized source. HAV is self-limited with no chronic carrier state, but approximately 10% to 15% of infected individuals develop a more prolonged or relapsing illness. It is the most frequent cause of hepatitis among children under 11 years of age.

Transfusion-related transmission, although rare, is caused by a blood donation from a recently infected, asymptomatic, viremic individual. The peak viremia occurs 2 weeks before onset of jaundice or elevation of hepatocellular enzymes and persists for a median period of 42 days (range, up to 59 days). The virus is quite resistant to many inactivation procedures, including the pathogen-reduction procedures being developed for cellular blood components (e.g., licensed psoralens or investigational riboflavin, both with ultraviolet [UV] irradiation) and fresh-frozen plasma (solvent/detergent and methylene blue). Transmission by clotting-factor concentrates treated with the solvent/detergent pathogen-reduction process occurred in the 1990s, but not thereafter. Plasma for further manufacture is routinely screened for HAV RNA by pooled NAT.

A 120-day deferral is recommended after exposure to HAV during community outbreaks to prevent transfusion transmission. Screening for HAV is not done for donations of blood for transfusion.

While the average number of annual HAV infections reported to CDC in recent years has declined substantially compared to 2000, fluctuations have occurred in the last 20 years due to large outbreaks. After a long downward trend, the first increase between 2012 and 2013 (1562 and 1781 reported cases, respectively) was due to a large multi-state outbreak associated with pomegranate arils imported from Turkey.[17] Between 2015 and 2016, the reported cases again increased by 44.4% from 1390 in 2015 to 2007 cases in 2016. The 2016 increase was due to two hepatitis A outbreaks, each of which was linked to imported foods. Substantial increases in incident cases of hepatitis A occurred in 2017 and 2018 (3366 and 12,474 reported cases, respectively) due to ongoing outbreaks reported to CDC among people who use drugs and people experiencing homelessness as well as outbreaks among MSM.[18,19]

Hepatitis E

The hepatitis E virus (HEV) is a small, nonenveloped single-stranded RNA virus in the *Hepeviridae* family. HEV was first recognized in the 1980s in Afghanistan among soldiers with unexplained hepatitis. There is a single serotype but at least four genotypes with differing geographic distributions and epidemiologic patterns. Genotypes 1 and 2 are generally associated with large, water-borne (fecal-orally transmitted) outbreaks in less developed tropical countries. Illness is usually self-limited but can be lethal in pregnant women, their fetuses, and patients with chronic liver disease. Genotypes 3 and 4 appear to be animal viruses that result in zoonotic infection of humans, most often through consumption of inadequately cooked pork products. Genotype 3 seems to be widely distributed and is present in developed countries, whereas genotype 4 seems to be more common in certain Asian countries. Transfusion-related transmission, mostly of serotype 3, has been well-documented in Japan, France, England, the Netherlands, and Spain.[20]

Recent studies suggest a wide range of seroprevalence rates, but some of the variability may be attributable to the differences in performance characteristics of the tests used and some to dietary habits. Most studies indicate a cohort effect, with prevalence rates increasing with age. Transfusion infectivity is logically associated with the presence of detectable viral RNA in the plasma and the frequency of this finding varies between 1 in 1000 to 1 in 10,000 donations.

In a large study in England,[21] 225,000 donations were tested and 79, or 1 in 2848, were found to be positive when tested for HEV RNA. For 43 of these, recipient tracing was possible and 18 (42%) showed evidence of transfusion-transmitted infection. A North American study screened 101,489 donations from Canada and the United States for HEV RNA and reported 14 positive donors, or 1 in 7300, all with low viral loads (ranging from 20 to 3080 copies/mL) consistent with a prior US study finding 7.7% of donors anti-HEV positive but only two of approximately 19,000 that were RNA positive.[22,23] Although the Canadian prevalence was fourfold higher than in the United States, quantitative and risk-based analysis found testing to not be cost effective. A current broader concern is the finding that highly immunosuppressed patients (such as solid-organ transplant recipients) do develop chronic HEV infections with long-term clinical sequelae, although these have not been specifically linked to infection via transfusion. While testing is standard in some European countries and Japan, where incidence rates are higher and transfusion transmissions well documented, in the United States and Canada routine testing is currently not recommended due to the absence of recognized transfusion transmission.

Non–A-E Hepatitis

Cases of posttransfusion hepatitis only rarely if ever occur but there remains speculation that undiscovered hepatitis agents exist. A small but consistent percentage of community-acquired hepatitis cases test negative for known hepatitis viruses, some cirrhosis is classified as "cryptogenic," an etiologic agent for hepatitis-associated aplastic anemia (Chapter 31) eludes description, and the cause of some cases of acute liver failure remains elusive. Several candidate agents have been proposed as non–A-E hepatitis viruses. None of these agents have been shown to be pathogenic and are instead likely commensal and nonpathogenic.

GBV-C (initially called hepatitis G virus) is a flavivirus with no confirmed disease association that is transmitted parenterally, including frequently by transfusion. Of interest, GBV-C infection may delay progression of disease in those co-infected with HIV, which has led to studies of the interactions between these viruses.[24]

From 1% to 4% of US blood donors are viremic compared with 15% to 20% of intravenous drug users who have detectable GBV-C RNA. Infection occurs frequently among those infected with HCV and HIV. More people have antibodies against the E2 envelope protein, in the absence of RNA, suggesting viral clearance. GBV-C has not been shown to cause liver disease or other morbidities, and hence there is no consideration of donor screening at this time. GBV-C is now referred to as a human pegivirus. A second human pegivirus has recently been described associated with HCV likely as a result of an acute parenteral co-infection event, but again, has not been associated with hepatitis or any pathology. This virus was identified by next generation sequencing.[25] Likely other such commensal agents will continue to be identified by the use of sophisticated molecular techniques (Chapter 3).

The torque teno virus (TTV) complex is a genetically diverse group of nonenveloped DNA viruses in the family *Circoviridae*, which was discovered in 1997. They cause viremia, and they are transmitted by transfusion, but they cause no recognized liver disease or other clinical illness.

SEN virus (SENV), another member of the *Circoviridae*, was described using degenerate polymerase chain reaction (PCR) primers while working with TTV. After an initial report associating SENV variants in two patients with transfusion-associated non–A-E hepatitis, subsequent epidemiologic studies have failed to link SENV with clinical hepatitis.

RETROVIRAL INFECTIONS

Human Immunodeficiency Virus

The HIVs type 1 and type 2 (HIV-1 and HIV-2) are retroviruses of the family *Retroviridae*, genus *Lentivirus*, and the etiologic agents of the acquired immunodeficiency syndrome (AIDS). They are enveloped viruses with two linear, positive-sense RNA molecules, 9.2 kb in length. The predominant transmission mechanisms are sexual, perinatal, and parenteral. An acute retroviral syndrome may be seen around 21 days after infection, although this period may range from 5 to 70 days and may involve fever, lymphadenopathy, and rash. If untreated, the incubation period for full-blown AIDS is measured in years. Molecular characterization divides HIV-1 into three groups: group M (main), group O (outliers), and group N (non-M/O). Group M subtype B infections predominate in the United States; only 3% of HIV-positive blood donors have non-B strains. Very rare group O infections have been detected in the United States among patients who were born, lived, or had sexual contact in endemic regions of West and Central Africa. HIV-2 infected persons in the United States are rare (less than 1% of HIV cases diagnosed annually) and have, for the most part, been infected after heterosexual transmission in West African emigrants or residents. HIV-2 disease requires a longer time to evolve and is less severe than that from HIV-1 There have been five confirmed cases of HIV-2 infection among blood donors in the United States.

In 2016 CDC estimated that a total of 1.1 million people in the United States 13 years or older were living with HIV infection, approximately 1 per 300 Americans, with 14% of those unaware of their infection. Of the approximately 38,000 new diagnoses in 2018, the highest number of new infections occurred in those 25 to 29 years old (20%), in predominately African Americans (40%). MSM continue to bear the greatest burden of HIV infection among all racial and ethnic groups. Although MSM represent 6% of the US population, in 2018 MSM accounted for nearly 82% of all new HIV infections in males and over 65% of all new HIV infections. Antiretroviral therapy for HIV treatment has improved dramatically since the advent of combination therapy in 1996, even against multidrug resistant viruses. HIV-associated morbidity and mortality have significantly been reduced so that HIV treatment has converted HIV infection to a chronic, versus a fatal, disease.

Antibody testing of blood donors detects both HIV-1 and its variants and HIV-2.[26] Antibody testing for HIV-1 began in 1985, with the addition of HIV-2 in 1992 (although HIV-1 tests before that time detected most HIV-2 infections arising from over 60% sequence homology between the two viruses) and for HIV-1 group O in 2006.

Both serologic testing and NAT are performed on every blood donation. First-generation antibody tests had a window period (time between infection and detection) of 45 days on average but has decreased significantly as tests became more sensitive leading to less than a 20-day window period. HIV-1 NAT, implemented in 1999 (initially in duplex tests with HCV performed in pools of 6 to 16 donations), detects RNA at a minimum concentration of approximately 5 copies per milliliter (50% lower limit of detection) leaving about a 9-day window period of risk in which an infected donor could donate and not be detected by any test in current use. Rare genetic variants of HIV may escape NAT detection when nucleotide sequence changes affect NAT primer or probe binding sites, but the vast majority of these infections will be detected serologically. Further, tests are now designed to detect two or more separate sequences representing different viral regions. Currently, the frequency of confirmed-positive test results for antibodies to HIV is 1.65 per 100,000 donations (1:61,000). Almost all of these are also RNA-positive as the finding of an infected donor with low-level RNA levels is very rare (on the order of 2%). One donation per 2.1 million is confirmed positive for RNA in the absence of antibody (NAT yield).[11]

To eliminate donations in the window period between exposure and test positivity, each blood donor is asked at each donation about exposure risks using questions developed in the early to mid-1980s, subsequent to the first reports of AIDS in hemophiliacs and transfusion recipients. These initial interventions targeted blood donations from homosexually active men and intravenous drug users, substantially reducing the transmission risk between 1983 and 1985. Five clusters of transfusion transmissions have been documented subsequent to the introduction of NAT, three before 2002 and one in 2008.[10,27] It is of interest to note that in two cases, although the transfused plasma component transmitted HIV, the corresponding red cell component did not. Modeling suggests a residual risk for HIV transmission persists at approximately one transmission per 1.5 million donations, because NAT cannot detect HIV infection during the immunosilent 9-day eclipse phase between infection and test reactivity. Processing and quarantine procedural errors do not now appear to be a risk for transmission of HIV via transfusion. Fourth-generation tests combine HIV-1/2 antibody and HIV antigen detection and detect almost 90% of infections detected by NAT. Their role is expanding worldwide even in countries that use HIV-1 NAT. In 2014 the CDC published revised guidelines for laboratory diagnosis of HIV infection (https://doi.org/10.15620/cdc.23447).[28]

Recent studies have shown that the predominant risk factors for HIV infection among 149 male infected blood donors surveyed retrospectively continues to be a history of MSM behavior (62% in cases vs. 2% in controls) or of a male having sex with an HIV-positive individual (26% in cases and none in controls). Of note, 50% of MSM activity occurred within the last 12 months. Although in the past, intravenous drug use was also a prominent risk factor, this has decreased in prominence (24% in cases vs. 5% in controls).[3]

In 2015, the Transfusion-Transmissible Infections Monitoring System (TTIMS) was established by the FDA, National Heart, Lung and Blood Institute (NHLBI), and the Office of the Assistant Secretary of Health (OASH) to establish a comprehensive monitoring program for transfusion-transmissible infections in the United States. Of primary focus for the first years of the program was the changing prevalence and incidence rates of HIV (as well as HCV and HBV) before and after MSM policy changes enacted by US blood centers following guidance changes by the FDA. Recent reports from the system show no marked changes in prevalence, incidence, residual risk, or recency of HIV infection during the 4 years surrounding policy changes, accounting for over 27.5 million donations from 9.4 million donors.[12,13,29–31] In April 2020, in response to the SARS-CoV-2 pandemic, the US FDA updated their 2015 guidance reducing the deferral period of MSM from 1 year since last MSM activity, as outlined in the 2015 guidance, to 3 months since last activity.[32] The TTIMS program will continue to monitor changes in screening and testing for TTIs following this policy change as well.

Current issues of concern related to HIV deferrals and testing include individuals on anti-retroviral therapy and those on post-exposure and pre-exposure prophylaxis. Initial reports show that individuals on ART or PrEP are part of the US donor population and that improved strategies need to be developed to encourage disclosure of these therapies and decrease test-seeking behaviors.[33]

Most consider the blood supply in the developed world to be at its highest historical safety level. This reflects incremental improvements in donor selection and history screening, blood testing, and process control that span four decades. For years, blood collection professionals and government regulators formulated blood safety policy decisions in an apparent aim to achieve a zero-risk blood supply. In part, this reflects the perceived delayed response of the transfusion medicine community in the early 1980s to the emergence of HIV in the blood supply and the recognition of the scope and severity of post-transfusion non-A, non-B hepatitis (subsequently hepatitis C [HCV]) following that. It reflects also the “dread fear” associated with transfusion-associated HIV. This reaction is seen when devastating, unpredictable, and stigmatizing events threaten potential victims who have little ability to escape the risk. This fear was validated by numerous transfusion-related HIV cases. It was amplified by widely publicized lawsuits, indictments, and criminal convictions of health ministers and policy makers in the 1980s and 1990s (*l’affaire du sang contaminé* in France addressing HIV and Canada’s Royal Commission of Inquiry on the Blood System into blood collection agencies’ response to non-A, non-B hepatitis risk) (Fig. 118.1). (See box on Blood Safety Decision Making.)

Human T-Lymphotropic Virus-1 and Virus-2

Human T-lymphotropic virus-1 (HTLV-1) and HTLV-2 are closely related delta retroviruses with 60% to 70% sequence homology and shared tropism for T-lymphocytes. In contrast to HIV, HTLV is rarely present in cell-free plasma and shows little active replication in infected humans. HTLV-1 is distributed worldwide, with endemic foci in southern Japan, the Caribbean, certain parts of South America, Africa, the Middle East, and Melanesia. HTLV-2 is endemic among Amerindians in both North and South America and African Pygmies. An epidemic of HTLV-2 infections has occurred over the past 40 to 50 years among intravenous drug users in the United States, Brazil, and Europe. Transmission of both HTLV-1 and HTLV-2 occurs by parenteral exposures, sexual contact, and by vertical transmission from mother to child during pregnancy and breastfeeding.

Diseases associated with HTLV-1 infection include adult T-cell leukemia/lymphoma (ATL), HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP), lymphocytic pneumonitis, uveitis, polymyositis, and arthritis. HTLV-2 does not appear to cause hematologic malignancies but has been associated with HAM/TSP and linked to a higher rate of common infections such as acute bronchitis, pneumonia, and urinary tract infections, suggesting a subtle immunomodulatory effect of the virus.[46]

ATL occurs in only 1% to 5% of infected persons following a latent period of decades. The illness is characterized by malignant lymphocytosis and leukemia, lymphadenopathy, hepatomegaly, abnormal liver function test results, splenomegaly, skin lesions, bone lesions, and hypercalcemia (Chapter 89). HAM/TSP occurs in approximately 2% of individuals infected with HTLV-1 and HTLV-2. Patients with transfusion-associated HAM/TSP develop neurologic symptoms rather more rapidly, at a median of 3.3 years after transfusion. This illness is characterized by slowly progressive chronic spastic paraparesis, lower limb weakness, urinary incontinence, impotence, sensory disturbances, low back pain, hyperreflexia, and impaired vibration sense.

Blood donor screening for HTLV-1 antibodies began in 1988, and more sensitive combination HTLV-1/2 assays that detect close to 100% of HTLV-2 infections were introduced in 1998. The prevalence of HTLV confirmed-positive donors decreased approximately 10-fold during the 1990s and continues to decrease to the current value of 0.002%. The rate is three-fold higher in female compared with male donors. Incident infections are rare in repeat donors, an observation that has led to one-time testing of donors in some European countries, but an incidence of 3 per million donor years of follow up is felt by some to be too high for adoption of this strategy in the United States. Cell-free components such as plasma and cryoprecipitate do not transmit HTLV, and less than 30% of infected cellular components transmit. The residual risk for transfusion-associated HTLV infection using contemporary serologic testing is approximately 1 per 3.3 million donations, but since the initiation of testing using these contemporary tests, there has not been a breakthrough HTLV infection by transfusion. The contribution of blood-donor serologic testing to this low residual risk is confounded by the effect of effective leukoreduction that reduces HTLV-1 copy numbers in red blood cell (RBC) concentrates by up to 6 logs, to below the infectious dose of 10^7 to 10^8 infected cells per unit (one of a number of arguments used in support of universal leukoreduction of blood components).

BLOOD SAFETY DECISION MAKING

Not surprisingly, from the 1980s until now, donor deferrals and blood-testing interventions have been rapidly, successively, and additively implemented for emerging and theoretical risks. Collection facilities introduced antibody testing to the HBcAg of HBV (anti-HBc) and ALT testing as surrogates for non-A, non-B hepatitis, HIV-1 p24 antigen testing, then NAT for hepatitis C and HIV and subsequently HBV, extensive deferrals for the risks attending transmissible spongiform encephalopathies (TSEs), NAT for West Nile virus (WNV), and antibody testing for *Trypanosoma cruzi*, among many others. The cost-benefit estimates for some of these interventions exceeded generally accepted thresholds by orders of magnitude, but this did not deter their adoption. In general, the implementation of these measures was undertaken in practice and in cost by the blood providers, but it is unlikely that this reactive approach can be sustained in the current health care–reform environment and in the face of declining funding for blood providers, at least in the United States.[34]

After HIV entered the blood supply, application of a stringent adaptation of the precautionary principle (originally promulgated for environmental protection, not transfusion safety) pushed decision making toward avoidance of all risks. The precautionary principle promotes implementation of measures to mitigate risk even if evidence of a risk is incomplete. It is supposed to be tempered by proportionality; that is, any measures adopted are to be proportional to the risk and with those used in similar circumstances, but some have argued that this has not been the case with blood safety measures, at least by the metric of cost-benefit. Nevertheless, although in potential conflict with evidence-based decision making, this approach resonated with policy advocates charged with transfusion safety. In contrast, when the risk for transfusion transmission of variant Creutzfeldt-Jakob (vCJD) disease (the human form of bovine spongiform encephalopathy, BSE) emerged as theoretical, modeling was used to balance the perceived risk against the impact of extensive donor deferrals on the adequacy of the blood supply and to arrive at a policy decision. Some argue that the magnitude of risk does not justify the stringency of the donor deferral policy, given the small risk in a country that was not BSE-endemic or lacked any BSE-contaminated materials in their food supply, but the process was the first to attempt to balance risk with adequacy. Subsequently, vCJD was shown to be transfusion transmissible.[35]

While screening donors for a risk such as vCJD does not have a large negative impact on availability of donations because of its distant impact on most US blood donors, universal screening for other disease agents is not feasible or warranted. For example, screening all donations for *T.* and Zika virus based on geographic exposure or theoretic local exposure can cause significant reduction in the number of collections without adding much value. Universal donation screening for *T. cruzi* antibodies has been replaced with one-time testing with the assumption that a negative test remains consistent throughout the donor's life.[36] Zika screening was challenging as regions of the United States became endemic for the disease after its introduction during the pandemic in 2015 to 2016. Following the pandemic, Zika virus testing (by NAT) was reduced from universal individual donation testing to minipool NAT using a model very similar to that used for WNV.[37,38]

Hemovigilance programs, such as the Serious Hazards of Transfusion (SHOT) in the United Kingdom and others in Canada, France, and a smaller program in the United States, have emerged, supplying evidence about a much broader range of transfusion hazards than just infections.[39] For example, a data-driven decision to minimize plasma transfusions from potentially alloimmunized female donors resulted in a dramatic reduction in transfusion-related acute lung injury (TRALI) in the United Kingdom, and studies in the United States have reproduced this finding. These systems provide an opportunity for monitoring the risks and benefits of new initiatives, (e.g., proactive pathogen-reduction). Pathogen-reduction processes (see box on Pathogen Reduction) offer the opportunity to abrogate most of the residual risk for all of the historically important transfusion-transmitted viral infections, bacterial contamination of platelets, babesiosis and malaria contaminated red cells, WNV infections, and Chagas disease. Pathogen-reduction could eliminate the often lengthy, reactive, iterative paradigm of emergence of a new pathogen in the population, recognition of a material threat to transfusion recipients, development of donor-deferral strategies followed by development and refinement of test systems that has characterized our historical approach. Critically, broadly active pathogen-reduction processes offer a layer of protection against unsuspected emergence of new agents.[40] If already in use, they would need only to be validated as active against a new agent or appropriate model agents. The challenge with this approach is broad-based availability (all components) and wide-scale acceptance, the impact on product quality, and the potential long-term toxicities that may not be apparent in premarketing clinical trials or implementation to date.[41–43]

More recently, as cost pressures for health care increase, transfusion professionals are questioning whether the zero-risk paradigm remains relevant. A consensus conference held in Toronto in October 2010 addressed concerns about "safety at any cost" and inconsistent decision-making practices affecting the blood supply. This initiative has led to a definitive effort to establish a framework for risk-based decision-making under the direction of the Alliance of Blood Operators (www.allianceofbloodoperators.org/abo-resources/risk-based-decision-making/rbdm-framework.aspx). The process involves risk identification, risk assessment, risk management, and risk communication, along with an assessment of risk tolerance, in the context of full and open communication with stakeholders.[44] Risk will never be zero, and there is now a realization that cost considerations,[45] politics, ideology, and public opinion cannot be ignored.

The deferral, notification, and counseling of healthy blood donors after a repeatedly reactive screening test for HTLVs has been problematic, and tens of thousands have been affected since screening started. The vast majority of such tests are false positive; a licensed confirmatory test is now available and will greatly improve this situation.

HUMAN HERPESVIRUS INFECTIONS

Human herpesviruses (HHVs) are enveloped, structurally complex double-stranded DNA viruses that cause common infectious diseases. Primary infection is followed by lifelong carrier states and the possibility of reactivation. They are classified in three subfamilies, *Alphaherpesvirinae, Betaherpesvirinae*, and *Gammaherpesvirinae*, of which the latter two contain the herpesviruses that are of greatest concern from a transfusion medicine standpoint. Members of the *Alphaherpesvirinae* subfamily, herpes simplex viruses (HSVs) and varicella-zoster virus (VZV), are rarely, if ever, associated with transfusion-transmitted infections. Transfusion-transmitted cytomegalovirus (CMV) is well recognized. Transmissions of Epstein-Barr virus (EBV) and HHVs 6 to 8 by blood are virtually nonexistent in the United States because of the use of primarily leukocyte-depleted (leukoreduced) blood products.

Cytomegalovirus

CMV, a beta herpesvirus, infects a wide range of cell types, including leukocytes of the monocyte-macrophage lineage and their progenitors. In the United States, in the population-based National Health and Nutrition Examination Survey (NHANES) III study, 58.9% of individuals more than 5 years old were seropositive, indicating prior CMV infection. Prevalence increases with age and is dependent on race/ethnicity and other demographic factors. The prevention of transfusion-transmitted CMV infection has depended on the use of "CMV safe" blood including a combination of leukoreduced and CMV-seronegative blood. There is, however, some evidence that acute infection may result in plasma viremia and thus the potential for transfusion infectivity.

Primary CMV infection in immunocompetent individuals is usually community acquired, often asymptomatic or associated with a mild, self-limited infectious mononucleosis syndrome. After virtually all infections, latent virus persists permanently in cellular reservoirs, allowing lifelong reactivation, and in the setting of transfusion or transplantation, the potential for viral transmission via non-leukoreduced cellular blood products, and allografts.

In immunosuppressed patients, CMV infection can cause severe morbidity and mortality from pneumonitis, hepatitis, gastroenteritis,

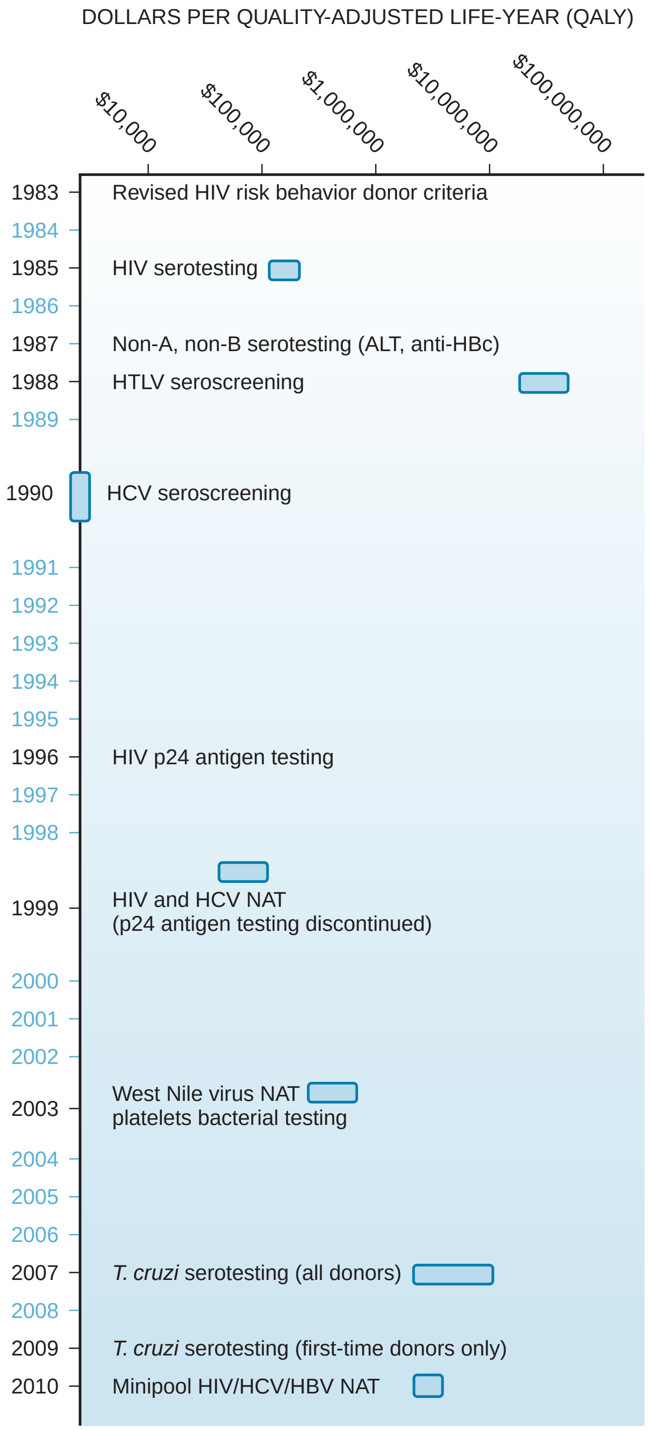


Figure 118.1 VARIOUS SAFETY MEASURES INTRODUCED DURING THE PAST THREE DECADES OF ENHANCED TRANSFUSION SAFETY. The X-axis displays costs associated with these advances per quality-adjusted life-year. Hepatitis C virus seroscreening saves costs. Estimates for platelet bacterial culturing and human immunodeficiency virus p24 antigen testing are not available. *ALT*, Alanine aminotransferase; *anti-HBc*, antibodies against HBcAg; *HBV*, hepatitis B virus; *HCV*, hepatitis C virus; *HIV*, human immunodeficiency virus; *HTLV*, human T-lymphotropic virus; *NAT*, nucleic acid testing; *QALY*, quality-adjusted life-year; *T. cruzi*, *Trypanosoma cruzi*.

retinitis, and other inflammatory conditions. CMV infection in low-birth-weight infants is associated with sepsis-like syndromes, respiratory distress, and liver and bone marrow dysfunction. Their infections can be acquired in utero, from breast milk, or from transfusion. Reported infant morbidity varies considerably but can approach 50% of seronegative low-birth-weight infants receiving unscreened, non-leukoreduced blood. CMV-seronegative marrow transplant patients are also susceptible to CMV infection, but the adoption of routine monitoring for CMV antigen or nucleic acid in transplant recipients and of preemptive antiviral therapy have decreased this risk (Chapters 94 and 110). Seronegative solid organ transplant recipients are also susceptible to symptomatic transfusion-transmitted CMV infections. CMV-seronegative, HIV-infected patients are another group at risk for transfusion-transmitted primary CMV infection. The historical risk for CMV infection in immunosuppressed recipients receiving CMV-unscreened, non-leukoreduced blood components varies from 13.5% to 53.3%. The residual risk for CMV infection in seronegative, immunocompetent patients who receive non-leukoreduced cellular blood components unscreened for presence of CMV antibodies is approximately 1%. CMV transmission strictly attributable to blood is rare to nonexistent with the combination of leukoreduction and CMV antibody screening. CMV antibody screening will be replaced by pathogen-reduction methods in the future.

Current practice in the United States is diverse. In a meta-analysis of 11 controlled trials in allogeneic marrow transplantation recipients, 12 of 829 recipients of seronegative units versus 24 of 878 receiving leukoreduced but unscreened units developed CMV infections.[47] Pooling of data from three studies of seronegative or leukoreduced components, in comparison with CMV unscreened components, demonstrated statistically identical 93.1% and 92.3% reduced risks of CMV infection, respectively. Thus, most clinicians consider leukoreduction and CMV seronegative blood to be equivalent; however, clinicians caring for neonates are more likely to require serologic screening. American Association of Blood Banks (AABB) recommends that each institution review its internal policies for blood use in patients vulnerable to CMV infection. The failure of clinicians to recognize recipients requiring CMV-safe transfusion and to request such components is an argument that has been advanced in support of universal pre-storage (versus bedside) leukoreduction.

CMV DNA blood donation screening by NAT may be considered an additional risk reduction measure for donors with seronegative window-period infections. However, current NAT assays would be expected to be equivalent to serologic screening in detecting latently infected blood donors. Any consideration of additional testing is complicated by the absence of randomized studies using contemporary leukoreduction methods and by our lack of understanding of the clinical impact of transfusion-transmitted CMV in an era of CMV monitoring and treatment. But with the advent of pathogen-reduction measures the need for any CMV donor screening will become obsolete. To this end, the Canadian National Advisory Committee on Blood and Blood Products released a consensus statement that leukoreduced products are equally safe as CMV-negative products in all populations except for intrauterine transfusion. Along with this statement, the Committee recommended discontinuing widespread CMV antibody testing and instead for hospitals to maintain a small inventory only for use in this special transfusion scenario.[48]

EPSTEIN-BARR VIRUS (HHV-4)

EBV, a gamma herpesvirus, is the causative agent of heterophile antibody-positive infectious mononucleosis and is etiologically associated with Burkitt lymphoma, nasopharyngeal carcinoma, and post-transplant lymphoproliferative disease (Chapters 59 and 87). Transfusion transmission of the virus is unusual because more than 90% of the adult population is infected, and second infection is prevented by host virus-specific cytotoxic T-lymphocytes, capable of lysing EBV-infected B lymphocytes when viral peptides are expressed on the lymphocyte surface. Rare cases of transfusion-transmitted EBV presenting as infectious mononucleosis have been described in immunocompetent recipients and in immunosuppressed patients following solid organ transplantation. Aggressive EBV-associated lymphoproliferative disorders have been observed in patients with immune injury after cord blood stem cell transplantation but have not been documented following transfusion. B lymphocytes are the likely source of transfusion-transmitted EBV infection, so leukoreduction is an attractive strategy to prevent infection in transfusion recipients. Nonclinical studies suggest that leukoreduction is capable of removing detectable EBV DNA from platelet concentrates and RBCs.

Human Herpesvirus-8

HHV8 is a gamma herpesvirus causally linked to Kaposi sarcoma, primary effusion lymphoma, and multicentric Castleman disease (Chapter 88). It is highly cell-associated with lymphocytes and monocyte-macrophages. Although generally spread person to person, including sexually,[49] it has been shown to be transmitted by transplantation. There is convincing evidence of HHV8 transmission by transfusion in sub-Saharan Africa, where the seroprevalence of HHV8 among blood donors is 40%, and fresh, non-leukoreduced blood transfusions are commonly used. The seroprevalence of HHV8 among US blood donors has been reported to be as high as 20% to 25%, but a multicenter study using well-characterized antibody tests on donations from five representative US regions estimated a seroprevalence of only 3.5% and showed no evidence of viremia by PCR. The possibility has been raised that transfusions in the United States have transmitted HHV8, but subsequently reported studies comparing transfused patients with un-transfused controls are not suggestive. Similar to what has been established for CMV infection, it is assumed that widespread leukoreduction in the United States mitigates the risk for HHV8 transmission by transfusion, but this has not been proven.

Other Herpesviruses (Herpes Simplex, Varicella-Zoster Virus, Human Herpesviruses 6 and 7)

Infection with these viruses is very common to ubiquitous. From 50% to 80% of the adult population is seroreactive to HSV-1 and/or HSV-2, up to 95% for VZV, more than 90% for HHV-6, and 70% to 90% for HHV-7. Primary infection is followed by lifelong latency for all HHVs, and reactivation from latency is described for all. There is no credible evidence of transfusion transmission of HSVs (causing orolabial and genital herpes and herpes encephalitis), VZV (chickenpox and shingles), or HHV-6 or HHV-7 (exanthem subitum or roseola infantum, multiorgan dissemination with reactivation in immunocompromised hosts).

Viremia occurs during both primary and reactivation infection with HSV and VZV, but transmission by blood has never been reported for these viruses. Although the lymphocyte association of HHV-6 and HHV-7, like CMV and EBV, suggests the possibility of transfusion transmission, well-documented case reports or series are not available. Case reports of HHV-6 transmission by hematopoietic stem cells used molecular methods to detect integrated donor HHV-6 DNA in engrafted donor cells and appear to represent transmission of latently infected cells rather than of active infection. Furthermore, transplant recipients may reactivate preexisting latent infection, thereby confounding the evaluation of potential transfusion transmission. HHV-6 and HHV-7 are highly leukocyte associated after primary infection, and leukoreduction would be expected to be effective by analogy to human CMV; however, cell-free viremia can be found during primary infection.

PARVOVIRUS

Human parvovirus B19 (erythrovirus) is a 19- to 23-nm–diameter nonenveloped, single-stranded DNA virus from the family *Parvoviridae*, as are adeno-associated viruses, human parvovirus 4 (PARV4), PARV5, and bocaviruses. B19 has at least three genotypes (Chapter 33). Genotype 1 infections appear predominantly in the United States and Europe. Genotype 2 infections appear confined to those born before 1973, and genotypes 3a and 3b infections occur in parts of West Africa. Infection with B19 is ubiquitous, with 50% of high school children demonstrating seropositivity, increasing to 90% in older adults. Approximately three-quarters of transfusion recipients have B19 IgG antibodies. Natural transmission occurs through the respiratory route most commonly and transplacentally to the fetus in up to 30% of women infected during pregnancy. Transfusion of blood and blood components, plasma derivatives, and organ transplantation are minor routes. In women infected during weeks 9 to 20 of pregnancy, fetal death occurs in 10% to 15% from hydrops fetalis. The low observed incidence of infection in transfusion recipients results from prior infection and immunity, co-infusion of neutralizing antibodies, and possibly because very high viral loads are uncommon in donor blood.

Parvovirus B19 causes erythema infectiosum, or fifth disease, oligoarthritis, and neurologic and myocardial infections. Following acute infection, viral replication leads to extremely high-titer viremia that declines at the time of IgM seroconversion approximately 9 days following infection. B19 may persist in bone marrow, liver, tonsils, and skin of healthy persons with no recognized clinical significance, and low levels of B19 DNA have been amplified from the blood of healthy people for several years after primary infection. The virus is highly tropic for erythroid progenitor cells using the P-blood group antigen, or globoside, as its receptor, causing aplastic crises in patients with sickle cell anemia, other inherited hemolytic diseases, and conditions associated with decreased RBC survival, malaria, and HIV/AIDS. Acute infection impairs erythropoiesis for 7 to 10 days, with complete cessation for 3 to 7 days, resulting in hemoglobin decreases (Chapter 33). Persistent infection, causing red cell aplasia, occurs in those who fail to develop neutralizing antibodies to the viral capsid protein 1. In these patients, the virus continues to circulate at high titer, greater than 10^{12} International Units (IU) or equivalent genome copies per mL. Patients at risk for persistent infection include those receiving chemotherapy and immunosuppression, organ transplant recipients, and those with HIV. Infection in these groups tends to respond favorably to intravenous immunoglobulin infusions.

B19 virus–impaired erythropoiesis appears to be multifactorial. B19 viral nonstructural protein 1 (NS1) mediates apoptosis via an 11-kDa protein that perturbs signal transduction and induces apoptosis. B19 viral DNA activates Toll-like receptor 9 inhibiting cell growth and is toxic for infected cells.

Blood and plasma-derivative transmission involves donations during the transient 1- to 2-week high-titer viremic period. The prevalence of B19 viremia has been reported as 0.003% to 0.84% in blood and plasma donors. In a study involving more than 12,100 B19 DNA–tested blood donations, given to surgical patients with a 78% pretransfusion B19 IgG seroprevalence, no B19 transmissions occurred from transfusions containing less than 10^6 IU per mL.[50] Hence most blood transfusion recipients are believed to be at low risk for an infectious exposure. However, careful studies in Japan have demonstrated some transmissions from components with comparatively low viral loads of around 10^5 IU per mL).[51,52]

By contrast, recipients of plasma-derived products made from manufacturing pools of thousands of donations have a significant risk because the virus survives partitioning, ethanol fractionation, and other anti-infective measures applied to derivatives, and a single high-titer viremic donation can overcome the neutralizing activity of antibody in the pool. Recipients of coagulation concentrates are at highest risk, whereas those of intravenous immunoglobulins and albumin are less so based on cohort studies of prevalence. In one report, 14% of viremic plasma donors had titers between 10^4 and 10^7 IU per mL, and one in 13,000 had titers greater than 10^7. In observational studies, seroconversion occurred among recipients of plasma derivatives with high titers, $10^{7.5}$ to $10^{8.5}$ IU per mL, treated with the solvent/detergent pathogen-reduction technique, suggesting that the protective effect of neutralizing anti-B19 antibodies is exceeded in the presence of high viral loads (see box on Pathogen Reduction). Since B19 is a nonenveloped virus, solvent/detergent pathogen-reduction treatment is ineffective in preventing B19 transmission in hemophilia patients receiving factor concentrates. Freeze-drying and dry-heat treatment (80°C [176°F] for 72 hours) inactivate more than 4.7 logs B19, but this is not always sufficient to prevent transmission.

PATHOGEN REDUCTION

Pathogen-reduction technology (PRT) offers a proactive strategy to address new threats; these technologies involve physical, chemical, and photochemical treatments of blood components to inactivate or decrease viral, bacterial, and parasite infectivity.[40,42]

Beginning in the 1980s, heat treatment, nanofiltration, and solvent/detergent treatment eliminated or reduced viral transmission in products derived from large-scale plasma fractionation such as albumin, immunoglobulin, and hemostatic factor concentrates.

In the past two decades, attention turned to whole blood–derived components: frozen plasma, platelets, and red cells. For these, PRT utilizes solvent-detergent, UV and methylene blue and visible light treatment for plasma; amotosalen (psoralen) and UVA light, and riboflavin (vitamin B_2) and UVB and UVA light for plasma and platelets; and riboflavin/UV light and S303 (a labile alkylating compound) for whole blood or red cells. Many European countries use PRT for platelets and plasma. Amotosalen/UVA-treated plasma and platelets (Intercept, Cerus Corporation) and solvent-detergent plasma (Octaplas, Octapharma) are FDA-approved and available in the United States. Because solvent-detergent does not inactivate nonenveloped viruses, Octapharma screens all incoming units in pools to prevent the introduction of HAV, HEV, and B19. Breakthrough HEV infections have been described with Intercept-treated plasma in France.[22]

Amotosalen/UVA (Intercept, Cerus) and riboflavin/UV (Terumo BCT) have been investigated to the greatest degree in North America. They provide significant antiviral activity against all agents for which tests are performed currently, human immunodeficiency virus (HIV), hepatitis B and C viruses (HBV and HCV), human T-lymphotropic virus (HTLV), West Nile virus (WNV), the parasite *Trypanosoma cruzi*, and cytomegalovirus, but the two methods appear to differ in their inactivation capacity. PRT also inactivates agents causing bacterial contamination of platelets; inactivates white blood cells to prevent transfusion-associated graft-versus-host disease; decreases formation and release of cytokines during storage, reducing febrile, nonhemolytic transfusion reactions; and abrogates white blood cell–induced alloantibody (e.g., human leukocyte antigen [HLA] antibody) formation mitigating alloimmune platelet refractoriness. Intercept-treated plasma (as mentioned) and platelets are FDA approved and available in the United States; clinical studies on pathogen-inactivated red cells (Cerus) and whole blood (Terumo BCT) are ongoing.

Methylene blue and visible light inactivate pathogens in plasma by targeting nucleic acids. However, this alters fibrinogen structure. Because it damages cell membranes, methylene blue is not used for platelets or red cells. It is not effective against hepatitis A or parvovirus and is not recommend for use in the treatment of thrombotic thrombocytopenic purpura.

Amotosalen/UVA targets nucleic acids. Platelets treated with these technologies have somewhat lower 1-hour post-transfusion corrected-count counts.[43] In clinical trials, mild and moderate bleeding frequency is increased, but not severe bleeding complications; the time between transfusions and the total number of platelet transfusions have not generally been different. Pulmonary toxicity similar to transfusion-related acute lung injury (TRALI) has been reported in clinical trials and in animal model experiments in which UV light has been implicated. Previous clinical trials in red blood cells (RBCs) were halted because of asymptomatic immunoreactivity against the red cell neoantigens believed to be the result of treatment and are being resumed with a reformulated process. Preliminary reports suggest riboflavin/UV causes functional impairment in red cells stored nearest the 42-day expiration Although there have been discussions about the potential for adverse reactions to treated products, extensive reviews of European data do not support the additional concern. Nevertheless, initial implementation of the Intercept platelet technology will be accompanied by Phase 4 studies in the United States.

Interest in PRT remains high because it reduces sepsis-related platelet transfusion complications and will eliminate the need for complex testing procedures to reduce bacterial risk related to contaminated platelets; inactivates parasites such as *Babesia microti* and *Plasmodium falciparum;* mitigates risks associated with recognized emerging pathogens such as dengue, chikungunya, and Zika viruses; and proactively decreases threats from unknown, emerging pathogens. It should be noted that inactivation capabilities differ greatly between the various technologies, and each must be evaluated for its intended use.

Currently, plasma derivative manufacturers use “in-process” NAT to identify and remove source (from paid and volunteer donors) and recovered (from volunteer whole blood donors) plasma units with the objective to assure that manufacturing pools have parvovirus B19 DNA titers no greater than 10^4 IU per mL. Approximately 1 per 10,000 plasma units is withheld from further processing. For volunteer whole blood donors, 1 per 6000 to 1 per 16,000 have titers greater than 10^6 IU per mL. Because individual whole-blood donations rarely cause recognized parvovirus infection, testing of single red cell units, platelets, or plasma for transfusion for B19 DNA is not currently required, although some advocate use of B19-tested blood components for those potentially at risk, including pregnant women, neonates, those with reduced RBC survival, and immunocompromised patients.

Of note, PARV4 and PARV5 have been detected in pooled plasma derivative samples, but the clinical significance is not known; limited clinical disease associations have been described.[53]

WEST NILE VIRUS

WNV is a mosquito-borne lipid-enveloped RNA virus in the Japanese encephalitis complex of the family *Flaviviridae*. Transmitted bird-to-bird primarily by culicine mosquito vectors, human infections occur incidentally. The first cases were identified in the West Nile district of Uganda in 1937. Subsequently, outbreaks occurred in the Middle East, South Africa, and Europe. The first North American cases appeared in New York City in 1999. Starting in July 2000, WNV cases spread to the Mid-Atlantic states. This was followed in the next 3 years by transcontinental dissemination. During the summer of 2002, a model suggesting a significant risk for WNV infection in blood donors was published,[54] and then four recipients of organ allografts from a single donor developed neuroinvasive WNV infection. Infection of the organ donor was traced to donor blood transfused before death from trauma. Transfusion transmission was documented in 23 recipients in 2002 against a background of 2946 cases of WNV-meningoencephalitis (WNME).[55] These events provoked a multidisciplinary effort, successfully engaging the transfusion medicine community, the manufacturers of blood donor–screening NAT platforms, and public health officials and regulators to develop and deploy, under an investigational new drug (IND) exemption, high-throughput screening tests in the 10 months before the 2003 transmission season began. WNV test implementation represents the benchmark for responsiveness to a serious emerging transfusion-transmitted infection in the United States.[56,57]

In 2003, 2946 WNME were reported and 714 healthy blood donors tested WNV RNA–positive. In 2004 case reports declined in the Northeast but extended westward into Arizona and Southern California. In 2005 more than 1300 WNME cases were reported, with approximately 25% occurring in California, relatively high activity in South Dakota, Nebraska, Louisiana, and Illinois, and low activity in the Northeast. In 2006 over 400 WNV RNA–positive blood donors were identified. Subsequently WNV activity was reported in Washington State for the first time, and high rates of infection were noted in Idaho, California, Nebraska, Illinois, Louisiana, and the Dakotas. By 2011 fewer than 150 blood donors tested positive with the highest frequencies in New York, Texas, Arizona, and California. The frequency of both WNME cases and RNA-positive blood donors continues to vary by year and remain unpredictable. In total, from 2002 to 2014 nearly 19,000 cases of WNME have been recorded with 4355 RNA-positive blood donors identified, which are related in magnitude by year.[38]

WNV RNA is believed to appear 1 to 3 days after a bite of an infected mosquito. In contrast to HIV and HCV, peak WNV levels are low (maximum observed 720,000 copies/mL, median 3500 copies/mL compared with 10^5 to 10^7 copies for HIV and HCV). In cohort studies of blood donors with positive WNV RNA tests, only 29% to 61% (retrospectively) describe any symptoms before or after donation consistent with WNV infection, compared with 3% to 20% of those not infected, demonstrating that the donor history is neither sensitive nor specific enough to prevent transfusion transmission.[58] Testing for antibodies also will not prevent transmission because IgM antibodies appear a median of 14 days and IgG antibodies a median of 17 days after infection and infectivity disappears rapidly with the development of antibody. These data explain the selection of WNV NAT for the testing strategy. To maximize efficiency, donation samples are tested in minipools containing aliquots from 6 to 16 donors, as is done for HCV, HBV, and HIV.

Despite minipool NAT, six transfusion-associated WNV cases occurred during 2003. The implicated donors were antibody-negative and had low-level RNA that escaped detection in their minipool (i.e., diluted) samples. At the end of 2003 an observation was made, even before the documentation of these transfusion transmissions, that the viral load in some donor samples is too low for detection by minipool NAT. Consequently, strategies have been developed to switch from minipool-NAT to NAT on individual donation aliquots (ID-NAT) in areas experiencing high WNV activity.[59] While ID-NAT year-round is cost prohibitive, its utility is likely to be greatest during WNV "season," that is June through December, in the United States. Alternatively, pathogen reduction would alleviate the residual risk of TT-WNV, thus further enhancing the safety of the blood supply.[60] In total, following the 23 cases of transfusion-transmitted WNV documented before the initiation of donation NAT screening, an additional 14 have been reported, most related to donations having only low levels of RNA; the last was in 2016. All implicated donations, except one in 2002, have been IgM negative. Blood centers must remain vigilant for appropriate conversion to individual donation-NAT for this strategy to be effective. A drawback of detecting RNA-reactive donations is that they are a lagging indicator and not an early warning system such as mosquito and bird activity, particularly during the onset of an epidemic period.[37]

DENGUE VIRUSES

This mosquito-borne infection is of great interest as an emerging pathogen with the potential to spread by transfusion. Forty percent of the world's population lives in areas with risk for dengue, including many areas visited by US travelers. It has spread rapidly in Latin America and the Caribbean since the 1980s. Dengue is endemic in Puerto Rico, the US Virgin Islands, and American Samoa, and there have been outbreaks in Hawaii, Texas, and Florida during the last 10 years. Dengue is caused by four related flaviviruses spread person to person by *Aedes aegypti* and *Aedes albopictus*, which are present in 16 and 35 US states, respectively. Over 2.3 million dengue clinical cases occurred in the Americas during 2015.

Most infections are asymptomatic, but illness ranges from undifferentiated fever to classic break-bone fever and severe dengue (dengue hemorrhagic fever and dengue shock syndrome). An approximately 7-day viremia is a feature of both asymptomatic and symptomatic infection, and asymptomatic blood donors from Hong Kong, Singapore, Brazil, and Puerto Rico have transmitted dengue to blood recipients in seven clusters. Although such reports are limited, compared with the high rates of vector-borne infection, there is no systematic surveillance for transfusion-transmitted dengue and its recognition in the face of widespread outbreaks is problematic.[61] RNA-positive, asymptomatic donors have been identified in Brazil, Central America, and Puerto Rico using NAT and antigen detection tests. Rates of donor RNA positivity in Puerto Rico are comparable to those found in US donors during the most active WNV seasons.[62,63] A recent study in Brazil documented transfusion transmission; however, when patient charts of those donors who received an RNA-positive unit and were shown to be infected were compared with control recipients who did not receive an RNA-positive unit, there was no reported increase in clinical illness.[64] This further illustrates the difficulty in identifying transfusion-transmitted dengue among severely ill patients who are frequently transfused.[63]

In the absence of significant outbreaks of locally-transmitted dengue in the continental United States, transfusion risk relates mainly to return of infected, asymptomatic or pre-symptomatic travelers to the United States from endemic areas. A 3- to 14-day incubation period precedes symptom onset. Deferral for travel to malarious areas (1 year for a US resident) offers some protection, but a large proportion of dengue-affected areas frequently visited from the United States are malaria-free and donor-travelers to those areas could potentially introduce the virus into the community and the blood supply. Careful surveillance and a high index of suspicion when sustained febrile illness occurs following transfusion are required to recognize transfusion-related dengue. Preliminary data on travelers from the United States to dengue-endemic destinations that are malaria-free suggest that 2- to 4-week deferral for travel to dengue-affected areas may have a greater adverse impact on blood donation than current malaria deferrals. To date, however, there are no instances of transfusion-transmitted dengue in the continental United States that are attributable to donor exposure through travel. The conditions for sustained spread of dengue exist in large areas of the United States: a source of infection from travelers and immigrants; a susceptible population; and competent vectors. Whether the recently identified outbreaks in the United States will continue or increase, and whether sustained transmission will begin is unknown. Transfusion-transmitted dengue has been identified by one group as one of the three highest-priority emerging infections posing a potential threat to transfusion recipients in the United States and Canada and has been the subject of discussions at the FDA's Blood Products Advisory Committee (see boxes on Emerging Infections and Xenotropic Murine Leukemia Virus–Related Virus: A Lesson Learned).

EMERGING INFECTIONS

The experiences with human immunodeficiency virus (HIV), new variant Creutzfeldt-Jakob disease (vCJD), and West Nile virus (WNV) have made it clear that planning for the emergence of new pathogens that may threaten the blood supply is critical in order to shorten the interval from emergence to mitigation. To that end, the characteristics of pathogens likely to enter the blood supply must be understood. These include the ability of the agent to establish an asymptomatic blood-borne phase, to survive under contemporary processing and storage conditions, to establish infection by the intravenous route, and to cause significant morbidity in a transfusion recipient.

Using these characteristics and a review of contemporary literature, the Transfusion Transmitted Diseases Committee of the American Association of Blood Banks (AABB), from 2005 to 2009 produced a compendium of infectious agents that might become relevant to transfusion medicine and attempted to prioritize those that were identified.[65] A series of 68 fact sheets was developed. The fact sheets provide transfusion medicine professionals and clinicians with an overview of the agents' phylogeny, epidemiology, clinical characteristics, and interventions that might be useful to protect the safety of the blood supply. Three agents were included in the highest priority stratum: *Babesia* spp., dengue viruses, and the vCJD prion; a section on each is included in this chapter. The fact sheets are freely available online, cover many of the agents discussed in this chapter, and have been updated when required (www.aabb.org/tm/eid/Pages/eidpost-pub.aspx).

Since the original publication, several new monographs have been added, reflecting an ongoing "horizon-scanning" initiative designed to alert the medical community to potential threats. These include yellow fever and the yellow fever vaccine viruses after the latter was transmitted to blood recipients when recent vaccine recipients failed to divulge that information during blood donation. Other fact sheets have been updated and rereleased to capture relevant new information (see box on Xenotropic Murine Leukemia Virus-Related Virus). The availability of these materials to the transfusion medicine and general medical communities is meant to increase awareness that unexpected infections in transfusion recipients should trigger consideration of that source and to provide background allowing the rational consideration of approaches to avert and minimize their spread.[66]

XENOTROPIC MURINE LEUKEMIA VIRUS–RELATED VIRUS: A LESSON LEARNED

The alleged association of the gamma retrovirus xenotropic murine leukemia virus–related virus (XMRV) with chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) is paradigmatic of the difficulties inherent in the real-time assessment of potential and emerging transfusion-transmissible infections. A group of CFS/ME patients and controls were evaluated using polymerase chain reaction (PCR), virus isolation, serology, and immunohistochemistry.[67] Evidence of the virus's presence was strongly associated with the clinical diagnosis, culturable virus was found in plasma, and the issue of transfusion transmission was appropriately raised.

Multiple subsequent studies from other groups over the ensuing 2 years failed to confirm the presence of XMRV in a variety of populations. Several publications suggested that PCR contamination underlies some positive results; however, data now indicate that XMRV arose as a recombinant of two murine leukemia viruses during passages in nude mice, leading to the conclusion that the initial findings were an artifact.[68] With these findings, the original publication was retracted. The absence of transfusion transmission and of the agent were also demonstrated in studies on the donor pool.[69] The amount of research and resulting publications on this recombinant laboratory-generated virus were simply another lesson in caution that is required prior to claims of a viral agent being a human pathogen, or in this case, being the result of recombinant contamination.

CHIKUNGUNYA VIRUS

Chikungunya virus is another tropical arbovirus transmitted by *Aedes* spp. mosquitoes. It is a togavirus of the alphavirus group and, although first recognized in Africa has been notably responsible for explosive outbreaks in the islands of the Indian Ocean and has most recently spread to the Caribbean, where more than 1.7 million clinical cases were reported from the end of 2013 to the middle of 2015, including RNA positivity in blood donors.[70] Although there have been no reported cases of transmission by transfusion, the similarity of early infection to that of dengue has resulted in significant concern. Notably, French authorities responded to the outbreak in La Réunion by halting local collection of red cells (providing for the island's needs by supplying blood from the French mainland) and by implementation of limited NAT and the use of pathogen reduction for platelets.[71] Other precautions that have been used have been to strengthen requirements of post-donation information from donors (a process that is enhanced by the high [50% to 80%] frequency of symptoms among infected subjects), along with deferrals for residence in affected areas. Chikungunya symptoms are similar to those of dengue, but without the impact upon the circulatory system. Arthralgia is, however, a prominent symptom and may be prolonged. Routine testing is not currently available.

ZIKA VIRUS

Zika virus is another tropical arbovirus of the flavivirus group. Again, first recognized in Africa, it has been circulating in the Pacific islands at relatively modest levels. In 2007 the first human outbreak was recognized on the island of Yap in Micronesia with subsequent spread in 2013 to French Polynesia.[72] Testing performed in Polynesia showed 2.8% of presenting donors to have Zika virus RNA. Within 2015 Zika virus and associated disease emerged in Brazil and began spreading beyond other South and Central American countries, Mexico, and the Caribbean including travel-associated cases in the United States.[73] By early 2016, the World Health Organization declared the Zika outbreak a Public Health Emergency of International Concern to increase surveillance and vector control and to expedite the development of diagnostics, treatment, and vaccine development. Given the similarity to and relationship with dengue, there are concerns about the spread of Zika virus and local transmission in the continental United States. At the height of the Zika epidemic in 2016, the number of cases within the US reached over 5100 and cases within US territories numbered over 36,500.[74] A particular concern is that infection in pregnant women is associated with an unexpectedly high and troubling incidence of microcephaly and other developmental abnormalities in fetuses.[75,76] Unlike the other mosquito-borne viruses, Zika appears to be sexually transmitted (and can be recovered from urine and semen).[77] Other than mosquito transmission, like dengue, transfusion transmission has been reported. As a result, the US FDA implemented universal, individual donation NAT for Zika virus RNA in 2016. The guidance was updated in July 2018 to allow for minipool NAT until certain triggers are met for individual testing based on regional virus activity or unless pathogen reduction technologies are used.[78]

SARS-COV-2 (COVID-19)

In late 2019, a novel coronavirus, SARS-CoV-2, was identified in the Wuhan province of China (Chapter 153). The disease associated with the virus (coronavirus disease 2019; COVID-19) ranges from asymptomatic to severe infection and has spread rapidly throughout the entire world, causing over 243 million cases of COVID-19 and over 5.0 million deaths, as of the time of writing, in an ongoing pandemic. Reports have shown detection of RNA in blood products,[79] however no evidence of transmissibility of SARS-CoV-2 through blood transfusion has been seen.[80,81] Despite this, the transfusion medicine community has remained vigilant of the ongoing developments and mutations of the virus, its associated disease, and potential blood product therapies. Antibody screening of all donations has been approved by the FDA to determine if a blood donor has had an antibody response to SARS-CoV-2 exposure, with many large blood collection organizations providing antibody-positive blood products to hospitals to treat patients with convalescent plasma, currently approved through EUA. Seroprevalence studies show a range of the US population with detectible antibodies from 1% to 23% in the general population and in 1.82% in the blood donor population.[82,83]

PANDEMIC INFLUENZA A

There was interest in the transfusion safety implications of influenza A even before the swine origin A-H1N1 pandemic of 2009 to 2010.[84] Viremia in donors, and the potential for infection of recipients, were discussed during meetings of an AABB pandemic influenza planning task force as early as 2008, based on old literature demonstrating the virus in the blood of experimentally infected volunteers and case reports of viral dissemination during human infection with highly pathogenic avian A-H5N1 virus. Subsequent studies have failed to identify influenza viruses in donors drawn in US communities during periods of high seasonal influenza activity or in donors with the onset of influenza-like illness immediately following donations.[85] The current consensus is that pandemic influenza is not likely a threat to blood recipients, but that disruption of the blood supply in a severe pandemic is a more important possibility, requiring careful advance planning.

BACTERIAL INFECTIONS

Cellular Product Contamination

Bacterial contamination of red cell and platelet components occurs primarily as a result of the inability to achieve complete skin disinfection at the donor phlebotomy site, where bacterial populations survive in skin appendages below the accessible surface and contaminate skin plugs made by the phlebotomy needle and enter the blood bag. Transient asymptomatic bacteremia at the time of blood donation, a break in technique during pooling the contents of or sealing blood containers, disruption of blood bag integrity or its contamination during manufacturing are other rare sources. Bacterial proliferation occurs more rapidly in platelet concentrates stored at room temperature than in red cells maintained at 4°C (39.2°F). As such, the FDA published updated recommendations in December 2020 to control the risk of bacterial contamination of products stored at room temperature.[86]

The rate of bacterial contamination in allogeneic platelets was estimated at 1 per 1000 to 3000 units with life-threatening septic reactions occurring in one per 15,000 to 100,000 recipients before routine implementation of culture-based tests to detect bacteria in apheresis platelets in 2004. These tests reduced contamination rates to approximately 1 per 5000 to 8500 platelet units. Septic reactions occur in approximately 30% of recipients receiving contaminated platelets. Bacterial contamination was responsible for 6 of 50 (12%) of transfusion-related deaths reported to the FDA in FY2018.[87] Prior years, including the period between 2014 and 2017, saw a smaller percentage of fatalities due to contamination of products, however, there is consensus that transfusion reactions are badly under recognized and under reported. Culturing fails to detect 50% to 75% of contaminated units. This undetected contamination occurs primarily when minimal bacterial levels are present when cultures are obtained, yielding culture aliquots without bacteria. Subsequently the few bacteria remaining in the blood bag can presumably grow out during storage, producing transfusions with significant bacterial levels. To minimize this, collection facilities generally hold apheresis platelets for approximately 24 hours after collection before sampling to allow low bacterial inocula to proliferate, improving the sensitivity of culture. As mentioned, improved skin antisepsis and diversion for laboratory testing of the first 40 mL of blood, which contains the skin plug, provide further reduction of contamination rates, transfusion reactions, and fatality rates by lowering the bacterial load that otherwise would enter the final component container.

Septic transfusion reactions should be suspected when one or more of the following occur within 1 to 4 hours of transfusion: temperature elevations greater than 1°C to 2°C (1.8°F to 3.6°F), chills and rigors, tachycardia more than 120 beats/min or an increase of 40 beats/min, or an increase or fall in systolic blood pressure of greater than 30 mm Hg. Additional signs and symptoms can include nausea, vomiting, diarrhea, bleeding, oliguria, or septic shock. Septic transfusion reactions may be confused with hemolytic transfusion reactions, febrile, nonhemolytic transfusion reactions, or TRALI.

Platelet concentrates contaminated with *Staphylococcus aureus*, *Serratia marcescens*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Streptococcus* species account for most clinically recognizable reactions. *Propionibacterium acnes* is a frequent culture isolate from platelets during storage when an anaerobic culture is also performed but is not believed to be responsible for a significant number of reactions. A clinically relevant case of *Clostridium perfringens*, another anaerobe, has been reported.[61] The bacterial dose in platelet concentrates and species virulence correlate with the clinical outcome. Bacterial concentrations greater than 10^5 colony-forming units (CFU)/mL are more likely to result in severe reactions or fatalities. Alternatively, low concentrations, 10^4 or fewer CFU/mL of skin organisms, frequently do not elicit reactions. Endotoxin elaboration by gram-negative organisms is associated with the most severe reactions, but the vast majority of gram-negative organisms are easily detected by bacterial culture.

In addition, bacteria grow at variable rates. *S. epidermidis* grows slowly, and *P. acnes* requires on average 3 days for detection. Because collection agencies continue bacterial culturing until the platelet unit expiration date or detection of growth, there is further opportunity to intercept released platelets that have not been transfused and to alert physicians about the potential risk for bacterial contamination in cases where the units have been administered. However, the vast majority of septic reactions are associated with false-negative culture tests because of sampling limitations as noted earlier. The overall rate of bacterial contaminated apheresis platelets is 1 per 5000 and for septic transfusion reactions is 1 per 107,000 distributed components.[88]

Before the mid-1980s, platelet concentrates were stored for 7 days. Following reports of septic reactions, the shelf life was reduced to 5 days to decrease the interval during which bacteria could proliferate. In the United States, blood suppliers distributed platelets from 2005 to 2008 with a 7-day shelf life following enrollment in an FDA-mandated post-market surveillance study (Post Approval Surveillance Study of Platelet Outcomes, Release Tested [PASSPORT]) that included testing for bacterial contamination by a sensitive culture method. Initial experience suggested that extended platelet storage coupled with bacterial testing substantially decreased platelet loss from outdating. However, the PASSPORT study found 1 per 4329 tested platelet products with negative bacterial cultures had bacterial growth when tested at the end of storage, 7 days later.[89] Septic transfusion reactions occurred in approximately 1 per 25,000 platelet transfusions, some following infusion of 3-day stored platelets, most with those stored 4 days or longer, leading to reinstatement of the 5-day platelet shelf life. A recent report of a cluster of four *Acinetobacter calcoaceticus/baumannii* complex septic transfusion reactions involving apheresis platelets includes one that occurred following pathogen reduction. Co-component sterility, successful inactivation of the cultured isolate, detection of photoproduct residuals in the treated platelets, and failure to amplify mitochondrial DNA in the treated platelets all suggest successful pathogen reduction. Unidentified environmental sources and inherent or acquired bag defects may have contributed to bacterial contamination following successful pathogen reduction.[90] Of significance, each of the four septic transfusion reactions that were part of this cluster were caused by genetically related strains of *Acinetobacter*, none of which implicated the apheresis platelets.[91] Several additional bacterial mitigation methods were used for the platelets in this case cluster demonstrating that even with the most stringent mitigations, risk still occurs. Additional mitigations included aerobic and anaerobic culture (one case), point-of-issue testing (two cases), and pathogen reduction (one case).

Alternative methods for detecting bacterial contamination include point-of-release testing in the hospital transfusion service. Considered a supplement to culture-based testing, this assay can be used shortly before issuing platelets for transfusion to detect units missed by early culture. This rapid qualitative immunoassay tests for the presence of conserved bacterial antigens (gram-negative lipopolysaccharide and gram-positive lipoteichoic acid). In one report using this test, 1 per 3000 platelet doses contained gram-positive organisms when issued 3 or more days after collection.[92] Other methods, under development for mitigating septic platelet events, include microcolorimetry, bacterial spore biosensors, real-time PCR, flow cytometry, and detection of other bacterial cell wall constituents. Pathogen-reduction technology using licensed psoralens (Intercept) or riboflavin plus UV light provides an opportunity for inactivating multiple pathogens before they can proliferate. Not all technologies, however, inactivate different bacterial isolates with the same efficiency.

RBC bacterial contamination rates approximate 1 per 30,000 units with adverse clinical outcomes occurring in 1 per 500,000 transfusions and fatalities occurring at a rate of 1 per 10 million. Most septic reactions occur in units stored for 4 weeks or longer, reflecting the delayed growth of bacteria at 4°C (39.2°F).

Bacteria isolated from contaminated red cell units include *S. marcescens*, *E. coli*, *Pseudomonas* species (especially *Pseudomonas fluorescens*), and *Yersinia enterocolitica*. The latter two are psychrophilic and known to proliferate in the cold. Gram-positive skin saprophytes account for most of the organism-contaminating platelet concentrates, with the remaining attributed to gram-negative organisms associated with occult bacteremia.

Immediate recognition of a platelet or red cell septic reaction and immediate discontinuation of the transfusion, supportive care, and antibiotic administration are the mainstays of therapy. Prevention relies on careful donor selection and scrupulous adherence to aseptic techniques and precautions from component collection, processing, transport, and storage to transfusion at the bedside. The Gram stain reveals bacteria in platelet concentrates contaminated with more than 10^5 to 10^6 CFU/mL.

A proportion of donors of platelet units contaminated with *Streptococcus bovis* or *Streptococcus infantarius* have been found to have colonic polyps or colon cancer.

SPIROCHETE INFECTIONS

Syphilis

US blood banks first screened donors with a serologic test for syphilis in 1938, and screening has been a regulatory requirement since 1958. It was the first test mandated for US donors and stood alone before the discovery of the Australia antigen for hepatitis almost two decades later. More than 100 transfusion syphilis cases were published before World War II, and many more certainly occurred. The last alleged case of transfusion-transmitted syphilis in the United States, however, was in 1966. Since then, hundreds of millions of components have been transfused in this country without another recognized case. There are multiple explanations for the disappearance of transfusion-transmitted syphilis in addition to improvements in testing: (1) the dramatic decline in the incidence of early syphilis in the United States over the decades, more than an order of magnitude, reducing the reservoir of donors able to transmit; (2) the end of direct donor-to-recipient transfusion combined with the loss of viability of *Treponema pallidum* in stored blood—the latter attributed to poor survival in refrigerated RBCs and to the high oxygen tension in platelets stored at higher temperatures; (3) the ubiquitous administration of antibiotics for trivial to serious viral, bacterial, and noninfectious clinical syndromes, especially to those sick enough to be transfused; (4) donor deferral for behavioral correlates of syphilis risk (e.g., male sex with males, occurrence of recent sexually transmitted infection in donors, exchange of drugs or money for sex, intravenous drug use); (5) passive surveillance for transfusion-transmitted syphilis compounded by the failure of recognition by physicians (who may have never seen syphilis, venereal or otherwise); and (6) donor illness during spirochetemia severe enough to prevent their presentation to donate blood or causing deferral. The relative contributions of each of these factors are unquantified.

Most blood collection facilities screen donors with an automated test for detecting treponemal antibodies (e.g., *T. pallidum* hemagglutination assay and *T. pallidum* particle agglutination assay); treponemal antibody confirmatory test usually follows using a variety of methods such as enzyme-linked immunosorbent assay (ELISA) or fluorescent treponemal antibody, absorbed (FTA-abs) test; most blood centers further test donors with treponemal antibody reactivity using a nontreponemal test (e.g., rapid plasma reagin [RPR]) to stage the reactivity as recent or past. Approximately 50% of donors with confirmed positive treponemal test results have evidence of a treated syphilis infection. Donations with reactive syphilis tests are generally discarded. Blood centers defer donors with positive treponemal confirmatory tests; donors may be reentered (regardless of their nontreponemal test results) 12 months after completion of treatment for syphilis. Treponemal and nontreponemal false positivity occurs in healthy donors caused by cross-reactivity with a wide variety of infectious diseases other than syphilis, in those with high-titer human leukocyte antigen (HLA) antibodies, after some immunizations, with autoimmune disease and other chronic inflammatory diseases, and have increasing prevalence with increasing age.

Most true treponemal antibody positives are the "serologic scar" of remote (often treated) infection and pose no risk to blood recipients. A recommendation to discontinue donor screening for syphilis was made in 1985 but not acted upon initially because of the perception that testing might be functioning as a surrogate for HIV or other transfusion-transmissible viruses.[93] This hypothesis has been rejected, but, because the role of syphilis testing in the elimination of transfusion syphilis is unclear, syphilis testing continues even though it has no apparent value. Although small PCR studies of reactive donors have been universally negative, rabbit inoculation studies using samples from confirmed reactive donors will likely be required to eliminate syphilis testing, and it seems unlikely the resources will be found to do a study of the required size.

Lyme Disease

Borrelia burgdorferi is the spirochete that causes Lyme disease, the most common tick-borne infection in North America and Europe. The agent was discovered in 1977 during investigations of an arthritis cluster in Connecticut children. Cases reported to the US CDC doubled between 1992 and 2006 to almost 20,000, continued to rise and have, since 2009, stabilized with annual reporting of confirmed cases between 22,000 and 29,000 (https://www.cdc.gov/lyme/datasurveillance/index.html, Lyme Disease Data and Surveillance). Geographic distribution of cases is highly focused, with the majority of reported cases occurring in 13 northeast, Mid-Atlantic, and north central states. *Ixodes scapularis*, the black-legged tick, transmits *B. burgdorferi* in those areas, and *Ixodes pacificus*, the western black-legged tick, transmits the infection along the Pacific coast. The ticks feed predominantly in late spring and early summer during the nymph stage. Emergence has been associated with environmental changes that increase deer and rodent reservoir populations and changing residential patterns putting humans in more intimate contact with the tick vectors. Rodents are the reservoir. Although deer are not infected, they transport and maintain the ticks. Birds may also play a role in transporting the vector ticks.

The characteristic erythema chronicum migrans ("bull's-eye") rash is present in 70% to 80% of cases within 30 days of infection. The wide variety of clinical findings includes malaise, fatigue, headache, myalgias, large joint arthralgias and arthritis, and neurologic and cardiac signs and symptoms. These may not immediately suggest Lyme disease in the absence of a typical rash or known tick exposure. Serologic testing supports the clinical diagnosis. Unfortunately, poor specificity is recognized with many screening methods, and a two-step approach using a sensitive EIA or immunofluorescence assay (IFA) followed by a confirmatory, more specific, immunoblot is recommended by the CDC.

B. burgdorferi spirochetemia occurs in 44% of patients with clinical Lyme disease and peaks 7 to 10 days after tick bite. The spirochete can survive in red cells, platelets, and frozen plasma for at least the duration of their routine storage. Transfusion transmission of *B. burgdorferi* has been demonstrated in a murine model. Despite these observations, no human transfusion-associated cases have been reported. This may reflect the relatively short spirochetemic phase and low levels of organisms, as well as deferral of donors with nonspecific illness or signs and symptoms of Lyme borreliosis. Although there are no official standards or guidance, it would be prudent for individuals with a history of Lyme disease to be deferred until well and treatment has been completed.

Other Tick-Borne Bacteria

Borrelia miyamotoi, the agent responsible for tick-borne relapsing fever, is transmitted by the same vector as Lyme disease, described above. First isolated and described in 1994, the spirochete has been responsible for influenza-like disease with relapsing episodes of spirochetemia and fever in Asia, Europe, and North America. To date, no transfusion-transmission of *B. miyamotoi* have been shown in humans. Two published studies have suggested a possible risk of human blood transfusion transmission by showing *B. miyamotoi* survival in human RBCs and platelets, as well as transmission in a mouse model.[94,95]

The rickettsia that cause human monocytic ehrlichiosis (HME) and human granulocytic anaplasmosis (HGA; formerly human granulocytic ehrlichiosis) are intracellular bacteria that survive in stored blood. *Ehrlichia chaffeensis* causes HME and is transmitted to humans by Lone Star tick (*Amblyomma americanum*) bites. Most cases occur in the south central and southeastern United States. *Anaplasma phagocytophilum* causes HGA and occurs predominantly in the northeastern and upper midwestern areas of the United States. *I. scapularis* and *I. pacificus* (the Lyme borreliosis vector) transmit the organism. Signs and symptoms include fever, chills, and headache, often associated with thrombocytopenia, leukopenia, and increased liver enzyme levels. Intracellular aggregates of microcolonies of bacteria referred to as morulae appear in monocytes in the HME, and inclusions appear in granulocytes in HGA. Seroprevalence studies in blood donors in endemic areas report 0.5% to 11.3% seropositivity for *A. phagocytophilum* antibodies. At least nine cases of transfusion-transmitted HGA from asymptomatic donors have been recognized after nonleukoreduced RBC transfusion; however, cases have occurred as well following leukoreduction including one from apheresis platelets.[96] Both *Ehrlichia* and *Anaplasma* spp. are white blood cell–associated, and it has been suggested that leukoreduction may mitigate their impact. The infectivity of *Orientia tsutsugamushi*, the rickettsial agent of scrub typhus, is reduced by up to 10^5 by filtration leukoreduction, but infectious *E. chaffeensis* survived in refrigerated red cells and could be isolated from the packed RBCs after filtration.

In a study of heavily tick-exposed military recruits in Arkansas, which included a look back to 10 recipients of transfused units from soldiers infected with *E. chaffeensis* and *Rickettsia rickettsii* (the agent of Rocky Mountain spotted fever [RMSF]), clinical illness did not occur in recipients. One possible RMSF seroconversion was reported. There is a single case report of transfusion-associated RMSF that involved a donor developing symptoms 3 days post-donation. The recipient developed symptoms 6 days post-transfusion.

PARASITIC INFECTIONS

Malaria

Five *Plasmodium* species (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi*) and occasionally others cause the protozoan disease malaria. In 2008 243 million cases occurred worldwide, with most in sub-Saharan Africa. Other endemic areas include parts of Asia and South America and more limited areas in Mexico, Central America, and the Caribbean. Of 1478 imported cases in the United States in 2009, 735 were acquired in Africa, 142 in Asia/Western Pacific, and 103 in the Americas.[97] West Africa accounted for most cases associated with Africa, and Honduras, Haiti, and Guyana for most of the cases from the Americas. During 2009 only two cases were acquired in Mexico, both *P. vivax*. In 2003 eight autochthonous infections occurred in Palm Beach, Florida, but none subsequently. Vectorial transmission, overwhelmingly the most common route, is by the bite of an infected female *Anopheles* mosquito. *Anopheles* mosquitoes generally feed between dusk and dawn, thereby limiting the risk to tourists who usually visit malaria areas during daylight hours. Almost three-quarters of those who acquired malaria were visiting friends or relatives in endemic areas. Missionaries and business travelers represent fewer than 20% of infected US citizens. Leisure travelers make up the small remainder.

P. falciparum accounts for 40% to 46% of US cases detected recently, *P. vivax* 11% to 20%, and *P. malariae* and *P. ovale* approximately 2% each. For the remainder, most are undetermined with a few mixed-species infections. *P. knowlesi* has not yet been reported.

Seventy percent of imported cases in the United States occur among returning travelers who were visiting friends or relatives, ethnically and racially distinct from the majority population of the United States (where malaria is not endemic), who return to their homelands (countries where malaria is endemic) to visit friends or relatives. The region of origin is very relevant to measures designed to prevent malaria transmission by transfusion because many of these individuals are semi-immune and therefore can be parasitemic while asymptomatic. In contrast, donors without malarial immunity who travel to endemic areas and become infected are nearly always symptomatic when they are parasitemic and would not be accepted as blood donors. Malaria symptoms occurred within 1 month of arrival in the United States in 84% of those infected with *P. falciparum* and 56% of those with *P. vivax* in 2009. The latter represents almost all cases of relapsing malaria. No acute onsets involving the four most frequent *Plasmodium* species occurred at an interval greater than 1 year.

Slightly over one hundred transfusion cases have been recognized and reported in the United States since 1963. Since 2000 there have been only 11 cases reported. In the majority of cases, transmission would have been prevented had the present donor deferral criteria been appropriately applied.

The incubation period for transfusion-associated malaria ranges from 8 to 90 days. *P. falciparum* has the shortest incubation time, mean 17 days (range, 8 to 36 days) and *P. malariae* the longest, mean 50 days (range, 8 to 90 days). Most cases involved RBC or whole-blood transfusion, although a few transmissions from platelet transfusions have occurred, presumably related to RBC contamination. Transmission from frozen plasma has not been reported.

Current strategies for reducing the low risk for transfusion transmission in the United States involve screening donors through the donor history questionnaire and deferral of residents of non-endemic areas who have traveled to malaria-endemic regions, as defined by the *CDC Health Information for International Travel* document, during the previous 3 months (with exemption for those donating products which will be pathogen-reduced), those with residence in endemic areas for 3 years since their last potential exposure in such regions, and those with a history of malaria for 3 years after resolution.[98] Apart from incomplete elicitation of malaria risk history, residual risk remains because transfusion transmissions of *P. falciparum*, *P. vivax*, and *P. ovale* have been reported 13, 27, and 7 years, respectively, after departure from malarious areas. Because *P. malariae* infection can persist for more than 70 years without symptoms, elimination of transfusion-induced malaria is a practical impossibility. At this time, licensed in vitro screening for at-risk donors using antibody tests, antigen detection, or NAT are unavailable in the United States. It is not clear that the limited commercial potential of assays to be used on a relatively small fraction of donors will justify the investment required to pass the stringent regulatory requirements of a blood donor screening assay. Donor deferral guidelines have been modified in 2014 to reduce the loss of more than 60,000 potential donors with travel to areas of Mexico where the malaria risk is minimal.[99]

Babesiosis

Babesia species that infect humans include *Babesia microti, Babesia duncani* (previously WA1 type), *Babesia divergens* (limited primarily to Europe), *B. divergens*–like (MO1 and EU1), and *Babesia venatorum. B. microti*, an intraerythrocytic protozoan, causes most human infections. The white-footed mouse, *Peromyscus leucopus*, serves as the reservoir and the deer or black-legged tick, *I. scapularis* (also the vector of Lyme borreliosis and HGA), as the vector. Transmission follows bites from infected ticks, primarily nymphs, from May through early September. The defined transmission period has less relevance for transfusion transmission because asymptomatic blood donors can be infected chronically.[100] Most cases occur in seven endemic states, including Massachusetts and its coastal islands, Rhode Island, Connecticut, New York, New Jersey, Wisconsin, and Minnesota. This geographic range has expanded recently, attributed to expansion of white-tailed deer, *Odocoileus virginianus*, populations. Although not a competent host, deer provide a blood meal and transportation for adult ticks to new areas.

The vast majority of both vectorial and transfusion-associated *Babesia* cases involve *B. microti. B. duncani* infections include those previously designated WA1. *B. divergens* cases occur predominantly in Europe. The incubation period varies from 1 to 9 weeks. The severity of illness associated with *Babesia* infections relates more to the infected individual's immune status than the *Babesia* species. The very young, older adults, persons who have had a splenectomy, and those with hereditary hemolytic disorders are at greatest risk for morbidity and mortality. Approximately one-third of infected subjects remain asymptomatic, and parasitemia may persist for more than 2 years. Typical symptoms resemble malaria and include fever, headache, chills, sweats, and arthralgia, myalgia, malaise, nausea, diarrhea, and hemolytic anemia. Parasitemia levels vary from 1% to 2% in otherwise healthy hosts to 85% in immunocompromised and asplenic patients. Case fatality rates approximate 5%.

Case reports of transfusion-associated *Babesia* infections include red cell units stored for up to 42 days. *Babesia* transmission has been reported following transfusion of cryopreserved red cells, and four cases involve whole blood–derived platelet transfusions presumably contaminated with red cells. At least 159 transfusion-associated *B. microti* cases have occurred, primarily in endemic regions, but cases in non-endemic states reflect the interstate movement of both blood components and blood donors and highlight the high index of suspicion required to recognize cases.[101] More than three-quarters of the reports occurred during the past decade. The all-cause mortality rate approaches 19%. Babesia represented the highest number of reported transfusion-related fatalities to the FDA from any single microbial infection in the periods of 2008 to 2012 and again in 2010 to 2014 including 8 of 21 (38%) and 4 of 15 (27%), respectively. The total number of transfusion-related fatalities in these periods was 198 and 176, respectively (www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/ReportaProblem/TransfusionDonationFatalities/). There are three reports of transfusion-transmission involving *B. duncani*, and single cases have been reported from Japan (*B. microti*–like) and Germany.

Babesiosis has been made a nationally notifiable infection as of 2011 and is included in the nascent biovigilance network of the National Healthcare Safety Network, so more accurate measurements of transfusion risk may become available in the future. In 2011 to 2013 up to 29 states reported a total of 3857 cases nationally of which 95% occurred in seven states in New England and the upper Midwest and increased to 98.5% in nine states (adding New Hampshire and Maine) (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6127a2.htm). Transfusion-transmission risk estimates in endemic states have occurred with the advent of investigational testing and are about 1 per 18,000 red cell units transfused in the highly endemic states of New England to an overall risk of approximately 1 per 100,000 units throughout the all nine endemic states mentioned (including New Hampshire and Maine, where an increasing number of transfusion-related cases have been reported).

Febrile patients must be queried for a transfusion history and babesiosis considered when transfusion has occurred. Diagnostic laboratory evaluation includes blood smear examination, which requires differentiation of *Babesia* spp. from *Plasmodium* infections. Tetrad or "Maltese cross" forms are diagnostic for *Babesia* but occur infrequently. Diagnostic testing most frequently involves indirect IFA testing for identifying persons with low-level parasitemia such as patients with chronic infections but remains positive for up to 5 years after resolution or cure of babesiosis. PCR testing is more sensitive for detecting acute infections before seroconversion.

While blood donation screening has proven to be highly effective[102]; testing was sought in the absence of universal pathogen reduction. IND testing was conducted using a NAT by PCR in conjunction with antibody assay as well as a more sensitive NAT-only assay. No transmission of *Babesia* was seen in any tested units. As a result, the NAT-only assay was approved for FDA licensure in 2019 and new guidance was released by FDA for screening donations in endemic areas (14 states, plus the District of Columbia).[103] Along with the new screening guidelines, FDA included new deferral guidance, which lessened the deferral period for reactive donors from indefinite to a 2-year deferral.

Leishmaniasis

Phlebotomine (Old World) and *Lutzomyia* (New World) sandfly bites transmit *Leishmania* infections to humans in most of the tropical and subtropical world. *Trypanosomatidae* of various species cause visceral infection also known as kala-azar (*L. donovani*, *L. infantum*, and others). Additional species are involved in cutaneous and mucocutaneous infections (*L. tropica*, *L. major*, *L. mexicana*, *L. braziliensis*, and others given a variety of local names). In addition, transplacental, sexual, and transfusion transmissions occur.[104] The promastigote form of the parasite resides in the gastrointestinal tract of sand flies and is inoculated into humans through a skin bite. In humans, promastigotes are phagocytosed by monocytes, where they transform into amastigotes that reproduce and reside in macrophages and the reticuloendothelial system. Organisms in monocytes and free amastigotes are released during refrigerated storage, transform back into extracellular promastigotes, and mediate transfusion transmission. *L. tropica* also survives in monocytes contained in frozen red cell preparations and in platelet concentrates stored at room temperature. At least 10 cases of transfusion-associated leishmaniasis attributed to *L. donovani* have been reported in endemic areas, mostly in young children or neonates. A probable case of platelet transfusion–transmitted *L. donovani* was reported in India. A presumed case of transfusion-transmitted *L. mexicana* in a renal transplant recipient was mistakenly diagnosed as Chagas disease because of serologic cross-reactivity between *Trypanosoma cruzi* and *Leishmania. L. infantum* DNA was amplified from 6% of peripheral blood mononuclear cells of blood donors with *Leishmania* antibodies in the Balearic Islands. Several animal model studies also demonstrate transmission by blood transfusion.

Asymptomatic infections occur frequently in healthy donors exposed in endemic areas, and the organisms may circulate in peripheral blood more than 1 year following exposure. Foxhounds infected with *Leishmania* species have been found in 18 US states and two Canadian provinces, but transmission has not extended to humans.

Following reports of *L. tropica*–related viscerotropic leishmaniasis in veterans of Operation Desert Storm, between August 1990 and December 1992, those serving in that theater of operations were deferred from blood donation for 1 year. The deferral period reflected the development of fever, malaise, abdominal pain, and intermittent diarrhea up to 7 months after return to the United States. *L. tropica* was found in the bone marrow of seven patients and in the lymph nodes in one. When intracellular amastigotes were seen in the peripheral blood of the one patient in whom this was studied following reports of cutaneous and visceral leishmaniasis among troops involved in the Afghanistan and Iraq wars, a similar 1-year deferral following departure from Iraq and Afghanistan was implemented. The military continues to enforce lifetime deferral for any clinical history of *Leishmania* infection.

The possibility of transmission to humans during military deployment and travel and the large number of military personnel returning from Iraq and Afghanistan raise concerns about future transfusion transmission. To date, there are no cases of transfusion-transmitted leishmaniasis in the United States. The widespread use of leukoreduction filters likely has a beneficial role because these filters reduce both intracellular and extracellular parasite concentrations by 3 to 4 logs.

Toxoplasmosis

Toxoplasmosis is caused by the obligate intracellular protozoan parasite *Toxoplasma gondii*, whose usual host is the domestic cat. The parasite is transmitted by exposure to cat feces, by eating raw or undercooked pork, goat, lamb, beef, or wild game, and congenitally. In the NHANES from 1999 to 2004, 24.8% of foreign-born US residents age 12 to 49 years of age, compared with 8.2% of US–born residents, had serologic evidence of infection; approximately 50% of those with antibody harbor parasites in tissue. Transfusion-associated disease has been described in immunocompromised patients receiving granulocyte concentrates from donors with chronic myeloid leukemia who would not qualify as blood donors currently. One possible transfusion-related case involves a platelet transfusion. Red cells and frozen plasma transmission have not been reported.

Chagas Disease

The protozoan parasite *T. cruzi* causes Chagas disease. The infection is widespread in Latin America; approximately 8 to 10 million people are affected. Humans become infected when bitten by *T. cruzi*–infected insects of the *Reduviidae* family (triatomine, assassin, kissing, or chinch bugs). Congenital transmission from mother to fetus, organ transplantation, blood transfusion, and ingestion are recognized routes as well. Once infection occurs, low-level, intermittently detectable parasitemia usually persists for life. Treatment with benznidazole or nifurtimox can reduce the risk for chronic sequelae, but neither of these drugs is FDA approved and access to only one of these is through an IND with the CDC. Treatment is most effective during the acute stage of infection. At least 18 mammalian species in the United States serve as reservoirs, including armadillos, opossums, and raccoons, and vectors are present in the lower two-thirds of the country; autochthonous vector transmission is rare but has been increasingly reported especially in rural areas of Texas. CDC estimates at least 20 states have had isolation of the insect vector and 17 states with the mammalian reservoir. An estimated 300,000 people in the United States and Canada are infected with *T. cruzi;* most are immigrants from Latin America. *T. cruzi* organisms remain viable in whole blood stored at refrigerator temperatures for 18 days, for longer than 8 months in citrated blood samples stored at room temperature, and following freezing and thawing. In South America, early data suggested that approximately 13% to 49% of recipients of fresh whole blood from parasitemic donors become infected. There has been concern that additional transfusion-associated Chagas disease cases will occur as immigration increases to the United States from Central and South America.

American trypanosomiasis, or Chagas disease, consists of an acute phase that varies from asymptomatic to manifestations that include fever, skin rash, and conjunctivitis with edema around the eyelids, lymphadenopathy, and hepatosplenomegaly. The acute phase usually resolves in 4 to 8 weeks unless severe myocarditis or meningoencephalitis intervenes. The latter is associated with fatal outcomes. Intracellular *T. cruzi* amastigotes remain in cardiac and skeletal muscle following the acute phase. Following an indeterminate stage of undetermined duration, chronic disease occurs in 20% to 30% of infected patients, manifesting as cardiac disease (initially conduction and left ventricular wall abnormalities), megacolon, or achalasia. Diagnosis is made on clinical and serologic grounds most often. Xenodiagnosis, hemoculture, and nucleic acid amplification tests are also available.

Donor risk factors for transfusion-transmitted *T. cruzi* infection include birth or residence in endemic regions such as Central America, South America, or Southeastern Mexico; living in dwellings with palm leaf–thatched roofs or mud walls, where vector insects reside; oral intake of contaminated foodstuffs; and receipt of unscreened blood transfusions in Latin America. Among donors who lived in poor housing or received a blood transfusion in endemic areas, 3% to 4% had *T. cruzi* antibodies. One study, conducted in California in the 1990s, found that 1 per 340 blood donors had a risk factor for Chagas disease. A large survey conducted from 1994 to 1998 in Los Angeles and Miami among immigrant blood donors from endemic areas found *T. cruzi* seroprevalence rates of 1 per 7500 and 1 per 9000, respectively. However, none of the 18 recipients in this survey who received blood from a seropositive donor, and who were available for testing, had evidence of infection.

A look-back study involving blood donations in Mexico made before determining the donors were *T. cruzi*–seropositive, found four of nine recipients of subsequently determined seropositive whole blood or platelets to be seropositive.

Since 1987, 30 years before testing started in the United States and Canada, seven cases of transfusion-associated Chagas disease were reported in those countries; symptoms developed 2 to 3 months after transfusion. In six cases, platelets were the implicated component; and the unit in the seventh case was not identified. In each of the six cases, the implicated donor emigrated from a *T. cruzi*–endemic region (Bolivia, Mexico, Paraguay, and Chile) between 16 and 33 years before the implicated donation. *T. cruzi* may separate with platelets, or room-temperature storage may favor parasite survival. In addition, acute Chagas disease has been reported in organ transplant recipients. More recently, two transfusion-related infections were identified in the United States as a result of recipient tracing (look-back) studies (but these represented fewer than 1% of all studied individuals exposed to transfusion of blood from infected donors; see later). Additional transfusion-associated cases have been reported from Spain, all derived from donors who had emigrated from South America for a total of 20 cases ever documented when combining all reported transfusion-associated cases from the United States, Canada, and Spain, the countries with the highest immigration rates from Latin America.[105]

The assumed increasing prevalence of *T. cruzi*–infected blood donors with immigration into the United States from Latin America, the reports of transfusion-associated cases, and some autochthonous infections triggered efforts to mitigate the risk for transfusion-transmitted Chagas disease in the United States. Questioning donors about region of birth or extended stay and transfusion in Chagas-endemic areas could interdict 75% of infected donors but would result in deferral of large numbers of non-infected donors. Leukoreduction decreases *T. cruzi* transmission in a mouse model by 50% to 70%, consistent with *Leishmania* and the rickettsia *Anaplasma*. Pathogen-reduction techniques successfully decrease *T. cruzi* viability in plasma and cellular products.

Serologic screening, used for many years in Latin America, was chosen as the preferred strategy for the United States. The first serologic test for screening blood donors, based on a *T. cruzi* whole-parasite lysate, achieved FDA licensure in December 2006, and voluntary screening commenced in early 2007. Approximately 1 per 30,000 donations showed confirmed reactive serologic results, a lower prevalence than estimated. In 2010 the FDA licensed a second assay that uses chimeric recombinant antigens and in 2011, a licensed supplemental test using the same antigen constructs was FDA licensed. Seropositive donors are overwhelmingly immigrants from *T. cruzi*–endemic countries with asymptomatic chronic infections, although a small number of seropositive donors may have acquired the infection in the United States via vertical transmission from infected immigrant mothers, or very rarely by autochthonous vectorial transmission. From the investigation of antibody-positive donors lacking any immigration or maternal risk factors, autochthonous transmission has been estimated to occur at the rate of 1 per 354,000 donors.[106]

Look-back studies involving recipients of prior donations from those found positive for *T. cruzi* antibodies on a subsequent donation showed rates of transfusion transmission much lower than predicted. Only two occurred (0.8%), both from the same apheresis platelet donor born in Argentina, among 253 recipients tested. In light of these findings, testing donors is now required only once rather that at each donation.[36,107] Concern that those tested once might contract *T. cruzi* during travel or within the United States seems unfounded in that no seroconversions have been observed in more than 4 million blood donors during 6 million person-years of follow up.[36] The unexpectedly low rate of transmission may relate to differences in blood component processing and storage in the United States compared with Latin America (absence of the transfusion of fresh whole blood), some underrepresentation of platelet donors in the look backs completed to date, or other biases. Although all donors are asked a question about having a history of Chagas disease, with the advent of sensitive testing, the question has no residual value and has been removed from the donor history questionnaire.[107,108]

TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES

The transmissible TSEs (prion diseases) are rare, lethal neurodegenerative diseases generally accepted to be caused by nucleic acid–free proteins called $\mathrm{PrP^{TSE}}$, as distinguished from the normal cellular protein, $\mathrm{PrP^{C}}$. The existence of additional factors has not been excluded, but current consensus supports the involvement of infectious proteins. $\mathrm{PrP^{TSE}}$ is an abnormal conformation of $\mathrm{PrP^{C}}$ that induces transformation (recruitment) of additional $\mathrm{PrP^{C}}$ to $\mathrm{PrP^{TSE}}$, resulting in deposits of insoluble aggregates in central nervous system tissue followed by progressive dementia and other characteristic neurologic findings. Non-variant Creutzfeldt-Jakob disease (CJD) is diagnosed at a rate of approximately one case per million population per year worldwide. It occurs as sporadic CJD (85% of cases), vertically transmitted familial diseases caused by germline mutations in the human *PRNP* gene (e.g., fatal familial insomnia and Gerstmann-Straüssler-Scheinker syndrome), and less commonly through iatrogenic transmission of infection (e.g., from dura mater implantation, contaminated surgical equipment, injection of human pituitary-derived growth hormone, or corneal transplant) and historically has been known to be spread horizontally (i.e., Kuru associated with consumption of human brain during ritual cannibalism).

There are no documented cases of transfusion transmission of sporadic CJD, the familial or iatrogenic forms, and cohort studies of intensively transfused patient groups have failed to establish any epidemiologic association in both the United Kingdom and the United States.[35,109] In an ongoing look-back effort, no cases of CJD have been observed among 826 recipients (of whom 154 were still alive as of December 31, 2014) of blood components from 65 donors subsequently diagnosed with CJD.[109] A case-control study of CJD patients, compared with control patients without CJD, found a fivefold increased risk for CJD with a history of transfusion more than 10 years before TSE onset, but the authors recognized critical sources of bias in their methods and their findings are unconfirmed. Nevertheless, as a result of the iatrogenic transmissions and long incubation period of the disease (as demonstrated in growth hormone transmissions), concern arose in the mid-1990s that CJD transmission could occur from asymptomatic donors to transfusion or derivative recipients. This theoretical risk resulted in lifetime donor deferral requirements for iatrogenic exposure to or a family history of classic CJD.

Like non-variant CJD, vCJD is a fatal, degenerative neurologic disease. It occurs in younger patients than classic CJD and has distinctive clinical, radiographic, histopathologic, and biochemical features. The first reports of vCJD from the United Kingdom were published in 1996. The etiologic agent is the same prion that causes BSE or "mad cow disease." Transmission of the BSE prion to humans occurred by consumption of beef and other bovine products containing infectious neural or reticuloendothelial tissue. In the United Kingdom the vCJD epidemic followed a massive epidemic of BSE in the 1980s and 1990s. The latter was traced to the recycling of material from dead sheep and cattle (offal) into feed for cattle. This practice was banned in 1988, and the vCJD outbreak subsided after a peak of 28 cases in 2000. The number of new vCJD diagnoses in the United Kingdom rose steadily in the first few years after 1995 from 3 in 1995 to 28 in 2000, but decreased since then to 5, 5, 5, 1, 3, 3, and 2 from 2005 to July 2011 and 2 additional since then totaling 177 definite or probable cases of vCJD reported in the United Kingdom.[110] An additional 52 cases have been diagnosed elsewhere, mostly in France and several other European countries. A few cases have been identified outside Europe, including four cases in the United States (two associated with exposure in the United Kingdom, one in Saudi Arabia, and one with international travel not linked to a country where other vCJD cases were seen), two from Canada, and one each from Japan, Taiwan, and Saudi Arabia. Most, but not all of the patients with non-European cases had more than 6 months' exposure in the United Kingdom during the BSE epidemic. Although it appears that the vCJD outbreak is waning (and is much smaller than initially feared), concern persists that there could be a second wave of the epidemic caused by delayed onset of the disease in infected individuals. This is based in part on observations of abnormal prion protein accumulation in random surgical tissue samples in the United Kingdom, which may represent preclinical vCJD disease. In one retrospective study, 3 of 12,674 appendectomy and tonsillectomy specimens from persons 20 to 29 years of age were positive. Furthermore, a critical polymorphism at codon 129 of the PrP^C gene coding for methionine or valine leads to variation in the susceptibility to and incubation period of human TSEs. All of the clinical cases reported to date are methionine homozygotes. Because only 37% of the general British population is homozygous for methionine, and are maximally susceptible to vCJD, a larger population (52%) of heterozygotes and valine homozygotes (11%) may be at risk for asymptomatic infection in a prolonged incubation period.

Four transfusion-transmitted infections with the vCJD prion have been reported in the United Kingdom. Although they may have developed vCJD independent of transfusion, this possibility is regarded highly unlikely based on epidemiologic and statistic considerations. Three of the four cases were diagnosed with clinical vCJD 6.5, 7.8, and 9 years after receiving nonleukocyte reduced RBCs or a blood component from two different donors who developed clinical symptoms of vCJD 40 and 21 months after donating. These three with symptomatic disease were homozygous for methionine at codon 129. The fourth case was heterozygous (methionine/valine) and had no clinical signs or symptoms of vCJD at death from unrelated causes but had abnormal prion protein aggregates in lymphoid (but not neural) tissues at autopsy and was considered to have preclinical infection. Five years before death the recipient received non-leukocyte reduced RBCs from a donor who developed clinical vCJD 18 months after donating. These four cases represent 6% of the 67 UK recipients who received blood components from 18 different donors subsequently diagnosed with vCJD (and an estimated 23% of exposed methionine homozygotes). In light of these cases and animal transfusion experiments, TSE transmission and disease from transfusion is no longer regarded as a theoretical event.[35] A single case reports a potential epidemiologic link between exposure to plasma derivatives and transmission of the agent to a recipient.

Prion-removal strategies (e.g., affinity filters) remain under evaluation, especially in the United Kingdom, but none is expected to be suitable for consideration for use in the United States in the immediate future. There are currently no blood donor screening tests for vCJD, leaving risk mitigation dependent on deferral of donors that could have asymptomatic infection. Initially, primarily on the basis of the prominent distribution of vCJD prion in reticuloendothelial tissue compared with classic prion protein (PrP^C), on subsequent reports of animal and human infection by transfusion, and then on the early observation that nearly all cases of vCJD were associated with potential exposure in the United Kingdom or to UK bovine products, the FDA adopted and has modified donor-deferral policies sequentially since 1999.[111] The first called for indefinite deferral of donors who had spent more than 6 months in the United Kingdom from 1980 to 1996, before control of the food chain and of recipients of bovine insulin from the UK. Models predicted that this would remove approximately 90% of the risk at a "cost" of deferring approximately 5% of otherwise eligible donors. These were subsequently expanded to include US military personnel and their dependents who spent certain durations on bases in the European Union where UK beef was imported during the BSE epidemic and recipients of transfusions in the United Kingdom and France during the peak risk periods of their BSE epidemics. Experience at US blood centers has largely confirmed the donor loss predictions of the FDA models. In the midst of the SARS-CoV-2 pandemic, the US FDA updated their donor deferral criteria using recent epidemiologic data published on risk of vCJD and CJD. This guidance allows for the removal of the following deferrals: Human Growth Hormone medication, blood relatives with CJD, time spent in countries other than the UK, France, and Ireland from 1980-present, time spent on military bases in Europe, and a history of injection of bovine insulin.[111]

Chronic wasting disease (CWD) of deer and elk is prevalent at rates as high as 15% in cervid populations in at least 24 states within the United States and Canada. Concern has been expressed that, given the popularity of hunting, there may be risk for exposure to and infection with the CWD prion during handling or consumption of infected animals. Apparent clusters of classic CJD in hunters have been alleged, but on full evaluation have not been shown to have a relationship to the CWD agent. There is currently no plan to intervene for this theoretical risk in hunters who donate blood beyond hygienic measures when handling cervids or their tissues.

FUTURE DIRECTIONS

The residual risk for transmitting HIV, HBV, and HCV is less than 1 per million units transfused, an extremely low rate. In contrast, platelet septic events are reported at a frequency of 1 per 100,000 units.

Transmission of known, emerging infectious agents such as dengue, Zika virus, *B. microti*, and those currently unrecognized remain problematic. Continued safety of the blood supply requires constant vigilance, early detection, reporting of untoward events, and excellent communication and cooperation between those providing blood components and those prescribing them.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Aubuchon JP, Prowse CV, eds. *Pathogen Inactivation: The Penultimate Paradigm Shift*. Bethesda MD: AABB Press; 2010.

Bennett JL, Blajchman MA, Delage G, et al. Proceedings of a consensus conference: Risk-based decision making for blood safety. *Transfus Med Rev*. 2011;25:267.

Berg MG, Lee D, Coller K, et al. Discovery of a novel human pegivirus in blood associated with hepatitis C virus co-infection. *PLoS Pathog*. 2015;11:e1005325.

Brennan CA, Yamaguchi J, Devare SG, et al. Expanded evaluation of blood donors in the United States for human immunodeficiency virus type 1 non-B subtypes and antiretroviral drug-resistant strains: 2005 through 2007. *Transfusion*. 2010;50:2707.

Busch MP. Cooley Award Lecture. Transfusion-transmitted viral infections: building bridges to transfusion medicine to reduce risks and understand epidemiology and pathogenesis. *Transfusion*. 2006;46:1624.

Centers for Disease Control and Prevention and Association of Public Health Laboratories: Laboratory Testing for the Diagnosis of HIV Infection: Updated Recommendations. Available at https://doi.org/10.15620/cdc.23447.

Centers for Disease Control and Prevention HIV transmission through transfusion—Missouri and Colorado, 2008. *MMWR*. 2010;59:1335.

Crowder LA, Schonberger LB, Dodd RY, Steele WR. Creutzfeldt-Jakob disease lookback study: 21 years of surveillance for transfusion transmission risk. *Transfusion*. 2017;57(8):1875–1878.

Custer B, Kessler D, Vahidnia F, et al. For the NHLBI Retrovirus Epidemiology Donor Study-II (REDS-II) Risk factors for retrovirus and hepatitis virus infections in accepted blood donors. *Transfusion*. 2014;55:1098–1107.

Dodd RY, Crowder LA, Haynes JM, et al. Screening blood donors for HIV, HCV and HBV at the American Red Cross: ten-year trends in prevalence, incidence and residual risk, 2007–2016. *Transfus Med Rev*. 2020;34(2):81–93.

FDA Final Rule Docket No FDA-2006N-0040. *Fed Regist*. 2015;80(99):29842–29906.

Hewitt PE, Ijaz S, Brailsford SR, et al. Hepatitis E virus in blood components: a prevalence and transmission study in southeast England. *Lancet*. 2014;384:1766–1773.

Jackson BR, Busch MP, Stramer SL, et al. The cost-effectiveness of NAT for HIV, HCV, and HBV in whole-blood donations. *Transfusion*. 2003;43:721.

Kleinman SH, Lelie N, Busch MP. Infectivity of human immunodeficiency virus-1, hepatitis C virus, and hepatitis B virus and risk of transmission by transfusion. *Transfusion*. 2009;49:2454–2489.

Kleinman S, Cameron C, Custer B, et al. Modeling the risk of an emerging pathogen entering the Canadian blood supply. *Transfusion*. 2010;50:2592.

Laperche S, Nübling CM, Stramer SL, et al. Sensitivity of hepatitis C virus core antigen and antibody combination assays in a global panel of window period samples. *Transfusion*. 2015;55:2489–2498.

Murphy EL, Glynn SA, Fridey J, et al. Increased prevalence of infectious diseases and other adverse outcomes in human T lymphotropic virus types I- and II-infected blood donors. Retrovirus Epidemiology Donor Study (REDS) Study Group. *J Infect Dis*. 1997;176:1468.

Orton SL, Stramer SL, Dodd RY, et al. Risk factors for HCV infection among blood donors confirmed to be positive for the presence of HCV RNA and not reactive for the presence of anti-HCV. *Transfusion*. 2004;44:275–281.

Petrik J, Lozano M, Seed CR, et al. Hepatitis E. *Vox Sang*. 2016;110:93–103.

Seed CR, Kiely P, Law M, et al. No evidence of a significantly increased risk of transfusion-transmitted human immunodeficiency virus infection in Australia subsequent to implementing a 12-month deferral for men who have had sex with men. *Transfusion*. 2010;50:2722.

Seghatchian J, Hervig T, Putter JS. Effect of pathogen inactivation on the storage lesion in red cells and platelet concentrates. *Transfus Apher Sci*. 2011;45:75.

Snyder EL, Stramer SL, Benjamin RJ. The safety of the blood supply—time to raise the bar. *N Engl J Med*. 2015;372:1882–1885.

Stramer SL, Dodd RY, Brodsky JP. The value of screening signal-to-cutoff ratios for hepatitis C virus antibody confirmation. *Transfusion*. 2013;53:1497–1500.

Stramer SL, Moritz ED, Foster GA, et al. Hepatitis E virus: seroprevalence and frequency of viral RNA detection among US blood donors. *Transfusion*. 2016;56:481–488.

Stramer SL, Notari EP, Krysztof DE, et al. Hepatitis B virus testing by minipool nucleic acid testing: does it improve blood safety? *Transfusion*. 2013;53:2449–2458.

Stramer SL, Wend U, Candotti D, et al. Nucleic acid testing to detect HBV infection in blood donors. *N Engl J Med*. 2011;364:236–247.

Taira R, Satake M, Momose S, et al. Residual risk of transfusion-transmitted hepatitis B virus (HBV) infection caused by blood components derived from donors with occult HBV infection in Japan. *Transfusion*. 2013;53:1393–1404.

Urwin PJM, Mackenzie JM, Llewelyn CA, et al. Creutzfeldt–Jakob disease and blood transfusion: updated results of the UK Transfusion Medicine Epidemiology Review Study. *Vox Sang*. 2016;110:310–316.

Vamvakas EC. Is white blood cell reduction equivalent to antibody screening in preventing transmission of cytomegalovirus by transfusion? A review of the literature and meta-analysis. *Transfus Med Rev*. 2005;19:181.

Xiang J, Wünschmann S, Diekema DJ, et al. Effect of coinfection with GB virus C on survival among patients with HIV infection. *N Engl J Med*. 2001;345:704.

Zou S, Dorsey KA, Notari EP, et al. Prevalence, incidence, and residual risk of human immunodeficiency virus and hepatitis C virus infections among United States blood donors since the introduction of nucleic acid testing. *Transfusion*. 2010;50:1495–1504.

Zou S, Stramer SL, Notari EP, et al. Current incidence and residual risk of hepatitis B infection among blood donors in the United States. *Transfusion*. 2009;49:1609–1620.

REFERENCES

1. Busch MP. Transfusion-transmitted viral infections: building bridges to transfusion medicine to reduce risks and understand epidemiology and pathogenesis. *Transfusion*. 2006;46(9):1624–1640. https://doi.org/10.1111/j.1537-2995.2006.00957.x.
2. Centers for Disease Control and Prevention. Viral Hepatitis: Surveillance for Viral Hepatitis—United States, 2017. Accessed 22 Dec 2019, http://www.cdc.gov/hepatitis/statistics/2017surveillance/pdfs/2017HepSurveillanceRpt.pdf
3. Custer B, Kessler D, Vahidnia F, et al. Risk factors for retrovirus and hepatitis virus infections in accepted blood donors. Research Support, N.I.H., Extramural. *Transfusion*. 2015;55(5):1098–1107. https://doi.org/10.1111/trf.12951.
4. Zou S, Stramer SL, Notari EP, et al. Current incidence and residual risk of hepatitis B infection among blood donors in the United States. *Transfusion*. 2009;49(8):1609–1620. https://doi.org/10.1111/j.1537-2995.2009.02195.x.
5. Stramer SL, Wend U, Candotti D, et al. Nucleic acid testing to detect HBV infection in blood donors. Evaluation Studies Research Support, Non-U.S. Gov't. *N Engl J Med*. 2011;364(3):236–247. https://doi.org/10.1056/NEJMoa1007644.
6. Taira R, Satake M, Momose S, et al. Residual risk of transfusion-transmitted hepatitis B virus (HBV) infection caused by blood components derived from donors with occult HBV infection in Japan. *Transfusion*. 2013;53(7):1393–1404. https://doi.org/10.1111/j.1537-2995.2012.03909.x.
7. FDA Final Rule: Docket No FDA-2006N-0040. *Fed Regist*. 2015;80(99):29842–29906.
8. Stramer SL, Notari EP, Krysztof DE, Dodd RY. Hepatitis B virus testing by minipool nucleic acid testing: does it improve blood safety? *Transfusion*. 2013;53(10 Pt 2):2449–2458. https://doi.org/10.1111/trf.12213.
9. Dodd RY, Nguyen ML, Krysztof DE, Notari EP, Stramer SL. Blood donor testing for hepatitis B virus in the United States: is there a case for continuation of hepatitis B surface antigen detection? *Transfusion*. 2018;58(9):2166–2170.
10. Kleinman SH, Lelie N, Busch MP. Infectivity of human immunodeficiency virus-1, hepatitis C virus, and hepatitis B virus and risk of transmission by transfusion. *Transfusion*. 2009;49(11):2454–2489. https://doi.org/10.1111/j.1537-2995.2009.02322.x.
11. Dodd RY, Crowder LA, Haynes JM, et al. Screening blood donors for HIV, HCV and HBV at the American Red Cross: ten-year trends in prevalence, incidence and residual risk, 2007–2016. *Transfus Med Rev*. 2020;34(2):81–93.
12. Steele WR, Dodd RY, Notari EP, et al. Prevalence of human immunodeficiency virus, hepatitis B virus, and hepatitis C virus in United States blood donations, 2015 to 2019: The Transfusion-Transmissible Infections Monitoring System (TTIMS). *Transfusion*. 2020;60:2327–2339. https://doi.org/10.1111/trf.16005.
13. Steele WR, Dodd RY, Notari EP, et al. HIV, HCV, and HBV incidence and residual risk in US blood donors before and after implementation of the 12-month deferral policy for men who have sex with men. *Transfusion*. 2021;61(3):839–850. https://doi.org/10.1111/trf.16250.
14. Orton SL, Stramer SL, Dodd RY, Alter MJ. Risk factors for HCV infection among blood donors confirmed to be positive for the presence of HCV RNA and not reactive for the presence of anti-HCV. *Transfusion*. 2004;44(2):275–281. https://doi.org/10.1111/j.1537-2995.2004.00623.x.
15. Stramer SL, Dodd RY, Brodsky JP. The value of screening signal-to-cutoff ratios for hepatitis C virus antibody confirmation. *Transfusion*. 2013;53(7):1497–1500. https://doi.org/10.1111/j.1537-2995.2012.03955.x.
16. Laperche S, Nübling CM, Stramer SL, et al. Sensitivity of hepatitis C virus core antigen and antibody combination assays in a global panel of window period samples. *Transfusion*. 2015;55(10):2489–2498. https://doi.org/10.1111/trf.13179.
17. Centers for Disease Control and Prevention. Multistate outbreak of hepatitis A virus infections linked to pomegranate seeds from Turkey (Final Update). Updated September 15, 2014. https://www.cdc.gov/hepatitis/outbreaks/2013/a1b-03-31/index.html
18. Centers for Disease Control and Prevention. Men who have sex with men and viral hepatitis. Updated August 24, 2020. https://www.cdc.gov/hepatitis/populations/msm.htm
19. Centers for Disease Control and Prevention. Widespread person-to-person outbreaks of hepatitis A across the United States. Updated May 3, 2021. https://www.cdc.gov/hepatitis/outbreaks/2017March-HepatitisA.htm
20. Petrik J, Lozano M, Seed CR, et al. Hepatitis E. *Vox Sang*. 2016;110(1):93–103. https://doi.org/10.1111/vox.12285.
21. Hewitt PE, Ijaz S, Brailsford SR, et al. Hepatitis E virus in blood components: a and transmission study in southeast England. *Lancet*. 2014;384(9956):1766–1773. https://doi.org/10.1016/S0140-6736(14)61034-5.
22. Delage G, Fearon M, Gregoire Y, et al. Hepatitis E virus infection in blood donors and risk to patients in the United States and Canada. *Transfus Med Rev*. 2019;33(3):139–145. https://doi.org/10.1016/j.tmrv.2019.05.017.
23. Stramer SL, Moritz ED, Foster GA, et al. Hepatitis E virus: seroprevalence and frequency of viral RNA detection among US blood donors. *Transfusion*. 2016;56(2):481–488. https://doi.org/10.1111/trf.13355.
24. Xiang J, Wünschmann S, Diekema DJ, et al. Effect of coinfection with GB virus C on survival among patients with HIV infection. *N Engl J Med*. 2001;345(10):707–714. https://doi.org/10.1056/NEJMoa003364.
25. Berg MG, Lee D, Coller K, et al. Discovery of a novel human pegivirus in blood associated with hepatitis C virus co-infection. *PLoS Pathog*. 2015;11(12):e1005325. https://doi.org/10.1371/journal.ppat.1005325.
26. Brennan CA, Yamaguchi J, Devare SG, Foster GA, Stramer SL. Expanded evaluation of blood donors in the United States for human immunodeficiency virus type 1 non-B subtypes and antiretroviral drug-resistant strains: 2005 through 2007. *Transfusion*. 2010;50(12):2707–2712. https://doi.org/10.1111/j.1537-2995.2010.02767.x.
27. Centers for Disease Control and Prevention HIV transmission through transfusion—Missouri and Colorado, 2008. *MMWR Morb Mortal Wkly Rep*. 2010;59(41):1335–1339.
28. Centers for Disease Control and Prevention, Association of Public Health Laboratories. Laboratory testing for the diagnosis of HIV infection: updated recommendations. June 27, 2014. https://doi.org/10.15620/cdc.23447
29. Steele WR, Notari EP, Nelson D, et al. Prevalence of human immunodeficiency virus, hepatitis B virus and hepatitis C virus in more than fifty percent of the United States blood supply: The Transfusion-Transmissible Infections Monitoring System (TTIMS). Presented at: Transfusion; September 2017; San Diego, CA.
30. Quiner C, Bruhn R, Grebe E, et al. Recently acquired infection among HIV-seropositive donors in the US from 2010–2018. *Transfusion*. 2020;60:2340–2347. https://doi.org/10.1111/trf.16032.
31. Grebe E, Busch MP, Notari EP, et al. HIV incidence in US first-time blood donors and transfusion risk with a 12-month deferral for men who have sex with men. *Blood*. 2020;136(11):1359–1367. https://doi.org/10.1182/blood.2020007003.
32. Food and Drug Administration. Guidance for industry: Revised Recommendations for Reducing the Risk of Human Immunodeficiency Virus Transmission by Blood and Blood Products. Updated August 2020. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/revised-recommendations-reducing-risk-human-immunodeficiency-virus-transmission-blood-and-blood.
33. Custer B, Quiner C, Haaland R, et al. HIV antiretroviral therapy and prevention use in US blood donors: a new blood safety concern. *Blood*. 2020;136(11):1351–1358. https://doi.org/10.1182/blood.2020006890.
34. Klein HG, Hrouda JC, Epstein JS. Crisis in the sustainability of the U.S. blood system. *N Engl J Med*. 2017;377(15):1485–1488. https://doi.org/10.1056/NEJMsb1706496.
35. Urwin PJM, Mackenzie JM, Llewelyn CA, Will RG, Hewitt PE. Creutzfeldt-Jakob disease and blood transfusion: updated results of the UK Transfusion Medicine Epidemiology Review Study. *Vox Sang*. 2016;110(4):310–316.
36. Dodd RY, Groves JA, Townsend RL, et al. Impact of one-time testing for *Trypanosoma cruzi* antibodies among blood donors in the United States. *Transfusion*. 2019;59(3):1016–1023. https://doi.org/10.1111/trf.15118.
37. Groves JA, Foster GA, Dodd RY, Stramer SL. West Nile virus activity in United States blood donors and optimizing detection strategies: 2014–2018. *Transfusion*. 2020;60(1):94–105. https://doi.org/10.1111/trf.15620.
38. Dodd RY, Foster GA, Stramer SL. Keeping blood transfusion safe from West Nile virus: American Red Cross experience, 2003 to 2012. *Transfus Med Rev*. 2015;29(3):153–161. https://doi.org/10.1016/j.tmrv.2015.03.001.
39. Kracalik I, Mowla S, Basavaraju SV, Sapiano MRP. Transfusion-related adverse reactions: data from the National Healthcare Safety Network Hemovigilance Module—United States, 2013–2018. *Transfusion*. 2021;61:1424–1434. https://doi.org/10.1111/trf.16362.
40. Snyder E, Stramer S, Benjamin RJ. The safety of the US blood supply—time to raise the bar. *N Engl J Med*. 2015;372:1882–1885.
41. Kleinman S, Cameron C, Custer B, et al. Modeling the risk of an emerging pathogen entering the Canadian blood supply. *Transfusion*. 2010;50(12):2592–2606. https://doi.org/10.1111/j.1537-2995.2010.02724.x.
42. AuBuchon JP, Prowse CV. *Pathogen Inactivation: The Penultimate Paradigm Shift*. Bethesda: AABB Press; 2010.

43. Seghatchian J, Hervig T, Putter JS. Effect of pathogen inactivation on the storage lesion in red cells and platelet concentrates. *Transfus Apher Sci*. 2011;45(1):75–84. https://doi.org/10.1016/j.transci.2011.06.006.
44. Leach Bennett J, Blajchman MA, Delage G, Fearon M, Devine D. Proceedings of a consensus conference: risk-based decision making for blood safety. *Transfus Med Rev*. 2011;25(4):267–292. https://doi.org/10.1016/j.tmrv.2011.05.002.
45. Jackson BR, Busch MP, Stramer SL, AuBuchon JP. The cost-effectiveness of NAT for HIV, HCV, and HBV in whole-blood donations. *Transfusion*. 2003;43(6):721–729. https://doi.org/10.1046/j.1537-2995.2003.00392.x.
46. Murphy EL, Glynn SA, Fridey J, et al. Increased prevalence of infectious diseases and other adverse outcomes in human T lymphotropic virus types I- and II-infected blood donors. Retrovirus Epidemiology Donor Study (REDS) Study Group. *J Infect Dis*. 1997;176(6):1468–1475. https://doi.org/10.1086/514143.
47. Vamvakas EC. Is white blood cell reduction equivalent to antibody screening in preventing transmission of cytomegalovirus by transfusion? A review of the literature and meta-analysis. *Transfus Med Rev*. 2005;19:181–199.
48. Canadian National Advisory Committee on Blood and Blood Products. NAC statement regarding appropriateness of use of Cytomegalovirus (CMV) sero negative versus CMV safe product (February 14, 2017). Updated Aug 7, 2018. 2021. https://nacblood.ca/en/resource/cytomegalovirus-cmv
49. Bhutani M, Polizzotto MN, Uldrick TS, Yarchoan R. Kaposi sarcoma-associated herpesvirus-associated malignancies: epidemiology, pathogenesis, and advances in treatment. *Semin Oncol*. 2015;42(2):223–246. https://doi.org/10.1053/j.seminoncol.2014.12.027.
50. Kleinman SH, Glynn SA, Lee TH, et al. A linked donor-recipient study to evaluate parvovirus B19 transmission by blood component transfusion. *Blood*. 2009;114(17):3677–3683. https://doi.org/10.1182/blood-2009-06-225706.
51. Satake M, Hoshi Y, Taira R, Momose SY, Hino S, Tadokoro K. Symptomatic parvovirus B19 infection caused by blood component transfusion. *Transfusion*. 2011;51(9):1887–1895. https://doi.org/10.1111/j.1537-2995.2010.03047.x.
52. Dodd RY. B19: benign or not? *Transfusion*. 2011;51(9):1878–1879. https://doi.org/10.1111/j.1537-2995.2011.03274.x.
53. Norja P, Hedman L, Kantola K, et al. Occurrence of human bocaviruses and parvovirus 4 in solid tissues. *J Med Virol*. 2012;84(8):1267–1273. https://doi.org/10.1002/jmv.23335.
54. Biggerstaff BJ, Petersen LR. Estimated risk of West Nile virus transmission through blood transfusion during an epidemic in Queens, New York City. *Transfusion*. 2002;42(8):1019–1026. https://doi.org/10.1046/j.1537-2995.2002.00167.x.
55. Pealer L, Marfin A, Petersen LR. Transmission of West Nile virus through blood transfusion in the United States in 2002. *N Engl J Med*. 2003;349:1236–1245.
56. Dodd RY. Emerging infections, transfusion safety, and epidemiology. *N Engl J Med*. 2003;349(13):1205–1206. https://doi.org/10.1056/NEJMp038138.
57. Petersen LR, Epstein JS. Problem solved? West Nile virus and transfusion safety. *N Engl J Med*. 2005;353(5):516–517. https://doi.org/10.1056/NEJMe058144.
58. Zou S, Foster GA, Dodd RY, Petersen LR, Stramer SL. West Nile fever characteristics among viremic persons identified through blood donor screening. *J Infect Dis*. 2010;202(9):1354–1361. https://doi.org/10.1086/656602.
59. Stramer SL, Fang CT, Foster GA, Wagner AG, Brodsky JP, Dodd RY. West Nile virus among blood donors in the United States, 2003 and 2004. *N Engl J Med*. 2005;353(5):451–459. https://doi.org/10.1056/NEJMoa044333.
60. Hayes C, Stephens L, Fridey JL, et al. Probable transfusion transmission of West Nile virus from an apheresis platelet that screened non-reactive by individual donor-nucleic acid testing. *Transfusion*. 2020;60(2):424–429. https://doi.org/10.1111/trf.15568.
61. Tomashek KM, Margolis HS. Dengue: a potential transfusion-transmitted disease. *Transfusion*. 2011;51(8):1654–1660. https://doi.org/10.1111/j.1537-2995.2011.03269.x.
62. Stramer SL, Linnen JM, Carrick JM, et al. Dengue viremia in blood donors identified by RNA and detection of dengue transfusion transmission during the 2007 dengue outbreak in Puerto Rico. Case Reports. *Transfusion*. 2012;52(8):1657–1666. https://doi.org/10.1111/j.1537-2995.2012.03566.x.
63. Matos D, Tomashek KM, Perez-Padilla J, et al. Probable and possible transfusion-transmitted dengue associated with NS1 antigen-negative but RNA confirmed-positive red blood cells. *Transfusion*. 2016;56(1):215–222. https://doi.org/10.1111/trf.13288.
64. Sabino EC, Loureiro P, Lopes ME, et al. Transfusion-transmitted dengue and associated clinical symptoms during the 2012 epidemic in Brazil. *J Infect Dis*. 2016;213(5):694–702. https://doi.org/10.1093/infdis/jiv326.
65. Stramer SL, Hollinger FB, Katz LM, et al. Emerging infectious disease agents and their potential threat to transfusion safety. *Transfusion*. 2009;49(Suppl 2):1S–29S. https://doi.org/10.1111/j.1537-2995.2009.02279.x.
66. Stramer SL, Dodd RY. Subgroup AT-TDEID. Transfusion-transmitted emerging infectious diseases: 30 years of challenges and progress. *Transfusion*. 2013;53(10 Pt 2):2375–2383. https://doi.org/10.1111/trf.12371.
67. Lombardi VC, Ruscetti FW, Das Gupta J, et al. Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome. *Science*. 2009;326(5952):585–589. https://doi.org/10.1126/science.1179052.
68. Paprotka T, Delviks-Frankenberry KA, Cingöz O, et al. Recombinant origin of the retrovirus XMRV. *Science*. 2011;333(6038):97–101. https://doi.org/10.1126/science.1205292.
69. Dodd RY, Hackett Jr. J, Linnen JM, et al. Xenotropic murine leukemia virus-related virus does not pose a risk to blood recipient safety. Evaluation Studies Research Support, Non-U.S. Gov't. *Transfusion*. 2012;52(2):298–306. https://doi.org/10.1111/j.1537-2995.2011.03450.x.
70. Chiu CY, Bres V, Yu G, et al. Genomic assays for identification of chikungunya virus in blood donors, Puerto Rico, 2014. *Emerg Infect Dis*. 2015;21(8):1409–1413. https://doi.org/10.3201/eid2108.150458.
71. Brouard C, Bernillon P, Quatresous I, et al. Estimated risk of Chikungunya viremic blood donation during an epidemic on Reunion Island in the Indian Ocean, 2005 to 2007. *Transfusion*. 2008;48(7):1333–1341. https://doi.org/10.1111/j.1537-2995.2008.01646.x.
72. Musso D, Nhan T, Robin E. Potential for Zika virus transmission through blood transfusion demonstrated during an outbreak in French Polynesia, November 2013 to February 2014. *Euro Surveill*. 2014;19:20761.
73. Fauci AS, Morens DM. Zika virus in the Americas—yet another arbovirus threat. *N Engl J Med*. 2016;374(7):601–604. https://doi.org/10.1056/NEJMp1600297.
74. Centers for Disease Control and Prevention. Zika virus: reporting and surveillance. Updated January 7, 2021. Accessed Jan 19, 2021, http://www.cdc.gov/zika/reporting/index.html
75. Hennessey M, Fischer M, Staples JE. Zika virus spreads to new areas—region of the Americas, May 2015–January 2016. *MMWR Morb Mortal Wkly Rep*. 2016;65(3):55–58. https://doi.org/10.15585/mmwr.mm6503e1.
76. Schuler-Faccini L, Ribeiro EM, Feitosa IML, et al. Possible association between Zika virus infection and microcephaly—Brazil, 2015. *MMWR Morb Mortal Wkly Rep*. 2016;65(3):1–4.
77. Musso D, Roche C, Robin E, Nhan T, Teissier A, Cao-Lormeau VM. Potential sexual transmission of Zika virus. *Emerg Infect Dis*. 2015;21(2):359–361. https://doi.org/10.3201/eid2102.141363.
78. Food and Drug Administration. Guidance for industry: revised recommendations for reducing the risk of Zika virus transmission by blood and blood components. Updated July 2018. Accessed August 30, 2018, http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/UCM518213.pdf
79. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet*. 2020;395(10223):497–506. https://doi.org/10.1016/S0140-6736(20)30183-5.
80. Cappy P, Candotti D, Sauvage V, et al. No evidence of SARS-CoV-2 transfusion transmission despite RNA detection in blood donors showing symptoms after donation. *Blood*. 2020;136(16):1888–1891. https://doi.org/10.1182/blood.2020008230.
81. Katz LM. Is SARS-CoV-2 transfusion transmitted? *Transfusion*. 2020;60(6):1111–1114. https://doi.org/10.1111/trf.15831.
82. Bajema KL, Wiegand RE, Cuffe K, et al. Estimated SARS-CoV-2 seroprevalence in the US as of September 2020. *JAMA Intern Med*. 2020 https://doi.org/10.1001/jamainternmed.2020.7976.
83. Dodd RY, Xu M, Stramer SL. Change in donor characteristics and antibodies to SARS-CoV-2 in donated blood in the US, June–August 2020. *JAMA*. 2020;324:1677–1679. https://doi.org/10.1001/jama.2020.18598.
84. Likos AM, Kelvin DJ, Cameron CM, et al. Influenza viremia and the potential for blood-borne transmission. *Transfusion*. 2007;47(6):1080–1088. https://doi.org/10.1111/j.1537-2995.2007.01264.x.
85. Stramer SL, Collins C, Nugent T, et al. Sensitive detection assays for influenza RNA do not reveal viremia in US blood donors. *J Infect Dis*. 2012;205(6):886–894. https://doi.org/10.1093/infdis/jir863.
86. Food and Drug Administration. Guidance for industry: bacterial risk control strategies for blood collection establishments and transfusion services to enhance the safety and availability of platelets for transfusion.

Updated December 2020. Accessed December 24, 2020, https://www.fda.gov/media/123448/download.

87. Food and Drug Administration. Fatalities reported to FDA following blood collection and transfusion: Annual summary for fiscal year 2015. Accessed March 4, 2017, http://transfusion.ru/2016/12-05-2.pdf
88. Eder AF, Meena-Leist CE, Hapip CA, Dy BA, Benjamin RJ, Wagner SJ. *Clostridium perfringens* in apheresis platelets: an unusual contaminant underscores the importance of clinical vigilance for septic transfusion reactions (CME). *Transfusion*. 2014;54(3 Pt 2):857–862. https://doi.org/10.1111/trf.12282. quiz 856.
89. Food and Drug Administration. Fatalities reported to FDA following blood collection and transfusion. 2018. https://www.fda.gov/media/136907/download
90. Fridey JL, Stramer SL, Nambiar A, et al. Sepsis from an apheresis platelet contaminated with *Acinetobacter calcoaceticus/baumannii* complex bacteria and *Staphylococcus saprophyticus* after pathogen reduction. *Transfusion*. 2020;60(9):1960–1969. https://doi.org/10.1111/trf.15951.
91. Jones SA, Jones JM, Leung V, et al. Sepsis attributed to bacterial contamination of platelets associated with a potential common source—multiple states, 2018. *MMWR Morb Mortal Wkly Rep*. 2019;68(23):519–523. https://doi.org/10.15585/mmwr.mm6823a2.
92. Jacobs MR, Smith D, Heaton WA, Zantek ND, Good CE. PGD Study Group. Detection of bacterial contamination in prestorage culture-negative apheresis platelets on day of issue with the Pan Genera Detection test. *Transfusion*. 2011;51(12):2573–2582. https://doi.org/10.1111/j.1537-2995.2011.03308.x.
93. Zou S, Notari EP, Fang CT, Stramer SL, Dodd RY. Current value of serologic test for syphilis as a surrogate marker for blood-borne viral infections among blood donors in the United States. *Transfusion*. Apr 2009;49(4):655–661. https://doi.org/10.1111/j.1537-2995.2008.02042.x.
94. Krause PJ, Hendrickson JE, Steeves TK, Fish D. Blood transfusion transmission of the tick-borne relapsing fever spirochete *Borrelia miyamotoi* in mice. *Transfusion*. 2015;55(3):593–597. https://doi.org/10.1111/trf.12879.
95. Thorp AM, Tonnetti L. Distribution and survival of *Borrelia miyamotoi* in human blood components. *Transfusion*. 2016;56(3):705–711. https://doi.org/10.1111/trf.13398.
96. Townsend RL, Moritz ED, Fialkow LB, Berardi V, Stramer SL. Probable transfusion-transmission of *Anaplasma phagocytophilum* by leukoreduced platelets. *Transfusion*. 2014;54(11):2828–2832. https://doi.org/10.1111/trf.12675.
97. Mali S, Tan KR, Arguin PM. Malaria surveillance—United States, 2009. *MMWR Surveill Summ*. 2011;60(3):1–15.
98. Food and Drug Administration. Guidance for industry: recommendations for donor questioning, deferral, reentry and product management to reduce the risk of transfusion-transmitted malaria. Updated April 2020. Accessed December 24, 2020, https://www.fda.gov/media/72243/download.
99. Spencer B, Kleinman S, Custer B, et al. Deconstructing the risk for malaria in United States donors deferred for travel to Mexico. *Transfusion*. 2011;51(11):2398–2410. https://doi.org/10.1111/j.1537-2995.2011.03158.x.
100. Leiby DA. Transfusion-transmitted Babesia spp.: bull's-eye on *Babesia microti*. *Clin Microbiol Rev*. 2011;24(1):14–28. https://doi.org/10.1128/CMR.00022-10.
101. Herwaldt BL, Linden JV, Bosserman E, Young C, Olkowska D, Wilson M. Transfusion-associated babesiosis in the United States: a description of cases. *Ann Intern Med*. 2011;155(8):509–519. https://doi.org/10.7326/0003-4819-155-8-201110180-00362.
102. Tonnetti L, Townsend RL, Dodd RY, Stramer SL. Characteristics of transfusion-transmitted Babesia microti, American Red Cross 2010–2017. *Transfusion*. 2019;59(9):2908–2912. https://doi.org/10.1111/trf.15425.
103. Food and Drug Administration. Guidance for industry: recommendations for reducing the risk of transfusion-transmitted babesiosis. Updated May 2019. Accessed December 23, 2020, https://www.fda.gov/media/114847/download.
104. Cardo LJ. Leishmania: risk to the blood supply. *Transfusion*. 2006;46(9):1641–1645. https://doi.org/10.1111/j.1537-2995.2006.00941.x.
105. Benjamin RJ, Stramer SL, Leiby DA, Dodd RY, Fearon M, Castro E. *Trypanosoma cruzi* infection in North America and Spain: evidence in support of transfusion transmission. *Transfusion*. 2012;52(9):1913–1921. https://doi.org/10.1111/j.1537-2995.2011.03554.x. quiz 1912.
106. Cantey PT, Stramer SL, Townsend RL, et al. The United States *Trypanosoma cruzi* Infection Study: evidence for vector-borne transmission of the parasite that causes Chagas disease among United States blood donors. *Transfusion*. 2012;52(9):1922–1930. https://doi.org/10.1111/j.1537-2995.2012.03581.x.
107. Food and Drug Administration. Guidance for industry: use of serological tests to reduce the risk of transmission of *Trypanosoma cruzi* infection in blood and blood components. Updated December 2017. Accessed August 24, 2018, https://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/UCM528600.pdf
108. Steele WR, Hewitt EH, Kaldun AM, Krysztof DE, Dodd RY, Stramer SL. Donors deferred for self-reported Chagas disease history: does it reduce risk? *Transfusion*. 2014;54(8):2092–2097.
109. Crowder LA, Schonberger LB, Dodd RY, Steele WR. Creutzfeldt-Jakob disease lookback study: 21 years of surveillance for transfusion transmission risk. *Transfusion*. 2017;57(8):1875–1878.
110. Maheshwari A, Fischer M, Gambetti P, et al. Recent US case of variant Creutzfeldt-Jakob disease-global implications. *Emerg Infect Dis*. 2015;21(5):750–759. https://doi.org/10.3201/eid2105.142017.
111. Food and Drug Administration. Guidance for industry: recommendations to reduce the possible risk of transmission of Creutzfeldt-Jakob disease and variant Cretuzfeldt-Jakob disease by blood and blood components. Updated August 2020. https://www.fda.gov/media/124156/download.

CHAPTER 119

PEDIATRIC TRANSFUSION MEDICINE

Bentley B. Rodrigue and Steven R. Sloan

A variety of neonatal and pediatric patients require blood component transfusions. This chapter focuses on aspects of blood-bank laboratory testing, blood products and components, transfusion indications, and potential adverse events that are specifically relevant to neonates and children.

PEDIATRIC BLOOD BANKING

Blood and Blood Components

Several different blood components, including whole blood, reconstituted whole blood, red blood cells (RBCs), platelets, plasma, and cryoprecipitated antihemophilic factor (CRYO) may be available from a blood bank for transfusion. Recently, additional components have become available in the United States. They are photochemically treated INTERCEPT platelets and plasma, and solvent detergent–treated plasma, Octaplas. The availability of specific component types varies between blood suppliers.

- Whole blood is infrequently used and may not be available from a particular blood bank or blood supplier, but it may be available on request and is used by some pediatric cardiac surgery services. Whole blood contains all RBCs, plasma, platelets, and an anticoagulant-preservative solution containing citrate, phosphate, dextrose, and possibly adenine.
- RBC units mostly contain RBCs but also contain some plasma and a preservative solution. Most RBC units contain an additive preservative solution that includes some combination of adenine, dextrose, and mannitol. Additive solutions are safe for relatively small ($\leq$20 mL/kg) transfusions. There are concerns over the safety of these additives given in large transfusions to neonates, and their safety in this setting has never been proven in a randomized clinical trial. In view of this concern, some hospital blood banks provide nonadditive RBC units or wash additive units intended for large transfusions to neonates. Because many blood centers provide only additive RBC units to hospital blood banks and washing an RBC unit takes approximately 1 hour, blood banks have been unable to supply RBC units without additives in many situations. Thus, many institutions now have significant experience transfusing large volumes of additive RBC units to neonates and have not noticed any problems.
- Two general types of platelet units are available in the United States, although any one blood bank or hospital may stock only one of these types. These two types, whole blood–derived platelets (platelets) and platelets collected by apheresis (pheresis platelets), differ in their size. A platelet unit contains approximately 5.5 to 10×10^{10} platelets in about 50 mL, whereas a pheresis platelet unit contains at least 3×10^{11} platelets in about 200 mL. It is often easier to use platelet units for small children because pheresis platelets usually need to be aliquoted to provide the correct dose. However, many blood centers exclusively provide only one type of platelet component. Recently, two modifications of platelet components were approved in the United States, although these had been in use in other countries for several years.
    - Platelets that have undergone pathogen inactivation using the INTERCEPT Blood System (Cerus Corporation, Concord, CA) that mixes amotosalen HCl, a synthetic psoralen compound that intercalates with nucleic acid and, on activation with ultraviolet (UV) light, cross-links pathogen and white blood cell DNA, inhibiting replication.[1] Almost all of the amotosalen, a potential carcinogen, is removed during processing. However, INTERCEPT platelets are contraindicated for neonatal patients treated with phototherapy devices emitting wavelengths <425 nm due to potential erythema from interaction between UV light and amotosalen. Fortunately, phototherapy devices currently used in the United States emit higher wavelength light in the 430 to 490 nm range.
    - Platelets stored in a platelet additive solution (PAS). PAS platelets contain very little plasma, reducing the risk of allergic reactions and transfusion-related acute lung injury (TRALI) but also lowering the dose of coagulation factors contained in the platelet component.
- Plasma is frozen to retain functional plasma proteins including clotting factors. Depending on the timing of freezing and thawing, the component may be called fresh frozen plasma (FFP) or another name. However, all of these plasma components contain all the necessary clotting factors. Two pathogen-inactivated plasma products that had been available in several countries are now available in the United States:
    - INTERCEPT (Cerus Corporation, Concord, CA) treated plasma using the same system as described above.
    - Octaplas (Octapharma, Hoboken, NJ) is a filtered pooled plasma product that is subjected to solvent/detergent treatment to inactivate lipid-enveloped viruses.[2] Its safety and efficacy in pediatric patients have not been evaluated, which would be important in neonates whose coagulation system regulation differs from adults. Specifically, Octaplas contains low concentrations of protein S and α2-antiplasmin, two inhibitors of the coagulation system that are present in low concentrations in neonates.[3,4]
- Cryoprecipitate is prepared from plasma and contains high concentrations of fibrinogen.
- Because pediatric patients require smaller doses of blood components, they often require only a portion of a component.
- RBCs are stored refrigerated and hence can be prepared in aliquots as needed if the blood bank has the necessary equipment. Alternatively, the blood center can collect RBC units into a collection system in which additional bags are attached for dispensing aliquots. Whole blood is also stored refrigerated, but its use is limited and it is almost never prepared in aliquots.
- All platelet components are stored at room temperature under constant agitation and can be prepared in aliquots when needed in the blood bank if the blood bank has the necessary equipment and supplies. However, 1 unit of whole blood–derived platelets does not contain many doses, even for infants, and most blood banks do not aliquot them.
- All plasma products are stored frozen and are not generally aliquoted after thawing. However, many blood centers will prepare plasma aliquots before freezing.
- Because even small infants rarely require less than 1 unit of cryoprecipitate, this component is rarely prepared in aliquots.

Directed Donations

Families sometimes prefer to donate blood for their children using a process known as directed donations, and some blood banks permit this. If this is done without medical reason, it offers no benefit and there are potential risks. Although directed donors need to go through the same screening and infectious disease testing process as all allogeneic blood donors, some studies suggest that directed donors have a slightly higher risk for infectious disease transmission.

In addition, directed donors may be a poor choice for immunologic reasons. For example, if a neonate has alloimmune thrombocytopenia or anemia, the pathologic antibody is a passively acquired maternal antibody directed against inherited paternal antigens. In this case, blood donated by the father would be recognized by the antibody in the baby's circulation and cleared just as the neonate's own platelets or erythrocytes are cleared. Another example in which immune concerns make directed donors a poor choice involves transplants. Some patients may require a future tissue or bone marrow transplant, and blood relatives often serve as the best donors for such transplants. However, prior transfusions from relatives may sensitize the patient's immune system to antigens present on the tissues of blood relatives, complicating those potential tissue or bone marrow transplants.

TECHNICAL CONSIDERATIONS/MECHANICAL DEVICES

Smaller pediatric patients require small transfusions administered at slow rates. Aliquots of components often need to be prepared. This can be performed by collecting blood into collection bags interconnected with sterile tubes or by attaching additional containers to a standard blood component by using a sterile docking device that produces a sterile weld between two separate tubing sets.

Blood components must be filtered to remove microaggregates before transfusion. For an adult patient, this is normally accomplished by transfusing the component through a filter contained within the blood administration set. These standard blood administration sets are not ideal for transfusing small patients because 20 to 40 mL of the component is lost in the dead space of the administration set. Pediatric microaggregate filters with much smaller dead space are available.

For nonbleeding patients, blood components should be transfused at a rate of no more than 5 mL/kg/h. For infants, this corresponds to a lower rate than can be regulated by most standard infusion pumps. Hence, these transfusions are usually performed using syringe pumps, with the blood component aliquot transferred to a syringe prior to the transfusion. Often, the blood bank prepares aliquots of a blood component through a pediatric microaggregate filter directly into a syringe, eliminating the need for bedside microaggregate filtration.

TRANSFUSION MEDICINE: GENERAL INDICATIONS AND DOSING

Indications for Red Blood Cells Transfusion in Neonates, Children, and Adolescents

Neonates Less Than 4 Months Old

RBC transfusions are more commonly administered to hospitalized neonates than any other pediatric patient age group, and RBCs are the component most often transfused in this population. Symptomatic anemia is the major indication for simple transfusion, and an RBC transfusion should be considered when the venous hemoglobin is less than 10 to 13 g/dL depending on the patient's clinical condition or when a neonate has lost approximately 10% of his/her blood volume. A transfusion dose of 10 to 15 mL/kg of RBCs should yield an increase in the neonate of 2 to 3 g/dL of hemoglobin after transfusion.

TABLE 119.1 **Guidelines for Transfusion of Red Blood Cells in Infants Less Than 4 Months of Age**

1. Hematocrit <20% with low reticulocyte count and symptomatic anemia (tachycardia, tachypnea, poor feeding)
2. Hematocrit <30% and
    a. On <35% oxygen hood, or
    b. On oxygen by nasal cannula, or
    c. On continuous positive airway pressure and/or intermittent mandatory ventilation on mechanical ventilation with mean airway pressure <6 cm of water, or
    d. With significant tachycardia or tachypnea (heart rate >180 beats/min for 24 h or respiratory rate >80 breaths/min for 24 h)
    e. With significant apnea or bradycardia (more than six episodes in 12 h or two episodes in 24 h requiring bag and mask ventilation while receiving therapeutic doses of methylxanthines), or
    f. With slow weight gain (<10 g/day observed over 4 days while receiving >100 kcal/kg/day)
3. Hematocrit <35% and
    a. On >35% oxygen hood, or
    b. On continuous positive airway pressure/intermittent mandatory ventilation with mean airway pressure >6–8 cm of water
4. Hematocrit <45% and
    a. On extracorporeal membrane oxygenation (ECMO), or
    b. With congenital cyanotic heart disease

RBC, Red blood cell.

Two randomized clinical trials of premature infants in neonatal intensive care units, examining restrictive versus liberal RBC transfusion practices, had conflicting results.[5,6] Therefore, most guidelines have been based on experience rather than evidence-based medicine (Table 119.1). A retrospective review investigating preoperative blood transfusions (PBTs) in surgical neonates revealed that when using a propensity score-matched model, PBTs are independently associated with increased morbidity and mortality, acknowledging that prospective studies are still needed.[7] Notably, previous data surrounding the contribution of RBC transfusion and anemia to necrotizing enterocolitis in very low birth weight (VLBW, ≦1500 g) infants has been inconsistent. However, a secondary, prospective, multicenter observational study has shown that it is severe anemia, and not transfusions themselves, that is the associated risk factor for necrotizing enterocolitis.[8] An ongoing study (http://clinicaltrials.gov/01702805) has the promise of providing definitive evidence to inform RBC transfusion practice in this population.[9]

With respect to blood product age, one prospective randomized clinical trial found equivalent clinical outcomes in neonates transfused with fresh RBCs stored for less than 7 days when compared to those transfused with standard-issue RBCs with a mean age of 14.6 days, although some have criticized the generalizability of the study results.[10,11]

Older Infants, Children, and Adolescents

RBC transfusion indications for infants older than 4 months and for young children are similar to those of adults. However, there are several noteworthy differences between children and adults: total blood volume, ability to tolerate blood loss, and age-specific hemoglobin levels (Table 119.2). In infants, RBC transfusions are primarily given for surgical losses, anemia of chronic diseases, and malignancies. Infants inherently have lower hemoglobin levels than adults and remain asymptomatic at lower hemoglobin concentrations, especially if the anemia occurs gradually. Even with these physiologic differences, general transfusion-trigger guidelines for pediatric intensive care unit patients are similar to those for adults, with a transfusion

TABLE 119.2 **Guidelines for Transfusion of Red Blood Cells in Patients More Than 4 Months of Age**

1. Emergency surgical procedure in patient with significant postoperative anemia
2. Preoperative anemia when other corrective therapy is not available
3. Intraoperative blood loss ≥15% total blood volume
4. Hematocrit <21%–24%
    a. In perioperative period, with signs and symptoms of anemia
    b. While on chemotherapy/radiotherapy
    c. Chronic congenital or acquired symptomatic anemia
5. Hematocrit <21%, hemodynamically stable patients >3 days old in the pediatric intensive care unit
6. Acute blood loss with hypovolemia not responsive to other therapy
7. Hematocrit <40% and
    a. With severe pulmonary disease
    b. On ECMO
8. Sickle cell disease (may require exchange transfusion)
    a. Cerebrovascular accident
    b. Acute chest syndrome
    c. Splenic sequestration
    d. Aplastic crisis
    e. Recurrent priapism
    f. Preoperatively when general anesthesia is planned (target hemoglobin 10 mg/dL)
9. Chronic transfusion programs for disorders of RBC production (e.g., β-thalassemia major and Diamond-Blackfan syndrome unresponsive to therapy)

ECMO, Extracorporeal membrane oxygenation; *RBC*, red blood cell.

TABLE 119.3 **Guidelines for Transfusion of Platelets in Neonates and Older Children**

Platelet Count <150,000/μL

1. Platelet count 5000–10,000/μL with failure of platelet production
2. Platelet count <25,000–30,000/μL in neonate with failure of platelet production
3. Platelet count <25,000–50,000/μL in stable premature infant
    a. With active bleeding, or
    b. Before an invasive procedure with failure of platelet production
4. Platelet count <50,000–100,000/μL in sick premature infant
    a. With active bleeding, or
    b. Before an invasive procedure in patient with DIC

Without Thrombocytopenia

1. Active bleeding with qualitative platelet defect
2. Unexplained excessive bleeding during cardiopulmonary bypass
3. Patient receiving ECMO with
    a. Platelet count <50,000–100,000/μL
    b. Higher platelet counts and bleeding

DIC, Disseminated intravascular coagulation; *ECMO*, extracorporeal membrane oxygenation.
Modified from Roseff SD, Luban NLC, Manno CS. Guidelines for assessing appropriateness of pediatric transfusion. *Transfusion*. 2002;42:1398; and Wong CC, Luban NLC. Intrauterine, neonatal, and pediatric transfusion. In: Mintz PD, ed. *Transfusion Therapy: Clinical Principles and Practice*. 2nd ed. Bethesda, MD: AABB Press; 2005:159.

trigger of 7 g/dL of hemoglobin for hemodynamically stable patients being shown to be safe for these patients.[12] This threshold has also been found to be safe for hematopoietic progenitor cell (HPC) transplant patients.[13] In pediatric intensive care unit patients, consensus has arrived at a strong recommendation to transfuse when hemoglobin drops to below 5 g/dL; however, at higher hemoglobin levels, there is less agreement regarding RBC transfusion practices.[14] The usual dose of RBCs is 10 to 15 mL/kg.

There is no evidence that pediatric patients benefit from transfusion of RBCs of a particular age. A multicenter, blinded, randomized clinical trial showed that among critically ill pediatric patients, fresh RBCs did not reduce the incidence of new or progressive multiple organ dysfunction syndrome (including mortality) when compared to the use of standard-issue RBCs.[15]

Platelets

Platelet transfusion support in pediatric patients is usually intended as a prophylactic strategy to prevent bleeding (Table 119.3). In sharp contrast to adults, who rarely develop spontaneous severe bleeding until their platelet counts fall below 10,000/μL, preterm infants with other complicating illnesses may bleed at higher platelet counts. The increased risk may be secondary to (1) lower levels of plasma coagulation factors, (2) natural anticoagulants that potentiate thrombin inhibition, (3) intrinsic or extrinsic platelet dysfunction, and (4) increased vascular fragility. Platelet counts and function in older children are similar to those of adults, and the indications for platelet transfusions do not differ from the indications for adults. The prophylactic platelet transfusion thresholds in premature infants have historically been quite controversial and based primarily on expert consensus rather than evidence-based medicine. However, a new multicenter randomized trial supports using a platelet-count threshold of 25,000/μL to guide the administration of prophylactic platelet transfusions in premature neonates who are not bleeding. In this study, it was shown that premature infants randomly assigned to receive prophylactic platelet transfusions at a threshold of 50,000/μL had a significantly higher rate of death or major bleeding event.[16]

Frozen Plasma and Cryoprecipitate

Frozen plasma is used in preterm and term infants most commonly to treat multiple factor deficiencies or vitamin K deficiency, a condition that can occur in infants not prophylactically given vitamin K after birth, especially if the mother ingested certain drugs during pregnancy such as warfarin, cephalosporins, or some anticonvulsants (Table 119.4). Dosing for all pediatric patients is 10 to 15 mL/kg. This should result in an increase in all factor activity of 15% to 20% unless there is a marked consumptive coagulopathy. As mentioned previously, solvent detergent-treated plasma, Octaplas, may not be as therapeutic for neonates as it has low concentrations of protein S and α2-antiplasmin.

Cryoprecipitate is used primarily to treat disorders resulting from a decrease in or dysfunction of fibrinogen or factor XIII deficiency. These indications are similar to those for adults. Children should receive 1 to 2 units of CRYO/10 kg patient weight. One unit or less of cryoprecipitate is usually sufficient to achieve hemostatic levels in infants and premature infants. The expected rise in fibrinogen should be 60 to 100 mg/dL.

Granulocytes

Granulocytes, whose efficacy, especially at low doses, is unproven, may be indicated for neutropenic children with infections unresponsive to standard antimicrobial therapy.[17] In addition, children with granulocyte dysfunction (e.g., chronic granulomatous disease [CGD]) severe infections may also benefit from granulocyte transfusions. However, patients with CGD are at significant risk for

TABLE 119.4 **Guidelines for Transfusion of Frozen Plasma and Cryoprecipitate in Neonates and Older Children**

Frozen Plasma

1. Support during treatment of DIC
2. Replacement therapy
   a. When specific factor concentrates are not available, including but not limited to, antithrombin, protein C or S deficiency, and factor II, factor V, factor X, and factor XI deficiencies
   b. During therapeutic plasma exchange when FFP is indicated
3. Reversal of warfarin in an emergency situation, such as before an invasive procedure with active bleeding. However, prothrombin complex concentrates are usually preferable.

Note: Frozen plasma is not indicated for volume expansion or enhancement of wound healing

Cryoprecipitate

1. Hypofibrinogenemia or dysfibrinogenemia with active bleeding
2. Hypofibrinogenemia or dysfibrinogenemia, undergoing an invasive procedure
3. Factor XIII deficiency with active bleeding or undergoing an invasive procedure in the absence of factor XIII concentrate
4. In the preparation of fibrin sealant
5. von Willebrand disease with active bleeding: cryoprecipitate is used in von Willebrand disease only when both of the following are true:
   a. 1-Deamino-8-D-arginine vasopressin is contraindicated, not available, or does not elicit response
   b. Virus-inactivated plasma-derived factor VIII concentrate (which contains von Willebrand factor) is not available

DIC, Disseminated intravascular coagulation; *FFP*, fresh frozen plasma.

developing anti-human leukocyte antigen (HLA) antibodies from granulocyte transfusions, potentially rendering granulocyte transfusions ineffective and complicating possible future hematopoietic cell transplants.[18]

TRANSFUSION MEDICINE: INDICATIONS IN UNIQUE PEDIATRIC POPULATIONS

Hemolytic Disease of the Fetus and Newborn

Hemolytic disease of the fetus and newborn (HDFN) occurs when the mother's immune system recognizes a foreign, paternally inherited antigen on fetal erythrocytes. The incidence of HDFN dramatically declined after the introduction of Rh immune globulin to prevent sensitization of the mother to rheumatic heart disease (RhD). Although the introduction of Rh immune globulin has prevented most cases of HDFN due to RhD sensitization, it has not totally eliminated HDFN due to RhD or reduced HDFN due to other antibodies.

Most significant cases of HDFN are due to antibodies recognizing antigens other than ABO system antigens. These antibodies should be detected in the blood-bank antibody screen of the blood specimen of the pregnant or postpartum woman. Also necessary for diagnosis, the corresponding antigens would be present on fetal or newborn erythrocytes or predicted to be present on fetal erythrocytes based on molecular testing of fetal DNA. The direct antiglobulin test (DAT) of the fetal or newborn's erythrocytes is usually positive, although it can be negative in transfused patients. Although antibody titers can be used to help predict the severity of the disease during pregnancy, they are generally not useful in the newborn. After birth, the severity of the anemia and the resulting hyperbilirubinemia serve as markers for the severity of HDFN. Rates of rise in bilirubin level are most helpful in determining whether an exchange transfusion will be necessary, with increases of 8 to 13 μmol/L/h despite phototherapy indicating that exchange transfusion will likely be necessary.[19]

RBCs transfused in utero to the fetus need to be compatible with the ABO type of the fetus and mother and hence are usually blood group O. They need to lack the antigen(s) to which the mother has made antibodies, and they need to be crossmatch-compatible with her serum. The RBCs should be irradiated to prevent transfusion-associated graft-versus-host disease (TA-GVHD), and cytomegalovirus (CMV)-safe to minimize the risk for transfusion-transmitted CMV. Relatively fresh RBCs also are usually chosen for these transfusions.

Reconstituted whole blood is usually used for neonatal exchange transfusions. The blood is prepared by removing most of the preservative solution from an RBC unit and adding plasma, usually so that the final hematocrit is 40% to 45%.

Neonatal Alloimmune Thrombocytopenia

The pathophysiology of neonatal alloimmune thrombocytopenia (NAIT) is similar to that of HDFN in that both involve immune-mediated attack and destruction of fetal and neonatal blood cells by the mother's immune system. Unlike HDFN, NAIT often affects a woman's first pregnancy. In addition, the antigens involved are due to polymorphisms on platelet-specific proteins. Although a variety of antigens can be implicated, the human platelet antigen-1 (HPA-1) protein is most frequently implicated in Whites, with approximately 70% to 80% of cases being due to women who lack the HPA-1a antigen making antibodies against HPA-1a that is expressed on fetal platelets.[20] When NAIT is suspected, maternal serum can be tested for antibodies to platelet antigens, and the parent's or patient's platelet antigens can be determined by molecular means in which their platelet antigens are indirectly determined from their DNA. An affected infant or fetus may require platelet transfusions. Although random platelets may be of some transient benefit, antigen-negative units are best. Maternal platelets lack the antigen, but because the plasma contains the pathogenic antibody, the platelet unit should be washed before transfusion. Alternatively, antigen-negative units may be available. Indeed, major blood centers usually have HPA-1a-negative units available.

Extracorporeal Membrane Oxygenation

Extracorporeal membrane oxygenation (ECMO) is an intervention in which whole blood is removed from the patient's venous circulation and circulated through a machine to remove carbon dioxide and replenish oxygen before being returned to the patient. This prolonged intervention is reserved for patients with more than 80% mortality risk and those who have been unresponsive to conventional ventilator support and medical treatment but still potentially can recover. In neonates and children, ECMO has become a lifesaving therapy in the treatment of multiple disorders; Table 119.5[21] lists indications for ECMO support in neonates and children. Standardized guidelines for transfusion practice have not been established, resulting in individualized centers establishing their own criteria. In order to prevent thrombosis and platelet activation in the extracorporeal circuit that is composed of extensive tubing, patients are anticoagulated, usually with heparin, resulting in a significant risk of bleeding.[22] Bleeding during ECMO is a common complication and may be caused by any of the following factors necessary for operating the ECMO circuit or resulting from the thrombogenic surface of the circuit: (1) systemic heparinization, (2) platelet dysfunction, (3) thrombocytopenia, (4) other coagulation defects, and (5) nonendothelial cell surface lining the circuitry. It is recommended that hospital transfusion services and ECMO staff be in close communication to agree on local protocols to ensure safe, efficient, and consistent care (Table 119.6 provides an

TABLE 119.5 Indications for Extracorporeal Membrane Oxygenation for Neonates and Pediatric Patients

Neonatal

- Meconium aspiration
- Respiratory distress syndrome
- Persistent pulmonary hypertension
- Congenital diaphragmatic hernia
- Sepsis

Pediatric

- Bacterial pneumonia
- Viral pneumonia
- Acute respiratory distress syndrome
- Burns
- Inhalation injuries
- Near drowning
- Sepsis

TABLE 119.6 Blood Product Protocols for Extracorporeal Membrane Oxygenation

Clinical Scenario	Urgency	Products	Storage
Cardiac arrest	5–10 min	2 units RBCs	<14 days
ECMO circuit disruption	5–10 min	2 units RBCs	<14 days
Progressive septic shock, nonneonate	30 min	2 units RBCs	<10 days
Neonate transferred for ECMO	1–2 h	2 units RBCs 1 unit FFP 1 unit platelets	<10 days
Cardiac ICU	30–60 min	2 units RBCs	<7 days
Gradual respiratory or cardiac failure on conventional support	Hours-days	2 units RBCs	<10 days

ECMO, Extracorporeal membrane oxygenation; *FFP*, fresh frozen plasma; *ICU*, intensive care unit; *RBC*, red blood cell.
Modified from protocols developed at The Children's Hospital of Philadelphia. Friedman DF, Montenegro LM. Extracorporeal membrane oxygenation and cardiopulmonary bypass. In: Hillyer CD, Strauss RG, Luban NLC, eds. *Handbook of Pediatric Transfusion Medicine*. London: Elsevier Academic Press; 2004:181.

example protocol from one institution).[21] Blood products for ECMO are typically ABO-and Rh-specific and crossmatch-compatible for priming. The RBC units for priming circuits are typically relatively fresh, irradiated, and CMV-seronegative and/or leukoreduced.

Trauma

Hemorrhagic shock requiring massive transfusion can occur in children. Prothrombin time (PT), activated partial thromboplastin time (aPTT), and platelet count abnormalities upon emergency room admission are strongly associated with mortality.[23] Transfusion management of a pediatric trauma patient often must be guided by the patient's estimated blood loss and associated signs and symptoms, such as hypotension or tachycardia, and bleeding. A prospective study found that a ratio of 1:1:1 of RBC to plasma to platelet units transfused did not decrease overall mortality but decreased hemorrhagic deaths in trauma patients.[24] The use of such a massive transfusion strategy has not been rigorously tested in pediatric patients. Although some have advocated adopting similar protocols for pediatric trauma centers, this has occurred in only a few places, most likely because there are little data on transfusions in pediatric trauma patients.[25]

Hemoglobinopathies

In patients with sickle cell disease (SCD), chronic transfusion therapy has been shown to reduce the risk for both primary and secondary stroke, by decreasing the hemoglobin S content of the patient's blood, as well as achieving a reduction in sickling, suppression of erythropoiesis, and preventing an increase in blood viscosity.[26,27] The risk for recurrent stroke has been reduced to less than 10% if hemoglobin levels are maintained between 8 and 9 g/dL and hemoglobin S levels below 30%. Simple or partial-exchange transfusion therapy can achieve this goal when performed approximately every 3 to 4 weeks. Chronic erythrocytapheresis has also been used for this therapy with an added mission to mitigate iron overload complications. Chronic transfusion treatment for stroke most times is an indefinite therapy; that is, once the patient is placed on it, cessation is not possible because it has been shown to lead to recurrent stroke.[26] Table 119.2 describes other indications for patients with SCD needing either simple or chronic RBC transfusion therapy. Products for patients with SCD should be screened at a minimum for hemoglobin S and should be leukocyte reduced to reduce the risk for febrile nonhemolytic transfusion reactions and to reduce the risk for HLA alloimmunization, resulting in platelet refractoriness that can complicate possible stem cell transplantation.

In children with SCD, it also is useful to prevent alloimmunization to minor RBC antibodies because most patients with severe SCD will require multiple RBC transfusions during their lifetime. Overall, the SCD patient has higher rates of alloimmunization than other chronically transfused patient groups. The antibodies most frequently produced are against common Rh, Kell, Duffy, and Kidd system antigens. Some sickle cell treatment centers perform thorough phenotype analysis of a patient's red cells before initiating transfusion therapy. This testing helps to reduce the rate of alloimmunization by allowing preferential selection of phenotypically similar units.[28] However, particularly for patients who are not yet alloimmunized, this process remains controversial because phenotypically compatible units may be difficult to obtain and expensive.[29] One common protocol followed for nonalloimmunized patients is pretransfusion phenotypic matching for C, E, and K antigens to reduce the incidence of alloimmunization. Once a patient has developed a red cell antibody, some centers extend matching to additional red cell antigens (Fy, Jk, S) to prevent further alloimmunization. However, care must be taken with some of these strategies as recent studies have found no benefit in alloimmunization rates by providing Rh-matched RBC units from minority donors.[30] The benefits of RBC transfusion therapy in SCD disease must constantly be weighed against the costs (e.g., iron overload, RBC alloimmunization, and increased donor exposure risks). As a result of these issues, some clinicians have proposed that a clinically successful course of transfusions that maintains the hemoglobin S below 30% could, after several years, be transitioned to a strategy of more limited transfusions with a hemoglobin S target of 40% to 50% to reduce the risks for iron overload.[26] Patients with SCD may also be at risk for delayed hemolytic transfusion reactions, the development of autoantibodies, and "hyperhemolytic" syndrome, a phenomenon in which a patient hemolyzes both the native and transfused erythrocytes following RBC transfusion. The mechanism is not well characterized.

Thalassemia

Thalassemia with severe anemia is usually treated with chronic transfusion therapy to improve tissue oxygenation and suppress extramedullary erythropoiesis in the liver, spleen, and bone marrow. This approach mitigates many of the complications caused by the ineffective erythropoiesis. In contrast to chronic transfusion regimens used to treat SCD, most β-thalassemia major patients requiring chronic transfusion therapy start at a very young age. The treatment goals in this population are characterized by (1) increasing oxygen-carrying capacity by anemia correction, (2) preventing progressive hypersplenism, (3) suppressing endogenous erythropoiesis, and (4) reducing gastrointestinal absorption of iron.[31] The target hemoglobin levels are usually 8 to 9 g/dL, where normal growth and development can occur in these patients. Super-transfusion protocols aim for higher target hemoglobin levels (11 to 12 g/dL) to reduce organomegaly from extramedullary hematopoiesis. Iron overload is a complication of this RBC transfusion protocol that cannot be prevented and must be treated with chelation therapy beginning early in childhood. In a recent report from the Centers for Disease Control and Prevention on transfusion complications in thalassemia patients in the thalassemia network in the United States from 2004 to 2012, iron-induced multi-organ dysfunction from transfusion was common despite chelation. Transfusion-transmitted disease pathogens were found in almost one-quarter of those patients monitored and almost 50% of patients experienced transfusion reactions including allergic, febrile nonhemolytic, and hemolytic. RBC alloimmunization was found in 19% of all patients with the most common antigens being E, Kell, and C. Years of transfusion was found to be the strongest predictor of alloantibody formation.[32] Phenotypic-matching protocols are used in some locations to prevent RBC alloimmunization, but data supporting or refuting this practice are scant and further research is needed.

Autoimmune Hemolytic Anemia

Autoimmune hemolytic anemia in children occurs predominantly in young children, with a median age of 3.8 years, and 53% are associated with other immunologic diseases, according to one study in which 74% of the cases had both immunoglobulin G (IgG) and C3d on their surface.[33] Like adult cases, most cases of autoimmune hemolytic anemia in children have antibody reactivity to all RBCs from all donors.

Children also may develop autoimmune hemolytic anemia associated with a Donath-Landsteiner antibody, which is rarely seen in adults. Classically, the Donath-Landsteiner antibody is associated with paroxysmal cold hemoglobinuria, a disease in which patients developed paroxysms of hemoglobinuria following exposure to cold temperatures.[34] However, some children with a Donath-Landsteiner antibody develop symptoms of anemia that are not obviously associated with exposure to cold. The syndrome often develops following an infection such as a respiratory infection.

The Donath-Landsteiner antibody is an IgG antibody that binds erythrocytes, usually recognizing the P antigen, at cold temperatures (<20°C [68°F]) and fixes early components of complement. The fixed complement does not cause significant hemolysis at cold temperatures, but upon warming, the entire complement complex binds and lyses the erythrocytes. Initial testing in the blood bank may be entirely normal or may reveal a positive DAT for C3. The Donath-Landsteiner antibody can be detected only by special testing in which the patient's plasma is incubated with erythrocytes in cold temperatures followed by warm temperatures.

Hematopoietic Cellular Transplant Patients

Transfusion support for HPC transplant patients is similar to that for adult patients, although there are few published studies on transfusion of pediatric HPC transplant patients. RBC transfusions are often not needed in these patients whose hemoglobin level is at least 7 g/dL.[13] While no studies in the modern era have been published on platelet transfusions in these patients, the use of a transfusion trigger of 10,000 platelets/μL, like that used for adults, seems reasonable for those patients without a significant bleeding risk other than thrombocytopenia.

Apheresis

Pediatric apheresis is used for many of the same indications as for adults. However, additional aspects need to be considered in treating pediatric patients. Many children require vascular access through a central vein because the peripheral veins of many children are too small. In addition, peripheral access requires patients to remain seated or reclining with limited arm movement for the procedure duration, and younger patients are often unable to comply with this given that procedures usually last at least 2 hours. During the procedures, children are susceptible to symptomatic hypocalcemia from citrate anticoagulation. For this reason, some pediatric centers use heparin to anticoagulate patients, although prophylactic cation replacement can successfully prevent most symptoms in pediatric apheresis patients.[35] Apheresis procedures subject the patient to volume shifts, which are usually a loss of less than 200 mL of fluid during the procedure and a gain of up to 300 mL at the end of the procedure. The actual fluid shifts depend on the machine, procedure, and parameters for that procedure. In some cases, the volume shifts may be too large to be safe for the patient. In these cases, most machines can be primed with 5% albumin, a RBC unit, or reconstituted whole blood. Using a prime solution, it is possible to minimize fluid shifts so that the patient is effectively volume neutral for the entire procedure. Procedures to accomplish this are usually only available at centers that perform apheresis on relatively large numbers of pediatric patients.

Special Processing and Prevention of Adverse Events in Pediatric Patients

Special processing of blood products is performed more frequently for children than for adults. This is especially true for the preterm infant population, specifically in VLBW infants weighing less than 1500 g and extremely low-birth-weight infants weighing less than 1000 g.

Leukocyte-Reduced Blood Components

Leukoreduced cellular blood products are used in greater than 80% of blood components transfused to children in the United States. The rationale behind the use in pediatric patients has primarily been extrapolated from adult studies. In general, leukoreduction reduces transmission of CMV,[36] decreases HLA alloimmunization,[37] and decreases the frequency of febrile nonhemolytic transfusion reactions. The benefits of leukoreduction for neonates differ from the benefits for older patients. While neonates younger than 4 months of age rarely develop transfusion reactions and rarely become HLA alloimmunized, some neonates, especially premature infants, are at risk for transfusion-transmitted CMV.[36] Additionally, one study of premature infants found that implementation of universal leukoreduction was associated with decreased incidences of retinopathy, prematurity, and bronchopulmonary dysplasia, and decreased length of hospitalization.[38]

Cytomegalovirus-Seronegative Blood Components

Some immunosuppressed pediatric patients, including premature infants, are at risk of significant disease from CMV infection. Transfusion-transmitted CMV can occur because many healthy blood donors have been infected at some point and their leukocytes harbor the virus. Approaches to reduce the risk of transfusion transmission of CMV include transfusion of blood components that test negative for antibodies to CMV (CMV seronegative), are leukoreduced, or both. Although each of these approaches is highly effective, none of these approaches provides zero risk of transfusion-transmitted CMV since blood from donors who were recently infected can lack antibodies to CMV and can contain cell-free virions that are not removed by leukoreduction. The risk of transfusion-transmitted CMV in VLBW infants (<1500 g) is less than 1% when the units are leukoreduced and CMV seronegative.[39] This approach has not been directly compared to using only CMV seronegative or using only leukoreduced units. However, results from older studies found that the residual risk for transfusion-transmitted CMV in adults from CMV seronegative blood components to be between 1% and 3% but current risks are likely lower, and one study found no infants acquiring transfusion-transmitted CMV-from transfusion of CMV seronegative blood components.[40] Leukoreduction is also very effective, reducing the rate of transfusion-transmitted CMV to less than 1% in immunosuppressed patients of all ages.[41] Some have recommended the combined approach of using leukoreduced CMV seronegative units for the prevention of transfusion-transmitted CMV for some of the most at-risk patients.[42] Despite confidence in the safety of using CMV seronegative units, it may not be practical and cost-effective to rely on CMV-seronegative donors as they often constitute less than or equal to one-half of the blood donor pool.

Irradiation

Mortality rates associated with TA-GVHD are as high as 80% to 90%, with no effective treatments. As a result, identifying neonates and children who are immunosuppressed is paramount, so irradiation of cellular components can be performed to prevent this noninfectious serious hazard of transfusion. There are differing expert opinions and practices, and local protocols based on patient populations, available equipment, and best practices at each institution should be followed. The following patients should receive irradiated cellular blood components: (1) premature infants with birth weight less than 1200 g, (2) any child with known or suspected cellular immune deficiency (e.g., severe combined immunodeficiency), (3) any child with significant immunosuppression due to chemotherapy or radiation treatment, (4) any child who receives blood components from blood relatives, and (5) any child who receives HLA-matched or crossmatched platelet components.

Washing

Washing removes the supernatant from RBC or platelet units and is usually performed to reduce the risk of allergic reactions related to plasma. For very young children, washing may also be performed to reduce the concentration of extracellular potassium or to remove anticoagulant-preservative solutions. Washing can also be used to remove pathologic antibodies from a donor unit. Most donor units lack such antibodies. However, if maternal RBC or platelet units are used to treat her newborn with HDFN or neonatal alloimmune thrombocytopenic purpura, respectively, then her plasma would contain the pathogenic antibodies and the units should be washed prior to transfusion. Indeed, because maternal blood contains pathologic antibodies in these settings, its use for these patients is not generally recommended. However, a maternal component may be used because it may be the only component available that lacks the antigen recognized by her antibodies

Volume Reduction

Volume reduction, involving removal of plasma, is primarily used for patients who cannot tolerate the volume of a blood component transfusion, such as some infants with compromised cardiac function. However, it may also be employed for infants and children who receive ABO-mismatched platelet transfusions because several deaths have been reported in the literature of children who have received out-of-group platelets (e.g., O platelet pheresis to A recipient).[43] Not all blood banks volume-reduce such platelets.

Reconstitution of RBCs for Neonatal Exchange Transfusion

During a neonatal exchange, the infant's whole blood is replaced with donor whole blood, which is usually reconstituted whole blood prepared from type-compatible RBCs and plasma. Typically, RBCs less than 5 to 7 days old are used to minimize the concentration of extracellular potassium and, theoretically, provide transfused RBCs that will have maximal in vivo benefit, although this has never been demonstrated. Because of theoretical concerns associated with large volumes of RBC additives transfused to premature infants, nonadditive units are sometimes selected or additives are removed from the RBCs prior to reconstitution. Most blood banks also provide RBC units that are negative for sickle hemoglobin to avoid the rare potential of intravascular sickling. RBC units should be leukoreduced, CMV-seronegative, or both to reduce the incidence of transfusion-transmitted CMV. The RBC unit should also be irradiated to prevent TA-GVHD if the infant is considered at risk of this by local criteria. If irradiated, the RBC or reconstituted whole blood should be irradiated just before the exchange to minimize the liberation of potassium from intracellular stores. Alternatively, the RBC unit can be irradiated prior to volume reduction or washing. The glucose load during an exchange transfusion can be high due to the fact that banked plasma contains added dextrose and because glucose is added to RBC preservative solutions. Hence, glucose levels should be monitored during the first few hours of the exchange.

The goal of most exchanges is to exchange twice the blood volume of the neonate. The volume needed can be calculated based on the fact that the blood volume of a neonate ranges from 100 mL/kg for the most premature to 85 mL/kg for term infants. Usually, one RBC unit is sufficient for a two-volume exchange. The reconstituted whole blood should have a hematocrit of approximately 40% to 50% and must be adequately mixed to maintain the intended hematocrit throughout the exchange transfusion.

The reconstituted whole blood should be transfused through a standard filter and an inline blood warmer. In general, no more than 5 mL/kg of body weight or 5% of the infant's blood volume is to be removed and replaced during a 2 to 5 minutes cycle. The exchange should be performed at a slow pace so as to not cause sudden hemodynamic changes that can result in cerebral blood flow shifts in intracranial pressure, precipitating an intraventricular hemorrhage.[44] A double-volume exchange transfusion typically takes 1.5 to 2 hours.

REFERENCES

1. FDA, INTERCEPT® Blood System for Platelets-Small Volume (SV) Processing Set. Intercept Package Insert; 2014.
2. Beeck H, Hellstern P. In vitro characterization of solvent/detergent-treated human plasma and of quarantine fresh frozen plasma. *Vox Sang*. 1998;74:219–223.
3. Albisetti M. The fibrinolytic system in children. *Semin Thromb Hemost*. 2003;29:339.
4. El Beshlawy A, Alaraby I, Abou Hussein H, et al. Study of protein C, protein S, and antithrombin III in newborns with sepsis. *Pediatr Crit Care Med*. 2019;11(1):52–59.
5. Bell EF, Strauss RG, Widness JA, et al. Randomized trial of liberal versus restrictive guidelines for red blood cell transfusion in preterm infants. *Pediatrics*. 2005;115(6):1685–1691.
6. Kirplani H, Whyte RK, Andersen C, et al. The Premature Infants in Need of Transfusion (PINT) study: a randomized, controlled trial of a restrictive (low) versus liberal (high) transfusion threshold for extremely low birth weight infants. *J Pediatr*. 2006;149(3):301–307.
7. Dukleska K, et al. Preoperative blood transfusions and morbidity in neonates undergoing surgery. *Pediatrics*. 2020;146(5):e20193718.
8. Patel RM, et al. Association of red blood cell transfusion, anemia, and necrotizing enterocolitis in very low-birth-weight infants. *JAMA*. 2016;315(9):889–897.
9. Nickel RS, Josephson CD. Neonatal transfusion medicine: five major unanswered research questions for the twenty-first century. *Clin Perinatol*. 2015;42(3):499–513.
10. Fergusson DA, Hébert P, Hogan DL, et al. Effect of fresh red blood cell transfusion on clinical outcomes in premature, very low-birth-weight infants: the ARIPI randomized trial. *JAMA*. 2012;308(14):1443–1451.
11. Fergusson D, Tinmouth A. Storage age of red blood cells for transfusion of premature infants. *JAMA*. 2013;309(6):544–545.

12. Lacroix J, Hébert PC, Hutchinson JS, et al. Transfusion strategies for patients in pediatric intensive care units. *N Engl J Med*. 2007;365(16):1609–1619.
13. Lightdale JR, Randolph AG, Tran CM, et al. Impact of a conservative red blood cell transfusion strategy in children undergoing hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2012;18(5):813–817.
14. Doctor A, Cholette JM, Remy KE. Recommendations on RBC transfusion in general critically ill children based on hemoglobin and/or physiologic thresholds from the pediatric critical care transfusion and anemia expertise initiative. *Pediatr Crit Care Med*. 2018;19(9S Suppl 1):S98–S113.
15. Spinella PC, Tucci M, Fergusson DA. Effect of fresh vs standard-issue red blood cell transfusions on multiple organ dysfunction syndrome in critically ill pediatric patients: a randomized clinical trial. *JAMA*. 2019;322(22):2179–2190.
16. Curley A, et al. Randomized trial of platelet-transfusion thresholds in neonates. *N Engl J Med*. 2019;380(3):242–251.
17. Price TH, Boeckh M, Harrison RW, et al. Efficacy of transfusion with granulocytes from G-CSF/dexamethasone treated donors in neutropenic patients with infection. *Blood*. 2015;126(18):2153–2161.
18. Heim KF, Fleisher T, Stroncek DF, et al. The relationship between alloimmunization and posttransfusion granulocyte survival; experience in a chronic granulomatous disease cohort. *Transfusion*. 2011;51(6):1154–1162.
19. Wennberg RP, Depp R, Heinrichs WL. Indications for early exchange transfusion in patients with erythroblastosis fetalis. *J Pediatr*. 1978;92(5):789–792.
20. Davoren A, Curtis BR, Aster RH, et al. Human platelet antigen-specific alloantibodies implicated in 1162 cases of neonatal alloimmune thrombocytopenia. *Transfusion*. 2004;44(8):1220–1225.
21. Friedman DF, et al. Extracorporeal membrane oxygenation and cardiopulmonary bypass. In: Hillyer SR, Luban NLC. CD, eds. *Handbook of Pediatric Transfusion Medicine*. London: Elsevier Academic Press; 2004.
22. Meliones JN, et al. Extracorporeal membrane oxygenation: the role of blood components. In: Chambers IL, ed. *Supporting the Pediatric Transfusion Recipient*. Bethesda, MD: AABB; 1994.
23. Hendrickson JE, Shaz BH, Pereira G, et al. Coagulopathy is prevalent and associated with adverse outcomes in transfused pediatric trauma patients. *J Pediatr*. 2012;160(2). p. 204-209.e3.
24. Holcome JB, Tilley BC, Baraniuk S, et al. Transfusion of plasma, platelets, and red blood cells in a 1:1:1 vs a 1:1:2 ratio and mortality in patients with severe trauma: the PROPPR randomized clinical trial. *JAMA*. 2015;313(5):471–482.
25. Dehmer JJ, Adamson W. Massive transfusion and blood product use in the pediatric trauma patient. *Semin Pediatr Surg*. 2010;19(4):286–291.
26. Adams RJ, Brambilla D, et al. Discontinuing prophylactic transfusions used to prevent stroke in sickle cell disease. *N Engl J Med*. 2005;353(26):2769–2778.
27. Adams RJ, McKie VC, Hsu L, et al. Prevention of a first stroke by transfusions in children with sickle cell anemia and abnormal results on transcranial Doppler ultrasonography. *N Engl J Med*. 1998;339(1):5–11.
28. Vichinsky EP, Luban N, Wright E, et al. Prospective RBC phenotype matching in a stroke-prevention trial in sickle cell anemia: a multicenter transfusion trial. *Transfusion*. 2001;41(9):1086–1092.
29. Castro O, Sandler SG, Houston-Yu P, et al. Predicting the effect of transfusing only phenotype-matched RBCs to patients with sickle cell disease: theoretical and practical implications. *Transfusion*. 2002;42(6):684–690.
30. Chou ST, Jackson T, Vege S, et al. High prevalence of red blood cell alloimmunization in sickle cell disease despite transfusions from Rh-matched minority donors. *Blood*. 2013;112(6):1062–1071.
31. Olivieri NF. The beta-thalassemias. *N Engl J Med*. 1999;341(2):99–109.
32. Vichinsky E, Neumayr L, Trimble S, et al. Transfusion complications in thalassemia patients: a report from the Centers for Disease Control and Prevention (CME). *Transfusion*. 2014;54(4):972–982.
33. Aladjidi N, Leverger G, Leblanc T, et al. New insights into childhood autoimmune hemolytic anemia: a French national observational study of 256 children. *Haematologica*. 2011;96(5):655–663.
34. Eder AF. Review: acute Donath-Landsteiner hemolytic anemia. *Immunohematol*. 2005;21(2):56–62.
35. Bolan CD, Yau YY, Cullis HC, et al. Pediatric large-volume leukapheresis: a single institution experience with heparin versus citrate-based anticoagulant regimens. *Transfusion*. 2004;44(2):229–238.
36. Gilbert GL, Hayes K, Hudson IL, James J. Prevention of transfusion-acquired cytomegalovirus infection in infants by blood filtration to remove leukocytes. Neonatal Cytomegalovirus Infection Study Group. *Lancet*. 1989;1(8649):1228–1231.
37. Trial to Reduce Alloimmunizations to Platelets Study Group. Leukocyte reduction and ultraviolet B irradiation of platelets to prevent alloimmunization and refractoriness to platelet transfusions. *N Engl J Med*. 1997;337(26):1861–1869.
38. Fergusson D, Hébert PC, Lee SK, et al. Clinical outcomes following institution of universal leukoreduction of blood transfusions for premature infants. *JAMA*. 2003;289(15):1950–1956.
39. Josephson CD, Caliendo AM, Easley KA, et al. Blood transfusion and breast milk transmission of cytomegalovirus in very low-birth-weight infants: a prospective cohort study. *JAMA Pediatr*. 2014;168(11):1054–1062.
40. Yeager AS, Grumet FC, Hafleigh EB, et al. Prevention of transfusion-acquired cytomegalovirus infections in newborn infants. *J Pediatr*. 1981;98(2):281–287.
41. Vamvakas EC. Is white blood cell reduction equivalent to antibody screening in preventing transmission of cytomegalovirus by transfusion? A review of the literature and meta-analysis. *Transfus Med Rev*. 2005;19(3):181–199.
42. Laupacis A, Brown J, Costello B, et al. Prevention of posttransfusion CMV in the era of universal WBC reduction: a consensus statement. *Transfusion*. 2001;41(4):560–569.
43. Josephson CD, Castillejo M-I, Grima K, et al. ABO-mismatched platelet transfusions: strategies to mitigate patient exposure to naturally occurring hemolytic antibodies. *Transfus Apher Sci*. 2010;42(1):83–88.
44. Bada HS, Chua C, Salmon JH, Hajjar W. Changes in intracranial pressure during exchange transfusion. *J Pediatr*. 1979;94(1):129–132.

CHAPTER 120

TRANSFUSION AND APHERESIS SUPPORT FOR SICKLE CELL DISEASE PATIENTS

John P. Manis

Transfusion of red cells in all patients is primarily triggered by the need to increase oxygen delivery to tissues in the setting of blood loss or anemia. For patients with sickle cell disease (SCD), there are additional considerations for transfusion including the need to remove sickle hemoglobin (HbS) containing cells in specific clinical settings. These settings include acute complications (e.g., acute chest syndrome) of SCD or for prophylaxis of vaso-occlusive episodes (VOEs) involving stroke.

Removal or dilution of endogenous HbS red cells by transfusion further benefits patients by preventing further HbS cell sickling and increasing tissue oxygenation, suppressing erythropoietin-induced bone marrow (BM) erythropoiesis, and lengthening the average red cell lifespan. Every transfusion decision, especially in SCD patients must carefully assess risks and benefits regarding when, what, and how to transfuse a red cell component. Patients with SCD experience higher rates of alloimmunization to red cell antigens, iron overload, and also require attention for potential changes in blood viscosity in response to transfusion therapy. Nevertheless, as in all patients with severe hemolytic anemia, selection and modification of red cell components should not delay transfusion in life-threatening emergencies.

Many accepted indications are based primarily on expert consensus, particularly when clinical trials are lacking. Overall, the use of transfusion therapy for SCD is increasing due to expanded indications, increased availability of erythrocytopheresis, and oral chelators to prevent transfusion-associated iron overload.

HEMODYNAMIC CONSIDERATIONS

The interplay of mutations causative for SCD leading to hemoglobin (Hb) polymerization resulting in increased red cell rigidity are described elsewhere (see Chapter 42). One of the main features of SCD is the changes of red cell shape in response in part to delayed polymerization of HbS resulting in a sickle shaped cell. The abnormally shaped red cells are poor at oxygen delivery to tissues and prone to further cell membrane damage resulting in both intravascular and extravascular hemolysis. In addition, the combination of abnormally shaped and damaged cells together with products of hemolysis promotes vasculopathy through endothelial cell dysfunction and vessel occlusion, especially in the capillary vasculature but also in large blood vessels, resulting in ischemic tissue injury, inflammation, and immune dysregulation. The most significant factor that promotes the formation of sickled red cells in SCD is hypoxia, leading to an accumulation of cells in the capillary and venous vasculature. In addition to hypoxia, other factors promote red cell deformation and hemolysis including acidosis and oxidative stress. Moreover, endothelial damage that results from the cycles of ischemic and reperfusion injury results in an environment that promotes further red cell damage and hemolysis. The loss of RBC deformability in SCD is the main factor responsible for the disease morbidity associated VOE.

Blood flow is governed by many factors including hematocrit (Hct), red blood cell shape, oxygenation, plasma proteins, and vascular elasticity. Studies of blood flow (hemorheology) over the past half century have pointed to altered dynamics in patients with SCD, owing largely to increased blood viscosity.[1] In turn, red cells, the largest cellular component of blood, largely govern viscosity with hematocrit and shape being the most important factors. A rise in Hct increases blood viscosity in all vessels and at all flow rates but is especially elevated in low flow vessels such as veins and capillaries. Increased viscosity produces increased resistance and pressure to blood flow, culminating in a drop of oxygen transport. While a drop in oxygen transport due to increased viscosity is seen at relatively high Hb concentrations (>20 g/dL) in cases of secondary polycythemia patients with normal red cells, patients with HbSS show a loss of oxygen delivery at a Hb concentration of about 10 to 12 g/dL. The viscosity of SCD patients is higher than that in patients with healthy red cells at the same hematocrit, which can further increase nearly ten-fold when deoxygenated. Deoxygenation of HbS containing red cells due to both a low flow state and a correspondingly longer time in a hypoxemic environment further promotes increased viscosity and irreversible sickling. Slower flow rates promote increased interactions between blood cells as well as between blood cells and endothelial cells that together with plasma proteins including coagulation and inflammatory factors lead to vaso-occlusion and tissue ischemia and injury. In fact, plasma viscosity increased in SCD is likely also due to these increased acute phase reactants and fibrinogen among other factors. Therefore, increased viscosity dramatically promotes irreversible sickling. However, blood viscosity (and similarly measures of this value alone) may not accurately represent all the forces that contribute to this pathologic process. Additional factors include shear rate, vessel size, and length of blood traversing small vessels. For these reasons, simple transfusion should be avoided in patients with Hb concentrations greater than 10 g/dL. Similarly, in SCD patients that receive transfusion and reach a higher than expected target Hb, phlebotomy to reduce the Hb concentration below 10 g/dL may be considered in some cases, especially in the setting of neurologic compromise.

A variety of additional factors alter the rheology of HbSS red cells, especially when additional genetic defects of Hb production are present. Alpha-thalassemia has been shown to ameliorate the complications seen in patients with HbSS disease, in part due to increased RBC deformability. In contrast, patients with Hb SC disease have increased blood viscosity but RBC deformability is not as severe as that found in SCD. Increased HbF concentrations, either through an inherited sequence modification (Hereditary Persistence of Fetal Hemoglobin [HPFH]), or pharmacologically (i.e., hydroxurea) significantly reduces the number of VOEs and improves the mortality of SCD.[2] In vitro studies have shown that HbF inhibits HbS polymerization, which improves membrane deformability and thus prevents the formation of sickled cells. In addition to red cell intrinsic factors, others affecting the endothelium also adversely influence SCD hemorheology and include products of hemolysis, reactive oxygen species, and nitric oxide concentration.[3] Taken together, one must consider all of these complex factors to plan for transfusion in order to balance the resultant increase of oxygen delivery with the possible increase in viscosity due to the remaining percentage of HbS containing cells.

CLINICAL CONSIDERATIONS FOR TRANSFUSION

Transfusion of healthy donor RBC in SCD serves to both increase oxygen delivery to tissues and to dilute endogenous HbS containing cells. This can be done in an acute setting with the hope of improving end organ damage (e.g., acute chest syndrome, multi-organ failure) or in a prophylactic fashion (e.g., stroke prevention or perioperative settings). Transfusion, however, has not been shown to reverse uncomplicated VOEs consisting primarily of boney pain and correspondingly is not indicated in patients who do not have symptoms of anemia. In those latter cases, transfusion will not improve pain but may prevent further complications of the initial VOE. Clinical trials of when and how to transfuse patients with SCD are few and consequently most clinicians rely on consensus papers and clinical practice (see the box on Indications for Red Cell Transfusion).

INDICATIONS FOR RED CELL TRANSFUSION

Clinical Indication	Simple Transfusion	Exchange Transfusion	Comments
Acute stroke	X	XXX	Exchange transfusion preferred
Primary stroke prevention	X	X	Maintain HbS <30% for at least 1 year
Secondary stroke prevention	X	X	
Severe acute chest	X	XXX	
Recurrent acute chest			Can consider prophylactic transfusion
Operative procedure (preoperatively)	X	X	For general anesthesia >1 h including abdominal surgery, tonsillectomy, orthopedic procedures
Multi-organ failure	X	XXX	
Splenic or hepatic sequestration	XXX	X	
Aplastic crisis	XXX	X	
Chronic transfusion and iron overload		X	
Pregnancy	X		For complicated pregnancy or patients that have been on hydroxycarbamide
Avascular necrosis			Transfuse only for symptomatic anemia
Vaso-occlusive crisis			Transfuse only for symptomatic anemia
Leg ulcers			Transfuse only for symptomatic anemia
Pain			Transfuse only for symptomatic anemia, may help for recurrent painful crises

ACUTE ANEMIA

SCD is a chronic hemolytic anemia that is compensated by increased erythropoiesis and reticulocytosis. Red cell survival is significantly reduced in SCD with HbS cells lasting about two weeks and those with HbF up to 6 weeks. Therefore, stress erythropoiesis is already present even at steady state in untransfused patients with SCD. Any additional insult or burden on red cell production may result in a severe anemia. The net Hb concentration varies significantly among patients with SCD depending on the homeostatic factors controlling erythropoiesis (e.g., erythropoietin, B12, folate) and physiologic adaptation to anemia. Therefore, no single Hb concentration value can define acute anemia, which should be evaluated by comparing the historical peripheral blood Hb concentration of each patient together with a clinical assessment of symptoms of anemia. In addition to the variability of Hb concentrations among patients, each patient has their own variability over time based on factors including hydration, again denoting the need to correlate laboratory values with symptoms. Generally, most SCD patients have a Hb concentration of approximately 8 to 10 g/dL and a Hct of approximately 20% to 30% with reticulocytosis in the range of 3% to 15%. A drop of Hb of 2 to 3 g/dL without a corresponding reticulocytosis and with clinical signs of anemia would warrant transfusion. This is especially important in children where blood flow to the brain increases from birth to early childhood and decreases during adolescence. Children with SCD are at greater risk for silent ischemia that may lead to stroke; therefore, a lower trigger should be strongly considered for transfusion.

Causes of acute anemia include red cell aplasia secondary to parvovirus infection, splenic sequestration or rupture, bleeding, or accelerated hemolysis that may be associated with infection. One of the most important causes of acute anemia in recently transfused SCD patients is delayed hemolytic transfusion reaction (DHTR) (discussed later) that should be evaluated in all such patients. In these clinical settings, there should be prompt consideration to transfuse as it is unlikely that increased erythropoiesis can raise the Hb concentration.

TRANSFUSION FOR COMPLICATIONS OF SICKLE CELL DISEASE

One of the more devastating outcomes in SCD are cerebrovascular accidents. Transfusion for this indication is divided into that for acute stroke and that for prophylaxis for primary or secondary events and are described in detail elsewhere (see Chapter 42). As stroke is a leading cause of morbidity and mortality in SCD, prevention of this complication is a major emphasis of patient care. Many studies have focused on identifying at risk children, with screening regimens using imaging techniques identifying those before neurological injury. The goal for patients at high risk or those with stroke is to decrease the overall percentage of HbS containing cells to at least less than 30%.[4] This is done in patients who cannot for any reason increase the percent of HbF containing cells (failure to respond) or in anticipation of maintenance therapy with hydroxyurea. The length of chronic transfusion therapy for patients is an unresolved question; however, most evidence supports indefinite chronic transfusion therapy. Given the various burdens of chronic transfusion therapy including vascular access, iron overload with simple transfusions and access to transfusion care, stem cell transplantation with matched related donors should be strongly considered. Exchange transfusion remains the mainstay of therapy for many patients in both acute and chronic settings. In acute stroke, simple transfusion is typically initiated given the ease of transfusion and the ability to use simple peripheral access. Exchange transfusion though is the standard of care and has been demonstrated to effectively prevent second strokes better than simple transfusion in children.

Acute chest syndrome (ACS) is another major complication of SCD with multiple underlying causes where exchange transfusion has been widely used to improve outcomes, though randomized control trials are lacking. Exchange transfusion in this setting allows for a rapid reduction of HbS concentrations in blood and an increase in oxygen-carrying HbA to help improve lung tissue perfusion, as the combination of small vessels in the setting of low oxygen tension can trigger sickling and concurrent vaso-occlusion. In children and adult patients with recurrent or frequent episodes of ACS, published reports supporting patients to be treated in chronic transfusion programs have been collected through secondary analyses of the stroke prevention trials including the reduction of painful VOEs. The latter findings underscore the lack of randomized trials to understand the role of transfusion therapy in many complications of SCD including multi-organ failure, priapism, peri-operative transfusion, or pulmonary hypertension. The most unresolved decisions are related to the use of prophylactic transfusions to avoid complications of SCD. For example, in pregnancy where there is an established high rate of SCD-related complications and death, there is varying evidence largely from case series to support prophylactic transfusion. This has supported the view that transfusion should be given "on-demand" for complications with prophylactic transfusion reserved for those patients with known recurrent complications of SCD or high-risk or complicated pregnancy.[5] In these and other settings, transfusion therapy can sometimes be used as an immediate therapy as it can rapidly restore healthy red cells that can subsequently act as a bridge to hydroxyurea or novel agents that may help modify SCD.[6] Correspondingly, for some patients who require a protective bridge therapy during high-risk clinical procedures (e.g., invasive surgery with general anesthesia or before BM transplantation), prophylactic transfusion may protect patients and improve morbidity from ACS or pain.[5]

Red cell transfusion remains a mainstay of therapy for SCD. There is a wide heterogeneity in the need for blood products among patients with SCD, but most patients will receive multiple transfusions over their lifetime. Transfusion can be divided into two general categories: simple transfusion or red cell exchange. Simple red cell transfusion introduces healthy donor red cells without any removal of patient blood in a similar fashion to any other blood transfusion. As in non-SCD patients, the objective is to increase the hemoglobin concentration to correspondingly raise the oxygen-carrying capacity blood. As HbA containing cells are transfused, simple transfusion can easily decrease the concentration of HbS-containing cells to levels sometimes approaching those of an exchange transfusion. For example, a severely anemic SCD patient (e.g., Hb 4 to 6) who has not been transfused in the past three months can decrease their HbS concentration to about 40% from 90% with a target Hb of 8 to 9 g/dL through simple transfusion. This net change approaches results that can otherwise be attained by red cell exchange, which typically takes longer to initiate. For this reason, simple transfusion should be initiated without delay in urgent clinical settings to patients with severe symptomatic anemia given the rapid relative decrease in sickle-containing red cells.

APHERESIS SUPPORTED RED CELL EXCHANGE

While simple transfusions may benefit patients with severe anemia, those who have Hb concentrations between 7 and 9 g/dL, levels frequently seen in SCD patients, require an alternate transfusion strategy. In these cases, patient red cells must be exchanged with healthy donor cells to avoid volume overload or hyperviscosity. Additionally, benefits of red blood cell exchange transfusion (RBCX) occur rapidly; therefore, this approach may be initiated as a bridging therapy for patients that will require hydroxyurea since it can take months to titrate the dose of hydroxyurea and assess its efficacy. Furthermore, an apheretic RBCX results in an isovolemic procedure that benefits patients with volume overload or hemodynamic instability. Manual exchange transfusion involves repeated cycles of phlebotomy followed by transfusion of blood. In these cases, up to 15% of blood may be removed at one time with adjustments made based on the calculated red cell mass and relative volume loss to the patient during the procedure. Manual exchanges are effective, but they are time-consuming and cumbersome, especially for adult patients with large blood volumes. The benefits of manual exchange transfusion include the wide availability of expertise and equipment to perform the procedure, though this is still best performed in dedicated medical units with expertise. An advantage for infants and children is that there is no need for specialized vascular access devices required for the procedure. Nevertheless, manual exchange transfusions can rarely achieve the lowest levels of remaining red cells that contain HbS as the exponential decrease of HbS concentrations requires the exchange of a relatively large blood volume with the concurrent use of significant donor blood. While most recommendations for chronic transfusion programs for stroke prevention is to keep the peak HbS concentration less than 30%, this is a difficult target to achieve. Lastly, manual exchange transfusion involves volume shifts associated with repeated cycles of phlebotomy and transfusion that can compromise hemodynamic stability in some patients.

Automated red blood cell exchange transfusion (aRBCX or erythrocytopheresis) can achieve the lowest concentration of HbS cells remaining in patients with SCD in a safe and rapid fashion. Apheresis instruments rely on the separation of red cells from other components of peripheral whole blood, usually by centrifugation, that can progressively exchange patients' red cells with donor red cells in either a continuous or discontinuous fashion. The overall procedure requires specialized instrumentation, dedicated staff with expertise, and is best managed with transfusion medicine supervision. Automated red cell exchange transfusion requires a relatively large amount of donor blood that is used to gradually deplete patient HbS containing blood.

The large amount of blood exchange during aRBCX requires precise vascular access using either steel intravenous needles or rigid apheresis grade catheters to withstand the intraluminal pressures required to draw and return blood at a fast rate. The use of peripheral veins is the preferred route for achieving vascular access, though this can be difficult for pediatric patients or those patients who have already undergone repeated venipuncture. Peripheral venous access is best achieved by experienced operators, especially those with the capability to use venous imaging techniques including ultrasound. The placement of an apheresis-compatible venous access device can be performed either episodically or implanted for long-term use. The use of catheters or implanted devices is associated with infection or thrombosis and must be carefully tailored for each patient in a chronic aRBCX program. The rate of catheter-related thrombosis has been long recognized to be higher in patients with SCD, though there is no consensus on the use of prophylactic anticoagulation or anti-platelet agents.[7] Lastly, the surgical establishment of an arterio-venous fistula can be considered in patients though there is a higher rate of complications when compared to patients undergoing hemodialysis, and moreover, some patients cannot tolerate the associated hemodynamic alterations.

Several patient-specific parameters are used to program apheresis instruments including the target hematocrit of the patient at the end of the procedure and the percentage of HbS containing cells remaining. There is no consensus on the end Hct target for patients undergoing aRBCX, though consideration is placed on not increasing the end hematocrit above levels resulting in hemodynamic compromise. Concerns for hyperviscosity commonly constrain the choice of end Hct to about 30%, though the loss of HbS containing red cells with the increasing HbA cells partially ameliorate the conditions seen in simple transfusion in SCD.[1] Furthermore, some patients may benefit from higher-end target hematocrits that may suppress endogenous hematopoiesis and potentially increase the time interval between exchange transfusions. Likewise, the target for the fraction of HbS cells remaining (FCR) is commonly set to 30%, largely derived from data surrounding chronic transfusion targets in stroke prevention. Lower targets may be beneficial in some patients, potentially increasing the aRBCX treatment interval. Lowering the FCR, however, significantly increases the number of healthy donor units required for exchange. This is due to the exponential increase in the amount of healthy red cells necessary to remove the reduced number of patient red cells as the aRBCX progresses. This significantly larger amount of donor blood increases red cell unit exposure and may not be possible for patients with highly specified red cell antigen needs. In certain clinical settings such as cardiac surgery, hypothermia and cardiopulmonary bypass and vascular constriction can potentially promote HbS cell sickling. In these cases, FCR values lower than 10% are chosen as targets. Lastly, estimating and entering the actual HbS fraction in the patient immediately prior to apheresis in the instrument algorithm can more precisely calculate and reduce the amount of donor blood necessary for aRBCX.

aRBCX apheresis instruments typically target the removal of red cells that sediment in the central part of the separation gradient, prompting investigation into altered methods of automated exchange to reduce the amount of donor blood needed or to target specific red cell populations.[8] Variations of the procedure to include intermittent aRBCX using a single needle is now available. Other modifications of the procedure include isovolemic hemodilution (or depletion exchange), where patients undergo depletion of peripheral blood to a specified nadir Hct with concomitant infusion of saline or albumin followed by an aRBCX procedure. This patient-specific strategy allows for the use of fewer units of donor red cells and results in an overall higher efficiency procedure. While the blood depletion period of the procedure is relatively brief, consensus criteria for patient selection for this strategy are not determined and may be unfavorable in those intolerant of anemia, hemodynamic alterations, or at higher risk of tissue damage resulting from reduced tissue oxygenation in acute settings (acute stroke or multi-organ failure).

Complications of aRBCX are relatively rare and mostly related to the method of vascular access with more cardiovascular-related complications found in SCD patients. One major concern in the field has been that the increased donor red cell unit exposure directly related to aRBCX may result in an increased risk of alloimmunization. An increase in red cell alloimmunization has not been observed in recent studies of aRBCX in SCD and moreover appears safer when compared to patients receiving episodic transfusion. Other infrequent outcomes associated with exposure to a large number of donor red cell units include allergic reaction, potential infection (e.g., babesia), and non-hemolytic transfusion reactions. A major benefit of aRBCX is the positive effect on iron balance, with a rapid decrease in the ferritin concentration in many patients. As aRBCX may especially affect serum ferritin values in the context of an acute phase reactant, measurement of tissue iron stores (e.g., liver iron concentration by MRI) suggests a relative benefit of aRBCX when compared to simple transfusion with chelation as a means to reduce total iron burden.

PREPARATIVE TRANSFUSION FOR STEM CELL TRANSPLANTATION

Greater attention is being positioned on curative therapies in select patients with SCD. Allogeneic stem cell transplant can deliver curative therapies for a subset of patients with SCD, with the best results and least toxicity achieved with matched sibling transplants. However, fewer than 25% of SCD patients in the United States have a suitable donor available, and fewer than one-third of the remaining patients have an appropriate unrelated matched donor. Allogeneic transplantation is frequently complicated by graft versus host disease, even more so when matched unrelated donors are used as a cell source. In addition, there are higher rates of toxicity that may be related to the conditioning regimens used for transplant, thus leading to the investigation of alternate conditioning-drug regimens. In addition to using allogeneic cells, a large effort has been placed in the use of genetically engineered autologous cells as a graft source for curative transplant. Using autologous cells eliminates the need for donors and eliminates the risk of graft versus host disease. Several approaches including lentiviral mediated gene addition of a modified beta globin gene or shRNA of Bcl11a to induce fetal Hb have encouraging results in clinical trials. Gene editing approaches using CRISPR-based techniques are also being trialed and hold large promise in the field.

The introduction of novel autologous cell therapies in SCD has led to observations involving the potential pathologic effects of the disease on the BM microenvironment and stem cell mobilization.[9] BM harvest was initially used as a source of stem cells in the cell therapies but far fewer than expected stem cells were recovered at harvest, and many patients failed to obtain an adequate gene therapy product. Preclinical studies have demonstrated that chronic tissue injury secondary to vaso-occlusive events can negatively influence the BM microenvironment and potentially even influence transplant outcomes. Strikingly, the transfusion of healthy red cells into a mouse model of SCD reversed many of the pathologic BM changes. These findings may in part explain the fewer numbers of progenitor cells obtained by BM harvest though future work is necessary to understand the role of red cell transfusion in the preparative regimen for transplantation. Presently, most transplant centers perform red cell exchange to keep the HbS less than 30% immediately prior to conditioning. This is done in large part to prevent VOE during the transplant and engraftment period.

One other factor that led to BM being used a cell source in autologous gene therapy is that G-CSF is relatively contraindicated in patients with SCD because it can trigger VOEs. While some patients have successfully weathered peripheral stem cell harvesting for autologous transplants for malignancy, increased morbidity and death have been reported. For these reasons, plerixafor alone has been used to mobilize SCD patients safely and efficiently in preparation for gene therapy. Standard apheresis collection techniques however have yielded fewer than expected stem cells with several consecutive days necessary to achieve target numbers of cells. Altered collection protocols are presently being explored to yield larger stem cell products. Recent studies have indicated that disease severity and increased hospitalization frequency within the preceding year correlated with poor collection yields. Patients undergoing mobilization and collection of stem cells have a preparative red cell exchange to prevent VOEs during the apheresis procedure. Whether red cell exchange improves mobilization and collection remains investigational.

SELECTION OF RED CELL COMPONENTS FOR TRANSFUSION

Red cell component selection in SCD demands careful consideration of both immediate and long-term requirements for safety, as this patient population will require frequent intervention with transfusion throughout their lifetime. Red cell alloimmunization is a major clinical hazard and cause of morbidity and mortality. For this reason, transfusion plans and associated considerations should be worked out well in advanced and individualized for each patient, as this will avoid delays when there is an acute need for red cell transfusion. Transfusion delays specific for SCD patients involve extended red cell antigen testing/matching, attentiveness to transfusion and alloantibody history, and the need to exclude sickle cell trait units. These latter activities may lead to a delay in dispensing red cells and subsequent transfusion; therefore, it is critical to communicate patient needs to blood bank physicians in urgent clinical settings of acute anemia. It is imperative that a detailed transfusion history be obtained from all institutions that have cared for the patient throughout their lifetime. The transfusion history should be obtained on initial hematology intake and updated frequently as key component of the medical record of the patient. Patients should be educated to ask about their transfusion history and to communicate restriction or outcomes to clinicians. This is especially important as transfusion restrictions for SCD patients vary widely among institutions and there is a lack of consensus in current clinical practice.

There are, however, several common considerations in the overall selection of red cell components for SCD. Allergic reactions and anaphylactic reactions to red cell transfusion do not occur more frequently in patients with SCD when compared to other frequently transfused patients; therefore, routine washing or premedication is not required. The use of leukoreduced red cell units leads to far fewer febrile non-hemolytic transfusion reactions that can avoid unnecessary evaluations and admission for SCD patients to exclude infection. Leukoreduction may also reduce alloimmunization to HLA antigens which may interfere with future clinical care. Irradiated blood products are frequently used to prevent transfusion-associated graft versus host disease; however, irradiation does reduce red cell lifespan, which may not be ideal in patients with few available blood units (rare blood type) or those that require chronic transfusion (e.g., RBCX program). Red cell age is a consideration in many transfusion programs and transfusing blood stored longer than 2 or 3 weeks is avoided. Lastly, sickle cell trait blood is excluded from use in SCD patients largely to avoid errors in calculating the percentage of HbS in peripheral blood by electrophoresis. Pathogen testing for red cell parasites (e.g., malaria or babesia) in endemic areas is sometimes performed as SCD patients are at greater risk of complications of infection. Parvovirus B19 is a pathogen that is not inactivated in the serum of most blood products and is particularly hazardous for patients with SCD who rely on stress erythropoiesis for maintenance. The diagnosis of parvovirus B19 infection should be considered in SCD patients with abrupt anemia and reticulocytopenia. Pathogen reduction techniques are under investigation in preventing both transfusion-transmitted infections and in avoiding irradiation by inactivating leukocytes.

TABLE 120.1 Pre-Transfusion Selection of Red Cells

- All patients should have red cell phenotype and preferably genotype in their medical record.
- Complete transfusion history in medical record from all facilities caring for patient.
- For patients who have not developed any alloantibodies: Transfuse red cells that are prophylactically matched for C, c, E, e, and K.
- For patients who have developed alloantibodies: Transfuse red cells excluding clinically significant antigens for which alloantibodies have been identified. Further prophylactic matching should include Fya/Fyb, Jka/Jkb, and S/s, in addition to C, c, E, e, and K.
- Transfusion plan should be documented in advance for patients with alloantibodies.
- Pre-storage leukoreduction to avoid febrile non-hemolytic transfusion reactions.
- Consider using red cell stored fewer than 15 days for patients on chronic transfusion programs.[a]
- Irradiation of red cells to prevent TA-GVHD.[a]

[a] Not evidence-based. RBC units stored longer or irradiated have not been shown to impact clinical outcomes. Decisions should be made based on local blood supply.
TA-GVHD, Transfusion-associated graft-versus-host-disease.

RED CELL ANTIGEN MATCHING

Alloimmunization resulting from mismatched RBC antigen is of enormous concern and consequence in individuals with SCD. Alloimmunization is far more common in SCD and commonly occurs if red cells are not fully matched for RH and Kell antigens. Patients with SCD are heavily allo-immunized as a group with prevalence estimates ranging from 10 to 66%.[10] There are multiple explanations for this higher than expected alloimmunization prevalence with a major factor being the red cell antigen differences between those in the healthy blood donor population and SCD patients. For example, African-Americans as a group are more likely to lack C, E, and K antigens, and accordingly, antibodies toward these antigens were the most common ones detected in SCD patients. However, even approaches to ethnically match donors and recipients have not eliminated alloimmunization. Other factors in SCD including those related to hemolysis or inflammation are associated with increased alloimmunization. Elevated free heme as a result of hemolysis is associated with an increased rate of alloimmunization.[10] Hemolysis can affect many immune cell functions and networks and therefore augment immune responses such as allo-immunization.[11] Free heme can also activate neutrophils and macrophages and enhance endothelial cell injury which again is associated with enhanced immune response. Pro-inflammatory markers including Il-6 and gamma interferon have been detected in SCD patients at higher levels and are associated with higher rates of alloimmunization patients with SCD, though the exact mechanisms affecting alloimmunization remain unknown.[12]

Various strategies of red cell antigen matching have been devised to prevent alloimmunization in SCD (Table 120.1). Expert panels have long recommended additional (to the universal ABO and D) prophylactic matching to include Rh (C, E or C/c, E/e), and K in patients with SCD.[5] Extending the crossmatch to other common red cell antigens including Fya/Fyb, Jka/Jkb, and S/s can further reduce alloimmunization though finding sufficient numbers of compatible units of red cells poses a significant challenge. Therefore, extended matching beyond Rh and K is usually reserved for those patients that have already formed a red cell antibody and are therefore at higher risk of forming additional alloantibodies. To enable antigen matching, an extended red cell antigen profile should be obtained in all SCD patients and be placed in the medical record. Traditional serologic testing for red cell antigen phenotypes has been largely supplanted by molecular analysis using DNA methods. Genotyping offers higher accuracy and additional information including detecting mutations in the GATA motif of the Duffy blood group genes that can identify patients that are not at risk for forming anti-Fy^b. Extended sequence analysis has yielded a large amount of sequence variation in all blood group antigens, but especially in those related to Rh.[13] A large amount of diversity exists in the Rh genes of patients of African descent. The two highly homologous Rh loci, *RHD* and *RHCE*, resulted from gene duplication and have resulted in variant RHD and RHCE alleles. Only a few frequent variants such as weak or partial D can be detected serologically; consequently, only DNA sequencing can distinguish specific genotypes. The sequence information can be used to understand alloimmunization to Rh antigens in SCD patients and sometimes can be used in matching donor red cell units. Cost and the ability to recruit the large number of blood donors now underrepresented in most blood banks have largely hindered extended genotype-guided red cell antigen matching, though this goal is currently being examined.[5]

COMPLICATIONS OF TRANSFUSION THERAPY

Alloimmunization is a major clinical problem for SCD patients with an accompanying burden to blood banking resources to find compatible blood in a timely fashion. Delays of finding appropriate blood can lead to poor outcomes. Given the relatively high prevalence of alloimmunization in SCD, most reactions to transfusion, including anaphylactic require close examination to exclude antibodies against RBC antigens. Identification of alloantibodies is crucial in preventing future hemolytic reactions that typically present clinically as a DHTR. Rapid determination of antigen specificity is necessary as most alloantibodies are short lived and proper documentation in the patient record is necessary to prevent DHTR. The presentation of patients with suspected DHTR is variable and is under-reported due to the lack of recognition.[14] Recent consensus guidelines have defined DHTR as a significant drop in hemoglobin within 21 days of transfusion, without an alternative cause. Associated evidence including an increase in the HgbS percentage and anemia can support the diagnosis of DHTR. Frequently, DHTR present with vaso-occlusive crises including bony pain or ACS, anemia, hemoglobinuria, and elevated markers of hemolysis in the blood (e.g., bilirubin or lactate dehydrogenase). Detection of alloantibodies by red cell screening assays yields results in as few as a third of cases. Assays to detect alloantibodies should be repeated over weeks after the transfusion to detect those with altered temporal kinetics. Therefore, a high degree of vigilance must be maintained to diagnose DHTR in all SCD patients undergoing transfusion.

Management of individuals with suspected DHTR is largely supportive and should focus on the treatment of the anemia and hydration. Transfusion should be avoided unless necessary and preferably after the inciting red cell antigen is identified to enable avoidance when selecting red cell units. In patients with severe DHTR, prophylactic immunosuppression with corticosteroids or intravenous immune globulin can be considered. Treatment with rituximab is investigational and may cause prolonged immunosuppression with associated morbidity. One of the most serious and potentially fatal complications of transfusion-associated hemolysis is hyperhemolysis or bystander hemolysis. Hyperhemolytic transfusion reactions (HHTR) entail alloimmune hemolysis of transfused red cells together with hemolysis of endogenous patient RBCs. HHTR is usually defined as a DHTR where the Hb concentration falls below the pretransfusion value. The mechanism of HHTR is poorly understood; however, there is increasing evidence that the complement pathway is integral in sustaining this syndrome. Immune complexes of alloantibodies and products of hemolysis can activate the classical and alternate complement pathways, respectively.[15] Treatment of HHTR is similar to that of DHTR with immunosuppression using glucocorticoids and supportive care. In steroid-refractory cases of HHTR, recent reports have suggested that complement inhibition using a monoclonal antibody targeting C5 may be helpful. Eculizimab treatment carries the associated risk of meningococcal infection, and anti-meningococcal prophylaxis may be considered. Given the high degree of hemolysis and the overall stress on the erythropoietic lineage, erythropoietin, iron, B12, and folate should be optimized to support the production of red cells. Mortality associated with HHTR has been estimated to be approximately 5% to 10%, and patients that have a history of HHTR are likely to have subsequent episodes of the syndrome. For this reason, hematopoietic stem cell transplant is sometimes considered.

IRON OVERLOAD

Iron overload is the most frequent result and complication of transfusion therapy. Identification and management of iron overload is discussed in detail elsewhere (see Chapters 37, 41, and 43). Iron overload is a prevalent problem in all patients undergoing chronic transfusion. Each milliliter of red cells contains up to one mg of iron, with a significant burden quickly accumulating especially in patients on chronic transfusion therapy. Excess iron stores cannot be eliminated, and chelation therapy is needed to remove the large amounts of iron present in transfused SCD patients. Interestingly, iron accumulates mostly in the liver in SCD in contrast to tissues such as the heart or endocrine organs in patients with thalassemia. Management of iron overload in SCD requires careful documentation of blood transfusion with a cumulative count in the patient record. Serial assessment of ferritin with tissue iron stores in the liver and sometimes the heart should be performed. Although patients undergoing chronic aRBCX treatment typically have a negative iron balance, not all have positive responses and tissue stores should be periodically examined.

REFERENCES

1. Connes P, Alexy T, Detterich J, et al. The role of blood rheology in sickle cell disease. *Blood Rev*. 2016;30:111–118.
2. Orkin SH, Bauer DE. Emerging genetic therapy for sickle cell disease. *Annu Rev Med*. 2019;70:257–271.
3. Kato GJ, Piel FB, Reid CD, et al. Sickle cell disease. *Nat Rev Dis Primers*. 2018;4:18010.
4. Kassim AA, Galadanci NA, Pruthi S, DeBaun MR. How I treat and manage strokes in sickle cell disease. *Blood*. 2015;125:3401–3410.
5. Chou ST, Alsawas M, Fasano RM, et al. American Society of Hematology 2020 guidelines for sickle cell disease: transfusion support. *Blood Adv*. 2020;4:327–355.
6. Osunkwo I, Manwani D, Kanter J. Current and novel therapies for the prevention of vaso-occlusive crisis in sickle cell disease. *Ther Adv Hematol*. 2020;11 2040620720955000.
7. Jeng MR, Feusner J, Skibola C, Vichinsky E. Central venous catheter complications in sickle cell disease. *Am J Hematol*. 2002;69:103–108.
8. Thibodeaux SR, Tanhehco YC, Irwin L, et al. More efficient exchange of sickle red blood cells can be achieved by exchanging the densest red blood cells: an ex vivo proof of concept study. *Transfus Apher Sci*. 2019;58:100–106.
9. Esrick EB, Bauer DE. Genetic therapies for sickle cell disease. *Semin Hematol*. 2018;55:76–86.
10. Fasano RM, Meyer EK, Branscomb J, et al. Impact of red blood cell antigen matching on alloimmunization and transfusion complications in patients with sickle cell disease: a systematic review. *Transfus Med Rev*. 2019;33:12–23.
11. Zhong H, Yazdanbakhsh K. Hemolysis and immune regulation. *Curr Opin Hematol*. 2018;25:177–182.
12. Hendrickson JE. Red blood cell alloimmunization and sickle cell disease: a narrative review on antibody induction. *Ann Blood*. 2020;5:33.
13. Lane WJ, Westhoff CM, Gleadall NS, et al. Automated typing of red blood cell and platelet antigens: a whole-genome sequencing study. *Lancet Haematol*. 2018;5:e241–e251.
14. Dean CL, Maier CL, Roback JD, Stowell SR. Multiple hemolytic transfusion reactions misinterpreted as severe vaso-occlusive crisis in a patient with sickle cell disease. *Transfusion*. 2019;59:448–453.
15. Roumenina LT, Bartolucci P, Pirenne F. The role of complement in post-transfusion hemolysis and hyperhemolysis reaction. *Transfus Med Rev*. 2019;33:225–230.

PART XI HEMOSTASIS AND THROMBOSIS

CHAPTER 121

OVERVIEW OF HEMOSTASIS AND THROMBOSIS

James C. Fredenburgh and Jeffrey I. Weitz

INTRODUCTION

Hemostasis preserves vascular integrity by balancing the physiologic processes that maintain blood in a fluid state under normal circumstances and prevent excessive bleeding after vascular injury. Preservation of blood fluidity depends on an intact vascular endothelium, and a complex series of regulatory pathways that maintain platelets in a quiescent state and keep the coagulation system in check. In contrast, arrest of bleeding requires rapid formation of hemostatic plugs at sites of vascular injury to prevent severe hemorrhage. Perturbation of hemostasis can lead to bleeding or thrombosis. Bleeding will occur if there is failure to seal vascular leaks either because of defective hemostatic plug formation, or because of premature breakdown of the plugs. In contrast, thrombosis may occur if prothrombotic stimuli are unregulated, or if thrombus resolution is attenuated.

Thrombosis can occur in arteries or veins and is a major cause of morbidity and mortality. Arterial thrombosis is the most common cause of acute coronary syndrome, ischemic stroke, and limb gangrene, whereas thrombosis in the deep veins of the leg leads to postthrombotic syndrome and pulmonary embolism, which can be fatal.

Most arterial thrombi form on top of disrupted atherosclerotic plaques, because plaque rupture exposes thrombogenic material in the plaque core to the blood.[1–3] This material then triggers platelet aggregation and fibrin formation, which results in the generation of a platelet-rich thrombus that temporarily or permanently occludes blood flow. Temporary occlusion of blood flow in coronary arteries may trigger unstable angina, whereas persistent obstruction causes myocardial infarction. The same processes can occur in the cerebral circulation, where temporary arterial occlusion may manifest as a transient ischemic attack, and persistent occlusion can lead to a stroke. Likewise, critical limb ischemia can occur if there is superimposed thrombosis on ruptured atherosclerotic plaques in the major arteries supplying blood to the lower extremities.[1]

In contrast to arterial thrombi, venous thrombi rarely form at sites of obvious vascular disruption.[3,4] Although they can develop after surgical trauma to veins, or secondary to indwelling venous catheters, they usually originate in the valve cusps of the deep veins of the calf or in the muscular sinuses, where there is stasis. Sluggish blood flow in these veins reduces the oxygen supply to the avascular valve cusps. Hypoxemia induces endothelial cells lining the valve cusps to become activated and express adhesion molecules onto their surfaces. Tissue factor–bearing leukocytes and microparticles adhere to these activated cells and induce coagulation. Impaired blood flow exacerbates local thrombus formation by reducing clearance of activated clotting factors and consumption of regulatory molecules. These responses constitute the three axes of Virchow's triad associated with development of thrombosis: stasis, hypercoagulability of the blood, and activation or disruption of the endothelium. A further complication of thrombosis is embolism, where venous or arterial thrombi can dislodge and travel to the lungs, brain, or other organs to produce pulmonary embolism, ischemic stroke, or systemic embolism, respectively.[1,4]

Arterial and venous thrombi contain platelets and fibrin, but the proportions differ. Arterial thrombi are rich in platelets because of the high shear on this side of the circulatory system. In contrast, venous thrombi, which form under low shear conditions, contain relatively few platelets, and consist mostly of fibrin and trapped red blood cells. Because of the predominance of platelets, arterial thrombi appear white, whereas venous thrombi appear red.

The antithrombotic drugs used for prevention and treatment of thrombosis target components of thrombi and include antiplatelet drugs, which inhibit platelets; anticoagulants, which attenuate coagulation; and fibrinolytic agents that induce fibrin degradation (see Chapter 143). With the predominance of platelets in arterial thrombi, strategies to inhibit or treat arterial thrombosis focus mainly on antiplatelet agents, although in the acute setting, strategies often include anticoagulants and fibrinolytic agents. When arterial thrombi are occlusive and rapid restoration of blood flow is imperative, mechanical and pharmacologic methods enable thrombus extraction, compression, or degradation. Parenteral anticoagulants are often given in conjunction with reperfusion therapy to prevent re-occlusion and clotting on guidewires and catheters. Anticoagulants may also be given for secondary prevention in patients with arterial thrombosis. Thus, when compared with placebos in stabilized patients with acute myocardial infarction, low-dose rivaroxaban administered on top of antiplatelet therapy reduced recurrent ischemic events and stent thrombosis.[5] Likewise, dual pathway inhibition with aspirin plus low-dose rivaroxaban is superior to either agent alone for prevention of major adverse cardiac and limb events in patients with coronary artery or peripheral artery disease.[6,7]

Anticoagulants are the mainstay for prevention and treatment of venous thromboembolism because fibrin is the predominant component of venous thrombi (see Chapter 143). Antiplatelet drugs are less effective than anticoagulants because of the limited platelet content of venous thrombi. Nonetheless, aspirin is as effective as rivaroxaban for extended thromboprophylaxis after hip or knee arthroplasty.[8] Selected patients with venous thromboembolism benefit from fibrinolytic therapy—for example, patients with massive pulmonary embolism achieve more rapid restoration of pulmonary blood flow with systemic, or catheter-directed, fibrinolytic therapy than with anticoagulant therapy alone. Certain patients with extensive deep vein thrombosis in the iliac and/or femoral veins may also have a better outcome with catheter-directed fibrinolytic therapy, and/or mechanical thrombus extraction in addition to anticoagulants.

This chapter provides an overview of hemostasis and thrombosis by highlighting the processes involved in platelet activation and aggregation, blood coagulation, and fibrinolysis.

HEMOSTATIC SYSTEM

The major components of the hemostatic system are the vascular endothelium, platelets, and the coagulation and fibrinolytic systems. The complex interplay between cellular and soluble factors is termed immunohemostasis. Although these highly integrated processes maintain normal hemostasis under physiological conditions, their dysregulation can lead to pathological responses (see box on Immunohemostasis).

Vascular Endothelium

A monolayer of endothelial cells lines the intimal surface of the circulatory tree and separates the blood from the prothrombotic subendothelial components of the vessel wall (see Chapter 122). As such, the vascular endothelium encompasses about 10^{13} cells and covers a vast surface area. Rather than serving as a static barrier, the healthy vascular endothelium is a dynamic organ (Fig. 121.1) that actively regulates hemostasis by inhibiting platelets, suppressing coagulation, promoting fibrinolysis, and modulating vascular tone and permeability.[3] Defective vascular function can lead to bleeding if the endothelium becomes more permeable to blood cells, if vasoconstriction does not occur, or if premature degradation of hemostatic plugs reopens repaired vasculature.

Platelet Inhibition

Endothelial cells synthesize prostacyclin and nitric oxide and release them into the blood.[11] These agents not only serve as potent vasodilators, but also inhibit platelet activation and subsequent aggregation by stimulating adenylate cyclase and increasing intracellular levels of cyclic adenosine monophosphate (cAMP). In addition, endothelial cells express CD39 on their surfaces, a membrane-associated ecto-adenosine diphosphatase (ADPase). By degrading adenosine diphosphate (ADP), which is a platelet agonist, CD39 attenuates platelet activation.

Anticoagulant Activity

Intact endothelial cells play an essential part in the regulation of thrombin generation through a variety of mechanisms. Endothelial cells produce heparan sulfate proteoglycans, which bind circulating antithrombin and accelerate the rate at which it inhibits thrombin and other coagulation enzymes. Tissue factor pathway inhibitor (TFPI), a naturally occurring inhibitor of coagulation, binds heparan sulfate on the endothelial cell surface.[12] Administration of heparin or low-molecular-weight heparin (LMWH) displaces glycosaminoglycan-bound TFPI from the vascular endothelium, and released TFPI may contribute to the antithrombotic activity of these drugs.

Endothelial cells regulate thrombin generation by expressing thrombomodulin and endothelial cell protein C receptor (EPCR) on their surfaces. Thrombomodulin binds thrombin and alters its substrate specificity such that thrombin no longer acts as a procoagulant but becomes a potent activator of protein C (see Chapter 125). Activated protein C (APC) serves as an anticoagulant by degrading activated factor V and factor VIII (factor Va and VIIIa, respectively), key cofactors involved in thrombin generation. Protein S acts as a cofactor in this reaction, and EPCR enhances this pathway by binding protein C and presenting it to the thrombin–thrombomodulin complex for activation. In addition to its role as an anticoagulant, APC also regulates inflammation and preserves the barrier function of the endothelium.[13]

Fibrinolytic Activity

The vascular endothelium promotes fibrinolysis by synthesizing and releasing tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), which initiate fibrinolysis by converting plasminogen to plasmin (see Chapter 125). Endothelial cells in most vascular beds synthesize t-PA constitutively and release it in response to stimuli such as thrombin or bradykinin. In contrast, perturbed endothelial cells produce u-PA in the settings of inflammation and wound repair.

IMMUNOHEMOSTASIS

The hemostatic system is not the sole mechanism through which the vasculature is protected from disruption. There is overwhelming evidence that inflammation plays an equally important part and that the two processes are tightly integrated and mutually dependent.[3,9] The terms immunohemostasis and thromboinflammation have been coined to capture this interplay. The inflammatory axis can be integrated into the cell-based model of hemostasis because leukocytes, platelets, and the endothelium are critical components of both processes. For example, tissue factor expression by monocytes is upregulated in response to inflammatory cytokines. The reductionist view of hemostasis and inflammation considers these pathways in isolation. However, it is now apparent that inflammation plays an integral part in stroke, deep-vein thrombosis, and myocardial infarction, processes previously attributed to dysregulation of hemostasis.[10] In agreement with their similar protective and reparative roles, both thrombosis and inflammation are initiated in response to tissue damage or pathogen infection.

Platelets provide an obvious link between the hemostatic and immune systems because they respond to bacterial or viral infection, as well as to ischemia and cancer. Because of their proximity, platelets are among the first cells to arrive at sites of injury where they interact with leukocytes and endothelial cells. Upon activation, platelets expose ligands that mediate their interaction with immune cells. Such ligands include P-selectin, intercellular adhesion molecule (ICAM)-2, macrophage-1 antigen (MAC-1), and CD40L. Furthermore, platelets express toll-like and Fc receptors and release soluble effectors such as cytokines, chemokines and platelet factor 4. Platelets also link the complement system to the coagulation and immune systems because platelets bind C3b via P-selectin and C3 via GPIb-alpha, and because they release bactericidal mediators such as thrombocidin. Consequently, platelets link hemostasis with innate and adaptive immunity.

Leukocytes provide another link in the thromboinflammation axis. Activated platelets and endothelial cells express P-selectin on their surface. Leukocytes bind P-selectin via P-selectin glycoprotein ligand-1 (PSGL-1), a counter receptor that is constitutively expressed on their surface. This interaction results in the formation of platelet-leukocyte aggregates and the tethering of leukocytes to the endothelium, interactions that promote the release of pro-inflammatory cytokines, reactive oxygen species, and proteolytic enzymes and facilitate neutrophil transmigration to sites of inflammation. Therefore, leukocytes contribute to innate immunity.

Leukocytes also contribute to coagulation. Activated monocytes express tissue factor, which triggers coagulation via the extrinsic pathway. Activated neutrophils undergo NETosis, which involves programmed release of neutrophil extracellular traps (NETs) that originate from decondensed chromatin and consist of DNA, histones, and metalloproteases such as myeloperoxidase (MPO) and elastase. The mesh-like structure of NETs provides a scaffold for platelet adhesion where histones can activate them and the DNA in NETs triggers coagulation via the contact system. Confirmation of the role of neutrophils and NETs in coagulation comes from the observations that neutrophil depletion or DNase administration attenuates thrombosis in murine models.

Enhanced understanding of thromboinflammation has identified new disease pathways and has revealed novel targets for directed therapy. The hypercoagulability in patients with novel coronavirus disease (COVID-19) illustrates the reciprocal and tight integration of thrombosis and inflammation (see Chapter 153). Such integration is also evident in chronic inflammatory diseases and explains why patients with inflammatory bowel disease, systemic lupus erythematosus and rheumatoid arthritis are at higher risk for thrombosis than those without these conditions. These revelations have prompted routine thromboprophylaxis for hospitalized patients with COVID-19, the use of global Janus kinase (JAK) inhibitors to attenuate inflammation in patients with rheumatic diseases, and the evaluation of novel agents such as soluble thrombomodulin for treatment of DIC.

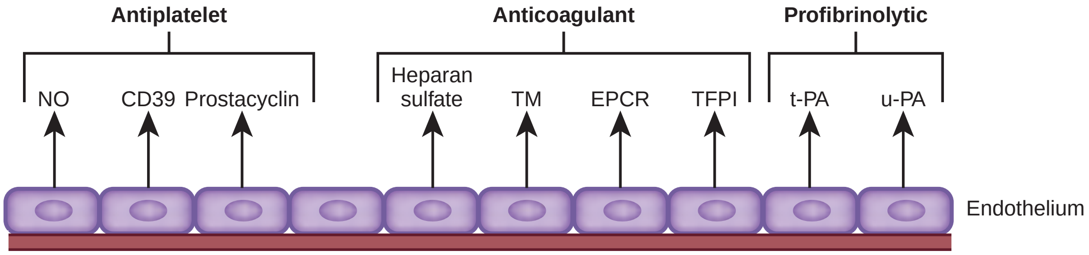


Figure 121.1 THE ANTITHROMBOTIC FUNCTIONS OF THE ENDOTHELIUM. The healthy endothelium has *(left)* antiplatelet activity because of synthesis and release of prostacyclin and nitric oxide *(NO)* and expression of CD39, a membrane-associated ectoADPase; *(center)* anticoagulant activity because of heparan sulfate proteoglycan-mediated activation of antithrombin and expression of thrombomodulin *(TM)* and endothelial protein C receptor *(EPCR)*, which are involved in protein C activation, and surface-bound tissue factor pathway inhibitor *(TFPI)*; and *(right)* profibrinolytic activity because of release of tissue and urokinase-type plasminogen activator (t-PA and u-PA, respectively).

Endothelial cells also produce type 1 plasminogen activator inhibitor (PAI-1), the major inhibitor of both t-PA and u-PA. Therefore, net fibrinolytic activity depends on the dynamic balance between the release of plasminogen activators and PAI-1. Fibrinolysis is localized to the endothelial cell surface because these cells express annexin II, a coreceptor for plasminogen and t-PA that promotes their interaction. Therefore, healthy vessels actively resist thrombus formation and help maintain platelets in a quiescent state.

Vascular Tone and Permeability

In addition to synthesizing potent vasodilators, such as prostacyclin and nitric oxide, endothelial cells also produce a group of counter-regulatory peptides known as endothelins that induce vasoconstriction. Endothelial cell permeability is influenced by the connections that join endothelial cells to their neighbors. Macromolecules traverse the endothelium via patent intercellular junctions, by endocytosis, or through transendothelial pores. Vasodilatation, severe thrombocytopenia, and high doses of heparin can increase endothelial permeability, which may contribute to bleeding. Bradykinin, generated through degradation of high molecular weight kininogen by kallikrein, also induces vascular leakage and inflammation. APC combats these effects by attenuating inflammation and vascular permeability, thereby enhancing the barrier function of the endothelium.

Platelets

Platelets are anucleate cellular particles released into the circulation after programmed fragmentation of bone marrow megakaryocytes (see Chapter 123). Because they are anucleate, platelets have limited capacity to synthesize proteins. Consequently, platelet protein composition is determined by the parent cell as well as by those factors endocytosed from the circulation. Thrombopoietin, a glycoprotein synthesized in the liver and kidneys, regulates megakaryocytic proliferation and maturation as well as platelet production. Once they enter the circulation, platelets have a life span of 7 to 10 days.

Damage to the intimal lining of the vessel exposes the underlying subendothelial matrix. Platelets home to sites of vascular disruption and adhere to the exposed matrix proteins (see Chapter 124). Adherent platelets undergo activation and not only release substances that recruit additional platelets to the site of injury, but also promote thrombin generation and subsequent fibrin formation (Fig. 121.2). A potent platelet agonist, thrombin amplifies platelet recruitment and activation. Activated platelets then aggregate to form a plug that seals the leak in the vasculature.[14] An understanding of the steps in these highly integrated processes helps pinpoint the sites of action of antiplatelet drugs, and rationalizes the utility of anticoagulants for the treatment of arterial thrombosis and venous thrombosis.

Adhesion

Platelets adhere to von Willebrand factor (vWF) and collagen that originate from endothelial cells and the subendothelium, respectively. The platelet monolayer promotes thrombin generation and subsequent fibrin formation. These events depend on constitutively expressed receptors on the platelet surface, α2β1 and glycoprotein (GP) VI, which bind collagen, and GPIbα and GPIIb/IIIa (αIIbβ3), which bind vWF. The platelet surface is crowded with receptors, but those involved in adhesion are the most abundant: every platelet has about 40,000 to 80,000 copies of GPIIb/IIIa and 25,000 copies of GPIbα. Receptors cluster in cholesterol-enriched subdomains, which render them more mobile, thereby increasing the efficiency of platelet adhesion and subsequent activation (see Chapter 124).

Under low shear conditions, such as in the venous circulation, collagen can capture and activate platelets on its own. Captured platelets undergo cytoskeletal reorganization that causes them to flatten and adhere more closely to the damaged vessel wall. Under high shear conditions in the arterial system, however, collagen and vWF must act in concert to support optimal platelet adhesion and activation. vWF synthesized by endothelial cells and megakaryocytes assembles into multimers that range from 550 to over 10,000 kDa. When released from storage in the Weibel-Palade bodies of endothelial cells or the α-granules of platelets, most of the vWF enters the circulation, but the vWF released from the abluminal surface of endothelial cells accumulates in the subendothelial matrix, where it binds collagen via its A3 domain. This surface-immobilized vWF can simultaneously bind platelets via its A1 domain. In contrast, circulating vWF does not react with unstimulated platelets. This difference in reactivity likely reflects vWF conformation; circulating vWF is in a coiled conformation that prevents access of its platelet-binding domain to vWF receptors on the platelet surface, whereas immobilized vWF assumes an elongated shape that exposes its A1 domain. Shear forces at sites of vascular injury also unfold vWF, thus contributing to the abundance of platelets in arterial thrombi. In this extended conformation, large vWF multimers serve as the molecular glue that tethers platelets to the damaged vessel wall with sufficient strength to withstand higher shear forces.[15] Large vWF multimers provide additional binding sites for collagen and heighten platelet adhesion because platelets have more vWF receptors than collagen receptors.

The size of the vWF multimers is regulated by ADAMTS13, a circulating metalloprotease and member of *A D*isintegrin *A*nd *M*etalloprotease with *T*hrombo*S*pondin type 1 repeats (ADAMTS) family.[15] Synthesized in hepatic stellate cells and found in other cells including endothelial cells, megakaryocytes and platelets, ADAMTS13 is secreted into plasma as a constitutively active enzyme. ADAMTS13 cleaves vWF, thereby preventing accumulation of ultra large multimers. Such cleavage is essential for normal hemostasis. Thus, deficiency of ADAMTS13 because of autoantibody formation or inherited mutations leads to thrombotic thrombocytopenic purpura (see Chapter 132), which is characterized by microvascular occlusion by platelet aggregates induced by ultra large vWF multimers.

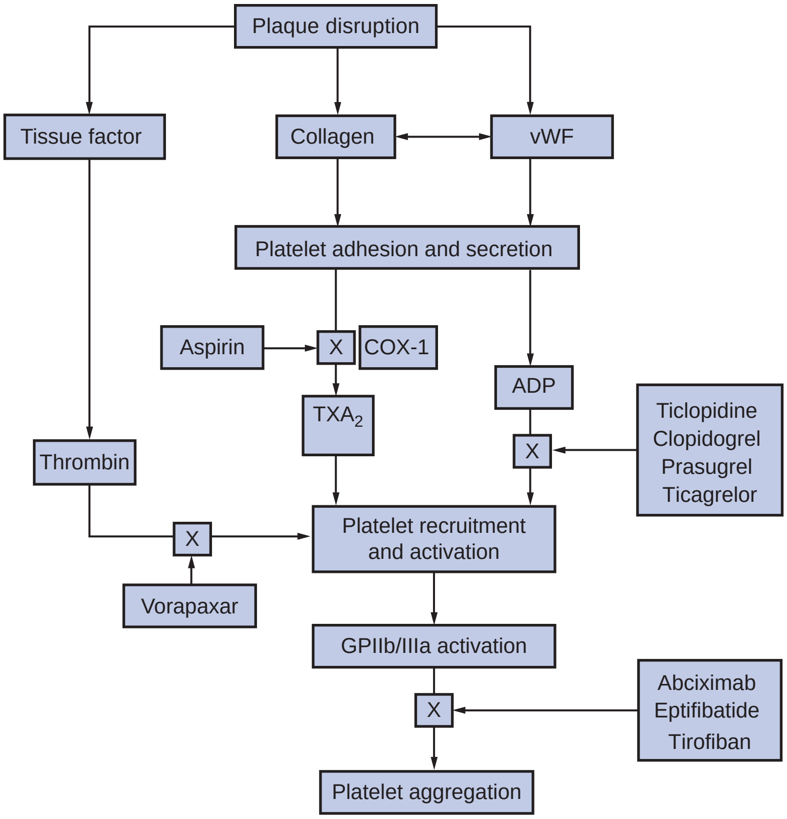


Figure 121.2 SITES OF ACTION OF ANTIPLATELET DRUGS. Aspirin inhibits thromboxane A2 *(TXA2)* synthesis by irreversibly acetylating cyclooxygenase-1 *(COX-1)*. Reduced TXA2 release attenuates platelet activation and recruitment to the site of vascular injury. Ticlopidine, clopidogrel, and prasugrel irreversibly block P2Y12, a key ADP receptor on the platelet surface, whereas ticagrelor is a reversible inhibitor of P2Y12. Abciximab, eptifibatide, and tirofiban inhibit the final common pathway of platelet aggregation by blocking fibrinogen and von Willebrand factor *(vWF)* binding to activated glycoprotein *(GP)* IIb/IIIa. Vorapaxar inhibits thrombin-mediated platelet activation by targeting protease activated receptor-1 *(PAR-1)*, the major thrombin receptor on platelets. *ADP*, Adenosine diphosphate.

Platelet adhesion to collagen or vWF results in platelet activation and secretion, the next steps in platelet plug formation.

Activation and Secretion

Adhesion to collagen and vWF initiates signaling pathways that result in platelet activation. These pathways induce cyclooxygenase-1 (COX-1)–dependent synthesis and release of thromboxane A2 and trigger the release of ADP from storage granules. Thromboxane A2 is a potent vasoconstrictor, and like ADP, locally activates ambient platelets and recruits them to the site of injury. This process results in expansion of the platelet plug. To activate platelets, thromboxane A2 and ADP must bind to their respective receptors on the platelet membrane. The thromboxane receptor (TP) is a G protein–coupled receptor that is found on platelets and on the endothelium, which explains why thromboxane A2 induces vasoconstriction as well as platelet activation.[16] ADP interacts with a family of G protein-coupled receptors on the platelet membrane. Most important of these is P2Y12, which is the target of the thienopyridines, but P2Y1 also contributes to ADP-induced platelet activation, and maximal ADP-induced platelet activation requires activation of both receptors. A third ADP receptor, P2X1, is an adenosine triphosphate (ATP)–gated calcium channel. Platelet storage granules contain ATP as well as ADP; ATP released during the platelet activation process may contribute to the platelet recruitment process in a P2X1-dependent fashion.

Although TP and the various ADP receptors signal through different pathways, they all trigger an increase in the intracellular calcium concentration in platelets. This, in turn, induces shape change via cytoskeletal rearrangement, granule mobilization and release, and subsequent platelet aggregation. Activated platelets promote coagulation by cycling phosphatidylserine from the inner membrane bilayer to the outer layer. Surface exposure of this anionic phospholipid is essential for assembly of coagulation factor complexes (see Chapter 125). Once assembled, these clotting factor complexes trigger a burst of thrombin generation and subsequent fibrin formation. In addition to converting fibrinogen to fibrin, thrombin amplifies platelet recruitment and activation, thus promoting expansion of the platelet plug. Thrombin binds to protease-activated receptor (PAR) types 1 and 4 (PAR1 and PAR4, respectively) on the platelet surface and cleaves their extended amino-termini, thereby generating new amino-termini that serve as tethered ligands that bind internally and activate the receptors (Fig. 121.3). Whereas low concentrations of thrombin cleave PAR1, PAR4 cleavage requires higher thrombin concentrations.[17] Cleavage of either receptor triggers platelet activation.

In addition to providing a surface on which clotting factors assemble, activated platelets also promote fibrin formation and subsequent stabilization by releasing factor V, factor XI, fibrinogen, and factor XIII (see Chapters 124 and 125). Thus, there is coordinated activation of platelets and coagulation, and the fibrin network that results from thrombin action helps anchor the platelet aggregates at the site of injury. Activated platelets also release adhesive proteins, such as vWF, thrombospondin, and fibronectin, which augment platelet adhesion at sites of injury, as well as growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), which promote wound healing. Platelet aggregation is the final step in the formation of the platelet plug.

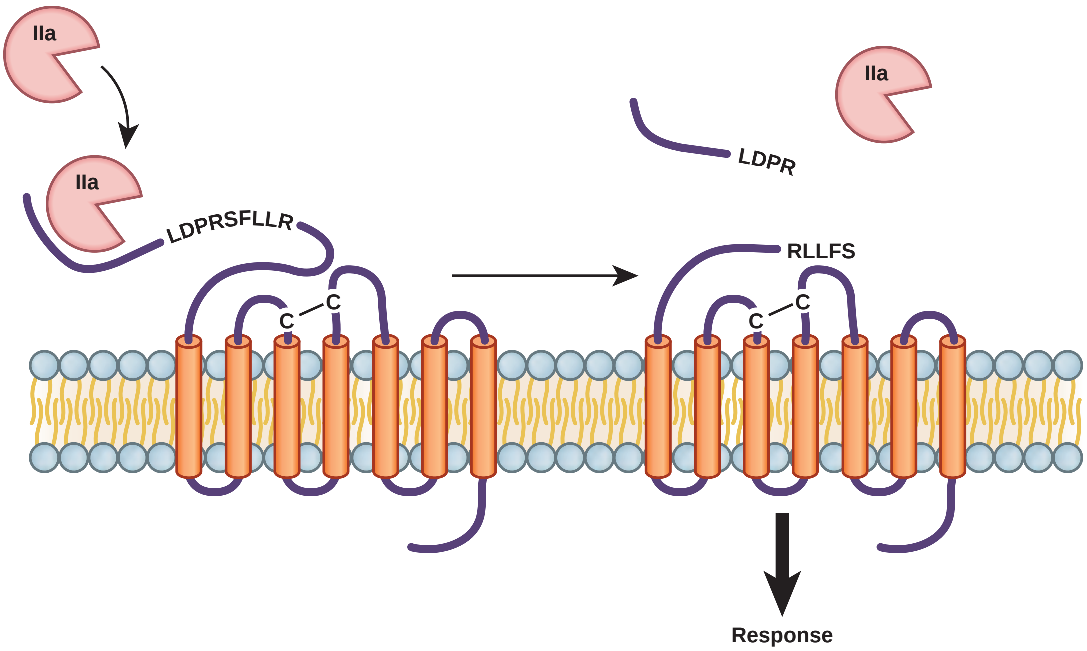


Figure 121.3 ACTIVATION OF PROTEASE-ACTIVATED RECEPTOR (PAR)-1 BY THROMBIN. Thrombin (IIa) binds to the amino terminus of the extracellular domain of PAR-1 where it cleaves a specific peptide bond. Cleavage of this bond generates a new amino-terminal sequence of Ser-Phe-Leu-Leu-Arg (SFLLR) that acts as a tethered ligand and binds to the body of the receptor, thereby activating it. Thrombin then dissociates from the receptor. Analogues of the first five or six amino acids of the tethered ligand sequences, known as thrombin receptor agonist peptides, can independently activate PAR-1.

Aggregation

GPIIb/IIIa mediates platelet-to-platelet linkages that result in the formation of clumps of platelets. On nonactivated platelets, GPIIb/IIIa exhibits minimal affinity for its ligands. Upon platelet activation, GPIIb/IIIa undergoes a conformational transformation, which reflects transmission of inside-out signals from its cytoplasmic domain to its extracellular domain.[18] This transformation enhances the affinity of GPIIb/IIIa for its ligands, fibrinogen, and, under high shear conditions, vWF (see Chapter 124). Cryptic Arg-Gly-Asp (RGD) peptide sequences located in fibrinogen and vWF, as well as a platelet-binding Lys-Gly-Asp (KGD) sequence in fibrinogen, mediate their interaction with GPIIb/IIIa. When subjected to high shear, circulating vWF elongates and exposes its platelet-binding domain, which enables its interaction with the conformationally activated GPIIb/IIIa. Similarly, initial binding to GPIIb/IIIa leads to a conformational change in fibrinogen that unmasks additional binding sites. Divalent fibrinogen and multivalent vWF molecules serve as bridges and bind adjacent platelets together. Once bound to GPIIb/IIIa, fibrinogen and vWF induce outside–inside signals that augment platelet activation and result in the activation of additional GPIIb/IIIa receptors, creating a positive feedback loop. Because GPIIb/IIIa acts as the final effector in platelet aggregation, it is a logical target for potent antiplatelet drugs (see Chapters 124 and 143). Fibrin, the ultimate product of the coagulation system, tethers the platelet aggregates together and anchors them to the site of injury.

Coagulation

Coagulation results in the generation of thrombin, which converts soluble fibrinogen to fibrin. Coagulation occurs through a series of concerted activation steps, wherein a nascent protease activates an inactive enzyme precursor (zymogen); a process that is repeated in a cascade-like fashion. The principal enzyme complexes are composed of a vitamin K–dependent enzyme and a non-enzyme cofactor assembled on anionic phospholipid membranes in a calcium-dependent fashion (see Chapter 125). Because each enzyme complex activates a substrate that becomes the enzyme component of the subsequent complex, a small stimulus can produce a robust response. Initially the small amount of thrombin generated activates non-enzyme cofactors and platelets. The activated platelets then provide an anionic surface on which the coagulation complexes assemble to promote rapid thrombin generation. Provision of an anionic phospholipid surface by activated platelets or other cells is essential to maximize the catalytic activity of these enzymatic complexes. The surface brings enzyme, cofactor, and substrate into proximity, thereby reducing the diffusional limitations of assembling soluble factors. The vitamin K-dependent factors, prothrombin and factors VII, IX, and X, are synthesized with post-translationally modified gamma-carboxy glutamic acid (Gla) residues at their amino termini that bear a greater negative charge. These Gla domains endow the factors with enhanced affinity for phosphatidylserine-rich membranes in a Ca^{2+}-dependent manner. Other proteins, the essential cofactors V and VIII, also have lipid-binding domains that promote their assembly into the activation complexes. The three predominant enzyme complexes involved in thrombin generation are extrinsic tenase, intrinsic tenase, and prothrombinase, named for their substrates, factor X and prothrombin (Fig. 121.4).

Coagulation can be divided into three phases: initiation, propagation, and termination.[19] These phases reflect the exquisite control over thrombin activity necessary to effect hemostasis. The initiation phase, which is predominantly mediated by extrinsic tenase, is responsible for generating the initial burst of thrombin required for platelet and coagulation factor activation. The propagation phase is largely mediated by intrinsic tenase and is responsible for the explosive thrombin generation necessary to sustain the coagulant response. The termination phase, which is mediated by numerous protease inhibitors, ensures that thrombin generation is localized and finite. Each of the activation complexes exhibits similar composition, assembly, and regulation.

Because initiation and propagation reactions require a membrane surface for assembly of factors, cells play a key role in coagulation. Tissue factor exposure on monocytes and extravascular cells and platelet membrane capacitation are recognized key steps. However, other cells also make important contributions. For example, damaged

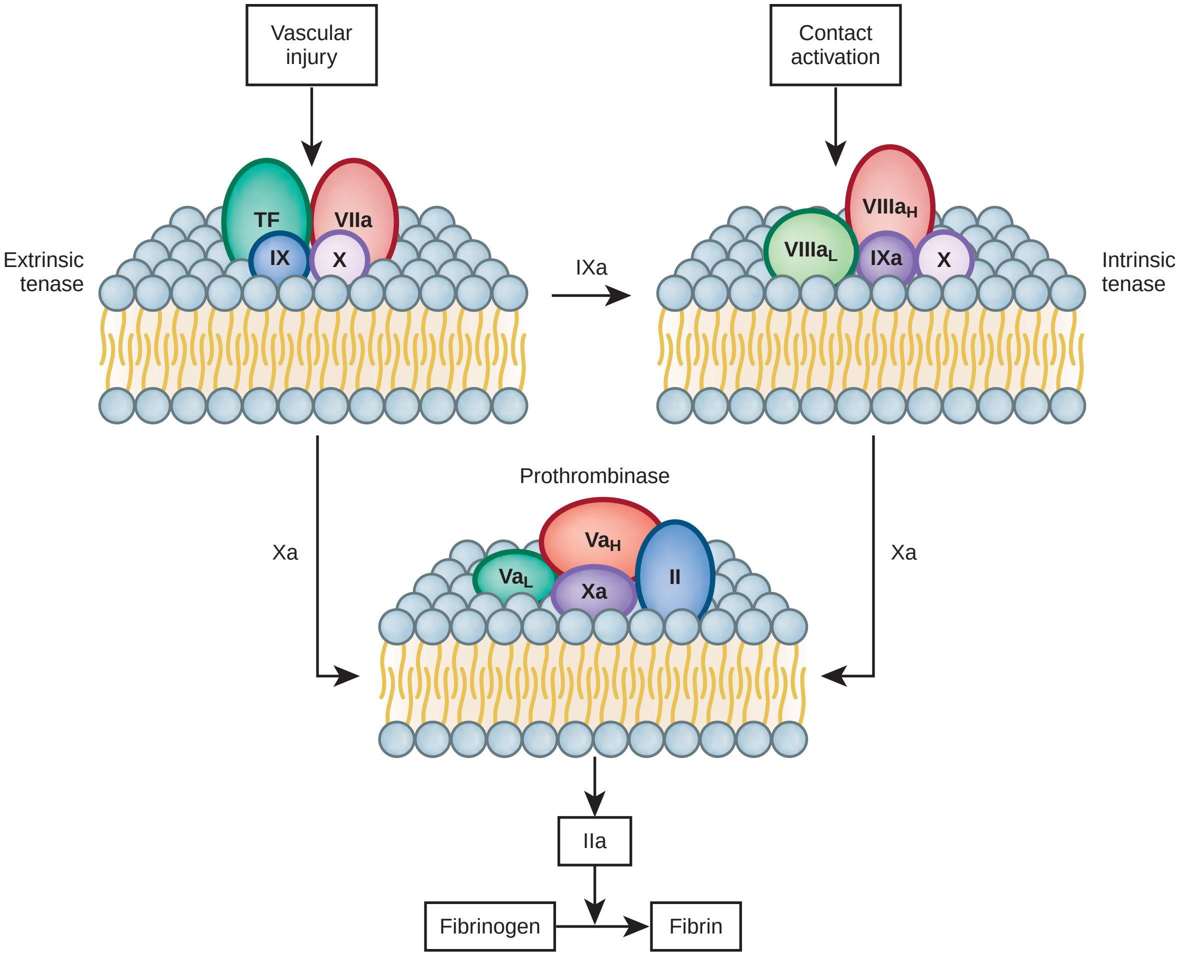


Figure 121.4 COAGULATION SYSTEM. Coagulation occurs through the action of discrete enzyme complexes, which are composed of a vitamin K–dependent enzyme and a non-enzyme cofactor. These complexes assemble on anionic phospholipid membranes in a calcium-dependent fashion. Vascular injury exposes tissue factor *(TF)*, which binds factor VIIa to form extrinsic tenase. Extrinsic tenase activates factors IX and X. Factor IXa binds to factor VIIIa to form intrinsic tenase, which activates factor X. The contact system also leads to activation of factor IX in response to exposure of anionic surfaces. Factor Xa binds to factor Va to form prothrombinase, which converts prothrombin (II) to thrombin (IIa). Thrombin then converts soluble fibrinogen into insoluble fibrin.

endothelium appears to support coagulation because mice deficient in PAR4, the major thrombin receptor on mouse platelets, still exhibit significant thrombus formation after laser injury. Red blood cells also bind to damaged endothelium and express procoagulant phospholipids on their surface, thereby providing additional sites for assembly of coagulation complexes. In addition, activated neutrophils extrude web-like structures known as neutrophil extracellular traps (NETs). Composed of nuclear DNA, histones, and metalloproteases, NETs promote coagulation by binding and activating platelets, trapping red blood cells, and activating the contact pathway. Thus, cells contribute to coagulation at numerous sites in the cascade.

Extrinsic Tenase

This complex forms upon exposure of tissue factor–expressing cells to the blood.[19] Tissue factor exposure occurs after atherosclerotic plaque rupture because the core of the plaque is rich in macrophages and other cells that express tissue factor. Denuding injury to the vessel wall also exposes tissue factor constitutively expressed by subendothelial fibroblasts and smooth muscle cells. In addition to cells in the vessel wall, activated circulating monocytes and monocyte-derived microparticles and extracellular vesicles (small membrane fragments) also provide a source of tissue factor. When tissue factor–bearing monocytes or microparticles bind to platelets or other leukocytes and their plasma membranes fuse, tissue factor transfer occurs. By binding to adhesion molecules expressed on activated endothelial cells or to P-selectin on activated platelets, these tissue factor–bearing cells, or microparticles, can initiate or augment coagulation. This phenomenon likely explains how venous thrombi develop in the absence of obvious vessel wall injury.

Tissue factor is an integral membrane protein that serves as a receptor for factor VIIa. Blood contains trace amounts of factor VIIa, which has negligible activity in the absence of tissue factor.[19] Although tissue factor is present on cell surfaces, it is proposed to exist in an encrypted, inactive form. The decryption step is thought to occur by a disulfide bond rearrangement catalyzed by protein-disulfide isomerase, and exposure of phosphatidylserine on the outer membrane surface. Factor VIIa binds tissue factor in a calcium-dependent fashion to form the extrinsic tenase complex, which is a potent activator of factors IX and X. Once activated, factor IXa and Xa serve as the enzyme components of intrinsic tenase and prothrombinase, respectively. Because sufficient levels of factor Xa and thrombin are formed in response to exposure of tissue factor, the extrinsic tenase complex is considered the essential mediator of the initiation phase.

Intrinsic Tenase

Factor IXa binds to factor VIIIa on anionic platelet or cell surfaces to form the intrinsic tenase complex.[20] Factor VIII circulates in blood in complex with vWF. Thrombin cleaves factor VIII, which converts it to its active form (factor VIIIa) and releases it from vWF. Activated platelets express binding sites for factor VIIIa. Once bound, factor

VIIIa binds factor IXa in a calcium-dependent fashion to form the intrinsic tenase complex, which then activates factor X. The loss in catalytic efficiency of intrinsic tenase that occurs with a deficiency of factors VIII or IX in hemophilia A and B, respectively, highlights their importance. Absence of the membrane or factor VIIIa almost completely abolishes enzymatic activity, and the catalytic efficiency of the complete complex is 10^6-fold greater than that of factor IXa alone. Because intrinsic tenase activates factor X at a rate 50- to 100-fold faster than extrinsic tenase, it plays a critical role in the amplification of factor Xa and subsequent thrombin generation.[21] Thus intrinsic tenase is crucial to the propagation phase of coagulation.

Contact Pathway

The contact pathway is so named because initial identification of its constituents (factors XII, XI, IX, and kallikrein) required contact with artificial agents such as ellagic acid or silica for activation. Such agents are used to trigger clotting when performing the activated partial thromboplastin time, a key global coagulation assay. Because of the reliance on artificial activators, the contact pathway lost prominence when the physiologic tissue factor pathway was identified. For many years, tissue factor exposure was regarded as the sole pathway for activation of coagulation and the contact system was thought to be unimportant for hemostasis because patients deficient in factor XII, prekallikrein, and high-molecular-weight kininogen do not have bleeding problems (see Chapters 125 and 135). Although patients with severe deficiency of factor XI can bleed after trauma or surgery, spontaneous bleeding is uncommon, and the plasma level of factor XI does not reliably predict the propensity for bleeding (see Chapter 135). The capacity of thrombin to feedback and activate factor XI may help to explain this phenomenon, thus re-igniting interest in factor XI because it bypasses the need for involvement of factor XII and the contact pathway. It also is possible that platelet-derived factor XI may be more important for hemostasis than circulating factor XI.[22]

There is increasing evidence that the contact pathway is important in thrombosis. Thus, catheters and other blood-contacting medical devices, such as stents, mechanical heart valves, and extracorporeal circuits trigger clotting through this mechanism. Factor XII, when bound to negatively charged or artificial surfaces, undergoes a conformational change that results in its activation. Factor XIIa converts prekallikrein to kallikrein in a reaction accelerated by high-molecular-weight kininogen, and factor XIIa and kallikrein both feedback to activate additional factor XII. Factor XIIa propagates coagulation by activating factor XI and generating factor XIa, the predominant activator of factor IX (Fig. 121.5).

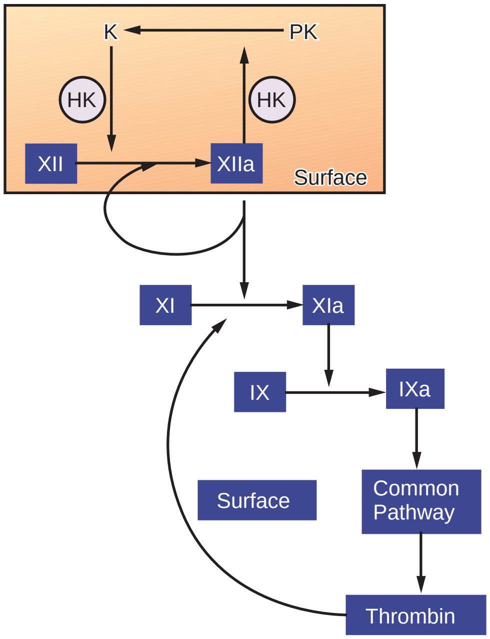


Figure 121.5 CONTACT SYSTEM. Factor XII (XII) is activated by contact with negatively charged surfaces. XIIa converts prekallikrein *(PK)* to kallikrein *(K)*, which can feed back to activate more XII. Likewise, XIIa also can feed back to amplify its own generation. About 75% of circulating PK is bound to high-molecular-weight kininogen *(HK)*, which localizes it to anionic surfaces and promotes PK activation. XIIa propagates clotting by activating XI, which then activates IX. The resultant IXa assembles into the intrinsic tenase complex, which activates X to initiate the common pathway of coagulation. Thrombin can feedback activate factor XI to further propagate coagulation.

This renewed interest in factor XI provided the stimulus to investigate the roles of factors XI and XII in thrombosis. Factor XI-deficient mice are protected from arterial injury–induced thrombosis to the same extent as factor IX-deficient mice, but do not experience bleeding, providing evidence of a clear demarcation between hemostasis and thrombosis. Mice lacking factor XII are also resistant to clot formation at sites of vascular injury. However, the physiologic mechanism for activation of the pathway remained unclear until studies showed that nuclear material released from neutrophils in the form of NETs, nucleic acids, and inorganic polyphosphates released from activated platelets or microorganisms activate coagulation in a contact pathway–dependent fashion.

Renewed interest in the contact pathway has spurred investigation of factors XI and XII as targets for new antithrombotic drugs. Agents targeting factors XI and XII include biological inhibitors, antisense oligonucleotides (ASO), antibodies, small molecule inhibitors, and aptamers. Both an ASO that reduces factor XI levels and an inhibitory antibody against factor Xa have been shown to reduce postoperative venous thrombosis in patients undergoing total knee arthroplasty, without an increase in bleeding. Similar interest in targeting the contact pathway is directed at device-related thrombosis. Thrombosis is a major cause of failure of blood-contacting medical devices, a problem that can lead to life-threatening complications including pulmonary embolism, coronary occlusion, and stroke, and activation of the contact pathway by the artificial surface is thought to be the primary cause. Therefore, the contact pathway has emerged as an attractive target for development of agents that reduce thrombosis with little impact on hemostasis.

The contact pathway may also contribute to the stability of arterial and venous thrombi.[23] NETs, and DNA and RNA released from damaged cells in atherosclerotic plaques, activate factor XII, and mice given DNA- or RNA-degrading enzymes exhibit attenuated thrombosis at sites of arterial injury. Polyphosphates released from activated platelets also activate factor XII, and may provide another stimulus for contact pathway activation. Mice deficient in factors XII or XI form small unstable thrombi at sites of arterial or venous damage, suggesting that factors XII and XI contribute to thrombogenesis (see Chapter 135). There is mounting evidence that the same is true in humans. Thus, patients with unstable angina have increased plasma levels of factor XIa that could reflect activation by factor XIIa, although activation by thrombin remains a possibility. The best evidence for the importance of the contact system comes from the results of phase 2 studies demonstrating that knock down of factor XI with an ASO or inhibition of factor XIa with a monoclonal antibody reduces the risk of postoperative venous thromboembolism in patients undergoing elective knee arthroplasty.[24,25] Therefore, the contact system is not only an important driver of thrombosis in blood-contacting medical devices, but may also contribute to thrombus propagation in situations where tissue factor initiates coagulation, such as after major surgery.

Prothrombinase

Being the only physiologic producer of thrombin, the prothrombinase complex is essential for hemostasis. Factor Xa binds to factor Va, its activated cofactor, on anionic phospholipid membrane surfaces in a calcium-dependent manner to form the prothrombinase complex. Activated platelets release factor V from their α-granules, and this platelet-derived factor V may play a more important role in hemostasis than its plasma counterpart.[26] Whereas plasma factor V requires thrombin activation to exert its cofactor activity, the partially activated factor V released from platelets already exhibits substantial cofactor activity. Activated platelets express specific factor Va binding sites on their surface, and bound factor Va serves as a receptor for factor Xa. The catalytic efficiency of factor Xa activation of prothrombin increases by 10^5-fold when factor Xa incorporates into the prothrombinase complex.[27] Prothrombin binds to the prothrombinase complex, where it undergoes conversion to thrombin in a reaction that releases prothrombin fragment 1.2 (F1.2). Plasma levels of F1.2, therefore, provide a marker of prothrombin activation. Prothrombin is the most abundant coagulation factor, and the efficiency of activation generates high local levels of thrombin.

Termination

Because thrombin clots fibrinogen, activates cells and platelets, and mediates anticoagulant and antifibrinolytic processes, it is imperative to regulate its activity and location. Thus, the termination phase plays a critical role in balancing the procoagulant forces. Two principal protease inhibitors modulate coagulation: TFPI and antithrombin. TFPI, which is located on platelets and microvascular endothelial cells, inhibits factor VIIa in a factor Xa-dependent manner. Because it binds TFPI, protein S serves both as a cofactor for this reaction and as a cofactor for APC -mediated inactivation of factors Va and VIIIa.[28] Adding to its anticoagulant role, TFPI also binds factor V and attenuates its assembly into the prothrombinase complex.[28] TFPI effectively halts tissue factor-mediated initiation of coagulation, but not before sufficient factor Xa is generated to initiate clotting.

The high levels of thrombin produced during the amplification phase are controlled by antithrombin. This serine protease inhibitor (serpin) also inactivates other coagulation proteases, including factors VIIa, IXa, Xa, and XIa. Although antithrombin is abundant, it exhibits only moderate inhibitory activity, except in the presence of cell-associated glycosaminoglycans, such as heparan sulfate. This is the biochemical basis for use of heparin, a structurally similar glycosaminoglycan derived from mast and intestinal mucosa cells that is used as an anticoagulant (see Chapter 143).

The protein C anticoagulant pathway that is catalyzed by thrombin mediates further regulation of thrombin generation. In the presence of endothelial surface bound thrombomodulin and EPCR, thrombin catalyzes the activation of protein C. APC is an important anticoagulant and anti-inflammatory mediator. In the context of hemostasis, APC inactivates FVa and FVIIIa to forms unable to act as cofactors in their respective activation complexes. Inactivation of FVIIIa by APC is promoted by intact FV and its activation intermediates, but not by FVa.[28]

The regulatory processes mediated by TFPI, antithrombin and APC ensure that thrombin generation is localized and limited. However, under conditions of hemostatic imbalance, sufficient thrombin is produced to overcome these checks and balances to ensure that coagulation occurs.

Fibrin Formation

Thrombin converts soluble fibrinogen into insoluble fibrin. Fibrinogen is a dimeric molecule, each half of which is composed of three polypeptide chains, the Aα, Bβ, and γ chains. Numerous disulfide bonds covalently link the chains together and join the two halves of the fibrinogen molecule (Fig. 121.6). Electron micrographic studies of fibrinogen reveal a trinodular structure with a central E domain flanked by two D domains. Crystal structures show symmetry of design with the central E domain, which contains the amino termini of the fibrinogen chains, joined to the lateral D domains by coiled-coil regions.

Fibrinogen circulates in a soluble form. Thrombin binds to the amino termini of the Aα and Bβ chains of fibrinogen, where it cleaves specific peptide bonds to release fibrinopeptide A and fibrinopeptide B and generates fibrin monomer (see Fig. 121.6). Because they are products of thrombin action on fibrinogen, plasma levels of these fibrinopeptides provide an index of thrombin activity. Fibrinopeptide release creates new amino termini that extend as knobs from the E domain of one fibrin monomer and insert into preformed holes in the D domains of other fibrin monomers. This creates long strands known as protofibrils, consisting of fibrin monomers noncovalently linked together in a half-staggered, overlapping fashion.[29]

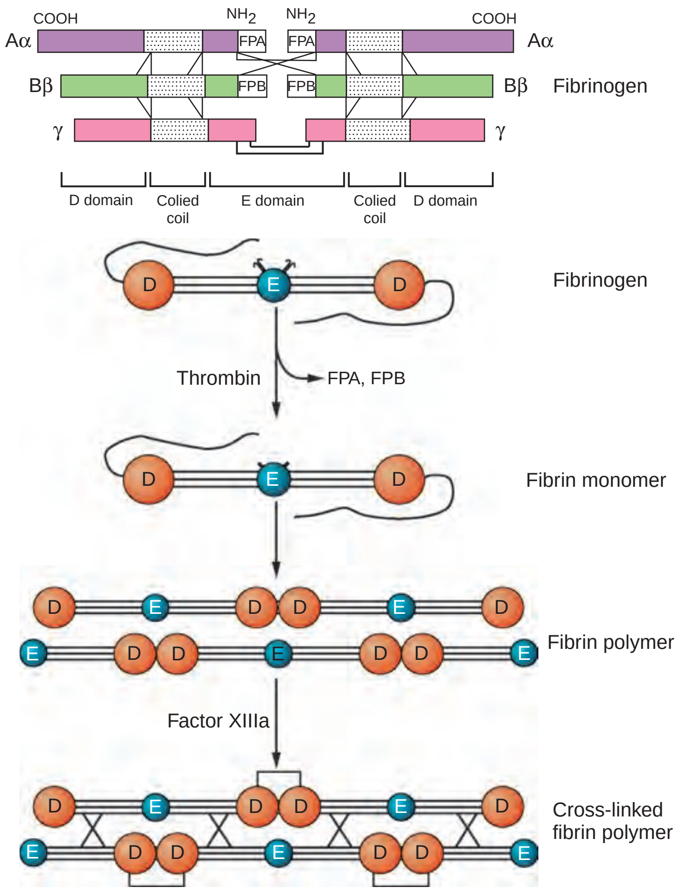


Figure 121.6 FIBRINOGEN STRUCTURE AND CONVERSION OF FIBRINOGEN TO FIBRIN. A dimeric molecule, each half of fibrinogen is composed of three polypeptide chains, Aα, Bβ, and γ. Numerous disulfide bonds (lines) covalently link the chains together and join the two halves of the fibrinogen molecule to yield a trinodular structure with a central E domain linked via the coiled-coil regions to two lateral D domains. To convert fibrinogen to fibrin, thrombin cleaves specific peptide bonds at the amino *(NH2)* termini of the Aα and Bβ chains of fibrinogen to release fibrinopeptide A *(FPA)* and fibrinopeptide B *(FPB)*, thereby generating fibrin monomer. Fibrin monomers polymerize to generate protofibrils arranged in a half-staggered overlapping fashion. By covalently cross-linking α and γ chains of adjacent fibrin monomers, factor XIIIa stabilizes the fibrin network and renders it resistant to degradation.

Noncovalently linked fibrin protofibrils lack tensile strength. The stability of the fibrin network is enhanced by platelets and procoagulant cells.[29] Platelets not only bind fibrin via GPIIb/IIIa and promote formation of a dense fibrin network, but they also release factor XIII. By covalently cross-linking α and γ chains of adjacent fibrin monomers, factor XIIIa stabilizes the fibrin in a calcium-dependent fashion and renders it relatively resistant to physical strain and degradation. A form of Factor XIII also circulates in blood as a heterodimer consisting of pairs of A and B subunits. The active and calcium binding sites on factor XIII are localized to the A subunit. Platelets contain large amounts of factor XIII in their cytoplasm, but platelet-derived factor XIII consists only of the A subunits (see Chapter 124). Both plasma and platelet factor XIII are activated by thrombin in a fibrin-dependent manner. Crosslinking of fibrin contributes to its tensile strength and traps erythrocytes, thereby contributing to the integrity of the thrombus.

Hemostasis depends on the dynamic balance between the formation of fibrin and its degradation. The fibrinolytic system mediates fibrin breakdown.

Fibrinolytic System

Fibrinolysis initiates when plasminogen activators convert plasminogen to plasmin, which then degrades fibrin into soluble fragments. Blood contains two immunologically and functionally distinct plasminogen activators, t-PA and u-PA. t-PA mediates intravascular fibrin degradation, whereas u-PA binds to a specific u-PA receptor (u-PAR) on the surface of cells, where it activates cell-bound plasminogen. Consequently, pericellular proteolysis during cell migration, and tissue remodeling and repair are the major functions of u-PA.[30]

Regulation of fibrinolysis occurs at several levels (see Chapter 125). The substrate of the fibrinolytic system, fibrin, serves a transient but essential stimulatory role that subsides as it degrades. The serpins, PAI-1, and to a lesser extent, PAI-2, inhibit the plasminogen activators, whereas α2-antiplasmin inhibits plasmin. Endothelial cells synthesize PAI-1, which inhibits both t-PA and u-PA, whereas monocytes and the placenta synthesize PAI-2, which specifically inhibits u-PA. Thrombin-activatable fibrinolysis inhibitor (TAFI) also modulates fibrinolysis and provides a link between fibrinolysis and coagulation.[30] Thrombosis can occur if there is impaired activation of the fibrinolytic system, whereas excessive activation leads to bleeding. Therefore, a review of the mechanisms of action of t-PA, u-PA, and TAFI is worthwhile.

Mechanism of Action of Tissue-Type Plasminogen Activator

t-PA, a serine protease, contains five discrete domains: a fibronectin-like finger domain, an epidermal growth factor (EGF) domain, two kringle domains, and a protease domain. Synthesized as a single-chain polypeptide, t-PA is converted into a two-chain form by plasmin. Single- and two-chain forms of t-PA convert plasminogen to plasmin. Native Glu-plasminogen is a single-chain polypeptide with a Glu residue at its amino-terminus. Plasmin cleavage near the amino-terminus generates Lys-plasminogen, a truncated form with a Lys residue at its new amino terminus.[31] t-PA cleaves a single peptide bond to convert single-chain Glu- or Lys-plasminogen into two-chain plasmin, composed of a heavy chain containing five kringle domains and a light chain containing the catalytic domain. Because its open conformation exposes the t-PA cleavage site, Lys-plasminogen is a better substrate than Glu-plasminogen, which assumes a circular closed conformation that renders this bond less accessible.

t-PA has little enzymatic activity in the absence of fibrin, but its activity increases by at least three orders of magnitude when fibrin is present. This increase in activity reflects the capacity of fibrin to serve as a template that binds t-PA and plasminogen and promotes their interaction. t-PA binds to fibrin via its finger and second kringle domains, whereas plasminogen binds fibrin via its kringle domains. Kringle domains are triple loop-like structures that bind Lys residues on fibrin and other proteins. As fibrin undergoes degradation, more Lys residues are exposed, which provide additional binding sites for t-PA and plasminogen. Consequently, degraded fibrin stimulates t-PA activation of plasminogen more than intact fibrin.

α2-Antiplasmin, another serpin, rapidly inhibits circulating plasmin by docking to its first kringle domain and then inhibiting the active site.[30] Because plasmin also binds to fibrin via its kringle domains, plasmin generated on the fibrin surface resists inhibition by α2-antiplasmin. This phenomenon endows fibrin-bound plasmin with the capacity to degrade fibrin. Factor XIIIa cross-links small amounts of α2-antiplasmin onto fibrin, which prevents premature fibrinolysis.[29]

Like fibrin, endothelial cells bind t-PA and plasminogen and markedly promote activation by colocalization of enzyme and substrate. Cell-surface binding is mediated by receptors such as annexin II, gangliosides, and α-enolase, as well as an orphan transmembrane protein expressed with a carboxy-terminal lysine residue. Plasminogen binds to exposed lysine residues on these receptors via its kringle domains. Lipoprotein(a) [LP(a)], which also possesses kringle domains, impairs cell-based fibrinolysis by competing with plasminogen for cell-surface binding. This phenomenon may explain the association between elevated LP(a) levels and atherosclerosis.

Mechanism of Action of Urokinase-Type Plasminogen Activator

Synthesized as a single-chain polypeptide, single-chain u-PA (scu-PA) has minimal enzymatic activity. Plasmin converts scu-PA into a two-chain form that is enzymatically active and capable of binding u-PAR, the u-PA receptor on cell surfaces. Further cleavage at the amino-terminus of two-chain u-PA yields a truncated, lower-molecular-weight form that lacks the u-PAR binding domain.

Two-chain forms of u-PA readily convert plasminogen to plasmin in the absence or presence of fibrin.[31] In contrast, scu-PA does not activate plasminogen in the absence of fibrin, but it can activate fibrin-bound plasminogen, because plasminogen adopts the readily activatable open conformation. Like the higher-molecular-weight form of two-chain u-PA, scu-PA binds to cell surface u-PAR, where plasmin can activate it. Many tumor cells elaborate u-PA and express u-PAR on their surface. As with fibrin and plasminogen receptors, colocalization of the reactants greatly promotes activation. Plasmin generated on these cancer cells endows them with the capacity for metastasis because plasmin readily degrades components of the extracellular matrix and activates growth factors and other degradative proteases.

Mechanism of Action of Thrombin-Activatable Fibrinolysis Inhibitor

TAFI, a procarboxypeptidase B–like molecule synthesized in the liver, circulates in blood in a latent form where thrombin bound to thrombomodulin can activate it to TAFIa (see Chapter 125). Unless bound to thrombomodulin, thrombin activates TAFI inefficiently.[32] TAFIa attenuates fibrinolysis by cleaving Lys residues from the carboxy termini of chains of degrading fibrin, thereby removing essential binding sites for plasminogen, plasmin, and t-PA, attenuating activation, and promoting plasmin inhibition. TAFI links fibrinolysis to coagulation because the thrombin–thrombomodulin complex not only activates TAFI, which attenuates fibrinolysis, but also activates protein C, which mutes thrombin generation.

TAFIa has a short half-life in plasma because the enzyme is unstable. Genetic polymorphisms can result in synthesis of more stable forms of TAFIa. Persistent attenuation of fibrinolysis by these variant forms of TAFIa may render patients susceptible to thrombosis.

DISORDERS OF HEMOSTASIS OR THROMBOSIS

A physiologic host defense mechanism, hemostasis focuses on arrest of bleeding by forming hemostatic plugs composed of platelets and fibrin at sites of vessel injury. In contrast, thrombosis reflects a pathologic process associated with intravascular thrombi that occlude the lumens of arteries or veins. Disorders are typically caused by disruption of one of the three axes of Virchow's triad: the vasculature, blood flow, or hypercoagulability of the blood. These processes are interdependent and integrated, and their dysregulation leads to immunothrombosis (see box on Immunohemostasis).

Hemostatic Disorders

Bleeding can occur if there is abnormal platelet plug formation and/or reduced thrombin generation and subsequent fibrin clot formation at the site of vascular injury, disorders of primary and secondary hemostasis, respectively. Bleeding also can occur if the platelet/fibrin clot is prematurely degraded because of excessive fibrinolysis; a disorder of tertiary hemostasis. The features distinguishing disorders of primary, secondary, and tertiary hemostasis are outlined in Table 121.1. Hemorrhagic disorders can be inherited or acquired, and the clinical and laboratory evaluation of such disorders is detailed in Chapters 126 and 127, respectively.

Disorders of Primary Hemostasis

Platelet plug formation, the first step in the arrest of bleeding at sites of injury, requires three key components: (a) an adequate number of functional platelets, (b) vWF, the molecular glue that mediates platelet adhesion to the damaged vessel wall even in the face of high shear, and (c) a normal blood vessel that constricts in response to injury (Table 121.2). Because the platelet plug provides the first line of defense against bleeding, patients with disorders of primary hemostasis often present with immediate bleeding after injury, and petechiae (pinpoint hemorrhages) may be noted. In addition to skin bleeding, mucocutaneous bleeding, which may manifest as epistaxis, bleeding gums, or hematochezia, is common as is excessive menstrual bleeding in women (see Chapter 126).

Disorders of primary hemostasis may be inherited or acquired.[33] Thrombocytopenia, or congenital or acquired disorders of platelet function, are common causes of bleeding. Thrombocytopenia can be the result of decreased production, which can occur because of failure, infiltration, or fibrosis of the bone marrow (see Chapters 30 and 31), increased platelet destruction, or abnormal distribution because of platelet pooling in the spleen (see Chapter 130). Increased destruction of platelets can occur via immune mechanisms, such as immune thrombocytopenic purpura (ITP), alloimmune thrombocytopenia, posttransfusion purpura, and drug-induced thrombocytopenia including heparin-induced thrombocytopenia (see Chapters 129, 130 and 131), or nonimmune mechanisms, which include microangiopathic disorders, such as thrombotic thrombocytopenic purpura and hemolytic uremic syndrome (see Chapter 132), as well as consumption because of activation of coagulation, such as occurs with disseminated intravascular coagulation (see Chapter 137).

TABLE 121.1 **Comparison of the Features of Disorders of Primary, Secondary, or Tertiary Hemostasis**

Features	Primary	Secondary	Tertiary
Components involved	Platelets, vWF, and vessel wall	Coagulation	Fibrinolysis factors
Site of bleeding	Skin and mucocutaneous and soft tissues	Muscles, joints, and deep tissues	Wounds and genitourinary tract
Physical findings	Petechiae and ecchymoses	Hematomas and hemarthroses	Hematuria and menorrhagia
Timing of bleeding	Immediate	Delayed	Delayed
Inheritance	Autosomal dominant	Autosomal or X-linked recessive	Autosomal recessive

vWF, von Willebrand factor.

TABLE 121.2 **Disorders of Primary Hemostasis**

Components Affected	Causes
Platelets	Quantitative or qualitative platelet disorders
vWF	Inherited or acquired deficiency or dysfunction of vWF

vWF, von Willebrand factor.

Platelet function disorders include disorders of platelet (a) adhesion, such as von Willebrand disease (see Chapter 133) and Bernard-Soulier syndrome (see Chapter 124); (b) thromboxane synthesis; (c) secretion, such as alpha or dense granule deficiency, or aspirin-like secretion defects; (d) aggregation, such as Glanzmann thrombasthenia (see Chapter 124); or (e) procoagulant activity (Scott syndrome) where the platelets fail to support clotting factor complex assembly (see Chapter 124). Acquired disorders of platelet function can occur in patients taking drugs that impair platelet function, such as aspirin or nonsteroidal anti-inflammatory drugs, or in patients with uremia, paraproteins, myelodysplastic, or myeloproliferative disorders (see Chapter 128).

Bleeding can also occur with inflammation or malformations of the blood vessels, or abnormalities of the connective tissue supporting the blood vessels. Inflammatory disorders include Henoch-Schonlein purpura (see Chapter 148) and the vasculitis that occurs with paraproteins or cryoglobulins, or in patients with systemic lupus erythematosus or other immune disorders.[34] Hereditary hemorrhagic telangiectasia is an inherited disorder associated with malformations of the capillaries. Telangiectatic vessels can often be seen in the oral and nasal cavities of patients with this disorder and bleeding episodes, primarily from the nose and gastrointestinal tract, are common. Abnormalities of the connective tissue matrix supporting the blood vessels include Marfan syndrome, Ehlers-Danlos syndrome, and pseudoxanthoma elasticum.[35] Patients with these disorders frequently report easy bruising.

Disorders of Secondary Hemostasis

Secondary hemostasis depends on rapid generation of sufficient amounts of thrombin to generate a fibrin mesh that not only consolidates the platelet aggregates that form at sites of vascular injury, but also is stable enough to provide a barrier that prevents leakage of blood from the damaged blood vessel.[29] Secondary hemostasis can be compromised by (a) impaired thrombin generation because of congenital or acquired deficiencies of coagulation factors or cofactors or intake of drugs that inhibit one or more steps in the coagulation pathways, (b) congenital or acquired fibrinogen deficiency or dysfunction, and/or (c) impaired cross-linking of fibrinogen because of congenital or acquired deficiency of factor XIII (Table 121.3).

Examples of inherited deficiencies of coagulation factors include hemophilia A and B, deficiencies of factor VIII and factor IX, respectively (see Chapter 134). Because of redundancy in the coagulation system, only patients with a factor VIII or factor IX level less than 1% have severe disease characterized by spontaneous bleeding or bleeding

TABLE 121.3 **Disorders of Secondary Hemostasis**

Components Affected	Causes
Coagulation factors	Congenital deficiency, autoantibodies, increased consumption, or drugs that attenuate thrombin generation or thrombin activity.
Fibrinogen	Decreased production, increased consumption or synthesis of an abnormal protein. Impaired fibrin polymerization because of fibrin(ogen) degradation products or paraproteins.
Fibrin cross-linking	Congenital or acquired factor XIII deficiency

with minimal trauma. Those with factor levels between 1% and 5% have an intermediate phenotype, whereas patients with factor VIII or IX levels above 5% usually have mild disease and bleed only with trauma or surgery. The frequency of bleeding episodes in patients with severe hemophilia can be reduced with prophylactic administration of the appropriate factor concentrate; such treatment is also administered to hemophiliacs with overt bleeding, or in preparation for surgery or other major interventions. Long-lasting factor VIII and IX molecules have been developed to reduce the frequency of factor replacement. The half-lives of these recombinant full-length or truncated proteins have been prolonged by conjugating them to hydrophilic polymers such as polyethylene glycol, or by fusing them with albumin or the Fc fragment of IgG1. Conjugation to polyethylene glycol protects the proteins from proteolytic degradation, whereas fusion technology creates new recycling pathways that diminish natural protein breakdown (see Chapter 134). Management of hemophilia becomes more complicated if patients develop inhibitory antibodies that attenuate or abolish the activity of the infused factor. Emicizumab, the first synthetic clotting factor, is a bispecific antibody that bypasses factor VIII by binding to factor IXa and bridging it to factor X. Emicizumab is now used for treatment of hemophilia A in patients with or without inhibitors (see Chapter 134).

Congenital deficiencies of prothrombin (factor II), factors V, VII, X, or XI (hemophilia C), or fibrinogen are less common causes of bleeding (see Chapter 135). In contrast, deficiencies of components of the contact pathway—factor XII, high-molecular-weight kininogen, and prekallikrein—are not associated with bleeding. The clinical and laboratory evaluation of such patients is detailed in Chapters 126 and 127, respectively, whereas their treatment is outlined in Chapters 115 and 135.

Acquired deficiencies of coagulation factors can result from decreased synthesis due to severe liver disease, vitamin K deficiency or intake of drugs that interfere with vitamin K metabolism, consumption because of excessive activation of coagulation (e.g., disseminated intravascular coagulation; see Chapter 137), or accelerated clearance due to adsorption by paraproteins or amyloid (see Chapters 91 and 92), or to autoantibodies that shorten the half-life, attenuate, or abolish clotting factor activity.

Congenital disorders of fibrinogen include absence or low levels of fibrinogen (afibrinogenemia and hypofibrinogenemia, respectively), or synthesis of a dysfunctional protein (dysfibrinogenemia). Acquired disorders of fibrinogen include decreased synthesis or production of an abnormal fibrinogen, increased fibrinogen consumption or the presence of inhibitors that interfere with fibrin polymerization, such as paraproteins, autoantibodies, particularly in patients with systemic lupus erythematosus or other immune disorders or elevated levels of fibrin(ogen) degradation products.

Stabilization of fibrin requires cross-linking of the α and γ chains of adjacent fibrin monomers to yield a polymer that is resistant to premature breakdown. Factor XIIIa performs this function by catalyzing the condensation of lysine residues on one chain with glutamic acid residues on another chain.[29] Congenital or acquired deficiency of factor XIII can impair cross-linking, resulting in bleeding. The hallmarks of severe factor XIII deficiency include umbilical stump bleeding in the neonatal period (see Chapters 135 and 148), intracranial hemorrhage with little or no trauma, recurrent soft tissue hemorrhages, and, in females, recurrent spontaneous miscarriages.

Disorders of Tertiary Hemostasis

Tertiary hemostasis depends on the generation of plasmin, which degrades fibrin and restores blood flow in damaged vessels. Premature lysis of fibrin in hemostatic plugs can lead to bleeding; this can occur systemically or can be localized (Table 121.4). Systemic fibrinolysis that occurs in the absence of activation of coagulation, the so-called primary hyperfibrinolysis, is rare but can occur with inherited deficiency of PAI-1 or α2-antiplasmin, the inhibitors of the plasminogen activators and plasmin, respectively, advanced liver disease, and some snakebites. More commonly, systemic hyperfibrinolysis is secondary to activation of coagulation by procoagulants such as tissue factor (e.g., in patients with metastatic cancer) or artificial surfaces (e.g., in cardiopulmonary bypass surgery or with cardiac assist devices). Examples of localized hyperfibrinolysis include menorrhagia or hematuria after prostatectomy triggered by excessive plasmin generation induced by the high concentrations of t-PA and u-PA in the uterus and genitourinary tract, respectively.

TABLE 121.4 Disorders of Tertiary Hemostasis

Component Affected	Causes
Plasminogen activators	Increased t-PA or u-PA release in the GU tract or other tissues.
Plasmin	Deficiency of PAI-1 or α2-antiplasmin, resulting in an increased plasmin concentration.

GU, Genitourinary; *PAI-1*, plasminogen activator inhibitor 1; *t-PA*, tissue plasminogen activator; *u-PA*, urokinase-type plasminogen activator.

Thrombotic Disorders

Thrombosis may occur in arteries, in the chambers of the heart, or in the veins. Factors contributing to thrombosis in these sites include endothelial injury or activation, reduced blood flow, and hypercoagulability of the blood, the so-called Virchow triad.

Arterial Thrombosis

Most arterial thrombi occur on top of disrupted atherosclerotic plaques. Plaques with a thin fibrous cap and a lipid-rich core are most prone to disruption. Erosion or rupture of the fibrous cap exposes thrombogenic material in the lipid-rich core to the blood, and triggers platelet activation and thrombin generation (see Chapter 142). The extent of plaque disruption and the content of thrombogenic material in the plaque determine the consequences of the event, regardless of whether it occurs in the cerebral circulation (see Chapter 144), the coronary circulation (see Chapter 145), or the major arteries of the legs (see Chapter 146), but host factors also contribute. Breakdown of regulatory mechanisms that limit platelet activation and inhibit coagulation can augment thrombosis at sites of plaque disruption.

Decreased production of nitric oxide and prostacyclin by diseased endothelial cells can trigger vasoconstriction and platelet activation. Proinflammatory cytokines lower thrombomodulin expression by endothelial cells, thereby sustaining thrombin generation, and stimulate PAI-1 expression, which inhibits fibrinolysis.

Products of blood coagulation contribute to atherogenesis, as well as its complications (see Chapter 142). Microscopic erosions in the vessel wall trigger the formation of tiny platelet-rich thrombi.[36] Activated platelets release PDGF and TGF-β, which promote a fibrotic response. Thrombin generated at the site of injury not only activates platelets and converts fibrinogen to fibrin, but also activates PAR-1 on smooth muscle cells and induces their proliferation, migration, and elaboration of extracellular matrix. Incorporation of thrombi into plaques promotes plaque growth, and decreased endothelial cell production of heparan sulfate that normally limits smooth muscle proliferation contributes to plaque expansion.[2] The multiple links between atherosclerosis and thrombosis have prompted the term atherothrombosis (see Chapter 142).

Intracardiac Thrombosis

Thrombi can form in the left ventricle after transmural myocardial infarction or with an aneurysm or dyskinetic ventricle, or in the left atrial appendage, particularly in patients with atrial fibrillation (see Chapter 147). Damage to the endothelium after myocardial infarction and abnormal blood flow are the major triggers for left ventricular thrombus formation. With rapid atrial fibrillation, there also is stasis and turbulent blood flow in the left atrial appendage, which is a long, blind-ended trabeculated pouch.[37] This may lead to localized activation of endothelial cells and subsequent loss of their anticoagulant phenotype, a process amplified by adhesion of leukocytes and subsequent elaboration of proinflammatory cytokines. The generation of thrombin creates a local hypercoagulable state that likely promotes thrombus formation on the abnormal endothelium. Embolization of these thrombi to the brain is a common cause of ischemic stroke and the major cause of mortality and morbidity in patients with atrial fibrillation.

Venous Thrombosis

The causes of venous thrombosis include those associated with hypercoagulability, which can be genetic or acquired, and the mainly acquired risk factors, such as advanced age, obesity, or cancer, which are associated with immobility (see Chapters 138 and 140). Inherited hypercoagulable states and these acquired risk factors combine to establish the intrinsic risk of thrombosis for each individual.[38] Superimposed triggering factors, such as surgery, pregnancy, or hormonal therapy, modify this risk, and thrombosis occurs when the combination of genetic, acquired, and triggering forces exceed a critical threshold.[39]

Some acquired or triggering factors entail a higher risk than others. For example, major orthopedic surgery, neurosurgery, multiple trauma, and metastatic cancer (particularly adenocarcinoma) are associated with the highest risk; prolonged bed rest, antiphospholipid antibodies (see Chapter 139), and the puerperium are associated with an intermediate risk; whereas pregnancy, obesity, long-distance travel, or the use of oral contraceptives or hormonal replacement therapy are mild risk factors. Up to half of the patients who present with venous thromboembolism before the age of 45 have inherited hypercoagulable disorders—so-called thrombophilia (see Chapter 138)—particularly those whose event occurred in the absence of risk factors or with minimal provocation, such as after minor trauma or a long-haul flight or with estrogen use.[39]

TREATMENT OF DISORDERS OF HEMOSTASIS AND THROMBOSIS

By the midpoint of the 20th century, two major anticoagulant drugs had been discovered, characterized, and given to humans for prevention or treatment of thrombotic disorders. Heparin and vitamin K antagonists, such as warfarin, dominated treatment regimens for decades. In the same era, determination of their mechanisms of action and dosing was aided by the discovery of the main players of the coagulation system, resulting from the development of sensitive functional assays to monitor their activity. Heparin and warfarin still represent effective members of the anticoagulant armamentarium; however, detailed understanding of the biochemistry and cell biology of hemostasis has directly contributed to the development of new therapies. Heparin derivatives with more predictable pharmacokinetics have improved therapy and reduced complications (see Chapter 143). Small molecule, direct inhibitors of thrombin and factor Xa have been developed. These direct oral anticoagulants include dabigatran, rivaroxaban, apixaban, and edoxaban; agents that are replacing conventional therapies (see Chapter 143). The resurgence of interest in the contact system as a potential mediator of thrombosis has led to the investigation of factors XI and XII as new targets for therapy.[40] On the hemostasis side, regimens to treat bleeding disorders include administration of factors VIIa, VIII, or IX, and prothrombin complex concentrate (see Chapters 134, 135 and 136). The new agents and treatment regimens highlight the therapeutic benefit that has resulted from our detailed understanding of hemostasis and thrombosis and provide optimism about further reducing cardiovascular disease as a leading cause of death.

REFERENCES

1. Bentzon JF, Otsuka F, Virmani R, et al. Mechanisms of plaque formation and rupture. *Circ Res*. 2014;114(12):1852–1866.
2. Spronk H, Padro T, Siland J, et al. Atherothrombosis and thromboembolism: position paper from the Second Maastricht Consensus Conference on Thrombosis. *Thromb Haemost*. 2018;118(02):229–250.
3. d'Alessandro E, Becker C, Bergmeier W, et al. Thrombo-inflammation in cardiovascular disease: an expert consensus document from the Third Maastricht Consensus Conference on Thrombosis. *Thromb Haemost*. 2020;120(4):538–564.
4. Wolberg AS, Rosendaal FR, Weitz JI, et al. Venous thrombosis. *Nat Rev Dis Primers*. 2015;1:15006.
5. Mega JL, Braunwald E, Wiviott SD, et al. Rivaroxaban in patients with a recent acute coronary syndrome. *N Engl J Med*. 2012;366(1):9–19.
6. Eikelboom JW, Connolly SJ, Bosch J, et al. Rivaroxaban with or without aspirin in stable cardiovascular disease. *N Engl J Med*. 2017;377(14):1319–1330.
7. Bonaca MP, Bauersachs RM, Anand SS, et al. Rivaroxaban in peripheral artery disease after revascularization. *N Engl J Med*. 2020;382(21):1994–2004.
8. Anderson DR, Dunbar M, Murnaghan J, et al. Aspirin or rivaroxaban for VTE prophylaxis after hip or knee arthroplasty. *N Engl J Med*. 2018;378(8):699–707.
9. Jackson SP, Darbousset R, Schoenwaelder SM. Thromboinflammation: challenges of therapeutically targeting coagulation and other host defense mechanisms. *Blood*. 2019;133(9):906–918.
10. Guo L, Rondina MT. The era of thromboinflammation: platelets are dynamic sensors and effector cells during infectious diseases. *Front Immunol*. 2019;10:2204.
11. Gimbrone Jr MA, García-Cardeña G. Endothelial cell dysfunction and the pathobiology of atherosclerosis. *Circ Res*. 2016;118(4):620–636.
12. Mast AE. Tissue factor pathway inhibitor: multiple anticoagulant activities for a single protein. *Arterioscl Thromb Vasc Biol*. 2016;36(1):9–14.
13. Loghmani H, Conway EM. Exploring traditional and nontraditional roles for thrombomodulin. *Blood*. 2018;132(2):148–158.
14. van der Meijden PEJ, Heemskerk JWM. Platelet biology and functions: new concepts and clinical perspectives. *Nat Rev Cardiol*. 2019;16(3):166–179.
15. South K, Lane DA. ADAMTS-13 and von Willebrand factor: a dynamic duo. *J Thromb Haemost*. 2018;16(1):6–18.
16. Burnstock G. Purinergic signaling in the cardiovascular system. *Circ Res*. 2017;120(1):207–228.
17. Nieman MT. Protease-activated receptors in hemostasis. *Blood*. 2016;128(2):169–177.
18. Durrant TN, van den Bosch MT, Hers I. Integrin α(IIb)β(3) outside-in signaling. *Blood*. 2017;130(14):1607–1619.
19. Grover SP, Mackman N. Tissue factor: an essential mediator of hemostasis and trigger of thrombosis. *Arterioscler Thromb Vasc Biol*. 2018;38(4):709–725.
20. Swieringa F, Kuijpers MJ, Lamers MM, et al. Rate-limiting roles of the tenase complex of factors VIII and IX in platelet procoagulant activity and formation of platelet-fibrin thrombi under flow. *Haematologica*. 2015;100(6):748–756.
21. Brummel-Ziedins KE, Orfeo T, Gissel M, et al. Factor Xa generation by computational modeling: an additional discriminator to thrombin generation evaluation. *PLoS One*. 2012;7(1):e29178.
22. Pathak M, Kaira BG, Slater A, et al. Cell receptor and cofactor interactions of the contact activation system and factor XI. *Front Med (Lausanne)*. 2018;5:66.
23. Székely O, Borgi M, Lip GYH. Factor XI inhibition fulfilling the optimal expectations for ideal anticoagulation. *Expert Opin Emerg Drugs*. 2019;24(1):55–61.
24. Weitz JI, Bauersachs R, Becker B, et al. Effect of osocimab in preventing venous thromboembolism among patients undergoing knee arthroplasty: the FOXTROT randomized clinical trial. *JAMA*. 2020;323(2):130–139.
25. Buller HR, Bethune C, Bhanot S, et al. Factor XI antisense oligonucleotide for prevention of venous thrombosis. *N Engl J Med*. 2015;372(3):232–240.
26. Gertz JM, Bouchard BA. Mechanisms regulating acquisition of platelet-derived factor V/Va by megakaryocytes. *J Cell Biochem*. 2015;116(10):2121–2126.
27. Mann KG. Thrombin generation in hemorrhage control and vascular occlusion. *Circulation*. 2011;124(2):225–235.
28. Dahlback B. Novel insights into the regulation of coagulation by factor V isoforms, tissue factor pathway inhibitor alpha, and protein S. *J Thromb Haemost*. 2017;15(7):1241–1250.
29. Kattula S, Byrnes JR, Wolberg AS. Fibrinogen and fibrin in hemostasis and thrombosis. *Arterioscl Thromb Vasc Biol*. 2017;37(3):e13–e21.
30. Urano T, Castellino FJ, Suzuki Y. Regulation of plasminogen activation on cell surfaces and fibrin. *J Thromb Haemost*. 2018;16(8):1487–1497.

31. Longstaff C, Kolev K. Basic mechanisms and regulation of fibrinolysis. *J Thromb Haemost*. 2015;13(Suppl 1):S98–S105.
32. Plug T, Meijers JC. Structure-function relationships in thrombin-activatable fibrinolysis inhibitor. *J Thromb Haemost*. 2016;14(4):633–644.
33. Montoro-García S, Schindewolf M, Stanford S, et al. The role of platelets in venous thromboembolism. *Semin Thromb Hemost*. 2016;42(3):242–251.
34. Swan D, Rocci A, Bradbury C, et al. Venous thromboembolism in multiple myeloma—choice of prophylaxis, role of direct oral anticoagulants and special considerations. *Br J Haematol*. 2018;183(4):538–556.
35. Malfait F. Vascular aspects of the Ehlers-Danlos syndromes. *Matrix Biol*. 2018;71-72:380–395.
36. Ten Cate H, Hackeng TM, García de Frutos P. Coagulation factor and protease pathways in thrombosis and cardiovascular disease. *Thromb Haemost*. 2017;117(7):1265–1271.
37. Ding WY, Gupta D, Lip GYH. Atrial fibrillation and the prothrombotic state: revisiting Virchow's triad in 2020. *Heart*. 2020;106(19):1463–1468.
38. Preston RJS, O'Sullivan JM, O'Donnell JS. Advances in understanding the molecular mechanisms of venous thrombosis. *Br J Haematol*. 2019;186(1):13–23.
39. Campello E, Spiezia L, Adamo A, et al. Thrombophilia, risk factors and prevention. *Expert Rev Hematol*. 2019;12(3):147–158.
40. Fredenburgh JC, Weitz JI. New anticoagulants: moving beyond the direct oral anticoagulants. *J Thromb Haemost*. 2021;19(1):20–29.

CHAPTER 122

BLOOD VESSELS

Aly Karsan and Janusz Rak

HEMOSTATIC, HEMATOPOIETIC, AND VASCULAR SYSTEMS AS A FUNCTIONAL CONTINUUM

Diseases affecting the bone marrow (BM) and peripheral blood are closely intertwined with the state of the vascular system,[1] which acts as a niche, conduit, and regulator of blood cells.[2] This is exemplified by the anatomic proximity and interactions among several related cellular populations, including hematopoietic stem and progenitor cells and their mature progeny, endothelial cells, perivascular tissues, and other components involved in hematopoiesis as well as blood vessel formation, repair, homeostasis, and patency.[2,3] Recent progress in understanding the molecular mechanisms involved in communications among these cells increasingly informs clinical practice and drug discovery efforts.[2,4,5] For instance, agents designed to block vascular growth (antiangiogenics) in solid tumors also elicit hematologic perturbations,[6] and have been considered in the treatment of hematopoietic malignancies with enhanced BM vascularity.[7] Indeed, the hematopoietic, hemostatic, and vascular compartments can be viewed as a functional continuum, both in health and in disease.

STRUCTURE AND FUNCTION OF THE VESSEL WALL

The circulatory system has traditionally been divided into the macrovasculature (vessels >100 μm in diameter) and the microvasculature, each endowed with distinct functional roles. The arterial system transports blood to tissues, resists changes in blood pressure proximally, and regulates blood flow distally. Veins return blood to the heart and act as capacitance vessels because they contain, at any given time, approximately 70% of the total blood volume. Venules with luminal diameters less than 50 μm are structurally similar to capillaries.[8] Capillaries and microvessels in general are particularly important in the exchange of gases, macromolecules, and cells between blood and tissue. Although large vessels play an important role in maintaining vascular tone, a significant proportion of peripheral resistance arises from the capillaries.[8] Capillary endothelial cells also have a metabolic role, as in the conversion of angiotensin and hydrolysis of lipoproteins. Finally, the formation of new vessels is initiated in the microvasculature.

Macrovasculature

Large vessels are composed of three layers: intima, media, and adventitia.[8] The intima comprises the endothelium and the subendothelium. The endothelial cells of large vessels contain distinct rod-shaped secretory organelles, called Weibel-Palade bodies, which contain von Willebrand factor (vWF) and other proteins, such as P-selectin and angiopoietin-2, that contribute to inflammation, angiogenesis, and tissue repair.[9] The abluminal face of the endothelium rests on a basement membrane, which supports the endothelial cell and can act as a secondary barrier against the extravasation of blood.[10] The subendothelial matrix contains occasional smooth muscle cells and scattered macrophages. Both smooth muscle cells and endothelial cells contribute to the extracellular matrix (ECM) of the intima, along with ECM components such as elastin and collagen.[11,12] In large vessels, the media is separated from the intima by a layer of elastin, the internal elastic lamina. Diseases associated with mutations in elastin include supravalvular aortic stenosis, Williams syndrome, and autosomal dominant *cutis laxa*.[13]

The medial layer is composed primarily of concentric layers of smooth muscle cells and their secreted matrix, which is a complex mix of glycoproteins and proteoglycans. This layer is responsible for the structural integrity of the wall and for maintaining vascular tone. Mutations of the fibrillin-1 gene, a microfilament protein in elastic fibers, result in the disruption of the media in Marfan syndrome.[14] Defects of type III collagen can cause aortic rupture in patients with Ehlers-Danlos syndrome type IV.[11] An attenuated band of elastic fibers, the external elastic lamina, separates the adventitia from the media.

The adventitia is composed of loose connective tissue, and the outer portion of the media contains an ECM scaffold containing fibroblasts, small nerves, progenitor cells, lymphatic vessels, and nutritive blood vessels, the *vasa vasorum*. The adventitia has been recognized as a dynamic environment important in the growth, disease, and repair of the artery. The external limit of the adventitial layer is loosely defined and becomes contiguous with the surrounding connective tissue of the organ.[8]

Microvasculature

Capillaries and postcapillary venules are composed of two main cell types: endothelial cells and pericytes.[15] The two cell types interact to contribute to basement membrane formation, maintenance, and remodeling. Long pericyte processes extend over the abluminal surface of each endothelial cell,[15] and reciprocal extensions of the endothelial cell make contact with the pericyte. Pericytes and endothelial cells form specialized junctions with each other at distinct points in the basement membrane. These adherens junctions connect the cytoskeleton of pericytes and endothelial cells, mediating contact inhibition through contractile forces. Gap junctions between the cytoplasm of pericytes and endothelial cells enable communication through the passage of metabolites and ionic currents. Like endothelial cells, pericytes are also heterogeneous and display a variety of functions including[15]: (1) contractile activity, which regulates blood flow; (2) multipotential capabilities resulting in differentiation to adipocytes, osteoblasts, phagocytes, and smooth muscle cells; and (3) regulation of capillary growth. In animal models and human disease (e.g., diabetic microangiopathy, hemangiomata), a lack of pericytes is associated with microaneurysms and disordered microvasculature.[16]

ENDOTHELIAL STRUCTURE, FUNCTION, AND HETEROGENEITY

The endothelium exhibits polarity manifested by the asymmetric distribution of cell surface glycoproteins and by the unidirectional secretion of some ECM proteins and mediators.[17] In cultured endothelial cells, apical–basal polarity is established before confluence, but intercellular junctions may have a role in maintaining polarity and cell viability in vivo.[18,19]

Four types of intercellular junctions between adjacent endothelial cells have been described: tight junctions, gap junctions, adherens junctions, and desmosomes (see Chapter 15). Their distribution varies along the vascular tree, with tight junctions occurring more frequently in the larger arteries and brain vasculature, correlating with a more stringent requirement for permeability control. The molecular structure of endothelial tight junctions is similar to that of epithelial cells, consisting of a network of fibrils, with the integral membrane components comprising occludin, claudin-5, and junctional adhesion molecules (JAMs), which associate with various structural and signaling proteins on the cytoplasmic face.[18]

The distribution of gap junctions tends to follow that of tight junctions. Connexin 37, connexin 40, and connexin 43 are gap junction proteins that have been detected in endothelial cells. Gap junctions mediate communication between adjacent endothelial cells and between endothelial cells and pericytes or smooth muscle cells; they also contribute to the endothelial barrier and vascular integrity.

Adherens junctions are formed by transmembrane glycoproteins called cadherins, which make the link between cell-to-cell contacts and the cytoskeleton, and thus sense and respond to mechanical forces at the intercellular interface and regulate permeability. Several different types of cadherins are expressed in endothelial cells. The endothelial-specific cadherin vascular endothelial cadherin (VE-cadherin [Cadherin-5]) is expressed on virtually all types of endothelium.[20] Like other cadherins, VE-cadherin forms homotypic contacts with VE-cadherin on adjacent cells. Within the cell, VE-cadherin complexes with catenins, which, through other proteins, contact the actin cytoskeleton. Homotypic engagement of VE-cadherin across adjacent cells results in density-dependent inhibition of endothelial proliferation. This process is thought to be mediated by the association of vascular endothelial growth factor receptor 2 (VEGFR-2) with VE-cadherin, which sequesters VEGFR-2 at the membrane and prevents its internalization into signaling compartments.[20]

Other membrane proteins located at inter-endothelial junctions include platelet endothelial cell adhesion molecule 1 (PECAM-1), which may be important in directing the formation of junctions, as well as nectins, JAMs, endothelial cell-selective adhesion molecule (ESAM), and integrins (particularly $\alpha 2\beta 1$ and $\alpha 5\beta 1$).

On the luminal side, endothelium is exposed to blood elements and circulating molecules such as cytokines and bacterial products. Engagement of endothelial receptors by these humoral factors activates a well-described series of responses, including the recruitment and transmigration of leukocytes and changes in endothelial cell coagulant activity (see Chapters 49 and 125).

Biomechanical forces resulting from pulsatile blood flow have been shown to mediate striking changes in endothelial morphology and metabolism. Vessels must withstand three types of physical forces: radial distension (tension), longitudinal stretch, and tangential shear stress. In response to unidirectional laminar flow (shear stress), endothelial cells reorganize their cytoskeletal architecture, rearrange focal contacts at the basal surface, and align in the direction of flow. After exposure to physical forces, some endothelial cell responses occur within seconds, such as activation of potassium channels and increased release of nitric oxide (NO), resulting in vasodilation. Other responses are related to changes in gene expression and occur within hours. Whereas unidirectional laminar flow at physiological levels is atheroprotective, disturbed flow at arterial branchpoints and bends predisposes to atherosclerotic plaque formation and thrombosis (see Chapter 142). Disturbed flow, which interferes with laminar shear stress signals, induces vascular pathology by differentially regulating mechanosensitive transcription factors that modulate inflammation, mitochondrial function, and glucose metabolism among other functions.[21,22]

Endothelial cells directionally secrete ECM proteins, such as collagens, elastin, fibronectin, laminins, and proteoglycans, to the abluminal face. The composition of the subendothelium varies depending on location in the vascular tree, age, and disease states. Endothelial cells bind to the ECM via integrins that link and integrate matrix proteins to the cytoskeleton at sites referred to as *focal contacts*. The integrins detected in resting endothelium include $\alpha 6\beta 1$, $\alpha 5\beta 1$, $\alpha 2\beta 1$, and $\alpha v\beta 3$. The ECM serves several important functions including: (1) barrier to macromolecules in the event of disruption of the endothelium; (2) sequestration of growth factors and mediation of high-affinity binding to endothelial cells (e.g., heparan sulfate binds to fibroblast growth factor [FGF]); (3) counter-structure for the binding of endothelial cell integrins, and (4) presentation of a prothrombotic surface that initiates coagulation after injury.[23]

Binding of endothelial cells to the ECM serves at least four purposes: (1) provision of a physical scaffold, or as a haptotactic agent, to induce endothelial cell migration[11]; (2) induction of integrins clustering at focal adhesion contacts to activate signaling cascades that promote survival or differentiation; fibronectin and vitronectin provide survival signals, whereas laminins signal differentiation; (3) maintenance of cell shape through integrin-mediated cell spreading provides an antiapoptotic signal independent of direct integrin-initiated signal transduction; and (4) anchoring the cell, thereby providing a mechanism whereby blood flow at the luminal surface of the endothelium creates shear stress, which transmits signals to cells.[24]

Despite their common features, quiescent endothelial cells in vivo represent heterogeneous phenotypic populations.[17] Endothelial cells from veins and arteries and from capillaries of different organs demonstrate structural, functional, and molecular heterogeneity.[17] Exposure to various physical forces and the different functions served by vessels of different caliber are reflected in different endothelial phenotypes. Intriguingly, phenotypic heterogeneity also exists between neighboring endothelial cells exposed to the same extracellular environment.[25,26] Matrix proteins, soluble factors from the organ, or heterotypic contacts with parenchymal cells, pericytes or smooth muscle cells are believed to be important factors in specifying endothelial cell phenotype. Conversely, increasing evidence indicates that endothelial cells in turn provide instructive morphogenic cues during organogenesis and in adults.[27] Specific examples of microvessels found in hematopoietic tissues are discussed in the following sections.

High Endothelial Venules

Lymphocyte migration into secondary lymphoid sites, such as lymph nodes, Peyer's patches, and chronically inflamed nonlymphoid tissues, occurs at specialized postcapillary venules called *high endothelial venules* (HEVs).[28] The endothelial cells of these venules exhibit a plump morphology, display intense biosynthetic activity, and are encircled by a continuous thick basal lamina formed from ECM components produced by surrounding pericyte-like cells called *fibroblastic reticular cells*. HEV endothelial cells express tissue-specific adhesion molecules and secrete a thick adhesion molecule-containing glycocalyx. These HEV adhesion molecules are referred to as addressins and comprise the L-selectin ligands mucosal addressin cellular adhesion molecule 1 (MAdCAM-1), glycosylation-dependent cellular adhesion molecule 1 (GlyCAM-1), endomucin, and nepmucin. Other HEV receptors implicated in lymphocyte trafficking include $\alpha 4\beta 7$ integrin, CD34, the chemokine receptor DARC (Duffy antigen receptor for chemokines) and the antiadhesive matrix protein Hevin. In addition to recruitment of immune cells, HEV can also influence the outcome of the immune response.[29]

Endothelium of Bone Marrow Sinuses

BM sinus endothelial cells are flat with loose interdigitated junctions and discontinuous basal lamina and are programmed to regulate the egress of hematopoietic cells. For example, if a red blood cell (RBC) that still is nucleated begins to enter the circulation, the body of the cell is allowed to cross and is released as a reticulocyte, while the nucleus is retained extravascularly. Adventitial reticular cells (similar to pericytes) also play an important role in controlling hematopoietic cell egress, and CXCL12 (SDF-1)–abundant reticular cells may also participate in hematopoietic stem cell maintenance.[30] Most quiescent hematopoietic stem cells in the mouse are closely associated with marrow sinusoids, although it is possible that some are also present in a periarteriolar location. CXCL12 is highly expressed in endothelial cells, and interactions with chemokine receptor CXC-chemokine receptor (CXCR)4 are essential for stem cell homing, mobilization, and transendothelial migration into the BM. The CXCR4 antagonist, Pleraxifor, is used to mobilize stem cells for autologous transplantation in patients with lymphoma or myeloma.[31] Vascular cell adhesion molecule 1 (VCAM-1) expressed on BM endothelial cells appears to be the major BM addressin for hematopoietic progenitor cells expressing VLA-4 (α4β1 integrin). Endothelial selectins have also been implicated in promoting hematopoietic stem and progenitor cell homing to the BM. Blockade or genetic targeting of E-selectin inhibits niche-mediated pro-survival signaling of acute myeloid leukemia (AML) blasts and synergizes with chemotherapy (see Chapter 60). Clinical trials with the small-molecule E-selectin mimetic Uproleselan (GMI-1271) are under way in AML.[32] Factors such as CD44 and matrix metalloproteinases (MMPs) and mechanical factors such as cytoskeletal rearrangement are also key to the homing process related to the endothelium.

PHYSIOLOGIC FUNCTIONS OF THE ENDOTHELIUM

Endothelium as a Barrier

The microvessels act to exchange material brought to tissues through circulation. The movement of lipophilic and low-molecular-weight hydrophilic substances between blood and tissue is virtually unimpeded (except for the brain), but the vessels are selectively permeable to macromolecules. This semi-selective barrier is necessary to maintain the fluid balance between intravascular and extravascular compartments, yet antibodies, hormones, cytokines, and other molecules must have access to the interstitial space to exert their function.

The movement of macromolecules across the vessel wall is governed by (1) hydrostatic and oncotic pressure gradients; (2) physicochemical properties of the molecule, such as size, shape, and charge; and (3) properties of the barrier. The barrier of the vessel wall is formed by the cellular components, endothelial cells, and pericytes, as well as by the charge and compactness of the matrix components, glycocalyx, and basement membrane. Macromolecules can pass either directly through the endothelial cell (transcellular path) or between adjacent endothelial cells (paracellular path).[33]

Although water mainly moves across endothelium paracellularly, a significant proportion (≤ 40%) uses the transcellular route and water-transporting membrane channels, the aquaporins. Macromolecular transport into cells can proceed by receptor-mediated systems, such as clathrin-coated pits, in which the molecules usually are targeted to the lysosome but may be transported through the cell. Caveolae are 50 to 100 nm membrane invaginations that are abundant in capillary endothelial cells, and participate in transcytosis, as well as in translocation of glycosylphosphatidylinositol-linked proteins into the cytoplasm and in transmembrane signaling. The known leakiness of tumor microvasculature led to the identification of a structure designated the vesiculovacuolar organelle (VVO).[34] These organelles are clusters of interconnecting uncoated vesicles and vacuoles that span the entire thickness of the vascular endothelium, thereby providing a transendothelial connection between the vascular lumen and the extravascular space. Their function is enhanced by vascular endothelial growth factor (VEGF), which is known to increase the permeability of vessels.

During inflammation, binding of neutrophils to the endothelium results in the generation of oxidants that can induce endothelial cell injury and increase permeability. Upon adhesion of neutrophils to the endothelium, leukocyte CD18 (β2 integrin)-mediated signals trigger the release of the neutrophil cationic protein called *heparin-binding protein/CAP37/azurocidin*, which in turn induces formation of gaps between endothelial cells and macromolecular efflux. Thrombin, a crucial hemostatic enzyme and inflammatory mediator, can increase endothelial permeability by several mechanisms resulting from activation of its receptor on endothelial cells.[33] Both an increase in transcellular vesicular and paracellular permeability play a role. The contraction and retraction of endothelial cells are accompanied by “loosening” of intercellular junctions and focal integrin contacts with the ECM. An increase in paracellular permeability results from post-translational modification of components of junctional proteins such as claudins and VE-cadherin. Thrombin may also alter the repulsive effect of the negatively charged glycocalyx. Pericyte contractility has been hypothesized as an additional mechanism for increasing permeability in inflammatory states.[33]

Endothelium as a Non-Thrombogenic Surface

The molecular mechanisms of hemostasis and thrombosis are addressed in Chapters 121 and 125. This section places the endothelium in the context of these processes, as shown in Fig. 122.1. Normal unperturbed endothelium presents a non-thrombogenic surface to

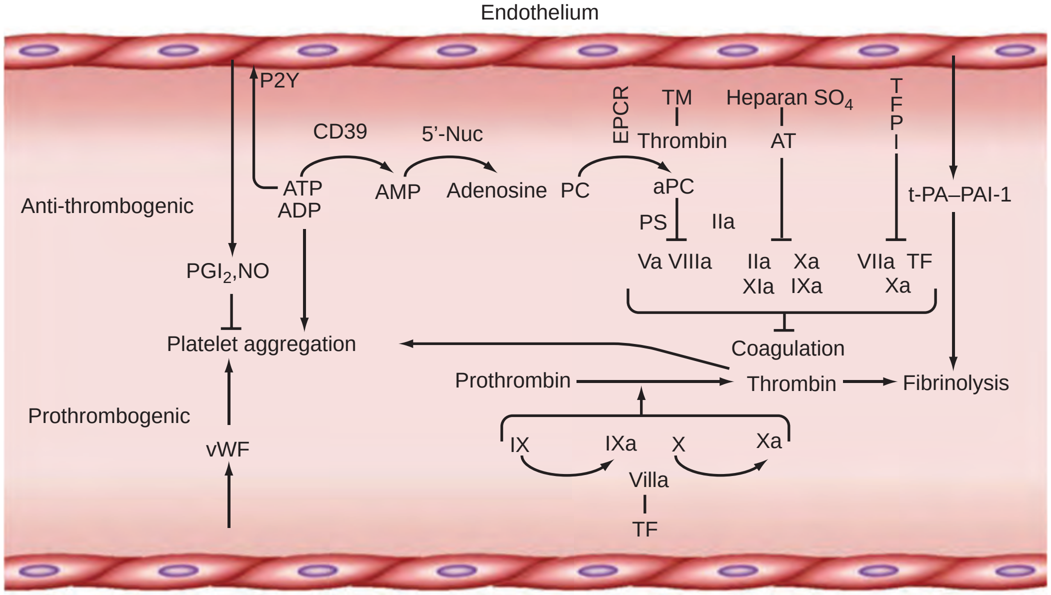


Figure 122.1 ENDOTHELIAL CELLS AS A PART OF THE HEMOSTATIC SYSTEM. In health endothelial cells expose antithrombogenic luminal surfaces due to several mechanisms, including modulation of platelet activity and the expression of tissue factor pathway inhibitor (TFPI) (see text). Perturbation of endothelial cell homeostasis by inflammation or injury may trigger the exposure of procoagulant subendothelial matrix, as well as procoagulant properties such as expression of tissue factor (TF), resulting in activation of the clotting cascade with resultant thrombin generation and activation of platelets (see text).

the circulation by inhibiting platelet aggregation, preventing the activation and propagation of coagulation, and enhancing fibrinolysis.[24] Conversely, when inflamed and injured or under conditions of disturbed flow, the endothelium may become thrombogenic.[35]

When near endothelial cells, platelets become unresponsive to agonists. This inhibition of platelet aggregation is accomplished by secretion of prostacyclin (prostaglandin I_2 [PGI_2]) and NO and by surface expression of an ecto-adenosine phosphatase (ADPase)/CD39/nucleoside triphosphate diphosphohydrolase (NTPDase-1). Prostacyclin is synthesized mainly by vascular endothelial and smooth muscle cells as a product of arachidonic acid metabolism. It inhibits platelet activation, secretion, and aggregation, as well as monocyte interaction with endothelial cells. It also causes vascular smooth muscle cell relaxation. NO also has a wide range of functions, including inhibition of platelet adhesion, activation, and aggregation. Most of the NO released from endothelial cells is elaborated abluminally, where it acts on the smooth muscle cell to cause vasodilation. However, some NO may enter the lumen and diffuse into platelets. Prostacyclin and NO can act synergistically to reverse platelet aggregation. The released platelet agonist, ADP, can be inactivated by endothelial membrane-associated CD39. Metabolism of ATP and ADP to adenosine monophosphate (AMP) by CD39 eliminates platelet recruitment and returns platelets to their resting state. Adenosine, which is generated by hydrolysis of AMP by ecto-5′-nucleotidase, inhibits platelet aggregation and promotes vasodilation. ATP/ADP can stimulate purinoreceptors on endothelial cells, resulting in the synthesis and release of PGI_2 and NO.[36]

Endothelial cells use three main pathways to inhibit thrombin generation and limit coagulation (see Chapters 121 and 125)[37]:

1. Antithrombin system: Heparan sulfate proteoglycans are secreted onto the luminal surface of endothelial cells and into the subendothelium. Heparan sulfates are capable of binding and activating antithrombin (AT), thereby accelerating inactivation of several procoagulant serine proteases, including thrombin, factor Xa, and factor IXa.
2. Protein C system: Thrombomodulin on the surface of endothelial cells binds thrombin. This coupling inhibits the coagulant properties of thrombin and increases its affinity for protein C, which it cleaves and activates. Activation of protein C by the thrombin–thrombomodulin complex is augmented by its binding to the endothelial cell protein C receptor (EPCR). Protein S, synthesized primarily by the endothelial cell, acts as a cofactor for activated protein C (APC). Independent of protein C, free protein S can inhibit the prothrombinase and intrinsic tenase complexes and interact directly with factors Va and VIIIa.
3. Tissue factor pathway inhibitor (TFPI): TFPI is a Kunitz-type serine protease inhibitor that modulates TF-initiated coagulation. TFPI binds to and directly inhibits the tissue factor (TF)–factor VIIa–factor Xa complex. It is mainly produced by and bound to endothelial cells, likely to surface glycosaminoglycans. There is also a plasma pool bound to low-density lipoprotein.

If coagulation occurs despite the many anticoagulant mechanisms, endothelial cells also provide proteins to promote fibrinolysis. Endothelium is a major source of tissue type plasminogen activator (t-PA). Approximately 40% of t-PA is bound to its inhibitor, PAI-1, which is also secreted by endothelial cells. Stresses such as exercise, acidosis, hypoxia, shear forces, increased venous pressure, and thrombin cause the release of t-PA, which converts plasminogen to plasmin, the key effector of fibrinolysis. Receptors for plasminogen and t-PA are present on the endothelial cell surface, enabling localized fibrinolytic activity.

Although intact endothelium is necessary to maintain blood in a fluid state and inhibit coagulation under normal conditions, injured endothelium can rapidly downregulate its anticoagulant functions and become procoagulant even without the overt vascular damage that occurs with trauma, surgery, or viral infection. Tissue injury or vascular pathology also leads to exposure of the underlying matrix, which is procoagulant by virtue of its binding to, and activation of platelets. Apoptotic endothelial cells become procoagulant through exposure of phosphatidylserine on their surfaces[38] and increased thrombin formation. These effects coupled with platelet adhesion to the injured endothelium contribute to thrombosis in diverse diseases.[37]

Even without endothelial death, perturbation of the vascular lining by inflammatory mediators converts the endothelium from a non-thrombogenic to a procoagulant surface because of downregulation of anticoagulant properties as well as induction of a procoagulant phenotype. Acute inflammation is associated with increased release of vWF, platelet-activating factor (PAF), and fibronectin, all of which may potentiate thrombus formation. Tumor necrosis factor (TNF), IL-1, and lipopolysaccharide increase endothelial cell expression of PAI-1 and decrease t-PA expression, thereby attenuating fibrinolysis, and downregulate thrombomodulin. Although they induce TF expression by cultured endothelial cells, evidence that they induce TF expression by endothelial cells in vivo is limited. *Instead*, circulating microparticles (also known as extracellular vesicles) generated by leukocytes, vascular or cancer cells have been shown to be a source of blood-borne TF and to contribute to coagulation. Although most microparticles are derived from platelets, RBCs, and monocytes, endothelial-derived microparticles may be an important source of circulating TF under conditions of massive activation.[39]

Endothelial Control of Vascular Tone

Vascular tone is orchestrated primarily by a balance between endothelium-derived vasodilators (NO, PGI_2, and various endothelium-derived hyperpolarizing factors [EDHFs]) and vasoconstrictors (endothelin-1 [ET-1], thromboxane [TXA_2] and superoxide).[40] In addition to inhibiting platelet aggregation, NO and PGI_2 serve as vasodilators. NO is produced by the conversion of L-arginine to L-citrulline by NO synthase (NOS). Three NOS genes exist, of which an inducible form (iNOS/NOS2) and a constitutively active endothelial form (eNOS/NOS-3) are expressed in endothelial cells. NOS2 may be responsible for the uncontrolled vasodilation seen in septic shock. NO release through NOS3 activation, mainly by unidirectional laminar flow-induced shear stress, is crucial for maintaining basal vasodilation. The action of NO on platelets (antiaggregatory), endothelial cells, and smooth muscle cells (relaxation) is caused by activation of guanylyl cyclase and formation of cyclic guanosine 3′,5′-cyclic monophosphate. Whereas NO is unstable, the formation of *S*-nitrosothiols in the presence of oxygen and thiols provides a stable reservoir of NO. Deoxygenation is accompanied by an allosteric change in *S*-nitrosohemoglobin that releases the NO group, thereby relaxing blood vessels and enabling sufficient blood flow to meet local oxygen requirements. Hemoglobin is a scavenger of NO, which may account for the vasoconstriction observed with the administration of cell-free, hemoglobin-based, RBC substitutes.

In addition to its antithrombotic role (above) endothelial PGI_2 acts locally to counterbalance the vasoconstriction induced by thromboxane A_2 (TXA_2). Whereas PGI_2 transduces a cellular signal by increasing the levels of cyclic AMP (cAMP), TXA_2 signals via the phosphoinositol pathway and lowers cAMP levels (see Chapter 123). The synthesis of prostaglandins is catalyzed by the action of cyclooxygenases (COX-1 and COX-2) on arachidonic acid. Aspirin irreversibly inhibits COX in both platelets and endothelial cells (see Chapters 123 and 124). However, its clinical effect is mainly exerted on platelets, which cannot synthesize new COX. Therefore, TXA_2 synthesis recovers only when new platelets enter the circulation, while endothelial COX restores PGI_2 levels within a few hours. In addition, platelets encounter aspirin before it is deacetylated by the liver and diluted by the venous circulation. The balance between the activity of PGI_2 and TXA_2 is important for vessel wall homeostasis, as evidenced by the fact that selective COX-2 inhibitors, which decrease the production of PGI_2 without affecting the production of TXA_2, increase the risk for cardiac events.

Endothelial cells release other relaxing factors (EDHFs), which act by increasing the membrane potential of smooth muscle cells. These factors comprise a variety of constitutive and inducible effectors including calcium-activated potassium channels, various cytochrome P450 and 15-lipoxygenase derived arachidonic acid metabolites, H_2O_2, and C-type natriuretic peptide.[41]

Endothelin 1 (ET-1) is a 21-amino acid peptide released preferentially at the abluminal surface of endothelial cells that exhibits potent vasoconstrictor activity. Of the three known ETs, only ET-1 is produced by endothelial cells. At least two receptors (ET-A and ET-B) bind all three ETs. Whereas ET-A is abundantly expressed on smooth muscle cells, ET-B is predominantly expressed on endothelial cells. The vasoconstrictor activity of ET-1 is preferentially mediated by ET-A G-protein–coupled receptors on smooth muscle cells. Engagement of the ET-B receptor on endothelial cells by ET-3 may paradoxically cause transient vasodilation. ET-1 may contribute to pregnancy-induced hypertension, reperfusion injury after ischemia, and pulmonary arterial hypertension. The dual ET receptor antagonist bosentan, and ET-A antagonist ambrisentan have been approved for treatment of the latter disease.

Another regulator of vascular tone is the superoxide anion. The source of this free radical may be the endothelium itself or inflammatory cells that have been recruited to sites of injury or inflammation. Interaction of superoxide radicals with NO produces peroxynitrite and reduces the concentration of NO. Peroxynitrite can oxidize low-density lipoprotein (LDL) and deleteriously modify other proteins, thereby causing endothelial dysfunction. Increased production of superoxide inhibits the synthesis of PGI_2 but not TXA_2.

The endothelium expresses angiotensin-converting enzyme (ACE) on its surface; this enzyme converts angiotensin I to angiotensin II, a potent vasoconstrictor. The interaction between ET, angiotensin II, and α-adrenergic agonists contributes to the pathogenesis of hypertension. An altered balance of the vasoactive substances described in this section has been proposed as a cause of endothelial dysfunction and the attendant vascular pathology observed in atherosclerosis, hypertension, and diabetes mellitus (see Chapter 142). Alteration of vascular function in these diseases may then perpetuate endothelial dysfunction and, consequently, worsen disease.[40]

INTERACTION OF BLOOD CELLS WITH THE VESSEL WALL

Leukocytes

In the absence of an inflammatory stimulus, neutrophils circulate freely and do not interact with the endothelium. This contrasts with continuous, low-level physiologic traffic of monocytes and lymphocytes across the vessel wall. Monocytes emigrate from the bloodstream to develop into tissue macrophages that may exhibit tissue- or organ-specific functions. To maintain immune surveillance of tissue, lymphocytes recirculate between blood and lymphatics, gaining entrance to the latter at the high endothelial venule of postcapillary venules in lymphoid tissue.[42]

Intravital microscopic studies have established a sequence of events involved in leukocyte emigration at extravascular sites of inflammation. Under conditions of flow, leukocytes first tether to, and then roll along, the endothelium of postcapillary venules adjacent to the site of inflammation. Some of the rolling leukocytes are activated and adhere firmly. The adherent leukocytes migrate along the endothelial surface

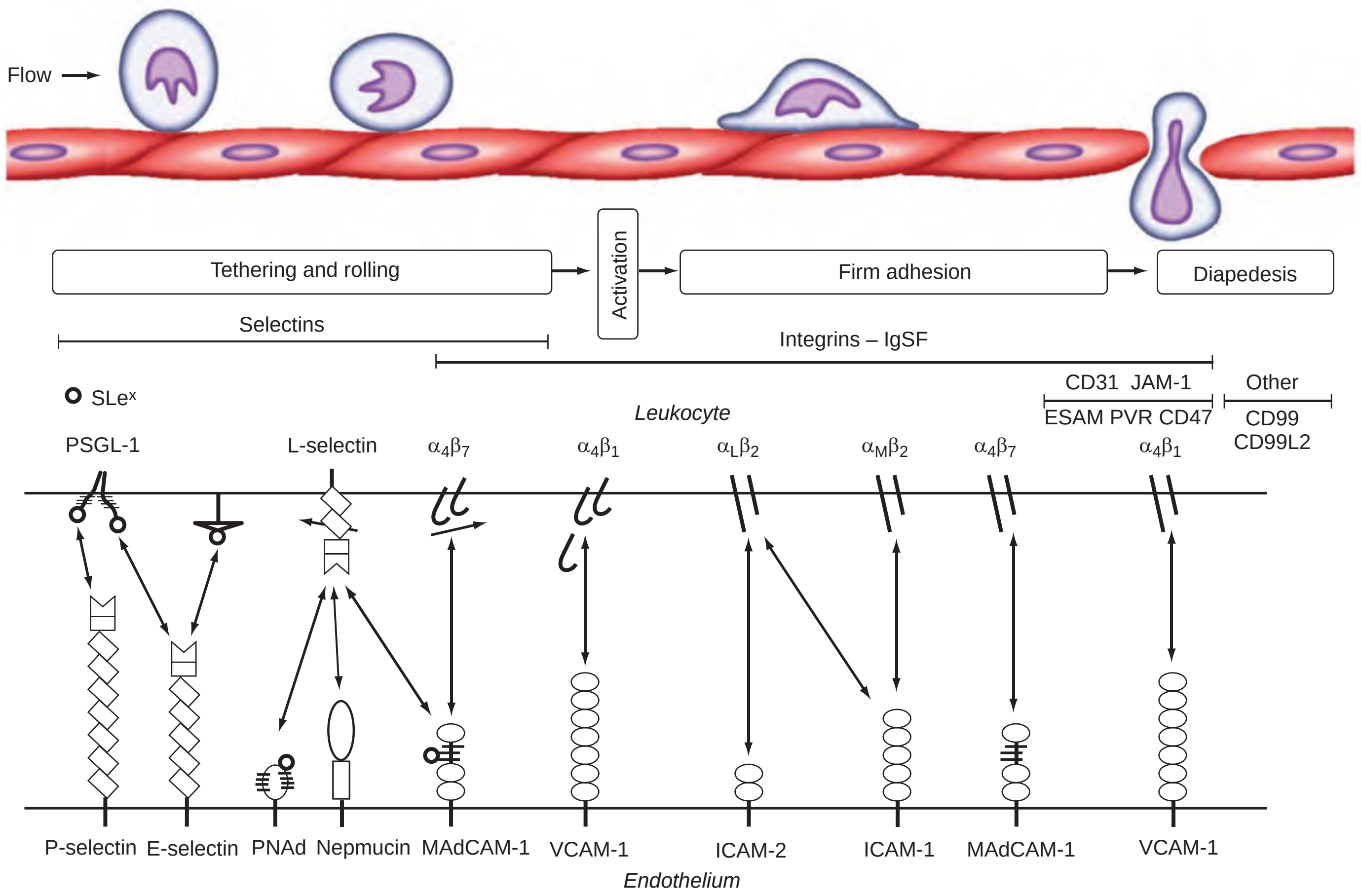


Figure 122.2 MOLECULAR MEDIATORS OF LEUKOCYTE-ENDOTHELIAL INTERACTIONS. The four-stage model of leukocyte-endothelial interactions (tethering, rolling, firm adhesion, and diapedesis) is predicated on diverse modes of adhesive interactions between the surfaces of the respective cells. These interactions are mediated by several classes of adhesion molecules including selectins, immunoglobulin family cell adhesion molecules and integrins (see text). *ESAM*, Endothelial cell-selective adhesion molecule; *ICAM-1*, intercellular adhesion molecule 1; *ICAM-2*, intercellular adhesion molecule 2; *JAM-1*, junctional adhesion molecule A; *MAdCAM-1*, mucosal addressin cell adhesion molecule 1; *PNAd*, N-terminal asparagine amidase; *PSGL-1*, P-selectin glycoprotein ligand 1; *PVR*, poliovirus receptor; *SLex*, sialyl-Lewis X antigen; *VCAM-1*, vascular cell adhesion molecule 1.

and diapedese between endothelial junctions to enter the extravascular tissue.[43] These steps in emigration (i.e., tethering, rolling, activation, firm adhesion, and diapedesis) are also involved in lymphocyte emigration at HEVs.[44] They result from the interaction between distinct leukocyte counterreceptors and endothelial receptors in an adhesion cascade (Fig. 122.2).[45,46] Rolling is observed only under flow conditions and is the consequence of shear forces acting on the leukocyte and adhesive interactions between selectin receptors and their glycoconjugate counter structures. It is initiated primarily by activation of the endothelium by extravascular stimuli such as bacterial-derived products or by endogenous mediators produced by the endothelium or cells in tissue. Early on, rolling is mediated by endothelial P-selectin, which is rapidly translocated from Weibel-Palade bodies to the luminal surface, and L-selectin on leukocyte microvilli. E-selectin is involved only at later time points because it is not constitutively expressed by endothelium but rather is induced over hours by de novo synthesis.

For leukocytes to circulate freely, their integrin receptors must be minimally adhesive, but they also must be able to rapidly increase binding at sites of inflammation. After tethering to endothelium by selectin interactions, leukocyte integrin receptors are activated by endothelial membrane-expressed PAF, endothelial membrane-bound chemokines, or locally secreted chemoattractants. Activation of leukocyte integrins involves changes in receptor affinity or affinity-independent receptor clustering, thereby promoting firm adhesion to endothelial ligands, which are members of the immunoglobulin gene superfamily (IgSF). These IgSF ligands are constitutively expressed (ICAM-1, ICAM-2), further upregulated (ICAM-1), or induced (VCAM-1) by inflammatory mediators. Subsequent leukocyte migration over the endothelium requires reversible adhesion caused by cyclic modulation of receptor avidity.

There are several caveats regarding the multistep model of initial selectin-mediated rolling and subsequent integrin-mediated firm adhesion. First, selectin-mediated rolling is not a prerequisite for emigration under conditions of reduced flow, as might occur at sites of inflammation. Second, the model was developed from observations in the systemic microcirculation where leukocyte emigration occurs in postcapillary venules under relatively low shear forces. However, selectins do not appear to play a major role in neutrophil emigration in the pulmonary microcirculation, where emigration occurs predominantly in capillaries, or in the liver microvasculature, where leukocytes emigrate primarily in sinusoids. Third, under some conditions, leukocytes can tether and roll via receptors other than selectins and α4 integrins (e.g., CD44 or VAP-1). Finally, several other adhesion pathways have been implicated in leukocyte adhesion to endothelium in vitro, and their roles in the adhesion cascade in vivo remain uncertain.[47]

While migrating on the endothelial surface, some leukocytes encounter an intercellular junction, diapedese between endothelial cells, enter extravascular tissue, and then migrate to the sites of inflammatory or immune reaction. This process depends on leukocyte integrin interactions with endothelial Ig superfamily ligands and on several junctional proteins, including PECAM-1 (CD31), JAM-1, CD99, CD99L2, ESAM, PVR, and CD47.[47–49] Diapedesis involves signaling by the leukocyte to the endothelial cell that triggers the opening of endothelial cell junctions. In some cases, leukocytes may emigrate directly through the body of an endothelial cell.[47]

Leukocyte recruitment is terminated by several mechanisms. Whereas E-selectin and P-selectin are removed from the endothelial cell surface by endocytosis, L-selectin is cleaved from leukocytes by a membrane protease. Decay of cytokine, chemokine, or chemoattractant generation leads to gradual resolution of endothelial adhesion molecule expression and integrin activation. Locally expressed mediators, such as NO, TGFβ, and Fas ligand, also inhibit further leukocyte adhesion to endothelium.[47]

The adhesion molecules involved in leukocyte trafficking from the bloodstream to tissue have emerged as important therapeutic targets. Extensive preclinical studies showed that blockade of leukocyte or endothelial adhesion molecules was efficacious in diverse disease models, prompting the development of adhesion antagonists. In spite of the seemingly sound biology, clinical development of these agents was largely unsuccessful, and progressive multifocal leukoencephalopathy, a rare viral infection of the central nervous sytem (CNS), was observed in patients treated with the α4-integrin antagonist natalizumab. However, approval of two integrin antagonists: efalizumab for treatment of psoriasis and natalizumab for treatment of multiple sclerosis[50] suggests a potential benefit for specific indications.

Platelets

Like neutrophils, non-activated platelets do not interact with unperturbed endothelium (see Chapter 124). After a vascular injury that produces endothelial denudation or retraction, platelets rapidly adhere to the exposed subendothelium. As discussed in greater detail in Chapters 123 and 124, at high shear rates, this initial adhesion does not require platelet activation and involves platelet glycoprotein Ib/V/IX (GPIb–V–IX) binding to vWF in the subendothelial matrix and platelet glycoprotein VI (GPVI) binding to collagen in the injured arterial wall. Platelet activation occurs after adhesion, leading to platelet spreading mediated by integrin receptor binding to matrix components and aggregation mediated by fibrinogen binding to activated GPIIb/IIIa (αIIbβ3).

In the context of inflammation, platelets are the first cells to adhere to the endothelium in postcapillary venules, where they recruit neutrophils and direct them through the endothelial barrier while maintaining vascular integrity. This process is mediated through cell-cell interactions and the release of chemokines.[51] Platelets can bind directly to activated endothelium in vivo via endothelial P-selectin and PECAM-1 and can roll on venular endothelium via interaction of platelet GPIbα with endothelial P-selectin. Platelets bind to HEVs in vivo via platelet P-selectin. In vitro, platelets adhere to intact endothelium via a platelet GPIIb/IIIa-dependent bridging mechanism involving platelet-bound adhesive proteins and the endothelial cell receptors ICAM-1, αVβ3 integrin, and GPIbα. Platelet adhesion to intact endothelium via these various pathways may contribute to thrombus formation in the circulation and may provide a link between thrombosis and inflammation in diseases such as atherosclerosis (see Chapter 142). Platelet-endothelial interactions also may contribute to the pathogenesis of thrombotic thrombocytopenic purpura (see Chapter 132). Normally, ultra-large vWF multimers remain attached to endothelium via P-selectin until they are cleaved by the plasma metalloproteinase ADAMTS-13. Failure of vWF multimer cleavage due to a deficiency of ADAMTS-13 may lead to spontaneous platelet adhesion to the endothelium and microvascular thrombosis in thrombotic thrombocytopenic purpura.[52]

Red Blood Cells

RBCs normally do not bind to endothelium unless damaged by sickle cell disease or infection with malaria parasites. The binding of sickle cells to postcapillary endothelium with secondary trapping of poorly deformable cells is thought to be an important pathogenic factor in vasoocclusive events (see Chapter 42).[53] Several interactions between sickle RBC receptors and endothelial ligands have been described, including α4β1/VCAM-1; α4β1/Lutheran blood group (basal cell adhesion molecule); and ICAM-4/αVβ3. The adhesive proteins thrombospondin (TSP-1) and vWF promote adhesion by serving as bridging factors between various sickle RBC and endothelial adhesion molecules. In addition to direct adhesion to endothelium, sickle RBC binding to adherent leukocytes appears to be an important mechanism for vascular occlusion in sickle cell disease.[54] Further, microparticles generated primarily from RBC, as well as free Hb, heme and iron released during hemolysis can activate endothelial cells and neutrophils to increase adhesion, potentially accelerating vasoocclusion.[55,56] Like leukocyte adhesion to endothelium in inflammatory and immune diseases, drugs targeting sickle RBC adhesive interactions with endothelial cells or leukocytes may prove useful in preventing or treating vaso-occlusive crises (see Chapter 43).[57,58]

Among human malarial parasites, only *Plasmodium falciparum* modifies the surface of the RBCs such that asexual parasites and gametocytes can adhere to the vascular endothelium. The binding of trophozoite- and schizont-infected RBCs to endothelium not only allows the parasite to escape destruction in the spleen but also may contribute to the pathogenesis of cerebral malaria. Multiple endothelial adhesion receptors have been implicated in mediating cytoadherence of infected RBCs, including P-selectin, ICAM-1, VCAM-1, and CD36.[53,59]

ENDOTHELIAL CELL ACTIVATION AND DYSFUNCTION

The endothelium is a dynamic and heterogeneous organ that responds to diverse stimuli, ranging from coagulation proteins and cytokines to hemodynamic forces and growth factors.[17] Activation of endothelial cells induces a complex proinflammatory and prothrombotic phenotype as well as expression of certain cytoprotective genes. Multiple transcription factors, particularly nuclear factor-κB (NFκB) and early growth response 1 (EGR-1), regulate these responses. Endothelial activation undoubtedly is an important event in host defense and repair, but it also may contribute to the pathogenesis of diverse disease states, ranging from sepsis to atherosclerosis.[60]

Endothelial dysfunction is characterized by a reduction in the bioavailability of vasodilators, particularly NO, leading to impairment of endothelium-dependent vasodilation, or by an increase in endothelium-derived contracting factors. Endothelial dysfunction is prominent in atherosclerosis but also has been described in diabetes, preeclampsia, hypertension, uremia, viral infections, and other diseases. In a broader sense, endothelial dysfunction encompasses proinflammatory and procoagulant changes, as well as apoptotic cell death.[61]

Several noninvasive approaches for assessing endothelial function have been developed. Endothelial vasodilatory responses can be evaluated by high-resolution ultrasound measurement of flow-mediated vasodilation or by using plethysmography to monitor changes in forearm blood flow during reactive hyperemia. Endothelial activation can be assessed in plasma by measuring the levels of biomarkers such as soluble endothelial adhesion molecules (e.g., sVCAM-1, sICAM-1, sE-selectin), endothelial coagulation proteins (e.g., vWF and thrombomodulin), or endothelial extracellular vesicles (EVs/MPs). The detection of circulating endothelial cells reflects significant vascular damage or cell death.[61]

A particularly aggressive form of endothelial activation and dysfunction has been noted with the emergence of zoonoses by the coronaviruses, SARS-CoV, MERS-CoV, and most virulently with SARS-CoV2, which causes COVID-19.[62,63] SARS-CoV-2 requires two receptors, ACE2 and the transmembrane protease TMPRSS-2, to bind and invade cells. Both receptors are expressed on the endothelium to varying degrees in different vascular beds. The endotheliopathy consequent to SARS-CoV-2 infection can result in vascular dysfunction, massive inflammation and coagulopathy.[64,65] Endothelial cells may be both an inducer and a target cell for coronavirus-induced cytokine release syndrome. The resulting impact on the integrity of the vasculature is clinically manifested in a subset of patients as stroke, thromboembolism, in situ thrombosis, or multiorgan failure. Immunothrombosis, which is the direct interaction of activated leukocytes with platelets and plasma coagulation factors, and endothelial apoptosis appear to contribute to the thrombotic events described in COVID-19. The nature of the underlying abnormality resembles a thrombotic microangiopathy, coupled with micro- and macrothrombosis and the induction of angiogenic responses in the affected vasculature.[64]

MOLECULAR REGULATORS OF VASCULAR GROWTH RESPONSES

Although vascular structures exhibit stability in adulthood, they are capable of anatomical changes through growth, remodeling, and regression. In this regard, the state of vascular networks is controlled by a web of intercellular communications, which are executed by soluble growth factors, adhesion molecules, ECM molecules, cell-cell contacts, the hemostatic system, various proteases, and the intercellular exchange of molecules (proteins, messenger RNA (mRNA), and microRNA).[2] A part of this circuitry entails the emission/uptake of EVs, including exosomes.[66] Of the involved mediators, some are essential for vascular growth and homeostasis, while others play more pleiotropic and context-dependent roles (see Table 122.1).[1,2,67]

Vascular Endothelial Growth Factors

Vascular endothelial growth factor-A (VEGF-A), which is also known as VEGF or vascular permeability factor (VPF), is indispensable for vascular development.[2,68,69] VEGF is the key member of a larger family of related polypeptides, including VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGFR-F, and placental growth factor (PlGF). Upon dimerization, these factors bind to their tyrosine kinase receptors (RTKs/VEGFRs), including VEGFR1/Flt-1, VEGFR2/KDR/Flk-1, and VEGFR3/Flt-4, often in conjunction with their neuropilin coreceptors (NRP1, NRP2),[70] as depicted in Fig. 122.3. For instance, VEGF-A interacts with VEGFR2, VEGFR1, and VEGFR3, whereas PlGF is selective for VEGFR1. The distribution of different VEGFRs on vascular (VECs) and lymphatic (LECs) endothelial cell subsets, as well as among endothelial progenitor cells (EPCs), hematopoietic, myeloid, and certain tumor cells, defines the known biologic activities of the various VEGF ligands. The effects of VEGF include stimulation of endothelial mitogenesis, migration, survival, morphogenesis, and vascular permeability (e.g., through formation of intercellular gaps, vesiculo-vacuolar organelles or transcellular structures know as *fenestrae*).[68] The signaling activity of VEGFR2 is crucial for these processes, whereas VEGFR1 is often expressed as a soluble splice variant (sFlt-1) that neutralizes VEGF (serves as a VEGF “sink”), thereby inhibiting angiogenesis.[2]

VEGF activity is also regulated by splicing of the corresponding mRNA, resulting in the generation of several protein isoforms, including VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206 (designations based on the number of amino acids).[2] Alternative splicing leads to the formation of a series of proangiogenic (VEGF-A_{xxx}a) and antiangiogenic (VEGF-A_{xxx}b) VEGF isoforms, the former being biologically predominant.[71] VEGF-A isoforms differ in their cell association, solubility, and ability to bind heparinoids, or to interact with neuropilins, all of which define the formation of extracellular gradients and related biologic responses to these factors. In this regard, VEGF165 is an especially potent inducer of angiogenesis (see Fig. 122.3).

VEGF-C and VEGF-D stimulate the growth of lymphatics (lymphangiogenesis) via activation of VEGFR3, while VEGF-B and PlGF interact with VEGFR1 and are involved in vascular pathologies and inflammation.[2]

Platelet-Derived Growth Factors

This family of VEGF-related growth factors consists of four members: PDGF-A, PDGF-B, PDGF-C, and PDGF-D, the homo- or heterodimers of which interact preferentially with one of the three known cellular RTKs, namely, PDGFRα, PDGFRβ, and PDGFRγ, each endowed with different cellular functions. For example, PDGF-BB is expressed by endothelial cells and mediates their capacity to attract mural cells harboring PDGFRβ.[72]

Prokineticins

This group of factors consists of the endocrine gland vascular endothelial growth factor/prokineticin 1 (EG-VEGF/PK1) and prokineticin 2 (Bv8/PK2) secreted by *Bombina variegata*, both of which interact with their respective G-protein-coupled receptors on endothelial cells (PK-R1 and PK-R2). These mediators induce VEGF-like effects in endothelial cells and may render tumors resistant to VEGF inhibition.[73]

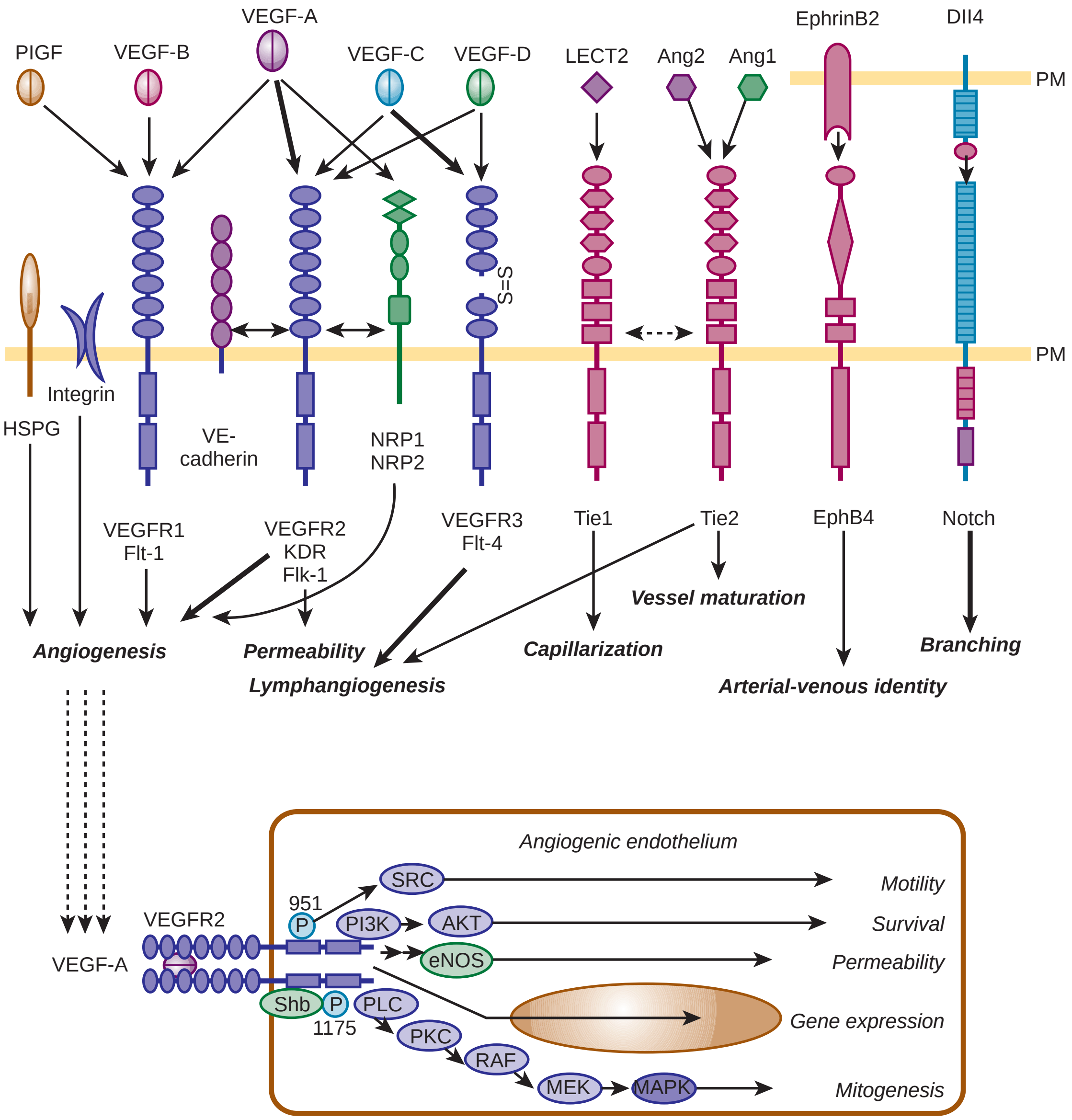

VEGF-A
PlGF
VEGF-B
VEGF-C
VEGF-D
LECT2
Ang2
Ang1
EphrinB2
Dll4
PM
S=S
PM
Integrin
HSPG
VE-
cadherin
NRP1
NRP2
VEGFR1
Flt-1
VEGFR2
KDR
Flk-1
VEGFR3
Flt-4
Tie1
Tie2
EphB4
Notch
Vessel maturation
Angiogenesis
Permeability
Capillarization
Branching
Lymphangiogenesis
Arterial-venous identity
Angiogenic endothelium
SRC
Motility
951
VEGFR2
P
PI3K
AKT
Survival
eNOS
Permeability
VEGF-A
Shb
P
PLC
Gene expression
1175
PKC
RAF
MEK
MAPK
Mitogenesis

Figure 122.3 ELEMENTS OF THE SIGNALING CIRCUITRY INVOLVED IN BLOOD VESSEL FORMATION AND ANGIOGENESIS. Receptors and co-receptors involved in angiogenic, lymphangiogenic, and regulatory signaling. *Bottom panel:* Outline of signaling pathways and their effector mechanisms downstream of VEGF-A/VEGF. *Dll4*, Delta-like 4; *eNOS*, endothelial nitric oxide synthase; *HSPG*, heparan sulfate proteoglycan; *MAPK*, mitogen-activated protein kinase; *NRP*, neuropilin; *PI3K*, phosphatidylinositol 3-kinase; *PLC*, phospholipase C; *PlGF*, placental growth factor; *PKC*, protein kinase C; *PM*, plasma membrane; *VE-cadherin*, vascular endothelial cadherin; *VEGF*, vascular endothelial growth factor; *SRC*, Rous sarcoma virus-related proto-oncogene.

Angiopoietins and TIE Receptors

Angiopoietins (ANGs) are encoded by three different genes (ANGPT1, 2, and 4), and products of which interact with the tyrosine kinase with immunoglobulin-like loops and epidermal growth factor homology domains-2 (TIE2 or TEK), a receptor tyrosine kinase (RTK), which is preferentially expressed by endothelial cells and some myeloid cells (see Fig. 122.3). They are members of a larger family including mouse ortholog of ANGPT4 (ANGPT3) and seven angiopoietin like proteins (ANGPTL), many with still poorly defined functions.[74] A receptor related to TIE2 and known as TIE1, interacts with leukocyte cell-derived chemotaxin 2 (LECT2) during the process of sinusoid capillarization[75,76] and acts by modulating TIE2 activity. Among TIE2 ligands, angiopoietin 1 (ANG1) emanates from perivascular cells and serves as the main receptor agonist to stabilize endothelial–mural cell interactions and to promote endothelial cell survival, vascular quiescence, and the nonpermeable state. ANG2, which is produced by VEGF-stimulated endothelium, exerts the opposite effect and stimulates pericyte detachment, permeability, vascular growth, or regression, as well as lymphangiogenesis.[2]

Notch Pathway

Delta-like (Dll1, 3, and 4) and Jagged (1 and 2) are membrane-bound ligands that activate Notch receptors (Notch 1–4) on adjacent cells. During vascular development and growth, Dll4 and Jagged 1 are expressed by subsets of endothelial and mural cells, respectively, and regulate their distinct functions within the capillary outgrowths (sprouts), as depicted in Figs. 122.3 and 122.4. Dll1 is also involved in circumferential vascular enlargement (arteriogenesis).[77]

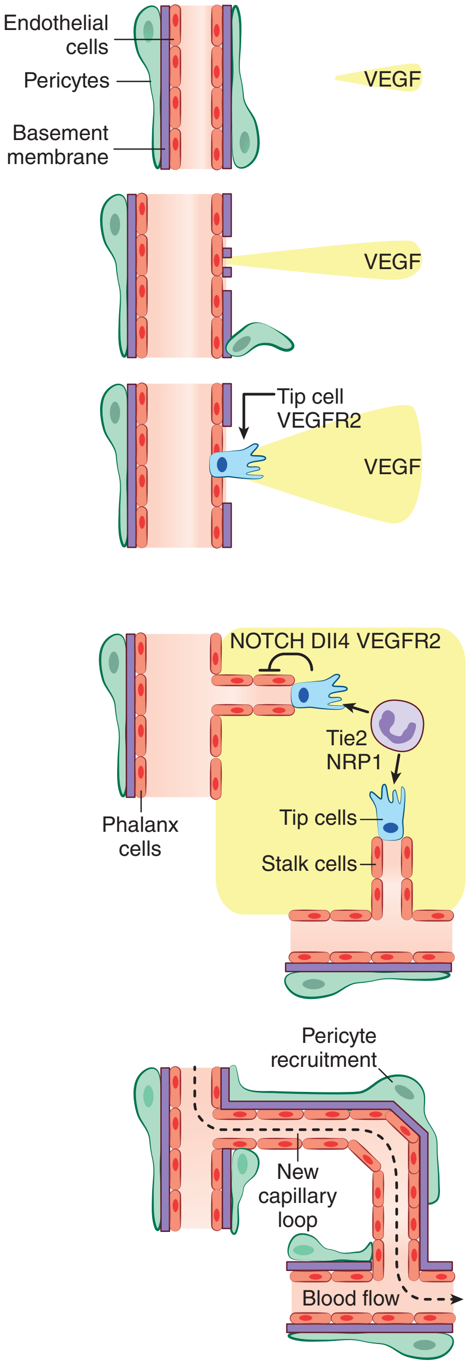
Ephrins and Eph Receptors

The arterial or venous identity of endothelial cells in the evolving microcirculation is preprogrammed by the expression of transmembrane guidance molecules, especially ephrin B2 and its EPHB4 receptor. Bidirectional signals emanating from these molecules, together with mechanosensory cues, govern the hierarchical arteriovenous vascular branching (*arborization*) that is essential for, and maintained by, proper blood flow. Other ephrins are implicated in endothelial–pericyte interactions and communication between blood vessels and tumor cells. They may also cooperate in VEGFR2 endocytosis and signaling.[2]

WNT and Guidance Factors

In addition to ephrins, several families of guidance molecules involved in the central nervous system also play important roles in vascular homeostasis and angiogenesis. Some of the crucial ligand-receptor pairs implicated in such interactions include WNT/Frizzled, Neuropilin (NRP)/Semaphorin, Slit/Robo, Sonic hedgehog (SSH)/Patched/Smoothened, and other pathways.[78,79] Some of the crucial effects of these interactions are exemplified by the role of WNT signaling in vascular permeability in brain tumors.[80]

Like the nervous system, the vasculature forms a highly ordered, branching network. The ordering of this patterned network is dependent on multiple attractive and repulsive cues, many of which are common to both the nervous and vascular systems.[81] As outlined in the following sections, the formation of new capillaries through the process of angiogenic sprouting involves the emergence of three distinct endothelial cell populations: the guiding cells (tip cells), following cells (stalk cells), and remaining cells (phalanx cells; see Fig. 122.4).[2]



Angiogenic gradient formation (“switch”)
Hypoxia, inflammation, oncogenic transformation
Increased expression of stimulators (VEGF)
Decreased expression of inhibitors

Endothelial (phalanx) cell stimulation
Basement membrane dissolution
Pericyte "drop out"
Circumferential extension (mother vessels)

Formation of endothelial tip cells
VEGF gradient sensing
Expression of tip cell markers
Metabolic adaptation (glycolysis)

Formation of endothelial sprouts
Directional migration of tip and stalk cells
Blockade of VEGFR2 expression on stalk cells
by tip cells via the Dll4/Notch pathway
Tip guidance (semaphorins, neuropilins, plexins, Robo4)

Extension of sprouts
Growth and migration of stalk cells and lumen formation

Myeloid cell–dependent anastomosis
Signalling by Tie2, Ang2, PlGF, PHD2, SDF-1a

Formation of new vascular loops
Connection and anastomosis of sprouts

Vascular maturation
Pericyte recruitment/vessel maturation (PDGFRb, Ang1)
Restoration of basement membrane (TIMPs, PAI-1)
Endothelial quiescence–phalanx cells (Notch)
Junction formation (VE-cadherin)
Anticoagulant surfaces

Blood flow
Resolution of hypoxia

Figure 122.4 SPROUTING ANGIOGENESIS. The change in the balance between angiogenesis stimulators and inhibitors (angiogenic switch) and especially the gradient of VEGF leads to sprouting angiogenesis. This process represents the core cellular program in formation of new capillary blood vessels from preexisting ones and begins with changes in the vessel wall (endothelial phalanx cells). The resulting formation of enlarged mother vessels, endothelial tip cells, proliferating stalk cells, capillary loops, and eventually anastomoses are depicted (details in the text[2]). *A*, Angiopoietin; *Dll4*, delta-like 4; *PAI-1*, plasminogen inhibitor; *TIMP*, tissue inhibitor of MMP; *VEGF*, vascular endothelial growth factor; *VEGFR*, vascular endothelial growth factor receptor.

Tip cells represent an important paradigm that illustrates the endothelial guidance apparatus. These cells express a distinctive profile of genes with substantially higher levels compared with stalk cells of platelet-derived growth factor (PDGF)-B, VEGFR-2, uncoordinated (Unc)5b, Delta-like (Dll)4, and VEGFR-3.[82] Whereas VEGF165 acts as an attractive cue to the tip cell of the endothelial sprout, Netrin-1 signals to Unc5b on the vasculature acting as a repulsive cue. Netrin-4 can also bind Neogenin, which in turn recruits and activates Unc5b to mediate repulsion. Interestingly, patterning and specification of small arteries along peripheral nerves in the skin of the embryonic limb involve nerve-derived VEGF; in other situations, neuronal patterning is dependent on the vasculature.[81,83] Thus, the congruent patterning of the neural and vascular systems likely is caused by the use of common signals and may require a cross-talk between the two systems.

Transforming Growth Factor β

Members of the transforming growth factor-β (TGFβ) family are multifunctional homodimeric peptides with diverse effects on cell proliferation, migration, differentiation, adhesion, and expression of cell adhesion molecules and ECM.[84] They are secreted as inactive precursors. After activation, they transmit signals to cells by binding to heteromeric complexes of type I and type II serine/threonine kinase receptors. In most cell types, the type I receptor engaged by TGFβ is the activin receptor-like kinase 5 (ALK5). However, in endothelial cells, TGFβ can bind and signal through ALK5 and ALK1. Contact between endothelial cells and periendothelial cells is required for the production of active TGFβ. Mice lacking TGFβ or TGFβ receptor type II exhibit similar defects in vasculogenesis and hematopoiesis. Endothelial proliferation is not affected, but poor contacts between endothelial and other cell layers in embryos of $TGF\beta^{-/-}$ mice result in a disorganized and reduced vascular network lacking capillary tubes. Mutations in two TGFβ receptors, ALK1 and the accessory TGFβ receptor endoglin, have been linked to the vascular disorder hereditary hemorrhagic telangiectasia. Disruption of TGFβ signaling likely plays a role in the telangiectasia seen in this disorder.[84]

Extracellular Matrix, Vascular Integrins, Cadherins, Cell Adhesion Molecules

Quiescent endothelial cells are anchored to the basement membrane, a structured layer of ECM composed mainly of laminin and collagen type IV. In contrast, growing (angiogenic) endothelial cells are surrounded by provisional ECM containing fibrin, vitronectin, fibronectin, and partially degraded collagens. Growth factors upregulate the expression of dimeric integrin receptors (αvβ3, αvβ5, α1β1, α2β1, α4β1, α5β1), which recognize specific motifs in ECM molecules (often the RGD sequence). Angiogenic growth factor receptors require integrin interactions for their signaling function.[2,85] The various functions of VE-cadherin/CD144, claudins (e.g., endothelial claudin 5) and connexins have been described earlier along with their role in endothelial cell interactions and adhesive communication with other cells.[5,86]

Structural and Functional Roles of the ECM During Vascular Growth Processes

Whereas interstitial collagens (e.g., collagen I) and provisional plasma-derived fibronectin–fibrin matrices stimulate endothelial tubular morphogenic events, laminin-rich matrices lead to endothelial differentiation and stabilization events.[87,88] Mice deficient in fibronectin die during embryogenesis and exhibit vascular defects. Type I collagen-deficient mice die of circulatory failure just before birth. Although most tumor vessels are covered by basement membrane, multiple structural abnormalities are noted in tumors consistent with ongoing vascular activation.[89] ECM proteins or their proteolytic fragments have been shown to inhibit angiogenesis.[1]

Adhesion Molecules Involved in Vascular Growth

Although it is universally accepted that integrins and integrin ligands function in angiogenesis, their exact actions remain unclear or debatable. In particular, substantial controversy surrounds the role of αvβ3 integrin.[90] Immunohistochemical studies localize this integrin to the tips of sprouting vessels. Neutralizing antibodies abrogate angiogenesis and induce vascular cell apoptosis in vivo, and inhibitory peptides or peptidomimetics blocking adhesive functions of integrin αvβ3 inhibit angiogenesis in a variety of animal models.[90] However, mice lacking αv show extensive angiogenesis, while both mice and humans (Glanzmann thrombasthenia) lacking β3 integrin also show normal angiogenesis.[91]

One of the integrin receptors for fibronectin, α5β1, has been shown to be necessary for vascular development, and α2β1 seems important for the formation of tubes by endothelial cells in vitro. However, there likely is a dynamic regulation of β1 integrins during angiogenesis because constitutive activation of this integrin inhibits endothelial sprouting in vitro and angiogenesis in vivo.[92] The junctional proteins VE-cadherin and PECAM-1, and possibly JAM-1, are expressed early in development and have a role in assembling the vasculature.[2]

Proteases

Proteases regulate the remodeling, growth, and invasion of new blood vessels. Some of these effects include the liberation of endothelial cells from their basement membrane confines, release of ECM-bound angiogenic growth factors, generation of regulatory peptides (e.g., angiogenesis inhibitors), clotting and fibrinolysis, intracellular signaling by protease-activated receptors, and numerous other steps involved in vascular maintenance and remodeling. For example, MMPs and their tissue inhibitors (TIMPs 1–4) participate in the controlled ECM/basement membrane breakdown that is required for invasiveness of the angiogenic endothelium.[93] The key enzymes in this group include: MMP-1, MMP-2, MMP-9, and MMP-14, coagulation factors (factor VIIa and thrombin) and their receptors (TF and PAR-1), plasminogen activators (u-PA) and their inhibitors (PAI-1), members of the disintegrin and metalloproteinase domain, and thrombospondin motif-containing families (ADAM and ADAMTS).[93,94]

Some of these effects are exemplified by the dissolution of subendothelial ECM by MMPs and heparanases, thus allowing endothelial cells to migrate at the initiation step of angiogenesis.[94,95] Matrix-bound growth factors are also released after ECM degradation. The balance between positive and negative regulators is the basis of tight control in this process. Tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA), by generating plasmin, can activate collagenases and other MMPs. Plasminogen activator inhibitors (PAIs) may block angiogenesis at this step. The MMPs are required for angiogenesis, and the tissue inhibitors of MMPs regulate their function.[96]

PLEIOTROPIC REGULATORS OF VASCULAR GROWTH

In addition to "professional" regulators of blood vessel formation and growth processes, several molecules with more pleiotropic biologic activity serve as stimulators or inhibitors of structural changes in the vasculature, either directly or indirectly (e.g., as inducers of VEGF). Examples of these diverse effectors are listed in Table 122.1 and include certain growth factors (FGF1, FGF2, HGF, TGFβ), chemokines (IL-8/CXCL8, SDF-1/CXCL12), secreted ECM proteins (TSP1, TSP2), proteolytic ECM fragments (tumstatin, angiostatin), phospholipids (sphingosine 1 phosphate [S1P]), and several other molecules.[1] The effects of these regulators on angiogenesis and other vascular growth responses are mediated by intracellular signaling pathways, including GTPases (Ras), kinases (src, Akt, PKC), transcription factors (ERG, HIF, MYC), microRNA species (miR17–92, miR155), and other effectors (see Fig. 122.3).[2] Among several classes of growth factors, polypeptides and lipids that exert vascular growth stimulating activities of special interest are factors central to wound healing programs, in which vascular growth plays a key role. Those activities are exemplified by

TABLE 122.1 **Molecular Regulators of Vascular Growth and Remodeling**

Regulator	Main Receptor(s)	Biologic Activity
Angiogenic Effectors Central to Endothelial and Mural Cell Function		
VEGF-A/VEGF	VEGFR2 (VEGFR3, VEGF1), NRP1	Stimulator of angiogenic functions, migration and survival of ECs, including the formation of tip cells
VEGF-C	VEGFR3 (VEGFR2)	Stimulator of angiogenesis (ECs) and lymphangiogenesis (LECs)
Ang 1	Tie2	Positive regulator of endothelial-mural interactions, EC survival, and vessel maturation
Ang 2	Tie2	Negative regulation of endothelial-mural interactions, stimulator of lymphangiogenesis
Dll4	Notch	Inhibitor of tip cell formation
Jag1	Notch	Stimulator of tip cell formation
EphrinB2	EPHB4	VEGFR internalization/signaling, arterial identity, tube formation
PDGF-B	PDGFRβ	Recruitment of mural cells, vessel maturation
TGFβ1	TGFβRII	Differentiation of mural cells, ECM formation
Integrins (αv, β1, β5)	ECM proteins	EC survival, migration morphogenesis
Stimulators Involved in Pathologic Angiogenesis		
PlGF	VEGFR1	Stimulates angiogenesis by interaction with ECs and BMDCs
Acidic FGF (FGF-1)	FGFRs 1–4	Stimulator of EC mitogenesis, survival, and angiogenesis
Basic FGF (FGF-2)	FGFRs 1–4	Stimulator of EC mitogenesis, survival, and angiogenesis
FGF-3	FGFRs 1–4	Stimulator of EC mitogenesis, survival, and angiogenesis
FGF-4	FGFRs 1–4	Stimulator of EC mitogenesis, survival, and angiogenesis
IL-8	CXCR1	Stimulator of ECs and inflammatory cells
IL-6	IL-6R	Stimulator of inflammatory angiogenesis
TNFα	TNFR1 (p55)	EC stimulator and VEGF inducer
Bv8	GPCR	Stimulator of endocrine and tumor ECs
PD-ECGF/TP	Unclear	Stimulator of angiogenesis
Angiogenin	170-kDa receptor	Stimulator of angiogenesis and tRNAse
MMP9	ECM proteins	Matrix metalloproteinase that breaks down the ECM and releases angiogenic growth factors

Endogenous Angiogenesis Inhibitors

Inhibitor	Biologic Activity
TSP-1	Interacts with the CD36 receptor, integrins, and other proteins, causing growth inhibition and apoptosis of angiogenic ECs
Endostatin	Proteolytic fragment of collagen XVIII with antiangiogenic activity
Angiostatin	Proteolytic fragment of plasminogen with antiangiogenic activity
Tumstatin	Proteolytic fragment of collagen IV alpha 3 chain
sFlt-1/sVEGFR1	Soluble splice variant of VEGFR1 neutralizing VEGF and blocking VEGFR2 signaling
VEGF165b	Splice variants of VEGF with antiangiogenic activity
PEX	Inhibitor of EC invasion and MMP activity
IFNα (β)	Inhibits release of angiogenic growth factors

Ang (1,2), Angiopoietin; *Bv8*, *Bombina variegata* protein; *Dll4*, delta-like 4; *FGF*, fibroblast growth factor; *GPCR*, G-protein–coupled receptor; *IFN*, interferon; *IL-6*, interleukin 6; *IL-8*, interleukin 8; *Jag1*, Jagged 1; *MMP9*, matrix metalloproteinase 9; *PD-ECGF/TP*, platelet-derived endothelial cell growth factor/thymidine phosphorylase; *PDGF*, platelet-derived growth factor; *PlGF*, placenta growth factor; *TNFα*, tumor necrosis factor alpha; *TSP-1*, thrombospondin 1; *VEGF*, vascular endothelial growth factor. See text and references for details.

FGFs, inflammatory cytokines, coagulation factors, and EVs, all of which emerged as increasingly well-defined and often actionable elements in the circuitry driving vascular growth and pathology.[2,66,86]

Fibroblast Growth Factors

The FGF family of growth factors encompasses 22 members and 3 subfamilies (FGF1/2/5, FGF3/4/6, FGF7/10/22, FGF8/17/18, and FGF9/16/20) interacting with three receptor tyrosine kinases (FGFR1–3) and heparin sulfate proteoglycan (HSPG) co-receptors.[97] While the initial focus of studies was on direct endothelial stimulating activity of FGF1/2, multiple cell types respond to FGFs and their vascular roles may be both direct and indirect. Evidence suggests that FGF receptors signal an inductive pathway by upregulating VEGFR-2 in differentiating mesoderm before vascular morphogenesis. FGF2 may also induce neovascularization in adults directly or through activation of the VEGF–VEGFR pathway. Consequently, FGFs represent viable targets for vascular growth stimulation and inhibition, the latter including cancer.[97]

Inflammatory Mediators

Inflammatory and immune system responses not only impact the state of endothelial cells (above) but also vascular growth processes. For example, multiple cytokines produced in the tumor microenvironment stimulate (IL-4, IL-6, IL-17), or mitigate (IFNγ) various aspects of angiogenesis. These processes are also influenced by proangiogenic chemokines (IL-8/CXCL8, SDF-1/CXCL12), angiostatic chemokines (CXCL9, CXCL10, CXCL11), and other products of infiltrating or resident cells, as extensively reviewed in the recent literature.[86]

Coagulation Factors

Blood clotting and blood vessel growth processes are intimately interrelated through shared components of the hemostatic machinery, such as platelets, proteins of the coagulation system, and fibrinolysis. TF represents a multifunctional paradigm that illustrates integration between blood clotting, intracellular signaling, inflammation, and healing (see Chapter 125).[3,98]

TF/F3/CD142 is a member of the cytokine receptor superfamily and a single-pass transmembrane receptor for coagulation factor VII/VIIa (FVII/FVIIa). In addition to its cofactor role, TF complexes with other clotting factors and interacts with G-protein–coupled protease-activated receptors (PAR-1 and PAR-2), thereby triggering intracellular signals and alterations in gene expression.[99] Among the affected genes are proangiogenic chemokines (IL-8/CXCL8, CXCL1) and cytokines (VEGF).[100,101] TF knock-out mice exhibit abnormalities of their large vessels and microvasculature secondary to defects in mesenchymal and peri-endothelial cell function.[102] Elevated TF expression in various tumors and the associated angiogenic responses have been reported.[103] Expression of mutant oncogenes (K-*ras*, *EGFR*) or lost tumor suppressor genes (*PTEN*, *p53*) lead to increased TF expression and activity, and this may link tumor angiogenesis and the hypercoagulable states manifested in cancer.[103]

PAR receptors (PAR-1–4) and their agonists have also been implicated in blood vessel formation. Abnormal development of the vasculature occurs in 50% of mice that are deficient in FV. The affected mice die in utero, and the 50% embryonic lethality is similar to that observed in thrombin receptor (PAR-1)-deficient mice that die without obvious coagulation defects.[104] Thrombin can promote angiogenesis through a mechanism that is independent of fibrin formation. For example, thrombin can stimulate the release of angiogenic factors (e.g., VEGF-A) from tumor cells and platelets, as well as induce VEGFR-2 on endothelial cells.[105] In addition, mechanisms intersecting with thrombin activation and function, such as APC and its endothelial receptor (EPCR) have been shown to contribute not only to the regulation of hemostasis, but also to inflammation, cell proliferation, apoptosis, tumor progression, and angiogenesis.[99] APC increases proliferation of vascular endothelial cells and angiogenesis by activation of mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and endothelial nitric oxide synthase (eNOS) pathways.[106]

Although clinical trials have suggested that treatment with low-molecular-weight heparins (LMWH) may improve the survival of cancer patients, these results require validation.[107] Many angiogenic growth factors (FGF, VEGF) contain heparin-binding motifs and heparanase-generated fragments of unfractionated heparin have anti-angiogenic effects in vitro. However, the mechanism responsible for the antitumor and antiangiogenic effects of these complex molecules needs to be defined.[108]

The potential involvement of fibrinolytic factors in angiogenesis has also been explored.[109] Plasminogen conversion to plasmin is regulated by several molecular mechanisms including tissue type (tPA) and urokinase (uPA) plasminogen activators and their inhibitors (PAI-1, -2, -3 and TAFIa). Pericellular proteolysis by plasmin changes the tissue microenvironment and promotes the invasion of cancer and endothelial cells, while cellular receptors for plasminogen and uPA (uPAR) interact with multiple signaling targets such as G-proteins and integrins, which may evoke cellular responses that can impact on blood vessel growth.[110]

Elements of the hemostatic system have also been implicated in such non-canonical processes as immunoregulation and cellular stemness. Examples of these linkages include changes in the expression of TF in stem versus non-stem brain tumor cell populations,[111] dependence of glioblastoma cell engraftment on PAR-1,[112] or elevated expression of platelet-activating protein, podoplanin (PDPN), in certain (albeit not all) tumor stem cell populations.[113] Strikingly, recruitment and retention of hematopoietic stem cells expressing EPCR have also been linked to PAR-1 signaling, further highlighting the emerging link between clotting factors, endothelial regulators, and cell fate.[114]

Extracellular Vesicles

Endothelial cells and other elements of the vessel wall, platelets, blood cells, and the various cells of the tissue microenvironment communicate not only through soluble factors but also by the exchange of complex (multimolecular) messages encapsulated in EVs. EV is an umbrella term comprising a spectrum of membrane-bound structures shed from cells through several mechanisms. For example, larger EVs (100 to 1000 nm in diameter) may directly bud from the plasma membrane as structures referred to as *microvesicles* (ectosomes or microparticles). In contrast, smaller EVs (30 to 150 nm in size), termed *exosomes*, originate within the cell, as secondary vesicles that are initially shed into the lumen of the late endosome-derived multivesicular body (MVBs). These small intraluminal vesicles (ILVs) are subsequently released from cells when MVBs contact the inner surface of the plasma membrane.[115] Other subsets of EVs include apoptotic bodies, large oncosomes, exosome-like membrane vesicles and several other structures generated through processes of membrane budding, membrane deformation associated with cell movement, cell fragmentation, and other processes. Different EV subsets carry distinct repertoires of bioactive and diagnostically important molecules including lipids, metabolites, proteins, nucleic acids (RNA, DNA), as well as, in some cases, also organelles, such as mitochondria.[115]

EVs protect and selectively transfer their molecular content between various “donor” and “recipient” cell populations within and outside of the vascular compartment. Platelets and RBCs are major sources of circulating EVs.[116] Other cellular subsets also contribute to the EV pool in plasma including endothelial, inflammatory and cancer cells. However, endothelial and myeloid cells are natural recipients of EVs shed to the circulation.[117] EVs (microparticles) play aforementioned important roles in hemostasis and thrombosis, in part as a source of phospholipids and as a mechanism transferring TF between cellular populations.[118]

Moreover, EVs may carry angiogenic growth factors (VEGF, FGF), inflammatory cytokines (IL-1), juxtacrine factors (ephrins, Dll4), enzymes (e.g., MMPs), extracellular matrix metalloproteinase inducer (EMMPRIN), and other bioactive cargo, including mRNA, microRNA, and DNA. Cancer-derived EVs contain active oncoproteins and oncogenic nucleic acids that may interact with and reprogram endothelial cells, stimulate their growth, trigger vascular permeability, and regulate angiogenesis.[66] Moreover, EVs are known to molecularly influence (educate) myeloid and stromal cells and platelets, thereby exerting wide-spread influences, including multiple facets of vascular growth processes.[66]

ENDOGENOUS INHIBITORS OF VASCULAR GROWTH

Multiple factors have been reported to negatively regulate vascular growth and morphogenesis.[1] Of interest is a class of endogenous angiogenesis inhibitors that originate as fragments of larger proteins that have little or no angiogenesis-related activity in their intact form. These include fragments of ECM proteins, such as fibronectin, collagen type IV α3 chain (tumstatin), and collagen type XIII (endostatin), as well as coagulation protein fragments such as cleaved plasminogen (angiostatin) and AT.[119] The mechanism of action of these molecules is poorly understood and depends on the properties of specific polypeptides, but the functional effect usually involves inhibition of endothelial proliferation or induction of apoptosis.

Other endogenous inhibitors include interferons, chemokines, interleukin-12 (IL-12), thrombospondin 1 (TSP-1), pigment epithelium-derived factor (PEDF), alternatively spliced isoforms of VEGF-A (VEGF-A_{xxx}b), soluble splice variant of VEGFR-1 (sVEGFR-1/sFLT-1) and several other entities.[2]

CELLULAR AND MOLECULAR PROGRAMS INVOLVED IN BLOOD VESSEL GROWTH AND HOMEOSTASIS

Angiogenesis stimulation and inhibition are but a small part of a complex vascular growth and remodeling machinery required for the formation, maintenance, and structural features of blood vessel networks as they respond to external and internal queues from embryogenesis to adulthood.

Prenatal Formation of the Vascular System

The human embryo develops the vascular system by the third week of gestation as a prerequisite for other organ systems and when its nutritional needs are no longer met by diffusion.[120] Vascular development proceeds in several phases. *Vasculogenesis* is the process whereby blood vessels form de novo through differentiation and self-assembly into a tubular structure of a subset of mesodermal precursors (hemangioblasts). The primary capillary plexus undergoes subsequent enlargement through *angiogenesis*, which as mentioned earlier, is the outgrowth of new capillaries from preexisting vascular channels, mainly due to physiological hypoxia. The resulting dense, but directionless initial vascular network undergoes pruning whereby some vessels regress, while others undergo enlargement, and become arteries or veins as a function of their position relative to the developing heart.[120,121] These processes involve vessel maturation whereby capillaries attract mural cell (pericyte) coverage and undergo remodeling through circumferential growth and vessel wall thickening. A similar process (arteriogenesis) is also operative during postnatal enlargement of preexisting collateral arterioles after occlusion of a supply artery.[120]

Postnatal Regulation of Vascular Growth

The vascular system is programmed to rapidly respond to changes in the microenvironment. Although these responses may be provoked by local factors (e.g., tissue injury or hypoxia), they are regulated at several levels, including locally, regionally, and systemically, and with the involvement of perivascular, vessel wall-associated, circulating, and BM-derived cells.[2] Cancer-related *neovascularization* exemplifies the diversity of processes that control cellular access to the perfused vascular space and illustrates the related terminology (Fig. 122.5). Indeed, an increase in vascular density or the presence of endothelial structures or blood within the tissue or tumor mass is not necessarily related to *angiogenesis* (see Fig. 122.4).[1] Instead, the vascular density and architecture represent a combination of parenchymal and vascular cell responses to metabolic demands and regulatory circuits operative in a given context. As mentioned earlier, these processes may include vasculogenesis-like recruitment of undifferentiated endothelial colony-forming cells (ECFC), formerly referred to as EPCs.[122] These cells are phenotypically similar to myeloid-derived, proangiogenic cells and may mediate vascular repair in response to endothelial damage (i.e., denudation) in larger vessels through several mechanisms.[122,123] Extravascular tissues may also actively increase their vascular access through processes of *vascular cooption* or *intussusception* whereby blood vessels may, respectively, become actively ensheathed or divided (split) by advancing external cellular masses.[124] Glomeruloid vessels, or *vascular tufting*, may also increase the proximity between the parenchyma and the vascular space through increased capillary looping directed by forces generated by pericytes.[125] The vascular wall may also undergo different forms of physiologic and pathologic *remodeling*, including circumferential lumen enlargement through growth, dilatation, or both. This is exemplified by capillary mother vessel formation prior to the onset of proper angiogenesis (see Fig. 122.5). The vascular wall thickening and diameter expansion, involving both endothelial and mural cells, and occurring during the formation of larger "feeding vessels" (or collaterals) upstream to tissue growth sites or ischemic regions, is often, as mentioned earlier, referred to as *arteriogenesis* or *arterio/venogenesis*.[126,127] Recruitment of pericytes to the newly assembled capillary endothelial tubes signifies the process of vascular maturation or stabilization.[2] Distinct mechanisms also regulate the programmed regression (*pruning*) of superfluous capillaries (e.g., during vascular *arborization*). Moreover, in cancer, new vascular structures are postulated to emerge as a consequence of the differentiation of cancer stem cells into endothelial-like or pericyte-like cellular populations (*vascular transdifferentiation*).[128,129] Cancer cells may also replace endothelial cells within the capillary walls of certain tumor types, giving rise to the aforementioned pseudo-endothelial lining (*vasculogenic mimicry*).[130] In the fibrotic liver, the porous endothelial lining of sinusoids is remodeled to resemble regular tight-walled capillaries (*capillarization*),[76] while conversely, the influence of VEGF and angiogenic growth factors in cancer leads to capillary *permeabilization* and leakiness.[131] Other (global) structural changes in the vessel wall or tree structure are simply referred to as *vascular remodeling*.[132] Each of these processes is driven by specialized cellular and molecular circuitry (program) and plays a unique role in vascular growth, homeostasis, and pathology, as outlined briefly in the following sections (see Fig. 122.5).[2,5,126,130]

Vasculogenesis

The existence and role of postnatal vasculogenesis are a subject of some debate. The process is thought to entail recruitment into sites of vascular growth, remodeling, and repair of cells with endothelial precursor (EPC)-like phenotype and properties resulting in their structural self-assembly, insertion into the vascular wall, or regulatory influence upon resident cells. These functions are now believed to reside in at least two BM-derived cellular populations, including the ECFCs, and myeloid-derived angiogenic cells (MACs).[122] While overt vasculogenesis occurs during early embryogenesis,[120] the role of this process during vessel growth in adults remains unclear and the structural contribution of BM-derived cells to pathological (tumor) vasculature is likely less than 5%.[133]

Angiogenesis

Postnatal angiogenesis represents the key mechanisms whereby new capillaries form under conditions of tissue growth, wound repair, inflammation, hypoxia, or proliferative disease (e.g., cancer). The new vascular structures emerge from preexisting endothelial channels mainly through a mechanism known as *sprouting angiogenesis* (see Fig. 122.4). In this case, the immediate triggering event usually involves the formation of a gradient of proangiogenic activity around hypoxic or activated cells. The resulting cascade of responses within the wall of the nearest capillary begins with local capillary distension to form an enlarged *mother vessel*. Although high concentrations of VEGF are sufficient to induce these changes,[131] the underlying molecular events usually involve a more global shift in levels of multiple angiogenesis inhibitors and stimulators ("angiogenic switch").[1] Some of these factors may upregulate VEGF in parenchymal, stromal, and inflammatory cells or act directly on the endothelium. Inflammatory cells often cluster at sites of angiogenesis prior to the formation of new blood vessels, where they contribute to the consolidation of the proangiogenic microenvironment or trigger vascular growth via VEGF-dependent and independent mechanisms.[2,5] Efficient sprouting involves metabolic reprogramming of endothelial cells, including the activation of glycolysis and upregulation of phosphofructokinase isoenzyme 3 (PFKFB3).[134]

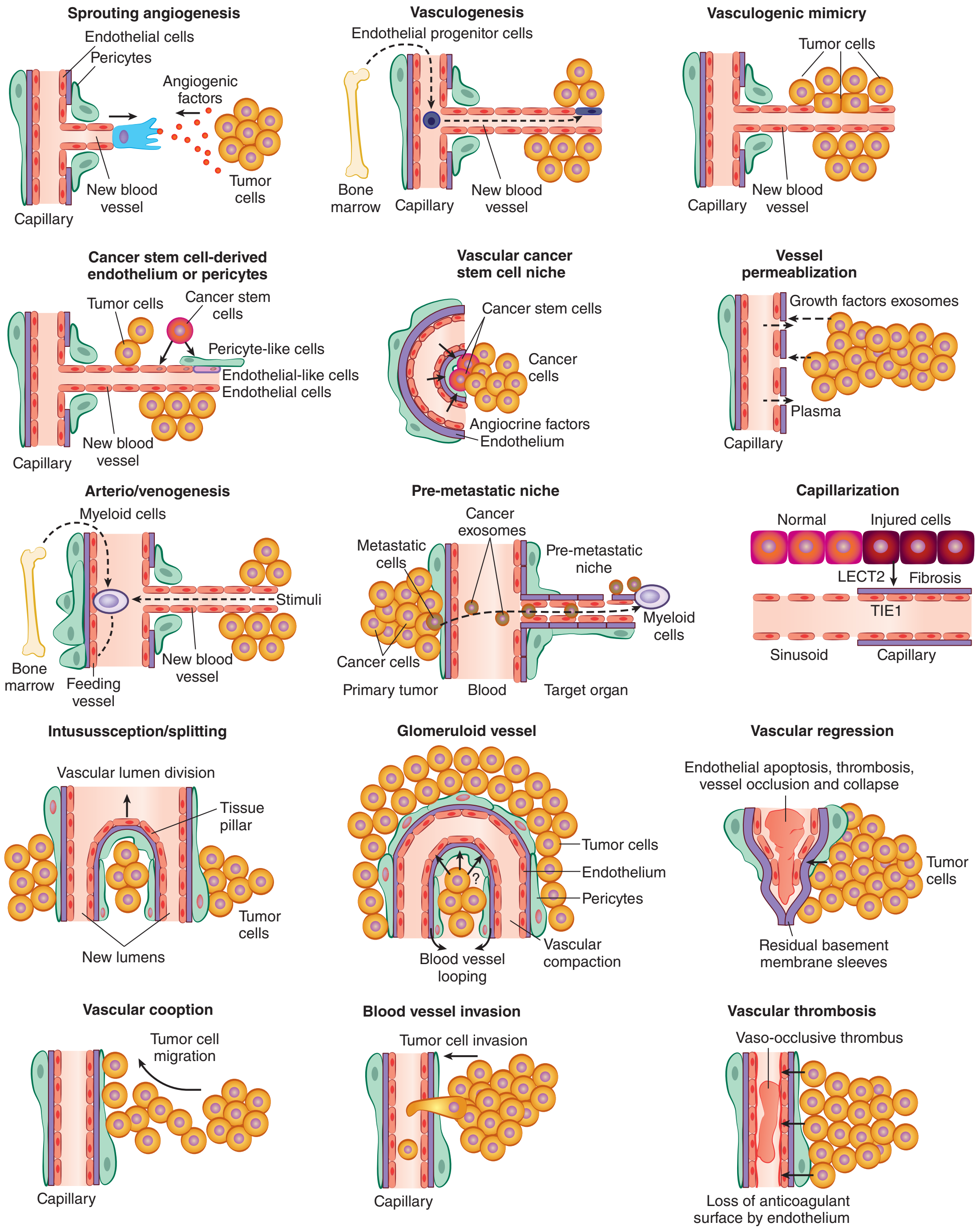

Sprouting angiogenesis
Endothelial cells
Pericytes
Angiogenic factors
New blood vessel
Tumor cells
Capillary
Vasculogenesis
Endothelial progenitor cells
Bone marrow
Capillary
New blood vessel
Vasculogenic mimicry
Tumor cells
Capillary
New blood vessel
Cancer stem cell-derived endothelium or pericytes
Tumor cells
Cancer stem cells
Pericyte-like cells
Endothelial-like cells
Endothelial cells
New blood vessel
Capillary
Vascular cancer stem cell niche
Cancer stem cells
Cancer cells
Angiocrine factors
Endothelium
Vessel permeablization
Growth factors exosomes
Plasma
Capillary
Arterio/venogenesis
Myeloid cells
Stimuli
Bone marrow
Feeding vessel
New blood vessel
Pre-metastatic niche
Cancer exosomes
Metastatic cells
Pre-metastatic niche
Myeloid cells
Cancer cells
Primary tumor
Blood
Target organ
Capillarization
Normal
Injured cells
LECT2
Fibrosis
TIE1
Sinusoid
Capillary
Intusussception/splitting
Vascular lumen division
Tissue pillar
Tumor cells
New lumens
Glomeruloid vessel
Tumor cells
Endothelium
Pericytes
?
Vascular compaction
Blood vessel looping
Vascular regression
Endothelial apoptosis, thrombosis, vessel occlusion and collapse
Tumor cells
Residual basement membrane sleeves
Vascular cooption
Tumor cell migration
Capillary
Blood vessel invasion
Tumor cell invasion
Capillary
Vascular thrombosis
Vaso-occlusive thrombus
Loss of anticoagulant surface by endothelium

Figure 122.5 PROCESSES OF VASCULAR GROWTH AND REMODELING IN PATHOLOGY. Neoplastic growths (also fibrosis or inflammation) can serve as paradigms for cellular programs regulating the proximity between parenchymal cells (including cancer cells) and the vascular lumen (see text for details). *Angiogenesis*, Recruitment of new capillary vessels through paracrine effects of angiogenic factors on endothelial cells of the preexisting vasculature; *Vasculogenesis*, recruitment of bone marrow-derived endothelial progenitor cells (EPCs) to the sites of vascular growth or repair (although EPC are essential during embryogenesis, postnatally their role is more limited as they lodge in, and contribute to, fragments of the newly formed vascular wall, or participate in the repair at sites of endothelial denudation); *Vasculogenic mimicry*, insertion and adaptation of non-endothelial cells into segments of the blood vessel lining; *Endothelial differentiation (or transdifferentiation) of cancer stem cells*, certain types of multipotential tumor-initiating (stem) cells may activate programs of endothelial or pericytic transdifferentiation and form segments of the newly formed vasculature; *Vascular (cancer) stem cell niche* is a site of proximity between endothelial cells and (cancer) stem cell populations, which receive paracrine (*angiocrine*) support and stimulation from endothelial cells; *Vessel permeabilization* occurs when the release of permeability factors (e.g., VEGF) from perivascular cells (e.g., cancer cells) leads to formation of endothelial cell fenestrae or other forms of trans- or para-endothelial permeability and leakage of plasma into the interstitium; *Arterio/venogenesis*, retrograde circumferential enlargement and remodeling of feeding vessels that supply expanding capillary networks with blood, a process involving bone marrow-derived myeloid cell recruitment through tissue-related stimuli and shear forces; *Pre-metastatic niche* is a modified local vascular and perivascular microenvironment, mostly through the exposure to cancer exosomes, prone to subsequent metastasis colonization in cancer; *Capillarization* is a change in the state of sinusoids resulting in their remodeling to capillary-like vessels, with the involvement of LECT2/TIE1 pathway; *Intussusception*, division of the vascular lumen into smaller tubes by ingrowth of external tissue pillars; *Glomeruloid vessel formation*, formation of bundles of vascular loops through forces (arrows) generated by endothelial, pericyte, and parenchymal cell interactions; *Vascular regression*, a process of regulated loss of endothelial viability, thrombosis, occlusion and collapse of a blood vessel; *Vascular cooption*, recruitment of cancer cells to the proximity of preexisting blood vessels; *Blood vessel invasion*, breaching of the blood vessel wall continuity by invading cancer cells; *Vascular thrombosis*, loss of patency, induction of intraluminal clotting as a result of changes in endothelial antithrombotic potential, physical damage, and loss of barrier function coupled with extravascular influence of highly coagulant cells (e.g., cancer cells).

Molecular Interplay Involved in Sprouting Angiogenesis

As mentioned earlier, VEGF plays a central and indispensable role in normal angiogenic sprouting. After exposure to this potent stimulator, ANG2 expression becomes upregulated in endothelial cells residing within the continuous monolayer of the capillary or mother vessel wall (phalanx cells). Endothelial ANG2 blocks tonic activation of the TIE2 receptor in the constitutive presence of ANG1, and by disrupting the key mechanism maintaining capillary structure leads to the detachment of pericytes. This is followed by local dissolution of the basement membrane (by MMPs), increase in vascular permeability, and extravasation of plasma proteins into the interstitium. The extravascular deposition of fibrin and the formation of a provisional ECM provide the scaffold for the formation of new capillaries.[2]

These processes liberate endothelial cells from physical constraints and initiate their directional migration in the form of a cellular column (angiogenic sprout) toward the source of the angiogenic stimulus (e.g., VEGF-expressing cells). Each sprout is composed of a single, specialized, leading endothelial cell (tip cell) equipped with hair-like, sensing projections (filopodia), which contain high concentrations of VEGFR2 and are rich in other regulators (e.g., PDGF-B, SDF-1, Apelin, and Dll4). In their gradient-seeking movement, tip cells are followed by a cohort of proliferating endothelial stalk cells, which express VEGFR1 and Notch. Stronger stimulation by VEGF results in VEGFR2-dependent upregulation of Dll4 in tip cells, allowing them to instruct their stalk cell counterparts (via Notch) to retain their phenotype, refrain from independent branching, and maintain VEGF signaling at lower levels. The latter is mediated, at least in part, by the upregulation of soluble VEGFR1, acting as a “VEGF sink.”

Exogenous blockade of the Dll4/Notch pathway leads to excessive generation of tip cells and sprouts (from stalk cells), resulting in the formation of an overly branched, hyperdense, nonperfused, and dysfunctional capillary networks (“nonproductive angiogenesis”).[135] Stimulation of Notch receptor by the Jagged 1 ligand modulates the effects of Dll4 on stalk cells and fine-tunes the capillary branching patterns.

The neighboring sprouts eventually anastomose to form new capillary loops. This process is mediated by interaction with myeloid cells expressing TIE-2 and NRP1 receptors.[27] Subsequent generation of the vascular lumen and resumption of blood flow occur through the formation of intercellular spaces between endothelial stalk cells.[2]

Vascular Maturation

To gain functionality, newly formed capillary loops must undergo maturation. This involves a buildup of a mural cell layer around the endothelial tube.[2] Responses of mural cells are triggered by secretion of PDGF-BB by endothelial tip cells, which attracts regional pericytes; a source of structural support and vessel-stabilizing, pro-survival ANG1 activity.[136] During these events, sphingosine 1 phosphate (S1P) regulates N-cadherin, which forms additional connections between endothelial cells and pericytes. Upon their attachment to the endothelial tube, pericytes assume a more mature phenotype under the influence of transforming growth factor beta 1 (TGFβ1).[136] Endothelial prolyl hydroxylase 2 (PHD2), an oxygen-sensing enzyme, also regulates pericyte recruitment. These mechanisms restore vascular integrity, mechanical resistance, endothelial cell survival, diminished dependence on VEGF, and restricted permeability.[2]

Capillarization

The formation of nonpermeable mature capillaries from permeable small sinusoid vessels (capillarization) may also accompany chronic pathology. For example, injured hepatocytes produce a mediator known as LECT2, which acts as a ligand for endothelial TIE1 receptor resulting in enhancement of TIE2 signaling, remodeling of sinusoids into capillaries, and progression of liver fibrosis.[76] It is possible that LECT2 and capillarization may also have roles in other contexts.

Lymphangiogenesis

The lymphatics comprise a low-flow, low-pressure system that collects extravasated fluid from the tissues and transfers it back to the venous system via the thoracic duct. Lymphatic vessels also serve an immune function by transporting lymphoid and antigen-presenting cells to lymphoid organs.[137,138] Lymphatic vessels share features with blood vessels, but they also exhibit differences such as the absence of basement membrane and filamentous attachment of endothelial cells to the surrounding tissue. While transcriptome of lymphatic endothelial cells exhibits 98% similarity to that of vascular endothelium, these cells also express distinctive markers (PROX1, VEGFR-3, LYVE-1, PDPN, CCL21), unique patterns of growth responses, and biological functions.[139]

Lymphatic network develops from venous endothelial cells that differentiate into lymphatic endothelium in response to specific molecular signals.[138] The branching and sprouting of lymphatics (lymphangiogenesis) resemble the process of angiogenesis. Lymphangiogenesis is triggered by VEGF-C and VEGF-D, both activating VEGFR-3 and also depend on the activity of ANG2 which, unlike in blood vessel capillaries, acts as a TIE2 agonist in lymphatic endothelium.[138] The α9β1 integrin and matrix interacting protein CCBE1 are necessary for proper lymphatic development, and the homeobox transcription factor PROX1 controls the program of lymphatic endothelial cell differentiation. Another regulator of early lymphatic endothelial cell specification is SOX18 (sex-determining Region Y Box 18), a transcriptional regulator of PROX1expression.[140]

Lymphatics play important roles in pathology. For example, they are the main conduits of lymphatic metastasis in cancer. Whether lymphatic vessel density in human tumors correlates with disease progression is not clear, but in animal models, induction of lymphangiogenesis and lymphatic dilation by VEGF-C or VEGF-D promotes lymph node metastasis.[137] Dysfunction of lymphatics in lymphedema can be caused by congenital defects, parasitic (filariasis) or neoplastic obstruction, or surgical resection causing retention of interstitial fluid in the tissue lacking functional drainage. Congenital lymphedema (Milroy disease) is linked to inactivating mutations of VEGFR-3.[138]

Vasculogenic Mimicry

Insertion of cancer cells into endothelial lining associated with change in their phenotype (vasculogenic mimicry), or a process of overt transdifferentiation of cancer stem cells to vascular-like cellular populations exemplify a possible involvement of non-endothelial lineages in the formation of vascular channels.[129] Vasculogenic mimicry occurs during normal placentation and in tumors, such as melanoma, or mouse teratoma. During these events, non-endothelial cells may adopt some of the morphologic and antigenic attributes of proper endothelium.[130] In human glioblastoma, cancer stem cells were reported to undergo endothelial or pericyte transdifferentiation, including the expression of corresponding lineage markers and some contribution to the vascular wall and perivascular invasion. The scope and functional role of these processes in human disease remain unclear and controversial.[128]

Vascular Cooption

Cancer cells may actively adopt a growth and invasion pattern around preexisting blood vessels.[124] This process (vascular cooption) is observed in highly vascularized organs, such as the lungs, liver, and brain, resulting in a nonangiogenic form of tumor access to blood vessel networks and resistance to antiangiogenic agents. Coopted vessels may undergo secondary structural alterations, which may result in their remodeling, formation of occlusive thrombi, and regression that can lead to ischemia.[124] The extent of the vessel cooption process in cancer can be considerably greater than previously thought and appears to be controlled by both the properties of the vasculature and the phenotype of cancer cells.[124]

Vascular Remodeling, Regression, and Endothelial Apoptosis in the Course of Vascular Growth

Even though the vasculature is laid down before circulation begins, hemodynamic forces are important for its maintenance and remodeling. Most of the vessels formed during vasculogenesis regress or are remodeled. A chronic decrease in blood flow results in narrowing of the vessel lumen. Remodeling, which involves loss of some vessels (pruning), as well as changes in lumen diameter and wall thickness, requires both cell death and proliferation (as well as remodeling of the ECM).[141] In addition to survival signals transmitted by integrins and growth factors also shear stress and mechano-signaling are important for endothelial survival and vessel healing after injury, as described in previous sections. While endothelial cells are exquisitely sensitive to death from detachment (anoikis), they also express several anti-apoptotic molecules to maintain viability when quiescent and when stressed or involved in vascular growth processes to maintain the intricate balance between their survival or elimination.[142]

Arteriogenesis

As mentioned earlier, this term refers to upstream circumferential enlargement of supply vessels that drain into a particular vascular bed, a process often triggered by the occlusion of the main supply vessel, or by expansion of the capillary volume (e.g. in cancer).[126] In adulthood, arteriogenesis is responsible for the formation of collateral arterial circulation to bypass pathological obstruction of arterial blood supply to tissues.[143] Remodeling of a preexisting collateral small arteriole is thought to be caused by flow-induced changes secondary to occlusion of the supply artery. The consequent increase in shear stress of blood forced through the collateral arteriole activates endothelial cells, resulting in myeloid cell (monocyte) recruitment and their infiltration into the media. Elaboration of various cytokines, growth factors, and proteases from monocytes and endothelial cells causes matrix degradation, smooth muscle cell proliferation, and rapid enlargement of the preexisting arteriole. Factors thought to promote arteriogenesis include FGF-2, PlGF, PDGF-BB, TGFβ1, monocyte chemoattractant protein 1 (MIP-1), and GM-CSF.[144]

MECHANISMS TRIGGERING BLOOD VESSEL GROWTH

Angiogenesis represents the predominant form of postnatal vascular growth and a paradigm of blood vessel responses to external cues. Cessation of physiological quiescence of endothelial cells and the angiogenic switch may occur in response to hypoxia, metabolic stress, growth factors, inflammatory mediators, or after activation of the coagulation system or changes in the secretome of adjacent cells that have undergone malignant transformation. These mechanisms are interdependent and often converge upon the regulation of the VEGF gene[2] but may also involve additional (or alternative) complex networks of molecular effectors. Although angiogenic responses may be morphologically similar, they exhibit highly context-dependent traits.[145]

The most studied network in this regard is the regulation of VEGF by the hypoxia response pathway. In this setting, VEGF transcription is controlled by dimeric hypoxia-inducible factors 1 and 2 (HIFs). Normally, the HIF1 alpha subunit (HIF-1α) is constitutively degraded by oxygen-dependent prolyl- and aspargyl hydroxylases, von Hippel–Lindau (VHL) ubiquitin ligase, and the proteasome.[146] Hypoxia blocks this process, resulting in elevated HIF activity and VEGF production by parenchymal, stromal, and inflammatory cells. Several other mechanisms of proangiogenic responses driven by hypoxia have also been described (e.g., NFκB, EGR1).[146]

In addition, exposure of cells to growth factors (EGF, FGF, HGF) and inflammatory cytokines (IL-6) may upregulate VEGF, and some of these effectors may also directly stimulate endothelial cells.[2] Activation of oncogenes (e.g., RAS) and loss of tumor suppressors (VHL) may lead to upregulation of VEGF and often pleiotropically affect other angiogenic growth factors in cancer cells, even under conditions of normoxia.[147] Oncogenic transformation also shuts down some of the angiogenic inhibitors, contributes to coagulopathy (through upregulation of TF and thrombin receptors), and enhances proangiogenic cellular vesiculation (biogenesis and emission of EVs loaded with vascular mediators). Indeed, oncogenic pathways often mimic, distort, or exacerbate the effects of hypoxia, inflammation, or microenvironmental stress.[147]

THERAPEUTIC IMPLICATIONS OF ENDOTHELIAL AND ANGIOGENIC RESPONSES TO DISEASE

Vascular events associated with cancer, hemangioma, viral infections, or vascular malformations have the potential to trigger hematologic consequences, either spontaneously or during therapeutic angiogenesis or antiangiogenesis. These linkages are still poorly understood but often include one or more of the following processes: (1) endothelial cell activation associated with intravascular upregulation of TF, adhesion molecules, and other mediators; (2) disruption of vascular wall continuity; (3) recruitment of inflammatory cells; (4) increased vascular permeability with resultant extravasation and activation of circulating coagulation factors; (5) platelet activation; (6) contact of procoagulant cell surfaces (e.g., metastatic cancer cells) with circulating blood; (7) flow perturbations and stasis; (8) indirect external effects (e.g., indwelling catheters or administration of chemotherapy); and (9) shedding of procoagulant, anticoagulant, or bioactive EVs (microparticles), and soluble factors into blood. In addition, blood vessel-directed therapies and their capacity to modulate or exacerbate some of these events represent an emerging challenge in hematology (see Chapters 94 and 151).

Revascularization Therapy

Ischemic heart muscle, peripheral artery disease, non-healing burns, and other causes of tissue hypoperfusion may benefit from controlled induction of angiogenesis or collateral circulation. Ongoing efforts are exploring the utility of recombinant growth factors (FGF, VEGF), gene therapy vectors, or cell therapy approaches (including mesenchymal stem cells) in inducing effective revascularization of affected tissues, as reviewed elsewhere.[148,149]

Antiangiogenic Therapy

The term “antiangiogenesis” is often used incorrectly to describe inhibition of all types of pathological vascular growth in diverse disease states collectively referred to as angiogenesis-dependent diseases.[1] While the nature of the vascular pathology in some of these conditions is not tantamount to angiogenesis, they all exhibit destructive endothelial cell responses that may be pharmacologically targetable. Examples of such diseases include neovascular age-related macular degeneration (NVAMD), retinopathy of prematurity (ROP), diabetic retinopathy (DR), vascular malformations, psoriasis, Kaposi sarcoma, cavernous hemangioma, arthritis endometriosis, and several forms of cancer.[1]

TUMOR ANGIOGENESIS AND ANTIANGIOGENESIS

The disorganized signaling cues during tumor angiogenesis may produce highly abnormal, leaky, tortuous, prothrombotic, and poorly perfused vasculature (vessel “abnormalization”), which may contribute to aberrant hemostasis.[2] Antiangiogenic agents may cause further perturbation by selective destruction of tumor-associated endothelial cells or by inhibition of their stimulatory circuitry.[1] Several classes of antiangiogenic compounds have been evaluated to date, including derivatives of natural angiogenesis inhibitors (e.g., endostatin or tumstatin), inhibitors of proangiogenic signaling pathways (antibodies, small-molecule agents), and agents that block proangiogenic inflammatory pathways (e.g., thalidomide analogs). Anticancer drugs may also be administered in low but frequent doses (metronomic chemotherapy) to target endothelial, immune, and regulatory cells preferentially.[1,4,150]

Bevacizumab (Avastin), a humanized monoclonal anti-VEGF antibody, was the first antiangiogenic agent to be approved (in 2004) for cancer treatment, and the drug is now used to treat colorectal, lung, kidney, recurrent brain tumors, and other cancers.[150] Another neutralizing antibody directed against VEGFR2 (Ramucirumab) has subsequently been introduced into oncology practice[151] along with anti-VEGF receptor-antibody chimeric inhibitor (Aflibercept). Several tyrosine kinase inhibitors (TKIs) with anti-VEGFR or multikinase activity (sunitinib, sorafenib, pazopanib, axitinib, regorafenib, nintedanib, lenvatinib, cabozantinib) are also in common use. Antiangiogenic activity was also documented in the case of inhibitors blocking oncogenic pathways that drive the VEGF production by cancer cells.[147] These agents include agents blocking signals generated by epidermal growth factor receptor (EGFR) and HER2 (cetuximab and trastuzumab, respectively), along with inhibitors of other oncogenic proteins.[147]

VEGF/VEGFR antagonists are mostly used in combination with chemotherapy, suggesting that they may serve as chemosensitizers, mediators of vascular normalization and immunomodulators.[2] Studies are underway to develop drugs that specifically target pathologic angiogenesis while sparing natural blood vessel growth (e.g., blockers of PlGF), or agents targeting pre-established tumor blood vessels (e.g., vascular disrupting agents [VDAs]).[4,150]

The overall objectives of antiangiogenic therapy in cancer are at least threefold: (1) to induce tumor hypovascularity, thereby causing hypoxic damage to cancer cells; (2) to deprive cancer (stem) cells of the paracrine growth stimulation and support rendered by angiogenic endothelial cells (anti-angiocrine effect)[147,152]; and (3) at lower doses, to induce vessel "normalization," resulting in the improved tumor perfusion associated with increased sensitivity to chemotherapy, radiation, and immunotherapy.[2] In experimental settings, promotion of blood vessel growth/dilatation combined with chemotherapy may exert an anticancer effect, and it is possible that rational and context-dependent modulation of the tumor vasculature may eventually replace global approaches of anti-angiogenesis.[153]

ANGIOGENESIS AND ANTIANGIOGENESIS IN HEMATOPOIETIC MALIGNANCIES

The vascular BM stroma plays a pivotal role in leukemogenesis.[154] Indeed, angiogenesis, increased vascular density, and increased levels of angiogenic growth factors have all been observed in the BM of patients with hematopoietic malignancies. VEGF may play multiple roles in this context, including as (1) a vascular growth stimulator; (2) a paracrine growth factor for leukemic stem cells[154]; and (3) an inducer of angiocrine interactions between these cells and the endothelial niche. Consequently, a wide spectrum of antiangiogenic agents, including VEGF antagonists (bevacizumab, sorafenib, sunitinib, cediranib), have been investigated as candidates for the treatment of hematologic disorders, such as acute myelogenous leukemia, chronic myelogenous leukemia, acute lymphoblastic leukemia, myelodysplastic syndrome, non-Hodgkin lymphoma, multiple myeloma, and others. Additional agents with antiangiogenic activity have also been explored, including bortezomib and anti-inflammatory antiangiogenics (thalidomide and lenalidomide).[154] While some of the latter agents are already in human use in hematologic malignancies (e.g., in mantle cell lymphoma, multiple myeloma, and myelodysplastic syndrome), the contribution of antiangiogenic effects to therapeutic efficacy is not clear.

HEMATOLOGIC COMPLICATIONS ASSOCIATED WITH BLOOD VESSEL-DIRECTED AGENTS

Manipulation of endothelial and mural cells during pro- and antiangiogenic therapy creates the potential for side effects.[155,156] The most extensive clinical experience in this regard is in cancer, where some toxicities (e.g., hypertension, proteinuria, fatigue, hypothyroidism) have been frequently observed, along with hematologic side effects such as thrombosis and/or bleeding. While cancer patients are prone to thrombosis,[157] antiangiogenic therapy increases the risk of venous thromboembolism or arterial thrombosis.[6] The risk varies depending on the agent and the type of cancer and is exacerbated by accompanying chemotherapy or hormonal therapy.[157] Thrombosis is of particular concern in patients with multiple myeloma who receive thalidomide derivatives in combination with anthracyclines and dexamethasone. In this case, the risk of thrombosis may be as high as 75%.[157] Bevacizumab may increase the risk of arterial thrombosis up to two-fold in patients with solid tumors, especially in the elderly with comorbid conditions. However, these estimates are variable (0.9% to 19.4% according to different studies) and drug-specific, because small-molecule TKIs acting on VEGFR are less likely to trigger venous thromboembolism (0% to 3%).[157]

Bleeding complications have also been recorded with several of these agents, ranging from minor to life threatening.[158] In general, however, antiangiogenic agents are relatively well tolerated, and their side effects are usually manageable with careful monitoring and standard supportive care.[6]

RELATIONSHIP BETWEEN THE VASCULATURE AND HEMATOPOIESIS

Hematopoietic cells and endothelial cells are intertwined in several ways. First, there is the likely existence of a common precursor, the hemangioblast, in the yolk sac (see *vasculogenesis*). Second, definitive hematopoietic stem cells emerge from specialized endothelial cells during embryogenesis in a process called endothelial-to-hematopoietic transition (see Chapter 9). Third, the endothelium is intimately involved in adult hematopoiesis, having a supportive role structurally and nutritionally (see also the section on BM Sinuses). Fourth, BM-derived myeloid cells play crucial regulatory roles in angiogenesis, arteriogenesis, and other processes of vascular growth and remodeling (see section on Cellular and Molecular Pathways Involved in Blood Vessel Formation). Finally, the endothelium organizes the controlled egress and ingress of hematopoietic cells in hematopoietic and other tissues (see Interaction of Blood Cells With the Vessel Wall).[159]

Between embryonic day 9.5 and 11.5 in the mouse and 4 to 6 weeks of gestation in the human, hemogenic endothelial cells undergo transdifferentiation to hematopoietic stem and progenitor cells.[160,161] This process of endothelial-to-hematopoietic transition occurs mainly in the ventral aspect of the dorsal aorta of the aorta-gonadal-mesonephros region, but also in other large arteries. Hemogenic endothelial cells express transcriptomes that are distinct from the surrounding vascular endothelial cells, and during embryogenesis, clusters of hematopoietic cells are observed to emerge from these cells. Hematopoietic stem cells thus generated then migrate to the fetal liver. Hemogenic endothelial cells initially express VE-cadherin, indicative of their endothelial origin, and sequentially upregulate the hematopoietic markers CD41, c-Kit, CD43, and CD45 during their transition to hematopoietic stem and progenitor cells. Runx1 is a critical transcription factor that identifies hemogenic endothelium and is required for their hematopoietic transdifferentiation. Additional key transcription factors driving this process include Spi1, Gfi1, FosB, and others.[162]

Mounting evidence indicates that hematopoietic stem and progenitor cells are not randomly distributed in the BM, but rather are spatially associated with blood vessels, the vascular niche. Endothelial cells, through paracrine and juxtacrine activity, in concert with other stromal and hematopoietic cells, provide a finely tuned system to modulate hematopoiesis in the BM such that differentiation, proliferation, migration, and self-renewal occur in a regulated fashion at steady state and in response to stress and injury.[30,163,164] Although there is minimal functional evidence in humans, several lines of evidence suggest that the human hematopoietic stem cell niche is like that in the mouse.[165] The physical proximity of endothelium and hematopoietic precursors within the marrow and the requirement of blood cells to transit through marrow endothelium to reach the circulation suggests an important role for endothelium. Both yolk sac and marrow endothelial cells support long-term proliferation and differentiation of hematopoietic cells in vitro. In addition to the expression of chemokines (CXCL12) and adhesion molecules (E-selectin, VCAM-1) (see also the section on BM Sinuses), marrow endothelial cells constitutively express high levels of IL-6, stem cell factor, granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF).[30] Like normal hematopoietic stem cells, leukemic stem cells dynamically interact with the BM stroma and vasculature, often in the context of increased angiogenic responses.[164]

Human endothelial cells express receptors for IL-3, stem cell factor, erythropoietin, and thrombopoietin, and show functional responses to IL-3 and erythropoietin. The shared responses to growth factors, combined with the importance of macrophages in angiogenesis and the production of cytokines by monocytes and macrophages, suggest that hematopoietic cells play a reciprocal role in maintaining the endothelium.

SUMMARY

The functional integration of the vascular system, BM, and circulating blood results in a high degree of biologic interdependence in health and disease. While this may not always be clinically obvious, the existence of these subtle links necessitates a greater consideration of blood vessel-regulating processes in the pathogenesis and therapy of hematologic disorders.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Aird WC. Endothelial cell heterogeneity. *Cold Spring Harb Perspect Med.* 2012;2:a006429.

Alitalo K. The lymphatic vasculature in disease. *Nat. Med.* 2011;17:1371–1380.

Augustin HG, Koh GY. Organotypic vasculature: from descriptive heterogeneity to functional pathophysiology. *Science.* 2017;357:eaal2379.

Barbier V, Erbani J, Fiveash C, et al. Endothelial E-selectin inhibition improves acute myeloid leukaemia therapy by disrupting vascular niche-mediated chemoresistance. *Nat Commun.* 2020;11:2042.

Batsivari A, Haltalli MLR, Passaro D, et al. Dynamic responses of the haematopoietic stem cell niche to diverse stresses. *Nat Cell Biol.* 2020;22:7–17.

Betsholtz C. Cell-cell signaling in blood vessel development and function. *EMBO Mol Med.* 2018;10.

Campbell WB, Gauthier KM. Inducible endothelium-derived hyperpolarizing factor: role of the 15-lipoxygenase-EDHF pathway. *J Cardiovasc Pharmacol.* 2013;61:176–187.

Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature.* 2011;473(7347):298–307.

Crane GM, Jeffery E, Morrison SJ. Adult haematopoietic stem cell niches. *Nat Rev Immunol.* 2017;17:573–590.

De Palma M, Biziato D, Petrova TV. Microenvironmental regulation of tumour angiogenesis. *Nat Rev Cancer.* 2017;17:457–474.

Dvorak HF. Tumors: wounds that do not heal—a historical perspective with a focus on the fundamental roles of increased vascular permeability and clotting. *Semin Thromb Hemost.* 2019;45:576–592.

Dzierzak E, Bigas A. Blood development: hematopoietic stem cell dependence and independence. *Cell Stem Cell.* 2018;22:639–651.

Folkman J. Angiogenesis: an organizing principle for drug discovery? *Nat. Rev Drug Discov.* 2007;6:273–286.

Jackson DG. Leucocyte trafficking via the lymphatic vasculature—mechanisms and consequences. *Front Immunol.* 2019;10:471.

Komarova YA, Kruse K, Mehta D, Malik AB. Protein interactions at endothelial junctions and signaling mechanisms regulating endothelial permeability. *Circ Res.* 2017;120:179–206.

Kreuger J, Phillipson M. Targeting vascular and leukocyte communication in angiogenesis, inflammation and fibrosis. *Nat Rev Drug Discov.* 2016;15:125–142.

Kuczynski EA, Vermeulen PB, Pezzella F, et al. Vessel co-option in cancer. *Nat Rev Clin Oncol.* 2019:16:469-493.

Lusis AJ. Genetics of atherosclerosis. *Trends Genet.* 2012;28:267–275.

Mondal R, Lahiri D, Deb S, et al. COVID-19: Are we dealing with a multisystem vasculopathy in disguise of a viral infection? *J Thromb Thrombolysis.* 2020:1–13.

Muller WA. Transendothelial migration: unifying principles from the endothelial perspective. *Immunol Rev.* 2016;273:61–75.

Nierodzik ML, Karpatkin S. Thrombin induces tumor growth, metastasis, and angiogenesis: evidence for a thrombin-regulated dormant tumor phenotype. *Cancer Cell.* 2006;10:355–362.

Peng Z, Shu B, Zhang Y, Wang M. Endothelial response to pathophysiological stress. *Arterioscler Thromb Vasc Biol.* 2019;39:e233–e243.

Pinho S, Frenette PS. Haematopoietic stem cell activity and interactions with the niche. *Nat Rev Mol Cell Biol.* 2019;20:303–320.

Rak J. *RAS* oncogenes and tumor-vasculature interface.. In: Thomas-Tikhonenko A, ed. *Cancer Genome and Tumor Microenvironment.* New York: Springer; 2009. p. 133–165.

Rayes J, Bourne JH, Brill A, Watson SP. The dual role of platelet-innate immune cell interactions in thrombo-inflammation. *Res Pract Thromb Haemost.* 2020;4:23–35.

Telen MJ, Malik P, Vercellotti GM. Therapeutic strategies for sickle cell disease: towards a multi-agent approach. *Nat Rev Drug Discov.* 2019;18:139–158.

van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol.* 2018;19:213–228.

Yau JW, Teoh H, Verma S. Endothelial cell control of thrombosis. *BMC Cardiovasc Disord.* 2015;15:130.

Zhang D, Xu C, Manwani D, Frenette PS. Neutrophils, platelets, and inflammatory pathways at the nexus of sickle cell disease pathophysiology. *Blood.* 2016;127:801–809.

REFERENCES

1. Folkman J. Angiogenesis: an organizing principle for drug discovery? *Nat Rev Drug Discov*. 2007;6:273–286.
2. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature*. 2011;473:298–307.
3. Dvorak FH, Rickles FR. Ch. 57. In: Coleman RB, ed. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*. Lippincott Company Williams & Wilkins; 2006:851–873.
4. Kerbel RS. Tumor angiogenesis. *N Engl J Med*. 2008;358:2039–2049.
5. Ferrara N. Role of myeloid cells in vascular endothelial growth factor-independent tumor angiogenesis. *Curr Opin Hematol*. 2010;17:219–224.
6. Hurwitz HI, et al. Venous thromboembolic events with chemotherapy plus bevacizumab: a pooled analysis of patients in randomized phase II and III studies. *J Clin Oncol*. 2011;29:1757–1764.
7. Wellbrock J, Fiedler W. Clinical experience with antiangiogenic therapy in leukemia. *Curr Cancer Drug Targets*. 2011;11:1053–1068. https://doi.org/10.2174/156800911798073078.
8. Pugsley MK, Tabrizchi R. The vascular system. An overview of structure and function. *J Pharmacol Toxicol*. 2000;44:333–340. https://doi.org/10.1016/s1056-8719(00)00125-8.
9. Valentijn KM, Sadler JE, Valentijn JA, Voorberg J, Eikenboom J. Functional architecture of Weibel-Palade bodies. *Blood*. 2011;117:5033–5043. https://doi.org/10.1182/blood-2010-09-267492.
10. Krüger-Genge A, Blocki A, Franke RP, Jung F.Vascular endothelial cell biology: an update. *Int J Mol Sci*. 2019;20 https://doi.org/10.3390/ijms20184411.
11. Ruoslahti E, Engvall E. Integrins and vascular extracellular matrix assembly. *J Clin Invest*. 1997;99:1149–1152. https://doi.org/10.1172/jci119269.
12. Majesky MW. Vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2016;36:e82–86. https://doi.org/10.1161/atvbaha.116.308261.
13. Duque Lasio ML, Kozel BA. Elastin-driven genetic diseases. *Matrix Biol*. 2018;71-72:144–160. https://doi.org/10.1016/j.matbio.2018.02.021.
14. Shovlin CL, Scott J. Inherited diseases of the vasculature. *Ann Rev Physiol*. 1996;58:483–507. https://doi.org/10.1146/annurev.ph.58.030196.002411.
15. Armulik A, Genové G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Develop Cell*. 2011;21:193–215. https://doi.org/10.1016/j.devcel.2011.07.001.
16. van Dijk CG, et al. The complex mural cell: pericyte function in health and disease. *Int J Cardiol*. 2015;190:75–89. https://doi.org/10.1016/j.ijcard.2015.03.258.
17. Aird WC. Endothelial cell heterogeneity. *Cold Spring Harb Perspect Med*. 2012;2:a006429. https://doi.org/10.1101/cshperspect.a006429.
18. Komarova YA, Kruse K, Mehta D, Malik AB. Protein interactions at endothelial junctions and signaling mechanisms regulating endothelial permeability. *Cir Res*. 2017;120:179–206. https://doi.org/10.1161/circresaha.116.306534.
19. Radeva MY, Waschke J. Mind the gap: mechanisms regulating the endothelial barrier. *Acta Physiol (Oxford)*. 2018;222 https://doi.org/10.1111/apha.12860.
20. Giannotta M, Trani M, Dejana E. VE-cadherin and endothelial adherens junctions: active guardians of vascular integrity. *Develop Cell*. 2013;26:441–454. https://doi.org/10.1016/j.devcel.2013.08.020.
21. Morbiducci U, et al. Atherosclerosis at arterial bifurcations: evidence for the role of haemodynamics and geometry. *Thromb Haemost*. 2016;115:484–492. https://doi.org/10.1160/th15-07-0597.
22. Peng Z, Shu B, Zhang Y, Wang M. Endothelial response to pathophysiological stress. *Arterioscler Thromb Vasc Biol*. 2019;39:e233–e243. https://doi.org/10.1161/atvbaha.119.312580.
23. Marchand M, Monnot C, Muller L, Germain S. Extracellular matrix scaffolding in angiogenesis and capillary homeostasis. *Semin Cell Develop Biol*. 2019;89:147–156. https://doi.org/10.1016/j.semcdb.2018.08.007.
24. Post A, Wang E, Cosgriff-Hernandez E. A review of integrin-mediated endothelial cell phenotype in the design of cardiovascular devices. *Ann Biomed Eng*. 2019;47:366–380. https://doi.org/10.1007/s10439-018-02171-3.
25. Vanlandewijck M, et al. A molecular atlas of cell types and zonation in the brain vasculature. *Nature*. 2018;554:475–480. https://doi.org/10.1038/nature25739.
26. Augustin HG, Koh GY. Organotypic vasculature: from descriptive heterogeneity to functional pathophysiology. *Science*. 2017;357 https://doi.org/10.1126/science.aal2379.
27. Marcu R, et al. Human organ-specific endothelial cell heterogeneity. *iScience*. 2018;4:20–35. https://doi.org/10.1016/j.isci.2018.05.003.
28. Pawlak JB, Caron KM. Lymphatic programing and specialization in hybrid vessels. *Fron Physiol*. 2020;11:114. https://doi.org/10.3389/fphys.2020.00114.
29. Allen E, et al. Combined antiangiogenic and anti-PD-L1 therapy stimulates tumor immunity through HEV formation. *Sci Trans Med*. 2017;9 https://doi.org/10.1126/scitranslmed.aak9679.
30. Crane GM, Jeffery E, Morrison SJ. Adult haematopoietic stem cell niches. *Nature Rev Immunol*. 2017;17:573–590. https://doi.org/10.1038/nri.2017.53.
31. Galán-Díez M, Cuesta-Domínguez Á, Kousteni S. The bone marrow microenvironment in health and myeloid malignancy. *Cold Spring Harb Perspect Med*. 2018;8 https://doi.org/10.1101/cshperspect.a031328.
32. Barbier V, et al. Endothelial E-selectin inhibition improves acute myeloid leukaemia therapy by disrupting vascular niche-mediated chemoresistance. *Nature Commun*. 2020;11:2042. https://doi.org/10.1038/s41467-020-15817-5.
33. Wettschureck N, Strilic B, Offermanns S. Passing the vascular barrier: endothelial signaling processes controlling extravasation. *Physiol Rev*. 2019;99:1467–1525. https://doi.org/10.1152/physrev.00037.2018.
34. Dvorak AM, Feng D. The vesiculo-vacuolar organelle (VVO). A new endothelial cell permeability organelle. *J Histochem Cytochem*. 2001;49:419–432. https://doi.org/10.1177/002215540104900401.
35. Chiu JJ, Chien S. Effects of disturbed flow on vascular endothelium: pathophysiological basis and clinical perspectives. *Physiol Rev*. 2011;91:327–387. https://doi.org/10.1152/physrev.00047.2009.
36. Coenen DM, Mastenbroek TG, Cosemans JMEM. Platelet interaction with activated endothelium: mechanistic insights from microfluidics. *Blood*. 2017;130:2819–2828. https://doi.org/10.1182/blood-2017-04-780825.
37. Yau JW, Teoh H, Verma S. Endothelial cell control of thrombosis. *BMC Cardiovasc Disord*. 2015;15:130. https://doi.org/10.1186/s12872-015-0124-z.
38. Bombeli T, Karsan A, Tait JF, Harlan JM. Apoptotic vascular endothelial cells become procoagulant. *Blood*. 1997;89:2429–2442.
39. Piccin A, Murphy WG, Smith OP. Circulating microparticles: pathophysiology and clinical implications. *Blood Rev*. 2007;21:157–171. https://doi.org/10.1016/j.blre.2006.09.001.
40. Sturtzel C. Endothelial cells. *Adv Med Exp Biol*. 2017;1003:71–91. https://doi.org/10.1007/978-3-319-57613-8_4.
41. Campbell WB, Gauthier KM. Inducible endothelium-derived hyperpolarizing factor: role of the 15-lipoxygenase-EDHF pathway. *J Cardiovasc Pharmacol*. 2013;61:176–187. https://doi.org/10.1097/FJC.0b013e31828165db.
42. Kreuger J, Phillipson M. Targeting vascular and leukocyte communication in angiogenesis, inflammation and fibrosis. *Nature Rev Drug Discov*. 2016;15:125–142. https://doi.org/10.1038/nrd.2015.2.
43. Schnoor M, Alcaide P, Voisin MB, van Buul JD. Crossing the vascular wall: common and unique mechanisms exploited by different leukocyte subsets during extravasation. *Mediators Inflamm*. 2015;2015:946509. https://doi.org/10.1155/2015/946509.
44. Jackson DG. Leucocyte trafficking via the lymphatic vasculature—mechanisms and consequences. *Front in Immunol*. 2019;10:471. https://doi.org/10.3389/fimmu.2019.00471.
45. Luster AD, Alon R, von Andrian UH. Immune cell migration in inflammation: present and future therapeutic targets. *Nature Immunol*. 2005;6:1182–1190. https://doi.org/10.1038/ni1275.
46. Ley K, Reutershan J. Leucocyte-endothelial interactions in health and disease. *Handbook of Experimental Pharmacology*. 2006:97–133. https://doi.org/10.1007/3-540-36028-x_4.
47. Hordijk PL. Recent insights into endothelial control of leukocyte extravasation. *Cell Mol Life Sci*. 2016;73:1591–1608. https://doi.org/10.1007/s00018-016-2136-y.
48. Strilic B, et al. Tumour-cell-induced endothelial cell necroptosis via death receptor 6 promotes metastasis. *Nature*. 2016;536:215–218. https://doi.org/10.1038/nature19076.
49. Muller WA. Transendothelial migration: unifying principles from the endothelial perspective. *Immunol Rev*. 2016;273:61–75. https://doi.org/10.1111/imr.12443.
50. Li H, Shi FH, Huang SY, Zhang SG, Chen ML. A review on clinical pharmacokinetics, pharmacodynamics, and pharmacogenomics of natalizumab: a humanized anti-α4 integrin monoclonal antibody. *Curr Drug Metabol*. 2018;19:1213–1223. https://doi.org/10.2174/1389200219666180427165841.
51. Rayes J, Bourne JH, Brill A, Watson SP. The dual role of platelet-innate immune cell interactions in thrombo-inflammation. *Res Pract Thromb Hemost*. 2020;4:23–35. https://doi.org/10.1002/rth2.12266.
52. Kremer Hovinga JA, Heeb SR, Skowronska M, Schaller M. Pathophysiology of thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. *J Thromb Hemost*. 2018;16:618–629. https://doi.org/10.1111/jth.13956.
53. Palomo M, Diaz-Ricart M, Carreras E. Is sickle cell disease-related neurotoxicity a systemic endotheliopathy? *Hematol Oncol Stem Cell Ther*. 2020;13:111–115. https://doi.org/10.1016/j.hemonc.2019.12.005.

54. Zhang D, Xu C, Manwani D, Frenette PS. Neutrophils, platelets, and inflammatory pathways at the nexus of sickle cell disease pathophysiology. *Blood*. 2016;127:801–809. https://doi.org/10.1182/blood-2015-09-618538.
55. Garnier Y, et al. Plasma microparticles of sickle patients during crisis or taking hydroxyurea modify endothelium inflammatory properties. *Blood*. 2020;136:247–256. https://doi.org/10.1182/blood.2020004853.
56. Guarda CCD, et al. Heme-mediated cell activation: the inflammatory puzzle of sickle cell anemia. *Expert Rev Hematol*. 2017;10:533–541. https://doi.org/10.1080/17474086.2017.1327809.
57. Kanter J, Lanzkron S. Innovations in targeted anti-adhesion treatment for sickle cell disease. *Clin Pharmacol Ther*. 2020;107:140–146. https://doi.org/10.1002/cpt.1682.
58. Telen MJ, Malik P, Vercellotti GM. Therapeutic strategies for sickle cell disease: towards a multi-agent approach. *Nature Rev Drug Discov*. 2019;18:139–158. https://doi.org/10.1038/s41573-018-0003-2.
59. Wautier JL, Wautier MP. Molecular basis of erythrocyte adhesion to endothelial cells in diseases. *Clin Hemorheol Microcir*. 2013;53:11–21. https://doi.org/10.3233/ch-2012-1572.
60. Lusis AJ. Genetics of atherosclerosis. *Trends Genet*. 2012;28:267–275.
61. Ungvari Z, et al. Endothelial dysfunction and angiogenesis impairment in the ageing vasculature. *Nature Rev Cardiol*. 2018;15:555–565. https://doi.org/10.1038/s41569-018-0030-z.
62. Pons S, Fodil S, Azoulay E, Zafrani L. The vascular endothelium: the cornerstone of organ dysfunction in severe SARS-CoV-2 infection. *Crit Care*. 2020;24:353. https://doi.org/10.1186/s13054-020-03062-7.
63. Mondal R, et al. COVID-19: Are we dealing with a multisystem vasculopathy in disguise of a viral infection? *J Thromb Thrombol*. 2020:1–13. https://doi.org/10.1007/s11239-020-02210-8.
64. Connors JM, Levy JH. COVID-19 and its implications for thrombosis and anticoagulation. *Blood*. 2020;135:2033–2040. https://doi.org/10.1182/blood.2020006000.
65. Al-Samkari H, et al. COVID-19 and coagulation: bleeding and thrombotic manifestations of SARS-CoV-2 infection. *Blood*. 2020;136:489–500. https://doi.org/10.1182/blood.2020006520.
66. Rak J. Extracellular vesicles - biomarkers and effectors of the cellular interactome in cancer. *Front Pharmacol*. 2013;4:21. https://doi.org/10.3389/fphar.2013.00021.
67. Betsholtz C. Cell-cell signaling in blood vessel development and function. *EMBO Mol Med*. 2018;10 https://doi.org/10.15252/emmm.201708610.
68. Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol*. 2002;20:4368–4380.
69. Ferrara N, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*. 1996;380:439–442.
70. Karaman S, Leppänen VM, Alitalo K. Vascular endothelial growth factor signaling in development and disease. *Development*. 2018;145 https://doi.org/10.1242/dev.151019.
71. Stevens M, Oltean S. Modulation of receptor tyrosine kinase activity through alternative splicing of ligands and receptors in the VEGF-A/VEGFR axis. *Cells*. 2019;8 https://doi.org/10.3390/cells8040288.
72. Heldin CH, Lennartsson J, Westermark B. Involvement of platelet-derived growth factor ligands and receptors in tumorigenesis. *J Inte Med*. 2018;283:16–44. https://doi.org/10.1111/joim.12690.
73. Negri L, Ferrara N. The prokineticins: neuromodulators and mediators of Inflammation and myeloid cell-dependent angiogenesis. *Physio Rev*. 2018;98:1055–1082. https://doi.org/10.1152/physrev.00012.2017.
74. Augustin HG, Koh GY, Thurston G, Alitalo K. Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. *Nat Rev Mol Cell Biol*. 2009;10:165–177.
75. Su T, Iwakiri Y. Novel endothelial LECT2/Tie1 signaling in liver fibrosis. *Hepatology*. 2020 https://doi.org/10.1002/hep.31183.
76. Xu M, et al. LECT2, a ligand for Tie1, plays a crucial role in liver fibrogenesis. *Cell*. 2019;178 https://doi.org/10.1016/j.cell.2019.07.021. 1478-1492.e1420.
77. Tetzlaff F, Fischer A. Control of blood vessel formation by notch signaling. *Adv Exp Med Biol*. 2018;1066:319–338. https://doi.org/10.1007/978-3-319-89512-3_16.
78. Fujiwara M, Ghazizadeh M, Kawanami O. Potential role of the Slit/Robo signal pathway in angiogenesis. *Vascular Med*. 2006;11:115–121. https://doi.org/10.1191/1358863x06vm658ra.
79. Caradu C, et al. Endogenous sonic hedgehog limits inflammation and angiogenesis in the ischaemic skeletal muscle of mice. *Cardiovasc Res*. 2018;114:759–770. https://doi.org/10.1093/cvr/cvy017.
80. Phoenix TN, et al. Medulloblastoma genotype dictates blood brain barrier phenotype. *Cancer Cell*. 2016;29:508–522. https://doi.org/10.1016/j.ccell.2016.03.002.
81. le Noble F, Klein C, Tintu A, Pries A, Buschmann I. Neural guidance molecules, tip cells, and mechanical factors in vascular development. *Cardiovas Res*. 2008;78:232–241. https://doi.org/10.1093/cvr/cvn058.
82. Gerhardt H, et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol*. 2003;161:1163–1177.
83. Carmeliet P. Blood vessels and nerves: common signals, pathways and diseases. *Nat Rev Genet*. 2003;4:710–720. https://doi.org/10.1038/nrg1158.
84. Goumans MJ, Ten Dijke P. TGF-β signaling in control of cardiovascular function. *Cold Spring Harb Perspect Biol*. 2018;10 https://doi.org/10.1101/cshperspect.a022210.
85. Guerrero PA, McCarty JH. Integrins in vascular development and pathology. *Adv Pharmacol*. 2018;81:129–153. https://doi.org/10.1016/bs.apha.2017.08.011.
86. De Palma M, Biziato D, Petrova TV. Microenvironmental regulation of tumour angiogenesis. *Nat Rev Cancer*. 2017;17:457–474. https://doi.org/10.1038/nrc.2017.51.
87. Davis GE, Senger DR. Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. *Circ Res*. 2005;97:1093–1107. https://doi.org/10.1161/01.RES.0000191547.64391.e3.
88. Halper J. Basic components of vascular connective tissue and extracellular matrix. *Adv Pharmacol*. 2018;81:95–127. https://doi.org/10.1016/bs.apha.2017.08.012.
89. Baluk P, Morikawa S, Haskell A, Mancuso M, McDonald DM. Abnormalities of basement membrane on blood vessels and endothelial sprouts in tumors. *Am J Pathol*. 2003;163:1801–1815. https://doi.org/10.1016/s0002-9440(10)63540-7.
90. Avraamides CJ, Garmy-Susini B, Varner JA. Integrins in angiogenesis and lymphangiogenesis. *Nat Rev Cancer*. 2008;8:604–617.
91. Reynolds LE, et al. Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. *Nat Med*. 2002;8:27–34.
92. Leong KG, et al. Activated notch4 inhibits angiogenesis: role of beta 1-integrin activation. *Mol Cell Biol*. 2002;22:2830–2841. https://doi.org/10.1128/mcb.22.8.2830-2841.2002.
93. Wang X, Khalil RA. Matrix metalloproteinases, vascular remodeling, and vascular disease. *Adv Pharmacol*. 2018;81:241–330. https://doi.org/10.1016/bs.apha.2017.08.002.
94. van Hinsbergh VWM, Engelse MA, Quax PH. Pericellular proteases in angiogenesis and vasculogenesis. *Arterioscler Thromb Vasc Biol*. 2006;26:716–728.
95. Vlodavsky I, et al. Heparanase: from basic research to therapeutic applications in cancer and inflammation. Drug Resistance Updates: Reviews and Commentaries in Antimicrobial and Anticancer. *Chemotherapy*. 2016;29:54–75. https://doi.org/10.1016/j.drup.2016.10.001.
96. Wang X, Khalil RA. Matrix metalloproteinases, vascular remodeling, and vascular disease. *Adv Pharmacol*. 2018;81:241–330. https://doi.org/10.1016/bs.apha.2017.08.002.
97. Presta M, Chiodelli P, Giacomini A, Rusnati M, Ronca R. Fibroblast growth factors (FGFs) in cancer: FGF traps as a new therapeutic approach. *Pharmacol Ther*. 2017;179:171–187. https://doi.org/10.1016/j.pharmthera.2017.05.013.
98. Wolberg AS, et al. Venous thrombosis. *Nat Rev Dise Primers*. 2015;1:15006. https://doi.org/10.1038/nrdp.2015.6.
99. Zelaya H, Rothmeier AS, Ruf W. Tissue factor at the crossroad of coagulation and cell signaling. *J Thromb Haemost*. 2018;16:1941–1952. https://doi.org/10.1111/jth.14246.
100. Albrektsen T, et al. Transcriptional program induced by factor VIIa-tissue factor, PAR1 and PAR2 in MDA-MB-231 cells. *J Thromb Haemost*. 2007;5:1588–1597.
101. Karpatkin S. Does hypercoagulability awaken dormant tumor cells in the host? *J Thromb Haemost*. 2004;2:2103–2106.
102. Carmeliet P, et al. Role of tissue factor in embryonic blood vessel development. *Nature*. 1996;383:73–75.
103. Tawil N, Bassawon R, Rak J. Oncogenes and clotting factors: the emerging role of tumor cell genome and epigenome in cancer-associated thrombosis. *Semin Thromb Hemost*. 2019;45:373–384. https://doi.org/10.1055/s-0039-1687891.
104. Coughlin SR. Protease-activated receptors in hemostasis, thrombosis and vascular biology. *J. Thromb Haemost*. 2005;3:1800–1814.

105. Nierodzik ML, Karpatkin S. Thrombin induces tumor growth, metastasis, and angiogenesis: evidence for a thrombin-regulated dormant tumor phenotype. *Cancer Cell*. 2006;10:355–362.
106. Uchiba M, et al. Activated protein C induces endothelial cell proliferation by mitogen-activated protein kinase activation in vitro and angiogenesis in vivo. *Circ Res*. 2004;95:34–41. https://doi.org/10.1161/01.res.0000133680.87668.fa.
107. Macbeth F, et al. Randomized phase III trial of standard therapy plus low molecular weight heparin in patients with lung cancer: fragmatic trial. *J Clin Oncol*. 2016;34:488–494. https://doi.org/10.1200/jco.2015.64.0268.
108. Sanford D, Naidu A, Alizadeh N, Lazo-Langner A. The effect of low molecular weight heparin on survival in cancer patients: an updated systematic review and meta-analysis of randomized trials. *J Thromb Haemost*. 2014;12:1076–1085. https://doi.org/10.1111/jth.12595.
109. Browder T, Folkman J, Pirie-Shepherd S. The hemostatic system as a regulator of angiogenesis. *J Biol Chem*. 2000;275:1521–1524.
110. Kwaan HC, Mazar AP, McMahon BJ. The apparent uPA/PAI-1 paradox in cancer: more than meets the eye. *Semin Thromb Hemost*. 2013;39:382–391. https://doi.org/10.1055/s-0033-1338127.
111. D'Asti E, et al. Tissue factor regulation by miR-520g in primitive neuronal brain tumor cells: a possible link between oncomirs and the vascular tumor microenvironment. *Am J Pathol*. 2016;186:446–459.
112. Auvergne R, et al. PAR1 inhibition suppresses the self-renewal and growth of A2B5-defined glioma progenitor cells and their derived gliomas in vivo. *Oncogene*. 2016;35:3817–3828. https://doi.org/10.1038/onc.2015.452.
113. Quintanilla M, Montero-Montero L, Renart J, Martin-Villar E. Podoplanin in inflammation and cancer. *Int J Mol Sci*. 2019;20 https://doi.org/10.3390/ijms20030707.
114. Gur-Cohen S, et al. PAR1 signaling regulates the retention and recruitment of EPCR-expressing bone marrow hematopoietic stem cells. *Nature Med*. 2015;21:1307–1317. https://doi.org/10.1038/nm.3960.
115. van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol*. 2018;19:213–228. https://doi.org/10.1038/nrm.2017.125. 2018.
116. Arraud N, et al. Extracellular vesicles from blood plasma: determination of their morphology, size, phenotype and concentration. *J Thromb Haemost*. 2014;12:614–627. https://doi.org/10.1111/jth.12554.
117. Verweij FJ, Hyenne V, Van Niel G, Goetz JG. Extracellular vesicles: catching the light in zebrafish. *Trends Cell Biol*. 2019 https://doi.org/10.1016/j.tcb.2019.07.007.
118. Del Conde I, Shrimpton CN, Thiagarajan P, Lopez JA. Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. *Blood*. 2005;106:1604–1611.
119. Folkman J. Endogenous angiogenesis inhibitors. *APMIS: Acta Pathol Microbiol Immunol Scand*. 2004;112:496–507. https://doi.org/10.1111/j.1600-0463.2004.apm11207-0809.x.
120. Potente M, Mäkinen T. Vascular heterogeneity and specialization in development and disease. *Nat Rev Mol Cell Biol*. 2017;18:477–494. https://doi.org/10.1038/nrm.2017.36.
121. Rossant J, Hirashima M. Vascular development and patterning: making the right choices. *Curr Opin Genet Dev*. 2003;13:408–412.
122. Medina RJ, et al. Endothelial progenitors: a consensus statement on nomenclature. *Stem Cells Transl Med*. 2017;6:1316–1320. https://doi.org/10.1002/sctm.16-0360.
123. Xu Q. The impact of progenitor cells in atherosclerosis. *Nat Clin Pract Cardiovasc Med*. 2006;3:94–101.
124. Kuczynski EA, Vermeulen PB, Pezzella F, Kerbel RS, Reynolds AR. Vessel co-option in cancer. *Nat Rev Clin Oncol*. 2019 https://doi.org/10.1038/s41571-019-0181-9.
125. Dome B, Hendrix MJ, Paku S, Tovari J, Timar J. Alternative vascularization mechanisms in cancer: pathology and therapeutic implications. *Am J Pathol*. 2007;170:1–15.
126. Nagy JA, Dvorak HF. Heterogeneity of the tumor vasculature: the need for new tumor blood vessel type-specific targets. *Clin Exp Metastasis*. 2012;29:657–662.
127. Heil M, Schaper W. Arteriogenic growth factors, chemokines and proteases as a prerequisite for arteriogenesis. *Drug News Perspect*. 2005;18:317–322.
128. Cheng L, et al. Glioblastoma stem cells generate vascular pericytes to support vessel function and tumor growth. *Cell*. 2013;153:139–152.
129. Ricci-Vitiani L, et al. Tumour vascularization via endothelial differentiation of glioblastoma stem-like cells. *Nature*. 2010;468:824–828.
130. Welti J, Loges S, Dimmeler S, Carmeliet P. Recent molecular discoveries in angiogenesis and antiangiogenic therapies in cancer. *J Clin Invest*. 2013;123:3190–3200.
131. Dvorak HF. Tumors: wounds that do not heal-a historical perspective with a focus on the fundamental roles of increased vascular permeability and clotting. *Semin Thromb Hemost*. 2019;45:576–592. https://doi.org/10.1055/s-0039-1687908.
132. Farnsworth RH, Lackmann M, Achen MG, Stacker SA. Vascular remodeling in cancer. *Oncogene*. 2014;33:3496–3505. https://doi.org/10.1038/onc.2013.304.
133. Peters BA, et al. Contribution of bone marrow-derived endothelial cells to human tumor vasculature. *Nat Med*. 2005;11:261–262.
134. Li X, Kumar A, Carmeliet P. Metabolic pathways fueling the endothelial cell drive. *Ann Rev Physiol*. 2019;81:483–503. https://doi.org/10.1146/annurev-physiol-020518-114731.
135. Thurston G, Noguera-Troise I, Yancopoulos GD. The Delta paradox: DLL4 blockade leads to more tumour vessels but less tumour growth. *Nat Rev Cancer*. 2007;7:327–331.
136. Gaengel K, Genove G, Armulik A, Betsholtz C. Endothelial-mural cell signaling in vascular development and angiogenesis. *Arterioscler Thromb Vasc Biol*. 2009;29:630–638.
137. Stacker SA, et al. Lymphangiogenesis and lymphatic vessel remodelling in cancer. *Nature Reviews Cancer*. 2014;14:159–172. https://doi.org/10.1038/nrc3677.
138. Alitalo K. The lymphatic vasculature in disease. *Nat Med*. 2011;17:1371–1380.
139. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Biol*. 2007;8:464–478. https://doi.org/10.1038/nrm2183.
140. Jha SK, Rauniyar K, Jeltsch M. Key molecules in lymphatic development, function, and identification. *Ann Anat*. 2018;219:25–34. https://doi.org/10.1016/j.aanat.2018.05.003.
141. Korn C, Augustin HG. Mechanisms of vessel pruning and regression. *Developme Cell*. 2015;34:5–17. https://doi.org/10.1016/j.devcel.2015.06.004.
142. Watson EC, Grant ZL, Coultas L. Endothelial cell apoptosis in angiogenesis and vessel regression. *Cell Mol Life Sci*. 2017;74:4387–4403. https://doi.org/10.1007/s00018-017-2577-y.
143. Heil M, Eitenmüller I, Schmitz-Rixen T, Schaper W. Arteriogenesis versus angiogenesis: similarities and differences. *J Cell Mol Med*. 2006;10:45–55. https://doi.org/10.1111/j.1582-4934.2006.tb00290.x.
144. Heil M, Schaper W. Influence of mechanical, cellular, and molecular factors on collateral artery growth (arteriogenesis). *Circ Res*. 2004;95:449–458.
145. Rohan RM, Fernandez A, Udagawa T, Yuan J, D'Amato RJ. Genetic heterogeneity of angiogenesis in mice. *FASEB*. 2000;14:871–876.
146. Rey S, Semenza GL. Hypoxia-inducible factor-1-dependent mechanisms of vascularization and vascular remodelling. *Cardiovasc Res*. 2010;86:236–242.
147. Rak J. *RAS* oncogenes and tumor-vasculature interface. In: Thomas-Tikhonenko A, ed. *Cancer Genome and Tumor Microenvironment*. New York: Springer; 2009. p.133–165.
148. Inampudi C, Akintoye E, Ando T, Briasoulis A. Angiogenesis in peripheral arterial disease. *Curr Opin Pharmacol*. 2018;39:60–67. https://doi.org/10.1016/j.coph.2018.02.011.
149. Simons M. Angiogenesis: where do we stand now? *Circulation*. 2005;111:1556–1566. https://doi.org/10.1161/01.cir.0000159345.00591.8f.
150. Cook KM, Figg WD. Angiogenesis inhibitors: current strategies and future prospects. *CA Cancer J Clin*. 2010;60:222–243.
151. Vennepureddy A, Singh P, Rastogi R, Atallah JP, Terjanian T. Evolution of ramucirumab in the treatment of cancer—a review of literature. *J Oncol Pharmacy Pract*. 2017;23:525–539. https://doi.org/10.1177/1078155216655474.
152. Rafii S, Butler JM, Ding BS. Angiocrine functions of organ-specific endothelial cells. *Nature*. 2016;529:316–325. https://doi.org/10.1038/nature17040.
153. Wong PP, Bodrug N, Hodivala-Dilke KM. Exploring novel methods for modulating tumor blood vessels in cancer treatment. *Curr Biol*. 2016;26:R1161–r1166. https://doi.org/10.1016/j.cub.2016.09.043.
154. Medinger M, Mross K. Clinical trials with anti-angiogenic agents in hematological malignancies. *J Angiogenes Res*. 2010;2:10.
155. Verheul HM, Pinedo HM. Possible molecular mechanisms involved in the toxicity of angiogenesis inhibition. *Nat Rev Cancer*. 2007;7:475–485. https://doi.org/10.1038/nrc2152.
156. Jayson GC, Kerbel R, Ellis LM, Harris AL. Antiangiogenic therapy in oncology: current status and future directions. *Lancet*. 2016;388:518–529. https://doi.org/10.1016/s0140-6736(15)01088-0.
157. Zangari M, et al. Thrombotic events in patients with cancer receiving antiangiogenesis agents. *J Clin Oncol*. 2009;27:4865–4873.

158. Elice F, Rodeghiero F. Bleeding complications of antiangiogenic therapy: pathogenetic mechanisms and clinical impact. *Thromb Res*. 2010;125(Suppl 2):S55–S57.
159. Lacaud G, Kouskoff V. Hemangioblast, hemogenic endothelium, and primitive versus definitive hematopoiesis. *Exp Hematol*. 2017;49:19–24. https://doi.org/10.1016/j.exphem.2016.12.009.
160. Ottersbach K. Endothelial-to-haematopoietic transition: an update on the process of making blood. *Biochem Soc Trans*. 2019;47:591–601. https://doi.org/10.1042/bst20180320.
161. Dzierzak E, Bigas A. Blood development: hematopoietic stem cell dependence and independence. *Cell Stem Cell*. 2018;22:639–651. https://doi.org/10.1016/j.stem.2018.04.015.
162. Gao L, et al. RUNX1 and the endothelial origin of blood. *Exp Hematol*. 2018;68:2–9. https://doi.org/10.1016/j.exphem.2018.10.009.
163. Pinho S, Frenette PS. Haematopoietic stem cell activity and interactions with the niche. *Nat Rev Mol Cell Biology*. 2019;20:303–320. https://doi.org/10.1038/s41580-019-0103-9.
164. Batsivari A, et al. Dynamic responses of the haematopoietic stem cell niche to diverse stresses. *Nat Cell Biol*. 2020;22:7–17. https://doi.org/10.1038/s41556-019-0444-9.
165. van Pel M, Fibbe WE, Schepers K. The human and murine hematopoietic stem cell niches: are they comparable? *Ann NY Acad Sci*. 2016;1370:55–64. https://doi.org/10.1111/nyas.12994.

CHAPTER 123

MEGAKARYOCYTE AND PLATELET STRUCTURE

Kellie R. Machlus and Joseph E. Italiano, Jr.

Platelets are small anucleate fragments that are formed from the cytoplasm of megakaryocytes and have a characteristic discoid shape. To assemble and release platelets, megakaryocytes become polyploid by endomitosis and follow a maturation program that results in the conversion of the bulk of their cytoplasm into multiple long processes called *proplatelets*. To produce its quota of 1000 to 2000 platelets, a megakaryocyte may release as many as 10 to 20 proplatelets. Platelets then form selectively at the ends of proplatelets. As platelets develop, their content of granules and organelles is delivered to them in a stream of individual particles moving from the megakaryocyte cell body to the nascent platelet buds at the proplatelet tips. Platelet formation can be arbitrarily divided into two phases. The first phase of megakaryocyte differentiation and maturation takes days to complete and requires megakaryocyte-specific growth factors such as thrombopoietin (TPO). Massive nuclear proliferation without cell division (endomitosis) to 16 to 32 N and enlargement of the megakaryocyte cytoplasm occur because the megakaryocyte is filled with cytoskeletal proteins, platelet-specific granules, and sufficient membrane to complete the platelet assembly process. The second phase is relatively rapid and can be completed in hours. During this phase, megakaryocytes generate platelets by remodeling their cytoplasm first into proplatelets, then preplatelets, which are released into the circulation, where they undergo repeated fission to generate discoid platelets.

MEGAKARYOCYTE DEVELOPMENT

Endomitosis

Hematopoietic stem cells, which are endowed with the genetic capacity to differentiate into all blood cell lineages, are induced down the pathway to become megakaryocytes by their exposure to growth factors such as TPO.[1] Megakaryocytes become polyploid (i.e., 4 N, 16 N, 32 N, 64 N) through repeated cycles of deoxyribonucleic acid (DNA) replication without cell division.[2–5] Normal ploidy ranges from 4 to 64 times the haploid DNA complement, but the majority of cells fall within three ploidy classes (8 N, 16 N, and 32 N), with 16 N being dominant (Fig. 123.1).[6,7] Ploidy number appears to be a predetermined event, possibly signifying genetic diversity among megakaryocyte populations. Megakaryocyte polyploidization results in a functional gene amplification whose likely function is an increase in protein synthesis.[8] This process, called *endomitosis*, is a shortened mitosis caused by a block in late anaphase, thereby bypassing cytokinesis.[9,10]

Cells undergoing diploid mitoses proceed through cytokinesis and complete abscission division, whereas megakaryocytes exhibit regression of the cleavage furrow and reenter G_1 as polyploid cells.[1] During polyploidization of megakaryocytes, the nuclear envelope breaks down, and an abnormal spherical mitotic spindle forms. The spindle has attached chromosomes that align from a position equidistant from the spindle poles. Sister chromatids segregate and move toward their respective poles (anaphase A). However, the spindle poles fail to move apart and do not undergo the microtubule-driven separation typically observed during anaphase B. Individual chromatids are not moved to the poles, and subsequently a single nuclear envelope encapsulates the entire set of sister chromatids.[9,10] In most cell types, checkpoints and feedback controls ensure that DNA replication and cell division are tightly coupled. Megakaryocytes appear to be an exception to this rule, indicating they have managed to deregulate this process. Proposed mechanisms for regulating endomitosis include a reduction in mitosis-promoting factor[11,12] or decreased expression of cyclin B.[12–15] Cyclins appear to play a critical role in directing endomitosis. Cyclin D3 is overexpressed in the G_1 phase of maturing megakaryocytes,[16] but triple knockout of cyclins D1, D2, and D3 in mice does not appear to affect megakaryocyte development.[17] In contrast, cyclin E–deficient mice exhibit a profound defect in megakaryocyte development.[18] The molecular programming involved in endomitosis is characterized by the mislocalization or absence of at least two critical regulators of mitosis: the chromosomal passenger proteins Aurora-B/AIM-1 and Survivin. AIM-1 (absent in melanoma 1), a serine/threonine kinase in the Aurora family that is implicated in mitosis, is downregulated as megakaryocyte polyploidization occurs, suggesting its loss may lead to the abortive mitosis and polyploidization.[19,20] However, while Aurora kinase A is required for hematopoiesis, it is dispensable for mouse megakaryocyte endomitosis and differentiation.[21] Whereas deletion of the anaphase promoting complex (APC)/C cofactor Cdc20 causes mitotic arrest and severe thrombocytopenia, lack of the kinases Aurora-B, Cdk1, or Cdk2 does not affect endomitosis of megakaryocytes or platelet counts. Deletion of Cdk1 forces a change to endocycles without mitosis, whereas polyploidization in the absence of cdk1 and cdk2 occurs in the presence of aberrant replication events. Notably, ablation of these kinases rescues defects in Cdc20-null megakaryocytes. These observations suggest that endomitosis can be functionally replaced by alternative polyploidization mechanisms in vivo. One explanation for endomitosis could be inhibition of microtubule-based forces in anaphase B. Spindle pole separation during anaphase B is thought to be powered by the sliding of antiparallel interdigitating microtubules past each other[22] mediated by the mitotic kinesin-like protein 1. This protein localizes at regions of overlapping microtubules during anaphase B, and studies in vitro indicate that it can slide microtubules past each other.[23] Therefore lack of spindle pole separation during endomitosis may result from failure of megakaryocytes to undergo normal spindle orientation and/or the absence of signals that localize or activate a kinesin motor molecule that provides force for sliding.

Cytoplasmic Maturation

Megakaryocytes, the largest of the hematopoietic cells, undergo a pronounced cytoplasmic maturation to attain their large volumes. Cytoplasmic maturation begins during endomitosis and increases considerably after all DNA amplification has ended (Fig. 123.2). Megakaryocytes enlarge dramatically as they mature, reaching sizes of 100 to 150 µm in diameter in culture and in bone marrow. During this process, the megakaryocyte cytoplasm rapidly fills with platelet-specific proteins, organelles, and membrane systems that ultimately are subdivided and packaged into platelets (Fig. 123.3). Their cytoplasmic space expands and, except for the most cortical regions, becomes densely filled with internal membranes that subsequently serve as the repository for the plasma membrane to be regurgitated for coating proplatelets as they extend.[24] This internal membrane system, which is one of the most striking features of mature megakaryocytes, has been referred to as the *demarcation membrane system* (DMS) or, more recently, the invaginated membrane system (IMS). The DMS, first described by Yamada in 1957, consists of an extensive, tortuous, branching network of membrane channels composed of flattened

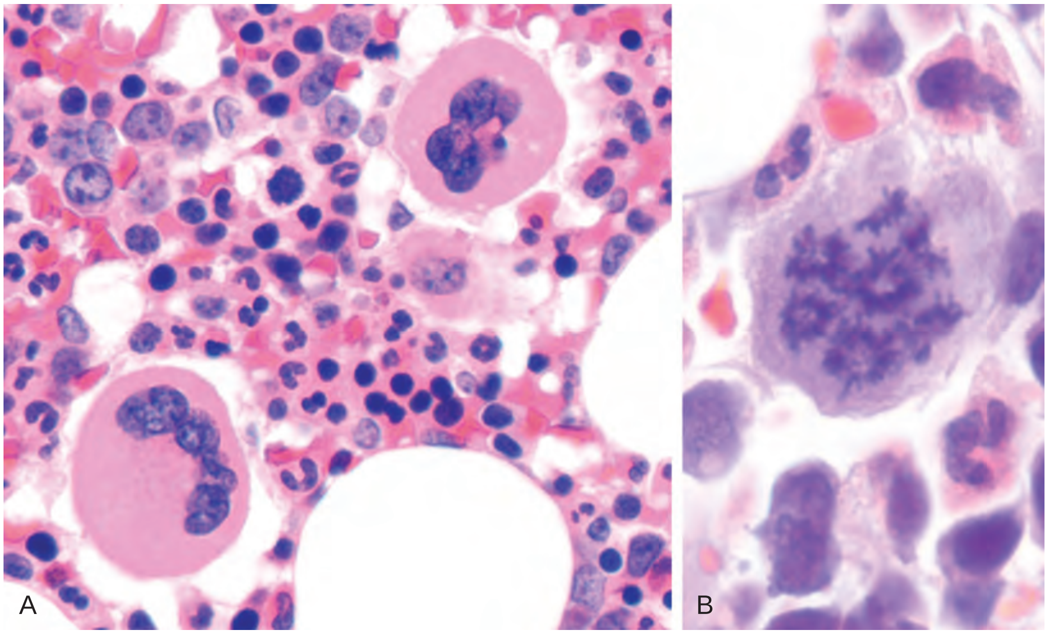


Figure 123.1 POLYPLOID MEGAKARYOCYTES IN THE BONE MARROW. Large polyploid megakaryocytes are seen in the bone marrow on a typical hematoxylin and eosin–stained slide and are recognized by their abundant pink cytoplasm and large nuclei (A). The degree of polyploidization is difficult to determine. Rarely, megakaryocytes can be seen in mitosis (B), and when chromosomes are aligned in metaphase plates, the high ploidy level become quite apparent. In the mitotic figure illustrated, the megakaryocyte is a 16 N form with eight 2 N metaphase plates.

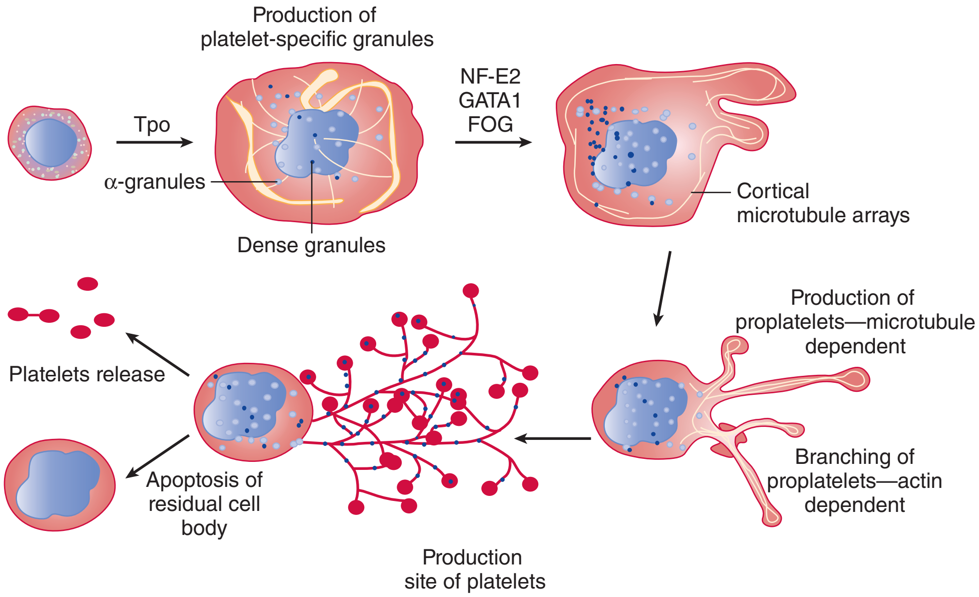

Production of platelet-specific granules
Tpo
α-granules
Dense granules
NF-E2
GATA1
FOG
Cortical microtubule arrays
Production of proplatelets—microtubule dependent
Branching of proplatelets—actin dependent
Production site of platelets
Platelets release
Apoptosis of residual cell body

Figure 123.2 SUMMARY OF THE MAJOR EVENTS THAT LEAD TO PLATELET FORMATION AND RELEASE FROM MEGAKARYOCYTES. Hematopoietic stem cells are converted into megakaryocytes by exposure to the specific growth factor thrombopoietin (TPO). TPO initiates a maturation program that amplifies the megakaryocyte DNA and leads to synthesis of platelet-specific proteins. In particular, cytoskeletal elements, membrane systems, and receptor proteins are made in bulk, and the megakaryocyte becomes filled with platelet-specific granules. Platelet production begins when microtubules aggregate in the cell cortex, and one pole of the megakaryocyte spontaneously elaborates pseudopodia. These begin as large blunt pseudopodia, which subsequently thin and branch into proplatelets. The branching reaction is dependent on a localized assembly of actin and is inhibited by drugs that disrupt actin filaments. Platelets are assembled primarily at the ends of the proplatelets. Intracellular organelles are delivered to the platelet buds along microtubule tracks in the shafts. Platelets are released from the ends of proplatelets. *FOG*, Friend of *GATA1*; *GATA1*, GATA-binding protein 1.

cisternae and tubules. Initially, the DMS was proposed to play an essential role in platelet formation by defining preformed “platelet territories” or “platelet fields” within the megakaryocyte cytoplasm.[25,26] Release of individual platelets was postulated to occur by massive fragmentation of the megakaryocyte cytoplasm along DMS fracture lines between these fields. However, studies demonstrating that platelets are primarily assembled and released from proplatelet ends (see Platelet Formation later in this chapter) are inconsistent with this notion and indicate instead that the DMS functions predominantly as a membrane reserve for proplatelet formation.[24] Direct visualization of mature DMSs containing phosphatidylinositol 4,5-bisphosphate suggests that it is the source of proplatelet membranes.[2] Studies by Eckly et al.[27] have begun to provide insights into how the DMS forms and matures. To develop the DMS, the megakaryocyte

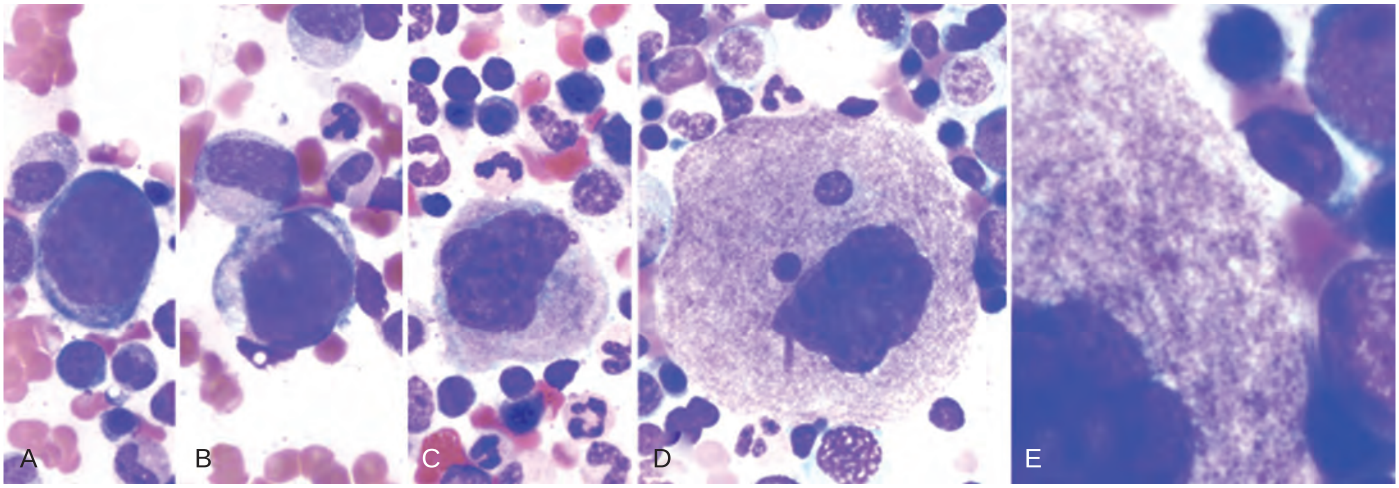


Figure 123.3 PLATELET PRODUCTION IN THE MEGAKARYOCYTE. (A) Immature polyploid megakaryoblast with little differentiation. (B) Megakaryocyte with early Golgi zone. (C) Early platelet production in cytoplasm. (D) Late-stage megakaryocyte with abundant internal membranes, organelles, and platelet-specific proteins. (E) Early formation of demarcation membranes.

plasma membrane enfolds at specific sites to generate a perinuclear pre-DMS. Next, the pre-DMS is expanded into its mature form by material added from Golgi-derived vesicles and by endoplasmic reticulum–mediated lipid transfer. This structural description is in line with studies on platelet glycosyltransferases, which arrive early in the forming DMS and eventually make their way to the megakaryocyte and platelet surface.[28] Thus far, only a handful of proteins that participate in DMS formation have been identified based on alteration in DMS structure in certain knockout mouse models. Membrane-deforming proteins that use F-BAR (Family of Bin-Amphysin-Rvs) domains to curve membranes or use guanosine triphosphate (GTP) as an energy source to bud vesicles from membranes appear to be necessary for normal megakaryocyte maturation and platelet release. Gross disruptions in DMS structure are found in megakaryocytes isolated from either filamin A–knockout, pascin2-knockout, dynamin 2–knockout, or Cdc42-interacting protein 4 (CIP4)-knockout mice. CIP4 is an F-BAR protein that induces membrane tubulation and localizes to membrane lipids via its BAR domain and interacts with the Wiskott-Aldrich syndrome protein (WASp).[29] CIP4$^{-/-}$ mice have mild thrombocytopenia, with a 25% decrease in the platelet count. While megakaryocyte numbers and ploidy are normal in CIP4$^{-/-}$ knockout mice, megakaryocytes isolated from these mice are less effective in producing proplatelets in vitro.

Dynamins are highly conserved, large mechanochemical GTPases involved in endocytosis and vesicle transport, and mutations in dynamin 2 have been associated with thrombocytopenia in humans. Dynamin 2–dependent endocytosis is required for megakaryocyte development in mice.[30] Filamin connects glycoprotein (GP)Ibα to the actin cytoskeleton and binds pacsin2, a molecule that deforms membranes. Pacsin2 has an F-BAR and SH3 domain that binds dynamin and N-WASp. Pacsin2 plays an important role in organizing internal membranes during megakaryocyte development and platelet production.

Maturing megakaryocytes, like other granulated cells, contain an abundance of ribosomes and rough endoplasmic reticulum, where protein synthesis occurs. During this phase of megakaryocyte development, the cytoplasm fills with cytoskeletal proteins, platelet-specific receptors and secretory granules, and normal cellular organelles such as mitochondria and lysosomes. A hallmark of mature megakaryocytes is their abundance of platelet-specific secretory granules. The two specific granules destined for platelets are α-granules and dense granules. α-Granules, the more abundant (approximately 40 to 80 per platelet) and larger of the two (200 to 500 nm in diameter), contain proteins that enhance platelet adhesion, promote cell-cell interactions, regulate angiogenesis, and stimulate vascular repair. α-Granules store matrix proteins and contain GP receptors in their membranes (Fig. 123.4A). The bulk of cellular P-selectin and a portion of $\alpha_{IIb}\beta_3$ and the GPIb/IX/V complex, a receptor for von Willebrand factor (vWF), are expressed in the membranes of α-granules. Adhesion molecules within the granules include, but are not limited to, vWF, fibrinogen, fibronectin, vitronectin, and thrombospondin. α-Granule proteins can be derived from different sources. Some proteins, such as α-thromboglobulin and vWF, are synthesized by megakaryocytes. However, fibrinogen, also a major component of α-granules, is not synthesized by megakaryocytes and is instead taken up from plasma by an endocytic mechanism requiring fibrinogen binding to $\alpha_{IIb}\beta_3$. Although little is known about the intracellular trafficking of proteins in megakaryocytes, experiments using cryosectioning and immunoelectron microscopy suggest that multivesicular bodies are an essential intermediate stage in the formation of platelet α-granules. During megakaryocyte development, large (≈0.5 μm) multivesicular bodies undergo a gradual transition from granules containing 30 to 70 nm internal vesicles to granules containing secretion concentrates.

The second and smaller type of platelet granule is the dense granule. Platelets contain relatively few dense granules (approximately 4 to 6 per platelet), which are approximately 150 nm in diameter. Dense granules have electron opaque cores, which give them a bull's-eye appearance, and function primarily to recruit additional platelets to sites of vascular injury. Dense granules contain soluble activating agents, such as serotonin and adenosine diphosphate (ADP), as well as divalent cations. A novel electron-dense tubular system–related compartment called the T-granule has been described in platelets. The T-granules contain Toll-like receptor 9 (TLR9), which links the innate and adaptive immune response during infectious inflammation. TLR9 colocalizes with protein disulfide isomerase, a vascular thiol isomerase, and is associated with either vesicle-associated membrane protein (VAMP) 7 or VAMP 8. T-granules are believed to be a remnant of the endoplasmic reticulum and are derived from the dense tubular system.[31]

When the megakaryocyte reaches a certain point of maturation, proplatelet production begins, and granules are sent into the proplatelets, destined for platelets.

REGULATION OF MEGAKARYOCYTE DEVELOPMENT

The development of megakaryocytes and the process of platelet biogenesis occur within a complex bone marrow environment where both cytokines and adhesive interactions play an essential role. Megakaryocytes are imprisoned within the subendothelial layer of the bone marrow sinuses, where development and platelet

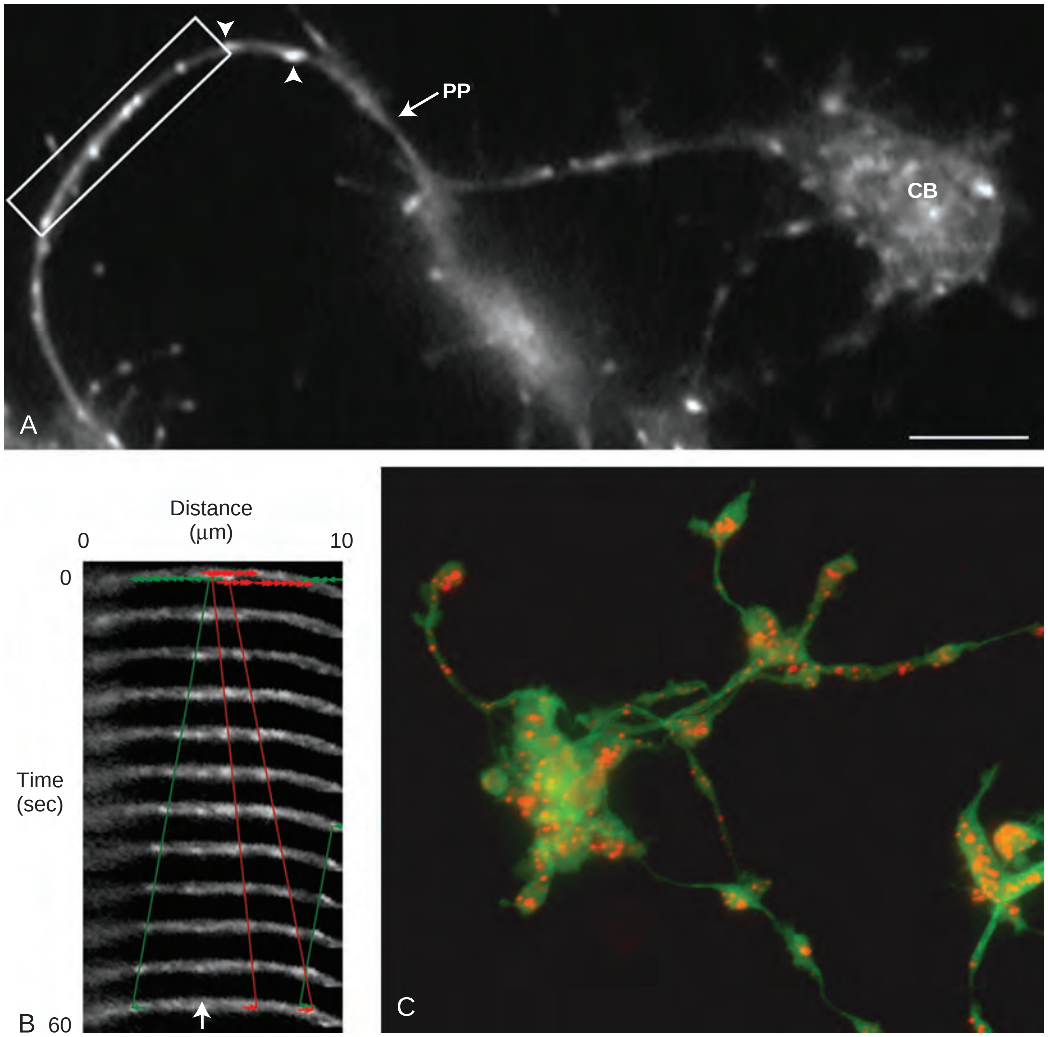


Figure 123.4 MICROTUBULE DYNAMICS DURING PROPLATELET FORMATION. (A) Visualization of plus-end microtubule assembly in living megakaryocytes expressing end-binding protein 3 (EB3)-green fluorescent protein (GFP). First frame from the time-lapse sequence (B) of a living megakaryocyte that was retrovirally directed to express EB3-GFP. The cell body (CB) is at the right of the micrograph, and proplatelets (PP) extend to the left. EB3-GFP labels growing microtubule plus ends in a characteristic "comet" staining pattern that has a bright front and a dim tail. (B) The kymograph shows movement over time. Images are every 5 seconds. EB3-GFP comets undergo bidirectional movements in PP, demonstrating that microtubules are organized as bipolar arrays. Some EB3-GFP comets move toward the tip and are highlighted in green; others that move toward the cell body are highlighted in red. (C) Distribution of α-granules in megakaryocytes and PP projections visualized by fluorescence microscopy. α-Granules are stained with Alexa 568 *(red)*–labeled anti–von Willebrand factor antibodies. The proplatelets have been co-stained with Alexa 488 *(green)* antitubulin antibodies to highlight the microtubules.

biogenesis are regulated at multiple levels by several cytokines. TPO, which is synthesized in the bone marrow and liver, is the principal regulator of thrombopoiesis. TPO also plays a central role in hematopoietic stem cell survival and proliferation. Circulating levels of TPO induce proliferation and maturation of megakaryocyte progenitors by binding to the c-Mpl (myeloproliferative leukemia protein) receptor and signaling induction. TPO regulates all stages of megakaryocyte development, from the hematopoietic stem cell stage through cytoplasmic maturation. TPO increases platelet production by increasing both the number and size of individual megakaryocytes. However, it is important to note that TPO plays no role in triggering megakaryocytes to make proplatelets or to release platelets. c-Mpl activation is regulated by a complex array of signaling molecules that turn on specific transcription factors (see Transcriptional Regulation of Platelet Formation later in this chapter) to drive megakaryocyte proliferation and maturation. Although TPO appears to function as the main regulator of megakaryocyte development, it is not exclusive in this action. The cytokine stem cell factor, granulocyte-macrophage colony-stimulating factor, FLT (fms related receptor tyrosine kinase 1) ligand, interleukin (IL)-1, IL-3, IL-6, IL-11, CCL5, and erythropoietin also can regulate megakaryocyte development but appear to function mainly in concert with TPO. Mice that lack TPO or its receptor c-Mpl have approximately 15% of the normal platelet count. The discovery of TPO in 1994 and the development of primary megakaryocyte or mouse embryonic stem cell cultures that can be induced to faithfully reconstitute platelet formation has provided systems for studying megakaryocytes in the act of making platelets in vitro. Megakaryocytes isolated from mouse fetal liver and incubated with TPO for 4 to 5 days mature into large polyploid cells that are capable of generating and releasing large numbers of platelets. In a similar fashion, mouse embryonic stem cells can be induced to mature into polyploid megakaryocytes in the presence of stromal cells and TPO, IL-6, and IL-11. This process requires 10 to 12 days, during which the conversion of embryonic stem cells into hematopoietic stem cells very likely occurs in the first half of the time and the maturation of hematopoietic stem cells into proplatelet-producing megakaryocytes in the second half. Human embryonic stem cells can be coaxed to differentiate into mature megakaryocytes,[3] although the process takes several more days in culture. Recently, platelets have been generated from induced human pluripotent stem cells in culture using a doxycycline-controlled c-MYC expression vector.[4]

PLATELET FORMATION

Proplatelets and the Cytoskeletal Mechanics of Platelet Formation

The discovery of TPO and the development of megakaryocyte cultures that reconstitute platelet formation in vitro have allowed visualization of megakaryocytes in the act of forming platelets.[5] The actual mechanical process of platelet production begins when mature megakaryocytes start to elaborate proplatelets (see Fig. 123.2 and Fig. 123.5). This process is distinguished by the erosion of one pole of the megakaryocyte cytoplasm (see Fig. 123.5). Multiple thick pseudopodia are extended and subsequently elongate to yield thin tubules. As these slender tubules grow, they branch repeatedly and develop periodic densities along their length that impart a beaded appearance.[5,6] One of the first insights into the cytoskeletal mechanics of platelet formation comes from the work of Tablin and colleagues, who showed that proplatelet formation is dependent on microtubules; that is, proplatelet elaboration is inhibited by microtubule poisons. Microtubule poisons are effective because the extension of proplatelets from the megakaryocyte is mediated by the assembly of microtubules and their reorganization into cortical bundles. Cortical bundles align in the shafts of proplatelets, and proplatelet elongation is driven by sliding movements between overlapping microtubules composed of these bundles.[7] The microtubule bundles form loops at the end of each proplatelet, and ultimately a single microtubule is rolled into a coil at the proplatelet end to define the platelet territory. Cytoplasmic tubulin in solution is an αβ dimer that reversibly polymerizes into microtubules, which are long, hollow cylinders with an outer diameter of 25 nm. Many studies have confirmed an essential role in platelet biogenesis for β_1 tubulin, a divergent and lineage-specific β tubulin that is a major component of the megakaryocyte proplatelet cytoskeleton and marginal microtubule coil of the platelet. β_1 Tubulin, which is expressed exclusively in platelets and megakaryocytes during the late stages of megakaryocyte development, is essential for production of normal numbers of platelets, as well as for the discoid shape of platelets. The evidence supporting the role of β_1 tubulin in these processes comes from several sources. First, messenger RNA (mRNA) subtraction between wild-type and nuclear factor, erythroid 2 (NF-E2)–deficient megakaryocytes identifies β_1 tubulin as a downstream effector of the megakaryocyte transcription factor NF-E2 that is absent in NF-E2–deficient megakaryocytes. Second, genetic elimination of the β_1-tubulin gene in mice results in thrombocytopenia.[6] Third, megakaryocytes isolated from β_1-tubulin knockout mice fail to form proplatelets in vitro and instead extend only a small number of blunt protrusions.

The first event that signals proplatelet production is the consolidation of microtubules into large bundles at the megakaryocyte cortex that subsequently are reorganized into parallel bundles in the shafts of the proplatelets (Fig. 123.6).[8] Microtubule bundles are thick near the body of the megakaryocyte as they enter the proplatelet shaft but become progressively thinner along the shaft, such that only 5 to 10 microtubules remain at the end of the proplatelet. Of note, the microtubule bundles that run down the proplatelet shaft make characteristic U turns in the tips and reenter the shaft, forming teardrop-shaped structures (Fig. 123.7). This creates a bipolar orientation of bundles near the proplatelet tip, a geometry required to explain the bidirectional granule and organelle traffic observed in proplatelets. The looped arrangement of microtubules in proplatelet tips also constrains the elongation mechanism used to grow proplatelets because of an insufficient number of free microtubule ends to nucleate this reaction.

Direct visualization of microtubule dynamics in living megakaryocytes using green fluorescent protein (GFP) technology has provided insights into how microtubules orient to power proplatelet elongation (see Fig. 123.4A and B). End-binding protein 3 (EB3), a microtubule plus end–binding protein associated only with growing microtubules, fused to GFP, was retrovirally expressed in murine megakaryocytes and used as a marker to localize microtubule plus ends and to follow plus end dynamics.[7] Immature megakaryocytes without proplatelets use a centrosomal-coupled microtubule nucleation/assembly reaction, which appears as a prominent starburst pattern when visualized with EB3-GFP. Microtubules assemble only from the centrosome and grow outward into the cell cortex, where they turn and run parallel to the cell edges. However, just before proplatelet production begins, centrosomal assembly ends and microtubules release and consolidate into the cortex as bundles. Fluorescence time-lapse microscopy of living, proplatelet-producing megakaryocytes expressing EB3-GFP reveals that as proplatelets elongate, microtubules assemble continuously throughout the entire proplatelet. EB3-GFP studies also reveal that microtubules polymerize in both directions in proplatelets (i.e., toward both the tips and cell body), demonstrating that microtubules composing the bundles have a mixed polarity. The cytoplasmic Ran-binding protein, RanBP10, is a β_1-tubulin–binding protein that appears to regulate the assembly of proplatelet microtubules.[9] Even though microtubules are continuously assembling at their plus ends in proplatelets, polymerization per se does not provide the force for proplatelet elongation. First, the rates of microtubule polymerization (average 10.2 μm/min) are approximately 10-fold faster than the proplatelet elongation rate. Second, proplatelet elongation continues when microtubule polymerization is blocked with drugs that inhibit net assembly, suggesting an alternative mechanism for proplatelet elongation. Third, proplatelets possess an inherent microtubule sliding mechanism. Cytoplasmic dynein, a minus-end microtubule molecular motor protein, localizes along the microtubules of the proplatelet and appears to directly contribute to microtubule sliding; inhibition of dynein through disassembly of the dynactin complex prevents proplatelet formation. Microtubule sliding can be reactivated in detergent-permeabilized proplatelets with adenosine triphosphate, which is known to support the enzymatic

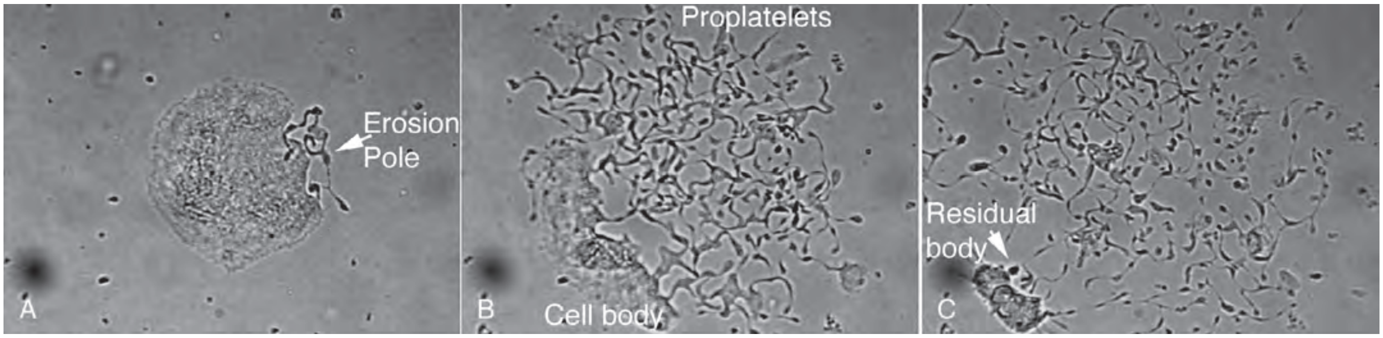


Figure 123.5 FORMATION OF PROPLATELETS BY A MOUSE MEGAKARYOCYTE. Time-lapse sequence of a maturing megakaryocyte showing the events that lead to elaboration of proplatelets in vitro. (A) Platelet production begins when the megakaryocyte cytoplasm starts to erode at one pole *(arrow)*. (B) The bulk of the megakaryocyte cytoplasm has been converted into multiple proplatelet processes that continue to lengthen and form swellings along their length. These processes are highly dynamic and undergo bending and branching. (C) Once the bulk of the megakaryocyte cytoplasm has been converted into proplatelets, the entire process ends in a rapid retraction that separates the released proplatelets from the residual cell body.

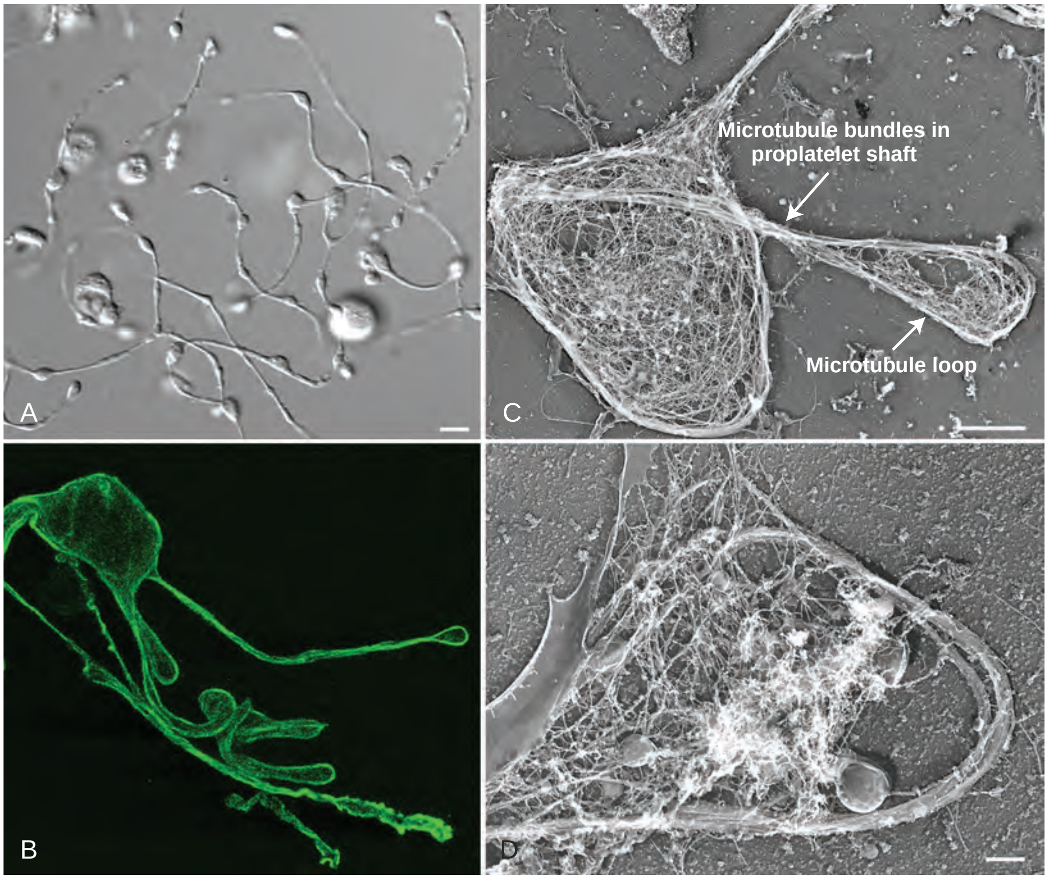


Figure 123.6 STRUCTURE OF PROPLATELETS. (A) Differential interference contrast image of proplatelets elaborated by mouse megakaryocytes in culture (bar = 5 μm). (B) Staining of proplatelets with Alexa 488 antitubulin immunoglobulin G reveals that the microtubules line the shaft of the proplatelet and form loops at the proplatelet tips (bar = 5 μm). (C, D) Organization of microtubules in the tips of proplatelets. (C) Microtubules form bundles in the proplatelet shafts (bar = 2 μm). (D) Microtubules loop in the proplatelet ends and reenter the proplatelet shafts (bar = 0.2 μm).

activity of microtubule-based molecular motors and activates elongation in permeabilized proplatelets that contain dynein and its regulatory complex dynactin. More recent analysis has indicated six types of behaviors that characterize the elaboration of proplatelets: elongation, branching, pausing, fusions, fragmentations, and retractions. While the average elongation rate for proplatelets over time is 1 μm/min, extension normally occurs in bursts and pauses. Burst rates greatly exceed the average rates, and under flow rates of less than 30 μm/min have been observed. These rates correlate well with the sliding rates of microtubules within the bundles. Fluorescence recovery after photobleaching studies have demonstrated that microtubule sliding drives proplatelet elongation and is dependent on cytoplasmic dynein.[32] Thus dynein-facilitated microtubule sliding appears to be the key event driving proplatelet elongation.

Nascent platelets assemble at the bulbous ends of proplatelets, as defined by the rolling of a single microtubule into a coil having the same diameter as the coil found in the mature platelet. Given that maturation of the platelet is limited to these sites; efficient platelet production requires a large number of proplatelet ends. In vitro, megakaryocytes use a unique mechanical process to repeatedly bifurcate the shafts of the proplatelet, thereby amplifying the number of ends. To accomplish this task, the shaft of elongating proplatelets is bent on itself and a new proplatelet grows out of the bend—a process that results in bifurcation of the shaft. Proplatelet elongation is mediated by microtubules, whereas actin mediates the bending and branching of proplatelet shafts. Actin filament assemblies decorate branch points, and agents that disrupt actin assembly, such as the cytochalasins, reduce proplatelet branching. One possibility is that in vitro proplatelet bending and branching are regulated by the actin-based molecular motor myosin. Myosin II is an adenosine triphosphatase (ATPase) motor that makes up 2% to 5% of the total platelet protein. Myosin II binds to actin filaments and generates force for contraction. Each myosin has two heads and a long, rod-like tail whose function is to permit the molecules to assemble into bipolar filaments. Of interest, a mutation in the tail domain of the nonmuscle myosin heavy chain A gene in humans results in several disorders, including May-Hegglin anomaly, Sebastian syndrome, and Fechtner syndrome. These rare autosomal platelet disorders are characterized by thrombocytopenia with giant platelets. Recent findings have implicated myosin IIA in restricting proplatelet production until megakaryocytes attain full maturity. The loss of myosin IIA function through targeted gene disruption in mice, through dominant inhibitory mutations in humans, or by manipulation of cultured megakaryocytes, appears to accelerate proplatelet production. Consequently, platelet production is inefficient and produces platelets that vary extensively in shape, content, and diameter. These findings also suggest that the Rho-ROCK-myosin light chain pathway regulates myosin IIA.

Experiments also indicate that protein kinase Cα associates with aggregated actin filaments in megakaryocytes and negatively regulates proplatelet formation.[33] Recently, we identified a novel protein, MARCKS (myristoylated alanine-rich C-kinase substrate), that is a downstream target of protein kinase Cα and regulates the process of actin polymerization in megakaryocytes.[34] MARCKS attaches to phosphatidylinositol 4,5-bisphosphate $(PIP)_2$ rafts, limiting their availability to bind WASp and blocking activation of the actin-related protein 2/3 complex (Arp2/3) actin nucleating complex. Upon phosphorylation, MARCKS is internalized and PIP_2 is free to bind WASp, thus activating the Arp2/3 complex and initiating actin polymerization. In this way, MARCKS phosphorylation may provide a fast way to activate or inhibit actin polymerization, when necessary, over the course of proplatelet production. However, the role of actin filament dynamics in platelet biogenesis remains unclear.

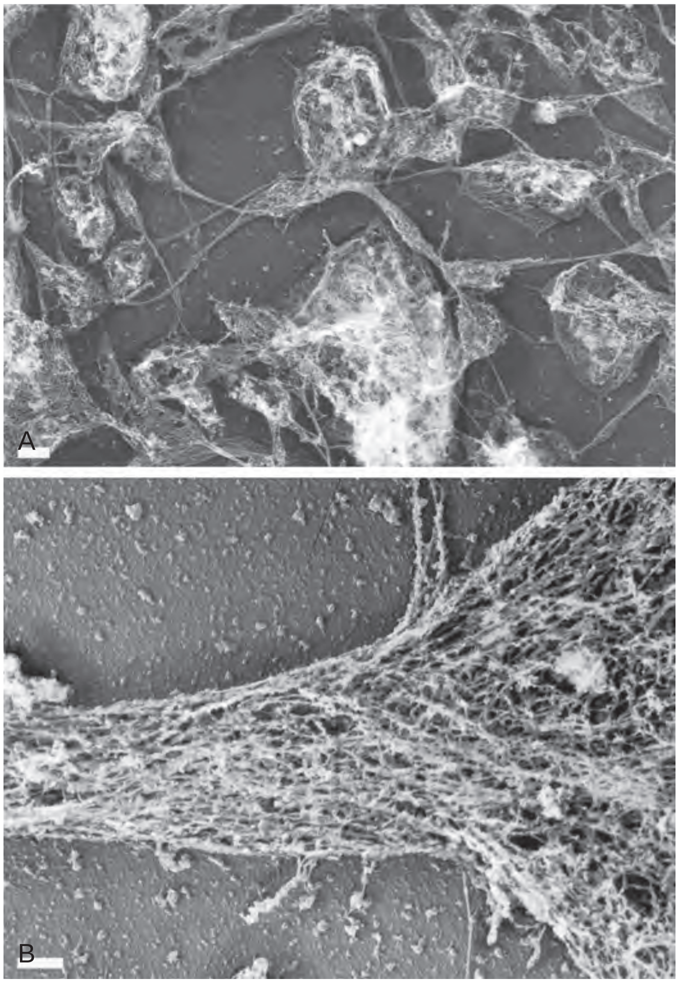


Figure 123.7 MEMBRANE SKELETON OF THE PROPLATELET. Representative electron micrographs of the detergent-insoluble proplatelet cytoskeleton. Proplatelets were permeabilized with 0.75% Triton X-100, 5 μM phallacidin, and 0.1% glutaraldehyde. Examination through electron microscopy reveals that the plasma membrane of the proplatelet tube is supported by a fibrous membrane skeleton that is similar in structure to the membrane skeleton of mature platelets. (A) This low-magnification field shows that an intact membrane skeleton laminates the underside and extends along the entire length of proplatelets (bar = 1 μm). (B) High-magnification, three-dimensional electron micrograph of the proplatelet membrane skeleton reveals a lattice-like network of elongated filamentous strands, similar in nature to the spectrin-based network in red blood cells and platelets. The membrane skeleton continuously laminates the underside of the proplatelet. A cytoplasmic bridge is shown *(left)* linking to a swelling *(right)* (bar = 200 nm).

Profilin is a small actin-binding protein that stimulates filament polymerization. It binds ADP, converting them to adenosine triphosphate (ATP) monomers, which interact only with the high-affinity end of actin filaments. Mice whose megakaryocytes/platelets specifically lack profilin 1 have macrothrombocytopenia, with counts reduced by 40% to 50%. Platelet turnover is accelerated, and the mature platelets have only a sparse internal actin cytoskeleton. Although their actin cytoskeleton is disrupted, platelets missing profilin have a hardy microtubule cytoskeleton with a thickened microtubule coil and more stable hyperacetylated microtubules.[35] In some ways, platelets from profilin 1–deficient mice are similar in behavior to platelets from patients with Wiskott-Aldrich syndrome or those from WASp- or WIP (WASp-interacting protein)-deficient mice. WASp is a signaling protein that promotes actin assembly, binding to Arp2/3 when activated by binding of GTP-Cdc42. However, most studies performed on human Wiskott-Aldrich syndrome platelets or platelets derived from WASp-deficient mice revealed normal and even hyperfunctionality for the WASp-null platelets.[36,37] Humans with Wiskott-Aldrich syndrome have low platelet counts as a consequence of accelerated platelet clearance, a process that also lowers the platelet count in WASp/WIP-deficient mice. Studies have shown that the microtubule coils in platelets from Wiskott-Aldrich syndrome patients are hyperacetylated, like those in platelets from profilin 1 deficient mice. However, in Wiskott-Aldrich syndrome, circulating platelets are small, and not large as observed in profilin 1–deficient mice.

Human Arp2/3 attaches to and is activated by WASp family proteins, where it acts as an initiation site for new actin filaments and is required for actin filament branching. Arp2/3 has two ARPC1 (actin-related protein C1) component isoforms, with ARPC1B prominently expressed in blood cells. Recently, a report was published on three individuals from two families with homozygous mutations in ARPC1B.[38] Patients present with defective Arp2/3 actin filament branching that is associated with multisystem disease including platelet abnormalities, cutaneous vasculitis, eosinophilia, and a predisposition to inflammatory diseases. The Arpc2 gene was knocked out in murine platelets, and as observed in the patients, this was associated with significant microthrombocytopenia as a result of premature release of platelets into the bone marrow and reduced platelet survival.[39] Not surprisingly, because WASp and Arp2/3 are binding partners, this phenotype largely phenocopies that of WASp/WIP-deficient mice.

Another actin-binding protein that plays a significant role in platelet generation and structure is filamin A. Filamin A is a large cytoplasmic protein that crosslinks actin filaments into arrays and attaches the vWF receptor (GPIb-IX-V) to the underlying actin cytoskeleton. Filamin subunits are elongated strands made up of 24 repeats, each approximately 100 amino acids in length that are folded into immunoglobulin G (IgG)-like beta barrels. There are three filamin genes on chromosomes 3, 7, and X. Filamin A (X) and filamin B (3) are expressed in platelets, with filamin A being present in a greater than 10-fold excess over filamin B. The second rod domain of filamin has a binding site for the cytoplasmic tail of GPIbα, and biochemical experiments have shown that the majority of platelet filamin (≧90%) is in complex with GPIbα. Knockout of the filamin A gene in the megakaryocyte lineage of mice leads to low platelet count with giant platelets.[40] Filamin A–null megakaryocytes release platelets more rapidly than wild-type megakaryocytes, and the severe macrothrombocytopenia appears to be caused by the rapid clearance of platelets and by the spontaneous formation of platelet microvesicles. These observations suggest that filamin A–null megakaryocytes prematurely produce large and fragile platelets that are rapidly removed from the bloodstream.

In addition to proteins that promote actin assembly and structural organization, there is also strong evidence that proteins that stimulate actin filament assembly play a major role in platelet production. Members of the actin-depolymerizing factor (ADF) cofilin family are known to play an essential role in temporally and spatially regulating the turnover of actin filaments in all species. The importance of filamentous actin severing proteins in platelet production has been established. Deletion of all ADF/n-cofilin dynamics in megakaryocytes virtually abolishes platelet formation and results in the formation of both giant and microparticle-like platelets with aberrant ultrastructure.

In addition to proteins that promote actin assembly and structural organization, there is also strong evidence that proteins that stimulate actin filament assembly play a major role in platelet production. Members of the ADF cofilin family play an essential role in temporally and spatially regulating the turnover of actin filaments in all mammals. Deletion of all ADF/n-cofilin dynamics in megakaryocytes virtually abolishes platelet formation and results in the formation of both giant and microparticle-like platelets that are characterized by aberrant ultrastructure.[41] Proplatelet production is highly reduced in ADF/n-cofilin–null megakaryocytes. Defective proplatelet production has also been observed in mice in which the small regulatory GTPases Rho, Cdc42, and Rac have been deleted in the megakaryocyte lineage.[42,43] All told, these observations suggest that in vivo actin filament turnover is an essential step in the end stages of platelet generation.

In addition to playing an essential role in proplatelet elongation, the microtubules lining the shafts of proplatelets serve a secondary function: transport of membrane, organelles, and granules into proplatelets and assembling platelets at proplatelet ends (see Fig. 123.4C). Organelles are sent individually from the cell body into the proplatelets, where they move bidirectionally until they are captured at proplatelet tips.[14] Immunofluorescence and electron microscopic studies indicate that organelles are intimately associated with microtubules, and actin poisons do not diminish organelle motion. Thus movement appears to involve microtubule-based forces. Bidirectional organelle movement is conveyed in part by the bipolar arrangement of microtubules within the proplatelet because kinesin-coated latex beads move in both directions over the microtubule arrays of permeabilized proplatelets. Of the two major microtubule motors, kinesin and cytoplasmic dynein, only the plus end–directed kinesin is localized in a pattern similar to organelles and granules and is therefore likely responsible for transporting these elements along microtubules. It appears that two mechanisms of organelle and granule movement are involved in platelet assembly: first, organelles and granules travel along microtubules and, second, the microtubules themselves slide bidirectionally in relation to other motile filaments, indirectly moving organelles along proplatelets in a piggyback manner.

Although the roles of microtubules and actin filaments in proplatelet development have been extensively studied, our understanding of the function of the membrane skeleton has only recently been established. High-resolution electron microscopy reveals that proplatelets have a dense spectrin-based membrane skeleton similar in structure to that of mature blood platelets. Nonerythroid spectrin subunits, alpha-II and beta-II spectrin, are predominately expressed in mouse megakaryocytes, proplatelets, and platelets, but erythroid alpha-I and beta-I spectrin isoforms are also expressed (see Fig. 123.6).[15] Assembly of spectrin tetramers is required for development of the DMS and proplatelet elaboration because expression of a spectrin tetramer–disrupting construct in megakaryocytes inhibits both processes. Furthermore, integration of this spectrin-disrupting construct into a permeabilized proplatelet system quickly destabilizes proplatelets, resulting in massive blebbing and swelling. Spectrin tetramers also stabilize the barbell-like shapes found in the penultimate stage in platelet production. Taken together, these studies suggest a role for spectrin in different steps of megakaryocyte development through its participation in the formation of demarcation membranes and in the maintenance of proplatelet structure.

Platelet Maturation at the Proplatelet Tip

Platelet maturation at proplatelet tips ends when a single microtubule detaches from the microtubule bundle and is rolled into a coil. To complete construction of mature platelets, once the fundamental cytoskeletal components have been delivered to and assembled in the platelet buds, the buds must fill with their organelle and granule content.

Granules are sent to nascent platelets on the microtubule tracks of the proplatelets. The concentration of this cargo in the platelet occurs by an end-trapping mechanism as granules and organelles, which enter the nascent platelet, continue to move in the tip but do not return to the proplatelet shaft.

Release of Mature Platelets

Details of how mature platelets release from the proplatelet tips are beginning to come into focus. In vitro, maturation of proplatelets ends in a rapid retraction that separates a variable portion of the proplatelets from the residual cell body, leaving behind a naked, denuded nucleus (see Fig. 123.5C). Activation of apoptotic pathways in the cell body has been shown to be coincident with this event. Junt and colleagues have used intravital fluorescence microscopy to visualize proplatelet production in the opened cranial marrow cavity of living mice.[16] Yellow fluorescent protein (YFP)-labeled megakaryocytes were seen to protrude proplatelets and release megakaryocyte fragments into the marrow sinusoids of living mice. Notably, these anucleate fragments typically exceed platelet dimensions, suggesting that platelet morphogenesis continues in the circulation. In line with these observations, we identified a previously unrecognized intermediate stage in platelet formation and release, which we termed the *preplatelet*.[17] Preplatelets are defined as discoid cells (3 to 10 μm) that retain the capacity to convert into barbell-shaped proplatelets and undergo fission into platelets. The conversion of preplatelets to barbell proplatelets is powered by microtubule-based forces. It is likely that the microtubule motors that drive proplatelet extension are involved in aspects of platelet release, as well as in the process of microtubule coiling. Sliding of an uncoiled portion of the microtubule relative to the rigid microtubule bundle in the proplatelet tip would provide a simple mechanism to effect platelet release and would explain the variable morphology of the small but reproducible percentage (<5%) of dumbbell-shaped platelets that are present in blood. The morphologic similarities between platelets that form new cell bodies and preplatelets are striking. Whether or not newly released platelets exhibit a preplatelet phenotype, which allows them to form barbell shapes and divide again, is not clear.

Location of Platelet Release

Megakaryocytes cultured in vitro outside the confines of the bone marrow can form developed proplatelets in solution, suggesting that direct interaction with the marrow environment is not essential for platelet production. However, the efficiency of platelet production in culture appears to be diminished relative to that observed in vivo, and the bone marrow environment, which contains a complex cell population, could influence platelet formation by direct cell contact or secretion of cytokines. It has been suggested that, by extending into the bone marrow sinusoids, proplatelets provide a mechanism for extension into the bone, allowing release of platelets directly into the circulation.[19,20] Scanning electron micrographs have revealed bone marrow megakaryocytes extending proplatelets through junctions in the endothelium into the sinusoidal lumen, supporting the concept that platelet production occurs in the bone marrow. Megakaryocytes have been observed in the lung, prompting the notion that platelets can be formed from their parent cells in the pulmonary circulation. Aschoff initially described pulmonary megakaryocytes in 1893 and suggested that they originated in the marrow, migrated into the bloodstream, and, because of their massive size, became lodged in the pulmonary capillaries, where they released platelets. This mechanism requires the migration of megakaryocytes from the bone marrow into the circulation, which had not been observed by intravital microscopy. Lefrancais[44] and colleagues used two-photon intravital microscopy to visualize the lung in mice in which platelet factor 4-Cre recombinase (PF4-Cre) drives membrane GFP expression in megakaryocytes and platelets. They observe large circulating GFP^+ cells passing through the lung microcirculation, where they produce GFP^+ proplatelet extensions in a flow-dependent manner. To investigate the derivation of these pulmonary megakaryocytes, they used an orthotopic single lung transplant model and found that the megakaryocytes producing platelets in the lung circulation are of extrapulmonary origin, the majority of which originated from the bone marrow. However, studies using accelerated models of thrombopoiesis in mice suggest that only a small fraction of platelet production occurs in the lung.[45] In addition, removal of a lung in mice or humans does not alter the platelet count. It is also worth noting that patients with pathologic conditions of the lung such as chronic obstructive pulmonary disease, pulmonary tuberculosis, and cystic fibrosis do not have decreased platelet counts; in fact, these patients often present with elevated platelet counts (thrombocytosis).[46–48] Together, these murine and human data suggest that the lung is not the primary site of platelet production.

Transcriptional Regulation of Platelet Formation

Megakaryocyte development and platelet formation are controlled by the coordinated action of transcription factors that specifically turn on the genes of megakaryocyte precursors or suppress the expression of genes that support other cell types.[22] Gene-targeting studies in mice have identified several genes that are crucial for megakaryocyte development and platelet formation. Leading the list of transcription factors that play an essential role in megakaryocyte maturation and platelet biogenesis is the basic leucine zipper heterodimer NF-E2. NF-E2 is a protein composed of a ubiquitously expressed 18- to 20-kDa small-Maf subunit and a p45 subunit that is restricted to erythroid and megakaryocytic lineages. Although NF-E2 was postulated to be a transcription factor that specifically drove the expression of genes essential for erythropoiesis, mice lacking p45 NF-E2 do not exhibit defects in erythropoiesis. Instead, mice deficient in the p45 subunit or two of the small-Maf subunits die of hemorrhage shortly after birth because of a complete lack of circulating platelets. Although megakaryocytes undergo normal endomitosis and proliferate in response to TPO, mice deficient in p45 NF-E2 produce increased numbers of megakaryocytes that are larger than normal, contain fewer granules, exhibit a highly disorganized DMS, and fail to generate proplatelets in vitro, a phenotype indicative of a late block in megakaryocyte maturation. Therefore NF-E2 appears to control the transcription of a limited number of genes involved in cytoplasmic maturation and platelet formation. Shivdasani and colleagues generated a subtracted complementary DNA (cDNA) library enriched in transcripts downregulated in NF-E2–knockout megakaryocytes. Using this approach, these investigators have started to identify the downstream targets of NF-E2 and to analyze their role in the terminal stages of megakaryocyte differentiation. Putative transcriptional targets of NF-E2 include β_1-tubulin, thromboxane synthase, and proteins that regulate inside-out signaling via $\alpha_{IIb}\beta_3$ integrin. The zinc finger protein GATA1 is also a transcription factor that plays a critical role in driving the expression of genes essential for megakaryocyte maturation. However, unlike NF-E2, which appears to drive the later stage of megakaryocyte development, *GATA1* functions at multiple stages of development. Initially, GATA proteins were thought to regulate red blood cell maturation because genetic disruption of the *GATA1* gene in mice results in embryonic lethality secondary to a block in erythropoiesis. However, several more recent observations also implicate *GATA1* as a regulator of megakaryocyte differentiation. First, forced expression of *GATA1* in the early myeloid cell line 416b induces megakaryocyte differentiation. Second, Shivdasani and colleagues used targeted mutagenesis of regulatory elements within the *GATA1* locus to generate mice with a selective loss of *GATA1* in the megakaryocyte lineage. These knockdown mice expressed sufficient amounts of *GATA1* in erythroid cells to circumvent the embryonic lethality caused by anemia. *GATA1* deficiency in megakaryocytes leads to severe thrombocytopenia. Platelet counts are reduced to approximately 15% of normal, and the small numbers of circulating platelets are round and larger than normal. These mice have increased numbers of small megakaryocytes that exhibit an accelerated rate of proliferation. The small cytoplasmic volume of *GATA1*-deficient megakaryocytes typically contains an excess of rough endoplasmic reticulum, very few platelet-specific granules, and an underdeveloped or disorganized DMS, suggesting that maturation of megakaryocytes is arrested in GATA1-deficient megakaryocytes.

A family with X-linked dyserythropoietic anemia and thrombocytopenia due to a mutation in *GATA1* has been described. A single-nucleotide substitution in the N-terminal zinc finger of *GATA1* inhibits the interaction of *GATA1* with its essential cofactor, friend of *GATA1* (FOG). Although megakaryocytes in affected family members are abundant, they are unusually small and exhibit several abnormal features, including an abundance of smooth endoplasmic reticulum, an underdeveloped DMS, and a lack of granules. These observations suggest an essential role for the *FOG1-GATA1* interaction in thrombopoiesis. Genetic elimination of FOG in mice unexpectedly results in specific ablation of the megakaryocyte lineage, suggesting a *GATA1*-independent role for FOG in the early stages of megakaryocyte development; therefore *GATA1* and FOG are required for megakaryocyte generation from a common bipotential progenitor.

The results from several knockout mice models also indicate a role for additional transcription factors in megakaryocyte development. Mice carrying a null mutation in *Fli-1*, a member of the ETS family of winged helix-turn-helix transcription factors that bind purine-rich sequences in gene promoters, exhibit defects in megakaryocyte development. Megakaryocytes cultured from mice lacking *Fli-1* contain reduced numbers of α-granules, disorganization of the demarcation membranes, and a reduction in size. Mice lacking the hematopoietic zinc finger (Hzf) protein, a transcription factor that is predominantly expressed in megakaryocytes, have reduced numbers of α-granules in megakaryocytes and platelets. Therefore Hzf may regulate the transcription of genes involved in the synthesis of α-granule components and/or their packaging into α-granules. SCL (stem-cell leukemia), a basic helix-loop-helix transcription factor initially identified in a subset of human T-cell leukemia with multilineage characteristics, also appears to be critical for megakaryopoiesis. Results of deletion of SCL in mice indicate that this transcription factor is required for proper erythroid and megakaryocyte development.

PLATELETS

Structure of the Resting Platelet

Megakaryocyte development culminates in the release of proplatelets that are remodeled into mature discoid platelets having dimensions of approximately 3.0 × 0.5 μm and a cytoplasmic volume of 7 fL.[23] The evolutionary explanation for the discoid shape of the platelet is unknown. Discoid shape may permit more efficient flow or dispersion of clot-promoting elements or may simply reflect the microtubule-based mechanism by which platelets are produced. In humans, platelets normally circulate for 7 to 10 days. Given that nearly 1 trillion platelets circulate in an adult human, an adult produces approximately 100 billion platelets each day.

The precise morphology of newly released platelets is unknown. However, when released into the circulation or maintained in culture, platelets have a reproducible structure. Although they are heterogeneous in size, platelets have discoid shapes with flat, featureless surfaces (Figs. 123.8A and 123.9A) that are interrupted only by pit-like openings into the open canalicular system (OCS). The OCS is an extensive system of internal membrane conduits that serves as a passageway to the outside world into which granular contents are released. It also is a reservoir of plasma membrane, membrane receptors, and proteins. For example, approximately 30% of the thrombin receptors are localized in the OCS of the resting platelet, awaiting movement to the surface when the cells are activated. Although continuous with the plasma membrane, not all proteins on the cell surface can enter the OCS. Factors controlling movement into the OCS remain to be defined but likely depend on the actin cytoskeleton. However, entry restriction occurs at the necks of OCS infoldings. The third function of the OCS is to serve as a source of redundant plasma membrane for cell spreading. OCS membrane initially is disgorged to the surface following cell activation. When cells are activated in solution, much of this membrane is subsequently reabsorbed into the remnants of the OCS.

A small thin zone of cytoplasm separates the plasma membrane of the resting platelet from a marginal microtubule coil and the general intracellular space, which contains all inclusion bodies and the internal cytoskeleton of the cell. This zone is filled with the spectrin-based membrane skeleton (see Fig. 123.9A). Beneath this zone sits a microtubule coil. Then follows the cytoplasmic space, which is filled with filaments of actin that embed granules, organelles, the OCS, and other specialized membrane systems such as smooth endoplasmic reticulum.

Platelets actively recruit other blood-borne cells to areas of vascular damage by releasing mediators packaged in intracellular granules (described earlier in Cytoplasmic Maturation) that initiate secondary homeostatic interactions and that express a “sticky” apical surface after the platelets adhere. In the resting platelet, granules are juxtaposed together and are in intimate association with the membranes of the OCS. The release reaction of platelet granules differs from that of other cells. Granules rarely fuse with the plasma membrane; instead, they exocytose into the OCS. Platelets also contain lysosomes and mitochondria, which are easily identified under the electron microscope by their internal system of membrane cristae.

Cytoskeleton of the Resting Platelet

Although both microtubule- and actin-based forces have been considered in the elaboration and branching of proplatelets, respectively, it is the integration of the microtubule and actin cytoskeletal elements that uniquely defines the shape of the mature platelet. One of the most distinguishing features of the resting platelet is its marginal microtubule coil (see Fig. 123.8). αβ-Tubulin dimers assemble into microtubule polymers under physiologic conditions. In resting platelets, tubulin is equally divided between dimer and polymer fractions. In many cell types, αββ-tubulin subunits are in a dynamic equilibrium with microtubules such that reversible cycles of assembly-disassembly of microtubules are observed. Microtubules are long, hollow polymers (24 nm in diameter) that are responsible for many types of cellular movements, such as segregation of chromosomes during mitosis and transport of organelles across the cell. The microtubule ring of the resting platelet, initially characterized in the late 1960s by White and Krivit, was described as a single microtubule approximately 100 µm long and is coiled 8 to 12 times inside the periphery of the platelet. However, recent work suggests that the marginal band is highly dynamic and consists of multiple microtubules with mixed polarity that undergo constant assembly and disassembly.[24] This may accommodate the shrinkage of microtubule coil diameters that occurs with aging of platelets. Antagonistic microtubule motors appear to keep the marginal microtubule coil in its resting state.[49] The primary function of the microtubule coil is to maintain the discoid shape of the resting platelet. Disassembly of platelet microtubules with drugs such as vincristine, colchicine, or nocodazole causes platelets to become round and to lose their discoid shape. Cooling the platelets also causes disassembly of the microtubule coil and loss of the discoid shape. Mice lacking β_1 tubulin, the major hematopoietic β-tubulin isoform, produce platelets that lack their characteristic discoid shapes and have defective marginal bands. Genetic elimination of β_1 tubulin in mice results in thrombocytopenia with circulating platelet counts less than 50% of normal. β_1-Tubulin–deficient platelets are spherical in shape, apparently due to shortened marginal bands with fewer coils. Normal platelets possess a marginal band that consists of 8 to 12 coils, whereas β_1-tubulin knockout platelets contain only two to three coils. A human β_1-tubulin functional substitution (AG→CC) inducing both structural and functional platelet alterations has been described. Of note, the Q43P β_1-tubulin variant was found in 10.6% of the general population and in 24.2% of 33 unrelated patients with undefined congenital macrothrombocytopenia. Electron microscopy revealed enlarged spherocytic platelets with a disrupted marginal band and structural alterations. Platelets with the Q43P β_1-tubulin variant showed mild platelet dysfunction with reduced ATP secretion, attenuated thrombin receptor–activating peptide (TRAP)-induced aggregation, and impaired adhesion to collagen under flow conditions.[50] A

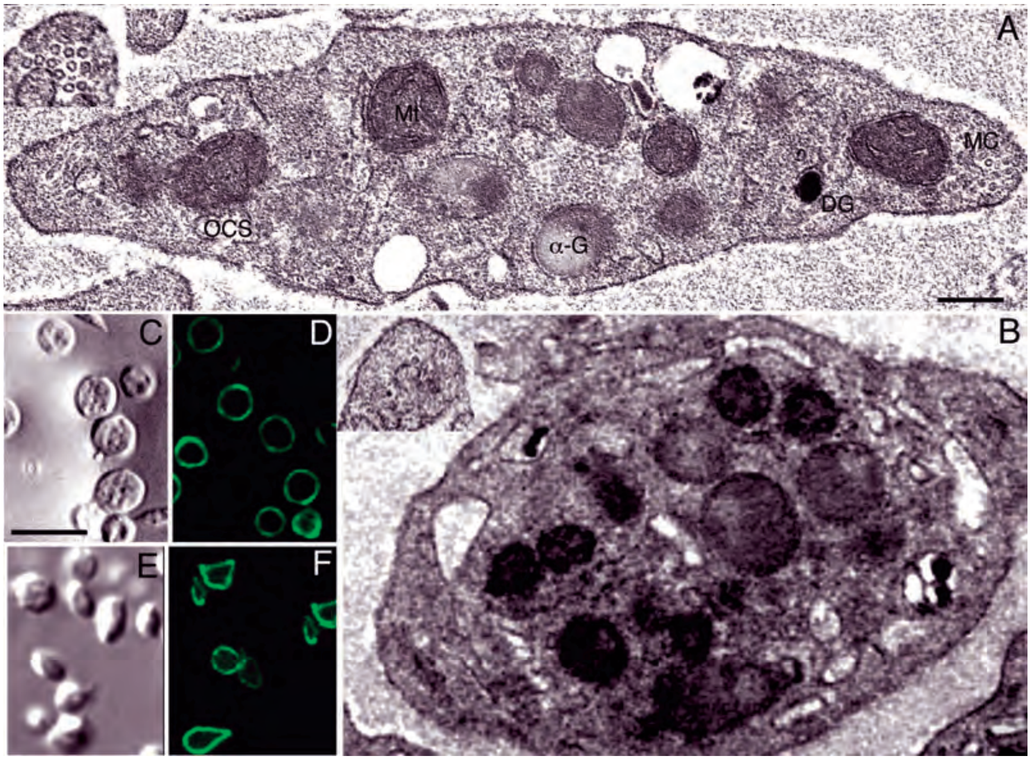


Figure 123.8 COMPARISON OF THE STRUCTURE OF NORMAL MOUSE PLATELETS (A, C, D) WITH THOSE LACKING B_1 TUBULIN (B, E, F). (A) Electron micrograph of a resting mouse platelet sectioned through its thin axis. The cut plane reveals the microtubule coil (MC) at the cell periphery. The *inset* shows a high-magnification cross-section through the MC of the resting platelet. The microtubule is wound 11 times in this platelet, forming the coil. The cytoplasmic space embeds mitochondria *(MT)*, α-granules (α-G), and dense granules *(DG)*. Spaces created by the open canalicular system *(OCS)* are apparent. (B) Electron micrograph of a thin section through a platelet isolated from a mouse lacking β_1 tubulin (bar = 0.2 µm). Platelets from these animals are spherical (E) and have only a rudimentary microtubule coil *(inset)*. In this platelet the microtubule is twisted twice. (C) Differential interference contrast image of resting platelets shows them to be flat discs. (D) MC of the resting mouse platelet. Staining of fixed mouse platelets with Alexa 488 antitubulin immunoglobulin G *(IgG)* reveals the MC. This coil resides at the periphery of the platelet. (E) Differential interference contrast image of platelets lacking β_1 tubulin. (F) Staining of fixed mouse β_1-tubulin–deficient platelets with Alexa 488 antitubulin IgG reveals the coil is defective and bent in a number of places throughout the platelets. (C–F are the same magnification; bar = 5 µm.)

more-than-doubled prevalence of the β_1-tubulin variant was observed in healthy subjects not undergoing ischemic events, raising the possibility that the variant confers an evolutionary advantage and a protective cardiovascular role.

Actin is the most abundant of all the platelet proteins, with 2 million molecules expressed per platelet. Of these molecules, 800,000 assemble to form the 2000 to 5000 linear actin polymers that exist in the resting cell (see Fig. 123.9A). The remainder of the actin is maintained in storage as a 1:1 complex with β_4 thymosin, which can be converted to filaments during platelet activation to drive cell spreading. All evidence indicates that the filaments of the resting platelet are interconnected at various points into a rigid cytoplasmic network because platelets express high concentrations of actin cross-linking proteins, including filamin and α-actinin.[7] Both filamin and α-actinin are homodimers in solution. Three filamin genes are located on chromosomes 3, 7, and X. Filamin A (X) and filamin B (3) are expressed in platelets. Filamin A is expressed at levels more than 10-fold higher than that of filamin B. Filamin subunits are elongated strands composed primarily of 24 repeats, each approximately 100 amino acids in length and folded into IgG-like β-barrels. Each strand has an N-terminus actin-binding site that shares homology with other actin-binding proteins, two rod domains that are end-to-end assemblies of the repeat units, interrupted by two hinge domains between repeats 15 and 16, and 23 and 24, and a C-terminus self-association site (Fig. 123.10B). Subunits assemble to form V-shaped bipolar molecules (i.e., the self-association site is the vertex of the V), and the actin-binding sites are on the free ends. Inclusion of the first hinge in filamin depends on alternative RNA splicing. Filamin now is recognized to be a prototype "scaffolding molecule" that collects binding partners and localizes them adjacent to the plasma membrane. Partners bound by filamin members include the small GTPases, RalA, Rac, Rho, and Cdc42, with RalA binding in a GTP-dependent manner; the exchange factors Trio and Toll; kinases such as PAK1; phosphatases; and transmembrane proteins. Most partner proteins are bound within the C-terminus portion of filamin.

Central to the structural organization of the resting platelet is an interaction between filamin and the cytoplasmic tail of the GPIbα subunit of the GPIb-IX-V complex.[26] The second rod domain (repeat 17) of filamin has a binding site for the cytoplasmic tail of GPIbα. The interaction between filamin A and GPIbα occurs at the atomic level. Repeat 17 of filamin A has a groove between certain β-sheet strands that forms a pocket for the GPIbα tail (see Fig. 123.10). Binding between filamin A and GPIbα is driven by entropic forces, and the alignment and specificity are provided by large residues that create a lock-and-key fit between the two molecules. The interaction between one filamin A subunit and GPIbα has an affinity of approximately 10 μM, whereas high-affinity binding (10 nM) occurs when each filamin A subunit in a molecule and both GPIbα chains in a vWF receptor are engaged. Studies have shown that the bulk of platelet filamin (>90%) is in complex with GPIbα. This interaction has three

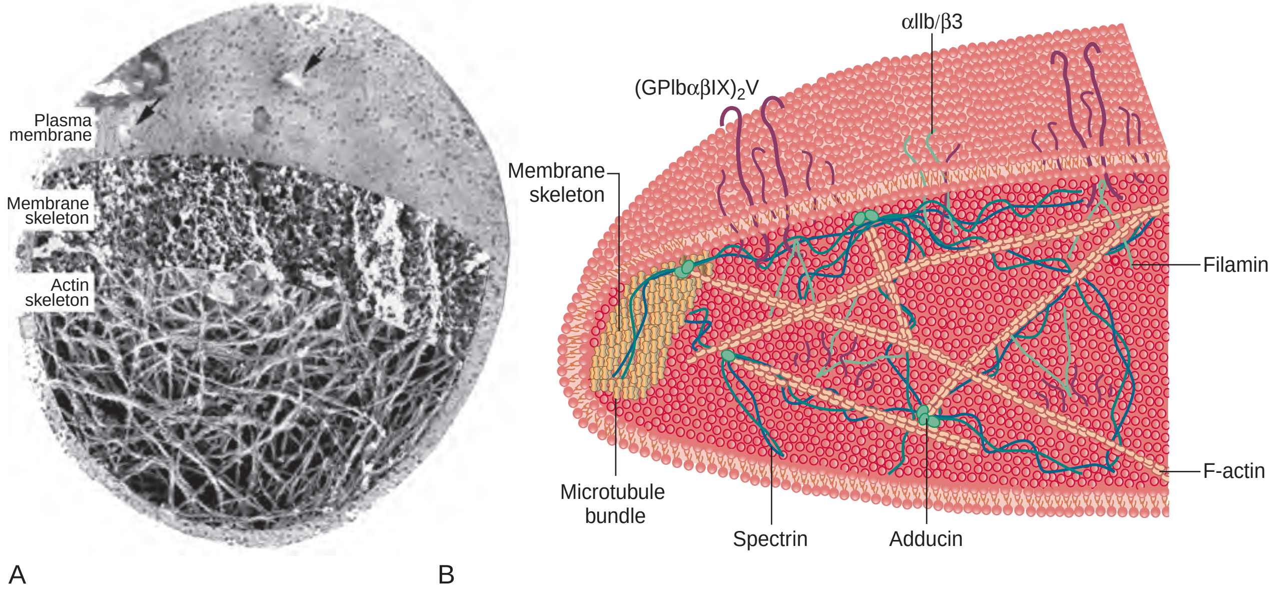


Figure 123.9 STRUCTURE OF THE RESTING HUMAN BLOOD PLATELET AND ITS ACTIN-BASED CYTOSKELETON. (A) Composite illustrating the major actin cytoskeletal layers of the resting platelet. *Plasma membrane*: The plasma membrane of the resting cell is flat and featureless, except for periodic invaginations that lead into the open canalicular system *(OCS) (arrows). Membrane skeleton*: The plasma membrane of the platelet is supported by a submembranous spectrin-based skeleton. This network is composed primarily of spectrin molecules, which are tetramers with actin binding sites at the ends. Actin filament ends dock on spectrin to complete the network. The association between spectrin and F actin is promoted by adducin. *Actin cytoskeleton*: As discussed earlier, the spectrin network is both directly and indirectly attached to the underlying actin filaments. Filament ends interconnect spectrin molecules, whereas the filamin links run from the filament sides to the plasma membrane receptor (GPIbαβIX)$_2$V. The cytoplasmic space has a dense filling of actin filaments. Actin filaments from the cell center radiate outward. As the filaments approach the plasma membrane, they turn and run in parallel with it. The actin filaments have been decorated using myosin subfragment 1 (S1), which gives them a twisted cable-like appearance in frozen samples. Myosin S1 labeling reveals the polarity of the actin filament. "Pointed" and "barbed" ends are definable. The ends of actin filaments are bound by the ends of spectrin molecules on the edges of the membrane network *(arrowhead)*. A microtubule coil composed of a single long microtubule resides just beneath the plasma membrane at the periphery of the thin axis of the platelet (not shown) (bar = 0.5 μm). (B) Schematic showing the structural features of the resting blood platelet cytoskeleton. Resting cells have discoid shapes. Structural elements that support this shape are (1) a marginal microtubule coil, (2) a spectrin-based membrane skeleton, and (3) a rigid network of cross-linked cytoplasmic actin filaments (only a small number of the actin filaments have been added to this illustration so that they will not obscure the rest of the structures in the cell). Platelets have a specialized membrane skeleton composed of spectrin, actin, and many associated proteins. Spectrin tetramers (200 nm long and 5 nm wide) have actin filament-binding sites at each molecular end. The membrane skeleton is held in compression between the plasma membrane and the cytoplasmic actin by filamin connections from the sides of actin filaments to the cytoplasmic tails of GPIbα subunit of the membrane glycoprotein complex that binds to von Willebrand factor (GPIbαβIX)$_2$V complex. Greater than 98% of the barbed ends of actin filaments are capped by adducin and capZ in the resting platelet.

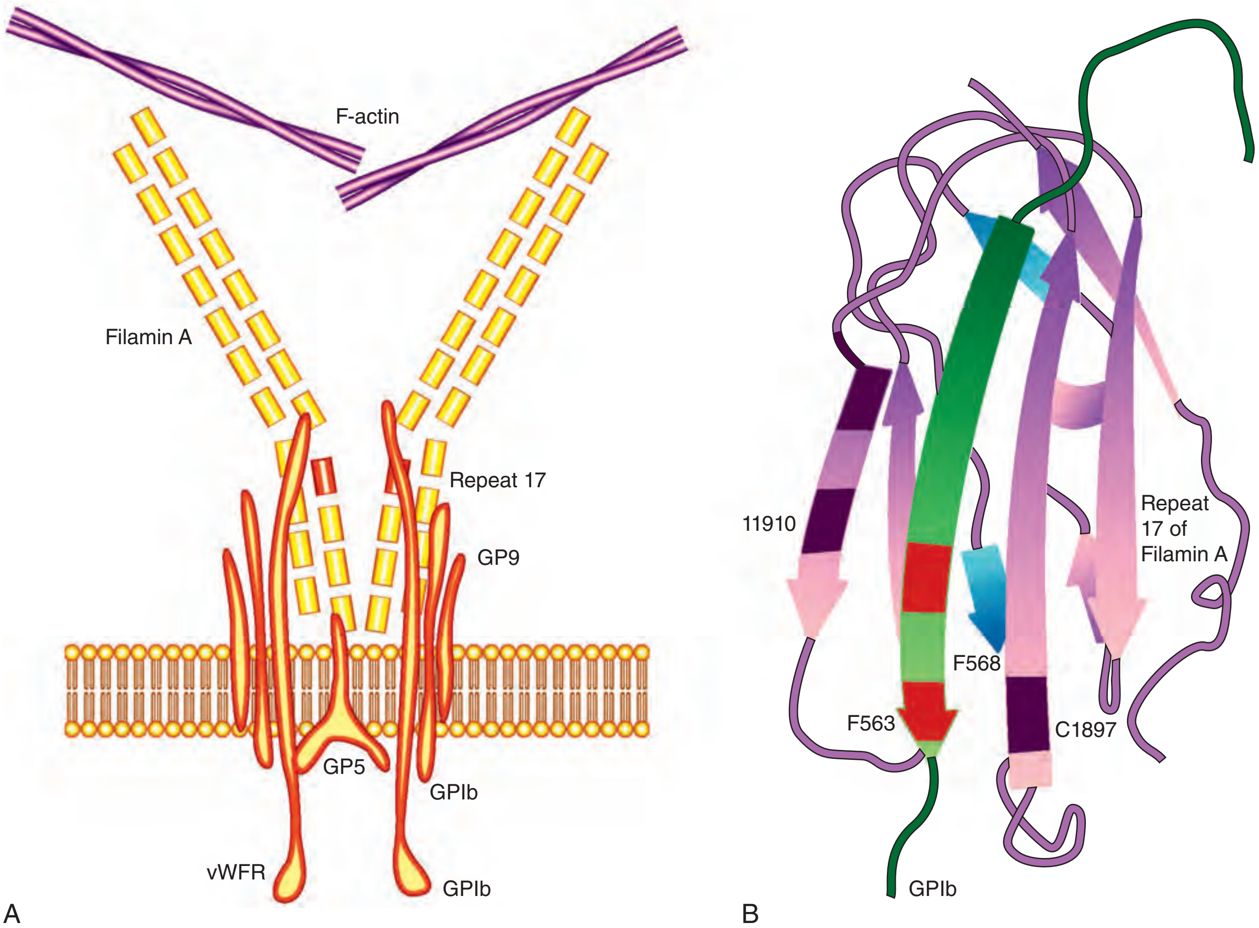


Figure 123.10 INTERACTION OF FILAMIN A WITH THE VON WILLEBRAND FACTOR RECEPTOR. (A) Model showing the orientation of filamin A when interacting with the GPIbα chain of the von Willebrand factor receptor (vWFR) and cytoplasmic actin filaments. For tight binding of filamin A to vWFR, both GPIbα chains of the receptor must be engaged by a single filamin A molecule. (B) Ribbon diagram showing the interface between filamin A repeat 17 and the filamin A binding region of the GPIbα tail (residues 556 to 577). Critical residues that provide the lock-and-key interaction between the two domains are indicated. *GP*, Glycoprotein.

consequences. First, it positions filamin's self-association domain and associated partner proteins at the plasma membrane while dangling filamin's actin binding sites into the cytoplasm. Second, because a large fraction of filamin is bound to actin, it aligns the GPIb-IX-V complexes on the surface of the platelet over the underlying filaments (see Fig. 123.9B). Third, because the filamin linkages between actin filaments and the GPIb-IX-V complex pass through the pores of the spectrin lattice, it restrains the molecular movement of the spectrin strands in this lattice and holds the lattice in compression. The filamin-GPIbα connection is essential for the formation and release of discoid platelets by megakaryocytes; platelets lacking this connection are large and fragile and are produced in low numbers. However, the role of the filamin-vWF receptor connection in platelet construction is unknown. Because a low number of Bernard-Soulier platelets form and release from megakaryocytes, it can be argued that this connection is a late event in the maturation process and is not required for platelet shedding. Both filamin and GPIbα are synthesized early, but linkage between the two may not occur until later, perhaps as late as the final stages of platelet shedding. Bernard-Soulier patients have an autosomal recessive bleeding disorder characterized by low platelet count with giant platelets, underscoring the importance of the filamin-GPIbα connection in stabilizing the membrane skeleton in platelet biology.

Aside from the erythrocyte, the platelet is the only cell whose membrane skeleton has been visualized at high resolution. Like the erythrocyte, the platelet membrane skeleton is a self-assembly of elongated spectrin strands (see Fig. 123.9) that interconnect through binding to actin filaments. Platelets express approximately 2000 spectrin molecules. Although considerably less is known about how the spectrin-actin network forms and is connected to the plasma membrane in the platelet relative to the erythrocyte, certain differences between the two membrane skeletons have been identified. First, the spectrin strands composing the platelet membrane skeleton interconnect using the ends of long actin filaments instead of short actin oligomers. These ends arrive at the plasma membrane originating from filaments in the cytoplasm. Hence the spectrin lattice is assembled into a continuous network by its association with actin filaments. Second, tropomodulins are not expressed at sufficiently high levels, if at all, to have a major role in capping the pointed ends of the platelet actin filaments. Instead, biochemical experiments have revealed that a substantial number (≈2000) of these ends are free in the resting platelet. Third, although little tropomodulin protein is expressed, α adducin and γ adducin are abundantly expressed and appear to cap many of the barbed ends of the filaments composing the resting actin cytoskeleton. Adducin is a key component of the membrane skeleton, forming a triad complex with spectrin and actin. Capping of barbed filament ends by adducin also serves the function of targeting them to the spectrin-based membrane skeleton because the affinity of spectrin for adducin-actin complexes is greater than for either actin or adducin alone. Platelet GPs involved in attaching spectrin to the membrane remain to be defined.

REFERENCES

1. Bluteau D, Lordier L, Di Stefano A, et al. Regulation of megakaryocyte maturation and platelet formation. *J Thromb Haemost*. 2009;7(1):227.
2. Schulze H, Korpal M, Hurov J, et al. Characterization of the megakaryocyte demarcation membrane system and its role in thrombopoiesis. *Blood*. 2006;107(10):3868.
3. Lu SJ, Li F, Yin H, et al. Platelets generated from human embryonic stem cells are functional in vitro and in the microcirculation of living mice. *Cell Res*. 2011;21(3):530–545.
4. Takayama N, Nishimura S, Nakamura S, et al. Transient activation of c-MYC expression is critical for efficient platelet generation from human induced pluripotent stem cells. *J Exp Med*. 2010;207(13):2817.
5. Italiano Jr JE, Lecine P, Shivdasani RA, et al. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J Cell Biol*. 1999;147(6):1299.
6. Schwer H, Lecine P, Tiwari S, et al. A lineage-restricted and divergent b tubulin isoform is essential for the biogenesis, structure and function of mammalian blood platelets. *Curr Biol*. 2001;11(8):579.
7. Patel S, Richardson J, Schulze H, et al. Differential roles of microtubule assembly and sliding in proplatelet formation by megakaryocytes. *Blood*. 2005;106(13):4076.
8. Patel S, Hartwig J, Italiano Jr J. The biogenesis of platelets from megakaryocyte proplatelets. *J Clin Invest*. 2006;115(12):3348.
9. Schulze H, Dose M, Korpal M, et al. RanBP10 is a cytoplasmic guanine nucleotide exchange factor that modulates noncentrosomal microtubules. *J Biol Chem*. 2008;283(20):14109.
10. Chen Z, Naveiras O, Balduini A, et al. The May-Hegglin anomaly gene MYH9 is a negative regulator of platelet biogenesis modulated by the Rho-ROCK pathway. *Blood*. 2007;110(1):171.
11. Eckly A, Strassel C, Freund M, et al. Abnormal megakaryocyte morphology and proplatelet formation in mice with megakaryocyte-restricted MYH9 inactivation. *Blood*. 2009;113(14):3182.
12. Leon C, Eckly A, Hechler B, et al. Megakaryocyte-restricted MYH9 inactivation dramatically affects hemostasis while preserving platelet aggregation and secretion. *Blood*. 2007;110(9):3183.
13. Chen Z, Shivdasani RA. Regulation of platelet biogenesis: insights from the May-Hegglin anomaly and other MYH9-related disorders. *J Thromb Haemost*. 2009.;7(suppl 1):272.
14. Richardson J, Shivdasani R, Boers C, et al. Mechanisms of organelle transport and capture along proplatelets during platelet production. *Blood*. 2005;106(13):4066.
15. Patel-Hett S, Wang H, Begonja AJ, et al. The spectrin-based membrane skeleton stabilizes mouse megakaryocyte membrane systems and is essential for proplatelet and platelet formation. *Blood*. 2011;118(6):1641–1652.
16. Junt T, Schulze H, Chen Z, et al. Dynamic visualization of thrombopoiesis within bone marrow. *Science*. 2007;317(5845):1767.
17. Thon JN, Montalvo A, Patel-Hett S, et al. Cytoskeletal mechanics of proplatelet maturation and platelet release. *J Cell Biol*. 2010;191(4):861.
18. Schwertz H, Köster S, Kahr WH, et al. Anucleate platelets generate progeny. *Blood*. 2010;115(18):3801.
19. Larson MK, Watson SP. A product of their environment: do megakaryocytes rely on extracellular cues for proplatelet formation? *Platelets*. 2006;17(7):435.
20. Larson MK, Watson SP. Regulation of proplatelet formation and platelet release by integrin alpha IIb beta3. *Blood*. 2006;108(5):1509.
21. Goldenson B, Kirsammer G, Stankiewicz MJ, et al. Aurora kinase A is required for hematopoiesis, but is dispensable for murine megakaryocyte endomitosis and differentiation. *Blood*. 2015;125(13):2141.
22. Dore LC, Crispino JD. Transcription factor networks in erythroid cell and megakaryocyte development. *Blood*. 2011;118(2):231–239.
23. Hartwig JH. The platelet: form and function. *Semin Hematol*. 2006.;43(suppl 1):S94.
24. Patel-Hett S, Richardson JL, Schulze H, et al. Visualization of microtubule growth in living platelets reveals a dynamic marginal band with multiple microtubules. *Blood*. 2008;111(9):4605.
25. Nakamura F, Stossel TP, Hartwig JH. The filamins: organizers of cell structure and function. *Cell Adh Migr*. 2011;5(2):160.
26. Nakamura F, Pudas R, Heikkinen O, et al. The structure of the GP1b-filamin A complex. *Blood*. 2005;107(5):1925.
27. Eckly A, Heijnen H, Pertuy F, et al. Biogenesis of the demarcation membrane system (DMS) in megakaryocytes. *Blood*. 2014;123(6):921.
28. Wandall HH, Rumjantseva V, Sørensen AL, et al. The origin and function of platelet glycosyltransferases. *Blood*. 2012;120(3):626.
29. Chen Y, Aardema J, Kale S, et al. Loss of the F-BAR protein CIP4 reduces platelet production by impairing membrane-cytoskeleton remodeling. *Blood*. 2013;22(10):1695.
30. Bender M, Giannini S, Grozovsky R, et al. Dynamin 2-dependent endocytosis is required for normal megakaryocyte development in mice. *Blood*. 2015;125(6):1014.
31. Thon JN, Peters CG, Machlus KR, et al. T granules in human platelets function in TLR9 organization and signaling. *J Cell Biol*. 2012;198(4):561–574.
32. Bender M, Thon JN, Ehrlicher AJ, et al. Microtubule sliding drives proplatelet elongation and is dependent on cytoplasmic dynein. *Blood*. 2015;125(5):860.
33. Rojnuckarin P, Kaushansky K. Actin reorganization and proplatelet formation in murine megakaryocytes: the role of protein kinase calpha. *Blood*. 2001;97(1):154–161.
34. Machlus Kr, Wu SK, Stumpo DJ, et al. Synthesis and dephosphorylation of MARCKS in the late stages of megakaryocyte maturation drive proplatelet formation. *Blood*. 2016;127(11):1468–1480.
35. Bender M, Stritt S, Nurden P, et al. Megakaryocyte-specific Profilin1-deficiency alters microtubule stability and causes a Wiskott-Aldrich syndrome-like platelet defect. *Nat Commun*. 2014;5:4746.
36. Snapper SB, Rosen FS, Mizoguchi E, et al. Wiskott-Aldrich syndrome protein-deficient mice reveal a role for WASP in T but not B cell activation. *Immunity*. 1998;9(1):81–91.
37. Sabri S, Foudi A, Boukour S, et al. Deficiency in the Wiskott-Aldrich protein induces premature proplatelet formation and platelet production in the bone marrow compartment. *Blood*. 2006;108(1):134–140.
38. Kahr WHA, Pluthero FG, Elkadri A, et al. Loss of the Arp2/3 complex component ARPC1B causes platelet abnormalities and predisposes to inflammatory disease. *Nat Commun*. 2017;3(8):14816.
39. Paul DS, Casari C, Wu C, et al. Deletion of the Arp2/3 complex in megakaryocytes leads to microthrombocytopenia in mice. *Blood Adv*. 2017;1(18):1398–1408.
40. Begonia JA, Hoffmeister KM, Hartwig JH, Falet H. FlnA-null megakaryocytes prematurely release large and fragile platelets that circulate poorly. *Blood*. 2011;118(8):2285–2295.
41. Bender M, Eckly A, Hartwig JH. ADF/n-cofilin-dependent actin turnover determines platelet formation and sizing. *Blood*. 2010;116(10):1767.
42. Pleines I, Dütting S, Cherpokova D, et al. Defective tubulin organization and proplatelet formation in murine megakaryocytes lacking Rac1 and Cdc42. *Blood*. 2013;122(18):3178.
43. Pleines I, Hagedorn I, Gupta S. Megakaryocyte-specific RhoA deficiency causes macrothrombocytopenia and defective platelet activation in hemostasis and thrombosis. *Blood*. 2012;119(4):1054.
44. Lefrançais E, Guadalupe OM, Caudrillier A, et al. The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature*. 2017;544(7648):105–109.
45. Davis RE, Stenberg PE, Levin J, Beckstead JH. Localization of megakaryocytes in normal mice and following administration of platelet antiserum, 5-fluorouracil, or radiostrontium: evidence for the site of platelet production. *Exp Hematol*. 1997;25(7):638–648.
46. Mikhailidis DP, Stead RJ, Barradas MA, et al. Platelet abnormalities in patients with cystic fibrosis and obligate heterozygotes. *Haematologica*. 1990;75(2):137–140.
47. Harrison MT, Short P, Williamson PA, et al. Thrombocytosis is associated with increased short and long term mortality after exacerbation of chronic obstructive pulmonary disease: a role for antiplatelet therapy? *Thorax*. 2014;69(7):609–615.
48. Şahin F, Yazar E, Yıldız P. Prominent features of platelet count, plateletcrit, mean platelet volume and platelet distribution width in pulmonary tuberculosis. *Multidiscip Respir Med*. 2012;7(1):38.
49. Diagouraga B, Grichine A, Fertin A, et al. Motor-driven marginal band coiling promotes cell shape change during platelet activation. *J Cell Biol*. 2014;204(2):177.
50. Freson K, De Vos R, Wittevrognel C, et al. The β1-tubulin Q43P functional polymorphism reduces the risk of cardiovascular disease in men by modulating platelet function and structure. *Blood*. 2005;106(7):2356.

CHAPTER 124

MOLECULAR BASIS OF PLATELET FUNCTION

Margaret L. Rand and Sara J. Israels

A primary physiological role of platelets is to support hemostasis at sites of vascular injury by forming a plug that arrests blood loss (Fig. 124.1). Normally, disc-shaped platelets circulate in the bloodstream without adhering to the endothelium of the vessel wall. When the endothelium is damaged, platelets adhere to the exposed subendothelial collagen and, at high shear, to collagen-immobilized von Willebrand factor (VWF). Platelet adhesion at the site of vessel wall damage initiates activation events via intracellular signaling pathways that trigger (1) reorganization of the actin cytoskeleton that results in a shape change from discs to irregular spheres with filopodia, and enables platelet spreading to increase surface contact; (2) secretion of dense granule and α-granule contents, including adenosine 5-diphosphate (ADP) from the dense granules; (3) formation and release of second messengers including thromboxane A_2 (TxA_2); and (4) exposure of phosphatidylserine (PS) on the platelet surface, thereby creating a procoagulant activated platelet surface for assembly of coagulation factor complexes that accelerate the generation of thrombin (see Chapters 121 and 125). Platelet agonists, including ADP, TxA_2, and thrombin, bind to their specific membrane receptors, initiating signaling pathways that convert integrin αIIbβ3 from a low-affinity resting state to a high-affinity activated state capable of binding its ligands. Divalent fibrinogen and multivalent VWF function as bridges between αIIbβ3 on adjacent activated platelets, resulting in aggregation and plug formation. This series of platelet responses is essential for the hemostatic function of platelets, and when impaired by congenital or acquired defects, bleeding can occur. However, if these same events take place on a ruptured atherosclerotic plaque, they can lead to the formation of a platelet-rich thrombus that can occlude the arterial lumen and lead to ischemia (see Chapter 121).

This chapter addresses the molecular basis of platelet activation and primary hemostatic plug formation. Significant progress has been made in defining the molecular mechanisms that govern platelet responses, facilitated by the study of patients with congenital defects of platelet function and/or number, and by the study of genetically modified animals. The field of platelet biology has been expanding to encompass the roles of platelets in non-hemostatic processes; these are reviewed briefly at the end of this chapter (see section on Platelets Beyond Hemostasis), and some are touched upon throughout the chapter. What we have learned about the molecular mechanisms of platelets in the setting of hemostasis and thrombosis is now being applied in new areas to address new questions.

MOLECULAR BASIS OF PLATELET ADHESION

Substrates for Platelet Attachment and Spreading

Platelet plug formation is initiated by contact of circulating platelets with proteins in the subendothelial extracellular matrix (ECM). Normal endothelium maintains an effective barrier that prevents circulating platelets from contact with these ECM proteins (see Chapter 122). In addition to the physical barrier, endothelial cells actively inhibit platelet activation by the release of nitric oxide, prostacyclin, and enzymes (CD39/CD73) involved in the metabolism of ADP.[1] At sites of vascular injury, where endothelium is lost or damaged and the ECM becomes exposed to flowing blood, platelets come into close contact with ECM adhesive proteins that promote initial attachment and subsequent activation.

The subendothelial matrix can be viewed as a dynamic and mutable interface that provides multiple substrates to support platelet adhesion (Table 124.1). These substrates include collagens,[2] immobilized VWF,[3] fibronectin,[4] laminin,[5] and thrombospondin-1.[6] Plasma proteins such as fibrinogen,[7] vitronectin,[8] and circulating VWF also interact with platelets after binding to matrix components. The roles of collagen and VWF are well defined, while the contributions of other adhesive proteins to the initial attachment of platelets are less well established. Studies of mice deficient in individual matrix proteins have been useful in determining the contribution of these proteins to the integrated function of the ECM in the formation, growth, and stabilization of the platelet plug.

Shear rates in flowing blood, which vary depending on vessel caliber, influence the contribution of specific matrix proteins. At low shear, platelets can be captured directly by collagens, but at higher shear rates ($>1000\,s^{-1}$), VWF becomes critical in ensuring efficient platelet attachment via the receptor glycoprotein (GP)Ibα (Fig. 124.2A) (see section on Platelet Adhesion Receptors). VWF, secreted from endothelial cells and recruited from plasma, binds to type VI collagen in the vascular matrix via the VWF A1 domain, and to types I and III collagen in both superficial and deeper layers of the matrix via the VWF A3 domain (see Chapter 133).[9]

Collagen types I, III, and VI exposed at sites of vascular injury are key substrates for direct platelet adhesion. Collagen is a fibrillar protein made up of tropocollagen monomers packed into units of five to generate microfibrils.[10] Collagens have motifs that are not only recognized by VWF but also by the platelet collagen receptors α2β1 and GPVI (see section on Platelet Adhesion Receptors).[2,11] At low shear, collagen can function independently of VWF to capture platelets at the matrix surface. However, under high shear, collagen and VWF, with their respective receptors, function in concert.[9,12]

Platelet adhesion is supported by other substrates in the matrix, and by plasma proteins that interact with the matrix. It is likely that these proteins fine-tune the primary hemostatic response depending on the site or severity of the injury. Plasma proteins including fibronectin,[4,13] vitronectin,[8] and fibrinogen (and its polymerized product fibrin), matrix protein laminin[5] and platelet α-granule protein thrombospondin-1[6] support platelet adhesion in experimental systems. It is likely that most of these proteins play an ancillary role in vivo via mechanisms that include enhancing VWF immobilization in the matrix (laminin), protecting large VWF multimers from degradation (thrombospondin-1), or bridging between platelet receptors and matrix collagen (fibronectin).

Platelet Adhesion Receptors

Nomenclature

The individual adhesive proteins serve as ligands for specific receptors on the platelet surface (see Table 124.1). Several nomenclature systems have been used to identify the membrane glycoproteins of the platelet, such that the same receptor may have multiple designations. The original and still widely used system designates the membrane glycoproteins according to their electrophoretic mobility upon sodium dodecyl sulfate polyacrylamide gel electrophoresis; protein separation is based on molecular weight, with lower molecular-weight proteins having a greater mobility than those with higher molecular

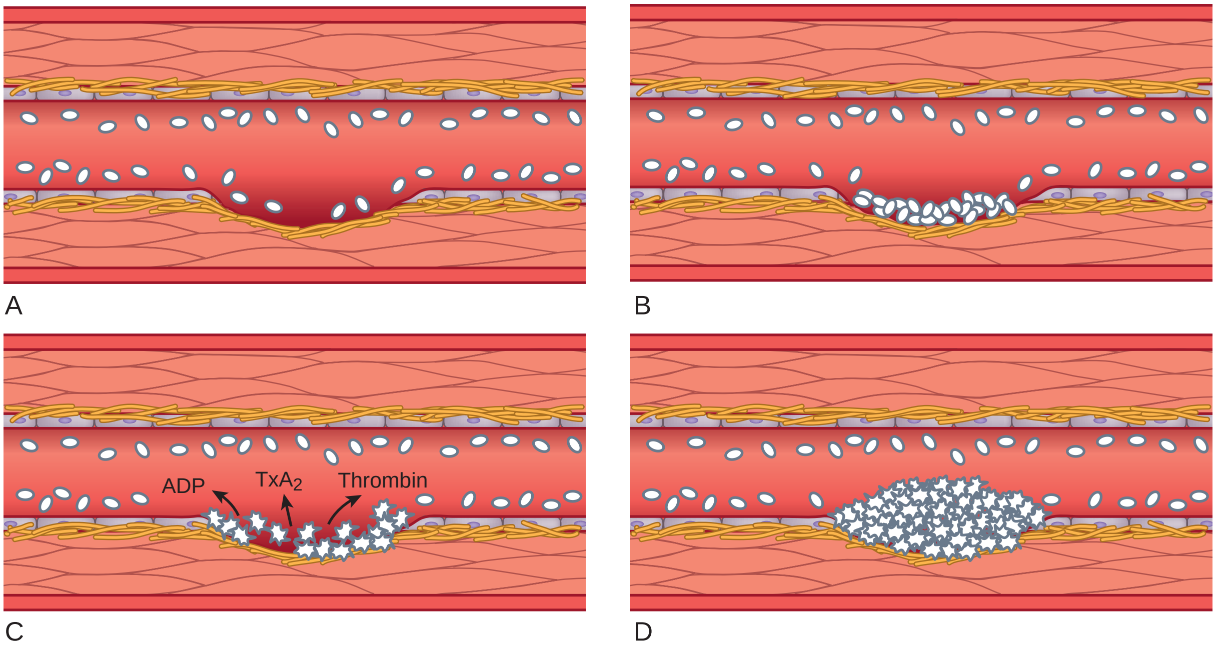


Figure 124.1 HEMOSTATIC RESPONSE OF PLATELETS TO VESSEL WALL INJURY. (A) Disruption of the endothelial cell lining of the blood vessel exposes constituents of the subendothelial extracellular matrix. (B) Platelets adhere to and spread on matrix constituents. (C) Activated platelets secrete ADP, synthesize and release thromboxane A_2 (TxA_2), and promote the generation of thrombin. (D) ADP, TxA_2, and thrombin activate and recruit additional platelets that aggregate to form a platelet plug.

TABLE 124.1 **Subendothelial Matrix Adhesive Proteins and Their Platelet Receptors**

Adhesive Protein	Receptor(s)
Collagen	GPIa–IIa (α2β1)[a] GPVI
von Willebrand factor	GPIb–IX–V GIIb–IIIa (αIIbβ3)
Fibronectin	GPIc–IIa (α5β1) GPIIb–IIIa (αIIbβ3)
Thrombospondin-1	αvβ3
Vitronectin	αvβ3 GPIIb–IIIa (αIIbβ3)
Laminin	α6β1

[a] Alternative name for receptor is given in brackets.

weight. Glycoproteins were designated as GPI, II, III, and so on, with GPI having the highest molecular weight. With greater resolution techniques, additional glycoproteins were identified, hence the subdivision designations, for example, GPIa, GPIb, GPIc; GPIIa, GPIIb. Many of the membrane glycoproteins on the platelet surface exist as noncovalent complexes, for example, GPIa–IIa, GPIb–IX–V, GPIIb–IIIa.

In addition to the GP nomenclature system, the αβ integrin nomenclature system is used. Several of the platelet membrane glycoproteins are members of the integrin family of adhesion receptors (see below), for example, GPIa–IIa is α2β1, GPIIb–IIIa is αIIbβ3. Some glycoproteins also have cluster of differentiation (CD) designations, for example, CD41 for GPIIb and CD61 for GPIIIa. Finally, some receptors have been named based on their function (e.g., the fibrinogen, fibronectin, and vitronectin receptors). Although functional designations can be appropriate from a descriptive standpoint, at least two different membrane glycoproteins on platelets serve as receptors for vitronectin and fibronectin (see Table 124.1), and several are receptors for collagen (see below). Beyond creating nomenclature complexity, redundancy of platelet receptors endows the platelet with the capacity to form multiple contacts with a single matrix constituent. Thus, a single ligand may initiate several distinct functional responses by engaging different receptors.

The Integrin Family of Adhesion Receptors

Integrins are members of a superfamily of broadly distributed adhesion receptors that mediate cell-matrix and cell-cell interactions. They are composed of noncovalently associated α and β transmembrane polypeptide subunits with large extracellular domains and short cytoplasmic tails. Of the 18 known α subunits, platelets express five (α2, α5, α6, αIIb, αv), and of the eight known β subunits, platelets express two (β1, β3), with a total of five different platelet integrins. It is estimated that half of the surface area of an activated platelet is occupied by integrin receptors. Many of the glycoprotein receptors on platelets that mediate cell-matrix adhesion are members of the integrin family (see Table 124.1). α2β1 is a receptor for subendothelial collagen (see below); α5β1 for fibronectin; α6β1 for laminin; αIIbβ3 for fibrinogen, VWF, and fibronectin; and αvβ3 for vitronectin. Of the integrins expressed on blood cells, αIIbβ3 is the most narrowly distributed and is restricted predominantly to platelets and megakaryocytes. It not only plays a prominent role in platelet aggregation (see section on Molecular Basis of Platelet Aggregation), but in many other platelet responses, including e.g., the association of platelets with tumor cells, an interaction involved in tumor metastasis, and with a variety of microorganisms, interactions involved in infection.

Role of GPIb–IX–V in Platelet Adhesion

GPIb–IX–V is a notable example of a major cell surface molecule involved in platelet adhesion that is not a member of the integrin family. GPIb is composed of an α (heavy) chain and two β (light) chains; the α and β chains both span the platelet membrane and are linked by disulfide bonds.[14] GPIX and GPV are smaller single-chain transmembrane polypeptides that are noncovalently associated with GPIb, and all three subunits are members of the leucine-rich repeat protein superfamily. GPIb and GPIX are necessary for the expression of the complex on the platelet surface. The GPIbα-GPIbβ–IX–V complex has a stoichiometry of 1:2:1:1 and exists in high copy number on the platelet surface (25,000 to 35,000 molecules per platelet) with the N-terminus of GPIbα being the major ligand-binding subunit of the complex. Platelet activation and aging are associated with the shedding of glycocalicin, a large proteolytic N-terminal fragment of GPIbα that is cleaved by (mostly) ADAM17. The cytoplasmic tail of GPIbα binds to a number of molecules, including 14-3-3ζ and filamin A (that binds to actin filaments in the cytoskeleton) (see section on the Platelet Cytoskeleton) that are important for GPIb-IX-V-mediated signaling.[15]

GPIb–IX–V plays a major role in hemostasis and thrombosis as a receptor for immobilized VWF in the subendothelium. Plasma VWF does not normally interact with platelet GPIb–IX–V, but at the high shear rates that occur in the arterial microcirculation, a conformational change in VWF bound to subendothelial collagen exposes a GPIbα binding site in its A1 domain. Thus, GPIb–IX–V binds to subendothelial VWF; this interaction is reversible with fast on and off rates, which allows for translocation (rolling) of platelets on the surface of the subendothelium (see Fig. 124.2A).[9,12] A polymorphism in the GPIbα gene leads to variable numbers of tandem repeats in the macroglycopeptide region (sialomucin "stalk") of the protein between the ligand-binding region and the transmembrane domain. The length of this region, which is determined by the number of tandem 13-amino acid repeats (one to four), may have a role in regulating platelet adhesion to VWF. With GPIb-IX-V being a major mechanoreceptor on platelets, a pulling force exerted on GPIbα by VWF binding activates a mechanosensory domain close to the transmembrane domain, thereby resulting in intracellular signaling.[14]

In addition to serving as a receptor for VWF in the subendothelium, GPIb–IX–V is also the high-affinity receptor for the soluble agonist thrombin (see section on Soluble Agonist Receptors). Other adhesive ligands for GPIbα include the counter-receptors P-selectin (CD62P), expressed on activated platelets and endothelial cells, and leukocyte integrin Mac-1 (αMβ2), which is involved in the formation of platelet-leukocyte conjugates.[15]

Platelets from patients with Bernard-Soulier syndrome (BSS) lack GPIb–IX–V because of variants in the *GP1BA, GP1BB*, or *GP9* genes that prevent the expression of the complex on the platelet surface. Decreased expression of GPIb–IX–V impairs platelet adhesion to the subendothelium and results in a bleeding diathesis (see section on Molecular Basis of Inherited Platelet Disorders).

Collagen Receptors

Subendothelial collagen has long been recognized as an important initiator of platelet responses, serving as a substrate for platelet adhesion, which ultimately results in platelet aggregation. Three different receptors are involved in platelet responses to collagen: the GPIb–IX–V complex, which binds to collagen via VWF (see above), and GPVI and α2β1 (GPIa–IIa), which directly bind to collagen.

GPVI, with 3000 to 4000 copies per platelet, is a transmembrane protein member of the immunoglobulin superfamily, with two extracellular immunoglobulin domains. Its cytoplasmic domain contains a proline-rich sequence that binds to tyrosine kinases involved in platelet signaling (see section on Activation Pathways).[9,16] GPVI is noncovalently associated with the Fc receptor γ chain (FcRγ) dimer, which is required for GPVI surface expression and for GPVI signaling via immunoreceptor tyrosine-based activation motifs (ITAMs) in the FcRγ cytoplasmic tails. There is in vitro and in vivo evidence of shedding of GPVI from the surface of activated platelets by the action of ADAM10 and ADAM17. Fibrin has recently been described as an additional ligand for GPVI, adding to the scope of the role of this receptor in platelet adhesion.[17,18] Deficiencies of GPVI can be associated with a mild bleeding diathesis (see section on Genetic Basis of Inherited Platelet Function Disorders).

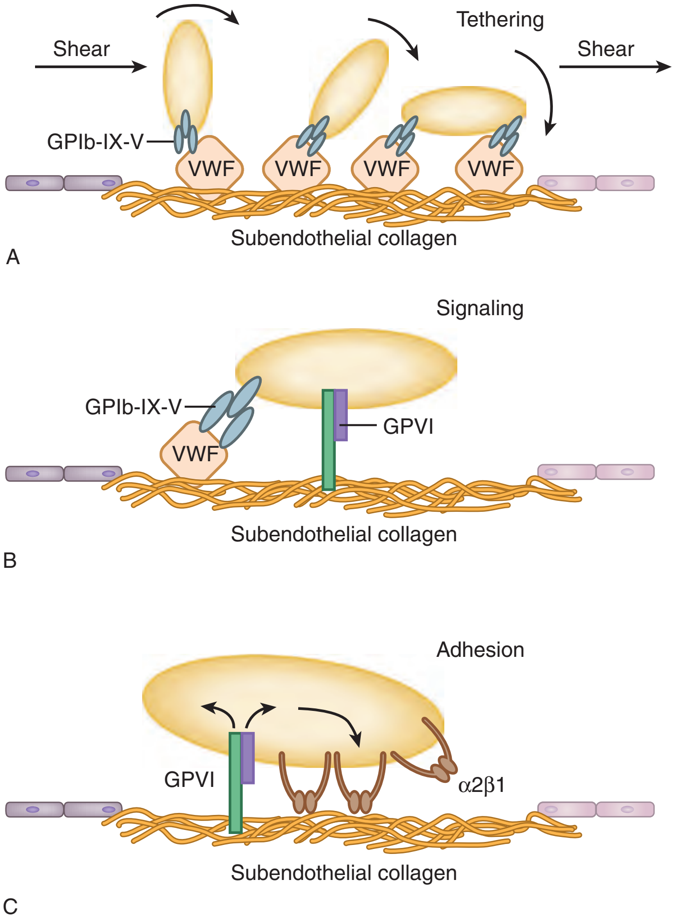


Figure 124.2 PLATELET ADHESION TO THE SUBENDOTHELIUM IS A MULTISTEP PROCESS. (A) Collagen-bound von Willebrand factor (VWF) in the subendothelium undergoes a conformational change when exposed to arterial shear stress such that glycoprotein (GP)Ibα of the GPIb–IX–V complex binds to the VWF A1 domain. These rapidly formed bonds are quickly broken and reestablished, causing platelet translocation, or rolling, along the subendothelium. (B) The translocation process slows platelet transit, allowing the signaling receptor GPVI to bind directly to collagen. (C) GPVI-mediated signal transduction pathways activate the integrin α2β1, enabling it to bind strongly to collagen. This final association between the platelet and collagen is stable, and allows the platelet to adhere firmly to the subendothelium.

α2β1, with approximately 4000 copies per platelet, is the major collagen-binding integrin on the platelet surface.[9,19] It undergoes a conformational change from a low- to a high-affinity state to allow it to bind to collagen. Silent polymorphisms in the α2 gene control its expression level, with higher plasma membrane levels associated with enhanced adhesion to collagen in vitro and an increased risk of cardiovascular disease.[20]

The adhesion of platelets to collagen is a complex, multistep process. An initial interaction of GPIb–IX–V with VWF in the subendothelium (see above) facilitates a direct, low-affinity interaction of GPVI with collagen (see Fig. 124.2B). Binding of GPVI to collagen is a potent stimulus to intracellular signaling pathways (see section on Activation Pathways). As a result of this signaling cascade, α2β 1 is activated to bind collagen with high affinity (see Fig. 124.2C), thereby forming a stable interaction. Alternatively, or in addition, low levels of constitutively activated α2β1 can initiate direct binding of platelets to collagen, thereby assisting GPVI to bind and to initiate activation signaling. At high shear, GPVI plays the major role in mediating stable adhesion, while at lower shear, α2β1 has a more significant role.[21,22]

CLEC-2

CLEC-2 (C-type lectin-like receptor 2) is a transmembrane glycoprotein that was originally described as the receptor responsible for platelet activation by the snake venom rhodocytin, inducing signaling events similar to those initiated by GPVI (see section on Activation Pathways).[16] The major endogenous ligand for CLEC-2 is the transmembrane mucin-like glycoprotein podoplanin, which is expressed on cells outside the vasculature[23]; the CLEC-2-podoplanin interaction does not appear to contribute to hemostasis and arterial thrombosis, although it is involved in inflammation-driven venous thrombosis. Critically, platelet CLEC-2 interaction with podoplanin on lymphatic endothelial cells regulates blood and lymphatic vascular separation during fetal development (see Chapter 122). The CLEC-2-podoplanin interaction on neuro-epithelium is important in blood vessel development in the embryonic brain.[16,24]

MOLECULAR BASIS OF PLATELET ACTIVATION

Soluble Agonist Receptors

Most agonist receptors on platelets are members of the superfamily of trimeric G protein-coupled receptors (GPCRs) that contain seven transmembrane spanning α-helices, four extracellular loops and domains, and four intracellular loops and domains. Upon binding of their ligands, intracellular signaling is initiated via G proteins (GTP-binding proteins) associated with the GPCRs at the inner plasma membrane, leading to diverse downstream responses (see section on Activation Pathways). The platelet GPCRs include receptors for thrombin, ADP, the arachidonic acid metabolite TxA_2, and epinephrine (Table 124.2).[25] Deficiencies or dysfunction of these GPCRs can result in bleeding diatheses ranging from mild to severe (see section on Molecular Basis of Inherited Platelet Disorders).

Thrombin Receptors

Thrombin receptors on platelets belong to the protease-activated receptor (PAR) family, GPCRs with a unique activation mechanism that involves cleavage of the N-terminal extracellular domain, thereby creating a new N-terminus that acts as a tethered ligand for the receptor. PAR1 and PAR4 are present on human platelets (PAR3 and PAR4 on mouse platelets); PAR1 is the high-affinity thrombin receptor (approximately 2500 copies per platelet), responding to thrombin at nanomolar concentrations, and PAR4 is a lower affinity receptor.[26] After thrombin cleavage of the N-terminus of PAR1, the tethered ligand (peptide sequence: Ser-Phe-Leu-Leu-Arg-Asn; SFLLRN) undergoes a conformational change and forms an intramolecular complex with a region also on the N-terminal extracellular domain of the receptor, referred to as ligand binding site-1. This triggers the G protein cycle of PAR1's associated G proteins. PAR4 is nonhomologous with PAR1 in both the peptide sequence of the tethered ligand (Gly-Tyr-Pro-Gly-Lys-Phe; GYPGKF) and its binding characteristics. Although PAR4 requires a higher concentration of thrombin for cleavage, once cleaved, it produces a more sustained signal than PAR1. GPIb–IX–V also possesses a high-affinity binding site for thrombin on GPIbα that facilitates PAR1 cleavage by thrombin.

Platelet PAR1 can be cleaved and activated by proteases other than thrombin, including several members of the membrane metalloproteinase family. These enzymes cleave PAR1 at sites different from the thrombin cleavage site, generating distinct tethered ligands, and distinct patterns of downstream G protein activation.[27]

ADP and ATP Receptors[28]

ADP is an important primary platelet agonist and amplifies other primary responses following its secretion from platelet dense granules.

TABLE 124.2 Platelet G Protein–Coupled Receptors, Their Associated G Proteins (A), and Their Effectors (B)

(A)

Agonist	GPCR	G Protein
Thrombin	PAR1	G_q, G_i (?), $G_{12/13}$
	PAR4	G_q, $G_{12/13}$
ADP	P2Y1	G_q
	P2Y12	G_i
TxA_2	TP	G_q, $G_{12/13}$
PGI_2	IP	G_s

(B)

G Protein Subunit(s)	Effector	Function
$G\alpha_q$	PLC-β	↑IP_3/DAG
$G\beta\gamma_i$	PLC-β	↑IP_3/DAG
	PI3K-γ	↑3-PPIs
$G\alpha_{12/13}$	p115-RhoGEF	RhoA activation
$G\alpha_s$	Adenylate cyclase	↑cAMP
$G\alpha_i$	Adenylate cyclase	↓cAMP

cAMP, Cyclic AMP; *DAG*, diacylglycerol; *GPCR*, G protein-coupled receptor; *IP*, prostaglandin I_2 (prostacyclin) receptor; *IP_3*, inositol-1,4,5-trisphosphate; *PAR*, protease-activated receptor; *PGI_2*, prostaglandin I_2 (prostacyclin); *PI*, phosphoinositide; *PI3K*, phosphatidylinositol 3-kinase; *PLC*, phospholipase C; *3-PPIs*, 3-phosphorylated phosphoinositides; *RhoA*, Ras homolog family member A; *TP*, thromboxane/prostanoid; *TxA_2*, thromboxane A_2.
Adapted from Brass LF, Newman DK, Wannermacher KM, et al. Signal transduction during platelet plug formation. In: Michelson AD, ed. *Platelets*. 3rd ed. San Diego: Academic Press; 2013;367–398, with permission.

Platelets have two ADP receptors: P2Y1 and P2Y12, which are GPCRs that are coupled to distinct G proteins. ADP binding to P2Y1 (approximately 150 copies per platelet), which is coupled to the α subunits of G_q and $G_{12/13}$, mediates a transient rise in intracellular calcium, and induces platelet shape change and rapidly reversible aggregation. ADP binding to P2Y12 (approximately 500 copies per platelet), which is coupled to the α subunit of G_i, results in inhibition of adenylyl cyclase, decreased levels of cyclic AMP (cAMP) and enhancement of the aggregation and secretion responses produced by other agonists. The ADP-P2Y12 receptor axis is key to positive feedback signaling that recruits circulating platelets to the primary platelet plug. Full aggregation responses to ADP in vitro require concurrent activation of both receptor pathways.[29]

Adenosine 5'-triphosphate (ATP) is an antagonist of P2Y1 and P2Y12, but an activator of P2X1, a ligand-gated ion channel, that causes rapid influx of Ca^{2+} from the external milieu and amplifies responses to other agonists. The effects of ADP and ATP are modulated in vivo by CD39, a nucleoside triphosphate diphosphohydrolase (NTPDase-1) expressed by endothelial and vascular smooth muscle cells that hydrolyzes both ATP and ADP, thereby maintaining homeostasis and preventing inappropriate platelet activation.[1]

Thromboxane A_2 Receptor

In humans, the TxA_2 thromboxane/prostanoid (TP) receptor exists in two isoforms, the result of alternative splicing (TPα and TPβ). TPα is the dominant form expressed on platelets, with approximately 1000 copies per platelet.[30]

Epinephrine Receptor

Epinephrine is a relatively weak agonist but plays a role in potentiating platelet activation by other agonists, manifested through its ability to inhibit cAMP formation. In humans, the epinephrine receptor is the α_{2A}-adrenergic receptor (approximately 300 copies per platelet).

Activation Pathways

Once a subendothelial or soluble agonist binds to its receptor on the platelet surface, intracellular signaling pathways (detailed in Fig. 124.3) are set in motion. These signaling pathways result in reorganization of the cytoskeleton and platelet shape change, storage granule exocytosis, TxA_2 synthesis, PS surface exposure, and αIIbβ3 activation.

The signaling pathways, initiated by collagen binding to the ITAM-linked receptor GPVI, and by thrombin, ADP, TxA_2, and epinephrine binding to their specific GPCRs, comprise a "molecular toolkit"[31] of signaling molecules—effectors, second messengers, and signal integrators—which will be considered briefly. Much has been learned about the signaling pathways from genetically modified mice. The reader is referred to comprehensive reviews of platelet signaling for more detail.[15,24,31–42]

Heterotrimeric G Proteins Are Early Response Elements for Most Soluble Platelet Agonists[36]

The GPCRs for soluble platelet agonists are constitutively associated at the inner plasma membrane with specific heterotrimeric G proteins (consisting of α, β, and γ subunits) of the families G_q, $G_{12/13}$, and G_i (see Table 124.2A). The receptor for the potent platelet inhibitory molecule, prostaglandin I_2 (PGI_2; prostacyclin) is also a GPCR and is coupled to G_s.

In the basal, resting state, guanosine 5'-diphosphate (GDP) is bound to the α subunit of the G protein. Upon ligand binding, a GPCR acts as a guanine nucleotide exchange factor (GEF), promoting exchange of GDP for guanosine 5'-triphosphate (GTP) on different classes of the α subunit. Sites on the α and βγ subunits are thereby exposed, allowing for activation of effector molecules including the β isoform of phospholipase C (PLCβ), the γ isoform of phosphatidylinositol 3-kinase (PI3K), and the small GTPase (see below) Rho-specific GEF, p115-RhoGEF (see Table 124.2B). Adenylate cyclase is activated via G_s and inhibited by G_i. Intrinsic GTPase activity

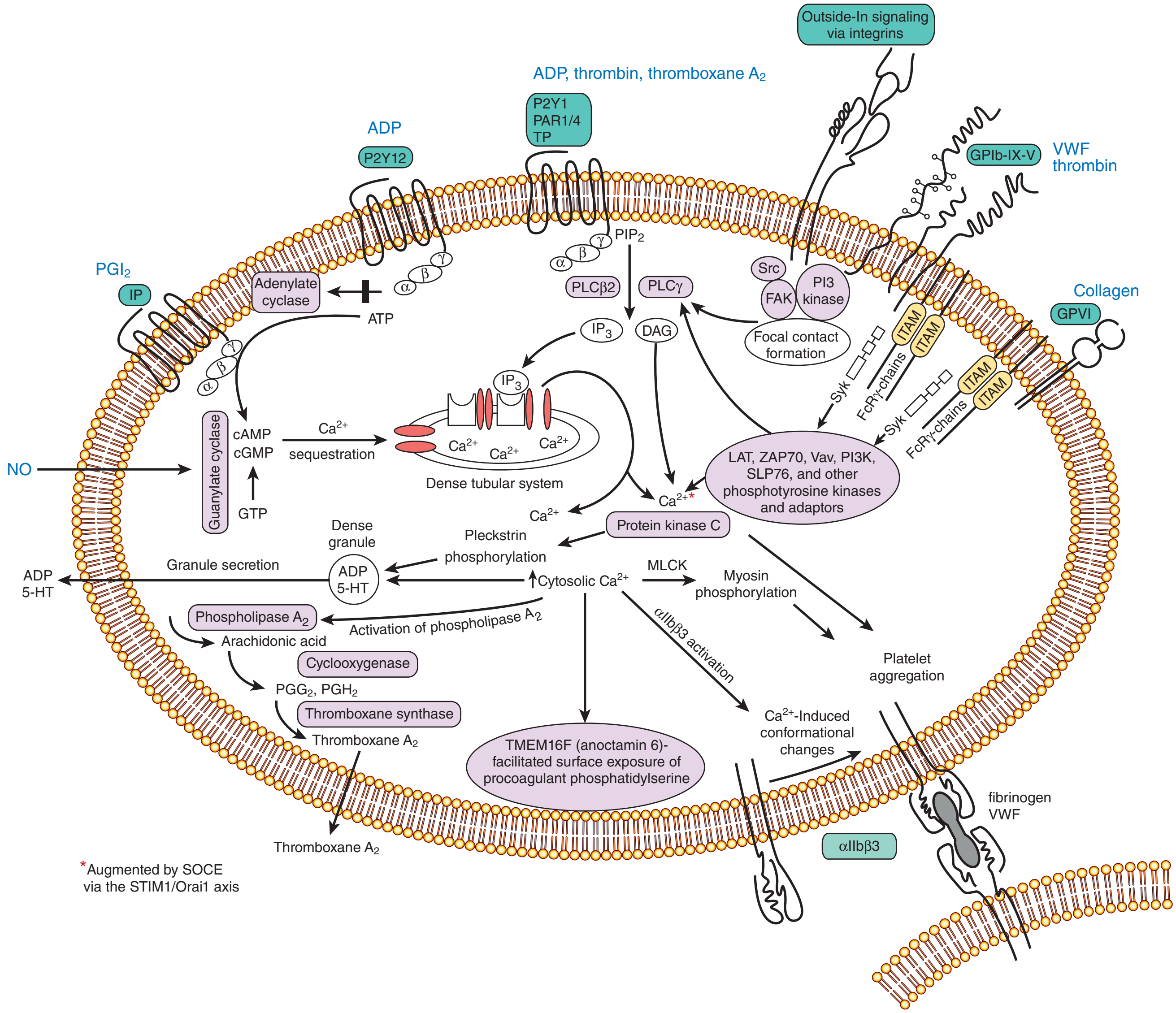

Outside-In signaling via integrins
ADP, thrombin, thromboxane A2
P2Y1
PAR1/4
TP
ADP
P2Y12
GPIb-IX-V
VWF
thrombin
PGI2
IP
Adenylate cyclase
ATP
α
β
γ
PIP2
PLCβ2
PLCγ
Src
FAK
PI3 kinase
Collagen
GPVI
Focal contact formation
ITAM
ITAM
Syk
FcRγ-chains
ITAM
ITAM
Syk
FcRγ-chains
IP3
DAG
IP3
Guanylate cyclase
cAMP
cGMP
GTP
Ca2+
sequestration
Ca2+
Ca2+
Ca2+
Dense tubular system
NO
LAT, ZAP70, Vav, PI3K, SLP76, and other phosphotyrosine kinases and adaptors
Ca2+*
Ca2+
Protein kinase C
Dense granule
Pleckstrin phosphorylation
Granule secretion
ADP 5-HT
ADP 5-HT
↑Cytosolic Ca2+
MLCK
Myosin phosphorylation
Phospholipase A2
Activation of phospholipase A2
Arachidonic acid
Cyclooxygenase
PGG2, PGH2
Thromboxane synthase
Thromboxane A2
αIIbβ3 activation
Platelet aggregation
Ca2+-Induced conformational changes
TMEM16F (anoctamin 6)-facilitated surface exposure of procoagulant phosphatidylserine
fibrinogen
VWF
αIIbβ3
Thromboxane A2
*Augmented by SOCE via the STIM1/Orai1 axis

Figure 124.3 PLATELET INTRACELLULAR SIGNALING PATHWAYS. *5-HT*, Serotonin (5-hydroxytryptamine); *cAMP*, cyclic AMP; *cGMP*, cyclic GMP; *DAG*, diacylglycerol; *GP*, glycoprotein; *IP*, prostaglandin I_2 (prostacyclin) receptor; *IP_3*, inositol-1,4,5-trisphosphate; *ITAM*, immunoreceptor tyrosine-based activation motif; *MLCK*, myosin light chain kinase; *NO*, nitric oxide; *Orai1*, calcium release-activated calcium channel protein 1; *PAR*, protease-activated receptor; *PGG_2*, prostaglandin G_2; *PGH_2*, prostaglandin H_2; *PGI_2*, prostaglandin I_2 (prostacyclin); *PIP_2*, phosphatidylinositol 4,5-bisphosphate; *PI3K*, phosphatidylinositol 3-kinase; *PLC*, phospholipase C; *SOCE*, store-operated Ca^{2+} entry; *STIM1*, stromal interaction molecule 1; *TP*, thromboxane/prostanoid receptor; *VWF*, von Willebrand factor.

of the α subunit, enhanced by GTPase-activating proteins (GAPs), specifically regulator of G-protein signaling (RGS) proteins,[43] hydrolyzes the α subunit-bound GTP to GDP, thus restoring the G protein to its inactive resting conformation.

Protein Tyrosine Kinases Activated by ITAM-linked Receptors Modulate Enzyme Activity and Allow for the Formation of Signaling Complexes[24,32,36]

GPVI is the most potent signaling collagen receptor. As an ITAM-linked receptor, clustering of GPVI upon binding to collagen results in phosphorylation of the constitutively associated FcRγ dimer by tyrosine kinases of the Src family (Fyn and Lyn) that are associated with the cytoplasmic domain of GPVI. Phosphorylation of tandem ITAM motifs enables binding of Syk via Src homology 2 (SH2) domains. Activation of the tyrosine kinase Syk leads to the phosphorylation of the adaptor protein LAT, which serves as the platform for the assembly of a signalosome—a complex of multiple signaling enzymes and adaptor molecules (e.g., SLP-76, Gads, Grb2) within membrane lipid rafts. The signalosome serves as a membrane scaffold for phosphorylation/activation of effectors including phospholipase Cγ (PLCγ) and the β isoform of PI3K.

The platelet receptor platelet-endothelial cell adhesion molecule (PECAM-)1 negatively regulates GPVI signaling via immunoreceptor tyrosine-based inhibitory motif (ITIM) sequences in its cytoplasmic tail by recruiting the tyrosine phosphatase SHP-2 and reducing LAT signalosome assembly.

The signaling pathway initiated by binding of GPIb–IX–V to VWF is similar to that of GPVI in that both involve activation of PLCγ via FcRγ, Fyn and Lyn, and Syk.[15,36] Binding of platelets to podoplanin via CLEC-2 also induces signaling events like those initiated through GPVI. However, rather than (tandem) ITAM phosphorylation, it involves phosphorylation of smaller hemITAMs in the cytoplasmic tails of dimerized receptors.

Phospholipase C Is Responsible for the Hydrolysis of Membrane PI-4,5P$_2$[36]

Activation of PLC, either the β-isoform (by thrombin, ADP, or TxA$_2$ via G$_q$) or the γ-isoform (by collagen, protein tyrosine kinases, and scaffold molecules) results in the hydrolysis of the inner plasma membrane leaflet phospholipid phosphatidylinositol-4,5-bisphosphate (PI-4,5P$_2$). The two second messengers that are the formed, soluble inositol-1,4,5-trisphosphate (IP$_3$) and membrane-associated diacylglycerol (DAG), lead, respectively, to increased cytosolic Ca^{2+} concentrations and to activation of a variety of proteins, most notably protein kinase C (PKC), a serine/threonine kinase.

Phosphatidylinositol 3-Kinase Forms 3-Phosphorylated Phosphoinositides[36,37]

Activation of PI3K isoforms, either γ (via G$_i$) or β (via the collagen signaling pathway), results in phosphorylation of PI-4,5P$_2$ to yield PI-3,4,5P$_3$. Inner membrane leaflet PIP$_3$ functions in signaling by interacting with proteins that contain binding motifs termed pleckstrin homology (PH) domains; an example of a major PIP$_3$ effector is Akt, that is activated to function as a serine/threonine kinase.

Serine/Threonine Kinases Regulate the Activity of Other Proteins Involved in Platelet Activation[33,36,44]

PKC is a central protein kinase in the PLC signaling pathway that phosphorylates the serine and threonine residues of many platelet proteins, including pleckstrin. Upon activation by DAG and Ca^{2+}, the conventional isoforms, PKCα and PKCβ, play important roles in platelet secretion and platelet spreading on fibrinogen, respectively. The serine/threonine kinase Akt (PKB) regulates platelet secretion and αIIbβ3 activation.

PKA and PKG are described in the section below on Cyclic Nucleotides.

The mitogen-activated PKs (MAPKs) ERK, JNK, and p38 are serine/threonine kinases that are activated following platelet stimulation with physiological agonists and are involved in platelet activation processes.[38]

Cytosolic Ca^{2+}[35,36,39]

An increase in the intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$) is a key event that triggers platelet activation and aggregation; Ca^{2+}-dependent responses include shape change, secretion, procoagulant surface exposure, TxA$_2$ formation, and αIIbβ3 activation. The basal [Ca^{2+}]$_i$ is maintained at approximately 0.05 μM and can increase to greater than 1 μM with strong stimulation. The initial increase results from IP$_3$-mediated release of Ca^{2+} from the dense tubular system (DTS) via IP$_3$-receptors. Upon depletion of this Ca^{2+} pool, store-operated Ca^{2+} entry (SOCE) occurs from the platelet exterior. Stromal interaction molecule 1 (STIM1), a protein in the DTS membrane, undergoes a conformational change when DTS Ca^{2+} is depleted, allowing it to bind and activate Orai1 (calcium release-activated calcium channel protein 1), the major SOCE Ca^{2+}-selective release-activated Ca^{2+} (CRAC) channel in the platelet plasma membrane.

Phospholipase A$_2$ Is Responsible for the Synthesis of TxA$_2$[31]

An increase in [Ca^{2+}]$_i$ is one of the main triggers for activation of cytosolic phospholipase A$_2$ (cPLA2), resulting in the formation of the proaggregatory prostanoid TxA$_2$. cPLA2 hydrolyzes the polyunsaturated fatty acid arachidonic acid from the C2 position of inner plasma membrane leaflet phospholipids, and arachidonic acid is converted to the cyclic endoperoxide intermediates prostaglandin (PG) G$_2$ and PGH$_2$ by cyclooxygenase (COX)-1. Thromboxane synthase in the platelet cytosol metabolizes PGH$_2$ to TxA$_2$, which diffuses out of the platelet and is available to activate additional platelets via the TP receptor.

Small GTPases[40–42]

In contrast to the heterotrimeric G proteins associated with GPCRs, platelets also contain small (i.e., low molecular weight) GTPases (belonging to a superfamily of monomeric G proteins) that are critical signaling integrators. Like heterotrimeric G proteins, they are activated by GEFs and inactivated by GAPs. Roles of specific small GTPases of the Rho family, involved in cytoskeletal dynamics, the Ras family, involved in cell adhesion, and the Rab family, involved in membrane trafficking, are described below in the sections Cytoskeletal Reorganization, Molecular Mechanisms of Aggregation and Secretion of Granules, respectively.

Cyclic Nucleotides Are Inhibitory[36]

PGI$_2$ and nitric oxide (NO), synthesized by the endothelium, aid in maintaining platelets in a resting state. PGI$_2$-mediated activation of adenylate cyclase via G$_s$ increases the intracellular level of cAMP. Nitric oxide (NO) diffuses into the cytoplasm and activates soluble guanylate cyclase, generating cGMP. These cyclic nucleotides activate PKA and PKG, respectively. Both serine/threonine kinases phosphorylate multiple substrates including filamin and the small GTPase Rap1. Elevated levels of cAMP and cGMP result in sequestration of intracellular Ca^{2+} in the DTS, and inhibition of cytoskeletal rearrangement, granule secretion, and aggregation.

In contrast, lowered levels of cAMP occur due to inhibition of adenylate cyclase via G_i; this is important in facilitating activation of platelets.

Reorganization of the Actin Cytoskeleton

Platelet plug formation requires platelets to undergo a rapid change from their resting discoid shape to active forms that spread over the damaged subendothelium and recruit additional platelets by providing an expanded surface area for platelet–platelet or platelet–leukocyte interactions. The discoid shape of the circulating platelet is maintained by an internal cytoskeleton composed of polymers of actin and tubulin and their associated proteins (see also Chapter 123). Shape change requires the remodeling of the resting cytoskeleton and the assembly of new cytoskeletal fibers to transform the platelet into its activated configuration.

The Resting Platelet Cytoskeleton

The resting platelet cytoskeleton maintains cell shape and integrity as the platelet encounters the high shear forces of blood flow in small vessels. The spectrin-based membrane skeleton (similar but not identical to that of erythrocytes) forms a contiguous network with actin filaments to support platelet ultrastructure. Actin is the single most abundant platelet protein (2 million copies per platelet), forming 2000 to 5000 linear actin polymer filaments that are cross-linked to form a rigid cytoplasmic network. Cross-linking proteins include filamin A and B, and α-actinin. The interaction between filamin A/B and the cytoplasmic tail of the VWF receptor GPIbα provides structural stability and the major link between the plasma membrane and the actin cytoskeleton.[45] Loss of this linkage in platelets deficient in either GPIbα (BSS) or filamin A results in loss of restraint of the spectrin lattice, swelling of the membrane skeleton, and large fragile platelets that are subject to rapid clearance from the circulation.[12]

Platelets contain a long microtubule wound 8 to 12 times into a coil that sits just beneath the plasma membrane, maintaining the discoid shape of the resting platelet. Microtubules are rigid polymers made up of αβ-tubulin heterodimeric subunits.[46] β1-tubulin deficient mice have platelets that are spherical and fail to develop a discoid shape due to aberrant microtubule assembly.[47] A heterozygous human variant in β1-tubulin, which may be present in as many as 10% of the general population, results in decreased levels of β1-tubulin and a subset of spherocytic platelets.[48]

Cytoskeletal Reorganization During Platelet Activation

The assembly and disassembly of the actin cytoskeleton allows platelets to spread. Following platelet activation, the platelet makes contact with the ECM, initially changing shape from disc to sphere and developing filopodia, followed by flattening and spreading of broad lamellae.[49] Granules and organelles are relocated to the center of the cell. The filopodia are filled with long actin filaments originating in the center of the cell. Increases in $[Ca^{2+}]_i$ initiated by signaling through G_q result in activation of gelsolin, a multidomain protein with binding sites for Ca^{2+}, actin, and phospholipid. Ca^{2+} binding alters the conformation of gelsolin such that it binds and cleaves actin filaments, leading to disassembly of the resting actin cytoskeleton and allowing the platelet to change shape. Signaling via $G_{12/13}$ and p115-RhoGEF activates the small GTPase RhoA that is also involved in regulating actin filament formation and myosin contraction. For example, actin filaments are stabilized by the activation of Rho-activated kinase (p160ROCK) and LIM-kinase.

Assembly of the activated platelet cytoskeleton doubles the actin filament content of the platelet by exposing nucleation ends on existing actin filaments (the result of severing of filaments by gelsolin) or creating new nucleation sites (by the Arp 2/3 complex). The Arp2/3 complex is enriched in the periphery of the activated platelet where actin assembly is occurring, and the complex is activated by proteins associated with cell adhesion sites and by Wiskott-Aldrich syndrome protein (WASp) family members.[50]

Platelets are also the force-generating component of clot retraction. αIIbβ3 is tethered to underlying actin filaments in association with cytoplasmic proteins talin, filamin, paxillin, zyxin, α-actinin, and vinculin. Association of cytoplasmic myosin with actin provides the motor for the contractile force. Platelets contain nonmuscle myosin IIA and IIB; myosin II is a hexamer made up of two heavy chains and four light chains (myosin light chains: MLCs). Assembly into bipolar filaments is the result of Ca^{2+}-activated phosphorylation of the 20-kDa MLCs by MLC kinase, enhanced by blocking MLC dephosphorylation through Rho kinase. *MYH9*-related thrombocytopenia is the result of variants in the gene for nonmuscle myosin heavy chain IIA (NMMHC-IIA) (see section on Molecular Basis of Inherited Platelet Disorders).[51]

Secretion of Granules

Platelet activation leads to the release of more than 300 diverse molecules that stimulate or inhibit platelets or other blood and vascular cells, covalently modify the mechanical properties of thrombi, regulate coagulation, contribute to cell adhesive events, and modulate wound healing, inflammation, and angiogenesis (see Chapters 122 and 125).

Most of the substances that are actively and selectively secreted from platelets are packaged in storage granules formed in megakaryocytes. Platelets contain three types of granules: dense (δ) granules, α-granules, and lysosomal granules (Fig. 124.4A).

Dense Granules

Dense granules belong to the family of lysosome-related organelles (LROs) that also includes melanosomes, cytotoxic T-cell granules, and neutrophil azurophilic granules. Platelets contain three to eight dense granules, which store high concentrations of cations (Ca^{2+}, Mg^{2+}, K^+), polyphosphate, nucleotides (ADP, ATP, GTP), and bioactive amines (serotonin and histamine); they are innately dense when viewed by electron microscopy due to their Ca^{2+} content. These granules undergo a complex maturation process that is regulated by biogenesis of LRO complexes (BLOCs), involving the delivery of specialized cargo by Rab GTPases,[52] and the modulation of zinc transport by the transmembrane protein TMEM163.[53] Variants in specific protein members of BLOCs 1, 2, and 3, or in the adaptor protein complex 3 (AP3) result in dense granule deficiency and associated LRO abnormalities in mice and in Hermansky-Pudlak syndrome (HPS) 1 to 10 in humans, characterized by mucocutaneous bleeding and oculocutaneous albinism (see section on Molecular Basis of Inherited Platelet Function Disorders).[54]

α-Granules

α-Granules are unique to platelets and are the most abundant granule type: 50 to 80 granules per platelet, taking up 10% of the platelet volume. They contain a large number of proteins that are either synthesized by megakaryocytes or taken up from plasma by endocytosis.[55] Genetic defects in *NBEAL2* and *VPS33B*, genes involved in α-granule synthesis, result in α-granule deficiency syndromes (see section on Molecular Basis of Inherited Platelet Disorders).[56–60] Proteomic studies have identified more than 300 unique proteins released by α-granules.[61] These proteins have diverse functions and can be classified as coagulants and anticoagulants (e.g., factors V and XI, antithrombin,

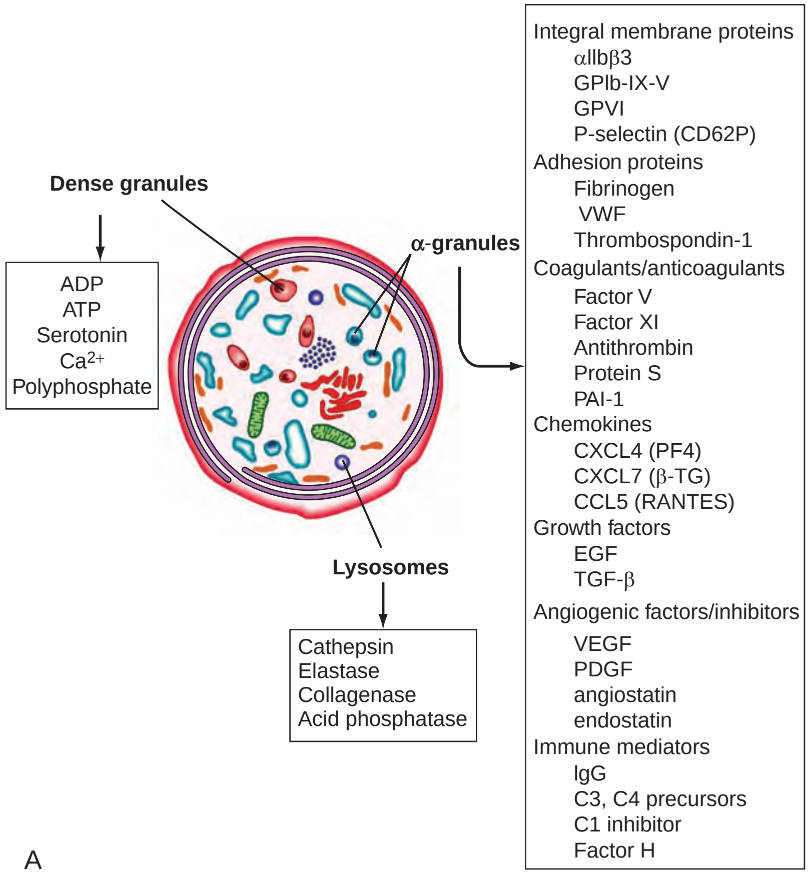

Dense granules
ADP
ATP
Serotonin
Ca2+
Polyphosphate
α-granules
Lysosomes
Cathepsin
Elastase
Collagenase
Acid phosphatase
Integral membrane proteins
αIIbβ3
GPIb-IX-V
GPVI
P-selectin (CD62P)
Adhesion proteins
Fibrinogen
VWF
Thrombospondin-1
Coagulants/anticoagulants
Factor V
Factor XI
Antithrombin
Protein S
PAI-1
Chemokines
CXCL4 (PF4)
CXCL7 (β-TG)
CCL5 (RANTES)
Growth factors
EGF
TGF-β
Angiogenic factors/inhibitors
VEGF
PDGF
angiostatin
endostatin
Immune mediators
IgG
C3, C4 precursors
C1 inhibitor
Factor H
A

Figure 124.4 (A) Platelet granule contents released by secretion. Examples of bioactive substances stored in platelet dense granules, α-granules, and lysosomes. *(Continued)*

protein S, plasminogen activator inhibitor-1); adhesion proteins (e.g., fibrinogen, VWF, thrombospondin-1); chemokines (e.g., CXC-chemokine ligand 4 [CXCL4; platelet factor 4], CXCL7 [β-thromboglobulin]); growth factors (e.g., epidermal growth factor, transforming growth factor-β); angiogenic factors (e.g., vascular endothelial growth factor, platelet-derived growth factor, angiostatin); and immune mediators (e.g., precursors of complement factors C3 and C4) (see Fig. 124.3A).[62] There appears to be heterogeneity in the cargo protein content of subsets of α-granules, with pro- and anti-angiogenic proteins stored in distinct subsets and differentially released.[63] Membrane-bound proteins in α-granules include integrins, immunoglobulin family receptors, leucine-rich repeat family receptors and tetraspanins. Most of these are present on the resting platelet plasma membrane, but following exocytosis, there is a marked increase in plasma membrane expression of some receptors including αIIbβ3 and GPVI. Other receptors, such as P-selectin (CD62P), are only expressed on the plasma membrane after platelet activation and granule exocytosis.[60]

Lysosomal Granules

Platelets have small numbers of primary and secondary lysosomes that contain enzymes involved in degradation of proteins, carbohydrates, and lipids. These enzymes include cathepsins, elastase, collagenases, galactosidase, glucuronidase, and acid phosphatase.

Mechanism of Granule Secretion

The secretion of platelet granule contents occurs through mechanisms analogous to those required for the exocytosis of granules from neurons and mast cells. The process is complex, and both the rate and extent of secretion are related to the strength of the activating stimulus: triggered rapidly and completely by strong agonists such as thrombin. Induction of secretion by weak agonists (e.g., ADP) occurs when the cells are brought into close contact, such as during aggregation,[64] and is dependent on TxA$_2$ to amplify the effect of the primary agonist. Dense granule release is the most rapid; lysosomal release is the slowest; α-granule release is the most variable. The bioactive molecules in the releasate affect the microenvironment of the platelet plug contributing to hemostasis and would healing.

In most cells, exocytosis occurs when vesicles fuse with the plasma membrane and release their contents into the extracellular milieu. In platelets, granules clustered centrally by platelet shape change also fuse with one another and with the open canalicular system (OCS), a system of invaginations of the plasma membrane extending into the interior of the platelet; the granule contents diffuse to the external environment. Membrane fusion is driven by the soluble *N*-ethylmaleimide–sensitive factor (NSF) attachment protein receptors (SNAREs). Platelets have the three basic components of SNARE machinery: tSNAREs (target receptors associated with OCS and plasma membrane), vSNAREs (vesicle-associated receptors associated with the granule membranes), and soluble regulators (including NSF and NSF-attachment proteins).[62,65] vSNAREs including vesicle-associated membrane proteins (VAMP2, VAMP3, VAMP7, VAMP8) interact with syntaxin isoforms (syntaxin 8, syntaxin 11) and synaptosomal-associated protein (SNAP)-23 on the target membranes to form four helix bundles that bring the vesicular and target membranes into proximity (see Fig. 124.4B). SNARE function is tightly regulated by chaperone proteins. NSF regulates membrane interaction by disassembling SNARE complexes on the same membrane so that they are available to form complexes with proteins on opposing membranes. The Sec1/Munc proteins (Munc18a, b, and c, and Munc13-4) and the small GTPase Rab 27 regulate granule docking,[65] a preliminary step to granule membrane fusion (see Fig. 124.4B). Conditional

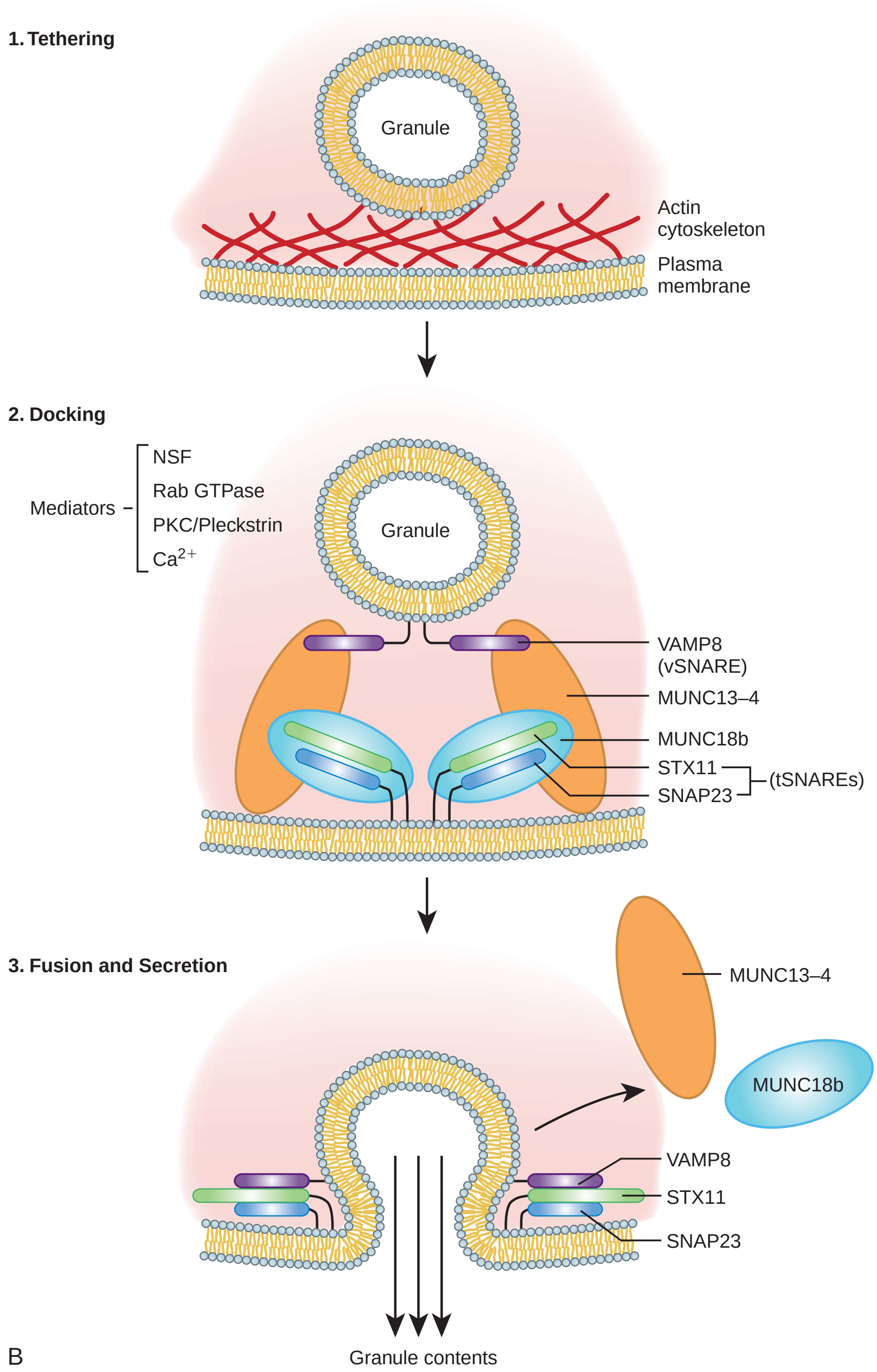

1. Tethering
Granule
Actin cytoskeleton
Plasma membrane
2. Docking
Mediators
NSF
Rab GTPase
PKC/Pleckstrin
Ca2+
Granule
VAMP8 (vSNARE)
MUNC13–4
MUNC18b
STX11
SNAP23
(tSNAREs)
3. Fusion and Secretion
MUNC13–4
MUNC18b
VAMP8
STX11
SNAP23
B
Granule contents

Figure 124.4 cont'd. (B) Molecular control of granule secretion. (1) Tethering: attachment of granules to the membrane cytoskeleton. (2) Docking: the engagement of vSNAREs on the granule membrane and tSNAREs on the target membrane to form a pre-fusion complex. In platelets, these docking proteins include VAMPS (represented by VAMP8) on α-granule and dense granule membranes, STX11/SNAP-23 complex on the plasma membrane and accessory proteins Munc13-4 and Munc18b/Munc18-2. (3) Fusion/secretion: involves the zippering of opposing SNAREs, applying force to fuse the granule and plasma membranes, resulting in release of the granule contents. Additional mediators of this active process include Rab GTPases, PKC, pleckstrin, Ca^{2+}, and NSF. *CXCL*, CXC-chemokine ligand; *EGF*, epidermal growth factor; *GP*, glycoprotein; *IgG*, immunoglobulin G; *NSF*, *N*-ethylmaleimide-sensitive factor; *PAI-1*, plasminogen activator inhibitor-1; *PDGF*, platelet-derived growth factor; *PF4*, platelet factor 4; *PKC*, protein kinase C; *RANTES*, regulated upon activation, normal T-cell expressed, and secreted; *SNAP-23*, synaptosomal-associated protein-23; *SNARE*, soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor; *STX11*, syntaxin 11; *TGF-β*, transforming growth factor-β; *tSNARE*, target SNARE; *VAMP8*, vesicle-associated membrane protein 8; *VEGF*, vascular endothelial growth factor; *vSNARE*, vesicle-associated SNARE; *VWF*, von Willebrand factor; *β-TG*, β-thromboglobulin.

knock-out studies in mice have demonstrated the contributions of vSNAREs and SNAP-23 to platelet secretion, bleeding and thrombosis.[66,67]

Activation pathways involving intracellular Ca^{2+}, Rab GTPase, and PKC isoforms regulate the SNARE complex interactions. PKC isoforms phosphorylate several of the SNARE proteins and their regulators, including Munc18c, syntaxin 4, and SNAP-23. The PKC substrate pleckstrin is also a critical mediator of granule exocytosis: pleckstrin-deficient mice exhibit markedly impaired platelet secretion.[68]

The important role of granule releasates in hemostasis is highlighted by the bleeding diathesis that occurs with granule deficiencies or defects of granule exocytosis. α-Granules secrete fibrinogen and VWF, which mediate platelet-platelet and platelet-ECM interactions. Of the total VWF, 20% is contained in α-granules, enriched in the most potent high-molecular-weight VWF multimers (see Chapter 132). ADP released from dense granules is essential for recruitment of circulating platelets to the primary hemostatic plug. Other mediators released from α-granules play key roles in additional functions including wound repair, angiogenesis, and host defense. However, these modulators can also be pathogenic, contributing to the inflammatory response, atherosclerosis, and tumor metastases (see Fig. 124.4A) (see section below on Platelets Beyond Hemostasis).[69]

Procoagulant Surface Exposure

The phospholipids of the plasma membrane of resting platelets are asymmetrically distributed between the leaflets of the membrane bilayer, with choline-containing phospholipids predominating in the outer cytoplasmic leaflet, and the minor anionic aminophospholipid PS predominating in the inner. Platelet adhesion to collagen and stimulation with thrombin synergistically result in a loss of the asymmetry, or "scrambling," of the membrane phospholipids, such that PS becomes exposed on the external plasma membrane leaflet.[70] PS translocated to the surface of activated platelets facilitates the assembly of the intrinsic tenase complex (factors VIIIa, IXa, and X) and prothrombinase complex (factors Va and Xa, and prothrombin), contributing to the burst of thrombin generation that occurs in the propagation phase of coagulation (see Chapter 125). It sustains the procoagulant response by concentrating the coagulation factors and protecting them from inactivation.[71] Specifically, the negatively charged γ-carboxyglutamate (Gla) residues of the vitamin K-dependent factors, factors VII(a), IX(a), X(a), and prothrombin, mediate Ca^{2+}-dependent binding of these factors to negatively-charged PS. There is recent evidence that plasminogen also binds to PS-exposing platelets, thereby linking coagulation and fibrinolysis.[72]

Exposure of PS on the platelet surface requires sustained increases in $[Ca^{2+}]_i$ via influx across the plasma membrane and activation of phospholipid scramblase (see Fig. 124.3), which rapidly and nonspecifically transports phospholipids between the membrane leaflets.[39,73] The transmembrane protein TMEM16F (anoctamin 6) has been shown to be essential for PS exposure,[74] but its specific role is not yet clear. Scott syndrome, a platelet disorder characterized by a defect in PS exposure, is associated with variants in *TMEM16F* (*ANO6*; see section on Molecular Basis of Inherited Platelet Disorders).[75] There is heterogeneity in the platelet response to stimulation: only a proportion of activated platelets expose PS (this population of platelets is variously described as being procoagulant, super-activated, coated [with covalently linked platelet secretion products (e.g., serotonin, fibrinogen, factor Va)], capped or having Sustained Calcium-Induced Platelet [SCIP] morphology). Besides supporting thrombin generation, PS-exposing platelets are characterized by inactive αIIbβ 3 and a distinct balloon-like morphology with loss of internal organelles. In vitro, they are readily identified by annexin A5 or lactadherin binding. The pathway that leads to rapid PS exposure on activated platelets can be distinguished from a slower intrinsic apoptotic pathway, which involves caspase activation.[73]

Platelet-Derived Extracellular Vesicles

PS exposure is accompanied by blebbing of extracellular vesicles (microparticles; microvesicles; ectosomes) from the plasma membrane[76]; Scott syndrome platelets are deficient in extracellular vesicle formation. These small (0.05 to 1 µm diameter) vesicles are distinct from exosomes, which are released from intracellular vesicles. Circulating extracellular vesicles are thought to originate from megakaryocytes as well as from platelets. The surface membranes of extracellular vesicles are heterogeneous in the expression of platelet membrane receptors such as αIIbβ3 and GPIb–IX–V, and activation markers such as CD62P, CD63, activated αIIbβ3, and surface-exposed PS. Extracellular vesicles can support hemostasis; platelets from patients with the very rare bleeding disorder Castaman syndrome have an isolated deficiency of extracellular vesicle generation. Increased circulating levels of extracellular vesicles have been reported in thrombotic conditions (such as myocardial infarction, thrombotic thrombocytopenic purpura, sickle cell disease, and diabetes), immune-mediated conditions (such as immune thrombocytopenia and heparin-induced thrombocytopenia), and in malignancy and inflammatory conditions. Whether they are active contributors or passive indicators of these conditions is uncertain.[76] Extracellular vesicles also play a role in platelet-cell communications, delivering bioactive molecules (e.g., cytokines, eicosanoids, RNA species) to target cells, even those inaccessible to platelets, as they can enter lymph, bone marrow and synovial fluid.[77] For example, extracellular vesicles formed by platelets activated via GPVI have been shown to contribute to the pathophysiology of rheumatoid arthritis, delivering IL-1 to synovial fibroblasts.

MOLECULAR BASIS OF PLATELET AGGREGATION

αIIbβ3 and Molecular Mechanisms of Aggregation

Integrin αIIbβ3 is the most abundant membrane protein on the platelet surface, with 40,000 to 80,000 copies per resting platelet, a very high receptor density for such a small cell. Platelet activation can produce an additional 10% increase in this number as a result of expression from internal pools, namely, α-granule membranes. On resting platelets, αIIbβ3 is in a low affinity bent conformation that is unable to bind its ligands. Activation of αIIbβ3 results in a change to a high affinity, extended conformation (Fig. 124.5), allowing it to bind divalent fibrinogen or multivalent VWF, which can bridge to other activated αIIbβ3 molecules on adjacent platelets, mediating platelet aggregation. The (extracellular) N-terminal portion of β3 contains three divalent cation-binding sites including the metal-ion dependent adhesion site (MIDAS); metal ions (Ca^{2+}) are required for ligand binding to αIIbβ3. Absence or dysfunction of αIIbβ3 on the platelet surface leads to Glanzmann thrombasthenia (GT), a disorder characterized by a moderate-to-severe bleeding diathesis and absent in vitro platelet aggregation responses to all agonists (see section on Molecular Basis of Inherited Platelet Disorders).

Activation of platelets with any of the agonists described in the preceding sections, including collagen, thrombin, ADP, and TxA_2, results in the conversion of αIIbβ3 from its resting to its activated state.[78] This transformation is a consequence of the intracellular signaling pathways set in motion when the agonist binds to its receptor (see section on Activation Pathways), leading to the final common pathway that results in aggregation; it occurs rapidly, on the order of seconds, after platelet exposure to an agonist. The signal is transmitted to the cytoplasmic tails of αIIbβ3 and through the transmembrane helices, ultimately inducing a change in the extracellular domain to render αIIbβ3 competent to bind its ligands. The signaling process responsible for this transformation is referred to as "inside-out" signaling through αIIbβ3.

Specifically, it is the agonist-induced increase in $[Ca^{2+}]_i$ and DAG formation that result in the final common pathway of integrin activation.[79] Via the Ca^{2+}- and DAG-regulated GEF CalDAG-GEFI, the small GTPase Rap1 (of the Ras family) is activated and promotes binding of the cytoskeletal protein talin to the cytoplasmic domain of β3.[80] Talin cooperates with kindlin-3, which also binds to the cytoplasmic domain of β3, to unclasp the complex between the αIIb and β3 cytoplasmic tails. This leads to a dissociation of the transmembrane complex of the αIIb and β3 subunits, inducing a conformational change in the extracellular domains. The bent conformation of αIIbβ3 is transitioned through intermediate configurations to an extended conformation that exposes the ligand-binding site (see Fig. 124.5) for fibrinogen and VWF.

Both fibrinogen and VWF bind to extracellular domains of activated αIIbβ3 via Arg-Gly-Asp (RGD) sequences. Fibrinogen contains two such sequences, RGD-Ser and RGD-Phe, in its Aα chains, and VWF contains one in its C1 domain. The RGD sequence is a broadly used recognition code in cellular adhesive reactions; it is found on a variety of proteins, and it is recognized by several other integrin receptors. Fibrinogen has a second binding sequence for activated αIIbβ3, Lys-Gln-Ala-Gly-Asp-Val (KQAGDV), in the C-terminus of its γ-chains, that is the primary one by which fibrinogen binds to activated αIIbβ3.[79] While fibrinogen binding mediates platelet aggregation at low shear, it is VWF binding that mediates aggregation at high shear.[81] Other ligands that bind αIIbβ3, mainly via an RGD sequence, include fibronectin, vitronectin, and thrombospondin-1.

αIIbβ3 transmits signaling information bidirectionally in that ligand binding induces further conformational changes to activated αIIbβ3; these result in exposure of binding sites on the cytoplasmic tails of the integrin for cytoskeletal and signaling proteins, and activation of protein kinases and phosphatases. This "outside-in" signaling is important in platelet responses such as full platelet spreading, irreversible aggregation, and clot stability and retraction.[82]

The close platelet-platelet contact brought about by aggregation facilitates binding of additional cell surface ligands on one platelet to cell surface receptors on adjacent platelets. Such binding can affect platelet activation and thrombus stability via initiation of intracellular signaling or formation of additional contacts between platelets. These ligand/receptor pairs include: ephrin/Eph kinases; semaphorin4D/CD72 and a member of the plexin B family; and growth-arrest specific gene 6 (Gas-6)/receptor tyrosine kinases Tyro3, Axl, and Mer.[31,81,83]

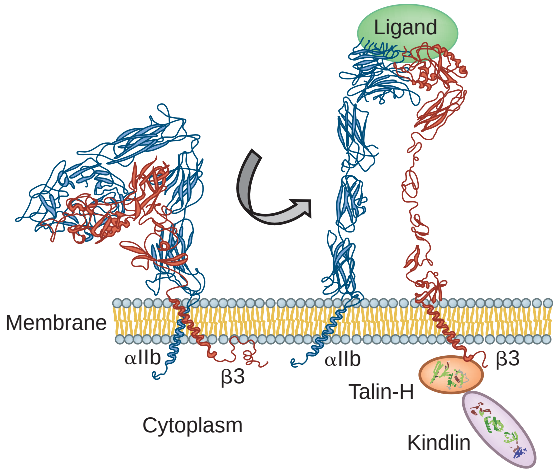


Figure 124.5 MODEL SHOWING THE ACTIVATION OF αIIbβ3. Signaling pathways induced by platelet agonists result in talin and kindlin binding to the cytoplasmic tail of β3 and inside-out signaling such that the integrin is transformed from its low-affinity bent conformation on the resting platelet to its high-affinity extended conformation on the activated platelet. The extended conformation has the ligand binding site exposed, allowing for the binding of fibrinogen or von Willebrand factor, and thus for platelet aggregation. Binding of these ligands initiates outside-in signaling, triggering further intracellular signal transduction and platelet responses. This model is based on the crystal structure of the extracellular domain of αvβ3, and the cytoplasmic tails on the nuclear magnetic resonance structure of αIIbβ3. *(Adapted, with permission, from Bledzka K, Smyth SS, Plow EF. Integrin αIIbβ3. From discovery to efficacious therapeutic target. Circ Res. 2013;112:1189.)*

Laboratory Evaluation of Aggregation

The in vitro aggregation response of platelets is most commonly assessed using aggregometry, considered to be the gold standard of platelet function testing.[84] In the aggregometer, platelet responses are measured in suspension, bypassing the initial adhesion response of platelets to the subendothelial ECM that occurs in vivo (see section on Platelet Adhesion). Optical or turbidometric aggregometry, now more often referred to as *light transmission aggregometry (LTA)*, was developed in the 1960s; in LTA, light transmission through a rapidly stirred suspension of platelets at 37°C is recorded upon addition of a soluble agonist (Table 124.3). With all agonists except epinephrine, there is an initial, transient, small decrease in light transmission from baseline due to actin cytoskeleton reorganization and platelet shape change from discs to more rounded forms with extended filipodia. This is followed by a larger increase in light transmission as the platelets aggregate. Depending on the agonist and its concentration, platelets may deaggregate, indicated by a subsequent decrease in light transmission (Fig. 124.6).

In clinical laboratories, platelet-rich plasma (PRP), obtained by low-speed centrifugation of a whole blood specimen anticoagulated

TABLE 124.3 **Agonists Used in the Laboratory Evaluation of Aggregation**

Agonist	In Vivo/In Vitro Mechanism of Action
Thrombin receptor-activating peptide (TRAP)[a]	SFLLRN (Ser-Phe-Leu-Leu-Arg-Asn), the peptide sequence of the new N-terminus of PAR-1 after thrombin cleavage (binds to (uncleaved) PAR-1 in vitro)
ADP	Released from platelet dense granules; acts synergistically with many other agonists (binds to P2Y1 and P2Y12)
Arachidonic acid	Is metabolized by the cyclooxygenase pathway to TxA_2 which is released from stimulated platelets and is rapidly metabolized to inactive TxB_2 in plasma (TxA_2 binds to TP)
U46619	Stable TxA_2 mimetic (binds to TP)
Collagen	In subendothelial extracellular matrix (binds to GPVI and α2β1)
Epinephrine[b]	A weak agonist that is not associated with platelet shape change and that potentiates other agonists; may allow for hormonal regulation of hemostasis (binds to α_{2A}-adrenergic receptor)
Ca^{2+}-ionophore	Directly mobilizes intracellular Ca^{2+} (does not bind to a receptor)
Ristocetin[c]	An antibiotic that changes the conformation of VWF, exposing the binding site for GPIbα of the GPIb–IX–V complex, allowing platelet agglutination by VWF

GP, Glycoprotein; *PAR*, protease-activated receptor; *TP*, thromboxane/prostanoid; *TxA_2*, thromboxane A_2; *TxB_2*, thromboxane B_2; *VWF*, von Willebrand factor.

[a] TRAP is used as a substitute for thrombin. Thrombin binds to GPIb–IX–V and cleaves PAR-1 and PAR-4, but is not often used in the assessment of platelet aggregation in citrated platelet-rich plasma as it causes fibrin clot formation.

[b] Low response to epinephrine occurs in a proportion of healthy individuals due to natural variation in receptor density, and is not necessarily indicative of a platelet function disorder.

[c] In contrast with the other agonists that stimulate aggregation, an energy-requiring process, ristocetin induces platelet agglutination via VWF, a process that does not require platelets to be metabolically active.

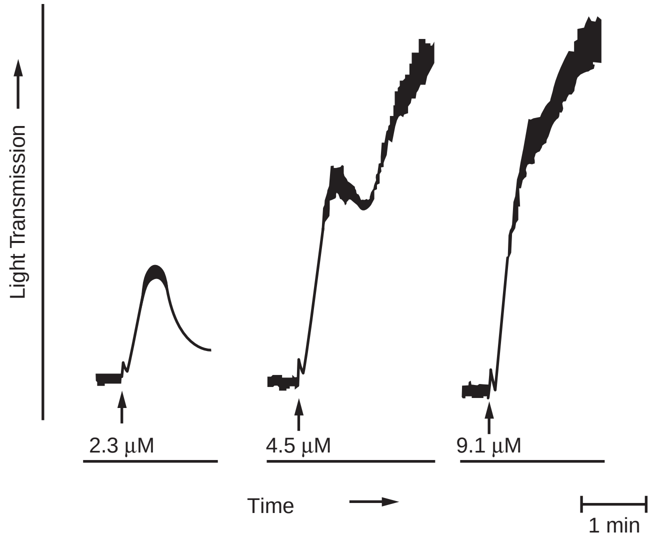


Figure 124.6 AGGREGATION RESPONSES OF PLATELETS IN CITRATED PLATELET-RICH PLASMA STIMULATED WITH ADP. (Note that the micromolar concentration of Ca^{2+} in citrated platelet-rich plasma is sufficient to allow aggregation to occur.) The *arrows* indicate the addition of increasing concentrations of ADP from left to right. Following addition of the agonist, platelet shape change is observed as a slight decrease in light transmission, followed by an increase in light transmission as the platelets aggregate. The lowest concentration of ADP (2.3 µM; *left*) induces primary aggregation followed by deaggregation, as indicated by the subsequent decrease in light transmission. The intermediate concentration of ADP (4.5 µM; *middle*) induces primary aggregation followed by a secondary wave of aggregation; the secondary wave occurs due to thromboxane A_2 formation by the activated platelets and subsequent granule content exocytosis, including ADP. At the highest concentration of ADP (9.1 µM; *right*), the two phases of aggregation merge so that only a single wave of aggregation is apparent. *(Adapted with permission from Rand ML, Leung R, Packham MA. Platelet function assays. Transfus Apher Sci. 2003;28:307.)*

with citrate, is routinely used in LTA assessment of platelet function (see Chapter 127). Citrate lowers the plasma concentration of Ca^{2+} to the micromolar range, which is still sufficient for aggregation to occur. In research laboratories, LTA of platelets isolated from plasma and suspended in balanced salt solutions (e.g., Tyrode's solution) is often used. In this case, Ca^{2+} must be added to the suspending medium to allow aggregation to occur. LTA measures platelet aggregation under conditions of low shear, in which aggregation is dependent on fibrinogen binding to activated αIIbβ3. This is in contrast with tests such as the Platelet Function Analyzer (PFA-100/200),[84,85] which measures aggregation under conditions of high shear (5000 to 6000 s^{-1}), in which aggregation is mediated by VWF binding to activated αIIbβ3.

Some of the commonly used platelet agonists are listed in Table 124.3. Of physiological relevance are the platelet-derived aggregating agents—ADP, and the arachidonic acid metabolite TxA_2—that provide a mechanism for stimulated platelets to recruit additional platelets to an aggregate in vivo. As described above, these agonists activate platelets by binding to specific receptors and triggering intracellular signaling pathways that ultimately converge to a set of common steps that permit the platelets to aggregate. Platelet aggregation, mediated by ligand binding to activated αIIbβ3, is energy and signal transduction dependent, and can be distinguished on this basis from platelet agglutination induced by ristocetin (see Table 124.3), mediated by VWF binding to GPIbα of the GPIb–IX–V complex, which does not require platelets to be metabolically active. Many preanalytical and analytical variables affect the results obtained by LTA and recommendations for clinical laboratory standardization have been published.[86–88]

Platelet aggregation can also be measured using whole-blood aggregometry. This test measures platelet aggregation in diluted, anticoagulated whole blood as the change in impedance or resistance between two electrodes when platelets adhere to the electrodes and aggregate in response to soluble agonists (see above and Table 124.3). Whole blood aggregometry has the advantages over LTA of requiring smaller blood volumes and less sample manipulation. Lumi-aggregometers provide simultaneous measurement of aggregation and dense granule secretion, specifically ATP release, via a luciferin-luciferase assay.

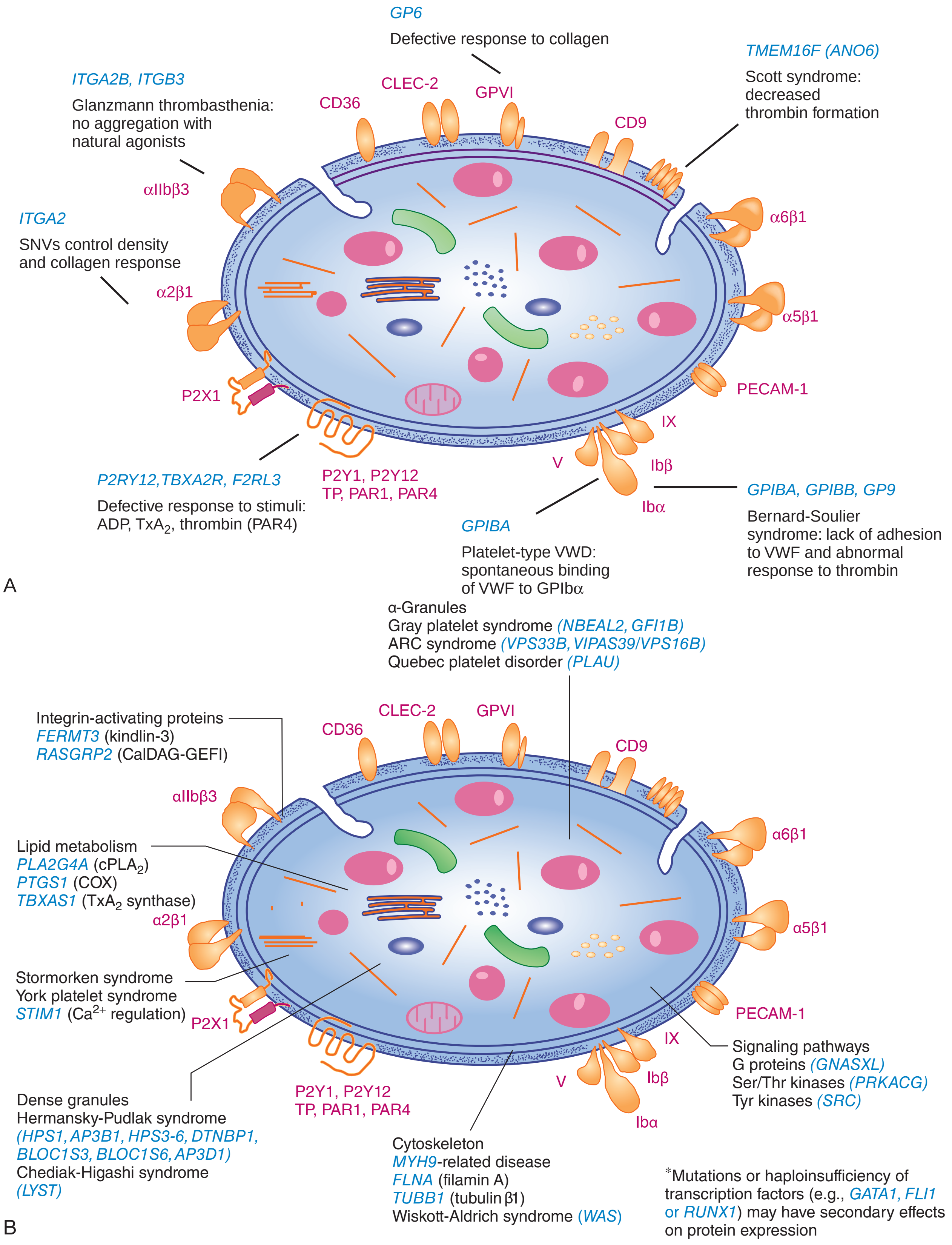
The information gained from aggregometry has been extremely useful in the diagnosis of platelet function disorders, whether inherited or acquired.[85,87,89] However, aggregation as measured in vitro does not always reflect platelet function in vivo; clinical bleeding and the aggregation response of platelets do not necessarily concur. This disparity reflects, in part, the importance of platelet adhesion under flow conditions in hemostatic plug/arterial thrombus formation, an effect that is not detected by aggregometry.[81] Tests that allow for measurement of platelet adhesion, activation, and aggregation on a variety of physiologically-relevant surfaces under flow conditions at variable shear rates, both physiological and pathological, and for measurement of multiple outcomes to characterize platelet responses will, in the future, provide additional information about platelet function.[90,91]

MOLECULAR BASIS OF INHERITED PLATELET DISORDERS

Inherited platelet disorders are a heterogeneous group of bleeding disorders involving defects in platelet function and/or number (usually thrombocytopenia). The bleeding symptoms experienced by patients are primarily mucocutaneous, such as epistaxis, bruising, bleeding from the oropharynx or gastrointestinal tract, heavy menstrual bleeding, and postpartum and surgical (including dental) bleeding (see Chapter 126). These symptoms can range from very mild to life threatening, depending on the disorder (severity may vary among individuals with the same disorder). While severe disorders such as GT and BSS are relatively easy to diagnose, milder disorders (e.g., those affecting secretion of granule contents) are more difficult to characterize and it is likely that they have a higher prevalence than previously appreciated[92]; the expected prevalence of clinically-significant predicted loss-of-function variants in platelet-associated genes is 0.3% in the general population.[93] Much has been learned about platelet development, structure and function, and the underlying genetic basis of specific disorders from the investigation of patients; indeed, the molecular defects in many of the disorders have now been identified (Fig. 124.7). Important insights have also been provided by characterization of mouse models in which platelet proteins have been altered genetically.[94–96] The reader is referred to comprehensive reviews of inherited disorders of platelets for more details.[97–100]

Inherited Platelet Function Disorders

Inherited platelet function disorders encompass defects in platelet adhesion, with deficiencies or dysfunction of receptors for subendothelial VWF or collagen; platelet activation, including deficiencies or dysfunction of receptors for soluble agonists, of cytoskeletal proteins, of signaling pathways, of dense and α-granule secretion, and of PS exposure; and platelet aggregation, with deficiencies or dysfunction of integrin αIIbβ3. Most of the disorders described below have an autosomal recessive pattern of inheritance, and thus affected individuals have homozygous or compound heterozygous gene variants. The disorders vary significantly in their severity, and some present with associated thrombocytopenia.


GP6
Defective response to collagen
TMEM16F (ANO6)
Scott syndrome:
decreased
thrombin formation
ITGA2B, ITGB3
Glanzmann thrombasthenia:
no aggregation with
natural agonists
CLEC-2
CD36
GPVI
CD9
αIIbβ3
ITGA2
SNVs control density
and collagen response
α2β1
α6β1
α5β1
PECAM-1
P2X1
IX
V
Ibβ
Ibα
P2RY12,TBXA2R, F2RL3
Defective response to stimuli:
ADP, TxA_2, thrombin (PAR4)
P2Y1, P2Y12
TP, PAR1, PAR4
GPIBA, GPIBB, GP9
Bernard-Soulier
syndrome: lack of adhesion
to VWF and abnormal
response to thrombin
GPIBA
Platelet-type VWD:
spontaneous binding
of VWF to GPIbα
A
α-Granules
Gray platelet syndrome (NBEAL2, GFI1B)
ARC syndrome (VPS33B, VIPAS39/VPS16B)
Quebec platelet disorder (PLAU)
Integrin-activating proteins
FERMT3 (kindlin-3)
RASGRP2 (CalDAG-GEFI)
CD36
CLEC-2
GPVI
CD9
αIIbβ3
Lipid metabolism
PLA2G4A ($cPLA_2$)
PTGS1 (COX)
TBXAS1 (TxA_2 synthase)
α2β1
α6β1
α5β1
Stormorken syndrome
York platelet syndrome
STIM1 (Ca^{2+} regulation)
P2X1
PECAM-1
IX
V
Ibβ
Ibα
Signaling pathways
G proteins (GNASXL)
Ser/Thr kinases (PRKACG)
Tyr kinases (SRC)
Dense granules
Hermansky-Pudlak syndrome
(HPS1, AP3B1, HPS3-6, DTNBP1,
BLOC1S3, BLOC1S6, AP3D1)
Chediak-Higashi syndrome
(LYST)
P2Y1, P2Y12
TP, PAR1, PAR4
Cytoskeleton
MYH9-related disease
FLNA (filamin A)
TUBB1 (tubulin β1)
Wiskott-Aldrich syndrome (WAS)
B

*Mutations or haploinsufficiency of transcription factors (e.g., *GATA1, FLI1* or *RUNX1*) may have secondary effects on protein expression

Figure 124.7 GENETIC CAUSES OF INHERITED PLATELET DISORDERS. (A) Surface membrane receptor defects and (B) granule and intracellular protein defects. *ARC*, Arthrogryposis-renal dysfunction-cholestasis; *$cPLA_2$*, cytosolic phospholipase A_2; *CalDAG-GEFI*, Ca^{2+} and diacylglycerol-regulated guanine nucleotide exchange factor I; *COX*, cyclooxygenase; *GP*, glycoprotein; *PAR*, protease-activated receptor; *PECAM*, platelet-endothelial cell adhesion molecule; *Ser*, serine; *SNV*, single-nucleotide variant; *Thr*, threonine; *TxA_2*, thromboxane A_2; *Tyr*, tyrosine; *VWD*, von Willebrand disease; *VWF*, von Willebrand factor. *(Adapted with permission from Nurden AT, Nurden P. Inherited disorders of platelet function. J Thromb Haemost. 2015;13[suppl 1]:S2.)*

Abnormalities of Platelet Adhesion

GPIb–IX–V[15]

In BSS, variants in *GP1BA, GP1BB*, or *GP9* result in defective platelet surface expression of GPIb–IX–V, and thus a decreased ability of platelets to adhere to subendothelial VWF, as well as a decreased in vitro response of platelets to thrombin and ristocetin. Frameshift and nonsense (type 1 variants), and missense (type 2 variants) alterations have been described[14,101]; correlation of genotype with the moderate to severe bleeding phenotype in BSS is not apparent. The macrothrombocytopenia that occurs in BSS is discussed in the section Inherited Thrombocytopenias.

The autosomal dominant platelet-type VWD[102] is the result of gain-of-function variants in the VWF binding site on GPIbα. Enhanced binding of plasma VWF to GPIbα leads to platelet agglutination and a bleeding phenotype; (macro)thrombocytopenia may be present. In vitro, enhanced agglutination to low-dose ristocetin is observed. Platelet-type VWD is clinically indistinguishable, but genetically distinguishable, from type 2B VWD, caused by gain-of-function variants in the GPIbα binding site of VWF.

Collagen Receptors[103]

There is variability in the platelet surface expression levels of GPVI and α2β1 that are determined by specific haplotypes in *GP6* and *ITGA2*, respectively. Very rare deficiencies of α2β1 have been reported, associated with a mild bleeding diathesis and decreased in vitro platelet responses to collagen.[20] Several patients, including some from unrelated Chilean families, with *GP6* variants leading to deficiencies in GPVI expression have been described; they also have a mild bleeding phenotype with decreased in vitro responses of platelets to collagen. Acquired GPVI deficiency can also occur, with ectodomain shedding (i.e., cleavage) of the receptor from the platelet surface in response to autoantibodies.

Abnormalities of Platelet Activation

Soluble Agonist Receptors

Rare patients have been described with defects in the gene for the ADP receptor P2Y12 (*P2RY12*) or in the gene for the TxA_2 TP α receptor (*TBXA2R*). The defects mimic the effects of antiplatelet drugs: for *P2RY12*, clopidogrel, prasugrel, ticagrelor, and cangrelor, that block P2Y12; and for *TBXA2R*, aspirin, which blocks TxA_2 formation. As such, decreased platelet responses in vitro to ADP, and to arachidonic acid and the stable thromboxane mimetic U46619, respectively, are observed in these patients, who have a mild bleeding diathesis. There has been a recent first description of a rare variant in *F2RL3*, the gene for the PAR4 thrombin receptor, which is associated with decreased in vitro platelet responses to thrombin and the PAR4-activating peptide.[104]

Cytoskeletal Defects

These are discussed below in the section on Inherited Thrombocytopenias.

Secretion Defects

There are two types of disorders associated with deficiencies in granule secretion: (1) those due to defective platelet granule formation, low numbers of granules or absent cargo; and (2) those due to defects in secretion machinery, where granules are present, but contents are not secreted following platelet activation.

Dense Granules[105]

HPS is characterized by oculocutaneous albinism and platelet-dense granule deficiency.[106] The distinct HPS-1 to HPS-10 subtypes arise from defects in 10 genes, respectively: *HPS1*, *AP3B1*, *HPS3–6*, *DTNBP1*, *BLOC1S3*, *BLOC1S6*, and *AP3D1*. The protein products of these genes interact in BLOCs or in adaptor protein complex-3 (AP-3) that are involved in dense granule biogenesis.[54] Some subtypes are also associated with pulmonary fibrosis and granulomatous colitis. Patients with Chediak-Higashi syndrome have severe immunological defects in addition to oculocutaneous albinism and dense granule deficiency; they have variants in the *LYST* gene, the product of which is a vesicular transport protein. Non-syndromic dense granule deficiencies are relatively common. They are heterogeneous and include qualitative dense granule defects and their underlying molecular bases are unknown. Syndromic and non-syndromic dense granule deficiencies are associated with mild-to-moderate bleeding; in vitro secretion responses as measured by lumi-aggregometry are reduced or absent.[107]

Normal numbers of granules with decreased secretion responses are indicative of defective granule secretion processes. For example, in vitro evidence of dense and α-granule secretion defects with normal granule cargo are observed in some forms of familial hemophagocytic lymphohistiocytosis (FHL). Variants for genes encoding SNARE proteins or their regulators have been identified: Munc13-4 (*UNC13D*) in FHL3,[108] syntaxin-11 (*STX11*) in FHL4,[109] and Munc18b/syntaxin binding protein 2 (*STXBP2*) in FHL5.[110]

α-Granules

The classic α-granule deficiency, gray platelet syndrome (GPS), is discussed in the section on Inherited Thrombocytopenias. Platelet α-granules are also absent in the multisystem disorder ARC (arthrogryposis-renal dysfunction-cholestasis) syndrome, which is lethal within the first year of life. Here, the gene defect is in *VPS33B*, which encodes a Sec1/Munc18 interacting protein involved in intracellular vesicular trafficking, or in *VIPAS39* (*VPS16B*), which encodes VPS33B-interacting protein. While α-granules are present in platelets from patients with the autosomal dominant Quebec platelet disorder (QPD), their contents are proteolytically degraded by plasmin generated by urokinase-type plasminogen activator (u-PA), with overexpression related to tandem duplication of *PLAU*, the u-PA gene.[111] Increased fibrinolysis after platelet secretion results in delayed bleeding after surgery or trauma; in vitro platelet responses to epinephrine are decreased.[107]

Signaling Pathways

Patients have been identified with apparent congenital disorders in a variety of signaling pathways involving G proteins (G_q, G_s, G_i), $cPLA_2$, and PLC, PKC-θ, Ca^{2+} mobilization, TxA_2 formation (including COX-1 and thromboxane synthase deficiencies), and granule secretion.[112] However, for the most part, the underlying genetic defects in these patients, who experience mild-to-moderate bleeding, remain unknown. The following are several disorders for which the underlying genetic defects are known. An insertion variant in the extra-large $G\alpha_s$ gene (*GNASXL*) has been associated with $G\alpha_s$ hyperfunction in platelets and a bleeding phenotype, neurological problems, and mild skeletal abnormalities. A gain-of-function variant in *SRC*, that encodes the tyrosine kinase Src, results in an autosomal dominant disorder characterized by thrombocytopenia, bleeding, abnormal platelet α-granules, myelofibrosis and bone pathologies.[113,114] A gain-of-function variant in *STIM1* resulting in a CRAC channelopathy has been shown in the autosomal dominant Stormorken syndrome, which is characterized by a mild bleeding diathesis, thrombocytopenia, and, in resting platelets, elevated $[Ca^{2+}]_i$ and surface PS exposure. The York platelet syndrome, characterized by thrombocytopenia, ultrastructural abnormalities in platelet organelles, and deficiency of Ca^{2+} storage in dense granules, has also been identified as a CRAC channelopathy due to the same gain-of-function variant in *STIM1* that is found in Stormorken syndrome. Variants in *PLA2G4A* encoding cPLA2, in *PTGS1* encoding COX, and in *TBXAS1* encoding thromboxane synthase (Ghosal syndrome) have been described.

PS Exposure

Scott syndrome, in which there is a defect in PS exposure and thus, platelet procoagulant activity, is associated with variants in *TMEM16F* (*ANO6*)[75]; TMEM16F (anoctamin 6) is essential for Ca^{2+}-dependent PS exposure. There is diminished platelet-derived extracellular vesicle formation. Decreased thrombin generation results in a moderate-to-severe bleeding phenotype; in vitro platelet aggregation responses are not affected and therefore not helpful in making the diagnosis.

Abnormalities of Platelet Aggregation

$\alpha IIb\beta 3$[115,116]

One of the best characterized platelet function disorders, GT, results from quantitative or qualitative defects of the fibrinogen/VWF receptor αIIbβ3, and thus the inability of platelets to aggregate. The bleeding phenotype ranges from mild to life-threatening hemorrhage, and in vitro there are absent aggregation responses to all agonists, while ristocetin-induced agglutination is intact. Type I GT is defined as less than 5% surface-expressed αIIbβ3; type II, as 5% to 25%; and variant, as normal expression levels of an αIIbβ3 complex that is nonfunctional. Approximately 200 variants have been described in the *ITGA2B* and *ITGB3* genes, most commonly missense and nonsense variants, and those involving splice variants and small deletions and insertions with frame shifts; genotype does not correlate with phenotype.[117] Rare variants result in macrothrombocytopenia (see section on Inherited Thrombocytopenias).

Defects in the gene for CalDAG-GEFI (*RASGRP2*)[118,119] or kindlin-3 *(FERMT3)* have been reported that prevent activation of αIIbβ3, with subsequent absence of platelet aggregation. Deficiency in kindlin-3 is the basis for leukocyte deficiency-adhesion III syndrome, a severe disorder that encompasses defective integrin β2 function in leukocytes and associated susceptibility to infections and poor wound healing, as well as severe bleeding.

Inherited Thrombocytopenias

Inherited thrombocytopenias vary in severity, platelet morphology, and presence or absence of associated platelet dysfunction. Some types are associated with congenital anomalies and some with predisposition to additional diseases, including malignancy. The advent of high throughput sequencing has greatly enhanced our understanding of known disorders, and the identification of previously unexplained disorders, by identifying causal gene variants; presently defects in upwards of 40 human genes are known to be associated with inherited thrombocytopenias.

The pathogenesis of inherited thrombocytopenias can best be understood in the context of platelet biogenesis and senescence, with defects occurring in megakaryocyte differentiation, maturation, proplatelet formation and platelet release, and in alterations in platelet survival (see Fig. 124.7; see also Chapter 123).[120,121]

Abnormalities of Megakaryocyte Differentiation

Defects in the maturation of hematopoietic stem cells into megakaryocytes result in deficiency or absence of bone marrow megakaryocytes. Examples of differentiation defects:

Congenital Amegakaryocytic Thrombocytopenia

Congenital amegakaryocytic thrombocytopenia (CAMT) is an autosomal recessive disease caused by variants in *MPL*, the gene encoding the thrombopoietin (TPO) receptor (Mpl). Binding of TPO to its receptor activates multiple signaling pathways including JAK2/STAT, Ras/MAPK, and PI3K.[122] Disease severity depends on the specific variant that results in either complete absence of Mpl or some residual Mpl. TPO signaling is essential for commitment and differentiation in megakaryocytes but also in the erythroid and myeloid lineages. Patients with CAMT, who have isolated thrombocytopenia at birth, usually develop trilineage bone marrow hypoplasia in childhood.[123]

Thrombocytopenia With Absent Radii

Thrombocytopenia with absent radii (TAR) is an autosomal recessive disorder characterized by absent radii and isolated thrombocytopenia, also present at birth. In contrast to CAMT, platelet counts improve with age and other cell lines are unaffected. The cause is compound inheritance of a low-frequency single-nucleotide variant and a rare null allele in *RBM8A*, a gene encoding the exon-junction complex subunit member Y14. This complex is involved in RNA processing and nuclear export. It is still unknown how this defect results in the TAR phenotype or megakaryocyte differentiation, although it may affect the response of hematopoietic progenitors to TPO.[124,125]

Amegakaryocytic Thrombocytopenia With Radio-Ulnar Synostosis

Amegakaryocytic thrombocytopenia with radio-ulnar synostosis (ATRUS) is the result of heterozygous defects in *HOXA11*, a member of the *HOX* gene family of transcription factors involved in embryonic development. These patients have thrombocytopenia from birth that does not improve with time, associated with fusion of the proximal radius and ulna.[123]

Abnormalities of Megakaryocyte Maturation

Altered maturation is characterized by immature, dysplastic or dysfunctional megakaryocytes. Many of these conditions are the result of defects in transcription factors involved in megakaryopoiesis (e.g., *RUNX1, FLI1, GATA1, ETV6*), and some are associated with platelet function defects and/or an increased risk of malignancy. Examples of maturation defects:

Familial Platelet Disorder With Predisposition to Myeloid Malignancy

Familial platelet disorder with predisposition to myeloid malignancy (FPDMM) is the result of heterozygous variants in *RUNX1* (also called *AML1* or *CBFA2*) that encodes the alpha subunit of the core-binding factor transcription complex. This complex regulates expression of genes involved in stem cell proliferation versus differentiation in hematopoiesis. The loss of this regulation results in failure of megakaryocyte maturation. *RUNX1* also regulates synthesis of specific proteins that can alter platelet function and result in abnormalities of platelet granule structure or granule secretion.[126] Germline *RUNX1* variants predispose to the development of myelodysplastic syndrome and acute *myeloid* leukemia in approximately 40% of individuals.[127]

Paris-Trousseau Thrombocytopenia and Jacobsen Syndrome

Paris-Trousseau thrombocytopenia and Jacobsen syndrome are disorders caused by deletions in chromosome 11q23-ter; the severity of the syndrome, which includes cardiac and facial defects, depends on the size of the deletion. Heterozygous loss of the transcription factor gene *FLI1* results in macrothrombocytopenia, abnormal α-granules and internal membrane structure (see Chapter 123).[126]

ANKRD26-Related Thrombocytopenia

ANKRD26-related thrombocytopenia (*ANKRD26*-RT) is an autosomal dominant inherited thrombocytopenia, the result of point variants in the 5′-UTR of *ANKRD26*. *ANKRD26* expression is normally downregulated during megakaryocyte maturation by the binding of RUNX1/FLI1 to the 5′-UTR. Lack of binding by these co-regulators because of the alterations in the 5′-UTR, leads to a persistent expression of ANKRD26, increased downstream signaling, and dysmegakaryopoiesis.[128] Affected individuals are at increased risk of developing myelodysplastic syndrome and some also have evidence of dysregulation of erythroid cell maturation.[129]

ETV6-Related Thrombocytopenia

Germline heterozygous variants in *ETV6* lead to autosomal dominant thrombocytopenia and a predisposition to lymphoid malignancy.[130] A transcriptional regulator, ETV6 normally dimerizes with FLI1, suggesting that abnormal interactions between ETV6 and FLI1 may contribute to defects in downstream expression of platelet proteins involved in regulation of cytoskeletal dynamics and platelet maturation.

GATA-1-Related Thrombocytopenias

These are X-linked diseases associated with anemia, indicating this transcription factor's involvement in both megakaryocytic and erythroid maturation (see Chapter 123). Variants in GATA-1 that interfere with binding to its cofactor FOG1 result in dyserythropoietic anemia with thrombocytopenia and defects in α-granule maturation.[97]

Gray Platelet Syndrome

Gray platelet syndrome (GPS) is the result of homozygous variants in *NBEAL2*, a regulator of membrane dynamics and vesicle trafficking, affecting α-granule development. The disorder is characterized by progressive macrothrombocytopenia, absent platelet α-granules, splenomegaly, immune dysregulation and myelofibrosis.[56–58,131] Platelets appear pale gray on stained blood films because of the absence of α-granules. The *Nbeal2*$^{-/-}$ mouse also shows evidence of abnormal megakaryocyte maturation,[132] defects in retention of α-granule cargo proteins,[133] and poor wound healing.

Heterozygous variants in *GFI1B*, encoding a transcription factor involved in megakaryopoiesis and erythropoiesis, have been identified in association with a phenotype similar to GPS. Affected individuals have macrothrombocytopenia and α-granule deficiency, and some have erythrocyte abnormalities.[134]

Abnormalities of Proplatelet Formation and Platelet Release

Defects in proplatelet formation and the conversion of proplatelets to platelets are the basis for this group of thrombocytopenias, the majority of which are macrothrombocytopenias.

Variants in genes encoding platelet cytoskeletal components (e.g., *MYH9, ACTN1, FLNA*), the microtubule system (e.g., *TUBB1*), or surface membrane receptors that interact with the cytoskeleton (e.g., *GP1BA*, *ITGA2B*, *ITGB3*) can lead to these diseases. Examples of defects in proplatelet formation and platelet release include:

MYH9-Related Disease[135]

Previously referred to as the May-Hegglin, Sebastian, and Fechtner syndromes and Epstein anomaly, *MYH9*-related disease is the result of autosomal dominant variants in *MYH9*, the gene that encodes NMMHC-IIA. NMM is involved in the generation of energy-driven skeletal forces involved in cytokinesis, cell motility, and shape change. MYH9 is composed of two main domains, N-terminal and tail domains. The N-terminus of the myosin heavy chain is composed of the head or motor domain that interacts with actin and binds ATP, and the neck domain that binds myosin light chains. The C-terminal tail domain is involved in dimerization of the heavy chains and in phosphorylation. Affected individuals have macrothrombocytopenia from birth, the result of decreased proplatelet formation related to abnormal distribution of actin-myosin cytoskeleton and abnormal migration on collagen that may lead to ectopic and ineffective proplatelet formation.

Neutrophils contain cytoplasmic clusters of MYH9 protein that can be identified by immunofluorescence microscopy. In some patients, these inclusions can be seen on May-Grünwald-Giemsa-stained blood films (Döhle-like bodies). Approximately 35% of affected individuals develop nephropathy that evolves to end-stage renal disease, 20% develop early-onset cataracts, and 50% develop sensorineural hearing loss.[51] There is a correlation between the clinical phenotype and the specific variant: motor domain variants are often associated with hearing loss and nephritis in addition to thrombocytopenia, while those in the tail domain are associated with moderate thrombocytopenia, only.

ACTN1- and FLNA-Related Thrombocytopenias

These result from variants in the genes that encode α-actinin or filamin A, both functioning as cross-linking proteins in the actin-based cytoskeleton. Filamin A is also critical in binding the cytoplasmic tail of GPIbα to the filamentous actin network and stabilizing αIIbβ3 in its resting state. Monoallelic variants of either gene result in abnormalities of proplatelet formation and variable degrees of macrothrombocytopenia.[45,121]

Bernard-Soulier Syndrome

In addition to the functional adhesion defect (described in the section on Abnormalities of Platelet Adhesion), patients with BSS have thrombocytopenia with giant platelets. Abnormalities in proplatelet formation resemble those seen in variants of cytoskeletal proteins, suggesting that the anchoring of the actin filament network by GPIbα to the plasma membrane is a required part of this process, disrupted by deficiency of the GPIb–IX–V transmembrane protein complex.[12,121] Most heterozygotes do not have macrothrombocytopenia, but there are exceptions where specific monoallelic variants in *GPIBA* (e.g., p.A156V, the Bolzano variant) or *GPIBB*[136] cause mild macrothrombocytopenia. Mild to moderate thrombocytopenia is also observed in 22q11.2 deletion syndrome (velocardiofacial/DiGeorge syndrome) in which there is a hemizygous (usually) somatic deletion at chromosome 22q11.2 that includes *GPIBB*; this syndrome can be associated with additional developmental defects.

ITGA2B/ITGB3-Related Thrombocytopenias

These are rare macrothrombocytopenias with functional defects associated with specific monoallelic variants in *ITGA2B* or *ITGB3* that are distinct from the variants that cause GT, described previously. These variants result in constitutive activation of αIIbβ3 and downstream effectors, defects in actin-myosin reorganization, and abnormalities of proplatelet formation.[137]

TUBB1-Related Thrombocytopenia

This is caused by variants in the gene encoding β1-tubulin that impact its association with α-tubulin and result in abnormalities in assembly of the microtubule coil.[138] This defect in the microtubule coil leads to production of fewer proplatelets with abnormal morphology. Platelets are spherical rather than discoid (see also Chapter 123). Platelet counts and bleeding manifestations are highly variable.

PRKACG-Related Macrothrombocytopenia

This is the result of a germ-line variant in the gene encoding the γ -catalytic subunit of the cAMP-dependent protein kinase (PK), PKA. There is a defect in proplatelet formation and a low level of filamin A in megakaryocytes.[139]

Wiskott-Aldrich Syndrome and Other Microthrombocytopenias

Wiskott-Aldrich syndrome (WAS) and X-linked thrombocytopenia (XLT), both microthrombocytopenias are caused by variants in the *WAS* gene. Pathogenic variants that result in non-expression of WASp cause WAS, which manifests as severe immune dysregulation, in addition to thrombocytopenia. XLT is caused by hypomorphic variants that decrease but do not abolish expression of the normal WASp. WASp is involved in regulation of actin polymerization; the defect in platelet production may be the result of ectopic platelet shedding by megakaryocytes.[120,140]

Recently, other causes of microthrombocytopenia have been identified including deficiency in ARPC1B (ARP 2/3 complex 1B), which is activated by the WASp complex and plays an important role in actin nucleation and polymerization. Germline variants in *ARPC1B* are associated with defects in platelet and T-cell function, leading to a severe immunodeficiency.[141,142]

PLATELETS BEYOND HEMOSTASIS

Although platelets were originally identified for roles in hemostasis and arterial thrombosis, they have become well-recognized for their involvement in a myriad of other physiological and pathological processes, including wound healing/tissue repair; infection, (innate and adaptive) immunity, and inflammation; (development and progression of) malignancy and tumor metastasis; (lymph)angiogenesis; and neurodegenerative diseases. Some of these have been briefly touched on in sections above (see The Integrin Family of Adhesion Receptors; CLEC-2; Secretion of Granules; and Platelet-Derived Extracellular Vesicles).

Many of the same mechanisms that play a role in hemostasis and thrombosis facilitate platelet participation in these non-hemostatic processes. Platelet receptors such as αIIbβ3, GPIb-IX-V, GPVI, CLEC-2, P-selectin, CD40/CD40L, FcγRIIA, and TLRs (Toll-like receptors) have been implicated in tumor progression and metastatic spread; in the immune response to bacterial challenge; in the progression of inflammatory conditions such as atherosclerosis and rheumatoid arthritis. The intense inflammation accompanying infection with the SARS-CoV-2 virus (COVID-19) is associated with platelet-platelet and platelet-leukocyte interactions that contribute to the significant incidence of thrombosis in this disease.[143,144] SARS-CoV-2 virions have been detected in megakaryocytes and platelets of COVID-19 patients, and platelets from these patients are hyper-reactive and have a distinct prothrombotic transcriptome profile.[144,145] Platelets are not directly activated by SARS-CoV-2 as they lack the ACE2 receptor for the virus; the mechanism may be anti SARS-CoV-2 immune complexes that bind and activate the FcγRIIA receptor leading to granule secretion and procoagulant surface exposure.[144,146]

The secretion of platelet granule contents, including adhesive proteins, growth factors and chemokines/cytokines, that serve to facilitate wound healing/tissue repair, also function in acute and chronic inflammatory disease; in angiogenesis, including tumor angiogenesis; in tumor cell activation and growth; and in the progression of Alzheimer disease. Platelet-derived extracellular vesicles contribute to inflammatory conditions such as rheumatoid arthritis and to the platelet-cancer nexus. Platelets contribute to host defense as they recognize bacteria, recruit traditional immune cells to sites of infection and secrete bactericidal mediators. They can bind, internalize and clear a variety of viruses. The reader is referred to comprehensive reviews of non-hemostatic roles of platelets for details.[24,147–154]

PLATELET AGGREGATION TESTING FOR THE DIAGNOSIS OF PLATELET DISORDERS

Platelet aggregometry is the mainstay in the work-up of platelet function disorders (PFDs). Abnormalities in aggregation tracings can point towards specific diagnoses and direct further testing.

Glanzmann Thrombasthenia

In this severe PFD in which there are quantitative or qualitative defects in integrin αIIbβ3 (GPIIb–IIIa), the receptor to which fibrinogen/von Willebrand factor (VWF) binds on activated platelets, there is absent aggregation response to all agonists. However, agglutination in response to ristocetin occurs, as it is independent of αIIbβ3.

Bernard-Soulier Syndrome

This disorder with defective surface expression of the adhesive VWF receptor GPIb–IX–V is characterized by a lack of agglutination to ristocetin. Note that in von Willebrand disease (VWD), there is an absent agglutination response to ristocetin because of deficient VWF.

Defects in Agonist/Adhesion Receptors

P2Y12: a defect in this ADP receptor results in decreased and reversible aggregation responses to ADP. TPα: thromboxane A_2 (TxA_2) receptor defects show decreased aggregation responses to arachidonic acid, collagen and the stable TxA_2 mimetic U46619. GPVI: a defect in this collagen receptor is characterized by a decreased aggregation response to collagen.

Storage Granule Deficiencies/Secretion Defects

In these disorders, secondary aggregation responses to ADP, collagen, epinephrine, and arachidonic acid can be reduced, but it should be noted that secretion defects may be present with a normal aggregation profile. In dense granule disorders, secretion as measured by lumi-aggregometry is decreased.

Platelet Type VWD/Type 2B VWD

These gain-of-function disorders in the VWF-binding site of GPIbα and in the GPIbα-binding site of VWF, respectively, are characterized by an abnormally increased agglutination response to a low concentration of ristocetin.

Effects of Antiplatelet Medications

Aggregation responses are also affected by antiplatelet medications. For example, individuals taking aspirin or other nonsteroidal anti-inflammatory drugs have decreased platelet aggregation responses to arachidonic acid, but not to U46619. Patients taking P2Y12 receptor blockers (i.e., clopidogrel, prasugrel, ticagrelor, or cangrelor) will have decreased and/or reversible aggregation responses to ADP, depending on the extent of inhibition of the receptor.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Ambrosio AL, Di Pietro SM. Storage pool diseases illuminate platelet dense granule biogenesis. *Platelets*. 2017;28(2):138–146.

Balduini A, Raslova H, Di Buduo CA, et al. Clinic, pathogenic mechanisms and drug testing of two inherited thrombocytopenias, ANKRD26-related thrombocytopenia and MYH9-related diseases. *Eur J Med Genet*. 2018; 61(11):715–722.

Banerjee M, Whiteheart SW. The ins and outs of endocytic trafficking in platelet functions. *Curr Opin Hematol*. 2017;24(5):467–474.

Bledzka K, Qin J, Plow EF. Integrin αIIbβ3. In: Michelson AD, ed. *Platelets*. ed 4. San Diego: Academic Press; 2019:227–241.

Botero JP, Lee K, Branchford BR, et al. Glanzmann thrombasthenia: genetic basis and clinical correlates. *Haematologica*. 2020;105(4):888–894.

Brass LF, Newman DK, Wannermacher KM, et al. Signal transduction during platelet plug formation. In: Michelson AD, ed. *Platelets*. ed 3. San Diego: Academic Press; 2013:367–398.

Cattaneo M. Inherited disorders of platelet function. In: Michelson AD, ed. *Platelets*. ed 4. San Diego: Academic Press; 2019:877–904.

Coenen DM, Heinzmann ACA, Karel MFA, et al. The multifaceted contribution of platelets in the emergence and aftermath of acute cardiovascular events. *Atherosclerosis*. 2021;319:132–141.

Di Paola J, Porter CC. ETV6-related thrombocytopenia and leukemia predisposition. *Blood*. 2019;134(8):663–667.

Gaseka A, Nieuwland R, Siljander PR-M. Platelet-derived extracellular vesicles. In: Michelson AD, ed. *Platelets*. ed 4. San Diego: Academic Press; 2019:401–416.

Harrison P, Lordkipanidze M. Testing platelet function. *Hematol Oncol Clin North Am*. 2013;27(3):411–441.

Italiano Jr. JE, Bergmeier W, Tiwari S, et al. Mechanisms and implications of platelet discoid shape. *Blood*. 2003;101(12):4789–4796.

Joshi S, Whiteheart SW. The nuts and bolts of the platelet release reaction. *Platelets*. 2017;28(2):129–137.

Koupenova M, Clancy L, Corkrey HA, et al. Circulating platelets as mediators of immunity, inflammation, and thrombosis. *Circ Res*. 2018;122(2):337–351.

Lee RH, Stefanini L, Bergmeier W. Platelet signal transduction. In: Michelson AD, ed. *Platelets*. ed 4. San Diego: Academic Press; 2019:329–348.

Li R. The glycoprotein Ib-IX-V complex. In: Michelson AD, ed. *Platelets*. ed 4. San Diego: Academic Press; 2019:193–211.

Lucotti S, Muschel RJ. Platelets and metastasis: new implications of an old interplay. *Front Oncol*. 2020;10:1350.

Nurden AT. The biology of the platelet with special reference to inflammation, wound healing and immunity. *Front Biosci (Landmark Ed)*. 2018;23(4): 726–751.

Nurden AT. Clinical significance of altered collagen-receptor functioning in platelets with emphasis on glycoprotein VI. *Blood Rev*. 2019;38:100592.

Nurden AT, Nurden P. Inherited thrombocytopenias: history, advances and perspectives. *Haematologica*. 2020;105(8):2004–2019.

Nurden P, Stritt S, Favier R, et al. Inherited platelet diseases with normal platelet count: phenotypes, genotypes and diagnostic strategy. *Haematologica*. 2021;106(2):337–350.

Puhm F, Boilard E, Machlus KR. Platelet extracellular vesicles: beyond the blood. *Arterioscler Thromb Vasc Biol*. 2021;41(1):87–96.

Rand ML, Reddy EC, Israels SJ. Laboratory diagnosis of inherited platelet function disorders. *Transfus Apher Sci*. 2018;57(4):485–493.

Rayes J, Watson SP, Nieswandt B. Functional significance of the platelet immune receptors GPVI and CLEC-2. *J Clin Invest*. 2019;129(1):12–23.

Reddy EC, Rand ML. Procoagulant phosphatidylserine-exposing platelets in vitro and in vivo. *Front Cardiovasc Med*. 2020;7:15.

Ribes A, Oprescu A, Viaud J, et al. Phosphoinositide 3-kinases in platelets, thrombosis and therapeutics. *Biochem J*. 2020;477(22):4327–4342.

Sang Y, Roest M, De Laat B, et al. Interplay between platelets and coagulation. *Blood Rev*. 2021;46:100733.

Senis YA, Mazharian A, Mori J. Src family kinases: at the forefront of platelet activation. *Blood*. 2014;124(13):2013–2024.

Wei AH, Schoenwaelder SM, Andrews RK, et al. New insights into the haemostatic function of platelets. *Br J Haematol*. 2009;147(4):415–430.

Willis Fox O, Preston RJS. Molecular basis of protease-activated receptor 1 signaling diversity. *J Thromb Haemost*. 2020;18(1):6–16.

REFERENCES

1. Atkinson B, Dwyer K, Enjyoji K, et al. Ecto-nucleotidases of the CD39/NTPDase family modulate platelet activation and thrombus formation: potential as therapeutic targets. *Blood Cells Mol Dis*. 2006;36(2):217–222.
2. Herr AB, Farndale RW:. Structural insights into the interactions between platelet receptors and fibrillar collagen. *J Biol Chem*. 2009;284(30):19781–19785.
3. Mendolicchio GL, Ruggeri ZM:. New perspectives on von Willebrand factor functions in hemostasis and thrombosis. *Semin Hematol*. 2005;42(1):5–14.
4. Cho J, Mosher DF:. Role of fibronectin assembly in platelet thrombus formation. *J Thromb Haemost*. 2006;4(7):1461–1469.
5. Inoue O, Suzuki-Inoue K, Ozaki Y. Redundant mechanism of platelet adhesion to laminin and collagen under flow: involvement of von Willebrand factor and glycoprotein Ib-IX-V. *J Biol Chem*. 2008;283(24):16279–16282.
6. Jurk K, Clemetson KJ, De Groot PG, et al. Thrombospondin-1 mediates platelet adhesion at high shear via glycoprotein Ib (GPIb): an alternative/backup mechanism to von Willebrand factor. *FASEB J*. 2003;17(11):1490–1492.
7. Savage B, Saldivar E, Ruggeri ZM:. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell*. 1996;84(2):289–297.
8. Reheman A, Gross P, Yang H, et al. Vitronectin stabilizes thrombi and vessel occlusion but plays a dual role in platelet aggregation. *J Thromb Haemost*. 2005;3(5):875–883.
9. Ruggeri ZM:. Platelet adhesion under flow. *Microcirculation*. 2009;16(1):58–83.
10. Shoulders MD, Raines RT:. Collagen structure and stability. *Annu Rev Biochem*. 2009;78:929–958.
11. Savage B, Almus-Jacobs F, Ruggeri ZM. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. *Cell*. 1998;94(5):657–666.
12. Bryckaert M, Rosa JP, Denis CV, et al. Of von Willebrand factor and platelets. *Cell Mol Life Sci*. 2015;72(2):307–326.
13. Ni H, Yuen PS, Papalia JM, et al. Plasma fibronectin promotes thrombus growth and stability in injured arterioles. *Proc Natl Acad Sci USA*. 2003;100(5):2415–2419.
14. Quach ME, Li R. Structure-function of platelet glycoprotein Ib-IX. *J Thromb Haemost*. 2020;18(12):3131–3141.
15. Li R. The glycoprotein Ib-IX-V complex. In: Michelson AD, ed. *Platelets*. ed 4. San Diego: Academic Press; 2019:193–211.
16. Haining EJ, Nicolson PLR, Onselaer M-B, et al. GPVI and CLEC-2. In: Michelson AD, ed. *Platelets*. ed 4. San Diego: Academic Press; 2019:213–226.
17. Mammadova-Bach E, Ollivier V, Loyau S, et al. Platelet glycoprotein VI binds to polymerized fibrin and promotes thrombin generation. *Blood*. 2015;126(5):683–691.
18. Alshehri OM, Hughes CE, Montague S, et al. Fibrin activates GPVI in human and mouse platelets. *Blood*. 2015;126(13):1601–1608.
19. Madamanchi A, Santoro SA, Zutter MM. alpha2beta1 Integrin. *Adv Exp Med Biol*. 2014;819:41–60.
20. Clemetson KJ, Clemetson JM. Platelet receptors. In: Michelson AD, ed. *Platelets*. ed 4. San Diego: Academic Press; 2019:169–192.
21. Watson SP, Farndale RW, Moroi M, et al. Platelet collagen receptors. In: Marder VJ, Aird WC, Bennett JS, eds. *Hemostasis and Thrombosis Basic Principles and Clinical Practice*. ed 6. Philadelphia: Lippincott Williams & Wilkins; 2013:420–430.
22. Savage B, Ruggeri ZM. The basis for platelet adhesion. In: Marder VJ, Aird WC, Bennett JS, eds. *Hemostasis and Thrombosis Basic Principles and Clinical Practice*. ed 6. Philadelphia: Lippincott Williams & Wilkins; 2013:400–409.
23. Quintanilla M, Montero-Montero L, Renart J, et al. Podoplanin in inflammation and cancer. *Int J Mol Sci*. 2019;20(3):707.
24. Rayes J, Watson SP, Nieswandt B. Functional significance of the platelet immune receptors GPVI and CLEC-2. *J Clin Invest*. 2019;129(1):12–23.
25. Versteeg HH, Heemskerk JW, Levi M, et al. New fundamentals in hemostasis. *Physiol Rev*. 2013;93(1):327–358.
26. Sidhu TS, French SL, Hamilton JR. Differential signaling by protease-activated receptors: implications for therapeutic targeting. *Int J Mol Sci*. 2014;15(4):6169–6183.
27. Willis Fox O, Preston RJS. Molecular basis of protease-activated receptor 1 signaling diversity. *J Thromb Haemost*. 2020;18(1):6–16.
28. Gachet C, Hechler B. Platelet purinergic receptors in thrombosis and inflammation. *Hamostaseologie*. 2020;40(2):145–152.
29. Cattaneo M. P2Y12 receptors: structure and function. *J Thromb Haemost*. 2015;13(Suppl 1):S10–S16.
30. Capra V, Back M, Angiolillo DJ, et al. Impact of vascular thromboxane prostanoid receptor activation on hemostasis, thrombosis, oxidative stress, and inflammation. *J Thromb Haemost*. 2014;12(2):126–137.
31. Brass LF, Newman DK, Wannermacher KM, et al. Signal transduction during platelet plug formation. In: Michelson AD, ed. *Platelets*. ed 3. San Diego: Academic Press; 2013:367–398.
32. Senis YA, Mazharian A, Mori J. Src family kinases: at the forefront of platelet activation. *Blood*. 2014;124(13):2013–2024.
33. Harper MT, Poole AW. Diverse functions of protein kinase C isoforms in platelet activation and thrombus formation. *J Thromb Haemost*. 2010;8(3):454–462.
34. Woulfe DS. Akt signaling in platelets and thrombosis. *Expert Rev Hematol*. 2010;3(1):81–91.
35. Varga-Szabo D, Braun A, Nieswandt B. STIM and Orai in platelet function. *Cell Calcium*. 2011;50(3):270–278.
36. Lee RH, Stefanini L, Bergmeier W. Platelet signal transduction. In: Michelson AD, Cattaneo M, Frelinger A, Newman P, eds. *Platelets*. San Diego: Academic Press; 2019:329–348.
37. Ribes A, Oprescu A, Viaud J, et al. Phosphoinositide 3-kinases in platelets, thrombosis and therapeutics. *Biochem J*. 2020;477(22):4327–4342.
38. Patel P, Naik UP. Platelet MAPKs-a 20+ year history: What do we really know? *J Thromb Haemost*. 2020;18(9):2087–2102.
39. Fernandez DI, Kuijpers MJE, Heemskerk JWM. Platelet calcium signaling by G-protein coupled and ITAM-linked receptors regulating anoctamin-6 and procoagulant activity. *Platelets*. 2021;32(7):863–871.
40. Aslan JE. Platelet Rho GTPase regulation in physiology and disease. *Platelets*. 2019;30(1):17–22.
41. Walsh TG, Li Y, Wersall A, et al. Small GTPases in platelet membrane trafficking. *Platelets*. 2019;30(1):31–40.
42. Stefanini L, Bergmeier W. RAP GTPases and platelet integrin signaling. *Platelets*. 2019;30(1):41–47.
43. Dehelian D, Gupta S, Wu J, et al. RGS10 and RGS18 differentially limit platelet activation, promote platelet production, and prolong platelet survival. *Blood*. 2020;136(15):1773–1782.
44. Guidetti GF, Canobbio I, Torti M. PI3K/Akt in platelet integrin signaling and implications in thrombosis. *Adv Biol Regul*. 2015;59:36–52.
45. Rosa JP, Raslova H, Bryckaert M. Filamin A: key actor in platelet biology. *Blood*. 2019;134(16):1279–1288.
46. Cuenca-Zamora EJ, Ferrer-Marin F, Rivera J, et al. Tubulin in platelets: when the shape matters. *Int J Mol Sci*. 2019;20(14):3484.
47. Italiano Jr. JE, Bergmeier W, Tiwari S, et al. Mechanisms and implications of platelet discoid shape. *Blood*. 2003;101(12):4789–4796.
48. Freson K, De Vos R, Wittevrongel C, et al. The TUBB1 Q43P functional polymorphism reduces the risk of cardiovascular disease in men by modulating platelet function and structure. *Blood*. 2005;106(7):2356–2362.
49. Zaninetti C, Sachs L, Palankar R. Role of platelet cytoskeleton in platelet biomechanics: current and emerging methodologies and their potential relevance for the investigation of inherited platelet disorders. *Hamostaseologie*. 2020;40(3):337–347.
50. Cerecedo D. Platelet cytoskeleton and its hemostatic role. *Blood Coagul Fibrinolysis*. 2013;24(8):798–808.
51. Kunishima S, Saito H. Advances in the understanding of MYH9 disorders. *Curr Opin Hematol*. 2010;17(5):405–410.
52. Aguilar A, Weber J, Boscher J, et al. Combined deficiency of RAB32 and RAB38 in the mouse mimics Hermansky-Pudlak syndrome and critically impairs thrombosis. *Blood Adv*. 2019;3(15):2368–2380.
53. Yuan Y, Liu T, Huang X, et al. A zinc transporter, transmembrane protein 163, is critical for the biogenesis of platelet dense granules. *Blood*. 2021;137(13):1804–1817.
54. Ambrosio AL, Di Pietro SM. Storage pool diseases illuminate platelet dense granule biogenesis. *Platelets*. 2017;28(2):138–146.
55. Banerjee M, Whiteheart SW. The ins and outs of endocytic trafficking in platelet functions. *Curr Opin Hematol*. 2017;24(5):467–474.
56. Albers CA, Cvejic A, Favier R, et al. Exome sequencing identifies NBEAL2 as the causative gene for gray platelet syndrome. *Nat Genet*. 2011;43(8):735–737.
57. Gunay-Aygun M, Falik-Zaccai TC, Vilboux T, et al. NBEAL2 is mutated in gray platelet syndrome and is required for biogenesis of platelet alpha-granules. *Nat Genet*. 2011;43(8):732–734.
58. Kahr WH, Hinckley J, Li L, et al. Mutations in NBEAL2, encoding a BEACH protein, cause gray platelet syndrome. *Nat Genet*. 2011;43(8):738–740.
59. Gissen P, Johnson CA, Morgan NV, et al. Mutations in VPS33B, encoding a regulator of SNARE-dependent membrane fusion, cause

arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome. *Nat Genet*. 2004;36(4):400–404.

60. Maynard DM, Heijnen HF, Horne MK, et al. Proteomic analysis of platelet alpha-granules using mass spectrometry. *J Thromb Haemost*. 2007;5(9):1945–1955.
61. Coppinger JA, Cagney G, Toomey S, et al. Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. *Blood*. 2004; 103(6):2096–2104.
62. Blair P, Flaumenhaft R. Platelet alpha-granules: basic biology and clinical correlates. *Blood Rev*. 2009;23(4):177–189.
63. Italiano Jr. JE, Richardson JL, Patel-Hett S, et al. Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. *Blood*. 2008; 111(3):1227–1233.
64. Reed GL. Platelet secretory mechanisms. *Semin Thromb Hemost*. 2004; 30(4):441–450.
65. Joshi S, Whiteheart SW. The nuts and bolts of the platelet release reaction. *Platelets*. 2017;28(2):129–137.
66. Joshi S, Banerjee M, Zhang J, et al. Alterations in platelet secretion differentially affect thrombosis and hemostasis. *Blood Adv*. 2018; 2(17):2187–2198.
67. Williams CM, Li Y, Brown E, et al. Platelet-specific deletion of SNAP23 ablates granule secretion, substantially inhibiting arterial and venous thrombosis in mice. *Blood Adv*. 2018;2(24):3627–3636.
68. Lian L, Wang Y, Flick M, et al. Loss of pleckstrin defines a novel pathway for PKC-mediated exocytosis. *Blood*. 2009;113(15):3577–3584.
69. Golebiewska EM, Poole AW. Platelet secretion: from haemostasis to wound healing and beyond. *Blood Rev*. 2015;29(3):153–162.
70. Zwaal RF, Comfurius P, Bevers EM. Surface exposure of phosphatidylserine in pathological cells. *Cell Mol Life Sci*. 2005; 62(9):971–988.
71. Sang Y, Roest M, De Laat B, et al. Interplay between platelets and coagulation. *Blood Rev*. 2021;46:100733.
72. Whyte CS, Swieringa F, Mastenbroek TG, et al. Plasminogen associates with phosphatidylserine-exposing platelets and contributes to thrombus lysis under flow. *Blood*. 2015;125(16):2568–2578.
73. Reddy EC, Rand ML. Procoagulant phosphatidylserine-exposing platelets in vitro and in vivo. *Front Cardiovasc Med*. 2020;7:15.
74. Suzuki J, Umeda M, Sims PJ, et al. Calcium-dependent phospholipid scrambling by TMEM16F. *Nature*. 2010;468(7325):834–838.
75. Millington-Burgess SL, Harper MT. Gene of the issue: ANO6 and Scott syndrome. *Platelets*. 2020;31(7):964–967.
76. Gaseka A, Nieuwland R, Siljander PR-M. Platelet-derived extracellular vesicles. In: Michelson AD, Cattaneo M, Frelinger A, Newman P, eds. *Platelets*. ed 4. San Diego: Academic Press; 2019:401–416.
77. Puhm F, Boilard E, Machlus KR. Platelet extracellular vesicles: beyond the blood. *Arterioscler Thromb Vasc Biol*. 2021;41(1):87–96.
78. Bledzka K, Smyth SS, Plow EF. Integrin alphaIIbbeta3: from discovery to efficacious therapeutic target. *Circ Res*. 2013;112(8):1189–1200.
79. Bledzka K, Qin J, Plow EF. Integrin αIIbβ3. In: Michelson AD, Cattaneo M, Frelinger A, Newman P, eds. *Platelets*. ed 4 San Diego: Academic Press; 2019:227–241.
80. Lagarrigue F, Paul DS, Gingras AR, et al. Talin-1 is the principal platelet Rap1 effector of integrin activation. *Blood*. 2020;136(10):1180–1190.
81. Jackson SP. The growing complexity of platelet aggregation. *Blood*. 2007;109(12):5087–5095.
82. Durrant TN, Van Den Bosch MT, Hers I. Integrin alphaIIbbeta3 outside-in signaling. *Blood*. 2017;130(14):1607–1619.
83. Law LA, Graham DK, Di Paola J, et al. GAS6/TAM pathway signaling in hemostasis and thrombosis. *Front Med (Lausanne)*. 2018;5:137.
84. Harrison P, Lordkipanidze M. Testing platelet function. *Hematol Oncol Clin North Am*. 2013;27(3):411–441.
85. Rand ML, Reddy EC, Israels SJ. Laboratory diagnosis of inherited platelet function disorders. *Transfus Apher Sci*. 2018;57(4):485–493.
86. Christie DJ, Avari T, Carrington LR, et al: Platelet function testing by aggregometry; Approved guideline. CLSI document H58-A, Clinical and Laboratory Standards Institute, Wayne, PA, 2008.
87. Harrison P, Mackie I, Mumford A, et al. Guidelines for the laboratory investigation of heritable disorders of platelet function. *Br J Haematol*. 2011;155(1):30–44.
88. Cattaneo M, Cerletti C, Harrison P, et al. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. *J Thromb Haemost*. 2013;11(6):1183–1189.
89. Gresele P, Falcinelli E, Bury L. Laboratory diagnosis of clinically relevant platelet function disorders. *Int J Lab Hematol*. 2018;40(suppl 1):34–45.
90. De Witt SM, Swieringa F, Cavill R, et al. Identification of platelet function defects by multi-parameter assessment of thrombus formation. *Nat Commun*. 2014;5:4257.
91. Brouns SLN, Van Geffen JP, Campello E, et al. Platelet-primed interactions of coagulation and anticoagulation pathways in flow-dependent thrombus formation. *Sci Rep*. 2020;10(1):11910.
92. Quiroga T, Goycoolea M, Panes O, et al. High prevalence of bleeders of unknown cause among patients with inherited mucocutaneous bleeding. A prospective study of 280 patients and 299 controls. *Haematologica*. 2007;92(3):357–365.
93. Oved JH, Lambert MP, Kowalska MA, et al. Population based frequency of naturally occurring loss-of-function variants in genes associated with platelet disorders. *J Thromb Haemost*. 2021;19(1):248–254.
94. Wei AH, Schoenwaelder SM, Andrews RK, et al. New insights into the haemostatic function of platelets. *Br J Haematol*. 2009;147(4):415–430.
95. Leon C, Dupuis A, Gachet C, et al. The contribution of mouse models to the understanding of constitutional thrombocytopenia. *Haematologica*. 2016;101(8):896–908.
96. Nagy M, Van Geffen JP, Stegner D, et al. Comparative analysis of microfluidics thrombus formation in multiple genetically modified mice: link to thrombosis and hemostasis. *Front Cardiovasc Med*. 2019;6:99.
97. Nurden AT, Nurden P. Inherited thrombocytopenias: history, advances and perspectives. *Haematologica*. 2020;105(8):2004–2019.
98. Nurden P, Stritt S, Favier R, et al. Inherited platelet diseases with normal platelet count: phenotypes, genotypes and diagnostic strategy. *Haematologica*. 2021;106(2):337–350.
99. Cattaneo M. Inherited disorders of platelet function. In: Michelson AD, ed. *Platelets*. ed 4. San Diego: Academic Press; 2019:877–904.
100. Al-Huniti A, Kahr WH. Inherited platelet disorders: diagnosis and management. *Transfus Med Rev*. 2020;34(4):277–285.
101. Savoia A, Kunishima S, De Rocco D, et al. Spectrum of the mutations in Bernard-Soulier syndrome. *Hum Mutat*. 2014;35(9):1033–1045.
102. Othman M, Kaur H, Emsley J. Platelet-type von Willebrand disease: new insights into the molecular pathophysiology of a unique platelet defect. *Semin Thromb Hemost*. 2013;39(6):663–673.
103. Nurden AT. Clinical significance of altered collagen-receptor functioning in platelets with emphasis on glycoprotein VI. *Blood Rev*. 2019;38:100592.
104. Norman JE, Cunningham MR, Jones ML, et al. Protease-activated receptor 4 variant p.Tyr157Cys reduces platelet functional responses and alters receptor trafficking. *Arterioscler Thromb Vasc Biol*. 2016; 36(5):952–960.
105. Dupuis A, Bordet JC, Eckly A, et al. Platelet delta-storage pool disease: an update. *J Clin Med*. 2020;9(8):2508.
106. Huizing M, Malicdan MCV, Wang JA, et al. Hermansky-Pudlak syndrome: mutation update. *Hum Mutat*. 2020;41(3):543–580.
107. Karampini E, Bierings R, Voorberg J. Orchestration of primary hemostasis by platelet and endothelial lysosome-related organelles. *Arterioscler Thromb Vasc Biol*. 2020;40(6):1441–1453.
108. Nakamura L, Bertling A, Brodde MF, et al. First characterization of platelet secretion defect in patients with familial hemophagocytic lymphohistiocytosis type 3 (FHL-3). *Blood*. 2015;125(2):412–414.
109. Ye S, Karim ZA, Al Hawas R, et al. Syntaxin-11, but not syntaxin-2 or syntaxin-4, is required for platelet secretion. *Blood*. 2012;120(12):2484–2492.
110. Al Hawas R, Ren Q, Ye S, et al. Munc18b/STXBP2 is required for platelet secretion. *Blood*. 2012;120(12):2493–2500.
111. Liang M, Soomro A, Tasneem S, et al. Enhancer-gene rewiring in the pathogenesis of Quebec platelet disorder. *Blood*. 2020;136(23):2679–2690.
112. Rao AK. Inherited platelet function disorders: overview and disorders of granules, secretion, and signal transduction. *Hematol Oncol Clin North Am*. 2013;27(3):585–611.
113. Turro E, Greene D, Wijgaerts A, et al. A dominant gain-of-function mutation in universal tyrosine kinase SRC causes thrombocytopenia, myelofibrosis, bleeding, and bone pathologies. *Sci Transl Med*. 2016; 8(328):328ra330.
114. De Kock L, Freson K. The (patho)biology of SRC kinase in platelets and megakaryocytes. *Medicina (Kaunas)*. 2020;56(12):633.
115. Nurden AT, Pillois X. ITGA2B and ITGB3 gene mutations associated with Glanzmann thrombasthenia. *Platelets*. 2018;29(1):98–101.
116. Botero JP, Lee K, Branchford BR, et al. Glanzmann thrombasthenia: genetic basis and clinical correlates. *Haematologica*. 2020;105(4):888–894.
117. Nurden AT, Pillois X, Fiore M, et al. Expanding the mutation spectrum affecting alphaIIbbeta3 Integrin in Glanzmann Thrombasthenia: Screening of the ITGA2B and ITGB3 genes in a large international cohort. *Hum Mutat*. 2015;36(5):548–561.
118. Westbury SK, Canault M, Greene D, et al. Expanded repertoire of RASGRP2 variants responsible for platelet dysfunction and severe bleeding. *Blood*. 2017;130(8):1026–1030.

119. Canault M, Alessi MC. RasGRP2 structure, function and genetic variants in platelet pathophysiology. *Int J Mol Sci*. 2020;21(3):1075.
120. Freson K, Wijgaerts A, Van Geet C. Update on the causes of platelet disorders and functional consequences. *Int J Lab Hematol*. 2014; 36(3):313–325.
121. Pecci A, Balduini CL. Lessons in platelet production from inherited thrombocytopenias. *Br J Haematol*. 2014;165(2):179–192.
122. Plo I, Bellanne-Chantelot C, Mosca M, et al. Genetic alterations of the thrombopoietin/MPL/JAK2 axis impacting megakaryopoiesis. *Front Endocrinol (Lausanne)*. 2017;8:234.
123. Geddis AE. Inherited thrombocytopenias: an approach to diagnosis and management. *Int J Lab Hematol*. 2013;35(1):14–25.
124. Albers CA, Paul DS, Schulze H, et al. Compound inheritance of a low-frequency regulatory SNP and a rare null mutation in exon-junction complex subunit RBM8A causes TAR syndrome. *Nat Genet*. 2012; 44(4):435–439. S1-2.
125. Manukjan G, Bosing H, Schmugge M, et al. Impact of genetic variants on haematopoiesis in patients with thrombocytopenia absent radii (TAR) syndrome. *Br J Haematol*. 2017;179(4):606–617.
126. Tijssen MR, Ghevaert C. Transcription factors in late megakaryopoiesis and related platelet disorders. *J Thromb Haemost*. 2013;11(4):593–604.
127. Schlegelberger B, Heller PG. RUNX1 deficiency (familial platelet disorder with predisposition to myeloid leukemia, FPDMM). *Semin Hematol*. 2017;54(2):75–80.
128. Bluteau D, Balduini A, Balayn N, et al. Thrombocytopenia-associated mutations in the ANKRD26 regulatory region induce MAPK hyperactivation. *J Clin Invest*. 2014;124(2):580–591.
129. Pecci A. Pathogenesis and management of inherited thrombocytopenias: rationale for the use of thrombopoietin-receptor agonists. *Int J Hematol*. 2013;98(1):34–47.
130. Di Paola J, Porter CC. ETV6-related thrombocytopenia and leukemia predisposition. *Blood*. 2019;134(8):663–667.
131. Sims MC, Mayer L, Collins JH, et al. Novel manifestations of immune dysregulation and granule defects in gray platelet syndrome. *Blood*. 2020;136(17):1956–1967.
132. Kahr WH, Lo RW, Li L, et al. Abnormal megakaryocyte development and platelet function in Nbeal2(−/−) mice. *Blood*. 2013;122(19):3349–3358.
133. Lo RW, Li L, Leung R, et al. NBEAL2 (Neurobeachin-Like 2) is required for retention of cargo proteins by alpha-granules during their production by megakaryocytes. *Arterioscler Thromb Vasc Biol*. 2018;38(10):2435–2447.
134. Monteferrario D, Bolar NA, Marneth AE, et al. A dominant-negative GFI1B mutation in the gray platelet syndrome. *N Engl J Med*. 2014; 370(3):245–253.
135. Balduini A, Raslova H, Di Buduo CA, et al. Clinic, pathogenic mechanisms and drug testing of two inherited thrombocytopenias, ANKRD26-related thrombocytopenia and MYH9-related diseases. *Eur J Med Genet*. 2018;61(11):715–722.
136. Sivapalaratnam S, Westbury SK, Stephens JC, et al. Rare variants in GP1BB are responsible for autosomal dominant macrothrombocytopenia. *Blood*. 2017;129(4):520–524.
137. Nurden AT, Pillois X, Fiore M, et al. Glanzmann thrombasthenia-like syndromes associated with macrothrombocytopenias and mutations in the genes encoding the alphaIIbbeta3 integrin. *Semin Thromb Hemost*. 2011;37(6):698–706.
138. Burley K, Westbury SK, Mumford AD. TUBB1 variants and human platelet traits. *Platelets*. 2018;29(2):209–211.
139. Manchev VT, Hilpert M, Berrou E, et al. A new form of macrothrombocytopenia induced by a germ-line mutation in the PRKACG gene. *Blood*. 2014;124(16):2554–2563.
140. Notarangelo LD, Ochs HD. Wiskott-Aldrich syndrome: a model for defective actin reorganization, cell trafficking and synapse formation. *Curr Opin Immunol*. 2003;15(5):585–591.
141. Brigida I, Zoccolillo M, Cicalese MP, et al. T-cell defects in patients with ARPC1B germline mutations account for combined immunodeficiency. *Blood*. 2018;132(22):2362–2374.
142. Kahr WH, Pluthero FG, Elkadri A, et al. Loss of the Arp2/3 complex component ARPC1B causes platelet abnormalities and predisposes to inflammatory disease. *Nat Commun*. 2017;8:14816.
143. Hottz ED, Azevedo-Quintanilha IG, Palhinha L, et al. Platelet activation and platelet-monocyte aggregate formation trigger tissue factor expression in patients with severe COVID-19. *Blood*. 2020;136(11):1330–1341.
144. Manne BK, Denorme F, Middleton EA, et al. Platelet gene expression and function in patients with COVID-19. *Blood*. 2020;136(11):1317–1329.
145. Barrett TJ, Bilaloglu S, Cornwell M, et al. Platelets contribute to disease severity in COVID-19. *J Thromb Haemost*. 2021;19(12): 3139–3153.
146. Althaus K, Zlamal J, Bakchoul T. Antibody-mediated platelet activation in COVID-19: A coincidence or a new mechanism of the dysregulated coagulation system? *J Thromb Haemost*. 2021;19(5):1171–1173.
147. Koupenova M, Clancy L, Corkrey HA, et al. Circulating platelets as mediators of immunity, inflammation, and thrombosis. *Circ Res*. 2018; 122(2):337–351.
148. Nurden AT. The biology of the platelet with special reference to inflammation, wound healing and immunity. *Front Biosci (Landmark Ed)*. 2018;23(4):726–751.
149. Portier I, Campbell RA. Role of platelets in detection and regulation of infection. *Arterioscler Thromb Vasc Biol*. 2021;41(1):70–78.
150. Antoniak S, Mackman N. Platelets and viruses. *Platelets*. 2021; 32(3):325–330.
151. Coenen DM, Heinzmann ACA, Karel MFA, et al. The multifaceted contribution of platelets in the emergence and aftermath of acute cardiovascular events. *Atherosclerosis*. 2021;319:132–141.
152. Lucotti S, Muschel RJ. Platelets and metastasis: new implications of an old interplay. *Front Oncol*. 2020;10:1350.
153. Orian JM, D'souza CS, Kocovski P, et al. Platelets in multiple sclerosis: early and central mediators of inflammation and neurodegeneration and attractive targets for molecular imaging and site-directed therapy. *Front Immunol*. 2021;12:620963.
154. D'Ambrosi S, Nilsson J, Wurdinger T. Platelets and tumor-associated RNA transfer. *Blood*. 2021;137(23):3181–3191.

CHAPTER 125

MOLECULAR BASIS OF BLOOD COAGULATION

Kathleen Brummel-Ziedins, Kenneth G. Mann, James C. Fredenburgh, and Jeffrey I. Weitz

Blood is the principal vehicle delivering oxygen and nutrients to the various tissues and organs of the body. Blood flow and the integrity of the vasculature are essential to life itself. The hemostatic process has evolved to provide damage recognition and protection from blood loss after perforation of the vasculature while at the same time preventing the systemic activation of the clotting system. However, pathologic occlusions are associated with dysregulation of the intravascular system, resulting in venous or arterial thrombosis. The fine line between vascular occlusion and hemostasis is defined by the complex interplay between pro- and anticoagulant materials provided by the blood, the vasculature, and subvascular elements. The appropriate functions occur as a consequence of intense focal development and regulation of enzymatic activity at sites of vascular injury.

The development of the inventory of components involved in plasma clotting were initially based on the most abundant procoagulant plasma proteins, notably prothrombin and fibrinogen, and extended during the past century with the identification of genetic abnormalities that led to bleeding and deviations in laboratory tests that evolved as the inventory of congenital defects expanded. In some instances, laboratory test results indicating a defect in the procoagulant system were not mirrored by hemostatic pathology. In a similar fashion, the congenital defects associated with thrombosis led to the discovery of anticoagulant proteins in blood and vascular counterparts associated with their presentation and activation.

The functional connections between procoagulant "factors" were developed by mixing and matching plasmas associated with different hemostatic disorders. This inventory and its connectivity were ratified and expanded by experiments performed with transgenically mutated mice.

The dynamics of the plasma coagulation process as expressed are a consequence of the molar concentrations of the pro- and anticoagulant components in blood and the vasculature, and the kinetic processes associated with the dynamics of both the activation and functions of the various proteins associated with the process.

The initial result of the activation of the procoagulant hemostatic process is the formation of a fibrin–platelet plug that forms the temporary seal of the vascular perforation in hemostasis. The generation of an occlusive fibrin–platelet plug blocking further flow through an element of the vasculature is thrombosis. In both instances, the fibrin–platelet scaffold is ultimately removed and substituted by vascular repair, new cells, and connective tissue. In thrombosis, the platelet–fibrin plug is removed mechanically or by biochemical intervention to restore flow to the flow-starved vascular bed.

The elements of a clotted fibrinogen–platelet plug are dissolved by the fibrinolytic system, a tightly regulated, dynamic system involving enzyme activation, feedback regulation, and blockade by a potent series of inhibitors. Just as there is an interplay between the pro- and anticoagulant components that brings about a blood clot in hemostasis, similar mechanisms occur when blood clots are dissolved via the fibrinolytic process, which is essential for tissue repair. This chapter describes the components of the pro- and anticoagulant system and the pro- and antifibrinolytic system, and the interplay between these systems. The common feature of both systems is the focal presentation of activity that is dependent on the presentation of surface-bound enzymatic complexes that can cleave their respective substrates.

Blood coagulation can best be understood if viewed as a choreographed system that starts from an inventory of the key players, the relationship or connectivity of these players, and the dynamic catalytic processes. These processes together keep blood in a fluid state but primed to react to vascular injury in an explosive manner. Therefore following sections describe the process of blood coagulation in terms of the inventory, the connectivity, and then the dynamics.

INVENTORY: PROCOAGULANT, ANTICOAGULANT, AND FIBRINOLYTIC PROTEINS, INHIBITORS AND RECEPTORS

Putting together an inventory of the blood coagulation components is still ongoing, but what we currently know to date began from initial observations that were made in the fifth century and recorded in the Babylonian Talmud. It was noted that if two male children died of bleeding after circumcision, the third should not be circumcised.[1] Over the centuries, many more hypotheses were made regarding what happens to blood when it escapes from the body.[2,3] The realization that clots stem blood loss only occurred in the 18th century.[2,3] The existence of thrombin, the key enzyme in blood coagulation, was recognized in the 19th century.[2,4]

In 1905, Paul Morawitz proposed the classic theory of coagulation.[5] He hypothesized that in the presence of calcium and thromboplastin, prothrombin was converted to thrombin, which in turn converted fibrinogen to the fibrin clot. These clotting factors were subsequently assigned Roman numerals, factor I (fibrinogen), factor II (prothrombin), factor III (thromboplastin; tissue factor membrane), and factor IV (calcium).[6] As more coagulation factors were introduced, they were assigned consecutive Roman numerals. The activated forms are distinguished by a lower case "a" after their Roman numeral designation. Therefore, the activated form of factor V becomes factor Va.

More complex descriptions of the coagulation system were as a "cascade" or "waterfall." Macfarlane[7] and Davie and Ratnoff[8] proposed that in an intrinsic pathway, involving only plasma, blood coagulated by a sequence of events in which the reactions occurred in a defined series leading to fibrin clot formation. Over the past six decades, the Morawitz/Davie and Macfarlane pathways have been significantly expanded (Fig. 125.1). Each reaction shares a similar mechanism in which an inactive zymogen protein is converted to an active enzyme and each "enzyme" is a surface-bound multiprotein complex consisting of a surface, divalent calcium ions (Ca^{2+}), a protease, and a cofactor. Although some facets of these initial descriptions are still valid, the emerging concept of coagulation and fibrinolysis centers on a complex network of highly interwoven concurrent processes. Procoagulant and fibrinolytic events occur simultaneously with many positive- and negative-feedback loops regulating the processes.

To fully understand the multiple simultaneous processes that occur to effect a hemostatic response, we will first inventory the key procoagulant, anticoagulant, and fibrinolytic participants. The inventory sections discuss the vitamin K–dependent protein family,

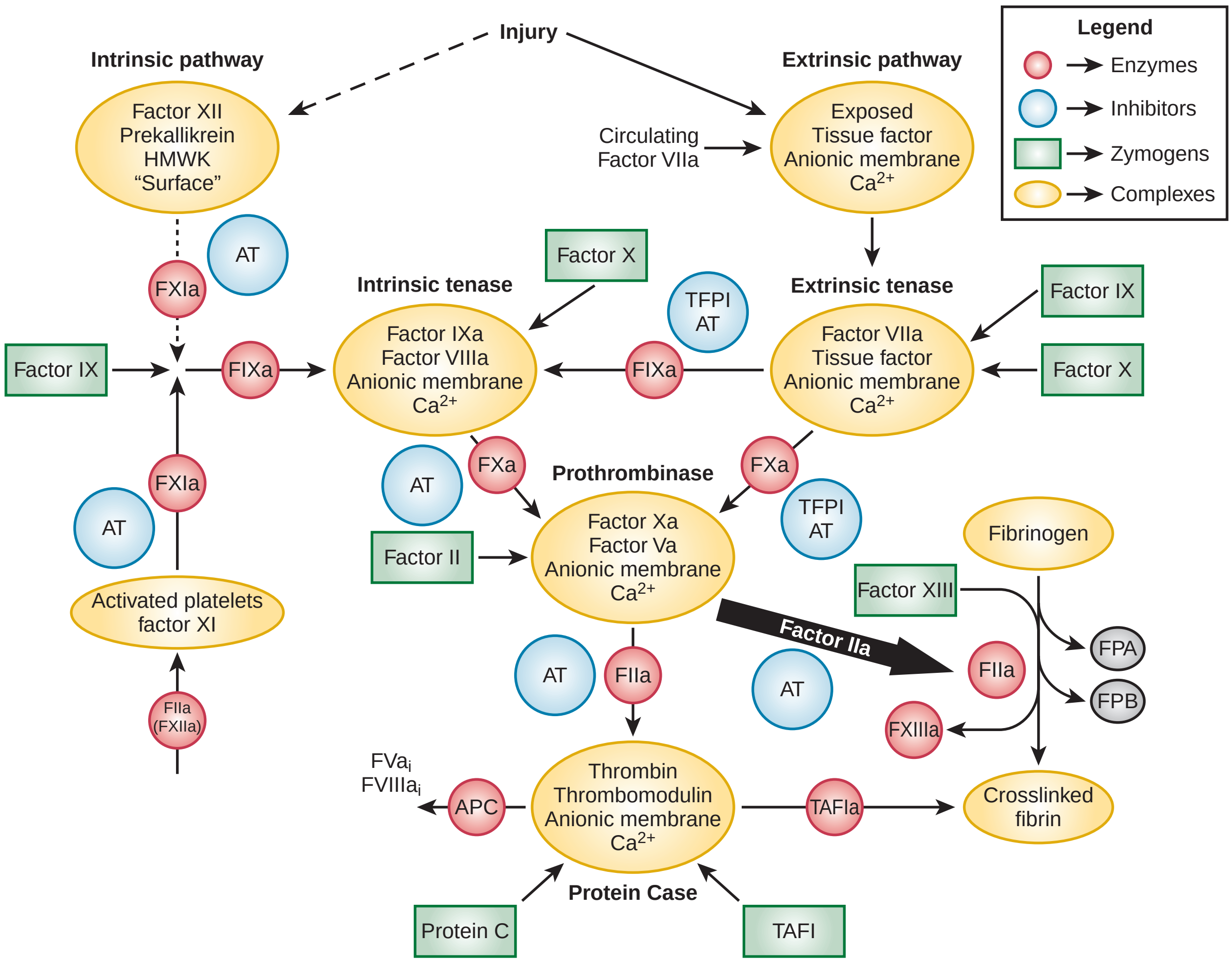

Legend
Enzymes
Inhibitors
Zymogens
Complexes
Injury
Intrinsic pathway
Factor XII
Prekallikrein
HMWK
"Surface"
Extrinsic pathway
Circulating
Factor VIIa
Exposed
Tissue factor
Anionic membrane
Ca^{2+}
AT
FXIa
Factor X
Intrinsic tenase
TFPI
AT
Extrinsic tenase
Factor IX
Factor IX
FIXa
Factor IXa
Factor VIIIa
Anionic membrane
Ca^{2+}
FIXa
Factor VIIa
Tissue factor
Anionic membrane
Ca^{2+}
Factor X
FXIa
AT
FXa
Prothrombinase
FXa
TFPI
AT
Fibrinogen
AT
Factor II
Factor Xa
Factor Va
Anionic membrane
Ca^{2+}
Factor XIII
Activated platelets
factor XI
Factor IIa
FPA
FIIa
AT
FIIa
AT
FPB
FIIa
(FXIIa)
FXIIIa
FVa_i
$FVIIIa_i$
APC
Thrombin
Thrombomodulin
Anionic membrane
Ca^{2+}
TAFIa
Crosslinked
fibrin
Protein Case
Protein C
TAFI

Figure 125.1 OVERVIEW OF HEMOSTASIS. Coagulation is initiated via two pathways, the primary extrinsic pathway *(right)* and the accessory (historically called the contact or intrinsic) pathway *(left)*. An illustration of the multistep processes is as follows: enzymes *(small circles)*, inhibitors *(large circles)*, zymogens *(boxes)*, or complexes *(ovals)*. The accessory pathway has no known bleeding etiology associated with it; thus, this pathway is considered an accessory to hemostasis. Upon injury to the vessel wall, tissue factor, the cofactor for the extrinsic tenase complex, is exposed to circulating factor (F) VIIa and forms the vitamin K–dependent complex extrinsic tenase. FIX and FX are converted to the serine proteases factor IXa (FIXa) and factor Xa (FXa), which then form the intrinsic tenase and prothrombinase complexes, respectively. The combined actions of intrinsic and extrinsic tenase complexes and the prothrombinase complex lead to an explosive burst of thrombin (FIIa). Thrombin not only functions as a procoagulant but also acts as an anticoagulant when complexed with the cofactor thrombomodulin in the protein Case complex. The product of the protein Case reaction, activated protein C (APC), inactivates the cofactors FVa and FVIIIa. The cleaved species, factor Vai (FVai) and factor VIIIai (FVIIIai), no longer support the respective procoagulant activities of the prothrombinase and intrinsic tenase complexes. When thrombin is generated through procoagulant mechanisms, thrombin cleaves fibrinogen, releasing fibrinopeptide A (FPA) and fibrinopeptide B (FPB), and activates FXIII to form a cross-linked fibrin clot. Thrombin–thrombomodulin also activates thrombin activatable fibrinolysis inhibitor (TAFI), which delays fibrin degradation by plasmin. The procoagulant response is downregulated by the stoichiometric inhibitors tissue factor pathway inhibitor (TFPI) and antithrombin (AT). TFPI attenuates the activity of extrinsic tenase, the trigger of coagulation. AT directly inhibits thrombin, FIXa, and FXa. The accessory pathway provides an alternate route for the generation of FIXa. Thrombin has also been shown to activate FXI. *HMWK*, High-molecular-weight kininogen.

cofactor proteins, the intrinsic accessory pathway proteins, endothelium, platelets, proteinase inhibitors, clot proteins, and fibrinolysis proteins.

The Vitamin K–Dependent Protein Family

Vitamin K–dependent proteins, synthesized in the liver, play a central role in blood coagulation through either procoagulant or anticoagulant mechanisms. The vitamin K–dependent protein family includes the zymogen procoagulant factors VII, IX, X, and prothrombin, and the anticoagulants protein C, protein S, and protein Z (Fig. 125.2 and Table 125.1). Except for protein S and protein Z, after cleavage to their active forms these proteins are serine proteases related to the trypsin and chymotrypsin superfamily. Peptide bond cleavage at specific sites converts the vitamin K–dependent zymogens to their active serine protease forms. All share noncatalytic domains, each of which is characterized by highly conserved regions that fold, independently from the rest of the molecule, into a characteristic three-dimensional shape. The domains of the vitamin K–dependent proteins are illustrated in Fig. 125.2. Several reviews have been written on vitamin K–dependent proteins.[9–11]

Vitamin K is essential for the biosynthesis of these clotting factors by participating in the cyclic oxidation and reduction of the enzyme that converts 9 to 13 N-terminal glutamate residues to γ-carboxy glutamate (Gla; Fig. 125.2; see reviews listed in the References[9,12–14]). This posttranslational modification to form Gla residues adds a net negative charge to the molecules that enables the vitamin K–dependent proteins to interact with Ca^{2+} and a membrane surface.[15,16] Blocking the formation of the Gla residues is the basis for anticoagulant therapy with coumarin (warfarin) derivatives, which are chemically similar in structure to vitamin K (Fig. 125.3). The Ca^{2+}

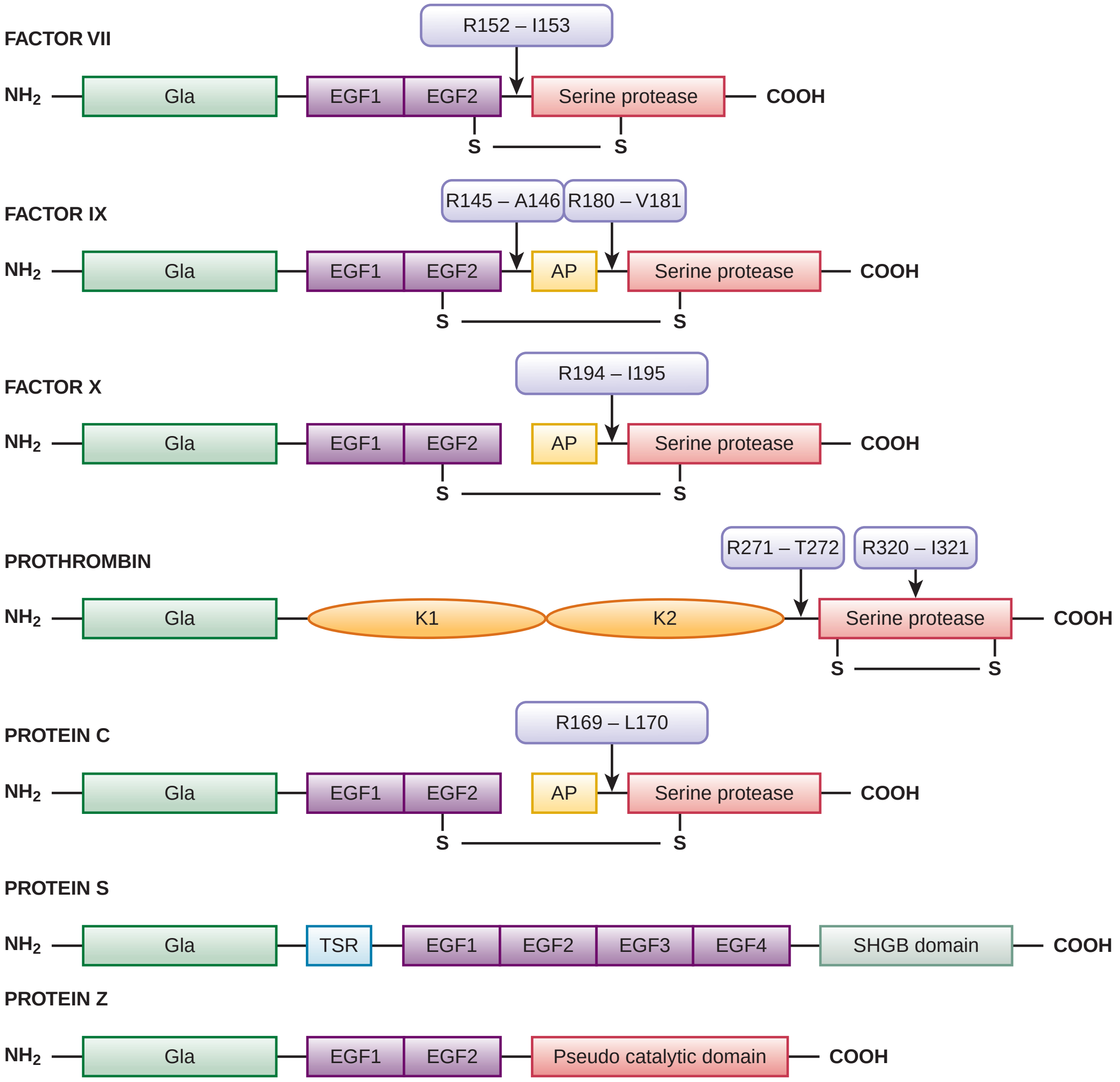

FACTOR VII
R152 – I153
NH2
Gla
EGF1
EGF2
Serine protease
COOH
S
S
FACTOR IX
R145 – A146
R180 – V181
NH2
Gla
EGF1
EGF2
AP
Serine protease
COOH
S
S
FACTOR X
R194 – I195
NH2
Gla
EGF1
EGF2
AP
Serine protease
COOH
S
S
PROTHROMBIN
R271 – T272
R320 – I321
NH2
Gla
K1
K2
Serine protease
COOH
S
S
PROTEIN C
R169 – L170
NH2
Gla
EGF1
EGF2
AP
Serine protease
COOH
S
S
PROTEIN S
NH2
Gla
TSR
EGF1
EGF2
EGF3
EGF4
SHGB domain
COOH
PROTEIN Z
NH2
Gla
EGF1
EGF2
Pseudo catalytic domain
COOH

Figure 125.2 SCHEMATIC REPRESENTATION OF THE VITAMIN K–DEPENDENT PROTEINS. The vitamin K–dependent proteins can be divided into two classes, procoagulant (factors II, VII, IX, and X) and anticoagulant (protein C, protein S, and protein Z). The building blocks for these proteins include an NH_2-terminal Gla domain, consisting of 9 to 13 Gla residues, followed by either an EGF–like domain in factor VII, factor IX, factor X, protein C, protein S, and protein Z, or a K domain in prothrombin. In protein S, a TSR precedes the EGF domain. Active sites are contained within the serine protease domain. Cleavage sites for the conversion of zymogens to their active forms are designated by boxes above the arrows. Factor IX, factor X, and protein C are activated by proteolytic removal of an AP. Protein S, which is not a serine protease precursor, contains an SHGB at the COOH-terminus. Protein Z also contains a "pseudo catalytic domain" in the COOH-terminus and does not function as a serine protease. Disulfide bonds (-S-S-) critical to the integrity of the two-chain zymogens or active forms are presented. *AP*, Activation peptide; *EGF*, epidermal growth factor; *K*, kringle; *SHGB*, sex hormone–binding globulin–like domain; *TSR*, thrombin-sensitive region.

binding association with this modification is also the basis for the anticoagulant activity of sodium citrate, a calcium chelator, found in the blue-top vacuum tubes used for clinical laboratory testing of clotting activity. The level of inhibition achieved with the same dose of warfarin varies among patients. Increased sensitivity to warfarin has been identified in patients when started after surgery.[17,18] Factors affecting the level of anticoagulation include dietary intake of vitamin K; liver function; concomitant medications that either reduce or enhance the warfarin effect[19]; common polymorphisms in the vitamin K epoxide reductase complex subunit 1 (VKORC1), which is responsible for vitamin K reduction; and polymorphisms in CYP2C9, which affect warfarin metabolism (see reviews listed in the References[20,21]). Therefore, genotyping may be helpful to personalize starting doses of warfarin. Ultimately, proper monitoring of warfarin therapy is essential. This is done using the prothrombin time (PT) with the assay sensitivity corrected using the international normalized ratio (INR).[22,23]

The NH_2-terminal Gla domains are followed by either a kringle domain in factor II or an epidermal growth factor–like domain (EGF) in factor VII, factor IX, factor X, protein C, protein S, and protein Z (see Fig. 125.2). Protein S is not a serine protease precursor and instead contains a thrombin-sensitive region before the EGF domain and a sex hormone–binding globulin–like domain (SHBG) in the COOH-terminus.[24] Protein Z contains a "pseudo catalytic domain" in the COOH-terminus and does not function as a serine protease enzyme.[25]

Vitamin K–dependent protein complexes are essential for establishing hemostatic balance (Fig. 125.4). Each complex is composed of

TABLE 125.1 Procoagulant, Anticoagulant, and Fibrinolytic Proteins, Inhibitors, and Receptors

Protein	Molecular Weight (kD)	Plasma Concentration		Plasma $t_{1/2}$ (Days)	Clinical Phenotype Associated with Deficiency	Functional Classification
		(nmol/L)	(μg/mL)			
Procoagulant Proteins and Receptors						
Factor XII	80	500	40	2–3	None	Protease zymogen
HMW kininogen	120	670	80		None	Cofactor
LMW kininogen	66	1300	90			Cofactor
Prekallikrein	85/88	486	42			Protease zymogen
Factor XI	160	30	4.8	2.5–3.3	Sometimes bleeding	Protease zymogen
Tissue factor	44			N/A		Cell-associated cofactor
Factor VII	50	10	0.5	0.25	Bleeding (occasionally thrombotic)	VKD protease zymogen
Factor X	59	170	10	1.5	Bleeding	VKD protease zymogen
Factor IX	55	90	5	1	Bleeding	VKD protease zymogen
Factor V	330	20	6.6	0.5	Bleeding[a]	Soluble procofactor
Factor VIII	285	1.1–1.5	0.3–0.4	0.3–0.5	Bleeding	Soluble procofactor
vWF	255	Varies	10		Bleeding	Carrier for factor VIII
Factor II	72	1400	100	2.5	Bleeding[b]	VKD protease zymogen
Fibrinogen	340	7400	2500	3–5	Bleeding[c]	Structural clot protein
Factor XIII	320	94	30	9–10	Bleeding	Transglutaminase zymogen
Anticoagulant Proteins, Inhibitors, and Receptors						
Protein C	62	65	4	0.33	Thrombotic	Proteinase zymogen
Protein S	69	300	20	1.75	Thrombotic	Inhibitory cofactor
Protein Z	62	47	2.9	2.5	Sometimes thrombotic	Inhibitory cofactor
Thrombomodulin	100	N/A	N/A	N/A		Cofactor/modulator
Tissue factor pathway inhibitor	40	1–4	0.1	minutes		Proteinase inhibitor
Antithrombin	58	2400	140	2.5–3	Thrombotic	Proteinase inhibitor
Heparin cofactor II	66	500–1400	33–90	2.5	Often thrombotic	Proteinase inhibitor
α_2-Macroglobulin	735	2700–4000	2–3000	<1 h		Proteinase inhibitor
α_1-Proteinase inhibitor	53	28,000–65,000	1500–3500	6		Proteinase inhibitor
Endothelial protein C receptor						Receptor
Fibrinolytic Proteins, Inhibitors, and Receptors						
Plasminogen	88	2300	210	2.2		Proteinase zymogen
t-PA	70	0.07	0.005	<5 min		Proteinase zymogen
u-PA	54	0.04	0.002	5 min		Proteinase zymogen
TAFI	58	75	4.5	10 min	Thrombotic	Carboxypeptidase
FSAP	64	190	12			Fibrinolytic zymogen
PAI-1	52	0.2	0.01	<10 min	Bleeding	Proteinase inhibitor
PAI-2	47/60	<0.070	<0.005	—		Proteinase inhibitor
α-Antiplasmin	70	500	70	2.6	Bleeding	Proteinase inhibitor

FSAP, Factor VII–activating protease; *HMW*, high-molecular-weight; *LMW*, low-molecular-weight; *PAI*, plasminogen activator inhibitor; *TAFI*, thrombin-activatable fibrinolysis inhibitor; *t-PA*, tissue plasminogen activator; *u-PA*, urinary plasminogen activator (urokinase); *u-PAR*, urokinase-type plasminogen activator receptor; *VKD*, vitamin K–dependent; *vWF*, von Willebrand factor.

[a] Factor V Leiden mutation associated with thrombosis.

[b] Prothrombin 20210A mutation associated with thrombosis.

[c] Some fibrinogen mutations associated with thrombosis.

a serine protease enzyme, a cofactor that functions as a surface receptor or enhancer for the enzyme Ca^{2+}, and a negatively charged membrane surface provided by activated or damaged cells (e.g., endothelial cells, monocytes, and platelets). There are four vitamin K–dependent complexes: the extrinsic tenase complex (factor VIIa–tissue factor), the intrinsic tenase complex (factor IXa–factor VIIIa), the prothrombinase complex (factor Xa–factor Va), and the anticoagulant protein Case complex (thrombin–thrombomodulin).

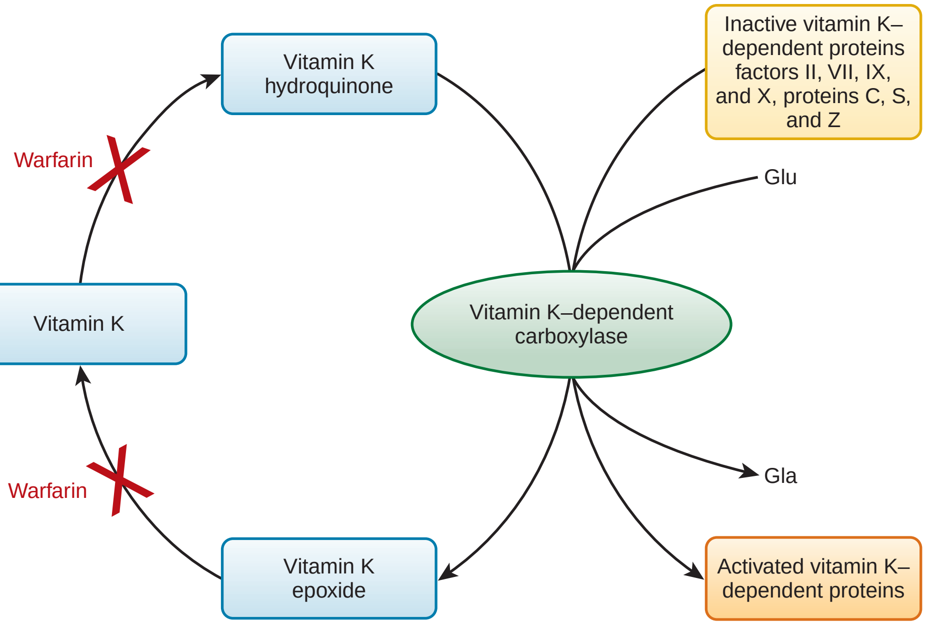

Vitamin K hydroquinone
Inactive vitamin K–dependent proteins factors II, VII, IX, and X, proteins C, S, and Z
Warfarin
Glu
Vitamin K
Vitamin K–dependent carboxylase
Warfarin
Gla
Vitamin K epoxide
Activated vitamin K–dependent proteins

Figure 125.3 VITAMIN K–DEPENDENT PROCESS AND WARFARIN EFFECT. Schematic representation of the mechanism of γ-carboxy glutamate generation by a vitamin K–dependent reaction cycle to produce an active protein is illustrated. The regeneration of vitamin K hydroquinone by the vitamin K–dependent reductases is inhibited by warfarin.

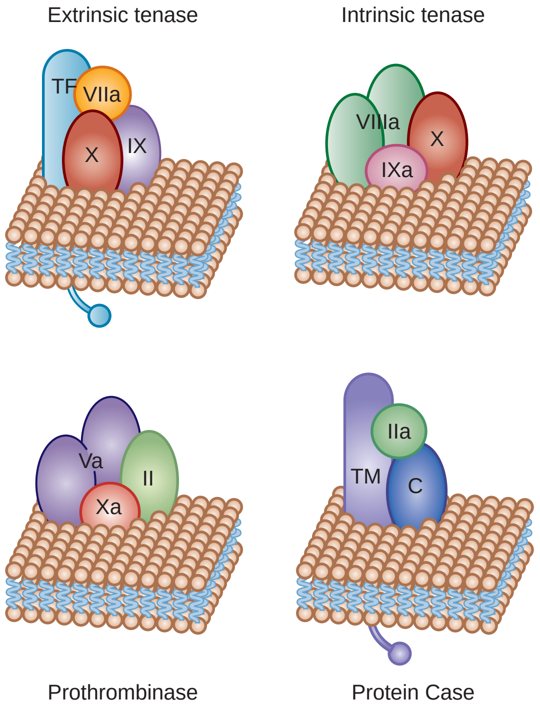


Figure 125.4 VITAMIN K–DEPENDENT COMPLEXES. Three procoagulant complexes (extrinsic tenase, intrinsic tenase, and prothrombinase) and one anticoagulant complex (protein Case) are illustrated. Each membrane complex consists of a vitamin K–dependent serine protease (factor VIIa [VIIa], factor IXa [IXa], factor Xa [Xa], or thrombin [IIa]) and a soluble or cell surface–associated cofactor (factor VIIIa [heavy and light chain $VIII_H$ and $VIII_L$], factor Va [heavy and light chain V_H and V_L], tissue factor [TF], or thrombomodulin [TM]). Each serine protease is shown in association with the appropriate cofactor protein and zymogen substrate(s) on the membrane surface. The membrane serves as a scaffold for the coagulation reactants, enhancing the reaction rates by 10^4- to 10^9-fold.

When the serine protease is associated with its respective cofactor on an appropriate membrane surface with Ca^{2+}, the specific reactions occur at a rate that is 10^4- to 10^9-fold faster than that of protease–substrate combination alone.[26] One way to visualize the importance of the assembly of these macromolecular complexes in the formation of the hemostatic plug is to note that if a healthy person takes 4 minutes for his or her blood to clot, then in the absence of membrane and cofactor, blood clot formation would take approximately 3.8 years.

Cofactor Proteins

There are two categories of procoagulant cofactor proteins: the cell-bound cofactors (tissue factor and thrombomodulin) and the soluble plasma-derived procoagulant procofactors (factor V and factor VIII with its circulating carrier von Willebrand factor [vWF]).

Cell-Bound Cofactors

Tissue Factor

Tissue factor is a transmembrane protein that functions as a nonenzymatic cofactor for factor VIIa in the extrinsic tenase complex[27] (see reviews listed in the References[28,29]; Fig. 125.5A). In the absence of injury or inflammatory stimuli, tissue factor is not expressed on cellular surfaces in direct contact with circulating blood (see the review listed in the References[30]). Presentation of tissue factor to the circulation is the event that triggers the primary procoagulant pathway of coagulation (see Fig. 125.1).[31–33] There are no known mutations or deficiencies of human tissue factor, and tissue factor deletion in mice is lethal during embryonic development,[34] leading to the speculation that tissue factor is essential for life.

Tissue factor activity is primarily regulated by controlling its presentation. The commonly accepted sources of functional tissue factor are the subendothelium exposed upon vascular damage or monocytes stimulated by cytokines. However, there is controversy regarding the source and presentation of active tissue factor and whether functional

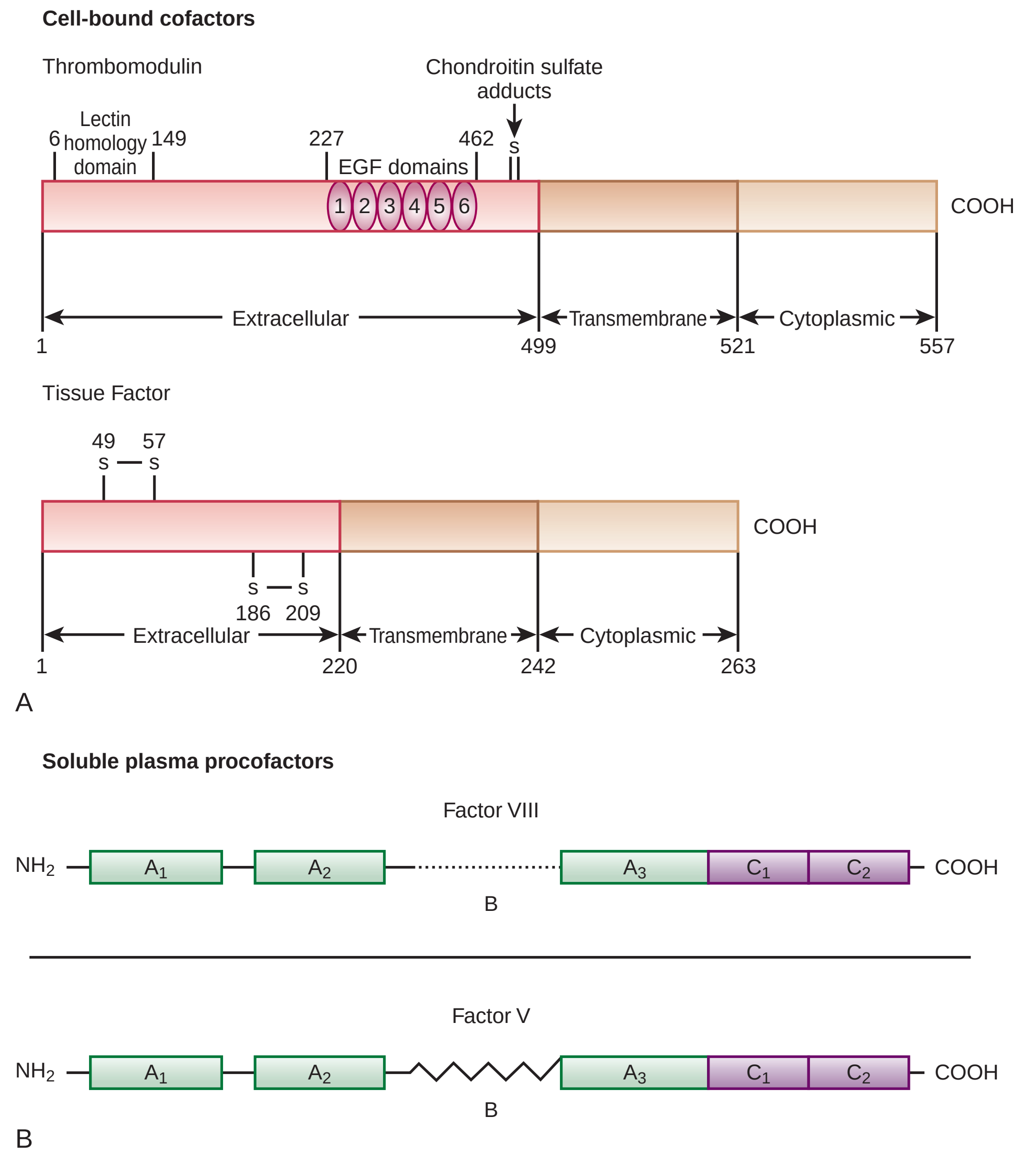

Cell-bound cofactors
Thrombomodulin
Chondroitin sulfate adducts
Lectin homology domain
6
149
227
462
S
EGF domains
1
2
3
4
5
6
COOH
Extracellular
Transmembrane
Cytoplasmic
1
499
521
557
Tissue Factor
49
57
S — S
COOH
S — S
186
209
Extracellular
Transmembrane
Cytoplasmic
1
220
242
263
A
Soluble plasma procofactors
Factor VIII
NH2
A1
A2
A3
C1
C2
COOH
B
Factor V
NH2
A1
A2
A3
C1
C2
COOH
B
B

Figure 125.5 CELL-BOUND AND SOLUBLE COFACTOR STRUCTURES . (A) Cell-bound cofactors: tissue factor and thrombomodulin. Tissue factor is composed of an extracellular domain (residues 1–219), a transmembrane domain (residues 220–242), and a cytoplasmic domain (residues 243–263). Two disulfide bonds (-S-S-) are identified. One cysteine (C^{245}) contains a thiol ester linkage to a fatty acid. Tissue factor, which is the cofactor for factor VIIa in the extrinsic tenase complex, is exposed on the subendothelial surface after injury. Thrombomodulin is an endothelial cell-surface glycoprotein composed of five distinct domains. These include a lectin-like domain (residues 6–149), a domain containing six EGF–like regions (residues 227–462), a small extracellular domain rich in threonine and serine residues (S^{472} and S^{474} have been identified as sites of chondroitin sulfate adducts), a membrane-spanning region (residues 499–521), and a cytoplasmic tail (residues 522–557). There are nine known glycosylation sites on the thrombomodulin molecule. Thrombomodulin functions as the cofactor in the protein Case complex and assists in the generation of activated protein C. (B) Soluble plasma procofactors: factor VIII and factor V. The linear domain structures (A1–A2–A3–C1–C2) are illustrated. The B regions (factor V, residues 709–1545; factor VIII, residues 740–1689) connecting the A2 and A3 domains are shown by *dotted* (factor VIII) or *jagged* (factor V) lines. *EGF*, Epidermal growth factor.

tissue factor circulates in blood in healthy and pathologic states.[35–40] Microparticle sources of blood-borne tissue factor are generally defined as submicron-sized cell-derived membrane fragments produced in response to activation or apoptosis. Their role in hemostasis is still debated.[28,35,41,42]

Thrombomodulin

Thrombomodulin is a type 1 transmembrane protein constitutively expressed on the surface of vascular endothelial cells (see Fig. 125.5A). Thrombomodulin is a high-affinity receptor for all thrombin forms and acts as a cofactor for the thrombin-dependent activation of protein C.[43] The endothelial cell protein C receptor (EPCR) provides cell-specific binding sites for both protein C and activated protein C (APC).[44–47] When bound to thrombomodulin, thrombin's procoagulant activities (e.g., its capacity to generate fibrin and activate factor V, factor VIII, factor XI, and platelets) are neutralized, and the rate of inactivation of thrombin by antithrombin is increased.[48–50] The generation of APC by the thrombin–thrombomodulin complex leads to inactivation of the procoagulant cofactors factor Va and factor VIIIa, thus suppressing thrombin formation.[51,52] Thrombin–thrombomodulin, or the "protein Case" (see Fig. 125.4) complex, also has an antifibrinolytic role via activation of the fibrinolysis inhibitor, thrombin-activatable fibrinolysis inhibitor (TAFI; see reviews listed in the References[53–56]).

Thrombomodulin activity on the surface of endothelial cells is decreased by inflammatory cytokines,[57,58] and this decrease may contribute to the hypercoagulation characteristic of inflammatory states.

Soluble Plasma Procofactors

Factor V

Factor V is a large single-chain glycoprotein (GP) that circulates in human plasma (see Table 125.1 and Fig. 125.5B) and is also contained in the α-granules of human platelets, with approximately 18% to 25% of the total factor V found in platelets.[59] The procofactor factor V is proteolyzed by α-thrombin to the active cofactor factor Va.[60] Factor Va functions both as a factor Xa receptor and positive modulator of factor Xa catalytic potential in the prothrombinase complex (see Fig. 125.4).[61] Factor Va is proteolytically inactivated by APC.[62,63] The importance of this regulatory mechanism is demonstrated by the "APC resistance" syndrome associated with factor V$^{\text{Leiden}}$.[64,65] Individuals with factor V$^{\text{Leiden}}$ have a G to A substitution at nucleotide 1691 in the factor V gene that results in an Arg^{506}→Gln mutation at the protein level.[66] Factor Va$^{\text{Leiden}}$ has normal cofactor activity as part of the prothrombinase complex. However, unlike normal factor Va, factor Va$^{\text{Leiden}}$ is more slowly inactivated by APC. The Arg^{506}→Gln mutation hinders the first step in the series of inactivating cleavages by APC. Cleaved factor Va$^{\text{Leiden}}$ retains limited cofactor activity and continues to promote thrombin generation. The identification, role in coagulation, and overall importance of factor V in hemostasis have been described in numerous reviews.[61,67]

Factor VIII

The soluble procofactor factor VIII, or antihemophilic factor (see Fig. 125.5B), circulates in plasma in complex with the large multimeric protein vWF.[68] vWF acts to regulate the plasma concentration of factor VIII. Factor VIII interaction with vWF requires the NH_2- and COOH-termini of the factor VIII light chain (A3, C1, and C2 domains), although a specific vWF binding site has been identified at residues 1673–1684 on the light chain.[68–71] Factor VIII is activated by thrombin cleavage at three sites (Arg^{372}, Arg^{740}, Arg^{1689}) to generate the heterotrimeric cofactor factor VIIIa.[72] The vWF binding site is removed from the factor VIII protein by thrombin cleavage at Arg^{1689}. After forming, vWF–free factor VIIIa forms a complex with the serine protease factor IXa, Ca^{2+}, and a membrane (provided by platelets), resulting in the intrinsic tenase complex (see Fig. 125.4). Factor VIIIa function is downregulated by the relatively rapid dissociation of the noncovalently associated A2 subunit, which is produced by the cleavages required for activation, and by cleavage by APC.[52] Factor VIII is homologous (40% identity) to the procofactor factor V.[73]

Deficiency of factor VIII, or hemophilia A, is a well-characterized bleeding disorder linked to the X chromosome (see Chapter 134).[74] Hemophilia A therefore occurs almost exclusively in males at a frequency of 1 in 5000 to 1 in 10,000 individuals.[75]

von Willebrand Factor

vWF has several key roles in coagulation. It is synthesized in endothelial cells and also contained in the α-granules of human platelets.[76] vWF is a large adhesive GP that circulates in plasma as a heterogeneous mixture of disulfide-linked multimers that range in size from dimers (M_r=600,000) to extremely large multimers of more than 20 million kDa. vWF has binding sites for factor VIII, heparin, collagen, platelet GPIb, and platelet GPIIb–IIIa.[77–90] vWF acts as the bridge between platelets to promote platelet aggregation. The primary platelet binding site for vWF is the GPIb–IX–V receptor complex (see Chapter 124). GPIb–IX–V is an active receptor on unstimulated platelets and serves to promote platelet aggregation and adhesion to vWF in the absence of platelet activation.[91]

vWF is a structural protein and is part of the subendothelial matrix. Endothelial cells secrete vWF multimers, which are larger than those found circulating in plasma.[92] The function of these large multimeric forms of vWF is to bind to and agglutinate blood platelets under high shear conditions. These large multimers of vWF are degraded by a specific metalloprotease called a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS)-13.[93–95] In familial and acquired thrombotic thrombocytopenic purpura, ultra-large vWF multimers are correlated with defective ADAMTS-13 activity (see Chapter 133).[96]

ABO blood type has a significant influence on vWF levels, with individuals of types A, B, or AB blood having much higher levels of vWF than those with type O blood.[97,98] vWF is also known for its role in ristocetin-induced platelet agglutination,[99,100] which is the basis of clinical assays for von Willebrand disease, a fairly common disorder that is estimated to occur in 1% to 2% of the general population (see Chapter 133).[101–104] vWF is also an acute-phase reactant, and vWF levels are elevated as a result of stress, pregnancy, or surgical trauma.[105–108]

The Intrinsic Accessory Pathway Proteins

The designation "intrinsic accessory pathway" emerged as the relationship between genetic deficiencies and bleeding phenotypes was established (see Fig. 125.1). Deficiencies of proteins associated with the intrinsic or accessory pathway (factor XII, prekallikrein, and high-molecular-weight kininogen [HMWK]) are not ordinarily associated with excessive bleeding, even after surgical challenge (see Chapters 121 and 135).[109–111] In contrast, deficiencies of the protein components of the extrinsic or primary pathway (prothrombin and factors V, VII, VIII, IX, and X) can lead to severe bleeding diatheses.[112–116] The physiologic role of the accessory pathway is therefore not clearly understood.[117]

Although the intrinsic pathway proteins have no defined role in normal hemostasis, these accessory pathway factors are thought to play a key role in disseminated intravascular coagulation associated with the systemic inflammatory response syndrome[118,119] and in the promotion of thrombus stability,[117,120,121] and may be a new target to control pathologic coagulation.[122] Also, the accessory pathway is important in cardiopulmonary bypass because of contact between blood components and synthetic surfaces.[119] Recent evidence suggests that biologic activation of the contact pathway system may be accomplished through assembly of these proteins on endothelial cell membranes and that prekallikrein is activated by an endothelial cell membrane cysteine protease rather than by factor XII.[123,124] Extracellular RNA derived from damaged or necrotic cells has also been found to participate in the activation of proteases of the contact pathway by providing a procoagulant cofactor template for factor XII- or XI-induced contact activation.[125] Polyphosphates, linear inorganic polymers of 60 to 100 phosphate residues, released from platelets have also been shown to activate fXII (see Chapter 121).[126]

In principle, factor XI represents an intersection point for the two pathways. Individuals with factor XI deficiency (hemophilia C) have a variable bleeding phenotype upon surgical challenge,[127,128] therefore establishing an essential role for factor XI in hemostasis (see Chapter 135). During the coagulation process, factor XIa formation appears to be catalyzed by thrombin as part of a positive-feedback loop stemming from thrombin generation.[129] Factor XIa then functions in the propagation phase of thrombin generation in association with the primary pathway by activation of factor IX.[130]

Three proteins, factor XII, prekallikrein, and HMWK, are required for activity of the intrinsic or accessory pathway. Factor XII and prekallikrein are zymogens that are activated to generate serine proteases, and HMWK is a nonenzymatic procofactor (Fig. 125.6). The activation of this pathway in vitro is accomplished when factor XII autoactivates to factor XIIa upon exposure to foreign surfaces, including kaolin, dextran sulfate, and sulfatides.[131–133] The substrates for factor XIIa, prekallikrein, and factor XI exist in noncovalent complex with HMWK and become activated to kallikrein and factor XIa, respectively.[134] Positive-feedback loops exist in which kallikrein cleaves HMWK, thereby releasing bradykinin and allowing more prekallikrein and factor XI to associate and activate.[135] This pathway is also negatively regulated by the cleavage of HMWK by factor XIa.[136]

Proteinase Inhibitors

Proteinases, enzymes that hydrolyze peptide bonds, are found in a wide array of biologic systems, including the blood coagulation

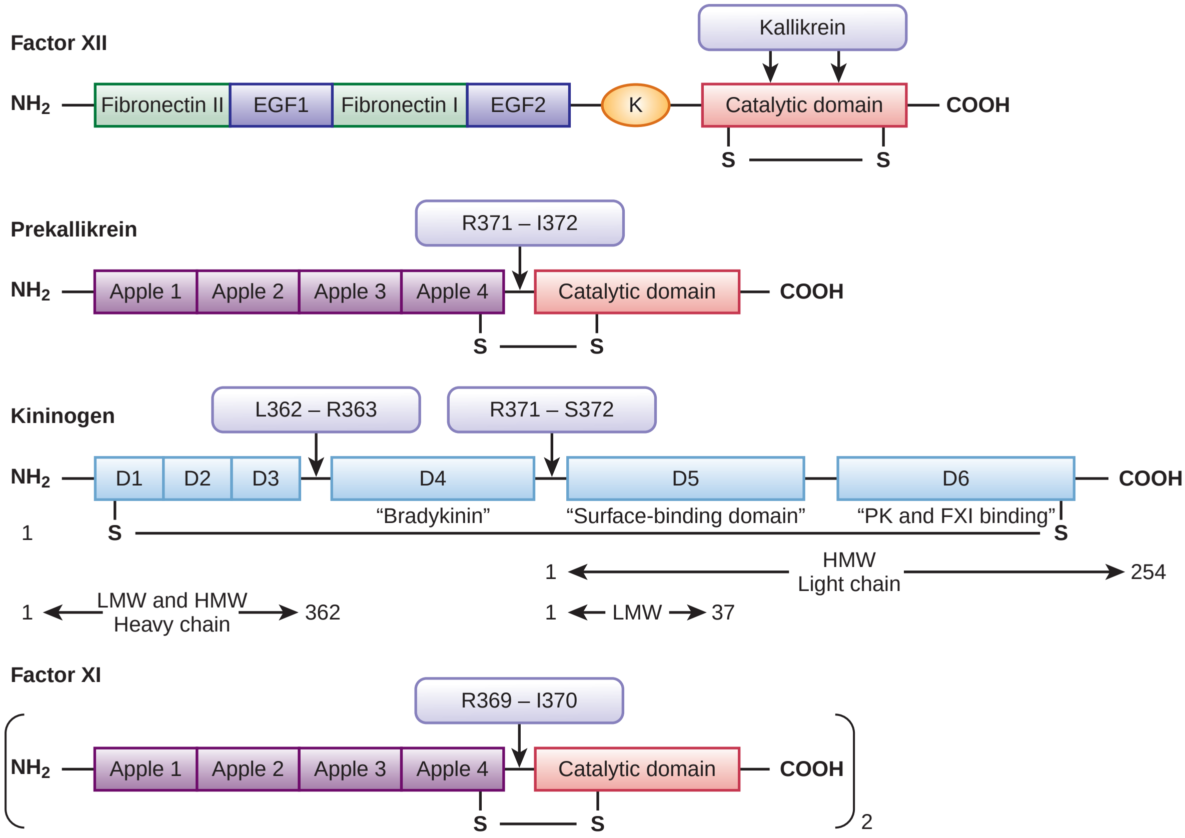


Figure 125.6 SCHEMATIC REPRESENTATION OF THE ACCESSORY PATHWAY (INTRINSIC) PROTEINS. Factor XII, prekallikrein, kininogen, and factor XI are shown with their various domains depicted. Cleavage sites for activation are identified with an arrow, and with the specific amino acid residues of the site shown. Key interchain disulfide bonds (-S-S-) are included. For the kininogens, horizontal arrows indicate the amino acid residues defining heavy and light chain regions of the activated forms of the cofactors. Factor XI is illustrated as a monomer. *EGF*, Epidermal growth factor; *FXI*, factor XI; *HMW*, high molecular weight; *LMW*, low molecular weight; *PK*, prekallikrein.

process (clot formation and fibrinolysis), digestive system, apoptotic cascades, and the immune system. To keep these systems in balance between activation and inhibition, a complex system of proteinase inhibitors has evolved. In blood, proteinase inhibitors constitute a significant percentage of circulating proteins. In general, proteinases that activate the coagulation and fibrinolytic cascades have highly defined substrate specificities. Coagulation is kept in check through the action of several specific and broad-spectrum proteinase inhibitors. Specific clot formation inhibitors are antithrombin, tissue factor pathway inhibitor (TFPI), heparin cofactor II, and protein C inhibitor. Together specific and broad-spectrum inhibitors function to localize, limit, and control hemostasis (see Chapter 121).

Antithrombin

Antithrombin is a member of the serpin proteinase inhibitory family and circulates in blood as a single-chain GP[137] (see Table 125.1; Fig. 125.7). Congenital antithrombin deficiency exhibits an autosomal dominant pattern of inheritance, with an incidence of 1 in 2000 to 1 in 5000.[138] Individuals with this deficiency have partial expression of antithrombin and are prone to venous thromboembolic disease.[139] The complete absence of antithrombin is lethal.

Antithrombin has a broad spectrum of inhibitory activity, with most of its target proteases participating in the coagulation cascade (see Fig. 125.1). It is primarily an inhibitor of the serine proteases thrombin, factor Xa, factor IXa, factor VIIa–tissue factor, factor XIa, factor XIIa, and kallikrein.[140–142] Heparin and heparan sulfate potentiate these reactions, and heparin is used for the prevention and treatment of thrombosis. When antithrombin is complexed with heparin, its rate of inhibition of several coagulation proteases is accelerated by up to 10,000-fold. The general mechanism of inhibition involves reaction of the active site of the enzyme with a peptide loop structure (the reactive center loop) of antithrombin, forming a tight, equimolar (1:1) complex. Inactivation proceeds through covalent bond formation between antithrombin and the protease followed by inactivating structural rearrangements of both antithrombin and the protease.

Antithrombin also displays antiproliferative and anti-inflammatory properties that primarily derive from its ability to inhibit thrombin. In addition, latent or cleaved forms of antithrombin have antiangiogenic activities.[143]

Tissue Factor Pathway Inhibitor

TFPI, formerly called *extrinsic pathway inhibitor* or *lipoprotein-associated coagulation inhibitor*, is a multivalent Kunitz-type plasma proteinase inhibitor. It circulates in plasma as a heterogeneous collection of partially proteolyzed forms (see Table 125.1 and Fig. 125.7).[144–147] Up to 90% of circulating TFPI is found associated with lipoproteins, primarily low-density lipoprotein.[144,148,149] Parenteral TFPI is cleared from the circulation mainly by the liver and has an unusually short half-life (minutes) compared with other proteinase inhibitors.

Many reviews of TFPI have been published.[150–160] The importance of TFPI in blood coagulation is best illustrated through transgenic mice with complete TFPI deficiency; the deficiency is embryonically lethal.[154] However, this lethality in mice can be rescued by heterozygous or homozygous factor VII deficiency.[161] This implies that diminishing the level of factor VII lessens the need for TFPI-mediated inhibition of the factor VIIa–tissue factor coagulation pathway during embryogenesis.[161] Similarly, the combination of low normal TFPI with factor V Leiden is lethal.[162,163] Mice with combined heterozygous TFPI deficiency and homozygous apolipoprotein E deficiency develop more extensive atherosclerosis burden,[164] raising the possibility that TFPI contributes to protection from atherosclerosis, as well as serving as a regulator of thrombosis.

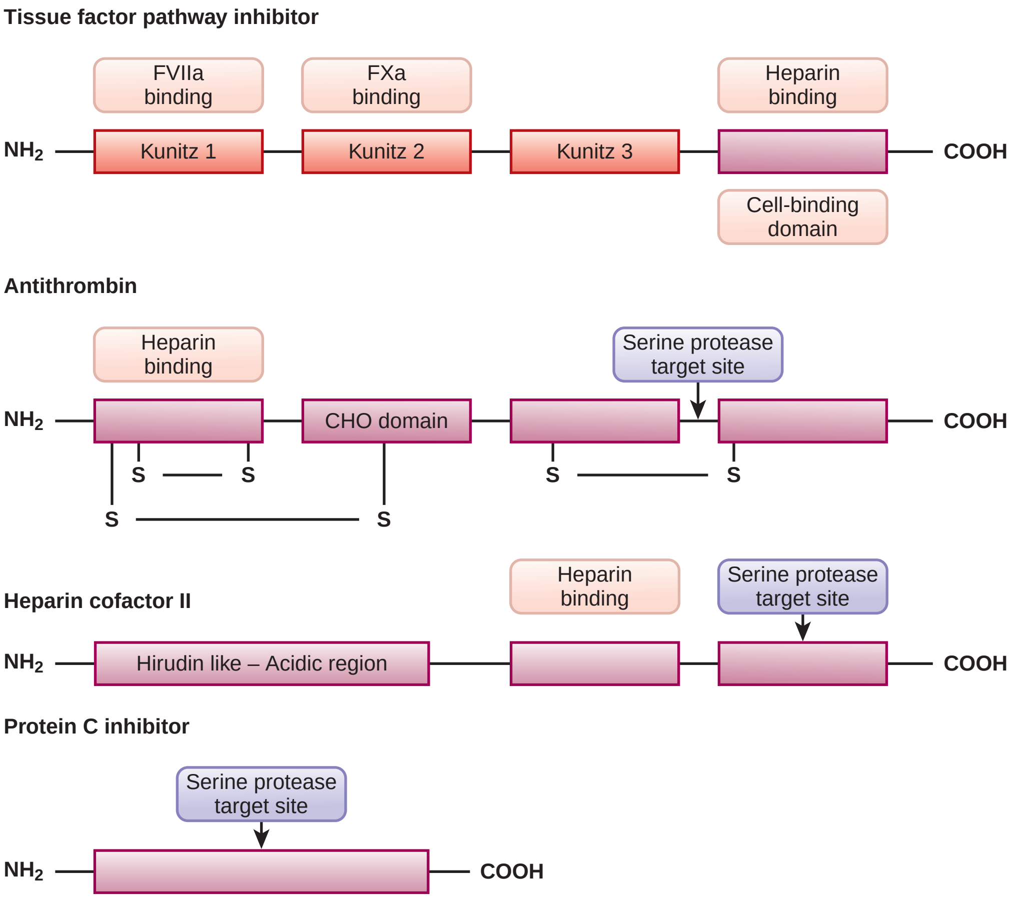


Figure 125.7 SCHEMATIC REPRESENTATION OF SEVERAL PROTEINASE INHIBITORS. Tissue factor pathway inhibitor (TFPI) contains three Kunitz domains. TFPI inhibits the serine proteases FVIIa and FXa, shutting down the extrinsic pathway of coagulation. Kunitz 1 domain binds FVIIa, and Kunitz 2 domain binds FXa. The COOH-terminus of TFPI contains a basic region, the cell-binding domain, which binds to heparin. Antithrombin (AT) contains two intrachain disulfide bonds (-S-S-) in its NH_2-terminus and one in its COOH-terminus with a carbohydrate-rich domain (CHO) in between. The region of interaction between the active sites of target proteases and AT is illustrated (reactive center loop). Heparin binding to AT occurs in the NH_2-terminus and enhances the rate of inhibition of serine proteases. Heparin cofactor II inhibits thrombin. Structurally, the inhibitor contains an NH_2-terminus hirudin-like region, a heparin or dermatan sulfate binding region, and a reactive center loop. The reactive site is at Leu^{444}. Protein C inhibitor is a serine protease inhibitor that inhibits several proteases, including activated protein C, thrombin, and FXa. It is also a potent inhibitor of the thrombin–thrombomodulin complex. The reactive bond (Arg^{354}) in the reactive center loop is shown. *FVIIa*, Factor VIIa; *FXa*, factor Xa.

TFPI is the principal stoichiometric inhibitor of the extrinsic factor tenase complex (factor VIIa–tissue factor).[165] Effective TFPI inhibition of the factor VIIa–tissue factor complex depends on the presence of factor Xa. Thus inhibition of the extrinsic factor tenase by TFPI occurs only after significant factor IXa and factor Xa formation. Inhibition by TFPI is achieved by formation of the stable quaternary, by tissue factor–factor VIIa–TFPI–factor Xa complex, and by formation of the factor Xa–TFPI complex directly.

Heparin Cofactor II

Heparin cofactor II is a member of the serpin family (see Fig. 125.7). The plasma concentration of heparin cofactor II is 0.5 to 1.4 μmol/L[166,167] (see Table 125.1). Its plasma half-life is approximately 2.5 days. Similar to antithrombin, heparin cofactor II inhibits thrombin in a reaction that is accelerated more than 1000-fold by heparin.[168] However, unlike antithrombin, the only coagulation enzyme inhibited by heparin cofactor II is thrombin.[169] The rate of thrombin inhibition by heparin cofactor II—in the absence or presence of heparin or heparin-like molecules—is significantly slower than by antithrombin under similar conditions. Considering that the plasma concentration of heparin cofactor II is 25% to 50% that of antithrombin and that low levels of heparin cofactor II are not strongly associated with thrombosis,[170] the physiologic role of heparin cofactor II as a systemic thrombin inhibitor has been questioned.

In vitro, heparin cofactor II inhibition of thrombin is accelerated by dermatan sulfate proteoglycans synthesized by fibroblasts and vascular smooth muscle cells.[171] Thus, heparin cofactor II may be uniquely suited to regulate extravascular thrombin in areas of vascular endothelium disruption in which heparin cofactor II would be exclusively stimulated by dermatan sulfate in the subendothelium. In addition, heparin cofactor II may participate in regulation of acute inflammation and wound healing by harboring a peptide chemotactic for neutrophils and monocytes that is released by leukocyte proteolysis.[172]

Heparin cofactor II may also have a role in protection from thrombosis during pregnancy. Increased levels of dermatan sulfate in the maternal and fetal circulation[173] along with increased levels of heparin cofactor II in pregnant women have been reported.[174,175] Low levels of thrombin–heparin cofactor II complexes are detected in normal plasma samples; elevated levels were detected in patients with disseminated intravascular coagulation.[176] Although inherited deficiency of heparin cofactor II has been associated with thrombosis, this is not always the case.[177–179]

Protein C Inhibitor

Protein C inhibitor is a member of the serine proteinase inhibitor family and is also known as *plasminogen activator inhibitor-3* (PAI-3). It circulates in blood at a concentration of 5 μg/mL[180,181] (see Table 125.1 and Fig. 125.7) and is cleared from the circulation with a half-life of 1 day. When in complex with a target (e.g., APC), it is cleared from circulation with a $t_{1/2}$ of 20 minutes.[182]

Protein C inhibitor is considered a nonspecific inhibitor in that its targets range from procoagulant (serine proteinases), anticoagulant, and fibrinolytic enzymes to plasma and tissue kallikreins, the sperm protease acrosin, and prostate-specific antigen.[183,184] The major target of protein C inhibitor, as its name suggests, appears to be APC.[182,185,186] Protein C inhibitor has been shown to regulate TAFI activation by inhibiting the thrombin–thrombomodulin complex.[187] Its importance as a dual regulator of coagulation and fibrinolysis remains unresolved.[188,189] Other targets for protein C inhibitor include human plasma kallikrein,[190] factor XIa,[190] factor Xa, and thrombin. Because there are no documented patients with a deficiency to date, the actual function of protein C inhibitor in vivo has yet to be elucidated.

α_2-Macroglobulin

α_2-Macroglobulin is a nonspecific proteinase inhibitor that targets a broad spectrum of protease substrates. It is present in human plasma at concentrations ranging from 2 to 4 μmol/L (2 to 3 mg/mL). α_2-Macroglobulin can also be found at higher concentrations in extravascular fluids.[191] This protease inhibitor can be produced in a variety of cells, including hepatocytes, fibroblasts, and macrophages.[192,193] Human α_2-macroglobulin circulates in plasma as a tetramer.[191–198] α_2-Macroglobulin has a unique mechanism of action, which accounts for its broad specificity. The initial step involves the "bait region" of α_2-macroglobulin.[199] After proteolysis in this bait region, α_2-macroglobulin undergoes conformational changes that trap the proteinase inside the molecule.[199] Consequently, α_2-macroglobulin inhibits a broad range of proteinases. It is distinctive in its capacity to inhibit members from each of four mechanistic classes of proteinases (serine, cysteine, and aspartic proteinases, and metalloproteinases). α_2-Macroglobulin functions as a secondary inhibitor of serine proteinases in plasma by inhibiting thrombin, kallikrein, and plasmin.[200,201] It may also be important in preventing thromboembolic events when there is a congenital deficiency of antithrombin or acquired deficiency in sepsis.[202,203] α_2-Macroglobulin also inhibits various growth factors and cytokines, including transforming growth factor-α (TGF-α),[204] interleukin (IL)-1β,[205] IL-6,[206] acidic fibroblast growth factor,[207] basic fibroblast growth factor,[207] tumor necrosis factor-α (TNF-α),[208] and IL-2.[209] Polymorphisms identified in α_2-macroglobulin have been thought to play a role in Alzheimer's disease.[210–212] Overall, the biologic role of α_2-macroglobulin in vivo is still being elucidated.

Reduced levels of α_2-macroglobulin in humans have been observed in individuals with chronic obstructive lung disease[213] and metastatic cancer.[214] Complete deficiency has not been reported, suggesting that absence of α_2-macroglobulin is incompatible with survival. Inactivation of the α_2-macroglobulin gene in mice has no obvious phenotype, but the mice are resistant to endotoxin challenge.[215] It has been suggested that α_2-macroglobulin serves as a neutralizer of TGF-α and an inducer of nitric oxide synthesis in mice.[216]

Endothelium

Blood cells and the vasculature are crucial to normal hemostasis. Multiple processes involving components of the vessel wall, circulating platelets, and plasma protein moieties interact to maintain blood fluidity (see Chapter 122). These must be precisely choreographed to allow the vasculature to perform its myriad complex physiologic activities (Fig. 125.8). The endothelium, the thin layer of cells that lines the interior of blood and lymphatic vessels, plays a key role because of its strategic interface among organs, tissues, and circulating blood. The cells that form the endothelium are called *endothelial cells*, those in direct contact with blood are called *vascular endothelial cells*, and those in direct contact with lymph are known as *lymphatic endothelial cells*. Vascular endothelial cells line the entire circulatory system (from the heart to the capillaries).

The endothelium varies in morphology and physiologic function in different parts of the vasculature (see Chapter 122). This complex cellular network not only provides a structural barrier to contain flowing blood but also regulates blood pressure, vascular tone, permeability, and processes involving other cells such as smooth muscle cells, leukocytes, and platelets, and deposits an intricate basement membrane and extracellular matrix.[217] In addition, the endothelium is involved in inflammatory and immune responses and angiogenesis. [218] Defects in vascular endothelium function, therefore, have profound physiologic implications. Excessive bleeding can result from structural abnormalities of the endothelial cell layer or supporting matrix. Impaired expression or secretion of PAI-1 by the endothelium likewise promotes bleeding through increased fibrinolytic activity.[219] Conversely, endothelial cells are also involved in mediating processes that promote atherosclerotic plaque formation and thrombotic pathologies.[220]

The early work of Ware and Seegers[221] identified the phospholipid requirements for coagulation. The biologic elements contributing to the phospholipid include damaged vascular tissue, activated platelets, and inflammatory cells. The contributions of the membrane to the formation and expression of procoagulant complexes are essential. However, the nature of the membranes that support procoagulant reactions is poorly understood. Mechanically damaged cells can provide the anionic membrane bilayer inner leaflet phospholipids, which can support general procoagulant complex formation; however, more subtle cellular activation events also generate selective complex-forming sites on intact cells. Activated platelet membranes express individual binding sites for the factor IXa–factor VIIIa and factor Xa–factor Va complexes. Hemorrhagic pathology is therefore associated with thrombocytopenia and is also displayed in a rare disease, Scott syndrome, which appears to result from the improper presentation of these platelet binding sites (see Chapter 124).[222] Binding sites have also been reported on several peripheral blood cells, especially activated monocytes. The vascular endothelium itself can provide binding sites after stimulation by cytokine growth factors.[223] The endothelium also provides the anticoagulant thrombomodulin, TFPI and heparan sulfate. EPCR provides cell-specific binding sites for both protein C and APC.[44,224] EPCR is downregulated by TNF-α.[44] Monocytes appear to express specific binding sites for APC that are distinct from the EPCR.[225] The cell-expressed binding sites may be important in the anti-inflammatory properties of APC.[46]

A further consequence of damage to the endothelium is the release of pathologic quantities of vWF, which promote platelet aggregation and adhesion to the subendothelium, and thus the formation of potentially fatal thrombi. Endothelial dysfunction is also linked with hypertension, diabetes, obesity, and hyperlipidemia.

Platelets

Platelets, or thrombocytes, are vital to procoagulant events and contribute to the fibrinolytic process as well. They are small, irregularly shaped, clear cell fragments, which are derived from megakaryocytes (see Chapter 123). The average lifespan of a platelet is approximately 5 to 9 days. Platelets are at the balance of bleeding or clotting events: when platelet numbers are low (thrombocytopenia), excessive bleeding can occur, and when platelet numbers are high (thrombocytosis), thrombosis can occur. Disorders that reduce the number of platelets but typically cause thrombosis instead of bleeding are heparin-induced thrombocytopenia and thrombotic thrombocytopenic purpura (see Chapters 131 and 132).

Like the endothelium, the undisturbed platelet presents a nonthrombogenic surface. Important components of platelet physiology are surface adhesion protein complexes and the platelet secretory granules: α-granules, lysosomes, and dense granules (see Chapter 124). Contents of the α-granules include procoagulant and adhesive proteins such as fibrinogen, fibronectin, thrombospondin, vWF, P-selectin, HMWK, platelet factor 4, osteonectin, factor V,[59] and factor XI.[226] Other α-granule contents, α_1-antitrypsin, protein S, TFPI, and platelet inhibitor of factor XI, are involved in anticoagulant activities.[227–229] The α-granule also contains proteins that meditate both pro- and antifibrinolytic processes. These proteins include plasminogen, α_2-antiplasmin, factor XIII, and PAI-1.[230–234] In the unstimulated platelet, the granule contents remain internalized and anionic phospholipid is sequestered in the inner leaflet of the plasma

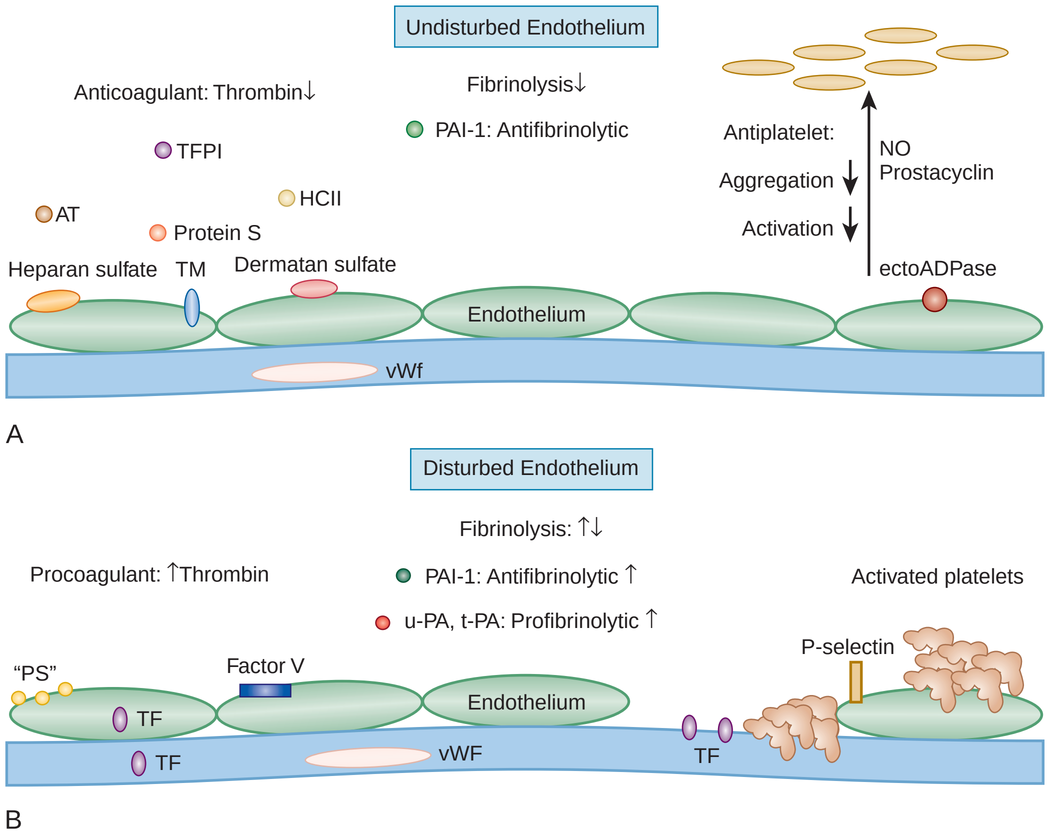


Figure 125.8 SCHEMATIC OF THE CHANGES THAT OCCUR TO THE ENDOTHELIUM UPON INJURY. Under normal conditions in the absence of injury or chemical stimulus (A), *the undisturbed endothelium* actively downregulates thrombin generation through the production of tissue factor pathway inhibitor (TFPI), antithrombin (AT), protein S, heparan sulfate, thrombomodulin (TM), and dermatan sulfate. The undisturbed endothelium is also antifibrinolytic and secretes plasminogen activator inhibitor-1 (PAI-1). In the absence of a stimulus, the endothelium likewise prevents platelet activation, secretion, and aggregation through the production of nitric oxide (NO), prostacyclin, and the membrane-associated protein ectoADPase. When the *endothelium is disturbed* (B), the endothelium becomes procoagulant and accelerates thrombin formation by exposing or expressing anionic phospholipid ("PS"), tissue factor (TF), and factor V. The fibrinolytic response is modulated by the release of both antifibrinolytic and profibrinolytic molecules. Urokinase plasminogen activator (u-PA) and tissue plasminogen activator (t-PA) are profibrinolytic and serve to activate plasminogen; PAI-1 inhibits both enzymes and is antifibrinolytic. Platelet activation, secretion, and aggregation are also promoted under conditions in which the endothelium is disrupted. von Willebrand factor (vWF) in the subendothelial matrix is exposed, allowing platelets to attach to the surface of the vessel. P-selectin likewise promotes platelet attachment.

membrane. Prostaglandin I2 (prostacyclin) and nitric oxide released from endothelial cells, the presence of CD39, and the inability of normal plasma vWF to bind spontaneously to the platelet surface are the inhibitory mechanisms that keep platelets unactivated.[235]

When the vascular system is perturbed, platelet plug formation occurs in stages. During the first stage, platelets adhere and are activated by exposure to collagen and vWF and other matrix components (Fig. 125.9). The cytoskeleton spreads and platelet–fibrinogen aggregates are formed, and the contents of the granules are secreted.[236–238] The activated platelets adhere to each other, endothelial cells, leukocytes, and components of the subendothelial matrix.[239] The phosphatidyl serine–rich internal face of cell membranes are exposed and present a highly procoagulant surface to the circulation.[240] In addition, activated platelets express specific receptors or binding sites for the assembly of the procoagulant multiprotein complexes (Fig. 125.10). There are approximately 3000 factor Va binding sites on the activated platelet membrane.[241] Factor Va forms part of the receptor for factor Xa. Factor Xa is also reported to bind to effector cell protease receptor-1 (EPR-1) molecules expressed on activated platelets.[241–244]

In the extension phase of platelet plug formation, where activated platelets accumulate on top of the initial monolayer of platelets bound to collagen, the presence of receptors on the platelet surface allows agonists such as thrombin, adenosine diphosphate, and thromboxane A_2 to recruit additional circulating platelets into the growing hemostatic plug (see Fig. 125.10). Subsequently, during the platelet plug formation perpetuation phase, close contacts between platelets promote the growth and stabilization of the hemostatic plug, in part through contact-dependent signaling mechanisms.[235]

Clot Proteins

A central event in blood coagulation is the conversion of soluble fibrinogen (factor I) to insoluble fibrin (see Fig. 125.1; see reviews listed in the References[245,246]). Fibrinogen functions in hemostasis to stem blood loss. It serves as a molecular bridge to support interplatelet aggregation, and it is the precursor of fibrin, which is the main component of the protein scaffolding of the forming hemostatic plug. Platelet aggregation critically depends on fibrinogen binding to activated platelets via the platelet fibrinogen receptor GPIIb/IIIa as well as fibrin adhesion (see Fig. 125.10). Fibrinogen/fibrin also regulates thrombin activity by interactions that include the proteolytic cleavage by thrombin of fibrinopeptides[247,248] to form a fibrin clot and thrombin exosite binding to fibrin, which potentially limits the diffusion of thrombin, thereby regulating clot propagation. The structure, stability, and duration of insoluble fibrin are controlled by an interplay between fibrin formation and fibrinolysis, which includes other molecular and cellular components.

The description of fibrinogen activation and fibrin assembly has been based on studies using citrated plasmas or purified proteins. The

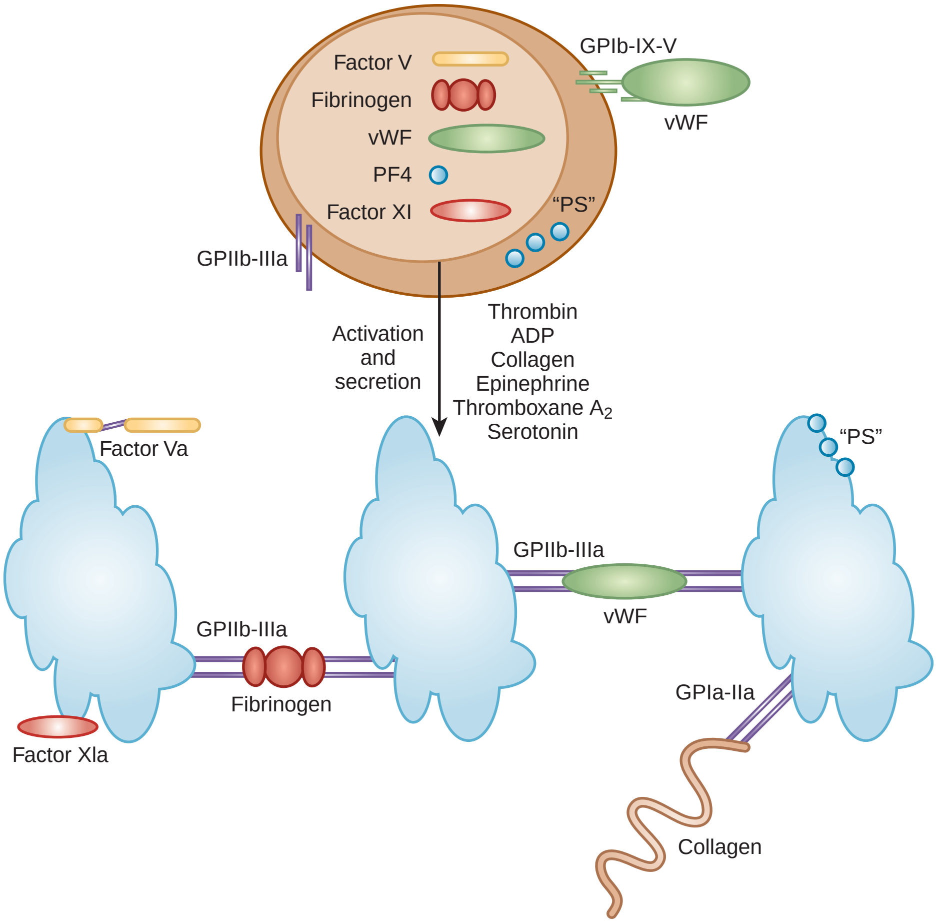


Figure 125.9 SCHEMATIC OF PLATELET ACTIVATION, SECRETION, AND AGGREGATION. Platelets have multiple functions in hemostasis. They serve as reservoirs of factor V, fibrinogen, von Willebrand factor (vWF), platelet factor 4 (PF4), and factor XI. Platelets also contribute a significant portion of the anionic phospholipid ("PS") necessary for membrane-dependent complex formation and function. In the unstimulated state, proteins and other molecules are sequestered in the platelet granules. Anionic phospholipid is found only in the inner leaflet of the platelet membrane and is not exposed to flowing blood. The GPIb–IX–V complex, which recognizes vWF, is an active receptor, but the GPIIb/IIIa receptor, which recognizes a variety of molecules, including fibrinogen and vWF, is not active. The GPIb–IX–V receptor likely allows unstimulated platelets to attach to exposed subendothelial vWF, thereby promoting procoagulant events before platelet activation. Upon activation by a variety of agonists, platelets secrete granule contents, become activated and bind factor V/Va and factor XI/XIa, and expose anionic phospholipid. The GPIIb/IIIa receptor serves to link platelets to each other and the vessel wall. Collagen receptors, such as GPIa–IIa, promote both platelet activation and aggregation. *ADP*, Adenosine diphosphate; *GP*, glycoprotein.

three main players in fibrinogen to fibrin conversion are the enzyme thrombin, the substrates fibrinogen, and the cross-linking transglutaminase factor XIII. Fibrinogen is composed of six polypeptide chains (two Aα, two Bβ, and two γ chains) that form two symmetrical half molecules (three chains each) with the NH_2-termini cross-linked to each other. The outside two domains of fibrinogen are composed of the Bβ and γ chains, and designated as the D domain. The central domain that contains the NH_2-termini of all the chains is designated as the E domain. From x-ray crystallographic data, fibrinogen has a trinodular structure aligned as D–E–D domains (Fig. 125.11).

The kinetics of fibrinogen cleavage by thrombin results in the hydrolysis of Arg–Gly bonds removing small, polar N-terminal fragments (fibrinopeptides [FPs]) from the NH_2-terminal of the Aα and Bβ chains Fig. 125.11.[249,250] Cleavage at the Arg–Gly bond of the Aα chain releases FPA and forms fibrin I. The release of two FPA peptides exposes a site in the E domain that interacts with a site in the D domain to form overlapping fibrils. Subsequent cleavage of the Arg–Gly bond on the Bβ chain releases FPB to form fibrin II, presumably increasing lateral aggregation of the protofibrils.[251]

An important enzyme for the structure and stability of the fibrin clot is transglutaminase factor XIIIa.[252–254] Its function is to cross-link fibrin and other adhesive proteins, including integrin receptors, providing a stable network. Only the Aα chain and the γ chain, which have donor (Gln) and acceptor (Lys) sites, participate in cross-linking of adjacent glutamyl and lysyl residues by factor XIIIa.[251,255–257]

Fibrinogen is also required for competent inflammatory reactions. Fibrinogen is an acute-phase reactant whose levels increase during inflammation.[258,259] In these situations, fibrinogen functions as a bridging molecule in cell-cell interactions.[260,261] Fibrin and fibrinogen constitute a matrix that modulates cellular responses in a variety of cell types, including endothelial cells, epithelial cells, leukocytes, platelets, and fibroblasts. Cellular receptors that bind fibrinogen and fibrin include the integrins $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, and $\alpha_5\beta_1$, and the cellular adhesion molecules intercellular adhesion molecule-1 and vascular endothelial cadherin.[262–265]

Fibrin clot-based assays have been extensively used in the clinical diagnosis of bleeding disorders (see Chapter 127). Two in vitro plasma tests, the PT[266,267] and the activated partial thromboplastin time (aPTT),[268] segregate the coagulation process into tissue factor–initiated or surface contact processes, respectively. The aPTT, which initiates coagulation by the introduction of a foreign surface, only examines the biologic constituents intrinsic to plasma. This assay is sensitive to isolated or combined deficiencies of factor XII, HMWK, prekallikrein, factor XI, factor VIII, factor IX, factor X, factor V, prothrombin, and fibrinogen. The PT assay is based on initiating coagulation via an extrinsic source of tissue factor (thromboplastin). The

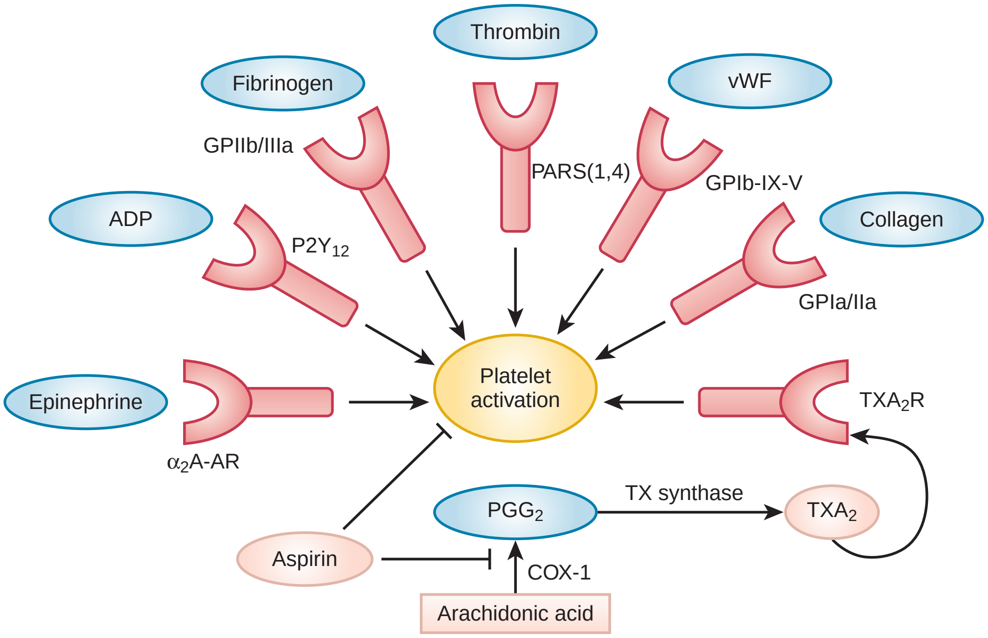

Thrombin
Fibrinogen
vWF
GPIIb/IIIa
PARS(1,4)
GPIb-IX-V
ADP
P2Y12
Collagen
GPIa/IIa
Epinephrine
Platelet activation
TXA2R
α2A-AR
TX synthase
PGG2
TXA2
Aspirin
COX-1
Arachidonic acid

Figure 125.10 SELECTED PLATELET RECEPTOR TARGETS AND THEIR RESPECTIVE INHIBITORY AGENTS. When a platelet encounters a break in the endothelium, it encounters molecules that trigger its activation, such as collagen, thromboxane A_2 (TXA_2), ADP, and thrombin. The ability of platelets to adhere, aggregate, respond to agonists, aid in coagulation, and bind fibrin are all processes mediated by the plasma membrane glycoproteins (GPs) of the platelet. The platelet GPIbIX–V constitutes the receptor for von Willebrand factor (vWF). GPIa/IIa mediates platelet–collagen interactions. GPIIb/IIIa is a receptor for fibrinogen. $P2Y_{12}$ is a chemoreceptor for ADP. The α_2A-adrenergic receptor (α_2A-AR) is expressed on platelets and binds the naturally occurring ligand epinephrine. Cyclooxygenase-1 (COX-1) is responsible for the formation of prostaglandins and thromboxane. COX-1 converts arachidonic acid to prostaglandin G_2 (PGG_2). Aspirin irreversibly inhibits COX-1. *TX*, Thromboxane; *TXA_2R*, thromboxane A_2 receptor.

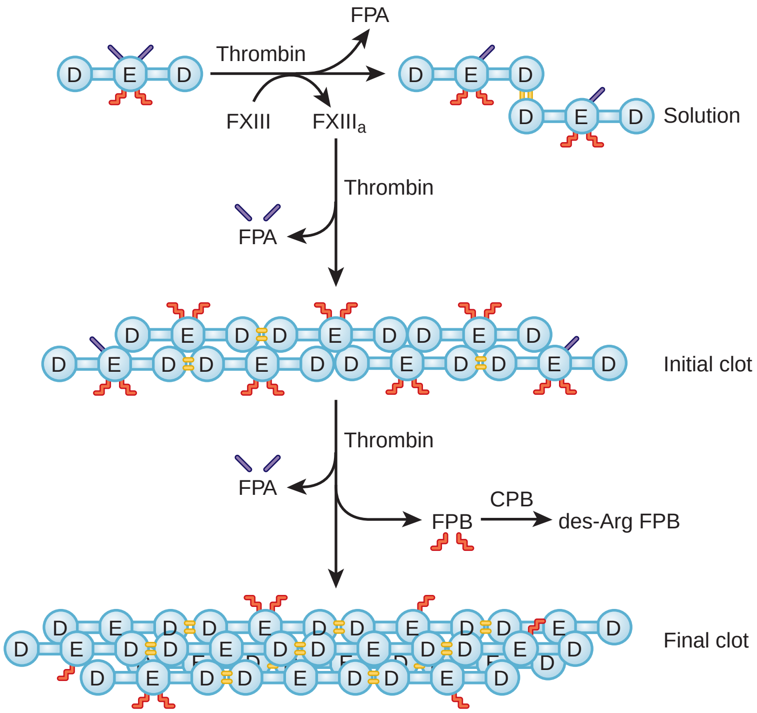

FPA
Thrombin
D E D
FXIII
FXIIIa
Solution
Thrombin
FPA
Initial clot
Thrombin
FPA
FPB
CPB
des-Arg FPB
Final clot

Figure 125.11 SCHEMATIC REPRESENTATION OF WHOLE-BLOOD FIBRIN FORMATION. At the start of clot formation, thrombin simultaneously acts on fibrinogen (D–E–D) and factor XIII (FXIII). A portion (≈40%) of fibrinopeptide A (FPA) is released from fibrinogen, and an initial clot is formed from the complementary overlap of the exposed sites between the E and D domains of adjacent fibrin molecules. Activated factor XIIIa (FXIIIa) simultaneously cross-links adjacent D domains (D=D). Thus, the initial soluble fibrin clot is composed of fibrinogen, fibrin, and γ–γ dimers with fibrinopeptide B (FPB) still attached. The initial clot is continuously acted on by thrombin, resulting in the release of the remaining FPA and some of the FPB to yield a final clot with the majority of FPB still attached. The released FPB is selectively acted on by a carboxypeptidase B–like enzyme (CPB; potentially activated thrombin-activatable fibrinolysis inhibitor a), which cleaves the C-terminal arginine to produce des-Arg FPB. The significance of this cleavage is still unclear. *(From Brummel KE, Butenas S, Mann KG. An integrated study of fibrinogen during blood coagulation. J Biol Chem. 1999;274:22862, with permission.)*

PT assay is sensitive to isolated or combined deficiencies of factor VII, factor X, factor V, prothrombin, and fibrinogen. Although these in vitro clotting assays help establish a basis for hemostasis, abnormal test results are not always mirrored by human pathology associated with bleeding or thrombosis.

Fibrinolysis Proteins

Clot formation is integrated with clot dissolution (fibrinolysis). Fibrinolysis, the elimination of blood clots, has two types, primary fibrinolysis, and secondary fibrinolysis. Whereas primary fibrinolysis is a normal body process, secondary fibrinolysis is the breakdown of clots caused by a medicine, a medical disorder, or some other cause.

The biochemical mechanisms of clot dissolution center on fibrin-specific activation of plasminogen to plasmin. The key proteins involved are plasminogen, the tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA), and the inhibitors (PAI-1, α_2-antiplasmin, and TAFIa). Plasminogen is the inactive precursor of the enzyme plasmin, which is the primary catalyst of fibrin degradation (Fig. 125.12).[269] The process of plasminogen activation can occur through three distinct pathways: (1) the intrinsic activator system (analogous to the contact system of blood coagulation), (2) the extrinsic activators (t-PA and u-PA), and (3) an exogenous activator system involving pharmacologic agents (fibrinolytic drugs). The primary pathway used in vivo appears to be the extrinsic pathway. However, both the intrinsic as well as exogenous activator systems can play important roles in human disease.

t-PA and u-PA, secreted by the endothelium, have unique structures and properties that affect the specificity and rate of plasmin generation (see Fig. 125.12).[270] After being generated, plasmin digests fibrin in a pattern that produces a collection of degradation products, including fragment X, fragment Y, and the core fragments, fragments D and E. The first step in degrading fibrin is the removal of the α chains, thus exposing the coiled coils. As these coils are cleaved, different-sized fragments are released.[271] Fibrinogen is represented as a trinodular structure (D–E–D domains) with each E domain and D domain separated by a coiled coil domain. Upon formation of fibrin,

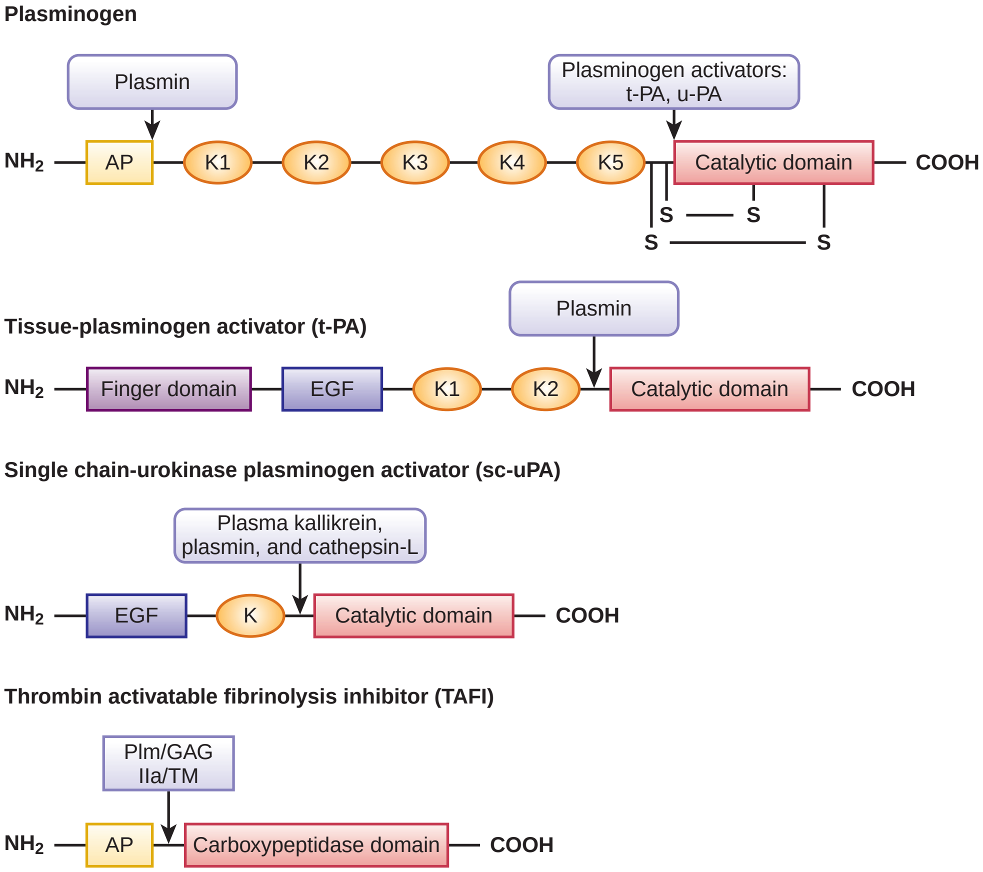

Plasminogen
Plasmin
Plasminogen activators: t-PA, u-PA
NH2
AP
K1
K2
K3
K4
K5
Catalytic domain
COOH
S
S
S
S
Tissue-plasminogen activator (t-PA)
Plasmin
NH2
Finger domain
EGF
K1
K2
Catalytic domain
COOH
Single chain-urokinase plasminogen activator (sc-uPA)
Plasma kallikrein, plasmin, and cathepsin-L
NH2
EGF
K
Catalytic domain
COOH
Thrombin activatable fibrinolysis inhibitor (TAFI)
Plm/GAG
IIa/TM
NH2
AP
Carboxypeptidase domain
COOH

Figure 125.12 SCHEMATIC OF FIBRINOLYTIC PROTEINS. Plasminogen is the inactive precursor of the enzyme plasmin, which is the primary catalyst of fibrin degradation. The domain structure of human plasminogen is represented by Kringle domains (K1–K5), a catalytic domain, and the arrows indicate the sites of proteolytic cleavage by plasmin and plasminogen activators (tissue plasminogen activator [t-PA] and urokinase plasminogen activator [u-PA]). Disulfide bonds are illustrated by -S-S-. t-PA is a serine proteinase and consists of an A and B chain. The A chain consists of a fibronectin finger-like domain, an EGF-like domain, and two kringle domains. The K2 domain and the finger domain of t-PA mediate the binding of t-PA to fibrin. The B-chain of t-PA contains the active site catalytic triad. Single-chain t-PA is an efficient plasminogen activator in the presence of fibrin and is converted to the two-chain form by cleavage of the peptide bond between R275 and I276. This cleavage is performed primarily by the action of plasmin during fibrinolysis. Single-chain u-PA (sc-uPA) is a serine protease and is an ineffective catalyst. Plasmin or plasma kallikrein can hydrolyze the K158–I159 peptide bond, converting sc-uPA into the fully active two-chain form (two-chain u-PA) composed of an epidermal growth factor (EGF) domain (EGF), a single kringle domain, a connecting peptide region, and the serine protease-type catalytic domain. Thrombin-activatable fibrinolysis inhibitor (TAFI): Activation of TAFI (TAFIa) yields an exopeptidase with carboxypeptidase B-like substrate specificity. TAFIa delays fibrinolysis by cleaving COOH-terminus Arg or Lys residues made available during partial plasmin digestion of the fibrin clot. Removing these residues attenuates the self-amplifying mechanism of fibrin-based plasmin formation wherein partial plasmin proteolysis of fibrin increases the number of binding sites (COOH-terminal lysine residues) available for efficient plasminogen activation. TAFI contains an activation peptide region and a carboxypeptidase domain. It is activated by the thrombin–thrombomodulin complexes (IIa/TM) and plasmin–glycosaminoglycan complexes (Plm/GAG) by hydrolysis of the R92–A93 bond.

cross-links occur between alternating molecules of fibrin at the D domain (D=D). Plasmin degrades fibrin, releasing various-sized fragments, the smallest of which is the D=D or D-dimer (M_r=180,000). The largest of these fragments is XXD, X=D–E–D, with a mass of 595,000.[272,273] Elevated levels of D-dimer are found in the blood of patients with various thrombotic and thrombolytic disorders (see Chapter 127).[274] These circulating fragments are cleared by other proteases or by the kidney and liver.

Inhibitors of the Fibrinolytic System

Plasminogen activation in blood is primarily inhibited by PAI-1, which targets u-PA and t-PA. PAI-1 also has a role in tissue remodeling by interfering with vitronectin-dependent processes of cell adhesion and migration.[275] Congenital deficiency of PAI-1 is rare, with homozygous individuals displaying abnormal bleeding in response to trauma.[276] Platelets contribute to the fibrinolytic process by binding t-PA and plasminogen and supporting plasmin generation. Increased levels of plasma PAI-1 delay fibrin removal by shortening the functional lifetime of plasminogen activators, thereby shifting hemostasis to a more thrombotic state.[277]

TAFI is a plasma zymogen with homology to procarboxypeptidases A and B (see Fig. 125.12).[55,278–280] Activation of TAFI yields the exopeptidase (TAFIa) with carboxypeptidase B–like substrate specificity. TAFIa catalyzes the removal of basic amino acids (arginine, lysine) from the COOH-termini of polypeptides. The activation of many of the cofactors and zymogens of the coagulation and fibrinolytic cascades results in the generation of functional proteins with COOH-terminus arginine or lysine residues. COOH-terminus lysine residues that appear in fibrin fragments degraded with plasmin have been identified as the major substrates for TAFIa. The physiologic activator of TAFIa is the thrombin–thrombomodulin complex, thus defining TAFIa as a coagulation-dependent activity.[281] Because TAFIa functions as an attenuator of fibrinolysis,[53] an adequate rate of TAFIa generation appears critical for the stabilization of the blood clot. Plasmas with specific deficiencies in the coagulation pathway exhibit reduced rates of thrombin production, decreased levels of TAFIa, and premature clot lysis.[257,282,283]

Fibrinolytic drugs are often given after a myocardial infarct or ischemic stroke to dissolve the fibrin clot blocking the coronary or cerebral artery (see Chapters 144, 145, and 146). Fibrinolytic drugs are also used in massive pulmonary embolism (see Chapter 140). Antifibrinolytics, such as aminocaproic acid (ε-aminocaproic acid) and tranexamic acid, are used as inhibitors of fibrinolysis.

CONNECTIVITY AND DYNAMICS IN HEMOSTASIS

In the healthy state, the hemostatic system is relatively quiescent, with the vascular endothelial cells, the blood, and the extravascular tissue functioning to maintain fluidity. Blood platelets remain in a quiescent state because of the endothelial cell lining of the blood vessel being an active anticoagulant that secretes small molecules and enzymes. The endothelium also provides constituent anticoagulant proteins, which inhibit the blood coagulation system. These vascular anticoagulant systems are both passive and dynamic in nature, and function in cooperation with plasma components. The blood supplies pro- and anticoagulant proteins in the plasma and platelets, which contribute to the coagulation reaction. If the endothelium becomes damaged, the pro- and anticoagulant levels become imbalanced, and cells that should remain in the blood can leak through blood vessels into adjacent body tissue, which triggers a response. The dimensions of the response are relevant to the injury. The extravascular compartment and blood interact to rapidly produce a vigorous local coagulation response, which attenuates blood loss and initiates the vascular repair process in four phases: initiation, propagation, termination, and elimination/fibrinolysis.[284–286]

Initiation

If vascular injury occurs, a measured response is triggered in that the extent of damage regulates platelet and fibrin deposition. Activated platelets provide the membrane surfaces upon which coagulation enzymes can be anchored, assembled, and expressed. Therefore, the activated platelet membrane provides both an initiating and limiting component to the extent of a coagulation reaction.[287] More vascular damage produces more anchored activated platelets, and more membrane allows the assembly of more coagulation enzymes, which ultimately results in increased fibrin formation.

When the vascular system is perturbed, the initial stages of the hemostatic response are triggered. The initial principal player is the extrinsic tenase complex (tissue factor–factor VIIa), which is composed of a cell membrane; tissue factor exposed by vascular damage or cytokine stimulation; Ca^{2+}; and the serine protease plasma factor VIIa, which is already present in its active form at 1% to 2% of the factor VII zymogen concentration (Fig. 125.13).[53,288] Before binding to tissue factor, the plasma serine protease factor VIIa is essentially inert from the catalytic perspective and thus impervious to the abundant protease inhibitors in plasma.[289] Factor VII also competes with factor VIIa for tissue factor binding, thus serving as a negative regulator that buffers the overall reaction.[290,291] Factor VII–activating protease (FSAP) has also been shown to activate factor VII in the absence of tissue factor.[292–294] The physiologic function of FSAP still is unclear, but most recently has been suggested to be involved in inflammation (see the review listed in the References[295]). The extrinsic tenase complex (tissue factor–factor VIIa) activates low levels of the zymogens factor X and factor IX to their respective serine protease enzymes factor Xa (≈10 pM) and factor IXa (≈1 pM).[296,297] Factor X is the more efficient and abundant substrate.[298,299]

The extrinsic tenase complex is under tight supervision by TFPI, which can bind both the complex and the product factor Xa (see Fig. 125.13).[300,301] TFPI, present in low abundance in blood, is released from the vasculature by heparin.[302] If the initiating procoagulant stimulus is sufficient to overcome the level of this anticoagulant response, a threshold is exceeded and downstream complexes can be formed.

The limited amounts of factor Xa that escape inhibition by TFPI and antithrombin bind to available membrane sites and can activate tiny amounts of prothrombin to thrombin (see Fig. 125.13).[303] The time period in which factor Xa directly generates picomolar amounts of thrombin[304] is referred to as the *initiation phase* of blood coagulation (Fig. 125.13). During the initiation phase, circulating blood cells and procoagulant proteins are activated,[305] the procoagulant elements necessary for the full procoagulant response are generated, and a preliminary fibrin network is formed.[306] Although this process is inefficient, this initial thrombin is essential for the acceleration of the process by serving as the activator of platelets through cleavage of protease–activated receptors (PAR1 and PAR4)[307] and the activation of the procofactors factor V and factor VIII.[129] Thrombin also activates factor XI to factor XIa,[130] initiating the accessory pathway that enhances factor IX activation.[308]

In analyses of tissue factor–induced activation of the coagulation process in whole blood, the initial period of thrombin generation (based on levels of thrombin–antithrombin complexes) illustrates that most catalyst formation occurs before fibrin clot formation (see Fig. 125.13).[305] The small amount of thrombin that is generated during the initiation phase is from the extrinsic factor tenase and is able to activate platelets,[309] factor XIII,[310] factor V,[311] and factor VIII,[312] and release some fibrinopeptide A and fibrinopeptide B[263] from fibrinogen to form fibrin. Less than 2% of the final thrombin produced is required to achieve the activation of these catalysts produced in blood to form the initial clot. However, activation of the catalysts is essential to generate the bulk of thrombin (≈95%) that is formed during the propagation phase of the reaction. The aggregated platelets and fibrin resulting from thrombin formation are the principal components of the initial vascular plug formation. It has recently been shown that a fraction of red blood

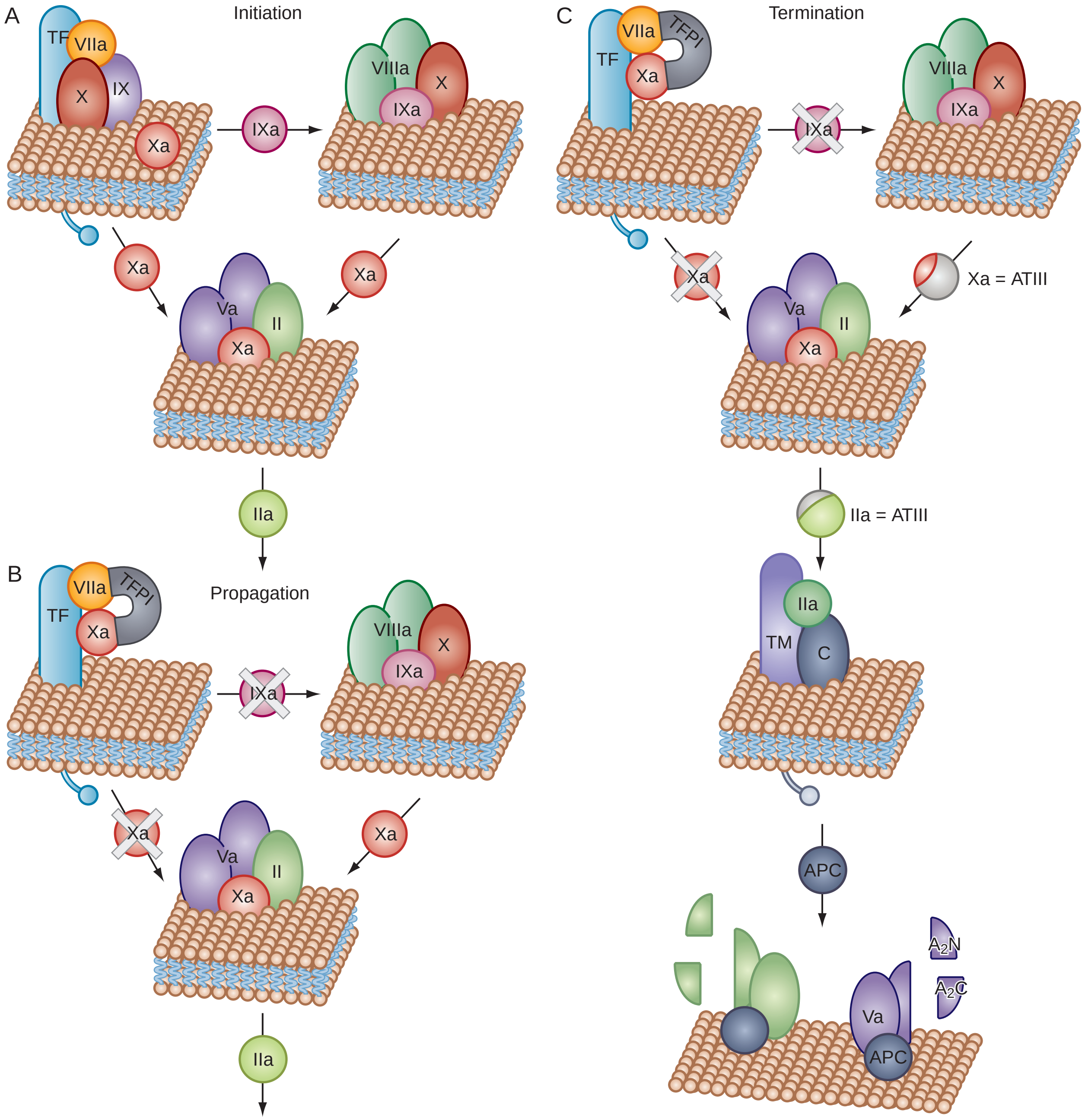


Figure 125.13 REGULATION OF THE DYNAMIC PROCOAGULANT RESPONSE. Initiation (A), propagation (B), and termination (C) of thrombin generation and the procoagulant response are illustrated. Four vitamin K–dependent complexes are shown: extrinsic tenase, intrinsic tenase, prothrombinase, and protein Case (C). The procoagulant response is regulated by the stoichiometric inhibitors antithrombin (AT) and TFPI. AT inhibits thrombin, factor Xa, and factor IXa that are free in solution. Tissue factor pathway inhibitor (TFPI) inhibits both factor Xa and the factor VIIa–TF–factor Xa complex. Activated protein C (APC) generated from the protein Case complex (thrombomodulin [TM]–thrombin [IIa]) inactivates factor Va and factor VIIIa by proteolysis of their heavy chains. Low levels of thrombin are required to initiate clot formation (initiation phase) and trigger the coagulation cascade response (propagation phase). The enzymes, cofactors, and inhibitors act together to generate a hemostatic response that can be divided into an initiation phase and a propagation and termination phase. During the initiation phase, factors X and IX are converted to their respective serine proteases factor Xa and factor IXa; low levels of thrombin are subsequently generated by factor Xa. This thrombin then can activate platelets and the procofactors factors V and VIII, which stimulate further thrombin generation during the propagation phase. Thrombin generation is attenuated by shutting down the initiation phase by means of the stoichiometric inhibitor of the extrinsic tenase complex, TFPI, followed by AT, which directly inhibits thrombin and factors Xa and IXa. *TF*, Tissue factor. *(From Mann KG. Coagulation Explosion. Burlington, VT: Vermont Business Graphics; 1997, with permission.)*

cells (≤1%) provide phosphatidyl serine–associated membrane receptors that can support the procoagulant complexes. These red blood cells are responsible for approximately 30% of all thrombin formation.[313]

After cofactor factor VIIIa is formed, it combines on activated platelets with the serine protease factor IXa that was generated by the tissue factor–factor VIIa complex to form the intrinsic tenase complex (see Fig. 125.13). This complex is the major activator of factor X; it is 50-fold more efficient than factor VIIa–tissue factor in catalyzing factor X activation.[299,314] The extrinsic tenase complex is under the control of TFPI.[315,316] In the absence of factor VIII (hemophilia A) or factor IX (hemophilia B), the intrinsic tenase complex cannot be assembled; thus no amplification of factor Xa generation occurs. This is the principal defect observed in hemophilia[317,318]:

initial production of factor Xa by the tissue factor–factor VIIa complex is inadequate to efficiently stem blood flow.

Factor Xa combines with factor Va on the activated platelet membrane surfaces at specific receptor sites to form the prothrombinase complex; the principal generator of thrombin (see Fig. 125.13).[319,320] This process serves as a major amplification loop of blood coagulation. The factor IXa and factor Xa constituent of prothrombinase and the intrinsic tenase complex are protected from inhibition by antithrombin and other plasma inhibitors when in the complexed form.

Propagation

When a sufficient stimulus is provided to overcome the antagonist–inhibitor threshold, the accumulating mass of activated platelets will support increasing intrinsic tenase and prothrombinase complex formation on their surfaces through specific platelet receptors, and the local inhibitor concentrations are overwhelmed (see Fig. 125.13). These platelet-bound catalysts execute the propagation phase of the reaction, during which massive amounts of thrombin are produced.[321] This phase of thrombin generation continues independent of the initially presented tissue factor if there is a continuous supply of blood to deliver new plasma procoagulant reactants, platelets, and fibrinogen to the site of perforation in the vascular endothelium.

Essential to the formation of the prothrombinase complex is the generation of factor Xa. Factor Xa is a unique regulatory enzyme in that it is formed through both the intrinsic tenase and the extrinsic tenase complexes. Under normal conditions, the concentration of factor Xa is the rate-limiting component of the prothrombinase complex. The other components of the complex, platelets (membrane surface–binding sites), and the cofactor (factor Va) are activated rapidly to produce a surplus that is ready for action.[319] The coagulation mechanism can become sensitive to factor V or platelets when confronted with congenital deficiencies, thrombocytopenia, platelet pathology, or pharmacologic interventions.[322,323]

The initial factor Xa is generated via the tissue factor–factor VIIa complex during the initiation phase. Additional factor Xa is

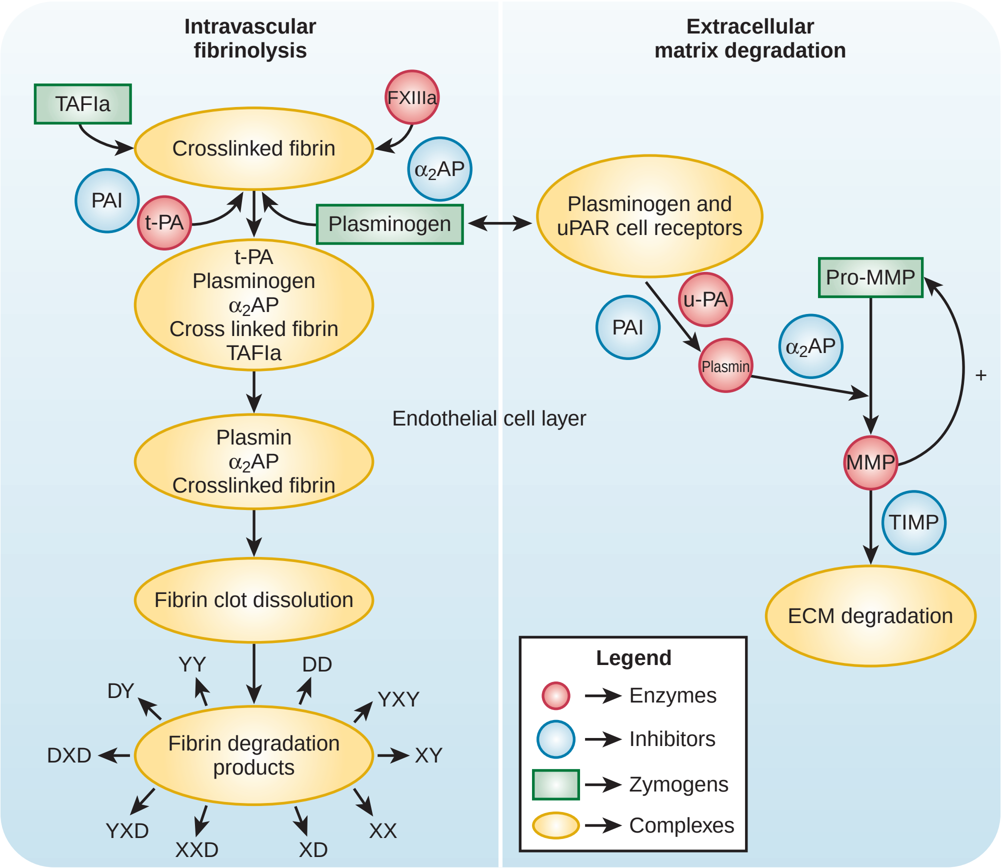


Figure 125.14 SCHEMATIC OF THE DYNAMIC INTERACTION BETWEEN THE PROTEINS AND INHIBITORS OF FIBRINOLYSIS. Cross-linked fibrin formation is integrated with fibrin clot dissolution and degradation of its products. Two pathways are shown, intravascular fibrinolysis and extracellular matrix, separated by an endothelial cell layer. The enzymes *(red circles)*, inhibitors *(blue circles)*, zymogens *(green boxes)*, and complexes *(large yellow ovals)* are illustrated in a simplified form to show this multi-component process. The key proteins of the fibrinolytic system *(left panel)* are plasminogen, the plasminogen activator t-PA, PAIs, α2-AP and TAFI, and the transglutaminase FXIIIa. t-PA and plasminogen both bind to the fibrin surface, cross-linked by FXIIIa, where t-PA is an effective catalyst of plasminogen conversion to plasmin. Initially, plasmin proteolysis of fibrin generates new COOH-terminus lysine residues, which function as higher affinity binding sites for plasminogen, setting up an amplifying loop of plasminogen activation. The formation of activated TAFI (TAFIa) results in the removal of the plasmin-generated COOH-terminus lysine residues, thus suppressing the rate of fibrin lysis. Opposing these events are antifibrinolytic mechanisms. Soluble and cross-linked α2-AP complexes with plasmin, rendering it inactive. PAI rapidly reacts with t-PA, thus reducing the concentration of the plasminogen activator. Fibrin degradation occurs by plasmin cleavage at the D-E-D domains of fibrin polymers to yield a variety of polymers as illustrated (for a definition of the D-E-D domain, see Fig. 125.11). Plasminogen can cross the endothelial cell layer and become converted to plasmin by u-PA *(right panel)*. Plasmin can convert latent MMPs (pro-MMPs) to their active form (MMPs). MMPs themselves can act in a positive-feedback mechanism to convert pro-MMPs to more MMPs, which ultimately degrade the extracellular matrix. Plasmin-mediated effects are inhibited by PAI and α2-AP. MMP-mediated effects are inhibited by TIMPs. *α2-AP*, α2-Antiplasmin; *ECM*, extracellular matrix; *FXIIIa*, activated factor XIII; *MMP*, matrix metalloproteinase; *PAI*, plasminogen activator inhibitor; *t-PA*, tissue plasminogen activator; *TAFI*, thrombin-activatable fibrinolysis inhibitor; *TIMP*, tissue inhibitors of metalloproteinases; *u-PA*, urokinase plasminogen activator.

then generated by the intrinsic tenase complex (factor IXa–factor VIIIa–membrane–Ca^{2+}). Initially, the concentration of the factor VIIa–tissue factor complex is higher than the concentration of the factor VIIIa–factor IXa complex, which requires activation and assembly. As time progresses, the contribution of the intrinsic tenase complex to factor Xa generation exceeds that of the extrinsic tenase.[324] The intrinsic tenase complex is kinetically more efficient and activates factor X at a 50- to 100-fold higher rate than the extrinsic tenase complex.[299,325,326] The burst of factor Xa that is generated overcomes the levels of factor Xa inhibitors, such as TFPI, and achieves maximal prothrombinase activity and propagation of the procoagulant response.[162,315] The bulk of thrombin (≈ 95%) is formed during the propagation phase after fibrin clot formation.[305,327] Without the intrinsic tenase complex being formed, as occurs in hemophilia A or B, factor Xa is not generated in levels sufficient to produce the propagation phase of thrombin generation.[317,328]

Because the presentation of a clot in a low tissue factor model depends on the generation of 10 to 30 nM thrombin,[305,327] at high tissue factor concentrations, tissue factor–factor VIIa generates factor Xa rapidly and masks the contribution of the factor VIIIa–factor IXa complex in clot end point assays. This is the case for the PT[266,267] in which the concentration of the initiator, thromboplastin (tissue factor and phospholipid), is chosen to produce a clot time of 11 to 15 seconds. This corresponds to a tissue factor concentration over 20 nM. In our whole-blood studies, a concentration of 5 pM tissue factor is used, which produces a clot time in the range of 5 minutes.[305,327] Therefore in hemophilia A, the PT does not reflect a change in clot time in this well-established hemorrhagic disease. The major defect occurs after clot time, during the propagation phase of thrombin generation, which is dramatically decreased.[317,321,329]

Termination

When blood flow has ceased because of the formation of a fibrin–platelet "dam," the overwhelming concentration of inhibitors present in blood, including TFPI and antithrombin, heparin cofactor II, α_2-macroglobulin, α_1-antitrypsin, and protein C inhibitor, can "catch up" and inhibit the various reactants as they dissociate from their respective complexes (see Fig. 125.13).[150,315,330–332]

In the intact vasculature surrounding the growing thrombus, procoagulant enzymes and cofactors escaping the wound site are rapidly quenched under normal circumstances by the stoichiometric and dynamic inhibitory systems of blood in cooperation with elements of the vascular endothelium. The free serine proteases (thrombin, factor IXa, and factor Xa) of the coagulation system in the plasma environment are rapidly inhibited by the surplus of antithrombin molecules. The reaction is accelerated by the interaction of antithrombin with heparan sulfate proteoglycans presented constitutively on the surface of vascular endothelial cells.[333]

Any thrombin escaping from the wound site may bind resident thrombomodulin molecules constitutively expressed by vascular endothelial cells. Thrombomodulin-bound thrombin is converted from a procoagulant enzyme to an anticoagulant enzyme.[334,335] The thrombin–thrombomodulin complex (protein Case) activates protein C, which in turn downregulates the intrinsic factor tenase and prothrombinase procoagulants by cleaving factor VIIIa and factor Va, respectively. The rates of APC inactivation of factors Va and VIIIa are enhanced by protein S. TAFI is also activated by protein Case and serves to delay clot lysis (see reviews listed in the References[53,336]). Cleavage of factor Va by APC and inhibition of thrombin generation also reduces thrombin–thrombomodulin–mediated TAFI activation.[337]

When operating properly, this system of blood leakage attenuation displays the appropriate level of procoagulant required to obstruct blood loss but is precluded from systemic activation of the coagulation system. The converse to hemostasis occurs when the damaging insult for the vasculature is internal to the vessel lumen.[338]

Elimination and Fibrinolysis

The hemostatic or pathologic thrombus is structurally composed of aggregated platelets and cross-linked fibrin. The steps in thrombin generation of a cross-linked fibrin clot are shown diagrammatically in Fig. 125.11. Other plasma proteins and blood cells are also trapped within the clot. Clot formation is integrated with clot dissolution by plasmin to maintain hemostatic balance. The plasminogen system has two roles: t-PA generates plasmin at the fibrin surface and governs fibrin homeostasis, and u-PA binds to a cellular u-PA receptor (u-PAR) and generates pericellular plasmin, which plays an important role in tissue remodeling and cellular migration (see Fig. 125.14).[339,340] The latter function is, to a great extent, mediated by plasmin activation of matrix metalloproteinases, which degrade ECM. t-PA and u-PA are secreted by vascular endothelial cells[341,342] and are regulated by cellular cytokines and components produced during the clotting cascade, including thrombin.

In the absence of fibrin, t-PA is a poor enzyme.[343–345] However, both t-PA and plasminogen bind to the fibrin surface with a resulting 100-fold enhancement in plasminogen activation. Thus t-PA activation of fibrinolysis is primarily initiated by and localized to fibrin.[343,345] The digestion of fibrin by plasmin[346] is seen in Fig. 125.14 and further described under the section Fibrinolysis Proteins.[274,346–349]

Fibrinolysis is regulated primarily by PAI-1,[350,351] PAI-2,[352] α_2-antiplasmin, and TAFI.[353] The antagonism between PAI-1 and the plasminogen activators u-PA and t-PA provides a threshold response of the fibrinolytic process in much the same way as the procoagulant–anticoagulant balance provides an activation threshold for the clotting process.[354–357] α_2-Antiplasmin is the primary inhibitor of plasmin.[358–360] Plasmin, when bound through its lysine binding sites to fibrin, reacts more slowly with α_2-antiplasmin than when free in solution because α_2-antiplasmin interacts with plasma plasmin by binding to the lysine binding sites.[358–362] In contrast, α_2-antiplasmin, bound covalently by factor XIIIa to the Aα chain of fibrin, blocks plasmin binding and appears to decrease plasminogen activation.[363] TAFIa functions in vitro as an antifibrinolytic factor by suppressing the positive-feedback pathway of fibrinolysis.[255,256,337]

The elimination phase begins the process of tissue repair by dissolving the fibrin–platelet clot generated in the earlier phases of hemostasis. The damaged vascular tissue not only requires plasmin to clear the fibrin clot, but also to initiate removal of damaged tissue to allow for cell migration into the injured area.[364,365] Plasmin activates a variety of matrix metalloproteinases that degrade subendothelial matrix components and extricate the damaged tissue.[365,366] These processes mark the beginnings of the final stages of the hemostatic response, repair, and regeneration.

The importance of the tight regulation of these processes is perhaps best illustrated by malfunctions of the hemostatic response. An inappropriate response can lead to one of two opposing but equally undesirable outcomes. Failure to form a sufficient hemostatic plug to arrest blood flow subsequent to vascular injury can result in pathologic hemorrhage. Excessive clot formation or failure to efficiently lyse a clot may result in thrombosis with consequent vascular obstruction. Under normal circumstances, the vascular endothelium together with the aforementioned positive- and negative-feedback loops within the procoagulant pathways prevent these negative outcomes by actively controlling the coagulation process until a triggering stimulus of sufficient magnitude threatens vascular integrity (Fig. 125.15). Initiation of the procoagulant response also initiates the fibrinolytic response simultaneously with repair and regeneration processes.

SUMMARY

This chapter describes the process of blood coagulation (extrinsic and intrinsic pathway) by dividing it into sections based on procoagulant, anticoagulant, and fibrinolytic enzymes, cofactors, and inhibitors in the overall process of fibrin formation and fibrin dissolution.

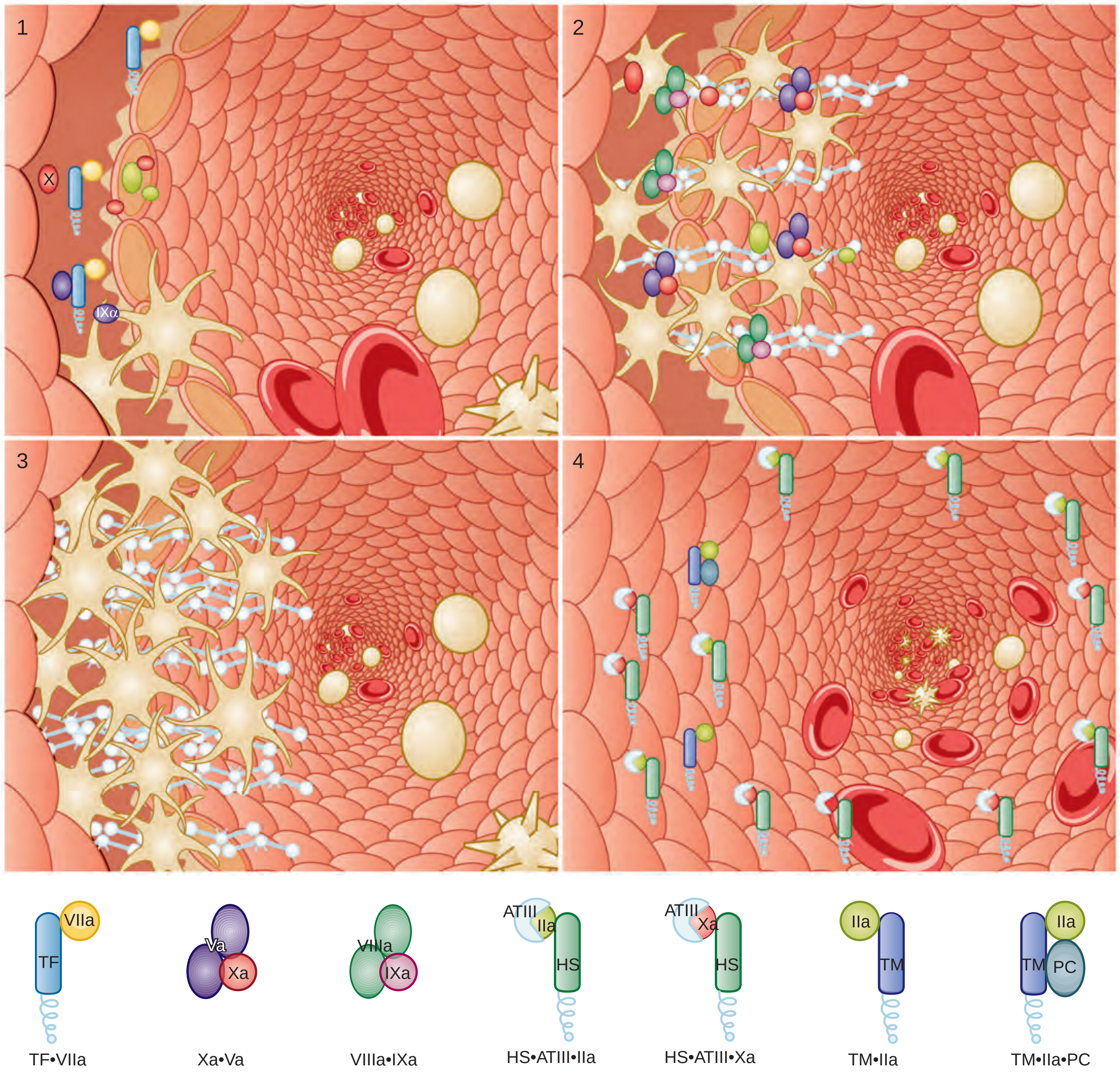


Figure 125.15 SCHEMATIC OF THE DYNAMIC EVENTS DURING TISSUE FACTOR–INITIATED BLOOD COAGULATION. A cross-section of a blood vessel showing the luminal space, endothelial cell layer, and extravascular region is presented at the site of perforation. The blood coagulation process in response is depicted in four stages. *Extrinsic tenase* complex, TF•VIIa; *prothrombinase* complex, Xa•Va; *intrinsic tenase*, VIIIa•IXa; antithrombin (AT)–endothelial cell heparan sulfate (HS) proteoglycan complex bound to thrombin or FXa, HS•AT•(IIa or Xa); protein C bound to thrombomodulin (TM)–thrombin, TM•IIa•PC. *Stage 1*. Perforation results in the delivery of blood, and with it circulating factor VIIa and platelets, to an extravascular space rich in membrane-bound tissue factor (TF). Platelets adhere to collagen and von Willebrand factor associated with the extravascular tissue, and TF binds factor VIIa, initiating the process of factor IX and factor X activation. Factor Xa activates small amounts of prothrombin to thrombin, which activates more platelets and converts factor V and factor VIII to factor Va and factor VIIIa. *Stage 2*. The reaction is propagated by platelet-bound intrinsic tenase and prothrombinase, with the former being the principal factor Xa generator. Initial clotting occurs and fibrin begins to fill in the void in cooperation with activated platelets. *Stage 3*. A barrier composed of activated platelets ladened with procoagulant complexes and enmeshed in fibrin scaffolding is formed. The reaction in the now-filled perforation is terminated by reagent consumption, attenuating further thrombin generation, but functional procoagulant enzyme complexes persist because they are protected from the dynamic inhibitory processes found on the intravascular face. *Stage 4*. View downstream of the perforation. Enzymes escaping from the plugged perforation are captured by antithrombin–heparan complexes, and the protein C (PC) system is activated by residual thrombin binding to endothelial cell TM, initiating the dynamic anticoagulant system. These intravascular processes work against occlusion of the vessel despite the continuous resupply of reactants across the intravascular face of the thrombus. *(From Orfeo T, Butenas S, Brummel-Ziedins KE, et al. The tissue factor requirement in blood coagulation. J Biol Chem. 2005;280:42887, with permission.)*

When all of the players are present, the overall process of blood coagulation and fibrinolysis is best described as a dynamic threshold-limited, complex, intertwined process that together promotes hemostasis.

ACKNOWLEDGMENTS

We the authors would like to thank Matthew Gissel in the preparation of this chapter. The authors were supported by the NIH-DOD TACTIC study 1-UM-1-HL120877-2, the Systems Biology program ARO-W911NF-10-1-0376, and by the United States Naval Health Research Center contract W911QY-15-C-0027.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Claesen K, Mertens JC, Leenaerts D, et al. Carboxypeptidase u (CPU, TAFIa, CPB2) in thromboembolic disease: What do we know three decades after its discovery? *Int J Mol Sci*. 2021;22:883.

Doolittle RF. Some important milestones in the field of blood clotting. *J Innate Immun*. 2016;8:23–29.

Grover SP, Mackman N. Tissue factor in atherosclerosis and atherothrombosis. *Atherosclerosis*. 2020;307:80–86.

Ito T, Thachil J, Asakura H, et al. Thrombomodulin in disseminated intravascular coagulation and other critical conditions—a multi-faceted anticoagulant protein with therapeutic potential. *Crit Care*. 2019;23:280.

Jaffer IH, Weitz JI. The blood compatibility challenge. Part 1: blood-contacting medical devices: the scope of the problem. *Acta Biomater*. 2019;94:2–10.

Kiouptsi K, Reinhardt C. Physiological roles of the von Willebrand factor-factor VIII interaction. *Subcell Biochem*. 2020;94:437–464.

Medcalf RL, Keragala CB. Fibrinolysis: a primordial system linked to the immune response. *Int J Mol Sci*. 2021;22

Mehic D, Colling M, Pabinger I, et al. Natural anticoagulants: a missing link in mild to moderate bleeding tendencies. *Haemophilia*. 2021;27:701–709.

Morrissey JH, Tajkhorshid E, Rienstra CM. Nanoscale studies of protein-membrane interactions in blood clotting. *J Thromb Haemost*. 2011;9(Suppl 1): 162–167.

Neubauer K, Zieger B. Endothelial cells and coagulation. *Cell Tissue Res*. 2022;387:391–398.

Preston RJS, O'Sullivan JM, O'Donnell JS. Advances in understanding the molecular mechanisms of venous thrombosis. *Br J Haematol*. 2019;186: 13–23.

Schmaier AH. The contact activation and kallikrein/kinin systems: pathophysiologic and physiologic activities. *J Thromb Haemost*. 2016;14: 28–39.

Schreuder M, Reitsma PH, Bos MHA. Blood coagulation factor Va's key interactive residues and regions for prothrombinase assembly and prothrombin binding. *J Thromb Haemost*. 2019;17:1229–1239.

Srivastava P, Gailani D. The rebirth of the contact pathway: a new therapeutic target. *Curr Opin Hematol*. 2020;27:311–319.

Versteeg HH, Heemskerk JW, Levi M, et al. New fundamentals in hemostasis. *Physiol Rev*. 2013;93:327–358.

Wolberg AS. Fibrinogen and factor XIII: newly recognized roles in venous thrombus formation and composition. *Curr Opin Hematol*. 2018;25: 358–364.

Wolberg AS, Aleman MM, Leiderman K, et al. Procoagulant activity in hemostasis and thrombosis: Virchow's triad revisited. *Anesth Analg*. 2012;114:275–285.

REFERENCES

1. Brinkhous KM. A short history of hemophilia, with some comments on the word "hemophilia.". In: Brinkhous KM, Hemker HC, eds. *Handbook of Hemophilia*. New York: American Elsevier; 1975.
2. Ratnoff OD. The evolution of knowledge about hemostasis. In: Ratnoff OD, Forbes CD, eds. *Disorders of Hemostasis*. Philadelphia: W.B. Saunders Company; 1996:1.
3. Owen Jr CA. *A History of Blood Coagulation*. Rochester, MN: Mayo Foundation for Medical Education and Research; 2001.
4. Owen CA. Older concepts of blood coagulation. In: Nichols WL, Bowie EJ, eds. *A History of Blood Coagulation*. Rochester, MN: Mayo Foundation for Medical Education and Research; 2001:7.
5. Morawitz P. Die chemie der blutgerrinnung. *Ergebn Physiol*. 1905;4:307.
6. Wright IS. The nomenclature of blood clotting factors. *Thromb Diath Haemorrh*. 1962;7:381.
7. Macfarlane RG. An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. *Nature*. 1964;202:498.
8. Davie EW, Ratnoff OD. Waterfall sequence for intrinsic blood clotting. *Science*. 1964;145:1310.
9. Oldenburg J, Marinova M, Muller-Reible C, et al. The vitamin K cycle. *Vitam Horm*. 2008;78:35.
10. Brenner B, Kuperman AA, Watzka M, et al. Vitamin K-dependent coagulation factors deficiency. *Semin Thromb Hemost*. 2009;35:439.
11. Chatrou ML, Reutelingsperger CP, Schurgers LJ. Role of vitamin K-dependent proteins in the arterial vessel wall. *Hamostaseologie*. 2011;31:251.
12. Nelsestuen GL, Shah AM, Harvey SB. Vitamin K-dependent proteins. *Vitam Horm*. 2000;58:355.
13. Berkner KL. Vitamin K-dependent carboxylation. *Vitam Horm*. 2008;78:131.
14. Bandyopadhyay PK. Vitamin K-dependent gamma-glutamylcarboxylation: an ancient posttranslational modification. *Vitam Horm*. 2008;78:157.
15. Nelsestuen GL, Kisiel W, Di Scipio RG. Interaction of vitamin K dependent proteins with membranes. *Biochemistry*. 1978;17:2134.
16. Schwalbe RA, Ryan J, Stern DM, et al. Protein structural requirements and properties of membrane binding by gamma-carboxyglutamic acid-containing plasma proteins and peptides. *J Biol Chem*. 1989;264:20288.
17. Schulman S, El Bouazzaoui B, Eikelboom JW, et al. Clinical factors influencing the sensitivity to warfarin when restarted after surgery. *J Intern Med*. 2008;263:412.
18. Lurie Y, Loebstein R, Kurnik D, et al. Warfarin and vitamin K intake in the era of pharmacogenetics. *Br J Clin Pharmacol*. 2010;70:164.
19. Cosgriff SW. The effectiveness of an oral vitamin K in controlling excessive hypothrombinemia during anticoagulant therapy. *Ann Intern Med*. 1956;45:14.
20. Stafford DW. The vitamin K cycle. *J Thromb Haemost*. 2005;3:1873.
21. Garcia AA, Reitsma PH. VKORC1 and the vitamin K cycle. *Vitam Horm*. 2008;78:23.
22. Hirsh J. Optimal intensity and monitoring warfarin. *Am J Cardiol*. 1995;75:39B.
23. Hirsh J, Dalen J, Anderson DR, et al. Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range. *Chest*. 2001; 119:8S.
24. Joseph DR, Baker ME. Sex hormone-binding globulin, androgen-binding protein, and vitamin K-dependent protein S are homologous to laminin A, merosin, and Drosophila crumbs protein. *FASEB J*. 1992;6:2477.
25. Prowse CV, Esnouf MP. The isolation of a new warfarin-sensitive protein from bovine plasma. *Biochem Soc Trans*. 1977;5:255.
26. Mann KG, Nesheim ME, Church WR, et al. Surface-dependent reactions of the vitamin K-dependent enzyme complexes. *Blood*. 1990;76:1.
27. Nemerson Y, Repke D. Tissue factor accelerates the activation of coagulation factor VII: the role of a bifunctional coagulation cofactor. *Thromb Res*. 1985;40:351.
28. Butenas S, Orfeo T, Mann KG. Tissue factor in coagulation: Which? Where? When? *Arterioscler Thromb Vasc Biol*. 2009;29:1989.
29. Williams JC, Mackman N. Tissue factor in health and disease. *Front Biosci (Elite Ed)*. 2012;4:358.
30. Butenas S, Orfeo T, Brummel-Ziedins KE, et al. Tissue factor in thrombosis and hemorrhage. *Surgery*. 2007;142:S2.
31. Nemerson Y. Tissue factor and hemostasis. *Blood*. 1988;71:1.
32. Edgington TS, Mackman N, Brand K, et al. The structural biology of expression and function of tissue factor. *Thromb Haemost*. 1991;66:67.
33. Nakagaki T, Foster DC, Berkner KL, et al. Initiation of the extrinsic pathway of blood coagulation: evidence for the tissue factor dependent autoactivation of human coagulation factor VII. *Biochemistry*. 1991;30:10819.
34. Toomey JR, Kratzer KE, Lasky NM, et al. Targeted disruption of the murine tissue factor gene results in embryonic lethality. *Blood*. 1996;88:1583.
35. Giesen PL, Rauch U, Bohrmann B, et al. Blood-borne tissue factor: another view of thrombosis. *Proc Natl Acad Sci USA*. 1999;96:2311.
36. Santucci RA, Erlich J, Labriola J, et al. Measurement of tissue factor activity in whole blood. *Thromb Haemost*. 2000;83:445.
37. Osterud B. The role of platelets in decrypting monocyte tissue factor. *Semin Hematol*. 2001;38:2.
38. Zillmann A, Luther T, Muller I, et al. Platelet-associated tissue factor contributes to the collagen-triggered activation of blood coagulation. *Biochem Biophys Res Commun*. 2001;281:603.
39. Butenas S, Bouchard BA, Brummel-Ziedins KE, et al. Tissue factor activity in whole blood. *Blood*. 2005;105:2764.
40. Morel O, Toti F, Hugel B, et al. Procoagulant microparticles: disrupting the vascular homeostasis equation? *Arterioscler Thromb Vasc Biol*. 2006;26:2594.
41. Falati S, Liu Q, Gross P, et al. Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin. *J Exp Med*. 2003;197:1585.
42. Chou J, Mackman N, Merrill-Skoloff G, et al. Hematopoietic cell-derived microparticle tissue factor contributes to fibrin formation during thrombus propagation. *Blood*. 2004;104:3190.
43. Esmon NL, Owen WG, Esmon CT. Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. *J Biol Chem*. 1982;257:859.
44. Fukudome K, Esmon CT. Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor. *J Biol Chem*. 1994;269:26486.
45. Bangalore N, Drohan WN, Orthner CL. High affinity binding sites for activated protein C and protein C on cultured human umbilical vein endothelial cells. Independent of protein S and distinct from known ligands. *Thromb Haemost*. 1994;72:465.
46. Esmon CT. Inflammation and the activated protein C anticoagulant pathway. *Semin Thromb Hemost*. 2006;32:49.
47. Rezaie AR. Regulation of the protein C anticoagulant and antiinflammatory pathways. *Curr Med Chem*. 2010;17:2059.
48. Hofsteenge J, Taguchi H, Stone SR. Effect of thrombomodulin on the kinetics of the interaction of thrombin with substrates and inhibitors. *Biochem J*. 1986;237:243.
49. Bourin MC, Boffa MC, Bjork I, et al. Functional domains of rabbit thrombomodulin. *Proc Natl Acad Sci USA*. 1986;83:5924.
50. Preissner KT, Delvos U, Muller-Berghaus G. Binding of thrombin to thrombomodulin accelerates inhibition of the enzyme by antithrombin III. Evidence for a heparin-independent mechanism. *Biochemistry*. 1987; 26:2521.
51. Kalafatis M, Rand MD, Mann KG. The mechanism of inactivation of human factor V and human factor Va by activated protein C. *J Biol Chem*. 1994;269:31869.
52. Eaton D, Rodriguez H, Vehar GA. Proteolytic processing of human factor VIII. Correlation of specific cleavages by thrombin, factor Xa, and activated protein C with activation and inactivation of factor VIII coagulant activity. *Biochemistry*. 1986;25:505.
53. Nesheim M. Fibrinolysis and the plasma carboxypeptidase. *Curr Opin Hematol*. 1998;5:309.
54. Bajzar L, Jain N, Wang P, et al. Thrombin activatable fibrinolysis inhibitor: not just an inhibitor of fibrinolysis. *Crit Care Med*. 2004;32:S320.
55. Mosnier LO, Bouma BN. Regulation of fibrinolysis by thrombin activatable fibrinolysis inhibitor, an unstable carboxypeptidase B that unites the pathways of coagulation and fibrinolysis. *Arterioscler Thromb Vasc Biol*. 2006;26:2445.
56. Heylen E, Willemse J, Hendriks D. An update on the role of carboxypeptidase U (TAFIa) in fibrinolysis. *Front Biosci*. 2011;17:2427.
57. Conway EM, Rosenberg RD. Tumor necrosis factor suppresses transcription of the thrombomodulin gene in endothelial cells. *Mol Cell Biol*. 1988; 8:5588.
58. Conway EM. Thrombomodulin and its role in inflammation. *Semin Immunopathol*. 2012;34:107.
59. Tracy PB, Eide LL, Bowie EJ, et al. Radioimmunoassay of factor V in human plasma and platelets. *Blood*. 1982;60:59.
60. Monkovic DD, Tracy PB. Activation of human factor V by factor Xa and thrombin. *Biochemistry*. 1990;29:1118.
61. Mann KG, Kalafatis M. Factor V: a combination of Dr Jekyll and Mr Hyde. *Blood*. 2003;101:20.
62. Esmon CT. The subunit structure of thrombin-activated factor V. Isolation of activated factor V, separation of subunits, and reconstitution of biological activity. *J Biol Chem*. 1979;254:964.

63. Mann KG, Hockin MF, Begin KJ, et al. Activated protein C cleavage of factor Va leads to dissociation of the A2 domain. *J Biol Chem*. 1997; 272:20678.
64. Dahlback B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci USA*. 1993;90:1004.
65. Castoldi E, Rosing J. APC resistance: biological basis and acquired influences. *J Thromb Haemost*. 2010;8:445.
66. Bertina RM, Koeleman BP, Koster T, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature*. 1994; 369:64.
67. Camire RM. A new look at blood coagulation factor V. *Curr Opin Hematol*. 2011;18:338.
68. Leyte A, Verbeet MP, Brodniewicz-Proba T, et al. The interaction between human blood-coagulation factor VIII and von Willebrand factor. Characterization of a high-affinity binding site on factor VIII. *Biochem J*. 1989;257:679.
69. Foster PA, Fulcher CA, Houghten RA, et al. An immunogenic region within residues Val1670-Glu1684 of the factor VIII light chain induces antibodies which inhibit binding of factor VIII to von Willebrand factor. *J Biol Chem*. 1988;263:5230.
70. Saenko EL, Shima M, Rajalakshmi KJ, et al. A role for the C2 domain of factor VIII in binding to von Willebrand factor. *J Biol Chem*. 1994;269: 11601.
71. Saenko EL, Scandella D. The acidic region of the factor VIII light chain and the C2 domain together form the high affinity binding site for von Willebrand factor. *J Biol Chem*. 1997;272:18007.
72. Butenas S, van 't Veer C, Mann KG. Evaluation of the initiation phase of blood coagulation using ultrasensitive assays for serine proteases. *J Biol Chem*. 1997;272:21527.
73. Jenny RJ, Pittman DD, Toole JJ, et al. Complete cDNA and derived amino acid sequence of human factor V. *Proc Natl Acad Sci USA*. 1987;84:4846.
74. Mannucci PM, Tuddenham EG. The hemophilias–from royal genes to gene therapy. *N Engl J Med*. 2001;344:1773.
75. Hoyer LW. Hemophilia A. *N Engl J Med*. 1994;330:38.
76. Koutts J, Walsh PN, Plow EF, et al. Active release of human platelet factor VIII-related antigen by adenosine diphosphate, collagen, and thrombin. *J Clin Invest*. 1978;62:1255.
77. Weiss HJ, Turitto VT, Baumgartner HR. Effect of shear rate on platelet interaction with subendothelium in citrated and native blood. I. Shear rate–dependent decrease of adhesion in von Willebrand's disease and the Bernard-Soulier syndrome. *J Lab Clin Med*. 1978;92:750.
78. Santoro SA. Adsorption of von Willebrand factor/factor VIII by the genetically distinct interstitial collagens. *Thromb Res*. 1981;21:689.
79. Ruggeri ZM, Bader R, de Marco L. Glanzmann thrombasthenia: deficient binding of von Willebrand factor to thrombin-stimulated platelets. *Proc Natl Acad Sci USA*. 1982;79:6038.
80. Fujimoto T, Hawiger J. Adenosine diphosphate induces binding of von Willebrand factor to human platelets. *Nature*. 1982;297:154.
81. Fujimoto T, Ohara S, Hawiger J. Thrombin-induced exposure and prostacyclin inhibition of the receptor for factor VIII/von Willebrand factor on human platelets. *J Clin Invest*. 1982;69:1212.
82. Ruggeri ZM, De Marco L, Gatti L, et al. Platelets have more than one binding site for von Willebrand factor. *J Clin Invest*. 1983;72:1.
83. Schullek J, Jordan J, Montgomery RR. Interaction of von Willebrand factor with human platelets in the plasma milieu. *J Clin Invest*. 1984; 73:421.
84. Sakariassen KS, Nievelstein PF, Coller BS, et al. The role of platelet membrane glycoproteins Ib and IIb-IIIa in platelet adherence to human artery subendothelium. *Br J Haematol*. 1986;63:681.
85. Bockenstedt P, Greenberg JM, Handin RI. Structural basis of von Willebrand factor binding to platelet glycoprotein Ib and collagen. Effects of disulfide reduction and limited proteolysis of polymeric von Willebrand factor. *J Clin Invest*. 1986;77:743.
86. Girma JP, Kalafatis M, Pietu G, et al. Mapping of distinct von Willebrand factor domains interacting with platelet GPIb and GPIIb/IIIa and with collagen using monoclonal antibodies. *Blood*. 1986;67:1356.
87. Pareti FI, Fujimura Y, Dent JA, et al. Isolation and characterization of a collagen binding domain in human von Willebrand factor. *J Biol Chem*. 1986;261:15310.
88. Cruz MA, Yuan H, Lee JR, et al. Interaction of the von Willebrand factor (vWF) with collagen. Localization of the primary collagen-binding site by analysis of recombinant vWF a domain polypeptides. *J Biol Chem*. 1995;270:10822.
89. Matsushita T, Sadler JE. Identification of amino acid residues essential for von Willebrand factor binding to platelet glycoprotein Ib. Charged-to-alanine scanning mutagenesis of the A1 domain of human von Willebrand factor. *J Biol Chem*. 1995;270:13406.
90. Goto S, Salomon DR, Ikeda Y, et al. Characterization of the unique mechanism mediating the shear-dependent binding of soluble von Willebrand factor to platelets. *J Biol Chem*. 1995;270:23352.
91. Zhang L, Castellino FJ. The binding energy of human coagulation protein C to acidic phospholipid vesicles contains a major contribution from leucine 5 in the gamma-carboxyglutamic acid domain. *J Biol Chem*. 1994;269: 3590.
92. Furlan M, Lammle B. Assays of von Willebrand factor-cleaving protease: a test for diagnosis of familial and acquired thrombotic thrombocytopenic purpura. *Semin Thromb Hemost*. 2002;28:167.
93. Fujikawa K, Suzuki H, McMullen B, et al. Purification of human von Willebrand factor-cleaving protease and its identification as a new member of the metalloproteinase family. *Blood*. 2001;98:1662.
94. Gerritsen HE, Robles R, Lammle B, et al. Partial amino acid sequence of purified von Willebrand factor-cleaving protease. *Blood*. 2001;98:1654.
95. Chung DW, Fujikawa K. Processing of von Willebrand factor by ADAMTS-13. *Biochemistry*. 2002;41:11065.
96. Tsai HM. ADAMTS13 and microvascular thrombosis. *Expert Rev Cardiovasc Ther*. 2006;4:813.
97. Gill JC, Endres-Brooks J, Bauer PJ, et al. The effect of ABO blood group on the diagnosis of von Willebrand disease. *Blood*. 1987;69:1691.
98. Percy ME, Rusk AC, Garvey MB, et al. Carrier detection in hemophilia A: ABO blood group, multiple measurements, and application of logistic discrimination. *Am J Med Genet*. 1988;31:871.
99. Weiss HJ, Rogers J, Brand H. Defective ristocetin-induced platelet aggregation in von Willebrand's disease and its correction by factor VIII. *J Clin Invest*. 1973;52:2697.
100. Moake JL, Olson JD, Troll Jr JH, et al. Interaction of platelets, von Willebrand factor, and ristocetin during platelet agglutination. *J Lab Clin Med*. 1980;96:168.
101. Berliner SA, Seligsohn U, Zivelin A, et al. A relatively high frequency of severe (type III) von Willebrand's disease in Israel. *Br J Haematol*. 1986; 62:535.
102. Rodeghiero F, Castaman G, Dini E. Epidemiological investigation of the prevalence of von Willebrand's disease. *Blood*. 1987;69:454.
103. Werner EJ, Broxson EH, Tucker EL, et al. Prevalence of von Willebrand disease in children: a multiethnic study. *J Pediatr*. 1993;123:893.
104. Lenk H, Nilsson IM, Holmberg L, et al. Frequency of different types of von Willebrand's disease in the GDR. *Acta Med Scand*. 1988;224:275.
105. Pottinger BE, Read RC, Paleolog EM, et al. von Willebrand factor is an acute phase reactant in man. *Thromb Res*. 1989;53:387.
106. Blann AD. von Willebrand factor antigen as an acute phase reactant and marker of endothelial cell injury in connective tissue diseases: a comparison with CRP, rheumatoid factor, and erythrocyte sedimentation rate. *Z Rheumatol*. 1991;50:320.
107. Stevens TR, James JP, Simmonds NJ, et al. Circulating von Willebrand factor in inflammatory bowel disease. *Gut*. 1992;33:502.
108. Ates E, Bakkaloglu A, Saatci U, et al. von Willebrand factor antigen compared with other factors in vasculitic syndromes. *Arch Dis Child*. 1994;70:40.
109. Hathaway WE, Belhasen LP, Hathaway HS. Evidence for a new plasma thromboplastin factor. I. Case report, coagulation studies and physicochemical properties. *Blood*. 1965;26:521.
110. Hoak JC, Swanson LW, Warner ED, et al. Myocardial infarction associated with severe factor-XII deficiency. *Lancet*. 1966;2:884.
111. Colman RW, Bagdasarian A, Talamo RC, et al. Williams trait. Human kininogen deficiency with diminished levels of plasminogen proactivator and prekallikrein associated with abnormalities of the Hageman factor-dependent pathways. *J Clin Invest*. 1975;56:1650.
112. Biggs R, Douglas AS, Macfarlane RG, et al. Christmas disease: a condition previously mistaken for haemophilia. *Br Med J*. 1952;2:1378.
113. Owen Jr CA, Cooper T, Parahemophilia AMA. *Arch Intern Med*. 1955;95:194.
114. Telfer TP, Denson KW, Wright DR. A new coagulation defect. *Br J Haematol*. 1956;2:308.
115. Hoyer LW. Molecular pathology and immunology of factor VIII (hemophilia A and factor VIII inhibitors). *Hum Pathol*. 1987;18:153.
116. Roberts HR, Escobar MA. Inherited disorders of prothrombin conversion. In: Colman RW, Marder VJ, Clowes AW, eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*. Philadelphia: Lippincott Williams & Wilkins; 2005:923.

117. Woodruff RS, Sullenger B, Becker RC. The many faces of the contact pathway and their role in thrombosis. *J Thromb Thrombolysis*. 2011;32:9.
118. Bone R. Sepsis and multiple organ failure: consensus and controversy. In: Lamy M, Thijs LG, eds. *Mediators of Sepsis*. New York: Springer-Verlag; 1992:3.
119. Colman RW. Contact activation pathway: inflammatory, fibrinolytic, anticoagulant, antiadhesive and antiangiogenic activites. In: Colman RW, Hirsh J, Marder VJ, eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*. Philadelphia: Lippincott Williams & Wilkins; 2001: 103.
120. Renne T, Pozgajova M, Gruner S, et al. Defective thrombus formation in mice lacking coagulation factor XII. *J Exp Med*. 2005;202:271.
121. Kleinschnitz C, Stoll G, Bendszus M, et al. Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis. *J Exp Med*. 2006;203:513.
122. Schousboe I. Pharmacological regulation of factor XII activation may be a new target to control pathological coagulation. *Biochem Pharmacol*. 2008; 75:1007.
123. Schmaier AH. Plasma contact activation: a revised hypothesis. *Biol Res*. 1998;31:251.
124. Rojkjaer R, Hasan AA, Motta G, et al. Factor XII does not initiate prekallikrein activation on endothelial cells. *Thromb Haemost*. 1998;80:74.
125. Kannemeier C, Shibamiya A, Nakazawa F, et al. Extracellular RNA constitutes a natural procoagulant cofactor in blood coagulation. *Proc Natl Acad Sci USA*. 2007;104:6388.
126. Muller F, Renne T. Platelet polyphosphates: the nexus of primary and secondary hemostasis. *Scand J Clin Lab Invest*. 2011;71:82.
127. Rapaport SI, Proctor RR, Patch MJ, et al. The mode of inheritance of PTA deficiency: evidence for the existence of major PTA deficiency and minor PTA deficiency. *Blood*. 1961;18:149.
128. Sidi A, Seligsohn U, Jonas P, et al. Factor XI deficiency: detection and management during urological surgery. *J Urol*. 1978;119:528.
129. Lawson JH, Kalafatis M, Stram S, et al. A model for the tissue factor pathway to thrombin. I. An empirical study. *J Biol Chem*. 1994;269:23357.
130. Gailani D, Broze GJ. Jr: Factor XI activation in a revised model of blood coagulation. *Science*. 1991;253:909.
131. Wiggins RC, Cochrane CC. The autoactivation of rabbit Hageman factor. *J Exp Med*. 1979;150:1122.
132. Silverberg M, Dunn JT, Garen L, et al. Autoactivation of human Hageman factor. Demonstration utilizing a synthetic substrate. *J Biol Chem*. 1980; 255:7281.
133. Schmaier AH. Assembly, activation, and physiologic influence of the plasma kallikrein/kinin system. *Int Immunopharmacol*. 2008;8:161.
134. Mandle RJ, Colman RW, Kaplan AP. Identification of prekallikrein and high-molecular-weight kininogen as a complex in human plasma. *Proc Natl Acad Sci USA*. 1976;73:4179.
135. Scott CF, Silver LD, Schapira M, et al. Cleavage of human high molecular weight kininogen markedly enhances its coagulant activity. Evidence that this molecule exists as a procofactor. *J Clin Invest*. 1984;73:954.
136. Scott CF, Silver LD, Purdon AD, et al. Cleavage of human high molecular weight kininogen by factor XIa in vitro. Effect on structure and function. *J Biol Chem*. 1985;260:10856.
137. Rosenberg RD, Damus PS. The purification and mechanism of action of human antithrombin-heparin cofactor. *J Biol Chem*. 1973;248:6490.
138. Perry DJ, Carrell RW. Molecular genetics of human antithrombin deficiency. *Hum Mutat*. 1996;7:7.
139. Harper PL, Luddington RJ, Daly M, et al. The incidence of dysfunctional antithrombin variants: four cases in 210 patients with thromboembolic disease. *Br J Haematol*. 1991;77:360.
140. Casu B, Oreste P, Torri G, et al. The structure of heparin oligosaccharide fragments with high anti-(factor Xa) activity containing the minimal antithrombin III-binding sequence. Chemical and ^{13}C nuclear-magnetic-resonance studies. *Biochem J*. 1981;197:599.
141. Olson ST, Shore JD. Demonstration of a two-step reaction mechanism for inhibition of alpha-thrombin by antithrombin III and identification of the step affected by heparin. *J Biol Chem*. 1982;257:14891.
142. Olson ST, Bjork I, Shore JD. Kinetic characterization of heparin-catalyzed and uncatalyzed inhibition of blood coagulation proteinases by antithrombin. *Methods Enzymol*. 1993;222:525.
143. O'Reilly MS, Pirie-Shepherd S, Lane WS, et al. Antiangiogenic activity of the cleaved conformation of the serpin antithrombin. *Science*. 1999;285:1926.
144. Novotny WF, Girard TJ, Miletich JP, et al. Purification and characterization of the lipoprotein-associated coagulation inhibitor from human plasma. *J Biol Chem*. 1989;264:18832.
145. Bajaj MS, Kuppuswamy MN, Saito H, et al. Cultured normal human hepatocytes do not synthesize lipoprotein-associated coagulation inhibitor: evidence that endothelium is the principal site of its synthesis. *Proc Natl Acad Sci USA*. 1990;87:8869.
146. Broze Jr GJ, Lange GW, Duffin KL, et al. Heterogeneity of plasma tissue factor pathway inhibitor. *Blood Coagul Fibrinolysis*. 1994;5:551.
147. Olivera BM. E.E. Just lecture, 1996. Conus venom peptides, receptor and ion channel targets, and drug design: 50 million years of neuropharmacology. *Mol Biol Cell*. 1997;8:2101.
148. Sanders NL, Bajaj SP, Zivelin A, et al. Inhibition of tissue factor/factor VIIa activity in plasma requires factor X and an additional plasma component. *Blood*. 1985;66:204.
149. Lesnik P, Vonica A, Guerin M, et al. Anticoagulant activity of tissue factor pathway inhibitor in human plasma is preferentially associated with dense subspecies of LDL and HDL and with Lp(a). *Arterioscler Thromb*. 1993; 13:1066.
150. Broze Jr GJ. Tissue factor pathway inhibitor. *Thromb Haemost*. 1995;74:90.
151. Sandset PM. Tissue factor pathway inhibitor (TFPI)—an update. *Haemostasis*. 1996;26:154.
152. Lindahl AK. Tissue factor pathway inhibitor: from unknown coagulation inhibitor to major antithrombotic principle. *Cardiovasc Res*. 1997;33:286.
153. Schwartz AL, Broze Jr GJ. Tissue factor pathway inhibitor endocytosis. *Trends Cardiovasc Med*. 1997;7:234.
154. Huang ZF, Higuchi D, Lasky N, et al. Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. *Blood*. 1997;90:944.
155. Golino P, Ragni M, Cimmino G, et al. Role of tissue factor pathway inhibitor in the regulation of tissue factor-dependent blood coagulation. *Cardiovasc Drug Rev*. 2002;20:67.
156. Kato H. Regulation of functions of vascular wall cells by tissue factor pathway inhibitor: basic and clinical aspects. *Arterioscler Thromb Vasc Biol*. 2002;22:539.
157. Amirkhosravi A, Meyer T, Amaya M, et al. The role of tissue factor pathway inhibitor in tumor growth and metastasis. *Semin Thromb Hemost*. 2007; 33:643.
158. Hackeng TM, Rosing J. Protein S as cofactor for TFPI. *Arterioscler Thromb Vasc Biol*. 2009;29:2015.
159. DelGiudice LA, White GA. The role of tissue factor and tissue factor pathway inhibitor in health and disease states. *J Vet Emerg Crit Care (San Antonio)*. 2009;19:23.
160. Kasthuri RS, Glover SL, Boles J, et al. Tissue factor and tissue factor pathway inhibitor as key regulators of global hemostasis: measurement of their levels in coagulation assays. *Semin Thromb Hemost*. 2010;36:764.
161. Chan JC, Carmeliet P, Moons L, et al. Factor VII deficiency rescues the intrauterine lethality in mice associated with a tissue factor pathway inhibitor deficit. *J Clin Invest*. 1999;103:475.
162. van 't Veer C, Golden NJ, Kalafatis M, et al. Inhibitory mechanism of the protein C pathway on tissue factor-induced thrombin generation. Synergistic effect in combination with tissue factor pathway inhibitor. *J Biol Chem*. 1997;272:7983.
163. Eitzman DT, Westrick RJ, Bi X, et al. Lethal perinatal thrombosis in mice resulting from the interaction of tissue factor pathway inhibitor deficiency and factor V Leiden. *Circulation*. 2002;105:2139.
164. Westrick RJ, Bodary PF, Xu Z, et al. Deficiency of tissue factor pathway inhibitor promotes atherosclerosis and thrombosis in mice. *Circulation*. 2001;103:3044.
165. Crawley JT, Lane DA. The haemostatic role of tissue factor pathway inhibitor. *Arterioscler Thromb Vasc Biol*. 2008;28:233.
166. Tollefsen DM, Majerus DW, Blank MK. Heparin cofactor II. Purification and properties of a heparin-dependent inhibitor of thrombin in human plasma. *J Biol Chem*. 1982;257:2162.
167. Griffith MJ, Carraway T, White GC, et al. Heparin cofactor activities in a family with hereditary antithrombin III deficiency: evidence for a second heparin cofactor in human plasma. *Blood*. 1983;61:111.
168. Tollefsen DM, Pestka CA, Monafo WJ. Activation of heparin cofactor II by dermatan sulfate. *J Biol Chem*. 1983;258:6713.
169. Parker KA, Tollefsen DM. The protease specificity of heparin cofactor II. Inhibition of thrombin generated during coagulation. *J Biol Chem*. 1985;260:3501.
170. Giri TK, Ahn CW, Wu KK, et al. Heparin cofactor II levels do not predict the development of coronary heart disease: the Atherosclerosis Risk in Communities (ARIC) study. *Arterioscler Thromb Vasc Biol*. 2005; 25:2689.
171. McGuire EA, Tollefsen DM. Activation of heparin cofactor II by fibroblasts and vascular smooth muscle cells. *J Biol Chem*. 1987;262:169.
172. Church FC, Pratt CW, Hoffman M. Leukocyte chemoattractant peptides from the serpin heparin cofactor II. *J Biol Chem*. 1991;266:704.
173. Andrew M, Mitchell L, Berry L, et al. An anticoagulant dermatan sulfate proteoglycan circulates in the pregnant woman and her fetus. *J Clin Invest*. 1992;89:321.
174. Massouh M, Jatoi A, Gordon EM, et al. Heparin cofactor II activity in plasma during pregnancy and oral contraceptive use. *J Lab Clin Med*. 1989;114:697.

175. Liu L, Dewar L, Song Y, et al. Inhibition of thrombin by antithrombin III and heparin cofactor II in vivo. *Thromb Haemost*. 1995;73:405.
176. Andersson TR, Sie P, Pelzer H, et al. Elevated levels of thrombin-heparin cofactor II complex in plasma from patients with disseminated intravascular coagulation. *Thromb Res*. 1992;66:591.
177. Simioni P, Lazzaro AR, Coser E, et al. Hereditary heparin cofactor II deficiency and thrombosis: report of six patients belonging to two separate kindreds. *Blood Coagul Fibrinolysis*. 1990;1:351.
178. Weisdorf DJ, Edson JR. Recurrent venous thrombosis associated with inherited deficiency of heparin cofactor II. *Br J Haematol*. 1991;77:125.
179. Corral J, Aznar J, Gonzalez-Conejero R, et al. Homozygous deficiency of heparin cofactor II: relevance of P17 glutamate residue in serpins, relationship with conformational diseases, and role in thrombosis. *Circulation*. 2004;110:1303.
180. Suzuki K, Nishioka J, Hashimoto S. Protein C inhibitor. Purification from human plasma and characterization. *J Biol Chem*. 1983;258:163.
181. Laurell M, Christensson A, Abrahamsson PA, et al. Protein C inhibitor in human body fluids. Seminal plasma is rich in inhibitor antigen deriving from cells throughout the male reproductive system. *J Clin Invest*. 1992; 89:1094.
182. Laurell M, Stenflo J, Carlson TH. Turnover of *I-protein C inhibitor and *I-alpha 1-antitrypsin and their complexes with activated protein C. *Blood*. 1990;76:2290.
183. Geiger M, Zechmeister-Machhart M, Uhrin P, et al. Protein C inhibitor (PCI). *Immunopharmacology*. 1996;32:53.
184. Seregni E, Botti C, Ballabio G, et al. Biochemical characteristics and recent biological knowledge on prostate-specific antigen. *Tumori*. 1996;82:72.
185. Heeb MJ, Espana F, Griffin JH. Inhibition and complexation of activated protein C by two major inhibitors in plasma. *Blood*. 1989;73:446.
186. Hoogendoorn H, Nesheim ME, Giles AR. A qualitative and quantitative analysis of the activation and inactivation of protein C in vivo in a primate model. *Blood*. 1990;75:2164.
187. Espana F, Vicente V, Tabernero D, et al. Determination of plasma protein C inhibitor and of two activated protein C-inhibitor complexes in normals and in patients with intravascular coagulation and thrombotic disease. *Thromb Res*. 1990;59:593.
188. Mosnier LO, Elisen MG, Bouma BN, et al. Protein C inhibitor regulates the thrombin-thrombomodulin complex in the up- and down regulation of TAFI activation. *Thromb Haemost*. 2001;86:1057.
189. Strandberg K, Astermark J, Bjorgell O, et al. Complexes between activated protein C and protein C inhibitor measured with a new method: comparison of performance with other markers of hypercoagulability in the diagnosis of deep vein thrombosis. *Thromb Haemost*. 2001;86:1400.
190. Meijers JC, Kanters DH, Vlooswijk RA, et al. Inactivation of human plasma kallikrein and factor XIa by protein C inhibitor. *Biochemistry*. 1988;27: 4231.
191. Sottrup-Jensen L. Alpha$_2$-macroglobulin and related thiol ester proteins. In: Putnam FW, ed. *The Plasma Proteins*. Olando: Academic Press; 1987:191.
192. Travis J, Salvesen GS. Human plasma proteinase inhibitors. *Annu Rev Biochem*. 1983;52:655.
193. Pizzo SV, Gonias SL. Receptor-mediated protease regulation. In: Conn PM, ed. *The Receptors*. Orlando: Academic Press; 1984:177.
194. Swenson RP, Howard JB. Structural characterization of human alpha2-macroglobulin subunits. *J Biol Chem*. 1979;254:4452.
195. Sottrup-Jensen L, Stepanik TM, Wierzbicki DM, et al. The primary structure of alpha 2-macroglobulin and localization of a Factor XIIIa cross-linking site. *Ann N Y Acad Sci*. 1983;421:41.
196. Kan CC, Solomon E, Belt KT, et al. Nucleotide sequence of cDNA encoding human alpha 2-macroglobulin and assignment of the chromosomal locus. *Proc Natl Acad Sci USA*. 1985;82:2282.
197. Carlsson-Bosted L, Moestrup SK, Gliemann J, et al. Three different conformational states of pregnancy zone protein identified by monoclonal antibodies. *J Biol Chem*. 1988;263:6738.
198. Christensen U, Simonsen M, Harrit N, et al. Pregnancy zone protein, a proteinase-binding macroglobulin. Interactions with proteinases and methylamine. *Biochemistry*. 1989;28:9324.
199. Barrett AJ, Starkey PM. The interaction of alpha 2-macroglobulin with proteinases. Characteristics and specificity of the reaction, and a hypothesis concerning its molecular mechanism. *Biochem J*. 1973;133:709.
200. Harpel PC. Human plasma alpha 2-macroglobulin. An inhibitor of plasma kallikrein. *J Exp Med*. 1970;132:329.
201. Harpel PC. Alpha2-plasmin inhibitor and alpha2-macroglobulin-plasmin complexes in plasma. Quantitation by an enzyme-linked differential antibody immunosorbent assay. *J Clin Invest*. 1981;68:46.
202. Mitchell L, Piovella F, Ofosu F, et al. Alpha-2-macroglobulin may provide protection from thromboembolic events in antithrombin III-deficient children. *Blood*. 1991;78:2299.
203. Abbink JJ, Nuijens JH, Eerenberg AJ, et al. Quantification of functional and inactivated alpha 2-macroglobulin in sepsis. *Thromb Haemost*. 1991; 65:32.
204. Huang SS, O'Grady P, Huang JS. Human transforming growth factor beta. alpha 2-macroglobulin complex is a latent form of transforming growth factor beta. *J Biol Chem*. 1988;263:1535.
205. Borth W, Luger TA. Identification of alpha 2-macroglobulin as a cytokine binding plasma protein. Binding of interleukin-1 beta to "F" alpha 2-macroglobulin. *J Biol Chem*. 1989;264:5818.
206. Matsuda T, Hirano T, Nagasawa S, et al. Identification of alpha 2-macroglobulin as a carrier protein for IL-6. *J Immunol*. 1989;142:148.
207. Dennis PA, Saksela O, Harpel P, et al. Alpha 2-macroglobulin is a binding protein for basic fibroblast growth factor. *J Biol Chem*. 1989;264:7210.
208. Wollenberg GK, LaMarre J, Rosendal S, et al. Binding of tumor necrosis factor alpha to activated forms of human plasma alpha 2 macroglobulin. *Am J Pathol*. 1991;138:265.
209. Legres LG, Pochon F, Barray M, et al. Evidence for the binding of a biologically active interleukin-2 to human alpha 2-macroglobulin. *J Biol Chem*. 1995;270:8381.
210. Kovacs DM. alpha2-macroglobulin in late-onset Alzheimer's disease. *Exp Gerontol*. 2000;35:473.
211. McGeer PL, McGeer EG. Polymorphisms in inflammatory genes and the risk of Alzheimer disease. *Arch Neurol*. 2001;58:1790.
212. Zappia M, Cittadella R, Manna I, et al. Genetic association of alpha2-macroglobulin polymorphisms with AD in southern Italy. *Neurology*. 2002;59:756.
213. Kruger U. [Chronic obstructive lung disease and alpha-2-macroglobulin deficiency in serum–case report]. *Pneumologie*. 1993;47:531.
214. Kanoh Y, Ohtani H, Koshiba K. [Studies on alpha 2 macroglobulin deficiency in association with cancer metastasis.]. *Nihon Rinsho Meneki Gakkai Kaishi*. 1997;20:30.
215. Umans L, Serneels L, Overbergh L, et al. Targeted inactivation of the mouse alpha 2-macroglobulin gene. *J Biol Chem*. 1995;270:19778.
216. Webb DJ, Wen J, Lysiak JJ, et al. Murine alpha-macroglobulins demonstrate divergent activities as neutralizers of transforming growth factor-beta and as inducers of nitric oxide synthesis. A possible mechanism for the endotoxin insensitivity of the alpha2-macroglobulin gene knock-out mouse. *J Biol Chem*. 1996;271:24982.
217. Griendling KK, Alexander RW. Endothelial control of the cardiovascular system: recent advances. *FASEB J*. 1996;10:283.
218. Rodgers GM. Endothelium and the regulation of hemostasis. In: Lee GR, Lickins J, Paraskevas F, eds. *Wintrobe's Clinical Hematology*. Baltimore: Williams & Wilkins; 1999:765.
219. Colman RW, Marder VJ, Salzman EW, et al. Overview of hemostasis. In: Colman RW, Hirsh J, Marder VJ, eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*. Philadelphia: Lippincott; 1994:3.
220. Makrides SC, Ryan US. Overview of the endothelium. In: Loscalzo J, Schafer AI, eds. *Thrombosis and Hemorrhage*. Baltimore: Williams & Wilkins; 1998:295.
221. Ware AG, Seegers WH. Two-stage procedure for the quantitative determination of prothrombin concentration. *Am J Clin Pathol*. 1949; 19:471.
222. Weiss HJ, Turitto VT, Baumgartner HR. Role of shear rate and platelets in promoting fibrin formation on rabbit subendothelium. Studies utilizing patients with quantitative and qualitative platelet defects. *J Clin Invest*. 1986;78:1072.
223. Bevilacqua MP, Pober JS, Majeau GR, et al. Interleukin 1 (IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. *J Exp Med*. 1984;160:618.
224. Esmon CT. Structure and functions of the endothelial cell protein C receptor. *Crit Care Med*. 2004;32:S298.
225. Hancock WW, Grey ST, Hau L, et al. Binding of activated protein C to a specific receptor on human mononuclear phagocytes inhibits intracellular calcium signaling and monocyte-dependent proliferative responses. *Transplantation*. 1995;60:1525.
226. Tuszynski GP, Bevacqua SJ, Schmaier AH, et al. Factor XI antigen and activity in human platelets. *Blood*. 1982;59:1148.
227. Bagdasarian A, Colman RW. Subcellula localization and purification of platelet alpha1-antitrypsin. *Blood*. 1978;51:139.
228. Schwarz HP, Heeb MJ, Wencel-Drake JD, et al. Identification and quantitation of protein S in human platelets. *Blood*. 1985;66:1452.
229. Novotny WF, Girard TJ, Miletich JP, et al. Platelets secrete a coagulation inhibitor functionally and antigenically similar to the lipoprotein associated coagulation inhibitor. *Blood*. 1988;72:2020.
230. McDonagh J, McDonagh Jr RP, Delage JM, et al. Factor XIII in human plasma and platelets. *J Clin Invest*. 1969;48:940.

231. Holt JC, Niewiaroswski S. Secretion of plasminogen by washed human platelets. *Circulation*. 1980;62:342.
232. Plow EF, Collen D. The presence and release of alpha 2-antiplasmin from human platelets. *Blood*. 1981;58:1069.
233. Erickson LA, Hekman CM, Loskutoff DJ. The primary plasminogen-activator inhibitors in endothelial cells, platelets, serum, and plasma are immunologically related. *Proc Natl Acad Sci USA*. 1985;82:8710.
234. Kruithof EK, Tran-Thang C, Bachmann F. Studies on the release of a plasminogen activator inhibitor by human platelets. *Thromb Haemost*. 1986;55:201.
235. Brass LF. Thrombin and platelet activation. *Chest*. 2003;124:18S.
236. White JG. Views of the platelet cytoskeleton at rest and at work. *Ann N Y Acad Sci*. 1987;509:156.
237. Hartwig JH. Platelet morphology. In: Loscalzo J, Schafer AI, eds. *Thrombosis and Hemorrhage*. Baltimore: Williams & Wilkins; 1998:207.
238. Parise LV, Boudignon-Proudhon C, Keely PJ, et al. Platelets in hemostatis. In: Foersten J, Lukens J, Paraskevas F, eds. *Wintrobe's Clinical Hematology*. Baltimore: Williams & Wilkins; 1999:661.
239. Nurden AT. Human platelet membrane glycoproteins. In: Bloom AL, Forbes CD, Thomas DP, eds. *Hemostasis and Thrombosis*. New York: Churchill Livingstone; 1994:115.
240. Bevers EM, Comfurius P, Zwaal RF. Changes in membrane phospholipid distribution during platelet activation. *Biochim Biophys Acta*. 1983;736:57.
241. Bouchard BA, Catcher CS, Thrash BR, et al. Effector cell protease receptor-1, a platelet activation-dependent membrane protein, regulates prothrombinase-catalyzed thrombin generation. *J Biol Chem*. 1997;272:9244.
242. Altieri DC, Edgington TS. Sequential receptor cascade for coagulation proteins on monocytes. Constitutive biosynthesis and functional prothrombinase activity of a membrane form of factor V/Va. *J Biol Chem*. 1989;264:2969.
243. Altieri DC, Edgington TS. Identification of effector cell protease receptor-1. A leukocyte-distributed receptor for the serine protease factor Xa. *J Immunol*. 1990;145:246.
244. Altieri DC, Stamnes SJ. Protease-dependent T cell activation: ligation of effector cell protease receptor-1 (EPR-1) stimulates lymphocyte proliferation. *Cell Immunol*. 1994;155:372.
245. Blomback B. Fibrinogen: evolution of the structure-function concept. Keynote address at fibrinogen 2000 congress. *Ann N Y Acad Sci*. 2001; 936:1.
246. Doolittle RF, Yang Z, Mochalkin I. Crystal structure studies on fibrinogen and fibrin. *Ann N Y Acad Sci*. 2001;936:31.
247. Bailey K, Bettelheim FR, Lorand L, et al. Action of thrombin in the clotting of fibrinogen. *Nature*. 1951;167:233.
248. Blomback B. Studies on the action of thrombotic enzymes on bovine fibrinogen as measured by N-terminal analysis. *Arkiv Kemi*. 1958;12:321.
249. Hirose S, Oda K, Ikehara Y. Biosynthesis, assembly and secretion of fibrinogen in cultured rat hepatocytes. *Biochem J*. 1988;251:373.
250. Lee SY, Lee KP, Lim JW. Identification and biosynthesis of fibrinogen in human uterine cervix carcinoma cells. *Thromb Haemost*. 1996;75:466.
251. Chung DW, Davie EW. gamma and gamma' chains of human fibrinogen are produced by alternative mRNA processing. *Biochemistry*. 1984;23:4232.
252. Laki K, Lorand L. On the solubility of fibrin clots. *Science*. 1948;108:280.
253. Lorand L. A study on the solubility of fibrin clots in urea. *Hung Acta Physiol*. 1948;1:192.
254. Lorand L. Factor XIII: structure, activation, and interactions with fibrinogen and fibrin. *Ann N Y Acad Sci*. 2001;936:291.
255. Sakharov DV, Plow EF, Rijken DC. On the mechanism of the antifibrinolytic activity of plasma carboxypeptidase B. *J Biol Chem*. 1997;272:14477.
256. Wang W, Boffa MB, Bajzar L, et al. A study of the mechanism of inhibition of fibrinolysis by activated thrombin-activable fibrinolysis inhibitor. *J Biol Chem*. 1998;273:27176.
257. Broze Jr GJ, Higuchi DA. Coagulation-dependent inhibition of fibrinolysis: role of carboxypeptidase-U and the premature lysis of clots from hemophilic plasma. *Blood*. 1996;88:3815.
258. de Maat MP. Effects of diet, drugs, and genes on plasma fibrinogen levels. *Ann N Y Acad Sci*. 2001;936:509.
259. Danesh J, Lewington S, Thompson SG, et al. Plasma fibrinogen level and the risk of major cardiovascular diseases and nonvascular mortality: an individual participant meta-analysis. *JAMA*. 2005;294:1799.
260. Sriramarao P, Languino LR, Altieri DC. Fibrinogen mediates leukocyte-endothelium bridging in vivo at low shear forces. *Blood*. 1996;88:3416.
261. Weisel JW. Fibrinogen and fibrin. *Adv Protein Chem*. 2005;70:247.
262. Wright SD, Weitz JI, Huang AJ, et al. Complement receptor type three (CD11b/CD18) of human polymorphonuclear leukocytes recognizes fibrinogen. *Proc Natl Acad Sci USA*. 1988;85:7734.
263. Mosesson MW. The roles of fibrinogen and fibrin in hemostasis and thrombosis. *Semin Hematol*. 1992;29:177.
264. Martinez J, Ferber A, Bach TL, et al. Interaction of fibrin with VE-cadherin. *Ann N Y Acad Sci*. 2001;936:386.
265. Podolnikova NP, Yakubenko VP, Volkov GL, et al. Identification of a novel binding site for platelet integrins alpha IIb beta 3 (GPIIbIIIa) and alpha 5 beta 1 in the gamma C-domain of fibrinogen. *J Biol Chem*. 2003;278: 32251.
266. Quick AJ, Stanley-Brown M, Bancroft FW. A study of the coagulation defect in hemophilia and in jaundice. *J Med Sci*. 1935;190:501.
267. Owren PA, Aas K. The control of dicumarol therapy and the quantitative determination of prothrombin and proconvertin. *Scand J Clin Lab Invest*. 1951;3:201.
268. Langdell RD, Wagner RH, Brinkhous KM. Effect of antihemophilic factor on one-stage clotting tests; a presumptive test for hemophilia and a simple one-stage antihemophilic factor assay procedure. *J Lab Clin Med*. 1953; 41:637.
269. Marder VJ, Francis CW. Plasmin degradation of cross-linked fibrin. *Ann N Y Acad Sci*. 1983;408:397.
270. Violand BN, Castellino FJ. Mechanism of the urokinase-catalyzed activation of human plasminogen. *J Biol Chem*. 1976;251:3906.
271. Hanna LS, Scheraga HA, Francis CW, et al. Comparison of structures of various human fibrinogens and a derivative thereof by a study of the kinetics of release of fibrinopeptides. *Biochemistry*. 1984;23:4681.
272. Lewis SD, Shields PP, Shafer JA. Characterization of the kinetic pathway for liberation of fibrinopeptides during assembly of fibrin. *J Biol Chem*. 1985;260:10192.
273. Mihalyi E. Clotting of bovine fibrinogen. Kinetic analysis of the release of fibrinopeptides by thrombin and of the calcium uptake upon clotting at high fibrinogen concentrations. *Biochemistry*. 1988;27:976.
274. Righini M, Perrier A, De Moerloose P, et al. D-Dimer for venous thromboembolism diagnosis: 20 years later. *J Thromb Haemost*. 2008;6: 1059.
275. Kjoller L, Kanse SM, Kirkegaard T, et al. Plasminogen activator inhibitor-1 represses integrin- and vitronectin-mediated cell migration independently of its function as an inhibitor of plasminogen activation. *Exp Cell Res*. 1997;232:420.
276. Fay WP, Parker AC, Condrey LR, et al. Human plasminogen activator inhibitor-1 (PAI-1) deficiency: characterization of a large kindred with a null mutation in the PAI-1 gene. *Blood*. 1997;90:204.
277. Huber K. Plasminogen activator inhibitor type-1 (part two): role for failure of thrombolytic therapy. PAI-1 resistance as a potential benefit for new fibrinolytic agents. *J Thromb Thrombolysis*. 2001;11:195.
278. Nesheim M, Bajzar L. The discovery of TAFI. *J Thromb Haemost*. 2005; 3:2139.
279. Bertina RM, van Tilburg NH, Haverkate F, et al. Discovery of thrombin activatable fibrinolysis inhibitor (TAFI). *J Thromb Haemost*. 2006;4:256.
280. Boffa MB, Koschinsky ML. Curiouser and curiouser: recent advances in measurement of thrombin-activatable fibrinolysis inhibitor (TAFI) and in understanding its molecular genetics, gene regulation, and biological roles. *Clin Biochem*. 2007;40:431.
281. Bajzar L, Morser J, Nesheim M. TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex. *J Biol Chem*. 1996;271:16603.
282. Minnema MC, Friederich PW, Levi M, et al. Enhancement of rabbit jugular vein thrombolysis by neutralization of factor XI. In vivo evidence for a role of factor XI as an anti-fibrinolytic factor. *J Clin Invest*. 1998;101:10.
283. Bouma BN, Mosnier LO, Meijers JC, et al. Factor XI dependent and independent activation of thrombin activatable fibrinolysis inhibitor (TAFI) in plasma associated with clot formation. *Thromb Haemost*. 1999;82:1703.
284. Mann KG. Thrombin generation in hemorrhage control and vascular occlusion. *Circulation*. 2011;124:225.
285. Mann KG: Coagulation Explosion, Part I—Initiation 2010. Available from: http://www.youtube.com/watch?v=RP1eLdkdJKg, (cited January 18, 2012).
286. Mann KG: Coagulation Explosion, Part II—Propagation 2010. Available from: http://www.youtube.com/watch?v=oj9E33BZMsI, (cited January 18, 2012).
287. Bouchard BA, Tracy PB. Platelets, leukocytes, and coagulation. *Curr Opin Hematol*. 2001;8:263.
288. Morrissey JH, Macik BG, Neuenschwander PF, et al. Quantitation of activated factor VII levels in plasma using a tissue factor mutant selectively deficient in promoting factor VII activation. *Blood*. 1993;81:734.
289. Komiyama Y, Pedersen AH, Kisiel W. Proteolytic activation of human factors IX and X by recombinant human factor VIIa: effects of calcium, phospholipids, and tissue factor. *Biochemistry*. 1990;29:9418.

290. van 't Veer C, Golden NJ, Mann KG. Inhibition of thrombin generation by the zymogen factor VII: implications for the treatment of hemophilia A by factor VIIa. *Blood*. 2000;95:1330.
291. van't Veer C, Mann KG. The regulation of the factor VII-dependent coagulation pathway: rationale for the effectiveness of recombinant factor VIIa in refractory bleeding disorders. *Semin Thromb Hemost*. 2000;26:367.
292. Choi-Miura NH, Tobe T, Sumiya J, et al. Purification and characterization of a novel hyaluronan-binding protein (PHBP) from human plasma: it has three EGF, a kringle and a serine protease domain, similar to hepatocyte growth factor activator. *J Biochem*. 1996;119:1157.
293. Romisch J, Feussner A, Vermohlen S, et al. A protease isolated from human plasma activating factor VII independent of tissue factor. *Blood Coagul Fibrinolysis*. 1999;10:471.
294. Romisch J. Factor VII activating protease (FSAP): a novel protease in hemostasis. *Biol Chem*. 2002;383:1119.
295. Stephan F, Aarden LA, Zeerleder S. FSAP, a new player in inflammation? *Hamostaseologie*. 2012;32:51.
296. Osterud B, Rapaport SI. Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation. *Proc Natl Acad Sci USA*. 1977;74:5260.
297. Jesty J, Silverberg SA. Kinetics of the tissue factor-dependent activation of coagulation Factors IX and X in a bovine plasma system. *J Biol Chem*. 1979;254:12337.
298. Bom VJ, Bertina RM. The contributions of Ca^{2+}, phospholipids and tissue-factor apoprotein to the activation of human blood-coagulation factor X by activated factor VII. *Biochem J*. 1990;265:327.
299. Lawson JH, Mann KG. Cooperative activation of human factor IX by the human extrinsic pathway of blood coagulation. *J Biol Chem*. 1991;266:11317.
300. Girard TJ, Warren LA, Novotny WF, et al. Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor. *Nature*. 1989;338:518.
301. Baugh RJ, Broze Jr GJ, Krishnaswamy S. Regulation of extrinsic pathway factor Xa formation by tissue factor pathway inhibitor. *J Biol Chem*. 1998;273:4378.
302. Novotny WF, Palmier M, Wun TC, et al. Purification and properties of heparin-releasable lipoprotein-associated coagulation inhibitor. *Blood*. 1991;78:394.
303. Rapaport SI. The extrinsic pathway inhibitor: a regulator of tissue factor-dependent blood coagulation. *Thromb Haemost*. 1991;66:6.
304. Orfeo T, Brufatto N, Nesheim ME, et al. The factor V activation paradox. *J Biol Chem*. 2004;279:19580.
305. Brummel KE, Paradis SG, Butenas S, et al. Thrombin functions during tissue factor-induced blood coagulation. *Blood*. 2002;100:148.
306. Mann KG, Brummel-Ziedins K, Orfeo T, et al. Models of blood coagulation. *Blood Cells Mol Dis*. 2006;36:108.
307. Coughlin SR, Vu TK, Hung DT, et al. Characterization of a functional thrombin receptor. Issues and opportunities. *J Clin Invest*. 1992;89:351.
308. Naito K, Fujikawa K. Activation of human blood coagulation factor XI independent of factor XII. Factor XI is activated by thrombin and factor XIa in the presence of negatively charged surfaces. *J Biol Chem*. 1991;266:7353.
309. Davey MG, Luscher EF. Actions of thrombin and other coagulant and proteolytic enzymes on blood platelets. *Nature*. 1967;216:857.
310. Lorand L, Konishi K. Activation of the fibrin stabilizing factor of plasma by thrombin. *Arch Biochem Biophys*. 1964;105:58.
311. Mann KG, Jenny RJ, Krishnaswamy S. Cofactor proteins in the assembly and expression of blood clotting enzyme complexes. *Annu Rev Biochem*. 1988;57:915.
312. Fay PJ. Subunit structure of thrombin-activated human factor VIIIa. *Biochim Biophys Acta*. 1988;952:181.
313. Whelihan MF, Zachary V, Orfeo T, et al. Prothrombin activation in blood coagulation:the erythrocyte contribution to thrombin generation. *Blood*. 2012;120:3837–3845.
314. Ahmad SS, Rawala-Sheikh R, Walsh PN. Components and assembly of the factor X activating complex. *Semin Thromb Hemost*. 1992;18:311.
315. van 't Veer C, Mann KG. Regulation of tissue factor initiated thrombin generation by the stoichiometric inhibitors tissue factor pathway inhibitor, antithrombin-III, and heparin cofactor-II. *J Biol Chem*. 1997;272:4367.
316. Hockin MF, Jones KC, Everse SJ, et al. A model for the stoichiometric regulation of blood coagulation. *J Biol Chem*. 2002;277:18322.
317. Cawthern KM, van't Veer C, Lock JB, et al. Blood coagulation in hemophilia A and hemophilia C. *Blood*. 1998;91:4581.
318. Butenas S, Brummel KE, Branda RF, et al. Mechanism of factor VIIa-dependent coagulation in hemophilia blood. *Blood*. 2002;99:923.
319. Tracy PB, Nesheim ME, Mann KG. Platelet factor Xa receptor. *Methods Enzymol*. 1992;215:329.
320. Nesheim ME, Taswell JB, Mann KG. The contribution of bovine Factor V and Factor Va to the activity of prothrombinase. *J Biol Chem*. 1979;254:10952.
321. Orfeo T, Brummel-Ziedins KE, Gissel M, et al. The nature of the stable blood clot procoagulant activities. *J Biol Chem*. 2008;283:9776.
322. Butenas S, Cawthern KM, van't Veer C, et al. Antiplatelet agents in tissue factor-induced blood coagulation. *Blood*. 2001;97:2314.
323. Butenas S, Branda RF, van't Veer C, et al. Platelets and phospholipids in tissue factor-initiated thrombin generation. *Thromb Haemost*. 2001;86:660.
324. Mann KG, Butenas S, Brummel K. The dynamics of thrombin formation. *Arterioscler Thromb Vasc Biol*. 2003;23:17.
325. Mann KG, Krishnaswamy S, Lawson JH. Surface-dependent hemostasis. *Semin Hematol*. 1992;29:213.
326. Butenas S, Mann KG. Kinetics of human factor VII activation. *Biochemistry*. 1996;35:1904.
327. Rand MD, Lock JB, van't Veer C, et al. Blood clotting in minimally altered whole blood. *Blood*. 1996;88:3432.
328. Brummel-Ziedins KE, Branda RF, Butenas S, et al. Discordant fibrin formation in hemophilia. *J Thromb Haemost*. 2009;7:825.
329. Brummel-Ziedins KE, Whelihan MF, Gissel M, et al. Thrombin generation and bleeding in haemophilia A. *Haemophilia*. 2009;15:1118.
330. Moncada S, Higgs EA, Vane JR. Human arterial and venous tissues generate prostacyclin (prostaglandin x), a potent inhibitor of platelet aggregation. *Lancet*. 1977;1:18.
331. Esmon CT, Owen WG. Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. *Proc Natl Acad Sci USA*. 1981;78:2249.
332. Busch C, Owen WG. Identification in vitro of an endothelial cell surface cofactor for antithrombin III. Parallel studies with isolated perfused rat hearts and microcarrier cultures of bovine endothelium. *J Clin Invest*. 1982;69:726.
333. Turk B, Brieditis I, Bock SC, et al. The oligosaccharide side chain on Asn-135 of alpha-antithrombin, absent in beta-antithrombin, decreases the heparin affinity of the inhibitor by affecting the heparin-induced conformational change. *Biochemistry*. 1997;36:6682.
334. Esmon CT, Esmon NL, Harris KW. Complex formation between thrombin and thrombomodulin inhibits both thrombin-catalyzed fibrin formation and factor V activation. *J Biol Chem*. 1982;257:7944.
335. Esmon NL, Carroll RC, Esmon CT. Thrombomodulin blocks the ability of thrombin to activate platelets. *J Biol Chem*. 1983;258:12238.
336. Bajzar L. Thrombin activatable fibrinolysis inhibitor and an antifibrinolytic pathway. *Arterioscler Thromb Vasc Biol*. 2000;20:2511.
337. Bajzar L, Nesheim ME, Tracy PB. The profibrinolytic effect of activated protein C in clots formed from plasma is TAFI-dependent. *Blood*. 1996;88:2093.
338. Tracy RP. Thrombin, inflammation, and cardiovascular disease: an epidemiologic perspective. *Chest*. 2003;124:49S.
339. Robbins KC, Summaria L, Hsieh B, et al. The peptide chains of human plasmin. Mechanism of activation of human plasminogen to plasmin. *J Biol Chem*. 1967;242:2333.
340. Pannell R, Gurewich V. Activation of plasminogen by single-chain urokinase or by two-chain urokinase—a demonstration that single-chain urokinase has a low catalytic activity (pro-urokinase). *Blood*. 1987;69:22.
341. Dichek D, Quertermous T. Thrombin regulation of mRNA levels of tissue plasminogen activator and plasminogen activator inhibitor-1 in cultured human umbilical vein endothelial cells. *Blood*. 1989;74:222.
342. Diamond SL, Eskin SG, McIntire LV. Fluid flow stimulates tissue plasminogen activator secretion by cultured human endothelial cells. *Science*. 1989;243:1483.
343. Hoylaerts M, Rijken DC, Lijnen HR, et al. Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. *J Biol Chem*. 1982;257:2912.
344. Zamarron C, Lijnen HR, Collen D. Kinetics of the activation of plasminogen by natural and recombinant tissue-type plasminogen activator. *J Biol Chem*. 1984;259:2080.
345. Loscalzo J. Structural and kinetic comparison of recombinant human single- and two-chain tissue plasminogen activator. *J Clin Invest*. 1988;82:1391.
346. Gaffney PJ, Brasher M. Subunit structure of the plasmin-induced degradation products of crosslinked fibrin. *Biochim Biophys Acta*. 1973;295:308.
347. Francis CW, Marder VJ, Barlow GH. Plasmic degradation of crosslinked fibrin. Characterization of new macromolecular soluble complexes and a model of their structure. *J Clin Invest*. 1980;66:1033.
348. Marder VJ. Physicochemical studies of intermediate and final products of plasmin digestion of human fibrinogen. *Thromb Diath Haemorrh*. 1970;39:187.
349. Larrieu MJ, Marder VJ, Inceman S. Effects of fibrinogen degradation products on platelets and coagulation. *Thromb Diath Haemorrh Suppl*. 1966;20:215.

350. Reilly CF, Hutzelmann JE. Plasminogen activator inhibitor-1 binds to fibrin and inhibits tissue-type plasminogen activator-mediated fibrin dissolution. *J Biol Chem*. 1992;267:17128.
351. Sharp AM, Stein PE, Pannu NS, et al. The active conformation of plasminogen activator inhibitor 1, a target for drugs to control fibrinolysis and cell adhesion. *Structure*. 1999;7:111.
352. Kawano T, Morimoto K, Uemura Y. Partial purification and properties of urokinase inhibitor from human placenta. *J Biochem*. 1970;67:333.
353. Eaton DL, Malloy BE, Tsai SP, et al. Isolation, molecular cloning, and partial characterization of a novel carboxypeptidase B from human plasma. *J Biol Chem*. 1991;266:21833.
354. Loskutoff DJ. Type 1 plasminogen activator inhibitor and its potential influence on thrombolytic therapy. *Semin Thromb Hemost*. 1988;14:100.
355. Levi M, Biemond BJ, van Zonneveld AJ, et al. Inhibition of plasminogen activator inhibitor-1 activity results in promotion of endogenous thrombolysis and inhibition of thrombus extension in models of experimental thrombosis. *Circulation*. 1992;85:305.
356. Braaten JV, Handt S, Jerome WG, et al. Regulation of fibrinolysis by platelet-released plasminogen activator inhibitor 1: light scattering and ultrastructural examination of lysis of a model platelet-fibrin thrombus. *Blood*. 1993;81:1290.
357. Fay WP, Eitzman DT, Shapiro AD, et al. Platelets inhibit fibrinolysis in vitro by both plasminogen activator inhibitor-1-dependent and -independent mechanisms. *Blood*. 1994;83:351.
358. Collen D, De C, Verstraete M. Immunochemical distinction between antiplasmin and alpha-antitrypsin. *Thromb Res*. 1975;7:245.
359. Collen D. Identification and some properties of a new fast-reacting plasmin inhibitor in human plasma. *Eur J Biochem*. 1976;69:209.
360. Moroi M, Aoki N. Isolation and characterization of alpha2-plasmin inhibitor from human plasma. A novel proteinase inhibitor which inhibits activator-induced clot lysis. *J Biol Chem*. 1976;251:5956.
361. Mullertz S, Clemmensen I. The primary inhibitor of plasmin in human plasma. *Biochem J*. 1976;159:545.
362. Wiman B, Collen D. On the kinetics of the reaction between human antiplasmin and plasmin. *Eur J Biochem*. 1978;84:573.
363. Sakata Y, Aoki N. Significance of cross-linking of alpha 2-plasmin inhibitor to fibrin in inhibition of fibrinolysis and in hemostasis. *J Clin Invest*. 1982;69:536.
364. Collen D. Ham-Wasserman lecture: role of the plasminogen system in fibrin-homeostasis and tissue remodeling. *Hematology Am Soc Hematol Educ Program*. 2001;1
365. Ellis V, Pyke C, Eriksen J, et al. The urokinase receptor: involvement in cell surface proteolysis and cancer invasion. *Ann N Y Acad Sci*. 1992;667:13.
366. Lijnen HR. Plasmin and matrix metalloproteinases in vascular remodeling. *Thromb Haemost*. 2001;86:324.

CHAPTER 126

EVALUATION OF THE PATIENT WITH SUSPECTED BLEEDING DISORDERS

Catherine P. M. Hayward and Alice D. Ma

Bruising and bleeding are common reasons for a hematology referral.[1] Some consults are for management of an acute bleed in someone who may have a hemorrhagic disorder. Such bleeds may be spontaneous or occur after surgery or trauma, and the potential bleeding disorder may be inherited or acquired. With acute bleeding problems, the approach to patients must be rapid and directed at urgent restoration of hemostasis. Some referrals are for evaluation after an unexpected bleeding episode or the diagnosis of a bleeding disorder in a family member. Some of these referrals may be urgent because of upcoming procedures.

The assessment of such patients must be individualized, including a detailed history of the current and all past bleeding events, a detailed family history, and a thorough review of all medications, including nonprescription and supplemental agents. The assessment of bleeding or bruising can be challenging because individuals without bleeding problems often experience minor bleeding symptoms.[2] Furthermore, most individuals referred for assessment of bleeding or bruising will have experienced some bleeding symptoms that may or may not reflect an underlying bleeding disorder.[1] Because many individuals referred to a hematologist for evaluation will be diagnosed with a bleeding disorder, there is a high pretest probability for a bleeding disorder among such patients.[1] The next step is laboratory evaluation, guided by the pretest probability of finding an underlying bleeding disorder.[1] It is important to note that laboratory studies can yield false-positive findings,[1,3] and external pressures (e.g., insurance coverage) may limit the diagnostic testing available to the patient and physician. Finally, the hematologist must formulate a differential diagnosis and plan for bleeding symptom management (e.g., control of heavy menstrual bleeding [HMB]), including strategies to minimize future bleeding risks from exposure to surgery and other invasive procedures). Fig. 126.1 provides a general guide to the steps involved in clinical assessment.

BLEEDING HISTORY EVALUATION

When evaluating a bleeding history, it is important to recognize that the presenting problems are influenced by the following factors:

1. The nature and severity of the defect, and the presence of single or multiple risk factors for bleeding
2. Whether the bleeding problem is congenital or acquired
3. Antecedent exposure to hemostatic challenges (e.g., surgery, dental extraction, menses, and childbirth) and the risk for bleeding with each of these challenges
4. The presence of other medical problems (e.g., renal, hepatic, or thyroid disease), including anemia
5. Variability in the bleeding symptoms experienced by individuals without bleeding disorders (e.g., nosebleeds, bruising) and by individuals with known bleeding disorders, even within families with the same defect
6. Local factors (e.g., sun-damage to the skin, vascular lesions, diverticular disease, or cancerous lesions in the gastrointestinal [GI] tract) and the possibility of nonaccidental trauma
7. Treatments that increase the risk for bleeding (e.g., antiplatelet agents, such as aspirin and nonsteroidal antiinflammatory drug [NSAID] used for pain control, anticoagulants)
8. Whether treatments were used to prevent or control bleeding
9. Whether treatments prescribed for other reasons may have reduced bleeding (e.g., reduced menstrual bleeding while on oral contraceptives to prevent pregnancy)

A detailed history of bleeding episodes, including assessment of the family history, is critical for an accurate assessment of whether a bleeding diathesis is present, as well as the nature of such a bleeding tendency. Questions should therefore be aimed at determining the likelihood of a bleeding disorder being present as well as the type of the putative bleeding diathesis (is this a disorder of primary or secondary hemostasis?) and its inheritance pattern. The history should include an orderly description of bleeding during infancy and childhood, including umbilical stump bleeding (characteristic of factor XIII deficiency [see Chapter 135]), bleeding with circumcision (characteristically seen in boys with severe hemophilia A or B [see Chapter 134]), bleeding with loss of deciduous teeth, and bleeding with childhood trauma and surgeries (see Chapters 148 and 155). Bleeding with dental procedures, including wisdom tooth removal, should be explored. Questions such as “Did you have to go back for stitches? Did you awaken with a pillow covered with blood? How many days of bleeding did you experience after your dental extractions?” are more specific than “Did you bleed with tooth removal?” Patients with milder bleeding disorders may only bleed with procedures involving mucosal surfaces, due to the high levels of fibrinolytic activity at these sites. Epistaxis may be a presenting symptom of von Willebrand disease (VWD) or hereditary hemorrhagic telangiectasia (HHT) and is especially notable if it does not stop with pressure and requires either cautery or a visit to the emergency department. Other bleeding episodes, whether spontaneous or provoked, should be elucidated. Bleeding into muscles and joints is characteristic of moderate or severe disorders of humoral clotting factors[4] and some fibrinolytic defects (e.g., Quebec platelet disorder[5]), whereas mucosal bleeding is typical of disorders of primary hemostasis.[6,7] Easy bruising is a complaint voiced by many persons without underlying bleeding disorders,[2] but certain historical features are worth noting. The new onset of bruising can herald a new thrombocytopenic disorder such as immune thrombocytopenia (ITP) (see Chapter 129) or acute leukemia (see Chapters 60, 63, 66, 67 and 68), or it can point to acquired hemophilia (see Chapter 134). Bruising which only occurs over the hands and forearms in older individuals suggests the presence of senile purpura. Some cases of bruising can be explained by fragile skin caused by sun exposure, cigarette smoking, or corticosteroid use. Other cases of bruising can be provoked by use of serotonin reuptake inhibitors or herbal supplements. Lastly, there are rare syndromic bruising disorders such as the Gardner-Diamond syndrome, mostly seen in young women with underlying psychiatric disorders, with a characteristic pattern and appearance to the bruises.[8]

Each individual surgical and major dental procedure undergone by the patient should be explored in depth.[9] The details of bleeding, including timing (immediate or delayed), the need for transfusion, comments by the surgeon concerning the characteristics of the bleeding (e.g., did it start on the day of the surgery/procedure or days later?), any known anatomic sources of bleeding, etc. can illuminate the bleeding diathesis. Immediate bleeding may be more characteristic of a disorder of primary hemostasis,[6] whereas delayed bleeding is seen more in patients with deficiencies in humoral clotting factors and fibrinolytic defects.[4,5] Bleeding in patients with an underlying

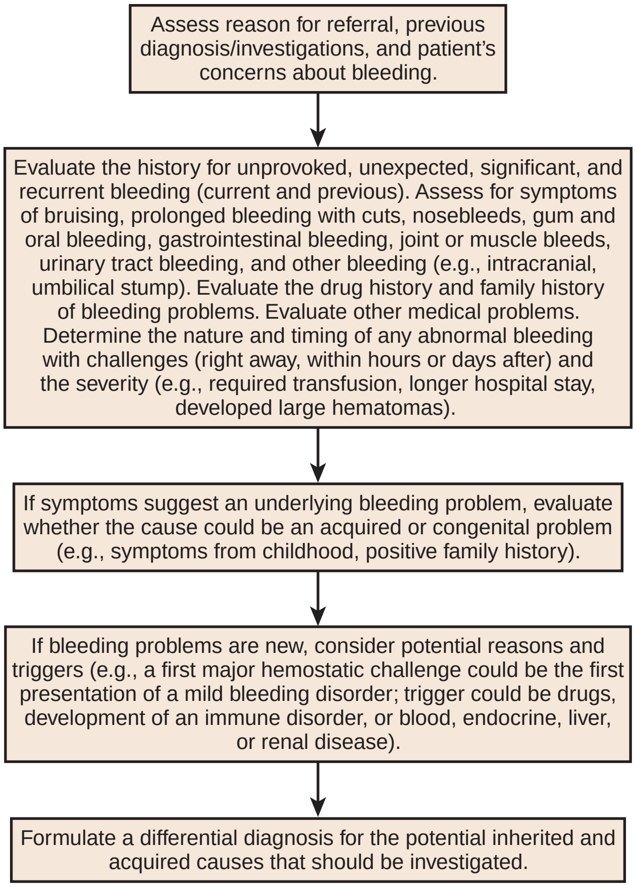

Assess reason for referral, previous diagnosis/investigations, and patient's concerns about bleeding.
Evaluate the history for unprovoked, unexpected, significant, and recurrent bleeding (current and previous). Assess for symptoms of bruising, prolonged bleeding with cuts, nosebleeds, gum and oral bleeding, gastrointestinal bleeding, joint or muscle bleeds, urinary tract bleeding, and other bleeding (e.g., intracranial, umbilical stump). Evaluate the drug history and family history of bleeding problems. Evaluate other medical problems. Determine the nature and timing of any abnormal bleeding with challenges (right away, within hours or days after) and the severity (e.g., required transfusion, longer hospital stay, developed large hematomas).
If symptoms suggest an underlying bleeding problem, evaluate whether the cause could be an acquired or congenital problem (e.g., symptoms from childhood, positive family history).
If bleeding problems are new, consider potential reasons and triggers (e.g., a first major hemostatic challenge could be the first presentation of a mild bleeding disorder; trigger could be drugs, development of an immune disorder, or blood, endocrine, liver, or renal disease).
Formulate a differential diagnosis for the potential inherited and acquired causes that should be investigated.

Figure 126.1 STEPS TO EVALUATE BLEEDING AND BRUISING PROBLEMS.

primary hemostatic defect is typically described as "diffuse oozing," without the readily identifiable bleeding source seen with a surgical mishap, such as a severed vessel. Patients with primary hemostatic defects often report that their bleeding occurred on the day of surgery,[6] rather than days later, which would suggest a coagulation factor deficiency or a fibrinolytic disorder. If a woman has bled with some procedures but not others, she should be asked if she was on estrogen-containing medications during the procedures in which she had good hemostasis, because estrogen can increase levels of von Willebrand factor (VWF), leading to normalization of hemostasis. Bleeding with some but not all procedures also suggests a mild rather than a severe disorder.[6]

Women should be carefully questioned about their menstrual history.[10,11] Duration and severity of flow[10,11] are more important than the presence or severity of cramping. Although HMB is medically defined as loss of more than 80 ml of blood per menstrual cycle,[11] few women are capable of determining this with any degree of precision. Pad or tampon use is imprecise as well because the number of sanitary products used may vary with the degree of fastidiousness of each patient. To that end, pictorial assessments of blood loss (depicting pads or tampons with varying degrees of saturation) have been devised, with scores given for numbers of products used and their saturation.[12] An underlying bleeding disorder is found in up to one-third of adolescents and adults who present with HMB.[10,13–16] Historical features correlated with a higher likelihood of an underlying bleeding disorder being found include "flooding" and "gushing" accidents, passage of clots larger than 1 cm, duration longer than 7 days, heavy flow greater than 3 days, and the development of iron deficiency.[6] While bleeding during pregnancy is less common in women with VWD, platelet function disorders, and most other bleeding disorders (exceptions include fibrinogen disorders, plasminogen activator inhibitor-1 [PAI-1] deficiency, and factor XIII deficiency), postpartum hemorrhage is more commonly seen compared with the general population.[6,7] Postpartum hemorrhage usually occurs 24 to 48 hours after delivery, and in women with bleeding problems, postpartum bleeding (lochia) can continue beyond the normal 6 weeks.[9]

A family history of bleeding should be carefully sought out, with consideration of whether consanguinity is culturally accepted by the family. The family history evaluation may require several visits to fully document, as familial memories are probed. A family history of bleeding with surgical procedures, bleeding requiring transfusions, and HMB leading to hysterectomy at a young age should be queried. However, a negative family history does not rule out a congenital bleeding disorder. Approximately one-third of all cases of hemophilia A arise from spontaneous mutations.[17] Many of the rare coagulation disorders, including deficiency of factors II, V, VII, and X (see Chapter 135), Glanzmann thrombasthenia (see Chapter 124)), and VWD type 2N (see Chapter 133), among others, are inherited in an autosomal recessive fashion,[18] and other family members may be entirely asymptomatic or bleeding problems may be limited to siblings. (See Case 1: Illustration of a Mild, Inherited Bleeding Problem.)

Certain medications and herbal and dietary supplements increase the risk of bleeding. The use of these agents may precipitate a hemorrhage in those with milder bleeding disorders. The use of aspirin and nonsteroidal antiinflammatory agents impairs primary hemostasis (see Chapter 128), and their use should be avoided prior to surgery or prior to evaluation of the hemostatic system. Their inclusion in over-the-counter products seems ubiquitous, and careful attention to cold and flu remedy use is warranted. In the Southeast United States, aspirin-containing remedies such as Goody powders or BC powders are not viewed as medications, and their use must be specifically queried.

Without treatment, severe bleeding disorders typically cause abnormal bleeding with all major hemostatic challenges.[19–21] In contrast, bleeding may not occur with every challenge in persons with milder defects.[6] Age influences the bleeding history by increasing the likelihood of exposures to hemostatic challenges and the development of sequelae such as arthropathy in patients with severe hemophilia[4] and Quebec platelet disorder.[5] A severe unexplained bleed with surgery may be considered suspicious of a bleeding problem. However, if the person reports that prior challenges did not result in bleeding, this narrows down the possibilities to a mild inherited bleeding disorder, an acquired bleeding disorder, an iatrogenic condition (e.g., bleeding while on anticoagulant therapy or a technical problem during surgery that caused bleeding), or a problem that is not related to a congenital or acquired bleeding disorder (e.g., a postpartum hemorrhage after a caesarean section from uterine atony or retained products of conception).

A bleeding disorder assessment needs to consider both familial and personal bleeding symptoms (see Case 2: Illustration of The Importance of Assessing Both Personal and Familial Bleeding Problems). However, a familial tendency toward bleeding may "pseudonormalize" bleeding symptoms and make recognition of abnormal bleeding patterns difficult. (See Case 3: Illustration of Delayed Recognition of Abnormal Bleeding.)

The timing of bleeding with challenges is evaluated to determine whether the bleeding problem reflects a common bleeding disorder, such as VWD, a platelet function disorder, an undefined mucocutaneous bleeding problem, or a rarer cause, such as a defect or

CASE 1: ILLUSTRATION OF A MILD, INHERITED BLEEDING PROBLEM

A 77-year-old man who is starting treatment for multiple myeloma was discovered to have a prolonged activated partial thromboplastin time (see Chapter 126). Review of his records indicated that the abnormality was present on a previous admission for spinal cord compression, which was treated with surgery. He required 4 units of packed red blood cells several days after this surgery because of delayed postoperative bleeding. There was no other bleeding history. He was found to have mild factor IX deficiency (see Chapter 134), unrelated to the myeloma, and his daughter proved to be a carrier of this defect.

CASE 2: ILLUSTRATION OF THE IMPORTANCE OF ASSESSING BOTH PERSONAL AND FAMILIAL BLEEDING PROBLEMS

A 24-year-old special forces paratrooper was evaluated for life-threatening bleeding. Following a routine practice jump, he felt a “pop” in his abdomen and developed progressive abdominal pain and generalized weakness. Upon evaluation, he had eventual syncope, a hemoglobin of 3 g/dL, and a CT scan showing a large abdominal wall hematoma. Arteriogram revealed extravasation of contrast material from a ruptured artery. Coil embolization was unsuccessful, and the patient became progressively hypotensive, hypothermic, and acidemic. Next of kin was contacted, and the patient’s father disclosed a history of hemophilia in the patient’s maternal uncle. The factor IX level was found to be 6%, and infusion of factor IX concentrate led to cessation of bleeding (see Chapter 134). Notably, the patient’s nickname during basic training was “Bruise.”

CASE 3: DELAYED RECOGNITION OF ABNORMAL BLEEDING

A 35-year-old woman presented with life-threatening hemorrhage following hysterectomy. She had a lifelong history of abnormal bleeding, including gum bleeding (“spat out a mouthful of blood” with brushing), abnormal bruising, and a personal and family history of heavy menses (during teenage years, mother and sister also had heavy menstrual bleeding, enough so that the mother would announce “time for new mattresses” every few years). This pattern of bleeding was felt to be “normal for the family.” The patient was eventually diagnosed with von Willebrand disease (see Chapter 133) and a qualitative platelet disorder (see Chapter 124).

CASE 4: ILLUSTRATION OF THE IMPORTANCE OF ASSESSING BLEEDING PROBLEMS OVER TIME

A 72-year-old man was referred for evaluation of a severe bleed after receiving a single dose of low-molecular-weight heparin for unconfirmed deep vein thrombosis. He had a history of a similar bleeding episode several years previously while on warfarin treatment for atrial fibrillation. There was no other bleeding history, and the patient subsequently developed a spontaneous iliopsoas bleed. He had undergone numerous surgeries earlier in life without any bleeding problems, and there was no family history of bleeding. The bleeding history suggested the possibility of an acquired bleeding problem, possibly acquired von Willebrand disease or an acquired factor deficiency. Diagnostic testing indicated that he had acquired factor XIII deficiency (see Chapter 135). This case illustrates the fact that there may be more than one risk factor for bleeding: in this case, several exposures to anticoagulants triggered bleeding in a patient with an acquired factor deficiency. On initial treatment of his iliopsoas bleed with factor XIII concentrate, there was partial neutralization of the infused factor followed by accelerated clearance, consistent with acquired factor XIII deficiency secondary to an autoantibody.

CASE 5: EVALUATION OF AN ISOLATED SYMPTOM—RECURRENT PREGNANCY LOSS WITH BLEEDING

A 32-year-old woman was referred for evaluation of a low fibrinogen level in the setting of acute placental abruption, resulting in a third pregnancy loss (this time in the third trimester). She had no prior bleeding history apart from having suffered three placental abruptions associated with severe bleeding that required transfusion. The family history was negative for bleeding problems. She had previously been investigated for thrombophilia but had not been tested for a bleeding disorder. The low fibrinogen level persisted over many months (levels of approximately 90 mg/dL), suggesting that the defect was inherited (see Chapter 135). She received fibrinogen concentrate for two subsequent pregnancies, which she carried to term and delivered without bleeding problems. This case illustrates the need to consider inherited disorders when the bleeding symptoms are unusual and severe, even if there is only one bleeding symptom. It also illustrates that prognosis is dependent on diagnosis and treatment.

deficiency in a coagulation factor or fibrinolytic protein (see Case 4: Illustration of the Importance of Assessing Bleeding Problems Over Time). Although patients will often know whether bleeding began on the day of a challenge[6] or if it started days later,[5] their recall of timing details is often better when procedures were done without general anesthesia (e.g., dental extractions, biopsies).

A newborn or child presenting with spontaneous intracranial hemorrhage or a large cephalohematoma should be investigated for severe underlying bleeding disorders, such as thrombocytopenia, hemophilia, factor XIII deficiency, other coagulation factor deficiencies, or a severe defect in platelets or VWF (see Chapter 148).[19,20,22,23] Trauma-related subdural or intracranial hemorrhage can also be a manifestation of a severe bleeding disorder.[22] In adults, ischemic strokes are more frequent than hemorrhagic strokes (see Chapter 144), although hemorrhagic strokes appear to predominate in persons with some types of bleeding disorders (e.g., Quebec platelet disorder[5]), and they can also reflect complications of antithrombotic drug treatment.

Excessive or prolonged bleeding after childbirth or pregnancy loss can be problematic for some women with bleeding problems.[6,10–12,24] In addition, severe fibrinogen disorders, PAI-1 deficiency, and factor XIII deficiency compromise carrying a pregnancy to term and need to be excluded if the patient has unexplained pregnancy losses that are associated with hemorrhagic placental abruption[19,25,26] (see Case 5: Evaluation of an Isolated Symptom—Recurrent Pregnancy Loss With Bleeding).

BLEEDING SCORE

Objective frameworks for the evaluation of bleeding symptoms have been developed to codify clinical impressions into a numeric score.[7,9,10,27] These bleeding assessment tools (BATs) were initially developed as research tools to quantify bleeding symptoms and severities. Several BATs and their subsequent versions have been refined. They have been studied and validated in specific populations, including those being evaluated for VWD, platelet disorders, symptoms of HMB, and other bleeding syptoms.[6,7,10,27,28]

Several other tools have been developed with refinements of the original “Vicenza bleeding score” with similar formats: in a grid format, specific bleeding symptoms (e.g., epistaxis, dental hemorrhage) are scored in severity from trivial to severe, and then summed to a final bleeding score. Unfortunately, despite these refinements, the shared weakness of these tools is poor sensitivity (as low as 40%), which has not been greatly improved.[29] Whether these tools are appropriate for use in the clinical setting is still debated, but because of their high negative predictive value, they are probably best for identifying patients who do not require further testing. In addition, the bleeding scores may be useful in predicting future bleeding: in a large, prospective study of VWD, a higher bleeding score is the strongest predictor of bleeding, even over other laboratory and clinical assessments.[30] Whether the bleeding score’s predictive power holds true in other mild bleeding disorders is not yet known, although some studies have not shown the scores to be predictive of future bleeding.[27] Finally, in response to the variability of bleeding scores utilized in clinical studies that make comparing these studies challenging, the International Society on Thrombosis and Haemostasis (ISTH) has proposed a new consensus BAT with the goal of expanding the understanding of bleeding rates and phenotypes more precisely in congenital bleeding disorders.[9] Despite the ease of use and the good negative predictive value of various BATs, the judgement of an experienced clinician remains supreme. BATs have also been used to estimate bleeding risks as likelihood or odds ratios (relative to unaffected relatives or the general population) for experiencing different kinds of bleeding symptoms (e.g., bleeding with surgery, menses longer than 7 days, joint bleeds) and also risks for needing certain types of treatments, such as blood transfusion.[5,6]

THE PHYSICAL EXAMINATION

A careful physical examination may provide useful clues to the etiology of the patient's bleeding (Table 126.1). Examining the skin may reveal petechiae, indicating thrombocytopenia, or the characteristic ecchymoses, scarring, and lax skin seen with senile purpura. Patients with scurvy have characteristic perifollicular hemorrhages and "corkscrew" hairs. Telangiectasias around the lips or on the fingertips may signal the presence of HHT. Some patients with vascular Ehlers-Danlos syndrome exhibit characteristic "cigarette paper" skin, which is thin, crepey, and translucent.[31] Bruises should be examined for their number, pattern, and age—if they are all the same color and lividity, they may have all occurred simultaneously. It is important to determine whether the pattern of distribution is indicative of self-infliction, as can be seen sometimes in some patients with Munchausen syndrome. Oculocutaneous albinism is associated with several platelet disorders, including the Hermansky-Pudlak syndrome and the Chédiak-Higashi syndrome (see Chapter 124).[18] Splenomegaly can be associated with thrombocytopenia and may indicate underlying cirrhosis. Other stigmata of liver disease, such as spider angiomata, gynecomastia, asterixis, and jaundice, also suggest that the patient may have an associated liver coagulopathy. Joint hypermobility and skin hyperelasticity may be found in hypermobile Ehlers-Danlos syndrome, although not all patients with this disorder manifest the skin findings.[31] A harsh systolic murmur may indicate severe aortic stenosis, which can cause an acquired type 2 VWD, with associated GI bleeding from arteriovenous malformations (see Chapter 133).[32] An enlarged tongue, carpal tunnel syndrome, and periorbital purpura may point to amyloidosis (see Chapter 93), which can be associated with an acquired deficiency of factor X and many other clotting proteins, including factor V, VWF, α_2-antiplasmin, and PAI-1.[33–36] See Case 6 for an illustration of physical examination leading to clues to the cause of worsening bleeding.

CASE 6: THE PHYSICAL EXAMINATION ILLUMINATED THE CAUSE OF WORSENING BLEEDING SYMPTOMS

A 58-year-old man with type 2A von Willebrand disease (see Chapter 133) was evaluated for worsening gastrointestinal (GI) bleeding. He had a history of multiple GI bleeds attributed to arteriovenous malformations. Despite thrice weekly infusions of von Willebrand concentrate, his transfusion requirements were increasing from 2 units every 3–4 months to every 7–10 days. Evaluation revealed a new harsh systolic murmur. Echocardiography showed large mitral and aortic vegetations, and blood cultures grew *Staphylococcus epidermidis*, likely from his indwelling catheter. After valve replacement, his transfusion requirements markedly decreased, perhaps as the result of improvement in shearing of high molecular weight von Willebrand factor (VWF) multimers.

EPIDEMIOLOGY AND GENETICS

An understanding of the epidemiology of bleeding problems requires distinction between symptoms that rarely represent a pathologic condition and do not require investigation or therapy (e.g., isolated "easy bruising," brief nosebleeds) and those that are more suspicious, require treatment, and/or are highly predictive of a bleeding problem (Table 126.2).[1–3] Some individuals have multiple risk factors for bleeding (e.g., low VWF levels, exposure to drugs that inhibit platelet function after a surgical procedure associated with a high risk for bleeding).[3] Although some symptoms, such as nosebleeds, easy bruising, and HMB, are quite prevalent in the general population (10% or more report these symptoms),[2] these problems are more frequent and more severe in individuals with bleeding disorders.[5–7,12] In addition, cohort studies have shown that females with platelet disorders have more mucocutaneous bleeding symptoms than affected males.[6] HMB requiring treatment is common among women, and

TABLE 126.1 Physical Exam Clues to Bleeding Manifestations

Corkscrew hairs, perifollicular hemorrhages (typical of scurvy)
Oral blood blisters, particularly if the patient has thrombocytopenia
Ecchymoses, hematomas, and skin pigmentation changes because of recurrent bleeds
Ecchymoses limited to sun-exposed areas of the skin
Signs of active bleeding from a site of trauma or an incision, including excessive blood loss into drains
Sequelae of previous bleeds in individuals known or suspected to have a severe bleeding disorder, such as muscle wasting and arthropathy, neurologic abnormalities from prior intracranial, or compartment syndrome bleeds
Pallor arising from anemia: the palms are usually notably pale when the hemoglobin is less than 10 g/dL
Signs of an underlying hematologic disorder, such as lymphadenopathy and/or splenomegaly
Signs of acute or chronic liver disease, such as jaundice, hepatomegaly, spider nevi, palmar erythema, or Dupuytren contractures
Signs of an endocrine disorder, such as hypothyroidism or Cushing syndrome
Vascular lesions such as telangiectasia on the face, lips, tongue, buccal mucosa, or fingers which can suggest hereditary hemorrhagic telangiectasia
Joint hypermobility and skin laxity suggesting a collagen disorder
Signs suggestive of a syndromic bleeding disorder (albinism, hearing impairment, absent radii)

TABLE 126.2 Differential Diagnosis of Bleeding Problems

Major Categories	Comments
No bleeding disorder	Symptoms do not reflect a bleeding disorder and have another explanation (e.g., a surgical bleed not caused by a bleeding disorder).
Possible bleeding disorder	The laboratory findings are nondiagnostic, and the bleeding history is considered equivocal (e.g., unexplained serious bleed with one surgical procedure; unexplained heavy menstrual bleeding without other bleeding problems).
Definite bleeding disorder, undefined or indeterminate type (commonly referred to as bleeding of unknown cause)	The bleeding history is consistent with a bleeding disorder; however, the laboratory findings are nondiagnostic. Commonly the bleeding history resembles mild to moderate defects in platelet function or von Willebrand factor. The diagnosis should only be made once an adequate evaluation for common bleeding disorders (e.g., for von Willebrand disease, platelet aggregation defects, and platelet dense granule deficiency) is completed. If testing is not complete, the classification should indicate the types of conditions excluded or not excluded (e.g., mild mucocutaneous bleeding problem, von Willebrand disease excluded, mild mucocutaneous bleeding problem, platelet dense granule deficiency not yet excluded).
Definite bleeding disorder with a defined cause	The symptoms and laboratory findings are considered diagnostic of a bleeding disorder. Tables 126.3 and 126.4 summarize many of the potential inherited and acquired causes.

TABLE 126.3 Differential Diagnosis of Congenital Bleeding Disorders

Disorder	Comments
Fibrinogen deficiency or dysfunction	Deficiencies can be mild-moderate hypofibrinogenemia or severe afibrinogenemia. Fibrinogen function is abnormal in dysfibrinogenemias, which can present with bleeding, thrombosis, or both. Functional fibrinogen levels are reduced much more than immunofibrinogen levels in dysfibrinogenemias, whereas fibrinogen function and immunofibrinogen levels are similarly reduced in hypofibrinogenemia.
X-linked coagulation factor deficiencies—hemophilia	Presentation is influenced by the degree of deficiency. Factor VIII deficiency is more common than factor IX deficiency. If factor VIII is low, von Willebrand disease (especially type 2N) should be excluded as the cause.
Rarer, coagulation factor deficiencies	Deficiencies can affect factors XI, V, II, VII, or X, and the presentation is dependent on the severity of the deficiency. Hereditary deficiencies of multiple coagulation factors are rare (e.g., of factors V and VIII, or multiple vitamin K–dependent coagulation factors for congenital defects impairing γ-carboxylation) and can easily be excluded by measuring multiple factors.
Fibrinolytic defects	Causes include disorders caused by loss of function, such as α_2-antiplasmin or PAI-1 deficiency, and by gain-of-function defects, such as Quebec platelet disorder (overexpression of urokinase plasminogen activator in megakaryocytes).
von Willebrand disease	Causes include quantitative (partial type 1 to severe type 3) and qualitative defects (loss of function in type 2M and 2A, gain of function in type 2B and platelet type). Type 1 von Willebrand disease can be confused with low von Willebrand factor levels (e.g., because of blood group O) and its diagnosis needs to consider the bleeding history. Type 1C is a quantitative disorder due to abnormal clearance of VWF.
Platelet disorders	These conditions can affect platelet number, function, or both. The most common type of platelet function disorder includes disorders that impair aggregation responses and/or cause dense granule deficiency. Disorders of platelet function are commonly subclassified by the nature of the defect, such as the following: 1. Defects of membrane receptors for adhesive proteins (e.g., Glanzmann thrombasthenia and Bernard-Soulier syndrome) or agonists (e.g., $P2Y_{12}$ deficiency) 2. Defects of signaling or secretion (the largest subcategory), which include disorders caused by transcription factor mutations 3. Cytoskeletal defects (e.g., MYH9-related disorders) 4. Storage pool disorders (e.g., gray platelet syndrome, dense granule deficiency, αγ-storage pool deficiency, Quebec platelet disorder) 5. Defects of procoagulant function (e.g., Scott syndrome)
Vascular disorders	Congenital vascular malformation, including hereditary hemorrhagic telangiectasia, Ehlers-Danlos syndrome.

PAI-1, Plasminogen activator inhibitor 1.

it can sometimes be life-threatening in those with severe bleeding disorders. With moderate or severe disorders, there is less uncertainty about the disease prevalence than there is for milder disorders (e.g., type 1 VWD), where there is ongoing debate about the diagnostic criteria that define a pathologic abnormality (see Chapter 133). There is less information on the prevalence of disorders that require complex tests for diagnosis and have many potential causes (e.g., platelet function disorders). Bleeding of unknown cause (BUC) (i.e., definite bleeding problems despite normal or nondiagnostic test findings) has emerged as a common cause of mucocutaneous and postsurgical bleeding.[37]

The process of referral for bleeding makes it likely that the person has had some bleeding symptoms that may or may not reflect an underlying congenital (Table 126.3) or acquired (Table 126.4) bleeding problem.[1] Because of this referral bias, the prevalence of bleeding disorders in tertiary referral clinics is much higher than in the general population. The prevalence of inherited bleeding disorders in the general population is quite low (from 0.00023% to more than 1% for VWD, with much lower estimates based on studies of bleeding disorder clinic cases compared with those derived from population screening studies that used higher cutoffs to define quantitative deficiencies; 0.005% to 0.01% for hemophilia; and much lower for other coagulation factor deficiencies).[38] Consequently, unselected screening is not recommended. Founder effects in populations and cultural acceptance of consanguinity can alter the prevalence of both common and rare inherited bleeding disorders.

A person with a first-degree relative with an autosomal dominant bleeding problem or with a sibling with a recessively inherited disorder has a higher pretest probability of an inherited bleeding disorder. When assessing the relative of someone with a positive family history of a bleeding problem, it is important to realize that the person may have the same condition, with or without additional defects, or a different bleeding problem. Furthermore, it is common for affected family members to show some variability in the severity of their bleeding symptoms. Index cases typically have more severe bleeding symptoms (illustrated in reference[6]). Accordingly, laboratory investigations are often used to assess family members at high risk, even in the absence of a remarkable personal bleeding history. Patients may appear to have syndromic findings, but it is important to determine whether the separate findings on history and physical examination are coinherited and thus represent the syndrome—or are independently inherited and thus nonsyndromic. (See Case 7: Symptoms Seemingly Linked, But Not.)

CASE 7: SYMPTOMS SEEMINGLY LINKED, BUT NOT

An 18-year-old woman from Puerto Rico presented for evaluation of potential Hermansky-Pudlak syndrome (see Chapter 123), due to a lifelong history of abnormal mucocutaneous and postprocedural bleeding as well as oculocutaneous albinism. On further questioning, there was a family history of both albinism and abnormal bleeding, but these two features were not linked, (i.e., some family members with albinism did not bleed, and others who bled did not have albinism). Genetic testing was negative for Hermansky-Pudlak syndrome, and the patient was subsequently given the diagnosis of a qualitative platelet disorder, based on platelet function testing.

TABLE 126.4 Differential Diagnosis of Acquired Bleeding Problems

Disorder	Comments
Drug induced	Aspirin, NSAIDs, other platelet function inhibitors (e.g., $P2Y_{12}$ and $\alpha_{IIb}\beta_3$ inhibitors), anticoagulants, fibrinolytic drugs, and antidepressants are common causes.
Acquired factor deficiencies	The causes can be immune (e.g., acquired factor VIII deficiency, acquired factor V deficiency) or nonimmune. Reductions in multiple factors can result from vitamin K deficiency, treatment with vitamin K antagonists, liver disease, hemodilution, and rarely snakebites. Severe acquired hypofibrinogenemia is commonly caused by a postpartum coagulopathy or severe liver disease but can also reflect hyperfibrinolysis. Prothrombin deficiency occurs with some lupus anticoagulants. Amyloidosis can cause an acquired factor X deficiency, which may be associated with reductions in other coagulation factors synthesized in the liver if the liver is involved.
Disseminated intravascular coagulation	The manifestations can include thrombocytopenia, consumption of coagulation factors, including fibrinogen, and impairment of hemostatic mechanisms from the fibrin/fibrinogen degradation products. Causes are wide ranging and include postpartum consumptive states, severe head injury, prostate and other cancers, and snakebites.
Acquired von Willebrand disease	The cause can be immune (often in association with an IgG paraprotein) or nonimmune (e.g., increased proteolysis of von Willebrand factor with stenotic aortic valvular disease).
Immune thrombocytopenia	Bleeding is usually influenced by the severity of the thrombocytopenia. Some autoantibodies interfere with platelet membrane receptor function, causing bleeding disproportionate to the thrombocytopenia.
Non–drug-induced, acquired platelet function disorders	The cause can be immune (see earlier) or nonimmune, typically from bone marrow disorders, although they can be secondary to Cushing syndrome or hypothyroidism.
Liver disease	Liver disease can cause thrombocytopenia, deficiencies of coagulation factors, hypofibrinogenemia and dysfibrinogenemia, and increased fibrinolysis. In mild liver disease, factor VII and sometimes factors XI and XII are low. Fibrinogen is often increased in early liver disease, and if low, the finding suggests severe liver disease.
Renal disease	Anemia is an important predictor of uremic bleeding. Uremic bleeding is typically associated with severe renal impairment.
Hypothyroidism	Hypothyroidism can cause an acquired von Willebrand disease and acquired defects in platelet function.
Cushing syndrome	This syndrome should be suspected when there are symptoms and findings suggestive of Cushing syndrome or treatment with systemic glucocorticoids.
Surgical bleeding	This is often a diagnosis of exclusion, although the procedural notes sometimes document that a technical problem was encountered that led to abnormal bleeding.
Vitamin K deficiency	Newborns are at risk, as are individuals with malabsorption and/or receiving broad-spectrum antibiotics that reduce vitamin K production by reducing gut bacteria. Older adults are also at greater risk for developing vitamin K deficiency because of reduced stores from poorer intake of vitamin K. If the patient does not respond to parenteral vitamin K, other causes should be considered.
Vitamin C deficiency (scurvy)	This diagnosis should be considered when there is lethargy with skin and gum bleeding (perifollicular hemorrhages, gum bleeding with swelling). The condition is rare in high-income countries. The cause is usually a very poor diet or malabsorption. Patients with end-stage renal disease who are nonadherent to vitamin repletion are at risk because vitamin C is removed with hemodialysis.

IgG, Immunoglobulin G; *NSAID*, nonsteroidal antiinflammatory drug.

LABORATORY MANIFESTATIONS

The general investigations that are appropriate for most assessments of bleeding problems include[1] a complete blood count with differential and peripheral smear,[2] a ferritin level to assess for iron deficiency,[3] a blood group and antibody screen, particularly for individuals with prior pregnancies or transfusions and upcoming surgery or major dental procedures, and[4] tests of renal function (creatinine) if antifibrinolytic therapy will be considered (because the dosage is dependent on renal function) and/or an MYH9-related disorder is suspected (see box on The Laboratory Manifestations of Bleeding Disorders).[1] The bleeding time test is no longer recommended because of its technical limitations and poor sensitivity to common bleeding disorders (see Chapter 127).[1] Electrolyte levels merit monitoring when repeated doses of desmopressin therapy are given and/or if the fluid balance is difficult to assess. Tests of liver function are warranted if the person has risks for transfusion-acquired, chronic liver disease. Tests for liver and thyroid disease (particularly hypothyroidism) can be helpful if the history suggests an acquired bleeding problem of unknown etiology.[1] Screening for Cushing syndrome (24-hour urine test for free cortisol) should be restricted to patients with acquired bleeding problems that suggest this possibility (e.g., bleeding associated with

THE LABORATORY MANIFESTATIONS OF BLEEDING DISORDERS

The laboratory manifestations of bleeding disorders can include abnormalities from the following:
1. The underlying hemostatic defect.
2. Bleeding complications (e.g., anemia, iron deficiency, coagulopathy secondary to hemodilution after resuscitation for a massive bleed, development of red cell antibodies after transfusion).
3. False-positive abnormalities (e.g., prolonged aPTT caused by incidental mild factor XII deficiency, which is found in approximately 1 of 200 patients (see Chapter 135), or a lupus anticoagulant (see Chapter 139), which can be a transient finding in approximately 5% of hospitalized patients).
4. Extremes of normal variation (e.g., slightly reduced von Willebrand factor levels in an individual who is blood group O, absent secondary aggregation with epinephrine in adjusted platelet rich–plasma aggregation studies).

obesity, the development of type 2 diabetes, hypertension, striae, and/or changes in physical appearance).[1]

The investigations for a bleeding problem typically also include screening tests (prothrombin time [PT], [aPTT], thrombin clotting time, and fibrinogen level), which are inexpensive tests, that detect acquired coagulopathies much more commonly than they detect inherited bleeding disorders because of differences in prevalence (see Chapter 126).[1] These investigations are useful as a baseline for individuals at risk for developing a dilutional coagulopathy from bleeding and as initial investigations of a possible inherited or acquired coagulation disorder. Abnormalities, if detected, require further evaluation to determine if the cause is a fibrinogen disorder or a deficiency of one or more coagulation factors.[1] Screening tests for VWD are warranted for individuals with a personal or familial history of mucocutaneous bleeding.[1] Platelet function disorders should be evaluated by aggregation tests and tests for dense granule deficiency, if available, because these tests are useful for assessing common bleeding disorders.[1,39] More detailed analyses for individuals with personal and family histories of bleeding disorders might include factor VIII measured by chromogenic assay.[1] Approximately 40% of individuals with mild hemophilia A have a discrepancy between the factor VIII activity measured by the one stage and chromogenic methods.[40] In some cases, the aPTT may be normal, the one stage factor VIII level may be normal, but the chromogenic factor VIII level can be low (see Case 8: Not All Cases of Hemophilia A Can Be Diagnosed with One Stage Factor VIII Assays). Chapter 127 provides more detail on the specific diagnostic tests that are appropriate for a laboratory workup of bleeding problems.

PROGNOSIS

The prognosis for bleeding problems depends on the severity and nature of the hemostatic defect, exacerbating factors, and whether these problems can be readily corrected (e.g., vitamin K to correct deficiency, discontinuing aspirin) or if they require hemostatic therapies, such as factor concentrates and/or drugs (e.g., desmopressin, tranexamic acid, or aminocaproic acid). Life expectancy is generally normal, even with the most severe bleeding disorders (e.g., severe hemophilia). The prognosis for some rare disorders significantly improves after puberty (e.g., factor IX Leyden). A few show progressive worsening over time (e.g., development of aplasia from congenital amegakaryocytic thrombocytopenia,[41] transformation of thrombocytopenia from an inherited RUNX1 mutation into myelodysplasia or acute myeloid leukemia[6]). Other disorders (e.g., acquired hemophilia) may go into complete remission after immunosuppressive treatment with risk for later relapses.[42]

CASE 8: NOT ALL CASES OF HEMOPHILIA A CAN BE DIAGNOSED WITH ONE STAGE FACTOR VIII ASSAYS

A 57-year-old woman presented for evaluation of bleeding following a face-lift. For several weeks after this procedure, she required extraction of facial hematomas. Her bleeding history was otherwise notable for absence of bleeding with laparoscopic hysterectomy or C-section together with a history of gum bleeding and bleeding with dental procedures. She had normal menses and no history of postpartum hemorrhage. She was on estrogen replacement therapy. Family history was notable for “factor VIII deficiency” in her father and a paternal uncle. Her father died of bleeding following a Whipple procedure without factor VIII repletion. Her sister had significant heavy menstrual bleeding, bleeding with multiple procedures, and postpartum hemorrhage. Her PT and aPTT were normal. Platelet function analyzer (PFA-100) testing showed normal closure times. One stage factor VIII activity was normal at 55%. Chromogenic factor VIII activity was low at 18%. Genetic analysis showed an *F8* c.5954G>A (p.Arg1985Gln), a pathogenic variant known to occur in the A3 region of the F8 gene, associated with mild hemophilia A and discrepant factor VIII assays.

Mild platelet function disorders, mild type 1 VWD, and many common undefined conditions that cause mucocutaneous bleeding usually respond well to prophylactic desmopressin and antifibrinolytic therapy, which is given to prevent bleeding with major surgical and dental procedures or bleeding with childbirth.[37,43,44] Nonetheless, it is important to have a specific diagnosis to manage situations in which desmopressin therapy alone is insufficient to control bleeding.

Women with bleeding disorders have a similar prognosis to men, although they often have a greater burden of symptoms because of HMB and childbirth-related bleeding, and they often report more mucocutaneous bleeding.[7,39] Their outcomes with pregnancy and childbirth appear to be more similar to individuals without bleeding disorders when they receive treatment to control bleeding with delivery.[6,24] However, some disorders have sufficiently good outcomes with childbirth that treatment for uncomplicated childbirth is not required (e.g., for Quebec platelet disorder[5]). Childbirth plans for women with bleeding disorders require consideration of the options for pain management and avoidance of procedures, such as spinal or epidural anesthesia, to limit bleeding risks unless the defect can be readily corrected.[45]

THERAPY

An individual’s bleeding history is an important consideration whenever formulating therapeutic plans to control and minimize bleeding risks. Some symptoms do not warrant therapy (e.g., bruising), whereas treatment is important for controlling HMB and for preventing and limiting challenge-related bleeding (e.g., from major or minor surgery and dental and endoscopic procedures, and traumas). For severe disorders, prophylactic treatment is warranted to prevent spontaneous bleeding and to limit challenge-related bleeding, which can be severe. The focus of therapy for some acquired conditions (e.g., acquired hemophilia) is immunosuppressive therapy to achieve a remission while managing any acute bleeding that warrants therapy.[42] For individuals with mild bleeding disorders, who have undergone multiple prior surgical procedures without bleeding, it may be appropriate to have treatment available (e.g., desmopressin on “standby”) that is to be administered if and when abnormal bleeding occurs, provided that the procedural-related risks of bleeding are small and readily managed (e.g., biopsy under local anesthetic) while waiting for the medication to have an effect.

For women with bleeding disorders, there are general treatments that can be considered for symptomatic management regardless of the type of bleeding disorder. For example, options for HMB management include oral contraceptives, antifibrinolytic drugs, and hormone-releasing intrauterine devices.[11] When HMB limits lifestyle and further pregnancies are not desired, surgical options (endometrial ablation, hysterectomy) may be preferred options, particularly when menopause is not imminent.

General management of bleeding often includes supportive care, correction and prevention of anemia (e.g., iron replacement for iron deficiency), and immunization against viruses that may be acquired by blood transfusion (e.g., hepatitis A and B) (see Case 9: Illustration of Changes in Bleeding Outcomes With Treatment). If the patient is immobilized or is undergoing a procedure with significant risks for thrombosis, there should be plans for thromboprophylaxis if the defect can be corrected during this treatment (e.g., by factor replacement therapy for hemophilia or VWD, fibrinolytic inhibitor therapy for Quebec platelet disorder) or for alternative thromboprophylaxis if this is not possible (e.g., use of pneumatic compression devices without anticoagulant drugs if the treatment will provide only brief hemostatic correction [e.g., desmopressin, platelet transfusions]).[46] Other measures to limit bleeding risks from bleeding disorders, with surgery, include the avoidance of intramuscular injections, spinal and epidural anesthesia, and NSAIDs for pain management. In older individuals with bleeding disorders and symptomatic atherosclerotic disease (e.g., angina), aspirin therapy may be appropriate, with increased vigilance for signs and symptoms of significant bleeding.[46]

CASE 9: ILLUSTRATION OF CHANGES IN BLEEDING OUTCOMES WITH TREATMENT

A 38-year-old woman was evaluated for a bleeding disorder. Her family physician had already excluded the possibility of von Willebrand disease. The patient had a long-standing history of massive bruises, often without recollection of trauma, prolonged nosebleeds requiring medical attention since early childhood, prolonged bleeding from minor cuts, and severe bleeding requiring blood transfusions with many surgical procedures. She had a history of recurrent iron deficiency anemia, HMB requiring medical therapies, and immediate postpartum bleeding. Her father had a history of bleeding problems, but the cause of the bleeding problem in the family was unknown. The history suggested an inherited disorder, possibly a platelet function disorder or a form of von Willebrand disease. The testing indicated that she had a platelet secretion defect with multiple aggregation abnormalities, with no evidence of von Willebrand disease. She underwent additional surgical procedures, using desmopressin treatment to reduce her bleeding risks, with no abnormal bleeding. Her HMB was controlled with tranexamic treatment. She self-administered desmopressin treatment to control nosebleeds, with good effect. This case illustrates that treatment affects bleeding outcomes and the importance of evaluating for common defects in hemostasis.

For more information on therapies for specific disorders, see the chapters on hemophilia (Chapter 134), rare coagulation factor deficiencies (Chapter 135), VWD (Chapter 133), and platelet disorders (Chapters 124 and 128).

SUMMARY

The evaluation of a patient with a suspected bleeding disorder is complex and potentially confusing. A thorough history (and perhaps in the future, a BAT score) and physical examination will help the clinician determine the pretest probability of an underlying bleeding diathesis and thus guide the laboratory investigation. Despite these tests, some patients with a convincing personal and family history of abnormal bleeding may have laboratory evaluations that remain unrevealing. Therapy should be guided toward replacing the deficient hemostatic element, either as needed or prophylactically, depending on the individual circumstances.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for practicing clinicians.

Borzutzky C, Jaffray J. Diagnosis and management of heavy menstrual bleeding and bleeding disorders in adolescents. *JAMA Pediatr*. 2020;174:186–194.

Brunet J, Badin M, Chong M, et al. Bleeding risks for uncharacterized platelet function disorders. *Res Pract Thromb Haemost*. 2020;4:799–806.

Brunet JG, Iyer JK, Badin MS, et al. Electron microscopy examination of platelet whole mount preparations to quantitate platelet dense granule numbers: implications for diagnosing suspected platelet function disorders due to dense granule deficiency. *Int J Lab Hematol*. 2018;40:400–407.

Castaman G, Giacomelli SH, Biasoli C, et al. Risk of bleeding and thrombosis in inherited qualitative fibrinogen disorders. *Eur J Haematol*. 2019;103:379–384.

Chalmers EA, Alamelu J, Collins PW, et al. Intracranial haemorrhage in children with inherited bleeding disorders in the UK 2003–2015: a national cohort study. *Haemophilia*. 2018;24:641–647.

Fasulo MR, Biguzzi E, Abbattista M, et al. The ISTH bleeding assessment tool and the risk of future bleeding. *J Thromb Haemost*. 2018;16:125–130.

Federici AB, Bucciarelli P, Castaman G, et al. The bleeding score predicts clinical outcomes and replacement therapy in adults with von Willebrand disease. *Blood*. 2014;123:4037–4044.

Gresele P, Orsini S, Noris P, et al. Validation of the ISTH/SSC bleeding assessment tool for inherited platelet disorders: a communication from the Platelet Physiology SSC. *J Thromb Haemost*. 2020;18:732–739.

Hayward CPM. How I investigate for bleeding disorders. *Int J Lab Hematol*. 2018;40(Suppl 1):6–14.

Hermans C, Kulkarni R. Women with bleeding disorders. *Haemophilia*. 2018;24(Suppl 6):29–36.

Jain S, Zhang S, Acosta M, Malone K, et al. Prospective evaluation of ISTH-BAT as a predictor of bleeding disorder in adolescents presenting with heavy menstrual bleeding in a multidisciplinary hematology clinic. *J Thromb Haemost*. 2020;18:2542–2550.

James P, Salomon O, Mikovic D, Peyvandi F. Rare bleeding disorders—bleeding assessment tools, laboratory aspects and phenotype and therapy of FXI deficiency. *Haemophilia*. 2014;20(Suppl 4):71–75.

Kallen ME, Dulau-Florea A, Wang W, Calvo KR. Acquired and germline predisposition to bone marrow failure: diagnostic features and clinical implications. *Semin Hematol*. 2019;56:69–82.

Kizilocak H, Young G. Diagnosis and treatment of hemophilia. *Clin Adv Hematol Oncol*. 2019;17:344–351.

Magnay JL, O'Brien S, Gerlinger C, Seitz C. Pictorial methods to assess heavy menstrual bleeding in research and clinical practice: a systematic literature review. *BMC Women's Health*. 2020;20:24.

Mauer AC, Khazanov NA, Levenkova N, et al. Impact of sex, age, race, ethnicity and aspirin use on bleeding symptoms in healthy adults. *J Thromb Haemost*. 2011;9:100–108.

Mauser-Bunschoten EP, Kadir RA, Laan ETM, et al. Managing women-specific bleeding in inherited bleeding disorders: a multidisciplinary approach. *Haemophilia*. 2021;27:463–469.

Megy K, Downes K, Simeoni I, et al. Curated disease-causing genes for bleeding, thrombotic, and platelet disorders: communication from the SSC of the ISTH. *J Thromb Haemost*. 2019;17:1253–1260.

Mumford AD, O'Donnell J, Gillmore JD, et al. Bleeding symptoms and coagulation abnormalities in 337 patients with AL-amyloidosis. *Br J Haematol*. 2000;110:454–460.

Orsini S, Noris P, Bury L, et al. Bleeding risk of surgery and its prevention in patients with inherited platelet disorders. *Haematologica*. 2017;102:1192–1203.

Rodeghiero F, Tosetto A, Abshire T, et al. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. *J Thromb Haemost*. 2010;8:2063–2065.

Rosen MW, Weyand AC, Pennesi CM, et al. Adolescents presenting to the emergency department with heavy menstrual bleeding. *J Pediatr Adolesc Gynecol*. 2020;33:139–143.

Saes JL, Verhagen MJA, Meijer K, et al. Bleeding severity in patients with rare bleeding disorders: real-life data from the RBiN study. *Blood Adv*. 2020;4:5025–5034.

Screening and management of bleeding disorders in adolescents with heavy menstrual bleeding: ACOG Committee Opinion, Number 785. *Obstet Gynecol*. 2019;134:e71-e83.

Sharma R, Flood VH. Advances in the diagnosis and treatment of Von Willebrand disease. *Hematology Am Soc Hematol Educ Program*. 2017;2017:379–384.

Sridharan M, Ali U, Hook CC, et al. The Mayo Clinic experience with psychogenic purpura (Gardner–Diamond syndrome). *Am J Med Sci*. 2019;357:411–420.

Srivaths LV, Zhang QC, Byams VR, et al. Differences in bleeding phenotype and provider interventions in postmenarchal adolescents when compared to adult women with bleeding disorders and heavy menstrual bleeding. *Haemophilia*. 2018;24:63–69.

Thomas W, Downes K, Desborough MJR. Bleeding of unknown cause and unclassified bleeding disorders; diagnosis, pathophysiology and management. *Haemophilia*. 2020;26(6):946–957.

Tiede A, Klamroth R, Scharf RE, et al. Prognostic factors for remission of and survival in acquired hemophilia A (AHA): results from the GTH-AH 01/2010 study. *Blood*. 2015;125:1091–1097.

Tosetto A, Castaman G, Rodeghiero F. Bleeders, bleeding rates, and bleeding score. *J Thromb Haemost*. 2013;11(Suppl 1):142–150.

REFERENCES

1. Hayward CPM. How I investigate for bleeding disorders. *Int J Lab Hematol.* 2018;40(Suppl 1):6–14.
2. Mauer AC, Khazanov NA, Levenkova N, Tian S, Barbour EM, Khalida C, et al. Impact of sex, age, race, ethnicity and aspirin use on bleeding symptoms in healthy adults. *J Thromb Haemost.* 2011;9:100–108.
3. Barbosa ACN, Montalvao SAL, Barbosa KGN, Colella MP, Annichino-Bizzacchi JM, Ozelo MC, et al. Prolonged APTT of unknown etiology: a systematic evaluation of causes and laboratory resource use in an outpatient hemostasis academic unit. *Res Pract Thromb Haemost.* 2019;3:749–757.
4. Kizilocak H, Young G. Diagnosis and treatment of hemophilia. *Clin Adv Hematol Oncol.* 2019;17:344–351.
5. McKay H, Derome F, Haq MA, Whittaker S, Arnold E, Adam F, et al. Bleeding risks associated with inheritance of the Quebec platelet disorder. *Blood.* 2004;104:159–165.
6. Brunet J, Badin M, Chong M, Iyer J, Tasneem S, Graf L, et al. Bleeding risks for uncharacterized platelet function disorders. *Res Pract Thromb Haemost.* 2020;4:799–806.
7. Gresele P, Orsini S, Noris P, Falcinelli E, Alessi MC, Bury L, et al. Validation of the ISTH/SSC bleeding assessment tool for inherited platelet disorders: a communication from the Platelet Physiology SSC. *J Thromb Haemost.* 2020;18:732–739.
8. Sridharan M, Ali U, Hook CC, Nichols WL, Pruthi RK. The Mayo Clinic experience with psychogenic purpura (Gardner–Diamond syndrome). *Am J Med Sci.* 2019;357:411–420.
9. Rodeghiero F, Tosetto A, Abshire T, Arnold DM, Coller B, James P, et al. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. *J Thromb Haemost.* 2010;8:2063–2065.
10. Jain S, Zhang S, Acosta M, Malone K, Kouides P, Zia A. Prospective evaluation of ISTH-BAT as a predictor of bleeding disorder in adolescents presenting with heavy menstrual bleeding in a multidisciplinary hematology clinic. *J Thromb Haemost.* 2020;18:2542–2550.
11. Screening and management of bleeding disorders in adolescents with heavy menstrual bleeding: ACOG Committee Opinion, Number 785. *Obstet Gynecol.* 2019;134:e71-e83.
12. Magnay JL, O'Brien S, Gerlinger C, Seitz C. Pictorial methods to assess heavy menstrual bleeding in research and clinical practice: a systematic literature review. *BMC Women's Health.* 2020;20:24.
13. Borzutzky C, Jaffray J. Diagnosis and Management of Heavy Menstrual Bleeding and Bleeding Disorders in Adolescents. *JAMA Pediatr.* 2020;174:186–194.
14. Rosen MW, Weyand AC, Pennesi CM, Stoffers VL, Bourdillon CM, George JS, et al. Adolescents presenting to the emergency department with heavy menstrual bleeding. *J Pediatr Adolesc Gynecol.* 2020;33:139–143.
15. O'Brien B, Mason J, Kimble R. Bleeding disorders in adolescents with heavy menstrual bleeding: the Queensland Statewide Paediatric and Adolescent Gynaecology Service. *J Pediatr Adolesc Gynecol.* 2019;32:122–127.
16. Srivaths LV, Zhang QC, Byams VR, Dietrich JE, James AH, Kouides PA, et al. Differences in bleeding phenotype and provider interventions in postmenarchal adolescents when compared to adult women with bleeding disorders and heavy menstrual bleeding. *Haemophilia.* 2018;24:63–69.
17. Kasper CK, Lin JC. Prevalence of sporadic and familial haemophilia. *Haemophilia.* 2007;13:90–92.
18. Megy K, Downes K, Simeoni I, Bury L, Morales J, Mapeta R, et al. Curated disease-causing genes for bleeding, thrombotic, and platelet disorders: communication from the SSC of the ISTH. *J Thromb Haemost.* 2019;17:1253–1260.
19. Peyvandi F, Menegatti M, Palla R. Rare bleeding disorders: worldwide efforts for classification, diagnosis, and management. *Semin Thromb Hemost.* 2013;39:579–584.
20. James P, Salomon O, Mikovic D, Peyvandi F. Rare bleeding disorders - bleeding assessment tools, laboratory aspects and phenotype and therapy of FXI deficiency. *Haemophilia.* 2014;20(Suppl 4):71–75.
21. Saes JL, Verhagen MJA, Meijer K, Cnossen MH, Schutgens REG, Peters M, et al. Bleeding severity in patients with rare bleeding disorders: real-life data from the RBiN study. *Blood Adv.* 2020;4:5025–5034.
22. Chalmers EA, Alamelu J, Collins PW, Mathias M, Payne J, Richards M, et al. Intracranial haemorrhage in children with inherited bleeding disorders in the UK 2003–2015: a national cohort study. *Haemophilia.* 2018;24:641–647.
23. Sanders YV, Fijnvandraat K, Boender J, Mauser-Bunschoten EP, van der Bom JG, de Meris J, et al. Bleeding spectrum in children with moderate or severe von Willebrand disease: relevance of pediatric-specific bleeding. *Am J Hematol.* 2015;90:1142–1148.
24. Hermans C, Kulkarni R. Women with bleeding disorders. *Haemophilia.* 2018;24(Suppl 6):29–36.
25. Castaman G, Giacomelli SH, Biasoli C, Contino L, Radossi P. Risk of bleeding and thrombosis in inherited qualitative fibrinogen disorders. *Eur J Haematol.* 2019;103:379–384.
26. Peyvandi F, Palla R, Menegatti M, Siboni SM, Halimeh S, Faeser B, et al. Coagulation factor activity and clinical bleeding severity in rare bleeding disorders: results from the European Network of Rare Bleeding Disorders. *J Thromb Haemost.* 2012;10:615–621.
27. Fasulo MR, Biguzzi E, Abbattista M, Stufano F, Pagliari MT, Mancini I, et al. The ISTH Bleeding Assessment Tool and the risk of future bleeding. *J Thromb Haemost.* 2018;16:125–130.
28. Boender J, Kruip MJHA, Leebeek FWG. A diagnostic approach to mild bleeding disorders. *J Thromb Haemost.* 2016;14:1507–1516.
29. Tosetto A, Castaman G, Rodeghiero F. Bleeders, bleeding rates, and bleeding score. *J Thromb Haemost.* 2013;11(Suppl 1):142–150.
30. Federici AB, Bucciarelli P, Castaman G, Mazzucconi MG, Morfini M, Rocino A, et al. The bleeding score predicts clinical outcomes and replacement therapy in adults with von Willebrand disease. *Blood.* 2014;123:4037–4044.
31. Artoni A, Bassotti A, Abbattista M, Marinelli B, Lecchi A, Gianniello F, et al. Hemostatic abnormalities in patients with Ehlers-Danlos syndrome. *J Thromb Haemost.* 2018;16:2425–2431.
32. Mehta R, Athar M, Girgis S, Hassan A, Becker RC. Acquired Von Willebrand Syndrome (AVWS) in cardiovascular disease: a state of the art review for clinicians. *J Thromb Thrombolysis.* 2019;48:14–26.
33. Quek JKS, Wong WH, Tan CW, Tha MH, Nagarajan C, Lee LH, et al. Acquired factor V deficiency in a patient with myeloma and amyloidosis. *Thromb Res.* 2018;164:1–3.
34. Harrison JS, Frazier SR, McConnell DD, Yasar SJ, Melnyk N, Salaru G. Evidence of both von Willebrand factor deposition and factor V deposition onto AL amyloid as the cause of a severe bleeding diathesis. *Blood Coagul Fibrinol.* 2017;28:342–347.
35. Sucker C, Hetzel GR, Grabensee B, Stockschlaeder M, Scharf RE. Amyloidosis and bleeding: pathophysiology, diagnosis, and therapy. *Am J Kidney Dis.* 2006;47:947–955.
36. Mumford AD, O'Donnell J, Gillmore JD, Manning RA, Hawkins PN, Laffan M. Bleeding symptoms and coagulation abnormalities in 337 patients with AL-amyloidosis. *Br J Haematol.* 2000;110:454–460.
37. Thomas W, Downes K, Desborough MJR. Bleeding of unknown cause and unclassified bleeding disorders; diagnosis, pathophysiology and management. *Haemophilia.* 2020;26(6):946–957.
38. Peyvandi F, Spreafico M. National and international registries of rare bleeding disorders. *Blood Transfus.* 2008;6(Suppl 2):s45–48.
39. Brunet JG, Iyer JK, Badin MS, Graf L, Moffat KA, Timleck M, et al. Electron microscopy examination of platelet whole mount preparations to quantitate platelet dense granule numbers: implications for diagnosing suspected platelet function disorders due to dense granule deficiency. *Int J Lab Hematol.* 2018;40:400–407.
40. Rodgers S, Duncan E. Chromogenic factor VIII assays for improved diagnosis of hemophilia A. *Methods Mol Biol.* 2017;1646:265–276.
41. Kallen ME, Dulau-Florea A, Wang W, Calvo KR. Acquired and germline predisposition to bone marrow failure: diagnostic features and clinical implications. *Semin Hematol.* 2019;56:69–82.
42. Tiede A, Klamroth R, Scharf RE, Trappe RU, Holstein K, Huth-Kuhne A, et al. Prognostic factors for remission of and survival in acquired hemophilia A (AHA): results from the GTH-AH 01/2010 study. *Blood.* 2015;125:1091–1097.
43. Orsini S, Noris P, Bury L, Heller PG, Santoro C, Kadir RA, et al. Bleeding risk of surgery and its prevention in patients with inherited platelet disorders. *Haematologica.* 2017;102:1192–1203.
44. Sharma R, Flood VH. Advances in the diagnosis and treatment of Von Willebrand disease. *Hematology Am Soc Hematol Educ Program.* 2017;2017: 379–384.
45. Mauser-Bunschoten EP, Kadir RA, Laan ETM, Elfvinge P, Haverman L, Teela L, et al. Managing women-specific bleeding in inherited bleeding disorders: a multidisciplinary approach. *Haemophilia.* 2021;27:463–469.
46. Martin K, Key NS. How I treat patients with inherited bleeding disorders who need anticoagulant therapy. *Blood.* 2016;128:178–184.

CHAPTER 127

LABORATORY EVALUATION OF HEMOSTATIC AND THROMBOTIC DISORDERS

Menaka Pai and Karen A. Moffat

This chapter provides a practical approach to the laboratory evaluation of hemostatic and thrombotic disorders. Any assessment of hemostatic or thrombotic disorders must start with a thorough history and physical examination. These can provide clues to guide subsequent laboratory testing, diagnosis, and management.

Physiologic hemostasis is a complex interplay of cellular or plasma elements: the adhesion of platelets to damaged endothelium, the aggregation of platelets to form a temporary plug, the successive activation of coagulation factors to form a stabilizing fibrin clot, clot retraction to repair endothelial damage, and finally clot breakdown through fibrinolysis (Fig. 127.1). Commonly available laboratory tests focus on the individual components of hemostasis by testing coagulation proteins, platelets, and fibrinolytic proteins; this chapter is organized into similar components to provide a structured laboratory approach to the patient with a hemostatic or thrombotic problem. Yet the clinician must be mindful that in the body, the components of hemostasis work together to form a product that is more than the sum of its parts.

LABORATORY EVALUATION OF COAGULATION PROTEINS

The Physiology Underlying Laboratory Evaluation of Coagulation Proteins

For over half a century, the process of fibrin clot formation has been conceptualized as a “coagulation cascade.” This is based on the waterfall hypothesis of Davie, Ratnoff, and MacFarlane, who almost simultaneously reported a sequence of proteolytic reactions starting with factor XII (Hageman factor) activation by surface contact and ending with thrombin’s proteolysis of fibrinogen to form fibrin.[1,2] However, upon its introduction, this hypothesis of successive necessary proteolytic reactions already appeared to be too simplistic. Nearly a decade earlier, Ratnoff had identified that deficiencies of factor XII were not associated with bleeding; others soon established that deficiency of factor XII’s cofactors (prekallikrein and high-molecular-weight kininogen) also did not result in a bleeding phenotype. In 1977, Osterud and Rappaport recognized that the factor VIIa/tissue factor complex can activate factor IX to factor IXa.[3] Broze later recognized that this complex cannot directly activate factor X, because of the presence of tissue factor pathway inhibitor (TFPI); factor IX activation is a prerequisite in vivo (see Chapter 124). Though deficiency of factor XII and its antecedents is not associated with bleeding, factor XI deficiency is (see Chapter 134). In 1991, Gailani and Broze explained this by proposing that factor XI can be activated independently of factor XII, as formed thrombin cycles back to activate factor XI and thus amplifies its own formation.[4] Factor XII, long considered to have no role in coagulation in vivo, has been found to play a role in thrombus formation and angiogenesis.[5] Our understanding of hemostasis has advanced still further in recent years, as blood coagulation research is increasingly performed under flow conditions with cellular elements in vitro and in animal models.

The physiologic “coagulation cascade” now appears to be an intricate system with built-in shortcuts and feedback loops. It is triggered by factor VIIa in complex with tissue factor, and results in the activation of zymogens that become serine proteases.[4] This system functions in concert with platelet activation and fibrinolysis (Fig. 127.2). However, clinical laboratory testing of coagulation proteins is not based on this current understanding—it follows the original Ratnoff-Davie-Macfarlane surface-activated coagulation cascade hypothesis.[1,2] These tests attempt to mimic in vivo processes by carrying them out in vitro, and thus do not capture the true complexity of physiologic hemostasis. They are still useful in diagnosing coagulation protein deficiencies and important bleeding disorders. However, they can be misleading, as they can also detect abnormalities of questionable clinical significance. The clinician must therefore understand the distinction between the complexity of physiologic hemostasis and the simplistic picture presented by laboratory tests.

As we proceed in reviewing tests for coagulation proteins, we can place them into three technical categories:

1. **Immunologic tests**, which include enzyme-linked immunosorbent assays (ELISA), immunoelectrophoresis, and immunoturbidometric (latex immuno-agglutination) tests. These tests are quantitative and detect specific proteins with polyclonal or monoclonal antibodies. Their sensitivity and specificity depend on the antibody used (polyclonal versus monoclonal) and the presence of interfering substances (e.g., rheumatoid factor, human anti-mouse antibody (HAMA), and other autoantibodies).
2. **Chromogenic or amidolytic assays**, which measure the activity of the serine proteases of the coagulation system as they react with synthetic peptides. The reaction (and thus the activity of serine protease) can be measured as the synthetic peptide releases a colored dye. Chromogenic assays are affected by the specificity of the peptide substrate. A disadvantage of these assays is that their narrow measure of an enzyme’s activity may not correlate with its biologic activity in vivo or its activity in clot-based assays.
3. **Clot-based or coagulation assays** are functional and compare the clotting potential of a patient’s plasma with control plasma with a known clotting potential. These tests are more difficult and time-consuming to perform than the others, and are susceptible to interference from other factors, such as anticoagulant effect and hemolysis-icteric-lipemic (HIL) interferences. However, they most closely approximate in vivo hemostasis.

SCREENING FOR COAGULATION PROTEIN DEFECTS: ACTIVATED PARTIAL THROMBOPLASTIN TIME, PROTHROMBIN TIME, AND THROMBIN CLOTTING TIME

The three assays most often used to screen for coagulation protein defects are: (1) the activated partial thromboplastin time (APTT), induced in vitro by surface-activation of prekallikrein, high-molecular-weight kininogen, and factor XII; (2) the prothrombin time (PT), induced in vitro by the addition of excess tissue factor; and (3) the thrombin clotting time (TCT), a test of fibrinogen integrity and thrombin inhibition. The PT, APTT, and TCT are clot-based assays that measure the rate of clot formation and are based on the

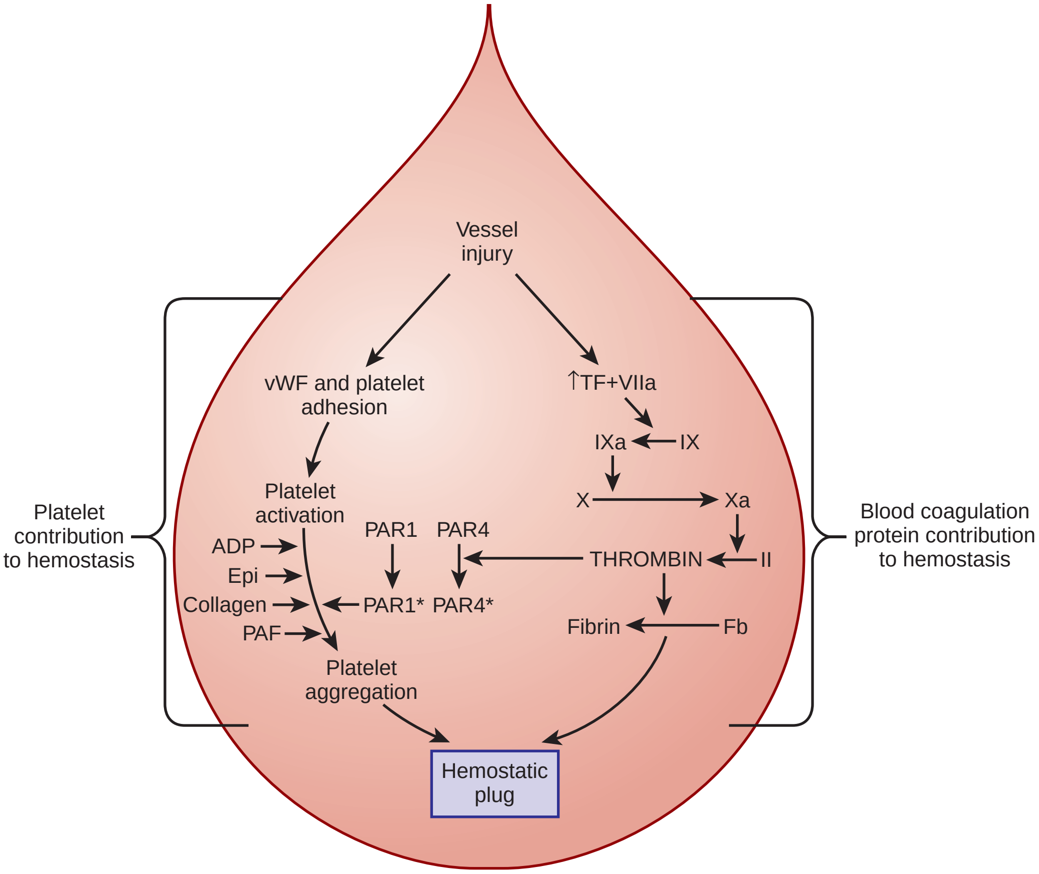


Figure 127.1 SCHEMATIC DIAGRAM OF HEMOSTASIS. Two equally important arms of activity contribute to physiologic hemostasis. The platelet contribution to hemostasis is as follows. When a vessel is injured, exposing collagen, platelets adhere to the injury site via vWF and, in high-shear areas, glycoprotein VI (not shown in figure). Upon adherence, the platelets are activated and release their granule contents. Released ADP and other granule contents recruit more platelets to the injury site. Simultaneously, subendothelial cell tissue factor exposed at the site of injury binds activated factor VII (VIIa) and activates factor IX to activated factor IX (IXa) and, sequentially, factor X to activated factor X (Xa), and prothrombin (II) to thrombin. Exposed collagen also promotes factor XII autoactivation and generation of additional thrombin in the milieu of activating platelets (not shown in figure). Thrombin stimulates more platelets, enhancing the platelet plug. Thrombin also proteolyzes fibrinogen to form fibrin monomer, which then polymerizes into a fibrin clot. These events occur on or about the activated platelet surface. *ADP*, Adenosine diphosphate; *Epi*, epinephrine; *Fb*, fibrinogen; *PAF*, platelet-activating factor; *PAR*, protease-activated receptor, *TF*, tissue factor; *vWF*, von Willebrand factor.

waterfall hypothesis of coagulation. They trigger a sequence of proteolytic reactions in the intrinsic, extrinsic, and common pathways (Fig. 127.3). The reactions culminate in the proteolysis of fibrinogen to form a fibrin clot, which causes soluble proteins to precipitate in the patient's plasma sample. These soluble proteins can be detected by either increased electric impedance or decreased optical clarity, depending on the instrumentation used to measure the result.

Any defect in one of the coagulation proteins along the pathway to clot formation will give an abnormal result (i.e., a delayed time to clot formation) (see Fig. 127.3). For example, factor XI deficiency will lead to a prolonged APTT. Furthermore, because clot formation depends on a series of reactions, any substance (e.g., inhibitory antibodies, anticoagulants) that interferes with the assay *downstream* from a specific coagulation protein will lead to an abnormal result. For example, profound fibrinogen deficiency will lead to a prolonged APTT and PT, even when the levels and function of all the upstream coagulation proteins are normal.

In the APTT assay, activation of the blood coagulation system occurs when factor XII and its cofactors encounter a negatively charged surface such as kaolin, celite, silica, or ellagic acid (hence the intrinsic pathway's alias as the "contact pathway"). This causes the protein to change shape, allowing its autoactivation and subsequent initiation of the cascade of proteolytic reactions seen in the coagulation system. The APTT measures many proteins that are critical to physiologic hemostasis, including factor VIII and factor IX. However, it also measures proteins like factor XII, prekallikrein, and high-molecular-weight kininogen that are not necessary for physiologic hemostasis. To perform the APTT assay, equal parts of a negatively charged surface and phospholipid mixture are incubated with patient plasma for a predetermined time. Calcium chloride is then added to recalcify the citrated plasma, and the time to clot formation is measured. The APTT assay measures all the proteins of the intrinsic system (prekallikrein, high-molecular-weight kininogen, factor XII, factor XI, factor IX, and factor VIII) and the proteins of the common pathway (factors X, V, prothrombin [factor II], and fibrinogen). These proteins have different threshold levels to which they must fall before the APTT shows an abnormality. For example, most commercial APTT reagents detect a decrease in factor VIII when the protein level decreases to 35% to 45% of normal (i.e., 0.35 to 0.45 U/mL). Alternatively, factor XII and high-molecular-weight kininogen levels must fall to 10% to 15% of normal before the APTT becomes significantly abnormal, however small decreases in these factors may cause slight prolongation. The sensitivity of the screening tests for detection of specific abnormalities varies with the factor being tested, the commercial reagent used in the assay, and the equipment platform for measurement. Each clinical laboratory should determine the level of decrease for each coagulation factor that produces an abnormal APTT with their current equipment and reagents.

In the PT assay, addition of excess tissue factor creates a nonphysiologic change in the normal stoichiometric relationship of

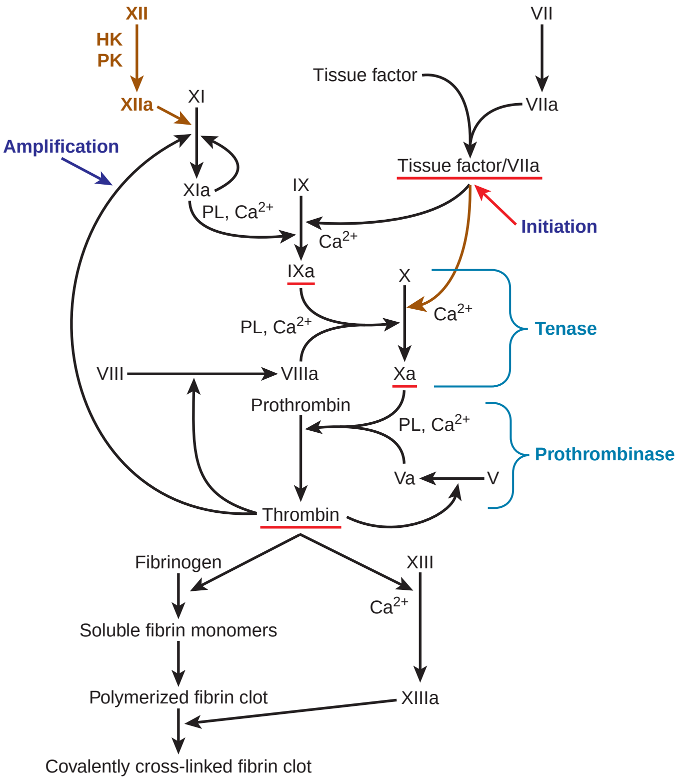


Figure 127.2 SCHEMATIC DIAGRAM OF PHYSIOLOGIC HEMOSTASIS. Formation of the tissue factor–activated factor VII complex (TF/VIIa) results in factor IX activation to activated factor IX (IXa). TF/VIIa does *not* normally activate factor X directly *(brown line)*. Activated factor IX activates factor X to activated factor X (Xa) in the presence of activated factor VIII (VIIIa), which is formed from prior thrombin activation of factor VIII (tenase). Xa in the presence of activated factor V (Va) activates prothrombin to thrombin (IIa) (prothrombinase). Thrombin proteolyzes fibrinogen to form a fibrin clot. If more thrombin is needed, thrombin can activate factor XI to activated factor XI (XIa), which then activates more factor IX to factor IXa, which makes more activated factor X and thrombin. If more thrombin-induced clot formation is needed, thrombin also activates thrombin-activatable fibrinolysis inhibitor, which inhibits fibrinolysis (pathway not shown). Factor XI also can be activated by activated factor XII, which is formed secondarily by the constitutive activation of prekallikrein in the presence of high-molecular-weight kininogen by contact activation in collagen-exposed injured vessels. These latter mechanisms are *not* constitutive for physiologic hemostasis. However, in non-physiological states, such as sepsis, clot formation in the intravascular compartment, or cardiopulmonary bypass, activated factor XII can activate factor XI to initiate hemostasis with thrombin formation. This latter mechanism is the basis of the activated partial thromboplastin time, a major screening test for hemostatic disorders. *HK*, High-molecular-weight kininogen; *PK*, prekallikrein; *PL*, phospholipid; *TF*, tissue factor; *Va*, activated factor V. *(Modified from Schmaier AH, Miller J. Coagulation and fibrinolysis. In: McPherson RA, Pincus MR, eds. Henry's Clinical Diagnosis and Management by Laboratory Methods. 22nd ed. Philadelphia: Elsevier; 2011:785.)*

coagulation factors, allowing factor VIIa to overcome the inhibitory effect of TFPI; the factor VIIa/tissue factor complex then activates factor X to factor Xa (this bypasses the usual physiologic requirement for this process to occur through factor IX activation). To perform the PT assay, one part patient plasma and two parts tissue thromboplastin (human or rabbit tissue-derived or recombinant human tissue factor) are added to phospholipid and calcium. The time required to clot formation is measured. The PT assay measures the extrinsic pathway of coagulation, which consists of factor VII and the proteins of the common pathway (factors X, V, II, and fibrinogen). Like the APTT, the PT becomes abnormal at different threshold levels for different factors, depending on the commercial reagent and the equipment platform. For example, factor VII levels must generally fall below 35% to 40% before the PT becomes abnormal.

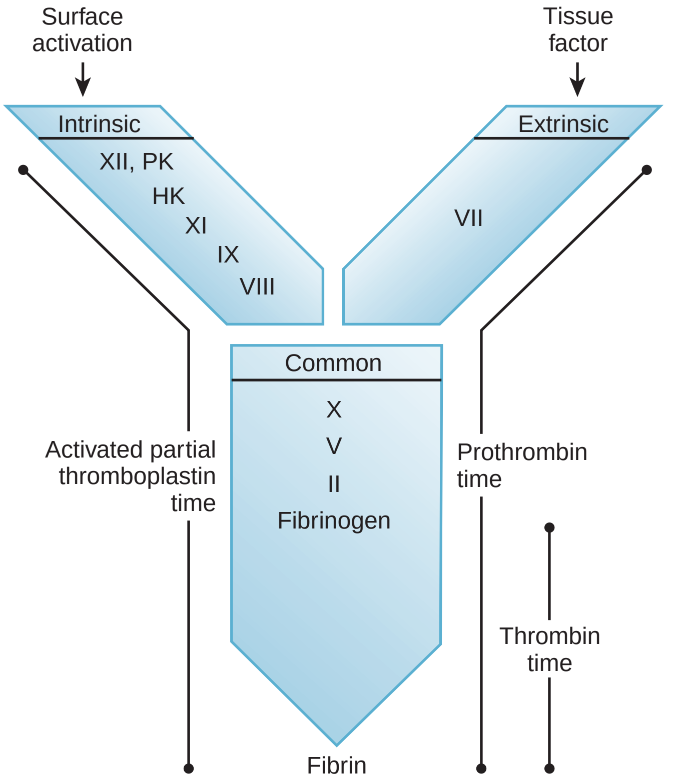


Figure 127.3 ORGANIZATION OF THE COAGULATION SYSTEM BASED ON CURRENT SCREENING ASSAYS. The intrinsic coagulation pathway consists of the protein factors XII, XI, IX, and VIII and prekallikrein and high-molecular-weight kininogen. The extrinsic coagulation pathway consists of tissue factor and factor VII. The common pathway of the coagulation system consists of factors X, V, II, and fibrinogen (I). The activated partial thromboplastin time requires the presence of every protein except tissue factor and factor VII. The prothrombin time requires factors VII, X, V, and II, and fibrinogen. The thrombin clotting time only tests the integrity of fibrinogen. *(Modified from Schmaier AH. Approach to the bleeding patient. In: Schmaier AH, Petruzzelli LM, eds. Hematology for the Medical Student. Philadelphia: Lippincott Williams and Wilkins; 2003:79.)*

The PT can also be used to monitor warfarin therapy if the test reporting is modified so it can be interpreted universally. Because of the plethora of commercially available PT reagents and coagulation instruments, it is impossible to know the normal range for the PT from any given laboratory. The international normalized ratio (INR) was thus developed to standardize the reporting of the PT and to create a universal benchmark for monitoring warfarin therapy. $\text{INR} = (\text{patient PT (sec)}/\text{mean normal laboratory PT (sec)})^{\text{ISI}}$, where the international sensitivity index (ISI) for a given thromboplastin reagent is a measure of its responsiveness to reductions in the levels of the vitamin K-dependent coagulation factors, factors II, VII, IX, and X. The ISI (provided by each reagent's manufacturer, but ideally locally validated) is based on the degree of variation of the thromboplastin reagent from the World Health Organization (WHO) reference thromboplastin. This reference has an arbitrarily assigned value of 1.0. INR derivation can be performed by developing a calibration line for any given thromboplastin, using orthogonal regression or linear regression, and incorporating the PT determined with the WHO standard.[6] Determination of the ISI requires samples from a minimum of 20 healthy donors and 60 patients on stable warfarin therapy with INR values ranging from 1.5 to 5.0.[7] The higher the ISI, the less sensitive the thromboplastin. In practice, changes in the INR are considered interchangeable with changes in the PT. However, the INR is correctly used only to characterize the degree of PT prolongation in samples from patients taking vitamin K antagonists such as warfarin.

In the TCT assay, exogenous thrombin is added to examine the integrity of its major substrate, fibrinogen. To perform this assay, purified thrombin is added to plasma, and the time to clot formation is measured. The TCT is thus a direct measure of the conversion of fibrinogen to fibrin (see Fig. 127.3). When performing this assay, it is essential to use the minimal amount of α-thrombin (3000 U/mg specific activity) that will reproducibly "clot" fibrinogen, usually 2 to 6 U/mL, aiming for a clotting time (CT) of approximately 20 seconds with pooled normal plasma to achieve maximal sensitivity and render the test clinically useful. This assay is distinguishable from the clottable fibrinogen assay (Clauss assay) by the amount of thrombin used. The clottable fibrinogen assay uses far more thrombin (100 U/mL) and determines the amount of functional fibrinogen in patient plasma compared with known levels of functional fibrinogen in calibration plasma.[8] A prolonged TCT, as indicated by values outside the 95% confidence interval for the time to clot of a large population of normal donor samples, suggests reduced fibrinogen levels (usually <100 mg/dL), abnormal fibrinogen function, or the presence of an inhibitor of the exogenous thrombin (e.g., heparin or a direct thrombin inhibitor). The TCT can also be prolonged if there is interference with fibrin polymerization, which can be caused by elevated levels of fibrinogen degradation products or the presence of a paraprotein. Some drugs, such as valproic acid, also prolong the TCT.[9]

EVALUATION OF SPECIFIC COAGULATION PROTEIN DEFECTS

When the APTT, PT, or TCT suggest a coagulation protein defect, the plasma concentration of the coagulation factors should be quantified. Factor assays determine the nature and degree of severity of coagulation protein defects and can also be used to monitor factor replacement therapy. Coagulation protein defects can take three forms: a true protein deficiency; an abnormal protein that cannot participate in its physiologic function(s); or an inhibitor that targets the active site of the protein or enhances its clearance. Inherited protein deficiencies and abnormalities can be caused by deletions, insertions, and missense/nonsense mutations in individual genes. Inhibitors are generally immunoglobulins, although abnormally produced endogenous heparin, fibronectin, or cryoglobulins can also serve as acquired inhibitors to coagulation proteins.

If a coagulation protein defect is suspected, clinical laboratory testing can be done with immunologic, chromogenic, or clot-based assays. Clot-based assays for coagulation proteins are functional: they will be abnormal with both true deficiencies and dysfunctional proteins. These take the form of one-stage assays that use the APTT (for factor XII, prekallikrein, high-molecular-weight kininogen, factors XI, IX, and VIII) or the PT (for factors VII, X, V, and II) as the platform. Specific factor–deficient plasma is the key to clot-based factor assays as it provides the required specificity for each assay. These assays are based on the principle that when plasma (either from a reference standard or from a patient) containing the factor is added to plasma completely deficient in that factor, it can "correct" (i.e., shorten) the prolonged CT. For example, a factor VIII assay requires APTT reagent and calcium chloride, WHO reference calibration plasma, factor VIII–deficient plasma (which has < 1 U/dL of factor VIII and normal levels of all other factors), and patient plasma. Several different dilutions of the reference plasma are set up, creating a range of known concentrations of factor VIII. Factor VIII–deficient plasma is added to each dilution and APTTs are performed according to laboratory protocol. A calibration curve is set up by plotting the known concentration of factor VIII (*x*-axis) against the time to clot in seconds (*y*-axis). This process is repeated, substituting patient plasma for reference plasma. If the patient plasma has decreased levels of factor VIII, it cannot compensate for the absence of factor VIII in the factor VIII–deficient plasma and the time to clot formation is prolonged compared with a similar dilution of reference plasma. By comparing the time to clot of the patient's plasma to the calibration curve, the level of factor VIII activity in the patient's plasma can be quantified.

Immunologic assays can be used to establish the quantity (as opposed to the quality) of coagulation proteins. When used together with clot-based tests, these assays can detect a protein with reduced function, but normal production. For example, an abnormal fibrinogen (dysfibrinogenemia) can be detected by measuring clottable fibrinogen and fibrinogen antigen on the same sample. If fibrinogen clottability (function) is less than 70% of the amount of fibrinogen antigen (production), the protein produced is likely functionally abnormal, however the cut-off for the ratio of activity to antigen is not well validated.

Chromogenic Factor Assays

There has been a significant growth in the development of replacement strategies for hemophilia A and B patients (see Chapter 133). These include extended half-life recombinant factor VIII and factor IX and in the case of FVIII, by-passing agents. The development and increasing use of the new replacement products introduce several challenges for both the clinician and the coagulation laboratory. The APTT-based (one-stage) factor assays are traditionally used for the diagnosis and monitoring of replacement in this patient population. Due to the heterogeneity of the extended half-life products, there is wide and significant variability in the activity levels between one-stage and two-stage or chromogenic factor assays. In response to this, the United Kingdom Haemophilia Centre Doctors' Organisation published a guideline for the laboratory measurement of factor replacement therapies. This guideline summarizes current, as well as soon to be released products and the recommended assay and reagents for laboratories to use.[10] Collaboration between the treating clinicians and the laboratory is paramount to ensure that the appropriate assays for monitoring the replacement products are available.

Evaluation of Specific Inhibitors of Coagulation Proteins

An inhibitor is generally suspected when a prolonged clot-based assay does not correct after mixing patient plasma in equal quantities with normal plasma (i.e., in an immediate 1:1 mix), or if an apparent factor deficiency is not consistent with the patient's clinical history. Screening for inhibitors is accomplished by using a 1:1 mix in a clot-based assay. If coagulation factor–deficient plasma is mixed with normal plasma, clotting will not be impaired, as the normal plasma will compensate for the deficient factor in the plasma and "correct" its prolonged CT. However, if inhibitor-containing plasma is mixed with normal plasma, clotting will be impaired, because the specific inhibitor will decrease the activity of the coagulation factor in the normal plasma as well. Specific inhibitors of coagulation proteins are generally time-dependent, that is, clotting may not be impaired immediately after the 1:1 mix is performed. However, if the mixture is incubated for 2 hours at 37°C, the CT will be prolonged.

Inhibitors of specific coagulation proteins increase the risk for bleeding. The most common acquired inhibitor is an antibody against factor VIII (see Chapters 133 and 134). These can occur as an alloantibody in persons with hemophilia, or as an autoantibody in those with previously normal hemostasis.[11] Patients with an autoantibody-type acquired factor VIII inhibitor present with a prolonged APTT and hemophilia-like bleeding. Both males and females can develop this type of inhibitor. It is characteristically seen in older adult patients, patients with B-cell malignancies, patients with connective tissue disorders such as systemic lupus erythematosus, and in the postpartum period (see Chapter 134). Management decisions in these patients are influenced by the severity of the bleeding and the titer of the inhibitor.[11,12] Quantitative measurement of factor VIII inhibitors is performed using the Bethesda method, often using the Nijmegen modifications for improved sensitivity and specificity. The Bethesda method is based on the observation that if a coagulation factor is incubated with plasma containing its specific inhibitor, the factor will be progressively neutralized. If the amount of factor added and the duration of incubation are standardized, the inhibitor potency can be measured based on how much of the factor is inhibited. One Bethesda unit is defined as the amount of factor VIII inhibitor that will neutralize 50% of 1 unit of factor VIII in normal plasma after 2 hours of incubation at 37°C. Normal pooled plasma is considered to contain 1 unit of factor VIII. The Bethesda assay is performed by incubating dilutions of the patient plasma with normal pooled plasma for 2 hours at 37°C. At the end of the incubation period, the factor VIII and the percentage residual factor activity is calculated and plotted against a standardized lin/log graph of Bethesda units versus residual factor VIII activity. The factor VIII percentage nearest to 50% (between 30% and 60%) is chosen for calculating the potency of the inhibitor to minimize overestimating (<50%) or underestimating (>50%) the inhibitor titre.[12] At 50% residual factor activity, the test plasma contains, by definition, 1 Bethesda inhibitor unit per mL. The Nijmegen modification allows for more accurate determination of inhibitor titers at low levels of factor VIII inhibition. Increases in pH and decreases in protein concentrations can lead to increased inactivation of factor VIII, potentially leading to false-positive results with the standard Bethesda assay. The Nijmegen modification buffers normal plasma with 0.1 M imidazole and uses immunodepleted factor VIII–deficient plasma in the control mixture.

Inhibitors directed against other coagulation factors are rare, and their associated hemorrhagic manifestations are variable, ranging from minor bleeding to life-threatening events (see Chapter 134). Autoantibodies against von Willebrand factor (vWF) can develop in the context of a paraproteinemia, whereas autoantibodies against prothrombin are a rare complication of systemic lupus erythematosus. Inhibitors to other factors can be measured with modifications of the Bethesda method.

Evaluation of Acquired Inhibitors of Coagulation Proteins

The most frequently detected acquired inhibitors are the antiphospholipid antibodies (APLAs) (see Chapter 138). APLAs represent antibodies directed against epitopes on proteins that bind phospholipids. They include the lupus anticoagulant (LAC), anticardiolipin antibodies (aCL), and anti-β_2 glycoprotein I antibodies (aβ_2GPI). LAC are often detected when they interfere with the APTT and PT, which they do to a variable extent. They also interfere in any phospholipid-dependent clotting assay. This effect is dependent on the nature and the concentration of phospholipid in the commercial reagent. LAC (like other APLAs) do not increase bleeding risk. APLAs may have no clinical significance at all, or they may predispose to thrombosis (see Chapter 138). Laboratory assays for APLAs fall into three categories: (1) clot-based assays that detect the LAC; (2) immunoassays that detect aCL; and (3) immunoassays that detect aβ_2GPI. There is currently a lack of standardization of these assays, and their findings do not always correlate with clinical symptoms. However, laboratory identification of APLAs is a key component of diagnosing antiphospholipid syndrome, an autoimmune syndrome that increases the risk of thrombotic and pregnancy complications.[13] Current laboratory principles of detecting APLAs are well described in Chapters 137 and 138. Highlights are outlined here.

LAC screening guidelines recommend the use of two laboratory tests based on different principles: the dilute Russell viper venom time, and a LAC-sensitive APTT, using an APTT reagent containing silica as the activator.[13] Both tests are phospholipid dependent. If at least one of the two tests suggests the presence of a LAC, a 1:1 mixing test using pooled normal plasma should be performed. Confirmatory testing must also be done with an assay that is less sensitive but more specific for LAC by increasing the phospholipid content. aCL and aβ_2GPI antibodies are detected using immunoassays. Traditionally, the ELISA technique was used. However, newer automated assays have been introduced; these are expected to improve test reproducibility, specificity, and accuracy of detection. At this time, aCL and aβ_2GPI testing is not well standardized, and there is a lack of uniformity in reference material used for calibration. Reporting is also not standardized. aCL assays are expressed in GPL (units of aCL immunoglobulin [Ig] G antibodies, calculated against the original Harris standards) or MPL (units of aCL IgM antibodies, calculated against the original Harris standards). aβ_2GPI can be expressed in U/mL, ng/mL, and μg/mL among others.

PRACTICAL APPROACH TO LABORATORY TESTING OF COAGULATION PROTEINS

The first step in interpreting laboratory tests of coagulation proteins is synthesizing the results of the PT, APTT, and TCT. When all three assays are performed simultaneously, they can give the clinician a high-level snapshot of the patient's coagulation factors, and inform a practical differential diagnosis (Table 127.1). An abnormality in one test suggests an abnormality in one branch of the Ratnoff-Davie-Macfarlane coagulation cascade, whereas abnormalities in more than one test suggest a problem in the common pathway of coagulation.

Isolated prolongation of the APTT is caused by abnormalities in the intrinsic pathway, only some of which are associated with an increased bleeding risk (see Table 127.1). It is essential to consider the APTT in the context of a thorough clinical bleeding assessment (see Chapter 126). If the prolonged APTT is associated with bleeding, then the differential diagnosis in decreasing likelihood of frequency is factor VIII deficiency; factor IX deficiency; or factor XI deficiency. Factor VIII and IX deficiencies are X-linked, and affect 1:5000 to 10,000 males and 1:25,000 to 30,000 females respectively.[14] Factor XI deficiency is an autosomal disorder that affects both males and females, and has a variable prevalence.[14] It causes a clinically significant bleeding disorder in 1 in 1 million individuals in the general population, but has a rate of heterozygosity as high as 8% to 9% in Ashkenazi Jews (see Chapters 133 and 134 for a full discussion of these factor deficiencies). If the isolated prolonged APTT is not associated with bleeding symptoms, the most common cause is an APLA, often found in otherwise healthy individuals. Coagulation protein deficiencies that prolong the APTT but are not associated with bleeding include factor XII, prekallikrein, and high-molecular-weight kininogen deficiency (see Table 127.1). It is important to consider these three proteins when evaluating isolated prolongation of the APTT to preclude misguided attempts to "correct" the prolonged APTT by giving patients unnecessary blood products or factor concentrates.

Isolated prolongation of the PT is caused by abnormalities in the extrinsic pathway (see Table 127.1). An isolated PT prolongation associated with bleeding usually indicates factor VII deficiency (see Chapter 134); these deficiencies are partial, as complete factor VII deficiency appears to be incompatible with life.[14] Sometimes, mild defects in common pathway proteins (e.g., factors II, V, and X) may initially appear as an isolated PT prolongation, though as they progress, they eventually prolong the APTT as well. This asynchronous prolongation occurs because PT reagents with an ISI approaching 1 are more sensitive to factor deficiencies, specifically factors VII, X, and II, than the APTT reagents. Conversely, the PT and APTT are relatively insensitive to mild defects in fibrinogen. With some reagents, APLAs may also prolong the PT.

When confronted with prolongation in both the PT and APTT, the hematologist should first consider clinical situations that broadly affect these tests: anticoagulant therapy, vitamin K deficiency, liver disease, or disseminated intravascular coagulation (DIC) (see Table 127.1). These conditions are far more common than isolated deficiencies of the factors in the common pathway (factors II, V, and X). Anticoagulants act by inhibiting one or many coagulation proteins in the common pathway, while vitamin K deficiency decreases the functional levels of factors II, VII, IX, and X. Vitamin K deficiency is

TABLE 127.1 **Synthesizing Results of Prothrombin Time, Activated Partial Thromboplastin Time, and Thrombin Clotting Time**

Test				
PT	**APTT**	**TCT**	**Possible Diagnoses**	**Next Steps**
N	N	N	Normal hemostasis, disorder of platelet function, factor XIII deficiency, disorder of vascular hemostasis, mild coagulation protein deficiency, mild vWD, disorder of fibrinolysis (α_2-antiplasmin deficiency/defect, plasminogen activator inhibitor-1 deficiency/defect)	Focused history and physical, medication review, evaluation of platelets, evaluation of fibrinolysis, evaluation of vWF
H	N	N	Factor VII deficiency, early oral anticoagulation, lupus anticoagulant (reagent dependent), mild factor II, V, or X deficiency, specific factor VII inhibitor	Focused history and physical, medication review, factor assays (VII, II, V, X), evaluation of specific factor inhibitor, evaluation of antiphospholipid antibodies
N	H	N	Factor VIII, IX, XI, XII, prekallikrein or HMWK deficiency, lupus anticoagulant, amyloid-adsorbed factor IX, specific factor inhibitor	Focused history and physical, factor assays (VIII, IX, XI, XII, PK, HMWK), evaluation of specific factor inhibitor, evaluation of antiphospholipid antibodies, evaluation of amyloidosis (initially, serum and urine protein electrophoresis with immunofixation, serum free light chain assay)
H	H	N	Multiple factor deficiency (e.g., liver failure, vitamin K deficiency, oral anticoagulants), factor V, X, or II deficiency, amyloid-adsorbed factor X, specific factor inhibitor	Focused history and physical, medication review, factor assays (V, X, II), evaluation of specific factor inhibitor, evaluation of liver productive function and liver injury (albumin, ALT, AST, GGT, alkaline phosphatase), evaluation of amyloidosis (initially, serum and urine protein electrophoresis with immunofixation, serum free light chain assay)
H	H	H	Anticoagulants, DIC, dilutional coagulopathy, liver disease, severe fibrinogen deficiency/disorder, inhibition of fibrin polymerization, hyperfibrinolysis	Focused history and physical, medication review, clottable fibrinogen and fibrinogen antigen, reptilase time, evaluation of fibrinolysis, evaluation of liver productive function and liver injury (albumin, ALT, AST, GGT, alkaline phosphatase)

ALT, Alanine aminotransferase; *APTT*, activated partial thromboplastin time; *AST*, aspartate aminotransaminase; *DIC*, disseminated intravascular coagulation; *GGT*, gamma glutamyltransferase; *H*, high/prolonged; *HMWK*, high-molecular-weight kininogen; *N*, normal; *PT*, prothrombin time; *TCT*, thrombin clotting time; *vWD*, von Willebrand disease; *vWF*, von Willebrand factor.

found in individuals receiving warfarin therapy, who are profoundly nutritionally depleted (caused by poor diet or the receipt of incomplete parenteral nutrition), who have altered gut microbiota as a result of intestinal surgery or antibiotic therapy, or, very rarely, who have defects in transport proteins or enzymes for vitamin K metabolism. The liver synthesizes most of the coagulation proteins, so liver disease can have a profound effect on laboratory tests of hemostasis by causing multiple factor deficiencies. Prekallikrein, factor XI, factor VII, and factor V production drop in hepatic failure, and fibrinogen synthesis becomes abnormal. DIC, an important cause of APTT and PT prolongation, is discussed in detail toward the end of this chapter (see also Chapter 136).

TCT prolongation can be caused by reduced fibrinogen levels (e.g., because of liver disease), abnormal fibrinogen function, or large amounts of thrombin inhibitors like heparin or a direct thrombin inhibitor. The TCT also detects defects in fibrinopeptide A and B release, as well as polymerization defects. The reptilase time uses Batroxobin (Reptilase), a purified enzyme from the snake *Bothrops atrox*, to "clot" fibrinogen by liberating only fibrinopeptide A. An abnormal TCT with a normal reptilase time indicates a fibrinopeptide B–release defect, an abnormality that results in long APTT and PT values but is not associated with bleeding. The reptilase time will not be altered by the presence of heparin and can thus be used to determine if a prolonged TCT is the result of heparin. Another practical way to determine if a prolonged TCT is a result of heparin is to use the protamine neutralization test. This test is based on the TCT, with varying amounts of protamine sulfate added to patient plasma before the addition of thrombin. Protamine sulfate can neutralize heparin, and thus normalize the TCT. The concentration of heparin in the plasma can be calculated from the amount of protamine sulfate required to neutralize the heparin.

When presented with a prolonged clot-based assay, the hematologist must also be able to differentiate between a coagulation protein deficiency and an inhibitor to a specific coagulation protein. Two approaches can be used to obtain a specific diagnosis. The first approach uses the immediate 1:1 mix, a test in which patient and normal pooled plasma are mixed in equal proportions. Mixing studies based on the PT or APTT are interpreted based on the fact that 50% levels of any coagulation factor will yield normal PT and APTT values. Only when the level drops below 50% will clot formation become prolonged, though the sensitivity of the assay then depends on the factor and the test reagent. Practically, this means that if a patient has less than 1% of a factor, and their plasma is mixed 1:1 with normal plasma, the PT or APTT will correct to normal. However, if a patient has something in their plasma that interferes with protein function in normal plasma (e.g., an inhibitor, an anticoagulant), the PT or APTT will remain prolonged. The immediate 1:1 mix can thus be used to screen for the presence of factor deficiency and inhibitors. This approach is far from perfect. No studies provide standardized evidence-based laboratory procedures for these screening assays, so the ratio of patient plasma to normal plasma used, the time of incubation from mixing to assay, and the sensitivity and specificity for assessing factor deficiencies and circulating anticoagulants are variable. This makes it difficult to translate the results of mixing studies into meaningful diagnostic data. If a factor deficiency or inhibitor is suspected, it is essential to perform the appropriate specific testing, as outlined previously.

Other comorbid illnesses may also result in a bleeding diathesis. For example, systemic amyloidosis is associated with decreases in plasma factor X or IX because of adsorption of the coagulation proteins onto the amyloid protein. In the case of factor X adsorption, the PT and APTT may be affected, while in the case of factor IX adsorption, only the APTT is affected. Hypergammaglobulinemic states seen with multiple myeloma or Waldenström macroglobulinemia (IgM) can be associated with inhibitors to coagulation protein function. Dysfibrinogenemias are common in these patients because fibrinogen readily binds the immunoglobulin.

LABORATORY EVALUATION OF PLATELETS AND VON WILLEBRAND FACTOR

Screening for Disorders of Primary Hemostasis

Disorders of platelets and vWF, the key components of primary hemostasis, are characterized by disproportionate bleeding after injury or surgery, petechiae, purpura, ecchymoses, mucosal bleeding (including epistaxis and gum bleeding), and heavy menstrual bleeding. Most patients have a family history of similar mucocutaneous bleeding. In the past, the Ivy bleeding time—a test in which the forearm is cut, and the time until bleeding stops is measured—was used to screen for disorders of primary hemostasis, including platelet function disorders (PFDs) and von Willebrand disease (vWD).[15] This test is no longer considered to be useful in the investigation of bleeding disorders. Bleeding times are invasive and operator dependent, can be affected by nonhematologic factors (e.g., Ehlers-Danlos syndrome, osteogenesis imperfecta, scurvy, skin quality, skin temperature, vascular function), and have low predictive value for surgical bleeding.[16] Bleeding times also are not useful for detecting platelet dysfunction in the setting of thrombocytopenia. Importantly, the bleeding time is neither sensitive nor specific enough to preclude further testing for vWF or platelet abnormalities.[17] A normal bleeding time should not reassure the clinician that an important bleeding disorder is not present.

Several automated analyzers have been proposed as replacements for the bleeding time, and potentially useful screening tools for disorders of primary hemostasis. The platelet function analyzer (PFA-100, Siemens, Deerfield, IL) has been best evaluated. The PFA-100 attempts to recreate the high shear conditions under which primary hemostasis occurs in vivo. Using citrated whole blood, it measures the closure time required for platelets to adhere to agonist-coated membranes and aggregate under high shear stress (5000 to 6000/s). Two cartridges are available: one with collagen–adenosine diphosphate (ADP) membranes, and the other with collagen–epinephrine coated membranes. Platelet function is measured as a function of the time needed to occlude the aperture, termed the *closure time*. Abnormalities can lead to prolongation of the closure time. The PFA-100 is more sensitive than the bleeding time in patients with previously diagnosed vWD, but less sensitive in those with mild platelet secretion disorders (which are the most frequent PFDs) and platelet storage pool deficiency. In a prospective study by Quiroga et al., the overall sensitivity of closure times in vWD and PFDs were 50% and 18%, respectively.[17] This suggests that the PFA-100 is only marginally more useful than the bleeding time. It is still not sensitive or specific enough to preclude further testing for vWF or platelet abnormalities and cannot definitively rule out important bleeding disorders.

At this time, laboratory screening tests for disorders of primary hemostasis have limited usefulness. Diagnosis of these disorders must be grounded in a thorough clinical history and physical examination, and ultimately relies on the performance of the specific and sensitive tests outlined in the following.

Evaluation of Platelets

The first step in the investigation of platelet disorders is determining the platelet count because thrombocytopenia alone may increase bleeding risk (thrombocytopenia is discussed in detail in Chapters 128 and 129). Obtaining a detailed list of medications the patient is taking, allopathic and naturopathic, prescription and over-the-counter, is also essential, as many drugs impact platelet number and function. Platelet function can also be altered in the setting of systemic disorders, such as chronic kidney disease, liver disease, myeloproliferative and myelodysplastic disorders, and malignancy. Inherited platelet disorders are often syndromic, and are associated with manifestations like albinism, bone changes, sensorineural hearing loss, renal disease, and immunodeficiency (see Chapter 126).

Platelet function can be evaluated with platelet aggregation studies. These tests measure the ability of agonists to cause in vitro platelet activation and aggregation. Two forms of platelet aggregation studies are available: light transmission aggregometry (LTA) in platelet-rich plasma and impedance aggregometry in platelet-rich plasma or whole blood. LTA in platelet-rich plasma is considered the gold standard for platelet function testing, whereas impedance aggregometry is less well characterized. Several recent guidelines have laid out standards on how to perform and interpret LTA.[18] Briefly, when a platelet agonist is added to platelet-rich plasma, aggregation occurs and light transmission increases. When factors like temperature, platelet count, and mixing speed are controlled, the amount and rate of this increase depends on how reactive platelets are to the added agonist. The increase in light transmission can be measured spectrophotometrically. Recommended agonists include ADP, epinephrine, arachidonic acid, collagen, and the thromboxane analogue U46619 at predetermined concentrations.[18] Ristocetin, an antibiotic that facilitates vWF binding to the glycoprotein Ib/IX/V complex, is also used in LTA to induce platelet agglutination. ADP and epinephrine induce a biphasic aggregation pattern; primary aggregation occurs in response to activation of the platelet glycoprotein IIb/IIIa receptor, then platelets degranulate and there is secondary aggregation. Conversely, arachidonic acid, thromboxane analogue, and collagen result in a monophasic aggregation pattern (see Chapter 123). Several defects of platelet function have well-characterized LTA patterns (Fig. 127.4).[19] Bernard–Soulier syndrome, a defect in glycoprotein Ib, results in normal aggregation to ADP and collagen, but absent agglutination to ristocetin (this pattern can also be seen in vWD.) Glanzmann thrombasthenia, a defect in glycoprotein IIb/IIIa, results in an absent primary response to ADP and collagen (see Chapter 123). Impaired aggregation to arachidonic acid suggests an aspirin effect, or cyclooxygenase deficiency. The abnormal arachidonic acid response can be further delineated with the use of thromboxane analogue U46619 (see Chapter 127). Platelet storage pool disorders can be diagnosed with LTA and studies that measure proteins within or released from platelet α-granules or dense granules. Tests of ATP secretion from platelets can complement and expand on LTA results, however reproducibility of these findings is poor.[20] ATP secreted by dense granules is measured using a sensitive luminescent (firefly luciferin-luciferase) assay for extracellular ATP. Platelet electron microscopy is increasingly used to evaluate the ultrastructure of platelets, including the dense granules. Finally, with appropriate monoclonal antibodies, flow cytometry can examine the surface of the platelet. Flow cytometry has a variety of uses, including detection of platelet activation by using antibodies to proteins expressed on the platelet surface (e.g., P-selectin, thrombospondin), diagnosis of platelet surface glycoprotein deficiencies, and measurement of dense granules (see Chapter 123).

Evaluation of von Willebrand Factor

vWD is discussed in detail in Chapter 132. It is diagnosed when patients have a history of mucocutaneous bleeding (usually with a positive family history), and quantitative or qualitative abnormalities of vWF. Laboratory testing for vWD includes the factor VIII assay, vWF antigen (vWF:Ag), and vWF activity (commonly measured with the ristocetin cofactor assay, vWF:Rco or with an automated assay).[21] Many laboratories also test vWF further with the collagen binding assay (vWF:CB), which is abnormal when there is a loss of high-molecular-weight vWF multimers or impaired binding of vWF to the vascular endothelium, the low-dose ristocetin-induced platelet aggregation assay (abnormal with loss-of-function and gain-of-function defects that characterize type 2B vWD and platelet-type pseudo-vWD), and vWF multimer analysis (which is useful in type 2A and 2M vWD).[21] Assays that measure factor VIII binding to vWF can identify patients with defects in factor VIII binding, such as type 2N vWD.[21] Highly specialized coagulation laboratories may also perform vWF gene sequence analysis and vWF propeptide analysis; the latter shows promise in detecting defects associated with accelerated vWF clearance (see Chapter 132).[21]

The diagnosis of vWD, particularly type 1 vWD, which is defined as a partial, quantitative deficiency of vWF, can be challenging.[15]

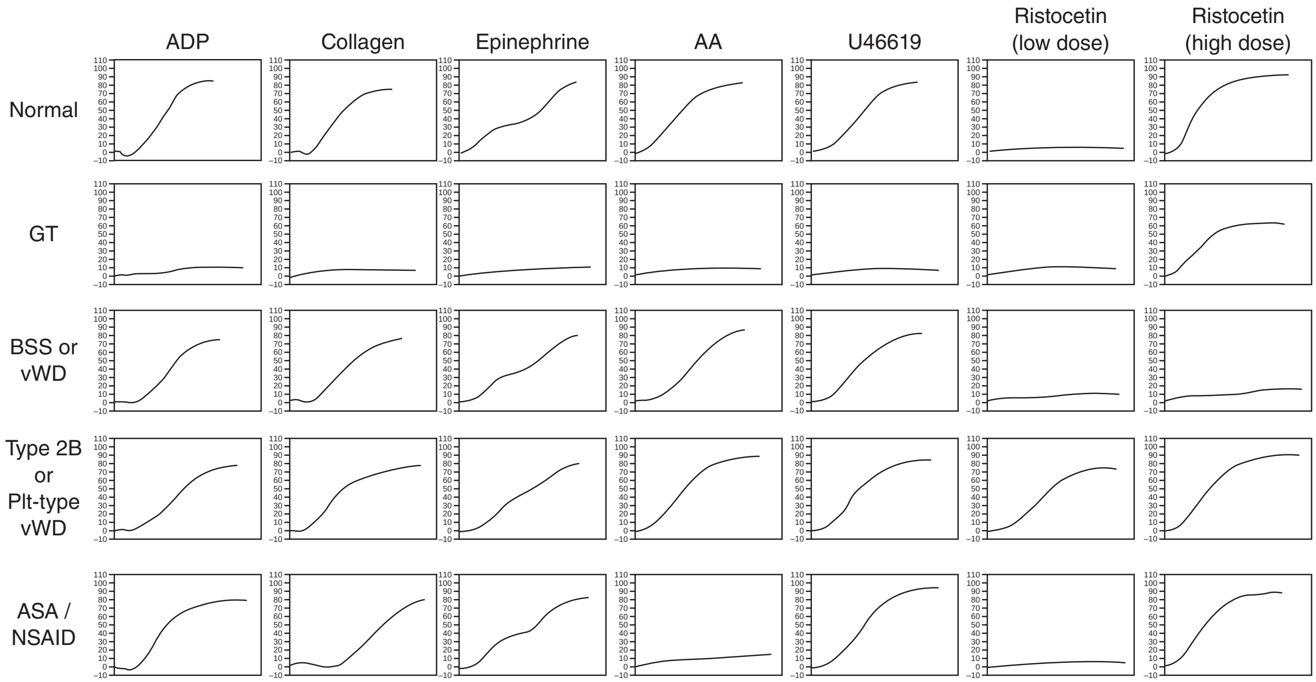


Figure 127.4 LIGHT TRANSMISSION AGGREGOMETRY PATTERNS. In Glanzmann thrombasthenia (GT) aggregation is absent or decreased with all agonists except high dose ristocetin. In Bernard-Soulier syndrome (BSS) and von Willebrand disease (vWD) there is an aggregation response with all agonists except ristocetin. In the presence of acetylsalicylic acid (ASA) or nonsteroidal antiinflammatory drugs (NSAID) aggregation is reduced with arachidonic acid (AA) but present with thromboxane analogue U46619. *ADP*, Adenosine diphosphate.

Studies have shown a high prevalence of bleeding symptoms in normal, healthy individuals. By definition, 2.5% of the population will also have a level of vWF:Ag that falls below the lower limit of laboratories' normal reference range. Thus, there is a danger of coincidentally associating low vWF with bleeding symptoms, and overdiagnosing type 1 vWD in the general population.[22] To further complicate matters, the normal range of plasma vWF is quite broad. vWF is an acute phase reactant, and can increase in patients with inflammation, infection, or stress. Levels also increase with pregnancy and with comorbid diseases like cancer. vWF levels increase with age and are lower in individuals with blood type O (see Chapter 132). It is recommended that the label of type 1 vWD only be applied when the clinical presentation is consistent, and when vWF levels are persistently low and <30 IU/dL.[15]

LABORATORY EVALUATION OF FIBRINOLYSIS

Disorders of fibrin cross-linking and fibrinolysis are not recognized with routine coagulation and platelet testing (see Table 127.1). Congenital factor XIII deficiency, a rare disease with a prevalence of 1 in 2 or 3 million people worldwide, is one example.[23] Screening tests for factor XIII deficiency aim to denature fibrin clots in acetic acid, monochloroacetic acid, or urea.[23] In the urea clot solubility test, clot formation is induced in patient plasma and in normal and factor XIII deficient plasma controls by addition of calcium with or without added thrombin. Clots are exposed to a 5 M urea solution and observed over time. The control clot should remain undissolved at 24 hours because fibrin clots cross-linked in the presence of thrombin and factor XIII are stable. If the patient has severe factor XIII–deficiency, the clot will dissolve rapidly. The urea clot solubility test and related assays are not standardized and have poor sensitivity; their detection limit is generally less than 5% of factor XIII activity. Suspected factor XIII deficiency must be confirmed by functional assays (which measure the release of ammonia during the transglutaminase reaction or incorporation of radioactive amines into proteins), or immunologic factor XIII antigen assays (see Chapter 134).[23]

Tests that provide a global picture of fibrinolysis can also be performed; they measure the combined effect of plasminogen activators (e.g., urokinase, tissue plasminogen activator) and inhibitors (e.g., α_2-antiplasmin, plasminogen activator inhibitor-1 [PAI-1]). Defects in the latter group of proteins can produce hyperfibrinolytic states and increased bleeding potential. In the euglobulin clot lysis assay, plasma is diluted and acidified at 4 °C. This causes precipitation of the acid insoluble or euglobulin fraction of plasma, which includes fibrinogen, plasminogen, plasminogen activators, and PAI-1. The precipitate is redissolved, and the fibrinogen is clotted by adding calcium. The time to spontaneous lysis of the fibrin clot is then monitored. Shortened lysis times are observed in hyperfibrinolytic states such as DIC, or when there is a deficiency of PAI-1; however, like the urea clot solubility test, the euglobulin clot lysis time is hampered by a lack of standardization and susceptibility to interference by other factors. Specialized coagulation laboratories may offer specific assays for plasminogen inhibitors as well.

OTHER ACTIVITIES FOR HEMOSTASIS LABORATORIES

Global Hemostasis Assays

Standard coagulation tests can measure the individual components of hemostasis. There is tremendous interest in evaluating hemostasis more globally, to accurately evaluate in vivo hemostatic function, sensitively and specifically diagnose hemostatic disorders, monitor treatment, and better predict clinical manifestations of disordered hemostasis. Global hemostasis assays aim not only to measure components of hemostasis, but also determine how they interact with each other. They do so by assessing the rate of thrombin generation, the quantity of thrombin generation, the formation of clots in whole blood, and/or the polymerization of fibrin.

Measurement of thrombin generation was first described in the early 1950s by MacFarlane and Biggs (who used whole blood), and

Pitney and Dacie (who used plasma). Over the years, methods were refined; ultimately, continuous measurement of thrombin generation was accomplished by using a thrombin-chromogenic substrate in defibrinated plasma, or a fluorogenic substrate in whole plasma. In general, modern thrombin generation assays (TGAs) use recalcified platelet-rich or platelet-poor plasma, and then trigger clotting with tissue factor. TGAs can capture not only the rate and amount of thrombin generated, but also the role of platelets (which act as an amplifying surface for thrombin generation) if they are performed in platelet-rich plasma. TGAs have been widely used in research. Clinically, they may play a role in thrombophilic states (e.g., antithrombin deficiency, protein C or S deficiency, activated protein C resistance, cancer-associated thrombosis) and in hemorrhagic tendencies (e.g., hemophilia, other factor deficiencies, cardiac surgery). In hemophilia, TGAs might more accurately predict bleeding risk and efficacy of inhibitor bypassing than traditional tests. TGAs are not yet used broadly in the clinical context; test procedures are not well standardized, and validated reference ranges for specific conditions have not been developed.

Viscoelastic assays like thromboelastography and thromboelastometry are increasingly used in the clinical context. Viscoelastic tests are based on the premise that the result of normal hemostasis is to rapidly create a strong, stable clot; changes in the speed of clot formation, or its strength or stability, may indicate abnormal hemostatic function. Thromboelastography was first described in 1948 by Hartert, who presented a method in which clotting was triggered in fresh whole blood with the addition of celite (an activator of the intrinsic pathway). The blood was then put into a continuously rotating cup, and a torsion wire was introduced into the system. As the clot gradually became stronger, the movement of the torsion wire dampened and ultimately stopped. A tracing of the torsion wire's movement over time reflected the velocity of clot formation, its maximal stability, and its gradual dissolution. This tracing was recorded onto light-sensitive photographic paper by a mirror-galvanometer; the image developed over hours to days.

Currently, two semi-automated commercially available viscoelastic devices allow results to be obtained in 10 to 15 minutes: the ROTEM-analyzer (TEM International, Munich, Germany), which uses a fixed cup with a rotating pin and optical detector, and the TEG-analyzer (Haemonetics Corp., Braintree, MA), which uses a torsion wire and a rotating cup. Both are kinetic tests, and their tracings measure clot formation over time. They also measure maximal clot strength, clot elasticity, and clot lysis. Both use citrated whole blood that is recalcified and added to a cuvette. An activator (e.g., tissue factor) is commonly used to standardize the test and speed up the assay. These analyzers can perform additional tests (using different activators or additives) to provide information like that of the APTT or PT, to neutralize heparin, to inhibit fibrinolysis, and to qualitatively analyze the functional fibrinogen component.

A TEG tracing has several components (Fig. 127.5). Initial clot formation, and the activity of coagulation factors, is captured by the reaction (R) time. As the clot forms, the TEG measures the kinetic (K) time, which reflects the interaction of coagulation factors, fibrin, and platelets. The maximal amplitude (MA) reflects platelet function and fibrinolysis. Once the clot begins to break down, the TEG gives information on lysis at 30 and 60 minutes (CL30 and CL60, respectively). These components reflect fibrinolysis. A ROTEM tracing provides similar information; the CT, clot formation time (CFT), maximum clot formation (MCF), and lysis time (LY) are essentially interchangeable with the R time, K time, MA, and CL, respectively.[24]

Viscoelastic methods are increasingly used at the bedside.[24] They have shown some promise in detecting coagulopathies and guiding the use of blood products and other prohemostatic therapy intraoperatively, in obstetric hemorrhage, in the trauma setting, and in the intensive care unit.[25] They may also be helpful in screening for hypercoagulable states, as some individuals with a history of thromboembolism demonstrate accelerated clot propagation. Viscoelastic methods do have some drawbacks. They are insensitive to platelet and vWF dysfunction, as well as to factor XIII problems. Assay standardization and variability of results continue to be a challenge.[26,27]

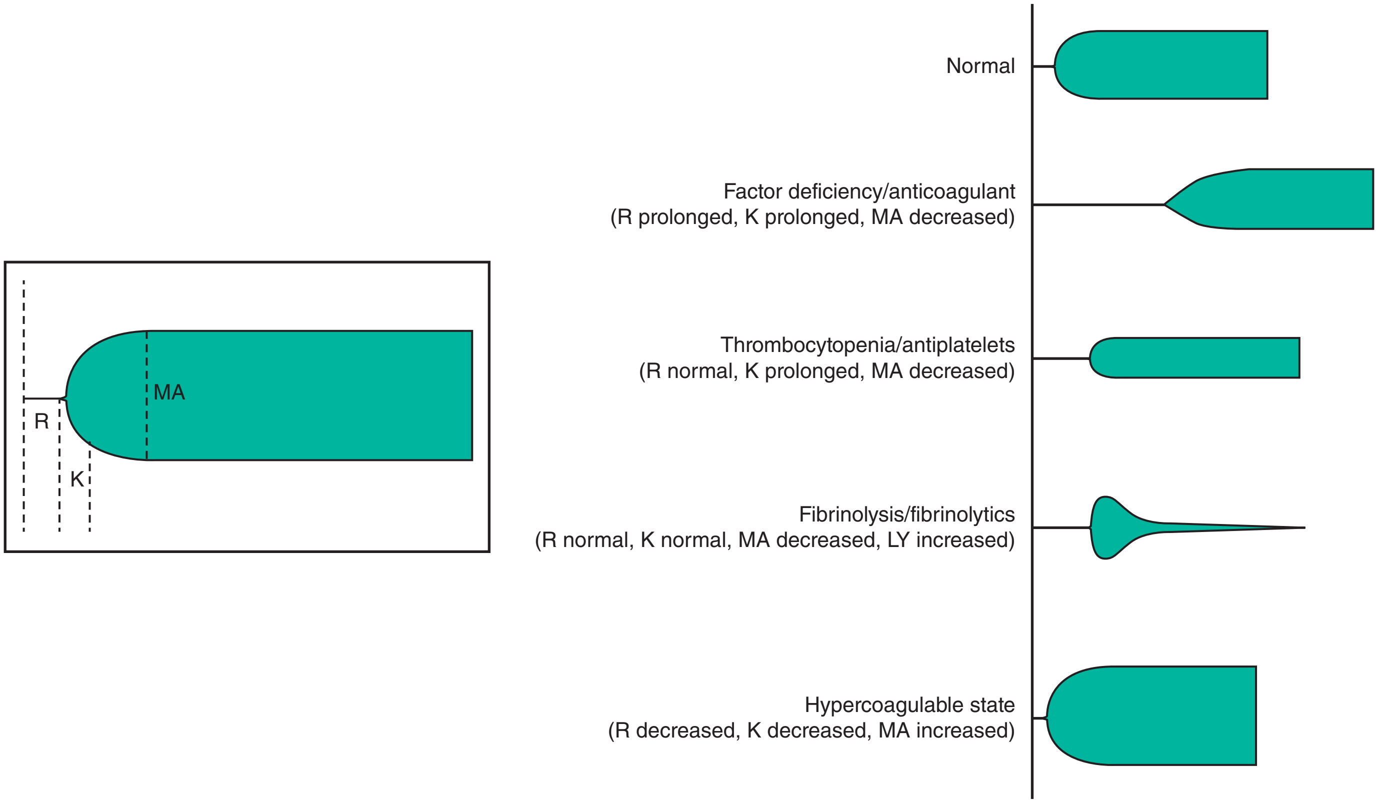

Normal
Factor deficiency/anticoagulant
(R prolonged, K prolonged, MA decreased)
MA
R
K
Thrombocytopenia/antiplatelets
(R normal, K prolonged, MA decreased)
Fibrinolysis/fibrinolytics
(R normal, K normal, MA decreased, LY increased)
Hypercoagulable state
(R decreased, K decreased, MA increased)

Figure 127.5 COMPONENTS OF A TEG (thromboelastogram) TRACING AND EXAMPLES OF DISORDERED COAGULATION. Initial clot formation and the activity of coagulation factors is captured by the reaction (R) time. The interaction of factors, fibrin, and platelets is captured by the kinetic (K) time. Platelet function and fibrinolysis are captured by the maximal amplitude (MA). Once the clot begins to break down, the TEG gives information on lysis at 30 and 60 minutes. Various disorders of coagulation can be detected using the TEG.

Evaluation of Prothrombotic States

In addition to the diagnosis of bleeding disorders, the hemostasis laboratory can help evaluate acquired and inherited prothrombotic states (see Chapter 137). It is known that certain prothrombotic states (e.g., antithrombin, protein C, and protein S deficiencies; antiphospholipid syndrome) may confer a higher risk for recurrent thrombosis. Patients with these abnormalities may benefit from long-term anticoagulation after their first episode of thrombosis. Less severe prothrombotic states (e.g., heterozygous factor V Leiden and prothrombin 20210 polymorphisms, functional defects in fibrinogen and plasminogen) do not appear to impact the risk of recurrence. Clinicians often consider testing for prothrombotic states in patients who develop thrombosis at a young age, who have a strong family history of thrombosis, who have thrombosis at an unusual site, or who experience recurrent or unexplained thrombosis. However, the utility of this testing is limited, as the results usually do not affect patient management or result in improved patient outcomes.[28] Testing can also result in potential harm, including inappropriate use of anticoagulation and undue patient anxiety. For these reasons, testing for prothrombotic states is not routinely recommended in unselected patients with thrombosis. Testing should only be undertaken with patients whose clinical presentation strongly suggests an underlying prothrombotic condition. Before testing is done, clinicians should consider how it will affect management, and if it is aligned with the patient's values and preferences. Clinicians should also understand the effect of anticoagulants on commonly ordered coagulation tests, to ensure results are not misinterpreted (Table 127.2).

Monitoring Anticoagulant Therapy

The coagulation laboratory plays an important role in the monitoring of patients on anticoagulant therapy (see Table 127.2). Historically, monitoring was limited to unfractionated heparin and warfarin, which are monitored using the clot-based APTT and PT/INR assays, respectively. However, coagulation laboratories are increasingly developing assays to monitor low-molecular-weight heparin (LMWH), fondaparinux, parenteral direct thrombin inhibitors, and the direct oral anticoagulants (DOACs), which include dabigatran, rivaroxaban, apixaban, and edoxaban (see Chapter 142). Therapeutic ranges for these agents are not clinically validated and may vary by laboratory and by indication. However, clinicians may consider measurement of on-therapy drug levels in patients who are obese, have kidney or hepatic insufficiency, experience thrombosis or bleeding while on therapy, or require urgent surgery.

The ability to neutralize the enzymatic activity of factor Xa or thrombin is the most reliable method to quantify the plasma levels of anticoagulants. Anti-factor Xa chromogenic assays can be used to measure factor Xa inhibitors like LMWH, fondaparinux, rivaroxaban, apixaban, and edoxaban, while anti-IIa (thrombin) or ecarin based chromogenic and clot-based assays can measure direct thrombin inhibitors like argatroban, bivalirudin, hirudin, and dabigatran. Standard curves for anticoagulants are constructed by combining known amounts of the anticoagulant and the factor it inhibits. Residual factor activity is inversely proportional to the concentration of drug in the sample. Anti-factor Xa assays to monitor LMWHs can be developed against WHO's International Standard for LMWH,

TABLE 127.2 **The Effect of Anticoagulants on Commonly Ordered Coagulation Tests**

Test	Warfarin	UFH	LMWH	Direct Thrombin Inhibitors (e.g., Dabigatran)	Factor Xa Inhibitors (e.g., Rivaroxaban, Apixaban, Edoxaban)
PT/INR (clot based)	Increases	No effect or increases	No effect	No effect or increases	No effect or increases
APTT (clot based)	Increases	Increases	No effect or increases	No effect or increases	No effect or increases
Thrombin time (clot based)	No effect	Increases	No effect or increases	Increases	No effect
Clauss fibrinogen (clot based)	No effect	No effect	No effect	Minimal effect to falsely decrease (if direct thrombin inhibitor level very high)	No effect
Antithrombin (chromogenic anti-Xa based)	No effect	Decreases	No effect to decreases	No effect	Falsely increases
Antithrombin (chromogenic anti-IIa based)	No effect	Decreases	No effect to decreases	Falsely increases	No effect
Protein C—functional (clot based)	Decreases	No effect (neutralizer in reagent)	No effect	Falsely increases	Falsely increases
Protein C—functional (chromogenic)	Decreases	No effect (neutralizer in reagent)	No effect	No effect	No effect
Protein S—free (latex immunoassay)	Decreases	No effect	No effect	No effect	No effect
Activated protein C (APC) resistance (clot based)	Unknown	Unknown	Unknown	Unknown, may be falsely normal	Unknown, may be falsely normal
Lupus anticoagulant—lupus sensitive APTT (clot based)	Falsely increases, may correct on mixing	Falsely increases, generally does not correct on mixing	Possibly falsely increases, generally does not correct on mixing	Falsely increases, generally does not correct on mixing	Falsely increases, generally does not correct on mixing
Lupus anticoagulant—DRVVT (dilute russell viper venom time) (clot based)	Falsely increases, does not correct on mixing	No effect	No effect	Falsely increases, does not correct on mixing	Falsely increases, does not correct on mixing

APTT, Activated partial thromboplastin time; *INR*, international normalized ratio; *PT*, prothrombin time.

which serves as a calibrator of drug levels. Other anticoagulants require unique calibrated assays.[29]

The DOACs also affect more commonly used clot-based assays. The TCT and APTT are more sensitive to dabigatran than the PT, and the TCT can be used to exclude the presence of dabigatran and its anticoagulant effect. The PT is sensitive to rivaroxaban (though sensitivity varies by laboratory reagent), while it is less sensitive to apixaban.[30] The PT should not be used to exclude clinically significant drug concentrations of either rivaroxaban or apixaban; appropriately calibrated anti-factor Xa assays are more appropriate.

It is important to understand the effect of anticoagulants on commonly ordered coagulation tests, not just to estimate their drug concentrations, but to ensure that these test results are not misinterpreted due to the presence of interfering drugs (see Table 127.2).

Evaluating Activated Coagulation States

Specialized coagulation laboratories can monitor activated coagulation states that result in acute hemostasis and thrombosis, such as antiphospholipid syndrome (see Chapter 138), heparin-induced thrombocytopenia (see Chapter 130), and warfarin–induced skin necrosis (see Chapter 142). The classic activated coagulation state is DIC (see Chapter 136). DIC is a clinicopathologic state characterized by excessive and dysregulated activation of coagulation and fibrinolysis, with consequent consumption of prothrombotic and antithrombotic factors. DIC is triggered by an underlying cause, such as sepsis, malignancy, trauma, obstetrical complications, or intravascular hemolysis. There are two forms of DIC: an *acute* and a *chronic* form. The acute form is characterized by overwhelming defibrination and a tendency to hemorrhage. The APTT and PT are elevated, and the fibrinogen level and platelet count are low. The more indolent chronic form is characterized by partially compensated coagulation and a tendency to thrombosis. The APTT, PT, and platelet count may be normal or slightly low, and the fibrinogen can be normal or even elevated.

The laboratory diagnosis of DIC requires evidence of simultaneous coagulation and fibrinolysis. Evaluation begins with a complete blood count, PT, APTT, and fibrinogen. The D-dimer assay is a useful adjunct to confirm fibrinolysis. D-dimers are insoluble degradation products that arise when fibrin is cross-linked by thrombin-activated factor XIII, and then degraded by plasmin. Though D-dimer elevation is a sensitive test for DIC, it is not specific for this condition; D-dimer elevation occurs in pregnancy, large vessel thrombosis, hepatic failure, and many other conditions. Isolated D-dimer elevation in the absence of another etiology can also suggest chronic DIC. Laboratory testing can be repeated serially to monitor the severity of DIC, and its response to treatment.

REFERENCES

1. Macfarlane RG. An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. *Nature*. 1964;202:498–499.
2. Davie EW, Ratnoff OD. Waterfall sequence for intrinsic blood clotting. *Science*. 1964;145:1310–1312.
3. Osterud B, Rapaport SI. Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation. *Proc Natl Acad Sci USA*. 1977;74:5260–5264.
4. Gailani D, Broze Jr GJ. Factor XI activation in a revised model of blood coagulation. *Science*. 1991;253:909–912.
5. Schmaier AH. Physiologic activities of the contact activation system. *Thromb Res*. 2014;133(Suppl 1):S41–S44.
6. Poller L, Ibrahim S, Keown M, et al. Simplified method for international normalized ratio (INR) derivation based on the prothrombin time/INR line: an international study. *Clin Chem*. 2010;56:1608–1617.
7. Poller L, Van Den Besselaar AM, Jespersen J, et al. The effect of sample size on fresh plasma thromboplastin ISI determination. *Br J Haematol*. 1999;105:655–663.
8. Verhovsek M, Moffat KA, Hayward CPM. Laboratory testing for fibrinogen abnormalities. *Am J Hematol*. 2008;83:928–931.
9. Hayward CPM, Moffat KA. Laboratory testing for bleeding disorders: strategic uses of high and low-yield tests. *Int J Lab Hematol*. 2013;35:322–333.
10. Gray E, Kitchen S, Bowyer A, et al. Laboratory measurement of factor replacement therapies in the treatment of congenital haemophilia: a United Kingdom Haemophilia Centre Doctors' Organisation guideline. *Haemophilia.*. 2020;26(1):6–16.
11. Green D. Factor VIII inhibitors: a 50-year perspective. *Haemophilia*. 2011;17:831–838.
12. Verbruggen B, Novakova I, Wessels H, et al. The Nijmegen modification of the Bethesda assay for factor VIII:C inhibitors: improved specificity and reliability. *Thromb Haemost*. 1995;73:247–251.
13. Pengo V, Tripodi A, Reber G, et al. Update of the guidelines for lupus anticoagulant detection. Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis. *J Thromb Haemost*. 2009;7:1737–1740.
14. Palla R, Peyvandi F, Shapiro AD. Rare bleeding disorders: diagnosis and treatment. *Blood*. 2015;125:2052–2061.
15. James PD, Connell NT, Ameer B, et al. ASH ISTH NHF WFH 2021 guidelines on the diagnosis of von Willebrand disease. *Blood Adv*. 2021;5(1):280–300.
16. Peterson P, Hayes TE, Arkin CF, et al. The preoperative bleeding time test lacks clinical benefit: College of American Pathologists' and American Society of Clinical Pathologists' position article. *Arch Surg*. 1998;133:134–139.
17. Quiroga T, Goycoolea M, Munoz B, et al. Template bleeding time and PFA-100 have low sensitivity to screen patients with hereditary mucocutaneous hemorrhages: comparative study in 148 patients. *J Thromb Haemost*. 2004;2:892–898.
18. Cattaneo M, Cerletti C, Harrison P, et al. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. *J Thromb Haemost*. 2013;11(6):1183–1189.
19. Hayward CPM, Pai M, Liu Y, et al. Diagnostic utility of light transmission platelet aggregometry: results from a prospective study of individuals referred for bleeding disorder assessments. *J Thromb Haemost*. 2009;7:676–684.
20. Badin MS, Graf L, Iyer JK, et al. Variability in platelet dense granule adenosine triphosphate release findings amongst patients tested multiple times as part of an assessment for a bleeding disorder. *Int J Lab Hematol*. 2016;38(6):648–657.
21. Ng C, Motto DG, Di Paola J. Diagnostic approach to von Willebrand disease. *Blood*. 2015;125:2029–2037.
22. Sadler JE. Von Willebrand disease type 1: a diagnosis in search of a disease. *Blood*. 2003;101:2089–2093.
23. Karimi M, Peyvandi F, Naderi M, Shapiro A. Factor XIII deficiency diagnosis: challenges and tools. *Int J Lab Hematol*. 2018;40(1):3–11.
24. Curry NS, Davenport R, Pavord S, et al. The use of viscoelastic haemostatic assays in the management of major bleeding: A British Society for Haematology Guideline. *Br J Haematol*. 2018;182(6):789–806.
25. Wikkelsø A, Wetterslev J, Møller AM, Afshari A. Thromboelastography (TEG) or thromboelastometry (ROTEM) to monitor haemostatic treatment versus usual care in adults or children with bleeding. *Cochrane Database Syst Rev*. 2016(8):CD007871.
26. Bolliger D, Tanaka KA. Point-of-care coagulation testing in cardiac surgery. *Semin Thromb Hemost*. 2017;43(4):386–396.
27. Kitchen DP, Jennings I, Kitchen S, et al. Bridging the gap between point-of-care testing and laboratory testing in hemostasis. *Semin Thromb Hemost*. 2015;41:272–278.
28. Middeldorp S, van Hylckama Vlieg A. Does thrombophilia testing help in the clinical management of patients? *Br J Haematol*. 2008;143:321–335.
29. Gosselin RC, Adcock DM, Bates SM, et al. International Council for Standardization in Haematology (ICSH) recommendations for laboratory measurement of direct oral anticoagulants. *Thromb Haemost*. 2018;118(03):437–450.
30. Sarode R. Direct oral anticoagulant monitoring: what laboratory tests are available to guide us? *Hematology Am Soc Hematol Educ Program*. 2019;2019(1):194–197.

CHAPTER 128

ACQUIRED DISORDERS OF PLATELET FUNCTION

Peter L. Gross and José A. López

Acquired disorders of platelet function are among the most common hematologic abnormalities, which reflects the sensitivity of platelets to external and internal perturbations. The clinical challenge in evaluating acquired disorders of platelet function is to determine whether observed derangements in platelet function pose a threat to the patient. Although altered platelet function can predispose patients to either hemostatic or thrombotic disorders, this chapter deals primarily, but not exclusively, with platelet disorders that may compromise hemostasis. We also try to help clinicians determine the clinical importance of these disorders, but the marked variation in bleeding risks associated with disorders of platelet function renders this a difficult task. Bleeding in patients with acquired platelet dysfunction is likely to occur less frequently and predictably than in those with severe inherited platelet disorders such as Bernard-Soulier syndrome or Glanzmann thrombasthenia (see Chapter 123) and may manifest only in the setting of trauma or surgery or in the presence of additional hemostatic defects. However, even some patients with inherited platelet disorders only bleed when stressed by surgery or trauma, making the distinction between inherited and acquired disorders less clear-cut.

The laboratory evaluation of these patients may illuminate these disorders (see Chapter 126) but may offer little concrete guidance in management. Acquired platelet defects (Table 128.1) may produce abnormal laboratory test results, such as a prolonged closure time on a platelet function analyzer (PFA) or abnormal aggregation in response to added agonists. Historically, the bleeding time was used to evaluate bleeding risk in patients with jaundice or uremia, but it measures hemostasis only in one vascular bed (the skin) and bears inherent inter-operator variability. Although platelet assays are useful research tools, defects quantified by laboratory tests, including the bleeding time, are not predictive of bleeding in these patients. Additionally, some acquired platelet disorders increase the risk of thrombosis rather than bleeding, a risk for which there is currently no effective screening test. Lastly, tests that depend on assessment of the function of platelets ex vivo do not assess the contributions of labile substances from the endothelium (see Chapter 122) and occasionally (as in platelet aggregometry) do not account for flow, a key component of in vivo platelet function.

DRUGS, FOODS, AND ADDITIVES THAT AFFECT PLATELET FUNCTION

The most common causes of acquired platelet dysfunction are drugs. In a large prospective study, 5649 unselected adult patients were screened preoperatively for hemostatic defects with the activated partial thromboplastin time (aPTT), prothrombin time (PT), platelet count, PFA-100 testing, and a questionnaire regarding bleeding history.[1] Bleeding history was positive in 628 patients (11.1%), and impaired hemostasis was verified in 256 (40.8%) of these patients. Of these 256 patients, 162 (63.28%) were found to have acquired platelet dysfunction. Antiplatelet drugs or nonsteroidal anti-inflammatory drugs (NSAIDs) were responsible for the acquired platelet dysfunction in 147 patients and antibiotics were responsible in 10 patients.

Numerous drugs affect platelet function (Table 128.2). For several, inhibition of platelet function is their intended effect; for most, platelet dysfunction is an unintended and undesired side effect. Drug-induced platelet function abnormalities do not usually cause a clinically significant problem in healthy individuals. However, these drugs may increase the bleeding risk with interventions (e.g., surgery, biopsy), trauma, or in the presence of other hemostatic defects, such as those associated with cirrhosis or uremia. Antiplatelet agents are discussed more fully in Chapter 143 The effects of these drugs on platelet function are defined by abnormalities of platelet aggregation, but their contribution to a risk of excessive bleeding is definitively established only for acetylsalicylic acid (ASA, aspirin), clopidogrel, prasugrel, ticagrelor, inhibitors of glycoprotein IIb/IIIa, and vorapaxar.

ANTIPLATELET DRUGS

Activation Receptor Antagonists

Adenosine Diphosphate (ADP) Receptor Antagonists

The thienopyridines selectively and irreversibly inhibit ADP-mediated platelet activation and aggregation.[2] ADP activates platelets by raising the concentration of cytoplasmic Ca^{2+} through influx from the extracellular fluid and mobilization from internal stores, and by decreasing the concentration of intracellular cyclic adenosine monophosphate (cAMP) coupled to inhibition of adenylyl cyclase. ADP receptors can be divided into two groups: the G protein–coupled receptors, termed P2Y, and the ionotropic receptors, P2X. Platelets contain two P2Y receptors, $P2Y_1$ and $P2Y_{12}$, complexed to the heterotrimeric G proteins G_q and G_{i2}, respectively. ADP binding to $P2Y_1$ is necessary for platelet aggregation but is not sufficient. Instead, $P2Y_1$ is responsible for ADP-induced platelet shape change, and its engagement by ADP triggers a transient aggregatory response. $P2Y_{12}$, coupled to inhibition of adenylyl cyclase, mediates the amplification of the aggregation response.[3] The thienopyridine derivatives have no effects on arachidonic acid metabolism and hence act synergistically with ASA to inhibit platelet function.

The thienopyridines are all prodrugs of similar structure that produce active metabolites that bind irreversibly to $P2Y_{12}$, inhibiting ADP-mediated aggregation for up to 10 days after withdrawal of the drug, paralleling the platelet life span.[4] Because most platelet agonists require ADP for their full activity, the thienopyridines inhibit platelet activation by all agonists except strong agonists at high concentrations.[5] Another hypothetical advantage over ASA is their inhibition of shear-induced platelet aggregation.[6]

There are three generations of thienopyridine drugs, represented by ticlopidine, clopidogrel, and prasugrel. Ticlopidine is rarely used because of potentially serious side effects including agranulocytosis, thrombocytopenia, and thrombotic thrombocytopenic purpura.[7] The non-thienopyridine $P2Y_{12}$ inhibitors, which include ticagrelor and cangrelor, are not prodrugs and serve as reversible inhibitors.

Thrombin Receptor Antagonists

Direct-Acting Agents

Thrombin, the most potent platelet agonist, activates platelets via protease activated receptor (PAR)-1 and PAR-2. Vorapaxar, a tricyclic

TABLE 128.1 Acquired Disorders of Platelet Function

Drugs, Foods, and Additives

- Drugs: see Table 128.2
- Food and additives: omega-3 fatty acids, ethanol, ginger, onion, garlic, black tree fungus, Gingko biloba, cumin, turmeric, tonic water, caffeine, pineapple, others

Clonal Disorders

- Clonal hematologic diseases
- Myeloproliferative neoplasms
- Paroxysmal nocturnal hemoglobinuria
- Paraproteinemias
- Leukemias and myelodysplastic syndromes
- Solid tumors

Systemic Metabolic Disorders

- End-stage renal disease
- Liver diseases
- Diabetes and hyperlipidemias

Platelet Dysfunction Related With Extracorporeal Circuits

Miscellaneous

- Hypothermia
- Trauma
- SARS-CoV-2

Tropical Diseases

- Scurvy
- Acquired platelet dysfunction with eosinophilia

himbacine derivative, is a PAR-1 antagonist with a long half-life (up to 8 days) and a slow off-rate. When used in combination with ASA, vorapaxar increased the risk of major bleeding compared with placebo. On its own, vorapaxar reduced the risk of major adverse cardiovascular events in patients with peripheral artery disease compared with placebo, but was associated with a small increase in the risk of bleeding.[8] Vorapaxar is contraindicated in patients with a history of stroke, transient ischemic attack, or intracranial hemorrhage.

Indirect Agents

The COMPASS trial showed that a combination of ASA plus low dose rivaroxaban reduced the risk of major adverse cardiovascular and limb events compared with ASA or rivaroxaban alone.[9] The mechanism responsible for the synergistic effect of ASA and rivaroxaban is unclear but likely reflects the capacity of rivaroxaban to attenuate thrombin generation and to inhibit PAR-1 activation by factor Xa.[10] Although these findings suggest that any anticoagulant that reduces factor Xa generation would have antiplatelet effects, when full dose apixaban was compared with placebo on top of antiplatelet therapy in patients with acute coronary syndrome, there was excessive bleeding with apixaban and no reduction in major adverse cardiovascular events.[11] Therefore, synergism depends on using the right dose of the anticoagulant in combination with antiplatelet therapy.

Experimental Agents

Thromboxane Receptor Antagonists

Thromboxane synthesis inhibitors and thromboxane receptor antagonists were developed to circumvent the non-platelet effects of cyclooxygenase (COX) inhibitors.[12] Development was halted when clinical trials revealed no advantages over ASA.

TABLE 128.2 Drug-Induced Platelet Dysfunction

Anti-Platelet Drugs

- Activation receptor antagonists
  - ADP receptor antagonists
  - $P2Y_{12}$ antagonists
  - Thienopyridines: clopidogrel, ticlopidine, prasugrel
  - Nonthienopyridines: ticagrelor, cangrelor, elinogrel
- Dual $P2Y_{12}$ and $P2Y_1$ inhibition: GLS-409
- Thrombin receptor antagonists
- Direct: vorapaxar, PZ-128, BMS-986120
- Indirect: anticoagulants (e.g., rivaroxaban)
- Thromboxane receptor antagonists: ridogrel, seratrodast, terutroban, sulotroban, etc.
  - GPI/IX/V antagonists: anfibatide
  - GP VI antagonists: trowaglerix derived peptides

Inactivation Receptor Agonists

- Prostanoid agonists: epoprostenol, iloprost, beraprost

Aggregation Inhibitors

- αIIbβ3 inhibitors
- parentral: abciximab, eptifibatide, tirofiban
- oral: sibrafiban, orbofiban, iotrafiban

Signaling Molecule Inhibitors

- COX inhibitors
  - Acetyl salicylic acid (ASA, brand name aspirin)
  - NSAIDs: ibuprofen, naproxen, indomethacin, etc.
- PDE inhibitors
  - Nonselective PDE inhibitors: pentoxifylline, caffeine, theophylline
  - PDE3 inhibitors: cilostazol, milrinone, anagrelide
  - PDE5 inhibitors: dipyridamole, sildenafil
- STK inhibitors: BI1002494
- 12-Lipoxygenase inhibitors: ML-355
- Classes of drugs that inhibit platelets
  - Cardiovascular agents
  - Calcium channel blockers: nifedipine, diltiazem, verapamil
  - β-Blockers: propranolol
  - Vasodilators: nitrates, nitroprusside
  - Diuretics: furosemide
  - Angiotensin II receptor antagonist: losartan, valsartan, and olmesartan
  - Antibiotics: β-lactams, amphotericin, hydroxychloroquine, nitrofurantoin
  - Antifungal drugs: miconazole, amphotericin B
  - Psychiatric drugs: TCAs, fluoxetine, chlorpromazine, promethazine, trifluoperazine
  - Oncologic drugs: mithramycin, daunorubicin, BCNU, asparaginase, vincristine, dasatinib, ibritunib
  - Anesthetics: dibucaine, procaine, halothane, sevoflurane, propofol
  - Plasma expanders: dextran, hydroxyl ethyl starch
- Heparins and thrombolytic agents
  - Miscellaneous: clofibrate, statins, cocaine, ketanserin, radiographic contrast agents, antihistamines, immunosuppressive drugs

ADP, Adenosine diphosphate; *BCNU*, carmustine; *COX*, cyclooxygenase; *NSAID*, nonsteroidal antiinflammatory drug; *PDE*, phosphodiesterase; *TCA*, tricyclic antidepressant.

GPVI Antagonists

Genetic deficiency of glycoprotein (GP) VI, a collagen receptor on platelet, is associated with a mild bleeding diathesis. GPVI antagonists include revacept, a fusion protein containing the extracellular domain of GPVI linked to an Fc fragment, antibodies against GPVI and peptide fragments of the *Tropidolaemus wagleri venom trowaglerix*.[13]

GP I/IX/V Antagonists

The GP I/IX/V complex serves as the primary platelet receptor for von Willebrand factor (vWF). Caplacizumab, which binds vWF and blocks its interaction with the receptor, is approved for the treatment of thrombotic thrombocytopenic purpura (see Chapter 132). Other agents that target the same interaction include ARC1779, an aptamer that binds vWF, and anfibatide, which binds GPIb.[13]

Activation Receptor Agonists

Adenyl Cyclase Stimulators or Prostacyclin Analogues

Epoprostenol (the synthetic salt of prostacyclin), prostacyclin (iloprost), and an orally active stable analogue (beraprost) are prostacyclin-like agents that are used for the treatment of pulmonary arterial hypertension and peripheral artery disease.[14] Despite the potent inhibition of platelet aggregation induced by prostacyclin in vitro, it has little or no effect on the bleeding time.

Aggregation Inhibitors

Parenteral $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) Inhibitors

The final common step in platelet aggregation is activation of the major platelet integrin $\alpha_{IIb}\beta_3$ to a ligand-competent form, which binds multivalent ligands such as fibrinogen or vWF, thereby cross-linking platelets into aggregates. Conventional antiplatelet agents such as ASA and clopidogrel each inhibit only one pathway leading to platelet aggregation, ASA preventing thromboxane production, and clopidogrel blocking the major ADP receptor. Dual therapy with ASA and clopidogrel improves the clinical benefit of antiplatelet therapy, but aggregation is still able to proceed through the action of other agonists such as thrombin. Because $\alpha_{IIb}\beta_3$ engagement is required for aggregation through all pathways, blocking this receptor inhibits platelet aggregation more effectively.

Inhibitors of $\alpha_{IIb}\beta_3$ work selectively and competitively to inhibit platelet aggregation.[15,16] These agents are intended to produce a transient effect akin to the defect in Glanzmann thrombasthenia, but there are important differences between the drug effect and the disease.

Although these agents share many similarities in their antithrombotic properties, they also have several clinically important pharmacokinetic and off-target effect differences. Three drugs are available: eptifibatide, tirofiban, and abciximab.

Abciximab

The first $\alpha_{IIb}\beta_3$ inhibitor approved for clinical use, abciximab (ReoPro), is a human-murine chimeric Fab fragment of a monoclonal antibody that targets the β_3 subunit of $\alpha_{IIb}\beta_3$ and therefore also reacts with another integrin that shares the β_3 subunit, $\alpha_V\beta_3$. Although $\alpha_{IIb}\beta_3$ expression is restricted to megakaryocytes and platelets, $\alpha_V\beta_3$ is expressed on platelets; at low levels; and in cells of the vascular wall, including endothelial cells and fibroblasts.[17] In addition to its antithrombotic properties, abciximab may also reduce infarct size, prevent stent thrombosis, and modulate inflammatory responses by virtue of β_3 subunit inhibition in other tissues.[15]

Abciximab administered intravenously as a standard 0.25 mg/kg bolus blocks approximately 80% of surface $\alpha_{IIb}\beta_3$ and inhibits platelet aggregation to a similar extent, although the extent of inhibition is variable.[18] This level of blockade increases the bleeding time only mildly. The bleeding time only becomes markedly prolonged when receptor blockade exceeds 90%. After the bolus dose, abciximab is infused at a dose of 0.125 μg/kg/min for 12 hours. Several trials have established this as a clinically effective regimen.[19] Plasma levels of free abciximab drop rapidly after its administration, with an initial half-life of approximately 30 minutes. Most of the drug is bound to platelets. This explains why patients with thrombocytosis may require larger weight-adjusted doses of abciximab to attain a therapeutic antiplatelet effect[19] and why patients with lower platelet counts treated with the usual dose of abciximab display more profound platelet inhibition. Abciximab is not excreted in the urine and is probably metabolized by the reticuloendothelial system when platelets carrying bound abciximab are cleared from the circulation. The dose of abciximab does not need adjustment in patients with renal impairment. Although a daily platelet turnover rate of 10% would predict that no abciximab would be detected in blood after 10 days of its administration, platelet-bound abciximab has been observed up to 3 weeks after its initial administration, suggesting platelet-to-platelet redistribution of the drug or release of new platelets from megakaryocytes that had previously bound the drug.[18] Because of the low plasma levels of unbound abciximab, the drug's inhibitory effect can be rapidly reversed by platelet transfusion, and hemostasis should be normal when the concentration of infused platelets exceeds 50,000/μL, as it is in normal individuals. However, because of the ability of abciximab to redistribute to the newly transfused platelets, the platelet-bound abciximab may produce a gradual inhibitory effect on the infused platelets;[18] in patients with severe or refractory bleeding, it may be necessary to infuse a very large dose of platelets, sufficient to leave 50% of $\alpha_{IIb}\beta_3$ receptors free, a number shown to be sufficient for normal hemostasis in studies of Glanzmann thrombasthenia heterozygotes. Without platelet transfusion, platelet aggregation generally returns to baseline levels within 12 to 24 hours after discontinuing abciximab.

Eptifibatide

Eptifibatide (Integrilin) is a cyclic heptapeptide based on the Lys-Gly-Asp (KGD) sequence found in barbourin, a platelet-inhibitory disintegrin from the venom of the Southeastern Pigmy rattlesnake, *Sistrurus miliarius barbourin*.[20] Eptifibatide differs pharmacokinetically from abciximab in several important ways.[21] For example, unlike abciximab, intravenous eptifibatide infusion produces high levels of unbound drug because of its lower affinity for the receptor. Platelet transfusion, therefore, is not a good method for acutely reversing the antiplatelet effect of eptifibatide because the newly transfused platelets are rapidly inhibited. However, its short plasma half-life (≈2.5 hours) allows for rapid clearance of eptifibatide and the reversal of its antiplatelet effect when administration is discontinued. Platelet aggregation returns to normal in approximately 4 hours, with the bleeding time normalizing within 1 hour.[21]

Tirofiban

Tirofiban (Aggrastat) is a peptidomimetic agent based on the Arg-Gly-Asp (RGD) sequence, with a pharmacokinetic profile similar to that of eptifibatide; neither cross-reacts with $\alpha_V\beta_3$.[22] As with eptifibatide, the maximum antiplatelet effect is seen at concentrations that leave a high concentration of unbound drug in the plasma. Thus, platelet transfusions are not often effective for reversal of the antiplatelet effect of tirofiban. However, its half-life is even shorter than that of eptifibatide (≈2 hours), and its antiplatelet effect diminishes rapidly after discontinuation of the infusion.

The major adverse effects of $\alpha_{IIb}\beta_3$ inhibitors are thrombocytopenia and bleeding complications. Up to 5% of patients receiving abciximab experience mild thrombocytopenia (<100,000/μL), and

1% develop profound thrombocytopenia (<20,000/μL).[23,24] The incidence of severe thrombocytopenia is lower with tirofiban and eptifibatide, being approximately 0.2%.[24,25] A decrease in the platelet count is appreciable within the first hours after administration. It is therefore essential that a platelet count be obtained 2 to 4 hours after initiating treatment. Most patients with severe thrombocytopenia respond well to platelet transfusions and their platelet counts usually recover within 5 days, although they may take more than 1 week to do so.

Bleeding complications with $\alpha_{IIb}\beta_3$ inhibitors are quite common, especially after femoral artery catheterization. Rates of major bleeding vary between 0.7% and 5.2% in different randomized controlled studies.[25,26] Intracranial bleeding is very rare (0.09%).[25] The risk of bleeding increases in patients older than 65 years of age; in patients weighing less than 50 kg; in patients taking anticoagulant, antiplatelet, or thrombolytic therapies; and in patients with inherited or acquired bleeding disorders such as hemophilia and uremia, respectively.[25,26]

Oral $\alpha_{IIb}\beta_3$ Inhibitors

Although several oral αIIbβ3 inhibitors were developed, they failed to prevent major adverse cardiovascular events and some were associated with increased mortality that was not related to bleeding.[27] A plausible but unproven explanation for the increased mortality is that these agents produce a prothrombotic state because they block platelet aggregation but not platelet activation, and the activated platelets can then promote thrombin generation.

Signaling Molecule Inhibitors

Acetyl Salicylic Acid (ASA, Brand Names Including Aspirin)

ASA has been in routine use worldwide for more than 100 years and is still the most common agent associated with platelet dysfunction.[28] The antipyretic and analgesic effect of willow bark was first recorded by Galen. In 1826 and 1828 Leroux and Buchner isolated a compound from willow bark that they called salicin (from *salix* = willow in Latin). In 1897, Felix Hoffman from the Bayer Company, inspired by his father's severe arthritis and the untoward side effects of salicylic acid, discovered a method to convert salicylic acid, the active compound in willow bark, to a compound with less gastrointestinal (GI) toxicity, acetyl salicylic acid.[29] ASA was widely used for rheumatic fever in the 1940s. In the 1950s, case reports and uncontrolled studies were published suggesting that ASA could prevent myocardial infarction and stroke.[30,31] In the 1960s, the effect of ASA on platelet activation was revealed.[30] The clinical importance of the antithrombotic effect of ASA was clearly demonstrated in a study of 22,071 physicians who received either 325 mg of ASA or placebo every other day over the course of 5 years.[32] Those receiving ASA had a 44% decreased incidence of myocardial infarction. In a large meta-analysis, it was shown that ASA reduced death from vascular events by 15% and nonfatal vascular events by 30%.[32] The primary mechanism by which ASA impairs hemostasis is through irreversible acetylation of the enzyme cyclooxygenase-1 (COX-1), an early enzyme in the synthetic pathway that produces the potent platelet agonist, thromboxane A_2 (TXA_2). Because platelets have a limited capacity to synthesize new proteins, a single low dose of ASA (30 to 100 mg)[33] or as little as 10 mg taken daily for 1 week can completely inhibit TXA_2 production and therefore impair the function of a cohort of platelets for their circulating life span.[34] ASA also acetylates the isoform of COX mostly present in endothelial cells, COX-2; COX-2 acetylation blocks the synthesis of prostacyclin, a strong inhibitor of platelet activation.[35] However, COX-2 is less sensitive than COX-1 to inhibition by ASA,[28] and endothelial cells, unlike platelets, are able to synthesize new COX-2.[36]

Platelets exposed to ASA either in vivo or in vitro predictably demonstrate impaired aggregation in response to epinephrine, ADP, arachidonic acid, and low concentrations of collagen and thrombin,[37] a result of defective TXA_2 production. These agonists have therefore been defined as weak agonists, requiring that TXA_2 diffuse out of the platelet, bind to its receptor on the platelet membrane, and reinforce aggregation by promoting secretion from α- and dense granules. ASA-treated platelets stimulated with these agonists demonstrate only a primary, reversible wave of aggregation without granule secretion. Stronger agonists (high concentrations of thrombin and collagen) do not require TXA_2 synthesis to cause platelet secretion and irreversible aggregation, and thus most hemostatic function is maintained in ASA-treated patients.

ASA may also have COX-independent actions that may affect coagulation. At the site of microvascular injury, platelets bind to exposed collagen and coagulation is initiated by tissue factor concurrently (see Chapter 121). It was reported that low-dose ASA (30 mg/day for 7 days) decreased thrombin formation in healthy volunteers.[38] Undas et al.[39] used the same microvascular injury model and showed that ASA at 75 mg/day for 7 days decreased the velocity of prothrombin consumption by 29%, thrombin generation by 29%, and delayed both activation and maximum cleavage of factor XIII by thrombin. Interestingly, high cholesterol levels (greater than 240 mg/dL) impaired the antithrombotic effect of low-dose ASA in a microvascular injury model.[40]

In contrast to the antiplatelet effect of ASA, which appears unrelated to the dose above small threshold doses, GI mucosal toxicity is dose related.[41] The mechanism of mucosal injury appears to be distinct from the effect on hemostasis, involving ionic trapping of ASA within gut mucosal cells[42] and diminished synthesis of protective gut prostaglandins.[43,44] The inhibitory effect of even a single dose of ASA on gastric prostaglandin synthesis is prolonged, with one study showing that gastric COX activity was still 57% suppressed 72 hours after a single 325 mg dose of ASA.[45] Bleeding may originate from discrete ulcers or diffuse mucosal damage and is more common to arise from the upper GI tract. The risk of bleeding is increased even with doses of ASA as low as 10 to 30 mg/day;[46] the protective effect of resistant coatings is unproven.[47] The GI risk of ASA is increased in elderly adults (older than 65 years of age) and in those with concomitant medical conditions, such as cardiovascular disease, as well as in those taking certain other medications, such as NSAIDs.[47] ASA appears to delay the healing of gastric ulcers, possibly because it also interferes with the release of growth factors from platelets, such as endostatin and vascular endothelial growth factor.[48]

Regular use of ASA (≥75 mg) impairs primary hemostasis dose-dependently.[33] ASA treatment is associated with a small but significant increase in mucocutaneous bleeding as evidenced by easy bruising, hematemesis, melena, epistaxis, and an increased frequency of blood transfusion surrounding surgeries. Because low doses have been shown to be effective in preventing thrombosis, it is likely that the use of low doses (100 mg/day or less) maintains the antithrombotic effects of ASA but reduces the risk of bleeding.

ASA may unmask mild hereditary disorders of platelet function.[49] For example, patients with mild von Willebrand disease often have a normal bleeding time that becomes markedly increased with ASA therapy.[49] The bleeding risk associated with ASA is increased in the presence of other hemostatic defects, or if ASA is given concomitantly with anticoagulant drugs.

ASA Resistance

The response to ASA therapy varies among individuals. Every day, the human body can produce approximately 10×10^{11} platelets, and this production can increase 10-fold if needed. The life span of platelets is 8 to 10 days, and every day nearly 10% to 12% of the platelets are replaced by new platelets. The capacity of the bone marrow to produce new platelets, the ASA dosage, the ASA exposure time (single doses or regular use), the ASA formulation, and individual parameters will determine the antihemostatic effect of ASA in any particular individual.

The issue of ASA resistance is hotly debated, with reported incidence rates varying from 0% to 57%.[50–53] The presence of ASA resistance is associated with high rates of cardiovascular events.[51,53]

The most important issue in determining whether laboratory ASA resistance is real is compliance. Schwartz et al.[54] evaluated 190 patients with a history of coronary disease and receiving ASA therapy. They investigated these patients on regular ASA therapy, after withdrawal of ASA for 7 days, and after observed ingestion of 325 mg of ASA. They found that 17 patients (9%) receiving regular ASA therapy had ASA resistance as defined by defective inhibition of arachidonic acid–stimulated platelet aggregation. After being observed to take the 325 mg dose of ASA, however, all but one patient displayed inhibition of aggregation. Similar results were observed in other studies.[55] Therefore, many investigators have identified noncompliance as the predominant cause of ASA resistance, and higher doses of ASA may eliminate the resistance. Although it has been shown that ASA inhibits platelet functions in a dose-dependent manner,[56] higher ASA doses failed to reduce the risk of thrombosis in large trials.[57]

Higher ASA resistance rates are reported in elderly patients, probably owing to the fact that increased gastric pH affects ASA absorption in patients taking enteric-coated forms of ASA.[33,58] Other causes of ASA resistance may include increased platelet turnover and increased COX-1 expression in new platelets[59] and overexpression of COX-2 in macrophages and endothelial cells.[60] It has also been reported that concomitant use of some NSAIDs such as ibuprofen and naproxen may prevent COX-1 inactivation in patients receiving low-dose ASA.[61,62] Similarly, increased platelet production after hemorrhage has been cited as a cause for ASA resistance.[63] Interindividual variation in the response to ASA might also be explained by genetic variations in the genes for COX-1 and COX-2, platelet integrins α_{IIb} or β_3, the ADP receptors $P2Y_1$ and $P2Y_{12}$, the TXA_2 receptor, and some coagulation proteins.[52,64–67] However, these studies showed conflicting results and have failed to confirm that ASA therapy tailored to account for genetic variation improves clinical outcomes.

In patients treated with ASA who experience recurrent arterial thrombosis, it is important to keep in mind that arterial thrombosis is a multifactorial process and that the inhibition of one, relatively minor, pathway of platelet activation may be insufficient to prevent recurrent events.

Assessment of the inhibitory effects of ASA on platelets might be important in two clinical situations: (1) in patients with arterial thrombosis, or at high risk for arterial thrombosis, to ensure that sufficient platelet inhibition is achieved by a given ASA dose; (2) for estimating the bleeding risks in patients who need surgery or who have experienced trauma.

The antiplatelet effect of ASA can be screened by different techniques, including light-transmission aggregometry, whole-blood aggregometry, PFA-100, VerifyNow ASA, measurement of serum/plasma or urinary TXB_2 metabolites, thromboelastography, flow cytometry, or other tests (see Chapters 124 and 127).

Phosphodiesterase Inhibitors

Prostacyclin increases the levels of cAMP, whereas nitric oxide (NO) increases the levels of cyclic guanosine monophosphate (cGMP). Elevation of cAMP or cGMP inhibits many pathways involving platelet activation, including shape change, $\alpha_{IIb}\beta_3$ activation, and degranulation. Phosphodiesterases (PDEs) catalyze the degradation of cAMP and cGMP, thus preventing platelet inhibition. Eleven PDE isoforms have been described, and three are expressed in platelets: PDE2, PDE3, and PDE5.[68] Many PDE inhibitors have antiplatelet effects.

Nonselective Phosphodiesterase Inhibitors

These include the methylxanthines, caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine), and pentoxifylline (3,7-dimethyl-1-(5-oxohexyl)xanthine). Caffeine, at a dosage of 250 mg orally three times a day for one week, was demonstrated to reduce platelet aggregation in healthy subjects.[69]

PDE3 inhibitors include anagrelide, cilostazol, and milrinone. Although anagrelide inhibits platelet aggregation in vitro, it also inhibits megakaryocyte maturation and proliferation, and causes thrombocytopenia in humans by a mechanism that is poorly understood.[70] Anagrelide is used to treat essential thrombocythemia (ET) (see Chapter 71).[71]

Cilostazol is a cyclic nucleotide PDE inhibitor with both platelet inhibitory and vasodilatory effects; its current clinical use for peripheral artery disease in the United States was preceded by its use in Japan and other Asian countries (see Chapter 146). In addition to selectively inhibiting PDE, cilostazol also inhibits adenine nucleotide uptake, a property not shared with other PDE inhibitors that further enhances its antiplatelet activity.[72]

Milrinone inhibits platelet aggregation and shape change. The drug is used for congestive heart failure.

PDE5 Inhibitors

Dipyridamole has several biologic effects, including PDE inhibition, and targets the PDE5 isoform that degrades cGMP. It also enhances the release and prevents the breakdown of prostaglandin I_2 (prostacyclin).[68,73] Primarily used for prevention of stroke, its usefulness was demonstrated by a meta-analysis of randomized trials showing that the combination of dipyridamole (particularly the modified release form) and low-dose ASA was more effective than ASA alone in secondary prevention of vascular events after transient ischemic attack or stroke (see Chapter 144).[74] Dipyridamole has been associated with a significantly increased risk of GI bleeding when used either as a single agent or in combination with ASA.[75]

Inhibitors of PDE5 are used for the treatment of erectile dysfunction.[76] PDE5 is expressed in platelets, and its inhibition results in increased levels of platelet cGMP, an effect similar to that of nitric oxide. One study examined the activation response of platelets drawn from patients taking sildenafil (Viagra) when stimulated with either ADP or low-dose thrombin. The results showed significantly reduced activation in response to ADP in sildenafil-treated platelets, suggesting that the drug impairs selected pathways of platelet function in vivo. Several reports of sildenafil-associated bleeding have appeared, including epistaxis,[77,78] hemorrhoidal bleeding,[79] spontaneous intracranial hemorrhage,[80] and acute variceal bleeding.[81]

SYK Inhibitors

Positioned downstream to GPVI, spleen tyrosine kinases (SYK) are crucial for platelet activation. BI002494 is a selective SYK inhibitor that has antithrombotic activity in rodent thrombosis models. The potential for off-target oncogenic effects[82] will need to be eliminated before further development.

12-Lipoxygenase Inhibitors

Lipoxygenases (LOXs) catalyze the oxygenation of polyunsaturated fatty acids, which leads to the synthesis of signaling molecules. 12-LOX, which is expressed in megakaryocytes and platelets, oxidizes arachidonic acid at carbon 12. Growing evidence suggests that 12-LOX is involved in platelet activation. In a murine thrombosis model, ML355, an oral 12-LOX inhibitor, attenuated thrombus formation with a minimal increase in bleeding.[83]

OTHER DRUGS THAT ADVERSELY AFFECT PLATELET FUNCTION

Nonsteroidal Antiinflammatory Drugs

In addition to ASA, many other drugs used for their antiinflammatory and analgesic properties can impair platelet function.[84] As with

ASA, their mechanism of action appears to be through inhibition of COX-1; but in contrast to ASA, these agents only temporarily affect COX-1 function, inhibiting the enzyme only as long as the active drug circulates. Among these agents, therefore, only drugs such as piroxicam, which has a plasma half-life of more than 2 days,[85] affect platelets for more than a few hours. Like with ASA, the most sensitive indicator of impaired platelet function is the inhibition of in vitro platelet aggregation and secretion. These agents induce little or no prolongation of the bleeding time, consistent with the bleeding time being a less sensitive measure of the ASA-induced defect.[84] PFA-100 may detect the defect, manifested as a prolonged closure time with the collagen/epinephrine cartridge.[86]

Reports of bleeding correlate with the effects of these agents on platelet function, suggesting that they increase the risk for excessive bleeding less than ASA. As with ASA, they may prolong the bleeding time in patients with severe hemophilia, but in two studies, therapeutic doses of ibuprofen had no effect on the bleeding time in 19 of 20 patients with hemophilia.[87] Therefore, the clinical approach to patients taking any drug that can inhibit COX-1 should be similar, but the reversibility of the effect of NSAIDs provides an added margin of safety when these agents are used. Because of the increased bleeding risk, these drugs should be stopped before surgery. The timing for cessation of NSAIDs is based on the half-lives of the individual drug. Indomethacin, ibuprofen, ketoprofen, and diclofenac all have short half-lives (2 to 6 hours) and discontinuing these drugs 1 day before surgery is sufficient. Naproxen, sulindac, diflunisal, and celecoxib have intermediate half-lives (7 to 15 hours) and should be stopped 2 or 3 days before surgery. Nabumetone, meloxicam, and piroxicam have very long half-lives (greater than 20 hours). The American College of Chest Physicians (ACCP) guidelines recommend discontinuing these drugs 10 days before surgery.[19] Analgesics such as acetaminophen and sodium or choline salicylate do not inhibit platelet function and have no adverse effects on hemostasis.[87]

Cardiovascular Drugs

Many cardiovascular drugs affect platelet functions through a variety of mechanisms.[88] Administration of nitroglycerin, isosorbide dinitrate, or nitroprusside can decrease platelet aggregation and secretion in vitro, but their effects in vivo are likely to be minimal and inconsistently observed.[84] The mechanism of platelet inhibition appears to involve the production of nitric oxide from the drug, with concomitant increases in platelet cAMP and, more markedly, cGMP.[89] β-adrenergic receptor blockers such as propranolol, metoprolol, atenolol, and pindolol have also been shown to inhibit platelet aggregation, apparently through mechanisms independent of β-adrenergic receptor blockade.[90,91] Several of these agents have been shown to blunt platelet aggregation to ADP and collagen,[92] and to decrease platelet TXA_2 production in response to agonists.[90] Blunting of serotonin uptake by platelets and inhibition of the response to serotonin have also been demonstrated. There are numerous reports of antiplatelet effects of calcium channel blockers, such as nifedipine, verapamil, and diltiazem. Most of these studies demonstrated inhibition of the aggregation of washed platelets in the presence of high concentrations (micromolar) of the drug. This effect is mainly observed with epinephrine as the agonist, and it does not appear to be related to the inhibition of calcium ion influx. Proposed mechanisms include inhibition of epinephrine binding to α2-adrenergic receptors, inhibition of the platelet response to TXA_2, and inhibition of serotonin-induced aggregation. In therapeutic doses, the calcium channel blockers do not prolong the bleeding time. At high concentrations, quinidine can act as an antagonist of platelet α2-adrenergic receptors. In one report, a patient taking 800 mg of quinidine and 650 mg of ASA daily developed melena and generalized petechiae with a normal platelet count and a bleeding time over 35 minutes.[93] In a subsequent study in two healthy volunteers, quinidine caused a mild prolongation of the bleeding time that was apparently potentiated by ASA. Quinidine and its stereoisomer, quinine, can also impair hemostasis by inducing drug-dependent antibodies that cause thrombocytopenia (see Chapter 129).

The commonly used angiotensin receptor antagonists losartan, valsartan, and olmesartan interact with the TXA_2 receptor and inhibit thromboxane-dependent platelet adhesion and aggregation.[94] Valsartan also inhibits the expression of platelet receptors including $\alpha_{IIb}\beta_3$ and GPIb.[95]

Antibiotics

Antibiotics can also affect platelet function. Those implicated most often are the penicillins and cephalosporins, which share a β-lactam ring structure. Some of these drugs produce predictable dose- and duration-related effects on the bleeding time.[96–98] Because the effect on the bleeding time is seen only in patients who are receiving large parenteral doses of antibiotics, this is a potential problem only for hospitalized patients. In a study of 74 hospitalized patients with a consistently prolonged bleeding time, the likely cause was penicillin in 39 patients (30 patients were receiving greater than 15,000,000 U/day of penicillin G, and 9 were receiving 6 to 8 g/day of ampicillin), and ASA or related drugs in seven patients.[98] Floxacillin (an isoxazolyl penicillin) was shown to significantly impair platelet function and cause severe bleeding when used in high doses in a patient with infective endocarditis.[99]

The structural properties that cause some, but not all, penicillins and cephalosporins to affect platelet function are unknown. The side-chain structure alters the antibacterial and pharmacologic properties of the penicillins and cephalosporins, and may also determine their effects on platelet function. It is postulated that the antibiotic associates with the platelet plasma membrane via a lipophilic mechanism where it perturbs receptor–agonist interactions or signal transduction.[100] The characteristic laboratory findings are a prolonged bleeding time and abnormal platelet aggregation studies that occur after several days of high-dose parenteral therapy.[97,98,101] These abnormalities do not usually subside until several days after the antibiotic is discontinued.

The frequency of clinically important bleeding in patients taking β-lactam antibiotics is low and is not predicted by a prolonged bleeding time; consequently, the causal relation to antibiotic treatment is unproven.[102] For each report implicating an antibiotic as a cause of hemorrhage, many more patients receive the same antibiotics in large doses without bleeding complications.[102]

Although antibiotic-induced platelet dysfunction usually is of little clinical importance, there are some exceptions to this rule. The frequency of clinically important hemorrhagic complications with moxalactam appears to be higher than with other antibiotics.[96] This drug has been demonstrated to inhibit ADP- and collagen-induced platelet aggregation in a dose-dependent fashion and to decrease TXA_2 generation,[103] which suggests that, like ASA, it acts on the thromboxane synthesis pathway. Furthermore, in contrast to most other β-lactam antibiotics, moxalactam contains a methylthiotetrazole-leaving group that has been implicated in the inhibition of the synthesis of the vitamin K–dependent coagulation factors.[104] Therefore, moxalactam-induced bleeding may be caused by the combination of deficiencies in vitamin K–dependent coagulation factors and impaired platelet function.

Nitrofurantoin, an antibiotic structurally unrelated to the β-lactam antibiotics, may mildly prolong the bleeding time and impair platelet aggregation at plasma concentrations in excess of 20 μM.[105]

Antifungal Drugs

Miconazole has been shown to inhibit platelet aggregation in rabbits;[106] but there is no evidence that the drug causes platelet dysfunction in humans. Concomitant use of miconazole and vitamin K antagonists may cause significant bleeding because miconazole is a strong inhibitor of CYP2C9 (see Chapter 143).[107]

Amphotericin B inhibits thrombin-induced platelet aggregation, serotonin uptake, and thrombin-induced serotonin release in vitro.[108] Studies have shown that platelet transfusion immediately after amphotericin B infusion caused poor platelet recovery and survival. An interval of at least 2 hours should separate platelet transfusion from amphotericin B therapy.[109]

Psychiatric Drugs

Platelets from patients taking tricyclic antidepressant drugs (imipramine, amitriptyline, nortriptyline) or phenothiazines (chlorpromazine, promethazine, trifluoperazine) can exhibit impaired in vitro aggregation and secretion responses to ADP, epinephrine, and collagen, but this effect is not associated with an increased risk for bleeding.[84] However, there are numerous reports of antiplatelet effects of the widely used selective serotonin reuptake inhibitors (SSRIs) such as paroxetine, sertraline, fluoxetine, and citalopram, which block the only means of platelet serotonin uptake for storage[110–114]; the effect of these drugs in decreasing platelet serotonin levels has been documented.[115,116] Prolonged bleeding times,[117] excessive bruising,[118] and defective platelet aggregation have all been noted in patients taking fluoxetine.[119] SSRI use increases perioperative and postoperative bleeding,[120] increases red cell transfusion rates during and after surgery,[121] and increases the risk of postpartum hemorrhage in pregnant women.[122] On the other hand, the cessation of the SSRI antidepressant drug may cause discontinuation syndrome or depression relapse. Thus, the benefit-risk profile should be considered before the cessation of an SSRI prior to elective surgery.

Additionally, the risk of GI bleeding is increased in patients taking SSRIs, particularly when they are combined with NSAIDs[123] or antiplatelet drugs.[124] A prospective population study showed that current use, but not previous use, of antidepressants, especially SSRIs was associated with a lower risk for myocardial infarction.[125] A systematic review found that patients with acute coronary syndromes with depression had a reduction in recurrent coronary events if they were on an SSRI.[126]

Oncologic Drugs

Administration of mithramycin has been associated with decreased platelet aggregation, a prolonged bleeding time, and mucocutaneous bleeding.[127] Daunorubicin and bis-chloroethyl-nitrosourea can both inhibit platelet aggregation and secretion when added to platelet-rich plasma, but these effects do not appear to be clinically important.[84] Vincristine inhibits platelet aggregation by interfering with the microtubule network.[128]

Tyrosine kinase inhibitors, which are used for the treatment of chronic myeloid leukemia, can cause thrombocytopenia and/or platelet dysfunction (see Chapter 69). Dasatinib and imatinib both have been demonstrated to impair platelet aggregation.[129] Dasatinib, which is specific for Src family kinase, may cause clinically relevant bleeding because of platelet dysfunction.[130]

Ibritunib is an orally administered Btk (Bruton type) kinase inhibitor used for the treatment of B-cell malignancies. It also inhibits signaling downstream of platelet GPVI (Tec [tyrosine kinases with a pleckstrin homology domain] and Src family kinases) and affects platelet adhesion to vWF under arterial flow, increasing the risk for bleeding. Acalabrutinib, a more selective Btk inhibitor, does not inhibit Src family kinases and was not associated with major bleeding in Phase II trials, suggesting that bleeding observed with ibrutinib may reflect off-target inhibition of Src family kinases. Importantly, vWF or FVIII overcame the inhibitory effect of ibrutinib on thrombin generation.[131,132]

Anesthetics

Although some local and general anesthetic agents have been shown to inhibit platelet function in vitro, this effect is generally produced at high drug concentrations.[133,134] Sevoflurane suppresses formation of TXA_2; propofol and nitrous oxide inhibit calcium mobilization.[133] Procaine inhibits calcium and P-selectin release from platelet storage pools.[135] Dibucaine is a calpain activator, induces platelet apoptosis, and inhibits platelet functions.[136] At usual doses, halothane reversibly inhibits platelet aggregation.[137]

Plasma Expanders

Dextrans are partially hydrolyzed branched polymers of glucose. Of the two preparations in clinical use, dextran 70 has an average molecular mass of 70 to 75 kDa and dextran 40 has an average molecular mass of 40 kDa. Both preparations are effective plasma expanders and can affect platelet function, but the high-molecular-weight molecules have a greater effect on hemostasis.[138] Dextran infusion also impairs platelet aggregation and platelet procoagulant activity, and can cause a modest reduction in plasma vWF concentration. However, dextran has no effect on platelet function when added directly to platelet-rich plasma in vitro.[139] Because of its effects on platelet function, dextran was explored as an antithrombotic agent, but it is no longer used for this purpose.

Hydroxyethyl starch, known as hetastarch, is a synthetic glucose polymer with an average molecular weight of 450,000 (range: 10,000 to 1,000,000) that is also used for plasma expansion. Hetastarch use has been associated with abnormal platelet function (albeit inconsistently), a problem more evident with the higher-molecular-weight forms.[140] Like dextran, it can prolong the bleeding time, particularly if administered at doses higher than 20 mL/kg in a 6% solution, and predisposes to bleeding if administered simultaneously with heparin or in the presence of a preexisting hemostatic defect.[141]

Heparins and Thrombolytic Agents

Although heparin is best known for its anticoagulant effect and its association with HIT and thrombosis (see Chapters 131 and 143), it also has the potential to affect platelet function. Heparin can bind to the platelet surface,[142] induce platelet aggregation and secretion,[143] and impair vWF-dependent platelet function.[144] Whether these phenomena contribute to heparin-induced bleeding is unknown. The prolonged bleeding time is probably the result of inhibition of thrombin generation, analogous to the slight but significant increase in bleeding times seen in patients with hemophilia.[143]

Paradoxically, heparin is also capable of inducing the association of vWF with the GPIb complex.[145] Whether this is restricted to certain individuals or occurs only at certain heparin concentrations is unclear, but it raises the possibility that this mechanism may contribute to heparin-induced thrombosis.

Bleeding during therapy with plasminogen activators is predominantly caused by fibrin degradation, hypofibrinogenemia, and increased levels of fibrin(ogen) degradation products, usually in the setting of structural lesions. At pharmacologic concentrations, streptokinase, urokinase, and tissue plasminogen activator (t-PA) may also impair platelet function through several potential mechanisms, all related to excessive production of plasmin.[146] First, high levels of fibrin(ogen) degradation products and low levels of fibrinogen can impair platelet aggregation. Second, the binding of plasminogen to the platelet surface may facilitate its conversion to plasmin.[147] Plasmin can degrade GPIbα (thereby impairing the interaction of the platelet with vWF[148]) and fibrinogen (thereby dispersing platelet aggregates).[149] Third, plasmin can inhibit platelet aggregation by blocking the release of arachidonic acid from platelet membranes, thereby limiting TXA_2 production.[150] The clinical importance of these observations is unknown.

In addition to decreasing platelet function, plasmin has been shown to directly activate platelets,[151–154] an effect that may contribute to vessel reocclusion after t-PA treatment for myocardial infarction.[155] Platelet activation is the result of plasmin-mediated cleavage of protease-activated receptors, particularly PAR4.[156]

Miscellaneous Drugs

The statins, which inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase, are widely used in patients with dyslipidemia.[157] These agents reduce the risk of cardiovascular events through a variety of mechanisms, including reducing the risk of atherothrombosis. Among its antithrombotic effects are decreases in platelet reactivity, partly brought about by its beneficial effects on endothelial cells, with increased production of nitric oxide and prostacyclin, and by direct effects on platelets.[158] Some of the effects of statins on platelets may be dose-related. Membrane cholesterol content, which is lowered by the statins, has been correlated with platelet reactivity,[159,160] with studies demonstrating that the content of cholesterol in platelet membranes correlates with localization of important adhesive and agonist receptors within membrane microdomains known as lipid rafts.[161,162] In addition to lowering plasma and membrane cholesterol levels, statins also reduce the activity of stimulatory signaling pathways involving the small guanyl triphosphate (GTP)–binding proteins Ras, Rho, and Rac, which require posttranslational prenylation to be targeted to cell membranes. Statins inhibit protein prenylation.[163] No bleeding complications have been associated with statin use, but a substudy of the Platelet Receptor in Ischemic Syndrome Management (PRISM) trial showed that their sudden withdrawal in the setting of acute coronary syndrome was associated with an increased cardiac risk compared with the risk in patients who continued to receive statins (relative risk: 2.93) or never received statins.[164] This increase in adverse cardiac events was partially attributed to increased platelet reactivity. A recent meta-analysis found no correlation between statin therapy and increased intracranial hemorrhage.[165]

Clofibrate, another lipid-lowering drug, diminishes platelet responsiveness to ADP, collagen, and epinephrine when given to patients with type II hyperbetalipoproteinemia, and can diminish the responsiveness of normal platelets to ADP and epinephrine in vitro.[84]

Cocaine accounts for many drug-related visits to emergency departments in the United States. Its use is associated with a large increase in the incidence of myocardial infarction in individuals who are otherwise at low risk, and it has been hypothesized that cocaine-induced platelet aggregation is a major contributor to this risk.[166] In vitro, however, cocaine inhibits platelet aggregation in response to several agonists and dissociates preformed aggregates.[167] Another important prothrombotic mechanism associated with cocaine use is the ability of cocaine and its long-acting metabolites to induce the release of vWF from endothelial cells.[168]

Ketanserin, which has been studied for its potential to prevent atherosclerotic complications, decreases platelet aggregation in response to serotonin. Antihistamines, some radiographic contrast agents, and immunosuppressive drugs can also impair platelet aggregation. The mechanisms responsible for these effects are unknown.

Foods and Food Additives

Certain foods and food additives can have important effects on platelet function, particularly when consumed in large quantities. In a classic study published in 1979, Dyerberg and Bang[169] reported that Greenland Inuit on traditional diets had markedly prolonged bleeding times compared with gender- and age-matched Danish control subjects (mean: 8.1 minutes vs. 4.8 minutes, respectively), and they correlated this finding with an impaired secondary wave of platelet aggregation to ADP and collagen, and high plasma levels of ω-3 fatty acids. The proposed mechanisms by which ω-3 fatty acids (eicosapentaenoic acid, C20:5ω-3; and docosahexaenoic acid, C22:6ω-3) interfere with platelet function is through competition with arachidonic acid for the 2-acyl position of membrane phospholipids or access to COX-1.[170,171] ω-3 Fatty acids not only reduce TXA_2 synthesis in response to platelet agonist stimulation by competing with the substrate arachidonic acid, but also by producing inhibitory eicosanoids. The latter mechanism was substantiated in the original study of an Inuit population by the demonstration that administration of ASA decreased the bleeding time in all subjects tested but not to normal levels.[169] Additionally, ω-3 fatty acids may increase the production of antiaggregatory prostaglandins by cells in the vessel wall.[169] In a general population age over 50, supplementation of ω-3 fatty acid did not reduce major adverse cardiac events.[172] However, in patients with hyperlipidemia, icosapent ethyl, a highly purified eicosapentaenoic acid ethyl ester, reduced major cardiovascular events by 22% (22.0% to 17.2%)[173] most likely by reducing plaque volume.[174] Icosapent ethyl was associated with a small increase in serious bleeding (2.1% to 2.7%).[173]

Ethanol, one of the most commonly and excessively ingested substances in the world, acts synergistically with ASA to prolong the bleeding time (see Chapter 126).[175] In addition, ethanol acts cooperatively with agents that block binding of fibrinogen to $\alpha_{IIb}\beta_3$ to further reduce platelet aggregation in response to several agonists, an effect not solely the result of reduced TXA_2 generation.[176] Ethanol can also impair collagen-induced platelet aggregation, secretion, arachidonate mobilization, and TXA_2 formation, but it did not inhibit platelet adhesion to deendothelialized rabbit aortae.[177]

Other food components or additives can also affect platelet function and increase the risk of minor bleeding. Easy bruising after eating Chinese food has been attributed to a platelet inhibitory effect of black tree fungus.[178] A component of onion extract can inhibit platelet arachidonic acid metabolism.[179] Ajoene, a component of garlic, is an inhibitor of fibrinogen binding to platelets and platelet aggregation.[180] Extracts from frequently consumed spices—cumin, turmeric, and clove—can decrease platelet thromboxane production and inhibit platelet aggregation.[181] Bromelain, an extract from pineapple, can inhibit ADP-induced platelet aggregation, and prolong both the PT and aPTT.[182] Parsley (*Petroselinum crispum*) may inhibit ADP-induced platelet aggregation.[183]

It can be concluded that platelets are sensitive to a variety of therapeutic and dietary compounds, the extent of inhibition primarily depending on the dose of the inhibitory agent.[84] However, an increased risk for clinically important bleeding has been demonstrated only for ASA and agents specifically designed to inhibit platelet function. Reports of increased bleeding with all other agents must be viewed with caution. Despite this qualification, it is prudent for clinicians to have a thorough understanding of the antiplatelet effects of prescribed drugs and to always consider the potential impacts of drug- or diet-induced platelet dysfunction, particularly in patients with coexisting hemostatic defects or those consuming large amounts of these agents.

Scurvy

Vitamin C deficiency results in defective collagen synthesis and abnormal bleeding from fragile capillaries. Platelet aggregation defects in patients with scurvy normalize after vitamin C supplementation.[184]

CLONAL DISORDERS

Hematological Clonal Disorders

Myeloproliferative Neoplasms

Myeloproliferative neoplasms (MPNs; see Chapters 69–73) are clonal disorders arising from hematopoietic stem cells affected by somatic mutations that cause abnormal production of mature myeloid cells. Philadelphia chromosome-negative MPNs, which include polycythemia vera (PV), ET, and primary myelofibrosis, are the most common MPNs. These disorders are characterized by varying degrees of leukocytosis or thrombocytosis; patients with PV usually have a markedly elevated hematocrit. Particularly in PV and ET, thrombosis or bleeding accounts for a high percentage of the associated morbidity, with thrombosis being the most common.

Bleeding in patients with MPN is primarily mucocutaneous. The bleeding time is prolonged in a small percentage of patients with

MPNs, but bleeding complications can occur even in patients with normal bleeding times. Routine plasma coagulation tests, such as the PT and aPTT, may be falsely prolonged if the red blood cell mass is increased. Because the plasma volume in these patients is reduced, it is important to adjust the citrate concentration in the tubes used to collect the blood for coagulation testing.

The mechanisms of bleeding are multifactorial in patients with MPN. Some bleeding episodes can in part be attributed to thrombosis, such as variceal bleeding resulting from thrombosis-induced portal hypertension.[185] Acquired platelet abnormalities, acquired coagulation factor (especially factor V) and vWF deficiencies,[186] antiplatelet drug usage, and drugs used for the clonal disease (such as anagrelide) may cause bleeding problems.

The platelets of patients with MPNs can show various morphologic abnormalities, including variations in size and shape, as well as reduced numbers of secretory granules. Platelet survival can be decreased in ET. The most common platelet abnormality is decreased aggregation and secretion in response to agonists, particularly epinephrine, ADP, and collagen.[187] These abnormalities are not simply the result of the high platelet count, because patients with reactive thrombocytosis have functionally normal platelets.[188] In what may appear to be a paradox, some patients demonstrate spontaneous in vitro platelet aggregation in platelet-rich plasma. Decreased platelet aggregation or secretion and decreased procoagulant activity may be caused by (1) agonist-induced release of arachidonic acid from membrane phospholipids; (2) conversion of arachidonic acid to prostaglandin endoperoxides or lipoxygenase products; (3) platelet responsiveness to TXA_2; (4) dense-granule or αα-granule contents; or (5) α2-adrenergic receptors. Some of the described abnormalities may also be a consequence of platelet activation in vivo or ex vivo during platelet preparation.

Specific platelet membrane abnormalities have also been reported, including deficiencies of GPIb and GPIX, causing an acquired form of the Bernard-Soulier syndrome (see Chapter 124)[189]; deficiencies of receptors for prostaglandin D_2[190]; c-MPL receptors[191]; and an increased number of Fc receptors.[192] Because MPNs are clonal in origin, the abnormal platelets can arise from a clone of abnormal megakaryocytes.[193] Alternatively, the findings may be the result of platelet hyperreactivity and previous activation.[194,195] It is important to emphasize several features about the platelet function defects reported in MPNs. First, no defect has consistently predicted the risk for bleeding or thrombosis. Second, no defect is unique to, and therefore predictive of, a particular MPN. Third, the relative frequency of the different defects varies widely. Therefore, the clinical importance of the abnormalities of platelet function in MPNs is unknown.

Acquired von Willebrand syndrome (see Chapter 133) can cause mucocutaneous bleeding or complicate surgical interventions in nonbleeding patients. In acquired von Willebrand syndrome, the largest multimers of vWF are absent, possibly as a consequence of their adsorption to binding sites on the elevated numbers of platelets,[196–200] or because of disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13) proteolysis of the large multimers.[201] In a prospective study of MPN patients, the incidence of acquired von Willebrand syndrome was 11%.[202] Although increased adsorption of plasma vWF multimers by platelets is the cause of the syndrome and decreased vWF:ristocetin cofactor activity/Ag or vWF:collagen binding/Ag ratio was found in patients with extreme thrombocytosis,[200] acquired von Willebrand syndrome has also been described in patients with platelet counts between 120 and $135 \times 10^9/\mu L$.[202]

Several intrinsic platelet function defects result, at least in part, from an increased sensitivity of the platelets to activation, a potential consequence of the JAK2 gain-of-function mutation. For example, a study demonstrated an increased risk of thrombosis in patients with chronic MPN when the mutant kinase was detected in platelets or in platelets and granulocytes.[193] Likewise, the JAK2 mutation in platelets correlated with increased platelet expression of tissue factor and P-selectin, decreased expression of CD41 and CD42b, and increased quantities of platelet–neutrophil aggregates, all indicators of a hyperreactive platelet phenotype.[194] Panova-Noeva et al.[195] evaluated the platelets from 140 patients with MPN (80 ET, 60 PV) for global procoagulant potential by measuring thrombin generation and expression of tissue factor and P-selectin on the platelet surface. They found that patients with the JAK2 V617F mutation had the highest values for thrombin generation and platelet tissue factor and P-selectin expression. These findings correlated with JAK2 V617F allele burden.[195]

Paroxysmal Nocturnal Hemoglobinuria

Paroxysmal nocturnal hemoglobinuria (PNH; see Chapter 32) is another clonal disorder that involves all blood cells. The hematopoietic stems cells and their progeny are defective in the synthesis of the glycosylphosphatidylinositol attachments required for the plasma membrane expression of some membrane proteins, leading to a defect in all glycosylphosphatidylinositol-linked proteins on blood cells,[203] including platelets.[204]

Thrombosis is a leading cause of mortality in PNH, affecting at least half of these patients. The platelet function abnormalities described in PNH range from hypersensitivity to agonists to dysfunction.[205] One study showed platelets to be hypersensitive to epinephrine, ADP, and collagen, as judged by their abilities to aggregate and to release ^{14}C serotonin.[206] The total release of nucleotides was also markedly increased over normal with all aggregating agents. By contrast, another study examining platelets from PNH patients showed them to be profoundly hyporeactive, as measured by defective clot formation, adhesion, and aggregation.[207] This finding was interpreted as being a consequence of chronic overstimulation of the platelets while they circulate. Platelet activation and increased platelet microparticle formation have also been demonstrated in PNH patients.[205,206,208]

Paraproteinemias

Although thrombotic complications can occur in patients with paraproteinemias because of hyperviscosity, bleeding complications also are seen. Platelet dysfunction is observed in approximately one-third of patients with immunoglobulin (Ig)A myeloma or Waldenström macroglobulinemia, in 15% of patients with IgG myeloma, and occasionally in patients with benign monoclonal gammopathy (see Chapters 90–92).[209,210] Additional hemostatic problems in these patients can be caused by the hyperviscosity syndrome,[211,212] a heparin-like coagulation inhibitor,[213] acquired von Willebrand syndrome,[200] or complications of amyloidosis (e.g., acquired factor X deficiency[214] or enhanced fibrinolysis).[215] Patients may have markedly abnormal results on laboratory tests (e.g., a prolonged thrombin time) with no evidence of clinical bleeding.[209]

Abnormalities of platelet function correlate with the concentration of the plasma paraprotein. Myeloma proteins can inhibit all platelet functions (aggregation, secretion, procoagulant activity, and clot retraction), and normal platelets can acquire these defects when incubated with the purified monoclonal immunoglobulin.[216,217] In some cases, specific interactions of the monoclonal protein have been described. One IgA myeloma protein inhibited the ability of a suspension of aortic connective tissue to aggregate normal platelets.[218] The bleeding time and bleeding symptoms of the patient from whom this paraprotein was isolated were corrected when the IgA myeloma protein was removed by plasmapheresis. One patient had a fatal hemorrhage from an IgG1κ paraprotein that bound GPIIIa (β_3 integrin) and inhibited platelet aggregation.[219] A number of reports have described acquired von Willebrand disease in patients with myeloma, benign monoclonal gammopathy, or chronic lymphocytic leukemia.[220] In some patients, the plasma concentration of vWF was decreased; in others, the larger multimers were deficient. The myeloma protein can either accelerate vWF clearance from plasma or interfere with its binding to platelet GPIb.

Easy bruising, epistaxis, periorbital purpura (in patients with amyloidosis), and GI hemorrhage are the most common bleeding symptoms associated with the paraproteinemias. Because bleeding appears to be related to high plasma paraprotein concentrations,[221] chemotherapy for the underlying plasma cell neoplasm should be given to effect a longer lasting reduction of the paraprotein. In emergency situations, plasmapheresis should be performed expeditiously; the effectiveness of this therapy can be evaluated by improvement of bleeding. Intravenous immunoglobulin (IVIG) infusions are effective in controlling bleeding in patients with plasma cell dyscrasias and acquired von Willebrand disease. IVIG can produce a clinical and laboratory response in 12 to 72 hours, and the effect usually persists for 1 to 3 weeks. In patients with severe bleeding, IVIG can be combined with plasmapheresis, desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP), and infusions of vWF concentrate or factor VIIa.[200,202,210,220]

Leukemias and Myelodysplastic Syndromes

Bleeding in patients with the leukemias and myelodysplastic syndromes (MDS) is almost always caused by thrombocytopenia, but abnormalities of platelet function have also been described. In acute myeloid leukemia and its variants, platelets may be larger than normal, abnormally shaped, and vary in their granule numbers (see Chapter 61). Abnormal platelet structure and function have been described, especially in association with acute megakaryoblastic leukemia (FAB M7),[222–224] with one study describing three patients with decreased aggregation to collagen, ADP, epinephrine, and the thromboxane analogue U46619, along with a decreased platelet serotonin content.[222] Platelet abnormalities can also be found in the MDS, with defective aggregation and glass bead retention most often associated with hypolobulated megakaryocytes and the 5q− syndrome.[224,225] Abnormal platelet function has also been described in association with B-cell malignancies, such as hairy cell leukemia, which can persist after splenectomy,[226] and with chronic lymphocytic leukemia,[227] in which the platelets exhibit reduced responses to GPVI agonists such as collagen and convulxin.

Also, acquired platelet GP defects have been reported in hematological malignancies, including acquired Glanzmann thrombasthenia in a patient with Hodgkin lymphoma[228] and acquired Bernard-Soulier syndrome in a child with MDS.[229]

Solid Tumors

Bleeding complications in patients with cancer are related to decreased platelet production caused by bone marrow infiltration, the myelosuppressive effects of chemotherapy and radiotherapy, sepsis, disseminated intravascular coagulation, microangiopathic hemolytic anemia, drug-induced thrombocytopenia, immune thrombocytopenia, or hypersplenism. Although several defects of platelet functions have been described in cancer patients, none are specific.[230]

SYSTEMIC METABOLIC DISORDERS

End-Stage Renal Disease

The pathogenesis of the hemorrhagic diathesis in patients with end-stage renal disease (ESRD) is complex (see Chapter 150). Factors such as platelet function abnormalities caused by uremic toxins, anemia, hemodialysis procedures (both the artificial circulation and the use of anticoagulation), and decreased drug clearance may impair hemostasis.

In ESRD patients both platelet hypofunction and platelet hyperreactivity can occur.[231]

Deficiencies in the number of GPIb complexes in uremic platelets have been reported, the number inversely correlating with the creatinine level.[232] Consistent with this finding, another study demonstrated that defective ristocetin-induced platelet aggregation of uremic platelet-rich plasma was not corrected by resuspending the platelets in normal platelet-poor plasma. Likewise, uremic platelets suspended in normal plasma were defective in their adhesion to deendothelialized rabbit vessels at high shear, a test of the competency of the interaction between GPIb and vWF.[233] Conversely, the same study demonstrated that plasma factors also influence platelet function by showing that normal platelets suspended in uremic plasma acquired an adhesion defect possibly related to an abnormal interaction of vWF with the subendothelium. This led to the suggestion that vWF may be abnormal in uremia. Two studies have demonstrated normal-to-elevated vWF antigen and activity (measured as ristocetin cofactor activity) in uremic patients but decreased levels of the largest, most hemostatically active multimers,[234,235] reflected in one study as a decreased ratio of activity to antigen level compared with the vWF from normal subjects.[234] Taken together, the various studies consistently indicate that the first step of platelet adhesion at sites of vessel wall injury is abnormal, a defect that could be clinically significant if coupled with other defects of platelet function.[231]

The platelets of uremic patients frequently exhibit reduced fibrinogen binding, aggregation, and secretion in response to a wide variety of agonists. This abnormality may persist when the platelets are removed from uremic plasma, and in some studies, uremic plasma has induced these defects in normal platelets. One potential mechanism is provided by the demonstration that fibrinogen fragment levels are elevated in patients with ESRD and bind to platelet $\alpha_{IIb}\beta_3$, inhibiting platelet aggregation.[236]

Uremic platelets can also exhibit a reduction in several of the biochemical responses necessary for aggregation and secretion, including the rise in cytoplasmic calcium ion concentration, release of arachidonic acid from membrane phospholipids, conversion of arachidonic acid to TXA_2, and dense-granule and α-granule secretion. Abnormal cytoskeletal assembly and deficient tyrosine phosphorylation have also been noted in uremic platelets, abnormalities only partially corrected by dialysis.[231,237]

The accumulation of dialyzable platelet-inhibitory substances in the plasma of uremic patients has long been recognized. The ability of uremic plasma to inhibit platelet function was demonstrated in the late 1960s by Horowitz and colleagues,[238] who showed reduced ADP-induced platelet aggregation and a prolonged Stypven clotting time, a measure of platelet procoagulant activity. This activity, previously called platelet factor 3, is vital for the support of coagulation factor complex assembly on the platelet surface and requires externalization of the anionic phospholipid phosphatidylserine. One uremic substance with platelet inhibitory properties described by Horowitz and coworkers[239] was guanidinosuccinic acid, which accumulates in uremic plasma as an alternative byproduct of L-arginine metabolism,[240] a consequence of the inhibitory effect of high urea levels on enzymes of the urea cycle. Similar to L-arginine, guanidinosuccinic acid is a precursor of nitric oxide, which is produced in increased quantities in the endothelial cells and platelets of uremic patients and experimental animals.[240] Consistent with an important role for nitric oxide in uremia, infusion of the nitric oxide synthesis inhibitor monomethyl L-arginine reduced the bleeding time in uremic rats.[241] Furthermore, suppression of nitric oxide production appears to correlate with the benefit of conjugated estrogens in improving the bleeding time in uremia.[242] Increased expression of vascular prostacyclin has also been described in uremic rats, a factor that would further depress in vivo platelet function.[243]

Dialysis itself may be associated with alterations in platelet function. For example, hemodialysis can reduce the responsiveness of platelets to agonists in vitro,[244] and chronic hemodialysis and peritoneal dialysis are associated with an increase in reticulated platelets, suggesting accelerated platelet turnover.[245] Of the two forms of dialysis, hemodialysis appears to have the greatest effect on platelet function, with one study noting abnormal cytoskeletal assembly and defective tyrosine phosphorylation to thrombin stimulation in the platelets of patients on hemodialysis, parameters that returned almost to normal with the institution of ambulatory peritoneal dialysis.[237] The cause of the defect associated with hemodialysis is likely to be the chronic low-level platelet activation associated with the procedure. In particular, polymerization and depolymerization of actin, release of granule proteins and their binding to the platelet surface, and shedding of membrane proteins may render the platelets relatively refractory to activation.

Uremia and artificial surfaces during hemodialysis also affect platelet messenger RNA (mRNA) and microRNA profiles and alter protein expression.[246]

As would be expected, platelets from uremic patients may be unusually sensitive to medicines that decrease platelet function. ASA has been reported to produce a greater prolongation of the bleeding time in uremic patients than in controls, an effect that may be attributable to more than the irreversible inhibition of COX-1.[247] Similarly, β-lactam antibiotics that prolong the bleeding time can also have a greater effect in uremic patients and may increase the risk of bleeding, particularly those antibiotics cleared by the kidney.[84,248]

Anemia, which does correlate with the severity of the renal failure, is an independent cause of a prolonged bleeding time,[248–251] and the defect correlates with the severity of the anemia. The relationship of the bleeding time to hematocrit in uremic patients has been confirmed by its correction with the transfusion of red blood cells and by treatment with erythropoietin.[231,251,252]

In contrast to the studies of platelet function performed to understand the possible increased risk for bleeding in patients with chronic renal failure, the coagulation and fibrinolytic systems have been interrogated to understand the increased risk for thrombotic complications, a major cause of mortality.[253]

Uremia-related contributions to a thrombotic tendency include endothelial dysfunction, increased tissue factor (TF) expression, increased microparticle generation, increased coagulation factors (fibrinogen, FXIIa, FVIIa) and proinflammatory markers, decreased natural anticoagulant levels, and changes related to renal replacement therapy as extracorporeal thrombin generation, decreased fibrinolysis, and membrane-induced platelet activation.[231,254]

Liver Disease

The bleeding diathesis observed with fulminant or end-stage liver disease (Chapter 150) is multifactorial, with contributing causes including thrombocytopenia, anemia, deficiencies in liver-synthesized coagulation factors, and excessive fibrinolysis.[255,256] Patients with chronic liver disease and hepatic cirrhosis of various causes have been reported to have prolonged bleeding times, and these disorders are associated with other platelet function abnormalities possibly related to a decrease in GPIb.[256] Although an association between prolonged bleeding time and GI hemorrhage has been demonstrated in some studies of cirrhotic patients,[255] other studies showed that the bleeding time and platelet aggregation abnormalities correlated best with the degree of thrombocytopenia,[257] suggesting that no specific platelet function defect exists in liver disease. An ASA-like defect has been reported in patients with severe liver disease, with defective aggregation and TXA_2 production.[258] Interestingly, one study showed that platelets obtained from the portal circulation of patients with hepatocellular cancer on a background of cirrhosis showed decreased and delayed aggregation in response to collagen compared with circulating platelets.[259] In addition to portal hypertension and esophageal varices, this finding raises the possibility that localized vascular bed factors (including increased prostacyclin) may contribute to variceal bleeding.

Because patients with cirrhosis often have a complicated hemostatic picture that includes thrombocytopenia, decreased fibrinogen levels, and prolonged PT and aPTT, one might expect this to uniformly produce a severe bleeding diathesis. However, recent studies have shown that the hemostatic defects in these patients are at least partially compensated through several mechanisms. The potential to bleed because of thrombocytopenia is reduced because vWF levels are often elevated and ADAMTS13 activity is decreased,[260–262] perhaps because of decreased synthesis by hepatic stellate cells. The decreased concentrations of procoagulant factors are balanced by the decreased concentrations of anticoagulant proteins.[263] Dysfibrinogenemia produces a less severe hemostatic defect because of decreased plasminogen levels.[264,265] This balance may easily be tipped in either direction, favoring either bleeding or thrombosis.

Although patients with cirrhosis may have platelet dysfunction, it is usually not associated with serious bleeding. Bleeding in these patients cannot be predicted with routine diagnostic tests, such as the platelet count and bleeding time.[261,266]

Hypothermia

Hypothermia is defined as a core body temperature below 35°C (95°F) and is classified as mild (32°C to 35°C), moderate (28°C to 32°C), severe (20°C to 28°C), or profound (below 20°C). The most common cause of hypothermia is exposure to cold weather or immersion in cold water, but hypothermia can also be caused by dehydration, severe trauma, massive transfusion, head injury, burns, sepsis, and drugs (e.g., alcohol, sedatives, and hypnotics). Age is an important risk factor because elderly adults and newborns are particularly prone to hypothermia. Hypothermia can be intentionally induced in some cardiac operations.[267]

Mild hypothermia is usually well-tolerated, but mortality increases when the core body temperature falls below 20°C. In animals, hypothermia causes thrombocytopenia because of platelet sequestration in the spleen and liver. Hypothermia inhibits platelet aggregation in response to thrombin and thromboxane, increases platelet expression of P-selectin, and decreases expression of the GPIb-IX-V complex.[268] Both the thrombocytopenia and the functional defects are reversible, returning to normal when the body temperature normalizes.[267]

Trauma

Trauma-associated hemorrhage is a leading cause of death worldwide. Platelets are dysfunctional in critically injured patients.[269] A recent publication described the phenomenon of platelet ballooning in response to trauma. Ballooned platelets are coated in histone H4, which can activate the coagulation system, and release procoagulant microparticles that bind to leukocytes. Platelets can be induced to undergo ballooning by in vitro exposure to histone H4; an effect that was independent of the histone H4 receptors (toll-like receptors 2 and 4), suggesting that histone H4 can directly affect the platelet membrane.[270] Consistent with this concept, the proteomes of dysfunctional platelets in trauma patients exhibit 16-fold higher levels of histone H4 than normal platelets, as well as higher levels of other proteins associated with inflammation.[271]

In patients with trauma-associated intracranial hemorrhage, neither platelet transfusion nor desmopressin improved outcome in those taking ASA or clopidogrel.[272]

PLATELET DYSFUNCTION RELATED WITH EXTRACORPOREAL CIRCUITS

Extracorporeal circuits such as cardiopulmonary bypass (CPB), extracorporeal membrane oxygenation (ECMO), continuous flow left ventricular assist devices (LVADs), and total artificial heart generate high shear stress and affect blood cells, endothelial cells, and plasma proteins.

Bleeding complications can be seen in 10% to 20% of the patients undergoing CPB, and hemostatic disturbances are responsible for bleeding in nearly half of those patients.[273,274] CPB activates both the coagulation and fibrinolytic systems and causes consumption coagulopathy. Besides the surgery itself, high shear stress and exposure of the blood to artificial surfaces activates FXII and FXI directly and induces blood cells to express TF.[273,275]

Typical findings after bypass surgery include a prolonged bleeding time (longer than expected for the degree of thrombocytopenia), decreased platelet aggregation, decreased platelet agglutination in response to ristocetin, and depletion of platelet α-granule and dense granule contents.[276]

As with hemodialysis, the platelet defect caused by CPB is most likely a consequence of platelet activation and fragmentation within the extracorporeal circuit. The severity of the platelet abnormalities correlates with the duration of the bypass procedure.[274] With uncomplicated surgery, platelet function returns to normal within 24 hours; however, a much longer time may be required in some patients, and the platelet count typically does not return to normal for several days.[277]

Thrombocytopenia is caused by hemodilution and deposition of platelets on the bypass circuit and, to a lesser extent, sequestration of damaged platelets in the liver. Platelet dysfunction during bypass may be caused by reversible adhesion and aggregation of platelets on fibrinogen adsorbed from plasma onto the bypass circuit material, mechanical trauma and shear stress, cardiotomy suction, trace concentrations of circulating thrombin and ADP, complement activation, hypothermia, blood conservation devices, bypass priming solutions, and, with bubble oxygenators, exposure of platelets to the blood–air interface.[84,273] CPB consistently induces the formation of platelet fragments or membrane "microparticles," evidence that the platelet surface membrane is subjected to severe mechanical stress and activation during the procedure.[278] Thus considerable platelet activation and aggregation occur during CPB, which leads to deleterious effects from substances released from the platelets and the new adhesive molecules exposed on their surfaces, and renders the platelets relatively refractory to activation by agonists.

Another surgical cause of platelet dysfunction relates to the use of deep hypothermic circulatory arrest in some surgeries. This procedure involves cooling the vital organs to temperatures between 15°C and 22°C (59°F and 71.6°F) for reducing oxygen requirements of major organs such as the heart, brain, and kidney.[267]

In patients with mechanical circulatory support such as LVADs or ECMO, continuous exposure of blood to nonbiological surfaces creates additional hemostatic problems.[279,280] Continuously elevated shear stress both elongates and degrades vWF multimers mechanically and facilitates the cleavage of high-molecular-weight vWF multimers by ADAMTS13, causing acquired von Willebrand disease.[280] Acquired von Willebrand disease (see Chapter 133) was less severe with an axial-flow LVAD, than with a centrifugal flow LVAD.[281] Increased shear stress created by these devices also causes platelet receptor shedding. In a study evaluating patients with ECMO and continuous flow LVADs, platelet surface receptor shedding was demonstrated by elevated soluble GPVI levels in plasma, and significantly reduced expression of GPVI and GPIbα on the platelet surface.[268] However, another study reported normal levels of GPIIb, GPIX, and GPIbα on the platelet surface with LVADs, but found reduced secretion from platelet α- and dense granules in response to thrombin.[281]

INFECTIOUS DISEASES

SARS-CoV-2

Infection with SARS-CoV-2 is associated with micro- and macrovascular thrombosis (see Chapter 153). Platelets from patients infected with SARS-CoV-2 show increased response to agonists likely as a result of increased MAP kinase activation and increased thromboxane production.[282,283]

Tropical Diseases

Numerous tropical infections cause thrombocytopenia. These will not be covered. Some tropical infections inhibit platelet activation with or without causing thrombocytopenia. Although a low platelet count portends a poor prognosis in patients with dengue fever, bleeding does not correlate with the platelet count. Platelet aggregation and dense-granule release are impaired in patients with dengue fever,[284] likely reflecting platelet exhaustion. The dengue protein DENV binds to platelets and activates them.

Platelets in patients with hantavirus infection exhibit impaired activation in response to multiple agonists.[285] Others have demonstrated increased platelet activation[286] in patients with hantavirus infection. For Lassa fever, Ebola infection, and Marburg infection, bleeding does not correlate with thrombocytopenia, implying platelet dysfunction. In Lassa fever a plasma inhibitor of platelet activation was found.[287] A rhesus model of Ebola infection reported impaired platelet activation to a wide range of agonists; plasma from infected monkeys did not transmit the platelet defect to other monkeys, implying that it was inherent to the platelets.[288]

Leptospirosis can cause thrombocytopenia, but bleeding is usually mild. An exception is hemorrhagic pneumonitis, which is a common cause of death in those with leptospirosis. Platelets from patients with leptospirosis had impaired activation to ADP and thrombin receptor-activating peptide (TRAP), and this dysfunction correlated with outcome better than platelet count did.[289]

Antiplatelet Antibodies

Immunoglobulins can bind to platelets in a specific or nonspecific fashion and can disrupt platelet function. In conditions such as immune thrombocytopenic purpura (ITP) (see Chapter 129), systemic lupus erythematosus (SLE) (see Chapter 139), and platelet alloimmunization, the antibodies can trigger accelerated platelet destruction and subsequent thrombocytopenia. Surviving platelets, known as stress platelets, display enhanced function.[290] Indeed, bleeding times in ITP may be shorter than expected for the degree of thrombocytopenia. Sometimes, however, the hemorrhagic tendency is out of proportion to the degree of thrombocytopenia or persists after the platelet count returns to normal, suggesting that the bound antibody is perturbing platelet function.[291]

In some patients with ITP or SLE, platelet dysfunction may be suspected because mucocutaneous bleeding symptoms occur despite platelet counts that are usually sufficient for normal hemostasis (greater than 50,000/μL), and the bleeding time may be longer than expected for the degree of thrombocytopenia. Patients with antiplatelet antibodies can exhibit defective platelet function in vitro even if they do not have prolonged bleeding times or clinical symptoms of excessive bleeding, a situation similar to what occurs with ASA ingestion or renal disease. For example, in two studies, 13 of 19 patients with ITP demonstrated impaired platelet aggregation to ADP, epinephrine, or collagen.[292,293] In two other studies, 22 of 35 patients with SLE were found to have decreased platelet aggregation in response to these agonists.[294,295] The platelet function abnormalities appear to be antibody-mediated because IgG purified from the plasma or eluted from the platelets of these patients inhibited the aggregation of normal platelets.

Platelet antibodies may affect several aspects of platelet function. The most frequently observed abnormality is the absence of platelet aggregation in response to low concentrations of collagen and the absence of the second wave of aggregation in response to ADP or epinephrine. This pattern is identical to the abnormalities caused by ASA, described earlier. In ITP and SLE, the abnormal aggregation may be related to a reduction in the contents of dense and α granules, or to an activation defect manifested by diminished synthesis of TXA_2.

Autoantibodies or alloantibodies usually impair the function of the antigen against which they are targeted. For example, alloantibodies (e.g., anti-PlA1) and autoantibodies that bind to αIIbβ3 produce a syndrome similar to Glanzmann thrombasthenia.[296–300] These antibody-associated thrombopathies can be severe.[301]

It is not surprising that many of the antiplatelet antibodies target $\alpha_{IIb}\beta_3$ because this is the most abundant receptor on the platelet surface and is required for all aggregation responses.[302] Antibodies that target GPIb have also been described, in one case producing a syndrome of severe refractory thrombocytopenia and a functional defect resembling that observed in Bernard-Soulier syndrome.[303] An interesting aspect of antibodies against GPIb is their association with more severe thrombocytopenia than that observed with antibodies directed against other antigens.[304] This finding is consistent with the observations that anti-GPIb antibodies inhibit megakaryopoiesis[305] and proplatelet formation in vitro.[306] Autoantibodies have been described that target GPIa (integrin α2),[307,308] GPIV,[308] and GPVI.[309–311]

Platelet-activating antibodies have been described. Of particular interest is the report of a patient who presented with immune thrombocytopenia and an antibody against CD9 (a member of the tetraspanin protein family) that could activate normal platelets.[312] After recovery of the platelet count, the patient relapsed, this time with an antibody described against the platelet Fc receptor, FcγRIIA. The latter antibody blocked the capacity of the first antibody to activate platelets, confirming that activation involved stimulation of the Fc receptor.

Platelets that have been activated and induced to secrete but have not been incorporated into aggregates are likely to be refractory to platelet agonists and deficient in secretory granule contents, essentially leading to an acquired storage pool deficiency, which has also been described in association with autoantibodies.[313]

Acquired Platelet Dysfunction With Eosinophilia

Acquired platelet dysfunction with eosinophilia is a self-limited disorder characterized by mucocutaneous bleeding and hyper-eosinophilia.[314,315] The platelet count is usually normal; however, the platelets are pale by light microscopy (as in gray platelet syndrome), and aggregation studies show an acquired storage pool defect. All reported cases have been from South-East Asia. In some cases, eosinophilia and platelet dysfunction returned to normal after anti-helminthic therapy, which suggests a parasitic infection.[316]

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Adili R, et al. First selective 12-LOX inhibitor, ML355, impairs thrombus formation and vessel occlusion in vivo with minimal effects on hemostasis. *Arterioscler Thromb Vasc Biol*. 2017;37(10):1828–1839.

Brennan Y, et al. Acquired platelet disorders. *Thromb Res*. 2020;196(2020):561–668.

Bye AP, et al. Severe platelet dysfunction in NHL patients receiving ibrutinib is absent in patients receiving acalabrutinib. *Blood Adv*. 2017;1(26):2610–2623.

Casari C, Bergmeier W. Acquired platelet disorders. *Thromb Res*. 2016;141(Suppl. 2):S73–S75.

Comer SP, et al. COVID-19 induces a hyperactive phenotype in circulating platelets. *PLoS Biol*. 2021;19(2):e3001109.

Eikelboom JW, et al. Rivaroxaban with or without aspirin in stable cardiovascular disease. *N Engl J Med*. 2017;377(14):1319–1330.

George JN, Shattil SJ. The clinical importance of acquired abnormalities of platelet function. *N Engl J Med*. 1991;324(27).

Lamb DJ, et al. Inhibition of SYK kinase does not confer a pro-proliferative or pro-invasive phenotype in breast epithelium or breast cancer cells. *Oncotarget*. 2020;11(14):1257–1272.

Manne BK, et al. Platelet gene expression and function in patients with COVID-19. *Blood*. 2020;136(11):1317–1329.

Petzold T, et al. Rivaroxaban reduces arterial thrombosis by inhibition of FXa-driven platelet activation via protease activated receptor-1. *Circ Res*. 2020;126(4):486–500.

St John A, et al. Plasma proteomic profile associated with platelet dysfunction after trauma. *J Thromb Haemost*. 2021;19(7):1666–1675.

Trugilho MR, de O, et al. Platelet proteome reveals novel pathways of platelet activation and platelet-mediated immunoregulation in dengue. *PLoS Pathog*. 2017;13(5):e1006385.

Vulliamy P, et al. Histone H4 induces platelet ballooning and microparticle release during trauma hemorrhage. *Proc Natl Acad Sci U S A*. 2019;116(35):17444–17449.

REFERENCES

1. Koscielny J, Ziemer S, Radtke H. A practical concept for preoperative identification of patients with impaired primary hemostasis. *Clin Appl Thromb Hemost*. 2004;10(195).
2. Michelson AD. P2Y12 antagonism: promises and challenges. *Arterioscler Thromb Vasc Biol*. 2008;28:s33–s38.
3. Dorsam RT, Kunapuli SP. Central role of the P2Y12 receptor in platelet activation. *J Clin Invest*. 2004;113(340).
4. Solet DJ, Zacharski LR, Plehn JF. The role of adenosine 5′-diphosphate receptor blockade in patients with cardiovascular disease. *Am J Med*. 2001;111(45).
5. Gachet C. ADP receptors of platelets and their inhibition. *Thromb Haemost*. 2001;86(222).
6. Taka T, Okano E, Seki J. Effects of clopidogrel on platelet activation and coagulation of non-anticoagulated rat blood under high shear stress. *Haemostasis*. 1999;29(189).
7. Curtin R, Fox D, Fitzgerald D. *Clopidogrel and Ticlopidine, Platelets*. San Diego, CA: Academic Press; 2002:787–801.
8. Morrow DA, et al. Vorapaxar in the secondary prevention of atherothrombotic events. *N Engl J Med*. 2012;366(15):1404–1413.
9. Eikelboom JW, et al. Rivaroxaban with or without aspirin in stable cardiovascular disease. *N Engl J Med*. 2017;377(14):1319–1330.
10. Petzold T, et al. Rivaroxaban reduces arterial thrombosis by inhibition of FXa-driven platelet activation via protease activated receptor-1. *Circ Res*. 2020;126(4):486–500.
11. Alexander JH, et al. Apixaban with antiplatelet therapy after acute coronary syndrome. *N Engl J Med*. 2011;365(8):699–708.
12. Gresele P, et al. Thromboxane synthase inhibitors, thromboxane receptor antagonists and dual blockers in thrombotic disorders. *Trends Pharmacol Sci*. 1991;12(4):158–163.
13. Gremmel T, et al. Novel aspects of antiplatelet therapy in cardiovascular disease. *Res Pract Thromb Haemost*. 2018;2(3):439–449.
14. Gomberg-Maitland M, Olschewski H. Prostacyclin therapies for the treatment of pulmonary arterial hypertension. *Eur Respir J*. 2008;31(891).
15. Ostrowska M, Adamski P, Koziński M. Off-target effects of glycoprotein IIb/IIIa receptor inhibitors. *Cardiol J*. 2014;21(458).
16. Armstrong PC, Peter K. GPIIb/IIIa inhibitors: from bench to bedside and back to bench again. *Thromb Haemost*. 2012;107(808).
17. Hodivala-Dilke KM, McHugh KP, Tsakiris DA, et al. Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. *J Clin Invest*. 1999;103(229).
18. Mascelli MA, Lance ET, Damaraju L. Pharmacodynamic profile of short-term abciximab treatment demonstrates prolonged platelet inhibition with gradual recovery from GP IIb/IIIa receptor blockade. *Circulation*. 1998;97(1680).
19. Douketis JD, Berger PB, Dunn AS. The perioperative management of antithrombotic therapy: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest*. 2008;133(299S).
20. Scarborough RM, Rose JW, Hsu MA. Barbourin. A GPIIb-IIIa-specific integrin antagonist from the venom of *Sistrurus* m. *barbouri*. *J Biol Chem*. 1991;266(9359).
21. Scarborough RM. Development of eptifibatide. *Am Heart J*. 1999;138(1093).
22. King S, Short M, Harmon C. Glycoprotein IIb/IIIa inhibitors: the resurgence of tirofiban. *Vascul Pharmacol*. 2016;78(10).
23. Tcheng JE, Kereiakes DJ, Lincoff AM. Abciximab readministration: results of the ReoPro readministration registry. *Circulation*. 2001;104(870).
24. Aster RH. Immune thrombocytopenia caused by glycoprotein IIb/IIIa inhibitors. *Chest*. 2005;127(53S).
25. Dasgupta H, Blankenship JC, Wood GC. Thrombocytopenia complicating treatment with intravenous glycoprotein IIb/IIIa receptor inhibitors: a pooled analysis. *Am Heart J*. 2000;140(206).
26. Boersma E, Harrington RA, Moliterno DJ. Platelet glycoprotein IIb/IIIa inhibitors in acute coronary syndromes: a meta-analysis of all major randomised clinical trials. *Lancet*. 2002;359(189).
27. Cannon CP. Oral glycoprotein IIb/IIIa inhibitions—great idea, but it didn't work. *Am J Med*. 2002;112(8):673–675.
28. Patrono C, Coller B, Dalen JE. Platelet-active drugs: the relationships among dose, effectiveness, and side effects. *Chest*. 1998;114(470S).
29. Mackowiak PA. Brief history of antipyretic therapy. *Clin Infect Dis*. 2000;31(suppl 5).
30. Miner J, Hoffhines A. The discovery of aspirin's antithrombotic effects. *Tex Heart Inst J*. 2007;34(179).
31. Undas A, Brummel-Ziedins KE, Mann KG. Antithrombotic properties of aspirin and resistance to aspirin: beyond strictly antiplatelet actions. *Blood*. 2007;109(2285).
32. Steering Committee of the Physician's Health Study Research Group Final report of the asprin component of the ongoing physicians' health study. *N Engl J Med*. 1989;321(1989).
33. Eikelboom JW, Hirsh J, Spencer FA. Antiplatelet drugs: antithrombotic therapy and prevention of thrombosis. *Chest*. 2012;141:e89S.
34. Kallmann R, Nieuwenhuis HK, Groot PG. Effects of low doses of aspirin, 10 mg and 30 mg daily, on bleeding time, thromboxane production and 6-keto-PGF1 a excretion in healthy subjects. *Thromb Res*. 1987;45(355).
35. Cipollone F, Rocca B, Patrono C. Cyclooxygenase-2 expression and inhibition in atherothrombosis. *Arterioscler Thromb Vasc Biol*. 2004; 24(246).
36. Weksler BB. Regulation of prostaglandin synthesis in human vascular cells. *Ann N Y Acad Sci*. 1987;509:142–148.
37. Weiss HJ, Aledort LM. Impaired platelet-connective-tissue reaction in man after aspirin ingestion. *Lancet*. 1967;2(495).
38. Kyrle PA, Westwick J, Scully MF. Investigation of the interaction of blood platelets with the coagulation system at the site of plug formation in vivo in man—effect of low-dose aspirin. *Thromb Haemost*. 1987;57(62).
39. Undas A, Brummel K, Musial J. Blood coagulation at the site of microvascular injury: effects of low-dose aspirin. *Blood*. 2001;98(2423).
40. Szczeklik A, Musial J, Undas A. Inhibition of thrombin generation by aspirin is blunted in hypercholesterolemia. *Arterioscler Thromb Vasc Biol*. 1996;16(948).
41. Roderick PJ, Wilkes HC, Meade TW. The gastrointestinal toxicity of aspirin: an overview of randomised controlled trials. *Br J Clin Pharmacol*. 1993;35(219).
42. Wolfe MM, Lichtenstein DR, Singh G. Gastrointestinal toxicity of nonsteroidal antiinflammatory drugs. *N Engl J Med*. 1999;340(1888).
43. Lee M, Cryer B, Feldman M. Dose effects of aspirin on gastric prostaglandins and stomach mucosal injury. *Ann Intern Med*. 1994;120(184).
44. Wallace JL, Ma L. Inflammatory mediators in gastrointestinal defense and injury. *Exp Biol Med (Maywood)*. 2001;226(1003).
45. Feldman M, Shewmake K, Cryer B. Time course inhibition of gastric and platelet COX activity by acetylsalicylic acid in humans. *Am J Physiol Gastrointest Liver Physiol*. 2000;279:G1113–G1120.
46. Cryer B, Feldman M. Effects of very low dose daily, long-term aspirin therapy on gastric, duodenal, and rectal prostaglandin levels and on mucosal injury in healthy humans. *Gastroenterology*. 1999;117(17).
47. Sibilia J, Ravaud P, Marck G. Risk factors for gastrointestinal bleeding associated with low-dose aspirin. *Presse Med*. 2003;32:S9.
48. Ma L, Elliott SN, Cirino G. Platelets modulate gastric ulcer healing: role of endostatin and vascular endothelial growth factor release. *Proc Natl Acad Sci USA*. 2001;98(6470).
49. Stuart MJ, Miller ML, Davey FR. The post-aspirin bleeding time: a screening test for evaluating haemostatic disorders. *Br J Haematol*. 1979;43(649).
50. Hovens MMC, Snoep JD, Eikenboom JCJ. Prevalence of persistent platelet reactivity despite use of aspirin: a systematic review. *Am Heart J*. 2007;153(175).
51. Snoep JD, Hovens MMC, Eikenboom JCJ. Association of laboratory-defined aspirin resistance with a higher risk of recurrent cardiovascular events: a systematic review and meta-analysis. *Arch Intern Med*. 2007;167(1593).
52. Verschuren JJ, Jukema JW. Pharmacogenetics of antiplatelet therapy: ready for clinical application? *Heart*. 2011;97(1268).
53. Krasopoulos G, Brister SJ, Beattie WS. Aspirin "resistance" and risk of cardiovascular morbidity: systematic review and meta-analysis. *BMJ*. 2008; 336(195).
54. Schwartz KA, Schwartz DE, Ghosheh K. Compliance as a critical consideration in patients who appear to be resistant to aspirin after healing of myocardial infarction. *Am J Cardiol*. 2005;95(973).
55. Lev EI, Patel RT, Maresh KJ. Aspirin and clopidogrel drug response in patients undergoing percutaneous coronary intervention: the role of dual drug resistance. *J Am Coll Cardiol*. 2006;47(27).
56. Gurbel PA, Bliden KP, DiChiara J. Evaluation of dose-related effects of aspirin on platelet function: results from the Aspirin-Induced Platelet Effect (ASPECT) study. *Circulation*. 2007;115(3156).
57. Mehta SR, Tanguay JF, Eikelboom JW. Double-dose versus standard-dose clopidogrel and high-dose versus low-dose aspirin in individuals undergoing percutaneous coronary intervention for acute coronary syndromes (CURRENT-OASIS 7): a randomised factorial trial. *Lancet*. 2010;376(1233).
58. Alberts MJ. Platelet function testing for aspirin resistance is reasonable to do: yes! *Stroke*. 2010;41(2400).
59. Guthikonda S, Lev EI, Patel R. Reticulated platelets and uninhibited COX-1 and COX-2 decrease the antiplatelet effects of aspirin. *J Thromb Haemost*. 2007;5(490).

60. Cipollone F, Fazia ML. COX-2 and atherosclerosis. *J Cardiovasc Pharmacol*. 2006;47(Suppl 1).
61. Patrono C, Baigent C, Hirsh J. Antiplatelet drugs: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest*. 2008;133(199).
62. Catella-Lawson F, Reilly MP, Kapoor SC. Cyclooxygenase inhibitors and the antiplatelet effects of aspirin. *N Engl J Med*. 2001;345(1809).
63. Hankey GJ, Eikelboom JW. Aspirin resistance. *Lancet*. 2006;367(9510):606–617.
64. Halushka MK, Walker LP, Halushka PV. Genetic variation in cyclooxygenase 1: effects on response to aspirin. *Clin Pharmacol Ther*. 2003;73(122).
65. Goodman T, Ferro A, Sharma P. Pharmacogenetics of aspirin resistance: a comprehensive systematic review. *Br J Clin Pharmacol*. 2008;66(222).
66. Voora D, Horton J, Shah SH. Polymorphisms associated with in vitro aspirin resistance are not associated with clinical outcomes in patients with coronary artery disease who report regular aspirin use. *Am Heart J*. 2011;162(166).
67. Würtz M, Kristensen SD, Hvas AM. Pharmacogenetics of the antiplatelet effect of aspirin. *Curr Pharm Des*. 2012;18(5294).
68. Gresele P, Momi S, Falcinelli E. Anti-platelet therapy: phosphodiesterase inhibitors. *Br J Clin Pharmacol*. 2011;72(634).
69. Cavalcante JW, J. Santos PR, Menezes MG. Influence of caffeine on blood pressure and platelet aggregation. *Arq Bras Cardiol*. 2000;75(97).
70. Thiele J, Kvasnicka HM, Schmitt-Graeff A. Effects of anagrelide on megakaryopoiesis and platelet production. *Semin Thromb Hemost*. 2006;32(352).
71. Andersson BS. Essential thrombocythemia: diagnosis and treatment, with special emphasis on the use of anagrelide. *Hematology*. 2002;7(173).
72. Kambayashi J, Liu Y, Sun B. Cilostazol as a unique antithrombotic agent. *Curr Pharm Des*. 2003;9(2289).
73. Marnett LJ, Siedlik PH, Ochs RC. Mechanism of the stimulation of prostaglandin H synthase and prostacyclin synthase by the antithrombotic and antimetastatic agent, nafazatrom. *Mol Pharmacol*. 1984;26(328).
74. Leonardi-Bee J, Bath PM, Bousser MG. Dipyridamole for preventing recurrent ischemic stroke and other vascular events: a meta-analysis of individual patient data from randomized controlled trials. *Stroke*. 2005;36(162).
75. Hallas J, Dall M, Andries A. Use of single and combined antithrombotic therapy and risk of serious upper gastrointestinal bleeding: population based case-control study. *BMJ*. 2006;333(726).
76. Rosen RC, Kostis JB. Overview of phosphodiesterase 5 inhibition in erectile dysfunction. *Am J Cardiol*. 2003;92(9M).
77. Bott SR, Shergill I, Arya M. Epistaxis after sildenafil. *J R Soc Med*. 2002;95(528).
78. Hicklin LA, Ryan C, Wong DK. Nose-bleeds after sildenafil (Viagra). *J R Soc Med*. 2002;95(402).
79. Sheikh RA, Yasmeen S, Prindiville TP. Hemorrhoidal bleeding associated with sildenafil. *Am J Gastroenterol*. 2001;96(2518).
80. Buxton N, Flannery T, Wild D. Sildenafil (Viagra)-induced spontaneous intracerebral haemorrhage. *Br J Neurosurg*. 2001;15(347).
81. Tzathas C, Christidou A, Ladas SD. Sildenafil (Viagra) is a risk factor for acute variceal bleeding. *Am J Gastroenterol*. 2002;97(1856).
82. Lamb DJ, et al. Inhibition of SYK kinase does not confer a pro-proliferative or pro-invasive phenotype in breast epithelium or breast cancer cells. *Oncotarget*. 2020;11(14):1257–1272.
83. Adili R, et al. First selective 12-LOX inhibitor, ML355, impairs thrombus formation and vessel occlusion in vivo with minimal effects on hemostasis. *Arterioscler Thromb Vasc Biol*. 2017;37(10):1828–1839.
84. George JN, Shattil SJ. The clinical importance of acquired abnormalities of platelet function. *N Engl J Med*. 1991;324(27).
85. McQueen EG, Facoory B, Faed JM. Non-steroidal anti-inflammatory drugs and platelet function. *N Z Med J*. 1986;99(358).
86. Favaloro EJ. Clinical application of the PFA-100. *Curr Opin Hematol*. 2002;9(407).
87. Thomas P, Hepburn B, Kim HC. Nonsteroidal anti-inflammatory drugs in the treatment of hemophilic arthropathy. *Am J Hematol*. 1982;12(131).
88. Blann AD, Nadar S, Lip GY. Pharmacological modulation of platelet function in hypertension. *Hypertension*. 2003;42(1):1–7.
89. Torfgård KE, Ahlner J. Mechanisms of action of nitrates. *Cardiovasc Drugs Ther*. 1994;8(701).
90. Petríková M, Jancinová V, Nosál R. Antiplatelet activity of carvedilol in comparison to propranolol. *Platelets*. 2002;13(479).
91. Dash D, Rao K. Effect of propranolol on platelet signal transduction. *Biochem J*. 1995;309(99).
92. Falciani M, Rinaldi B, D'Agostino B. Effects of nebivolol on human platelet aggregation. *J Cardiovasc Pharmacol*. 2001;38(922).
93. Lawson D, Mehta J, Mehta P. Cumulative effects of quinidine and aspirin on bleeding time and platelet a 2-adrenoceptors: potential mechanism of bleeding diathesis in patients receiving this combination. *J Lab Clin Med*. 1986;108(581).
94. Sato Y, Fujii S, Imagawa S. Platelet aggregability in patients with hypertension treated with angiotensin II type 1 receptor blockers. *J Atheroscler Thromb*. 2007;14(31).
95. Serebruany VL, Pokov AN, Malinin AI. Valsartan inhibits platelet activity at different doses in mild to moderate hypertensives: Valsartan Inhibits Platelets (VIP) trial. *Am Heart J*. 2006;151(92).
96. Brown RB, Klar J, Lemeshow S. Enhanced bleeding with cefoxitin or moxalactam. Statistical analysis within a defined population of 1493 patients. *Arch Intern Med*. 1986;146(2159).
97. Sattler FR, Weitekamp MR, Ballard JO. Potential for bleeding with the new beta-lactam antibiotics. *Ann Intern Med*. 1986;105(924).
98. Wisløff F, Godal HC. Prolonged bleeding time with adequate platelet count in hospital patients. *Scand J Haematol*. 1981;27(45).
99. Rau J, Simon M, Sander M. Severe bleeding as a result of platelet inhibition caused by floxacillin treatment for endocarditis. *J Thorac Cardiovasc Surg*. 2013;146:e63–e65.
100. Shattil SJ, Bennett JS, McDonough M. Carbenicillin and penicillin G inhibit platelet function in vitro by impairing the interaction of agonists with the platelet surface. *J Clin Invest*. 1980;65(329).
101. Schulz C, Beckerath OV, Okrojek R. Platelet dysfunction and inhibition of multiple electrode platelet aggregometry caused by penicillin. *Thromb J*. 2010;8(13).
102. Haburchak DR, Head DR, Everett ED. Postoperative hemorrhage associated with carbenicillin administration. Report of two cases and review of the literature. *Am J Surg*. 1977;134(630).
103. Mihara S, Kakushi H, Shike T. Effects of latamoxef on in vitro and ex vivo thromboxane A2 generation in human platelets. *Thromb Res*. 1988; 49(215).
104. Lipsky JJ. Antibiotic-associated hypoprothrombinaemia. *J Antimicrob Chemother*. 1988;21(281).
105. Rossi EC, Levin NW. Inhibition of primary ADP-induced platelet aggregation in normal subjects after administration of nitrofurantoin (furadantin). *J Clin Invest*. 1973;52(2457).
106. Ishikawa S, Manabe S, Wada O. Miconazole inhibition of platelet aggregation by inhibiting cyclooxygenase. *Biochem Pharmacol*. 1986;35(1787).
107. Miki A, Ohtani H, Sawada Y. Warfarin and miconazole oral gel interactions: analysis and therapy recommendations based on clinical data and a pharmacokinetic model. *J Clin Pharm Ther*. 2011;36(642).
108. Pastakia KB, Brownson NE, Terle DA. Amphotericin B induced abnormalities in human platelets. *Clin Mol Pathol*. 1996;49:M301–M307.
109. Hussein MA, Fletcher R, Long TJ. Transfusing platelets 2 h after the completion of amphotericin-B decreases its detrimental effect on transfused platelet recovery and survival. *Transfus Med*. 1998;8(43).
110. Fuller RW. Serotonin uptake inhibitors: uses in clinical therapy and in laboratory research. *Prog Drug Res*. 1995;45:167–204.
111. Sauer WH, Berlin JA, Kimmel SE. Effect of antidepressants and their relative affinity for the serotonin transporter on the risk of myocardial infarction. *Circulation*. 2003;108(32).
112. Tseng YL, Chiang ML, Huang TF. A selective serotonin reuptake inhibitor, citalopram, inhibits collagen-induced platelet aggregation and activation. *Thromb Res*. 2010;126(517).
113. Reikvam AG, Hustad S, Reikvam H. The effects of selective serotonin reuptake inhibitors on platelet function in whole blood and platelet concentrates. *Platelets*. 2012;23(299).
114. Bismuth-Evenzal Y, Gonopolsky Y, Gurwitz D. Decreased serotonin content and reduced agonist-induced aggregation in platelets of patients chronically medicated with SSRI drugs. *J Affect Disord*. 2012;136(99).
115. Blardi P, Lalla Ad, Leo A. Serotonin and fluoxetine levels in plasma and platelets after fluoxetine treatment in depressive patients. *J Clin Psychopharmacol*. 2002;22(131).
116. Menys VC, Smith CC, Lewins P. Platelet 5-hydroxytryptamine is decreased in a preliminary group of depressed patients receiving the 5-hydroxytryptamine re-uptake inhibiting drug fluoxetine. *Clin Sci*. 1996;91(87).
117. Humphries JE, Wheby MS, VandenBerg SR. Fluoxetine and the bleeding time. *Arch Pathol Lab Med*. 1990;114(727).
118. Pai VB, Kelly MW. Bruising associated with the use of fluoxetine. *Ann Pharmacother*. 1996;30(786).
119. Alderman CP, Moritz CK, Ben DI. Tovim, Abnormal platelet aggregation associated with fluoxetine therapy. *Ann Pharmacother*. 2002;26(1517).
120. Roose SP, Rutherford BR. Selective serotonin reuptake inhibitors and operative bleeding risk: a review of the literature. *J Clin Psychopharmacol*. 2016;36(704).

121. Sajan F, Conte JV, Tamargo RJ. Association of selective serotonin reuptake inhibitors with transfusion in surgical patients. *Anesth Analg*. 2016;123(21).
122. Hanley GE, Smolina K, Mintzes B. Postpartum hemorrhage and use of serotonin reuptake inhibitor antidepressants in pregnancy. *Obstet Gynecol*. 2016;127(553).
123. Anglin R, Yuan Y, Moayyedi P. Risk of upper gastrointestinal bleeding with selective serotonin reuptake inhibitors with or without concurrent nonsteroidal anti-inflammatory use: a systematic review and meta-analysis. *Am J Gastroenterol*. 2014;109(811).
124. Labos C, et al. Risk of bleeding associated with combined use of selective serotonin reuptake inhibitors and antiplatelet therapy following acute myocardial infarction. *CMAJ*. 2011;183(16):1835–1843.
125. Noordam R, Aarts N, Leening MJ. Use of antidepressants and the risk of myocardial infarction in middle-aged and older adults: a matched case-control study. *Eur J Clin Pharmacol*. 2016;72(211).
126. Fernandes N, et al. The impact of SSRIs on mortality and cardiovascular events in patients with coronary artery disease and depression: systematic review and meta-analysis. *Clin Res Cardiol*. 2021;110(2):183–193.
127. Ahr DJ, Scialla SJ, Kimbali Jr DBJ. Acquired platelet dysfunction following mithramycin therapy. *Cancer*. 1978;41(448).
128. White JG, Rao GH. Effects of a microtubule stabilizing agent on the response of platelets to vincristine. *Blood*. 1982;60(474).
129. Quintás-Cardama A, Han X, Kantarjian H. Tyrosine kinase inhibitor-induced platelet dysfunction in patients with chronic myeloid leukemia. *Blood*. 2009;114(261).
130. Kostos L, Burbury K, Srivastava G. Gastrointestinal bleeding in a chronic myeloid leukaemia patient precipitated by dasatinib-induced platelet dysfunction: case report. *Platelets*. 2015;26(809).
131. Levade M, David E, Garcia C. Ibrutinib treatment affects collagen and von Willebrand factor-dependent platelet functions. *Blood*. 2014;124(3991).
132. Bye AP, et al. Severe platelet dysfunction in NHL patients receiving ibrutinib is absent in patients receiving acalabrutinib. *Blood Adv*. 2017;1(26):2610–2623.
133. Kozek-Langenecker SA. The effects of drugs used in anaesthesia on platelet membrane receptors and on platelet function. *Curr Drug Targets*. 2002;3(247).
134. Gibbs NM. The effect of anaesthetic agents on platelet function. *Anaesth Intensive Care*. 1991;19(495).
135. Watala C, Boncler M, Golanski J. Release of calcium and P-selectin from intraplatelet granules is hampered by procaine. *Thromb Res*. 1999;94(1).
136. Zhang W, Liu J, Sun R. Calpain activator dibucaine induces platelet apoptosis. *Int J Mol Sci*. 2011;12(2125).
137. Dalsgaard-Nielsen J, Gormsen J. Effects of halothane on platelet function. *Thromb Haemost*. 1980;44(143).
138. Langdell RD, Adelson E, Furth FW. Dextran and prolonged bleeding time; results of a sixty-gram, one-liter infusion given to one hundred sixty-three normal human subjects. *J Am Med Assoc*. 1958;166(346).
139. Weiss HJ. The effect of clinical dextran on platelet aggregation, adhesion, and ADP release in man: in vivo and in vitro studies. *J Lab Clin Med*. 1967;69(37).
140. de Jonge E, Levi M. Effects of different plasma substitutes on blood coagulation: a comparative review. *Crit Care Med*. 2001;29(1261).
141. Mishler JM. Synthetic plasma volume expanders—their pharmacology, safety and clinical efficacy. *Clin Haematol*. 1984;13(75).
142. Horne MK, Chao ES. Heparin binding to resting and activated platelets. *Blood*. 1989;74(238).
143. Salzman EW, Rosenberg RD, Smith MH. Effect of heparin and heparin fractions on platelet aggregation. *J Clin Invest*. 1980;65(64).
144. Sobel M, McNeill PM, Carlson PL. Heparin inhibition of von Willebrand factor-dependent platelet function in vitro and in vivo. *J Clin Invest*. 1991;87(1787).
145. Perrault C, Ajzenberg N, Legendre P. Modulation by heparin of the interaction of the A1 domain of von Willebrand factor with glycoprotein Ib. *Blood*. 1999;94(4186).
146. Coller BS. Platelets and thrombolytic therapy. *N Engl J Med*. 1990;322(33).
147. Miles LA, Ginsberg MH, White JG. Plasminogen interacts with human platelets through two distinct mechanisms. *J Clin Invest*. 1986;77(2001).
148. Adelman B, Michelson AD, Loscalzo J. Plasmin effect on platelet glycoprotein Ib-von Willebrand factor interactions. *Blood*. 1985;65(32).
149. Loscalzo J, Vaughan DE. Tissue plasminogen activator promotes platelet disaggregation in plasma. *J Clin Invest*. 1987;79(1749).
150. Schafer AI, Adelman B. Plasmin inhibition of platelet function and of arachidonic acid metabolism. *J Clin Invest*. 1985;75(456).
151. Watabe A, Ohta M, Matsuyama N. Characterization of plasmin-induced platelet aggregation. *Res Commun Mol Pathol Pharmacol*. 1997;96(341).
152. Kimura M, Andersen TT, Fenton JW. Plasmin-platelet interaction involves cleavage of functional thrombin receptor. *Am J Physiol*. 1996;271:C54–C60.
153. Winters KJ, Santoro SA, Miletich JP. Relative importance of thrombin compared with plasmin-mediated platelet activation in response to plasminogen activation with streptokinase. *Circulation*. 1991;84(1552).
154. Penny WF, Ware JA. Platelet activation and subsequent inhibition by plasmin and recombinant tissue-type plasminogen activator. *Blood*. 1992;79(91).
155. Parise P, Hauert J, Iorio A. Streptokinase and rt-PA activate platelets by a different way: implications on the rethrombosis rate after their administration in myocardial infarction. *J Lab Clin Med*. 1995;125(212).
156. Quinton TM, Kim S, Derian CK. Plasmin-mediated activation of platelets occurs by cleavage of protease-activated receptor 4. *J Biol Chem*. 2004;279(18434).
157. Maron DJ, Fazio S, Linton MF. Current perspectives on statins. *Circulation*. 2000;101(207).
158. Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. *Arterioscler Thromb Vasc Biol*. 2001;21(1712).
159. Shattil SJ, Anaya-Galindo R, Bennett J. Platelet hypersensitivity induced by cholesterol incorporation. *J Clin Invest*. 1975;55(636).
160. Tandon N, Harmon JT, Rodbard D. Thrombin receptors define responsiveness of cholesterol-modified platelets. *J Biol Chem*. 1983;258(11840).
161. Shrimpton CN, Borthakur G, Larrucea S. Localization of the adhesion receptor glycoprotein Ib-IX-V complex to lipid rafts is required for platelet adhesion and activation. *J Exp Med*. 2002;196(1057).
162. Munday AD, Gaus K, López JA. The platelet glycoprotein Ib-IX-V complex anchors lipid rafts to the membrane skeleton: implications for activation-dependent cytoskeletal translocation of signaling molecules. *J Thromb Haemost*. 2010;8(163).
163. Liao JK. Isoprenoids as mediators of the biological effects of statins. *J Clin Invest*. 2002;110(285).
164. Heeschen C, Hamm CW, Laufs U. Withdrawal of statins increases event rates in patients with acute coronary syndromes. *Circulation*. 2002;105(1446).
165. Hackam DG, Woodward M, Newby LK. Statins and intracerebral hemorrhage: collaborative systematic review and meta-analysis. *Circulation*. 2011;124(2233).
166. Callahan KP, Malinin AI, Atar D. Platelet activation as a universal trigger in the pathogenesis of acute coronary events after cocaine abuse. *Swiss Med Wkly*. 2001;131(487).
167. Jennings LK, White MM, Sauer CM. Cocaine-induced platelet defects. *Stroke*. 1993;24(1352).
168. Hobbs WE, Moore EE, Penkala RA. Cocaine and specific cocaine metabolites induce von Willebrand factor release from endothelial cells in a tissue-specific manner. *Arterioscler Thromb Vasc Biol*. 2013;33(1230).
169. Dyerberg J, Bang HO. Haemostatic function and platelet polyunsaturated fatty acids in Eskimos. *Lancet*. 1979;2(433).
170. Kim DN, Eastman A, Baker JE. Fish oil, atherogenesis, and thrombogenesis. *Ann N Y Acad Sci*. 1995;748(474).
171. Schoene NW. Vitamin E and omega-3 fatty acids: effectors of platelet responsiveness. *Nutrition*. 2001;17(793).
172. Manson JE, et al. Marine n-3 fatty acids and prevention of cardiovascular disease and cancer. *N Engl J Med*. 2019;380(1):23–32.
173. Bhatt DL, et al. Cardiovascular risk reduction with icosapent ethyl for hypertriglyceridemia. *N Engl J Med*. 2019;380(1):11–22.
174. Budoff MJ, et al. Effect of icosapent ethyl on progression of coronary atherosclerosis in patients with elevated triglycerides on statin therapy: final results of the EVAPORATE trial. *Eur Heart J*. 2020;41(40):3925–3932.
175. Deykin D, Janson P, McMahon L. Ethanol potentiation of aspirin-induced prolongation of the bleeding time. *N Engl J Med*. 1982;306(852).
176. Rand ML, Jakubowski JA, Fisher MJ. Ethanol enhances the inhibitory effect of an oral GPIIb/IIIa antagonist on human platelet function. *J Lab Clin Med*. 2002;140(391).
177. Nguyen A, Packham MA, Rand ML. Effects of ethanol on platelet responses associated with adhesion to collagen. *Thromb Res*. 1999;95(303).
178. Hammerschmidt DE. Szechwan purpura. *N Engl J Med*. 1980;302(1191).
179. Srivastava KC. Onion exerts antiaggregatory effects by altering arachidonic acid metabolism in platelets. *Prostaglandins Leukot Med*. 1986;24(43).
180. Apitz-Castro R, Badimon JJ, Badimon L. Effect of ajoene, the major antiplatelet compound from garlic, on platelet thrombus formation. *Thromb Res*. 1992;68(145).
181. Srivastava KC. Extracts from two frequently consumed spices—cumin (*Cuminum cyminum*) and turmeric (*Curcuma longa*)—inhibit platelet aggregation and alter eicosanoid biosynthesis in human blood platelets. *Prostaglandins Leukot Essent Fatty Acids*. 1989;37(57).
182. Kaur H, Corscadden K, Lott C. Bromelain has paradoxical effects on blood coagulability: a study using thromboelastography. *Blood Coagul Fibrinolysis*. 2016;27(745).

183. Chaves DS, Frattani FS, Assafim M. Phenolic chemical composition of *Petroselinum crispum* extract and its effect on haemostasis. *Nat Prod Commun*. 2011;6(961).
184. Dey F, Moller A, Kemkes-Matthes B. Reduced platelet aggregation in a boy with scurvy. *Klin Padiatr*. 2012;224(448).
185. Barbui T, Barosi G, Grossi A. Practice guidelines for the therapy of essential thrombocythemia. A statement from the Italian Society of Hematology, the Italian Society of Experimental Hematology and the Italian Group for Bone Marrow Transplantation. *Haematologica*. 2004;89(215).
186. Ashrani AA, Tefferi A, Pruthi RK. Acquired factor V deficiency in myeloproliferative neoplasms: a Mayo Clinic series of 33 patients. *Br J Haematol*. 2015;171(875).
187. Carobbio A, Thiele J, Passamonti F. Risk factors for arterial and venous thrombosis in WHO-defined essential thrombocythemia: an international study of 891 patients. *Blood*. 2011;117(5857).
188. Ginsburg AD. Platelet function in patients with high platelet counts. *Ann Intern Med*. 1975;82(506).
189. Berndt MC, Kabral A, Grimsley P. An acquired Bernard-Soulier-like platelet defect associated with juvenile myelodysplastic syndrome. *Br J Haematol*. 1988;68(97).
190. Cooper B, Schafer AI, Puchalsky D. Platelet resistance to prostaglandin D2 in patients with myeloproliferative disorders. *Blood*. 1978;52(618).
191. Horikawa Y, Matsumura I, Hashimoto K. Markedly reduced expression of platelet c-Mpl receptor in essential thrombocythemia. *Blood*. 1997;90(4031).
192. Moore A, Nachman RL. Platelet Fc receptor. Increased expression in myeloproliferative disease. *J Clin Invest*. 1981;67(1064).
193. Toyama K, Karasawa M, Yamane A. JAK2-V617F mutation analysis of granulocytes and platelets from patients with chronic myeloproliferative disorders: advantage of studying platelets. *Br J Haematol*. 2007;139(64).
194. Falanga A, Marchetti M, Vignoli A. V617F JAK-2 mutation in patients with essential thrombocythemia: relation to platelet, granulocyte, and plasma hemostatic and inflammatory molecules. *Exp Hematol*. 2007;35(702).
195. Panova-Noeva M, Marchetti M, Spronk HM. Platelet-induced thrombin generation by the calibrated automated thrombogram assay is increased in patients with essential thrombocythemia and polycythemia vera. *Am J Hematol*. 2011;86(337).
196. Castaman G, Lattuada A, Ruggeri M. Platelet von Willebrand factor abnormalities in myeloproliferative syndromes. *Am J Hematol*. 1995;49(289).
197. van Genderen PJ, Leenknegt H, Michiels JJ. Acquired von Willebrand disease in myeloproliferative disorders. *Leuk Lymphoma*. 1996;22(suppl 1): 79–82.
198. van Genderen PJ, Leenknegt H, Michiels JJ. The paradox of bleeding and thrombosis in thrombocythemia: Is von Willebrand factor the link? *Semin Thromb Hemost*. 1997;23:385.
199. van Genderen PJ, Budde U, Michiels JJ. The reduction of large von Willebrand factor multimers in plasma in essential thrombocythaemia is related to the platelet count. *Br J Haematol*. 1996;93(962).
200. Tiede A, Rand JH, Budde U. How I treat the acquired von Willebrand syndrome. *Blood*. 2011;117(6777).
201. Shim K, Anderson PJ, Tuley EA. Platelet-vWF complexes are preferred substrates of ADAMTS13 under fluid shear stress. *Blood*. 2008;111(651).
202. Mohri H, Motomura S, Kanamori H. Clinical significance of inhibitors in acquired von Willebrand syndrome. *Blood*. 1998;91(3623).
203. Bessler M, Mason PJ, Hillmen P. Paroxysmal nocturnal haemoglobinuria (PNH) is caused by somatic mutations in the PIG-A gene. *EMBO J*. 1994;13(110).
204. Vu T, Griscelli-Bennaceur A, Gluckman E. Aplastic anaemia and paroxysmal nocturnal haemoglobinuria: a study of the GPI-anchored proteins on human platelets. *Br J Haematol*. 1996;93(586).
205. Fu R, Meng Y, Wang Y. The dysfunction of platelets in paroxysmal nocturnal hemoglobinuria. *Thromb Res*. 2016;148(50).
206. Steinberg D, Carvalho AC, Chesney CM. Platelet hypersensitivity and intravascular coagulation in paroxysmal nocturnal hemoglobinuria. *Am J Med*. 1975;59(845).
207. Grünewald M, Grünewald A, Schmid A. The platelet function defect of paroxysmal nocturnal haemoglobinuria. *Platelets*. 2004;15(145).
208. Gralnick HR, Vail M, McKeown LP. Activated platelets in paroxysmal nocturnal haemoglobinuria. *Br J Haematol*. 1995;91(697).
209. Lackner H, Hunt V, Zucker MB. Abnormal fibrin ultrastructure, polymerization, and clot retraction in multiple myeloma. *Br J Haematol*. 1970;18(625).
210. Edelstein C, Scanu AM. Precautionary measures for collecting blood destined for lipoprotein isolation. *Methods Enzymol*. 1986;128(151).
211. Mehta J, Singhal S. Hyperviscosity syndrome in plasma cell dyscrasias. *Semin Thromb Hemost*. 2003;29(467).
212. Kwaan HC. Hyperviscosity in plasma cell dyscrasias. *Clin Hemorheol Microcirc*. 2013;55(75).
213. Palmer RN, Rick ME, Rick PD. Circulating heparan sulfate anticoagulant in a patient with a fatal bleeding disorder. *N Engl J Med*. 1984;310(1696).
214. Furie B, Greene E, Furie BC. Syndrome of acquired factor X deficiency and systemic amyloidosis in vivo studies of the metabolic fate of factor X. *N Engl J Med*. 1977;297(81).
215. Meyer K, Williams EC. Fibrinolysis and acquired alpha-2 plasmin inhibitor deficiency in amyloidosis. *Am J Med*. 1985;79(394).
216. Kasturi J, Saraya AK. Platelet functions in dysproteinaemia. *Acta Haematol*. 1978;59(104).
217. Djunic I, Elezovic I, Ilic V. The effect of paraprotein on platelet aggregation. *J Clin Lab Anal*. 2014;28(141).
218. Vigliano EM, Horowitz HI. Bleeding syndrome in a patient with IgA myeloma: interaction of protein and connective tissue. *Blood*. 1967;29(823).
219. DiMinno G, Coraggio F, Cerbone AM. A myeloma paraprotein with specificity for platelet glycoprotein IIIa in a patient with a fatal bleeding disorder. *J Clin Invest*. 1986;77(157).
220. Rinder MR, Richard RE, Rinder HM. Acquired von Willebrand's disease: a concise review. *Am J Hematol*. 1997;54(139).
221. Perkins HA, MacKenzie MR, Fudenberg HH. Hemostatic defects in dysproteinemias. *Blood*. 1970;35(695).
222. Pogliani EM, Colombi M, Cofrancesco E. Platelet dysfunction in acute megakaryoblastic leukemia. *Acta Haematol*. 1989;81(1).
223. Hendrick AM, Shah S, Raich PC. Platelet dysfunction in acute megakaryoblastic leukemia. *Arch Pathol Lab Med*. 1984;108(63).
224. Psaila B, Bussel JB, Frelinger AL. Differences in platelet function in patients with acute myeloid leukemia and myelodysplasia compared to equally thrombocytopenic patients with immune thrombocytopenia. *J Thromb Haemost*. 2011;9(2302).
225. Lintula R, Rasi V, Ikkala E. Platelet function in preleukaemia. *Scand J Haematol*. 1981;26(65).
226. Cortelazzo S, Viero P, D'Emilio A. Platelet dysfunction in splenectomized patients with hairy cell leukemia. *Eur J Cancer Clin Oncol*. 1986; 22(935).
227. Bellucci S, Huisse MG, Boval B. Defective collagen-induced platelet activation in two patients with malignant haemopathies is related to a defect in the GPVI-coupled signalling pathway. *Thromb Haemost*. 2005;93(130).
228. Raman V, Quillen K, Sloan JM. Acquired Glanzmann thrombasthenia associated with Hodgkin lymphoma: rapid reversal of functional platelet defect with ABVD (adriamycin/bleomycin/vinblastine/dacarbazine) chemotherapy. *Clin Lymphoma Myeloma Leuk*. 2014;14:e51–e54.
229. Frigeni M, Galli M. Childhood myelodysplastic syndrome associated with an acquired Bernard-Soulier-like platelet dysfunction. *Blood*. 2014;124(2609).
230. Avvisati G, Tirindelli MC, Annibali O. Thrombocytopenia and hemorrhagic risk in cancer patients. *Crit Rev Oncol Hematol*. 2003;48:S13–S16.
231. Oudemans-van Straaten HM. Hemostasis and thrombosis in continuous renal replacement treatment. *Semin Thromb Hemost*. 2015;41(91).
232. Salvati F, Liani M. Role of platelet surface receptor abnormalities in the bleeding and thrombotic diathesis of uremic patients on hemodialysis and peritoneal dialysis. *Int J Artif Organs*. 2001;24(131).
233. Castillo R, Lozano T, Escolar G. Defective platelet adhesion on vessel subendothelium in uremic patients. *Blood*. 1986;68(337).
234. Gralnick HR, McKeown LP, Williams SB. Plasma and platelet von Willebrand factor defects in uremia. *Am J Med*. 1988;85(806).
235. Casonato A, Pontara E, Vertolli UP. Plasma and platelet von Willebrand factor abnormalities in patients with uremia: lack of correlation with uremic bleeding. *Clin Appl Thromb Hemost*. 2001;7(81).
236. Thekkedath UR, Chirananthavat T, Leypoldt JK. Elevated fibrinogen fragment levels in uremic plasma inhibit platelet function and expression of glycoprotein IIb–IIIa. *Am J Hematol*. 2006;81(915).
237. Díaz-Ricart M, Estebanell E, Cases A. Abnormal platelet cytoskeletal assembly in hemodialyzed patients results in deficient tyrosine phosphorylation signaling. *Kidney Int*. 2000;57(1905).
238. Horowitz HI, Cohen BD, Martinez P. Defective ADP-induced platelet factor 3 activation in uremia. *Blood*. 1967;30(331).
239. Horowitz HI, Stein IM, Cohen BD. Further studies on the platelet-inhibitory effect of guanidinosuccinic acid and its role in uremic bleeding. *Am J Med*. 1970;49(336).
240. Noris M, Remuzzi G. Uremic bleeding: closing the circle after 30 years of controversies? *Blood*. 1999;94(2569).
241. Remuzzi G, Perico N, Zoja C. Role of endothelium-derived nitric oxide in the bleeding tendency of uremia. *J Clin Invest*. 1990;86(1768).
242. Noris M, Todeschini M, Zappella S. 17beta-estradiol corrects hemostasis in uremic rats by limiting vascular expression of nitric oxide synthases. *Am J Physiol Renal Physiol*. 2000;279:F626–F635.
243. Zoja C, Vigano G, Bergamelli A. Prolonged bleeding time and increased vascular prostacyclin in rats with chronic renal failure: effects of conjugated estrogens. *J Lab Clin Med*. 1988;112(380).

244. Sloand JA, Sloand EM. Studies on platelet membrane glycoproteins and platelet function during hemodialysis. *J Am Soc Nephrol*. 1997;8(799).
245. Himmelfarb J, Holbrook D, McMonagle E. Increased reticulated platelets in dialysis patients. *Kidney Int*. 1997;51(834).
246. Plé H, Maltais M, Corduan A. Alteration of the platelet transcriptome in chronic kidney disease. *Thromb Haemost*. 2012;108(605).
247. Gaspari F, Vigano G, Orisio S. Aspirin prolongs bleeding time in uremia by a mechanism distinct from platelet cyclooxygenase inhibition. *J Clin Invest*. 1987;79(1788).
248. Eberst ME, Berkowitz LR. Hemostasis in renal disease: pathophysiology and management. *Am J Med*. 1994;96(168).
249. Hellem AJ, Borchgrevink CF, Ames SB. The role of red cells in haemostasis: the relation between haematocrit, bleeding time and platelet adhesiveness. *Br J Haematol*. 1961;7(42).
250. Fernandez F, Goudable C, Sie P. Low haematocrit and prolonged bleeding time in uraemic patients: effect of red cell transfusions. *Br J Haematol*. 1985;59(139).
251. Blajchman MA, Bordin JO, Bardossy L. The contribution of the haematocrit to thrombocytopenic bleeding in experimental animals. *Br J Haematol*. 1994;86(347).
252. Cases A, Escolar G, Reverter JC. Recombinant human erythropoietin treatment improves platelet function in uremic patients. *Kidney Int*. 1992;42(668).
253. Sagripanti A, Barsotti G. Bleeding and thrombosis in chronic uremia. *Nephron*. 1997;75(125).
254. Lutz J, Menke J, Sollinger D. Haemostasis in chronic kidney disease. *Nephrol Dial Transplant*. 2014;29(29).
255. Violi F, Leo R, Basili S. Association between prolonged bleeding time and gastrointestinal hemorrhage in 102 patients with liver cirrhosis: results of a retrospective study. *Haematologica*. 1994;79(61).
256. Sanchez-Roig MJ, Rivera J, Moraleda JM. Quantitative defect of glycoprotein Ib in severe cirrhotic patients. *Am J Hematol*. 1994;45(10).
257. Stein SF, Harker LA. Kinetic and functional studies of platelets, fibrinogen, and plasminogen in patients with hepatic cirrhosis. *J Lab Clin Med*. 1982;99(217).
258. Laffi G, Cominelli F, Ruggiero M. Altered platelet function in cirrhosis of the liver: impairment of inositol lipid and arachidonic acid metabolism in response to agonists. *Hepatology*. 1988;8(1620).
259. Kunihiro N, Kawai B, Sanjo A. Platelet aggregation and coagulation and fibrinolysis parameters in both portal and systemic circulations in patients with cirrhosis and hepatocellular carcinoma. *Hepatol Res*. 2001;19(52).
260. Lisman T, Bongers TN, Adelmeijer J. Elevated levels of von Willebrand factor in cirrhosis support platelet adhesion despite reduced functional capacity. *Hepatology*. 2006;44(53).
261. Mannucci PM. Abnormal hemostasis tests and bleeding in chronic liver disease: are they related? No. *J Thromb Haemost*. 2006;4(721).
262. Reuken PA, Kussmann A, Kiehntopf M. Imbalance of von Willebrand factor and its cleaving protease ADAMTS13 during systemic inflammation superimposed on advanced cirrhosis. *Liver Int*. 2015;35(37).
263. Tripodi A, Primignani M, Chantarangkul V. An imbalance of pro- vs anti-coagulation factors in plasma from patients with cirrhosis. *Gastroenterology*. 2009;137(2105).
264. Lisman T, Bakhtiari K, Pereboom IT. Normal to increased thrombin generation in patients undergoing liver transplantation despite prolonged conventional coagulation tests. *J Hepatol*. 2010;52(355).
265. Lisman T, Caldwell SH, Burroughs AK. Hemostasis and thrombosis in patients with liver disease: the ups and downs. *J Hepatol*. 2010;53(362).
266. Tripodi A. Hemostasis abnormalities in cirrhosis. *Curr Opin Hematol*. 2015;22(406).
267. van Poucke S, Stevens K, Marcus AE. Hypothermia: effects on platelet function and hemostasis. *Thromb J*. 2014;12(31).
268. Michelson AD, Barnard MR, Khuri SF. The effects of aspirin and hypothermia on platelet function in vivo. *Br J Haematol*. 1999;104(64).
269. Kutcher ME, et al. Characterization of platelet dysfunction after trauma. *J Trauma Acute Care Surg*. 2012;73(1):13–19.
270. Vulliamy P, et al. Histone H4 induces platelet ballooning and microparticle release during trauma hemorrhage. *Proc Natl Acad Sci U S A*. 2019;116(35):17444–17449.
271. St John A, et al. Plasma proteomic profile associated with platelet dysfunction after trauma. *J Thromb Haemost*. 2021;19(7):1666–1675.
272. Glass NE, et al. Platelet dysfunction in patients with traumatic intracranial hemorrhage: Do desmopressin and platelet therapy help or harm? *Am J Surg*. 2022;223(1):131–136.
273. Yavari M, Becker RC. Coagulation and fibrinolytic protein kinetics in cardiopulmonary bypass. *J Thromb Thrombolysis*. 2009;27(95).
274. Espinosa A, Stenseth R, Videm V. Comparison of three point-of-care testing devices to detect hemostatic changes in adult elective cardiac surgery: a prospective observational study. *BMC Anesthesiol*. 2014;14(80).
275. Hathcock JJ. Flow effects on coagulation and thrombosis. *Arterioscler Thromb Vasc Biol*. 2006;26(1729).
276. Kestin AS, Valeri CR, Khuri SF. The platelet function defect of cardiopulmonary bypass. *Blood*. 1993;82(107).
277. van Poucke S, Stevens K, Wetzels R. Early platelet recovery following cardiac surgery with cardiopulmonary bypass. *Platelets*. 2016;1(2016).
278. George JN, Pickett EB, Saucerman S. Platelet surface glycoproteins: studies on resting and activated platelets and platelet membrane microparticles in normal subjects, and observations in patients during adult respiratory distress syndrome and cardiac surgery. *J Clin Invest*. 1986;78(340).
279. Baumann KL, Massicotte MP. Mechanical circulatory support: balancing bleeding and clotting in high-risk patients. *Hematology Am Soc Hematol Educ Program*. 2015;2015(61).
280. Bartoli CR, Restle DJ, Zhang DM. Pathologic von Willebrand factor degradation with a left ventricular assist device occurs via two distinct mechanisms: mechanical demolition and enzymatic cleavage. *J Thorac Cardiovasc Surg*. 2015;149(281).
281. Geisen U, et al. Platelet secretion defects and acquired von Willebrand syndrome in patients with ventricular assist devices. *J Am Heart Assoc*. 2018;7(2).
282. Manne BK, et al. Platelet gene expression and function in patients with COVID-19. *Blood*. 2020;136(11):1317–1329.
283. Comer SP, et al. COVID-19 induces a hyperactive phenotype in circulating platelets. *PLoS Biol*. 2021;19(2):e3001109.
284. Trugilho MRO, et al. Platelet proteome reveals novel pathways of platelet activation and platelet-mediated immunoregulation in dengue. *PLoS Pathog*. 2017;13(5):e1006385.
285. Laine O, et al. Hantavirus infection-induced thrombocytopenia triggers increased production but associates with impaired aggregation of platelets except for collagen. *Thromb Res*. 2015;136(6):1126–1132.
286. Connolly-Andersen AM, et al. Increased thrombopoiesis and platelet activation in hantavirus-infected patients. *J Infect Dis*. 2015;212(7):1061–1069.
287. Cummins D, et al. A plasma inhibitor of platelet aggregation in patients with Lassa fever. *Br J Haematol*. 1989;72(4):543–548.
288. Fisher-Hoch SP, et al. Haematological and biochemical monitoring of Ebola infection in rhesus monkeys: implications for patient management. *Lancet*. 1983;2(8358):1055–1058.
289. Tunjungputri RN, et al. Platelet dysfunction contributes to bleeding complications in patients with probable leptospirosis. *PLoS Negl Trop Dis*. 2017;11(9):e0005915.
290. Cortelazzo S, Viero P, Casarotto C. Bleeding on patients with autoimmune thrombocytopenic purpura and normal platelet count. *Scand J Haematol*. 1984;32(403).
291. Clancy R, Jenkins E, Firkin B. Qualitative platelet abnormalities in idiopathic thrombocytopenic purpura. *N Engl J Med*. 1972;286(622).
292. Heyns AD, Fraser J, Retief FP. Platelet aggregation in chronic idiopathic thrombocytopenic purpura. *J Clin Pathol*. 1978;31(1239).
293. Regan MG, Lackner H, Karpatkin S. Platelet function and coagulation profile in lupus erythematosus. Studies in 50 patients. *Ann Intern Med*. 1974;81(462).
294. Dorsch CA, Meyerhoff J. Mechanisms of abnormal platelet aggregation in systemic lupus erythematosus. *Arthritis Rheum*. 1982;25(966).
295. van Leeuwen EF, Leeksma OC, van Mourik JA. Effect of the binding of anti-Zwa antibodies on platelet function. *Vox Sang*. 1984;47(280).
296. Balduini CL, Bertolino G, Noris P. Defect of platelet aggregation and adhesion induced by autoantibodies against platelet glycoprotein IIIa. *Thromb Haemost*. 1992;68(208).
297. Balduini CL, Grignani G, Sinigaglia F. Severe platelet dysfunction in a patient with autoantibodies against membrane glycoproteins IIb–IIIa. *Haemostasis*. 1987;17(98).
298. Meyer M, Kirchmaier CM, Schirmer A. Acquired disorder of platelet function associated with autoantibodies against membrane glycoprotein IIb–IIIa complex–1. Glycoprotein analysis. *Thromb Haemost*. 1991;65(491).
299. Spangenberg P, Kirchmaier CM, Schirmer A. Functional studies on platelets of a patient with an acquired disorder of platelet function associated with autoantibodies against membrane glycoprotein IIB/IIIA complex. *Thromb Res*. 1993;69(435).
300. McMillan R, Tani P, Millard F. Platelet-associated and plasma anti-glycoprotein autoantibodies in chronic ITP. *Blood*. 1987;70(1040).
301. Tuffigo M, Lazaro E, James C. Successful use of recombinant factor VIIa in a patient with acquired Glanzmann thrombasthenia. *Haemophilia*. 2015;21:e116–e118.
302. Berchtold P, Muller D, Beardsley D. International study to compare antigen-specific methods used for the measurement of antiplatelet autoantibodies. *Br J Haematol*. 1997;96(477).
303. Devine DV, Currie MS, Rosse WF. Pseudo-Bernard-Soulier syndrome: thrombocytopenia caused by autoantibody to platelet glycoprotein Ib. *Blood*. 1987;70(428).

304. Nomura S, Yanabu M, Soga T. Analysis of idiopathic thrombocytopenic purpura patients with antiglycoprotein IIb/IIIa or Ib autoantibodies. *Acta Haematol*. 1991;86(25).
305. Chang M, Nakagawa PA, Williams SA. Immune thrombocytopenic purpura (ITP) plasma and purified ITP monoclonal autoantibodies inhibit megakaryocytopoiesis in vitro. *Blood*. 2003;102(887).
306. Takahashi R, Sekine N, Nakatake T. Influence of monoclonal antiplatelet glycoprotein antibodies on in vitro human megakaryocyte colony formation and proplatelet formation. *Blood*. 1999;93(1951).
307. Deckmyn H, Chew SL, Vermylen J. Lack of platelet response to collagen associated with an autoantibody against glycoprotein Ia: a novel cause of acquired qualitative platelet dysfunction. *Thromb Haemost*. 1990;64(74).
308. Beer JH, Rabaglio M, Berchtold P. Autoantibodies against the platelet glycoproteins (GP) IIb/IIIa, Ia/IIa, and IV and partial deficiency in GPIV in a patient with a bleeding disorder and a defective platelet collagen interaction. *Blood*. 1993;82(820).
309. Sugiyama T, Okuma M, Ushikubi F. A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia. *Blood*. 1987;69(1712).
310. Deckmyn H, van Houtte E, Vermylen J. Disturbed platelet aggregation to collagen associated with an antibody against an 85- to 90-Kd platelet glycoprotein in a patient with prolonged bleeding time. *Blood*. 1992;79(1466).
311. Moroi M, Jung SM, Okuma M. A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. *J Clin Invest*. 1989;84(1440).
312. Yanabu M, Nomura S, Fukuroi T. Platelet activation induced by an antiplatelet autoantibody against CD9 antigen and its inhibition by another autoantibody in immune thrombocytopenic purpura. *Br J Haematol*. 1993; 84(694).
313. Cortelazzo S, Casarotto C, D'Emilio A. Acquired storage pool deficiency with increased platelet-associated IgG in a patient with compensated chronic idiopathic thrombocytopenic purpura. *Haematologica*. 1984; 69(81).
314. Lee ACW. Unusual hematologic disease affecting Caucasian children traveling to Southeast Asia: acquired platelet dysfunction with eosinophilia. *Hematol Rep*. 2012;4:e5.
315. Yadav DD, Nayar PS, Manchanda RV. Acquired platelet dysfunction with eosinophilia (APDE) syndrome: a case report. *Indian J Hematol Blood Transfus*. 2016;32(235).
316. Shih MY, et al. Acquired platelet dysfunction with eosinophilia in two patients. *Pediatr Neonatol*. 2020;61(3):346–347.

CHAPTER 129

DISEASES OF PLATELET NUMBER: IMMUNE THROMBOCYTOPENIA, NEONATAL ALLOIMMUNE THROMBOCYTOPENIA, AND POSTTRANSFUSION PURPURA

Michelle P. Zeller, Shuoyan Ning, Donald M. Arnold, and Caroline Gabe

Platelets are anucleate cells that are required for primary hemostasis. A normal platelet count is 150,000 to 400,000 platelets per microliter of blood, with a normal lifespan of 7 to 10 days for circulating platelets. The body clears 10^{11} platelets per day by balancing complex proapoptotic and antiapoptotic mechanisms. Exogenous reagents and environmental changes can also impact the rate of platelet clearance[1] by the cells of the reticuloendothelial system (RES), including the spleen.

Platelet production is stimulated by thrombopoietin (TPO), a hormone that is constitutively secreted by the liver. TPO binds to c-Mpl, its receptor on platelets, hematopoietic progenitor cells, and bone marrow (BM) megakaryocytes. When bound to c-Mpl, TPO is internalized, degraded, and removed from the circulation; thus, when the platelet count is low, free TPO levels are high and more platelets are produced. In contrast, when platelet counts are high, circulating TPO levels are low and platelet production declines. This primitive feedback system is very effective at maintaining the platelet count at a stable level. Evidence in mice has shown that the Ashwell-Morell receptor on murine hepatocytes binds platelets that have lost sialic acid residues on their surface.[2] Binding activates a Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway, resulting in increased hepatic TPO messenger RNA (mRNA) expression and TPO production. Targeting this pathway may become a therapeutic approach for the treatment of thrombocytopenic disorders such as immune thrombocytopenia (ITP).

Immune-mediated platelet disorders disrupt normal regulation of platelet number because of antibody-mediated or cell-mediated platelet destruction or platelet underproduction. Antibodies that target self (autoimmune) or nonself (alloimmune) antigens on platelets can cause severe thrombocytopenia. ITP is an autoimmune disorder characterized by antibodies directed against platelet glycoproteins (GPs). Neonatal alloimmune thrombocytopenia (NAIT) is a thrombocytopenic syndrome caused by platelet alloantibodies. Posttransfusion purpura (PTP) has features of both alloantibody- and autoantibody-mediated processes. These platelet disorders have related immunologic features with distinct clinical characteristics (Table 129.1). The pathophysiology, clinical manifestations, and management of these disorders are discussed in this chapter.

IMMUNE THROMBOCYTOPENIA

ITP is a common autoimmune disease characterized by a platelet count less than 100×10^9/L that can be associated with an increased risk of bleeding. Increased platelet destruction resulting from platelet autoantibodies is a hallmark of ITP, but many patients with the clinical syndrome do not have detectable autoantibodies. In addition to platelet destruction, platelet underproduction is also a feature of ITP. Platelet underproduction may also be mediated by immune mechanisms that target megakaryocytes in the BM.[3] Conventional treatments, including corticosteroids, intravenous immunoglobulin (IVIg), immunosuppressant medications, and splenectomy, are aimed at reducing the autoimmune attack on platelets and/or megakaryocytes. Likewise, fostamatinib targets platelet destruction by inhibiting Syk (spleen tyrosine kinase), which is required for platelet phagocytosis. On the other hand, TPO receptor agonists (RAs) are megakaryocyte growth factors that work by increasing platelet production.

Epidemiology

The natural history of ITP differs in children and adults. In most children, ITP presents acutely and resolves within several weeks, often not requiring any intervention. Seasonal variability suggests that viral infections may trigger the disease in many children. Conversely, ITP in adults tends to be insidious in onset and is more often characterized by a chronic or remitting and relapsing course.

Incidence and Prevalence of Immune Thrombocytopenia

For children, the incidence of acute ITP is estimated at 1.9 to 6.4 per 100,000 per year.[4] Nearly 70% of childhood ITP occurs between the ages of 1 and 10 years, with the peak prevalence between 4 and 6 years. Most studies in children report an overall male predominance in early childhood and equalization or reversal to female predominance in older children.[5,6] For adults, incidence estimates range from 2 to 5 per 100,000 per year.[4–8] A retrospective analysis from the United Kingdom described a bimodal distribution for men, with peak incidences before the age of 18 years and between 75 and 84 years of age.[7] Relatively stable incidence rates were found in women up to the age of 60 years with a steady increase thereafter. The incidence of ITP has been reported to double in patients older than 60 years of age. The estimated overall prevalence of ITP in adults is 9.5 per 100,000, and it ranges from 4.1 per 100,000 in those between 19 and 24 years of age to 16 per 100,000 in those between 55 and 64 years of age.[5] There is a female predominance that is less apparent in older age groups.

Pathophysiology

ITP is caused by increased platelet destruction and impaired platelet production, and the principal mechanism is thought to be due to autoantibodies. The antibody hypothesis began with the observation that blood from patients with ITP was able to cause a reduction in the platelet count in healthy volunteers. In one of the first experiments, William Harrington infused blood from ITP patients into normal volunteers and observed a decrease in the platelet counts in most recipients.[9] The circulating factor responsible for this effect was first identified as platelet-associated immunoglobulin G (PAIgG). With the development of more specific assays, it was determined that the antibodies were directed against platelet GPs, specifically GPIIbIIIa and GPIbIX. GP-specific assays for the detection of autoantibodies exhibited improved specificity but had low sensitivity (50% to 66%), meaning that many ITP patients had a negative test and only ITP

TABLE 129.1 **Antibody-Mediated Thrombocytopenic Disorders Caused by Autoantibodies (Immune Thrombocytopenia), Alloantibodies (Neonatal Alloimmune Thrombocytopenia) or Potentially Both (Posttransfusion Purpura)**

	Immune Thrombocytopenia	Neonatal Alloimmune Thrombocytopenia	Posttransfusion Purpura
Immune reaction	Autoimmune	Alloimmune	Features of both alloimmunity and autoimmunity
Incidence	5 per 100,000 population	40 per 100,000 births (or 1 per 2500)	1 per 100,000 blood transfusions
Principal antigenic target	GPIIb/IIIa	HPA-1a	HPA-1a plus autoantigens
Nature of the antibody	Intermittent	Persistent (past 1 year)	Persistent often at high titers
Mode of sensitization	Autoantibody	Alloantibody	Features of alloantibodies and autoantibodies
Sensitizing event	Mostly unknown; some viral illnesses, chronic infection	Exposure to fetal platelet antigens early in first pregnancy	Blood transfusion (RBCs or platelets) 5–10 days earlier
Bleeding frequency	Uncommon	Common	Very common
Epidemiology	Higher incidence in children and elderly adults; female predominance in early adulthood	Majority affects fetus or newborn carrying the HPA-1a antigen	Almost all are HPA-1bb women sensitized by previous transfusion or pregnancy

GP, Glycoprotein; *HPA*, human platelet antigen; *RBC*, red blood cell.

patients had a positive test. Anti-GP autoantibodies target platelets for destruction in the RES by binding to the Fc-receptor on phagocytic cells, particularly the spleen. Peptides from phagocytosed platelets may be processed and presented to specific T cells, which in turn stimulate B cells to produce additional platelet autoantibodies. This process, known as epitope spreading, may explain why patients have circulating autoantibodies targeting a variety of platelet antigens. Antibody-induced platelet destruction in ITP can also be caused by complement activation and platelet apoptosis.

The low sensitivity of platelet antibody testing (anti-GPIIbIIIa or anti-GPIbIX) may be explained by the presence of autoantibodies against other target proteins (e.g., anti-GPV, which was found in 64.7% of patient samples in one cohort study[10]), low-avidity platelet antibodies, or the sequestration of autoantibodies in other body compartments. In a study of 18 patients with ITP, 10 (56%) had antiplatelet antibodies detectable in BM aspirate fluid, including 5 patient who did not have detectable circulating antibodies.[3] Similarly, a study of spleen tissue from patients with ITP identified antibody-secreting cells as the major splenic B-cell population in patients who were refractory to rituximab.[11]

In addition to accelerated platelet destruction, platelet production also may be impaired in ITP. Evidence supporting reduced platelet production derives from radiolabeled autologous platelet studies demonstrating normal or reduced platelet turnover[12] and from clinical studies that have consistently demonstrated the capacity of TPO-RAs to increase platelet counts in patients with severe thrombocytopenia. Megakaryocytes also express GP receptors, which render them targets of ITP autoantibodies. Indeed, in vitro studies demonstrated suppression of megakaryocyte growth and maturation when the cells were incubated with IgG from ITP patients.[13–15]

In addition to the effect of autoantibodies, cytotoxic T cells from ITP patients may have direct cytolytic effects on platelets. Some patients with active ITP but without detectable platelet autoantibodies had $CD8^+$ T cells that induced platelet lysis in vitro.[16,17] In contrast, $CD8^+$ T cells from patients in remission did not show significant platelet reactivity. In another study, despite demonstrating the increased cytotoxic potential of $CD8^+$ T cells in a sample of patients with ITP, no correlation was found between this cytotoxic potential and the presence or absence of platelet antibodies.[18] Furthermore, compared with cells from controls, $CD3^+$ cells from ITP patients exhibited increased expression of genes involved in cell-mediated cytotoxicity, including tumor necrosis factor-α (TNF-α), perforin, and granzyme A and B, and $CD8^+$ T cells exhibited increased expression of FasL (Fas–Fas ligand) and TNF-α.[17]

Another mechanism of thrombocytopenia in ITP is non–FcR-mediated platelet destruction. Platelet autoantibodies have been shown to cause desialylation of platelet GPs, leading to recognition and clearance of platelets in the liver by the Ashwell-Morell receptors.[2,19] Antibody-mediated desialylation may also impair platelet production.[2]

In the broadest sense, autoimmunity develops because of a breakdown in regulatory checkpoints that occurs during development or maturation of the immune system. Although the precise events that trigger the loss of self-tolerance to platelet GPs are largely unknown, patients with ITP have been shown to exhibit several immune alterations to platelet antigens including dysfunctional cellular immunity because of T helper (Th)0/Th1 polarization, decreased regulatory T-cell function, and autoreactive platelet-specific cytotoxic T cells. In addition, ITP patients may have increased circulating levels of cytokines and soluble factors that promote the survival of self-reactive T and B cells, including B-cell activating factor, a proliferation-inducing ligand, and B-cell lymphoma-2–interacting mediator of cell death. Reduced levels of proapoptotic cytokines that regulate self-reactive T cells, including Fas, interferon-γ, interleukin-2 receptor β (IL2RB), Bax, and caspases 8 and A20, have also been demonstrated.

Primary and Secondary Immune Thrombocytopenia

Primary ITP, previously called idiopathic thrombocytopenic purpura, occurs in the absence of an identifiable cause. Secondary ITP occurs because of an underlying cause such as infection, pregnancy, drugs, or lymphoproliferative disease (see box on Causes of Secondary ITP).

Infection may stimulate the formation of platelet-reactive autoantibodies. Cross-reactive antibodies (molecular mimicry) have been identified in *Helicobacter pylori*, human immunodeficiency virus (HIV), and hepatitis C virus (HCV) infections. Regarding *H. pylori*, a meta-analysis that included 788 patients showed that *H. pylori* eradication resulted in higher platelet counts in ITP patients compared with untreated or noneradicated controls.[20] Another systematic review evaluating 696 patients reported that 43% of treated patients achieved platelet counts greater than 100×10^9/L; however, the effect was highly dependent on geographic location, with the beneficial effect mainly observed in patients from Japan.[21] There is no clear guidance on when to screen for *H. pylori*. Guidelines from the American Society for Hematology[22,23] suggest that screening be considered for patients with ITP for whom eradication therapy would be

CAUSES OF SECONDARY IMMUNE THROMBOCYTOPENIA

Infections—HIV, HCV, *Helicobacter pylori*

Autoimmune disorders—SLE, APS, RA, Evan syndrome

Lymphoproliferative disorders

Drugs[a]—quinine, quinidine, rifampin, abciximab, vancomycin

[a] Examples of drugs that can induce ITP.

APS, Antiphospholipid syndrome; *HCV*, hepatitis C virus; *HIV*, human immunodeficiency virus; *ITP*, immune thrombocytopenia; *RA*, rheumatoid arthritis; *SLE*, systemic lupus erythematosus.

used. The most recent ITP consensus report recommends testing for *H. pylori* in adults with typical ITP, in those with digestive symptoms, and those from areas of high prevalence.[24]

ITP also may present in the setting of pregnancy. Mild ITP may be difficult to differentiate from incidental thrombocytopenia of pregnancy, and pregnancy-related vascular disorders, such as preeclampsia, microangiopathy caused by HELLP syndrome (characterized by hemolysis, elevated liver enzymes, and a low platelet count), and acute fatty liver must be excluded. In these pregnancy-related disorders, platelet counts tend to be mildly reduced and hypertension is common. Incidental thrombocytopenia of pregnancy (also called *gestational thrombocytopenia*) represents a physiologic change in platelet count that occurs late in pregnancy and is associated with a mild reduction in platelet count, which does not respond to immune-modulating therapy. In contrast, pregnancy-associated ITP may present at any time during pregnancy, and the thrombocytopenia can range from mild to severe. Typically, platelet counts increase with ITP-specific therapies such as IVIg or corticosteroids. Platelet count thresholds for instituting treatment are the same as those for women with ITP who are not pregnant (e.g., platelet count less than 20×10^9/L). Vaginal deliveries are thought to be safe for mothers with ITP, even if the platelet count is very low, and most clinicians try to maintain the count greater than 20 to 30×10^9/L. A higher platelet count is generally recommended for caesarean section delivery (50×10^9/L) and epidural anesthesia (70 to 80×10^9/L). Although evidence is lacking, IVIg and corticosteroids are generally safe in pregnancy, but corticosteroids can be associated with hypertension, gestational diabetes, intrauterine growth restriction, and other pregnancy-associated morbidities. Splenectomy is rarely performed during pregnancy because most women can successfully be managed with less-aggressive treatments. Immunosuppressant medications, such as azathioprine, have been used in pregnancy but should be reserved for refractory pregnancy-associated ITP with bleeding. Rituximab is not recommended during pregnancy, although it is likely to be safe. Rituximab may interfere with fetal and neonatal B-cell development, which may lead to increased susceptibility to infections. TPO-RAs may be considered in exceptional circumstances during pregnancy if other treatments have failed and the risk of severe bleeding is high. For mothers with active ITP or even with ITP that is in remission, there is a 10% risk of thrombocytopenia in the newborn because of passive transfer of maternal antiplatelet autoantibodies.

Clinical and Laboratory Features of Immune Thrombocytopenia

Thrombocytopenia

Thrombocytopenia is the defining feature of ITP. The international working group on standardization of terminology in ITP established a platelet count less than 100×10^9/L as the cutoff for the diagnosis (Table 129.2).[25] Primary ITP remains a diagnosis of exclusion because there is no specific test to confirm the diagnosis. Investigations for patients with thrombocytopenia are used to rule out nonimmune causes, including pseudothrombocytopenia, myelodysplastic syndromes, thrombotic microangiopathies, liver disease, splenomegaly, certain drugs, ethanol abuse, or hereditary thrombocytopenia; and secondary causes of ITP such as infection, concomitant autoimmune disease, or lymphoproliferative disorders. Data from a Canadian ITP Registry showed that one in seven patients suspected of having primary ITP was misdiagnosed during their disease.[26] Response to ITP-specific treatment (e.g., high-dose IVIg or high-dose corticosteroids) is generally considered a good indicator of immune-mediated thrombocytopenia.

TABLE 129.2 **Standardized Terminology and Definitions for Immune Thrombocytopenia Proposed by the International Working Group (Vicenza Consensus Conference) in 2009 and ASH Guideline 2019**

Terminology	Definition
ITP	Immune thrombocytopenia (rather than idiopathic or immune thrombocytopenic purpura)
Platelet threshold for ITP diagnosis	$<100 \times 10^9$/L
Primary ITP	ITP with no associated cause (diagnosis of exclusion)
Secondary ITP	ITP in the setting of an underlying cause (see box on Causes of Secondary ITP)
Newly diagnosed ITP	Designation for patients at diagnosis (rather than "acute" ITP) up to 3 months of the diagnosis
Persistent ITP	Sustained or recurrent thrombocytopenia lasting 3–12 months
Chronic ITP	Thrombocytopenia lasting >12 months
Complete response	Achievement of a platelet count of $\geq 100 \times 10^9$/L in the absence of bleeding
Response	Achievement of a platelet count of $\geq 30 \times 10^9$/L and at least doubling baseline in the absence of bleeding
Early response	Achievement of a platelet count $\geq 30 \times 10^9$/L and at least doubling baseline at 1 week
Initial response	Achievement of a platelet count $\geq 30 \times 10^9$/L and at least doubling baseline at 1 month
Durable response	Achievement of a platelet count $\geq 30 \times 10^9$/L and at least doubling baseline at 6 months
Corticosteroid-dependent	Ongoing need for continuous prednisone >5 mg/day (or corticosteroid equivalent) or frequent courses of corticosteroids to maintain a platelet count $\geq 30 \times 10^9$/L and/or to avoid bleeding
Refractory ITP	Failure to achieve a response or relapse after splenectomy[a] and requirement for treatment(s) to minimize the risk of clinically significant bleeding
Remission	Platelet count $>100 \times 10^9$/L at 12 months

[a] Splenectomy failure may not be applicable in children.

ITP, Immune thrombocytopenia.

Clinical Features

The clinical presentation of ITP can range from asymptomatic thrombocytopenia to severe or life-threatening bleeding. When bleeding occurs, bleeding symptoms characteristic of ITP ("platelet-type bleeding") include skin bleeding (i.e., bruises, nonpalpable purpura, or petechiae), oral hemorrhagic blood blisters or oral petechiae, epistaxis, menorrhagia, or gastrointestinal bleeding manifested usually by melena. In addition to oral purpura, hematuria may be an important warning sign for major bleed, as demonstrated in a recent retrospective cohort study.[27] The most severe complication of ITP is intracerebral hemorrhage (ICH).

Investigations of Patients With Suspected Immune Thrombocytopenia

Patients presenting with newly identified thrombocytopenia require a careful history and physical examination to uncover the underlying cause of the thrombocytopenia and to assess the risk of bleeding. If splenomegaly is present on physical examination, the thrombocytopenia may be caused by hypersplenism, and imaging is warranted. A panel of laboratory tests are needed to identify possible causes of the thrombocytopenia (see box on Laboratory Basic Evaluation for Patients With Suspected ITP). A complete blood count and review of the blood film are required to exclude other hematologic conditions, such as hereditary forms of thrombocytopenia (e.g., May-Hegglin) or pseudothrombocytopenia (Fig. 129.1). HIV and HCV testing should be performed in patients with suspected ITP, and *H. pylori* testing should be considered in patients from certain countries (e.g., Japan). Hepatitis B testing at baseline is reasonable if immunosuppressive treatments such as rituximab are being considered and to exclude the possibility of false-positive anti–hepatitis B core antibody testing after IVIg.[28] There are insufficient data to support routine screening for antinuclear antibodies or antiphospholipid antibodies unless other signs and symptoms of systemic lupus erythematosus or antiphospholipid syndrome are present. BM aspiration and biopsy should be done for patients with abnormalities in other cell lines such as anemia, leukopenia, or macrocytosis; however, most patients with typical ITP do not require BM examination. Quantitative Ig levels may be useful in children to exclude common variable immune deficiency. Thyroid testing can uncover subclinical hypothyroidism or hyperthyroidism that can lead to mild thrombocytopenia. Coagulation tests such as

LABORATORY BASIC EVALUATION FOR PATIENTS WITH SUSPECTED IMMUNE THROMBOCYTOPENIA

Tests of Potential Utility for Patients With Suspected ITP

ASH Guideline 2019	International Consensus Report 2019	Other Tests That May be Required
Complete blood count and reticulocyte count	Complete blood count and reticulocyte count	*Helicobacter pylori*[a]
Peripheral blood film	Peripheral blood film	Bone marrow examination[b]
HIV	HIV	
HCV	HCV	
Further test according to history and CBC	HBV	
	Quantitative Ig level measurement	
	Blood group (Rh)	

[a]For patients with ITP in certain geographical regions (e.g., Asia)
[b]Could be appropriate in those relapsing after remission, in patients not responding to initial treatment options, or if other abnormalities are detected in the blood count or morphology.
ASH, American Society of Hematology; *CBC*, Complete blood count; *HCV*, hepatitis C virus; *HIV*, human immunodeficiency virus; *Ig*, immunoglobulin; *ITP*, immune thrombocytopenia.

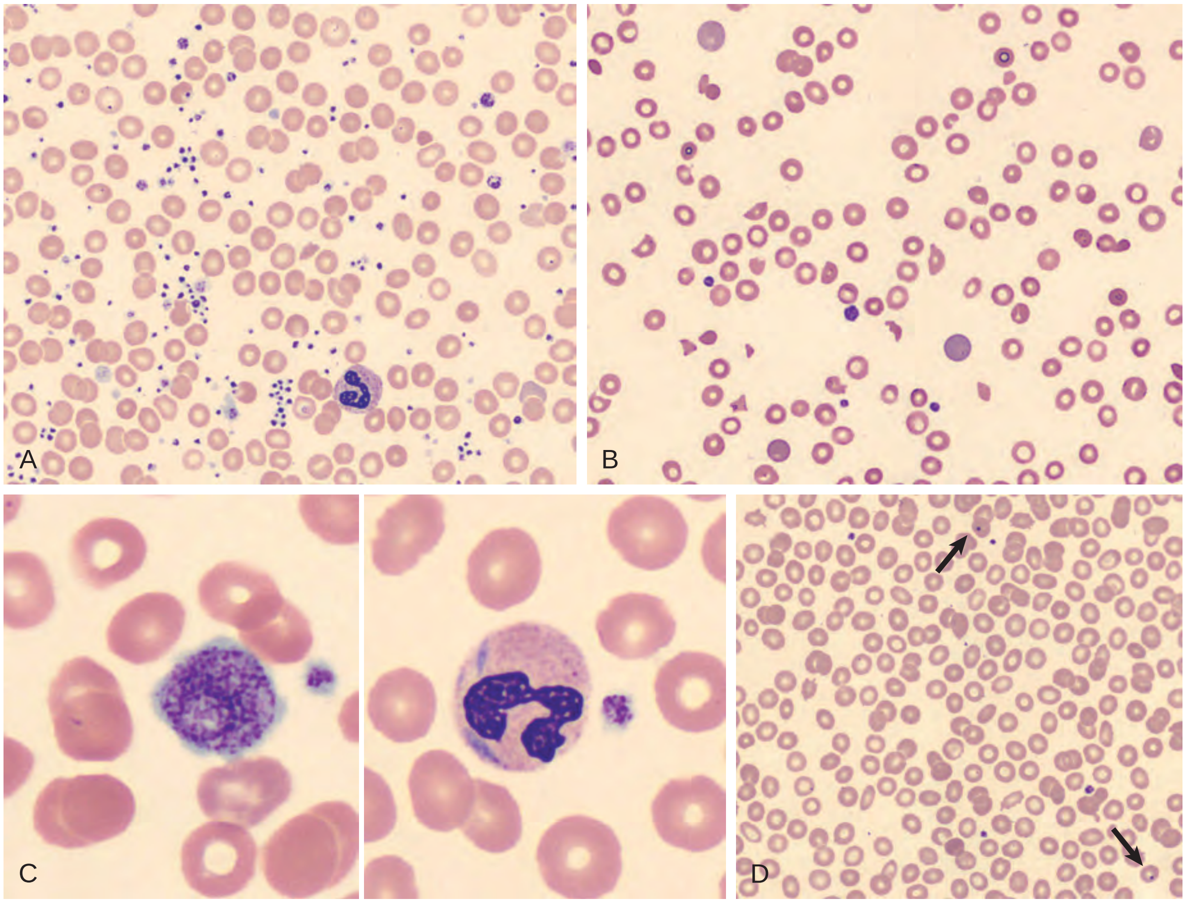


Figure 129.1 BLOOD FILM EXAMINATIONS FROM PATIENTS WITH THROMBOCYTOPENIA. (A) Pseudothrombocytopenia caused by EDTA (ethylenediaminetetraacetic acid) showing marked platelet clumping. (B) Schistocytes (fragmented red blood cells) and reticulocytosis in a patient with thrombotic thrombocytopenic purpura. (C) Macrothrombocyte *(left panel)* and neutrophil-containing cytoplasmic inclusions (Döhle bodies, *right panel*) in a patient with May-Hegglin anomaly. (D) A patient with immune thrombocytopenia with low platelets and postsplenectomy Howell-Jolly bodies *(arrows)*.

D-dimer, partial thromboplastin time (PTT), and fibrinogen may be helpful in excluding chronic disseminated intravascular coagulation in patients with malignancy (see Chapter 126). Antiplatelet antibody tests are rarely used in routine clinical practice.

Prognosis

Clinical Outcomes: Mortality, Bleeding, and Quality of Life

Chronic ITP has been associated with a risk of death that is up to four times higher than that in the general population. ITP patients are more likely to die of bleeding, infection, and hematologic malignancies. In a systematic review of prospective studies, the incidence of ICH was 1.4% for adults (95% confidence interval [CI], 0.9 to 2.1) and 0.4% for children (95% CI, 0.2 to 0.7).[29] The proportion of patients with severe (non-ICH) bleeding was 10% for adults (95% CI, 4.1 to 17.1) and 20% for children (95% CI, 10.0 to 32.9). Risk factors for severe bleeding include severe thrombocytopenia, previous bleeding, and older age. Another recent study also identifies male sex and heavily pretreated ITP as potential risk factors for major bleeding.[27] Some deaths are attributable to adverse effects of treatment rather than the disease. Quality of life is affected, at least in part, because of the prevalence of fatigue that appears to be independent of platelet count levels.

Treatment

Most patients with platelet counts greater than 30 × 10^9/L and without bleeding can be managed safely with observation alone. This is especially true for children with ITP who are at low risk of serious bleeding. Furthermore, in up to 80% of cases, childhood ITP resolves within 6 months with no treatment. If patients present with bleeding, such as epistaxis or mucosal hemorrhage, treatment is required. For adults, a period of observation is reasonable if there is no evidence of bleeding and the platelet count is stable and greater than 30 × 10^9/L, because some patients will have spontaneous remission.[30] The American Society of Hematology (ASH) 2019 guidelines recommend treatment for newly diagnosed ITP patients who are asymptomatic or have minor mucocutaneous bleeding with a platelet count less than 30 × 10^9/L.

Treatment Options

Corticosteroids with or without IVIg are first-line treatments for patients with newly diagnosed ITP. Treatment options for second-line therapies include rituximab, splenectomy, TPO-RAs, fostamatinib, and immune suppressant medications. We recommend an approach that uses the least toxic treatments with the aim of resolving bleeding or preventing severe bleeding and that aligns with patient preferences to optimize health-related quality of life (Fig. 129.2, and see Box on Treatment Options for ITP and box on First-Line Therapy).

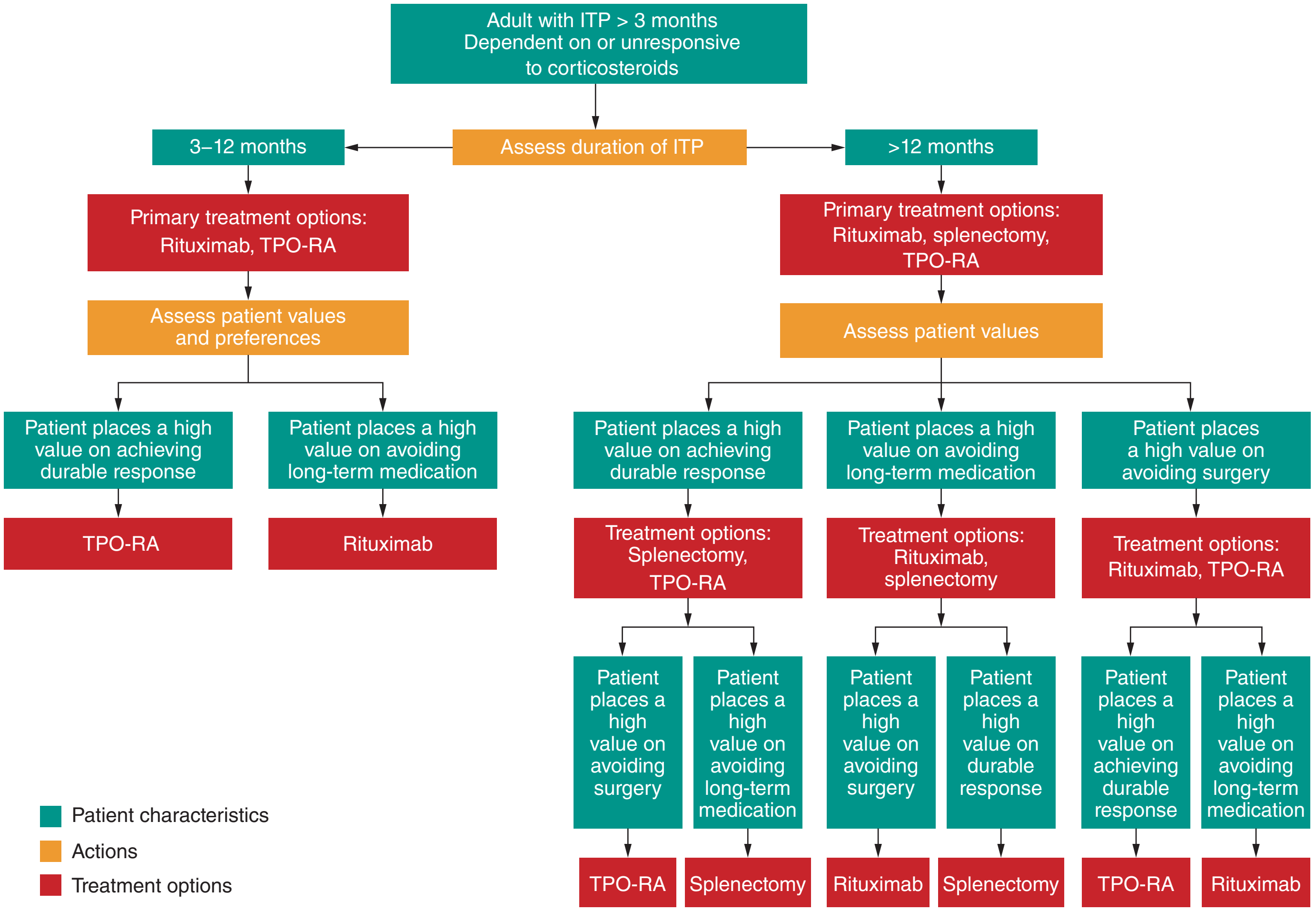

Adult with ITP > 3 months
Dependent on or unresponsive to corticosteroids
Assess duration of ITP
3–12 months
>12 months
Primary treatment options: Rituximab, TPO-RA
Assess patient values and preferences
Patient places a high value on achieving durable response
Patient places a high value on avoiding long-term medication
TPO-RA
Rituximab
Primary treatment options: Rituximab, splenectomy, TPO-RA
Assess patient values
Patient places a high value on achieving durable response
Patient places a high value on avoiding long-term medication
Patient places a high value on avoiding surgery
Treatment options: Splenectomy, TPO-RA
Treatment options: Rituximab, splenectomy
Treatment options: Rituximab, TPO-RA
Patient places a high value on avoiding surgery
Patient places a high value on avoiding long-term medication
Patient places a high value on avoiding surgery
Patient places a high value on durable response
Patient places a high value on achieving durable response
Patient places a high value on avoiding long-term medication
TPO-RA
Splenectomy
Rituximab
Splenectomy
TPO-RA
Rituximab
Patient characteristics
Actions
Treatment options

Figure 129.2 Selection of second-line therapy in adults with immune thrombocytopenia (ITP) should be individualized based on duration of disease and patient values and preferences. Other factors that may influence treatment decisions include frequency of bleeding sufficient to require hospitalization or rescue medication, comorbidities, compliance, medical and social support networks, cost, and availability of treatments. Patient education and shared decision-making is encouraged. *TPO-RA*, Thrombopoietin receptor agonist. *(From Neunert C, Terrell DR, Arnold DM, et al. American Society of Hematology 2019 guidelines for immune thrombocytopenia. Blood Adv. 2019;3(23):3829-3866.)*

TREATMENT OPTIONS FOR IMMUNE THROMBOCYTOPENIA

First Line

IVIg

Corticosteroids

Anti-D

Second Line

Rituximab

TPO agonists

Splenectomy

Fostamatinib

Immunosuppressive/immunomodulatory medications (mycophenolate, cyclosporine, azathioprine and others)

ITP, Immune thrombocytopenia; *IVIg*, intravenous immunoglobulin; *TPO*, thrombopoietin.

FIRST-LINE THERAPY

An 8-year-old girl is brought to the emergency department because her mother noticed bruising on her legs. She has had a sore throat and fever for the past 7 days but is otherwise well and not taking any medications. On physical examination, there are a few small bruises on her legs but no petechiae on her skin or purpura in her mouth. Neurologic examination findings are normal, and the spleen is not palpable. The platelet count is 23 × 10^9/L. The presumed diagnosis is ITP without significant bleeding; thus the decision is made to observe the child in the hospital with no specific treatment. The next day, the platelet count is 33 × 10^9/L. On day 2, it is 37 × 10^9/L, and the child is discharged home. One week later, the platelet count is 66 × 10^9/L, and 1 month later, it is up to 155 × 10^9/L.

Corticosteroids

The conventional starting dose is predniso(lo)ne at 0.5 to 2 mg/kg (maximum dose 80 mg, even in patients weighing more than 80 kg) for 2 weeks, to a maximum of 3 weeks; or dexamethasone 40 mg/day for 4 days, repeated monthly for up to three cycles. In general, 60% to 70% of adults with acute ITP achieve an initial response with corticosteroids. If a response occurs (e.g., platelet count imp >50 × 10^9/L), the predniso(lo)ne dose should be tapered, aiming to stop by 6 weeks. The course of corticosteroids should be tapered and stopped even if the platelet count drops during the period of drug treatment to avoid prolonged exposure to corticosteroids. If there is no response to the initial dose within 2 weeks, the predniso(lo)ne should be tapered rapidly over 1 week and stopped to limit the side effects. Sustained platelet count responses (platelet count >100 × 10^9/L at 6 months) with corticosteroids are infrequent in practice but have been reported to be as high as 47% in some studies. The risk of relapse increases with longer duration of follow-up. Low-dose prednisone (0.5 mg/kg/day followed by a taper) may be as effective as the conventional dose for initial ITP treatment, but long-term remissions are rare.

High-dose dexamethasone is also effective, and in one study that included 125 adults with ITP, approximately 40% of patients had a sustained response that lasted 2 to 5 years.[31] Repeated cycles of high-dose dexamethasone (once per month for 6 months) may result in even higher rates of durable remissions, although this effect may simply reflect the total corticosteroid exposure. High-dose dexamethasone is associated with side effects including hypertension, muscle weakness, insomnia, and impaired cognition. In a systematic review of randomized trials comparing high-dose dexamethasone and prednisone in adults with previously untreated ITP ($n = 533$), treatment with dexamethasone resulted in improved overall (79% vs. 59%; $P = 0.048$) and complete platelet count responses (64% vs. 36%; $P = 0.040$) without excess toxicity.[32] Platelet count responses occurred more rapidly with high-dose dexamethasone than with prednisone (within 14 days); however, sustained responses at 6 months were similar between the groups.

Intravenous Immunoglobulin and Rh-Immune Globulin (Anti-D)

The predominant mechanism of action of high-dose IVIg and anti-D is thought to be via RES blockade. Individuals with low plasma IgG levels exhibit more rapid clearance of sensitized red blood cells (RBCs) (indicating enhanced RES capacity) than those with high levels of plasma IgG, such as those achieved with high-dose IVIg. A competitive model of RES clearance would also explain why anti-D administration to Rhesus (Rh)-positive individuals is effective in improving platelet counts in patients with ITP. Thus IgG-sensitized RBCs compete for Fc receptor occupancy. Other potential mechanisms of action of IVIg or anti-D include antiidiotypic antibodies, stimulation of proinflammatory and antiinflammatory cytokines, upregulation or downregulation of various Fc receptors, and the induction of soluble immune complexes. In a mouse model of ITP, transfer of IVIg-primed dendritic cells recapitulated the effect of IVIg.

IVIg (1 g/kg on 1 or 2 consecutive days or 0.4 g/kg/day for 5 days) or intravenous (IV) anti-D (50 to 75 mg/kg once), where available, may be appropriate options for patients with bleeding or at high risk for bleeding, for those who require a surgical procedure (as the response is usually achieved in a few days), or for those who are unresponsive to corticosteroids. Based on the results of a meta-analysis of randomized controlled trials that included 410 children, the probability of achieving a platelet count greater than 20 × 10^9/L at 48 hours was higher with IVIg than with corticosteroids (relative risk for corticosteroids, 0.74; 95% CI, 0.65 to 0.85).[33] Similar results were observed in adults. Common side effects of IVIg include headache, hypertension, and chills. Hemolysis, thrombosis, renal impairment, and neutropenia are rare complications.

Anti-D and IVIg have similar efficacy in children. Hemolysis is expected with anti-D administration, and rarely, intravascular hemolysis can be severe or even fatal. Consequently, the US Food and Drug Administration (FDA) has issued a black box warning about the use of anti-D for the treatment of ITP, and the drug has been removed from certain European markets. In general, the use of anti-D is restricted to patients who have not undergone splenectomy, are Rh-positive, and have a negative direct antiglobulin test.

Second-Line Therapy

Randomized trials comparing different treatment options for second-line therapy for ITP are lacking; therefore the decision as to which therapy to choose depends on the goals of therapy (remission vs. maintenance), patient characteristics, patient preference, access, and costs. Despite advances in medical care and new pharmacologic treatment options, splenectomy remains the treatment that is most likely to induce a durable remission; however, the use of splenectomy has declined in recent years. TPO-RAs including romiplostim, eltrombopag, and avatrombopag; rituximab; and fostamatinib are reasonable second line treatments for ITP.

Splenectomy

In a systematic review, approximately two-thirds of patients achieved a platelet count response after splenectomy, usually within days.[34] Despite the high success rate with splenectomy, patients (and physicians) are often reluctant to undertake an invasive procedure such as splenectomy when pharmacologic alternatives are available. Only younger age has been identified as a predictor of splenectomy success, although some investigators have found a correlation between prior response to IVIg and a splenic uptake on radiolabeled platelet sequestration studies.[35]

With currently available minimally invasive surgical techniques, complications after splenectomy are uncommon. The overall mortality rate is approximately 1% with open surgery and about 0.2% after laparoscopic splenectomy. The most frequent perioperative complications include pneumonia, subphrenic abscess or pleural effusion

(4%), major bleeding (1.5%), and thromboembolism (1%). With laparoscopic techniques, patients have less postoperative pain, shorter hospital stays, and fewer wound complications.

Because the spleen is involved in clearance of encapsulated bacteria, asplenic individuals are at risk for infection with *Streptococcus pneumoniae, Neisseria meningitides*, and *Haemophilus influenzae* type b. Therefore all patients undergoing splenectomy should receive vaccinations against these bacteria at least 2 weeks before surgery. Poor compliance and vaccine failures contribute to the ongoing risk of serious postsplenectomy infections. The lifetime risk of overwhelming postsplenectomy infection is estimated to be 1% to 3%, with the risk being higher in children younger than 15 years of age and in patients with hematologic malignancies. Although the risk of an infection requiring hospitalization was highest in the first 90 days after splenectomy in a cohort of 3812 patients in Denmark,[36] the risk remained 2.5 times higher than that in the general population even after 90 days. Because approximately 20% of patients can achieve a remission within the first year of the ITP diagnosis, it is prudent to wait at least 12 months before splenectomy.

Splenectomy can lead to a high rate of durable remission. In a systematic review, 1731 of 2623 adults (66%) with ITP achieved a complete response after splenectomy at a median follow-up of 28 months (range 1 to 153 months), and this response rate was maintained for 10 years or more.[34]

Disadvantages of splenectomy include a lack of validated predictors of response, surgical risk with 30-day mortality and complication rates of 0.2% and 9.6% for laparoscopic splenectomy, respectively, and 1.0% and 12.9% for open splenectomy, respectively, and an increased risk of postsplenectomy infection, and vascular thrombosis.[34] In the past decade since the introduction of new pharmacologic options including the TPO-RAs, the use of splenectomy has continued to decline.

Rituximab

Rituximab has been widely used in patients with various autoimmune diseases, including ITP. Rituximab is an anti-CD20 monoclonal antibody that targets and destroys CD20$^+$ B lymphocytes, some of which are likely involved in autoantibody production. Data correlating cellular profiles with clinical outcomes suggest that the efficacy of rituximab reflects improvement in T-cell function and reversion of T-cell abnormalities; processes downstream to its direct effect on B-cell depletion.

In a systematic review of 19 observational studies that enrolled 313 ITP patients of whom 46% had not undergone splenectomy, rates of complete response (platelet count $>150 \times 10^9$/L) and overall response (platelet count $>50 \times 10^9$/L) with rituximab after a median follow-up of 9.5 months were 44% (95% CI, 30 to 58) and 63% (95% CI, 53 to 73), respectively.[37] The typical rituximab regimen was 375 mg/m^2 administered by IV infusion once weekly for 4 consecutive weeks. The median time to response was 5.5 weeks, with some responses occurring much later (up to 8 to 12 weeks), and a median duration of response of 10.5 months. Several studies have shown that patients who are earlier in their disease course (<12 months), female patients, younger patients (<40 years), and patients with underlying autoimmune conditions may be more likely to achieve a complete response after rituximab.[38] Other observational studies have reported lower rates of durable remission, ranging from 24% at 12 months to 35% at 57 months. A meta-analysis of five randomized trials demonstrated that complete platelet count response was more frequent with rituximab plus standard of care than with standard of care alone (relative risk 1.4, 95% CI, 1.1 to 1.8); however, there was limited evidence for sustained platelet count responses beyond 6 to 12 months.[39]

In a prospective observational study that included 60 adults with ITP who had not undergone splenectomy and who had a median of two prior therapies, 24 (40%) achieved a platelet count greater than 50×10^9/L and at least twice their baseline value at 1 year, and 20 (33%) maintained their platelet count response at 2 years with rituximab treatment.[40] In a follow-up study of 72 adults and 66 children with chronic ITP who achieved an initial response to rituximab, 21% to 26% maintained a treatment-free response for at least 5 years.[41] Low-dose rituximab (100 mg/week for 4 weeks) has been shown to be effective in ITP, but the frequency of durable responses is uncertain.

Minor infusion-related side effects of rituximab occur in approximately 30% of patients with ITP and include hypotension, rash, sore throat, fever, and rigors. Severe or fatal infusion reactions are rare in patients treated for autoimmune diseases. Serum sickness, which is characterized by arthropathy, fever, and low serum complement levels, may be more common in children than in adults and often necessitates treatment interruption. Progressive multifocal leukoencephalopathy (PML) is a rapidly fatal neurologic syndrome caused by reactivation of latent JC virus in the brain. Rare reports have linked PML with rituximab treatment.

Rituximab also interferes with the response to polysaccharide vaccines. This is of potential concern in patients who may subsequently undergo splenectomy and supports the practice of administering immunizations before initiating rituximab therapy. Rituximab has a role for patients with ITP who wish to avoid long-term therapy and the surgical risks of splenectomy; however, the durability of response may be less than that with the TPO-RAs or splenectomy.

Thrombopoietin Receptor Agonists

Drugs aimed at increasing platelet production by stimulating the c-Mpl receptor have been investigated for the treatment of thrombocytopenia. Initial studies with pegylated recombinant human megakaryocyte growth and development factor were halted because of the development of cross-reactive antibodies against endogenous TPO, which led to severe and sustained thrombocytopenia in some healthy volunteers. Although research with these specific agents was halted, the findings prompted the development of second-generation TPO-RAs that have no homology to endogenous TPO. Two TPO-RAs were approved for the treatment of patients with chronic ITP in 2008: romiplostim and eltrombopag. Since then, their use has increased progressively. In 2019 avatrombopag, another TPO-RA, was licensed for use in patient with chronic ITP. For this class of medications, the effect on platelet count is generally sustained if the TPO-RA is continued. When they are stopped, platelet counts tend to fall rapidly back to baseline levels; however, in recent studies, it has been reported that 10% to 30% of patients achieve a durable response after treatment discontinuation.[42,43]

Romiplostim (administered as a once-weekly subcutaneous injection) is a synthetic peptibody consisting of four peptides linked to an IgG Fc fragment. The molecule binds the c-Mpl receptor at the same location as endogenous TPO and stimulates megakaryocyte proliferation and platelet production through intracellular JAK/STAT and mitogen-activated protein kinase signaling. In a phase III trial, a durable platelet count response (defined as the achievement of a platelet count of 50×10^9/L or higher for 6 or more of the last 8 weeks of treatment) was achieved in 41 of 83 patients (49%) receiving romiplostim compared with 1 of 42 (2%) patients receiving placebo.[44] In a subsequent randomized trial that compared romiplostim plus standard of care with standard of care alone, romiplostim was associated with more platelet count responses, fewer treatment failures, fewer splenectomies, less bleeding, and better quality of life.[45] Weekly doses of romiplostim ranged from 1 to 10 μg/kg; however, most patients achieved a suitable response with a dose of 3 μg/kg. Weekly doses were titrated up or down to maintain the platelet count in the appropriate range (30 to 100×10^9/L).

Eltrombopag (administered as an oral daily tablet, 50 to 75 mg daily, >2 hours before or 4 hours after products containing polyvalent cations) is a small molecule, nonpeptide TPO-RA. Eltrombopag also activates the c-Mpl receptor, but unlike romiplostim, eltrombopag binds to the transmembrane domain of the receptor and does not compete with circulating TPO for binding. In a phase III trial, eltrombopag was associated with an eightfold increase in platelet count response compared with placebo throughout the 6-month treatment period.[46] The time to response was 1 to 2 weeks (like romiplostim), and there was minimal need for dose titration. Durable responses were achieved in 57 of 95 patients (60%) receiving maintenance eltrombopag and in only 4 of 39 patients (10%) receiving placebo.

Avatrombopag is another TPO-RA licensed for the treatment of chronic ITP in adults that binds to the transmembrane region of the TPO receptor and activates signal transduction pathways leading to increased platelet production, like eltrombopag. Unlike eltrombopag, avatrombopag does not interact with food or cations and has not been associated with liver enzyme elevations. A phase III study comparing avatrombopag with placebo in patients with chronic ITP demonstrated the safety and efficacy of avatrombopag, with a platelet response rate of 66% versus 0%, respectively.[47]

TPO-RAs are generally well tolerated but have been associated with headache, fatigue, and insomnia. The development of BM reticulin in patients with ITP was an early concern; however, this problem was rarely encountered in prospective studies and the changes improved with discontinuation of the medication. TPO-RAs have also been associated with thromboembolic events (independent of platelet count), with a rate of 7% for patients receiving romiplostin and 4% for eltrombopag. This risk may be somewhat higher than the baseline risk of thromboembolism in patients with ITP. Eltrombopag has been associated with serum liver function test abnormalities in approximately 10% of patients. Treatment-related serious adverse events were infrequent even after prolonged exposure to romiplostim ($n = 292$)[48] or eltrombopag ($n = 299$).[49]

A recent randomized trial evaluated the use of eltromobopag versus IVIg as treatments for ITP during the perioperative period ($n = 76$).[50] Eltrombopag was not inferior to IVIg and is therefore a reasonable alternative for raising the platelet count before surgery and during the immediate postoperative period. One patient had a treatment-related pulmonary embolism after eltrombopag.

Fostamatinib

Fostamatinib is a splenic tyrosine kinase inhibitor approved in 2018 by the FDA for treatment of chronic ITP. In a randomized trial of second-line therapy for patients with ITP for a median duration of ITP of 8.5 years, many of whom had failed splenectomy, TPO agents, and/or rituximab, fostamatinib was associated with a stable response (platelets $\geq 50 \times 10^9$/L for 4 of 6 weeks) in 18% of patients and on overall response (platelets $\geq 50 \times 10^9$/L at any time) in 43% of patients. The initial dose of fostamatinib was 100 mg twice daily. Response occurred in a median of 15 days. Diarrhea and hypertension were the most frequently reported side effects.[51,52]

Immune Suppressant Medications

Immune suppressant medications, such as mycophenolate, azathioprine, and cyclosporine, have variable response (varying from 25% to 70%), usually of short duration and delayed time to response (4 to 8 weeks). This group of medications is often reserved for chronic refractory ITP patients following TPO-RAs. However, a recent randomized trial comparing corticosteroids to a combination of mycophenolate mofetil and corticosteroids suggested that the addition of mycophenolate may result in a better response and lower risk of relapse or refractory disease, despite a decrease in quality of life.[53] Immune suppressant medications can sometime be more effective when given in combination as show in a retrospective study of 19 patients treated with the combination of azathioprine, mycophenolate mofetil, and cyclosporine. Overall response in that group of patients was 74%, and median time to response was 2 months (interquartile range, 1.3 to 4.5).[54]

Treatment of Refractory Immune Thrombocytopenia

As suggested by the International Working Group on standardization of terminology in ITP, the term *refractory ITP* is used to define patients who have failed splenectomy or relapsed thereafter and either exhibit severe thrombocytopenia or have a risk of bleeding that necessitates therapy (see Table 129.2).[25]

Evidence to help guide management of patients with chronic refractory ITP after splenectomy is limited, and treatment has been largely unsatisfactory. The overarching principle of therapy for this population is to prevent bleeding with the achievement of a stable, although not necessarily normal, platelet count. Combination therapy may be more effective than single agent treatment for achievement of this goal.

In randomized trials, patients with ITP who had not undergone splenectomy tended to show better platelet count responses to TPO-RAs than those who had; however, the difference was small, and response rates in patients who had undergone splenectomy approached 50%. Although some patients included in the trials had failed up to five prior therapies, the results may not be applicable to all patients with refractory ITP.

Before the availability of TPO-RAs, a systematic review identified rituximab, azathioprine, and cyclophosphamide as the agents most often associated with complete responses in patients with refractory ITP.[55] Good response rates have also been reported with a combination of cyclosporine, azathioprine, and mycophenolate, as mentioned earlier. Similarly, a combination of IVIg, IV methylprednisolone, vincristine, or IV anti-D followed by maintenance therapy with danazol and azathioprine has shown good response rates.[56] Other treatment options include low-dose or alternate-day corticosteroids, repeated doses of IVIg, high-dose chemotherapy, dapsone, and even the combination of romiplostin and eltrombopag. High-dose chemotherapy followed by stem cell transplantation has also been used successfully in this population, but with the advent of TPO-RAs, transplantation is no longer used.

COVID-19 and ITP

The coronavirus (SARS-Cov-2) pandemic raised several challenges for investigation of thrombocytopenia and management of ITP. Although thrombocytopenia may be present in up to 36% of patients hospitalized with COVID-19,[57] it is usually mild and only a minority of patients will present significant thrombocytopenia, which may be due to numerous potential causes. A retrospective study showed a very low incidence of ITP secondary to COVID-19 (0.34%) usually presenting after the first week of symptoms and with an excellent response to standard treatment (corticosteroids and IVIg).[58]

In the outpatient setting, corticosteroids have been discouraged as a treatment for ITP because of the potential risk for new or worsening COVID-19 infection. A practical guidance statement from the British Society of Haematology favored the use of TPO-RAs over immune-suppressive agents and suggested minimizing the dose and duration of corticosteroids.[59]

Rare thrombocytopenic syndromes have been described with the COVID-19 vaccines. Vaccine-induced immune thrombotic thrombocytopenia is a well-characterized immune-mediated thrombotic disorder caused by anti-PF4 antibodies that develop after exposure to the COVID-19 adenoviral vector vaccines.[60] A separate syndrome is vaccine-associated ITP, which has been reported after any of the COVID-19 vaccines and has a clinical presentation that is similar to primary ITP.[61] The frequency of vaccine-associated ITP has been estimated to occur be 1.13 per 100,000 doses,[62] but underreporting is likely. Patients with preexisting ITP have an increased risk of worsening thrombocytopenia after COVID-19 vaccination. In a prospective analysis of 52 patients with chronic ITP, 12% of patients had a drop in platelet count post vaccination, which was independent of remission status, concurrent ITP treatment, or vaccine type. All patients responded to rescue therapy with corticosteroids with or without IVIg.[63]

NEONATAL ALLOIMMUNE THROMBOCYTOPENIA

NAIT, also referred to as fetal and neonatal alloimmune thrombocytopenia (FNAIT), is an uncommon but serious fetal and neonatal thrombocytopenic disorder. Severe thrombocytopenia can lead to bleeding in utero or, during the neonatal period, resulting in death or significant morbidity. The incidence of NAIT has been estimated to range from 1:1000 to 1:10,000 births, although underdiagnosis is common. NAIT is an important condition to accurately recognize because treatment may prevent recurrences in any subsequent pregnancies.

Clinical Presentation

Thrombocytopenia is the key feature of NAIT. Thrombocytopenia may be profound in fetuses and neonates affected by NAIT, and in severe cases, the platelet count may be less than 10 to 20 × 10^9/L shortly after birth. In the neonatal setting, thrombocytopenia typically occurs in an otherwise healthy newborn without other causes for thrombocytopenia, outlined in Table 129.3, although the presence of other causes of thrombocytopenia does not exclude a diagnosis of NAIT. Thrombocytopenia will often worsen hours to days after delivery, likely reflecting increased RES function in the newborn, particularly within the lungs. Without treatment, neonatal thrombocytopenia may last for days, but it can be severe and persist for weeks before counts recover once the antiplatelet antibody levels decline. Clinical features depend on the severity of the thrombocytopenia. Bleeding symptoms range from petechiae and bruising to gastrointestinal, pulmonary, or retinal bleeding. Intracranial hemorrhage (ICH) is the most feared complication of NAIT; occurring in 10% to 20% of patients, ICH can occur in utero or after birth and carries significant mortality and morbidity. Predictors of ICH include a history of a sibling with ICH, severe thrombocytopenia, and children born to multigravidas.

Pathophysiology

In NAIT, fetal platelets contain a platelet antigen inherited from the father which the mother lacks. This prompts maternal IgG antibody formation against fetal/paternal platelet-specific antigens. Fetal and neonatal thrombocytopenia in NAIT thus reflects the clearance of IgG-sensitized fetal platelets by maternal alloantibodies; maternal IgG antibodies cross the placenta, bind fetal platelets, and cause clearance of the antibody-bound platelets by the RES, resulting in fetal thrombocytopenia. The syndrome can be considered analogous to the destruction of fetal RBCs in hemolytic disease of the fetus and newborn (HDFN) with several differences. First, NAIT often presents in a first pregnancy, possibly due to early maternal sensitization to paternally derived antigens expressed on fetal platelets. In contrast, it is uncommon for HDFN to occur in a first pregnancy without previous sensitization to paternally derived fetal RBC antigens. Second, pregnant women at risk for HDFN (e.g., with RBC antibodies) can be identified by routine antenatal antibody screens. Furthermore, anti-RhD immune globulin can be given prepartum and postpartum to RhD-negative women to reduce the risk of RhD sensitization. To date, screening programs for NAIT have not been widely implemented because women at risk are not readily identifiable before sensitization has occurred and therapies to prevent maternal alloimmunization are not available at this time (see box on Neonatal Alloimmune Thrombocytopenia Versus Hemolytic Disease of the Fetus and Newborn).

TABLE 129.3 Differential Diagnosis of Thrombocytopenia in Newborns

- **Perinatal Hypoxemia**
- **Placental Insufficiency**
- **Congenital Infection**
  - Sepsis
  - Toxoplasmosis
  - Rubella
  - Cytomegalovirus
- **Autoimmune**
  - Maternal immune thrombocytopenia
  - Maternal systemic lupus erythematosus
- **Disseminated Intravascular Coagulation**
- **Maternal Drug Exposure**
- **Congenital Heart Disease**
- **Hereditary Thrombocytopenia**
  - MYH9 macrothrombocytopenia (including May-Hegglin anomaly)
  - Thrombocytopenia absent radii syndrome
  - Amegakaryocytic thrombocytopenia
  - Wiskott-Aldrich syndrome
  - Fanconi anemia
- **Hemangioma With Thrombocytopenia**
  - Kasabach-Merritt syndrome
- **Bone Marrow Infiltration**
  - Congenital leukemia

Laboratory Investigation of Suspected Neonatal Alloimmune Thrombocytopenia

The diagnosis of NAIT is established by documenting the presence of platelet-specific antigen incompatibility between mother and infant (or mother and father) and the presence of maternal antiplatelet alloantibodies directed against the incompatible human platelet antigen (HPA) (Fig. 129.3).[64] Diagnosis first requires allele-specific genotyping using polymerase chain reaction technology to identify a maternal-fetal (or maternal-paternal) antigenic mismatch. In the prenatal setting where fetal HPA status is unclear (e.g., paternal testing is unavailable, or if the father is heterozygous for the implicated antigen), chorionic villous sampling or amniocentesis may be pursued to establish fetal HPA status but are associated with procedure-related risks, including miscarriage. Noninvasive approaches using maternal cell-free DNA to establish fetal HPA status can also be used but may not be widely available.[65]

Serologic confirmation for the complete array of maternal alloantibodies is more difficult for two reasons. First, the technology is complex, and relatively few laboratories perform these tests. In general, most commercial assays detect platelet alloantibodies directed against only a limited number of antigens. This limitation necessitates the use of more specific assays, such as monoclonal antibody immobilization of platelet antigen assays (MAIPAs) or antigen capture assays; however, even these tests are limited by the shortage of monoclonal antibodies required to capture the different target proteins that express the alloantigens (e.g., HPA-15 on CD109). An alternative method uses radioimmunoprecipitation (RIP), which can detect all the known alloantibodies described to date. Second, up to 25% of HPA-1a–negative women with NAIT have no detectable antibodies using currently available laboratory methods. More recent studies have used surface plasmon resonance to identify alloantibodies in women suspected of having NAIT but who tested negative in conventional immunoassays. Low-affinity anti–HPA-1a antibodies were

NEONATAL ALLOIMMUNE THROMBOCYTOPENIA VERSUS HEMOLYTIC DISEASE OF THE FETUS AND NEWBORN

NAIT can be viewed as the platelet equivalent of HDFN with some important differences: (1) maternal sensitization by fetal platelet antigens can occur early in the first trimester; (2) NAIT can affect first pregnancies; (3) women at risk for NAIT are not easily identifiable before sensitization and thus are not amenable to universal screening programs; and, (4) specific therapies to prevent platelet antigen sensitization are lacking (unlike HDFN due to anti-D, where Rh-immune globulin is available to prevent alloimmunization).

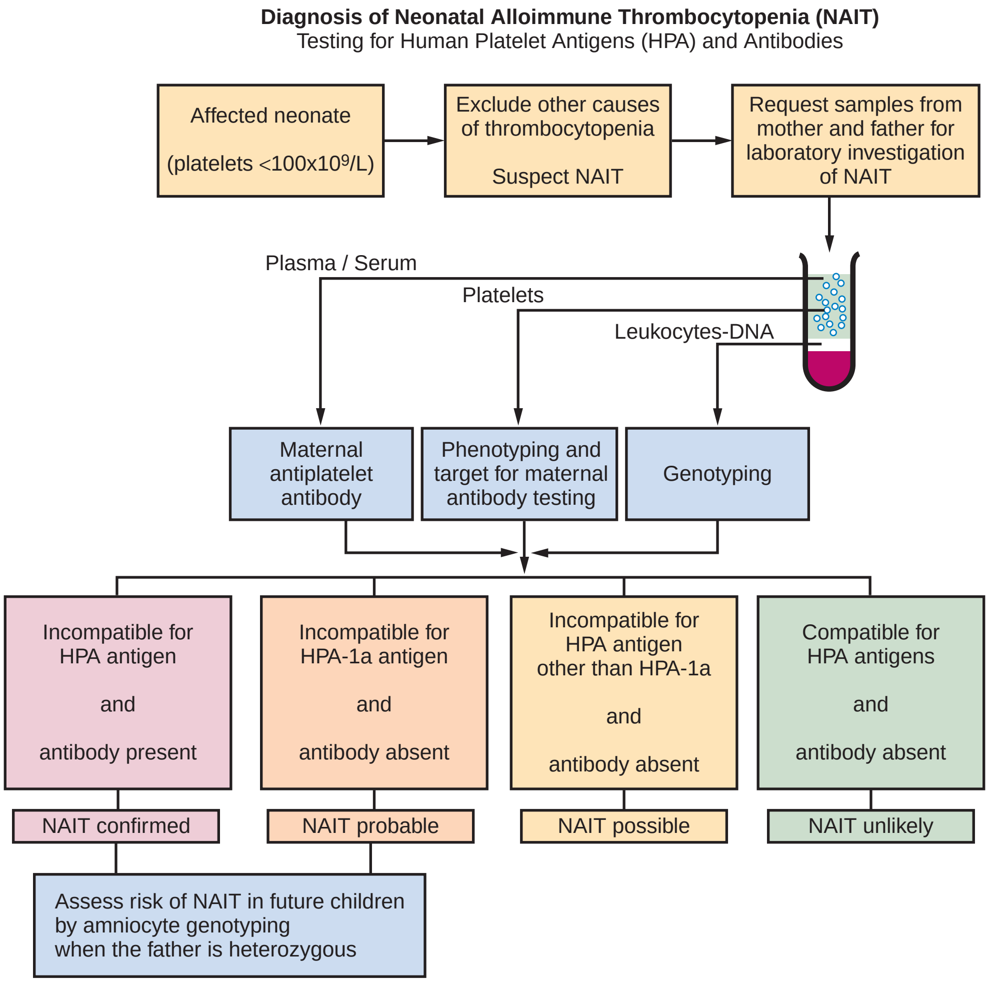

Diagnosis of Neonatal Alloimmune Thrombocytopenia (NAIT)
Testing for Human Platelet Antigens (HPA) and Antibodies
Affected neonate
(platelets <100x10⁹/L)
Exclude other causes of thrombocytopenia
Suspect NAIT
Request samples from mother and father for laboratory investigation of NAIT
Plasma / Serum
Platelets
Leukocytes-DNA
Maternal antiplatelet antibody
Phenotyping and target for maternal antibody testing
Genotyping
Incompatible for HPA antigen and antibody present
Incompatible for HPA-1a antigen and antibody absent
Incompatible for HPA antigen other than HPA-1a and antibody absent
Compatible for HPA antigens and antibody absent
NAIT confirmed
NAIT probable
NAIT possible
NAIT unlikely
Assess risk of NAIT in future children by amniocyte genotyping when the father is heterozygous

Figure 129.3 DIAGNOSTIC TESTING ALGORITHM FOR INVESTIGATION OF NEONATAL ALLOIMMUNE THROMBOCYTOPENIA AND MANAGEMENT RECOMMENDATIONS BASED ON RESULTS OF TESTING. This algorithm is currently used by the McMaster University Platelet Immunology Reference Laboratory, 2017. Maternal blood samples are tested for platelet antigens (phenotyping and polymerase chain reaction genotyping) and platelet alloantibodies. Amniocentesis and fetal genotyping are recommended when the father is known to be heterozygous for the incompatible antigen. *HPA*, Human platelet antigen; *NAIT*, neonatal alloimmune thrombocytopenia. *(Modified from Arnold DM, Smith JW, Kelton JG. Diagnosis and management of neonatal alloimmune thrombocytopenia. Transfus Med Rev. 2008;22:255, with permission.)*

detected in some, suggesting that standard immunoassays may be limited in their capacity to detect such antibodies.[66] In addition, low-incidence platelet-specific alloantigens expressed on platelets only from the paternal lineage may account for fetomaternal incompatibility. Detection of alloantibodies in these cases requires that maternal serum be tested against paternal platelets whenever possible.

The most common platelet alloantigen implicated in NAIT is HPA-1a. This important alloantigen is defined by a leucine (HPA-1a) to proline (HPA-1b) substitution at amino acid 33 on GPIIIa. Maternal incompatibility to HPA-1a is implicated in more than 80% of patients with NAIT. These women lack the common HPA-1a antigen (i.e., their genotype is HPA-1bb), and during pregnancy they are immunized with fetal HPA-1a antigen inherited from the father. Other commonly implicated antigens are HPA-5a5b on GPIa/IIa and HPA-15a15b on the glycosylphosphatidylinositol-anchored protein CD109. Only 6 of the 28 platelet antigen systems have been defined by maternal alloantibodies against both alleles; these include the HPA 1, 2, 3, 4, 5, and 15 systems. There are several other low-frequency alleles, the majority of which are expressed on platelet GPIIb/IIIa and are usually found within a single family. Although maternal immunization to low-frequency antigens is implicated in some cases of NAIT, these antigens account for a minority of the NAIT cases that remain unresolved after investigation for common HPA antigens.[67] Frequently, discrepancies in human leukocyte antigen (HLA) and ABO, which are also expressed on platelets, are found during the course of investigations for NAIT; however, their significance is uncertain. A database of genetically confirmed alloantigens is maintained by the European Bioinformatics Institute (http://www.ebi.ac.uk).

Management

Postnatal Management of Infants After Delivery

When NAIT is suspected, treatment should be initiated before confirmatory test results are available if thrombocytopenia is severe (platelet count <30 × 10^9/L) or if there is evidence of bleeding. It is important to appreciate that thrombocytopenia at birth can worsen over the next few days among patients with NAIT. Platelet transfusions can be administered to increase neonatal platelet counts. HPA alloantigen-compatible platelets are more effective than random donor platelets in achieving higher posttransfusion platelet counts, but if HPA antigen-selected platelets are unavailable, random donor platelets can still be administered because they frequently produce adequate platelet count increments.[68] High-dose IVIg (1 to 2 g/kg) may be considered as an adjunct to platelet transfusion. Current guidelines recommend that all neonates suspected of NAIT undergo routine cranial ultrasound within 24 hours of delivery.

Antenatal Management of Mothers in Subsequent Pregnancies

Women with a previously affected infant with NAIT are at high risk of having another affected infant, and thus careful management during subsequent pregnancies is required. Paternal HPA genotyping should be performed for the antigen(s) implicated in previous NAIT case(s), and in cases of paternal antigen heterozygosity or if antigen status is unknown/unavailable, fetal antigenic testing can be performed by amniocentesis or cell-free DNA to determine if the fetus is at risk and whether treatment is required.

Antenatal treatment options for at-risk mothers during subsequent pregnancies range from careful observation, IVIg with or without corticosteroids, to fetal blood sampling (FBS) and intrauterine platelet transfusion (IUPT). Invasive strategies such as FBS and IUPT are associated with heightened rates of complications, including fetal death and premature labor without benefits in clinical outcomes[69]; therefore a noninvasive approach is recommended. Mainstay antenatal therapy for women with a previously affected infant is high-dose IVIg (1 to 2 g/kg) administered weekly throughout pregnancy. The optimal timing at which antenatal IVIg should start is 12 to 16 weeks for women with a child affected by fetal/neonatal ICH and between 20 and 22 weeks for women without an ICH affected fetus/neonate.[70] A systematic review summarizing the results of four randomized trials and 22 observational studies found no consistent evidence supporting the addition of corticosteroids.[69] Corticosteroids (prednisone) are given at some centers in combination with IVIg to mothers at high risk, such as those with a previously affected infant with ICH or severe thrombocytopenia, or if the response to IVIg is suboptimal. Dexamethasone has an associated risk with oligohydramnios and is not recommended in current guidelines.[70]

Antenatal Fetal Monitoring of At-Risk Fetuses

Serial ultrasonography is indicated for fetal surveillance. Ultrasound provides a simple, noninvasive method for identifying fetal bleeds at early stages. FBS by percutaneous cannulation of the umbilical or intrahepatic vein with or without IUPT was associated with an 11% rate of complications in a systematic review (54 complications in 497 treated pregnancies), with 26% leading to fetal loss.[69] Furthermore, a platelet transfusion protocol based on the detection of fetal thrombocytopenia would necessitate frequent FBS procedures because of the short (7-day) lifespan of transfused platelets. As such, routine FBS with or without IUPT is not recommended. Among HPA-1a immunized women, serial measurement of anti-HPA-1a antibody levels may be useful to predict the degree of fetal thrombocytopenia.[71]

Mode of Delivery

There is no evidence that planned cesarean section is safer than uncomplicated vaginal delivery for infants with NAIT. A planned delivery is important to ensure that personnel and resources, including antigen-compatible platelet transfusions, are readily available. Invasive fetal procedures (scalp blood sampling and electrodes) or assisted deliveries (forceps, vacuum) should be avoided if the fetal platelet count is unknown.

Population Screening for Neonatal Alloimmune Thrombocytopenia

Universal NAIT screening programs for all pregnant women are challenging because of the difficulty in early identification of at-risk women, the low prevalence of NAIT, and the lack of specific and proven treatments for the prevention of alloimmunization. Screening algorithms also must account for the large difference between the number of women lacking an antigen and the number who develop NAIT; about 2% of women will be identified as HPA-1bb, whereas only 1 in 20 of these will have an affected child because of HPA-1a immunization.[72] As such, universal screening programs are not currently available, but there are ongoing research initiatives targeting the development of prophylactic therapies to prevent HPA-1a alloimmunization.[73] However, sisters of women with a history of NAIT should undergo screening because they are likely to possess similar HPA genotypes. HLA DRB3*01:01 typing may be useful because its absence in non–HPA-1a mothers without an affected fetus/neonate and without HPA-1a antibodies confers a low risk of alloimmunization.[74]

POSTTRANSFUSION PURPURA

PTP is a rare thrombocytopenic syndrome that is provoked by an immune-mediated reaction against HPAs, most frequently HPA-1a. PTP presents 5 to 10 days following exposure to platelets or platelet antigenic material in blood transfusions with profound thrombocytopenia and clinically significant bleeding.

Epidemiology

PTP is rare with an unknown incidence; however, estimates place incidence around 1 to 2 per 100,000 transfusions. Data from the Serious Hazards of Transfusion (SHOT) surveillance program from the United Kingdom reveal decreased incidence of PTP over the past decade (Fig. 129.4).[75] This decrease may reflect universal leukoreduction, which was implemented in the United Kingdom in 1999. The average annual incidences of PTP in the years preceding 1996–99 and following the implementation of universal leukoreduction between 2000 and 2005 were 10.3 and 2.3 cases per year, respectively. Since the implementation of universal leukoreduction, there has also been a shift from RBCs to platelets as the inciting transfusion event, which may be attributable to the reduction in platelet contamination of red cell products with leukoreduction methods. One study estimated that the frequency of PTP corresponds to 1.8 per 100,000 transfusions.[76] In this study, platelet containing transfusions posed a significantly higher risk than RBC transfusions.

The majority of PTP cases are reported in women, the majority of whom had a history of pregnancy; 52/53 confirmed cases of PTP were women according to SHOT data. In a report of 61 patients, the sensitizing event among women was pregnancy alone in 58%, pregnancy and/or transfusion in 34%, and transfusion alone in 7.5%. Males had associated transfusion histories in 55%, with some having no known exposure. PTP has not been reported in children.

Clinical Presentation

PTP presents with severe thrombocytopenia (platelets $<10 \times 10^9$/L) and bleeding, which may include petechiae, purpura, mucosal hemorrhage, hematuria, and rarely ICH. The platelet drop can occur over 24 hours. The thrombocytopenia is often refractory to platelet transfusions even with antigen-negative platelets. Mortality because of severe thrombocytopenia and bleeding has been estimated to be 5% to 20%. The thrombocytopenia occurs 5 to 10 days after blood transfusion and typically resolves within weeks but occasionally can be prolonged and severe and persist for months. Other causes of thrombocytopenia with similar clinical presentation include primary ITP, drug-induced ITP, sepsis, disseminated intravascular coagulation, and thrombotic thrombocytopenic purpura; however, association with transfusion and bleeding severity suggests a diagnosis of PTP. Patients with PTP may present with features that overlap with heparin-induced thrombocytopenia (HIT), and positive HIT testing in the context of anti–HPA antibodies has been reported; however, thrombocytopenia is

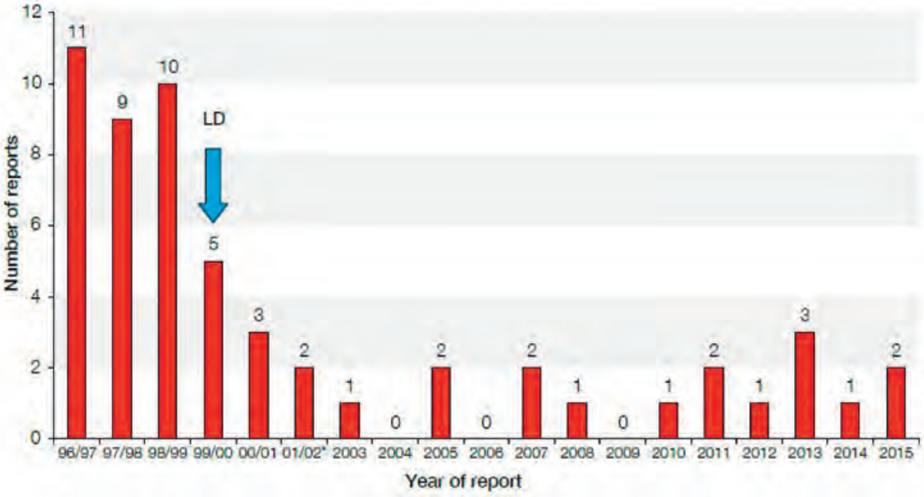


Figure 129.4 CONFIRMED CASES OF POSTTRANSFUSION PURPURA REPORTED BY THE UK SERIOUS HAZARDS OF TRANSFUSION (SHOT) SURVEILLANCE PROGRAM. Universal leukoreduction began in the United Kingdom in late 1999 and coincided with a decrease in new cases of posttransfusion purpura. *(From* www.shotuk.org.*)*

CLINICAL PRESENTATION OF POSTTRANSFUSION PURPURA

On postoperative day 8 after spinal surgery, a 63-year-old woman has a platelet count of 3×10^9/L. During the operation, she received 3 units of non–leuko-reduced packed RBCs because of intraoperative bleeding. She is receiving intravenous ampicillin and dalteparin thromboprophylaxis. On physical examination, she has extensive oral mucosal purpura and petechiae on both lower extremities. HIT testing results (anti-PF4/heparin enzyme-linked immunosorbent assay and serotonin release assay) are negative (see Chapter 130). The ampicillin is stopped, and she is treated with IVIg (2 g/kg) and platelet transfusions. Three days later, the platelet count is 4×10^9/L, and the patient develops melena. The presumed diagnosis is posttransfusion purpura; therefore HPA-1a–negative platelets and high-dose parenteral corticosteroids are administered, and daily plasma exchange is initiated. One week later, the thrombocytopenia and bleeding symptoms resolve. Platelet antibody testing reveals the presence of anti–HPA-1a antibodies.

typically less severe with HIT, and bleeding is rare (see Chapter 131). (See box on Clinical Presentation of Posttransfusion Purpura.)

Diagnosis

Diagnosis of PTP is confirmed by presence of platelet-specific alloantibodies. Most patients with PTP lack the common HPA-1a platelet antigen and are homozygous for HPA-1b. These patients develop anti-HPA 1a antibodies from sensitizing events such as previous pregnancies or transfusions. Anti–HPA 1a antibodies may persist for many years in these patients. In addition, platelet antibodies with specificities other than HPA-1a may also be found.[77] Direct and indirect methods for identifying platelet-specific antibodies can be used.[78]

Pathophysiology

PTP is mediated by an anamnestic antibody response. Most patients who develop PTP were exposed to a sensitizing event against platelet antigens that are absent on the patient's platelets. PTP occurs after reexposure to the same antigen when present in transfused blood products. The most common scenario involves a middle-aged or older multiparous woman who is HPA 1b-1b.[78]

Platelets contain abundant amounts of GPIIb/IIIa (see Chapter 123); thus even small numbers of platelets or platelet microparticles contained within RBC concentrates can be immunogenic and can lead to the development of PTP. Genotypic analyses have shown that HLA-II alleles DRβ3*0101 and DQβ1*0201 are commonly associated with this disorder. Similar to NAIT, the frequency of immunization depends on platelet alloantigen discrepancy plus the presence of certain immune response genes.

The most intriguing feature of PTP is that the patient's own antigen-compatible platelets are destroyed. Furthermore, platelet reactive antibodies can be eluted from both antigen-positive and antigen-negative platelets. Although the mechanism is still poorly understood, theories to explain this "innocent bystander" phenomenon include immune complex formation, passive antigen adsorption, and autoantibody formation.

Immune complexes can form if the anti–HPA-1a antibodies bind soluble antigen. Alternatively, platelet alloantigens contained within the transfused blood product may be passively adsorbed onto autologous platelets, converting them from antigen negative to antigen positive and rendering them targets for immune destruction. Immune complexes may then bind to platelets through Fc receptors, causing platelet destruction. Anti–HPA-1a antibodies have been shown to induce platelet activation through release of platelet-derived RANTES (regulated on activation, normal, T-cell expressed and secreted), a proinflammatory and immunomodulatory chemokine involved in multiple immunologic processes, including Ig synthesis and regulation of Th1/Th2 cytokine homeostasis. In some patients with PTP, the alloantibodies have been shown to interfere with cell-fibrinogen interaction, thereby increasing the risk of bleeding.

The stimulation of specific anti–HPA alloantibodies could in turn initiate the formation of platelet-reactive *autoantibodies*. Production of pan-reactive antibodies has been shown to correspond with the period of greatest thrombocytopenia. Serologic analyses of PTP cases demonstrated the presence of reactive IgG and IgM antibodies against GPIIb/IIIa, GPIX, and GPIa/IIa; however, only HPA-specific IgG antibodies persisted.[79] The presence of autoantibodies can help to explain the destruction of both donor and recipient platelets.

Management

Treatment of PTP should be initiated even before serologic test results for anti–HPA-antigens are available. The primary goal of treatment is to abbreviate the period of severe thrombocytopenia and minimize the risk of bleeding. Multiple treatments are often administered simultaneously or in rapid succession because of potential severity of sequelae.

Patients with PTP require admission to hospital for close observation, supportive treatments, and rapid management of bleeding symptoms should they occur. Nonessential transfusions should be discontinued. The mainstay of therapy is high-dose IVIg, which is followed by a platelet count increase after 3 to 4 days.[80] High-dose corticosteroids have also been tried as a means of inhibiting RES phagocytosis and reducing IgG synthesis. Patients with bleeding should be transfused with HPA-compatible platelets. While awaiting the results of antigen typing, HPA-1a–negative platelet transfusions can be administered to reduce further antibody production. Patients with PTP may be at increased risk of transfusion reactions such as fever, dyspnea, and allergic reactions. Plasma exchange should be considered in patients who do not respond to IVIg and corticosteroids.

A review of the literature supported use of IVIg (1B) and plasma exchange (2B) in management of PTP; and use of leukocyte reduction (2A), avoidance of unnecessary transfusion (2C), and if transfusion is required, HPA-compatible or washed cellular products (2C)[81] as preventative measures.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Arnold DM, Heddle NM, Cook RJ, et al. Perioperative oral eltrombopag versus intravenous immunoglobulin in patients with immune thrombocytopenia: a non-inferiority, multicentre, randomised trial. *Lancet Haematol*. 2020; 7(9):e640–e648.

Arnold DM, Nazi I, Santos A, et al. Combination immunosuppressant therapy for patients with chronic refractory immune thrombocytopenic purpura. *Blood*. 2010;115(1):29–31.

Arnold DM, Smith JW, Kelton JG. Diagnosis and management of neonatal alloimmune thrombocytopenia. *Transfus Med Rev*. 2008;22(4):255–267.

Bakchoul T, Bertrand G, Krautwurst A, et al. The implementation of surface plasmon resonance technique in monitoring pregnancies with expected fetal and neonatal alloimmune thrombocytopenia. *Transfusion*. 2013;53(9): 2078–2085.

Baker JM, Shehata N, Bussel J, et al. Postnatal intervention for the treatment of FNAIT: a systematic review. *J Perinatol*. 2019;39(10):1329–1339.

Cheng G, Saleh MN, Marcher C, et al. Eltrombopag for management of chronic immune thrombocytopenia (RAISE): a 6-month, randomised, phase 3 study. *Lancet*. 2011;377(9763):393–402.

Delaney M, Wendel S, Bercovitz RS, et al. Transfusion reactions: prevention, diagnosis, and treatment. *Lancet*. 2016;388(10061):2825–2836.

Ghevaert C, Rankin A, Huiskes E, et al. Alloantibodies against low-frequency human platelet antigens do not account for a significant proportion of cases of fetomaternal alloimmune thrombocytopenia: evidence from 1054 cases. *Transfusion*. 2009;49(10):2084–2089.

Hawkins J, Aster RH, Curtis BR. Post-transfusion purpura: current perspectives. *J Blood Med*. 2019;10:405–415.

Kamphuis MM, Paridaans N, Porcelijn L, et al. Screening in pregnancy for fetal or neonatal alloimmune thrombocytopenia: systematic review. *BJOG*. 2010;117(11):1335–1343.

Kjær M, Bertrand G, Bakchoul T, et al. Maternal HPA-1a antibody level and its role in predicting the severity of fetal/neonatal alloimmune thrombocytopenia: a systematic review. *Vox Sang*. 2019;114(1):79–94.

Kjær M, Geisen C, Akkök ÇA, et al. Strategies to develop a prophylaxis for the prevention of HPA-1a immunization and fetal and neonatal alloimmune thrombocytopenia. *Transfus Apher Sci*. 2020;59(1):102712.

Kuter DJ, Bussel JB, Lyons RM, et al. Efficacy of romiplostim in patients with chronic immune thrombocytopenic purpura: a double-blind randomised controlled trial. *Lancet*. 2008;371(9610):395–403.

Lieberman L, Greinacher A, Murphy MF, et al. Fetal and neonatal alloimmune thrombocytopenia: recommendations for evidence-based practice, an international approach. *Br J Haematol*. 2019;185(3):549–562.

Menis M, Forshee RA, Anderson SA, et al. Posttransfusion purpura occurrence and potential risk factors among the inpatient US elderly, as recorded in large Medicare databases during 2011 through 2012. *Transfusion*. 2015; 55(2):284–295.

Mithoowani S, Gregory-Miller K, Goy J, et al. High-dose dexamethasone compared with prednisone for previously untreated primary immune thrombocytopenia: a systematic review and meta-analysis. *Lancet Haematol*. 2016;3(10):e489–e496.

Mueller-Eckhardt C, Kiefel V. High-dose IgG for post-transfusion purpura-revisited. *Blut*. 1988;57(4):163–167.

Neunert C, Terrell DR, Arnold DM, et al. American Society of Hematology 2019 guidelines for immune thrombocytopenia [Erratum in *Blood Adv*. 2020;4(2):252]. *Blood Adv*. 2019;3(23):3829–3866.

Ouzegdouh Mammasse Y, Chenet C, Drubay D, et al. A new efficient tool for non-invasive diagnosis of fetomaternal platelet antigen incompatibility. *Br J Haematol*. 2020;190(5):787–798.

Provan D, Arnold DM, Bussel JB, et al. Updated international consensus report on the investigation and management of primary immune thrombocytopenia. *Blood Adv*. 2019;3(22):3780–3817.

Rodeghiero F, Stasi R, Gernsheimer T, et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. *Blood*. 2009;113(11):2386–2393.

Sainio S, Javela K, Tuimala J, Haimila K. Maternal HLA genotyping is not useful for predicting severity of fetal and neonatal alloimmune thrombocytopenia. *Br J Haematol*. 2017;176(1):111–117.

Shrestha S, Nazy I, Smith JW, et al. Platelet autoantibodies in the bone marrow of patients with immune thrombocytopenia. *Blood Adv*. 2020;4(13):2962–2966.

Taaning E, Tonnesen F. Pan-reactive platelet antibodies in post-transfusion purpura. *Vox Sang*. 1999;76(2):120–123.

Williamson LM, Stainsby D, Jones H, et al. The impact of universal leukodepletion of the blood supply on hemovigilance reports of posttransfusion purpura and transfusion-associated graft-versus-host disease. *Transfusion*. 2007;47(8):1455–1467.

Winkelhorst D, Murphy MF, Greinacher A, et al. Antenatal management in fetal and neonatal alloimmune thrombocytopenia: a systematic review. *Blood*. 2017;129(11):1538–1547.

Woelke C, Eichler P, Washington G, Flesch BK. Post-transfusion purpura in a patient with HPA-1a and GPIa/IIa antibodies. *Transfus Med*. 2006;16(1):69–72.

REFERENCES

1. Quach ME, Chen W, Li R. Mechanisms of platelet clearance and translation to improve platelet storage. *Blood*. 2018;131(14):1512–1521. https://doi.org/10.1182/blood-2017-08-743229.
2. Li J, van der Wal DE, Zhu G, et al. Desialylation is a mechanism of Fc-independent platelet clearance and a therapeutic target in immune thrombocytopenia. *Nat Commun*. 2015;6:7737. https://doi.org/10.1038/ncomms8737.
3. Shrestha S, Nazy I, Smith JW, Kelton JG, Arnold DM. Platelet autoantibodies in the bone marrow of patients with immune thrombocytopenia. *Blood Adv*. 2020;4(13):2962–2966. https://doi.org/10.1182/bloodadvances.2020001846.
4. Terrell DR, Beebe LA, Vesely SK, Neas BR, Segal JB, George JN. The incidence of immune thrombocytopenic purpura in children and adults: a critical review of published reports. *Am J Hematol*. 2010;85(3):174–180. https://doi.org/10.1002/ajh.21616.
5. Segal JB, Powe NR. Prevalence of immune thrombocytopenia: analyses of administrative data. *J Thromb Haemost*. 2006;4(11):2377–2383. https://doi.org/10.1111/j.1538-7836.2006.02147.x.
6. Yong M, Schoonen WM, Li L, et al. Epidemiology of paediatric immune thrombocytopenia in the General Practice Research Database. *Br J Haematol*. 2010;149(6):855–864. https://doi.org/10.1111/j.1365-2141.2010.08176.x.
7. Schoonen WM, Kucera G, Coalson J, et al. Epidemiology of immune thrombocytopenic purpura in the General Practice Research Database [Erratum in *Br J Haematol*. 2009 Sep;147(1):157]. *Br J Haematol*. 2009;145(2):235–244. https://doi.org/10.1111/j.1365-2141.2009.07615.x.
8. Abrahamson PE, Hall SA, Feudjo-Tepie M, Mitrani-Gold FS, Logie J. The incidence of idiopathic thrombocytopenic purpura among adults: a population-based study and literature review. *Eur J Haematol*. 2009;83(2):83–89. https://doi.org/10.1111/j.1600-0609.2009.01247.x.
9. Harrington WJ, Minnich V, Hollingsworth JW, Moore CV. Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. *J Lab Clin Med*. 1951;38(1):1–10.
10. Vollenberg R, Jouni R, Norris PAA, et al. Glycoprotein V is a relevant immune target in patients with immune thrombocytopenia. *Haematologica*. 2019;104(6):1237–1243. https://doi.org/10.3324/haematol.211086.
11. Mahévas M, Patin P, Huetz F, et al. B cell depletion in immune thrombocytopenia reveals splenic long-lived plasma cells. *J Clin Invest*. 2013;123(1):432–442. https://doi.org/10.1172/JCI65689.
12. Stoll D, Cines DB, Aster RH, Murphy S. Platelet kinetics in patients with idiopathic thrombocytopenic purpura and moderate thrombocytopenia. *Blood*. 1985;65(3):584–588.
13. McMillan R, Wang L, Tomer A, Nichol J, Pistillo J. Suppression of in vitro megakaryocyte production by antiplatelet autoantibodies from adult patients with chronic ITP. *Blood*. 2004;103(4):1364–1369. https://doi.org/10.1182/blood-2003-08-2672.
14. Yang L, Wang L, Zhao CH, et al. Contributions of TRAIL-mediated megakaryocyte apoptosis to impaired megakaryocyte and platelet production in immune thrombocytopenia. *Blood*. 2010;116(20):4307–4316. https://doi.org/10.1182/blood-2010-02-267435.
15. Chang M, Nakagawa PA, Williams SA, et al. Immune thrombocytopenic purpura (ITP) plasma and purified ITP monoclonal autoantibodies inhibit megakaryocytopoiesis in vitro. *Blood*. 2003;102(3):887–895. https://doi.org/10.1182/blood-2002-05-1475.
16. Zhao C, Li X, Zhang F, Wang L, Peng J, Hou M. Increased cytotoxic T-lymphocyte-mediated cytotoxicity predominant in patients with idiopathic thrombocytopenic purpura without platelet autoantibodies. *Haematologica*. 2008;93(9):1428–1430. https://doi.org/10.3324/haematol.12889.
17. Zhang F, Chu X, Wang L, et al. Cell-mediated lysis of autologous platelets in chronic idiopathic thrombocytopenic purpura. *Eur J Haematol*. 2006;76(5):427–431. https://doi.org/10.1111/j.1600-0609.2005.00622.x.
18. Vrbensky JR, Arnold DM, Kelton JG, et al. Increased cytotoxic potential of $CD8^+$ T cells in immune thrombocytopenia. *Br J Haematol*. 2020;188(5):e72–e76. https://doi.org/10.1111/bjh.16334.
19. Marini I, Zlamal J, Faul C, et al. Autoantibody-mediated desialylation impairs human thrombopoiesis and platelet lifespan. *Haematologica*. 2021;106(1):196–207. https://doi.org/10.3324/haematol.2019.236117.
20. Franchini M, Cruciani M, Mengoli C, Pizzolo G, Veneri D. Effect of *Helicobacter pylori* eradication on platelet count in idiopathic thrombocytopenic purpura: a systematic review and meta-analysis. *J Antimicrob Chemother*. 2007;60(2):237–246. https://doi.org/10.1093/jac/dkm195.
21. Stasi R, Sarpatwari A, Segal JB, et al. Effects of eradication of *Helicobacter pylori* infection in patients with immune thrombocytopenic purpura: a systematic review. *Blood*. 2009;113(6):1231–1240. https://doi.org/10.1182/blood-2008-07-167155.
22. Neunert C, Lim W, Crowther M, et al. The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia. *Blood*. 2011;117(16):4190–4207. https://doi.org/10.1182/blood-2010-08-302984.
23. Neunert C, Terrell DR, Arnold DM, et al. American Society of Hematology 2019 guidelines for immune thrombocytopenia [Erratum in *Blood Adv*. 2020;4(2):252]. *Blood Adv*. 2019;3(23):3829–3866. https://doi.org/10.1182/bloodadvances.2019000966.
24. Provan D, Arnold DM, Bussel JB, et al. Updated international consensus report on the investigation and management of primary immune thrombocytopenia. *Blood Adv*. 2019;3(22):3780–3817. https://doi.org/10.1182/bloodadvances.2019000812.
25. Rodeghiero F, Stasi R, Gernsheimer T, et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. *Blood*. 2009;113(11):2386–2393.
26. Arnold DM, Nazy I, Clare R, et al. Misdiagnosis of primary immune thrombocytopenia and frequency of bleeding: lessons from the McMaster ITP Registry. *Blood Adv*. 2017;1(25):2414–2420. https://doi.org/10.1182/bloodadvances.2017010942.
27. Mithoowani S, Cervi A, Shah N, et al. Management of major bleeds in patients with immune thrombocytopenia. *J Thromb Haemost*. 2020;18(7):1783–1790. https://doi.org/10.1111/jth.14809.
28. Arnold DM, Crowther MA, Meyer RM, et al. Misleading hepatitis B test results due to intravenous immunoglobulin administration: implications for a clinical trial of rituximab in immune thrombocytopenia. *Transfusion*. 2010;50(12):2577–2581. https://doi.org/10.1111/j.1537-2995.2010.02766.x.
29. Neunert C, Noroozi N, Norman G, et al. Severe bleeding events in adults and children with primary immune thrombocytopenia: a systematic review. *J Thromb Haemost*. 2015;13(3):457–464.
30. Schifferli A, Holbro A, Chitlur M, et al. A comparative prospective observational study of children and adults with immune thrombocytopenia: 2-year follow-up. *Am J Hematol*. 2018;93(6):751–759. https://doi.org/10.1002/ajh.25086.
31. Cheng Y, Wong RSM, Soo YOY, et al. Initial treatment of immune thrombocytopenic purpura with high-dose dexamethasone. *N Engl J Med*. 2003;349(9):831–836.
32. Mithoowani S, Gregory-Miller K, Goy J, et al. High-dose dexamethasone compared with prednisone for previously untreated primary immune thrombocytopenia: a systematic review and meta-analysis. *Lancet Haematol*. 2016;3(10):e489–e496.
33. Beck CE, Nathan PC, Parkin PC, et al. Corticosteroids versus intravenous immune globulin for the treatment of acute immune thrombocytopenic purpura in children: a systematic review and meta-analysis of randomized controlled trials. *J Pediatr*. 2005;147(4):521–527.
34. Kojouri K, Vesely SK, Terrell DR, George JN. Splenectomy for adult patients with idiopathic thrombocytopenic purpura: a systematic review to assess long-term platelet count responses, prediction of response, and surgical complications. *Blood*. 2004;104(9):2623–2634. https://doi.org/10.1182/blood-2004-03-1168.
35. Amini SN, Nelson VS, Sobels A, Schoones JW, Zwaginga JJ, Schipperus MR. Autologous platelet scintigraphy and clinical outcome of splenectomy in immune thrombocytopenia: a systematic review and meta-analysis. *Crit Rev Oncol Hematol*. 2020;153:103040. https://doi.org/10.1016/j.critrevonc.2020.103040.
36. Thomsen RW, Schoonen WM, Farkas DK, Riis A, Fryzek JP, Sørensen HT. Risk of venous thromboembolism in splenectomized patients compared with the general population and appendectomized patients: a 10-year nationwide cohort study. *J Thromb Haemost*. 2010;8(6):1413–1416. https://doi.org/10.1111/j.1538-7836.2010.03849.x.
37. Arnold DM, Dentali F, Crowther MA, et al. Systematic review: efficacy and safety of rituximab for adults with idiopathic thrombocytopenic purpura. *Ann Intern Med*. 2007;146(1):25–33. https://doi.org/10.7326/0003-4819-146-1-200701020-00006.
38. Lucchini E, Zaja F, Bussel J. Rituximab in the treatment of immune thrombocytopenia: what is the role of this agent in 2019? *Haematologica*. 2019;104(6):1124–1135. https://doi.org/10.3324/haematol.2019.218883.
39. Chugh S, Lim W, Crowther MA, et al. Rituximab plus standard of care for treatment of primary immune thrombocytopenia: a systematic review and meta-analysis. *Lancet Haematol*. 2015;2:e75–e81.
40. Godeau B, Porcher R, Fain O, et al. Rituximab efficacy and safety in adult splenectomy candidates with chronic immune thrombocytopenic purpura: results of a prospective multicenter phase 2 study. *Blood*. 2008;112(4):999–1004. https://doi.org/10.1182/blood-2008-01-131029.

41. Patel VL, Mahevas M, Lee SY, et al. Outcomes 5 years after response to rituximab therapy in children and adults with immune thrombocytopenia. *Blood*. 2012;119(25):5989–5995.
42. Ghadaki B, Nazi I, Kelton JG, Arnold DM. Sustained remissions of immune thrombocytopenia associated with the use of thrombopoietin receptor agonists. *Transfusion*. 2013;53(11):2807–2812.
43. Mahévas M, Fain O, Ebbo M, et al. The temporary use of thrombopoietin-receptor agonists may induce a prolonged remission in adult chronic immune thrombocytopenia. Results of a French observational study. *Br J Haematol*. 2014;165(6):865–869. https://doi.org/10.1111/bjh.12888.
44. Kuter DJ, Bussel JB, Lyons RM, et al. Efficacy of romiplostim in patients with chronic immune thrombocytopenic purpura: a double-blind randomised controlled trial. *Lancet*. 2008;371(9610):395–403.
45. Kuter DJ, Rummel M, Boccia R, et al. Romiplostim or standard of care in patients with immune thrombocytopenia. *N Engl J Med*. 2010;363(20):1889–1899.
46. Cheng G, Saleh MN, Marcher C, et al. Eltrombopag for management of chronic immune thrombocytopenia (RAISE): a 6-month, randomised, phase 3 study. *Lancet*. 2011;377(9763):393–402.
47. Jurczak W, Chojnowski K, Mayer J, et al. Phase 3 randomised study of avatrombopag, a novel thrombopoietin receptor agonist for the treatment of chronic immune thrombocytopenia. *Br J Haematol*. 2018;183(3):479–490. https://doi.org/10.1111/bjh.15573.
48. Kuter DJ, Bussel JB, Newland A, et al. Long-term treatment with romiplostim in patients with chronic immune thrombocytopenia: safety and efficacy. *Br J Haematol*. 2013;161(3):411–423. https://doi.org/10.1111/bjh.12260.
49. Saleh MN, Bussel JB, Cheng G, et al. Safety and efficacy of eltrombopag for treatment of chronic immune thrombocytopenia: results of the long-term, open-label EXTEND study. *Blood*. 2013;121(3):537–545. https://doi.org/10.1182/blood-2012-04-425512.
50. Arnold DM, Heddle NM, Cook RJ, et al. Perioperative oral eltrombopag versus intravenous immunoglobulin in patients with immune thrombocytopenia: a non-inferiority, multicentre, randomised trial. *Lancet Haematol*. 2020;7(9):e640–e648. https://doi.org/10.1016/S2352-3026(20)30227-1.
51. Bussel J, Arnold DM, Grossbard E, et al. Fostamatinib for the treatment of adult persistent and chronic immune thrombocytopenia: results of two phase 3, randomized, placebo-controlled trials. *Am J Hematol*. 2018;93(7):921–930. https://doi.org/10.1002/ajh.25125.
52. Bussel JB, Arnold DM, Boxer MA, et al. Long-term fostamatinib treatment of adults with immune thrombocytopenia during the phase 3 clinical trial program. *Am J Hematol*. 2019;94(5):546–553. https://doi.org/10.1002/ajh.25444.
53. Bradbury CA, Pell J, Hill Q, et al. Mycophenolate mofetil for first-line treatment of immune thrombocytopenia. *N Engl J Med*. 2021;385(10):885–895. https://doi.org/10.1056/NEJMoa2100596.
54. Arnold DM, Nazi I, Santos A, et al. Combination immunosuppressant therapy for patients with chronic refractory immune thrombocytopenic purpura. *Blood*. 2010;115(1):29–31. https://doi.org/10.1182/blood-2009-06-222448.
55. Vesely SK, Perdue JJ, Rizvi MA, Terrell DR, George JN. Management of adult patients with persistent idiopathic thrombocytopenic purpura following splenectomy: a systematic review. *Ann Intern Med*. 2004;140(2):112–120. https://doi.org/10.7326/0003-4819-140-3-200402030-00012.
56. Boruchov DM, Gururangan S, Driscoll MC, Bussel JB. Multiagent induction and maintenance therapy for patients with refractory immune thrombocytopenic purpura (ITP). *Blood*. 2007;110(10):3526–3531. https://doi.org/10.1182/blood-2007-01-065763.
57. Yue H, Bai X, Wang J, et al. Clinical characteristics of coronavirus disease 2019 in Gansu province, China. *Ann Palliat Med*. 2020;9(4):1404–1412. https://doi.org/10.21037/apm-20-887.
58. Kewan T, Gunaratne TN, Mushtaq K, Alayan D, Daw H, Haddad A. Outcomes and management of immune thrombocytopenia secondary to COVID-19: cleveland clinic experience. *Transfusion*. 2021;61(7):2014–2018. https://doi.org/10.1111/trf.16368.
59. Pavord S, Thachil J, Hunt BJ, et al. Practical guidance for the management of adults with immune thrombocytopenia during the COVID-19 pandemic. *Br J Haematol*. 2020;189(6):1038–1043. https://doi.org/10.1111/bjh.16775.
60. Huynh A, Kelton JG, Arnold DM, et al. Antibody epitopes in vaccine-induced immune thrombotic thrombocytopaenia. *Nature*. 2021;596(7873):565–569. https://doi.org/10.1038/s41586-021-03744-4.
61. Lee E-J, Beltrami-Moreira M, Al-Samkari H, et al. SARS-CoV-2 vaccination and ITP in patients with de novo or preexisting ITP. *Blood*. 2022;139(10):1564–1574. https://doi.org/10.1182/blood.2021013411.
62. Simpson CR, Shi T, Vasileiou E, et al. First-dose ChAdOx1 and BNT162b2 COVID-19 vaccines and thrombocytopenic, thromboembolic and hemorrhagic events in Scotland. *Nat Med*. 2021;27(7):1290–1297. https://doi.org/10.1038/s41591-021-01408-4.
63. Kuter DJ. Exacerbation of immune thrombocytopenia following COVID-19 vaccination. *Br J Haematol*. 2021;195(3):365–370. https://doi.org/10.1111/bjh.17645.
64. Arnold DM, Smith JW, Kelton JG. Diagnosis and management of neonatal alloimmune thrombocytopenia. *Transfus Med Rev*. 2008;22(4):255–267.
65. Ouzegdouh Mammasse Y, Chenet C, Drubay D, et al. A new efficient tool for non-invasive diagnosis of fetomaternal platelet antigen incompatibility. *Br J Haematol*. 2020;190(5):787–798. https://doi.org/10.1111/bjh.16593.
66. Bakchoul T, Bertrand G, Krautwurst A, et al. The implementation of surface plasmon resonance technique in monitoring pregnancies with expected fetal and neonatal alloimmune thrombocytopenia. *Transfusion*. 2013;53(9):2078–2085.
67. Ghevaert C, Rankin A, Huiskes E, et al. Alloantibodies against low-frequency human platelet antigens do not account for a significant proportion of cases of fetomaternal alloimmune thrombocytopenia: evidence from 1054 cases. *Transfusion*. 2009;49(10):2084–2089.
68. Baker JM, Shehata N, Bussel J, et al. Postnatal intervention for the treatment of FNAIT: a systematic review. *J Perinatol*. 2019;39(10):1329–1339. https://doi.org/10.1038/s41372-019-0360-7.
69. Winkelhorst D, Murphy MF, Greinacher A, et al. Antenatal management in fetal and neonatal alloimmune thrombocytopenia: a systematic review. *Blood*. 2017;129(11):1538–1547. https://doi.org/10.1182/blood-2016-10-739656.
70. Lieberman L, Greinacher A, Murphy MF, et al. Fetal and neonatal alloimmune thrombocytopenia: recommendations for evidence-based practice, an international approach. *Br J Haematol*. 2019;185(3):549–562. https://doi.org/10.1111/bjh.15813.
71. Kjær M, Bertrand G, Bakchoul T, et al. Maternal HPA-1a antibody level and its role in predicting the severity of fetal/neonatal alloimmune thrombocytopenia: a systematic review. *Vox Sang*. 2019;114(1):79–94. https://doi.org/10.1111/vox.12725.
72. Kamphuis MM, Paridaans N, Porcelijn L, et al. Screening in pregnancy for fetal or neonatal alloimmune thrombocytopenia: systematic review. *BJOG*. 2010;117(11):1335–1343. https://doi.org/10.1111/j.1471-0528.2010.02657.x.
73. Kjær M, Geisen C, Akkök ÇA, et al. Strategies to develop a prophylaxis for the prevention of HPA-1a immunization and fetal and neonatal alloimmune thrombocytopenia. *Transfus Apher Sci*. 2020;59(1):102712. https://doi.org/10.1016/j.transci.2019.102712.
74. Sainio S, Javela K, Tuimala J, Haimila K. Maternal HLA genotyping is not useful for predicting severity of fetal and neonatal alloimmune thrombocytopenia. *Br J Haematol*. 2017;176(1):111–117. https://doi.org/10.1111/bjh.14385.
75. Williamson LM, Stainsby D, Jones H, et al. The impact of universal leukodepletion of the blood supply on hemovigilance reports of posttransfusion purpura and transfusion-associated graft-versus-host disease. *Transfusion*. 2007;47(8):1455–1467. https://doi.org/10.1111/j.1537-2995.2007.01281.x.
76. Menis M, Forshee RA, Anderson SA, et al. Posttransfusion purpura occurrence and potential risk factors among the inpatient US elderly, as recorded in large Medicare databases during 2011 through 2012. *Transfusion*. 2015;55(2):284–295.
77. Woelke C, Eichler P, Washington G, Flesch BK. Post-transfusion purpura in a patient with HPA-1a and GPIa/IIa antibodies. *Transfus Med*. 2006;16(1):69–72. https://doi.org/10.1111/j.1365-3148.2005.00633.x.
78. Hawkins J, Aster RH, Curtis BR. Post-transfusion purpura: current perspectives. *J Blood Med*. 2019;10:405–415. https://doi.org/10.2147/JBM.S189176.
79. Taaning E, Tønnesen F. Pan-reactive platelet antibodies in post-transfusion purpura. *Vox Sang*. 1999;76(2):120–123.
80. Mueller-Eckhardt C, Kiefel V. High-dose IgG for post-transfusion purpura-revisited. *Blut*. 1988;57(4):163–167.
81. Delaney M, Wendel S, Bercovitz RS, et al. Transfusion reactions: prevention, diagnosis, and treatment. *Lancet*. 2016;388(10061):2825–2836. https://doi.org/10.1016/S0140-6736(15)01313-6.

CHAPTER **130**

THROMBOCYTOPENIA CAUSED BY HYPERSPLENISM, PLATELET DESTRUCTION, OR SURGERY/HEMODILUTION

Theodore E. Warkentin

Thrombocytopenia is defined as a platelet count below the lower limit of the normal range ($\approx 150 \times 10^9$/L). Sometimes, an expanded definition of thrombocytopenia is appropriate. For example, an abrupt drop in the platelet count can signify the onset of a disorder such as heparin-induced thrombocytopenia (HIT) or bacteremia even if the platelet count remains above 150×10^9/L.[1] This is especially relevant in the second or third week after surgery because patients usually have platelet counts that peak at levels two to three times higher than their usual preoperative value (postoperative thrombocytosis).[2]

In the clinical evaluation of a patient with thrombocytopenia, three questions must be asked. First, could the patient have pseudothrombocytopenia? Second, what is the most likely explanation for the thrombocytopenia? Third, what are the risks posed by the causative disorder and the severity of the thrombocytopenia? For example, severe thrombocytopenia caused by drug-dependent antibodies or platelet-reactive autoantibodies is often associated with bleeding. By contrast, thrombocytopenia caused by HIT antibodies or attributable to disseminated intravascular coagulation (DIC) secondary to adenocarcinoma is associated with thrombosis (see Chapters 131 and 137, respectively). Often, the underlying cause of the thrombocytopenia (e.g., bacteremia, cancer, cirrhosis), rather than the thrombocytopenia itself, poses the greater risk.

Thrombocytopenia can be caused by any of five general mechanisms: (1) platelet underproduction, (2) increased platelet destruction, (3) increased platelet consumption, (4) platelet sequestration, and (5) hemodilution. Platelet underproduction usually occurs in association with underproduction of other blood cell lines, which results in bicytopenia or pancytopenia. Thrombocytopenia caused by increased platelet destruction develops when immune-mediated mechanisms, most often platelet-reactive antibodies, cause the rate of platelet loss to surpass the ability of the bone marrow (BM) to produce platelets. In contrast, increased platelet consumption refers to pathological enhancement of physiological mechanisms, with resulting increased platelet loss. Examples include increased thrombin-mediated platelet consumption (DIC) and increased platelet-von Willebrand factor interactions (thrombotic microangiopathy [TMA]). Table 130.1 lists some causes of platelet destruction and consumption. Thrombocytopenia from platelet sequestration is caused by platelet redistribution from the circulation into an enlarged splenic vascular bed. Hemodilution is characterized by a decreased number of platelets, as well as red blood cells (RBCs) and white blood cells (WBCs) as a result of the administration of colloid, crystalloid, or platelet-poor blood products.

Sometimes, pathophysiological mechanisms overlap. For example, immune HIT is caused by antibodies (destruction) that directly cause platelet activation and that also trigger DIC (consumption). Advanced liver disease can feature severe thrombocytopenia as a result of hypersplenism (sequestration), hyperfibrinolysis (consumption), and decreased thrombopoietin production (underproduction).

In the postoperative period, platelet count changes reflect several processes, including initial hemodilution (immediate platelet count decrease) and increased platelet consumption (first 2 to 4 days), at which point the platelet count begins to rise because of increased platelet production. When the platelet count reaches its postoperative peak—usually about 14 days after surgery—platelet production decreases somewhat, and the platelet count returns to baseline (Fig. 130.1).[2]

APPROACH TO PATIENTS WITH THROMBOCYTOPENIA

History and Physical Examination

Certain information should be ascertained, including (1) the location and severity of bleeding (if any); (2) the temporal profile of the hemostatic defect (acute, chronic, or relapsing), particularly the temporal relationship with potential proximate triggers (e.g., new drugs, recent infection); (3) the presence of symptoms of a secondary illness, such as a neoplasm, infection, or an autoimmune disorder such as systemic lupus erythematosus (SLE); (4) history of recent medication use, alcohol ingestion, or transfusion; (5) presence of risk factors for certain infections, particularly human immunodeficiency virus (HIV) infection or viral hepatitis; and (6) family history of thrombocytopenia.

As part of the physical examination, evidence of hemostatic impairment should be sought, as well as secondary causes of thrombocytopenia. The signs of platelet-related bleeding include petechiae and purpura. Petechiae typically occur in the dependent regions of the body or on traumatized areas. Spontaneous mucous membrane bleeding (wet purpura), epistaxis, hematuria, and gastrointestinal bleeding indicate a more serious hemostatic defect. Although petechiae are common in patients whose platelet counts are less than 10 to 20×10^9/L, most patients with platelet counts over 50×10^9/L have no signs of hemostatic impairment. The physical examination may provide an explanation for the thrombocytopenia. For example, enlarged lymph nodes may indicate a viral infection, such as infectious mononucleosis or HIV infection, or a neoplastic process. An enlarged spleen raises the possibility of hypersplenism.

Timing of Onset and Severity of Thrombocytopenia

Many thrombocytopenic disorders, particularly those involving an immune pathogenesis, exhibit characteristic temporal features that can aid in the diagnosis. For example, if the platelet count begins to fall 5 to 10 days (median, 6 to 7 days) after starting a new drug or after a blood transfusion and reaches a nadir of less than 20×10^9/L a few days later, the diagnosis of drug-induced immune thrombocytopenia (D-ITP) or posttransfusion purpura (PTP), respectively, should be considered (Fig. 130.2).[3] Patients with these disorders typically have mucocutaneous bleeding and are at risk for fatal intracranial hemorrhage.

A similar temporal profile is also characteristic of typical-onset HIT (see Chapter 130), although in that disorder the platelet count falls below 20×10^9/L in only 5% to 10% of affected patients (see Fig. 130.2); in approximately 80% to 90% of patients, the platelet count nadir ranges from 20 to 150×10^9/L; and in the remainder, the platelet count nadir never falls below 150×10^9/L despite a large reduction in the platelet count.[4] When the platelet count falls abruptly after drug administration, the possibility of rapid-onset thrombocytopenia caused by preexisting drug-dependent antibodies should be considered, as is well described with HIT. Indeed, so-called rapid-onset HIT is the presenting feature of this adverse drug reaction in 25% to 30% of cases.[5] Rapid-onset thrombocytopenia is also a feature of glycoprotein (GP) IIb/IIIa (αIIbβ3) antagonist-induced ITP (see Chapter 129).[6]

TABLE 130.1 Mechanisms of Platelet Destruction or Consumption

Type of Thrombocytopenia	Specific Example(s)
Platelet Destruction (Immune-Mediated)	
Autoantibody-mediated platelet destruction by RES	Primary and secondary idiopathic (immune) ITP
Alloantibody-mediated platelet destruction by RES	NAIT, PTP, PAT; alloimmune platelet transfusion refractoriness
Drug-dependent, antibody-mediated platelet destruction by RES	Drug-induced immune ITP (e.g., quinine, vancomycin)
Platelet activation by binding of IgG Fc of drug-dependent IgG to platelet FcγIIa receptors	Heparin-induced thrombocytopenia (HIT)[a]; protamine (heparin)-induced thrombocytopenia (PHIT)[a]
Platelet Consumption (Pathologically Enhanced Physiological Mechanisms)	
Platelet consumption through platelet activation by thrombin or proinflammatory cytokines	DIC; septicemia or systemic inflammatory response syndromes; HIT[a], PHIT[a]
Hyperfibrinolysis	Disorders of hyperfibrinolysis (e.g., certain malignancies, advanced liver disease) include element of platelet consumption
Platelet consumption via ingestion by macrophages (hemophagocytosis)	Infections, certain malignant lymphoproliferative disorders
Platelet consumption through platelet interactions with altered vWF[c]	TMA disorders, e.g., iTTP,[b] HUS
Platelet losses on artificial surfaces	CPB, use of intravascular catheters
Decreased platelet survival associated with cardiovascular diseases	Congenital and acquired heart disease, cardiomyopathy, pulmonary embolism

CPB, Cardiopulmonary bypass surgery; *DIC*, disseminated intravascular coagulation; *HIT*, heparin-induced thrombocytopenia; *PHIT*, protamine (heparin) induced thrombocytopenia; *HUS*, hemolytic uremic syndrome; *IgG*, immunoglobulin G; *ITP*, idiopathic (immune) thrombocytopenic purpura; *iTTP*, immune-mediated thrombotic thrombocytopenic purpura; *NAIT*, neonatal alloimmune thrombocytopenia; *PAT*, passive alloimmune thrombocytopenia; *PTP*, posttransfusion purpura; RES, reticuloendothelial system; *TMA*, thrombotic microangiopathy; *VWF*, von Willebrand factor.

[a] HIT and PHIT have overlapping features of antibody-mediated platelet activation (destruction) and DIC (consumption).

[b] Although platelet destruction is not directly caused by antibodies, immune mechanisms can explain enhanced VWF-platelet interactions (e.g., autoimmune clearance of VWF-cleaving metalloprotease, associated with iTTP).

Occasionally, thrombocytopenia worsens in the first few days after surgery; this can occur with multiorgan system failure (e.g., cardiogenic or septic shock, with lactic acidemia) (see Fig. 130.2). If the patient develops concomitant DIC and hypotension, there is a high risk for ischemic limb injury secondary to microvascular thrombosis ("symmetric peripheral gangrene"), especially if the patient has "shock liver" (acute ischemic hepatitis), which is a risk factor for severe depletion of protein C and antithrombin, important endogenous anticoagulants (see Chapter 125).[7,8] Another potential contributory factor for severe natural anticoagulant depletion and risk for limb gangrene in susceptible patients is colloid transfusion (albumin, high-dose IVIG), which lacks coagulation factors including protein C and antithrombin.[9] If the platelet count falls to very low levels and is accompanied by microangiopathic hemolysis, the possibility of postoperative thrombotic thrombocytopenic purpura (TTP) should be considered (see Chapter 132).[10]

Rarely, abrupt thrombocytopenia, with or without complicating thrombosis, can occur in post-cardiac or vascular surgery patients following protamine administration (to reverse intraoperative heparin anticoagulation).[11] This disorder is caused by platelet-activating anti-protamine (heparin) antibodies ("protamine [heparin]-induced thrombocytopenia"). Because these antibodies are transient, at-risk patients include those with recent exposure to both heparin and protamine, such as patients who previously underwent cardiac or vascular surgery (in the recent past) or who may have received preoperative unfractionated or low-molecular-weight heparin while at the same time receiving protamine-containing insulin products.

Mild to moderate platelet count decreases that occur soon after transfusion of blood products are common and can be explained by hemodilution; however, a marked platelet count fall after transfusion may be the result of passive alloimmune thrombocytopenia (PAT)[12,13] or sepsis because of contaminated blood products (see Fig. 130.2).

Other characteristic temporal features of thrombocytopenia include postenterohemorrhagic *Escherichia coli*–associated hemolytic uremic syndrome (HUS); thrombocytopenia and microangiopathic hemolysis that begin approximately 1 week after a prodromal diarrheal illness; and fungemia-associated thrombocytopenia (onset, 1 to 3 weeks after complex illness involving indwelling catheters and broad-spectrum antibiotic use). In contrast, thrombocytopenia of insidious onset that progresses over several years suggests chronic liver disease, with evolution to portal hypertension and associated splenomegaly (e.g., cirrhosis secondary to alcohol or hepatitis C) or a slowly progressive BM disorder (e.g., myelodysplasia).

Laboratory Evaluation

Laboratory evaluation of patients with thrombocytopenia includes evaluation of the complete blood count (CBC), examination of the peripheral blood film, and obtaining relevant ancillary studies, which sometimes includes BM aspirate/biopsy.

The blood film is examined to exclude pseudothrombocytopenia, which is characterized by in vitro platelet clumping. This phenomenon, which is evident in approximately 1 in 1000 blood samples, is most often caused by naturally occurring GPIIb/IIIa-reactive autoantibodies that induce aggregation of platelets in the presence of the calcium-chelating anticoagulant ethylenediamine tetraacetic acid (EDTA). Because the platelet aggregates are not counted by the electronic particle counter, the automated platelet count appears falsely low. The correct platelet count usually can be determined by collecting the blood into sodium citrate or heparin or by performing the count on nonanticoagulated finger prick samples; maintaining the blood sample at 37°C often attenuates platelet clumping. EDTA-dependent pseudothrombocytopenia has no pathologic significance other than potentially placing a patient in jeopardy for inappropriate treatment for thrombocytopenia that does not exist. A much less common (1 in 10,000 blood samples) antibody-mediated pseudothrombocytopenic disorder is platelet satellitism, in which rosette-like clusters of platelets surround the neutrophils. This entity is produced by immunoglobulin G (IgG) antibodies that recognize EDTA-induced cryptic epitopes on both platelet GPIIb/IIIa and neutrophil FcγIII receptors.

BM examination can be helpful for platelet production assessment, particularly if megakaryocytes are reduced or abnormal in appearance. Examination of the BM can be diagnostic in some disorders (e.g., leukemia, metastatic tumor, Gaucher disease, megaloblastic anemia).

Besides the CBC, commonly performed blood tests to evaluate thrombocytopenia include: prothrombin time/international normalized ratio (PT/INR), partial thromboplastin time (PTT), fibrinogen, D-dimer (screening tests for DIC), serum enzyme levels such as lactic dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine phosphokinase (CPK) (evaluation of hemolysis, shock liver, muscle injury), blood cultures (bacteremia, fungemia). More specific assays appropriate in selected patients include testing for HIT or non-HIT drug-dependent platelet-reactive antibodies (e.g., using

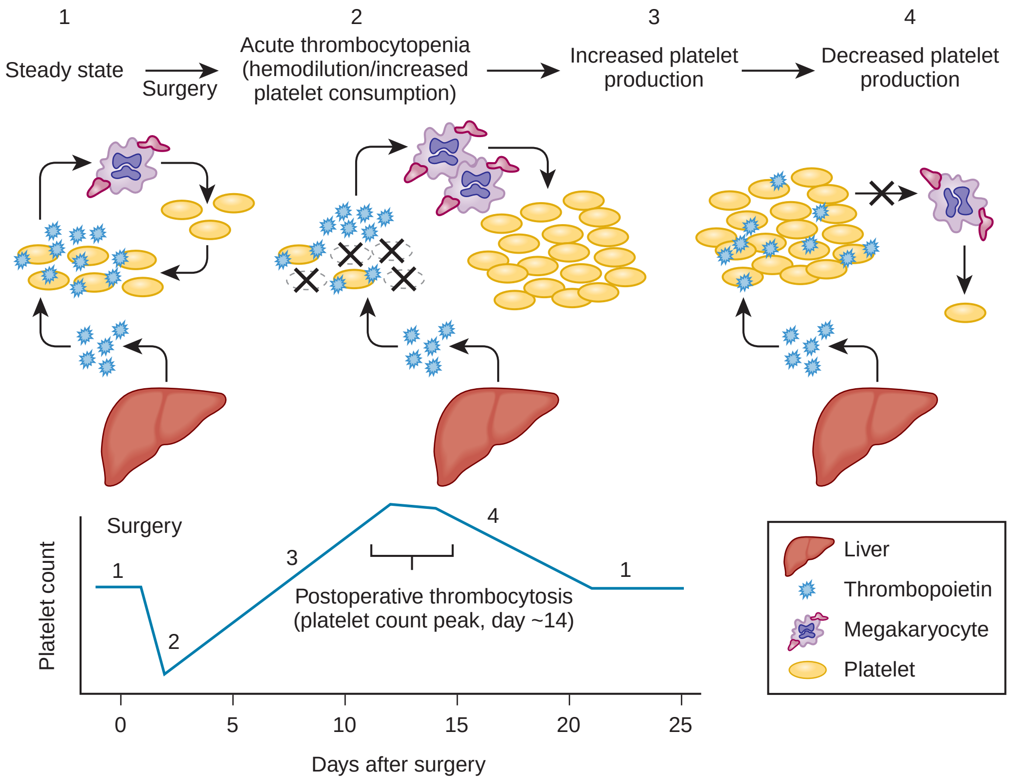

1
Steady state
Surgery
2
Acute thrombocytopenia (hemodilution/increased platelet consumption)
3
Increased platelet production
4
Decreased platelet production
Platelet count
Surgery
1
2
3
4
1
Postoperative thrombocytosis (platelet count peak, day ~14)
0
5
10
15
20
25
Days after surgery
Liver
Thrombopoietin
Megakaryocyte
Platelet

Figure 130.1 POSTSURGERY PLATELET COUNT CHANGES. Initial platelet count declines result from hemodilution and increased platelet consumption, with the platelet count nadir occurring between postoperative days 1 to 4 (median, day 2). There is constitutive production of thrombopoietin (TPO) by the liver. TPO binds to platelets and megakaryocytes via a specific receptor (c-Mpl, not shown), and receptor-bound TPO is removed from the circulation and degraded. The level of circulating TPO is thus inversely related to the mass of platelets and megakaryocytes. In early postsurgery thrombocytopenia, fewer TPO binding sites are available, resulting in high free TPO levels, which stimulate megakaryocyte proliferation and differentiation and lead to increased platelet production. With subsequent thrombocytosis, the high platelet mass acts as a "sink" for removing TPO, with decreased stimulus for platelet production. Thus after acute postsurgery thrombocytopenia, TPO levels rise about twofold, leading to increased platelet production that begins on days 2 to 4, with resulting thrombocytosis that generally peaks at approximately day 14 (postoperative thrombocytosis) and returns to baseline by about day 21. *(Reprinted, with modifications, with permission, from Arnold DM, Warkentin TE. Thrombocytopenia and thrombocytosis. In: Wilson WC, Grande CM, Hoyt DB, eds. Trauma: Critical Care. Vol. 2. New York: Informa Healthcare; 2007:983.)*

the monoclonal antibody immobilization of platelet antigens [MAIPA] test), platelet-reactive alloantibodies, antiphospholipid antibodies (nonspecific inhibitor, anti-cardiolipin antibodies, anti-β_2-glycoprotein 1 antibodies), quantitative immunoglobulin determination and serum protein electrophoresis, viral serology (HIV, hepatitis C, etc.), tests for hemolysis (direct Coombs test, haptoglobin levels, etc.).

In exceptional circumstances, when the mechanism of chronic thrombocytopenia is unclear, an autologous platelet survival study using ^{111}In-labeled platelets may be informative. Three patterns can be seen: (1) normal platelet survival and recovery (underproduction), (2) marked reduction in the platelet life span (increased destruction or consumption), and (3) reduced recovery but a normal or near-normal life span (sequestration). However, platelet survival studies are rarely performed.

Therapy

The risk of bleeding in patients with thrombocytopenia can be reduced by avoiding drugs that impair hemostasis (e.g., alcohol, antiplatelet agents, anticoagulants) and invasive procedures (e.g., intramuscular injections). If drug-induced thrombocytopenia is suspected, as many medications as possible, especially those started within the preceding 5 to 14 days, should be stopped. Life-threatening bleeding episodes should be treated with platelet transfusion regardless of the mechanism of the thrombocytopenia.

The underlying cause and anticipated natural history of the thrombocytopenic disorder influence the decision about prophylactic platelet transfusion. As a rule, patients with chronic thrombocytopenic disorders characterized by increased platelet destruction (e.g., chronic immune thrombocytopenia [ITP]) or chronic underproduction (e.g., aplastic anemia or myelodysplasia) can tolerate long periods of severe thrombocytopenia without major bleeding (see Chapter 113). In addition, prophylactic platelet transfusions can trigger alloimmunization against human leukocyte antigen (HLA) or platelet antigens, thereby jeopardizing future therapeutic platelet transfusions. Consequently, prophylactic platelet transfusions are seldom indicated for such patients except when they are at risk of bleeding because of trauma or major surgery. When platelets are given, the platelet count should be maintained above 50×10^9/L ($>100\times10^9$/L, if possible, for central nervous system procedures). Invasive procedures such as thoracentesis, paracentesis, and liver biopsy are not usually associated with excess bleeding if the platelet count is greater than 50×10^9/L.

Prophylactic platelet transfusions should not be given to patients with strongly suspected or confirmed HIT, TTP, HUS, and, possibly, DIC, because they may exacerbate platelet-mediated thrombotic complications, and, particularly with HIT, mucocutaneous bleeding is uncommon. However, bleeding from severe thrombocytopenia, or other patient-specific considerations, may justify platelet transfusion even in these disorders.

ANATOMY AND PHYSIOLOGY

The Spleen: Anatomy and Function

The spleen is a small, well-perfused organ that receives about 5% of the total cardiac output. In adults, the spleen weighs between 150 and 200 g and measures approximately 11 cm in length.

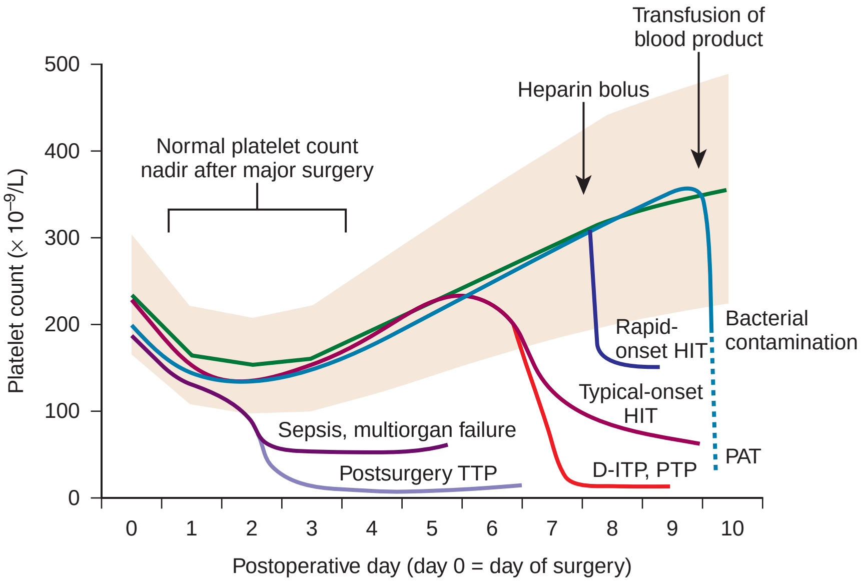


Figure 130.2 TIMING OF ONSET AND SEVERITY OF THROMBOCYTOPENIA: IMPLICATIONS FOR DIFFERENTIAL DIAGNOSIS. The usual postoperative platelet count nadir is seen between postoperative days 1 to 3 (inclusive). Early and progressive platelet count declines often reflect severe postoperative complications such as sepsis and multiorgan failure; severe thrombocytopenia plus microangiopathic hemolysis can (rarely) indicate postsurgery thrombotic thrombocytopenic purpura. Thrombocytopenic disorders that begin approximately 1 week after surgery are often immune mediated: moderate thrombocytopenia can indicate heparin-induced thrombocytopenia (HIT), both "typical onset" or (if heparin is not being given) "delayed onset"; very severe thrombocytopenia can indicate drug-induced immune thrombocytopenia (D-ITP) or (rarely) posttransfusion purpura. An abrupt decline in platelet count after receiving a heparin bolus in a patient who has received heparin within the past 7 to 100 days can indicate "rapid-onset" HIT; thrombocytopenia that begins abruptly after transfusion of a blood product can indicate sepsis from bacterial contamination or (rarely) passive alloimmune thrombocytopenia caused by transfusion of platelet-reactive alloantibodies. *D-ITP*, Drug-induced immune thrombocytopenia; *HIT*, heparin-induced thrombocytopenia; *PAT*, passive alloimmune thrombocytopenia; *PTP*, posttransfusion purpura; *TTP*, thrombotic thrombocytopenic purpura. *(Reprinted, with permission, from Greinacher A, Warkentin TE. Acquired non-immune thrombocytopenia. In: Marder VJ, Aird WC, Bennett JS, et al., eds. Hemostasis and Thrombosis: Basic Principles and Clinical Practice. 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:796.)*

The anatomy of the spleen is uniquely suited for its function; progressive branching of the splenic artery into trabecular and central arteries helps separate the plasma from the cellular elements (see Chapter 156). The central arteries arise perpendicularly from the trabecular arteries and skim the plasma layer from the cells. Soluble antigens in the plasma are delivered to the white pulp, where phagocytic cells process them and antibody production is initiated.

A cell-rich, hemoconcentrated fraction of the blood is delivered to the red pulp. Some of this blood flows directly to the splenic veins (the closed system), but most moves into the splenic cords (the open system). Here, the cellular elements percolate through a meshwork of reticulum fibers, reticuloendothelial cells, and supporting cells to reach the splenic sinuses. The cells enter the sinuses by passing through narrow fenestrations in the basement membrane of the endothelial cells lining the sinuses. The blood exits through the splenic vein into the portal system. Because the veins in the portal system lack valves, any increase in portal pressure is transmitted to the splenic microcirculation.

The spleen plays many important roles. It is the largest lymphoid organ in the body and contributes to host defense by clearing microorganisms and antibody-coated cells. The spleen is also important for antibody synthesis, especially antibodies directed against soluble antigens. The filtering function of the spleen includes (1) culling (removal of damaged or senescent cells and bacteria), (2) pitting (removal of RBC inclusion bodies or parasites), and (3) remodeling (reticulocyte sequestration and maturation). The spleen also serves as a reservoir of platelets (accommodating about one-third of the platelet mass in normal individuals). By contrast, the human spleen contains less than 2% of the total RBC mass, although in some animals (dogs and cats), the spleen is a much more important RBC reservoir.

Physiologic Platelet Sequestration

Radiolabeled platelet studies have shown that approximately 30% of the total platelet mass exists as a freely exchangeable pool in the spleen. Because the normal platelet life span is 9 to 10 days, platelets spend approximately one-third of their lives, or 3 days, within the spleen. In patients with hypersplenism, up to 90% of the platelets can be found in the spleen.

After labeled platelets are injected, there is accumulation in both the liver and the spleen. An initial, irreversible phase of hepatic uptake occurs. This equilibrates during the first 5 minutes and may reflect hepatic clearance of platelets damaged during the labeling procedure. Simultaneously, there is a slow increase in activity over the spleen that peaks in about 20 minutes. Splenic platelet uptake depends on input (spleen blood flow) and output (clearance).

The splenic platelet pool size can decrease and the platelet count increase with intravenous infusions of epinephrine in normal persons and in patients with splenomegaly. By contrast, isoprenaline increases the splenic pool size. Splenic blood flow increases with increasing spleen size, although perfusion (flow per unit of tissue volume) falls. Blood flow can increase in some inflammatory disorders (e.g., SLE) without an increase in spleen size. A marked increase or decrease in splenic perfusion alters the proportion of platelets within the spleen.

Fig. 130.3 shows why approximately 30% of the platelets are normally present in the spleen.[2] Because about 5% of cardiac output goes to the spleen and because the average splenic transit time (i.e., the time for the platelet to pass through the spleen) is approximately 10 minutes—compared with the usual average time of 1 minute for a platelet to make a complete circulatory pass—approximately one-third of the platelets are within the spleen (i.e., 5%×10 minutes/95%×1 minute, or a ratio of 50 : 95, or ≈1:2). With hypersplenism, the splenic blood flow can increase up to fivefold (i.e., from 5% to 25% of total blood flow). Thus, even without an increase in splenic transit time, 70% or more of the platelets can be reversibly sequestered within the spleen.

The most important determinant of the splenic platelet pool is the spleen size. The measurement of spleen size can thus be helpful in predicting the degree of thrombocytopenia expected from excess platelet pooling in the spleen. For example, if 90% of the platelet pool is in the spleen (i.e., 10% outside the spleen), the platelet count

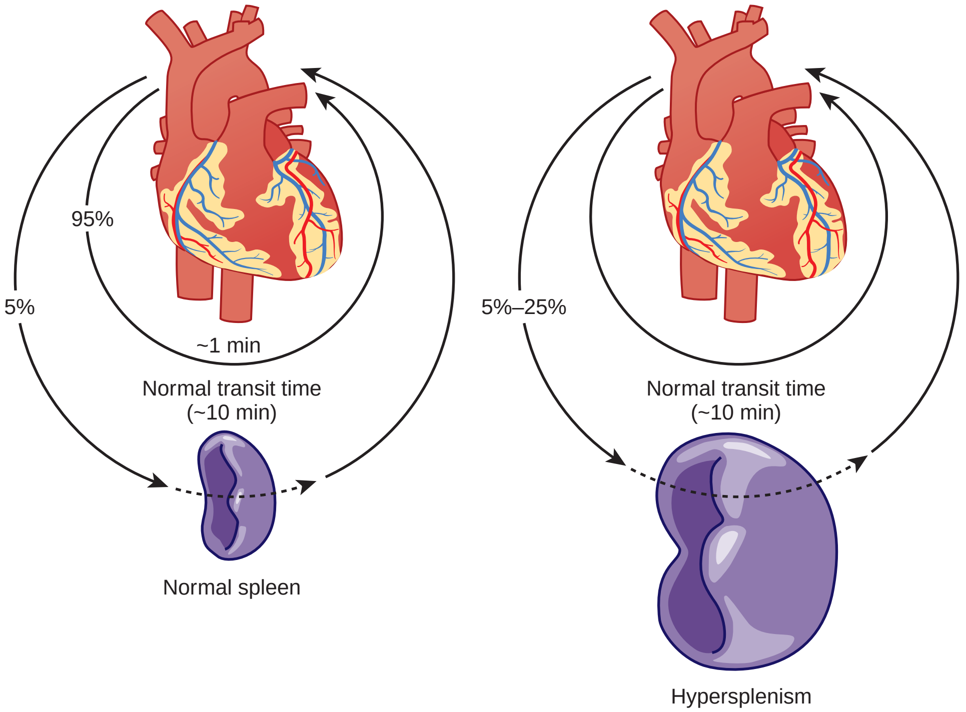


Figure 130.3 PHYSIOLOGIC AND PATHOLOGIC PLATELET SPLENIC SEQUESTRATION. Normally, about 5% of cardiac output is to the spleen; however, a platelet that enters the spleen spends about 10 minutes there (splenic transit time = 10 minutes). In contrast, it usually takes only about 1 minute for a platelet to make a circulatory pass elsewhere. Thus, at any one time about one-third of the platelets are located within the spleen: (5% × 10 minutes):(95% × 1 minute), or an approximate 1:2 ratio. In hypersplenism, the splenic blood flow can increase by a factor of 5, that is, from 5% to 25% of total blood flow per minute. Thus, even without increase in splenic transit time, up to 70% or more of the platelets can be reversibly sequestered within the spleen. *(From Arnold DM, Warkentin TE. Thrombocytopenia and thrombocytosis. In: Wilson WC, Grande CM, Hoyt DB, eds. Trauma: critical care. Vol. 2. New York: Informa Healthcare USA; 2007:983.)*

will be reduced by sevenfold because normally, 70% of platelets lie outside the spleen. Consequently and as a general rule, even if the spleen is massively enlarged, severe thrombocytopenia ($<20 \times 10^9$/L) is unusual. On the other hand, mild thrombocytopenia may be explained by mild splenomegaly that may not be detectible on physical examination but can be seen with imaging studies.

PATHOLOGIC PLATELET SEQUESTRATION: HYPERSPLENISM

Definition

Hypersplenism is a syndrome characterized by splenomegaly and any or all of the following cytopenias: anemia, leukopenia, or thrombocytopenia. Implicit in the definition is that the cytopenias will correct after splenectomy. Although splenomegaly is almost always present in hypersplenism, some patients with splenomegaly do not have hypersplenism. Hypersplenism usually is the result of an identifiable pathologic process, but rarely, the cause of the splenomegaly remains elusive, and the hypersplenism is termed *primary*.

Pathogenesis

A list of disorders producing splenomegaly and hypersplenism is presented in Table 130.2. An increase in the size of the spleen can be caused by several mechanisms. Increased workload of the spleen can be caused by immunologic stress (infection, inflammation, or an autoimmune disorder) or by increased RBC removal (RBC membrane disorders, hemoglobinopathies). Portal hypertension also increases the size of the spleen, producing congestive splenomegaly. Benign and malignant infiltrative disorders may increase splenic size (infiltrative splenomegaly) and cause hypersplenism. Some of these disorders produce thrombocytopenia by more than just hypersplenism (e.g., BM infiltration with tumor, immune-mediated platelet clearance). Thus the demonstration of an enlarged spleen does not necessarily mean that the cytopenias are solely the result of hypersplenism.

Thrombocytopenia of hypersplenism is caused primarily by increased splenic platelet pooling. A massively enlarged spleen can hold more than 90% of the total platelet mass. Without altered platelet production, the total body platelet mass usually is normal, and the platelet life span is near normal. Usually, the splenic transit time remains normal (≈10 minutes), but the absolute number of platelets retained within the enlarged spleen is increased. All the sequestered platelets remain part of the exchangeable pool. In hypersplenism, the thrombocytopenia is moderately severe (platelet counts of 50×10^9/L to 150×10^9/L). Severe thrombocytopenia ($<20 \times 10^9$/L) suggests another diagnosis, or (in the setting of advanced liver disease) concomitant contributors such as hyperfibrinolysis and decreased (hepatically synthesized) thrombopoietin production. It is unusual for patients with hypersplenism alone to have evidence of hemostatic impairment attributable to thrombocytopenia or to need specific interventions to raise the platelet count. Plasma volume expansion occurs in hypersplenism, but hemodilution plays a relatively minor role in the thrombocytopenia.

The neutropenia of hypersplenism is caused by an increase in the marginated granulocyte pool, a portion of which is located in the spleen. The neutropenia of hypersplenism is usually asymptomatic.

Diagnosis

Thrombocytopenia is likely to be caused by hypersplenism when (1) splenomegaly is present, (2) the thrombocytopenia is mild to

TABLE 130.2 **Differential Diagnosis of Splenomegaly and Hypersplenism**

Infections
Acute
Viral (viral hepatitis, infectious mononucleosis, CMV infection)
Bacterial (septicemia, salmonellosis, brucellosis, splenic abscess)
Parasite (toxoplasmosis)
Subacute and Chronic
Subacute bacterial endocarditis
Tuberculosis
Malaria
Kala-azar
Fungal disease
Inflammation
Felty syndrome
SLE
Serum sickness
Rheumatic fever
Sarcoidosis
ALPS
Congestive Splenomegaly
Intrahepatic
Cirrhosis
Extrahepatic
Portal vein obstruction
Splenic vein obstruction
Hepatic vein occlusion (Budd-Chiari syndrome)
Chronic Passive Congestion
Heart failure
Hematologic Disorders
RBC disorders: hemolytic anemias, thalassemia, sickle cell disorders
Neoplasia
Malignant
MPDs
Myeloid metaplasia
Polycythemia rubra vera
Essential thrombocythemia
Chronic leukemia
Chronic myeloid leukemia
Chronic lymphocytic leukemia
Hairy cell leukemia
Lymphoma
Acute leukemia
Malignant histiocytosis
Benign
Hamartoma
Hemangioma
Lymphangioma
Fibroma
Storage Diseases
Gaucher disease
Niemann-Pick disease
Miscellaneous
Amyloidosis
Cysts

ALPS, Autoimmune lymphoproliferative syndrome; *CMV*, cytomegalovirus; *MPD*, myeloproliferative disorder; *RBC*, red blood cell; *SLE*, systemic lupus erythematosus.

moderate in severity, (3) moderately reduced neutrophil counts and low-normal hemoglobin levels are found, and (4) there is no or minimal evidence of impaired hematopoiesis on BM examination. Ultrasonography, computed tomography, and radionuclide imaging are of comparable sensitivity for documenting splenomegaly, and an imaging study should be performed if splenomegaly is not evident on physical examination. The mean platelet volume is often slightly decreased in hypersplenism, but this finding is not sufficiently specific to be diagnostically useful. An ^{111}In-labeled platelet survival study can be diagnostic of hypersplenism, demonstrating reduced platelet recovery and a normal platelet life span. Determining the cause of the splenomegaly is usually the most important issue.

Therapy

Several maneuvers can improve or correct the cytopenias attributable to hypersplenism, including total or partial splenectomy; partial splenic embolization; and in patients with congestive splenomegaly, surgical or transjugular intrahepatic portosystemic shunting. However, cytopenias secondary to hypersplenism and thrombocytopenia are almost never of sufficient severity to justify such treatment. Consequently, the decision to perform one of these interventions usually depends on other considerations. For example, splenectomy should be considered for pain relief or early satiety associated with massive splenomegaly (e.g., in myelo- or lymphoproliferative disorders) or for splenomegaly of unknown origin (for investigation of possible splenic lymphoma).

Short-term complications from splenectomy include infections, bleeding, and thromboembolism. The major long-term risk associated with splenectomy is overwhelming septicemia; this risk can be reduced by vaccination. All patients should be vaccinated against pneumococci, meningococci, and *Haemophilus* species at least 2 weeks before elective splenectomy. Moreover, "booster" doses of pneumococcus and meningococcus vaccines are recommended after 5 years.[14] Splenectomy for congestive hypersplenism in the setting of portal hypertension is associated with high morbidity and mortality rates. Splenectomy is also associated with high morbidity (50%) and mortality (10% to 15%) rates in myeloid metaplasia and does not alter the natural history of this disorder. Thus, splenectomy is usually performed for palliation of intractable symptoms.

Splenectomy in Gaucher disease usually corrects the cytopenias, relieves abdominal discomfort, and improves growth in children. Partial, rather than total, splenectomy has been used to avoid shifting the deposition of glucocerebroside from the spleen to the bones. Often, however, splenomegaly and hypersplenism recur after partial splenectomy. Enzyme replacement therapy can reduce the morbidity from hypersplenism (see Chapter 54).

DRUG-INDUCED THROMBOCYTOPENIC SYNDROMES

Many drugs can cause thrombocytopenia. Some drugs (e.g., anticancer chemotherapeutic agents, linezolid,[15] valproic acid[16]) cause dose-dependent thrombocytopenia, generally through myelosuppressive mechanisms. An important disorder encountered by hematologists is unexpected thrombocytopenia caused by immunologic (idiosyncratic) mechanisms.[17–20] The frequency of these reactions varies considerably among drugs, but is generally very rare (<1:10,000) for commonly used drugs such as acetaminophen, indomethacin, naproxen, quinine, and trimethoprim-sulfamethoxazole (TMP-SMX). The most frequently implicated drugs are vancomycin, TMP-SMX, piperacillin, rifampin, and oxaliplatin.

Immunologic drug-induced thrombocytopenia can occur through different mechanisms (Fig. 130.4). For example, thrombocytopenia can occur when the Fab terminus of the pathogenic IgG binds to a complex composed of drug (or drug metabolite) and a platelet membrane component (typically, platelet GPIIb/IIIa or GPIb/IX/V). The Fc portions of the pathogenic IgG molecules do

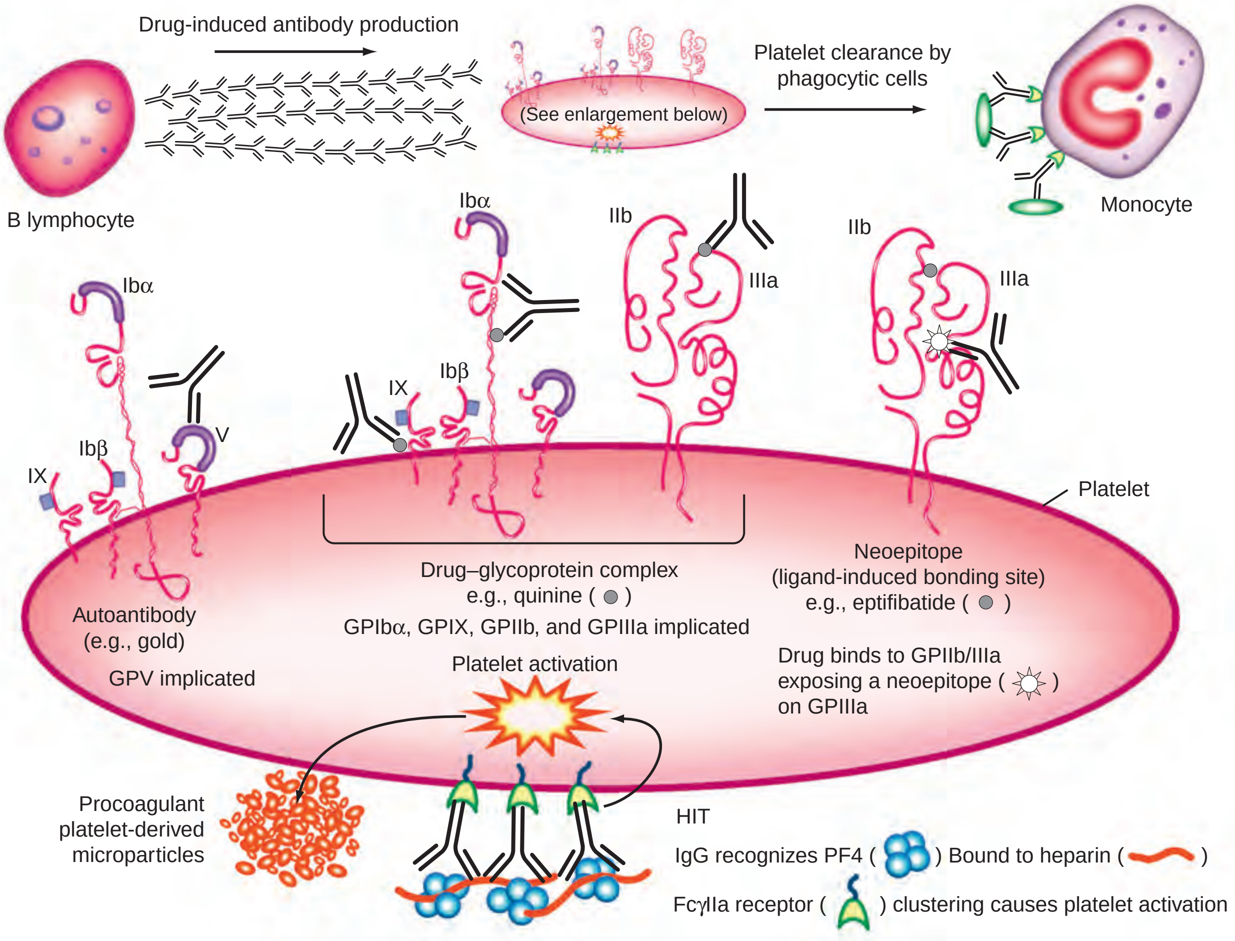

Drug-induced antibody production
(See enlargement below)
Platelet clearance by phagocytic cells
B lymphocyte
Monocyte
Ibα
IIb
IIIa
IIb
IIIa
Ibα
Ibβ
IX
V
Ibβ
IX
Platelet
Neoepitope
(ligand-induced bonding site)
e.g., eptifibatide (●)
Drug–glycoprotein complex
e.g., quinine (●)
GPIbα, GPIX, GPIIb, and GPIIIa implicated
Autoantibody
(e.g., gold)
GPV implicated
Platelet activation
Drug binds to GPIIb/IIIa
exposing a neoepitope (☼)
on GPIIIa
Procoagulant
platelet-derived
microparticles
HIT
IgG recognizes PF4 ( ) Bound to heparin ( )
FcγIIa receptor ( ) clustering causes platelet activation

Figure 130.4 MECHANISMS OF DRUG-INDUCED IMMUNE THROMBOCYTOPENIA. Four immune thrombocytopenic syndromes are illustrated. On the bottom of the schematic platelet, heparin-induced thrombocytopenia (HIT) is illustrated, indicating that immunoglobulin G (IgG) antibodies bind to complexes of platelet factor 4 (PF4) and heparin, with the Fc regions of the antibodies binding to the platelet FcγIIa receptors, resulting in platelet activation (including generation of procoagulant, platelet-derived microparticles). On the *top* of the schematic platelet, three mechanisms are illustrated that lead to increased platelet clearance by phagocytic cells. From left to right, these are (1) autoantibody-induced immune thrombocytopenia (e.g., gold-induced anti-glycoprotein V [GPV] antibodies). (2) Drug-dependent antibodies reactive against drug (or drug metabolite)–platelet glycoprotein complex(es) (e.g., quinine-induced thrombocytopenia in which drug-dependent antibodies against GPIbα, GPIX, GPIIb, and GPIIIa have been implicated, resulting in an antibody/drug/glycoprotein ternary complex), and (3) antibodies against neoepitope(s) formed in the presence of a drug (e.g., eptifibatide-induced immune thrombocytopenia caused by formation of a ligand-induced binding site elsewhere on the GPIIb/IIIa complex after eptifibatide binding). Note that preexisting (naturally occurring) antibodies can explain abrupt-onset thrombocytopenia in a patient receiving eptifibatide for the first time.

not bind to platelets but interact with Fc receptors on phagocytic cells of the reticuloendothelial system, which ingest the platelets, leading to accelerated platelet clearance. Severe thrombocytopenia (platelet counts $<20 \times 10^9$/L) is typically observed. This mechanism is exemplified by quinine- and vancomycin-induced thrombocytopenia. A complicating issue is that quinine and quinidine can also rarely cause thrombocytopenia with a clinical picture of microangiopathic hemolysis and/or DIC.

Sometimes, the Fab terminus binds to a neoepitope on platelet GPIIb/IIIa induced by the drug, and the drug itself is not part of the neoepitope. This mechanism is exemplified by the GPIIb/IIIa receptor antagonists, eptifibatide and tirofiban, which bind to the Arg-Gly-Asp recognition site on GPIIb/IIIa, forming a neoepitope to which the "fiban-dependent" antibodies bind. In contrast, the GPIIb/IIIa receptor antagonist, abciximab (a human/murine chimeric Fab fragment specific for GPIIIa) can lead to severe immune thrombocytopenia if the patient has antibodies (preexisting or acquired) that recognize the murine component of the chimeric antibody.

Another distinct form of drug-induced thrombocytopenia is exemplified by HIT (see Chapter 130). In this disorder, the Fab portion of the pathogenic IgG binds to platelet factor 4 (PF4), an α-granule protein that is immunogenic when complexed to heparin or certain other polyanions. The Fc portions of the IgG molecules bind to platelet FcγIIa receptors, initiating intense platelet activation (see Fig. 130.4). Perhaps because HIT is a platelet activation syndrome or because of the relatively low number of HIT antibodies that bind to platelet surfaces, the thrombocytopenia is typically mild to moderate rather than severe (see Fig. 131.3 in Chapter 131).

DRUG-INDUCED IMMUNE THROMBOCYTOPENIA

A large number of drugs can cause a syndrome that mimics acute ITP (D-ITP); however, there are relatively few drugs—approximately two dozen—for which causation is well established on both clinical and serologic grounds (Table 130.3).[21–24] Typically, severe thrombocytopenia (platelet count usually $<20 \times 10^9$/L), together with petechiae and purpura, develop within approximately 1 week (although occasionally much longer) after initiation of therapy with the responsible drug. D-ITP is much less common than HIT. For example, a relatively "common" cause of this syndrome is TMP-SMX (co-trimoxazole),

TABLE 130.3 List of Drugs Implicated in Drug-Induced Immune Thrombocytopenia

Drugs Common to All Three Lists

Abciximab,[a,b] acetaminophen, amiodarone, ampicillin, eptifibatide,[a,c] ethambutol, carbamazepine,[a] ceftriaxone,[a] ciprofloxacin, furosemide, haloperidol, oxaliplatin,[a] naproxen, phenytoin,[a] piperacillin,[a] quinine,[a] quinidine,[a] ranitidine, rifampin,[a] sulfisoxazole, tirofiban,[a,c] trimethoprim-sulfamethoxazole,[a] vancomycin[a]

Oklahoma Website[21,22]: Definite/Probable	Milwaukee (per OHU Website)[23]	Arnold[24]: Definite/ Probable
Aceclofenac	Amitriptyline	Amlodipine
Acetazolamide	Amoxicillin	Cefamandole
Acetyldigoxin	Argatroban	Diazepam
Acyclovir	Aspirin	Gold[d]
Adalimumab	Atenolol	Lorazepam
Adefovir	Azacitidine	Mirtazapine
Alatrofloxacin	Aztreonam	Penicillin
Albendazole	Bumetanide	Rosiglitazone
Alfuzocin	Bupropion	Tranilast
Alprenolol	Cefadroxil	
Aminoglutethimide	Cefazolin	
Aminosalicylic acid	Cefepime	
Amlodipine	Cefpodoxime	
Amphotericin B	Ceftazidime	
Anakinra	Celecoxib	
Aspirin	Cephalexin	
Atorvastatin	Clindamycin	
Bismuth	Cyclosporine	
Bisoprolol	Daptomycin	
Butoconazole	Diphenhydramine	
Captopril	Dobutamine	
Carvedilol	Doxycycline	
Cefamandole	Esomeprazole	
Chlorothiazide	Ethosuximide	
Chlorpheniramine	Exenatide	
Chlorpromazine	Felbamate	
Chlorpropamide	Felodipine	
Cilastatin/imipenem	Fentanyl	
Cimetidine	Fexofenadine	
Citalopram	Hydroxychloroquine	
Citrus flavonoids	Ibrutinib	
Clarithromycin	Irinotecan	
Clidinium/chlordiazepoxide	Lamotrigine	
Clopidogrel	Lansoprazole	
Cranberry juice	Leucovorin	
Dabigatran	Levofloxacin	
Dactinomycin/actinomycin	Lisinopril	
Danazol	Loracarbef	
Desferoxamine	Lorazepam	
Desipramine	Losartan	
Dexamethasone	Methylprednisolone	
Diatrizoate meglumine	Metronidazole	
Diazepam	Nafcillin	
Diazoxide		
Diclofenac		
Diethylstilbestrol		
Difluoromethylornithine (eflornithine)		

TABLE 130.3 List of Drugs Implicated in Drug-Induced Immune Thrombocytopenia—cont'd

Drugs Common to All Three Lists

Abciximab,[a,b] acetaminophen, amiodarone, ampicillin, eptifibatide,[a,c] ethambutol, carbamazepine,[a] ceftriaxone,[a] ciprofloxacin, furosemide, haloperidol, oxaliplatin,[a] naproxen, phenytoin,[a] piperacillin,[a] quinine,[a] quinidine,[a] ranitidine, rifampin,[a] sulfisoxazole, tirofiban,[a,c] trimethoprim-sulfamethoxazole,[a] vancomycin[a]

Oklahoma Website[21,22]: Definite/Probable	Milwaukee (per OHU Website)[23]	Arnold[24]: Definite/ Probable
Diflunisal	Nitrofurantoin	
Digitoxin	Nizatidine	
Digoxin	Olanzapine	
Diltiazem	Olmesartan	
Dipyridamole	Ondansetron	
Doxepin	Oxaprozin	
Drospirenone/ ethinylestradiol	Oxcarbazepine	
Etanercept	Pantoprazole	
Etretinate	Papaverine	
Ezetimibe	Paroxetine	
Famotidine	Phenobarbital	
Felbamate	Piperacillin/ Tazobactam*	
Fenoprofen	Procainamide	
Feprazone	Propoxyphene	
Finasteride	Propranolol	
Flucloxacillin	Propylthiouracil	
Fluconazole	Protamine	
Fluoxetine	Quetiapine	
Formestane	Rifaxim	
Gabapentin	Rituximab	
Gentamicin	Sertraline	
Glucagon	Simvastatin	
Glyburide	Spironolactone	
Gold[d]	Tacrolimus	
Hepatitis B vaccine	Trazodone	
Human Papillomavirus vaccine	Trimethoprim	
Hydrochlorothiazide	Valproic acid	
Hydrochlorothiazide/ triamterene	Verapamil	
Iloprost	Voriconazole	
Imatinib	Zolmitriptan	
Imipramine		
Inamrinone		
Indinavir		
Indomethacin		
Infliximab		
Influenza vaccine		
Interferon-α		
Iocetamic acid		
Iopamidol		
Iopanoic acid		
Ioversol		
Ipilimumab		
Irinotecan		
Iron dextran		

TABLE 130.3 List of Drugs Implicated in Drug-Induced Immune Thrombocytopenia—cont'd

Drugs Common to All Three Lists

Abciximab,[a,b] acetaminophen, amiodarone, ampicillin, eptifibatide,[a,c] ethambutol, carbamazapine,[a] ceftriaxone,[a] ciprofloxacin, furosemide, haloperidol, oxaliplatin,[a] naproxen, phenytoin,[a] piperacillin,[a] quinine,[a] quinidine,[a] ranitidine, rifampin,[a] sulfisoxazole, tirofiban,[a,c] trimethoprim-sulfamethoxazole,[a] vancomycin[a]

Oklahoma Website[21,22]: Definite/Probable	Milwaukee (per OHU Website)[23]	Arnold[24]: Definite/ Probable
Isoniazid		
Isotretinoin		
Itraconazole		
Jui (herbal medicine)		
Lamivudine		
Lamotrigine		
Leucovorin		
Levamisole		
Levetiracetam		
Levofloxacin		
Lithium		
Lopinavir/ritonavir		
Losartan		
Lupinus termis bean		
Mebhydrolin		
Meclofenamate		
Meloxicam		
Meningococcal C vaccine		
Meprobamate		
Melperone		
Meropenem		
Mesalamine		
Methazolamide		
Methicillin		
Methyldopa		
Methylphenidate		
Methylprednisolone		
Mexiletine		
Minoxidil		
Mirtazapine		
Molsidomine		
Morphine		
Moxifloxacin		
Nalidixic acid		
Naphazoline		
Niacin (vitamin B3)		
Nimesulide		
Nitroglycerin		
Octreotide		
Oseltamivir/probenecid		
Oxcarbazepine		
Oxprenolol		
Oxyphenbutazone		
Oxytetracycline		
Pantoprazole		
Peginterferon α2b		
Penicillin		
Pentoxifylline		
Phenobarbital		
Piperacillin/tazobactam		
Piperazine		
Primidone		
Procainamide		
Pyrazinamide		
Quetiapine		
Rifabutin		
Risperidone		
Rituximab		
Rosiglitazone		
Rosuvastatin		
Sho-saiko-to		
Simvastatin		
Sodium stibogluconate		
Spironolactone		
Sulfadiazine		
Sulfamethoxypyridazine		
Sulfapyridine		
Sulfasalazine		
Sulfathiazole		
Tahini (pulp sesame seeds)		
Tamoxifen		
Teicoplanin		
Terbinafine		
Thalidomide		
Thiothixene		
Tiagabine		
Ticlopidine		
TNFα/INFγ		
Tolmetin		
Tranilast		
Trastuzumab		
Vitamin A palmitate		
Zoledronic acid		

Heparin, as a trigger of heparin-induced thrombocytopenia (HIT), a distinct disorder, is not included in this table.

D-ITP, Drug-induced immune thrombocytopenia; *IFN*, interferon; *TNF*, tumor necrosis factor.

[a] More than 10 patients identified in Milwaukee lab.

[b] Subtype of D-ITP where patient's antibodies (either naturally occurring or that are formed after treatment with abciximab) bind to platelet GPIIb/IIIa after binding of abciximab (chimeric human-murine Fab that recognizes GPIIb/IIIa) to platelets.

[c] Subtype of D-ITP caused by fiban-dependent antibodies (either naturally occurring or formed after treatment with eptifibatide or tirofiban) that recognize neoepitopes on platelet GPIIb/IIIa that form after binding of fiban drug to GPIIb/IIIa.

[d] Gold induces autoantibodies, not typical drug-dependent antibodies.

even though it occurs in only approximately 1 in 25,000 patients who receive this drug combination.

Important exceptions to these generalizations occur with D-ITP that results from GPIIb/IIIa receptor antagonists. These reactions are relatively common (affecting about 0.5% to 1% of patients) and usually occur within hours of first use because of preexisting, naturally occurring antibodies. Another exception is carbimazole-induced thrombocytopenia, in which mild thrombocytopenia is explained by the presence of relatively small quantities of the platelet GP target (platelet endothelial cell adhesion molecule-1 [PECAM-1]) on the platelet surface.[25] Besides the atypical immune-mediated syndromes of HIT and GPIIb/IIIa antagonist thrombocytopenia, the most common drugs (in absolute terms) implicated in the causation of classic D-ITP syndrome are quinine (outpatients) and vancomycin (inpatients).[26]

Pathogenesis

Drug-dependent binding of the Fab component of IgG to platelet GP leads to platelet destruction. This occurs because the IgG-sensitized platelets are recognized by Fc receptors of phagocytic cells. For quinine- and quinidine-induced ITP, both the GPIIb/IIIa and GPIb/IX/V complexes have been implicated as targets for the drug-dependent IgG (see Fig. 130.4). By contrast, for sulfa antibiotic- and naproxen-induced ITP, the GPIIb/IIIa complex is predominantly involved. Sometimes, drug metabolites form the antigen rather than the parent drug. A trimolecular complex is formed among IgG Fab, the drug (or metabolite), and the platelet GP. In contrast to HIT, platelet Fc receptors are not involved in D-ITP pathogenesis. Drug-dependent IgG binding is remarkably heterogeneous with respect to binding affinity, number of binding sites per platelet, and the range of drug concentrations required. A study of quinine-induced ITP identified two different types of antibodies: quinine-dependent antibodies that bound to platelets in the presence of drug and quinine-specific antibodies that reacted with quinine-conjugated albumin.[27] The pathophysiologic implications of this latter group of antibodies remain unclear.

The fundamental mechanism that accounts for antibody formation in a small proportion of patients is unknown. One group has proposed that drug-dependent platelet-reactive antibodies are derived from a pool of naturally occurring autoantibodies with inherently weak (nonpathological) affinity for certain platelet membrane GPs.[17,27] However, if a certain drug is able to enhance antibody–antigen interaction, and if B cells expressing such antibodies are induced to proliferate and undergo affinity maturation in such a patient, the resulting antibody can destroy the platelets in the presence of the drug.

Clinical Features

Patients with D-ITP typically present with petechiae, purpura, and severe thrombocytopenia (platelet count often $<20 \times 10^9$/L). Systemic symptoms, such as fever and chills, may occur in patients with abrupt-onset thrombocytopenia. Usually, the thrombocytopenia becomes clinically apparent 1 to 2 weeks after initiation of the drug, but the thrombocytopenia can start after a patient has been taking a drug for several years. Typically, the platelet count begins to rise a few days after stopping the implicated drug, but occasionally several weeks are required for recovery, possibly because of the generation of drug-independent IgG (platelet autoantibodies).

Sometimes, drug exposure is relatively obscure. Among outpatients, the physician needs to inquire about potential exposure to quinine. Quinine is widely available—for example, as an ingredient in tonic water, as an additive to street drugs, and in some countries as therapy for leg cramps. Vancomycin is a relatively common cause of D-ITP in hospitalized inpatients. Most often, this occurs after the usual intravenous administration, but some reports have implicated exposure via orthopedic cement or with peritoneal administration of vancomycin.

Rarely, distinct drug-dependent IgG molecules destroy RBCs or WBCs in addition to platelets. For example, both platelet- and leukocyte-reactive quinidine-dependent IgG molecules have been detected in a patient with quinidine-induced bicytopenia. Sometimes, the immune process is directed against a pluripotent hematopoietic stem cell, resulting in pancytopenia accompanied by BM aplasia or hypoplasia (e.g., gold-, carbamazepine-, or quinidine-induced pancytopenia). A BM aspirate should be performed in patients with suspected drug-induced bicytopenia or pancytopenia because a hypoplastic BM may be indicative of drug-induced aplastic anemia.

Diagnosis

A high index of clinical suspicion is required to make the diagnosis. Moreover, clinicians need to evaluate which drug (often among several that the patient is receiving) is most likely to explain a diagnosis of D-ITP.[21–24] The clinician should focus on drugs that have been started 5 to 10 days before the onset of the platelet count decrease (Fig. 130.5A). Also, with drugs such as quinine, in which there may be intermittent exposure (e.g., consumption of gin and tonic), a very recent exposure (previous 1 or 2 days) usually explains the abrupt onset of symptomatic thrombocytopenia.

After the physician has identified one or more potential agents, the next step is to determine whether these drugs have previously been implicated as causes of D-ITP. The box Search Strategies When Investigating a Patient With Possible Drug-Induced Immune Thrombocytopenic Purpura includes some strategies for identifying drugs implicated in D-ITP[28–31].

SEARCH STRATEGIES WHEN INVESTIGATING A PATIENT WITH POSSIBLE DRUG-INDUCED IMMUNE THROMBOCYTOPENIC PURPURA

Three sources of information as to whether a drug has been implicated as a cause of D-ITP:

- **PubMed search** (http://www.pubmed.ncbi.nlm.nih.gov): (name of drug) and (thrombocytopenia). By way of example, the author encountered a patient who developed severe thrombocytopenia 5 days after starting treatment with mirtazapine. Searching (mirtazapine) and (thrombocytopenia) in December 2014 identified two reports[28,29] of mirtazapine-induced D-ITP syndrome. By 2020, mirtazapine-induced D-ITP was an established entity.[30]
- **Drug-induced thrombocytopenia website** (http://www.ouhsc.edu/platelets/ditp.html): Investigators at the University of Oklahoma published a comprehensive survey of drugs implicated in D-ITP using clinical criteria[21]; a website maintained by these investigators is updated every 2 years (last updated, 2018[22]).
- **Database from drug-dependent platelet-reactive antibody testing** at the BloodCenter of Wisconsin, 1995–present (http://www.ouhsc.edu/platelets/InternetPostingLab2_18_11Frames.htm): the BloodCenter of Wisconsin maintains a website reporting its experience in detecting drug-dependent platelet-reactive antibodies.[31]

Demonstration of drug-dependent binding of IgG to platelets in vitro can be important for diagnosis (see Fig. 130.5B). Labeled Ig-specific probes (e.g., flow cytometry assays) or GP capture techniques (e.g., MAIPA) can be used. In other cases, D-ITP test results are negative, but the diagnosis still seems likely based on clinical features and supporting literature.

There are certain caveats in diagnostic testing. First, metabolites must sometimes be used instead of the parent drug to detect the IgG.

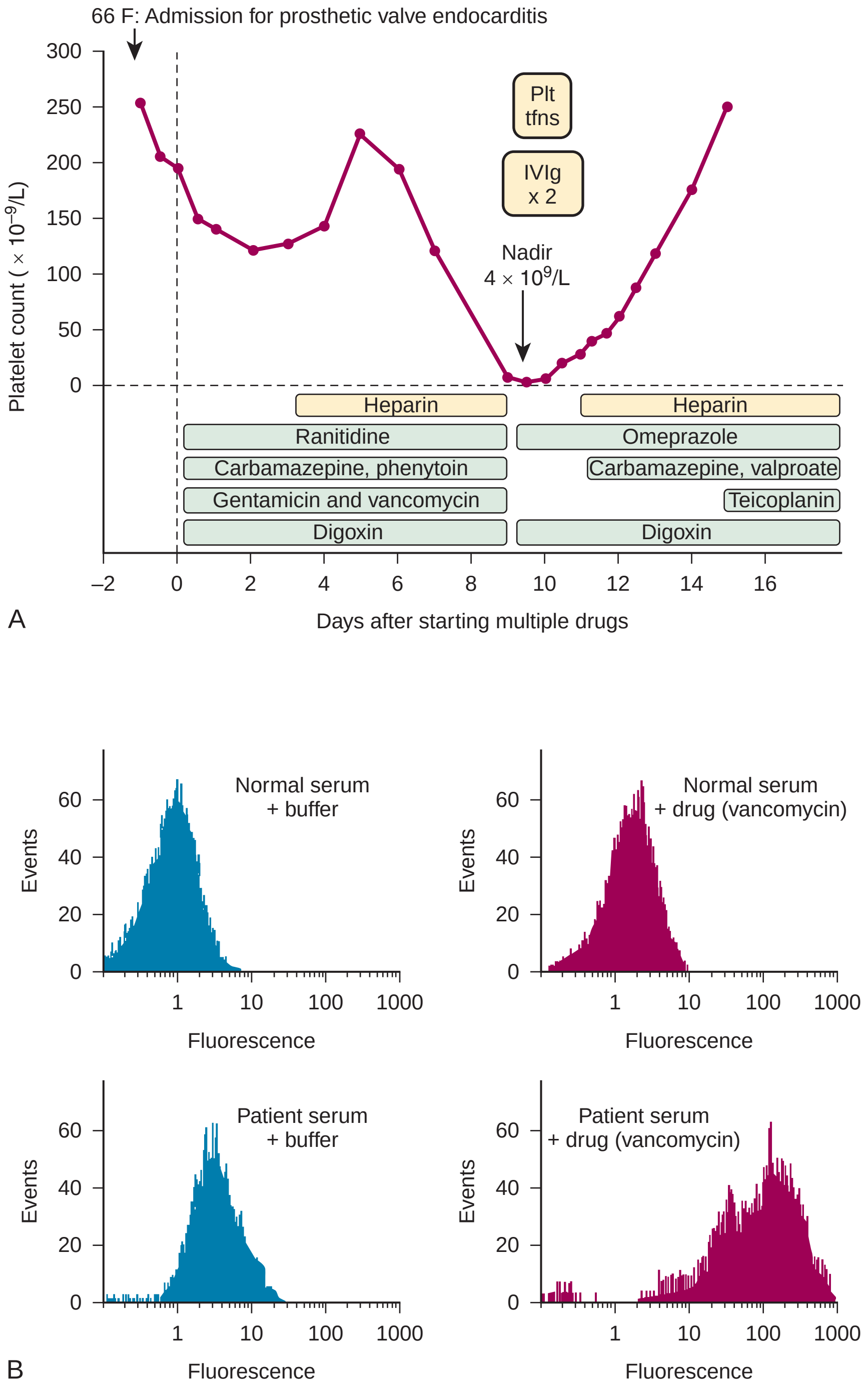


Figure 130.5 DRUG-INDUCED IMMUNE THROMBOCYTOPENIA (D-ITP) SECONDARY TO VANCOMYCIN. (A) Timeline of drug-induced immune thrombocytopenia (D-ITP). A 66-year-old woman was admitted for prosthetic valve endocarditis 5 months after undergoing mitral valve replacement. The initiation of multiple new drugs and the onset 6 days later of progressively severe thrombocytopenia (platelet count nadir, 4×10^9/L on day 9) suggested D-ITP syndrome. However, the timing fit several drugs (ranitidine, carbamazepine, phenytoin, gentamicin, vancomycin, and digoxin). (B) Drug-dependent binding of antibodies was demonstrated using patient serum and vancomycin. Also, test results for heparin-induced thrombocytopenia antibodies were negative. Thus the diagnosis of vancomycin-induced D-ITP syndrome was made based on clinical and serological grounds. The patient received treatment with high-dose intravenous immunoglobulin (IVIG) and platelet transfusions. *Plt tfns*, Platelet transfusions.

Second, the target drug (or metabolite) must be included in the wash buffer used in these assays. Third, despite these maneuvers, the sensitivity of in vitro assays is relatively low, and the diagnosis of D-ITP must often be made on clinical grounds. Sometimes, the diagnosis is confirmed by inadvertent or deliberate reexposure to the suspected drug. However, deliberate drug challenge is not often performed because of its potential risk.

An intriguing approach to identify drug-dependent platelet-reactive antibodies was reported by investigators at the Milwaukee Blood Center.[32] They used nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (which lack xenoantibodies, thereby allowing infused human platelets to circulate) to identify drug-dependent antibodies in patient sera, including antibodies that only recognize drug metabolites (presumably, mice produce the same or similar drug metabolites as humans, which are recognized by the drug-dependent antibodies).

Management

As many drugs as possible should be discontinued in patients with suspected D-ITP. If further drug treatment is necessary, an alternate, immunologically non-cross-reactive substitute should be used. Spontaneous improvement in the platelet count usually begins within a few days of discontinuing the offending drug, although in some cases, complete recovery may take 2 weeks or longer. Platelet transfusions should be given to patients with life-threatening bleeding. High-dose intravenous immunoglobulin (IVIg), 1 g/kg given over 6 to 8 hours, with a second dose 1 or 2 days later if required, may be helpful in some situations. Corticosteroids appear to be relatively ineffective for treatment of D-ITP.

DRUG-INDUCED IMMUNE THROMBOCYTOPENIA OF RAPID ONSET

A rapid onset of thrombocytopenia (within hours) can occur if a patient with preexisting drug-dependent antibodies is (re)exposed to the drug.[33] This situation is relatively common in HIT (≈25% of patients identified)[5] because repeated treatment with heparin is common and heparin use itself can result in the complication (HIT-associated thrombosis) that might lead to further use of heparin. Because HIT antibodies are transient, however, rapid-onset HIT occurs in patients with recent heparin exposures, usually within the past 100 days.[5] By contrast, repeated episodes of quinine-induced thrombocytopenia of abrupt onset can occur many months or even years apart because these antibodies persist for much longer. Thrombocytopenia of rapid onset is commonly seen with GPIIb/IIIa receptor antagonists (see next section).

THROMBOCYTOPENIA CAUSED BY GLYCOPROTEIN IIB/IIIA RECEPTOR ANTAGONISTS

Several thrombocytopenic syndromes have been reported with use of GPIIb/IIIa receptor antagonists (abciximab, eptifibatide, tirofiban) administered during percutaneous coronary intervention (e.g., angioplasty, stenting): (1) rapid-onset, severe thrombocytopenia within 12 hours of drug administration (in 0.4% to 2% of patients), (2) rapid-onset pseudothrombocytopenia (in approximately 1% of patients exposed to abciximab [ReoPro]), (3) rapid-onset thrombocytopenia within 12 hours of a second exposure to a GPIIb/IIIa antagonist, and (4) delayed-onset thrombocytopenia beginning 4 to 7 days after drug administration (rare).[6] The frequency of rapid-onset thrombocytopenia is higher among patients who receive a second course of therapy, especially if it follows the initial exposure by only a few weeks. Thrombocytopenia is typically severe (median platelet count nadir, about 5 × 10^9/L to 10 × 10^9/L), but clinical effects vary dramatically, ranging from absence of petechiae or other signs of bleeding (in 50% of patients) to fatal hemorrhage (in less than 5%). Some patients develop anaphylactoid reactions accompanying the abrupt platelet count declines. The antibodies may cause thrombosis in some patients, likely because the pathogenic antibodies also activate platelets.[6] Fig. 130.6 illustrates an example of delayed-onset, abciximab-induced thrombocytopenia; in such patients, high-titer antibodies triggered by abciximab recognize

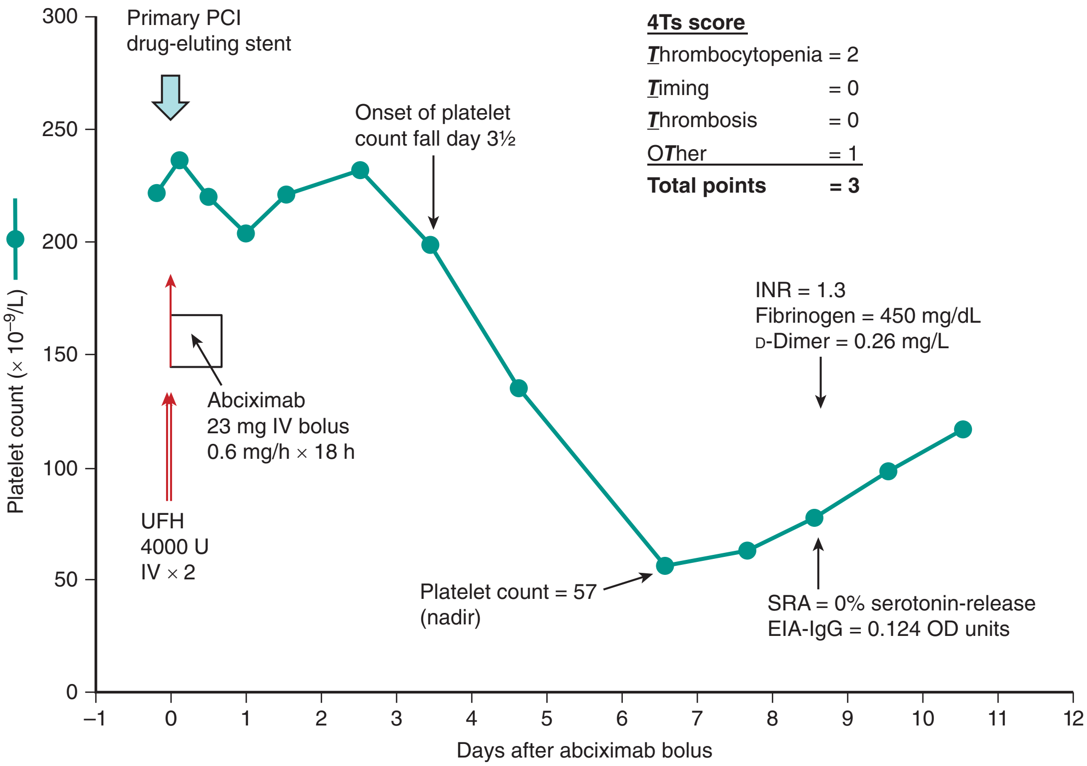


Figure 130.6 DELAYED-ONSET ABCIXIMAB-INDUCED IMMUNE THROMBOCYTOPENIA. Moderate thrombocytopenia (platelet count nadir, 57 × 10^9/L) in an elderly patient started several days after percutaneous coronary intervention (PCI) during which unfractionated heparin (UFH) and abciximab were administered. This sequence of events suggested immune thrombocytopenia triggered either by heparin (delayed-onset heparin-induced thrombocytopenia [HIT]) or abciximab. The unusually early onset of thrombocytopenia (day 3½), the normal D-dimer (reference range <0.50 mg/L), and negative HIT antibody assays ruled out HIT. *EIA*, Enzyme-immunoassay; *INR*, international normalized ratio; *IV*, intravenous, *OD*, optical density; *PCI*, percutaneous coronary intervention; *SRA*, serotonin-release assay; *U*, units.

platelets to which abciximab remains irreversibly bound several days post-exposure.

These syndromes are caused by at least three mechanisms. First, antibodies of IgG (and possibly IgM) class can bind to neoepitopes on the GPIIb/IIIa complex generated by these drugs (or their metabolites); that is, ligand-induced binding sites (see Fig. 130.4). Up to 5% of humans and nonhuman primates have naturally occurring IgG antibodies that will bind to GPIIb/IIIa receptors in the presence of drug, which could explain why rapid-onset thrombocytopenia occurs so frequently with these agents. A second mechanism is applicable to abciximab, a chimeric Fab fragment comprised of murine GPIIb/IIIa-reactive sequences and human framework sequences. Interestingly, although a high frequency of normal persons (74%) have antibodies that recognize platelets coated with abciximab, these "normal" antibodies were shown to differ from those detected in patients in whom thrombocytopenia developed after a second exposure to abciximab: whereas the pathogenic antibodies recognized murine sequences within abciximab, the "normal" (nonpathogenic) antibodies were specific for the carboxyl terminus (papain cleavage site) of Fab fragments prepared from normal human IgG. Drug-dependent antibodies can also be generated about 1 week after exposure. The antibodies differ among patients with respect to the precise neoepitopes recognized and display variable degrees of cross-reactivity among the different GPIIb/IIIa receptor antagonists ("fibans"). This explains why a repeat treatment course with another GPIIb/IIIa receptor antagonist may not necessarily cause thrombocytopenia. A third mechanism involves eptifibatide-dependent antibodies that activate platelets via their FcγIIa receptors.[6]

Some naturally occurring antibodies only bind to GPIIb/IIIa when the calcium concentration is low, thus explaining pseudothrombocytopenia—falsely low platelet count estimates caused by ex vivo aggregation of platelets in blood samples collected into calcium-chelating anticoagulants, especially EDTA.

Platelet transfusions are indicated in patients who are bleeding, although their efficacy has not been established. Platelet transfusions are most likely to be effective for treating abciximab-induced thrombocytopenia because the drug binds so tightly to GPIIb/IIIa that little abciximab is free to bind to the transfused platelets. Platelet transfusions are not indicated in pseudothrombocytopenia, which underscores the importance of reviewing the blood film when a low platelet count is reported after treatment with one of these agents.

Various in vitro assays using flow cytometry or enzyme-linked immunosorbent assay (ELISA) have been developed to detect these antibodies. Because some pathogenic antibodies are naturally occurring, it is theoretically possible to identify patients at high risk for rapid-onset thrombocytopenia in elective situations (see box on Approach to Patients With Thrombocytopenia Following Percutaneous Coronary Intervention).

APPROACH TO PATIENTS WITH THROMBOCYTOPENIA FOLLOWING PERCUTANEOUS CORONARY INTERVENTION

Acute-onset thrombocytopenia

Four diagnoses should be considered in patients who develop thrombocytopenia within minutes or a few hours after a PCI and have received one or more of the following agents: (1) platelet GPIIb/IIIa inhibitor (e.g., abciximab, eptifibatide, tirofiban), (2) heparin, or (3) iodinated contrast agent.

- **GPIIb/IIIa inhibitor–induced pseudothrombocytopenia**. The patient has no symptoms or signs of bleeding, and platelet aggregates are seen in the blood film. The platelet count is falsely reported as low by the automated particle counter, which fails to count aggregated platelets. No treatment is required.
- **GPIIb/IIIa inhibitor–induced thrombocytopenia**. The platelet count falls abruptly, often to profoundly reduced levels (typical nadir, $<20 \times 10^9$/L). Hemostatic impairment is variable, ranging from petechiae to fatal hemorrhages; occasionally, patients develop anaphylactoid reactions or even associated thrombosis. Treatment involves stopping all platelet antagonists and anticoagulants and giving platelets if the patient has signs of bleeding. Prophylactic platelet transfusions can also be considered if the platelet count is very low (e.g., $<10 \times 10^9$/L). Testing for drug-dependent antibodies can be accomplished using flow cytometry or ELISA.
- **Rapid-onset HIT**. In patients who have preexisting HIT antibodies because of recent heparin exposure (generally within the past 100 days), rapid-onset HIT can occur when heparin is given during PCI. This is much less common than GPIIb/IIIa inhibitor–induced thrombocytopenia or pseudothrombocytopenia, so presumptive treatment of HIT with a nonheparin anticoagulant is rarely indicated in this situation. The platelet count nadir is usually much higher than with GPIIb/IIIa inhibitor–induced thrombocytopenia.
- **Radiocontrast-induced ITP**. Very rarely, patients who have previously received iodinated contrast can develop abrupt-onset, severe thrombocytopenia after exposure to contrast during PCI. Platelet transfusions (with or without high-dose IVIg) are appropriate for a bleeding patient.

Delayed-onset thrombocytopenia

Fig. 130.6 illustrates the clinical and laboratory course of a patient who developed thrombocytopenia approximately 1 week after PCI. The differential diagnosis consisted of delayed-onset HIT versus delayed-onset abciximab-induced thrombocytopenia. Ultimately, the diagnosis of HIT was discarded because (a) the onset of thrombocytopenia (day 3½) was too soon for HIT; (b) normal D-dimer argued against HIT-associated hypercoagulability, and (c) testing for HIT antibodies was negative. Thus, the patient most likely had delayed-onset, abciximab-induced thrombocytopenia.

ELISA, Enzyme-linked immunosorbent assay; *HIT,* heparin-induced thrombocytopenia; *ITP*, induced thrombocytopenia; *IVIg*, intravenous immunoglobulin; *PCI*, percutaneous coronary intervention.

Malignancy

Thrombocytopenia complicating malignant disorders most frequently results from antineoplastic treatment or BM replacement by tumor. However, certain thrombocytopenic syndromes have been associated with malignancy, including autoimmune thrombocytopenia, DIC, and TMA.

ITP attributable to platelet GP-reactive autoantibodies can complicate neoplastic lymphoproliferative diseases such as Hodgkin disease, non-Hodgkin lymphoma, chronic lymphocytic leukemia, and multiple myeloma. Sometimes, the thrombocytopenia responds to treatment of the neoplasm, although in some patients (particularly those with Hodgkin disease), the thrombocytopenia is indistinguishable from ITP and is not related to the activity of the lymphoma.

DIC occurs with certain malignancies, particularly adenocarcinoma of the pancreas, stomach, lung, colon, breast, and prostate (see Chapter 137). Some patients present with venous or, less commonly, arterial thrombosis as the first clinical manifestation of their malignancy. In these patients, the presence of thrombocytopenia is an important clue that should prompt investigations for DIC, such as measurement of fibrinogen and D-dimer levels. In the author's experience, the platelet count typically rises to normal or even elevated levels with heparin therapy because heparin ameliorates the DIC process; however, recurrent thrombocytopenia and thrombosis can occur within hours of discontinuing the heparin therapy (Fig. 130.7).[34] Cancer patients with DIC and venous thrombosis are also at increased risk for warfarin-associated venous limb gangrene.[35,36] The role of various tumor-associated procoagulant substances—such as "cancer procoagulant" (a 68-kDa cysteine proteinase that activates factor X independently of tissue factor/factor VIIa) and cancer-associated tissue factor (which is expressed on tumor cells and circulating microparticles)—suggest pathophysiologic parallels with microthrombosis in HIT because both cancer-associated DIC and acute HIT are risk factors for phlegmasia cerulea dolens and venous limb gangrene during anticoagulation with warfarin. Thus, cancer patients with DIC should receive heparin (especially LMWH) rather than

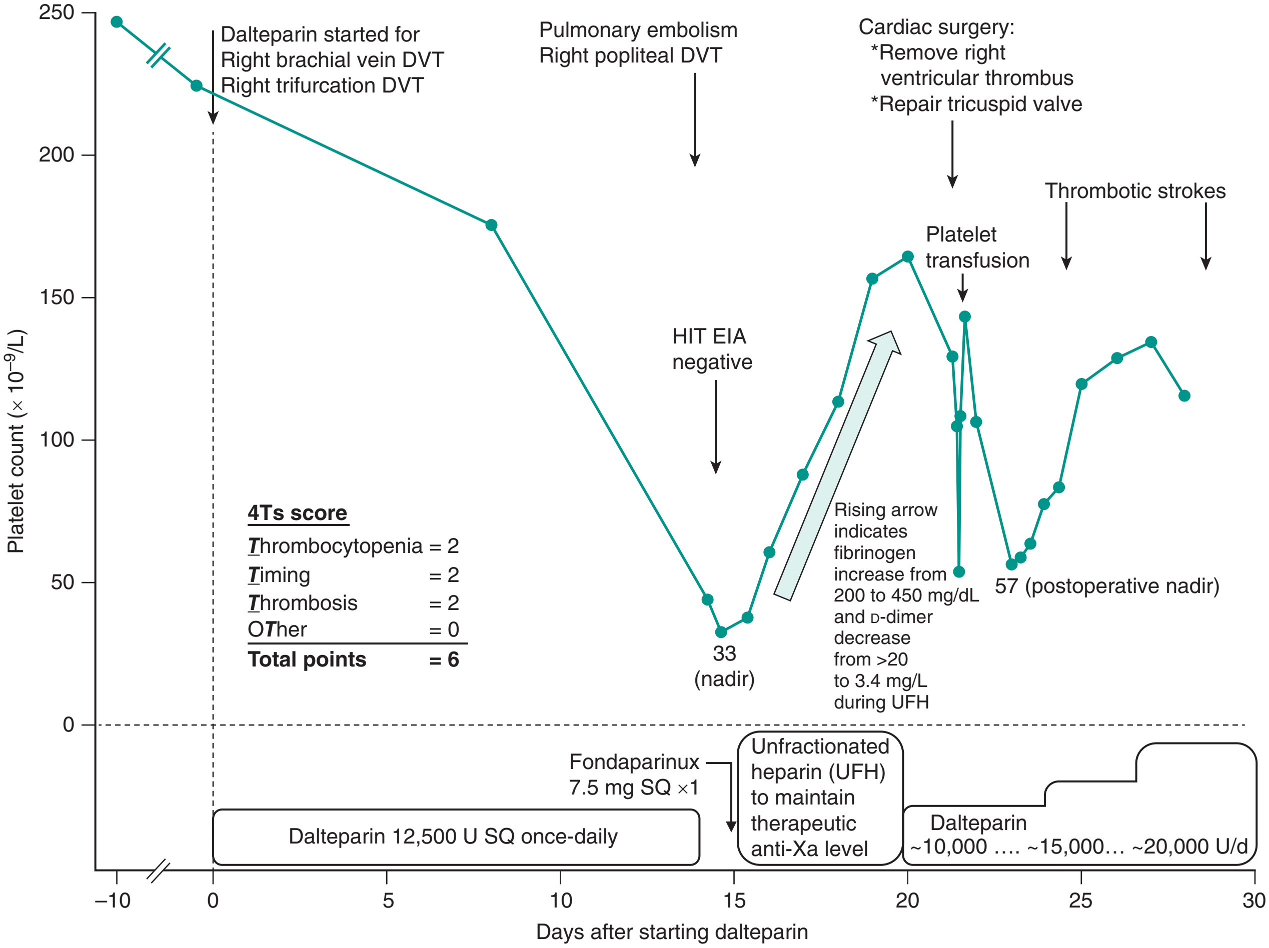


Figure 130.7 CANCER-ASSOCIATED DISSEMINATED INTRAVASCULAR COAGULATION MIMICKING HEPARIN-INDUCED THROMBOCYTOPENIA. This patient was diagnosed with metastatic adenocarcinoma of the lung (malignant pericardial effusion, cerebral, and adrenal metastases), and received dalteparin for brachial and trifurcation deep-vein thrombosis (DVT). Severe thrombocytopenia occurred approximately 2 weeks later. Despite a strong clinical picture for heparin-induced thrombocytopenia (HIT) based on a high 4Ts score (6 points), testing for HIT antibodies was negative by enzyme-linked immunosorbent assay (ELISA). Resumption of heparin resulted in correction of the thrombocytopenia and improvement in markers of disseminated intravascular coagulation (DIC) (rising fibrinogen and falling D-dimer levels). The diagnosis was cancer-associated DIC, with exacerbation of thrombocytopenia when heparin was held or was subtherapeutic. *DVT*, Deep-vein thrombosis; *EIA*, enzyme-immunoassay; *SQ*, subcutaneous; *SRA*, serotonin-release assay; *UFH*, unfractionated heparin.

warfarin anticoagulation (see box on Diagnostic Considerations in the Patient with Limb Ischemia and Thrombocytopenia in Chapter 131). Sometimes, nonbacterial thrombotic endocarditis (“marantic” endocarditis) with associated embolic phenomena (e.g., strokes, splenic infarcts) and DIC can occur in association with metastatic cancer.[37,38]

DIC with hemorrhagic manifestations is characteristically seen in some patients with prostate cancer and in many patients with acute promyelocytic leukemia. It is crucial to recognize promyelocytic leukemia because treatment with all-*trans*-retinoic acid induces differentiation of the malignant cells, thereby rapidly reducing the life-threatening bleeding risks attributable to hyperfibrinolysis. Further, antifibrinolytic therapy (e.g., tranexamic acid), which is generally *contraindicated* in DIC states—can be used to improve hemostasis in hyperfibrinolysis secondary to prostate cancer or promyelocytic leukemia.

A destructive thrombocytopenic disorder that resembles HUS or TTP has been described in patients with advanced cancer. In some patients, mitomycin, gemcitabine, or other drugs may have contributed to the microangiopathy.[39–41] DIC is not usually present. Some patients respond transiently to plasmapheresis, but for many, response to any therapy is poor.

HEMODILUTION AND PLATELET CONSUMPTION AFTER SURGERY

Platelet count declines of 30% to 70% occur universally after major surgery and reflect the combined effects of hemodilution and increased platelet consumption. Such hemodilution-associated thrombocytopenia is especially prominent after cardiac surgery and is proportional to the amount of fluids (crystalloid, colloid, blood products) administered. The platelet count fall is abrupt and is evident immediately after surgery. Dilutional coagulopathy also is responsible for the minor to moderate prolongations in coagulation test results that occur transiently after surgery.

Perioperative hemodilution is usually accompanied by increased platelet consumption related to the effects of surgery. This helps to explain why the postsurgery platelet count usually continues to decline over the next 1 to 3 days, with the postoperative nadir (lowest platelet count value) usually occurring at a median of postoperative day 2, with a range between postoperative days 1 through 4 (Fig. 130.8).[2] Subsequently, there is a rise in the platelet count that peaks at approximately day 14 at levels often two to three times the patient’s preoperative baseline before it returns to baseline over the next 2

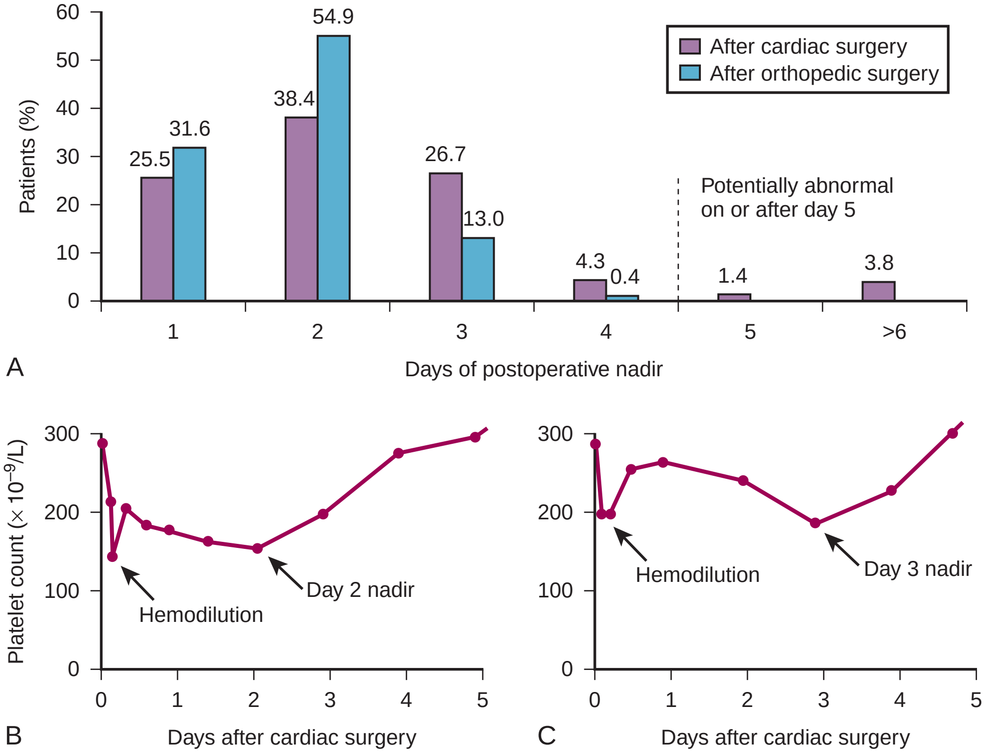


Figure 130.8 EARLY POSTOPERATIVE PLATELET COUNT DECLINES. (A) Distribution of early postoperative count nadirs. For both orthopedic and cardiac surgery patients, day 2 represents the most common day for the postoperative platelet count nadir to occur (data exclude day 0); beyond postoperative day 4, it is likely that a superimposed thrombocytopenic disorder is occurring. (B and C) Representative postcardiac surgery platelet count declines. Both patients illustrate early hemodilution effects (day 0) and subsequent additional early platelet count declines with nadirs on day 2 (B) and day 3 (C). Neither patient received platelet transfusions. *(From Greinacher A, Warkentin TE. Acquired non-immune thrombocytopenia. In: Marder VJ, Aird WC, Bennett JS, et al., eds. Hemostasis and Thrombosis: Basic Principles and Clinical Practice. 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2013:796.)*

weeks (≈day 28). As described earlier in this chapter (see Fig. 130.1), these platelet count changes reflect thrombopoietin physiology.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Arnold DM, Kukaswadia S, Nazi I, et al. A systematic evaluation of laboratory testing for drug-induced thrombocytopenia. *J Thromb Haemost*. 2013;11:169.

Arnold DM, Warkentin TE. Thrombocytopenia and thrombocytosis. In: Wilson WC, Grande CM, Hoyt DB, eds. *Trauma: Critical Care*. 2. New York: Informa Healthcare USA; 2007:983.

Asopa S, Patel A, Khan OA, et al. Non-bacterial thrombotic endocarditis. *Eur J Cardiothorac Surg*. 2007;32:696.

Aster RH, Bougie DW. Drug-induced immune thrombocytopenia. *N Engl J Med*. 2007;357:580.

Aster RH, Curtis BR, Bougie DW, et al. Thrombocytopenia associated with the use of GPIIb/IIIa inhibitors: position paper of the ISTH working group on thrombocytopenia and GPIIb/IIIa inhibitors. *J Thromb Haemost*. 2006;4:678.

Aster RH, Curtis BR, McFarland JG, et al. Drug-induced immune thrombocytopenia: pathogenesis, diagnosis, and management. *J Thromb Haemost*. 2009;7:911.

Bakchoul T, Jouni R, Warkentin TE. Protamine (heparin)-induced thrombocytopenia: a review of the serological and clinical features associated with anti-protamine/heparin antibodies. *J Thromb Haemost*. 2016;14:1685.

Bakchoul T, Marini I. Drug-associated thrombocytopenia. *Hematology Am Soc Hematol Educ Program*. 2018;2018:576.

Bell WR, Starksen NF, Tong S, Porterfield JK. Trousseau's syndrome. Devastating coagulopathy in the absence of heparin. *Am J Med*. 1985;79:423.

Bonanni P, Grazzini M, Niccolai G, et al. Recommended vaccinations for asplenic and hyposplenic adult patients. *Hum Vaccin Immunother*. 2017;13:359.

Bougie DW, Wilker PR, Aster RH. Patients with quinine-induced immune thrombocytopenia have both "drug-dependent" and "drug-specific" antibodies. *Blood*. 2006;108:922.

Buoli M, Serati M, Botturi A, Altamura AC. The risk of thrombocytopenia during valproic acid therapy: a critical summary of available clinical data. *Drugs R D*. 2018;18:1.

George JN, Nester CM. Syndromes of thrombotic microangiopathy. *N Engl J Med*. 2014;371:654.

Greinacher A, Warkentin TE. Acquired non-immune thrombocytopenia. In: Marder VJ, Aird WC, Bennett JS, eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*. 6th ed Philadelphia: Lippincott Williams & Wilkins; 2013:796.

Liu X, Sahud MA. Glycoprotein IIb/IIIa complex is the target in mirtazapine-induced immune thrombocytopenia. *Blood Cell Mol Dis*. 2003;30:241.

Mitta A, Curtis BR, Reese JA, George JN. Drug-induced thrombocytopenia: 2019 update of clinical and laboratory data. *Am J Hematol*. 2019;94:E76.

Natsumoto B, Yokota K, Omata F, Furukawa K. Risk factors for linezolid-associated thrombocytopenia in adult patients. *Infection*. 2014;42:1007.

Saleem R, Reese JA, George JN. Drug-induced thrombotic microangiopathy: an updated systematic review, 2014–2018. *Am J Hematol*. 2018;93:E241.

Shih AW, Lam AS, Warkentin TE. Levofloxacin-induced acute immune-mediated thrombocytopenia of rapid-onset. *J Pharm Pract*. 2018;31:234.

Vayne C, Guéry EA, Rollin J, et al. Pathophysiology and diagnosis of drug-induced immune thrombocytopenia. *J Clin Med*. 2020;9:2212.

Von Drygalski A, Curtis BR, Bougie DW, et al. Vancomycin induced immune thrombocytopenia. *N Engl J Med*. 2007;356:904.

Warkentin TE. Drug-induced immune-mediated thrombocytopenia—from purpura to thrombosis. *N Engl J Med*. 2007;356:891.

Warkentin TE. Ischemic limb gangrene with pulses. *N Engl J Med*. 2015;373:642.

Warkentin TE, Cook RJ, Sarode R, et al. Warfarin-induced venous limb ischemia/gangrene complicating cancer: a novel and clinically distinct syndrome. *Blood*. 2015;126:486.

Warkentin TE, Kelton JG. Temporal aspects of heparin-induced thrombocytopenia. *N Engl J Med*. 2001;344:1286.

Warkentin TE, Ning S, Lim W. Colloid transfusion, natural anticoagulant depletion, and symmetric peripheral gangrene. *N Engl J Med*. 2020;383(16):1592.

Warkentin TE, Smith JW. The alloimmune thrombocytopenic syndromes. *Transfus Med Rev*. 1997;11:296.

Warkentin TE, Roberts RS, Hirsh J, Kelton JG. An improved definition of immune heparin-induced thrombocytopenia in postoperative orthopedic patients. *Arch Intern Med*. 2003;163:2518.

Zmaili MA, Alzubi JM, Kocyigit D, et al. A contemporary 20-year Cleveland Clinic experience of non-bacterial thrombotic endocarditis: etiology, echocardiographic imaging, management, and outcomes. *Am J Med*. 2021;134(3):361.

REFERENCES

1. Warkentin TE, Roberts RS, Hirsh J, Kelton JG. An improved definition of immune heparin-induced thrombocytopenia in postoperative orthopedic patients. *Arch Intern Med*. 2003;163:2518.
2. Arnold DM, Warkentin TE. Thrombocytopenia and thrombocytosis. In: Wilson WC, Grande CM, Hoyt DB, eds. *Trauma: Critical Care*. 2. New York: Informa Healthcare USA; 2007:983.
3. Greinacher A, Warkentin TE. Acquired non-immune thrombocytopenia. In: Marder VJ, Aird WC, Bennett JS, eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*. 6th ed Philadelphia: Lippincott Williams & Wilkins; 2013:796.
4. Warkentin TE. Heparin-induced thrombocytopenia: pathogenesis and treatment. *Br J Haematol*. 2003;121:535.
5. Warkentin TE, Kelton JG. Temporal aspects of heparin-induced thrombocytopenia. *N Engl J Med*. 2001;344:1286.
6. Aster RH, Curtis BR, Bougie DW, et al. Thrombocytopenia associated with the use of GPIIb/IIIa inhibitors: position paper of the ISTH working group on thrombocytopenia and GPIIb/IIIa inhibitors. *J Thromb Haemost*. 2006;4:678.
7. Siegel DM, Cook RJ, Warkentin TE. Acute hepatic necrosis and ischemic limb necrosis. *N Engl J Med*. 2012;367:879.
8. Warkentin TE. Ischemic limb gangrene with pulses. *N Engl J Med*. 2015;373:642.
9. Warkentin TE, Ning S, Lim W. Colloid transfusion, natural anticoagulant depletion, and symmetric peripheral gangrene. *N Engl J Med*. 2020;383(16):1592.
10. Tordon B, Warkentin TE, Moore JC, et al., Post-cardiac surgery thrombotic thrombocytopenic purpura: a presence of anti-ADAMTS13 autoantibodies at preoperative baseline. *Platelets*. 2022;33(3):479.
11. Bakchoul T, Jouni R, Warkentin TE. Protamine (heparin)-induced thrombocytopenia: a review of the serological and clinical features associated with anti-protamine/heparin antibodies. *J Thromb Haemost*. 2016;14:1685.
12. Warkentin TE, Smith JW, Hayward CPM, et al. Thrombocytopenia caused by passive transfusion of anti-glycoprotein Ia/IIa alloantibody (anti-HPA-5b). *Blood*. 1992;79:2480.
13. Warkentin TE, Smith JW. The alloimmune thrombocytopenic syndromes. *Transfus Med Rev*. 1997;11:296.
14. Bonanni P, Grazzini M, Niccolai G, et al. Recommended vaccinations for asplenic and hyposplenic adult patients. *Hum Vaccin Immunother*. 2017;13:359.
15. Natsumoto B, Yokota K, Omata F, Furukawa K. Risk factors for linezolid-associated thrombocytopenia in adult patients. *Infection*. 2014;42:1007.
16. Buoli M, Serati M, Botturi A, Altamura AC. The risk of thrombocytopenia during valproic acid therapy: a critical summary of available clinical data. *Drugs R D*. 2018;18:1.
17. Aster RH, Bougie DW. Drug-induced immune thrombocytopenia. *N Engl J Med*. 2007;357:580.
18. Warkentin TE. Drug-induced immune-mediated thrombocytopenia—from purpura to thrombosis. *N Engl J Med*. 2007;356:891.
19. Arnold DM, Nazi I, Warkentin TE, et al. Approach to the diagnosis and management of drug-induced immune thrombocytopenia. *Transfus Med Rev*. 2013;27:137.
20. Bakchoul T, Marini I. Drug-associated thrombocytopenia. *Hematology Am Soc Hematol Educ Program*. 2018;2018:576.
21. George JN, Raskob GE, Shah SR, et al. Drug-induced thrombocytopenia: a systematic review of published case reports. *Ann Intern Med*. 1988;129:886.
22. Mitta A, Curtis BR, Reese JA, George JN: Drug-induced thrombocytopenia: 2019 update of clinical and laboratory data. Am J Hematol 94:E76, 2019.
23. www.ouhsc.edu/platelets/ditp; last accessed September 8, 2020.
24. Arnold DM, Kukaswadia S, Nazi I, et al. A systematic evaluation of laboratory testing for drug-induced thrombocytopenia. *J Thromb Haemost*. 2013;11:169.
25. Kroll H, Sun QH, Santoso S. Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a target glycoprotein in drug-induced thrombocytopenia. *Blood*. 2000;96:1409.
26. Von Drygalski A, Curtis BR, Bougie DW, et al. Vancomycin-induced immune thrombocytopenia. *N Engl J Med*. 2007;356:904.
27. Bougie DW, Wilker PR, Aster RH. Patients with quinine-induced immune thrombocytopenia have both “drug-dependent” and “drug-specific” antibodies. *Blood*. 2006;108:922.
28. Liu X, Sahud MA. Glycoprotein IIb/IIIa complex is the target in mirtazapine-induced immune thrombocytopenia. *Blood Cell Mol Dis*. 2003;30:241.
29. Stuhec M, Alisky J, Malesic I. Mirtazapine associated with drug-related thrombocytopenia: a case report. *J Clin Psychopharmacol*. 2014;34:662.
30. Vayne C, Guéry EA, Rollin J, et al. Pathophysiology and diagnosis of drug-induced immune thrombocytopenia. *J Clin Med*. 2020;9:2212.
31. Aster RH, Curtis BR, McFarland JG, et al. Drug-induced immune thrombocytopenia: pathogenesis, diagnosis, and management. *J Thromb Haemost*. 2009;7:911.
32. Bougie DW, Nayak D, Boylan B, et al. Drug-dependent clearance of human platelets in the NOD/scid mouse by antibodies from patients with drug-induced immune thrombocytopenia. *Blood*. 2010;116:3033.
33. Shih AW, Lam AS, Warkentin TE. Levofloxacin-induced acute immune-mediated thrombocytopenia of rapid-onset. *J Pharm Pract*. 2018;31:234.
34. Bell WR, Starksen NF, Tong S, Porterfield JK. Trousseau’s syndrome. Devastating coagulopathy in the absence of heparin. *Am J Med*. 1985;79:423.
35. Warkentin TE. Venous limb gangrene during warfarin treatment of cancer-associated deep venous thrombosis. *Ann Intern Med*. 2001;135(8 Pt 1):589.
36. Warkentin TE, Cook RJ, Sarode R, et al. Warfarin-induced venous limb ischemia/gangrene complicating cancer: a novel and clinically distinct syndrome. *Blood*. 2015;126:486.
37. Asopa S, Patel A, Khan OA, et al. Non-bacterial thrombotic endocarditis. *Eur J Cardiothorac Surg*. 2007;32:696.
38. Zmaili MA, Alzubi JM, Kocyigit D, et al. A contemporary 20-year Cleveland Clinic experience of non-bacterial thrombotic endocarditis: etiology, echocardiographic imaging, management, and outcomes. *Am J Med*. 2021;134(3):361–369.
39. George JN, Nester CM. Syndromes of thrombotic microangiopathy. *N Engl J Med*. 2014;371:654.
40. Al-Nouri ZL, Reese JA, Terrell DR, et al. Drug-induced thrombotic microangiopathy: a systematic review of published reports. *Blood*. 2015;125:605.
41. Saleem R, Reese JA, George JN. Drug-induced thrombotic microangiopathy: an updated systematic review, 2014–2018. *Am J Hematol*. 2018;93:E241.

CHAPTER 131

HEPARIN-INDUCED THROMBOCYTOPENIA

Theodore E. Warkentin

Heparin-induced thrombocytopenia (HIT) is the most important drug-induced immune-mediated cytopenia for several reasons. First, heparin is a widely used anticoagulant (see Chapter 143). Second, HIT is relatively common, occurring in approximately 1% to 3% of postoperative patients, and 0.2% to 0.5% of medical patients, who receive unfractionated heparin (UFH) derived from porcine intestine for 7 to 14 days.[1] Third, HIT frequently causes life- and limb-threatening venous or arterial thrombosis. Fourth, there are several pitfalls of HIT management, including the potential for thrombocytopenia and/or thrombosis to worsen despite stopping heparin, the high risk for warfarin-associated microthrombosis (most often manifesting as venous limb gangrene), and the potential for failure of approved direct thrombin inhibitor (DTI) therapy because of the confounding of activated partial thromboplastin time (aPTT)–monitored dosing that results from HIT-associated coagulopathies. Finally, various off-label therapies are gaining acceptance for treating HIT, including parenteral anticoagulants (fondaparinux, danaparoid), oral agents (rivaroxaban, apixaban), and even high-dose intravenous immunoglobulin (IVIG) to interrupt HIT antibody-induced platelet activation.

HIT is caused by platelet-activating immunoglobulin G (IgG) antibodies that bind to multimolecular complexes of cationic platelet factor 4 (PF4)—a member of the CXC subfamily of chemokines—bound to (anionic) heparin through electrostatic interactions.[2,3] Although anti-PF4/heparin antibodies are frequently triggered by heparin therapy, relatively few patients develop clinically evident HIT. Indeed, a major current problem with HIT is its "overdiagnosis"[4]: only 5% to 15% of patients who are referred for antibody testing have a serologic profile that supports a diagnosis of HIT.[5] The challenge for the clinician is to discern which of the (many) patients who develop thrombocytopenia in association with heparin therapy have HIT, a conundrum magnified by the observation that at most 50% of anti-PF4/heparin antibody-positive patients have "true" HIT, as indicated by the presence of platelet-activating IgG antibodies.[5]

EPIDEMIOLOGY

Table 131.1 lists risk factors for HIT. Patients at highest risk for HIT are those with multiple interacting risk factors (e.g., females given postoperative thromboprophylaxis with UFH for 2 weeks [frequency approximately 5%]).[6] Even higher frequencies (approximately 10%) are reported in patients with ventricular assist devices who are receiving therapeutic doses of UFH. Ironically, even though the risk for HIT appears to be somewhat higher in women (odds ratio, 1.5 to 2.0),[6] HIT is rare in pregnancy, particularly with the use of low-molecular-weight heparin (LMWH). The synthetic antithrombin-binding sulfated pentasaccharide, fondaparinux, although similarly immunizing as LMWH, is much less likely than LMWH to cause HIT, likely because fondaparinux does not usually increase the platelet-activating potential of HIT antibodies.[7] Indeed, fondaparinux is an effective treatment for HIT (discussed later).

Only a minority of patients who form anti-PF4/heparin antibodies following heparin treatment develop clinically evident HIT.[8] The proportion of antibody-positive patients who develop HIT ranges from as high as one-third (e.g., postorthopedic surgery thromboprophylaxis with UFH [nowadays rarely used for this indication]) to as few as 1 in 50 (postcardiac surgery patients).[9] Notably, the risk for HIT in postcardiac surgery patients who receive UFH thromboprophylaxis is only approximately 1% even though as many as 50% to 80% of patients develop detectable anti-PF4/heparin antibodies within 2 weeks of surgery.[10] In general, those at the highest risk for HIT are the subgroup of patients whose anti-PF4/heparin antibodies evince strong platelet-activating properties in vitro.[11] However, patient-dependent susceptibility factors are also important, because not all patients who form platelet-activating antibodies develop HIT.

PATHOBIOLOGY

Fig. 131.1 illustrates several features of HIT pathogenesis. Heparin binds reversibly and saturably to platelets and can weakly activate platelets in vitro through potentiation of $\alpha_{IIb}\beta_3$-mediated outside-inside signaling.[12] In general, the direct platelet-activating effects of heparin, as well as its immunogenicity, are proportional to its molecular mass and degree of sulfation, and associated ability to form ultra-large antigenic complexes, respectively; consequently, LMWH is less likely to cause HIT than UFH.[13] Antigen complexes are formed at relatively narrow PF4:heparin molar ratios, suggesting that immunization could reflect attainment of particular immunizing ratios of PF4 and heparin in vivo ("stoichiometric model of immunization").[14] A unique laboratory characteristic of HIT is that high heparin concentrations (10 to 100 units/mL) inhibit platelet activation by the pathogenic IgG[15,16]; this laboratory feature is exploited in diagnostic testing for HIT (see Diagnosis). The high overall frequency of anti-PF4/heparin immunization could reflect innate immunity, given that PF4/heparin ultra-large antigenic complexes bind to "natural" IgM in blood, activating the classical pathway of complement, with the complement-coated PF4/heparin/IgM complexes binding to B-cells via the complement receptor CD21, triggering the immune response.[17]

Platelet activation by HIT antibodies occurs because of platelet-activating HIT-IgG cross-link platelet FcγIIa receptors.[18] This leads to "strong" platelet activation, including the formation of procoagulant platelet-derived microparticles.[19] However, the high thrombotic risk of HIT likely reflects "pancellular" activation beyond platelets alone.[20,21] This includes HIT antibody-induced activation of endothelium and monocytes (with cell surface expression of tissue factor)[22,23] and activation of neutrophils (including resulting "netosis").[24,25] Moreover, injured vessels release von Willebrand factor (vWF) "strings" to which PF4 binds, resulting in additional platelet activation through interaction with HIT antibodies.[26]

The pancellular activation results in excess thrombin generation, which contributes to the pathogenesis of some of the unusual sequelae of HIT, which can include thrombosis involving large veins and arteries, as well as microvascular thrombosis (e.g., warfarin-associated venous limb gangrene.[27] HIT can also be complicated by overt (decompensated) disseminated intravascular coagulation (DIC).[28]

There is a characteristic timeline that underlies the HIT immune response (Fig. 131.2).[29,30] Formation of pathogenic IgG antibodies is surprisingly fast (detectable 4 to 5 days following initial heparin administration), even in patients who have never previously been exposed to heparin. Bacterial cell walls, which are negatively charged, bind PF4, and there is evidence that bacterial infection could be responsible for preimmunization against PF4-dependent antigens.[31] This could explain both the high frequency of anti-PF4/heparin immunization and of clinical HIT (i.e., HIT could represent a misdirected antibacterial immune response).[31] Although heparin-treated

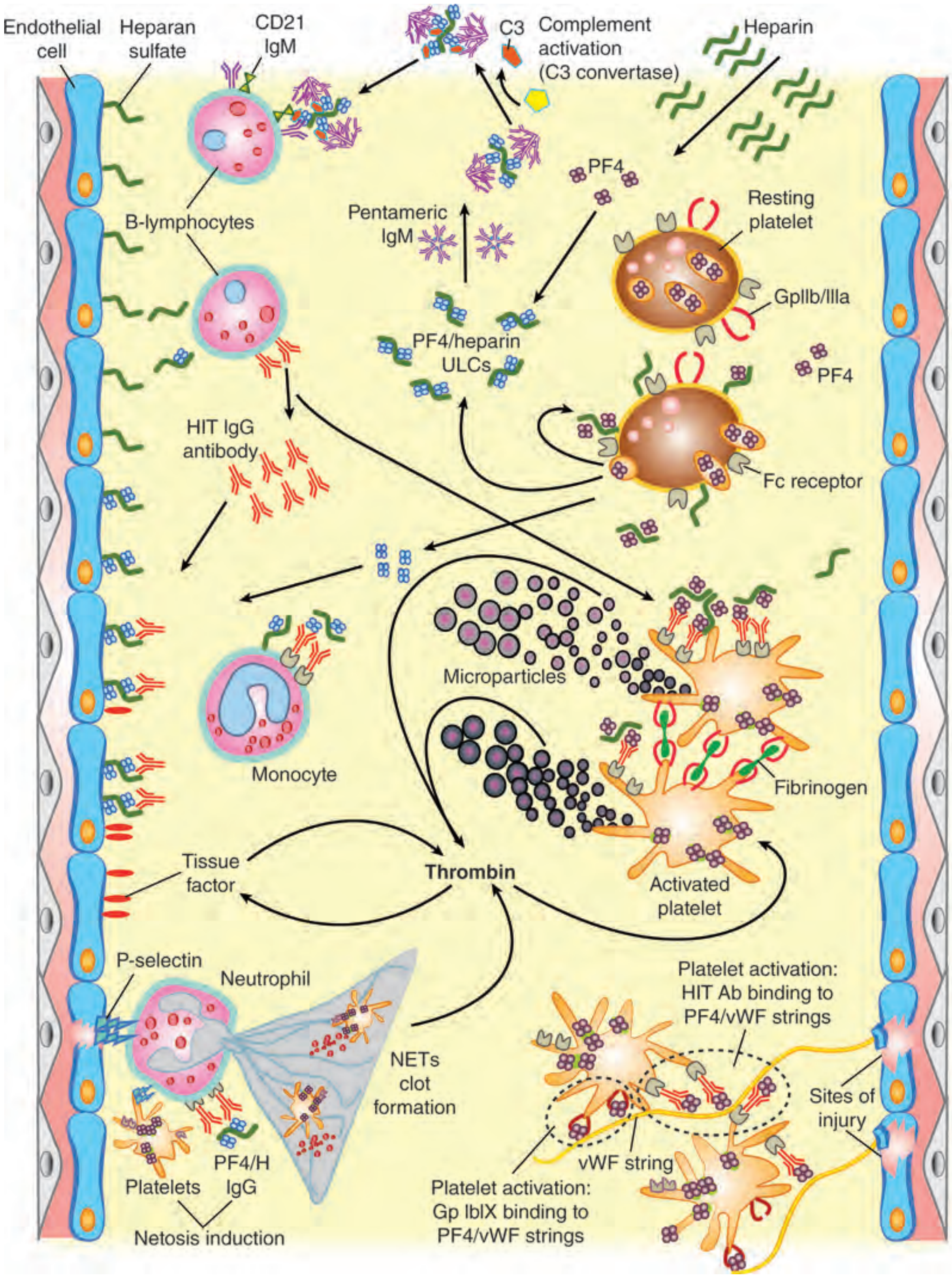

Endothelial cell
Heparan sulfate
CD21
IgM
C3
Complement activation (C3 convertase)
Heparin
PF4
B-lymphocytes
Pentameric IgM
Resting platelet
GpIIb/IIIa
PF4/heparin ULCs
PF4
HIT IgG antibody
Fc receptor
Microparticles
Monocyte
Fibrinogen
Tissue factor
Thrombin
Activated platelet
P-selectin
Neutrophil
Platelet activation: HIT Ab binding to PF4/vWF strings
NETs clot formation
Sites of injury
vWF string
PF4/H IgG
Platelets
Netosis induction
Platelet activation: Gp IbIX binding to PF4/vWF strings

Figure 131.1 PATHOGENESIS OF HEPARIN-INDUCED THROMBOCYTOPENIA: PANCELLULAR ACTIVATION. Heparin produces mild platelet activation, resulting in the release of platelet factor 4 (PF4) from platelet α-granules and the formation of immunogenic PF4/heparin complexes, including ultralarge complexes (ULCs). The ULCs also are recognized by natural IgM (shown in its pentameric form), which activate complement via C1q (not shown), that is, the classical complement pathway; the resulting PF4/heparin/IgM/C3 complexes bind to B lymphocytes via CD21 (complement receptor 2). B lymphocytes generate immunoglobulin G (IgG) antibodies that recognize the PF4/heparin complexes, which—if platelet-activating—are known as heparin-induced thrombocytopenia (HIT) antibodies; the Fc "tails" of the IgG bind to platelet FcγIIa receptors, resulting in Fc receptor clustering and consequent "strong" platelet activation. Platelet-derived microparticles are generated that accelerate thrombin generation. The HIT antibodies also recognize PF4 bound to endothelial heparan sulfate, leading to immunoinjury that causes endothelial activation, including the release of tissue factors. HIT antibodies also activate monocytes to generate tissue factors. Recent studies indicate that HIT antibody-induced neutrophil activation results in "netosis", that is, formation of neutrophil extracellular traps, contributing to immunothrombosis. Platelet-neutrophil and endothelial-neutrophil interactions are mediated through P-selectin. HIT antibodies also recognize PF4 bound to von Willebrand factor (vWF) "strings" released at sites of arterial injury, also contributing to localized platelet activation. All these events contribute to the "pancellular" activation underlying the prothrombotic diathesis of HIT.

TABLE 131.1 **Risk Factors for Heparin-Induced Thrombocytopenia**

Heparin type	Unfractionated > low-molecular-weight heparin > fondaparinux
Patient type	Postoperative (major > minor surgery) > medical > obstetric/pediatric
Dose[a]	Prophylactic dose > therapeutic dose > flushes
Duration	11–14 days[b] > 5–10 days > 4 days or fewer
Sex	Female > male

[a] Importance of heparin dose is uncertain because of confounding effect of patient type (e.g., postoperative patients tend to receive prophylactic-dose heparin whereas medical patients [e.g., with venous thromboembolism] are more likely to receive therapeutic-dose heparin); nevertheless, reported frequencies of heparin-induced thrombocytopenia (HIT) are relatively high in patients given postoperative prophylactic-dose heparin.

[b] Heparin exposure beyond 14 days does not usually increase the risk of HIT beyond that of an 11- to 14-day exposure.

patients can form heparin-dependent antibodies of the IgM or IgA subclass, these appear unlikely to cause HIT.[8]

As noted, thrombocytopenia develops in only a minority of patients who form HIT antibodies.[1] The variable risk for HIT in different patient populations may reflect variations in the susceptibility of platelets to activation by HIT-IgG and/or differences in the levels and immunoglobulin class composition of HIT antibodies. Poorly defined clinical factors also influence the risk for HIT, which occurs more often in surgical patients than in medical or obstetric patients.[6,32] Major trauma was more likely than minor trauma to be associated with HIT antibody formation and clinical HIT in one study.[33] There is indirect evidence that the formation of stoichiometrically optimal complexes of PF4/heparin influences the risk for immunization.[14] The clinical situation influences the type of HIT-associated thrombosis: venous thromboembolism typically develops in orthopedic patients with HIT, whereas thrombosis develops equally often in arteries and veins in cardiovascular patients with HIT.[1,32] Genetic factors likely play a role, too: HIT-associated thrombosis is more likely to occur in patients who bear the arginine (R) allele within a platelet Fc receptor polymorphism (H131R),[32,34,35] perhaps because this allele better allows for endogenous IgG2 subclass antibodies to inhibit the interaction of HIT antibodies with Fc receptors.

CLINICAL AND LABORATORY MANIFESTATIONS

Most patients with HIT have moderate thrombocytopenia; The median platelet count nadir is approximately 60 to 70 × 10^9/L; for 90% of patients, the platelet count nadir is greater than 20 × 10^9/L (Fig. 131.3).[32,36] In the rare patient whose platelet count falls to less than 10 × 10^9/L, there is often evidence of overt DIC, and red cell fragments and circulating normoblasts may be evident on examination of the blood smear. Thrombosis can occur in HIT patients even if there is a minimal decrease in the platelet count, and even if the platelet count nadir never falls below 150 × 10^9/L.[1,36] Neutrophil counts have also been reported to be elevated in patients with HIT, particularly those who develop thrombotic complications.[37]

The platelet count fall usually begins 5 to 10 days after the initiation of an immunizing heparin exposure and while the patient continues to receive heparin (typical-onset HIT).[38] Usually the immunizing trigger is heparin given during surgery (e.g., cardiac or vascular surgery) or that is started soon after surgery (e.g., postoperative thromboprophylaxis). Interestingly, starting LMWH thromboprophylaxis before elective surgery (as is more frequently done in Europe) is less likely to be associated with antibody formation compared with postoperative first-dose administration.[14]

HIT is recognized in approximately one-quarter of patients when the platelet count fall occurs abruptly after restarting UFH or LMWH (rapid-onset HIT). Invariably, such patients have been exposed to heparin within the previous 5 to 100 days, because HIT antibodies are transient and are only detectable for several weeks after an immunizing heparin exposure.[38] Consequently, rapid-onset HIT, which is caused by the administration of heparin to a patient with preformed HIT antibodies, is strongly associated with recent heparin exposure.

HIT is a highly prothrombotic condition (relative risk for thrombosis, ~15)[39]: Approximately 40% to 70% of HIT patients (with diagnosis confirmed by both PF4-dependent immunoassay and platelet activation assay) develop thrombosis.[32,36,40] Both venous (deep vein thrombosis and/or pulmonary embolism) and arterial (especially aortic and iliofemoral arterial thrombosis, ischemic stroke, or myocardial infarction) thrombosis can occur (venous:arterial thrombosis ratio, ~4:1).[32,40] Other complications include cerebral venous sinus thrombosis,[41] necrotizing skin lesions at the sites of subcutaneous heparin injection[42] and acute inflammatory/cardiorespiratory (anaphylactoid)[43,44] or transient memory disturbances after intravenous heparin bolus administration to sensitized persons.[45] Unexplained abdominal/flank pain or hypotension in a patient with HIT suggests adrenal infarction (often with associated adrenal hemorrhage), which if bilateral can lead to acute adrenal failure; the adrenal necrosis results from adrenal vein thrombosis.[46,47] Bowel infarction secondary to mesenteric artery thrombosis or mesenteric/portal vein thrombosis has also been reported.

Patients with HIT have an unusual predisposition to develop ischemic limb necrosis. Indeed, approximately 2% to 5% of such patients develop some degree of limb necrosis necessitating amputation. Historically, ischemic limb necrosis complicating HIT was linked to arterial thrombosis featuring platelet-rich clots.[48] However, microvascular thrombosis in a limb with deep-vein thrombosis, known as venous limb gangrene, is the most common explanation for ischemic limb loss in HIT.[27,49] Occasionally multiple limbs are involved. (See the box on Diagnostic Considerations in the Patient With Limb Ischemia and Thrombocytopenia.[50–53])

AUTOIMMUNE HIT

Recently, the term “autoimmune HIT” (aHIT) has been introduced into the HIT lexicon (Table 131.2).[54] The unifying concept is the detectability of HIT antibodies that activate platelets strongly in vitro

TABLE 131.2 **Autoimmune Heparin-Induced Thrombocytopenia Syndromes**

aHIT Syndrome	Definition
Delayed-onset HIT	HIT-related platelet count fall that begins or worsens after stopping heparin
Persisting HIT	HIT-related platelet count fall that requires prolonged time for platelet count recovery (at least 1 week[a])
Heparin “flush” HIT	HIT triggered by exposure only to small concentrations (“flushes”) of heparin
Spontaneous HIT syndrome	HIT without proximate heparin exposure; patients often have knee replacement surgery (without heparin exposure) or preceding infection
Fondaparinux-associated HIT	HIT caused by fondaparinux (most such patients have aHIT antibodies that further enhance platelet activation in the presence of fondaparinux (“cross-reactivity”)
Severe HIT with overt DIC	Some patients with severe HIT (marked thrombocytopenia, multiple thrombi, and/or overt DIC) who do not meet the above 5 definitions of aHIT have detectable aHIT antibodies

[a] No accepted definition, but platelet count recovery time exceeding 1 or 2 weeks suggests the diagnosis of “persisting” HIT (also known as “refractory” HIT).

aHIT, Autoimmune Heparin-Induced Thrombocytopenia; *DIC*, disseminated intravascular coagulation; *HIT*, heparin-induced thrombocytopenia.

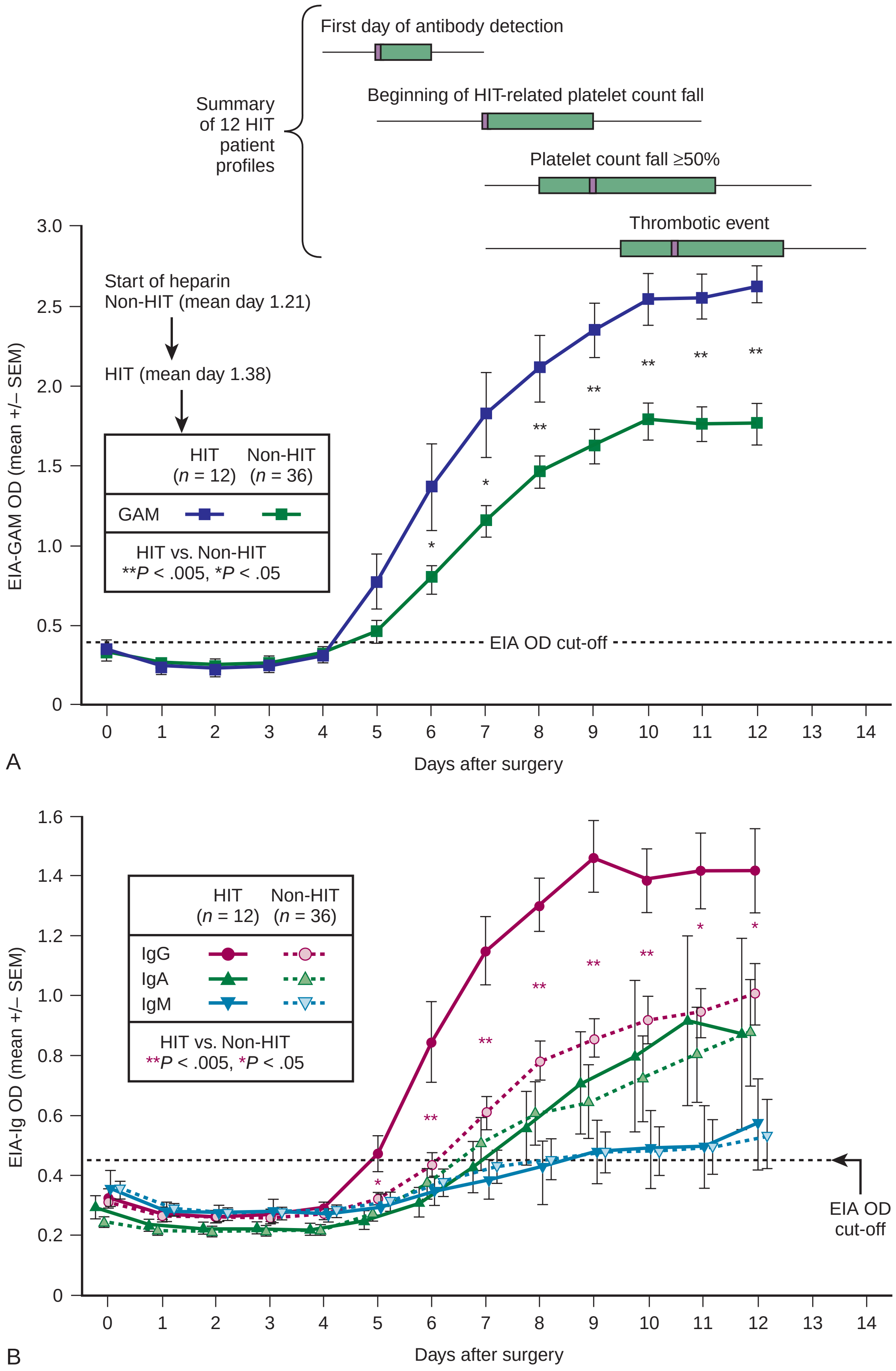


Figure 131.2 CHARACTERISTIC TIMELINE OF HEPARIN-INDUCED THROMBOCYTOPENIA. Anti-platelet factor 4 (PF4)/heparin antibodies (by enzyme immunoassay [EIA]) per postoperative day in 12 patients with heparin-induced thrombocytopenia (HIT) and 36 seropositive non-HIT control patients. (A) Mean (± standard error of the mean [SEM]) optical density (OD) of anti-PF4/heparin antibodies detected using a commercial immunoassay (EIA-GAM) that detects antibodies of all three immunoglobulin classes (IgG, IgA, IgM). HIT patients are indicated by blue squares and seropositive non-HIT controls by green squares. On each day beginning on postoperative day 6, there is a significant difference in the mean of the OD levels between the patients with HIT and the seropositive non-HIT controls ($P < .05$ by nonpaired t-test). At the top of the figure, summary data for 12 HIT patient profiles are shown for four key events (the first day of antibody detection, beginning of HIT-related platelet count fall, platelet count fall ≥50%, and thrombotic event), summarized as median (small purple squares within rectangles), interquartile range (rectangles), and range (ends of thin black lines). (B) Mean (±SEM) OD values of anti-PF4/heparin antibodies detected using an in-house immunoassay (EIA-Ig) that detects antibodies of the individual immunoglobulin classes, IgG (*red circles*), IgA (*green triangles*), and IgM (*blue inverted triangles*) for HIT (*solid symbols*) and non-HIT (*open symbols*). On each postoperative day beginning on day 5, there is a significant difference in the mean of the OD units for the IgG immunoassay between the patients with HIT and the seropositive non-HIT controls (**$P < .005$ for days 6 to 10; *$P < .05$ for days 5, 11, and 12). *(Fro* *ood. 2009;113:4693.)*

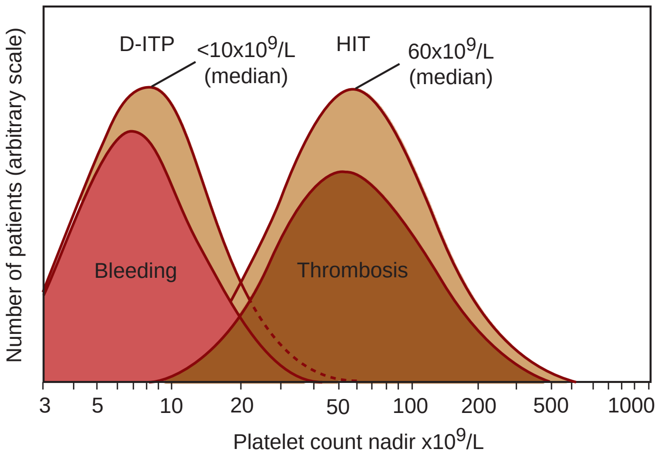


Figure 131.3 SEVERITY OF THROMBOCYTOPENIA IN DRUG-INDUCED IMMUNE THROMBOCYTOPENIA: A COMPARISON OF HEPARIN-INDUCED THROMBOCYTOPENIA VERSUS OTHER DRUG-INDUCED IMMUNE THROMBOCYTOPENIA DISORDERS. Whereas drug-induced immune thrombocytopenia (D-ITP) is strongly associated with petechiae and purpura, heparin-induced thrombocytopenia (HIT) is strongly associated with thrombosis. Note: The heights of the D-ITP and HIT curves are not drawn to scale, because HIT is more common than all DITP disorders combined. *(From Warkentin TE. Drug-induced immune-mediated thrombocytopenia—from purpura to thrombosis. N Engl J Med. 2007;356:891.)*

DIAGNOSTIC CONSIDERATIONS IN THE PATIENT WITH LIMB ISCHEMIA AND THROMBOCYTOPENIA

Concurrence of limb ischemia/necrosis and thrombocytopenia suggests one of several hematologic emergencies:

- HIT. Occlusion of large lower-limb arteries by platelet-rich “white clots” is characteristic of HIT. The major clue is an otherwise unexplained platelet count fall that begins 5 or more days after the initiation of heparin, with an absence of arterial pulses. Urgent thromboembolectomy may be limb-sparing.[50] Sensitive assays for HIT antibodies give strongly positive results. See subsequent entry for warfarin.
- Warfarin-induced phlegmasia cerulea dolens/venous limb gangrene. Coumarin anticoagulants, such as warfarin, can lead to venous ischemia (phlegmasia cerulea dolens) or venous limb gangrene in patients with DIC caused by HIT[27] or adenocarcinoma.[51] Limb loss can occur even though the limb pulses are palpable.
- Adenocarcinoma-associated DIC. Severe venous or arterial thrombosis can develop in patients with metastatic adenocarcinoma who have DIC.[51] This often occurs within hours after stopping heparin. A clinical clue is an otherwise unexplained rise in platelet count that occurs with initial or repeated heparin therapy.
- Sepsis-associated microvascular thrombosis. Acquired natural anticoagulant depletion (e.g., markedly reduced antithrombin or protein C levels), especially in the setting of acute ischemic hepatitis (“shock liver”), can complicate DIC associated with sepsis or cardiogenic shock, leading to bilateral acral limb ischemia or necrosis involving feet and (sometimes) fingers/hands (“symmetric peripheral gangrene”).[49,52] These patients can be misdiagnosed as having HIT. Optimal treatment is unknown but can include heparin anticoagulation (with anti-factor Xa monitoring), antithrombin concentrates, and plasma infusion.
- Septic embolism. Rarely, infective endocarditis or aneurysmal thrombosis leads to the constellation of thrombocytopenia and acute limb ischemia.
- Antiphospholipid syndrome. Autoimmune thrombocytopenia and hypercoagulability can interact to produce acute limb ischemia and thrombocytopenia in patients with antiphospholipid syndrome (particularly, catastrophic antiphospholipid syndrome, or CAPS).[53]

even in the absence of heparin; the clinical correlate is frequently that of a patient whose platelet count fall occurs or persists despite absence (or discontinuation of) heparin.

The first reported aHIT syndrome, “delayed-onset HIT,” indicates patients who develop thrombocytopenia that begins at least 5 days after a brief exposure to heparin or that worsens despite stopping heparin.[41,55] Sometimes, thrombocytopenia persists for several weeks despite stopping heparin (“persisting” or “refractory” HIT).[56,57] Other patients have developed HIT despite the sole exposure to heparin being heparin “flushes.”[58] On exceptionally rare occasions, a transient prothrombotic disorder that resembles HIT clinically and serologically occurs without any apparent preceding heparin exposure; triggers of so-called “spontaneous HIT syndrome” include knee replacement surgery (perhaps because of the release of heparin-like glycosaminoglycans from knee cartilage) and infection.[47,59–61] Some patients with fondaparinux-associated HIT have aHIT antibodies whose platelet-activating properties are enhanced in the presence of fondaparinux.[62] Finally, some patients with multiple venous and/or arterial thrombi, or who have unusually low platelet counts with DIC, have a laboratory picture of aHIT. aHIT syndromes are important to recognize, as they have certain treatment implications, including the efficacy of adjunctive therapy with high-dose IVIG.

DIFFERENTIAL DIAGNOSIS

Only approximately 10% of patients who undergo laboratory investigations for clinically suspected HIT have a serologic profile that supports the diagnosis (i.e., detectable heparin-dependent, platelet-activating IgG antibodies that recognize PF4/heparin complexes). The differential diagnosis includes postoperative thrombocytopenia (hemodilution/platelet consumption), thrombocytopenia of critical illness, and septicemia, among others. Some non-HIT disorders mimic HIT so closely that they warrant the term pseudo-HIT.[63] One example of pseudo-HIT is adenocarcinoma-associated DIC, in which the combination of progressive thrombocytopenia and severe venous limb ischemia/necrosis that occurs during the transitioning of patients from heparin (either UFH or LMWH) to warfarin may suggest a diagnosis of HIT.[51] Another disorder that can mimic HIT is the antiphospholipid syndrome: here, thrombocytopenia, thrombosis, and a false-positive PF4-dependent enzyme immunoassay (EIA) caused by anti-PF4 (not anti-PF4/heparin) antibodies can be present.[64] Recently, protamine (heparin)-induced thrombocytopenia as a potential explanation for post-cardiac surgery thrombocytopenia/thrombosis has been reported (see Chapter 130).

CLINICAL SCORING SYSTEMS

The pretest probability of HIT can be estimated using validated scoring systems. The 4Ts system represents a mnemonic that is based on ***T***hrombocytopenia, ***T***iming (of onset of thrombocytopenia or thrombosis), ***T***hrombosis (or other clinical sequelae of HIT), and no o***T***her explanation for thrombocytopenia, with each of the 4Ts scoring as an integer of 0, 1, or 2 points (thus the maximum score is 8 points) (Table 131.3).[65,66] The presence of platelet-activating HIT antibodies is unlikely (<3%) with a low score (≤3 points), but relatively probable (approximately 65%) with a high score (≥6). An intermediate score (4 or 5) indicates a clinical profile compatible with HIT, but also with other disorders, such as sepsis; here the frequency of platelet-activating HIT antibodies is still only approximately 10% to 20%.

Another scoring system is called the HIT Expert Probability (HEP) score (Table 131.4).[67] In the initial evaluation, a HEP score of 4 points or higher indicated an approximately 50% probability of HIT, whereas a score of 3 points or lower was associated with only a 3% probability of HIT.

It is unlikely that one scoring system offers major advantages over the other. In general, low scores in either system indicate a low

TABLE 131.3 **4Ts Scoring System: Estimating the Pretest Probability of Heparin-Induced Thrombocytopenia**

	Points (0, 1, or 2 for Each of Four Categories: Maximum Possible Score = 8)		
	2	**1**	**0**
Thrombocytopenia (acute)	Platelet fall >50% (nadir $\geq 20 \times 10^9$/L) and no surgery within preceding 3 days	Nadir, 10–19 × 10^9/L; or any 30%–50% fall; or, >50% fall within 3 days of surgery	Nadir, <10 × 10^9/L; or any <30% fall
Timing[a] of platelet count fall, thrombosis, or other sequelae	Clear onset between days 5–10; or $\leq$1 day (if prior heparin exposure in the past 5–30 days)	Consistent with day 5–10 fall, but not clear (e.g., missing platelet counts); or $\leq$1 day (heparin exposure within past 31–100 days); or platelet fall after day 10	Platelet count fall begins in $\leq$4 days without recent heparin exposure
Thrombosis or other clinical sequelae	New proven thrombosis; skin necrosis[b]; anaphylactoid reaction after IV heparin bolus; adrenal hemorrhage	Progressive or recurrent thrombosis; erythematous skin lesions[b]; suspected thrombosis (awaiting confirmation with imaging)	None
O***T***her cause for thrombocytopenia is not evident	No other explanation for platelet count fall is evident	Possible other cause is evident	Definite other cause is present
Pretest probability score: 6–8 = HIGH; 4–5 = INTERMEDIATE; 0–3 = LOW			

The scoring system shown here has undergone minor modifications from previously published scoring systems.

[a] First day of immunizing heparin exposure is considered on day 0; immunizing heparin is usually that given during or soon after surgery (e.g., unfractionated heparin [UFH] during cardiac surgery is more immunogenic than UFH given during preceding acute coronary syndrome or with heart catheterization); the day the platelet count *begins* to fall is considered the day of onset of thrombocytopenia.

[b] Skin lesions occurring at heparin injection sites.

Reprinted with permission from Warkentin TE. Clinical picture of heparin-induced thrombocytopenia. In: Warkentin TE, Greinacher A (eds.), *Heparin-Induced Thrombocytopenia.* 4th ed. New York: Informa Healthcare USA; 2007:21–66.

probability of HIT, whereas high scores indicate approximately a 50 : 50 chance of HIT. Thus laboratory testing is crucial to establishing a diagnosis of HIT.

LABORATORY DIAGNOSIS

Assays for HIT antibodies can be classified as platelet "activation" (or "functional") and PF4-polyanion "antigen" assays (or immunoassays).[68] Activation assays that measure serotonin release from ^{14}C-labeled, washed platelets (i.e., the serotonin-release assay [SRA]) are quite sensitive and specific for detecting clinically significant HIT antibodies.[11,15,16] Important quality control maneuvers include the selection of platelet donors whose platelets respond well to Fc receptor stimulation, as well as the inclusion of negative and positive HIT sera of variable reactivity to ensure that the test platelets identify weaker HIT sera.[69] The characteristic activation profile produced by HIT serum includes increased platelet activation at low heparin concentrations (0.05 to 0.3 units/mL), but low background platelet activation at high heparin concentrations (10 to 100 units/mL).[15,16] Strong platelet activation induced by HIT sera even in the absence of pharmacologic heparin is a feature of delayed-onset HIT and other aHIT syndromes.[54,55]

Recently, the concept of "SRA-negative HIT" has been posited.[70,71] Such patients can be identified by using a PF4-enhanced platelet activation assay, such as the PF4-SRA (where high concentrations of PF4 are added, rather than heparin)[72] or the P-selectin expression assay.[73] One of these tests should be performed when the SRA gives an unexpected negative result in an immunoassay-positive patient who otherwise appears likely to have HIT.

Three enzyme(-linked) immuno(sorbent) assays (EIAs or ELISAs) are commercially available for detecting antibodies that recognize PF4 bound to heparin, to the polyanion, polyvinyl sulfonate, or PF4 (from platelet lysate) bound to protamine. These assays are very sensitive for detecting HIT antibodies but are much more likely than washed platelet activation assays to detect clinically insignificant anti-PF4/heparin antibodies. Thus results of laboratory tests must be interpreted in the clinical context—that is, HIT is a "clinicopathologic" syndrome.[74]

Activation and antigen assays are not 100% concordant, and there are advantages if reference laboratories are able to perform both types of assay. In general, serum or plasma from patients with clinical HIT has strongly positive assay results, whereas clinically insignificant antibodies give weaker results.[5] Indeed, if the EIA yields only a weakly positive result (e.g., 0.40 to 1.0 absorbance units), the corresponding probability of "true" HIT is 5% to 10% at most. In contrast, if the EIA yields a strongly positive result (>2.0 units), the probability of HIT exceeds 90%.[5]

One conceptual way to relate activation and antigen assays is through the "iceberg" model (Fig. 131.4), where a positive platelet activation assay better predicts for clinically relevant antibodies (the portion of iceberg above the waterline). Recently, the iceberg model has been adapted for rapid immunoassays (discussed subsequently).[75]

Rapid HIT immunoassays provide results within an hour of sample acquisition. Five rapid immunoassays are available depending on jurisdictional approval and local availability (Table 131.5). One assay, the latex immunoturbidimetric assay (LIA), is a functionalized immunoassay, as it detects HIT antibodies through competitive inhibition of a HIT-like monoclonal antibody.[76] Another assay, the chemiluminescence immunoassay (CLIA), is IgG-specific, and has a very good overall sensitivity-specificity profile.[77] Another assay, the lateral flow-immunoassay (LFI), is usually interpreted qualitatively (positive/negative), although if read by a scanner, a strong-positive result points to a high likelihood of clinically relevant antibodies being present.[78] The particle gel immunoassay (PaGIA), which uses blood bank methodology, is another qualitative assay that can, however, be modified to a semiquantitative assay by determining the titer of a positive result.[79] Finally, a recent systematic review/meta-analysis of the particle immunofiltration assay (PIFA) showed the assay to provide little diagnostic information, and its use is not recommended.[80] As a general rule, assays with relatively high sensitivity for HIT (PaGIA, LFI, CLIA, LIA) are useful for ruling out the diagnosis, especially in low or intermediate probability situations. In contrast, a positive rapid immunoassay points broadly to a 50-50 probability of HIT in most clinical circumstances.

TABLE 131.4 **Heparin-Induced Thrombocytopenia Expert Probability Scoring System**

Clinical Feature	Score
1. Magnitude of fall in platelet count (measured from peak platelet count to nadir platelet count since heparin exposure)	
a. <30%	–1
b. 30%–50%	1
c. >50%	3
2. Timing of fall in platelet count	
For patients in whom typical-onset HIT is suspected	
a. Fall begins <4 days after heparin exposure	–2
b. Fall begins 4 days after heparin exposure	2
c. Fall begins 5–10 days after heparin exposure	3
d. Fall begins 11–14 days after heparin exposure	2
e. Fall begins >14 days after heparin exposure	–1
For patients with previous heparin exposure in the last 100 days in whom rapid-onset HIT is suspected	
f. Fall begins <48 h after heparin reexposure	2
g. Fall begins >48 h after heparin reexposure	–1
3. Nadir platelet count	
a. $\leq 20 \times 10^9$/L	–2
b. $>20 \times 10^9$/L	2
4. Thrombosis (Select no more than one)	
For patients in whom typical-onset HIT is suspected	
a. New VTE or ATE ≥4 days after heparin exposure	3
b. Progression of preexisting VTE or ATE while receiving heparin	2
For patients in whom rapid-onset HIT is suspected	
c. New VTE or ATE after heparin exposure	3
d. Progression of preexisting VTE or ATE while receiving heparin	2
5. Skin necrosis	
a. Skin necrosis at subcutaneous heparin injection sites	3
6. Acute systemic reaction	
a. Acute systemic reaction after intravenous heparin bolus	2
7. Bleeding	
a. Presence of bleeding, petechiae, or extensive bruising	–1
8. Other causes of thrombocytopenia (Select all that apply)	
a. Presence of a chronic thrombocytopenic disorder	–1
b. Newly initiated nonheparin medication known to cause thrombocytopenia	–2
c. Severe infection	–2
d. Severe DIC (defined as fibrinogen <100 mg/dL and D-dimer >5.0 μg/mL	–2
e. Indwelling intraarterial device (e.g., IABP, VAD, ECMO)	–2
f. Cardiopulmonary bypass within previous 96 h	–1
g. No other apparent cause	3

In its initial evaluation, a HEP score of ≥4 points indicated an approximately 50% probability of HIT, whereas a score of ≤3 points was associated with only a 3% probability of HIT.

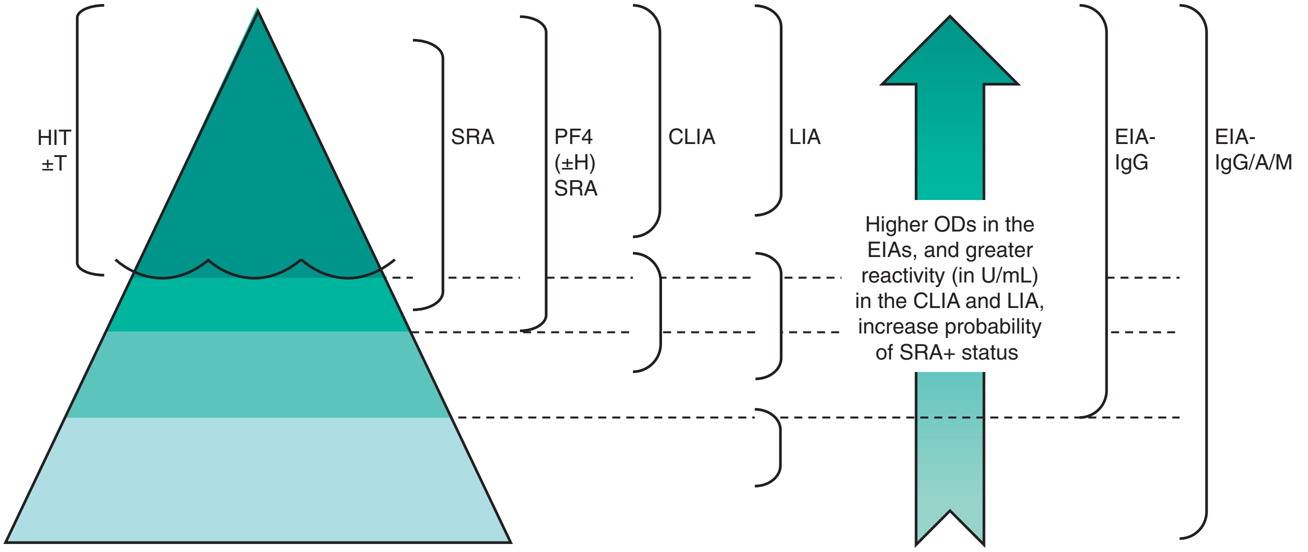
ATE, Arterial thromboembolism; *DIC*, disseminated intravascular coagulation; *ECMO*, extracorporeal membrane oxygenation; *HIT*, heparin-induced thrombocytopenia; *IABP*, intraaortic balloon pump; *VAD*, ventricular assist device; *VTE*, venous thromboembolism.

Reprinted with permission from Cuker A, Arepally G, Crowther MA, et al. The HIT Expert Probability (HEP) Score: a novel pre-test probability model for heparin-induced thrombocytopenia based on broad expert opinion. *J Thromb Haemost* 2010;8:2642.

However, for those rapid immunoassays that provide semiquantitative output, a strong-positive result points to a positive diagnosis of HIT in the appropriate clinical context;[76,77] this is analogous to a patient with a strong-positive EIA result (e.g., >2.0 OD units). Recently, the combined use of two rapid immunoassays—PaGIA and CLIA,[81] or LIA and CLIA[82]—have been reported. Fig. 130.4 shows how data can be interpreted with the results of dual LIA/CLIA testing.

PROGNOSIS

Approximately half of all patients with SRA-positive HIT have clinically evident thrombosis at the time of initial diagnosis of HIT.[40] Among patients without thrombosis at diagnosis, there appears to be at a high risk (approximately 25% to 50%) for subsequent thrombosis if heparin is simply discontinued.[40] It is increasingly accepted


HIT
±T
SRA
PF4
(±H)
SRA
CLIA
LIA
Higher ODs in the EIAs, and greater reactivity (in U/mL) in the CLIA and LIA, increase probability of SRA+ status
EIA-
IgG
EIA-
IgG/A/M

LIA or CLIA-IgG result (U/mL)	<1.00	1.00–4.99	5.00–15.99	≥16.00
Probability of HIT (SRA+ status) per LIA result	<1%	~30%	~70%	~90%
Probability of HIT (SRA+ status) per CLIA result	<1%	~50%	~90%	>95%

Dual LIA and CLIA analysis (6 point scale)	0	1	2	3	4	5	6
Probability of HIT (SRA+ status)	<1%	~5%	~30%	~60%	~80%	~95%	>97%
Probability of platelet-activating Abs	<3%	~10%	~55%	~85%	~98%		

Figure 131.4 ICEBERG MODEL OF HEPARIN-INDUCED THROMBOCYTOPENIA (HIT). The part of the iceberg that protrudes above the waterline indicates patients with HIT±T (HIT with or without thrombosis). The large iceberg represents the totality of antibodies detected by polyspecific PF4-dependent EIA (EIA-IgG/A/M), with a large subgroup comprising patients with detectable PF4-reactive antibodies of IgG class (EIA-IgG). The great majority of HIT cases are detected by serotonin-release assay (SRA), yielding SRA-positive (SRA+) results, and with most SRA-negative HIT detectable by a modified (PF4-enhanced) SRA, either the PF4-SRA or the PF4/H-SRA (collectively indicated as "PF4(±H)-SRA"). The latex immunoturbidimetric assay (LIA) and chemiluminescence-immunoassay (CLIA) both have relatively high sensitivity for HIT, with the CLIA (an IgG-specific immunoassay) correlating more closely with EIA-IgG than the LIA (which can also detect IgA and IgM class antibodies). Although the model implies 100% sensitivity of the EIA-IgG and EIA-IgGAM, in reality, an occasional HIT case will be negative in one commercial EIA but test positive in another. The model also indicates that the probability of HIT increases with increasing reactivity by EIA or by LIA and/or CLIA (including interpreting both tests together). For the 6-point scale, 0, 1, 2, or 3 points, respectively, are given for LIA and CLIA results that are <1.00, 1.00–4.99, 5.00–15.99, and ≥16.00. Note that "Probability of platelet-activating antibodies" refers to the probability of a sample testing positive by any platelet activation assay (e.g., conventional SRA, PF4-SRA, or PF4/H-SRA). *(From Warkentin TE. How I diagnose and manage HIT. Hematology Am Soc Hematol Educ Program. 2011;2011:143–149.)*

that further anticoagulation is indicated in most patients strongly suspected as having "isolated" HIT—that is, HIT recognized because of thrombocytopenia rather than because of HIT-associated thrombosis. Indeed, it is a recommendation of the American Society of Hematology (ASH) guidelines to give therapeutic-dose anticoagulation for isolated HIT.[83]

THERAPY

Although it is standard practice to discontinue heparin in patients strongly suspected of having HIT, including stopping heparin "flushes" of intravascular catheters (with substitution of saline flushes), heparin cessation is not necessarily beneficial, for two reasons: (1) heparin likely has an anticoagulant effect even in patients with HIT, and (2) HIT antibodies often cause ongoing platelet activation and hypercoagulability in the absence of pharmacologic heparin. In addition, HIT antibody levels can decline even with continued heparin.[84] The recognition that patients with HIT have increased thrombin generation[27] provides a rationale for the use of nonheparin anticoagulant agents that rapidly inhibit thrombin or its generation.

Currently, there are two types of anticoagulant therapeutic approaches that are used to treat HIT: (1) exclusive or predominant factor Xa inhibitors, either parenteral "indirect" (i.e., antithrombin-dependent) factor Xa inhibitors (danaparoid, fondaparinux) or "direct" factor Xa inhibitors (rivaroxaban, apixaban), and (2) DTIs (argatroban, bivalirudin, dabigatran). In the author's opinion, factor Xa inhibitors—although not approved in the United States for treatment of HIT—are the preferred treatment option for HIT, because they have numerous advantages over DTIs (Table 131.6). In addition, because approximately 90% of patients suspected of HIT do not have HIT,[5] another advantage for factor Xa inhibitors is that these agents, unlike "niche" DTIs such as argatroban and bivalirudin, are safe and effective for the prevention and treatment of thrombosis in numerous non-HIT settings. Another recent conceptual development in HIT treatment is the growing recognition that platelet activation can be rapidly inhibited by the administration of high-dose IVIG, making this a rational adjunctive treatment in several clinical situations.

TABLE 131.5 Rapid Immunoassays for Heparin-Induced Thrombocytopenia

Rapid Immunoassay	Comments
Latex immune-turbidimetric assay (LIA)	Functionalized immunoassay (see text); high sensitivity and moderate specificity; semiquantitative assay (result in U/mL); performed on coagulation instrument (requires plasma); available in the United States
Chemiluminescence immunoassay (CLIA)	High sensitivity and high specificity; detects IgG class antibodies; semiquantitative assay (result in U/mL); available in the United States
Particle gel immunoassay (PaGIA)	High sensitivity and moderate specificity; semiquantitative assay (if performed using diluted serum); not available in the United States
Lateral flow immunoassay (LFI)	High sensitivity and moderate specificity; semiquantitative assay (if read using a scanner); not available in the United States
Particle immunofiltration assay (PIFA)	Poor sensitivity and specificity (not recommended for use); qualitative results only (positive/negative); no longer available in the United States

High sensitivity generally means at least 90%–95%; moderate specificity for most assays indicates a positive predictive value (PPV) of approximately 50% overall irrespective of pretest probability; however, PPV can approach 100% if the test result is strongly positive (e.g., LIA or CLIA >16 U/mL; positive PaGIA at 1 in 16 dilution.

Indirect Factor Xa Inhibitors: Danaparoid and Fondaparinux

Danaparoid is an anticoagulant heparinoid (mixture of glycosaminoglycans) with predominant inhibitory activity against factor Xa that is effective for treating HIT; however, danaparoid was never approved for HIT treatment in the United States (although it was approved in many other countries, including Canada and the European Union) and was discontinued in the United States in April 2002. Danaparoid was shown to be safe and effective for the treatment of HIT in an open-label randomized controlled trial (vs. dextran 70)[85] and in a retrospective comparison against ancrod (defibrinogenating snake venom) and/or vitamin K antagonist therapy.[86]

In recent years, however, physicians have increasingly used fondaparinux as an off-label therapy for HIT. The advantages of fondaparinux include its long half-life, the fact that it does not require coagulation monitoring nor does it influence the aPTT or international normalized ratio (INR), its low potential to cross-react with HIT antibodies, and low cost. In addition, because relatively few patients with suspected HIT actually have this diagnosis, the wide experience with fondaparinux and its approval for the prevention and treatment of thrombosis in non-HIT settings constitute other advantages. To date, the experience with fondaparinux to treat HIT has been favorable.[87–90] Also, fondaparinux is much less likely than UFH or LMWH to precipitate rapid-onset HIT in patients who have unrecognized HIT antibodies.[91] Although fondaparinux thromboprophylaxis has been associated with rare cases of de novo HIT,[62,92] this should not deter from its use as a treatment of HIT because the potential to trigger immunization is "dissociated" from whether an anticoagulant potentiates antibody-induced platelet activation,[7] and fondaparinux has low potential to "cross-react" with HIT antibodies and thus to exacerbate HIT[91] (although this phenomenon has been reported[56,60]). Dosing information for danaparoid and fondaparinux is shown in Table 131.7.

Indirect Factor Xa Inhibitors: Rivaroxaban, Apixaban, Edoxaban

In theory, oral direct factor Xa inhibitors, such as rivaroxaban and apixaban, should be effective for the treatment of HIT. Indeed, anecdotal experience with rivaroxaban[46,60,93,94] and apixaban[93,95] has been generally favorable, and the ASH HIT treatment guidelines[83] include these direct oral anticoagulants (DOACs) as anticoagulant options for treating HIT. Dosing for two oral Xa inhibitors is shown in Table 131.8.

Direct Thrombin Inhibitors: Bivalirudin, Argatroban

Bivalirudin (Angiomax) is a 20-amino-acid thrombin inhibitor modeled after hirudin (natural anticoagulant produced by the medicinal leech) that is composed of two peptide fragments that recognize the active site of thrombin and its fibrinogen-binding site, linked by a tetraglycine spacer.[96] The half-life of bivalirudin is about 25 minutes, and only minor dose adjustments are needed in patients with renal insufficiency because bivalirudin is only partially cleared by the kidneys. It is approved as an alternative to heparin in patients undergoing percutaneous coronary intervention (PCI), including those with HIT.[97] Its short half-life and predominantly extrarenal elimination are reasons why it is an option for intraoperative anticoagulation in patients undergoing cardiac surgery when heparin is contraindicated because of acute or recent HIT. Published experience using bivalirudin off-label to treat HIT appears favorable.[98]

Argatroban (marketed as Argatroban in the United States and as Novastan elsewhere) is a small-molecule (527-Da) DTI that undergoes predominantly hepatobiliary excretion and is, therefore, suitable for use without dose adjustment in patients with renal failure (the dose is reduced by three-quarters in patients with hepatic insufficiency).[99] Argatroban is approved in the United States for both treatment and prevention of thrombosis in HIT. It is given in the same dosage for both indications (usual starting infusion rate of 2 μg/kg/min without an initial bolus). However, lower starting doses are usually given (e.g., 0.5 to 1.2 μg/kg/min), especially in critically ill patients.[100] Argatroban (in substantially higher doses) is also approved for anticoagulation during PCI in patients with acute HIT or a history of HIT.[101] Its half-life is 40 to 50 minutes. Argatroban prolongs the INR more than bivalirudin,[102] and a higher-than-usual target INR during warfarin co-therapy[103] (which depends on the thromboplastin reagent used to measure the INR) can be expected (see the section on Caveats in Treatment of Heparin-Induced Thrombocytopenia). Argatroban reduced the frequency of a composite endpoint (new thrombosis, limb amputation, and all-cause mortality) from 56.5% (in historical controls) to 43.8% and 41.5% in two studies of patients with HIT complicated by thrombosis; new thrombosis event rates were reduced from 34.8% to 19.4% and 13.1%, respectively.[104] In patients with isolated HIT, argatroban reduced the rate of new thrombosis from 22.4% to 6.9%, and the combined event rate of new thrombosis, limb amputation, and all-cause mortality from 38.8% to 26.9%.[104] Unfortunately, outcome data are not available for the subgroup of argatroban-treated patients who tested positive for HIT antibodies; thus, the comparison with (antibody-positive) historical controls may overestimate any benefit with argatroban (antibody status unknown). Dosing information for the parenteral DTIs, bivalirudin and argatroban is shown in Table 131.9.

In theory, dabigatran, an oral thrombin inhibitor, should also be effective for the treatment of HIT. To date, experience in treating HIT has been minimal.

High-Dose Intravenous Immunoglobulin

Increased plasma IgG concentrations inhibit HIT antibody-induced platelet activation through FcγIIa receptors.[105] High-dose intravenous immunoglobulin (IVIG) is emerging as an approach to rapidly inhibit platelet activation, leading in most cases to rapid platelet

TABLE 131.6 **Comparison of the Three Classes of Anticoagulants Used to Treat Heparin-Induced Thrombocytopenia**

	Indirect (AT-Dependent) Factor Xa Inhibitors: Danaparoid, Fondaparinux	Direct Factor Xa Inhibitors: Rivaroxaban, Apixaban	Direct Thrombin Inhibitors: Argatroban, Bivalirudin
Half-life	✓ Long (danaparoid, 25 h[a]; fondaparinux, 17 h): reduces the risk of rebound hypercoagulability	✓ Intermediate (rivaroxaban, ~12 h[b]; apixaban, ~12 h): reduces the risk of rebound hypercoagulability	Short (<2 h): potential for rebound hypercoagulability
Dosing	✓ Both prophylactic- and therapeutic-dose regimens[c]	✓ Both prophylactic- and therapeutic-dose regimens[c]	Prophylactic-dose regimens are less well established
Monitoring	✓ Direct (anti-Xa levels): accurate drug levels obtained	✓ Direct (anti-Xa levels): accurate drug levels obtained (not widely available)	Indirect (aPTT): risk for DTI underdosing resulting from aPTT elevation caused by non-DTI factors, (e.g., HIT-associated DIC, liver dysfunction, etc.)
Effect on INR	✓ No significant effect: thus simplifies overlap with warfarin	✓ Increases with rivaroxaban; however, as rivaroxaban and apixaban can be used for long-term anticoagulation, no need for warfarin overlap	Increases INR: argatroban > bivalirudin; complicates warfarin overlap
Protein C pathway	✓ Adverse effect unlikely (thrombin remains available to activate protein C)	✓ Adverse effect unlikely (thrombin remains available to activate protein C)	Thrombin inhibition could impair thrombin-mediated activation of the protein C pathway
Reversibility of action	✓ Irreversible inhibition: AT forms a covalent bond with factor Xa	Reversible inhibition	Reversible inhibition
Efficacy and safety in non-HIT settings	✓ Treatment and prophylaxis of VTE (danaparoid, fondaparinux) and ACS (fondaparinux)	✓ Treatment and prophylaxis of VTE (rivaroxaban, apixaban), ACS (rivaroxaban), and chronic CAD or PAD (rivaroxaban)	Not established for most non-HIT settings (exception: bivalirudin established for PCI)
Platelet activation	✓ Danaparoid inhibits platelet activation by HIT antibodies	No effect	No effect
Inhibition of clot-bound factor Xa or thrombin	No effect	✓ Inhibition of both free and clot-bound factor Xa	✓ Inhibition of both free and clot-bound thrombin
Drug clearance	Predominantly renal	Renal component and metabolism (e.g., rivaroxaban and apixaban both metabolized by CYP3A4)	Predominantly hepatobiliary: argatroban; renal component: bivalirudin
Cost	✓ Relatively low[d] (fondaparinux)	✓ Relatively low[d]	Relatively high[d]

A check mark (✓) indicates favorable feature in comparison to drug classes (author's opinion).

[a] For danaparoid, half-lives of its anti-thrombin (anti-IIa) and its thrombin generation inhibition activities (2–4 h and 3–7 h, respectively) are shorter than for its anti-Xa activity (approximately 25 h).

[b] Mean terminal elimination half-life 5–9 h in the young but 11–13 h in the elderly.

[c] Although therapeutic dosing is recommended for HIT, the availability of prophylactic-dose regimens increases flexibility when managing potential non-HIT situations when diagnostic certainty is not high.

[d] Another cost consideration is that a patient can be discharged to home on subcutaneous danaparoid or fondaparinux, or oral rivaroxaban or apixaban, whereas an additional 5–7 in-hospital days may be required for parenteral DTI-warfarin overlap before discharge from hospital.

ACS, Acute coronary syndrome; *aPTT*, activated partial thromboplastin time; *AT*, antithrombin; *CAD*, coronary artery disease; *DIC*, disseminated intravascular coagulation; *DTI*, direct thrombin inhibitor; *HIT*, heparin-induced thrombocytopenia; *INR*, international normalized ratio; *PAD*, peripheral arterial disease; *PCI*, percutaneous coronary intervention; *VTE*, venous thromboembolism.

count increase and probable decrease in thrombotic risk (for review, see reference 106). Currently, high-dose IVIG is recommended as adjunctive therapy (together with therapeutic-dose anticoagulation) for treating aHIT disorders, such as delayed-onset HIT,[107] persisting (refractory) HIT,[108] heparin "flush" associated HIT,[109] or spontaneous HIT syndrome.[110] In addition, IVIG has been used prior to planned heparin reexposure in patients who continue to have circulating platelet-activating HIT antibodies.[111,112]

Caveats in Treatment of Heparin-Induced Thrombocytopenia

Warfarin and other vitamin K antagonists (coumarins) should not be used during the acute thrombocytopenic phase of HIT.[27] A particularly high-risk situation is HIT associated with deep vein thrombosis, especially if there is overt (decompensated) DIC. If such patients are treated with warfarin, there is a risk for progression to venous limb gangrene.[27,104] The laboratory marker for this unusual syndrome is a high INR (generally, greater than 4.0), which corresponds to the combination of a marked reduction in the level of protein C together with increased thrombin generation (as evidenced by elevated levels of thrombin-antithrombin complexes) during warfarin therapy.[27] Although warfarin (in theory) should be safe in a patient whose thrombin generation is controlled (e.g., using a DTI), it is important to delay the initiation of warfarin until there is a substantial resolution of the thrombocytopenia (to more than 150×10^9/L).[83,104] Warfarin treatment should be delayed because its prolongation of the aPTT may result in *underdosing* of the DTI, and because stopping the DTI before resolution of the HIT during warfarin overlap can lead to limb loss caused by fulminant venous limb gangrene.

Several case observations suggest that aPTT confounding of DTI therapy can be a factor explaining the progression of thrombosis in patients with severe HIT.[4,113] For example, patients can develop progressive thrombocytopenia and HIT-associated consumptive coagulopathy despite stopping heparin (Fig. 131.5). In such patients, aPTT

TABLE 131.7 Treatment Schedules for Danaparoid and Fondaparinux

Anticoagulant	Therapeutic Dosing Protocol for HIT-Associated Thrombosis[a]	Anticoagulant Monitoring	Clearance	Half-Life (h)	Comment
Danaparoid	Initial bolus, 2250 units[b] IV; accelerated infusion (400 units/h × 4 h, 300 units/h × 4 h; then 200 units/h IV, subsequently adjusted by antifactor Xa levels)	Antifactor Xa levels (target, 0.5–0.8 units/mL)	Renal (minor)	25	Widely approved for HIT treatment (although not in the United States); not available in the United States; low risk for in vivo cross-reactivity; prophylactic-dose therapy[c] may be appropriate when clinical suspicion for HIT is low
Fondaparinux	7.5 mg[d] subcutaneous once daily	Anti–Xa factor levels (target levels not well established)	Renal (major)	17	Not approved for HIT treatment (although increasingly used as off-label therapy). Prophylactic-dose therapy[e] may be appropriate when clinical suspicion for HIT is low, or if there is renal insufficiency

[a] Therapeutic dosing is usually appropriate for strongly suspected or confirmed HIT (including “isolated” HIT, i.e., HIT without apparent thrombosis), or when thrombosis is documented.

[b] Adjust IV danaparoid bolus for body weight: <60 kg, 1500 units; 60–75 kg, 2250 units; 75–90 kg, 3000 units; >90 kg, 3750 units.

[c] Prophylactic-dose regimen, 750 units subcutaneous every 8 h (for renal failure, reduce to 750 units every 12 h).

[d] Five milligrams for body weight <50 kg and 10 mg for body weight >100 kg; the author sometimes gives 10 mg as the first and/or second dose (rather than 7.5 mg) for severe HIT. Because HIT treatment is usually started in the afternoon, the author usually gives the second dose (and subsequent doses) at 8 AM (i.e., the interval between first and second doses is often only 14–20 h rather than 24 h), which helps to achieve steady-state therapy more quickly. Dose reduction and antifactor Xa monitoring (if available) are appropriate if being used in a patient with renal insufficiency.

[e] Prophylactic-dose regimen, 2.5 mg subcutaneous every day (assumes normal renal function).

HIT, Heparin-induced thrombocytopenia; *IV*, intravenous.

TABLE 131.8 Treatment Schedules for Rivaroxaban and Apixaban (Off-Label Treatment for Heparin-Induced Thrombocytopenia)

Anticoagulant	Therapeutic Dosing Protocol[a]	Anticoagulant Monitoring	Clearance	Half-Life (h)	Comment
Rivaroxaban	15 mg twice-daily p.o. for 21 days; then 20 mg once-daily p.o.[b]	Not generally required	33% renal	~12[c]	Use with caution if CrCl 15–29 mL/min; avoid if CrCl <15 mL/min or if coadministering dual inhibitors of CYP3A4 and P-glycoprotein
Apixaban	10 mg twice-daily p.o. for 7 days[d]; then, 5 mg twice-daily p.o.[b]	Not generally required	25% renal	~12[c]	Use with caution if CrCl 15–29 mL/min; avoid if CrCl <15 mL/min or coadministering dual inhibitors of CYP3A4 and P-glycoprotein

[a] Therapeutic dosing is usually appropriate for strongly suspected or confirmed HIT (including “isolated” HIT, i.e., HIT without apparent thrombosis), or when thrombosis is documented.

[b] Duration of anticoagulation is usually at least 3–6 months for patients with HIT-associated thrombosis, but for patients with isolated HIT, anticoagulation can generally be stopped once the platelet count has recovered to a stable plateau, and if no evidence for thrombosis or other indication for anticoagulation is apparent.

[c] The half-life of both rivaroxaban and apixaban is increased by 30%–50% in patients with CrCl 15–29 mL/min.

[d] Apixaban is given at a dose of 10 mg twice daily for 7 days in patients with acute venous thromboembolism, but it is reasonable to continue the higher dose for more than 7 days in patients with aHIT who have a persistently low platelet count (i.e., “persisting” or “refractory” HIT).

aHIT, Autoimmune heparin-induced thrombocytopenia; *HIT*, heparin-induced thrombocytopenia.

TABLE 131.9 Treatment Schedules for Bivalirudin and Argatroban

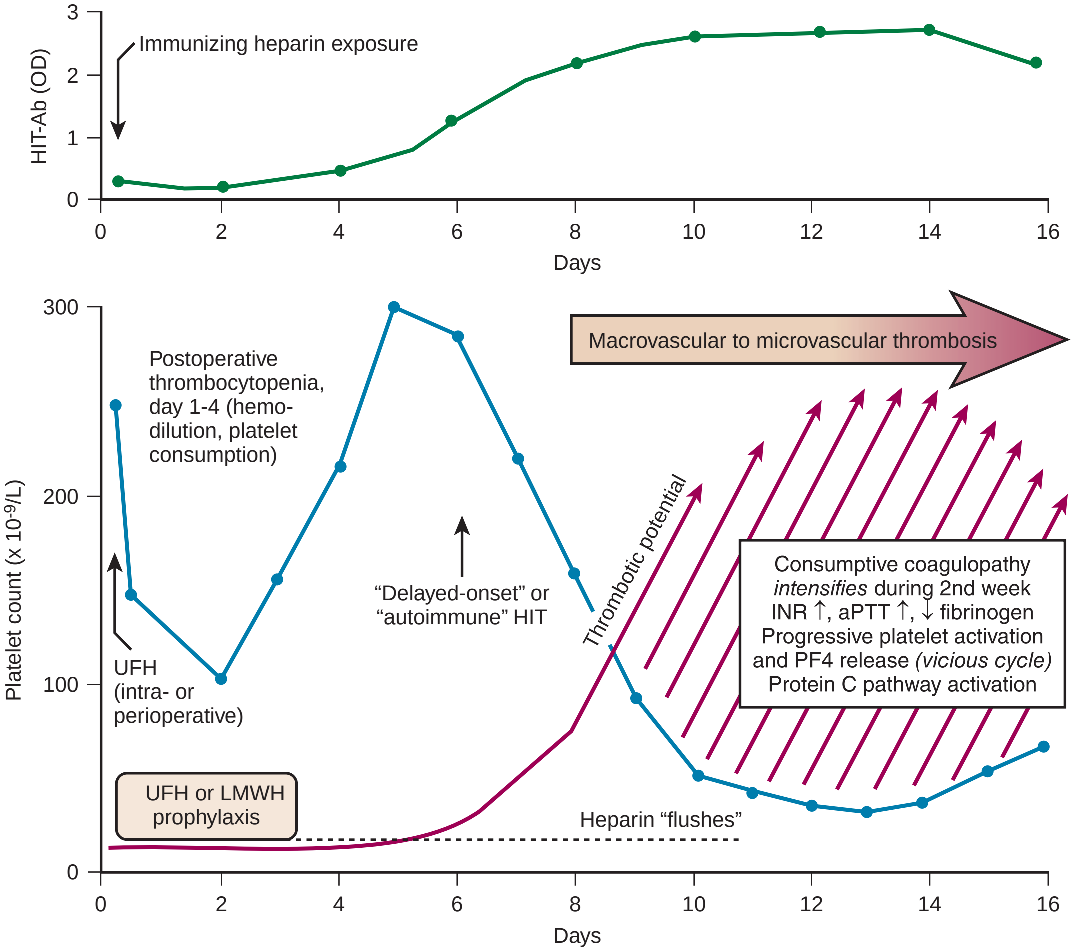
Anticoagulant	Dosing Protocol for HIT-Associated Thrombosis	Anticoagulant Monitoring[a]	Clearance	Half-Life (min)	Comment
Bivalirudin	No bolus; initial infusion rate: 0.15–0.20 mg/kg/h	1.5–2.5 × baseline aPTT	Enzymic (80%); renal (20%)	25	Off-label treatment for HIT (although approved for PCI in patients with HIT); minor prolongation of INR (compared with argatroban)
Argatroban	No bolus; initial infusion rate: 2 μg/kg/min	1.5–3.0 × baseline aPTT (maximum aPTT, 100 s)	Hepatobiliary	40–50	Initial dose 0.5 μg/kg/min in hepatic insufficiency[d]; moderate or marked prolongation of INR, which complicates overlap with warfarin anticoagulation

Dosing protocols shown are appropriate for most patients with strongly suspected or confirmed HIT whether or not complicated by thrombosis. (Dosing for bivalirudin and argatroban is substantially different when given for PCI.)

[a] In general, the patient’s baseline aPTT should be used for calculating target range, when appropriate; otherwise, the mean laboratory normal range can be used.

[d] Reduced initial dosing (e.g., 0.5–1.2 μg/kg/min) is also appropriate for patients in intensive care units, with cardiac failure, or early postcardiac surgery.

aPTT, Activated partial thromboplastin time; *HIT*, heparin-induced thrombocytopenia; *INR*, international normalized ratio; *PCI*, percutaneous coronary intervention.



Indirect (AT-mediated) factor Xa inhibitors (danaparoid, fondaparinux)	1. Long half-life achieves stable levels of anticoagulation. 2. No interference with activation of the protein C pathway. 3. Specific assays accurately measure drug levels (anti-Xa activity). 4. Inhibition of HIT-Ab-induced platelet activation (danaparoid only).
Direct thrombin inhibitors (DTIs) (hirudin, argatroban, bivalirudin)	1. aPTT monitoring can be unreliable, e.g., DIC, liver disease, warfarin. 2. Short half-lives — risk for "rebound" hypercoagulability. 3. Interfere with thrombin-induced activation of protein C pathway. 4. Reversible DTIs (argatroban, bivalirudin) could block thrombin inhibition by AT.
Vitamin K antagonists (warfarin, acenocoumarol, phenprocoumon)	1. Vitamin K antagonists do not inhibit thrombin or its generation. 2. Predispose to protein C depletion (vitamin K–dependent factor). 3. Raise the aPTT, thus risking DTI underdosing or interruption. 4. Long half-lives, thus any adverse effects could persist for days.

Figure 131.5 CONCEPTUAL FRAMEWORK OF HEPARIN-INDUCED THROMBOCYTOPENIA (HIT): FOCUS ON HEPARIN-INDEPENDENT PLATELET ACTIVATION AND DELAYED-ONSET ("AUTOIMMUNE") HIT. The *upper panel* shows the timeline of HIT antibody (HIT-Ab) formation, as judged by optical density units in an antiplatelet factor 4/polyanion enzyme immunoassay (EIA). The *middle panel* illustrates a platelet count decline in the absence of heparin (or with small amounts of heparin, e.g., heparin flushes) indicating delayed-onset HIT, with the intensification of HIT-associated hypercoagulability from day 7 to 14, especially after stopping heparin. Patients with this clinical profile often have HIT-associated consumptive coagulopathy (overt disseminated intravascular coagulopathy [DIC]) and are at risk for confounding of activated partial thromboplastin time (aPTT)–monitored direct thrombin inhibitor (DIT) treatment. The *lower panel* compares different classes of anticoagulants for expected effects on HIT-associated hypercoagulability, including the risk for aPTT confounding in the setting of HIT-associated DIC. *AT*, Antithrombin; *INR*, international normalized ratio; *LMWH*, low-molecular-weight heparin; *PF4*, platelet factor 4; *aPTT*, activated partial thromboplastin time; *UHF*, unfractionated heparin. *(From Warkentin TE. Agents for the treatment of heparin-induced thrombocytopenia. Hematol Oncol Clin North Am. 2010;24:755.)*

monitoring of DTI therapy can fail because a brief course of DTI can abruptly lead to supratherapeutic aPTT levels—not because of anticoagulant overdosing but because of the combination of DTI and consumptive coagulopathy—and interruption/cessation of DTI therapy can be associated with rapid progression of microvascular thrombosis. In this setting, factor Xa inhibitors (danaparoid, fondaparinux, rivaroxaban, apixaban) may be more effective because they do not require aPTT monitoring. Alternatively, DTI levels can be measured directly, but these assays are rarely performed in North American laboratories.

Using sensitive assays for HIT antibodies, LMWH reacts similarly to UFH in vitro. Furthermore, because LMWH can lead to worsening of clinical HIT, these agents should not be used to treat HIT.[83,104]

Although primary treatment for HIT should include an anticoagulant that inhibits thrombin or reduces its generation, certain treatment adjuncts can be used in special situations. These include surgical thromboembolectomy, high-dose IVIG (discussed above), antiplatelet drugs such as aspirin, and therapeutic plasma exchange.[114]

These caveats, along with recommendations for the management of patients with suspected HIT, are summarized in the box on Diagnosis and Treatment of Heparin-Induced Thrombocytopenia.

PLATELET COUNT MONITORING FOR HEPARIN-INDUCED THROMBOCYTOPENIA

The frequency of platelet count monitoring in patients receiving heparin should reflect the overall risk of HIT (i.e., the preparation, the dose, and the clinical situation).[83,104] At least alternate-day platelet count monitoring should be considered in patients at relatively high risk for developing HIT (e.g., after orthopedic or cardiac surgery). Monitoring at least two or three times a week should be considered for patients at moderate risk for developing HIT (e.g., medical patients receiving UFH, postoperative patients receiving LMWH). The frequency of HIT is low with LMWH,[1,6,13] particularly in medical patients or during pregnancy, and routine platelet count monitoring may not be required in such settings.[1,6] In addition, HIT rarely begins 14 or more days after the initiation of a course of heparin,[38] so routine monitoring beyond this period is not required.

ANTICOAGULATION AND PREVIOUS HEPARIN-INDUCED THROMBOCYTOPENIA

HIT antibodies are transient and usually are undetectable several weeks or a few months after an episode of HIT.[38] If HIT antibodies are no longer detected, it is appropriate to use heparin for a brief indication in situations where other anticoagulants have drawbacks, for example, cardiac surgery.[38,104,115,116] Heparin should be avoided preoperatively (e.g., argatroban or bivalirudin can be used for heart catheterization). The risk of recurrent HIT appears to be low (approximately 2%) but is possible beginning approximately one week post-reexposure for cardiac or vascular surgery even if postoperative heparin is not given, because of the possibility of regenerating strong HIT antibodies that can activate platelets in the absence of pharmacologic heparin.[116] Alternatively, if a patient with recent HIT remains SRA+ and heparin reexposure is desired (e.g., for urgent cardiac surgery), therapeutic plasma exchange can be used to remove HIT antibodies preoperatively; in this situation, achieving a negative SRA is the appropriate serologic endpoint (as the EIA can remain positive even when platelet-activating antibodies are no longer detectable by SRA).[114] Another option (favored by the author) is to give high-dose IVIG shortly prior to the planned surgery so that intraoperative heparin is less likely to trigger acute platelet activation by the residual HIT antibodies.[111,112]

Patients with acute or recent HIT who require urgent heart surgery and still have detectable antibodies have been successfully treated with alternate anticoagulant approaches (e.g., bivalirudin, or heparin plus an antiplatelet agent such as epoprostenol or tirofiban), but each of these approaches has disadvantages. For example, epoprostenol causes hypotension, and there is limited experience with its use in cardiac surgery. Bivalirudin is a potential option for use in cardiac surgery, but special surgical and anesthesiologic maneuvers are required (because bivalirudin undergoes proteolysis, thus posing a risk for clotting of stagnant blood in the extracorporeal circuit). This author favors considering reexposure to heparin after HIT mitigating strategies (e.g., awaiting seroreversion, therapeutic plasma exchange, preoperative administration of high-dose IVIG, intraoperative administration of antiplatelet agents).[106,111,112]

A diagnostic and therapeutic approach to HIT is shown in the box on Diagnosis and Treatment of Heparin-Induced Thrombocytopenia.

DIAGNOSIS AND TREATMENT OF HEPARIN-INDUCED THROMBOCYTOPENIA

HIT should be clinically suspected when thrombocytopenia occurs during (or soon after) heparin therapy and the temporal profile of the decrease in platelet count is consistent with immune sensitization to heparin. Four relevant questions, the 4Ts, should be asked:

***T*hrombocytopenia:** Does the patient have thrombocytopenia? A greater than 50% fall in the platelet count can indicate HIT even if the platelet count has not fallen to below 150×10^9/L. (Note: Severe thrombocytopenia, such as a platelet count below 10×10^9/L, is only rarely caused by HIT.)

***T*iming:** Is the timing of the platelet count fall consistent with immune sensitization? In HIT the platelet count usually begins to fall 5–10 days after starting heparin (the first day of heparin is day 0); a more rapid fall in the platelet count can occur if the patient is already sensitized from heparin exposure within the past 100 days.

***T*hrombosis:** Does the patient have thrombosis or other sequelae of HIT? (Note: In addition to venous and arterial thrombosis, patients with HIT can develop necrotizing skin lesions at heparin injection sites or systemic inflammatory or cardiorespiratory reactions that begin 5–30 minutes after an IV heparin bolus.)

O*T*her: Are there other explanations for the thrombocytopenia? HIT is less likely when there is compelling clinical evidence for another cause of the thrombocytopenia, such as positive blood cultures or critical illness.

Steps in Management of Clinically Suspected Heparin-Induced Thrombocytopenia

1. Confirm that thrombocytopenia is present (repeat complete blood count), test for DIC, and test for HIT antibodies, preferably using a platelet activation test such as the SRA.
2. Assess clinically and radiologically for thrombosis (e.g., compression ultrasound for lower-limb deep venous thrombosis).
3. Stop all heparin, including heparin flushes.
4. Initiate treatment with an alternative anticoagulant, generally in therapeutic doses if HIT is strongly suspected (options: danaparoid[a,b] fondaparinux,[c] rivaroxaban,[c] apixaban,[c] argatroban,[c] bivalirudin[c]).
5. Although initial treatment decisions are made on clinical grounds, results of testing for HIT antibodies can influence subsequent treatment, including the decision to resume heparin if HIT has been ruled out. In some centers, the availability of rapid HIT assays allows for treatment decisions pending receipt of test results.

Caveats

- Do not use LMWH to treat HIT.
- Do not start (or continue) warfarin or other coumarins until the platelet count has returned to normal (>150×10^9/L), and give only in low initial doses (warfarin, 5 mg or less) and overlap with a parenteral anticoagulant for at least 5 days.
- Do give vitamin K (e.g., 5–10 mg IV over 30–60 min) if HIT is diagnosed after warfarin has already been given, so as to reduce the risk for coumarin necrosis and to avoid underdosing of DTI because of aPTT prolongation by warfarin.
- Beware of confounding[4,113] of aPTT-monitored DTI therapy in patients with severe HIT complicated by HIT-associated consumptive coagulopathy (DIC) or who have other explanations for an elevated aPTT, such as warfarin therapy, liver disease, or antiphospholipid syndrome.
- Do not give prophylactic platelet transfusions (platelet transfusions are appropriate if the patient is bleeding or if there is diagnostic uncertainty).
- Do not use inferior vena cava filters in patients with acute HIT; there is a high risk for progressive lower-limb venous thrombosis and even progression to venous limb gangrene, perhaps because anticoagulants used to treat HIT are not good at inhibiting surface-associated thrombin generation, and also because the presence of filter might discourage therapeutic-dose anticoagulation.

---

[a] Off-label treatment for HIT in the United States (although approved for the treatment of HIT in numerous other jurisdictions).

[b] Withdrawn from the US market but available in many other countries.

[c] Off-label treatment for HIT is listed as a treatment option in the American Society of Hematology (ASH) HIT treatment guidelines.[83]

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Amiral J, Bridey F, Dreyfus M, et al. Platelet factor 4 complexed to heparin is the target for antibodies generated in heparin induced thrombocytopenia. *Thromb Haemost*. 1992;68:95.

Cuker A, Arepally GM, Chong BH, et al. American Society of Hematology 2018 guidelines for management of venous thromboembolism: heparin-induced thrombocytopenia. *Blood Adv*. 2018;22:3360.

Greinacher A, Selleng K, Warkentin TE. Autoimmune heparin-induced thrombocytopenia. *J Thromb Haemost*. 2017;15:2099.

Gruel Y, Vayne C, Rollin J, et al. Comparative analysis of a French prospective series of 144 patients with heparin-induced thrombocytopenia (FRIGTIH) and the literature. *Thromb Haemost*. 2020;120:1096.

Khandelwal S, Ravi J, Rauova L, et al. Polyreactive IgM initiates complement activation by PF4/heparin complexes through the classical pathway. *Blood*. 2018;132:2431.

Lo GK, Juhl D, Warkentin TE, et al. Evaluation of pretest clinical score (4T's) for the diagnosis of heparin-induced thrombocytopenia in two clinical settings. *J Thromb Haemost*. 2006;4:759.

Martel N, Lee J, Wells PS. Risk for heparin-induced thrombocytopenia with unfractionated heparin and low-molecular-weight heparin thromboprophylaxis: a meta-analysis. *Blood*. 2005;106:2710.

Perdomo J, Leung HHL, Ahmadi Z, et al. Neutrophil activation and NETosis are the major drivers of thrombosis in heparin-induced thrombocytopenia. *Nat Commun*. 2019;10:1322.

Schindewolf M, Steindl J, Beyer-Westendorf J, et al. Use of fondaparinux off-label or approved anticoagulants for management of heparin-induced thrombocytopenia. *J Am Coll Cardiol*. 2017;70:2636.

VanderVeer EA, Torbiak RP, Prebtani APH, et al. Spontaneous heparin-induced thrombocytopenia syndrome presenting as bilateral adrenal infarction after knee arthroplasty. *BMJ Case Rep*. 2019;12:e232769.

Warkentin TE. Drug-induced immune-mediated thrombocytopenia—from purpura to thrombosis. *N Engl J Med*. 2007;356:891.

Warkentin TE. Ischemic limb gangrene with pulses. *N Engl J Med*. 2015;373:642.

Warkentin TE. Microvascular thrombosis and ischaemic limb losses in critically ill patients. *Hämostaseologie*. 2019;39:6.

Warkentin TE, Basciano PA, Knopman J, et al. Spontaneous heparin-induced thrombocytopenia syndrome. *Blood*. 2014;123:3651.

Warkentin TE, Davidson BL, Büller HR, et al. Prevalence and risk of preexisting heparin-induced thrombocytopenia antibodies in patients with acute VTE. *Chest*. 2011;140:366–373.

Warkentin TE, Elavathil LJ, Hayward CPM, et al. The pathogenesis of venous limb gangrene associated with heparin-induced thrombocytopenia. *Ann Intern Med*. 1997;127:804.

Warkentin TE, Greinacher A, Koster A, et al. Treatment and prevention of heparin-induced thrombocytopenia: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th edition). *Chest*. 2008;133:340S.

Warkentin TE, Kelton JG. Delayed-onset heparin-induced thrombocytopenia and thrombosis. *Ann Intern Med*. 2001;135:502.

Warkentin TE, Kelton JG. A 14-year study of heparin-induced thrombocytopenia. *Am J Med*. 1996;101:502.

Warkentin TE, Kelton JG. Temporal aspects of heparin-induced thrombocytopenia. *N Engl J Med*. 2001;344:1286.

Warkentin TE, Nazy I, Sheppard JI, et al. Serotonin-release assay-negative heparin-induced thrombocytopenia. *Am J Hematol*. 2020;95:38.

Warkentin TE, Pai M, Linkins LA. Direct oral anticoagulants for treatment of HIT: update of Hamilton experience and literature review. *Blood*. 2017;130:1104.

Warkentin TE, Sheppard JI, Moore JC, et al. Studies of the immune response in heparin-induced thrombocytopenia. *Blood*. 2009;113:4963.

Warkentin TE, Sheppard JI, Moore JC, et al. Quantitative interpretation of optical density measurements using PF4-dependent immunoassays. *J Thromb Haemost*. 2008;6:1304.

Warkentin TE, Sheppard JI, Smith JW, et al. Combination of two complementary automated rapid assays for diagnosis of heparin-induced thrombocytopenia (HIT). *J Thromb Haemost*. 2020;18:1435.

REFERENCES

1. Linkins LA, Lee DH. Frequency of heparin-induced thrombocytopenia. In: Warkentin TE, Greinacher A, eds. *Heparin-induced thrombocytopenia*. ed 5. Boca Raton, FL: CRC Press; 2013:110.
2. Amiral J, Bridey F, Dreyfus M, et al. Platelet factor 4 complexed to heparin is the target for antibodies generated in heparin induced thrombocytopenia. *Thromb Haemost*. 1992;68:95.
3. Greinacher A, Pötzsch B, Amiral J, et al. Heparin-associated thrombocytopenia: isolation of the antibody and characterization of a multimolecular PF4-heparin complex as the major antigen. *Thromb Haemost*. 1994;71:247.
4. Warkentin TE. HIT paradigms and paradoxes. *J Thromb Haemost*. 2011;9:105.
5. Warkentin TE, Sheppard JI, Moore JC, et al. Quantitative interpretation of optical density measurements using PF4-dependent immunoassays. *J Thromb Haemost*. 2008;6:1304.
6. Warkentin TE, Sheppard JI, Sigouin CS, et al. Gender imbalance and risk factor interactions in heparin-induced thrombocytopenia. *Blood*. 2006;108:2937.
7. Warkentin TE, Cook RJ, Marder VJ, et al. Anti-platelet factor 4/heparin antibodies in orthopedic surgery patients receiving antithrombotic prophylaxis with fondaparinux or enoxaparin. *Blood*. 2005;106:3791.
8. Warkentin TE, Sheppard JI, Moore JC, et al. Laboratory testing for the antibodies that cause heparin-induced thrombocytopenia: how much class do we need? *J Lab Clin Med*. 2005;146:341.
9. Warkentin TE, Sheppard JI, Horsewood P, et al. Impact of the patient population on the risk for heparin-induced thrombocytopenia. *Blood*. 2000;96:1703.
10. Warkentin TE, Sheppard JI, Sun JCJ, et al. Anti-PF4/heparin antibodies and venous graft occlusion in postcoronary artery bypass surgery patients randomized to postoperative unfractionated heparin or fondaparinux thromboprophylaxis. *J Thromb Haemost*. 2013;11:253.
11. Warkentin TE. How I diagnose and manage HIT. *Hematology Am Soc Hematol Educ Program*. 2011;2011:143.
12. Gao C, Boylan B, Fang J, et al. Heparin promotes platelet responsiveness by potentiating αIIbβ3-mediated outside-in signaling. *Blood*. 2011;117:4946.
13. Martel N, Lee J, Wells PS. Risk for heparin-induced thrombocytopenia with unfractionated heparin and low-molecular-weight heparin thromboprophylaxis: a meta-analysis. *Blood*. 2005;106:2710.
14. Warkentin TE, Cook RJ, Marder VJ, et al. Anti-PF4/heparin antibody formation post-orthopedic surgery thromboprophylaxis: the role of non-drug risk factors and evidence for a stoichiometry-based model of immunization. *J Thromb Haemost*. 2010;8:504.
15. Sheridan D, Carter J, Kelton JG. A diagnostic test for heparin-induced thrombocytopenia. *Blood*. 1986;67:27.
16. Warkentin TE, Arnold DM, Nazi I, et al. The platelet serotonin-release assay. *Am J Hematol*. 2015;90:564.
17. Khandelwal S, Ravi J, Rauova L, et al. Polyreactive IgM initiates complement activation by PF4/heparin complexes through the classical pathway. *Blood*. 2018;132:2431.
18. Kelton JG, Sheridan D, Santos A, et al. Heparin-induced thrombocytopenia: laboratory studies. *Blood*. 1988;72:925.
19. Warkentin TE, Hayward CPM, Boshkov LK, et al. Sera from patients with heparin-induced thrombocytopenia generate platelet-derived microparticles with procoagulant activity: an explanation for the thrombotic complications of heparin-induced thrombocytopenia. *Blood*. 1994;84:3691.
20. Warkentin TE. An overview of the heparin-induced thrombocytopenia syndrome. *Semin Thromb Hemost*. 2004;30:273.
21. Arepally GM, Cines DB. Pathogenesis of heparin-induced thrombocytopenia. *Transl Res*. 2020;225:131.
22. Madeeva D, Cines DB, Poncz M, et al. Role of monocytes and endothelial cells in heparin-induced thrombocytopenia. *Thromb Haemost*. 2016;116:806.
23. Hayes V, Johnston I, Arepally GM, et al. Endothelial antigen assembly leads to thrombotic complications in heparin-induced thrombocytopenia. *J Clin Invest*. 2017;127:1090.
24. Gollomp K, Kim M, Johnston I, et al. Neutrophil accumulation and NET release contribute to thrombosis in HIT. *JCI Insight*. 2018;3:399445.
25. Perdomo J, Leung HHL, Ahmadi Z, et al. Neutrophil activation and NETosis are the major drivers of thrombosis in heparin-induced thrombocytopenia. *Nat Commun*. 2019;10:1322.
26. Johnston I, Sarkar A, Hayes V, et al. Recognition of PF4-VWF complexes by heparin-induced thrombocytopenia antibodies contribute to thrombus propagation. *Blood*. 2020;135:1270.
27. Warkentin TE, Elavathil LJ, Hayward CPM, et al. The pathogenesis of venous limb gangrene associated with heparin-induced thrombocytopenia. *Ann Intern Med*. 1997;127:804.
28. Warkentin TE. Heparin-induced thrombocytopenia in critically ill patients. *Semin Thromb Hemost*. 2015;41:49.
29. Warkentin TE, Sheppard JI, Moore JC, et al. Studies of the immune response in heparin-induced thrombocytopenia. *Blood*. 2009;113:4963.
30. Greinacher A, Kohlmann T, Strobel U, et al. The temporal profile of the anti-PF4/heparin immune response. *Blood*. 2009;113:4970.
31. Krauel K, Weber C, Brandt S, et al. Platelet factor 4 binding to lipid A of Gram-negative bacteria exposes PF4/heparin-like epitopes. *Blood*. 2012;120:3345.
32. Gruel Y, Vayne C, Rollin J, et al. Comparative analysis of a French prospective series of 144 patients with heparin-induced thrombocytopenia (FRIGTIH) and the literature. *Thromb Haemost*. 2020;120:1096.
33. Lubenow N, Hinz P, Thomaschewski S, et al. The severity of trauma determines the immune response to PF4/heparin and the frequency of heparin-induced thrombocytopenia. *Blood*. 2010;115:1797.
34. Carlsson LE, Santoso S, Baurichter G, et al. Heparin-induced thrombocytopenia: new insights into the impact of the FcγRIIa-R-H131 polymorphism. *Blood*. 1998;92:1526.
35. Rollin J, Pouplard C, Sung HC, et al. Increased risk of thrombosis in FcγRIIA 131RR patients with HIT due to defective control of platelet activation by plasma IgG2. *Blood*. 2015;125:2397.
36. Warkentin TE. Drug-induced immune-mediated thrombocytopenia—from purpura to thrombosis. *N Engl J Med*. 2007;356:891.
37. Hui M, Sheppard JI, Li N, et al. Neutrophil and monocyte counts in heparin-induced thrombocytopenia. *Thromb Haemost*. 2019;119:941.
38. Warkentin TE, Kelton JG. Temporal aspects of heparin-induced thrombocytopenia. *N Engl J Med*. 2001;344:1286.
39. Warkentin TE. HITlights: a career perspective on heparin-induced thrombocytopenia. *Am J Hematol*. 2012;87(Suppl. 1):S92.
40. Warkentin TE, Kelton JG. A 14-year study of heparin-induced thrombocytopenia. *Am J Med*. 1996;101:502.
41. Warkentin TE, Bernstein RA. Delayed-onset heparin-induced thrombocytopenia and cerebral thrombosis and after a single administration of unfractionated heparin. *N Engl J Med*. 2003;348:1067.
42. Warkentin TE. Heparin-induced skin lesions. *Br J Haematol*. 1996;92:494.
43. Warkentin TE, Greinacher A. Heparin-induced anaphylactic and anaphylactoid reactions: two distinct but overlapping syndromes. *Expert Opin Drug Saf*. 2009;8:129.
44. Avram AT, Blostein MD, Hirsch AM, et al. Venous limb gangrene and pulseless electrical activity (PEA) cardiac arrest during management of deep-vein thrombosis and progressive limb ischemic necrosis following vascular surgery. *Am J Hematol*. 2020;95:712.
45. Warkentin TE, Hirte HW, Anderson DR, et al. Transient global amnesia associated with acute heparin-induced thrombocytopenia. *Am J Med*. 1994;97:489.
46. Warkentin TE, Safyan EL, Linkins LA. Heparin-induced thrombocytopenia presenting as bilateral adrenal hemorrhages. *N Engl J Med*. 2015;372:492.
47. VanderVeer EA, Torbiak RP, Prebtani APH, et al. Spontaneous heparin-induced thrombocytopenia syndrome presenting as bilateral adrenal infarction after knee arthroplasty. *BMJ Case Rep*. 2019;12:e232769.
48. Warkentin TE. Heparin-induced thrombocytopenia-associated thrombosis: from arterial to venous to venous limb gangrene. *J Thromb Haemost*. 2018;16:2128.
49. Warkentin TE. Ischemic limb gangrene with pulses. *N Engl J Med*. 2015;373:642.
50. Warkentin TE, Pai M, Cook RJ. Intraoperative anticoagulation and limb amputations in patients with immune heparin-induced thrombocytopenia who require vascular surgery. *J Thromb Haemost*. 2012;10:148.
51. Warkentin TE, Cook RJ, Sarode R, et al. Warfarin-induced venous limb ischemia/gangrene complicating cancer: a novel and clinically distinct syndrome. *Blood*. 2015;126:486.
52. Warkentin TE. Microvascular thrombosis and ischaemic limb losses in critically ill patients. *Hämostaseologie*. 2019;39:6.
53. James TE, Martin LJ, Warkentin TE, et al. Catastrophic antiphospholipid syndrome refractory to high-dose intravenous immunoglobulin responsive to therapeutic plasma exchange. *Platelets*. 2021;32:828.
54. Greinacher A, Selleng K, Warkentin TE. Autoimmune heparin-induced thrombocytopenia. *J Thromb Haemost*. 2017;15:2099.
55. Warkentin TE, Kelton JG. Delayed-onset heparin-induced thrombocytopenia and thrombosis. *Ann Intern Med*. 2001;135:502.
56. Warkentin TE. Clinical picture of heparin-induced thrombocytopenia (HIT) and its differentiation from non-HIT thrombocytopenia. *Thromb Haemost*. 2016;116:813.
57. Padmanabhan A, Jones CG, Pechauer SM, et al. IVIg for treatment of severe refractory heparin-induced thrombocytopenia. *Chest*. 2017;152:478.
58. Mian H, Warkentin TE, Sheppard JI, et al. Autoimmune HIT due to apheresis catheter heparin flushes for stem cell harvesting before autotransplantation for myeloma. *Blood*. 2017;130:1679.

59. Warkentin TE, Basciano PA, Knopman J, et al. Spontaneous heparin-induced thrombocytopenia syndrome. *Blood*. 2014;123:3651.
60. Poudel DR, Ghimire S, Dhital R, et al. Spontaneous HIT syndrome post-knee replacement surgery with delayed recovery of thrombocytopenia: a case report and literature review. *Platelets*. 2017;28:614.
61. Moores G, Warkentin TE, Farooqi MAM, et al. Spontaneous heparin-induced thrombocytopenia syndrome presenting as cerebral venous sinus thrombosis. *Neurology: Clin Pract*. 2020 Jan 14. https://doi.org/10.1212/CPJ.0000000000000805.
62. Warkentin TE, Sheppard JI, Manheim JC. HIT complicating fondaparinux prophylaxis: fondaparinux-dependent platelet activation as a marker for fondaparinux-induced HIT. *Thromb Haemost*. 2014;112:1319.
63. Warkentin TE. Pseudo-heparin-induced thrombocytopenia. In: Warkentin TE, Greinacher A, eds. *Heparin-Induced Thrombocytopenia*. ed 4. New York, NY: Informa Healthcare USA; 2007:261.
64. Pauzner R, Greinacher A, Selleng K, et al. False-positive tests for heparin induced thrombocytopenia in patients with antiphospholipid syndrome and systemic lupus erythematosus. *J Thromb Haemost*. 2009;7:1070.
65. Lo GK, Juhl D, Warkentin TE, et al. Evaluation of pretest clinical score (4 T's) for the diagnosis of heparin-induced thrombocytopenia in two clinical settings. *J Thromb Haemost*. 2006;4:759.
66. Warkentin TE, Linkins LA. Non-necrotizing heparin-induced skin lesions and the 4T's score. *J Thromb Haemost*. 2010;8:1483.
67. Cuker A, Arepally G, Crowther MA, et al. The HIT Expert Probability (HEP) Score: a novel pre-test probability model for heparin-induced thrombocytopenia based on broad expert opinion. *J Thromb Haemost*. 2010;8:2642.
68. Warkentin TE. Laboratory diagnosis of heparin-induced thrombocytopenia. *Int J Lab Hematol*. 2019;41(Suppl. 1):15.
69. Warkentin TE, Hayward CP, Smith CA, et al. Determinants of donor platelet variability when testing for heparin-induced thrombocytopenia. *J Lab Clin Med*. 1992;120:371.
70. Warkentin TE, Nazy I, Sheppard JI, et al. Serotonin-release assay-negative heparin-induced thrombocytopenia. *Am J Hematol*. 2020;95:38.
71. Pandya KA, Johnson EG, Davis GA, et al. Serotonin release assay (SRA)-negative HIT, a newly recognized entity: implications for diagnosis and management. *Thromb Res*. 2018;172:169.
72. Nazi I, Arnold DM, Warkentin TE, et al. Distinguishing between anti-platelet factor 4/heparin antibodies that can and cannot cause heparin-induced thrombocytopenia. *J Thromb Haemost*. 2015;13:1900.
73. Padmanabhan A, Jones CG, Curtis BR, et al. A novel PF4-dependent platelet activation assay identifies patients likely to have heparin-induced thrombocytopenia/thrombosis. *Chest*. 2016;150:506.
74. Warkentin TE, Greinacher A, Gruel Y, et al. Laboratory testing for heparin-induced thrombocytopenia: a conceptual framework and implications for diagnosis. *J Thromb Haemost*. 2011;9:2498.
75. Warkentin TE. Challenges in detecting clinically relevant HIT antibodies. *Hämostaseologie*. 2020;40:472.
76. Warkentin TE, Sheppard JI, Linkins LA, et al. Performance characteristics of an automated latex immunoturbidimetric assay [HemosIL® HIT-Ab(PF4-H)] for the diagnosis of immune heparin-induced thrombocytopenia. *Thromb Res*. 2017;153:108.
77. Warkentin TE, Sheppard JI, Linkins LA, et al. High sensitivity and specificity of an automated IgG-specific chemiluminescence immunoassay for diagnosis of HIT. *Blood*. 2018;132:1345.
78. Sachs UJ, von Hesberg J, Santoso S, et al. Evaluation of a new nanoparticle-based lateral-flow immunoassay for the exclusion of heparin-induced thrombocytopenia (HIT). *Thromb Haemost*. 2011;106:1197.
79. Nellen V, Sulzer I, Barizzi G, et al. Rapid exclusion or confirmation of heparin-induced thrombocytopenia: a single-center experience with 1,291 patients. *Haematologica*. 2012;97:89.
80. Warkentin TE, Cook RJ, Greinacher A. Limitations of the particle immunofiltration assay test for diagnosis of heparin-induced thrombocytopenia. *Am J Hematol*. 2020;95:E250.
81. Marchetti M, Barelli S, Zermatten MG, et al. Rapid and accurate Bayesian diagnosis of heparin-induced thrombocytopenia. *Blood*. 2020;135:1171.
82. Warkentin TE, Sheppard JI, Smith JW, et al. Combination of two complementary automated rapid assays for diagnosis of heparin-induced thrombocytopenia (HIT). *J Thromb Haemost*. 2020;18:1435.
83. Cuker A, Arepally GM, Chong BH, et al. American Society of Hematology 2018 guidelines for management of venous thromboembolism: heparin-induced thrombocytopenia. *Blood Adv*. 2018;22:3360.
84. Shih AW, Sheppard JI, Warkentin TE. Platelet count recovery and seroreversion in immune HIT despite continuation of heparin: further observations and literature review. *Thromb Haemost*. 2017;117:1868.
85. Chong BH, Gallus AS, Cade JF, et al. Prospective randomised open-label comparison of danaparoid with dextran 70 in the treatment of heparin-induced thrombocytopenia with thrombosis: a clinical outcome study. *Thromb Haemost*. 2001;86:1170.
86. Lubenow N, Warkentin TE, Greinacher A, et al. Results of a systematic evaluation of treatment outcomes for heparin-induced thrombocytopenia in patients receiving danaparoid, ancrod, and/or coumarin explain the rapid shift in clinical practice during the 1990s. *Thromb Res*. 2006;117:507.
87. Warkentin TE, Pai M, Sheppard JI, et al. Fondaparinux treatment of acute heparin-induced thrombocytopenia confirmed by the serotonin-release assay: a 30-month, 16-patient case series. *J Thromb Haemost*. 2011;9:2389.
88. Kang M, Alahmadi M, Sawh S, et al. Fondaparinux for the treatment of suspected heparin-induced thrombocytopenia: a propensity score-matched study. *Blood*. 2015;125:924.
89. Schindewolf M, Steindl J, Beyer-Westendorf J, et al. Use of fondaparinux off-label or approved anticoagulants for management of heparin-induced thrombocytopenia. *J Am Coll Cardiol*. 2017;70:2636.
90. Linkins LA, Hu G, Warkentin TE. Systematic review of fondaparinux for heparin-induced thrombocytopenia: when there are no randomized controlled trials. *Res Pract Thromb Haemost*. 2018;2:678.
91. Warkentin TE, Davidson BL, Büller HR, et al. Prevalence and risk of preexisting heparin-induced thrombocytopenia antibodies in patients with acute VTE. *Chest*. 2011;140:366–373.
92. Warkentin TE. Fondaparinux: does it cause HIT? Can it treat HIT? *Expert Rev Hematol*. 2010;3:567.
93. Warkentin TE, Pai M, Linkins LA. Direct oral anticoagulants for treatment of HIT: update of Hamilton experience and literature review. *Blood*. 2017;130:1104.
94. Farasatinasab M, Zarei B, Moghtadaei M, et al. Rivaroxaban as an alternative agent for heparin-induced thrombocytopenia. *J Clin Pharmacol*. 2020;60:1362.
95. Ezekwudo DE, Chacko R, Gbadamosi B, et al. Apixaban for treatment of confirmed heparin-induced thrombocytopenia: a case report and review of literature. *Exp Hematol Oncol*. 2017;6:21.
96. Warkentin TE, Greinacher A, Koster A. Bivalirudin. *Thromb Haemost*. 2008;99:830.
97. Mahaffey KW, Lewis BE, Wildermann NM, et al. The anticoagulant therapy with bivalirudin to assist in the performance of percutaneous coronary intervention in patients with heparin-induced thrombocytopenia (ATBAT) study: main results. *J Invasive Cardiol*. 2003;15:611.
98. Joseph L, Casanegra AI, Dhariwal M, et al. Bivalirudin for the treatment of patients with confirmed or suspected heparin-induced thrombocytopenia. *J Thromb Haemost*. 2014;12:1044.
99. Hursting MJ, Murray PT. Argatroban anticoagulation in renal dysfunction: a literature analysis. *Nephron Clin Pract*. 2008;109:c80.
100. Beiderlinden M, Treschan TA, Görlinger K, et al. Argatroban anticoagulation in critically ill patients. *Ann Pharmacother*. 2007;41:749.
101. Lewis BE, Hursting MJ. Direct thrombin inhibition during percutaneous coronary intervention in patients with heparin-induced thrombocytopenia. *Expert Rev Cardiovasc Ther*. 2007;5:57.
102. Warkentin TE, Greinacher A, Craven S, et al. Differences in the clinically effective molar concentrations of four direct thrombin inhibitors explain their variable prothrombin time prolongation. *Thromb Haemost*. 2005;94:958.
103. Hursting MJ, Lewis BE, Macfarlane DE. Transitioning from argatroban to warfarin therapy in patients with heparin-induced thrombocytopenia. *Clin Appl Thromb Hemost*. 2005;11:279.
104. Warkentin TE, Greinacher A, Koster A, et al. Treatment and prevention of heparin-induced thrombocytopenia: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th edition). *Chest*. 2008;133:340S.
105. Greinacher A, Liebenhoff U, Kiefel V, et al. Heparin-associated thrombocytopenia: the effects of various intravenous IgG preparations on antibody mediated platelet activation—a possible new indication for high dose i.v. IgG. *Thromb Haemost*. 1994;71:641.
106. Warkentin TE. High-dose intravenous immunoglobulin for the treatment and prevention of heparin-induced thrombocytopenia. *Exp Rev Hematol*. 2019;12:685.
107. Arcinas LA, Manji RA, Hrymak C, et al. Autoimmune heparin-induced thrombocytopenia and venous limb gangrene after aortic dissection repair: in vitro and in vivo effects of intravenous immunoglobulin. *Transfusion*. 2019;59:1924.
108. Park BD, Kumar M, Nagalla S, et al. Intravenous immunoglobulin as an adjunct therapy in persisting heparin-induced thrombocytopenia. *Transfus Apher Sci*. 2018;57:561.
109. McKenzie DS, Anuforo J, Morgan J, et al. Successful use of intravenous immunoglobulin G to treat refractory heparin-induced thrombocytopenia with thrombosis complicating peripheral blood stem cell harvest. *J Investig Med High Impact Case Rep*. 2018;6 2324709618755414.

110. Mohanty E, Nazir S, Sheppard JI, et al. High-dose intravenous immunoglobin to treat spontaneous heparin-induced thrombocytopenia syndrome. *J Thromb Haemost*. 2019;17:841.
111. Warkentin TE, Climans TH, Morin PA. Intravenous immune globulin to prevent heparin-induced thrombocytopenia. *N Engl J Med*. 2018;378:1845.
112. Koster A, Nazy I, Birschmann IE, et al. High-dose IVIG plus cangrelor platelet "anesthesia" during urgent heparin-CPB in a patient with recent SRA-negative HIT-thrombosis with persisting heparin-activating antibodies. *Res Pract Thromb Haematol*. 2020;23:1060.
113. Warkentin TE. Anticoagulant failure in coagulopathic patients: PTT confounding and other pitfalls. *Expert Opin Drug Saf*. 2014;13:25.
114. Warkentin TE, Sheppard JI, Chu FV, et al. Plasma exchange to remove HIT antibodies: dissociation between enzyme-immunoassay and platelet activation test reactivities. *Blood*. 2015;125:195.
115. Warkentin TE, Anderson JAM. How I treat patients with a history of heparin-induced thrombocytopenia. *Blood*. 2016;128:348.
116. Warkentin TE, Sheppard JI. Serological investigation of patients with a previous history of heparin-induced thrombocytopenia who are reexposed to heparin. *Blood*. 2014;123:2485.

CHAPTER 132

THROMBOTIC THROMBOCYTOPENIC PURPURA AND THE HEMOLYTIC UREMIC SYNDROMES

Gemlyn George and Kenneth D. Friedman

In 1924, Moschowitz reported a case of a 16-year-old girl who died of a previously undescribed illness characterized by microangiopathic hemolytic anemia (MAHA), petechiae, hemiparesis, and fever.[1] Postmortem examination revealed numerous hyaline thrombi, most prevalent in the terminal arterioles and capillaries of the heart and kidneys. In 1936, four similar cases were reported by Baehr and colleagues, who proposed that the hyaline thrombi were secondary to agglutinated platelets. In 1947, Singer suggested that the term thrombotic thrombocytopenic purpura (TTP) be used to describe this disorder. In 1955, Gasser used the term hemolytic uremic syndrome (HUS) to describe a related syndrome consisting of Coombs-negative hemolytic anemia, thrombocytopenia, and renal failure. The physiology of these two disorders remained a mystery until 1982 when "unusually large" multimers of von Willebrand factor (VWF) were discovered in the plasma of patients with relapsing TTP.[2] The clinical features of Shiga toxin-associated HUS (ST-HUS) were described in five young children who presented with renal failure following a diarrheal illness in 1962.[3] An association between HUS and *Shigella* infections was recognized in 1975 and *E. coli* that produce Shiga toxin (Stx) were identified in 1983. These disorders are now collected under the rubric of *thrombotic microangiopathies (TMA)*, based on their shared features of MAHA, thrombocytopenia, and microvascular thrombotic lesions with resultant organ dysfunction.

MAHA and thrombocytopenia are cardinal signs/features of all TMA syndromes, leading to overlapping clinical and pathologic features. However, a multitude of different pathogenic pathways lead to vascular endothelial injury in these conditions (Fig. 132.1). Most cases of TTP appear to be due to deficiency of a disintegrin-like and metalloprotease with thrombospondin type1 motif, family member 13 (ADAMTS13) either through an autoantibody or a congenital deficiency. This deficiency results in failure to control the interaction of VWF with platelets and subsequent organ dysfunction is a consequence of platelet-rich thrombi formation in the microcirculation (and occasionally in large vessels). The TMA syndrome with MAHA, thrombocytopenia, and renal failure not associated with diarrhea or severe ADAMTS13 deficiency had been called "atypical" hemolytic uremic syndrome (aHUS). This is mainly attributable to a defect in the regulation of the complement mechanism and unregulated deposition of complement factor C3b on cellular surfaces and these are currently designated as complement-mediated HUS (CM-HUS). A rare autosomal recessively inherited form of TMA occurs in patients with defects in the gene encoding diacylglycerol kinase epsilon (DGKε), which results in a shift of endothelial cells to a prothrombotic phenotype. Similarly, mutations in the methylmalonic aciduria and homocystinuria type C (MMACHC) gene results in methylmalonic aciduria and homocystinuria progressing to TMA with renal failure.

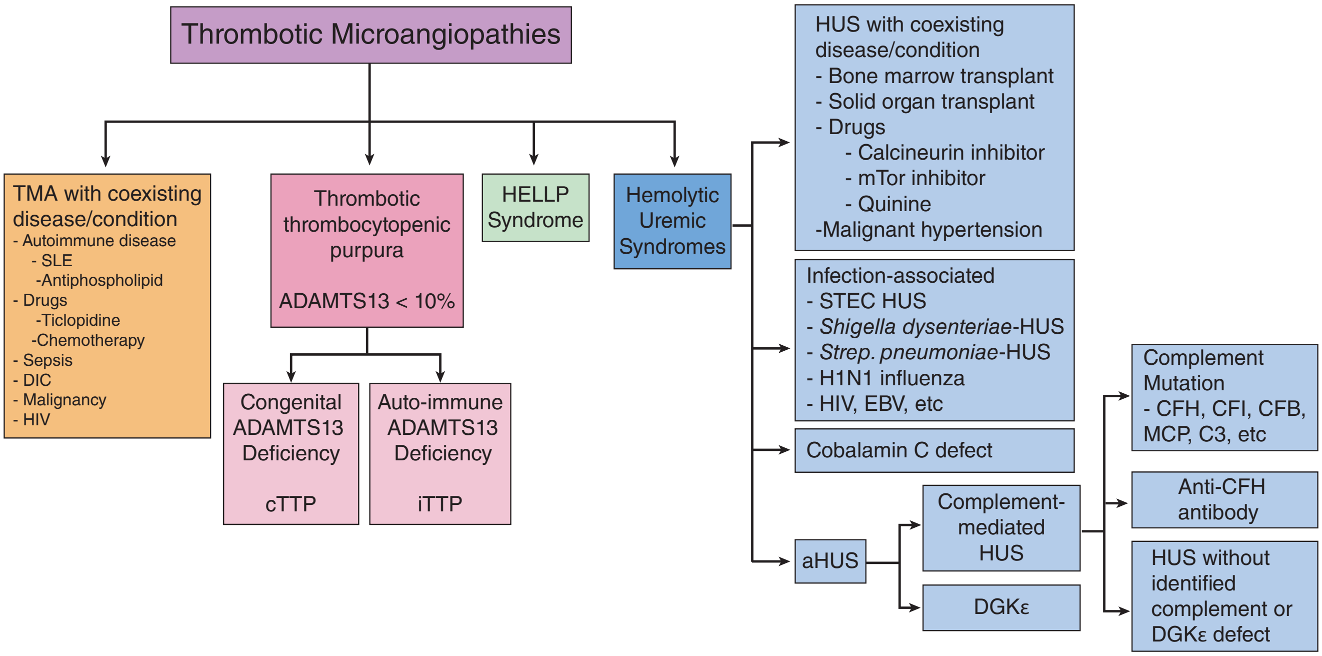

Thrombotic Microangiopathies
TMA with coexisting disease/condition
- Autoimmune disease
- SLE
-Antiphospholipid
- Drugs
-Ticlopidine
-Chemotherapy
- Sepsis
- DIC
- Malignancy
- HIV
Thrombotic thrombocytopenic purpura
ADAMTS13 < 10%
Congenital ADAMTS13 Deficiency
cTTP
Auto-immune ADAMTS13 Deficiency
iTTP
HELLP Syndrome
Hemolytic Uremic Syndromes
HUS with coexisting disease/condition
- Bone marrow transplant
- Solid organ transplant
- Drugs
- Calcineurin inhibitor
- mTor inhibitor
- Quinine
-Malignant hypertension
Infection-associated
- STEC HUS
- Shigella dysenteriae-HUS
- Strep. pneumoniae-HUS
- H1N1 influenza
- HIV, EBV, etc
Cobalamin C defect
aHUS
Complement-mediated HUS
DGKε
Complement Mutation
- CFH, CFI, CFB, MCP, C3, etc
Anti-CFH antibody
HUS without identified complement or DGKε defect

Figure 132.1 CLASSIFICATION OF THE VARIOUS FORMS OF THROMBOTIC MICROANGIOPATHIES. *aHUS*, Atypical hemolytic uremic syndrome; *C3*, complement factor 3; *CFB*, complement factor B; *CFH*, complement factor H; *CFI*, complement factor I; *cTTP*, congenital thrombotic thrombocytopenic purpura; *DGKε*, diacylglycerol kinase ε; *DIC*, disseminated intravascular coagulation; *EBV*, Epstein-Barr virus; *HELLP*, hemolysis, elevated liver function tests, and low platelet count; *HIV*, human immunodeficiency virus; *iTTP*, immune thrombotic thrombocytopenic purpura; *MCP*, membrane cofactor protein; *SLE*, systemic lupus erythematosus; *STEC*, Shiga toxin-producing *E. coli*; *TMA*, Thrombotic microangiopathy. *(Adapted from Fakouri F, Zuber J, Frémeaux-Bacchi V, et al. Haemolytic uraemic syndrome. Lancet. 2017;390:681–696.)*

Finally, in hemolytic uremic syndrome due to Stx-producing *E. coli* (STEC-HUS), endothelial injury is caused by Stx and inflammatory cytokines, perhaps inciting disease with increased frequency in individuals with specific genetic predispositions. STEC-HUS is the main mechanism underlying infection-associated HUS (IA-HUS), but several other infections may lead to TMA through other mechanisms. With improved understanding of the pathogenesis of TMA and the availability of targeted therapies, distinction among the various TMA syndromes is important.

DIFFERENTIAL DIAGNOSIS

The differential diagnosis of MAHA and thrombocytopenia is extensive (Fig. 132.2). Vascular damage secondary to severe sepsis, autoimmune disorders (i.e., systemic lupus erythematosus, scleroderma, antiphospholipid syndrome), septic or tumor emboli, immune complex-mediated vasculitis (e.g., infective endocarditis), malignant hypertension, complications of pregnancy (severe pre-eclampsia, eclampsia, HELLP syndrome), cryoglobulinemia, infection with rickettsia, or, more rarely, hemorrhage-inducing viral organisms, may all lead to thrombotic microangiopathy. Occasionally, patients with disseminated intravascular coagulation secondary to malignancy or sepsis present with microangiopathy of sufficient severity to be confused with primary TMA (see Chapter 137). In the setting of renal transplantation, a biopsy may be required to distinguish TMA from allograft rejection or recurrence of a preexisting renal vascular disorder. Occasional patients present with acute pancreatitis, acute respiratory distress syndrome, memory and personality changes, or other poorly defined neurologic symptoms, which have a broad differential diagnosis.

There are clues that have suggested that STEC-HUS examination should include the patient's age at the time of presentation, ingestion of undercooked ground beef or other food that might have been contaminated by cattle, contact with farm animals, or concurrent clinical presentation by another family member. History of asynchronous presentation in a sibling suggests inherited TTP or CM-HUS. Historic approaches to distinguishing between TTP from CM-HUS relied on the age at presentation and the types of symptoms. However, as demonstrated by trials showing the utility of eculizumab, CM-HUS is not uncommonly diagnosed in adults. Similarly, there is significant end-organ dysfunction overlap between these two disorders. While the kidney remains a major target organ in CM-HUS, renal injury of sufficient severity to require dialysis may occur in up to 10% of patients with TTP. Neurologic symptoms are reported in 25% to 79% of patients diagnosed with TTP but may also be seen in 10% to 30% of patients with CM-HUS.

Choosing the appropriate therapy depends on an accurate diagnosis. Once MAHA and thrombocytopenia have been confirmed by examination of the peripheral smear, other systemic disorders (e.g., sepsis, progression of already established disease or complications of drug therapies) must be excluded. (See box on Management of Complement-Mediated Hemolytic Uremic Syndrome.)

The probability of TTP can be calculated using scoring systems (discussed below) in addition to ADAMTS13 activity testing. In the setting of rapidly progressing renal failure, complement-mediated TMA should be considered because early use of eculizumab has

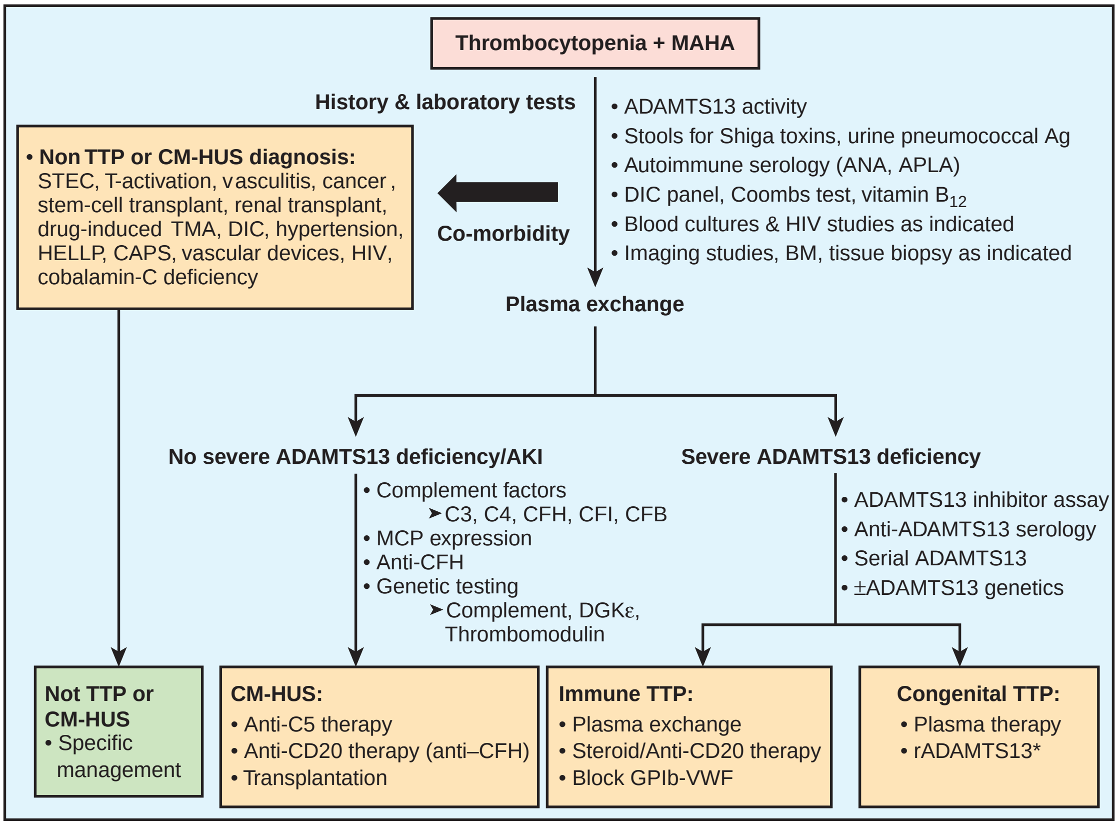

Thrombocytopenia + MAHA
History & laboratory tests
• ADAMTS13 activity
• Stools for Shiga toxins, urine pneumococcal Ag
• Autoimmune serology (ANA, APLA)
• DIC panel, Coombs test, vitamin B12
• Blood cultures & HIV studies as indicated
• Imaging studies, BM, tissue biopsy as indicated
• Non TTP or CM-HUS diagnosis:
STEC, T-activation, vasculitis, cancer, stem-cell transplant, renal transplant, drug-induced TMA, DIC, hypertension, HELLP, CAPS, vascular devices, HIV, cobalamin-C deficiency
Co-morbidity
Plasma exchange
No severe ADAMTS13 deficiency/AKI
• Complement factors
➤C3, C4, CFH, CFI, CFB
• MCP expression
• Anti-CFH
• Genetic testing
➤Complement, DGKε, Thrombomodulin
Severe ADAMTS13 deficiency
• ADAMTS13 inhibitor assay
• Anti-ADAMTS13 serology
• Serial ADAMTS13
• ±ADAMTS13 genetics
Not TTP or CM-HUS
• Specific management
CM-HUS:
• Anti-C5 therapy
• Anti-CD20 therapy (anti–CFH)
• Transplantation
Immune TTP:
• Plasma exchange
• Steroid/Anti-CD20 therapy
• Block GPIb-VWF
Congenital TTP:
• Plasma therapy
• rADAMTS13*

Figure 132.2 AN APPROACH TO DIAGNOSIS AND MANAGEMENT OF THROMBOTIC MICROANGIOPATHIES. *ADAMTS13*, A disintegrin and metalloproteinase with thrombospondin type 1 motifs, member 13; *ANA*, antinuclear antibody; *APLA*, antiphospholipid antibodies; *BM*, bone marrow; *CAPS*, catastrophic antiphospholipid syndrome; *CFB*, complement factor B; *CFH*, complement factor H; *CFI*, complement factor I; *CM-HUS*, complement-mediated hemolytic uremic syndrome; *DGKε*, diacyl glycerol kinase ε; *DIC*, Disseminated intravascular coagulation; *GPIb*, platelet glycoprotein Ib; *HELLP*, hemolysis, elevated liver function tests, and low platelet count; *MCP*, membrane cofactor protein; *STEC*, Shiga toxin-producing *E. coli.*; *THBD*, thrombomodulin; *VWF*, von Willebrand factor; Anti-C5 therapy refers to monoclonal antibody therapy such as eculizumab; Anti-CD20 therapy refers to monoclonal antibody therapy such as rituximab. Green and orange boxes indicate diagnoses or therapies. * indicates therapy under investigation. *(Modified from Zuber J, Fakhouri F, Roumenina LT, et al. Use of eculizumab for atypical haemolytic uraemic syndrome and C3 glomerulopathies. Nat Rev Nephrol. 2012;8:643–657.)*

been shown to prevent or reverse kidney injury. STEC infection should be ruled out as soon as possible if infection-ssociated HUS is considered, utilizing appropriate microbiologic studies (stool culture, immunoassay for Stx or fecal PCR). Children and young adults should be tested for a cobalamin C defect because defects in cobalamin metabolism may produce a similar hematologic picture (including thrombocytopenia, presence of bizarre microcytic red cells on the peripheral blood film, and elevations of lactate dehydrogenase [LDH] and bilirubin).

THROMBOTIC THROMBOCYTOPENIC PURPURA

Epidemiology

Acquired TTP or immune mediated (iTTP) accounts for 95% of all TTP cases and occurs with an estimated annual incidence of two to six cases per million (variation based on registry), but the incidence appears to be increasing, perhaps because of increased awareness. iTTP has a higher incidence in adults than in children and has a female/male ratio of 2–3:1 and a peak incidence in the fourth decade. Additional risk factors include blood group O, obesity, and African ancestry, which is also associated with an increased risk of relapse. Acquired TTP can occur in association with predisposing conditions such as autoimmune diseases, malignancy, infections, drugs, and pregnancy. TTP has been reported in approximately 0.3% of HIV-infected patients, usually in those with advanced disease.

Pregnancy is a common precipitating factor for both immune TTP (12% to 31% of individuals) and inherited severe ADAMTS13 deficiency.[4] Clinical symptomatic TTP most frequently develops in the second or third trimester, with the decrease in ADAMTS13 levels and increase in VWF and factor VIII that occur in normal pregnancy perhaps acting as precipitants.

Congenital TTP (cTTP), also known as Upshaw-Schülman syndrome, is less common than immune TTP. In general, most estimates of cTTP range from 0.5 to 2 cases per million. This incidence varies based on the region.

Pathobiology

TTP occurs when there is an inherited or acquired deficiency of ADAMTS13 (generally defined as less than 10%), leading to increased levels of "unusually large" VWF multimers that induce platelet aggregation in the microvasculature (Fig. 132.3).

TTP was first linked to VWF by Moake et al., who identified unusually large VWF multimers between disease flares in the plasma of patients with chronic relapsing TTP. He proposed that such patients lacked a plasma enzyme that cleaves these unusually large multimers and that accumulation of abnormally large multimers caused uncontrolled intravascular platelet aggregation, thrombosis, and tissue infarction. Subsequently, two separate investigators identified the plasma metalloprotease that degrades VWF multimers by cleaving the Tyr^{1605}-Met^{1606} bond in the VWF A2 domain (see Chapter 133). This metalloprotease is lacking in patients with TTP, including a subset with a presumably inherited form of TTP, and mixing studies demonstrated the presence of an immunoglobulin inhibitor in many of the non-inherited cases. It was also demonstrated that patients with clinically suspected HUS were not deficient in the metalloprotease, supplying the first clear evidence that the pathogenesis of TTP differs from that of HUS.

The breakthrough in the identification of the VWF cleaving protease happened in 2001 when genome-wide linkage analysis revealed that Upshaw-Schülman syndrome is caused by mutations in the ADAMTS13 gene on chromosome 9q34 (discussed within the inherited TTP section).[5] Almost simultaneously, three other groups working with the VWF-degrading protease (isolated from plasma or from commercial factor VIII/VWF concentrate) identified it as a member of the ADAMTS (A Disintegrin-like and Metalloprotease [reprolysin type] with ThromboSpondin type 1 motif) family of metalloproteases.; the designation 13 indicates that it was the 13th

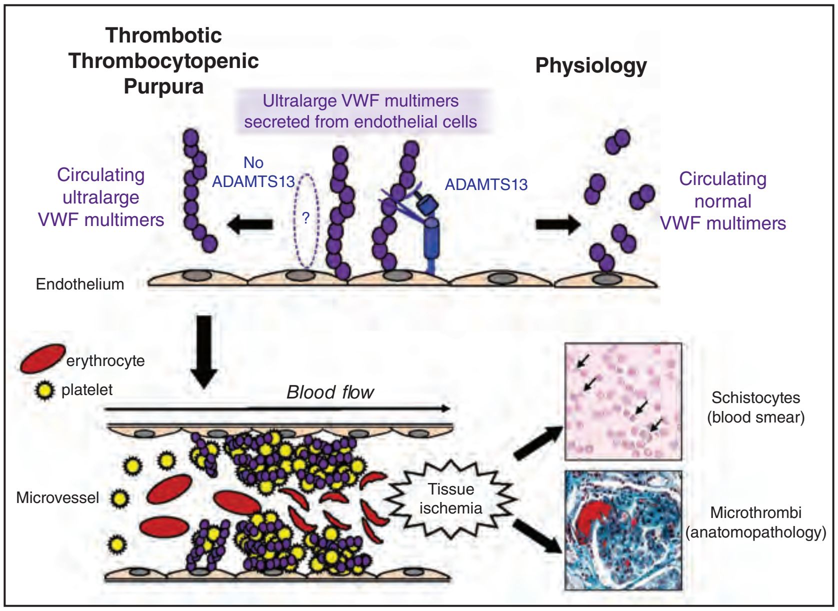

Thrombotic Thrombocytopenic Purpura
Physiology
Ultralarge VWF multimers secreted from endothelial cells
Circulating ultralarge VWF multimers
No ADAMTS13
?
ADAMTS13
Circulating normal VWF multimers
Endothelium
erythrocyte
platelet
Blood flow
Microvessel
Tissue ischemia
Schistocytes (blood smear)
Microthrombi (anatomopathology)

Figure 132.3 PATHOPHYSIOLOGY OF THROMBOTIC THROMBOCYTOPENIC PURPURA (TTP). In physiologic conditions, ultralarge von Willebrand factor (VWF) multimers released from endothelial cells are cleaved by ADAMTS13 into smaller VWF multimers, that are less adhesive to platelets. In TTP, because of absence of functional ADAMTS13 (either absent by congenital defect or inhibitor by specific autoantibodies), ultralarge VWF multimers are released into the blood and bind spontaneously to platelets to form aggregates within the arterial and capillary microvessels, The VWF-platelet aggregates are large enough to form microthrombi inducing tissue ischemia, platelet consumption and microangiopathic hemolytic anemia (schistocytes on blood smear). *(From Joly B, Coppo P, Veyradier A. Thrombotic thrombocytopenic purpura. Blood. 2017;129:2836–2846.)*

member of the ADAMTS family to be described. ADAMTS13 consists of multiple domains (Metalloprotease, Disintegrin-like domain, Thrombospondin type 1 repeat, Cysteine rich domain and Spacer domain, seven thrombospondin type 1 repeats, and two CUB [Complement components C1r and C1s, sea urchin protein Uegf, and Bone morphogenic protein-1] domains). The protein circulates in a folded conformation with the CUB domains interacting with the spacer domain. This folded structure suggests an autoinhibitory role in which the CUB domains shield exosites on ADAMTS13 to prevent unregulated enzyme activity and VWF proteolysis.

Endothelial cells secrete large VWF multimers that adhere to exposed collagen through their A3 domains. When VWF is tethered to collagen under high shear, these multimers elongate and reveal the platelet binding site for Gp1bα, which is in the A1 domain (see Chapter 123). This process facilitates platelet recruitment to the site of injury. While ADAMTS13 is generally maintained in a closed conformation with its CUB domains interacting and shielding the spacer domain, shear forces can open the conformation. Reciprocally induced conformational change in VWF and ADAMTS13 results in exposure of exosites on the enzyme and docking sites on VWF, culminating in regulated ADAMTS13-mediated cleavage of VWF in its A2 domain.[6] Such cleavage reduces the length of VWF multimers and curtails its capacity to form thrombi particularly in areas of high shear (e.g., small arterioles and capillaries).

The factors that trigger sporadic episodes of TTP by causing endothelial damage or activation remain undefined. Some patients with congenital deficiencies of ADAMTS13 may not develop TTP until adulthood, or not at all despite chronic ADAMTS13 deficiency. The latter observations suggest that ADAMTS13 deficiency should be considered an important predisposing factor, but not the sole cause of this syndrome. This concept is supported by studies of ADAMTS13-deficient mice, in which a TTP-like syndrome develops spontaneously in some genetic backgrounds (high VWF levels) but requires a triggering factor, such as Stx, in other strains.

Antibodies (abs) against ADAMTS13 work by inhibiting the proteolytic activity of ADAMTS13 on VWF or by increasing the clearance of ADAMTS13 from plasma by forming immune complexes. Most inhibitory immunoglobulins are directed against the spacer domain of ADAMTS13, but antibodies directed against carboxyl-terminal CUB1-2 and TSP2-8 domains also have been described. Confounding this finding were reports from serologic studies indicating that anti-ADAMTS13 IgG may be present in 5% of healthy individuals, 20% of patients with a TMA other than TTP, 13% of patients with SLE, and 5% of patients with antiphospholipid syndrome.[7] From a mechanistic standpoint, administration of the antibody alone resulted in a clinical syndrome resembling iTTP in baboon models.[8] Work from Roose et al., also suggests that the conformational state of ADAMTS13 is important in the pathogenesis of the disease.[9] Normally, ADAMTS13 is folded on itself in a "closed" and inactive conformation. However, ADAMTS13 opens when it docks with VWF. ADAMTS13 assumes an open conformation in the acute phase of the disease. This is important because, in the closed conformation, the spacer sites of ADAMTS13 are not accessible to autoantibodies. The factors that trigger the open conformation have yet to be discovered; however, one could hypothesize that there is a dynamic process, and that the presence of antibody stabilizes ADAMTS13 in the open conformation. From a clinical perspective, both high antibody levels and lower antigen levels are associated with mortality rates of 17% and 18%, respectively, while the combination appears to have a synergistic effect with a mortality rate of 27%.[10]

Many theories have been proposed to explain the onset of iTTP. The most prominent of these is the link between infections and iTTP. Proposed mechanisms include a two-hit model, cross reaction with $CD4^+$ T cells targeting microbiome derived peptides, variants in TLR-9 being more prone to producing Th1 cytokines and activation of the alternate complement pathway through interaction with unusually large VWF multimers.[11] Since the HLA system has been implicated in the development of autoimmune disease, research efforts have been directed to evaluation of the HLA system as a factor in the development of iTTP. The first association was reported in the 1990s with the antigens DR52 and DR53 from the HLA-DRB3 and HLA-DRB4 genes, respectively.[12] In 2010, two European studies demonstrated that HLA class 2 loci DRB1*11, DRB1*15, DQB1*03, and DQB1*06 were predisposing haplotypes for iTTP.[13] Interestingly, HLA-DRB1*04 and its associated HLA-DRB4-encoded serotype HLA-DR53 were shown to be protective against the development of iTTP. A Japanese group recently reported an association between HLA alleles DRB1*08:03, DRB3/4/5*blank, DQA1*01:03, and DQB1*06:01 and iTTP.[14] The increased incidence of iTTP in persons of African origin may reflect the low prevalence of the protective allele DRB1*04 in this ethnic group.[15]

Clinical Manifestations

The classic pentad of signs and symptoms of TTP syndrome include MAHA, thrombocytopenia, neurologic impairment, fever, and renal dysfunction. The complete pentad was most often described before the routine use of therapeutic plasma exchange. Earlier recognition of this syndrome and the established efficacy of plasma exchange, however, have led to the appreciation that MAHA and thrombocytopenia, in the absence of an obvious precipitating condition, are sufficient to make a presumptive diagnosis of TTP. In fact, at diagnosis less than 10% of patients present with the full pentad.

Approximately 10% to 40% of patients with TTP recall an upper respiratory tract infection or flu-like syndrome in the weeks preceding the diagnosis. Patients may present with malaise, fatigue, fever, or other nonspecific symptoms days to weeks in duration and are unresponsive to antibiotics or symptomatic management. The frequency of end-organ involvement is variable with the renal system, GI system, central nervous system, and cardiac system most often affected.

The central nervous system is the most often affected visceral organ at presentation with symptoms ranging from headache or transient confusion to severe symptoms such as seizures, TIA or stroke. Most patients have normal findings on imaging. Gastrointestinal symptoms occur in 35% to 40% of patients and include nausea, vomiting, abdominal pain or diarrhea. Acute renal failure requiring dialysis occurs in up to 5% to 15% of patients and severe renal dysfunction is more common in HUS. An elevated troponin may be identified in up to 70% of patients with TTP and is associated with worse prognosis.

Scoring systems incorporating clinical and laboratory parameters have been developed to enable prompt identification of patients with thrombotic microangiopathy who may benefit from plasma exchange (Table 132.1). These include the French score, the Bentley score and the PLASMIC score. The French score, which was derived from a cohort of 214 patients, has three components, (thrombocytopenia, creatinine level, and ANA) and has a negative predictive value of 93% with a score of zero.[16] The PLASMIC score contains seven components, and stratifies patients into low-, intermediate-, and high-risk categories.[17] It was developed to predict the likelihood of ADAMTS13 activity less than 10%. A high PLASMIC score (six to seven) is predictive of ADAMTS13 activity ≤10 with a sensitivity of 94%. Similarly, a low PLASMIC score predicts the absence of severe ADAMTS13 deficiency 98% of the time. A high probability score with either the French or the PLASMIC scoring system supports immediate initiation of plasma exchange.

Older adults (greater than 60 years) may have a distinct presentation compared with young adults. Atypical neurological symptoms such as confusion, headache, delirium, behavioral changes, TIAs, and stroke are commonly seen. Older patients present with greater renal impairment and less severe thrombocytopenia and anemia compared with younger patients. Consequently, the sensitivity of the PLASMIC score may be lower in older individuals, which can lead to a delay in diagnosis. Critically, the scoring systems developed for use in TTP were validated with populations containing an over representation of younger patients. In a study by the French group for TMA, only 61% of older patients with severe ADAMTS13 deficiency were as high risk using the French score.[18]

Laboratory Manifestations

The presence of schistocytes on the peripheral blood smear is a characteristic laboratory finding of TTP (Fig. 132.4). In most TMAs, schistocytes account for more than 1% of all erythrocytes. A finding of two or more schistocytes per high-power field is suggestive of MAHA. However, there is no threshold number below which the possibility of TTP can be excluded. More subtle signs of RBC fragmentation may be seen early in the course of the disease. Thrombocytopenia in TTP reflects deposition of platelets in microthrombi. In a series of 70 patients with TTP, the mean platelet count on presentation was 10,000.

TABLE 132.1 **PLASMIC and French Score Prediction of Likelihood of Severe ADAMTS13 Deficiency of Thrombotic Thrombocytopenic Purpura in Cases of Suspected Thrombotic Thrombocytopenic Purpura**

Parameter	French Score	PLASMIC Score
Platelet count	<30,000/μL: +1	<30,000/μL: +1
Serum creatinine level	<2.26 mg/dL: +1	<2.0 mg/dL: +1
Markers of hemolysis • Indirect bilirubin >2 mg/dL • Undetectable haptoglobin • Reticulocyte count >2.5%	[a]	+1
No active cancer in previous year	[a]	+1
No history of solid organ or stem cell transplant	[a]	+1
International normalized ratio (INR) < 1.5	[a]	+1
Mean corpuscular volume (MCV) < 90 fL	—	+1
Likelihood of severe ADAMTS13 deficiency (< 10%)	**Score = 0: 2%** **Score = 1: 70%** **Score = 2: 94%**	**Score = 0–4: 0%–4%** **Score = 5: 9%–24%** **Score = 6–7: 62%–82%**

[a] French score assumes microvascular hemolysis (schistocytes present) and absence of clinical evidence for associated cancer, transplantation or disseminated intravascular coagulation, and did not incorporate mean red cell volume.

TTP, Thrombotic thrombocytopenic purpura.

Adapted from Bendapudi PK, Hurwitz S, Fry A, et al. Derivation and external validation of the PLASMIC score for rapid assessment of adults with thrombotic microangiopathies: a cohort study. *Lancet Haematol*. 2017;4(4):e157-e164; and Zheng XL, Vesely SK, Cataland SR, et al. ISTH guidelines for the diagnosis of thrombotic thrombocytopenic purpura. *J Thromb Haemost*. 2020;18:2486–2495.

ADAMTS13 activity testing is critical for the diagnosis of TTP. However, therapy should not be delayed while waiting for results of this test if the clinical suspicion for TTP is high. An activity level of less than 10% confirms the diagnosis of TTP in patients with MAHA and thrombocytopenia without any other obvious cause. ADAMTS13 activity less than 10% has been noted in rare cases of sepsis and systemic cancer and thus cannot be used exclusively as a diagnostic test; the clinical context must also be considered.

ADAMTS13 activity assays involve incubation of plasma with substrate full-length VWF multimers or truncated peptides containing the ADAMTS13 cleavage site. The substrate undergoes proteolysis by ADAMTS13 in the test plasma and enzyme activity is measured by detecting the cleavage product or by measuring the residual VWF. The first generation of assays used VWF as the substrate with urea or guanidine hydrochloride to expose the cleavage site in the VWF A2 domain. Current methods for ADAMTS13 level measurement utilize VWF peptides that undergo rapid cleavage when exposed to ADAMTS13, with endpoints based upon detection of fluorescence due to separation of emitter and quencher adducts that become separated through the cleavage process (fluorescence resonance energy transfer [FRET]–based assays utilize substrates such as VWF-VWF73 peptide), immunologic detection of the cleaved end of a solid-phase adherent residual peptide or a surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. The antigen assays quantify the ADAMTS13 protein levels in plasma which includes both free active enzyme as well as inactive ADAMTS13 in complex with autoantibody.

The measurement of ADAMTS13 inhibitor levels using a functional inhibitor assay and/or autoantibody assay should also be performed when possible because the results are useful for predicting the clinical course and for distinguishing between iTTP and cTTP (see discussion in pathophysiology). Functional inhibitor assay is performed by mixing patient plasma with a source of ADAMTS13 (such as normal pooled plasma) and measuring the residual ADAMTS13 content. Inhibitor units are reported in a fashion analogous to how Bethesda units are quantified in the setting of hemophilia; one inhibitor unit is defined as the amount of inhibitor that will neutralize 50% of the ADAMTS13 activity when mixed into an equal volume of normal pooled plasma. Neutralizing antibodies interfere with ADAMTS13 proteolytic activity whereas non-neutralizing antibodies promote ADAMTS13 clearance from plasma. Several factors can

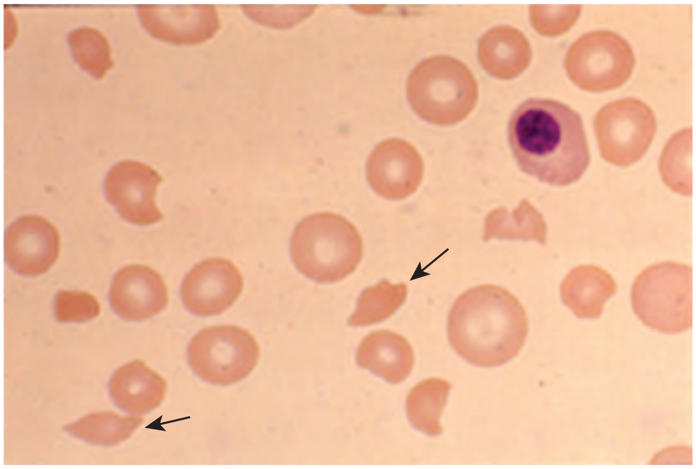


Figure 132.4 PERIPHERAL BLOOD FILM OBTAINED FROM A 28-YEAR-OLD WOMAN WHO PRESENTED WITH FEVER, EPISTAXIS, AND ALTERED MENTAL STATUS. Note the absence of platelets and the presence of a nucleated erythrocyte and schistocytes *(arrows)*, consistent with a microangiopathic process.

affect the detected ADAMTS13 activity and inhibitor. Samples for testing cannot be collected in EDTA because it chelates the metal ions required for ADAMTS13 activity. Other interfering substances include hyperbilirubinemia and severe lipemia, which interfere with the FRET-based assay. Antibody assays quantify anti-ADAMTS13 antibody levels (usually IgG but may also be IgM and IgA) using ELISA, western blotting, or immunoprecipitation. ADAMTS13 inhibitor assays identify and quantify anti-ADAMTS13 antibodies that functionally inhibit ADAMTS13 in vitro. If severe ADAMTS13 deficiency persists and inhibitor or antibody assays are negative, a diagnosis of cTTP should be considered and ADAMTS13 sequencing should be performed.

Inherited ADAMTS13 Deficiency (cTTP)

TTP was initially considered an acquired disorder, with the term TTP suggested in 1947 (after the discovery of systemic microvascular thrombosis) to distinguish this disorder from immune thrombocytopenic purpura (ITP). The hereditary occurrence of TTP was first described in 1975 by Wallace et al., who noted an autosomal recessive inheritance pattern.[19] A series of reports, including one by Upshaw, reporting improvement in clinical outcomes with plasma infusion resulted in the eponymous name. Subsequently, genome-wide linkage analysis revealed that Upshaw-Schülman syndrome is caused by mutations in the ADAMTS13 gene on chromosome 9q34.

ADAMTS13 is mainly synthesized by hepatic stellate cells but also by vascular endothelial cells and renal glomerular podocytes. Small amounts of functional ADAMTS13 are present in platelets. More than 200 mutations that can cause inherited TTP have been reported, and they involve almost all structural domains of ADAMTS13. Most of these mutations are single amino acid missense substitutions and most patients are carriers of compound heterozygous mutations. Mutations affecting the highly conserved N-terminal domains of ADAMTS13 are associated with lower residual enzyme activity, more severe clinical phenotype, and probably presentation of disease earlier in life. Expression studies have shown that missense mutations usually prevent the secretion of ADAMTS13, although some also impair catalytic activity. Specific mutations have been noted in population clusters with studies in Norway identifying the mutation p.R1060W and the insertion c.4143_4144dupA in approximately 0.3% to 1% of the population.[20] Similarly, other clusters have been noted in regions around the Baltic Sea, central Europe, Japan, and in Scandinavia. Because some of these mutations are associated with residual ADAMTS13 activity, the disease is often identified during pregnancy with patients sometimes presenting with severe pre-eclampsia. However, residual activity is not the only determinant of age at first disease manifestation.

Patients with cTTP are at risk for manifestation at any time in life. In general, however, the risk is highest at birth and with pregnancy. A 2011 study describing the natural history of cTTP in Japan identified 43 cases in patients ranging in age from childhood to 79 years.[21] Of these cases, 42% were diagnosed during childhood, 36% between the ages of 15 and 45 (all female, most commonly presenting during pregnancy), and 12% beyond 45 years of age. Likewise, a registry from the United Kingdom also reported a large variation in age at symptom onset ranging from childhood to adulthood.[22]

Newborns with TTP may present with hyperbilirubinemia, anemia, and severe thrombocytopenia. The pathophysiology of the presentation is due to an abrupt increase in systemic vascular resistance after circulation to the placenta stops in the setting of a high hematocrit and ultra large VWF multimers. Plasma infusion is the treatment of choice in this setting. Children who survive often remain asymptomatic for years.

In the Japanese cohort of 43 patients, 9 of 20 women were diagnosed with TTP during pregnancy. Antecedent pregnancies were associated with severe complications. cTTP should be considered in women presenting with pre-eclampsia at less than 25 weeks of gestation. Patients with cTTP have a high risk of stroke and TIA. In a cohort of 73 patients in the United Kingdom, 19% of patients had a stroke and another 8% a TIA.[23] The reported median age at presentation was 19 years; however, cases have been reported in patients over 60 years of age. Strokes can occur in the absence of hemolysis or thrombocytopenia. It has been estimated that 25% of strokes may be embolic with an undetermined source (see Chapter 144).[24] Interestingly, rates of myocardial infarction are much lower compared with that of stroke.[25] Acute kidney injury and chronic kidney disease have also been reported in patients with cTTP. As men do not experience the stress of pregnancy, recurrent stroke in males of young age should raise the possibility of hereditary TTP.

The absence of a functional ADAMTS13 inhibitor or antibody is characteristic of cTTP. False positive results with FRET-based functional assays can occur with severe hemolysis and a high hemoglobin concentration. The diagnosis of cTTP is further suspected if ADAMTS13 deficiency persists when the patient is in clinical remission and is confirmed by genetic analysis. As noted earlier, CNS symptoms may occur without thrombocytopenia or evidence of hemolysis; patients may also present with AKI. Fortunately, from a therapeutic perspective, plasma therapy results in rapid clinical recovery. In pregnant women with cTTP, monitoring of blood counts and prophylactic administration of plasma decrease the incidence of pregnancy complications and fetal loss, fostering a safer antenatal course.

The half-life of ADAMTS13 activity in patients receiving prophylactic plasma infusions is 2.5 to 3.5 days. Treatment intervals between plasma infusions have been empirically determined and depend on clinical symptoms and maintaining a normal platelet count. As a result, there is a wide variation in the recommended treatment intervals between infusions. Plasma derived factor VIII/VWF concentrates containing ADAMTS13 (Koate DVI and BPL 8Y) have been used for the treatment and prophylaxis of hereditary TTP. As a cautionary note, inhibitory alloantibodies can develop in patients receiving plasma therapy. In a phase 1 study, recombinant, fully glycosylated ADAMTS13 expressed in Chinese hamster ovarian cells (BAX 930; SHP 655; rADAMTS-13) was safe and well tolerated.[26] This product is under investigation in a multicenter study with results expected in 2023.

Multiple questions remain in the field of cTTP. From an epidemiological perspective, additional studies with case finding strategies need to be performed. In addition, strategies to optimize replacement therapy protocols using plasma derived or recombinant ADAMTS13 are required to avoid complications of pregnancy, preserve renal function and prevent large vessel occlusive disease (stroke and myocardial infarction).

TREATMENT OF THROMBOTIC THROMBOCYTOPENIC PURPURA

The goal of treatment in TTP is to prevent microvascular obstruction and replenish ADAMTS13 levels. Depending on the underlying etiology of ADAMTS13 deficiency—inherited or acquired (autoimmune)–replacement or replacement with immunosuppression +/− interruption of thrombus formation forms the thrust of therapy. The clinical presentation of TTP can be dramatic. When hemolysis and thrombocytopenia raise the specter of TTP, the PLASMIC score is useful to make a presumptive diagnosis and guide therapy. The reason for haste is the high mortality rate, which in the absence of therapy can exceed 90%, and the fact that the clinical situation can rapidly deteriorate.

The initial approach to TTP through the mid-70s focused on splenectomy, antiplatelet agents, high doses of glucocorticoids and whole blood exchange transfusions. The proposed rationale for exchange transfusion was to remove “a toxic substance.” Byrnes and Khurana established that plasma provides the critical factor for the treatment of TTP, ushering in a naive era in its treatment. (See box on Management of Acute Thrombotic Thrombocytopenic Purpura.)

MANAGEMENT OF ACUTE THROMBOTIC THROMBOCYTOPENIC PURPURA

- A high index of suspicion is necessary to make a diagnosis of thrombotic thrombocytopenic purpura (TTP)
  - Evaluate pretest probability of TTP based upon clinical assessment and risk assessment tool (PLASMIC or French Score)
  - Send ADAMTS13 assays (activity and either inhibitor or IgG antibody) before initiating therapy if such testing is available. In an appropriate clinical setting, an ADAMTS13 activity result <10% confirms a diagnosis of TTP
  - If pre-test probability is high, begin therapy for TTP. If pretest probability is intermediate or low, initiation of treatment of TTP is based upon clinical judgement while awaiting ADAMTS13 studies.
- Initial treatment of an acute episode of TTP:
  - Start plasma-based therapy. Plasma exchange (1.0–1.5 plasma volume) is recommended. Plasma infusion may be considered until exchange can be initiated, or potentially in a patient with known congenital TTP (cTTP)
  - In a patient with suspected or confirmed iTTP, begin corticosteroid therapy.
  - Anti-VWF therapy with caplacizumab is guided by ADAMTS13 activity assay results. Consider anti-VWF therapy with caplacizumab before ADAMTS13 results return if results are pending and patient has a high pretest probability, or after ADAMTS13 activity result returns at <10% for patients with low or intermediate pretest probability (use clinical judgement for activity result 10%–20%).
  - Consider early immunotherapy (e.g., rituximab) if iTTP is confirmed with ADAMTS13 inhibitor or anti-ADAMTS13 IgG assay, or if such diagnosis is highly suspected.
  - Platelet transfusion should be reserved for life-threatening bleeding. Packed red cell transfusion can be safely transfused in TTP.
- Subsequent treatment of an acute episode of TTP:
  - Continue plasma-based therapy until patient achieve clinical remission based on clinical symptoms, lactate dehydrogenase, and platelet count.
  - In treatment responders:
    - Continue anti-VWF therapy with caplacizumab for 30-day course or based upon ADAMTS13 activity results.
    - In patients with iTTP, complete immunotherapy treatment course.
  - In treatment non-responders, consider investigation for confounding conditions (such as infection). Alternative/additional therapies should also be considered.
- Subsequent follow-up:
  - For patients with iTTP, continued monitoring of ADAMTS13 activity is suggested as pre-emptive immunotherapy retreatment has been shown to decrease incidence of clinical disease relapse.
  - For patients with cTTP, ADAMTS13 replacement therapy (with plasma) prevents relapses and improves outcomes of pregnancy. Indications for chronic or episodic prophylactic replacement therapy remain to be defined, and clinical judgement is required.

Initial Treatment

Plasma Exchange in Thrombotic Thrombocytopenic Purpura

With the use of plasma infusion and plasma exchange, the survival of patients with TTP has increased to over 80% to 90%. The overarching debate in the late 70s and early 80s was the effectiveness of plasma infusion versus plasma exchange. In a nationwide trial conducted in Canada over a 7-year period, investigators addressed this question by randomizing patients to treatment with aspirin and dipyridamole and either plasma exchange or plasma infusion with fresh frozen plasma (FFP).[27] At 6 months, complete remissions were seen in 78% of patients treated with exchange versus 31% of those treated with plasma infusion. This study confirmed the superiority of plasma exchange over plasma infusion likely reflecting the fact that plasma exchange results in both the removal of the IgG inhibitor of ADAMTS13 and replacement of the deficient protein. In contrast, plasma infusion (or infusion of ADAMTS13 concentrate) suffices for patients with congenital ADAMTS13 deficiency. Large volumes of plasma are more easily administered by exchange, and unless a genetic deficiency of ADAMTS13 has been documented, plasma infusion should be reserved for situations in which exchange is not immediately available. The recovery of ADAMTS13 levels may lag behind other indicators of clinical response, and the utility of monitoring ADAMTS13 levels during acute treatment has not been established (see below concerning anti-VWF therapy with caplacizumab).

Plasma (either FFP or thawed plasma) is used for plasma exchange. There is no clear advantage for the use of cryo-poor plasma (CPP), which is depleted of VWF. These findings are consistent with a study showing similar concentrations and stability of ADAMTS13 during storage at 1°C to 5°C in CPP and FFP. Pilot studies suggest that solvent-detergent treated plasma (SDP), which contains ADAMTS13 at concentrations approximately 20% lower than those in FFP is as efficacious as FFP and is associated with fewer allergic/urticarial reactions. In the UK, SDP is used for all patients with TTP.[28]

Treatment is generally initiated with the goal of exchanging 1 to 1.5 plasma volumes (40 to 60 mL/kg) daily, although the optimal regimen has not been determined. Some experts advocate increased volumes of exchange if the initial response is poor. Neurologic improvement occurs most rapidly, often within hours to days. The serum LDH level typically falls by 50% within 3 days in responders, and the platelet count begins to rise at a mean of 5 days, though normalization may take several weeks. Improvement in renal function and disappearance of schistocytes occur more slowly.

Daily plasma exchange should be continued until neurologic symptoms have resolved and both a normal serum LDH and platelet count have been achieved; many experts recommend an additional 2 to 3 days of plasma exchange thereafter. Approximately 85% to 90% of patients exhibit a clinical and laboratory response to plasma exchange within 3 weeks, most often within 10 days (mean: 16 days; range: 3 to 36 days). However, 20% to 40% of patients will experience an exacerbation of disease within 30 days after stopping plasma exchange, whereas approximately 30% will relapse at later dates, usually within the first year. Limited data are available to support the decision between abrupt discontinuation or “tapering” of plasma exchange after remission.

Complication rates associated with plasma exchange therapy have been reported to be as high as 30%, but recent reports suggest a lower rate. Most adverse events are related to central venous catheter insertion, infection, allergic reactions to plasma, and occasionally thrombosis.

Plasma exchange is effective for patients with or without demonstrated inhibitors, and clinical responses frequently occur despite persistence of both the inhibitor and severe ADAMTS13 deficiency, although patients with high titer inhibitors may respond more slowly and relapse more often. Both congenital and acquired ADAMTS13 deficiency is characterized by unpredictable periods of stability between relapses, probably reflecting exacerbation of the disease by co-morbid conditions that activate or damage the endothelium, thereby increasing the release of unusually large VWF multimers, and triggering microvascular thrombosis.

Immunosuppression

Immunosuppression with glucocorticoids and anti-CD20 monoclonal antibody (rituximab) are a cornerstone of therapy in patients with iTTP. Glucocorticoids aid recovery by reducing production of autoantibodies to ADAMTS13. Therapy is typically started with oral prednisone at a dose of 1 to 2 mg/kg/day; a higher dose of methylprednisolone (1000 mg daily) may be used for critical illness. Prednisone was compared to cyclosporine in a randomized control trial and was found to be more effective in increasing ADAMTS13 activity and suppressing antibodies (study stopped after an interim analysis).

Anti-CD20 therapy with rituximab has been shown to reduce the risk of exacerbations and relapse and to hasten the response to therapy. Consequently rituximab is often given before plasma exchange is completed. Rituximab should ideally be given after plasma exchange to minimize its removal by the procedure, but its use should not delay plasma exchange. Rituximab has been shown to effectively deplete $CD20^+$ lymphocytes within 24 hours of infusion even with ongoing plasma exchange therapy.

The data supporting the utility of rituximab come mainly from observational studies. The STAR trial evaluated the benefit of adding rituximab to plasma exchange and glucocorticoids as initial treatment. Unfortunately, the trial was stopped because of poor accrual.[29] Data from a phase 2 study suggested that rituximab was safe and effective when given within 3 days of an acute TTP admission.[30] Compared with historical controls, inpatient stay was reduced by 7 days, and 10% of patients given rituximab relapsed compared with 57% in the historical control population. The dose of intravenous rituximab used for acquired TTP has generally followed the traditional schedule of 375 mg/m² once weekly for 4 consecutive weeks. A prospective multicenter single arm phase 2 trial using a fixed dose of 100 mg weekly for four doses showed response rates similar to those achieved with the higher dose.[31] Another approach pioneered by the French Thrombotic Microangiopathy group has been to use an adaptive dose of rituximab in response to B cell depletion.[23] Rituximab use in the acute phase of TTP prompts a high response rate (greater than 90% in the first month) and extends the time to relapse. The median time to relapse across studies conducted in the United Kingdom and France and using data from the Oklahoma TTP registry indicate a median time to relapse between 24 and 29 months.

Anti–von Willebrand Factor Therapy (Caplacizumab)

Microvascular thrombosis, the hallmark of TTP, occurs because of unregulated interaction of ultra-large VWF with platelets. Interruption of that process with anti-VWF therapy has the potential to halt microvascular obstructions and organ ischemia. Caplacizumab is a humanized, bivalent, variable domain only immunoglobulin fragment that targets the A1 domain of VWF and blocks its interaction with platelet glycoprotein lb-IX-V. In the randomized controlled phase 3 HERCULES trial, 145 patients with acquired TTP were randomly assigned to receive caplacizumab or placebo daily until 30 days after the last daily plasma exchange (plasma exchange was performed daily until at least 2 days after normalization of the platelet count).[32] All patients received daily plasma exchange and glucocorticoids and could receive other immunosuppressive therapies (slightly less than half received rituximab). Caplacizumab was associated with faster normalization of the platelet count, fewer days of plasma exchange, fewer exacerbations in the 30 days after stopping plasma exchange (4% vs. 38%) and a shorter course of hospitalization (10 vs. 14 days). Caplacizumab was associated with more bleeding (65% vs. 48%) which resolved spontaneously. In addition, a composite outcome of TTP related death, recurrence of TTP or a major thromboembolic event occurred in 12% of patients in the caplacizumab group and in 49% of those in the placebo group. Normalization of markers of organ dysfunction occurred sooner with caplacizumab than with placebo. The findings of more rapid platelet count response, reduced organ damage and conservation of resources, prompted the recent ISTH guidelines to suggest that caplacizumab be initiated in all patients with a high probability of having TTP in whom confirmation via ADAMTS13 assay is anticipated.[33] Based upon the initial phase 2 Titan and the phase 3 Hercules trial experience, it is recommended that caplacizumab be continued for 30 days after discontinuation of plasma exchange. Relapses were noted in patients who had severely suppressed ADAMTS13 activity below 10% at the end of the 30-day period after the last plasma exchange. As supported by the HERCULES trial data, some groups recommend monitoring ADAMTS13 activity level at that point and continuing caplacizumab therapy until ADAMTS13 levels recover to above 20% to prevent exacerbations of disease. Successful treatment of TTP with caplacizumab without concomitant plasma therapy has been reported in several individual cases and a recent case series.[34] Situations prompting this approach have included patients with contraindications to plasma therapy (such as history of severe allergic reactions to plasma or inadequate venous access) and patient refusal of plasma therapy (including religious objection to blood products). Because plasma exchange is the standard of care for TTP and use of caplacizumab alone is off-label, the decision to omit plasma-based therapy must be shared between the patients and their physicians. Trials of caplacizumab alone are needed in patients with TTP.

Other Modalities

Before the introduction of plasma therapy, splenectomy was the first-line treatment for TTP and induced remission in up to 50% of patients. Currently, splenectomy is reserved for patients who are refractory to all other modes of treatment. Antiplatelet agents have fallen out of favor and are now part of the historical construct of TTP treatment. Similarly, immunosuppressive agents like cyclophosphamide and vincristine are no longer used now that rituximab is available. Other adjunctive therapies that have fallen out of favor include azathioprine, mycophenolate mofetil, staphylococcal protein A immunoadsorption, and bortezomib. However, these may still be considered in refractory cases or in patients with iTTP whose ADAMTS13 levels fail to rise with rituximab.

Historically, platelet transfusions have not been recommended for patients with TTP because of their potential to provoke fatal thrombotic events. Data from the Oklahoma TTP-HUS registry challenged this dogma.[35] In a cohort of 54 consecutive TTP patients (of whom 47 had ADAMTS13 activity less than 10%), 33 patients received platelet transfusions. There was no difference in the incidence of death or severe neurologic events. The authors of the study concluded that there was “uncertain evidence of harm” with platelet transfusion in TTP. Although platelet transfusion might be considered prior to vascular access device placement, catheters for apheresis can safely be inserted even in the face of severe thrombocytopenia (less than 10,000). Therefore, based on accumulating evidence, platelet transfusion in TTP patients should be reserved for those with life-threatening bleeding.

Subsequent Therapy and Monitoring Response

Once a patient with TTP is stabilized, subsequent treatment is dictated by close monitoring for disease relapse and by immunosuppression. Disease response to therapy can be assessed using the platelet count. A good response is a platelet count over 150,000/μL for at least 3 days or a stable plateau. In addition to the platelet count, weekly monitoring of the LDH and ADAMTS13 level is suggested but well-established protocols do not exist. Schistocytes may persist for some time after the thrombocytopenia has resolved. Ultimately, normalization of the platelet count and improvement in symptoms are the most reliable indicators of response to therapy, while an increase in ADAMTS13 levels indicates a biochemical response.

Plasma exchange can be stopped once the platelet count is over 150,000 for at least 2 days. Plasma exchange may be stopped abruptly or may be tapered off, but the tapering is not of proven benefit. In the first month after discharge from the hospital, patients with TTP should have frequent blood counts and monitoring for symptoms (e.g., starting three times weekly and gradually tapering to once a week by the end of the first month if there has been no relapse). A relapse is generally preceded by a drop in the platelet counts below 100,000/μL. More frequent monitoring of symptoms and blood counts should be considered if the platelet count falls below 150,000. If the platelet count falls below 100,000 and ADAMTS13 activity is low or symptoms suggestive of a relapse (e.g., neurologic symptoms) develop, plasma therapy and/or further treatment with immunosuppression or anti-VWF therapy is indicated. If there is no relapse in the first month, glucocorticoids are generally tapered over 2 to 3 weeks while the rituximab course is completed. ADAMTS13 levels should be measured periodically after stopping plasma exchange until levels normalize. Patients with intermediate ADAMTS13 levels (20%—the lower limit of normal) may merit more frequent monitoring. Patients are at an increased risk for relapse in the first year with illness or surgery serving as potential triggers. ADAMTS13 levels should be checked every 3 months for the first year with more frequent if the level falls below 40%.

The use of immunotherapy and anti-VWF therapy (caplacizumab and rituximab) reduces the risk of exacerbations and relapses. Rituximab has decreased the frequency of relapse to about 13% with most relapses occurring over a year after plasma exchange is discontinued. The use of preemptive rituximab is supported by the results of a study from the French TMA group[36]; 48 of 233 patients had an ADAMTS13 level less than 10% and 30 were treated with rituximab preemptively. Whereas the 18 patients not treated preemptively had a median relapse free survival of 9 years, patients given rituximab did not reach a median relapse-free survival. This report was followed by a second study demonstrating a decrease of relapse from 74% with a median follow-up of 7 years versus 15% with a follow-up of approximately 3 years.

Therapy for patients with a history of iTTP whose ADAMTS13 level fall over time remains uncertain. Some experts suggest a single dose of rituximab followed by re-evaluation of the ADAMTS13 level in a month and withholding further therapy if the level is greater than 40% and giving additional weekly doses if the level remain less than 40%. Other experts suggest "maintenance" doses of rituximab, including the use of lower doses. The UK TTP registry demonstrated equivalence among 3 dose levels (375 mg/m^2 once per week for 4 weeks, 200 mg once per week for 4 weeks, 500 mg once per week for 4 weeks) with no differences in time for the recovery of ADAMTS13 levels and need for retreatment. If the patient has had multiple relapses with no effect of maintenance rituximab on ADAMTS13 levels, other immunosuppressive agents or splenectomy could be considered.

Patients with acquired TTP that does not respond to initial treatment with plasma exchange and immunosuppression within 4 days or with ongoing disease activity, as evidenced by thrombocytopenia or neurological symptoms, are considered refractory. In these patients, therapy may be intensified using a higher dose of glucocorticoids (1 g IV of methylprednisolone daily for 3 days) and addition of caplacizumab and rituximab (if not given already). Alternate diagnoses that could contributing to the refractory state need to be considered. These include infection, occult malignancy, or medications. With refractory disease, cyclophosphamide, bortezomib, cyclosporine, mycophenolate may be considered.

Patients with iTTP are susceptible to subsequent relapses and must be educated about the need to seek immediate medical care should symptoms arise. They also are at higher risk for hypertension, major depressive disorders, autoimmune diseases (SLE), and stroke compared with age and sex matched controls.

Special Scenarios

In patients with religious considerations that preclude them from accepting blood products, caplacizumab has been used successfully. Factor VIII concentrates that contain ADAMTS13 have been used in cTTP. Plasmapheresis with albumin as replacement fluid may be considered to reduce the ADAMTS13 autoantibody while waiting for immunosuppression effect to take hold. For patients presenting with TTP during pregnancy, retrospective data suggest that plasma exchange is the initial treatment of choice. These pregnancies are at a higher risk for intrauterine fetal demise due to thrombosis of the decidual arterioles.

INFECTION-ASSOCIATED HEMOLYTIC UREMIC SYNDROME

Clinical Manifestations

Infection-associated Stx-STEC-HUS is one of the main causes of acute renal failure in children.[37] Patients generally present with the triad of hemolytic anemia, thrombocytopenia, and acute kidney injury. STEC-HUS occurs most commonly after *E. coli* O157:H7–induced gastroenteritis, with rare cases reported after infection of the urinary tract or skin, or after infection with organisms other than *E. coli*. Based on data from O157:H7 outbreaks in which the time of exposure can be determined, the sudden onset of abdominal pain and watery diarrhea begins on average 4 days (range: 2 to 12 days) after toxin exposure.[38] Abdominal pain may be severe and precede the onset of diarrhea. In the absence of fever, STEC-HUS may be difficult to differentiate from inflammatory bowel disease, appendicitis, ischemic colitis, or intussusception. Bloody diarrhea generally ensues on the second day, accompanied in some cases by nausea and vomiting, though up to one-third of patients do not report blood in the stool. Fever is typically absent or mild. Colonoscopy reveals edematous colonic mucosa with occasional ulceration and pseudomembrane formation.

E. coli–associated hemorrhagic gastroenteritis is complicated by HUS in 8% to 18% of sporadic cases, and in over 20% in certain epidemic outbreaks. Therefore, the disease should be suspected in a patient who presents with characteristic clinical manifestations after an episode of bloody diarrhea, although the prototypic history of a preceding hemorrhagic gastroenteritis may be absent in up to 30% of cases. Young children and older adults are at greatest risk. When bloody diarrhea first develops, the platelet count, creatinine, and hematocrit are typically normal. However, as infection progresses, platelet counts often fall. HUS typically develops 7 days (range, 5 to 13 days) after the onset of diarrhea.[39,40] Patients often present with oliguria or other evidence of renal impairment; 50% to 75% of patients require dialysis, at least temporarily. The severity of MAHA varies considerably, but many affected individuals require red cell transfusion. Thrombocytopenia is common, with a median platelet count of 30,000/μL in one study but may be mild or absent in up to 30% of cases at presentation. Up to 25% of patients develop neurologic manifestations, which may include irritability and somnolence and less commonly, confusion, paresis, and seizures. The mortality rate is estimated at 3% to 5%.

Epidemiology

STEC-HUS accounts for at least 90% of cases of infection associated HUS.[41] In one study conducted in the United States, the median age of patients with STEC-HUS was 4 years; 55% of patients were younger than 5 years of age, 33% between 5 and 17 years, 6% between 18 and 44 years, and 6% older than 45 years of age. The annual incidence of STEC-HUS is estimated to be 2 to 3 per 10^5 in children under age 5. Shiga toxin-producing *E. coli* (STEC) accounts for 80% to 86% of cases in the United States and Europe. The most common serotype of *E. coli* associated with HUS is O157:H7, although other serotypes including O26:H11, O103:H2, O111:NM, O21:H19, O145:NM, and O104:H4 have been reported. The major reservoir of STEC is domestic cattle; approximately 2% to 3% harbor STEC in their gastrointestinal tract at the time of slaughter, and meat can become contaminated during processing. The organism has also been isolated from deer, sheep, goats, horses, dogs, birds, and flies. Large outbreaks have been reported in association with ingestion of contaminated ground beef, although contamination of poultry, cheese, fruits, and vegetables has also been reported. Other cases have been attributed to ingestion of contaminated water or unpasteurized apple cider or milk. The incidence of STEC-HUS may be increasing because of changes in food production methods. Industrial farming generates large quantities of potentially contaminated manure that periodically enters streams, where it can contaminate feed and uncooked vegetables. A 2011 outbreak of HUS in Northern Germany that affected more than 3000 individuals was caused by enteroaggressive *E. coli* O104:H4; the infection vector was traced to bean sprouts with evidence of intra-household transmission.[42] Similarly, in 2018, an outbreak related to contaminated romaine lettuce affected 135 people, resulting in 85 hospitalizations and 15 cases of HUS. Carriage of STEC may be asymptomatic, and fecal-oral transmission may contribute to epidemic spread in day care centers, nursing homes, and petting zoos. Aerosolization in barns has been implicated in outbreaks at fairs.

STEC strains are also associated with sporadic, nonepidemic cases of HUS. The disease occurs more frequently during the summer and autumn in temperate climates. Infection by Stx-producing *Shigella dysenteriae* serotype 1 is associated with HUS in developing countries and carries a higher mortality.

Pathobiology

STEC-HUS is caused by endothelial cell damage initiated by Stxs and the inflammatory cytokines they induce. The type of Stx, as well as other virulence factors, contribute to risk.

After ingestion, the noninvasive enteropathic *E. coli* colonize the terminal ileum and follicle-associated epithelium of Peyer patches, after which they colonize the colon. Once colonization occurs, STEC bacteria express numerous virulence factors by horizontally transmitted gene cassettes termed pathogenicity islands. One pathogenicity island contains the locus of enterocyte effacement (LEE), which contains genes for the adhesin intimin, as well as a type III secretion system (TTSS) that mediates transfer of bacterial proteins directly into enterocytes. Their capacity to cause HUS is mediated by two 70-kD bacterial exotoxins. The toxins are transported across the intestinal epithelium through specific para- and intercellular mechanisms and then circulate, most likely by low affinity binding to the surface of neutrophils and platelets, before being transferred to higher affinity receptors expressed on glomerular endothelial cells that are upregulated by proinflammatory cytokines.

The toxin produced by these strains of *E. coli* is identical to Shigella toxin and is therefore generally referred to as *Shiga-like toxin 1 (Stx1)*. Most strains of pathogenic *E. coli* produce a second homologous toxin, Stx2, which is associated with a higher risk for HUS. The toxins are carried on a lysogenic bacteriophage capable of infecting other strains of *E. coli*. Intact, 70-kD Stx holotoxins consist of a 32-kD A subunit responsible for inactivating ribosomal activity, and five 7.7-kD B receptor-binding subunits that aid in binding to the target receptor. The toxin binds to terminal Gala1-4Galb (galabiose) residues on globosyltriaosylceramide (Gb_3; also known as *CD77* and the human blood group *P^k antigen*). Increased expression of Gb_3 on glomerular microvascular and cerebral endothelium compared with other vascular beds contributes to their heightened sensitivity to apoptosis, cytotoxicity, upregulation of integrins, and procoagulant activity. The higher levels of Gb_3 expression in children compared with adults may explain why children are particularly prone to develop HUS.

After binding to Gb_3, Stx1, and Stx2 are internalized and transported in a retrograde manner to the endoplasmic reticulum and translocated into the cytosol. The A subunit is proteolyzed to a 27-kD A1 subunit that binds the 60 S ribosomal subunit and cleaves adenine 4323 from the 28 S ribosomal RNA. This prevents elongation factor 1-dependent binding of aminoacyl tRNA, which inhibits protein synthesis and leads to ribotoxic stress response, changes in cellular function, and apoptosis. Stx alters the usual balance between intestinal absorption and secretion to a net excretory phenotype. *E. coli* lipopolysaccharide and Stx also stimulate leukocyte and intrarenal expression of proinflammatory cytokines (including IL-1, IL-1β, IL-6, and IL-8; MCP-1; and interferon-γ), which exacerbate toxin- and leukocyte-induced endothelial dysfunction. Changes in endothelial function include upregulation of expression of Stx receptors, which further sensitizes cells to Stx-induced injury, increased expression of P-selectin and tissue factor, increased release of VWF, and down-regulation of thrombomodulin expression. Early in the course of the disease, Stx also acts in concert with lipopolysaccharide to trigger a procoagulant state that involves platelet activation.

It remains unclear why a minority of patients infected with STEC bacteria develop STEC-HUS.[43] Activation of complement may contribute to the pathogenesis of Stx-HUS. Stx2 directly activates the alternative pathway of complement and binds to SCR 6 to 8 and 19 and 20 of complement factor H (CFH), which mediate surface recognition of complement C3, blocking the ability of factor H to inhibit complement activation on cell surfaces. It has been hypothesized that subtle allotypic variation in CFH may modulate the sensitivity of CFH to Stx2-induced dysregulation in susceptible patients.[43]

Laboratory Manifestations

Similar to TTP, STEC-HUS is characterized by MAHA and thrombocytopenia. Thrombocytopenia tends to be less severe than observed that in TTP, and renal disease is the major clinical manifestation.

Renal biopsy, although rarely required for diagnosis in endemic areas, illustrates the importance of endothelial damage. Involved glomeruli show widening of the subendothelial space, which is filled with cellular debris and fibrin. Glomerular capillary endothelial cells are swollen and occasionally detached, leading to obliteration of the capillary lumens.

Though clinical features are often sufficient for the diagnosis of STEC-HUS, bacteriologic confirmation should be sought through stool culture, testing for Stx toxin (e.g., by ELISA), stool analysis for Stx structural genes, or acute and convalescent serologies. Stool should be tested as soon as possible after the onset of diarrhea. Because of the importance of expedited diagnosis, obtaining a rectal swab is suggested over waiting for a cup stool specimen. In a study of children with diarrhea, rectal swabs had a higher pathogen yield than cup stool specimens.[44] The recovery rate for STEC appears to be at least 90% during the first 6 days but less than 33% thereafter. *E. coli* O157:H7 ferments sorbitol slowly, appearing as colorless overnight colonies on sorbitol-MacConkey agar; sorbitol-negative cultures may be further characterized using commercially available O157:H7-specific antisera. Methods to detect Stx toxins or their structural genes improve diagnostic sensitivity. However, enzyme immunoassay (EIA) may fail to detect 10% to 15% of *E. coli* O157:H7 that are detected through culture[45]; thus, EIA is used in addition rather than instead of culture. Molecular panels that detect the genes for Stx 1 and 2 can deliver a result within several hours and can distinguish between these two toxins as well as other enteric pathogens.[46] If the lab report does not specify which Stx genes are present, it is critical to request that information, as the presence of Stx 2 represents a much higher risk of HUS than if this toxin is not present.

Antibody titers against *E. coli* O157:H7 antigens and Stx rise after infection, persist for 8 to 12 weeks, and may be useful to confirm STEC infection in selected cases; however, they are not widely available. In some cases, further evaluation may be desired to differentiate STEC-HUS from the other TMA, such as autoimmune disease, aHUS and TTP.

Imaging studies, such as computed tomography are rarely indicated in children suspected of STEC; however in adults in whom immunologic intestinal disorders may present as bloody diarrhea, CT imaging may be of value.

Prognosis

The outcome of patients with STEC-HUS is generally favorable, although STEC-HUS remains the most common cause of acute renal failure in children and up to 60% of children require dialysis during the acute phase. However, STEC-HUS is usually self-limited and as a result of advances in supportive care, mortality has been reduced to 3% to 5%, with death usually due to severe involvement of the central nervous system, intestine, or myocardium. Renal insufficiency generally resolves within 2 to 3 weeks, although some patients have prolonged anuria, requiring several months before recovery. Despite a favorable short-term outcome, many children with STEC-HUS develop chronic renal insufficiency over time. In one report of 29 patients with "typical" childhood HUS followed for 15 to 28 years, seven developed chronic renal failure and 12 developed hypertension, proteinuria, or reduced glomerular filtration rate, whereas only 10 showed no residual abnormalities. Therefore, 50% to 60% of patients have complete recovery with long-term preservation of renal function. Long-term outcomes are worse in older patients. Proteinuria persisting for more than 1 year after the initial episode of TMA is likewise associated with progressive renal disease.

Therapy

Treatment of STEC-HUS is supportive.[47] Because high risk of STEC causes severe illness, patients presenting with acute bloody diarrhea require careful evaluation, particularly if they present with absence of fever. Observation over a course of at least 6 hours is suggested to determine if symptoms of pain or diarrhea are abating, to suggest a more benign situation. If STEC is suspected, supportive therapy is important to initiate early.[48] Volume expansion within the first 4 days of the onset of diarrhea is performed to maintain renal perfusion as it significantly reduces the incidence of progression to oliguric HUS. Early and aggressive volume expansion with isotonic crystalloid is suggested. The volume administered is adjusted based upon age of the patient and in adults, by whether there are cardiopulmonary morbidities. However, no therapy has been shown to prevent HUS or to reduce the severity of kidney injury once established. In randomized trials, neither plasma infusion nor exchange was of benefit in children or adults with STEC-HUS. Corticosteroids, heparin, urokinase, aspirin, dipyridamole, antifibrinolytic drugs, and intravenous immunoglobulin are ineffective. Antimotility agents and narcotics delay clearance of *E. coli* and toxin from the GI tract and may increase the risk for progression to thrombotic microangiopathy; therefore, these agents should be avoided. Nonsteroidal anti-inflammatory agents may reduce renal blood flow and should also be avoided. Most experts believe that antibiotics increase the risk for progression to HUS, probably by lysing bacteria, releasing Stx and inducing bacteriophages on which Stx genes are expressed. This conclusion is supported by a large meta-analysis that included mostly observations studies in children, showing a trend towards higher risk of HUS with antibiotic use.[49] Therefore, routine use of antibiotic is discouraged.

Activation of complement may contribute to the pathogenesis of STEC-HUS. Increased levels of markers of alternative pathway activation and C5 convertase, as well as soluble C5b-C9 have been observed in patients with STEC-HUS. An uncontrolled retrospective study of 33 children with severe complications of STEC suggested a potential benefit in patients with severe neurologic complications. However, anticomplement therapy with anti-C5 monoclonal antibody eculizumab did not appear to improve 1-year renal outcome in that single center matched cohort study. Similarly, during the 2011 German O105H4 STEC-HUS epidemic, eculizumab was administered most commonly to patients with the most severe organ compromise and those often late in the course of disease. The outcome in these patients was similar to that in untreated patients.[50] Finally, during that German outbreak, immunoadsorption was undertaken in a small cohort of patients with severe neurologic compromise; while clinical benefit was reported, most of these patients also received other therapeutic interventions.[51] Additional data on the utility of eculizumab and immunoadsorption are needed before their routine use can be recommended.

Isolation of patients with STEC-HUS should be considered because shedding of the pathogenic bacteria may continue long after cessation of diarrhea.

ATYPICAL HEMOLYTIC UREMIC SYNDROME

Epidemiology

HUS is defined by MAHA and kidney injury is generally the predominant clinical feature. Broadly, this can be subdivided into infection associated HUS or complement-mediated HUS. Historically, children with HUS without diarrhea were described as having "atypical" HUS to make a distinction from HUS with a diarrheal prodrome caused by enteric infection. As a result, the term atypical HUS is composed of clinical HUS with dysregulation of the alternative complement pathway (CM HUS), mutations in the DGKε (diacylglycerol kinase ε) pathway (not thought to directly affect complement pathway regulation) and HUS without identified complement or DGKε mutation or anti-CFH antibody (see Fig. 132.2).

In children with HUS, the proportion with STEC-HUS is 85% to 90% while atypical HUS is 5% to 10%. The frequency of atypical HUS is not precisely documented in adults as the diagnosis in adults can be challenging and adults can present with any of the TMA syndromes associated with acute kidney injury-both genetic and acquired.

Abnormalities in complement regulation, most commonly due to mutations in complement regulatory proteins have been implicated in up to 50% of cases.[52] Inheritance is autosomal dominant with an estimated penetrance of 50%. aHUS can occur in siblings with or without identical genetic abnormalities. Clinical conditions that increase complement activation may unmask the genetic propensity to develop aHUS. The incidence of aHUS in the United States has been estimated at two per million based on the incidence of diarrhea negative HUS including *S pneumoniae* HUS, whereas European data suggest an annual incidence of 0.23 to 0.42 cases per million.[53]

Pathophysiology

The complement system is part of the innate immune system, enhancing the ability of antibodies and phagocytic cells clear microbes and damaged cells. It consists of multiple proteins synthesized by the liver, circulating in the blood as inactive precursors.

The complement system monitors the environment, protecting the intravascular space against bacterial invasion. The initial deposition of C3b can be mediated by the classical pathway by immune complexes, by the lectin pathways via interaction of bacterial carbohydrates with lectin-binding proteins, or via spontaneous hydrolysis of C3 to C3a and C3b (Fig. 132.5). C3b formation is then amplified by a positive feedback loop of the alternative pathway. C3b is deposited on almost all biological surfaces in contact with plasma resulting in its deposition on both pathogen surfaces as well as upon healthy host cells. This process wherein a small amount of autoactivated C3 is always present is called C3 tick-over. On healthy human cells, this process is terminated by control proteins (such as complement factors H, I, and CD46). However, on foreign or damaged cells, the alternative pathway is engaged when this activated C3 binds factor B. Bound factor B undergoes proteolytic cleavage by factor D to produce fragments Bb and Ba. This is further stabilized by properdin (p), creating a convertase complex C3bBbP. This "C3 convertase" cleaves more C3 to C3b setting an amplification loop in motion.

C3b binds to the C3 convertase to form C3bBbC3b which is the "C5 convertase." Like the stabilization process with C3 convertase, properdin also becomes part of C5 convertase, stabilizing the enzyme. C5 convertase goes on to cleave more C5 releasing C5a which acts as an anaphylatoxin while C5b initiates formation of the lytic membrane attack complex, built thought the cooperative actions of C6, C7, and C8 and resulting in polymerization of a pore through the cell membrane composed of multiple units of C9.[54]

To protect host cells from "collateral damage" as a consequence of complement C3b deposition, the process of complement activation on healthy cells is regulated through control proteins anchored onto membranes and in the fluid phase. The most prominent regulators of

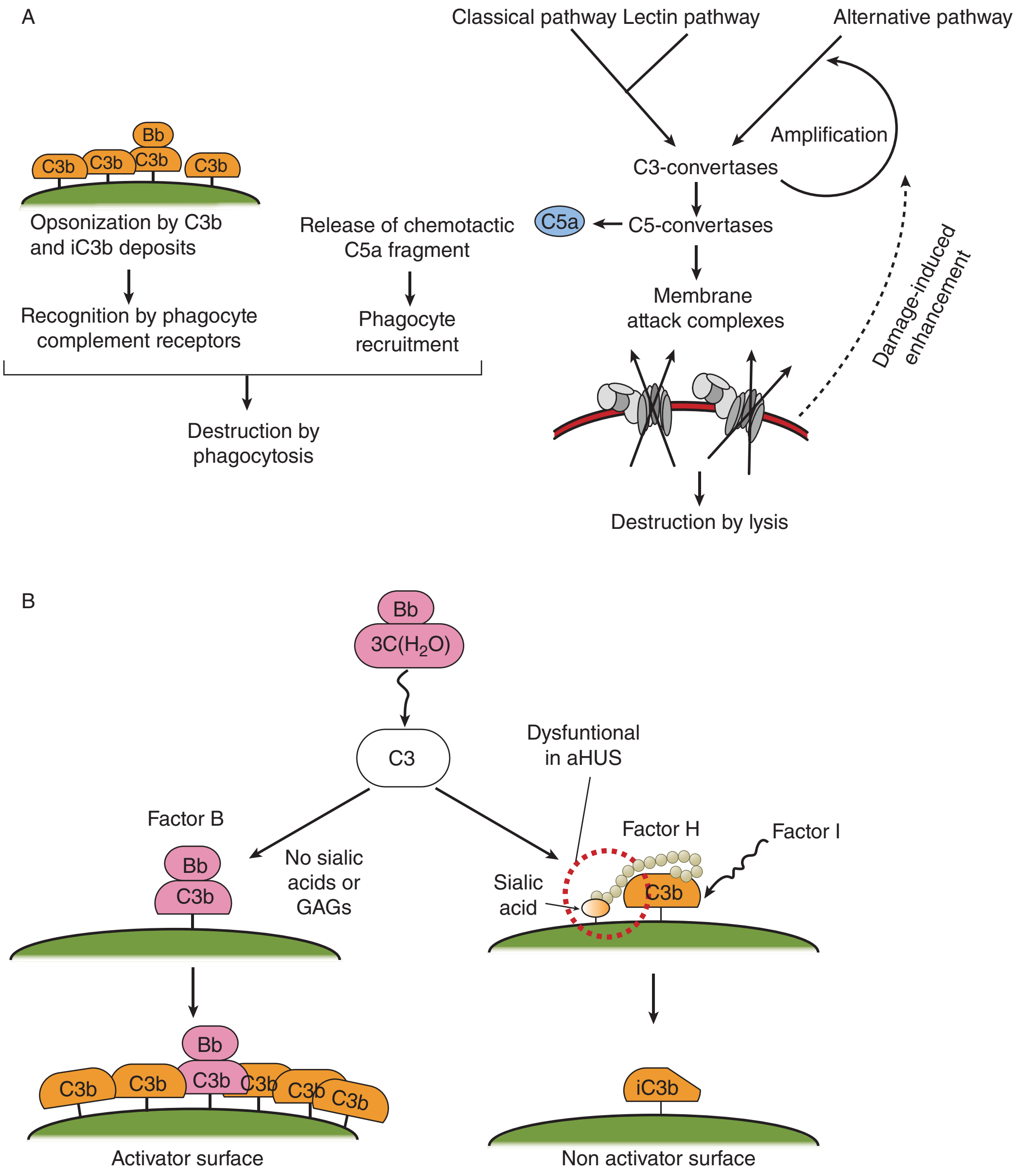

A
Classical pathway Lectin pathway
Alternative pathway
Amplification
C3-convertases
Bb
C3b
C3b
C3b
C3b
Opsonization by C3b
and iC3b deposits
Release of chemotactic
C5a fragment
C5a
C5-convertases
Damage-induced
enhancement
Membrane
attack complexes
Recognition by phagocyte
complement receptors
Phagocyte
recruitment
Destruction by
phagocytosis
Destruction by lysis
B
Bb
3C(H2O)
C3
Dysfuntional
in aHUS
Factor B
Bb
C3b
No sialic
acids or
GAGs
Factor H
Factor I
Sialic
acid
C3b
Bb
C3b
C3b
C3b
C3b
C3b
C3b
iC3b
Activator surface
Non activator surface

Figure 132.5 CONSEQUENCES OF COMPLEMENT ACTIVATION. (A) The complement system can be activated via three pathways: classical, lectin, and alternative. All pathways lead to formation of powerful enzymes, the C3-convertases, followed by activation of the terminal cascade. The main effector functions of complement (promotion of opsonophagocytosis by opsonization and chemotaxis and formation of lytic membrane attack complexes) aim to destroy harmful agents such as microbes. Lysis of target cells can lead to damage-induced enhancement of complement activation. (B) Alternative pathway activation is based on continuous, low-level covalent deposition of C3b molecules onto practically all surfaces in contact with plasma. If the C3b molecule is allowed to form an enzyme (shown in red), new C3b deposits will be formed around the enzyme leading to rapid amplification of the activation. If the regulator factor H binds to C3b, the convertase enzyme is inactivated, and no complement activation follows. The simultaneous interaction of factor H with both C3b and cell surface sialic acids (or possibly glycosaminoglycans [GAGs]) is essential for proper regulation on self-red cells, platelets, and endothelial cells. If this fails, disbalance between activation and regulation may lead to pathogenesis of atypical HUS. iC3b, C3b molecule incapable of forming an enzyme with factor B. *(From Jokiranta TS. HUS and atypical HUS. Blood. 2017;129(21):2847–2856.)*

the alternate pathway are complement factor I (CFI), CFH which are plasma based proteins along with CD35, CD46, CD55, and CD59 which are membrane proteins. CFI is synthesized in the liver and functions by cleaving C3b to inactive C3b and dissociating the C3 and C5 convertases. Foreign targets that do not have membrane-bound regulators or cannot bind soluble regulators are attacked. Similarly, CFH inhibits the alternate pathway by binding to C3b, both when deposited on cells and in the fluid phase, through competition with factor B for C3b binding. Factor H also acts as a cofactor for CFI. CD46, previously known as membrane cofactor protein (MCP), is a widely expressed transmembrane regulator that serves as a cofactor for CFI. Intact MCP is critical in preventing C3 activation on glomerular endothelium. In addition to the above, variants for genes involved in the coagulation pathway including thrombomodulin, vitronectin (*VTN*), and plasminogen have been reported in patients with aHUS; however, the pathogenic contribution of these protein remains to be confirmed.

Mutations in these inhibitory proteins (CFI, CHH, and CD46) or gain of function mutations in C3 or complement factor B (CFB) or autoantibodies to alternate pathway proteins (usually anti-CFH) provide a “first hit” which puts the individual at risk. Subsequently, a “second hit” through an inflammatory process propagates complement activation which then creates a vicious cycle leading to endothelial injury and a prothrombotic state.[55]

Genetics

Earlier reports suggested that genetic mutations account for at least 50% to 60% of aHUS or CM-HUS (Table 132.2). Of these, the most common belong to CFH with 20% to 30 % of all cases (40% to 45% in familial cases and 10% to 20% in sporadic form).[52] Clinically, the appearance of the syndrome is sporadic despite the presence of pathogenic variants in the patient and one of the parents suggesting incomplete penetrance. Up to 3% of patients have combined pathogenic variants of more than one complement protein, potentially increasing the risk of disease. However, many of the mutations reported were published before more rigorous criteria from the American College of Molecular Genetics were established, and the pathogenicity of reported sequence variants remains to be substantiated though either larger family or population studies, or through demonstration of functional defects through expression experiments.

More than 193 potentially pathogenic variants have been identified in the *CFH* gene (as indicated by the FH aHUS mutation database). The majority of *CFH* mutations in patients with aHUS are heterozygous and cluster in the C-terminal, of the protein, and the region responsible for CFH binding to the cell surface glycosaminoglycan.[56] As a result, there is a lower density of CFH on cell surfaces and diminished cofactor activity for degradation of C3b that has become adherent to the cell surface. Most abnormal CFH forms are secreted in plasma; it is hypothesized that mutant CFH interacts with normal CFH and inhibits its binding to endothelial cells resulting in a dominant negative effect. Thus, even though individuals with heterozygous mutations may have normal circulating CFH antigen, they are predisposed to development of the aHUS phenotype. As most patients are heterozygous for the mutation, serum CFH and C3 levels are often normal. In patients with the homozygous *CFH* mutation, they may have a quantitative CFH deficiency, failure to control C3, and very low C3 levels. In a French cohort of patients with aHUS, patients with *CFH* mutations had the worst clinical outcome with a significantly worse renal prognosis when compared to other subgroups.[53] In addition to mutations in genes encoding *CFH*, factor H autoantibodies have been described in 10% of patients, these patients more often had them since childhood.[57] These antibodies have been shown to block the C-terminal recognition domain of factor H—the same region in which functionally significant mutations are present. Adjacent to the gene encoding for CHF are a series of genes encoding for a series of homologous proteins, CFHR1, CFHR2, CFHR3, CFHR4, and CFHR5. Homozygous deletion of gene encoding factor H–related protein 1 (*CFHR1*) either through homozygous or compound heterozygous deletion of region encoding for *CFHR3-CFHR1* or *CFHR1-CFHR4* were noted in these patients, suggesting that congenital absence of CFHR1 protein predisposes to risk for development of autoantibody to CFH with development of aHUS.

TABLE 132.2 **Complement Genes and Proteins Identified in Atypical Hemolytic Uremic Syndrome**

Mutated Gene	Mechanism of Disease	Prevalence (%)	ESRD/Death 3–10 Years After Onset (%)
CFH	Loss of complement regulation	24–28	70–80
CFHR1/3 deletion with anti-factor H antibodies	Loss of complement regulation	3–10	30–70
CFI	Loss of complement regulation	4–8	60–70
MCP (CD46)	Loss of complement regulation	5–9[a]	<20
THBD	Loss of complement regulation	0–5	50–60
CFB	Gain of complement activation	0–4	70
C3	Gain of complement activation	2–8	60–70
DGKε	Prothrombotic state	0–3	46
None identified		30–48	50

[a] MCP mutations are frequently found in combination with other mutations (up to 22%).

CFHR, Complement factor H-related gene; *DGKε*, diacylglycerol kinase epsilon gene; *ESRD*, end-stage renal disease; *MCP*, membrane cofactor protein gene; *THBD*, thrombomodulin gene.

Prevalence and prognosis data relates to publications before the introduction of eculizumab, have been adapted from multiple authors, including Noris M, Fremeaux-Bacchi V, and Bresin E. Adapted from Jokiranta TS. HUS and atypical HUS. *Blood.* 2017;129(21):2847–2856.

Mutations in CD46 or MCP account for 5% to 15% of HUS cases.[58] These patients tend to do well if they present in childhood. Adults however have a significantly higher rate of end-stage renal disease (ESRD). Patients who undergo a renal transplant for this condition have a low rate of recurrence in the allograft, a finding attributed to the fact that CD46 is a cell surface protein which is expressed on the surface of the normal renal allograft cells. Mutations in factor I are mostly heterozygous and occur in 4% to 10% of cases. These patients have an intermediate prognosis with 50% progressing to ESRD or death in 2 years.[59] Complement factor 3 mutations account for 2% to 10% of cases and present with severe disease. "Gain of function" in C3 variants is attributed to failure of variant C3b to be effectively regulated by CFH, CD46 and CFI. *CFB* mutations are gain of function mutations and are seen in 1% to 4% of all cases. *CFB* mutations lead to enhanced formation or greater stability in C3 convertase on cell surfaces.[52]

Mutations in *DGKε* which encodes DGKε were described in children with disease onset before 12 months of age.[60] DGKε is a lipid kinase, present on endothelial cells, podocytes, and platelets, that catalyzes the phosphorylation of diacylglycerol substrates to phosphatidic acid. Loss of DGKε function results in enhanced activation of protein kinase C which in turn results in the upregulation of prothrombotic factors (VWF, tissue factor and plasminogen activator inhibitor). As *DGKε* sits outside the complement pathway, there is no pathogenic rational to suspect that complement directed therapy would be effective in affected patients. However, some reports have shown remission if there are coexisting mutations with genes from the complement pathway.

Vitronectin is a protein present in serum and in the extracellular matrix that is involved in complement activation, coagulation, cell adhesion, and wound healing. It inhibits the terminal complement cascade by blocking the formation of the membrane attack complex. Mutations in the gene encoding for vitronectin (*VTN*) have been recently implicated in aHUS.[61]

Clinical Manifestations

The clinical symptoms of HUS are non-specific and includes fatigue, low urine output, edema, and dyspnea. Though aHUS is generally not associated with a prodrome of bloody diarrhea, a history of recent gastroenteritis may be obtained from up to 30% of patients, making distinction from STEC HUS difficult. A triggering event may be seen in roughly 30% of adults and half of children. The hallmark of the disease is the classic triad of thrombocytopenia, hemolytic anemia, and acute kidney injury. Correspondingly, evidence of microangiopathy on the peripheral blood film is a universal finding with an elevated reticulocyte count and LDH. Renal damage can be present with nephrotic range proteinuria with up to 75% of adult patients requiring dialysis.[53,62] Some patients can also develop accelerated hypertension, complicating the initial diagnosis. It has been suggested that in the absence of another cause for a renal thrombotic microangiopathy (such as STEC-HUS, systemic lupus erythematosus, scleroderma, medication effect, complications of transplantation), the presence of a creatinine over 2.3 mg/dL and a platelet count over 30,000/μL argues against a diagnosis of TTP and favors aHUS. However, cohort studies in patients with TTP indicated that some patients did require dialysis; suggesting that these disorders may be indistinguishable on clinical grounds alone, thereby highlighting the importance of ADAMTS13 assessment. Extra renal manifestations are present in 8% to 25% of patients at presentation, and can include neurological symptoms (~10%), GI involvement (~5%), cardiac involvement[63] (10% as myocardial infarction or ischemic cardiomyopathy) or ulcerative-necrotic skin lesions.

Typically, aHUS follows a chronic relapsing course, often complicated by hypertension and renal insufficiency. Moreover, exacerbations are often preceded by infection, pregnancy, or other seemingly incidental events. Affected patients have a high risk for recurrence after renal allografting, and HUS occasionally presents in related renal allograft donors after surgery. The disorder may resemble TMA associated with pregnancy, cancer, and chemotherapy, which have similar clinical presentations, but different natural histories.

Laboratory Testing

aHUS is characterized by MAHA and thrombocytopenia. Compared with TTP, thrombocytopenia and anemia may be less severe and renal insufficiency more prominent. Reports suggest that between 6% and 15% of children do not have the full triad of thrombocytopenia, anemia, and renal dysfunction at presentation.

Diagnosis of aHUS requires several criteria, including: (1) the absence of other diseases associated with TMA, (2) the absence of criteria for pathogen-associated post-infectious HUS (i.e., negative stool culture and/or Stx assays), and (3) the absence of criteria for TTP (i.e., ADAMTS13 greater than 10%, no anti-ADAMTS13 antibodies). Recommended studies include obtaining levels of C3, C4, CFH, and CFI, flow cytometry studies for CD46, and serologic studies for anti-CFH antibodies before initiating plasma exchange therapy. However, it is important to note that decreased C3 is only found in 30% to 40% of patients and that decreased CFH or CFI antigen is only seen in 50% or 30% of patients with mutations in these genes respectively, and between 30% and 60% of patients with auto-antibodies to CFH.[53] Also, decreased levels of C3 and decreased MCP expression may be observed in the acute phase of STEC-HUS. Due to variable penetrance, only 20% to 30% of patients will have a family history of aHUS. Screening for mutations *in CFH, CFI, MCP, C3, CFB, THBD*, and recombination events involving CFH related genes (CFHR1-5) complete the diagnostic work up. Recently, two research teams have suggested that assays with cell-based endpoints have the potential to be informative in the diagnosis and management of patients with aHUS due to defects on complement regulation. One group has proposed an assay utilizing a recombinant cell line in which the PIG-a gene has been modified, resulting in failure of the cells to link control proteins CD55 and CD59 to the cell surface, rendering the cells susceptible to complement-mediated killing when exposed to the plasma of patients with defects in the alternative pathway of complement activation.[64] The second assay utilized cultures of microvascular endothelial cells that are activated with ADP and then exposed to patient plasma. Deposition of C5b9 complex is then assessed by confocal microscopy.[65] Such assays may hold promise for confirming failure of complement regulation to help affirm a diagnosis of aHUS, allow evaluation of the efficacy of anticomplement therapy, assessment of the risk for discontinuation of anticomplement therapy in an aHUS patient who has been stabilized with anticomplement therapy, and potentially identify other disease states in which failure to control complement is considered a potential pathogenic mechanism. Two diseases in that last category that are being studies are pre-eclampsia and catastrophic antiphospholipid syndrome (see Chapters 139 and 149). While cell-based assays hold promise, even if proven sensitive and specific, there remain multiple hurdles in making such assays generally available for patient diagnosis and management. As a result, these assays have not yet been universally adopted in diagnostic testing.

Prognosis

Atypical HUS has been historically associated with a poor prognosis, especially in older adults and those with severe renal dysfunction. The historic mortality rate approaches 25% and 50%, with many surviving patients developing chronic renal insufficiency. However, identification of the central role of control of complement and DGKε in the pathogenesis of aHUS allows prognostication based on genotype. Before the advent of specific anticomplement therapy, the prognosis of patients with genetic defects of factor H or factor I deficiency was poor, with a rate of recurrence and progression to ESRD or death of 70%, whereas MCP deficiency appeared to carry a better prognosis, with estimated progression to ESRD between 15% and 20%.[41] Aggressive plasma-based treatment is associated with improved hematologic parameters and may slow but does not prevent progression to renal failure. Anticomplement treatment with eculizumab (and the second-generation longer half-life ravulizumab), a humanized monoclonal antibody directed at complement factor C5, has been shown to halt disease activity and improve renal function. In fact, some participants in the single armed trials were able to discontinue dialysis therapy. Eculizumab is ineffective in patients with disease due to DGKε mutations unless there are coexisting mutations in the

MANAGEMENT OF COMPLEMENT-MEDIATED HEMOLYTIC UREMIC SYNDROME

- A high index of suspicion is necessary to make a diagnosis of complement-mediated hemolytic uremic syndrome (CM-HUS)
    - CM-HUS is a systemic disorder
        - Advanced renal failure, hypertension and increased vascular permeability suggest a diagnosis of CM-HUS rather than thrombotic thrombocytopenic purpura (TTP).
        - Neurologic and gastrointestinal symptoms are common.
- CM-HUS is a diagnosis of exclusion:
    - TTP should be excluded by ADAMTS13 assay
    - IA-HUS should be excluded with stool culture and/or tests for Shiga toxin.
    - Other causes of microangiopathic hemolytic anemia should be excluded with history, coagulation studies, infectious disease studies, serologies, etc.
    - Plasma studies of C3, C4, and complement proteins (CFH, CHI, CFB) and autoantibody to CFH may be normal, as may genetic studies (CFH, CFI, CFB, C3, MCP, THBD, and DGKε). Initiation of therapy should not be delayed while awaiting these study results.
- Treatment of suspected acute CM-HUS:
    - Plasma exchange is recommended until a diagnosis of TTP is excluded.
    - Anti-complement therapy (such as eculizumab or ravulizumab) is currently the therapy of choice for CM-HUS.
        - Vaccination to prevent meningococcal infection is required; antibiotic coverage should be initiated pending immunization effect and chronic antibiotic prophylaxis should be considered.
        - Early treatment reduced the risk of long-term renal impairment.
        - Thrombocytopenia and hemolysis tend to respond early, but renal recovery may be delayed.
- Subsequent follow-up:
    - Long-term anticomplement therapy is currently recommended. If anti-complement therapy is discontinued, close follow-up to detect early relapse with re-initiation of therapy (and potential prophylaxis during high-risk intervals) is suggested.

CFB, Complement factor B; *CFH*, complement factor H; *CFI*, complement factor I; *DGKε*, diacylglycerol kinase epsilon; *IA-HUS*, infection-associated HUS; *MCP*, membrane cofactor protein.

complement pathway. As might be expected with an intracellular protein deficiency, no recurrences have been observed in the few reported DGKε cases where renal transplantation was performed.

Therapy

Historically, patients presenting with TMA (either TTP or aHUS) were offered a trial of plasma exchange therapy. Plasma exchange may still be indicated in patients where there is uncertainty about the diagnosis of TTP or aHUS pending the results of assays for ADAMTS13, or until more definitive anticomplement therapy is available. This approach is also being questioned in patients presenting with platelet counts over 30,000/μL and creatinine levels over 2.3 mg/dL, findings that are observed more often in association with aHUS than with TTP. Although plasma therapy replaces missing soluble regulators of the alternative mechanism of activation of complement and may ameliorate thrombocytopenia, it does not appear to halt renal disease progression, as evidenced by a high incidence of ESRD in pediatric patients treated with this approach. Plasma-based therapy is at best temporizing and is associated with a high rate of complications.

Eculizumab is a recombinant humanized monoclonal immunoglobulin that binds C5 with high affinity and inhibits its cleavage to C5a (a proinflammatory mediator) and C5b (the first component of the C5b-9 membrane attack complex). In this way, it interrupts the pathway leading to endothelial cell damage, but it does not interrupt the activation of C3 as shown by sensitive biomarker studies. It has been studied in four prospective trials (one in children, three in adults).[66,67] Identification of complement gene mutations were not required in these studies. However, they did assess the ADAMTS13 to screen out patients with thrombotic microangiopathy due to TTP. Eculizumab did ameliorate disease activity in the majority of treated patients, whether or not they were refractory to plasma therapy. Several patients with severe renal insufficiency were able to discontinue dialysis. The increase in GFR was more pronounced in children compared to adults, with early drug initiation more likely to be associated with recovery of renal function.

Anticomplement therapy is the treatment of choice for patients with established aHUS or with a high clinical likelihood in an appropriate setting (e.g., postpartum status, positive family history, younger than 2 years of age). Patients with markers of active TMA (thrombocytopenia, LDH, and haptoglobin) respond rapidly to therapy. The results of trials and accumulating registry data suggest that the sooner treatment is initiated, the better the chance of recovery of renal function. Current data also suggest that remissions are sustained. While on therapy, complement activity through CH50 should be monitored (less than 10% for complete suppression).

Anticomplement therapy is associated with meningococcal infections with an annual rate of 0.5%.[68] Patients should be vaccinated for *Neisseria meningitis* and potentially for *S. pneumoniae* and *Haemophilus influenza* type b. Cases of meningococcal disease have been identified in eculizumab recipients despite vaccination. As a result, prophylaxis with antibiotics during the treatment period is not unreasonable.

Discontinuation of anticomplement therapy can be attempted in patients who have had an excellent response to therapy.[69] However, this requires close follow-up with initially weekly blood work and twice weekly urinalysis, with early re-introduction of therapy if markers indicate disease reactivation. An increased sC5b-9 plasma level at eculizumab discontinuation, female gender and the presence of a rare complement gene variant were associated with a higher relapse rate. Higher relapse rates have been noted in patients with *CFH* and CD46 mutations whereas those without complement variants seem to have the lowest risk.[70,71] Plasma exchange with immunosuppression is an established treatment for anti CFH antibody HUS—there have been case reports that suggest eculizumab can induce remission in this subtype.

Ravulizumab is a second generation C5 inhibitor modified to enhance endosomal dissociation of C5 and recycling to the vascular compartment through the neonatal Fc receptor pathway.[72] As a result, it achieves inhibition of complement-mediated hemolysis with an extended dosing interval. The terminal half-life is approximately four times that of eculizumab. FDA approval was obtained based on data from 58 adult and 16 pediatric patients with aHUS in single-arm trials. The most serious side effects reported were hypertension, pneumonia, and abdominal pain.

Generally, patients with aHUS who progress to ESRD are considered candidates for transplantation. Living-related donor transplants are not recommended, except possibly in the case of DGKε-associated disease, because of the risk for aHUS in the donor's remaining kidney. There are reports of aHUS developing in family-related renal donors within months of donation.

Since CFH, CFI, factor B, and C3 are soluble plasma proteins, deficiency or dysfunction is not corrected by renal allografting alone. The outcome of renal transplantation in aHUS has been poor, with a high rate of failure due to recurrent disease. In fact, the failure rate can approach 60% to 70% and failure often occurs within the first month after transplantation. The underlying genetic defect is predictive of relapse risk, being as high as 80% with mutations in CFH, C3 and factor B, but as low as 20% in patients with defects in MCP or in those without genetic abnormalities.[73] Before the introduction of eculizumab, combined liver and kidney transplantation was recommended; however the 1-year survival for this complicated procedure was disappointing. Anecdotal reports suggest that eculizumab prophylaxis may prevent post-transplantation recurrence and evidence is accumulating to support such therapy in conjunction with renal allografting.

Going forward, multiple unmet needs remain to be addressed. At this time, diagnosis of aHUS remains a clinical diagnosis of exclusion; generation of a practical assay to affirm a diagnosis of aHUS remains to be developed. Ongoing clinical studies may identify prognostic factors that might help identify which patients could safely discontinue anticomplement therapy, as well as provide informative prognostic data for the family members of patients with identified genetic variants that predispose to development of disease. There are a number of trials with agents targeting C5 incorporating multiple approaches including RNAi, small molecule inhibitors, and long-acting formulations.[74] Unfortunately, agents that target C5 are associated with risk for development of meningococcal disease. C3 inhibitors are also in clinical trials and have demonstrated clinical safety so far. Additional agents that specifically target the alternative pathway though inhibition of either complement factors B or D are also in development.

OTHER THROMBOTIC MICROANGIOPATHIC DISORDERS

Post-Transplantation Thrombotic Microangiopathy

TMA can occur in three transplant-related settings: (1) recurrent disease after transplantation for a TMA-related disorder, (2) in association with the use of immunosuppressive medications (calcineurin inhibitors [cyclosporine A and tacrolimus] and inhibitors of the mammalian target of rapamycin [mTOR inhibitors sirolimus and everolimus]), and (3) after hematopoietic stem cell transplantation.

Recurrent Disease and "De-Novo Thrombotic Microangiopathies" After Renal Transplantation

As indicated above, there is a high risk of developing recurrent aHUS after renal transplantation in patients with disorders of complement regulation. However, it is important to note that the diagnosis of recurrent TMA depends on the correct diagnosis of the index disorder. Patients with suspected de-novo TMA that is subsequently attributed to aHUS may have initially presented with hypertension, renal failure, and nephrosclerosis. In a French series of renal transplant recipients whose initial diagnosis was not aHUS, 7 of 24 of those diagnosed with de-novo aHUS after renal transplantation were shown to have mutations of CFH or CFI. In the majority of these individuals, renal failure was attributed to hypertension or chronic glomerulonephritis. No mutations were identified in a control group of renal transplant recipients who did not develop TMA, suggesting that underlying defects of complement should be considered when TMA develops in the post-renal transplant setting. Prophylactic anticomplement therapy is undertaken prior to transplantation in all patients with complement-mediated TMA due genetic deficiency or dysfunction of CFH or CFI. Some centers use a combination of eculizumab and plasmapheresis. In patients with ESRD due to TMA related to mutations of complement factors produced by the liver (i.e., CFH) a combined kidney liver transplant can correct the ESRD along with the underlying etiology. Most cases of de-novo TMA post-transplant are attributable to drug toxicity (calcineurin inhibitor), rejection, viral infection (such as parvovirus, CMV and hepatitis C), or a combination of these processes. Antibody-mediated rejection (ABMR) may be confirmed by demonstrating the presence of MHC antibody, or by renal biopsy evidence of peritubular capillary C4d staining. In patients with de novo TMA, who were subsequently diagnosed with a genetic mutation or having progressive disease despite addressing reversible causes, anti-complement therapy such as eculizumab is a treatment option. In the absence of anticomplement therapy, plasma exchange every 48 hours can also be performed. In patients found to have a pathogenic mutation, anticomplement therapy may be continued indefinitely. In patients without a pathogenic mutation and who have a precipitating cause, eculizumab discontinuation can be attempted with close monitoring for recurrence.

Immunosuppressive Medications

Cyclosporine, tacrolimus, and sirolimus have been implicated as causes of drug-induced thrombotic microangiopathy. TMA may be more likely to occur with cyclosporine compared to tacrolimus. Cyclosporine A and tacrolimus cause endothelial dysfunction and increased platelet aggregation by inhibiting prostacyclins. In most cases, discontinuation of these agents results in reversal of HUS. In retrospective studies, the incidence of biopsy-proven thrombotic microangiopathy was highest with combination of cyclosporine and sirolimus compared with other regimens and combinations.

Recent evidence suggests that mTor (mammalian target of rapamycin) inhibitors, such as sirolimus and everolimus, can also be associated with post-transplant TMA. It is likely that mTor inhibitors impair endothelial function, but the mechanism remains to be elucidated.

Hematopoietic Stem Cell Transplantation

TMA occurs in approximately 6% of patients who undergo allogeneic bone marrow transplantation; the incidence is estimated to be 0.1% to 1% after autologous marrow or stem cell transplantation. In a prospective study of 77 pediatric patients undergoing a HSCT, 34 patients developed TMA of whom 65% had a genetic variant in at least one gene as opposed to 9% without TMA.[75] Variants in three or more genes was associated with a mortality rate of 71%. The pathophysiology is assumed to reflect systemic endothelial cell injury from a three hit process where patients are susceptible due to a genetic predisposition, undergo stress from the transplant leading to activation of complement and formation of microthrombi leading to end organ dysfunction.

Risk factors include the use of an unrelated or mismatched donor, total body irradiation as part of the pre-transplant conditioning regimen, calcineurin inhibitor treatment, systemic cytomegalovirus or other infection, older age, and graft-versus-host disease (GVHD).

The Overall–TMA criteria by Cho et al uses a broad definition for TMA and incorporates the presence of schistocytes, an elevated LDH, normal coagulation studies, negative Coombs' test result, concurrent renal or neurological dysfunction unexplained by other mechanisms, progressive anemia, thrombocytopenia, and decreased levels of serum haptoglobin.[76] This is coupled with ADAMTS13 levels to distinguish it from TTP. In the pediatric and young adult population, Jodele et al., have proposed a new set of diagnostic criteria incorporating a renal biopsy with an elevated serum LDH, hypertension, proteinuria, new thrombocytopenia, new anemia, presence of schistocytes, and elevated levels of serum concentration of soluble C5b-9 as evidence for terminal complement activation.[77] In general, the diagnosis of HSCT induced TMA (HSCT-TMA) is challenging as fragmented red cells and thrombocytopenia are common after bone marrow transplantation, and because the differential diagnosis of fever, renal failure, and neurologic complications is extensive. The majority of cases occur within the first 100 days with a median of 32 days. Kidneys are a major target organ although it can affect any major organ (Lungs, GI, brain).

The management of HSCT-TMA involves eliminating precipitating factors. This starts with withdrawal or substitution of immunosuppressive medication especially calcineurin inhibitors. Alternative causes should be sought, and aggressive treatment of infection and GVHD should be employed in all but the most overt cases. Plasma exchange while effective in TTP has not been particularly effective in HSCT-TMA with response rates of less than 50%. Among other agents, defibrotide has been used with reported success in a few cases. The mechanism of action is believed to be through the prevention of platelet aggregation and platelet activation in the micro endothelial vasculature. In the pediatric and young adult population, eculizumab has been shown to work with remarkable efficacy in one series, with a 1 year survival of 62% versus 9% in the group treated with other modalities.[78] Patients received a median of 14 doses with all patients discontinuing eculizumab without recurrence of TMA. There have been other efforts to block other mediators of the complement pathway, namely mannose-binding protein-associated serine protease 2 (MASP-2) which when activated cleaves complement proteins C4 and C2 into C4a, C4b, C2a, and C2b. In the clinical trial space, narsoplimab, a MASP-2 inhibitor was evaluated in a phase 2 study for patients with a HUS, TTP, HCT associated TMA. The study met the primary efficacy point showing that 56% of patients receiving at least one dose achieved a complete response to status.

Cancer and Chemotherapy-Associated Thrombotic Microangiopathy

TMA may occur in the course of some patients with disseminated malignant neoplasms and large tumor burdens, most commonly adenocarcinoma of the gastrointestinal tract (mucin producing tumors), breast, or lung. It can occur as a result of bone marrow involvement, metastatic disease or due to a medication used for treatment. Patients generally present with an abrupt onset of moderate to severe MAHA and thrombocytopenia. Renal insufficiency and neurologic dysfunction occur less commonly than in idiopathic or chemotherapy-induced thrombotic microangiopathy and may result from concurrent metabolic disturbances, central nervous system metastases, stroke, or hemorrhage.

Laboratory evidence of DIC is found in 25% to 80% of patients based on findings of elevated fibrin degradation products or more sensitive measures of increased fibrinogen turnover. However, the observation that TMA occurs in only 5% of patients with disseminated malignancy and DIC suggests the involvement of additional factors, such as microvascular occlusion, intimal proliferation induced by tumor emboli within the pulmonary vasculature, or formation of an incompletely endothelialized tumor vasculature that predisposes to platelet adhesion, activation, and aggregation. Direct injury of endothelial vessels from the above processes in the marrow vessels can result in the release of ultralarge VWF multimers. Severe deficiency of ADAMTS13 is not typical. Outcomes of patients who develop TMA in the setting of chemotherapy or advanced cancer are poor because the TMA tends to be unrelenting and the comorbidity of the malignancy is significant. Survival is generally measured in weeks. The only effective therapy is reduction of the tumor burden, a goal often not attainable. A role for plasma exchange has not been established.

Specific cancer chemotherapeutic agents have also been implicated in the development of TMA. Patients are often on therapy for a malignancy at the time TMA develops, making attribution to the neoplasm versus its therapy difficult to determine. However, unlike cancer-associated TMA, most patients with chemotherapy-associated TMA may not have a large tumor burden. Implicated agents include mitomycin-C, gemcitabine, platinum-based chemotherapy, bleomycin, docetaxel, 5-fluourouracil, interferon-α and VEGF inhibitors, immunotherapy either alone or in combination. Chemotherapy-associated TMA is presumed to result from direct toxicity to the endothelium. Mitomycin inhibits the production of prostacyclin by umbilical vein endothelium, and infusion of mitomycin-C into the rat kidney induces histologic changes like those found with chemotherapy-induced TMA. Alternatively, anti-VEGF antibodies and other VEGF inhibitors have revealed a critical dependence of podocytes and glomerular endothelial cells on VEGF. Toxicity of these agents includes a thrombotic microangiopathy localized primarily to the kidney and manifested mainly by hypertension and proteinuria.

Patients with chemotherapy-associated TMA generally present with moderate to severe MAHA, thrombocytopenia, and renal insufficiency. Approximately 15% to 20% of such patients develop neurologic dysfunction. Compared to patients with TTP, cancer associated TMA may have greater pulmonary involvement with pulmonary edema and a new or exacerbated hypertension.

ADAMTS13 levels are normal or mildly reduced in chemotherapy-associated TMA. Plasma exchange has not been shown to be beneficial; however, case series raise the possibility that eculizumab may have utility in gemcitabine associated TMA.

Streptococcus Pneumonia

HUS associated with *Streptococcus* pneumonia occurs mainly in infants and young children, and rarely affects adults. It accounts for between 4% and 5% of all HUS cases United States. The incidence of pneumococcal associated HUS has decreased since the introduction of 13 Valent vaccine.

Several mechanisms have been proposed for the pathogenesis of pneumococcal associated HUS. The prevailing theory used to be centered around N-acetyl neuraminidase (sialidase) which is released during a pneumococcal infection. This cleaves sialic acid on the glycocalyx of the cell. It had been proposed that desialylation by neuraminidase results in the exposure of Thomsen-Friedenreich antigen (T antigen) on platelets, RBCs and glomeruli resulting in agglutination of red cells and hemolysis.[79]

An alternative proposal is desialylation by neuraminidase disrupts CFH binding sites on cell surfaces resulting in unregulated complement activation.[80] More recently, it has been proposed that increased expression of pneumococci surface proteins that bind plasminogen leading to plasmin generation resulting in fibrinogen degradation and complement activation leading to endothelial cell damage.[81]

Disordered Cobalamin Metabolism

Mutations in the MMACHC gene, which results in methylmalonic aciduria and homocystinuria, can result in atypical HUS. Clinically, these patients present with neurocognitive disease from their childhoods have microcephaly, seizures, ataxia, hypotonia, developmental delay, and a diagnosis of atypical HUS.

The diagnosis should be suspected in the presence of megaloblastic anemia accompanied by marked increase of homocysteine and low methionine levels, very high rates of urinary excretion of homocysteine and methylmalonic acid. The diagnosis is confirmed by genetic testing of the *MMACHC* gene.

Therapy includes parenteral administration of hydroxocobalamin, betaine, carnitine supplements, and leucovorin calcium with early initiation being critical for a good prognosis.

Miscellaneous Drug-Associated Thrombotic Microangiopathy

Although TMA has been associated with more than 75 drugs, the nine drugs that account for 75% of the cases include clopidogrel, estrogen/progesterone, gemcitabine, interferon, mitomycin, quinine, tacrolimus, and ticlopidine. The mechanisms by which these agents induce TMA may differ, as may the natural history and response to therapy. Whereas mitomycin C, gemcitabine, and Cyclosporine appear to induce TMA in a cumulative dose-dependent manner, quinidine and thienopyridines (ticlopidine and clopidogrel) induce thrombotic microangiopathy through either idiosyncratic, immune-mediated mechanisms or by acute endothelial toxicity.

The first report of quinine-associated TMA described the course of three patients with a syndrome resembling idiopathic HUS.[82] Patients generally present with severe MAHA, platelet counts below 50,000/μL and renal insufficiency, usually requiring dialysis. Neurologic dysfunction occurs in a minority of patients, but it can be severe; granulocytopenia and lymphopenia have been reported. In earlier reports, the prognosis was favorable once quinine was withdrawn and plasma exchange instituted. However, in another series, 17 of 225 patients with HUS had quinine-associated thrombotic microangiopathy, 4 of whom died, and 7 survivors were left with chronic renal failure.[83] Although this disorder occurs most commonly after the ingestion of quinine tablets, cases have been reported after exposure to quinine in beverages such as tonic water or herbal health supplements (cinchona). A careful history is sometimes necessary to establish the link between quinine ingestion and the patient's clinical disease. The pathogenesis may involve idiosyncratic, quinine-dependent antibodies reactive with platelet glycoproteins IIb/IIIa and Ib/IX and related antigens on endothelial cells and neutrophils that promote neutrophil aggregation and binding to endothelial cells in a drug-dependent manner. Patients with quinine-induced TMA have significant early mortality, though with appropriate supportive care and treatment of the acute episode, the disorder does not recur without re-exposure to quinine.

The thienopyridines are another common cause of drug-induced TTP. TTP occurs in a higher percentage of patients exposed to ticlopidine, with an incidence ranging from 1:600 to 1:4814 patient exposures in contrast to an estimated incidence of 4 per 1,000,000 to 1 per 8500 to 26,000 exposures to clopidogrel. No cases of clopidogrel-associated TTP were encountered in the CAPRIE and CURE studies, each of which enrolled more than 6200 patients. Despite the low incidence, the frequency with which clopidogrel is prescribed rendered it the most commonly reported drug associated with TTP in the FDA MedWatch database, accounting for more than 30% of all cases. The pathogenesis and natural history of TTP associated with ticlopidine and clopidogrel differ. Reductions in ADAMTS13 activity below 15% occur in 85% of patients with ticlopidine-associated TTP but in only 15% of patients with clopidogrel-associated TTP. Reduced ADAMTS13 reflects the development of anti-ADAMTS13 antibodies. TTP generally develops within 2 to 12 weeks of starting ticlopidine and responds relatively quickly to plasma exchange. Spontaneous relapses may occur. In contrast, 90% of patients exposed to clopidogrel, but only 10% of patients exposed to ticlopidine develop a syndrome characterized primarily by MAHA and renal insufficiency, without severe thrombocytopenia; these individuals have normal levels of ADAMTS13, usually develop disease within the first 2 weeks of exposure, and may require several weeks of plasma exchange to achieve remission. Whether or not plasma exchange actually improves the clinical outcome is uncertain. Spontaneous relapses are uncommon.

HIV-Associated Thrombotic Microangiopathy

An association between HIV infection and TMA has been recognized since 1984. TMA occurred in up to 7% of patients hospitalized with HIV infection. Conversely, the incidence of HIV infection in patients with TMA varied from 15% to 50% in endemic areas. In contrast, TMA is rare in HIV patients who are treated with highly active antiretroviral therapy (HAART). The mechanism of disease remains unclear, but endothelial injury has been hypothesized. Coexisting opportunistic infections may be a contributing cofactor. TMA that occurs in patients with advanced or untreated HIV often responds quickly to the initiation of antiretroviral therapy and can relapse if therapy is discontinued.

In some patients with HIV infection, the associated immune dysfunction may predispose to the formation of autoantibodies against ADAMTS13. This can lead to severe ADAMTS13 deficiency and TTP, despite adequate treatment for HIV. These patients may respond to plasma exchange.

Pregnancy-Associated Thrombotic Microangiopathy

The differential diagnosis of MAHA associated with gestation is complex. MAHA is seen in many conditions unique to pregnancy, such as pre-eclampsia, acute fatty liver associated with DIC, and HELLP syndrome (an acronym for *h*emolysis, *e*levated *l*iver function tests, and *l*ow *p*latelet count) in addition to aHUS and TTP.[84] The severity of the renal and neurologic abnormalities, and the time during gestation at which the signs and symptoms of TMA first appear may provide the clues needed to prevent critical delays in therapy.

For patients with congenital severe ADAMTS13 deficiency, pregnancy has a very high likelihood to trigger an acute severe episode, unmasking cTTP. The French TMA Reference Center reported an incidence of 24% of cTTP in pregnant patients presenting with their first TTP episode. For patients with an acquired autoimmune TTP, pregnancy may also increase risk of relapse. The syndrome occurs more commonly in the later half of pregnancy or in the postpartum period. Persistence or worsening thrombocytopenia, MAHA, and end-organ injury after delivery is strongly suggestive of a TMA. Pregnancy is often the first presentation for patients with cTTP. As part of the diagnosis, finding ADAMTS13 activities of less than 10% is crucial (ADAMTS13 levels decrease during pregnancy and may fall below 50% in many pregnant women close to term).

aHUS also occurs with increased frequency in association with pregnancy. The disorder primarily affects primiparas who present with MAHA, thrombocytopenia, renal insufficiency, and hypertension primarily in the postpartum period[85]—this later onset may help to distinguish HUS from other causes of pregnancy-associated MAHA and thrombocytopenia, such as pre-eclampsia and HELLP, which occur late in gestation or soon after delivery. Worsening renal function despite blood pressure control and delivery of the child should raise the suspicion for aHUS. Anticomplement therapy should be initiated promptly to preserve organ function (kidneys) if a complement-mediated process is suspected. Currently, the fetal effects of anticomplement therapy is unknown, but there does not appear to be increased fetal risks. Delivery does not treat complement-mediated TMA and these patients will need continued treatment with anticomplement therapy.

The HELLP syndrome complicates approximately 0.5% to 0.9% of pregnancies and approximately 10% to 20% of cases of pre-eclampsia. The pathogenesis is attributed to aberrant placental development, but the details remain unclear. Patients often present with abdominal pain and/or gastrointestinal symptoms, and concomitant hypertension and proteinuria. Seventy percent of cases occur prior to delivery, and most of the post-partum cases occur within 48 hours of parturition. Diagnosis is based on evident microangiopathic changes on the blood smear, thrombocytopenia, and abnormal liver function studies. The differential diagnosis is broad, and includes acute fatty liver of pregnancy, TTP, aHUS, vasculitis, DIC, and infection. The timing of development of symptoms may assist in the diagnosis because TTP tends to occur earlier in pregnancy, whereas aHUS often occurs post-partum. In one study, ADAMTS13 was decreased but not severely deficient in women with HELLP syndrome, with a reported mean of 31%.[86] Patients with HELLP usually have thrombocytopenia at that is less severe than what is seen in TTP and is usually more than 50,000. In addition, elevated transaminases are more commonly associated with HELLP than with aHUS or TTP. Initial management is to stabilize the patient, treat hypertension and prepare for delivery—delivery is considered curative for HELLP syndrome.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Alwan F, Vendramin C, Liesner R, et al. Characterization and treatment of congenital thrombotic thrombocytopenic purpura. *Blood.* 2019;133(15):1644–1651.

Bendapudi PK, Hurwitz S, Fry A, et al. Derivation and external validation of the PLASMIC score for rapid assessment of adults with thrombotic microangiopathies: a cohort study. *Lancet Haematol.* 2017;4(4):e157–e164.

Benhamou Y, Assié C, Boelle P-Y, et al. Development and validation of a predictive model for death in acquired severe ADAMTS13 deficiency-associated idiopathic thrombotic thrombocytopenic purpura: the French TMA Reference Center experience. *Haematologica.* 2012;97(8):1181–1186.

Brodsky RA. Complement in hemolytic anemia. *Blood.* 2015;126(22):2459–2465.

Davis TK, McKee R, Schnadower D, Tarr PI. Treatment of Shiga toxin-producing *Escherichia coli* infections. *Infect Dis Clin North Am.* 2013;27(3):577–597.

Fakhouri F, Zuber J, Frémeaux-Bacchi V, Loirat C. Haemolytic uraemic syndrome. *Lancet.* 2017;390(10095):681–696.

Fakhouri F, Scully M, Provôt F, et al. Management of thrombotic microangiopathy in pregnancy and postpartum: report from an international working group. *Blood.* 2020;136(19):2103–211717.

Fakhouri F, Fila M, Hummel A, et al. Eculizumab discontinuation in children and adults with atypical hemolytic-uremic syndrome: a prospective multicenter study. *Blood.* 2021;137(18):2438–2449.

Frémeaux-Bacchi V, Fakhouri F, Garnier A, et al. Genetics and outcome of atypical hemolytic uremic syndrome: a nationwide French series comparing children and adults. *Clin J Am Soc Nephrol.* 2013;8(4):554–562.

Gavriilaki E, Anagnostopoulos A, Mastellos DC. Complement in thrombotic microangiopathies: unraveling Ariadne's thread into the labyrinth of complement therapeutics. *Front Immunol.* 2019;10:337.

Hie M, Gay J, Galicier L, et al. Preemptive rituximab infusions after remission efficiently prevent relapses in acquired thrombotic thrombocytopenic purpura. *Blood.* 2014;124(2):204–210.

Hrdinová J, D'Angelo S, Graça NAG, et al. Dissecting the pathophysiology of immune thrombotic thrombocytopenic purpura: interplay between genes and environmental triggers. *Haematologica.* 2018;103(7):1099–1109.

Jodele S, Dandoy CE, Lane A, et al. Complement blockade for TA-TMA: lessons learned from a large pediatric cohort treated with eculizumab. *Blood.* 2020;135(13):1049–1057.

Legendre CM, Licht C, Muus P, et al. Terminal complement inhibitor eculizumab in atypical hemolytic-uremic syndrome. *N Engl J Med.* 2013;368(23):2169–2181.

Noris M, Remuzzi G. Atypical hemolytic-uremic syndrome. *N Engl J Med.* 2009;361(17):1676–1687.

Prevel R, Roubaud-Baudron C, Gourlain S, et al. Immune thrombotic thrombocytopenic purpura in older patients: prognosis and long-term survival. *Blood.* 2019;134(24):2209–2217.

Rock GA, Shumak KH, Buskard NA, et al. Comparison of plasma exchange with plasma infusion in the treatment of thrombotic thrombocytopenic purpura. Canadian Apheresis Study Group. *N Engl J Med.* 1991;325(6):393–397.

Roose E, Schelpe A-S, Tellier E, et al. Open ADAMTS13, induced by antibodies, is a biomarker for subclinical immune-mediated thrombotic thrombocytopenic purpura. *Blood.* 2020;136(3):353–361.

Sadler JE. Pathophysiology of thrombotic thrombocytopenic purpura. *Blood.* 2017;130(10):1181–1188.

Scully M, Knöbl P, Kentouche K, et al. Recombinant ADAMTS-13: first-in-human pharmacokinetics and safety in congenital thrombotic thrombocytopenic purpura. *Blood.* 2017;130(19):2055–2063.

Scully M, McDonald V, Cavenagh J, et al. A phase 2 study of the safety and efficacy of rituximab with plasma exchange in acute acquired thrombotic thrombocytopenic purpura. *Blood.* 2011;118(7):1746–1753.

Scully M, Thomas M, Underwood M, et al. Thrombotic thrombocytopenic purpura and pregnancy: presentation, management, and subsequent pregnancy outcomes. *Blood.* 2014;124(2):211–219.

Völker LA, Brinkkoetter PT, Knöbl PN, et al. Treatment of acquired thrombotic thrombocytopenic purpura without plasma exchange in selected patients under caplacizumab. *J Thromb Haemost.* 2020;18(11):3061–3066.

Zheng XL, Vesely SK, Cataland SR, et al. ISTH guidelines for the diagnosis of thrombotic thrombocytopenic purpura. *J Thromb Haemost.* 2020;18(10):2486–2495.

REFERENCES

1. Moschcowitz ELI. Hyaline thrombosis of the terminal arterioles and capillaries: a hitherto undescribed disease. *Proc NY Pathol Soc*. 1924:21–24.
2. Moake JL, Rudy CK, Troll JH, et al. Unusually large plasma factor VIII: von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. *N Engl J Med*. 1982;307(23):1432–1435.
3. Javett SN, Senior B. Syndrome of hemolysis, thrombopenia and nephropathy in infancy. *Pediatrics*. 1962;29:209–223.
4. Scully M, Thomas M, Underwood M, et al. Thrombotic thrombocytopenic purpura and pregnancy: presentation, management, and subsequent pregnancy outcomes. *Blood*. 2014;124(2):211–219.
5. Levy GG, Nichols WC, Lian EC, et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature*. 2001;413(6855):488–494.
6. Sadler JE. Pathophysiology of thrombotic thrombocytopenic purpura. *Blood*. 2017;130(10):1181–1188.
7. Grillberger R, Casina VC, Turecek PL, Zheng XL, Rottensteiner H, Scheiflinger F. Anti-ADAMTS13 IgG autoantibodies present in healthy individuals share linear epitopes with those in patients with thrombotic thrombocytopenic purpura. *Haematologica*. 2014;99(4):e58–e60.
8. Vanhoorelbeke K, De Meyer SF. Animal models for thrombotic thrombocytopenic purpura. *J Thromb Haemost*. 2013;11(Suppl 1):2–10.
9. Roose E, Schelpe A-S, Tellier E, et al. Open ADAMTS13, induced by antibodies, is a biomarker for subclinical immune-mediated thrombotic thrombocytopenic purpura. *Blood*. 2020;136(3):353–361.
10. Alwan F, Vendramin C, Vanhoorelbeke K, et al. Presenting ADAMTS13 antibody and antigen levels predict prognosis in immune-mediated thrombotic thrombocytopenic purpura. *Blood*. 2017;130(4):466–471.
11. Hrdinová J, D'Angelo S, Graça NAG, et al. Dissecting the pathophysiology of immune thrombotic thrombocytopenic purpura: interplay between genes and environmental triggers. *Haematologica*. 2018;103(7):1099–1109.
12. Joseph G, Smith KJ, Hadley TJ, et al. HLA-DR53 protects against thrombotic thrombocytopenic purpura/adult hemolytic uremic syndrome. *Am J Hematol*. 1994;47(3):189–193.
13. Scully M, Brown J, Patel R, McDonald V, Brown CJ, Machin S. Human leukocyte antigen association in idiopathic thrombotic thrombocytopenic purpura: evidence for an immunogenetic link. *J Thromb Haemost*. 2010;8(2):257–262.
14. Sakai K, Kuwana M, Tanaka H, et al. HLA loci predisposing to immune TTP in Japanese: potential role of the shared ADAMTS13 peptide bound to different HLA-DR. *Blood*. 2020;135(26):2413–2419.
15. Martino S, Jamme M, Deligny C, et al. Thrombotic thrombocytopenic purpura in black people: impact of ethnicity on survival and genetic risk factors. *PLoS One*. 2016;11(7):e0156679.
16. Benhamou Y, Assié C, Boelle P-Y, et al. Development and validation of a predictive model for death in acquired severe ADAMTS13 deficiency-associated idiopathic thrombotic thrombocytopenic purpura: the French TMA Reference Center experience. *Haematologica*. 2012;97(8):1181–1186.
17. Bendapudi PK, Hurwitz S, Fry A, et al. Derivation and external validation of the PLASMIC score for rapid assessment of adults with thrombotic microangiopathies: a cohort study. *Lancet Haematol*. 2017;4(4):e157–e164.
18. Prevel R, Roubaud-Baudron C, Gourlain S, et al. Immune thrombotic thrombocytopenic purpura in older patients: prognosis and long-term survival. *Blood*. 2019;134(24):2209–2217.
19. Wallace DC, Lovric A, Clubb JS, Carseldine DB. Thrombotic thrombocytopenic purpura in four siblings. *Am J Med*. 1975;58(5): 724–734.
20. von Krogh AS, Quist-Paulsen P, Waage A, et al. High prevalence of hereditary thrombotic thrombocytopenic purpura in central Norway: from clinical observation to evidence. *J Thromb Haemost*. 2016;14(1):73–82.
21. Fujimura Y, Matsumoto M, Isonishi A, et al. Natural history of Upshaw-Schulman syndrome based on ADAMTS13 gene analysis in Japan. *J Thromb Haemost*. 2011;9(Suppl 1):283–301.
22. Alwan F, Vendramin C, Liesner R, et al. Characterization and treatment of congenital thrombotic thrombocytopenic purpura. *Blood*. 2019;133(15):1644–1651.
23. Benhamou Y, Paintaud G, Azoulay E, et al. Efficacy of a rituximab regimen based on B cell depletion in thrombotic thrombocytopenic purpura with suboptimal response to standard treatment: results of a phase II, multicenter noncomparative study. *Am J Hematol*. 2016;91(12):1246–1251.
24. Sonneveld MAH, de Maat MPM, Portegies MLP, et al. Low ADAMTS13 activity is associated with an increased risk of ischemic stroke. *Blood*. 2015;126(25):2739–2746.
25. Borogovac A, George JN. Stroke and myocardial infarction in hereditary thrombotic thrombocytopenic purpura: similarities to sickle cell anemia. *Blood Adv*. 2019;3(23):3973–3976.
26. Scully M, Knöbl P, Kentouche K, et al. Recombinant ADAMTS-13: first-in-human pharmacokinetics and safety in congenital thrombotic thrombocytopenic purpura. *Blood*. 2017;130(19):2055–2063.
27. Rock GA, Shumak KH, Buskard NA, et al. Comparison of plasma exchange with plasma infusion in the treatment of thrombotic thrombocytopenic purpura. Canadian Apheresis Study Group. *N Engl J Med*. 1991;325(6):393–397.
28. Blombery P, Scully M. Management of thrombotic thrombocytopenic purpura: current perspectives. *J Blood Med*. 2014;5:15–23.
29. Uhl L, Kiss JE, Malynn E, Terrell DR, Vesely SK, George JN. Rituximab for thrombotic thrombocytopenic purpura: lessons from the STAR trial. *Transfusion*. 2017;57(10):2532–2538.
30. Scully M, McDonald V, Cavenagh J, et al. A phase 2 study of the safety and efficacy of rituximab with plasma exchange in acute acquired thrombotic thrombocytopenic purpura. *Blood*. 2011;118(7):1746–1753.
31. Zwicker JI, Muia J, Dolatshahi L, et al. Adjuvant low-dose rituximab and plasma exchange for acquired TTP. *Blood*. 2019;134(13):1106–1109.
32. Scully M, Cataland SR, Peyvandi F, et al. Caplacizumab treatment for acquired thrombotic thrombocytopenic purpura. *N Engl J Med*. 2019;380(4):335–346.
33. Zheng XL, Vesely SK, Cataland SR, et al. ISTH guidelines for the diagnosis of thrombotic thrombocytopenic purpura. *J Thromb Haemost*. 2020;18(10):2486–2495.
34. Völker LA, Brinkkoetter PT, Knöbl PN, et al. Treatment of acquired thrombotic thrombocytopenic purpura without plasma exchange in selected patients under caplacizumab. *J Thromb Haemost*. 2020;18(11): 3061–3066.
35. Swisher KK, Terrell DR, Vesely SK, Kremer Hovinga JA, Lämmle B, George JN. Clinical outcomes after platelet transfusions in patients with thrombotic thrombocytopenic purpura. *Transfusion*. 2009;49(5):873–887.
36. Hie M, Gay J, Galicier L, et al. Preemptive rituximab infusions after remission efficiently prevent relapses in acquired thrombotic thrombocytopenic purpura. *Blood*. 2014;124(2):204–210.
37. Jokiranta TS. HUS and atypical HUS. *Blood*. 2017;129(21):2847–2856.
38. Bell BP, Griffin PM, Lozano P, Christie DL, Kobayashi JM, Tarr PI. Predictors of hemolytic uremic syndrome in children during a large outbreak of *Escherichia coli* O157:H7 infections. *Pediatrics*. 1997;100(1):E12.
39. Freedman SB, Eltorki M, Chui L, et al. Province-wide review of pediatric Shiga toxin-producing *Escherichia coli* case management. *J Pediatr*. 2017;180:184–190. e1.
40. Mody RK, Gu W, Griffin PM, et al. Postdiarrheal hemolytic uremic syndrome in United States children: clinical spectrum and predictors of in-hospital death. *J Pediatr*. 2015;166(4):1022–1029.
41. Fakhouri F, Zuber J, Frémeaux-Bacchi V, Loirat C. Haemolytic uraemic syndrome. *Lancet (London, England)*. 2017;390(10095):681–696.
42. Frank C, Werber D, Cramer JP, et al. Epidemic profile of Shiga-toxin-producing *Escherichia coli* O104:H4 outbreak in Germany. *N Engl J Med*. 2011;365(19):1771–1780.
43. Orth-Höller D, Würzner R. Role of complement in enterohemorrhagic *Escherichia coli*-induced hemolytic uremic syndrome. *Semin Thromb Hemost*. 2014;40(4):503–507.
44. Freedman SB, Xie J, Nettel-Aguirre A, et al. Enteropathogen detection in children with diarrhoea, or vomiting, or both, comparing rectal flocked swabs with stool specimens: an outpatient cohort study. *Lancet Gastroenterol Hepatol*. 2017;2(9):662–669.
45. Qin X, Klein EJ, Galanakis E, et al. Real-time PCR assay for detection and differentiation of Shiga toxin-producing *Escherichia coli* from clinical samples. *J Clin Microbiol*. 2015;53(7):2148–2153.
46. Anderson NW, Tarr PI. Multiplex nucleic acid amplification testing to diagnose gut infections: challenges, opportunities, and result interpretation. *Gastroenterol Clin North Am*. 2018;47(4):793–812.
47. Davis TK, McKee R, Schnadower D, Tarr PI. Treatment of Shiga toxin-producing *Escherichia coli* infections. *Infect Dis Clin North Am*. 2013;27(3):577–597.
48. Grisaru S, Xie J, Samuel S, et al. Associations between hydration status, intravenous fluid administration, and outcomes of patients infected with Shiga toxin-producing *Escherichia coli*: a systematic review and meta-analysis. *JAMA Pediatr*. 2017;171(1):68–76.
49. Freedman SB, Xie J, Neufeld MS, et al. Shiga toxin-producing *Escherichia coli* infection, antibiotics, and risk of developing hemolytic uremic syndrome: a meta-analysis. *Clin Infect Dis*. 2016;62(10):1251–1258.
50. Lapeyraque A-L, Malina M, Frémeaux-Bacchi V, et al. Eculizumab in severe Shiga-toxin-associated HUS. *N Engl J Med*. 2011;364(26):2561–2563.
51. Greinacher A, Friesecke S, Abel P, et al. Treatment of severe neurological deficits with IgG depletion through immunoadsorption in patients with

Escherichia coli O104:H4-associated haemolytic uraemic syndrome: a prospective trial. *Lancet*. 2011;378(9797):1166–1173.
52. Nester CM, Barbour T, de Cordoba SR, et al. Atypical aHUS: state of the art. *Mol Immunol*. 2015;67(1):31–42.
53. Frémeaux-Bacchi V, Fakhouri F, Garnier A, et al. Genetics and outcome of atypical hemolytic uremic syndrome: a nationwide French series comparing children and adults. *Clin J Am Soc Nephrol*. 2013;8(4):554–562.
54. Bubeck D. The making of a macromolecular machine: assembly of the membrane attack complex. *Biochemistry*. 2014;53(12):1908–1915.
55. Brodsky RA. Complement in hemolytic anemia. *Blood*. 2015;126(22):2459–2465.
56. Frémeaux-Bacchi V, Miller EC, Liszewski MK, et al. Mutations in complement C3 predispose to development of atypical hemolytic uremic syndrome. *Blood*. 2008;112(13):4948–4952.
57. Moore I, Strain L, Pappworth I, et al. Association of factor H autoantibodies with deletions of CFHR1, CFHR3, CFHR4, and with mutations in CFH, CFI, CD46, and C3 in patients with atypical hemolytic uremic syndrome. *Blood*. 2010;115(2):379–387.
58. Noris M, Brioschi S, Caprioli J, et al. Familial haemolytic uraemic syndrome and an MCP mutation. *Lancet*. 2003;362(9395):1542–1547.
59. Noris M, Remuzzi G. Atypical hemolytic-uremic syndrome. *N Engl J Med*. 2009;361(17):1676–1687.
60. Lemaire M, Frémeaux-Bacchi V, Schaefer F, et al. Recessive mutations in DGKε cause atypical hemolytic-uremic syndrome. *Nat Genet*. 2013;45(5):531–536.
61. Bu F, Zhang Y, Wang K, et al. Genetic analysis of 400 patients refines understanding and implicates a new gene in atypical hemolytic uremic syndrome. *J Am Soc Nephrol*. 2018;29(12):2809–2819.
62. Noris M, Caprioli J, Bresin E, et al. Relative role of genetic complement abnormalities in sporadic and familial aHUS and their impact on clinical phenotype. *Clin J Am Soc Nephrol*. 2010;5(10):1844–1859.
63. Noris M, Remuzzi G. Cardiovascular complications in atypical haemolytic uraemic syndrome. *Nat Rev Nephrol*. 2014;10(3):174–180.
64. Gavriilaki E, Yuan X, Ye Z, et al. Modified Ham test for atypical hemolytic uremic syndrome. *Blood*. 2015;125(23):3637–3646.
65. Noris M, Galbusera M, Gastoldi S, et al. Dynamics of complement activation in aHUS and how to monitor eculizumab therapy. *Blood*. 2014;124(11):1715–1726.
66. Licht C, Greenbaum LA, Muus P, et al. Efficacy and safety of eculizumab in atypical hemolytic uremic syndrome from 2-year extensions of phase 2 studies. *Kidney Int*. 2015;87(5):1061–1073.
67. Legendre CM, Licht C, Muus P, et al. Terminal complement inhibitor eculizumab in atypical hemolytic-uremic syndrome. *N Engl J Med*. 2013;368(23):2169–2181.
68. McNamara LA, Topaz N, Wang X, Hariri S, Fox L, MacNeil JR. High risk for invasive meningococcal disease among patients receiving eculizumab (Soliris) despite receipt of meningococcal vaccine. *MMWR Morb Mortal Wkly Rep*. 2017;66(27):734–737.
69. Fakhouri F, Fila M, Hummel A, et al. Eculizumab discontinuation in children and adults with atypical hemolytic-uremic syndrome: a prospective multicenter study. *Blood*. 2021;137(18):2438–2449.
70. Wijnsma KL, Duineveld C, Volokhina EB, van den Heuvel LP, van de Kar NCAJ, Wetzels JFM. Safety and effectiveness of restrictive eculizumab treatment in atypical haemolytic uremic syndrome. *Nephrol Dial Transplant*. 2018;33(4):635–645.
71. Fakhouri F, Fila M, Provôt F, et al. Pathogenic variants in complement genes and risk of atypical hemolytic uremic syndrome relapse after eculizumab discontinuation. *Clin J Am Soc Nephrol*. 2017;12(1):50–59.
72. Stern RM, Connell NT. Ravulizumab: a novel C5 inhibitor for the treatment of paroxysmal nocturnal hemoglobinuria. *Ther Adv Hematol*. 2019;10:204062071987472.
73. Abbas F, El Kossi M, Kim JJ, Sharma A, Halawa A. Thrombotic microangiopathy after renal transplantation: current insights in de novo and recurrent disease. *World J Transplant*. 2018;8(5):122–141.
74. Gavriilaki E, Anagnostopoulos A, Mastellos DC. Complement in thrombotic microangiopathies: unraveling Ariadne's thread into the labyrinth of complement therapeutics. *Front Immunol*. 2019;10:337.
75. Jodele S, Zhang K, Zou F, et al. The genetic fingerprint of susceptibility for transplant-associated thrombotic microangiopathy. *Blood*. 2016;127(8):989–996.
76. Cho B-S, Yahng S-A, Lee S-E, et al. Validation of recently proposed consensus criteria for thrombotic microangiopathy after allogeneic hematopoietic stem-cell transplantation. *Transplantation*. 2010;90(8):918–926.
77. Jodele S, Davies SM, Lane A, et al. Diagnostic and risk criteria for HSCT-associated thrombotic microangiopathy: a study in children and young adults. *Blood*. 2014;124(4):645–653.
78. Jodele S, Dandoy CE, Lane A, et al. Complement blockade for TA-TMA: lessons learned from a large pediatric cohort treated with eculizumab. *Blood*. 2020;135(13):1049–1057.
79. Spinale JM, Ruebner RL, Kaplan BS, Copelovitch L. Update on *Streptococcus pneumoniae* associated hemolytic uremic syndrome. *Curr Opin Pediatr*. 2013;25(2):203–208.
80. Syed S, Hakala P, Singh AK, et al. Role of pneumococcal NanA neuraminidase activity in peripheral blood. *Front Cell Infect Microbiol*. 2019;9:218.
81. Meinel C, Spartà G, Dahse H-M, et al. Streptococcus pneumoniae from patients with hemolytic uremic syndrome binds human plasminogen via the surface protein PspC and uses plasmin to damage human endothelial cells. *J Infect Dis*. 2018;217(3):358–370.
82. Gottschall JL, Elliot W, Lianos E, McFarland JG, Wolfmeyer K, Aster RH. Quinine-induced immune thrombocytopenia associated with hemolytic uremic syndrome: a new clinical entity. *Blood*. 1991;77(2):306–310.
83. Kojouri K, Vesely SK, George JN. Quinine-associated thrombotic thrombocytopenic purpura-hemolytic uremic syndrome: frequency, clinical features, and long-term outcomes. *Ann Intern Med*. 2001;135(12):1047–1051.
84. Fakhouri F, Scully M, Provôt F, et al. Management of thrombotic microangiopathy in pregnancy and postpartum: report from an international working group. *Blood*. 2020;136(19):2103–2117.
85. Fakhouri F, Roumenina L, Provôt F, et al. Pregnancy-associated hemolytic uremic syndrome revisited in the era of complement gene mutations. *J Am Soc Nephrol*. 2010;21(5):859–867.
86. Lattuada A, Rossi E, Calzarossa C, Candolfi R, Mannucci PM. Mild to moderate reduction of a von Willebrand factor cleaving protease (ADAMTS-13) in pregnant women with HELLP microangiopathic syndrome. *Haematologica*. 2003;88(9):1029–1034.

CHAPTER 133

STRUCTURE, BIOLOGY, AND GENETICS OF VON WILLEBRAND FACTOR

Paula James, Orla Rawley, and Mackenzie Bowman

Von Willebrand factor (VWF) is an adhesive multimeric plasma glycoprotein (GP) that mediates platelet adhesion to injured subendothelium via GPIbα, and binds and stabilizes factor VIII (FVIII) in the circulation, protecting it from proteolytic degradation. This important multifunctional protein was named after the Finnish physician Dr. Erik von Willebrand, who first described von Willebrand disease (VWD) in 1926. In the original publication, he described a severe mucocutaneous bleeding problem in a family living on the Åland archipelago in the Baltic Sea.[1] The index case, a young woman named Hjördis, bled to death during her fourth menstrual period at the age of 13. Dr. von Willebrand referred to the condition as *pseudohemophilia*, but noted that in contrast to hemophilia, this condition affected both genders, with females typically being more severely affected.

In the mid-1950s, it was recognized that VWD was usually accompanied by a reduced level of FVIII activity, and that the bleeding phenotype could be corrected by the infusion of normal plasma.[2] In the early 1970s, the critical immunologic distinction between FVIII and VWF was made, and since that time significant progress has been made in our understanding of the molecular pathophysiology of this disorder.[3] Cloning and characterization of the *VWF* gene in the 1980s has facilitated further investigation into the function of VWF and the genetic basis of VWD.[4–7]

FUNCTIONS OF VON WILLEBRAND FACTOR

VWF is a multifunctional adhesive protein that plays an important role in both primary hemostasis and blood coagulation. In primary hemostasis, VWF initiates platelet adhesion at the site of endothelial injury, whereas in coagulation, VWF stabilizes FVIII in the circulation (Fig. 133.1).

Platelet Adhesion

At the site of endothelial injury, VWF adheres to exposed collagen. The hemostatically important forms of collagen include types I, III, and VI, but VWF preferentially binds to type III collagen. The interactions of VWF with collagen are predominately mediated by the VWF A3 domain (Fig. 133.2).[8] Once immobilized, VWF is subjected to the high shear rates of the arterial circulation and undergoes a conformational change that exposes the platelet GPIbα binding site within the VWF A1 domain.[9] High affinity, rapid, and reversible interaction between VWF and GPIbα tethers platelets to the endothelium, where they roll until they are immobilized and activated by direct platelet-collagen binding, which is mediated by two collagen receptors on platelets, GPVI and the integrin α2β1 (or GPIa/IIa).[10,11] The Arg-Gly-Asp (RGD) sequence within the VWF C1 domain also contributes to platelet adhesion by interacting with GPIIb-IIIa of activated platelets.[12]

Factor VIII Stabilization

VWF binds FVIII through the VWF D′D3 domains and protects it from proteolytic degradation, thereby prolonging its half-life. In the absence of VWF, FVIII has a half-life of approximately two hours in contrast to a half-life of 12 to 20 hours when bound to VWF.

BASAL VON WILLEBRAND FACTOR LEVELS

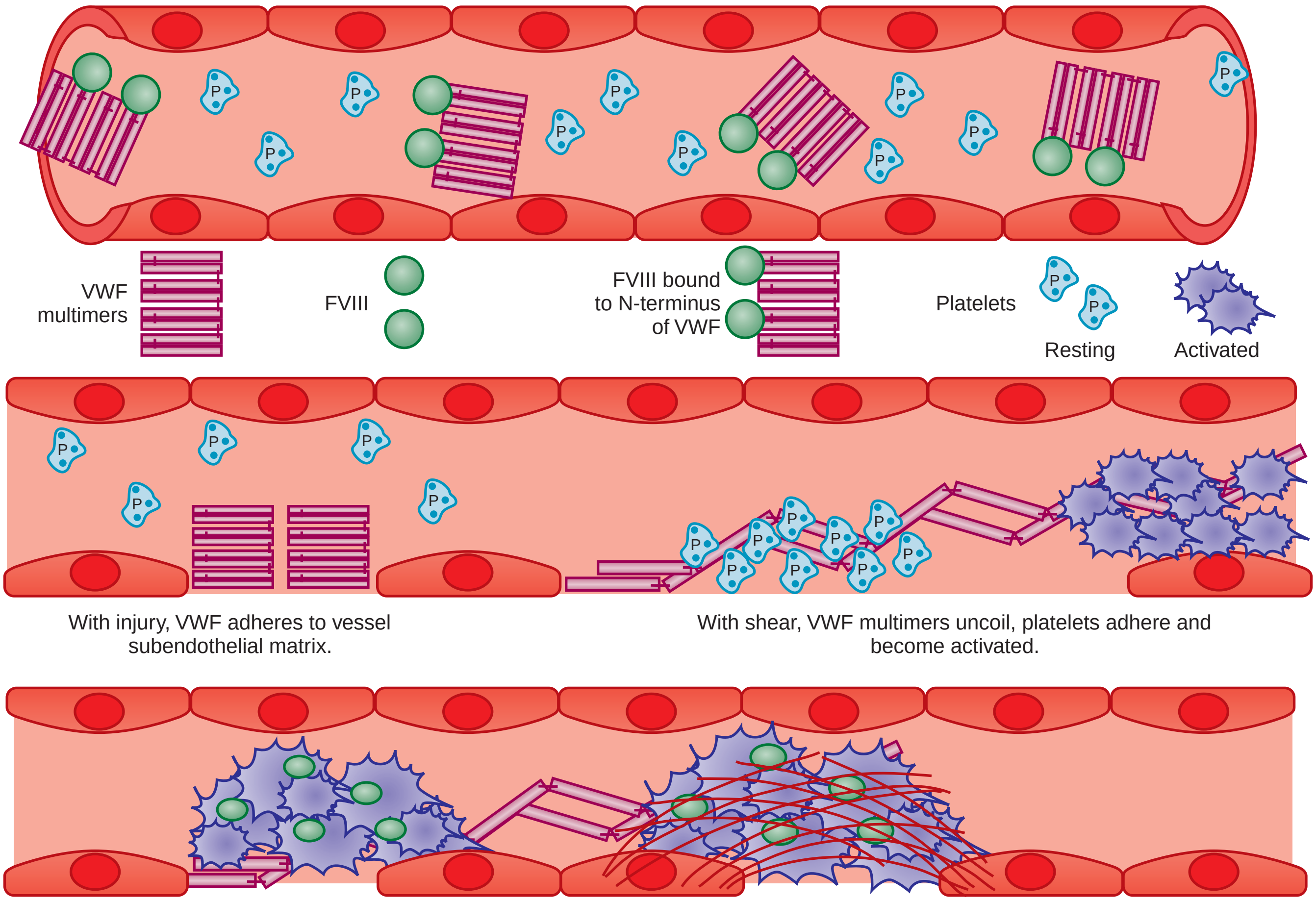
The normal level for VWF is highly variable and ranges from 50 to 200 IU/dL. Factors that contribute to the variable VWF levels include ABO genotype (see section on ABO blood groups later), Secretor genotype, race, and age. Plasma VWF concentrations have been reported to be approximately 20% higher in subjects homozygous for the Secretor (Se) allele as compared with those who are heterozygous.[13] VWF levels in African Americans are 15% higher than in those of European descent. Increased age has also been associated with higher VWF levels,[14] with studies suggesting that the levels may increase by 3.5 IU/dL per decade.[15]

Within individuals, VWF levels often vary over time as a result of β-adrenergic stimuli, drugs, or more sustained physiologic factors, such as pregnancy, hypothyroidism, chronic illness, or long-term use of certain medications.[16,17] The vasopressin analogue, 1-deamino-(8-D-arginine)-vasopressin (DDAVP, desmopressin) transiently and reliably increases VWF and FVIII levels—a fact that, in addition to an acceptable side effect profile, has led to the use of DDAVP as a first-line treatment in certain types of VWD and mild hemophilia A.[18] Several physiologic stressors involving β-adrenergic stimulation, such as exercise, surgery, and psychological distress, can produce an acute increase in VWF levels.[19] VWF levels remain elevated for up to 6 days after surgery, suggesting that after the acute secretory response, there is upregulation of VWF production. A sustained elevation of VWF levels can occur with chronic diseases, such as hyperthyroidism, renal failure, diabetes, liver disease, atherosclerosis, chronic inflammatory states, and cancer.[16,20–23] Conversely, acquired VWD, characterized by qualitative or quantitative VWF defects, can occur with certain medications, such as valproic acid,[24] and with some chronic medical conditions (see section later on acquired VWD).

VWF levels are increased by estrogen.[17] In premenopausal women, VWF levels vary in a cyclical fashion, with the lowest levels occurring in the early follicular phase of the menstrual cycle (days 1 to 7), and peak values occurring during the luteal phase. Oral contraceptives increase VWF levels and dampen the cyclical variation. This dose-dependent effect is mediated by the estrogen component and is evident with ethynylestradiol doses of 0.5 μg or higher. Lower estrogen doses have little or no effect on VWF levels. VWF levels increase in pregnancy starting in the second trimester and achieve levels threefold higher than baseline values by the end of the third trimester.[25] VWF levels return to baseline 1 to 3 weeks after delivery.

VON WILLEBRAND FACTOR GENE

Located on the short arm of chromosome 12 at p13.3, the *VWF* gene spans 178 kb and is composed of 52 exons that range in size from 1.3 kb (exon 28) to 40 bp (exon 50) (see Fig. 133.2A). There is a partial, unprocessed pseudogene, *VWFP*, located on the long arm of chromosome 22 at q 11.2, measuring 21 to 29 kb, which duplicates the *VWF* gene sequence for exons 23 to 34 with 97% sequence homology.[26] Gene conversion events involving the pseudogene contribute to the variant spectrum of VWD. The *VWF* gene is highly polymorphic. Analysis of the 1000 Genomes database has revealed more than 2700 single nucleotide variants (SNVs) in the normal population alone.[27] These variants are found throughout the coding



With injury, VWF adheres to vessel subendothelial matrix.

With shear, VWF multimers uncoil, platelets adhere and become activated.

Activated platelets expose phosphatidyl serine and bind FVIII to facilitate clotting.

Bleeding ceases by platelet-fibrin plug sealing vascular injury and is followed by thrombolysis and tissue repair.

Figure 133.1 FUNCTION OF VON WILLEBRAND FACTOR (VWF). Role of VWF in mediating the initial events in the hemostatic process. VWF is the carrier protein for FVIII *(top)*. After endothelial injury, VWF adheres to the exposed subendothelium, where it is uncoiled by the shear forces, thereby exposing GPIbα binding sites that interact with platelets *(middle)*. The bound platelets are activated and the GPIIb-IIIa complex is exposed on the platelet surface. Interaction of fibrinogen and VWF with GPIIb-IIIa then consolidates the platelet adhesive event and initiates platelet aggregation *(bottom)*. *FVIII*, Factor VIII; *GP*, glycoprotein. *(Used with the permission of Robert Montgomery.)*

sequence as well as in intronic regions, and appear to occur at a rate of ~2.5 variants per individual. The majority of these are rare variants, the pathological significance of which remain unclear, but a significant portion are associated with non-synonymous coding sequence changes, splice site changes or insertion/deletions. An International Society on Thrombosis and Haemostasis Scientific and Standardization Committee (ISTH-SSC) database of both benign and pathogenic variants is maintained by the European Association for Haemophilia and Allied Disorders (https://dbs.eahad.org/upcoming-vwf-db/). The high degree of polymorphism, the large size, and the presence of the pseudogene render full *VWF* gene sequencing and data interpretation challenging.

VWF expression is tightly restricted to endothelial cells and megakaryocytes. Cell-specific transcriptional regulation is complex and poorly understood. A 734-base pair region, spanning approximately 500 bases of the 5′-flanking region and 247 bases of the first exon, functions as a promoter and includes a minimal core promoter, as well as negative and positive regulatory regions.[28] The positive regulatory region confers cell-specificity by relieving inhibition in specific cells, thereby allowing transcription of the *VWF* gene.

DOMAIN STRUCTURE

Encoded VWF messenger ribonucleic acid is 8.8 kb in length and the translated pre-pro-VWF molecule contains 2813 amino acids (AA), comprising a 22 AA signal peptide, a 741 AA propeptide, and a 2050 AA mature subunit that possesses all the adhesive sites required for hemostatic activity. The AA sequence is cysteine rich, with cysteine residues constituting 8.3% of the total amino acid content. Cysteine residues are mainly found clustered in the N- and C-termini where they are involved in extensive intra- and inter-molecular disulfide bonding. Cysteines are least abundant in the VWF A domains, which contain just six cysteine residues. Both the A1 and A3 domains take on the form of disulfide-bonded loops, while the A2 domain possesses a rare vicinal disulfide that contributes to domain stability.[29]

The VWF AA sequence exhibits considerable internal homology and comprises a series of repeated domains (A, C, and D), which make up approximately 90% of the precursor. These repeated domain structures are highly homologous to domain structures found in many unrelated proteins. For example, the VWF A domain is the prototype for a protein superfamily and appears in up to 22 human proteins, such as leukocyte adhesion receptors, collagen receptors, and cartilage matrix protein.[30] Likewise, homologues of the VWF C, D, and cysteine knot (CK) domains exist throughout the genome and are found in proteins with various functions. This suggests that the *VWF* gene is the product of a complex series of partial gene duplications.

Based on the homologous internal repeat structure, the domain composition of VWF is given as NH2-SP-D1-D2-D'-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK-COOH.[31] The D domains are further divided into VWD, C8, TIL, and E subdomains. D' is a truncated assembly containing only the TIL and E subdomains. The D1,

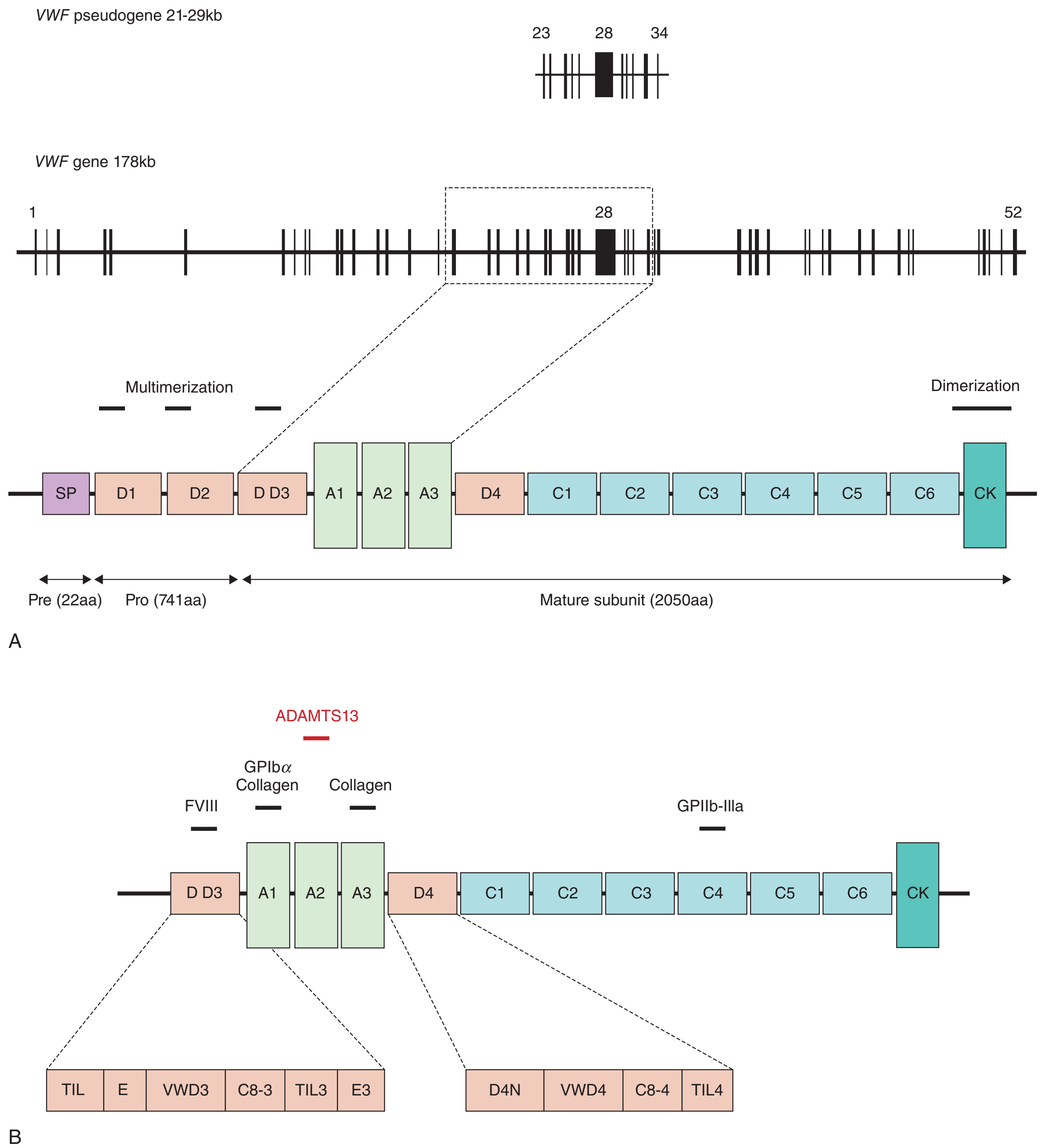

VWF pseudogene 21-29kb
23
28
34
VWF gene 178kb
1
28
52
Multimerization
Dimerization
SP
D1
D2
D D3
A1
A2
A3
D4
C1
C2
C3
C4
C5
C6
CK
Pre (22aa)
Pro (741aa)
Mature subunit (2050aa)
A
ADAMTS13
GPIbα
Collagen
Collagen
FVIII
GPIIb-IIIa
D D3
A1
A2
A3
D4
C1
C2
C3
C4
C5
C6
CK
TIL
E
VWD3
C8-3
TIL3
E3
D4N
VWD4
C8-4
TIL4
B

Figure 133.2 VON WILLEBRAND FACTOR GENE, PRIMARY TRANSLATIONAL PRODUCT AND PROTEIN. (A) The *VWF* gene is located on chromosome 12 at p13.3, spans 178 kb and includes 52 exons. The *VWF* pseudogene is located on chromosome 22 and replicates exons 23 to 34. The VWF mRNA is 8.8 kb in length. Pre-pro-VWF contains 2813 amino acids (AA) with a 22-AA signal peptide, a 741-AA propeptide, and a 2050-AA mature subunit. (B) The mature subunit consists of repeated domains (A–D), several of which have distinct functions and binding sites. *ADAMTS13*, A disintegrin and metalloproteinase with a thrombospondin type I motif member 13; *FVIII*, factor VIII.

D2, and D3 assemblies comprise VWD, C8, TIL, and E subdomains, while the D4 assembly lacks the E subdomain, but carries a unique subdomain structure designated D4N (see Fig. 133.2B).

The functional significance of many of the individual VWF domain structures has been established. The signal peptide (S) targets pre-pro-VWF to the endoplasmic reticulum (ER), where it is promptly cleaved. The CK domains of adjacent VWF monomers form disulfide bonds, resulting in tail-to-tail dimers that initiate the multimerization process.[32] The VWF propeptide (D1D2) plays a critical role in VWF multimer assembly by promoting the formation of head-to-head oligomers via intermolecular disulfide bonding between D3 domains of adjacent VWF dimers.[33,34]

Several functional sites that are critical for the regulation of hemostatic activity have also been identified. The D′D3 assembly constitutes the FVIII interactive region of VWF, with the bipartite FVIII binding site encompassing the noncontiguous TIL′ and C8-3 subdomains.[35] The hemostatically active A domains comprise several key binding and regulatory sites. Binding sites for platelet GPIbα, heparin, and collagens I, III, and VI have been localized to the A1 domain, though the major collagen III binding site localizes to the A3 domain.[8,36,37] The A2 domain contains the cryptic cleavage site for the regulatory protease ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13), with the scissile bond located between Tyr1605-Met1606.[38] The platelet GPIIb/IIIa binding site is found within the RGD motif of the C4 domain.[12]

BIOSYNTHESIS

VWF is exclusively synthesized in endothelial cells and megakaryocytes and undergoes a complex series of co- and post-translational modifications, including dimerization, glycosylation, sulfation, and ultimately, multimerization. The fully processed protein is then either released into the circulation or is stored in specialized organelles: the Weibel-Palade bodies (WPBs) of endothelial cells or the α-granules of platelets.

Dimerization of pro-VWF monomers occurs in the ER through the formation of disulfide bonds between CK domain monomers in a tail-to-tail fashion. The location of these intersubunit disulfide bonds has been localized to a subset of cysteine residues (Cys2771, Cys2773, and/or Cys2811).[32] In addition, the signal peptide is cleaved and most of the intrachain disulfide bonds are formed in the ER. Only pro-VWF dimers are then transported to the Golgi apparatus. Exiting the ER appears to be the rate-limiting step in VWF biosynthesis.

In the Golgi, pro-VWF dimers make up the basic building blocks for further multimerization. Here, the propeptide (D1D2) is cleaved by a propeptide processing protease, likely furin, between AA 763 to 764. The propeptide continues to be essential for VWF multimerization because under acidic conditions, it serves as an endogenous chaperone that promotes additional disulfide bond formation between D3 domains of adjacent dimers in a head-to-head orientation.[34,39,40] The inter-subunit disulfide bonds are likely mediated by the alignment of the cysteine pairs Cys1099 and Cys1142.[41] The VWF subunit is approximately 250 kDa, whereas resulting disulfide-linked multimers can be more than 20,000 kDa.

The mature VWF subunit is heavily glycosylated with the carbohydrate component making up approximately 20% of the monomeric mass. VWF expresses both N- and O-linked glycan structures which are important regulators of its functional activity, modifying its plasma half-life as well as its susceptibility to regulatory proteolysis by ADAMTS13.[42] In the ER, N-linked glycosylation begins as a co-translational event and 13 N-linked high-mannose-containing oligosaccharide chains are added to each VWF subunit. In addition, the propeptide has four potential N-glycosylation sites. The mannose-rich core is remodeled by a series of glycosyltransferases and glycosidases as the protein transits the ER, facilitating interaction with resident ER chaperones that direct protein folding and subsequent dimerization. Dimerization is absolutely dependent on the addition of N-linked glycans.[43]

Post-translational modification occurs in the Golgi with the addition of 10 O-linked oligosaccharides to the pro-VWF dimer. O-linked glycans are assembled via sequential addition of monosaccharide units rather than by transfer of a preassembled core. N-linked glycans undergo further processing to produce complex-type carbohydrate structures and sulfation of specific N-linked glycans (Asn1147 and Asn1231) occurs in the trans-Golgi network. Finally, both the N- and O-linked glycan structures are modified with capping terminal sialic acid moieties and unusually, approximately 13% of the N-linked and 1% of the O-linked glycans are modified to express ABO(H) antigenic determinants. Overall, the VWF glycome represents an extremely heterogeneous population of sugars. To date, over 300 distinct VWF N-linked glycan structures alone have been described.[44]

Interestingly, platelet-VWF exists as a natural glycoform and has distinct functional characteristics.[45] Terminal sialic expression on the N-linked glycans of platelet-VWF is reduced twofold and although H-antigen expression is comparable to that of its plasma-derived counterpart, further modification of the H antigen to produce group A or B determinants does not occur. As a direct consequence of its differential glycosylation, platelet-VWF is significantly more resistant to ADAMTS13-mediated proteolysis than plasma-derived VWF.

STORAGE AND SECRETION

The majority of the VWF in endothelial cells consists of low-molecular-weight (LMW) multimers that are constitutively secreted. The more biologically active high-molecular-weight (HMW) VWF multimers are preferentially targeted for storage in endothelium-specific cytoplasmic granules, the WPBs. The biogenesis of WPBs is driven by VWF itself. In addition to VWF and the VWF propeptide (VWFpp), numerous other proteins that are essential to hemostasis and inflammation, and to modulation of vascular tone and angiogenesis, are stored in WPBs including P-selectin, CD63, interleukin-8 (IL-8), tissue plasminogen activator, and angiopoietin-2 (Ang-2).[46] WPBs have a characteristic cigar-like shape, measuring 0.2 μm in width and 5 μm in length (Fig. 133.3), and are composed of tightly packed tubules, measuring 150 to 200 Å in cross-section.[47] Platelet VWF is stored within similar tubules that are found in the periphery of α-granules, and constitutes approximately 15% to 20% of total circulating VWF. Tubular packing condenses the length of VWF multimers by 50-fold and is dependent on the acidic pH within the trans-Golgi, as well as the propeptide (D1D2) and the NH_2-terminal region (D′D3A1). Large VWF multimers, which can be up to 100 μm in length, are reversibly packaged into coils. Upon WPB exocytosis, the filamentous strings of VWF are secreted into the circulation, where they rapidly unfurl and are capable of binding to platelets. If the tubular structure is perturbed, WPBs release short, tangled VWF that does not support platelet binding to the endothelium.

VWF is secreted via three pathways—constitutive, basal, and regulated release.[48] Regulated VWF release is via an acute pathway that is triggered by agonist-mediated activation of the endothelium. Basal secretion from WPBs occurs continuously and is likely in response to background levels of agonists and, possibly, also due to mechanical stimulation from the circulating blood. The continuous basal release of VWF is likely to be the source of circulating plasma VWF, whereas the acute regulated release of VWF provides a localised dose of ultra-large VWF (UL-VWF) to initiate primary hemostasis at times of injury.

A variety of agonists can induce the secretion of VWF from endothelial cells.[46] These agonists include histamine, thrombin, fibrin, the terminal complement proteins C5b-9, and β-adrenergic agonists. These agonists work via two main pathways to raise the intracellular levels of either (1) Ca^{2+} or (2) cyclic adenosine monophosphate (cAMP). Upon endothelial stimulation, WPBs release their contents through one of three modes of exocytosis, depending on the type and amount of protein that is required. VWF is released via full fusion of WPB with the plasma membrane. The freshly secreted unusually large, or UL-VWF, multimers are highly active and can spontaneously bind platelets. Some of the released protein remains associated with the endothelial membrane and some self-associates.

ADAMTS13

ADAMTS13 is a constitutively active plasma protease that cleaves circulating VWF between Tyr1605-Met1606 in the A2 domain.[49] Its main target is the UL-VWF multimers, which spontaneously bind platelet GPIbα. ADAMTS13 cleaves UL-VWF multimers when there is sufficient shear-stress to unfold the A2 domain to expose the cryptic cleavage site.[50] UL-VWF multimers that appear in plasma after WPB secretion are cleaved within 2 hours by ADAMTS13 to form LMW, or less hemostatically active multimers. ADAMTS13-mediated proteolysis of VWF is

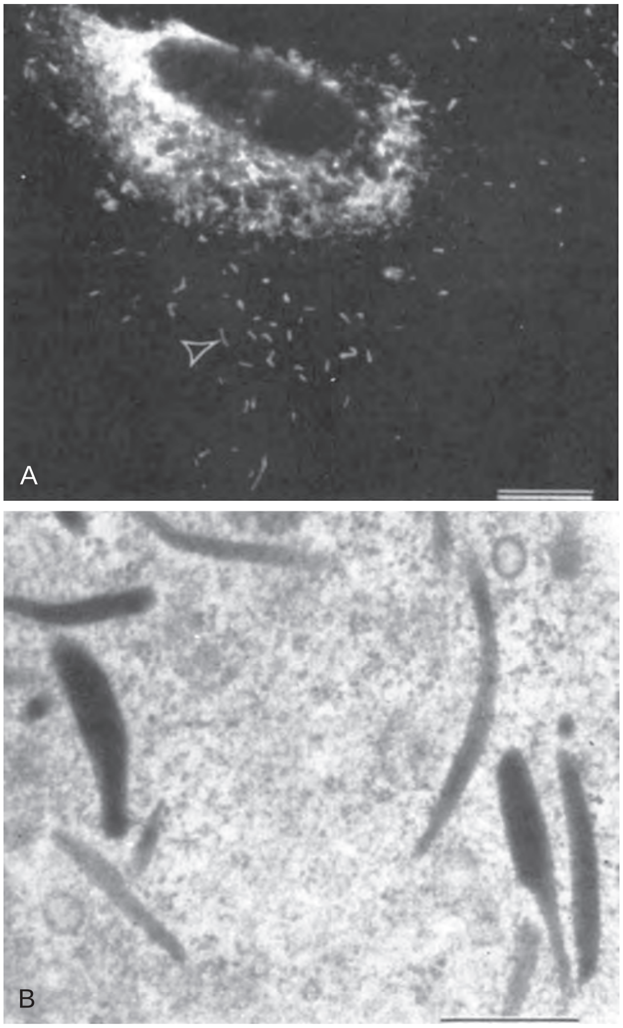


Figure 133.3 WEIBEL-PALADE BODIES OF ENDOTHELIAL CELLS. (A) Immunofluorescence staining of a human umbilical vein endothelial cell (HUVEC) with anti-VWF antibody. VWF is present in the perinuclear region, where it is synthesized, and in the Weibel-Palade bodies *(arrowhead)* throughout the cytoplasm. Bar = 10 µm. (B) Electron micrograph of Weibel-Palade bodies of the same origin. Bar = 0.5 µm.

responsible for the characteristic "triplet" pattern of the satellite bands that flank each main multimer band on multimer analysis gels (Fig. 133.4).

Susceptibility to ADAMTS13 proteolysis is influenced by glycosylation and specific VWF sequence variants.[51] De-glycosylated plasma-derived VWF is proteolyzed more rapidly than fully glycosylated VWF, and blood group O VWF is cleaved more rapidly than non-O VWF in the order O ≥ B ≥ A ≥ AB. Moreover, Bombay-VWF is proteolyzed even more rapidly than group O VWF.[52] In addition, although terminal α2–6 linked sialic acid expression protects VWF from spurious proteolysis by plasma cysteine and serine proteases, it specifically targets VWF for proteolysis by ADAMTS13.[53] Single-nucleotide variants, such as the c.4751 A > G variant giving rise to p.Tyr1584Cys in the VWF A2 domain, have also been shown to affect the susceptibility of VWF to proteolysis.[54]

Alterations in the balance between ADAMTS13 activity and VWF proteolysis can lead to several disease states. Congenital or acquired deficiency of ADAMTS13 can result in thrombotic thrombocytopenic purpura (see Chapter 132). Conversely, enhanced proteolysis can give rise to a bleeding phenotype. For example, in a subtype of type 2 A VWD, variant VWF exhibits enhanced susceptibility to ADAMTS13 cleavage which results in loss of large VWF multimers.

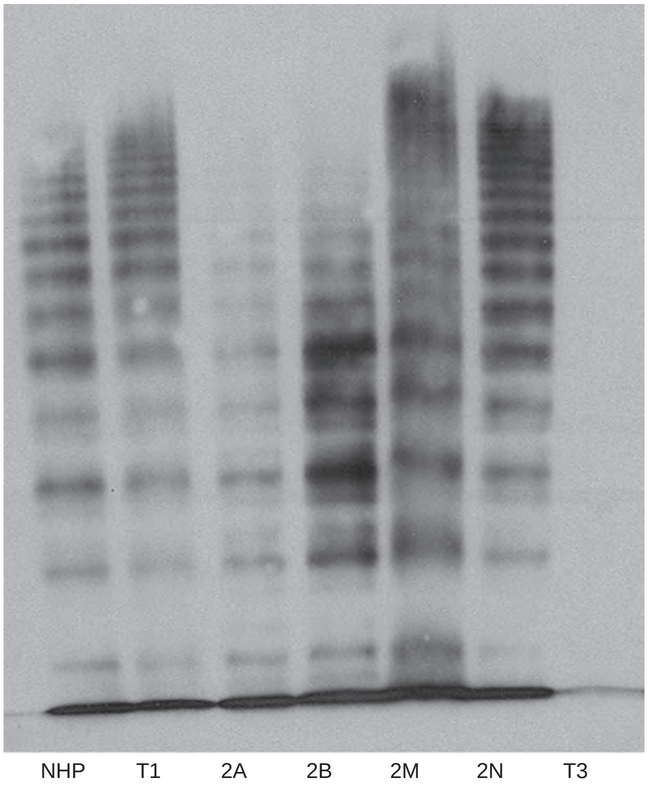


Figure 133.4 EXAMPLE OF A MULTIMER ANALYSIS (2.25% AGAROSE GEL). Lane 1 normal human plasma (NHP) represents normal plasma multimer patterns with a characteristic "triplet" pattern of satellite bands flanking each main multimer. Lanes 2 to 7 show the plasma von Willebrand factor (VWF) multimer analysis for patients with the different subtypes of von Willebrand disease (VWD) (T1, type 1; 2 A, type 2 A; 2B, type 2B; 2 M, type 2 M; 2 N, type 2 N; T3, type 3). Type 2 A and type 2B VWD both show variable loss of high-molecular-weight multimers. Types 1, 2 M, and 2 N demonstrate the presence of high-molecular-weight multimers. Finally, with type 3 VWD, there is a complete absence of VWF.

CLEARANCE

VWF clearance is a complex multifactorial process that involves numerous endocytic receptors expressed on a variety of cell-types.[55] Hepatic and splenic macrophages, as well as sinusoidal endothelial cells, represent the cell types thought to contribute most significantly to VWF clearance, though hepatocytes also have an established role. Following the identification of the asialoglycoprotein receptor (ASGPR) as the first receptor capable of mediating VWF removal from the plasma, several additional VWF clearance receptors were described. Macrophage receptors LRP1, SR-AI, MGL, and endothelial receptors CLEC4M, stabilin-2 and SCARA5 have all been shown to bind and endocytose VWF.[56–62] Although the exact molecular mechanisms underlying each of these VWF clearance pathways have not been fully elucidated, it is clear that both glycosylation and *VWF* sequence variation modulate VWF uptake. Blood group O individuals (see the following section on ABO Blood Groups) exhibit increased VWF clearance compared to non-O blood types displaying shorter VWF survival following DDAVP administration.[63] Additionally, a growing number of missense sequence variants have been associated with accelerated VWF clearance. To date >30 variants have been described. Most of these cluster around the VWF D3 and A1 domains, but variants have been described throughout the entirety of the coding sequence. Accelerated clearance has been associated with type 1, type 2 A, and type 2B phenotypes.

The VWFpp is co-secreted with mature VWF in a 1:1 stoichiometric ratio. After secretion, the VWFpp dissociates from VWF and circulates with a half-life of 2 to 3 hours.[64] In contrast, VWF circulates with a half-life of approximately 12 to 20 hours. These discordant clearance rates mean that the ratio between VWFpp and VWF can be used as a surrogate marker for altered VWF clearance.[65] A ratio >2.2 is considered indicative of accelerated or pathological VWF clearance.

ABO BLOOD GROUPS

ABO blood groups differ only with respect to a single terminal sugar residue. Despite this relatively minor modification to the glycan chain, ABO blood group expression has a significant quantitative effect on plasma VWF levels.[55] Blood group O individuals have plasma VWF levels that are on average 25% lower than those with non-O blood types: the mean VWF level in blood group O subjects is 74.8 IU/dL as compared to 105.9 IU/dL, 116.9 IU/dL, and 123.3 IU/dL in blood group A, B, and AB subjects, respectively.[63] Plasma VWF levels are further reduced in individuals with the rare Bombay phenotype.[52] An accumulating body of evidence suggests that differential VWF clearance is responsible for this quantitative effect, though the receptor responsible for mediating this phenomenon has not been identified. Blood group O individuals have a significantly shorter VWF half-life following DDAVP infusion compared to non-O individuals (Group O vs non-O: 10.0 vs 25.5 hours respectively). In addition, group O individuals also have significantly elevated VWFpp/VWF:Ag, indicating an increased rate of VWF clearance.[65] Clinically, the ABO-related variance in VWF levels is relevant; patients with blood group O are significantly overrepresented in "low VWF" or type 1 VWD populations.[66,67] However, in laboratory practice, blood group stratified VWF reference ranges are not employed.

AREAS OF ONGOING INVESTIGATION

The role of VWF in the regulation of vascular endothelial growth factor-dependent angiogenesis is an ongoing area of investigation. Such regulation occurs directly through its interaction with integrins on endothelial cells and, indirectly, via regulation of WPB formation, secretion of Ang-2, and other constituents.[68] Accumulating evidence suggests that VWF may regulate several aspects of cancer cell biology including tumor metastasis.[69] Additionally, the functional role of VWF in mediating venous thrombosis is an active area of investigation, particularly in the context of pathological immunothrombosis.[70,71] Genome-wide association studies (GWAS) over the past decade have identified a number of variants in non-*VWF* loci that influence plasma VWF levels. These include *STAB2* (stabilin-2), *SCARA5* (scavenger receptor class A member 5), *TC2N* (tandem C2 domains, nuclear), *STXBP5* (syntaxin-binding protein 5), *STX2* (syntaxin-2), and *CLEC4M* (C-type lectin domain family 4 member M).[72] The majority of these are either genes that modify VWF glycosylation, secretion from endothelial cells or platelets, or clearance from the plasma. Studies are currently investigating the effect of these genes on VWF pathobiology.

VON WILLEBRAND DISEASE

VWD is caused by deficient, or defective, plasma VWF and represents the most common inherited bleeding disorder. Bleeding symptoms reflect the defect in primary hemostasis: mucocutaneous bleeding, especially epistaxis and heavy menstrual bleeding. When FVIII levels are sufficiently low, the bleeding phenotype overlaps with that of mild to moderate hemophilia; patients may experience joint or muscle bleeds. The current VWD classification recognizes three types.[73] Type 1 VWD is characterized by quantitative deficiency of VWF, type 2 VWD is characterized by qualitative defects, and type 3 VWD is characterized by an almost complete absence of VWF.

Epidemiology

VWD is the most common inherited bleeding disorder. However, because VWF levels are variable in the population and symptom severity ranges from infrequent, mild bleeding to frequent or severe bleeds, the reported prevalence depends on the diagnostic criteria and the study population. In two large epidemiologic studies, the prevalence of VWD was approximately 1% in healthy school-age children based on low VWF activity, and a personal and family history of bleeding symptoms.[74,75] The prevalence of VWD in individuals who present to a primary care physician with bleeding symptoms is approximately 0.1%,[76] whereas in patients whose bleeding symptoms are sufficiently severe to warrant referral to specialized centers, the prevalence is 20 to 113 per million.[77]

Classification and Pathophysiology

The 2006 International Society on Thrombosis and Haemostasis (ISTH) VWD classification relies on the VWF protein phenotype, which in turn often reflects the underlying pathophysiology and has implications for treatment.[73] Type 1 VWD is a partial quantitative deficiency; type 2 (with four subtypes: 2A, 2B, 2M, and 2N) is a qualitative defect; and type 3 is a virtual deficiency of VWF (Table 133.1). The diagnosis and categorization of VWD into types can be achieved with laboratory testing. The differentiation among type 2 subtypes may require referral to a specialized laboratory. The current classification does not incorporate genotypic data: the diagnosis of VWD is not limited to individuals with variants within the *VWF* gene. *VWF* sequence variants may not be identified in VWD patients because of the complexity of the *VWF* gene or because of variants in other genes, such as those affecting secretion or clearance, which also lead to a VWD phenotype. A third level of classification denoted by roman numerals (e.g., VWD type 2A IIA) indicates specific phenotypes, and is a remnant of an older classification system that is mainly used in the research setting.

The American Society of Hematology (ASH), the ISTH, National Hemophilia Foundation (NHF), and the World Federation of Hemophilia (WFH) are currently collaborating to develop guidelines for the diagnosis and management of VWD. These guidelines, once finalized, will provide clinicians with an up-to-date consensus on VWD.

Von Willebrand Disease Type 1

Type 1 VWD, a quantitative deficiency of VWF, represents approximately 70% of VWD cases. The VWF is functionally normal, and patients have normal VWF multimers. Functional assays of VWF, such as VWF:RCo, are decreased in proportion to the decrease in

TABLE 133.1 **Classification of von Willebrand Disease**

Type	Description
1	Partial quantitative deficiency of VWF. Mild abnormalities in multimer structure or distribution may occur.
2	Qualitative VWF defects.
2A	Decreased VWF-dependent platelet adhesion and deficiency of HMW VWF multimers.
2B	Increased affinity for platelet GPIbα.
2M	Decreased VWF-dependent platelet adhesion with a normal multimer distribution.
2N	Decreased affinity for FVIII.
3	Almost complete deficiency of VWF.

FVIII, Factor FVIII; *GPIbα*, glycoprotein Ibα; *HMW*, high molecular weight; *VWF*, von Willebrand factor.

VWF:Ag, and the ratio of functional activity as compared with VWF:Ag is normal (i.e., VWF:RCo/VWF:Ag ratio is >0.7).

Sequence variants have been identified in approximately 65% of individuals with type 1 VWD and occur throughout the *VWF* gene.[78–80] Fully penetrant, dominantly inherited missense variants are more often identified when VWF:Ag and VWF:RCo levels are less than 25 IU/dL. In contrast, incompletely penetrant, dominantly inherited missense variants, such as p.Tyr1584Cys and p.Arg924Gln, are identified in approximately 50% of individuals whose VWF:Ag and VWF:RCo levels are above 25 IU/dL. The extent to which incompletely penetrant *VWF* variants contribute to the bleeding phenotype in individuals with VWF levels of approximately 50 IU/dL is not clear, and genetic analyses in such cases are difficult to interpret.

Missense variants may affect VWF levels by reducing secretion and/or increasing clearance. The most frequently reported genetic variant is a missense variant that results in the substitution of tyrosine with cysteine at amino acid 1584 (p.Tyr1584Cys), which is found in 10% to 20% of type 1 VWD patients.[67] Intracellular retention is a common mechanism for type 1 VWD pathogenicity, and can result from missense variants in various VWF domains. Haploinsufficiency from a heterozygous null allele results in reduced VWF expression in a small proportion of cases. A common heterozygous in-frame large deletion of exons 4 and 5 was reported in a cohort of type 1 VWD patients in the United Kingdom,[81] and this, and similar partial gene deletions, may contribute to the spectrum of variants in a minority of cases. A well-described pathophysiologic mechanism for type 1 VWD is increased VWF clearance, referred to as type 1 C (C for increased clearance), although this designation is not included in the ISTH classification. Patients typically have very low VWF levels, an increased VWFpp/VWF:Ag ratio, and a marked but short-lived response to DDAVP.[55,82] Of note, the half-life of VWF/FVIII concentrates is normal in these individuals. Missense variants mainly cluster around the D3 and A1 domains and can reduce the half-life of VWF up to 15-fold. p.Arg1205His, which is known as the "Vicenza" variant, is the most common, most severe, and best characterized of these variants.[83] Because of the transient response to DDAVP, the utility of this medication for treatment of major bleeds is questioned in this subgroup of patients.

VWF levels increase with age in normal individuals and the same phenomenon is seen in patients with type 1 VWD (but not type 2 or 3).[84] The increase has been reported as 3.5 IU/dL VWF:Ag and 7.1 IU/dL FVIII:C per decade. The effect of this increase on bleeding phenotype remains unclear and requires further investigation.

Von Willebrand Disease Type 2

Type 2 VWD is characterized by a qualitative defect of VWF activity and is further classified into subtypes that affect VWF-platelet interactions (2 A, 2B, and 2 M) and that affect VWF binding to FVIII (2 N) (Fig. 133.5).

Type 2A

Type 2A VWD is the most common type 2 subtype, accounting for approximately 10% of all VWD cases. Type 2 A VWD is usually inherited as a dominant trait and is characterized by a lack of HMW and/or intermediate-molecular-weight (IMW) multimers, which are the most hemostatically active. This results in a disproportionately low functional activity compared with the antigen level (i.e., VWF:RCo/VWF:Ag ratio of <0.7). The FVIII level may be low or normal. The multimer profile shows a loss of HMW and sometimes IMW multimers. This subtype may encompass missense variants that impair dimer (CK domain) or multimer assembly (recessive variants in the D1 and D2 domains), disrupt intersubunit disulfide bonds (D3 and D2 domains), enhance susceptibility to ADAMTS13-mediated proteolysis (A2 and A1 domains), and/or result in intracellular retention of VWF, particularly the HMW multimers (D3, A1, and A2).[85]

Type 2B

Type 2B VWD is the result of gain-of-function variants within the GPIbα binding site on VWF. Missense variants are in exon 28, in or close to the A1 domain. This results in spontaneous binding of VWF to platelets without the need for a VWF-collagen interaction. The VWF-platelet interactions selectively deplete the HMW multimers by increasing ADAMTS13 proteolysis. The increased binding of mutant VWF to platelets also triggers the formation of platelet aggregates, which are removed from the circulation resulting in thrombocytopenia. Altered megakaryocytopoiesis characterized by giant platelets with abnormal ultrastructure contributes to the thrombocytopenia.

The laboratory profile reveals a decreased VWF:RCo/VWF:Ag ratio and absence of HMW multimers, but in contrast to Type 2 A, ristocetin-induced platelet aggregation (RIPA) reveals increased sensitivity to low doses of ristocetin. Although these features may be present to varying degrees in the majority of patients, not all cases

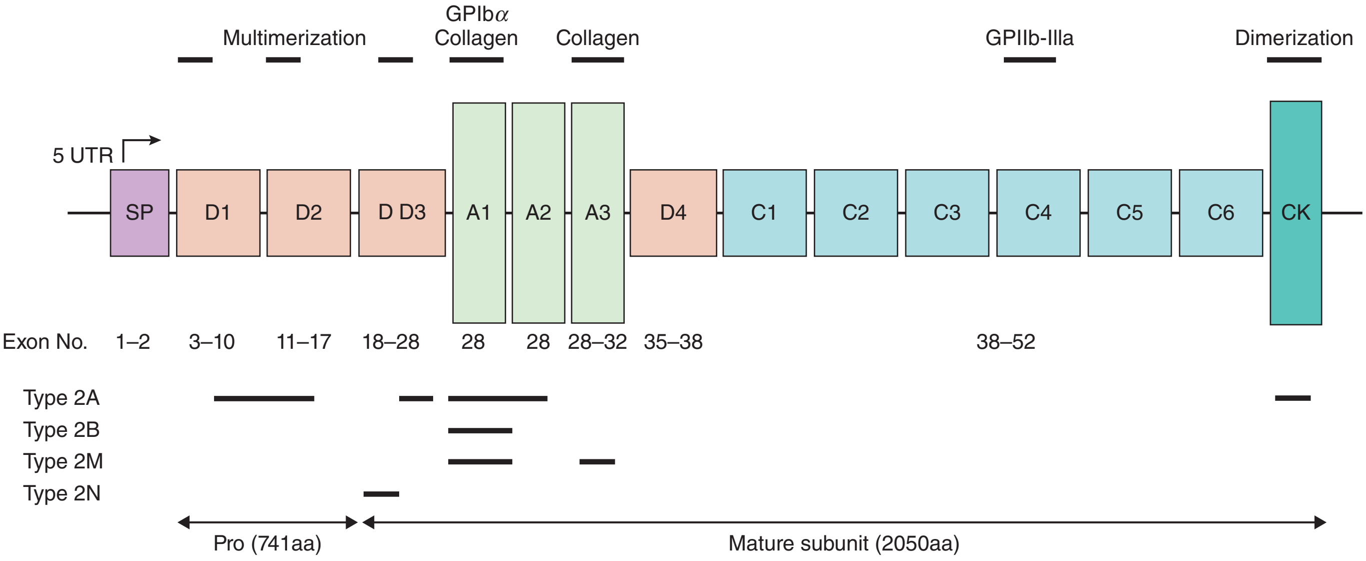


Figure 133.5 FUNCTIONAL DOMAINS OF VON WILLEBRAND FACTOR AND LOCATION OF TYPE 2 VON WILLEBRAND DISEASE (VWD) VARIANTS. VWF protein comprises a large N-terminal propeptide and mature subunit. Repeated protein domains are designated A through D. Highlighted are binding sites for factor VIII, platelet GPIb, collagen, and the areas critical for dimerization and multimerization. Sites of the common variants that result in type 2 VWD are shown. *Arrow* indicates translational start site. *FVIII*, Factor VIII; *GP*, glycoprotein; *VWF*, von Willebrand factor.

demonstrate these classic features. For example, variants affecting p.Pro1266Leu may enhance GPIbα binding (RIPA) without inducing thrombocytopenia or HMW multimer loss.[86]

Type 2M

Type 2M VWD (the M refers to multimer) is characterized by a loss of function variant within the VWF GPIbα binding site. The laboratory workup shows a reduced ratio of VWF:RCo to VWF:Ag, but a normal multimer pattern. A number of missense variants are reported in exon 28, and there are case reports of variants in exons 27, 30 to 31, and 52. VWF exhibits reduced affinity for GPIbα because of variants in the A1 domain that alter protein conformation,[87] but the HMW multimers are normal. Rare variants in the A3 domain that impair the VWF-collagen interaction are also classified as 2M VWD.[88] In these cases, VWF:RCo may be normal, and the diagnosis requires VWF collagen binding assays (VWF:CB).

Type 2N

Type 2N VWD (the N referring to Normandy, where the first cases were reported) has been described as an autosomal form of hemophilia A, and is an important consideration in the differential diagnosis of individuals of either sex who present with low FVIII levels.[89] The affinity of VWF for FVIII is reduced because of variants in the FVIII binding site, or conformational changes that impair the VWF-FVIII interaction. The characteristic laboratory feature is a disproportionate decrease in the FVIII level relative to the VWF level (which may be low or normal) with a resultant reduction in the FVIII:C/VWF:Ag ratio. The majority of patients with VWD type 2N have a normal multimer profile, but occasional cases will demonstrate loss of HMW multimers. The majority (≈ 80%) of missense variants are located in exons 18 to 20 (D′ and D3) with a much lower proportion of variants in exons 17 and 24 to 27. Type 2N exhibits autosomal recessive inheritance, and affected individuals are either homozygous, compound heterozygous for missense variants, or compound heterozygous for a missense variant and a variant resulting in a null allele. Definitive diagnosis requires evidence of reduced FVIII binding to VWF (VWF:FVIIIB) or identification of causative variants in the FVIII binding region of the *VWF* gene.

Von Willebrand Disease Type 3

Type 3 VWD is defined by a virtual absence of VWF. The inheritance of type 3 VWD is autosomal recessive in about half of type 3 patients and autosomal codominant in the remainder: approximately 50% of carriers will be symptomatic and meet the criteria for type 1 VWD.[90] This condition is characterized by prolongation of the aPTT, undetectable levels of VWF:Ag and VWF:RCo, and FVIII levels less than 10 IU/dL (i.e., less than 10% of normal). A recent study observed measurable VWFpp (median = 72 IU/dL) in a subset of type 3 VWD patients despite very low VWF:Ag levels, indicating that in those cases, VWF is produced and secreted, but rapidly cleared from the circulation.[91] Therefore, the levels of VWFpp may distinguish between type 3 VWD and severe type 1 VWD patients.

Variants associated with type 3 VWD are found throughout the coding region of *VWF*, including the propeptide. Up to 80% of type 3 VWD patients have two null alleles and produce little or no VWF. Null alleles can result from a variety of variants, with nonsense variants accounting for about one-third. Approximately 20% of alleles carry missense variants and are predominantly located in the D1–D2 (exons 3 to 11) and D4–CK (exons 37 to 52) domains. These variants may impair dimer or multimer formation, resulting in intracellular VWF retention and decreased secretion into plasma. Large deletions, predominantly resulting in frameshift variants affecting one or more exons, contribute to approximately 12% of the type 3 VWD variant spectrum. Because there is little or no circulating VWF, patients with these variants may develop alloantibodies against infused VWF.[92]

Low von Willebrand Factor

For many years, there has been debate regarding which cut-off level of VWF should be used for the diagnosis of type 1 VWD. Individuals with mild to moderate reductions in plasma VWF levels (30 to 50 IU/dL) may be considered in a distinct category labeled “low VWF levels” instead of type 1 VWD (levels <30 IU/dL). These individuals display variable bleeding phenotypes and often do not have *VWF* gene sequence variations. A recent large cohort study from Ireland showed that low VWF levels can be associated with significant bleeding which is predominantly due to reductions in VWF synthesis and/or secretion.[93] The underlying pathobiology of low VWF levels remains largely undefined, which poses challenges for the diagnosis and management of these individuals. The ASH/ISTH/NHF/WFH updated guidelines for the diagnosis and management of VWD will be especially critical for the diagnosis and management of this group of patients.

Clinical Manifestations

The bleeding history depends on disease severity; type 3 VWD is often diagnosed early in life, whereas mild type 1 VWD may not be diagnosed until adulthood. Individuals with VWD primarily complain of excessive mucocutaneous bleeding, such as spontaneous, recurrent epistaxis, and prolonged bleeding after dental cleaning or extraction. In addition, prolonged or excessive bleeding after surgery or trauma is often reported. Affected females frequently experience heavy menstrual bleeding from the time of menarche and can have prolonged or excessive bleeding after childbirth.[94] Musculoskeletal bleeding is unusual, except in type 2N or type 3 VWD when the FVIII:C level may be below 10 IU/dL. Bleeding assessment tools (BATs) help to standardize and quantify the bleeding history (see Chapter 126). The occurrence, frequency and severity of various bleeding symptoms are assessed, with each symptom evaluated using a common scoring system.[95] A bleeding score is then generated by summing the score of all bleeding symptoms for a given individual. BATs are helpful for making a VWD diagnosis. In addition, high bleeding scores are predictive of the risk of future bleeding (see Chapter 126).[96]

EXAMPLE OF THE PRESENTATION AND IMPACT OF TYPE 1 VON WILLEBRAND DISEASE

J.C., a 26-year-old female, presents with delayed postpartum hemorrhage (PPH) after the delivery of her first child. The pregnancy and delivery were unremarkable. One week postpartum, the patient experienced heavy bleeding, and required a dilation and curettage. At the same time, she was started on tranexamic acid, which was continued for 2 weeks. By approximately 3 weeks postpartum, there was complete cessation of bleeding. A bleeding history was significant for the following: excessive postsurgical bleeding following tonsillectomy and adenoidectomy, which required red blood cell transfusion; excessive bleeding post-dental extractions, requiring consultation with the dentist and repacking; and heavy menstrual bleeding from the time of menarche, which had been well controlled with a combined oral contraceptive pill. There was no family history of VWD. Investigations revealed VWF:Ag 29 IU/dL, VWF:RCo 28 IU/dL, and FVIII:C 60 IU/dL. Multimer gel was unremarkable. A DDAVP challenge demonstrated that the patient was a responder. She became pregnant again 4 years later. At the time of delivery, she was treated with tranexamic acid and a dose of DDAVP after the cord was clamped. Her postpartum course was uncomplicated.

This case illustrates several important points. First, type 1 VWD is a mild to moderate bleeding disorder. Patients often do not have significant symptoms on a regular basis, or symptoms may be masked or ameliorated by concomitant combined oral contraceptive use, which is common in this patient demographic. A careful bleeding history focusing on past hemostatic challenges, and subsequent complications is vital in making the diagnosis. A high index of suspicion is required for unusual bleeding complications, such as delayed PPH. The importance of making the diagnosis is highlighted here. With little burden of prophylactic treatment, bleeding complications can be avoided.

Type 1 VWD accounts for up to 70% of VWD. It typically manifests as mild mucocutaneous bleeding; however, symptoms may be more severe if VWF levels are below 15 IU/dL. Epistaxis and bruising are common symptoms in children. Heavy menstrual bleeding is the most common finding in women of reproductive age.

Type 2 VWD accounts for about 25% of all VWD. The relative frequency of the subtypes is 2A > 2N > 2M > 2B in European populations. Individuals with type 2A, 2B, and 2M VWD usually present with mild to moderate mucocutaneous bleeding, but bleeding episodes can be severe, particularly when VWF:RCo is very low or absent. In many patients with type 2B VWD, thrombocytopenia can develop or worsen with infection, surgery, pregnancy, or treatment with DDAVP. The symptoms of type 2N VWD are similar to those seen in mild hemophilia A (see Chapter 134) because both disorders are associated with reduced levels of FVIII:C.

Type 3 VWD is the rarest subtype and accounts for less than 5% of VWD. Prevalence estimates range from 0.55 to 6 per million, with higher rates seen with consanguineous marriage.[97] Type 3 VWD manifests with severe bleeding, including excessive mucocutaneous bleeding and musculoskeletal bleeding.

Penetrance

In autosomal dominant type 1 VWD, variants resulting in plasma VWF level less than 25 IU/dL are often fully penetrant, whereas those resulting in higher VWF levels are often incompletely penetrant.[78] Variants responsible for autosomal dominant types of VWD (2A, 2B, and 2M) are often fully penetrant. Thus, in contrast with the variably positive family histories in patients with type 1 VWD, those with type 2 VWD usually have a positive family history.

Laboratory Investigations

The laboratory evaluation for VWD involves a battery of qualitative and quantitative measurements of VWF and FVIII that should be interpreted by a physician with experience in this area given the heterogeneity of possible results (Table 133.2).[98]

Screening Tests

The complete blood cell count may show microcytic anemia because of iron deficiency or thrombocytopenia in type 2B VWD. The aPTT is often normal but may be prolonged if the FVIII:C level is reduced below 30 to 40 IU/dL, as can be seen with severe type 1, type 2N, or type 3 VWD. The prothrombin time is normal in VWD. Although some laboratories may also include a skin bleeding time and platelet function analysis (PFA closure time) in their evaluation of an individual with suspected VWD, these tests lack sensitivity in persons with mild bleeding or specificity for VWD.

Confirming a Diagnosis of von Willebrand Disease

The following specific factor assays should be performed even if the screening tests are normal.

Von Willebrand Factor: Antigen

This assay determines the quantity of VWF protein antigen in the plasma and is performed using an enzyme-linked immunosorbent assay (ELISA) or latex immunoassay (LIA). The normal range (which should be determined independently by each laboratory) is approximately 50 to 200 IU/dL.

Von Willebrand Factor Activity

Until recently, the VWF:RCo assay was the gold standard for the measurement of VWF platelet-binding activity. The VWF:RCo activity assay measures the capacity of VWF to agglutinate platelets in response to ristocetin. However, due to the high coefficient of variation within and between laboratories, the VWF:RCo assay is gradually being phased out in favor of the VWF:GPIbM activity assay.[99] This assay demonstrates improved precision and sensitivity compared with the VWF:RCo assay, and is not subject to the ristocetin-associated artifactual reductions in activity for *VWF* sequence variants p.Pro1467Ser and p.Asp1472His. The normal range for these assays is approximately 50 to 200 IU/dL.

Factor VIII: C Level

The functional FVIII assay determines the activity of FVIII in clot-based assays. The normal range is approximately 50 to 150 IU/dL.

Several analytic variables can complicate the diagnosis of VWD. Based on established reference ranges, approximately 2.5% of the normal population will have low VWF levels. In addition, assay variability, particularly for VWF:RCo, renders differentiation of type 1 VWD from type 2 VWD difficult.

VWF:RCo and VWF:Ag determined by LIA have limited sensitivity, which may result in the misdiagnosis of type 3 VWD as type 1 or type 2 VWD. Finally, inappropriate sample handling can lead to decreases in VWF:Ag, VWF:RCo, and FVIII:C, with VWF:RCo predominantly affected. All these factors must be considered when

TABLE 133.2 **Table of Investigations**

VWD Type	VWF:RCo IU/dL[a]	VWF:Ag IU/dL[a]	RCo/Ag IU/dL[a]	FVIII:C IU/dL[a]	Multimer Pattern[b]	Other
1	Low	Low	Equivalent	~1.5× VWF:Ag	Normal	
2A	Low	Low	VWF:RCo < VWF:Ag	Low or normal	Abnormal↓ HMWM	
2B	Low	Low	VWF:RCo < VWF:Ag	Low or normal	Abnormal↓ HMWM	↑ RIPA[c] (↓ platelet count)
2M	Low	Low	VWF:RCo < VWF:Ag	Low or normal	Normal	
2N	Normal/low	Normal/low	Equivalent	<30	Normal	↓ VWF:FVIIIB[d]
3	Absent	Absent	NA	<10	Absent	

[a] Relative to the reference range (approximate values); VWF:RCo (50–200 IU/dL); VWF:Ag (50–200 IU/dL); FVIII:C (50–150 IU/dL).

[b] HMWM, High-molecular-weight multimers.

[c] Increased agglutination at low concentrations of ristocetin.

[d] The ability of VWF to bind and protect FVIII is reduced. VWF and FVIII levels can look exactly like those in males with mild hemophilia A or in symptomatic hemophilia A carrier females.*Ag*, Antigen, *FVIII:C*, FVIII level; *NA*, not applicable; *RCo*, ristocetin cofactor; *RIPA*, ristocetin-induced platelet aggregation; *VWD*, von Willebrand disease; *VWF:FVIIIB*, FVIII-binding assay.

FACTORS TO CONSIDER WHEN INTERPRETING VON WILLEBRAND DISEASE TEST RESULTS

	Considerations	Results
Preanalytical	When was the sample collected and processed? Was there a significant delay before samples were run? Were samples frozen in a timely fashion?	VWF:RCo may be decreased resulting in a false-positive diagnosis of type 2 VWD
Analytical	Convention of established references	False-positive diagnosis of VWD in 2.5% of population
	High degree of assay variability for VWF:RCo	False-positive diagnosis of type 2 in a type 1 patient may result in the misdiagnosis of type 3 for type 1 or type 2 VWD
	A high lower limit of detection for certain VWF:Ag and VWF:RCo assays, in particular LIA-based assays	
Patient factors	Drugs (OCP, HRT, or valproic acid)	False negative or positive
	ABO type	False negative
	Pregnancy	Reversible acquired von Willebrand syndrome
	Hypothyroidism	
	Comorbid illness (e.g., valvular heart disease, lymphoma)	

Ag, Antigen; *HRT*, hormone replacement therapy; *LIA*, latex immunoassay; *OCP*, oral contraceptive pill; *RCo*, ristocetin cofactor; *VWD*, von Willebrand disease; *VWF*, von Willebrand factor.

interpreting VWF laboratory results, and at least two sets of tests using fresh samples are needed to confirm the diagnosis of VWD. Diagnostic testing should be avoided in stressed, ill, or pregnant patients (see box on Factors to Consider When Interpreting von Willebrand Disease Test Results).

Discriminating Tests to Identify von Willebrand Disease Subtype

Von Willebrand Factor Multimer Analysis

Sodium dodecyl sulfate (SDS)-agarose electrophoresis is used to assess VWF oligomers in plasma (see Fig. 133.4). Normal plasma contains multimers composed of over 40 VWF subunits. The largest multimers have molecular weights more than 20,000 kDa. Multimers are classified as HMW, IMW, and LMW by counting bands 1 to 5 as LMW, 6 to 10 as IMW, and those above 10 as HMW.[100] HMW and/or IMW multimers are decreased or missing in types 2 A and 2B VWD. Infrequently, circulating UL-VWF multimers may be apparent in the plasma. These are associated with pathological conditions such as thrombotic thrombocytopenic purpura and have also been observed in VWD Vicenza.

Low-Dose Ristocetin-Induced Platelet Aggregation

The RIPA assay tests the capacity of VWF to agglutinate platelets with varying concentrations of ristocetin. In contrast to the VWF:RCo (which evaluates the interaction between the patient's VWF and formalin-fixed platelets), the low-dose RIPA assay evaluates the sensitivity of the patient's platelets to low-dose ristocetin. In cases of type 2B or platelet-type VWD (PT-VWD), the platelet membrane is "overloaded" with high-affinity mutant VWF, resulting in abnormal platelet agglutination with low ristocetin concentrations. In some cases of type 2B VWD, all variables except low-dose RIPA may be normal. RIPA at normal ristocetin concentrations should be normal in type 1 VWD unless VWF levels are below 10 to 20 IU/dL.

Binding of Factor VIII by von Willebrand Factor

The VWF:FVIIIB ELISA determines the ability of VWF to bind FVIII and is useful for the diagnosis of type 2 N VWD. There are no standard units for the output of this test.

Collagen Binding Assay

The VWF:CB ELISA determines the ability of VWF to bind collagen and is dependent on HMW VWF multimers. Consequently, the test helps to identify functional VWF discordance (i.e., to distinguish between types 1 and 2 VWD).[101] Reduced collagen binding reflects the loss of HMW multimers, or can reflect a specific collagen-binding deficiency (type 2 M VWD). The normal range is approximately 50 to 200 IU/dL.

Von Willebrand Factor Propeptide/Antigen Ratio

An increased ratio of steady-state plasma VWFpp to VWF:Ag identifies patients with variants that cause accelerated VWF clearance.[65] A ratio >2.2 is considered indicative of an accelerated VWF clearance phenotype.

Desmopressin Responsiveness

DDAVP administration releases VWF stores from endothelial cells. The pattern of DDAVP response in VWD subtypes (Table 133.3) may help to assign VWD subtype. In addition, a decrease in the duration of the DDAVP response may indicate increased VWF clearance.

Genotyping

The identification of a variant is not necessary for the diagnosis of VWD. However, genotyping should be considered when specialized testing with the VWF:FVIIIB assay is unavailable and type 2 N VWD is suspected. Genotyping is also useful to discriminate between type 2B VWD and PT-VWD, and for prenatal assessment and alloantibody risk assessment in type 3 VWD. In mild-type 1 VWD, the likelihood of finding a pathological variant is low because variants are not localized to a particular domain or exon and the results are of little clinical utility.

Differential Diagnosis

Hemophilia A

Both type 2 N VWD and mild hemophilia A (caused by variants in *F8*) result in reduced levels of FVIII:C (approximately 5 to 40 IU/dL) with normal or borderline low levels of VWF. Although the VWF:FVIIIB test distinguishes between the two disorders, the test is not widely available and the results may be equivocal.

In families with reduced FVIII:C, an X-linked pattern of inheritance helps identify those with mild hemophilia A. When family history is uninformative and VWF levels and function are normal, it may be preferable to perform sequence analysis of the *F8* gene before the *VWF* gene, even in symptomatic females who are simplex cases (i.e., a single occurrence in a family), because *F8* variant and skewed X-chromosome inactivation (lyonization) are often responsible for symptoms. *F8* variants may be detected in more than 50% of cases referred for "possible 2 N VWD or hemophilia A." When *F8* variants are absent, or if the VWF level and function are also abnormal, *VWF* can be analyzed.

Platelet-Type von Willebrand Disease

PT-VWD, also called *pseudo VWD*, mimics type 2B VWD but is caused by variation in platelet *GPIBA*. The disorders can be distinguished by mixing patient platelets or plasma with control plasma or platelets and using aggregometry or flow cytometry to identify the defective component.[103] However, these assays are technically challenging. In the absence of variants in exon 28 of *VWF*, variants in exon 2 of *GPIBA* are identified in approximately 10% of persons misdiagnosed with type 2B VWD. To date, a number of missense variants have been identified including those more commonly affecting GPIbα AA p.Gly249 and p.Met255, as well as a 27 bp in-frame deletion c.1345_1371del27 (p.Pro449_Ser457del). Misdiagnosis of PT-VWD may result in ineffective treatment of patients. VWF concentrate is needed to correct the reduced VWF level, but platelet transfusion may also be required if there is significant thrombocytopenia. The half-life of replaced VWF is reduced in PT-VWD because of binding to abnormal GPIbα. Consequently, VWF concentrate must be administered more frequently.

Acquired von Willebrand Syndrome

This mild to moderate bleeding disorder is a result of an acquired deficiency or dysfunction of VWF. Exclusion of a lifelong personal and family history of bleeding is an important aspect of the diagnosis. Although acquired von Willebrand syndrome (AVWS) was thought to be uncommon, cohort studies suggest that the prevalence may be significantly underestimated.[104] When selected patient populations were screened, approximately 10% of patients with hematologic disorders, approximately 79% with aortic stenosis, and up to 100% with left ventricular assist devices were diagnosed with AVWS. The median age of diagnosis is 62 years, but the disorder may occur in any age group (range 2 to 96 years). AVWS has diverse pathology and may result from autoantibodies that impair VWF function or increase its clearance, adsorption of HMW VWF multimers to malignant cells or platelets, proteolytic cleavage of VWF after shear stress-induced unfolding, or decreased VWF synthesis. Diseases that have been implicated include (1) lymphoproliferative disorders and plasma cell dyscrasias, including monoclonal gammopathy of unknown significance, multiple myeloma, and Waldenström macroglobulinemia; (2) autoimmune disorders, including systemic lupus erythematosus, scleroderma, and antiphospholipid antibody syndrome; (3) aortic stenosis and ventricular septal defects, which can trigger shear-induced conformational changes that increase VWF proteolysis; (4) thrombocytosis, including myeloproliferative neoplasms that lead to a type 2 phenotype; (5) Wilms tumor or lymphoproliferative disorders that can be associated with increased VWF clearance by aberrant binding to tumor cells; (6) decreased VWF synthesis, for instance, with hypothyroidism, and drugs including valproic acid, ciprofloxacin, griseofulvin, and hydroxyethyl starch. The treatment goals can be divided into two categories: (1) treatment or prevention of bleeding and (2) induction of long-term remission.[105] The agents used for prevention and treatment of bleeding in AVWS overlap with those used in VWD and include DDAVP or VWF-containing concentrates, which can transiently increase VWF levels. Other options include recombinant factor VIIa (rFVIIa), antifibrinolytic agents, intravenous immunoglobulin, or plasmapheresis for AVWS associated with monoclonal gammopathies. Often a combination of agents is required to affect hemostasis. Maneuvers to induce long-term remission will depend on the underlying pathogenic etiology of AVWS. Whenever possible, treatment of the underlying disorder should be considered and may result in remission of the AVWS.

Management of von Willebrand Disease

The approach to the management of VWD is summarized in Fig. 133.6.

Evaluations Following Initial Diagnosis

To establish the extent of disease in an individual diagnosed with VWD, the following evaluations are recommended: (1) a personal and family history of bleeding to help predict severity and tailor treatment (use of a standardized BAT can be helpful); (2) a joint and muscle evaluation for those with type 3 VWD (musculoskeletal bleeding is rare in types 1 and 2 VWD); (3) screening for hepatitis B and C, as well as HIV if the diagnosis is type 3 VWD, or if the individual received blood products or plasma-derived clotting factor concentrates before 1985 (this screening should be followed by vaccinations for hepatitis A and B); (4) determination of serum iron and ferritin (to assess iron stores), because many individuals with VWD are iron deficient, particularly women with heavy menstrual bleeding; and (5) gynecologic evaluation for women with heavy menstrual bleeding. Individuals with VWD benefit from referral to a comprehensive bleeding disorders program for education, treatment, and genetic counseling.

TABLE 133.3 **Desmopressin Responsiveness in the Various Subtypes of von Willebrand Disease**

VWD Type	VWF:RCo	VWF:Ag	RCo/Ag	FVIII:C	VWF:CB	VWF:CB/VWF:Ag
1	Increase	Increase	Remains >0.7	Increase	Increase	Remains >0.7
2 A	No/little change	Increase	Remains <0.7	Increase	No/little change	Remains <0.7
2 M (GPIB binding dysfunction)	No/little change	Increase	Remains <0.7	Increase	Increase	Remains >0.7
3	No/little change	No/little change		No/little change	No/little change	

Ag, Antigen; *CB*, collagen binding assays; *FVIII:C*, factor VIII level; *GPIB*, glycoprotein 1B; *RCo*, ristocetin cofactor; *VWD*, von Willebrand disease; *VWF*, von Willebrand factor.
Modified from Favaloro EJ. Rethinking the diagnosis of von Willebrand disease. *Thromb Res.* 2011;127 Suppl:S17–21.[102]

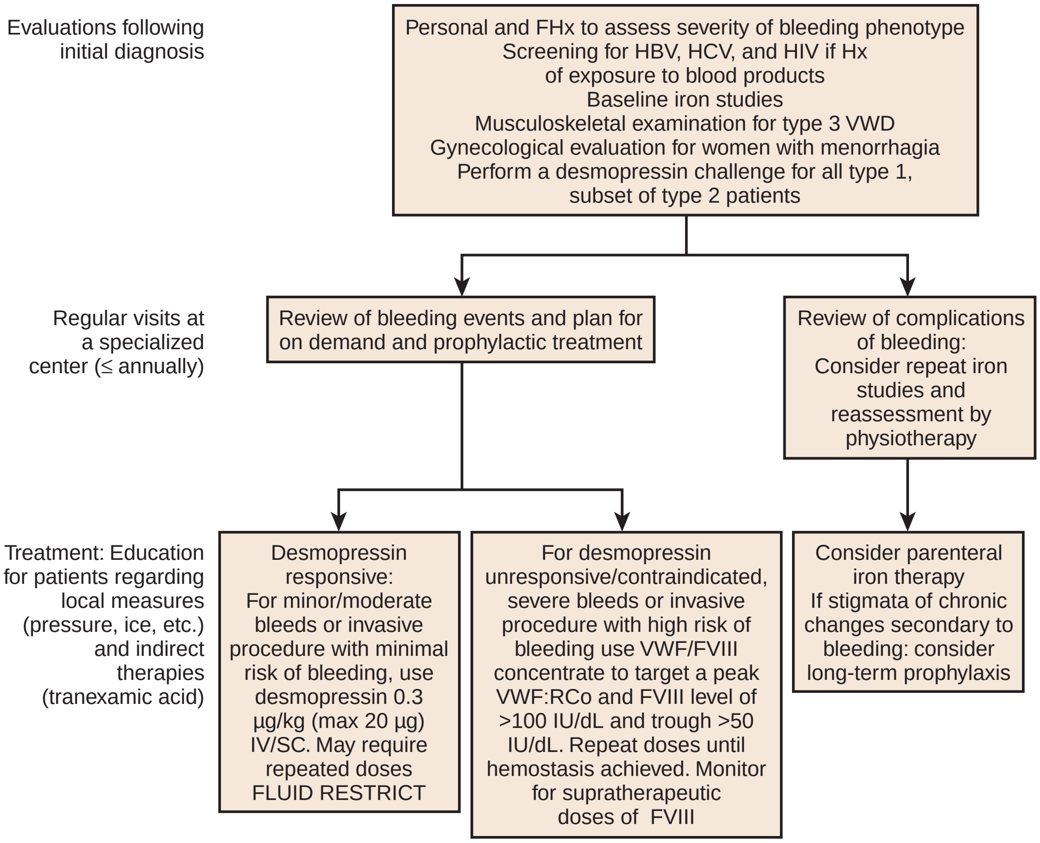

Evaluations following initial diagnosis
Personal and FHx to assess severity of bleeding phenotype
Screening for HBV, HCV, and HIV if Hx of exposure to blood products
Baseline iron studies
Musculoskeletal examination for type 3 VWD
Gynecological evaluation for women with menorrhagia
Perform a desmopressin challenge for all type 1, subset of type 2 patients
Regular visits at a specialized center (≤ annually)
Review of bleeding events and plan for on demand and prophylactic treatment
Review of complications of bleeding: Consider repeat iron studies and reassessment by physiotherapy
Treatment: Education for patients regarding local measures (pressure, ice, etc.) and indirect therapies (tranexamic acid)
Desmopressin responsive: For minor/moderate bleeds or invasive procedure with minimal risk of bleeding, use desmopressin 0.3 µg/kg (max 20 µg) IV/SC. May require repeated doses FLUID RESTRICT
For desmopressin unresponsive/contraindicated, severe bleeds or invasive procedure with high risk of bleeding use VWF/FVIII concentrate to target a peak VWF:RCo and FVIII level of >100 IU/dL and trough >50 IU/dL. Repeat doses until hemostasis achieved. Monitor for supratherapeutic doses of FVIII
Consider parenteral iron therapy
If stigmata of chronic changes secondary to bleeding: consider long-term prophylaxis

Figure 133.6 APPROACH TO THE MANAGEMENT OF VON WILLEBRAND DISEASE (VWD). *FHx*, Family history; *FVIII*, factor VIII; *HBV*, hepatitis B virus; *HCV*, hepatitis C virus; *HIV*, human immunodeficiency virus; *Hx*, history; *IV*, intravenous; *RCo*, ristocetin cofactor; *SC*, subcutaneous; *VWF*, von Willebrand factor.

Treatment of von Willebrand Disease

The management of VWD can be divided into three main categories: (1) localized measures to stop or minimize bleeding; (2) pharmacologic agents that provide indirect hemostatic benefit; and (3) treatments that directly increase plasma VWF and FVIII levels.[98]

Localized Measures

The importance of localized measures to control bleeding in VWD, such as the application of direct pressure to a site of bleeding or injury, should not be understated. Biting down on a piece of gauze may halt bleeding from a tooth socket, and application of a compression bandage and cold pack to an injured limb may reduce subsequent hematoma formation. With epistaxis, patients may benefit from a stepwise action plan that escalates from pressure to packing after a certain time period. In selected cases, nasal cautery may be required for prolonged or excessive epistaxis.

Several topical hemostatic agents that are predominately used to achieve surgical hemostasis may have a limited role in the treatment of VWD and bleeding; these include gelatin foam/matrix, topical thrombin, and fibrin sealants.

Indirect Therapies

Fibrinolytic inhibitors (e.g., tranexamic acid), which inhibit the conversion of plasminogen to plasmin, can be used either as sole therapy, or as adjuncts to DDAVP or VWF/FVIII concentrates, and may be particularly useful to control mucosal bleeding in the oral cavity or gastrointestinal (GI) or genitourinary tracts.[98] The most common adverse events to tranexamic acid are GI side-effects and headache. Tranexamic acid is contraindicated in disseminated intravascular coagulation and bleeding from the upper urinary tract, where it can lead to obstruction by clots. Hormonal treatments (i.e., the combined oral contraceptive pill) are effective for the treatment of menorrhagia. Nonmedical treatments, such as levonorgestrel-releasing intrauterine systems or endometrial ablation, may be useful in select patients with VWD.

Desmopressin

Most individuals with type 1 VWD and some with type 2 VWD respond to intranasal, intravenous, or subcutaneous treatment with DDAVP, which promotes release of stored VWF and raises levels 3- to 10-fold.[18] Peak effects are achieved 30 and 90 minutes after intravenous and intranasal delivery, respectively. The usual parenteral dose is 0.3 µg/kg infused intravenously in approximately 50 mL of normal saline over approximately 30 minutes. The dose of the highly concentrated intranasal preparation is 150 µg for children under 50 kg and 300 µg for larger children and adults. It is important to note that highly concentrated products (e.g., Stimate) deliver 150 µg per spray, a much higher concentration than that used to treat enuresis.

After VWD diagnosis, a DDAVP challenge is advisable to assess VWF response. VWF and FVIII levels should be determined before, and at several points, after DDAVP administration (e.g., at baseline and at 1- and 4-hours post-administration). A three-fold increase in VWF and FVIII levels to at least 30 IU/dL (30%) is usually considered adequate for situations such as dental procedures, minor surgery, or the treatment of epistaxis or menorrhagia.

DDAVP is safe and generally well tolerated. Common side effects include facial flushing and headache. Tachycardia, lightheadedness, and mild hypotension can occur. The most serious side effects are severe hyponatremia and seizures. Reduction of fluid intake for 24 hours after DDAVP administration is an important precaution to prevent water intoxication. Serum sodium levels should be monitored in patients receiving repeated doses of DDAVP. In addition, DDAVP should be used with caution in those younger than 2 years of age because of a higher risk for hyponatremia.[98] There are case reports of DDAVP precipitating coronary artery vasospasm and acute myocardial infarction.[106,107] Therefore, DDAVP should be used with caution in patients with a history of coronary artery disease or in the older adult. In persons who are intolerant to DDAVP or have a poor VWF response, clotting factor concentrate is required.

An important limitation in the use of DDAVP is the development of tachyphylaxis with repeated administration. When given repeatedly at intervals of less than 24 hours, the magnitude of the VWF and FVIII increments can fall to approximately 70% of those obtained with the initial dose. For practical purposes, a single dose of DDAVP before dental extractions or minor procedures is usually sufficient. Although repeated doses can be given at 12 or 24 hours, the potential for tachyphylaxis must be considered. Additionally, in situations where repeat dosing is considered, more prolonged fluid restriction is required.

Although most type 1 VWD patients respond adequately to DDAVP, type 3 VWD patients typically do not respond to this drug, and the response in type 2 VWD patients is variable.[108] Type 2 A patients often exhibit an adequate response and may benefit from a DDAVP trial. Type 2 M patients typically do not respond well to DDAVP. DDAVP is generally contraindicated in type 2B VWD because of the transient thrombocytopenia that accompanies the release of mutant VWF. In type 2 N VWD, DDAVP produces a two- to ninefold increase in FVIII, but the increase persists for approximately 3 hours. Therefore, DDAVP should be reserved for situations in which a transient rise in FVIII is sufficient.

Von Willebrand Factor/Factor VIII Concentrates

VWF/FVIII concentrates are required for patients who do not have an adequate response, experience significant side effects, or have contraindications to DDAVP. Because of tachyphylaxis and the risk of hyponatremia with DDAVP, patients with severe bleeding or those requiring major or repeated surgery often need VWF replacement therapy. Purified, viral-inactivated, plasma-derived VWF/FVIII is the product most frequently used (e.g., Humate-P, Wilate). The quantity of ristocetin cofactor activity (VWF:RCo) relative to FVIII:C varies by product; Humate-P contains 2.4 VWF:RCo units for each 1 FVIII:C unit, whereas Wilate contains a 1:1 ratio. Both products contain a full spectrum of VWF multimers, including HMW multimers, and closely resemble normal plasma. Highly purified FVIII concentrates (monoclonal antibody purified and recombinant) should not be used to treat VWD because they lack VWF.

Dosing recommendations are provided either in VWF:RCo (North America) or FVIII:C (Europe) units and are weight-based; repeat infusions can be given every 8 to 24 hours, depending on the clinical situation. The goal is to maintain VWF:RCo and FVIII:C at more than 100 IU/dL at peak and at more than 50 IU/dL at trough until hemostasis is achieved. With VWF/FVIII concentrates, the FVIII:C response is higher and more sustained than predicted from the dose because of the stabilizing effect of exogenous VWF on endogenous FVIII. Details regarding dosing can be found in the product inserts. VWF:RCo and FVIII:C levels should be measured in patients receiving repeat infusions, not only to ensure adequate hemostasis, but also to monitor for supraphysiological levels of FVIII because thromboembolic events have been associated with high FVIII levels. The overall incidence of thrombotic events is low, and most cases occur in surgical patients with other risk factors. Therefore, mechanical and anticoagulant thromboprophylaxis should be considered on a case-by-case basis. Adverse reactions to VWF/FVIII concentrates are rare but include allergic and anaphylactic symptoms, such as urticaria, chest tightness, rash, pruritus, and edema.

VWF/FVIII concentrates are effective in over 97% of events.[109] In the rare event that infusion of a VWF/FVIII concentrate is ineffective at stopping bleeding transfusion of platelet concentrates may be beneficial, presumably because they facilitate the delivery of small amounts of platelet VWF to the site of vascular injury.

Recently, a new recombinant VWF (rVWF) concentrate was approved in Europe and North America for use in adults diagnosed with VWD.[109] In contrast to traditional plasma-derived products, the rVWF preparation is FVIII-free, and thus, may be independently administered if baseline FVIII:C is adequate (>40%). Whether administered alone or co-administered with recombinant FVIII, rVWF is cleared with a significantly longer half-life than its plasma-derived counterpart. UL-VWF multimers which are lacking in plasma-derived products due to the proteolytic activity of ADAMTS13, are present in the recombinant product. These UL-VWF multimers are susceptible to endogenous ADAMTS13 and are rapidly proteolyzed into LMW forms. While phase 3 studies have demonstrated the hemostatic efficacy and safety of rVWF in the context of on-demand treatment and perioperative management,[110–112] ongoing studies are evaluating long-term use of rVWF for prophylaxis as well as its use in pediatric patients. Undoubtedly, rVWF represents an important advance in the treatment options for VWD.

Prophylaxis

Short-term prophylaxis with a combination of an antifibrinolytic, DDAVP, and/or VWF/FVIII concentrates, in anticipation of a defined bleeding challenge, such as surgery, is the standard of care in the treatment of VWD.[98] The role of long-term continuous prophylaxis, defined as primary if initiated before long-term sequelae have developed (e.g., joint damage), or secondary if initiated after the development of chronic changes, is less established in VWD than it is in severe hemophilia. Individuals with severe cases of VWD, particularly type 3 VWD and certain patients with severe type 1 or type 2 VWD, may experience recurrent joint bleeds, as well as severe and frequently recurrent nasal/oral, GI, or menstrual bleeding. The resultant anemia, hospitalizations, and absences from school or work may have a significant impact on quality of life. Several cohort studies, including the VWD International Prophylaxis (VIP) study, and case studies suggest that both primary and secondary long-term prophylaxis improve quality of life, alleviate anemia, reduce hospitalizations, and prevent chronic joint disease.[113] Complications are unusual aside from rare cases of VWF inhibitor formation (approximately 3% of type 3 VWD patients). There still, however, remains no international consensus regarding the specific indications, schedules, and dosing of prophylactic regimens.

Pediatric Issues

Prenatal diagnosis for pregnancies at increased risk (generally only for type 3 VWD) is possible by analysis of DNA extracted from fetal cells obtained by chorionic villus sampling at 11 to 13 weeks of gestation or amniocentesis at 15 to 18 weeks of gestation. The disease-causing allele(s) of an affected family member must be identified before prenatal testing. Preimplantation genetic diagnosis may be available for families in which the disease-causing variant(s) have been identified.

The diagnosis and care of infants and children with VWD requires special consideration. An accurate assessment of hemorrhagic symptoms is a key component in the diagnosis of VWD, but it often presents a significant challenge in the pediatric population. Because young children may have been exposed to few hemostatic challenges and may be prepubertal, the bleeding history may be unimpressive. The effect this negative bleeding history may have on perceived risk for bleeding is highlighted in two of the previously published bleeding scores, which assign a negative value to a lack of bleeding symptoms, and are based on the accumulation of bleeding symptoms or complications. Finally, the initial diagnostic assessment of an infant is complicated by the fact that VWF levels are higher in the neonatal period. Consequently, phenotypic testing of VWD should be delayed until later in childhood (see Fig. 133.6).

DDAVP should be avoided in children younger than the age of two because of the potential difficulty in restricting fluids. Infant males should be circumcised only after consultation with a pediatric hemostasis specialist. Typically, only patients with type 3 VWD experience spontaneous musculoskeletal bleeding, such as that seen in patients with severe hemophilia, and should be considered for long-term prophylaxis with VWF/FVIII concentrates, as discussed in the preceding section (see box on Assessment of a Neonate).[114]

ASSESSMENT OF A NEONATE

A baby boy was born to a mother with known type 2B VWD. On the day of birth, investigations demonstrated the following: VWF:Ag 78 IU/dL, VWF:RCo 61 IU/dL, and FVIII:C 69 IU/dL (unclear whether these were done on the mother or the baby). The mother was informed that the results were all within the normal range and that her newborn infant was not affected by type 2B VWD. When he was approximately 18 months of age, the mother noted excessive mucocutaneous bleeding. Blood work at that time demonstrated: VWF:Ag 50 IU/dL, VWF:RCo 20 IU/dL, and FVIII:C 51 IU/dL. Multimer analysis revealed a lack of high-molecular-weight multimers. Finally, genotyping revealed that the boy was heterozygous for the same variant, p.Arg1306Trp, that his mother carried.

This case illustrates several important points. Neonatal levels of VWF are increased over baseline; if investigations are performed, they should be interpreted using appropriate reference ranges. Alternatively, if a phenotypic diagnosis is being sought, it is preferable to postpone investigations, decreasing the likelihood of pretest analytical variables, such as difficulty in collecting an appropriate blood sample from a neonate, and the difficulties with interpretation of the results. Finally, in cases in which the *VWF* variant is known, genotyping is diagnostic and also the most straightforward.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Abshire TC, Federici AB, Alvárez MT, et al. Prophylaxis in severe forms of von Willebrand's disease: results from the von Willebrand disease prophylaxis network (VWD PN). *Haemophilia*. 2013;19(1):76–81.

Bowen DJ. An influence of ABO blood group on the rate of proteolysis of von Willebrand factor by ADAMTS13. *J Thromb Haemost*. 2003;1(1):33–40.

Bowman M, Tuttle A, Notley C, et al. The genetics of Canadian type 3 von Willebrand disease: further evidence for co-dominant inheritance of mutant alleles. *J Thromb Haemost*. 2013;11(3):512–520.

Canis K, Mckinnon TAJ, Nowak A, et al. Mapping the N-glycome of human von Willebrand factor. *Biochem J*. 2012;447(2):217–228.

Casari C, Lenting PJ, Wohner N, et al. Clearance of von Willebrand factor. *J Thromb Haemost*. 2013;11(SUPPL.1):202–211.

Castaman G, Lethagen S, Federici AB, et al. Response to desmopressin is influenced by the genotype and phenotype in type 1 von Willebrand disease (VWD): results from the European Study MCMDM-1VWD. *Blood*. 2008;111(7):3531–3539.

Castaman G, Tosetto A, Rodeghiero F. Pregnancy and delivery in women with von Willebrand's disease and different von Willebrand factor mutations. *Haematologica*. 2010;95(6):963–969.

Cumming A, Grundy P, Keeney S, et al. An investigation of the von Willebrand factor genotype in UK patients diagnosed to have type 1 von Willebrand disease. *Thromb Haemost*. 2006;96(5):630–641.

Eikenboom J, Van Marion V, Putter H, et al. Linkage analysis in families diagnosed with type 1 von Willebrand disease in the European study, molecular and clinical markers for the diagnosis and management of type 1 VWD. *J Thromb Haemost*. 2006;4(4):774–782.

Federici AB. The use of desmopressin in von Willebrand disease: the experience of the first 30 years (1977–2007). *Haemophilia*. 2008;14(suppl 1):5–14.

Federici AB, Bucciarelli P, Castaman G, et al. The bleeding score predicts clinical outcomes and replacement therapy in adults with von Willebrand disease. *Blood*. 2014;123(26):4037–4044.

Federici AB, Rand JH, Bucciarelli P, et al. Acquired von Willebrand syndrome: data from an international registry. *Thromb Haemost*. 2000;84(2):345–349.

Gallinaro L, Cattini MG, Sztukowska M, et al. A shorter von Willebrand factor survival in O blood group subjects explains how ABO determinants influence plasma von Willebrand factor. *Blood*. 2008;111(7):3540–3545.

Giannini S, Cecchetti L, Mezzasoma AM, Gresele P. Diagnosis of platelet-type von Willebrand disease by flow cytometry. *Haematologica*. 2010;95(6):1021–1024.

Goodeve AC. The genetic basis of von Willebrand disease. *Blood Rev*. 2010;24(3):123–134.

Haberichter SL, Balistreri M, Christopherson P, et al. Assay of the von Willebrand factor (VWF) propeptide to identify patients with type 1 von Willebrand disease with decreased VWF survival. *Blood*. 2006;108(10):3344–3351.

Haberichter SL, Castaman G, Budde U, et al. Identification of type 1 von Willebrand disease patients with reduced von Willebrand factor survival by assay of the VWF propeptide in the European study: molecular and clinical markers for the diagnosis and management of type 1 VWD (MCMDM-1VWD). *Blood*. 2008;111(10):4979–4985.

Halimeh S, Krümpel A, Rott H, et al. Long-term secondary prophylaxis in children, adolescents and young adults with von Willebrand disease. Results of a cohort study. *Thromb Haemost*. 2011;105(4):597–604.

James AH, Jamison MG. Bleeding events and other complications during pregnancy and childbirth in women with von Willebrand disease. *J Thromb Haemost*. 2007;5(6):1165–1169.

James PD, Notley C, Hegadorn C, et al. The mutational spectrum of type 1 von Willebrand disease: results from a Canadian cohort study. *Blood*. 2007;109(1):145–154.

Lavin M, Aguila S, Schneppenheim S, et al. Novel insights into the clinical phenotype and pathophysiology underlying low VWF levels. *Blood*. 2017;130(21):2344–2353.

McGrath RT, Van den Biggelaar M, Byrne B, et al. Altered glycosylation of platelet-derived von Willebrand factor confers resistance to ADAMTS13 proteolysis. *Blood*. 2013;122(25):4107–4110.

Nichols WL, Hultin MB, James AH, et al. Von Willebrand disease (VWD): evidence-based diagnosis and management guidelines, the National Heart, Lung, and Blood Institute (NHLBI) Expert Panel report (USA). *Haemophilia*. 2008;14(2):171–232.

Peyvandi F, Kouides P, Turecek PL, et al. Evolution of replacement therapy for von Willebrand disease: from plasma fraction to recombinant von Willebrand factor. *Blood Rev*. 2019;38:100572.

Rodeghiero F, Tosetto A, Abshire T, et al. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. *J Thromb Haemost*. 2010;8(9):2063–2065.

Sadler JE, Budde U, Eikenboom JCJ, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand factor. *J Thromb Haemost*. 2006;4(10):2103–2114.

Sanders YV, Giezenaar MA, Laros-van Gorkom BAP, et al. Von Willebrand disease and aging: an evolving phenotype. *J Thromb Haemost*. 2014;12(7):1066–1075.

Smith NL, Chen M-H, Dehghan A, et al. Novel associations of multiple genetic loci with plasma levels of factor VII, factor VIII, and von Willebrand factor: the CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium. *Circulation*. 2010;121(12):1382–1392.

Springer TA. Biology and physics of von Willebrand factor concatamers. *J Thromb Haemost*. 2011:130–143.

Tiede A, Rand JH, Budde U, Ganser A, Federici AB. How I treat the acquired von Willebrand syndrome. *Blood*. 2011;117(25):6777–6785.

REFERENCES

1. von Willebrand EA. Uber hereditare pseudohamophilie [On hereditary pseudohemophilia]. *Acta Med Scand*. 1931;76:521–549.
2. Nilsson IM, Blomback M, Jorpes E, Blomback B, Johansson SA. Von Willebrand's disease and its correction with human plasma fraction 1–0. *Acta Med Scand*. 1957;159(3):179–188.
3. Zimmerman TS, Ratnoff OD, Powell AE. Immunologic differentiation of classic hemophilia (factor 8 deficiency) and von Willebrand's disease, with observations on combined deficiencies of antihemophilic factor and proaccelerin (factor V) and on an acquired circulating anticoagulant against antihemophilic factor. *J Clin Invest*. 1971;50(1):244–254.
4. Ginsburg D, Handin RI, Bonthron DT, et al. Human von Willebrand factor (vWF): isolation of complementary DNA (cDNA) clones and chromosomal localization. *Science*. 1985;228(4706):1401–1406.
5. Lynch DC, Zimmerman TS, Collins CJ, et al. Molecular cloning of cDNA for human von Willebrand factor: authentication by a new method. *Cell*. 1985;41(1):49–56.
6. Sadler JE, Shelton-Inloes BB, Sorace JM, et al. Cloning and characterization of two cDNAs coding for human von Willebrand factor. *Proc Natl Acad Sci U S A*. 1985;82(19):6394–6398.
7. Verweij CL, de Vries CJ, Distel B, et al. Construction of cDNA coding for human von Willebrand factor using antibody probes for colony-screening and mapping of the chromosomal gene. *Nucleic Acids Res*. 1985;13(13):4699–4717.
8. Lankhof H, van Hoeij M, Schiphorst ME, et al. A3 domain is essential for interaction of von Willebrand factor with collagen type III. *Thromb Haemost*. 1996;75(6):950–958.
9. Schneider SW, Nuschele S, Wixforth A, et al. Shear-induced unfolding triggers adhesion of von Willebrand factor fibers. *Proc Natl Acad Sci. U S A*. 2007;104(19):7899–7903.
10. Ikeda Y, Handa M, Kawano K, et al. The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. *J Clin Invest*. 1991;87(4):1234–1240.
11. Nieswandt B, Brakebusch C, Bergmeier W, et al. Glycoprotein VI but not alpha2beta1integrin is essential for platelet interaction with collagen. *EMBO J*. 2001;20(9):2120–2130.
12. Beacham DA, Wise RJ, Turci SM, Handin RI. Selective inactivation of the Arg-Gly-Asp-Ser (RGDS) binding site in von Willebrand factor by site-directed mutagenesis. *J Biol Chem*. 1992;267(5):3409–3415.
13. O'Donnell J, Boulton FE, Manning RA, Laffan MA. Genotype at the secretor blood group locus is a determinant of plasma von Willebrand factor level. *Br J Haematol*. 2002;116(2):350–356.
14. Conlan MG, Folsom AR, Finch A, et al. Associations of factor VIII and von Willebrand factor with age, race, sex, and risk factors for atherosclerosis. The Atherosclerosis Risk in Communities (ARIC) Study. *Thromb Haemost*. 1993;70(3):380–385.
15. Sanders YV, Giezenaar MA. Laros-van Gorkom BAP, et al. Von Willebrand disease and aging: an evolving phenotype. *J Thromb Haemost*. 2014;12(7):1066–1075.
16. Liu L, Wang X, Lin Z, Wu H. Elevated plasma levels of VWF:Ag in hyperthyroidism are mediated through beta-adrenergic receptors. *Endocr Res*. 1993;19(2–3):123–133.
17. Kadir RA, Economides DL, Sabin CA, Owens D, Lee CA. Variations in coagulation factors in women: effects of age, ethnicity, menstrual cycle and combined oral contraceptive. *Thromb Haemost*. 1999;82(5):1456–1461.
18. Federici AB. The use of desmopressin in von Willebrand disease: the experience of the first 30 years (1977–2007). *Haemophilia*. 2008;14(Suppl 1):5–14.
19. Small M, Tweddel AC, Rankin AC, et al. Blood coagulation and platelet function following maximal exercise: effects of beta-adrenoceptor blockade. *Pathophysiol Haemost Thromb*. 1984;14(3):262–268.
20. Casonato A, Pontara E, Vertolli UP, et al. Plasma and platelet von Willebrand factor abnormalities in patients with uremia: lack of correlation with uremic bleeding. *Clin Appl Thromb*. 2001;7(2):81–86.
21. Meigs JB, O'Donnell CJ, Tofler GH, et al. Hemostatic markers of endothelial dysfunction and risk of incident type 2 diabetes: the Framingham Offspring Study. *Diabetes*. 2006;55(2):530–537.
22. Ferro D, Quintarelli C, Lattuada A, et al. High plasma levels of von Willebrand factor as a marker of endothelial perturbation in cirrhosis: relationship to endotoxemia. *Hepatology*. 1996;23(6):1377–1383.
23. Wang W-S, Lin J-K, Lin T-C, et al. Plasma von Willebrand factor level as a prognostic indicator of patients with metastatic colorectal carcinoma. *World J Gastroenterol*. 2005;11(14):2166–2170.
24. Kreuz W, Linde R, Funk M, et al. Valproate therapy induces von Willebrand disease type I. *Epilepsia*. 1992;33(1):178–184.
25. Castaman G, Tosetto A, Rodeghiero F. Pregnancy and delivery in women with von Willebrand's disease and different von Willebrand factor mutations. *Haematologica*. 2010;95(6):963–969.
26. Mancuso DJ, Tuley EA, Westfield LA, et al. Human von Willebrand factor gene and pseudogene: structural analysis and differentiation by polymerase chain reaction. *Biochemistry*. 1991;30(1):253–269.
27. Wang QY, Song J, Gibbs RA, et al. Characterizing polymorphisms and allelic diversity of von Willebrand factor gene in the 1000 genomes. *J Thromb Haemost*. 2013;11(2):261–269.
28. Jahroudi N, Lynch DC. Endothelial-cell-specific regulation of von Willebrand factor gene expression. *Mol Cell Biol*. 1994;14(2):999–1008.
29. Luken BM, Winn LYN, Emsley J, Lane DA, Crawley JTB. The importance of vicinal cysteines, C1669 and C1670, for von Willebrand factor A2 domain function. *Blood*. 2010;115(23):4910–4913.
30. Perkins SJ, Smith KF, Williams SC, et al. The secondary structure of the von Willebrand factor type A domain in factor B of human complement by Fourier transform infrared spectroscopy. Its occurrence in collagen types VI, VII, XII and XIV, the integrins and other proteins by averaged structure predictions. *J Mol Biol*. 1994;238(1):104–119.
31. Springer TA. Biology and physics of von Willebrand factor concatamers. *J Thromb Haemost*. 2011:130–143.
32. Katsumi A, Tuley EA, Bodó I, Sadler JE. Localization of disulfide bonds in the cystine knot domain of human von Willebrand factor. *J Biol Chem*. 2000;275(3):25585–25594.
33. Mayadas TN, Wagner DD. Vicinal cysteines in the prosequence play a role in von Willebrand factor multimer assembly. *Proc Natl Acad Sci U S A*. 1992;89(8):3531–3535.
34. Zhou Y-F, Eng ET, Nishida N, et al. A pH-regulated dimeric bouquet in the structure of von Willebrand factor. *EMBO J*. 2011;30(19):4098–4111.
35. Dong X, Leksa NC, Chhabra ES, et al. The von Willebrand factor D′D3 assembly and structural principles for factor VIII binding and concatemer biogenesis. *Blood*. 2019;133(14):1523–1533.
36. Cruz MA, Diacovo TG, Emsley J, Liddington R, Handin RI. Mapping the glycoprotein Ib-binding site in the von Willebrand factor A1 domain. *J. Biol. Chem*. 2000;275(25):19098–19105.
37. Fujimura Y, Titani K, Holland LZ, et al. A heparin-binding domain of human von Willebrand factor. Characterization and localization to a tryptic fragment extending from amino acid residue Val-449 to Lys-728. *J Biol Chem*. 1987;262:1734–1739.
38. Dent JA, Berkowitz SD, Ware J, Kasper CK, Ruggeri ZM. Identification of a cleavage site directing the immunochemical detection of molecular abnormalities in type IIA von Willebrand factor. *Proc Natl Acad Sci U S A*. 1990;87(16):6306–6310.
39. Journet AM, Saffaripour S, Wagner DD. Requirement for both D domains of the propolypeptide in von Willebrand factor multimerization and storage. *Thromb Haemost*. 1993;70(6):1053–1057.
40. Purvis AR, Sadler JE. A covalent oxidoreductase intermediate in propeptide-dependent von Willebrand factor multimerization. *J Biol Chem*. 2004;279(48):49982–49988.
41. Purvis AR, Gross J, Dang LT, et al. Two Cys residues essential for von Willebrand factor multimer assembly in the Golgi. *Proc Natl Acad Sci U S A*. 2007;104(10):15647–15652.
42. Millar CM, Riddell AF, Brown SA, et al. Survival of von Willebrand factor released following DDAVP in a type 1 von Willebrand disease cohort: influence of glycosylation, proteolysis and gene mutations. *Thromb Haemost*. 2008;99(5):916–924.
43. Wagner DD, Mayadas T, Marder VJ. Initial glycosylation and acidic pH in the Golgi apparatus are required for multimerization of von Willebrand factor. *J Cell Biol*. 1986;102(4):1320–1324.
44. Canis K, Mckinnon TAJ, Nowak A, et al. Mapping the N-glycome of human von Willebrand factor. *Biochem J*. 2012;447(2):217–228.
45. McGrath RT, Van Den Biggelaar M, Byrne B, et al. Altered glycosylation of platelet-derived von Willebrand factor confers resistance to ADAMTS13 proteolysis. *Blood*. 2013;122(25):4107–4110.
46. McCormack JJ, Lopes da Silva M, Ferraro F, Patella F, Cutler DF. Weibel-Palade bodies at a glance. *J Cell Sci*. 2017;130(21):3611–3617.
47. Weibel ER, Palada GE. New cytoplasmic components in arterial endothelia. *J Cell Biol*. 1964;23(1):101–112.
48. Giblin JP, Hewlett LJ, Hannah MJ. Basal secretion of von Willebrand factor from human endothelial cells. *Blood*. 2008;112(4):957–964.
49. Majerus EM, Zheng X, Tuley EA, Sadler JE. Cleavage of the ADAMTS13 propeptide is not required for protease activity. *J Biol Chem*. 2003;278:46643–46648.
50. Dong JF. Cleavage of ultra-large von Willebrand factor by ADAMTS-13 under flow conditions. *J Thromb Haemost*. 2005;3(8):1710–1716.
51. Bowen DJ. An influence of ABO blood group on the rate of proteolysis of von Willebrand factor by ADAMTS13. *J Thromb Haemost*. 2003;1(1):33–40.

52. O'Donnell JS, McKinnon TAJ, Crawley JTB, Lane DA, Laffan MA. Bombay phenotype is associated with reduced plasma-VWF levels and an increased susceptibility to ADAMTS13 proteolysis. *Blood*. 2005;106(6):1988–1991.
53. McGrath RT, McKinnon TAJ, Byrne B, et al. Expression of terminal α2-6-linked sialic acid on von Willebrand factor specifically enhances proteolysis by ADAMTS13. *Blood*. 2010;115(13):2666–2673.
54. Bowen DJ, Collins PW. An amino acid polymorphism in von Willebrand factor correlates with increased susceptibility to proteolysis by ADAMTS13. *Blood*. 2004;103(3):941–947.
55. Casari C, Lenting PJ, Wohner N, Christophe OD, Denis CV. Clearance of von Willebrand factor. *J Thromb Haemost*. 2013;11(Suppl.1):202–211.
56. Grewal PK, Uchiyama S, Ditto D, et al. The Ashwell receptor mitigates the lethal coagulopathy of sepsis. *Nat Med*. 2008;14(6):648–655.
57. Rastegarlari G, Pegon JN, Casari C, et al. Macrophage LRP1 contributes to the clearance of von Willebrand factor. *Blood*. 2012;119(9):2126–2134.
58. Wohner N, Muczynski V, Mohamadi A, et al. Macrophage scavenger receptor SR-AI contributes to the clearance of von Willebrand factor. *Haematologica*. 2018;103(4):728–737.
59. Ward SE, O'Sullivan JM, Drakeford C, et al. A novel role for the macrophage galactose-type lectin receptor in mediating von Willebrand factor clearance. *Blood*. 2018;131(8):911–916.
60. Rydz N, Swystun LL, Notley C, et al. The C-type lectin receptor CLEC4M binds, internalizes, and clears von Willebrand factor and contributes to the variation in plasma von Willebrand factor levels. *Blood*. 2013;121(26):5228–5237.
61. Swystun LL, Lai JD, Notley C, et al. The endothelial cell receptor stabilin-2 regulates VWF-FVIII complex half-life and immunogenicity. *J Clin Investig*. 2018;128(9):4057–4073.
62. Swystun LL, Ogiwara K, Lai JD, et al. The scavenger receptor SCARA5 is an endocytic receptor for von Willebrand factor expressed by littoral cells in the human spleen. *J Thromb Haemost*. 2019;17(8):1384–1396.
63. Gallinaro L, Cattini MG, Sztukowska M, et al. A shorter von Willebrand factor survival in O blood group subjects explains how ABO determinants influence plasma von Willebrand factor. *Blood*. 2008;111(7):3540–3545.
64. Borchiellini A, Fijnvandraat K, ten Cate JW, et al. Quantitative analysis of von Willebrand factor propeptide release in vivo: effect of experimental endotoxemia and administration of 1-deamino-8-ᴅ-arginine vasopressin in humans. *Blood*. 1996;88(8):2951–2958.
65. Haberichter SL, Balistreri M, Christopherson P, et al. Assay of the von Willebrand factor (VWF) propeptide to identify patients with type 1 von Willebrand disease with decreased VWF survival. *Blood*. 2006;108(10):3344–3351.
66. Goodeve A, Eikenboom J, Castaman G, et al. Phenotype and genotype of a cohort of families historically diagnosed with type 1 von Willebrand disease in the European study, Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMDM-1VWD). *Blood*. 2007;109(1):112–121.
67. James PD, Notley C, Hegadorn C, et al. The mutational spectrum of type 1 von Willebrand disease: Results from a Canadian cohort study. *Blood*. 2007;109(1):145–154.
68. Starke RD, Ferraro F, Paschalaki KE, et al. Endothelial von Willebrand factor regulates angiogenesis. *Blood*. 2011;117(3):1071–1080.
69. O'Sullivan JM, Preston RJS, Robson T, O'Donnell JS. Emerging roles for von Willebrand factor in cancer cell biology. *Semin Thromb Hemost*. 2018;44(2):159–166.
70. Brill A, Fuchs TA, Chauhan AK, et al. Von Willebrand factor-mediated platelet adhesion is critical for deep vein thrombosis in mouse models. *Blood*. 2011;117(4):1400–1407.
71. Brill A, Fuchs TA, Savchenko AS, et al. Neutrophil extracellular traps promote deep vein thrombosis in mice. *J Thromb Haemost*. 2012;10(1):136–144.
72. Smith NL, Chen M-H, Dehghan A, et al. Novel associations of multiple genetic loci with plasma levels of factor VII, factor VIII, and von Willebrand factor: the CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium. *Circulation*. 2010;121(12):1382–1392.
73. Sadler JE, Budde U, Eikenboom JCJ, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand factor. *J Thromb Haemost*. 2006;4(10):2103–2114.
74. Rodeghiero F, Castaman G, Dini E. Epidemiological investigation of the prevalence of von Willebrand's disease. *Blood*. 1987;69(2):454–459.
75. Werner EJ, Broxson EH, Tucker EL, et al. Prevalence of von Willebrand disease in children: a multiethnic study. *J Pediatr*. 1993;123(6):893–898.
76. Bowman M, Hopman WM, Rapson D, et al. A prospective evaluation of the prevalence of symptomatic von Willebrand disease (VWD) in a pediatric primary care population. *Pediatr Blood Cancer*. 2010;55(1):171–173.
77. Bloom AL. Von Willebrand factor: clinical features of inherited and acquired disorders. *Mayo Clin Proc*. 1991;66(7):743–751.
78. Goodeve AC. The genetic basis of von Willebrand disease. *Blood Rev*. 2010;24(3):123–134.
79. Eikenboom J, Van Marion V, Putter H, et al. Linkage analysis in families diagnosed with type 1 von Willebrand disease in the European study, molecular and clinical markers for the diagnosis and management of type 1 VWD. *J Thromb Haemost*. 2006;4(4):774–782.
80. Cumming A, Grundy P, Keeney S, et al. An investigation of the von Willebrand factor genotype in UK patients diagnosed to have type 1 von Willebrand disease. *Thromb. Haemost*. 2006;96(5):630–641.
81. Sutherland MS, Cumming AM, Bowman M, et al. A novel deletion mutation is recurrent in von Willebrand disease types 1 and 3. *Blood*. 2009;114:1091–1098.
82. Haberichter SL, Castaman G, Budde U, et al. Identification of type 1 von Willebrand disease patients with reduced von Willebrand factor survival by assay of the VWF propeptide in the European study: molecular and clinical markers for the diagnosis and management of type 1 VWD (MCMDM-1VWD). *Blood*. 2008;111(10):4979–4985.
83. Casonato A, Pontara E, Sartorello F, et al. Reduced von Willebrand factor survival in type Vicenza von Willebrand disease. *Blood*. 2002;99(1):180–184.
84. Sanders YV, van der Bom JG, Isaacs A, et al. CLEC4M and STXBP5 gene variations contribute to von Willebrand factor level variation in von Willebrand disease. *J Thromb Haemost*. 2015;13(6):956–966.
85. Jacobi PM, Gill JC, Flood VH, et al. Intersection of mechanisms of type 2A VWD through defects in VWF multimerization, secretion, ADAMTS-13 susceptibility, and regulated storage. *Blood*. 2012;119(19):4543–4553.
86. Holmberg L, Dent JA, Schneppenheim R, et al. Von Willebrand factor mutation enhancing interaction with platelets in patients with normal multimeric structure. *J Clin Invest*. 1993;91(5):2169–2177.
87. Flood VH, Gill JC, Christopherson PA, et al. Critical von Willebrand factor A1 domain residues influence type VI collagen binding. *J Thromb Haemost*. 2012;10(7):1417–1424.
88. Riddell AF, Gomez K, Millar CM, et al. Characterization of W1745C and S1783A: 2 novel mutations causing defective collagen binding in the A3 domain of von Willebrand factor. *Blood*. 2009;114(16):3489–3496.
89. Nishino M, Girma JP, Rothschild C, Fressinaud E, Meyer D. New variant of von Willebrand disease with defective binding to factor VIII. *Blood*. 1989;74(5):1591–1599.
90. Bowman M, Tuttle A, Notley C, et al. The genetics of Canadian type 3 von Willebrand disease: further evidence for co-dominant inheritance of mutant alleles. *J Thromb Haemost*. 2013;11(3):512–520.
91. Sanders YV, Groeneveld D, Meijer K, et al. von Willebrand factor propeptide and the phenotypic classification of von Willebrand disease. *Blood*. 2015;125(19):3006–3013.
92. Mannucci PM, Federici AB. Antibodies to von Willebrand factor in von Willebrand disease. *Adv Exp Med Biol*. 1995;386:87–92.
93. Lavin M, Aguila S, Schneppenheim S, et al. Novel insights into the clinical phenotype and pathophysiology underlying low VWF levels. *Blood*. 2017;130(21):2344–2353.
94. James AH, Jamison MG. Bleeding events and other complications during pregnancy and childbirth in women with von Willebrand disease. *J Thromb Haemost*. 2007;5(6):1165–1169.
95. Rodeghiero F, Tosetto A, Abshire T, et al. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. *J Thromb Haemost*. 2010;8(9):2063–2065.
96. Federici AB, Bucciarelli P, Castaman G, et al. The bleeding score predicts clinical outcomes and replacement therapy in adults with von Willebrand disease. *Blood*. 2014;123(26):4037–4044.
97. Eikenboom JC. Congenital von Willebrand disease type 3: clinical manifestations, pathophysiology and molecular biology. *Best Pract Res. Haematol*. 2001;14(2):365–379.
98. Nichols WL, Hultin MB, James AH, et al. Von Willebrand disease (VWD): evidence-based diagnosis and management guidelines, the National Heart, Lung, and Blood Institute (NHLBI) Expert Panel report (USA). *Haemophilia*. 2008;14(2):171–232.
99. Bodó I, Eikenboom J, Montgomery R, et al. Platelet-dependent von Willebrand factor activity. Nomenclature and methodology: communication from the SSC of the ISTH. *J Thromb Haemost*. 2015;13(7):1345–1350.
100. Budde U, Schneppenheim R, Eikenboom J, et al. Detailed von Willebrand factor multimer analysis in patients with von Willebrand disease in the European study, molecular and clinical markers for the diagnosis and management of type 1 von Willebrand disease (MCMDM-1VWD). *J Thromb Haemost*. 2008;6(5):762–771.
101. Flood VH, Gill JC, Friedman KD, et al. Collagen binding provides a sensitive screen for variant von Willebrand disease. *Clin Chem*. 2013;59(4):684–691.

102. Favaloro EJ. Rethinking the diagnosis of von Willebrand disease. *Thromb Res*. 2011;127(Suppl:):S17–S21.
103. Giannini S, Cecchetti L, Mezzasoma AM, Gresele P. Diagnosis of platelet-type von Willebrand disease by flow cytometry. *Haematologica*. 2010;95(6):1021–1024.
104. Federici AB, Rand JH, Bucciarelli P, et al. Acquired von Willebrand syndrome: data from an international registry. *Thromb Haemost*. 2000;84(2):345–349.
105. Tiede A, Rand JH, Budde U, Ganser A, Federici AB. How I treat the acquired von Willebrand syndrome. *Blood*. 2011;117(25):6777–6785.
106. Adachi Y, Sakakura K, Akashi N, et al. Coronary spastic angina induced after oral desmopressin (DDAVP) administration. *Intern Med*. 2016;55(24):3603–3606.
107. Pape E, Béné J, Buchdahl A-L, et al. Desmopressin-related myocardial infarction in a patient with Wegener's granulomatosis: a case report and review of the literature. *J Mal Vasc*. 2013;38(1):43–46.
108. Castaman G, Lethagen S, Federici AB, et al. Response to desmopressin is influenced by the genotype and phenotype in type 1 von Willebrand disease (VWD): results from the European Study MCMDM-1VWD. *Blood*. 2008;111(7):3531–3539.
109. Peyvandi F, Kouides P, Turecek PL, et al. Evolution of replacement therapy for von Willebrand disease: from plasma fraction to recombinant von Willebrand factor. *Blood Rev*. 2019;38:100572.
110. Gill JC, Castaman G, Windyga J, et al. Hemostatic efficacy, safety, and pharmacokinetics of a recombinant von Willebrand factor in severe von Willebrand disease. *Blood*. 2015;123(17):2038–2046.
111. Peyvandi F, Mamaev A, Wang JD, et al. Phase 3 study of recombinant von Willebrand factor in patients with severe von Willebrand disease who are undergoing elective surgery. *J Thromb Haemost*. 2019;17(1):52–62.
112. Mannucci PM, Kempton C, Millar C, et al. Pharmacokinetics and safety of a novel recombinant human von Willebrand factor manufactured with a plasma-free method: a prospective clinical trial. *Blood*. 2013;122(5):648–657.
113. Abshire TC, Federici AB, Alvárez MT, et al. Prophylaxis in severe forms of von Willebrand's disease: results from the von Willebrand disease prophylaxis network (VWD PN). *Haemophilia*. 2013;19(1):76–81.
114. Halimeh S, Krümpel A, Rott H, et al. Long-term secondary prophylaxis in children, adolescents and young adults with von Willebrand disease: results of a cohort study. *Thromb Haemost*. 2011;105(4):597–604.

CHAPTER 134

HEMOPHILIA A AND B

Manuel Carcao, Keith Gomez, Davide Matino, and Glenn F. Pierce

Hemophilia is the most common, severe inherited bleeding disorder recognized in humans. The X-linked mode of inheritance has been appreciated since biblical times, and the previous occurrence of the disease in the European royal family has added further interest in this condition.

Hemophilia is subdivided into hemophilia A and B; the former being a deficiency or absence of factor (F) VIII while the latter is a deficiency or absence of FIX. Both are single-gene disorders. Ultimately both disorders result in a similar bleeding pattern whose severity is generally proportional to the amount of active factor VIII (FVIII) or FIX that the person produces. As both types of hemophilia result in deficiency of a coagulation protein, for the past more than half-century treatment has involved replacement of the missing protein (either FVIII or FIX) either episodically (on demand) to treat bleeds or prophylactically to prevent them. In the last 5 to 10 years, the management of both hemophilia A and B has transformed with the availability of coagulation protein replacement products with longer half-lives and with the introduction of emicizumab, a FVIII mimetic.

Additionally, clinical trials on novel therapies that aim at rebalancing coagulation in patients with hemophilia by targeting natural anticoagulants are ongoing and progress in gene therapy over the last decade is poised to provide a cure for hemophilic patients with imminent licensing of gene therapy products.

Therefore, the management of hemophilia is rapidly evolving.

EPIDEMIOLOGY

The prevalence of hemophilia is expected to be the same in all populations globally, apart from rare clusters of mild-moderate disease in areas where founder variants have been propagated (e.g., mild hemophilia A in Twillingate, Newfoundland; mild/moderate hemophilia B in Old Order Amish, United States).[1,2] In many parts of the developing world, the true prevalence of the condition is unknown because of inadequate diagnostic facilities, but from experience in the developed world, it can be assumed that the incidence of hemophilia A and hemophilia B in males at birth is approximately one in 4000 (slightly higher than the previous estimate of one in 5000) and one in 20,000, respectively.[3,4]

Due to the X-linked recessive mode of inheritance, most affected subjects are male. However, for every male with hemophilia there are likely two carrier females (including, in most cases, the mother of the affected male as well as potentially some sisters of the male and his daughters). In addition to mothers and sisters of boys with hemophilia there are likely many other women who are carriers but are never diagnosed because they are not related to any male with hemophilia. Hence the hemophilia carrier state is almost certainly much more common than hemophilia in males. Most carriers without affected sons/brothers will likely never be investigated, as most (particularly women with non-O blood type) will not experience significant bleeding because their FVIII or FIX levels are in the normal range or, if their FVIII levels are low, will be misdiagnosed as having von Willebrand disease (VWD) rather than being carriers of hemophilia.[5] For further discussion on hemophilia carrier detection see the box on Hemophilia Carrier Detection and Prenatal Diagnosis. Even though most carrier females have clotting factor levels in a normal range, some hemophilia carrier women have levels low enough to cause mild, moderate, or even severe disease due to lyonization (X-linked inactivation). A new nomenclature reclassifies these women as having mild, moderate, or severe hemophilia in a similar manner to hemophilic males.[6]

HEMOPHILIA CARRIER DETECTION AND PRENATAL DIAGNOSIS

The X-linked recessive nature of hemophilia results in most affected subjects being males. Females, however, carry and transmit the hemophilic trait and can sometimes also have clinical manifestations of the disease. Female carriers of hemophilia rarely have factor levels low enough to cause moderate or severe hemophilia; however, this can occur under the genetic circumstances listed in Table 134.1.

There are four ways in which hemophilia carriers can be identified. The first three apply to women with a high a priori likelihood of being a carrier based on a positive family history of hemophilia while the last applies to those without a family history of hemophilia.

Determining Carrier Status in Women With a Family History of Hemophilia

First, considering the X-linked transmission of the disease, pedigree analysis will determine the carrier status of some women. Thus, all daughters of hemophilic fathers and all women with an affected son and another first-degree male relative (another son, brother, uncle, cousin) are obligate carriers.

The second mode of carrier detection involves laboratory testing. In a minority (≈20%–30%) of carriers, the PTT may be prolonged and plasma levels of FVIII, or FIX may be reduced. These findings, together with a family history of hemophilia, are predictive of a carrier state. The etiology of low factor levels in carrier females is multifactorial and includes a woman's blood type and VWF levels as well as the ratio of inactivation of the hemophilic and normal X chromosomes; a random process that occurs early in embryonic development. If there is markedly skewed inactivation of the normal X chromosome, the plasma level of FVIII or FIX may be correspondingly reduced, and the carrier female may have a bleeding disorder. It is important to determine the FVIII or FIX levels in all carrier females to inform the risk of bleeding with surgical procedures or childbirth. However, carriers rarely have a prolonged PTT or low plasma levels of FVIII or FIX. Therefore, a normal factor level does not rule out the carrier state. With hemophilia A, a low FVIII to VWF ratio is a good predictor of the carrier state.[5]

The third and most definitive approach to carrier diagnosis is to use molecular genetics to identify the causative FVIII or FIX mutation. The results of such testing can be available within a few days if the information is needed urgently. Genetic carrier determination enhances family counseling for hemophilic kindreds and enables optimal preparation for delivery and future planning.

Determining Carrier Status in Women With no Family History of Hemophilia

Very rarely a hemophilia carrier may be identified in a family in which there is no history of hemophilia. This would likely occur if the woman had abnormal bleeding associated with a low FVIII or FIX level. Although estimates of the percentage of hemophilia carriers with excessive bleeding (most often heavy menstrual bleeding) vary, it is likely in the 20%–30% range. Therefore, most de novo carriers will never be diagnosed or may only be diagnosed if they have affected sons or grandsons with hemophilia. Without a family history of hemophilia, it is difficult to confirm a diagnosis of hemophilia carriership even in a woman with excessive bleeding and a low FVIII or FIX level. Often carriers of hemophilia A are incorrectly labeled as having type 1 VWD particularly if they have an O blood type and, as a result low VWF levels. In this setting, a FVIII level that is much lower than the VWF level may be helpful.

Ideally, carrier detection should be undertaken before the woman contemplates starting a family. In many countries, testing for carrier status of a genetic disease is prohibited before adolescence to ensure that the subject can meaningfully participate in discussions of testing options.

Prenatal diagnosis of hemophilia begins with determination of the fetal sex, which can usually be determined by ultrasound examination. As levels of fetal DNA in maternal blood increase late in pregnancy examination for the presence of Y chromosome sequences in maternal blood enables definitive sex determination. If the fetus is female, no additional studies are required. If the fetus is male, fetal DNA for molecular genetic analysis can be isolated from chorionic villus samples obtained after 10 weeks of gestation or from amniocytes obtained by amniocentesis from 12 to 34 weeks. The risk of miscarriage with either procedure is approximately 1%. If the studies are being used to inform decisions about therapeutic abortion, they should be performed as early as possible. Determination of the hemophilic status of the fetus will also help planning for delivery, although consensus about optimal obstetric management of an affected baby is lacking.

Over the past decade, international studies indicate that the number of reported persons with hemophilia has increased by approximately 2% each year. The likely reasons for this trend include increasing awareness and improved diagnosis of hemophilia, increasing longevity of persons with hemophilia due to improved treatment and decreased mortality from human immunodeficiency virus (HIV) and hepatitis C. This also leads to greater propagation of hemophilia mutations as affected males are more likely to reach adulthood and "pass their genes" to the next generation. Potentially there may be other reasons to explain the increase in hemophilia.

TABLE 134.1 **Genetic Mechanisms Causing Hemophilia in Females**

- *F8* or *F9* mutation homozygosity
- *F8* or *F9* mutation compound heterozygosity
- Extreme skewing of X inactivation process
- X/O karyotype: Turner syndrome
- X/autosome translocation

The clinical signs and symptoms and inheritance patterns for hemophilia A and B are identical, and it was not until the early 1950s that the two forms of hemophilia were differentiated. Subsequently, in the early 1980s, the two genes encoding FVIII, and FIX were cloned, and the specific variants responsible for hemophilia began to be determined.

FACTOR VIII BIOLOGY: GENETICS, STRUCTURE, FUNCTION, AND PATHOPHYSIOLOGY

Factor VIII Gene and FVIII Expression

The FVIII gene (*F8*) encoding the FVIII protein is located at Xq28, the most distal band of the long arm of the X chromosome (Fig. 134.1). It is a large and complex structure, 186 kb in length, consisting of 9 kb of exonic DNA arranged into 26 exons and 177 kb of intronic DNA in 25 introns. Most exons are small, ranging in size from 69 base pairs (exon 5; the smallest) to 3106 base pairs (exon 14; the largest–encoding the central B domain).

Within intron 22 of the *F8* gene there are two additional coding elements, *F8A* and *F8B*. Although its function is unclear, the *F8A* sequence within intron 22 is frequently involved in intrachromosomal recombination that is described later. The *F8B* transcript is expressed in the same orientation as the native *F8* mRNA, and this transcript comprises an initial intron 22 sequence that is spliced onto exons 23 to 26 of *F8*. As with F8A, the F8B transcript is also ubiquitously expressed. Although the function of the protein is unknown because it contains the phospholipid binding region of FVIII it may play a role in membrane binding.

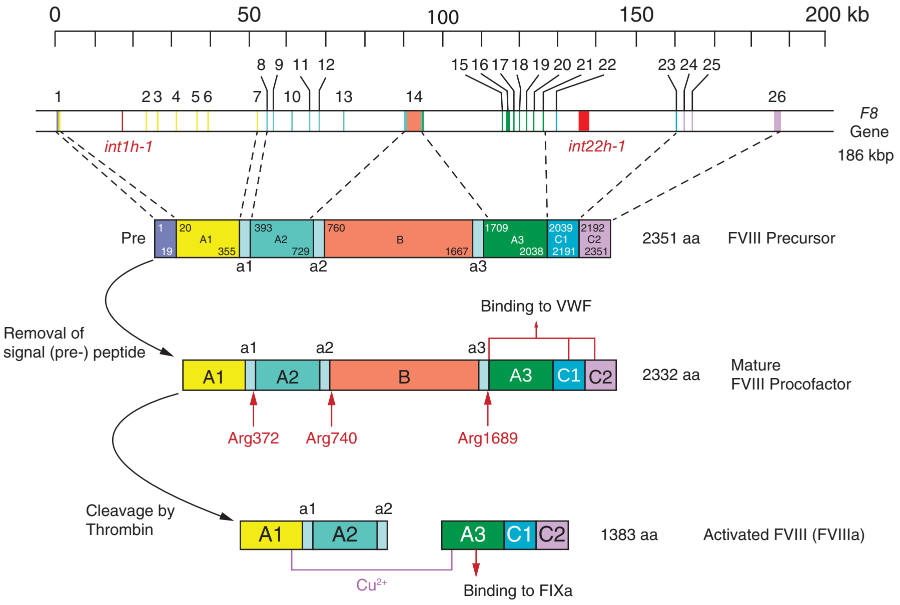


Figure 134.1 DERIVATION OF ACTIVATED FACTOR VIII FROM THE *F8* GENE. Top panel shows the *F8* gene with exons (1–26) colored according to the domains translated from them. Exon numbers are given above the gene. The primary translated product is the factor VIII (FVIII) precursor. Start and end amino acid numbers for each domain are shown. Removal of the signal peptide (Pre) generates the mature FVIII co-factor (2332 aa) that circulates in plasma. Cleavage by thrombin after the arginine residues (Arg372, 740, and 1689) shown in red activates the protein by removing the B and a3 domains.

Factor VIII Expression

Transcription of the *F8* gene is regulated by a promoter that contains binding sites for both tissue-specific and ubiquitous transcription factors. The mRNA transcribed from *F8* is 9 kb in length and contains 7053 nucleotides of coding (i.e., translated) sequence and short 5′ and long 3′ untranslated sequences. Two reports using different conditional knockout approaches in mice have shown that the vascular endothelium is the predominant site of FVIII expression in this animal model.[7,8] There is strong evidence that the liver sinusoidal endothelium is the source of FVIII production.[9] This fits with the recognition that hemophilia can be cured by orthotopic liver transplantation. Other endothelial cell vascular beds, such as those found in lymph nodes, and spleen, may also contribute to FVIII production.[10]

The transcription factors that regulate constitutive production of FVIII are not well defined but may include hepatocyte nuclear factor 1 (HNF1) and members of the CCAAT-enhancer-binding protein (C/EBP) and HNF4 families. FVIII also is an acute phase reactant, and studies have shown that FVIII expression can be induced through nuclear factor kappa-B (NFκB) and C/EBP. Working through these transcription factors, interleukin 6 can elevate FVIII expression as part of an acute systemic inflammatory response. FVIII levels are strongly influenced by circulating levels of its carrier protein, von Willebrand Factor (VWF). Both proteins are lower in blood group O individuals due to higher endogenous clearance of VWF and of the FVIII attached to it.[11] This impacts on the pharmacokinetics of FVIII administration—lasts longer in non-O blood type individuals than in O blood type individuals.

Biosynthesis of Factor VIII

FVIII biosynthesis has been extensively investigated in vitro using cell types that do not normally express this protein, such as Baby Hamster Kidney (BHK), Chinese Hamster Ovarian (CHO), and Human Embryonic Kidney (HEK 293) cells, among others. Little work has been performed on sinusoidal endothelial cells, the predominant cell type responsible for endogenous FVIII synthesis. More recently, transgene-produced FVIII in hepatocytes following gene therapy results in in-vivo expression of subnormal to normal circulating levels in this heterologous cell type.[12] The biosynthesis of transgenic FVIII has also not been well characterized.

After production of the primary polypeptide chain and cleavage of the 19 amino acid signal peptide, the protein undergoes a series of posttranslational modifications, including N- and O-linked glycosylation mainly in the B domain and sulfation of tyrosine residues (see Fig. 134.1). Furthermore, when the nascent protein transits the endoplasmic reticulum (ER), it interacts with ER chaperones, including calreticulin and immunoglobulin binding protein (BiP). Although these interactions limit the transport of misfolded or aggregated forms of the protein in heterologous cells with high levels of FVIII expression, their relevance in native FVIII-producing cells is unknown. Indeed, under conditions of high-level FVIII expression, cells can activate a classical unfolded protein response (UPR) and can succumb to apoptotic cell death. The relatively unique challenge of processing FVIII has implications for gene therapy where high expression of B-domain-deleted FVIII in a small number of heterologous hepatocytes may trigger an UPR.[13]

Efficient transit through the ER–Golgi boundary requires an interaction with glycans within the B domain of the protein. Absence of these glycans or lack of a specific cargo receptor complex that transports proteins (e.g., LMAN also known as ERGIC-53, multiple coagulation factor deficiency 2 [MCFD2]) markedly reduces levels of both secreted FVIII and FV, the latter resulting in the autosomal recessive disorder of combined deficiency of FVIII and FV (Fig. 134.2).[14]

Factor VIII Protein Structure

The DNA sequence of the *F8* gene predicts a translated single chain polypeptide with a molecular weight of 260 kDa consisting of 2351 amino acid residues, including a 19-residue signal peptide. Upon translocation to the ER, the signal peptide is cleaved. The remaining polypeptide is 2332 residues long.

The FVIII protein consists of three types of domains: the three A domains, which have a 35% to 40% amino acid sequence homology to ceruloplasmin and to FV; a central B domain with no known homologues; and two C-terminal domains that are also 35% to 40% homologous to ceruloplasmin and to FV (see Fig. 134.1). There are also three small "a" linker regions (each between 20 and 40 amino acids) consisting of predominantly acidic residues. The sequence of these domains within the protein is NH2-A1-a1-A2-a2-B-a3-A3-C1-C2-COOH. Tyrosine residues in the a2 and a3 domains, when sulfated, contribute to the cofactor function of FVIII and, with residues in the C1 domain, enable its interaction with VWF. Given the structural similarities between FVIII and FV it is not surprising that both function as cofactors for serine protease enzymes in the coagulation cascade: FVIII in the intrinsic tenase complex to generate FXa, and FV in the prothrombinase complex to generate thrombin. Of note, the FVIII B domain is not required for cofactor function; its principal role is to facilitate trafficking and secretion of the nascent polypeptide, and B-domain-deleted FVIII is fully active.

Subsequent proteolysis of the FVIII polypeptide chain in the Golgi generates a heavy chain of A1-A2-B domains with a mass of 90 kDa and a light chain consisting of the A3-C1-C2 domains with a mass of 80 kDa (see Fig. 134.1).

Circulating FVIII exists as a heterodimer of the NH_2-terminus heavy chain and the COOH-terminus light chain. The heavy and light chains are bound to one another at the A1 and A3 domains by noncovalent bonds that are divalent metal ion dependent. The involved ion is likely copper, which has been found in association with the FVIII A3 domain.

Storage, Secretion, Circulation, and Clearance of Factor VIII

After translation and ER/Golgi processing, FVIII is secreted into the circulation, where it forms a tight noncovalent complex with its multimeric partner VWF. Whereas the plasma concentration of FVIII is 100 to 200 ng/mL (≈1 nM), the concentration of VWF is approximately 10 μg/mL (50 nM); thus, the molar ratio of FVIII to VWF in the FVIII-VWF complex is about 1 to 50. The majority of VWF in plasma is synthesized and secreted by vascular endothelial cells. VWF binds strongest to the A3 and C1 regions of FVIII through sequences in the D' region (D'D3) of the mature VWF monomer.[15] The site at which FVIII and VWF first interact has been debated. However, confirmation that FVIII is expressed by endothelial cells suggest that at least some of the circulating FVIII may interact with VWF before secretion and may be co-stored with VWF in Weibel-Palade bodies.

Circulating levels of FVIII vary three-fold in the normal population (~50% to 150%), likely due to a combination of variability in synthesis and probably more importantly, variability in the clearance of FVIII mediated by VWF clearance. This in turn is related to ABO blood type as well as to other mechanisms leading to proteolysis and clearance of circulating FVIII/VWF.[16] In plasma, VWF protects FVIII from proteolysis by lipid-binding proteases including FXa. Without this interaction—for example, in cases of type 3 VWD (in which VWF is absent) or type 2N VWD (in which mutations occur in the FVIII binding region of VWF—D' region)—the plasma half-life of FVIII is markedly reduced and, consequently, plasma levels of FVIII may be very low.

VWF also protects FVIII from intracellular proteolysis by blocking binding of FVIII to cell surface lectin domain and scavenger

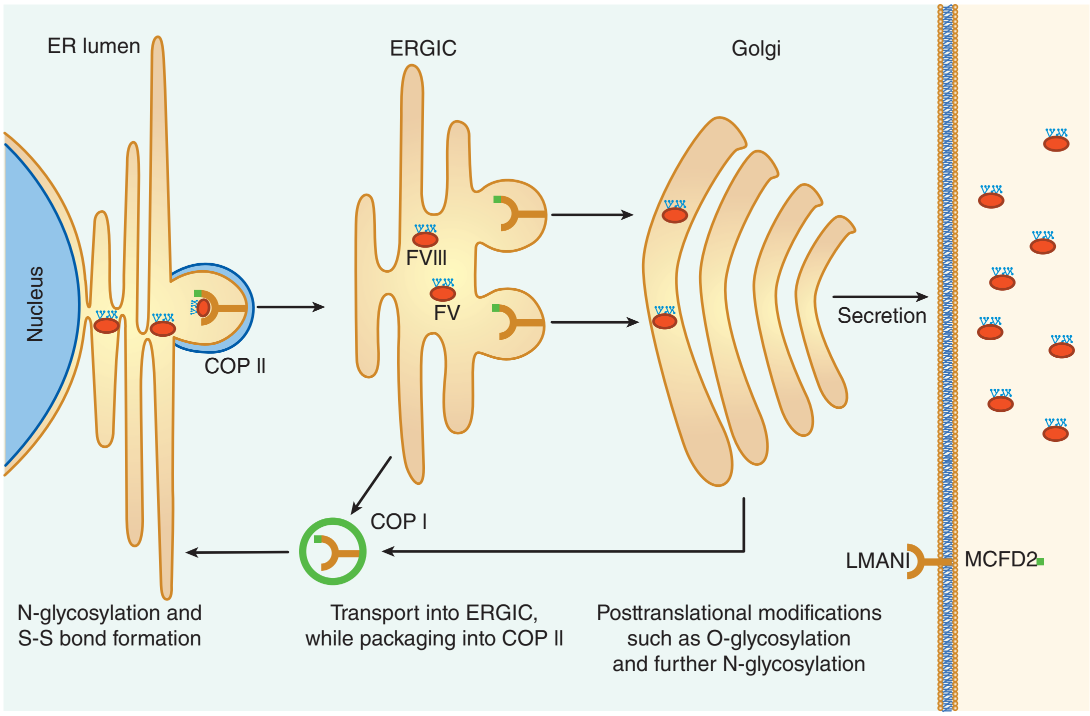


Figure 134.2 COMBINED FV AND FVIII DEFICIENCY. Factor V (FV) and factor VIII (FVIII) (which share substantial homology) are synthesized on the rough endoplasmic reticulum (ER) and translocated into the ER lumen, where both proteins undergo N-linked glycosylation, folding, and quality control. Proteins that pass the quality control are packaged into coat protein complex-II (COPII) vesicles that bud from distinct regions of the ER. After budding, COPII vesicles undergo fusion to form the ER–Golgi intermediate compartment (ERGIC). From there, specific proteins (lectin mannose binding protein type 1 [LMAN1; also called ERGIC-53] and multiple coagulation factor deficiency 2 [MCFD2]) are required for proper secretion of these factors into the blood stream. Homozygous or compound heterozygous mutations lead to the rare autosomal recessive disorder of combined FV and FVIII deficiency. COP1 recycles back to ER different transport factors.

receptors which target it for lysosomal degradation. VWF, with or without FVIII, is degraded via binding to lectin and scavenger receptors on the surfaces of Kupffer cells, hepatocytes, and endothelial cells. As the vast majority of FVIII circulates bound to VWF, FVIII much like VWF is cleared by VWF-specific catabolic pathways.[17] However, VWF-free circulating FVIII, representing 2% to 5% of the total circulating FVIII, has accelerated clearance (6- to 8-fold greater), thus representing a physiological VWF-independent clearance pathway. Thus, most of the clearance and half-life of FVIII is regulated by the clearance of VWF. This places a ceiling on the potential gain in half-life achieved by bioengineering FVIII molecules via Fc fusion or PEGylation technologies. Knowing this, one extended half-life (EHL) FVIII (efanesoctocog alpha; Sanofi) still in development has overcome this ceiling effect on FVIII half-life extension by engineering FVIII to no longer bind to endogenous VWF.[18]

VWF may also protect FVIII from recognition by the immune system in persons with hemophilia A. Endocytic uptake into antigen-presenting cells for presentation to CD4$^+$ T cells occurs largely through the C1–C2 domains, which are blocked when FVIII is bound to VWF.[19] All of this is in keeping with some clinical evidence suggesting a lower rate of neutralizing antibody development (inhibitors) with plasma-derived FVIII containing VWF than with recombinant FVIII.[20]

Activation and Coagulant Function of FVIII

FVIII plays a critical role in the propagation (amplification) phase of coagulation. The physiological activator of FVIII is thrombin, which proteolytically cleaves FVIII at three sites: Arg372 at the NH_2-terminus of the A2 domain, Arg740 at the NH_2-terminus of the B domain, and Arg1689 at the NH_2-terminus of the A3 domain (see Fig. 134.1). These cleavages release FVIII from VWF and result in the formation of a noncovalently associated A1-A2-A3/C1/C2 heterotrimeric activated FVIII (FVIIIa) molecule. FVIII can also be activated by FXa and FIXa, although the physiological contribution of activation by these proteases is less clear.

In its activated form, FVIIIa provides essential cofactor activity in the intrinsic tenase complex where FIXa is the serine protease and FX is the substrate (Fig. 134.3).[21] This reaction takes place on a procoagulant phospholipid surface, which in normal hemostasis is likely the activated platelet. Participation of FVIIIa in the complex enhances the catalytic efficiency of this reaction about 200,000-fold, and thus severe FVIII deficiency profoundly reduces the rate of FXa generation and renders this reaction biologically futile. The cofactor role of FVIIIa is assumed to be based on it serving as a “scaffold” protein that optimally aligns the enzymatic and substrate components of the complex (FIXa-FVIIIa-FX) on the phospholipid surface. The cofactor binds to the phospholipid surface through hydrophobic residues in the FVIII C1 domain. Emicizumab was developed by capitalizing on the scaffolding effect of FVIIIa. Serving as a FVIII mimetic, emicizumab is a bispecific monoclonal antibody that binds to both FIXa and FX and bridges them together thus substituting for the cofactor role of FVIIIa.

FVIIIa is inactivated via two processes (see Chapters 120 and 124). The predominant mechanism is through spontaneous dissociation of the A2 domain. The second is via activated protein C–mediated proteolysis at Arg336 and Arg562 in the FVIIIa heavy chain (see Fig. 134.1).

PATHOPHYSIOLOGY OF HEMOPHILIA A

Hemophilia A is a disorder characterized by congenital deficiency of FVIII. Almost all patients with hemophilia A have *F8* gene mutations. Because the *F8* gene is in the most distal band

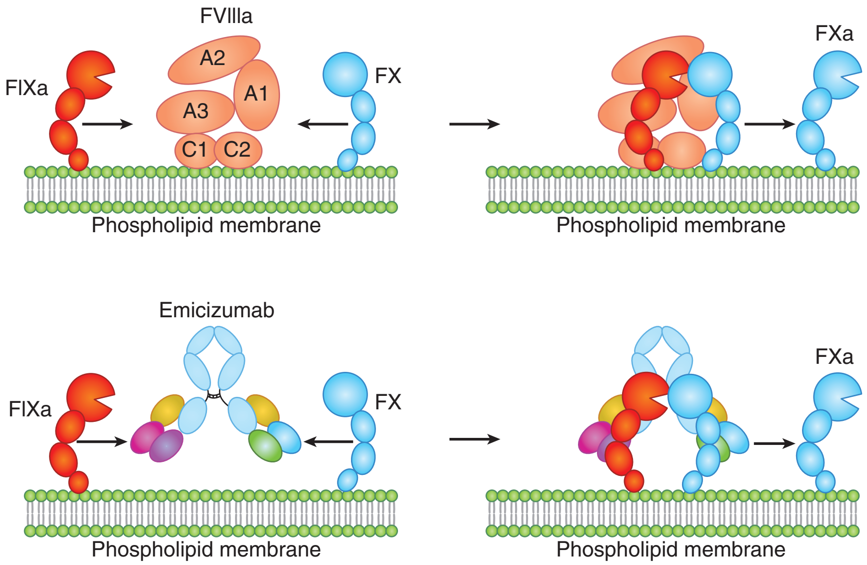


Figure 134.3 MECHANISM OF ACTION OF EMICIZUMAB. Activated factor VIII (FVIIIa) serves as an essential cofactor in the intrinsic tenase complex by binding activated factor IX (FIXa) (the enzyme) and factor X (FX) (the substrate) and promoting their interaction on the surface of activated platelets. FVIIIa binds to the phospholipid surface through residues in the FVIII C1 domain. Like FVIIIa, emicizumab binds to both FIXa and FX and promotes their interaction. Unlike FVIIIa, emicizumab does not bind to the phospholipid membrane.

(Xq28) on the X chromosome, hemophilia A follows an X-linked inheritance pattern. As a result, most affected individuals are male. Severe and moderately severe cases of hemophilia A are unusual in females but can result from the genetic mechanisms listed in Table 134.1.

Approximately 50% of cases of hemophilia A are caused by sporadic mutations and occur without a family history of the disorder. The sporadic mutation most commonly occurs in a female making her a carrier. However, because most carriers do not have excessive bleeding, the "new carrier" is unlikely to ever be diagnosed, or if diagnosed, not until later in life if she has an affected son or grandson.

The molecular genetic basis of hemophilia A has been extensively characterized over the past 35 years. A comprehensive Internet database of hemophilia A mutations is maintained and updated at https://f8-db.eahad.org/.

Approximately 45% of cases of severe hemophilia A are the result of a recurrent mutation, an inversion mutation involving sequences in intron 22 of the gene (Fig. 134.4). The mechanism responsible for this mutation involves an intrachromosomal recombination event in which there is an exchange between the F8A gene in intron 22 and one of two extragenic copies of this sequence that are located 5′ and telomeric to *F8*. The consequence of this event is inversion of the *F8* gene between exons 1 and 22 and although two distinct *F8* transcripts are expressed from separate promoters (exons 1 to 22 and exons 23 to 26), a contiguous FVIII protein sequence is not synthesized. The origin of this recurrent mutation is almost exclusively in the male germline (i.e., in sperm cells) where the single X chromosome has no partner to pair with, thereby potentially facilitating the F8A-mediated intrachromosomal recombination. The origin of this mutation in male germline explains why hemophilia often arises in daughters of older men whose sperm cells carry a greater number of mutations.[22]

A second recurrent inversion mutation involving intron 1 of the *F8* gene is responsible for approximately 2% of severe hemophilia A cases. Other mutations responsible for hemophilia A include more than 3000 DNA variants with an array of missense, nonsense, frameshift, insertion or deletion, duplication, and splicing mutations.[23] In addition, transcriptional mutations have been identified in the F8 promoter region. All regions of the gene are potential mutation targets but certain sequences, such as the CpG dinucleotide in arginine codons, are more prone to mutation because of spontaneous methylation of the 5′ cytosine and spontaneous deamination to thymine. Indeed, the recent identification of mutations deep within introns of the gene, which may alter *F8* mRNA splicing, suggests that almost all hemophilia A mutations are likely to be found within or adjacent to the *F8* gene.

Extensive genotype-phenotype studies in hemophilia A reveal a good correlation between null mutations (mutations that result in no production of factor) and severe disease and between non-null (largely missense) mutations and moderate or mild phenotypes. The phenotype of the disease remains generally consistent with specific mutations. One exception may be mutations within the B domain, which is dispensable for FVIII activity but is important for secretion of the protein after it undergoes post-translational modifications. Proteins synthesized with putative hemophilia causative B domain variants have normal activity, suggesting that many may be polymorphisms.[24]

In addition to its utility for family counseling, the *F8* genotype is also a strong predictor for the development of anti-FVIII antibodies (Table 134.2). Multidomain deletions are associated with the highest risk (≈75%) of inhibitor development, while most missense and splicing mutations are associated with the lowest risk (<10%). The largest group is those with an average risk of 20% to 30%; this includes patients with inversion, nonsense, and insertion/deletion mutations. Even within these categories there is significant variability in the risk of inhibitor development; for example, some missense mutations in the C2 and C3 domains are associated with much higher risk of inhibitor development than missense mutations in other domains.[25]

FACTOR IX BIOLOGY: GENETICS, STRUCTURE, FUNCTION, AND PATHOPHYSIOLOGY

FIX Gene and FIX Expression

The gene that encodes FIX (*F9)* was cloned and characterized by two groups in the early 1980s. The gene is located on the X chromosome at cytogenetic band Xq27 and spans 34 kb of genomic sequence (Fig. 134.5). The eight exons (previously designated as exons A to H

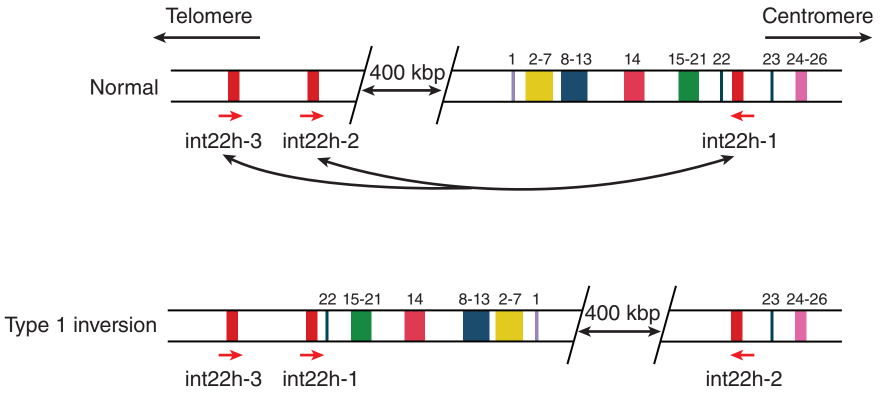


Figure 134.4 INTRON 22 INVERSION RESPONSIBLE FOR APPROXIMATELY 40% OF CASES OF SEVERE HEMOPHILIA A. The top panel shows the normal F8 gene with exons clustered and colored according to the domains they are translated into. Intron 22 contains the sequence highlighted in red, int22h-1, which has 2 homologous copies positioned 400 kbp telomeric to the gene but orientated in the opposite direction. When homologous recombination occurs during meiosis, the int22h-1 sequence can recombine with one of the homologous copies: int22h-2 or int22h-3. Because the sequences are orientated in opposite directions, there is inversion of the intervening sequence such that exons 1–22 of F8 are facing the wrong direction and are separated from exons 23–26 by 400 kbp as shown in the lower panel. This leads to disruption of the gene and abolition of transcription.

TABLE 134.2 **Factor VIII Mutant Genotype and Inhibitor Risk in Previously Untreated Patients With Hemophilia A**

Large insertion/deletions	≈75%
Light chain nonsense mutations	30%–40%
Inversion mutations (I-22 and I-1)	20%–25%
Small insertion/deletions	15%–25%
Small non-A run insertions/deletions	15%–20%
Heavy chain nonsense mutations	10%–20%
Factor VIII missense mutations	<10%
Small A run insertions/deletions	<5%
Splicing mutations	<5%

and now as exons 1 to 8) of the *F9* gene encode an mRNA transcript of 2.8 kb that is exclusively expressed in hepatocytes.

FIX Protein

FIX circulates in plasma at a concentration of approximately 5 μg/mL (90 nM). The mature circulating protein consists of 415 amino acids and has a molecular weight of 57 kDa (see Fig. 134.5). The protein is initially synthesized as a pre-pro-polypeptide with a short signal sequence, which facilitates entry into the ER, and a pro-polypeptide sequence, which interacts with γ-glutamylcarboxylase in the ER. The interaction with this microsomal enzyme catalyzes the post-translational conversion of the 12 amino-terminal glutamic acid residues of mature FIX into γ-carboxyglutamyl residues (the GLA domain). This post-translational modification enables FIX to form calcium-dependent interactions with procoagulant phospholipid membranes (see Chapter 125). A second post-translational modification at residue 64 in the first epidermal growth factor (EGF)–like domain of the mature polypeptide involves β-hydroxylation of asparagine. This modification also facilitates interactions with calcium and phospholipid membranes.

The molecular arrangement of FIX consists of an amino-terminal GLA domain followed by two EGF-like domains, an activation peptide, and the carboxyl-terminus catalytic domain, which is similar to the organization of procoagulant FVII and FX and the anticoagulant protein C (see Chapter 125). The characteristic feature of all serine proteases is the catalytic triad which in FIX is comprised of His267-Asp315-Ser411. These three residues form the active site of the enzyme which is responsible for cleavage of the Arg234-Ile235 scissile bond in FX that is required for its activation.

The FIX zymogen is activated by two processes; proteolytic cleavage mediated by FXIa and by a similar process involving the FVIIa–tissue factor complex. The activation of FIX involves sequential proteolysis of two peptide bonds at Arg191-Ala192 and Arg226-Val227 (see Fig. 134.5). These cleavages release the FIX activation peptide and result in the formation of the disulfide-linked FIXa enzyme with an NH_2-terminus light chain and a COOH-terminus heavy chain. Whereas the light chain binds to procoagulant phospholipid surfaces through a calcium-mediated process involving the GLA domain, the C-terminus catalytic domain cleaves and activates the substrate FX. This reaction involves the so-called intrinsic tenase complex in which FVIIIa participates as an essential cofactor. Absence of FVIIIa in the tenase complex reduces the catalytic efficiency of the reaction by approximately 200,000-fold.

FIXa is inhibited through an interaction with the natural anticoagulant protein antithrombin (AT). The fate of FIXa–AT complexes is incompletely understood, but specific AT–protease receptors on the surface of hepatocytes participate in this process.

PATHOPHYSIOLOGY OF HEMOPHILIA B

To date, all variants responsible for hemophilia B have involved the *F9* gene. Genetic analyses of large numbers of patients with hemophilia B have detected variants in the *F9* gene in more than 98% of cases, and it may well be that the remaining variants are located within intronic sequences that are not readily accessible using standard techniques (Fig. 134.6).[23]

In marked contrast to hemophilia A, in which approximately 50% of variants resulting in severe disease are caused by two recurrent gene inversion events, approximately 75% of variants in hemophilia B are missense substitutions. There are no common recurrent *F9* variants, although mild forms of hemophilia B may show a founder effect in some populations.

Despite the predominance of missense variants, multiple other variants can produce hemophilia B ranging from large gene deletions to a mix of nonsense, insertion or deletion, and splicing changes. The full array of *F9* variants responsible for hemophilia B can be viewed in an internet database at http://f9-db.eahad.org/.

The most biologically remarkable hemophilia B variant was discovered in Leyden and is eponymously named after that city. FIX deficiency with this variant results from disruption of a 40-bp region of the *F9* promoter around the hepatic transcription factor binding sites

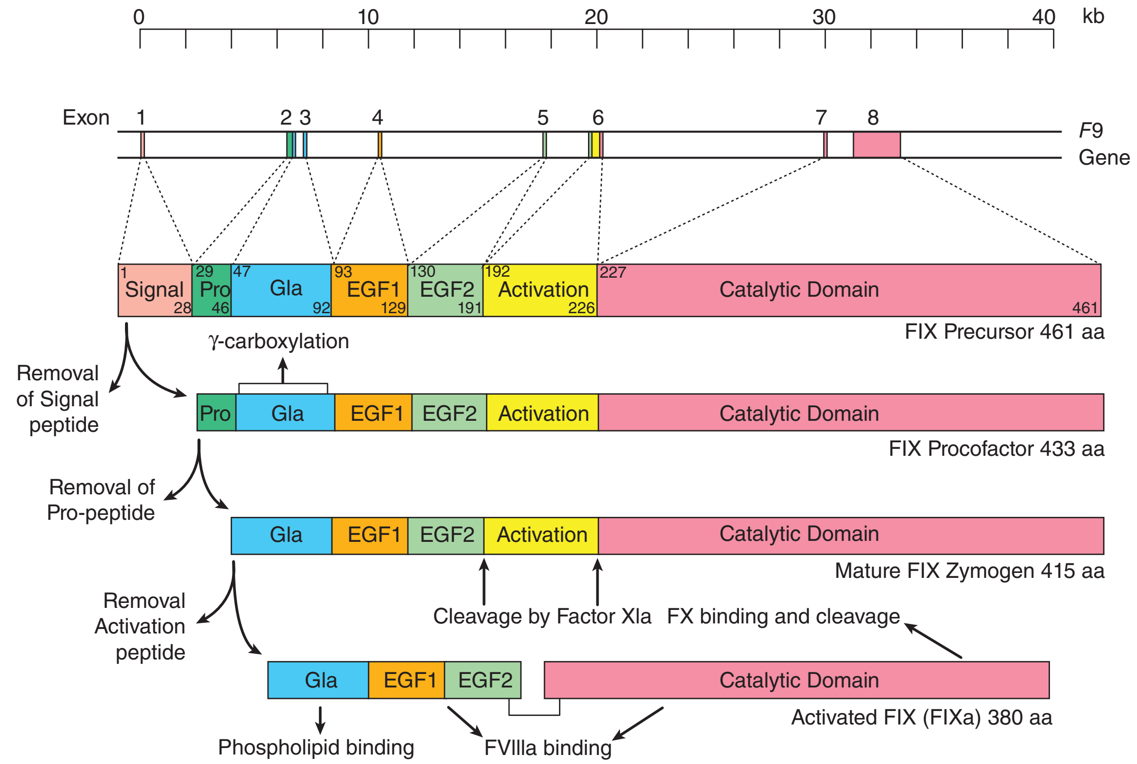

0
10
20
30
40
kb
Exon
1
2 3
4
5
6
7
8
F9
Gene
1
Signal
28
29
Pro
46
47
Gla
92
93
EGF1
129
130
EGF2
191
192
Activation
226
227
Catalytic Domain
461
FIX Precursor 461 aa
γ-carboxylation
Removal of Signal peptide
Pro
Gla
EGF1
EGF2
Activation
Catalytic Domain
FIX Procofactor 433 aa
Removal of Pro-peptide
Gla
EGF1
EGF2
Activation
Catalytic Domain
Mature FIX Zymogen 415 aa
Removal Activation peptide
Cleavage by Factor XIa
FX binding and cleavage
Gla
EGF1
EGF2
Catalytic Domain
Activated FIX (FIXa) 380 aa
Phospholipid binding
FVIIIa binding

Figure 134.5 DERIVATION OF ACTIVATED FIX FROM THE *F9* GENE. The top panel shows the *F9* gene with exons (1–8) colored according to the domains translated from them. Exon numbers are given above the gene. The primary translated product is the FIX precursor. Start and end amino acid numbers for each domain are shown. Removal of the signal peptide generates the FIX procofactor. The propeptide directs γ-carboxylation of glutamic acid residues in the Gla- domain. Removal of the propeptide produces the mature 415 amino acid protein. Cleavage by FXIa after Arg191 and Arg226 removes the activation peptide leaving the active protease. *EGF*, Epidermal growth factor-like domain.

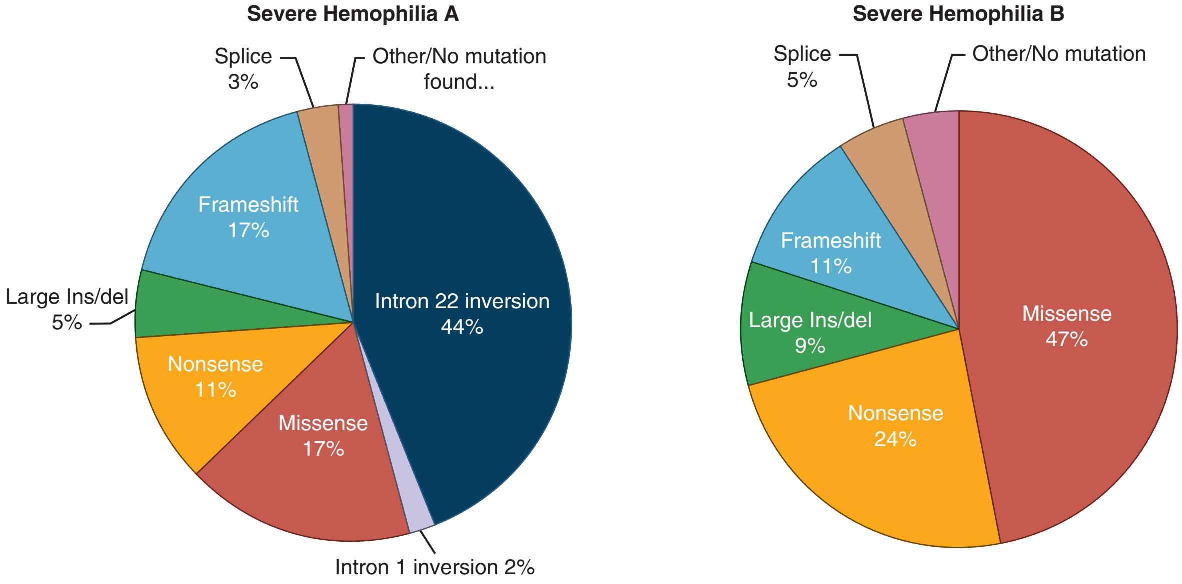


Figure 134.6 FREQUENCIES OF DIFFERENT TYPES OF F8 AND F9 VARIANTS IN SEVERE HEMOPHILIA A AND SEVERE HEMOPHILIA B. For severe hemophilia A (panel A), inversion variants (I-22 and I-1) account for 44% of all cases while missense variants account for about 17%. For severe hemophilia B (panel B), almost 50% of cases are caused by missense variants.

which are responsible for gene expression during early childhood.[26] If the variant spares an androgen responsive element in the promoter, gene expression normalizes with increasing androgen (testosterone) levels in puberty and beyond.[27] In this form of hemophilia B, FIX levels gradually increase from less than 5% at birth to greater than 30% by early adulthood. Because of the low FIX levels early in life, boys with this disorder often have bleeding episodes consistent with moderate hemophilia B. After puberty, FIX levels and bleeding phenotype improve or even resolve to the extent that the label of hemophilia is often removed from these patients.

Since the cloning of the *F9* gene in the 1980s, the association of *F9* gene deletions with FIX antibody formation has been well recognized. While most missense mutations causing hemophilia B are associated with a low risk of inhibitor development, large deletion mutations are associated with a higher risk (30% to 50% risk). In addition to neutralizing FIX enzymatic function, these antibodies can also be associated with anaphylactic or anaphylactoid reactions to FIX infusion.[28] Antibodies usually develop in young children (1 to 2 years old) after 10 to 20 exposures to FIX concentrate. In such patients, FIX concentrate use is generally no longer of benefit and attempts at inducing immunologic tolerance to FIX may be complicated by the development of nephrotic syndrome. Further discussion about inhibitors in hemophilia B is found later.

Hemophilia B is the bleeding disorder which in the past was associated with many of the royal families of Europe. Although the clinical picture in these individuals was consistent with severe hemophilia, the precise diagnosis was not reported until 2009. The "Royal" *F9* variant is a splicing abnormality occurring at the 3′ end of intron 3 that creates a new splice acceptor sequence that results in a novel out-of-frame transcript with 11 new amino acids followed by a premature stop codon.[29] This variant is predicted to result in nonsense-mediated decay of the aberrant FIX mRNA, which would inevitably result in a severe phenotype.

Missense variants elsewhere in the *F9* gene have been informative with regard to our detailed understanding of protein structure and function. Variants at both activation peptide cleavage sites have been described, and as with many genes, recurrent "hotspot" variants have been documented at arginine codons with CpG dinucleotide sequences.

HEMOPHILIA DIAGNOSIS

The initial diagnosis of hemophilia depends on the family context. In families in which hemophilia has previously been identified, family counseling can determine the risk of hemophilia transmission and usually results in a diagnosis being made in utero or early in neonatal life. In contrast, when there is no family history of the disease, the diagnosis will not be made until there are signs of bleeding, and the timing of this depends on the extent of the factor deficiency (i.e., disease severity). With severe deficiency, the diagnosis is usually made in the first 1 to 2 years of life, but with moderate disease, the diagnosis may be made later, and with mild hemophilia, the diagnosis may be delayed until well into adulthood when bleeding occurs after a surgical intervention.

Phenotypic Diagnosis of Hemophilia

The diagnosis of hemophilia involves measurement of FVIII or FIX coagulant levels in platelet-poor plasma using a functional clotting assay. Although most clinical hemostasis laboratories use a one-stage PTT (i.e., clot-based) test to quantify FVIII, some laboratories use either a two-stage or a chromogenic assay that measures the generation of FXa in a purified system (see Chapter 127). In these latter assays, the incubation time is often longer, which may influence test results. For example, some missense mutations causing mild hemophilia A result in the synthesis of an unstable FVIII with enhanced dissociation of the A2 domain from the remainder of the protein (Fig. 134.7). These unstable mutants can occasionally be missed when a one-stage FVIII assay is used but are readily apparent with two-stage or chromogenic assays that use a prolonged incubation time. Consequently, when a diagnosis of mild/moderate hemophilia A is made using a one-stage assay, it is advisable to also perform a chromogenic assay to better determine the extent of the deficiency.

The severity of hemophilia is based on the clotting factor level (severe, <1% [<0.01 IU/mL]; moderate, 1% to 5% [0.01 to 0.05 IU/mL]; and mild, >5% to 40% [>0.05 to 0.40 IU/mL]). When making a diagnosis of hemophilia B, caution must be taken in neonates because levels of FIX can be low in newborns because of their immature glutamylcarboxylase system. FIX levels double in the first few years of life and continue to rise slowly until puberty.[30] Consequently, a child initially labeled as having moderate hemophilia may be reclassified as having mild hemophilia later in life and a child initially diagnosed as having mild hemophilia B may later have normal FIX levels. In general, a FIX level greater than 20% in a neonate is considered normal as is a FIX level greater than 30% at 12 months of age.

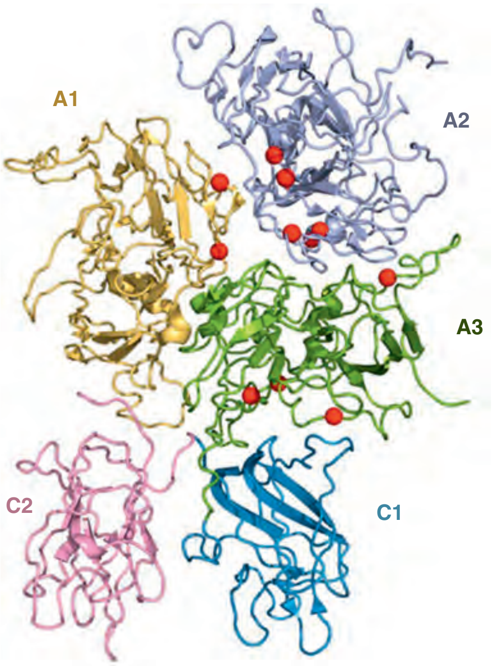


Figure 134.7 RIBBON REPRESENTATION OF THE STRUCTURE OF B-DOMAIN DELETED FACTOR VIII (FVIII). Domains are colored and labeled. Red spheres mark the positions of variants that result in hemophilia A with classical assay discrepancy (one-stage assays give a falsely higher value than chromogenic assays). These variants are clustered at the inter-domain boundaries mostly between the A2 and A1 or A3 domains. Mutations of these residues weaken the covalent bonds that tether the A2 domain to the cup created by A1/A3 resulting in more rapid dissociation and loss of activity. Because dissociation is time-dependent, the mutant proteins have higher activity in clotting assays where incubation times are short than in chromogenic assays where incubation times are longer.

Genetic Diagnosis of Hemophilia

The other diagnostic strategy that can be used for the diagnosis of hemophilia involves DNA analysis. Since the cloning of the FVIII and FIX genes in the early 1980s, molecular genetic approaches to hemophilia diagnosis have advanced dramatically. There are now greater than 3000 different *F8* mutations associated with hemophilia A and greater than 1000 *F9* mutations documented in hemophilia B.[23]

The strategy for genetic analysis depends on the type of hemophilia and the severity of the phenotype. For example, all patients with severe hemophilia A should be screened initially for the recurrent F8 intron 22 and intron 1 inversion mutations. In contrast, most patients with hemophilia B require full-sequence analysis of the entire FIX promoter, coding region, and splice sites.

If a familial mutation has already been identified in the FVIII or FIX gene, prenatal diagnosis can be accomplished with chorionic villus sampling or by amniocentesis. Outside of the prenatal context, mutation testing in hemophilia can be used for carrier detection for family planning purposes and as one component of the risk analysis for inhibitor development (see box on Hemophilia Carrier Detection and Prenatal Diagnosis). Determination of the hemophilic genotype is now regarded as standard of care.

TABLE 134.3 Differential Diagnosis of a Low Factor VIII Level

1. FVIII <10%
   - Severe or moderately severe hemophilia A
   - Severe type 1 VWD or type 3 VWD
   - Type 2N VWD
   - Acquired hemophilia A
   - Acquired VWD
2. FVIII: 10%–40%
   - Mild hemophilia A
   - VWD (any subtype)
   - Combined FVIII and FV deficiency

FV, Factor V; *FVIII*, factor VIII; *VWD*, von Willebrand disease.

Differential Diagnosis of Hemophilia

The initial clinical suspicion of hemophilia usually arises from signs and symptoms of excessive bleeding, a family history of a bleeding problem, or abnormal coagulation test results.

The initial laboratory screening test suggestive of hemophilia is a prolonged PTT. If the PTT normalizes after mixing with control plasma, factor levels are then measured to pinpoint the cause of the prolonged PTT. Although FVIII or FIX deficiency are associated with a prolonged PTT, deficiencies of FXI or FXII or combined deficiencies of various coagulation factors are other possibilities (see Chapter 135).

A low plasma FVIII level is not synonymous with a diagnosis of hemophilia A. Several disorders are associated with low FVIII levels (Table 134.3). Levels of FVIII below 10% are most often the result of inherited or acquired hemophilia A (in adults) or a severe form of inherited or acquired VWD (severe type 1 or type 3 VWD). Determination of VWF antigen and VWF activity levels is essential to differentiate these conditions. Levels of FVIII below 10% can also occur with type 2N VWD (see Chapter 133). This diagnosis can be confirmed with FVIII binding studies or by genotypic analysis of the FVIII binding codons of VWF (exons 17 to 25, which encode the D′D3 regions of the VWF protein).[31] Levels of FVIII between 10% and 50% are likely the result of either mild hemophilia A or VWD (types 1 or one of the type 2 variants: 2A, 2B, 2M, or 2N). Assessment of VWF levels (antigen and activity) must be undertaken in these cases. Rarely, mild or moderate FVIII deficiency can be co-inherited with FV deficiency. Combined inherited deficiency of FVIII and FV (levels are usually between 5% and 20%) has a prevalence of approximately 1 per million. This rare inherited trait is caused by recessive mutations in one of two genes involved in protein transport across the ER-Golgi interface: lectin mannose binding protein type 1 (LMAN1) or MCFD2.[14]

Isolated low plasma levels of FIX are almost always caused by congenital hemophilia B. In contrast to acquired hemophilia A, autoantibody development against FIX (i.e., acquired hemophilia B) is rarely encountered. Other situations in which FIX deficiency is found usually involve concomitant reductions in the other vitamin K–dependent clotting factors, such as occurs with vitamin K antagonists, vitamin K deficiency, or severe liver disease (see Chapter 126). A much less common cause of a mild deficiency of all the vitamin K–dependent proteins (FII, VII, IX, and X) is hereditary combined vitamin K-dependent clotting factors deficiency (VKCFD), an autosomal recessively inherited defect in the γ-glutamylcarboxylase (*GGCX*) gene, coding for the γ-carboxylase enzyme required for the posttranslational modification of these proteins.[32]

CLINICAL FEATURES OF HEMOPHILIA

The symptoms and signs of hemophilia A and B are identical and involve a propensity for prolonged and excessive bleeding (see Chapter 126). The bleeding tendency in hemophilia is determined in large part by the baseline activity level of the deficient or defective clotting factor. Thus, in severe hemophilia (A and B), in which the baseline clotting factor activity level is below 1% (0.01 IU/mL), pathological bleeding can occur in the neonatal period, especially intracranial bleeding from the delivery process. However, most frequently, severe disease manifests with easy bruising or soft tissue or joint bleeding beginning at around 6 to 12 months of age when children become more mobile. Excessive bleeding after circumcision can prompt diagnosis in the perinatal period. Without clotting factor prophylaxis, the annual incidence of bleeding increases in the first 5 to 6 years of life and plateaus at an average of 20 to 40 bleeds per patient per year.

TABLE 134.4 Reasons for Differences in Bleeding Phenotype

1. Differences in factor levels
2. Differences in mutations causing hemophilia: null vs. non-null mutations
3. Co-inheritance of other bleeding disorders
   - Von Willebrand disease
   - mild functional platelet disorders
4. Co-inheritance of prothrombotic disorders
   - factor V Leiden
   - prothrombin G20210A mutation
   - low levels of protein C, protein S, or antithrombin
5. Differences in the pharmacokinetic handling of factor. In the case of hemophilia A this includes the effect of ABO blood group and endogenous levels of von Willebrand factor (for patients on factor prophylaxis)
6. Differences in the pharmacokinetics and pharmacodynamics of emicizumab (for those patients on emicizumab)
7. Differences in levels of physical activity
8. Differences in the structural integrity of joints, making patients more/less susceptible to joint bleeds and joint damage

Most patients with mild hemophilia (factor levels of >5% [>0.05 IU/mL]) only experience bleeding with trauma or surgery and the disease may go undiagnosed until later in life.

The bleeding phenotype in patients with moderate hemophilia (FVIII or FIX levels between 1% and 5% [0.01 and 0.05 IU/mL]) can resemble that of patients with severe or mild hemophilia. Thus, the severity and frequency of bleeding may correlate poorly with the factor activity level and even patients with FVIII or FIX levels less than 1% or with the same mutation may have variable phenotypes for the reasons listed in Table 134.4. All these reasons for phenotypic variability may explain why some patients bleed much less than would be expected based on their factor levels while others bleed much more. These differences may also explain why some patients with joint bleeds develop arthropathy and others do not.

Hemophilia A and B are characterized by similar types of bleeds. Although not conclusively proven, several studies suggest that with similar factor levels, the bleeding phenotype in hemophilia A is less severe than that in hemophilia B as evidenced by a younger median age for the first joint bleed and for start of prophylaxis, as well as increased use of prophylaxis and greater need for joint arthroplasty in patients with severe hemophilia A than in those with severe hemophilia B.[33] Likewise, in children with moderate disease, a higher proportion with hemophilia A than with hemophilia B require prophylaxis. Although the reasons for these differences are incompletely understood, the genetics of the disorders are a likely cause; most patients with severe hemophilia A have null mutations and produce no FVIII, whereas most of those with hemophilia B have non-null mutations and synthesize small amounts of FIX. In addition, in patients with hemophilia B, the stress-induced release of FVIII with bleeds may augment the hemostatic potential of the residual FIX as FVIII is a cofactor for FIX.

Although bleeding is the hallmark of hemophilia, the types of bleeds and issues facing patients vary depending on age. This is particularly true in newborns where issues related to the birthing process can occur, which are not encountered later in life. Also, the problems in infancy, which is the highest risk period for development of inhibitors and the time when prophylaxis protocols are established, are different from those encountered later in life.

Hemophilia in Newborns

The hazards of hemophilia are greatest in the neonatal period, particularly for those with moderate or severe disease. Newborns with hemophilia can be born to mothers who are known or suspected hemophilia carriers or can be born to mothers who are not known to be carriers as there is no family history of hemophilia. The latter scenario, which is encountered in about 50% of cases,[34] is associated with the highest risk of bleeding because no precautions are taken. The optimal method for delivery of children with known or suspected severe hemophilia is uncertain. Most experts recommend atraumatic vaginal delivery to avoid the maternal risks associated with cesarean section and because delivery by cesarean section does not eliminate the risk of intracranial hemorrhage (ICH) (Fig. 134.8). Vacuum extraction or forceps delivery should be avoided because they increase the risk of extracranial bleeding (e.g., subgaleal hemorrhage and cephalohematoma) and ICH. Fetal blood sampling is not a significant risk factor for ICH.

Intracranial Hemorrhage in Newborns

The incidence of ICH in newborns with severe hemophilia is approximately 3% to 4%,[35,36] which is 40- to 80-fold higher than the risk in the non-hemophilic population. If a newborn male suspected of having hemophilia has any sign of ICH (e.g., unequal pupils, seizures, vomiting, or lethargy), the appropriate factor concentrate should be promptly administered.

If signs of ICH occur in newborn boys without a family history of hemophilia, the PTT should be determined along with central nervous system imaging. If the PTT is prolonged and imaging reveals ICH, the levels of FVIII, FIX, and VWF should be urgently quantified. While waiting for the test results, fresh plasma (10 mL/kg) can be given. Alternatively, because FVIII deficiency is more common than FIX deficiency, a FVIII concentrate can be administered, and the PTT determined 10 to 15 minutes later. If the patient has hemophilia A or type 3 VWD, the PTT will correct; with type 3 VWD, the correction will be transient because of the short half-life of FVIII in this disorder (see Chapter 133). If the PTT fails to correct, the child likely has FIX deficiency, and a FIX concentrate can then be given. If FVIII concentrate is given, fresh plasma can be administered while waiting for the PTT results to cover the possibility of hemophilia B.

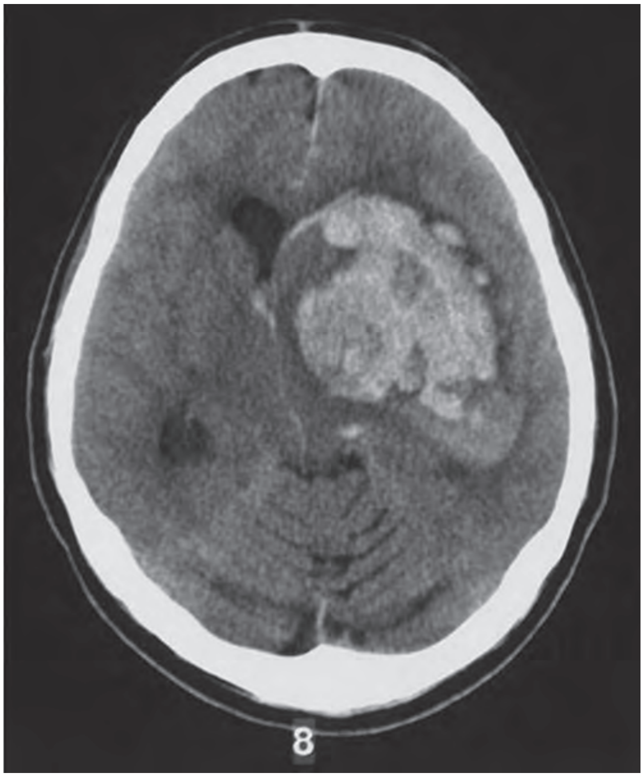


Figure 134.8 INTRACRANIAL BLEEDING IN HEMOPHILIA. A computed tomography scan shows an intracranial bleed in a person with hemophilia. This complication most often occurs after trauma with an incidence of five per 1000 per year in patients younger than 5 years of age and in 1% to 2% per year in persons 55 years of age or older.

Circumcision in Newborns

Surprisingly, excessive bleeding after circumcision only occurs in about half of boys with severe hemophilia. Consequently, the lack of excessive bleeding after circumcision does not exclude the diagnosis of hemophilia. In children suspected of having hemophilia, the diagnosis should be confirmed before circumcision. If hemophilia is confirmed and circumcision is desired, the appropriate factor concentrate should be administered before the procedure. A single dose prior to circumcision is usually sufficient although some clinicians give more after the procedure.[37]

Bleeding Manifestations With Hemophilia

The bleeding pattern in severe hemophilia is distinct and is rarely seen with other bleeding disorders. Although all types of bleeds can occur in hemophilia, musculoskeletal bleeds (hemarthrosis, muscle bleeds, and hematomas) are the most common and are responsible for most of the long-term complications. Other common sites for bleeding include the oral cavity, gastrointestinal (GI) and genitourinary tracts, and intracranial bleeding. Additionally, insufficiently treated muscle or soft tissue bleeds may result in pseudotumor formation (see box

HEMOPHILIC PSEUDOTUMOR

With no or with inadequate treatment, incomplete resorption of blood after repeated hemorrhage into muscles can result in the formation of pseudotumors—walled-off cystic structures surrounded by a fibrous membrane that can become multivacuolated and calcified over time. These cystic lesions can expand and destroy adjacent structures leading to bone fractures and deformities. Another rarer type of pseudotumor seen mostly in adults arises within the bone secondary to repeated subperiosteal bleeding. This type of pseudotumor usually occurs in the long bones of the lower extremities and in the pelvis. Distal pseudotumors in the hand are more common in children and are associated with pain from rapid growth or nerve compression.

Pseudotumors are usually diagnosed with ultrasonography or MRI and can be misdiagnosed as a neoplasm (e.g., Ewing sarcoma or osteosarcoma) or an infection (e.g., osteomyelitis or tuberculous abscess). Biopsy is contraindicated because of the potential for bleeding or infection. Small pseudotumors, particularly distal ones or pseudotumors in patients with inhibitors, can be treated conservatively with aggressive prophylactic therapy with clotting factor replacement (there are no data on long-term prophylaxis with emicizumab in this setting) along with immobilization of the affected limb.

If prophylactic therapy alone is insufficient, surgical excision is needed. This carries potential morbidity and even mortality and should only be undertaken by skilled surgeons in conjunction with appropriate hemophilia specialists. Embolization and radiation have been successful for treatment of small pseudotumors of the hand.[38]

COMPARTMENT SYNDROME

A compartment syndrome arises when a bleed (usually trauma-induced) occurs into a closed (encapsulated) space, such as the forearm or calf. The capsule restricts exit of blood, thereby raising the pressure within the compartment with resultant vascular compression, obstruction of blood flow, tissue ischemia, and the potential for nerve and muscle damage. Compartment syndrome is characterized by severe pain and swelling, limb pallor, paresthesia, and reduced limb movement. This condition requires urgent factor replacement and surgical decompression (fasciotomy) may be required. Without treatment, compartment syndrome may lead to permanent neuropathy, tissue necrosis, and even loss of the limb.

on Hemophilic Pseudotumor) or compartment syndrome (see box on Compartment Syndrome). Finally, bleeds can occur with surgery, dental procedures, or trauma.

Hemarthrosis

Joint bleeds account for about 75% of all bleeding events in hemophilia and are a hallmark of the disorder. Although bleeds can occur in any joint, the most commonly involved are the ankles, knees, and elbows, the so-called *index joints*. Less commonly involved joints are the shoulders, hips, and wrists. The least involved are the joints in the hands and feet.

The first onset of joint bleeding is highly variable. Although the median age for the first joint bleed is 1.8 years, some children experience their first bleed earlier as they begin to ambulate and others have it later in the fourth, fifth, or sixth years of life.[39] Without prophylaxis, hemarthroses, which can be spontaneous or related to trauma, become more common with age. The trauma may be imperceptible, particularly in infants who are unable to vocalize the occurrence of trauma. Even minimal trauma during sleep can trigger awakening with joint pain and swelling.

In patients with severe/moderate hemophilia, bleeding into joints continues until hemostatic therapy is given or until the extravascular pressure within the joint increases to the point where bleeding vessels are occluded. With the latter scenario, the joint pain may be excruciating. Although bleeding may stop spontaneously in patients with mild hemophilia, there still can be substantial accumulation of blood in the joint. Therefore, joint bleeds should be treated promptly with factor concentrates to reduce the risk of long-term complications. Joint aspiration to remove blood may be of benefit with bleeds into large joints such as the hips (see box on Hip Pain in Hemophilia).

With repeated episodes of joint bleeding, most older children, adolescents, and adults with hemophilia can identify early intraarticular bleeding before the classical signs appear through a feeling of warmth and tingling in the affected joint.

Joint damage occurs through at least three mechanisms: iron toxicity, inflammation, and mechanical distension of the joint. Repeated joint bleeding causes inflammation and hyperplasia of the synovial tissue (*synovitis*) which is the first step in the development of hemophilic arthropathy.[41]

Repeated bleeding into the same joint three times over a 6-month period qualifies that joint as a target joint.[42] Such joints are susceptible to further bleeding unless prophylaxis is given resulting in a vicious cycle that ultimately leads to destructive joint damage.

Early stage hemophilic arthropathy is characterized by synovial hyperplasia, extensive destruction of articular cartilage, progressive loss of joint space, cystic changes within the subchondral bone, osteoporosis, and atrophy of surrounding muscles. The final stages of hemophilic arthropathy are associated with deformed and dysfunctional joints with wasting of the muscles in the affected limb (Fig. 134.9). At this late stage, joint bleeds are less frequent because synovial hypertrophy is less prominent.

HIP PAIN IN HEMOPHILIA: HIP JOINT BLEED VERSUS ILIOPSOAS MUSCLE BLEED

Pain in the hip joint region can be caused by several conditions, for example, hip joint bleed, iliopsoas muscle bleed, bleed into surrounding muscles, retroperitoneal bleed, or appendicitis. Bleeds into the hip joint and iliopsoas muscle are serious and difficult to differentiate. Both cause pain and restricted movement of the hip joint with a tendency to maintain the leg in a partially flexed position, the position of lowest pressure. Consequently, ultrasonography or MRI is essential for accurate diagnosis; plain radiographs are insufficient.

Iliopsoas muscle bleeds can rapidly expand because there is no surrounding connective tissue to restrict their growth. This can lead to a significant decrease in the hemoglobin level and a need for blood transfusion. Furthermore, pressure on the femoral nerve can cause paresthesia, hyperesthesia, or weakness of the quadriceps muscle.

Treatment of iliopsoas muscle bleeds or bleeding into the hip joint involves prolonged administration of factor concentrates and immobilization and subsequent physical therapy, often necessitating hospitalization.[40]

The management of an acute hip joint bleed is somewhat different from management of bleeds in other joints because of the vascular anatomy of the hip joint, which renders the head of the femur vulnerable to ischemia. Persistent pain despite appropriate factor replacement may indicate ischemia in the femoral head and ultrasound-guided joint aspiration by an experienced interventional radiologist or surgeon should be considered. Graded physiotherapy should be instituted when there is symptomatic improvement. Follow-up imaging studies (MRI, bone scan, or both) should be considered for assessment of avascular necrosis.

Over the past 10 to 20 years, clinical and radiographic scoring systems have been developed to objectively evaluate the status of the joints over time (Table 134.5). Clinical scores are more readily available, do not require expensive radiological equipment, and better reflect the extent of joint disability. Plain radiographs are relatively inexpensive and widely available but are insensitive to early soft tissue changes and involve radiation exposure. Magnetic resonance imaging (MRI) is the most sensitive test for detection of early soft tissue changes and is not associated with radiation exposure but is more expensive than plain x-rays and less available. In addition, young children may require sedation to avoid movement during image acquisition. Ongoing studies are evaluating the utility of point-of-care ultrasonography for evaluation of hemophilic arthropathy.[48] Ultrasonography has the advantages of being inexpensive, not requiring sedation and being able to differentiate between synovitis and hemosiderin deposition. However, it is operator dependent, interpretation of its findings can be subjective, and it fails to identify joint structures such as cartilage. Some of these problems may be overcome with automated ultrasonography but more work is needed.

Soft Tissue Hemorrhages and Muscle Bleeds

Patients with hemophilia are prone to excessive and prolonged soft tissue and muscle bleeding. Superficial hematomas (bruises) may resolve spontaneously without the need for treatment, and as such, bruising is not an indication for treatment. However, in moderate and severe hemophilia, soft tissue hematomas often undergo progressive enlargement and may need to be treated. Furthermore, some soft tissue bleeds (e.g., retroperitoneal bleeds or hematomas of the neck) can cause extensive blood loss and be life or organ threatening because of their propensity to expand, thus causing compression of adjacent organs, blood vessels, and nerves and the airway in the case of a neck hematoma.

The muscles most often involved are, in descending order of frequency, the calf, thigh, buttocks, and forearm. Bleeds into these locations can lead to compartment syndrome, which is an emergency (see box on Compartment Syndrome).

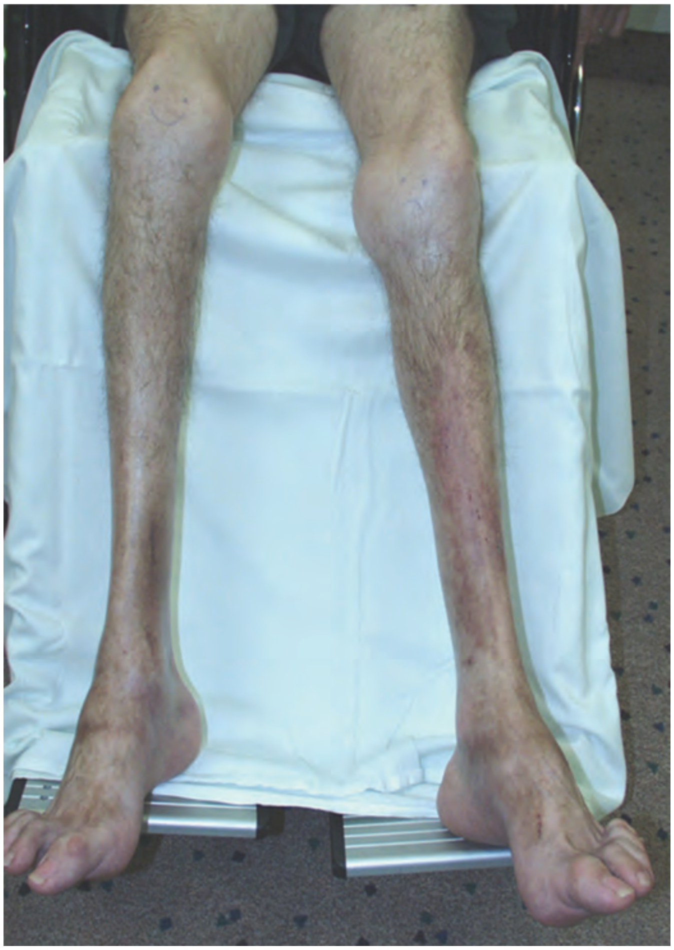


Figure 134.9 CLINICAL OUTCOME OF CHRONIC SEVERE HEMOPHILIC ARTHROPATHY. The legs of a 55-year-old patient with severe hemophilia A who is a wheelchair user. After multiple hemarthroses, the ankles and knees show deformities, and the leg muscles are atrophic because of disuse.

TABLE 134.5 Clinical and Radiological Evaluations in Hemophilia A and B

	Scores	Reference
Clinical	WFH Joint score (Gilbert score); this has largely been supplanted by the Hemophilia Joint Health Score (HJHS)	43
	HJHS	44
Plain radiography	Pettersson	45
Ultrasound	Head-US (Hemophilia Early Arthropathy Detection with Ultrasound)	46
MRI	Single compatible IPSG MRI scoring system	47

IPSG, International Prophylaxis Study Group; *MRI*, magnetic resonance imaging; *WFH*, World Federation of Hemophilia.

Mucous Membrane Bleeding

While epistaxis is uncommon in hemophilia, oral bleeding frequently occurs. Bleeding from the frenulum after falling forward is often one of the first manifestations of hemophilia as is bleeding in the tongue. Such bleeds can result in significant blood loss and swelling to the point of airway obstruction. Excessive bleeding with loss of deciduous teeth and eruption of secondary dentition can occur but is uncommon. Fortunately, most oral bleeds can be managed with antifibrinolytic agents such as tranexamic acid or epsilon aminocaproic acid although factor replacement may be needed for more serious cases. For dental surgery, particularly if a nerve block is required, factor replacement or DDAVP (desmopressin) must be given to raise the factor level to at least 30% of normal.

Hematuria

Hematuria is usually spontaneous, episodic, and painless but may also occur with trauma. Rarely, hematuria can be caused by renal calculi, which are thought to be more common in males with hemophilia than in those without hemophilia. Consensus on the optimal management of hematuria in patients with hemophilia is lacking, but increased fluid intake and bed rest are recommended. If bleeding continues or is particularly severe, prophylactic therapy (e.g., daily factor replacement) should be given. The value of steroids is controversial and use of antifibrinolytic agents is contraindicated because of the risk of ureteral obstruction by clots. Fortunately, hematuria in patients with hemophilia is not associated with progressive loss of renal function, and as such, its natural history is probably benign.

Gastrointestinal Bleeding

GI bleeding can occur in adults with hemophilia, particularly in association with chronic use of nonsteroidal anti-inflammatory drugs (NSAIDs) for arthropathy. Life-threatening upper GI bleeding can occur because of ruptured esophageal varices in patients with portal hypertension secondary to long-standing hepatitis C. Bleeds are usually treated with high doses of factor concentrate for several days together with antifibrinolytic agents.

Neurologic Bleeding

ICH, the most feared hemorrhagic event, remains a leading cause of death and morbidity (mental retardation, seizure disorders, and motor dysfunction), particularly in neonates and very young children with hemophilia.[49] The incidence of ICH is highest in neonates and is associated with the birthing process, particularly with traumatic vaginal delivery. ICH is also seen in young children with severe hemophilia, particularly those not receiving prophylaxis or in patients with high titer inhibitors not receiving emicizumab/bypassing agent prophylaxis.[50] In older adults, ICH is again more common, particularly in those with coexisting HIV or hepatitis C who have thrombocytopenia secondary to portal hypertension-induced splenomegaly or HIV-associated immune thrombocytopenia. ICH is rare in patients with mild or moderate hemophilia.

ICH may be subdural, epidural, subarachnoid, or intracerebral. Intraspinal (usually epidural) bleeding is rare, but when it occurs, can cause spinal cord compression. Most cases of ICH occur after trauma, but the trauma may be trivial, such that the ICH appears to occur spontaneously. Patients with ICH usually present with neurologic symptoms soon after trauma. Typical neurologic symptoms include drowsiness, loss of consciousness, seizures, headache, and unexplained vomiting.[51,52] Patients with any of these symptoms should immediately be given a bolus of the appropriate factor concentrate to raise the factor level to about 100% or a bypassing agent if there is a high titer inhibitor; this should be done prior to any diagnostic procedure (e.g., computed tomography or MRI). If ICH is confirmed, patients should be hospitalized for 10 to 14 days during which time they should be maintained on factor concentrate (by continuous infusion or frequent bolus infusion) to maintain factor levels over 100%. With emicizumab, the duration of treatment with aggressive factor replacement may be reduced.

Surgery and Bleeding

Except for circumcision in neonates, excessive bleeding is almost inevitable in patients with severe or moderate hemophilia who undergo surgery without adequate replacement therapy. The bleeding can be acute or delayed for several hours or days. In addition to bleeding, surgery in such patients is characterized by poor wound healing because of poor clot formation, prolonged bleeding, and wound infection. For surgical management of hemophilia see later section in this chapter.

CLINICAL MANAGEMENT OF HEMOPHILIA

The key aspects of hemophilia management are preventive therapy, treatment of bleeds when they occur, managing complications of bleeding, and dealing with the psychosocial issues of an inherited condition.

The management of hemophilia has undergone dramatic changes in the last few years. As a lifelong, expensive, and disabling illness, the management of patients with hemophilia is complex and has been and will likely continue to be best undertaken through comprehensive care at hemophilia treatment centers (HTC) with multidisciplinary teams of health professionals. These teams should include a physician (usually a hematologist) with specialist training in hemophilia, a dedicated nurse, a physical therapist, and a social worker. Additional members needed for consultation include an orthopedic surgeon, a dentist, a genetic counselor, an obstetrician/gynecologist, a psychologist, a rheumatologist, a urologist, and (increasingly, for the management of elderly persons with hemophilia) a cardiologist. Fortunately, the role of the orthopedic surgeon in the management of hemophilia has been reduced in countries where prophylactic therapy is provided.

Over the course of their lifetime, patients with hemophilia and their families will face many psychological and physical challenges. Children with hemophilia are often overprotected, which can lead to difficulties with adjustment to the school environment and to society in general as they age. Hemophilia impacts the choice of sports and recreational activities that are undertaken and some studies report poorer academic performance and behavioral and emotional problems in school-aged children with hemophilia compared with those without.[53] The causes may be multifactorial, including silent ICH. Children with hemophilia may resent their unaffected siblings and peers and they may harbor suppressed anger regarding their condition. Hemophilia can have an impact on vocational choices and opportunities for adults with arthropathy. Furthermore, those with HIV or hepatitis C have additional issues to deal with.

Hemophilia has an impact on the entire family. The child's mother may experience guilt knowing that as a carrier she passed the hemophilic mutation to her son. This can contribute to marital breakdown, although it is unclear whether marital discord is more frequent in parents of a child with versus without hemophilia. Regardless, persons with hemophilia and their families require ongoing education, counseling, and support.

Preventive Therapy

Preventing Trauma and Promoting Activity

Prior to the availability of effective prophylaxis, trauma prevention tactics were used to reduce the frequency of bleeding. One such strategy involves restriction from participation in high-risk sports (e.g., ice hockey, football, rugby, and martial arts). Even with effective prophylaxis, high-risk sports may be problematic for patients with severe hemophilia. Several other sports (e.g., soccer, basketball, baseball, and tennis) also entail a certain amount of risk, but for the child's psychological well-being, have generally been permitted if the child is on prophylaxis or receives prophylactic therapy prior to the activity. Other sports considered to be relatively safe include road cycling (with a protective helmet), running, and swimming. Ideally, persons with hemophilia should be encouraged to keep active throughout their lives because osteoporosis and obesity are growing problems in this population because of inactivity. All patients with severe and moderately severe hemophilia should be told to wear helmets for bicycle riding, rollerblading, skateboarding, and downhill skiing. There is controversy regarding the use of helmets (outside of sports) in very young children (ages 1 to 2.5 years) as they are learning to walk and are unsteady on their feet and prone to falling. It is important to advise families on the need for good dental hygiene to prevent or reduce the need for dental procedures (extractions, root canals), which entail a risk of bleeding. Unfortunately, dental care, including regular cleaning by a dentist, is often not subsidized by society and as such may be an expensive undertaking for families.

Vaccinations

Children with hemophilia have normal immune systems and should receive all routine vaccines. Vaccines should be given intramuscularly or subcutaneously using the smallest gauge needle possible (#25 or #27) and pressure should be applied at the injection site for 5 minutes. Without prophylaxis, there is a risk of hematoma formation after intramuscular injections. Subcutaneously administered vaccines appear to produce the same immune response as those given intramuscularly.[54] The enhanced safety of subcutaneous injections renders this the preferred route. Currently approved COVID-19 vaccines however require intramuscular administration.

In the past it was important that patients with hemophilia receive hepatitis B vaccinations at a young age. However, none of the current therapies (currently available plasma-derived factor concentrates, recombinant factor concentrates—both standard and EHL and nonfactor therapies) transmit hepatitis B and hence the urgency for hepatitis B vaccination has lessened. Nonetheless, vaccination should be considered because patients with hemophilia may acquire hepatitis B from blood transfusions required for surgery or trauma.

Avoidance of Aspirin and Other Medications That Increase Risk of Bleeding

The risk of bleeding in persons with hemophilia can be exacerbated by aspirin, other antiplatelet agents such as clopidogrel, and NSAIDs. Therefore, these agents should be avoided if possible. Cyclooxygenase-2 inhibitors have less of an effect on platelet function than traditional NSAIDs (see Chapter 128). If antiplatelet agents are required, coordinated care with an experienced cardiologist and a hemophilia specialist is needed.

Haemostatic Replacement Therapy

Standard Half-Life Clotting Factor Concentrates

Until the 1970s, plasma was the only source of clotting factors for replacement. Plasma contains only small amounts of FVIII and FIX. Consequently, large volumes of plasma are needed for replacement, which can lead to fluid overload. Cryoprecipitate, which contains high concentrations of FVIII, was commonly used for treatment of hemophilia A in the 1960s and 1970s. However, neither plasma nor cryoprecipitate is virally inactivated. In addition, storage issues and convenience and volume of administration have rendered these products obsolete in most countries. It should be noted that due to their low cost and wide availability in many parts of the world plasma and cryoprecipitate have continued to be used into the 21st century.

Plasma-derived clotting factor concentrates (CFCs), which are derived from thousands of units of plasma, first became available in the late 1960s. In the early 1970s and 1980s, it was evident that these concentrates were contaminated with hepatitis C and HIV.

Consequently, a generation of hemophilia patients worldwide died of HIV/AIDS or from complications of hepatitis C. This tragedy prompted development of effective viral inactivation methods for treatment of plasma-derived products such as solvent detergent, pasteurization, vapor treatment, nanofiltration, and dry heating, and the development of recombinant CFCs. Currently administered plasma-derived CFCs are not associated with any known viral transmission, although a small theoretical risk remains. These are classified as high purity (containing only the desired factor and very little else) or intermediate purity (containing the desired factor but with other factors present). In the case of FVIII, intermediate-purity products generally contain VWF (e.g., Alphanate [Grifols], Humate [CSL-Behring], and Wilate [Octapharma]). Intermediate-purity FIX products are prothrombin complex concentrates (PCC) (e.g., Beriplex [CSL Behring], Octaplex [Octapharma], and Proplex [Takeda]), which, in addition to FIX, contain other vitamin K dependent factors (FII, FVII, FX, along with protein C and S). In general, PCCs are no longer used for the management of bleeding in patients with hemophilia B because of the risk of thrombosis.

Recombinant FVIII was developed in the late 1980s. First-generation products were stabilized with human albumin in the final product. Albumin was eliminated in second-generation products, but these products were still exposed to human plasma-derived proteins during the manufacturing process. Third-generation products have no exposure to human or animal proteins and are stabilized with sucrose. Recombinant CFCs have traditionally been more expensive than their plasma-derived counterparts, but they have supplanted plasma-derived CFCs for hemophilia treatment because of enhanced safety and smaller volumes of infusion. BeneFix (Pfizer) was the first commercially available recombinant FIX, but two other standard half-life (SHL) recombinant FIX products are now available (Rixubis [Takeda], Ixinity [Emergent]).

The efficacy of factor concentrates primarily reflects their pharmacokinetic properties such as recovery and half-life. Until recently all available plasma-derived and recombinant CFCs have had similar pharmacokinetic properties; these have been referred to as SHL CFCs.

The recovery of factor concentrate refers to the increase in circulating factor activity level achieved immediately after infusion of a given amount of factor. With all FVIII concentrates, administration of 1 IU/kg of FVIII increases the FVIII level by approximately 2%. Therefore, to achieve a FVIII level of 100% in an individual with severe hemophilia, a dose of 50 IU/kg is needed. The volume of distribution for FIX is two- to threefold greater than that for FVIII. Therefore, a FIX dose of 1 IU/kg has traditionally been thought to increase the FIX level by 1%. However, with this dose, the recovery of recombinant FIX (BeneFix; Pfizer) only increases the FIX level by 0.7% to 0.8%. The recovery of EHL FIX concentrates is more variable with some behaving more like FVIII with recoveries of 1.5% per 1 U/kg administered.

The half-life determines the duration of activity after administration of the clotting factor. In general, SHL FVIII concentrates have shorter half-lives than SHL FIX concentrates (8 to 12 hours vs. 18 to 24 hours) and all the SHL concentrates have similar pharmacokinetic properties. Consequently, the choice of SHL FVIII or FIX concentrate was primarily influenced by cost and availability, and the desire to avoid the potential risks of plasma-derived products.

A major limitation of SHL CFCs is the need for frequent intravenous administration because of their short half-lives. With the requirement for every-other-day injection with some high-dose prophylaxis regimens, adherence can be problematic as evidenced by development of joint disease despite prophylaxis.[55]

Extended Half-Life FVIII and FIX Concentrates (Table 134.6)

Several EHL FVIII and FIX concentrates are now available. Although all EHL concentrates are recombinant proteins, the technology used to extend the half-life differs among products. The two main methods involve fusion technology and PEGylation. Both technologies work by interfering with the natural clearance of FVIII or FIX by the mononuclear phagocyte system and by sinusoidal endothelial cells. Attempts to prolong the half-life of FVIII by increasing its binding to VWF yielded minimal prolongation (Afstyla; CSL-Behring).

TABLE 134.6 **Extended Half-Life FVIII and FIX Concentrates**

Product	Technology	Manufacturer	$T_{1/2}$ vs. Native FVIII/FIX
Extended Half-Life FIX			
rFIXFc (Alprolix)	Fusion protein with Fc fragment of IgG1	Sanofi/Sobi	3X
rIX-FP (Idelvion)	Fusion protein with albumin	CSL-Behring	>5X
N9-GP (Rebinyn/ Refixia)	Site-specific glycoPEGylation (40 kDa PEG)	Novo-Nordisk	>5X
Extended Half-Life FVIII			
FVIII-Fc (Eloctate/ Elocta)	B domain deleted FVIII fused to a monomeric Fc fragment of IgG	Sanofi/Sobi	1.5–1.7X
BAY-94-9027 (JIVI)	Site specific PEGylation (60 kDa PEG) of a B domain deleted FVIII	Bayer	1.4–1.6X
BAX 855 (Adynovate/ Adynovi)	Controlled PEGylation (20 kDa branched chain PEG) of a full-length FVIII	Takeda	1.4–1.6X
N8-GP (Esperoct)	Single site specific glycoPEGylation (40 kDa PEG) of a B domain truncated FVIII	Novo Nordisk	1.6X

FVIII, Factor VIII; *PEG*, polyethylene glycol; $T_{1/2}$, half-life; *kDa*, kilodalton.
Adapted and updated from Carcao M. Changing paradigm of prophylaxis with longer acting factor concentrates. *Haemophilia.* 2014;20(suppl. 4):99.

Fusion technology involves molecular linking of the cDNA sequences of clotting factors to the cDNA of proteins (the Fc portion of immunoglobulin or albumin) that have long half-lives because they bind to the neonatal Fc receptor in endothelial endosomes. This protects them and whatever is attached to them from degradation within endothelial cells and recycles the proteins back into the plasma. There are currently 2 EHL fusion FIX concentrates—in one, the recombinant FIX is bound to recombinant Fc (Alprolix; Sanofi/Sobi), while in the other, it is bound to recombinant albumin (Idelvion; CSL Behring). There is also one Fc fusion FVIII (Eloctate/Elocta; Sanofi/Sobi).

For PEGylation, polyethylene glycol (PEG) molecules of different sizes are bound to either recombinant FVIII or FIX. There is one pegylated FIX product (Rebinyn/Refixia; Novo Nordisk) and 3 pegylated or glycoPEGylated FVIII products (Adynovate/Adynovi, Takeda; Esperoct, Novo Nordisk; Jivi, Bayer).

Until recently, recombinant FVIII was derived from full-length cDNA constructs. However, most of the EHL FVIII concentrates (including Eloctate/Elocta, Esperoct and Jivi) are B domain deleted; only Adynovate/Adynovi is full length.

The recovery of newer EHL FVIII products is like that of SHL FVIII. In contrast, the recovery of newer EHL FIX products is more variable with Refixia/Rebinyn and Idelvion yielding greater recovery than Alprolix, which may reflect differences in extravascular distribution. Compared with their SHL counterparts, the half-life of FIX is

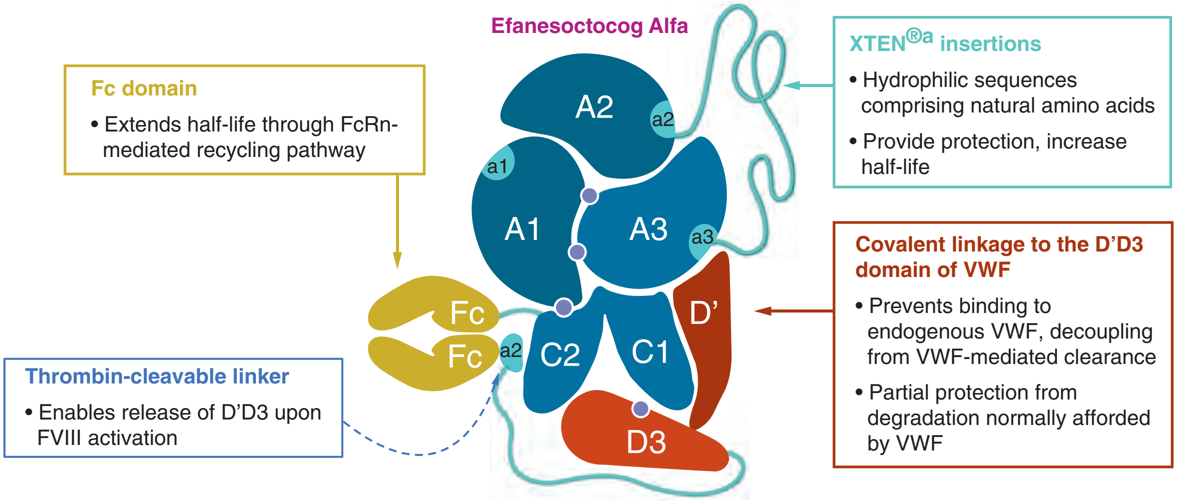

Efanesoctocog Alfa
Fc domain
• Extends half-life through FcRn-mediated recycling pathway
XTEN®a insertions
• Hydrophilic sequences comprising natural amino acids
• Provide protection, increase half-life
Covalent linkage to the D'D3 domain of VWF
• Prevents binding to endogenous VWF, decoupling from VWF-mediated clearance
• Partial protection from degradation normally afforded by VWF
Thrombin-cleavable linker
• Enables release of D'D3 upon FVIII activation
A2
a2
a1
A1
A3
a3
Fc
Fc
a2
C2
C1
D'
D3

Figure 134.10 EFANESOCTOCOG ALFA (BIVV001; SANOFI); A NOVEL EXTENDED HALF-LIFE RFVIII-VWF-XTEN FUSION PROTEIN. Consisting of a D'D3 molecule fused to rFVIII, efanesoctocog does not bind to endogenous von Willebrand factor (VWF) thereby eliminating VWF mediated clearance.[18] To prolong the half-life two Fc molecules and two XTEN polypeptides (144 aa and 288 aa) composed of natural hydrophilic amino acids are fused to the protein.

extended three- to fivefold while that of FVIII is extended by about 1.5-fold likely reflecting the dominant role of VWF in FVIII clearance.[18,56,57] All EHL FVIII products have similar pharmacokinetic properties.[58,59] This is not the case for EHL FIX where the half-life of Alprolix is shorter than that of the other two EHL FIX products. New evidence suggests that extravascular FIX sequesters in the basement membrane of blood vessels, where it may contribute to hemostasis when there is a breach in the vessel wall. Consequently, higher plasma levels of FIX may be required to achieve hemostasis with EHL FIX products that are confined only to the intravascular compartment. This phenomenon may explain the breakthrough bleeding reported with Idelvion.[60]

EHL products, particularly the newer EHL FIX products, enabler fewer infusions, achievement of higher trough levels, and improved patient satisfaction.[61] EHL FIXs allow for infusions as infrequently as once every 7 to 14 days while still obtaining sufficient levels of protection to prevent bleeds. For the most part, EHL FVIII products still need to be given at least twice per week to achieve trough levels around 2% to 3%.

Ongoing research is focused on novel methods for extending the half-life of FVIII or to enable subcutaneous delivery. BIVV001 (efanesoctocog alfa; Sanofi) is a novel EHL FVIII in which the FVIII has been engineered such that it no longer binds to VWF but has been linked to rFc and has 2 XTEN polypeptides composed of natural hydrophilic amino acids. These modifications result in a half-life 4-fold longer than that of SHL FVIII and may enable once-weekly therapy for patients with hemophilia A (Fig. 134.10).[18]

Prophylaxis

If hemostatic treatment is given only on demand (also referred to as episodic therapy), meaning only after a bleed occurs, recurrent bleeds will, over time, lead to disabling joint disease. Recognizing that on-demand "therapy" does not prevent joint disease, prophylaxis is the current standard of care. Until recently, prophylaxis referred to the regular replacement of CFC to prevent or reduce the risk of bleeding. With the availability of emicizumab, the definition of prophylaxis has changed. In many parts of the world, however, no prophylaxis is available, or if prophylaxis is available, it is restricted to SHL CFC.

The longest experience with the use of prophylaxis comes from European centers in Sweden, the Netherlands, and Germany. This experience began over 50 years ago. However, the first randomized study comparing prophylaxis with on-demand therapy was performed less than 20 years ago when Manco–Johnson and colleagues demonstrated 90% fewer bleeds in very young children started on prophylaxis prior to 2.5 years of age (i.e., primary prophylaxis) vs those treated on demand. Furthermore, they showed that after only a few years, children given prophylaxis exhibited less joint damage than those treated on demand.[62] Cohort studies and a few randomized studies confirm the benefits of prophylaxis and demonstrate reduced bleeding, prevention, or reduction of joint damage, reduced hospitalization rates, reduced need for orthopedic surgery, improved work and school attendance, and enhanced quality of life.[37,53,63–65] All prophylaxis regimens (from high to low dose) have demonstrated superiority over on-demand therapy.[37,66,67] One other potential (but not conclusively proven) benefit of prophylaxis is that early initiation of prophylaxis may reduce the risk of inhibitor development, at least in the subgroup of patients with severe hemophilia with "good risk" *F8* mutations.[68,69]

Consequently, prophylaxis is now considered the standard of care for all persons with severe hemophilia and for those with moderate hemophilia with a severe phenotype.[37]

Individualization of Prophylaxis

The concept of individualization of prophylaxis has evolved over time with the recognition that patients differ in their needs for prophylaxis with some patients requiring more intense prophylaxis than others.[70,71]

Until the advent of EHL factor concentrates, full-dose prophylaxis involved the administration of 25 to 40 IU/kg of conventional (i.e., SHL) FVIII every other day (minimum, 3 days/week) for hemophilia A and the same amount of FIX 2 days/week for hemophilia B. Less intense "intermediate-dose" prophylaxis regimens involved the administration of 15 to 25 U/kg 2 to 3 times a week, and low-dose prophylaxis regimens called for the administration of 10 to 15 IU/kg one or two times a week. For the most part, the full-dose regimen maintained trough levels over 1% while trough levels below 1% occurred with the lower-dose regimens at least some of the time.

Although EHL concentrates, particularly EHL-FIX, enable the achievement of higher trough levels, full-, intermediate-, and low-dose prophylaxis regimens with these agents have yet to be defined.

Contributing to interpatient differences in prophylaxis needs is the variability in the pharmacokinetics of factor concentrates from person to person. In general, children exhibit lower recoveries of clotting factors. There are large inter-individual variations in CFC half-life. In the case of FVIII, it is most affected by the patient's endogenous VWF level, which in turn depends on the ABO blood group. The half-lives of both FVIII and FIX increase with age for reasons that are poorly understood.

Prophylaxis with CFC (particularly SHL CFC) prophylaxis is burdensome for patients, especially young children, because of the need for frequent injections and costly for healthcare systems. Patients, particularly young children, receiving full-dose prophylaxis with CFCs often require a central venous access device (CVAD) or an arterial-venous fistulas (AVF) for access. The need for an access device may lead to delays in initiating prophylaxis or its administration at a suboptimal frequency to avoid the need for frequent venipuncture.[72,73]

When to Start Prophylaxis? Primary, Secondary, or Tertiary Prophylaxis

There is abundant evidence that starting prophylaxis early in life is optimal for prevention of joint disease.[37] This is referred to as primary prophylaxis; prophylaxis started at a very young age (usually 2 years of age or earlier) before joint disease has developed (generally, before or immediately after the first joint bleed). In contrast, secondary and tertiary prophylaxis refer to prophylaxis started at a later age (after the age of 2 years) or after several joint bleeds have occurred, and prophylaxis started after there is established joint disease, respectively. The term prophylaxis also encompasses short-term prophylaxis regimens given after surgery or ICH.

In the past, starting young children on prophylaxis was onerous because it involved training families/care givers and then the patient to administer factor in the home (referred to as home care—see box on Home Care).

Because of the need for venous access, many patients were started on less intensive prophylaxis regimens (e.g., once per week) and gradually escalated to full-dose regimens to obviate the need for a CVAD. EHL concentrates, particularly EHL FIX, enable administration of prophylaxis once per week or even once every two weeks without needing to escalate. Now, many young children with hemophilia A are started on emicizumab (discussed in more detail later), which is delivered subcutaneously. Emicizumab may enable earlier initiation of prophylaxis because infrequent subcutaneous injections are well tolerated even by very young children.

Because of the benefits of commencing prophylaxis early (i.e., primary prophylaxis) in children with severe hemophilia, the World Health Organization, World Federation of Hemophilia (WFH), and many other national hemophilia organizations have endorsed primary prophylaxis as the standard of care for such children.[37,62] This endorsement has extended to include patients with moderate hemophilia, especially those with a baseline factor level of 1% to 3%.[74]

HOME CARE

Hemophilia treatment (both prophylactic therapy and therapy to treat bleeds) is best administered in the patient's home. Having a patient or other caregiver able to administer therapy enables prompt treatment of bleeds and facilitates compliance with CFC prophylaxis. In the past, when intravenous clotting factor replacement was the only option for prophylaxis, hemophilia centers focused on teaching patients and families how to administer them. Such teaching is labor intensive and time consuming and usually requires collaboration between patients, their families, and dedicated hemophilia clinic nurses. If delivery via a peripheral vein was impossible a CVAD was inserted or an AVF was created. This approach enabled most boys with hemophilia older than 3 years of age to be treated at home by their parents (via peripheral veins or CVAD/AVFs) or in some cases by a visiting nurse. Once they reached the age of 8–12, most boys could be taught how to self-administer factor via a peripheral vein. Emicizumab has replaced factor infusion for prophylaxis in many infants and children. Because it is given subcutaneously, the need for venous access devices has been eliminated. Although families or patients are taught how to inject emicizumab subcutaneously, they will be inevitably less likely to learn how to administer intravenous therapies for management of breakthrough bleeds.

Although most children in Europe and North America are receiving prophylaxis, it is less routinely used in less affluent countries.[71] However, the uptake of prophylaxis has increased world-wide with the recognition that even intermediate- or low-dose regimens provide better results than on-demand therapy, and with the availability of EHL CFCs, which enable less frequent intravenous infusions, and with emicizumab which does away with the need for intravenous infusions altogether.

The perception that prophylaxis is less essential in adults than in children or adolescents has fortunately been changing and an increasing number of adults, at least in more affluent countries, are now on prophylaxis. In a 2013 survey of prophylaxis use in high-income countries (North America, Europe, Australia, and New Zealand) it was shown that 59% of adults with severe hemophilia A and 49% of those with severe hemophilia B were on regular prophylaxis.[75] Those proportions are likely much higher now with the introduction of EHL CFCs and emicizumab. It is now accepted that the gains of prophylaxis in childhood are rapidly lost if prophylaxis is stopped in adulthood.[37]

EHL CFCs and emicizumab have transformed prophylaxis, but there are many remaining questions. For example, how early in life should prophylaxis start, and should it start with emicizumab or with CFCs? Will EHL CFCs and emicizumab eliminate the need for CVADs? If emicizumab is used for prophylaxis, will patients be able to treat bleeds at home with CFCs, and if not will delays in obtaining treatment adversely affect joint health or quality of life?

Emicizumab

Emicizumab is a humanized bispecific murine monoclonal antibody that mimics the function of FVIII by binding FIXa and FX and bridging them together.[76] By bypassing FVIII, emicizumab can be used in patients with hemophilia A with or without inhibitors (see Fig. 134.3). Although emicizumab mimics the function of FVIIIa, there are differences that impact the outcome of FVIII administration on top of emicizumab (Table 134.7).[77]

In clinical trials (HAVEN 1, 2, 3, and 4), emicizumab provided effective prophylaxis against bleeding in patients with or without inhibitors and eliminated the development of target joints (Table 134.8). These trials have also shown reduced bleeding rates compared to patients on prophylaxis (both bypassing agent prophylaxis in patients with inhibitors and FVIII prophylaxis in those without inhibitors).[76,78,79,81] In addition to preventing bleeds, emicizumab may enable patients to safely undergo minor procedures such as CVAD removal or circumcision without additional hemostatic coverage. Nonetheless, because some bleeds have been reported in such patients, many clinicians choose to administer FVIII or a bypassing agent in these settings.

In addition to its efficacy, emicizumab has advantages over FVIII. These include its capacity to be administered subcutaneously and its half-life of about 29 days,[82] which enables dosing every 1 to 4 weeks.[76,78,80] Consequently, emicizumab is less burdensome than prophylaxis with FVIII prophylaxis in patients without inhibitors or bypassing agents in those with inhibitors.[79]

Emicizumab provides a steady-state level of hemostatic protection which may convert severe hemophilia to a mild state. The thrombin generation provided by emicizumab was in one small study found to be equivalent to that of FVIII levels of 10% to 40%.[83] Between patient variability in FVIII equivalency was also reported in the pivotal HAVEN-1 trial where steady state emicizumab levels in 112 subjects varied (median 50 ug/mL; range: 25 to 80 ug/mL), which correlated with a mean FVIII-like activity of 20.3% (95% CI, 12.3% to 28.4%).[84]

The protection provided by emicizumab is sufficient to protect most patients from bleeding most of the time but may not be adequate for those engaging in high-impact activities. If breakthrough bleeding occurs, intravenously administered hemostatic therapy is needed as emicizumab cannot be used to treat bleeds. FVIII is safe to administer to patients taking emicizumab prophylaxis. Emicizumab binds less

TABLE 134.7 Main Differences Between FVIII and Emicizumab

	FVIIIa	Emicizumab
Sites of interaction	Multiple sites	Single site
Affinity for FIXa) and FX	High affinity	Low affinity
Specificity of binding	Specific for FIXa and FX	No distinction between zymogen and enzyme (binds FIX and FIXa and FX and FXa)
Cofactor activity	Full cofactor activity • Promotes phospholipid binding • Stabilizes FIXa active site • Bridges FIXa to FX	Partial cofactor activity • Bridges FIXa to FX
Rate limiting step	Concentration of FVIIIa (Enzyme and substrate in excess)	Concentration of enzyme and substrate (Emicizumab in excess)
On/off mechanism	Yes	No
Level of self-regulation	High	Low

FVIIIa, Activated FVIII.
Adapted from Lenting PJ, Denis CV, Christophe OD. Emicizumab, a bispecific antibody recognizing coagulation factors IX and X: how does it actually compare to factor VIII? *Blood*. 2017;130(23):2463–2468.

TABLE 134.8 Main Features of the HAVEN 1–4 Multicenter Clinical Trials

	HAVEN 1[78]	HAVEN 2[79]	HAVEN 3[76]	HAVEN 4[80]
Population	Adults/ adolescents (≥12 years)	Pediatric (<12 years)	Adults/ adolescents (≥12 years)	Adults/ adolescents (≥12 years)
Inhibitor status	Positive	Positive	Negative	Positive (17%) Negative (83%)
N =	113	88	152	48
Emicizumab Regimen	1.5 mg/kg QW	1.5 mg/kg QW 3.0 mg/kg Q2W 6.0 mg/kg Q4W	1.5 mg/kg QW 3.0 mg/kg Q2W	6.0 mg/kg Q4W
Exposure duration, median (IQR), weeks	109 (92–167)	92 (68–124)	163 (108–170)	151 (84–153)
ABR (treated bleeds) (medians)	2.9	0.3	1.5	2.4

ABR, Annualized bleeding rate; *IQR*, interquartile range; *N*, number; *QW*, weekly; *Q2W*, every 2 weeks; *Q4W*, every 4 weeks.

avidly to the FIXa-FX complex than FVIIIa. Thus, if FVIII is given, it will preferentially bind to FIXa and FX displacing emicizumab.

FVIII levels in patients taking emicizumab cannot be monitored with routine one-stage clot-based or chromogenic assays. Such assays yield spurious FVIII levels of 500% or more. Although alternative assays have been developed, they are not widely available.[85]

Minor limitations of emicizumab include injection site reactions (pain and swelling particularly with larger volumes) and a low risk (<1%) of formation of neutralizing anti-drug antibodies.

Emicizumab is an option for patients with hemophilia A without inhibitors (in very young children it is likely the preferred option to avoid the need for intravenous therapy) and is the preferred prophylactic therapy for any hemophilia A patient who develops a high titer inhibitor. In older children without inhibitors, it is unclear whether emicizumab will remain the preferred option over intravenously CFCs, if very long half-life products such as BIVV001 (efanesoctocog alfa) are available.

Other FVIII mimetics (Mim8; Novo Nordisk and a second-generation emicizumab; Roche) are undergoing clinical investigation.[86] These appear to be more potent than emicizumab and potentially may convert severe hemophilia to a non-hemophilic state by providing the equivalent of FVIII levels greater than 40% at all times.

Rebalancing Therapies (Fig. 134.11)

Clinical trials exploring the efficacy and safety of therapies targeting natural anticoagulants (tissue factor pathway inhibitor [TFPI], AT, and the protein C/S pathways) are ongoing. These agents promote hemostasis by rebalancing the procoagulant to anticoagulant balance. Since that balance differs between individuals, clinical trials are assessing the dosing required to reverse the coagulation deficiency without inducing a prothrombotic state.

All these novel agents are, like emicizumab, delivered by subcutaneous injection. One major advantage of these rebalancing therapies is that, unlike emicizumab, they can be used in HB patients with inhibitors, which although rare is a condition that currently represents a very big unmet need. However, these agents are not yet approved and have had setbacks in clinical development and hence these agents may never become available.

Anti-Tissue Factor Pathway Inhibitor

Two monoclonal antibodies against TFPI are under investigation (marstacimab; Pfizer, and concizumab; Novo Nordisk). The antibodies are administered subcutaneously in regimens varying from daily to weekly. Drug-related thrombosis halted development of a third antibody against TFPI (befovacimab, Bayer)[87]; thrombosis has also been reported with concizumab.

Fitusiran (Sanofi)

Fitusiran is a small interfering RNA, which reduces the hepatic synthesis of AT, thereby enhancing thrombin generation and promoting hemostasis. Fitusiran is administered subcutaneously on a weekly or even monthly basis. In a phase 2 study in patients with hemophilia A or B, with or without inhibitors, fitusiran (50 or 80 mg once-monthly do) reduced AT levels by 80% and was associated with a median annual bleeding rate (ABR) of 1 in patients without inhibitors ($n = 19$). In the 14 patients with an inhibitor, fitusiran reduced the median ABR from 38 (pre-fitusiran) to 0.[88,89] No thrombotic episodes were observed. The phase 3 program for fitusiran was temporarily halted when a fitusiran-treated patient with hemophilia A without an inhibitor suffered a fatal cerebral vein thrombosis after being given 3 doses

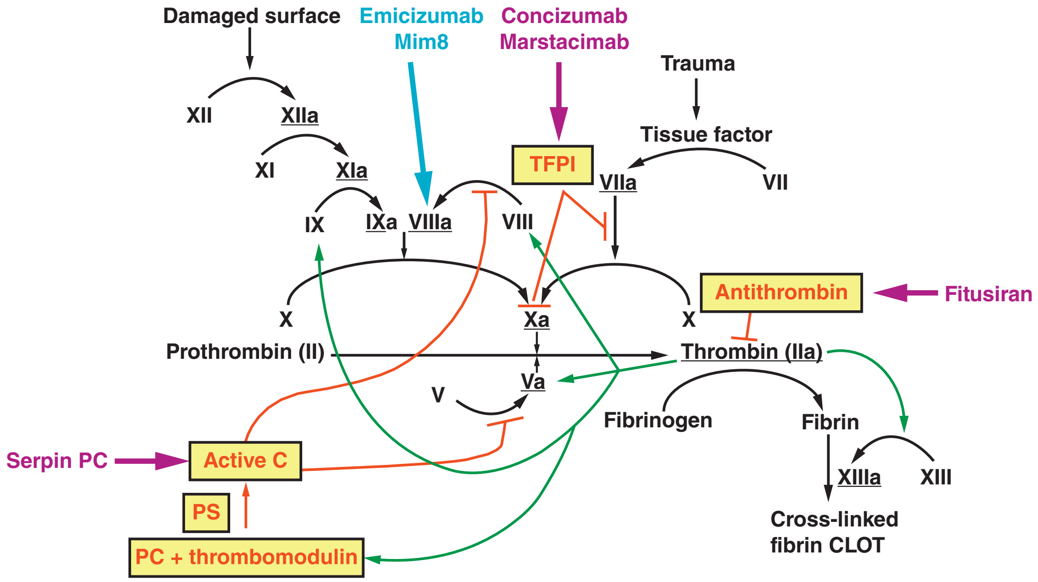


Figure 134.11 LOCATION OF ACTION OF NEW THERAPIES. Shown in *green* and *red arrows* are positive and negative feedback loops, respectively, provided by natural anticoagulants (highlighted in *yellow*: antithrombin, tissue factor pathway inhibitor (TFPI), activated protein C/protein S [PC/PS] and thrombomodulin). Activated factors are denoted by a little "a" and underlined. Factor VIII mimetics (emicizumab and Mim8; shown in *blue*) and rebalancing agents under development (shown in *purple*).

(31 to 46 U/kg) of FVIII. This event prompted a risk-mitigation strategy recommending the use of low doses of FVIII or bypassing agents in the event of bleeds. After another thrombotic event, the fitusiran dose was reduced to target an AT level of 15% to 35% and the trials are ongoing.

Other Rebalancing Agents

Additional rebalancing agents in development target other natural anticoagulants such as activated protein C or protein S.

Treatment of Bleeds

When a bleed occurs in a patient with hemophilia, immediate treatment is required to halt further bleeding. Rapid treatment prevents expansion of the bleed, accelerates healing, and reduces the risk of permanent disability. The longer the delay in providing treatment, the more bleeding that will ensue and, consequently, the longer it will take to completely resorb the blood. Blood within a joint is damaging to the synovium as well as to cartilage. Rapid treatment of joint bleeds will minimize damage to the joint and prompt treatment of muscle bleeds will limit expansion and reduce the risk of pseudotumor formation or compartment syndrome. Rapid treatment of ICH may be lifesaving and will minimize morbidity.

Even with the availability of emicizumab, treatment of bleeds still involves intravenous infusion of the missing factor. The capacity to administer rapid treatment is enhanced if the patient/family can administer factor at home (see box on Home Care).

Treatment of Bleeds With Factor Concentrates

The dose and duration of substitution therapy for treating or preventing bleeds depend on the severity of the bleed or the extent of the surgery.[37] Table 134.9 shows the desired factor levels for various types of bleeds and surgeries. After the initial bolus of factor, repeat doses of SHL concentrates have traditionally been needed. However, one dose of an EHL factor concentrate is often sufficient. For major bleeds, such as an ICH, or for major surgeries, such as joint replacement, 10 to 14 days of full factor coverage may be required, but for less severe bleeds, such as an uncomplicated hemarthrosis, 1 to 3 treatments (depending on the pharmacokinetics of the factor) are usually sufficient. For minor bleeds or minor surgeries (e.g., dental extraction) one treatment may be sufficient. For patients who require additional doses to treat a bleed or to ensure peri-surgical hemostasis, the frequency of treatment depends on the pharmacokinetics of the individual factor. Because of variable pharmacokinetics, monitoring trough factor levels every few days is useful to avoid high factor levels, which can be associated with thrombosis, or low levels, which can be associated with bleeding, and to detect inhibitor development.

An alternative method for maintaining hemostasis is to administer clotting factors by continuous intravenous infusion. The benefits of this approach include reduced overall factor consumption and elimination of the peaks and troughs associated with repeated bolus administration. In general, a FVIII infusion rate of 2 to 3 U/kg/h (with SHL FVIII or 2 U/kg with EHL FVIII) is sufficient once a FVIII level of 100% has been achieved with a bolus of 50 U/kg. Given the longer half-lives of EHL FVIII and particularly EHL FIX, continuous infusion of these products is not often necessary. Although concern has been raised about the possibility of inhibitor development with continuous infusion therapy, particularly in patients with mild or moderate hemophilia A, definitive evidence that continuous infusion poses a risk for inhibitor development is lacking.[90]

Treatment of Bleeds With Desmopressin

In the 1970s, Mannucci and colleagues showed that DDAVP (1-deamino-8 D-arginine vasopressin, desmopressin, Ferring Pharmaceuticals, Langley, United Kingdom), a synthetic analogue of the natural antidiuretic hormone vasopressin, raises circulating levels of VWF and FVIII:C.[91] DDAVP induces the release of endogenous VWF and FVIII from storage in Weibel-Palade bodies in endothelial cells. It also increases platelet adhesiveness and thus shortens the bleeding time independent of its effect on raising FVIII:C and VWF levels. Because DDAVP is not blood-derived or a factor concentrate, there is no risk of infectious complications or inhibitor development. DDAVP is less costly than factor concentrates and has few, generally mild and transient adverse events such as flushing, headache, mild tachycardia, and hypotension. DDAVP has antidiuretic activity and thus carries a risk of hyponatremia and seizures, particularly if given to very young children or to those

TABLE 134.9 **Target Factor Level for Different Types of Bleeds and Duration of Treatment**

Site of Bleed		Peak Level Desired (%)	Treatment Duration (Days)
Oral mucosa		>30	1 (plus tranexamic acid)
Epistaxis		>30	1 (plus tranexamic acid)
Joint or muscle		>50	1–3
Iliopsoas	Initial	80–100	1–2
	Maintenance	30–60	3–5
GU (hematuria)		40–50	3–5
GI	Initial	80–100	1–3 (plus tranexamic acid)
	Maintenance	30–50	4–7 (plus tranexamic acid)
CNS	Initial	80–100	1–7
	Maintenance	30–50	8–14
Surgery (minor)	Pre-op	50–80	
	Post-op	30–80	1–5 (plus tranexamic acid)
Surgery (major)	Pre-op	80–100	
	Post-op	60–80	1–3 (plus tranexamic acid)
		40–60	4–6 (plus tranexamic acid)
		30–50	7–14 (plus tranexamic acid)

Note that there is no significant difference in desired factor levels needed between hemophilia A and B. Of course, doses and frequency of dosing will differ between hemophilia A and hemophilia B and between different factor concentrates (standard half-life and extended half-life). Also note that for patients on emicizumab or rebalancing therapies guidelines need to be adapted according to more recent studies and recommendations.

CNS, Central nervous system; *GI*, gastrointestinal; *GU*, genitourinary; *rFIX*, recombinant factor IX; *rFVIII*, recombinant factor VIII.

Adapted from Srivastava A, Santagostino E, Dougall A. Treatment of specific hemorrhages. In: WFH Guidelines for the Management of Hemophilia, 3rd edition. *Haemophilia*. 2020;26(Suppl 6):1–58.

with excessive fluid intake. Consequently, many clinicians avoid DDAVP in children below the age of 3, administer it only once per day, and monitor serum sodium levels and fluid intake when it is administered in hospital. The effectiveness of DDAVP decreases with repeated administration (tachyphylaxis) because of exhaustion of VWF/FVIII stores. Therefore, DDAVP should not be given for more than 3 consecutive days.

Most patients with mild hemophilia respond to DDAVP with a doubling or tripling of their FVIII:C levels to 30% or greater. In patients with moderate hemophilia, DDAVP rarely increases FVIII:C levels into a hemostatically effective range. All individuals with mild hemophilia A should be assessed for DDAVP responsiveness. This involves determining FVIII and VWF levels before and 1 hour after DDAVP administration; unlike in cases of type 1 VWD it is not necessary to measure levels 4 hours after dosing.

DDAVP can be administered intravenously, subcutaneously, or intranasally. The usual dose for intravenous administration is 0.3 μg/kg given in 50 mL of normal saline over 30 minutes. The same dose can be given subcutaneously, which enables home administration. For intranasal administration, the usual dose is 150 μg (1 nasal puff) for patients less than 50 kg and 300 μg (1 nasal puff into each nostril) for patients ≥ 50 kg. The maximum effect of DDAVP is achieved within 30 minutes when given intravenously or subcutaneously and within 60 minutes when given intranasally. In general, the safest approach to avoid hyponatremia is to restrict fluids in patients receiving DDAVP and to administer only isotonic solutions such as normal saline.

Because DDAVP increases platelet adhesiveness, its use in patients with risk factors for coronary or cerebral artery thrombosis may result in angina, myocardial infarction, or even stroke. Hence, DDAVP should be used with caution in elderly men with hemophilia. DDAVP can transiently exacerbate bleeding because it also induces the release of tissue plasminogen activator. Consequently, DDAVP is often administered in conjunction with an antifibrinolytic agent.[92]

Antifibrinolytic Agents

Antifibrinolytic agents, such as tranexamic acid or epsilon aminocaproic acid, are useful in the management of mucosal bleeding (e.g., oropharynx, nose, GI tract, and uterine-vaginal lining) and are generally used in the context of oral bleeding or dental surgery. Oral tranexamic acid (25 mg/kg every 6 to 8 hours) should be started 24 hours before the scheduled procedure whereas intravenous tranexamic acid (10 mg/kg every 8 hours) can be given immediately before the procedure. Tranexamic acid tablets each containing 500 mg can be crushed and dissolved in liquids for administration to young children. The usual dosage of epsilon aminocaproic acid is 75 mg/kg every 6 hours. In general, antifibrinolytic agents should be administered for 3 to 7 days after a bleeding episode or an invasive procedure. Longer durations of therapy are required for tonsillectomy–adenoidectomy because bleeding often occurs about 7 to 10 days post-procedure when the eschar detaches. The major contraindication to the use of antifibrinolytic agents is hematuria.

Fibrin Sealants

Fibrin sealants, which enhance local hemostasis and may promote wound healing, are particularly useful for dental procedures, circumcision, and after excision of hemophilic pseudotumors.

Adjunctive Management Strategies for Joint Bleeds

In the management of joint bleeds, appropriate hemostatic support is critical, but other important measures include appropriate joint rest, analgesia, and graduated physiotherapy. The application of rest, ice (for 20 minutes every 3 to 4 hours), compression, and elevation (RICE protocol) for the first 24 hours after a joint bleed is beneficial. The role of joint aspiration is controversial but may be useful for management of persistent pain in large-volume hemarthrosis or for exclusion of septic arthritis. Orthotics and physical therapy are important components of long-term management. Ankle guards and arch supports are useful for patients with ankle arthropathy to improve gait and weight bearing capacity; shoe lifts equalize lower extremity length if there is a discrepancy, and muscle strength training enhances the mechanics of joint movement and helps to protect and stabilize the joint.

Surgery in Hemophilia

With appropriate management (factor replacement/desmopressin with or without other adjunctive hemostatic agents), intra- and post-operative hemorrhages can be prevented in patients with hemophilia thus allowing surgery to be performed safely. Ideally, a multidisciplinary team is required to undertake surgery in a safe manner; physicians with expertise in the management of hemophilia patients should always be involved. Some surgeries might need to be adapted for patients with hemophilia (e.g., bioprosthetic heart valves are preferable to mechanical valves to avoid the need for anticoagulant therapy).

Patients with hemophilia may need to undergo surgeries that individuals without hemophilia typically undergo including dental extraction, circumcision, tonsillectomy, and appendectomy as well as surgeries related to the disease such as CVAD insertion or removal and pseudotumor excision. Surgery can be done safely if patients receive therapy (generally factor concentrate in those without inhibitors and bypassing agents in those with inhibitors). For most surgeries, the goal of therapy is to "normalize" hemostasis during and after the procedure.

The most common surgical procedures in hemophilia are related to long term complications of joint bleeds. These include surgical or arthroscopic synovectomy, nonsurgical (chemical or radionucleotide) synovectomy, arthrodesis procedures, and joint replacement. Such procedures are now uncommon in children who started prophylaxis early in life.

An open surgical or arthroscopic synovectomy removes inflamed and thickened synovial tissue that is a source of joint bleeding. Although synovectomy may reduce the frequency of bleeding, it does not improve joint mobility and may even worsen it.

Radionucleotide synovectomy involves injection of a radioisotope (e.g., yttrium^{90}, chromic phosphate P^{32}) into the joint space to obliterate synovial tissue. Compared with surgical synovectomy, radionucleotide synovectomy is less invasive, is associated with a shorter hospital stay and reduced clotting factor coverage, and is less costly than surgical synovectomy. Radionucleotide synovectomy is particularly useful for patients with inhibitors because there is a lower risk of bleeding. However, the long-term safety of radionucleotide synovectomy in hemophilia is uncertain. Chemical synovectomy (e.g., using rifampicin) requires repeated injections to achieve results like radionucleotide synovectomy. Although radionucleotide synovectomy has replaced chemical synovectomy in many countries, this is not true world-wide.[93]

Arthrodesis (surgical joint fixation) is particularly useful for painful joints with greatly compromised mobility in which joint replacement is problematic (e.g., ankles).

Joint replacement (particularly of the knee, hip, and more recently, ankle) is still commonly performed in adults with hemophilia. The elbow, which is not amenable to arthrodesis or replacement, is often the most difficult joint to manage in patients with hemophilia. The need for joint surgery has lessened with earlier and more universal uptake of prophylaxis in higher-income countries.

Complications of Treatment

Until the mid-1970s, the major impediment to optimal management of patients with hemophilia was the lack of readily administered therapies to prevent or treat bleeds. By the 1980s, such treatments were available, and prophylaxis programs were initiated in many countries. Unfortunately, the 1980s was the era of HIV and hepatitis C, and most patients with hemophilia treated before 1985 became infected with both, with smaller numbers also becoming infected with hepatitis A and B. Many of these patients have died. This tragedy prompted widespread implementation of blood donor screening programs and viral inactivation processes and accelerated the development of recombinant CFCs.

The management of HIV and hepatitis C has advanced since the 1980s. HCV can now be eradicated, and HIV can be fully suppressed in most individuals.

Other Comorbidities in Patients With Hemophilia

With progressive improvements in hemophilia care, persons with hemophilia are living longer, and morbidities associated with aging, such as hypertension and coronary artery disease, are now complicating management. This phenomenon has prompted the development of guidelines for use of antiplatelet regimens for secondary prevention in such patients.[37]

Limitations to Treatment for Hemophilia

Currently, the major impediments in the management of patients with hemophilia are cost, risk of inhibitor development, and to a lesser degree than in the past, the need for repeated IV access.

1. **Cost:** Worldwide, cost is a major issue. Currently available treatments for hemophilia (factor concentrates and emicizumab) are expensive, and prophylaxis regimens with these products pose a considerable cost. However, the cost of factor concentrates has decreased with the introduction of EHL concentrates and emicizumab and in some countries through tendering systems. With other newer products (efanesoctocog and other FVIII mimetics such as Mim8 in development) the cost of currently available products may decrease. Lower pricing would enable less-affluent countries to provide prophylaxis.
2. **Inhibitor development:** The development of an immune response against the replacement therapy (FVIII or FIX concentrates) remains a problem.
3. **Need for frequent intravenous access leading to the need for CVADs:** One of the most difficult aspects of providing hemophilia care has been the need for frequent intravenous administration of factor concentrates leading to in many cases the insertion of CVADs. These have traditionally been very useful for the management of young children with severe hemophilia and in particular those children who develop high titer inhibitors and thus require immune tolerance induction (ITI) therapy (see section on Inhibitors). CVADs are not without their complications, including infections, thrombosis, and malfunction. Several studies have shown that radiographically proven CVAD-associated thrombosis develops in up to 50% of patients with hemophilia with these devices. Such thrombi may impair CVAD function, thereby necessitating their removal. Additionally, to insert CVADs necessitates a surgical procedure and eventually such CVADs will need to be removed necessitating more surgery. Now with EHL factors (especially EHL FIX and in the years to come other even more EHL FVIIIs like efanesoctocog) and with emicizumab there is less need to insert CVADs in young children, and so CVADs will certainly now have a diminished role in hemophilia management.

INHIBITORS IN HEMOPHILIA

The formation of neutralizing antibodies, or inhibitors against FVIII or FIX, has for some time been the most important complication of therapy in hemophilia now that available replacement products are free from the risk of transmission of infectious agents such as HCV or HIV. Inhibitors render factor concentrate replacement therapy ineffective. As a result, patients who develop inhibitors have until recently suffered from increased risk of bleeding, decreased quality of life, and increased risk of mortality. The advent of emicizumab, and potentially in the future other non-factor therapies, is fortunately changing this.

Autoantibodies to FVIII or to FIX can be detected in non-hemophilic persons, presumably reflecting incomplete immunologic tolerance to antigens in these proteins.[94] Pathologic *autoantibodies* can occur in autoimmune disorders such as acquired hemophilia. Persons with hemophilia A or B, on the other hand, can generate *alloantibodies* to epitopes present on infused factor molecules. Because these people have *F8* or *F9* mutations that result in the synthesis of altered FVIII or FIX or in the total absence of these proteins, these alloantibodies can be considered the products of physiologic immune responses to exogenous antigens. In both hemophilia A and B, inhibitors are IgG molecules, with IgG4 subclass being the predominant isotype.

Epidemiology and Clinical Manifestations of Inhibitors

Inhibitors arise more frequently in patients with severe hemophilia A than in those with severe hemophilia B (about 30% vs. up to 10%)

treated with replacement therapy. Inhibitors are less frequent in patients with non-severe hemophilia, particularly in hemophilia B, although some specific F8 genotypes can be associated with an increased risk of inhibitors even with moderate or mild hemophilia A.[95]

Most inhibitors arise within the first 10 to 20 exposures to either FVIII or FIX and consequently, most inhibitors are detected in very young children—these are referred to as previously untreated patients (PUPs).[96] If PUPs get to 50 exposures to factor (and hence are relabeled as being PTPs, previously treated patients) without developing an inhibitor, they are at a low (but not zero) risk of developing an inhibitor later in life.

In severe forms of hemophilia, inhibitor development usually occurs early in life because prophylactic therapy with FVIII or FIX is started at a young age and patients will quickly receive 10 to 20 exposures. This may change with the introduction of emicizumab, which may delay inhibitor development by years or even decades.[80,97] Even now, some patients with severe hemophilia from less-affluent countries with limited access to factor replacement therapy develop inhibitors at later ages because of reduced exposure to replacement products.

Inhibitor surveillance practices are evolving now that many PUPs are receiving emicizumab prophylaxis and have only limited exposure to factor concentrates in the context of managing bleeds or surgery. There is concern (not proven) that such patients being only exposed to factor in "danger" settings such as bleeds or surgery may be at a higher risk of inhibitor development. Research regarding inhibitor reduction strategies in this population (for example, with regular low-dose exposure of FVIII to elicit tolerization) is ongoing (NCT04030052).

Inhibitors inactivate the infused factor, which decreases or abolishes its effectiveness for prophylaxis or for treatment of bleeds. Inhibitors in patients with mild or moderate hemophilia may transform such patients to a more severe phenotype because they neutralize the individual's endogenous factor.

Diagnosis of Inhibitors

Clinical

The presence of an inhibitor may be suspected if the recovery of FVIII or FIX is less than expected after infusion (see Chapter 127) or if there is an anaphylactic reaction to FIX concentrate; anaphylactic reactions to FVIII concentrate are rare. Alternatively, they may be suspected by finding reduced factor levels immediately after infusion of factor.

Laboratory

Inhibitors are diagnosed, quantified, and classified with the Bethesda assay (see Chapter 126). This is a standard laboratory test that measures the amount of inhibitory activity against FVIII or FIX. Patient plasma is incubated with normal plasma for 2 hours at 37°C, and residual factor activity is then calculated. As a control, normal plasma is diluted with buffer, and factor activity is determined and compared with the result in patient plasma (Fig. 134.12). The Nijmegen modification of the Bethesda titer differs from the "classical" Bethesda assay in two respects: the normal plasma used is buffered to pH 7.4 with imidazole, and the control mixture is made by diluting normal plasma with FVIII deficient plasma, rather than with buffer.[98] The Nijmegen modification is more specific for detection of low-titer inhibitors than the classical Bethesda assay (i.e., it results in fewer false-positive results) and, if available, is the preferred method.

The Bethesda titer is measured in Bethesda units (BU), where 1 BU is the amount of inhibitory activity required to reduce the coagulant activity of the target factor in plasma by 50%. Consequently, an inhibitor titer of 1 BU will reduce factor activity by 50% while a titer of 2 BU will reduce activity by 75%, etc. Consequently, inhibitor titers of 5 BU or more will reduce factor activity by at least 97%, thereby rendering infusion of the factor therapeutically useless. Using the Bethesda assay, inhibitors are classified as high titer (≥5 BU) and low titer (<5 BU). This separation is important because low-titer inhibitors may be transient and bleeds in patients with low-titer inhibitors may respond to high doses of FVIII or FIX. In contrast, high titer inhibitors are usually persistent and bleeds in patients with such inhibitors usually require treatment with bypassing agents. Additionally, the higher the inhibitor titer, the less likely the success of inhibitor eradication strategies.

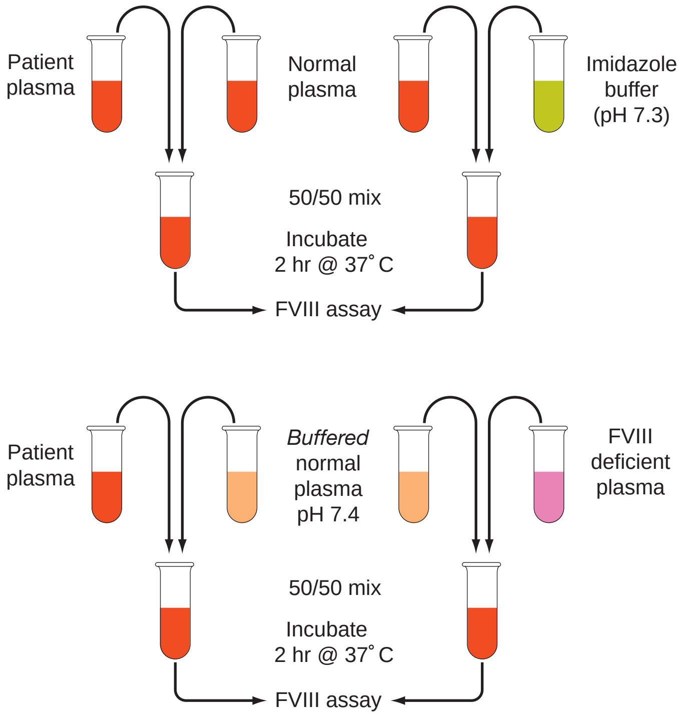


Figure 134.12 THE BETHESDA AND NIJMEGEN-MODIFIED BETHESDA ASSAYS. In the classic Bethesda assay *(top)*, patient plasma is incubated with normal plasma for 2 hours at 37°C, and residual factor activity is then quantified. As a control, normal plasma is diluted with buffer, and factor activity is determined and compared with that measured in the patient sample. The Nijmegen modification of the Bethesda assay *(bottom)* differs in two respects: first, normal plasma is buffered to pH 7.4 with imidazole, and second, the control mixture is made by diluting normal plasma with factor VIII (FVIII) deficient plasma, rather than buffer. A heat inactivation step can be used to inactivate residual FVIII in the patient plasma.

INHIBITORS IN HEMOPHILIA A

Pathophysiology of Inhibitor Development in Hemophilia A

FVIII is a peculiar protein, in that immunologic tolerance to FVIII in people without hemophilia A is not always complete. Rarely, because of autoimmune disease, immunologic tolerance to FVIII fails, and autoantibodies develop; this is known as acquired hemophilia A. Antibodies to FVIII, detectable by ELISA, can be found in the plasma of some individuals without congenital FVIII deficiency. T cells that are reactive to FVIII can also be found in normal individuals, although this reactivity is transient and less intense than that in hemophilia A patients.[99,100]

Only about one-third of patients with hemophilia A exposed to FVIII develop inhibitors. Reasons for inhibitor development are incompletely understood although some of the known risk factors for inhibitor development suggest possible explanations. For example, patients with large *F8* gene deletions and other mutations (referred to as null mutations) that cause an absence of any synthesized FVIII peptide (also referred to as cross-reactive material [CRM] negative) are at increased risk, suggesting that patients with some synthesized protein (CRM positive) may be tolerant to the epitopes that are produced. It has also been hypothesized that exposure to maternal FVIII *in utero* via maternal-fetal hemorrhage or early in life induces immunologic tolerance in the fetus.[101] However, the risk of inhibitor development is unaffected by intrauterine procedures such as amniocentesis[102] or breastfeeding.[103]

The development of FVIII inhibitors reflects a classical T-cell dependent immune response that starts with antigen presenting cells (APCs), mostly dendritic cells (DCs), which endocytose the encountered FVIII. How FVIII enters the DCs is unclear but the macrophage mannose receptor (MMR) on DCs appears to be involved.[104] After endocytosis, FVIII is processed into peptides and together with MHC class II molecules is presented on the surface of the APC to naive CD4$^+$ T cells in secondary lymphoid organs (spleen and lymph nodes).[105] In addition to the FVIII-derived peptide/MHC complex, co-stimulatory signals (ligands and receptors on the surface of T cells and APCs and pro-inflammatory stimuli such as cytokines) are necessary to fully activate naive T cells and enable binding to FVIII epitopes. If the recognition of FVIII-derived peptide by the T-cell receptor leads to the activation of specific T cells, these will migrate and activate B cells that have internalized the FVIII protein through their B-cell receptor and can present peptides in the MHC class II complex to the activated T cells. Activated naive FVIII-specific T cells consequently differentiate into effector helper CD4$^+$ T cells, which in turn activate FVIII-specific B cells, allowing them to undergo class switching and differentiate into FVIII-specific memory B cells or into plasma cells (that can be very long-lived) that produce low affinity anti-FVIII antibodies (Fig. 134.13).[104]

This differentiation is dependent on binding of the B-cell receptor, a membrane receptor in the immunoglobulin superfamily of receptors, to the antigen for which it has specificity, and subsequent interaction with a CD4$^+$ helper T cell. Numerous lines of evidence support the important role of helper T cells in the genesis of inhibitors. Thus, T-cell proliferation in response to FVIII in hemophilia A patients is greater in those with inhibitors than in those without.[106] Furthermore, blockade of CD3, a component of the T-cell receptor complex, decreases inhibitor formation in vitro,[107] as does stimulation of cytotoxic T-lymphocyte antigen 4 (CTLA-4), an inhibitory receptor involved in the downregulation of T-cell stimulation.[108] Finally, inhibitor titers often decrease in hemophilia A patients with HIV, particularly those with very low CD4$^+$ T-cells counts[109] and effective inhibitor development requires co-stimulation of T cells by APCs via the CD40/CD40 ligand or CD28/B7 pathway.[110]

Upon subsequent exposure to FVIII, memory B cells generated during the primary immune response are rapidly restimulated to proliferate and differentiate into antibody-secreting plasma cells that secrete high-affinity neutralizing antibodies.

The need for costimulatory signals in the process of forming antibodies fits with the "danger theory" proposed by P. Matzinger. The theory proposes that exposure to exogenous antigens can result in immunologic tolerance or in an immunologic response, depending on the state of the immune system at the time of exposure. Inhibitor development is more likely to occur when the immune system is exposed to FVIII in the setting of danger, a situation characterized by tissue injury such as occurs with large bleeds, infection, or surgery.[111] These events could trigger the immune system to activate a defense response to the infused clotting factor and potentially lead to an immune response to FVIII. The same theory suggests that if patients are exposed to FVIII in the absence of these danger signals, the immune system may initiate a response that leads to tolerance through the generation of T regulatory cells (Tregs). The possible role of "danger signals" or "alarm signals" in the development of inhibitors in hemophilia patients has stimulated a large body of research.[112] Investigations in hemophilic mice, and clinical observations in hemophilia A patients challenge the danger signal hypothesis, as discussed below in the section Treatment-Related and Modifiable Risk Factors in Hemophilia A. Recent evidence highlights the role of counteractive tolerogenic responses and not "danger signals" per se in inhibitor development.[113]

Tregs that suppress the activity of helper T cells may determine whether an individual patient will be immunologically reactive or

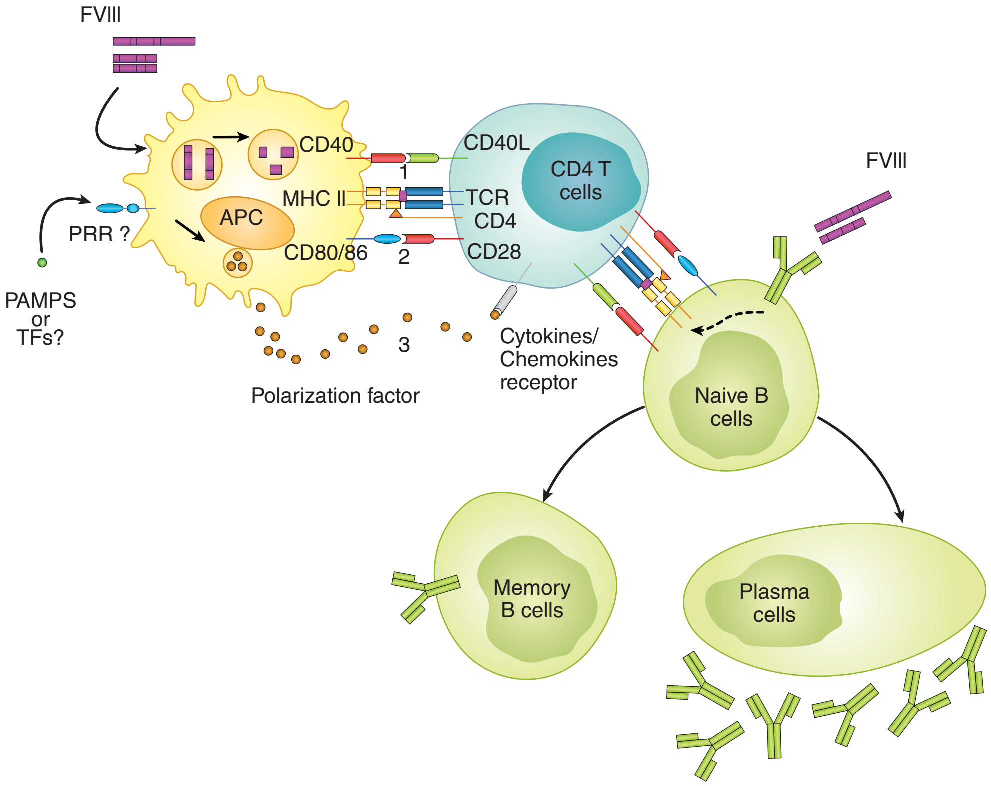


Figure 134.13 DEVELOPMENT OF FACTOR VIII (FVIII) INHIBITORS. The process starts with antigen presenting cells (APCs), which endocytose FVIII. The engulfed FVIII is processed into peptides and presented on the surface of the APC along with MHC class II molecules to CD4$^+$ T cells in the spleen and lymph nodes. Co-stimulatory signals (such as cytokines) are required for full activation of naive T cells capable of binding FVIII epitopes. T cells migrate and activate naive B cells, which undergo class switching and differentiate into FVIII-specific memory B cells or plasma cells that produce anti-FVIII antibodies.

tolerant to FVIII. T-cell proliferation in response to FVIII stimulation has been observed in Treg-depleted peripheral blood from normal (nonhemophiliac) human subjects.[99] Hemophilia A mice had lower inhibitor titers in response to FVIII when co-infused with Tregs taken from wild-type mice.[114] These observations are consistent with a role for Tregs in determining the immunologic response of hemophilia A patients to exogenous FVIII, and in tolerance to FVIII in normal individuals.[115]

FVIII inhibitors in patients with hemophilia A are predominantly of the IgG1 and IgG4 subclasses. Longitudinal analysis of the characteristics of total FVIII-binding antibody in PUPs exposed to FVIII confirms the results of previous studies and shows that high-affinity IgG1 antibodies appear first, followed by IgG3 and IgG4 anti-FVIII antibodies in patients who developed high titer inhibitors.[116]

FVIII epitopes for inhibitory antibodies have been identified in several locations in the FVIII molecule: for example, in a site in the A2 domain that is involved in the function of the tenase complex; in the FIX-binding site in the A3 domain; and in the phospholipid-binding and VWF-binding sites in the C2 domain.[116–118] The localization of these epitopes to functionally important regions of the FVIII molecule explains why these antibodies inhibit the coagulant activity of FVIII. In contrast, antibodies directed against the non-functional B domain do not appear to have inhibitory activity.

Inhibitors may be classified by the kinetics of their in vitro ability to inactivate FVIII. *Type I* inhibitors are characterized by a linear relationship between the antibody concentration and the logarithm of the residual FVIII activity; at high antibody concentrations, the inhibition of FVIII is near total. FVIII inhibitors in patients with congenital hemophilia A usually have type I kinetics. Inhibitors with *type II* kinetics do not display a linear relationship, and even high antibody concentrations do not result in complete inhibition of FVIII activity in vitro. Type II inhibitors are commonly found in acquired hemophilia A.[98] Whether an inhibitor has Type I or Type II kinetics may depend on the epitopes targeted, which could explain why antibodies with Type II kinetics are more common in acquired hemophilia A where antibodies tend to be oligoclonal and directed against epitopes less likely to result in Type I kinetics.

Other Immune Responses to FVIII

Several other immunologic reactions to FVIII are possible. These include *anaphylactic reactions*, *catalytic antibodies*, and *non-neutralizing antibodies* (NNAs). Except for anaphylactic reactions, which are of obvious importance, the clinical significance of other reactions is not clear.

Anaphylactic Reactions

Anaphylactic reactions (type 1 hypersensitivity reactions) to FVIII are rare and vary in severity. Such reactions have been reported in association with infusion of both plasma-derived and recombinant FVIII concentrates.119 Interestingly, evidence of IgE mediation is not a universal finding suggesting that some reactions may be mediated by complement activation, immune complexes, or through other mechanisms or that the trigger may be an excipient in these products, and not the FVIII.120

Because of the rarity of anaphylactic reactions to FVIII, their optimal management is unclear. Acutely, the usual supportive care for anaphylaxis should be provided. Subsequent exposures to FVIII-containing products should be attempted with caution, and with consideration of pre-medication.

The association of anaphylactic reactions to FVIII with inhibitor development is not clear. This contrasts with the situation in hemophilia B, in which anaphylaxis to FIX concentrates is often associated with inhibitor development. The reasons for these differences are unknown. It is possible that distribution of FIX to extravascular tissues is more likely to provoke such a reaction than intravascular distribution of FVIII. It is also possible that therapeutic doses of FIX, which contain more protein than therapeutic doses of FVIII, are a more potent stimulus for anaphylaxis.

Non-neutralizing Antibodies

Some antibodies to FVIII do not affect coagulant activity because they are directed against non-functional epitopes. These NNAs are detectable with ELISA but not with the Bethesda assay. NNAs to FVIII have been reported in approximately 15% to 30% of patients and can occur with any disease severity.[121] NNAs against FVIII may also be detected in healthy individuals. It has been proposed that NNAs in persons with hemophilia, particularly those of high titer, accelerate clearance, thereby shortening the half-life of infused factor, but this has not been a consistent finding.[122]

There are many unanswered but important questions about NNAs, not least of which is the issue of their proposed ability to alter the kinetics of infused factor and the clinical importance of this phenomenon. It is also unknown whether NNAs are a predictor or precursor of neutralizing antibodies (i.e., inhibitors) in some patients. In patients who have undergone immune tolerance induction (ITI; discussed later), it is not known whether persistent NNAs should be considered a form of failure of ITI, or whether persistence predicts a risk of inhibitor recurrence.[116]

Epidemiology

The reported incidence and prevalence of FVIII inhibitors in hemophilia A patients vary widely depending on the patient population studied, frequency of testing, and sensitivity of inhibitor assay.

A systematic review[123] that included several studies of PUPs estimated that the cumulative incidence of inhibitors varied from 0% to 39%. In PUPs with severe hemophilia A, the average inhibitor incidence was 23.1%, which is consistent with more recent estimates from large studies.[20,68,124] Patients with severe hemophilia A who develop FVIII inhibitors are most likely to do so early in the course of their treatment: inhibitor detection has been reported after a median of 9 to 36 EDs, and the incidence of inhibitor development plateaus at 50 EDs.[68,123]

In the same systematic review, the incidence of inhibitor development in persons with moderate hemophilia A was 7.9%. More recent data on mild/moderate hemophilia A patients with at least 100 EDs found an incidence of 13%, suggesting that inhibitors occur later in patients with non-severe hemophilia A, in one report at a median age of approximately 50 years and after a median of 30 EDs with a substantial proportion occurring between 50 and 100 EDs.[95]

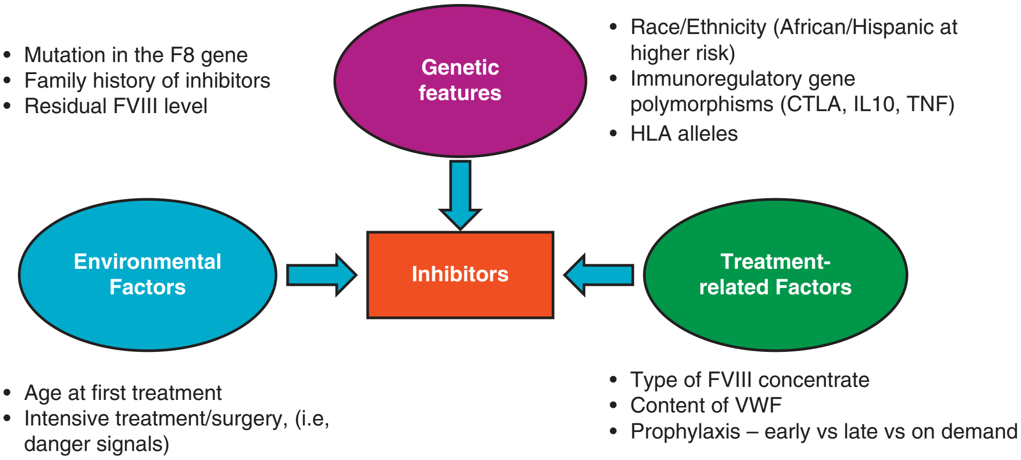
Genetic (Non-Modifiable) Risk Factors for Inhibitor Development in Hemophilia A (Fig. 134.14)

F8 genotype, which correlates with hemophilia severity, is the most important risk factor for FVIII inhibitors, with large deletions, nonsense mutations, and inversions of introns 1 and 22 associated with a higher risk than missense mutations. Even in mild/moderate hemophilia A, certain mutations in the A3 and C2 domains in the FVIII light chain confer an increased risk of inhibitor development, consistent with the observation that inhibitors are frequently directed against epitopes in these domains.[25]

Several other genetic loci also influence inhibitor risk, including polymorphisms in *TNF, CTLA4, IL10*, and *FCGR2A*. Some MHC II alleles may be involved as well. Ethnicity (African American or Hispanic) and a family history of inhibitors are also associated with increased inhibitor risk, supporting the concept of genetic determinants of risk.

Treatment-Related and Modifiable Risk Factors

How patients are treated may also influence inhibitor risk. Younger age at first FVIII exposure had been thought to be associated with increasing risk but this was likely due to the confounding effect of intense treatment at first exposure, which is itself a risk factor.[68,69]



Mutation in the F8 gene
Family history of inhibitors
Residual FVIII level
Genetic features
Race/Ethnicity (African/Hispanic at higher risk)
Immunoregulatory gene polymorphisms (CTLA, IL10, TNF)
HLA alleles
Environmental Factors
Inhibitors
Treatment-related Factors
Age at first treatment
Intensive treatment/surgery, (i.e, danger signals)
Type of FVIII concentrate
Content of VWF
Prophylaxis – early vs late vs on demand

Figure 134.14 RISK FACTORS FOR THE DEVELOPMENT OF INHIBITORS IN HEMOPHILIA A. *FVIII*, Factor VIII; *VWF*, von Willebrand factor.

There is some evidence that administering FVIII in a non-inflammatory state, that is, in a prophylactic setting, may reduce inhibitor development in contrast to administering it in the setting of a bleed.[68] This effect may be most pronounced in patients with low-risk *F8* mutations while it may have little or no impact in those with high-risk F8 mutations.[69] The role of prophylaxis in reducing inhibitor development may be related to its associated reduction in the need for intense exposure to FVIII for treatment of bleeds, intense exposure being defined as exposure to FVIII on 3 or more consecutive days.[69]

Avoidance of exposure to FVIII with other antigens, particularly antigens in adjuvant containing childhood vaccines, has been proposed to decrease risk.[125,126] More recent data suggests that this is not the case. In a hemophilia A mouse model, concurrent exposure to FVIII and influenza vaccine antigens results in competition for the immune system's "attention" between the antigens and results in no increase in inhibitor development.[127] Likewise, the PedNet group reported no increase in the risk of inhibitor development when routine childhood vaccines were given in close temporal proximity to FVIII.[128]

A prospective cohort study designed to test the hypothesis that starting low-frequency FVIII prophylaxis early would reduce the risk of inhibitor development by minimizing danger signals was stopped early because 42.1% of patients developed inhibitors.[129]

Surgery has been associated with inhibitor development, both in general[130] and when it represents the first exposure to FVIII.[68] In a meta-analysis of observational studies, the increased risk of inhibitor development associated with surgery was independent of the risk associated with the intensity of FVIII treatment.[130] Minor surgery, such as CVAD placement, may confer less risk than major surgery.[68]

Continuous infusion of FVIII independent of intensity of exposure to FVIII has also been suggested as a risk factor in the setting of mild/moderate hemophilia.[90]

Although these observations provide some support for the "danger signal" hypothesis of immunogenicity, epidemiological data suggest a more complex pathogenesis. Chronic inflammation associated with recurrent bleeding as well as acute hemarthrosis after knee injury in FVIII-deficient mice failed to increase the immune response to exogenous FVIII.[131] Overall, recent data suggest that the FVIII-specific immune response reflects the immune system's inability to develop a suppressive antigen-specific tolerogenic response, rather than excessive activation of the immune system in response to FVIII administration.[113,132]

The rate of inhibitor development has generally been assumed to be similar among the various plasma-derived and recombinant products. The results of some studies challenge this concept. The Study on Inhibitors in Plasma-Product Exposed Toddlers (SIPPET) study, a randomized comparison of inhibitor incidence across several brands of rFVIII and several VWF containing pdFVIII concentrates in PUPs, found a 1.87-fold increase in the incidence of inhibitors in patients treated with rFVIII.[20] This effect was particularly pronounced in patients with *F8* mutations predicted to result in a low risk of inhibitors, in whom the use of pdFVIII virtually eliminated the occurrence of inhibitors. Other studies however, including the PedNet study, found no significant difference in inhibitor development between plasma-derived and recombinant FVIII concentrates. However, the PedNet group and several others reported a higher rate of inhibitor development with a second-generation recombinant FVIII than with a third-generation recombinant FVIII.[20,124] The mechanistic underpinnings of these observations are not understood. Although the findings of these observational studies have been questioned, the results resulted in greater use of plasma-derived FVIII and reduced use of the second-generation recombinant FVIII product. With the current switch to EHL FVIIIs and emicizumab, the potential ramifications of these earlier studies have lessened.

While most rFVIII products have been manufactured using non-human mammalian cells (BHK or CHO cells), two rFVIII products (a SHL product—Nuwiq and an EHL product—FVIII-Fc [Eloctate/Elocta]) are manufactured in a human cell line (HEK293) and therefore bear "human-like" post-translational modifications, which could reduce the risk of inhibitor development. Although the pivotal PUP studies with these products reported a slightly lower rate of high titer inhibitors than that observed with rFVIII expressed in non-human cells, the differences were not statistically significant.[133] (See box on Choosing A Factor Concentrate for PUPs with Severe Hemophilia A to Minimize Inhibitor Risk.)

MANAGEMENT OF INHIBITORS IN HEMOPHILIA A

There are several management considerations in patients with FVIII inhibitors. These include the management of bleeds or surgery, the delivery of effective prophylaxis, and the initiation of an inhibitor elimination strategy. In the past few years with the introduction of emicizumab much has changed about the management of patients with inhibitors to FVIII.

Treatment of Bleeds

Patients with low titer inhibitors (<5 BU) can often be treated with FVIII using conventional doses, or more commonly, higher doses. Those with high titer inhibitors (≥5 BU) will not respond to typical factor replacement and require a "bypassing agent"—activated prothrombin complex concentrate (aPCC) or recombinant activated factor VII (rFVIIa).

aPCC is a plasma-derived product that contains factors II, VII, IX, and X, as well as the activated forms of these factors. The efficacy of aPCC for treatment of bleeds and for provision of effective hemostasis during surgical procedures has been established in patients with FVIII inhibitors.[134,135] Typical dosing is 85 IU/kg, given in a schedule

CHOOSING A FACTOR CONCENTRATE FOR PREVIOUSLY UNTREATED PATIENTS WITH SEVERE HEMOPHILIA A TO MINIMIZE INHIBITOR RISK

The choice of FVIII concentrate for a patient with severe hemophilia A is one of the most important medical decisions made for patients. There are several factors that can go into this decision, including the FVIII products that are available, the knowledge and comfort that the prescribing clinician has with those products, the pharmacokinetic properties of the FVIII (SHL vs. EHL), and patient/family preference.

It is uncertain whether the choice of FVIII affects the risk of inhibitor development. There are studies that support and studies that refute the premise that plasma-derived VWF-containing FVIII concentrates are associated with less inhibitor development. There are however no studies suggesting a higher rate of inhibitor development with plasma-derived FVIII. Even if plasma-derived FVIII concentrates were conclusively shown to have a lower incidence of inhibitor development some patients, particularly those in families impacted by HIV and hepatitis C infection, may not be comfortable with pdFVIII concentrates; many clinicians likely share this discomfort.

There is hope that EHL rFVIII being “shielded” by linkage to Fc or PEG may be associated with a lower risk of inhibitor development than “older” rFVIII products. This possibility requires confirmation in a comparative study. As a randomized comparison is unlikely to occur, the community will need to wait for large population-level data from adverse events surveillance programs to determine this.

With availability of emicizumab, PUPs are likely to start on it at an early age so that their exposure to FVIII will be limited to treatment of bleeds or surgery. Having a FVIII that results in less inhibitor development would be of crucial importance in such patients.

dependent on the clinical situation: in general, no more than every 6 to 8 hours. aPCC contains trace amounts of FVIII that can be sufficient to trigger an anamnestic response in patients with FVIII inhibitors; for this reason, clinicians avoid aPCC until the inhibitor titer has decreased (see below).

rFVIIa activates FX through the extrinsic pathway without the need for FVIII. For treatment of acute bleeds in patients with FVIII inhibitors, rFVIIa has been shown to be effective at a dose of 90 μg/kg; repeated doses may be required because the half-life of rFVIIa is only about 2 hours.[136] A single higher dose of 270 μg/kg is equally effective and safe and requires less frequent intravenous access, so this may be considered for bleeds that are anticipated to require more aggressive management, such as bleeding into target joints.[137] rFVIIa provides effective hemostasis during surgery.[138] Although rFVIIa has been administered by continuous infusion, bolus doses are preferred to generate bursts of thrombin (see Chapter 125).

Patients with high titer inhibitors not on emicizumab can be treated with either aPCC or rFVIIa. A randomized crossover comparison of rFVIIa and aPCC demonstrated similar overall efficacy in controlling acute joint bleeds with the two products, but their effectiveness was rated differently by approximately 30% of patients.[134] Currently, no laboratory test has been shown to predict clinical response to these agents. The choice of product for a particular patient, then, is a matter of clinical judgment, patient preference, trial-and-error, and their safety when given to patients on emicizumab.

For patients with severe or refractory bleeding, simultaneous or sequential use of rFVIIa and aPCC can be considered. However, there is a risk of serious thrombotic complications with this approach, even in younger patients. This risk may reflect the accumulation of aPCC components with long half-lives (such as FX or prothrombin) or potentiation of the effects of rFVIIa by aPCC components.[139] Combination therapy should be reserved, therefore, for severe or life-threatening bleeds for which single-agent therapy is insufficient.

Antifibrinolytics (epsilon-aminocaproic acid or tranexamic acid) may be useful adjuncts to bypassing agents, for management of bleeds or in the context of surgery.[140] Anti-fibrinolytics are thought to be safe in combination with rFVIIa, but their safety in combination with aPCC is less certain.[141]

Plasma-derived porcine FVIII, which can evade FVIII inhibitors because it lacks key epitopes against which antibodies are directed, was used in the past. A recombinant porcine FVIII concentrate (Obizur, Takeda) is now available. Although originally developed for use in acquired hemophilia A, there are reports of its successful use in patients with congenital hemophilia A with inhibitors. The plasma-derived porcine FVIII concentrate is no longer available because of adverse reactions such as thrombocytopenia and allergy, and concerns about potential infectious contaminants.

Prophylaxis in Patients With Inhibitors to FVIII

For patients with severe hemophilia without inhibitors, prophylactic therapy is the standard of care.[37] However, for patients with inhibitors, particularly high-titer inhibitors, prophylaxis with conventional doses of FVIII or FIX concentrates is ineffective. Until recently, prophylaxis for such patients relied on bypassing agents (rFVIIa or aPCC). Although effective for prophylaxis in this setting, they are less effective than prophylaxis with clotting factor replacement in patients without inhibitors.[142,143] Consequently, rFVIIa and aPCC have been supplanted by emicizumab.

Emicizumab provides effective prophylaxis against bleeding in patients with inhibitors and is superior to bypassing agents (HAVEN 1, 2). The effectiveness of emicizumab for prophylaxis in young children with inhibitors (HAVEN 2) was dramatic; 77% of children treated with emicizumab experienced no bleeding, and there was a 99% reduction in the annualized bleeding rate in 15 children who had previously been receiving bypassing agent prophylaxis.[79]

The superior prophylaxis provided by emicizumab and its convenience of administration have rendered it the preferred therapy for patients with high titer inhibitors.

Patients with inhibitors taking emicizumab can have breakthrough bleeds. Such bleeds need to be treated with bypassing agents. Although rFVIIa appears to be safe in this setting, venous thromboembolism and thrombotic microangiopathy (TMA) have been reported with aPCC, particularly if the daily dose of aPCC exceeds100 U/kg or if it is given for more than one day. This prompted an FDA black box warning against the use of aPCC in patients taking emicizumab except for those with life-threatening conditions where close clinical monitoring and low doses of aPCC are recommended.

Eradication of Inhibitors in Hemophilia A (ITI)

When a patient develops a FVIII inhibitor, he, his family, and their clinician must decide whether to attempt eradication with ITI therapy. In the past, the decision to undertake ITI was relatively straightforward because without it, patients would remain inhibitor positive resulting in increased morbidity and mortality and a significantly increased cost of care. The decision is less straightforward now that emicizumab is available.

ITI, which consists of regular and frequent administration of high doses of replacement factor concentrate, is the basic therapeutic strategy for eliminating inhibitory antibodies. In addition to factor concentrates, ITI regimens may include immunosuppressive agents or procedures such as plasmapheresis to deplete existing antibodies. ITI regimens (Bonn, Malmo, Dutch, high dose, low dose) vary with respect to the intensity and frequency of FVIII exposure and in the use and choice of concomitant immune suppression (in particular, rituximab).[144,145]

For patients (especially young children), the burden of frequent (usually daily) intravenous infusion of factor for a year or longer is considerable and for healthcare systems, the cost of ITI is enormous. CVADs are essential for undertaking ITI in young children. Despite the drawbacks of ITI, it is recommended over the alternative of allowing a child to remain with an inhibitor life-long.

The need for ITI is less clear now that emicizumab is available. Patients unlikely to respond to ITI such as those with very high inhibitor titers, those who failed to respond to previous attempts at ITI, or those unable to tolerate frequent doses of replacement therapy, may elect to take emicizumab instead of undergoing ITI. However, even with emicizumab, there may be benefits of ITI because, if successful, it allows the use of FVIII to treat breakthrough bleeds instead of rFVIIa, and it enhances access to trials of gene therapy. Finally, it is hypothesized that regular FVIII exposure may improve bone health.[146] Even if ITI is chosen, most patients are likely to be given concomitant emicizumab because it enables less intensive ITI regimens (i.e., non-daily) which reduces the need for CVADs. The use of ITI in conjunction with emicizumab is still being investigated for safety and efficacy.[147,148]

Low titer inhibitors (i.e., <5 BU) may resolve spontaneously, with or without continued exposure to FVIII. Patients with low titer inhibitors may be observed, and treatment to eliminate the inhibitor initiated only if the inhibitor titer increases. Patients with low titer inhibitors should still have a planned strategy for treating breakthrough bleeding and consideration should be given to prophylaxis.

The mechanisms through which ITI eradicates FVIII inhibitors are largely unknown. The relative proportions of different IgG subclasses may change with ITI, and the disappearance of anti-FVIII IgG4 is associated with success.[149] In a mouse model of hemophilia A, exposure to high concentrations of FVIII prevents FVIII-specific memory B cells from differentiating into antibody-secreting plasma cells in a T-cell-independent manner. Inhibition of B-cell differentiation involves caspase activation, suggesting that FVIII induces memory B cells to undergo apoptosis.[150] If these findings are correct, ITI is likely to be most successful if started soon after inhibitor development. However, even after successful ITI, some patients have residual anti-FVIII antibodies detectable by ELISA and FVIII-reactive T cells. Anti-idiotypic antibodies that neutralize anti-FVIII antibodies may account for the reduced FVIII inhibition[151,152] and emergence of T and B regulatory cells may restore tolerance to FVIII.[153]

Typically, ITI is considered successful if the patient has no detectable inhibitor by Bethesda assay (≤0.6 BU) and if infused FVIII displays normal pharmacokinetics (recovery and half-life).[144] The overall success rate of ITI ranges from 60% to 80%.[154] Several patient-related and treatment-related factors predict the success of ITI. The most important patient-related factor is the peak Bethesda inhibitor titer (prior to ITI); the lower the peak titer, the greater the chance of success with ITI. A low peak Bethesda titer during ITI may also predict success. Although a low Bethesda titer (<10 BU) at the start of ITI has been found to be a good predictor of ITI success this does not necessarily imply that clinicians should wait to start ITI until the inhibitor titer drops to less than 10 BU in those patients who develop peak titers of greater than 10 BU pre-ITI. A short interval between the detection of an inhibitor and initiation of ITI also increases the likelihood of success; consequently, some clinicians start ITI early, even if the inhibitor titer is greater than 10 BU.

The nature of the *F8* mutation may be an important predictor of ITI success. Missense mutations and small deletions and insertions confer a greater chance of success than large deletions, inversions, splice site mutations, or nonsense mutations.[155] Patient ethnicity may play a role; African ancestry is associated not only with a higher risk of inhibitor development but also with reduced rates of successful ITI.[156]

The influence of FVIII dose on ITI success is controversial. In the International ITI trial which randomized patients with “good risk” features to a high-dose regimen (200 IU/kg daily) or a lower-dose regimen (50 IU/kg given three times per week), the overall success rate was similar between the two regimens but the response was more rapid with the high-dose regimen.[144] This trial was stopped early because there were more bleeds with the low-dose regimen. Therefore, high-dose ITI regimens have been preferred both in patients with and without good risk features for ITI success.

Given the expense of FVIII concentrates, there is interest in low-dose ITI regimens that are not only more affordable but may be sufficient to prevent breakthrough bleeds in patients taking concomitant emicizumab.[157]

Some experts speculate that plasma-derived concentrates that contain VWF or EHL FVIII concentrates may be more effective for ITI, particularly for patients who have failed to respond to ITI with other products or who have poor risk features for successful ITI. This speculation is based on the concept that FVIII in these products is shielded by binding to VWF or by linkage to Fc or by PEGylation.[158,159] In addition, the Fc component of rFVIII-Fc may have antigen-specific effects on T cells and B cells.[160–162] Despite such speculation, there is no conclusive evidence that some products are better than others for ITI.

Rituximab (monoclonal anti-CD20, a B cell-depleting antibody) has been used successfully in conjunction with FVIII in patients with inhibitors refractory to conventional ITI.[145] Other immunosuppressive agents can also be considered, but their utility is uncertain.[147]

ITI fails in 20% to 40% of patients at first attempt, and even if successful, up to 30% of patients will show a recurrence of their inhibitor.[163] Although repeated course of ITI, addition of immunosuppressive agents, or changes in the FVIII product were used in such patients, these are rarely needed now that effective prophylaxis for patients with inhibitors is enabled with emicizumab.

The preceding discussion primarily applies to patients with severe hemophilia A. The situation is quite different for patients with moderate or mild disease who develop inhibitors. Many such patients will experience a spontaneous remission of their inhibitor without specific therapy. Other patients may require treatment to eliminate the inhibitor, but there is no clear advantage of ITI over a variety of immunosuppressive therapies. Eradication of inhibitors in patients with mild or moderate disease may be beneficial if the inhibitor is of sufficient titer to increase the risk of bleeding. However, as with severe hemophilia A the likelihood of undertaking ITI in mild/moderate hemophilia is lessened with emicizumab.

Prophylaxis of Patients During Immune Tolerance Induction

Patients who are receiving FVIII for ITI are in essence receiving a form of prophylactic therapy. This was seen in the International ITI study where patients receiving high-dose ITI (larger and more frequent doses of FVIII) experienced fewer bleeds than those placed on the low-dose regimen. This is suggestive of some short-lived protective activity of FVIII.[144] Although high titer inhibitors should theoretically inactivate even high doses of FVIII, there may be a short period of time before complete neutralization by antibodies which may be helpful to shut down bleeding that may have commenced.

However, many patients on ITI, particularly when their inhibitor titer remains high, continue to bleed. In the past, such patients were often put on bypassing agent prophylaxis. Given the cost, inconvenience, and limited effectiveness of prophylaxis with bypassing agents, emicizumab has taken over and it can be argued that all patients with inhibitors should now receive emicizumab whether they are on ITI or not.[164,165] If emicizumab and ITI are used together, a low-dose ITI regimen may suffice.[164] In patients who are tolerized after ITI, the duration of or the need for ongoing exposure to FVIII to prevent recurrent inhibitor formation is uncertain.[166]

INHIBITORS IN HEMOPHILIA B

Pathophysiology

Severe hemophilia B is less common than severe hemophilia A and a lower percentage of hemophilia B patients develop inhibitors. Therefore, less is known about FIX inhibitors than FVIII inhibitors.

Epitopes for FIX inhibitors have been identified in the γ-carboxyglutamic acid domain, the serine protease domain, and in the activation peptide region, where FIX is activated by FXIa (see Fig. 134.5). As is the case with inhibitory FVIII antibodies, the specificity of these antibodies for epitopes in functionally important regions of the FIX molecule explains their ability to inhibit FIX coagulant activity.

Because FIX inhibitors are antibodies of the IgG subclass (mainly IgG4), they are products of interaction between FIX-specific B cells and helper T cells. The contributions of co-stimulatory molecules, immunoregulatory genes, and Tregs to the pathogenesis of FIX inhibitors is unknown.

Epidemiology and Risk Factors for Inhibitor Development in Hemophilia B

The overall incidence of inhibitors in hemophilia B is between 3% and 5%. However, in a cohort of 154 severe hemophilia B patients, the PedNet network recently reported a cumulative inhibitor incidence at 75 EDs of 9.3% for all inhibitors and 5% for high-titer inhibitors.[167] Although the incidence in this cohort is higher than previously reported, the incidence of inhibitors in hemophilia B is still approximately one-quarter of that in hemophilia A.

Most patients (50% to 70%) who develop inhibitors develop high titer inhibitors and 50% or more of these exhibit allergic reactions to FIX products.[168] Because anaphylaxis may be the first manifestation of an inhibitor in patients with hemophilia B, it is recommended that the first 10 to 20 doses of FIX replacement be given in a medical facility where the patient can be observed. This is particularly important for patients with severe hemophilia B associated with FIX null mutations (large deletions or nonsense mutations) because they are at highest risk of inhibitor development (risk of 25% to 40%). In contrast, very few patients with severe hemophilia caused by missense mutations and virtually no patients with mild or moderate hemophilia develop inhibitors.[167,169]

The high proportion of missense mutations in patients with hemophilia B, which result in measurable amounts of FIX antigen, likely explains the lower risk of inhibitor formation compared with hemophilia A.[170] In addition, the sequence homology between FIX and other vitamin K dependent clotting factors may contribute to the diminished risk.

A family history of inhibitor development is a risk factor in hemophilia B; this is likely related to the underlying high-risk mutations in such families but could be associated with co-inheritance of other immunological determinants relevant to the immune response such as HLA alleles.

No significant associations between inhibitor development and treatment-related factors, such as type of FIX product, intensity of FIX exposure, or surgery, has been demonstrated to date.

Clinical Manifestations

The presence of an inhibitor should be suspected if the response to replacement therapy is less than expected. The morbidity with inhibitors in hemophilia B includes anaphylaxis, uncontrollable bleeding, and nephrotic syndrome, which can occur in combination. Allergic reactions can occur in up to 50% of patients with inhibitors.[28] Nephrotic syndrome, which occurs most frequently during ITI in patients with a history of allergic reactions to FIX, may not respond to corticosteroids, thereby necessitating the interruption of ITI.[171]

MANAGEMENT OF INHIBITORS IN HEMOPHILIA B

The goals of treatment of patients with hemophilia B and inhibitors are to prevent and treat bleeds and eradicate the inhibitor.

For treatment of bleeds, FIX concentrates can be used in patients with low-titer inhibitors without an allergic phenotype. It is unknown whether EHL FIX concentrates are more effective than SHL concentrates in this setting. Patients with high titer inhibitors require treatment with bypassing agents (aPCC or rFVIIa) for prevention and treatment of bleeds. Although aPCC and rFVIIa are equally effective and associated with a low risk of adverse events, the use of rFVIIa is preferred because the presence of FIX in aPCC may trigger allergic reactions or increase the titer of the inhibitor through induction of an anamnestic response.

Eradication of Inhibitors in Hemophilia B

Patients with hemophilia B and inhibitors are at higher risk for the development of life-threatening bleeding and severe arthropathy mainly because such patients do not benefit from conventional prophylaxis with FIX replacement therapy and prophylaxis with bypassing agents is less effective and less convenient. Furthermore, emicizumab (a FVIII mimetic) is not an option for such patients. Until rebalancing therapies are available, ITI remains an important part of management of high-titer inhibitors.

The experience with ITI in hemophilia B is limited to case series and case reports. ITI without immunosuppressive therapy is less effective in hemophilia B than it is in hemophilia A. In the North American Immune Tolerance Registry, the success rate of ITI in patients with hemophilia B was only 31%, and adverse reactions to therapy (including allergic reactions and nephrotic syndrome) were 10 times more frequent in hemophilia B than in hemophilia A.[172] Consequently, when ITI is considered in hemophilia B, concomitant immunosuppressive therapy is usually given with rituximab, cyclosporine A, or mycophenolate mofetil or some combinations of these.[173,174]

Because of the poor success rate with ITI and the potential complications, the decision to undertake it must be made with caution. If it is undertaken, the dose of FIX needs to be increased gradually with monitoring for allergic reactions and nephrotic syndrome. A recent report suggests that up to 50% of inhibitors to FIX can be eradicated particularly if concomitant immunosuppressive therapy is given.[174]

GENE THERAPY FOR HEMOPHILIA

Since the cloning of the *F8* and *F9* genes in the early 1980s, hemophilia has been a leading candidate for the application of gene therapy. The rationale for gene therapy in hemophilia includes the following: even small increments in FVIII or FIX levels are of clinical benefit; regulation of FVIII or FIX levels is not critical as long as they do not reach supra-physiologic levels for extended periods of time; the site of coagulation transgene expression does not need to be restricted provided that sufficient amounts of the protein enter the circulation; and animal models of hemophilia are available for preclinical evaluation of gene transfer approaches.

Modes of Transgene Delivery

The overall concept of gene therapy or gene transfer is quite simple—a normal functioning gene is delivered into host cells where the gene is then expressed, and the missing protein is produced. A key component of any gene transfer strategy is the development of a delivery system that enables efficient transfer of the therapeutic transgene into the recipient cell of choice. After 40 years of investigation, viral vectors remain the most effective means of achieving high-level gene transfer. Despite efforts to enhance the efficiency of non-viral delivery methods such as lipid encapsulation, use of physicochemical conjugates, or hydrodynamic injection, these approaches still have limitations that preclude their advancement. Development of lipid envelopes or nanoparticles has enabled successful RNA delivery to the cell cytoplasm, but methods to deliver therapeutic amounts of DNA to the nucleus remain elusive.

Hemophilia gene transfer studies have used two main types of viral vectors to deliver the gene into the host: retroviruses and adeno-associated viruses (AAV) (Table 134.10). A single hemophilia A patient treated with an adenoviral vector experienced significant hematological toxicity from a well-recognized innate immune response to adenovirus, and no further clinical studies have been undertaken with this vector type.[175]

Initial retroviral studies were performed with replication-defective γ-retroviral vectors, and one human clinical study in hemophilia A also used this vector system. The major problem with these vectors is the requirement for recipient cell replication to facilitate nuclear entry of the vector. In contrast, lentiviral vectors are equally capable of transducing both post-mitotic and replicating cells (see Table 134.10). For this reason, more recent studies have used lentiviral vector

TABLE 134.10 Comparison of Adeno-Associated Virus and Lentivirus Gene Therapies for Hemophilia

	AAV	Lentivirus
Size	~25 nm	~80–100 nm
Packaging capacity	~4.7 kb (ssDNA)	9 kb (ssRNA)
Transduction	Dividing and non-dividing cells[a]	Dividing and non-dividing cells
Integration	Primarily episomal, small % integrates, large absolute numbers[b]	Integrating near active transcription sites
Expression	Years if host immunity controlled	Lifelong
Immunogenicity	High humoral and T-cell responses	Humoral; strong innate responses
Manufacturing	Trial and commercial scale up achieved	Scale up for in vivo difficult
Clinical use	Intravenous delivery underway	Ex vivo and intravenous delivery planned
Pre-existing immunity	Widespread with most AAV serotypes (~50% in adults)	Very low
Use in children	Not likely to be durable due to liver growth and loss of episomes	Yes
Repeat dosing	No	No
Toxicities	Transaminitis, complement activation (may be serotype dependent), risk of insertional mutagenesis	Risk of insertional mutagenesis, oncogenesis

[a] Serotype-dependent.
[b] At total doses of 1E14–E16, 0.1%–1.0% integration results in millions of events per liver.
AAV, Adeno-associated virus.

protocols in which the vector construct is usually derived from elements of the HIV virus. The other major difference between lentiviral gene delivery and gene transfer with other viral vectors is that lentiviruses integrate their genome into the recipient cell genome as a natural part of their life cycle.[176] Although viral vectors do not possess the structural components that enable further rounds of viral replication, there is a risk of insertional mutagenesis, which can trigger activation of adjacent oncogenes or inactivation of tumor suppressor loci. Studies performed in the past 15 years indicate that lentiviral integrations are not random but tend to cluster in transcriptionally active sites and more often occur within introns and coding regions of genes rather than in the upstream regulatory regions where γ-retroviruses insert. Therapeutic levels of FVIII and FIX have been achieved in hemophilic dogs and in non-human primates after in vivo delivery, where lentiviral vectors localize largely in the liver.[177] Lentiviral vectors delivering the *F8* gene also have been utilized ex vivo in hematopoietic stem cells, where, depending on the specificity of the promoter, they may induce FVIII production in specific hematopoietic lineages. Autologous hematopoietic stem cells are mobilized, removed from the patient, transduced with the vector, and grown in sufficient quantities to reinfuse into the patient after at least partial bone marrow ablation to make room for the modified cells. This procedure has been pioneered for management of cancer and more recently in gene therapy for hemoglobinopathies, but carries significant morbidity risks due to partial bone marrow ablation. Both ex vivo and in vivo delivery of lentivirus delivering coagulation genes are expected to enter the clinic shortly. Lentiviral vectors are challenging to scale up and produce in sufficient quantities for in vivo delivery.[178]

Currently, the lead candidates for hemophilia gene transfer are various AAV serotypes. AAV is a small, non-pathogenic, human parvovirus with a small, single-stranded DNA genome of approximately 4.8 kb (see Table 134.10). Infection in humans, which for some serotypes of the virus is frequent, is not associated with clinical disease. Wild-type AAV requires a helper virus, usually adenoviruses or herpesviruses, to establish an infection in the host, as it borrows machinery from both the host and the replication competent viruses to replicate. For use in gene therapy, wild-type AAV genomes are exchanged for a construct of the therapeutic transgene and associated regulatory elements. The various serotypes of AAV have distinct tissue tropisms based upon specific cell surface receptor interactions, but much of the specificity is achieved by tissue or cell specific promoters and/or local administration. When administered intravenously, all serotypes localize predominantly in the liver due to the volume of blood that passes through the liver and the well-recognized fenestrated vessels that permit ready exchange between hepatocytes and the circulation. Several serotypes have been used in successful hemophilia clinical trials to produce subnormal to normal levels of circulating FVIII or FIX.

Upon receptor-mediated entry into recipient cells, AAV is trafficked through endosomes.[179] Some escape and enter the nucleus via an unidentified pathway, where the viral capsid is removed and single stranded DNA forms double-stranded DNA via second strand synthesis and annealing of positive and negative strands. Over time, the inverted terminal repeats (ITRs) flanking both ends of the transgene form circular extrachromosomal stable episomes, which in turn, form repeating episomal units, termed concatemers within the nucleus. These are stable in non-dividing cells but may be lost during cell division.

A small proportion of the virus integrates into the recipient genome at transcriptionally active sites.[180] Since $1E^{12}$-$6E^{13}$ vector genomes/kg are administered to achieve therapeutic effects in hemophilia, even a small number of insertions in the liver or other organs represents a large number, and a theoretical concern for oncogenesis. Although insertional mutagenesis may pose a risk of hepatocellular carcinoma, this has not been seen in large animal models of hemophilia or in patients but has been seen in various mouse strains. A limitation of AAV vectors is their small packaging capacity. Although this is not a problem for *F9* (cDNA ≈1.4 kb), even B domain deleted forms of *F8* exceed the 4.8 kb capacity of AAV vectors. Recent data suggest that when partial genomes of *F8* are packaged into AAV with 5′ and 3′ truncations, they anneal in the nucleus and form intact transcriptionally active transgenes.[181]

Clinical Trials of Hemophilia Gene Therapy

Over 500 patients with hemophilia have already received gene therapy in clinical trials. Despite early failures, recent AAV *F8* and *F9* trials have shown efficacy. Initial *F9* trials targeting skeletal muscle were halted because of lack of efficacy.[175] More recent trials have targeted the liver, and intravenous delivery of AAV8-FIX has resulted in a median level of FIX expression of 5% for at least 8 years with the higher dose.[182] Both a Phase 1/2 and a Phase 3 trial of AAV5-FVIII have converted severe patients into moderate, mild, or occasionally normal phenotypes, however the results are not predictable nor durable, in contrast to the FIX results.[183,184]

With the gain-of-function Padua FIX variant (Arg338Leu; R338L) now being widely used in clinical trials, even higher levels of FIX expression (7-fold higher) can be achieved.

FVIII gene therapy has historically been more challenging due to the larger size of even the B-domain deleted transgene and the well-recognized difficulty heterologous cells have in producing the recombinant protein.[185] Like the FIX clinical trials, FVIII trials have yielded results from zero (no effect) to greater than 100% factor activity. Even low levels of factor activity can have a dramatic effect on bleeding events and hence quality of life, however they do not relieve the patient of dependence on clotting factor replacement for significant trauma and surgeries. Of more concern is the loss of FVIII expression seen in patients, raising the question of the transient nature of what

TABLE 134.11 **Features of Adeno-Associated Virus-Mediated Gene Therapy**

- Wild-type AAV is a non-pathogenic virus.
- AAV integrates into the host genome at a rate of 0.1%–1%. Genotoxicity in some mouse strains, not observed in humans.
- Manageable occasional dose-dependent hypersensitivity reactions during AAV delivery.
- Innate immunity contributes to an adaptive T-cell dose dependent immune response.
- Vector re-administration cannot be achieved with different vector serotypes due to high homology between capsids and cross-reactive antibodies.
- Cytotoxic T-cell responses to capsid protein presentation on hepatocytes can kill transduced hepatocytes and limit the duration of expression.
- Transgene size is limited to ~4.8 kb but truncated genes may anneal in the nucleus to form full-length transcriptional units (i.e., factor VIII [FVIII]).
- Durability of FVIII response may be affected by ER stress response induced by FVIII translation, not observed with FIX and other transgenes.
- Responses in participants in clinical trials have shown high variability, lack of predictability for both FVIII and FIX, and for FVIII, unreliability and lack of durability.

AAV, Adeno-associated virus; *ER*, endoplasmic reticulum.

is expected to be curative. Since AAV, even other serotypes cannot be readministered due to the development of high and long-lived antibody titers, informed decision making is a necessity. Assay discrepancies in both FVIII and FIX gene therapy trials show that chromogenic assays yield a result that is 60% of one-stage assay results. Like FIX Padua, the transgene FVIII results in faster activation of the FVIII and the Tenase complex, producing a larger effect in the one-stage assay than the chromogenic assay, which is thought to be a better reflection of the true FVIII activity.[186] For FVIII, reasons for the discrepancy may include codon optimization resulting in slight variations in the folding of the newly synthesized molecule, or production in a heterologous cell type. The root cause of the discrepancy has not been identified.

Despite these clinical advances, many hurdles persist (Table 134.11).[187] The most important is an immune response against the vector capsid,[188] which necessitates exclusion of the 25% to 60% of patients with evidence of prior AAV exposure. Rates vary significantly by age and geographic population differences. Innate immunity to single stranded DNA involving toll-like receptors plays a role early in the host immune response.[188] Furthermore, even without prior AAV immunity, many patients develop a cytotoxic T-cell–mediated response that results in transient transaminitis and a reduction in transgene expression. This phenomenon is most evident with FIX gene therapy where steroid prophylaxis appears to be beneficial. Steroids appear to be less effective with FVIII gene therapy and other immunosuppressive drugs may be necessary to treat patients with very high levels of transaminases. Causes of transaminase elevations in hemophilia A trial participants may include cytotoxic T cells, but also may include the development of a UPR and concomitant hepatocyte stress.

Other concerns include durability and variability of the responses, and long-term safety (Table 134.12).[175] Full assessment of durability requires longer follow-up.[183] Rare patients exhibit a transient cytokine storm-like response to vector infusion, usually but not always manageable without stopping the infusion. More than 50% to 80% of patients in some trials are on prednisone or other immunosuppressives for an average of 8 months, with attendant side effects.[184] Although long-term data are limited, and integration into the genome occurs, AAV vectors appear to be relatively safe.

The future for hemophilia gene therapy as a curative therapy is promising but more research is required. Several Phase 3 trials of gene therapy for hemophilia A and B are ongoing or completed and represent the first step toward a cure. For hemophilia B, the results may last for many years. Better viral and nonviral delivery systems that enhance safety and durability and result in less variability are needed (see Table 134.12).[190]

TABLE 134.12 **Hurdles and Potential Solutions to Successful Adeno-Associated Virus Gene Therapy for Hemophilia**

Hurdles in AAV Gene Therapy	Problem	Potential Solutions
Pre-existing immunity	Half of patients seropositive to most primate-derived capsids	Use non-primate capsids (e.g., AAV5), deimmunized AAVs through high-throughput capsid screening, pre-treatment with agents to inhibit Ab production (i.e., rituximab), repeated plasmapheresis
Post-injection innate immunity	Some patients have acute reaction, CpGs may stimulate adaptive immunity	Manage anaphylactoid reactions with drugs, decrease CpG content
Post-injection adaptive immunity	Prevents repeat dosing due to B- and T-cell immunity	Administer immunosuppressive agents, use lower doses of vector to ameliorate
Liver transaminases	Even small elevations indicate hepatocyte stress or death due to cytotoxic T cells or cellular stress	Prednisone reactively or prophylactically, investigate root causes, use lower doses of vector
Integration	May cause oncogenesis	Identify causes of integration, use lower doses of vector
Loss of expression	Cause(s) unknown, dose-dependent	Use lower doses of vector, investigate causes
Variable response	Individuals respond to vector differently	Identify variables and drugs to boost transduction events

AAV, Adeno-associated virus; *CpGs*, 5′-C-phosphate-G-3′; oligodeoxynucleotides in AAV vectors.[189]

HEMOPHILIA IN THE WORLD—ECONOMIC CONSIDERATIONS

Although hemophilia is a worldwide disorder found in every ethnic group and race, its management highlights inequalities across countries. The inequality largely reflects the high cost of factor concentrates and non-factor therapies. The cost of plasma-derived or recombinant factor concentrates remains out of reach for many countries. This results in either no treatment or the use of cryoprecipitate or fresh frozen plasma, if available, to treat hemophilia A and B, respectively.[4] At the start of the 21st century, it was estimated that 70% to 80% of the world's hemophilia population received no therapy because of the high cost of treatment and many were undiagnosed as resources (particularly laboratory testing and clinical expertise) were not available in most countries.

With increased availability of therapy for hemophilia in affluent countries, the life span and quality of life of individuals with hemophilia have advanced, thereby widening the disparities between higher- and lower-income countries.[3] The recognition that even low-dose prophylaxis is beneficial enables care to a broader range of patients.[71] With time and the development of new products, the cost of hemophilia care has decreased. Some of this decrease reflects tender systems of purchase mainly in high-income countries. In less resourced countries cost of care remains stubbornly very expensive.

Humanitarian aid programs that provide factor concentrates and other hemophilia treatment products to countries in need have been initiated by organizations such as the WFH and industry partners.[191] The WFH program provides annually 20,000 patients in over 70 countries with access to clotting factors for prophylaxis, treatment of bleeds, or for perioperative management. Although this program has advanced access to treatment, the gaps in care remain wide in many countries.[37] Ultimately, if gene therapy can provide stable, predictable, and durable levels of FVIII and FIX cost effectively, it may solve the problem of inadequate supplies of protein replacement therapies for most of the world. Many unresolved issues must be solved before curative gene therapy is available on a widespread basis.[190]

ACKNOWLEDGMENT

We wish to thank Paul Moorehead for his initial contributions to this chapter.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Andersson NG, et al. Intracranial haemorrhage in children and adolescents with severe haemophilia A or B—the impact of prophylactic treatment. *Br J Haematol*. 2017;179(2):298–307.

Astermark J, et al. A randomized comparison of bypassing agents in hemophilia complicated by an inhibitor: the FEIBA NovoSeven comparative (FENOC) study. *Blood*. 2007;109(2):546–551.

Astermark J, et al. The B-natural study-the outcome of immune tolerance induction therapy in patients with severe haemophilia B. *Haemophilia*. 2021;27(5):802–813.

Blanchette VS, et al. Definitions in hemophilia: communication from the SSC of the ISTH. *J Thromb Haemost*. 2014;12(11):1935–1939.

Carcao MD, et al. Correlation between phenotype and genotype in a large unselected cohort of children with severe hemophilia A. *Blood*. 2013;121(19):3946–3952.

Eckhardt CL, et al. Factor VIII gene (F8) mutation and risk of inhibitor development in nonsevere hemophilia A. *Blood*. 2013;122(11):1954–1962.

Feldman BM, et al. Tailored frequency-escalated primary prophylaxis for severe haemophilia A: results of the 16-year Canadian Hemophilia Prophylaxis Study longitudinal cohort. *Lancet Haematol*. 2018;5(6):e252–e260.

Gouw SC, et al. Factor VIII products and inhibitor development in severe hemophilia A. *N Engl J Med*. 2013;368(3):231–239.

Gouw SC, et al. Intensity of factor VIII treatment and inhibitor development in children with severe hemophilia A: the RODIN study. *Blood*. 2013;121(20):4046–4055.

Hay CRM, DiMichele DM. The principal results of the international immune tolerance study: a randomized dose comparison. *Blood*. 2012;119(6):1335–1344.

Iorio A, et al. Establishing the prevalence and prevalence at birth of hemophilia in males: a meta-analytic approach using national registries. *Ann Intern Med*. 2019;171(8):540–546.

Konkle BA, et al. BIVV001 fusion protein as factor VIII replacement therapy for hemophilia A. *N Engl J Med*. 2020;383(11):1018–1027.

Leissinger C, et al. Anti-inhibitor coagulant complex prophylaxis in hemophilia with inhibitors. *N Engl J Med*. 2011;365(18):1684–1692.

Mahlangu J, et al. Emicizumab prophylaxis in patients who have hemophilia A without inhibitors. *N Engl J Med*. 2018;379(9):811–822.

Male C, et al. Inhibitor incidence in an unselected cohort of previously untreated patients with severe haemophilia B: a PedNet study. *Haematologica*. 2021;106(1):123–129.

Manco-Johnson MJ, et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *N Engl J Med*. 2007;357(6):535–544.

Nathwani AC, et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med*. 2014;371(21):1994–2004.

Oldenburg J. Optimal treatment strategies for hemophilia: achievements and limitations of current prophylactic regimens. *Blood*. 2015;125(13):2038–2044.

Oldenburg J, et al. Emicizumab prophylaxis in hemophilia A with inhibitors. *N Engl J Med*. 2017;377(9):809–818.

Ozelo MC, et al. Valoctocogene roxaparvovec gene therapy for hemophilia A. *N Engl J Med*. 2022;386(11):1013–1025.

Pasi KJ, et al. Multiyear follow-up of AAV5-hFVIII-SQ gene therapy for hemophilia A. *N Engl J Med*. 2020;382(1):29–40.

Pasi KJ, et al. Targeting of antithrombin in hemophilia A or B with RNAi therapy. *N Engl J Med*. 2017;377(9):819–828.

Peyvandi F, et al. A randomized trial of factor VIII and neutralizing antibodies in hemophilia A. *N Engl J Med*. 2016;374(21):2054–2064.

Rangarajan S, et al. AAV5-factor VIII gene transfer in severe hemophilia A. *N Engl J Med*. 2017;377(26):2519–2530.

Srivastava A, et al. WFH Guidelines for the Management of Hemophilia, 3rd edition. *Haemophilia*. 2020;26(Suppl 6):1–158.

Stonebraker JS, et al. The World Federation of Hemophilia Annual Global Survey 1999–2018. *Haemophilia*. 2020;26(4):591–600.

van den Berg HM, et al. Timing of inhibitor development in more than 1000 previously untreated patients with severe hemophilia A. *Blood*. 2019;134(3):317–320.

van Galen KPM, et al. A new hemophilia carrier nomenclature to define hemophilia in women and girls: communication from the SSC of the ISTH. *J Thromb Haemost*. 2021;19(8):1883–1887.

Young G, et al. A multicenter, open-label phase 3 study of emicizumab prophylaxis in children with hemophilia A with inhibitors. *Blood*. 2019;134(24):2127–2138.

Young G. Management of children with hemophilia A: how emicizumab has changed the landscape. *J Thromb Haemost*. 2021;19(7):1629–1637.

REFERENCES

1. Scully M-F, Stoffman J, Boyd S. The unusual pattern of hereditary bleeding disorders in the province of Newfoundland and Labrador-Canada's most Eastern province. *Transfus Apher Sci*. 2018;57(6):713–716.
2. Sharathkumar A, et al. Variability in bleeding phenotype in Amish carriers of haemophilia B with the 31008 C-->T mutation. *Haemophilia*. 2009;15(1):91–100.
3. Iorio A, et al. Establishing the prevalence and prevalence at birth of hemophilia in males: a meta-analytic approach using national registries. *Ann Intern Med*. 2019;171(8):540–s546.
4. Stonebraker JS, et al. The World Federation of Hemophilia Annual Global Survey 1999–2018. *Haemophilia*. 2020;26(4):591–600.
5. Labarque V, et al. Utility of factor VIII and factor VIII to von Willebrand factor ratio in identifying 277 unselected carriers of hemophilia A. *Am J Hematol*. 2017;92(6). E94-e96.
6. van Galen KPM, et al. A new hemophilia carrier nomenclature to define hemophilia in women and girls: communication from the SSC of the ISTH. *J Thromb Haemost*. 2021;19(8):1883–1887.
7. Do H, et al. Expression of factor VIII by murine liver sinusoidal endothelial cells. *J Biol Chem*. 1999;274(28):19587–19592.
8. Everett LA, et al. Murine coagulation factor VIII is synthesized in endothelial cells. *Blood*. 2014;123(24):3697–3705.
9. Fahs SA, et al. A conditional knockout mouse model reveals endothelial cells as the principal and possibly exclusive source of plasma factor VIII. *Blood*. 2014;123(24):3706–3713.
10. Pan J, et al. Patterns of expression of factor VIII and von Willebrand factor by endothelial cell subsets in vivo. *Blood*. 2016;128(1):104–109.
11. Gallinaro L, et al. A shorter von Willebrand factor survival in O blood group subjects explains how ABO determinants influence plasma von Willebrand factor. *Blood*. 2008;111(7):3540–3545.
12. Rangarajan S, et al. AAV5-factor VIII gene transfer in severe hemophilia A. *N Engl J Med*. 2017;377(26):2519–2530.
13. Fong S, et al. Induction of ER stress by an AAV5 BDD FVIII construct is dependent on the strength of the hepatic-specific promoter. *Mol Ther Methods Clin Dev*. 2020;18:620–630.
14. Zheng C, Zhang B. Combined deficiency of coagulation factors V and VIII: an update. *Semin Thromb Hemost*. 2013;39(6):613–620.
15. Chiu P-L, et al. Mapping the interaction between factor VIII and von Willebrand factor by electron microscopy and mass spectrometry. *Blood*. 2015;126(8):935–938.
16. Swystun LL, et al. Genetic determinants of VWF clearance and FVIII binding modify FVIII pharmacokinetics in pediatric hemophilia A patients. *Blood*. 2019;134(11):880–891.
17. Swystun LL, et al. The endothelial lectin clearance receptor CLEC4M binds and internalizes factor VIII in a VWF-dependent and independent manner. *J Thromb Haemost*. 2019;17(4):681–694.
18. Konkle BA, et al. BIVV001 fusion protein as factor VIII replacement therapy for hemophilia A. *N Engl J Med*. 2020;383(11):1018–1027.
19. Gangadharan B, et al. The C1 and C2 domains of blood coagulation factor VIII mediate its endocytosis by dendritic cells. *Haematologica*. 2017;102(2):271–281.
20. Peyvandi F, et al. A randomized trial of factor VIII and neutralizing antibodies in hemophilia A. *N Engl J Med*. 2016;374(21):2054–2064.
21. Yada K, Nogami K. Spotlight on emicizumab in the management of hemophilia A: patient selection and special considerations. *J Blood Med*. 2019;10:171–181.
22. Haldane JBS. The mutation rate of the gene for haemophilia, and its segregation ratios in males and females. *Ann Eugen*. 1947;13(4):262–271.
23. Johnsen JM, et al. Novel approach to genetic analysis and results in 3000 hemophilia patients enrolled in the my life, our future initiative. *Blood Adv*. 2017;1(13):824–834.
24. Ogata K, et al. Most factor VIII B domain missense mutations are unlikely to be causative mutations for severe hemophilia A: implications for genotyping. *J Thromb Haemost*. 2011;9(6):1183–1190.
25. Eckhardt CL, et al. Factor VIII gene (F8) mutation and risk of inhibitor development in nonsevere hemophilia A. *Blood*. 2013;122(11):1954–1962.
26. Reijnen MJ, et al. Hemophilia B Leyden: substitution of thymine for guanine at position -21 results in a disruption of a hepatocyte nuclear factor 4 binding site in the factor IX promoter. *Blood*. 1993;82(1):151–158.
27. Picketts DJ, Mueller CR, Lillicrap D. Transcriptional control of the factor IX gene: analysis of five cis-acting elements and the deleterious effects of naturally occurring hemophilia B Leyden mutations. *Blood*. 1994;84(9):2992–3000.
28. DiMichele D. Inhibitor development in haemophilia B: an orphan disease in need of attention. *Br J Haematol*. 2007;138(3):305–315.
29. Rogaev EI, et al. Genotype analysis identifies the cause of the "royal disease. *Science*. 2009;326(5954):817.
30. Attard C, et al. Developmental hemostasis: age-specific differences in the levels of hemostatic proteins. *J Thromb Haemost*. 2013;11(10):1850–1854.
31. Mazurier C. von Willebrand disease masquerading as haemophilia A. *Thromb Haemost*. 1992;67(4):391–396.
32. Napolitano M, Mariani G, Lapecorella M. Hereditary combined deficiency of the vitamin K-dependent clotting factors. *Orphanet J Rare Dis*. 2010;5:21.
33. Castaman G, Matino D. Hemophilia A and B: molecular and clinical similarities and differences. *Haematologica*. 2019;104(9):1702–1709.
34. Carcao MD, et al. Correlation between phenotype and genotype in a large unselected cohort of children with severe hemophilia A. *Blood*. 2013;121(19):3946–3952. S1.
35. Ljung R, et al. Haemophilia in the first years of life. *Haemophilia*. 2008;14(Suppl 3):188–195.
36. Zanon E, Pasca S. Intracranial haemorrhage in children and adults with haemophilia A and B: a literature review of the last 20 years. *Blood Transfus*. 2019;17(5):378–384.
37. Srivastava A, et al. WFH Guidelines for the Management of Hemophilia, 3rd edition. *Haemophilia*. 2020;26(Suppl 6):1–158.
38. van Dijk K, et al. Variability in clinical phenotype of severe haemophilia: the role of the first joint bleed. *Haemophilia*. 2005;11(5):438–443.
39. Valentino LA. Blood-induced joint disease: the pathophysiology of hemophilic arthropathy. *J Thromb Haemost*. 2010;8(9):1895–1902.
40. Blanchette VS, et al. Definitions in hemophilia: communication from the SSC of the ISTH. *J Thromb Haemost*. 2014;12(11):1935–1939.
41. Bakeer N, et al. Musculoskeletal ultrasound in hemophilia: results and recommendations from a global survey and consensus meeting. *Res Pract Thromb Haemost*. 2021;5(5):e12531.
42. Rodriguez-Merchan EC, De la Corte-Rodriguez H. Iliopsoas hematomas in people with hemophilia: diagnosis and treatment. *Expert Rev Hematol*. 2020;13(8):803–809.
43. Rodriguez-Merchan EC. Hemophilic pseudotumors: diagnosis and management. *Arch Bone Jt Surg*. 2020;8(2):121–130.
44. Kulkarni R, Lusher JM. Intracranial and extracranial hemorrhages in newborns with hemophilia: a review of the literature. *J Pediatr Hematol Oncol*. 1999;21(4):289–295.
45. Andersson NG, et al. Intracranial haemorrhage in children and adolescents with severe haemophilia A or B—the impact of prophylactic treatment. *Br J Haematol*. 2017;179(2):298–307.
46. Traivaree C, et al. Intracranial bleeding in haemophilia beyond the neonatal period—the role of CT imaging in suspected intracranial bleeding. *Haemophilia*. 2007;13(5):552–559.
47. Andersson NG, et al. Long-term follow-up of neonatal intracranial haemorrhage in children with severe haemophilia. *Br J Haematol*. 2020;190(2):e101–e104.
48. Shapiro AD, et al. Defining the impact of hemophilia: the academic achievement in children with hemophilia study. *Pediatrics*. 2001;108(6):e105.
49. Ragni MV, et al. Safety and immunogenicity of subcutaneous hepatitis A vaccine in children with haemophilia. *Haemophilia*. 2000;6(2):98–103.
50. Oldenburg J. Optimal treatment strategies for hemophilia: achievements and limitations of current prophylactic regimens. *Blood*. 2015;125(13):2038–2044.
51. Collins PW, et al. Recombinant long-acting glycoPEGylated factor IX in hemophilia B: a multinational randomized phase 3 trial. *Blood*. 2014;124(26):3880–3886.
52. Mahlangu J, et al. Defining extended half-life rFVIII—a critical review of the evidence. *Haemophilia*. 2018;24(3):348–358.
53. Carcao MD, et al. Comparative pharmacokinetics of two extended half-life FVIII concentrates (eloctate and adynovate) in adolescents with hemophilia A: is there a difference? *J Thromb Haemost*. 2019;17(7):1085–1096.
54. Solms A, et al. Direct comparison of two extended half-life PEGylated recombinant FVIII products: a randomized, crossover pharmacokinetic study in patients with severe hemophilia A. *Ann Hematol*. 2020;99(11):2689–2698.
55. Sidonio Jr. RF, Batsuli G. Plasma factor IX: the tip of the iceberg? *Haemophilia*. 2021;27(3):329–331.
56. Wells JR, et al. Exploring the impact of infusion frequency in hemophilia A: exit interviews with patients participating in BAY 94-9027 extension studies (PROTECT VIII). *Patient*. 2019;12(6):611–619.
57. Manco-Johnson MJ, et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *N Engl J Med*. 2007;357(6):535–544.

58. Berntorp E. Prophylactic therapy for haemophilia: early experience. *Haemophilia*. 2003;9(Suppl 1):5–9. discussion 9.
59. Oladapo AO, et al. Health-related quality of life assessment in haemophilia patients on prophylaxis therapy: a systematic review of results from prospective clinical trials. *Haemophilia*. 2015;21(5):e344–e358.
60. Manco-Johnson MJ, Soucie JM, Gill JC. Prophylaxis usage, bleeding rates, and joint outcomes of hemophilia, 1999 to 2010: a surveillance project. *Blood*. 2017;129(17):2368–2374.
61. Tang L, et al. Short-term low-dose secondary prophylaxis for severe/moderate haemophilia A children is beneficial to reduce bleed and improve daily activity, but there are obstacle in its execution: a multi-centre pilot study in China. *Haemophilia*. 2013;19(1):27–34.
62. Wu R, et al. Low dose secondary prophylaxis reduces joint bleeding in severe and moderate haemophilic children: a pilot study in China. *Haemophilia*. 2011;17(1):70–74.
63. Gouw SC, van der Bom JG, Marijke van den Berg H. Treatment-related risk factors of inhibitor development in previously untreated patients with hemophilia A: the CANAL cohort study. *Blood*. 2007;109(11):4648–4654.
64. Gouw SC, et al. Intensity of factor VIII treatment and inhibitor development in children with severe hemophilia A: the RODIN study. *Blood*. 2013;121(20):4046–4055.
65. Feldman BM, et al. Tailored frequency-escalated primary prophylaxis for severe haemophilia A: results of the 16-year Canadian Hemophilia Prophylaxis Study longitudinal cohort. *Lancet Haematol*. 2018;5(6):e252–e260.
66. Gouider E. Show me the evidence: effectiveness of low-dose prophylaxis. *Haemophilia*. 2020;26(Suppl 3):9–10.
67. Bedoya MA, et al. Implantable venous access devices in children with severe hemophilia: a tertiary pediatric institutional experience. *Pediatr Radiol*. 2020;50(8):1148–1155.
68. Santagostino E, Mancuso ME. Barriers to primary prophylaxis in haemophilic children: the issue of the venous access. *Blood Transfus*. 2008;6(Suppl 2):s12–s16. Suppl 2.
69. Collins PW, et al. Clinical phenotype of severe and moderate haemophilia: Who should receive prophylaxis and what is the target trough level? *Haemophilia*. 2021;27(2):192–198.
70. Carcao MD, et al. An International Prophylaxis Study Group (IPSG) survey of prophylaxis in adults with severe haemophilia. *Haemophilia*. 2017;23(5):e447–e450.
71. Mahlangu J, et al. Emicizumab prophylaxis in patients who have hemophilia A without inhibitors. *N Engl J Med*. 2018;379(9):811–822.
72. Lenting PJ, Denis CV, Christophe OD. Emicizumab, a bispecific antibody recognizing coagulation factors IX and X: how does it actually compare to factor VIII? *Blood*. 2017;130(23):2463–2468.
73. Callaghan MU, et al. Long-term outcomes with emicizumab prophylaxis for hemophilia A with or without FVIII inhibitors from the HAVEN 1–4 studies. *Blood*. 2021;137(16):2231–2242.
74. Oldenburg J, et al. Emicizumab prophylaxis in hemophilia A with inhibitors. *N Engl J Med*. 2017;377(9):809–818.
75. Young G, et al. A multicenter, open-label phase 3 study of emicizumab prophylaxis in children with hemophilia A with inhibitors. *Blood*. 2019;134(24):2127–2138.
76. Yoneyama K, et al. A pharmacometric approach to substitute for a conventional dose-finding study in rare diseases: example of phase III dose selection for emicizumab in hemophilia A. *Clin Pharmacokinet*. 2018;57(9):1123–1134.
77. Pipe SW, et al. Efficacy, safety, and pharmacokinetics of emicizumab prophylaxis given every 4 weeks in people with haemophilia A (HAVEN 4): a multicentre, open-label, non-randomised phase 3 study. *Lancet Haematol*. 2019;6(6):e295–e305.
78. Kizilocak H, et al. Determining the approximate factor VIII level of patients with severe haemophilia A on emicizumab using in vivo global haemostasis assays. *Haemophilia*. 2021;27(5):730–735.
79. Schmitt C, et al. Pharmacokinetics and pharmacodynamics of emicizumab in persons with hemophilia A with factor VIII inhibitors: HAVEN 1 study. *Thromb Haemost*. 2021;121(3):351–360.
80. Lowe A, et al. Effects of emicizumab on APTT, FVIII assays and FVIII inhibitor assays using different reagents: results of a UK NEQAS proficiency testing exercise. *Haemophilia*. 2020;26(6):1087–1091.
81. Østergaard H, et al. A factor VIIIa-mimetic bispecific antibody, Mim8, ameliorates bleeding upon severe vascular challenge in hemophilia A mice. *Blood*. 2021;138(14):1258–1268.
82. Mahlangu JN. Progress in the development of anti-tissue factor pathway inhibitors for haemophilia management. *Front Med (Lausanne)*. 2021;8:670526.
83. Pasi KJ, et al. Targeting of antithrombin in hemophilia A or B with RNAi therapy. *N Engl J Med*. 2017;377(9):819–828.
84. Pasi KJ, et al. Targeting of antithrombin in hemophilia A or B with investigational siRNA therapeutic fitusiran-results of the phase 1 inhibitor cohort. *J Thromb Haemost*. 2021;19(6):1436–1446.
85. Sharathkumar A, et al. Intensive exposure to factor VIII is a risk factor for inhibitor development in mild hemophilia A. *J Thromb Haemost*. 2003;1(6):1228–1236.
86. Mannucci PM. Desmopressin (DDAVP) in the treatment of bleeding disorders: the first 20 years. *Blood*. 1997;90(7):2515–2521.
87. Leissinger C, et al. Desmopressin (DDAVP) in the management of patients with congenital bleeding disorders. *Haemophilia*. 2014;20(2):158–167.
88. Rodriguez-Merchan EC. Differing approaches to diagnosis and treatment of musculoskeletal complications of haemophilia in resource-rich and resource-limited settings. *Blood Coagul Fibrinolysis*. 2012;23(7):575–579.
89. Whelan SF, et al. Distinct characteristics of antibody responses against factor VIII in healthy individuals and in different cohorts of hemophilia A patients. *Blood*. 2013;121(6):1039–1048.
90. Eckhardt CL, et al. Inhibitor development and mortality in non-severe hemophilia A. *J Thromb Haemost*. 2015;13(7):1217–1225.
91. van den Berg HM, et al. Timing of inhibitor development in more than 1000 previously untreated patients with severe hemophilia A. *Blood*. 2019;134(3):317–320.
92. Shima M, et al. A multicentre, open-label study of emicizumab given every 2 or 4 weeks in children with severe haemophilia A without inhibitors. *Haemophilia*. 2019;25(6):979–987.
93. Verbruggen B. Diagnosis and quantification of factor VIII inhibitors. *Haemophilia*. 2010;16(102):20–24.
94. Kamaté C, et al. Depletion of CD4$^+$/CD25 high regulatory T cells may enhance or uncover factor VIII-specific T-cell responses in healthy individuals. *J Thromb Haemost*. 2007;5(3):611–613.
95. Reding MT, et al. Sensitization of CD4$^+$ T cells to coagulation factor VIII: response in congenital and acquired hemophilia patients and in healthy subjects. *Thromb Haemost*. 2000;84(4):643–652.
96. Gupta N, et al. Regulation of immune responses to protein therapeutics by transplacental induction of T cell tolerance. *Sci Transl Med*. 2015;7(275):275ra21.
97. Santagostino E, et al. Environmental risk factors for inhibitor development in children with haemophilia A: a case-control study. *Br J Haematol*. 2005;130(3):422–427.
98. Astermark J, et al. Non-genetic risk factors and the development of inhibitors in haemophilia: a comprehensive review and consensus report. *Haemophilia*. 2010;16(5):747–766.
99. Delignat S, et al. Inhibitor formation in congenital hemophilia A: an immunological perspective. *Semin Thromb Hemost*. 2018;44(6):517–530.
100. Schep SJ, et al. An update on the 'danger theory' in inhibitor development in hemophilia A. *Expert Rev Hematol*. 2019;12(5):335–344.
101. Ettinger RA, et al. Lineages of human T-cell clones, including T helper 17/T helper 1 cells, isolated at different stages of anti-factor VIII immune responses. *Blood*. 2009;114(7):1423–1428.
102. Waters B, Lillicrap D. The molecular mechanisms of immunomodulation and tolerance induction to factor VIII. *J Thromb Haemost*. 2009;7(9):1446–1456.
103. Qian J, et al. Prevention and treatment of factor VIII inhibitors in murine hemophilia A. *Blood*. 2000;95(4):1324–1329.
104. Bray GL, et al. Loss of high-responder inhibitors in patients with severe hemophilia A and human immunodeficiency virus type 1 infection: a report from the multi-center hemophilia cohort study. *Am J Hematol*. 1993;42(4):375–379.
105. Reipert BM, et al. Modulation of factor VIII-specific memory B cells. *Haemophilia*. 2010;16(102):25–34.
106. Matzinger P. The danger model: a renewed sense of self. *Science*. 2002;296(5566):301–305.
107. Pratt KP, Arruda VR, Lacroix-Desmazes S. Inhibitors-recent insights. *Haemophilia*. 2021;27(Suppl 3):28–36.
108. Varthaman A, Lacroix-Desmazes S. Pathogenic immune response to therapeutic factor VIII: exacerbated response or failed induction of tolerance? *Haematologica*. 2019;104(2):236–244.
109. Smith BM, et al. Antigen-specific in vitro expansion of factor VIII-specific regulatory T cells induces tolerance in hemophilia A mice. *J Thromb Haemost*. 2020;18(2):328–340.
110. Schmidt A, Königs C. Regulatory T cells and their potential for tolerance induction in haemophilia A patients. *Hamostaseologie*. 2016;36(Suppl. 2):S5–S12.
111. Reipert BM, et al. The prospective hemophilia inhibitor PUP study reveals distinct antibody signatures prior to FVIII inhibitor development. *Blood Adv*. 2020;4(22):5785–5796.
112. Kahle J, et al. Frequency and epitope specificity of anti-factor VIII C1 domain antibodies in acquired and congenital hemophilia A. *Blood*. 2017;130(6):808–816.

113. Markovitz RC, et al. The diversity of the immune response to the A2 domain of human factor VIII. *Blood*. 2013;121(14):2785–2795.
114. Kadar JG, Schuster J, Hunzelmann N. IgE-mediated anaphylactic reaction to purified and recombinant factor VIII in a patient with severe haemophilia A. *Haemophilia*. 2007;13(1):104–105.
115. Montalvão SAL, et al. Allergic reaction in a cohort of haemophilia A patients using plasma-derived factor VIII (FVIII) concentrate is rare and not necessarily triggered by FVIII. *Haemophilia*. 2015;21(4):e281–e285.
116. Abdi A, et al. Prevalence and incidence of non-neutralizing antibodies in congenital hemophilia A—a systematic review and meta-analysis. *Front Immunol*. 2020;11:563.
117. Batsuli G, et al. High-affinity, noninhibitory pathogenic C1 domain antibodies are present in patients with hemophilia A and inhibitors. *Blood*. 2016;128(16):2055–2067.
118. Wight J, Paisley S. The epidemiology of inhibitors in haemophilia A: a systematic review. *Haemophilia*. 2003;9(4):418–435.
119. Gouw SC, et al. Factor VIII products and inhibitor development in severe hemophilia A. *N Engl J Med*. 2013;368(3):231–239.
120. Kurnik K, et al. New early prophylaxis regimen that avoids immunological danger signals can reduce FVIII inhibitor development. *Haemophilia*. 2010;16(2):256–262.
121. Auerswald G, Bidlingmaier C, Kurnik K. Early prophylaxis/FVIII tolerization regimen that avoids immunological danger signals is still effective in minimizing FVIII inhibitor developments in previously untreated patients—long-term follow-up and continuing experience. *Haemophilia*. 2012;18(1):e18–e20.
122. Lai JD, et al. Concurrent influenza vaccination reduces anti-FVIII antibody responses in murine hemophilia A. *Blood*. 2016;127(26): 3439–3449.
123. Platokouki H, et al. Vaccinations are not associated with inhibitor development in boys with severe haemophilia A. *Haemophilia*. 2018;24(2): 283–290.
124. Auerswald G, et al. The EPIC study: a lesson to learn. *Haemophilia*. 2015;21(5):622–628.
125. Eckhardt CL, et al. Surgery and inhibitor development in hemophilia A: a systematic review. *J Thromb Haemost*. 2011;9(10):1948–1958.
126. Peyron I, et al. Haemarthrosis and arthropathy do not favour the development of factor VIII inhibitors in severe haemophilia A mice. *Haemophilia*. 2015;21(1):e94–e98.
127. Matino D, et al. IDO1 suppresses inhibitor development in hemophilia A treated with factor VIII. *J Clin Invest*. 2015;125(10):3766–3781.
128. Liesner RJ, et al. Simoctocog alfa (Nuwiq) in previously untreated patients with severe haemophilia A: final results of the NuProtect study. *Thromb Haemost*. 2021;121(11):1400–1408.
129. Astermark J, et al. A randomized comparison of bypassing agents in hemophilia complicated by an inhibitor: the FEIBA NovoSeven comparative (FENOC) study. *Blood*. 2007;109(2):546–551.
130. Négrier C, et al. SURgical interventions with FEIBA (SURF): international registry of surgery in haemophilia patients with inhibitory antibodies. *Haemophilia*. 2013;19(3):e143–e150.
131. Key NS, et al. Home treatment of mild to moderate bleeding episodes using recombinant factor VIIa (Novoseven) in haemophiliacs with inhibitors. *Thromb Haemost*. 1998;80(6):912–918.
132. Santagostino E, et al. A prospective randomized trial of high and standard dosages of recombinant factor VIIa for treatment of hemarthroses in hemophiliacs with inhibitors. *J Thromb Haemost*. 2006;4(2):367–371.
133. Santagostino E, et al. Recombinant activated factor VII in the treatment of bleeds and for the prevention of surgery-related bleeding in congenital haemophilia with inhibitors. *Blood Rev*. 2015;29(Suppl 1):S9–S18.
134. Ingerslev J, Sørensen B. Parallel use of by-passing agents in haemophilia with inhibitors: a critical review. *Br J Haematol*. 2011;155(2):256–262.
135. Tran HTT, et al. Tranexamic acid as adjunct therapy to bypassing agents in haemophilia A patients with inhibitors. *Haemophilia*. 2014;20(3): 369–375.
136. Valentino LA, Holme PA. Should anti-inhibitor coagulant complex and tranexamic acid be used concomitantly? *Haemophilia*. 2015;21(6):709–714.
137. Leissinger C, et al. Anti-inhibitor coagulant complex prophylaxis in hemophilia with inhibitors. *N Engl J Med*. 2011;365(18):1684–1692.
138. Young G, et al. Safety and efficacy of recombinant activated coagulation factor VII in congenital hemophilia with inhibitors in the home treatment setting: a review of clinical studies and registries. *Am J Hematol*. 2017;92(9):940–945.
139. Hay CRM, DiMichele DM. The principal results of the International Immune Tolerance Study: a randomized dose comparison. *Blood*. 2012;119(6):1335–1344.
140. Biswas M, et al. Combination therapy for inhibitor reversal in haemophilia A using monoclonal anti-CD20 and rapamycin. *Thromb Haemost*. 2017;117(1):33–43.
141. Cadé M, et al. FVIII at the crossroad of coagulation, bone and immune biology: Emerging evidence of biological activities beyond hemostasis. *Drug Discov Today*. 2022;27(1):102–116.
142. Carcao M, et al. The changing face of immune tolerance induction in haemophilia A with the advent of emicizumab. *Haemophilia*. 2019; 25(4):676–684.
143. Ettingshausen CE, Sidonio Jr. RF. Design of an international investigator-initiated study on MOdern Treatment of Inhibitor-positiVe pATiEnts with haemophilia A (MOTIVATE). *Ther Adv Hematol*. 2021; 12 p. 20406207211032452.
144. van Helden PM, et al. IgG subclasses of anti-FVIII antibodies during immune tolerance induction in patients with hemophilia A. *Br J Haematol*. 2008;142(4):644–652.
145. Schep SJ, et al. Review of immune tolerance induction in hemophilia A. *Blood Rev*. 2018;32(4):326–338.
146. Sakurai Y, et al. Association of anti-idiotypic antibodies with immune tolerance induction for the treatment of hemophilia A with inhibitors. *Haematologica*. 2004;89(6):696–703.
147. Lacroix-Desmazes S, et al. Tolerating factor VIII: recent progress. *Front Immunol*. 2019;10:2991.
148. Schep SJ, et al. Role of regulatory cells in immune tolerance induction in hemophilia A. *Hemasphere*. 2021;5(5):e557.
149. Di Minno G, et al. Predictors of inhibitor eradication by primary immune tolerance induction in severe haemophilia A with high responding inhibitors. *Haemophilia*. 2022;28(1):55–64.
150. Coppola A, et al. Factor VIII gene (F8) mutations as predictors of outcome in immune tolerance induction of hemophilia A patients with high-responding inhibitors. *J Thromb Haemost*. 2009;7(11):1809–1815.
151. Callaghan MU, et al. Immune tolerance induction in 31 children with haemophilia A: is ITI less successful in African Americans? *Haemophilia*. 2011;17(3):483–489.
152. Li Z, et al. Low-dose immune tolerance induction alone or with immunosuppressants according to prognostic risk factors in Chinese children with hemophilia A inhibitors. *Res Pract Thromb Haemost*. 2021;5(5):e12562.
153. Carcao M, et al. Recombinant factor VIII Fc fusion protein for immune tolerance induction in patients with severe haemophilia A with inhibitors-a retrospective analysis. *Haemophilia*. 2018;24(2):245–252.
154. Blumberg RS, Lillicrap D. Tolerogenic properties of the Fc portion of IgG and its relevance to the treatment and management of hemophilia. *Blood*. 2018;131(20):2205–2214.
155. Krishnamoorthy S, et al. Recombinant factor VIII Fc (rFVIIIFc) fusion protein reduces immunogenicity and induces tolerance in hemophilia A mice. *Cell Immunol*. 2016;301:30–39.
156. Georgescu MT, et al. Recombinant factor VIII Fc inhibits B Cell activation via engagement of the FcγRIIB receptor. *Front Immunol*. 2020;11:138.
157. Meeks SL, Lacroix-Desmazes S. Emerging benefits of Fc fusion technology in the context of recombinant factor VIII replacement therapy. *Haemophilia*. 2020;26(6):958–965.
158. Antun A, et al. Inhibitor recurrence after immune tolerance induction: a multicenter retrospective cohort study. *J Thromb Haemost*. 2015; 13(11):1980–1988.
159. Batsuli G, et al. Immune tolerance induction in paediatric patients with haemophilia A and inhibitors receiving emicizumab prophylaxis. *Haemophilia*. 2019;25(5):789–796.
160. Mancuso ME, et al. Health-related quality of life and caregiver burden of emicizumab in children with haemophilia A and factor VIII inhibitors-results from the HAVEN 2 study. *Haemophilia*. 2020;26(6):1009–1018.
161. Young G. Management of children with hemophilia A: how emicizumab has changed the landscape. *J Thromb Haemost*. 2021;19(7):1629–1637.
162. Male C, et al. Inhibitor incidence in an unselected cohort of previously untreated patients with severe haemophilia B: a PedNet study. *Haematologica*. 2021;106(1):123–129.
163. Berntorp E, et al. Inhibitor treatment in haemophilias A and B: summary statement for the 2006 international consensus conference. *Haemophilia*. 2006;12(Suppl 6):1–7.
164. Franchini M, Santoro C, Coppola A. Inhibitor incidence in previously untreated patients with severe haemophilia B: a systematic literature review. *Thromb Haemost*. 2016;116(1):201–203.
165. Rallapalli PM, et al. An interactive mutation database for human coagulation factor IX provides novel insights into the phenotypes and genetics of hemophilia B. *J Thromb Haemost*. 2013;11(7):1329–1340.
166. Chitlur M, et al. Inhibitors in factor IX deficiency a report of the ISTH-SSC international FIX inhibitor registry (1997–2006). *Haemophilia*. 2009;15(5):1027–1031.
167. Dimichele D. The North American Immune Tolerance Registry: contributions to the thirty-year experience with immune tolerance therapy. *Haemophilia*. 2009;15(1):320–328.

168. Beutel K, et al. ITI with high-dose FIX and combined immunosuppressive therapy in a patient with severe haemophilia B and inhibitor. *Hamostaseologie*. 2009;29(2):155–157.
169. Astermark J, et al. The B-Natural study—the outcome of immune tolerance induction therapy in patients with severe haemophilia B. *Haemophilia*. 2021;27(5):802–813.
170. Pierce GF. Uncertainty in an era of transformative therapy for haemophilia: addressing the unknowns. *Haemophilia*. 2021;27(Suppl 3):103–113.
171. Ferrari G, Thrasher AJ, Aiuti A. Gene therapy using haematopoietic stem and progenitor cells. *Nat Rev Genet*. 2021;22(4):216–234.
172. Milani M, et al. Phagocytosis-shielded lentiviral vectors improve liver gene therapy in nonhuman primates. *Sci Transl Med*. 2019;11(493):eaav7325.
173. Milani M, et al. Genome editing for scalable production of alloantigen-free lentiviral vectors for in vivo gene therapy. *EMBO Mol Med*. 2017; 9(11):1558–1573.
174. Riyad JM, Weber T. Intracellular trafficking of adeno-associated virus (AAV) vectors: challenges and future directions. *Gene Ther*. 2021;28(12):683–696.
175. Wang D, Tai PWL, Gao G. Adeno-associated virus vector as a platform for gene therapy delivery. *Nat Rev Drug Discov*. 2019;18(5):358–378.
176. Pasi KJ, et al. Multiyear follow-up of AAV5-hFVIII-SQ gene therapy for hemophilia A. *N Engl J Med*. 2020;382(1):29–40.
177. Nathwani AC, et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med*. 2014;371(21):1994–2004.
178. Pasi KJ, et al. Persistence of haemostatic response following gene therapy with valoctocogene roxaparvovec in severe haemophilia A. *Haemophilia*. 2021;27(6):947–956.
179. Ozelo MC, et al. Valoctocogene roxaparvovec gene therapy for hemophilia A. *N Engl J Med*. 2022;386(11):1013–1025.
180. Hetz C, Zhang K, Kaufman RJ. Mechanisms, regulation and functions of the unfolded protein response. *Nat Rev Mol Cell Biol*. 2020;21(8): 421–438.
181. Rosen S, et al. Activity of transgene-produced B-domain-deleted factor VIII in human plasma following AAV5 gene therapy. *Blood*. 2020; 136(22):2524–2534.
182. Butterfield JSS, et al. A molecular revolution in the treatment of hemophilia. *Mol Ther*. 2020;28(4):997–1015.
183. Shirley JL, et al. Immune responses to viral gene therapy vectors. *Mol Ther*. 2020;28(3):709–722.
184. Kaczmarek R, et al. Eliminating Panglossian thinking in development of AAV therapeutics. *Mol Ther*. 2021;29(12):3325–3327.
185. Pierce GF, et al. First-year results of an expanded humanitarian aid programme for haemophilia in resource-constrained countries. *Haemophilia*. 2018;24(2):229–235.
186. Gilbert MS. Prophylaxis: musculoskeletal evaluation. *Semin Hematol*. 1993;30(3 Suppl 2):3–6.
187. Feldman BM, et al. Validation of a new pediatric joint scoring system from the International Hemophilia Prophylaxis Study Group: validity of the hemophilia joint health score. *Arthritis Care Res*. 2011; 63(2):223–230.
188. Pettersson H, et al. Radiologic evaluation of prophylaxis in severe haemophilia. *Acta Paediatr Scand*. 1981;70(4):565–570.
189. Prasetyo M, et al. Correlation between hemophilia early arthropathy detection with ultrasound (HEAD-US) score and hemophilia joint health score (HJHS) in patients with hemophilic arthropathy. *PLoS One*. 2021;16(4):e0248952.
190. Tolend M, et al. Critical appraisal of the International Prophylaxis Study Group magnetic resonance image scale for evaluating haemophilic arthropathy. *Haemophilia*. 2020;26(4):565–574.
191. Konkle BA, et al. BAX 335 hemophilia B gene therapy clinical trial results: potential impact of CpG sequences on gene expression. *Blood*. 2021; 137(6):763–774.

CHAPTER 135

RARE COAGULATION FACTOR DEFICIENCIES

David Gailani, Benjamin F. Tillman, and Allison P. Wheeler

The term "rare coagulation factor deficiency" refers to disorders of thrombin and/or fibrin formation caused by mutations in a single gene, other than those for von Willebrand factor (see Chapter 133), factor VIII, or factor IX (see Chapter 134), that reduce plasma activity of one or more coagulation proteins.[1,2] The most common inherited deficiencies that cause defects in plasma coagulation are those for factor VIII (hemophilia A) and factor IX (hemophilia B), with frequencies of 1 in 10,000 and 1 in 30,000 male births respectively (see Chapter 134). In comparison, severe deficiency of fibrinogen, the protease zymogens prothrombin or factors VII, X, or XI, the cofactor factor V, or the transaminase factor XIII are estimated to occur in one in 500,000 to 2 million individuals (Table 135.1). These conditions are inherited primarily as autosomal recessive conditions, implying carrier frequencies ($\sim$1 in 1000 persons) that are 10-fold higher than the carrier frequencies for alleles causing X-linked hemophilia A or B. The rarity of these disorders is therefore due to their recessive nature and not low allele frequency. This is important to keep in mind because partial (heterozygous) deficiencies of these proteins are relatively common and may contribute to bleeding symptoms. As with any recessive trait, incidences are up to 10-fold higher in areas where consanguinity is common.[2]

Fig. 135.1A shows a scheme reflecting current understanding of the major enzyme reactions involved in thrombin generation and fibrin formation. Factor VIIa binds to tissue factor in the wall of a damaged blood vessel, forming a complex that activates factor X to Xa, which converts prothrombin to thrombin in the presence of factor Va. Mice lacking prothrombin, factor VII, factor X, or factor V die in utero or soon after birth, demonstrating the importance of these reactions. Thrombin converts fibrinogen to fibrin. Factor IX is also activated by factor VIIa/tissue factor and, with factor VIIIa, sustains thrombin generation by activating factor X. In some situations, factor IX activation by factor XIa is required. The older model shown in Fig. 135.1B shows the order of reactions during coagulation in the prothrombin time (PT) and activated partial thromboplastin time (aPTT) assays (see Chapter 127). Here factor XI activation requires factor XII, prekallikrein, and high molecular weight kininogen. However, deficiencies of these proteins do not result in abnormal bleeding, indicating that other mechanisms exist for factor XI activation. In Fig. 135.1A thrombin activates factor XI. Finally, factor XIII is activated by thrombin and cross-links fibrin monomers within a fibrin polymer, increasing the tensile strength of fibrin (see Fig. 135.1A).

Rare coagulation factor deficiencies represent 3% to 5% of inherited plasma coagulation factor deficiencies. This chapter contains separate sections describing inherited and acquired deficiency states for each factor. A number from the Online Mendelian Inheritance in Man (OMIM) database is given in the title for each section on an inherited deficiency. Updated lists of mutations associated with factor deficiencies can be found at several websites such as http://www.clotbase.bicnirrh.res.in and http://www.hgmd.org. The rarity and clinical heterogeneity of the inherited disorders described in this chapter, combined with limited availability of testing, present challenges for establishing evidence-based treatment guidelines.[1,2] Common symptoms include bleeding from mucosal surfaces, heavy menstrual bleeding, and hemorrhage with invasive procedures and childbirth. Bleeding involving the central nervous system or umbilical cord stump are most frequent in severe deficiencies of fibrinogen, factors X, and factor XIII, while gastrointestinal bleeding is a particular problem in factor X deficiency. Hemarthroses can occur with afibrinogenemia and severe deficiency of factors II or X. The World Federation of Hemophilia (WHF, www.wfh.org) and the International Rare Bleeding Disorder Database (RBDD, www.rbdd.org) collect information for the rare coagulation factor deficiencies (Fig. 135.2).[2,3] The European Network of Rare Bleeding Disorders (EN-RBD) has subclassified these conditions based on clinical features to facilitate development of evidence-based diagnostic and treatment strategies (Table 135.2). They noted strong associations between bleeding severity and plasma factor levels in fibrinogen, factor X, and factor XIII deficiencies, weaker associations for factor V or factor VII deficiency, and very weak association in factor XI deficiency.

Table 135.3 summarizes treatment recommendations. Several therapeutic advances could improve treatment of rare bleeding disorders soon. Single factor concentrates are now available for fibrinogen and factors VII, X, XI, and XIII, although availability for some is limited.[1,2] A new factor V concentrate awaits clinical testing. Most of these preparations are derived from human plasma; recombinant versions are currently available only for factor VIIa and the A domain of factor XIII. Strategies for enhancing thrombin generation and activity such as siRNAs to reduce plasma antithrombin, the bispecific factor IX/factor X antibody emicizumab, and the anti-tissue factor pathway inhibitor antibody concizumab have shown promise in patients with hemophilia (see Chapter 134), and may turn out to be useful in some rare coagulation factor deficiencies.

INHERITED FIBRINOGEN DEFICIENCY (OMIM 202400)

Fibrinogen was first purified from plasma in the late 19th century. Fibrinogen and *fibrin* were designated *factor I* and *Ia*, respectively, by the International Committee for Nomenclature of Blood Clotting. Inherited absence of fibrinogen (*afibrinogenemia*) was described in 1920 and has an estimated incidence of 1 in 1 million people (see Table 135.1).[4] Partial fibrinogen deficiency is called *hypofibrinogenemia*. Fibrinogen is synthesized in hepatocytes as a 340,000-Dalton protein composed of two trimers, each containing an $A\alpha$, $B\beta$, and γ chain (Fig. 135.3) that are encoded by separate genes (*FGA, FGB*, and *FGG*) within a 50-kilobase span on chromosome 4. Thrombin converts fibrinogen to fibrin by cleaving fibrinopeptides A and B from the $A\alpha$ and $B\beta$ chains respectively (see Chapters 121 and 125). Fibrinogen also binds glycoprotein IIb/IIIa, facilitating platelet aggregation. Fibrinogen in platelet α-granules is taken up from plasma by a glycoprotein IIb/IIIa-dependent mechanism. Between 8% and 15% of plasma fibrinogen contains at least one γ chain that is a product of an alternatively spliced mRNA called γ'-fibrinogen. γ'-fibrinogen modulates thrombin and factor XIII activity and influences clot architecture.

The normal plasma fibrinogen concentration is 1.5 to 4.0 g/L (150 to 400 mg/dL). Afibrinogenemic patients have levels less than 0.1 g/L as determined by clotting and immunoreactive assays, due to homozygosity or compound heterozygosity for fibrinogen gene mutations. Hypofibrinogenemia is caused by heterozygosity for such mutations. The first mutation causing fibrinogen deficiency was

TABLE 135.1 **Properties of Plasma Coagulation Factors and Characteristics of Deficiency States**

Protein	Factor Plasma Half-Life (h)	Vitamin K Dependent Modification	Factor Level in Pregnancy	Incidence of Severe Inherited Deficiency	Autosomal Inheritance Pattern of Deficiency	Other Names for Deficiency State	Bleeding Diathesis in Severe Deficiency	Screening Tests in Deficiency	
								PT	PTT
Fibrinogen	72–120	No	↑	~1:1 × 10^6	Recessive or Dominant	Afibrinogenemia	Severe	↑	↑
Prothrombin	60–100	Yes	↔	~1:2 × 10^6	Recessive	Hypoprothrombinemia	Severe	↑	↑
Factor V	12–14	No	↔	~1:1 × 10^6	Recessive	Parahemophilia	Moderate to Severe	↑	↑
Factor VII	3–4	Yes	↑	~1:5 × 10^5	Recessive	Serum prothrombin conversion accelerator deficiency	Moderate to Severe	↑	↔
Factor X	20–40	Yes	↑	~1:1 × 10^6	Recessive	Stuart-Prower factor deficiency	Severe	↑ ↔	↑ ↔
Factor XI	45–52	No	Inconsistent	~1:1 × 10^6	Recessive or Dominant	Hemophilia C, plasma thromboplastin antecedent deficiency	Asymptomatic to Moderate	↔	↑
Factor XII	60	No	↑	Unknown	Recessive	Hageman trait	Asymptomatic	↔	↑
Prekallikrein	Not known	No	↔	Unknown	Recessive	Fletcher trait	Asymptomatic	↔	↑
High molecular weight kininogen	170	No	↔	Unknown	Recessive	Flaujeac trait, Williams trait, Fitzgerald trait	Asymptomatic	↔	↑
Factor XIII	150	No	↓	1:2 × 10^6	Recessive	Fibrin stabilizing factor deficiency	Severe	↔	↔

↑ Increase in coagulation factor; ↓ decrease; ↔ no change.

reported in 1999. Over 200 *FGA, FGB*, or *FGG* deletions, frameshifts, and point mutations have been identified in afibrinogenemic and hypofibrinogenemic patients' (https://site.geht.org/base-de-donnees-fibrinogene).[4] *FGA* is most affected. Missense mutations are more prevalent in *FGB* and *FGG*, and cluster in the polypeptide C-termini, affecting D-domain formation (see Fig. 135.3) and interfering with secretion. In afibrinogenemia, fibrinogen is not secreted due either to a lack of synthesis of a fibrinogen chain (Aα, Bβ, or γ) or a defect that prevents secretion. Fibrinogen polypeptides that are not secreted are usually degraded in hepatocytes. However, a few *FGG* mutations such as Gly284Arg (fibrinogen Brescia), Arg375Trp (fibrinogen Aguadilla), and Thr314Pro (fibrinogen Al DuPont) lead to mutant γ-chain accumulation of hepatocytes in the endoplasmic reticulum (ER) of hepatocytes and cause hepatic dysfunction and cirrhosis in a process like that associated with α_1-antitrypsin deficiency (ER storage disease).

Most hypofibrinogenemic patients are asymptomatic, but some bleed excessively with trauma or surgery, particularly with fibrinogen levels less than 0.5 g/L.[1,2,4] Spontaneous bleeding is rare with levels greater than 0.7 g/L and bleeding with surgery is unusual with levels greater than 1.0 g/L. Most (85%) afibrinogenemic patients bleed from mucosal surfaces. Heavy menstrual bleeding and bleeding from the skin, gastrointestinal tract, and genitourinary tract are common. Hemarthroses and muscle hematomas were frequent in one series (54% and 72%, respectively), but were less so (25%) in another study. Hemarthrosis-related arthropathy is less pronounced than in hemophilia A or B. Intracranial bleeding occurs in up to 10% of patients, providing justification for prophylaxis. Intracranial and umbilical cord stump bleeds are common in infancy. Afibrinogenemic patients may be prone to spontaneous splenic rupture. Pregnancy loss, usually in the first trimester, is common in afibrinogenemic women in the absence of factor replacement.[1,2,4] Fibrinogen-deficient mice also have difficulty sustaining pregnancy, confirming the importance of maternal fibrinogen to fetal viability. Pre- and postpartum bleeding are common, and intraabdominal bleeds from ruptured corpus luteal cysts have been reported.

Curiously, arterial and venous thrombosis can occur in afibrinogenemic patients. While some events are likely precipitated by known risk factors or by fibrinogen replacement, no identifiable cause is evident in many instances. By binding thrombin, fibrinogen and fibrin down regulate thrombin activity (a property previously referred to as "antithrombin I" activity), which may explain why thrombosis occurs.

The PT and aPTT assays use fibrin formation as an endpoint and will be infinitely prolonged in afibrinogenemia. In hypofibrinogenemia, the PT and aPTT may be normal because they are relatively insensitive to low fibrinogen levels. The von Clauss method is commonly used for quantifying the plasma fibrinogen concentration and is based on measuring time to clot formation after addition of a standard amount of thrombin (see Chapter 127). The assay is not reliable at low fibrinogen levels (<50 mg/dL) and may give falsely low readings with fibrinogen variants that polymerize slowly (dysfibrinogenemias), in situations where high levels of substances that interfere with fibrin polymerization are present (paraproteins, fibrin degradation products), or if the sialic acid content of fibrinogen is high (newborns, liver disease). Thus, it is important to

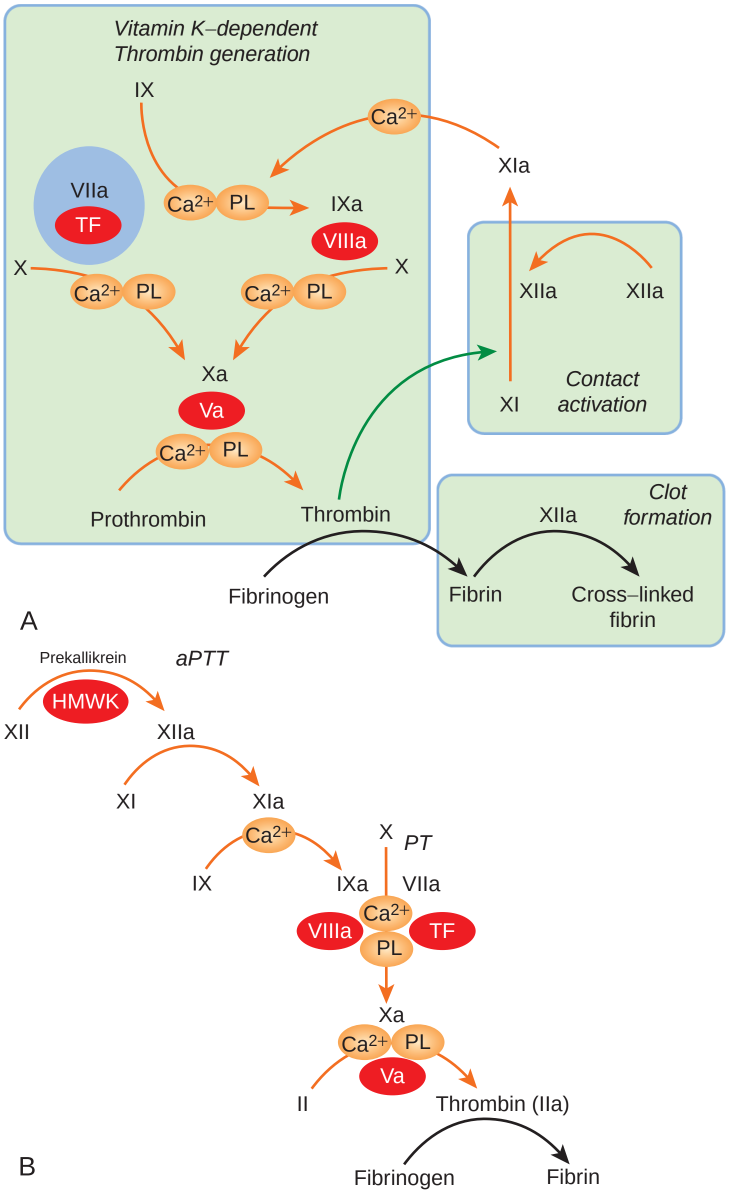

Vitamin K–dependent
Thrombin generation
IX
Ca2+
XIa
VIIa
TF
Ca2+
PL
IXa
VIIIa
X
Ca2+
PL
Ca2+
PL
X
XIIa
XIIa
Xa
Va
Contact
activation
XI
Ca2+
PL
Prothrombin
Thrombin
Clot
formation
XIIa
Fibrinogen
Fibrin
Cross–linked
fibrin
A
Prekallikrein
aPTT
HMWK
XII
XIIa
XI
XIa
Ca2+
X
PT
IX
IXa
VIIa
Ca2+
VIIIa
TF
PL
Xa
Ca2+
PL
Va
II
Thrombin (IIa)
B
Fibrinogen
Fibrin

Figure 135.1 MODELS OF PLASMA COAGULATION. Unactivated plasma factors are indicated by Roman numerals and activated factors by Roman numerals followed by a lowercase letter a. Enzymes are indicated by black lettering and cofactors by white lettering in red ovals. Orange arrows indicate reactions involving enzyme activation. Some reactions require calcium ions (Ca^{2+}) or phospholipid (PL) to proceed optimally. The green arrow indicates feedback activation of factor XI by thrombin. Black arrows indicate fibrin formation. (A) A model of tissue factor (TF) initiated hemostasis. Please read the introduction to this chapter for a description of the events leading to the formation and stabilization of a fibrin clot that are depicted in this figure. (B) The cascade/waterfall model of hemostasis. This model serves as the basis for the prothrombin time (PT) and activated partial thromboplastin time (aPTT) assays. In the PT assay a reagent containing TF is added to plasma, and the factor VIIa/TF complex induces clot formation through activation of factor X. In the aPTT assay a reagent containing a negatively charged substance initiates clot formation through activation of factor XII (contact activation; see Fig. 134.5 for details). *HK*, High-molecular-weight kininogen; *PK*, prekallikrein; *TF*, tissue factor.

demonstrate absence of immunoreactive fibrinogen to confirm a diagnosis of afibrinogenemia. In hypofibrinogenemia, functional and antigenic fibrinogen levels are proportionately decreased. A functional level that is low relative to the antigen level indicates dysfibrinogenemia.[5] Results for the template bleeding time and standard platelet aggregometry are usually abnormal in afibrinogenemia. However, aperture closure times in the PFA-100 platelet function screen are often normal, as this test depends on von Willebrand factor and not fibrinogen to support platelet aggregation. Afibrinogenemic patients have low erythrocyte sedimentation rates and develop little induration with skin tests for delayed hypersensitivity because of the absence of fibrin deposition.

Fresh frozen plasma (FFP) contains fibrinogen, but large volumes are required to correct significant fibrinogen deficiency. Cryoprecipitate is used most often in the United States to treat low fibrinogen levels (see Table 135.3). Each unit of cryoprecipitate has ~ 300 mg of fibrinogen. The plasma volume of a patient with a normal hematocrit is ~ 40 mL/kg body mass. Administering cryoprecipitate to an afibrinogenemic patient at 1 unit/5 kg of body mass increases plasma fibrinogen to ~1 g/L. The fibrinogen concentrate RiaSTAP (CSL Behring, marketed as Haemocomplettan in some European countries) was approved by the United States Food and Drug Administration (FDA) in 2009 for treating bleeding and for prophylaxis in severe fibrinogen deficiency. In 2017 the FDA approved another fibrinogen

TABLE 135.2 Plasma Factor Levels Based on European Network of Rare Bleeding Disorders Categories

Disorder	EN-RBD Severity Category			Clinical Correlation With Factor Level
	Severe	Moderate	Mild	
Fibrinogen deficiency	Undetectable	0.1–1.0 gm/dL	>1.0 g/dL	Strong
Prothrombin deficiency	Undetectable	≤0.1 IU/mL (≤10%)	>0.1 IU/mL (>10%)	Strong
Factor V deficiency	Undetectable	<0.1 IU/mL (<10%)	≥0.1 IU/mL (≥10%)	Weak
Factor VII deficiency	<0.1 IU/mL (<10%)	0.1–0.2 IU/mL (10%–20%)	>0.2 IU/mL (>20%)	Weak
Factor X deficiency	<0.1 IU/mL (<10%)	0.1–0.4 IU/mL (10%–40%)	>0.4 IU/mL (>40%)	Strong
Factor XI deficiency	—	—	—	Very weak
Factor XIII deficiency	Undetectable	<0.3 IU/mL (≤30%)	≥0.3 IU/mL (>30%)	Strong
Combined factor V and VIII deficiency	<0.2 IU/mL (<20%)	0.2–0.4 IU/mL (20%–40%)	>0.4 IU/mL (>40%)	Weak
Vitamin K-dependent factor deficiencies	—	—	—	Weak

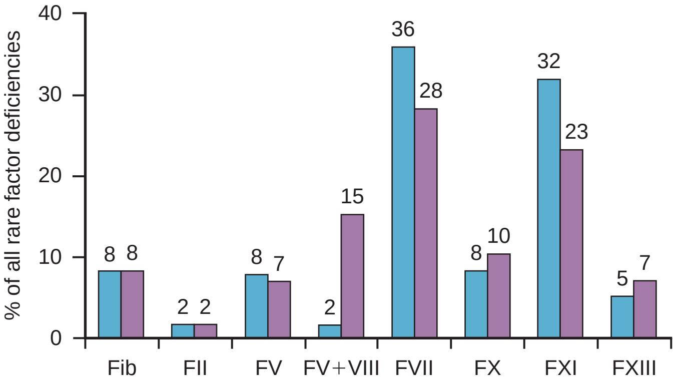


Figure 135.2 WORLDWIDE PREVALENCE OF RARE BLEEDING DISORDERS. Shown are data for each factor deficiency as a percent of total rare bleeding disorders from data collected by the World Federation of Hemophilia (WFH, *blue bars*) and the International Rare Bleeding Disorders Database (RBBD, *purple bars*). The difference in percentage for combined factor V and factor VIII deficiency between the surveys may reflect cases of factor V deficiency with mild hemophilia A being classified as combined deficiencies in the RBDD survey. (*Adapted from Peyvandi F, Menegatti M, Palla R. Rare bleeding disorders: worldwide efforts for classification, diagnosis, and management. Semin Thromb Haemost. 2013;39:579, with permission.)*

concentrate, Fibryga (Octapharma), for treating acute bleeding in adults and adolescents with inherited fibrinogen deficiency.[6] RiaSTAP and Fibryga have undergone viral inactivation. In 15 patients given RiaSTAP (70 mg/kg), the median plasma fibrinogen concentration 1-hour post-infusion was 1.3 g/L (half-life of 77.1 hours). Pharmacokinetic parameters for Fibryga are similar, but clearance appears to be slower. In patients with inherited fibrinogen deficiency, RiaSTAP was effective in 26 of 26 bleeding episodes, 10 of 11 surgical procedures, and all 90 prophylactic administrations. Subsequent studies involving trauma, cardiothoracic surgery, and obstetrical hemorrhage confirmed its ability to improve coagulation and reduce blood loss. In a randomized trial, Fibryga was non-inferior to cryoprecipitate in terms of the number of transfused blood components,[7] leading to expansion of its approval to include treatment of acquired fibrinogen deficiency in 15 European countries. Low-molecular-weight heparin may be administered with fibrinogen concentrate to reduce the risk of thrombosis. The required dose of concentrate can be determined using the following formula:

$$\text{Dose}(\text{mg/kg body weight}) = \frac{\text{Target Level }(\text{mg/dL})\quad \text{Measured Level }(\text{mg/dL})}{1.7(\text{kg body weight/dL})} \tag{5.1}$$

For significant bleeding, the United Kingdom Haemophilia Centre Doctors Organization guidelines recommend that the plasma fibrinogen concentration be maintained at 1.0 g/L until hemostasis is achieved and above 0.5 g/L until wound healing is complete. A similar strategy makes sense for managing surgical patients. A review of replacement therapy and outcomes for 50 patients with inherited fibrinogen deficiency generally agrees with these recommendations. A fibrinogen concentration of 0.5 to 1.0 g/L was adequate for the prevention or treatment of bleeding in nonsurgical or obstetrical settings, while 1.0 to 2.0 g/L was effective for the prevention of bleeding during surgery. This review reported thrombotic episodes (related or unrelated to replacement) in 30% of patients. As the half-life of transfused fibrinogen is ~3 days, dosing every 2 to days is usually adequate in the absence of consumption. Increased dosing frequency may be needed for massive hemorrhage, major surgery, or advanced pregnancy, and monitoring of fibrinogen levels is recommended to facilitate dose adjustments. The use of thromboelastometry to monitor fibrinogen concentrate dosing is popular in surgical settings.

Long-term prophylaxis may be useful for preventing initial bleeding in young patients or for preventing recurrence, particularly after central nervous system hemorrhage. Administering concentrate at 20 to 30 mg/kg every 7 to 14 days to maintain plasma levels of ~0.5 g/L is recommended. Prophylactic fibrinogen administration is required to maintain pregnancies in afibrinogenemic women and to reduce postpartum hemorrhage. Therapy should be initiated early in pregnancy because fetal loss in the first trimester is common. One study suggested that initiating therapy before conception is beneficial. Although some authors recommend maintaining fibrinogen levels above 0.5 g/L during pregnancy and the peripartum period, others suggest a higher level (1.0 g/L) based on reports of fetal loss with levels near 0.5 g/L.

The increased use of fibrinogen concentrates has prompted concerns about adverse events such as thromboembolism, hypersensitivity reactions, and viral transmission. An analysis of 27 years of pharmacovigilance data identified 21 cases of suspected viral transmission (1 per 124,300), 28 cases of thromboembolism (1 per 93,300), and 20 cases of hypersensitivity (1 per 130,600 doses). These data and recent experience from multiple centers suggest an acceptable safety profile.

Antifibrinolytic therapy with ε-amino caproic acid is an effective substitute for blood products for management of some mucosal

TABLE 135.3 Treatment Considerations for Rare Coagulation Factor Deficiencies

Protein	Blood Product Sources	Concentrate (Manufacturer)	Minimum Plasma Level Required for Hemostasis	Minimum Plasma Level Required for Surgery	Dose of Preferred Treatment for Surgery or Bleeding	Dosing Frequency for Surgery or Bleeding	Prophylaxis for Severe Deficiency	Pregnancy: Suggested Plasma Levels for Invasive Procedures and Delivery
Fibrinogen	CRYO FFP	RiaSTAP (CSL Behring)[a] FIBRYGA (Octapharma)[a]	>50 mg/dL	100 mg/dL until healing complete	20–30 mg/kg[b] 15–20 mL FFP/kg 1 bag CRYO/10 kg	Q 24–72 h depending on consumption	Keep level 50 mg/dL for recurrent bleeders	Maintain at 100 mg/dL throughout pregnancy
Prothrombin	FFP	PCC[a]	~5%	30%	20–30 factor IX units/kg	Q 48–72 h	40 factor IX units/kg Q 5–6 days	20%–30%
Factor V	FFP[a] platelets	None	~10%	25%	20 mL FFP/kg then 5–10 mL/kg q12 h	Q 12–24 h	Not usually required	15%–25%
Factor VII	FFP	NovoSeven[a] (recomb. factor VIIa, Novo Nordisk); SevenFACT (recomb. Factor VIIa, HEMA Biologics)[c]; PCC	5%–10%	15%–25%	15–30 μg factor VIIa/kg	Q 2–12 h	20–30 μg/kg 2–3 times per week	10%–20%
Factor X	FFP	PCC[a]; Coagadex (Factor X concentrate Bio Products Laboratory—FDA-submitted)	~10%	25%–40%	PCC: 15–20 factor IX units/kg FX: 25 factor X units/kg	Q 24 h	15–20 factor X units/kg Q weekly. 40–50 FX units twice weekly	10%–20%
Factor XI	FFP[a]	Hemoleven (LFB Biomedicaments); Factor XI concentrate (Bio Products Laboratory)	15%	30%–40%	FFP 20 mL/kg[d] FXI concentrate 15–20 units/kg	Q 1–2 days	Not required	May withhold treatment unless bleeding. 20%–40% if bleeding occurs
Factor XIII	CRYO FFP	Corifact[a] (Formerly Fibrogammin P, CSL Behring) Recombinant Factor XIII (Novo Nordisk—Investigational)	1%–2%	20%–50%	20–30 factor XIII units/kg	Single dose may be sufficient	40 factor XIII units/kg q 28 days	250 units/week through week 22, then 500 units/week with a 1000 unit bolus during labor

CRYO, Cryoprecipitate; *FFP*, fresh frozen plasma; *PCC*, prothrombin complex concentrate.

[a] Preferred treatment for inherited deficiency

[b] Assuming baseline fibrinogen is not known.

[c] FDA approved for the treatment of adults and adolescents (12 years old and older) with hemophilia A or B with inhibitors, but not currently approved for treatment of patients with inherited factor VII deficiency.

[d] Plasma concentration of factors can vary significantly, however 15 to 20 mL/kg should result in an increase of 10% to 20% factor XI activity. We recommend assessment of factor activity prior to surgical procedures and in the setting of significant bleeding if feasible.

bleeds and dental extractions (see box on Adjuncts to Factor Replacement in Inherited Factor Deficiencies). There remain concerns that such therapy increases the risk of thrombosis, particularly in patients with a history of thrombosis and also during pregnancy, surgery, or immobilization. However, the therapy is well tolerated by most patients. Fibrin sealant may be useful for tooth extraction and estrogen/progesterone therapy may be useful for controlling heavy menstrual bleeding.

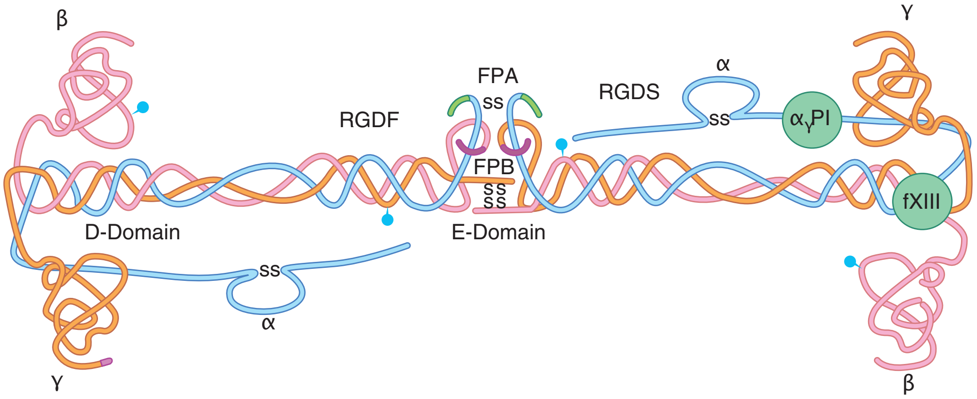


Figure 135.3 MODEL OF HUMAN FIBRINOGEN. A fibrinogen molecule consists of two trimers, each containing an Aα-chain *(blue)*, Bβ-chain *(pink)*, and γ-chain *(orange)*. The amino-termini of the six chains that compose the whole molecule are linked by disulfide bonds in the central E domain, and the C-termini of the polypeptides form two nodular D domains at opposite ends of the molecule. Fibrinopeptides A and B reside in the E domain and are removed by the proteolytic activity of thrombin during conversion of fibrinogen to fibrin monomer. *(Adapted from Mosesson MW. The roles of fibrinogen and fibrin in hemostasis and thrombosis. Semin Hematol. 1992;29:177, with permission.)*

ADJUNCTS TO FACTOR REPLACEMENT IN INHERITED FACTOR DEFICIENCIES

The antifibrinolytic agents ε-amino caproic acid and tranexamic acid can be effective adjuncts to factor replacement when treating inherited or acquired bleeding disorders and are useful alternatives to replacement therapy for mild bleeding or minor procedures. These drugs inhibit clot dissolution by interfering with plasminogen activation and plasmin activity and are particularly effective when bleeding involves tissues with high fibrinolytic activity such as the oral and nasal cavity. They are also useful for treating menorrhagia, limiting blood loss with surgery, controlling epistaxis, and reducing some types of gastrointestinal bleeding. A typical loading dose of ε-amino caproic acid is 100 mg/kg (4 g maximum) followed by 50 mg/kg (2 g maximum) every 6 h. If bleeding subsequently occurs, the dose can be increased, but should not exceed 24 g in 24 h. ε-amino caproic acid is available in oral and intravenous formulations. Identical dosing for either preparation may be used due to excellent bioavailability. Tranexamic acid may be administered at doses up to 1300 mg every 6 h.

For dental extraction in factor VIII or IX deficiency, many centers utilize a single 50%–100% dose of factor concentrate followed by 7 days of ε-amino caproic acid and it is reasonable to use this approach for patients with some rare bleeding disorders. Patients with factor XI deficiency do well with antifibrinolytic agents alone for tooth extraction (see box on Managing Factor XI Deficient Patients). Prolonged therapy with anti-fibrinolytics should be undertaken with caution in patients who are not mobile, who have a history of thrombosis, or who have significant urogenital bleeding. These drugs interfere with urokinase-mediated fibrinolysis in the genitourinary tract and can lead to thrombotic occlusion of the ureter. Antifibrinolytic agents in combination with activated prothrombin complex concentrates or recombinant factor VIIa have been used in some patients, but this should be done with caution because of the potential for increasing thrombotic risk. Patients may develop nausea or vertigo with high doses of ε-amino caproic doses and, in rare cases, rhabdomyolysis.

Recombinant factor VIIa may stop or prevent hemorrhage in rare bleeding disorders. The mechanism by which this agent contributes to hemostasis is not completely understood, particularly when used in patients with deficiencies of common pathway factors (prothrombin and factors V and X). For patients with rare bleeding disorders and an antibody inhibitor directed against the missing factor, factor VIIa may be the treatment of choice. Doses range from 15 to 120 μg/kg every 2–6 h depending upon the severity of bleeding. Because of the potential for thromboembolism with factor VIIa, we recommend frequent re-evaluation and reduction to the lowest effective dose. Doses of 90 μg/kg every 2–3 h have been used in patients with inherited factor V deficiency or combined deficiencies of factors V and VIII, with acquired factor X deficiency associated with amyloidosis, and with antibody-mediated acquired prothrombin deficiency associated with lupus anticoagulants. When compared with FFP, the volume of infused material is smaller with factor VIIa, and the risks for complications such as of fever, urticaria, transfusion-related acute lung injury (TRALI), and anaphylaxis are lower. Caution must be exercised when using factor VIIa in older patients with cardiovascular disease as arterial thrombosis can result, particularly when therapy is combined with antifibrinolytic therapy or prothrombin complex concentrate.

INHERITED DYSFIBRINOGENEMIA (OMIM 134820 Aα CHAIN, 134830 Bβ CHAIN, 134850 γ CHAIN)

In *dysfibrinogenemia*, structural variants of fibrinogen circulate in plasma.[5] Cases in which the dysfunctional protein is present at low concentration are referred to as *hypodysfibrinogenemia*. The first family with dysfibrinogenemia (15 amino acid insertion after Gln350 in the γ chain [fibrinogen Paris I]) was described in 1964. The true incidence of inherited dysfibrinogenemia is unknown, because the majority of affected individuals are asymptomatic.

Inherited dysfibrinogenemias are mostly autosomal dominant traits due to missense mutations in a fibrinogen gene (https://site.geht.org/base-de-donnees-fibrinogene/). Amino acid substitutions that affect fibrinopeptide release, fibrin polymerization or cross-linking, or fibrin degradation are described. The diagnosis is established by a measurement of fibrinogen using a rate-based clotting assay that is low relative to immunoreactive fibrinogen. The functional defects most often reported are influenced by the assays available in clinical laboratories and are unlikely to represent the full spectrum of mutations causing fibrinogen dysfunction. Variants easily detected in clinical laboratories typically have defects in fibrinopeptide release (*FGA*-Arg16His and *FGA*-Arg16Cys [fibrinogens Bicêtre and Metz]) or polymerize slowly (*FGG*-Ser434Asn [fibrinogen Caracas II], *FGG*-Arg275Cys and *FGG*-Arg275His). Indeed, ~45% of mutations in the dysfibrinogenemia database involve substitutions at *FGA*-Arg16 or *FGG*-Arg275, reflecting the ease with which such variants are detected by commonly used assays. Hypodysfibrinogenemia may be caused by single mutations that interfere with both fibrinogen assembly/secretion and function, or compound heterozygosity for reduced synthesis of one allele (usually a null mutation in *FGA* or *FGB*) and a dysfunctional second allele. As with dysfibrinogenemia, missense mutations are most common in hypodysfibrinogenemia.

Most individuals with dysfibrinogenemia are asymptomatic. In a multicenter study of 101 patients with inherited dysfibrinogenemia (with genotypes), 58% were identified incidentally because of an abnormal coagulation test. Over a mean follow-up of 8.8 years, the incidences of major bleeding and thrombosis after diagnosis were 2.5 and 18.7 per 1000 patient years, respectively, with estimated cumulative incidences of 19.2% and 30.1% at 50 years of age. There was no clear association between symptoms and fibrinogen level, fibrinogen functional abnormalities, or gene mutations, findings consistent with older observations that common substitutions such as *FGA*-Arg16His and *FGA*-Arg16Cys occur in asymptomatic individuals as well as in patients with bleeding or thrombosis.

Bleeding symptoms tend to be mild, with epistaxis, easy bruising, and heavy menstrual bleeding being common. More serious bleeding events including soft tissue hematomas, hemarthroses, postoperative hemorrhage, and bleeding during and after pregnancy occur, but are rarer. Major bleeding seems to occur primarily between the ages of 20 and 40 years, partly due to hemostatic challenges related to childbirth. Abnormal wound healing and spontaneous abortion have been reported.

Thrombosis primarily involves the venous circulation, although arterial events can occur. The mean ages for venous and arterial events in one study (34 and 49 years) were lower than for the general population. There is a high prevalence of VTE at the time of diagnosis, with an incidence during follow-up like that for carriers of the factor V Leiden polymorphism. Mutations clustering at the C-terminus of the Aα chain and near the thrombin cleavage site on the Bβ chain are associated with venous thrombosis as are abnormalities in fibrin polymerization, cross-linking, clot structure, and susceptibility to fibrinolysis. The Dusart syndrome caused by *FGA*-Arg554Cys (fibrinogen Paris V) was associated with venous thrombosis and sudden death from pulmonary embolism in adolescents and young adults in several families. Dysfibrinogenemia was reported in 5 of 33 patients with chronic thromboembolic pulmonary hypertension, with the *FGB*-Pro235Leu substitution found in three unrelated patients. Dysfibrinogenemias can alter fibrin polymer structure and its susceptibility to lysis, suggesting that the variants interfere with clot dissolution. Despite these associations, a review of 2376 patients with venous thrombosis found dysfibrinogenemia in less than 1%. Therefore, testing for abnormal fibrinogen in patients with venous thrombosis is not widely recommended (see Chapter 138).

As discussed in the section on inherited fibrinogen deficiency, maternal fibrinogen is required to maintain pregnancy. Obstetrical and postpartum complications such as spontaneous miscarriage, placental abruption, and postpartum hemorrhage occur in women with inherited dysfibrinogenemia, particularly those with a past history or family history of such complications. There are few guidelines for management of pregnant women with dysfibrinogenemia.

A group of mutations in the C-terminus of the fibrinogen Aα chain is associated with autosomal dominant hereditary amyloidosis. The amyloid deposits contain fragments of the variant fibrinogen. The kidneys are affected first, but visceral and nerve involvement may occur. Renal grafts become involved with amyloid, and liver transplantation may be a better treatment option. The allele for one responsible mutation, *FGA*-Glu526Val, is relatively common and may account for 5% of patients with apparent sporadic amyloid.

Dysfibrinogenemia is usually diagnosed because of abnormalities on routine coagulation tests such as the PT or aPTT. The thrombin time is often used to screen for dysfibrinogenemia, although its sensitivity is not established. The thrombin time involves measuring the time to clot formation after addition of a relatively small amount of thrombin to plasma (see Chapter 127). Specificity for dysfibrinogenemia is low because heparin, thrombin inhibitors (argatroban, dabigatran, hirudin), elevated levels of fibrin degradation products, paraproteins, and low fibrinogen levels all prolong the thrombin time. The reptilase time has been used as an alternative screen and is useful in combination with the thrombin time (see Chapter 127). Clot formation is induced with an enzyme from the venom of *Bothrops jararaca* or *Bothrops atrox* that cleaves fibrinopeptide A (but not fibrinopeptide B) from fibrinogen, and which is not sensitive to heparin or direct thrombin inhibitors. The plasma concentration of fibrinogen as determined by the von Clauss method (see "Inherited Fibrinogen Deficiency") may be falsely low in some forms of dysfibrinogenemia. Levels of immunoreactive fibrinogen are usually normal but are decreased in hypodysfibrinogenemia. Fibrin degradation products may appear to be elevated because variant fibrinogens may be incompletely incorporated into the clot, leading to the false impression that disseminated intravascular coagulation (DIC) is present (see Chapter 137).

Most dysfibrinogenemic patients are asymptomatic. When symptoms occur, there is little correlation with coagulation assay abnormalities, which renders it difficult to make general therapeutic recommendations. The patient's personal and family history can help to guide therapy. Active bleeding can be treated with replacement therapy as in afibrinogenemia and such treatment may be indicated in some patients before invasive procedures. In general, patients with thrombosis and dysfibrinogenemia should be treated in the same manner as other patients with thrombosis. There are no data on which to formulate recommendations as to duration of anticoagulant therapy; thus, past history, family history, coexisting conditions, and the nature (unprovoked or provoked by pregnancy or surgery) and severity of the thrombotic event are taken into consideration (see Chapters 140 and 141). As with any thrombotic event, the risk for bleeding associated with prolonged anticoagulant therapy needs to be considered. Recurrent spontaneous abortions have been associated with dysfibrinogenemia in several families and pregnancies have been carried to term using replacement therapy. While some investigators recommend fibrinogen replacement early in pregnancy, as in afibrinogenemic patients, the prothrombotic nature of the peripartum period may dictate against this approach in certain patients.

Acquired Disorders of Fibrinogen

Acquired hypofibrinogenemia occurs in DIC and primary fibrinolysis (see Chapter 137). Fibrinogen levels are usually normal or increased in liver disease, but levels less than 1 g/L may be seen in cirrhosis with hepatic failure or fulminant hepatic necrosis and indicate a poor prognosis. Patients receiving L-asparaginase for hematologic malignancies may develop severe hypofibrinogenemia (<0.2 g/L), while other coagulation factors are normal or only slightly reduced.

Acquired dysfibrinogenemia is common in liver disease, with 80% to 90% of patients with cirrhosis or liver failure showing some evidence of fibrinogen dysfunction. Increased sialic acid content like that seen with neonatal fibrinogen (see Chapter 148) decreases the rate of fibrin polymerization in the von Clauss assay, reducing the apparent fibrinogen concentration. Increased sialic acid content probably does not contribute significantly to abnormal hemostasis. Similarly, monoclonal paraproteins in patients with multiple myeloma may interfere with fibrin polymerization but usually do not cause abnormal hemostasis. Acquired dysfibrinogenemia also has been associated with malignancies and bone marrow transplantation.

Alloantibodies to fibrinogen have been described in persons with afibrinogenemia after exposure to fibrinogen containing products, including fibrin sealant and fibrinogen concentrates. While clinically significant in some persons, the frequency of this occurrence is rare, likely because many afibrinogenemic patients have trace amounts of circulating fibrinogen. Anti-fibrinogen antibodies were reported in only two afibrinogenemic patients after receiving fibrinogen concentrate and the incidence of antibody development may be lower than in patients receiving cryoprecipitate.

Autoantibodies against fibrinogen have been described in various clinical scenarios, including pregnancy, malignancy, and autoimmune disorders. Like inherited fibrinogen abnormalities, the antibodies can result in clinically silent laboratory changes or be associated with hemostatic and/or thrombotic abnormalities.

INHERITED PROTHROMBIN DEFICIENCY (OMIM 176930)

More than a century ago, Morawitz proposed that insoluble fibrin is formed from fibrinogen through the activity of fibrin ferment or *thrombin*. Thrombin was generated from a precursor, prothrombin, by thrombokinase (probably factor Xa). Prothrombin and thrombin are designated *factors II* and *IIa*. Total *prothrombin deficiency* is probably not compatible with life. Complete absence of the protein has not been observed in a human,[8] and prothrombin-deficient mice succumb to bleeding in utero or shortly after birth. Severe deficiency of plasma prothrombin activity caused by reduced plasma prothrombin antigen (*hypoprothrombinemia*) or circulating dysfunctional prothrombin (*dysprothrombinemia*) affects ~1 in 2 million people (see Table 135.1).[8] However, prothrombin deficiency is the third most common coagulation factor disorder in Puerto Rico (carrier frequency ~1 in 700), accounting for 6% of inherited bleeding disorders other than von Willebrand disease at the University of Puerto Rico Hemophilia Center. In the North American Rare Bleeding Disorder Registry, 62% of patients with prothrombin deficiency were of Latin American origin, possibly reflecting the prevalence of the Arg457Gln variant prothrombin Puerto Rico I. Globally almost 70% of patients with prothrombin deficiency have Latin or Hispanic ancestry.[8]

Prothrombin is a 72,000 Da protein that is converted to thrombin by factor Xa in complex with factor Va on phospholipid surfaces (see Fig. 135.1A). Thrombin catalyzes multiple procoagulant events including cleavage of fibrinopeptides A and B from fibrinogen (see Fig. 135.3), activation of factors V, VIII, XI, and XIII, and cleavage of protease activated receptors on a variety of cells including platelets (see Chapters 121 and 125). Thrombin binds to thrombomodulin on endothelial cells, downregulating coagulation by activating protein C and attenuating fibrinolysis by activating thrombin-activatable fibrinolysis inhibitor (TAFI).

Prothrombin deficiency is an autosomal recessive disorder that manifests as hypoprothrombinemia (reduced activity and antigen; cross-reactive material negative [CRM−] deficiency), dysprothrombinemia (activity reduced relative to antigen; CRM+ deficiency), or a combination of both.[8] Plasma prothrombin activity is typically 1% to 10% of normal in hypoprothrombinemia and 1% to 20% in dysprothrombinemia. Heterozygotes for either condition have 40% to 60% of normal activity. About 50 prothrombin gene (*F2*) mutations have been described in patients with prothrombin deficiency, three-quarters of which are missense mutations. In dysprothrombinemia, missense mutations typically cause defects in prothrombin conversion to thrombin (e.g., pArg457Gln [prothrombin Puerto Rico I] and pArg271Cys [prothrombin Madrid]), or result in functionally defective thrombin (e.g., Arg418Trp [prothrombin Tokushima]). A subset of recently identified dysprothrombinemias, caused by missense mutations in *F2* exon 14, alter thrombin regulation. Substitution of prothrombin Arg596 with leucine (prothrombin Yukuhasi), glutamine (prothrombin Belgrade), or tryptophan (prothrombin Padua 2) interferes with thrombin-antithrombin complex formation, resulting in increased circulating thrombin and a prothrombotic state that has been referred to as "antithrombin resistance."[9] In contrast to other dysprothrombinemias, these CRM+ variants do not cause excessive bleeding despite the apparent low plasma prothrombin activity. Prothrombin deficiency can be inherited in combination with deficiencies of other vitamin K–dependent proteins (see "Combined Deficiency of Vitamin K–Dependent Proteins").

Severe hypoprothrombinemia is inevitably associated with bleeding that may be life threatening. A committee of the International Society on Thrombosis and Haemostasis (ISTH) proposed classifying prothrombin deficiency as severe (<5% of normal activity), moderate (5% to 10% activity) or mild (>10% activity). This system has been adopted in the EN-RBD survey (see Table 135.2).[3] Central nervous system hemorrhage was reported in 8% to 12% of prothrombin-deficient patients, and in 20% with prothrombin levels less than 1% of normal activity.[3,8] Soft tissue hematomas, bruising (60%), and hemarthroses (42%) are common.[8] The bleeding diathesis can present at circumcision in neonates, with trauma or surgery, or as easy bruising, epistaxis, heavy menstrual bleeding, or gastrointestinal hemorrhage. Heterozygotes occasionally have excessive bleeding with surgery or tooth extraction, but most are asymptomatic.[8] Bleeding tends to be less severe in dysprothrombinemia and some variants are particularly mild. Homozygosity for the Arg67His variant causes a significant reduction in plasma prothrombin activity (<20% of normal) but relatively few symptoms.

In the PT and aPTT assays, prothrombin is converted to thrombin by factor Xa in complex with factor Va on phospholipid surfaces (see Fig. 135.1B), and severe prothrombin deficiency will prolong the clotting time in both tests (see Chapter 127). Some PT and aPTT reagents are relatively insensitive to reductions in prothrombin, and mild deficiencies may be missed. The diagnosis is usually confirmed, and the degree of deficiency established, by a modified PT assay using prothrombin-deficient plasma. A prothrombin antigen level is needed to distinguish between hypoprothrombinemia (activity and antigen are equivalent) and dysprothrombinemia (activity is less than antigen). Prothrombin deficiency must be distinguished from deficiencies of fibrinogen, factor V, or factor X, which also prolong the PT and aPTT (see Chapter 127).

Prothrombin complex concentrates (PCCs) (see Table 135.3) are the preferred treatment for prothrombin deficiency. PCCs come in four-factor forms containing prothrombin, factors VII, IX and X (e.g., Kcentra), and three-factor forms (prothrombin, and factors IX and X, e.g., Profilnine). Four-factor concentrates often include heparin, antithrombin, and/or proteins C and protein S to reduce thrombotic risk. PCCs are derived from human plasma and undergo viral inactivation. They were originally approved for use in factor IX deficiency, and their activities are still standardized in factor IX units. Prothrombin activity in PCCs is comparable to, or higher than, the factor IX activity. Hemostatic levels of prothrombin are estimated to be 20% to 40% of normal for major surgery or trauma, but 10% to 15% may be adequate for milder challenges.[8] A dose of 20 to 30 factor IX units/kg usually produces plasma prothrombin levels of 20% to 30% of normal in a severely deficient patient. The half-life of prothrombin is ~3 days and dosing every 2 to 3 days can maintain adequate levels until healing is complete. Alternatively, one fourth of the loading dose each day should keep the level therapeutic (>10%). Prophylactic infusion of PCC every 5 to 6 days prevented spontaneous bleeding in one severely deficient patient. PCC administration has been associated with thrombosis, although a meta-analysis of studies using PCCs to reverse the effect of warfarin suggests the risk is less than 2%. Similar rates of thrombosis have been reported when PCCs are used to reverse direct oral anticoagulants. It is reasonable to maintain the factor VII, IX, and X levels at less than 150% of normal to reduce risk. Dental procedures or minor bleeding may respond to antifibrinolytic therapy. Prothrombin deficiency may also be treated with FFP for bleeding episodes or surgical intervention (15 to 20 mL/kg loading dose followed by 3 mL/kg/day). Because of the long half-life, additional doses may not always be needed. Cryoprecipitate is not a source of prothrombin and plasma prothrombin levels do not increase after infusion or inhalation of desmopressin (DDAVP).

Acquired Prothrombin Deficiency

Acquired prothrombin deficiency occurs with warfarin therapy, poisoning with rodenticides such as brodifacoum, vitamin K deficiency, liver disease, and DIC. In these situations, other coagulation factors are also reduced. Cephalosporins, particularly those with N-methylthiotetrazole side chains, can also decrease prothrombin levels.

There are no reports of neutralizing antibodies forming after replacement therapy in inherited prothrombin deficiency, consistent with patients having at least trace amounts of circulating prothrombin. Phospholipid-dependent anti-prothrombin antibodies are common in patients with lupus anticoagulants and/or the antiphospholipid syndrome (see Chapter 139). They do not compromise hemostasis. More rarely, patients with a lupus anticoagulant or systemic lupus erythematosus develop antibodies that enhance prothrombin clearance, leading to true deficiency. This acquired hypoprothrombinemia occurs primarily in children under 10 years of age, usually in association with lupus anticoagulants after a viral illness. The episodes typically resolve spontaneously, but excessive bleeding can occur.[10] Intravenous immunoglobulin has been effective in reducing prothrombin clearance in this population.[10] In contrast to lupus anticoagulants, which prevent correction of prolonged clotting times when patient plasma is mixed with normal plasma, the abnormal clotting time caused by acquired hypoprothrombinemia does correct.

Treatment of antibody-mediated acquired prothrombin deficiency associated with lupus anticoagulants in patients with autoimmune diseases often requires immune suppression, because the condition does not resolve spontaneously. Steroids are effective in most patients, although relapse is common during weaning or after stopping treatment. Treatment with azathioprine or cyclophosphamide has successfully eradicated the antibody. Rituximab also is effective and is likely the preferred therapy. In a rare case of quinidine-induced lupus anticoagulant with a concomitant antiprothrombin antibody, cessation of the drug led to spontaneous resolution of acquired prothrombin deficiency, but not the lupus anticoagulant. The low prothrombin activity in these patients may protect them from thrombosis related to their lupus anticoagulant, as suggested by reports of thrombosis after successful eradication of the antiprothrombin antibody.

INHERITED FACTOR V DEFICIENCY (OMIM 227400)

In 1943, Quick reported that a labile plasma factor distinct from prothrombin was required for coagulation. At the same time, Owren noted that a patient with a lifelong bleeding diathesis lacked a plasma factor that, unlike prothrombin, did not adsorb onto aluminum hydroxide. Owren's patient was deficient in the labile factor described by Quick, which is now called *factor V*. Moderate to severe inherited *factor V deficiency* (1% to 10% of normal plasma level) occurs in 1 in 1 million persons (see Table 135.1).[11] Full-length factor V is the 330,000-Dalton precursor of the cofactor *factor Va*, which facilitates prothrombin activation by factor Xa on phospholipid surfaces (see Fig. 135.1).[11] During coagulation, the B-domain of factor V is removed by thrombin or factor Xa to produce factor Va (Fig. 135.4). An ~250,000 Da form of factor V lacking 703 amino acids from the B-domain is a minor constituent of normal plasma (see Fig. 135.4). This truncated form, referred to as factor V-short, is the product of an alternatively spliced mRNA. Eighty percent of factor V in blood is in plasma, with the remainder in platelet α-granules. In humans, platelet factor V is primarily of plasma origin. Megakaryocytes take up the protein in a process requiring low-density lipoprotein receptor-related protein-1 (LRP-1) and convert it to a partially activated form (see Chapters 123 and 124).

Severe factor V deficiency is an autosomal recessive trait with undetectable (<1% of normal) plasma factor V. In moderate deficiency the level is between 1 and 10% of normal (see Table 135.2).[11] More than 150 factor V gene (*F5*) mutations have been described in factor V deficient patients. Frameshifts and splice variants are common and are distributed throughout the gene. Nonsense mutations show a predilection for the B domain while missense mutations cluster in the A2 and C2 domains, resulting in polypeptides that are degraded within the cell and low levels of plasma factor V antigen (CRM− deficiency). Only two *F5* mutations have been reported in which plasma antigen exceeds activity (CRM+ mutations) The Ala221Val (factor V New Brunswick) and His147Arg substitutions appear to reduce factor Va activity by affecting protein stability. A few patients with abnormalities specific to platelet factor V have been described (see Chapter 123). In the Quebec platelet disorder, an autosomal dominant condition with low platelet factor V and normal plasma factor V levels, excessive proteolysis of α-granule proteins is caused by overexpression of urokinase. Platelet factor V activity is also reduced in Factor V New York, but here the platelet factor V protein appears normal and the mechanism for the disorder is unknown. Combined factor V and factor VIII deficiency is caused by mutations in proteins required for secretion of both proteins (see "Combined Factor V and Factor VIII Deficiency").

The autosomal dominant East Texas bleeding disorder is caused by an A2440G substitution in *F5* exon 13 (factor V East Texas), that increases mRNA encoding factor V-short (see Fig. 135.4), leading to increased plasma levels of this truncated form of the protein. In this disorder plasma levels of tissue factor pathway inhibitor (TFPI), an important coagulation regulator, are 10-fold higher than normal. The B-domain of full-length factor V contains basic and acid regions that likely bind to each other (see Fig. 135.4). In factor V-short, the basic region is missing, leaving the acidic region free to bind TFPI. It is thought that TFPI-mediated inhibition of factor Xa and the factor VIIa-tissue factor complex contribute to symptoms in East Texas bleeding disorder. A similar bleeding disorder was observed in a Dutch family with a C2588G substitution in *F5* exon 13, leading to loss of 632 B domain amino acids (factor V Amsterdam). These patients also have markedly elevated TFPI levels.

Symptoms in patients with severe factor V deficiency are variable and the correlation between bleeding and plasma factor V levels is relatively weak (see Table 135.2).[11] Significant, and even life-threatening bleeding occurs, but the frequency tends to be less than in patients lacking factor VIII or factor IX. Several factors may contribute to the variability. Some patients with mild bleeding have sufficient platelet factor V to support thrombin generation. This implies that their factor V is unstable in plasma but can still be taken up by platelets. Patients with the most severe bleeding may lack plasma and platelet factor V. In one such individual treated with plasma, infused factor V was taken up by megakaryocytes and was detectable in platelets 2 weeks after it was no longer detectable in plasma. Many patients with factor V deficiency have reduced plasma TFPI levels that may partly offset the need for factor V. Mucosal bleeding is the primary abnormality in inherited factor V deficiency, with 60% of patients experiencing epistaxis, heavy menstrual bleeding, or oral bleeding. Hematomas and hemarthroses occur in up to 25% of patients,[11] but debilitating arthropathy is infrequent. Severe bleeding in the central nervous system or gastrointestinal tract occurs, but is rare.[11]

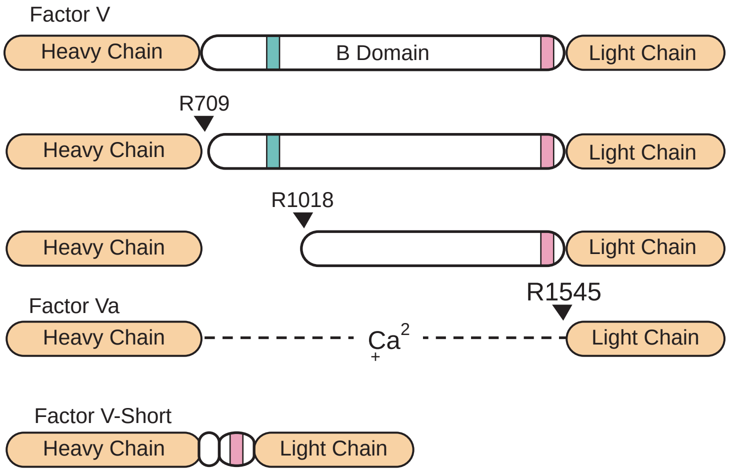


Figure 135.4 SCHEMATIC DIAGRAMS OF HUMAN FACTOR V AND FACTOR Va. In factor V, the heavy and light chains *(orange)* are separated by a central B domain (*white*, amino acids 710 to 1545) that contains a basic region (*blue*, amino acids 963 to 1008) and acidic region (*pink*, amino acids 1493 to 1537). Removal of the B domain by thrombin to generate factor Va involves sequential cleavages after Arginine 709, 1018, and 1545. Factor V-Short is the product of an alternatively spliced factor V mRNA that lacks residues 756 through 1458, which comprise most of the B domain including the basic region. In factor V-Short, the loss of the B domain basic region leaves the acidic region free to bind to tissue factor pathway inhibitor (TFPI).

Postpartum hemorrhage is common and there may be an increased incidence of miscarriage. Trauma, surgery, and dental extraction are associated with a high bleeding risk in untreated patients. Bleeding with surgery involving the urogenital tract, nose, or mouth may be particularly problematic because of high local fibrinolytic activity. Mild factor V deficiency (>10% of normal level) is not usually associated with excessive bleeding, although 10% to 15% of patients report some symptoms.

Thrombosis has been reported in factor V deficient patients. In most cases, the deficiency may not protect the patient. Indeed, except for deficiency of prothrombin or factor X, thrombosis has been reported in all severe coagulation factor deficiency states. However, the situation with factor V may be more complex. Mild to moderate factor V deficiency can occur in patients with the procoagulant polymorphism factor V Arg506Gln (factor V Leiden). If Arg506Gln and a mutation causing factor V deficiency are on opposite alleles (in-*trans*), most plasma factor V will have the Gln506 substitution. This is referred to as pseudo-homozygosity for activated protein C resistance, and it may substantially increase the risk of thrombosis beyond that typical for carriers of Arg506Gln (see Chapter 138). These patients usually do not bleed excessively, despite reduced plasma factor V antigen.

Factor V is required for normal prothrombin activation by factor Xa (see Fig. 135.1B). Therefore, factor V deficiency prolongs the PT and aPTT. The diagnosis and severity are usually established with a modified PT assay using factor V-deficient plasma (see Chapter 127). With severe deficiency, the template bleeding time may be prolonged, primarily in patients with concomitant reductions in platelet factor V. Patients with factor V deficiency should be tested for factor VIII deficiency so that the combined deficiency disorder is not missed (see "Combined Factor V and Factor VIII Deficiency"). In patients with East Texas bleeding disorder or factor V Amsterdam, the prolonged PT and aPTT are due to the inhibitory effects of TFPI and the PT and aPTT will not completely correct on mixing with normal plasma. The plasma TFPI level will be elevated, and factor V activity is usually normal.

FFP is the mainstay for treating bleeding and preparing for surgery in factor V deficient patients (see Table 135.3). The minimum factor V level required for hemostasis is 10% to 15% of normal.[11] This can be achieved in patients with less than 1% plasma factor V activity by administering 15 to 20 mL/kg FFP, followed by 5 mL/kg every 12 hours. A plasma level of 25% is recommended for major surgery. Estimates of the factor V plasma half-life vary, but 12 to 14 hours is assumed for replacement purposes. Infusing 20 mL/kg FFP over 3 to 4 hours before surgery, followed by 5 to 10 mL/kg every 12 hours is usually adequate. Infusions should be continued for 7 to 10 days to permit wound healing. Fluid overload from large volumes of FFP may be a problem in some patients. Plasma exchange has been used successfully to prepare factor V deficient patients at risk for fluid overload for surgery. Mucosal bleeding from the nose and mouth may respond to antifibrinolytic agents and superficial lacerations usually respond to local pressure. A factor V concentrate (manufactured by Kedrion) prepared from cryo-supernatant corrects clotting abnormalities in factor V deficient plasma in vitro, but it has not been extensively tested in patients. Cryoprecipitate is not a source of factor V and plasma levels do not respond to administration of DDAVP. Platelets contain factor V and may be particularly useful in patients with severe bleeding or with factor V inhibitors (see "Acquired Factor V Deficiency").[11] However, platelet transfusions are not recommended as routine first-line treatment because of the possibility of developing antiplatelet alloantibodies (see Chapter 123).

Heavy menstrual bleeding is common in factor V-deficient women. Symptoms can be managed with antifibrinolytic therapy (ε-amino caproic acid 50 to 60 mg/kg every 4 to 6 hours, or tranexamic acid 15 mg/kg every 6 to 8 hours), oral contraceptives, levonorgestrel-releasing intrauterine devices, replacement therapy, or surgical intervention (endometrial ablation or hysterectomy). Replacement should be adjusted to maintain a factor V level of 10% to 15% of normal. Factor V-deficient women may have significant bleeding with childbirth and should be treated in a similar manner.

Acquired Factor V Deficiency

Plasma factor V levels may be reduced along with other coagulation factors in patients with liver disease or DIC (see Chapter 137). Alloantibody inhibitors to factor V have been reported in a few severely deficient patients after replacement therapy and in patients treated with older topical bovine thrombin preparations that also contained bovine factor V. The latter situation occurs less frequently with newer recombinant human thrombin preparations. Acquired factor V deficiency caused by factor V autoantibodies has been reported in patients after surgery, blood transfusion, or therapy with β-lactam or aminoglycoside antibiotics, and in patients with cancer, myeloproliferative disorders, and autoimmune diseases. Rare patients with factor V deficiency in the setting of amyloidosis have been reported. Bleeding in patients with antibodies to factor V may be severe, although some patients are asymptomatic. When acquired factor V deficiency responds poorly to FFP infusion, antibodies that neutralize factor V activity may not be identifiable. Instead, it is likely that non-neutralizing antibodies that enhance the clearance of infused factor V are present.

Neutralizing factor V inhibitors cause prolongation of the PT and aPTT that does not correct when patient plasma is mixed with normal plasma. Factor V inhibitor titers are established using the Bethesda method used for inhibitors to factors VIII and IX (see Chapter 127). Unlike inhibitors to factor VIII, which typically take 1 to 2 hours to fully inactivate their target in a mixing study with normal plasma, factor V inhibitors inhibit factor V almost immediately.[12] The thrombin time should be normal, except when the inhibitor is induced by bovine thrombin, where antibodies against thrombin may also be present.

Most factor V inhibitors are transient. Steroids, immunosuppressive therapies, and exchange transfusions have been used in a few patients.[12] Platelet transfusion may be useful. Platelet factor V may either be protected from inhibition or be sufficiently different in structure from its plasma counterpart to not cross-react with antibodies directed against factor V. The effectiveness of recombinant factor VIIa (rfVIIa) and PCCs have not been clearly established. While the defect caused by factor V deficiency would hypothetically render therapy with factor VIIa ineffective, this agent has been used with apparent success in at least two patients with severe factor V deficiency requiring surgery.

INHERITED FACTOR VII DEFICIENCY (OMIM 227500)

Factor VII deficiency was first reported by Alexander and colleagues in 1951. Severe factor VII deficiency is the most common of the nonhemophilic coagulation factor deficiencies (see Table 135.1), with an estimated prevalence of 1 in 300,000 to 500,000 persons.[13] The heterozygote frequency may be ~1 in 350. *Factor VII* is the 50,000 Da precursor of the protease *factor VIIa*. Factor VII and VIIa bind to tissue factor and initiate coagulation through activation of factors X and IX (see Fig. 135.1A). Factor VII levels correlate weakly with clinical presentation (see Table 135.2). Factor VII activity levels of less than 10%, 10% to 20%, and greater than 20% have been proposed as criteria to classify deficiency as severe, moderate, or mild, respectively. However, many patients with levels less than 10% will not have significant bleeding, even with invasive procedures. Mice lacking factor VII develop normally in utero but succumb to bleeding at birth. An infant born lacking factor VII died from intracranial hemorrhage 12 days after birth, while another survived with replacement therapy. Thus, low levels of factor VII appear to be necessary to sustain life. Although factor VII deficiency is considered an autosomal recessive trait, bleeding symptoms are reported in up to 36% of patients with heterozygous deficiency. A classification system based on clinical presentation has also been proposed.

Nearly 300 factor VII gene (*F7*) mutations associated with factor VII deficiency have been described (www.factorvii.org and www.hgmd.cf.ac.uk). Most are missense mutations. Although most unique variants were identified in single families, some are widespread. In surveys of patients from Europe and Latin America, Ala244Val accounted for 84% of abnormal *F7* alleles in 88 unrelated Jewish patients, and in 14% and 7% of abnormal *F7* alleles in Germany and France, respectively. CRM+ variants are common. It is important to recognize that some factor VII variants demonstrate variable activity in PT assays, depending on the species of origin of the tissue factor used in the assay. The name "Factor VII Padua" has been applied to these variants, many of which have an Arg304Gln substitution that causes a significant defect in the interaction with rabbit tissue factor, but not with human or ox tissue factor. These variants are not associated with bleeding.

Factor VII deficiency may occur in combination with deficiencies of other vitamin K–dependent proteins (see "Combined Deficiency of Vitamin K–Dependent Proteins"). It may also present in combination with factor X deficiency from loss of both genes due to a chromosome 13q34 deletion. Factor VII deficiency has been reported in cases of trisomy 8 and in conjunction with abnormalities of bilirubin metabolism, intellectual impairment, microcephaly, epicanthus, cleft palate, and patent ductus arteriosus in the 13q deletion syndrome.

The clinical spectrum of bleeding in patients with factor VII deficiency is broad. Significant bleeding tends to be greatest with severe deficiency and patients with levels less than 1% of normal may have a syndrome similar to severe hemophilia, with spontaneous joint and soft tissue bleeding and hemarthrosis-related arthropathy.[13] The best predictor of bleeding risk seems to be the nature of bleeding (or its absence) at the time of diagnosis. Central nervous system hemorrhage is relatively common (4% to 17%). Patients with factor VII activity ≥ 10% of normal tend to have milder symptoms such as epistaxis, heavy menstrual bleeding, and bruising. Although most patients with factor VII levels ≥20% of normal have few symptoms, significant bleeding occasionally occurs. In one study, 36% of heterozygotes (factor VII activity 21% to 69%) reported bleeding problems involving skin and mucous membranes, while another study reported a greater risk for subcutaneous bleeding. One study with long-term data for heterozygotes and their family members found no statistically significant difference in bleeding between affected and unaffected family members, with affected individuals having factor activity levels between 35% and 65%. Excessive bleeding often complicates dental extraction and surgery on the oropharynx or urogenital tract in untreated patients, while abdominal surgery and hysterectomy are associated with fewer problems. This may reflect the different levels of fibrinolytic activity in these tissues. Postpartum bleeding is uncommon in factor VII-deficient women because factor VII levels rise late in pregnancy in all but the most severely deficient patients.[14] The risk of postpartum hemorrhage correlates poorly with FVII levels. This finding has led some to suggest that rfVIIa be used if there is excessive bleeding.

Factor VII deficiency has been associated with thrombosis.[15] A study of 33 reported cases of thrombosis (6 arterial, 27 venous) included 15 patients with molecular studies. Nearly all patients had other thrombotic risk factors, usually acquired, and four had inherited thrombophilia. Of the 15 patients with completed *F7* genotyping, 11 had either Arg304Gln (Factor VII Padua) or Ala294Val substitutions, both of which cause CRM+ deficiency. Those with factor VII Padua had no bleeding history, while mild bleeding was reported with pAla294Val. Treatment with vitamin K antagonists is problematic because the baseline PT is abnormal due to the mutation. Low-molecular-weight heparin or direct oral anticoagulants have been used successfully in small numbers of patients.

In factor VII deficiency, the PT is prolonged and the aPTT is normal (see Chapter 127). The diagnosis is confirmed, and the severity determined, with a modified PT assay using factor VII-deficient plasma. With sensitive thromboplastin reagents, the PT may be prolonged with factor VII levels at the lower end of the normal range. When assessing patients for factor VII deficiency, the composition of the thromboplastin reagent used to trigger clotting in the PT assay is important. Factor VII Padua-type variants interact poorly with the rabbit tissue factor-based thromboplastins widely used in North America. They function better with human tissue factor and best with ox tissue factor. The prevalence of factor VII Padua-type variants is low, but they have a worldwide distribution.

Major considerations when treating factor VII-deficient patients include the short plasma half-life of factor VII (3 to 4 hours), the relatively low recovery of infused material (possibly due to a large volume of distribution), and rapid clearance in children. A plasma factor VII level of 10% to 15% of normal is probably the minimum required for surgery, with 15% to 25% being adequate in most cases. Of 157 surgeries performed on 83 factor VII-deficient patients with a mean baseline activity of 5% who did not receive prophylaxis, 15.3% had bleeding complications. These data suggest that supplementing patients with baseline levels less than 10% should prevent bleeding. Factor replacement can be accomplished with several products (see Table 135.3). FFP is widely available, but its effectiveness is limited because of the large volumes required. Four-factor PCCs contain variable amounts of factor VII. However, these products are not favored, because they contain other vitamin K–dependent factors in higher concentrations than factor VII, which could increase the risk of thrombosis. rfVIIa is the preferred therapy for treating or preventing bleeding in factor VII deficiency. A registry of factor VII-deficient patients recorded the replacement therapy given for various bleeding events (hemarthrosis, soft-tissue hematomas, epistaxis, gum bleeding, menorrhagia, and others) and found that the majority responded adequately to a single relatively large dose of rfVIIa (60 μg/kg). Patients with heavy menstrual bleeding often required additional doses. Prophylaxis for invasive procedures may require treatment every 2 to 3 hours. In a study of 41 surgeries in 34 factor VII-deficient subjects receiving rfVIIa, bleeding occurred in only three instances, and in those the rfVIIa dose was considered low. The minimum treatment that adequately prevented bleeding was 13 μg/kg/dose, with no less than three doses given on the day of surgery. However, some patients may do well with less. In a report of patients with a mild to moderate risk of procedure-related bleeding, two doses on the day of surgery followed by once-daily dosing thereafter controlled bleeding. Continuous infusion rfVIIa has been used to cover surgery and may reduce the total amount of factor administered by 70% to 90% compared with bolus infusions. Caution should be exercised when treating patients with factor VII Padua variants with rfVIIa because replacement is often unnecessary and excessive replacement can increase the risk of thrombosis.

Patients with severe factor VII deficiency who have had life-threatening bleeding may benefit from secondary prophylaxis, particularly if bleeding involved the central nervous system (associated with 50% to 70% mortality). FFP and plasma-derived factor VII concentrates have been used, but rfVIIa is preferred. Despite its short half-life, rfVIIa can be effective at doses of 20 to 30 μg/kg given two to three times per week. The large volume of distribution for factor VII/VIIa may explain this effect. Not all bleeding episodes require factor replacement. Minor injuries may be controlled with local measures. Antifibrinolytic agents may be effective for minor bleeding, dental surgery, or procedures involving mucous membranes. Fibrin sealant may also be effective in some situations. Cryoprecipitate is not a source of factor VII and plasma levels do not respond to administration of DDAVP. An infant with severe inherited factor VII deficiency was cured with liver transplantation.

Acquired Factor VII Deficiency

Reduced levels of factor VII occur with warfarin therapy, poisoning with rodenticides such as brodifacoum, liver disease, DIC, biliary tract disease, vitamin K deficiency, and cephalosporin therapy. In most of these situations, other vitamin K–dependent factors are decreased, although the effect on factor VII is often greater because of its short half-life. Acquired factor VII deficiency has been reported as a paraneoplastic syndrome with atrial myxoma and Wilms tumor. Trisomy 8, which has been associated with inherited factor VII deficiency, has also been observed in a case of acquired factor VII deficiency in acute myeloid leukemia which resolved with chemotherapy and disappearance of the karyotypic abnormality. Other scenarios rarely associated with low factor VII include hematopoietic stem cell transplantation, aplastic anemia, and sepsis.

Alloantibody inhibitors to factor VII have been reported in deficient patients after replacement therapy. In a recent registry report, inhibitors were noted in 4 of 225 patients with activity levels less than 4% who received replacement therapy. All were high titer responders and three developed in infancy.

INHERITED FACTOR X DEFICIENCY (OMIM 227600)

Inherited *factor X deficiency* was first described by Duckert et al. in 1955 and was originally called *Stuart-Prower factor* after two patients who were missing the protein. Patient Stuart was thought to have factor VII deficiency, but mixing his plasma with factor VII-deficient plasma corrected the clotting defect. Patient Prower had multiple coagulation assay abnormalities, including a prolonged thromboplastin generation test and PT. The prevalence of severe factor X deficiency is ~1 in 1 million persons (see Table 135.1).[16] *Factor X* is a 58,000 Da protein that is the precursor of the protease *factor Xa*. Factor X may be activated by the factor VIIa/tissue factor complex or by a complex of factor IXa and factor VIIIa (see Fig. 135.1A). Factor Xa converts prothrombin to thrombin in the presence of factor Va, phospholipid, and calcium ions, and also converts factor V to factor Va.

Inherited factor X deficiency is an autosomal recessive trait. Factor X-deficient mice die in utero or shortly after birth from bleeding, indicating the protein is necessary for life. Humans with the severest form of factor X deficiency (activity <1% of normal) most likely have a trace of factor X in their plasma. More than 140 factor X gene (*F10*) mutations have been identified in factor X-deficient patients (http://www.hgmd.cf.ac.uk/ac/gene). Most are missense mutations, and many are associated with CRM+ variants.[16] A three-part classification system has been proposed. Type I deficiencies involve CRM− variants, and include patient Stuart (homozygous for Val298Met). Type II deficiencies are associated with CRM+ variants lacking activity and include patient Prower (compound heterozygote for Arg287Trp and Asp282Asn). Type III deficiencies include variants that are activated normally by Russell viper venom but not by factor VIIa/tissue factor or factor IXa/VIIIa (Factor X Friuli), variants defective only in activation by factor VIIa/tissue factor, variants defective only in activation by factor IXa/VIIIa, and variants with higher activity in chromogenic assays than in clotting assays. Factor X deficiency can be inherited with deficiencies of other vitamin K–dependent proteins (see "Combined Deficiency of Vitamin K–dependent Proteins") and in combination with factor VII deficiency when both genes are lost due to a chromosome 13 q34 deletion. In contrast to other vitamin K-dependent clotting factors (prothrombin, and factors VII and IX), there are no known factor X mutations associated with an increased risk of thrombosis.

A classification system for factor X deficiency has been proposed based on plasma activity (severe, <10%; moderate, 10% to 40%; mild, >40% of normal activity) (see Table 135.2). Bleeding in factor X deficiency is severe and occurs earlier in life in patients with the lowest plasma levels (<1%). There is an impression that factor X-deficient patients bleed more severely than patients with other rare inherited coagulopathies. In a series of 102 factor X-deficient patients, the most frequent symptom was easy bruising (55%) followed by hematoma (43%). Epistaxis and hemarthrosis were common. Intracranial hemorrhage was seen in patients homozygous for the pGly380Arg mutation. Among homozygotes, hemarthrosis was common, and all women suffered from heavy menstrual bleeding. Umbilical stump bleeding occurred in 28% of newborns. Moderate to mildly affected persons (activity >10%) may have increased bruising or bleeding with trauma. While heterozygotes tend to be asymptomatic, up to one third may have excessive bleeding from mucous membranes with invasive procedures or with childbirth.

Factor X is a component of the common pathway of coagulation (see Fig. 135.1B). Its absence prolongs the PT and aPTT, and must be distinguished from deficiencies of fibrinogen, prothrombin, or factor V. Definitive diagnosis and determination of severity are established using a modified PT or aPTT assay with factor X-deficient plasma. Some factor X variants preferentially prolong either the PT or the aPTT.

Factor X-deficient patients are treated with FFP, PCC, or newer plasma-derived factor X concentrates for most bleeding episodes (see Table 135.2).[2] The half-life of factor X is 20 to 40 hours. A trough level of 10% to 20% is usually sufficient for hemarthroses and soft tissue bleeding. FFP administered as a loading dose of 10 to 20 mL/kg followed by 3 to 6 mL/kg every 12 to 24 hours usually keeps trough levels above 10% to 20%.[16] Higher factor X levels may be required for severe bleeding or surgery, and accumulation of factor X in plasma can be achieved by increasing the transfusion frequency to every 12 hours. PCCs with a factor X:IX ratio of ~1:1 will increase plasma factor X levels by 1.5% for each factor IX unit/kg body weight. A dose of 15 to 20 factor IX units/kg every 1 to 2 days has been suggested for major surgery.[16] The factor X concentrate Coagadex is administered at a dose of 25 to 40 IU/kg twice weekly for prophylaxis. For control of bleeding, once-daily administration of 25 to 30 IU/kg is recommended. For surgery, the manufacturer suggests preoperative factor X levels of 70 to 90 IU/dL (70% to 90% of normal activity (see https://www.coagadex.com/download/coagadex-pi.pdf), which is a rather high level, based on data from a limited number of patients. While thrombosis has not been reported in patients with inherited factor X deficiency, there is a risk of thrombosis or DIC with PCC use, and it is recommended that plasma factor X levels greater than 50% of normal be avoided. Factor X levels rise modestly in pregnancy, but severely affected patients require prophylaxis with invasive procedures and at delivery to prevent hemorrhage. A factor X level of 20% to 40% is recommended. For patients with severe factor X deficiency and recurrent hemorrhage, prophylactic PCC or factor X concentrate is effective.[16] Minor bleeding can be treated with local measures and/or antifibrinolytics. Cryoprecipitate lacks factor X, and DDAVP infusion does not affect factor X levels. Severe factor X deficiency was cured by liver transplantation in a child.

Acquired Factor X Deficiency

Acquired factor X deficiency occurs with warfarin therapy, poisoning with rodenticides such as brodifacoum, vitamin K deficiency, liver disease, and DIC, all of which reduce levels of other coagulation factors. Factor X deficiency has also been reported with malignancies, infections, burns, proteinuria, and medications.

Acquired factor X deficiency may accompany systemic amyloidosis, occurring in 8.7% to 14% of patients with AL amyloidosis.[17] Factor X binds to amyloid fibrils, which reduces the plasma half-life of the protein. It can be difficult to distinguish this condition from inherited factor X deficiency in the laboratory because factor X-dependent assays correct after mixing with normal plasma in both conditions. The absence of a lifelong bleeding disorder, findings of a serum M-protein, signs of amyloidosis, and histologic confirmation of amyloid in tissues point toward AL amyloidosis. A poor response to factor X infusion in the absence of an inhibitor also distinguishes the conditions.

Patients with acquired factor X deficiency and amyloidosis have variable responses to infusions of products containing factor X rendering individual pharmacokinetic studies of factor X replacement therapy important. Treatment may include chemotherapy, splenectomy, plasma exchange, PCCs, activated factor VIIa or factor X concentrate. Optimal hemostatic management for invasive procedures has not been determined. In one series, complications occurred in only 13% of procedures and there was a poor correlation between the risk for bleeding and the factor X level.

As with prothrombin deficiency, there are no reports of acquired alloantibody inhibitors to factor X after replacement therapy in patients with inherited factor X deficiency, consistent with the notion that these patients have trace amounts of plasma factor X at baseline. There are rare reports of acquired autoantibody factor X inhibitors, most of which resolve after treatment of underlying conditions.

INHERITED FACTOR XI DEFICIENCY (OMIM 264900)

In 1953, Rosenthal and colleagues described members of a family with abnormal hemostasis, prolonged time to clot formation in glass tubes, and a normal PT. In mixing studies, patient plasma shortened the clotting times of hemophilia A and B plasmas, indicating that the missing factor was distinct from factors VIII and IX. Unlike the X-linked hemophilias, the new disorder (sometimes called hemophilia C) was transmitted as an autosomal trait. The missing factor was called *plasma thromboplastin antecedent* and subsequently *factor XI*. Early studies estimated that severe *factor XI deficiency* (<15% of normal plasma level) occurs in 1 per million persons (see Table 135.1); however, recent exome-based analyses indicate higher frequencies of 20.3 in 10^6 in East Asians, 12.9 in 10^6 in non-Finnish Europeans, 4.6 in 10^6 in persons of Latin origin, and 2 in 10^6 in Africans and South Asians.[18] The condition is most common in persons of Ashkenazi Jewish ancestry (incidence 1 in 450).[18] Factor XI is the 160,000 Da precursor of the protease *factor XIa*, which contributes to clotting by activating factor IX (see Fig. 135.1A). The protein is a homodimer,[18] a feature that has implications for inheritance of factor XI deficiency. Factor XI is activated by factor XIIa in the aPTT assay (see Fig. 135.1B); however, it is probably activated in vivo by other proteases such as thrombin (see Fig. 135.1A), because factor XII deficiency does not cause abnormal bleeding.

Factor XI deficiency in the Jewish population is an autosomal recessive condition, with two point mutations in the factor XI gene (*F11*) accounting for greater than 90% of mutant alleles. Glu117Stop is found in Jewish patients from different ethnic backgrounds and in non-Jewish patients (particularly South Asians) and is at least 2500 years old. Homozygotes lack plasma factor XI protein. Phe283Leu occurs primarily in Jews of European ancestry and is likely of more recent origin. It causes a defect in dimer formation and results in poor secretion. Homozygotes have ~10% of normal plasma factor XI activity. Compound heterozygotes for Glu117Stop and Phe283Leu have ~3% normal activity, while heterozygotes for either mutation have activities of 50% to 60%. The allele frequencies of Glu117Stop and Phe283Leu in Ashkenazi Jews (2.2% and 2.5%, respectively) indicate a carrier frequency for an abnormal factor XI allele of ~5%.

More than 250 human *F11* mutations have been identified (http://www.hgmd.cf.ac.uk/, www.factorxi.org), in addition to Glu117Stop and Phe283Leu. Several are recurrent and ethnic-specific. For example, a Phe223Leu substitution accounts for 23.5% of mutated alleles in Africans, while Gln263Stop and Leu424Cys-fs-Stop are responsible for 28.2% and 20.5% of mutations in East Asians, and Ala412Thr represents 25% of mutations in persons of Latin origin.[18] In most deficient patients, factor XI activity and antigen are comparably reduced (CRM− deficiency). CRM+ mutations are rare. A three-category scheme has been proposed for classifying CRM− factor XI deficiency. Category one contains mutations that prevent protein synthesis (e.g., Glu117Stop), while mutations in category two interfere with dimer formation (e.g., Phe283Leu). Inheritance in both categories follows a recessive pattern because mutant polypeptides do not interfere with the product of the normal *F11* allele in heterozygotes. Category three includes mutations that impair secretion but do not prevent dimer formation (e.g., Gly400Val). In heterozygotes, mutant and wild type polypeptides form non-secretable heterodimers, trapping normal protein in the cell. This phenomenon likely accounts for families in which severe to moderate factor XI deficiency appears to be a dominant trait.

Bleeding in severe factor XI deficiency is usually injury-related and occurs most frequently in tissues with robust fibrinolytic activity, such as the oral and nasal cavities and the urinary tract.[18] Injury to these areas causes excessive bleeding in two-thirds of patients, regardless of genotype. Bleeding with injury at other locations is less frequent and tends to occur in those with the lowest factor XI levels.[18] Bleeding may start at the time of injury or be delayed by hours, and oozing from tooth extraction may persist for days. Excessive bleeding with skin laceration, circumcision, appendectomy, and orthopedic surgery is infrequent. Spontaneous bleeding, except for heavy menses, is uncommon. In clinical trials evaluating factor XI inhibitors for thromboprophylaxis after knee replacement surgery, bleeding was uncommon even in those with less than 5% factor XI activity before or after surgery.

Bleeding correlates poorly with plasma factor XI activity (see Table 135.2). Patients with severe deficiency may not bleed excessively, even with surgery, and a patient may exhibit different bleeding tendencies over time. Opinions differ regarding the bleeding risk associated with mild deficiency (plasma level 20% to 50%). Some studies describe minimal bleeding with tooth extraction, tonsillectomy, nasal surgery, and urologic surgery, while others report

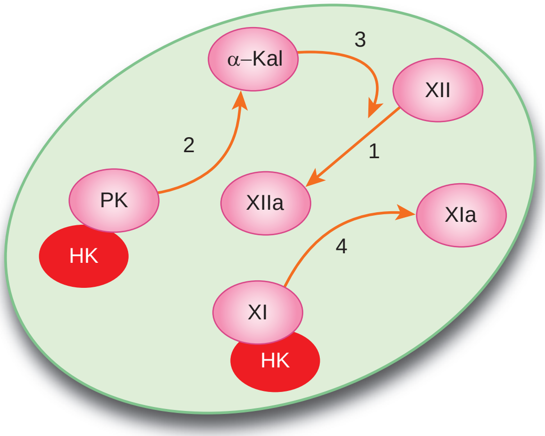


Figure 135.5 CONTACT ACTIVATION. In the aPTT assay, contact activation is initiated by conversion of factor XII (XII) to factor XIIa (XIIa) (reaction 1) when plasma is exposed to a negatively charged surface *(light green disk)*. Factor XIIa converts prekallikrein (PK) to the active protease plasma kallikrein (Kal) (reaction 2), and kallikrein reciprocally activates additional factor XII (reaction 3). Factor XIIa initiates coagulation through conversion of factor XI (XI) to factor XIa (XIa) (reaction 4). PK and factor XI require high molecular weight kininogen (HK) to bind properly to the surface. Factor XIa ultimately promotes thrombin generation and fibrin clot formation through activation of factor IX (see Fig. 135.1).

difficulty distinguishing severe and mild deficiency on clinical grounds. In a study of 45 families, the odds ratios for excessive bleeding were 13.0 and 2.6 for homozygotes and heterozygotes respectively. Thus, mild deficiency may confer a slightly increased risk for bleeding, but considerably less that in severe deficiency. One study indicated that ristocetin cofactor activity is significantly lower in symptomatic patients with heterozygous factor XI deficiency, compared with non-bleeders, suggesting that von Willebrand factor levels influence bleeding propensity. Thrombin generation and fibrinolysis assays have shown promise in predicting the bleeding propensity in factor XI deficiency, but this requires confirmation in larger studies.

In the aPTT assay, factor XI is bound to the contact surface through high molecular weight kininogen and is converted to factor XIa by factor XIIa (see Fig. 135.1B and Fig. 135.5). Factor XIa then activates factor IX. Therefore, factor XI deficiency prolongs the aPTT, but not the PT. Diagnosis and severity are established by a modified aPTT assay using factor XI-deficient plasma. The aPTT is often normal in mild (heterozygous) factor XI deficiency.

Perioperative therapy should be individualized for factor XI-deficient patients (see box on Managing Factor XI–Deficient Patients). In situations where replacement of factor XI is deemed appropriate, FFP or factor XI concentrate (Hemoleven or FXI Concentrate; see Table 135.2) have been used.[1,2,18,19] Plasma exchange has been used in some cases to avoid fluid overload. Cryoprecipitate does not contain factor XI. The half-life of factor XI is 45 to 52 hours, facilitating daily or every-other-day dosing. Hemoleven has orphan drug-status in the United States. A 3-year post-marketing analysis of the concentrate indicated it was effective for treating bleeding and as prophylaxis for surgery, invasive procedures, and pregnancy.[19] In the past, factor XI concentrate use could be associated with thrombosis and DIC, mostly in older patients with cardiovascular disease receiving doses greater than 30 units/kg.[19] More recent analyses suggest that patients receiving 20 to 30 units/kg may still have thrombotic events. This has led to a recommendation that initial doses do not exceed 10 to 15 U/kg. Concentrates should be used sparingly (or avoided) in patients with a history of thrombosis or risk factors for thrombosis,[1,2] and antifibrinolytic therapy should probably not be used concomitantly with concentrate. There has been a shift in management strategy for factor XI-deficient patients at some centers, with less reliance on factor replacement and greater use of rfVIIa and antifibrinolytic drugs (see box on Managing Factor XI–Deficient Patients). rfVIIa at relatively low doses (10 to 15 μg/kg), with or without antifibrinolytic therapy, was found to be effective in factor XI-deficient patients with alloantibody inhibitors against factor XI, and similar strategies were subsequently used successfully in deficient patients without inhibitors.[20] The bispecific antibody emicizumab, which is used in patients with hemophilia A with neutralizing inhibitors, enhances coagulation in factor XI–deficient plasma, but has not been tested in factor XI–deficient patients.

MANAGING FACTOR XI–DEFICIENT PATIENTS

Selecting treatment for patients with factor XI deficiency is challenging because of the poor correlation between symptoms and plasma factor levels. While patients of Jewish ancestry with severe deficiency have more bleeding problems than those with mild deficiency, this does not appear to hold for the general population. When preparing patients with severe factor XI deficiency (factor XI level <15 of normal) for an invasive procedure it is important to keep in mind that (1) a negative bleeding history does not indicate a low risk with subsequent procedures, (2) certain procedures are associated with lower risk of bleeding than others, and (3) patients with very low factor XI levels (<1%) frequently develop neutralizing inhibitors after replacement therapy. These observations have led to refinements in treatment recommendations that rely less on factor replacement and more on antifibrinolytic therapy and rfVIIa.

Antiplatelet drugs should be stopped 1 week before surgery. The prothrombin time and platelet count should be normal, and the possibility of co-existing hemostatic abnormalities investigated. Patients with factor XI levels greater than 40%–50% of normal generally do not experience abnormal bleeding and a history of excessive bleeding in such an individual suggests other hemostatic abnormalities are present. If the patient has been exposed to factor XI in the recent past, the possibility that a neutralizing inhibitor is present should be considered.

Traditionally, factor replacement has been used for major surgery in most factor XI–deficient patients, with treatment initiated prior to the procedure. Patients undergoing surgery on areas at high risk for bleeding such as the oropharynx, nasopharynx, or urinary tract are treated with FFP or factor XI concentrate to keep the plasma trough factor XI level greater than 40% of normal for at least 7 days. Administering FFP at 15 mL/kg every 1–2 days is the most common way to achieve this. A similar strategy has been applied for patients undergoing neurosurgery, head and neck surgery, cardiothoracic procedures, and major abdominal or pelvic surgery. For nasal surgery and oral procedures such as tonsillectomy, supplementing FFP with antifibrinolytic therapy should be considered. Antifibrinolytics must be used with caution in patients receiving factor XI concentrate because of the potential for thrombotic events. For prostatectomy and other surgery on the lower urinary tract, flushing the bladder with saline containing an antifibrinolytic agent may be beneficial. For some minor surgeries, patients may receive replacement to maintain levels of 30% for 5 days. However, a “Wait and See” strategy in which replacement is withheld unless bleeding occurs appears to be appropriate for circumcision, appendectomy, some orthopedic surgery, and with normal vaginal deliveries. In a phase II trial in which antisense oligonucleotide therapy was used to reduce plasma factor XI as prophylaxis to prevent venous thrombosis with knee arthroplasty, patients underwent surgery with factor XI levels as low as a few percent of normal without excessive bleeding. This demonstrates that factor XI probably plays a minor role in hemostasis during certain types of procedures.

Low doses of recombinant factor VIIa (rFVIIa, 15 to 30 μg/kg) administered every 2–4 h in conjunction with antifibrinolytic therapy has been used successfully instead of replacement therapy in patients without factor XI inhibitors undergoing surgery. In some centers, rFVIIa-based strategies are now preferred over replacement therapy for most indications. Benefits of this approach include the smaller volumes of infusion and the avoidance of induction of anti-factor XI alloantibodies, which frequently develop in patients with very low plasma factor XI levels (<1% of normal) after replacement therapy. In patients with factor XI alloantibodies, hemostasis remained intact after major surgery (including an aortic dissection) with administration of a single dose of rFVIIa (15 to 30 μg/kg) followed by antifibrinolytic therapy. The higher doses of rFVIIa typically used to treat patients with factor VIII inhibitors are not required in factor XI-deficient patients (with or without inhibitors) and should be avoided to keep the thrombotic risk low. In pregnancy, there are few data evaluating the safety of epidural anesthesia in the absence of some sort of coverage. While many physicians consider this situation an indication for factor replacement, coverage with a single dose of rFVIIa has been used successfully.

Alternatives to factor replacement are recommended in several situations. Tooth extraction or skin biopsies can be managed with anti-fibrinolytic drugs alone (ε-amino caproic acid 4 g loading dose and 2 g every 6 h thereafter or tranexamic acid 1300 mg every 6 h), starting 12 h before the procedure and continuing for 7 days. Dental procedures such as scaling or root canal can be performed safely using ε-amino caproic acid or tranexamic acid mouthwash prepared from the intravenous formulation three to four times daily with or without systemic antifibrinolytic therapy. Fibrin sealants can be used in place of fibrinolytic therapy for skin biopsy or resection of skin lesions.

Patients with factor XI deficiency can experience thromboembolic episodes. Aspirin or clopidogrel can be used in factor XI-deficient patients with myocardial infarction or other manifestations of atherosclerosis. Direct oral anticoagulants (DOACs) may be used for atrial fibrillation or venous thromboembolism. If these conditions are treated with warfarin, the INR should preferably not exceed 2.5.

Circumcision, orthopedic surgery, and appendectomy carry low bleeding risks in patients with severe factor XI deficiency, and replacement can often be withheld unless bleeding occurs.[18] A similar “wait and see” approach has been proposed for factor XI–deficient women during labor and delivery, which is associated with a relatively low (~ 20%) rate of excessive bleeding.[21] However, others have advocated replacement for childbirth. Mild factor XI deficiency (plasma level 20% to 70% of normal) does not appear to increase bleeding risk during childbirth. rfVIIa has been used to cover epidural block in deficient women during labor and delivery.[21] Dental procedures such as tooth extraction can be covered with antifibrinolytic therapy. The effectiveness of DDAVP in mild factor XI deficiency is not established. There are a few reports that levels increase in response to this drug.

Acquired Factor XI Deficiency

Factor XI levels are reduced in liver disease and DIC but are not affected by vitamin K deficiency or warfarin therapy. Mild to moderate factor XI deficiency occurs in ~25% of patients with Noonan syndrome and is common in carbohydrate-deficient glycoprotein syndrome, a group of inherited disorders involving defects in glycosylation of secreted glycoproteins.

Alloantibody inhibitors to factor XI are common after replacement therapy, particularly in patients with no circulating factor XI. In one study, one-third of individuals homozygous for Glu117Stop developed neutralizing antibodies, often after a single exposure to plasma. While factor XI-deficient patients with inhibitors do not usually have increased spontaneous bleeding, the antibodies interfere with replacement therapy during surgery. rfVIIa may be used in patients with factor XI antibodies as coverage for surgery. Some physicians prefer it to factor replacement, even in patients without inhibitory antibodies, to avoid inducing alloantibody formation.[20]

Factor XI autoantibodies have been reported in patients with autoimmune disorders (e.g., systemic lupus erythematosus, rheumatoid arthritis, Crohn disease, or membranoproliferative glomerulonephritis) and malignancies (e.g., chromic myelomonocytic and lymphocytic leukemia, or gastrointestinal adenocarcinoma). Significant bleeding is rare, but if it occurs can be treated with rfVIIa and/or antifibrinolytic therapy.

DEFICIENCIES OF FACTOR XII, PREKALLIKREIN OR HIGH MOLECULAR WEIGHT KININOGEN

Factor XII, prekallikrein, and high molecular weight kininogen (HK) form the plasma kallikrein-kinin system, a mechanism for generating vasoactive kinins such as bradykinin. The KKS is required for normal factor XI activation in the "contact phase" that initiates coagulation in the aPTT assay (see Figs. 135.1B and 135.5). Patients lacking a KKS component have a prolonged aPTT, but do not have abnormal hemostasis. While no specific therapies are required to prepare patients deficient in factor XII, prekallikrein, or HK for invasive procedures, the conditions must be identified to distinguish them from deficiencies of factors VIII, IX, or XI, which also prolong the aPTT, but do cause abnormal hemostasis.

Factor XII Deficiency (OMIM 234000)

In 1955, Ratnoff and Colopy described asymptomatic individuals with a novel abnormality of surface-induced coagulation. The missing plasma component was called *Hageman factor* after the index case and later designated *factor XII*. The incidence of severe *factor XII deficiency* is not known, as many deficient persons likely go undiagnosed. *Factor XIIa*, the activated form of factor XII, is an 80,000 Da protease that activates factor XI and prekallikrein in the contact phase of the aPTT assay (see Fig. 135.5).[22] A C/T polymorphism referred to as 46 C/T (or -4C/T) in the 5′-untranslated region of the factor XII gene (*F12*) strongly influences plasma factor XII levels. The frequency of *F12* 46T is high (73%) in Japanese and Han Chinese individuals, who have lower factor XII levels than members of other ethnic groups such as Caucasians (20%).

More than 50 *F12* gene mutations have been reported in factor XII-deficient individuals (http://www.hgmd.cf.ac.uk/ac/gene). Activity and antigen are usually reduced in parallel (CRM− deficiency), and CRM+ variants are rare. Factor XII deficiency is not associated with spontaneous or excessive post-traumatic bleeding and most cases are identified by the incidental finding of a prolonged aPTT. There is conflicting literature regarding an association between factor XII deficiency and thrombosis. Two studies reported inverse relationships between plasma factor XII levels and the risk for cardiovascular events; however, most analyses indicate no increased thrombotic risk for severe or partial deficiency. A meta-analysis examining the *F12* 46 C/T polymorphism suggests that a correlation with venous thrombosis or myocardial infarction, if present, is weak. Low factor XII levels have been linked to pregnancy loss or failure of in vitro fertilization. Interestingly, factor XII deficient mice do not exhibit abnormalities in reproduction. It is possible that anti-factor XII antibodies that are often associated with antiphospholipid antibody syndrome, rather than true factor XII deficiency, are responsible for pregnancy-related complications in humans. A few *F12* mutations have been associated with detrimental phenotypes. Arginine or lysine substitutions for Thr309 introduce a novel cleavage site for several proteases resulting in a truncated factor XII protein that accelerates prekallikrein activation and bradykinin generation.[22] This leads to a form of hereditary angioedema, characterized by episodic soft tissue swelling primarily involving the face, airway, limbs, or gastrointestinal tract. A Trp268Arg substitution also leading to a truncated form of factor XII has been associated with an autoinflammatory syndrome.[22]

Factor XIIa initiates the sequence of reactions required for clot formation in the aPTT assay (see Fig. 135.3). Factor XII undergoes autocatalytic conversion to factor XIIa on contact surfaces. Factor XIIa then activates prekallikrein to α-kallikrein, which activates additional factor XII (see Fig. 135.3). Factor XIIa also activates factor XI to factor XIa. In severe factor XII deficiency, the aPTT is very long (greater than the assay upper limit). Definitive diagnosis is established using a modified aPTT with factor XII-deficient plasma. Antiphospholipid antibodies may disproportionately affect factor XII measurements in aPTT-based assays. Factor XII antibodies are present in up to half of plasmas with anti-phospholipid antibodies and may account for many cases of presumed mild factor XII deficiency.

Prekallikrein Deficiency (OMIM 229000)

In 1965, Hathaway and coworkers described asymptomatic members of a family with long plasma clotting times that corrected on prolonged incubation with glass. The missing plasma component, initially called *Fletcher factor* after the index cases, was subsequently shown to be *prekallikrein*. Prekallikrein is the 95,000 Da precursor of the protease *plasma kallikrein*, which activates factor XII and cleaves HK to release bradykinin.

Severe *prekallikrein deficiency* is an autosomal recessive trait, presenting in the homozygous or compound heterozygous forms with plasma prekallikrein levels that are usually less than 1% of normal. The condition was thought to be rare; however, recent work indicates it is relatively common in individuals of African ancestry,[23] with a causative frameshift mutation (451dupT) in the prekallikrein gene (*Klkb1*) present in 1% to 1.5% of such individuals. This implies a prevalence for severe CRM− deficiency of ~1:5000 to 7000 in this population. In comparison, the Cys548Tyr substitution in persons of European ancestry has an allele frequency of ~0.1%.[23] These findings are consistent with older observations that Black PK-deficient individuals are usually CRM−, while PK-deficient individuals of European descent or Japanese individuals are usually CRM+. Mild prekallikrein deficiency is probably missed because a low level of prekallikrein activity is sufficient to normalize clotting in most aPTT assays. Partial prekallikrein deficiency (plasma level 10% to 50% of normal) is seen with severe deficiency of HK. This is likely due to increased clearance because most prekallikrein normally circulates in a complex with HK.

Severe prekallikrein deficiency presents as a prolongation of the aPTT in a person without a history of excessive bleeding. While thrombotic events have been described in deficient patients, it is not clear that the deficiency is contributory. Indeed, studies in mice suggest that reducing prekallikrein levels attenuates thrombus formation. Recent analyses raise the possibility that the incidence of hypertension is increased in prekallikrein deficient individuals, possibly related to reduced bradykinin generation.

In the aPTT, prekallikrein is converted to kallikrein by factor XIIa. Kallikrein activates additional factor XII (see Fig. 135.5). Severe prekallikrein deficiency causes a prolonged aPTT, and the diagnosis is confirmed using a modified aPTT with prekallikrein deficient plasma. The prolonged aPTT in prekallikrein deficiency may be shortened by increasing the incubation time with the contact reagent, which allows factor XII autoactivation to compensate for the prekallikrein deficiency. This distinguishes prekallikrein deficiency from deficiencies of factors VIII, IX, XI XII, and HK, where the prolonged aPTT does not correct with prolonged incubation. aPTT reagents that use ellagic acid as the contact activator are relatively insensitive to prekallikrein and may fail to identify some severely deficient patients.

High Molecular Weight Kininogen Deficiency (OMIM 228960)

In 1974, Schiffman and Lee reported that a plasma factor distinct from prekallikrein is required for factor XI activation by factor XIIa. In 1975, asymptomatic individuals from three families were described with in vitro abnormalities of coagulation, fibrinolysis, and kinin generation who lacked the unknown factor. The condition, originally named for the affected families (*Fitzgerald trait, Williams trait, and Flaujeac trait*), was subsequently determined to be due to deficiency of *high molecular weight kininogen (HK)*. The disorder appears to be rare. HK is a 110,000 Da multi-domain glycoprotein containing the vasoactive peptide bradykinin. Prekallikrein and factor XI circulate in plasma in complex with HK. In humans, low molecular weight kininogen (LK) and HK are products of a single kininogen gene (*Kng1*). LK is not required for in vitro coagulation. HK deficiency is an autosomal recessive trait. Depending on the genetic abnormality, patients may have isolated HK deficiency or combined HK and LK deficiency. HK deficient patients are usually identified by the incidental finding of a prolonged aPTT and they do not bleed excessively.

In the aPTT assay, HK facilitates binding of prekallikrein and factor XI to the contact surface, enhancing their activation by factor XIIa (see Fig. 135.5). Severe deficiency is characterized by a very prolonged aPTT. Definitive diagnosis is established using a modified aPTT with HK-deficient plasma. Partial prekallikrein deficiency is common in severe HK deficiency, likely due to increased catabolism of prekallikrein when it is not in complex with HK.

INHERITED FACTOR XIII DEFICIENCY (OMIM 134570 [A SUBUNIT] AND 134580 [B SUBUNIT]

In 1944, Robbins demonstrated that fibrin formed from purified fibrinogen was soluble in weak acid, while fibrin formed in the presence of serum or plasma was not. He proposed that plasma contained a *fibrin-stabilizing factor*. In 1960, Duckert and colleagues described the first patient with deficiency of this factor, which was subsequently designated *factor XIII*. The incidence of severe *factor XIII deficiency* is estimated at 1 in 2 million (see Table 135.1).[24] In blood, factor XIII is distributed between plasma and platelets, with a small amount in monocytes. The plasma protein is a 330,000 Da tetramer composed of two catalytic A subunits and two carrier B subunits. Factor XIII in platelets, monocytes, and placenta has only A subunits. The A subunit of the plasma protein appears to be synthesized primarily in hematopoietic cells, while the B subunit is synthesized in the liver. Factor XIII is converted to the transglutaminase *factor XIIIa* by thrombin. Factor XIIIa catalyzes formation of γ-glutamyl-ε-lysyl bonds between fibrin monomers (see Fig. 135.1A), resulting in a fibrin mesh resistant to dissolution in mild acid or urea (see Chapters 121 and 125). Factor XIIIa also cross-links fibrin to extracellular matrix and cytoskeletal proteins, thereby enhancing clot adherence to injury sites.

Inherited factor XIII deficiency is an autosomal recessive condition.[24] The current classification scheme recognizes factor XIII-A and factor XIII-B defects. Factor XIII-A deficiency is divided into type I (quantitative) and type II (qualitative) deficiencies. In patients with apparent combined FXIII-A and -B deficiency, low levels of the A subunit are likely the result of rapid clearance because of the absence of the stabilizing B subunit. Most cases of inherited factor XIII deficiency are due to mutations in the A subunit gene, with less than 5% in the B subunit gene.[24] More than 100 different mutations have been reported in the factor XIII-A gene (*F13A1*), with about 20 mutations in the factor XIII-B gene (*F13B*).[4] The incidence of *F13A1* and *F13B* mutations affecting plasma activity may be higher than suspected, as screening tests tend to pick up only severely decreased plasma activity (<5% of normal). A few mutations show evidence of a founder effect, with the *F13A1* IVS5-1 G>A splice-site defect and Arg661Stop nonsense mutations each identified in ten apparently unrelated individuals.[4]

Delayed bleeding from the umbilical stump occurs in 80% to 90% of newborns with severe factor XIII deficiency (activity ≤5% of normal) and is considered a diagnostic feature of the disorder. Ecchymoses, soft tissue hematomas, and prolonged bleeding with trauma are common, and recurrent soft tissue bleeding may lead to formation of hemorrhagic cysts (pseudotumors).[24] Hemarthroses is less frequent than in factor VIII or IX deficiency. Bleeding that is delayed 12 to 36 hours post-injury is characteristic of factor XIII deficiency, although hemorrhage is immediate in some cases. Bleeding at the time of invasive procedures may be minimal, but delayed hemorrhage often occurs. Intracranial bleeding is more frequent in factor XIII deficiency than in other inherited coagulation disorder, with an incidence as high as 40% to 60% in the first two decades of life.[4,24] This justifies prophylactic replacement. Delayed wound healing has been observed, possibly related to a defect in angiogenesis. Spontaneous abortions occur with most pregnancies in untreated factor XIII–deficient women,[24] possibly due to abnormal formation of the cytotrophoblastic shell and poor attachment of the placenta to the uterus. The issue of excessive bleeding in patients with milder (>5% of normal) factor XIII deficiency is more controversial. In at least two reports, patients heterozygous for factor XIII deficiency reported excessive bleeding, although the studies lacked control groups. Experience with pregnant women with severe factor XIII deficiency indicates that the factor XIII level needs to be higher than 10% to reliably prevent pregnancy loss.[24,25] This suggests that clinically significant hemostatic problems may occur in patients with levels between 5% and 10% of normal.

The PT and aPTT measure time to fibrin clot formation, but do not assess clot stability. Results of these assays are normal in factor XIII-deficient patients. Clots formed in plasma lacking factor XIII are soluble in 5 M urea or 1% monochloroacetic acid, while clots from normal plasma are stable for at least 24 hours. Solubility in 5 M urea (urea clot stability assay) is often used to screen for factor XIII deficiency or inhibitors. Plasma clots lacking α_2-antiplasmin are also soluble in the urea clot stability assay and can be identified with specific assays. Solubility tests have low sensitivity for detecting factor XIII deficiency, failing to detect some samples with less than 2% of normal activity and probably almost all samples with levels ≥5% of normal. Chromogenic assays that quantitatively measure factor XIIIa activity are available and are replacing the urea clot stability assays in many institutions (see Chapter 127). These assays can also detect and quantify neutralizing factor XIII inhibitors, using a mixing method with normal plasma. The ISTH recommends starting an evaluation with a quantitative assay for factor XIII activity. If abnormal, this is followed by measurements of FXIII-A and B antigens, assessment for autoantibodies with a mixing study to assess for neutralizing antibodies and binding assays for non-neutralizing antibodies. Depending on the results, additional molecular genetics testing to identify causative mutations should be performed. Implementation of this strategy has been limited by the technical expertise required and the expense.[24]

The plasma half-life of factor XIII is 10 to 14 days,[24] facilitating prophylactic replacement therapy. While some consider a level as low as 5% adequate for hemostasis, others suggest that levels greater than 10% are required to reduce bleeding.[24,26] An assessment by the Prospective Rare Bleeding Disorders Database included a 3-year prospective assessment of 29 patients that demonstrated a linear relationship between factor XIII activity and bleeding symptoms.[26] An activity of less than 15% was associated with a significant increase in spontaneous major bleeding. The FranceCoag network assessed 33 patients and concluded that prophylaxis should be considered during pregnancy or at diagnosis if factor XIII is less than 10%, due to the increased risk of life-threatening umbilical or intracranial hemorrhage during infancy. [25] Both plasma and cryoprecipitate contain factor XIII; however, the factor XIII concentrate Corifact (formerly Fibrogammin; see Table 135.2) is preferred for replacement.[24] Corifact contains factor XIII purified from human plasma and is pasteurized. Recombinant factor XIII-A subunit (Tretten) received FDA approval in December 2013 to prevent bleeding in patients with inherited factor XIII A-subunit deficiency. Tretten is produced in yeast and contains no human blood components. In a prospective study, prophylactic replacement with recombinant factor XIII to maintain activity at greater than 0.05 to 0.2 IU/mL (5% to 20% of normal activity) prevented spontaneous bleeding without adverse effects. A subsequent study in pediatric patients (<6 years old) with inherited factor XIII A subunit deficiency also demonstrated efficacy as prophylactic treatment.[27]

Dosing regimens are the same for plasma derived and recombinant factor XIII. A dose of 40 units/kg every 4 weeks is recommended for prophylaxis. Although factor XIII concentrate is preferred for prophylaxis or treatment of acute bleeding, if it is not available plasma (10 to 20 mL/Kg every 4 to 6 weeks) or cryoprecipitate (1 unit for every 10 to 20 kg body weight every 3 to 4 weeks) can be used. A dose of 20 to 30 units/kg/day should be used with major surgery to keep the plasma activity 20% to 50% of normal. For minor surgery, 10 to 20 units/kg/day for 2 to 3 days is sufficient. Bleeding can be treated with 10 to 30 units/kg/day depending on severity. In pregnancy replacement should be started early, preferably before gestational week 5, because decidual bleeding at the site of implantation will occur without replacement. The optimal plasma factor XIII concentration in pregnancy is not established, but greater than 10% of normal is recommended.[24] This can be achieved by infusing 250 units of concentrate every 7 days through week 22 of gestation, then 500 units per week until delivery, with a 1000-unit bolus during labor.

Acquired Factor XIII Deficiency

Plasma factor XIII levels decrease in DIC and liver disease, but significant deficiency is rare in liver disease and may indicate a poor prognosis. While increased consumption or decreased production of factor XIII may result in bleeding, it can also be clinically silent because relatively small amounts of the protein are required for hemostasis. Acquired deficiency has been reported in leukemia, Crohn's disease, ulcerative colitis, and Henoch-Schönlein purpura, but levels are usually greater than 30% of normal and replacement is not required.

Alloantibodies to factor XIII induced by replacement therapy in factor XIII-deficient patients are very uncommon with fewer than five patients currently reported in the medical literature.[28] Patients with antibodies to the A subunit demonstrate bleeding symptoms that are refractory to replacement therapy. Immunosuppressive agents have been used in attempts to eliminate the antibodies. In one case an alloantibody to the B subunit was identified, resulting in increased bleeding and minimal response to factor XIII infusions.

Autoantibodies, most often IgG, to factor XIII can be directed against either the A or B subunits. Approximately 100 cases have been reported and the frequency may be increasing, perhaps because of the superiority of quantitative testing over older clot solubility tests. Antibodies to the A subunit are more common (approximately 90%). Neutralizing antibodies can block factor XIII activation (subclassified as Aa inhibitors), factor XIIIa activity (Ab inhibitors), or factor XIIIa fibrin binding capacity, while non-neutralizing antibodies may increase protein clearance from plasma. Some antibodies have both capacities. Antibodies to the B subunit are non-neutralizing and result in rapid clearance of the factor XIII tetramer.

Autoantibodies to factor XIII are most commonly seen in the elderly and have been linked to the use of certain medications (isoniazid, penicillin, phenytoin, amiodarone, procainamide), and to medical conditions such as cancer, lymphoproliferative disorders, and rheumatologic conditions such as systemic lupus erythematosus.[28] In 50% of cases there is no clear association with another process. Symptoms range from trauma-induced joint and soft tissue bleeding to catastrophic intracranial hemorrhage. Diagnosis requires a high index of suspicion as these patients frequently have normal PT/aPTT and platelet count evaluations. A thorough hemostatic evaluation is required to rule out other bleeding diatheses. The ISTH has developed recommendations for diagnostic criteria including possible, probable, and definite indicators for the disorder. Complete laboratory evaluation for factor XIII antibody inhibitors may be complex, usually requiring quantitative factor XIII functional assays, plasma mixing studies, inhibitor assays, binding assays, and clearance studies. [28]

Concentrates of plasma or recombinant factor XIII are recommended for treating acute bleeding in patients with factor XIII autoantibodies because these preparations have larger amounts of the protein than cryoprecipitate or FFP. rfVIIa, anti-fibrinolytic agents, and plasma-exchange/immunoadsorption have been used to treat bleeding. Immunosuppressive agents such as prednisone, rituximab and cyclophosphamide have been used to eradicate pathogenic antibodies.

INHERITED DEFICIENCIES INVOLVING MULTIPLE COAGULATION FACTORS

Numerous cases of inherited deficiencies of more than one coagulation factor have been described. While most represent chance coinheritance of distinct deficiencies, several are unique familial syndromes (Table 135.4). Such syndromes are likely caused by abnormalities in intracellular protein processing or alterations in posttranslational modification. Two inherited conditions, combined factor V and VIII

TABLE 135.4 **Combined Familial Deficiency States**

Type	Deficient Factors	OMIM Designation	Underlying Cause
1	V and VIII	227300	Mutations in *LMAN1* or *MCFD2* genes
2	VIII and IX	134510	Unknown
3	II, VII, IX, X, protein C, and protein S	277450 (gamma-glutamyl carboxylase) 607473 (vitamin K oxidoreductase)	Mutations in γ-glutamyl carboxylase *(GGCX)* or vitamin K oxidoreductase *(VKORC1)* genes
4	VII and VIII	134430	Unknown
5	VIII, IX, and XI	134520	Unknown
6	IX and XI	134540	Unknown
	VII and X	www.omim.org/entry/227600	Gene deletion 13q34

OMIM, Online Mendelian Inheritance in Man.

LABORATORY TESTING IN RARE COAGULATION FACTOR DEFICIENCIES

When deficiency of a coagulation factor is being considered, it is important to keep in mind that (1) acquired conditions causing multiple factor deficiencies are more common than inherited deficiency of a single factor, and (2) common inhibitors of coagulation, such as lupus anticoagulants, heparins, and the direct oral anticoagulants (DOACs) interfere with coagulation factor assays. Unexplained prolongation of the PT or PTT should be evaluated in a qualified laboratory. Plasma should be prepared from blood collected by venipuncture. In our experience, the common practice of collecting blood from central venous catheters/ports or peripheral intravenous catheters frequently introduces fluids or drugs that adversely affect clotting assays and contribute to misdiagnosis. We use the thrombin time assay and factor Xa-based assays to screen samples for the presence of heparins or DOACs prior to evaluation.

The evaluation of a plasma sample with a prolonged PT and/or aPTT should start by repeating the abnormal test on a mixture of patient and normal plasma to determine if the abnormality is related to a clotting factor deficiency (clotting time becomes normal after mixing) or an inhibitor that interferes with clotting (clotting time remains prolonged after mixing). The mixing study should be performed with and without incubation (2 h), as some antibody inhibitors demonstrate a time-dependent pattern of inhibition. Slight (a few seconds) prolongations of the PT or PTT can be difficult to evaluate with a mixing study. We evaluate such samples with assays for lupus anticoagulants prior to measuring specific levels of coagulation factors.

The antibodies to clotting factors that most physicians are familiar with neutralize factor activity, leading to a prolonged PT and/or aPTT that do not correct on mixing with normal plasma. However, non-neutralizing antibodies can cause severe factor deficiency. These antibodies enhance clearance of a clotting factor from the plasma *in vivo* and often are not detected in a mixing study. A failure to respond to replacement therapy in the absence of a clearly measurable inhibitor suggests a non-neutralizing antibody is present. In our practice, we have observed severe acquired deficiencies of prothrombin, factor V, factor X, factor XI, and factor XIII caused by non-neutralizing antibodies.

If the level of a vitamin K–dependent protein (prothrombin or factors VII, IX or X) is low, levels of factor V and at least one other vitamin K–dependent factor should be measured. If multiple vitamin K–dependent factors are low and factor V is normal, a process affecting vitamin K is likely. If factor V is also low, liver disease or DIC should be considered. Tests for hepatic function (albumin) or injury (transaminases) can facilitate interpretation of the coagulation factor studies. Distinguishing DIC from liver disease can be difficult, because results of standard tests such as the PT, PTT, platelet count, fibrinogen, and D-dimer may be abnormal in both conditions. Measuring factor V and factor VIII may be useful in this situation, because both are often low in DIC, while factor VIII is normal or elevated in liver disease.

It is likely that the apparent rarity of inherited factor XIII deficiency is partly due to the insensitivity of clot solubility assays still used in many institutions to screen for this disorder. Such assays detect only the most severe deficiencies. Quantitative measurements of factor XIII activity are preferable.

Patients with factor XI, factor XII, prekallikrein, or high-molecular-weight kininogen deficiency may require anticoagulation for treatment or prophylaxis for thromboembolism. Assays based on contact activation such as the aPTT or the activated clotting time (ACT) cannot be used for monitoring therapy with heparin, or the direct thrombin inhibitors argatroban or bivalirudin, in these patients because baseline aPTT and ACT clotting times are prolonged. Chromogenic heparin assays based on factor Xa inhibition are widely available for monitoring heparin and should be used in place of the aPTT in patients with these deficiencies. Alternatively, low molecular weight heparin, fondaparinux, or a DOAC, which do not generally require monitoring, can be used instead of unfractionated heparin, argatroban, or bivalirudin.

deficiency and deficiency of vitamin K–dependent factors, are best characterized and are discussed below. When considering the possibility of multiple coagulation factor deficiencies, it is important to recognize that common nonspecific inhibitors such as lupus anticoagulants can produce artifactual effects that may lead to the false impression that more than one coagulation factor is deficient (see box on Laboratory Testing in Rare Coagulation Factor Deficiencies). Similarly, potent antibody inhibitors directed at a single coagulation factor, such as factor VIII, occasionally are strong enough to interfere with assays for other coagulation factors.

Combined Factor V and Factor VIII Deficiency (OMIM 227300)

Inherited combined deficiency of factor V and factor VIII was first reported in 1954. It is an autosomal recessive trait caused by mutations in either the *LMAN1* or *MCFD2* gene. The protein products of these genes, mannose-binding lectin, and multiple combined factor deficiency protein respectively, form a cargo receptor that transports factors V and VIII from the ER to the ER-Golgi intermediate compartment. The incidence of this disorder is thought to be 1 in 1 million persons in the general population, although the allele frequency is particularly high (~1%) in Tunisian Jews originating from a community on the island of Djerba.

Deficiency of the *LMAN1* or *MCFD2* gene products reduces factor V and factor VIII secretion, lowering plasma levels of the proteins to 5% to 30% of normal. In Tunisian Jews, a T-to-C substitution at a donor splice site in *LMAN1* intron 9 is responsible. A history of consanguinity, co-segregation of factor deficiencies, and similar reductions in factor V and VIII favor the familial disorder over chance inheritance of distinct deficiencies. Affected individuals bleed primarily after trauma. Epistaxis, gingival bleeding, easy bruising, and menorrhagia are common. Hemarthrosis unrelated to trauma may occur in 20% of patients, but bleeding from the GI tract or intracranial hemorrhage is less common.[29] Postpartum hemorrhage occurs in most affected women and invasive procedures, including tooth extraction, are usually accompanied by bleeding in the absence of factor replacement.

The PT and aPTT are both prolonged in combined factor V and factor VIII deficiency. As isolated factor V deficiency also prolongs these tests, a patient with a low factor V level should also have factor VIII measured to avoid missing the combined deficiency. Patients with mucosal bleeding and menorrhagia may respond to antifibrinolytic therapy. For more significant bleeds, or in preparation for surgery or tooth extraction, combinations of FFP and factor VIII concentrate have traditionally been recommended. DDAVP can be used to raise the factor VIII level but will have little effect on factor V. Trough levels of ~50% for factor VIII and 25% for factor V have been recommended for surgery. Plasma exchange may be used to increase factor V in situations in which volume overload from large volumes of FFP is a concern. rfVIIa was used successfully to stop bleeding in one surgery patient. Counterintuitively, a recent study suggests that lower levels of factor V improve thrombin generation and may ameliorate bleeding in patients with this condition, possibly by lowering the plasma level of the coagulation inhibitor TFPI.[29] As the factor V level in plasma and platelets may be adequate in most of these patients, strategies for raising factor VIII alone with administration of DDAVP or factor VIII concentrate may be effective for many patients.

Combined Deficiency of Vitamin K-Dependent Proteins (OMIM 277450 and 607473)

In 1966, McMillan and Roberts described a newborn girl with prolonged PT and aPTT, low levels of prothrombin and factors VII, IX, and X, and no evidence of liver disease or malabsorption. She responded partially to large doses of vitamin K. Subsequently, low protein C and protein S levels were reported in association with mutations in the *GGCX* gene for the ER enzyme *γ-glutamyl carboxylase* in other patients with the syndrome. γ-glutamyl carboxylase catalyzes post-translation modifications in proteins involved in several processes including coagulation (prothrombin; factors VII, IX, and X, protein C and protein S) and bone biology (osteocalcin and matrix Gla protein).[30] Specifically, the enzyme catalyzes addition of carboxyl groups to the N-terminal Gla-domains of certain proteins in a reaction that requires reduced vitamin K as a cofactor. The reactions result in formation of vitamin K 2,3-epoxide that must be reduced by *vitamin K epoxide reductase complex subunit 1* (*VKORC1*) to replenish the vitamin K pool. Reduced activity of γ-glutamyl carboxylase or vitamin K epoxide reductase due to mutations in the *GGCX* or *VKORC1* genes can impair γ-carboxylation, resulting in reduced functional levels (10% to 50% of normal) of all vitamin K–dependent coagulation factors and a bleeding tendency that may be severe. More than 40 disease-causing mutations have been identified in the human *GGCX* gene.[30] Although it is generally thought they produce autosomal recessive conditions, severe bleeding was reported in one neonate who appeared to be heterozygous for a *GGCX* mutation.

Combined deficiencies of prothrombin and factors VII, IX, and X cause prolongation of the PT and aPTT. Protein C and protein S are also reduced. The abnormalities correct on mixing with normal plasma. Failure to correct suggests that a nonspecific inhibitor such as a lupus anticoagulant is present and is interfering with the factor assays (see box on Laboratory Testing in Rare Coagulation Factor Deficiencies). Patients with deficiencies of vitamin K–dependent proteins should be evaluated for liver disease, malabsorption, and exposure to vitamin-K antagonists such as warfarin or the rodenticide brodifacoum. Toxicology screens can identify these agents in blood long after ingestion because of their long half-lives. Some patients have adequate clinical response to vitamin K_1 (10 mg weekly), but others require unusually high doses. Nonresponders or responders with significant bleeding episodes can be treated with FFP or PCC. Interestingly, some *GGCX* mutations are associated with skeletal, skin (pseudoxanthoma elasticum-like syndrome), or cardiovascular abnormalities due to failure of properly γ-carboxylated proteins other than those involved in coagulation.[30] These abnormalities may predominate in certain patients over abnormalities in blood coagulation, depending on the causative mutation.

Vitamin K–dependent proteins are reduced in patients taking warfarin, as a result of poisoning with rodenticides such as brodifacoum, and with vitamin K deficiency. In 2013 a cluster of cases of severe vitamin K deficiency causing hemorrhagic disease in newborns were linked to failures to administer prophylactic vitamin K after birth. In 2018 an outbreak of a coagulopathy, first identified in Illinois, was connected to smoking brodifacoum-laced synthetic marijuana. Patients presented with profound coagulopathy and no history of anticoagulant use. Subsequent plasma analysis confirmed the presence of brodifacoum. The levels of vitamin K–dependent proteins are also low in liver failure, in conjunction with other proteins synthesized in the liver (such as factor V), and in malabsorption syndromes. An acquired antibody that resulted in fatal bleeding in a patient with a lymphoproliferative disorder bound to an epitope on the Gla-domains of prothrombin and factors IX and X , likely interfering with the function of all three proteins.

REFERENCES

1. Jain S, Acharya SS. Management of rare coagulation disorders in 2018. *Transf. Apheresis Sci*. 2018;57:705–712.
2. Menegatti M, Peyvandi F. Treatment of rare factor deficiencies other than hemophilia. *Blood*. 2019;133(5):415–424.
3. Peyvandi F, DiMichele D, Bolton-Maggs PHB, et al. Classification of rare bleeding disorders (RBDs) based on the association between coagulant factor activity and clinical bleeding severity. *J Thromb Haem*. 2012;10:1938.
4. de Moerloose P, Schved JF, Nugent D. Rare coagulation disorders: fibrinogen, factor VII and factor XIII. *Haemophilia*. 2016;22(Suppl 5):61–65.
5. Casini A, Blondon M, Lebreton A, et al. Natural history of patients with congenital dysfibrinogenemia. *Blood*. 2015;125:553.
6. Ross C, Rangarajan S, Karimi M, et al. Pharmacokinetics, clot strength and safety of a new fibrinogen concentrate: randomized comparison with active control in congenital fibrinogen deficiency. *J Thromb Haemost*. 2018;16(2):253–261.
7. Callum J, Farkouh ME, Scales DC, et al. Effect of fibrinogen concentrate vs cryoprecipitate on blood component transfusion after cardiac surgery: the FIBRES randomized clinical trial. *JAMA*. 2019;322(20):1–11.
8. Lancellotti S, Basso M, de Crisofaro R. Congenital prothrombin deficiency: an update. *Semin Thromb Hemostasis*. 2013;39:596–606.
9. Girolami A, Ferrari S, Cosi E, et al. Congenital prothrombin defects: they are not only associated with bleeding but also with thrombosis: a new classification is needed. *Hematology*. 2018;23(2):105–110.
10. Sarker T, Roy S, Hollon W, Rajpurkar M. Lupus anticoagulant acquired hypoprothrombinemia syndrome in childhood: two distinct patterns and review of the literature. *Haemophilia*. 2015;21:754–760.
11. Tabibian S, Shiravand Y, Shams M, et al. A comprehensive overview of coagulation factor V and congenital factor V deficiency. *Semin Thromb Hemost*. 2019;45(5):523–543.
12. Yanagiya R, Kanouchi K, Toubai T, et al. Plasma exchange as an initial treatment for severe bleeding induced by acquired factor V deficiency: a case report and mini literature review. *Acta Haematol*. 2020:1–5.
13. Robinson KS. An overview of inherited factor VII deficiency. *Transfus Apher Sci*. 2019;58(5):569–571.
14. Winikoff R, Scully MF, Robinson KS. Women and inherited bleeding disorders—a review with a focus on key challenges for 2019. *Transfus Apher Sci*. 2019;58(5):613–622.
15. Girolami A, Ferrari S, Cosi E, et al. Vitamin K-dependent coagulation factors that may be responsible for both bleeding and thrombosis (FII, FVII, and FIX). *Clin Appl Thromb Hemost*. 2018;24(9_suppl):42S–47S.
16. Menegatti M, Peyvandi F. Factor X deficiency. *Sem Thromb Hemost*. 2009;35(4):407.
17. Thompson CA, Kyle R, Gertz M, et al. Systemic AL amyloidosis with acquired factor X deficiency: a study of perioperative bleeding risk and treatment outcomes in 60 patients. *Am J Hematol*. 2010;85:171.
18. Asselta R, Paraboschi EM, Rimoldi V, et al. Exploring the global landscape of genetic variation in coagulation factor XI deficiency. *Blood*. 2017;130 :e1–e6.
19. Bauduer F, de Raucourt E, Boyer-Neumann C, et al. Factor XI replacement for inherited factor XI deficiency in routine clinical practice: results of the HEMOLEVEN prospective 3-year postmarketing study. *Haemophilia*. 2015;21:481.
20. Salomon O, Budnik I, Avishai E, et al. Single low dose of rFVIIa combined with antifibrinolytic agent is a simple and safe treatment for factor XI-deficient patients undergoing surgery. *Thromb Haemost*. 2019;119(12):1927–1932.
21. Wheeler AP, Hemingway C, Gailani D. The clinical management of factor XI deficiency in pregnant women. *Expert Rev Hematol*. 2020;13(7):719–729.
22. Maas C, Renné T. Coagulation factor XII in thrombosis and inflammation. *Blood*. 2018;131(17):1903–1909.
23. Barco S, Sollfrank S, Trinchero A, et al. Severe plasma prekallikrein deficiency: clinical characteristics, novel KLKB1 mutations, and estimated prevalence. *J Thromb Haemost*. 2020;18(7):1598–1617.
24. Karimi M, Peyvandi F, Naderi M, Shapiro A. Factor XIII deficiency diagnosis: challenges and tools. *Int J Lab Hematol*. 2018;40(1):3–11.
25. Bouttefroy S, Meunier S, Milien V, et al. Congenital factor XIII deficiency: comprehensive overview of the FranceCoag cohort. *Br J Haematol*. 2020;188(2):317–320.
26. Menegatti M, Palla R, Boscarino M, et al. Minimal factor XIII activity level to prevent major spontaneous bleeds. *J Thromb Haemost*. 2017;15(9):1728–1736.
27. Kerlin BA, Inbal A, Will A, et al. Recombinant factor XIII prophylaxis is safe and effective in young children with congenital factor XIII-A deficiency: international phase 3b trial results. *J Thromb Haemost*. 2017;15(8):1601–1606.
28. Muszbek L, Pénzes K, Katona É. Auto- and alloantibodies against factor XIII: laboratory diagnosis and clinical consequences. *J Thromb Haemost*. 2018;16(5):822–832.
29. Shao Y, Wu W, Xu G, et al. Low factor V level ameliorates bleeding diathesis in patients with combined deficiency of factor V and factor VIII. *Blood*. 2019;134(20):1745–1754.
30. Hao Z, Jin D, Chen X, et al. γ-glutamyl carboxylase mutations differentially affect the biological function of vitamin K-dependent proteins. *Blood*. 2020;137:533–543.

CHAPTER 136

TRANSFUSION THERAPY FOR COAGULATION FACTOR DEFICIENCIES

Elizabeth Roman and Catherine S. Manno

This chapter reviews products available to treat deficiencies of plasma coagulation proteins. The development of blood component therapy and subsequently protein concentrates that are enriched in particular coagulation factors and other proteins made possible the effective treatment of bleeding episodes in patients with hemophilia and other diatheses. In the 1940s, a collaborative effort funded by the US government was undertaken among protein scientists with the goal of rapidly developing a method to isolate albumin from human plasma to provide a lyophilized intravascular volume expander for use in the military. As part of this effort, Dr. Edwin Cohn developed an ethanol fractionation procedure that was amenable to large-scale manufacturing.[1] Building on the foundation of the Cohn fractionation procedure (see Chapter 115), the first coagulation factor concentrates were developed in the mid-1960s and provided a safer and more effective treatment for patients with the X-linked coagulation deficiencies, hemophilia A and B. Given the limited human plasma resource as a raw material for the production of all but a few coagulation protein concentrates, manufacturers of human plasma–based products attempted to derive the maximum yield from each pool of plasma by deriving more products from these processes.

The development of recombinant products was fueled by infectious disease transmission through derived products. Currently licensed products are produced in mammalian cell culture to optimize necessary posttranslational modifications required for biologic activity. These recombinant expression processes are complicated and expensive. Transgenic recombinant technology has been explored as a way to decrease or eliminate reliance on the human plasma resource.

Recently, recombinant technology has made substantial progress in the development of long-acting factor VIII and IX preparations, which are effective in preventing as well as treating bleeding episodes; however, the advances have been more significant in the extension of the factor IX half-life, rather than factor VIII. By multiple mechanisms the half-lives of these coagulation proteins have been extended, profoundly impacting the daily life of patients with hemophilia who use factor concentrate as prophylaxis, as prolonged half-life factors have reduced the number of infusions to maintain factor efficacy.

During the last decade, several non-factor concentrates, novel agents with preferred routes of administration and improved dosing schedules have become available for patients with hemophilia with and without inhibitors. In addition, results from gene therapy for both factor VIII and IX deficiencies are promising and may lead to a dramatic shift in the future paradigm treatment for these patients. Viral vectors enable the liver cells to make a functional copy of the missing factor, eliminating the need for routine prophylactic therapy and reducing the number of spontaneous or trauma-related bleeding episodes.

HEMOPHILIA A AND B

The hemophilias are X-linked disorders caused by deficiencies of either factor VIII (hemophilia A, or classic hemophilia) or factor IX (hemophilia B, or Christmas disease). The genes for these coagulation factors are located in close proximity to the long arm of the X chromosome. Whereas hemophilia A affects 1:5000 males, hemophilia B affects 1:30,000. This difference in incidence is roughly correlated with the size of the genes, and more than 30% of cases arise from spontaneous mutations.

The major morbidity of the severe hemophilias A and B is arthropathy, a result of recurrent joint bleeding developing over the course of years in those with inhibitors or those who are untreated or undertreated. The major cause of hemorrhagic mortality is bleeding into critical closed spaces (e.g., intracranial).[2] Central nervous system (CNS) bleeding occurs in 3% to 14% of patients, and mortality from CNS hemorrhage ranges from 20% to 50%[3–5] with neurologic sequelae (including seizures, motor impairment, or mental retardation) observed in 40% to 50% of survivors.[3] CNS bleeding episodes occur predominantly in patients with severe disease (<1% factor level).[3] A more detailed discussion of the hemophilias and the molecular biology of factors VIII and IX can be found in Chapter 134.

TRANSFUSION THERAPY FOR HEMOPHILIA A AND B

History of Transfusion for Hemophilia

Transfusion was first proposed by Schönlein and his student Hopf in 1832 as a treatment for “bleeders” who were suffering from exsanguinating hemorrhage, and these two were likely the first to have used the term *Haemophilie* to describe the disease.[6,7] The first effective transfusion-based intervention for hemophilia is credited to Samuel Lane who, in 1840, infused 10 to 12 ounces of fresh human blood into a 12-year-old boy with postoperative hemorrhage after eye surgery for correction of a squint.[8] Subsequently, a variety of interventions, using the infusion of human and animal blood and blood derivatives, were used in the therapy of hemorrhage in patients with congenital bleeding diatheses (Table 136.1). Citrated plasma was first used in 1923 for the treatment of hemophilia by Feissly in a father-to-son transfusion.[9] Development of modern blood banking in the 1930s and expansion of transfusion during and after World War II allowed for more widespread use of whole blood and subsequently frozen plasma in the treatment of hemophilia. Because of limited availability, the use of whole blood and components of whole blood for the treatment of hemophilia and other diseases was initially confined to larger metropolitan areas. In addition, volume constraints associated with the quantity of whole blood or plasma needed to achieve therapeutic levels of coagulation limited their usefulness.[31]

The advent of modern transfusion therapy for hemophilia came with the observation that the cold-insoluble precipitate remaining after the thawing of frozen plasma at 4°C contains high concentrations of factor VIII.[32] Application of this procedure to the separation of components of the whole blood[18] allowed for the production of a reduced-volume blood product known as *cryoprecipitate*. Cryoprecipitate derived from a single whole blood collection contains approximately 125 units of factor VIII and quickly replaced frozen plasma as the therapy of choice for the treatment of bleeding episodes in hemophilia A in the 1960s. The availability of cryoprecipitate made the treatment of bleeding episodes by patients in their homes, rather than at a hospital, a reality. In addition, the development of quantitative assays for factor VIII[14] and factor IX[13] meant that the two diseases could now be distinguished and the effects of transfusion therapy on circulating levels of factors could be more accurately assessed.

TABLE 136.1 **Development of Transfusion Therapy for Hemophilia**

1832	Schönlein proposes transfusion for exsanguination[7]
1840	Lane transfuses whole blood to stop postoperative bleeding in hemophilia[8]
1905	Weil reports use of human serum to treat hemophilia[9]
1911	Addis fractionates plasma by acid method[10]
1923	Feissly uses citrated plasma in ABO blood group-mismatched father-to-son transfusion for hemophiliaia[11]
1930s–1940s	Development of modern blood banking. Availability of whole blood and frozen plasma for therapy (allows levels of approximately 5%)
1946	Cohn develops ethanol fractionation of plasmaia[1]
1949	Graham uses FFP in canine hemophilia modelia[12]
1945–1960	Fractionation of plasmas with AHF activity
1952	Biggs distinguishes hemophilia B from hemophilia Aia[14]
1953	Graham, Langdell, and Brinkhous develop quantitative assays to measure AHFia[14,15]
1958	Barium precipitation of plasma to enrich for factor IX[16,17]
1963	Wagner uses glycine precipitation to partially purify factor VIII
1964	Pool develops clinically useful cryoprecipitate for factor VIII deficiency (allows levels of >20%)[18]
1966	Johnson uses PEG to partially purify factor VIII[19]
1967	Brinkhous develops glycine and PEG method to produce large-scale high potency factor VIII product (allows levels of 100%)[20]
1965–1970	Home infusion therapy
1969	Hoag produces large-scale prothrombin complex concentrate for factor IX deficiency[21]
1970s	HBsAg assay is developed
1978–1985	HIV contaminates blood supply and factor concentrates
1979–1986	Heat treatment of factor concentrates reduces transmission of hepatitis B and HIV[22,23]
1985	Assay for HIV is licensed
1982	Immunoaffinity method of purification for factor VIII[24,25]
1986	S/D method of treating infusible protein solutions to inactivate enveloped viruses[26,27]
1992–1993	First recombinant factor VIII concentrates are licensed[28,29]
1998	Recombinant factor IX concentrate is licensed[30]
1999	Nucleic acid amplification testing of blood donor
1999	Recombinant factor VIIa approved for hemophilia A and B with inhibitor
2007	Recombinant factor VIIa approved for acquired hemophilia
2011	First plasma-derived factor XIII product approved by the FDA
2011	Successful report of AAV mediated gene therapy for factor IX deficiency

AAV, Adeno-associated virus; *AHF*, Human antihemophilic factor; *FDA*, Food and Drug Administration; *FFP*, fresh-frozen plasma; *HBsAg*, hepatitis B surface antigen; *HIV*, human immunodeficiency virus; *PEG*, polyethylene glycol; *S/D*, solvent/detergent.

Before the discovery of plasma cryoprecipitate, significant advances had been made in the fractionation of plasma using ethanol,[1] glycine,[20] polyethylene glycol,[19] a combination of glycine and polyethylene glycol,[20] and calcium or barium[16,21,33] to precipitate plasma proteins. These techniques, in conjunction with cold precipitation of frozen plasma, laid the groundwork that resulted in the production of the first factor VIII and factor IX concentrates for clinical use.[20,21] These concentrates could be lyophilized and stored at temperatures up to 4°C with extended stability. Infusion of factor concentrates resulted in high circulating levels of factor VIII and factor IX without the complication of volume overload and paved the way for intensive infusion therapy for serious and life-threatening bleeding complications such as intracranial, retroperitoneal, and retropharyngeal hemorrhages and to prevent bleeding following surgery. Because they were produced from large pools of single plasma donations (>1000), initial concentrates were nearly universally contaminated with viral pathogens such as hepatitis B and non-A, non-B hepatitis (hepatitis C). Initial attempts to attenuate viral transmission using pasteurization and dry heat, instituted by manufacturers in the late 1970s and early 1980s,[22,34,35] were found to limit the transmission of hepatitis B. Eventually, these techniques were found to inactivate the human immunodeficiency virus (HIV).[23,36,37] Before the widespread application of these techniques, however, the majority of patients with severe hemophilia treated with concentrates between 1978 and 1985 were infected with HIV and hepatitis C virus. This tragic consequence of infusion therapy helped fuel the development of modern strategies to reduce the risk of viral transmission by products derived from human plasma. These strategies include[1] careful screening of potential donors for risk factors leading to infection with transfusion-transmissible infections,[2] more vigilant surveillance of the blood donor base for the appearance of new pathogens,[3] development and implementation of testing specifically for markers of infectious agents,[4] purification strategies that reduce viral contamination in final products, and[5] physical and chemical viral inactivation methods to treat infusible products. Development and refinement in techniques of molecular biology in the 1970s and 1980s resulted in the cloning of the genes for many plasma proteins, including factor VIII and factor IX.[38–40] Within the next decade, the production and licensure of biologically active recombinant factor VIII and factor IX products had become a reality.[28,41–43] Concentrates of these recombinant products have been shown to be effective and have not been associated with the transmission of pathogens. Further development of recombinant products centered on the removal of all human and animal proteins in the production and formulation to further reduce the risk of inadvertent contamination with emerging pathogens, such as variant prions,[44,45] and newly discovered agents, such as hepatitis G virus and other transfusion-transmitted viruses.[46,47] In addition, episodic supply constraints incurred in the manufacture of recombinant proteins in mammalian cell culture systems resulting in supply shortages of recombinant factor VIII[38] have led to greater interest in transgenic production of human plasma proteins compared with mammalian cell culture. With transgenic systems, raw material from which the protein of interest is purified (milk, plant tissue) can be produced in abundance.

Infusion Regimens and Dosing for Hemophilia

Traditionally, the mainstay of therapy for hemophilia involved the treatment of bleeding episodes with infusing products capable of replacing the missing factor VIII or IX. This so-called on-demand therapy is effective in staunching hemorrhages, but not before tissue damage has occurred. Bleeding is especially destructive in the synovium, where a vicious cycle develops in which the initial bleed results in a proliferative inflammatory response and hypertrophy of synovial tissues that then become more susceptible to further trauma and bleeding. The result in the short term is repeated bleeds into the same joint, resulting in what is referred to as a "target joint," and eventually chronic joint destruction or hemophilic arthropathy. Patients with chronic arthropathy often require surgical intervention, including synovectomy, debridement, joint replacement, or even joint fusion.

With the availability of factor concentrates that allowed for the attainment of high plasma levels of factor VIII or IX, prophylactic therapy became possible. This approach was pioneered by Swedish treaters who demonstrated that the use of prophylactic regimens,[49] wherein trough factor levels maintained at greater than 1% of normal, reduces the incidence of arthropathy and CNS hemorrhage.[29] Greater availability of virally safe factor concentrates has allowed for the initiation of prophylactic regimens in early childhood. This "primary" form of prophylaxis has become the standard of care in developed countries. For prophylaxis, the National Hemophilia Foundation Medical and Scientific Advisory Council recommends infusion of factor VIII 25 to 50 U/kg 3 times a week or every other day for hemophilia A and factor IX 40 to 100 U/kg 2 or 3 times a week for hemophilia B.[50] Prophylaxis is not universally practiced, however, owing to the high cost of factor concentrates, the requirement for frequent intravenous (IV) infusion, and the need for the placement of central venous catheters in some patients, especially small children, to obtain IV access. The cost factor makes primary prophylaxis and even on-demand treatment prohibitive to more than 60% of the hemophilia patients in the world.[51]

To understand how prophylaxis was instituted in the United States, a survey of hemophilia treatment centers in March 2010 was conducted: 62 centers responded, and 32% (or 20 centers) initiated prophylaxis on a once-weekly schedule, 21% (13 centers) on a twice-weekly schedule, and 47% on a thrice-weekly schedule.[52] This survey demonstrated the diversity in practice and deviation from the recommendation from the National Hemophilia Foundation. Alternative schedules for prophylaxis have been investigated. For example, the Canadian Hemophilia Primary Prophylaxis Study, a small, prospective, multicenter study, evaluated a tailored prophylaxis regimen in 25 patients with severe hemophilia A; patients were started at 50 U/kg once a week and were escalated to 30 U/kg twice weekly and then 25 U/kg on alternate days if one of the three situations occurred: development of a target joint, four bleeds in 3 months, or five or more bleeds occurred into any one joint. This seemed to be well-tolerated, resulting in 1.2 bleeds per year while maintaining good joint function; long-term follow-up of tailored prophylaxis is needed, but it may be a cost-effective and central line-sparing option.[53]

Dosing regimens for the treatment of bleeding episodes in hemophilia have also evolved paralleling the availability of high-concentration pathogen-safe replacement products. Although no universal regimen for "on-demand" treatment has been established, certain trends prevail. In general, for non-life-threatening bleeding episodes, the goal of therapy is to achieve a plasma factor VIII or IX level of between 30% and 100%. For life-threatening bleeds or prophylaxis for surgical procedures, the goal is a level of 100% to be maintained by repeated bolus infusions or continuous infusion for a duration of 10 to 14 days or longer, depending on the severity of the bleed or surgical intervention.

The majority of prophylaxis regimens aim at achieving a trough factor level of approximately 1%. Primary prophylaxis, instituted in young children, is aimed at preventing any joint bleeding episodes that would eventually result in chronic arthropathy. Secondary prophylaxis refers to limited or prolonged periods of prophylactic therapy, instituted after a serious bleed or the development of repeated bleeding into a single joint (target joint); tertiary prophylaxis entails the initiation of prophylaxis subsequent to the onset of joint disease. The landscape of prophylaxis regimens is changing and the novel agents, with less frequent, subcutaneous administration may replace the more traditional practice; as of August 2020, the current Medical and Scientific Advisory Council of the National Hemophilia Foundation did not recommend a preferred prophylaxis regimen.[54]

To prevent the development of a target joint and chronic arthropathy, many hemophilia treatment centers have recently adopted a regimen of two, three, or more infusions after hemarthrosis (aggressive on-demand treatment). Specific dosing regimens for bleeding episodes have been developed by treaters and treatment centers. Although slight variations in indications and target plasma levels of factor VIII and factor IX among treatment centers exist, representative dosing regimens are similar and are presented in Table 136.3.[55,56]

TREATMENT OF HEMOPHILIA

Products Available for Treatment of Factor VIII Deficiency

Factor VIII Concentrates

In high-income countries, the current standard of care for the treatment and prevention of bleeding episodes in patients with severe hemophilia A and in patients with a mild or moderate disease who do not respond to 1-deamino 8-D arginine vasopressin (DDAVP) is the infusion of recombinant or plasma-derived factor VIII. Recovery of recombinant factor VIII ranges from 1.5% to 2.5%/IU/kg so that dosing assumes a rise in plasma factor VIII activity of 2% for every 1 IU/kg infused. Available concentrates are optimized to enhance viral clearance during purifications and undergo one or more viral inactivation steps during manufacture. Plasma-derived factor VIII concentrates, treated with multiple purification and viral inactivation steps, are also available and have an excellent recent record of safety.

Recombinant Factor VIII Products

Highly purified recombinant factor VIII concentrates have been licensed in North America, Europe, and Japan since the early 1990s. These are either full-length or B domain–deleted molecules (the B domain is not required for activity in coagulation) that are expressed in mammalian cell culture (Chinese hamster ovary, embryonic hamster, or human kidney cell lines) and are purified using immunoaffinity techniques. The development of these recombinant products was fueled primarily by concerns regarding the safety of the human blood donor pool and the viral epidemics that occurred within the hemophilia population with the use of early plasma-derived products. As with highly purified plasma-derived factor VIII concentrates, the first-generation recombinant products contain animal or human proteins in cell culture and final formulation for stability. "Second-generation" products (Kogenate FS, Bayer, Refacto, Pfizer) contain animal or human proteins in cell culture only. Third-generation products

(Advate, Takeda Pharmaceutical Company Limited; Xyntha, Pfizer) do not have human proteins or other additives in the cell culture or as a stabilizer.[57,58] Recombinant production methods that do not rely on the human plasma resource theoretically should provide for unlimited supply.

The first extended half-life recombinant factor VIII product, Eloctate, Biogen Idec, synthesized in a human embryonic kidney cell line, was US Food and Drugs Administration (FDA) approved in June 2014. It is a B domain–deleted factor VIII covalently linked to human immunoglobulin G1; this Fc fusion protein has extended the half-life of factor VIII: in adults, almost 20 hours, 12 to 17 years old almost 16.5 hours, 6 to 11 years old about 14.5 hours and 2 to 5 years old, 12 hours.[59] Almost all of the patients (99%) receiving Eloctate for 6 months were able to use the product every 3 days or longer to maintain a factor VIII level of 1% to 3%.[59] Eloctate, Biogen Idec, has been used on-demand, for prophylaxis and in the perioperative setting. For prophylaxis, Biogen Idec recommends 50 IU/kg every 4 days and suggests personalizing the dose based on response, 25 to 65 IU/kg at 3 to 5-day intervals.[59] The package insert also indicates that children less than 6 years old, may require more frequent or higher doses (up to 80 IU/kg).[59] For minor and moderate bleeds, Eloctate 20 to 30 IU/kg can be given every 24 to 48 hours; however, in children less than 6 years old, Eloctate should be given every 12 to 24 hours.[59] For major bleeding episodes, Eloctate 40 to 50 IU/kg should be given every 12 to 24 hours and in children less than 6 years old, Eloctate should be given every 8 to 24 hours.[59] Many other long-acting factor VIII products are currently available.

Intermediate- and High-Purity Plasma-Derived Concentrates

Intermediate-purity plasma-derived concentrates are prepared from cryoprecipitated plasma or fresh-frozen plasma (FFP), from which factor VIII is further purified using precipitation, gel permeation, ion exchange, or affinity chromatography, often in combination. Specific factor VIII coagulant activity in these products ranges from 2 to more than 100 IU/mg of protein, and many of the methods used also copurify significant amounts of von Willebrand factor (vWF), making them useful for the treatment of some patients with von Willebrand disease (vWD; see later discussion on the treatment of vWD). More highly purified plasma-derived concentrates are produced using heparin ligand or immunoaffinity purification methods and have specific activities ranging from 140 to greater than 3000 IU/mg. To stabilize the factor VIII molecule, the majority of these products are formulated by adding human albumin before lyophilization: Alphanate (Grifols USA) and Koate-DVI (Talecris Biotherapeutics).

Emicizumab-kxwh (Hemlibra, Genentech)

Emicizumab is a humanized bispecific antibody that binds both IX and X, essentially mimicking the factor VIII function and is given subcutaneously. In 2017, the results of the phase 3 HAVEN-1 study demonstrated that weekly subcutaneous dosing in patients >12 years old with inhibitors had a significant reduction in annualized bleeding rate (ABR); group A patients were receiving bypass agents on demand and given weekly emicizumab; group B did not receive emicizumab and group C patients were receiving prophylaxis with bypass agents plus emicizumab. There was an 87% reduction in ABR in group A vs group B.[60] The treatment was well tolerated with the most common side effect being local injection site reactions (15%). However, three patients receiving high-dose activated prothrombin complex concentrate (aPCC) with emicizumab prophylaxis developed thrombotic microangiopathy (TMA). In the HAVEN-2 study, a phase 3 study of children 2 to 12 years old with hemophilia A and inhibitors, patients were randomly assigned to three different dosing schedules: group A received emicizumab 1.5 mg/kg weekly, group B 3 mg/kg every 2 weeks and Group C 6 mg/kg weekly monthly; ABR was 0.3, 0.2, and 2.2 respectively.[61] The most common side effects were local reactions and nasopharyngitis. There were no reports of TMA or thrombosis. Two patients developed anti-drug antibodies: in one patient there was a loss of drug efficacy and in the other, the antibody resolved without intervention. Patients without inhibitors have also benefited from emicizumab prophylaxis, as demonstrated in the HAVEN 3 study,

TABLE 136.2 Dosing Regimens for Traditional Factor Replacement in Bleeding and Prophylaxis in Hemophilia

Site	Factor Level (%)	Dose Hemophilia A (U/kg)	Dose Hemophilia B (U/kg)	Duration of Treatment	Comments
Joint	30–70	15–35	30–70	1–3 days	Splinting, temporary splinting, no weight bearing
Life threatening (e.g., intracranial, retropharyngeal, retroperitoneal)	80–100	40–50	80–100	1–14 days	Antifibrinolytic therapy with retropharyngeal bleeds
Soft tissue	30–50	15–25	30–50	2–5 days	Higher levels can be used for compartment syndrome
Surgery	80–100	40–50	80–100	10–14 days (or shorter for minor procedures)	Significant blood loss can occur into large muscles of the lower extremity and the iliopsoas
Oral	20–50	10–25	20–50	1–2 days	Antifibrinolytic therapy
Gastrointestinal[a]	30–100	15–50	30–100	2–3 days	Should be evaluated for source
Genitourinary[b]	30–50	15–25	30–50	1–2 days	Avoid antifibrinolytic therapy
Prophylaxis[c]	50	25	50	qod or 3×/week	

[a] Depending on severity.

[b] Painless spontaneous hematuria often requires no treatment other than fluid intake. Persistence requires treatment and evaluation.

[c] Use of a schedule of 25 U/kg qod and a dose of 40 U/kg with an interval of 2 days between the next dose may increase compliance by decreasing infusions to three per week.

qod, Every other day.

Data from DiMichele D. Hemophilia 1996: New approach to an old disease. *Pediatr Clin North Am.* 1996;43:709; Mannucci PM. Haemophilia treatment protocols around the world: towards a consensus. *Haemophilia.* 1998;4:421; and Lusher J. *Treatment of Congenital Coagulopathies*, AABB Press; 1999.

evaluating weekly (group A), every other week prophylaxis (group B), no emicizumab (group C), and weekly dosing with prophylactic factor VIII (group D); there was a 96% and 97% reduction in the ABR for groups A and B, with over 50% of participants not having any bleeds. Local reactions were the most common and there was no TMA, thrombosis, antidrug antibodies or inhibitors.[62] HAVEN-4 included patients with and without inhibitors receiving monthly emicizumab (a small cohort received 4 weekly doses prior to starting the monthly dosing) and the ABR was 2.4, with side effects being similar to those seen in HAVEN-3.[63] Emicizumab can be given from infancy to adults at a loading dose of 3 mg/kg weekly for four doses; thereafter it can be given weekly (1.5 mg/kg), every 2 weeks (3 mg/kg), or monthly (6 mg/kg). Since different vial sizes cannot be mixed and volume may exceed the maximum for a single injection, patients need to receive multiple injections for one dose but compared to 104 to 156 (twice–thrice weekly) IV administrations/year, patient satisfaction remains high. There are two black box warnings for emicizumab: TMA with the use of high-dose aPCC and thrombosis. For surgery or breakthrough bleeding, patients may need factor VIII replacement or a bypassing agent.

Clotting based coagulation tests, including activated partial thromboplastin time (aPTT), factor VIII levels (other factor levels), Bethesda assays, aPTT-based activated protein C resistance and activated clotting time will not be accurate in patients who are using emicizumab (PTT can be shortened-normal) and secondary to the long half-life of emicizumab, these abnormalities in clotting assays may persist for up to 6 months after the last dose; therefore, chromogenic (with bovine factor IXa and Xa) or immune-based assays for Factor VIII and Bethesda monitoring can be used.[64] In the HAVEN trials, drug monitoring levels or coagulation assays were not used for clinical decision making and using animal models, emicizumab approximates a factor VIII level of 10% to 20%. If an inhibitor is suspected, anti-drug antibodies may be sent (commercial test not available); if an emicizumab level is needed, emicizumab calibrators and controls are available from r^2 Diagnostics.[65]

Novel Direction for Factor VIII Deficiency

Many of the new products, like emicizumab, improve global hemostasis without the replacement of the Factor VIII product. Fitusiran, Sanofi, is in clinical trials and is a small inhibitory messenger RNA blocking antithrombin production and enhancing thrombin generation. This drug is given monthly, subcutaneously, and has been studied in patients with moderate or severe hemophilia A or B with or without inhibitors and has not yet been licensed. The Phase 1 study assessed pharmacokinetics and pharmacodynamics of weekly versus monthly dosing, demonstrating that there was a dose-dependent reduction in antithrombin with the monthly dosing; no thrombotic events were reported.[66] A Phase 2 study of fitusiran at 50 and 80 mg, showed promising results with an ABR of 0.84 and suppression of antithrombin.[67] Two thrombotic events were reported in patients using fitusiran: an atrial thrombosis and a fatal cerebral sinus venous thrombosis, in a patient receiving concurrent high-dose Factor VIII replacement; therefore, trials were temporarily halted and bleeding management guidelines for patients on fitusiran were published in December 2017. The current Phase 3 fitusiran trial was put on hold by Sanofi in October 2020 due to additional reports of thrombotic events.

Another novel therapeutic being evaluated in clinical trials in subjects with hemophilia A or B with or without inhibitors is concizumab, Novo Nordisk, a humanized monoclonal antibody to the K2 domain of tissue factor pathway inhibitor (TFPI), which impedes binding of TFPI to the active site of Factor Xa. In a Phase 2 trial, patients were given daily subcutaneous doses, with potential dose escalation if bleeding occurred; ABR was reduced by 78% in patients with inhibitors and three patients developed anti-drug antibodies without clinical correlations.[68] In March 2020, concizumab trials were halted due to three thrombotic events but trials resumed in August 2020.[69]

Gene Therapy for Factor IX Deficiency

A landmark paper was published in December 2011 describing a phase 1 trial of the first six patients with severe factor IX deficiency who received one peripheral IV injection of an adeno-associated virus (AAV) expressing a factor IX gene, at three different doses. This vector was selected as it does not integrate into the host genome and a capsid of AAV serotype 8 was added to avoid immunogenicity. Another advantage of using this serotype is the propensity for it to home to the liver. Sixty-seven percent of the patients in the trial no longer needed prophylaxis and the other 33% needed less factor. Patients who received the highest dose experienced a transient elevation of the alanine aminotransferase, between weeks 7 and 10, which resolved with prednisolone; this effect correlates with finding AAV8-capsid specific T cells in the peripheral blood.[70] These authors published an update on the long-term outcome of 10 men (22 to 64 years old) treated with gene therapy using the AAV8 construct; the additional four patients received the high dose of the vector and had on average 5% factor IX levels, with a decrease in bleeding episodes, sustained over 4 years.[71]

More recent trials in gene therapy take advantage of a point mutation in the factor IX gene, known as Factor IX Padua, which synthesizes about eightfold higher Factor IX than wild type; factor IX–R338L was engineered with a liver-specific promoter and infused at a dose of 5×10^{11} vector genomes/kg in 10 men with severe hemophilia B with ABR reduction from 11.1 to 0.4 after infusion and average factor IX levels of 33.7 ± 18.5%. Two patients had an asymptomatic elevation of liver enzymes, which resolved after a short course of prednisone.[72]

Gene Therapy for Factor VIII Deficiency

Due to the large size of the factor VIII gene, advances in gene therapy for hemophilia A have been much slower than for hemophilia B, with the first report for gene therapy in hemophilia A coming 8 years after the landmark hemophilia B publication. Nine men with severe hemophilia A received a single dose of AAV vector encoding a B domain deleted human factor VIII (AAV5-hVIII-SQ, BMN-280, BioMarin) at three doses with one receiving a low dose, one intermediate dose, and the remaining seven at a high dose, with the patients in the low and intermediate groups having factor VIII less than 3 IU/dL and 6/7 high-dose participants had >50 IU/dL, which persisted at 1 year. After the first patient in the high-dose group had an elevated ALT, the remainder of the group were given prophylactic prednisone. There were no neutralizing antibodies.[73] In 2020, there was a 3-year follow-up of 15 patients who had received a dose of AAV5-hVIII-SQ; 13 of the 15 patients at 2 to 3 years who received a dose of 2 or 4×10^{13} vector genomes/kg had factor VIII levels in the moderate range (mean 13% to 20%) with ABR of 0 and reduction of factor VIII from 139 to 155 infusions/year to 0 to 0.5.[74] This product is not yet licensed in the United States, awaiting further safety data from an additional 2-year follow-up.

1-Deamino 8-D Arginine Vasopressin

The preferred product for the treatment of patients with mild or moderate hemophilia A is the synthetic octapeptide DDAVP, a vasopressin analog. DDAVP causes a release of factor VIII (and vWF) from endothelial cells, raising plasma factor VIII by approximately threefold (range 2- to 12-fold) in patients with hemophilia in whom the disease is caused by decreased production or secretion of a functional protein or a protein that has decreased activity. To be effective, DDAVP relies on a partial quantitative deficiency of factor VIII; thus, patients with severe hemophilia will not benefit from its use if the causative mutation results in no synthesis, secretion, or a nonfunctional protein. In a retrospective study assessing response to a DDAVP challenge in mild or moderate hemophilia, 57% of patients with mild hemophilia had a positive response, and several who failed the initial challenge had a response after a mean of 6 years, increasing the response rate to 71% in the mild group.[75] The response to DDAVP in an individual patient is typically reproducible, and effective response must be documented before its routine use or as prophylaxis for bleeding in surgical procedures (see box on 1-Deamino 8-D Arginine Vasopressin Trial). IV and intranasal preparations are available. The IV product has been used in a subcutaneous route of administration. The intranasal preparation more easily allows a patient to administer the compound on an as-needed basis in a home therapy regimen. The phenomenon of tachyphylaxis, the decreased effectiveness of repeated doses of the same compound, occurs after several, typically three, consecutive doses.

Injectable DDAVP (Sanofi Aventis) is available in 4 μg/mL. The recommended dose is 0.3 μg/kg, mixed in 30 mL normal saline (for children <10 kg, 10 mL normal saline), infused IV slowly over 30 minutes. This dose can be repeated after 12 to 24 hours. DDAVP nasal spray Stimate (CSL Behring) is available in a metered-dose pump that delivers 0.1 mL (150 μg) per activation (spray). The dose is one activation for patients weighing less than 50 kg and two activations in separate nostrils for those weighing more than 50 kg. In general, only three consecutive doses of DDAVP should be used unless otherwise advised by an experienced hemophilia treater. Because DDAVP is a vasopressin analog, there is a risk of fluid retention with its use. Changes in fluid balance can result in hyponatremia and seizures, especially when DDAVP is used in individuals on nonfluid-restricted or salt-restricted diets (e.g., elderly or very young patients or surgical patients undergoing fluid replacement with solutions with concentrations <0.9% sodium). For this reason, DDAVP is not recommended for children younger than 2 years of age. Caution is advised with the use of DDAVP in patients at risk for arterial thrombosis because there have been reports of myocardial infarction and cerebral thrombosis with its use.[76]

1-DEAMINO 8-D ARGININE VASOPRESSIN TRIAL

1. Collect citrated plasma from the patient immediately before DDAVP infusion for testing with the post infusion blood specimen.
2. Administer DDAVP IV (0.3 μg/1 kg) in 25–50 mL normal saline.
3. Wait approximately 30 min after the infusion, carefully observing the patient for possible adverse side effects (increased blood pressure, facial flushing, signs or symptoms of hyponatremia).
4. Collect post-DDAVP infusion specimen in sodium citrate at 60, 120, and 240 min.
5. Compare the pre- and post-DDAVP factor VIII and vWF:Ag levels to confirm a therapeutic response, threefold increase from baseline (for mild or moderate hemophilia, response is defined as twofold increase in factor VIII: C levels or an absolute level above 0.31 U/mL at 1 h).[75]

Plasma/Cryoprecipitate

In communities where virally inactivated factor VIII concentrates are not available, cryoprecipitate provides an effective alternative to therapy with concentrates for hemophilia A and vWD patients. Cryoprecipitate is a small-volume product (10 to 15 mL) enriched in factor VIII, vWF, fibrinogen, fibronectin, and factor XIII. Dosing can be calculated assuming approximately 80 to 150 IU of factor VIII per bag of cryoprecipitate (derived from a 450-mL single whole blood donor collection unit). Thus, a typical 1750-IU dose (50% correction for a 70-kg patient) would require between 10 and 21 bags or units.

Products Available for Treatment of Factor IX Deficiency

Factor IX Concentrates

As with replacement therapy for factor VIII deficiency, intermediate- and high-purity products derived from plasma (Alphanine SD, Grifols; and Mononine, CLS Behring) and recombinant products for the treatment of factor IX deficiency are available. All of these concentrates also undergo at least one viral inactivation or exclusion step in manufacturing. The volume of distribution of factor IX is approximately twice the plasma volume. This can be explained in part by the affinity of factor IX for type IV collagen present in the extracellular matrix.[77] Recovery of factor IX after the infusion is therefore approximately 1%/IU/kg. The recovery observed with recombinant factor IX is approximately 20% lower than that observed with plasma-derived factor IX.[78] This is likely caused by differences in posttranslational modifications between recombinant factor IX and plasma-derived factor IX.

Intermediate-purity factor IX products are generally purified from plasma using anion-exchange chromatography or calcium or barium precipitate adsorption. These methods select proteins that contain highly negatively charged epitopes. The vitamin K-dependent (VKD) coagulation factors, by virtue of their gamma-carboxyglutamic acid–rich domains, are such proteins; thus, these techniques copurify varying amounts of the other VKD coagulation factors, factor VII, factor X, and prothrombin with factor IX. Hence, the products are referred to as *prothrombin complex concentrates* (PCCs). The levels of vitamin K proteins in these products vary and can be obtained from the manufacturer. In addition, the PCCs are contaminated with trace amounts of activated forms of VKD factors. The presence of these activated factors is the likely explanation for thromboses that have occurred with the use of these intermediate-purity products, typically in the setting of postoperative immobility or hepatic dysfunction.[79] To minimize the risk of thrombosis with the use of PCCs, it is advisable to achieve a peak factor IX level no higher than 50%.[80] Some clinicians add small amounts of heparin to infusions of these products to prevent thrombosis, and some manufacturers have formulated PCCs with heparin or antithrombin III.

High-purity factor IX products are produced from plasma using techniques of ligand affinity or immunoaffinity chromatography and typically have specific activities greater than 150 IU/mg. Three single high-purity recombinant factor IX products have been licensed in the United States (BeneFIX, Wyeth; Ixinity, Aptevo Biotherapeutics LLC; and Rixubis, Baxalta Incorporated). Because specific products have different recovery results, consulting the package insert and performing recovery studies are recommended; for example, 1 IU BeneFIX/kg will increase the circulating activity of factor IX by 0.8 ± 0.2 and 0.7 ± 0.3 IU/dL in adults and pediatric (younger than 15 years old) patients, respectively.[82] Also, the initial dose of BeneFIX should be given under direct medical supervision because the potential for an allergic reaction is significant.[81] Thrombotic complications have not been reported with these high-purity products; some thrombotic events have been associated with continuous administration, which is not an approved method.[81] In patients with a history of allergy and inhibitors, irreversible nephrotic syndrome has been reported during immune tolerance induction (ITI).[82,83]

In communities where factor IX concentrates are not available, plasma may provide a means of replacing factor IX in hemophilia B patients during bleeding episodes. Each unit of FFP derived from a 450-mL whole blood collection contains approximately 200 IU of factor IX. Replacement to therapeutic levels of factor IX with FFP is difficult because of the complication of volume overload. In some areas, S/D plasma provides a virally inactivated alternative to FFP.

There are three extended half-life recombinant factor IX products licensed in the United States (Alprolix, Biogen Inc, Idelvion, CSL Behring LLC and Rebinyn, Novo Nordisk). Alprolix, synthesized in a human embryonic kidney cell line and purified by nanofiltration is covalently linked to human immunoglobulin G1; this Fc fusion protein has extended the half-life of factor IX: in adults, almost 86.5 hours, 12 to 17-years old about 83.5 hours, 6 to 11 years old about 72 hours and 2 to 5 years old almost 66.5 hours.[84] Some patients with factor IX deficiency may only need factor IX twice a month with this product. It should be noted that kaolin-based assays may underestimate the factor IX level.[84]

Other Useful Adjuncts for Treatment of Bleeding in Hemophilia A and B

The antifibrinolytic agents tranexamic acid (oral–Lysteda, Ferring Pharmaceuticals Inc; IV Cyklokapron, Pfizer) and ε-aminocaproic acid (EACA) (Amicar, Xanodyne Pharmaceuticals) are sometimes used in conjunction with clotting factor replacement therapy or DDAVP in the treatment of bleeding that occurs with hemophilia and other bleeding disorders. These agents interact with the lysine-binding site of plasminogen and enhance its activation. The more important effect of lysine analogs is that their occupancy of this site inhibits binding of the zymogen, plasminogen, to fibrin, which is necessary for full fibrinolytic activity. Antifibrinolytics are useful in the setting of mucosal bleeding and may substantially reduce the need for clotting factor concentrates in the setting of oral mucosal bleeding with tooth extractions. These agents are administered preoperatively and continued postoperatively until wound healing occurs. Although tranexamic acid is more potent, it is less readily absorbed when given orally. Tranexamic acid is administered at 25 mg/kg orally or 10 mg/kg IV preoperatively, and therapy can be continued postoperatively using oral rinses with a 5% solution. EACA is used at a dose of 100 mg/kg preoperatively followed by 100 mg/kg every 6 hours either orally or IV (maximum dose, 24 g/day). The dosing of these medications should be reduced in patients with renal insufficiency.

INHIBITORS OF FACTOR VIII AND FACTOR IX

A major complication in the treatment of patients with hemophilia is the development of inhibitory antibodies against the infused factors that interfere with factor activity. Rarely, antibodies may develop that do not affect activity but increase the clearance of factor VIII or factor IX. The incidence of factor VIII inhibitors is approximately 30%, and in patients receiving recombinant products inhibitors typically develop within the first 20 exposures to replacement therapy.[85,86] Results of the Survey of Inhibitors in Plasma-Product Exposed Toddlers (SIPPET) trial showed that patients who received recombinant products were two-fold more likely to develop inhibitors than those treated with plasma-derived products.[87] In another analysis of the SIPPET data, inhibitor production was found to be the highest in the first 10 exposure days with recombinant products occurring earlier (first 5 exposure days) and lasting longer than for plasma-derived products (exposure days 6 to 10).[88]

Patients with low-titer, transient inhibitors can be managed with increased doses of factor VIII or novel agents. Recently, a prospective, longitudinal study of 25 patients with severe hemophilia A evaluated the immune response during the first 50 exposure days to Advate; four distinct signatures were seen: subgroup 1 had no antibodies to factor VIII; subgroup 2 had non-neutralizing IgG1 antibodies; subgroup 3 had transient IgG1 antibodies and subgroup 4 developed high-affinity IgG1 antibodies, followed by IgG3 which was the harbinger of persistent antibodies and production of IgG4 antibodies. The seven patients in subgroup 4 all had high or moderate risk factor 8 mutations.[89] Inhibitors to factor IX occur less frequently, with an estimated incidence of approximately 1% to 3%.[90] Patients with factor IX antibodies may have anaphylactic reactions when treated with high-purity products.[91,92]

Patients with high-titer inhibitors to either factor VIII or factor IX do not usually achieve hemostasis after factor replacement; however, in the era of new agents, there are alternatives that lead to enhanced thrombin generation without factors. Management of patients who develop inhibitors involves two approaches: (1) acute treatment of bleeding episodes and (2) ITI therapy, whereby the patient is treated frequently (often daily) with factor VIII or factor IX in an effort to suppress the production of inhibitors by the immune system (similar to allergic desensitization therapy).

Treatment of acute bleeding episodes can be accomplished by three methods. For patients with low-titer inhibitors (<5 Bethesda units [BU], a laboratory measurement of inhibitor activity), hemostasis can be accomplished with large doses of factor VIII or factor IX (e.g., as high as 200 IU/kg given at frequent intervals) in an effort to neutralize or "override" the circulating inhibitor. A neutralization approach is usually ineffective for patients with high-titer inhibitors (>5 to 10 BU) and is expensive because of the large amounts of the factor required. The peak inhibitor titers pre-immune tolerance therapy and during treatment are the most significant prognostic features.[93] These patients and patients with low-titer inhibitors who fail to respond to override therapy can be treated using products that act to "bypass" the inhibitor. There are three current bypass agents approved; one is aPCCs (FEIBA, Takeda Pharmaceutical Company Limited), which contain factors VII, X, and prothrombin in addition to factor IX that enhance the production of thrombin generation by activating the coagulation pathway at points further down than the factor VIIIa/factor IXa step.

The other two are recombinant activated factor VII (rFVIIa) (NovoSeven, Novo Nordisk) was licensed in Europe and the United States with an indication for use as a bypassing agent in the treatment of patients with hemophilia A and hemophilia B and inhibitors, or acquired hemophilia (also approved for bleeding and perioperative management in Glanzmann thrombasthenia, who are refractory to platelet transfusion and congenital factor VII deficiency) and SEVENFACT, HEMA Biologics). The dose of NovoSeven for patients with the inhibitor is 90 μg/kg every 2 hours. SEVENFACT is given every 3 hours compared with every 8 to 12 hours for PCCs or aPCCs. Thrombosis has also been reported with the use of rFVIIa[80,94,95] and PCCs.[83]

Despite the success achieved with ITI regimens (80% to 90%),[96] "bypass" agents are still needed in patients undergoing tolerization because an interval potentially as long as 2 to 3 years exists between the time that induction is initiated and the inhibitor is suppressed. Bleeding episodes that occur before the achievement of tolerance usually require treatment with one or another of the bypassing agents.

Leissinger et al. demonstrated in hemophilia A patients with high-titer inhibitors the superiority of prophylactic thrice-weekly FEIBA (Baxter) at 85 U/kg over on-demand therapy.[88] In a prospective, randomized, crossover study with a 3-month washout between the two arms, which were each 6 months, the prophylaxis regimen prevented the progression of joint disease in high-titer inhibitor patients; and is now recommended for prophylaxis in high-titer inhibitor patients (see box Treatment of Life-Threatening Bleeding Episodes in Patients With Inhibitors Against Factor VIII or Factor IX).

Treatment of life-threatening bleedings in the context of a high-titer inhibitor represents a significant challenge. The effect of non-factor VIII concentrates (bypass agents) when the inhibitor titer is greater than 10 BU (precluding the use of factor VIII), is difficult to monitor in the laboratory because plasma factor levels do not reflect hemostasis. Adjunctive therapies are frequently used in this setting to lower the titer of the antibody to allow for treatment of the patient with factor VIII concentrates. Aggressive regimens are used in which circulating antibodies are depleted using plasmapheresis or staphylococcal protein A immunoadsorption, and reduction of antibody production is attempted using immunomodulatory agents such as steroids, cyclophosphamide, and IV infusion of immunoglobulin G (IgG).[98–101] These strategies for acutely reducing antibody titers are also used by some hemophilia treaters at the outset of ITI. In a phase II trial, Leissinger et al. evaluated the safety and efficacy of rituximab, without concurrent ITI, to reduce high-titer inhibitor levels in hemophilia A patients,[102] although the study was designed to enroll 43 patients, only 23 were enrolled and the Data Safety Monitoring Board closed the study because of poor accrual over 4 years. Three of 16 (18.8%) had a major response (inhibitor titer <5 BU) and one with a minor response (initial inhibitor titer <5 BU but increased after factor VIII exposure).[103] Several case reports or series of patients with severe hemophilia have demonstrated a greater advantage from rituximab when used in conjunction with ITI.[104,105] In a recent review of inhibitor eradication with rituximab, Franchini and Mannucci concluded that the combination of ITI with rituximab in mild to moderate hemophilia is likely to be more beneficial[106] than when used without simultaneous factor VIII. Also, rituximab has been used successfully in the setting of acquired hemophilia A, mostly with concurrent immunosuppression.[105]

TREATMENT OF LIFE-THREATENING BLEEDING EPISODES IN PATIENTS WITH INHIBITORS AGAINST FACTOR VIII OR FACTOR IX

Concentrates

1. Factor VIII containing concentrates in high doses (as high as 150–200 IU/kg) if inhibitor titer is low (<5–10 BU). High-dose continuous infusion of factor VIII (≈10 IU/kg/h) after a high-dose bolus may be useful.
2. Recombinant factor VIIa—NovoSeven. Dose is 90 μg/kg (or a dose up to 320 μg/kg) administered every 2 h. Risk of thrombosis exists with this product.
3. Activated PCCs at a dose of 50–75 IU/kg every 8–12 h. Risk of thrombosis exists with this product.

Immunomodulation

1. Antibody depletion
   a. Plasmapheresis
   b. Extracorporeal immunoadsorption of plasma (staphylococcal protein A column therapy and other methods)
2. Suppression of antibody production
   a. High-dose steroids (equivalent of prednisone 80 mg/day)
   b. Cyclophosphamide (10–15 mg/kg load and 2–3 mg/kg/day)
   c. Intravenous immunoglobulin (1 g/kg daily for 2 days)
   d. More aggressive regimens that may include vincristine, azathioprine, cyclosporine, or interferon-γ

Conservative Measures

1. Immobilization
2. Compression
3. Local application of hemostatic agents
4. Antifibrinolytics
5. Avoid venipunctures, intramuscular injections, arterial puncture, and lumbar punctures
6. Avoid use of medications that inhibit platelet function (ASA, NSAIDs)
7. DDAVP may be effective in some patients with low-titer inhibitors

ASA, Aspirin; *BU*, Bethesda unit; *NSAID*, nonsteroidal anti-inflammatory drug; *PCC*, prothrombin complex concentrate.

Patients with inhibitors to factor VIII or factor IX can also be treated with novel agents for prophylaxis; although most treaters would try one attempt at ITI.[93]

VON WILLEBRAND DISEASE

vWD is an autosomal dominant bleeding disorder first described by Erik von Willebrand in 1926, who named this bleeding diathesis *pseudohemophilia*. vWD is relatively common, with a prevalence of up to 2% of the general population.[107] The penetrance and severity of the disease vary depending on the type of vWD (type 1, 2, or 3); the specific mutation; the number of affected genes; the patient's blood type; and numerous drug, hormonal, and stress-related parameters. These factors, particularly the subtype of vWD and the patient's response to DDAVP, influence the recommended treatment for acute bleeding episodes or prophylaxis against bleeding.

Although the disease had been described in the 1920s, the primary defect had earlier been attributed to either platelets or the vessel wall. An understanding of the missing protein in vWD and its relationship to factor VIII was not appreciated until the 1950s. Correction of the defect with transfusion of plasma, but not with platelet concentrates alone, defined the disease as one involving a missing plasma protein factor (vWF), not a platelet or vessel wall defect.[108] A correlation between vWD and factor VIII activity had previously been observed—the observation that a plasma fraction from patients lacking factor VIII (hemophilia A) could correct the defect in vWD in addition to the autosomal inheritance pattern, which helped further differentiate hemophilia A from vWD.[108] A more detailed discussion of the clinical features and pathophysiology of vWD and the molecular biology of vWF can be found in Chapter 133.

Transfusion Therapy for von Willebrand Disease

The specific treatment for vWD varies with the bleeding symptoms and signs and with the subtype of vWD (see Chapter 133). Treatment is guided further by laboratory results indicating the potential success of increasing vWF with DDAVP and the clinical experience with a particular patient and his or her natural biologic family members who have vWD. When possible, attempts should be made to treat the patient without exposing him or her to plasma-derived products.

Nonprotein-Based Treatment

DDAVP is a synthetic octapeptide homolog of vasopressin that results in the release of stored vWF from Weibel Palade bodies of the endothelium (see section on treatment of hemophilia A). DDAVP is often effective for type 1 disease and may be useful in certain patients with type 2 disease. It is not appropriate for use in patients with type 3 disease in whom no stores of vWF exist. Before the use of DDAVP, a DDAVP trial should be conducted to document its efficacy in an individual patient. The trial is performed as described for hemophilia A. The phenomenon of tachyphylaxis is also observed when DDAVP is used to treat vWD. For bleeding that does not respond to DDAVP or in patients in whom a poor response is observed with DDAVP, other modalities (typically protein based) must be used.

As described for hemophilia A, the antifibrinolytic agent EACA or tranexamic acid are often administered in the setting of dental surgery to inhibit fibrinolysis. Care should be taken when these agents are administered in patients with a predisposition to thrombosis.

Estrogens upregulate vWF synthesis and may be useful, especially in women. Therapy with estrogens should also help ameliorate menorrhagia in affected symptomatic patients.

Plasma Protein–Based Therapy

Factor VIII Concentrates

Some intermediate-purity factor VIII concentrates are manufactured from plasma using methods that copurify significant amounts of vWF. Many of the products listed in Table 136.3 contain vWF. Humate-P (CLS Behring GmbH) and Wilate (Octapharma), which is 1:1 vWF:RCo/factor VIII, are currently licensed for the treatment of vWD. The multimeric pattern of VWF in these concentrates varies, and no product has the pattern of multimers that is present in normal plasma.[109] Because many of these products are effective clinically, the importance of these differences in multimer pattern remains to be determined. In addition, when using multiple doses of these products, factor VIII and vWF:RCo should be monitored because there is an increased risk of thrombotic events with the accumulation of factor VIII.

von Willebrand Factor Concentrates (Plasma-Derived and Recombinant)

Several chromatography-purified plasma-derived concentrates enriched in vWF have been studied by manufacturers in Europe.[110,111] Preliminary reports suggest that these are amenable to viral inactivation steps and may be effective both in vitro and in vivo.[109] To date, none of these have been developed as widely licensed products. Recombinant vWF (VONVENDI, Takeda Pharmaceutical Company Limited.) which is albumin- and plasma-free and synthesized in Chinese hamster ovaries was approved in 2015. In patients with severe vWD, a phase 3 multicenter trial of recombinant vWF, given alone or with Advate, was effective in treating all 22 patients with bleeding episodes; no thrombosis or inhibitors occurred during the study.[112] This can be used for treatment, prophylaxis, or in the perioperative setting; by itself, recombinant vWF can raise plasma factor VIII levels by 40% within 6 hours, with some individual variability.[113]

Cryoprecipitate

Cryoprecipitate was the mainstay of plasma-based therapy for bleeding in patients with vWD until the availability of virally inactivated intermediate-purity factor VIII concentrates with preserved functional vWF protein. These factor VIII concentrates are the products of choice for the treatment of bleeding in patients with types 1 and 2 vWD who are unresponsive to DDAVP and for bleeding in patients with type 3 vWD. Owing to the small potential for viral contamination of cryoprecipitate, manufacture of which does not include steps to inactivate viral pathogens, cryoprecipitate is currently indicated only when virally safe concentrates containing vWF are unavailable.

ACQUIRED FACTOR VIII AND VON WILLEBRAND FACTOR DEFICIENCY

Antibodies that inhibit the activity of factor VIII can develop in previously normal patients. Most of these patients have no underlying disease; however, factor VIII antibodies can arise in the setting of autoimmune disorders or the postpartum period. Although approximately one-third of inhibitors disappear spontaneously, the mortality rate among affected patients is significant.

In adults, acquired vWD is a rare autoimmune disorder that can occur in association with other autoimmune disorders or lymphoproliferative disease. Antibodies may interact with the epitopes on vWF required for its normal activity or result in increased clearance of antibody–vWF complexes.

In children, acquired vWD is extremely rare but has been reported in the context of Wilms tumor, hypothyroidism, and congenital heart disease and some medications; with treatment of the underlying disorder, vWF normalizes.

Treatment

Therapy for those patients with relatively high inhibitor titers (>5 BU) includes bypassing agents (FEIBA or rFVIIa) to stop bleeding and immunosuppression to reduce inhibitor levels. Plasmapheresis or immunoadsorption can be used to reduce the circulating levels of inhibitors. Immunosuppressive agents and IV IgG are useful adjuncts to abrogate the production of the autoantibody. DDAVP can also be effective in some patients with low-titer inhibitors. Unlike inhibitors that develop with hemophilia A, exposure to factor VIII usually does not result in increased antibody titers.[114] Conservative measures to control hemorrhage should also be used in conjunction with the above, including immobilization and compression, topical or local hemostatic agents, and antifibrinolytic therapy. The use of venipunctures, intramuscular injections, and drugs with platelet inhibitory activity should be minimized or avoided.

OTHER COAGULATION PROTEIN DEFICIENCIES

Approximately 15% of inherited bleeding disorders are caused by deficiencies of coagulation factors other than factor VIII, factor IX, or vWF. Inherited deficiencies of fibrinogen and factors II, V, VII, X, XI, and XIII may result in bleeding, requiring treatment. The genes for these factors are not located on the X chromosome; thus two gene defects are typically required for symptomatic disease. Consanguinity is frequent in affected kindreds. The characteristics of these deficiencies are presented in Table 136.3. Hereditary deficiencies of factor

TABLE 136.3 **Other Coagulation Deficiencies**

Factor	Incidence	Inheritance	Chromosome	Half-Life	Target Plasma Level	Treatment Product[a]
I (fibrinogen)	$1–2/10^6$		4	2–4 days	50–100 mg/dL	Concentrates
Afibrinogenemia		AR				Cryoprecipitate
Hypofibrinogen		AR or AD				
Dysfibrinogen		AR or AD				
II (prothrombin)	$<1/10^6$	AD	11	3 days	30%	PCCs Plasma
V	$1/10^6$	AR	1	36 hours	25%	Plasma
VII	$2/10^6$	AR	13	3–6 hours	25%	rFVIIa Concentrates PCCs Plasma
X	$2/10^6$	AR	13	40 hours	10%–25%	Concentrate PCC Plasma
XI		AR or AD	4	80 hours	20%–40%	Concentrates Plasma
XIII	$<1/10^6$	AR	1.6	9 days	5%	Concentrates Cryoprecipitate Plasma

[a] Products are listed in order of viral safety.

AD, Autosomal dominant; *AR*, autosomal recessive; *PCC*, prothrombin complex concentrates.

Data from Cohen AJ, Kessler CM. Treatment of inherited coagulation disorders. *Am J Med.* 99:675, 1995.

XII, prekallikrein, and high-molecular-weight kininogen have not been described to result in bleeding diatheses.

The mainstays of therapy for these disorders are cryoprecipitate (for fibrinogen deficiency) and plasma. In some countries, concentrates enriched in the missing protein have been available. An S/D-treated pooled plasma product is also available in some regions. Pooled S/D-treated plasma has the advantage over FFP in that it is virally inactivated but has the drawback of exposing the patient to a large number of donors (2500) with each dose.

Fibrinogen Deficiency

Bleeding disorders can result from low to absent levels of fibrinogen (hypofibrinogenemia or afibrinogenemia) or a protein with abnormal function (dysfibrinogenemia). The inheritance pattern for the former is autosomal recessive and for the latter is autosomal dominant. Dysfibrinogenemias can result in bleeding or hypercoagulability. The gene for fibrinogen is located on chromosome 4. Because fibrinogen is required for the formation of a fibrin clot, it is surprising that afibrinogenemic patients survive gestation and birth. Diagnosis is made when patients present with bleeding from the umbilical stump, intracranial hemorrhage, or mucosal bleeding. Hemarthroses can occur but are less common than observed with the hemophilias. Wound healing may be delayed. Increased incidence of fetal wastage in patients with afibrinogenemia and hypofibrinogenemia is observed, and term gestation is rarely achieved without replacement of fibrinogen.[115–117] Because the substrate for clot formation, fibrinogen, is missing or deficient, the prothrombin time (PT), aPTT, thrombin clotting time (TCT), and coagulation assays that have fibrin formation as their endpoints are all prolonged. Replacement of fibrinogen is accomplished with cryoprecipitate with a goal of achieving a plasma level between 50 and 100 mg/dL and at least 60 mg/dL for maintenance of pregnancy.[117] (Each bag of cryoprecipitate contains approximately 200 to 300 mg of fibrinogen.) The biologic half-life of fibrinogen is between 2 and 4 days. Virally inactivated, highly purified fibrinogen concentrates are available in Europe, China, Japan, and the United States, RiaSTAP, CSL Behring and Fibryga, Octapharma, which is nano-filtered, are available. These fibrinogen concentrates offer many advantages over cryoprecipitate, including viral inactivation, standardized fibrinogen content, smaller infusion volume and expedited time to treat the patient as reconstitution is swift, since it is stored as a lyophilized powder, and there is no need for cross-matching.[118] Adverse reactions to treatment include the development of fibrinogen antibodies; allergic reactions; and, paradoxically, thrombosis.

Prothrombin Deficiency

Congenital prothrombin deficiency is a rare autosomal recessive disorder estimated to be present at a rate of 0.5 cases per million. The disease has been described with both homozygous and heterozygous defects (including compound heterozygotes). No reports of aprothrombinemia appear in the literature, suggesting that a complete lack of the protein is incompatible with normal development. Hemorrhagic symptoms include bruising; hemarthroses; intracranial, mucosal, and deep-tissue bleeding; and menorrhagia.[119] Correlation between bleeding and prothrombin levels is poor. Both the PT and the aPTT are prolonged, and the thrombin time is normal. Treatment of prothrombin deficiency includes plasma at a dose of 15 to 25 mL/kg followed by 3 mL/kg every 12 to 24 hours to achieve levels of approximately 30%. The half-life of prothrombin is approximately three days. PCCs contain prothrombin and other VKD factors and can be used to treat prothrombin-deficient patients who are undergoing major surgery or life-threatening bleedings. Caution should be exercised because these have been associated with thrombosis.

Factor V Deficiency

Inherited deficiency of factor V occurs in fewer than 1:1,000,000 people with an autosomal recessive inheritance pattern (homozygous or compound heterozygous), resulting in factor V levels less than 20%. Symptoms include bleeding from the umbilical stump and mucous membranes, ecchymoses, menorrhagia, postpartum bleeding, and intracranial hemorrhage. Hemarthroses can occur but are less common than severe hemophilia. The correlation between bleeding and factor V levels is poor. No factor V–enriched plasma concentrates are available. Because factor V is in the common pathway of coagulation, in deficiency states, both the PT and the aPTT are prolonged. Treatment of bleeding involves the infusion of plasma at 15 to 20 mL/kg with a goal of achieving levels of 20%. The half-life of factor V is approximately 36 hours. Because platelets contain stored factor V,[120] they have been used to treat bleeding. Platelet infusion may result in the production of platelet-specific antibodies.

Factor VII Deficiency

Factor VII deficiency occurs in approximately 1:500,000 people; it is autosomally recessive and of the rarely inherited coagulation disorders, factor VII deficiency is the most common. Severe bleeding occurs with levels below 1%, and symptoms in severely affected patients are similar to those observed with severe hemophilia. Intracranial hemorrhage may occur in up to 16% of cases and neonates after vaginal delivery. The PT is prolonged, but the aPTT and thrombin times are normal. PCCs contain factor VII and may provide a benefit over plasma because they are virally inactivated. As with the use of these products for factor VIII and factor IX inhibitors, thrombosis has been reported. Recombinant activated factor VII (NovoSeven, Novo Nordisk) concentrate is effective in the treatment of bleeding with a congenital factor VII deficiency.[121–124] This can be used at a dose of 10 to 40 μg/kg every 4 to 6 hours, which is much lower than the dose used for patients with hemophilia and inhibitors.[125] Inhibitors to factor VIIa have developed in patients with congenital factor VII deficiency.[121]

Factor X Deficiency

Congenital deficiency of factor X is a rare condition resulting from homozygous or compound heterozygous defects in the autosomal genes for factor X located on chromosome 13. Bleeding correlates with factor X levels, and symptoms include epistaxis, menorrhagia, hemarthrosis, intracranial or gastrointestinal hemorrhage, hematuria, and umbilical cord bleeding. Because factor X is a component of prothrombinase (the first step in the common pathway), diagnosis is suggested by both a prolonged PT and a prolonged aPTT but a normal TCT. Treatment is with plasma products (FFP or S/D-treated plasma) at approximately 15 to 25 mL/kg followed by 5 mL/kg every 24 hours with a goal of plasma factor X activity levels of 20%. PCCs can be used; however, they contain variable amounts of factor X. As with the use of PCCs for other indications, the risk of thrombosis is increased. Coagdex, Bio Products Laboratory, is a plasma-derived factor X concentrate, approved in 2015 for prophylaxis, treatment of bleedings and perioperative management in mild-moderate factor X deficiency. There is a registry study NCT03161626, which collects data about patients with severe factor X deficiency and major surgery. For prophylaxis, Coagdex can be given twice weekly (children <12, 40 units/kg, age ≥12, 25 units/kg) but levels should be monitored to assess individual response.[126]

Factor XI Deficiency

Congenital factor XI deficiency (sometimes called hemophilia C) is an autosomal disorder with a recessive pattern of inheritance and is particularly common in Ashkenazi Jews in whom two specific mutations account for the approximately 8% prevalence of an abnormal factor XI gene.[127] The factor XI gene is located on chromosome 4. Bleeding symptoms are variable and include postsurgical or traumatic bleeding or heavy menses in women. Spontaneous hemorrhage and musculoskeletal bleeding are not typical of the disorder, and these characteristics distinguish this disorder from hemophilia A and B. Plasma levels of factor XI do not correlate with bleeding symptoms unlike the factor levels in hemophilia A and B. The aPTT is prolonged with factor XI deficiency; PT and thrombin time are normal. The bleeding risk in patients with factor XI deficiency depends on the surgical site and the fibrinolytic activity, if low prophylaxis may not be needed.[128] Plasma (FFP and S/D-treated plasma) provides the mainstay of therapy, and dosing takes into consideration the long half-life of 60 to 80 hours; however, some patients may have difficulty tolerating the large fluid volume of FFP. Although the half-life of factor XI in S/D plasma is comparable to that of FFP, decreased levels of factor XI in the product (≈30% to 50%) have been reported.[129] Especially in those patients with absent factor XI protein, inhibitors have developed after exposure to factor XI products. Two virally reduced or inactivated factor XI concentrates have been developed using affinity chromatography or cation-exchange chromatography.[130] These two concentrates, licensed in France (Hemoleven, LFB Biomedicaments France) and England (FXI concentrate Bio Products Laboratory). Both concentrates have been demonstrated to be effective, but there have been reports of thromboembolic complications with their use, presumably because of contaminant activated factor IX. Both products are now formulated with antithrombin and heparin, but caution is advised with their use in elderly patients and in patients with preexisting cardiovascular disease. In addition, because of thromboembolic risk, when using factor XI concentrates, antifibrinolytics should not be used concurrently. The recommended dose of these concentrates is 10 to 15 IU/kg.[128] There are no recombinant XI products available.

Because bleeding occurs in patients with factor XI deficiency more often in settings in which fibrinolysis is more active (oral mucosa, urinary tract), antifibrinolytic agents can be useful adjuncts in the treatment of such bleeding episodes or used as sole agents to prevent bleeding with dental procedures. Fibrin glue can provide a useful adjunct or be used solely in topical bleeding (dental extractions, circumcisions). DDAVP has been reported as useful for the prevention of surgical bleeding in factor XI deficiency.[131] The mode of effect of the DDAVP is unclear but is likely caused by the coexistence of mild vWF deficiency.

Factor XIII Deficiency

Factor XIII is a transglutaminase that catalyzes the cross-linking of γ-glutamyl and ε-lysyl groups of fibrin monomers, stabilizing the forming clot by decreasing its susceptibility to fibrinolysis. Inherited deficiency of factor XIII is autosomal recessive and results from defects in the genes for the two subunits of factor XIII located on chromosome 1 (B subunit) and chromosome 6 (A subunit). The majority of reported defects involve the A subunit. Umbilical bleeding in homozygotes may be observed at birth. Soft tissue bleedings, hemarthroses, excessive intracranial bleeding (relative to other coagulation deficiencies), pseudotumors, and bleeding during surgery are observed in affected patients. Surgery is also complicated by poor wound healing. Affected male patients may have oligospermia, and female patients may have recurrent miscarriages. Coagulation screening test results (PT, aPTT, and TCT) are normal. Solubility of clots is increased with factor XIII deficiency; thus, the diagnosis can be made by incubating clots in the presence of solubilizing agents such as 5 M urea or 1% to 2% chloroacetic acid. This qualitative test fails to detect mild and moderate deficiency; therefore, it is not recommended to perform quantitative factor XIII testing when suspecting a deficiency. Factor XIII has a long half-life (9 to 15 days). Previously, FFP (2 to 3 mL/kg) or cryoprecipitate (1 bag/10 to 20 kg) were the only options for treating patients with factor XIII deficiency. However, in February 2011, the first plasma-derived factor XIII concentrate (Corifact, CSL Behring) was approved by the FDA for prophylaxis; this concentrate is given intravenously to maintain a trough level of 5% to 20% with the initial recommended dose being 40 IU/kg every 28 days.[132] Because of the long half-life of factor XIII, the replacement can be used on a prophylactic basis (dosing every 3 to 4 weeks) to prevent intracranial hemorrhage and other bleeding episodes. The United Kingdom Haemophilia Centre Doctors' Organisation's current guidelines recommend lifetime prophylaxis for those patients with a severe factor XIII deficiency and a personal or family history of bleeding.[125] Dosing with factor XIII concentrate is 10 to 20 IU/kg every 4 to 6 weeks, with lower doses for prophylaxis and higher doses for the treatment of bleeding episodes, as well as escalating the dose throughout pregnancy.[125]

Recombinant FXIII-A2 (Tretten, Novo Nordisk) was approved in 2013. For prophylaxis in patients with congenital factor XIII A–subunit deficiency, recombinant FXIII-A2 35 IU/kg can be given monthly, targeting a trough >10%; this product cannot be used for patients with a B-subunit deficiency.[133] During the phase 1 extension study, no patients developed inhibitors or had thrombotic complications; twelve patients who underwent minor surgery eight patients receiving recombinant FXIII-A2 within the last 7 days and four within the last 10 to 21 days, did not require additional doses of factor.[134]

OTHER PLASMA-DERIVED PROTEIN CONCENTRATES

As discussed in Chapter 115, plasma fractionation processes also allow for the isolation of plasma components other than the coagulation proteins. Immunoglobulins are isolated by a variety of techniques from the Cohn fraction II–III. α_1-Antitrypsin and antithrombin are produced from fraction IV. As with other VKD proteins, protein C may be isolated from Cohn fraction II.

Anticoagulant Proteins (Protein C and Antithrombin)

Brief mention should be made of plasma-derived concentrates enriched in protein C and recombinant antithrombin III (Thrombate III, Grifols). These products have been used for a variety of indications, but both are licensed for the treatment of congenital deficiencies that result in thrombosis. Protein C concentrate has also been used in the treatment of purpura fulminans with meningococcemia. Antithrombin III in combination with defibrotide has been used for the successful treatment of veno-occlusive disease in patients who develop this complication after undergoing bone marrow transplantation,[135] heparin resistance in patients undergoing cardiopulmonary bypass, extracorporeal membrane oxygenation (ECMO), and sepsis. In addition, antithrombin III has been used in patients with acute lymphoblastic leukemia who are receiving asparaginase. There is recombinant topical thrombin (Recothrom, Baxter) which has been used in the surgical setting.

Although early studies showed some efficacy in using activated protein C to treat sepsis.[136] a Cochrane review reported that in five randomized controlled trials, including 5101 patients, activated protein C did not decrease the risk of death and was associated with an increased risk of bleeding.[137] Ceprotin, Baxter, is protein C concentrate that has been used for patients with severe protein C deficiency.

Immunoglobulins

The use of immunoglobulins (both IV and hyperimmune products) for a variety of diseases is discussed elsewhere in this book.

α_1-Protease Inhibitor (Antitrypsin)

α_1-Protease inhibitor (or antitrypsin) concentrates (Prolastin-C, Grifols; Aralast, Bayer Corporation; Zemaira, CLS Behring) are available for the prophylactic treatment of people with congenital deficiency of α_1-antitrypsin. α_1-Antitrypsin inhibits neutrophil elastase, which is thought to be responsible for the alveolar damage resulting in emphysema. These replacement products, administered IV, are presumed to act to delay the progression of congenital emphysema associated with this protease inhibitor deficiency.

FUTURE DIRECTIONS

Long-acting factor products have now been approved and have improved quality of life while reducing the number of weekly infusions and limiting the need for venous access devices. Novel delivery methods and rebalancing therapies for hemophilia, such as monthly subcutaneous injections are now widely used, greatly enhancing the quality of life. The possibility of future treatment with an oral agent may become a reality. Alternatively, curative treatment with gene therapy or gene editing may drastically reduce the need for factor replacement or rebalancing therapies. This continues to be a most promising time for the management of patients with hemophilia and rare inherited factor deficiencies.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Bolton-Maggs PHB, Perry DJ, Chalmers EA, et al. The rare coagulation disorders–review with guidelines for management from the United Kingdom Haemophilia Centre Doctors' Organisation. *Haemophilia*. 2004;10:593.

Brand-Staufer B, Carcao M, Kerlin BA, et al. Pharmacokinetic characterization of recombinant factor XIII (FXIII)-A2 across age groups in patients with FXIII A-subunit congenital deficiency. *Haemophilia*. 2015;21(3):380–385.

Ehrenforth S, Kreutz W, Scharrer I, et al. Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs. *Lancet*. 1992;339:594.

Feldman BM, Pai M, Rivard GE, et al. Tailored prophylaxis in severe hemophilia A: interim results from the first 5 years of the Canadian Hemophilia Primary Prophylaxis Study. *J Thromb Haemost*. 2006;4(6):1228–1236.

Franchini M, Mannucci PM: Inhibitor eradication with rituximab in haemophilia: where do we stand? *Br J Haematol*. 2014;165(5):600–608.

Green D. Complications associated with the treatment of haemophiliacs with inhibitors. *Haemophilia*. 1999;5(Suppl 3):11–17.

High KA. Factor IX: molecular structure, epitopes, and mutations associated with inhibitor formation. *Adv Exp Med Biol*. 1995;386:79–86.

Lancellotti S, Basso M, De Cristofaro R. Congenital prothrombin deficiency: an update. *Semin Thromb Hemost*. 2013;39(6):596–606.

Leissinger C, Gringeri A, Antmen B, et al. Anti-inhibitor coagulant complex prophylaxis in hemophilia with inhibitors. *N Engl J Med*. 2011;365:1684.

Lusher JM, Arkin S, Abildgaard CF, et al. Recombinant factor VIII for the treatment of previously untreated patients with hemophilia A: safety, efficacy, and development of inhibitors. Kogenate Previously Untreated Patient Study Group. *N Engl J Med*. 1993;328:453.

Mannucci PM: Haemophilia treatment protocols around the world: towards a consensus. *Haemophilia*. 1998;4(4):421.

Mannucci PM, Carlsson S, Harris AS:. Desmopressin, surgery and thrombosis. *Thromb Haemost*. 1994;71(1):154–155.

Mariani G, Testa MG, Di Paolantonio T, et al. Use of recombinant, activated factor VII in the treatment of congenital factor VII deficiencies. *Vox Sang*. 1999;77(3):131–136.

Mumford AD, Ackroyd S, Alikhan R, et al. Guideline for the diagnosis and management of the rare coagulation disorders: a United Kingdom Haemophilia Centre Doctors' Organization guideline on behalf of the British Committee for Standards in Haematology. *Br J Haematol*. 2014;167(3):304–326.

Nathwani AC, Reiss UM, Tuddenham EGD, et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med*. 2014;371(21):1994–2004.

Nathwani AC, Tuddenham EGD, Rangarajan S, et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med*. 2011;365(25):2357–2365.

Nilsson IM, Berntorp E, Zettervall O. Induction of immune tolerance in patients with hemophilia and antibodies to factor VIII by combined treatment with intravenous IgG, cyclophosphamide, and factor VIII. *N Engl J Med*. 1988;318:947.

Oldenburg J, Mahlangu JN, Kim B, et al. Emicizumab prophylaxis in hemophilia A with inhibitors. *N Engl J Med*. 2017;377(9):809–818.

Ragni MV, Fogarty PJ, Josephson NC, et al. Survey of current prophylaxis practices and bleeding characteristics of children with severe haemophilia A in US haemophilia treatment centres. *Haemophilia*. 2011;18:63–68.

Roberts HR: The treatment of hemophilia: past tragedy and future promise. *N Engl J Med*. 1989;321:1188.

Santagostino E, Young G, Escuriola Ettingshausen CE, et al. Inhibitors: a need for eradication? *Acta Haematol*. 2019;141(3):151–155.

White GC, Beebe A, Nielsen B. Recombinant factor IX. *Thromb Haemost*. 1997;78:261.

REFERENCES

1. Cohn EJ, Strong LE, et al. Preparation and properties of serum and plasma proteins; a system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. *J Am Chem Soc*. 1946;68:459–475.
2. Gilchrist GSPDRR. *Neurologic Complications in Hemophilia*. New York: Raven Press; 1989.
3. Eyster ME, Gill FM, Blatt PM, Hilgartner MW, Ballard JO, Kinney TR. Central nervous system bleeding in hemophiliacs. *Blood*. 1978;51(6):1179–1188.
4. Andes WA, Wulff K, Smith WB. Head trauma in hemophilia. A prospective study. *Arch Intern Med*. 1984;144(10):1981–1983.
5. Bray GL, Luban NL. Hemophilia presenting with intracranial hemorrhage. An approach to the infant with intracranial bleeding and coagulopathy. *Am J Dis Child*. 1987;141(11). 1215–127.
6. Schönlein J. Haemorrhaphilie (erbliche Anlange zu Bltutngen) (vol 2) Schönlein's Vorlesungen niedergeschriben und herausgegeben von einem seiner Zühorer;1832.
7. Hopff F. *Ueber die Haemophilie oder die erbliche Anlage zu toetlichen Blutungen*. Wuerzburg: CW Becker; 1828.
8. Lane S. Haemorrhagic diathesis: successful transfusion of blood. *Lancet*. 1840;35(896):185–188.
9. Weil P. L'Hémophilie, pathologie et sérotherapie. *Press Med*. 1905
10. Addis T. The pathogenesis of hereditary haemophilia. *J Path Bact*. 1911;15(4):427–452.
11. Feissly R. Etudes sur l'hémophilie. *Bull Mem Soc Med Hop Paris*. 1778;47:1923.
12. Graham JB, Buckwalter JA. Canine hemophilia; observations on the course, the clotting anomaly, and the effect of blood transfusions. *J Exp Med*. 1949;90(2):97–111.
13. Biggs R, Douglas AS, Macfarlane RG, Dacie JV, Pitney WR, Merskey Christmas disease: a condition previously mistaken for haemophilia. *Br Med J*. 1952;2(4799):1378–1382.
14. Graham JB, Collins Jr. DL, Godwin ID, Brinkhous KM. Assay of plasma antihemophilic activity in normal heterozygous (hemophilia) and prothrombinopenic dogs. *Proc Soc Exp Biol Med*. 1951;77(2):294–296.
15. Langdell RD, Wagner RH, Brinkhous KM. Effect of antihemophilic factor on one-stage clotting tests; a presumptive test for hemophilia and a simple one-stage antihemophilic factor assy procedure. *J Lab Clin Med*. 1953;41(4):637–647.
16. Didisheim P, Loeb J, Blatrix C, Soulier JP. Preparation of a human plasma fraction rich in prothrombin, proconvertin, Stuart factor, and PTC and a study of its activity and toxicity in rabbits and man. *J Lab Clin Med*. 1959;53(2):322–330.
17. Birk G. Zur Therapie der Hämophilie B. *Klinisch Wochenenschr*. 1958;36:240.
18. Pool JG, Shannon AE. Production of high-potency concentrates of antihemophilic globulin in a closed-bag system. *N Engl J Med*. 1965;273(27):1443–1447.
19. Johnson AJ, Newman J, Howell MB, Puszkin S. Two large-scale procedures for purification of human antihemophilic factor (AHF). *Blood*. 1966;28:1011.
20. Brinkhous KM, Shanbrom E, Roberts HR, Webster WP, Fekete L, Wagner RH. A new high-potency glycine-precipitated antihemophilic factor (AHF) concentrate. Treatment of classical hemophilia and hemophilia with inhibitors. *JAMA*. 1968;205(9):613–617.
21. Hoag MS, Johnson FF, Robinson JA, Aggeler PM. Treatment of hemophilia B with a new clotting-factor concentrate. *N Engl J Med*. 1969;280(11):581–586.
22. Schimpf K, Mannucci PM, Kreutz W, Brackmann HH, Auerswald G, Ciavarella N, et al. Absence of hepatitis after treatment with a pasteurized factor VIII concentrate in patients with hemophilia and no previous transfusions. *N Engl J Med*. 1987;316(15):918–922.
23. Schimpf K, Brackmann HH, Kreuz W, Kraus B, Haschke F, Schramm W, et al. Absence of anti-human immunodeficiency virus types 1 and 2 seroconversion after the treatment of hemophilia A or von Willebrand's disease with pasteurized factor VIII concentrate. *N Engl J Med*. 1989;321(17):1148–1152.
24. Fass DN, Knutson GJ, Katzmann JA. Monoclonal antibodies to porcine factor VIII coagulant and their use in the isolation of active coagulant protein. *Blood*. 1982;59(3):594–600.
25. Fulcher CA, Zimmerman TS. Characterization of the human factor VIII procoagulant protein with a heterologous precipitating antibody. *Proc Natl Acad Sci U S A*. 1982;79(5):1648–1652.
26. Horowitz B, Wiebe ME, Lippin A, Stryker MH. Inactivation of viruses in labile blood derivatives. I. Disruption of lipid-enveloped viruses by tri(n-butyl)phosphate detergent combinations. *Transfusion*.. 1985;25(6):516–522.
27. Horowitz B, Wiebe ME, Lippin A, Vandersande J, Stryker MH. Inactivation of viruses in labile blood derivatives. II. Physical methods. *Transfusion*.. 1985;25(6):523–527.
28. Schwartz RS, Abildgaard CF, Aledort LM, Arkin S, Bloom AL, Brackmann HH, et al. Human recombinant DNA-derived antihemophilic factor (factor VIII) in the treatment of hemophilia A. recombinant Factor VIII Study Group. *N Engl J Med*. 1990;323(26):1800–1805.
29. Teitel JM. Safety of coagulation factor concentrates. *Haemophilia*. 1998;4(4):393–401.
30. Roth DA, Kessler CM, Pasi K, et al. Human recombinant factor IX: safety and efficacy studies in hemophilia B patients previously treated with plasma-derived factor IX concentrates. *Blood*.. 2001;98(13):3600–3606.
31. Owen C. *Infusion Therapy in Hemophilia A and B*. New York: American Elsevier Publishing Company; 1975.
32. Pool JG, Robinson J. Observations on plasma banking and transfusion procedures for haemophilic patients using a quantitative assay for antihaemophilic globulin (AHG). *Br J Haematol*. 1959;5(1):24–30.
33. Aggeler PM, Spaet TH, Emery BBE. Purification of plasma thromboplastin factor B (plasma thromboplastin component) and its identification as a beta2 globulin. *Science*. 1954;119(3101):806.
34. Schulman S, Wiechel B. Hepatitis, epidemiology and liver function in hemophiliacs in Sweden. *Acta Med Scand*. 1984;215(3):249–256.
35. McDougal JS, Martin LS, Cort SP, Mozen M, Heldebrant CM, Evatt BL. Thermal inactivation of the acquired immunodeficiency syndrome virus, human T lymphotropic virus-III/lymphadenopathy-associated virus, with special reference to antihemophilic factor. *J Clin Invest*. 1985;76(2):875–877.
36. Roberts HR. The treatment of hemophilia: past tragedy and future promise. *N Engl J Med*. 1989;321(17):1188–1190.
37. Mannucci PM, Schimpf K, Abe T, Aledort LM, Anderle K, Brettler DB, et al. Low risk of viral infection after administration of vapor-heated factor VIII concentrate. International Investigator Group. *Transfusion*. 1992;32(2):134–138.
38. Gitschier J, Wood WI, Goralka TM, Wion KL, Chen EY, Eaton DH, et al. Characterization of the human factor VIII gene. *Nature*. 1984;312(5992):326–330.
39. Vehar GA, Keyt B, Eaton D, Rodriguez H, O'Brien DP, Rotblat F, et al. Structure of human factor VIII. *Nature*. 1984;312(5992):337–342.
40. Yoshitake S, Schach BG, Foster DC, Davie EW, Kurachi K. Nucleotide sequence of the gene for human factor IX (antihemophilic factor B). *Biochemistry*. 1985;24(14):3736–3750.
41. Bray GL, Gomperts ED, Courter S, Gruppo R, Gordon EM, Manco-Johnson M, et al. A multicenter study of recombinant factor VIII (recombinate): safety, efficacy, and inhibitor risk in previously untreated patients with hemophilia A. The Recombinate Study Group. *Blood*. 1994;83(9):2428–2435.
42. Lusher JM, Arkin S, Abildgaard CF, Schwartz RS. Recombinant factor VIII for the treatment of previously untreated patients with hemophilia A. Safety, efficacy, and development of inhibitors. Kogenate Previously Untreated Patient Study Group. *N Engl J Med*. 1993;328(7):453–459.
43. White 2nd GC, Courter S, Bray GL, Lee M, Gomperts ED. A multicenter study of recombinant factor VIII (Recombinate) in previously treated patients with hemophilia A. The Recombinate Previously Treated Patient Study Group. *Thromb Haemost*. 1997;77(4):660–667.
44. Lee DC, Stenland CJ, Miller JL, Cai K, Ford EK, Gilligan KJ, et al. A direct relationship between the partitioning of the pathogenic prion protein and transmissible spongiform encephalopathy infectivity during the purification of plasma proteins. *Transfusion*. 2001;41(4):449–455.
45. Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, Alperovitch A, et al. A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet*. 1996;347(9006):921–925.
46. Linnen J, Wages Jr. J, Zhang-Keck ZY, Fry KE, Krawczynski KZ, Alter H, et al. Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissible agent. *Science*. 1996;271(5248):505–508.
47. Nishizawa T, Okamoto H, Konishi K, Yoshizawa H, Miyakawa Y, Mayumi M. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. *Biochem Biophys Res Commun*. 1997;241(1):92–97.
48. Ongoing recombinant factor VIII shortage contingency recommendations. Advisory NHFM 371;2000.
49. Ljung RC. Can haemophilic arthropathy be prevented? *Br J Haematol*. 1998;101(2):215–219.
50. National Hemophilia Foundation. Medical and Scientific Advisory Council (MASAC) Recommendation concerning prophylaxis: regular administration of clotting factors to prevent bleeding #179. 2007.

51. Lillicrap D. In: Thomas AE, Halsey C, eds. *New Advances in the Treatment of Children with Hemophilia*. Basel: Karger; 2014:67–80.
52. Ragni MV, Fogarty PJ, Josephson NC, Neff AT, Raffini LJ, Kessler CM. Survey of current prophylaxis practices and bleeding characteristics of children with severe haemophilia A in US haemophilia treatment centres. *Haemophilia*. 2012;18(1):63–68.
53. Feldman BM, Pai M, Rivard GE, Israels S, Poon MC, Demers C, et al. Tailored prophylaxis in severe hemophilia A: interim results from the first 5 years of the Canadian Hemophilia Primary Prophylaxis Study. *J Thromb Haemost*. 2006;4(6):1228–1236.
54. Foundation NH. Medical and Scientific Advisory Council (MASAC) Recommendation concerning products licensed for the treatment of hemophilia and other bleeding disorders #263. 2020.
55. DiMichele D. Hemophilia 1996. New approach to an old disease. *Pediatr Clin North Am*. 1996;43(3):709–736.
56. Mannucci PM. Haemophilia treatment protocols around the world: towards a consensus. *Haemophilia*. 1998;4(4):421.
57. Parti R, Schoppmann A, Lee H, Yang L. Stability of lyophilized and reconstituted plasma/albumin-free recombinant human factor VIII (ADVATE rAHF-PFM). *Haemophilia*. 2005;11(5):492–496.
58. Osterberg T, Fatouros A, Neidhardt E, Warne N, Mikaelsson M. B-domain deleted recombinant factor VIII formulation and stability. *Semin Hematol*. 2001;38(2 Suppl 4):40–43.
59. Eloctate Prescribing Information: Biogen Idec. Available from: https://products.sanofi.us/Eloctate/Eloctate.pdf.
60. Oldenburg J, Mahlangu JN, Kim B, Schmitt C, Callaghan MU, Young G, et al. Emicizumab prophylaxis in hemophilia A with inhibitors. *N Engl J Med*. 2017;377(9):809–818.
61. Young G, Liesner R, Chang T, Sidonio R, Oldenburg J, Jimenez-Yuste V, et al. A multicenter, open-label phase 3 study of emicizumab prophylaxis in children with hemophilia A with inhibitors. *Blood*. 2019;134(24):2127–2138.
62. Mahlangu J, Oldenburg J, Paz-Priel I, Negrier C, Niggli M, Mancuso ME, et al. Emicizumab prophylaxis in patients who have hemophilia A without inhibitors. *N Engl J Med*. 2018;379(9):811–822.
63. Pipe SW, Shima M, Lehle M, Shapiro A, Chebon S, Fukutake K, et al. Efficacy, safety, and pharmacokinetics of emicizumab prophylaxis given every 4 weeks in people with haemophilia A (HAVEN 4): a multicentre, open-label, non-randomised phase 3 study. *Lancet Haematol*. 2019;6(6):e295–e305.
64. Hemlibra Prescribing Information: Genetech; 2018. Available from: https://www.gene.com/download/pdf/hemlibra_prescribing.pdf.
65. Jenkins PV, Bowyer A, Burgess C, Gray E, Kitchen S, Murphy P, et al. Laboratory coagulation tests and emicizumab treatment A United Kingdom Haemophilia Centre Doctors' Organisation guideline. *Haemophilia*. 2020;26(1):151–155.
66. Pasi KJ, Rangarajan S, Georgiev P, Mant T, Creagh MD, Lissitchkov T, et al. Targeting of antithrombin in hemophilia A or B with RNAi therapy. *N Engl J Med*. 2017;377(9):819–828.
67. Pasi KJ, Georgiev P, Mant T, et al. Fitusiran, an investigational RNAi therapeutic targeting antithrombin for the treatment of hemophilia: interim results from a phase 2 extension study in patients with hemophilia A or B with and without inhibitors. *Res Pract Thromb Haemost*. 2017;1:25.
68. Shapiro AD, Angchaisuksiri P, Astermark J, Benson G, Castaman G, Chowdary P, et al. Subcutaneous concizumab prophylaxis in hemophilia A and hemophilia A/B with inhibitors: phase 2 trial results. *Blood*. 2019;134(22):1973–1982.
69. Concizumab clinical trial resumed. The Pharma Letter. 2020. https://www.thepharmaletter.com/article/concizumab-clinical-trial-lifted.
70. Nathwani AC, Tuddenham EGD, Rangarajan S, Rosales C, McIntosh J, Linch DC, et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med*. 2011;365(25):2357–2365.
71. Nathwani AC, Reiss UM, Tuddenham EGD, Rosales C, Chowdary P, McIntosh J, et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med*. 2014;371(21):1994–2004.
72. George LA, Sullivan SK, Giermasz A, Rasko JEJ, Samelson-Jones BJ, Ducore J, et al. Hemophilia B gene therapy with a high-specific-activity factor IX variant. *N Engl J Med*. 2017;377(23):2215–2227.
73. Rangarajan S, Walsh L, Lester W, Perry D, Madan B, Laffan M, et al. AAV5-factor VIII gene transfer in severe hemophilia A. *N Engl J Med*. 2017;377(26):2519–2530.
74. Pasi KJ, Rangarajan S, Mitchell N, Lester W, Symington E, Madan B, et al. Multiyear follow-up of AAV5-hFVIII-SQ gene therapy for hemophilia A. *N Engl J Med*. 2020;382(1):29–40.
75. Revel-Vilk S, Blanchette VS, Sparling C, Stain AM, Carcao MD. DDAVP challenge tests in boys with mild/moderate haemophilia A. *Br J Haematol*. 2002;117(4):947–951.
76. Mannucci PM, Carlsson S, Harris AS. Desmopressin, surgery and thrombosis. *Thromb Haemost*. 1994;71(1):154–155.
77. Cheung WF, van den Born J, Kühn K, Kjellén L, Hudson BG, Stafford DW. Identification of the endothelial cell binding site for factor IX. *Proc Natl Acad Sci U S A*. 1996;93(20):11068–11073.
78. White 2nd GC, Beebe A, Nielsen B. Recombinant factor IX. *Thromb Haemost*. 1997;78(1):261–265.
79. Blatt PM, Lundblad RL, Kingdon HS, McLean G, Roberts HR. Thrombogenic materials in prothrombin complex concentrates. *Ann Intern Med*. 1974;81(6):766–770.
80. Aledort LM. rFVIIa—its thrombogenicity. *Thromb Haemost*. 2000;84(3):522–523.
81. BeneFIX Prescribing Information 2020. Available from: http://labeling.pfizer.com/showlabeling.aspx?id=492.
82. Warrier I. Management of haemophilia B patients with inhibitors and anaphylaxis. *Haemophilia*. 1998;4(4):574–576.
83. Kohler M. Thrombogenicity of prothrombin complex concentrates. *Thromb Res*. 1999;95(4 suppl 1):S13–S17.
84. Alprolix Prescribing Information 2017. Available from: https://products.sanofi.us/Alprolix/alprolix.html.
85. Ehrenforth S, Kreuz W, Scharrer I, Linde R, Funk M, Güngör T, et al. Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs. *Lancet*. 1992;339(8793):594–598.
86. Scharrer I, Bray GL, Neutzling O. Incidence of inhibitors in haemophilia A patients—a review of recent studies of recombinant and plasma-derived factor VIII concentrates. *Haemophilia*. 1999;5(3):145–154.
87. Cannavò A, Valsecchi C, Garagiola I, Palla R, Mannucci PM, Rosendaal FR, et al. Nonneutralizing antibodies against factor VIII and risk of inhibitor development in severe hemophilia A. *Blood*. 2017;129(10):1245–1250.
88. Peyvandi F, Cannavò A, Garagiola I, Palla R, Mannucci PM, Rosendaal FR, et al. Timing and severity of inhibitor development in recombinant versus plasma-derived factor VIII concentrates: a SIPPET analysis. *J Thromb Haemost*. 2018;16(1):39–43.
89. Reipert BM, Gangadharan B, Hofbauer CJ, Berg V, Schweiger H, Bowen J, et al. The prospective Hemophilia Inhibitor PUP Study reveals distinct antibody signatures prior to FVIII inhibitor development. *Blood Adv*. 2020;4(22):5785–5796.
90. High KA. Factor IX: molecular structure, epitopes, and mutations associated with inhibitor formation. *Adv Exp Med Biol*. 1995;386:79–86.
91. Warrier I, Ewenstein BM, Koerper MA, Shapiro A, Key N, DiMichele D, et al. Factor IX inhibitors and anaphylaxis in hemophilia B. *J Pediatr Hematol Oncol*. 1997;19(1):23–27.
92. Warrier I, Lusher JM. Development of anaphylactic shock in haemophilia B patients with inhibitors. *Blood Coagul Fibrinolysis*. 1998;9 Suppl 1:S125–S128.
93. Santagostino E, Young G, Escuriola Ettingshausen C, Jimenez-Yuste V, Carcao M. Inhibitors: a need for eradication? *Acta Haematol*. 2019;141(3):151–155.
94. Green D. Complications associated with the treatment of haemophiliacs with inhibitors. *Haemophilia*. 1999;5(Suppl 3):11–17.
95. SEVENFACT Package Insert 2020. Available from: https://www.fda.gov/media/136610/download.
96. DiMichele DM. Inhibitors in haemophilia: a primer. *Haemophilia*. 2000;6(suppl 1):38–40.
97. Leissinger C, Gringeri A, Antmen B, Berntorp E, Biasoli C, Carpenter S, et al. Anti-inhibitor coagulant complex prophylaxis in hemophilia with inhibitors. *N Engl J Med*. 2011;365(18):1684–1692.
98. Nilsson IM. The management of hemophilia patients with inhibitors. *Transfus Med Rev*. 1992;6(4):285–293.
99. Nilsson IM, Berntorp E, Zettervall O. Induction of immune tolerance in patients with hemophilia and antibodies to factor VIII by combined treatment with intravenous IgG, cyclophosphamide, and factor VIII. *N Engl J Med*. 1988;318(15):947–950.
100. Nilsson IM, Hedner U. Immunosuppressive treatment in haemophiliacs with inhibitors to factor VIII and factor IX. *Scand J Haematol*. 1976;16(5):369–382.
101. Nilsson IM, Jonsson S, Sundqvist SB, Ahlberg A, Bergentz SE. A procedure for removing high titer antibodies by extracorporeal protein-A-sepharose adsorption in hemophilia: substitution therapy and surgery in a patient with hemophilia B and antibodies. *Blood*. 1981;58(1):38–44.
102. Rituximab to Treat Severe Hemophilia A (RICH) 2013. Available from: https://clinicaltrials.gov/ct2/show/NCT00331006.
103. Leissinger C, Josephson CD, Granger S, Konkle BA, Kruse-Jarres R, Ragni MV, et al. Rituximab for treatment of inhibitors in haemophilia A. A phase II study. *Thromb Haemost*. 2014;112(3):445–458.
104. Carcao M, St Louis J, Poon MC, Grunebaum E, Lacroix S, Stain AM, et al. Rituximab for congenital haemophiliacs with inhibitors: a Canadian experience. *Haemophilia*. 2006;12(1):7–18.

105. Collins PW, Mathias M, Hanley J, Keeling D, Keenan R, Laffan M, et al. Rituximab and immune tolerance in severe hemophilia A: a consecutive national cohort. *J Thromb Haemost*. 2009;7(5):787–794.
106. Franchini M, Mannucci PM. Inhibitor eradication with rituximab in haemophilia: where do we stand? *Br J Haematol*. 2014;165(5):600–608.
107. Rodeghiero F, Castaman G, Dini E. Epidemiological investigation of the prevalence of von Willebrand's disease. *Blood*. 1987;69(2):454–459.
108. Nilsson IM, Blomback M, Jorpes E, Blomback B, Johansson SA. Von Willebrand's disease and its correction with human plasma fraction 1-0. *Acta Med Scand*. 1957;159(3):179–188.
109. Mannucci PM, Tenconi PM, Castaman G, Rodeghiero F. Comparison of four virus-inactivated plasma concentrates for treatment of severe von Willebrand disease: a cross-over randomized trial. *Blood*. 1992;79(12):3130–3137.
110. Burnouf-Radosevich M, Burnouf T. Chromatographic preparation of a therapeutic highly purified von Willebrand factor concentrate from human cryoprecipitate. *Vox Sang*. 1992;62(1):1–11.
111. Goudemand J, Mazurier C, Marey A, Caron C, Coupez B, Mizon P, et al. Clinical and biological evaluation in von Willebrand's disease of a von Willebrand factor concentrate with low factor VIII activity. *Br J Haematol*. 1992;80(2):214–221.
112. Gill JC, Castaman G, Windyga J, Kouides P, Ragni M, Leebeek FWG, et al. Hemostatic efficacy, safety, and pharmacokinetics of a recombinant von Willebrand factor in severe von Willebrand disease. *Blood*. 2015;126(17):2038–2046.
113. VONVENDI Prescribing Information 2019. Available from: https://www.shirecontent.com/PI/PDFs/VONVENDI_USA_ENG.pdf.
114. Feinstein D. Inhibitors in Hemophilia. Third ed: Philadelphia: Churchill Livingstone.
115. Goodwin TM. Congenital hypofibrinogenemia in pregnancy. *Obstet Gynecol Surv*. 1989;44(3):157–161.
116. Ness PM, Budzynski AZ, Olexa SA, Rodvien R. Congenital hypofibrinogenemia and recurrent placental abruption. *Obstet Gynecol*. 1983;61(4):519–523.
117. Inamoto Y, Terao T. First report of case of congenital afibrinogenemia with successful delivery. *Am J Obstet Gynecol*. 1985;153(7):803–804.
118. Franchini M, Lippi G. Fibrinogen replacement therapy: a critical review of the literature. *Blood Transfus*. 2012;10(1):23–27.
119. Lancellotti S, Basso M, De Cristofaro R. Congenital prothrombin deficiency: an update. *Semin Thromb Hemost*. 2013;39(6):596–606.
120. Breederveld K, Giddings JC, ten Cate JW, Bloom AL. The localization of factor V within normal human platelets and the demonstration of a platelet-factor V antigen in congenital factor V deficiency. *Br J Haematol*. 1975;29(3):405–412.
121. Mariani G, Testa MG, Di Paolantonio T, Molskov Bech R, Hedner U. Use of recombinant, activated factor VII in the treatment of congenital factor VII deficiencies. *Vox Sang*. 1999;77(3):131–136.
122. White B, O'Connor H, Smith OP. Successful use of recombinant VIIa (Novoseven) and endometrial ablation in a patient with intractable menorrhagia secondary to FVII deficiency. *Blood Coagul Fibrinolysis*. 2000;11(2):155–157.
123. Hunault M, Bauer KA. Recombinant factor VIIa for the treatment of congenital factor VII deficiency. *Semin Thromb Hemost*. 2000;26(4):401–405.
124. Roberts HR. Clinical experience with activated factor VII: focus on safety aspects. *Blood Coagul Fibrinolysis*. 1998;9 suppl 1:S115–S118.
125. Mumford AD, Ackroyd S, Alikhan R, Bowles L, Chowdary P, Grainger J, et al. Guideline for the diagnosis and management of the rare coagulation disorders: a United Kingdom Haemophilia Centre Doctors' Organization guideline on behalf of the British Committee for Standards in Haematology. *Br J Haematol*. 2014;167(3):304–326.
126. Coagdex Prescribing Information 2015. Available from: https://www.coagadex.com/download/Coagadex_USPI_09-2018.pdf.
127. Seligsohn U, Peretz H. Molecular genetics aspects of factor XI deficiency and Glanzmann thrombasthenia. *Haemostasis*. 1994;24(2):81–85.
128. Shapiro A. The use of prophylaxis in the treatment of rare bleeding disorders. *Thromb Res*. 2020;196:590–602.
129. Inbal A, Epstein O, Blickstein D, Kornbrot N, Brenner B, Martinowitz U. Evaluation of solvent/detergent treated plasma in the management of patients with hereditary and acquired coagulation disorders. *Blood Coagul Fibrinolysis*. 1993;4(4):599–604.
130. Seligsohn U. Factor XI deficiency. *Thromb Haemost*. 1993;70(1):68–71.
131. Castaman G, Ruggeri M, Rodeghiero F. Clinical usefulness of desmopressin for prevention of surgical bleeding in patients with symptomatic heterozygous factor XI deficiency. *Br J Haematol*. 1996;94(1):168–170.
132. Corifact Prescribing Information 2019. Available from: http://cslbehring.vo.llnwd.net/o33/u/central/PI/US/Corifact/EN/Corifact-Prescribing-Information.pdf.
133. Brand-Staufer B, Carcao M, Kerlin BA, et al. Pharmacokinetic characterization of recombinant factor XIII (FXIII)-A2 across age groups in patients with FXIII A-subunit congenital deficiency. *Haemophilia*. 2015;21(3):380–385.
134. Carcao M, Altisent C, Castaman G, et al. Recombinant FXIII (rFXIII-A2) prophylaxis prevents bleeding and allows for surgery in patients with congenital FXIII A-subunit deficiency. *Thromb Haemost*. 2018;118(3):451–460.
135. Haussmann U, Fischer J, Eber S, et al. Hepatic veno-occlusive disease in pediatric stem cell transplantation: impact of pre-emptive antithrombin III replacement and combined antithrombin III/defibrotide therapy. *Haematologica*. 2006;91(6):795–800.
136. Bernard GR, Vincent JL, Laterre PF, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. *N Engl J Med*. 2001;344(10):699–709.
137. Marti-Carvajal AJ, Sola I, Lathyris D, Cardona AF. Human recombinant activated protein C for severe sepsis. *Cochrane Database Syst Rev*. 2011(3):CD004388.

CHAPTER 137

DISSEMINATED INTRAVASCULAR COAGULATION

Marcel Levi

A variety of disorders, including infectious or inflammatory conditions and malignant disease, will lead to activation of coagulation. In many cases, this activation of coagulation will not lead to clinical complications and will not even be detected by routine laboratory tests, but can only be measured with sensitive molecular markers for activation of coagulation factors and pathways.[1,2] However, if activation of coagulation is sufficiently strong, the platelet count may decrease and global clotting times may become prolonged. In its most extreme form, systemic activation of coagulation is known as disseminated intravascular coagulation (DIC). DIC is characterized by the simultaneous occurrence of widespread (micro)vascular thrombosis, thereby compromising blood supply to various organs, which may contribute to organ failure.[3,4] Because of ongoing activation of the coagulation system and other factors, such as impaired synthesis and increased degradation of coagulation proteins and protease inhibitors, consumption of clotting factors and platelets may occur, resulting in diffuse bleeding.

In view of the multiple, often contrasting mechanisms that occur in patients with DIC, a consensual definition of DIC had been a matter of debate. In 2001, the subcommittee on DIC of the International Society on Thrombosis and Haemostasis proposed a definition that reflects the central role of the microvascular milieu (i.e., endothelial cells, blood cells, and the plasma protease system, in the pathogenesis of DIC). This definition of DIC reads as follows: “DIC is an acquired syndrome characterized by the intravascular activation of coagulation without a specific localization and arising from different causes. It can originate from and cause damage to the microvasculature, which if sufficiently severe, can produce organ dysfunction.”[5]

The diagnosis of DIC may be hampered by the non-specific nature of many indicators of coagulation activation, although newly developed scoring algorithms based on readily available routine laboratory parameters show promising diagnostic accuracy.[5] Owing to the complexity of the clinical presentation, the variable, and unpredictable course, and the multitude of therapies given to patients with DIC, properly conducted clinical trials are difficult to perform and even to devise. Management relies on limited evidence from clinical trials in combination with small studies employing surrogate outcome endpoints and experience from case series, as well as from an understanding of the underlying pathophysiologic mechanisms.[6]

EPIDEMIOLOGY

Activation of coagulation in concert with inflammatory activation can result in microvascular thrombosis, which contributes to multiple organ failure in patients with severe sepsis.[7] In support of this concept, postmortem findings in patients with coagulation abnormalities and DIC on the background of severe sepsis include diffuse bleeding, hemorrhagic necrosis of tissues, microthrombi in small blood vessels, and thrombi in mid-size and larger arteries and veins. Ischemia and necrosis were invariably the results of fibrin deposition in small and mid-size vessels. Importantly, intravascular thrombi appear to be the driver of the organ dysfunction. Fibrin deposition in various organs also is a characteristic finding in animal models of DIC. Thus, experimental bacteremia or endotoxemia causes intra- and extravascular fibrin deposition in the kidneys, lungs, liver, brain, and other organs. Amelioration of the hemostatic defect with various interventions in these models reduces fibrin deposition, improves organ function, and, in some cases, reduces mortality. Finally, the results of clinical studies also support the concept that the extent of activation of the coagulation system is an important determinant of clinical outcome. DIC has been shown to be an independent predictor of organ failure and mortality.[8] In a consecutive series of patients with severe sepsis, 43% of patients with DIC were compared with 27% without DIC. In this study, the severity of the coagulopathy was directly related to mortality.[9]

In addition to microvascular thrombosis and organ dysfunction, coagulation abnormalities may have other harmful consequences. Thrombocytopenia in patients with sepsis places them at increased risk of bleeding. For example, critically ill patients with a platelet count of less than 50×10^9/L have a four to fivefold higher risk for bleeding than those with higher platelet counts.[10] Although the overall risk of intracerebral bleeding in patients in the intensive care unit (ICU) is less than 0.5%, up to 88% of patients with this complication have platelet counts less than 100×10^9/L. The use of anticoagulants in patients with thrombocytopenia further increases the risk of bleeding. Regardless of the cause, multivariate analyses indicate that thrombocytopenia is an independent predictor of ICU mortality and increases the risk of death by 1.9- to 4.2-fold. In particular, thrombocytopenia that persists for more than 4 days after ICU admission, or a 50% or greater decrease in the platelet count during the ICU stay is associated with a four- to sixfold increase in mortality. In fact, the platelet count appears to be a stronger predictor of ICU mortality than composite scoring systems, such as the Acute Physiology and Chronic Evaluation (APACHE) II or Multiple Organ Dysfunction Score (MODS). Decreased levels of coagulation factors, as reflected by prolonged global coagulation times, also increase the risk of bleeding. Prolongation of the prothrombin time (PT) or activated partial thromboplastin time (aPTT) to over 1.5 times the control is associated with an increased risk of bleeding and mortality in critically ill patients.

PATHOBIOLOGY

Traditionally, DIC was thought to be the result of activation of both the extrinsic and intrinsic pathways of coagulation. The classical concept was that the extrinsic pathway was initiated by a tissue-derived component, which activated factor VII, leading to the direct conversion of prothrombin to thrombin. This process would proceed as long as there was tissue damage from systemic infection, trauma, placental abruption, or malignancy. In contrast, the intrinsic or contact pathway of coagulation was initiated by contact activation of factor XII which, together with its cofactors, kallikrein and kininogen, then activated factor XI with subsequent activation of factor IX. The initiators of contact activation were poorly understood until recently but were thought to include collagen and artificial surfaces. In recent years, the molecular mechanisms of coagulation pathways have been defined (Fig. 137.1) (see Chapters 120 and 124). This has provided new insight into the pathogenesis of DIC. In general, current thinking is that thrombin and fibrin generation in patients with DIC is largely driven via the extrinsic pathway; the role of the contact system is uncertain.

Tissue Factor-Factor VII(a) Pathway

The extrinsic pathway is initiated by the tissue factor (TF)-factor VIIa complex. TF is a membrane-bound 4.5 kD protein that is constitutively expressed on cells that are mostly in tissues not in direct contact

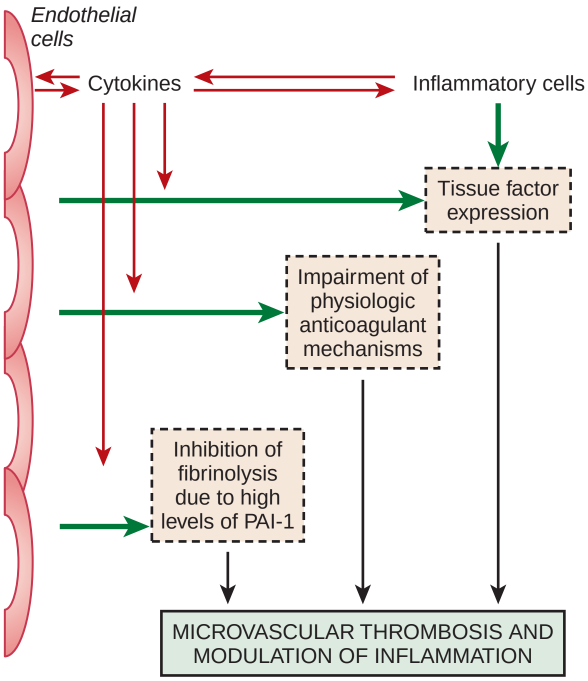

Endothelial cells
Cytokines
Inflammatory cells
Tissue factor expression
Impairment of physiologic anticoagulant mechanisms
Inhibition of fibrinolysis due to high levels of PAI-1
MICROVASCULAR THROMBOSIS AND MODULATION OF INFLAMMATION

Figure 137.1 PATHWAYS INVOLVED IN THE ACTIVATION OF COAGULATION IN DIC. Both perturbed endothelial cells and activated mononuclear cells may produce proinflammatory cytokines that induce tissue factor expression, thereby initiating coagulation. In addition, downregulation of physiological anticoagulant mechanisms and inhibition of fibrinolysis promotes intravascular fibrin deposition. *PAI-1*, Plasminogen activator inhibitor, type 1.

with blood, such as the adventitial layer of larger blood vessels. Subcutaneous tissue also contains substantial amounts of TF. When expressed on the cell surface, TF interacts with factor VII, either in its zymogen or activated form. The TF-factor VIIa complex catalyzes the activation of both factor IX and factor X. Factors IXa and Xa enhance the activation of factors X and prothrombin, respectively. In cells in contact with the blood, TF is induced by mediators such as cytokines, C reactive protein, and advanced glycosylation end products. Inducible TF is predominantly expressed by monocytes and macrophages. Monocyte TF expression is enhanced in the presence of platelets and granulocytes in a P-selectin-dependent manner. This may reflect nuclear factor-kappa B (NFκB) activation that occurs when activated platelets bind to neutrophils or mononuclear cells. These cell-cell interactions also stimulate the production of IL-1b, IL-8, monocyte chemotactic protein 1 (MCP-1), and TNFα. Under cell culture conditions, cytokines such as TNFα, and IL-1, can induce TF expression by vascular endothelial cells, but the in vivo relevance of this finding is uncertain. Studies in vivo suggest that IL-6 is the dominant inducer of TF expression by mononuclear cells.

Increased monocyte TF expression and procoagulant activity has been demonstrated in DIC associated with sepsis, cancer, or coronary disease. Expression of TF appears to be localized to certain organs and vascular beds, but it is uncertain whether its expression is under genetic control in an organ-specific manner. With trauma, such as extensive surgery, brain injury, or burns, it is likely that constitutively expressed TF at the site of injury is the primary source of procoagulant material, but direct support for this concept is lacking.

The Intrinsic Pathway

The role of the intrinsic pathway in the pathogenesis of DIC is uncertain. Negatively-charged substances, such as phospholipids, polyphosphates, and glycosaminoglycans, are potential activators of the contact pathway (see Chapters 120 and 124). Studies in patients with suspected DIC have identified elevated levels of markers of activation of the contact system. In patients with meningococcal septicemia, there was a negative correlation between plasma factor XII levels and the levels of factor XIIa-C1 inhibitor complexes. Although this finding implies consumption of factor XII and subsequent downstream activation of factor XI, an alternative explanation is that there is a negative acute-phase effect with reduced synthesis of factor XII in conjunction with thrombin-mediated activation of factor XI. However, blockade of the contact system with a factor XIIa-directed antibody failed to prevent DIC in a baboon model of *Escherichia coli* sepsis but diminished the development of lethal hypotension. These findings provide reasonable support for the current view that the contact pathway does not contribute to DIC but may play important roles in proinflammatory mechanisms related to vascular permeability, vascular proliferation (kininogen induces smooth muscle cell proliferation), and enhancement of fibrinolysis.[11]

Cytokines and Other Amplification Pathways

Activation of blood coagulation requires several cofactors (see Chapters 120 and 124). For the development of DIC, the surfaces of cell remnants or intact cells, inflammatory mediators, and coagulation proteins are required. The stimulus for activation depends on the underlying disease and may range from bacterial cell compounds, such as endotoxin, TF on host or cancer cells, other cancer cell procoagulants, TF, or fat or amniotic fluid. Each of these triggers interacts with other mediators: TF assembles on anionic phospholipid surfaces, which can be provided by activated platelets, leukocytes, or by cancer cells. Cytokines interact with receptors and induce signaling pathways that induce TF expression and other proinflammatory components via the NFκB complex.

Endotoxin is a lipopolysaccharide compound of gram-negative bacteria that induces the sepsis syndrome and DIC. Gram-negative bacteria liberate endotoxin from their membrane, which interacts with cell surfaces via various pathways. In blood, endotoxin directly binds to CD14 on monocytes and binds to endothelial cells after complexing with lipopolysaccharide-binding protein (LBP) and the Toll-like receptor 4 (TLR 4) complex. Through these interactions, endotoxin induces signaling pathways that culminate in NFκB activation and initiates the expression of proinflammatory cytokines and TF. Likewise, exotoxin such as lipoteichoic acid (LTA) from gram-positive bacteria can induce proinflammatory cytokine expression in a similar manner.

The molecular mechanisms underlying endotoxin-induced activation of coagulation have been studied in primates and baboons. In endotoxin or *E. coli* models of sepsis, inhibition of the TF pathway abolishes the activation of coagulation, thereby highlighting the importance of TF. IL-6 is an important mediator of procoagulant effects, whereas TNFα is involved in the fibrinolytic response to endotoxin. Inhibition of TF with tissue factor pathway inhibitor (TFPI) reduces IL-6 levels in the baboon model, suggesting that there is extensive crosstalk between coagulation and inflammatory mediators (see the following section). Monocytes that express TF bind factor VII(a), shed TF, or bind to the damaged vessel wall. After interacting with platelets, circulating monocytes can trigger DIC. Microvesicles may accelerate this process, and the complex interaction among cells, membrane fragments, soluble mediators, and plasma proteins may trigger the DIC syndrome. The severity and duration of the consumptive process are mainly determined by the potency of the triggers and the capacity of inhibitory mechanisms.

Coagulation Protease Crosstalk Promotes Inflammation

In addition to activating coagulation protein zymogens, coagulation proteases also interact with specific cell receptors and trigger signaling pathways that elicit proinflammatory mediators.[7] Factor Xa, thrombin, and the factor VIIa-TF complex have such effects. Factor Xa injection into rats induces localized inflammation, probably as a result of its interaction with effector-cell protease receptor-1 (EPR-1) and not because of thrombin generation. Exposure of cultured endothelial cells to factor Xa stimulates the production of MCP-1, IL-6, and IL-8 and upregulates the expression of adhesion proteins that tether neutrophils to the cell surface. Further evidence for the crosstalk between inflammation and coagulation comes from the observations that IL-6 and IL-8 elicit TF-dependent procoagulant activity in monocytes and the identification of IL-6 as the critical mediator of procoagulant activity either on its own or after endotoxin challenge in vivo. Therefore, cytokine production induced by factor Xa may be an important driver of coagulation in DIC.

In addition to its procoagulant functions, thrombin has a variety of non-coagulant effects. Thrombin induces the release of MCP-1 and IL-6 from fibroblasts, epithelial cells, and mononuclear cells in vitro. Thrombin also induces IL-6 and IL-8 production by endothelial cells, which has a procoagulant effect that is TF-dependent. Cell activation by thrombin is likely mediated by protease-activated receptors. The factor VIIa-TF complex also activates cells by binding protease-activated receptor-2 (PAR-2).

Direct evidence of the in vivo relevance of these phenomena comes from a study showing that recombinant factor VIIa infusion in volunteers induces an increase in plasma levels of IL-6 and IL-8. Although the concentrations of factor VIIa infused far exceed those found in patients with sepsis, it is possible that factor VIIa-induced cytokine production is of physiological importance. Thus, this information adds to the concept that several coagulation proteases induce proinflammatory mediators that augment procoagulant activity and amplify the consumptive process. Endogenous anticoagulant pathways are essential to regulate these proteases and prevent uncontrolled DIC.

Endogenous Anticoagulant Pathways in Disseminated Intravascular Coagulation

The development of DIC is counteracted by several mechanisms (see Chapters 120 and 124). First, coagulation inhibitors regulate the coagulation mechanism. These inhibitors include antithrombin (AT), the protein C pathway, and TFPI (Fig. 137.2).[7] AT, which complexes and inhibits thrombin and factor Xa, is one of the most important inhibitors, and reduced AT levels are a characteristic of DIC. Reductions in AT levels reflect a combination of reduced protein synthesis increased clearance through the formation of protease-AT complexes and AT degradation by neutrophil elastase. In addition, cytokines may impair proteoglycan synthesis in the vessel wall, thereby reducing the availability of heparan sulfate for the potentiation of AT activity.

In animal models of experimental bacteremia, AT concentrate infusion increases survival, reduces the severity of DIC, and lowers the levels of IL-6 and IL-8. Therefore, in addition to its anticoagulant function, AT may also have an anti-inflammatory effect.

Activated protein C and its cofactor protein S form a second line of defense. Thrombin binds to thrombomodulin on the endothelial cell surface, and the thrombin-thrombomodulin complex converts protein C to its active form, activated protein C (APC).[12] In addition, the thrombin-thrombomodulin complex converts thrombin activatable fibrinolytic inhibitor (TAFI) into its activated form, TAFIa. APC inactivates factors Va and VIIIa by proteolytic cleavage, thus down regulating the coagulation cascade. Endothelial cells, primarily those of large blood vessels, express endothelial protein C receptor (EPCR) on their surface. EPCR binds protein C and presents it to the thrombin-thrombomodulin complex for activation; a process that amplifies APC generation about 20-fold.

APC has anti-inflammatory effects on mononuclear cells and granulocytes, which may be distinct from its anticoagulant activity. Administration of APC prevents thrombin-induced thromboembolism in mice, mainly through its antithrombotic effect. In addition, several studies have demonstrated the anti-inflammatory effects of APC.

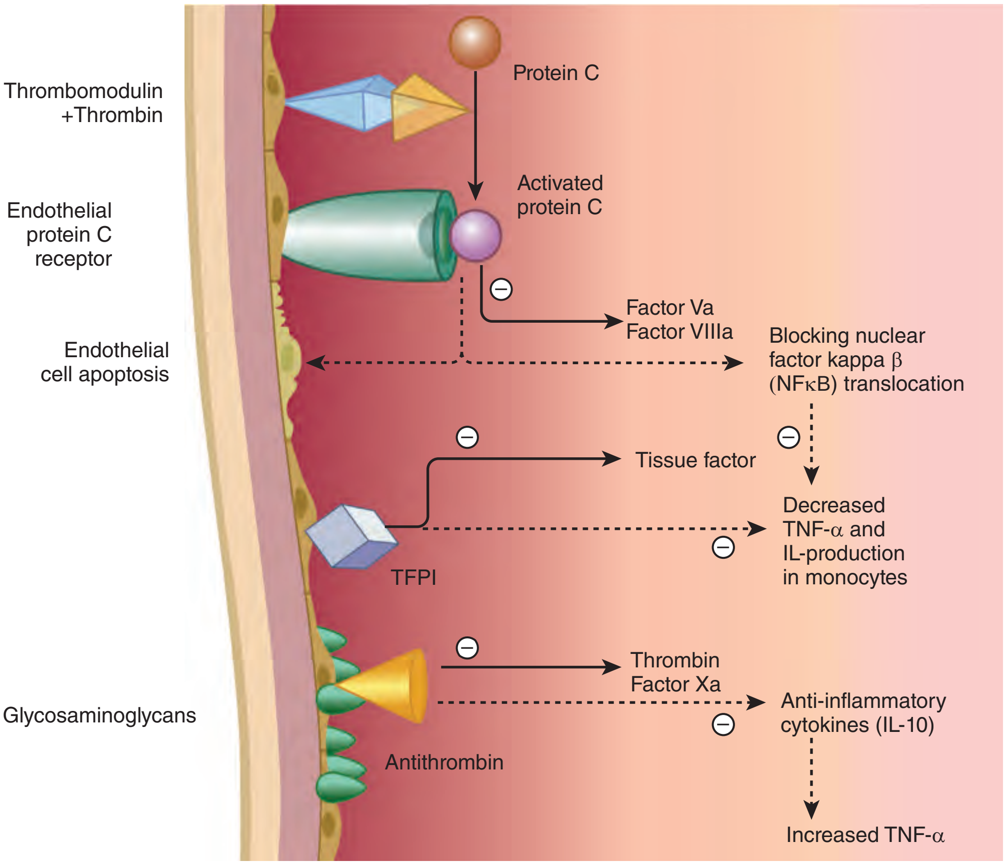


Figure 137.2 PHYSIOLOGICAL ANTICOAGULANT MECHANISMS IN DIC. Physiological anticoagulant mechanisms (activated protein C system, tissue factor pathway inhibitor *[TFPI]*, and antithrombin) are not only involved in blocking thrombin generation and thrombin activity but also affect inflammatory pathways.

Defects in the protein C pathway enhance the vulnerability to inflammatory reactions and DIC. In patients with DIC, lower levels of protein C and protein S are associated with increased mortality. Mice with one allele targeted disruption of the protein C gene, causing heterozygous protein C deficiency, have more severe DIC and a greater inflammatory response than wild-type mice. Blockade of the activity of protein C by infusion of C4 binding protein converted a sublethal model of *E. coli* in baboons into a lethal model. Blockade of EPCR with a neutralizing monoclonal antibody also increased mortality in the *E. coli* baboon model. In contrast, infusion of PC in the same model protected the baboons from fatal DIC. Thus, the protein C pathway appears to be an important host defense against sepsis and DIC.

With systemic inflammation, TNFα and interleukin-1 may down regulate the expression of thrombomodulin by endothelial cells as they do in cell culture experiments. Although thrombin upregulates EPCR expression, by activating metalloproteases, thrombin may also induce EPCR shedding from the endothelium.

Thrombomodulin plays a central role in the pathogenesis of DIC.[13] First, thrombomodulin is essential for the activation of protein C by thrombin; as such, thrombomodulin plays a key role in the anticoagulant and anti-inflammatory properties of the protein C system. Second, thrombomodulin binds thrombin, thereby preventing it from exerting prothrombotic and proinflammatory effects. Third, thrombomodulin-bound thrombin activates TAFI, which suppresses bradykinin activity and complement activation. In DIC, the expression of thrombomodulin is downregulated and activation of protein C is impaired.[14] Thus, histological analysis of skin biopsies from patients with meningococcal sepsis reveals decreased endothelial expression of thrombomodulin in vessels with and without thrombosis.

TFPI is another inhibitor of coagulation. TFPI, which can be either endothelial cell–associated or lipoprotein-bound in plasma, inhibits the TF factor VIIa complex by forming a quaternary complex in which factor Xa is the fourth component.

The importance of TFPI in sepsis is uncertain because the majority of patients with sepsis have normal levels of TFPI. The relevance of TFPI in DIC is illustrated by three lines of experimentation. First, depletion of TFPI sensitized rabbits to DIC induced by TF infusion. Second, TFPI infusion protected against the harmful effects of *E. coli* in primates. TFPI not only inhibited DIC in baboons challenged with lethal amounts of *E. coli* but prevented mortality and improved organ function. Third, TFPI infusion blocked the procoagulant effects of endotoxin in human volunteers.

Although normal levels and functions of coagulation inhibitors are important for protection against DIC, there is no evidence that patients with congenital deficiencies of coagulation inhibitors are at higher risk for DIC. Additional work is needed to examine the impact of these deficiencies on the interaction between coagulation and inflammation.

Fibrinolysis

In experimental models of DIC, there is an initial enhancement in fibrinolysis followed by marked impairment caused by the release of type 1 plasminogen activator inhibitor (PAI-1). The molecular mechanisms responsible for these findings include TNFα and IL-1 induced decrease in tPA release by endothelial cells and increase in PAI-1 production and TNFα-induced increase in urokinase-type plasminogen activator (uPA) production. Endotoxin and TNFα stimulate PAI-1 production in the liver, kidneys, lungs, and adrenal glands of mice. The net procoagulant state is illustrated by a late rise in fibrin breakdown fragments after *E. coli* challenge in baboons.

Experimental data also suggest that enhanced fibrinolysis clears fibrin from organs. Endotoxin-induced fibrin formation in the kidneys and adrenal glands occurs when PAI-1 levels increase. Furthermore, endotoxin does not induce fibrin formation in PAI-1 knockout mice, and fibrin deposition is increased in mice with functionally inactive thrombomodulin (TMProArg mutation). These experiments demonstrate that fibrinolytic action is required to reduce the extent of intravascular fibrin formation.

Fibrinolytic activity is regulated by PAI-1, the principal inhibitor of this system. Recent studies have shown that a functional mutation in the PAI-1 gene, the 4 G/5 G polymorphism, not only influences the plasma level of PAI-1 but was also linked to clinical outcomes in patients with meningococcal septicemia. Patients with the 4 G/4 G genotype had significantly higher plasma PAI-1 levels and an increased risk of death compared with those without this polymorphism. The PAI-1 polymorphism does not influence the risk of contracting meningitis, but likely increases the risk of fatal septic shock in patients with meningococcal infection. These studies provide the first evidence that genetically determined differences in fibrinolytic activity influence the risk of complications from gram-negative infection. In cohorts of patients with DIC, high levels of PAI-1 are one of the best predictors of mortality. These data suggest that DIC contributes to mortality in patients with sepsis. High levels of PAI-1 in such patients may contribute to mortality or may simply be a marker of severe inflammation because PAI-1 is an acute-phase protein.

CLINICAL MANIFESTATIONS

The clinical manifestation of DIC may vary depending on the underlying disorder. At one end of the spectrum is the acute severe DIC that often occurs in the setting of sepsis, major trauma, obstetric calamities, and severe immunologic responses. Diffuse multiorgan bleeding, hemorrhagic necrosis, microvascular thrombosis, and thrombi in medium and large blood vessels are common findings at autopsy. Occasionally, patients with unequivocal clinical and laboratory evidence of DIC do not have confirmatory postmortem findings. Conversely, some patients whose clinical and laboratory findings were not consistent with DIC have typical autopsy findings. The lack of correlation among clinical, laboratory, and pathologic findings may reflect partly postmortem changes in the blood (e.g., hyperfibrinolysis) in some cases but is mostly unexplained. Diffuse microthrombi are most frequently found in the lungs and kidneys, followed by the brain, heart, liver, spleen, adrenal glands, pancreas, and gut. Immunohistology and ultrastructural analysis indicate that most thrombi consist of fibrin and platelets. In addition, activated mononuclear cells and evidence of inflammation are frequently present. In cases of persistent DIC, organization and endothelialization of the microthrombi are often observed.

Acute tubular necrosis is more frequent than renal cortical necrosis in patients with DIC. Clinically, thrombotic occlusive events occur first as a result of microthrombi that obstruct the microcirculation of organs. These thrombi result from clots that form either in the circulation or *in situ* in arterioles, capillaries, or venules. Circulatory obstruction produces organ hypoperfusion and can lead to ischemia, infarction, and necrosis. The process is disseminated throughout the microcirculation; therefore, all organs are potentially vulnerable.

In contrast to acutely ill patients with severe DIC, some patients have mild or subclinical disease manifested only by laboratory abnormalities. Subacute or chronic DIC generally occurs in patients with malignancy, particularly those with mucin-producing adenocarcinomas or acute promyelocytic leukemia (see Chapters 60 and 63). Patients with acute promyelocytic leukemia frequently present with hemorrhage, whereas thrombotic manifestations predominate in those with adenocarcinoma. In addition, patients with solid tumors may develop nonbacterial endocarditis with subsequent systemic arterial embolism and infarction.

Another cause of subacute or chronic DIC is the retained dead fetus syndrome. These patients may be asymptomatic or may present with mild to moderate skin and mucous membrane bleeding.

It is important to stress that DIC is not a disease but is always secondary to an underlying disorder that causes the activation of coagulation. The underlying disorders most commonly associated with DIC are listed in Box 137.1 and are described in detail in the following section.

BOX 137.1 Clinical Conditions That Are Most Frequently Complicated by Disseminated Intravascular Coagulation

- Sepsis/severe infection
- Trauma/burn/heatstroke
- Malignancy
  - Solid tumors
  - Acute leukemia
- Obstetrical conditions
  - amniotic fluid embolism
  - Abruptio placentae
  - HELLP syndrome
- Vascular abnormalities
  - Kasabach-Merrit syndrome
  - Other vascular malformations
  - Aortic aneurysms
- Severe allergic/toxic reactions
- Severe immunologic reactions (e.g., transfusion reaction)

HELLP, hemolysis, elevated liver enzymes, and low platelet count.

Disseminated Intravascular Coagulation in Infectious Disease

Systemic infections are among the most common causes of DIC. Immunocompromised patients, asplenic patients whose ability to clear bacteria (particularly pneumococci) is impaired, and newborns whose anticoagulant systems are immature are particularly prone to infection-induced DIC. Infections may be superimposed on trauma or malignancy, which themselves are potential triggers of DIC. In addition, infections can aggravate bleeding and thrombosis by directly inducing thrombocytopenia, hepatic dysfunction, and shock, which can lead to diminished blood flow in the microcirculation.

Clinically overt DIC occurs in 30% to 50% of patients with gram-negative or gram-positive sepsis. Extreme examples of sepsis-related DIC are streptococcus A toxic shock syndrome and meningococcemia. Streptococcus A toxic shock syndrome is characterized by deep tissue infection, vascular collapse, vascular leakage, and multiple organ dysfunction. M protein released from streptococci forms complexes with fibrinogen which bind to β_2 integrins on neutrophils leading to their activation. Meningococcemia, a fulminant gram-negative infection, is characterized by extensive hemorrhagic necrosis, DIC, and shock. The most frequent gram-negative infections associated with DIC are caused by *Pseudomonas aeruginosa, E. coli*, and *Proteus vulgaris*. Patients with these infections may only have laboratory evidence of activated coagulation or they may present with severe DIC.

Activation of the coagulation system has also been documented with nonbacterial pathogens such as viruses, protozoa (malaria), and fungi. Common viral infections, such as influenza, varicella, rubella, and rubeola, are rarely associated with DIC. Some viral infections can cause "hemorrhagic fever" characterized by fever, hypotension, bleeding, and renal failure. Laboratory evidence of DIC can accompany Korean, rift valley, and dengue-related hemorrhagic fever. Protozoan infections, such as cerebral malaria, may be associated with overt DIC. In these cases, secondary deficiency of a disintegrin-like metalloprotease with thrombospondin type 1 repeats (ADAMTS-13), the von Willebrand cleaving protease, may occur.[15] Such deficiency may also complicate other types of sepsis-induced DIC and may enhance platelet-vessel wall interactions, thereby contributing to microvascular thrombosis.

An extreme form of DIC manifests as purpura fulminans, a severe, often lethal form of DIC in which extensive areas of the skin over the extremities and buttocks undergo hemorrhagic necrosis. The disease predominantly affects infants and children and is rare in adults. Diffuse microthrombi in small blood vessels leads to necrosis, and vasculitis may also be found in biopsies of skin lesions. The disorder can occur 2 to 4 weeks after mild infection, such as scarlet fever, varicella, or rubella, or can occur during an acute viral or bacterial infection in patients with acquired or hereditary deficiencies of protein C or protein S (see Chapters 120 and 137). The syndrome mimics neonatal homozygous protein C or protein S deficiency; where purpura fulminans, with or without extensive thrombosis, develops soon after birth.

Disseminated Intravascular Coagulation With Trauma, Brain Injury, Burns, and Heatstroke

The time interval between trauma and medical intervention correlates with the development and extent of DIC. Experience during battle reveals that fast evacuation and prompt medical care reduce the risk of DIC. Extensive exposure of TF to the blood and hemorrhagic shock are the likely triggers of DIC in patients with trauma. However, cytokines may also contribute because cytokine levels in trauma patients are similar to those in patients with sepsis.[16] Plasma levels of TNFα, IL-1β, PAI-1, TF, neutrophil elastase, and soluble thrombomodulin can be elevated in trauma patients with evidence of DIC and predictive of major organ dysfunction and death. Laboratory evidence of severe DIC, reduced fibrinolytic activity, and perhaps low AT levels are predictive of poor outcomes in such patients.

In patients with head injuries, DIC is associated with a high rate of mortality. A DIC score is predictive of prognosis in such patients and can add information beyond the Glasgow coma score. Brain injury likely triggers DIC because of the abundant TF in the brain. Specimens of contused brain tissue obtained during surgery in patients with head injury and autopsy specimens of liver, lungs, kidneys, and pancreas reveal microthrombi in arterioles and venules.

Bleeding, laboratory tests indicative of DIC, and microthrombi in biopsy specimens from undamaged skin have been described in patients with extensive burns. Studies using labeled fibrinogen or platelets indicate local consumption in burned areas as well as systemic consumption. TF at sites of burned tissue, systemic inflammatory response syndrome induced by the burn, and superimposed infections can all trigger DIC. Local activation of coagulation in the bronchoalveolar compartment may contribute to acute lung injury in these patients.

Severe hemorrhage and multiple organ failure, indicative of DIC, may accompany heatstroke.[17] Diffuse fibrin deposition and hemorrhagic infarction are found in fatal human cases. DIC associated with profound fibrin(ogen)olysis is evident in patients with heatstroke. The possible triggers of DIC in patients with heatstroke include endothelial cell damage and TF release from heat-damaged tissues. In a series of 18 critically ill patients with heatstroke during the 2003 heatwave in Western Europe,[17] high levels of IL-6 and IL-8 were noted along with activation of white blood cells, as evidenced by upregulation of β2-integrin and increased production of reactive oxygen species. All patients had evidence of systemic activation of coagulation and DIC was present in about one-third. The extent of activation of inflammation and coagulation was correlated with the severity of the heatstroke.

Disseminated Intravascular Coagulation With Obstetric Complications

Placental abruption occurs in 0.2% to 0.4% of pregnancies and is a leading cause of perinatal death.[18] Older multiparous women and those with hypertensive disorders of pregnancy are thought to be at highest risk. About 10% of women with placental abruption develop DIC. Amniotic fluid activates coagulation in vitro, and the degree of placental separation correlates with the extent of DIC, suggesting that leakage of thromboplastin-like material from the placental system is responsible for DIC. Only severe DIC results in shock and fetal death.

Amniotic fluid embolism is a rare but serious complication of pregnancy and delivery. A maternal mortality rate of 86% was reported in a 1979 review of 272 cases, but in a more recent population-based study, the maternal mortality was only 26%. Patients predisposed to amniotic fluid embolism are multiparous women whose pregnancies are postmature with large fetuses and women undergoing tumultuous labor after pharmacologic or surgical induction.

Amniotic fluid is introduced into the maternal circulation through tears in the chorioamniotic membrane, rupture of the uterus, and injury of uterine veins. TF in amniotic fluid is the likely trigger of DIC. Mechanical obstruction of pulmonary blood vessels by fetal debris, meconium, and other particulate matter in the amniotic fluid enhances local fibrin–platelet thrombus formation and fibrinolysis. Occlusion of the pulmonary arteries and an acute anaphylactoid response reminiscent of severe systemic inflammatory response syndrome induce the sudden onset of dyspnea, cyanosis, acute cor pulmonale, left ventricular dysfunction, shock, and convulsions. These symptoms are followed within minutes to several hours by severe bleeding in one-third of patients. Diffuse bleeding occurs from the atonic uterus, puncture sites, gastrointestinal tract, and other organs.

Some studies report activation of coagulation and DIC in women with preeclampsia and eclampsia. In a large series of patients, there was a correlation between the clinical severity of preeclampsia and eclampsia and the platelet count and level of fibrin(ogen) degradation products. Also consistent with DIC were results of assays of thrombin generation and activation of fibrinolysis, such as (thrombin-antithrombin) TAT complexes, D-dimer, and levels of Bβ1–42. Despite these observations, heparin is of uncertain benefit. HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count) is another complication of pregnancy-induced hypertension. Liver biopsy evidence of fibrin deposition in hepatic blood vessels and laboratory tests are consistent with DIC, but HELLP syndrome is thought to be caused by thrombotic microangiopathy.

Disseminated Intravascular Coagulation With Malignancy

Patients with solid tumors are vulnerable to risk factors and additional triggers of DIC that can aggravate thromboembolism and bleeding.[19] Risk factors include older age, advanced cancer, and use of chemotherapy or antiestrogen therapy. Triggers include septicemia, immobilization, and impaired liver function because of metastatic disease. Microangiopathic hemolytic anemia frequently is induced by DIC in patients with cancer and is particularly severe in patients with widespread mucin-secreting adenocarcinomas. Solid tumor cells can express a variety of procoagulant molecules including TF and cancer procoagulant, a cysteine protease with factor X activating properties. In breast cancer, TF is expressed by vascular endothelial cells as well as tumor cells. TF also appears to be involved in tumor metastasis and angiogenesis.

Cancer procoagulant is an endopeptidase found in extracts of neoplastic cells and in the plasma of patients with solid tumors. The role of this enzyme in the pathogenesis of cancer-related DIC is unclear. Interactions of P- and L-selectins with mucin from mucinous adenocarcinomas can induce the formation of platelet microthrombi and provide a third mechanism for cancer-related thrombosis. Depending on the rate and quantity of exposure or influx of TF-bearing vesicles, DIC can be overt or covert.

There are numerous reports of DIC and hyperfibrinolysis in patients with acute leukemia. In 161 consecutive patients with acute myeloid leukemia, DIC was diagnosed in 52. In patients with acute lymphoblastic leukemia, DIC was diagnosed in 15% to 20% of patients.[20] In patients with acute promyelocytic leukemia (APL), DIC is found in more than 90% of patients at the time of diagnosis or with remission induction therapy. APL cells express TF and cancer procoagulant, which can initiate coagulation. In addition, they release IL-Iβ and TNFα, compromising the protein C anticoagulant pathway by downregulating thrombomodulin expression. APL cells also express large amounts of annexin II, augmenting the conversion of plasminogen to plasmin. The net result of these processes is DIC and hyperfibrinolysis that can lead to fatal bleeding. All-*trans*-retinoic acid, which is used for induction and maintenance therapy of APL, inhibits the deleterious effect of APL cells and reduces the risk of DIC and bleeding.

Disseminated Intravascular Coagulation With Vascular Disorders

Rarely, vascular anomalies can trigger DIC. Localized consumption of platelets and fibrinogen in large aortic aneurysms can lead to coagulation abnormalities and bleeding.[21] In a series of patients with aortic aneurysms, 40% had elevated levels of fibrin(ogen) degradation products, but only 4% had laboratory evidence of DIC or bleeding.[21] Factors that predispose such patients to DIC include large aneurysms, particularly those with dissection or expansion. Coagulation in these cases is likely triggered by the abundant TF in atherosclerotic plaques.

Kasabach and Merritt were the first to describe bleeding in association with giant cavernous hemangiomas, benign tumors found in newborns or children that can evolve into convoluted masses of abnormal vascular channels that sequester and consume platelets and fibrinogen. Localized pain may occur because of thrombosis within the hemangiomas and the consumptive process may lead to chronic DIC and bleeding after trauma or with surgery. Cavernous hemangiomas may regress spontaneously; some respond to radiation or laser therapy.

Disseminated Intravascular Coagulation With Liver Disease

The levels of most coagulation factors, endogenous anticoagulants, and components of the fibrinolytic system are reduced in patients with severe liver disease reflecting reduced synthesis. In addition, the capacity of the liver to clear factors activated clotting factors and t-PA is decreased. Thrombocytopenia is common because of hypersplenism and decreased hepatic production of thrombopoietin. The similarities between the hemostatic defects with liver disease and those with DIC have evoked controversy as to the contribution of DIC to the coagulopathy of liver disease. Several laboratory and clinical observations support the concept that DIC accompanies hepatic disorders. These include reduced half-life of radiolabeled fibrinogen that is reversed with heparin administration, failure of replacement therapy to significantly increase the levels of hemostatic factors suggesting ongoing consumption, and increased levels of markers of activation of coagulation. All these findings are consistent with increased thrombin generation. Against the DIC hypothesis is the observation that microthrombi are found in only 2% of the tissues from patients who die of liver disease, and the fact that the increase in fibrinogen turnover can be explained by extravascular accumulation. Current thinking is that DIC is rare in patients with liver disease but that such patients are sensitive to the triggers of DIC because of the decreased synthetic capacity of the diseased liver and its inability to clear activated clotting factors. In patients undergoing peritoneovenous shunting for severe ascites, those with underlying liver disease are more likely to develop DIC than those with normal liver function.

Disseminated Intravascular Coagulation With Toxic Reactions or Snake Bites

The venom of certain snakes, particularly vipers and rattlesnakes, can produce a DIC-like coagulopathy.[22] Prominent among these species are *Vipera, Echis* (*E. carinatus* or *E. coloratus*), *Aspis, Crotalus, Bothrops*, and *Agkistrodon*. Venoms of these snakes contain enzymes or peptides that: (1) release fibrinopeptide A (*Agkistrodon rhodostoma*); (2) activate prothrombin even in the absence of calcium (*E. carinatus*); (3) activate factors X and V (Russell viper venom); (4) degrade fibrinogen (*Agkistrodon acutus*); (5) induce platelet aggregation; (6) inhibit platelet aggregation because of the presence of arginine-glycine-aspartic acid-containing peptides; (7) activate protein C; and (8) damage endothelial cells, which leads to bleeding, tissue ischemia, and edema. Interestingly, victims of snake bites rarely have excessive bleeding or thrombosis despite the abnormal coagulation tests and DIC-like picture.

LABORATORY MANIFESTATIONS

Thrombocytopenia or a rapidly declining platelet count is a diagnostic hallmark of DIC. However, only 35% to 44% of critically ill patients develop thrombocytopenia (platelet count $<150 \times 10^9/L$). Consequently, the specificity of thrombocytopenia for the diagnosis of DIC is limited.[10] A platelet count less than $100 \times 10^9/L$ is seen in 50% to 60% of DIC patients, whereas 10% to 15% of patients have a platelet count less than $50 \times 10^9/L$. In surgical or trauma patients with DIC, over 80% have platelet counts less than $100 \times 10^9/L$.

Consumption of coagulation factors in DIC leads to reduced levels of coagulation factors. Impaired coagulation factor synthesis due to impaired liver function or vitamin K deficiency and loss of coagulation proteins as a result of massive bleeding may also contribute. Although the accuracy of one-stage clotting assays for the diagnosis of DIC has been challenged (because of the presence of activated coagulation factors), there is good correlation between the levels of coagulation factors and the severity of the DIC. A low level of coagulation factors is reflected by prolonged coagulation screening tests, such as the PT or aPTT. A prolonged PT or aPTT is found in 14% to 28% of intensive care patients and in more than 95% of patients with DIC.

Plasma levels of factor VIII are paradoxically increased in most patients with DIC, probably due to the massive release of von Willebrand factor from the endothelium in combination with the acute-phase behavior of factor VIII. Recent studies have pointed to a relative decrease in the von Willebrand cleaving protease ADAMTS-13, thereby causing high concentrations of ultra-large von Willebrand multimers in plasma, which may facilitate platelet-vessel wall interactions and lead to thrombotic microangiopathy and subsequent organ dysfunction.[15]

Although measurement of fibrinogen levels has been advocated as a useful tool for the diagnosis of DIC, the levels are not helpful in many cases.[23] Fibrinogen is an acute-phase protein, and plasma levels can remain within the normal range despite ongoing consumption. In a consecutive series of patients with DIC, a low fibrinogen level had a sensitivity of only 28% for the diagnosis of DIC, and only patients with severe DIC had low levels.

Markers of Fibrin Generation and Degradation

Plasma levels of fibrin degradation products (FDP) are frequently used for the diagnosis of DIC. Increased levels of FDP were found in 42% of consecutive patients in the intensive care unit, in 80% of trauma patients, and in 99% of patients with sepsis and DIC. FDP can be quantified by rapid turnaround ELISA or latex agglutination assays. None of the available FDP assays discriminates between degradation products of fibrinogen and crosslinked fibrin, which may cause spuriously high results. The specificity of high levels of FDP for DIC is therefore limited and elevated FDP levels may be found in many other conditions including trauma, recent surgery, inflammation, and venous thromboembolism. Because FDP are metabolized in the liver and cleared via the kidneys, FDP levels are influenced by liver and kidney function.

The D-dimer assay better differentiates between degradation of crosslinked fibrin and fibrinogen than the FDP assay because D-dimer is exclusively derived from crosslinked fibrin (see Chapter 126). Fibrin is crosslinked by factor XIII that is activated by thrombin (see Chapters 120 and 124). Therefore, D-dimer is a marker of both fibrin formation and degradation.

Although D-dimer levels are high in patients with DIC, they are also elevated in patients with venous thromboembolism, recent surgery, or inflammatory conditions. Theoretically, measurement of soluble fibrin or fibrin monomers could be helpful to diagnose intravascular fibrin formation in DIC. Indeed, initial clinical studies suggest that an increase in the concentration of soluble fibrin above a defined threshold is diagnostic of DIC. Unfortunately, the problem so far is that a reliable test is not routinely available for quantitating soluble fibrin in plasma.

Endogenous Coagulation Inhibitors

Plasma levels of physiological coagulation inhibitors such as protein C and antithrombin may be useful indicators of ongoing activation of coagulation.[24] Low levels of these coagulation inhibitors are found in 40% to 60% of critically ill patients and in 90% of those with DIC.

Levels of protein C correlate with the severity of the DIC, and low levels are a predictor of poor outcome. In patients with meningococcal septicemia, protein C levels are markedly reduced, which may explain why such patients are prone to purpura fulminans. The decrease in protein C levels in such patients reflects reduced protein C activation because of downregulation of thrombomodulin and reduction in the levels of free protein S as well as protein C consumption.

Antithrombin levels also are a predictor of outcome in patients with sepsis and DIC. Antithrombin levels are reduced in such patients because of impaired synthesis (negative acute-phase response), increased consumption, and degradation by neutrophil elastase.

Fibrinolytic Markers

Fibrinolytic activity in DIC can be monitored by measuring plasma levels of plasminogen and α2-antiplasmin. Low levels may indicate consumption of these proteins. Plasmin generation is best assessed by measuring the levels of plasmin-α2-antiplasmin (PAP) complexes, which are moderately elevated in patients with DIC. However, because the concentration of α2-antiplasmin is half of that of plasminogen, plasmin can be generated in concentrations that overwhelm its major inhibitor. Under these circumstances, backup inhibitors such as α2-macroglobulin, α1-antitrypsin, and C1-inhibitor take over. The apparent insufficient fibrinolytic activity in patients with DIC is attributed to high levels of the inhibitor of plasminogen activation, PAI-1. Indeed, plasma levels of PAI-1 are often elevated in patients with DIC and are strongly correlated with an unfavorable outcome. A functional mutation in the *PAI1* gene, the 4 G/5 G polymorphism, is associated with increased levels of PAI-1 and a worse outcome in patients with meningococcal septicemia and associated DIC.

Point of Care Tests

Thromboelastography (TEG) can be done at the bedside and is popular in acute care settings.[25] The theoretical advantage of TEG over conventional coagulation assays is that it also provides an index of platelet function and fibrinolytic activity. TEG is increasingly employed in critically ill patients with hypercoagulable states, including DIC.[25] Procoagulant and anticoagulant states identified by TEG in patients with DIC are correlated with organ dysfunction and survival,[26,27] although it remains uncertain whether TEG offers advantages over the usual coagulation assays.[28] In a systematic review that included two randomized controlled trials and 16 observational studies in patients with sepsis, TEG was helpful for correctly identifying an endogenous fibrinolytic state.[29] The authors also found a correlation between hypocoagulability and mortality. The use of TEG for the diagnosis of DIC has not been rigorously evaluated although supporters believe that the assay may help appraise the state of coagulation in patients with critical illness.[30,31] However, TEG appears overly sensitive to interventions such as the administration of fibrinogen, and the therapeutic benefit of which is uncertain.

Diagnostic Algorithm for Disseminated Intravascular Coagulation

A simple scoring system for the diagnosis of DIC has been developed by the subcommittee on DIC of the International Society on Thrombosis and Haemostasis (ISTH) (Box 137.2).[5] The score can be calculated using routinely available laboratory tests (i.e., platelet count, PT), a fibrin-related marker (usually D-dimer), and fibrinogen. Tentatively, a score of 5 or more is compatible with DIC, whereas

BOX 137.2 Diagnostic Algorithm for the Diagnosis of Overt Disseminated Intravascular Coagulation[a]

1. Presence of an underlying disorder known to be associated with DIC (see Table 130.2)
   (no = 0, yes = 2) ☐
2. Score global coagulation test results
   - platelet count *(>100 = 0; <100 = 1; <50 = 2)*
   - level of fibrin markers (e.g., D-dimer, fibrin degradation products) ☐
   - (no increase: 0; moderate increase: 2; strong increase: 3)[b] ☐
   - prolonged prothrombin time
   - *(<3s. = 0; >3s. but <6s. = 1; >6s. = 2)* ☐
   - fibrinogen level
     (>1.0g/L = 0; <1.0g/L = 1) ☐
3. Calculate score
4. If ≥5: compatible with overt DIC; repeat scoring daily
   If <5: suggestive (not affirmative) for non-overt DIC; repeat next 1–2 days; ☐

[a] According to the Scientific Standardization Committee of the International Society of Thrombosis and Haemostasis.[5]

[b] Strong increase >5 × upper limit of normal; moderate increase is > upper limit of normal but <5 × upper limit ©of normal.

a score of less than 5 may be indicative but is *not* affirmative for non-overt DIC. More refined scoring systems have been developed for the diagnosis of non-overt DIC and are now being evaluated. A recent study showed that the INR can be used in place of PT prolongation, further facilitating international exchange and standardization. By using receiver-operating characteristic curves, the optimal cut-off for D-dimer was identified, thereby optimizing the sensitivity and the negative predictive value of the score. Prospective studies show that the DIC score has a sensitivity of 93% and a specificity of 98%. Studies in a series of patients with specific underlying disorders causing DIC (e.g., cancer or obstetric complications) show similar results. The severity of DIC according to this scoring system is related to mortality in patients with sepsis.[9] Linking prognostic determinants from critical care measurement scores such as APACHE-II to DIC scores is an important means of assessing prognosis in critically ill patients. Similar scoring systems have been developed and evaluated in Japan. The major difference between the international and Japanese scoring systems is a slightly higher sensitivity of the Japanese algorithm, which may reflect differences in the patient population because the Japanese series include more patients with hematological malignancies.

DIFFERENTIAL DIAGNOSIS

The diagnosis of DIC is complicated because patients with DIC may have other causes for coagulation abnormalities. For example, septic patients with DIC may have liver failure and vitamin K deficiency.

The differential diagnosis of thrombocytopenia in patients with suspected DIC is shown in Table 137.1. Sepsis itself is a risk factor for thrombocytopenia in critically ill patients, and the severity of sepsis correlates with the extent of thrombocytopenia. The principal factors that contribute to thrombocytopenia in patients with sepsis are impaired platelet production, increased consumption or destruction, or sequestration of platelets in the spleen or along the endothelial surface. Impaired production of platelets in the bone marrow may seem contradictory in view of the high levels of platelet production-stimulating proinflammatory cytokines, such as TNFα and IL-6, and the high concentrations of thrombopoietin in patients with sepsis. However, hemophagocytosis, phagocytosis of megakaryocytes and other hematopoietic cells by monocytes and macrophages, is common in severe sepsis and may be a result of high levels of macrophage colony-stimulating factor (M-CSF).

TABLE 137.1 Differential Diagnosis of Thrombocytopenia in Suspected Disseminated Intravascular Coagulation

Differential Diagnosis	Additional Diagnostic Clues
DIC	Prolonged aPTT and PT, increased FDP or D-dimer, low levels of antithrombin or protein C
Sepsis without DIC	Positive (blood) cultures, positive sepsis criteria, hemophagocytosis observed on bone marrow examination
Massive blood loss	Severe bleeding, low hemoglobin, prolonged aPTT, and PT,
Thrombotic microangiopathy	Schistocytes observed on blood smear, Coombs negative hemolysis, fever, neurologic symptoms, renal insufficiency, coagulation tests usually normal, ADAMTS-13 levels decreased
Heparin-induced thrombocytopenia	History of heparin use, venous or arterial thrombosis, positive HIT test (usually immunoassay for heparin-platelet factor 4 antibodies), increase in platelet count after cessation of heparin; coagulation tests usually normal
Immune thrombocytopenia	Antiplatelet antibodies, normal or increased number of megakaryocytes in bone marrow aspirate, thrombopoietin *(TPO)* level decreased; coagulation tests usually normal
Drug-induced thrombocytopenia	Decreased number of megakaryocytes in bone marrow aspirate or detection of drug-induced antiplatelet antibodies, increase in platelet count after cessation of the drug; coagulation tests usually normal

aPTT, Activated partial thromboplastin time; *FDP*, fibrin degradation products; *HIT*, heparin-induced thrombocytopenia; *PT*, prothrombin time.

Heparin-induced thrombocytopenia (HIT) is another cause of a low platelet count and thrombosis in critically ill patients (see Chapter 130). In a consecutive series of critically ill patients receiving heparin, HIT occurred in 1%. The risk of HIT is higher with unfractionated than with low-molecular-weight heparin (LMWH). Thrombosis may occur in 25% to 50% of patients with HIT (with fatal thrombosis in 4% to 5%). The diagnosis of HIT is based on the detection of HIT antibodies in combination with the occurrence of thrombocytopenia in a patient receiving heparin, with or without concomitant arterial or venous thrombosis.

Thrombocytopenia is a hallmark of thrombotic microangiopathies include syndromes such as thrombotic thrombocytopenic purpura, hemolytic-uremic syndrome, malignant hypertension, chemotherapy-induced microangiopathic hemolytic anemia, and the HELLP syndrome (see Chapter 131). A common feature of these disorders is endothelial damage, triggering platelet adhesion and aggregation, thrombin generation, and impaired fibrinolysis. The consequences of severe endothelial dysfunction include thrombocytopenia, mechanical fragmentation of red cells with hemolytic anemia, and thrombotic occlusion of the microvasculature of organs such as the kidney and brain leading to renal failure and neurologic dysfunction, respectively.

Drug-induced thrombocytopenia is another frequent cause of thrombocytopenia in critically ill patients (see Chapter 129). Thrombocytopenia may be caused by drug-induced myelosuppression, such as caused by cytostatic agents, or by immune-mediated mechanisms. The diagnosis of drug-induced thrombocytopenia is complicated in patients suspected of having DIC because they often are taking multiple medications and have numerous potential causes for thrombocytopenia. Drug-induced thrombocytopenia is often diagnosed based upon the timing of initiation of a new agent in relation to the development of thrombocytopenia, after exclusion of other causes of thrombocytopenia. Rapid normalization of the platelet count after discontinuation of the suspected agent is highly suggestive of drug-induced thrombocytopenia.

Prolongation of global coagulation tests may reflect a deficiency of one or more coagulation factors (Table 137.2). In addition, but more rarely, it may reflect the presence of an inhibiting autoantibody

TABLE 137.2 Differential Diagnosis of Prolonged Activated Partial Thromboplastin Time and/or Prothrombin Time in Suspected Disseminated Intravascular Coagulation

Test Result	Cause
PT prolonged, aPTT normal	Factor VII deficiency Mild vitamin K deficiency Mild liver insufficiency Low doses of vitamin K antagonists
PT normal, aPTT prolonged	Factor VIII, IX, or XI deficiency Unfractionated heparin Inhibitory antibody and/or antiphospholipid antibody Factor XII or prekallikrein deficiency
PT and aPTT prolonged	Factor X, V, II or fibrinogen deficiency Severe vitamin K deficiency Vitamin K antagonists Global clotting factor deficiency • Decreased synthesis: liver failure • Increased loss: massive bleeding, DIC

aPTT, Activated partial thromboplastin time; *PT*, prothrombin time.

(see Chapter 134). Factor deficiency can be distinguished from an inhibitory by mixing patient plasma with control plasma. If the addition of control plasma corrects the abnormal test, coagulation factor deficiency is likely, whereas if there is no correction, an inhibiting antibody is likely to be present (see Chapter 126). The lupus anticoagulant can prolong the aPTT, and antiphospholipid syndrome may be associated with thrombocytopenia and thrombosis (see Chapter 138).

In general, acquired deficiencies of coagulation factors may be due to impaired synthesis, massive loss, or increased turnover (consumption). Impaired synthesis is often due to liver disease or vitamin K deficiency. Vitamin K deficiency may be caused by poor nutrition in combination with the use of antibiotics that affect intestinal flora. The PT is sensitive to both conditions because this test is dependent on plasma levels of factor VII (the vitamin K-dependent coagulation factor with the shortest half-life). Liver failure may be differentiated from vitamin K deficiency by measuring the level of factor V, which is not vitamin K dependent. In fact, factor V levels are incorporated into scoring systems for acute liver failure.

Uncompensated loss of coagulation factors may occur after massive bleeding, for example, trauma patients or patients undergoing major surgical procedures. This is particularly common when crystalloid, colloid, and red cells replacement is given without concomitant administration of plasma and cryoprecipitate. Depletion types of coagulopathy may exacerbate the bleeding and massive transfusion may trigger systemic activation of inflammatory pathways, thereby exacerbating the coagulopathy. In hypothermic patients (e.g., trauma patients), global coagulation tests may underestimate the extent of derangement in coagulation because the assays are performed at 37°C.

THERAPY

Management of DIC depends on the treatment of the underlying disorder that triggered the DIC. For sepsis-induced DIC, treatment includes aggressive use of intravenous organism-directed antibiotics and source control (e.g., by surgery or drainage). Other examples of treatment of the underlying condition include cancer surgery or chemotherapy, uterus evacuation in patients with abruptio placentae, resection of an aortic aneurysm, and debridement of crushed tissue in cases of trauma. In addition to treating the underlying cause of DIC, supportive treatment aimed at coagulopathy may be helpful (Box 137.3).[6]

BOX 137.3 Mainstays of Supportive Treatment of Disseminated Intravascular Coagulation

Modality	Details	Expectations/Rationale
Treating the underlying disorder	Dependent on the primary diagnosis	Inhibit or block the trigger of disseminated intravascular coagulation (DIC)
Antithrombotic agents	Prophylactic heparin to prevent venous thromboembolism (Low dose) therapeutic heparin in case of confirmed thromboembolism or if the clinical picture is dominated by (micro) vascular thrombosis and associated organ failure	Risk of venous thromboembolism is increased in critically ill patients, trauma patients, or patients with cancer Prevent fibrin formation; tip the balance within the microcirculation toward anticoagulant mechanisms and physiologic fibrinolysis; allow reperfusion of the skin, kidneys, and brain
Transfusion	Infuse platelets, plasma, and fibrinogen (cryoprecipitate) if there is overt bleeding or a high risk of bleeding	Bleeding should be controlled over the course of hours Platelet count, coagulation tests, and fibrinogen should return toward normal
Anticoagulant factor concentrates	Recombinant human activated protein C may be effective in sepsis and DIC (24 μg/kg/h for 4 days)	Restore anticoagulation in the microvascular environment and may have anti-inflammatory activity
Fibrinolytic inhibitors	Tranexamic acid (e.g., 500–1000 mg—q8–12 h or ε-aminocaproic acid 1000–2000 mg q8–12 h)	May be useful if there is hyperfibrinolysis; Bleeding ceases, but there is a risk of microvascular thrombosis and renal failure

Platelet and Plasma Transfusion

Low levels of platelets and coagulation factors increase the risk of bleeding. However, plasma or platelet substitution therapy should not be given on the basis of laboratory results alone; such therapy is only indicated if there is bleeding or if an invasive procedure is needed.[6] The presumed efficacy of treatment with plasma, fibrinogen concentrate, cryoprecipitate, or platelets is not based on randomized controlled trials but is rational therapy in patients with or at risk of bleeding. The concept that the administration of blood components "adds fuel to the fire" has never been proven.

Replacement therapy for thrombocytopenia consists of 5 to 10 units of platelet concentrate to increase the platelet count to 20 to 30 × 10^9/L, or to 50 × 10^9/L in patients requiring invasive procedures. Major problems with the administration of fresh-frozen plasma in patients with DIC who are bleeding are volume overload and capillary leak, which can lead to pulmonary edema, predispose patients to adult respiratory distress syndrome (ARDS), and induce ascites. Coagulation factor concentrates, such as prothrombin complex concentrate, may overcome the problem of volume overload but do not contain essential factors, such as factor V. Moreover, caution is advocated with the use of prothrombin complex concentrates in DIC because traces of activated factors may trigger thrombosis. Specific deficiencies of coagulation factors, such as fibrinogen, can be corrected by the administration of purified coagulation factor concentrates.

Cryoprecipitate can be used to rapidly raise the levels of fibrinogen and von Willebrand factor when DIC is accompanied by bleeding and the fibrinogen level is less than 1 g/L. Cryoprecipitate has at least four to five times more fibrinogen per mL than fresh-frozen plasma. Nonetheless, fresh-frozen plasma contains fibrinogen in sufficient amounts for treatment of mild to moderate hypofibrinogenemia.

Anticoagulant Treatment

Heparin therapy in patients with DIC remains controversial. Experimental studies suggest that heparin can at least partially inhibit the activation of coagulation in DIC. However, a beneficial effect of heparin in patients with DIC has not been demonstrated in controlled clinical trials. Also, the safety of heparin is debatable in DIC patients who are prone to bleeding. A large trial in patients with severe sepsis showed a non-significant benefit of low dose heparin on 28-day mortality and no major safety concerns.[32]

There is consensus that heparin is beneficial in some categories of DIC, such as metastatic carcinomas, purpura fulminans, and aortic aneurysm (prior to resection). Heparin also is indicated for treating thromboembolic complications in large vessels and before surgery in patients with chronic DIC. Heparin administration may be helpful in patients with acute DIC when intensive blood component replacement fails to control bleeding or when thrombosis threatens to cause irreversible tissue injury (e.g., acute cortical necrosis of the kidney or digital gangrene).

Heparin should be used cautiously. In patients with chronic DIC because of metastatic carcinoma or aortic aneurysm, infusion of unfractionated heparin at a dose of 500 to 750 U/hour without a bolus injection has been advocated. If no response is obtained within 24 hours, escalating dosages can be used. In hyperacute DIC cases, such as amniotic fluid embolism, septic abortion, and purpura fulminans, an intravenous heparin bolus of 5000 to 10,000 U heparin may be given along with replacement therapy. Some experts, however, do not administer a bolus dose of heparin even under these circumstances. Continuous heparin infusion of 500 to 1000 U/hour may be necessary to maintain the benefit until the underlying disease responds to treatment. Apart from these considerations, current guidelines dictate the universal use of prophylactic doses of heparin or low-molecular-weight heparin to prevent venous thromboembolic events in critically ill patients.[6]

Theoretically, the best anticoagulant for the treatment of DIC would be directed against TF. To this end, agents such as recombinant TFPI, inactivated factor VIIa, and recombinant nematode anticoagulant protein c2 (NAPc2) have been evaluated. Phase II trials of recombinant TFPI in patients with sepsis showed promising results, but phase III trials in patients with severe sepsis or severe pneumonia and organ failure did not show an overall survival benefit with TFPI.[33]

Recombinant human soluble thrombomodulin binds thrombin to form a complex that activates protein C (see Chapters 120 and 124). In a phase III randomized double-blind clinical trial in patients with DIC, soluble thrombomodulin had a better effect on bleeding and coagulation parameters than heparin. A systematic review and meta-analysis of mostly retrospective studies of soluble thrombomodulin in severe sepsis demonstrated a pooled relative risk of 0.81 (95% confidence interval [CI] 0.62 to 1.06) in three trials that included a total of 838 patients, and a relative risk of 0.59 (95% CI 0.45 to 0.77) in nine observational studies that included 571 patients.[34]

In a double-blind, placebo-controlled, phase III study, 816 patients with severe sepsis-associated coagulopathy were randomized to soluble thrombomodulin or placebo.[35] The absolute risk reduction in 28-day mortality with thrombomodulin was 26.8%, which was 2.55% lower (95% CI, −3.68 to 8.77) than the mortality with placebo. The benefit of thrombomodulin was greatest in patients with high levels of thrombin generation or D-dimer; a finding that needs prospective validation.

Physiological Anticoagulant Factor Concentrates

Restoration of the levels of physiological anticoagulants in DIC may be a rational approach.[36,37] Based on successful preclinical studies, the use of AT concentrate\s and heparin in patients with DIC was evaluated in patients with sepsis, septic shock, or both. The trials have shown some benefit in terms of improvement of laboratory parameters, shortening of the duration of DIC, or improvement in organ function. In several small clinical trials, high dose AT produced a modest, but not significant. reduction in mortality. A large randomized controlled trial also showed no significant reduction in mortality with AT concentrate in patients with sepsis,[38] although a post-hoc subgroup analysis suggested some benefit in patients who did not receive concomitant heparin. In a small randomized trial in patients with burns and DIC, AT administration decreased mortality, reduced multiple organ failure, and improved coagulation parameters compared with placebo.

Because abnormalities of the protein C system contribute to the pathogenesis of DIC, therapy with APC was predicted to be beneficial.[39] A dose-ranging study suggested that an APC dose of 24 μg/kg per hour was optimal, as evidenced by a decrease in D-dimer levels. A subsequent phase III trial comparing APC with placebo in patients with sepsis was stopped early because all-cause mortality at 28 days was reduced from 31% to 25% with APC. Patients with "overt DIC" (see Diagnosis) benefited more from APC than those without overt DIC[9] with relative risk reductions in mortality of 38% and 18%, respectively. Despite these findings, meta-analyses of published literature concluded that the evidence supporting the use of APC, even in patients with severe disease, was not strong. A subsequent series of negative trials in specific populations of patients with severe sepsis and the fact that APC administration increases the risk of bleeding led to the withdrawal of APC from the market.[40]

Fibrinolytic Inhibitors

Most guidelines recommend against the use of anti-fibrinolytic agents, such as ε-aminocaproic acid or tranexamic acid, in patients with DIC. This is because these drugs block already suppressed endogenous fibrinolysis and may further compromise tissue perfusion. In support of this concept, there are reports of severe thrombosis in DIC patients treated with these agents. However, in patients with DIC accompanied by primary hyperfibrinolysis, as can occur with acute promyelocytic leukemia, giant cavernous hemangioma, heatstroke, and metastatic carcinoma of the prostate, the use of fibrinolytic inhibitors can be considered if there is serious bleeding that does not respond to replacement therapy and evidence of hyperfibrinolysis. In these situations, it is important to replace depleted blood components before initiating treatment with fibrinolytic inhibitors.

REFERENCES

1. Levi M, Scully M. How I treat disseminated intravascular coagulation. *Blood*. 2018;131:845–854.
2. Levi M. Disseminated intravascular coagulation. In: Kaushansky K, Lichtman M, Prchal J, Levi M, Press O, Burns L, eds. *Williams Hematology*. 9th ed Philadelphia: McGraw Hill; 2016.
3. Levi M, ten Cate H. Disseminated intravascular coagulation. *N Engl J Med*. 1999;341:586–592.
4. Levi M. Disseminated intravascular coagulation. *Crit Care Med*. 2007;35:2191–2195.
5. Taylor Jr FB, Toh CH, Hoots WK, et al. Towards definition, clinical and laboratory criteria, and a scoring system for disseminated intravascular coagulation. *Thromb Haemost*. 2001;86:1327–1330.
6. Levi M, Toh CH, Thachil J, Watson HG. Guidelines for the diagnosis and management of disseminated intravascular coagulation. *Br J Haematol*. 2009;145:24–33.
7. Levi M, van Der PT. Inflammation and coagulation. *Crit Care Med*. 2010;38:S26–S34.
8. Fourrier F, Chopin C, Goudemand J, et al. Septic shock, multiple organ failure, and disseminated intravascular coagulation. Compared

patterns of antithrombin III, protein C, and protein S deficiencies. *Chest*. 1992;101:816–823.
9. Dhainaut JF, Yan SB, Joyce DE, et al. Treatment effects of drotrecogin alfa (activated) in patients with severe sepsis with or without overt disseminated intravascular coagulation. *J Thromb Haemost*. 2004;2:1924–1933.
10. Levi M, Opal SM. Coagulation abnormalities in critically ill patients. *Crit Care*. 2006;10:222.
11. Colman RW, Schmaier AH. Contact system: a vascular biology modulator with anticoagulant, profibrinolytic, antiadhesive, and proinflammatory attributes. *Blood*. 1997;90:3819–3843.
12. Esmon CT. The regulation of natural anticoagulant pathways. *Science*. 1987;235:1348–1352.
13. Gando S, Levi M, Toh CH. Disseminated intravascular coagulation. *Nat Rev Dis*. 2016;2:16037.
14. Faust SN, Levin M, Harrison OB, et al. Dysfunction of endothelial protein C activation in severe meningococcal sepsis. *N Engl J Med*. 2001;345:408–416.
15. Levi M, Scully M, Singer M. The role of ADAMTS-13 in the coagulopathy of sepsis. *J Thromb Haemost*. 2018;16:646–651.
16. Gando S, Nakanishi Y, Tedo I. Cytokines and plasminogen activator inhibitor-1 in posttrauma disseminated intravascular coagulation: relationship to multiple organ dysfunction syndrome. *Crit Care Med*. 1995;23:1835–1842.
17. Huisse MG, Pease S, Hurtado-Nedelec M, et al. Leucocyte activation: the link between inflammation and coagulation during heatstroke. A study of patients during the 2003 heat wave in Paris. *Crit Care Med*. 2008;36:2288–2295.
18. Levi M. Disseminated intravascular coagulation (DIC) in pregnancy and the peri-partum period. *Thromb Res*. 2009;123(Suppl 2). S63–S64.
19. Levi M. Disseminated intravascular coagulation in cancer: an update. *Semin Thromb Hemost*. 2019;45:342–347.
20. Barbui T, Falanga A. Disseminated intravascular coagulation in acute leukemia. *Semin Thromb Hemost*. 2001;27:593–604.
21. Fisher Jr. DF, Yawn DH, Crawford ES. Preoperative disseminated intravascular coagulation associated with aortic aneurysms. A prospective study of 76 cases. *Arch Surg*. 1983;118:1252–1255.
22. Isbister GK. Snake bite doesn't cause disseminated intravascular coagulation: coagulopathy and thrombotic microangiopathy in snake envenoming. *Semin Thromb Hemost*. 2010:444–451.
23. Levi M, de Jonge E, Meijers J. The diagnosis of disseminated intravascular coagulation. *Blood Rev*. 2002;16:217–223.
24. Levi M, Sivapalaratnam S. Disseminated intravascular coagulation: an update on pathogenesis and diagnosis. *Exp Rev Hematol*. 2018;11: 663–672.
25. Dempfle CE, Borggrefe M. Point of care coagulation tests in critically ill patients. *Semin Thromb Hemost*. 2008;34:445–450.
26. Johansson PI, Stensballe J, Vindelov N, et al. Hypocoagulability, as evaluated by thrombelastography, at admission to the ICU is associated with increased 30-day mortality. *Blood Coagul Fibrinolysis*. 2010;21:168–714.
27. Park MS, Martini WZ, Dubick MA, et al. Thromboelastography as a better indicator of hypercoagulable state after injury than prothrombin time or activated partial thromboplastin time. *J Trauma*. 2009;67:266–275.
28. Collins PW, Macchiavello LI, Lewis SJ, et al. Global tests of haemostasis in critically ill patients with severe sepsis syndrome compared to controls. *Br J Haematol*. 2006;135:220–227.
29. Muller MC, Meijers JC, Vroom MB, Juffermans NP. Utility of thromboelastography and/or thromboelastometry in adults with sepsis: a systematic review. *Crit Care*. 2014;18:R30.
30. Brenner T, Schmidt K, Delang M, et al. Viscoelastic and aggregometric point-of-care testing in patients with septic shock—cross-links between inflammation and haemostasis. *Acta Anaesthesiol Scand*. 2012;56:1277–1290.
31. Daudel F, Kessler U, Folly H, et al. Thromboelastometry for the assessment of coagulation abnormalities in early and established adult sepsis: a prospective cohort study. *Crit Care*. 2009;13:R42.
32. Levi M, Levy M, Williams MD, et al. Prophylactic heparin in patients with severe sepsis treated with drotrecogin alfa (activated). *Am J Respir Crit Care Med*. 2007;176:483–490.
33. Abraham E, Reinhart K, Opal S, et al. Efficacy and safety of tifacogin (recombinant tissue factor pathway inhibitor) in severe sepsis: a randomized controlled trial. *JAMA*. 2003;290:238–247.
34. Yamakawa K, Aihara M, Ogura H, et al. Recombinant human soluble thrombomodulin in severe sepsis: a systematic review and meta-analysis. *J Thromb Haemost*. 2015;13(4):508–519.
35. Vincent JL, Francois B, Zabolotskikh I, et al. Effect of a recombinant human soluble thrombomodulin on mortality in patients with sepsis-associated coagulopathy: the SCARLET randomized clinical trial. *JAMA*. 2019;321:1993–2002.
36. Levi M, de Jonge E, van der Poll T. Rationale for restoration of physiological anticoagulant pathways in patients with sepsis and disseminated intravascular coagulation. *Crit Care Med*. 2001;29:S90–S94.
37. de Jonge E, van der Poll T, Kesecioglu J, Levi M. Anticoagulant factor concentrates in disseminated intravascular coagulation: rationale for use and clinical experience. *Semin Thromb Hemost*. 2001;27:667–674.
38. Warren BL, Eid A, Singer P, et al. Caring for the critically ill patient. High-dose antithrombin III in severe sepsis: a randomized controlled trial. *JAMA*. 2001;286:1869–1878.
39. Levi M. Activated protein C in sepsis: a critical review. *Curr Opin Hematol*. 2008;15:481–486.
40. Ranieri VM, Thompson BT, Barie PS, et al. Drotrecogin alfa (activated) in adults with septic shock. *N Engl J Med*. 2012;366:2055–2064.

CHAPTER 138

HYPERCOAGULABLE STATES

Julia A. M. Anderson and Jeffrey I. Weitz

Arterial and venous thromboses are common problems for all clinicians. Some patients with thrombosis have an underlying hypercoagulable state. These hypercoagulable states can be divided into three categories: inherited disorders, acquired disorders, and those that are mixed in origin.

Inherited hypercoagulable states, also known as thrombophilic disorders, can be due to loss of function of natural anticoagulant pathways or gain of function in procoagulant pathways (Table 138.1). Acquired hypercoagulable states represent a heterogeneous group of disorders in which the risk for thrombosis appears to be higher than that in the general population. These include such diverse risk factors as a prior history of thrombosis, obesity, pregnancy, cancer and its treatment, antiphospholipid antibody syndrome, drug-induced thrombosis such as heparin-induced thrombocytopenia or thrombosis associated with chemotherapeutic agents, or myeloproliferative disorders. The pathogenesis of thrombosis in these situations is likely multifactorial in origin. Mixed disorders are those with both inherited and acquired components, for example, hyperhomocysteinemia. Although severe hyperhomocysteinemia and associated homocystinuria are rare genetic disorders, most cases of mild to moderate hyperhomocysteinemia result from acquired folate and/or vitamin B_{12} deficiency superimposed on common genetic mutations in biochemical pathways involved in methionine metabolism.

Genetic hypercoagulable states and acquired risk factors combine to establish an intrinsic risk for thrombosis for each individual. This risk can be modified by extrinsic or environmental factors, such as surgery, immobilization, or hormonal therapy, which also increase the risk for thrombosis. When the intrinsic and extrinsic forces exceed a critical threshold, thrombosis occurs (Fig. 138.1). Appropriate thromboprophylaxis can prevent the thrombotic risk from exceeding this critical threshold, but breakthrough thrombosis can still occur if procoagulant stimuli overwhelm protective mechanisms.

This chapter describes the inherited, acquired, and mixed hypercoagulable states, details their laboratory evaluation, and provides practical advice for the management of these conditions.

INHERITED HYPERCOAGULABLE STATES

Inherited disorders are found in up to half of patients who present with venous thromboembolism before the age of 40, particularly those whose event occurred either in the absence of well-recognized risk factors, such as surgery or immobilization, or with minimal provoking factors, such as minor trauma, long-distance flight, or estrogens. Patients with inherited thrombophilic disorders often have a family history of thrombosis. Of greatest significance is a family history of sudden death due to pulmonary embolism or a history of multiple family members requiring long-term anticoagulation therapy because of recurrent thrombosis. Patients who present with venous thrombosis in unusual sites, such as the cerebral or mesenteric veins, those with recurrent thrombosis, and patients who develop skin necrosis upon initiation of warfarin therapy should also be suspected of having an inherited hypercoagulable state.

From a pathophysiologic perspective, inherited hypercoagulable states fall into two categories. First are those associated with loss of function of endogenous anticoagulant proteins. These include deficiencies of antithrombin, protein C, and protein S. The second category involves gain of function in procoagulant pathways. These disorders include factor V Leiden and the FIIG20210A mutation, as well as increased levels of procoagulant proteins, such as factors VIII, IX, and XI. Each of these conditions will be briefly described.

Loss of Function of Endogenous Anticoagulants

Antithrombin Deficiency

Antithrombin, a single-chain glycoprotein with a molecular weight of 52,000 Da, is a member of the serine proteinase inhibitor (serpin) superfamily. Antithrombin is synthesized in the liver and endothelial cells and plays a critical part in regulating coagulation by forming a 1:1 covalent complex with thrombin, factor Xa, and other activated clotting factors. Once covalent complexes are generated, they are cleared from the circulation via the liver. The rate of antithrombin interaction with its target proteases is accelerated by heparin by 1000-fold. Heparan sulfate proteoglycan, which coats the vasculature, is the physiologic counterpart of medicinal heparin.

Newborn infants have approximately 50% of normal adult antithrombin levels, and much lower levels are found in preterm infants because of liver immaturity; adult levels are attained at 6 months.

Antithrombin deficiency can be inherited or acquired, and congenital deficiency of antithrombin was the first reported inherited risk factor for venous thromboembolism. Congenital antithrombin deficiency is relatively rare, occurring in about 1 in 2000, and can be one of two types (Table 138.2), both of which are inherited in an autosomal-dominant fashion and affect both sexes equally. Type I deficiency, which represents the classic deficiency state, is the result of reduced synthesis of biologically normal antithrombin. Heterozygotes with this condition have parallel reductions in antithrombin antigen and activity with levels reduced to about 50% of normal. A heterogeneous group of nonsense mutations, small deletions, insertions, or single-base substitutions are the molecular cause of most cases, although gene deletions can also be responsible. An antithrombin mutation database compiled by members of the Plasma Coagulation Inhibitors Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Hemostasis summarizes the mutations and can be accessed on the Imperial College London website (https://www.imperial.ac.uk/immunology-inflammation/research/haematology/haemostasis-and-thrombosis/database/#database) or in the human gene mutation database (www.hgmd.cf.ac.uk).

Type II antithrombin deficiencies are characterized by normal levels of antithrombin with impaired functional activity due to the presence of a variant protein. This condition is mainly caused by missense mutations that result in single amino acid substitutions. The clinical consequences of type II antithrombin deficiency depend on the location of the mutation, which may involve the reactive center loop or the heparin-binding domain. For example, some mutations in the reactive center loop of antithrombin slow its interaction with target proteases and are characterized by reduced antithrombin activity in the absence or presence of heparin. In contrast, mutations in the heparin-binding domain are associated with reduced antithrombin activity in the presence of heparin but normal activity in its absence. Unlike other inherited forms of antithrombin deficiency, which are embryonic lethal in the homozygous state, mutations in the

TABLE 138.1 Classification of Hypercoagulable States

Hereditary	Mixed	Acquired
Loss of Function		
Antithrombin deficiency	Hyperhomocysteinemia	Previous venous thromboembolism
Protein C deficiency	Obesity	Pregnancy, puerperium
Protein S deficiency	Cancer	Drug-induced: Heparin-induced thrombocytopenia Prothrombin complex concentrates L-asparaginase Hormonal therapy
Gain of Function		
Factor V Leiden	Postoperative	
Prothrombin FII G20210A	Myeloproliferative disorders	
Elevated factor VIII, IX, or XI		

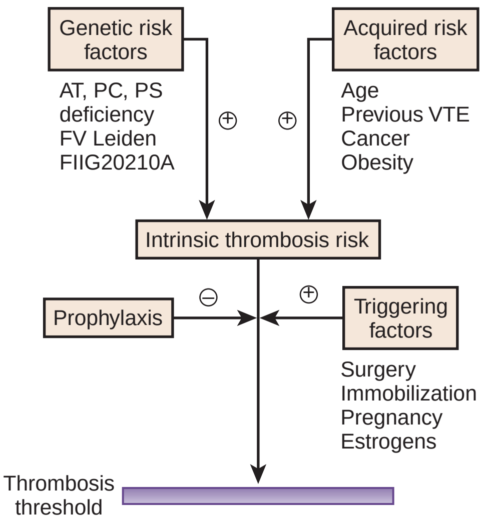


Figure 138.1 THROMBOSIS THRESHOLD. Genetic and acquired risk factors continue to determine an intrinsic risk for thrombosis for each individual. This risk is increased by extrinsic or environmental factors and decreased by thromboprophylaxis. If the intrinsic and extrinsic forces exceed a critical threshold at which thrombin generation overwhelms the protective mechanisms, thrombosis will result. *AT*, Antithrombin; *PC*, protein C; *PS*, protein S; *VTE*, venous thromboembolism. *(From Anderson JA, Weitz JI. Hypercoagulability and uncommon vascular diseases. In: Jaff MR, White CJ, eds. Vascular Disease: Diagnostic and Therapeutic Approaches. Minneapolis, MN: Cardiotext Publishing; 2011.)*

TABLE 138.2 Types of Inherited Antithrombin Deficiency

Type	Antigen	Activity (No Heparin)	Activity (With Heparin)
I	Low	Low	Low
II (active site defect)	Normal	Low	Low
II (heparin-binding site defect)	Normal	Normal	Low

heparin-binding domain only have clinical consequences in individuals homozygous for these mutations and do not increase the risk for thrombosis in the heterozygous state.

Because of the wide variety of heritable forms of antithrombin deficiency, functional antithrombin assays are the preferred method for screening. Most functional assays use synthetic substrates to monitor the rates at which added thrombin or factor Xa are inhibited in patient plasma. However, the assays differ in terms of whether bovine or human thrombin is used and whether heparin is added. Defects in the heparin-binding domain of antithrombin will be detected only in the presence of heparin. When antithrombin deficiency is identified with functional assays, immunologic assays are performed to distinguish between type I and type II deficiency.

Congenital antithrombin deficiency can be associated with spontaneous venous thromboembolism, but thrombosis often occurs in the setting of pregnancy and the puerperium; with the use of estrogen-containing oral contraceptives; or after major trauma or surgery. Thrombotic events are rare in children, with events typically occurring from mid to late teenage years and into the early twenties. The European Prospective Cohort on Thrombophilia (EPCOT) study compared the risks of a first episode of venous thromboembolism in asymptomatic individuals with antithrombin, protein C, or protein S deficiency with that in subjects with factor V Leiden over a 6-year period. The annual incidence of venous thromboembolism was highest in those with antithrombin deficiency.[1] Likewise, in a cohort of Italian patients, the risk of venous thrombosis was higher with antithrombin deficiency than with other thrombophilic defects.[2] The most common sites for venous thrombosis in patients wit antithrombin deficiency are the deep veins of the leg, but thrombosis can occur in mesenteric veins, as well as the renal and retinal veins. The risk for recurrence is high,[3] particularly in those with lower antithrombin levels,[4] and the risk varies depending on the subtype of antithrombin deficiency.

Acquired antithrombin deficiency can reflect decreased antithrombin synthesis, increased consumption, or enhanced clearance (Table 138.3). Decreased synthesis can occur in patients with severe hepatic disease, particularly cirrhosis, or in those given L-asparaginase, the latter because of drug-induced retention of antithrombin within the endoplasmic reticulum. Increased thrombin generation can result in antithrombin consumption. Disorders associated with excessive thrombin generation include acute thrombosis, disseminated intravascular coagulation (DIC), severe sepsis, polytrauma, disseminated malignancy, extensive burns, or prolonged extracorporeal circulation. Heparin treatment can reduce antithrombin levels up to 20% by enhancing its clearance. Severe antithrombin deficiency can also occur in some patients with nephrotic syndrome because of the loss of protein in the urine.

Users of oral contraceptive pills or hormone replacement therapy may have moderate reductions in antithrombin levels; during pregnancy, the antithrombin levels do not fall significantly but decreases are found in preeclampsia and pregnancy-induced hypertensive illnesses.

TABLE 138.3 Causes of Acquired Antithrombin Deficiency

Decreased Synthesis	Increased Consumption	Enhanced Clearance
Hepatic cirrhosis	Major surgery	Heparin
Severe liver disease	Acute thrombosis	Nephrotic syndrome
L-asparaginase	Disseminated intravascular coagulation Severe sepsis Multiple trauma Malignancy Prolonged extracorporeal circulation	

Protein C Deficiency

The protein C pathway is an important natural anticoagulant pathway. This on-demand pathway is activated when thrombin is generated (Fig. 138.2). Thrombin binds to thrombomodulin, a transmembrane thrombin receptor found on the surface of endothelial cells. Once bound to thrombomodulin, the substrate specificity of thrombin is altered so that it no longer serves as a procoagulant enzyme, but becomes a potent activator of protein C, a vitamin K–dependent glycoprotein. Thus, thrombin bound to thrombomodulin activates protein C 1000-fold more efficiently than free thrombin. The endothelial cell protein C receptor (EPCR), another transmembrane receptor on the endothelial cell surface, binds protein C and presents it to the thrombin-thrombomodulin complex for activation, thereby producing an additional 20-fold increase in the rate of protein C activation. The physiologic importance of thrombomodulin and EPCR is highlighted by the fact that deficiency of either receptor results in embryonic lethality in mice.

Activated protein C (APC) dissociates from this activation complex and serves as an anticoagulant by proteolytically degrading and inactivating factors Va and VIIIa, thereby attenuating thrombin generation. For efficient inactivation of factors Va and VIIIa, APC must bind to protein S, its cofactor. This interaction facilitates APC binding to activated cell surfaces, particularly the platelet surface, where factors Va and VIIIa are localized.

APC has a half-life in the circulation of about 15 minutes, whereas thrombin has a half-life of about 10 seconds. APC is inhibited by protein C inhibitor and α_1-proteinase inhibitor (α_1-antitrypsin), both of which are relatively slow inhibitors. Only the activity of protein C inhibitor is enhanced by heparin, but both inhibitors appear to contribute to APC inhibition in vivo.

Protein C deficiency can be inherited or acquired. Like antithrombin deficiency, protein C deficiency is inherited in an autosomal-dominant fashion and has a proven association with venous thrombosis.[5] Based on studies in healthy blood donors, heterozygous protein C deficiency can be found in 1 in 200 to 500 of the adult population, but many of these individuals do not have a history of thrombosis. Thus, the phenotypic expression of hereditary protein C deficiency is highly variable and may depend on other, as yet unrecognized, modifying factors. In contrast to antithrombin deficiency in which the homozygous state is embryonic lethal, homozygous or doubly heterozygous protein C deficiency can occur. The prevalence of homozygous protein C deficiency is estimated to be 1 in 160,000 to 360,000 births. Newborns with these disorders may present with purpura fulminans characterized by widespread thrombosis.

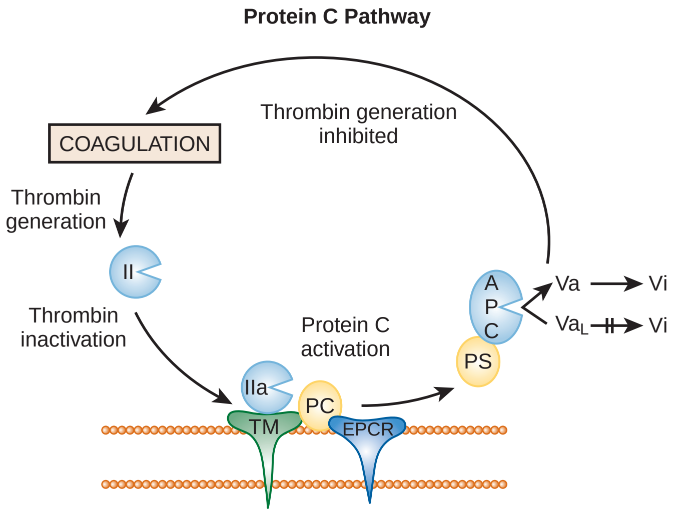


Figure 138.2 PROTEIN C PATHWAY. Activation of coagulation triggers thrombin (IIa) generation. Excess thrombin binds to thrombomodulin *(TM)* on the endothelial cell surface. Once bound, the substrate specificity of thrombin is altered so that it no longer acts as a procoagulant but becomes a potent activator of protein C *(PC)*. Endothelial protein C receptor *(EPCR)* binds PC and presents it to thrombomodulin-bound thrombin, where it is activated. Activated protein C *(APC)*, together with its cofactor, protein S *(PS)*, binds to the activated platelet surface and proteolytically degrades factor Va *(Va)* into inactive fragments *(Vi)*. Because factor Va is a critical component of the prothrombinase complex, factor Va inactivation by APC attenuates thrombin generation. Because factor Va Leiden *(FVa$_L$)* is resistant to inactivation by APC, patients with the factor V Leiden mutation have reduced capacity to regulate thrombin generation. *(From Anderson JA, Weitz JI. Hypercoagulability and uncommon vascular diseases. In: Jaff MR, White CJ, eds. Vascular Disease: Diagnostic and Therapeutic Approaches. Minneapolis, MN: Cardiotext Publishing; 2011.)*

Individuals with heterozygous protein C deficiency can develop skin necrosis upon initiation of treatment with vitamin K antagonists, such as warfarin. Typically, skin lesions are found on the extremities, breasts, or trunk. Starting as erythematous macules, the central regions of the cutaneous lesions become purpuric and then necrotic over a period of hours unless protein C is administered. Biopsies reveal fibrin thrombi within the vessels of the skin associated with interstitial hemorrhage. The skin lesions are clinically and histologically similar to those seen in infants with purpura fulminans and are attributable to the transient hypercoagulable state that is induced by warfarin, thereby explaining why they occur early during the course of warfarin therapy. The half-life of protein C is short and similar to that of factor VII. Starting warfarin in patients with protein C deficiency causes a further reduction in protein C levels, particularly if loading doses of warfarin are given. Thus, the activity of the natural anticoagulant protein C pathway is compromised before warfarin lowers the levels of vitamin K–dependent procoagulant proteins, particularly prothrombin and factor X, into the range required for its antithrombotic effects. Warfarin-induced skin necrosis has also been reported in association with acquired deficiency of protein C.

Hereditary protein C deficiency can be further delineated into two subtypes using immunologic and functional assays (Table 138.4). Most functional assays use Protac, a protease isolated from the venom of the copperhead snake, to activate protein C in plasma. The enzymatic activity of APC can then be determined directly using an APC-directed synthetic substrate, or it can be indirectly quantified by measuring the extent of prolongation of the activated partial thromboplastin time (aPTT). The most common form of hereditary protein C deficiency is the classic or type I deficiency state. This disorder reflects the reduced synthesis of a normal protein and is characterized by a parallel reduction in protein C antigen and activity, resulting in a quantitative deficiency due to reduced synthesis or stability of protein C. A variety of genetic defects can produce type I protein C deficiency, including promoter mutations, splice-site abnormalities, in-frame deletions, frameshift deletions, in-frame insertions, and frameshift insertions in the protein C gene *(PROC)*, but missense or nonsense mutations are the most common.

Type II protein C deficiency reflects synthesis of a dysfunctional protein and is characterized by normal protein C antigen with reduced functional activity, a qualitative deficiency. Most type II protein C deficiency states are caused by point mutations. Mutations in the active site of APC reduce its activity against synthetic substrates and decrease its capacity to prolong the aPTT. In contrast, mutations that affect other protein C domains essential for its activity may reduce its anticoagulant activity but may not affect its capacity to cleave synthetic substrates activity. Therefore, coagulation-based functional assays are preferred when screening patients for protein C deficiency.

Diagnosis of protein C deficiency is complicated. Protein C circulates in human plasma at an average concentration of 4 μg/mL.

TABLE 138.4 **Types of Inherited Protein C Deficiency**

Type	Antigen	Activity
I	Low	Low
II	Normal	Low

Plasma protein C antigen levels are widely distributed in healthy adults such that 95% of the values range from 70% to 140%. Furthermore, protein C levels increase with age, particularly in postmenopausal women.[6] The wide range of values renders the establishment of a normal range difficult. Levels less than 55%, however, are likely to reflect deficiency, whereas those between 55% and 70% are considered borderline and may be consistent with a deficiency state or the lower end of the normal distribution. Acquired causes of protein C deficiency must be excluded, and to document the presence of protein C deficiency, it is necessary to repeat the testing. Family studies may also be helpful to highlight the autosomal-dominant pattern of inheritance.

Acquired protein C deficiency can be due to decreased synthesis or increased consumption. Decreased synthesis can occur with liver disease or with warfarin therapy. Warfarin decreases functional activity more than immunologic activity. Newborns have protein C levels 20% to 40% lower than those of adults, and premature infants have even lower levels. Protein C consumption can occur with severe sepsis, with DIC, and after surgery. Reduced protein C levels have also been reported in cancer patients receiving cyclophosphamide, methotrexate, 5-fluorouracil, or L-asparaginase. A particularly severe form of acquired protein C deficiency has been described in association with meningococcal septicemia. In contrast to antithrombin, which is excreted in the urine of patients with nephrotic syndrome, the levels of protein C are normal or elevated in patients with nephrotic syndrome.

Protein S Deficiency

Protein S serves as a cofactor for APC and enhances its capacity to inactivate factors Va and VIIIa. In addition, protein S may have direct anticoagulant activity by inhibiting prothrombin activation through its capacity to bind anionic phospholipid, factor Va, or factor Xa, components of the prothrombinase complex. The importance of the direct anticoagulant activity of protein S is uncertain.

In the circulation, about 60% of total protein S is bound to C4b-binding protein, an acute phase complement component. Because only 40% of the protein S that is free is functionally active, only patients with low free protein S levels are prone to venous thrombosis. Therefore, the diagnosis of protein S deficiency requires measurement of both free and bound forms of protein S. Total protein S levels can be measured immunologically under conditions that dissociate protein S from C4b-binding protein. The free fraction can then be quantified with a monoclonal antibody that only recognizes free protein S, while the functional activity of protein

S can be measured using an APC cofactor assay. This assay depends on the prolongation of the aPTT when diluted patient plasma is added to protein S–depleted plasma containing APC and factor Va.

Protein S deficiency can be inherited or acquired. Heterozygous protein S deficiency is inherited in an autosomal-dominant manner; the prevalence varies between 1% and 7% among patients with thrombotic events. There is an association with unprovoked venous thromboembolism.[7] Based on measurements of total and free protein S antigen and protein S activity, three subtypes of inherited protein S deficiency have been identified (Table 138.5). Type I or classic deficiency results from the decreased synthesis of a normal protein and is characterized by reduced levels of total and free protein S antigen together with reduced protein S functional activity. Molecular analysis of protein S deficiency is complicated because there are two homologous protein S genes, one of which is likely a pseudogene. Nonetheless, most cases of type I protein S deficiency are caused by partial gene deletions, missense mutations, base pair insertions or deletions, premature stop codons, or mutations affecting a splice site in the gene encoding protein S *(PROS1)*. Type II protein S deficiency is characterized by normal levels of total and free protein S associated with reduced protein S activity. This type of deficiency is uncommon, and most of the causative mutations encode protein S domains involved in its interaction with APC.

TABLE 138.5 **Types of Inherited Protein S Deficiency**

Type	Total Protein S	Free Protein S	Protein S Activity
I	Low	Low	Low
II	Normal	Normal	Low
III	Normal	Low	Low

Type III protein S deficiency is characterized by normal levels of total protein S, but low levels of free protein S are associated with reduced protein S activity. The molecular basis of this type of deficiency appears to be similar to that of the type I deficiency states. In fact, type I and type III protein S deficiency are likely to be manifestations of the same disease because they often coexist in families. Thus, younger family members present with type I deficiency, whereas older family members have type III deficiency because protein S levels increase with age.

Acquired protein S deficiency can be due to decreased synthesis, increased consumption, loss, or shift of free protein S to the bound form. Decreased synthesis can occur in patients with severe liver disease, in those given L-asparaginase, and in patients given vitamin K antagonists. Increased consumption of protein S occurs in patients with acute thrombosis or in those with DIC. Patients with nephrotic syndrome can lose free protein S in their urine, causing decreased protein S activity. Total protein S levels in these patients are often normal because the levels of C4b-binding protein increase, shifting more protein S to the bound form. C4b-binding protein levels also increase in pregnancy and with the use of oral contraceptives. This shifts more protein S to the bound form and lowers the levels of free protein S and protein S activity. The pathophysiologic consequences of this phenomenon are uncertain. An association between antiphospholipid antibodies and acquired protein S deficiency has been reported in patients with severe forms of varicella zoster virus infection complicated by purpura fulminans. In healthy neonates, the total protein S antigen levels are 15% to 30% of normal, and the C4b-binding protein is significantly reduced to less than 20%, such that the free form of protein S predominates and the functional levels are only slightly reduced compared with normal adult levels.

Gain of Function Mutations

Gain of function mutations include factor V Leiden, FIIG20210A, elevated levels of procoagulant proteins, and other less well-characterized genetic disorders. Gain of function mutations are more prevalent in the general population than those associated with loss of function.

Factor V Leiden

In 1993, Dahlback and colleagues described three families with a history of venous thromboembolism. Affected family members exhibited limited prolongation of the aPTT when APC was added to their plasma. Accordingly, this phenotype was designated APC resistance (APCR). Bertina and colleagues demonstrated that APCR co-segregated with the factor V gene and was due to a single-base substitution, guanine to adenine at position 1691, that produced an Arg 506 Gln mutation at one of the APC cleavage sites on factor Va. This mutation, which is designated factor V Leiden, endows activated factor V Leiden with a 10-fold longer half-life in the presence of APC than its wild-type counterpart.

The factor V Leiden mutation is responsible for most cases of APCR. Other causes are mutations at Arg 306, another APC cleavage site. Arg 306 is replaced by a Gly residue in factor V Hong Kong and by a Thr residue in factor V Cambridge. Neither of these mutations is strongly associated with thrombosis.

The factor V Leiden mutation is inherited in an autosomal-dominant fashion. The prevalence of the mutation ranges from 2% to 5% in Whites but it is rare in Asians and Africans. This racial difference likely reflects a founder effect with the mutation arising 20,000 to 30,000 years ago, after the divergence of non-African from African and Caucasoid from Mongoloid subpopulations. The prevalence of factor V Leiden homozygosity is about 1 in 2500. The risk for thrombotic complications is lower with factor V Leiden than it is with deficiencies of antithrombin, protein C, or protein S;[8] and in the heterozygous state, factor V Leiden does not appear to be a strong risk factor for recurrent venous thrombosis.[9] The risk for thrombosis is higher in homozygotes than in heterozygotes. Acquired APC resistance may be caused by hormonal changes during pregnancy or by the administration of estrogens, such as oral contraceptive pills or hormone replacement therapy.

A diagnosis of APCR is established using a functional assay based on the ratio of the aPTT after APC addition divided by that determined before APC addition. Second-generation tests, which add dilute patient plasma to factor V-deficient plasma, are more specific for factor V Leiden. A normal functional test excludes factor V Leiden; a positive functional test for APCR should be confirmed with a genetic test for the factor V Leiden mutation. Some laboratories only use the genetic test for the diagnosis of factor V Leiden.

FII G20210A Mutation

After extensive screening of 28 families with unexplained venous thromboembolism, Poort and colleagues identified a heterozygous G to A nucleotide transition at position 20210 in the 3′-untranslated region of the prothrombin gene in five of the probands. This mutation, FII G20210A, results in elevated levels of prothrombin. Elevated levels of prothrombin, in turn, may increase the risk for thrombosis by enhancing thrombin generation or by inhibiting factor Va inactivation by APC.

The mechanism by which the FII G20210A mutation causes increased prothrombin levels appears to vary. Enhanced protein synthesis may result from more efficient 3′-end formation, increased messenger RNA stability, increased translation efficiency, or some combination of these mechanisms. An intronic FII gene polymorphism, A19911G, which influences splicing efficiency, may modulate the effect of the FII G20210A mutation such that heterozygous carriers of both mutations have a greater risk for thrombosis than those with only the FII G20210A mutation.

Like the factor V Leiden mutation, the prevalence of the FII G20210A mutation is higher in whites and low in Asians, American Indians, and African Americans. A founder effect likely explains the higher prevalence in whites. The mutation may have provided a survival advantage based on a protective effect with childbirth or severe sepsis.

FII G20210A is found in 1% to 6% of Whites. The mutation is more common in southern Europe than in northern Europe, a gradient opposite to that of factor V Leiden. Rare individuals homozygous for the FII G20210A mutation have been identified. In the Leiden Thrombophilia Study, 6.2% of venous thrombosis patients and 2.3% of healthy matched controls had the FII G20210A mutation.[10] The mutation independently confers a 2.8-fold increased risk for venous thrombosis, with no gender bias; a risk lower than that with antithrombin, protein C, or protein S deficiency. The abnormality confers a weaker increased risk of venous thrombosis than protein C, protein S, or antithrombin deficiencies.

Laboratory diagnosis of FIIG 20210 A depends on genetic screening after PCR amplification of the 3′-untranslated region of the FII gene. Although FII G20210A heterozygotes have 30% higher levels of prothrombin than non-carriers, the wide range of prothrombin levels in healthy individuals precludes the use of this phenotype to identify carriers.

Elevated Levels of Procoagulant Proteins

Elevated levels of factor VIII and other coagulation factors, including factors XI, IX, and VII, have been implicated as independent risk factors for thrombosis. Although the molecular bases for the high levels of these coagulation factors have yet to be identified, genetic mechanisms are likely responsible because the hereditability of these quantitative abnormalities is high.

Other Hereditary Disorders

The dysfibrinogenemias represent a heterogeneous group of disorders characterized by abnormal fibrinogen structure and are diagnosed by low functional and/or immunologic levels of fibrinogen, in association with prolonged thrombin and reptilase times. Acquired causes of dysfibrinogenemia, such as liver disease, must be excluded in the diagnostic work-up. Most congenital dysfibrinogenemias are asymptomatic and are often identified as incidental findings when coagulation testing is performed for other reasons. Up to 40% of the known dysfibrinogenemias are associated with a bleeding diathesis. Approximately 15 variant fibrinogens, which represent less than 10% of known dysfibrinogenemias, have been reported to be associated with thrombotic complications, including fibrinogen Marburg, Caracas V, Chapel Hill III, Hannover II, Nijmegen, New York I, Christchurch II and III, and Milano III. The exact mechanism by which these dysfibrinogenemias increase the risk for thrombosis depends on the nature of the fibrinogen defect. Most affect the C-terminal domain of the Aα chains or the thrombin cleavage site on the Bβ chains. Some biochemical defects have been further characterized, such as defects in the release of fibrinopeptides A or B by thrombin, impaired binding of thrombin or tissue plasminogen activator to fibrin, or resistance to lysis by plasmin. It is likely that acquired and/or other hereditary factors contribute to the thrombosis that occurs in patients with dysfibrinogenemia.

ACQUIRED HYPERCOAGULABLE STATES

Acquired hypercoagulable states include antiphospholipid syndrome and cancer, as well as pregnancy and estrogen therapy (oral contraception or hormone replacement therapy). These disorders can occur in isolation or can be superimposed on hereditary hypercoagulable states. Heparin-induced thrombocytopenia is an immune-mediated adverse drug reaction and is a strong, independent risk factor for arterial and venous thrombosis.

Lupus Anticoagulants and the Antiphospholipid Syndrome

First described in a study by Wasserman and colleagues in 1906 among patients with positive serologic tests for syphilis, antiphospholipid antibodies are a heterogeneous group of autoantibodies directed against proteins that bind phospholipid. Antibodies can be categorized into those that prolong phospholipid-dependent coagulation assays, known as the lupus anticoagulant (LA), or anticardiolipin antibodies (ACLs), which target cardiolipin. A subset of ACL recognizes other phospholipid-bound proteins, particularly β_2-glycoprotein I.

Patients who have thrombosis in association with an LA and/or ACL (antibodies of the immunoglobulin [Ig]G or IgM subclass directed against cardiolipin or β_2-glycoprotein 1) are diagnosed with antiphospholipid syndrome (APS). The criteria for diagnosis of APS were updated in 2006.[11] APS is considered primary when it occurs in isolation and secondary when it is associated with autoimmune disorders, such as systemic lupus erythematosus or other connective tissue diseases. Clinical manifestations of APS include one or more episodes of thrombosis, one or more unexplained fetal deaths at 10 or more weeks of gestation, or three or more first-trimester miscarriages (less than 10 weeks of gestation); placental thrombosis is hypothesized to be the cause of the pregnancy-related complications that characterize APS. Intrauterine growth retardation, preeclampsia, and eclampsia have also been associated with APS. APS can also occur with cancer, with some infections, and with drugs such as phenothiazines, phenytoin, hydralazine, or amoxicillin. Thrombosis in APS patients can be arterial, venous, or placental.

To make the diagnosis of APS, at least one clinical criterion and one laboratory criterion must be met.[12] Laboratory diagnosis of APS requires the presence of LA or ACL on tests taken 12 weeks apart. Tests for LA are well standardized, and an LA is associated with a higher risk of thrombosis than ACL.

The LA is detected using phospholipid-dependent clotting tests. Most screening assays are based on the aPTT. aPTT reagents differ in their sensitivity for detection of LA, and many laboratories have adopted less sensitive aPTT reagents for routine aPTT testing. LA is suspected when the aPTT is prolonged. To explore the cause of the prolonged aPTT, patient plasma is mixed with normal plasma, and LA is suspected if the aPTT remains prolonged. The diagnosis is confirmed by demonstrating that the addition of excess hexagonal-phase phospholipid normalizes the aPTT, thereby documenting the phospholipid dependence of the abnormal test result.[13] In addition to the aPTT, a battery of phospholipid-dependent clotting tests is often used for the diagnosis of LA. These include the dilute Russell viper venom time and kaolin clotting time.

ACL antibodies are detected using immunoassays. Only ACL of medium to high titer and of the IgG or IgM subclass are associated with thrombosis, and the risk is higher with antibodies against β_2 glycoprotein-1 than against cardiolipin. For ACL, the amount of IgG or IgM antibody binding to cardiolipin-coated platelets is expressed in standardized GPL or MPL units, with 1 unit representing the cardiolipin-binding capacity of 1 μg/mL affinity-purified antiphospholipid antibody from reference sera. The extent of antibody binding is influenced by both the titer of the antibody and its affinity for cardiolipin. The lack of standardization of ACL assays renders it difficult to compare results between laboratories.

ACL antibodies are found in 3% to 10% of healthy individuals. They also are common with certain infections (such as coronavirus-19 [COVID-19], mycobacterial pneumonia, malaria, or parasitic disorders) and after exposure to some medications. Often, these antibodies are of low titer and are transient. ACL antibodies are detected in about 30% to 50% of patients with systemic lupus erythematosus. Of these, 10% to 20% also have LA.

The mechanism by which antiphospholipid antibodies trigger thrombin generation is unclear. In cell cultures, these antibodies can directly activate endothelial cells and induce the expression of adhesion molecules that can tether tissue factor-bearing leukocytes or microparticles onto their surface. Antiphospholipid antibodies also have been shown to (a) activate platelets, (b) interfere with the protein C pathway, (c) inhibit antithrombin catalysis by vessel wall heparan sulfate, (d) impair fibrinolysis, and (e) enhance transmigration of oxidized low density lipoprotein (LDL) into the vessel wall, thereby promoting atherothrombosis. Whether these mechanisms are operative in vivo has yet to be established. In contrast to most hypercoagulable states, APS can be associated with spontaneous arterial thrombosis, as well as with venous thromboembolism. Arterial thrombosis can manifest as an ischemic stroke or transient ischemic attack. Thrombosis of the sagittal sinus or other cerebral veins, a form of venous thrombosis, is another cause of stroke in these patients.

Heparin-Induced Thrombocytopenia

A clinicopathologic syndrome, heparin-induced thrombocytopenia (HIT) is diagnosed based on clinical features[14] and laboratory detection of HIT antibodies (see Chapter 131). The risk for HIT is higher with unfractionated heparin than with low-molecular-weight heparin (LMWH) and almost never occurs with fondaparinux. HIT is more common in surgical patients than in medical patients and occurs more frequently in women.

Typical clinical features of HIT include thrombocytopenia and thrombosis (arterial or venous). Less common features include necrotic skin lesions at the site of subcutaneous heparin injection, acute systemic reactions to heparin, and rarely, DIC (see Chapter 137).[15] Thrombocytopenia is the most common finding, occurring in 90% of patients. Typically, the platelet count falls 5 to 10 days after heparin is started.[16] However, thrombocytopenia can occur earlier if the patient has been exposed to heparin in the past 3 months. Rarely, the onset of HIT is delayed and occurs several days after stopping heparin.

HIT is an autoimmune-like disorder and is caused by heparin-dependent, platelet-activating antibodies of the IgG subclass. These antibodies are directed against neoantigens that are exposed on platelet factor 4 (PF4) when it forms a complex with heparin. By binding to FcγII receptors on platelets, these antibodies trigger platelet activation. Activated platelets and platelet-derived microparticles provide an anionic phospholipid surface on which coagulation factors assemble and promote thrombin generation. This produces an aggressive hypercoagulable state and explains why 30% to 70% of HIT patients develop thrombosis.

The diagnosis of HIT is supported by assays that capitalize on the platelet-activating properties of HIT antibodies. Functional assays, such as the platelet serotonin release assay or heparin-induced platelet activation assay, detect antibody-induced platelet activation in the presence of heparin and are the gold standard for diagnosis of HIT. In contrast, although enzyme immunoassays for the detection of antibodies against PF4/heparin complexes are more sensitive than functional assays, they are less specific because only a small subset of these antibodies has the capacity to produce HIT. Consequently, a negative immunoassay is useful to exclude the diagnosis of HIT, but a positive test should be confirmed with a functional assay.

When the diagnosis of HIT is established, heparin must be stopped, and an alternative anticoagulant should be given. Options include direct thrombin inhibitors (such as argatroban or bivalirudin) or parenteral factor Xa inhibitors (such as fondaparinux or danaparoid) or oral factor Xa inhibitors (such as rivaroxaban or apixaban). Treatment with parenteral agents should be continued until the platelet count returns to baseline levels, at which point an oral factor Xa inhibitor or warfarin can be initiated.

Cancer and Its Treatment

About 25% of patients who present with venous thromboembolism have cancer. Cancer patients who develop venous thromboembolism have reduced survival compared with those without this complication. Patients with brain tumors, pancreatic cancer, and advanced ovarian, lung, gastrointestinal tract, or prostate cancer have particularly high rates of venous thromboembolism.[17] Treatment with chemotherapy, hormonal therapy, and biologic agents, such as erythroid stimulating agents and antiangiogenic drugs, and surgery further increases the risk for venous thromboembolism. Additional risk factors include indwelling central venous catheters and major abdominal, pelvic, or orthopedic surgery.

The pathogenesis of thrombosis in cancer patients is multifactorial in origin and represents a complex interplay among the tumor, patient characteristics, and the host hemostatic system. Tumors can initiate coagulation by expressing tissue factor or cysteine proteases on their surface or by shedding tissue factor-bearing microparticles. In addition to its role in coagulation, tissue factor also acts as a cell-signaling molecule that promotes tumor proliferation and spread.

Patient factors that contribute to venous thromboembolism include immobility and venous stasis secondary to extrinsic compression of major veins by tumor. Surgical procedures, indwelling central venous catheters, and chemotherapy can produce vessel wall injury. In addition, tamoxifen, selective estrogen receptor modulators (SERMs), L-asparaginase, and other drugs may induce an acquired hypercoagulable state by reducing the levels of natural anticoagulant proteins.

L-Asparaginase and combination chemotherapeutic regimens, such as breast cancer regimens of cyclophosphamide, methotrexate, and 5-fluorouracil increase the risk for thrombosis. The incidence of thromboembolic events in children receiving L-asparaginase for treatment of acute lymphocytic leukemia ranges from 1.1% to 36.7% depending on whether catheter-related events are included. The mechanism likely involves the decreased synthesis of antithrombin, protein C, and protein S, in addition to the retention of antithrombin within the endoplasmic reticulum.

Patients with multiple myeloma and other plasma cell dyscrasias are at increased risk for arterial and venous thrombosis. The reason for this is unclear but may include acquired activated protein C resistance, elevated levels of factor VIII and/or von Willebrand factor, and the influence of the paraprotein on blood viscosity and fibrinolysis. Patients treated with thalidomide or lenalidomide are at high risk for venous thromboembolism, particularly when these drugs are given in combination with dexamethasone. Current guidelines recommend low-dose aspirin or LMWH thromboprophylaxis for patients receiving these agents.[18,19] Rivaroxaban or apixaban may be an alternative to LMWH in these patients.

Although a proportion of patients who present with unprovoked venous thromboembolism have occult cancer, extensive screening for cancer is not recommended.[20] A careful history should be taken to identify any symptoms suggestive of underlying cancer. If such symptoms are present, further investigation is warranted. If there are no symptoms suggestive of underlying cancer, patients should be encouraged to undergo age-appropriate screening tests for breast, cervical, colon, or prostate cancer.

Myeloproliferative Disorders

The most common Philadelphia chromosome-negative (Ph-neg) myeloproliferative disorders, essential thrombocythemia (ET) and polycythemia vera (PV), are associated with an increased risk for thrombosis, especially arterial thrombosis, and venous thrombosis affecting the splanchnic vessels, including the hepatic and portal veins, which can lead to the Budd–Chiari syndrome, or the mesenteric veins. Thrombosis involving the microcirculation is common in patients with ET, and patients may complain of erythromelalgia, characterized by burning pain, redness and swelling of the fingers and toes, transient visual defects, or recurrent headache. Although ET and PV may evolve to myelofibrosis or transform into acute myeloid leukemia, fatal cardiovascular events are a leading cause of mortality. The reported cumulative risk for thrombosis ranges from 2.5% to 5.0% per patient-year in PV and from 1.9% to 3% per patient-year in ET, depending on the patient risk category. Age over 60 years and a previous history of thrombosis are risk factors for serious thrombosis, whereas usual cardiovascular risk factors, such as hypertension, diabetes, dyslipidemia, and smoking, may place patients at intermediate risk.

The pathogenesis of thrombosis in patients with myeloproliferative disorders is multifactorial in origin and includes leukocytosis, leukocyte activation, rheologic abnormalities due to raised red cell mass in PV, enhanced platelet activation, and a prothrombotic endothelial phenotype.

An acquired point mutation in the Janus kinase 2 gene (*JAK2* V617F) is found in up to 95% of patients with Ph-neg PV and in 50% to 60% of those with ET. The *JAK*2 gene encodes a cytoplasmic tyrosine kinase that is critical for signaling between type 1 cytokine receptors and intracellular proliferation mechanisms. Clinical observations, supported by laboratory studies, suggest an association between the *JAK*2 mutation and increased leukocyte and platelet activation, particularly in reticulated platelets, and there is a correlation between the burden of the mutant allele and thrombin generation. Acquired activated protein C resistance secondary to a reduction in free protein S levels has also been demonstrated in patients with ET and PV. Overall, there is little to support routine *JAK*2 screening unless splenomegaly or an elevated hemoglobin, white blood cell, and/or platelet count raises the possibility of an underlying myeloproliferative disorder. However, it may be justifiable to check for the *JAK*2 mutation in patients with unexplained splanchnic or mesenteric vein thrombosis, even in the absence of these findings.

Current management of PV and ET is aimed at the prevention of major cardiovascular events and is based on the patient's risk category. Low-risk patients with PV are managed with phlebotomy, whereas high-risk patients are given cytoreductive therapy. Low-dose aspirin (75 to 100 mg daily) is recommended for all PV patients regardless of risk category, and such therapy is highly effective for the treatment of the microcirculatory disturbances in patients with ET.

Paroxysmal Nocturnal Hemoglobinuria

A rare but serious disorder, paroxysmal nocturnal hemoglobinuria (PNH) is associated with intravascular hemolysis and cytopenia. PNH is caused by the clonal expansion of a hematopoietic stem cell that has a somatic mutation in the X-linked *PIG-A* gene, which encodes cell surface proteins that serve as phosphatidylinositol anchors (see Chapter 32). Patients with PNH may have life-threatening thrombosis that can be difficult to recognize when it affects the splanchnic or cerebral veins. Severe persistent abdominal pain or headache in patients with PNH should prompt appropriate radiologic investigations to exclude thrombosis. Although patients with PNH who have documented thrombosis should be treated with anticoagulants, eculizumab, a monoclonal antibody complement inhibitor, is highly effective and the only licensed treatment for PNH.[21]

Pregnancy

Pregnancy is an independent risk factor for venous thromboembolism: the risk for venous thromboembolism in pregnant women is up to six-fold higher than that in age-matched nonpregnant women. About 1 in 1000 pregnancies are complicated by venous thromboembolism, and the risk is highest in the postpartum period. Thus, venous thromboembolic disease is the leading cause of maternal morbidity and mortality and is estimated to account for 12% of the fatalities in pregnancy.[22]

The individual risk for venous thromboembolism in pregnancy and the puerperium, defined as the 6-week period after delivery, is influenced by patient-related factors. These factors include age over 35 years, body mass index over 29, cesarean delivery, prolonged immobilization, obesity, and thrombophilia or family history of venous thromboembolism. Multiparity, ovarian hyperstimulation, and a past history or family history of venous thromboembolism are other risk factors.

Over 90% of deep vein thrombosis in pregnancy occurs in the left leg because the enlarged uterus further compresses the left iliac vein by placing pressure on the overlying right iliac and ovarian arteries. A similar mechanism likely explains the isolated left iliofemoral thrombosis that can occur in pregnancy.

Hypercoagulability of the blood occurs in pregnancy and reflects a combination of venous stasis and changes in the hemostatic system. The enlarging uterus reduces venous blood flow from the lower extremities. This is not the only mechanism responsible for venous stasis because blood flow from the lower extremities begins to decrease by the end of the first trimester, likely reflecting hormonally induced venous dilatation. Systemic factors also contribute to hypercoagulability. Thus, the levels of circulating procoagulant proteins increase in the third trimester of pregnancy. These include factor VIII, fibrinogen, and von Willebrand factor, among others. Coincidentally, suppression of natural anticoagulant pathways and decreased fibrinolytic activity occur. Thus, there is an acquired resistance to activated protein C that is related, at least in part, to reduced levels of free protein S. The net effect of these changes is enhanced thrombin generation, in addition to the release of tissue factor from the uteroplacental circulation, as evidenced by elevated levels of prothrombin fragments and thrombin/antithrombin complexes. Platelet activation and increased platelet turnover occur, and mild thrombocytopenia, likely secondary to consumption, occurs in 8.3% of women at term. The altered levels of hemostatic proteins normalize 4 to 6 weeks after delivery.

About half of the episodes of venous thromboembolism in pregnancy occur in women with thrombophilia. The risk for venous thromboembolism in women with thrombophilic defects depends on the type of abnormality and the presence of other risk factors. The risk appears to be highest in women with a positive family history of venous thromboembolism who are homozygous for the factor

V Leiden or FIIG 20210 A mutation. There is also an increased risk in women with antithrombin, protein C, or protein S deficiency and a positive family history and a lower risk in those who are heterozygous for the factor V Leiden or FIIG 20210 A mutation. The risk of venous thromboembolism during pregnancy is similar in all three trimesters and begins in early pregnancy. In general, the daily risk is higher in the postpartum period than it is during pregnancy. Therefore, if thromboprophylaxis is given during pregnancy, it must be administered throughout the pregnancy and continued for at least 6 weeks postpartum.

Assisted Conception and Ovarian Hyperstimulation Syndrome

The overall risk for venous thrombosis in women undergoing ovarian hyperstimulation is small (estimated as 0.1% per treatment cycle). Often, thrombosis affects veins of the upper extremities or the jugular veins; the explanation for this phenomenon remains unclear. Thrombophilia testing is not routinely recommended in women undergoing ovarian stimulation because the predictive value is low.

Hormonal Therapy

Oral contraceptives, estrogen replacement therapy, and SERMs are all associated with an increased risk for thrombosis. The relatively high risk for venous thromboembolism associated with early oral contraceptives prompted the development of low-dose formulations containing reduced doses of estrogen and progestin. Currently available low-estrogen combination oral contraceptives contain 20 to 50 μg of ethinylestradiol and one of several different progestogens. Even these low-dose combination contraceptives are associated with a three- to four-fold increased risk for venous thromboembolism compared with the risk in non-users. In absolute terms, this translates to an incidence of 3 to 4 per 10,000. In contrast, progesterone-only methods of contraception, including pills, cutaneous implants, and progesterone-releasing intrauterine devices, are associated with little or no risk of thrombosis.[23]

Although smoking increases the risk for myocardial infarction and stroke in women taking oral contraceptives, it is unclear whether smoking affects the risk for venous thromboembolism. In contrast, obesity increases the risk for both arterial and venous thrombosis, and the risk of venous thrombosis increases steeply in those over the age of 40 years. The risk for venous thromboembolism is highest during the first 3 months of oral contraceptive use and persists only for the duration of use.

Case-control studies suggest that the risk for venous thromboembolism is 20- to 30-fold higher in women with inherited thrombophilia who use oral contraceptives than the risk for non-users with thrombophilia or users without these defects. Despite the increased risk, however, routine screening for thrombophilia is not indicated in women considering the use of oral contraceptives. Based on the estimated incidence and case fatality rate of thrombotic events, it is estimated that 400,000 women would need to be screened to detect 20,000 carriers of factor V Leiden. Oral contraceptives would need to be withheld in all these women to prevent a single death. For less prevalent thrombophilic defects, even larger numbers of women would need to be screened. Based on these considerations, routine screening cannot be recommended.

Oral contraceptive pills may cause prothrombotic side effects by inducing modest increases in levels of procoagulant factors (such as factors VII, VIII, X, prothrombin, and fibrinogen) and decreases in the levels of anticoagulant proteins (such as antithrombin and protein S). Acquired APC resistance is an almost universal finding in women taking oral contraceptives; the clinical significance of this phenomenon is uncertain.

There is good evidence that oral hormonal replacement therapy with conjugated equine estrogen (with or without a progestogen) increases the risk for myocardial infarction, ischemic stroke, and venous thrombosis. Carriers of the factor V Leiden mutation receiving hormone replacement therapy have a significantly increased risk for venous thromboembolism. Data from the Heart and Estrogen Replacement Study (HERS) and the Estrogen Replacement and Atherosclerosis Trial indicate that heterozygous carriers of the factor V Leiden mutation who were taking hormone replacement therapy had a 14-fold higher risk for venous thromboembolism compared with non-carriers receiving placebo. Based on this information, the use of oral hormone replacement preparations has markedly decreased. Use of transdermal hormone replacement therapy does not appear to increase the risk of venous thrombosis.[24]

SERMs are estrogen-like compounds. The prototypical SERM is tamoxifen, which serves as an estrogen antagonist in the breast, but has an estrogen agonist effect in other tissues, such as bone and uterus. Like estrogens, tamoxifen increases the risk for venous thromboembolism three- to fourfold. The risk is higher in postmenopausal women, particularly when they are receiving systemic combination chemotherapy.

Aromatase inhibitors are replacing tamoxifen for the treatment of estrogen receptor-positive breast cancer. These agents are associated with a lower risk for venous thromboembolism than is tamoxifen. Raloxifene, a SERM used to prevent osteoporosis, increases the risk for venous thromboembolism threefold compared with placebo. Therefore, this agent is contraindicated for the prevention of osteoporosis in women with a prior history of venous thromboembolism.

Prior History of Venous Thromboembolism

A history of previous venous thromboembolism places patients at risk for recurrence. Those with unprovoked venous thromboembolism have a particularly high risk for recurrence when anticoagulant treatment is stopped. Their risk for recurrence is about 10% at 1 year and 30% at 5 years. This risk occurs regardless of whether there is an underlying thrombophilic defect, such as factor V Leiden or the FIIG 20210 A mutation.

The risk for recurrent venous thromboembolism is lower in patients whose incident event occurred in association with a well-recognized and transient risk factor, such as major surgery or prolonged immobilization. These patients have a risk for recurrence of about 4% at 1 year and 10% at 5 years. Patients at highest risk for recurrence are those homozygous for factor V Leiden or the FII G20210A mutation and those with APS, advanced malignancy, or inherited deficiencies of antithrombin, protein C, or protein S. The risk for recurrence in such patients is likely to be 15% at 1 year and up to 50% at 5 years.[25]

COMBINED INHERITED AND ACQUIRED HYPERCOAGULABLE STATES

Hyperhomocysteinemia is the prototypical hypercoagulable state that occurs due to a combination of inherited and acquired factors. Homocysteine is an intermediate sulfur-containing amino acid that acts as a methyl group donor during the metabolism of methionine, an essential amino acid derived from the diet. The interconversi of 5-methyltetrahydrofolate, a methyl group donor, vitamin B_{12} and folate, cofactors in the interconversion, and the enzyme methionine synthase. Increased levels of homocysteine can be the result of increased production or reduced metabolism. Severe hyperhomocysteinemia and cysteinuria are rare and are usually caused by deficiency in the enzyme, cystathione β-synthetase. More common is mild to moderate hyperhomocysteinemia. This can be caused by genetic mutations in methyltetrahydrofolate reductase (MTHFR) when they are accompanied by the nutritional deficiency of folate, vitamin B_{12}, or vitamin B_6. Common polymorphisms in MTHFR, C677T, and A1298C are associated with reduced enzymatic activity and increased thermolability. The cofactor requirements are therefore increased with these mutations.

Hyperhomocysteinemia also can be associated with certain drugs, such as methotrexate, theophylline, cyclosporine, and most anticonvulsants, as well as some chronic diseases, such as end-stage renal disease, severe hepatic dysfunction, and hypothyroidism.

A fasting serum homocysteine level over 15 mmol/L is considered elevated. Although elevated levels were a common finding, routine fortification of flour with folic acid has resulted in lower homocysteine levels in the general population. Elevated serum levels of homocysteine have been associated with an increased risk for arterial thrombosis (myocardial infarction, stroke, and peripheral arterial disease) and venous thromboembolism.

Elevated levels of homocysteine can be reduced by the administration of folate with vitamin B_{12} and vitamin B_6. Randomized trials, however, have shown that the reduction of homocysteine levels with vitamin therapy does not reduce the risk for recurrent cardiovascular events in patients with coronary artery disease or stroke, nor does it lower the risk for recurrent venous thromboembolism; whether reduction of homocysteine levels is of benefit in peripheral artery disease with bypass grafts is less certain. Nonetheless, based on these negative trials and the declining incidence of hyperhomocysteinemia, the enthusiasm for screening for hyperhomocysteinemia has waned.

CLINICAL EVALUATION OF PATIENTS WITH HYPERCOAGULABLE STATES

A carefully taken history is essential to evaluate a patient with a history of thrombosis. The patient's age at the time of thrombosis, the location of the thrombosis, and results of objective diagnostic tests should be recorded. A historical record of previous thromboses and sites, and potential risk factors, such as recent surgery, trauma, prolonged immobility, pregnancy, or estrogen use should be noted. A family history of thrombosis, especially in first-degree relatives, may point to a heritable thrombophilic defect; systemic symptoms, such as anorexia, weight loss, change in bowel habits, or gastrointestinal bleeding, may suggest an underlying malignant condition.

A full physical examination should be conducted with attention to abdominal or pelvic masses, lymphadenopathy, and skin changes such as skin necrosis and livedo reticularis. Basic investigations are necessary to exclude acquired causes of thrombosis and to assess the patient's general health and tolerance to anticoagulation (see box on Routine Investigations to Evaluate a Patient with Venous Thrombosis).

THROMBOPHILIA SCREENING

In the past, the indications for thrombophilia screening were somewhat controversial, and the implications of such tests were often misinterpreted. It is now apparent that testing for heritable thrombophilia does not predict the likelihood of recurrence in unselected patients with symptomatic venous thrombosis. For patients with a first episode of venous thromboembolism, thrombophilia screening is indicated only if the results influence the duration of treatment or have an impact on family counseling regarding the use of estrogen-containing compounds. It is reasonable to screen patients whose first episode of thrombosis occurred before the age of 40 years, patients with thrombosis in an unusual site, such as cerebral or mesenteric veins, and those with two or more first-degree relatives, with unprovoked thrombosis; retinal vein thrombosis is not an indication for such screening. Screening should not be done when patients are taking an anticoagulant. If indefinite anticoagulation therapy is planned, screening is generally not required. Neonates and children with purpura fulminans should be urgently tested for protein C or S deficiency, as should adults who develop skin necrosis in association with vitamin K antagonists (see box on When to Perform a Thrombophilia Screen).

ROUTINE INVESTIGATIONS TO EVALUATE A PATIENT WITH VENOUS THROMBOSIS

Test	Abnormality	Diagnostic Information
Complete blood count	Elevated hematocrit Increased white count Increased platelet count Leukopenia Thrombocytopenia	Myeloproliferative disorder (e.g., essential thrombocythemia, polycythemia vera); cytopenias may be found in paroxysmal nocturnal hemoglobinuria; if thrombocytopenia associated with heparin administration, consider heparin-induced thrombocytopenia
Blood film	Leukoerythroblastic changes	Underlying neoplasm invading bone marrow
Liver function tests	Abnormal tests	May point to malignancy
Renal function	Impaired renal function	Assess prior to anticoagulation with heparin or low-molecular-weight heparin or direct oral anticoagulants
Urinalysis	Proteinuria	Nephrotic syndrome; may be associated with venous thromboembolism or renal vein thrombosis
PT and aPTT	Prolonged PT and aPTT	To enable safe anticoagulation to proceed if required Need to exclude lupus anticoagulant

aPTT, Activated partial thromboplastin time; *PT*, prothrombin time.

WHEN TO PERFORM A THROMBOPHILIA SCREEN

Clinical Scenario

- First episode of unprovoked venous thromboembolism in individuals younger than 40 years of age
- Thrombosis in an unusual site (e.g., cerebral or mesenteric vein thrombosis)
- Two or more first-degree relatives with unprovoked thrombosis
- Three or more early pregnancy losses, or one or more fetal deaths after 10 weeks, gestation

LABORATORY EVALUATION OF THROMBOPHILIA

Screening should include functional assays for antithrombin and protein C, an immunoassay for free protein S, testing for activated protein C resistance using the modified APC sensitivity ratio with DNA testing for the factor V Leiden mutation if the screening test is positive, DNA testing for the FIIG 20210 A gene mutation, and phospholipid-based clotting tests to detect an LA and enzyme immunoassay for ACL (see box on Essential Tests for Thrombophilia Screening). The benefits and potential harms of thrombophilia testing should always be discussed in advance with the patient because the psychological impact of knowing that they are a carrier of a genetic defect is one of the disadvantages of testing and because test results may also impact the cost of life insurance.

The timing of thrombophilia screening is critical. Levels of natural anticoagulants may be lower at the time of an acute thrombotic event; additionally, heparin can reduce the level of antithrombin, while vitamin K antagonists, such as warfarin, reduce the levels of protein

ESSENTIAL TESTS FOR THROMBOPHILIA SCREENING

- Basic coagulation screen
  - International normalized ratio (INR): to exclude the warfarin effect—warfarin will lower protein C and S levels
  - Activated partial thromboplastin time (aPTT): to exclude the heparin effect—heparin will lower antithrombin levels
- Functional assay for antithrombin (with heparin to detect type II defects)
- Functional assay for protein C
- Functional assay for protein S (immune assays for total and free protein S)
- APC resistance assay: with the genetic test for factor V Leiden for confirmation of abnormal results
- Genetic test for FIIG 20210A gene mutation
- Anticardiolipin and β_2-glycoprotein-1 antibodies (IgG and IgM) and lupus anticoagulant assay

C and protein S. Therefore, testing is best performed well after the acute event and when anticoagulant treatment has been discontinued. During pregnancy, protein S levels fall, complicating the diagnosis of protein S deficiency.

MANAGEMENT OF THROMBOSIS IN PATIENTS WITH HYPERCOAGULABLE STATES

Thrombosis treatment is usually divided into two overlapping stages, initial treatment, and extended therapy. The impact of hypercoagulable states on these two stages is discussed separately, as is their impact on the duration of anticoagulant therapy and recommendations for the prevention of recurrence.

Initial Treatment

With few exceptions, the management of initial thrombotic events in patients with hypercoagulable states is no different from the management of these events in patients without underlying hypercoagulable disorders. The exceptions are purpura fulminans in newborns with homozygous protein C or protein S deficiency, warfarin-induced skin necrosis, heparin-induced thrombocytopenia, and thrombosis in patients with severe antithrombin deficiency. Newborns with purpura fulminans require protein C or protein S concentrates or enough fresh frozen plasma to increase the levels of protein C or protein S.

Patients with antithrombin deficiency can usually be managed with LMWH or unfractionated heparin, although higher doses may be needed to achieve therapeutic anticoagulation. As an alternative, oral factor Xa inhibitors such as apixaban or rivaroxaban can be considered. Patients with severe antithrombin deficiency may require antithrombin concentrates to increase plasma levels of antithrombin to a point where heparin or LMWH can be used for treatment. Antithrombin concentrates are commercially available in plasma-derived and recombinant forms. The aim of treatment with antithrombin concentrate is to initially increase antithrombin activity to greater than 120% of the normal level (based on an expected 1.4% increase above baseline activity level per IU/kg of antithrombin administered) and to then maintain antithrombin activity at over 80% of the normal level. Plasma antithrombin levels need to be monitored to ensure that levels over 80% are maintained; antithrombin has a half-life of 2 to 4 days.

Treatment of heparin-induced thrombocytopenia involves discontinuation of heparin (including all forms of heparin such as heparin flushes of indwelling central venous catheters) and the initiation of an alternative anticoagulant, such as argatroban, fondaparinux, or an oral factor Xa inhibitor. Once the platelet count returns to its baseline level in those given a parenteral anticoagulant, the patient can be given either a vitamin K antagonist at a low initiating dose or a direct oral anticoagulant.

In patients with triple positive antiphospholipid antibody syndrome (LA, ACL, and antibody against β2-glycoprotein 1), or those with APS complicated by arterial thrombosis, direct oral anticoagulants such as rivaroxaban have been found to be less effective than vitamin K antagonists.[26–28] For those with single or dual positive APS, there is insufficient data to guide advice on the optimal anticoagulant to use initially and longer term. However, in 2019, the European Medicines Agency (EMA) and the MHRA (UK) advised against the use of all direct oral anticoagulants in people with APS of any type. Initial treatment is usually with LMWH or fondaparinux, followed by extended use of a vitamin K antagonist.

In asymptomatic subjects with laboratory evidence of ACL or LA, but no history of venous or arterial thrombosis, appropriate thromboprophylaxis should be given at the time of thrombotic challenge, such as major surgical procedures, prolonged immobilization, or pregnancy and the puerperium. There is no indication for primary preventive treatment with anticoagulants or antiplatelet agents in such individuals.

Extended Therapy

Extended treatment of thrombosis in patients with hypercoagulable states is like that of patients without these underlying disorders. Caution is needed when starting patients with protein C or protein S deficiency on warfarin or other vitamin K antagonists to prevent skin necrosis. Warfarin should not be started in these patients until therapeutic anticoagulation has been fully achieved with heparin or LMWH. Once started, low doses of warfarin should be given to prevent precipitous decreases in the levels of protein C or protein S; the heparin or LMWH should only be stopped when the international normalized ratio (INR) has been therapeutic for at least two consecutive days.

In patients with APS, randomized trials have shown that usual-intensity warfarin (target INR of 2.0 to 3.0) is as effective as higher-intensity warfarin. The risk for major bleeding is lower with usual-intensity warfarin than it is with higher-intensity regimens. A target INR of 2.5 with an INR range from 2.0 to 3.0 is appropriate for patients with other hypercoagulable states.

For patients with cancer-associated thrombosis, compared with warfarin, LMWH reduces the risk for recurrent venous thromboembolism without increasing bleeding,[29] lacks interactions with chemotherapy and other medicines, and holds favorable pharmacokinetics for with-holding prior to invasive procedures. The major drawbacks of LMWH have been cost and its requirement for parenteral administration. Direct oral anticoagulants appear to be efficacious and may take over as the mainstay of treatment for patients with cancer-associated thrombosis; evidence from clinical trials demonstrate non-inferiority compared to LMWH, although there are several outstanding issues, such as drug-drug interactions, and bleeding risk in certain tumor types.[30–32]

Duration of Treatment

The presence of a hypercoagulable state has no influence on the duration of anticoagulant treatment in patients whose venous thromboembolic event occurred in the setting of a well-recognized and transient risk factor, such as major surgery or prolonged immobilization due to medical illness. These patients are treated with anticoagulants for a minimum period of 3 months.

For those with unprovoked venous thromboembolism, a minimum of 3 months of anticoagulation treatment is recommended, at which point individualized assessment of the risk of recurrence without anticoagulation and the risk of bleeding with anticoagulation should be undertaken. Unless the risk of bleeding is high, most patients should receive extended anticoagulation therapy. Heterozygosity for factor V Leiden or the FIIG20210A mutation does not influence the risk for recurrence. In contrast, patients with deficiency of antithrombin, protein C, or protein S or those homozygous for factor V Leiden or the FII G20210A mutation appear to be at higher risk for recurrence and likely should receive longer-term anticoagulation treatment. Likewise, patients with APS with a persistent ACL or LA are also at high risk for recurrence and require extended treatment with a vitamin K antagonist.

The decision to continue anticoagulation indefinitely requires consideration of the site and severity of the first episode of venous thromboembolism, information on whether or not the event was provoked, assessment of the risk for anticoagulant-related bleeding, and other risk factors such as an elevated D-dimer level after stopping anticoagulant therapy.[25] There is mounting evidence that patients with VTE provoked by minor transient risk factors such as a flight or a brief period of immobilization and those with minor persistent risk factors such as obesity or inflammatory bowel disease have a risk of recurrence of up to 8% at one year if anticoagulation therapy is stopped. Therefore, extended anticoagulation may need to be considered in such patients taking into account adherence and patient preference.

Treatment and Prevention of Thrombosis During Pregnancy

Thrombophilic disorders have no influence on the treatment of venous thrombosis during pregnancy. Due to its better bioavailability and longer half-life, LMWH is the anticoagulant of choice; fondaparinux is reserved for women with an allergy to heparin. LMWH can be given once or twice daily in a weight-adjusted fashion. The need for dose adjustments and monitoring during the course of pregnancy has been a controversial area, but consensus-based recommendations advise against the need for monitoring anti-factor Xa levels. Direct oral anticoagulants and vitamin K antagonists should not be used in pregnancy because these drugs have the potential to cross the placenta.

No studies have addressed the optimal duration of anticoagulation for pregnancy-related venous thrombosis. In total, treatment should be given for 3 to 6 months from the time of diagnosis. Warfarin and LMWH can be safely administered in nursing mothers, with no detectable anticoagulant effect in breast milk. In contrast, direct oral anticoagulants should not be used in nursing mothers because low levels of drugs have been detected in breast milk.

Women with a past history of unprovoked or recurrent venous thromboembolism, those homozygous for factor V Leiden or the FII G20210A mutation, and those with deficiencies of antithrombin, protein C, or protein S and a family history of venous thrombosis should receive antepartum prophylaxis with LMWH. Postpartum, LMWH or warfarin should be given for 6 weeks. Postpartum treatment with LMWH or warfarin for 6 weeks is likely adequate for women with a history of venous thrombosis secondary to a well-defined risk factor. Prophylaxis during pregnancy, as well as postpartum, should be considered for women who developed venous thromboembolism after taking oral contraceptives, particularly if they have underlying thrombophilia. Women with thrombophilic defects but no prior history of venous thromboembolism or family history of the same likely do not require antepartum prophylaxis or postpartum treatment, but definitive data are lacking. A summary of these recommendations is provided in Table 138.6.[33]

TABLE 138.6 Management of Women With a History of Venous Thrombosis During Pregnancy and the Puerperium

Clinical History	Thrombophilia	Antepartum	Postpartum[a]
Prior VTE due to a transient risk factor	No	Surveillance	Yes
Prior VTE due to pregnancy or estrogens	Yes or no	Prophylactic LMWH	Yes
Prior idiopathic VTE	Yes or no	Prophylactic LMWH	Yes
Recurrent VTE	Yes or no	Treatment dose LMWH	Resume long-term anticoagulation
No prior VTE Positive family history	Antithrombin deficiency; homozygous FII G20210A; or Factor V Leiden; or dual heterozygosity for both mutations	Prophylactic or intermediate dose LMWH	Yes

[a]Postpartum prophylaxis involves a 6-week course of prophylactic doses of LMWH or dose-adjusted warfarin (target INR: 2.0–3.0).
LMWH, Low-molecular-weight heparin; *VTE*, venous thromboembolism.

Thrombophilia and Fetal Loss

About 30% of women have at least one fetal loss, and approximately 5% of women of reproductive age experience recurrent fetal loss. Women with hereditary or acquired thrombophilia have a two- to five-fold increased risk for fetal loss. Screening for APS is recommended if there is a history of three or more miscarriages before 10 weeks' gestation. Although once-daily LMWH in prophylactic doses, with or without aspirin, is often prescribed for women with recurrent fetal loss on the background of an underlying thrombophilic defect, such therapy is of no benefit and is costly and potentially harmful.

CONCLUSIONS AND FUTURE DIRECTIONS

Inherited or acquired hypercoagulable states can now be identified in up to 50% of patients with venous thromboembolism thanks to an increased understanding of the regulation of coagulation. The role of these disorders in the pathogenesis of arterial thrombosis is less clear, and further research is necessary to identify those individuals vulnerable to arterial thrombosis after plaque rupture. Despite an improved ability to diagnose hypercoagulable states, the impact of this information on clinical decisions remains limited. Common congenital hypercoagulable states increase the risk for a first thrombotic episode but appear to have little impact on the risk for recurrence. Identification of biomarkers for patients at risk for recurrent thrombosis and elucidating new hypercoagulable states are goals for the future.

REFERENCES

1. Vossen CY, Conard J, Fontcuberta J, et al. Risk of a first venous thrombotic event in carriers of a familial thrombophilic defect. The European Prospective Cohort on Thrombophilia (EPCOT). *J Thromb Haemost*. 2005;3:459.
2. Rossi E, Ciminello A, Za T, et al. In families with inherited thrombophilia the risk of venous thromboembolism is dependent on the clinical phenotype of the proband. *Thromb Haemost*. 2011;106(4):646–654.
3. Vossen CY, Walker ID, Svensson P, et al. Recurrence rate after a first venous thrombosis in patients with familial thrombophilia. *Arterioscler Thromb Vasc Biol*. 2005;25(9):1992–1997.
4. Di Minno MN, Dentali F, Lupoli R, et al. Mild antithrombin deficiency and risk of recurrent venous thromboembolism: a prospective cohort study. *Circulation*. 2014;129(4):497–503.

5. Lijfering WM, Brouwer JL, Veeger NJ, et al. Selective testing for thrombophilia in patients with first venous thrombosis: results from a retrospective family cohort study on absolute thrombotic risk for currently known thrombophilic defects in 2479 relatives. *Blood*. 2009;113(21):5314–5322.
6. Franchi F, Biguzzi E, Martinelli I, et al. Normal reference ranges of antithrombin, protein C and protein S: effect of sex, age and hormonal status. *Thromb Res*. 2013;132(2):e152–e157.
7. Pintao MC, Ribeiro DD, Bezemer ID, et al. Protein S levels and the risk of venous thrombosis: results from the MEGA case-control study. *Blood*. 2013;122(18):3210–3219.
8. Vossen CY, Conard J, Fontcuberta J, et al. Familial thrombophilia and lifetime risk of venous thrombosis. *J Thromb Haemost*. 2004;2(9): 1526–1532.
9. Lijfering WM, Middeldorp S, Veeger NJ, et al. Risk of recurrent venous thrombosis in homozygous carriers and double heterozygous carriers of factor V Leiden and prothrombin G20210A. *Circulation*. 2010;121(15):1706–1712.
10. van der Meer FJ, Koster T, Vandebroucke JP, et al. The Leiden Thrombophilia Study (LETS). *Thromb Haemost*. 1997;78:631.
11. Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost*. 2006;4:295.
12. Favaloro EJ, Wong RCW. Antiphospholipid antibody testing for the antiphospholipid syndrome: a comprehensive practical review including a synopsis of challenges and recent guidelines. *Pathology*. 2014;46(6):481–495.
13. Devreese KM. Testing for antiphospholipid antibodies: advances and best practices. *Int J Lab Hematol*. 2020;42(Suppl):49–58.
14. Lo GK, Juhl D, Warkentin TE, et al. Evaluation of pretest clinical score (4T's) for the diagnosis of heparin-induced thrombocytopenia in two clinical settings. *J Thromb Haemost*. 2006;4(4):759–765.
15. Cuker A, Arepally GM, Chong BH, et al. American Society of Hematology 2018 guidelines for management of venous thromboembolism: heparin-induced thrombocytopenia. *Blood Adv*. 2018;2(22):3360–3392.
16. Cuker A. Clinical and laboratory diagnosis of heparin-induced thrombocytopenia: an integrated approach. *Semin Thromb Hemost*. 2014;40(1):106–114.
17. Lee AYY. Management of thrombosis in cancer: primary prevention and secondary prophylaxis. *Br J Haematol*. 2006;128:291.
18. Zamagni E, Brioli A, Tacchetti P, et al. Multiple myeloma, venous thromboembolism, and treatment-related risk of thrombosis. *Semin Thromb Hemost*. 2011;37:209.
19. Carrier M, Le Gal G, Lee AYY. Rates of venous thromboembolism in multiple myeloma patients undergoing immunomodulatory therapy with thalidomide or lenalidomide; a systematic review and meta-analysis. *J Thromb Haemost*. 2011;9:653.
20. Carrier M, Lazo-Langner A, Shivakumar S, et al. Screening for occult cancer in unprovoked venous thromboembolism. *N Engl J Med*. 2015;373(8):697–704.
21. Hill A, DeZern AE, Kinoshita T, et al. Paroxysmal nocturnal haemoglobinuria. *Nat Rev Dis Primers*. 2017;3:17028.
22. Ewins K, Ní Ainle F. VTE risk assessment in pregnancy. *Res Pract Thromb Haemost*. 2020;4(2):183–192.
23. Middeldorp S. Thrombosis in women: what are the knowledge gaps in 2013? *J Thromb Haemost*. 2013;11(Suppl 1):180–191.
24. Canonico M, Plu-Bureau G, Lowe GDO, et al. Hormone replacement therapy and risk of venous thromboembolism in postmenopausal women: systematic review and meta-analysis. *BMJ*. 2008;336(7655):1227–1231.
25. Kearon C, Akl EA. Duration of anticoagulant therapy for deep vein thrombosis and pulmonary embolism. *Blood*. 2014;123(12):1794–1801.
26. Pengo V, Denas G, Zoppellaro G, et al. Rivaroxaban vs warfarin in high-risk patients with antiphospholipid antibody syndrome. *Blood*. 2018;132(13):1365–1371.
27. Ordi-Ros J, Saez-Comet L, Perez-Conesa M, et al. Rivaroxaban versus vitamin K antagonist in antiphospholipid syndrome: meta-analysis of randomized controlled trials. *Ann Intern Med*. 2019;171(10):685–694.
28. Dufrost V, Wahl D, Zuily S. Direct oral anticoagulants in antiphospholipid antibody syndrome: meta-analysis of randomized controlled trials. *Autoimmun Rev*. 2021;20(1)102711.
29. Lee AYY, Levine MN, Baker RI, et al. Low-molecular-weight heparin versus coumarin for the prevention of recurrent venous thromboembolism in patients with cancer. *N Engl J Med*. 2003;349(2):146–153.
30. Young AM, Marshall A, Thirlwall J, et al. Comparison of an oral factor xa inhibitor with low molecular weight heparin in patients with cancer with venous thromboembolism: results of a randomized trial (SELECT-D). *J Clin Oncol*. 2018;36:2017–2123.
31. Agnelli G, Becattini C, Meyer G, et al. Apixaban for the treatment of venous thromboembolism associated with cancer. *N Engl J Med*. 2020;382:1599–1607.
32. Raskob GE, van Es N, Verhamme P, et al. Edoxaban for the treatment of cancer-associated venous thromboembolism. *N Engl J Med*. 2018;378:615–624.
33. Bates SM, Greer IA, Middeldorp S, et al. VTE, thrombophilia, antithrombotic therapy, and pregnancy: antithrombotic therapy and prevention of thrombosis, 9th ed: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest*. 2012;141(2 Suppl):e691S–e736S.

CHAPTER 139

ANTIPHOSPHOLIPID SYNDROME

Lucia R. Wolgast and Jacob H. Rand

Antiphospholipid (aPL) syndrome (APS) is an autoimmune thrombophilic syndrome for which definitive disease mechanisms have not been established. The condition is defined and diagnosed by a constellation of clinical and laboratory criteria, detailed below. This chapter reviews the current understanding of pathogenic mechanisms, the current diagnostic tests for the condition, its clinical manifestations, and treatment approaches.

DEFINITION OF ANTIPHOSPHOLIPID SYNDROME

APS is an autoimmune thrombophilic condition in which patients develop antibodies against plasma proteins that bind to phospholipids. Diagnostic criteria (referred to as the Sydney criteria, detailed in Table 139.1) have been developed to provide consistency for clinical trials. These require that patients have documented evidence of vascular thrombosis and/or obstetric complications attributable to placental vascular insufficiency along with persistent abnormalities of one or more of aPL laboratory tests. The latter include elevated anticardiolipin (aCL) IgG or IgM antibodies, anti-β_2-glycoprotein I (anti-β2GPI) IgG or IgM antibodies, and positive lupus anticoagulant (LA) tests. The diagnosis is established only after the laboratory abnormalities have been confirmed 12 or more weeks after initial testing.

It is important for clinicians to realize that these formal criteria were intended to provide a uniformly rigorous definition of APS for standardizing clinical research. In real-world clinical practice settings, some patients may be diagnosed with presumptive APS without meeting the strict investigational criteria.

Patients with systemic lupus erythematosus (SLE) may develop APS, in which case they are considered to have **secondary APS**, while other patients may have a "stand-alone" form of the disorder, **primary APS**. Occasional patients will present with **catastrophic APS (CAPS)**, which manifests as disseminated thrombosis in large and small vessels with resulting multiorgan failure (criteria detailed in Table 139.2).

ANTIGENIC SPECIFICITIES OF ANTIPHOSPHOLIPID ANTIBODIES

The complexity of this autoimmune disorder is readily apparent by the observation that the addition of APS plasma to artificial phospholipid membranes results in the formation of proteinaceous complexes. This phenomenon has been visualized using atomic force microscopy (Fig. 139.1). Analysis of the composition of these complexes has revealed the presence of aPL antibodies and a variety of bound plasma proteins. It remains to be established whether the protein targets of the antibodies vary depending on patient manifestations.

The major protein recognized by aPL antibodies is β_2-glycoprotein I (β2GPI), a 50 kDa glycoprotein member of the complement control protein (CCP) superfamily. The protein consists of five homologous CCP domains, each consisting of ~60–amino acids, with a fifth domain that includes a phospholipid-binding site near the carboxy-terminus of the protein (Fig. 139.2). β2GPI binds to membranes via cationic residues, which interact with the anionic polar heads of membrane phospholipids adjacent to a hydrophobic loop that inserts into the lipid bilayer. β2GPI bound to phospholipid has a "J-shaped" fishhook conformation with the binding site for phospholipid in domain V, near the carboxy-terminus "barb" of the hook. The domain I epitope, which is cryptic in the unbound conformation of the protein, is exposed when β2GPI binds to phospholipid bilayers. Elevated levels of IgG antibodies against the domain I Gly40-Arg43 epitope have been correlated with an increased risk for thrombosis.

Several additional proteins have been identified as targets for autoantibodies in APS patients. These include prothrombin (factor II), factor V, protein C, protein S, annexin A2, annexin A5, tissue factor pathway inhibitor (TFPI), high and low molecular weight kininogen, factor VII/VIIa, plasmin, vimentin, and others. APS antibodies that bind to acidic glycosphingolipids known as sulfatides have also been described.

PATHOGENIC EFFECTS OF ANTIPHOSPHOLIPID ANTIBODIES

Multiple pathogenic mechanisms have been postulated to explain the thrombotic and pregnancy complications in APS (Table 139.3).

Activation of Complement

There is convincing evidence that complement activation plays a role in the APS disease process. When incubated with APS plasma, phospholipid vesicles activate more complement than when incubated with control plasma, as evidenced by more C5a and sC5b-9 generation (Fig. 139.3). Complement activation likely reflects the formation of APS macro-immune complexes on these vesicles. The modified Ham test has also been used to demonstrate complement activation; 69% of APS sera collected within 1 year of a thrombotic event and 86% of CAPS sera tested positive, whereas only 6.8% of SLE sera were positive. Complement activation is induced by anti-β2GPI antibodies, primarily through the classical pathway and germline variants in complement regulatory genes have been found in patients with thrombotic APS and CAPS. The role of complement activation is buttressed by findings in a mouse model of APS antibody-induced pregnancy loss where genetic deletion of C3 or treatment of wild-type mice with a C3 convertase inhibitor protected against pregnancy complications.

Inhibition of Anticoagulant and Fibrinolytic Mechanisms

aPL antibodies accelerate coagulation reactions on endothelial cells and trophoblasts by disrupting the antithrombotic shield provided by annexin A5. Annexin A5 forms a two-dimensional crystalline array over phospholipid bilayers, which shields anionic phospholipids on cell membranes and blocks coagulation proteins from binding and assembling on the cell membrane, thereby preventing phospholipid-dependent coagulation enzyme reactions. Annexin A5 is highly expressed by endothelial cells and localizes to the apical membrane of

TABLE 139.1 Sydney Investigational Criteria for the Diagnosis of the Antiphospholipid Syndrome

Clinical

- Vascular thrombosis (one or more episodes of arterial, venous, or small vessel thrombosis). For histopathologic diagnosis, there should be no evidence of inflammation in the vessel wall.
- Pregnancy morbidities attributable to placental insufficiency, including: (1) three or more otherwise unexplained recurrent spontaneous miscarriages before 10 weeks of gestation, (2) one or more fetal losses after the 10th week of gestation, (3) stillbirth, and (4) episode of preeclampsia, preterm labor, placental abruption, intrauterine growth restriction, or oligohydramnios that are otherwise unexplained.

Laboratory

- Medium or high titer aCL or anti-β2GPI IgG and/or IgM antibody present on two or more occasions, at least 12 weeks apart, measured by standard ELISAs.
- Lupus anticoagulant, on two or more occasions, at least 12 weeks apart, detected according to the guidelines of the ISTH SSC Subcommittee on Lupus Anticoagulants and Phospholipid-Dependent Antibodies.

Adapted from Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *Thromb Haemost.* 2006;4:295–306.
"Definite APS" is considered to be present if at least one of the clinical criteria and one of the laboratory criteria are met.
aCL, Anticardiolipin; *aPL*, antiphospholipid; *β2GPI*, *β*2-glycoprotein I; *ELISA*, enzyme-linked immunosorbent assay; *Ig*, immunoglobulin.

TABLE 139.2 Proposed Criteria for the Classification of Catastrophic Antiphospholipid Syndrome

1. Evidence of involvement of three or more organs, systems and/or tissues[a]
2. Development of manifestations simultaneously or in less than a week
3. Confirmation by histopathology of small vessel occlusion in at least one organ or tissue[b]
4. Laboratory confirmation of the presence of antiphospholipid antibodies (lupus anticoagulant and/or anticardiolipin antibodies)[c]

Definite catastrophic APS

- All four criteria

Probable catastrophic APS

- All four criteria, except for only two organs, systems and/or tissues involvement
- All four criteria, except for the absence of laboratory confirmation at least 6 weeks apart due to the early death of a patient never previously tested for aPL prior to the catastrophic APS event
- Criteria 1, 2, and 4
- Criteria 1, 3, and 4 and the development of a third event in more than a week but less than a month, despite anticoagulation.

[a] Usually, clinical evidence of vessel occlusions, confirmed by imaging techniques when appropriate. Renal involvement is defined by a 50% rise in serum creatinine, severe systemic hypertension (N180/100 mmHg) and/or proteinuria (N500 mg/24 h).
[b] For histopathological confirmation, significant evidence of thrombosis must be present, although, in contrast to Sydney criteria, vasculitis may be present.
[c] If the patient had not been previously diagnosed as having an APS, the laboratory confirmation requires that the presence of antiphospholipid antibodies must be detected on two or more occasions at least 6 weeks apart (not necessarily at the time of the event), according to the proposed preliminary criteria for the classification of definite APS.
aPL, Antiphospholipid; *APS*, antiphospholipid syndrome.
Adapted from Asherson RA, Cervera R, de Groot PG, et al. Catastrophic antiphospholipid syndrome: international consensus statement on classification criteria and treatment guidelines. *Lupus.* 2003;12:530–534.

placental syncytiotrophoblasts where maternal blood interfaces with fetal cells. Pregnant mice treated with anti-annexin A5 antibodies develop placental necrosis, fibrosis, and pregnancy loss, while pregnant annexin A5-null mice experience placental infarction and have reduced litter sizes. Anti-β2GPI IgG/β2GPI immune complexes disrupt the annexin A5 antithrombotic shield by competitively displacing the protein from the surfaces of cell membranes (Fig. 139.4). A mechanistic assay has been developed to assess disruption of annexin A5 anticoagulant activity.

aPL antibodies inhibit several steps in the protein C pathway. These include: (1) reducing protein C activation by the thrombomodulin-thrombin complex, (2) inhibiting assembly of the protein C complex, (3) inhibiting activated protein C (APC) directly or via its cofactor protein S, (4) binding to factors Va and VIIIa and protecting them from proteolysis by APC, and (5) reducing levels of protein C and protein S. Some aPL antibodies bind protein C or S. APC resistance has been described in APS plasmas and has been correlated with anti-β2GPI domain I antibodies.

Autoantibodies directed against TFPI have also been reported in APS patients; these antibodies may inhibit the capacity of TFPI to downregulate activation of factor IX and factor X by the factor VIIa-tissue factor complex. TFPI has a thrombogenic role by serving as a cofactor for promoting aPL-mediated expression of tissue factor by monocytes.

aPL antibodies impact fibrinolytic mechanisms in multiple ways including (1) binding to annexin A2 and reducing its capacity to serve as a receptor for tissue plasminogen activator (t-PA) and plasminogen, (2) reducing the catalytic activity of plasmin or t-PA, (3) increasing the levels of plasminogen activator inhibitor-1, (4) inhibiting autoactivation of factor XII, and (5)reducing plasmin generation because antibodies against β2GPI attenuate its capacity to serve as a cofactor for t-PA-mediated activation of plasminogen.

Activation of Platelets, Monocytes, and Neutrophils

Recent evidence from a mouse model suggests that aPL-induced thrombosis results from platelet activation that promotes endothelial activation and fibrin formation. aPL antibodies can induce platelet aggregation, an effect that may be promoted via signaling through apolipoprotein E receptor 2′ (ApoER2′) with the β2GPI binding site for ApoER2′ on platelets localized to its domain V. Since β2GPI has a dampening effect on platelet adhesion by interfering with the platelet–von Willebrand factor interaction, aPL antibodies increase platelet adhesion in flow systems by interfering with this dampening. Additionally, proteomic studies support a role for platelet disulfide isomerase family members in promoting aPL-mediated thrombosis.

aPL antibodies increase the expression of tissue factor and other cytokines by monocytes—a process that occurs through activation of the p38MAPK and MEK-1/ERK pathways. A recent unexpected finding was that TFPI primes the monocytes for the aPL-mediated signaling that results in tissue factor expression. aPL antibodies may also promote mitochondrial dysfunction and oxidative stress in monocytes, thereby inducing a proinflammatory state.

Finally, anti-β2GPI/β2GPI complexes can promote neutrophils to undergo NETosis, because neutrophil extracellular traps (NETs) can initiate clotting (see Chapter 121). The activation of the TLR4/MyD88/MAPKs axis was shown to be involved in the formation of NETs, which then promoted coagulation via tissue factor expression on the NETs along with platelet aggregation. This mechanism was further supported by the proteomic studies.

Effects on Vascular Endothelium

aPL antibodies can bind to, injure, and/or activate cultured vascular endothelial cells. Antibody binding to β2GPI on the endothelial surface may trigger signaling cascades that promote the expression of tissue factor and adhesion molecules.

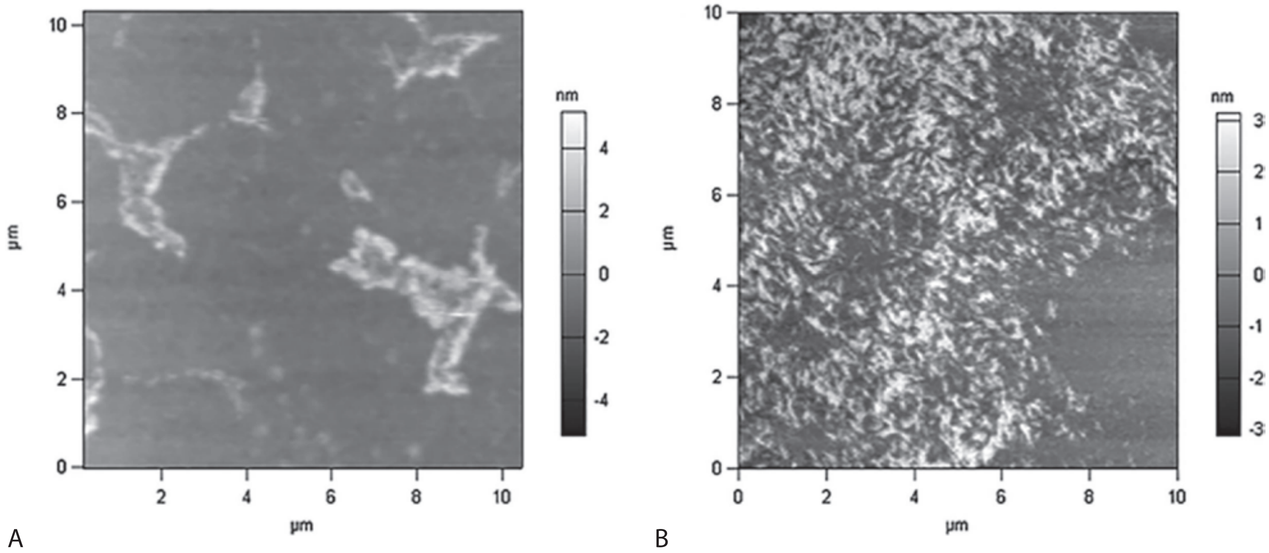

10
8
6
4
2
0
µm
2
4
6
8
10
µm
nm
4
2
0
-2
-4
A
10
8
6
4
2
0
µm
0
2
4
6
8
10
µm
nm
3
2
1
0
-1
-2
-3
B

Figure 139.1 Atomic force microscopy images of antiphospholipid syndrome (APS) macromolecular complexes compared with non-APS control plasma. Atomic force microscopy images demonstrating the interaction of human plasma with a planar phospholipid layer formed on mica. (A) Plasma samples from APS patients form complex heterogeneous macromolecular complexes on phospholipid layers, (B) control plasmas deposit proteins that form a more homogeneous matted surface. *(Adapted from Rand JH, Taatjes DJ. Reimagining the antiphospholipid syndrome, an enigmatic thrombophilic disorder, through the looking glass of microscopic imaging. Histochem Cell Biol. 2018; 150:529–543.)*

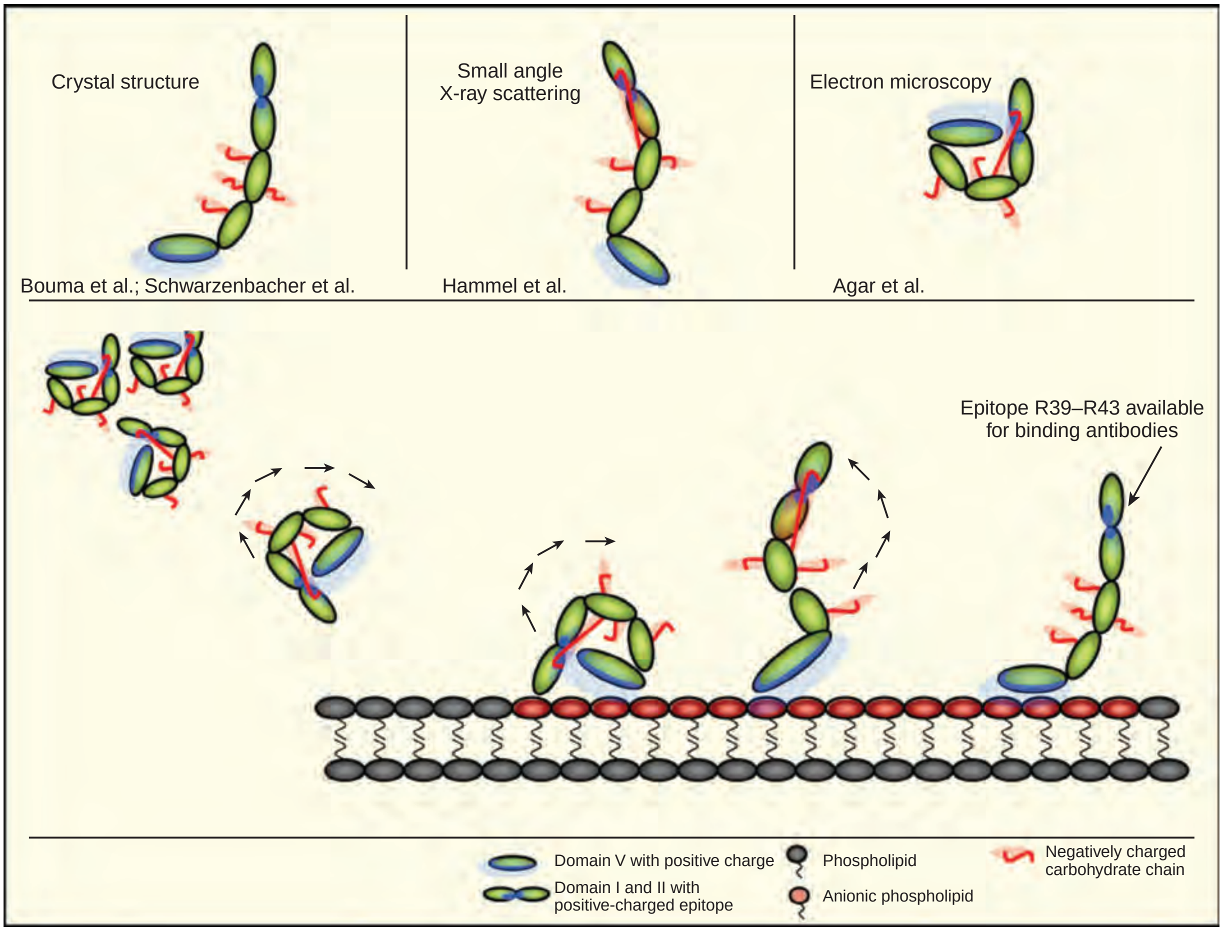

Crystal structure
Bouma et al.; Schwarzenbacher et al.
Small angle
X-ray scattering
Hammel et al.
Electron microscopy
Agar et al.
Epitope R39–R43 available
for binding antibodies
Domain V with positive charge
Domain I and II with
positive-charged epitope
Phospholipid
Anionic phospholipid
Negatively charged
carbohydrate chain

Figure 139.2 Models for proposed β2GPI structures. By X-ray crystallography, β2GPI exhibits a J-shaped structure. However, small angle X-ray scattering (SAXS) of β2GPI in solution reveals an S-shaped conformation with a carbohydrate chain at the interface between domains I and II. In contrast to the SAXS structure, transmission electron microscopy of negatively stained unbound β2GPI mounted on grids exhibits a circular conformation, whereas it exhibits a "J" shaped structure when bound to phospholipid. The arginine 39-arginine 43 epitope on domain I recognized by thrombogenic aPL antibodies is cryptic in the proposed free-β2GPI conformations and is exposed when the protein binds to phospholipid. *(From de Laat B, de Groot PG. Autoantibodies directed against domain I of beta2-glycoprotein I. Curr Rheumatol Rep. 2011;13(1):70–76.)*

TABLE 139.3 **Proposed Pathogenic Mechanisms of Antiphospholipid Syndrome**

- I. Activation of Complement
    - A. Stimulated by formation of antiphospholipid syndrome (APS) macro-immune complexes
    - B. Augmented by germline variants of complement proteins.
- II. Inhibition of Anticoagulant and Fibrinolytic Mechanisms
    - A. Disruption of the annexin A5 anticoagulant shield
    - B. Inhibition of the protein C pathway—via reduced activation of protein C, inhibition of assembly of protein C complex, direct inhibition of proteins C and/or S, protecting factors Va and VIIIa from proteolysis, reduction of protein C and/or S levels, or prevention of protein C and EPCR binding
    - C. Interference with tissue factor pathway inhibitor (TFPI)
    - D. Interference with fibrinolysis via blocking of annexin A2, direct inhibition of plasmin, increase of PAI-1, or autoactivation of XIIa, inhibiting β2GPI cofactor activity
- III. Activation of Platelets, Monocytes and Neutrophils
    - A. Activation of platelets via ApoER2’
    - B. Interference of β2GPI in regulating von Willebrand factor (vWF)–mediated platelet adhesion
    - C. Activation of monocytes/macrophages, with increased tissue factor and cytokine expression
    - D. Activation of monocytes causes mitochondrial dysfunction and oxidative stress
    - E. Release of neutrophil extracellular traps (NETosis)
- IV. Effects on Vascular Endothelium
    - A. Direct injury and subsequent anti-β2GPI binding on endothelial cells
    - B. Signaling via annexin A2/TLR4/ApoER2 inducing proadhesive prothrombotic phenotype
    - C. Induction of adhesion molecules and tissue factor on endothelial cells and cytokine release
    - D. mTORC pathway-mediated vasculopathy
    - E. Release of procoagulant microparticles by endothelial cells and platelets
- V. Effects on Trophoblasts and Endometrial Cells
    - A. Abnormal trophoblast proliferation, migration and invasiveness, increased trophoblast apoptosis, and reduced secretion of HCG and adhesion molecules
    - B. Disruption in the differentiation of decidual endometrial cells
    - C. Disruption of maternal spiral artery transformation and maturation

Like its role in activating platelets, ApoER2′ expressed on the endothelial surface may also be a target for anti-β2GPI/β2GPI complexes to trigger tissue factor and adhesion molecule expression. Evidence from ApoER2′$^{-/-}$ knockout mice supports a role for the protein in mediating APS thrombosis.

Annexin A2 is an endothelial surface receptor for t-PA and plasminogen which serves as a receptor for β2GPI on vascular endothelium. In addition to aPL antibody-mediated inhibition described above, annexin A2 may play a role in mediating the effects of aPL antibodies on the signaling steps that promote thrombosis. Annexin A2 is not a transmembrane protein and so the signaling co-receptors, Toll-like receptor-2 (TLR2) and Toll-like receptor-4 (TLR4), have been implicated as triggers of the signaling cascade. Downstream signaling appears to involve tumor necrosis factor receptor-associated factor 6 (TRAF6) and myeloid differentiation factor 88 (MyD88). Tissue factor expression is mediated by p38 mitogen-activated protein kinase (MAPK). This mechanism is supported by the finding that a mutation in murine TLR-4 that disrupts lipopolysaccharide (LPS) binding attenuated the prothrombotic state in mice injected with aPL antibodies.

aPL antibodies may contribute to other vascular lesions by stimulating the mammalian target of the rapamycin complex (mTORC) pathway. Endothelial cells and platelets activated by aPL antibodies release microparticles containing procoagulant proteins and nucleic acids.

Effects on Trophoblasts and Endometrial Cells

In mice, in addition to disruption of the annexin A5 anticoagulant shield at the maternal-fetal interface, aPL-mediated promotion of tissue factor expression induces trophoblast injury and fetal death. Tissue factor contributed to the C5a-induced oxidative burst in neutrophils that promoted trophoblast and fetal injury in APS.

aPL antibodies may induce trophoblast proliferation, migration, and invasiveness, increase trophoblast apoptosis, and reduce the secretion of human chorionic gonadotropin (HCG) and adhesion molecules. Activation of MYD88 by TLR4 has been linked to increased levels of proinflammatory cytokines (IL-8, IL-1β, monocyte chemoattractant proteins) that influence trophoblast survival. Downregulation of signal transducer and activator of transcription 3 (STAT3) phosphorylation decreases IL-6 expression and reduces trophoblast migration. aPL antibodies have been implicated in the disruption of maternal spiral artery transformation and maturation, as well as differentiation of decidual endometrial cells through activation of signaling pathways such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). Defective placentation may result in impaired blastocyte implantation, thereby leading to fetal loss.

Genetic and Proteomic Studies in Antiphospholipid Syndrome

Genetic factors appear to play a role in the development of aPL antibodies even though familial APS is rare. One study of seven families, which included 30 individuals who met consensus criteria for APS, concluded that the inheritance pattern of aPL antibodies appeared to be autosomal dominant, however no specific linkages could be identified. A study done with peripheral blood mononuclear cells from aPL antibody-positive patients found a gene expression pattern that appeared to correlate with a predisposition for thrombosis. Some of the identified genes encoded proteins that are known to be involved in thrombosis, including apolipoprotein E (ApoE), factor X, and thromboxane. Other genes were not connected with the disease process, such as hypoxia-inducible factor-1alpha (HIF-1α), zinc finger proteins, matrix metalloproteinase 19 (MMP19), interleukin 22 (IL22) receptor, and hematopoietic progenitor cell antigen (CD34) precursor. As mentioned above, germline variants of complement proteins have recently been associated with thrombotic APS and CAPS.

Proteomic studies have revealed a series of proteins that are differentially expressed in monocytes of APS patients with a history of thrombosis. These include annexin A1, annexin A2, ubiquitin Nedd8, Rho A protein, protein disulfide isomerase, and Hsp60. Proteomic analysis of platelets and plasmas from LA-positive patients with a history of thromboembolism supports the role of PDI and NETosis in APS-associated thrombosis. PDI family members were upregulated in platelets and plasma of these patients compared with controls. Leukocyte elastase inhibitor (SERPINB1), an antagonist of NET formation, was decreased and citrullinated histone H3, a NET-specific marker, was increased.

ANTIPHOSPHOLIPID ASSAYS

Paths to Development

The recognition of APS stemmed from observations of two laboratory anomalies. One of these, the biologic false-positive serologic test for syphilis (STS), caused by antibodies against cardiolipin

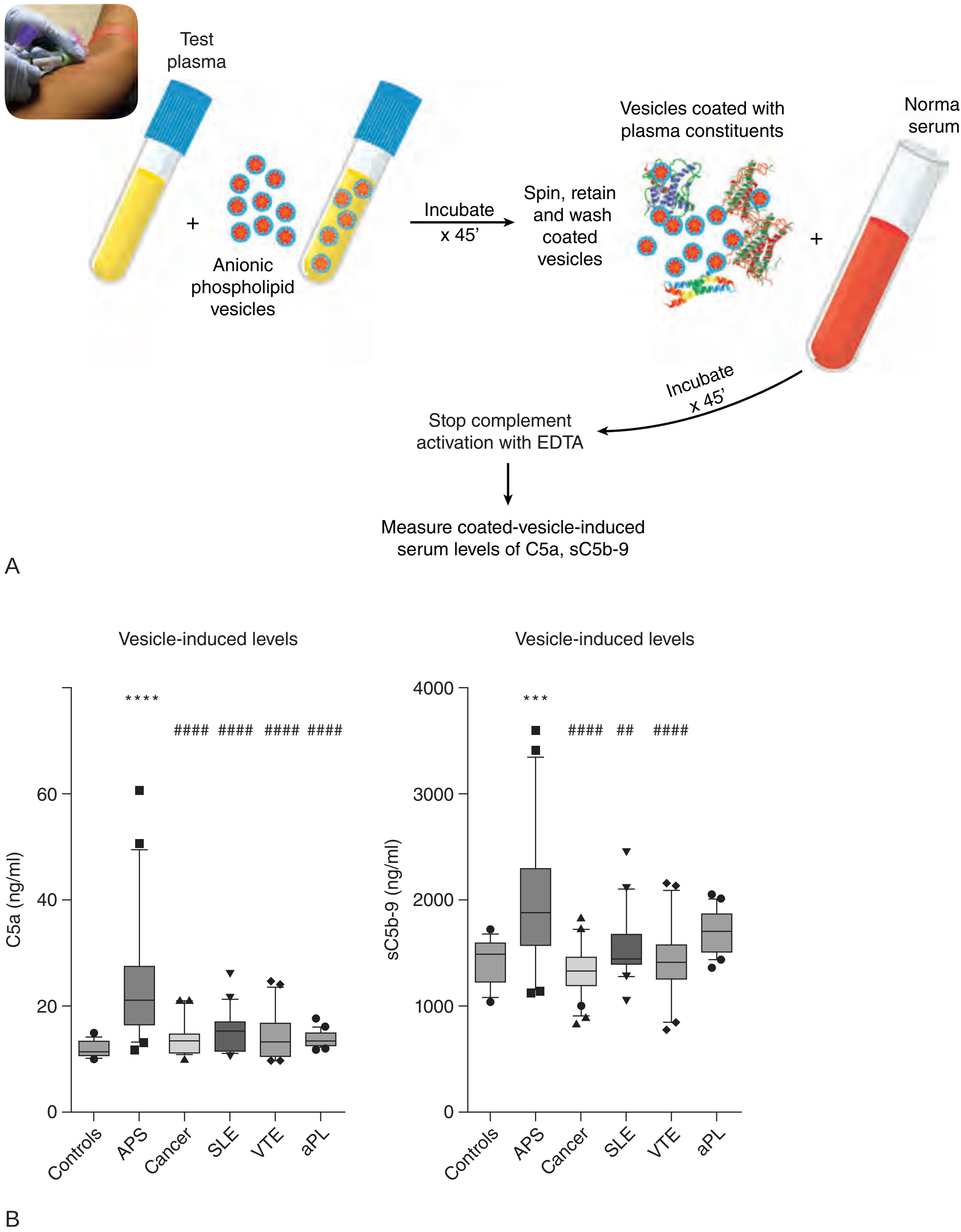


Figure 139.3 Two-stage assay for detecting complement activation in antiphospholipid syndrome (APS). (A) Pictorial representation of a method to measure complement activation generated in serum by phospholipid vesicles pre-incubated with patient plasmas. (B) Complement activation after exposure of patient plasma-coated vesicles to normal serum. Anionic phospholipid vesicles were coated with plasma from healthy controls, patients with APS, cancer, systemic lupus erythematosus (SLE), venous thromboembolism (VTE), or asymptomatic patients with positive antiphospholipid (aPL) antibodies, after which complement activation was triggered and levels of C5a (*left*) and sC5b-9 (*right*) were quantified. Only statistically significant differences are shown. $^{*}P < .05$, $^{***}P < .0005$, $^{****}P < .0001$ versus controls. $^{\#}P < .05$, $^{\#\#}P < .005$, $^{\#\#\#}P < .0005$, $^{\#\#\#\#}P < .0001$ versus APS. *(Adapted from Rand JH, Wu X-X, Wolgast LR, Lei V, Conway EM. A novel 2-stage approach that detects complement activation in patients with antiphospholipid antibody syndrome. Thromb Res. 2017;156:119–125.)*

(diphosphatidylglycerol), formed the basis for an immunoassay that prompted the identification of "anticardiolipin antibody syndrome," which was then renamed "antiphospholipid antibody syndrome." The other anomaly was the finding that some patients had a prolonged activated partial thromboplastin time (aPTT) due to a unique inhibitor of blood coagulation. The first patients identified with this anomaly had SLE, which led to the misnomer "LA". This brief history is helpful for appreciating that the current aPL tests, which were empirically derived from these two anomalies, are limited because they do not directly measure a disease mechanism. Nevertheless, these assays have become the mainstay markers of this disorder and have proven to be useful as surrogate reporters of thrombotic risk.

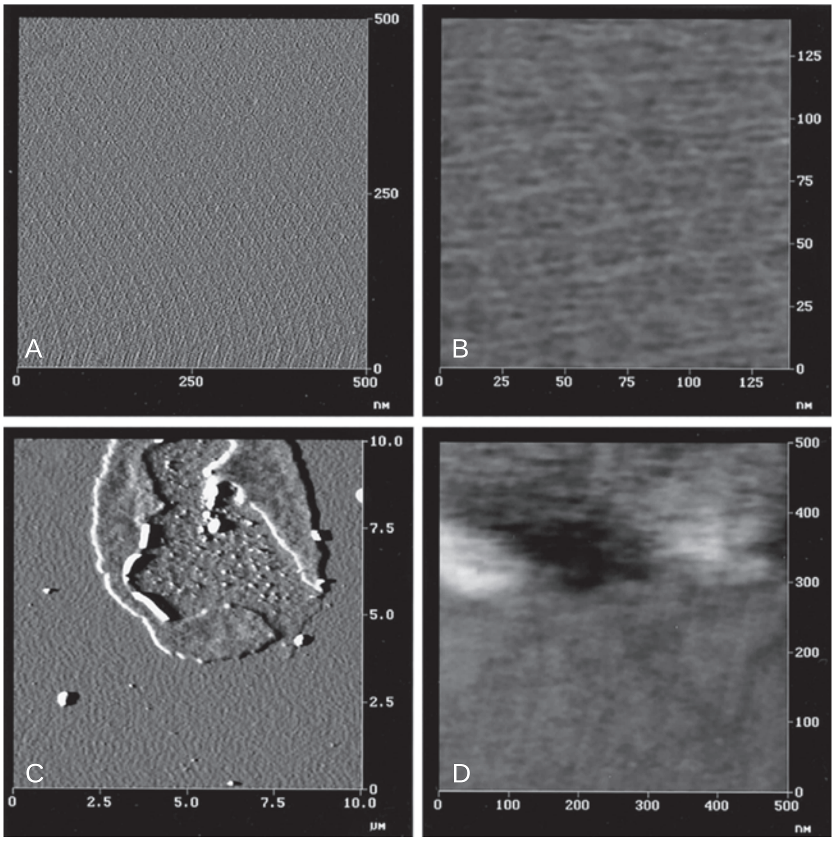


Figure 139.4 Sequential effects of β_2-glycoprotein I (β_2GPI) and antiphospholipid syndrome (APS) monoclonal antibodies on Annexin A5 crystal structure. Atomic force microscopy images from a dynamic imaging experiment showing the effect of APS monoclonal antibodies on a preformed annexin A5 crystalline lattice. The addition of β2GPI alone has no discernible effect on the lattice as observed using amplitude imaging (A) and at higher magnification with height imaging (B). After addition of APS monoclonal antibodies (anti-β2GPI IgG), circular deformities occur indicating disruption of the crystal lattice as seen in an amplitude image (C). A height image (D) within the circular deformities shows dark areas representing portions of the surface that have lost annexin A5 coverage, near light areas representing elevated structures, presumably antibody–antigen complexes. *(Reprinted from Rand JH, Taatjes DJ. Reimagining the antiphospholipid syndrome, an enigmatic thrombophilic disorder, through the looking glass of microscopic imaging. Histochem Cell Biol. 2018;150(5):529–543.)*

The current Sydney criteria for the diagnosis of APS identify LA, aCL, IgG and IgM, and anti-β2GPI IgG and IgM as the key diagnostic assays. To qualify for the diagnosis of APS, positive results must be obtained on two or more occasions at least 12 weeks apart (see Table 139.1).

"Criteria" Antiphospholipid Syndrome Assays

Lupus Anticoagulant Tests

LA tests are performed in a variety of configurations, all of which share the purpose of detecting antibody-mediated inhibition of phospholipid-dependent blood coagulation reactions.

An updated guidance on LA testing was published by the LA Subcommittee of the International Society on Thrombosis and Haemostasis Scientific and Standardization Committee (ISTH/SCC) in 2020.

LA tests include the dilute Russell viper venom time (dRVVT), modifications of the aPTT with LA-sensitive and LA-insensitive reagents, the kaolin clotting time (KCT), the dilute prothrombin time (dPT) which was previously known as the tissue thromboplastin inhibition time, the hexagonal phase array test, and the ecarin-taipan clotting time. The dRVVT and the silica clotting time, a form of the aPTT, are the most widely performed tests. Interlaboratory comparison studies demonstrate agreement on identification of plasmas containing strongly positive LA activity, but disagreement about samples with weaker activity (these are missed in approximately half the cases) and misdiagnosis of coagulation factor-deficient LA-negative plasmas as being LA-positive.

Despite the limitations, LA positivity is more sensitive and specific for the presence of aPL antibodies than aCL and anti-β2GPI assays. In a systemic review of 7000 patients and 25 studies, the presence of an LA was found to have a strong association with thrombosis, both in patients with and without SLE and regardless of site and type of thrombosis. Also, LA had a stronger association than aCL antibodies for thrombosis. Meanwhile, medium to high titer aCL antibodies were associated with cerebral stroke and myocardial infarction and not deep venous thrombosis. The mean odds ratios (ORs) for thrombosis were 11 for LA, 3 for high-titer aCL antibodies, and 1.6 for elevated levels of aCL antibodies. [LRW2] Furthermore, the Predictors of Pregnancy Outcome: Biomarkers in Antiphospholipid Antibody Syndrome and Systemic Lupus Erythematosus (PROMISSE) study demonstrated that of the available aPL markers, a positive LA was the single strongest predictor of adverse pregnancy outcome in patients with APS. However, routine LA screening is unlikely to benefit the general population since the high relative risks reported in these studies should be seen in the context of the low baseline risks of thrombosis and adverse pregnancy outcomes in disease-free individuals. Nevertheless, LA testing and aPL assay testing may be beneficial to ascertain risk in patients with a high baseline thrombotic risk, such as patients with SLE or a previous thrombosis.

Patient Selection for Lupus Anticoagulant Testing

The recommendations for patient selection for LA testing were expanded in the 2020 ISTH/SSC guidance and are summarized in Table 139.4.

Prior to ordering testing for patients meeting the ISTH/SSC guidance, clinicians should consider that it is preferable for patients

TABLE 139.4 **Patient Selection for Lupus Anticoagulant Testing**

1. LA testing should be performed together with testing for anticardiolipin (aCL) and anti-β_2 glycoprotein I (aβ2GPI) to assess the risk profile in patients who are likely to have antiphospholipid syndrome (APS):
    - younger patients (<50 years) with unprovoked venous thromboembolism (VTE)
    - VTE at unusual sites
    - younger patients (<50 years) with ischemic stroke, transient ischemic attack, or other evidence of brain ischemia
    - arterial thrombosis in other sites in younger patients (<50 years)
    - microvascular thrombosis
    - recurrent VTE unexplained by subtherapeutic anticoagulation, patient nonadherence, or malignancy
    - pregnancy morbidity: fetal loss after 10 weeks, recurrent early (first trimester) miscarriages, prematurity (<34 weeks' gestation) associated with severe (pre)eclampsia, HELLP syndrome, placental insufficiency (fetal growth restriction), stillbirth
    - systemic lupus erythematosus: testing for lupus anticoagulant (LA) is part of the diagnostic criteria and contributes to risk assessment
2. LA testing could be considered in the following situations:
    - immune thrombocytopenia, particularly with presence of arthralgias or arthritis, hair loss, sun sensitivity, mouth ulcers, rash, or thromboembolism
    - livedo reticularis, particularly with presence of symptoms of other systemic autoimmune diseases or mild thrombocytopenia
    - younger patients (<50 years) with non-criteria clinical manifestations (i.e., those not included in the Sydney criteria) such as cognitive dysfunction and valvular heart disease with presence of evidence of other systemic autoimmune diseases
    - patients of younger age (<50 years) following provoked VTE when the provoking environmental factor is disproportionally mild
    - patients with unexplained prolonged aPTT as an incidental finding

not to be tested in the setting of an acute thrombotic event and that any such testing should be interpreted with caution because false positive and negative results can occur. Whenever possible, LA testing should not be performed in patients receiving anticoagulant treatment because false positives and negatives can occur. When there is a need to perform testing, it is best to suspend anticoagulant treatment when feasible. If patients are tested during treatment with low molecular weight heparin (LMWH), it is recommended that samples be taken, when possible, at least 12 hours after the last dose of LMWH and that the anti-Xa activity be checked concurrently. Direct oral anticoagulants (DOACs) are especially problematic in causing false positive LA tests and, whenever possible, should be held prior to testing.

Choosing LA assays.The ISTH/SCC has proposed specific criteria for standardizing the diagnosis of LA. When APS is suspected on clinical grounds, it is recommended that laboratories perform two different versions of LA tests. The dRVVT is widely used in clinical laboratories and is believed to be specific for detecting LA in patients at high risk for thrombosis who are not receiving anticoagulant therapy. aPTT tests performed with silica as an activator and low PL content were recommended as the second test of choice because of their sensitivity for LA, however, aPTT assays utilizing ellagic acid as the activator may also be used (see Chapter 127).

Dilute Russell Viper Venom Time. The dRVVT is considered one of the most sensitive LA tests. The assay uses Russell viper venom (RVV) in a system containing limiting quantities of diluted rabbit brain phospholipid. RVV activates factor X, leading to clotting. aPL antibodies which prolong the dRVVT by interfering with the assembly of the prothrombinase complex, an effect reversed by the addition of excess phospholipid (sometimes referred to as a "confirmatory test"). To ensure that prolongation of the clotting time is not the result of a factor deficiency, the procedure includes mixing of patient plasma with control plasma. Anticoagulant therapy with heparin, warfarin or DOACs can yield falsely abnormal test results.

Activated Partial Thromboplastin Time. The finding of a prolonged aPTT in an otherwise healthy individual without a history of bleeding is often attributable to a LA. aPTT reagents vary in their sensitivity to LA, so it is important to know the characteristics of the particular reagent that is being utilized. The LA needs to be differentiated from antibodies directed against specific coagulation factors (see Chapter 127) and from anticoagulant medications. In addition to utilizing specific assays to exclude these possibilities, the clinician can check whether the aPTT is normal if a LA-insensitive aPTT reagent is used. Mixing and incubating the test plasma with normal plasma may be helpful in differentiating LAs from coagulation factor inhibitors. aPTTs performed on mixtures of normal plasma and plasma containing a factor VIII inhibitor usually require incubation for 1 to 2 hours at 37°C to exhibit prolongation, whereas LA-containing plasmas typically prolong the aPTT immediately, without requiring incubation. The clinician should be aware that both types of anticoagulants (i.e., LA and specific coagulation factor inhibitors) may coexist and yield a confusing laboratory picture. LAs may also cause artifactual decreases in contact activation pathway coagulation factor levels because those assays are based on the aPTT; these patients are sometimes misdiagnosed as having multiple coagulation factor deficiencies. This problem can be identified by noting whether there is non-parallelism between dilutions of the plasma and the standard curve used for the assay, or by using an aPTT reagent that is insensitive to LA.

Anticardiolipin IgG and IgM Antibody Assays

The development of the quantitative aCL antibody radioimmunoassay and subsequent enzyme-linked immunosorbent assay (ELISA) was the single advance that led to the identification of APS as a new syndrome. Although aCL ELISAs are sensitive, they are not specific for APS, especially when present at low positive levels or in patients with certain infections. Patient with syphilis can have a false positive aCL antibody test. Alternatively, patients with aCL positive antibodies can have a false positive syphilis Venereal Disease Research Laboratory (VDRL) test since cardiolipin is used as an antigen target to detect non-specific antibodies released by spirochetes. Testing guidelines recommend using β2GPI-dependent cardiolipin targets to avoid detection of non-cofactor related aCL produced by infections or drugs.

aCL may be detected by ELISA or newer methods such as chemiluminescence analysis (CLIA) or multiline dot assay (MLDA). The cutoffs for medium to high aCL titers (i.e., 99th percentile) are greater than 40 IgG Phospholipid Units (GPL)/IgM Phospholipid Units (MPL) in ELISA and greater than 20 Chemiluminescent Units (CU) in CLIA.

High levels of aCL antibodies are associated with an increased risk of venous thromboembolism, myocardial infarction, stroke, and recurrent pregnancy loss. In a 10-year follow-up study, 50% of initially asymptomatic patients with persistently elevated aCL antibodies went on to develop APS. The presence of elevated titers of aCL antibodies 6 months after an episode of venous thromboembolism is a predictor of an increased risk of recurrence and of death.

It is very important for the clinician to recognize that **the majority of patients with elevated levels of aCL antibodies on routine screening do not have APS**. Many individuals have elevated antibody levels in response to infections; these antibodies have not been associated with thrombotic complications. In various studies, the prevalence of positive immunoassays in the asymptomatic "normal" population has range from 3% to nearly 20%. Therefore, it is important to repeat testing after 12 weeks to determine whether there is persistent positivity.

Anti-β2-Glycoprotein I IgG and IgM Antibody Assays

β2GPI is the major protein cofactor recognized by aPL antibodies. The protein is composed of five homologous domains and assumes a closed conformation in solution and an open conformation when bound to phospholipid membranes (see Fig. 139.2). Anti-β2GPI antibodies that bind to the circular closed form generally recognize epitopes between domains one and five, while in the open form, anti-β2GPI antibodies interact with the exposed domain one. Anti-β2GPI ELISAs are less sensitive but more specific for APS than aCL antibody assays. Although antibodies against β2GPI are usually found in conjunction with aCL and anti-phosphatidylserine antibodies, some patients with APS only have antibodies against β2GPI. Despite their higher specificity (98%), anti-β2GPI assays cannot be relied upon as a stand-alone test for APS because of their low sensitivity (40% to 50%) and concurrent testing for both antibodies, along with LA is advised. Antibodies against β2GPI can be detected by ELISA, CLIA, or MLDA. The determined cutoffs for medium to high titers (i.e., 99th percentile) are greater than 40 GPL/MPL in ELISA and greater than 20 CU in CLIA.

In a systematic literature review, 34 of 60 studies showed a significant association between anti-β2GPI antibodies and thrombosis. Anti-β2GPI antibodies were more often associated with venous than arterial thrombotic events and were independent risk factors for venous thrombosis.

Considerations and Limitations of Laboratory Immunoassays

Although a variety of assays are used, including more advanced and automated systems, there is no consensus on a "gold standard" for the detection of aCL and anti-β2GPI antibodies. Studies have shown poor agreement between commercially available immunoassays for detecting aCL and anti-β2GPI IgG/IgM antibodies. Therefore, it is recommended to use the same testing system for patient diagnosis and follow-up. In addition, guidelines recommend laboratories determine their own 99th percentile cutoffs for in-house assays using a population of at least 40 healthy volunteers.

The interpretation of immunoassays should also take into consideration the identified isotype. The aCL and anti-β2GPI IgG isotype have a stronger association with thrombosis than IgM. Although aCL and anti-β2GPI IgM isotypes have been associated with pregnancy morbidity and thrombosis in some reports, these associations have not been confirmed due to lack of paired IgG and IgM results. To not "miss" diagnosing APS patients, the guidelines advise continued measurement of IgG and IgM, with the presence of aCL and anti-β2GPI of the same isotype reinforcing the probability of APS.

Multipositivity for Antiphospholipid Tests and Other Scoring Systems for Clinical Risk

Strong positivity for more than one of the APS criteria assays (LA, aCL, and anti-β2GPI) has been correlated with increased risk for developing clinical events in several retrospective studies and in one prospective study. APS patients who are positive for all three tests (LA, aCL, and anti-β2GPI), referred to as "triple-positive" patients, have the highest risk of thrombosis. This risk is progressively lessened in double-positive (LA-negative) and single-positive patients.

In pregnant women with APS, triple APS assay positivity, with or without previous thromboembolism, is associated with a higher probability of adverse neonatal outcome than double or single aPL antibody-positivity and no thrombosis history. Multipositivity, but not single positivity, for aPL antibodies was associated with antenatal and postnatal deep vein thrombosis (DVT).

Both symptomatic and asymptomatic patients with persistent triple-positive laboratory results (LA-positive, aCL and anti-β2GPI of high titer, double-positive for LA, aCL and/or anti-β2GPI of high titer, or single positive for LA or persistently high aPL titers) should be considered high-risk for future manifestations of APS. Finally, single positivity for aCL IgG or IgM in low-medium titers or anti-β2GPI IgG or IgM in low-medium titers should be considered lower risk for APS but treatment may be warranted if such patients develop thrombosis or pregnancy complications or have other high-risk factors.

The global APS score (GAPSS) is a clinical scoring system that assigns points for the presence of six features—arterial hypertension (1 point), hyperlipidemia (3 points), LA (4 points), aCL antibodies (5 points), anti-β2GPI antibodies (4 points), and antiphosphatidylserine/prothrombin (aPS/PT) antibodies (3 points). GAPSS scores ≥ 10 have the best diagnostic accuracy. Higher GAPSS values are also associated with recurrent thrombotic events. A complementary version, the adjusted GAPSS (aGAPSS), which excludes aPS/PT, was also found to be higher in patients with recurrent thrombosis (arterial or venous) than in those without recurrence. Patients with recurrent arterial, but not venous, thrombosis had higher aGAPSS. A meta-analysis of both the GAPSS and aGAPSS scoring systems showed the highest scores in patients with arterial thrombosis and recurrence. Complement markers including platelet bound C4d (measured by flow cytometry) and low C3 have been included in some composite risk scores.

"Non-Critera" Antiphospholipid Syndrome Assays

Clinicians may consider testing selected patients with assays that are not included within the formal diagnostic criteria. An example would be a patient with SLE or another autoimmune disorder who has clinical manifestations that suggest APS but also has negative assays for aCL and aβ2GPI IgG and IgM along with negative LA tests.

IgA Antibodies Against Cardiolipin and β_2-Glycoprotein I

Although aPL antibodies of IgA isotype (either aCL or anti-β2GPI) were not included in the international consensus statement on the criteria for APS classification, testing for the IgA isotype (particularly IgA anti-β2GPI) was recommended in cases where APS is suspected but the IgG and IgM tests are negative. Anti-β2GPI IgA antibodies were reported to be associated with thrombosis. A recent retrospective case-control study of 56 patients with isolated anti-β2GPI IgA found that patients with this marker had significantly more thromboembolic events and higher polyclonal IgA levels than controls.

ANTIPHOSPHOLIPID SYNDROME TESTING PEARLS

- Laboratory testing for aPL antibodies should not be included in test panels for screening healthy individuals.
- Testing should be limited to patients with the thrombotic and/or pregnancy manifestations of the disorder or those with autoimmune diseases such as SLE.
- Weakly positive tests for aPL are unlikely to be clinically relevant.
- Transient aPL antibodies can result from infection (including HIV and COVID-19), liver disease, cancer, and medications such phenothiazines, quinine, procainamide, oral contraceptives, and TNF inhibitors.
- Asymptomatic triple-positive patients appear to be at high risk for thrombosis and should be considered for prophylactic therapy.

Anti-β2GPI IgA was associated with an increased risk of thromboembolic events in patients with SLE. The significance of aCL IgA for the development of thrombotic complications is still under investigation. In a study of 192 APS patients, 42% were positive for aCL IgA, while in 90 seronegative APS patients, 12% were positive for aCL IgA.

Antiphosphatidylserine/Prothrombin Assays

Prothrombin is considered the second major cofactor for aPL antibodies after β2GPI. Antibodies against phosphatidylserine are reported to correlate with APS more than aCL antibodies, particularly in patients with arterial thrombosis. Accordingly, this noncriteria assay is included in the GAPSS scoring systems. The data for individual antiprothrombin (aPT) and antiphosphatidylserine (aPS) assays have been mixed in terms of their utility for identifying patients with APS and their correlation with other APS criteria assays. Antibodies against the combined phosphatidylserine-prothrombin complex (aPS/PT) have been shown to be closely associated with APS and LA. The presence of aPS/PT antibodies was strongly correlated with the presence of LA, had a higher sensitivity and specificity than antiprothrombin antibodies alone for the diagnosis of APS, and exhibited comparable sensitivity and specificity to aCL for the diagnosis of APS. In a recent study that included 168 APS patients, 86% were positive for aPS/PT antibodies and 50% of the seronegative APS patients were positive. There was a significant association of aPS/PT IgG with thrombotic events and aPS/PT IgG and IgM with pregnancy loss. It has been proposed that the addition of aPS/PT IgG/IgM assays to current APS criteria could classify an additional 12% patients as having APS and an additional 43% patients as having obstetric APS. The apparent high diagnostic performance of this test may render it useful for identifying patients who are currently classified as APS negative, as an aid for risk assessment, and as an adjunct in situations where LA testing is unavailable.

Annexin A5 Resistance Assay and Anti-Annexin Assays

Annexin A5 (often referred to as annexin V) is a calcium-dependent protein that binds to anionic phospholipids on syncytiotrophoblast and vascular endothelium and forms a protective shield. This shield is disrupted by anti-β2GPI/β2GPI complexes, thereby exposing more anionic phospholipids and accelerating coagulation reactions. The annexin A5 resistance (A5R) assay was designed to measure disruption of the annexin A5 shield. The failure of annexin A5 to sufficiently prolong coagulation times because of interference by aPL antibodies is referred to as A5R. This assay has been correlated with an increased risk for thrombosis and pregnancy complications in patients with aPL. A study of 750 such patients demonstrated significantly lower A5R values in APS patients with a history of thrombosis than in aPL antibody-positive patients without a history of thrombosis, aPL antibody-negative patients with a thrombosis history, and healthy controls (Fig. 139.5). Lower A5R values also correlated with multipositivity for APS criteria assays and increased thrombotic risk. Clinicians should be aware that this assay falls into the category of laboratory developed tests (LDTs) and is not commercially available.

Anti-annexin A2 antibodies are significantly elevated in APS patients compared with healthy donors and patients with SLE without thrombosis.

Anti-Domain I of β_2-Glycoprotein I Assay

This immunoassay identifies IgG antibodies against a specific amino acid sequence, G40-R30, within domain I (D1) of β2GPI. Anti-D1 antibodies correlate better thrombosis and pregnancy loss than other aPL antibodies. Anti-D1 and LA positivity are associated with venous thrombosis in APS patients. A recent analysis of 198 samples from patients with a variety of autoimmune disorders revealed that the 52 patients with anti-β2GPI IgG antibodies could be divided into two groups: one whose antibodies recognized D1 alone and another

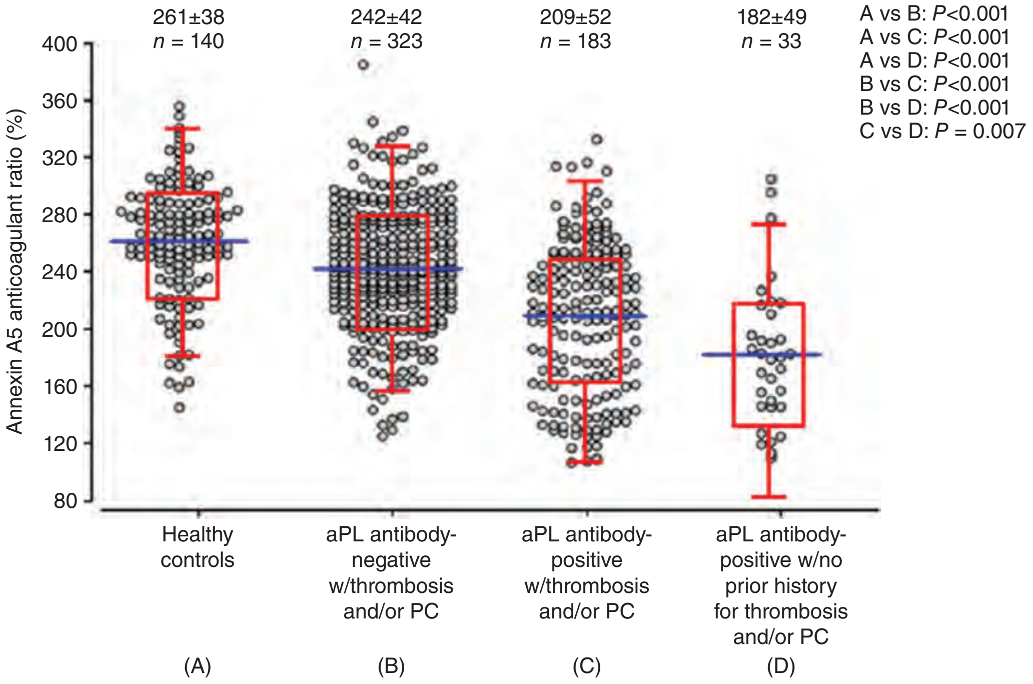


Figure 139.5 Antiphospholipid (aPL) antibody-positive patients have lower annexin A5 anticoagulant ratio (A5R) values and an increased risk of thrombosis and/or pregnancy complications. In patients with a history of thrombosis and/or pregnancy complications (PC), aPL antibody-positive patients had a significantly lower mean A5R value (mean ±SD, 209 ±52%; *n* = 183) than aPL antibody-negative patients (mean ± SD, 242 ± 42%; *n* = 323; *P* < .001) and disease-free patients (mean ± SD, 261 ±38%; *n* = 140; *P* < .001). Lower A5R values have been associated with an increased risk of thrombosis and/or pregnancy complications. In patients with no prior history of thrombosis and/or PC, aPL antibody-positive patients also had significantly lower A5R values (mean ± SD: 182 ± 49%; *n* = 33). Based on the findings in the prospective cohort, this group may be at increased risk of a future thrombosis and/or PC. *(From Wolgast LR, Arslan AA, Wu X-X, Beyda JN, Pengo V, Rand JH. J Thromb Haemost. 2017;15:1412–1421.)*

whose antibodies reacted with all domains; only the former group had positivity for LA and an increased risk of thrombosis.

Anti-D1 has a sensitivity of 85% and a specificity over 99% for APS, which may render it a valuable test for APS diagnosis. In a recent study, patients tested for anti-D1 IgG and aPS/PT IgG/IgM showed high positive predictive value for the diagnosis of APS. Positivity for anti-D1 was also associated with triple positivity.

Complement Testing

The modified Ham test, a functional indicator of complement activation, was reported to be positive in 88% of patients with CAPS, 69% of APS patients within a year of a thrombotic event, and 36% of APS patients overall, but only in 7% of patients with SLE. In 201 women with obstetric APS, 49% had decreased levels of complement proteins, either low C4 or C3 levels or a reduction in both. In pregnant APS patients, low C3 and/or C4 levels and evidence of complement activation (high plasma C5a and C5b-9 levels) were correlated with adverse pregnancy outcomes. Anti-C1q antibodies also may contribute to recurrent pregnancy loss and APS.

Genomic and Biomarker Testing in Antiphospholipid Syndrome

A recent study identified 16 genes that appear to contribute to thrombotic primary APS. These genes include platelet factor 4 variant 1 (PF4V1), selectin P (SELP) , TLR2 TLR4, serpin family member 1 (SERPINE1), apolipoprotein H (APOH, a synonym for β2GPI), integrin subunit alpha 2 (ITGA2) , platelet glycoprotein Ib alpha chain (GP1BA) , coagulation factor II thrombin receptor (F2R)), F2R like trypsin receptor 1 (F2RL1), TFPI, coagulation factor III tissue factor (F3), vascular endothelial growth factor A (VEGFA), fms-related tyrosine kinase1 (FLT1), and TNF-ALPHA.

Familial studies show a consistent genetic predisposition for APS with DR4 and DRw53 antigens. Population studies reveal an association between several HLA antigens and both primary APS and APS associated with SLE, although other studies suggest that these are distinct entities.

aPL antibodies modulate the expression of several prothrombotic genes in monocytes and endothelial cell lines. They induce the phosphorylation of MEK1/ERK proteins, p38 MAP kinase-dependent nuclear translocation, and activation of NFκB/Rel proteins, which upregulate the expression of tissue factor, IL-6, IL-8, and the inducible nitric oxide synthase (iNOS). Proteomic studies reveal that aPL antibodies alter the protein profile of APS monocytes, including dysregulated expression of annexin A1 (AnxA1), annexin A2 (AnxA2), ubiquitin-like protein Nedd8, Rho A protein, protein disulfide isomerase (PDI), and heat shock protein-60 (Hsp60). These proteins are associated with a hypercoagulable state, as well as with autoimmune-related responses.

APS patients have been reported to have polymorphisms in components of the hemostatic system that may promote thrombosis, including PAI-1, prothrombin, TFPI, TNFα, GP1b, GPIa/IIa, P-selectin, PSGL-1, β2GPI, and TLR4.

CLINICAL MANIFESTATIONS OF THE ANTIPHOSPHOLIPID SYNDROME

The clinical manifestations of APS can be categorized into “criteria manifestations” that are included in the formal definition of the disorder, and “non-criteria manifestations.” The criteria manifestations include thrombotic events, obstetric APS, and CAPS, while the non-criteria manifestations include other pathologic conditions that have been associated with aPL antibodies but have not yet been included in the classification of “definite APS” by the expert consensus groups.

Criteria Manifestations of Antiphospholipid Syndrome

Systemic Vascular Thrombosis

The most common manifestations of APS are deep vein thrombosis of the lower extremity, pulmonary embolism, and stroke/transient ischemic attack. These may occur spontaneously or in the presence of predisposing factors such as estrogen therapy, oral contraceptives, pregnancy, the postpartum state, surgery, or trauma. The usual age at the initial presentation with thrombosis is 35 to 45 years. Except for patients with SLE, men and women are equally susceptible to thrombotic manifestations. Not surprisingly, occasional patients with APS-associated venous thrombosis—but generally not with arterial thrombosis—have concurrent hereditary thrombophilic conditions such as heterozygosity for the factor V Leiden variant. Other sites of venous thrombosis include the thoracic veins (superior vena cava, subclavian or jugular veins), cerebral and/or retinal veins, and abdominal and/or pelvic veins. Sites of arterial thromboembolic events include myocardial infarction, nonatherosclerotic coronary artery occlusion, pulmonary hypertension attributed to thrombotic occlusion, and occlusions of abdominal, renal, and retinal arteries. In one study of patients with aPL antibodies and radiologic evidence of thrombosis, 59% had thrombosis limited to the venous circulation, 28% had only arterial thrombosis, and 13% had both types of events.

The most significant risk factor for recurrent thromboembolism in APS is a history of a prior thromboembolic event. The risk of recurrence after a first episode of venous thromboembolism in patients with aCL antibodies is about 30% at 4 years and is likely to be higher in those with a LA or with anti-β2GPI D1 antibodies.

Stroke and Transient Ischemic Attack

Ischemic stroke occurs relatively frequently in APS and can be an initial manifestation in approximately 25% of APS patients. APS should be suspected when young patients present with a transient ischemic attack or a stroke without other risk factors. Children presenting with an APS-associated stroke tend to have venous rather than arterial occlusion with 7% of children presenting with cerebral vein thrombosis in a multinational childhood registry. The CAPS registry reported that 56% of CAPS patients had cerebral manifestations and stroke was responsible for 19% of CAPS-related deaths.

Elevated levels of any of the three APS criteria assays (aCL, β2GPI, and LA) are associated with an increased risk for stroke. In women with LA positivity and other modifiable risk factors such as smoking or use of oral contraceptives, the risk of stroke is increased (from 43- to 201-fold with oral contraceptives and 87-fold with smoking). SLE and APS patients with valvular heart lesions are also at increased risk of cerebrovascular disease. An echocardiography should be considered for APS patients with stroke since a high proportion of have cardiac valvular abnormalities (see below).

Increased risk of recurrence has been demonstrated in multi-positive APS patients with an LA and either aCL or β2GPI antibodies. In addition to the immediate impact, recurrent stroke may lead to multi-infarct dementia that can mimic other causes of dementia.

Reproductive Manifestations

Pregnant women with APS tend to present with a history of recurrent (i.e., three or more) miscarriages. Studies have shown that up to 38% of pregnant women with recurrent fetal loss have elevated aPL antibodies. Pregnant women with elevated aPL antibodies have significantly more obstetric complications, including pre-eclampsia, placental abruption, miscarriage, prematurity, intrauterine fetal demise, intrauterine growth restriction, and oligohydramnios, than aPL antibody-negative pregnant women. In approximately half of the patients, the pregnancy loss occurs in the first trimester; other patients present with later loss, mostly in the second trimester, but some even later, including stillbirth. Pregnant women with APS are also more prone to develop deep vein thrombosis during pregnancy or the puerperium. CAPS occurs rarely in pregnant patients.

As mentioned earlier, routine aPL antibody screening of asymptomatic obstetrical patients is not warranted because of the high frequency of false positives with the current tests and the absence of any specified treatment for asymptomatic positives. Most studies have estimated the prevalence of aPL antibodies among general obstetric populations to be approximately 5% or less and most of these aPL antibody-positive patients are asymptomatic. A history of previous pregnancy loss, obstetrical complications, or thrombosis is a better predictor for future pregnancy loss than the laboratory abnormalities.

Studies have been mixed as to whether aPL antibodies cause infertility; it has been suggested that aPL antibodies may impair trophoblastic differentiation, proliferation, and migration, which can lead to implantation failure. However, the current consensus is that aPL antibodies do not contribute to early reproductive failure (i.e. infertility) and that patients with infertility do not need to be screened for aPL antibodies.

Non-Criteria Manifestations of Antiphospholipid Syndrome

Cardiovascular Manifestations

Approximately 65% of patients with APS have heart valve abnormalities detected by echocardiography. These include leaflet thickening, irregular nodules and/or non-bacterial vegetations (i.e., Libman-Sacks endocarditis), and severe valve dysfunction (regurgitation, stenosis) in the absence of a history of rheumatic fever or infective endocarditis. Valvular heart disease in APS patients can lead to heart failure, valve replacement and death. Emboli from valve vegetations may result in stroke. APS should be considered in patients without the more typical risk factors for coronary artery disease and in patients with evidence of thrombotic or embolic coronary occlusion who lack angiographic evidence of atherosclerotic disease.

Deposits of immunoglobulins, including aCL antibodies, and complement components are commonly found on the affected valves.

In women who used oral contraceptives or smoked, the presence of a LA increased the risk for myocardial infarction (5- to 22-fold with oral contraceptives and 34-fold with smoking). Antiprothrombin antibodies were reported to be a predictor of myocardial infarction in middle-aged men, particularly those with additional risk factors.

Neurologic Manifestations

In addition to stroke, other neurologic abnormalities associated with aPL antibodies include migraine, seizures, chorea, Guillain-Barré syndrome, transient global amnesia, dementia, diabetic peripheral neuropathy, orthostatic hypotension, acute sensorineural hearing loss, and transverse myelitis. Approximately 40% to 60% of multiple sclerosis patients are positive for at least one aPL antibody. The presence of aPL antibodies in multiple sclerosis patients may reflect disease progression or a disease flare.

Patients with psychotic disorders have an increased prevalence of LA and aCL antibodies, even in the absence of treatment with antipsychotic drugs, such as chlorpromazine, that can be associated with these antibodies. Approximately 25% to 32% of patients with acute psychosis have been reported to be aPL antibody positive. Patients with chronic schizophrenia have an increased frequency of aPL antibodies. Older patients with a history of cerebral ischemia and triple aPL antibody positivity are at greatest risk for neuropsychiatric disorders.

Cutaneous Manifestations

Cutaneous manifestations may be the first sign of APS. Livedo reticularis is relatively common, occurring in 24% of a series of 1000 aPL patients, and occasionally presents in a necrosing form. Noninflammatory vascular thrombosis is the most frequent histopathologic feature. Necrotizing vasculitis, livedoid vasculitis, superficial vein thrombosis, cutaneous ulceration and necrosis, erythematous macules, purpura, ecchymoses, painful skin nodules, subungual splinter hemorrhages, anetoderma (macular atrophy), discoid lupus erythematosus, and cutaneous T-cell lymphoma have all been reported.

Pulmonary Manifestations

Patients with APS may present with in situ thrombosis in pulmonary vessels and pulmonary hypertension. Studies have shown a high rate of aPL antibody positivity (15%) in patients with chronic thromboembolic pulmonary hypertension. Most patients with CAPS have dyspnea, and most of these individuals have ARDS.

Hepatic and Gastrointestinal Manifestations

The liver is the most commonly affected abdominal organ in APS. Occlusion of hepatic vessels supplying the biliary tree may present as acute acalculous cholecystitis with gallbladder necrosis.

aPL antibody levels are frequently elevated in patients with chronic liver disease. In one prospective study of patients with liver disease, approximately half of the patients with alcoholic liver disease and one third of the patients with chronic hepatitis C had elevated aPL antibody levels. The frequency was even higher in patients with more severe cirrhosis. Primary biliary cirrhosis has also been associated with aPL antibodies.

Gastrointestinal manifestations of APS include esophageal necrosis with perforation, intestinal ischemia and infarction, pancreatitis, colonic ulceration, and giant gastric ulcerations. APS has been reported in patients with mesenteric inflammatory veno-occlusive disease and with mesenteric and portal vein obstruction.

Renal and Adrenal Abnormalities

Renal involvement in APS presents with a variety of manifestations, including renal artery thrombosis or stenosis, renal vein thrombosis, allograft loss due to thrombosis after kidney transplantation, and injury to the renal microvasculature, also known as APS nephropathy. This nephropathy is characterized by fibrous intimal hyperplasia, focal cortical atrophy, and thrombotic microangiopathy. The most frequent clinical features are hypertension, microscopic hematuria, proteinuria (ranging from mild to nephritic levels), and renal insufficiency.

CLINICAL PEARLS

- APS is an acquired thrombophilic disorder associated with venous or arterial thrombosis and/or pregnancy complications attributable to placental insufficiency accompanied by l antiphospholipid antibodies in the blood.
- The syndrome is identified by persistent positivity of criteria laboratory tests including high titers of aCL and anti-β2GPI IgG or IgM antibodies detected by immunoassays and by positive LA.
- Rare patients have a CAPS in which there is disseminated thrombosis in large and small vessels, often after a triggering event such as infection or surgery, and often with multiorgan ischemia and infarction.
- Selected patients may be suspected of having APS without necessarily meeting the strict investigational criteria.

With respect to endocrine manifestations, in one series hypothyroidism was found in 22% of APS patients and thyroid antibodies in 18%. Adrenal insufficiency has been reported as the most significant endocrine manifestation of APS and adrenal crisis as a complication of adrenal insufficiency has been reported in SLE and APS patients. Acute adrenal failure secondary to bilateral infarction of the adrenal glands has been reported as a first manifestation of primary APS. In a recent study of 105 cases of SLE and APS patients with adrenal insufficiency, the mortality rate was 4%.

Retinal Abnormalities

As many as 80% of APS patients have ocular involvement. In absence of known risk factors, APS should be suspected in patients with recurrent ocular thrombosis and in younger patients with central retinal artery or vein occlusion. An ophthalmologic assessment is recommended for all patients with APS. The diagnosis of APS retinopathy should be suspected in patients with diffuse retinal vaso-occlusion, particularly when characterized by involvement of arteries and veins, neovascularization at presentation, and symptoms of systemic rheumatologic disease. Elevated aPL antibody levels were present in 5% to 33% of patients with retinal vein occlusion. Cilioretinal artery occlusion, optic neuropathy, and severe vaso-occlusive retinopathy have been described with APS.

Hematological Abnormalities

Approximately 20% to 53% of patients with APS have varying degrees of thrombocytopenia. The decrease in platelet count is generally mild or moderate, and is rarely significant enough to cause bleeding complications or affect anticoagulant therapy. Thrombocytopenia in patients with elevated aPL antibodies has been associated with arterial thrombosis.

Patients with aPL antibodies can develop antibodies against glycoproteins on platelets (GPIIb/IIIa, GPIb/IX, GPIa/IIa, and GPIV) and are at risk for immune thrombocytopenic purpura (ITP). ITP patients who are triple positive for aPL antibodies have also been shown to have lower platelet counts and are at risk for thrombosis with concurrent steroid therapy (see Chapter 129). In a prospective cohort study, the 5-year thrombosis-free survival rates for aPL antibody-positive and aPL antibody-negative ITP patients were 39% and 98%, respectively, suggesting that thrombocytopenia itself is not protective against thrombosis.

By themselves, aPL antibodies are not associated with defective hemostasis. Therefore, patients with APS who exhibit a bleeding tendency should be evaluated for a concurrent acquired coagulopathy. In APS patients with normal platelet counts, the differential diagnosis for a bleeding tendency should include hypoprothrombinemia, acquired thrombocytopathy, acquired factor VIII inhibitor, and acquired von Willebrand syndrome (see Chapters 126 and 127).

Catastrophic Antiphospholipid Syndrome

Rare patients present with CAPS, which is characterized by severe widespread vascular occlusion and a high mortality. The formal diagnosis of CAPS requires evidence of involvement of at least three organs, systems and/or tissues, development of manifestations simultaneously or within 1-week, histopathological evidence of small vessel occlusion, and presence of aPL antibodies (Table 139.2).

In contrast to the diagnostic criteria for APS, the diagnostic criteria for CAPS accept histological evidence of vasculitis together with thrombosis. Patients with CAPS may present with severe multiorgan ischemia/infarction usually with concurrent microvascular thrombosis, extensive venous thromboembolism, respiratory failure, stroke, abnormal liver enzymes, renal impairment, adrenal insufficiency, and cutaneous infarction. The respiratory failure is usually due to acute respiratory distress syndrome (ARDS) and diffuse alveolar hemorrhage. There may be laboratory evidence of disseminated intravascular coagulation (see Chapter 137).

According to the CAPS Registry at the time of writing, most CAPS patients are female (69%) and in their late 30 s (mean age of 38 years), but patients can present at any age (range 0 to 85 years). In 46% of CAPS cases, the catastrophic event was their first manifestation of APS. Precipitating factors for CAPS include infections (present in 49% of the cases), surgical procedures (17%), malignancies (16%), anticoagulation withdrawal or low international normalized ratio (8%), pregnancy complications (8%), drugs (5%), and active SLE(3%); 60% of patients had primary APS, 30% had SLE, 4% had SLE-like disease, and 6% had other autoimmune diseases.

The most frequently affected organs were the kidneys (73% of episodes), followed by the lungs (60%), brain (56%), heart (50%), and skin (47%) (Fig. 139.6). Other organs affected included peripheral vessels, intestine, spleen, adrenal glands, pancreas, retina, and bone marrow.

Thrombocytopenia was detected in 67% of cases and one third of patients had evidence of hemolysis and 11% had DIC. About 70% of CAPS patients are positive for at least one aPL antibody. The mortality rate for patients in the CAPS Registry was 35% and the only identified predictive factor for adverse outcome was underlying SLE. Fortunately, relapse is rare in survivors.

Coronavirus Disease–Associated Antiphospholipid Syndrome

There have been several reports of patients infected with severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) and ensuing COVID-19 who have developed antiphospholipid antibodies. This is intriguing because vascular thrombosis is a frequent complication in these patients. In one series from France, 50 out of 58 critically ill COVID-19 patients tested positive for LA. However, these results are difficult to evaluate because testing was performed in the acute setting where false positives are common and where elevated levels of C-reactive protein (CRP) can mimic LA. In most studies, tests were done at a single time point and levels were above the usual reference ranges but below those considered clinically significant. Therefore, the relevance of these findings remain uncertain.

Pediatric Antiphospholipid Syndrome

APS is a significant cause of thrombosis in the pediatric population. In a European registry, children with primary APS tended to be younger and to have a higher frequency of arterial thrombosis than those with secondary APS. Patients with secondary APS were more likely to have venous thrombotic events associated with hematologic and skin manifestations. Compared with adult APS, pediatric primary APS patients are more likely to develop other autoimmune diseases, present with cerebral thrombotic events (i.e., cerebral vein thrombosis or ischemic stroke) and develop more non-criteria manifestations such as Evans syndrome, Raynaud phenomenon, and chorea. Furthermore, the rate of recurrent thrombosis is higher in pediatric APS than in adult APS. CAPS has been reported in children but is rare. Infection induced aPL antibodies are the most common form in pediatric patients.

aPL antibodies have been found in up to 30% of offspring of mothers with APS. However, thrombosis is rare in newborns delivered from mothers with APS, and only a few cases have been reported, mostly associated with other prothrombotic factors. Other complications in neonates born to APS mothers include prematurity, neurodevelopment abnormalities and learning disabilities.

In a recent European prospective study, 17% of neonates born to APS mothers were premature; however, no specific complications were found during the 5-year follow-up.

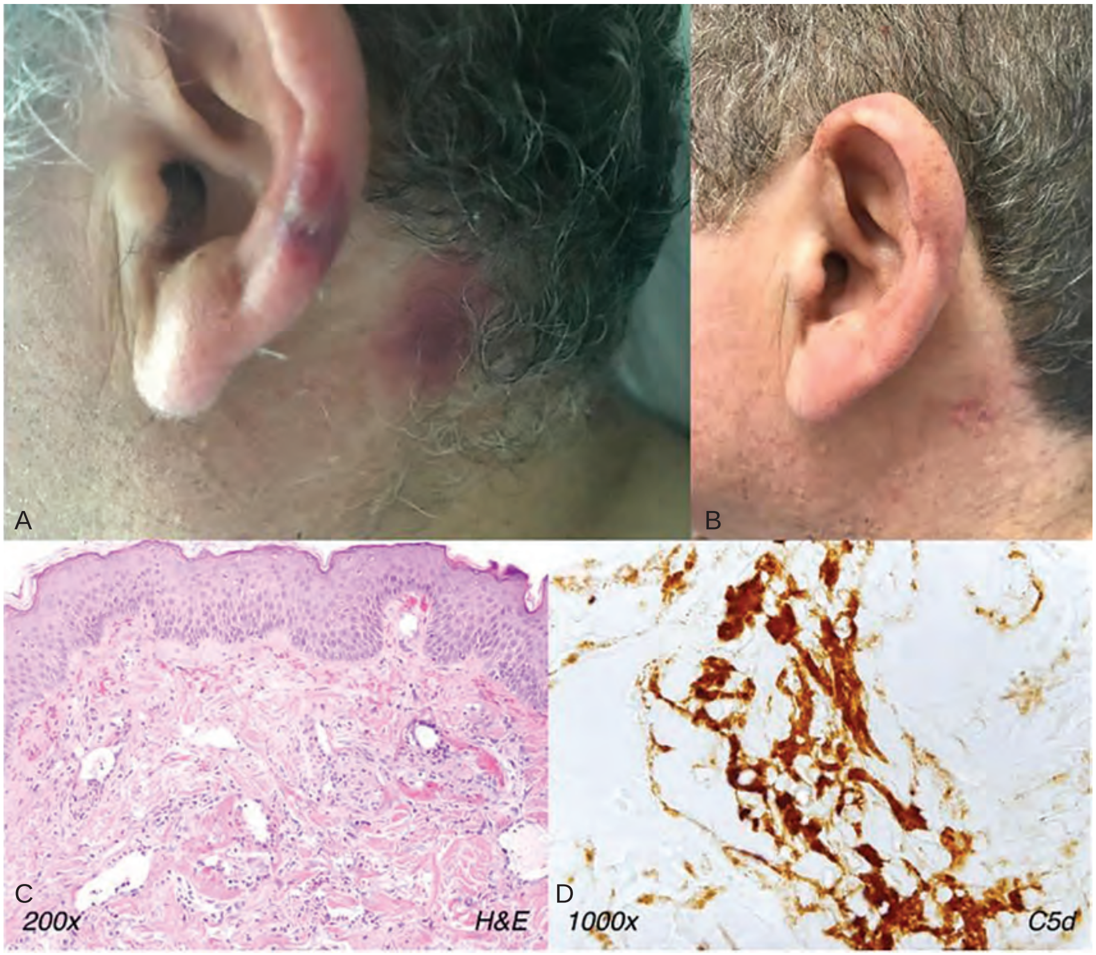


Figure 139.6 Catastrophic antiphospholipid syndrome (CAPS) manifesting as ear cartilage thrombosis successfully treated with eculizumab. (A) Ear cartilage thrombosis. (B) Resolution after treatment with complement inhibitor. (C) Ear cartilage biopsy (H&E staining) showing pale pink thrombi. (D) Immunohistochemistry staining with C5b-9 showing vessel deposition. *(From Nauseef JT, Lim HI, DeSancho MT. Successful outcome with eculizumab treatment in a patient with antiphospholipid syndrome presenting with an unusual thrombotic storm. J Thromb Thrombolysis. 2021;52(2):597–600.)*

The Single Hub and Access point for pediatric Rheumatology in Europe (SHARE) initiative was launched to develop diagnostic criteria and management regimens for pediatric APS.

TREATMENT OF ANTIPHOSPHOLIPID SYNDROME

There is general agreement that patients with recurrent or spontaneous thrombosis require long-term anticoagulant therapy with warfarin and that patients with recurrent spontaneous pregnancy losses require antithrombotic therapy with heparin and aspirin for most of the gestational period and with warfarin or LMWH for the puerperium (see Chapter 149). There are differences of opinion regarding the approach to treatment of patients with a single thrombotic event, a history of a remote thrombotic events (i.e., greater than 5 years previously), or patients with thrombotic events provoked by major transient risk factors such as surgery, pregnancy, or estrogens. Recently, the European League Against Rheumatism (EULAR) summarized treatment recommendations for patients with definite APS (Table 139.5).

There is controversy regarding the use of DOACs in APS. A prospective, randomized study that compared warfarin with rivaroxaban in patients with triple positive APS patients and a history of thrombosis reported more recurrent thrombotic events and more major bleeding events with rivaroxaban. A recent metanalysis confirmed these findings. It remains unclear whether these findings are only applicable to multipositive APS patients and to rivaroxaban. Nonetheless, until more data are available, it would be prudent to avoid DOACs in APS patients. The 2019 EULAR recommendations include avoiding rivaroxaban in patients with triple positivity or arterial events, but states that DOACs may be considered in patients with difficulty achieving a target international normalized ratio (INR) of 2 to 3 despite warfarin compliance or who have contraindications to warfarin. However, switching treatment from warfarin to a DOAC due to low compliance should be avoided.

Thrombosis

For the acute management of thrombotic APS, unfractionated heparin (UFH) or LMWH should be overlapped with a vitamin K antagonist such as warfarin (see Chapter 143). The parenteral anticoagulant can be stopped when the INR is therapeutic. The dose of warfarin should be adjusted to achieve an INR between 2.0 and 3.0. A recent review on warfarin for treatment of venous and arterial thrombosis in APS patients, showed no benefit of higher-intensity warfarin. It is also recommended that other cardiovascular risk factors should be addressed to minimize the risk of recurrence, including dyslipidemia, smoking, obesity, arterial hypertension, and diabetes.

Anticoagulation for thrombotic APS, even in patients with a single thrombotic event, should generally be continued indefinitely. Although many studies are underway on patients with low-risk aPL profiles, there have been no definitive studies reporting safe termination of anticoagulation in thrombotic APS patients.

TABLE 139.5 Recommendations for Treatment of Antiphospholipid Syndrome in Adults

APS Category	Clinical Manifestation/ Indication	Treatment	Duration
Definite APS with venous thrombosis	First unprovoked venous thrombosis	VKA with target INR 2–3 is recommended	Long term
	Provoked venous thrombosis	VKA with target INR 2–3 is recommended	Same duration as non-APS patients, however longer term if patient is triple aPL antibody positive
	Recurrent venous thrombosis, despite VKA treatment INR 2–3	Consider one of the following: • Addition of low-dose aspirin • Increase VKA INR to 3–4 • Change to low molecular weight heparin	Long term
Definite APS with arterial thrombosis	First arterial thrombosis	VKA with target INR 2–3 or INR 3–4 is recommended, considering bleeding and recurrence riskVKA with target INR 2–3 plus low-dose aspirin may also be considered	Long term
	Recurrent arterial thrombosis	Consider one of the following: • Increase VKA INR to 3–4 • Addition of low-dose aspirin • Change to low molecular weight heparin	Long term
Pregnant women with obstetric APS only (no prior thrombotic events) with or without SLE	History of ≥ 3 recurrent spontaneous miscarriages <10th week gestation	Combination treatment with low-dose aspirin and heparin at prophylactic doses	Duration of pregnancy, continue prophylactic dose of heparin for 6 weeks after delivery
	History of fetal loss ≥ 10 week of gestation	Combination treatment with low-dose aspirin and heparin at prophylactic doses	Duration of pregnancy, continue prophylactic dose of heparin for 6 weeks after delivery
	History of delivery <34 weeks gestation due to eclampsia or severe pre-eclampsia, or due to placental insufficiency	Treatment with low-dose aspirin alone or combination treatment with low-dose aspirin and heparin at prophylactic doses, if high-risk profile or other risk factors	Duration of pregnancy, if on heparin, continue prophylactic dose of heparin for 6 weeks after delivery
	Recurrent pregnancy complications despite combination treatment with LDA and heparin at prophylactic doses	Consider one of the following: • Increase heparin dose to therapeutic dose • Addition of hydroxychloroquine • Low-dose prednisone in the first trimester • IVIG may be considered in select cases	Duration of pregnancy, if on heparin, continue prophylactic dose of heparin for 6 weeks after delivery
Pregnant women with non-criteria obstetric APS	History of two recurrent spontaneous miscarriages <10th week gestation	Treatment with low-dose aspirin alone or combination treatment with low-dose aspirin and heparin at prophylactic doses if high-risk profile or other risk factors	Duration of pregnancy, if on heparin, continue prophylactic dose of heparin for 6 weeks after delivery
	History of delivery ≥34 weeks gestation due to eclampsia or severe pre-eclampsia	Treatment with low-dose aspirin alone or combination treatment with low-dose aspirin and heparin at prophylactic doses if high-risk profile or other risk factors	Duration of pregnancy, if on heparin, continue prophylactic dose of heparin for 6 weeks after delivery
Pregnant women with a history of thrombotic APS	History of arterial or venous thrombosis	Combination treatment with low-dose aspirin and heparin at therapeutic doses if high-risk profile or other risk factors	Duration of pregnancy, switch from VKA to heparin as soon as pregnancy is confirmed (ideally before 6th week gestation)
Catastrophic antiphospholipid syndrome prevention	For all aPL antibody–positive individuals and thrombotic APS patients	Prompt treatment of infections Avoid interruption in anticoagulation medication Minimize low INR levels	Long term
Catastrophic antiphospholipid syndrome	First-line treatment	Triple combination therapy with glucocorticoids, heparin and plasma exchange, or IVIG. Treat all triggering factors.	

Note: Direct oral anticoagulants (DOACs) should not be used in patients with triple aPL antibody positivity and arterial events, except in extraordinary circumstances where clinical judgement may prevail.

aPL, Antiphospholipid; *APS*, antiphospholipid syndrome; *VKA*, vitamin K antagonist.

Adapted and modified from Tektonidou MG, Andreoli L, Limper M, et al. EULAR recommendations for the management of antiphospholipid syndrome in adults. *Ann Rheum Dis*. 2019;78:1296–1304.

TREATMENT PEARLS AND HIGH-RISK aPL ANTIBODY PROFILE IN ASYMPTOMATIC PATIENTS

Treatment Pearls

- Long-term anticoagulant therapy with warfarin is recommended for APS patients with thrombosis.
- DOACs are **not** recommended.
- Aspirin and heparin are recommended for pregnant APS patients and for VTE prophylaxis postpartum.
- CAPS is associated with high mortality; in addition to anticoagulants, patients with CAPS often require plasmapheresis and immunosuppressive agents; some patients may benefit from complement inhibitors.
- Asymptomatic patients with high-risk aPL antibody profiles should be considered for prophylactic therapy with low-dose aspirin.

High-Risk aPL Antibody Profile in Asymptomatic Patients

The presence (on two or more occasions at least 12 weeks apart) of:

- LA-positive (measured according to ISTH guidelines)
- Double positive (any combination of LA, aCL, or anti-β2GPI antibodies)
- Triple positive (LA, aCL, and anti-β2GPI antibodies)
- Persistently high titers of aCL and/or anti-β2GPI antibodies

Stroke

In APS patients with stroke, high-intensity warfarin or concomitant use of aspirin or other antiplatelet drugs has not been shown to be of benefit over usual intensity warfarin alone.

Pregnancy Complications

The current approach to treating pregnant women with APS and recurrent pregnancy loss or other aPL antibody associated complications of pregnancy includes daily low-dose aspirin (75 to 81 mg/day) and prophylactic doses of either UFH or LMWH although LMWH is preferred (see Chapters 149). Heparin is withheld when labor begins or 24 hours prior to a cesarean section. Anticoagulation with either LMWH or warfarin (although most patients and physicians prefer LMWH for convenience, despite the need for injection) is then resumed for 6 weeks due to the increased risk of venous thromboembolism (VTE) in this period. In obstetric APS patients who are planning to become pregnant, low-dose aspirin should be started prior to conception and heparin (LMWH or UFH) should be added as soon as pregnancy is confirmed. Women with a history of thrombotic APS, should be switched from warfarin to a combination treatment of low-dose aspirin and heparin at therapeutic dosage during pregnancy.

Anticoagulant Monitoring Considerations

For patients treated acutely with intravenous UFH, care must be taken to determine whether the patient has a preexisting LA that can interfere with aPTT monitoring of heparin. This problem can be circumvented by using an LA-insensitive aPTT reagent or by measuring anti-Xa levels. An alternative approach would be to treat with weight-based dose of LMWH.

In some patients with APS, an LA can artifactually elevate the prothrombin time and INR. A multicenter study reported that all but one of the commercial thromboplastins in use at nine centers provided acceptable INR values for APS patients with LA. New thromboplastins should be checked for their responsiveness to LA prior to their use for monitoring warfarin therapy in patients with APS. Chromogenic factor X (CFX) assays can be used as an alternative to the INR for APS patients, especially for patients with a prolonged baseline prothrombin time prior to initiating warfarin therapy, those with a persistently positive LA, and those with recurrent VTE. Therapeutic CFX values range from 20% to 40%; thus, a CFX of 40% would approximate an INR of 2.0, and a CFX of 20% would approximate an INR of 3.0.

Catastrophic Antiphospholipid Syndrome

Patients with CAPS require aggressive treatment because of the high mortality. Treatment for CAPS is directed toward the thrombotic events and suppression of the cytokine cascade. Conventional anticoagulant therapy is usually insufficient and treatment modalities may include anticoagulation with heparin, immunosuppressive therapy with high-dose steroids, intravenous immunoglobulin (IVIG) cyclophosphamide, azathioprine, or rituximab. Plasmapheresis may be a helpful adjunct in some patients. Triple therapy, including anticoagulation, steroids, and either IVIG, plasma exchange, or both is recommended. The increased application of this triple therapy strategy has resulted in a significant reduction in mortality rate to 30% in CAPS.

In addition, concurrent treatment of precipitating factors is also recommended (e.g., infection or malignancy). For refractory CAPS, either rituximab (B cell depletion) or eculizumab (complement inhibition) may be considered.

Asymptomatic Antiphospholipid Antibody-Positive Patients

Asymptomatic patients with no previous thrombosis but a high-risk aPL antibody profile should be considered for long term prophylaxis with low-dose aspirin, especially if there are other thrombotic risk factors (see boxes on Treatment Pearls). Furthermore, aPL antibody carriers should be considered for thromboprophylaxis with usual doses of LMWH in high-risk situations, such as surgery, prolonged immobilization, and the puerperium. In addition to low-dose aspirin, aPL-antibody carriers with SLE should be considered for treatment with hydroxychloroquine as primary prophylaxis. (See boxes on Treatment Pearls and High-Risk aPL Antibody Profile in Asymptomatic Patients.)

A meta-analysis of seven observational studies of 460 asymptomatic aPL carriers reported that low-dose aspirin reduced the risk of first thrombosis half compared with nonuse. It is also recommended that thrombosis risk factors such as hypertension and dyslipidemia should be treated and patients should be counseled on the increased risk of thrombosis with oral contraceptives, pregnancy, estrogen replacement, surgery, or immobilization. Treatment recommendations for asymptomatic patients with low- and high-risk aPL antibody profiles are summarized in Table 139.6.

Other Treatments for Antiphospholipid Syndrome

Hydroxychloroquine

Hydroxychloroquine (HCQ), a synthetic antimalarial drug with anti-inflammatory properties, is a standard of care for the treatment of SLE. HCQ has also been associated with a reduction in the risk of thrombosis in APS and SLE. The potential effectiveness of this treatment is supported by the results of an animal model of aPL antibody thrombosis, and the evidence that HCQ disrupts aPL IgG-β2GPI complexes and reverses the inhibitory effect of aPL antibodies on annexin A5 on phospholipid bilayers and on human placental syncytiotrophoblasts. In a longitudinal cohort study that included 272 patients with APS and 152 taking HCQ (17 of 272 were on warfarin, 203 were on prednisolone, 112 on azathioprine, 38 on aspirin) there were fewer thrombotic complications in those taking HCQ (OR 0.17, 95% CI 0.07 to 0.44; $P < .0001$). In asymptomatic aPL antibody-positive patients with SLE, primary prophylaxis with aspirin and HCQ appeared to reduce the frequency of thrombotic events. A 2017 study showed that long term use of HCQ significantly reduced aPL antibody levels and the rate of recurrence. These studies suggest that

TABLE 139.6 Recommendations for Primary Prevention of Antiphospholipid-Positive Asymptomatic Adults

Clinical History	aPL-Antibody Profile Risk	Treatment
Asymptomatic	High risk*	Low-dose aspirin 75–100 mg/day recommended
SLE and no history of thrombosis or PC	High risk[a]	Low-dose aspirin 75–100 mg/day recommended with hydroxychloroquine treatment
	Low risk[b]	Low-dose aspirin 75–100 mg/day recommended with hydroxychloroquine treatment
Non-pregnant women with a history of obstetric APS only (with or without SLE)	Low[b] and high risk[a]	Low-dose aspirin 75–100 mg/day after adequate risk/benefit evaluation
Pregnant women with no history of thrombosis or PC (with or without SLE)	High risk[a]	Low-dose aspirin 75–100 mg/day should be considered

[a] High-risk aPL Antibody Profile: The presence (in 2 or more occasions at least 12 weeks apart) of lupus anticoagulant (measured according to ISTH guidelines), or of double (any combination of lupus anticoagulant, aCL antibodies or anti-β2GPI antibodies), triple (all three subtypes) aPL positivity, or the presence of persistently high aPL titers.

[b] Low-risk aPL Antibody Profile: Isolated aCL or anti-β2GPI antibodies at low-medium titers, particularly if transiently positive.

aPL, Antiphospholipid; *APS*, antiphospholipid syndrome; *PC*, pregnancy complication; *SLE*, systemic lupus erythematosus.

Adapted and Modified from Tektonidou MG, Andreoli L, Limper M et al. *Ann Rheum Dis* 2019;78:1296–1304.

HCQ is a useful adjunct to anticoagulant therapy. An ongoing study (HIBISCUS: Hydroxychloroquine for the secondary prevention of thrombotic and obstetrical events in primary antiphospholipid syndrome) is exploring the benefits of HCQ. EULAR guidelines state that HCQ could be used in women with refractory obstetric APS not responding to standard therapy.

Rituximab

Rituximab is an anti-CD20 chimeric monoclonal antibody approved for the treatment of rheumatoid arthritis. It has been shown to be effective in treating non-criteria manifestations refractory to conventional therapy. The task force recommends using Rituximab in difficult to treat APS patients with hematological and microangiopathic manifestations.

Eculizumab

Eculizumab is an anti-C5 monoclonal antibody that inhibits terminal complement activation. The drug was reported to be effective in selected patients with refractory CAPS and evidence of microangiopathic hemolytic anemia (see Fig. 139.6). A study of eculizumab in CAPS patients with end stage renal disease is underway (clinicaltrials.gov identifier NCT01029587).

SUGGESTED READINGS

Abreu MM, Danowski A, Wahl DG, et al. The relevance of "non-criteria" clinical manifestations of antiphospholipid syndrome: 14th International Congress on Antiphospholipid Antibodies Technical Task Force Report on Antiphospholipid Syndrome Clinical Features. *Autoimmun Rev*. 2015;14(5):401–414.

Alijotas-Reig J, Esteve-Valverde E, Ferrer-Oliveras R, et al. The European Registry on Obstetric Antiphospholipid Syndrome (EUROAPS): a survey of 1000 consecutive cases. *Autoimmun. Rev*. 2019;18(4):406–414.

Belizna C, Pregnolato F, Abad S, et al. HIBISCUS: Hydroxychloroquine for the secondary prevention of thrombotic and obstetrical events in primary antiphospholipid syndrome. *Autoimmun. Rev*. 2018;17(12):1153–1168.

Bradacova P, Slavik L, Ulehlova J, et al. Current promising biomarkers and methods in the diagnostics of antiphospholipid syndrome: a review. *Biomedicines*. 2021;9(2):166.

Breen KA, Sanchez K, Kirkman N, et al. Endothelial and platelet microparticles in patients with antiphospholipid antibodies. *Thromb Res*. 2015;135(2):368–374.

Cervera R, Rodríguez-Pintó I, Espinosa G. The diagnosis and clinical management of the catastrophic antiphospholipid syndrome: a comprehensive review. *J Autoimmun*. 2018 Aug;92:1–11.

Chaturvedi S, Braunstein EM, Yuan X, et al. Complement activity and complement regulatory gene mutations are associated with thrombosis in APS and CAPS. *Blood*. 2020;135(4):239–251.

Chayoua W, Kelchtermans H, Moore GW, et al. Detection of anti-cardiolipin and anti-β2glycoprotein I antibodies differs between platforms without influence on association with clinical symptoms. *Thromb Haemost*. 2019;119(5):797–806.

Cohen H, Cuadrado MJ, Erkan D, et al. 16th International Congress on Antiphospholipid Antibodies Task Force Report on Antiphospholipid Syndrome Treatment Trends. *Lupus*. 2020;29(12):1571–1593.

de Jesús GR, Sciascia S, Andrade D, et al. Factors associated with first thrombosis in patients presenting with obstetric antiphospholipid syndrome (APS) in the APS alliance for clinical trials and international networking clinical database and repository: a retrospective study. *BJOG*. 2019;126(5):656–661.

Duarte-García A, Pham MM, Crowson CS, et al. The epidemiology of antiphospholipid syndrome: a population-based study. *Arth Rheumatol (Hoboken, NJ)*. 2019;71(9):1545–1552.

Devreese KMJ, de Groot PG, de Laat B, et al. Guidance from the Scientific and Standardization Committee for lupus anticoagulant/antiphospholipid antibodies of the International Society on Thrombosis and Haemostasis: update of the guidelines for lupus anticoagulant detection and interpretation. *J Thromb Haemost*. 2020;18(11):2828–2839.

El Hasbani G, Khamashta M, Uthman I. Antiphospholipid syndrome and infertility. *Lupus*. 2020;29(2):105–117.

Erkan D, Aguiar CL, Andrade D, et al. 14th International Congress on Antiphospholipid Antibodies: task force report on antiphospholipid syndrome treatment trends. *Autoimmun Rev*. 2014;13:685–696.

Favaloro EJ, Mohammed S, Curnow J, Pasalic L. Laboratory testing for lupus anticoagulant (LA) in patients taking direct oral anticoagulants (DOACs): potential for false positives and false negatives. *Pathology*. 2019;51(3):292–300.

Hollerbach A, Müller-Calleja N, Ritter S, et al. Platelet activation by antiphospholipid antibodies depends on epitope specificity and is prevented by mTOR inhibitors. *Thromb Haemost*. 2019;119(7):1147–1153.

Islam MA, Khandker SS, Alam F, et al. Genetic risk factors in thrombotic primary antiphospholipid syndrome: a systematic review with bioinformatic analyses. *Autoimmun. Rev*. 2018;17(3):226–243.

Iuliano A, Galeazzi M, Sebastiani GD. Antiphospholipid syndrome's genetic and epigenetic aspects. *Autoimmun Rev*. 2019 Sep;18(9):102352.

Kim MY, Guerra MM, Kaplowitz E, et al. Complement activation predicts adverse pregnancy outcome in patients with systemic lupus erythematosus and/or antiphospholipid antibodies. *Ann. Rheum. Dis*. 2018;77(4):549–555.

Lopez-Pedrera C, Barbarroja N, Patiño-Trives AM, et al. New biomarkers for atherothrombosis in antiphospholipid syndrome: genomics and epigenetics approaches. *Front Immunol*. 2019;10:764.

Müller-Calleja N, Hollerbach A, Ritter S, et al. Tissue factor pathway inhibitor primes monocytes for antiphospholipid antibody-induced thrombosis. *Blood*. 2019;134(14):1119–1131.

Nakamura H, Oku K, Amengual O, et al. First-line, non-criterial antiphospholipid antibody testing for the diagnosis of antiphospholipid syndrome in clinical practice: a combination of anti-β2-glycoprotein I domain I and anti-phosphatidylserine/prothrombin complex antibodies tests. *Arthritis Care Res (Hoboken)*. 2018;70(4):627–634.

Pengo V, Ruffatti A, Legnani C, et al. Incidence of a first thromboembolic event in asymptomatic carriers of high-risk antiphospholipid antibody profile: a multicenter prospective study. *Blood*. 2011;118(17):4714–4718.

Radin M, Sciascia S, Erkan D, et al. The adjusted global antiphospholipid syndrome score (aGAPSS) and the risk of recurrent thrombosis: results from the APS ACTION cohort. *Semin Arthritis Rheum*. 2019;49(3):464–468.

Rand JH, Wu X-X, Wolgast LR, et al. A novel 2-stage approach that detects complement activation in patients with antiphospholipid antibody syndrome. *Thromb. Res*. 2017;156:119–125.

Rand JH, Taatjes DJ. Reimagining the antiphospholipid syndrome, an enigmatic thrombophilic disorder, through the looking glass of microscopic imaging. *Histochem Cell Biol*. 2018;150(5):529–543.

Sanchez-Redondo J, Espinosa G, Varillas Delgado D, Cervera R. Recurrent thrombosis with direct oral anticoagulants in antiphospholipid syndrome: a systematic literature review and meta-analysis. *Clin Ther*. 2019;41(9):1839–1862.

Scambi C, Ugolini S, Tonello M, et al. Complement activation in the plasma and placentas of women with different subsets of antiphospholipid syndrome. *Am J Reprod Immunol*. 2019;82(6):e13185.

Schreiber K, Breen K, Parmar K, et al. The effect of hydroxychloroquine on haemostasis, complement, inflammation and angiogenesis in patients with antiphospholipid antibodies. *Rheumatology (Oxford)*. 2018;57(1):120–124.

Sciascia S, Khamashta MA, Bertolaccini ML. New tests to detect antiphospholipid antibodies: antiprothrombin (aPT) and anti-phosphatidylserine/prothrombin (aPS/PT) antibodies. *Curr Rheumatol Rep*. 2014;16(5):415.

Soybilgic A, Avcin T. Pediatric APS: state of the art. *Curr Rheumatol Rep*. 2020;22(3):9.

Taatjes DJ, Bouffard N, Turkovich Mvon, et al. Visualization of macro-immune complexes in the antiphospholipid syndrome by multi-modal microscopy imaging. *Micron*. 2017;100:23–29.

Tektonidou MG, Andreoli L, Limper M, et al. EULAR recommendations for the management of antiphospholipid syndrome in adults. *Ann Rheum Dis*. 2019;78(10):1296–1304.

Tinti MG, Carnevale V, Inglese M, et al. Eculizumab in refractory catastrophic antiphospholipid syndrome: a case report and systematic review of the literature. *Clin Exp Med*. 2019;19(3):281–288.

Tortosa C, Cabrera-Marante O, Serrano M, et al. Incidence of thromboembolic events in asymptomatic carriers of IgA antiβ2 glycoprotein-I antibodies. *PLoS. One*. 2017;12(7):e0178889.

Weinstein A, Alexander RV, Zack DJ. A review of complement activation in SLE. *Curr Rheumatol Rep*. 2021;23(3):16.

Wolgast LR, Arslan AA, Wu X-X, et al. Reduction of annexin A5 anticoagulant ratio identifies antiphospholipid antibody-positive patients with adverse clinical outcomes. *J Thromb Haemost*. 2017;15:1412–1421.

You Y, Liu Y, Li F, et al. Anti-β2GPI/β2GPI induces human neutrophils to generate NETs by relying on ROS. *Cell Biochem Funct*. 2019;37(2):56–61.

CHAPTER 140

VENOUS THROMBOEMBOLISM

Noel C. Chan and Jeffrey I. Weitz

Venous thromboembolism (VTE) encompasses deep vein thrombosis (DVT) and pulmonary embolism (PE). DVT commonly affects the deep veins of the legs, and infrequently involves veins at other anatomical sites (such as upper extremity, splanchnic, or cerebral veins). Thrombotic occlusion of the deep veins impairs drainage of blood, thereby resulting in pain and swelling distal to the obstruction. Embolization of thrombus from deep veins in the leg to the pulmonary arteries is the leading cause of PE. About two-thirds of patients with symptomatic VTE present with DVT, whereas the remainder present with PE,[1] and about 1 in 20 patients with DVT and 1 in 10 with PE die within 30 days.[2] In patients who survive, long-term complications, such as post-thrombotic syndrome (PTS) and chronic thromboembolic pulmonary hypertension (CTEPH), are important causes of morbidity in up to 50% of patients with DVT and 4% of those with PE, respectively.[3] Thrombosis can also affect superficial veins. Although the condition is self-limiting when superficial veins in the arms are involved as a complication of intravenous catheters or drug delivery, thrombi in the superficial veins of the lower extremities, particularly those in the thigh close to the saphenofemoral junction, can propagate into the deep veins, thereby resulting in DVT and PE.

EPIDEMIOLOGY

VTE affects 1 to 2 in 1000 individuals annually and is the third most common cause of vascular death after myocardial infarction and stroke.[1,4] In the United States, about 900,000 people are diagnosed with VTE each year, with more than half related to hospitalization or institutionalization.[5] Consequently, VTE is considered the number one cause of preventable death in hospitalized patients.[6]

The incidence of VTE increases with age, from 1 case per 10,000 persons per year in individuals younger than 40 years of age to 5 to 10 cases per 1000 persons per year in those 80 years of age or older.[4] Although men and women are affected equally overall, the incidence is higher in women of childbearing age than in men of similar age; for those 45 years of age or older, the incidence of VTE is higher in men than in women.[4] The incidence of VTE is higher in Whites and African Americans and is lower in Asians, Asian Americans, and Native Americans.[1,4] The incidence of VTE is twofold higher in high-income countries than in low-income countries even after adjustments for baseline characteristics and risk factors.[7]

PATHOGENESIS OF VENOUS THROMBOEMBOLISM

Except in pregnancy (see Chapter 141), DVT usually starts in the deep veins of the calf where it forms in the valve pockets. Although most calf DVT spontaneously resolves, there is propagation into more proximal veins in about 20% of the cases.[3,8] These more proximal clots have the potential to break off and embolize to the lungs, resulting in PE. Thus, PE can be detected in about 40% of patients with proximal DVT and at least 50% of patients with PE have evidence of DVT.

DVT in pregnancy usually involves the iliofemoral vein, spares the distal veins, and affects the left leg in greater than 80% of cases (see Chapter 141).[9] Localization to the left iliofemoral veins likely reflects a May-Thurner-like syndrome, as the enlarging uterus exaggerates the compression of the left iliac vein where it is crossed by the right iliac artery.

The pathogenesis of VTE involves an interplay among hypercoagulability of the blood, sluggish blood flow, and damage or activation of the vessel wall, which are the components of the so-called Virchow triad.[10] Hypercoagulability of the blood triggers a systemic increase in thrombin generation, whereas stasis and damage or activation of the vessel wall explain why DVT usually occurs in the deep veins of the leg.[11]

Hypercoagulability

Many hereditary and acquired disorders are associated with hypercoagulability. Hereditary disorders that are potent risk factors for VTE include deficiencies of natural anticoagulants such as antithrombin, protein C, and protein S (discussed in Chapters 125 and 138).[12] With reduced levels of these endogenous anticoagulants, there is increased thrombin generation and the potential for clot formation. Moderate genetic risk factors for VTE include factor (F) V Leiden and the prothrombin gene mutation. FV Leiden, an FV variant that is resistant to inactivation by activated protein C, is found in about 5% of individuals of European descent.[13] The prothrombin gene mutation, which is found in about 3% of those of European descent, is a single nucleotide polymorphism in the 3′ untranslated region that increases prothrombin synthesis.[14] Delayed inactivation of FVa Leiden and elevated prothrombin levels lead to increased thrombin generation. Although these inherited thrombophilic disorders increase the risk of the first episode of VTE, their impact on the risk of recurrence is unclear. Consequently, routine thrombophilia testing is not recommended in patients with VTE because such tests rarely influence long-term management (see Chapter 138).[15]

Up to 50% patients with the first episode of VTE have no identifiable major risk factors and are described as having unprovoked VTE. The remainder develops VTE secondary to risk factors and these episodes are considered provoked. Acquired risk factors for VTE can be divided into those that are transient or persistent and major or minor.[16] Examples of major transient risk factors are surgery, trauma and hospitalization for COVID-19, whereas minor transient risk factors include immobilization, long-distance travel, pregnancy, and estrogen therapy. Common examples of major persistent risk factors are cancer and antiphospholipid syndrome, whereas minor persistent risk factors include advanced age, obesity, renal impairment, and chronic inflammatory disorders such as inflammatory bowel disease and rheumatoid arthritis. Patients with both hereditary and acquired risk factors are at particularly high risk of VTE because risks tend to be multiplicative rather than additive. Local factors explain why DVT usually starts in the calf.

Stasis, Hypoxia, and Endothelial Dysfunction

Most venous thrombi originate in the valve pockets of the calf veins where there is stasis and hypoxia.[17,18] Consistent with slow blood flow, contrast dye lingers in the valve pockets after venography.[17] Hypoxia occurs because the valve leaflets are avascular and the oxygen tension in the valve pockets rapidly decreases when blood flow stops because of immobility.[18] Endothelial cells lining the valve pockets express antithrombotic proteins such as thrombomodulin and endothelial protein C receptor on their surface (see Chapter 125).[19] Expression is downregulated by local hypoxia or inflammation and by reduced expression of the oscillatory shear-dependent transcription factors that endow perivalvular venous endothelial cells with an antithrombotic phenotype (see Chapters 121 and 122).[20] In addition, stasis and hypoxia induce a pro-inflammatory and prothrombotic endothelial phenotype by the upregulation of adhesion molecules such as P- and E-selectin, intercellular adhesion molecule 1 (ICAM-1), and von Willebrand factor. Therefore, stasis and hypoxia in the valve pockets of the veins in the lower extremities promote a hypercoagulable microenvironment, which explains why DVT starts in this site. In contrast, upper extremity DVT is often triggered by central venous catheters or by extrinsic compression of the arm veins (e.g., Paget-Schroetter syndrome).[21] Indwelling catheters or extrinsic compression retard blood flow, whereas delivery of chemotherapy, or antibiotics via the catheters, induces local activation of the endothelium. Regardless of whether DVT occurs in the upper or lower extremities, however, the initiators of coagulation are likely to be similar.

Initiators of Coagulation in Venous Thromboembolism

Tissue factor initiates coagulation in patients with VTE, but the source of the tissue factor remains elusive.[22] Overt damage to the vessel wall is rarely seen with DVT. However, hypoxia and inflammatory cytokines upregulate tissue factor expression by endothelial cells and circulating monocytes and induce endothelial cell expression of adhesion molecules such as P-selectin. The importance of endothelial cell tissue factor is uncertain, but P-selectin tethers tissue, factor-bearing monocytes, and microparticles onto their surface via interaction with P-selectin glycoprotein ligand.[23] By preventing such binding, P-selectin inhibitors attenuate venous thrombosis in animal models. Statins and proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors attenuate tissue factor expression by monocytes, which may explain why they reduce the risk of recurrent VTE.[24–26] Therefore, adhesion of tissue factor expressing leukocytes and microparticles to the activated endothelium appears to be an important driver of DVT.

In addition to tissue factor, emerging data suggest that the contact system also plays a part in the initiation of coagulation in VTE.[27–29] Neutrophil extracellular traps (NETs) extruded from activated neutrophils trapped within venous thrombi provide a scaffold for platelet adhesion and activate FXII, thereby initiating coagulation via the intrinsic pathway (see Chapters 121 and 125).[30] Likewise, inorganic polyphosphate released from activated platelets enhances FXII activation.[31] Thrombin feedback activation of FXI, a reaction enhanced by polyphosphate, amplifies coagulation via the intrinsic pathway. With increasing evidence that the contact system is involved in the initiation of VTE, FXI has emerged as a target for new anticoagulants that have the potential to be safer than those that inhibit FXa or thrombin (see Chapter 143).[32]

There is mounting evidence that platelets play a part in the pathogenesis of VTE.[33] Venous thrombi consist of a central core that lies adjacent to the vessel wall and is rich in fibrin and trapped red blood cells and an overlying shell composed of platelet-rich layers. There is evidence from mouse models that platelets are important in the initiation and propagation of venous thrombosis. Thus, P-selectin-dependent recruitment of platelets and leukocytes is essential for thrombus formation after incomplete ligation of the inferior vena cava because inhibition or depletion of platelets or neutrophils suppressed thrombus formation in this model.[34] Likewise, platelet depletion abrogated spontaneous venous thrombosis induced by concomitant knockdown of antithrombin and protein C. In addition, antiplatelet agents such as aspirin or clopidogrel attenuated thrombosis in a murine venous stasis model.[35,36] Findings in mice may be translatable to humans because patients with acute VTE have evidence of systemic platelet activation and increased levels of platelet-derived soluble P-selectin.[37] Therefore, platelets likely contribute to VTE, which explains why aspirin is effective for its prevention, albeit less so than anticoagulants.[38]

Stabilization, Embolization, and Resolution of Venous Thrombi

In patients with DVT, the risk of PE appears to be dependent on the size and the stability of the thrombus. The importance of thrombus size is highlighted by the fact that calf DVT rarely causes PE, whereas up to 50% of patients with proximal DVT have associated PE. Clot stability also influences the risk of PE. Thrombin activates FXIII, which stabilizes the clot and renders it more resistant to lysis by crosslinking the fibrin polymers and by facilitating retention of red blood cells in the thrombus.[39,40] Enhanced thrombin generation and subsequent FXIII activation in patients with FV Leiden could explain the epidemiological evidence that DVT is more common than PE in such patients. This concept is supported by the results of studies in mice.[41] Thus, treatment with dabigatran after ferric chloride-induced femoral vein thrombosis enhanced PE by suppressing thrombin-mediated FXIII activation. Conversely, FXIII supplementation reduced the risk of PE without promoting thrombus growth.

Thrombus resolution depends on fibrinolysis, which occurs when plasminogen is converted to plasmin. Degradation of thrombi by plasmin likely explains why most calf DVT resolve spontaneously. Migration of leukocytes into the thrombus promotes fibrinolysis and contributes to early thrombus resolution by releasing clot digesting enzymes.[42] Impaired fibrinolysis has been linked to recurrent VTE and up to 50% of patients with proximal DVT have ultrasound evidence of residual vein occlusion 1 year after their index event. Reduced blood flow contributes to PTS, which is the major long-term complication of DVT.

CLINICAL MANIFESTATIONS OF VENOUS THROMBOEMBOLISM

Deep Vein Thrombosis

DVT typically causes swelling, pain, and erythema of the affected extremity. DVT in the proximal veins of the lower extremity is more likely to be symptomatic than DVT confined to the calf veins. Extensive thrombosis involving the iliac and femoral veins can cause phlegmasia cerulea dolens, an uncommon limb-threatening syndrome characterized by severe leg pain, swelling, venous gangrene, and arterial ischemia with loss of distal pulses.

The differential diagnosis of patients with suspected DVT includes musculoskeletal disorders (muscle or tendon strains or tears), lymphatic obstruction, venous insufficiency, a ruptured popliteal (Baker) cyst, cellulitis, sciatica, muscle hematoma, and post-thrombotic syndrome.

Pulmonary Embolism

The most common symptoms and signs of PE include dyspnea on exertion, chest pain which is often pleuritic in nature, tachypnea, and cough. Many patients with PE have concomitant symptoms and signs of DVT. Less common symptoms and signs include fever, hemoptysis, cyanosis, hypotension, and shock. Other non-specific symptoms include palpitations, anxiety, and lightheadedness.

Patients with severe PE often have dyspnea at rest and hypotension, and they may present with syncope because of hypoxemia and low cardiac output. Central and peripheral cyanosis can occur, and the jugular veins may be distended if right heart failure develops. The second heart sound can be widely split, and the pulmonic component may be loud because of delayed emptying of the right ventricle.

A right ventricular heave may be present with severe PE because of acute pulmonary hypertension.

When patients present with chest symptoms suggestive of PE, the differential diagnosis includes pulmonary disorders, such as pneumonia, an exacerbation of chronic obstructive lung disease or asthma, pleurisy secondary to connective tissue disease, cardiac disorders such as heart failure, acute coronary syndrome, or pericarditis; and musculoskeletal disorders such as rib fracture.

DIAGNOSIS OF VENOUS THROMBOEMBOLISM

In patients with suspected VTE, rapid and accurate diagnosis is essential because failure to institute anticoagulant therapy in those with VTE can result in thrombus progression and PE, which can be fatal, whereas unnecessary anticoagulation in those without VTE can cause bleeding. Clinical diagnosis of DVT or PE is unreliable because no individual symptom or sign is sufficiently predictive for confirming or excluding the diagnosis. Therefore, non-invasive imaging tests are often needed.

Compression ultrasound is used for the diagnosis of DVT and CT pulmonary angiography (CTPA) or, less frequently, ventilation-perfusion (V/Q) lung scanning is used for the diagnosis of PE. The challenge with the reliance on imaging tests for ruling out VTE is that most patients with suspected VTE do not have it. Of those with suspected DVT and PE, the diagnosis is confirmed in only about 20% and 5%, respectively.[43–45] Therefore, relying on imaging in all patients with suspected VTE is problematic because of the cost to healthcare systems, the unnecessary exposure to radiation for many patients with suspected PE, and the potential for overdiagnosis of events of uncertain clinical significance, such as calf DVT and subsegmental PE. Instead, a sequential diagnostic approach that starts with the determination of the pretest probability of DVT or PE using validated risk assessment models enhances reproducibility and testing efficiency because it guides the need for imaging, thereby minimizing unnecessary testing.

Although clinical risk assessment tools categorize patients according to their pretest probability of VTE with reasonable accuracy, they should not be used as a standalone to exclude or confirm VTE diagnosis. Instead, risk assessment models should be combined with D-dimer testing, which if negative, excludes the diagnosis of VTE in patients with a “VTE-unlikely” pretest probability. If the D-dimer test is positive or if VTE is likely, the diagnosis of DVT or PE requires confirmation with compression ultrasonography or lung imaging (CTPA or V/Q scan), respectively.

Diagnostic Tests

D-Dimer

D-dimer is a plasmin-derived breakdown product of cross-linked fibrin and is a biomarker that reflects coagulation activation and fibrinolysis (see Chapters 121 and 125).[46] D-dimer levels are elevated in most patients with VTE. D-dimer assays vary in their sensitivity and specificity. Enzyme-linked immunosorbent and advanced turbidimetric D-dimer assays have sensitivities greater than 95%. With highly sensitive assays, the combination of a negative D-dimer test and a ‘VTE-unlikely’ pretest probability effectively rules out DVT or PE, thereby obviating the need for imaging. However, the test is nonspecific because D-dimer levels are increased in a variety of conditions, including malignancy, inflammatory disorders, and infections. Therefore, patients with a positive D-dimer test require imaging to confirm a diagnosis of VTE. Consequently, the D-dimer test is most useful as a tool to rule out suspected VTE.

Compression Ultrasonography

Compression ultrasonography with or without Doppler imaging is the most widely used non-invasive test for the diagnosis of DVT.[47] Noncompressibility of the proximal leg veins on ultrasonography is diagnostic of DVT in symptomatic patients and is an indication for treatment. Compared with venography, venous compression ultrasonography has a sensitivity and specificity for detection of proximal DVT (femoral or popliteal veins) of about 95%; the sensitivity and specificity for detecting calf DVT are lower.[48] Consequently, some centers examine only the veins above the trifurcation in the calf, whereas others examine both the proximal and calf veins. A normal whole leg ultrasound effectively excludes the diagnosis of DVT because the risk of missing DVT is less than 2% at 3 months. If only the proximal veins are examined, the ultrasound should be repeated in 1 week to exclude extension of possible calf DVT into the proximal veins.

Venography

Although ascending contrast venography remains the gold standard for diagnosis of DVT, the test is rarely performed because of its expense, discomfort to the patient, and potential for complications. A constant intraluminal filling defect is diagnostic and DVT can be excluded in patients with a normal, adequately performed venogram. Minor side effects of venography include local pain, nausea; serious adverse reactions, such as anaphylaxis, are rare.

Computed Tomography Pulmonary Angiography

Multidetector CTPA has largely replaced V/Q lung scanning for diagnosing PE because of its wide availability, the rapidity of its results, and a sensitivity and specificity sufficient for its use as a standalone test. In contrast to V/Q scanning, CTPA not only permits direct visualization of thrombi in the pulmonary arteries of patients with PE but also provides an alternative diagnosis in many of those without PE. In a meta-analysis of 16 studies that included 4392 participants, the pooled estimates for the sensitivity and specificity of CTPA for PE were 94% and 98%, respectively.[49] A CTPA showing thrombus in segmental or more proximal pulmonary arteries provides evidence of PE, whereas a negative CTPA excludes the diagnosis. Compared with V/Q scanning, CTPA detects more isolated subsegmental thrombi in about 1% to 5% of patients, but it is unclear whether isolated subsegmental defects are benign or associated with outcomes like those of more proximal thrombi. A normal D-dimer level and/or a normal bilateral venous compression ultrasound examination may help to exclude VTE in this setting.

Ventilation/Perfusion Lung Scan

This two-part test consists of a ventilation phase and a perfusion phase. The ventilation phase involves inhalation of an aerosol form of radioactive isotopes of xenon or technetium to assess air delivery to the various parts of the lung. The perfusion phase involves intravenous injection of technetium-labeled macroaggregates of albumin, which enables assessment of blood flow within the lungs once the aggregates lodge in the pulmonary microcirculation. Images for both parts of the test are acquired using a gamma counter. Areas of the lung affected by PE do not light up on the perfusion scan because the macroaggregates of albumin fail to reach sites where the pulmonary arteries are occluded. Areas with abnormal perfusion because of PE will ventilate normally, yielding a ventilation-perfusion mismatch. A normal V/Q lung scan excludes the diagnosis of PE, but only 25% of patients with suspected PE have a normal scan.[49] One or more segmental or larger perfusion defects that ventilate normally characterize a high probability lung scan, which establishes the diagnosis of PE. However, even with single-photon emission CT technology, only about 10% of patients with suspected PE have a high-probability scan. The remaining 65% of lung scans exhibit smaller areas of mismatch or matched defects, and fall within the non-high probability category. Because up to 40% of patients with non-high probability scans have PE, such patients often require additional investigations to exclude the diagnosis.

Although CTPA has largely replaced V/Q lung scanning for the diagnosis of PE, the lung scan is the diagnostic test of choice for patients with renal impairment or a history of allergy to contrast media, and for women younger than 40 years of age to reduce radiation exposure to the breasts. For the diagnosis of PE in pregnancy, lung scanning produces less maternal and fetal radiation exposure and is sometimes preferred over CTPA (see Chapter 141).

Pulmonary Angiography

Although direct pulmonary angiography is the reference standard for establishing the presence or absence of PE, it is an invasive test associated with a mortality rate of 0.2%, particularly in patients with hemodynamic compromise or respiratory failure. Because of the widespread availability of CTPA and V/Q scanning, pulmonary angiography is rarely undertaken.

Diagnostic Strategies for Suspected Deep Venous Thrombosis

Diagnostic algorithms for the noninvasive diagnosis of suspected DVT are presented in Fig. 140.1. The diagnosis of DVT requires an integrated approach because the clinical symptoms and signs are not specific, and clinical risk assessment models or D-dimer testing used in isolation are not sufficiently predictive to confirm or exclude the diagnosis. When assessing outpatients with suspected DVT, clinical prediction rules assign a pretest probability score (DVT likely or unlikely) based on the clinical manifestations and the presence or absence of risk factors. The Wells clinical prediction rule for DVT assigns a risk category based on the following factors: active cancer (or cancer treated within the previous 6 months), calf swelling (≥3 cm compared with the asymptomatic leg), swelling of superficial veins (non-varicose), unilateral pitting edema, tenderness along the course of the deep veins, recent immobilization (≥3 days), or major surgery (within 12 weeks), and absence of an alternative diagnosis.[50]

Acute DVT can be excluded in patients with a DVT-unlikely pretest probability and a negative D-dimer result. Patients with a positive D-dimer result or a DVT-likely pretest probability should proceed to objective testing. If compression ultrasonography cannot be promptly performed, patients can be empirically anticoagulated pending diagnostic testing on a subsequent day. A positive ultrasound result in a patient with a DVT-likely pretest probability confirms the diagnosis of DVT. Serial ultrasonography should be considered if the clinical suspicion of DVT remains high despite a negative initial leg ultrasound and no alternative diagnosis is identified.

Among inpatients with suspected DVT, imaging is required because the performance of clinical prediction models such as the Wells score is lower in hospitalized patients than in outpatients and upfront D-dimer testing is associated with a high rate of false positives because of comorbidities.

Diagnostic Strategy for Suspected Pulmonary Embolism

A diagnostic algorithm for evaluation of patients with suspected PE is shown in Fig. 140.1. After a history and physical examination, electrocardiogram (ECG), and chest X-ray, the pretest probability of PE should be assessed using a validated clinical prediction model. The Wells clinical prediction rule assigns a pretest probability based on the presence of the following: clinical signs of DVT, recent immobilization or surgery, heart rate above 100 beats/min, previous history of VTE, hemoptysis, active malignancy, and absence of an alternative diagnosis.[51]

A PE-unlikely pretest probability combined with a negative D-dimer test excludes PE. Patients with a positive D-dimer test or a high pretest probability require imaging. A negative CTPA or a normal V/Q scan rules out PE. An intraluminal filling defect in a segmental or more proximal pulmonary artery on the CTPA, or one or more segmental (or larger) perfusion defects with normal ventilation on the V/Q scan, establishes a diagnosis of PE. The diagnosis of PE cannot be excluded if perfusion defects correspond with abnormalities on the chest X-ray. CTPA is the preferred diagnostic test when there are chest X-ray abnormalities, but if this cannot be done, bilateral leg compression ultrasonography may be helpful to rule out DVT.

EVALUATION FOR UNDERLYING MALIGNANCY

Cancer is a risk factor for VTE. A comprehensive history should be taken, and physical examination performed in patients with

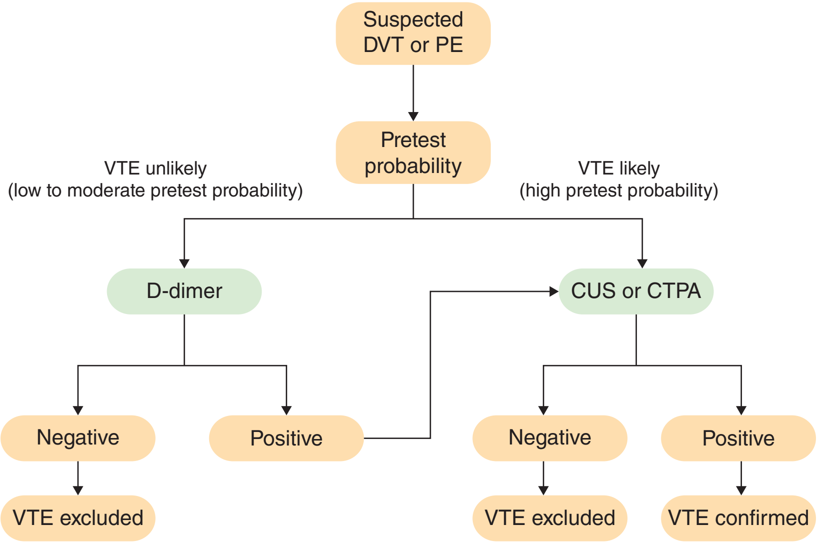


Figure 140.1 DIAGNOSTIC APPROACH IN PATIENTS WITH SUSPECTED DEEP VEIN THROMBOSIS (DVT) OR PULMONARY EMBOLISM (PE). The first step is an assessment of the pretest probability using a validated DVT or PE clinical prediction model. Patients categorized as VTE unlikely (low or moderate pretest probability) should undergo D-dimer testing. A negative D-dimer excludes the diagnosis, whereas those with a positive D-dimer test result or those categorized as VTE likely (high pretest probability) should undergo diagnostic imaging with compression ultrasound (CUS) or computed tomography pulmonary angiography (CTPA) to rule out or confirm DVT and PE, respectively.

documented VTE, along with routine tests such as blood counts, tests of liver function, urinalysis, and stool for occult blood.[52] Age- and sex-specific cancer screening tests (e.g., colonoscopy and mammography) should be performed over the next few months. If these tests are negative, the likelihood of finding occult cancer within a year of diagnosis of VTE is less than 5%. Therefore, routine abdominal and pelvic CT scanning or positron emission tomography is not recommended because such testing is expensive and does not increase the rate of cancer diagnosis or improve outcomes.

TREATMENT OF VENOUS THROMBOEMBOLISM

Anticoagulant therapy is the mainstay for the treatment of acute VTE to prevent thrombosis progression and fatal PE, and to minimize the risk of long-term complications. Unless the risk of bleeding is high, anticoagulation therapy should be started immediately in patients with a likely pretest probability of PE or DVT even while awaiting confirmatory imaging tests. Once the diagnosis is established, patients with contraindications to anticoagulant therapy may require the insertion of an inferior vena cava filter.

Most patients with acute DVT or with low-risk PE can receive out-of-hospital treatment because such treatment is as safe as inpatient treatment.[53,54] In patients with PE, risk stratification using prediction rules such as the Pulmonary Embolism Severity Index (PESI) can help to identify the 50% of those who can safely be treated as outpatients.[55] In non-low-risk patients with PE without evidence of hemodynamic instability, further risk stratification is recommended. The presence of right ventricular (RV) dysfunction as identified by imaging (echocardiogram or CTPA) or by biochemical assays (cardiac biomarkers such as troponin or B-type natriuretic peptide) predicts higher early mortality risk.[54,56] These higher risk patients should be observed in the hospital to ensure that they remain hemodynamically stable. Patients with evidence of both RV dysfunction on imaging and elevated cardiac biomarkers require close monitoring for hemodynamic decompensation and consideration for reperfusion therapy.

The risk of recurrent VTE is highest in the first few weeks after diagnosis and progressively decreases thereafter. Consequently, VTE treatment has been divided into three stages: initial therapy for the first 1 to 3 weeks, the goals of which are to prevent thrombus extension and fatal pulmonary embolism; therapy for the subsequent 3 months, which is often termed long-term therapy and is given to treat the VTE and to reduce the risk of complications, such as PTS and chronic thromboembolic pulmonary hypertension; and extended therapy beyond 3 months, which is administered when the risk of recurrent VTE without anticoagulant therapy exceeds the risk of major bleeding with continued therapy.

Severe Pulmonary Embolism

Patients with acute PE associated with hemodynamic compromise, defined as systolic blood pressure below 90 mm Hg for more than 15 minutes, or with cardiopulmonary arrest should be considered for pharmacologic, mechanical, or surgical reperfusion therapy in addition to anticoagulation therapy.[54,56] Pharmacologic reperfusion therapy involves the systemic administration of a fibrinolytic agent such as activase (tissue plasminogen activator) or tenecteplase (see Chapter 143). Up to 13% of patients given systemic fibrinolytic therapy experience a major bleed, and the rate of intracranial or fatal bleeding can reach 1.8%. Consequently, systemic fibrinolytic therapy is only justified in patients with severe pulmonary embolism without contraindications. The risk of bleeding is likely to be lower with catheter-directed or pharmacomechanical thrombolysis than with systemic fibrinolysis because lower doses of fibrinolytic agents are used with these techniques. A surgical pulmonary embolectomy is an option for patients with severe PE secondary to large proximal emboli who are at high risk for bleeding with systemic fibrinolytic therapy or who have failed such treatment.

Extensive Iliofemoral Deep Vein Thrombosis

Patients with iliofemoral DVT who present with phlegmasia or with severe leg pain and swelling may be candidates for catheter-directed or pharmacomechanical thrombolysis. Randomized clinical trials comparing these procedures with anticoagulation therapy alone have yielded mixed results.[57,58] The interventions produce a more rapid resolution of the pain and swelling but are associated with an increased risk for bleeding and have a variable effect on the risk of PTS. Therefore, catheter-directed or pharmacomechanical thrombolysis should be reserved for patients with limb-threatening DVT or severe leg pain and swelling who are at low risk of bleeding.

Anticoagulant Therapy for Acute Venous Thromboembolism

Guidelines give preference to direct oral anticoagulants (DOACs) over warfarin for the treatment of most patients with VTE (Table 140.1)[54]; special populations are discussed below. The DOACs include dabigatran, which inhibits thrombin, and apixaban, edoxaban, and rivaroxaban, which inhibit FXa (see Chapter 143). Apixaban and

TABLE 140.1 **Anticoagulant Dosing Regimens for Treatment of Venous Thromboembolism**

Anticoagulant Regimens	Venous Thromboembolism Treatment Stage		
	Initial Therapy	Long-Term Therapy	Extended Therapy
Unfractionated heparin	80 IU/kg iv bolus, then 18 IU/kg/h, with dose adjustment aiming for a hospital-specific aPTT target range	N/A	N/A
Enoxaparin	1 mg/kg twice daily or 1.5 mg/kg once daily		
Dalteparin	100 IU/kg twice daily or 200 IU/kg once daily	150 IU/kg once daily (for cancer-associated VTE)	
Tinzaparin	175 IU/kg once daily		
Warfarin	Dose adjusted to a target INR of 2.0–3.0		
Apixaban	10 mg twice daily for 7 days, followed by 5 mg twice daily	5 mg twice daily	2.5 mg twice daily
Dabigatran	After an initial 5-day course of LMWH: 150 mg twice daily Consider 110 mg twice daily if: age >75 years or creatinine clearance 30–49 mL/min		
Edoxaban	After an initial 5-day course of LMWH: 60 mg once daily 30 mg once daily if creatinine clearance 15–50 mL/min, body weight ≤60 kg or concomitant use of potent P-glycoprotein inhibitor		
Rivaroxaban	15 mg twice daily for 21 days followed by 20 mg daily		10 mg once daily

rivaroxaban are the agents most frequently used for acute VTE treatment because unlike dabigatran or edoxaban, they do not require an initial 5-day course of low-molecular-weight heparin (LMWH) prior to their initiation.

The risk of recurrence is higher in the first few weeks after the diagnosis of VTE than it is later in the course of the disease. Consequently, the initial doses of apixaban and rivaroxaban are higher than subsequent doses (see Table 140.1). Thus, apixaban is given at a starting dose of 10 mg twice daily for 7 days followed by 5 mg twice daily thereafter, whereas rivaroxaban is started at a dose of 15 mg twice daily for 21 days, followed by 20 mg once daily thereafter. If dabigatran or edoxaban is chosen, a minimum 5-day course of treatment with a parenteral anticoagulant is needed prior to their initiation at doses of 150 mg twice daily and 60 mg once daily, respectively. The dose of edoxaban is reduced to 30 mg once daily if the creatinine clearance is between 15 and 50 mL/min, the body weight is 60 kg or less, or there is concomitant use of potent P-glycoprotein inhibitors such as verapamil or dronedarone.

Warfarin is an alternative to DOACs for VTE treatment in patients taking medications such as phenytoin, carbamazepine, or ritonavir, those with antiphospholipid syndrome (see Chapter 139) or end-stage kidney disease, or for patients with body weights over 150 kg because of limited information on the efficacy of DOACs in such individuals. If warfarin is used, treatment starts with its administration together with a rapid-acting parenteral anticoagulant such as unfractionated heparin, LMWH, or fondaparinux. The drugs are overlapped for at least 5 days and the parenteral anticoagulant is stopped when the international normalized ratio (INR) is 2.0 or more for two consecutive days.

Unfractionated heparin is the parenteral agent of choice for patients with a creatinine clearance less than 30 mL/min because LMWH and fondaparinux are cleared by the kidneys. Heparin should be administered by continuous intravenous infusion and dosed using a weight-based nomogram. The dose of heparin is adjusted based on the results of the activated partial thromboplastin time (aPTT). The aPTT is determined 6 hours after starting heparin or after changing the infusion rate; once daily aPTT determination is sufficient once a therapeutic value is achieved (see Table 140.1). Rapid achievement and maintenance of a therapeutic aPTT are important to reduce the risk for recurrent VTE.

Up to 25% of patients with acute VTE have resistance to unfractionated heparin, which is defined as a requirement for higher-than-expected doses to achieve a therapeutic aPTT. If available, anti-factor Xa level monitoring is recommended in such patients. In addition to coagulation monitoring, the platelet count should be measured at least two or three times per week because of the risk for heparin-induced thrombocytopenia (see Chapter 131).

LMWH is administered in weight-based doses using prefilled syringes. Dosage regimens differ with various LMWH formulations, but once-daily administration is as effective and safe as twice-daily administration (see Table 140.1). LMWH does not require coagulation monitoring, but the determination of anti-factor Xa levels can be considered for patients with a creatinine clearance less than 30 mL/min, those with morbid obesity, and pregnant women in the second and third trimester who may require adjustment as their weight changes. Anti-factor Xa levels are usually determined on blood samples drawn 4 hours after subcutaneous injection; therapeutic ranges of 0.6 to 1.0 IU/mL for twice-daily dosing and 1.0 to 2.0 IU/mL for once-daily dosing have been proposed.

Fondaparinux is a synthetic analog of the pentasaccharide sequence required for the binding of heparin to antithrombin. The drug is given subcutaneously once daily in a weight-adjusted manner: 5 mg for a body weight below 50 kg; 7.5 mg for body weights of 50 to 100 kg, and 10 mg for body weights greater than 100 kg.

Long-Term Anticoagulant Therapy

All VTE patients require anticoagulant therapy for at least 3 months, at which point the decision to stop or extend treatment is made based on the balance between the risk of recurrence if treatment is stopped and the risk of bleeding if it is not.[54] Patient preference also needs consideration.

A 3-month course of treatment is sufficient for patients whose VTE was provoked by major transient and reversible risk factors, such as surgery or trauma, provided that these risk factors have resolved. The risk of recurrence after stopping anticoagulation treatment in such patients is about 1% at 1 year and 5% at 5 years. Women who develop VTE with estrogen therapy also can be treated for 3 months if hormonal treatment is withdrawn.

Patients with unprovoked VTE or persistent major risk factors, such as active cancer, are at higher risk for recurrence if anticoagulant therapy is stopped (at least 10% at 1 year and 30% at 5 years) and, therefore, require extended treatment unless they are at high risk for bleeding.

In patients with unprovoked VTE, an elevated D-dimer level 1 month after stopping anticoagulant therapy identifies those at higher risk for recurrence.[59] Even with a normal D-dimer level, however, men have a 1-year risk of recurrence of about 9%, which is too high to justify stopping anticoagulation therapy. In women with unprovoked VTE, the risk of recurrence in those with a normal D-dimer level 1 month after stopping anticoagulant therapy is about 5% at 1 year; only women with unprovoked VTE who are less than 65 years of age, not obese, have no evidence of PTS and have a normal D-dimer level should consider stopping anticoagulation therapy.

For extended anticoagulation therapy beyond 6 months, the doses of apixaban and rivaroxaban can be reduced to 2.5 mg twice daily and 10 mg once daily. If warfarin is used, the dose still needs to be adjusted to achieve a target INR of 2 to 3; adjustment to a lower target INR of 1.5 to 2 was associated with a higher rate of recurrence.

Side Effects of Anticoagulant Therapy

Bleeding is the most common side effect of anticoagulant therapy. Major bleeding, which includes intracranial, gastrointestinal, or retroperitoneal bleeding and leads to hospitalization, transfusion, and death, occurs 1% to 3% per year of patients treated with warfarin. The risk of major bleeding is lower with DOACs than with warfarin. With both warfarin and DOACs, the risk for major bleeding increases with older age, the presence of comorbid conditions (e.g., hypertension, renal impairment, previous gastrointestinal bleeding, or cancer), and the use of concomitant drugs, particularly antiplatelet agents.

An approach to the management of major bleeding is detailed in Fig. 140.2. If serious bleeding occurs with heparin or LMWH, protamine sulphate completely reverses the anticoagulant effect of heparin and partially reverses that of LMWH (see Chapter 143). For serious bleeding with warfarin, vitamin K should be given by slow intravenous infusion along with four-factor prothrombin complex concentrate to replenish the missing vitamin K-dependent clotting factors. Dabigatran is rapidly reversed with idarucizumab, a monoclonal antibody fragment that binds dabigatran with high affinity.[60] Andexanet alfa, a recombinant variant of factor Xa, reverses the anticoagulant effects of apixaban, rivaroxaban, and edoxaban as well as heparin and LMWH.[61] If andexanet alfa is not available, four-factor prothrombin complex concentrate is often given based on data from cohort studies suggesting that it is effective. Ciraparantag, a small synthetic molecule that binds DOACs and heparin, is undergoing evaluation for DOAC reversal.[62]

Heparin-induced thrombocytopenia is a life- and limb-threatening complication of heparin that occurs less frequently with LMWH and almost never with fondaparinux (see Chapter 131). Heparin and LMWH are associated with a reversible elevation in the levels of transaminases. Skin necrosis (see Chapters 137 and 142) and blue toes (see Chapter 142) are rare complications with warfarin.

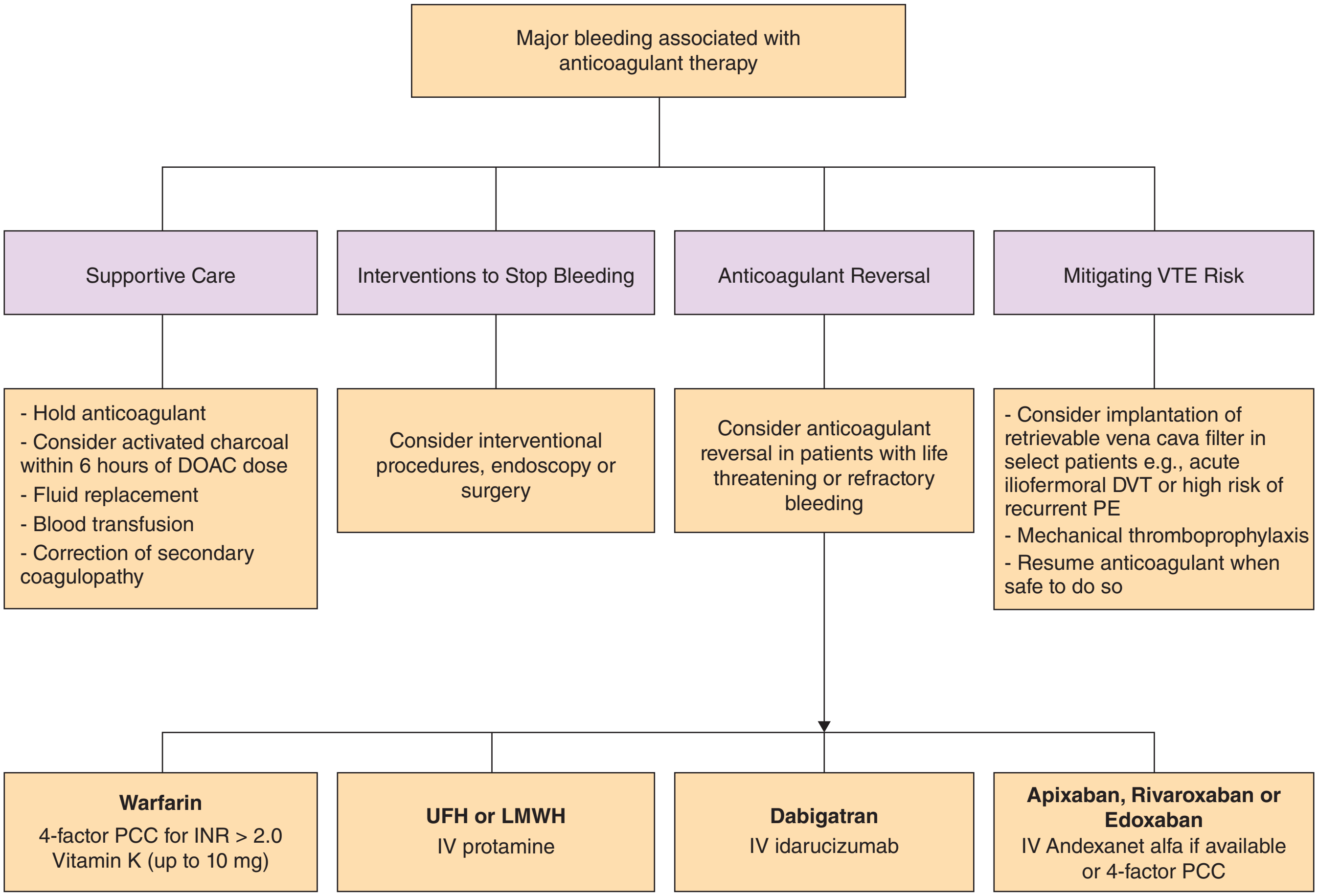

Major bleeding associated with anticoagulant therapy
Supportive Care
Interventions to Stop Bleeding
Anticoagulant Reversal
Mitigating VTE Risk
- Hold anticoagulant
- Consider activated charcoal within 6 hours of DOAC dose
- Fluid replacement
- Blood transfusion
- Correction of secondary coagulopathy
Consider interventional procedures, endoscopy or surgery
Consider anticoagulant reversal in patients with life threatening or refractory bleeding
- Consider implantation of retrievable vena cava filter in select patients e.g., acute iliofermoral DVT or high risk of recurrent PE
- Mechanical thromboprophylaxis
- Resume anticoagulant when safe to do so
Warfarin
4-factor PCC for INR > 2.0
Vitamin K (up to 10 mg)
UFH or LMWH
IV protamine
Dabigatran
IV idarucizumab
Apixaban, Rivaroxaban or Edoxaban
IV Andexanet alfa if available or 4-factor PCC

Figure 140.2 MANAGEMENT OF BLEEDING IN PATIENTS TAKING ANTICOAGULANTS. *DOAC*, Direct oral anticoagulant; *FEIBA*, factor eight inhibitor bypass agent; *LMWH*, low-molecular-weight heparin; *PCC*, prothrombin concentrate complex; *UFH*, unfractionated heparin.

VENOUS THROMBOEMBOLISM MANAGEMENT IN SPECIFIC POPULATIONS

Cancer-Associated Venous Thromboembolism

Cancer and its treatment increase the risk for VTE, and VTE is the second most common cause of death after tumor progression in cancer patients. Reduced mobility, venous compression by tumor central venous catheters, expression of tissue factor and other procoagulants by cancer cells, and chronic inflammation contribute to the increased risk of VTE in cancer patients. Therefore, the index of suspicion should be high in cancer patients who present with symptoms and signs suggestive of DVT or PE. Imaging for the staging of cancer may reveal incidental PE or DVT, which also requires treatment.

In patients with cancer-associated VTE, LMWH reduces the risk of recurrence more effectively than warfarin without significantly increasing the risk of bleeding. However, LMWH is more expensive than warfarin, and adherence is limited because of the need for daily subcutaneous injections. DOACs are more effective than LMWH for the treatment of cancer-associated VTE but are associated with more bleeding in patients with gastroesophageal cancer, unresected colorectal cancer, or urothelial cancers. Thrombocytopenia (platelet count below 50,000/μL) and metastatic disease to the brain, particularly with melanoma or renal cell cancer, also increase the risk for bleeding.

LMWH may be a better choice than DOACs for VTE treatment in patients at risk of bleeding. A prophylactic dose of LMWH can be used if the platelet count is between 30,000 and 50,000/μL, and the full dose is used once the count rises above 50,000/μL. If there is no bleeding after several weeks of treatment with LMWH, switching to a DOAC can be considered.

Patients with cancer-associated VTE require continued anticoagulation treatment if they have active cancer or are receiving ongoing cancer treatment.[63] The lower dose regimens of apixaban and rivaroxaban used for secondary prevention have not been evaluated in patients with active cancer. Until more data are available, therefore, the full dose regimens should be used.

Venous Thromboembolism in Pregnancy

Warfarin and DOACs are contraindicated in pregnancy. The approach to the prevention, diagnosis, and treatment of VTE in pregnancy is detailed in Chapter 141.

Antiphospholipid Syndrome

Antiphospholipid syndrome is associated with a hypercoagulable state and an increased risk of venous and arterial thrombosis (see Chapter 138 and 139). In patients with VTE in the setting of triple-positive antiphospholipid syndrome, defined by the presence of a lupus anticoagulant and increased levels of anticardiolipin and anti-β2-glycoprotein I antibodies, randomized trials revealed a higher risk of recurrence with DOACs than with warfarin.[64,65] Therefore, warfarin should be used for treatment in these patients (see Chapter 139). Patients with antiphospholipid syndrome have a high risk of recurrence if anticoagulation therapy is stopped. Therefore, indefinite anticoagulation is recommended.

Diagnosis and Treatment of Recurrent Venous Thromboembolism

The diagnosis of recurrent VTE is challenging.[45] A history of DVT increases the pretest probability of recurrence and reduces the diagnostic specificity of ultrasonography because, with proximal DVT, residual ultrasound abnormalities are found in up to 50% of patients 1 year after the index event. Consequently, compression ultrasonography in such patients is useful only if the comparison with a previous examination shows the involvement of a new venous segment. An elevated D-dimer level also suggests recurrence.

For patients with suspected recurrent PE, a normal D-dimer level helps to exclude the diagnosis. If the D-dimer test is positive, imaging with CTPA should be undertaken. CTPA is preferred over V/Q scanning for diagnosis of recurrence because filling defects detected by CTPA usually resolves with anticoagulation treatment, whereas perfusion defects detected by V/Q scanning are often persistent.

When assessing patients with suspected recurrent VTE who are on anticoagulant therapy, the adequacy of such treatment is an important consideration (e.g., nonadherence, subtherapeutic INR values, interruptions for procedures or surgery). Recurrent thrombosis despite adequate therapeutic anticoagulation occurs most commonly in patients with cancer, antiphospholipid syndrome (see Chapters 138 and 139), or heparin-induced thrombocytopenia (see Chapter 131).

Strategies for managing patients with recurrent VTE despite anticoagulation include LMWH, higher-intensity warfarin, or higher doses of DOACs. The anticoagulant dose can be increased to full dose in patients who were receiving lower dose regimens of LMWH, apixaban, or rivaroxaban for secondary prophylaxis. If recurrent VTE occurs despite full-dose LMWH, the drug can be given twice daily instead of once daily, and the dose can be increased by 25%; the anti-factor Xa level should be quantified to ensure that a therapeutic level is achieved. LMWH also is an option for the treatment of breakthrough VTE in patients who were taking full doses of DOACs; such patients can be considered for a switch back to DOACs after at least a month of successful treatment with LMWH. Patients with breakthrough thrombosis despite warfarin can be switched to a DOAC, or the dose of warfarin can be increased to obtain a higher target INR (e.g., 2.5 to 3.5).

Vena Cava Filters

Inferior vena cava filters, which are inserted percutaneously, are useful for patients at high risk for PE who have contraindications for anticoagulation therapy, such as recent surgery or major bleeding, or for those with a recent diagnosis of VTE who require major surgery (see Fig. 140.2). Retrievable filters should be used in these cases, and the devices should be removed as soon as anticoagulant therapy can safely be administered.

Long-Term Complications

Post-Thrombotic Syndrome

The initial pain and swelling in patients with DVT are caused by venous obstruction and inflammation. Acute obstruction usually resolves with anticoagulation therapy once recanalization occurs, and collateral venous channels develop. Venous hypertension develops if there is residual venous obstruction and incompetence of the venous valves. Venous hypertension, the hallmark of PTS, develops in up to 50% of patients with proximal DVT, usually 6 months to 2 years after the event.[66] The syndrome is characterized by dependent pain and swelling that is worse at the end of the day and is relieved with leg elevation.

Compression stockings do not prevent PTS. Therefore, their use can be delayed until the acute inflammatory process and outflow obstruction have subsided. Lifestyle alterations such as frequent leg elevation, avoidance of prolonged standing or sitting, and occasional use of analgesics will relieve symptoms in many patients. If symptoms are severe, a lightweight stocking can be helpful until the edema improves. A stocking that applies 30 to 40 mm Hg of pressure at the ankle can be prescribed if symptoms persist or worsen despite these measures.

Venous ulcers, the most severe complication of PTS, typically occur in the perimalleolar area. The best management is prevention by application of graduated compression stockings if skin changes develop in association with leg swelling. Treatment of ulcers consists of an emollient and regular leg wrapping. After the ulcer heals, graduated compression stockings should be prescribed. Surgical closure or removal of incompetent saphenous veins does not accelerate ulcer healing time compared with dressings alone but reduces the risk of recurrence.

Chronic Thromboembolic Pulmonary Hypertension

A rare complication of PE, CTEPH develops in 0.5% to 5% of patients over the course of months or years when emboli in major pulmonary arteries are replaced by fibrous tissue that becomes incorporated into the vessel walls, thereby narrowing or obstructing them.[67] Chronic obstruction of the pulmonary vascular bed increases pulmonary arterial resistance and can lead to right heart failure. Although patients may be asymptomatic at first, they experience increasing dyspnea on exertion and hypoxemia as the disease progresses. CTEPH should be suspected in patients with pulmonary hypertension, and the diagnosis is established with a combination of echocardiography, V/Q scanning, and CTPA.

Patients with CTEPH require lifelong anticoagulation therapy. Definitive treatment involves surgical thromboendarterectomy to remove the occluding material from the pulmonary arteries. For patients too sick for surgery, balloon pulmonary angioplasty may be considered. Medical therapy focuses on the treatment of right heart failure and the use of prostacyclin analogs or soluble guanylate cyclase stimulators.

PSYCHOSOCIAL CONSIDERATIONS

Long-term studies suggest that up to 50% of patients with PE report functional limitations and reduced quality of life for many years after their index event. The term post-PE syndrome has been used to describe patients with dyspnea, exercise limitation, and impaired quality of life that persists for more than 3 months despite anticoagulation therapy. The syndrome encompasses patients with CTEPH, chronic thromboembolic disease (CTED), and dyspnea with functional limitations but without evidence of pulmonary vascular disease.[68]

CTEPH is defined by the presence of a mean pulmonary artery pressure of 25 mm Hg or greater, mismatched perfusion defects on the V/Q scan, and specific findings on CTPA or magnetic resonance angiography. Patients with CTED have exercise limitations and imaging findings like those with CTEPH but do not meet the criteria for pulmonary hypertension. The incidence of CTED is unknown, but it likely affects less than 5% of patients with PE. Post-PE syndrome, persistent dyspnea with functional limitation, is common and its pathophysiology is poorly understood. CTEPH can be excluded with a normal V/Q scan and echocardiogram and other causes of dyspnea should be explored. A cardiopulmonary rehabilitation program or self-monitored exercise program may be beneficial. With ongoing symptoms, stopping anticoagulation therapy is problematic because patients may present with suspected recurrent PE and require repeated imaging tests. More work on this entity is needed.

PREVENTION OF VENOUS THROMBOEMBOLISM

More than half of the VTE events that occur each year in the United States are related to recent hospitalization or institutionalization, and up to 75% of all fatal PEs are associated with recent hospitalization. Therefore, VTE is the number one cause of preventable death in hospitalized patients. Consequently, the risk of VTE should be assessed in all hospitalized patients and if indicated, appropriate thromboprophylaxis should be administered.[69,70]

Despite the plethora of large, randomized trials demonstrating the efficacy and safety of mechanical and pharmacologic measures for reducing the risk of VTE in hospitalized patients, prophylaxis remains underused. Factors that increase the risk for DVT include surgery (particularly major orthopedic surgery and neurosurgery), major trauma, prolonged bed rest or immobilization, previous history of VTE, cancer, heart failure, paralysis or paresis, morbid obesity, advanced age, admission for COVID-19 or other forms of pneumonia, and elevated D-dimer levels.

Unfractionated heparin or LMWH is the mainstay of in-hospital thromboprophylaxis. Mechanical prophylaxis (anti-embolic stockings and intermittent pneumatic compression) should be used as an adjunct to pharmacologic prophylaxis or as standalone prophylaxis in patients at high risk for bleeding. Thromboprophylaxis should be continued until hospital discharge and extended for 30-days after discharge in patients undergoing hip or knee arthroplasty, surgery for hip fracture, or major pelvic or abdominal surgery for cancer. Selected medically ill patients may also benefit from extended thromboprophylaxis including those with elevated D-dimer levels or severe COVID-19 infection. Low-dose rivaroxaban (10 mg once daily) can be used in place of LMWH in such patients to simplify out-of-hospital thromboprophylaxis.

ATTRIBUTIONS

The section on Pathogenesis of VTE is an adaptation of *Recent advances in understanding, diagnosing and treating venous thrombosis (F1000Research 2020, 9 [Faculty Rev]:1206)* by Noel C. Chan and Jeffrey I. Weitz, and is used under the terms of a CC BY 4.0 license.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Abbattista M, Capecchi M, Martinelli I. Treatment of unusual thrombotic manifestations. *Blood*. 2020;135(5):326–334.

Chan NC, Eikelboom JW, Weitz JI. Evolving treatments for arterial and venous thrombosis: role of the direct oral anticoagulants. *Circ Res*. 2016;118(9):1409–1424.

Chan NC, Weitz JI. Recent advances in understanding, diagnosing and treating venous thrombosis. *F1000Res*. 2020;9 F1000 Faculty Rev-1206.

Chopard R, Albertsen IE, Piazza G. Diagnosis and treatment of lower extremity venous thromboembolism: a review. *JAMA*. 2020;324(17):1765–1776.

Connors JM. Thrombophilia testing and venous thrombosis. *N Engl J Med*. 2017;377(12):1177–1187.

de Winter MA, Vlachojannis GJ, Ruigrok D, et al. Rationale for catheter-based therapies in acute pulmonary embolism. *Eur Heart J Suppl*. 2019;21 (Suppl I):I16–I22.

Global COVID-19 Thrombosis Collaborative Group Endorsed by the ISTH, NATF, ESVM, and the IUA, Supported by the ESC Working Group on Pulmonary Circulation and Right Ventricular Function. COVID-19 and thrombotic or thromboembolic disease: implications for prevention, antithrombotic therapy, and follow-up: JACC state-of-the-art review. *J Am Coll Cardiol*. 2020;75(23):2950–2973.

Heit JA. Epidemiology of venous thromboembolism. Nature Reviews. *Cardiology*. 2015;12(8):464–474.

Kahn SR. The post-thrombotic syndrome. *Hematology Am Soc Hematol Educ Program*. 2016;2016(1):413–418.

Kearon C, Kahn SR. Long-term treatment of venous thromboembolism. *Blood*. 2020;135(5):317–325.

Klok FA, Delcroix M, Bogaard HJ. Chronic thromboembolic pulmonary hypertension from the perspective of patients with pulmonary embolism. *J Thromb Haemost*. 2018;16:1040–1051.

Lyman GH, Carrier M, Ay C, et al. American Society of Hematology 2021 guidelines for management of venous thromboembolism: prevention and treatment in patients with cancer. *Blood Adv*. 2021 Feb;5(4):927–974.

Nicholson M, Chan N, Bhagirath V, Ginsberg J. Prevention of venous thromboembolism in 2020 and beyond. *J Clin Med*. 2020;9(8):2467.

Piazza G. Advanced management of intermediate- and high-risk pulmonary embolism: JACC focus seminar. *J Am Coll Cardiol*. 2020;76(18):2117–2127.

Renner E, Barnes GD. Antithrombotic management of venous thromboembolism: JACC focus seminar. *J Am Coll Cardiol*. 2020;76(18):2142–2154.

Thukral S, Vedantham S. Catheter-based therapies and other management strategies for deep vein thrombosis and post-thrombotic syndrome. *J Clin Med*. 2020;9(5):1439.

Weitz JI, Chan NC. Novel antithrombotic strategies for treatment of venous thromboembolism. *Blood*. 2020;135(5):351–359.

Weitz JI, Fredenburgh JC, Eikelboom JW. A test in context: D-dimer. *J Am Coll Cardiol*. 2017;70(19):2411–2420.

REFERENCES

1. White RH. The epidemiology of venous thromboembolism. *Circulation*. 2003;107(23 suppl 1):I4–I8.
2. Heit JA, Silverstein MD, Mohr DN, Petterson TM, O'Fallon WM, Melton 3rd LJ. Predictors of survival after deep vein thrombosis and pulmonary embolism: a population-based, cohort study. *Arch Intern Med*. 1999;159(5):445–453.
3. Kearon C. Natural history of venous thromboembolism. *Circulation*. 2003;107(23_suppl_1):I-22–I-30.
4. Heit JA. Epidemiology of venous thromboembolism. *Nat Rev Cardiol*. 2015;12(8):464–474.
5. Raskob GE, Silverstein R, Bratzler DW, Heit JA, White RH. Surveillance for deep vein thrombosis and pulmonary embolism: recommendations from a national workshop. *Am J Prev Med*. 2010;38(4 suppl):S502–S509.
6. Henke PK, Kahn SR, Pannucci CJ, et al. Call to action to prevent venous thromboembolism in hospitalized patients: a policy statement from the American Heart Association. *Circulation*. 2020;141(24):e914–e931.
7. Siegal DM, Eikelboom JW, Lee SF, et al. Variations in incidence of venous thromboembolism in low-, middle-, and high-income countries. *Cardiovasc Res*. 2020;117(2):576–584.
8. Philbrick JT, Becker DM. Calf deep venous thrombosis. A wolf in sheep's clothing? *Arch Intern Med*. 1988;148(10):2131–2138.
9. Chan W-S, Spencer FA, Ginsberg JS. Anatomic distribution of deep vein thrombosis in pregnancy. *CMAJ*. 2010;182(7):657–660.
10. Reitsma PH, Versteeg HH, Middeldorp S. Mechanistic view of risk factors for venous thromboembolism. *Arterios cler Thromb Vasc Biol*. 2012;32(3):563–568.
11. Esmon CT. Basic mechanisms and pathogenesis of venous thrombosis. *Blood Rev*. 2009;23(5):225–229.
12. Seligsohn U, Lubetsky A. Genetic susceptibility to venous thrombosis. *N Engl J Med*. 2001;344(16):1222–1231.
13. Dahlbäck B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci U S A*. 1993;90(3):1004–1008.
14. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood*. 1996;88(10):3698–3703.
15. Connors JM. Thrombophilia testing and venous thrombosis. *N Engl J Med*. 2017;377(12):1177–1187.
16. Anderson Jr. FA, Spencer FA. Risk factors for venous thromboembolism. *Circulation*. 2003;107(23 Suppl 1):I9–I16.
17. McLachlin AD, McLachlin JA, Jory TA, Rawling EG. Venous stasis in the lower extremities. *Ann Surg*. 1960;152(4):678–685.
18. Hamer JD, Malone PC, Silver IA. PO2 in venous valve pockets: its possible bearing on thrombogenesis. *Br J Surg*. 1981;68(3):166–170.
19. Brooks EG, Trotman W, Wadsworth MP, et al. Valves of the deep venous system: an overlooked risk factor. *Blood*. 2009;114(6):1276–1279.
20. Welsh JD, Hoofnagle MH, Bamezai S, et al. Hemodynamic regulation of perivalvular endothelial gene expression prevents deep venous thrombosis. *J Clin Invest*. 2019;129(12):5489–5500.
21. Joffe HV, Goldhaber SZ. Upper-extremity deep vein thrombosis. *Circulation*. 2002;106(14):1874–1880.
22. Manly DA, Boles J, Mackman N. Role of tissue factor in venous thrombosis. *Ann Rev Physiol*. 2011;73:515–525.
23. Vandendries ER, Furie BC, Furie B. Role of P-selectin and PSGL-1 in coagulation and thrombosis. *J Thromb Haemost*. 2004;92(3):459–466.
24. Owens 3rd AP, Passam FH, Antoniak S, et al. Monocyte tissue factor-dependent activation of coagulation in hypercholesterolemic mice and monkeys is inhibited by simvastatin. *J Clin Invest*. 2012;122(2):558–568.
25. Pai M, Evans NS, Shah SJ, Green D, Cook D, Crowther MA. Statins in the prevention of venous thromboembolism: a meta-analysis of observational studies. *Thromb Res*. 2011;128(5):422–430.
26. Glynn RJ, Danielson E, Fonseca FA, et al. A randomized trial of rosuvastatin in the prevention of venous thromboembolism. *N Engl J Med*. 2009;360(18):1851–1861.
27. Grover SP, Mackman N. Intrinsic pathway of coagulation and thrombosis. *Arterioscler Thromb Vasc Biol*. 2019;39(3):331–338.
28. Büller HR, Bethune C, Bhanot S, et al. Factor XI antisense oligonucleotide for prevention of venous thrombosis. *N Engl J Med*. 2015;372(3):232–240.
29. Weitz JI, Bauersachs R, Becker B, et al. Effect of osocimab in preventing venous thromboembolism among patients undergoing knee arthroplasty: the FOXTROT randomized clinical trial. *JAMA*. 2020;323(2):130–139.
30. Thålin C, Hisada Y, Lundström S, Mackman N, Wallén H. Neutrophil extracellular traps: villains and targets in arterial, venous, and cancer-associated thrombosis. *Arterioscler Thromb Vasc Biol*. 2019;39(9):1724–1738.
31. Müller F, Mutch NJ, Schenk WA, et al. Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. *Cell*. 2009;139(6):1143–1156.
32. Weitz JI, Chan NC. Novel antithrombotic strategies for treatment of venous thromboembolism. *Blood*. 2020;135(5):351–359.
33. Lehmann M, Schoeman RM, Krohl PJ, et al. Platelets drive thrombus propagation in a hematocrit and glycoprotein VI-dependent manner in an in vitro venous thrombosis model. *Arterioscler Thromb Vasc Biol*. 2018;38(5):1052–1062.
34. von Brühl ML, Stark K, Steinhart A, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J Exp Med*. 2012;209(4):819–835.
35. Tarantino E, Amadio P, Squellerio I, et al. Role of thromboxane-dependent platelet activation in venous thrombosis: aspirin effects in mouse model. *Pharmacol Res*. 2016;107:415–425.
36. Herbert JM, Bernat A, Maffrand JP. Importance of platelets in experimental venous thrombosis in the rat. *Blood*. 1992;80(9):2281–2286.
37. Chirinos JA, Heresi GA, Velasquez H, et al. Elevation of endothelial microparticles, platelets, and leukocyte activation in patients with venous thromboembolism. *J Am Coll Cardiol*. 2005;45(9):1467–1471.
38. Weitz JI, Lensing AWA, Prins MH, et al. Rivaroxaban or aspirin for extended treatment of venous thromboembolism. *N Engl J Med*. 2017;376(13):1211–1222.
39. Kim PY, Vu TT, Leslie BA, Stafford AR, Fredenburgh JC, Weitz JI. Reduced plasminogen binding and delayed activation render γ'-fibrin more resistant to lysis than γA-fibrin. *J Biol Chem*. 2014;289(40):27494–27503.
40. Aleman MM, Byrnes JR, Wang JG, et al. Factor XIII activity mediates red blood cell retention in venous thrombi. *J Clin Invest*. 2014;124(8):3590–3600.
41. Shaya SA, Gani DM, Weitz JI, Kim PY, Gross PL. Factor XIII prevents pulmonary emboli in mice by stabilizing deep vein thrombi. *Thromb Haemost*. 2019;119(6):992–999.
42. Wakefield TW, Myers DD, Henke PK. Mechanisms of venous thrombosis and resolution. *Arterioscler Thromb Vasc Biol*. 2008;28(3):387–391.
43. Dronkers CEA, Ende-Verhaar YM, Kyrle PA, et al. Disease prevalence dependent failure rate in diagnostic management studies on suspected deep vein thrombosis: communication from the SSC of the ISTH. *J Thromb Haemost*. 2017;15(11):2270–2273.
44. Dronkers CEA, van der Hulle T, Le Gal G, et al. Towards a tailored diagnostic standard for future diagnostic studies in pulmonary embolism: communication from the SSC of the ISTH. *J Thromb Haemost*. 2017;15(5):1040–1043.
45. Lim W, Le Gal G, Bates SM, et al. American Society of Hematology 2018 guidelines for management of venous thromboembolism: diagnosis of venous thromboembolism. *Blood Adv*. 2018;2(22):3226–3256.
46. Weitz JI, Fredenburgh JC, Eikelboom JW. A test in context: D-dimer. *J Am Coll Cardiol*. 2017;70(19):2411–2420.
47. Needleman L, Cronan JJ, Lilly MP, et al. Ultrasound for lower extremity deep venous thrombosis. *Circulation*. 2018;137(14):1505–1515.
48. Bhatt M, Braun C, Patel P, et al. Diagnosis of deep vein thrombosis of the lower extremity: a systematic review and meta-analysis of test accuracy. *Blood Adv*. 2020;4(7):1250–1264.
49. Patel P, Patel P, Bhatt M, et al. Systematic review and meta-analysis of test accuracy for the diagnosis of suspected pulmonary embolism. *Blood Adv*. 2020;4(18):4296–4311.
50. Wells PS, Anderson DR, Bormanis J, et al. Value of assessment of pretest probability of deep-vein thrombosis in clinical management. *Lancet*. 1997;350(9094):1795–1798.
51. Wells PS, Anderson DR, Rodger M, et al. Excluding pulmonary embolism at the bedside without diagnostic imaging: management of patients with suspected pulmonary embolism presenting to the emergency department by using a simple clinical model and d-dimer. *Ann Intern Med*. 2001;135(2):98–107.
52. Delluc A, Antic D, Lecumberri R, Ay C, Meyer G, Carrier M. Occult cancer screening in patients with venous thromboembolism: guidance from the SSC of the ISTH. *J Thromb Haemost*. 2017;15(10):2076–2079.
53. Aujesky D, Roy PM, Verschuren F, et al. Outpatient versus inpatient treatment for patients with acute pulmonary embolism: an international, open-label, randomised, non-inferiority trial. *Lancet*. 2011;378(9785):41–48.
54. Ortel TL, Neumann I, Ageno W, et al. American Society of Hematology 2020 guidelines for management of venous thromboembolism: treatment of deep vein thrombosis and pulmonary embolism. *Blood Adv*. 2020;4(19):4693–4738.

55. Aujesky D, Obrosky DS, Stone RA, et al. Derivation and validation of a prognostic model for pulmonary embolism. *Am J Respir Crit Care Med*. 2005;172(8):1041–1046.
56. Konstantinides SV, Meyer G, Becattini C, et al. 2019 ESC Guidelines for the diagnosis and management of acute pulmonary embolism developed in collaboration with the European Respiratory Society (ERS): The Task Force for the diagnosis and management of acute pulmonary embolism of the European Society of Cardiology (ESC). *Eur Heart J*. 2019;41(4):543–603.
57. Enden T, Haig Y, Kløw N-E, et al. Long-term outcome after additional catheter-directed thrombolysis versus standard treatment for acute iliofemoral deep vein thrombosis (the CaVenT study): a randomised controlled trial. *Lancet*. 2012;379(9810):31–38.
58. Vedantham S, Goldhaber SZ, Julian JA, et al. Pharmacomechanical catheter-directed thrombolysis for deep-vein thrombosis. *N Engl J Med*. 2017;377(23):2240–2252.
59. Kearon C, Parpia S, Spencer FA, et al. Long-term risk of recurrence in patients with a first unprovoked venous thromboembolism managed according to d-dimer results: a cohort study. *J Thromb Haemost*. 2019;17(7):1144–1152.
60. Pollack CV, Reilly PA, van Ryn J, et al. Idarucizumab for dabigatran reversal—full cohort analysis. *N Engl J Med*. 2017;377(5):431–441.
61. Connolly SJ, Crowther M, Eikelboom JW, et al. Full study report of andexanet alfa for bleeding associated with factor Xa inhibitors. *N Engl J Med*. 2019;380(14):1326–1335.
62. Ansell J, Laulicht BE, Bakhru SH, et al. Ciraparantag, an anticoagulant reversal drug: mechanism of action, pharmacokinetics, and reversal of anticoagulants. *Blood*. 2021;137(1):115–125.
63. Lyman GH, Carrier M, Ay C, et al. American Society of Hematology 2021 guidelines for management of venous thromboembolism: prevention and treatment in patients with cancer. *Blood Adv*. 2021;5(4):927–974.
64. Pengo V, Denas G, Zoppellaro G, et al. Rivaroxaban vs warfarin in high-risk patients with antiphospholipid syndrome. *Blood*. 2018;132(13): 1365–1371.
65. Ordi-Ros J, Sáez-Comet L, Pérez-Conesa M, et al. Rivaroxaban versus vitamin K antagonist in antiphospholipid syndrome: a randomized noninferiority trial. *Ann Intern Med*. 2019;171(10):685–694.
66. Kahn SR, Comerota AJ, Cushman M, et al. The postthrombotic syndrome: evidence-based prevention, diagnosis, and treatment strategies. *Circulation*. 2014;130(18):1636–1661.
67. Hoeper MM, Mayer E, Simonneau G, Rubin LJ. Chronic Thromboembolic Pulmonary Hypertension. *Circulation*. 2006;113(16):2011–2020.
68. Klok FA, Delcroix M, Bogaard HJ. Chronic thromboembolic pulmonary hypertension from the perspective of patients with pulmonary embolism. *J Thromb Haemost*. 2018;16(6):1040–1051.
69. Anderson DR, Morgano GP, Bennett C, et al. American Society of Hematology 2019 guidelines for management of venous thromboembolism: prevention of venous thromboembolism in surgical hospitalized patients. *Blood Adv*. 2019;3(23):3898–3944.
70. Schünemann HJ, Cushman M, Burnett AE, et al. American Society of Hematology 2018 guidelines for management of venous thromboembolism: prophylaxis for hospitalized and nonhospitalized medical patients. *Blood Adv*. 2018;2(22):3198–3225.

CHAPTER 141

PREVENTION AND TREATMENT OF VENOUS THROMBOEMBOLISM IN PREGNANCY

Leslie Skeith and Shannon M. Bates

Pregnant persons and those who are postpartum are at increased risk of venous thromboembolism (VTE). Although pregnancy-related VTE can affect any part of the venous circulation, as in the non-pregnant population, it most commonly manifests as deep vein thrombosis (DVT) or pulmonary embolism (PE). Management of pregnancy-related VTE is complicated as the diagnosis, prevention, and treatment of pregnancy-related VTE must consider fetal, as well as maternal, well-being. Moreover, there are limited high-quality data to help clinicians manage this patient population.

EPIDEMIOLOGY

VTE is a cause of significant maternal morbidity and mortality globally. The incidence of pregnancy-related VTE has been estimated at 1.2 per 1000 deliveries (95% confidence interval [CI], 0.6 to 1.8).[1] Although this absolute risk is low, VTE is responsible for 3.2% of all maternal deaths.[2] In higher-income countries, where death from obstetrical hemorrhage is less common, that figure is 13.8%.[2]

In a meta-analysis of 20 studies, the pooled incidence of VTE was similar in each of the antepartum and postpartum periods at 0.6 per 1000 pregnant persons.[1] Given the shorter length of the postpartum period, the daily risk is substantially higher after delivery (15- to 35-fold increase in relative risk) than during pregnancy (5- to 10-fold relative risk increase).[3] The daily VTE risk is highest in the first three to six weeks after delivery, although a small absolute risk increase may be seen up to 12 weeks after delivery.[1,4]

Risk Factors

The most important risk factor for pregnancy-associated VTE is a prior history of venous thrombosis, which increases the risk of VTE antepartum to 4.2% (95% CI, 0.3% to 6.0%) and postpartum to 6.5% (95% CI, 4.3% to 9.7%).[5,6] The risk of antepartum recurrence has been reported to vary according to provoking factors present at the initial event, with estimated risks of 6.4% (95% CI, 3.9% to 10.4%), 3.6% (95% CI, 1.4% to 8.9%), and 1.1% (95% CI, 0.2% to 5.8%) in those with a history of hormonal-associated, unprovoked, and provoked (nonhormonal temporary risk factor) events, respectively.[5,6]

Inherited thrombophilias are also important risk factors, especially when accompanied by a family history of VTE (Table 141.1).[5] While there have been many studies evaluating the association between hereditary thrombophilias and pregnancy-related VTE, the impact of acquired thrombophilias has been less well investigated in this setting. The most common acquired thrombophilia is antiphospholipid syndrome (see Chapter 139). There are few data on the risk of pregnancy-associated VTE in those with antiphospholipid antibody positivity and no prior thrombosis history; however, in those with obstetrical antiphospholipid syndrome, it has been estimated at 0% (95% CI, 0% to 1.2%) antepartum and 1.1% (95% CI, 0.2%% to 6.2%) postpartum.[7]

Many clinical risk factors have been shown to increase the risk of pregnancy-associated VTE[5]; however, most, including cesarean delivery, have only a small effect with absolute risks far less than 1% in each of the antepartum and postpartum periods.[5,8] How combinations of independent risk factors might affect overall VTE risk has not been well studied, although one might reasonably expect that combinations of factors might result in more significant risks. Factors with estimated antepartum or postpartum VTE risks greater than 1% are listed in Table 141.2.

PATHOPHYSIOLOGY

The pathophysiology of pregnancy-associated VTE can be related to Virchow's triad.[6] Changes in hemostasis include alterations in levels of coagulation factors and natural inhibitors of coagulation that favor thrombosis (increased levels of factor VIII and factor X, von Willebrand factor, and fibrinogen, as well as decreased protein S levels) and a reduction in fibrinolytic activity (increased activity of plasminogen activator inhibitors-1 and -2 and decreased tissue plasminogen activator activity). VTE risk is also heightened by decreased lower extremity venous flow resulting from hormonally mediated increases in lower limb venous distensibility and capacity, as well as by compression of the pelvic veins by the gravid uterus and of the left iliac vein by the right iliac artery. Damage to the endothelium may occur from venous distension and at the time of delivery.

CLINICAL MANIFESTATIONS

The clinical manifestations of pregnancy-associated VTE can be similar to those in the non-pregnant population and include lower extremity pain, tenderness, and swelling for DVT and chest pain, dyspnea, tachypnea, tachycardia, palpitations, hemoptysis, pre-syncope/syncope, and apprehension for PE. Symptoms or signs of lower extremity DVT occur in less than 25% of pregnant persons with proven PE.

Differences in VTE presentation during pregnancy include a predilection for left leg DVT (greater than 80% of cases during pregnancy) and for isolated iliac and/or femoral vein thrombosis.[9] These latter events usually present with back, flank, or buttock pain and swelling and discoloration of the entire leg.

DIFFERENTIAL DIAGNOSIS

The differential diagnosis for DVT includes venous insufficiency, superficial vein thrombosis, cellulitis, hematoma, ruptured Baker's cyst, congestive heart failure, and tumor. The differential diagnosis for PE is extensive and includes pneumonia, pleurisy, congestive heart failure, pneumothorax, musculoskeletal pain, and myocardial infarction. Of course, symptoms like those of VTE are common even in uncomplicated pregnancies and are not usually due to DVT or PE. It is, therefore, important to ask about sudden changes or worsening in symptoms and to be vigilant for associated features of VTE out of keeping with normal pregnancy.

Studies in which pregnant women with symptoms suggestive of DVT or PE underwent appropriate testing report a prevalence of VTE of less than 10%. However, healthcare providers must have a high index of suspicion for VTE when a pregnant woman presents

TABLE 141.1 **Asymptomatic Hereditary Thrombophilia and Risk of Pregnancy-Associated Venous Thromboembolism**

Thrombophilia	Antepartum Risk	Postpartum Risk
Positive Family VTE History [Absolute risk (95% CI)[a]]		
Antithrombin deficiency	2.70% (95% CI, 0%–8.53%)	4.83% (95% CI, 0%–15.65%)
Protein C deficiency	1.63% (95% CI, 0%–5.02%)	1.06 (95% CI, 0%–4.09%)
Protein S deficiency	0% (95% CI, 0%–1.46%)	1.76% (95% CI, 0%–5.99%)
Factor V Leiden, heterozygous	0.50% (95% CI, 0.06%–1.21)	0.62% (95% CI, 0%–1.90%)
Factor V Leiden, homozygous	6.88% (95% CI, 1.04%–15.83%)	5.87% (95% CI, 0.57%–14.44%)
Prothrombin mutation, heterozygous	0 (95% CI, 0%–0.73%)	0.95% (95% CI, 0%–3.26%)
Prothrombin mutation, homozygous	No data, likely at least as high as in those with no family history	No data, likely at least as high as in those with no family history
Combined thrombophilia	0 (95% CI, 0%–2.35%), although risk likely at least as high as in those with no family history	1.06% (95% CI, 0%–5.30%)
Negative Family VTE History [Risk Ratio (95% CI); Estimated absolute risk[b]]		
Antithrombin deficiency	OR: 6.09 (95% CI, 1.58–23.43) Absolute risk: 0.36%	OR: 6.09 (95% CI, 1.58–23.43) Absolute risk: 0.36%
Protein C deficiency	OR: 4.8 (95% CI, 2.2–10.6) Absolute risk: 0.3%	OR: 4.8 (95% CI, 2.2–10.6) Absolute risk: 0.3%
Protein S deficiency	OR 3.2 (95% CI, 1.5–6.9) Absolute risk: 0.2%	OR 3.2 (95% CI, 1.5–6.9) Absolute risk: 0.2%
Factor V Leiden, heterozygous	OR: 4.6 (95% CI, 2.6–7.9) Absolute risk: 0.28%	OR: 4.6 (95% CI, 2.6–7.9) Absolute risk: 0.28%
Factor V Leiden, homozygous	OR: 34.4 (95% CI, 9.9–120.1) Absolute risk: 2.06%	OR: 34.4 (95% CI, 9.9–120.1) Absolute risk: 2.06%
Prothrombin mutation, heterozygous	OR: 5.43 (95% CI, 3.66–8.03) Absolute risk: 0.32%	OR: 5.43 (95% CI, 3.66–8.03) Absolute risk: 0.32%
Prothrombin mutation, homozygous	OR: 26.4 (95% CI, 1.2–559.3) Absolute risk: 1.6%	OR: 26.4 (95% CI, 1.2–559.3) Absolute risk: 1.6%
Combined thrombophilia (for factor V Leiden and prothrombin gene mutation, compound heterozygosity)	OR: 47 (95% CI, 26–84) Absolute risk: 2.82%	OR: 47 (95% CI, 26–84) Absolute risk: 2.82%

[a] Risks from family cohort studies, estimates of absolute risk (95% CI).

[b] Risks from case control studies, absolute risk estimated by OR × baseline VTE risk of 0.6/1000 pregnancies.

CI, Confidence interval; *OR*, odds ratio.

Data from Bates SM, Rajasekhar A, Middeldorp S, et al. American Society of Hematology 2018 guidelines for management of venous thromboembolism: venous thromboembolism in the context of pregnancy. *Blood Adv.* 2018;2(22):6–8.

with these symptoms since missing a diagnosis exposes the patient to the risk of fatal PE.

LABORATORY MANIFESTATIONS

While D-dimer levels are instrumental in guiding the investigation of suspected VTE in the nonpregnant population, there is less of a role for D-dimer measurements in the pregnant population. The majority of pregnant patients have an elevated D-dimer, especially as pregnancy progresses. However, D-dimer values are significantly higher in pregnant patients who have confirmed DVT, compared with pregnant patients without DVT.[10] While higher D-dimer cutpoints or trimester specific D-dimer levels have been proposed to improve the utility of D-dimer values during pregnancy, these strategies have not been validated. D-dimer levels should not be used in isolation to evaluate a pregnant woman with suspected DVT or PE. A systematic meta-analysis that investigated the safety of D-dimer to rule out VTE in pregnant persons reported a high sensitivity and negative predictive value for the assay[11]; the authors suggested that their results suggest that D-dimer can safely rule out VTE during pregnancy when the disease prevalence is consistent with a low/intermediate or unlikely pre-test probability. However, the number of studies (three prospective, one retrospective) and subjects with VTE ($n = 69$) were small and only two of the included studies were prospective management studies. Recent advances in the use of D-dimer in combination with clinical prediction rules in pregnant patients are described below.

Diagnosing Suspected Deep Vein Thrombosis in Pregnancy

Imaging with a compression Doppler ultrasound of the leg is the recommended first test to evaluate suspected DVT in pregnancy. Because the majority of DVTs in pregnancy start in the pelvic veins and progress distally, ultrasonography should include evaluation of the iliac veins. If there is high clinical suspicion for isolated iliac vein thrombus after an initial negative leg ultrasound, then magnetic resonance imaging (MRI) of the pelvic veins should be considered where available.[5] In a large database study, there was no increased risk of pregnancy loss, congenital anomaly, neoplasm or hearing loss

TABLE 141.2 Clinical Risk Factors for Pregnancy-Associated Venous Thromboembolism With Estimated Risks of Greater Than 1%

Clinical Risk Factor	Adjusted Odds Ratio (95% CI) from Case Control Studies and Estimated Absolute Risk (%)[a]	Absolute Risk % (95% CI)
Antepartum		
Prior venous thromboembolism		4.2% (95% CI, 0.3%–6.0%)
• Hormonal–related		6.4% (95% CI, 3.9%–10.4%)
• Unprovoked		3.6% (95% CI, 1.4%–8.9%)
• Non-hormonal provoking factor		1.1% (95% CI, 0.2%–5.8%)
Immobility[b] with prepregnancy BMI $\geq$25 kg/m^2	OR: 62.3 (95% CI: 11.5–337.0) Estimated absolute risk: 3.8%	
Dermatomyositis	OR: 24.07 (95% CI: 5.93–97.70) Estimated absolute risk: 1.9%	
Severe ovarian hyperstimulation syndrome or ovarian hyperstimulation syndrome requiring hospitalization		1.6%–6.6%
Myeloid leukemia	OR: 20.75 (95% CI: 6.61–65.12) Estimated absolute risk: 1.3%	
Postpartum		
Prior venous thromboembolism		6.5% (95% CI, 4.3–9.7%)
Immobility[b] with prepregnancy BMI $\geq$25 kg/m^2	OR: 40.1 (95% CI: 8.0–201.5) Estimated absolute risk: 2.4%	
Infection[c] following vaginal delivery	OR: 20.2 (95% CI: 6.4–63.5) Estimated absolute risk: 1.2%	

[a] Absolute risk estimated by OR × baseline VTE risk of 0.6/1000 pregnancies.
[b] Immobility classified as strict bedrest for $\geq$1 week in the antepartum period.
[c] Infection defined as clinical signs/symptoms with fever and elevated white blood cell count.
BMI, Body mass index; *CI*, confidence interval; *OR*, odds ratio.
Data from Bates SM, Rajasekhar A, Middeldorp S, et al. American Society of Hematology 2018 guidelines for management of venous thromboembolism: venous thromboembolism in the context of pregnancy. *Blood Adv.* 2018;2(22):6–8; and Bates SM, Middeldorp S, Rodger M, et al. Guidance for the treatment and prevention of obstetric-associated venous thromboembolism. *J Thromb Thrombolysis.* 2016;41(1):92–128.

in children with first trimester MRI exposure.[12] However, non-gadolinium techniques should be used and gadolinium avoided when possible due to reported side effects of pregnancy loss, rheumatological, inflammatory, or infiltrative skin disorders and the rare complication of nephrogenic systemic fibrosis.[12] If the initial ultrasound is negative, then a repeat clinical assessment and serial leg ultrasounds with iliac imaging three to seven days later is recommended to ensure no progressive DVT is missed.[5] If isolated iliac vein thrombosis is suspected and MRI is not available, a repeat ultrasound can be performed sooner, and consideration should be given to empiric anticoagulation until a negative follow-up test is obtained. A strategy of a single whole leg ultrasound (including the calf veins) with imaging of the iliac veins is used in some centers, but the upper bound of the 95% CI around the risk estimate for missed VTE (1.4%; 95% CI, 0.3% to 4.9%) was greater than that generally accepted.[5] In a systematic review of eight studies that evaluated the utility of serial ultrasonography after a single negative proximal or whole leg ultrasound with iliac vein imaging in pregnant patients with suspected DVT, the false negative frequency for a single ultrasound was 1.3% (95% CI, 0.4% to 2.6%), and a follow-up DVT was diagnosed on fewer than 1% of serial tests.[13] There were insufficient data to compare a whole leg versus proximal ultrasound strategy. Given the relatively small sample size, high upper bound 95% CI, and the lack of a confirmatory patient management study showing the safety of a single proximal ultrasound with iliac vein imaging, a serial ultrasound approach that includes imaging of the iliac veins is still recommended in most cases. However, this information can be used to counsel patients in shared decision making regarding the need to return for follow-up tests.

Diagnosing Suspected Pulmonary Embolism in Pregnancy

A clinical prediction rule to evaluate suspected DVT in pregnancy called the LEFt rule (Left leg, Edema of calf circumference of 2 centimeters or over, and a First Trimester presentation)[9] has been developed. Research is ongoing to prospectively validate the use of the LEFt rule in combination with D-dimer (LEaD: clinicaltrials.gov ID NCT02507180) in pregnant persons with suspected DVT.

Until recently, there were no validated clinical prediction rules to evaluate suspected PE in the pregnant population and, consequently, advanced chest imaging was the recommended first test in all patients. Ventilation/perfusion (V/Q) lung scanning and computed tomography pulmonary angiography (CTPA) have similar diagnostic accuracy for PE and frequency of nondiagnostic test results in pregnancy.[5] While the fetal radiation dose is typically higher with V/Q scanning than with CTPA, levels with both are below the threshold of radiation exposure of 50 mGy (5 cGy). Above this level, there is up to a twofold increase in the risk of childhood malignancy; only in higher doses (100 to 200 mGy) are major malformations seen.[5,8] V/Q scanning is often the preferred test, if available locally, because there is less radiation exposure to the breast tissue and a theoretically lower breast cancer risk over time, compared to that with CTPA scanning.[5,8] Newer generation CT scanners and different CTPA scan protocols may minimize the amount of radiation exposure, and some scintigraphy protocols include perfusion only, lower radioisotope dose, or no accompanying chest radiograph. However, how these protocols affect diagnostic accuracy of PE is less well known and they may lead to a higher proportion of non-diagnostic scans. Some diagnostic algorithms and guidelines suggest completing bilateral compression doppler leg ultrasounds prior to any chest imaging, however, the yield for detecting asymptomatic DVT is very low, and imaging may still miss thrombosis of the pelvic veins.

Two recent large prospective studies have demonstrated the safe use of clinical prediction rules in combination with D-dimer testing in the pregnant population. In the first, women with none of the YEARS criteria (clinical signs of DVT, hemoptysis, or PE as the most likely diagnosis) and D-dimer < 1000 ng/mL or one or more YEARS criteria and D-dimer < 500 ng/mL had chest imaging withheld.[14] Patients with clinical signs of DVT first underwent compression ultrasonography of the symptomatic leg, and if DVT was present then treatment was initiated, and no chest imaging performed. Among the 195 patients that avoided CT chest imaging, symptomatic VTE was diagnosed during three months of follow-up in 0.51% (95% CI, 0.09% to 2.9%). Furthermore, among the 494 patients in whom DVT was not diagnosed at baseline, CTPA was avoided in close to 40% of patients.[14] In the second study, CTPA was safely avoided in 12% of the study patients with a low or intermediate clinical pretest probability of PE using the revised Geneva score and D-dimer less than 500 ng/mL (frequency of symptomatic VTE after three months of follow-up was 0% [95% CI, 0% to 8.0%]).[15]

There are limitations to these clinical prediction rules that require further study. Clinical gestalt is still required with the YEARS algorithm for the "PE as the most likely diagnosis" criterion. The revised Geneva score also applies imperfectly to pregnant persons as it includes an item for "age greater than 65 years." Nevertheless, data from these prospective studies suggest that D-dimer results in combination with clinical pretest probability can safely exclude PE in a significant proportion in pregnant patients without the need for radiologic imaging.

If a clinical prediction score is used for suspected PE in pregnancy, we recommend use of the YEARS algorithm (including lower extremity ultrasounds in those with leg symptoms) to avoid advanced chest imaging in the largest proportion of patients. Of the two strategies described in the text, the YEARS algorithm had the most robust estimate for VTE frequency after three months of follow-up and it has also been retrospectively validated using data from the other study described in the text that used the revised Geneva score in combination with D-dimer.

PROGNOSIS

The case fatality rate of pregnancy-related VTE has been estimated at 0.68% (95% CI, 0.41% to 0.96%), while that for PE is 2.00% (95% CI, 1.44 to 2.56).[1] Patients who develop iliofemoral thrombosis, which is more common in pregnancy, are more likely to develop post-thrombotic syndrome, a condition that can be associated with chronic swelling, discomfort, discoloration and, less commonly, leg ulceration. In a large case control study, 42% of women with pregnancy-associated DVT had some degree of post-thrombotic syndrome with strong predictors that included proximal DVT in the postpartum period, advanced age, and smoking.[16] Patients rarely develop chronic thromboembolic pulmonary hypertension (CTEPH) and, more commonly, have a continued reduction in exercise capacity after PE (post-PE syndrome), although data on both in the pregnant population are lacking. PE can have significant psychosocial effects, including anxiety, post-traumatic or a panic-like syndrome. Younger patients typically report a greater impact than older patients,[17] and it would be reasonable to hypothesize that this would be even greater during stressful periods like pregnancy and postpartum.

THERAPY

Anticoagulant Use During Pregnancy

While unfractionated heparin (UFH) and low molecular weight heparin (LMWH) do not cross the placenta and are safe for the fetus, LMWH is preferred because of its improved bioavailability, longer drug half-life, more predictable dose response, and improved safety profile (see Chapter 143).[5] Side effects of LMWH include antepartum bleeding (~1.4%), early postpartum bleeding (~1.9%), adverse skin reactions, wound hematomas, and rarely heparin-induced thrombocytopenia (HIT; see Chapter 131).[18] Longer-term use of therapeutic-dose LMWH may lead to reduced bone mineral density, but this has not been found with prophylactic doses of LMWH.[5,8] Because of the rare but serious risk of epidural hematoma and associated hemiplegia, it is recommended that neuraxial anesthesia be delayed at least 24 hours after the last dose of therapeutic LMWH and 12 hours after the last dose of prophylactic LMWH.[5,8]

In the setting of previous or active HIT, use of danaparoid is recommended if it is available because it does not cross the placenta and is safe for the fetus (see Chapter 131). As fondaparinux does cross the placenta in small amounts and safety data are primarily limited to case series with exposure later in pregnancy, its use should be avoided (especially early in pregnancy) unless there is no safe alternative. Switching to a different LMWH formulation improves skin reactions in many cases.

Vitamin K antagonists (VKAs) like warfarin carry a known risk of congenital anomalies, fetal hemorrhage, and pregnancy loss and so should be avoided in pregnancy for VTE indications, as safe and effective alternatives like LMWH are available.[8] Warfarin embryopathy typically occurs with exposure between 6 and 12 weeks' gestation and includes nasal hypoplasia, stippled epiphyses and, less commonly, limb hypoplasia. Other congenital anomalies including central nervous system malformations have been reported with VKA use during pregnancy.[8]

Direct oral anticoagulants (DOACs) cross the placenta and animal exposure studies indicate a risk of reproductive toxicity, including implantation loss, congenital malformations, altered ossification, and hemorrhage. To date, limited clinical data (predominantly with rivaroxaban) do not show a specific pattern of fetal anomalies, with rates of congenital anomalies similar to those seen in the general pregnant population; however, more research is still needed.[19] Based on guidance from the International Society on Thrombosis and Haemostasis (ISTH), exposure to a DOAC in pregnancy does not warrant elective termination of pregnancy but pregnant persons with DOAC exposure should be switched to LMWH as soon as possible.[20]

Patients receiving long-term VKA or DOAC therapy should be counselled about associated fetal risks with exposure and the need to seek medical attention as soon as possible with a positive pregnancy test. A pregnancy-specific anticoagulant management plan should be developed prior to any planned pregnancy.[6] Options include conversion to LMWH before conception is attempted (which may result in extended LMWH exposure) and performance of frequent pregnancy tests with substitution of LMWH as soon as possible once pregnancy is achieved. Given the paucity of data regarding the risk of embryopathy with DOAC therapy, ISTH guidance recommends a transition to LMWH or VKA prior to conception.[20]

Anticoagulant Therapy

In a patient with a new confirmed DVT or PE diagnosed in pregnancy, initial weight-based therapeutic dose LMWH is recommended. A systematic review involving the nonpregnant population and limited data in pregnancy suggest no difference in bleeding or efficacy with once or twice daily dosing regimens and guidance documents suggest that either approach is acceptable.[5,8] The recent American Society of Hematology (ASH) guidelines have suggested against the routine use of anti-Xa level monitoring given resource and cost implications, lack of test reliability, lack of clinical outcome data, and absence of validated anti-Xa level therapeutic ranges in pregnancy.[5]

There is wide practice variation with respect to LMWH dosing and the role of anti-Xa level monitoring in pregnancy. Consideration may be given to using peak anti-Xa levels to direct dosing in selected cases such as those with antithrombin deficiency and early in therapy in those with extensive VTE, recurrent VTE while on therapeutic dose anticoagulation, or extreme over or under body weight although clinical outcome data are lacking. There is a limited role for checking trough anti-Xa levels, but this can be considered in renal insufficiency to exclude drug accumulation.

Interventional Management of Venous Thromboembolism in Pregnancy

Evidence for the use of systemic or catheter directed thrombolysis in this patient population is limited to case reports and case series. Because of concerns about both maternal bleeding and fetal complications, pregnancy is often considered a relative contraindication to thrombolysis; however, patients should not be denied potentially life-saving therapy just because they are pregnant. Systemic thrombolysis in pregnancy should be reserved for patients with life-threatening hemodynamic instability or limb-threatening compromise.[5] There are limited data on the use of catheter-directed thrombolysis in pregnancy because of the associated radiation risks. Extrapolation from randomized controlled data in the non-pregnant population highlights an important bleeding risk. Given high risks of major obstetrical bleeding with use thrombolytic therapy following delivery, if available, thrombectomy may be preferred over systemic thrombolysis during the immediate postpartum period.

Inferior vena cava (IVC) filter placement is technically more challenging during pregnancy because of anatomic changes, including dilation of the IVC, and concerns about fetal radiation exposure during fluoroscopy. Filter thrombosis and IVC penetration may be more common in pregnancy and there are important long-term implications with failure of filter retrieval. Consequently, IVC filter placement should be avoided when possible and filter placement is generally reserved for those patients in whom anticoagulant therapy cannot be safely given or must be withheld (e.g., for delivery) within two to four weeks of newly diagnosed VTE.

Prevention of Venous Thromboembolism

In pregnant persons with prior VTE, a detailed history of their event and discussion about the role of thromboprophylaxis is needed. In those with a prior unprovoked or hormone-associated VTE, the risk of recurrent VTE is sufficiently high that LMWH prophylaxis is recommended during pregnancy and for 6 weeks postpartum (Tables 141.2 and 141.3).[5–7] In someone with a previous event associated with a major provoking risk factor (e.g., hospitalization, major surgery), the risk of recurrence appears lower and prophylaxis is recommended only in the postpartum period with clinical vigilance during pregnancy. Where the provoking event may have been more modest (e.g., long haul flight or minor illness) or there are additional risk factors

TABLE 141.3 Prophylaxis Recommendations (American Society of Hematology)

Risk Factor	Antepartum	Postpartum
Prior hormonal-related VTE	Recommend yes	Recommend yes
Prior unprovoked VTE	Recommend yes	Recommend yes
Prior VTE with resolved major transient risk factor	Suggest no	Recommend yes
Asymptomatic Thrombophilia with Positive Family VTE History		
Antithrombin deficiency	Recommend yes	Recommend yes
Protein C deficiency	Suggest no	Suggest yes
Protein S deficiency	Suggest no	Suggest yes
Factor V Leiden, heterozygous	Suggest no	Suggest no
Factor V Leiden, homozygous	Suggest yes	Suggest yes
Prothrombin mutation, heterozygous	Suggest no	Suggest no
Prothrombin mutation, homozygous	No formal recommendation, individual panel members yes	No formal recommendation, individual panel members yes
Combined thrombophilia	Suggest yes	Suggest yes
Asymptomatic Thrombophilia with Negative Family VTE History		
Antithrombin deficiency	Suggest no	Suggest no
Protein C deficiency	Suggest no	Suggest no
Protein S deficiency	Suggest no	Suggest no
Factor V Leiden, heterozygous	Suggest no	Suggest no
Factor V Leiden, homozygous	Suggest yes	Suggest yes
Prothrombin mutation, heterozygous	Suggest no	Suggest no
Prothrombin mutation, homozygous	Suggest no	Suggest yes
Combined thrombophilia	Suggest yes	Suggest yes

VTE, Venous thromboembolism.
Recommendations from Bates SM, Rajasekhar A, Middeldorp S, et al. American Society of Hematology 2018 guidelines for management of venous thromboembolism: venous thromboembolism in the context of pregnancy. *Blood Adv.* 2018;2(22):6–8; based on a VTE risk threshold antepartum of 2% and postpartum of 1%.

(e.g., known thrombophilia), a discussion about the potential risks and benefits of thromboprophylaxis during pregnancy and the postpartum period is needed, with a focus on patient values and preferences.

For patients with inherited thrombophilia but no personal history of VTE, clinical practice guideline recommendations vary based on limited data and different VTE risk thresholds chosen for thromboprophylaxis.[5] Prophylaxis recommendations are typically based on thrombophilia severity and family VTE history. Antepartum prophylaxis is typically reserved for those with more severe thrombophilias, with clinical vigilance typically recommended for patients with mild thrombophilias (e.g., heterozygosity for the factor V Leiden or prothrombin gene mutation). Prophylaxis is more commonly provided during the postpartum period as the daily VTE risk is higher and the burden of prophylaxis is lower given the shorter time frame. The ASH guidelines prophylaxis recommendations are summarized in Table 141.3.[5]

The optimal LMWH dose for prevention of VTE in pregnancy is unknown, with standard prophylactic and intermediate dosing regimens commonly used both antepartum and postpartum. A randomized controlled trial comparing a prophylactic versus an intermediate LMWH dose strategy will provide guidance (Highlow study NTR3894). In patients with severe thrombophilias such as marked antithrombin deficiency, therapeutic doses of LMWH are often used for prophylaxis, even in the absence of a VTE history.

Management of Anticoagulant Therapy at Delivery and Postpartum

An individualized, multidisciplinary approach with shared decision making is important when making decisions about delivery plans and anesthetic options. Risks of bleeding around the time of delivery and of recurrent VTE with anticoagulant interruption, as well as the importance placed by both patients and healthcare providers on avoiding induction of labor and preserving access to regional anesthesia, must be considered. In North America, induction of labor with planned interruption of LMWH is commonly used to increase access to neuraxial anesthesia and decrease the risk of bleeding during delivery, especially for those on therapeutic-dose LMWH. This strategy was recommended in the ASH guidelines for those receiving therapeutic LMWH.[5] Those guidelines suggested against scheduled delivery with discontinuation of anticoagulants in women receiving prophylactic LMWH, given the shorter time interval required before safe insertion of an epidural catheter. Other strategies include holding LMWH and allowing spontaneous delivery or stopping LMWH ahead of the expected delivery date (which may be associated with an increased risk of recurrent VTE). Some centers substitute prophylactic UFH for prophylactic LMWH close to term to increase the likelihood of access to regional anesthesia with spontaneous labor. A switch to twice daily LMWH or intravenous UFH prior to induction to limit the time off therapeutic anticoagulation may be used in women with more recently diagnosed VTE (e.g., within one month of delivery). Postpartum, anticoagulation resumption should be done cautiously as initiating therapeutic anticoagulation too quickly may lead to bleeding complications.

Anticoagulants and Breastfeeding

VKAs, UFH, LMWH, danaparoid, and fondaparinux are considered safe to use while breastfeeding.[5] VKAs and UFH do not cross into breast milk. A small amount of LMWH is detected in breast milk but is considered safe due to a very low oral drug bioavailability. No or very low levels of danaparoid are detected in breast milk and there are no published data on the excretion of fondaparinux into breast milk; however, both agents have low oral availability and so significant absorption by breastfeeding infants is unlikely.[8] Low to moderate levels of rivaroxaban and apixaban have been detected in breast milk and their use should be avoided in breastfeeding women.[5,21]

COVID-19 AND PREGNANCY

Venous Thromboembolism Risk in Pregnant Persons With COVID-19

There is an increased risk of venous and arterial thrombosis in pregnant patients with COVID-19 infection, compared to those without COVID-19.[22,23] The risk of a thrombotic event in pregnant women with COVID-19 appears to be less than 1% (~0.2% to 0.3%),[22–25] but data are still evolving. Additional factors may increase this thrombotic risk further, including traditional risk factors for pregnancy-associated VTE and severity of COVID-19 illness. There is less information known about the risk of thrombosis in the postpartum period for pregnant patients with COVID-19.[25] A proportion of COVID-19-affected pregnancies have evidence of coagulopathy with laboratory abnormalities including thrombocytopenia, prolonged prothrombin time (PT) and activated partial thromboplastin time (aPTT), elevated D-dimer and hyper-fibrinogenemia that reflects an immunothrombotic state.[24,26] How coagulopathy is associated with thrombosis in pregnancies with COVID-19 remains an active area of study.

Prevention of Venous Thromboembolism in Pregnant Individuals With COVID-19

To date, there are no high-quality pregnancy-specific data to guide the role of thromboprophylaxis in pregnant patients with COVID-19.[26] Large randomized controlled trials that studied the role of prophylactic, intermediate, or therapeutic-dose LMWH for the prevention of VTE and other outcomes in the setting of COVID-19 infection have systemically excluded pregnant patients.[26] As a result, currently decisions need to be made by extrapolating data from these randomized trials and from non-COVID-19 pregnancy cohort and case-control studies that include other pregnancy-associated VTE risk factors. Recent trials of non-pregnant patients have found that therapeutic LMWH may be beneficial in moderately ill hospitalized patients, but not in severely ill patients.[27] Careful consideration and multidisciplinary decision-making should be used when deciding on anticoagulant dosing around delivery and the immediate postpartum period, as drawbacks of therapeutic LMWH include risk of bleeding and reduced access to neuraxial anesthesia.

FUTURE DIRECTIONS

Areas of active research include validating and optimizing diagnostic algorithms for suspected DVT and PE in pregnancy; evaluating strategies for prevention of postpartum VTE; optimizing VTE anticoagulant management during pregnancy and the postpartum periods, as well as around the time of delivery; evaluating how the impact of COVID-19 is similar to and differs from that in the non-pregnant population; and studying how best to incorporate patient values and preferences in shared decision making around anticoagulation in pregnancy.

REFERENCES

1. Kourlaba G, Relakis J, Kontodimas S, et al. A systematic review and meta-analysis of the epidemiology and burden of venous thromboembolism among pregnant women. *Int J Gynecol Obstet*. 2016;132(1):4–10.
2. Say L, Chou D, Gemmill A, et al. Global causes of maternal death: a WHO systematic analysis. *Lancet Glob Heal*. 2014;2(6):323–333.
3. Pomp ER, Lenselink AM, Rosendaal FR, Doggen CJM. Pregnancy, the postpartum period and prothrombotic defects: risk of venous thrombosis in the MEGA study. *J Thromb Haemost*. 2008;6(4):632–637.

4. Kamel H, Navi BB, Sriram N, et al. Risk of a thrombotic event after the 6-week postpartum period. *N Engl J Med*. 2014;370:1307–1315.
5. Bates SM, Rajasekhar A, Middeldorp S, et al. American Society of Hematology 2018 guidelines for management of venous thromboembolism: venous thromboembolism in the context of pregnancy. *Blood Adv*. 2018;2:6–8.
6. Rodger M. Pregnancy and venous thromboembolism: "TIPPS" for risk stratification. *Hematol Am Soc Hematol Educ Progr*. 2014;2014(1):387–392.
7. Skeith L. Preventing venous thromboembolism during pregnancy and postpartum: crossing the threshold. *Hematology*. 2017;2017(1):160–167.
8. Bates SM, Middeldorp S, Rodger M, et al. Guidance for the treatment and prevention of obstetric-associated venous thromboembolism. *J Thromb Thrombolysis*. 2016;41(1):92–128.
9. Chan W-S, Lee A, Spencer FA, et al. Predicting deep vein thrombosis in pregnancy: out in "LEFt" field? *Ann Intern Med*. 2009;151:85–92.
10. Chan WS, Lee A, Spencer FA, et al. D-dimer testing in pregnant patients: towards determining the next "level" in the diagnosis of deep vein thrombosis. *J Thromb Haemost*. 2010;8(5):1004–1011.
11. Bellesini M, Robert-Ebadi H, Combescure C, et al. D-dimer to rule out venous thromboembolism during pregnancy: a systematic review and meta-analysis. *J Thromb Haemost*. 2021;19(10):2454–2467.
12. Ray JG, Vermeulen MJ, Bharatha A, et al. Association between MRI exposure during pregnancy and fetal and childhood outcomes. *JAMA*. 2016;316(9):952.
13. Al Lawati K, Aljazeeri J, Bates SM, et al. Ability of a single negative ultrasound to rule out deep vein thrombosis in pregnant women: a systematic review and meta-analysis. *J Thromb Haemost*. 2020;18:373–380.
14. van der Pol LM, Tromeur C, Bistervels IM, et al. Pregnancy-adapted YEARS algorithm for diagnosis of suspected pulmonary embolism. *N Engl J Med*. 2019;280:1139–1149.
15. Righini M, Robert-Ebadi H, Elias A, et al. Diagnosis of pulmonary embolism during pregnancy a multicenter prospective management outcome study. *Ann Intern Med*. 2018;169(11):766–773.
16. Wik HS, Jacobsen AF, Sandvik L, Sandset PM. Prevalence and predictors for post-thrombotic syndrome 3 to 16 years after pregnancy-related venous thrombosis: a population-based, cross-sectional, case-control study. *J Thromb Haemost*. 2012;10:840–847.
17. Hunter R, Lewis S, Noble S, et al. "Post-thrombotic panic syndrome": a thematic analysis of the experience of venous thromboembolism. *Br J Health Psychol*. 2017;22(1):8–25.
18. Romualdi E, Dentali F, Rancan E, et al. Anticoagulant therapy for venous thromboembolism during pregnancy: a systematic review and a meta-analysis of the literature. *J Thromb Haemost*. 2013;11(2):270–281.
19. Beyer-Westendorf J, Tittl L, Bistervels I, et al. Safety of direct oral anticoagulant exposure during pregnancy: a retrospective cohort study. *Lancet Haematol*. 2020;7:e884–e891.
20. Cohen H, Arachchillage DR, Middeldorp S, et al. Management of direct oral anticoagulants in women of childbearing potential: guidance from the SSC of the ISTH. *J Thromb Haemost*. 2016;14:1673–1676.
21. Zhao Y, Arya R, Couchman L, Patel JP. Are apixaban and rivaroxaban distributed into human breast milk to clinically relevant concentrations? *Blood*. 2020;136(15):1783–1785.
22. Servante J, Swallow G, Thornton JG, et al. Haemostatic and thromboembolic complications in pregnant women with COVID-19: a systematic review and critical analysis. *BMC Pregnancy Childbirth*. 2021;21(1):1–14.
23. Jering KS, Claggett BL, Cunningham JW, et al. Clinical characteristics and outcomes of hospitalized women giving birth with and without COVID-19. *JAMA Intern Med*. 2021;181(5):714–717.
24. Kazi S, Othman M, Khoury R, et al. ISTH Registry on Pregnancy and COVID-19 Associated Coagulopathy (COV-PREG-COAG)—First Report. Res Pract. *Thromb Haemost*. 2021;5(Suppl 2).
25. Gabrieli D, Cahen-Peretz A, Shimonovitz T, et al. Thromboembolic events in pregnant and puerperal women after COVID-19 lockdowns: a retrospective cohort study. *Int J Gynecol Obstet*. 2021;155(1):95–100.
26. D'Souza R, Malhamé I, Teshler L, et al. A critical review of the pathophysiology of thrombotic complications and clinical practice recommendations for thromboprophylaxis in pregnant patients with COVID-19. *Acta Obstet Gynecol Scand*. 2020;00:1–11.
27. Sholzberg M, Costa BR, Tang GH, et al. Randomized trials of therapeutic heparin for COVID-19: a meta-analysis. *Res Pract Thromb Haemost*. 2021;5:e12638.

CHAPTER 142

ATHEROTHROMBOSIS

Daisy Sahoo, Moua Yang, and Roy L. Silverstein

Morbidity and mortality from atherosclerosis, the pathologic process underlying acute myocardial infarction, sudden death, stroke, and limb loss, represent an enormous burden on society and health care systems (see Chapters 144–146). Even though death rates from heart attack and stroke have dropped precipitously over the past 48 years (68% and 77%, respectively),[1] cardiovascular disease (CVD) is still the number one cause of death in the United States, accounting for more than 30% of all deaths—approximately 2200 per day (or one every 40 seconds). Unfortunately, as the developing world adopts a more “Western” lifestyle marked by a high-fat, calorie-rich diet and limited physical activity, these statistics are becoming the norm worldwide. The annual financial burden of cardiovascular disease in the United States in 2015 was estimated by the American Heart Association (AHA) to be $351 billion, including $138 billion due to lost productivity. These costs are predicted to double over the ensuing 15 years, as analyses of population survey data predict a 30% increase in the number of people with CVD during this time. Importantly, improved treatments for acute complications of atherosclerosis also come with the burden of increased chronic complications (e.g., heart failure).

The increasing prevalence of other metabolic disorders such as the dual world-wide epidemics of type II diabetes and obesity[2] increase risk of adverse thrombotic events and are associated with increased mortality from CVD, both directly and indirectly. Furthermore, the global SARS-CoV-2 pandemic that began in early 2020 has produced a large population of individuals with post-infection complications, so-called “long-COVID-19.” Some of these complications are cardiovascular in nature, but the long-term impact on CVD prevalence remains unknown at this time. Thus, concerns have been raised that reduction in mortality due to cardiovascular disease will eventually stop or even reverse direction.

Much of our knowledge of the epidemiology of cardiovascular disease comes from large-scale, long-term population studies, the most important being the Framingham Heart Study sponsored by the National Heart, Lung, and Blood Institute (NHLBI). This ongoing prospective study began in 1948 with a cohort of ≈5200 men and women between the ages of 30 and 62 in Framingham, Massachusetts, and has since added two subsequent generations and several other cohorts. Through this study and others elsewhere in the United States and Europe, major risk factors for cardiovascular disease have been identified, including cigarette smoking, low-density lipoprotein (LDL) cholesterol, family history, systolic blood pressure, male sex, waist circumference, menopause, physical inactivity, body mass index, high-sensitivity C-reactive protein (hsCRP), and coronary artery calcification scores. High-density lipoprotein (HDL) was identified as a protective factor. Based on these data, risk scores have been developed to guide physicians and patients, and public health campaigns developed targeting hypertension, elevated cholesterol, and lifestyle modifications. These have clearly contributed to the reductions in cardiovascular mortality, although they cannot account for the entire decrement. Cardiovascular risk can also be altered by dietary modifications. For example, Mediterranean diets and foods rich in fish oils like the omega-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid have been shown to reduce cardiovascular mortality and improve cardiovascular outcomes. Furthermore, racial and ethnic disparities are well described in cardiovascular risk, with Blacks having higher rates than all other groups.

PATHOBIOLOGY

Atherosclerosis is caused by the progressive formation of arterial plaques, which are characterized by accumulation of lipids, in particular cholesterol and its derivatives, and inflammatory cells in the tunica intima of large- and medium-sized arteries (Fig. 142.1). Although occlusion of blood flow by plaque encroachment into the lumen can cause ischemia of downstream tissues, most of the severe clinical events associated with atherosclerosis derive from acute or subacute thrombosis at a site of an unstable, inflamed, or ruptured plaque or at a site of recent mechanical or pharmacologic intervention, a process termed atherothrombosis. The past 45 years have seen a remarkable increase in our understanding of the basic pathophysiologic mechanisms that govern plaque formation, plaque progression and acute arterial thrombosis.[3] Translation of this knowledge into diagnostic, preventive, and therapeutic strategies has been impressive, but there is still much unmet need for improvement. This chapter will summarize the current prevailing models of atherogenesis and atherothrombosis, highlighting some key knowledge gaps and potential new therapeutic targets.

CENTRAL ROLE OF LIPOPROTEINS IN ATHEROGENESIS

Epidemiologic and clinical trial data, along with numerous animal model studies, show unequivocally that elevated levels of plasma lipids, especially LDL cholesterol, are essential for plaque development. Atherosclerotic lesions can be induced in “athero-resistant” animals, including mice and rabbits, by manipulating their diets and/or genomes to cause hypercholesterolemia. The development of mouse genetic models nearly 30 years ago has led to mechanistic insights into the underlying pathobiology of plaque formation. In humans, raising LDL cholesterol levels increases the risk for atherosclerosis proportionally, while lowering levels either by lifestyle change or

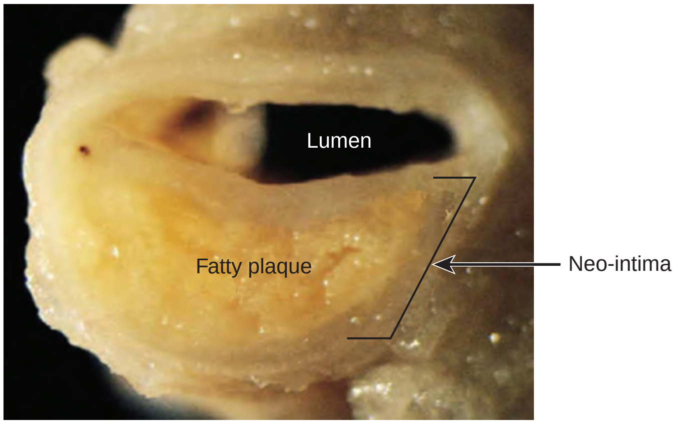


Figure 142.1 ATHEROMATOUS PLAQUE. Cross-sectional view of a human artery taken from an autopsy, showing accumulation of yellow-colored fatty materials in neointima.

pharmacologic intervention reduces risk. In general, lowering LDL cholesterol levels by 10% reduces risk for cardiovascular events by 25% and cardiovascular death by 10%.

Normally, cholesterol levels are tightly controlled in response to diet and cellular needs by a complex transcriptional pathway mediated by sterol response element-binding protein 2 (SREBP2). At low levels of cellular cholesterol, SREBP2 is activated and binds to specific DNA sequences in target genes known as *sterol response elements* to activate their transcription. The two key target genes are *HMGCR* and *LDLR*, which encode 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase (the rate-limiting enzyme in cholesterol biosynthesis) and the LDL receptor and their activation increases cholesterol production and cellular LDL uptake, respectively. At high levels of cellular cholesterol, SREBP2 is inactive, cholesterol biosynthesis is turned off, and LDL receptor expression is downregulated. As shown in Fig. 142.2, statin drugs decrease cellular cholesterol biosynthesis by inhibiting HMGCoA reductase and thereby inducing an increase in LDL receptor expression and driving down plasma LDL cholesterol levels.

Multiple randomized clinical trials have demonstrated efficacy of statin class drugs in reducing risk for cardiovascular events in high-risk individuals. Initial studies focused on secondary prevention in patients with a history of a previous atherosclerotic event, but later trials demonstrated efficacy as part of a primary prevention strategy in subjects with LDL cholesterol greater than 190 mg/dL or in subjects with "normal" LDL cholesterol (70 to 190 mg/dL) in the setting of a high-risk score. These studies, along with those showing benefit of lowering LDL cholesterol to levels well below "normal" (e.g., to 70 mg/dL in high-risk individuals),[4] are consistent with the concept that "normal" levels of LDL cholesterol, as defined by population means in the Western world, are not reflective of a true normal biology.

Genetic studies of rare individuals with extremely low LDL cholesterol levels identified a null mutation in a gene known as *PCSK9* that encodes a serine protease involved in downregulating LDL receptor expression. Individuals with this mutation seem to be otherwise normal, despite nearly absent LDL, suggesting that targeting this enzyme could represent an effective strategy for cholesterol-lowering therapeutics. Indeed, humanized monoclonal antibodies inhibiting PCSK9 function were quickly tested through clinical trials (OSLER and ODYSSEY) and approved by the FDA in 2015 as second line therapy for hypercholesterolemia.[5] The trials showed that these agents are well-tolerated and lower LDL cholesterol by approximately 60%, decrease cardiovascular events by half, and long-term efficacy and safety were demonstrated in at least one study. Emerging information on expression patterns of PCSK9 and regulation of other cellular receptors by this enzyme points to potential for off-target effects, and, thus, the need for long term follow-up of individuals on this therapeutic modality.

Niemann-Pick C1-like1 protein is expressed on the surface of enterocytes and hepatocytes and facilitates cholesterol absorption. Ezetimibe, a well-tolerated FDA-approved drug, inhibits this protein and lowers LDL cholesterol by approximately 20%. Early enthusiasm for this drug waned when studies failed to show improved cardiovascular outcomes, raising doubts about the relationship between biochemical endpoints and clinical efficacy, but a more recent trial of Ezetimibe in combination with a statin showed a 10% reduction in cardiovascular events at 7 years associated with a mean decrease in LDL cholesterol of 16 mg/dL compared with statin alone.[6] The most recent clinical guidelines published by AHA and the American College of Cardiology (2018) recommend considering non-statin drugs, including Ezetimibe and PCSK9 inhibitors, in higher risk individuals whose LDL cholesterol levels remain above 70 mg/dL after maximizing statin therapy.

Peripheral cells have the capacity to eliminate excess cholesterol through a process known as *reverse cholesterol transport (RCT)*. In this pathway, summarized in Fig. 142.3, post-lysosomal trafficking of intracellular cholesterol to the plasma membrane, mediated in part by the actions of acyl-CoA cholesterol acyltransferase (ACAT) and Niemann-Pick type C (NPC) protein, allows the cell surface adenosine triphosphate (ATP)–binding cassette (ABC) proteins ABCA1 and ABCG1 to transport excess cholesterol to apolipoprotein A (apoA)-containing lipoproteins, either nascent HDL (via ABCA1) or mature

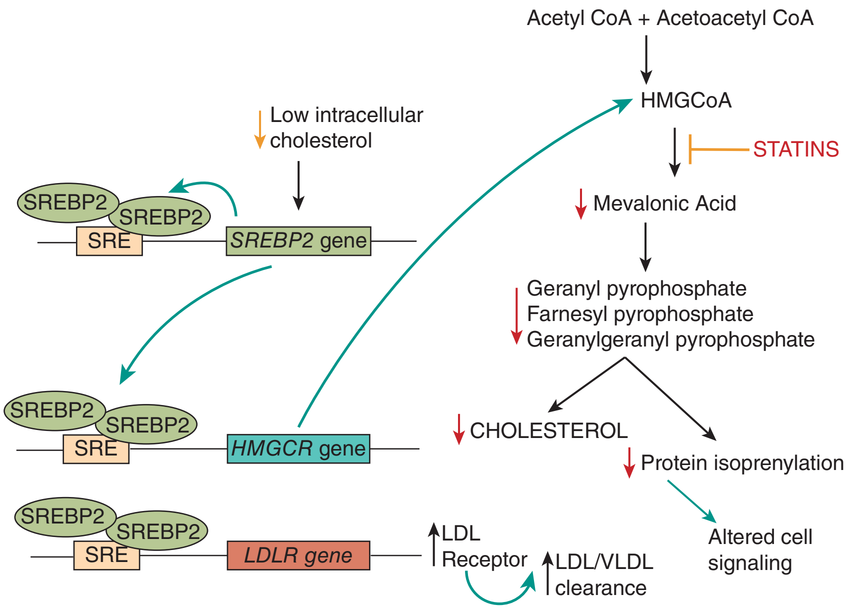


Figure 142.2 STATINS TARGET CHOLESTEROL AND ISOPRENYLATION PATHWAYS. The statin class of drugs inhibits the intracellular enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase. This enzyme converts HMGCoA to mevalonic acid, which is a precursor in the biosynthesis of the isoprenoids geranyl and farnesyl pyrophosphates, which in turn are precursors of cholesterol, as well as intermediates in isoprenyl modification of proteins, including small-molecular-weight G proteins. Since HMGCoA reductase is the rate-limiting step in these pathways, its inhibition by statins leads to decreases in cholesterol biosynthesis, hepatic VLDL production, and protein isoprenylation *(red arrows)*. Low levels of intracellular cholesterol activate the sterol response element-binding protein 2 (*SREBP2*) gene, which encodes the SREBP2 transcription factor *(green ovals)* that binds to sterol response elements (SRE) in multiple genes, including those encoding HMGCoA reductase (*HMGCR*) and the low-density lipoprotein (LDL) receptor (*LDLR*). The ensuing increased expression of LDL receptor results in increased clearance of apoB-containing lipoproteins (LDL and very low-density lipoprotein [VLDL]) from plasma, further lowering plasma cholesterol levels.

HDL (via ABCG1). HDL then "delivers" the cholesterol back to the liver, where it is selectively taken up by hepatocytes through a protein known as scavenger receptor B1 (SRB1) and ultimately secreted into bile and excreted in feces. The role of HDL in RCT probably accounts for its association with lowered risk for cardiovascular disease, but HDL particles also contain anti-inflammatory and antioxidant proteins that may further contribute to lowering atherosclerosis risk.

Pharmacologic and lifestyle approaches to enhance RCT have received significant attention as potential anti-atherosclerosis strategies. HDL levels can be raised by physical exercise, as well as by moderate alcohol ingestion, and these are both associated with lower cardiovascular risk. The only FDA-approved pharmacologic agent capable of raising HDL is niacin, but high doses are required, and these are often poorly tolerated because of side effects such as facial flushing. A combination of high-dose niacin with a prostaglandin D_2 receptor antagonist to block facial flushing and an extended-release form of niacin have been developed, but although both raise HDL levels, neither decreased cardiovascular risk.

The circulating plasma enzyme, cholesteryl ester transfer protein (CETP), functions to transfer cholesteryl esters from HDL to very low-density lipoprotein (VLDL) and LDL (see Fig. 142.3) in exchange for triglycerides. Inhibiting CETP raises HDL levels (and lowers LDL) significantly in humans, but multiple large phase III randomized clinical trials with pharmacologic CETP inhibitors failed to show efficacy despite raising HDL levels and at least one agent was associated with excess mortality.

Failure of CETP inhibitors and niacin led to doubts about the value of targeting HDL for anti-atherosclerosis therapy. An emerging concept, however, is that HDL particles are sensitive to oxidative modification in the pro-atherogenic milieu, and that these modifications can render HDL particles incompetent to function in RCT.[7] Simply raising HDL in this milieu, therefore, might not impact RCT. In this regard, novel assays to assess HDL function—so-called cholesterol efflux capacity—have been developed[8] and show potential as biomarkers for RCT in future drug development efforts. One such potential new target is microRNA-33a (miR-33a) which is encoded by a DNA sequence embedded in the SREBP2 gene and was found to repress several key genes involved in cholesterol homeostasis (see Fig. 142.3), including *ABCA1* and *ABCG1*, as well as genes involved in fatty acid oxidation and glucose metabolism.[9] Blocking miR-33a in mice increased HDL levels and inhibited atherosclerosis, suggesting that this could be a good target for therapeutic development. Other strategies under development to enhance RCT include direct infusion of cholesterol acceptors from ABCA1 or G1, including recombinant apoA or small apoA mimetic amphipathic peptides.

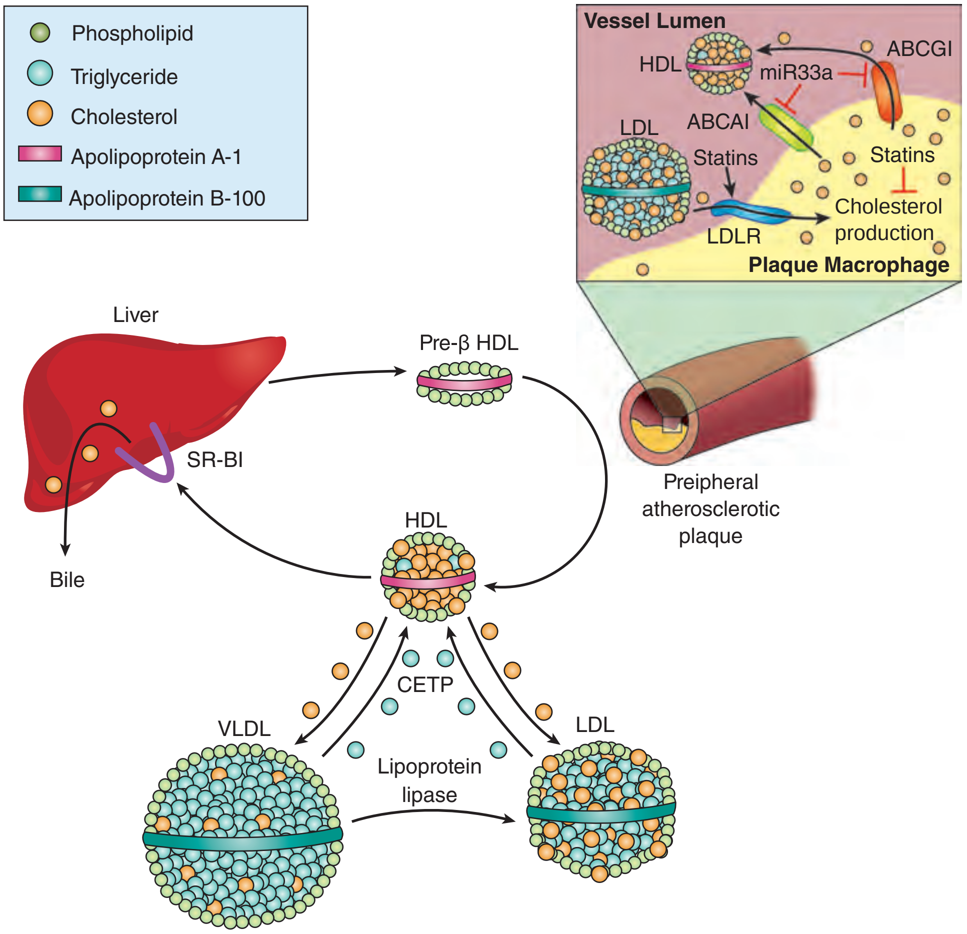


Figure 142.3 REVERSE CHOLESTEROL TRANSPORT. Cells in the periphery, including macrophage foam cells *(inset)*, have the capacity to unload intracellular cholesterol via two members of the adenosine triphosphate–binding cassette (ABC) transporter family: ABCA1 and ABCG1. The former transfers cholesterol to apoA and/or nascent high-density lipoprotein (HDL) particles, whereas the latter transfers cholesterol to mature HDL. HDL interacts with scavenger receptor B1 (SRB1) on liver cells, and through a process known as *selective cholesterol uptake*, the cholesterol within the HDL is internalized, where it can ultimately be reused or excreted via bile. The cholesterol in HDL can exchange with triglycerides in low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) particles through the action of an enzyme, cholesterol ester transfer protein (CETP). Inhibitors of CETP thus raise plasma HDL levels and lower LDL levels. LDL and VLDL are normally cleared by cells in the periphery via internalization by LDL receptors. Statin drugs (inset), by inhibiting HMGCoA reductase, lower intracellular cholesterol levels and raise LDL receptor levels, thus lowering plasma LDL. A microRNA (miR33a) encoded within the SREBP gene blocks expression of ABCA1 and ABCG1. *(Courtesy of Hayley Powers, MCW.)*

Multiple epidemiologic and genetic studies have shown positive associations between circulating triglyceride levels and coronary artery disease risk (see Chapter 145). Genetic variants in the gene encoding lipoprotein lipase, a key regulator of triglyceride metabolism, have also been associated with circulating triglyceride levels and with coronary artery disease risk, as have genetic variants in the *APOC3* and *APOA5* genes, which encode apolipoproteins in triglyceride-rich lipoproteins that critically regulate lipoprotein lipase in a reciprocal manner. Common variants in the *ANGPTL3* gene, another regulator of lipoprotein lipase activity, have also been identified.[10] Therapeutic interventions targeting the lipoprotein lipase pathway, including APOC3 antisense oligonucleotides or monoclonal antibody therapy against ANGPTL3 are under development.

FOAM CELL FORMATION AND THE FATTY STREAK

Atherosclerotic plaque develops in a geographically discontinuous manner very slowly and continuously evolves over many decades.[3] Autopsy studies of motor vehicle accident victims showed early "pre-atherosclerotic" lesions present in the aorta of otherwise healthy young children. These small superficial lesions consist predominantly of lipid-laden macrophages, so-called *foam cells*, and are termed *fatty streaks*. These are considered precursors of atherosclerotic plaque.

The initial event in formation of the fatty streak and atherosclerotic plaque is most likely transudation of apolipoprotein B–containing lipoproteins across the endothelial cell monolayer into the arterial wall (Fig. 142.4). A transcytosis mechanism mediated by scavenger receptors (SRs) expressed on the luminal cell surface of endothelial cells may also play a role.[11] These lipoproteins are predominantly LDL although remnant lipoproteins derived from chylomicrons produced by the intestinal epithelium are also found. The primary sites of lipoprotein entry are at arterial branch points or curvatures, explaining the discontinuous nature of atherosclerotic lesions. These locations are where normal laminar blood flow (and T-cell activity) is disturbed. Normal laminar flow activates a specific genetic "protection" program in endothelial cells that enhances barrier function and promotes an anti-inflammatory, antioxidant, and antiatherogenic phenotype. Loss of these shear-dependent signals disrupts the normal endothelial architecture and increases permeability, allowing unregulated entry of lipoproteins into the tunica intima. Within the intima, the lipoproteins become "trapped" by specific interactions with normal components of the vessel wall, such as glycosaminoglycans, and undergo structural modifications, including aggregation, oxidation, and in the setting of hyperglycemia, glycation.

Just as data show an unequivocal role for LDL cholesterol in atherogenesis, equally compelling studies in humans and animal models show that mononuclear phagocytes are necessary for plaque formation and progression, and that atherosclerosis is associated with a chronic inflammatory state in the vessel wall and in the systemic circulation.[12] Targeted genetic mutations that block monocyte development from hematopoietic precursors or that prevent monocyte trafficking into tissues dramatically protect mice from experimental atherosclerosis. The earliest and most prominent cell types in human and mouse atherosclerotic lesions are monocytes and macrophages. Initial monocyte entry into the arterial intima is in response to poorly understood cues related to endothelial cell dysfunction. Environmental and genetic influences, such as hypertension, angiotensin II production, cigarette smoke, diabetes, metabolic syndrome, and chronic periodontal infection may contribute to generalized endothelial dysfunction, but it is likely that the modified lipoproteins, particularly oxidized LDL (oxLDL) trapped in the vessel wall, play a major initiating role.[13] OxLDL activates an endothelial cell receptor known as LOX1 to induce expression of monocyte adhesion molecules and this response is amplified in the presence of angiotensin II. Other components of the "dysfunctional" endothelial cell phenotype include von Willebrand

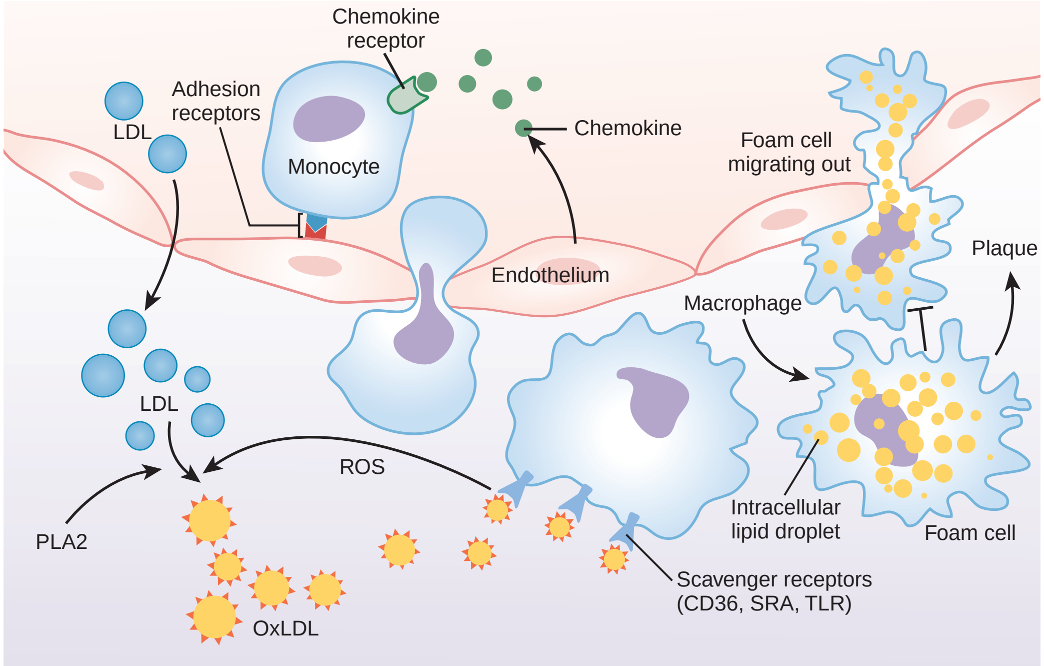


Figure 142.4 FOAM CELL FORMATION IS AN EARLY EVENT IN ATHEROGENESIS. Low-density lipoprotein (LDL) particles, which are transudated through a dysfunctional endothelium at arterial branch points or areas of disrupted shear, enter the intima and become trapped within the extracellular matrix. In the presence of inflammation, the LDL particles become oxidized or otherwise modified and contribute to endothelial dysfunction by interacting with receptors on endothelial cells, such as LOX1. Monocytes, in response to chemokines secreted by activated endothelial cells and/or inflammatory leukocytes and platelets, adhere to the endothelium via selectin family adhesion receptors, as well as β2 integrins and their counter receptors (ICAM and VCAM). The monocytes then diapedese into the intima, where they differentiate into inflammatory macrophages that generate oxidant stress within the vessel wall and release proinflammatory mediators. Scavenger receptors, in particular CD36, expressed on macrophages recognize and internalize oxidized LDL (oxLDL), leading to a feed-forward loop of increased LDL oxidation and increased scavenger receptor expression, resulting ultimately in the formation of cholesterol-laden foam cells. These accumulate in the intima, in part because of signals induced by oxLDL that inhibit migration, forming plaque. *ROS*, Reactive oxygen species.

factor release from Weibel-Palade bodies and altered homeostasis of nitric oxide and eicosanoid pathways (see Chapter 122).

Monocyte recruitment to the vessel wall (see Fig. 142.4) begins with their capture and rolling along the activated endothelium mediated by specific interaction of selectin family adhesion molecules (L-selectin on circulating monocytes and P-selectin on activated endothelial cells) with their counter receptors, including PSGL1. Subsequent signals induced by endothelial-derived chemokines, including CCL5/RANTES, CCL2/MCP1, IL-8, and CXCL1, lead to further recruitment of monocytes and facilitate firm adhesion of the rolling monocytes to the vessel wall. The latter is mediated by interaction of ICAM1 and VCAM on the activated endothelial surface with specific integrin family counter receptors on monocytes, β2 integrins for ICAM1 and α4β1 for VCAM. The adherent monocytes then go through diapedesis via disrupted endothelial junctions and enter the intima. Recent studies have suggested that a particular subset of circulating "inflammatory" monocytes, defined by high expression of the Ly6C antigen (in mice) or CD14 (in humans), are the predominant source of entering cells. The normal vasculature also contains a small number of resident macrophages, as well as "patrolling" monocytes.[14] The latter are distinct from the $Ly6C^+/CD14^{hi}$ cells and exhibit a less inflammatory phenotype.

The fate of monocytes within the intima is probably determined in part by lineage commitment programs carried by the entering monocytes and in part by local environmental cues. Most, however, seem to polarize toward the so-called M1 inflammatory macrophage phenotype. Investigators have, however, detected dendritic cell-like phenotypes (expressing CD11c), M2-like cells that express arginase and eNOS and are highly phagocytic and anti-inflammatory, and proangiogenic VEGF-expressing macrophage phenotypes within plaque. In addition to monocytes, lymphocytes,[15] particularly T cells, also enter the intima, where they contribute to plaque formation by secreting cytokines and other mediators. Of these infiltrating lymphocytes, interferon-γ producing Th1 cells and interleukin-17 producing Th17 cells have been shown to promote atherosclerosis, while the Treg cell subset is protective.

Reactive oxygen species (ROS) generated by the inflammatory milieu within the atherogenic vessel wall modify the trapped LDL by oxidizing both protein and lipid moieties (see Fig. 142.4), creating what are called collectively oxLDL and affecting the 2,3-diphosphoglycerate (2,3-DPG, also known as 2,3-bisphosphoglycerate or 2,3-BPG) levels.[13] Modified LDL lose their affinity for the LDL receptor but gain affinity for a genetically unrelated family of receptors known as SRs, including SRA1 and CD36, which are present at high levels on the macrophage surface. These receptors are part of the innate immune system, and their recognition of specific structures within oxLDL presumably relates to their mimicry of similar structures found on pathogenic organisms. CD36, for example,[16] also recognizes mycobacterial and *Staphylococcus* cell wall components, certain fungal structures, and falciparum malaria–infected erythrocytes. TLR family members, including TLR2, TLR4, and TLR6, can also recognize modified LDL and can partner with CD36 in these functions. Unlike the LDL receptor, which is downregulated in the setting of excess intracellular cholesterol, SR expression is not regulated in that manner, and, in fact, is increased by the presence of oxLDL. This, in part, results from internalization of oxidized fatty acids that serve as ligands for the PPAR family of transcription factors, particularly PPARγ, which is a major positive regulator of CD36 expression.[17] Thus, over many months and years, the continued entry and oxidation of LDL in the intima coupled with upregulated expression of SRs lead to massive intracellular accumulation of cholesterol and formation of lipid-laden foam cells.

OxLDL-SR interactions initiate a cascade of events in macrophages, including internalization of the bound oxLDL, activation of proinflammatory pathways, and activation of transcriptional pathways. The CD36-oxLDL interaction, in addition to promoting ROS formation, also decreases expression of endogenous antioxidant pathways and inhibits macrophage migration. Furthermore, recognition of oxLDL by CD36 initiates metabolic repurposing from mitochondrial respiration to a glycolytic-like state. Although not mirroring the Warburg effect in cancer cells, CD36-mediated metabolic repurposing results in mitochondrial dysfunction, ROS production, and phenotypic polarization toward a proinflammatory state. These events result in a feed forward loop that increases leukocyte recruitment into the vessel wall, inhibits macrophage migration out of the vessel wall, and increases oxLDL formation. The net effect is formation and accumulation of lipid-laden foam cells and proinflammatory immune cells, which together form plaque (see Fig. 142.4).

Although abundant data from animal models and correlative human studies support the oxidative stress hypothesis, considerable controversy remains because interventional trials of antioxidant therapy in humans have generally failed to prevent the complications of atherosclerosis.[18] These trials, however, are difficult to interpret because of lack of convincing evidence that the tested "antioxidant" therapies target the relevant vascular or circulating oxidant pathways. These oxidant systems include NADPH oxidase, xanthine oxidase, myeloperoxidase (MPO), uncoupled nitric oxide synthase, and dysfunctional mitochondrial electron transport chain. MPO is of particular interest because it generates a highly specific oxidized phospholipid ligand for CD36 from LDL and because circulating MPO levels correlate with risk for atherosclerosis and acute cardiovascular events.

Another potential problem with the antioxidant clinical trials is that the choice of antioxidants may have been flawed. Most used vitamin E formulations containing mainly α-tocopherol. Recent studies showed that tocopherols, in addition to having activity as antioxidants, have important cell-signaling functions mediated by specific cellular receptors. Therapy with formulations containing primarily α-tocopherol may downregulate endogenous γ-tocopherol levels, leading to imbalance in natural tocopherol signaling pathways. Furthermore, tocopherols are lipid-based structures that are themselves subject to oxidation, producing lipid peroxides that can promote further oxidative stress. Nevertheless, it is also possible that pathways of cholesterol uptake unrelated to LDL oxidation and SR expression, such as by micro- and macropinocytosis of "native" LDL or aggregated LDL, play a significant role in foam cell formation.

Important unresolved issues in the pathogenesis of early atherosclerotic lesions include understanding why macrophages do not exit the vessel wall and travel to regional nodes after ingesting modified LDL, as they would after ingesting exogenous pathogens, and why cholesterol efflux cannot keep up with LDL uptake to maintain homeostasis. OxLDL inhibition of macrophage migration (see Fig. 142.4) may explain the former, suggesting that blocking oxLDL-mediated signaling pathways might have therapeutic potential by facilitating macrophage exit from developing plaque. Indeed, in experimental animals, transplantation of atherosclerotic aortae from hypercholesterolemic animals into normal recipients induces migration of lipid-laden macrophages out of the vessel wall and plaque regression. The second issue might relate to differential gene regulation of SRs compared with cholesterol efflux transporters, and/or to intracellular cholesterol trafficking that might make it inaccessible to efflux transporters. In addition, as noted previously, HDL particles, like LDL, are sensitive to oxidative modification which may render them dysfunctional in RCT and, thus, lose their atheroprotective properties.[7]

An intriguing recent development in understanding the cellular composition of plaque comes from lineage marker studies which show that a surprisingly large percentage of foam cells expressing macrophage markers are derived from a smooth muscle cell lineage.[19] These studies suggest that local cues in plaque might interact with intimal or adventitial smooth muscle cells to redirect their genetic programs toward a macrophage-like phenotype. It is possible that such cells do not possess the full migratory capacity of macrophages, and this could contribute to the trapping of foam cell in plaque.

LESION EVOLUTION: REMODELING AND THE VULNERABLE PLAQUE

In humans, plaque develops and evolves very slowly over many decades, explaining in part why animal models in which advanced lesions develop rapidly over weeks and months may not accurately reflect the human condition. Accumulation of foam cells is only one component of the atherogenic process. Early on, signals from macrophages (Fig. 142.5) and the injured endothelium, primarily platelet-derived growth factor (PDGF), stimulate adventitial smooth muscle cells to migrate into the intima and to proliferate.[3] These cells contribute to the mass of the lesions, secrete collagen and other factors that change the nature of the intimal extracellular matrix, and may even contribute to the burden of foam cells. Lymphocytes infiltrating from the circulation and mast cells from the adventitia also contribute to the inflammatory milieu and matrix remodeling. Platelet antigens are also readily detectable in plaque, suggesting that platelets enter the intima, where they may contribute to matrix remodeling and the inflammatory state by secreting PDGF, transforming growth factor-β, and other growth factors, along with platelet factor 4, which is a potent chemokine, also known as *CXCL4*, for monocytes. Animal studies suggest that platelets may facilitate monocyte recruitment, serving as a bridge between the endothelium and circulating monocytes (see Fig. 142.5).[20] Sophisticated single-cell imaging studies in mice showed that monocytes continue to traffic through the "shoulders" of even advanced stage lesions.

Plaque generally grows in an eccentric manner within the intima and in certain instances can create significant obstruction to blood flow (Fig. 142.6, *left*). In such cases, as oxygen demand increases, tissue ischemia results, leading to angina and/or lower extremity claudication. Recently, in vivo studies using advanced imaging techniques, however, demonstrate that in most cases, the vessel wall remodels as plaque grows (see Fig. 142.6, *right*) so that the arterial lumen is mostly preserved, and the bulk of the atheromatous mass is abluminal. These studies show that traditional two-dimensional angiographic techniques vastly underestimate plaque burden. Of note, such studies along with careful histopathologic examinations, have led to the concept that the "quality" of the plaque may be more important than its quantity in predicting cardiovascular outcomes. Some plaques, particularly those with thick fibrous caps and cellular cores seem to be "stable" (Fig. 142.7, *left*), whereas others, particularly those with thin fibrous caps, abundant leukocytes, and necrotic, lipid-rich cores seem to be "unstable" and vulnerable to erosion and rupture (see Fig. 142.7, *right*).[21] Rupture refers to the sudden loss of integrity of the fibrous cap with release of plaque material into the lumen, often followed by acute occlusive thrombosis. Erosion is a more subtle concept referring to loss of endothelial cells at the shoulder of the lesion or minimal leakage of plaque through a partially disrupted cap. Plaque erosion may lead to sub-occlusive thrombus formation and/or intraplaque hemorrhage and thrombosis. Repeated cycles of erosion and intraplaque hemorrhage/thrombosis may account for the apparent stepwise growth of some lesions.

Widespread use of statins and adoption of lifestyles compatible with cardiovascular risk reduction may be responsible for a shift in the natural history of coronary plaque development that lowers risk of catastrophic rupture and occlusive thrombosis, but promotes erosions, micro-rupture and the formation of mini-thrombi, so-called "non-ST elevation" events. Understanding the factors that contribute to plaque vulnerability is thus an extremely important topic of research,[21] but one that is difficult to model in animals. Some key features that have emerged are the degree of angiogenesis within the plaque, balance of matrix-degrading enzymes and enzyme inhibitors, extent of apoptosis of cells within the plaque, deposition of calcium within the plaque, and level of systemic and local inflammation.

Plaque angiogenesis can be visualized in real time by ultrafast CT and MRI.[22] Based on analogy to tumor growth, it is not surprising that growing plaque requires a blood supply and that the neovessels within plaque may, like their counterparts in cancer, be leaky and unstable (see Chapter 122). This neovascular instability may contribute to plaque instability by facilitating entry of inflammatory cells, platelets, and plasma components, such as fibrinogen, cell-derived microvesicles (MV) and exosomes. In animal models, treatment with antiangiogenic agents significantly slows plaque growth. In some disease states such as diabetes, accelerated atherosclerosis may reflect a "microvascular" disease of the vasa vasora.

Integrity of the fibrous cap is maintained by a balance between collagen synthesis by smooth muscle cells and fibroblasts and collagen lysis by matrix-degrading enzymes. The predominant enzymes

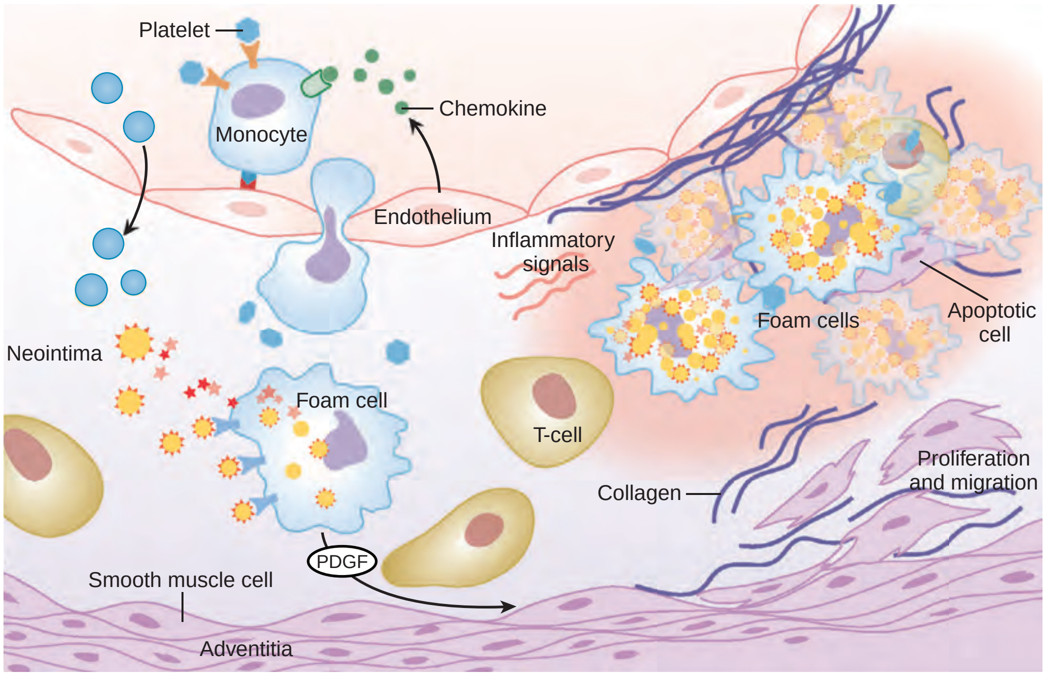


Figure 142.5 LATER EVENTS IN ATHEROGENESIS. Fatty streaks formed in the vessel wall via accumulation of foam cells (see Fig. 142.4) evolve over many years into complex plaque. In response to chemokines, monocytes continue to enter plaque, perhaps accompanied by platelets. T cells, B cells, and other inflammatory cells also accumulate. Cholesterol loading of macrophages induces apoptosis and apoptotic cells accumulate because of dysfunction of normal efferocytotic clearance pathways. Smooth muscle cells respond to platelet-derived growth factor (PDGF), TGFβ, and other signals and proliferate and migrate into the neointima. These cells produce collagen and other matrix components, contributing to plaque growth and formation of a fibrous cap. To support plaque growth, an angiogenic response is elicited from vasa vasora within the adventitia.

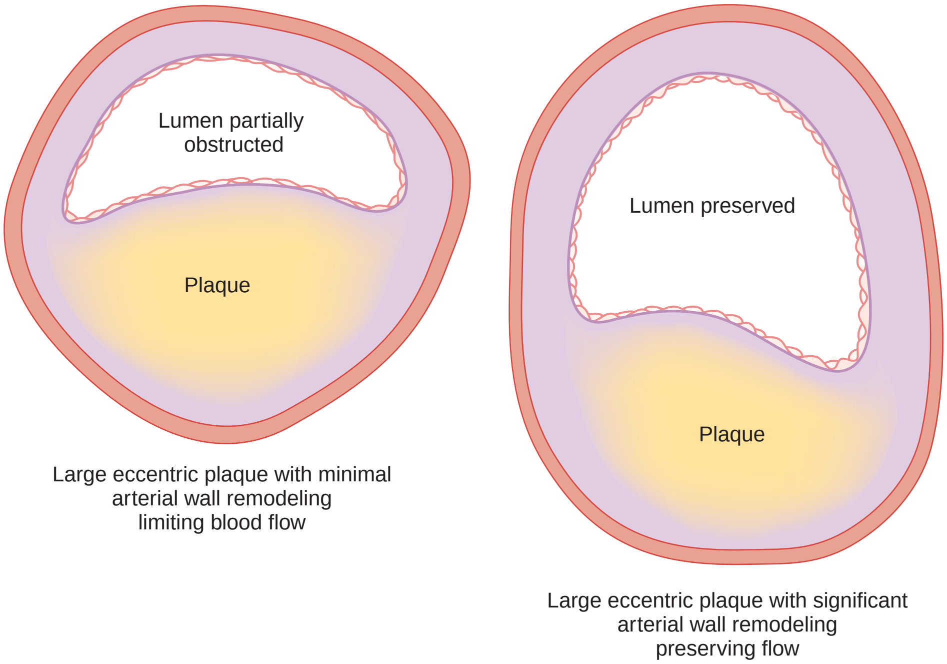

Lumen partially obstructed
Plaque
Large eccentric plaque with minimal arterial wall remodeling limiting blood flow
Lumen preserved
Plaque
Large eccentric plaque with significant arterial wall remodeling preserving flow

Figure 142.6 VESSEL WALL REMODELING CAN PRESERVE ARTERIAL LUMEN. On the left is a cross-section cartoon image of an artery containing a large eccentric plaque *(orange)*. The vessel has undergone minimal remodeling. The atheromatous lesion is extending into the arterial lumen and would be visible on an angiogram as an obstructing lesion. On the right is a lesion of similar mass, but its formation was accompanied by significant vessel wall remodeling so that the lesion extends mainly into the vessel wall, preserving the lumen. On an angiogram this would be nearly invisible.

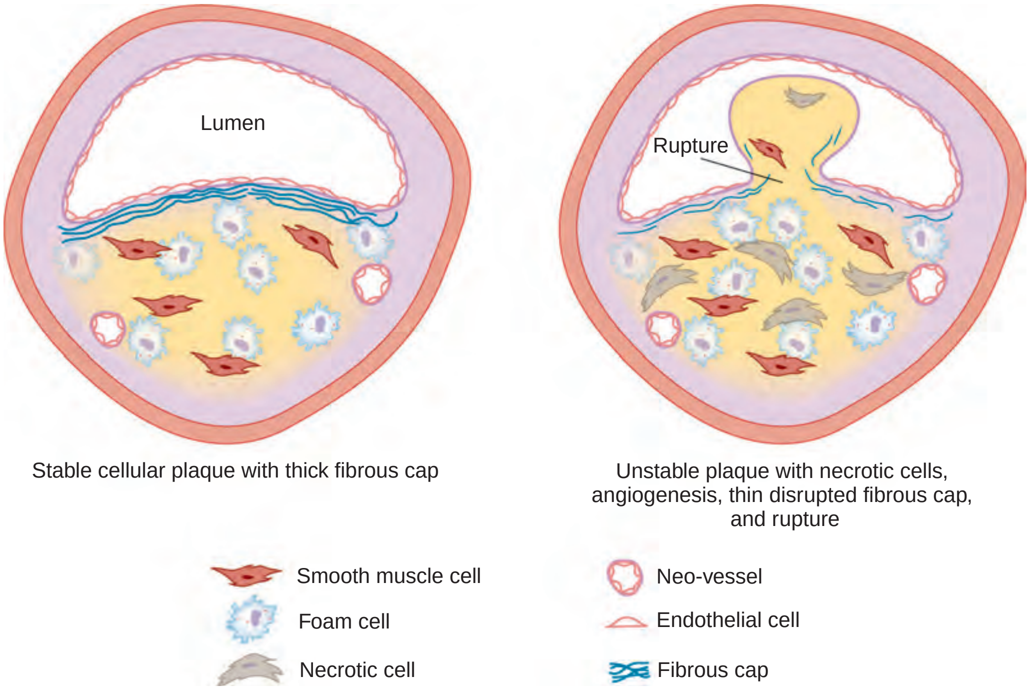

Lumen
Stable cellular plaque with thick fibrous cap
Rupture
Unstable plaque with necrotic cells, angiogenesis, thin disrupted fibrous cap, and rupture
Smooth muscle cell
Foam cell
Necrotic cell
Neo-vessel
Endothelial cell
Fibrous cap

Figure 142.7 UNSTABLE PLAQUE IS PRONE TO RUPTURE. On the left is a cross-section cartoon image of an artery with a plaque containing abundant smooth muscle cells and foam cells and a thick fibrous cap. On the right is a similar-size plaque, but with a thin fibrous cap that has ruptured allowing plaque contents to extrude into the lumen. This plaque contains smooth muscle cells and foam cells, as in the stable plaque on the left, but there is abundant angiogenesis, along with apoptotic and necrotic cells.

are members of the large family of zinc-dependent matrix metalloproteinases (MMPs) capable of degrading most matrix components, including collagen. These enzymes are tightly regulated by a network of activators and inhibitors and in settings in which activation exceeds inhibition, excessive matrix degradation may occur. Metalloproteases of the ADAM (a disintegrin and metalloprotease domain) family and ADAMTS (a disintegrin and metalloprotease domain with thrombospondin structural homology domains) family may also contribute to plaque instability. Although several clinical trials have studied MMP inhibitors in atherosclerosis, none have shown clear benefit to date.

A prominent feature of advanced atherosclerotic lesions is the presence of apoptotic cells, mostly of macrophage and smooth muscle cell origin. The nature of the pro-apoptotic signals within plaque is incompletely understood, but excess intracellular cholesterol can initiate the endoplasmic reticulum stress response leading to apoptosis.[23] OxLDL signaling through SRs and/or TLRs can also induce apoptosis. In most inflammatory sites, apoptotic leukocytes are quickly removed by phagocytes in a process known as *efferocytosis*. The efferocytotic macrophages are generally thought to be of the M2, anti-inflammatory type; thus, their engagement by apoptotic cells not only removes the apoptotic cell from the microenvironment, but also directly contributes to downregulation of the inflammatory state by inducing secretion of anti-inflammatory cytokines and effectors. In atherosclerotic plaque, this process seems to be inefficient so that apoptotic cells accumulate, contributing to the lipid load and releasing potentially toxic contents. In mouse models, TNFα is a major driver of inefficient efferocytosis and interfering with CD47, an anti-efferocytic protein on the surface of macrophages, improves clearance of apoptotic cells and plaque debris and ameliorates atherosclerosis.[24]

Although therapeutic interventions to stabilize vulnerable plaque or prevent plaques from becoming vulnerable have not yet materialized in humans, several imaging approaches have been studied in attempt to develop useful biomarkers to identify vulnerable plaque and therefore to identify patients who might benefit from aggressive antithrombotic and lipid-lowering therapeutic interventions. Neovascular imaging by CT or MRI are promising technologies, as are plaque characterization by optical coherence tomography, high resolution MRI, linear infrared imaging, and thermography.[22]

PLAQUE RUPTURE AND ACUTE ARTERIAL THROMBOSIS

The devastating complications of atherosclerosis, including acute coronary syndromes (ACS), stroke, gangrene, and sudden death result primarily from acute and subacute thrombosis occurring at the site of plaque rupture (Fig. 142.8). As described in later chapters in this section, treatment and prevention strategies using aggressive antiplatelet, anticoagulant, fibrinolytic, and/or mechanical approaches have been remarkably effective at reducing major cardiovascular events, but tremendous unmet needs still exist, particularly in the areas of primary and secondary prevention of thrombosis (see Chapters 143–146).

Pathophysiologic mechanisms underlying acute arterial thrombosis center on two key concepts: (1) exposure of prothrombotic materials to the local circulation as a consequence of plaque rupture acts as a thrombotic trigger, and (2) advanced atherosclerosis is associated with a systemic prothrombotic state that accelerates or enhances pathologic thrombosis.[25] The former mechanism undoubtedly plays a major role. Immunohistochemical studies have convincingly shown that plaque contains abundant tissue factor (TF), and acute anticoagulation therapy directed toward blocking thrombin generation or inhibiting thrombin has proven to be effective in ACS (see Chapter 145). Plaque TF is derived mainly from smooth muscle cells, fibroblasts, and activated macrophages. Studies have also suggested that platelets can synthesize TF RNA transcripts after stimulation by inflammatory mediators and thus may also contribute to the plaque procoagulant load; however functional TF expression by platelets is not clearly established. A significant proportion of plaque TF may be in the form of membrane-bound MV derived from apoptotic and/or activated cells (see next section for details).[26] Exposure of flowing blood to TF exposed in, or released from, ruptured plaque leads to rapid activation of factor Xa, thrombin generation, and activation of platelets (see Chapters 124 and 125). In addition to TF, other components of plaque that become exposed to blood at sites of rupture include collagen and oxidized phospholipids, both of which can activate platelets directly. Thus aggressive antiplatelet therapy with aspirin, ADP receptor P2Y12 inhibitors, glycoprotein αIIbβ3 inhibitors, and protease activated receptor-1 (PAR1) inhibitors are mainstays of the pharmacologic approach to treatment of ACS (see Chapters 143 and 145); and aspirin and P2Y12 inhibitors are of proven efficacy for secondary prevention.

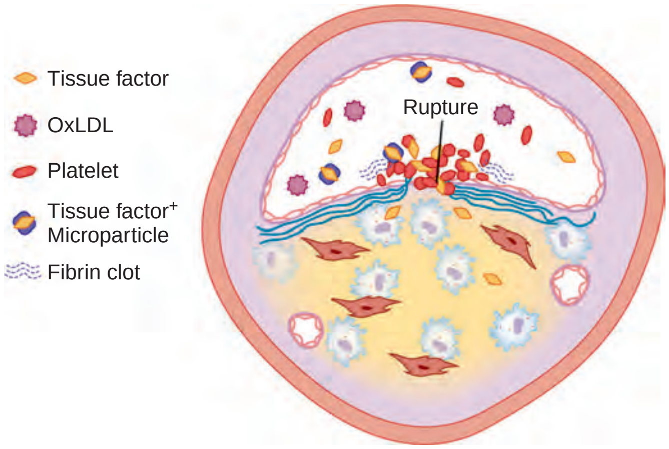


Figure 142.8 ACUTE PLATELET-RICH THROMBUS AT SITE OF PLAQUE RUPTURE. An atherosclerotic artery with a ruptured plaque (as described in Fig. 142.7) serves as a nidus for formation of an acute thrombus. Tissue factor (TF) contained in plaque or expressed on circulating microparticles (MPs) leads to thrombin generation and formation of fibrin at the site of rupture. Platelets accumulate at the rupture site and are activated by plaque components, such as collagen, forming an aggregate that, if large enough, can obstruct blood flow. Blood in patients with advanced atherosclerosis is prothrombotic, in part because of oxidized low-density lipoprotein (LDL)–induced release of TF-positive MPs from monocytes. Oxidized LDL (oxLDL) also interacts with platelets to make them more sensitive to activation and aggregation by plaque contents.

HYPERLIPIDEMIA, ATHEROSCLEROSIS, AND A SYSTEMIC PROTHROMBOTIC STATE

Platelet hyper-reactivity has long been thought to play a role in acute atherothrombosis. Clinical studies support an association between in vitro platelet reactivity and prognosis in patients with coronary disease, and it was shown during the 1970s that platelets from patients with familial hypercholesterolemia were more sensitive to activation by epinephrine or ADP. Similar findings were seen in platelets from mouse models of hyperlipidemia, such as the *apoe null* strain fed a high-fat diet. Of note, hyperlipidemic mice show accelerated time to form occlusive thrombi after experimental arterial injury compared with controls. This phenotype is likely the result of both enhanced platelet accumulation and fibrin deposition. In that context, much attention has been paid to identifying and characterizing receptors on the platelet surface that recognize specific classes of lipids and lipoproteins.[25] The receptors fall into two main groups: pattern recognition innate immune receptors, including CD36, SRA, TLR4, and LDL receptor–like protein (LRP)-8 (also known as *apoER2*), and G-protein coupled receptors, including receptors for lysophosphatidic acid (LPA), platelet-activating factor, and thromboxane (see Chapter 124).

CD36 (also known as *platelet glycoprotein IV* or *IIIb*) is of particular interest because of its known function on macrophages as a high affinity signaling receptor for oxLDL and because oxidized phospholipids are readily detectable in circulating plasma of experimental animals and patients with atherosclerosis and hyperlipidemia. Indeed, studies showed that oxLDL binds to CD36 on the surface of platelets in a specific, concentration- and time-dependent manner. At high concentrations, such as might occur during acute plaque rupture, oxLDL/CD36 signals activate platelets; however, at lower concentrations such as those observed in the circulation of individuals with atherosclerosis, oxLDL/CD36 signals augment platelet aggregation responses to low doses of “classic” platelet agonists, such as ADP and collagen. CD36 signaling in platelets involves multiple coordinated pathways including generation of intracellular ROS. In addition, CD36 also blocks natural anti-activation, inhibitory pathways in platelets mediated by cyclic nucleotides. Intracellular crosstalk between CD36 and pathways downstream of the collagen receptor GPVI may also augment procoagulant responses and thereby enhance platelet-dependent fibrin formation and secondary hemostasis in the atherogenic microenvironment (see Chapter 124).

Atherosclerotic plaque and so-called “minimally” oxidized LDL particles contain LPA, biologically active lipids generated by the action of phospholipases on LDL and cell membrane lipids. Platelets express at least three different G-protein-coupled LPA receptors, but the in vivo relevance of this system has not been demonstrated. Ex vivo studies, however, demonstrated that at high concentrations, minimally oxidized LDL sensitized platelets to aggregate in response to other agonists. Epidemiologic studies have also associated levels of soluble phospholipase A2 with cardiovascular risk. In addition to participating in LPA biosynthesis, this family of enzymes generates free fatty acids from phospholipids that serve as substrates for cyclooxygenase and lipoxygenases, producing both pro- and antiatherogenic eicosanoids.

In addition to platelet hyper-reactivity, it is also quite likely that systemic dysregulation of the coagulation cascade contributes to the prothrombotic state associated with advanced atherosclerosis. This is consistent with studies showing that atherosclerosis is associated with increased risk for venous thromboembolic disorders. Elevated levels of circulating D-dimer and thrombin-antithrombin complexes have been reported in patients with ACS, and D-dimer levels tend to track with hsCRP. Large-scale population studies have identified elevated fibrinogen and factor VIII levels as risk factors for atherosclerosis. A key initiator of thrombin generation in these settings may be circulating TF (see Fig. 142.8) in the form of TF-expressing MVs.[26] MVs are 200 to 1000 nm diameter vesicular fragments that bud off from cells during either activation or apoptosis. MVs generated from platelets, monocytes, erythrocytes, leukocytes, or endothelial cells during vascular injury become incorporated into developing thrombi in vivo. MVs, mostly of platelet origin, can be detected in the circulation of normal human subjects, but markedly increased numbers of circulating MVs have been reported in patients with a variety of inflammatory and prothrombotic disorders including ACS. Furthermore, MVs characteristically have PS externalization on the surface which aids in the capacity to bind to platelet CD36 and enhance platelet reactivity via the same mechanism as oxLDL.

It is important to note that MVs derived from other cells (e.g., monocytes, macrophages, smooth muscle cells, and tumor cells) are an additional source of circulating TF. A mechanistic link between atherogenesis and thrombosis may be oxLDL, which in vitro can induce TF-positive MV generation from blood-derived monocytes. Of interest, a large clinical trial (JUPITER) testing the efficacy of statins in subjects with normal LDL, but elevated levels of hsCRP, showed that rates of venous thrombosis, as well as coronary events, were significantly decreased. In animal models, statins decrease circulating levels of oxLDL and MV levels, as well as levels of other prothrombotic and proinflammatory biomarkers, even in the absence of lowering cholesterol, suggesting that oxLDL-mediated prothrombotic activities may be important targets for thrombo-prevention in patients with atherosclerosis. These studies also support those showing that statins may have anti-inflammatory and antithrombotic activities independent of lipid lowering. Since HMGCoA reductase is also the limiting step in the biosynthesis of isoprenoids, it has been postulated that statins may dampen signaling pathways mediated by small molecular weight G proteins (e.g., Ras) in which membrane targeting by protein isoprenylation is required (see Fig. 142.2).

In mice, the contact activating system and intrinsic coagulation pathway have been shown to participate in arterial thrombosis after injury and to have proatherogenic properties.[27] Targeting these systems is attractive in that patients with known deficiencies in factor XII or contact system proteins that have no obvious phenotype and do not have a bleeding diathesis, thus, presumably an antithrombotic effect could be achieved without altering normal hemostasis (see Chapters 125, 135, and 143).

CROSS TALK BETWEEN COAGULATION AND INFLAMMATION SYSTEMS IMPACT ATHEROGENESIS

As noted previously, atherosclerosis can be thought of as a chronic, low-grade inflammatory disease of the artery wall. Clinical studies based on biomarkers such as hsCRP, MPO, and IL6, suggest that advanced atherosclerosis is associated with a systemic state of chronic inflammation and that the degree of inflammation reflects the level of risk for major complications, possibly because the more inflamed the plaque, the more vulnerable the lesion. It is also now clear that mediators of inflammation, such as IL1, LPA, oxLDL, and circulating MVs may interact with platelets and the coagulation cascade to promote a hypercoagulable, prothrombotic state. Furthermore, mediators of coagulation, such as thrombin, TF/factor VIIa complexes, and factor Xa, may contribute to the proinflammatory state by interacting with protease-activated receptors on endothelial cells and leukocytes. Activated platelets can contribute to inflammation by facilitating monocyte adhesion to endothelium and by secreting proinflammatory molecules. Thus, bidirectional crosstalk between the inflammation and coagulation systems enhance both pathways and promotes atherosclerosis.

Neutrophils also contribute to platelet activation and venous thrombosis through formation of procoagulant neutrophil extracellular traps (NETs), but the contributions of neutrophils to atherothrombosis is not well-established. Clinical studies have suggested a link between platelet-neutrophil complex formation and cardiovascular disease, and in aged mice, NETs have been shown to promote atherosclerosis and plaque erosion through a mitochondrial oxidant stress pathway.

Not surprisingly, patients with systemic autoimmune inflammatory disorders, including rheumatoid arthritis, Wegener’s granulomatosis, and lupus erythematosus have been shown to have accelerated atherosclerosis, as assessed by biomarkers such as ultrasound evidence of carotid artery intima-medial thickening, as well as major cardiovascular events. Obesity, a risk factor for atherosclerosis, is now known to be a systemic inflammatory disorder with evidence of inflammation readily detectable within obese central adipose tissue. Obese patients also have elevated, circulating levels of coagulation and inflammation biomarkers, including plasminogen activator inhibitor-1 (PAI-1).

Given these observations, there has been renewed interest in exploring the use of anti-inflammatory agents and antithrombotic agents to slow the progress of atherosclerosis. The approach has been tempered by clinical studies showing small increases in atherosclerotic complications in patients taking COX inhibitors (especially those with preferential activity against COX2), but this may relate to the specific target and the complication of altered eicosanoid production, perhaps changing the balance, away from anti-atherothrombotic end products, such as prostacyclin and resolvins, and toward pro-atherothrombotic products, such as thromboxane, HETE, and lipoxins. Also, although aspirin and ADP receptor antagonists reduce risk for major cardiovascular events, no studies have shown an impact on burden of atheroma.

A randomized clinical trial (CANTOS) explored the efficacy of blocking the IL-1β pro-inflammatory signaling pathway with an FDA-approved monoclonal antibody, canakinumab, in more than 10,000 patients with previous myocardial infarction and elevated CRP levels. The study showed significant reductions in hsCRP levels along with a modest reduction in the primary composite outcome of non-fatal MI, stroke, or cardiovascular death. A trial of the nonspecific anti-inflammatory agent methotrexate (CIRT) showed no cardiovascular benefit, while a secondary prevention trial using colchicine (COLCOT) showed a modest benefit. These studies demonstrate proof of principle for targeting inflammation pathways in atherothrombosis. However, currently available approaches are cumbersome, expensive, and associated with long term risks that may not be acceptable for most patients.

GUT MICROBIOME AND ATHEROTHROMBOSIS

A very promising avenue of research in atherothrombosis is the gut microbiome. Elegant studies using mouse models and human subjects have identified a "metaorganismal" pathway that contributes both to the development of plaque[28] and to the prothrombotic state associated with atherosclerosis.[29] Diets rich in phosphatidylcholine, choline, and carnitine can be metabolized by specific genera of microbes to generate trimethylamine (TMA) through microbial choline TMA lyases. TMA enters the portal circulation and is then converted to trimethylamine n-oxide (TMAO) in the liver by flavin monooxidase enzymes. TMA and TMAO levels in blood associate strongly with both risk of cardiovascular disease and arterial thrombosis in human subjects, and TMAO was shown to promote platelet activation in vitro and thrombosis in animal models. Mechanistically, TMAO increases intracellular calcium levels in platelets; however, the receptor for TMAO has not yet been identified. Importantly, diet influences the constitution of the gut microbiome, as well as the ability to generate TMA and TMAO. Human subjects on vegan diets have reduced capacity to generate TMA/TMAO than do carnivores and omnivores, and this can be restored by adding back meat, eggs, or carnitine to the diet. Interestingly, atherothrombotic risk in mice and humans could be transmitted from susceptible to resistant strains by fecal microbiome transplant, and resistant strains could be converted to susceptible ones by antibiotic or dietary manipulation of the microbiome. Furthermore, transient knockdown or genetic overexpression of flavin monooxygenase in mice promotes the complementary TMAO levels and prothrombotic states in vivo. Choline analogs were subsequently shown to inhibit microbial TMA lyase and lower TMAO levels, thus decreasing platelet reactivity. These studies suggest that microbial enzymes and/or ecosystems could be targeted for atherothrombosis treatment and prevention strategies and this remains an active area of research.

HEMATOPOIESIS AND ATHEROTHROMBOSIS

The bone marrow niche is sensitive to hyperlipidemia. Atherogenic-prone LDL receptor null mice fed a high fat diet show signs of elevated prothrombotic immature reticulated platelets, thrombocytosis, and increased platelet reactivity. Production of these cells could be related to modulation of the thrombopoietin pathway by cholesterol in megakaryocytes or megakaryocyte progenitors. In mice or humans with diabetes and hyperlipidemia, levels of reticulated platelets are increased. Although the mechanisms of elevated platelet production in these settings is not well understood, it could contribute to thrombotic risk in atherogenic conditions.

Whole genome and whole exome sequencing have revealed that clonal hematopoiesis of indeterminate potential (CHIP), that is expansion of somatic blood cell clones in the absence of other hematologic abnormality, is quite common and increases in prevalence progressively with age. While initial interest in CHIP focused on its relationship to development of malignant hematopoiesis, recent studies revealed a significant association with atherosclerotic cardiovascular disease.[30] Prospective and retrospective case-control studies showed two- to fourfold increase in the risk of coronary heart disease and myocardial infarction, with mutations in *DNMT3A*, *TET2*, *ASXL1*, and *JAK2* each associated with increased risk. Bone marrow transplant studies in atherogenic mice using donors deficient in at least some of these genes also showed enhanced atherogenesis. Mechanisms underlying this phenomenon are under intense investigation and may include disordered macrophage function, abnormal cellular metabolism, and thrombocytosis.

PLAQUE REGRESSION

Pharmacologic approaches to decrease the amount of arterial plaque in patients with established atherosclerosis has proved to be an elusive goal. Intravenous ultrasound has shown that aggressive lowering of LDL cholesterol with high-dose statins and rigorous dietary alterations can halt plaque progression and, in some cases, cause some plaque regression.[4] As noted previously, strategies to enhance RCT or to induce macrophage emigration from plaque are being developed, and strategies to stabilize plaque by targeting the neovasculature, cellular apoptosis, and efferocytosis are being considered. Specific antioxidant approaches based on fundamental understanding of the oxidation processes relevant to atherosclerosis remain viable as well, and much effort is being put into developing new animal models to study plaque regression.

ADDITIONAL FUTURE DIRECTIONS

Enthusiasm for large-scale genome-wide association studies (GWAS) as discovery platforms to identify novel genes and pathways that could be targeted with therapeutic or preventive effect has waned as multiple expensive studies have failed to produce impressive results. The most consistent data to come from GWAS identified a large genomic region on chromosome 9p21 as a risk site, but identification of specific genes has not yet been accomplished with certainty. More recently, approaches moving beyond GWAS have coupled whole genome or whole exome sequencing of highly selective subjects (such as genetically isolated Icelanders[31] or subjects with high or low lipoprotein levels) followed by deep sequencing of specific candidate genes in larger cohorts. This approach has yielded new data identifying potential novel anti-atherothrombosis targets; for example, loss of function variants of asialoglycoprotein receptor-1, NPC1L1, and APOC3 were found to be associated with decreased levels of non-HDL cholesterol and decreased risk of coronary artery disease.

An area of research that has generated intriguing data of potential relevance to atherosclerosis diagnostics and therapeutics is the potential role of noncoding RNAs, such as microRNA (miR), long non-coding RNAs (LncRNA), and circular RNAs. MiRs are small non-coding RNAs that regulate gene expression in a sequence-specific manner by post-transcriptional mechanisms. Dozens of miRs that are expressed in highly regulated, tissue-specific manners, and that regulate expression of genes involved in all aspects of atherosclerosis, including hepatic lipoprotein homeostasis; macrophage, endothelial cell, and smooth muscle cell and mechanosensory functions; and cytokine expression have been identified.[9] Therapeutic interventions based on specific miRs are under development, including, as noted previously, an inhibitor of miR-33 to raise HDL levels and promote RCT. Unbiased "omics" studies to identify expression levels of miR-mRNA pairs that associate with cardiovascular risk or with circulating lipoprotein levels also show promise for both biomarker development and therapeutic target discovery.

Although B cells are not prominent cellular constituents of plaque, B1 subclass cells responsible for innate antibody generation, are present in plaque from humans and experimental animals. These cells produce innate IgM antibodies, including a prevalent species called E06 that reacts with oxLDL. E06 and other autoantibodies against plaque constituents may be protective, perhaps by facilitating clearance of oxLDL via pathways that do not lead to foam cell formation, and/or by interfering with oxLDL-mediated activation of circulating monocytes and platelets.[15] These studies were exemplified by the reduction of atherosclerosis in hyperlipidemic murine models expressing a single chain variant of E06. Based on these observations, passive immunization strategies against oxLDL are also being considered.

REFERENCES

1. Roger VL, Sidney S, Fairchild AL, et al. Recommendations for cardiovascular health and disease surveillance for 2030 and beyond: a policy statement from the American Heart Association. *Circulation*. 2020;141:e104–e119.
2. Benjamin EJ, Muntner P, Alonso A, et al. Heart disease and stroke statistics—2019 update: a report rrom the American Heart Association. *Circulation*. 2019;139:e56–e528.

3. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature*. 2011;473:317–325.
4. Nicholls SJ, Ballantyne CM, Barter PJ, et al. Effect of two intensive statin regimens on progression of coronary disease. *N Engl J Med*. 2011;365:2078–2087.
5. Sabatine MS, Giugliano RP, Wiviott SD, et al. Efficacy and safety of evolocumab in reducing lipids and cardiovascular events. *N Engl J Med*. 2015;372:1500–1509.
6. McPherson R, Hegele RA. Ezetimibe: rescued by randomization (clinical and mendelian). *Arterioscler. Thromb. Vasc. Biol*. 2015;35:e13–e15.
7. Undurti A, Huang Y, Lupica JA, et al. Hazen, Modification of high density lipoprotein by myeloperoxidase generates a pro-inflammatory particle. *J. Biol. Chem*. 2009;284:30825–30835.
8. Rohatgi A, Khera A, Berry JD, et al. HDL cholesterol efflux capacity and incident cardiovascular events. *N Engl J Med*. 2014;371:2383–2393.
9. Feinberg MW, Moore KJ. MicroRNA regulation of atherosclerosis. *Circ. Res*. 2016;118:703–720.
10. Bauer RC, Khetarpal SA, Hand NJ, Rader DJ. Therapeutic targets of triglyceride metabolism as informed by human genetics. *Trends Mol. Med*. 2016;22:328–340.
11. Huang L, Chambliss KL, Gao X, et al. Shaul, SR-B1 drives endothelial cell LDL transcytosis via DOCK4 to promote atherosclerosis. *Nature*. 2019;569:565–569.
12. Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. *Cell*. 2011;145:341–355.
13. Steinberg D, Witztum JL. Oxidized low-density lipoprotein and atherosclerosis. *Arterioscler. Thromb. Vasc. Biol*. 2010;30:2311–2316.
14. Narasimhan PB, Marcovecchio P, Hamers AAJ, Hedrick CC. Nonclassical monocytes in health and disease. *Annu. Rev. Immunol*. 2019;37:439–456.
15. Hedrick CC. Lymphocytes in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol*. 2015;35:253–257.
16. Silverstein RL, Febbraio M. CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior. *Sci. Signal*. 2009;2:re3.
17. Nagy L, Tontonoz P, Alvarez JG, et al. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. *Cell*. 1998;93: 229–240.
18. Mitra S, Deshmukh A, Sachdeva R, et al. Mehta, Oxidized low-density lipoprotein and atherosclerosis implications in antioxidant therapy. *Am. J. Med. Sci*. 2011;342:135–142.
19. Owsiany KM, Alencar GF, Owens GK. Revealing the origins of foam cells in atherosclerotic lesions. *Arterioscler. Thromb. Vasc. Biol*. 2019;39:836–838.
20. Massberg S, Brand K, Grüner S, et al. A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. *J. Exp. Med*. 2002;196:887–896.
21. Finn AV, Nakano M, Narula J, et al. Concept of vulnerable/unstable plaque. *Arterioscler. Thromb. Vasc. Biol*. 2010;30:1282–1292.
22. Dweck MR, Doris MK, Motwani M, et al. Imaging of coronary atherosclerosis—evolution towards new treatment strategies. *Nat. Rev. Cardiol*. 2016;13:533–548.
23. Scull CM, Tabas I. Mechanisms of ER stress-induced apoptosis in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol*. 2011;31:2792–2797.
24. Kojima Y, Volkmer J-P, McKenna K, et al. CD47-blocking antibodies restore phagocytosis and prevent atherosclerosis. *Nature*. 2016;536:86–90.
25. Podrez EA, Byzova TV, Febbraio M, et al. Platelet CD36 links hyperlipidemia, oxidant stress and a prothrombotic phenotype. *Nat. Med*. 2007;13:1086–1095.
26. Rautou PE, Vion AC, Amabile N, et al. Microparticles, vascular function, and atherothrombosis. *Circ. Res*. 2011;109:593–606.
27. Borissoff JI, Spronk HM, ten Cate H. The hemostatic system as a modulator of atherosclerosis. *N Engl J Med*. 2011;364:1746–1760.
28. Koeth RA, Wang Z, Levison BS, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat. Med*. 2013;19:576–585.
29. Zhu W, Gregory JC, Org E, et al. Gut microbial metabolite TMAO enhances platelet hyperreactivity and thrombosis risk. *Cell*. 2016;165:111–124.
30. Jaiswal S, Natarajan P, Ebert BL. Clonal hematopoiesis and atherosclerosis. *N Engl J Med*. 2017;377:1401–1402.
31. Nioi P, Sigurdsson A, Thorleifsson G, Helgason H, Agustsdottir AB, et al. Variant ASGR1 associated with a reduced risk of coronary artery disease. *N Engl J Med*. 2016;374:2131–2141.

CHAPTER 143

ANTITHROMBOTIC DRUGS

Iqbal H. Jaffer and Jeffrey I. Weitz

Arterial or venous thromboembolism is a major cause of morbidity and mortality. Arterial thrombosis is the most common cause of acute myocardial infarction, ischemic stroke, and limb gangrene, whereas deep vein thrombosis can lead to pulmonary embolism, which can be fatal, and to post-thrombotic syndrome. Most arterial thrombi are superimposed on disrupted atherosclerotic plaque because plaque rupture exposes thrombogenic material in the plaque core to the blood.[1] This material then triggers platelet aggregation and fibrin formation, which results in the generation of a platelet-rich thrombus that can temporarily or permanently occlude blood flow (see Chapter 143). In contrast to arterial thrombi, venous thrombi rarely form at sites of obvious vascular disruption.[1] Although they can develop after surgical trauma to veins or secondary to indwelling central venous catheters, venous thrombi usually originate in the valve cusps of the deep veins of the calf or in the muscular sinuses, where they are triggered by stasis. Sluggish blood flow in these veins reduces the oxygen supply to the avascular valve cusps. Endothelial cells lining the valve cusps become activated and express adhesion molecules on their surface. These adhesion molecules tether tissue factor–bearing leukocytes and micro particles to the surface of activated endothelial cells, where the tissue factor triggers coagulation (see Chapter 121). Local thrombus formation is exacerbated by reduced clearance of activated clotting factors because of impaired blood flow. If the calf vein thrombi extend into more proximal veins of the leg, thrombus fragments can dislodge, travel to the lungs, and produce a pulmonary embolism.

Arterial and venous thrombi are composed of platelets and fibrin, but the proportions differ. Arterial thrombi are rich in platelets because of the high shear in the injured arteries. In contrast, venous thrombi, which form under low-shear conditions, contain relatively few platelets and are composed predominantly of fibrin and trapped red cells. Because of the predominance of platelets, arterial thrombi appear white, whereas venous thrombi are red in color, reflecting the trapped red cells.

Antithrombotic drugs are used for the prevention and treatment of thrombosis. Targeting the components of thrombi, these agents include (1) antiplatelet drugs, which inhibit platelets; (2) anticoagulants, which attenuate coagulation; and (3) fibrinolytic agents, which induce fibrin degradation (Fig. 143.1). With the predominance of platelets in arterial thrombi, strategies to inhibit or treat arterial thrombosis are focused mainly on antiplatelet agents, although in the acute setting they often include anticoagulants and fibrinolytic agents. The observations that the addition of low-dose rivaroxaban, an oral factor Xa inhibitor, to dual-antiplatelet therapy reduces recurrent ischemic events in patients with acute coronary syndrome, and its addition to aspirin reduces the risk of major adverse coronary and limb events in patients with coronary or peripheral artery disease, highlight the potential usefulness of anticoagulants on top of antiplatelet agents for secondary prevention (see Chapter 145 and 146).

Anticoagulants are the mainstay of prevention and treatment of venous thromboembolism because fibrin is the predominant component of venous thrombi. Antiplatelet drugs are less effective than anticoagulants in this setting because of the limited platelet content of venous thrombi. Fibrinolytic therapy is used in selected patients with venous thromboembolism. For example, patients with severe pulmonary embolism as evidenced by hypotension benefit from systemic or catheter-directed fibrinolytic therapy. Catheter-directed fibrinolytic therapy also can be used as an adjunct to anticoagulants for the treatment of patients with deep vein thrombosis involving the iliac and femoral veins.

This chapter is focused on antithrombotic agents. In addition to describing antiplatelet, anticoagulant, and fibrinolytic drugs that are in current use, new agents in advanced stages of development also are discussed.

ANTIPLATELET DRUGS

Role of Platelets in Arterial Thrombosis

In healthy vasculature, circulating platelets are maintained in an inactive state by nitric oxide (NO) and prostacyclin released by endothelial cells lining the blood vessels (see Chapter 121). In addition, endothelial cells also express CD39—an ecto-ADPase—on their surface, which degrades adenosine diphosphate (ADP) released from activated platelets. When the vessel wall is damaged, the release of these substances is impaired and the subendothelial matrix is exposed. Platelets adhere to exposed collagen and to von Willebrand factor via glycoprotein (GP) VI and GPIb–IX, respectively; receptors are constitutively expressed on the platelet surface (see Chapter 124). Adherent platelets undergo a change in shape, secrete ADP from their dense granules, and synthesize and release thromboxane A_2. Released ADP and thromboxane A_2, which are platelet agonists, activate ambient platelets and recruit them to the site of vascular injury.

Disruption of the vessel wall also exposes tissue factor–expressing cells to the blood. Tissue factor initiates coagulation (see Chapter 125). Activated platelets potentiate coagulation by binding clotting factors and supporting the assembly of activation complexes that enhance thrombin generation. In addition to converting fibrinogen to fibrin, thrombin amplifies its own generation and serves as a potent platelet agonist, thereby recruiting additional platelets to the site of injury.

When platelets are activated, GPIIb/IIIa ($\alpha_{IIb}\beta_3$), the most abundant receptor on the platelet surface, undergoes a conformational change that enables it to ligate fibrinogen (see Chapter 124). Divalent fibrinogen molecules bridge adjacent platelets together to form platelet aggregates. Fibrin strands, generated through the action of thrombin, then weave these aggregates together to form a platelet–fibrin mesh.

Antiplatelet drugs target various steps in this process (Fig. 143.2). The commonly used drugs include aspirin, thienopyridines (clopidogrel and prasugrel), ticagrelor, cangrelor, dipyridamole, GPIIb/IIIa antagonists, and vorapaxar. Each is briefly described in the following.

Aspirin

The most widely used antiplatelet agent is aspirin. As a cheap and effective antiplatelet drug, aspirin serves as the foundation of most antiplatelet strategies.

Mechanism of Action

Aspirin produces its antithrombotic effect by irreversibly acetylating and inhibiting platelet cyclooxygenase-1 (COX-1), a critical enzyme in the biosynthesis of thromboxane A_2 (see Fig. 143.2). At high doses (about 1 g/day), aspirin also inhibits COX-2, an inducible COX isoform found in endothelial cells and inflammatory cells. In endothelial cells, COX-2 initiates the synthesis of prostacyclin, a potent vasodilator, and inhibitor of platelet aggregation.

Indications

Aspirin is widely used for secondary prevention of cardiovascular events in patients with coronary artery disease, cerebrovascular disease, or peripheral vascular disease. Compared with placebo, aspirin produces about a 25% reduction in the risk of cardiovascular death, myocardial infarction, or stroke in these patients. Aspirin is of limited benefit for primary prevention in subjects without established cardiovascular disease.[2] If used for primary prevention, aspirin should be restricted for those at high risk of cardiovascular events.[2]

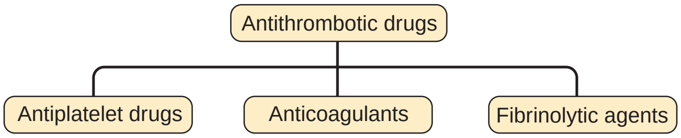

Antithrombotic drugs
Antiplatelet drugs
Anticoagulants
Fibrinolytic agents

Figure 143.1 CLASSIFICATION OF ANTITHROMBOTIC DRUGS.

Dosages

Aspirin is usually administered at doses of 75 to 325 mg once daily. There is no evidence that higher-dose aspirin is more effective than lower aspirin doses, and some analyses suggest reduced efficacy with higher doses. Because the side effects of aspirin are dose-related, daily aspirin doses of 75 to 150 mg are recommended for most indications. When rapid platelet inhibition is required, an initial aspirin dose of at least 160 mg should be given.

Side Effects

The most common side effects are gastrointestinal and range from dyspepsia to erosive gastritis or peptic ulcers with associated bleeding. These side effects are, at least to some extent, dose related. The use of enteric-coated or buffered aspirin in place of plain aspirin does

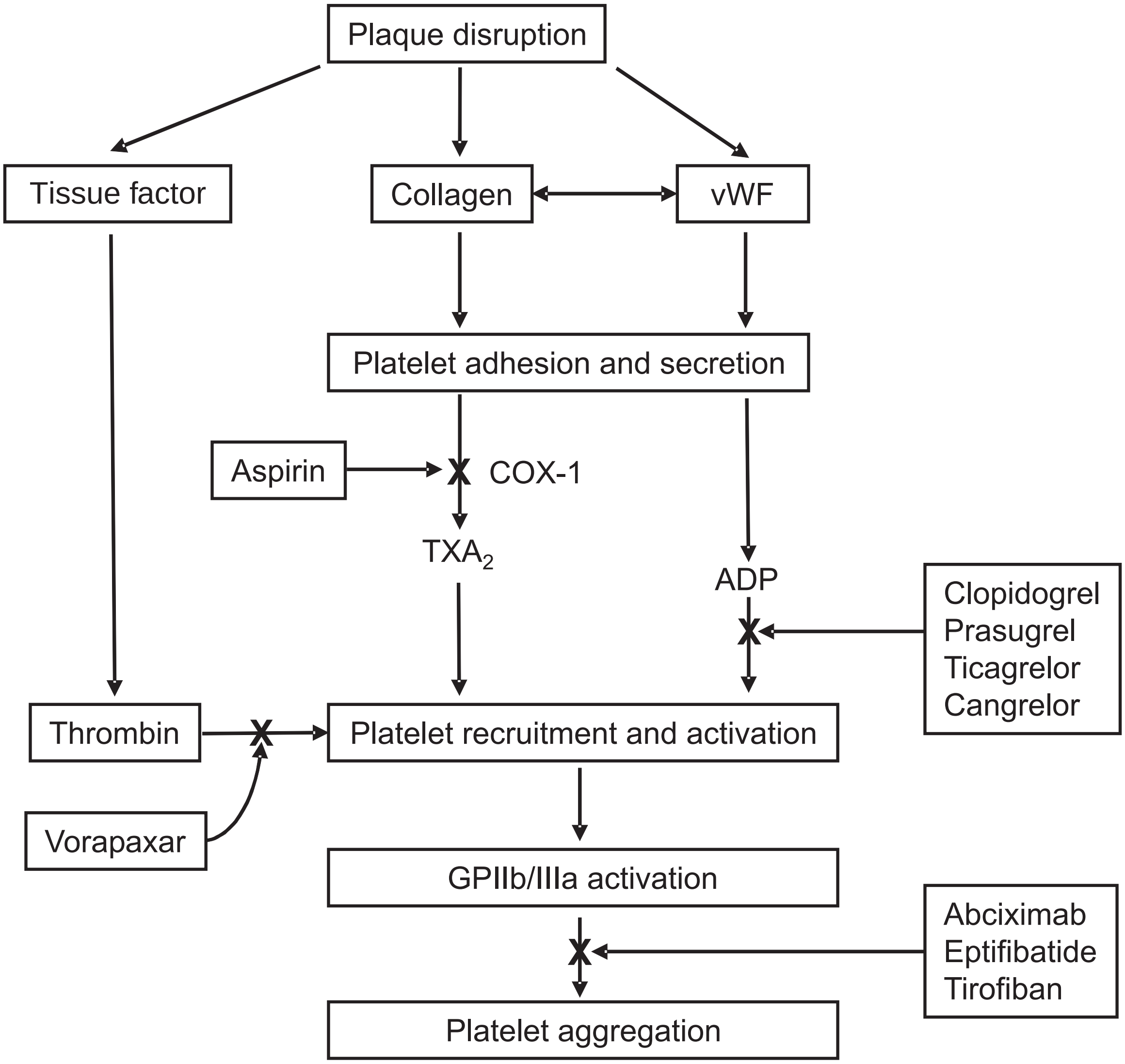

Plaque disruption
Tissue factor
Collagen
vWF
Platelet adhesion and secretion
Aspirin
COX-1
TXA2
ADP
Clopidogrel
Prasugrel
Ticagrelor
Cangrelor
Thrombin
Platelet recruitment and activation
Vorapaxar
GPIIb/IIIa activation
Abciximab
Eptifibatide
Tirofiban
Platelet aggregation

Figure 143.2 SITES OF ACTION OF ANTIPLATELET DRUGS. Aspirin inhibits thromboxane A_2 (TXA_2) synthesis by irreversibly acetylating cyclooxygenase-1 (COX-1). Reduced TXA_2 release attenuates platelet activation and recruitment to the site of vascular injury. Clopidogrel, prasugrel, ticagrelor, and cangrelor block $P2Y_{12}$, a key adenosine diphosphate (ADP) receptor on the platelet surface. Therefore, these agents also attenuate platelet recruitment. Vorapaxar inhibits type 1 protease-activated receptor (PAR-1), the major thrombin receptor on platelets. Abciximab, eptifibatide, and tirofiban inhibit the final common pathway of platelet aggregation by blocking fibrinogen binding to activated glycoprotein (GP) IIb/IIIa. *vWF*, von Willebrand factor.

not eliminate the prostaglandin-mediated gastrointestinal side effects. The risk of major bleeding with aspirin ranges from 1% to 3% per year and is higher when aspirin is used in conjunction with anticoagulants, such as warfarin. When dual antiplatelet therapy is used, low-dose aspirin should be given (75 to 100 mg daily). Eradication of *Helicobacter pylori* infection and concomitant administration of proton pump inhibitors may reduce the risk of upper gastrointestinal bleeding, particularly in patients with a history of peptic ulcer disease.

Aspirin should not be administered to patients with aspirin allergy characterized by bronchospasm. This problem occurs in about 0.3% of the general population and is more common in those with chronic urticaria, asthma, nasal polyps, or chronic rhinitis. Hepatic and renal toxicity is observed with aspirin overdose.

Aspirin Resistance

The term *aspirin resistance* has been used to describe both clinical and laboratory phenomena.[3] Clinical aspirin resistance is defined as the failure of aspirin to protect patients from ischemic vascular events. This is not a helpful definition, because it is made after a breakthrough adverse cardiovascular event occurs. Furthermore, it is not realistic to expect aspirin, which blocks only thromboxane A_2–induced platelet activation, to prevent all vascular events.

Aspirin resistance also has been described biochemically as a failure of the drug to produce its expected inhibitory effects on tests of platelet function, such as thromboxane A_2 synthesis or arachidonic acid–induced platelet aggregation. Poor adherence is a common cause of apparent aspirin resistance (see Chapter 128). Even in those taking the drug, however, the tests used for the diagnosis of aspirin resistance are not well standardized. Furthermore, there is no definitive evidence that these tests identify patients at risk of recurrent vascular events, or that resistance can be reversed either by giving higher doses of aspirin or by adding other antiplatelet drugs. Until such information is available, testing for aspirin resistance remains a research tool.

ADP Receptor Antagonists

$P2Y_{12}$ is the major ADP receptor on platelets. Agents that inhibit $P2Y_{12}$ include oral agents such as the thienopyridines and ticagrelor, and cangrelor, which is a parenteral inhibitor.

Thienopyridines

The thienopyridines include clopidogrel and prasugrel, as well as ticlopidine, which is rarely used.

Mechanism of Action

The thienopyridines are structurally related drugs that selectively inhibit ADP-induced platelet aggregation by irreversibly blocking $P2Y_{12}$ (see Fig. 143.2). Clopidogrel and prasugrel are prodrugs that must be metabolized by the hepatic cytochrome P450 (CYP) enzyme system to acquire activity. Consequently, their onset of action is delayed unless loading doses are given. The metabolic activation of prasugrel is more efficient than that of clopidogrel. Consequently, prasugrel produces a more rapid, greater, and more uniform $P2Y_{12}$ blockade than clopidogrel.

Indications

When compared with aspirin in patients with recent ischemic stroke, myocardial infarction, or peripheral arterial disease, clopidogrel reduced the risk of cardiovascular death, myocardial infarction, and stroke by 8.7%. Therefore, clopidogrel is slightly more effective than aspirin, but it is more expensive. Clopidogrel and aspirin are often combined to capitalize on their capacity to block complementary pathways of platelet activation. For example, dual antiplatelet therapy with the combination of aspirin plus clopidogrel is recommended after stent implantation in the coronary arteries for management of acute coronary syndrome (see Chapter 145) or in the arteries of the leg for management of acute limb ischemia (see Chapter 146).

The combination of clopidogrel and aspirin also is effective in patients with unstable angina. Thus in 12,562 such patients, the risk of cardiovascular death, myocardial infarction, or stroke was 9.3% in those randomized to the combination of clopidogrel and aspirin and 11.4% in those given aspirin alone, a 20% relative risk reduction.[4] However, combining clopidogrel with aspirin increases the risk of major bleeding to about 2% per year. This bleeding risk persists even if the daily dose of aspirin is 100 mg or less. Therefore, the combination of clopidogrel and aspirin should be used only when there is a clear benefit. For example, this combination has not proven to be superior to clopidogrel alone in patients with acute ischemic stroke or to aspirin alone for primary prevention in those at risk for cardiovascular events.

When compared with clopidogrel in 13,608 patients with acute coronary syndromes undergoing percutaneous coronary intervention (PCI), prasugrel reduced the combined rate of cardiovascular death, myocardial infarction, or stroke from 12.1% to 9.9%, a decrease driven mainly by a reduction in nonfatal myocardial infarction.[5] However, prasugrel increased the rate of major bleeding and fatal bleeding, particularly in patients over the age of 65 years, in those weighing less than 60 kg, and in those with a history of stroke.[5] Therefore, prasugrel should not be used in patients with these characteristics. Prasugrel was compared with clopidogrel in 7243 aspirin-treated patients under the age of 75 years with unstable angina or myocardial infarction without ST-segment elevation. At a median follow-up of 17 months, the primary endpoint, the composite of cardiovascular death, myocardial infarction, and stroke had occurred in 13.9% of the prasugrel group and in 16% of the clopidogrel group, a difference that was not statistically significant.[6] Rates of major bleeding and intracranial bleeding were similar with the two treatment regimens. Therefore, prasugrel has no advantage over clopidogrel in medically managed patients with acute coronary syndrome.

Dosing

Clopidogrel is given once daily at a dose of 75 mg. Because its onset of action is delayed for several days, loading doses of clopidogrel are given when rapid ADP receptor blockade is desired. For example, patients undergoing coronary artery stenting are often given a loading dose of 600 mg, which produces inhibition of ADP-induced platelet aggregation within 6 hours. Prasugrel is given as a loading dose of 60 mg followed by 10 mg once daily. Patients older than 75 years or weighing less than 60 kg should receive a daily prasugrel dose of 5 mg.

Side Effects

Gastrointestinal and hematologic side effects, other than bleeding, are rare with clopidogrel and prasugrel.

Clopidogrel Resistance

There is considerable between-patient variability in the capacity of clopidogrel to inhibit ADP-induced platelet aggregation.[7] Reduced responsiveness to clopidogrel is termed *clopidogrel resistance* and platelets from patients with this phenomenon exhibit high reactivity to ADP. Reduced responsiveness to clopidogrel reflects, at least in part, genetic polymorphisms in CYP2C19, which is critical for metabolic activation of clopidogrel in the liver. Loss-of-function *CYP2C19*2* and *CYP2C19*3* alleles are found in 2% of Whites, 4% of Blacks, and 14% of Asians, and the levels of the active metabolite of clopidogrel are reported to be up to 33% lower in carriers of either of these alleles than in those with healthy alleles. Patients with clopidogrel resistance are reported to have a higher risk of ischemic complications after PCI, including myocardial infarction and stent thrombosis. Based on these findings, some experts recommend pharmacogenetic testing and point-of-care assessment of platelet reactivity to predict or assess clopidogrel responsiveness. However, attempts to manage such patients with higher doses of clopidogrel or with additional antiplatelet drugs have not resulted in improved outcomes. Instead, patients with clopidogrel resistance may benefit from a switch to prasugrel, which produces more uniform inhibition of ADP-induced platelet aggregation, or to ticagrelor.

Ticagrelor

An orally active agent belonging to the cyclopentyl-triazolopyrimidine class, ticagrelor acts as a direct inhibitor of $P2Y_{12}$.

Mechanism of Action

Ticagrelor reversibly binds to $P2Y_{12}$ at a location distinct from the ADP binding site and blocks ADP-mediated receptor activation in a noncompetitive fashion, likely through an allosteric mechanism. Because it does not require metabolic activation, ticagrelor has a more rapid onset of action than clopidogrel or prasugrel.

Indications

When compared with clopidogrel in 18,624 patients with acute coronary syndromes, ticagrelor reduced the rate of cardiovascular death, myocardial infarction, or stroke from 11.7% to 9.8%.[8] All-cause mortality was also reduced with ticagrelor compared with clopidogrel (4.5% and 5.9%, respectively; $P < .001$), but ticagrelor produced more major bleeding not related to bypass surgery (2.8% and 2.2%, respectively).[8] An invasive strategy was planned for 72% of the patients entered in the trial. In this subset, the primary composite endpoint occurred in 9% of patients randomized to ticagrelor and in 10.7% of those given clopidogrel, a 16% relative risk reduction.[9] Rates of major bleeding were similar with ticagrelor and clopidogrel (11.6% and 16.5%, respectively; $P = .88$). Therefore ticagrelor also was superior to clopidogrel in patients undergoing coronary interventions.

If cost is not an issue and patients are compliant with twice-daily dosing, ticagrelor is a reasonable alternative to clopidogrel in patients with acute coronary syndrome, regardless of whether they are managed medically or undergo PCI with stent implantation. For these indications, ticagrelor should be given for at least 1 year. It also is reasonable to use ticagrelor in place of clopidogrel in patients with clopidogrel resistance or in those who develop in-stent thrombosis despite clopidogrel therapy.

Dosing

Ticagrelor is given as a 180-mg oral loading dose followed by 90 mg twice daily. The dose does not need adjustment in patients with renal impairment, but caution is needed in patients with hepatic impairment or in those receiving potent inhibitors or inducers of CYP3A4 because ticagrelor is metabolized in the liver via CYP3A4. Ticagrelor is usually administered in conjunction with aspirin; the daily aspirin dose should not exceed 100 mg.[10]

Side Effects

In addition to bleeding, the most common side effects of ticagrelor include dyspnea, which can develop in up to 15% of patients, and bradyarrhythmia. The dyspnea, which tends to occur soon after initiating ticagrelor, is usually self-limited and mild in intensity but can be persistent and may necessitate drug discontinuation in some patients. Although the exact mechanism responsible for these side effects is unknown, they may be adenosine-mediated because ticagrelor inhibits its reuptake.

Cangrelor

Cangrelor is the only available parenteral inhibitor of $P2Y_{12}$.

Mechanism of Action

Cangrelor is a rapidly acting reversible inhibitor of $P2Y_{12}$. It has an immediate onset of action after intravenous administration, a half-life of 3 to 5 minutes, and an offset of action within 1 hour.

Indications

Cangrelor is licensed for use in patients undergoing PCI, in whom it produces rapid ADP receptor blockade in those who have not received pretreatment with clopidogrel, prasugrel, or ticagrelor and are not receiving a GPIIb/IIIa inhibitor.

Dosing

Cangrelor is administered as a 30 μg/kg intravenous bolus before PCI followed by an infusion of 4 μg/kg/min for at least 2 hours or for the duration of PCI, whichever is longer. When transitioning to oral $P2Y_{12}$ inhibitor therapy, ticagrelor can be given at a loading dose of 180 mg at any time during the cangrelor infusion or immediately after discontinuation. In contrast, loading doses of prasugrel or clopidogrel (60 and 600 mg, respectively) should be given only after cangrelor is stopped because cangrelor blocks the interaction of their active metabolites with $P2Y_{12}$.

Side Effects

The major side effect of cangrelor is bleeding.

Dipyridamole

Dipyridamole is a relatively weak antiplatelet agent on its own. An extended-release formulation of dipyridamole combined with low-dose aspirin, a preparation known as Aggrenox, is used for the prevention of stroke in patients with transient ischemic attacks.

Mechanism of Action

By inhibiting phosphodiesterase, dipyridamole blocks the breakdown of cyclic AMP (cAMP). Increased levels of cAMP reduce intracellular calcium and inhibit platelet activation. Dipyridamole also blocks the uptake of adenosine by platelets and other cells. This produces a further increase in local cAMP levels because the platelet adenosine A_2 receptor is coupled to adenylate cyclase (Fig. 143.3).

Indications

Dipyridamole plus aspirin was compared with aspirin or dipyridamole alone, or with placebo, in patients with an ischemic stroke or transient ischemic attack. The combination reduced the risk of stroke by 22.1% compared with aspirin alone and by 24.4% compared with dipyridamole alone. In a second trial, researchers compared dipyridamole plus aspirin with aspirin alone for secondary prevention in patients with ischemic stroke. Vascular death, stroke, or myocardial infarction occurred in 13% of patients given combination therapy and in 16% of those treated with aspirin alone. When Aggrenox was compared with clopidogrel, however, there was no difference in efficacy,

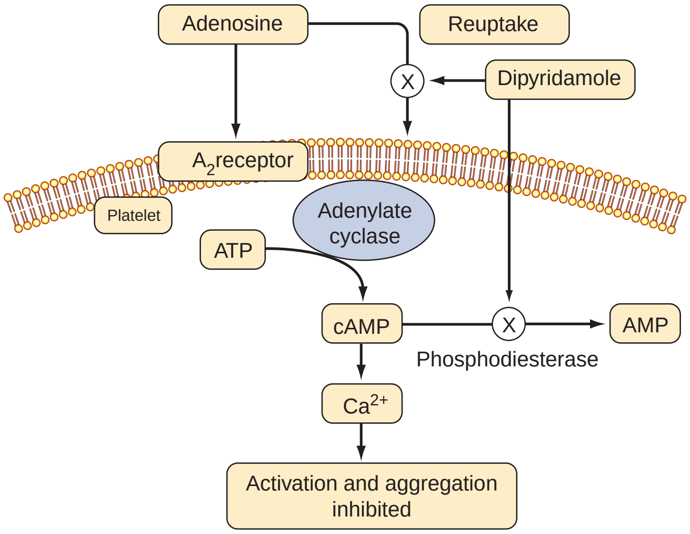


Figure 143.3 MECHANISM OF ACTION OF DIPYRIDAMOLE. Dipyridamole increases levels of cyclic adenosine monophosphate (cAMP) in platelets by blocking the reuptake of adenosine and by inhibiting phosphodiesterase-mediated cAMP degradation. By promoting calcium uptake, cAMP reduces intracellular levels of calcium. This, in turn, inhibits platelet activation and aggregation. *AMP*, Adenosine monophosphate; *ATP*, adenosine triphosphate.

and there was more intracranial bleeding with Aggrenox.[11] Although Aggrenox is used for stroke prevention, because of its vasodilatory effects and the paucity of data supporting the use of dipyridamole in patients with symptomatic coronary artery disease, Aggrenox should not be used for stroke prevention in such patients.

Dosing

Aggrenox is given twice daily. Each capsule contains 200 mg of extended-release dipyridamole and 25 mg of aspirin.

Side Effects

Because dipyridamole has vasodilatory effects, it must be used with caution in patients with coronary artery disease. Gastrointestinal complaints, headache, facial flushing, dizziness, and hypotension also can occur. These symptoms often subside with continued use of the drug.

GPIIb/IIIa Receptor Antagonists

As a class, parenteral GPIIb/IIIa receptor antagonists have an established niche in patients with acute coronary syndromes. The three agents in this class are abciximab, eptifibatide, and tirofiban.

Mechanism of Action

A member of the integrin family of adhesion receptors, GPIIb/IIIa is found on the surface of platelets and megakaryocytes. With about 40,000 to 80,000 copies per platelet, GPIIb/IIIa is the most abundant receptor (see Chapter 124). Consisting of a noncovalently linked heterodimer, GPIIb/IIIa is inactive on resting platelets. When platelets are activated, inside-outside signal transduction pathways trigger a conformational activation of the receptor. Once activated, GPIIb/IIIa binds adhesive molecules, such as fibrinogen and, under high shear conditions, von Willebrand factor. Binding is mediated by Arg-Gly-Asp (RGD) sequences found on fibrinogen and von Willebrand factor, as well as by the Lys-Gly-Asp (KGD) sequence located within a unique dodecapeptide domain on the γ-chains of fibrinogen. Once bound, fibrinogen and/or von Willebrand factor bridge adjacent platelets together to induce platelet aggregation.

Although abciximab, eptifibatide, and tirofiban all target the GPIIb/IIIa receptor, they are structurally and pharmacologically distinct (Table 143.1). Abciximab is a Fab fragment of a humanized murine monoclonal antibody directed against the activated form of GPIIb/IIIa. Abciximab binds to the activated receptor with high affinity and blocks the binding of adhesive molecules. In contrast to abciximab, eptifibatide, and tirofiban are synthetic small molecules. Eptifibatide is a cyclic heptapeptide that binds GPIIb/IIIa because it incorporates the KGD motif, whereas tirofiban is a nonpeptidic tyrosine derivative that acts as an RGD mimetic. Abciximab has a long half-life and can be detected on the surface of platelets for up to 2 weeks. Eptifibatide and tirofiban have shorter half-lives.

In addition to targeting the GPIIb/IIIa receptor, abciximab also inhibits the closely related $\alpha_v\beta_3$ receptor, which binds vitronectin, and $\alpha_M\beta_2$, a leukocyte integrin. In contrast, eptifibatide and tirofiban are specific for GPIIb/IIIa. Inhibition of $\alpha_v\beta_3$ and $\alpha_M\beta_2$ may endow abciximab with antiinflammatory and/or antiproliferative properties that extend beyond platelet inhibition.

Indications

These agents are used in high-risk patients undergoing PCI, particularly those with ST-segment elevation acute myocardial infarction who have not been adequately pretreated with antiplatelet drugs or in those requiring rapid platelet inhibition. Tirofiban and eptifibatide are occasionally used upstream to intervention in high-risk patients with unstable angina.

TABLE 143.1 **Features of GPIIb/IIIa Antagonists**

Feature	GPIIb/IIIa Antagonists		
Generic name	Abciximab	Eptifibatide	Tirofiban
Trade name	ReoPro	Integrilin	Aggrastat
Description	Fab fragment of humanized mouse monoclonal antibody	Cyclical KGD-containing heptapeptide	Nonpeptidic RGD mimetic
Specific for GPIIb/IIIa	No	Yes	Yes
Plasma half-life	Short (min)	Long (2.5 h)	Long (2.0 h)
Platelet-bound half-life	Long (days)	Short	Short
Renal clearance	No	Yes	Yes
Dosing	0.25 mg/kg bolus followed by a 12-h infusion of 10 µg/min	Two 180 µg/kg boluses given 10 min apart	25 µg/kg boluses followed by an 18-h infusion of 0.15 µg/kg/min
Adjustment for renal impairment	No	Yes	Yes

KGD, Lys-Gly-Asp; *RGD*, Arg-Gly-Asp.

Dosing

All the GPIIb/IIIa antagonists are given as an intravenous bolus followed by an infusion. Because they are cleared by the kidneys, the doses of eptifibatide and tirofiban must be reduced in patients with renal impairment.

Side Effects

In addition to bleeding, thrombocytopenia is the most serious complication. Thrombocytopenia is immune-mediated and is caused by antibodies directed against neoantigens on GPIIb/IIIa that are exposed upon antagonist binding (see Chapter 129). With abciximab, thrombocytopenia occurs in up to 5% of patients. Thrombocytopenia is severe in about 1% of these individuals. Thrombocytopenia is less common with the other two agents, occurring in about 1% of treated patients.

Vorapaxar

An orally active inhibitor of type 1 protease-activated receptor (PAR-1), vorapaxar inhibits the major thrombin receptor on platelets.[12,13]

Mechanism of Action

Thrombin is a potent platelet activator that acts through PARs (see Chapter 124).[14] PAR-1 is the highest-affinity thrombin receptor on platelets; PAR-1 also is expressed on endothelial cells and atherosclerotic plaques.[15] Vorapaxar is a synthetic analog of himbacine, which is a natural product. Given orally, vorapaxar is rapidly absorbed from the gastrointestinal tract and inhibits platelet PAR-1 in a reversible manner; however, because of its long half-life (8 to 12 days) and its high-affinity interaction with PAR-1, vorapaxar is effectively an irreversible inhibitor of PAR-1.

Indications

Vorapaxar is indicated for the reduction of thrombotic cardiovascular events in patients with a history of myocardial infarction or peripheral artery disease and no history of intracranial hemorrhage, stroke, or transient ischemic attack. The drug is often administered in conjunction with aspirin and/or clopidogrel. Vorapaxar is contraindicated in patients with a prior history of intracranial bleeding, stroke, or transient ischemic attack.

Dosing

Vorapaxar is given orally at a dose of 2.08 mg once daily.

Side Effects

The major side effect is bleeding, including intracranial and fatal bleeding.

ANTICOAGULANTS

There are both parenteral and oral anticoagulants. Currently available parenteral anticoagulants include heparin, low-molecular-weight heparin (LMWH), fondaparinux, and direct thrombin inhibitors (hirudin, bivalirudin, and argatroban). The oral anticoagulants include the vitamin K antagonists, of which warfarin is the agent most often used in North America, and the direct oral anticoagulants (DOACs), which are also termed non-vitamin K antagonist oral anticoagulants (NOACs) that target thrombin (dabigatran etexilate) or factor Xa (rivaroxaban, apixaban, and edoxaban).[14]

Parenteral Anticoagulants

Heparin

A sulfated polysaccharide, heparin is isolated from mammalian tissues rich in mast cells. Most commercial heparin is derived from porcine intestinal mucosa and is a polymer of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine residues.

Mechanism of Action

Heparin acts as an anticoagulant by activating antithrombin (previously known as *antithrombin III*) and accelerating the rate at which antithrombin inhibits clotting enzymes, particularly thrombin and factor Xa (see Chapter 121). Antithrombin, the obligatory plasma cofactor for heparin, is a 58,000-Da single-chain polypeptide that is a member of the serine protease inhibitor (serpin) superfamily. Synthesized in the liver and circulating in plasma at a concentration of 2.6 ± 0.4 μM, antithrombin acts as a suicide substrate for its target enzymes.

To activate antithrombin, heparin binds to the serpin via a unique pentasaccharide sequence that is found on one-third of the chains of commercial heparin (Fig. 143.4); heparin chains lacking this pentasaccharide sequence have little or no anticoagulant activity. Once bound to antithrombin, heparin induces a conformational change in the reactive center loop of antithrombin that renders it more readily accessible to its target proteases. This conformational change enhances the rate at which antithrombin inhibits factor Xa by at least two orders of magnitude, but it has little effect on the rate of thrombin inhibition by antithrombin (Fig. 143.5). To catalyze thrombin inhibition, heparin serves as a template that binds antithrombin and thrombin simultaneously. The formation of this ternary complex brings the enzyme in close apposition to the inhibitor, thereby promoting the formation of a stable covalent thrombin–antithrombin complex.

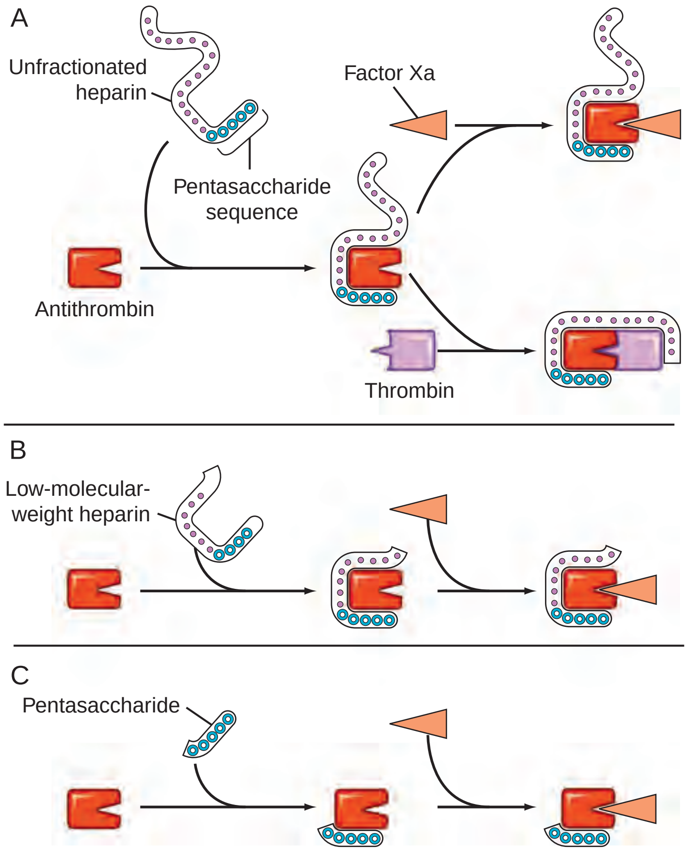


Figure 143.4 MECHANISM OF ACTION OF HEPARIN, LOW-MOLECULAR-WEIGHT HEPARIN (LMWH), AND FONDAPARINUX. (A) Heparin binds to antithrombin via its pentasaccharide sequence. This induces a conformational change in the reactive center loop of antithrombin that accelerates its interaction with factor Xa. To potentiate thrombin inhibition, heparin must simultaneously bind to antithrombin and thrombin. Only heparin chains composed of at least 18 saccharide units, which correspond to a molecular weight of 5400, are of sufficient length to perform this bridging function. With a mean molecular weight of 15,000, all the heparin chains are long enough to do this. (B) LMWH has greater capacity to potentiate factor Xa inhibition by antithrombin than thrombin does because, with a mean molecular weight of 4500 to 5000, at least half of the LMWH chains are too short to bridge antithrombin to thrombin. (C) The synthetic pentasaccharide fondaparinux only accelerates factor Xa inhibition by antithrombin because it is too short to bridge antithrombin to thrombin.

Only pentasaccharide-containing heparin chains composed of at least 18 saccharide units (which correspond to a molecular weight of 5400) are of sufficient length to bridge thrombin and antithrombin together. With a mean molecular weight of 15,000 and a range of 5000 to 30,000, most of the chains of unfractionated heparin are long enough to produce this bridging effect. Consequently, by definition, heparin has equal capacity to promote the inhibition of thrombin and factor Xa by antithrombin and is assigned an anti-factor Xa to anti-factor IIa (thrombin) ratio of 1:1.

In addition to activating antithrombin, heparin also catalyzes heparin cofactor II. This 66,000-Da serpin, which is found in plasma at a concentration of 1.2 ± 0.4 μM, is a specific inhibitor of thrombin (see Chapter 125). Two features account for the specificity of heparin cofactor II. First, the reactive site of heparin cofactor II contains the sequence Leu444–Ser445, a peptide bond that is not

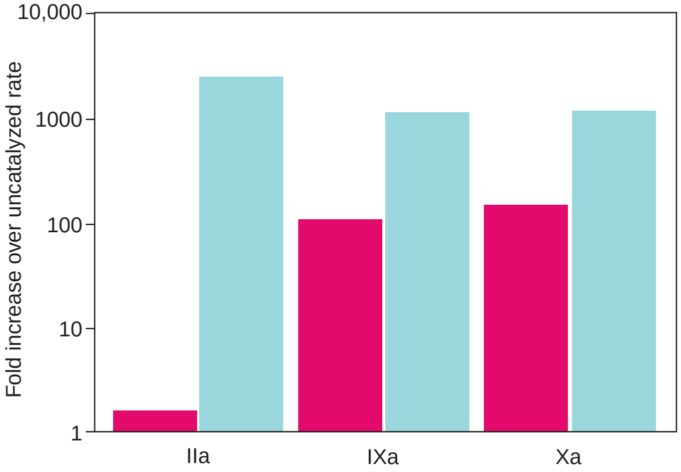


Figure 143.5 COMPARISON OF THE STIMULATORY EFFECTS OF PENTASACCHARIDE AND UNFRACTIONATED HEPARIN ON CATALYSIS OF ANTITHROMBIN-MEDIATED INHIBITION OF THROMBIN (IIa), FACTOR IXa (IXa), AND FACTOR Xa (Xa). Second-order rate constants of inhibition of factor IIa, IXa, or Xa by antithrombin, measured in the presence of heparin *(green bars)* or fondaparinux *(pink bars)* were divided by those determined in the absence of glycosaminoglycan and are plotted as fold increases over the uncatalyzed rate of inhibition. *(From Wiebe EM, Stafford AR, Fredenburgh JC, et al. Mechanism of catalysis of inhibition of factor IXa by antithrombin in the presence of heparin or pentasaccharide. J Biol Chem. 2003;278(37):35767–35774.)*

readily susceptible to cleavage by coagulation proteases other than thrombin. Second, heparin cofactor II possesses a unique anionic sequence at its N-terminal. In its unactivated state, this sequence forms an intramolecular bond with the positively charged glycosaminoglycan binding site located on the body of heparin cofactor II. When heparin or dermatan sulfate, a glycosaminoglycan that interacts only with heparin cofactor II, binds to heparin cofactor II, this N-terminal sequence is displaced which enables its tethering to a positively charged domain on thrombin known as *exosite 1*. This tethering interaction occurs only with thrombin and facilitates thrombin inhibition by heparin cofactor II (Fig. 143.6).

Maximum catalysis of heparin cofactor II by heparin requires heparin chains composed of at least 26 saccharide units, which corresponds to a molecular weight of 7800. Longer heparin chains activate heparin cofactor II to a greater extent than shorter chains because the longer chains are of sufficient length, not only to bind to heparin cofactor II, but also to bind to thrombin to form a ternary complex. The formation of this ternary complex brings thrombin and heparin cofactor II into close apposition.

The interaction of heparin with heparin cofactor II is not mediated by the antithrombin-binding pentasaccharide sequence. Because heparin lacks a specific heparin cofactor II binding domain, heparin's affinity for heparin cofactor II is lower than that for antithrombin. Consequently, 10-fold higher heparin concentrations are needed to promote thrombin inhibition by heparin cofactor II than thrombin inhibition by antithrombin. Therefore, heparin cofactor II only contributes to the anticoagulant activity of heparin when the drug is given in high doses.

Heparin causes the release of tissue factor pathway inhibitor (TFPI) from the endothelium. A factor Xa-dependent inhibitor of tissue factor–bound factor VIIa, TFPI may contribute to the antithrombotic activity of heparin. Longer heparin chains induce the release of more TFPI than shorter chains.

Pharmacology

Heparin must be given parenterally. It is usually administered subcutaneously or by continuous intravenous infusion. When used for therapeutic purposes, the intravenous route is most often employed.

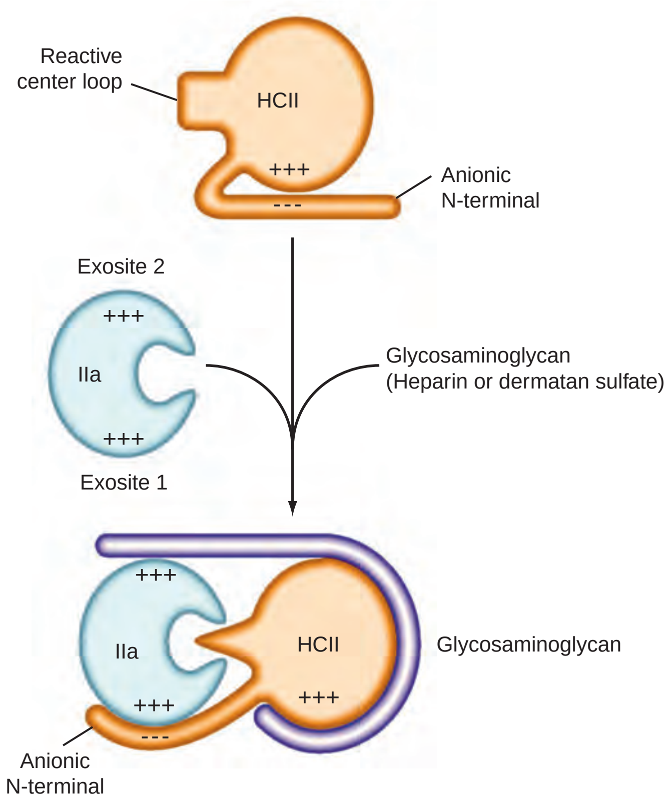


Figure 143.6 CATALYSIS OF HEPARIN COFACTOR II (HCII) BY GLYCOSAMINOGLYCANS. In its unactivated form, the acidic N-terminal tail of HCII is tethered to the positively charged glycosaminoglycan-binding site on the body of the serpin. The binding of heparin or dermatan sulfate to this binding site displaces the N-terminal tail, thereby facilitating the interaction of this anionic domain with exosite 1 on thrombin. Glycosaminoglycan binding also evokes a conformational change in the reactive center loop of HCII that contributes to the formation of the HCII–thrombin complex. Longer heparin chains bind not only to HCII but also to the heparin-binding domain on thrombin, so-called exosite 2. Heparin-mediated bridging of HCII to thrombin enhances the rate of inhibition. Consequently, longer heparin chains that contain at least 26 saccharide units promote thrombin inhibition by HCII to a greater extent than shorter chains. Unlike heparin, dermatan sulfate does not need to bind to thrombin for maximal enhancement in the rate of thrombin inhibition, because shorter dermatan sulfate fragments are just as active as longer ones.

If heparin is given subcutaneously for treatment of thrombosis, the dose of heparin must be high enough to overcome the limited bioavailability associated with this method of delivery.

After entering the circulation, heparin binds to a variety of plasma proteins other than antithrombin, which decreases its anticoagulant activity. The levels of heparin-binding proteins vary between patients because some of these proteins are acute-phase reactants whose levels are elevated in ill patients, whereas others, such as high-molecular-weight multimers of von Willebrand factor, are released when platelets or endothelial cells are activated by thrombin. Activated platelets also release platelet factor 4 (PF4), a highly cationic protein that binds heparin with high affinity. A large amount of PF4 in the vicinity of platelet-rich arterial thrombi can neutralize the anticoagulant activity of heparin.

Because the levels of heparin-binding proteins vary between patients, the anticoagulant response to fixed or weight-adjusted doses of heparin is unpredictable. Consequently, coagulation monitoring is essential to ensure that a therapeutic response is obtained when heparin is administered for treatment purposes.

Heparin is cleared through a combination of a rapid, saturable, and a much slower first-order mechanism. The saturable phase of heparin clearance reflects binding to endothelial cell receptors and macrophages.

Bound heparin is internalized and depolymerized. The slower, nonsaturable mechanism of clearance is largely renal. At therapeutic doses, a large proportion of heparin is cleared through the rapid, saturable, dose-dependent mechanism. The complex kinetics of clearance renders the anticoagulant response to heparin nonlinear at therapeutic doses, with both the intensity and duration of effect rising disproportionately with increasing dose. Thus, the apparent biological half-life of heparin increases from approximately 30 minutes after an intravenous bolus of 25 units/kg, to 60 minutes with an intravenous bolus of 100 units/kg, to 150 minutes with a bolus of 400 units/kg.

Monitoring

Heparin therapy can be monitored using the activated partial thromboplastin time (aPTT), anti-factor Xa level, or the activated clotting time (ACT). Although the aPTT is the test most often employed for this purpose, there are problems with this assay. aPTT reagents vary in their sensitivity to heparin, and the type of coagulometer used for testing can influence the results. Consequently, laboratories must establish a therapeutic aPTT range with each reagent–coagulometer combination by measuring the aPTT and anti-factor Xa level in plasma samples collected from heparin-treated patients. For most of the aPTT reagents and coagulometers in current use, therapeutic heparin levels are achieved with a two- to threefold prolongation of the aPTT.

Anti-factor Xa levels also can be used to monitor heparin therapy. With this test, therapeutic heparin levels range from 0.3 to 0.7 units/mL. Although this test is gaining in popularity, anti-factor Xa assays have yet to be standardized, and results can vary between laboratories.

The ACT is used to monitor heparin when high doses are given (>100 units/kg) to patients undergoing PCI or cardiac surgery with cardiopulmonary bypass (CPB) and those requiring extracorporeal membrane oxygenation (ECMO). The ACT varies depending on which device is used. For PCI, the target ACT is 250 to 300 seconds in patients not receiving GPIIb/IIIa inhibitors and 200 to 250 seconds if a GPIIb/IIIa inhibitor is given. For CPB, a target ACT longer than 480 seconds is desirable, whereas for ECMO, the target ACT ranges from 140 to 240 seconds.

Up to 25% of heparin-treated patients with venous thromboembolism require more than 35,000 units/day to achieve a therapeutic aPTT. These patients are considered heparin resistant. It is useful to measure anti-factor Xa levels in these patients because many will have a therapeutic anti-factor Xa level despite a subtherapeutic aPTT. This dissociation in test results occurs because elevated plasma levels of fibrinogen and factor VIII, both of which are acute-phase proteins, shorten the aPTT but have no effect on anti-factor Xa levels. Heparin therapy in patients who exhibit this phenomenon is best monitored using anti-factor Xa levels instead of the aPTT. Patients with congenital or acquired antithrombin deficiency and those with unusually high levels of heparin-binding proteins often require very high doses of heparin to achieve a therapeutic aPTT or anti-factor Xa level. If there is good correlation between the aPTT and the anti-factor Xa levels, either test can be used to monitor heparin therapy.

Dosing

For prophylaxis, heparin is usually given in fixed doses of 5000 units subcutaneously two or three times daily. With these low doses, coagulation monitoring is unnecessary. In contrast, monitoring is essential when heparin is given in therapeutic doses because a subtherapeutic anticoagulant response has been associated with a higher risk of recurrent thrombosis. Fixed-dose or weight-based heparin nomograms are used to standardize heparin dosing and to shorten the time required to achieve a therapeutic anticoagulant response. At least two heparin nomograms have been validated in patients with venous thromboembolism and both reduced the time required to achieve a therapeutic aPTT. Weight-adjusted heparin nomograms also have been evaluated in patients with acute coronary syndrome.

Unmonitored twice-daily subcutaneous heparin proved as effective and safe as once-daily LMWH for initial treatment of venous thromboembolism in one study.[15] High doses of heparin were used; treatment was started with a dose of 333 units/kg followed by 250 units/kg twice daily thereafter. This regimen may be an option for patients with renal insufficiency where LMWH or fondaparinux is problematic.

TABLE 143.2 Pharmacokinetic and Biophysical Limitations of Heparin

Limitations	Mechanism
Poor bioavailability at low doses	Limited absorption of long heparin chains
Dose-dependent clearance	Binds to endothelial cells
Variable anticoagulant response	Binds to plasma proteins whose levels vary from patient to patient
Reduced activity in the vicinity of platelet-rich thrombi	Neutralized by platelet factor 4 released from activated platelets
Limited activity against factor Xa incorporated in the prothrombinase complex and thrombin bound to fibrin	Reduced capacity of heparin–antithrombin complex to inhibit factor Xa bound to activated platelets and thrombin bound to fibrin

Limitations

Heparin has pharmacokinetic and biophysical limitations (Table 143.2). The pharmacokinetic limitations reflect heparin's propensity to bind in a pentasaccharide-independent fashion to cells and plasma proteins. Heparin binding to cells explains its dose-dependent clearance, whereas binding to plasma proteins results in a variable anticoagulant response and can lead to heparin resistance.

The biophysical limitations of heparin reflect the inability of the heparin–antithrombin complex to (1) inhibit factor Xa when it is incorporated into the prothrombinase complex, the complex that converts prothrombin to thrombin, and (2) inhibit thrombin bound to fibrin. Consequently, factor Xa bound to activated platelets within platelet-rich thrombi has the potential to generate thrombin, even in the face of heparin. Once this thrombin binds to fibrin, it too is protected from inhibition by the heparin–antithrombin complex. Clot-associated thrombin can then trigger thrombus growth by locally activating platelets and amplifying its own generation through feedback activation of factors V, VIII, and XI. Further compounding the problem is the potential for heparin neutralization by the high concentrations of PF4 released from activated platelets within the platelet-rich thrombus.

Side Effects

The most common side effect of heparin is bleeding. Other complications include thrombocytopenia, osteoporosis, and elevated levels of transaminases.

Bleeding

The risk of heparin-induced bleeding increases with higher heparin doses. Concomitant administration of drugs that affect hemostasis, such as antiplatelet or fibrinolytic agents, increases the risk of bleeding, as does recent surgery or trauma. Heparin-treated patients with serious bleeding can be given protamine sulfate to neutralize the heparin. Protamine sulfate, a mixture of basic polypeptides originally isolated from salmon sperm, binds heparin with high affinity, and the resultant protamine-heparin complexes are then cleared. Typically, 1 mg of protamine sulfate neutralizes 100 units of heparin. Protamine sulfate is given intravenously. Anaphylactoid reactions to protamine sulfate can occur, and drug administration by slow intravenous infusion is recommended to reduce the risk of these problems.

Thrombocytopenia

Heparin can cause thrombocytopenia. Heparin-induced thrombocytopenia (HIT) is an antibody-mediated process that is triggered by antibodies directed against neoantigens on PF4 that are exposed when heparin binds to this protein (Table 143.3). These antibodies, which usually are of the immunoglobulin G subtype, bind simultaneously to the heparin–PF4 complex and to platelet Fc receptors (see Chapter 131). Such binding activates the platelets and generates

TABLE 143.3 Features of Heparin-Induced Thrombocytopenia

Features	Details
Thrombocytopenia	Platelet count of 100,000/µL or less or a decrease in platelet count of 50% or more
Timing	Platelet count falls 5–10 days after starting heparin
Type of heparin	More common with unfractionated heparin than LMWH
Type of patient	More common in surgical patients than medical patients; more common in women than in men
Thrombosis	Venous thrombosis more common than arterial thrombosis

LMWH, Low-molecular-weight heparin.

TABLE 143.4 Management of Heparin-Induced Thrombocytopenia

Stop all heparin

Give an alternative anticoagulant, such as argatroban, bivalirudin, danaparoid, fondaparinux or rivaroxaban.

Do not give platelet transfusions.

Do not give warfarin until the platelet count returns to its baseline level. If warfarin is administered, give vitamin K to restore the INR to normal.

Evaluate for thrombosis, particularly deep vein thrombosis.

INR, International normalized ratio.

platelet microparticles. Circulating microparticles are prothrombotic because they express anionic phospholipids on their surface and can bind clotting factors, thereby promoting thrombin generation.

HIT can be associated with thrombosis, either arterial or venous. Venous thrombosis, which manifests as deep vein thrombosis and/or pulmonary embolism, is more common than arterial thrombosis. Arterial thrombosis can manifest as ischemic stroke or acute myocardial infarction. Rarely, platelet-rich thrombi in the distal aorta or iliac arteries can cause critical limb ischemia.

The diagnosis of HIT is established using enzyme-linked assays to detect antibodies against heparin–PF4 complexes or with platelet activation assays. Enzyme-linked assays are sensitive, but they can be positive in the absence of any clinical evidence of HIT. The most specific diagnostic test is the serotonin release assay. This test is performed by quantifying serotonin release when washed platelets loaded with labeled serotonin are exposed to patient serum in the absence or presence of varying concentrations of heparin. If the patient serum contains the HIT antibody, heparin addition induces platelet activation and subsequent serotonin release.

Management of HIT is outlined in Table 143.4. Heparin should be stopped in patients with suspected or documented HIT, and an alternative anticoagulant should be administered to prevent or treat thrombosis.[16] The agents most often used for this indication are parenteral direct thrombin inhibitors, such as argatroban or bivalirudin, or parenteral factor Xa inhibitors, such as fondaparinux or danaparoid. Oral factor Xa inhibitors, such as rivaroxaban, have been used successfully to manage HIT.

Patients with HIT, particularly those with associated thrombosis, often have evidence of increased thrombin generation that can lead to consumption of protein C. If these patients are given warfarin without a concomitant parenteral anticoagulant to suppress thrombin generation, the further decrease in protein C levels induced by warfarin can trigger skin necrosis. To avoid this problem, patients with HIT should be treated with a direct thrombin inhibitor or fondaparinux until the platelet count returns to normal levels. At this point, low-dose warfarin therapy can be introduced, and the thrombin inhibitor or fondaparinux can be discontinued when the anticoagulant response to warfarin has been therapeutic for at least 2 days. HIT also is covered extensively in Chapter 131.

TABLE 143.5 Advantages of Low-Molecular-Weight Heparin Over Heparin

Advantages	Consequence
Better bioavailability and longer half-life after subcutaneous injection	Can be given subcutaneously once or twice daily for both prophylaxis and treatment
Dose-independent clearance	Simplified dosing
Predictable anticoagulant response	Coagulation monitoring is unnecessary in most patients
Lower risk of heparin-induced thrombocytopenia	Safer than heparin for short- or long-term administration
Lower risk of osteoporosis	Safer than heparin for extended administration

Osteoporosis

Treatment with therapeutic doses of heparin for over 1 month can cause a reduction in bone density. This complication has been reported in up to 30% of patients given long-term heparin therapy, and symptomatic vertebral fractures occur in 2% to 3% of these individuals.

Elevated Levels of Transaminases

Therapeutic doses of heparin frequently cause a modest elevation in the serum levels of hepatic transaminases, without a concomitant increase in the level of bilirubin. The levels of transaminases rapidly return to normal when the drug is stopped. The mechanism responsible for this phenomenon is unknown.

Low-Molecular-Weight Heparin

Consisting of smaller fragments of heparin, LMWH is prepared from unfractionated heparin by controlled enzymatic or chemical depolymerization. The mean molecular weight of LMWH is about 5000, one-third the mean molecular weight of unfractionated heparin. LMWH has several advantages over heparin (Table 143.5) and is used instead of heparin for most indications.

Mechanism of Action

Like heparin, LMWH exerts its anticoagulant activity by activating antithrombin. With a mean molecular weight of 5000, which corresponds to about 17 saccharide units, at least about half of the pentasaccharide-containing chains of LMWH are too short to bridge thrombin to antithrombin (see Fig. 143.4). However, these chains retain the capacity to accelerate factor Xa inhibition by antithrombin because this activity is largely the result of the conformational changes in antithrombin evoked by pentasaccharide binding. Consequently, LMWH catalyzes factor Xa inhibition by antithrombin more than thrombin inhibition. Depending on their unique molecular weight distributions, LMWH preparations have anti-factor Xa to anti-factor IIa ratios ranging from 2:1 to 4:1.

Pharmacology

Although usually given subcutaneously, LMWH also can be administered intravenously if a rapid anticoagulant response is needed. LMWH has pharmacokinetic advantages over heparin. These advantages reflect the fact that shorter heparin chains bind less avidly to endothelial cells, macrophages, and heparin-binding plasma proteins. Reduced binding to endothelial cells and macrophages eliminates the rapid, dose-dependent, and saturable mechanism of clearance that is a characteristic of unfractionated heparin. Instead, the clearance of LMWH is dose-independent, and its plasma half-life is longer. Based on the measurement of anti-factor Xa levels, LMWH has a plasma half-life of about 4 hours. LMWH is cleared almost exclusively by the kidneys, and the drug can accumulate in patients with renal insufficiency.

LMWH exhibits about 90% bioavailability after subcutaneous injection. Because LMWH binds less avidly than heparin to heparin-binding proteins in plasma, LMWH produces a more predictable dose response, and resistance to LMWH is rare. With a longer half-life and more predictable anticoagulant response, LMWH can be given subcutaneously once or twice daily without coagulation monitoring, even when the drug is given in treatment doses. These properties render LMWH more convenient than unfractionated heparin. Capitalizing on this feature, studies in patients with venous thromboembolism have shown that home treatment with LMWH is as effective and safe as in-hospital treatment with continuous intravenous infusions of heparin. Outpatient treatment with LMWH streamlines care, reduces health care costs, and increases patient satisfaction.

Monitoring

In most patients, LMWH does not require coagulation monitoring. If monitoring is necessary, anti-factor Xa levels must be measured because most LMWH preparations have little effect on the aPTT. Therapeutic anti-factor Xa levels with LMWH range from 0.5 to 1.2 units/mL when measured 3 to 4 hours after drug administration. When LMWH is given in prophylactic doses, peak anti-factor Xa levels of 0.2 to 0.5 units/mL are desirable.

Indications for LMWH monitoring include renal insufficiency and morbid obesity. LMWH monitoring in patients with a creatinine clearance of 50 mL/min or less is advisable to ensure that there is no drug accumulation. Although weight-adjusted LMWH dosing appears to produce therapeutic anti-factor Xa levels in patients who are overweight, this approach has not been evaluated extensively in those with morbid obesity. It may also be advisable to monitor the anticoagulant activity of LMWH during pregnancy because dose requirements can change, particularly in the third trimester. Monitoring also is important in high-risk settings, such as in patients with mechanical mitral valves who are given LMWH for the prevention of valve thrombosis.

Dosing

The doses of LMWH recommended for prophylaxis or treatment vary depending on the LMWH preparation. For prophylaxis, once-daily subcutaneous doses of 4000 to 5000 units are often used, whereas doses of 2500 to 3000 units are given when the drug is administered twice daily. For treatment of venous thromboembolism, a dose of 150 to 200 units/kg is given if the drug is administered once daily. If a twice-daily regimen is employed, a dose of 100 units/kg is given. In patients with unstable angina, LMWH is given subcutaneously on a twice-daily basis at a dose of 100 to 120 units/kg.

Side Effects

The major complication of LMWH is bleeding. Meta-analyses suggest that the risk of major bleeding is lower with LMWH than with unfractionated heparin. HIT and osteoporosis are less common with LMWH than with unfractionated heparin.

Bleeding

The risk of bleeding with LMWH is increased with concomitant use of antiplatelet or fibrinolytic drugs. Recent surgery, trauma, or underlying hemostatic defects also increase the risk of bleeding with LMWH.

Although protamine sulfate can be used as an antidote for LMWH, protamine sulfate incompletely neutralizes the anticoagulant activity of LMWH because it binds only the longer chains of LMWH. Because longer chains are responsible for the catalysis of thrombin inhibition by antithrombin, protamine sulfate completely reverses the anti-factor IIa activity of LMWH. In contrast, protamine sulfate only partially reverses the anti-factor Xa activity of LMWH because the shorter pentasaccharide-containing chains of LMWH do not bind to protamine sulfate. Consequently, patients at high risk for bleeding may be more safely treated with continuous intravenous unfractionated heparin than with subcutaneous LMWH. In addition to the potential for complete reversal with protamine sulfate, the short half-life of intravenous heparin also is an advantage if major bleeding occurs.

Thrombocytopenia

The risk of HIT is about fivefold lower with LMWH than with heparin. LMWH binds less avidly to platelets and causes less PF4 release. Furthermore, with a lower affinity for PF4 than heparin, LMWH is less likely to induce the conformational changes in PF4 that trigger the formation of HIT antibodies. LMWH should not be used to treat patients with HIT, because most HIT antibodies exhibit cross-reactivity with LMWH, and there are case reports of thrombosis when patients with HIT were treated with LMWH.

Osteoporosis

The risk of osteoporosis is lower with long-term LMWH than with heparin. For extended treatment, therefore, LMWH is a better choice than heparin because of the lower risk of both osteoporosis and HIT.

Fondaparinux

A synthetic analog of the antithrombin-binding pentasaccharide sequence, fondaparinux differs from LMWH in several ways (Table 143.6). Fondaparinux is licensed for (1) thromboprophylaxis in medical, general surgical, and high-risk orthopedic patients and (2) as an alternative to heparin or LMWH for initial treatment of patients with established venous thromboembolism. In some countries, fondaparinux also is approved as an alternative to heparin or LMWH for the treatment of patients with acute coronary syndrome.

Mechanism of Action

As a synthetic analog of the antithrombin-binding pentasaccharide sequence found in heparin and LMWH, fondaparinux has a molecular weight of 1728. Fondaparinux binds only to antithrombin (see Fig. 143.4) and is too short to bridge thrombin to antithrombin. Consequently, fondaparinux catalyzes factor Xa inhibition by antithrombin and does not enhance the rate of thrombin inhibition.

TABLE 143.6 Comparison of Low-Molecular-Weight Heparin and Fondaparinux

Features	LMWH	Fondaparinux
Molecular mass (Da)	4500–6000	1728
Catalysis of factor Xa inhibition	Yes	Yes
Catalysis of thrombin inhibition	Yes	No
Bioavailability after subcutaneous administration (%)	90	100
Plasma half-life (h)	4	17
Renal excretion	Yes	Yes
Induces release of TFPI	Yes	No
Neutralized by protamine sulfate	Partially	No

LMWH, Low-molecular-weight heparin; *TFPI*, tissue factor pathway inhibitor.

Pharmacology

Fondaparinux exhibits complete bioavailability after subcutaneous injection. With no binding to endothelial cells or plasma proteins, the clearance of fondaparinux is dose-independent, and its plasma half-life is about 17 hours. The drug is given subcutaneously once daily. Because fondaparinux is cleared unchanged via the kidneys, it is contraindicated in patients with a creatinine clearance of less than 30 mL/min, and it should be used with caution in those with a creatinine clearance less than 50 mL/min.

Fondaparinux produces a predictable anticoagulant response after administration in fixed doses because it does not bind to plasma proteins. The drug is given at a dose of 2.5 mg once daily for the prevention of venous thromboembolism. For initial treatment of established venous thromboembolism, fondaparinux is given at a dose of 7.5 mg once daily. The dose can be reduced to 5 mg once daily for those weighing less than 50 kg and increased to 10 mg once daily for those weighing more than 100 kg. When given in these doses, fondaparinux is as effective as heparin or LMWH for the initial treatment of patients with deep vein thrombosis or pulmonary embolism and produces similar rates of bleeding.

Although not licensed for this indication in the United States, fondaparinux has been used in place of heparin or LMWH in patients with acute coronary syndrome. When the prophylactic dose of fondaparinux (2.5 mg daily) was compared with treatment doses of enoxaparin in patients with non–ST-segment elevation acute coronary syndrome, there was no difference in the rate of cardiovascular death, myocardial infarction, or stroke at 9 days.[17] However, the rate of major bleeding was 50% lower with fondaparinux than with enoxaparin, a difference that likely reflects the fact that the dose of fondaparinux was lower than that of enoxaparin. In patients with acute coronary syndrome who require PCI, there is a risk of catheter thrombosis with fondaparinux unless adjunctive heparin is given.

Side Effects

Fondaparinux does not cause HIT, and, in contrast to LMWH, there is no cross-reactivity of fondaparinux with HIT antibodies. Although fondaparinux has been used successfully for HIT treatment, large clinical trials are lacking, and the drug is not licensed for this indication.

The major side effect of fondaparinux is bleeding. There is no antidote for fondaparinux. Protamine sulfate has no effect on the anticoagulant activity of fondaparinux because it fails to bind to the drug. Andexanet alfa reverses fondaparinux but is not licensed for this indication. Recombinant activated factor VII reverses the anticoagulant effects of fondaparinux in volunteers, but it is unknown whether this agent will control fondaparinux-induced bleeding.

Parenteral Direct Thrombin Inhibitors

Heparin and LMWH are indirect inhibitors of thrombin because their activity is mediated by antithrombin. In contrast, direct thrombin inhibitors do not require a plasma cofactor; instead, these agents bind directly to thrombin and block its interaction with its substrates. Approved parenteral direct thrombin inhibitors include hirudin derivatives (lepirudin and desirudin), argatroban, and bivalirudin (Table 143.7). Lepirudin and desirudin are no longer available. Argatroban is licensed for the treatment of patients with HIT, whereas bivalirudin is approved as an alternative to heparin in patients undergoing cardiac surgery or PCI, including those with HIT.

Argatroban

A univalent inhibitor that targets the active site of thrombin, argatroban is metabolized in the liver. Consequently, this drug must be used with caution in patients with hepatic insufficiency. Argatroban is not cleared via the kidneys, so this drug is safer than lepirudin for patients with HIT who have renal insufficiency.

TABLE 143.7 Comparison of the Properties of Hirudin, Bivalirudin, and Argatroban

	Hirudin	Bivalirudin	Argatroban
Molecular mass (Da)	7000	1980	527
Site(s) of interaction with thrombin	Active site and exosite 1	Active site and exosite 1	Active site
Renal clearance	Yes	No	No
Hepatic metabolism	No	No	Yes
Plasma half-life (min)	60	25	45

Argatroban is administered by continuous intravenous infusion and has a plasma half-life of about 45 minutes. The aPTT is used to monitor its anticoagulant effect, and the dose is adjusted to achieve an aPTT 1.5 to 3 times the baseline value, but not to exceed 100 seconds. Argatroban also prolongs the international normalized ratio (INR), a feature that can complicate the transitioning of patients from argatroban to warfarin. This problem can be circumvented by using the levels of factor X to monitor warfarin in place of the INR. Alternatively, the argatroban infusion can be stopped for 2 to 3 hours before INR determination.

Bivalirudin

A synthetic 20-amino acid analog of hirudin, bivalirudin is a divalent thrombin inhibitor. Thus the N-terminal portion of bivalirudin interacts with the active site of thrombin, whereas its C-terminal tail binds to exosite 1. Bivalirudin has a plasma half-life of 25 minutes, the shortest half-life of all the parenteral direct thrombin inhibitors. Bivalirudin is degraded by peptidases and is partially excreted via the kidneys. When given in high doses for PCI or cardiac surgery, the anticoagulant activity of bivalirudin is monitored using the ACT. With lower doses, its activity can be assessed using the aPTT.

Studies comparing bivalirudin with heparin plus GPIIb/IIIa antagonists suggest that bivalirudin produces less bleeding. This feature plus its short half-life makes bivalirudin an attractive alternative to heparin in patients undergoing PCI, and it is licensed for this indication. Bivalirudin also is approved for the management of patients with HIT who require PCI or cardiac surgery.

ORAL ANTICOAGULANTS

Current oral anticoagulant practice dates to the mid-to late 1930s when the vitamin K antagonists were discovered because of investigations into the cause of hemorrhagic disease in cattle. Characterized by a decrease in prothrombin levels, this disorder is caused by the ingestion of hay containing spoiled sweet clover. Hydroxycoumarin, which was isolated from bacterial contaminants in the hay, interferes with vitamin K metabolism, thereby causing a syndrome like vitamin K deficiency. A variety of coumarin derivatives is available. The agent most widely used in North America is warfarin.

The repertoire of oral anticoagulants has expanded with the introduction of the DOACs. These agents include dabigatran, which inhibits thrombin, and rivaroxaban, apixaban, and edoxaban, which inhibit factor Xa.[14] The DOACs are licensed for thromboprophylaxis after hip or knee replacement, stroke prevention in patients with atrial fibrillation, and for treatment and secondary prevention of venous thromboembolism.

Warfarin

A water-soluble vitamin K antagonist, warfarin was initially developed as a rodenticide. Like other vitamin K antagonists, warfarin interferes with the synthesis of the vitamin K-dependent clotting proteins, which include prothrombin (factor II) and factors VII, IX, and X. Warfarin also reduces the synthesis of the vitamin K-dependent anticoagulant proteins, proteins C, S, and Z.

Mechanism of Action

All the vitamin K-dependent clotting factors possess glutamic acid residues at their N-termini. A posttranslational modification adds a carboxyl group to the γ-carbon of these residues to generate γ-carboxyglutamic acid. This modification is essential for the expression of the activity of these clotting factors because it permits their calcium-dependent binding to negatively charged phospholipid surfaces. The γ-carboxylation process is catalyzed by a vitamin K-dependent carboxylase. Thus, vitamin K from the diet is reduced to vitamin K hydroquinone by vitamin K reductase (Fig. 143.7). Vitamin K hydroquinone serves as a cofactor for the carboxylase enzyme, which in the presence of carbon dioxide replaces the hydrogen on the γ-carbon of glutamic acid residues with a carboxyl group. During this process, vitamin K hydroquinone is oxidized to vitamin K epoxide, which is then reduced to vitamin K by vitamin K epoxide reductase (VKOR).

Warfarin inhibits VKOR, thereby blocking the γ-carboxylation process. This results in the synthesis of vitamin K-dependent clotting proteins that are only partially γ-carboxylated. Warfarin acts as an anticoagulant because these partially γ-carboxylated proteins have reduced or absent biologic activity. The onset of action of warfarin is delayed until the newly synthesized clotting factors with reduced activity gradually replace their fully active counterparts.

The antithrombotic effect of warfarin depends on a reduction in the functional levels of factor X and prothrombin, clotting factors that have half-lives of 24 and 72 hours, respectively. Because of the delay in achieving an antithrombotic effect, initial treatment with warfarin is supported by concomitant administration of a rapidly acting parenteral anticoagulant, such as heparin, LMWH, or fondaparinux, in patients with established thrombosis or at high risk for thrombosis. This strategy also helps to prevent warfarin-induced skin necrosis (see later).

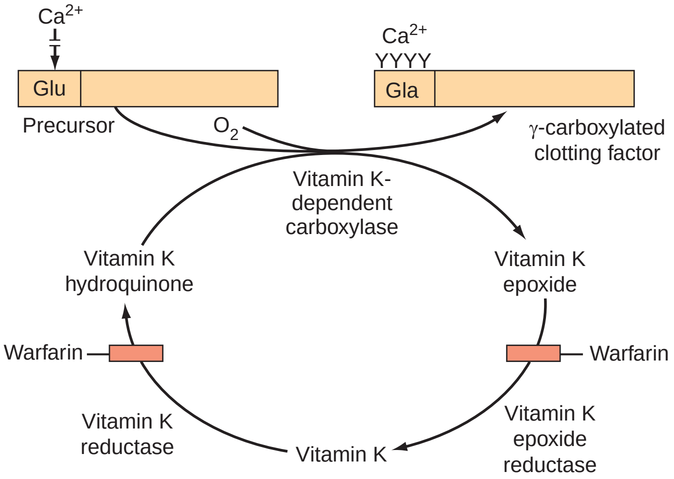


Figure 143.7 THE VITAMIN K CYCLE AND ITS INHIBITION BY WARFARIN. Dietary vitamin K is reduced by vitamin K reductase to generate vitamin K hydroquinone. Vitamin K hydroquinone serves as a cofactor for the vitamin K–dependent carboxylase that converts glutamic acid residues at the N-termini of the vitamin K–dependent precursors to γ-carboxyglutamic acid residues, thereby creating the so-called Gla domain. By binding calcium, the Gla domain is critical for the interaction of the vitamin K-dependent clotting factors with negatively charged phospholipid membranes. During vitamin K-dependent carboxylation, vitamin K is oxidized to vitamin K epoxide. Vitamin K epoxide is then converted to vitamin K by vitamin K epoxide reductase. Vitamin K antagonists, such as warfarin, interfere with this cycle by inhibiting vitamin K epoxide reductase and vitamin K reductase. Of these two enzymes, vitamin K antagonists more readily block vitamin K epoxide reductase than vitamin K reductase. Consequently, supplemental vitamin K can overcome the inhibitory effects of vitamin K antagonists.

Pharmacology

Warfarin is a racemic mixture of R and S isomers; of these, the S isomer is more active. Warfarin is rapidly and almost completely absorbed from the gastrointestinal tract. Levels of warfarin in the blood peak about 90 minutes after drug administration. Racemic warfarin has a plasma half-life of 36 to 42 hours, and over 97% of circulating warfarin is bound to albumin. Only unbound warfarin is biologically active.

Warfarin accumulates in the liver, where the two isomers are metabolized via distinct pathways. Oxidative metabolism of the more active S isomer is mediated by CYP2C9.[18] Two relatively common variants, *CYP2C9*2* and *CYP2C9*3*, have reduced activity. Patients with these variants require a lower maintenance dose of warfarin. The target of warfarin is VKOR. Polymorphisms in the C1 subunit of this enzyme (VKORC1) also can render patients less or more responsive to the anticoagulant effects of warfarin.[18] These findings have prompted a recommendation that patients starting on warfarin should be tested for these polymorphisms and that this information should be incorporated into their warfarin-dosing algorithms. Whether this approach will increase the efficacy and/or safety of warfarin therapy is uncertain.

In addition to genetic factors, diet, drugs, and various disease states influence the anticoagulant effect of warfarin. Fluctuations in dietary vitamin K intake affect the activity of warfarin. A wide variety of drugs can alter the absorption, clearance, or metabolism of warfarin. Because of the variability in the anticoagulant response to warfarin, coagulation monitoring is essential to ensure that a therapeutic response is obtained.

Monitoring

Warfarin therapy is most often monitored using the prothrombin time, a test that is sensitive to reductions in the levels of prothrombin, factor VII, and factor X.[19] The test is performed by adding thromboplastin, a reagent that contains tissue factor, phospholipid, and calcium, to citrated plasma and determining the time to clot formation. Thromboplastins vary in their sensitivity to reductions in the levels of the vitamin K-dependent clotting factors. Consequently, less sensitive thromboplastins will prompt the administration of higher doses of warfarin to achieve a target prothrombin time. This is problematic because higher doses of warfarin increase the risk of bleeding.

The INR was developed to circumvent many of the problems associated with the prothrombin time assay. To calculate the INR, the patient's prothrombin time is divided by the mean normal prothrombin time, and this ratio is then multiplied by the international sensitivity index (ISI), an index of the sensitivity of the thromboplastin used for prothrombin time determination to reductions in the levels of the vitamin K-dependent clotting factors. Highly sensitive thromboplastins have an ISI of 1.0. Most current thromboplastins have ISI values that range from 1.0 to 1.4.

For most indications, warfarin is administered in doses that produce a target INR of 2.0 to 3.0.[19] An exception is patients with mechanical heart valves in the mitral position, where a target INR of 2.5 to 3.5 is recommended.[20] Vitamin K antagonists have a narrow therapeutic window. Thus, studies in atrial fibrillation demonstrate an increased risk of cardioembolic stroke when the INR falls below 1.7 and an increase in bleeding with INR values over 4.5. Likewise, a study with patients receiving long-term warfarin therapy for unprovoked venous thromboembolism demonstrated a higher rate of recurrent venous thromboembolism when the warfarin dose was adjusted to achieve a target INR of 1.5 to 1.9 than with a target INR of 2.0 to 3.0. Rates of major bleeding were similar.

Dosing

Warfarin is usually started at a dose of 5 to 10 mg. The dose is then titrated to achieve the desired target INR. Because of its delayed onset of action, patients with established thrombosis or those at high risk for thrombosis are given concomitant treatment with a rapidly acting parenteral anticoagulant, such as heparin, LMWH, or fondaparinux. Initial prolongation of the INR reflects a reduction in the functional levels of factor VII. Consequently, concomitant treatment with the parenteral anticoagulant should be continued until the INR has been therapeutic for at least 2 consecutive days. A minimum 5-day course of parenteral anticoagulation is recommended to ensure that the levels of prothrombin have been reduced into the therapeutic range with warfarin.

Because warfarin has a narrow therapeutic window, frequent coagulation monitoring is essential to ensure that a therapeutic anticoagulant response is obtained. Even patients with stable warfarin dose requirements should have their INR determined every 2 to 4 weeks.[20] More frequent monitoring is necessary when new medications are introduced because many drugs enhance or reduce the anticoagulant effects of warfarin.

Side Effects

Like all anticoagulants, the major side effect of warfarin is bleeding. A rare complication is skin necrosis. Warfarin crosses the placenta and can cause fetal abnormalities. Consequently, warfarin should be avoided during pregnancy, particularly during the first trimester.

Bleeding

At least half of the bleeding complications with warfarin occur when the INR exceeds the therapeutic range. Bleeding complications may be mild, such as epistaxis or hematuria, or more severe, such as retroperitoneal or gastrointestinal bleeding. Life-threatening intracranial bleeding also can occur.

To minimize the risk of bleeding, the INR should be maintained in the therapeutic range. In asymptomatic patients whose INR is between 3.5 and 4.5, warfarin should be withheld until the INR returns to the therapeutic range. If the INR is over 4.5, a therapeutic INR can be achieved more rapidly by the administration of low doses of sublingual vitamin K.

Patients with serious bleeding should be given 5 to 10 mg of vitamin K by slow intravenous infusion. Additional vitamin K should be given until the INR is in the normal range. Treatment with vitamin K should be supplemented with prothrombin complex concentrate to replace the missing vitamin K–dependent clotting factors.[20]

Warfarin-treated patients who experience bleeding when their INR is in the therapeutic range require investigation into the cause of the bleeding. Those with gastrointestinal bleeding often have underlying peptic ulcer disease or a tumor. Similarly, investigation of hematuria or uterine bleeding in patients with a therapeutic INR may unmask a tumor of the genitourinary system.

Skin Necrosis

A rare complication of warfarin, skin necrosis usually is seen 2 to 5 days after initiation of therapy. Well-demarcated erythematous lesions form on the thighs, buttocks, breasts, or toes. Typically, the center of the lesion becomes progressively necrotic. Examination of skin biopsies taken from the border of these lesions reveals thrombi in the microvasculature.

Warfarin-induced skin necrosis is seen in patients with congenital or acquired deficiencies of protein C or protein S. Initiation of warfarin therapy in these patients produces a precipitous fall in plasma levels of proteins C or S, thereby eliminating this important anticoagulant pathway before warfarin exerts an antithrombotic effect through lowering of the functional levels of factor X and prothrombin. The resultant procoagulant state triggers thrombosis. Why the thrombosis is localized to the microvasculature of fatty tissues is unclear.

Treatment involves discontinuation of warfarin and reversal with vitamin K if needed. An alternative anticoagulant, such as heparin, LMWH, or a DOAC such as rivaroxaban or apixaban, should be given. Protein C concentrates or recombinant activated protein C can be given to patients with protein C deficiency to accelerate healing of the skin lesions; fresh frozen plasma may be of value for those with protein S deficiency. Occasionally, skin grafting is necessary when there is extensive skin loss.

Because of the potential for skin necrosis, patients with known protein C or protein S deficiency require overlapping treatment with a parenteral anticoagulant when initiating warfarin therapy. Warfarin should be started in low doses in these patients, and the parenteral anticoagulant should be continued until the INR is therapeutic for at least 2 to 3 consecutive days.

Pregnancy

Warfarin crosses the placenta and can cause fetal abnormalities or bleeding. The fetal abnormalities include a characteristic embryopathy, which consists of nasal hypoplasia and stippled epiphyses. The risk of embryopathy is highest if warfarin is given in the first trimester of pregnancy. Central nervous system abnormalities also can occur with exposure to coumarins at any time during pregnancy. Finally, maternal administration of warfarin produces an anticoagulant effect in the fetus that can cause bleeding. This is of particular concern at delivery when trauma to the head during passage through the birth canal can lead to intracranial bleeding. Because of these potential problems, warfarin is rarely used in pregnancy, particularly in the first and third trimesters. Instead, heparin, LMWH, or fondaparinux can be given during pregnancy for prevention or treatment of thrombosis.

Warfarin does not pass into breast milk. Consequently, warfarin can safely be administered to nursing mothers.

Special Problems

Patients with a lupus anticoagulant or those who need urgent or elective surgery present special challenges. Although observational studies have suggested that patients with thrombosis complicating the antiphospholipid syndrome required higher-intensity warfarin regimens to prevent recurrent thromboembolic events, randomized trials demonstrated that targeting an INR of 2.0 to 3.0 is as effective as higher-intensity treatment and produces less bleeding. Monitoring warfarin therapy can be problematic in patients with antiphospholipid antibody syndrome if the lupus anticoagulant prolongs the baseline INR.

If patients receiving long-term warfarin treatment require an elective invasive procedure, warfarin can be stopped 5 days before the procedure to allow the INR to return to normal levels. Those at high risk for recurrent thrombosis can be bridged with once- or twice-daily subcutaneous injections of LMWH when the INR falls below 2.0. The last dose of LMWH should be given 12 to 24 hours before the procedure, depending on whether LMWH is administered twice or once daily, respectively. After the procedure, warfarin can be restarted.

Direct Oral Anticoagulants

The DOACs have a rapid onset of action and half-lives that permit once- or twice-daily administration. Designed to produce a predictable level of anticoagulation, DOACs are more convenient to administer than warfarin because they are given in fixed doses without the need for routine monitoring of coagulation. As a class, the DOACs are at least as effective as warfarin and produce less serious bleeding, particularly less intracranial hemorrhage.[21,22]

Mechanism of Action

DOACs are small molecules that bind reversibly to the active site of their target enzyme. Table 143.8 summarizes the pharmacologic features of these agents.

Dosages

For prevention of stroke in patients with nonvalvular atrial fibrillation, rivaroxaban is given at a dosage of 20 mg once daily, with a reduction to 15 mg once daily in patients with a creatinine clearance of 15 to 49 mL/min; dabigatran is given at a dosage of 150 mg twice daily, with a reduction to 75 mg twice daily in those with a creatinine

TABLE 143.8 **Comparison of the Pharmacological Properties of Dabigatran, Rivaroxaban, Apixaban, and Edoxaban**

	Dabigatran	Rivaroxaban	Apixaban	Edoxaban
Target	Thrombin (IIa)	Factor Xa	Factor Xa	Factor Xa
Active drug	No	Yes	Yes	Yes
Onset time (h)	0.5–2	2–4	3–4	1–3
Half-life (h)	12–17	5–13	~12	10–14
Renal excretion (%)	80	33	27	50

clearance of 15 to 30 mL/min; apixaban is given at a dosage of 5 mg twice daily, with a reduction to 2.5 mg twice daily for patients with at least two of the "ABC" criteria (i.e., *a*ge over 80 years, *b*ody weight under 60 kg, and *c*reatinine over 1.5 g/dL); and edoxaban is given at a dosage of 60 mg once daily for patients with a creatinine clearance of 50 to 95 mL/min and with a reduction to 30 mg once daily for patients with any one of the following criteria: creatinine clearance 15 to 50 mL/min, a body weight of 60 kg or less, or use of potent P-GP inhibitors, such as verapamil or quinidine.

Dabigatran, rivaroxaban, apixaban, and edoxaban are also licensed for the treatment of patients with VTE. Dabigatran and edoxaban are started after patients have received at least a 5-day course of treatment with a parenteral anticoagulant such as LMWH; dabigatran is given at a dose of 150 mg twice daily provided the creatinine clearance is over 30 mL/min, and the dosage regimen for edoxaban is identical to that used in patients with atrial fibrillation. In contrast, rivaroxaban and apixaban can be given in all-oral regimens; rivaroxaban is started at a dose of 15 mg twice daily for 21 days and is then reduced to 20 mg once daily thereafter, whereas apixaban is started at a dose of 10 mg twice daily for 7 days and is then reduced to 5 mg twice daily thereafter. For long-term secondary prevention, the dosage of apixaban can be lowered to 2.5 mg twice daily and the dose of rivaroxaban can be lowered to 10 mg once daily, doses that have safety profiles similar to those of placebo and aspirin, respectively.

Dabigatran, rivaroxaban, and apixaban are licensed for thromboprophylaxis after elective hip or knee replacement surgery; edoxaban is not licensed for this indication except in Japan. Thromboprophylaxis is started after surgery and is often continued for 30 days in patients undergoing hip replacement and for 10 to 14 days in patients undergoing knee replacement. In lower-risk patients undergoing hip or knee replacement surgery, a 5-day course of rivaroxaban followed by a 30-day course of aspirin at a dose of 81 mg daily appears to be as effective and safe as extended thromboprophylaxis with rivaroxaban. Dabigatran is given at a dose of 220 mg once daily, whereas rivaroxaban and apixaban are given at doses of 10 mg once daily and 2.5 mg twice daily, respectively.

Very low dose rivaroxaban (2.5 mg twice daily) in combination with aspirin (81 mg once daily) is licensed for lowering the risk for major adverse cardiovascular events in patients with chronic coronary or peripheral artery disease (see Chapter 145 and 146). None of the other DOACs is approved for this indication.

Monitoring

Although administered without routine monitoring, it is helpful to determine the anticoagulant activity of the DOACs in some situations,[23] such as assessment of adherence, detection of accumulation or overdose, identification of bleeding mechanisms, and determination of activity before surgery or intervention. For qualitative assessment of anticoagulant activity, the prothrombin time can be used for factor Xa inhibitors and the aPTT for dabigatran. Rivaroxaban and edoxaban prolong the prothrombin time more than apixaban does. In fact, because apixaban has such a limited effect on the prothrombin time, anti-factor Xa assays are needed to assess its activity.[23] The effect of the drugs on tests of coagulation varies depending on the reagents used to perform the tests. Chromogenic anti-factor Xa assays and the diluted thrombin clotting time or ecarin clotting or chromogenic assays with appropriate calibrators provide quantitative assays to measure plasma levels of the factor Xa inhibitors and dabigatran, respectively.

Side Effects

As with any anticoagulant, bleeding is the most common side effect of the DOACs. Although the risk of intracranial bleeding is lower with the DOACs than with warfarin, the risk for gastrointestinal bleeding is higher with dabigatran (at the 150-mg, twice-daily dose), rivaroxaban, and edoxaban (at the 60-mg, once-daily dose) than with warfarin.[22] Dyspepsia occurs in up to 10% of patients treated with dabigatran; this problem improves with time and can be minimized by taking the drug with food.

Periprocedural Management

Like warfarin, the DOACs must be stopped before surgical procedures associated with a moderate or high risk for bleeding.[24,25] The drugs should be withheld for 1 to 2 days or longer if renal function is impaired. After surgery, patients should receive thromboprophylaxis with LMWH until hemostasis is restored, at which point the DOACs can be restarted.

Cardiac procedures such as atrial fibrillation ablation or pacemaker implantation can safely be performed without interruption of the DOACs. However, it may be prudent to hold the dose on the morning of the day of the procedure to avoid intervention at peak drug levels.

Management of Bleeding

With minor bleeding, withholding one or two doses of drug is usually sufficient. With more serious bleeding, the approach is similar to that with warfarin, except that vitamin K administration is of no benefit; the anticoagulant and any antiplatelet drugs should be withheld, the patient should be resuscitated with fluids and blood products as necessary, and the bleeding site should be identified and managed. Coagulation testing will determine the extent of anticoagulation, and renal function should be assessed so that the half-life of the drug can be calculated.[26] Timing of the last dose of anticoagulant is important, and oral-activated charcoal may help prevent absorption of drugs administered in the past 4 hours, particularly in cases of overdose. If bleeding continues or is life-threatening, or if it occurs in a critical organ (e.g., the eye) or in a closed space (e.g., the pericardium or retroperitoneum), reversal of the anticoagulant should be considered.

Idarucizumab is licensed for dabigatran reversal in patients with serious bleeding or in those requiring urgent surgery or intervention. A humanized antibody fragment, idarucizumab binds dabigatran with 350-fold higher affinity than that of dabigatran for thrombin to form an essentially irreversible complex that is cleared by the kidneys. Idarucizumab, which is given intravenously as a 5-g bolus, rapidly reverses the anticoagulant effects of dabigatran and normalizes the aPTT, diluted thrombin time, or ecarin clot time.[27,28]

Andexanet alfa is available for reversal of rivaroxaban, apixaban, and edoxaban. A recombinant variant of factor Xa without catalytic activity, andexanet serves as a decoy to sequester oral factor Xa inhibitors until they are cleared from the circulation.[29–32] Low- or high-dose intravenous andexanet regimens are used. The low dose regimen starts with a bolus of 400 mg followed by an infusion of 4 mg/min for up to 120 minutes, whereas the high-dose regimen starts with a bolus of 800 mg followed by an infusion of 8 mg/min for up to 120 minutes. The low-dose regimen is used for reversal of doses of rivaroxaban or apixaban of 10 or 5 mg or less, respectively, or for any dose of rivaroxaban or apixaban if the last dose was taken more than 8 hours prior to presentation. The high-dose regimen is used to reverse rivaroxaban or apixaban doses over 10 and 5 mg, respectively, if the last dose was taken less than 8 hours since the presentation, or for reversal if the dose of rivaroxaban or apixaban or the timing of the last dose is unknown. Andexanet alfa is expensive and is not available in all countries and in all hospitals. Because of its cost, andexanet alfa is often reserved for reversal in patients with intracranial bleeds or for bleeds into a closed space such as retroperitoneal or pericardial bleeds. If andexanet is unavailable, the results of prospective cohort studies suggest that 4-factor prothrombin complex concentrate (25 to 50 units/kg) also is effective at restoring hemostasis. If there is continued bleeding, activated prothrombin complex concentrate (50 units/kg) or recombinant factor VIIa (90 µg/kg) can be considered.[31,32]

Neither andexanet alfa nor 4-factor prothrombin complex concentrate has been evaluated for reversal in patients requiring urgent surgery or intervention. Furthermore, andexanet alfa not only reverses oral factor Xa inhibitors but also reverses heparin and LMWH. This could be problematic in patients who require cardiac surgery or vascular surgery, procedures where heparin is used routinely. To circumvent this problem, most surgical procedures and interventions can be done without reversal and 4-factor prothrombin complex concentrate can be given if necessary. For patients requiring surgery to stop bleeding, such as those with a ruptured aortic aneurysm or with bleeding secondary to polytrauma, upfront 4-factor prothrombin concentrate administration can be considered.

At an earlier stage of development than andexanet, ciraparantag is a synthetic, cationic small molecule that binds rivaroxaban, apixaban, and edoxaban, as well as dabigatran, heparin, LMWH, and fondaparinux.[33–35] When given as an intravenous bolus to volunteers who took rivaroxaban, apixaban, or edoxaban, ciraparantag reduced the whole-blood clotting time in a concentration-dependent manner.[34,35] Additional studies are needed before ciraparantag will be approved.

Pregnancy

As small molecules, the DOACs can pass through the placenta. Consequently, these agents are contraindicated in pregnancy, and when used by women of childbearing potential, appropriate contraception is important. Small amounts of rivaroxaban pass into breast milk, and it is unknown whether the other DOACs also do so. Therefore, DOACs should not be used in nursing mothers.

Novel Anticoagulants in Development

Although the DOACs are a major advance over warfarin, the search for more effective and safer anticoagulants continues. Evidence that factor XI and factor XII—components of the contact system—are important for thrombosis but less so for hemostasis has prompted the development of anticoagulants that target these factors.[36,37] Ongoing studies are evaluating antisense oligonucleotides, inhibitory antibodies, and small molecule inhibitors of factor XI.

FIBRINOLYTIC DRUGS

Role of Fibrinolytic Therapy

Used to degrade thrombi, fibrinolytic drugs can be administered systemically, or they can be delivered via catheters directly into the substance of the thrombus. Systemic delivery is used for treatment of acute myocardial infarction, acute ischemic stroke, and most cases of massive pulmonary embolism. The goal of therapy is to produce rapid thrombus dissolution, thereby restoring antegrade blood flow. In the coronary circulation, restoration of blood flow reduces morbidity and mortality by limiting myocardial damage, whereas in the cerebral circulation, rapid thrombus dissolution decreases the neuronal death and brain infarction that produce irreversible brain injury. For patients with massive pulmonary embolism, the goal of fibrinolytic therapy is to restore pulmonary artery perfusion.

Peripheral artery thrombi and thrombi in the proximal deep veins of the leg are most often treated using a catheter-directed approach. Catheters with multiple side holes can be used to deliver fibrinolytic drugs. In some cases, intravascular devices that fragment and extract the thrombus are used to hasten treatment.

Mechanism of Action

Commonly used fibrinolytic agents include streptokinase, recombinant tissue-type plasminogen activator (rt-PA), which is also known as alteplase or activase, and two recombinant derivatives of rt-PA, tenecteplase and reteplase. All of these agents act by converting the proenzyme, plasminogen, to plasmin, the active enzyme (Fig. 143.8). Plasmin then degrades the fibrin matrix of thrombi, thereby producing soluble fibrin degradation products.

Endogenous fibrinolysis is regulated at two levels. Plasminogen activator inhibitors, particularly the type 1 form, known as PAI-1, prevent excessive plasminogen activation by regulating the activity of tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Once plasmin is generated, plasmin inhibitors, particularly α_2-antiplasmin, regulate it. The plasma concentration of plasminogen is twofold higher than that of α_2-antiplasmin. Consequently, with pharmacologic doses of plasminogen activators, the concentration of plasmin that is generated can exceed that of α_2-antiplasmin. In addition to degrading fibrin, unregulated plasmin also can degrade fibrinogen and other clotting factors. This process, which is known as the systemic lytic state, reduces the hemostatic potential of the blood and increases the risk of bleeding.

The endogenous fibrinolytic system is geared to localize plasmin generation to the fibrin surface. Both plasminogen and t-PA bind to fibrin to form a ternary complex that promotes efficient plasminogen activation. In contrast to free plasmin, plasmin generated on the fibrin surface is relatively protected from inactivation by α_2-antiplasmin, a feature that promotes fibrin dissolution. Furthermore, C-terminal lysine residues exposed as plasmin degrades fibrin serve as binding sites for additional plasminogen and t-PA molecules. This creates positive feedback that enhances plasmin generation. When used pharmacologically, the various plasminogen activators capitalize on these mechanisms to a lesser or greater extent.

There are two pools of plasminogen: circulating plasminogen and fibrin-bound plasminogen (Fig. 143.9). Plasminogen activators that preferentially activate fibrin-bound plasminogen are considered fibrin specific. In contrast, nonspecific plasminogen activators do not discriminate between fibrin-bound and circulating plasminogen. Activation of circulating plasminogen results in the generation of unopposed plasmin that can trigger the systemic lytic state. Alteplase and its derivatives are fibrin-specific plasminogen activators, whereas streptokinase is a nonspecific agent.

Streptokinase

Unlike other plasminogen activators, streptokinase is not an enzyme and does not directly convert plasminogen to plasmin. Instead, streptokinase forms a 1:1 stoichiometric complex with plasminogen.

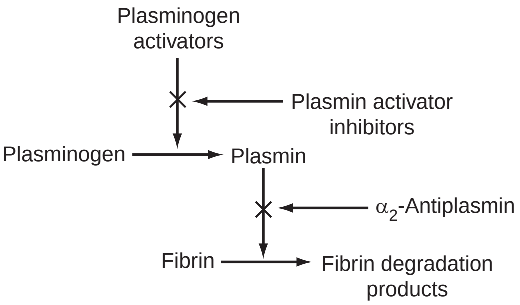


Figure 143.8 FIBRINOLYTIC SYSTEM AND ITS REGULATION. Plasminogen activators convert plasminogen to plasmin. Plasmin then degrades fibrin into soluble fibrin degradation products. The system is regulated at two levels. Type 1 plasminogen activator inhibitor (PAI-1) inhibits the plasminogen activators, whereas α_2-antiplasmin serves as the major inhibitor of plasmin.

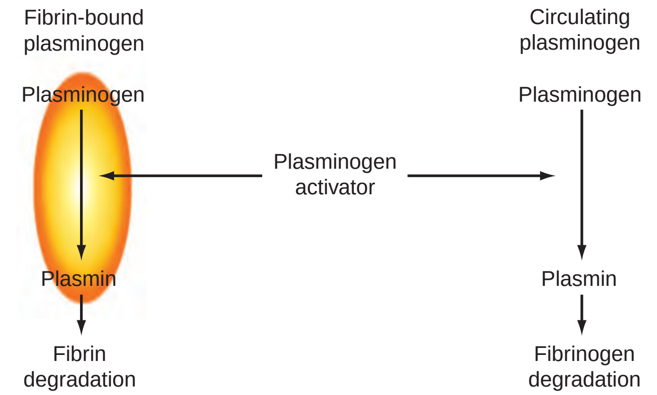


Figure 143.9 CONSEQUENCES OF ACTIVATION OF FIBRIN-BOUND OR CIRCULATING PLASMINOGEN. The fibrin specificity of plasminogen activators reflects their capacity to distinguish between fibrin-bound and circulating plasminogen. This, in turn, reflects their affinity for fibrin. Plasminogen activators with high affinity for fibrin preferentially activate fibrin-bound plasminogen. This results in the generation of plasmin on the fibrin surface. Fibrin-bound plasmin, which is protected from inactivation by α_2-antiplasmin, degrades fibrin to yield soluble fibrin degradation products. In contrast, plasminogen activators with little or no affinity for fibrin do not distinguish between fibrin-bound and circulating plasminogen. Activation of circulating plasminogen results in systemic plasminemia and subsequent degradation of fibrinogen and other clotting factors.

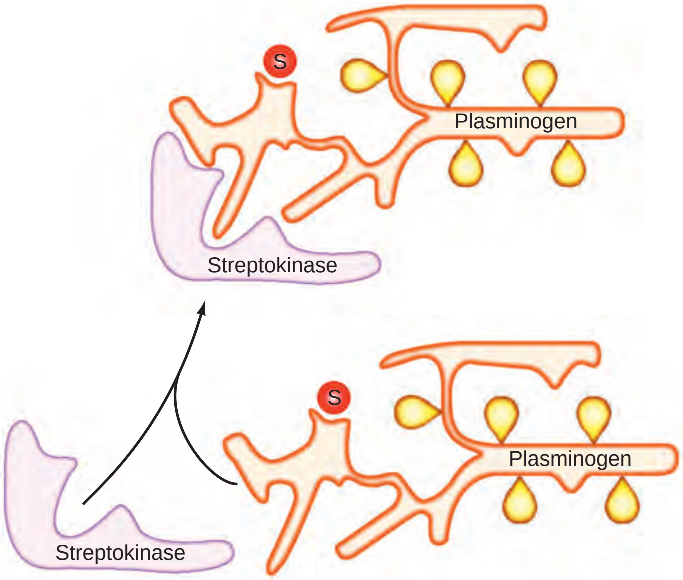


Figure 143.10 MECHANISM OF ACTION OF STREPTOKINASE. Streptokinase binds to plasminogen and induces a conformational change in plasminogen that exposes its active site. The streptokinase–plasmin(ogen) complex then serves as the activator of additional plasminogen molecules.

Formation of this complex induces a conformational change in plasminogen that exposes its active site (Fig. 143.10). This conformationally altered plasminogen then converts additional plasminogen molecules to plasmin.

Streptokinase has no affinity for fibrin, and the streptokinase–plasminogen complex activates both free and fibrin-bound plasminogen. Activation of circulating plasminogen generates sufficient amounts of plasmin to overwhelm α_2-antiplasmin. Unopposed plasmin not only degrades fibrin in the occlusive thrombus but also induces a systemic lytic state.

When given systemically to patients with acute myocardial infarction, streptokinase reduces mortality. For this indication, the drug is usually given as an intravenous infusion of 1.5 million units over 30 to 60 minutes. Patients who receive streptokinase can develop antibodies against the drug, as can patients with prior streptococcal infection. These antibodies can reduce the effectiveness of streptokinase.

Allergic reactions occur in about 5% of patients treated with streptokinase. These may manifest as a rash, fever, chills, and rigors. Although anaphylactic reactions can occur, these are rare. Transient hypotension is common with streptokinase and has been attributed to plasmin-mediated release of bradykinin from kallikrein. The hypotension usually responds to leg elevation and administration of intravenous fluids and low doses of vasopressors, such as dopamine or norepinephrine.

Alteplase (Activase)

A recombinant form of single-chain t-PA, alteplase has a molecular weight of 68,000. Alteplase is rapidly converted into its two-chain form by plasmin. Although single- and two-chain forms of t-PA have equivalent activity in the presence of fibrin, in its absence, single-chain t-PA has 10-fold lower activity.

Alteplase consists of five discrete domains (Fig. 143.11). The N-terminal A-chain of two-chain alteplase contains four of these domains: residues 4 through 50 make up the finger domain, a region that resembles the finger domain of fibronectin; residues 50 through 87 are homologous with epidermal growth factor; and residues 92 through 173 and 180 through 261, which have homology to the kringle domains of plasminogen, are designated as the first and second kringle domains, respectively. The fifth alteplase domain is the protease domain; it is located on the C-terminal B-chain of two-chain alteplase.

The interaction of alteplase with fibrin is mediated by the finger domain and, to a lesser extent, by the second kringle domain. The affinity of alteplase for fibrin is considerably higher than that for fibrinogen. Consequently, the catalytic efficiency of plasminogen activation by alteplase is two to three orders of magnitude higher in the presence of fibrin than in the presence of fibrinogen. This phenomenon helps to localize plasmin generation to the fibrin surface.

Although alteplase preferentially activates plasminogen in the presence of fibrin, alteplase is not as fibrin selective as was first predicted. Its fibrin specificity is limited because, like fibrin, (DD)E, the major soluble degradation product of cross-linked fibrin, binds alteplase and plasminogen with high affinity. Consequently, (DD)E is as potent as fibrin as a stimulator of plasminogen activation by alteplase. Whereas plasmin generated on the fibrin surface results in thrombolysis, plasmin generated on the surface of circulating (DD)E degrades fibrinogen. Fibrinogenolysis results in the accumulation of fragment X, a high-molecular-weight clottable fibrinogen degradation product. Incorporation of fragment X into hemostatic plugs formed at sites of vascular injury renders them susceptible to lysis. This phenomenon may contribute to alteplase-induced bleeding.

A trial comparing alteplase with streptokinase for treatment of patients with acute myocardial infarction demonstrated significantly lower mortality with alteplase than with streptokinase, although the absolute difference was small. The greatest benefit was seen in patients less than 75 years of age with anterior myocardial infarction who presented less than 6 hours after symptom onset.

For treatment of acute myocardial infarction or acute ischemic stroke, alteplase is given as an intravenous infusion over 60 to 90 minutes. The total dose of alteplase usually ranges from 90 to 100 mg. Allergic reactions and hypotension are rare, and alteplase is not immunogenic.

Tenecteplase

A genetically engineered variant of t-PA, tenecteplase was designed to have a longer half-life than t-PA and to be resistant to inactivation by PAI-1. To prolong its half-life, a new glycosylation site was added

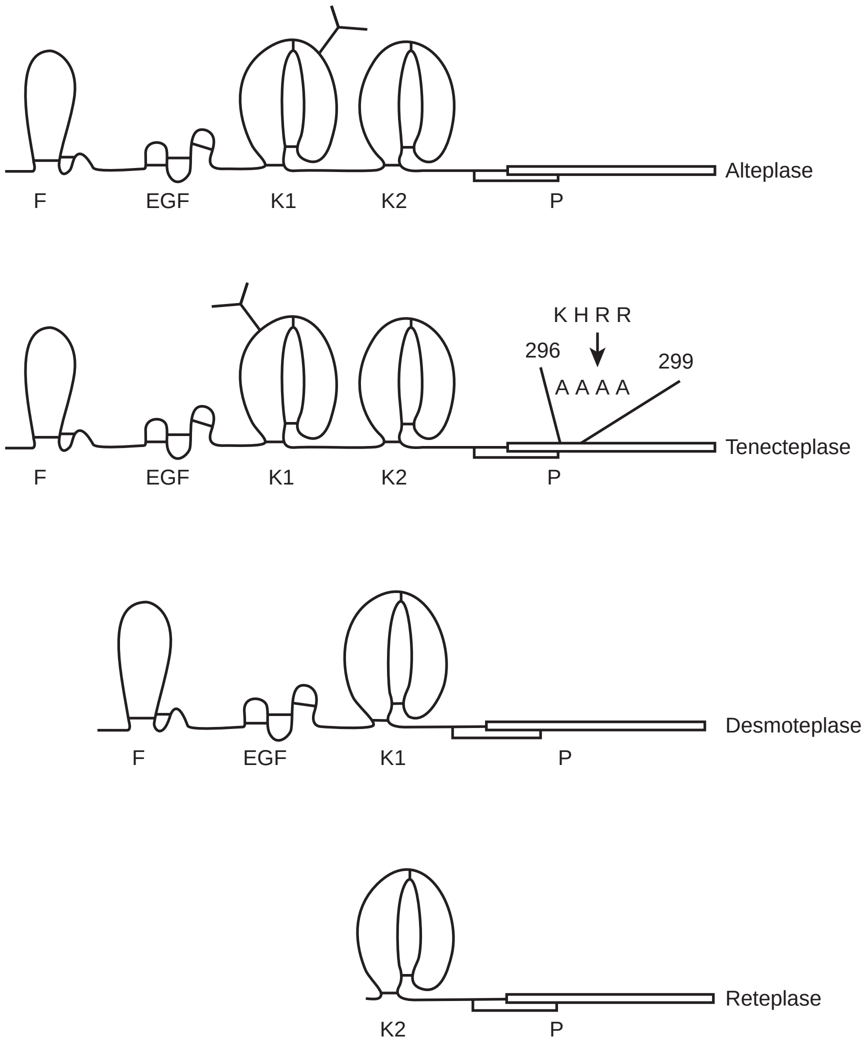


Figure 143.11 DOMAIN STRUCTURES OF ALTEPLASE (TISSUE-TYPE PLASMINOGEN ACTIVATOR [T-PA]), TENECTEPLASE, DESMOTEPLASE, AND RETEPLASE. The finger (F), epidermal growth factor (EGF), first and second kringle (K1 and K2, respectively), and protease (P) domains are illustrated. The glycosylation site (Y) on K1 has been repositioned in tenecteplase to endow it with a longer half-life. In addition, a tetra-alanine substitution in the protease domain renders tenecteplase resistant to inhibition by type 1 plasminogen activator inhibitor. Desmoteplase differs from alteplase and teneteplase in that it lacks a K2 domain. Reteplase is a truncated variant that lacks the F, EGF, and K1 domains.

to the first kringle domain (see Fig. 143.11). Because addition of this extra carbohydrate side chain reduced fibrin affinity, the existing glycosylation site on the first kringle domain was removed. To render the molecule resistant to inhibition by PAI-1, a tetra-alanine substitution was introduced at residues 296 to 299 in the protease domain, the region responsible for the interaction of t-PA with PAI-1.

Tenecteplase is more fibrin specific than t-PA. Although both agents bind to fibrin with similar affinity, the affinity of tenecteplase for (DD)E is significantly lower than that of t-PA. Consequently, (DD)E does not stimulate systemic plasminogen activation by tenecteplase to the same extent as t-PA. As a result, tenecteplase produces less fibrinogenolysis than t-PA.

For coronary fibrinolysis, tenecteplase is given as a single intravenous bolus. In a large phase III trial in which almost 17,000 patients were enrolled, the 30-day mortality rate with single-bolus tenecteplase was similar to that with accelerated dose t-PA.[38] Although rates of intracranial hemorrhage also were similar with both treatments, patients given tenecteplase had fewer noncerebral bleeds and a reduced need for blood transfusions compared with those treated with t-PA. The improved safety profile of tenecteplase likely reflects its enhanced fibrin specificity. These properties have prompted studies comparing tenecteplase with alteplase in patients with acute ischemic stroke.[39]

Reteplase

A recombinant t-PA derivative, reteplase is a single-chain variant that lacks the finger, epidermal growth factor, and first kringle domains (see Fig. 143.11). This truncated derivative has a molecular weight of 39,000. Reteplase binds fibrin more weakly than t-PA because it lacks the finger domain. Because it is produced in *Escherichia coli*, reteplase is not glycosylated. This endows it with a plasma half-life longer than that of t-PA. Consequently, reteplase is given as two intravenous boluses, which are separated by 30 minutes. Clinical trials have demonstrated that reteplase is at least as effective as streptokinase for treatment of acute myocardial infarction, but the agent is not superior to t-PA.[40]

Desmoteplase

A recombinant form of the full-length plasminogen activator isolated from the saliva of the vampire bat, desmoteplase (see Fig. 143.11) is more fibrin specific than t-PA.[41] Although preliminary studies with desmoteplase for treatment of acute ischemic stroke were promising, further development has been halted.

CONCLUSIONS AND FUTURE DIRECTIONS

Arterial and venous thrombosis reflects a complex interplay among the vessel wall, platelets, the coagulation system, and the fibrinolytic pathways. Activation of coagulation also triggers inflammatory pathways that may contribute to thrombogenesis. A better understanding of the biochemistry of blood coagulation and advances in structure-based drug design have identified new targets and resulted in the development of novel antithrombotic drugs. Well-designed clinical trials have provided detailed information on which drugs to use and when to use them. Despite these advances, however, arterial and venous thromboembolic disorders remain a major cause of morbidity and mortality. Therefore the search for better targets and more potent or more convenient antiplatelet, anticoagulant, and fibrinolytic drugs continues.

REFERENCES

1. Mackman N. Triggers, targets and treatments for thrombosis. *Nature*. 2008;451:914–918.
2. Sutcliffe P, Connock M, Gurung T, et al. Aspirin for prophylactic use in the primary prevention of cardiovascular disease and cancer: a systematic review and overview of reviews. *Health Technol Assess*. 2013;17(43):1–253.
3. Fitzgerald R, Pirmohamed M. Aspirin resistance: effect of clinical, biochemical and genetic factors. *Pharmacol Ther*. 2011;130:213–225.
4. Yusuf S, Zhao F, Mehta SR, et al. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. *N Engl J Med*. 2001;345:494–502.
5. Wiviott SD, Braunwald E, McCabe CH, et al. Prasugrel versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med*. 2007;357:2001–2015.
6. Roe MT, Armstrong PW, Fox KAA, et al. Prasugrel versus clopidogrel for acute coronary syndromes without revascularization. *N Engl J Med*. 2012;367:1297–1309.
7. Gurbel PA, Tantry US. Clopidogrel response variability and the advent of personalised antiplatelet therapy. A bench to bedside journey. *Thromb Haemost*. 2011;106:265–271.
8. Wallentin L, Becker RC, Budaj A, et al. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med*. 2009;361:1045–1057.
9. Cannon CP, Harrington RA, James S, et al. Comparison of ticagrelor with clopidogrel in patients with a planned invasive strategy for acute coronary syndromes (PLATO): a randomised double-blind study. *Lancet*. 2010;375:283–293.
10. Mahaffey KW, Wojdyla DM, Carroll K, et al. Ticagrelor compared with clopidogrel by geographic region in the Platelet Inhibition and Patient Outcomes (PLATO) trial. *Circulation*. 2011;124:544–554.
11. Sacco RL, Diener H-C, Yusuf S, et al. Aspirin and extended-release dipyridamole versus clopidogrel for recurrent stroke. *N Engl J Med*. 2008;359:1238–1251.
12. Tricoci P, Huang Z, Held C, et al. Thrombin-receptor antagonist vorapaxar in acute coronary syndromes. *N Engl J Med*. 2012;366:20–33.
13. Morrow DA, Braunwald E, Bonaca MP, et al. Vorapaxar in the secondary prevention of atherothrombotic events. *N Engl J Med*. 2012;366:1404–1413.
14. Eikelboom JW, Weitz JI. New anticoagulants. *Circulation*. 2010;121:1523–1532.
15. Kearon C, Ginsberg JS, Julian JA, et al. Comparison of fixed-dose weight-adjusted unfractionated heparin and low-molecular-weight heparin for acute treatment of venous thromboembolism. *JAMA*. 2006;296(8):935–942.
16. Cuker A, Cines DB. How I treat heparin-induced thrombocytopenia. *Blood*. 2012;119:2209–2218.
17. Yusuf S, Mehta SR, Chrolavicius S, et al. Comparison of fondaparinux and enoxaparin in acute coronary syndromes. *N Engl J Med*. 2006;354:1464–1476.
18. Manolopoulos VG, Ragia G, Tavridou A. Pharmacogenetics of coumarinic oral anticoagulants. *Pharmacogenomics*. 2010;11:493–496.
19. Ageno W, Gallus AS, Wittkowsky A, et al. Oral anticoagulant therapy: antithrombotic therapy and prevention of thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest*. 2012;141(2 Suppl):e44S–e88S.
20. Whitlock RP, Sun JC, Fremes SE, et al. Antithrombotic and thrombolytic therapy for valvular disease: antithrombotic therapy and prevention of thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest*. 2012;141(2 Suppl):e576S–e600S.
21. Ruff CT, Giugliano RP, Braunwald E, et al. Comparison of the efficacy and safety of new oral anticoagulants with warfarin in patients with atrial fibrillation: a meta-analysis of randomised trials. *Lancet*. 2014;383:955–962.
22. van Es N, Coppens M, Schulman S, et al. Direct oral anticoagulants compared with vitamin K antagonists for acute venous thromboembolism: evidence from phase 3 trials. *Blood*. 2014;124:1968–1975.
23. Gosselin RC, Adcock DM, Douxfils J. An update on laboratory assessment for direct oral anticoagulants (DOACs). *Int J Lab Hematol*. 2019;41(S1):33–39.
24. Spyropoulos AC, Al-Badri A, Sherwood MW, et al. Periprocedural management of patients receiving a vitamin K antagonist or a direct oral anticoagulant requiring an elective procedure or surgery. *J Thromb Haemost*. 2016;14(5):875–885.
25. Barnes GD, Mouland E. Peri-procedural management of oral anticoagulants in the DOAC era. *Prog Cardiovasc Dis*. 2018;60(6):600–606.
26. Tomaselli GF, Mahaffey KW, Cuker A, et al. ACC expert consensus decision pathway on management of bleeding in patients on oral anticoagulants: a report of the American College of Cardiology Solution Set Oversight Committee. *J Am Coll Cardiol 76(5):594-622*. 2020(2020).
27. Pollack Jr. CV, Reilly PA, van Ryn J, et al. Idarucizumab for dabigatran reversal - full cohort analysis. *N Engl J Med*. 2017;377:431–441.
28. Greinacher A, Thiele T, Selleng K. Reversal of anticoagulants: an overview of current developments. *Thromb Haemost*. 2015;113:931.
29. Siegal DM, Curnutte JT, Connolly SJ, et al. Andexanet alfa for the reversal of factor Xa inhibitor activity. *N Engl J Med*. 2015;373:2413–2424.
30. Connolly SJ, Crowther M, Eikelboom JW, et al. Full study report of andexanet alfa for bleeding associated with factor Xa inhibitors. *N Engl J Med*. 2019;380:1326–1335.
31. Thomas S, Makris M. The reversal of anticoagulation in clinical practice. *Clin Med (Lond)*. 2018;18(4):314–319.
32. Dhakal P, Rayamajhi S, Verma V, et al. Reversal of anticoagulation and management of bleeding in patients on anticoagulants. *Clin Appl Thromb Hemost*. 2017;23(5):410–415.
33. Ansell JE, Laulicht BE, Bakhru SH, et al. Ciraparantag, an anticoagulant reversal drug: mechanism of action, pharmacokinetics, and reversal of anticoagulants. *Blood.*. 2021;137(1):115–125.
34. Ansell JE, Bakhru SH, Laulicht BE, et al. Ciraparantag reverses the anticoagulant activity of apixaban and rivaroxaban in healthy elderly subjects. *Eur Heart J*. 2022 Mar 7;43(10):985–992.
35. Ansell JE, Bakhru SH, Laulicht BE, et al. Single-dose ciraparantag safely and completely reverses anticoagulant effects of edoxaban. *Thromb Haemost*. 2017;117(2):238–245.
36. Fredenburgh JC, Weitz JI. New anticoagulants: moving beyond the direct oral anticoagulants. *J Thromb Haemost*. 2021;19(1):20–29.
37. Hsu C, Hutt E, Bloomfield DM, et al. Factor XI inhibition to uncouple thrombosis from hemostasis: JACC review topic of the week. *J Am Coll Cardiol*. 2021;78(6):625–631.
38. Van De Werf F, Adgey J, Ardissino D, et al. Single-bolus tenecteplase compared with front-loaded alteplase in acute myocardial infarction: the ASSENT-2 double-blind randomised trial. *Lancet*. 1999;354:716–722.
39. Parsons M, Spratt N, Bivard A, et al. A randomized trial of tenecteplase versus alteplase for acute ischemic stroke. *N Engl J Med*. 2012;366:1099–1107.
40. Topol EJ, Ohman EM, Armstrong PW, et al. Survival outcomes 1 year after reperfusion therapy with either alteplase or reteplase for acute myocardial infarction: results from the Global Utilization of Streptokinase and t-PA for Occluded Coronary Arteries (GUSTO) III trial. *Circulation*. 2000;102:1761–1765.
41. Medcalf RL. Desmoteplase: discovery, insights and opportunities for ischaemic stroke. *Br J Pharmacol*. 2012;165:75–89.

CHAPTER 144

STROKE

Emer McGrath, Michelle Canavan, and Martin O'Donnell

Stroke is the second leading cause of mortality worldwide and a leading cause of acquired adult disability. Primary stroke subtypes include ischemic stroke, intracerebral hemorrhage (ICH), and subarachnoid hemorrhage (SAH). Each stroke subtype has differing etiologies, outcomes, and management strategies. In the past 30 years, there have been major advances in the diagnosis (emergence of widely available neuroimaging) and treatment of acute stroke. In addition, there is increased awareness of the importance of covert stroke (stroke on neuroimaging without a history of acute clinical stroke). This chapter provides an overview of stroke, with a primary focus on ischemic stroke, which is the most common cause of stroke worldwide.

DEFINITION

Stroke is defined by the World Health Organization (WHO) as "rapidly developing clinical signs of focal (or global) disturbance of cerebral function, lasting 24 hours or longer or leading to death with no apparent cause other than that of vascular origin." This definition is conventionally considered to include ischemic stroke, ICH, and SAH. However, due to advances in our knowledge about the nature, timing, and clinical presentation of stroke and its mimics, as well as significant advances in neuroimaging, in 2013 the American Heart Association (AHA)/American Stroke Association (ASA) proposed an updated definition of central nervous system (CNS) infarction (including hemorrhagic infarction) defined as "brain, spinal cord, or retinal cell death attributable to ischemia, based on: (1) pathological, imaging, or other objective evidence of cerebral, spinal cord, or retinal focal ischemic injury in a defined vascular distribution; or (2) clinical evidence of cerebral, spinal cord, or retinal focal ischemic injury based on symptoms persisting ≥24 hours or until death, and other etiologies have been excluded." According to the AHA/ASA, "a transient ischemic attack (TIA) is defined as a transient episode of neurologic dysfunction caused by focal brain, spinal cord, or retinal ischemia, without acute infarction."

EPIDEMIOLOGY

Frequency

The WHO estimates that approximately 14 million people suffer a stroke each year, and of these, more than five million die and another five million people are left permanently disabled. Although age-specific stroke mortality rates worldwide have declined between 1990 and 2016, predominantly due to a decline in high-income countries, the reduction in age-standardized stroke incidence rates has been far less over that time period, indicating that the global burden of stroke remains high. Indeed, the estimated global lifetime risk of stroke among those aged 25 years or older increased from 22.8% in 1990 to 25% in 2016, and the incidence of stroke in some lower-income regions (e.g., east Asia and Southern sub-Saharan Africa) increased between 1990 and 2016. Worldwide, stroke shows significant geographical variation, in terms of incidence (and temporal trends), case fatality, and case mix (i.e., stroke subtypes). Overall, because of the aging population and population growth, the absolute numbers of individuals with prevalent stroke, stroke-related mortality, and global stroke-related disability remain high and continue to increase.

Traditional Risk Factors for Stroke

Both ischemic stroke and ICH are associated with several potentially modifiable risk factors, including hypertension, diabetes mellitus, smoking, poor diet, and physical inactivity. The INTERSTROKE study, which included 6000 participants from 22 countries, reported that 10 key risk factors are associated with 90% of the population-attributable risk (PAR) of ischemic stroke, namely hypertension, smoking, waist-to-hip ratio, diet, physical activity level, diabetes mellitus, alcohol intake, psychosocial stress/depression, and cardiac causes such as atrial fibrillation and ratio of apolipoprotein B to apolipoprotein A1 (Table 144.1). Of these risk factors, five were associated with 80% of the PAR for all stroke (hypertension, smoking, abdominal obesity, physical inactivity, and diet), and each was an important risk factor for both ischemic and hemorrhagic stroke. Thus a large proportion of stroke is potentially preventable through population-based interventions aimed at modifying these risk factors. Hypertension is the strongest risk factor for both ischemic stroke and ICH and, arguably, the most modifiable through lifestyle intervention (e.g., reducing salt intake) and use of antihypertensive medications.

PATHOBIOLOGY

Stroke can be classified into ischemic stroke (Fig. 144.1) and hemorrhagic stroke. Hemorrhagic stroke is further subtyped into ICH (Fig. 144.2) and SAH. In North America and Europe, approximately 87% of strokes are due to ischemia, with the remaining 13% due to hemorrhage.

Etiological Classification of Ischemic Stroke

Unlike acute coronary syndrome, which is primarily due to large vessel atherosclerosis (see Chapter 145), the underlying mechanisms for ischemic stroke are more heterogeneous. The most used etiological classification is the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification system which focuses on the pathophysiological mechanism of ischemic stroke and is based on clinical features and the results of diagnostic investigations (brain imaging, cardiac investigations, and neurovascular imaging). The five etiological subcategories in the TOAST classification system include large-artery stroke, cardioembolism, small-artery occlusion (or lacunar ischemic stroke), stroke of other determined etiology, and stroke of undetermined etiology.

Large Artery Stroke

Large artery stroke is usually a consequence of atherosclerosis in the extracranial (carotid or vertebral) and/or intracranial arteries (e.g., middle cerebral or basilar artery), with plaque rupture and thrombus formation. Ischemic stroke may result from artery-to-artery thromboembolism with distal occlusion or, less commonly, by acute occlusion with resultant hypoperfusion (e.g., watershed infarction). Large vessel atherosclerosis accounts for approximately 20% of all ischemic stroke in high-income countries and is predominantly extracranial in origin. Intracranial atherosclerosis has been reported to account for

TABLE 144.1 Traditional Risk Factors for Ischemic Stroke

Risk Factor	OR (99% CI)
Hypertension (self-reported history)	2.37 (2.00–2.79)
Current smoking[a]	2.32 (1.91–2.81)
Diabetes mellitus	1.60 (1.29–1.99)
Ratio of ApoB to Apo A1	2.40 (1.86–3.11)
Obesity (waist to hip ratio)	1.69 (1.38–2.07)
Regular physical activity	0.68 (0.51–0.91)
Diet risk score	1.34 (1.09–1.65)
Alcohol consumption[a]	
1–30 drinks per month	0.79 (0.63–1.00)
>30 drinks per month	1.41 (1.09–1.82)
Psychosocial factors	
Psychosocial stress	1.30 (1.04–1.62)
Depression	1.47 (1.19–1.83)

[a] Comparator for current smoker and alcohol intake is never or former

Apo, Apolipoprotein; *CI*, confidence interval; *OR*, odds ratio.; *CI*, confidence interval; *Apo*, Apolipoprotein.

Reprinted with permission from Elsevier. O'Donnell MJ, Xavier D, Liu L, et al. Risk factors for ischaemic and intracerebral haemorrhagic stroke in 22 countries (the INTERSTROKE study): a case-control study. *Lancet*. 2010;376:112–123.

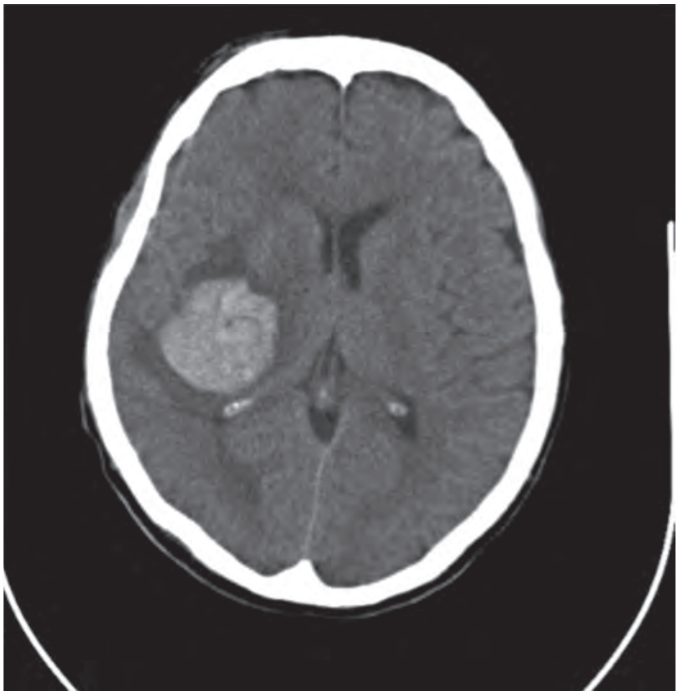


Figure 144.1 COMPUTED TOMOGRAPHY OF BRAIN WITH RIGHT INTRACEREBRAL HEMORRHAGE.

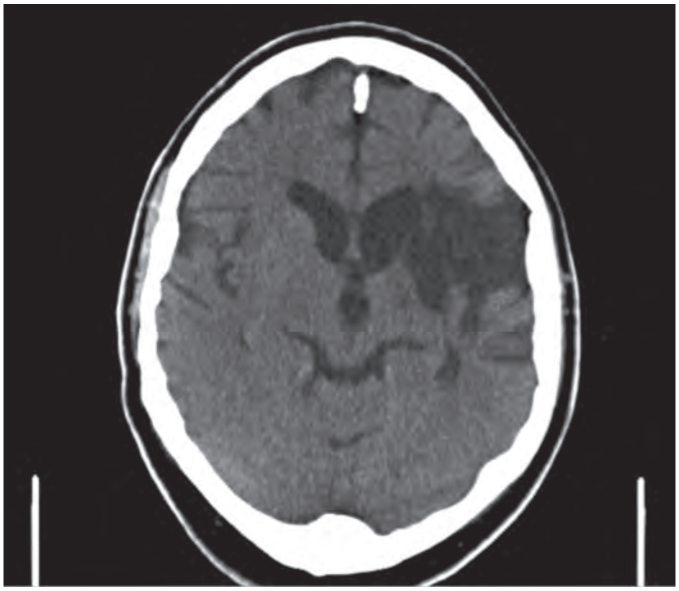


Figure 144.2 COMPUTED TOMOGRAPHY OF BRAIN WITH LEFT HEMISPHERIC ISCHEMIC STROKE.

up to 33% to 50% of ischemic strokes in parts of Asia (e.g., China and Thailand), but it is a much less common cause of acute stroke in North America and Europe. Arterial dissection, the third leading cause of ischemic stroke in young people, can lead to ischemic stroke by either local occlusion or distal thromboembolism. Predisposing factors for dissection include trauma and underlying collagen vascular disorders, such as fibromuscular dysplasia and Ehlers-Danlos syndrome. Less common large vessel mechanisms of ischemic stroke include moyamoya disease, Fabry disease, and large vessel arteritis (e.g., Takayasu arteritis and giant cell arteritis).

Cardioembolism

Approximately 14% to 30% of all ischemic strokes are caused by cardioembolism, with marked regional variation (see Chapter 14). Several conditions predispose to cardioembolism, originating from the venous system (paradoxical embolism), intracardiac (e.g., atrial fibrillation), or postcardiac (aortic arch disease).

Precardiac

Paradoxical emboli occur when emboli that arise in the venous circulation (e.g., from a deep vein thrombus) cross into the arterial circulation through a patent foramen ovale (PFO), atrial septal defect (ASD), or a pulmonary arteriovenous malformation (AVM). A PFO is found in up to 20% of the general population. Several observational studies have reported an association between first ischemic stroke and PFO, particularly in younger patients, and percutaneous PFO closure reduces the risk of recurrent stroke in patients younger than 60 years of age, indicating that PFO is a risk factor for recurrent ischemic stroke. Atrial septal aneurysm, a protrusion of part of the atrial septum through the fossa ovalis into the right or left atria, is associated with an increased risk of ischemic stroke, particularly when it occurs simultaneously with a PFO.

Intracardiac

Left-sided cardiac sources of emboli include left atrial thrombus secondary to atrial fibrillation or flutter (see Chapter 146), left ventricle thrombus after a transmural myocardial infarction (see Chapter 145), akinetic segments of myocardium with a low ejection fraction in the setting of an old myocardial infarction, cardiac tumors such as left atrial myxoma, and abnormalities of the mitral valve (both native and artificial). Atrial fibrillation or flutter is a significant risk factor for stroke, associated with a fivefold increase in risk. In North America and Europe, approximately 25% of all ischemic strokes are attributed to atrial fibrillation, a proportion that increases with older age (see Chapter 147). After acute myocardial infarction, mural thrombi can arise in the presence of left ventricular aneurysms, akinetic segments of left ventricular myocardium or new-onset atrial fibrillation or flutter. Congestive heart failure is an independent risk factor for stroke and is associated with a twofold to threefold increase in the relative risk of ischemic stroke. Valvular heart disease can involve both native (e.g., rheumatic heart disease) and prosthetic heart valves. Of native valvular disease, mitral stenosis has the strongest association with ischemic stroke; mitral annular calcification and mitral valve prolapse have weaker associations. Mitral stenosis is also commonly associated with atrial fibrillation, further increasing the risk of ischemic stroke. Emboli can also arise from valvular vegetations in nonbacterial thrombotic endocarditis or infective endocarditis. Mechanical mitral and aortic valves are associated with a sufficiently high risk for ischemic stroke that indefinite oral anticoagulant therapy is indicated. Rheumatic heart disease still accounts for half of all cases of endocarditis in some regions of the world (e.g., India and Africa). Several epidemiological studies have shown a link between ischemic stroke and *Trypanosoma cruzi* infection (Chagas disease) in South America.

Postcardiac

Atherosclerotic plaques in the aortic arch proximal to the left subclavian artery can be postcardiac sources of emboli, either atheromatous debris or platelet emboli, which then enter the cerebral circulation resulting in ischemic stroke. Severe aortic arch atheromas (>4 mm in diameter) are associated with a fourfold increase in the risk of ischemic stroke and peripheral embolism. Although anatomically a large vessel source, aortic arch disease is usually included in cardiac causes of ischemic stroke because it is typically identified via transesophageal echocardiography.

Small Vessel Disease

Approximately 20% of all ischemic strokes are due to lacunar or small vessel infarcts. Lacunar infarcts are the result of occlusion of small, deep penetrating arteries, such as the lenticulostriate branches of the anterior cerebral and middle cerebral arteries. The terminal pathophysiological mechanism underlying small artery occlusion is believed to be local thrombosis secondary to microatheroma (lipid laden macrophages, cholesterol deposits, and subintimal fibroblast proliferation) and lipohyalinosis (the intermediate stage between fibrinoid necrosis and microatheroma, which has characteristics of both arterial atheromatous lipid deposits and arteriolar hyalinization disease). Growing evidence supports a concept that damage to the glycocalyx by factors such as hyperglycemia, hypertension, and smoking contributes to vascular endothelial damage (see Chapter 142). Other causes of small artery occlusion include microemboli from atherosclerotic plaques, polycythemia vera (see Chapter 70), antiphospholipid antibodies (see Chapter 139), amyloid angiopathy (see Chapter 93), cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL), Sneddon syndrome, and various types of small vessel arteritis. The combination of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) is an inherited progressive disorder characterized by mitochondrial dysfunction and early onset of stroke, typically before the age of 40. The mitochondrial angiopathy hypothesis suggests that the lesions are secondary to ischemia which is caused by mitochondrial and vascular dysfunction of cerebral small arteries.

Ischemic Stroke of Other Determined Etiology

Cerebral Venous Sinus Thrombosis

Cerebral venous sinus thrombosis accounts for less than 1% of ischemic strokes and typically affects younger people. The superior sagittal, transverse, and cavernous sinuses are those most commonly affected by thrombosis. Venous thrombosis results in localized edema and venous infarction, which often becomes hemorrhagic and may raise intracranial pressure (ICP). Reported risk factors for cerebral venous sinus thrombosis include inherited thrombophilia (see Chapter 138); acquired prothrombotic states such as antiphospholipid syndrome (see Chapter 139), pregnancy and the puerperium (see Chapter 141) and hypercoagulability of malignancy; infections such as otitis, sinusitis, and mastoiditis; chronic inflammatory conditions such as granulomatosis with polyangiitis and sarcoidosis; and trauma such as a head injury, dehydration, and injury to the jugular veins or sinuses during neurosurgical procedures.

Intracerebral Hemorrhage

ICH accounts for approximately 10% to 15% of all strokes (a larger proportion is reported in middle- and low-income countries) and can be classified as either primary or secondary, depending on the underlying cause. Primary ICH accounts for approximately 80% to 90% of cases and is the result of spontaneous rupture of small intracerebral blood vessels, usually damaged by chronic hypertension (and other vascular risk factors) or amyloid angiopathy. Antithrombotic therapy, particularly anticoagulant therapy, is an important risk factor for ICH (see Chapter 143). Secondary ICH occurs as a result of vascular abnormalities (ruptured saccular aneurysm, AVM), tumors (e.g., cavernous angioma, intracerebral neoplasm), impaired coagulation (e.g., due to use of oral anticoagulants or bleeding disorders such as hemophilia [see Chapter 134] or von Willebrand disease [see Chapter 133]), hemorrhagic transformation of ischemic stroke, septic emboli, vasculitis, moyamoya disease, and alcohol or illicit drug use (e.g., cocaine, amphetamines).

Subarachnoid Hemorrhage

SAH refers to bleeding within the subarachnoid space, which is the space between the arachnoid and pia mater. It accounts for approximately 3% to 5% of all strokes and is mostly caused by rupture of an intracranial aneurysm (approximately 80% to 85% of cases). Idiopathic nonaneurysmal perimesencephalic hemorrhage accounts for approximately 10% of cases, while the remaining 5% are due to rare causes such as inflammatory lesions of cerebral arteries (e.g., mycotic aneurysm, polyarteritis nodosa, primary angiitis), noninflammatory lesions of intracerebral vessels (e.g., arterial dissection, cerebral AVMs, cerebral amyloid angiopathy, cerebral venous thrombosis, moyamoya disease), vascular lesions of the spinal cord (saccular aneurysm of the spinal artery, spinal AVM), coagulopathy (e.g., hemophilia, von Willebrand disease), sickle cell disease (see Chapters 42 and 43), tumors (e.g., malignant glioma), trauma, and drug use (e.g., cocaine, anticoagulants and sympathomimetic agents).

Covert Stroke

Clinically overt stroke is considered to represent only a fraction of all episodes of cerebral infarction. The advent of contemporary magnetic resonance imaging (MRI) sequences has identified a large burden of subclinical cerebrovascular disease, which includes covert infarction, white matter hyperintensities, enlarged perivascular spaces, cerebral atrophy, and microbleeds. Covert stroke is common; for example, a systematic review of eight population-based studies reported a prevalence of silent brain infarcts in an older population of 8% to 28%. Moreover, covert stroke has been associated with an increased risk of cognitive decline, dementia, depression, and gait impairment. (See box on What Is Cryptogenic Ischemic Stroke?)

Hematological Disorders and Ischemic Stroke

Inherited Thrombophilia

In general, studies have reported either no association or a modest association between inherited thrombophilia and ischemic stroke (see

WHAT IS CRYPTOGENIC ISCHEMIC STROKE?

In some cases, the cause of stroke cannot be definitively determined and the stroke is classified as "stroke of undetermined etiology." A stroke may be classified in this category when one of the following two conditions is met: (1) an extensive evaluation is negative, which includes large vessel imaging and complete cardiovascular assessment; or (2) the diagnostic evaluation is incomplete. The most important determinant of the proportion of patients labeled as having cryptogenic stroke is the extent of the etiological diagnostic testing, including transesophageal echocardiography. In studies where an extensive etiological work-up was undertaken, the proportion of patients designated as having cryptogenic stroke is small (5%–15%). In older patients with complete evaluation, paroxysmal atrial fibrillation is suspected to be the most common underlying cause of ischemic stroke in older adults with "cryptogenic" ischemic stroke.

Chapter 138). Meta-analyses of observational studies have failed to demonstrate a significant association between the factor V Leiden mutation and ischemic stroke in adults, although some small case-control studies have reported an increased risk of ischemic stroke in those with the factor V Leiden mutation using oral contraceptives. Two large prospective population studies, the Physicians' Health Study and the Cardiovascular Health Study, failed to demonstrate a significant association between the prothrombin gene mutation G20210A and ischemic stroke risk, although a large meta-analysis of 19 case control studies reported a modest association. There is no prospective evidence to definitively support an association between protein C, protein S, or antithrombin deficiency and ischemic stroke risk in adults.

Antiphospholipid Syndrome

Antiphospholipid syndrome is reviewed in Chapter 139. Stroke is the main acute arterial thrombotic complication of antiphospholipid syndrome and is reported to be the initial clinical manifestation in 13% of patients subsequently diagnosed with antiphospholipid syndrome. However, the overall association between antiphospholipid antibodies (without other features of the syndrome) and ischemic stroke is less certain. Anticardiolipin antibodies have been associated with ischemic stroke in some studies, especially in young women, but not in others. The lupus anticoagulant is a more potent risk factor for ischemic stroke. For example, in the Risk of Arterial Thrombosis in Relation to Oral Contraceptives (RATIO) study, the odds ratio (OR) for an association between lupus anticoagulant and ischemic stroke was 43.1 (95% confidence interval [CI], 12.2 to 152.2) compared with an OR of 2.3 (95% CI, 1.4 to 3.7) for anti–β_2-glycoprotein I antibodies and no significant association for anticardiolipin antibodies. Current AHA guidelines recommend antiplatelet therapy for secondary prevention of ischemic stroke in patients with cryptogenic ischemic stroke who do not meet the criteria for antiphospholipid syndrome but who have positive antiphospholipid antibodies. For patients with cryptogenic ischemic stroke who meet the diagnostic criteria for antiphospholipid syndrome and who have persistently moderate to high antiphospholipid antibody titers for longer than 12 weeks, oral anticoagulant therapy for secondary prevention of ischemic stroke can be considered (see Chapter 139). The most recent American College of Chest Physicians (ACCP) guidelines recommend oral anticoagulant therapy with a vitamin K antagonist dose-adjusted to achieve a target international normalized ratio (INR) of 2 to 3 for the latter group. Oral factor Xa inhibitors such as rivaroxaban or apixaban are not currently recommended as alternatives to warfarin (see Chapter 143) in this patient population because a recent randomized trial (TRAPS) was stopped early because of an increased risk of recurrent thrombotic events, including stroke, in patients treated with rivaroxaban compared with warfarin.

Sickle Cell Disease

Sickle cell disease is associated with an increased risk of stroke, particularly in those homozygous for the sickle cell gene (see Chapters 42 and 43). The risk of stroke is 11% at 20 years of age, 15% at 30 years, and 24% at 45 years. The most common mechanism underlying ischemic stroke in sickle cell disease is believed to be large artery arteriopathy secondary to hyperplasia of the intima from repeated endothelial injury.

Myeloproliferative Disorders

Myeloproliferative disorders, such as polycythemia vera and essential thrombocytopenia (see Chapters 70 and 71), are uncommon conditions associated with an increased risk of stroke. In a randomized controlled trial of daily aspirin in 518 patients with polycythemia vera, the rate of combined ischemic and hemorrhagic stroke over 3 years was 1.2% in the aspirin group compared with 3.8% in the control group. Defective platelet function in these disorders also results in an increased risk of ICH (see Chapter 128).

Thrombotic Thrombocytopenic Purpura

Thrombotic thrombocytopenic purpura is an uncommon systemic disorder associated with an increased risk of ischemic stroke (see Chapter 132). It is characterized by a pentad of thrombocytopenia, microangiopathic hemolytic anemia, fever, renal failure, and neurological abnormalities, including ischemic stroke.

Paraproteinemia

Paraproteinemia, including Waldenström macroglobulinemia (Chapter 92), multiple myeloma (see Chapter 91), and POEMS syndrome (characterized by a plasma cell proliferative disorder, typically myeloma, along with polyneuropathy, organomegaly, endocrinopathy and skin changes) are also associated with an increased risk of ischemic stroke and hemorrhagic stroke (due to thrombocytopenia).

Genetic Risk Factors

A substantial body of evidence from twin, family, and animal studies supports a genetic contribution to stroke risk. Based on genome-wide association studies (GWAS), many alleles with small effect sizes together contribute to an increased stroke risk. A meta-analysis of GWAS from more than 12,000 patients with ischemic stroke identified three loci with genome-wide significance for ischemic stroke: PITX2 and ZFHX3, previously associated with an increased risk of atrial fibrillation, were associated with an increased risk of ischemic stroke; and HDAC9 was associated with an increased risk of large artery stroke. Another meta-analysis reported that APOEε2 carrier status was associated with an increased burden of white matter hyperintensities (a marker for prior cerebrovascular disease) and risk of ischemic stroke. A third more recent meta-analysis of 12 GWAS reported four significant loci, including the previously reported PITX2 and ZFHX3 loci for cardioembolic stroke and HDAC9 for large artery stroke, along with ABO for all ischemic strokes.

The concordance rate for stroke is 65% higher in monozygotic twins than in dizygotic twins, with a range of 12.8% to 19.0% in monozygotic twins and 3.6% to 13.0% in dizygotic twins. A family history of stroke increases the risk of ischemic stroke by approximately 75% and the presence of a first-degree relative with a history of hemorrhagic stroke is associated with a sixfold increase in risk of hemorrhagic stroke. Several monogenic disorders have been associated with an increased risk of ischemic stroke, including Marfan syndrome and Ehlers-Danlos syndrome (predisposing to extracranial artery dissection), Fabry disease, familial moyamoya disease, homocystinuria, Menkes disease, sickle cell disease, hereditary endotheliopathy with retinopathy, nephropathy, and stroke (HERNS), and MELAS. Rare mendelian forms of stroke have been described such as CADASIL and CARASIL, which manifest as small vessel ischemic stroke. However, most monogenic disorders are infrequent and thus have limited impact on the wider population despite their marked effect on individual risk in young populations.

CLINICAL MANIFESTATIONS

Clinical Presentation

Stroke is a clinical diagnosis, supported by the results of neuroimaging (computed tomography [CT] or MRI of brain). CT of the brain may be normal in patients with acute ischemic stroke and cases of MRI-negative stroke also occur. With advances in neuroimaging, an increasing proportion of patients presenting with transient neurological deficits (previously diagnosed as TIA) have small infarcts identifiable on high-quality MRI. Clinically, stroke is characterized by the sudden onset of a focal neurological deficit lasting more than 24 hours or leading to death due to a presumed vascular cause. Common presenting features are lateralizing weakness of the upper extremities and/or lower extremities, facial weakness, speech abnormalities (aphasia, dysarthria), visual loss (monocular visual loss or homonymous hemianopia), reduced level of consciousness, ataxia, diplopia, vertigo, and headache (ICH). Several clinical conditions can mimic stroke. Among patients admitted to a hospital with a suspected stroke, only approximately two-thirds are subsequently diagnosed with stroke. Common stroke mimics include migraine, seizure, syncope, hypoglycemia, primary or secondary brain tumors, transient global amnesia, and toxic-metabolic disturbances with delirium.

Ischemic Stroke Versus Intracerebral Hemorrhage

Ischemic stroke and ICH can have similar presentations. Some clinical features are more typically associated with ICH (e.g., coma, neck stiffness, seizures, vomiting, and headache) than ischemic stroke; however, neuroimaging is required to discriminate between ischemia and hemorrhage. Therefore emergent CT or MRI of brain is mandatory in all patients presenting with suspected acute stroke.

Measuring Stroke Severity

Many scales are available for measuring stroke severity, including the Scandinavian Stroke Scale, the Canadian Stroke Scale, and the National Institutes of Health Stroke Scale (NIHSS). The NIHSS is the scale most widely used in clinical practice. Other scales (e.g., Hunt and Hess, and Fisher) are used to measure stroke severity in patients with SAH.

Risk of Stroke After Transient Ischemic Attack

The ABCD2 score is a validated clinical score for predicting the risk of stroke in patients presenting with TIA and was designed for use in primary care settings. Points are assigned for each of the following risk factors: age older than 60 years (1 point); blood pressure 140/90 mmHg or greater on first evaluation (1 point); clinical symptoms of focal weakness (2 points) or speech impairment without weakness (1 point); duration of 10 to 59 minutes (1 point) or 60 minutes or more (2 points), and diabetes mellitus (1 point). In combined validation cohorts, the 2-day risk of stroke was 0% for scores of 0 or 1, 1.3% for scores of 2 or 3, 4.1% for scores of 4 or 5, and 8.1% for scores of 6 or 7.

INVESTIGATIONS

Computed Tomography of the Brain

Neuroimaging is required to distinguish between ischemic and hemorrhagic stroke. On CT of the brain, early features of ischemia can include loss of distinction between the gray and white matter border, sulcal effacement, loss of definition of the insula, evolving areas of hypodensity, and hyperdense vessel sign due to thromboembolism in a vessel (e.g., middle cerebral artery, basilar artery). Scoring systems (e.g., Alberta Stroke Program Early CT Score [ASPECTS] score) may be used to quantify the burden of ischemia, which predicts the risk of ICH in patients receiving thrombolysis. SAH is not reliably excluded by CT alone. If the index of suspicion for SAH is high, a lumbar puncture for cerebrospinal fluid (CSF) evaluation is required to reliably exclude this diagnosis.

Magnetic Resonance Imaging

MRI is superior to CT for detecting acute ischemia; however, patient contraindications or lack of availability outside specialist stroke centers may limit its use in the acute setting. Diffusion-weighted imaging (DWI) may identify acute ischemia within 1 hour of symptom onset. For diagnosis of ICH, MRI with gradient related echo or susceptibility-weighted imaging has been reported to yield results that exhibit 96% concordance with those of CT of the brain.

Neurovascular Imaging

Extracranial and intracranial stenosis may be detected using ultrasound, CT angiography, magnetic resonance angiography, or conventional angiography. Ultrasound is a noninvasive, relatively inexpensive, and widely available imaging modality but is dependent on ultrasonographer experience and skill. Transcranial doppler may be used to detect intracranial large vessel stenosis and arterial vasospasm after aneurysmal SAH; it can also be used to risk-stratify patients with carotid stenosis based on the presence of high-intensity transient signals (HITSs) due to microemboli.

Cardiac Work-Up

An electrocardiogram can identify atrial fibrillation/flutter or evidence of previous myocardial infarction, whereas in-hospital telemetry monitoring can identify a larger proportion of patients with paroxysmal atrial fibrillation (see Chapter 147). Based on the recent CRYSTAL AF and EMBRACE trials, all patients with cryptogenic ischemic stroke or TIA should have ambulatory cardiac monitoring for at least 30 days after ischemic stroke to improve rates of detection of paroxysmal atrial fibrillation. Longer durations of cardiac monitoring result in higher diagnostic yield, but the optimal duration is unclear. Transthoracic echocardiography (e.g., to detect left ventricular thrombus, left atrial dilatation as a marker of occult atrial fibrillation, or PFO), has a low yield in patients with no prior cardiac history and a normal cardiac examination. The routine use of transesophageal echocardiography is controversial; however, this is an essential investigation in patients with suspected endocarditis. A PFO can be identified with a bubble study when performing transthoracic or transesophageal echocardiography. If a PFO is detected and additional work-up is negative (i.e., cryptogenic stroke), the possibility of deep vein thrombosis in the lower extremities or pelvis should be explored by compression ultrasonography and magnetic resonance venography, respectively. Although transesophageal echocardiography identifies potential etiologies of ischemic stroke in a relatively large proportion of patients, such as PFO, atrial septal aneurysm, or aortic arch disease, the treatment implications of some of these findings are less certain. All patients presenting with stroke should have vascular risk factor profiling, including measurement of blood pressure, fasting lipids, blood glucose, and hemoglobin A1c (HbA1c). All patients should have a screening complete blood count, and troponin level and the coagulation profile should be checked. Other useful tests, depending on clinical suspicion, include erythrocyte sedimentation rate, antiphospholipid antibody panel, blood cultures, antineutrophilic cytoplasmic antibodies, antinuclear antibodies, homocysteine, lipoprotein a, lumbar puncture with CSF analysis for infectious or inflammatory vasculopathies, and CT of the chest, abdomen, and pelvis in cases of suspected hypercoagulability of malignancy.

THERAPY

A complete review of the management of stroke is beyond the scope of this chapter. Instead, we provide an overview of the acute and chronic management of stroke.

Reperfusion Therapy for Acute Ischemic Stroke

Thrombolysis for Acute Ischemic Stroke

Intravenous tissue plasminogen activator (tPa) is the most rigorously evaluated thrombolytic intervention in acute ischemic stroke. Current guidelines recommend administration of tPa in patients with acute ischemic stroke presenting within 3 hours of symptom onset (Table 144.2). Based on the results of the ECASS III trial, current guidelines recommend administration of tPa to eligible patients up to 4.5 hours after symptom onset, provided none of the following additional exclusion criteria are met: age older than 80 years, severe stroke (NIHSS >25), history of both prior stroke and diabetes mellitus, use of oral anticoagulant therapy at time of acute stroke regardless of INR, or imaging evidence of ischemic injury involving more than one-third of the middle cerebral artery territory. The main complication of tPa is ICH. In the Third International Stroke Trial (IST-3), which enrolled patients up to 6 hours after symptom onset, there was an increased risk of spontaneous ICH and death at 7 days in the intervention group compared with the control group, although total mortality was similar between the two groups at 6 months. Based on the results of the WAKE-UP trial, AHA guidelines now recommend use of MRI in individuals with stroke symptoms on awakening or with an unclear time of onset greater than 4.5 hours from last known well time to identify individuals with diffusion-positive, fluid attenuated inversion recovery (FLAIR)-negative lesions who can benefit from intravenous thrombolysis within 4.5 hours of recognition of stroke symptoms.

Mechanical Thrombectomy

Several randomized trials have demonstrated benefit of early intra-arterial (IA) mechanical thrombectomy in addition to intravenous thrombolysis compared with intravenous thrombolysis alone in patients with acute ischemic stroke due to large proximal artery occlusion. Current guidelines recommend IA mechanical thrombectomy for patients meeting the following criteria: (1) small infarct size (e.g., an ASPECTS score ≥6) with no evidence of hemorrhage on neuroimaging, (2) evidence of a causative proximal large artery occlusion in the anterior circulation on vessel imaging (via CT or magnetic resonance angiography), (3) persistent and potentially disabling neurologic deficit with NIHSS of 6 or greater, (4) symptom onset within the past 6 hours, (5) prestroke modified Rankin score of 0 to 1 (indicating minimal to no disability), and (6) rapid access to a stroke center with the necessary expertise in use of stent retriever devices. Mechanical thrombectomy can also be considered in carefully selected patients with evidence of diffusion perfusion mismatch on neuroimaging or mismatch between clinical deficits and imaging features up to 24 hours from the time of onset of stroke symptoms, including individuals with posterior circulation strokes. Compared with intravenous thrombolysis alone, IA treatment resulted in significantly higher rates of functional independence at 90 days, with no significant difference in the risk of symptomatic ICH or death between treatment groups. The number needed to treat for one additional person to reach functional independence ranged from 3 to 7.5. Most patients who are eligible for mechanical thrombectomy should be treated with intravenous thrombolysis prior to thrombectomy. However, patients who are not eligible for intravenous thrombolysis (e.g., patients with infective endocarditis) should still be considered for mechanical thrombectomy if they meet the aforementioned eligibility criteria.

TABLE 144.2 Eligibility Criteria for Thrombolysis in Acute Ischemic Stroke

Eligibility Criteria
Diagnosis of ischemic stroke causing measurable neurological deficit
The neurological signs should not be minor and isolated. Caution should be exercised in treating a patient with major deficits.
Onset of symptoms <3 h before beginning treatment
The neurological signs should not be clearing spontaneously
The symptoms of stroke should not be suggestive of subarachnoid hemorrhage
The patient or family members should understand the potential risks and benefits from treatment.
Contraindications to Thrombolysis
Evidence of intracranial hemorrhage on CT
Head trauma or prior stroke in previous 3 months
Myocardial infarction in the previous 3 months
Gastrointestinal or urinary tract hemorrhage in previous 21 days
Arterial puncture at a noncompressible site in the previous 7 days
Major surgery in the previous 14 days
History of previous intracranial hemorrhage
Elevated blood pressure (systolic >185 mmHg and diastolic >110 mmHg)
Evidence of active bleeding or acute trauma (fracture) on examination
Taking an oral anticoagulant with INR ≥1.7
If receiving heparin in previous 48 h, aPTT must be in normal range:
Platelet count ≥100,000 mm^3
Blood glucose concentration ≥50 mg/dL (2.7 mmol/L)
Seizure with postictal residual neurological impairments
CT shows a multilobar infarction (hypodensity >{1/3} cerebral hemisphere).

aPTT, activated partial thromboplastin time; *CT*, Computed tomography; *INR*, international normalized ratio.

Intra-Arterial Fibrinolysis

Rescue IA fibrinolysis is an option for recanalization in patients with large-artery occlusion who have not responded to mechanical thrombectomy or intravenous tPa, although use of IA t-Pa is currently not FDA approved.

Acute Stroke Unit

Organized stroke unit (OSU) care in a dedicated, geographically identified ward with multidisciplinary teams that exclusively manage patients with stroke is associated with improved outcomes in patients with acute stroke. A meta-analysis of 26 trials ($n = 5592$) investigating OSU care reported a reduction in death (OR 0.86; 95% CI 0.76 to 0.98) and death or dependency at 1 year (OR 0.82; 95% CI

0.73 to 0.92), independent of age, sex, or stroke severity. Organized stroke care is associated with improved outcome for several reasons, including a reduced risk of medical complications such as deep vein thrombosis (due to earlier mobilization) and a reduced risk of aspiration pneumonia, fever, urinary tract infection, falls, and delirium. Prompt evaluation of swallowing function and compliance with speech pathology safe swallowing guidelines help to reduce the risk of aspiration pneumonia in patients with stroke.

Blood Pressure in Acute Stroke

Although hypertension is the most important risk factor for both ischemic and hemorrhagic stroke, the optimal approach to managing blood pressure in the acute setting remains uncertain. Several randomized controlled trials have evaluated blood pressure lowering in acute ischemic stroke, and meta-analyses of these trials indicate no benefit of lowering blood pressure in the acute phase of ischemic stroke. Current AHA guidelines recommend cautious introduction of antihypertensive therapy in those with an initial blood pressure greater than 220/120 mmHg, unless there is an alternative indication for blood pressure lowering (e.g., hypertensive encephalopathy, aortic dissection) or the patients have received thrombolytic therapy, when a blood pressure target of less than 180/105 mmHg is recommended for the initial 24 hours post treatment. The INTERACT II trial enrolled patients with spontaneous ICH within 6 hours of symptom onset and systolic blood pressure between 150 and 220 mmHg. There was no difference in the risk of death or major disability between patients randomized to intensive treatment (target systolic blood pressure <140 mmHg) compared with less than 180 mmHg, although it was safe and an ordinal analysis of functional outcomes (modified Rankin score) suggested improved functional outcomes with a blood pressure target of less than 140 mmHg. In the ATACH phase III trial of patients with ICH within 4.5 hours of symptom onset, an acute blood pressure lowering target of 110 to 139 mmHg was not superior to a target of 140 to 179 mmHg for reduction in death and disability.

Antithrombotic Therapy in Acute Ischemic Stroke

Based on the results of two large randomized controlled trials, the IST and Chinese Acute Stroke Trial (CAST), early antiplatelet therapy with aspirin reduces the risk of recurrent stroke and death in patients with acute ischemic stroke. In general, therapeutic parenteral anticoagulant therapy is not indicated in acute ischemic stroke, except for cerebral venous sinus thrombosis. Therapeutic heparin is commonly used in patients with acute ischemic stroke and extracranial carotid or vertebral artery dissection, or symptomatic extracranial vertebral or carotid artery atherosclerotic stenosis with crescendo TIAs from artery-to-artery embolism while awaiting carotid endarterectomy (CEA). Other potential situations when anticoagulation may be considered in acute ischemic stroke include acute basilar artery thrombosis, acute left ventricular thrombus, or small volume cardioembolic strokes in patients with atrial fibrillation who were not on therapeutic anticoagulation at the time of stroke, although the evidence guiding clinical decision making on antithrombotic therapy in these patients is limited. The CADISS trial, which included 250 patients with symptomatic carotid and vertebral artery dissection, reported no difference in the risk of recurrent stroke or death between patients randomized to antiplatelet and anticoagulant therapy. However, significant limitations of this study include a small sample size, failure to confirm a diagnosis of dissection in 20% of participants, and low rates of recurrent stroke. Consequently, the generalizability of the findings is limited.

Carotid Endarterectomy and Stenting

The European Carotid Surgery Trial (ECST) and the North American Symptomatic Carotid Endarterectomy Trial (NASCET) are the largest trials to evaluate CEA in patients with recent ischemic stroke and TIA. Based on the results of these trials, current guidelines recommend CEA in patients with symptomatic 70% to 99% stenosis of the ipsilateral carotid artery and estimated life expectancy of 5 years or longer, provided that the perioperative morbidity and mortality are estimated to be less than 6%. Although patients with 50% to 69% stenosis also derive benefit from CEA, it is only recommended for selected patients based on age, comorbidities, time from stroke/TIA, and operative risk. Time from symptom onset to CEA is the primary determinant of the absolute risk reduction obtained with this procedure, with maximal benefit realized when CEA is performed within the first 2 weeks after symptom onset. Although CEA is the gold standard for patients with symptomatic significant carotid stenosis, carotid angioplasty and stenting is an alternative option for patients with greater than 70% stenosis considered to be a high operative risk for CEA.

Intracranial Stenting

Two trials to date have failed to show a benefit of intracranial stenting in patients with symptomatic significant (≥70%) intracranial stenosis. The SAMMPRIS trial reported no benefit of endovascular stenting (self-expanding stent) in addition to aggressive medical therapy compared with aggressive medical therapy alone. The trial was stopped early due to a higher rate of stroke and death at 30 days in the endovascular group. The VISSIT trial, which compared a balloon-expandable stent in addition to medical therapy with medical therapy alone, reported similar results, with higher rates of stroke or death at 30 days and at 1 year in the stented group.

Prevention of Venous Thromboembolism

See Chapters 140 and 141.

Acute Management of Intracerebral Hemorrhage

Management of ICH includes both medical and surgical components. Initial strategies include monitoring appropriate patients in an intensive care, intubation of those with reduced consciousness, discontinuation of antithrombotic therapy, immediate reversal of anticoagulant therapy (see Chapter 143), and administration of antipyretics for fever, insulin for hyperglycemia, and venous thromboembolism prophylaxis (e.g., intermittent pneumatic compression). Raised ICP can occur because of the hemorrhage itself or due to secondary edema. Management of increased ICP includes elevation of the head of the bed to 30 degrees, analgesia, and sedation. More aggressive therapies include osmotic diuresis with mannitol or hypertonic saline, drainage of CSF using a ventricular catheter (ventriculostomy), hyperventilation, neuromuscular blockade, and induction of pharmacologic coma with barbiturates or propofol. Antiepileptic therapy should be used for seizure control, but prophylaxis is not recommended in current guidelines. The STICH I and STITCH II surgical trials showed no benefit of early surgery compared with medical management within 48 to 96 hours of onset of spontaneous ICH. However, uncertainty remains in some patient groups, and current AHA guidelines suggest craniotomy for those with supratentorial hematoma with neurologic deterioration or coma, large hematoma with significant midline shift, or elevated ICP that is refractory to medical management. Surgical evacuation is recommended for patients with cerebellar hemorrhage greater than 3 cm in diameter (approximately 14 cm^3 in volume) or for those with evidence of neurological deterioration, brainstem compression, or hydrocephalus. Ventriculostomy with external drainage may be required for patients with intraventricular extension of ICH and a deteriorating level of consciousness. Based on the results of the large multicenter FAST trial, current guidelines suggest against the use of recombinant factor VIIa for acute ICH that is not associated with warfarin use outside of clinical trials. In the recent TICH-2 trial, use of tranexamic acid compared with placebo did not result in improved 90-day functional status or reduced mortality.

Stroke Rehabilitation

Intensive rehabilitation should commence as early as possible after acute stroke. Animal models of neuroplasticity suggest that training results in upregulation of growth-promoting factors primarily in the first 4 weeks after acute stroke, but recovery can continue for months or years after the acute event. High levels of patient (and family) motivation and engagement are associated with better outcomes from stroke rehabilitation. Substantial evidence supports a well-coordinated multidisciplinary team care (nursing, physical therapy, occupational therapy, speech therapy, dietician, and social work) approach for optimal delivery of stroke rehabilitation. This care can be provided in the stroke unit setting or by early supported discharge teams in the patient's own home. Both strategies have been shown to improve independence in stroke patients. For older patients with stroke, physical rehabilitation in a long-term care facility has been shown to improve independence.

Secondary Prevention of Ischemic Stroke

CASE STUDY

A 72-year-old man presented with right-sided weakness and expressive aphasia 4 weeks ago. CT scan of brain revealed an ischemic stroke in the left hemisphere. He was admitted to a stroke unit and was discharged home after completing a rehabilitation program. Investigations included ultrasound of the carotids (<20% stenosis of carotids), Holter monitoring (no arrhythmia), and transthoracic echocardiography with bubble study (small PFO). He is known to have hypertension; his serum glucose and HbA1c were normal. He was initiated on aspirin at the time of diagnosis. His blood pressure in clinic today is 152/92 mmHg, and he is currently receiving perindopril (8 mg daily). His fasting low-density lipoprotein (LDL) level was 140 mg/dL.

Antithrombotic Therapy

Current guidelines recommend aspirin, clopidogrel, or combination aspirin-dipyridamole for secondary prevention of noncardioembolic ischemic stroke. The MATCH trial, which compared clopidogrel (75 mg/day) plus aspirin (75 mg/day) with aspirin alone in patients with recent ischemic stroke or TIA, found no reduction in the risk of major vascular events over 18 months of follow-up but an increased risk of life-threatening and major bleeding in patients treated with combination therapy. Similar results were noted in the SPS3 trial, which included patients with recent symptomatic lacunar infarction. The subsequently published CHANCE trial reported a lower risk of recurrent stroke at 90 days in patients with TIA or minor ischemic stroke (NIHSS≤3) treated with the combination of clopidogrel (loading dose of 300 mg followed by 75 mg per day for 90 days) plus aspirin (75 mg/day for first 21 days) compared with aspirin alone. The international POINT trial also reported similar findings, with a reduced risk of major ischemic vascular events (ischemic stroke, myocardial infarction, and ischemic vascular death) with the combination of clopidogrel (initial dose 600 mg then 75 mg/day for 90 days) plus aspirin (50 to 325 mg/day) versus aspirin alone. In patients with atrial fibrillation or mechanical heart valves, oral anticoagulation is generally recommended for secondary prevention of ischemic stroke (see box on When Should Oral Anticoagulant Therapy Be Started After Acute Ischemic Stroke in Patients With Atrial Fibrillation?). The direct thrombin inhibitor dabigatran and the oral factor Xa inhibitor apixaban have been shown to be superior to warfarin, while the oral factor Xa inhibitors rivaroxaban and edoxaban have been shown to be noninferior to warfarin for the prevention of stroke and systemic embolism in patients with atrial fibrillation (see Chapter 143). These agents are commonly used in clinical practice and offer several advantages over warfarin, including a more rapid onset of action, more predictable anticoagulant effect enabling fixed daily dosing without the need for routine coagulation monitoring, and a lower risk of food and drug interactions. In addition, the risk of ICH is lower (albeit a small absolute reduction in risk) with the newer anticoagulants compared with warfarin. Oral anticoagulants are not superior to aspirin for the secondary prevention of stroke in patients with noncardioembolic (WARSS and ESPRIT), intracranial stenosis (WASID), or embolic stroke of undetermined source (NAVIGATE ESUS and RE-SPECT ESUS).

WHEN SHOULD ORAL ANTICOAGULANT THERAPY BE STARTED AFTER ACUTE ISCHEMIC STROKE IN PATIENTS WITH ATRIAL FIBRILLATION?

We initiate all patients on aspirin in the acute setting. In patients with atrial fibrillation who present with an acute transient ischemic attack or minor ischemic stroke in the absence of a large area of acute infarction, we usually initiate anticoagulant therapy immediately if the decision has been made to use anticoagulation. For those with severe stroke or moderate stroke with a large area of infarction, initiating aspirin is recommended without anticoagulant therapy in the acute phase. Anticoagulation can be cautiously introduced 7–14 days after stroke onset provided that hemorrhagic transformation of the infarct has been excluded on neuroimaging in those suspected to be at increased risk of ICH. For patients who have had large infarcts, evidence of hemorrhagic transformation, progression of stroke, or uncontrolled hypertension, further delays beyond 14 days after stroke may be required prior to initiation of therapy with oral anticoagulation. We stop aspirin once a therapeutic anticoagulant effect is achieved (immediately with introduction of a direct oral anticoagulant), unless there is a compelling indication for combining aspirin with an anticoagulant (e.g., recent coronary stent [see Chapter 145]). We do not bridge with treatment doses of heparin, and bridging therapy is unnecessary with the rapidly acting direct oral anticoagulants.

Lipid Modification

Current guidelines recommend statin therapy in patients with LDL of 100 mg/dL or greater. The SPARCL trial, which compared atorvastatin 80 mg daily with placebo, reported a reduced risk of recurrent stroke over 5 years of follow-up in the atorvastatin group. In the recent TST trial, there was a reduced risk of major cardiovascular events (ischemic stroke, myocardial infarction, need for urgent coronary or carotid revascularization, or cardiovascular death) in patients with large vessel atherosclerosis randomized to an LDL target of 70 mg/dL or less compared with an LDL target of 90 to 110 mg/dL.

Blood Pressure

PROGRESS, the largest trial of antihypertensive therapy for secondary stroke prevention, compared perindopril (with or without indapamide) with placebo. Combination therapy reduced blood pressure by 12/5 mmHg and stroke risk by 43% and produced a greater reduction in blood pressure and stroke risk compared with perindopril alone. Current guidelines recommend a target blood pressure of 140/90 mmHg or less for secondary stroke prevention.

Patent Foramen Ovale Closure

Based on evidence from several randomized controlled trials (CLOSE, REDUCE, RESPECT extended follow-up), PFO closure is currently considered an effective management option for prevention of recurrent stroke in carefully selected patients with cryptogenic ischemic stroke based on European consensus guidelines. Percutaneous PFO closure may be more effective than antiplatelet therapy alone in those 60 years of age or younger or those with an embolic appearing nonlacunar stroke on neuroimaging which is confirmed cryptogenic in origin following comprehensive stroke work-up (i.e., estimated high probability of a causal role of PFO with no other evident cause of stroke).

Lifestyle Modification

Lifestyle modification remains a cornerstone of stroke prevention. Important components include smoking cessation, moderate physical activity, weight reduction in overweight or obese patients, adopting a healthy heart diet with increased fruit and vegetable intake and reduced salt intake in those consuming high-salt diets, and moderate alcohol consumption.

PROGNOSIS

Prognosis after stroke depends on age, underlying stroke etiology, severity of the neurological deficit and level of dependence, and comorbidity burden. In a large cohort of more than 10,000 US patients hospitalized with ischemic stroke, 1- and 4-year mortality rates were 24.5% and 41.3%, respectively, with rates of recurrent stroke of 8.0% and 18.1% at 1 year and 4 years, respectively. Early death usually occurs due to neurological complications (e.g., cerebral edema, raised ICP) or medical complications of dependency and immobilization (e.g., aspiration pneumonia). Longer-term mortality is usually due to cardiovascular disease. Two-thirds of stroke survivors are left with chronic residual disability. In addition, patients with stroke are at an increased risk of myocardial infarction, venous thromboembolism, urinary tract infection, hip fracture, pneumonia, and subsequent rehospitalization. Common complications of stroke in the longer term include seizure disorders, cognitive impairment and dementia, depression, and chronic pain syndromes (e.g., central poststroke pain).

FUTURE DIRECTIONS

To date, our knowledge of the epidemiology of stroke has been derived from studies in North America and Europe, with comparatively fewer studies in middle- and low-income countries, although most of the global burden of stroke occurs in these regions. In addition, several large ongoing epidemiological collaborative studies (International Stroke Genetics Consortium) will clarify the role of genetics in the pathogenesis of stroke. Population-based interventions to reduce the burden of stroke will be an important focus of future research and will include evaluation of interventions to reduce salt intake and use of combination cardioprotective therapies (e.g., Polypill) in high-risk populations. For acute ischemic stroke, further studies are underway evaluating the use of tenecteplase as a more convenient alternative to tPa (alteplase) for thrombolysis. The role of mechanical thrombectomy compared with the combination of intravenous thrombolysis plus mechanical thrombectomy in patients with proximal vessel occlusions will also need to be evaluated, as well as the possibility of expanding the eligibility criteria for endovascular therapy, both within and beyond current time windows. The optimal blood pressure goal following endovascular therapy also remains to be determined.

LIST OF TRIAL ACRONYMS

ATACH	Antihypertensive Treatment of Acute Cerebral Hemorrhage
CADISS	Cervical Artery Dissection in Stroke Study
CHANCE	Clopidogrel with Aspirin in Acute Minor Stroke or Transient Ischemic Attack
CRYSTAL AF	Cryptogenic Stroke and Underlying Atrial Fibrillation
ECASS	European Cooperative Acute Stroke Study
ESPRIT	European/Australasian Stroke Prevention in Reversible Ischaemia Trial
FAST	Factor Seven for Acute Hemorrhagic Stroke Trial
INTERACT	Intensive Blood Pressure Reduction in Acute Cerebral Haemorrhage Trial
NAVIGATE ESUS	New Approach Rivaroxaban Inhibition of Factor Xa in a Global Trial Versus ASA to Prevent Embolism in Embolic Stroke of Undetermined Source
POINT	Platelet-Oriented Inhibition in New TIA and Minor Ischemic Stroke Trial
PROGRESS	Perindopril Protection Against Recurrent Stroke Study (PROGRESS)
RE-SPECT ESUS	Randomized, Double-Blind Evaluation in Secondary Stroke Prevention Comparing the Efficacy and Safety of the Oral Thrombin Inhibitor Dabigatran Etexilate versus Acetylsalicylic Acid in Patients With Embolic Stroke of Undetermined Source
SAMMPRIS	Stenting versus Aggressive Medical Therapy for Intracranial stenosis
SPARCL	Stroke Prevention by Aggressive Reduction in Cholesterol Levels Trial
SPS3	Secondary Prevention of Small Subcortical Strokes Trial
STITCH	Surgical Trial in IntraCerebral Hemorrhage
TICH	Tranexamic Aid for Hyperacute Primary IntraCerebral Hemorrhage
TRAPS	Trial of Rivaroxaban in AntiPhospholipid Syndrome
TST	Treat Stroke to Target
VISSIT	Vitesse Intracranial Stent Study for Ischemic Stroke Therapy
WARSS	Warfarin-Aspirin Recurrent Stroke Study
WASID	Warfarin-Aspirin Symptomatic Intracranial Disease Trial

SUGGESTED READINGS

Amarenco P, Kim JS, Labreuche J, et al. A comparison of two LDL cholesterol targets after ischemic stroke. *N Engl J Med*. 2019;382:9–19.

Brott TG, Calvet D, Howard G, et al. Long-term outcomes of stenting and endarterectomy for symptomatic carotid stenosis: a preplanned pooled analysis of individual patient data. *Lancet Neurol*. 2019 Apr;18(4):348–356.

Campbell BCV, Ma H, Ringleb PA, et al. Extending thrombolysis to 4.5–9 h and wake-up stroke using perfusion imaging: a systematic review and meta-analysis of individual patient data. *Lancet*. 2019 Jul 13;394(10193):139–147.

Dia A, Cifu AS, Shah AP. Management of patients with a patent foramen ovale with history of stroke or TIA. *JAMA*. 2021;325(1):81–82.

Global Burden of Disease 2016 Stroke Collaborators Global, regional, and national burden of stroke, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol*. 2019;18(5):439–458.

Hemphill III JC, Greenberg SM, Anderson CS, et al. Guidelines for the management of spontaneous intracerebral hemorrhage: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke*. 2015;46(7):2032–2060.

Johnston SC, Easton JD, Farrant M, Barsan W, et al. Clopidogrel and aspirin in acute ischemic stroke and high-risk TIA. *N Engl J Med*. 2018;379: 215–225.

Johnston SC, Amarenco P, Denison H, et al. Ticagrelor and aspirin or aspirin alone in acute ischemic stroke or TIA. *N Engl J Med*. 2020;383:207–217.

Kleindorfer DO, Towfighi A, Chaturvedi S, et al. 2021 guideline for the prevention of stroke in patients with stroke and transient ischemic attack: a guideline from the American Heart Association/American Stroke Association. *Stroke*. 2021;52:e364–e467.

Lip GYH, Banerjee A, Boriani G, et al. Antithrombotic therapy for atrial fibrillation: CHEST guideline and expert panel report. *Chest*. 2018 Nov;154(5):1121–1201.

O'Donnell MJ, Chin SL, Rangarajan S, et al. Global and regional effects of potentially modifiable risk factors associated with acute stroke in 32 countries (INTERSTROKE): a case-control study. *Lancet*. 2016;388: 761–765.

Ma H, Campbell BCV, Parsons MW, et al. Thrombolysis guided by perfusion imaging up to 9 hours after onset of stroke. *N Engl J Med*. 2019;380:1795–1803.

Moullaali TJ, Wang X, Martin RH, et al. Blood pressure control and clinical outcomes in acute intracerebral haemorrhage: a preplanned pooled analysis of individual participant data. *Lancet Neurol*. 2019 Sep;18(9): 857–864.

Pan Y, Elm JJ, Li H, et al. Outcomes associated with clopidogrel-aspirin use in minor stroke or transient ischemic attack: a pooled analysis of clopidogrel in high-risk patients with acute non-disabling cerebrovascular events (CHANCE) and platelet-oriented inhibition in new TIA and minor ischemic stroke (POINT) trials. *JAMA Neurol*. 2019;76:1466.

Powers WJ, Rabinstein AA, Ackerson T, et al. Guidelines for the early management of patients with acute ischemic stroke: 2019 update to the 2018 guidelines for the early management of acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke*. 2019;50:e344–e418.

RESTART Collaboration Effects of antiplatelet therapy after stroke due to intracerebral haemorrhage (RESTART): a randomised, open-label trial. *Lancet*. 2019 Jun 29;393(10191):2613–2623.

Wang T, Luo J, Wang X, et al. Endovascular therapy versus medical treatment for symptomatic intracranial artery stenosis. *Cochrane Database Syst Rev*. 2020 Aug 11;8(8):CD013267.

Yang P, Zhang Y, Zhang L, et al. Endovascular thrombectomy with or without intravenous alteplase in acute stroke. *N Engl J Med*. 2020;382:1981–1993.

CHAPTER 145

ACUTE CORONARY SYNDROMES

John W. Eikelboom and Jeffrey I. Weitz

Acute coronary syndrome (ACS) leads to millions of hospital admissions worldwide each year and is a leading cause of death. Antithrombotic therapies are a cornerstone in the immediate and long-term management of ACS, reducing the risk of myocardial infarction (MI) and death in both medically and invasively managed patients. This chapter reviews fibrinolytic, antiplatelet, and anticoagulant therapies in the treatment of patients with ACS and provides a practical guide to several common hemostatic and thrombotic problems encountered in this patient population.

CLASSIFICATION

The clinical classification of ACS is based on the electrocardiogram (ECG) at the time of presentation and blood levels of cardiac biomarkers (troponin, creatine kinase). Patients with ST-segment elevation on the ECG and elevated cardiac biomarkers are diagnosed with ST-segment elevation MI (STEMI). Patients with non–ST-segment elevation (NSTE) ACS are subdivided according to whether or not they have elevated blood levels of cardiac biomarkers; those with elevated cardiac biomarkers are diagnosed with non–ST-segment elevation MI (NSTEMI), and those without elevated cardiac biomarkers are diagnosed with unstable angina.[1–3]

PATHOPHYSIOLOGY

Atherothrombosis plays a central role in the pathogenesis of ACS.[4] Hypertension, dysglycemia, dyslipidemia, and toxins contained in tobacco cause endothelial injury. Lipid accumulation promotes an inflammatory response characterized by the recruitment of macrophages, smooth muscle cells, and fibroblasts to the site of injury and the formation of increasingly complex and unstable plaques with a necrotic core and fibrous cap. Disruption of the fibrous cap by shear forces and its degradation by enzymatic and cellular processes expose the plaque contents to the blood. Platelets adhere to exposed subendothelial proteins and become activated and aggregate. Exposed tissue factor induces thrombin generation on cellular surfaces, further promoting the formation of a platelet-fibrin thrombus that can occlude coronary blood flow. These processes are described in more detail in Chapter 142.

The clinical manifestations of coronary atherothrombosis are influenced by the extent and duration of obstruction to blood flow and the presence or absence of collateral circulation. Patients with small plaques that do not significantly impair blood flow generally remain asymptomatic. Patients with significant flow-limiting plaques may develop ischemic symptoms (e.g., chest pain, breathlessness) during exertion when myocardial oxygen demand exceeds supply. Patients with acute plaque disruption with superimposed thrombus formation that completely obstructs coronary blood flow typically present with STEMI unless there is a robust collateral circulation. If obstruction to blood flow is transient or partial, patients typically present with NSTE ACS. If myocardial ischemia results in ventricular fibrillation, sudden cardiac death supervenes.

ANTITHROMBOTIC MANAGEMENT

The goal of antithrombotic therapies in patients with ACS is to prevent new thrombus formation at the site of plaque disruption and facilitate lysis of intracoronary thrombus. Antithrombotic drugs are also used to prevent thrombus formation on the guide wires, catheters, and stents used to open occluded arteries and to prevent and treat left ventricular thrombus formation.

Although the pathophysiology is similar irrespective of whether patients present with STEMI or NSTE ACS, only STEMI patients benefit from immediate reperfusion therapy. This difference reflects the fact that most patients with NSTEMI do not have an occluded infarct-related coronary artery. Instead, they develop MI because of transient coronary artery occlusion or because of distal embolization of the thrombus.

REPERFUSION THERAPY FOR ST-SEGMENT ELEVATION MYOCARDIAL INFARCTION

Effective approaches to coronary reperfusion include mechanical (primary percutaneous coronary intervention [PCI]) and pharmacologic (fibrinolytic therapy) methods.

Primary Percutaneous Coronary Intervention

Primary PCI is preferred over fibrinolytic therapy in patients with STEMI because it produces higher patency rates and does not cause intracranial bleeding. Unlike fibrinolysis, which only treats the thrombus, primary PCI also allows treatment of the underlying atherosclerotic plaque. In high-income countries, most patients with STEMI are treated with primary PCI. In middle- and low-income countries, many centers lack the facilities and expertise to perform urgent coronary interventions, and only a minority of STEMI patients worldwide is treated with primary PCI.[5,6] Patients undergoing primary PCI are routinely treated with antiplatelet and anticoagulant therapy (discussed in sections on Antiplatelet Therapy and Anticoagulant Therapy).

Fibrinolytic Therapy

Fibrinolytic drugs are plasminogen activators that initiate fibrinolysis by converting plasminogen to plasmin. Plasmin degrades fibrin resulting in clot lysis and recanalization of thrombotic occlusion. Restoration of coronary blood flow limits infarct size and improves myocardial function and survival.

The pharmacologic characteristics of the fibrinolytic drugs most used in the management of ACS are summarized in Table 145.1. Streptokinase was the first agent to be evaluated in large-scale randomized controlled trials. A non–fibrin-specific agent, streptokinase, indirectly activates plasminogen, whereas the more fibrin-specific agents alteplase, reteplase, and tenecteplase directly convert plasminogen to plasmin. Reteplase and tenecteplase have longer half-lives than alteplase, enabling them to be given by double- or single-bolus injection, respectively, which simplifies administration. The direct-acting fibrinolytic agents are more fibrin specific than streptokinase because they bind to fibrin, where they convert fibrin-bound plasminogen to plasmin. Consequently, these agents produce a less marked systemic lytic state than streptokinase, which has no fibrin affinity. Avoidance of a systemic lytic state, which is characterized by a reduction in the

TABLE 145.1 Pharmacologic Characteristics of Fibrinolytic Drugs Used in the Management of Patients With ST-Segment Elevation Myocardial Infarction

Characteristic	Streptokinase	Alteplase	Reteplase	Tenecteplase
Fibrin specificity[a]	–	++	+	+++
Dose	1.5 million units	100 mg	20 U	30–50 mg
Administration	Infusion over 30–60 min	Bolus 15 mg; then infusion 0.75 mg/kg (maximum, 50 mg) over 30 min, 0.5 mg/kg (maximum, 35 mg) over the next 60 min	Double bolus, 10 U over 2 min; then repeat 10 U bolus after 30 min	Single weight-adjusted bolus, <60 kg = 30 mg, 60–69 kg = 35 mg, 70–79 kg = 40 mg, 80–89 kg = 45 mg, ≥90 kg = 50 mg
Half-life (min)	18–23	3–4	18	20
Adjunctive antiplatelet therapy[b]	Aspirin	Aspirin	Aspirin	Aspirin
Adjunctive anticoagulant therapy	Heparin in patients at high risk of thromboembolism[c]	Heparin 60 U/kg bolus (maximum, 4000 U); 12 U/kg/h (maximum, 1000 U/h)	Heparin 60 U/kg bolus (maximum, 4000 U); 12 U/kg/h (maximum, 1000 U/h)	Heparin 60 U/kg bolus (maximum, 4000 U); 12 U/kg/h (maximum, 1000 U/h)

[a] Less fibrin specificity is associated with more systemic fibrinogen depletion.

[b] Fibrinolytic drugs were evaluated on a background of aspirin, but the addition of clopidogrel to aspirin was subsequently shown to provide incremental benefit.

[c] High-risk patients include those with large or anterior myocardial infarction, atrial fibrillation, known left ventricular thrombus, or previous thromboembolism.

circulating level of fibrinogen, is an important potential advantage of fibrin-specific drugs because this can be expected to be associated with a lower risk of bleeding complications.

Initial trials with streptokinase established the efficacy of fibrinolytic therapy for reduction in the risk of MI and death; subsequent trials compared the efficacy and safety of newer fibrinolytic drugs with those of streptokinase or alteplase.

Streptokinase

Streptokinase is a single-chain polypeptide derived from β-hemolytic *Streptococcus* cultures. After intravenous (IV) administration, streptokinase binds to plasminogen, and the resulting streptokinase–plasminogen enzymatic complex converts plasminogen to plasmin. Because plasmin nonspecifically degrades circulating fibrinogen as well as fibrin, streptokinase produces a systemic lytic state. Streptokinase induces the formation of anti-streptokinase antibodies and can cause allergic reactions, particularly with repeated administration. Severe reactions are rare, but rash, shivering, pyrexia, and mild hypotension occur in up to 10% of patients. It is uncertain whether neutralizing antibodies reduce the efficacy of streptokinase.

The GISSI-1 (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico 1) trial, which was conducted in 11,806 patients with STEMI, demonstrated that compared with no lytic therapy, streptokinase (1.5 million units over 1 hour) significantly reduced 21-day in-hospital mortality (10.7% vs. 13.0%; $P = .0002$). A similar reduction in mortality was seen when the same dose of streptokinase was compared with no lytic therapy in 17,187 patients with suspected MI in the ISIS-2 (International Studies of Infarct Survival 2) trial (9.2% vs. 12.0%; $P < .00001$).

Alteplase

Alteplase is a recombinant tissue-type plasminogen activator that directly converts plasminogen to plasmin. Although more fibrin-specific than streptokinase, alteplase still induces a systemic lytic state. It has a short circulating half-life of 3 to 4 minutes, which necessitates its administration by a bolus followed by a continuous IV infusion.

The ISIS-3 ($n = 41{,}299$) and GISSI-2 ($n = 20{,}891$) trials found no benefit of alteplase over streptokinase; findings possibly explained by the suboptimal use of heparin in conjunction with a short-acting fibrinolytic agent (heparin was given subcutaneously after a delay of 4 to 12 hours) and lack of front-loading of alteplase. The GUSTO-1 (Global Utilization of Streptokinase and Tissue Plasminogen Activator to Treat Occluded Arteries 1) trial ($n = 41{,}021$) demonstrated that front-loaded alteplase (15 mg bolus, followed by 0.75 mg/kg [maximum, 50 mg] as an infusion over 30 minutes and then 0.50 mg/kg [maximum, 35 mg] over 60 minutes [maximum, 100 mg over 90 minutes]) plus IV heparin (5000 U IV bolus, followed by 1000 U/hour as an infusion, with the dose titrated to achieve an activated partial thromboplastin time [aPTT] of 60 to 85 seconds) compared with streptokinase (1.5 million units over 1 hour) with or without IV heparin, reduced 30-day mortality (6.3% vs. 7.3%; $P = .001$). The mortality benefits of alteplase were greatest in patients younger than the age of 75 years and those with anterior MI. Despite its enhanced fibrin specificity, alteplase was not associated with less bleeding than streptokinase.

The greatest benefits of fibrinolytic therapy are seen in patients treated within 1 hour of symptom onset; there is a much smaller benefit or no benefit if treatment is commenced more than 6 hours after symptom onset.[7]

Reteplase

Reteplase is a second-generation non-glycosylated deletion mutant of alteplase. Reteplase is less fibrin-specific than alteplase but has a longer half-life (18 minutes) that enables administration by double-bolus IV injection.

The INJECT trial ($n = 6010$) demonstrated that reteplase and streptokinase were associated with similar 35-day rates of mortality (9.0% vs. 9.5%), in-hospital stroke (1.2% vs. 1.0%), and major bleeding (0.7% vs. 1.0%), although reteplase was associated with a twofold higher rate of intracranial bleeding (0.8% vs. 0.4%). The GUSTO-III trial ($n = 15{,}059$) demonstrated that reteplase (two 10-mg IV bolus injections given 30 minutes apart) and front-loaded alteplase were associated with similar 30-day rates of mortality (7.5% vs. 7.2%) and stroke (1.6% vs. 1.7%).

Tenecteplase

Tenecteplase is a third-generation multiple point mutant of alteplase. Tenecteplase has a half-life of 20 minutes and is given by a single bolus injection. Tenecteplase is the most fibrin-specific fibrinolytic drug approved for clinical use.

The ASSENT-2 (Assessment of the Safety and Efficacy of a New Thrombolytic) study, which enrolled 16,949 STEMI patients, showed that tenecteplase and alteplase were associated with similar 30-day rates of mortality (6.2% vs. 6.2%) and stroke (1.8% vs. 1.7%). Tenecteplase did not reduce the risk of intracerebral bleeding compared with alteplase (1% vs. 1%) but reduced the rate of major noncerebral bleeding (4.7% vs. 5.9%; $P < .0002$), possibly reflecting its enhanced fibrin specificity.

Adjunctive Antithrombotic Therapy in Patients Receiving Fibrinolytic Drugs

A compelling rationale exists to administer adjunctive antithrombotic therapy to STEMI patients treated with fibrinolytic therapy. Platelet-rich thrombi that form after plaque rupture are relatively resistant to degradation, and the use of concomitant antiplatelet and anticoagulant therapy may help to promote clot lysis. Fibrinolytic drugs have an early activating effect on platelets, and the plaque rupture site remains prothrombotic after successful reperfusion therapy. Evidence in support of the efficacy of adjuvant antiplatelet and anticoagulant therapy in patients with STEMI is summarized in the sections on antiplatelet and anticoagulant therapy, respectively.

Intracranial Bleeding

The most important side effect of fibrinolytic therapy is intracranial bleeding, which affects up to 1% of patients and, in the majority of cases, is either fatal or permanently disabling.[8] Risk factors for intracranial bleeding include increasing age (particularly in patients older than 75 years of age), history of prior stroke, uncontrolled blood pressure, female sex, and low body weight. Bolus dose fibrinolytic therapy may be associated with a higher risk of intracranial bleeding than fibrinolytic therapy administered by continuous IV infusion (see Table 145.2 and box on Case 1: Bleeding After Fibrinolytic Therapy).[9]

ANTIPLATELET THERAPY

Aspirin and oral adenosine diphosphate (ADP) receptor antagonists, such as clopidogrel, prasugrel, and ticagrelor are the foundation antiplatelet therapies for the management of ACS. The use of glycoprotein (GP) IIb/IIIa antagonists has decreased since the introduction of the ADP receptor antagonists, but they retain a role in high-risk ACS patients, particularly STEMI patients undergoing primary PCI.[5,6,10] In 2015, the US Food and Drug Administration approved cangrelor, a short-acting parenteral ADP receptor antagonist, for ACS patients undergoing PCI who have not received an oral ADP receptor antagonist and who are not given a GPIIb/IIIa inhibitor. The pharmacologic characteristics of antiplatelet drugs commonly used in the management of ACS are summarized in Table 145.3 (oral drugs) and 145.4 (IV drugs).

Oral Antiplatelet Drugs

Aspirin

Aspirin inhibits platelets by irreversibly blocking the platelet enzyme cyclooxygenase-1, thereby preventing the formation of thromboxane A_2, a potent platelet agonist and vasoconstrictor. Despite having a half-life of only 20 minutes, the antiplatelet effect of aspirin lasts for the lifetime of the platelet (5 to 10 days) because platelets are anucleate and lack the machinery to synthesize new enzymes.[11]

TABLE 145.2 Management of Major Bleeding in Patients With ST-Segment Elevation Myocardial Infarction Treated With Fibrinolytic Therapy

Steps	Approach	Considerations
Stop treatment	Stop fibrinolytic drug	Recovery of fibrinogen levels can take 24–48 h after stopping streptokinase
	Stop antiplatelet drugs	Antiplatelet effects of aspirin and clopidogrel last for the life span of platelets (5–10 days)
	Stop anticoagulant	Heparin has a half-life of about 40 min; LMWH has a half-life of 3-6 h
Local measures	Local pressure	Sustained local pressure (e.g., for 30 min) may be required
	Endoscopy and local injection[a]	Can be used for upper or distal lower GI bleeding
	Embolization[a]	Used in rare situations such as life-threatening pulmonary or intraabdominal bleeding
Laboratory evaluation	Fibrinogen	Levels may be undetectable (<100 mg/dL)
	PT and aPTT	Results are uninterpretable if fibrinogen levels are low (<100 mg/dL)
	Anti-Xa level	PT (INR) and aPTT are elevated with warfarin; aPTT is prolonged with therapeutic doses of heparin but not with LMWH or fondaparinux
	Crossmatch blood	Chromogenic anti-factor Xa assay for LMWH and fondaparinux; this test is unaffected by low fibrinogen levels
		To restore blood volume and maintain hemoglobin
Reversal of fibrinolytic effect	Cryoprecipitate	Recommended initial dose is 10 U; monitored by repeating fibrinogen level
	FFP	The half-life of fibrinogen is about 4 days
		Requires large volumes (e.g., 2 L) to restore fibrinogen levels
Reversal of antiplatelet effect	Platelet transfusion	Increases the number of functional platelets but does not reverse the antiplatelet effects of aspirin and clopidogrel
		Also see text under heading "Antiplatelet Therapy"
Reversal of anticoagulant effect	Protamine sulphate	Protamine reverses heparin and partially reverses LMWH; it has no effect on fondaparinux (also see Table 145.6)

[a] Also requires restoration of adequate hemostasis.

aPTT, Activated partial thromboplastin time; *FFP*, fresh frozen plasma; *GI*, gastrointestinal; *INR*, international normalized ratio; *LMWH*, low-molecular-weight heparin; *PT*, prothrombin time.

CASE 1: BLEEDING AFTER FIBRINOLYTIC THERAPY

A 63-year-old man with a history of hypertension presents to a rural hospital with sudden onset of severe retrosternal chest pain. An electrocardiogram (ECG) performed in the emergency department demonstrates 4-mm ST-segment elevation in the anterior leads. The nearest hospital with a cardiac catheterization laboratory is more than 6 h away. After treatment with aspirin (325 mg loading dose), clopidogrel (300 mg loading dose), and an intravenous (IV) infusion of front-loaded alteplase, he has a rapid resolution of his pain and the ECG reveals normalization of the ST-segment elevation. Toward the end of the alteplase infusion, he complains of abdominal pain, becomes hypotensive, and develops melena. Hemoglobin is 10.2 g/dL, the activated partial thromboplastin time (aPTT) is 89 s, the international normalized ratio (INR) is 1.7, and the fibrinogen level is 80 mg/dL.

Aspirin and clopidogrel are held; the patient receives fibrinogen concentrate and is started on an IV infusion of a proton pump inhibitor. He undergoes urgent upper gastrointestinal tract endoscopy with an injection of a bleeding ulcer. He does not receive platelets because of concerns about the risk of recurrent myocardial infarction (MI). No further bleeding occurs, and after 48 h he resumes aspirin. Clopidogrel is restarted 1 week later after repeat endoscopy demonstrates the healing of the ulcer.

Comment

Fibrinolytic trials in patients with acute MI report a 1%–6% incidence of major bleeding and a 10% incidence of moderate bleeding during the first 30 days. The most common sources of major bleeding are the gastrointestinal tract and procedure-related bleeding. The principles of management of major bleeding in ST-segment elevation MI (STEMI) patients treated with fibrinolytic therapy are summarized in Table 145.2. Steps include (1) stop antithrombotic therapies; (2) use local measures when possible to control bleeding; (3) draw blood to measure fibrinogen, prothrombin time (PT), aPTT, and possibly anti-Xa levels (to measure the anticoagulant effect of low-molecular-weight heparin [LMWH]) and for blood typing and cross-match; and (4) administer therapies to mitigate or reverse the effects of fibrinolytic, antiplatelet, and anticoagulant drugs.

The extent and duration of the systemic lytic state induced by fibrinolytic drugs are determined by their fibrin specificity. Whereas streptokinase produces profound and sustained depletion of fibrinogen (<100 mg/dL) that lasts for up to 24-48 h, alteplase and other more fibrin-specific agents exert less pronounced and more short-lived effects on fibrinogen levels. The aPTT and PT can be markedly prolonged in patients with hypofibrinogenemia. Normal coagulation can be restored by increasing the fibrinogen level to at least 100 mg/dL. This can readily be achieved by infusing fibrinogen concentrate (dose [mg/kg] = [target level (mg/dL) - measured level (mg/dL)] divided by 1.7 or 1.8 [mg/dL per mg/kg body weight]), or 70 mg/kg body weight if fibrinogen level unknown) or cryoprecipitate (recommended dose: 10 units). Although fresh frozen plasma also contains fibrinogen, large volumes are needed to increase the fibrinogen concentration to the desired range.

Platelet dysfunction caused by antiplatelet therapy and fibrinolytic therapy cannot be specifically reversed, but the infusion of donor platelets might help to restore platelet function. Platelet transfusion is generally reserved for patients with life-threatening bleeding because of concerns about the risk of recurrent MI. A single unit of single donor platelets can be expected to increase the platelet count by 50–60 × 10^9/L. Even a small number of functional (non-aspirinated) platelets (e.g., 10%–20%) is sufficient to generate enough thromboxane A_2 to overcome the effects of aspirin, but a much larger number of transfused platelets is needed to overcome the antiplatelet effects of clopidogrel and prasugrel. Platelet infusions are relatively ineffective if given within 24 h of the last dose of ticagrelor. In this case, bleeding was controlled with fibrinogen concentrate and local measures, and platelet transfusion was not required.

Multiple randomized trials have demonstrated the efficacy of aspirin for the prevention of recurrent MI, stroke, or death in patients with ACS.[12] The ISIS-2 investigators evaluated the efficacy and safety of aspirin in 17,187 STEMI patients who were also randomized to receive or not to receive fibrinolytic therapy. Aspirin (162 mg/day), compared with no aspirin, significantly reduced 35-day mortality (9.4% vs. 11.8%; $P < .00001$) as well as nonfatal reinfarction (1.0% vs. 2.0%) and stroke (0.3% vs. 0.6%) without increasing bleeding. The Antithrombotic Trialists' Collaboration (ATTC) pooled the data from 15 randomized trials of antiplatelet therapy (predominantly with aspirin) involving 19,302 acute MI patients who were also treated with fibrinolytic therapy. Compared with placebo or no antiplatelet therapy, antiplatelet therapy given for a mean duration of 1 month significantly reduced the composite outcome of MI, stroke, or death (10.4% vs. 14.2%; $P < .0001$).

At least four randomized controlled trials have demonstrated the efficacy of aspirin in patients with NSTE ACS. Pooled data from four trials that included a combined total of 3096 patients indicate that aspirin compared with placebo or no aspirin significantly reduced the composite endpoint of MI, stroke, or vascular death (6.4% vs. 12.5%; $P = .0005$).

The efficacy and safety of aspirin in combination with dipyridamole have been evaluated in PCI patients in one randomized controlled trial involving 376 patients. Aspirin (300 mg) plus dipyridamole (75 mg given three times daily except during a 24-hour period around the time of the procedure when dipyridamole was given intravenously) reduced the risk of MI by more than half compared with placebo (1.6% vs. 6.9%; $P = .01$). These results led to the adoption of aspirin as standard therapy for patients undergoing PCI.

The CURRENT OASIS-7 (Clopidogrel Optimal Loading Dose Usage to Reduce Recurrent EveNTs/Optimal Antiplatelet Strategy for InterventionS) trial explored the optimal dose of aspirin in 25,086 ACS patients with or without ST-segment elevation on the presenting ECG and included 17,263 patients undergoing early PCI.[13] After an initial aspirin loading dose, patients were randomized to receive aspirin at a dose of 300 to 325 mg/day or 75 to 100 mg/day for 30 days. The two doses were associated with similar rates of MI, stroke, or death at 30 days (4.2% vs. 4.4%), but the higher aspirin dose increased the rate of gastrointestinal bleeding (0.4% vs. 0.2%; $P = .04$). Based on these results, the optimal dose of aspirin for the management of patients with ACS (after an initial loading dose of 160 to 325 mg) appears to be 75 to 100 mg/day.

Clopidogrel

Clopidogrel undergoes metabolic activation in the liver to form the active moiety that irreversibly blocks P2Y12, the major ADP receptor on the platelet surface, as evidenced by a decrease in ADP-induced platelet activation and aggregation. Like aspirin, the antiplatelet effect of clopidogrel lasts for the lifetime of the platelet. Clopidogrel is an effective antiplatelet drug for the prevention of MI and death when used in combination with aspirin but has a slow onset of action and a variable antiplatelet effect, which may limit its effectiveness.

The COMMIT (Clopidogrel and Metoprolol in Myocardial Infarction Trial) trial, which involved 45,582 patients with STEMI, all of whom were treated with aspirin (162 mg/day), demonstrated that compared with placebo, 30 days of treatment with clopidogrel (75 mg/day) reduced the risk of MI, stroke, or death (9.2% vs. 10.1%; $P = .002$) with no increase in major bleeding (0.58% vs. 0.55%).[14] There was no age restriction to enrollment in the COMMIT trial, and clopidogrel was given without a loading dose. In the CLARITY (Clopidogrel as Adjunctive Reperfusion Therapy) trial ($n = 3491$), which was conducted in parallel with COMMIT, clopidogrel was given as a loading dose of 300 mg followed by 75 mg/day for 2 to 8 days was compared with placebo in patients receiving fibrinolysis. Clopidogrel reduced the risk of MI or death (15% vs. 21.7%; $P < .001$). Consistent benefits of clopidogrel were demonstrated in the 1863 patients who underwent PCI after mandated angiography.

The CURE (Clopidogrel in Unstable Angina to Prevent Recurrent Events) trial, which included 12,562 patients with NSTE ACS, all of whom were treated with aspirin (recommended dose: 75 to 325 mg/day), demonstrated that compared with placebo, a median of 9 months of treatment with clopidogrel (300 mg loading dose followed by 75 mg/day) reduced the risk of MI, stroke, or death (9.3% vs. 11.4%; $P < .001$) at the cost of an increase in major bleeding (3.7%

TABLE 145.3 **Pharmacologic Characteristics of Oral Antiplatelet Drugs Commonly Used in the Management of Acute Coronary Syndrome**

		ADP Receptor Antagonists		
Characteristic	**Aspirin**	**Clopidogrel**	**Prasugrel**	**Ticagrelor**
Class	COX inhibitor	Thienopyridine (second generation)	Thienopyridine (third generation)	Cyclopentyl triazolopyrimidine
Target	COX-1	P2Y12	P2Y12	P2Y12
Dose	162–325-mg loading dose; 75–325 mg/day maintenance dose	300–600-mg loading dose; 75 mg/day maintenance dose	60-mg loading dose; 10 mg/day maintenance dose	180-mg loading dose; 90 mg bid maintenance dose
Prodrug	No	Yes	Yes	No
Time to effect[a]	<1 h	4–6 h[b]	<1 h	<1 h
Drug half-life	20 min	Minutes	Minutes	12 h
Reversible	No	No	No	Yes

[a] After loading dose.

[b] Increased antithrombotic benefit was seen after the first hour in patients enrolled in the COMMIT trial who did not receive a loading dose, but the maximum effect is not seen until after 4-6 h.

ADP, Adenosine diphosphate; *bid*, twice daily; *COX*, cyclooxygenase.

vs. 2.7%; $P = .001$).[15] Consistent benefits of clopidogrel were seen in the 2,658 patients who underwent PCI regardless of whether or not they underwent coronary stenting.

Prompted by concerns that some patients achieve suboptimal inhibition of platelet function with conventional doses of clopidogrel, the CURRENT-OASIS 7 trial compared the efficacy and safety of a higher loading and maintenance dose of clopidogrel (600-mg loading dose followed by 150 mg/day for 6 days and 75 mg/day for 23 days thereafter) with standard-dose clopidogrel (300-mg loading dose and 75 mg daily thereafter) in 25,086 patients with ACS referred for an invasive management strategy.[13] Both regimens were associated with similar rates of the primary outcome, MI, stroke, or vascular death (4.2% vs. 4.4%), but the higher dose regimen was associated with an increase in major bleeding (2.5% vs. 2.0%; $P = .01$). However, in the 17,263 patients who underwent PCI, the higher dose regimen reduced MI, stroke, or vascular death (3.9% vs. 4.5%; $P = .039$) and stent thrombosis (0.7% vs. 1.3%; $P = .0001$) compared with the lower dose regimen.

Prasugrel

Prasugrel is a third-generation thienopyridine that is more potent than clopidogrel and has a more rapid onset of action. Prasugrel is a prodrug, but unlike clopidogrel, it requires only single-step bioconversion to form the active metabolite that irreversibly blocks the platelet P2Y12 receptor.

The TRITON-TIMI 38 (Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition With Prasugrel–Thrombolysis In Myocardial Infarction 38) trial evaluated the efficacy and safety of prasugrel in 13,608 ACS patients with or without ST-segment elevation who were undergoing PCI, all of whom were treated with aspirin (recommended dose: 75 to 162 mg/day).[16] Compared with clopidogrel (300 mg followed by 75 mg once daily thereafter), prasugrel (60 mg followed by 10 mg once daily thereafter) continued for a median of 14.5 months reduced the risk of MI, stroke, or cardiovascular death (9.9% vs. 12.1%; $P < .001$) and stent thrombosis (1.1% vs. 2.4%; $P < .001$) but did not reduce mortality and increased noncoronary artery bypass graft (CABG) major bleeding (2.4% vs. 1.8%; $P = .03$) as well as intracranial and fatal bleeding.

The TRILOGY ACS[17] (Targeted Platelet Inhibition to Clarify the Optimal Strategy to Medically Manage Acute Coronary Syndromes) trial evaluated the efficacy and safety of prasugrel in 9326 aspirin-treated patients with NSTE ACS who were managed noninvasively. At a median of 17 months of follow-up, prasugrel (30 mg followed by 10 mg once daily in patients less than 75 years or 5 mg once daily for those 75 years or older or who weighed less than 60 kg) and clopidogrel (300 mg followed by 75 mg once daily) were associated with similar rates of MI, stroke or cardiovascular death (13.9% vs. 16.0%; $P = .21$) and similar rates of major bleeding.

Ticagrelor

Ticagrelor is a nonthienopyridine P2Y12 receptor antagonist. Like prasugrel, ticagrelor is more potent and has a more rapid onset of action than clopidogrel, but unlike both clopidogrel and prasugrel, ticagrelor binds reversibly to the P2Y12 receptor.

The PLATO trial evaluated the efficacy and safety of ticagrelor in 18,624ACS patients with or without ST-segment elevation.[18] Compared with clopidogrel (300 or 600 mg loading dose followed by 75 mg once daily), ticagrelor (180 mg loading dose followed by 90 mg twice daily) reduced the risk of MI, stroke, or vascular death by 16% (9.8% vs. 11.7%; $P < .001$) and produced a significant reduction in vascular death (4.0% vs. 5.1%; $P = .001$) and stent thrombosis (1.3% vs. 1.9%; $P = .009$) (see box on Case 2: Stent Thrombosis). There was no increase in major bleeding (11.6% vs. 11.2%) or intracranial or fatal bleeding, but ticagrelor increased non-CABG major bleeding (4.5% vs. 3.8%; $P = .03$).

The ISAR-REACT 5 trial evaluated the relative efficacy and safety of ticagrelor and prasugrel in 4018 patients who presented with ACS and for whom invasive evaluation was planned. Compared with prasugrel (60 mg followed by 10 mg once daily thereafter), ticagrelor (180 mg loading dose followed by 90 mg twice daily) was associated with a significantly higher rate of MI, stroke or death (9.3% vs. 6.9%, $P = .006$) at 1 year, with no significant difference in individual components of this primary outcome, stent thrombosis or major bleeding (5.4% vs. 4.8%, $P = .46$).

Duration of Dual Antiplatelet Therapy

Results of the CURE, TRITON-TIMI 38, and PLATO studies support the recommendation to treat patients with recent ACS with dual antiplatelet for a minimum of 12 months. Building on these results, the PEGASUS-TIMI 54 trial demonstrated in 21,162 post-MI patients who successfully completed 12 months of treatment with dual antiplatelet therapy that extended-duration ticagrelor (60 or 90 mg twice daily; median 1.7 years) compared with placebo on a background of aspirin reduced CV death, MI or stroke (60 mg twice

CASE 2: STENT THROMBOSIS

A 49-year-old man with a history of type 2 diabetes presents with non–ST-segment elevation myocardial infarction and undergoes percutaneous coronary intervention (PCI) with the placement of a drug-eluting stent in the left anterior descending coronary artery. He receives a 325-mg loading dose of aspirin and a 180 mg loading dose of ticagrelor and is discharged on aspirin 81 mg/day and ticagrelor 90 mg twice daily. He presents to the emergency department 3 weeks later with recurrent chest pain and anterior ST-segment elevation on the ECG. He is taken urgently to the cardiac catheterization laboratory where a diagnosis of stent thrombosis is confirmed, and he undergoes repeat PCI with thrombus aspiration.

Further history reveals that he discontinued ticagrelor after leaving the hospital because he felt that he was taking too many pills. He is counselled regarding the importance of adherence to antiplatelet therapy to protect his stent and is discharged on indefinite dual antiplatelet therapy with aspirin and ticagrelor.

Comment

Stent thrombosis is a potentially life-threatening complication of PCI, affecting 1%–2% of patients during the first year and presenting in almost all cases like death or myocardial infarction.[19] Risk factors for stent thrombosis can be categorized as patient-related, technical (procedure, stent, or lesion), or drug-related. The single most important predictor of stent thrombosis is premature discontinuation of antiplatelet therapy. In patients treated with clopidogrel, high on-treatment platelet reactivity during treatment has also emerged as a predictor of stent thrombosis and is affected by clinical (e.g., age, diabetes, renal insufficiency) and genetic factors. Carriers of reduced-function *CYP2C19* alleles have low levels of the active metabolite of clopidogrel, diminished platelet inhibition, and an increased risk of stent thrombosis. Unlike clopidogrel, which undergoes two-step, cytochrome P450 (CYP)-dependent metabolic bioconversion in the liver, prasugrel and ticagrelor are not affected by CYP polymorphisms and consistently produce greater and more consistent inhibition of ADP-induced platelet aggregation than standard doses of clopidogrel. Higher doses of clopidogrel (e.g., 225 or 300 mg/day) in patients heterozygous for *CYP2C19*2* alleles produce levels of platelet inhibition like those seen with standard (75 mg) doses but have not been evaluated in clinical outcome studies.

Concerns regarding the risk of stent thrombosis caused by poor clopidogrel response[20] have been alleviated by the routine use of ticagrelor or prasugrel in preference to clopidogrel. However, the lack of persistence or adherence remains an important cause of stent thrombosis.

daily vs. placebo: 7.77% vs. 9.04%, $P = .004$; 90 mg twice daily vs. placebo: 7.85% vs. 9.04%, $P = .008$). Both doses of ticagrelor increased TIMI major bleeding but not intracranial bleeding. The DAPT trial demonstrated in 9961 stented patients who had successfully completed 12 months of treatment with dual antiplatelet therapy that extended duration clopidogrel or prasugrel (median 18 months) compared with control on a background of aspirin reduced stent thrombosis (0.4% vs. 1.4%, $P < .001$) as well as death, MI, or stroke (4.3% vs. 5.9%, $P < .001$) at the cost of an increase in GUSTO bleeding. The greatest benefits were evident in patients with a history of MI. Accordingly, guidelines recommend extended dual antiplatelet therapy for up to an additional 36 months in patients with recent ACS who were successfully treated for the first year and did not have major bleeding.

Intravenous Antiplatelet Drugs

Glycoprotein IIb/IIIa Inhibitors

Three GPIIb/IIIa inhibitors are available for clinical use (see Table 145.4). Abciximab is a humanized version of a Fab fragment of a murine antibody directed against GPIIb/IIIa; tirofiban is a nonpeptide tyrosine derivative that selectively binds to GPIIb/IIIa, and eptifibatide is a synthetic disulfide-linked cyclic heptapeptide with high specificity for GPIIb/IIIa.

The efficacy and safety of GPIIb/IIIa inhibitors have been extensively evaluated in patients with ACS. A meta-analysis restricted to trials ($n = 6$) involving at least 1000 patients suggests a modest effect of these agents for the prevention of MI, urgent revascularization, or vascular death in patients with NSTE ACS (10.8% vs. 11.8%; $P = .015$).[21] Pooled data from a meta-analysis of 16 randomized trials involving 10,085 STEMI patients undergoing primary PCI demonstrated that adjunctive GPIIb/IIIa blockade did not reduce 30-day mortality (2.8 vs. 2.9%; $P = .75$) or reinfarction (1.5 vs. 1.9%; $P = .22$) and increased major bleeding (4.1 vs. 2.7%; $P = .0004$). However, meta-regression analysis confirmed the impression from individual trials of a mortality benefit of IV GPIIb/IIIa inhibitors in those at highest risk.[22]

Cangrelor

Cangrelor is a rapid-acting IV P2Y12 receptor antagonist that has been compared with clopidogrel or placebo in three randomized trials that included patients with NSTE ACS. An individual patient meta-analysis of the three trials[23] demonstrated that compared with control, cangrelor reduced the risk of ischemia-driven revascularization, stent thrombosis, MI, or death at 48 hours (3.8% vs. 4.7%, $P = .0007$) at the cost of an increase in GUSTO mild bleeding (16.8% vs. 13.0%, $P < .0001$) but no increase in more severe bleeding.

ANTICOAGULANT THERAPY

Anticoagulant therapy is effective for the initial and long-term management of patients with ACS with or without ST-segment elevation.[5,6,10] The pharmacologic characteristics of parenteral anticoagulants used in the management of ACS are summarized in Table 145.5. Early trials demonstrated that heparin and aspirin were similarly effective for the prevention of MI, but aspirin was adopted as the foundation antithrombotic agent because it caused less bleeding than heparin. Subsequent randomized trials demonstrated that the use of heparin on top of aspirin provided additive benefit and that low-molecular-weight heparin (LMWH), fondaparinux, and bivalirudin provided advantages over heparin.

TABLE 145.4 Pharmacologic Characteristics of Intravenous Antiplatelet Drugs Used in the Management of Acute Coronary Syndrome

	GPIIb/IIIa Inhibitors			ADP Receptor Antagonist
Characteristic	**Abciximab**	**Eptifibatide**	**Tirofiban**	**Cangrelor**
Class	Fab fragment	Nonpeptide	Cyclic heptapeptide	Nonthienopyridine
Onset	Rapid	Rapid	Rapid	Rapid
Drug half-life	10–30 min	2 h	2.5 h	3–6 min
Reversibility of platelet inhibition	Slow	Rapid	Rapid	Rapid
Excretion	Unknown	40%–70% renal	50% renal	Dephosphorylation

TABLE 145.5 Pharmacologic Characteristics of Parenteral Anticoagulants Commonly Used in the Management of Patients With Acute Coronary Syndrome

	Unfractionated Heparin	Enoxaparin	Bivalirudin	Fondaparinux
Route of administration	IV	SC (first dose IV[a])	IV	SC (first dose IV[a])
Frequency of dosing	Continuous IV infusion	Twice daily; once daily if CrCl <30 mL/min	Continuous IV infusion	Once-daily injection
Clearance	Primarily nonrenal	Renal	Renal, proteolytic cleavage	Renal
Use in ACS patients with moderate renal impairment	Yes	Yes (dose reduction)	Yes (dose reduction)	Yes[b]
Use in ACS patients undergoing dialysis	Yes	No experience	Yes (dose reduction)	No experience[c]
Routine laboratory monitoring	Yes	No	No[d]	No
Dose	Adjust dose according to the results of the aPTT	Fixed weight-adjusted	Fixed weight-adjusted	Fixed
Accumulation in renal failure	No	Yes	Yes	Yes
Non-anticoagulant side effects	Allergy, HIT	HIT (rare)	—	—
Nonbleeding contraindications	Allergy, immune HIT	Allergy, immune HIT	Allergy	Allergy
Antidote	Protamine sulfate Andexanet alfa reverses anti-Xa component but is not approved for this indication	Protamine sulfate partially reverses Andexanet alfa reverses but is not approved for this indication	No	No Andexanet alfa reverses but is not approved for this indication

[a] The first dose of enoxaparin was given by the intravenous route in the TIMI-11B (Thrombolysis In Myocardial Infarction 11B) and EXTRACT-TIMI 25 (Enoxaparin and Thrombolysis Reperfusion for Acute Myocardial Infarction Treatment, Thrombolysis in Myocardial Infarction 25) studies. The first dose of fondaparinux was given by the intravenous route in the OASIS-6 (Optimal Antiplatelet Strategy for InterventionS 6) trial.

[b] Acute coronary syndrome patients with creatinine up to 265 µmol/L were eligible for inclusion in the OASIS-5 and -6 trials (equivalent to an estimated creatinine clearance of 15–20 mL/min in a 70-kg patient who is 70 years of age).

[c] Fondaparinux is contraindicated in patients with venous thromboembolism who have severe renal impairment.

[d] Monitoring and dose adjustment required in patients with creatinine clearance below 30 mL/min.

ACS, Acute coronary syndromes; *aPTT*, activated partial thromboplastin time; *CrCl*, creatinine clearance; *HIT*, heparin-induced thrombocytopenia; *IV*, intravenous; *SC*, subcutaneous.

The pharmacologic characteristics of oral anticoagulants that have been demonstrated to be effective in the initial and long-term management of ACS are summarized in Table 145.6.[24] Warfarin is effective for the prevention of major cardiovascular events for the long-term management of patients with ACS who are also treated with aspirin, but no adequately powered randomized controlled trials have evaluated the efficacy and safety of warfarin in the context of dual antiplatelet therapy. Two direct oral anticoagulants, rivaroxaban and apixaban, have been evaluated in phase III trials for long-term secondary prevention in ACS patients,[25,26] and rivaroxaban has been approved for this indication in Europe.

Heparin

Heparin is derived from porcine intestinal mucosa and is administered by IV or subcutaneous injection. It inhibits coagulation by binding to antithrombin to induce a conformational change that converts antithrombin from a slow to a rapid inhibitor of thrombin and factor Xa.

Heparin has several limitations, including immune-mediated platelet activation, which leads to heparin-induced thrombocytopenia, and nonspecific protein binding, resulting in a variable anticoagulant response and the need for routine coagulation monitoring. Despite these limitations, heparin remains widely used because it has important advantages over more recently introduced parenteral anticoagulants. First, the anticoagulant effect of heparin can be rapidly and completely reversed with protamine sulfate. Second, heparin is suitable for use in patients with renal failure because it is predominantly cleared via extra-renal routes. Third, unlike LMWH and fondaparinux, heparin is also highly effective for the prevention of contact activation of coagulation induced by guide wires, catheters and stents.[27]

Meta-analysis of four randomized controlled trials involving 1239 STEMI patients treated with aspirin and fibrinolytic therapy demonstrated that short-term IV heparin (given for about 1 week) did not significantly reduce death or reinfarction compared with placebo or no heparin, but increased bleeding.[28] However, these trials were underpowered to demonstrate the benefit of heparin. Definitive data concerning the efficacy of heparin in STEMI patients treated with fibrinolysis come from a meta-analysis of 26 trials involving 73,000 patients, in which heparin, given either intravenously or subcutaneously, reduced mortality and reinfarction compared with placebo or no heparin.[29] Consistent benefits were evident in a subset of five trials in which patients also received aspirin; heparin compared with placebo or no heparin reduced both mortality (8.6% vs. 9.1%; $P = .03$) and reinfarction (3.0% vs. 3.3%; $P = .04$).

Short-term heparin therapy is also beneficial when added to aspirin in patients with NSTE ACS. A meta-analysis of six trials involving 1353 patients demonstrated that compared with placebo or no heparin, up to 7 days of IV heparin reduced death or MI (4.5% vs. 7.4%; $P = .045$) with a nonsignificant excess of major bleeding.[30] Heparin has not been rigorously evaluated in ACS patients undergoing PCI but is routinely used in this setting.

Low-Molecular-Weight Heparin

LMWH is derived from heparin by enzymatic or chemical methods, yielding shorter polysaccharide chains that produce a more predictable anticoagulant effect than heparin and a lower propensity for heparin-induced thrombocytopenia. LMWH is given in fixed weight-adjusted doses without routine coagulation monitoring. The anticoagulant effects of LMWH can be partially reversed with protamine sulfate.

TABLE 145.6 Pharmacological Characteristics of Warfarin and New Oral Anticoagulants Evaluated in Phase III Trials for the Long-Term Management of Acute Coronary Syndrome

Characteristic	Warfarin	Rivaroxaban	Apixaban
Target	VKORC1	Factor Xa	Factor Xa
Prodrug	No	No	No
Bioavailability (%)	100	80	60
Dosing	Variable, once daily	Fixed, 2.5 or 5 mg twice daily[a]	Fixed, 5 mg twice daily (2.5 mg twice daily in selected patients)
Half-life	Mean: 40 h (range: 20–60 h)	7–11 h	12 h
Renal clearance (%)	Nil	66[b]	25
Routine coagulation monitoring	Yes (INR)	No	No
Drug interactions	Multiple	Potent inhibitors of CYP3A4 and P-gp[c]	Potent inhibitors of CYP3A4 and P-gp[c]
Antidote	Yes (vitamin K, PCC, FFP)	Yes[d]	Yes[d]
Approved for ACS management	Yes	Yes, in Europe	No

[a] A once-daily regimen was tested in atrial fibrillation.

[b] Half of renally cleared rivaroxaban is cleared as unchanged drug and half as inactive metabolites.

[c] Potent inhibitors of both CYP3A4 and P-glycoprotein include azole antifungals (e.g., ketoconazole, itraconazole, voriconazole, posaconazole) and protease inhibitors, such as ritonavir. Potent inhibitors of CYP3A4 include azole antifungals, macrolide antibiotics (e.g., clarithromycin), and protease inhibitors (e.g., atanazavir).

[d] Andexanet alfa is approved in Europe and United States for use as an antidote for rivaroxaban and apixaban.

ACS, Acute coronary syndromes; *CYP-3A4*, cytochrome P450 3A4; *FFP*, fresh frozen plasma; *fXa*, activated factor X; *INR*, international normalized ratio; *PCC*, prothrombin complex concentrates; *P-gp*, P-glycoprotein; *VKORC1*, C1 subunit of vitamin K epoxide reductase.

The CREATE trial ($n = 15{,}570$) was the largest of four trials that evaluated the efficacy and safety of various LMWH preparations in patients with STEMI also treated with aspirin and fibrinolysis.[31] Approximately half of the patients in the CREATE trial were treated with dual antiplatelet therapy. The pooled data ($n = 16{,}943$) demonstrated that compared with placebo, initial LMWH continued for up to 7 days reduced the risk of reinfarction (1.6% vs. 2.2%; $P < .05$) and death (7.8% vs. 8.7%; $P < .05$) at the cost of an increase in major bleeding (1.1% vs. 0.4%; $P < .05$).

Most of the trials compared LMWH with heparin for the treatment of STEMI tested enoxaparin. Pooled data from eight trials involving 27,758 patients demonstrated that compared with heparin, initial LMWH reduced the risk of MI (2.1% vs. 3.9%; $P < .05$); rates of death were similar (5.3% vs. 5.8%) and there was more major bleeding (2.2% vs. 1.6%).[32] Consistent results were evident at 30 days.

The Fragmin during Instability in Coronary artery disease 1 (FRISC-1) trial evaluated the efficacy and safety of dalteparin in patients with NSTE ACS. Pooled data from FRISC-1 ($n = 1539$) and a second much smaller phase II study demonstrated that compared with placebo or no LMWH, LMWH reduced the risk of MI or death (1.6% vs. 5.2%; $P < .0001$) with no significant increase in major bleeding.

Multiple trials have compared LMWH with heparin in patients with NSTE ACS. A pooled analysis of five trials involving 12,171 patients demonstrated that LMWH and heparin were associated with similar rates of MI or death (2.2% vs. 2.3%) and major bleeding.[30] A subsequent meta-analysis involving 21,946 patients with NSTE ACS demonstrated that compared with heparin, enoxaparin reduced the risk of death or MI (10.1% vs. 11.0%; $P < .05$) with no increase in major bleeding.[31] The latter meta-analysis included some trials that did not involve the use of GPIIb/IIIa inhibitors and others in which they were widely used. The proportion of patients who underwent an invasive management strategy also varied across the trials.

Fondaparinux

Fondaparinux is a synthetic pentasaccharide that contains the essential five-sugar chain that binds antithrombin with high affinity and enhances the rate at which it inhibits factor Xa. Fondaparinux has a half-life of 17 hours, which enables once-daily subcutaneous administration and produces an even more predictable anticoagulant effect than LMWH and does not appear to cause heparin-induced thrombocytopenia. Protamine sulfate has no effect on the anticoagulant activity of fondaparinux.

Fondaparinux has been evaluated in patients with ACS in three large phases III randomized controlled trials. The OASIS-6 trial evaluated fondaparinux in 12,092 STEMI patients treated with single or dual antiplatelet therapy and fibrinolytic therapy.[33] Compared with placebo or heparin, fondaparinux (2.5 mg/day) reduced the risk of reinfarction or death (9.7% vs. 11.2%; $P = .008$) with consistent effects in patients with or without an indication for heparin therapy and in patients who received aspirin alone or the combination of aspirin and clopidogrel. Fondaparinux did not increase major bleeding. However, fondaparinux was associated with no benefit and a trend for harm in patients undergoing primary PCI.

The OASIS-5 trial evaluated fondaparinux in 20,078 patients with NSTE ACS treated with aspirin with or without clopidogrel. Fondaparinux (2.5 mg once daily) and enoxaparin (1 mg/kg twice a day) given for a mean of 6 days were associated with similar rates of the primary outcome of refractory ischemic, MI, or death at 9 days (5.8% vs. 5.7%), but there was a lower rate of major bleeding with fondaparinux (2.2% vs. 4.1%; $P < .001$).[34] At 30 days, fondaparinux was associated with a reduced rate of death compared with enoxaparin (2.9% vs. 3.5%; $P = .02$), which appeared to be explained primarily by the lower rate of bleeding. In 6238 invasively managed patients, fondaparinux and enoxaparin were associated with similar rates of the primary outcome but fondaparinux was associated with a small excess of catheter-related thrombosis (0.9% vs. 0.4%) and a reduction in major bleeding (2.4% vs. 5.1%; $P < .00001$). The risk of catheter thrombosis was largely prevented by the administration of IV heparin at the time of intervention.

The FUTURA OASIS-8 (Fondaparinux Trial With Unfractionated Heparin During Revascularization in Acute Coronary Syndromes) study compared the effect of standard-dose heparin (85 units/kg bolus with additional boluses based on an activated clotting time dosing algorithm) with reduced-dose heparin (50 units/kg without activated clotting time adjustment) on the primary outcome, a composite of bleeding or major vascular site access complications, in 2026 patients with NSTE ACS who were treated with fondaparinux and scheduled to undergo PCI within 72 hours.[35] The incidences of the primary outcome and catheter-related thrombosis were similar in the two heparin dose groups, but death, MI, or target vessel revascularization occurred more often in the low-dose heparin group. These data support the use of standard-dose heparin in patients with NSTE ACS who are treated with fondaparinux and scheduled to undergo PCI.

Bivalirudin

Bivalirudin is an IV direct thrombin inhibitor that binds thrombin and prevents it from interacting with its substrates. Bivalirudin has a half-life of only 25 minutes after IV injection, making the lack of an antidote less problematic than it is for longer-acting anticoagulants. It is about 20% renally cleared, and the half-life is prolonged in patients with renal impairment. Bivalirudin does not cause heparin-induced thrombocytopenia.

The efficacy and safety of direct thrombin inhibitors in ACS patients and those undergoing PCI were examined in the Direct Thrombin Inhibitor Trialists' Collaboration meta-analysis that included data from 11 trials and 35,970 patients.[36] The pooled data indicated a significant benefit of direct thrombin inhibitors (including bivalirudin) compared with heparin for the prevention of MI or death. Clopidogrel and GPIIb/IIIa inhibitors were not used in the trials included in this meta-analysis.

The HORIZONS AMI (Harmonizing Outcomes With Revascularization and Stents in Acute Myocardial Infarction) trial evaluated the use of bivalirudin in 3602 STEMI patients undergoing PCI who were treated with the combination of aspirin and clopidogrel.[37] Compared with heparin plus GPIIb/IIIa, bivalirudin plus provisional GPIIb/IIIa inhibitor was associated with similar rates of the primary outcome, MI, target vessel revascularization, stroke, or death (5.4% vs. 5.5%) at 30 days, but bivalirudin significantly reduced major bleeding (4.9% vs. 8.3%; $P < .0001$).

The EUROMAX (European Ambulance Acute Coronary Syndrome Angiography) trial[38] evaluated the use of prehospital bivalirudin in 2218 participants treated with aspirin and a P2Y12 receptor antagonist (more than 50% received ticagrelor or prasugrel) being transported for primary PCI. Compared with heparin (unfractionated or low-molecular-weight) plus optional GPIIb/IIIa inhibitor, bivalirudin reduced the risk of death or non-CABG major bleeding (5.1% vs. 8.5%; $P = .001$), a difference that was primarily driven by a reduction in major bleeding (2.6% vs. 6.0%; $P < .001$). Bivalirudin was associated with an increase in stent thrombosis within 24 hours (1.1% vs. 0.2%; $P = .007$).

The single-center, open-label HEAT-PPCI (unfractionated heparin versus bivalirudin in primary percutaneous coronary intervention) trial[39] evaluated the use of bivalirudin in 1829 participants treated with aspirin and a P2Y12 receptor antagonist (almost 90% received ticagrelor or prasugrel) undergoing emergency angiography. Compared with heparin (13% also received a GPIIb/IIIa inhibitor), bivalirudin (15% also received a GPIIb/IIIa inhibitor) was associated with an increase in the composite of death, stroke, reinfarction, or unplanned target lesion revascularization (8.7% vs. 5.7%; $P = .01$). The rates of major bleeding were similar (3.5% vs. 3.1%; $P = .59$), but there was more stent thrombosis with bivalirudin (3.4% vs. 0.9%; $P = .001$)

The BRIGHT (Bivalirudin vs. Heparin With or Without Tirofiban) trial[40] evaluated the use of bivalirudin continued for 30 minutes to 4 hours after PCI in 2194 participants with acute MI. Compared with heparin alone or heparin plus a GPIIb/IIIa inhibitor, bivalirudin reduced the risk of death, reinfarction, stroke, ischemia-driven target vessel revascularization, or any bleeding (8.8% vs. 13.2%; $P = .008$, and 8.8% vs. 17%; $P < .001$), primarily driven by a reduction in bleeding (4.1% vs. 7.5% vs. 12.3%; $P < .001$) with similar rates of major adverse cardiac or cerebral events (5.0% vs. 5.8% vs. 4.9%; $P = .74$) and stent thrombosis (0.6% vs. 0.9% vs. 0.7%; $P < .77$). Prolonged bivalirudin therapy after PCI might explain the lack of excess stent thrombosis that was seen in most of the earlier bivalirudin trials.

The ACUITY (Acute Catheterization and Urgent Intervention Triage Strategy) trial evaluated the use of bivalirudin in 13,819 patients with NSTE ACS undergoing PCI who were treated with the combination of aspirin plus clopidogrel as well as a GPIIb/IIIa inhibitor.[41] Bivalirudin was non-inferior to heparin or enoxaparin plus a G IIb/IIIa inhibitor for the prevention of MI, unplanned revascularization, or death at 30 days (7.8% vs. 7.3%) but was associated with a lower rate of major bleeding (3.0% vs. 5.7%; $P < .001$).

A meta-analysis of 16 trials involving 33,958 participants[42] showed that compared with heparin-based treatment regimens, bivalirudin was associated with increased major adverse cardiac events and stent thrombosis and a reduction in major bleeding, with no difference in death.

Oral Anticoagulation

Warfarin is an oral vitamin K antagonist that has been evaluated in the long-term management of patients with ACS who are treated with aspirin but has not been rigorously tested on a background of dual antiplatelet therapy. Furthermore, the efficacy and safety of the combination of aspirin plus warfarin have not been compared with those of dual antiplatelet therapy.

The results from a meta-analysis of 14 trials involving 25,307 patients demonstrated that compared with placebo or no warfarin, long-term warfarin (target INR, 2.0 to 3.0) reduced the risk of MI, ischemic stroke or death (9.4% vs. 12.3%; $P < .0001$) at the cost of a twofold increase in major bleeding (2.6% vs. 1.1%; $P < .00001$).[43]

Rivaroxaban and apixaban are oral direct factor Xa inhibitors that have undergone evaluation in phase III trials for the long-term management of patients with ACS. The ATLAS TIMI-51 trial compared rivaroxaban (2.5 or 5 mg twice a day) with placebo in 15,526 ACS patients, the majority of whom were also treated with dual antiplatelet therapy with aspirin plus clopidogrel.[25] Treatment was started a median of 4.7 days after onset of symptoms and was continued for a mean of 13 months. Rivaroxaban significantly reduced the risk of MI, stroke, or cardiovascular death (8.9% vs. 10.7%; $P = .008$) at the cost of an increase in non-CABG major bleeding (2.1% vs. 0.6%; $P < .001$) and intracranial bleeding (0.6% vs. 0.2%; $P = .009$).

The COMPASS trial evaluated the same doses of rivaroxaban shown to be effective in the ATLAS TIMI 51 trial for long term management in 27,395 patients with chronic coronary artery disease (CAD) or peripheral artery disease (PAD). During a mean of 23 months of follow up, the combination of rivaroxaban plus aspirin compared with aspirin alone reduced CV death, stroke or MI (4.1% vs. 5.4%, $P < .001$), and mortality (3.4% vs. 4.1%, $P = .01$) at the cost of an increase in major bleeding (3.1% vs. 1.9%, $P < .001$).

Based on the results of the ATLAS and COMPASS trials, rivaroxaban (2.5 mg twice daily) has been approved in Europe for post ACS patients, and in North America, Europe, and elsewhere for the long term management of patients with chronic CAD or PAD.

The APPRAISE trial compared apixaban (5 mg twice daily; 2.5 mg twice daily in selected patients) with placebo in patients with ACS. The trial was stopped early because of an excess of bleeding with no significant reduction in ischemic events.[26]

The oral direct thrombin inhibitor dabigatran etexilate has been tested in a phase II trial in ACS patients but has not undergone evaluation in a phase III trial (see Table 145.7 and box on Case 3: Coronary Artery Stenting in a Patient Who Requires Anticoagulant Therapy).

CONCLUSIONS AND FUTURE DIRECTIONS

Antiplatelet and anticoagulant drugs are effective for the prevention of MI and death across the spectrum of patients with ACS, including STEMI patients treated with fibrinolytic therapy and those undergoing PCI. Aggressive antithrombotic treatment regimens involving multiple antiplatelet drugs given in combination with an anticoagulant during the acute phase have substantially reduced the risk of early and late recurrent ischemic events and stent thrombosis in ACS patients but at the cost of an increased risk of bleeding. Clinicians require detailed knowledge of the pharmacology and side effects of antithrombotic therapies used in the management of patients with

ACS to optimize clinical outcomes. Comparative effectiveness studies are required to better identify the combinations of antithrombotic drugs that maximize the net clinical benefit for patients with ACS by minimizing the risk of both thrombotic and bleeding events.

TABLE 145.7 Strategies Aimed at Minimizing the Risk of Bleeding in Patients Treated With Combinations of Antiplatelet and Anticoagulant Therapies

Proposed Approach	Rationale
Aspirin maintenance dose ≤100 mg/day	Higher aspirin maintenance doses increase bleeding, and there is no evidence that they improve efficacy
PPI with a preference for agents that interfere less with CYP 2C19 (e.g., pantoprazole)	Much of the excess bleeding is from the GI tract. The use of acid-suppressive agents that interfere less with CYP 2C19 minimizes the potential for a negative interaction with clopidogrel
Preference for a direct oral anticoagulant	All direct oral anticoagulants are associated with lower rates of life-threatening bleeding than warfarin
Manage warfarin in a specialized anticoagulation clinic	Compared with usual care, specialist clinics achieve a greater time in the therapeutic range of the INR
Avoid NSAIDs	NSAIDs are a common cause of upper GI bleeding
Avoid prasugrel and ticagrelor	Prasugrel and ticagrelor are more potent than clopidogrel and cause more bleeding

CYP, Cytochrome P450; *GI*, gastrointestinal; *INR*, international normalized ratio; *NSAID*, nonsteroidal antiinflammatory drug; *PPI*, proton pump inhibitor.

CASE 3: CORONARY STENTING IN PATIENTS WHO REQUIRE ANTICOAGULANT THERAPY

A 55-year-old woman with a recent anterior myocardial infarction who underwent primary PCI with implantation of a drug-eluting stent in the left anterior descending coronary artery is found to have a left ventricular thrombus on transthoracic echocardiogram. She is taking aspirin and ticagrelor. She is switched from ticagrelor to clopidogrel and is started on intravenous heparin, which is overlapped with warfarin until an international normalized ratio of 2 is achieved. At the time of discharge from the hospital, aspirin is stopped and she continues on dual antithrombotic therapy with clopidogrel and warfarin.

Comment

Anticoagulation is indicated for the management of left ventricular thrombosis, and dual antiplatelet therapy is indicated for the management of patients with drug-eluting stents. The combination of an anticoagulant with dual antiplatelet therapy (i.e., triple therapy) is associated with a 2%–3% incidence of major bleeding during the first 30 days and a 4%–12% incidence during the first year.[44] Randomized trials have demonstrated that dual antithrombotic therapy with the combination of dabigatran etexilate (110 or 150 mg twice daily), apixaban (2.5 or 5.0 mg twice daily), rivaroxaban (15 or 20 mg once daily) or edoxaban (60 or 30 mg once daily), and clopidogrel substantially reduce bleeding compared with triple therapy in patients who have an indication for both anticoagulation and antiplatelet therapy after coronary artery stenting.[45–48]

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Amsterdam EA, Wenger NK, Brindis RG, et al. 2014 AHA/ACC guideline for the management of patients with non-ST-elevation acute coronary syndromes: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *J Am Coll Cardiol*. 2014;64:e139.

Antithrombotic Trialists' Collaboration. Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. *Lancet*. 2009;373:1849.

Boersma E, Harrington RA, Moliterno DJ, et al. Platelet glycoprotein IIb/IIIa inhibitors in acute coronary syndromes: a meta-analysis of all major randomised clinical trials. *Lancet*. 2002;359:189.

Bonaca MP, Bhatt DL, Cohen M, et al. Long-term use of ticagrelor in patients with prior myocardial infarction. *N Engl J Med*. 2015;372:1791.

Chan NC, Weitz JI. Antithrombotic agents. *Circ Res*. 2019;124:426.

Collins R, MacMahon S, Flather M, et al. Clinical effects of anticoagulant therapy in suspected acute myocardial infarction: systematic overview of randomised trials. *BMJ*. 1996;313:652.

Davì G, Patrono C. Platelet activation and atherothrombosis. *N Engl J Med*. 2007;357:2482.

Eikelboom JW, Connolly SJ, Bosch J, et al. Rivaroxaban with or without aspirin in stable cardiovascular disease. *N Engl J Med*. 2017;377:1319.

Eikelboom JW, Quinlan DJ, Mehta SR, et al. Unfractionated and low-molecular-weight heparin as adjuncts to thrombolysis in aspirin-treated patients with ST-elevation acute myocardial infarction: a meta-analysis of the randomized trials. *Circulation*. 2005;112:3855.

Holmes MV, Perel P, Shah T, et al. CYP2C19 genotype, clopidogrel metabolism, platelet function, and cardiovascular events: a systematic review and meta-analysis. *JAMA*. 2011;306:2704.

Fibrinolytic Therapy Trialists' (FTT) Collaborative Group. Indications for fibrinolytic therapy in suspected acute myocardial infarction: collaborative overview of early mortality and major morbidity results from all randomised trials of more than 1000 patients. *Lancet*. 1994;343:311.

Levine GN, Bates ER, Blankenship JC, et al. 2015 ACC/AHA/SCAI focused update on primary percutaneous coronary intervention for patients with ST-elevation myocardial infarction: an update of the 2011 ACCF/AHA/SCAI guideline for percutaneous coronary intervention and the 2013 ACCF/AHA guideline for the management of ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines and the Society for Cardiovascular Angiography and Interventions. *Circulation*. 2015;133:1135.

Marchini JF, Manica A, Croce K. Stent thrombosis: understanding and managing a critical problem. *Curr Treat Options Cardiovasc Med*. 2012;14:91.

Mauri L, Kereiakes DJ, Yeh RW, et al. Twelve or 30 months of dual antiplatelet therapy after drug-eluting stents. *N Engl J Med*. 2014;371:2155.

Mega JL, Braunwald E, Wiviott SD, et al. Rivaroxaban in patients with a recent acute coronary syndrome. *N Engl J Med*. 2012;366:9.

Patel SC, Mody A. Cerebral hemorrhagic complications of thrombolytic therapy. *Prog Cardiovasc Dis*. 1999;42:217.

Patrono C, García Rodríguez LA, Landolfi R, et al. Low-dose aspirin for the prevention of atherothrombosis. *N Engl J Med*. 2005;353:2373.

Rath D, Geisler T. Optimal antiplatelet and anticoagulation strategies in acute coronary syndromes. *Herz*. 2020;45(6):528–536.

Sibbing D, Angiolillo DJ, Huber K. Antithrombotic therapy for acute coronary syndrome: past, present and future. *Thromb Haemost*. 2017;117:1240.

Schüpke S, Neumann F-J, Menichelli M, et al. Ticagrelor or prasugrel in patients with acute coronary syndromes. *N Engl J Med*. 2019;381:1524.

Steg PG, Bhatt DL, Hamm CW, et al. Effect of cangrelor on periprocedural outcomes in percutaneous coronary interventions: a pooled analysis of patient-level data. *Lancet*. 2013;382:1981.

REFERENCES

1. White HD, Chew DP. Acute myocardial infarction. *Lancet*. 2008;372:570.
2. Sami S, Willerson JT. Contemporary treatment of unstable angina and non-ST-segment-elevation myocardial infarction (part 1). *Tex Heart Inst J*. 2010;37:141.
3. Sami S, Willerson JT. Contemporary treatment of unstable angina and non-ST-segment-elevation myocardial infarction (part 2). *Tex Heart Inst J*. 2010;37:262.
4. Davì G, Patrono C. Platelet activation and atherothrombosis. *N Engl J Med*. 2007;357:2482.
5. O'Gara PT, Kushner FG, Ascheim DD, et al. ACC/AHA guideline for the management of patients with ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *J Am Coll Cardiol*. 2013;61:e78–e140.
6. Levine GN, Bates ER, Blankenship JC, et al. 2015 ACC/AHA/SCAI focused update on primary percutaneous coronary intervention for patients with ST-elevation myocardial infarction: an update of the 2011 ACCF/AHA/SCAI Guideline for Percutaneous Coronary Intervention and the 2013 ACCF/AHA Guideline for the Management of ST-Elevation Myocardial Infarction. *J Am Coll Cardiol*. 2016;67:1235.
7. Indications for fibrinolytic therapy in suspected acute myocardial infarction: collaborative overview of early mortality and major morbidity results from all randomised trials of more than 1000 patients. Fibrinolytic Therapy Trialists' (FTT) Collaborative Group. *Lancet*. 1994; 343:311.
8. Patel SC, Mody A. Cerebral hemorrhagic complications of thrombolytic therapy. *Prog Cardiovasc Dis*. 1999;42:217.
9. Mehta SR, Eikelboom JW, Yusuf S. Risk of intracranial haemorrhage with bolus versus infusion thrombolytic therapy: a meta-analysis. *Lancet*. 2000;356:449.
10. Anderson JL, Adams CD, Antman EM, et al. ACCF/AHA focused update incorporated into the ACC/AHA 2007 guidelines for the management of patients with unstable angina/non-ST-elevation myocardial infarction: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *Circulation*. 2011;123:e426.
11. Patrono C, García Rodríguez LA, Landolfi R, et al. Low-dose aspirin for the prevention of atherothrombosis. *N Engl J Med*. 2005;353:2373.
12. Antithrombotic Trialists' Collaboration. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *BMJ*. 2002;324:71.
13. Mehta SR, Bassand JP, Chrolavicius S, et al. Dose comparisons of clopidogrel and aspirin in acute coronary syndromes. *N Engl J Med*. 2010;363:930.
14. Chen ZM, Jiang LX, Chen YP, et al. Addition of clopidogrel to aspirin in 45,852 patients with acute myocardial infarction: randomised placebo-controlled trial. *Lancet*. 2005;366:1607.
15. Yusuf S, Zhao F, Mehta SR, et al. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. *N Engl J Med*. 2001;345:494.
16. Wiviott SD, Braunwald E, McCabe CH, et al. Prasugrel versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med*. 2001;357:2007.
17. Roe MT, Armstrong PW, Fox KAA, et al. Prasugrel versus clopidogrel for acute coronary syndromes without revascularization. *N Engl J Med*. 2012;367:1297.
18. Wallentin L, Becker RC, Budaj A, et al. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med*. 2009;361:1045.
19. Marchini JF, Manica A, Croce K. Stent thrombosis: understanding and managing a critical problem. *Curr Treat Options Cardiovasc Med*. 2012;14:91.
20. Holmes MV, Perel P, Shah T, et al. CYP2C19 genotype, clopidogrel metabolism, platelet function, and cardiovascular events: a systematic review and meta-analysis. *JAMA*. 2011;306:2704.
21. Boersma E, Harrington RA, Moliterno DJ, et al. Platelet glycoprotein IIb/IIIa inhibitors in acute coronary syndromes: a meta-analysis of all major randomised clinical trials. *Lancet*. 2002;359:189.
22. De Luca G, Navarese E, Marino P. Risk profile and benefits from Gp IIb–IIIa inhibitors among patients with ST-segment elevation myocardial infarction treated with primary angioplasty: a meta-regression analysis of randomized trials. *Eur Heart J*. 2009;30:2705.
23. Steg PG, Bhatt DL, Hamm CW, et al. Effect of cangrelor on periprocedural outcomes in percutaneous coronary interventions: a pooled analysis of patient-level data. *Lancet*. 1981;382:2013.
24. Eikelboom JW, Weitz JI. New anticoagulants. *Circulation*. 2010;121:1523.
25. Mega JL, Braunwald E, Wiviott SD, et al. Rivaroxaban in patients with a recent acute coronary syndrome. *N Engl J Med*. 2011;366:9.
26. Alexander JH, Lopes RD, James S, et al. Apixaban with antiplatelet therapy after acute coronary syndrome. *N Engl J Med*. 2011;365:699.
27. Hirsh J, O'Donnell M, Eikelboom JW. Beyond unfractionated heparin and warfarin: current and future advances. *Circulation*. 2007;116:552.
28. Eikelboom JW, Quinlan DJ, Mehta SR, et al. Unfractionated and low-molecular-weight heparin as adjuncts to thrombolysis in aspirin-treated patients with ST-elevation acute myocardial infarction: a meta-analysis of the randomized trials. *Circulation*. 2005;112:3855.
29. Collins R, MacMahon S, Flather M, et al. Clinical effects of anticoagulant therapy in suspected acute myocardial infarction: systematic overview of randomised trials. *BMJ*. 1996;313:652.
30. Eikelboom JW, Anand SS, Malmberg K, et al. Unfractionated heparin and low-molecular-weight heparin in acute coronary syndrome without ST elevation: a meta-analysis. *Lancet*. 2000;355:1936.
31. Yusuf S, Mehta SR, Xie C, et al. Effects of reviparin, a low-molecular-weight heparin, on mortality, reinfarction, and strokes in patients with acute myocardial infarction presenting with ST-segment elevation. *JAMA*. 2005;293:427.
32. Quinlan DJ, Eikelboom JW. Low-molecular-weight heparin as an adjunct to thrombolysis in ST elevation myocardial infarction. *Arch Intern Med*. 2009;169:1163.
33. Yusuf S, Mehta SR, Chrolavicius S, et al. Effects of fondaparinux on mortality and reinfarction in patients with acute ST-segment elevation myocardial infarction: the OASIS-6 randomized trial. *JAMA*. 2006;295:1519.
34. Yusuf S, Mehta SR, Chrolavicius S, et al. Comparison of fondaparinux and enoxaparin in acute coronary syndromes. *N Engl J Med*. 2006;354:1464.
35. Steg PG, Jolly SS, Mehta SR, et al. Low-dose vs standard-dose unfractionated heparin for percutaneous coronary intervention in acute coronary syndromes treated with fondaparinux: the FUTURA/OASIS-8 randomized trial. *JAMA*. 2010;304:1339.
36. Direct Thrombin Inhibitor Trialists' Collaborative Group. Direct thrombin inhibitors in acute coronary syndromes: principal results of a meta-analysis based on individual patients' data. *Lancet*. 2002;359:294.
37. Stone GW, Witzenbichler B, Guagliumi G, et al. Bivalirudin during primary PCI in acute myocardial infarction. *N Engl J Med*. 2008;358:2218.
38. Steg PG, van 't Hof A, Hamm CW, et al. Bivalirudin started during emergency transport for primary PCI. *N Engl J Med*. 2013;369:2207.
39. Shahzad A, Kemp I, Mars C, et al. Unfractionated heparin versus bivalirudin in primary percutaneous coronary intervention (HEAT-PPCI): an open-label, single centre, randomised controlled trial. *Lancet*. 2014;384:1849.
40. Han Y, Guo J, Zheng Y, et al. Bivalirudin vs heparin with or without tirofiban during primary percutaneous coronary intervention in acute myocardial infarction: the BRIGHT randomized clinical trial. *JAMA*. 2015;313:1336.
41. Stone GW, McLaurin BT, Cox DA, et al. Bivalirudin for patients with acute coronary syndromes. *N Engl J Med*. 2006;355:2203.
42. Cavender MA, Sabatine MS. Bivalirudin versus heparin in patients planned for percutaneous coronary intervention: a meta-analysis of randomised controlled trials. *Lancet*. 2014;384(9943):599–606.
43. Andreotti F, Testa L, Biondi-Zoccai GGL, et al. Aspirin plus warfarin compared to aspirin alone after acute coronary syndromes: an updated and comprehensive meta-analysis of 25,307 patients. *Eur Heart J*. 2006;27:519.
44. Angiolillo DJ, Goodman SG, Bhatt DL, et al. Antithrombotic therapy in patients with atrial fibrillation treated with oral anticoagulation undergoing percutaneous coronary intervention: a North American perspective-2018 update. *Circulation*. 2018;138:527–536.
45. Gibson CM, Mehran R, Bode C, et al. Prevention of bleeding in patients with atrial fibrillation undergoing PCI. *N Engl J Med*. 2016;375:2423.
46. Cannon CP, Bhatt DL, Oldgren J, et al. Dual antithrombotic therapy with dabigatran after PCI in atrial fibrillation. *N Engl J Med*. 2019;377:1513.
47. Lopes RD, Heizer G, Aronson R, et al. Antithrombotic therapy after acute coronary syndrome or PCI in atrial fibrillation. *N Engl J Med*. 2019;380:1509.
48. Vranckx P, Valgimigli M, Eckardt L, et al. Edoxaban-based versus vitamin K antagonist-based antithrombotic regimen after successful coronary stenting in patients with atrial fibrillation (ENTRUST-AF PCI): a randomised, open-label, phase 3b trial. *Lancet*. 2019;394(10206):1335.

CHAPTER 146

PERIPHERAL ARTERY DISEASE

Stanislav Henkin and Mark A. Creager

Peripheral artery disease (PAD) is an important manifestation of systemic atherosclerosis with significant morbidity and mortality.PAD affects the lower extremities and is defined as a stenosis or occlusion in the aorta or the arteries supplying blood to the legs, including the iliac, femoral, popliteal, or infrapopliteal vessels (peroneal, posterior tibial, and anterior tibial arteries). Stenosis is typically caused by atherosclerosis. Nonatherosclerotic causes of vascular disease also can obstruct the peripheral arteries (see later discussion). There are two major clinical consequences of PAD. First, PAD can cause leg symptoms that include intermittent claudication, which impairs walking ability and diminishes the quality of life, and rest pain, which occurs when there is critical limb ischemia (CLI). Second, as an atherosclerotic disorder, PAD is associated with as much as a four- to six-fold increased risk of cardiovascular death, myocardial infarction (MI), and stroke. This chapter reviews the epidemiology, pathophysiology, and management of PAD.

EPIDEMIOLOGY

Prevalence and Incidence

The prevalence of PAD has been determined from several epidemiologic studies. Early studies determined the prevalence of PAD from the presence of symptoms, such as intermittent claudication, or a history of peripheral revascularization. Many patients with PAD are asymptomatic, and the use of noninvasive diagnostic testing, specifically the measurement of the ankle-brachial index (ABI), has provided further clarification of the overall prevalence of the disease.[1–3] In most of these studies, an ABI of 0.90 or less was used to define PAD. The prevalence of PAD in adults 40 years of age or older is estimated to be 7.2%, accounting for approximately 8.5 million adults in the United States alone.[4] In addition, PAD is estimated to affect as many as 202 million individuals worldwide, with increasing prevalence in both high and low-middle income countries.[4,5] There is a sharp increase in the prevalence of PAD with increasing age, approximating 16.8% of women and 19.8% of men older than age 65 years in the German Epidemiological Trial on ABI (GetABI study).[6] There is a similar trend in the US, as ~20% of adults older than 70 years have PAD, with a higher prevalence among those with a history of diabetes or smoking. Recent studies have also shown higher PAD prevalence in populations of lower socioeconomic status, including lower education and lower income levels, as well as a disproportionate burden among some race/ethnicity groups, particularly blacks, as compared with Hispanic or non-Hispanic white populations.[7,8]

The incidence of PAD, which is largely based on the development of symptomatic disease, is less well established. Data from the Framingham Heart Study show an incidence rate of intermittent claudication of less than 0.4 per 1000 per year in younger men (35 to 45 years) and a rate as high as 6 per 1000 per year in older men (older than 65 years). The incidence of symptomatic PAD is lower in women in most age groups, although the estimates are more comparable in the oldest age group. Estimates of the incidence of PAD based on ABI are less commonly reported. One such study reports an incidence of 1.7 per 1000 person-years for ages 40 to 54 years, 1.5 per 1000 person-years for ages 55 to 64 years, and 17.8 per 1000 person-years for ages 65 years and older. When the diagnosis of PAD is based on ABI alone, the differences in incidence between men and women are less evident. In the Cardiovascular Health Study, for example, there are no gender differences in the incidence of PAD based on ABI after adjusting for cardiovascular risk factors. At the other end of the spectrum, CLI represents approximately 11% of the patients with PAD.[9] The incidence of CLI is estimated to be approximately 2% per year in the US or 400 to 1000 per million individuals per year.[3,9]

Risk Factors

Atherosclerotic risk factors, including smoking, diabetes, hypertension, hyperlipidemia, renal insufficiency, and inflammation, contribute to the development of PAD (see Chapter 142).

In virtually all population-based studies, smoking has been one of the strongest risk factors for PAD. The risk is highest for current smokers compared with nonsmokers, with two to four times increased odds of PAD and the risk of PAD increases in a dose-dependent manner relative to the number of cigarettes smoked and the duration of tobacco use. In the Women's Health Study, smoking more than 15 cigarettes per day increased the risk of incident PAD approximately 17-fold, and the risk was lower in former smokers than in active smokers.[10] In the Edinburgh Artery Study, smoking was two to three times more likely to cause lower extremity PAD compared with coronary artery disease (CAD).[11]

Diabetes is also a potent risk factor for PAD and increases the risk of PAD by two- to four-fold. Data from the Rotterdam study and the San Luis Valley Diabetes study reveal that upwards of 12% to 20% of individuals with PAD have coexisting diabetes.[12,13] Moreover, the risk of PAD increases depending on the duration and severity of diabetes. In the Strong Heart Study, individuals with PAD had a more than two-fold higher prevalence of diabetes compared with those without PAD, and diabetes tended to be of longer duration and associated with higher glycosylated hemoglobin levels.[14] Patients with PAD who have concomitant diabetes are also more likely to develop intermittent claudication and ischemic ulceration and to require major amputation compared with those without diabetes.

Hypertension is a more modest risk factor for the development of PAD compared with its importance as a risk factor for coronary and cerebrovascular disease.[1] Although evidence suggests that hypertension increases the prevalence of PAD by 1.5- to 2.2-fold, the association of hypertension with an incident or symptomatic PAD is less clear. Among Native Americans in the Strong Heart Study, those with PAD had a higher mean systolic blood pressure, and the prevalence of PAD was significantly higher in those with established hypertension.[14] In the Framingham Heart Study, hypertension increased the risk of developing intermittent claudication.[15] In the Women's Health study, the risk of incident PAD increased by 43% with every 10 mm Hg increase in systolic blood pressure.[16] In the Atherosclerosis Risk in Communities (subjects with diabetes) study, individuals with systolic blood pressure greater than 130 mm Hg were significantly more likely to develop PAD.[17] In the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial, both low (<120 mm Hg) and high (>160 mm Hg) systolic blood pressures were associated with PAD events (PAD-related hospitalization, PAD-related procedure, outpatient PAD medical therapy, or PAD-related death).[18]

Dyslipidemia, specifically elevated total cholesterol, low-density lipoprotein (LDL) cholesterol and triglycerides, and reduced high-density lipoprotein (HDL) cholesterol, is associated with PAD. Epidemiologic studies, such as the Cardiovascular Health Study and the Framingham Heart Study, have shown that a 10-mg/dL increase in total cholesterol increases the risk of PAD by 5% to 10%. In the Strong Heart study, individuals with PAD had significantly higher levels of total cholesterol, triglycerides, and LDL cholesterol than those without. The Whitehall study also demonstrated that an elevated total cholesterol level was associated with symptoms of intermittent claudication.[19]

Chronic renal insufficiency has been recognized to be significantly associated with PAD in several studies. In the NHANES, renal insufficiency (defined as a glomerular filtration rate <60 mL/min) was associated with a 2.5-fold higher odds of PAD even after adjustment for other cardiovascular risk factors.[20] In the Heart and Estrogen/Progesterone Replacement (HERS) study, renal insufficiency was also associated with an increased risk of incident peripheral vascular events, including revascularization and amputation.[21] In The Chronic Renal Insufficiency Cohort study (CRIC), women less than 70 years old were 50% more likely than men to develop PAD.[22] Moreover, renal insufficiency or albuminuria increases the mortality risk in patients with PAD irrespective of other risk factors, including diabetes.

Several nontraditional factors have been associated with PAD. Markers of systemic inflammation, such as C-reactive protein (CRP), are elevated in patients with PAD. In the Physicians' Health Study, the risk of developing symptomatic PAD was approximately two-fold higher in those in the highest CRP quartile compared with those in the lowest quartile.[23] Although several markers of inflammation, such as soluble intercellular adhesion molecule 1, chemokine ligand 2, galactin-3, and others have been studied in patients with PAD, none are routinely measured in clinical practice. Insulin resistance is associated with both prevalent PAD as shown in data from the NHANES study and with incident PAD as demonstrated in the Cardiovascular Health Study.

PATHOBIOLOGY

Atherosclerosis is a progressive vascular disease characterized by lipid accumulation and the formation of plaque in the arterial walls (see Chapter 145). The pathophysiology of atherosclerosis includes endothelial dysfunction, vascular inflammation, and cellular proliferation. Early in the atherogenic process, recruitment of inflammatory cells and accumulation of lipids promote the development of a lipid-rich atheroma. Inflammation promotes the elaboration of proteases that weaken the vessel wall and allow positive remodeling with the outward expansion of the arterial wall to accommodate the intimal expansion that occurs as a result of plaque formation. Although positive remodeling initially preserves the arterial lumen, continued plaque growth results in progressive narrowing of the lumen, which then limits blood flow and oxygen supply to target organs. This process may be enhanced by biomechanical factors, such as turbulent blood flow, particularly in areas of altered shear stress. This phenomenon is of particular significance at branch points along the arterial tree, which is predisposed to atherosclerotic plaque formation.

Pathologic studies primarily involving coronary arteries have shown that atherosclerotic plaque formation is a dynamic biologic process that exhibits marked heterogeneity; some plaques remain "stable," while others have a more "unstable" pathophysiology. Stable atherosclerotic plaques may be asymptomatic, or symptoms can occur with exertion if demand exceeds supply. On the other hand, "vulnerable" or unstable plaques are prone to acute rupture, and superimposed thrombi may cause sudden arterial insufficiency, and cause events such as MI, or theoretically acute limb ischemia (see Chapters 142 and 145). Whereas atherosclerotic plaque contributes importantly to PAD, recent pathologic studies have demonstrated an important role of thrombosis detected in amputated limbs of patients with CLI.[24] Specifically, in almost 75% of patients with CLI, thrombosis contributed to luminal stenosis. Significant atherosclerosis, often with associated thrombus, was found in most lesions in the femoral-popliteal arteries, whereas thrombus without significant atherosclerosis was found in the majority of infrapopliteal arteries, indicative of embolism from a more proximal site, or thrombosis in situ. It is notable that a recent genome-wide association study showed an association between PAD and Factor V Leiden (FVL; F5 p.R506Q), further highlighting the potential pathophysiologic role of thrombosis in PAD.[25]

Although PAD is mostly caused by atherosclerosis, other causes include thromboembolism, vasculitides (e.g., thromboangiitis obliterans, giant cell arteritis, Takayasu arteritis), trauma, popliteal artery entrapment, cystic adventitial disease, fibromuscular dysplasia, and endofibrosis of the iliac artery (Table 146.1). Nonvascular causes of leg pain should also be considered in the differential diagnosis, including lumbosacral spine disease (causing pseudoclaudication), venous diseases, hip, knee, or ankle osteoarthritis, chronic compartment syndrome, myositis, and others (Table 146.2).

TABLE 146.1 Nonatherosclerotic Causes of Peripheral Artery Disease

- Thromboembolism
- Atheroembolism
- Vasculitides
- Large vessel vasculitides, such as giant cell arteritis and Takayasu arteritis
- Small vessel vasculitides, such as thromboangiitis obliterans (Buerger disease)
- Trauma
- Popliteal artery entrapment
- Cystic adventitial disease
- Fibromuscular dysplasia
- Iliac artery endofibrosis

TABLE 146.2 Nonarterial Causes of Leg Pain (Differential Diagnosis for Intermittent Claudication Symptoms)

- Lumbar radiculopathy
- Spinal stenosis
- Hip or knee osteoarthritis
- Myositis
- Venous claudication

CLINICAL MANIFESTATIONS

Most patients with PAD are asymptomatic at presentation. Typical claudication symptoms are present in only 10% to 35% of patients and CLI in approximately 11%.[1] The classic symptoms of PAD include intermittent claudication and rest pain, the latter occurring in patients with CLI. Intermittent claudication is defined as exertional discomfort in the muscles of the lower extremities that is variably described as pain, aching, burning, fatigue, or heaviness. Symptoms arise with leg exercise, typically walking, and are relieved after a predictable duration of rest (usually <10 minutes). Intermittent claudication occurs with effort and not at rest, and symptoms do not abate until activity ceases; a change in position is unnecessary. Although many patients with PAD report atypical symptoms, most have impaired walking ability exemplified by reduced walking speed or distance. CLI arises when there is inadequate perfusion to meet the resting metabolic demands of the tissues. Patients with CLI have pain at rest, typically affecting the toes, feet, or both; they may have accompanying tissue loss with nonhealing ulcers, tissue necrosis, or gangrene (Fig. 146.1).

DIAGNOSIS

The diagnosis of PAD is often evident from the history and physical examination. An important diagnostic feature is diminished or absent pulses in the legs. The examiner should palpate the femoral,

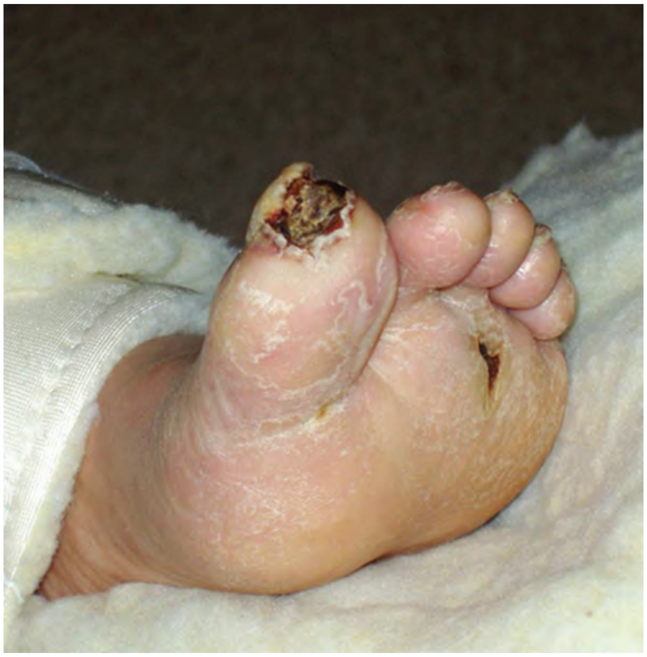


Figure 146.1 Ulceration and gangrene of the foot representative of critical limb ischemia in a patient with peripheral artery disease.

popliteal, dorsalis pedis, and posterior tibial pulses. The absence of selected pulses provides insight into the location of critical stenoses. The groin should be auscultated for femoral artery bruits, which may be indicative of turbulent flow from atherosclerotic plaque. Other findings suggestive of PAD include pallor of the soles of the feet upon leg elevation and the development of rubor when the feet are then placed in the dependent position. Signs of chronic limb ischemia include muscle atrophy; hair loss; thickened nails; and in severe stages, cyanosis, pallor, and coolness of the skin of the feet.

Ankle–Brachial Index

The ABI is a simple, non-invasive test for the diagnosis of PAD.[26] Normally, when measured in the supine position, the systolic blood pressure in the legs is the same as that in the arms. However, pulse wave amplification may yield a higher systolic pressure at the ankle. Therefore, the ratio of the ankle to the brachial systolic pressure, designated as the ABI, should be 1.0 or slightly higher. A diminution of the ankle systolic blood pressure relative to the brachial artery pressure indicates a stenosis or occlusion in the aorta or arteries of the lower extremities.

The ABI is determined by measuring the systolic blood pressure in both arms (brachial arteries) and both ankle arteries (dorsalis pedis and posterior tibial arteries) after the patient has been in the supine position for at least 5 to 10 minutes.[1] To measure these pressures, sphygmomanometric cuffs are sequentially inflated at each location to suprasystolic pressures. The onset of systole with subsequent cuff deflation is determined with a Doppler device that is placed over the artery. The ABI for each leg is calculated by dividing the higher of the two ankle pressures by the higher of the two arm pressures. Considering the intrinsic variability in blood pressure over time, an ABI of 0.90 or less is indicative of PAD. At this threshold, the ABI has excellent sensitivity (90%) and specificity (>95%) compared with angiography. An ABI of 0.91 to 1.0 is considered borderline.[1,2] Vascular calcification, as often occurs in patients with diabetes or renal insufficiency, may preclude accurate determination of systolic blood pressure at the ankle. For this reason, an ABI that is markedly elevated (e.g., >1.4) is considered inaccurate and indicative of vascular calcification. In this circumstance, other simple noninvasive diagnostic tests, such as assessment of the toe–brachial index, pulse volume recording (PVR), or continuous wave Doppler may be useful to detect PAD. In some cases of PAD, the ABI is normal at rest. This is particularly common in patients who have a proximal disease, such as iliac artery stenosis, and an extensive collateral circulation. In such cases, measurement of the ABI after walking will detect a decrease in the ankle systolic pressure relative to brachial artery systolic pressure, thereby revealing the presence of PAD.

Noninvasive Testing and Imaging for Diagnosis of Peripheral Artery Disease

Several other noninvasive tests may help in the diagnosis of PAD and the identification of sites of stenosis. These tests include segmental pressures with PVR or continuous wave Doppler, duplex ultrasonography, computed tomography angiography (CTA), magnetic resonance angiography (MRA), and conventional contrast angiography. When measuring segmental leg pressures, systolic blood pressure measurements are obtained at multiple levels in the leg, typically in the upper thigh, lower thigh, upper calf, ankle, and across the metatarsal region of the foot. Systolic blood pressures in these sites are then compared with the higher of the arm systolic blood pressures. A significant drop in blood pressure (>20 mm Hg) from one level to the next can localize arterial stenosis with a high degree of precision. An upper thigh pressure that is lower than the arm pressure indicates stenosis in the distal aorta or the iliac or femoral arteries (or both). PVRs, using a plethysmographic instrument that records the change in volume with each arterial pulsation, or continuous wave Doppler with a hand-held Doppler instrument can be obtained at each level. A normal PVR waveform resembles an arterial waveform with a brisk upstroke and a prominent dicrotic notch in the downstroke (Fig. 146.2). Abnormal waveforms, which appear distal to a hemodynamically significant stenosis, have a parvus et tardus appearance with a blunted upstroke and decreased pulse amplitude. The normal Doppler waveform is made up of three components, including rapid antegrade flow during systole, transient reversal of flow during early diastole, and slow antegrade flow during late diastole.

Duplex ultrasonography is used both for the diagnosis of PAD and for the surveillance of bypass grafts or stents after revascularization procedures. Color Doppler can identify abnormal flow with turbulence and Doppler aliasing suggesting an area of stenosis (Fig. 146.3). Pulse-Doppler sampling can then confirm flow acceleration in a diseased segment. A peak systolic velocity (PSV) in a diseased segment that is more than twice the PSV in the proximal segment indicates a hemodynamically significant stenosis of at least 50%. Duplex ultrasonography has been shown to be up to 95% accurate and reproducible with sensitivity and specificity of 88% to 92% and 79% to 97%, respectively, compared with angiography.[27,28] However, duplex ultrasonography is a time-consuming and operator-dependent procedure.

The most used imaging modalities for diagnosis of PAD are MRA and CTA. The two tests have comparable diagnostic accuracy for identification of arterial stenosis. MRA has been shown to have a sensitivity of 95% and specificity of 97% to detect stenosis greater than 50%, and CTA was shown to have a sensitivity of 91% and specificity of 91%.[29]

MRA takes advantage of the inherent magnetic properties of human tissue. Pulsed magnetic sequences cause protons within cells to spin and align, generating a frequency of energy that can be detected by the scanner. Various tissues have different frequencies that allow delineation of the structures and tissues within the body. The addition of the paramagnetic contrast agent gadolinium allows selective imaging of moving blood (Fig. 146.4). This flow-related enhancement of the vasculature produces angiographic images. Although MRA has the advantage of using nonionizing radiation, it has several limitations. For example, MRA cannot be performed in patients with some implanted cardiac devices or other metal objects. Although MRA can be used in patients with vascular stents, ferromagnetic metals in the stents may produce artifacts that limit the assessment of the stented vessel. Claustrophobia may also preclude MRA.

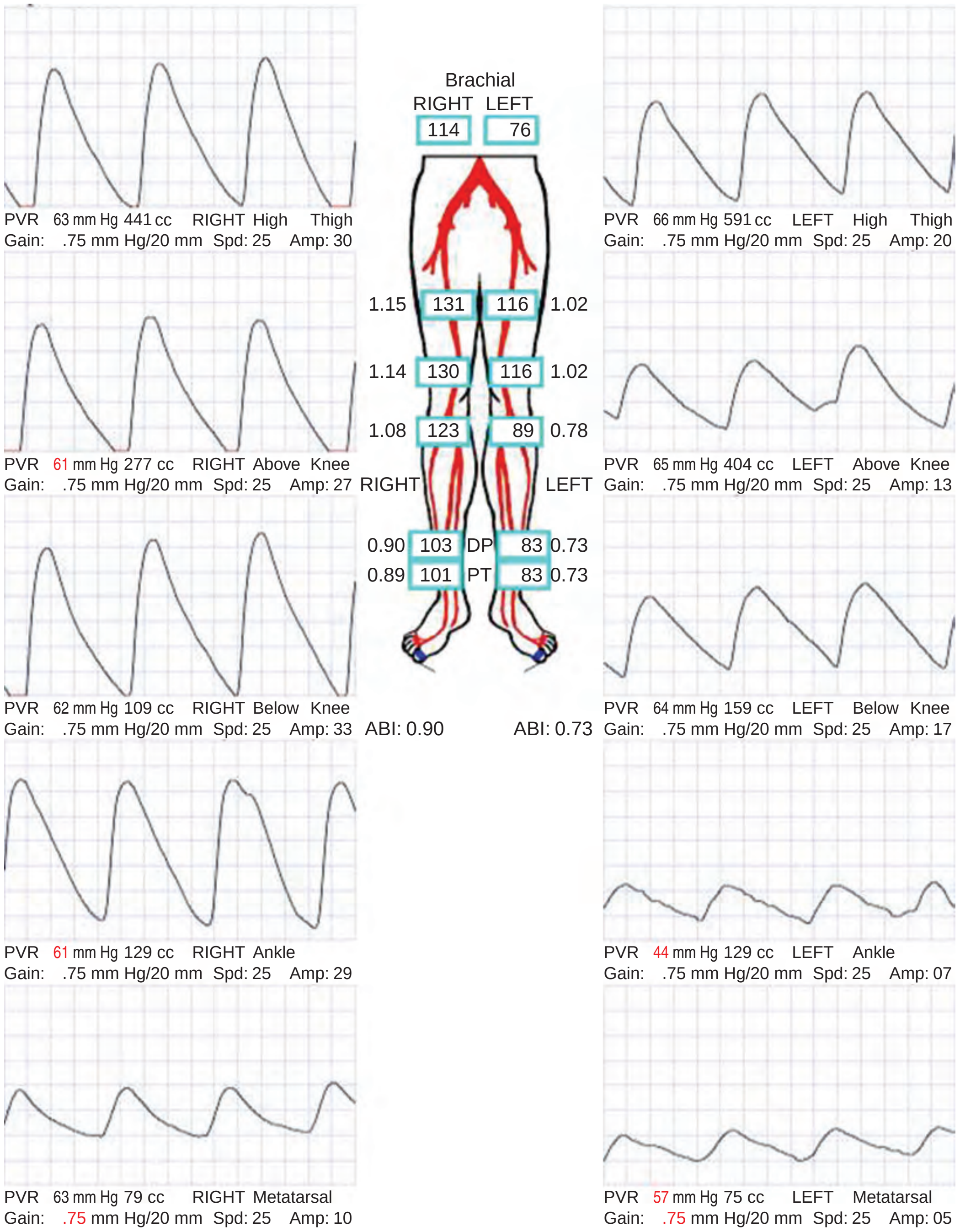

Brachial
RIGHT LEFT
114
76
PVR 63 mm Hg 441 cc RIGHT High Thigh
Gain: .75 mm Hg/20 mm Spd: 25 Amp: 30
PVR 66 mm Hg 591 cc LEFT High Thigh
Gain: .75 mm Hg/20 mm Spd: 25 Amp: 20
1.15 131
116 1.02
1.14 130
116 1.02
1.08 123
89 0.78
PVR 61 mm Hg 277 cc RIGHT Above Knee
Gain: .75 mm Hg/20 mm Spd: 25 Amp: 27
RIGHT
LEFT
PVR 65 mm Hg 404 cc LEFT Above Knee
Gain: .75 mm Hg/20 mm Spd: 25 Amp: 13
0.90 103 DP 83 0.73
0.89 101 PT 83 0.73
PVR 62 mm Hg 109 cc RIGHT Below Knee
Gain: .75 mm Hg/20 mm Spd: 25 Amp: 33
ABI: 0.90
ABI: 0.73
PVR 64 mm Hg 159 cc LEFT Below Knee
Gain: .75 mm Hg/20 mm Spd: 25 Amp: 17
PVR 61 mm Hg 129 cc RIGHT Ankle
Gain: .75 mm Hg/20 mm Spd: 25 Amp: 29
PVR 44 mm Hg 129 cc LEFT Ankle
Gain: .75 mm Hg/20 mm Spd: 25 Amp: 07
PVR 63 mm Hg 79 cc RIGHT Metatarsal
Gain: .75 mm Hg/20 mm Spd: 25 Amp: 10
PVR 57 mm Hg 75 cc LEFT Metatarsal
Gain: .75 mm Hg/20 mm Spd: 25 Amp: 05

Figure 146.2 SEGMENTAL LEG PRESSURE MEASUREMENTS AND PULSE VOLUME RECORDINGS. The ankle–brachial index (ABI) is normal in the right leg. The greater than 20-mm Hg drop in systolic blood pressure between the lower thigh and the calf in the left leg suggests stenosis involving the distal left superficial femoral artery, popliteal artery, or both. There is also evidence of blunting of the pulse volume recording with a parvus et tardus waveform. The significant difference in brachial artery systolic blood pressure is suggestive of left subclavian artery stenosis. *Amp*, Amplitude; *DP*, dorsalis pedis; *PT*, posterior tibial; *PVR*, pulse volume recordings; *Spd*, speed.

Like MRA, CTA also has excellent specificity and sensitivity for detection of arterial stenosis. The advent of large-volume imaging with multidetector scanners enables rapid image acquisition and high resolution. Advantages of CTA include the capacity to rapidly visualize the entire arterial tree and to delineate vascular calcification and intraluminal thrombus. Limitations of the test include the exposure to ionizing radiation and the need for iodinated contrast agents, which is problematic in patients with renal impairment. In addition, extensive arterial calcification may prevent accurate determination of the degree of stenosis.

MRA and CTA have largely supplanted conventional catheter-based angiography for PAD diagnosis. Nonetheless, invasive contrast angiography remains the gold standard for the diagnosis of PAD. Catheter-based angiography is most useful in situations in which concurrent endovascular interventions are planned or in preparation for surgical revascularization. Limitations to catheter-based angiography include the invasive nature of the procedure, the need to administer iodinated contrast, and the radiation exposure. Potential complications include arteriovenous fistula or pseudoaneurysm formation at the access site, atheroembolism, dissection, and contrast-induced renal insufficiency. Alternatives to iodine-based contrast agents, such as carbon dioxide and gadolinium, can be used when administration of iodinated contrast is contraindicated.

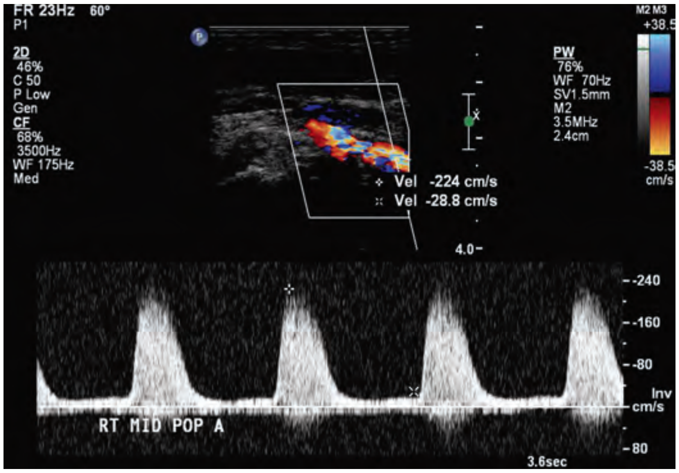


Figure 146.3 DUPLEX ARTERIAL ULTRASOUND DEMONSTRATING DOPPLER INTERROGATION OF THE POPLITEAL ARTERY. Turbulence of color Doppler flow indicates possible stenosis, confirmed by elevated peak systolic velocity, monophasic waveform, and spectral broadening.

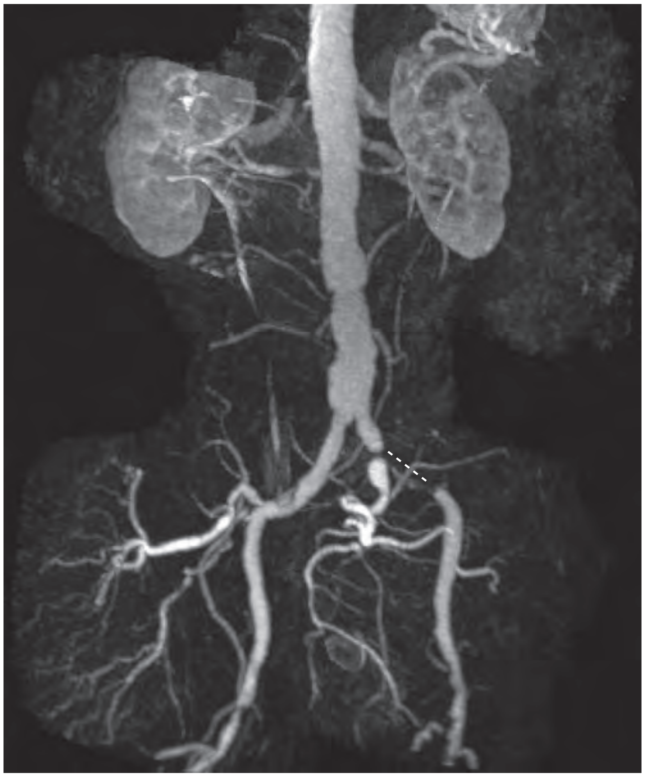


Figure 146.4 Representative projection imaging from a gadolinium-enhanced magnetic resonance angiogram demonstrating segmental occlusion of the left common iliac artery (indicated by *dashed white line*), as well as nonobstructive atherosclerotic disease in the distal aorta and more distally in the external iliac arteries.

PROGNOSIS

Prognostic considerations are broadly divided into limb outcomes and overall cardiovascular outcomes. Limb prognosis is dependent on the severity of symptoms at initial presentation; concurrent risk factors, such as cigarette smoking and diabetes; and the likelihood of successful revascularization in those with threatened limb viability. Among patients with claudication symptoms, leg discomfort remains stable in the majority (≈70% to 80%), worsens in about 10% to 20%, and progresses to CLI in less than 1%.[1,9] Data from the Reduction of Atherothrombosis for Continued Health (REACH) registry, which enrolled patients with established PAD on the basis of clinical symptoms, revealed that patients with abnormal ABI and prior revascularization had a 23.6% rate of new adverse limb events over 4 years including new revascularization procedures and ischemic amputations.[30] These data are consistent with findings from the TRA2 P-TIMI 50 trial, which evaluated the impact of vorapaxar (see Chapter 142) in patients with PAD, where the rate of peripheral artery revascularization in the placebo-treated group was 22.2% over a median period of 3 years.[31] Prognosis is the worst for those with CLI, where mean amputation-free survival at 1 year is only 50%. Outcomes are worse for patients with PAD who continue to smoke or have coexisting comorbidities (diabetes, renal insufficiency, CAD); such patients have even higher rates of ischemic ulceration and amputation.

Given the high risk of concomitant coronary and cerebral artery disease, individuals with PAD are also at increased risk of MI, stroke, and cardiovascular death (see Chapters 144 and 145). The mortality rate is increased two- to four-fold in patients with PAD (ABI <0.9) compared with those without PAD, as confirmed in a meta-analysis of 16 cohort studies from the ABI collaboration.[32] Furthermore, a graded association has been noted with lower ABI associated with increased mortality.[32] Also, individuals with high ABI (≥1.4) but without baseline cardiovascular disease are three times as likely to die from cardiovascular disease compared with those with normal ABI.[33] In patients with PAD, the risk of MI is increased by 20% to 60%, and the risk of stroke is increased by 40%. In the TRA 2°P-TIMI 50 trial, the 3-year event rate for the composite of cardiovascular death, MI, or stroke was 7.6% in individuals with PAD alone, and 12.8% in individuals with concomitant PAD and CAD.[34] In the COMPASS trial, the adverse cardiovascular event rate in the PAD group for the same composite outcome was 7% over a median of 21 months.[35]

THERAPY

The major goals in the management of patients with PAD are to (1) reduce the risk of cardiovascular morbidity and mortality and (2) improve lower extremity symptoms and preserve limb viability. Aggressive cardiovascular risk factor modification is indicated for all patients with PAD. This includes treatment of dyslipidemia, hypertension, and diabetes (including regular foot care) and the use of antiplatelet therapy. Encouragement of healthy lifestyle habits remains a cornerstone of the management of individuals with PAD; these include smoking cessation, a diet enriched in fruits and vegetables and limited in saturated fats, and regular physical activity.[1]

Dyslipidemia

Treatment of dyslipidemia with statins decreases major cardiovascular events in patients with all manifestations of atherosclerosis (see Chapter 142). The 4S study (Scandinavian Simvastatin Survival Study) was one of the first to demonstrate a clear benefit of lipid-lowering therapy for secondary prevention in patients with atherosclerosis and dyslipidemia.[36] Subsequently, the Heart Protection Study demonstrated that statin therapy was associated with a 22% relative risk (RR) reduction in adverse cardiovascular events in patients with vascular disease, including PAD.[37] Recent practice guidelines recommend the use of high-intensity statin therapy, such as atorvastatin 80 mg daily or rosuvastatin 20 mg daily, for individuals with established atherosclerotic vascular disease, including those with PAD to lower cholesterol (LDL <70 mg/dL) and to reduce the risk of atherosclerotic events in this population. Statin use is not only associated with lower cardiovascular event rates, but also with approximately 18% lower rates of adverse limb outcomes, including worsening symptoms, peripheral revascularization, and amputations. Individuals with recurrent atherosclerotic events or LDL ≥70 mg/dL may benefit from ezetimibe or a PCSK9 inhibitor in addition to a maximally tolerated statin.[38] In the Further Cardiovascular Outcomes Research with PCSK9 Inhibition in Subjects with Elevated Risk (FOURIER) trial, the PCSK9 inhibitor evolocumab reduced the composite outcome of cardiovascular death, MI, stroke, unstable angina, or coronary revascularization by 21% in individuals with PAD. Importantly, individuals with PAD had a larger absolute risk reduction in key outcomes compared to those without PAD.[39]

The appropriate management of non-LDL cholesterol (HDL and triglycerides) is less clear. Although reduced HDL levels are a risk factor for atherosclerosis, studies with medications that increase HDL levels and lower triglyceride levels have yielded conflicting results (see Chapter 142). The VA-HIT study (Veterans Affairs High-Density Lipoprotein Intervention Trial) showed that gemfibrozil reduced the risk of fatal coronary disease or nonfatal MI by 22% over a median of 5.1 years in patients with known CAD.[40] However, in a study of patients with PAD, bezafibrate did not affect the rate of coronary events or stroke.[41] In addition, in the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study, fenofibrate did not reduce coronary events in diabetics, although its use was associated with fewer nonfatal MIs and revascularization procedures.[42] Although niacin also reduces lipid levels, its efficacy for reducing cardiovascular events has been called into question based on recent studies showing no added benefit in patients with established atherosclerotic vascular disease.[43] Subgroup analyses showed no meaningful differences in the subset of patients with established PAD.

Hypertension

Treatment of hypertension is a critical component of the management of patients with atherosclerotic PAD. Such treatment reduces the risk of stroke, MI, and congestive heart failure. Management of hypertension should follow current guidelines.[44] Although there is concern that lower systemic blood pressure may exacerbate symptoms of claudication or CLI, the majority of patients experience no change in their symptoms. Several studies have found that antihypertensive drugs reduce cardiovascular events in patients with PAD. The HOPE (Heart Outcomes Prevention) study demonstrated a 22% reduction in cardiovascular events with ramipril in patients with atherosclerotic disease, including those with PAD.[45] ONTARGET (Ongoing Telmisartan Alone and in Combination with Ramipril Global Endpoint Trial) demonstrated that telmisartan and ramipril produced similar reductions in cardiovascular death, MI, stroke, or hospitalization for heart failure (16.7% and 16.5%, respectively; RR, 1.01; 95% confidence interval, 0.94 to 1.09), albeit at the cost of greater risk of hypotension, syncope, and renal insufficiency when the combination of telmisartan and ramipril was used.[46] In a substudy of the Appropriate Blood Pressure Control in Diabetes (ABCD) trial, intensive blood pressure lowering in patients with PAD was associated with a 65% relative reduction in the risk of MI, stroke, or cardiovascular death compared with modest blood pressure-lowering therapy.[47] The INVEST study (International Verapamil-SR/Trandolapril Study) demonstrated that a calcium channel blocker-based antihypertensive strategy was comparable to a beta-blocker-based strategy in patients with concomitant PAD.[48] The ALLHAT trial (Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial) noted a J-shaped relationship between systolic blood pressure and peripheral vascular events, with best outcomes in individuals with systolic blood pressure between 120 and 129 mm Hg.[18]

Diabetes

In patients with PAD with concomitant diabetes, the goals of diabetes care include control of hyperglycemia and attention to diabetic foot care. Not only is diabetes associated with adverse limb outcomes (increased risk of ischemic ulceration and amputation in patients with PAD), but it also is associated with increased mortality. Aggressive glycemic control reduces both macrovascular events (MI, stroke, and death) and microvascular events (nephropathy, neuropathy, and retinopathy) in patients with type I diabetes. Although aggressive glycemic control reduces microvascular events in individuals with type II diabetes, reductions in macrovascular events have not been shown. Several studies, such as the ADVANCE (Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation),[49] Action to Control Cardiovascular Risk in Diabetes[50] and Veterans Affairs Diabetes trials,[51] have failed to show a reduction in cardiovascular events with aggressive treatments aimed at lowering the hemoglobin A1c to 6%. Likewise in patients with type II diabetes who were at risk for cardiovascular events, the PROactive study (Prospective Pioglitazone Clinical Trial in Macrovascular Events) failed to show a reduction in the primary composite endpoint of cardiovascular or limb outcomes (lower rates of extremity revascularization or amputation) with pioglitazone. However, the study did show a significant 16% reduction in the secondary endpoint, a composite of total mortality, nonfatal MI, or stroke, with pioglitazone.[52] Several recent studies have evaluated the cardiovascular effects of newer classes of glucose-lowering agents including the glucagon-like peptide-1 (GLP-1) analogues, such as semaglutide and liraglutide; dipeptidyl peptidase-4 inhibitors (DPP-4), such as saxagliptin; and sodium-glucose co-transporter-2 (SGLT-2) agents, such as empagliflozin, canagliflozin, and dapagliflozin. These studies, as well as meta-analyses, have suggested that SGLT-2 inhibitors and GLP-1 agonists have beneficial effects in lowering cardiovascular mortality and major adverse cardiac events, while DPP-4 inhibitors do not have a significant effect on these outcomes.[53–57] Although the optimal glycemic control required to reduce macrovascular complications in patients with type II diabetes remains unclear, guidelines recommend treatments that maintain the level of hemoglobin A1c below 7%.

Foot care is a critical component of diabetes management, particularly in those with PAD. Diabetes increases the risk of foot injury because of the associated peripheral neuropathy and decreased sensation, thereby increasing the risk of ulcer formation. Resting limb ischemia and amputation are more frequent in patients with PAD with diabetes than in those without diabetes. Therefore careful attention to foot care is imperative to prevent skin breakdown, infections, ulceration, and amputation.

Antiplatelet Therapy

Antiplatelet therapy is recommended for patients with atherosclerosis, including patients with symptomatic PAD.[1] The Antiplatelet Trialists' Collaboration meta-analysis showed that antiplatelet therapy produces a 22% to 32% reduction in the RR of stroke, MI, or vascular death in patients with high-risk vascular disease, including prior stroke or transient ischemic attack or MI.[58] In a subset of patients with evidence of PAD based on symptoms of claudication or prior lower extremity bypass or angioplasty, antiplatelet therapy also produced a 22% RR reduction. The meta-analysis included studies that evaluated a variety of antiplatelet agents, such as aspirin, dipyridamole, picotamide, and ticlopidine. However, the benefits of aspirin have been called into question with a recent meta-analysis showing no significant effect of aspirin on cardiovascular events and a higher risk of major bleeding in patients with PAD, especially in asymptomatic patients.[59] The POPADAD (Prevention Of Progression of Arterial Disease and Diabetes) study,[60] which randomized patients with ABI below 0.99 and no known cardiovascular disease to 100 mg of aspirin or placebo, showed no significant difference between the groups. The Aspirin for Asymptomatic Atherosclerosis (AAA) trial explored whether aspirin (100 mg/day) was of benefit in asymptomatic individuals with PAD diagnosed solely on the basis of an abnormal ABI.[61] Compared with placebo, aspirin had no effect on the composite endpoint of fatal or nonfatal MI, stroke, or revascularization.

When clopidogrel was compared with aspirin in the CAPRIE trial, clopidogrel was associated with an 8% RR reduction in cardiovascular events for the entire study population of patients with recent MI, ischemic stroke, and PAD, and an even higher 24% RR reduction in

PAD subgroup.[62] The CHARISMA study showed no benefit of dual antiplatelet therapy with aspirin plus clopidogrel in a population of patients with established CAD, cerebrovascular disease, or PAD or those with risk factors for these disorders.[63] In a post-hoc analysis that focused on patients with symptomatic or asymptomatic PAD, there also was no benefit of dual antiplatelet therapy for the primary endpoint. However, the risk of MI and repeat hospitalization for ischemic events was lower with dual antiplatelet therapy (HR, 0.63; 95% CI, 0.42 to 0.96 and HR, 0.81; 95% CI, 0.68 to 0.95, respectively).[64]

Newer antiplatelet agents have also been evaluated in patients with PAD. The TRA2P-TIMI 50 study found that the addition of vorapaxar, a protease-activated receptor-1 antagonist, significantly reduced the composite endpoint of cardiovascular death, MI, or stroke by 13% in patients with established atherosclerosis manifesting as a prior MI, ischemic stroke, or established PAD.[65] Among the subset of patients with PAD, vorapaxar did not significantly affect the composite endpoint but did reduce the risk of hospitalization for acute limb ischemia by 42% ($P = .006$) and peripheral artery revascularization by 16% ($P = .017$), albeit with an increased risk of bleeding (HR 1.62, $P = .001$).[31] More recently, the EUCLID study compared ticagrelor (90 mg twice daily) with clopidogrel in patients with symptomatic PAD. There was no statistically significant difference between the two therapies in reducing a composite endpoint including cardiovascular death, MI, or ischemic stroke (HR 1.02, $P = .65$), and no difference in rates of acute limb ischemia (HR 1.03, $P = .85$). Additionally, there was no difference bleeding risk between clopidogrel and ticagrelor.[66] The PEGASUS-TIMI 54 study also demonstrated the benefit of ticagrelor in reducing major cardiovascular events among patient with PAD.[67]

Anticoagulant Therapy

A combination of anticoagulant therapy with warfarin and aspirin was initially evaluated in the Warfarin Antiplatelet Vascular Evaluation (WAVE) trial, which randomized patients to antiplatelet agent alone versus a combination of warfarin (target international normalized ratio 2.0 to 3.0) and antiplatelet therapy in patients with PAD. There was no difference in major adverse cardiac events between groups, with a significantly higher risk of life-threatening bleeding in the warfarin arm. Thus, full-dose anticoagulation is not recommended for treatment of PAD.[68] More recently, the combination of low-dose rivaroxaban (2.5 mg twice daily) in addition to aspirin in patients with the stable atherosclerotic vascular disease was examined in the COMPASS trial. Individuals receiving rivaroxaban and aspirin, compared with aspirin alone, had a 24% RR reduction in cardiovascular death, stroke, or MI, albeit at a significantly higher risk of major bleeding (HR 1.7, 95% CI 1.4 to 2.05).[69] A similar trend was seen in the PAD subgroup, including a significant reduction in major adverse limb events, including limb revascularization or amputation.[70] The VOYAGER PAD (Vascular Outcomes Study of ASA Along with Rivaroxaban in Endovascular or Surgical Limb Revascularization for PAD) trial, low-dose rivaroxaban in addition to aspirin also reduced major adverse cardiac and limb events after lower extremity revascularization, but with a higher risk of bleeding.[71] Based on these data, low-dose rivaroxaban may be considered in patients at a high risk of vascular events and low risk of bleeding. A small trial compared edoxaban with clopidogrel after endovascular therapy for PAD; there was no difference in rates of restenosis or reocclusion between the two groups.[72] There are no published trials that have evaluated either dabigatran or apixaban in patients with PAD. Thus, the results of COMPASS and VOYAGER PAD with rivaroxaban cannot be extrapolated to other direct oral anticoagulants.

Treatment of Lower Extremity Symptoms

Treatments that target the symptoms and signs of PAD are implemented to improve function and mobility and preserve limb viability. Established treatments can be broadly categorized into supervised exercise therapy, pharmacotherapy, and revascularization. Therapeutic angiogenesis has also been explored as a potential therapeutic option.

Exercise Therapy

Supervised exercise therapy improves walking distance by as much as 100% to 150% in patients with PAD. Patients are recommended to walk on a treadmill or track three to five times per week for a duration of at least 45 minutes per session for 3 to 6 months. Exercise should continue until patients develop moderate to severe claudication; after a rest period, they should resume walking with the cycle repeated until the session is over. As patients improve, walking speed and treadmill grade can be increased. Studies have shown that home-based exercise therapy is also effective when home activities are monitored and quantified[73] or when paired with periodic and facilitated group behavioral training.[74] The CLEVER (Claudication: Exercise Versus Endoluminal Revascularization) trial showed that a 6-month supervised exercise training program in patients with aortoiliac disease and claudication produced a greater improvement in walking time than optimal medical therapy alone or stenting.[75] In contrast, in the IRONIC (Invasive Revascularization or Not in Intermittent Claudication) study, improvement in disease-specific quality-of-life measures was greater for stenting than supervised exercise therapy in patients with life-limiting claudication at 1 year after intervention,[76] but not at 5 years after intervention.[77] While prior studies had evaluated the benefits of supervised exercise therapy in comparison to revascularization, the ERASE study assessed the impact of exercise therapy in addition to endovascular revascularization. The study showed that combination treatment resulted in greater walking distance and quality of life at 1 year than supervised exercise therapy alone.[78] That the combination of supervised exercise and endovascular intervention improves quality of life has been confirmed in a meta-analysis, while no difference in either the quality of life or future revascularization or amputation was seen between supervised exercise alone versus endovascular intervention.[79] It remains clear that physical activity should be recommended as a part of a multifaceted approach to improving symptoms in patients with PAD.

The mechanisms responsible for the benefits of exercise remain unclear. Potential mechanisms include collateral blood vessel development because of upregulation of angiogenic growth factors, endothelium-dependent vasodilation because of enhanced nitric oxide bioavailability, more efficient walking biomechanics, and improved skeletal muscle metabolism.

Pharmacologic Therapies

Although many medications have been evaluated, few have improved the symptoms of claudication in patients with PAD. Only two medications are approved by the Food and Drugs Administration: cilostazol and pentoxifylline. Cilostazol is a phosphodiesterase 3 (PDE3) inhibitor with both vasodilator and antiplatelet properties. The precise mechanism by which cilostazol confers a benefit in patients with PAD is unknown. Several randomized trials have shown that compared with placebo, cilostazol produces an approximately 50% increase in walking time and improves the perceived quality of life.[80] Because other PDE3 inhibitors (e.g., milrinone) have been linked to increased mortality in patients with congestive heart failure, cilostazol should not be used in this subpopulation of patients with PAD. However, cilostazol has not been associated with an increase in mortality. Evidence for the efficacy of pentoxifylline, a hemorheological agent, is less robust than that for cilostazol, and pentoxifylline is less likely to be of clinical benefit.

Statins have also been explored for the treatment of claudication to exploit their pleiotropic effects, such as attenuation of inflammation. One study showed an improvement in pain-free walking time with atorvastatin but no significant increase in maximal walking time.[81] Other studies revealed an increase in walking time with simvastatin.[82,83] Another study, however, showed no benefit of niacin plus lovastatin on walking times compared with placebo.[84]

Revascularization

Lower extremity revascularization is reserved for patients with CLI or lifestyle-limiting claudication symptoms despite maximal medical therapy. For those with CLI, prompt revascularization is necessary. Revascularization in such patients may alleviate resting limb pain, accelerate the healing of ulcers, and reduce infection. In patients with claudication, revascularization, in addition to supervised exercise and medical therapy, can lessen leg discomfort and improve the quality of life. Options for revascularization include endovascular (percutaneous) intervention or open surgical revascularization.

Endovascular intervention, which includes percutaneous transluminal balloon angioplasty (PTA) and endovascular stenting, is increasingly being used as a less invasive option for revascularization in patients with PAD (Fig. 146.5). The advances in the technology of balloon-expandable and self-expanding stents have widened the population of patients with suitable anatomic lesions that stand to benefit from these procedures. Eligible patients include those with severe or disabling symptoms of claudication and those with limb-threatening ischemia. Clinical outcomes for endovascular revascularization depend on the type and length of the lesions. Treatment of stenoses is more likely to be successful than treatment of total occlusions, and success is influenced by a variety of morphologic characteristics, as delineated in the TransAtlantic Inter-Society Consensus (TASC) Working Group classification for iliac and femoropopliteal lesions.[3] Durability and patency are greatest for iliac artery lesions; the likelihood of long-term patency with endovascular interventions is lower with the disease in more distal arteries. Patency rates decrease with increasing lesion length, the presence of diffuse disease or multiple lesions, and poor run-off, as well as other adverse patient characteristics, such as diabetes, active smoking, and renal failure.[1]

For aortoiliac interventions, endovascular treatment affords excellent long-term patency, especially when combined with stenting. Five-year patency rates are approximately 94% and are comparable to rates achieved with surgical intervention. Results are less durable for femoropopliteal PTA with studies showing a variable benefit of standard nitinol stents in the femoropopliteal arteries depending on lesion length and other factors.[85] Compared with bare-metal stents, drug-eluting stents (DES) have shown promise for femoral artery revascularization with improved survival free of major vascular events (death, amputation, or revascularization) with paclitaxel-eluting DES.[86] There are also studies showing benefits of the drug-eluting balloon over standard (uncoated) balloon angioplasty for femoropopliteal artery revascularization resulting in reduced rates of restenosis and target vessel revascularization with comparable safety.[87] However, recent meta-analyses has called into question the safety of paclitaxel-coated balloons and stents, suggesting a higher risk of all-cause mortality, though other analyses have not identified this risk.[88–91] Thus, the operator must weigh the decision between improved vascular outcomes but possible higher all-cause mortality with DES; in this case, a detailed risk/benefit discussion with the patient is of utmost importance.

Endovascular treatment of infrapopliteal artery stenosis is typically reserved for patients with limb ischemia. In the PARADISE (Preventing Amputations Using Drug Eluting Stents) study, a prospective, nonrandomized study of 106 patients, the 3-year amputation-free survival rate after DES implantation for treatment of limb ischemia involving the infrapopliteal vessels was 68%.[92] In the IN.PACT DEEP study, infrapopliteal artery revascularization with a drug-eluting balloon was non-inferior to standard balloon angioplasty with respect to the primary endpoints of target lesion revascularization and late lumen loss at 5 years; importantly the safety of paclitaxel-coated balloon angioplasty was similar to PTA.[93]

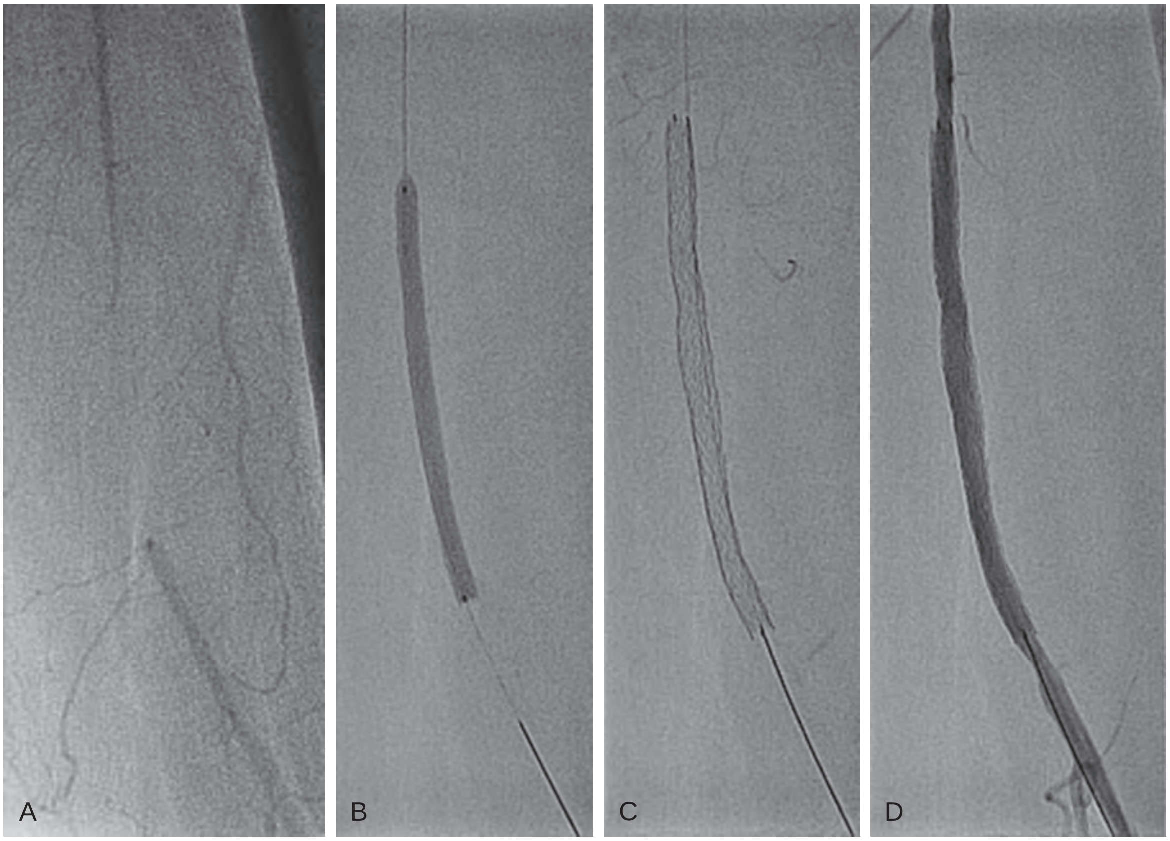


Figure 146.5 CONVENTIONAL CONTRAST ANGIOGRAPHY ILLUSTRATING USE OF A SELF-EXPANDING NITINOL STENT TREATMENT OF AN OCCLUSION OF THE SUPERFICIAL FEMORAL ARTERY. (A) Occlusion in the mid-superficial femoral artery. (B) Balloon angioplasty. (C) Stent deployment in the lesion. (D) Angiogram after stent deployment. *(From Thukkani AK, Kinlay S. Endovascular intervention for peripheral artery disease. Circ Res. 2015;116(9):1599–1613.)*

Surgical revascularization remains the gold standard for peripheral revascularization with the choice of operation depending on the location of the stenosis. Options include (1) aortoiliac or aortofemoral reconstruction for proximal disease involving the aorta or iliofemoral vessels, (2) femoral-popliteal bypass (either above- or below-knee popliteal) for superficial femoral artery or popliteal artery disease, and (3) femoral-distal (tibial or peroneal) bypass for distal arterial stenosis. Aortobiiliac or aortobifemoral bypass graft surgery for aortoiliac occlusive disease ("inflow") produces excellent long-term results with 5-year patency rates ranging from 85% to 90%. Surgical treatment of infrainguinal disease ("outflow") also produces durable results, although outcomes depend on the type of bypass conduit used. Vein grafts are the most durable; femoral-popliteal vein bypass grafts have an expected 5-year patency rate of approximately 66%. The 5-year patency rate for prosthetic grafts, such as polytetrafluoroethylene (PTFE), is lower, about 47%.[1] Limitations of surgical interventions include the need for general anesthesia and the attendant risk of cardiovascular events and death associated with major noncardiac surgery in patients with atherosclerosis. Given the potential for coexistent cardiovascular disease, preoperative assessment is important to identify and limit the risk of cardiovascular events in vascular surgery patients.[94]

Whereas revascularization is indicated for most patients with CLI to preserve limb viability, the comparative efficacy and safety of endovascular revascularization and surgical reconstruction for CLI are not known. A contemporary registry has sought to evaluate the comparative safety and effectiveness of surgical and endovascular interventions in patients with symptomatic PAD.[95] The BEST-CLI Trial (Best Endovascular versus Best Surgical Therapy in Patients with Critical Limb Ischemia) is an ongoing trial that is comparing the relative benefits of surgical versus endovascular treatment in this population.[96]

FUTURE DIRECTIONS

A better understanding of the factors that contribute to intermittent claudication and CLI is needed to craft innovative and durable therapeutic interventions for the management of the limb complications of PAD. In addition, greater knowledge is needed about the unique contributors to atherogenesis and thrombosis in the lower extremities compared with other arterial beds. Further understanding of the downstream effects of compromised blood flow, such as changes in skeletal muscle energetics and impaired neural function, will inform the design and development of novel pharmacotherapies for individuals with PAD. Finally, more comparative efficacy studies are needed to clarify ongoing debates with respect to the relative benefits of exercise training versus invasive therapies; endovascular versus surgical revascularization; and the impact of therapeutic angiogenesis and cell-based therapies in the treatment of PAD. Greater knowledge dissemination regarding PAD diagnosis and risks associated with PAD remains key to early detection and implementation of risk factor modification therapies to improve the care of this high-risk population.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Aboyans V, Criqui MH, Abraham P, et al. Measurement and interpretation of the ankle-brachial index: a scientific statement from the American Heart Association. *Circulation*. 2012;126(24):2890–2909.

Aboyans V, Ricco J-B, Bartelink M-LEL, et al. 2017 ESC guidelines on the diagnosis and treatment of peripheral arterial diseases, in collaboration with the European Society for Vascular Surgery (ESVS): document covering atherosclerotic disease of extracranial carotid and vertebral, mesenteric, renal, upper and lower extremity arteriesEndorsed by: the European Stroke Organization (ESO)The Task Force for the Diagnosis and Treatment of Peripheral Arterial Diseases of the European Society of Cardiology (ESC) and of the European Society for Vascular Surgery (ESVS). *Eur Heart J*. 2018;39(9):763–816.

Ambler GK, Waldron C-A, Contractor UB, et al. Umbrella review and meta-analysis of antiplatelet therapy for peripheral artery disease. *Br J Surg*. 2020;107(1):20–32.

Anand SS, Bosch J, Eikelboom JW, et al. Rivaroxaban with or without aspirin in patients with stable peripheral or carotid artery disease: an international, randomised, double-blind, placebo-controlled trial. *Lancet*. 391(10117):219-229.

Bedenis R, Stewart M, Cleanthis M, et al. Cilostazol for intermittent claudication. *Cochrane Database Syst Rev*. 2014(10):CD003748 Published online October 31, 2014.

Bonaca MP, Bauersachs RM, Anand SS, et al. Rivaroxaban in peripheral artery disease after revascularization. *N Engl J Med*. 2020;382(21):1994–2004.

Djerf H, Millinger J, Falkenberg M, et al. Absence of long-term benefit of revascularization in patients with intermittent claudication: five-year results from the IRONIC randomized controlled trial. *Circ Cardiovasc Interv*. 2020;13(1):e008450.

Eikelboom JW, Connolly SJ, Bosch J, et al. Rivaroxaban with or without aspirin in stable cardiovascular disease. *N Engl J Med*. 2017;377(14):1319–1330.

Fakhry F, Spronk S, van der Laan L, et al. Endovascular revascularization and supervised exercise for peripheral artery disease and intermittent claudication: a randomized clinical trial. *JAMA*. 2015;314(18):1936.

Fleisher LA, Fleischmann KE, Auerbach AD, et al. 2014 ACC/AHA guideline on perioperative cardiovascular evaluation and management of patients undergoing noncardiac surgery: executive summary: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *Circulation*. 2014;130(24):2215–2245.

Gerhard-Herman MD, Gornik HL, Barrett C, et al. 2016 AHA/ACC guideline on the management of patients with lower extremity peripheral artery disease: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *J Am Coll Cardiol*. 2017;69(11):e71–e126.

Grenet G, Ribault S, Nguyen GB, et al. GLUcose COntrol safety & efficacy in type 2 DIabetes, a systematic review and NETwork meta-analysis. *PLoS One*. 2019;14(6):e0217701.

Hiatt WR, Fowkes FGR, Heizer G, et al. Ticagrelor versus clopidogrel in symptomatic peripheral artery disease. *N Engl J Med*. 2017;376(1):32–40.

Katsanos K, Spiliopoulos S, Kitrou P, et al. Risk of death following application of paclitaxel-coated balloons and stents in the femoropopliteal artery of the leg: a systematic review and meta-analysis of randomized controlled trials. *J Am Heart Assoc*. 2018;7(24).

Klarin D, Lynch J, Aragam K, et al. Genome-wide association study of peripheral artery disease in the Million Veteran Program. *Nat Med*. 2019;25(8):1274–1279.

Lu Y, Ballew SH, Tanaka H, et al. 2017 ACC/AHA blood pressure classification and incident peripheral artery disease: The Atherosclerosis Risk in Communities (ARIC) Study. *Eur J Prev Cardiol*. 2020;27(1):51–59.

Murphy TP, Cutlip DE, Regensteiner JG, et al. Supervised exercise, stent revascularization, or medical therapy for claudication due to aortoiliac peripheral artery disease: the CLEVER study. *J Am Coll Cardiol*. 2015;65(10):999–1009.

Narula N, Dannenberg AJ, Olin JW, et al. Pathology of peripheral artery disease in patients with critical limb ischemia. *J Am Coll Cardiol*. 2018;72(18):2152–2163.

Nehler MR, Duval S, Diao L, et al. Epidemiology of peripheral arterial disease and critical limb ischemia in an insured national population. *J Vasc Surg*. 2014;60(3):686–695.e2.

Norgren L, Hiatt WR, Dormandy JA, et al. Inter-society consensus for the management of peripheral arterial disease (TASC II). *J Vasc Surg*. 2007;45(1):S5–S67.

Pandey A, Banerjee S, Ngo C, et al. Comparative efficacy of endovascular revascularization versus supervised exercise training in patients with intermittent claudication: meta-analysis of randomized controlled trials. *JACC Cardiovasc Interv*. 2017;10(7):712–724.

Secemsky EA, Kundi H, Weinberg I, et al. Association of survival with femoropopliteal artery revascularization with drug-coated devices. *JAMA Cardiol*. 2019;4(4):332.

Secemsky EA, Kundi H, Weinberg I, et al. Drug-eluting stent implantation and long-term survival following peripheral artery revascularization. *J Am Coll Cardiol*. 2019;73(20):2636–2638.

Shu J, Santulli G. Update on peripheral artery disease: epidemiology and evidence-based facts. *Atherosclerosis*. 2018;275:379–381.

Tsai TT, Rehring TF, Rogers RK, et al. The contemporary safety and effectiveness of lower extremity bypass surgery and peripheral endovascular interventions in the treatment of symptomatic peripheral arterial disease. *Circulation*. 2015;132(21):1999–2011.

REFERENCES

1. Gerhard-Herman MD, Gornik HL, Barrett C, et al. 2016 AHA/ACC guideline on the management of patients with lower extremity peripheral artery disease: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *J Am Coll Cardiol*. 2017;69(11):e71–e126. https://doi.org/10.1016/j.jacc.2016.11.007.
2. Aboyans V, Ricco J-B, Bartelink M-LEL, et al. 2017 ESC guidelines on the diagnosis and treatment of peripheral arterial diseases, in collaboration with the European Society for Vascular Surgery (ESVS): document covering atherosclerotic disease of extracranial carotid and vertebral, mesenteric, renal, upper and lower extremity arteries Endorsed by: the European Stroke Organization (ESO) The Task Force for the Diagnosis and Treatment of Peripheral Arterial Diseases of the European Society of Cardiology (ESC) and of the European Society for Vascular Surgery (ESVS). *Eur Heart J*. 2018;39(9):763–816. https://doi.org/10.1093/eurheartj/ehx095.
3. Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FGR. Inter-society consensus for the management of peripheral arterial disease (TASC II). *J Vasc Surg*. 2007;45(1):S5–S67. https://doi.org/10.1016/j.jvs.2006.12.037.
4. Benjamin EJ, Muntner P, Alonso A, et al. Heart Disease and Stroke Statistics-2019 Update: a report from the American Heart Association. *Circulation*. 2019;139(10):e56–e528. https://doi.org/10.1161/CIR.0000000000000659.
5. Shu J, Santulli G. Update on peripheral artery disease: epidemiology and evidence-based facts. *Atherosclerosis*. 2018;275:379–381. https://doi.org/10.1016/j.atherosclerosis.2018.05.033.
6. Meves SH, Diehm C, Berger K, et al. Peripheral arterial disease as an independent predictor for excess stroke morbidity and mortality in primary-care patients: 5-year results of the getABI study. *Cerebrovasc Dis*. 2010;29(6):546–554. https://doi.org/10.1159/000306640.
7. Pande RL, Creager MA. Socioeconomic inequality and peripheral artery disease prevalence in US adults. *Circ Cardiovasc Qual Outcomes*. 2014;7(4):532–539. https://doi.org/10.1161/CIRCOUTCOMES.113.000618.
8. Allison MA, Ho E, Denenberg JO, et al. Ethnic-specific prevalence of peripheral arterial disease in the United States. *Am J Prev Med*. 2007;32(4):328–333. https://doi.org/10.1016/j.amepre.2006.12.010.
9. Nehler MR, Duval S, Diao L, et al. Epidemiology of peripheral arterial disease and critical limb ischemia in an insured national population. *J Vasc Surg*. 2014;60(3):686–695.e2. https://doi.org/10.1016/j.jvs.2014.03.290.
10. Conen D, Everett BM, Kurth T, et al. Smoking, smoking cessation, [corrected] and risk for symptomatic peripheral artery disease in women: a cohort study. *Ann Intern Med*. 2011;154(11):719–726. https://doi.org/10.7326/0003-4819-154-11-201106070-00003.
11. Leng GC, Lee AJ, Fowkes FG, Lowe GD, Housley E. The relationship between cigarette smoking and cardiovascular risk factors in peripheral arterial disease compared with ischaemic heart disease. The Edinburgh Artery Study. *Eur Heart J*. 1995;16(11):1542–1548. https://doi.org/10.1093/oxfordjournals.eurheartj.a060775.
12. Hiatt WR, Marshall JA, Baxter J, et al. Diagnostic methods for peripheral arterial disease in the San Luis Valley Diabetes Study. *J Clin Epidemiol*. 1990;43(6):597–606. https://doi.org/10.1016/0895-4356(90)90164-k.
13. Meijer WT, Hoes AW, Rutgers D, Bots ML, Hofman A, Grobbee DE. Peripheral arterial disease in the elderly: The Rotterdam Study. *Arterioscler Thromb Vasc Biol*. 1998;18(2):185–192. https://doi.org/10.1161/01.atv.18.2.185.
14. Fabsitz RR, Sidawy AN, Go O, et al. Prevalence of peripheral arterial disease and associated risk factors in American Indians: the Strong Heart Study. *Am J Epidemiol*. 1999;149(4):330–338. https://doi.org/10.1093/oxfordjournals.aje.a009817.
15. Murabito JM, D'Agostino RB, Silbershatz H, Wilson PWF. Intermittent claudication. A risk profile from The Framingham Heart Study. *Circulation*. 1997;96(1):44–49. https://doi.org/10.1161/01.CIR.96.1.44.
16. Powell TM, Glynn RJ, Buring JE, Creager MA, Ridker PM, Pradhan AD. The relative importance of systolic versus diastolic blood pressure control and incident symptomatic peripheral artery disease in women. *Vasc Med*. 2011;16(4):239–246. https://doi.org/10.1177/1358863X11413166.
17. Lu Y, Ballew SH, Tanaka H, et al. 2017 ACC/AHA blood pressure classification and incident peripheral artery disease: The Atherosclerosis Risk in Communities (ARIC) Study. *Eur J Prev Cardiol*. 2020;27(1):51–59. https://doi.org/10.1177/2047487319865378.
18. Itoga NK, Tawfik DS, Lee CK, Maruyama S, Leeper NJ, Chang TI. Association of blood pressure measurements with peripheral artery disease events. *Circulation*. 2018;138(17):1805–1814. https://doi.org/10.1161/CIRCULATIONAHA.118.033348.
19. Smith GD, Shipley MJ, Rose G. Intermittent claudication, heart disease risk factors, and mortality. The Whitehall Study. *Circulation*. 1990;82(6):1925–1931. https://doi.org/10.1161/01.cir.82.6.1925.
20. O'Hare AM, Glidden DV, Fox CS, Hsu C-Y. High prevalence of peripheral arterial disease in persons with renal insufficiency: results from the National Health and Nutrition Examination Survey 1999-2000. *Circulation*. 2004;109(3):320–323. https://doi.org/10.1161/01.CIR.0000114519.75433.DD.
21. O'Hare AM, Vittinghoff E, Hsia J, Shlipak MG. Renal insufficiency and the risk of lower extremity peripheral arterial disease: results from the Heart and Estrogen/Progestin Replacement Study (HERS). *J Am Soc Nephrol*. 2004;15(4):1046–1051. https://doi.org/10.1097/01.asn.0000119574.27772.fd.
22. Wang GJ, Shaw PA, Townsend RR, et al. Sex differences in the incidence of peripheral artery disease in the chronic renal insufficiency cohort. *Circ Cardiovasc Qual Outcomes*. 2016;9(2 Suppl 1):S86–93. https://doi.org/10.1161/CIRCOUTCOMES.115.002180.
23. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Plasma concentration of C-reactive protein and risk of developing peripheral vascular disease. *Circulation*. 1998;97(5):425–428. https://doi.org/10.1161/01.cir.97.5.425.
24. Narula N, Dannenberg AJ, Olin JW, et al. Pathology of peripheral artery disease in patients with critical limb ischemia. *J Am Coll Cardiol*. 2018;72(18):2152–2163. https://doi.org/10.1016/j.jacc.2018.08.002.
25. Klarin D, Lynch J, Aragam K, et al. Genome-wide association study of peripheral artery disease in the Million Veteran Program. *Nat Med*. 2019;25(8):1274–1279. https://doi.org/10.1038/s41591-019-0492-5.
26. Aboyans V, Criqui MH, Abraham P, et al. Measurement and interpretation of the ankle-brachial index: a scientific statement from the American Heart Association. *Circulation*. 2012;126(24):2890–2909. https://doi.org/10.1161/CIR.0b013e318276fbcb.
27. Whelan JF, Barry MH, Moir JD. Color flow Doppler ultrasonography: comparison with peripheral arteriography for the investigation of peripheral vascular disease. *J Clin Ultrasound*. 20(6):369-374. https://doi.org/10.1002/jcu.1870200602.
28. Eiberg JP, Grønvall Rasmussen JB, Hansen MA, Schroeder TV. Duplex ultrasound scanning of peripheral arterial disease of the lower limb. *Eur J Vasc Endovasc Surg*. 2010;40(4):507–512. https://doi.org/10.1016/j.ejvs.2010.06.002.
29. Collins R, Burch J, Cranny G, et al. Duplex ultrasonography, magnetic resonance angiography, and computed tomography angiography for diagnosis and assessment of symptomatic, lower limb peripheral arterial disease: systematic review. *BMJ*. 2007;334(7606):1257. https://doi.org/10.1136/bmj.39217.473275.55.
30. Kumbhani DJ, Steg PG, Cannon CP, et al. Statin therapy and long-term adverse limb outcomes in patients with peripheral artery disease: insights from the REACH registry. *Eur Heart J*. 2014;35(41):2864–2872. https://doi.org/10.1093/eurheartj/ehu080.
31. Bonaca MP, Scirica BM, Creager MA, et al. Vorapaxar in patients with peripheral artery disease: results from TRA2{degrees}P-TIMI 50. *Circulation*. 2013;127(14):1522–1529. https://doi.org/10.1161/CIRCULATIONAHA.112.000679. 1529e1-6.
32. Fowkes FGR, Murray GD, Butcher I, et al. Ankle brachial index combined with Framingham risk score to predict cardiovascular events and mortality: a meta-analysis. *JAMA—Assoc*. 2008;300(2):197–208. https://doi.org/10.1001/jama.300.2.197.
33. Velescu A, Clara A, Martí R, et al. Abnormally high ankle–brachial index is associated with all-cause and cardiovascular mortality: The REGICOR Study. *Eur J Vasc Endovasc Surg*. 2017;54(3):370–377. https://doi.org/10.1016/j.ejvs.2017.06.002.
34. Qamar A, Morrow DA, Creager MA, et al. Effect of vorapaxar on cardiovascular and limb outcomes in patients with peripheral artery disease with and without coronary artery disease: analysis from the TRA 2°P-TIMI 50 trial. *Vasc Med*. 2020;25(2):124–132. https://doi.org/10.1177/1358863X19892690.
35. Anand SS, Bosch J, Eikelboom JW, et al. Rivaroxaban with or without aspirin in patients with stable peripheral or carotid artery disease: an international, randomised, double-blind, placebo-controlled trial. *Lancet*. 391(10117):219-229. Published online November 10, 2017. https://doi.org/10.1016/S0140-6736(17)32409-1.
36. No authors listed. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet*. 1994;344(8934):1383–1389. https://doi.org/10.1016/S0140-6736(94)90566-5.
37. Heart Protection Study Collaborative Group MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. *Lancet*. 2002;360(9326):7–22. https://doi.org/10.1016/S0140-6736(02)09327-3.

38. Grundy SM, Stone NJ, Bailey AL, et al. 2018 AHA/ACC/AACVPR/AAPA/ABC/ACPM/ADA/AGS/APhA/ASPC/NLA/PCNA guideline on the management of blood cholesterol: Executive Summary: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *Circulation*. 2019;139(25):3168–3209. https://doi.org/10.1161/CIR.0000000000000624.
39. Bonaca MP, Nault P, Giugliano RP, et al. Low-density lipoprotein cholesterol lowering with evolocumab and outcomes in patients with peripheral artery disease: insights from the FOURIER trial (Further Cardiovascular Outcomes Research With PCSK9 Inhibition in Subjects With Elevated Risk). *Circulation*. 2018;137(4):338–350. https://doi.org/10.1161/CIRCULATIONAHA.117.032235.
40. Rubins HB, Robins SJ, Collins D, et al. Gemfibrozil for the secondary prevention of coronary heart disease in men with low levels of high-density lipoprotein cholesterol. Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial Study Group. *N Engl J Med*. 1999;341(6):410–418. https://doi.org/10.1056/NEJM199908053410604.
41. Meade T, Zuhrie R, Cook C, Cooper J. Bezafibrate in men with lower extremity arterial disease: Randomised controlled trial. *Br Med J*. 2002;325(7373):1139–1141. https://doi.org/10.1136/bmj.325.7373.1139.
42. Keech A, Simes RJ, Barter P, et al. Effects of long-term fenofibrate therapy on cardiovascular events in 9795 people with type 2 diabetes mellitus (the FIELD study): randomised controlled trial. *Lancet*. 2005;366(9500):1849–1861. https://doi.org/10.1016/S0140-6736(05)67667-2.
43. Landray MJ, Haynes R, Hopewell JC, et al. Effects of extended-release niacin with laropiprant in high-risk patients. *N Engl J Med*. 2014;371(3):203–212. https://doi.org/10.1056/NEJMoa1300955.
44. Whelton PK, Carey RM, Aronow WS, et al. 2017 ACC/AHA/AAPA/ABC/ACPM/AGS/APhA/ASH/ASPC/NMA/PCNA guideline for the prevention, detection, evaluation, and management of high blood pressure in adults: a report of the American College of Cardiology/American Heart Association Task Force on clinical practice guidelines. *Hypertension*. 2018;71(6):e13–e115. https://doi.org/10.1161/HYP.0000000000000065.
45. Östergren J, Sleight P, Dagenais G, et al. Impact of ramipril in patients with evidence of clinical or subclinical peripheral arterial disease. *Eur Heart J*. 2004;25(1):17–24. https://doi.org/10.1016/j.ehj.2003.10.033.
46. Yusuf S, Teo KK, Pogue J, et al. Telmisartan, ramipril, or both in patients at high risk for vascular events. *N Engl J Med*. 2008;358(15):1547–1559. https://doi.org/10.1056/NEJMoa0801317.
47. Mehler PS, Coll JR, Estacio R, Esler A, Schrier RW, Hiatt WR. Intensive blood pressure control reduces the risk of cardiovascular events in patients with peripheral arterial disease and type 2 diabetes. *Circulation*. 2003;107(5):753–756. https://doi.org/10.1161/01.CIR.0000049640.46039.52.
48. Bavry AA, Anderson RD, Gong Y, et al. Outcomes among hypertensive patients with concomitant peripheral and coronary artery disease: findings from the INternational VErapamil-SR/Trandolapril STudy. *Hypertension*. 2010;55(1):48–53. https://doi.org/10.1161/HYPERTENSIONAHA.109.142240.
49. Patel A, MacMahon S, Chalmers J, et al. Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes. *N Engl J Med*. 2008;358(24):2560–2572. https://doi.org/10.1056/NEJMoa0802987.
50. Gerstein HC, Miller ME, Byington RP, et al. Effects of intensive glucose lowering in type 2 diabetes. *N Engl J Med*. 2008;358(24):2545–2559. https://doi.org/10.1056/NEJMoa0802743.
51. Duckworth W, Abraira C, Moritz T, et al. Glucose control and vascular complications in veterans with type 2 diabetes. *N Engl J Med*. 2009;360(2):129–139. https://doi.org/10.1056/NEJMoa0808431.
52. Dormandy JA, Charbonnel B, Eckland DJ, et al. Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. *Lancet*. 2005;366(9493):1279–1289. https://doi.org/10.1016/S0140-6736(05)67528-9.
53. Marso SP, Daniels GH, Brown-Frandsen K, et al. Liraglutide and cardiovascular outcomes in type 2 diabetes. *N Engl J Med*. 2016;375(4):311–322. https://doi.org/10.1056/NEJMoa1603827.
54. Husain M, Birkenfeld AL, Donsmark M, et al. Oral semaglutide and cardiovascular outcomes in patients with type 2 diabetes. *N Engl J Med*. 2019;381(9):841–851. https://doi.org/10.1056/NEJMoa1901118.
55. Verma S, Mazer CD, Al-Omran M, et al. Cardiovascular outcomes and safety of empagliflozin in patients with type 2 diabetes mellitus and peripheral artery disease: a subanalysis of EMPA-REG OUTCOME. *Circulation*. 2018;137(4):405–407. https://doi.org/10.1161/CIRCULATIONAHA.117.032031.
56. Gutierrez JA, Scirica BM, Bonaca MP, et al. Prevalence and outcomes of polyvascular (coronary, peripheral, or cerebrovascular) disease in patients with diabetes mellitus (from the SAVOR-TIMI 53 trial). *Am J Cardiol*. 2019;123(1):145–152. https://doi.org/10.1016/j.amjcard.2018.09.014.
57. Grenet G, Ribault S, Nguyen GB, et al. GLUcose COntrol Safety & Efficacy in type 2 DIabetes, a systematic review and NETwork meta-analysis. *PLoS One*. 2019;14(6):e0217701. https://doi.org/10.1371/journal.pone.0217701.
58. Baigent C, Sudlow C, Collins R, Peto R. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *Br Med J*. 2002;324(7329):71–86. https://doi.org/10.1136/bmj.324.7329.71.
59. Ambler GK, Waldron C-A, Contractor UB, Hinchliffe RJ, Twine CP. Umbrella review and meta-analysis of antiplatelet therapy for peripheral artery disease. *Br J Surg*. 2020;107(1):20–32. https://doi.org/10.1002/bjs.11384.
60. Belch J, MacCuish A, Campbell I, et al. The Prevention Of Progression of Arterial Disease And Diabetes (POPADAD) trial: factorial randomised placebo controlled trial of aspirin and antioxidants in patients with diabetes and asymptomatic peripheral arterial disease. *BMJ*. 2008;337(oct16 2). https://doi.org/10.1136/bmj.a1840. a1840-a1840.
61. Fowkes FGR, Price JF, Stewart MCW, et al. Aspirin for prevention of cardiovascular events in a general population screened for a low ankle brachial index: a randomized controlled trial. *JAMA—J Am Med Assoc*. 2010;303(9):841–848. https://doi.org/10.1001/jama.2010.221.
62. CAPRIE Steering Committee A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). *Lancet*. 1996;348(9038):1329–1339. https://doi.org/10.1016/S0140-6736(96)09457-3.
63. Bhatt DL, Fox KAA, Hacke W, et al. Clopidogrel and aspirin versus aspirin alone for the prevention of atherothrombotic events. *N Engl J Med*. 2006;354(16):1706–1717. https://doi.org/10.1056/NEJMoa060989.
64. Cacoub PP, Bhatt DL, Steg PG, Topol EJ, Creager MA. Patients with peripheral arterial disease in the CHARISMA trial. *Eur Heart J*. 2008;30(2):192–201. https://doi.org/10.1093/eurheartj/ehn534.
65. Morrow DA, Braunwald E, Bonaca MP, et al. Vorapaxar in the secondary prevention of atherothrombotic events. *N Engl J Med*. 2012;366(15):1404–1413. https://doi.org/10.1056/NEJMoa1200933.
66. Hiatt WR, Fowkes FGR, Heizer G, et al. Ticagrelor versus clopidogrel in symptomatic peripheral artery disease. *N Engl J Med*. 2017;376(1):32–40. https://doi.org/10.1056/NEJMoa1611688.
67. Bonaca MP, Bhatt DL, Storey RF, et al. Ticagrelor for prevention of ischemic events after myocardial infarction in patients with peripheral artery disease. *J Am Coll Cardiol*. 2016;67(23):2719–2728. https://doi.org/10.1016/j.jacc.2016.03.524.
68. Warfarin Antiplatelet Vascular Evaluation Trial Investigators Anand S, Yusuf S, et al. Oral anticoagulant and antiplatelet therapy and peripheral arterial disease. *N Engl J Med*. 2007;357(3):217–227. https://doi.org/10.1056/NEJMoa065959.
69. Eikelboom JW, Connolly SJ, Bosch J, et al. Rivaroxaban with or without aspirin in stable cardiovascular disease. *N Engl J Med*. 2017;377(14):1319–1330. https://doi.org/10.1056/NEJMoa1709118.
70. Anand SS, Bosch J, Eikelboom JW, et al. Rivaroxaban with or without aspirin in patients with stable peripheral or carotid artery disease: an international, randomised, double-blind, placebo-controlled trial. *Lancet*. 2018;391(10117):219–229. https://doi.org/10.1016/S0140-6736(17)32409-1.
71. Bonaca MP, Bauersachs RM, Anand SS, et al. Rivaroxaban in peripheral artery disease after revascularization. *N Engl J Med*. 2020;382(21):1994–2004. https://doi.org/10.1056/NEJMoa2000052.
72. Moll F, Baumgartner I, Jaff M, et al. Edoxaban plus aspirin vs dual antiplatelet therapy in endovascular treatment of patients with peripheral artery disease: results of the ePAD trial. *J Endovasc Ther*. 2018;25(2):158–168. https://doi.org/10.1177/1526602818760488.
73. Gardner AW, Parker DE, Montgomery PS, Scott KJ, Blevins SM. Efficacy of quantified home-based exercise and supervised exercise in patients with intermittent claudication: a randomized controlled trial. *Circulation*. 2011;123(5):491–498. https://doi.org/10.1161/CIRCULATIONAHA.110.963066.
74. McDermott MM, Liu K, Guralnik JM, et al. Home-based walking exercise intervention in peripheral artery disease: a randomized clinical trial. *JAMA*. 2013;310(1):57. https://doi.org/10.1001/jama.2013.7231.
75. Murphy TP, Cutlip DE, Regensteiner JG, et al. Supervised exercise, stent revascularization, or medical therapy for claudication due to aortoiliac peripheral artery disease: the CLEVER study. *J Am Coll Cardiol*. 2015;65(10):999–1009. https://doi.org/10.1016/j.jacc.2014.12.043.
76. Nordanstig J, Taft C, Hensäter M, Perlander A, Österberg K, Jivegård L. Improved quality of life after 1 year with an invasive versus a noninvasive treatment strategy in claudicants: one-year results of the Invasive Revascularization Or Not in Intermittent Claudication (IRONIC) trial. *Circulation*. 2014;130(12):939–947. https://doi.org/10.1161/CIRCULATIONAHA.114.009867.

77. Djerf H, Millinger J, Falkenberg M, Jivegård L, Svensson M, Nordanstig J. Absence of long-term benefit of revascularization in patients with intermittent claudication: five-year results from the IRONIC randomized controlled trial. *Circ Cardiovasc Interv*. 2020;13(1). https://doi.org/10.1161/CIRCINTERVENTIONS.119.008450.
78. Fakhry F, Spronk S, van der Laan L, et al. Endovascular revascularization and supervised exercise for peripheral artery disease and intermittent claudication: a randomized clinical trial. *JAMA*. 2015;314(18):1936. https://doi.org/10.1001/jama.2015.14851.
79. Pandey A, Banerjee S, Ngo C, et al. Comparative efficacy of endovascular revascularization versus supervised exercise training in patients with intermittent claudication: meta-analysis of randomized controlled trials. *JACC Cardiovasc Interv*. 2017;10(7):712–724. https://doi.org/10.1016/j.jcin.2017.01.027.
80. Bedenis R, Stewart M, Cleanthis M, Robless P, Mikhailidis DP, Stansby G. Cilostazol for intermittent claudication. *Cochrane Database Syst Rev*. 2014;2014(10):CD003748. https://doi.org/10.1002/14651858.CD003748.pub4.
81. Mohler 3rd ER, Hiatt WR, Creager MA. Cholesterol reduction with atorvastatin improves walking distance in patients with peripheral arterial disease. *Circulation*. 2003;108(12):1481–1486. https://doi.org/10.1161/01.CIR.0000090686.57897.F5.
82. Aronow WS, Nayak D, Woodworth S, Ahn C. Effect of simvastatin versus placebo on treadmill exercise time until the onset of intermittent claudication in older patients with peripheral arterial disease at six months and at one year after treatment. *Am J Cardiol*. 2003;92(6):711–712. https://doi.org/10.1016/S0002-9149(03)00833-6.
83. Mondillo S, Ballo P, Barbati R, et al. Effects of simvastatin on walking performance and symptoms of intermittent claudication in hypercholesterolemic patients with peripheral vascular disease. *Am J Med*. 2003;114(5):359–364. https://doi.org/10.1016/S0002-9343(03)00010-X.
84. Hiatt WR, Hirsch AT, Creager MA, et al. Effect of niacin ER/lovastatin on claudication symptoms in patients with peripheral artery disease. *Vasc Med*. 2010;15(3):171–179. https://doi.org/10.1177/1358863X09360579.
85. Kansal A, Long CA, Patel MR, Jones WS. Endovascular treatment of femoro popliteal lesions. *Clin Cardiol*. 2019;42(1):175–183. https://doi.org/10.1002/clc.23098.
86. Dake MD, Ansel GM, Jaff MR, et al. Durable clinical effectiveness with paclitaxel-eluting stents in the femoropopliteal artery: 5-year Results of the Zilver PTX randomized trial. *Circulation*. 2016;133(15):1472–1483. https://doi.org/10.1161/CIRCULATIONAHA.115.016900.
87. Rosenfield K, Jaff MR, White CJ, et al. Trial of a paclitaxel-coated balloon for femoropopliteal artery disease. *N Engl J Med*. 2015;373(2):145–153. https://doi.org/10.1056/NEJMoa1406235.
88. Katsanos K, Spiliopoulos S, Kitrou P, Krokidis M, Karnabatidis D. Risk of death following application of paclitaxel-coated balloons and stents in the femoropopliteal artery of the leg: a systematic review and meta-analysis of randomized controlled trials. *J Am Heart Assoc*. 2018;7(24). https://doi.org/10.1161/JAHA.118.011245.
89. Ouriel K, Adelman MA, Rosenfield K, et al. Safety of paclitaxel-coated balloon angioplasty for femoropopliteal peripheral artery disease. *JACC Cardiovasc Interv*. 2019;12(24):2515–2524. https://doi.org/10.1016/j.jcin.2019.08.025.
90. Secemsky EA, Kundi H, Weinberg I, et al. Drug-eluting stent implantation and long-term survival following peripheral artery revascularization. *J Am Coll Cardiol*. 2019;73(20):2636–2638. https://doi.org/10.1016/j.jacc.2019.02.020.
91. Secemsky EA, Kundi H, Weinberg I, et al. Association of survival with femoropopliteal artery revascularization with drug-coated devices. *JAMA Cardiol*. 2019;4(4):332. https://doi.org/10.1001/jamacardio.2019.0325.
92. Feiring AJ, Krahn M, Nelson L, Wesolowski A, Eastwood D, Szabo A. Preventing leg amputations in critical limb ischemia with below-the-knee drug-eluting stents. *J Am Coll Cardiol*. 2010;55(15):1580–1589. https://doi.org/10.1016/j.jacc.2009.11.072.
93. Zeller T, Micari A, Scheinert D, et al. The IN.PACT DEEP clinical drug-coated balloon trial: 5-year outcomes. *JACC Cardiovasc Interv*. 2020;13(4):431–443. https://doi.org/10.1016/j.jcin.2019.10.059.
94. Fleisher LA, Fleischmann KE, Auerbach AD, et al. 2014 ACC/AHA guideline on perioperative cardiovascular evaluation and management of patients undergoing noncardiac surgery: executive summary: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *Circulation*. 2014;130(24):2215–2245. https://doi.org/10.1161/CIR.0000000000000106.
95. Tsai TT, Rehring TF, Rogers RK, et al. The contemporary safety and effectiveness of lower extremity bypass surgery and peripheral endovascular interventions in the treatment of symptomatic peripheral arterial disease. *Circulation*. 2015;132(21):1999–2011. https://doi.org/10.1161/CIRCULATIONAHA.114.013440.
96. Menard MT, Farber A, Assmann SF, et al. Design and rationale of the best endovascular versus best surgical therapy for patients with critical limb ischemia (BEST-CLI) trial. *J Am Heart Assoc*. 2016;5(7). https://doi.org/10.1161/JAHA.116.003219.

CHAPTER 147

ATRIAL FIBRILLATION

Monika Kozieł Siołkowska, Tatjana S. Potpara, and Gregory Y. H. Lip

Atrial fibrillation (AF) is the most prevalent sustained cardiac arrhythmia, affecting approximately 3% of adults worldwide.[1] Its prevalence and incidence are rising continuously with the increasingly aging population. AF is associated with impaired prognosis, increased risk of all-cause mortality, stroke, heart failure, and hospital admissions, in addition to poor quality of life.

Importantly, AF should not be considered in isolation regarding the process of prevention, detection, and management. Rather, a more holistic approach and integrated care is needed to consider underlying comorbidities and cross-disease sequelae of AF.

In this chapter, we present a brief overview of the overall aspects of AF, with particular focus on the issues relating to stroke and systemic embolism (SSE).

EPIDEMIOLOGY

There have been progressive increases in the incidence and prevalence of AF, and the burden of this condition is estimated to increase further. This increase seems to be associated with an aging population, conditions predisposing to AF, and better detection of silent AF. AF is commonly accompanied by conditions, such as hypertension, heart failure, coronary artery disease (CAD), valvular heart disease, diabetes mellitus, obesity, or chronic kidney disease (CKD).[2] Given that AF is a growing epidemic, the societal impact and cost of this disease will continue to increase.

Pathobiology and Classification of Atrial Fibrillation

AF can be classified based on etiology, depending on whether it is present in patients without structural heart disease or occurs in association with other conditions. AF is defined as paroxysmal if it is self-terminating within 7 days, persistent if it is continuous greater than 7 days or permanent if it is chronic and accepted by the patient and physician. In case of permanent AF, rhythm control strategy is not a management option.

The pathogenesis of AF involves progressive structural changes in the atria, including atrial fibrosis, hypocontractility, fatty infiltration, inflammation, ischemia, vascular remodeling, ion channel dysfunction, and Ca^{2+} instability.

Although there is an overlap, pulmonary vein triggers seem to play a dominant role in younger patients with relatively normal hearts and short episodes of AF, and an abnormal atrial tissue substrate may play a more important role in patients with structural heart disease and persistent/permanent AF. After a period of continuous AF, electrical remodeling occurs, further facilitating the continuance of AF ("AF begets AF"). These changes are initially reversible if sinus rhythm is restored but may become permanent and related to structural changes if AF continues (left atrial dilatation, cardiac fibrosis, and impairment of systolic/diastolic function). Of note is that AF, especially if early-onset, may have a genetic component, independent of cardiovascular comorbidities.

CLINICAL MANIFESTATIONS

Symptoms

Patients with AF experience worse quality of life than healthy controls. AF is associated with symptoms including lethargy, dyspnea, palpitations, chest tightness, sleeping disorders, and psychosocial distress. However, these symptoms may reflect other concomitant diseases. The arrhythmia may be symptomatic or asymptomatic ("silent AF"), and AF patients may have both symptomatic and asymptomatic episodes. Importantly, silent AF is common, with severe consequences, such as stroke and/or death.

The modified European Heart Rhythm Association (EHRA) symptom scale has been proposed to assess symptom severity in patients with AF (Table 147.1).

Stroke and Thromboembolism

Patients with AF have an elevated risk of stroke (a four- to five-fold increase), but this risk is not homogeneous and is dependent upon stroke risk factors (see Chapter 143). Ischemic strokes associated with AF usually result from cardioembolism to a large cerebral artery, and, therefore, AF-related strokes tend to be larger and more often fatal or result in greater disability, worse functional outcomes, and greater mortality compared with strokes from other causes.[3] The arrhythmia is also associated with greater cognitive decline and increased risk of vascular dementia. Cognitive disturbances may occur in the absence of a clinically overt stroke because of multiple asymptomatic cerebral emboli.

In patients with AF, thrombi commonly form within the LAA due to stagnant flow and decreased emptying of this blind-ended structure. Most atrial thrombi in nonvalvular AF (approximately 90%) are formed within the LAA, which explains why, on the background of anticoagulant therapy, surgical occlusion of the LAA at the time of cardiac surgery reduced the risk of stroke by 33% compared with no occlusion.[5] Thrombus formation in AF is consistent with the fulfilment of the Virchow triad of thrombogenesis, including intra-atrial stasis, endothelial dysfunction, and a prothrombotic or hypercoagulable state, due to elevated levels of D-dimer, P-selectin, and von Willebrand factor.[6]

Heart Failure, Other Consequences and Death

Heart failure and AF coexist in many patients, and they can cause and exacerbate each other. Rapid ventricular rate in AF may precipitate signs and symptoms of acute heart failure in patients with an underlying heart failure with preserved LVEF. Poorly controlled ventricular rate in AF may also result in development of heart failure with reduced LVEF in individuals with previously normal left ventricular function (so-called tachycardiomyopathy), with or without clinically overt symptoms and signs of heart failure. Patients with AF and heart failure (with either preserved or reduced LVEF) have

CASE 1: CONCOMITANT ATRIAL FIBRILLATION AND RISK OF STROKE

A 76-year-old woman with chronic heart failure and coronary artery disease who was taking warfarin for stroke prevention because of a history of paroxysmal AF was referred to cardiology because of a history of palpitations and progressive exertional breathlessness. Decompensation of chronic heart failure was diagnosed. Laboratory tests showed poor quality of anticoagulation control (the time in therapeutic range [TTR] of the international normalized ratio was 50%). The electrocardiogram (ECG) confirmed AF with mean ventricular rate of 130 beats/min. On transthoracic echocardiography (TTE), the left ventricular ejection fraction (LVEF) was reduced (30%), no significant valvular disease was revealed, and the left atrium was enlarged (46 mm). Intravenous diuretics were administered to improve the patient's clinical status. Coronary angiography revealed no critical stenoses in coronary arteries. Transesophageal echocardiography (TEE) showed a thrombus in the left atrial appendage (LAA) measuring 2 cm. Warfarin was stopped and a non-vitamin K antagonist oral anticoagulant (NOAC) was initiated. After one month, a follow-up TEE showed disappearance of the LAA thrombus and a successful electrical cardioversion (ECV) was performed. Optimal pharmacological therapy was implemented. Three months later, the LVEF increased to 40%. Multiple ECGs confirmed sinus rhythm.

Comment

In the context of other cardiovascular comorbidities, identifying symptoms of AF may be challenging, since AF commonly complicates other conditions, especially those that have a structural impact on the heart, such as heart failure, hypertension, or valvular disease. The need for early identification of AF and its appropriate management according to guidelines should be emphasized to reduce adverse outcomes. Paroxysmal AF with a rapid ventricular rate has been associated with clinical deterioration of heart failure. Thrombus in the LAA was related to poor quality of anticoagulation with warfarin. Thus, NOACs were started due to their better efficacy/safety ratio, convenience of use, predictable anticoagulant effects without the need for routine coagulation monitoring, and fewer food and drug interactions compared with vitamin K antagonists (VKAs).[4]

worse symptoms, poorer prognosis, and increased mortality compared with individuals with AF without heart failure.[7] Regardless of the development of heart failure, AF is associated with a twofold increased risk of all-cause mortality in women and a 1.5-fold increase in men.

DIAGNOSIS

Advances in diagnostic technologies facilitate the development of more precise therapeutic approaches. The diagnosis of AF is based on ECG showing the absence of P waves, fibrillatory waves between QRS complexes, and irregular R-R intervals. Importantly, prolonged ECG monitoring may increase the probability of detecting undiagnosed, silent AF.

Cardiac electronic implantable devices with an atrial lead enable detection of atrial high-rate episodes (AHRE). An increasing body of evidence shows that AHRE are associated with an elevated risk of AF and AF-related thromboembolism.[8] Thus, individuals with AHRE require further assessment for AF with regular ECG monitoring.[9] In stroke survivors, additional non-invasive ECG monitoring or implantable loop-recorders should be considered to detect silent AF.

Key Investigations

Cardiac ultrasound in AF provides important information on risk stratification, cardiac function, complications, prognosis, and management of associated conditions. Whereas TTE is useful in all patients with AF, TEE allows evaluation of LAA thrombus in individuals being considered for cardioversion or catheter ablation. Intracardiac echocardiography, computed tomography, or magnetic resonance complement TEE and play additional roles in the management of patients with AF.

TABLE 147.1 Symptom Scoring in Atrial Fibrillation

mEHRA Score	Symptoms	Description
1	None	No symptoms related to AF
2a	Mild	Normal daily activity not affected by symptoms associated with AF
2b	Moderate	Normal daily activity not affected, but patient troubled by symptoms
3	Severe	Normal daily activity affected
4	Disabling	Normal daily activity discontinued

AF, Atrial fibrillation.

Blood tests may be useful in initial assessment of patients with AF, and they should include tests for renal, thyroid, and hepatic function, in addition to serum electrolytes, full blood count, and tests of coagulation, such as prothrombin time/INR and activated partial thromboplastin time.

Numerous biomarkers have been associated with the risk of stroke and bleeding in AF and have contributed to our understanding of pathophysiological mechanisms involved in complications of AF. Moreover, many biomarkers are non-specifically related to multiple outcomes, both cardiovascular and non-cardiovascular. Thus, they may be better used to rule out (rather than rule in) in relation to management decision-making.

Differential Diagnoses

Other atrial rhythms may be similar to AF on an ECG, but these can often be distinguished by the presence of discrete P waves (e.g., atrial tachycardias). Atrial flutter, in its typical form, is defined by a sawtooth pattern of regular atrial activation, visible as flutter waves in leads II, III, aVF, and V1. Atrial flutter commonly appears with 2:1 atrioventricular block, resulting in a ventricular rate of approximately 150 beats/min (unless rate control medications have been used). It should be emphasized that atrial flutter is also associated with SSE, and the requirements for anticoagulation are the same as those for AF. Supraventricular tachycardias can be easily distinguished from other narrow complex tachycardias (and often effectively treated) using intravenous adenosine. A regular wide QRS complex tachycardia should be medicated urgently as ventricular tachycardia, unless a high probability of a bundle-branch block pattern or ventricular pre-excitation is assumed.

An Integrated Approach to Atrial Fibrillation Management

The Atrial fibrillation Better Care (ABC) pathway for integrated management is a simple strategy (A–avoid stroke, B–better symptom management, C–cardiovascular and comorbidity risk reduction) that improves detection and reminds clinicians of the simple decision-making steps for treatment of AF in a holistic manner ("Easy as ABC …") (Fig. 147.1).[10]

"A" Avoid Stroke/Anticoagulation

Stroke prevention is a fundamental part of the management of patients with AF. The first step is to identify low-risk patients (those with CHA_2DS_2-VASc [congestive heart failure, hypertension, age ≥75 years, diabetes, stroke/transient ischemic attack, vascular disease, age 65 to 74 years, sex category] score of 0 in men or 1 in women), in whom antithrombotic therapy is not indicated (Table 147.2).[11] The subsequent step is to offer stroke prevention to patients with one or more non-sex CHA_2DS_2-VASc stroke risk factors, and stroke prevention means oral anticoagulation, either a well-managed VKA or a non-vitamin K antagonist oral anticoagulant (NOAC) (see later sections).

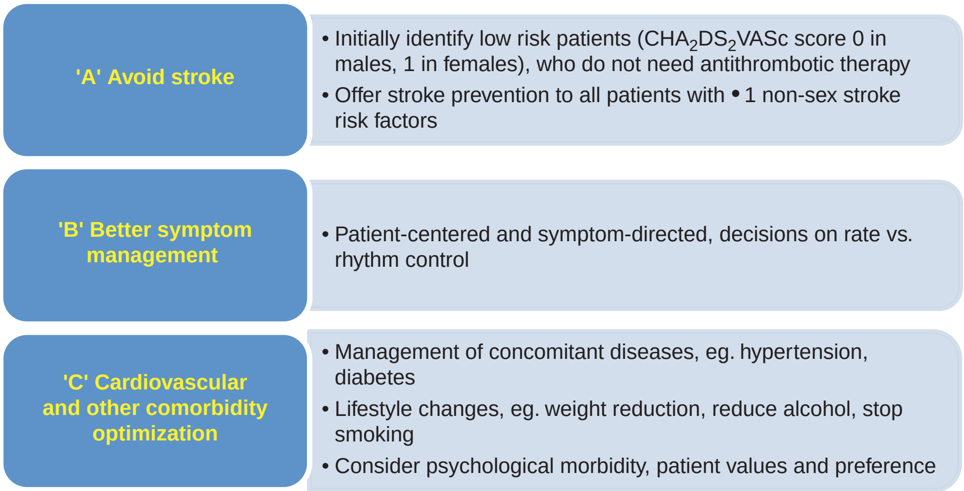
Evaluation of bleeding risk should be performed in all patients. The HAS-BLED (hypertension, abnormal renal/liver function, stroke, bleeding history or predisposition, labile INR, elderly, drugs or alcohol concomitantly) score was originally derived and proposed for bleeding risk assessment in patients with AF (Table 147.3).[12] The HAS-BLED score should be used to draw attention to modifiable bleeding risk factors (e.g., uncontrolled blood pressure, concomitant use of nonsteroidal anti-inflammatory drugs [NSAIDs] or antiplatelet agents in an anticoagulated patient, labile INRs), and to flag "high risk" patients for early follow-up review (e.g., 4 weeks rather than 4 to 6 months) (Fig. 147.2). Such a proactive strategy with appropriate use of dynamic bleeding risk assessment and follow-up was associated with lower risk of bleeding events and improved anticoagulation uptake, compared with usual care.[13]

"B" Better Symptom Control

The next aspect of AF management is patient-centered, symptom directed decision on rate or rhythm control. In general, there is little difference in AF-related clinical outcomes between rate or rhythm control.

"C" Cardiovascular Risk Factor and Comorbidity Management

Multiple cardiovascular risk factors and comorbidities commonly coexist with AF. As part of integrated holistic AF care, comorbidities should be addressed, for example, management of heart failure,


'A' Avoid stroke
'B' Better symptom management
'C' Cardiovascular and other comorbidity optimization

- Initially identify low risk patients (CHA_2DS_2VASc score 0 in males, 1 in females), who do not need antithrombotic therapy
- Offer stroke prevention to all patients with • 1 non-sex stroke risk factors

- Patient-centered and symptom-directed, decisions on rate vs. rhythm control

- Management of concomitant diseases, eg. hypertension, diabetes
- Lifestyle changes, eg. weight reduction, reduce alcohol, stop smoking
- Consider psychological morbidity, patient values and preference

Figure 147.1 THE ABC PATHWAY FOR INTEGRATED CARE MANAGEMENT OF ATRIAL FIBRILLATION.

TABLE 147.2 **Risk Stratification for Stroke and Systemic Thromboembolism**

CHA_2DS_2-VASc Score	
Clinical Characteristic	**Points**
CHF or LVEF ≤40%	1
Hypertension	1
Age ≥75 years	2
Diabetes	1
Stroke/TIA	2
Vascular disease	1
Age 65–74 years	1
Sex category (female)	1
Cumulative score	Range 0–9

For the CHA2DS2-VASc score, estimated stroke and thromboembolism event rates at 1 year follow-up are 0.78% (0 points), 2.01% (1 point), 3.71% (2 points), 5.92% (3 points), 9.27% (4 points), 15.26% (5 points), 19.74% (6 points), 21.50% (7 points), 22.38% (8 points), 23.64% (9 points); see Olesen JB, Lip GY, Hansen ML et al. Validation of risk stratification schemes for predicting stroke and thromboembolism in patients with atrial fibrillation: nationwide cohort study. *Br Med J.* 2011;342:d124.
Vascular disease = prior myocardial infarction, peripheral artery disease, and/or aortic plaque.
CHF, Congestive heart failure; *LVEF*, left ventricular ejection fraction; *TIA*, transient ischemic attack.

TABLE 147.3 **Risk Stratification for Bleeding: The HAS-BLED Score**

HAS-BLED Score	
Clinical Characteristic	**Points**
Hypertension	1
Abnormal renal/liver function	1 or 2
Stroke	1
Bleeding	1
Labile INRs	1
Elderly (age >65 years)	1
Drugs or alcohol	1 or 2
Cumulative score	Range 0–9

Hypertension = systolic blood pressure >160 mm Hg; abnormal renal function = dialysis, transplant, creatinine >2.6 mg/dL or >200 µmol/L; abnormal liver function = cirrhosis or bilirubin >2x normal with AST/ALT/AP >3x normal; labile INR = unstable/high INRs, time in therapeutic range <60%; drugs = antiplatelet agents, nonsteroidal anti-inflammatory drugs; alcohol = eight or more drinks/week.
ALT, Alanine aminotransferase; *AP*, alanine phosphatase; *AST*, aspartate aminotransferase; *INR*, international normalized ratio.

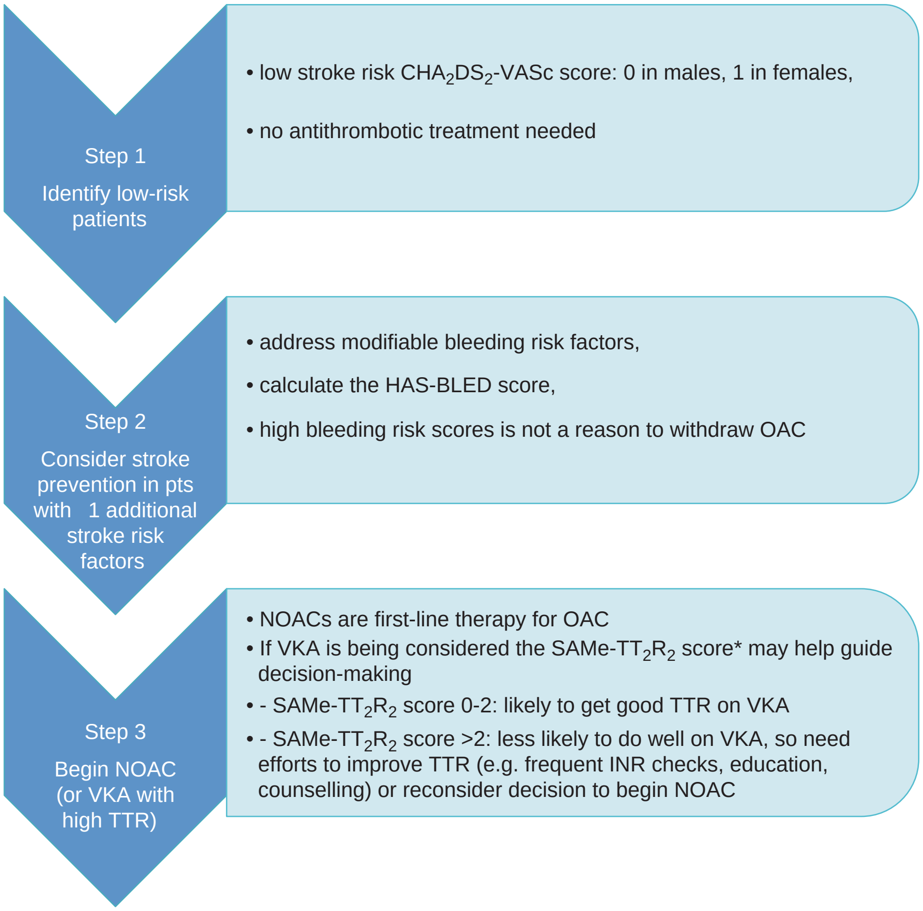

Step 1
Identify low-risk patients
• low stroke risk CHA$_2$DS$_2$-VASc score: 0 in males, 1 in females,
• no antithrombotic treatment needed
Step 2
Consider stroke prevention in pts with 1 additional stroke risk factors
• address modifiable bleeding risk factors,
• calculate the HAS-BLED score,
• high bleeding risk scores is not a reason to withdraw OAC
Step 3
Begin NOAC (or VKA with high TTR)
• NOACs are first-line therapy for OAC
• If VKA is being considered the SAMe-TT$_2$R$_2$ score* may help guide decision-making
• - SAMe-TT$_2$R$_2$ score 0-2: likely to get good TTR on VKA
• - SAMe-TT$_2$R$_2$ score >2: less likely to do well on VKA, so need efforts to improve TTR (e.g. frequent INR checks, education, counselling) or reconsider decision to begin NOAC

Figure 147.2 STRATEGIES FOR STROKE PREVENTION. *SAMe-TT$_2$R$_2$ (sex [female], age [<60 years]), medical history (≥2 from: hypertension, diabetes mellitus, coronary artery disease/ myocardial infarction, peripheral artery disease, congestive heart failure, previous stroke, pulmonary disease, and hepatic or renal disease), treatment (interacting drugs, e.g., amiodarone), tobacco use (within 2 years), race (non-White).

hypertension, diabetes mellitus, sleep apnea, and underlying cardiac ischemia. Lifestyle changes, including weight reduction, regular exercise, and limitation of alcohol and stimulant use, should be pursued. If possible, education and counseling should be implemented.

Hypertension is common in AF patients. Hypertension has an impact on the pathogenesis, management, and prognosis of AF. As a modifiable risk factor, control of hypertension carries a potential for AF prevention, lowering of AF recurrence rates, and avoiding the devastating complications, such as stroke. Thus, optimal blood pressure control should be an integral part of the management of AF patients. In case of rate control strategy, beta-blockers or non-dihydropyridine calcium channel blockers should be considered. Stroke prevention with OAC is recommended in AF individuals with hypertension. Importantly, hypertension substantially elevates the risk of intracerebral hemorrhage in AF patients on OAC. Thus, efforts should be made towards achieving a target blood pressure of less than 130/80 mm Hg in patients receiving OAC.[14]

Management compliant with the ABC approach has been associated with a reduced rate of cardiovascular events and lower healthcare costs when compared with noncompliance with the ABC pathway.[15] The prospective cluster randomized mAFA II trial randomized AF patients to the mAFA intervention arm (based on the ABC pathway) versus a usual care arm.[11,16] The intervention arm was associated with significantly reduced risks of rehospitalization (1.2% vs. 4.5%; hazard ratio [HR]: 0.32; 95% confidence interval [CI]: 0.17 to 0.60; $P < .001$) and composite of stroke/thromboembolism, all-cause death and rehospitalization (1.9% vs. usual care, 6.0%; HR: 0.39; 95% CI: 0.22 to 0.67; $P < .001$).[17]

Rate Control

Rate control is usually adequate to improve symptoms related to AF, although the evidence for the optimal type and intensity of rate control is scarce. Pharmacological agents for maintaining acute or long-term rate control include digoxin, beta-blockers, diltiazem or verapamil, or combination therapy.

Patients with acute, new-onset AF often require heart rate control with a preference for beta-blockers or rate-limiting calcium antagonists, such as diltiazem or verapamil. In sedentary individuals, digoxin may be considered. Diltiazem or verapamil is contraindicated in patients with left ventricular systolic dysfunction, where beta blockers and/or digoxin may be considered. However, beta-blockers do not decrease all-cause mortality in individuals with AF and heart failure when compared with placebo.[18] Conventional approaches to maintain a strict heart rate (<80 beats/min) have not proved to be better than a more lenient approach, for both prognosis and symptom control.

Rhythm Control

Clinical trials that compared rhythm control with rate control alone have not shown superiority of one strategy over another for clinical outcomes.[19] Thus, a rhythm control strategy is recommended to improve AF-related symptoms in patients who remain symptomatic on optimal rate control therapy. Cardioversion, catheter/surgical ablation, or combination therapy is frequently effective when antiarrhythmic drugs have failed. All antiarrhythmic agents have possible side effects (for example, thyroid dysfunction, corneal microdeposits, and pulmonary fibrosis with amiodarone use; proarrhythmic effects of Class I antiarrhythmics). The initial choice of antiarrhythmic drug should be preceded by evaluation of the safety of antiarrhythmic therapy.[19]

As a non-drug option, electrical cardioversion (ECV) is a common method of converting AF to sinus rhythm, especially in hemodynamically unstable patients with AF. Pre-treatment with antiarrhythmic drugs may increase the efficacy of ECV. Catheter ablation of AF involves radiofrequency ablation or cryoablation to the endocardium, typically including isolation of the pulmonary veins. This rhythm control approach is a second-line treatment in patients with AF and failure of or intolerance to antiarrhythmic drug therapy. In selected patients, catheter ablation in AF patients with heart failure significantly reduces recurrent AF.

Surgical ablation (targeting the pulmonary veins or Cox-Maze approach) is usually performed in patients undergoing cardiac surgery for other reasons (e.g., coronary artery bypass grafting or valve surgery), often in combination with surgical left atrial appendage exclusion. Each method of rhythm control strategy is associated with recurrence of AF, which can be often asymptomatic. Due to risk of recurrent AF, long-term anticoagulation should not be stopped in patients with risk factors for stroke.

PREVENTION OF STROKE AND THROMBOEMBOLISM

Thromboembolic risk is not homogeneous among AF patients, and it depends on the presence of various stroke risk factors, such as prior stroke or transient ischemic attack, hypertension, age, structural heart disease, and diabetes mellitus.[11] The more common and validated stroke risk factors have been used to formulate various stroke risk stratification schema, and the CHA$_2$DS$_2$-VASc score is the most used (see Table 147.2). The default should be to offer stroke prevention, unless the patient is low risk, and no antithrombotic therapy is required. Thus, the CHA$_2$DS$_2$-VASc score should be used to initially identify patients with "low stroke risk" (CHA$_2$DS$_2$-VASc score 0 in men, or 1 in women) who do not require antithrombotic therapy. The subsequent step is to offer stroke prevention to AF patients with one or more non-sex CHA$_2$DS$_2$-VASc stroke risk factors (CHA$_2$DS$_2$-VASc score ≥ 1 in a male, ≥ 2 in a female). In most cases of AF associated with valvular heart disease (such as mitral stenosis), there are rarely any reasons not to offer antithrombotic therapy to patients due to the high baseline risk of SSE, regardless of other patient characteristics.[20]

Oral Anticoagulation

Adjusted-dose warfarin is associated with a 64% relative risk (RR) reduction in stroke and a 26% reduction in all-cause mortality, compared with placebo or control.[21] Aspirin was associated with a nonsignificant reduction (19%) in stroke. A target INR range of 2.0 to 3.0 is recommended, and the average TTR should be less than 70%. There is no evidence that lower INR range targets are beneficial, despite such practice being common in Asian countries.[22]

We are now in the era of the NOACs, which offer relative efficacy, safety, and convenience compared with VKAs. In the meta-analysis by Ruff et al.,[23] NOACs were compared with warfarin using data from the Randomized Evaluation of Long-Term Anticoagulation Therapy (RE-LY), Rivaroxaban Once Daily Oral Direct Factor Xa Inhibition Compared with Vitamin K Antagonism for Prevention of Stroke and Embolism Trial in AF (ROCKET-AF), Apixaban for the Prevention of Stroke in Subjects With AF (ARISTOTLE), and the Effective Anticoagulation with Factor Xa Next Generation in AF–Thrombolysis in Myocardial Infarction 48 (ENGAGE AF-TIMI 48) clinical trials; NOACs were shown to have a better risk-benefit profile, with significant decreases in stroke or systemic embolic events (RR 0.81, 95% CI 0.73–0.91, $P < .0001$), intracranial hemorrhage (RR 0.49, 95% CI 0.38–0.64, $P < .0001$), and all-cause mortality (RR 0.90, 95% CI 0.85–0.95, $P = .0003$) compared with warfarin. The rate of major bleeding with NOACs was similar to that with warfarin, but gastrointestinal bleeding (GIB) was more frequent with NOACs than with warfarin.

CASE 2: INTERVENTIONAL APPROACH TO ATRIAL FIBRILLATION

A 68-year-old man with hypertension and paroxysmal AF was referred to cardiology with a 4-month history of palpitations and impaired exercise tolerance. He had been admitted to his local emergency department three times due to AF with rapid ventricular response that had required cardioversion. Treatment with a beta-blocker and propafenone had not decreased the frequency of symptoms and the patient complained of profound lethargy associated with higher doses of beta-blocker. The patient was prescribed dabigatran 150 mg twice daily for stroke prevention (see Chapter 142). Blood tests were within reference range. On TTE, LVEF was impaired (45%), no significant valvular disease was revealed, and the left atrium was enlarged (44 mm). After discussing available management options and the procedural risk, cryoballoon ablation of AF was performed. Dabigatran was continued without interruption. The procedure was uncomplicated, and the patient was discharged the following day. At 3 months, the patient remained asymptomatic. His CHA$_2$DS$_2$-VASc score was 2, and the patient continued using dabigatran post-ablation.

Comment

In some patients, symptomatic AF may significantly impair the quality of life, and rhythm control may be a preferred treatment choice. With failure of pharmacological rhythm control therapy, the patient was offered catheter ablation. Catheter ablation of AF performed in experienced centers is usually more effective than antiarrhythmic drug therapy, especially for symptom relief and quality of life improvement. There is no high-quality evidence confirming that AF catheter ablation prevents major cardiovascular outcomes. Thus, in selected patients with symptomatic, paroxysmal AF who prefer an interventional strategy, catheter ablation may be considered as first-line therapy. A higher probability of sinus rhythm maintenance and lower procedure-related complications can be expected in younger patients without structural heart disease, with a short history of AF, and frequent, short episodes of AF. Complication rates are low in experienced centers (1 in 100 risk of cardiac tamponade and 1 in 500 risk of stroke), and 1-year freedom from AF ranges from 50% to 80% in experienced centers.

Regardless of the apparently successful maintenance of sinus rhythm, oral anticoagulation should be continued in patients with two or more CHA$_2$DS$_2$-VASc risk factors, equivalent to an estimated stroke rate of over 3%–4% per year.

The randomized trial data are also complemented by effectiveness and safety data from large "real world" observational cohorts.[24,25] In the largest analysis, ARISTOPHANES (Anticoagulants for Reduction in Stroke: Observational Pooled Analysis on Health Outcomes and Experience in Patients) NOACs were associated with reduced rates of SSE compared with warfarin (HR 0.64, 95% CI 0.58 to 0.70 for apixaban; HR 0.82, 95% CI 0.71 to 0.95 for dabigatran; HR 0.79, 95% CI 0.73 to 0.85 for rivaroxaban) and similar rates of major bleeding (HR 0.60, 95% CI 0.56 to 0.63 for apixaban; HR 0.71, 95% CI 0.65 to 0.78 for dabigatran; HR 1.06, 95% CI 1.02 to 1.10 for rivaroxaban).[24] The FDA-sponsored analysis, by Graham et al.,[26] showed a reduced risk of thromboembolic stroke, intracranial hemorrhage, and mortality among patients on standard doses of NOACs compared with warfarin. Dabigatran and apixaban were associated with a better benefit-harm profile than warfarin.

Compared with warfarin, NOACs have a rapid onset/offset of action, fewer drug interactions, and no effect of dietary vitamin K intake on their activity. The predictable dose-related anticoagulant effect of NOACs enable their use in fixed doses without the need for routine coagulation monitoring, thus simplifying the therapy (Table 147.4). In NOAC-eligible AF patients, international AF guidelines recommend NOACs in preference to VKAs.[11] Patients with nonvalvular AF given VKAs should have the dose adjusted to maintain the INR within the range of 2.0 to 3.0, with a TTR of $\geq 70\%$. The TTR is influenced by various clinical factors, and the more common ones have been used to formulate the SAMe-TT$_2$R$_2$ score, where a score of greater than 2 is associated with a likelihood of having a poor TTR,[27,28] whereby interventions to improve TTR

TABLE 147.4 Characteristics of NOACs

	Dabigatran	Apixaban	Edoxaban	Rivaroxaban
Mechanism	Oral direct thrombin inhibitor	Oral direct factor Xa inhibitor	Oral direct factor Xa inhibitor	Oral direct factor Xa inhibitor
Prodrug	Yes	No	No	No
Standard dose	150 mg twice daily	5 mg twice daily	60 mg once daily	20 mg once daily
Reduced dose	110 mg twice daily; 75 mg twice daily in United States	2.5 mg twice daily	30 mg once daily	15 mg once daily
Bioavailability	3%–7%	50%	62%	66% without food, 80%–100% with food
Renal clearance of absorbed dose	80%	27%	50%	35%
Plasma protein binding	35%	87%	55%	95%
Dialyzability	50%–60%	14%	Not available	Not available
Liver metabolism: CYP3A4 involved	No	Yes (25%)	Minimal (<4% of elimination)	Yes (18%)
Absorption with food	No effect	No effect	6%–22%	≥39%
Elimination half-life	12–17 h	12 h	10–14 h	5–9 h (young), 11–13 h (elderly)

should be implemented (e.g., more frequent INR checks or frequent follow up), or, more ideally, switching to a NOAC should be considered. Efforts to improve prescription of OAC include avoiding under-dosing, which requires education of health care providers; implementation of local guidelines; interdisciplinary medical care programs educating both care providers and patients; and persuasive interventions utilizing peer-group experts.[29]

Of note is that VKAs are currently the only therapy with established safety in patients with AF and rheumatic mitral valve disease and/or mechanical heart valves.[20]

Secondary Stroke Prevention

The highest risk of recurrent stroke occurs in the early phase after a first stroke or TIA (see Chapter 143). Data on the optimal timing of OAC (re)initiation after an AF-related stroke are scarce and conflicting. In AF patients with acute ischemic stroke, OAC should be usually started within 2 weeks of acute ischemic stroke, but the optimal timing within this period is not established. Patients with AF and acute ischemic stroke without contraindications to OAC should be managed with long-term oral anticoagulation.

Prevention of recurrent stroke with OAC is effective but requires a multidisciplinary approach, including stroke physicians and cardiologists, to carefully select appropriate patients and minimize the risk of hemorrhagic transformation. All NOACs are associated with a lower risk of intracranial hemorrhage than VKAs; hence, if a patient had a stroke while on warfarin, clinicians may consider switching to a NOAC. Such patients also require assessment of other causes of stroke, such as uncontrolled blood pressure or significant carotid disease. If on warfarin, we should ensure that the TTR is greater than 70% and, if on a NOAC, whether they were on the label-adherent dose and there was good persistence and adherence to therapy.[30]

Nonpharmacologic Strategies for Stroke Prevention

Several non pharmacological options are available for stroke prevention in AF in patients with history of bleeding who have absolute contraindications OAC. These include LAA occlusion, surgical LAA occlusion, or permanent carotid filters.

Surgical LAA occlusion may be performed during cardiac surgery undertaken for other indications. Among AF patients after cardiac surgery, the majority of whom continued to receive ongoing OAC, the risk of ischemic stroke or systemic embolism was lower with LAA occlusion during the surgery than without it.[5]

Occlusion of the LAA may be considered for stroke prevention in AF patients with absolute contraindications for long-term OAC.[31] A candidate for LAA occlusion should ideally have no contraindications to dual antiplatelet therapy (DAPT) for at least several weeks, followed by permanent single antiplatelet therapy (SAPT). In patients with contraindications to antiplatelet therapy, percutaneous endocardial/epicardial or minimally invasive surgical epicardial LAA occlusion may be considered. In randomized trials,[32] LAA occlusion was non-inferior to VKA for stroke prevention in individuals with AF and moderate stroke risk, with a likelihood of lower bleeding rates during follow-up. In AF patients undergoing cardiac surgery, surgical LAA occlusion or exclusion may be considered for stroke prevention. However, residual LAA flow or incomplete LAA exclusion can contribute to higher risk of stroke. A novel approach using permanent, bilateral common carotid artery (CCA) filters has been proposed to capture emboli and prevent major cerebral stroke in patients with contraindications to OAC.

Periprocedural Anticoagulation

Interruption of anticoagulation temporarily increases the thromboembolic risk, whereas non-interrupted anticoagulation increases the risk of bleeding associated with surgical procedures.[33] Individuals scheduled for low bleeding risk procedures (e.g., dental and cutaneous procedures) can continue oral anticoagulation (warfarin within the target INR range and probably NOACs, as well),[34] whereas AF patients at high or moderate thromboembolic risk should limit the period without anticoagulation to the shortest possible interval with or without bridging with low-molecular-weight heparin (LMWH) in case of procedures requiring a temporary OAC interruption. When interruption of OAC is required, bridging does not appear to be beneficial, except in patients with mechanical heart valves.

ANTICOAGULANT-RELATED BLEEDING

There are various risk factors for bleeding, and the more common risk factors have been used to formulate bleeding risk scores. Of the various bleeding risk stratification schema, the HAS-BLED is a user-friendly tool for clinicians, which stratifies patients into two categories of risk: high, low-intermediate (see Table 147.3).[35] Due to the dynamic nature of bleeding, bleeding risk evaluation needs to be repeated regularly.

Many studies have reported less intracranial hemorrhage and life-threatening bleeding with NOACs than with warfarin. Patients who suffered a major bleeding event on NOACs had a more favorable outcome than those on VKAs.[36,37]

In the 2018 EHRA Practical Guide on the use of NOACs in patients with AF,[4] recommended management of bleeding in individuals treated with NOACs depends on the following factors: the type of bleeding (minor, major non-life threatening, or major life threatening), timing of the last NOAC intake, dosing regimen, renal function, concomitant medication, and other aspects influencing hemostasis.

In AF patients with severe acute bleeding, it is recommended to withhold OAC until the underlying cause is identified and managed. Each case will require an individualized approach and decision to restart OAC therapy after consideration of the risks, benefits, alternatives, and patient's values. Notably, the re-initiation of OACs after a major bleeding in patients with AF has been associated with a positive clinical benefit in comparison to withholding OACs.[38]

Resumption of OAC after bleeding should be considered in all eligible patients by a multidisciplinary AF team. Complex decisions concerning the discontinuation and re-initiation of OAC should also be discussed by a multidisciplinary team, analysing the risk of recurrent stroke and bleeding, and taking into consideration different prevention options.[9] In case of bleeding due to secondary or reversible causes, OAC can be safely reinitiated once the cause of the bleed has been corrected. The risks for recurrent ICH or recurrent GIB are often similar between OAC restarters and non-restarters.[39]

Reversal of Anticoagulation

The management of bleeding remains a clinical challenge in the setting of anticoagulation. The most important aspect is to eliminate the source of bleeding. In patients on VKAs, physicians have many years of experience with reversing the agent with several therapies, such as intravenous vitamin K, fresh frozen plasma (FFP), and prothrombin complex concentrate (PCC). When rapid reversal is required, PCC is preferred over FFP; recombinant factor VIIa is not recommended (see Chapter 142).

Idarucizumab can be useful in case of life-threatening bleeds with dabigatran. Andexanet alfa is an option for reversal of oral factor Xa inhibitors.[40] PCC may also support hemostasis in such patients.[41]

Given their short half-lives, time appears to be the most important factor in bleeding associated with NOACs. This emphasizes the significance of asking patients about the exact time of the last NOAC intake and ascertaining factors affecting plasma concentrations and hemostasis (e.g., age, comorbidities, and concomitant use of antiplatelet drugs).[4]

FUTURE DIRECTIONS

Current gaps in the evidence on management of AF include screening for AF using patient-operated ECG devices, smartphones, and other technologies, comparison of different rate control therapies, defining the place of rhythm control with hybrid approaches to restore sinus rhythm, and establishing how best to manage patients with AF and concomitant heart failure. Problems, such as optimal stroke prevention in patients with severe renal dysfunction, LAA occlusion for stroke prevention, and anticoagulation in patients after major bleeding, especially ICH or GIB, require further research.

Whereas stroke prevention remains to be a cornerstone of AF management, the risk of death in patients with AF remains unacceptably high. Further attention to these areas is crucial, considering the rapidly increasing incidence and prevalence of AF, as well as the burden that this condition is placing on patients and health care systems.

REFERENCES

1. Haim M, Hoshen M, Reges O, et al. Prospective national study of the prevalence, incidence, management and outcome of a large contemporary cohort of patients with incident non-valvular atrial fibrillation. *J Am Heart Assoc*. 2015;4(1):e001486.
2. Allan V, Honarbakhsh S, Casas J-P, et al. Are cardiovascular risk factors also associated with the incidence of atrial fibrillation? A systematic review and field synopsis of 23 factors in 32 population-based cohorts of 20 million participants. *Thromb Haemost*. 2017;117(5):837–850.
3. Freedman B, Potpara TS, Lip GYH. Stroke prevention in atrial fibrillation. *Lancet*. 2016;388(10046):806–817.
4. Steffel J, Verhamme P, Potpara TS, et al. The 2018 European Heart Rhythm Association Practical Guide on the use of non-vitamin K antagonist oral anticoagulants in patients with atrial fibrillation: executive summary. *Europace*. 2018;20(8):1231–1242.
5. Whitlock RP, Belley-Cote EP, Paparella D, et al. Left atrial appendage occlusion during cardiac surgery to prevent stroke. *N Engl J Med*. 2021;384(22):2081–2091.
6. Donal E, Lip GYH, Galderisi M, et al. EACVI/EHRA Expert Consensus Document on the role of multi-modality imaging for the evaluation of patients with atrial fibrillation. *Eur Heart J Cardiovasc Imaging*. 2016;17(4):355–383.
7. Kotecha D, Chudasama R, Lane DA, et al. Atrial fibrillation and heart failure due to reduced versus preserved ejection fraction: a systematic review and meta-analysis of death and adverse outcomes. *Int J Cardiol*. 2016;203:660–666.
8. Khan AA, Boriani G, Lip GYH. Are atrial high rate episodes (AHREs) a precursor to atrial fibrillation? *Clin Res Cardiol*. 2020;109(4):409–416.
9. Kirchhof P, Benussi S, Kotecha D, et al. 2016 ESC guidelines for the management of atrial fibrillation developed in collaboration with EACTS. *Eur Heart J*. 2016;37(38):2893–2962.
10. Lip GYH. The ABC pathway: an integrated approach to improve AF management. *Nat Rev Cardiol*. 2017;14(11):627–628.
11. Lip GYH, Banerjee A, Boriani G, et al. Antithrombotic therapy for atrial fibrillation: CHEST guideline and expert panel report. *Chest*. 2018;154(5):1121–1201.
12. Pisters R, Lane DA, Nieuwlaat R, et al. A novel user-friendly score (HAS-BLED) to assess 1-year risk of major bleeding in patients with atrial fibrillation: the Euro Heart Survey. *Chest*. 2010;138(5):1093–1100.
13. Guo Y, Lane DA, Chen Y, Lip GYH. Regular bleeding risk assessment associated with reduction in bleeding outcomes: the mAFA-II randomized trial. *Am J Med*. 2020 pii: S0002-9343(20)30274–6.
14. Williams B, Mancia G, Spiering W, et al. 2018 ESC/ESH Guidelines for the management of arterial hypertension. *Eur Heart J*. 2018;39(33):3021–3104.
15. Proietti M, Romiti GF, Olshansky B, et al. Improved outcomes by integrated care of anticoagulated patients with atrial fibrillation using the simple ABC (atrial fibrillation better care) pathway. *Am J Med*. 2018;131(11):1359–1366. e6.
16. Proietti M, Laroche C, Opolski G, et al. 'Real-world' atrial fibrillation management in Europe: observations from the 2-year follow-up of the EURObservational Research Programme-Atrial Fibrillation General Registry Pilot Phase. *Europace*. 2017;19(5):722–733.
17. Guo Y, Lane DA, Wang L, et al. Mobile health technology to improve care for patients with atrial fibrillation. *J Am Coll Cardiol*. 2020;75:1523–1534.
18. Kotecha D, Holmes J, Krum H, et al. Efficacy of β blockers in patients with heart failure plus atrial fibrillation: an individual-patient data meta-analysis. *Lancet*. 2014;384(9961):2235–2243.
19. Dan G-A, Martinez-Rubio A, Agewall S, et al. Antiarrhythmic drugs-clinical use and clinical decision making: a consensus document from the European Heart Rhythm Association (EHRA) and European Society of Cardiology (ESC) Working Group on Cardiovascular Pharmacology, endorsed by the Heart Rhythm Society (HRS), Asia–Pacific Heart Rhythm Society (APHRS) and International Society of Cardiovascular Pharmacotherapy (ISCP). *Europace*. 2018;20(5):731–732an.
20. Lip GYH, Collet JP, de Caterina R, et al. Antithrombotic therapy in atrial fibrillation associated with valvular heart disease: executive summary of a joint consensus document from the European Heart Rhythm Association

(EHRA) and European Society of Cardiology Working Group on Thrombosis, Endorsed by the ESC Working Group on Valvular Heart Disease, Cardiac Arrhythmia Society of Southern Africa (CASSA), Heart Rhythm Society (HRS), Asia Pacific Heart Rhythm Society (APHRS), South African Heart (SA Heart) Association and Sociedad Latinoamericana de Estimulación Cardíaca y Electrofisiología (SOLEACE). *Thromb Haemost*. 2017;117(12):2215–2236.

21. Hart RG, Pearce LA, Aguilar MI. Meta-analysis: antithrombotic therapy to prevent stroke in patients who have nonvalvular atrial fibrillation. *Ann Intern Med*. 2007;146(12):857–867.
22. Pandey AK, Xu K, Zhang L, et al. Lower versus standard INR targets in atrial fibrillation: a systematic review and meta-analysis of randomized controlled trials. *Thromb Haemost*. 2020;120(3):484–494.
23. Ruff CT, Giugliano RP, Braunwald E, et al. Comparison of the efficacy and safety of new oral anticoagulants with warfarin in patients with atrial fibrillation: a meta-analysis of randomized trials. *Lancet*. 2014;383(9921):955–962.
24. Lip GYH, Keshishian A, Li X, et al. Effectiveness and safety of oral anticoagulants among nonvalvular atrial fibrillation patients. *Stroke*. 2018;49(12):2933–2944.
25. Hohnloser SH, Basic E, Hohmann C, Nabauer M. Effectiveness and safety of non-vitamin K oral anticoagulants in comparison to phenprocoumon: data from 61,000 patients with atrial fibrillation. *Thromb Haemost*. 2018;118(3):526–538.
26. Graham DJ, Baro E, Zhang R, et al. Comparative stroke, bleeding, and mortality risks in older medicare patients treated with oral anticoagulants for nonvalvular atrial fibrillation. *Am J Med*. 2019;132(5):596–604.
27. Bernaitis N, Clark G, Kohja S, et al. The SAMe-TT$_2$R$_2$ score predicts warfarin control in an Australian population with atrial fibrillation. *J Clin Med*. 2019;8(6):882.
28. Zulkifly H, Lip GYH, Lane DA. Use of the SAMe-TT$_2$R$_2$ score to predict anticoagulation control in atrial fibrillation and venous thromboembolism patients receiving vitamin K antagonists: a review. *Heart Rhythm*. 2018;15(4):615–623.
29. Pritchett RV, Bem D, Turner GM, et al. Improving the prescription of oral anticoagulants in atrial fibrillation: a systematic review. *Thromb Haemost*. 2019;119(2):294–307.
30. Hohnloser SH, Basic E, Nabauer M. Changes in oral anticoagulation therapy over one year in 51,000 atrial fibrillation patients at risk for stroke: a practice-derived study. *Thromb Haemost*. 2019;119(6):882–893.
31. Glikson M, Wolff R, Hindricks G, et al. EHRA/EAPCI expert consensus statement on catheter-based left atrial appendage occlusion—an update. *Europace*. 2019;22(2):184.
32. Reddy VY, Doshi SK, Sievert H, et al. Percutaneous left atrial appendage closure for stroke prophylaxis in patients with atrial fibrillation: 2.3-year follow-up of the PROTECT AF (watchman left atrial appendage system for embolic protection in patients with atrial fibrillation) trial. *Circulation*. 2013;127(6):720–729.
33. Douketis JD, Murphy SA, Antman EM, et al. Peri-operative adverse outcomes in patients with atrial fibrillation taking warfarin or edoxaban: analysis of the ENGAGE AF-TIMI 48 trial. *Thromb Haemost*. 2018;118(6):1001–1008.
34. Steffel J, Verhamme P, Potpara TS, et al. The 2018 European Heart Rhythm Association Practical Guide on the use of non-vitamin K antagonist oral anticoagulants in patients with atrial fibrillation. *Eur Heart J*. 2018;39(16):1330–1393.
35. Borre ED, Goode A, Raitz G, et al. Predicting thromboembolic and bleeding event risk in patients with non-valvular atrial fibrillation: a systematic review. *Thromb Haemost*. 2018;118(12):2171–2187.
36. Hylek EM, Held C, Alexander JH, et al. Major bleeding in patients with atrial fibrillation receiving apixaban or warfarin: the ARISTOTLE trial (Apixaban for reduction in stroke and other thromboembolic events in atrial fibrillation): predictors, Characteristics, and clinical outcomes. *J Am Coll Cardiol*. 2014;63(20):2141–2147.
37. Piccini JP, Garg J, Patel MR, et al. Management of major bleeding events in patients treated with rivaroxaban vs. warfarin: results from the ROCKET AF trial. *Eur Heart J*. 2014;35(28):1873–1880.
38. Staerk L, Lip GYH, Olesen JB, et al. Stroke and recurrent hemorrhage associated with antithrombotic treatment after gastrointestinal bleeding in patients with atrial fibrillation: nationwide cohort study. *BMJ*. 2015;351:h5876.
39. Proietti M, Romiti GF, Romanazzi I, et al. Restarting oral anticoagulant therapy after major bleeding in atrial fibrillation: a systematic review and meta-analysis. *Int J Cardiol*. 2018;261:84–91.
40. Connolly SJ, Milling Jr. TJ, Eikelboom JW, et al. Andexanet alfa for acute major bleeding associated with factor Xa inhibitors. *N Engl J Med*. 2016;375(12):1131–1141.
41. Majeed A, Ågren A, Holmström M, et al. Management of rivaroxaban- or apixaban-associated major bleeding with prothrombin complex concentrates: a cohort study. *Blood*. 2017;130(15):1706–1712.

CHAPTER 148

BLEEDING AND CLOTTING DISORDERS IN PEDIATRICS

Nasrin Samji, Anthony K. C. Chan, and Mihir D. Bhatt

The neonatal and pediatric stages are periods of rapid physiologic changes, some of which affect the hemostatic system. The hemostatic system is a dynamic system that evolves gradually from birth into the mature adult form. Evaluation of bleeding and clotting disorders in children requires an understanding of the evolution of physiologic normal values for age, the congenital disorders that present in early life, and the common clinical settings that affect hemostasis and thrombosis risks in childhood. The rapid evolution of the blood coagulation system after birth leads to a dynamic group of age-dependent reference ranges for the levels of the various components that should be considered physiologically normal. Developmental hemostasis is the term applied to the evolution of the hemostatic and fibrinolytic systems through infancy and childhood into adult age.

DEVELOPMENTAL HEMOSTASIS

The coagulation system in children provides innate protection from thrombosis without an increased risk for bleeding. The hemostatic system evolves throughout childhood and most rapidly during the neonatal period. Changes in the plasma concentrations of proteins involved in blood coagulation result in a dynamic group of reference ranges for preterm and term infants. Although different from adult values, these reference ranges are neither abnormal nor pathologic. The relative rarity of hemorrhagic or thrombotic complications in this population suggests that the neonatal coagulation system is physiologically replete.

Laboratory Evaluation

The pioneering work of the late Dr. Maureen Andrew paved the way for research into developmental hemostasis. The concept of developmental hemostasis is now widely accepted, and her seminal papers describing the reference ranges for healthy premature and full-term neonates and children are still widely quoted.[1–3] Laboratory evaluation of thrombosis or bleeding in neonates and children must take into account the age-related reference ranges, which differ significantly from adult levels. In addition, because coagulation assay results vary depending on the type of analyzer and reagents used, caution is needed when prior publications of defined reference ranges for neonates are used.[4]

Although research has looked into age-specific differences in the quantitative levels of hemostatic proteins,[5] functional assays that are reagent and analyzer-specific are still predominantly used for hemostatic diagnosis in the pediatric population. Changes in functional protein levels correspond with changes in global tests of coagulation. The prothrombin time (PT), expressed in seconds, in full-term and premature neonates is like that in children and adults, despite the fact that neonates have relative deficiencies of the vitamin K-dependent coagulation factors. The activated partial thromboplastin time (aPTT) is prolonged in newborns, which is attributed to relative deficiencies in the contact factors (see Chapter 126). Thromboelastography reaction time is significantly lower in neonates compared with children and adults. The thrombin time in neonates is like that in children and adults when measured in the presence of calcium, which compensates for the unique fetal form of fibrinogen that has increased sialic acid content.

Blood Coagulation Proteins

The synthesis of fetal and neonatal coagulation proteins begins at approximately 10 weeks of gestation, and plasma concentrations of these proteins increase with gestational age. Maternal coagulation proteins cannot cross the placenta; however, maternal drugs, such as warfarin and phenytoin, can affect the synthesis of fetal vitamin K-dependent coagulation proteins.

In the healthy newborn, plasma levels of procoagulant factors, such as factor (F)VII, FIX, FX, and prothrombin (the vitamin K-dependent coagulation factors) and FXI, FXII, prekallikrein, and high-molecular-weight kininogen (HMWK) (the contact factors), are about 50% lower than adult values (Table 148.1).[1,2] The levels rise as the infant ages, and they reach about 80% of normal adult values by 6 months of age but remain decreased throughout childhood.[6] The levels of fibrinogen, FV, FVIII, and FXIII in neonates are similar to those in adults and remain so throughout childhood.[2,6] The level of von Willebrand factor (vWF) in newborns is about two-fold higher than that in adults and the level of vWF gradually decreases over the first 6 months of life.[2] In contrast, the levels of antithrombin (AT) are lower than adult levels in the first 3 months of life and are comparable to the levels seen in patients with heterozygous AT deficiency (see Chapter 138). The physiologic ranges for coagulation factors in healthy newborns who have received intramuscular vitamin K after delivery are shown in Table 148.2.

Regulation of Thrombin

Thrombin regulation in neonatal plasma is like that in plasma from adults who are receiving therapeutic doses of anticoagulants. The concentration of prothrombin is lower in neonates than that in adults. However, the extent of thrombin generation depends on the experimental system; thrombin generation in plasma from neonates has been reported to be comparable to that in adult plasma provided that thrombomodulin is added.[7,8]

Thrombin is inhibited by AT, α_2-macroglobulin, and heparin cofactor II. In neonates, the α_2-macroglobulin concentration is two-fold higher than that in adults. The overall capacity of newborn plasma to inhibit thrombin is like that of adult plasma due in part to the increased binding of thrombin by α_2-macroglobulin. Thrombin inhibition by heparin cofactor II is catalyzed by a dermatan sulfate-like proteoglycan, which is produced by the placenta and is found in both maternal and fetal plasma.

Regulation of thrombin generation is accomplished by upstream inhibition of the clotting proteins in the prothrombinase and tenase complexes (see Chapters 121 and 125). Plasma concentrations of protein C are low at birth and gradually increase to adult levels by 6 months of age. Although the total concentration of protein S is low at birth, the functional activity of protein S is comparable to that in adults because low levels of C4b-binding protein result in more free protein S. The interaction of protein S with activated protein C in neonatal plasma may be limited by the elevated levels of α_2-macroglobulin. Free tissue factor pathway inhibitor levels in neonates are

TABLE 148.1 Common Hematologic Manifestations of COVID-19

Component	Neonatal Versus Adult Level	
Primary hemostasis	↔	Platelet count
	↑	vWF
Coagulation factors	↓	FII, FVII, FIX, FX
	↓	FXI, FXII
	↓ to ↔	FV, FXIII
	↔	Fibrinogen
	↔	FVIII
Anticoagulant factors	↓	TFPI, AT, PC, PS
	↑	α_2M
Fibrinolysis	↓	Plasminogen
	↔ to ↑	PAI

α_2M, α_2-Macroglobulin; *AT*, antithrombin; *F*, factor; *PAI*, plasminogen activator inhibitor; *PC*, protein C; *PS*, protein S; *TFPI*, tissue factor pathway inhibitor; *vWF*, von Willebrand factor.
Modified from Guzzetta NA, Miller BE. Principles of hemostasis in children: models and maturation. *Paediatr Anaesth.* 2011;21:3.

lower than in adults, although total levels of tissue factor pathway inhibitor in neonatal plasma are like those in adult plasma.

The Fibrinolytic System

Although the levels of some components of the fibrinolytic system in neonates and children are different from those in adults, the clinical relevance of this finding is probably minimal. The fibrinolytic system regulates fibrin deposition by generating plasmin, which solubilizes fibrin. At birth, the fibrinolytic system has all the key components, but there are important age-related differences in the quantity and quality of the fibrinolytic proteins and enzymes. Plasma concentrations of plasminogen, tissue plasminogen activator (tPA), and α_2-antiplasmin (α_2AP) are decreased, whereas plasma concentrations of plasminogen activator inhibitor 1 (PAI-1) are increased. Furthermore, plasmin generation and overall fibrinolytic activity are decreased.[9] The capacity to generate plasmin in newborn plasma is generally reduced compared with adult plasma, which likely reflects the decreased plasminogen concentration. Despite lower levels of fibrinolytic components, the newborn fibrinolytic system is still effective.

The euglobulin clot lysis time is a global assay of fibrinolytic activity but reflects only part of the physiologic fibrinolytic potential. Maneuvers that induce the release of endogenous fibrinolytic components, such as venous occlusion, desmopressin infusion, or exercise, provide more sensitive measures of in vivo fibrinolytic activity.[9]

Plasminogen levels are lower in neonates and children than in adults, and fetal plasminogen binds to cellular receptors with lower affinity because of its increased sialic acid and mannose content. Whereas healthy neonates have lower plasmin-generating potential than adults, baseline levels of tPA can increase up to eightfold with illness. Neonates with severe plasminogen deficiency have only a minimally increased risk for thrombosis, and most of their clinical findings are the result of impaired extravascular fibrinolysis. The major plasmin inhibitors circulate at near adult levels in the neonate. In children, plasminogen activator inhibitor (PAI-1) is elevated, which results in fibrinolytic suppression during childhood.

Imbalances in the fibrinolytic system, whether hereditary or acquired, can lead to thrombotic or bleeding complications. Hereditary disorders, although rare, include plasminogen deficiency, PAI-1 deficiency, and α_2AP deficiency. The efficacy and safety of fibrinolytic therapy may be influenced by age-dependent differences in the fibrinolytic system. Data are lacking on the optimal doses of fibrinolytic agents for the pediatric population, especially for neonates. tPA is the agent of choice, with the most common dose and duration of treatment being 0.5 mg/kg/h infused over 6 hours.[10]

Platelets

Platelet production starts around the end of the first trimester of gestation and reaches adult levels in the middle of the third trimester. Therefore, platelet counts in term and premature newborns and children are not different from those in adults. Similarly, mean platelet volume and platelet ultrastructure in neonates and children closely resemble those in adults. Yet, all the main platelet functions are deficient at birth. Neonatal platelets exhibit reduced phospholipid metabolism, calcium mobilization, granule secretion, and aggregation in response to agonists compared with platelets from children or adults.[11] Although thrombocytopenia is common in neonates, thrombocytosis is rare and is associated with prematurity.

There are two proposed theories behind the reduced platelet function at birth. One hypothesis suggests that platelet activation and degranulation at the time of labor and delivery leads to exhausted circulating platelets; the other suggests that intrinsic platelet peculiarities account for the reduced reactivity of neonatal platelets. Studies of platelets from neonates reveal reduced aggregation in response to agonists, such as ADP, epinephrine, collagen, thrombin, and thromboxane analogues. This is especially true when platelets from preterm infants are studied. Flow cytometry studies using labeled monoclonal antibodies against platelet activation markers also reveal that platelets from cord blood or from neonates on the first postnatal day are hyporeactive compared with adult platelets.[11] This decreased responsiveness is transient, and the platelets usually regain normal reactivity 10 to 14 days after delivery.

Despite the reduced platelet reactivity at birth, platelet adhesion may be enhanced because of higher levels of functionally more potent high-molecular-weight vWF multimers in the plasma. Consistent with this concept, the bleeding time and the platelet function analyzer (PFA-100) closure time are shorter in neonates than in children and adults.[11] In healthy neonates, enhanced platelet adhesion immediately after delivery may compensate for the reduced platelet activation in response to agonists. Nonetheless, sick neonates may be at increased risk for bleeding.[11]

The PFA-100 closure time is preferred over the bleeding time as a means of assessing platelet-related hemostasis. Bleeding time is an in vivo screening test that reflects the interaction between platelets and the blood vessel wall, but the results are as variable in neonates as they are in children and adults (see Chapter 127). Although the PFA-100 will not detect mild defects in platelet function, such defects rarely cause serious bleeding in neonates.

The Vessel Wall

Studies in vitro and in neonatal animals suggest that the endothelium in neonates has greater antithrombotic potential than that in adult vessels. In both rabbit venous and aortic models, neonatal endothelium expresses more heparan sulfate proteoglycans than adult endothelium, which results in greater AT-mediated anticoagulant activity in rabbit pups compared with adult rabbits. Circulating levels of endothelial cell adhesion markers vary with age, implying dynamic expression and/or secretion of these proteins as a function of age (see Chapter 122).

BLEEDING DISORDERS IN PEDIATRICS

Significant bleeding in children should prompt clinical evaluation. In sick children, acquired factor deficiencies or thrombocytopenia are frequently to blame, but rare congenital factor deficiencies can also manifest with bleeding. Initial empirical therapy consists of platelet

TABLE 148.2 Reference Values (Ranges) for Common Coagulation Tests and Blood Coagulation Protein Levels by Age, Comparing Two Comprehensive Prospective Studies With Different Methodologies

	Day 1		Day 3 (ref 3) vs. Day 5 (ref 2)		1 month–1 year		Adult (measured)	
	Ref 3	**Ref 2**	**Ref 3**	**Ref 2**	**Ref 3**	**Ref 2**	**Rel 3**	**Ref 2**
Prothrombin time (s)	15.6	13	14.9	12.4	13.1	12.3	13	12
	(14.4–16.4)	(11.6–14.4)	(13.5–16.4)	(10.5–13.9)	(11.5–15.3)	(10.7–13.9)	(11.5–14.5)	(11–14)
PTT (s)	38.7	42.9	36.3	42.6	39.3	35.5	33.2	33
	(34.3–44.8)	(31.3–54.5)	(29.5–42.2)	(25.4–59.8)	(35.1–46.3)	(28.1–42.9)	(28.6–38.2)	(27–40)
Thrombin time (s)	N/A	23.5	N/A	23.1	17.1	24.3	16.6	N/A
		(19–28.3)		(18–29.2)	(16.3–17.6)	(19.4–29.2)	(16.2–17.2)	
Fibrinogen (mg/dL)	280	283	330	312	242	251	310	278
	(192–374)	(225–341)	(283–401)	(237–387)	(82–383)	(150–387)	(190–430)	(156–400)
Prothrombin (%)	54	48	62	63	90	88	110	108
	(41–69)	(37–59)	(50–73)	(48–78)	(62–103)	(60–116)	(78–138)	(70–146)
Factor V (%)	81	72	122	95	113	91	118	106
	(64–103)	(54–90)	(92–154)	(70–120)	(94–14)	(55–127)	(78–152)	(62–150)
Factor VII (%)	70	66	86	89	128	87	129	105
	(52–88)	(47–85)	(67–107)	(62–116)	(83–160)	(47–127)	(61–199)	(67–143)
Factor VIII (%)	182	1(X)	159	88	94	73	160	99
	(105–329)	(61–139)	(83–274)	(55–121)	(54–145)	(53–109)	(52–290)	(50–149)
Factor IX (%)	48	53	72	53	71	86	130	109
	(35–56)	(34–72)	(44–97)	(34–72)	(43–121)	(36–139)	(59–254)	(55–163)
Factor X (%)	55	40	60	49	95	78	124	106
	(46–67)	(26–54)	(46–75)	(34–64)	(77–122)	(38–118)	(96–171)	(70–152)
Factor XI (%)	30	38	57	55	89	86	112	97
	(7–41)	(24–52)	(24–79)	(39–71)	(62–125)	(49–134)	(67–196)	(67–127)
Factor XII (%)	58	53	53	47	79	77	115	108
	(43–80)	(33–73)	(14–80)	(29–65)	(20–135)	(39–115)	(35–207)	(52–164)
Antithrombin III (%)	76	63	74	67	109	104	96	100
	(58–90)	(51–75)	(60–89)	(54–80)	(72–134)	(84–124)	(66–124)	(74–126)
Protein C activity (%)	36	35	44	42	71	59	104	96
	(24–44)	(26–44)	(28–54)	(31–53)	(31–112)	(37–81)	(74–164)	(64–128)
Protein S activity (%)	36	36	49	50	102	87	75	81
	(28–47)	(24–48)	(33–67)	(36–64)	(29–162)	(55–119)	(54–103)	(60–113)
D-dirner (pg/mL)	1.47	N/A	1.34	N/A	0.22	N/A	0.18	N/A
	(0.41–2.47)		(0.58–2.74)		(0.11–0.42)		(0.05–0.42)	

N/A, Not available; *PTT*, partial thromboplastin time.
Modified from Monagle P, Barnes C, Ignjatovic V, et al. Developmental haemostasis: impact for clinical haemostasis laboratories. *Thromb Haemost.* 2006;95:362; and Andrew M, Paes B, Milner R, et al. Development of the human coagulation system in the full-term infant. *Blood.* 70:165, 1987 (range inferred from published statistical documentation).

and/or factor supplementation, which is often administered while diagnostic studies are under way.

Evaluation of the Bleeding Child

Age, gender, presentation, birth and neonatal history, surgical history, family history, and medication use are each important in the evaluation of a child with hemorrhagic complications. Bleeding symptoms, such as epistaxis, occur in healthy children and do not always necessitate evaluation for an underlying bleeding disorder. It is important to consider frequency, duration, and severity of these symptoms to identify which children require testing. Table 148.3 highlights bleeding phenotypes in children that are concerning for an underlying bleeding disorder. Along with personal history, family history, such as bleeding tendencies, parental ethnicity, and history of consanguinity, may help to identify congenital bleeding disorders. A negative family history does not exclude an inherited bleeding disorder, especially X-linked and autosomal recessive disorders. In hemophilia, for example, up to one-third of patients have a negative family history.[12] Pregnancy and delivery history, such as maternal infection, drug use, or immune thrombocytopenia (ITP), can provide important clues in cases of neonatal bleeding.

On physical examination, the location and characteristics of the bleeding (e.g., procedural, mucosal, cutaneous, intracranial, visceral), whether diffuse or localized, and the general appearance of the child as sick or well, may help to identify the underlying etiology of the hemorrhage. In ill-appearing children, disseminated intravascular

TABLE 148.3 **Bleeding Phenotypes Concerning for an Underlying Bleeding Disorder**

Umbilical stump bleeding in a newborn
Greater than expected bleeding post-circumcision
Mucocutaneous bleeding lasting >30 min or requiring surgical intervention (ex. cauterization)
Greater than expected bleeding after surgery or minor procedures (e.g., dental extraction)
New petechiae or purpura in an otherwise healthy child
Spontaneous intracranial hemorrhage
Spontaneous gastrointestinal bleeding
Spontaneous joint, muscle or soft tissue bleeding
Heavy menstrual bleeding in a female with prior history of mucocutaneous bleeding

coagulation (DIC) or liver disease may result in acquired factor deficiencies (see Chapter 137). These disorders tend to present with diffuse bleeding. Well-appearing children are more likely to have localized bleeding or ecchymoses because of thrombocytopenia, von Willebrand disease, platelet function defects, or a rare inherited factor deficiency.

Laboratory evaluation of bleeding in children should include sepsis evaluation and determination of the platelet count, PT/international normalized ratio (INR), aPTT, thrombin time, and fibrinogen concentration (Table 148.4). If the test results are normal, children with a history of bleeding should have levels of FXIII, α_2AP, and PAI-1 activity determined because deficiencies of these proteins are not detected with routine screening. The approach to laboratory screening in neonates is summarized in Fig. 148.1, which has been modified from Blanchette and Rand.[13] Platelet function should be evaluated when primary hemostatic defects are suspected.[14] In children with a family history of hemophilia or when hemophilia is suspected, the levels of FVIII and FIX should be determined regardless of the extent of aPTT prolongation.

Thrombocytopenia

The following factors are important to consider when evaluating children with thrombocytopenia: congenital or acquired, sick or well, maternal antibody or drug (in case of neonates), and platelet size. Thrombocytopenia is defined as a platelet count less than 150 × 10^9/L.[15] Causes of thrombocytopenia in children include decreased platelet production, increased platelet consumption, and/or hypersplenism (Table 148.5). Other contributing factors include infection, placental insufficiency, genetic disorders, medications, DIC, or immune deficiency. Rare causes of decreased platelet production in children include primary congenital platelet or marrow disorders and infiltrative disorders.

Neonatal Thrombocytopenia

Platelet counts in the range of 100 to 150 × 10^9/L are common in healthy neonates; these mostly reflect transient thrombocytopenia and require no further investigation unless there is an ongoing decrease in the count. More severe thrombocytopenia (platelet count <50 × 10^9/L) in neonates rarely manifests with bleeding, particularly in the absence of maternal antiplatelet antibodies (see Chapter 129). The estimated prevalence of thrombocytopenia is in the range of 1% to 5% of all newborns. The prevalence of thrombocytopenia increases to 22% to 35% in neonates admitted to the neonatal intensive care unit (NICU), with the rates increasing with decreasing gestational age. With severe thrombocytopenia, platelet transfusion may be necessary to treat or decrease the risk of bleeding.

TABLE 148.4 **Differential for Bleeding Disorders Based on Coagulation Tests**

Prolonged PT/INR, Normal APTT	Normal PT/INR, Prolonged APTT	Prolonged PT/INR and APTT	Normal PT/INR and APTT
• Factor VII deficiency • Warfarin/rodenticide poisoning	• Hemophilia A or B (Factors VIII or IX deficiency) • Factor XI or XII deficiency • Contact factor (HMWK or prekallikrein deficiency) • vWD • Heparin effect • Lupus anticoagulant	• Infection/sepsis • Disseminated intravascular coagulation • Vitamin K deficiency • Factor II, V, or X deficiency • Low fibrinogen	• Factor XIII deficiency • vWD • Platelet function disorders • Fibrinolytic disorders • Connective tissue disorders

APTT, Activated partial thromboplastin time; *HMWK*, high-molecular-weight kininogen; *INR*, international normalized ratio; *PT*, prothrombin time; *vWD*, von Willebrand disease.

Neonatal Alloimmune Thrombocytopenia

Neonatal alloimmune thrombocytopenia is the platelet equivalent of hemolytic disease of the newborn. It affects approximately 1 in 2000 pregnancies. When fetal platelets express paternal antigens (HPA) that the mother lacks, transplacental passage of maternal immunoglobulin G directed against fetal platelets can occur and usually results in severe neonatal thrombocytopenia (platelet count <30 × 10^9/L). This may result in major bleeding, particularly ICH, which can occur in 10% to 20% of untreated pregnancies.[15] Incompatibilities for HPA-1a account for the majority of cases of neonatal alloimmune thrombocytopenia in populations of European ancestry. About half of the cases of neonatal alloimmune thrombocytopenia occur in the first pregnancy because fetal platelets pass into the maternal circulation early in the pregnancy. Treatment options include transfusion of antigen-negative platelets if available (HPA-1a ± 5b). Although large, randomized studies are lacking, the threshold count for platelet transfusion in most studies is 30 × 10^9/L. High-dose intravenous immunoglobulin and/or a trial of random donor platelets can be given if compatible platelets are unavailable. The recommended total dose of intravenous immunoglobulin is 1 to 2 g/kg administered either at a dose of 0.4 g/kg daily for 3 to 5 days or at a dose of 1 g/kg daily for 1 or 2 days.[16]

Immune Thrombocytopenia

ITP is a common cause of symptomatic thrombocytopenia in children with an annual incidence of 1 to 6.4 per 100,000.[17] Although ITP can occur at any age, its incidence peaks in children 2 to 5 years of age (see Chapter 129). ITP is classified as newly diagnosed (<3 months), persistent (3 to 12 months), or chronic (>12 months) based on the duration of symptoms and the thrombocytopenia.[18] ITP generally presents in an otherwise healthy child, who may have a history of preceding viral illness with a sudden onset of petechial rash, bruising, and/or bleeding. While bleeding symptoms are variable, the majority of children have mucosal bleeding (40%), and only 3% have serious bleeding requiring blood transfusion and/or hospitalization. Intracranial hemorrhage (ICH) is rare and occurs in less than 1% of children with ITP.[19] The diagnosis of ITP is based on typical presentation, isolated thrombocytopenia (platelets <100,000 × 10^9/L), and an absence of features suggestive of an alternate diagnosis

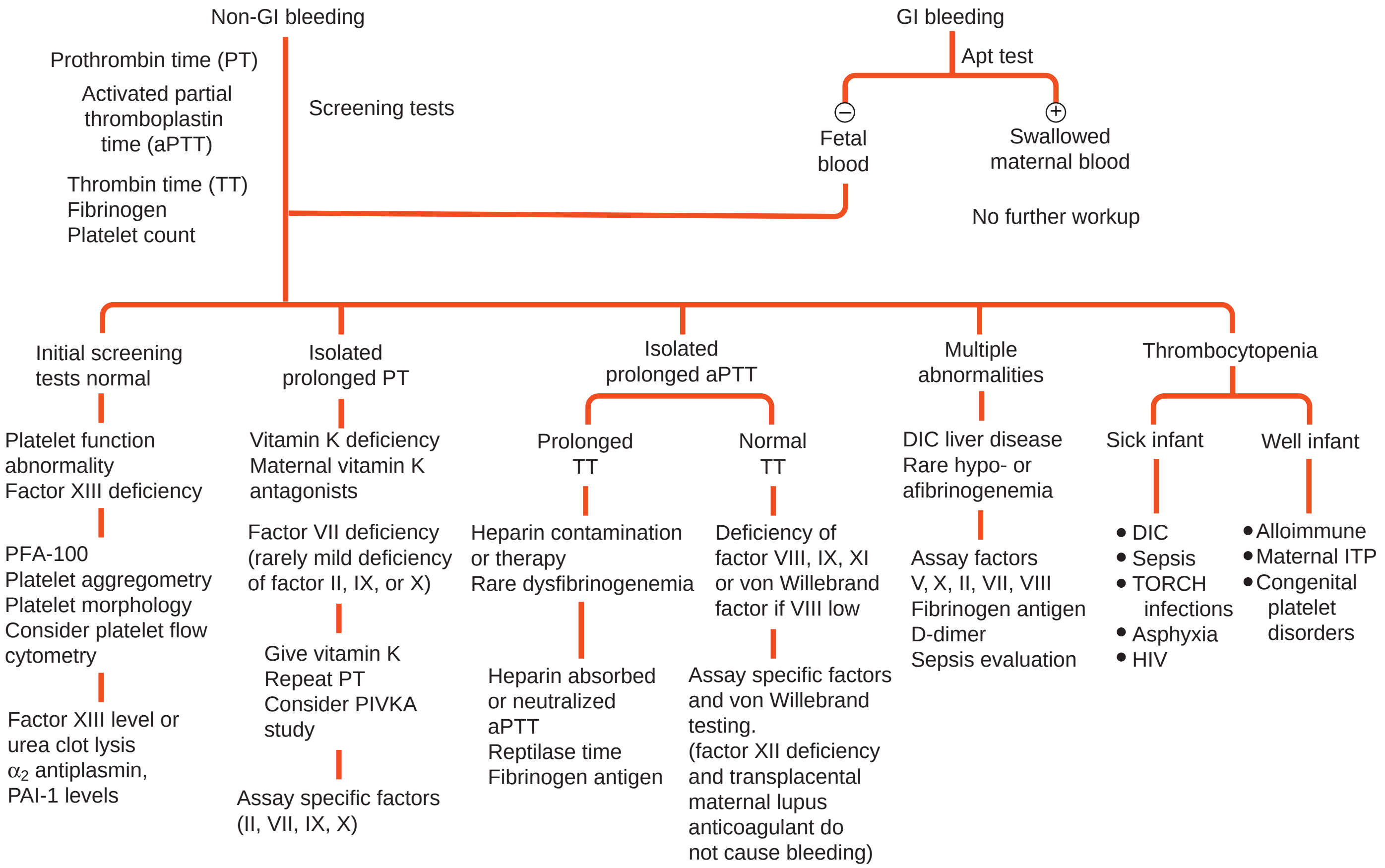

Non-GI bleeding
Prothrombin time (PT)
Activated partial thromboplastin time (aPTT)
Thrombin time (TT)
Fibrinogen
Platelet count
Screening tests
GI bleeding
Apt test
⊖
Fetal blood
⊕
Swallowed maternal blood
No further workup
Initial screening tests normal
Platelet function abnormality
Factor XIII deficiency
PFA-100
Platelet aggregometry
Platelet morphology
Consider platelet flow cytometry
Factor XIII level or urea clot lysis
α2 antiplasmin,
PAI-1 levels
Isolated prolonged PT
Vitamin K deficiency
Maternal vitamin K antagonists
Factor VII deficiency (rarely mild deficiency of factor II, IX, or X)
Give vitamin K
Repeat PT
Consider PIVKA study
Assay specific factors (II, VII, IX, X)
Isolated prolonged aPTT
Prolonged TT
Heparin contamination or therapy
Rare dysfibrinogenemia
Heparin absorbed or neutralized
aPTT
Reptilase time
Fibrinogen antigen
Normal TT
Deficiency of factor VIII, IX, XI or von Willebrand factor if VIII low
Assay specific factors and von Willebrand testing.
(factor XII deficiency and transplacental maternal lupus anticoagulant do not cause bleeding)
Multiple abnormalities
DIC liver disease
Rare hypo- or afibrinogenemia
Assay factors V, X, II, VII, VIII
Fibrinogen antigen
D-dimer
Sepsis evaluation
Thrombocytopenia
Sick infant
• DIC
• Sepsis
• TORCH infections
• Asphyxia
• HIV
Well infant
• Alloimmune
• Maternal ITP
• Congenital platelet disorders

Figure 148.1 DIAGNOSTIC APPROACH TO THE BLEEDING NEONATE. *DIC*, Disseminated intravascular coagulation; *GI*, gastrointestinal; *HIV*, human immunodeficiency virus; *ITP*, immune thrombocytopenia purpura; *PAI-1*, plasminogen activator inhibitor 1; *TORCH*, toxoplasmosis, other agents, rubella, cytomegalovirus, herpes simplex. *(Modified from Blanchette VS, Rand ML. Platelet disorders in newborn infants: diagnosis and management. Semin Perinatol. 1997;21:53.)*

(e.g., signs and symptoms of malignancy or autoimmune conditions, abnormal white count or hemoglobin on CBC, and abnormal peripheral blood smear among others). The management of newly diagnosed ITP patients is based on severity of bleeding symptoms, degree of thrombocytopenia, and medical and social history. Options include observation alone or one of glucocorticoids, IVIG, or anti-D immune globulin. All patients should be advised to restrict high-risk activities and to avoid antiplatelet and anticoagulant medications. Most children with ITP recover within 3 to 6 months, while 10% to 20% go on to have chronic ITP. Risk factors for chronic ITP include older age, lack of preceding infection, and less severe thrombocytopenia. Options for managing chronic ITP include rituximab, thrombopoietin receptor agonists (romiplostim, eltrombopag), splenectomy, and other immunosuppressive medications (see Chapter 129).

Platelet Function Disorders

Qualitative platelet disorders are rarely associated with overt bleeding in young age. Patients presenting with bleeding who have normal coagulation test results and platelet counts require further investigation. Bleeding may be from mucocutaneous sites, sites of blood sampling and procedures, or may present as cephalohematoma and umbilical stump bleeding in neonates. Platelet function disorders range from defects in several structures and signaling pathway as outlined in Table 148.6. Only the most severe genetic disorders of platelet function present in the neonatal period. These include Glanzmann thrombasthenia and Bernard-Soulier syndrome (see Chapter 124).

Platelet function is affected by commonly used medications, such as aspirin and non-steroidal anti-inflammatory drugs (e.g., ibuprofen, naproxen). Herbal medicines, such as ginkgo biloba, are also known to alter platelet function and can cause bleeding symptoms. In neonates, common medications, such as nitric oxide, prostaglandin E_2, and indomethacin, can affect platelet function (see Chapter 128).

It is challenging to diagnose platelet function disorders in neonates and young children because of the technical limitations of platelet function assays and the need for large volumes of blood for testing (see Chapter 127). Initial screening for suspected platelet function disorders can be performed using the PFA-100 analyzer, followed by evaluation of platelet morphology and platelet aggregation responses using light transmission aggregometry. Flow cytometry can be used to evaluate specific surface glycoproteins and electron microscopy studies can be used to assess platelet granule morphology (see Chapter 124). Absent platelet aggregation in response to all agonists and deficiency of αIIbβ3 on flow cytometry are diagnostic for Glanzmann thrombasthenia (see Chapter 124). Flow cytometry also provides definitive diagnostic information about Bernard-Soulier syndrome, dense granule deficiency, and Scott syndrome.

Platelet transfusions are often given if the patient is bleeding. However, the potential risk of HLA allosensitization if normal platelets are given to patients with congenital deficiency of platelet surface antigens must be weighed against the severity of bleeding when functional defects are suspected. It is recommended that platelet transfusion be restricted to patients with Glanzmann thrombasthenia. Recombinant activated FVII (rFVIIa) has been used to manage serious bleeding and avoid the need for platelet transfusion. Other adjunctive local measures include topical thrombin, fibrin sealants, and antifibrinolytic medications, such as tranexamic acid.

TABLE 148.5 Classification of Thrombocytopenia

Decreased Production		Increased Destruction/Consumption		
Congenital	**Acquired**	**Immune**	**Non-immune**	**Sequestration/ Consumption**
Small platelets: • WAS • XLT *Normal platelets:* • TAR • CAMT • ATRUS • Fanconi anemia • Inborn errors of metabolism (e.g., isovaleric acidemia, Pearson syndrome) • Trisomy 21 *Large platelets:* • BSS • GPS • MYH-9-related disorders	*Neonate-specific:* • Placental insufficiency • IUGR • Fetal hypoxia *All children*: • Infection • Drugs (e.g., chemotherapy agents, valproic acid) • Aplastic anemia • Myelodysplastic syndrome • Marrow infiltration (e.g., leukemia, neuroblastoma) • Nutritional deficiency	*Autoimmune:* • NAT • Immune thrombocytopenia • Autoimmune conditions (e.g., SLE, ALPS, APA syndrome) *Alloimmune:* • NAIT • Post-transfusion purpura *Drug-induced:* • Heparin-induced thrombocytopenia	• Infection • DIC • HUS/TTP • Drug-induced thrombotic microangiopathy • vWD-type 2B • Platelet-type vWD • Congenital heart disease • Artificial surfaces (e.g., ECMO, dialysis, pharesis)	• Hypersplenism • Thrombosis • Hemangioma (e.g., Kasabach-Merritt syndrome) • Burns • Hypothermia

ALPS, Autoimmune lymphoproliferative syndrome; *APAS*, antiphospholipid antibody; *ATRUS*, amegakaryocytic thrombocytopenia with radioulnar synostosis; *BSS*, Bernard-Soulier syndrome; *CAMT*, congenital amegakaryocytic thrombocytopenia; *DIC*, disseminated intravascular coagulation; *ECMO*, extracorporeal membrane oxygenation; *GPS*, Gray platelet syndrome; *HUS/TTP*, hemolytic uremic syndrome/thrombotic thrombocytopenic purpura; *IUGR*, intrauterine growth restriction; *MYH*, myosin heavy chain; *NAT*, neonatal autoimmune thrombocytopenia; *NAIT*, neonatal alloimmune thrombocytopenia; *SLE*, systemic lupus erythematosus; *TAR*, thrombocytopenia with absent radii; *vWD*, von Willebrand disease; *WAS*, Wiskott-Aldrich syndrome; *XLT*, X-linked thrombocytopenia.

Inherited Coagulation Disorders

Isolated Prolonged Prothrombin Time/INR

Isolated prolonged PT/INR can be secondary to factor VII deficiency or vitamin K antagonists (e.g., warfarin). Inherited FVII deficiency is a rare, autosomal recessive condition with a strong gene dosage effect (see Chapter 135). Neonatal bleeding can occur in severe cases (i.e., homozygosity or compound heterozygosity for two mutations in the FVII gene). FVII deficiency can be associated with microcephaly or midline defects because disruption of chromosome 13q can lead to loss of adjacent genes. As a vitamin K-dependent protein, FVII deficiency can occur in association with deficiencies of the other vitamin K-dependent clotting proteins with abnormalities of the vitamin K pathways. Low doses of rFVIIa can be used for treatment of isolated FVII deficiency; FFP and/or vitamin K can be used for management of combined deficiencies.

Isolated Prolonged Activated Partial Thromboplastin Time

Isolated prolonged aPTT can be secondary to deficiencies of factors VIII, IX, XI, XII, HMWK, or prekallikrein and specific or nonspecific antibodies (e.g., lupus anticoagulant) affecting these factors (see Chapters 127 and 139). Evaluation for FVIII, FIX, and FXI deficiencies should be undertaken immediately in any child with bleeding symptoms and isolated prolongation of the aPTT or a positive family history. Deficiencies of FXII, HMWK, and prekallikrein are associated with prolonged aPTT but do not contribute to bleeding symptoms (see Chapter 135). Neonates have a physiologically prolonged aPTT compared with older children and adults; hence, age-appropriate reference ranges must be utilized (see Table 159.2).[1,2]

Prolonged Prothrombin Time/INR and Activated Partial Thromboplastin Time

The association of bleeding with prolongation of both the PT/INR and the aPTT in otherwise healthy children may indicate vitamin K deficiency or congenital deficiencies of prothrombin, FV or FX, as well as rare combined deficiencies (see Chapters 127 and 135). Routine prophylactic administration of vitamin K to newborns can complicate the diagnosis of neonatal bleeding related to vitamin K deficiency.[20] More commonly, combined PT and aPTT prolongation occurs in sick children with DIC or severe hepatic disease. Combined factor deficiencies are rare but must be considered when the laboratory findings or clinical course is confusing. Autosomal recessive mutations in LMAN1 (ERGIC-53) or MCFD2 can lead to combined FV and FVIII deficiency because of defective intracellular processing of the factors (see Chapter 135). Mutations in γ-glutamyl carboxylase are associated with inherited combined deficiencies of all the vitamin K-dependent proteins. FVII deficiency has rarely been reported in combination with FV, FVIII, FIX, FX, FXI, and protein C defects, as reviewed by Girolami et al.[21]

Hemophilia

Hemophilia is one of the most common inherited coagulation disorders, as discussed in detail in Chapter 134. Hemophilia A is caused by FVIII deficiency and hemophilia B is caused by FIX deficiency. In newborns with a family history, cord blood samples can be used for diagnosis of hemophilia A, thereby avoiding the need for peripheral venipuncture. In term and preterm infants, FVIII levels are within the normal adult range despite physiologic differences in the hemostatic system. Thus, confirmation of hemophilia A is possible in the neonatal period. However, because the levels of the vitamin K-dependent clotting factors, including FIX, are reduced at

TABLE 148.6 Inherited Disorders of Platelet Function

Defects in Receptors for Adhesive Proteins
Bernard-Soulier syndrome,[a] platelet-type von Willebrand disease (GP-Ib-IX-V complex)
Glanzmann thrombasthenia[a] (GP-IIb/IIIa, $\alpha IIb\beta 3$)
GP-Ia/IIa, $\alpha 2\beta 1$
GPVI
GPIV
Defects in Soluble Agonist Receptors
Thromboxane A2 receptor
α_2-adrenergic receptor
P2Y$_{12}$ receptor
Defects in Platelet Granules
δ-storage pool deficiency, Hermansky-Pudlak syndrome, Chediak-Higashi syndrome, thrombocytopenia with absent radii syndrome (δ-granules)
Gray platelet syndrome, Quebec platelet disorder, Paris-Trousseau-Jacobsen syndrome (α-granules)
α,δ-storage pool deficiency (α- and δ–granules)
Defects in Signal-Transduction Pathways
Primary secretion defects
Abnormalities of the arachidonic acid/TxA$_2$ pathway
G$_{\alpha q}$ deficiency
Partial selective PLC-β_2 deficiency
Defects in pleckstrin phosphorylation
Defects in Ca^{2+} mobilization
Abnormalities of Cytoskeleton
MYH9-related disorders (May-Hegglin anomaly, Sebastian syndrome, Fechtner syndrome, Epstein syndrome)
Wiscott-Aldrich syndrome
X-linked thrombocytopenia
Abnormalities of Membrane Phospholipids
Scott syndrome
Stormorken syndrome

Modified from Israels SJ, El-Ekiaby M, Quiroga T, et al. Inherited disorders of platelet function and challenges to diagnosis of mucocutaneous bleeding. *Haemophilia.* 2010;16:152.

[a] Can manifest in neonatal period.

birth (and even lower in preterm infants), diagnosis of hemophilia B is difficult until older age.

The reported incidence of hemophilia A and B are 1/5000 males and 1/20,000 males, respectively.[12] Hemophilia is more common in males than females, given the location of both the FVIII and FIX gene on the X chromosome. However, severe FVIII or FIX deficiency can, albeit rarely, present in females, such as in Turner syndrome or with extreme Lyonization. Approximately one-third of cases of hemophilia are the result of spontaneous mutations; consequently, a family history may be lacking. Evaluation of hemophilia carrier status in mothers of unknown status (e.g., single prior affected child or positive history in maternal grandfather) can be performed genetically because factor levels in carriers are variable and can overlap with normal levels. The bleeding phenotype in hemophilia patients can vary from mucocutaneous bleeding to spontaneous joint and muscle bleeding. Most bleeding episodes in newborn males occur after circumcision or umbilical stump separation, although ICH is reported in approximately 3.5% of newborns with hemophilia,[22] and cephalhematoma is common. Joint bleeding is unusual in infancy until the child starts to bear weight. Children with hemophilia A or B are treated with recombinant FVIII or FIX concentrates, respectively. Routine prophylaxis is rarely indicated in infancy unless there is significant bleeding. Children generally start prophylactic treatment around 1 year of age. For further details of hemophilia treatment and prognosis, see Chapter 134.

von Willebrand Disease

vWD is the most common inherited bleeding diathesis with an estimated prevalence of 1%. It is described in detail in Chapter 133. There are three types of vWD: type 1 is a partial quantitative defect, type 2 is a qualitative defect, and type 3 is a complete quantitative defect. Children with vWD most commonly present with mucocutaneous bleeding or bleeding after dental/surgical procedures. vWD is rarely associated with neonatal bleeding. In a case series that included 55 newborns with vWD, there were no bleeding complications, although cases of scalp hematomas and bleeding after vitamin K injection or umbilical stump detachment were reported. A family history of vWD, especially type 2 vWD, should prompt vWD evaluation in children before circumcision and possibly before intramuscular injections. The laboratory testing for vWD includes vWF antigen, vWF activity, factor VIII assay, and vWF multimer distribution (if required). The diagnosis of vWD may be difficult in infancy because vWF concentrations are high at birth, and there is a large proportion of high-molecular-weight vWF multimers (see Chapter 127). Children with a diagnosis of type 3 vWD should be tested for mutations that predispose them to inhibitory alloantibody formation before aggressive replacement with exogenous vWF.[23] Treatment of children with severe vWD involves administration of plasma-derived concentrates that contain vWF (e.g., Humate-P or Alphanate), dosed initially at 40 to 60 ristocetin cofactor units/kg.

FXIII Deficiency

FXIII is a transglutaminase that cross-links fibrin, thereby rendering it resistant to lysis. FXIII deficiency is inherited in an autosomal recessive manner, and the prevalence of FXIII deficiency is estimated to be 1 in 3 to 5 million (see Chapter 135). Homozygotes usually have FXIII levels of less than 1% and have a severe bleeding diathesis. Patients with heterozygous FXIII deficiency are usually asymptomatic but have reduced levels of FXIII.[24] Children with FXIII deficiency may present with umbilical bleeding a few days after birth, a frequent finding that occurs in 80% of cases. More severe bleeding, including ICH, occurs in 25% to 30% of patients, a frequency higher than that in patients with hemophilia. ICH is the major cause of death and disability in children with FXIII deficiency, and ICH in a child with no other risk factors should prompt a search for FXIII deficiency. FXIII deficiency is not detected with screening PT, aPTT, or thrombin time assays. Specific assays for FXIII or urea clot solubility testing are used for diagnosis (see Chapter 127). The clot solubility test is sensitive to very low levels of FXIII (<1%) but is normal if FXIII levels are in the 1% to 3% range. Consequently, FXIII immunoassays are perferable.[24] Treatment of FXIII deficiency includes FFP, cryoprecipitate, or FXIII concentrates. Recombinant FXIII concentrate contains the alpha subunit only, whereas plasma-derived FXIII concentrate contains both alpha and beta subunits. Most patients have alpha subunit deficiency and can, therefore, be treated with either. However, patients with beta subunit–related FXIII deficiency require plasma derived FXIII concentrate. Prognosis is excellent, but affected patients face a lifelong risk for bleeding and those with severe deficiency require prophylaxis. In 2013, the US Food and Drug Administration approved a new recombinant FXIII A subunit for use for routine prophylaxis in patients with FXIII deficiency.

Other Rare Inherited Factor Deficiencies

Autosomal recessive deficiencies, in either homozygous or compound heterozygous state, are grouped as rare coagulation disorders that can manifest as severe bleeding diatheses. Severe deficiencies of fibrinogen, FV, FVII, FX, and FXIII (isolated or in combination) are the most likely disorders to present with bleeding conditions. One common feature of these disorders is the association with ICH.[25] Deficiency of fibrinogen can manifest with bleeding in the soft tissues, bleeding after circumcision, or bleeding after umbilical stump detachment. The diagnosis can be established with fibrinogen assays, and treatment involves replacement with cryoprecipitate or, if available, fibrinogen concentrate (see box on Recommended Dosing for Transfusion in Neonatal Hemorrhage). Severe FXI deficiency is more prevalent in Ashkenazi Jews and can present with bleeding in newborns after hemostatic challenges, such as circumcision. FV and prothrombin deficiencies are the other rare autosomal recessive homozygous deficiencies that can cause hemorrhagic symptoms.[14] Autosomal recessive deficiencies of α_2AP and PAI-1 have also been associated with bleeding in childhood and adult age. Not all deficiencies result in a bleeding diathesis.

RECOMMENDED DOSING FOR TRANSFUSION IN NEONATAL HEMORRHAGE

PRBC: 10–15 mL/kg single-donor PRBC infused over 4 h
Platelets[a]: 10 mL/kg raises platelet count by 75,000 (goal >50,000 if bleeding, >20,000 if not bleeding)
FFP: 10–20 mL/kg every 6–12 h for purpura fulminans
Cryoprecipitate: 0.15 units/kg raises fibrinogen about 100 mg/dL (goal >150 mg/dL if bleeding, >50 mg/dL if not bleeding)
vWF: 40–60 ristocetin cofactor units/kg of plasma-derived FVIII/vWF preparations
Factor VIII: for hemophilia A—50 units/kg load, then 25 units/kg every 12 h; recombinant factor preferred (monitor FVIII)
Factor IX: for hemophilia B—80–100 units/kg daily; recombinant factor preferred (monitor FIX)
Factor VIIa: for severe factor VII deficiency—20–30 mcg/kg every 6–12 h

[a] Volume limits transfusion of platelets by the "unit" in small neonates. Practices vary—follow institutional guidelines for volume dosing or volume reduction.
FFP, Fresh frozen plasma; *PRBC*, packed red blood cells; *vWF*, von Willebrand factor.

Liver Disease

Liver disease in children may be caused by viral hepatitis, parenteral nutrition, cholestasis, hypoxic injury, or metabolic disease. Rare disorders that cause liver failure include hereditary tyrosinemia, neonatal hemochromatosis, and hemophagocytic lymphohistiocytosis. Liver dysfunction can affect the hemostatic balance resulting in activation of the coagulation and fibrinolytic systems, reduced synthesis of coagulation factors, poor clearance of activated hemostatic components, thrombocytopenia, platelet dysfunction, loss of coagulation proteins into ascites fluid, and failure to utilize vitamin K.[26]

Due to reduced synthesis of multiple coagulation proteins, laboratory workup typically reveals a prolonged PT and aPTT. Acute liver disease also results in elevated liver enzyme levels, direct hyperbilirubinemia, and elevated ammonia concentrations. The platelet count may be reduced, especially if hypersplenism is present, and platelet dysfunction is common. Hypofibrinogenemia is a late manifestation of liver disease, and elevated levels of fibrin degradation products and D-dimer mainly reflect delayed hepatic clearance. Assays of FV, FVII, and FVIII can help distinguish between liver disease, vitamin K deficiency, and consumptive coagulopathy (e.g., DIC). FV and FVIII are not vitamin K-dependent clotting factors. FV is synthesized in the liver, whereas FVIII is synthesized in multiple cell types, and the levels of both in children are similar to those in adults. Deficiency of all three implies consumption, whereas decreased levels of FV and FVII with a normal FVIII level suggest liver disease (see Chapter 137).

Treatment should include replacement with FFP and/or cryoprecipitate, as well as platelet transfusion. Fibrinogen concentrate has been used as an alternative to cryoprecipitate. Patients with biliary atresia or other cholestatic liver failure syndromes may also benefit from parenteral vitamin K. The outcome is dependent on treatment of the underlying cause of liver disease.

Vitamin K Deficiency and Bleeding in Neonates and Infants

Vitamin K is an essential cofactor for γ-glutamyl carboxylase, the enzyme required for posttranslational carboxylation of prothrombin, FVII, FIX, and FX, and proteins C, S, and Z. Many newborns are deficient in vitamin K. Risk factors for bleeding with vitamin K deficiency include maternal malabsorption, maternal intake of drugs that impair vitamin K metabolism, exclusive breastfeeding, and neonatal malabsorption. Classification is based on the time of presentation. Early vitamin K deficiency–associated bleeding occurs in the first 24 to 48 hours of life and is associated with maternal intake of drugs (e.g., phenytoin, barbiturates, antibiotics), which cross the placenta and impair vitamin K synthesis. Classical vitamin K deficiency–associated bleeding manifests from days 2 to 7 of life and is related to low placental transfer of vitamin K, low concentrations of vitamin K in breast milk, lack of gastrointestinal bacterial flora, and poor oral intake. Gastrointestinal bleeding is the most common presentation, but procedural bleeding, bruising, or ICH can occur. Late-onset vitamin K deficiency–related bleeding can occur at up to 12 weeks of age, usually in association with exclusive breastfeeding or with neonatal fat malabsorption, and often manifests as catastrophic bleeding, including ICH. Congenital vitamin K deficiency is an autosomal recessive disorder that occurs because of mutations in the genes encoding γ-glutamyl carboxylase or the vitamin K2,3–epoxide reductase complex. Neonates with this disorder often have severe bleeding, including ICH. Genotype analysis is necessary to confirm the defects.[27]

Prophylaxis with a single dose of vitamin K (0.5 to 1 mg) in the delivery room prevents early- and classic-onset bleeding. Although oral dosing may be effective, because of the potential for impaired absorption, regurgitation, or noncompliance, parenteral administration by the intramuscular, subcutaneous, or intravenous route is preferred. Additional doses of oral vitamin K should be given at days 7 and 28 in breastfed infants. Vitamin K–induced coagulopathy should be treated with vitamin K, which can be given by slow intravenous infusion or by subcutaneous injection (to minimize anaphylactoid reactions). If there is bleeding, fresh frozen plasma (FFP) at a dose of 10 to 15 mL/kg body weight can be given. The overall prognosis of vitamin K–deficiency bleeding is good.

Intraventricular Hemorrhage in Neonates

Intraventricular hemorrhage (IVH) is associated with significant morbidity and mortality in the newborn period, particularly in premature infants. In the United States, it is estimated that approximately 12,000 premature infants and 20% to 25% of very low-birth-weight infants develop IVH each year. With improvements in neonatal care, the incidence of IVH is decreasing.

The etiology of IVH is multifactorial and includes prematurity of the cerebral vasculature and ischemia-reperfusion injury related to ventilatory support, blood pressure lability, and extracorporeal membrane oxygenation (ECMO). Other risk factors for IVH include vaginal delivery, severe respiratory distress syndrome (RDS), low Apgar scores, pneumothorax, hypoxia, hypercapnia, seizures, patent ductus arteriosus, and infection. Many of these risk factors induce IVH by altering cerebral blood flow. Coagulopathy and thrombocytopenia can contribute to IVH, but their role in the pathogenesis of IVH is uncertain. One study reported hypofibrinogenemia, thrombocytopenia, or prolonged clotting times in 11 of 15 neonates with IVH and in only 5 of 35 unaffected newborns. Hemorrhage complicating cerebral vein thrombosis may explain some cases of IVH (especially in full-term infants), and heterozygosity for the FV Leiden mutation was reported in 18% of neonates with grade 2 to 4 IVH compared with 3% of controls. Cerebellar hemorrhage should raise suspicion of organic acidemia, such as methylmalonic, propionic, or isovaleric acidemia.

Vitamin K, indomethacin, AT, FFP, FXIII, tranexamic acid, and ethamsylate has been evaluated for prevention of IVH with mixed results. rFVIIa may be useful for treatment of IVH, but additional studies are needed to determine its efficacy and safety.

CLOTTING DISORDERS IN PEDIATRICS

The incidence of thromboembolism (TE) has a bi-modal peak in children, with an increased occurrence in newborns and adolescents. Initial registry data from Canada and Denmark estimated the incidence of neonatal TE to be 2.4 per 1000 admissions to neonatal intensive care and 4.1 per 100,000 live births, respectively.[28,29] In the contemporary era, it is reported to be as high as 15 per 1000 neonatal intensive care admissions.[30] Pediatric TE is uncommon with a reported incidence of 0.07 to 0.14 per 10,000 for venous TE,[29,31] the majority of which are provoked.[32] Due to improved diagnostic modalities, increased awareness, greater use of central venous lines (CVLs), the incidence is reportedly rising.[32–34] Pediatric TE is more common in hospitalized children (venous TE incidence: 30 to 58 per 10,000 hospital admissions).[33,35] Idiopathic venous TE is rare with an incidence of 3% to 4% and is frequently recognized in older age groups, post-pubescence, and in the presence of at least two prothrombotic risk factors.[29,31]

Important risk factors for the development of TE include vascular catheterization, infection, surgery, immobilization, thrombophilia, and co-morbidities, such as congenital heart disease, nephrotic syndrome, necrotizing enterocolitis, and asphyxia.[4] Laboratory workup often reveals a hypercoagulable state, such as decreased levels of AT, protein C, or protein S; defective fibrinolysis; hyperactive platelets; elevated levels of clotting factors (i.e., fibrinogen, FVII, FVIII); and the presence of antiphospholipid antibodies or thrombophilic defects, such as FV Leiden or the prothrombin gene mutation.[1,2,4] A family history of TE or miscarriages may indicate a hereditary thrombophilia.

Consensus guidelines have been developed for the management of TE.[10] Heparin, either unfractionated heparin (UFH) or low-molecular-weight heparin (LMWH), remains the mainstay of treatment in newborns and children. Direct oral anticoagulants (DOACs) are now being increasingly utilized in children. Dabigatran and rivaroxaban are now approved in the USA and Canada, respectively, for the treatment of venous TE (VTE) in children. If the thrombus is limb- or life-threatening, thrombolytic therapy may be considered. Long-term sequelae of TE in children include incomplete thrombus resolution, post-thrombotic syndrome, pulmonary insufficiency, pulmonary hypertension, recurrence, organ dysfunction, paradoxical embolism, and mortality.[36] Duration of anticoagulation depends on the individuals age, persistence of risk factors, thrombus resolution, and location of the thrombus. Most children are treated between 6 weeks to 6 months with anticoagulation. Provoked VTE in children is treated for 3 months like in adults. However, the practice will likely change in the near future with the recent publication of a randomized controlled trial showing non-inferior outcomes with shorter duration of treatment (6 weeks) in children with provoked VTE and no history of severe anticoagulant deficiencies or prior thrombosis.[37] Recurrence ranges from 3% in neonates to 12% in older children, although this number is as high as 21% in children diagnosed with first unprovoked venous TE.[38]

Risk Factors for Pediatric Thrombosis

Virchow's triad of endothelial damage, hypercoagulability, and venous stasis are the cornerstones of development of TE in childhood. Risk factors can be inherited or acquired and may be patient-, disease-, or treatment-related (Table 148.7). Unlike adults, most childhood TEs are associated with multiple risk factors. A registry in the Netherlands reported at least two risk factors were present in 81% of children diagnosed with VTE between 1 month and 18 years of age.[29] Acquired risk factors include central venous access devices, malignancy, trauma, prematurity, severe infection, inflammatory disease, dehydration, and reduced mobility (see Table 148.7). Acquired thrombophilia often reflects chronic diseases states or treatments, which result in high levels of inflammatory cytokines, release or increased expression of tissue factor, diminished fibrinolysis, and imbalance of natural anticoagulants and procoagulants.[39]

Indwelling Catheters

Central venous and arterial access is essential for the advanced care of neonates, infants, and children. CVLs are the single most important acquired risk factor for developing TE in childhood, and are responsible for approximately one-third of all cases of deep vein thrombosis (DVT) in children, mostly in the upper extremites.[31] Indwelling vascular catheters are also the most common acquired thrombotic risk factor in neonates. In a literature review by Park et al.,[40] the incidence of TE in neonates with central venous catheters was 9.2%. Duration of catheter implantation, infusion of blood products and malposition of umbilical venous catheters were found to be important risk factors. The most frequently reported sites of thrombosis in neonates are hepatic veins, right atrium, and inferior vena cava.[41] During childhood, CVL-related thrombosis is frequently diagnosed in the superior vena cava and in the femoral and iliac veins.[29]

The incidence of catheter-associated DVT is influenced by the method of detection. When catheters are used for total parenteral nutrition, it is estimated that DVT is diagnosed in 1% on clinical grounds, in 35% by echocardiography, and in 75% by venography. While contrast venography is regarded as the reference standard for the diagnosis of thrombosis in neonates and children with central venous catheters, ultrasonography is more commonly used because it is noninvasive, easy to perform at the bedside, and it does not expose patients to ionizing radiation.

DVT often causes pain, swelling, and discoloration of the affected limb. Loss of catheter patency, evidence of collateral circulation, and/or unexplained thrombocytopenia should raise the suspicion of DVT. Prospective imaging studies performed before central access catheter removal demonstrate thrombi in up to 86% of patients.[29] Treatment often begins with removal of the catheter, although consideration of anticoagulation before catheter removal is warranted, especially in children with right-to-left intracardiac or intrapulmonary shunting. Small catheter-associated thrombi may resolve without specific therapy. Larger thrombi or those in locations associated with greater morbidity (e.g., sinovenous, renal, portal) may warrant short courses of heparin or thrombolytic agents.[10] Platelets should never be administered through arterial catheters because of the potential risk for TE.

Hereditary Thrombophilia

Inherited thrombophilia plays an important role in development of TE in childhood. It is associated with first onset pediatric venous TE with odds ratios between 2.63 and 8.73.[38,42] The most common inherited thrombophilias include deficiencies of AT, protein C, protein S, Factor V Leiden, and prothrombin gene mutation.[42] Other inherited thrombophilic conditions include elevated lipoprotein(a), maternal anticardiolipin antibodies, and nonspecific inhibitors (see Chapter 138). Studies have shown an increased risk of recurrent VTE in children with thrombophilia, particularly in individuals with two or more genetic abnormalities.[38] However, most individuals diagnosed with thrombosis and thrombophilia have at least one clinical predisposing risk factor, demonstrating that thrombophilia alone may be insufficient to trigger thrombosis in this population.[42]

Hereditary thrombophilia should be suspected in patients with spontaneous and extensive TE, ischemic skin lesions, or purpura fulminans within the neonatal period. Suspicion should be highest in children with a family history of thrombosis, particularly unprovoked VTE.[39] The Subcommittee for Perinatal and Pediatric Thrombosis

TABLE 148.7 Risk Factors for Thrombosis in Neonates and Children

Patient-Related	
Age (<1 year old and ≥11)	
Post-pubertal	
Obesity (BMI > 95th percentile)	
Smoking	
Pregnancy	
Post-partum	
Anatomic anomalies (May-Thurner or thoracic outlet syndrome)	
Prematurity	
Inherited	
Thrombophilia	
• Antithrombin deficiency	
• Prothrombin gene mutation	
• Protein C deficiency	
• Protein S deficiency	
• Factor V Leiden	
Family history of VTE	
Disease-Related	
Malignancy (highest risk in leukemia, lymphoma, sarcoma)	
Inflammatory bowel disease	
Nephrotic syndrome	
Severe infection	
Congenital heart disease	
Trauma requiring ICU admission	
Altered/immobility (>72 h)	
Antiphospholipid antibodies	
Dehydration	
Treatment-Related	
Central venous/arterial catheters	
Orthopedic procedures	
ICU admission	
Surgery	
Total parenteral nutrition	
Medication	
• Chemotherapy (asparaginase, cisplatin, doxorubicin)	
• Steroids	
• Combined oral contraception	
Perinatal Risk Factors	
Maternal-associated conditions:	Neonatal risk factors:
Maternal infection	Central venous catheter
Placental disease	Sepsis
Maternal diabetes	Dehydration
Antiphospholipid antibody syndrome	Perinatal asphyxia
Hypertension	Congenital heart disease
Metabolic syndrome	Prematurity
C-section delivery	
Inherited thrombophilia	
Premature rupture of membranes	

of the Scientific and Standardization Committee of the International Society on Thrombosis and Hemostasis recommended that pediatric patients with spontaneous TE be tested for a full panel of genetic and acquired thrombophilic defects.[43] However, because of the difficulty in obtaining a large volume of blood from neonates, one can consider performing these tests in stages unless the result of the testing has immediate impact on patient management. On initial presentation of TE, DNA-based assays can be performed. Testing for levels of natural coagulation inhibitors should be delayed until 6 months of age, when the levels approach those in adults, and until anticoagulant treatment is discontinued.

Diagnosis

Thrombosis can be asymptomatic or symptomatic with clinical manifestations dependent on the location. For example, cerebral vein thrombosis can present with headache, vomiting, focal neurologic deficits, or seizures. Patients with pulmonary embolism (PE) may present with shortness of breath and chest pain, while extremity DVT usually present with edema of the affected limb, pain, and skin discoloration.[29] Noninvasive tests are used to diagnose DVT, although historically, venography was the gold standard. Compression doppler ultrasonography is the most widely used noninvasive imagining modality, and it can be performed at the bedside of critically ill children. However, inability to compress the vessels of the upper central venous system, apart from the jugular veins, reduces the utility of this imaging modality. Cross-sectional imagining, such as magnetic resonance angiography/venography and computed tomography angiography/venography, are the primary imaging tools for assessment of veins and arteries in the brain, chest, and abdomen. Additionally, these imaging modalities are useful in assessing the clot burden or identifying anatomic abnormalities that may predispose patients to thrombosis, such as May-Thurner or thoracic outlet syndrome (TOS). The utility of biomarkers, such as D-dimer, which is useful in adults, requires further research in pediatrics.

Specific Neonatal Thrombotic Syndromes

Renal Vein Thrombosis

The renal vein is one of the most common sites of neonatal thrombosis. The incidence of renal vein thrombosis (RVT) is estimated to be 0.5 per 1000 NICU admissions and 2.2 to 2.6 per 100,000 live births.[28,41,44] RVT is more common in males than in females and in the left renal vein, although 28% to 44% occur bilaterally. Two-thirds of neonates with RVT presents within 3 days postnatally, and 7% of neonates can present with RVT in utero.[43] Most common clinical manifestations of RVT include hematuria, palpable flank mass, and thrombocytopenia. Neonates may also have co-existing hypertension, proteinuria, renal failure, adrenal hemorrhage, and anemia. RVT is associated with prematurity, umbilical venous catheters, diabetic mothers, asphyxia, and infections. Although FV Leiden, the prothrombin gene mutation, and elevated lipoprotein(a) have been associated with RVT, they are unlikely to be causal in most cases. Most infants with RVT will experience complete, cortical, or segmental infarction of the affected kidney and/or hypertension.[26] It is estimated that neonatal RVT increase risk of chronic kidney disease, hypertension, or death by 15.5-fold.[44]

Diagnosis is reliably confirmed by Doppler ultrasound examination. There are no evidence-based treatment guidelines, and most cases of RVT result in loss of renal tissue regardless of the treatment.[45] Neonates with RVT should be followed for persistent hypertension and progressive renal insufficiency. Unilateral RVT without uremia or clot extension into the inferior vena cava can be managed with heparin or LMWH. Treatment should be given for at least 3 months if there is extension into the inferior vena cava. Bilateral RVT with renal failure should be treated with thrombolytic therapy, followed by heparin or LMWH.[10] There is an ongoing registry by International Pediatric Thrombosis Network on neonatal RVT that shall help to derive more information on this entity.

Portal Vein Thrombosis

The true incidence of portal vein thrombosis (PVT) is unknown, but it is estimated to range from 1% to 43% of neonates with umbilical venous catheters or 36 per 1000 NICU admissions.[46,47] The wide variation in incidence reflects differences in imaging protocols. Major risk factors for PVT include umbilical venous catheters and sepsis/omphalitis. The role of inherited thrombophilia in PVT is controversial. There are multiple studies reporting on the association of inhibitor protein deficiencies (protein C, protein S, and AT) with PVT; however, it is difficult to ascertain whether the deficiencies are genetic or acquired because the testing is done after the diagnosis of PVT has been established.[46] Long-term complications of PVT include lobar atrophy of the liver and portal hypertension with associated gastrointestinal bleeding. Complications are more frequent with ectopic umbilical venous catheter placement (below or in the liver) or when thrombi are occlusive. Ultrasonography may reveal evidence of prior PVT with cavernous transformation of the portal vein and subsequent splenomegaly and reversal of portal flow.[48] Spontaneous resolution of PVT is common, but detection of PVT is important, even in asymptomatic patients, because PVT can lead to portal hypertension, which may manifest up to 10 years later.[48] Neonates with umbilical venous catheters should be monitored by ultrasonography, and catheter removal and/or anticoagulation therapy should be considered for PVT.

Cerebral Sinovenous Thrombosis

Based on data from the Canadian Pediatric Ischemic Stroke Registry, the prevalence of cerebral sinovenous thrombosis (CSVT) is 0.67 per 100,000 children, with 43% occurring in the neonatal period.[49] The clinical presentation may be relatively silent, or patients may manifest with diffuse neurologic changes, seizures, and IVH. The most frequently involved vessels are the superior and lateral sinuses, and up to one-third of cases have associated venous infarction and subsequent hemorrhage.[26] Up to 31% of full-term neonates with IVH have associated CSVT, suggesting that the pathophysiology of IVH in full-term neonates is different from that in premature infants.

Risk factors for neonatal CSVT include perinatal asphyxia, diffuse hypoxic injury, dehydration, infection, congenital heart disease, and severe illness, with ECMO now recognized as a specific risk factor. The frequency of hereditary thrombophilia in neonates with CSVT ranges from 20% to 40%, with FV Leiden and MTHFR C677T occurring most often. In contrast to purpura fulminans, no cases of congenital deficiencies of protein C, protein S, nor AT have been reported in association with CSVT. Multiple risk factors (maternal, neonatal, perinatal, or prothrombotic) are found in over half of neonates with CSVT.[10] Although the diagnosis of CSVT in neonates is often made by transcranial ultrasonography, magnetic resonance venography is the most sensitive diagnostic test. Based on data from the Canadian Pediatric Ischemic Stroke Registry, neonatal CSVT is associated with cerebral parenchymal infarcts in 42% of cases, and 83% of these are hemorrhagic infarcts.[49]

Aside from treatment of the underlying conditions, when relevant, there are no standard treatment guidelines. Nonetheless, good results have been obtained with anticoagulant therapy, and UFH or LMWH is given if there is no significant ICH. Extended treatment with LMWH or a vitamin K antagonist is recommended for a minimum of 6 weeks and no longer than 3 months.[10] The goal of anticoagulation is not to prevent recurrence; rather to prevent progression and minimize neurologic sequelae. In a study by Kenet et al.,[50] none of the 75 neonates with CSVT had a recurrence after a median follow-up of 36 months. For those with significant hemorrhage, radiologic monitoring in 5 to 7 days is recommended, and anticoagulation should be initiated if there is evidence of thrombus propagation.[10] Anticoagulation is associated with a high rate of hemorrhage, so treatment plans should be individualized. In older patients, punctate hemorrhage behind a cerebral venous infarct is not an absolute contraindication to anticoagulation, but studies in neonates are lacking. Nevertheless, in the Canadian registry, 36% of neonates were treated with UFH or LMWH, mostly for 3 months, and no cases of death or neurologic compromise from hemorrhage were reported.[49] Overall, neurologic impairment was reported in up to two-thirds of cases, and approximately 2% died.

Purpura Fulminans

Purpura fulminans is characterized by disseminated purpuric lesions often associated with bullae and necrosis. The histopathology of these lesions reveals diffuse cutaneous microthrombi with surrounding hemorrhage. Diffuse thrombosis, including stroke, retinal infarcts, limb gangrene, and DIC, can occur in purpura fulminans. Causes include severe protein C, protein S, or AT deficiency, either acquired as a complication of sepsis or inherited as homozygous or compound heterozygous conditions. Some infants with severe protein C deficiency do not develop TE until adulthood, suggesting that additional factors influence the neonatal presentation. Treatment with heparin and replacement with protein C concentrate or FFP are indicated. Long-term anticoagulation is often needed.[51]

Ischemic Stroke

Perinatal acute ischemic stroke (AIS) is an important cause of cerebral palsy, epilepsy, and cognitive impairment. Perinatal AIS mostly occurs in full-term neonates with a prevalence of 29 to 93 cases per 100,000 live births. Presenting features include seizures and lethargy. In the neonatal period, AIS often presents with focal or generalized seizures, although pathologic hand preference before 1 year of age is most common if the stroke was asymptomatic in the newborn period. Ischemic injury is usually detected by magnetic resonance angiography, and unilateral lesions favor the left hemisphere. Diffusion-weighted magnetic resonance imaging is superior to cranial ultrasonography or computed tomography scanning.[10]

Risk factors for perinatal AIS are different from TE risk factors in older infants and children because maternal and placental factors play a more important role, and some events even occur in utero.[52] The most common acquired risk factors for perinatal AIS are perinatal asphyxia, fetal distress, chorioamnionitis or other infections, preeclampsia, congenital heart disease, and dehydration. The contribution of congenital thrombophilia to perinatal AIS risk is unclear, although maternal anticardiolipin antibodies may be present for a brief period. In most cases of perinatal AIS, a hypercoagulable state is not detected. One potential mechanism of stroke is embolism of placental thrombi via the umbilical vein and through the patent foramen ovale of the fetus or neonate. Evaluation of placental pathologic conditions is important because demonstration of placental thrombi or abruption may be indicative of a maternal prothrombotic state. Furthermore, if placental thrombi are seen on histological examination, the risk for recurrent events is especially low. Long-term developmental outcomes depend on the extent of the stroke and location of the lesion; strokes involving Broca and Wernicke areas, the internal capsule, or the basal ganglia have a poor outcome.

Treatment depends on whether the stroke is embolic or nonembolic in origin. For nonembolic AIS, current guidelines recommend against anticoagulation or aspirin for neonates with first AIS, especially if the infarct is large or there is evidence of hemorrhage. Anticoagulant or aspirin therapy is recommended for neonates with recurrent AIS.[10] In a patient with a documented cardioembolic stroke, anticoagulant therapy is suggested.

Specific Pediatric Thrombotic Syndromes

Pulmonary Embolism

PE in children is rare, with an incidence of 0.1 to 0.9 per 100,000 overall[29] and 9 to 57 per 100,000 hospitalization children.[31] The epidemiology and pathophysiology are significantly different from that of the adult population. Most PE occurs in neonates. In adolescents, females may have a higher predilection possibly due pregnancy and hormonal therapy. Additionally, Black children have a twofold higher risk compared with White children. PE is associated with DVT in 30% to 60% of cases. Unprovoked PE in the pediatric population is rare, with more than 95% of children have an underlying condition that places them at risk of VTE.[53]

Severe PE can present with symptoms, such as pleuritic chest pain, dyspnea, hemoptysis, persistent tachycardia, hypoxemia, and fever. Massive PE causes complete obstruction of pulmonary flow and can present with concomitant hemodynamic instability or sudden death. Delayed diagnosis is not uncommon as symptoms are non-specific resulting in preliminary investigation of more common childhood illnesses.[53] Computed tomography pulmonary angiography (CTPA) is the diagnostic imaging modality of choice; however, diagnostic work-up should also assess clinical severity to guide management. Traditionally, ventilation perfusion (V/Q) lung scanning was used for the diagnosis of PE. However, such scans require cooperation of the child, and abnormalities may not be specific for PE.[53–55]

Treatment of PE is adapted from adult data and smaller pediatric studies. Pharmacologic treatment includes anticoagulation with either UFH or LMWH, followed by LMWH, vitamin K antagonist, or a DOAC. LMWH is the preferred agent in children without a significant bleeding risk. Thrombolysis, thrombectomy, or embolectomy is indicated in the setting of massive PE with associated hemodynamic instability. Thrombolysis can be catheter-directed or systemic. CHEST guidelines recommend anticoagulation for at least 3 months, or until resolution of precipitating risk factor for secondary PE.[10]

Anatomic Abnormality–Associated Thrombotic Syndromes

Anatomic abnormalities within the pediatric population increase the risk of DVT due to compression of major vessels, which results in reduced blood flow, endothelial perturbation and proliferation, collagen and elastin deposition, and, ultimately, partial or complete vessel occlusion.[56] This can result in extensive and life-threatening thrombosis, which may result in the need for thrombolysis, surgical intervention, or long-term anticoagulation.

May–Thurner Syndrome

May–Thurner syndrome (MTS) is an anatomic variant wherein the left iliac vein is compressed between the overlying right common iliac artery and the lumbar vertebrae. Iliac vein compression causes obstructed venous return and subsequent development of symptomatic DVT. MTS should be considered in adolescents with left-sided iliofemoral thrombosis. The risk of DVT in MTS is relatively low, with an incidence of 2% to 5%, and is often associated with prothrombotic risk factors, such as immobility, hormonal therapy, obesity, recent surgery, inflammatory syndromes, or underlying thrombophilia.[56–58] Diagnosis of DVT is initially made with compression Doppler ultrasound; however, diagnosing MTS requires cross-sectional imaging, such as MR or CT venography, or angiography, to identify the anatomic compression and to visualize the detailed vascular anatomy. There is no established treatment approach to MTS, and treatment is based on the extent of DVT and risk to life/limb. Treatment with anticoagulation, such as LMWH, is used to reduce the risk of progression, embolization, and recurrence. Catheter directed thrombolytic therapy has been utilized in this population without life or limb threatening ischemia and has shown to be effective in the treatment, but not in the prevention of recurrent thrombus.[57] Literature regarding stenting of the vasculature is limited, and complications, such as stent migration secondary to growth, stent narrowing, and thrombosis, should be considered if selecting this option.[57] Children and adolescents should be followed long term for the risk of recurrence and long-term side effects, such as post-thrombotic syndrome.[58]

Thoracic Outlet Syndrome

TOS, also known as Paget-Shroetter syndrome or axillary-subclavian vein effort thrombosis, is a neurovascular bundle disorder resulting from compression by the clavicle, first rib, and scalene muscles. Vascular damage is thought to be a consequence of repetitive venous injury related to arm use, hyperextension of the neck, or hyperabduction of the upper limb, which often occurs in activities that involve repetitive upper extremity movements, such as swimming, weightlifting, and baseball pitching.[59–61] Patients with TOS may present with symptoms of neck discomfort, upper limb weakness, sensory loss, or vascular symptoms, such as swelling, pain, distended superficial veins, heaviness of the limb, and discoloration of the skin of the affected extremity. A high index of suspicion is required in healthy active children and adolescents who present with these symptoms.[60] Clinical signs include a positive Wright maneuver, elevated arm stress test (EAST),[59] and Adson's test.[62] Doppler ultrasound with compression is the initial diagnostic modality of choice. However, due to technical limitations, contrast-enhanced CT or MR venography with the arm positioned in the resting and overhead position is suggested to provide increased accuracy.[60] Management is controversial, and there is no standard approach for treatment of children with TOS. Re-establishing venous flow and prevention of thrombus propagation is the preliminary aim of treatment. Therapeutic options include anticoagulation, thrombolysis, thrombectomy, and balloon angioplasty.[63] Long-term management options involve surgical decompression of the thoracic inlet, first rib resection, as well as supportive management, such as physiotherapy.[62] Timing of surgery is controversial and complications, such as hemothorax and pneumothorax, can occur.[61,63] Prolonged follow-up is required to assess for thrombus recurrence.

Principles of Therapy

Treatment of DVT in childhood is largely extrapolated from the adult literature and practice. As with other age-groups, therapeutic modalities available to children include supportive care, anticoagulation, thrombolytic agents, and surgical thrombectomy. The British Hemostasis and Thrombosis Task Force, American College of Chest Physicians, and the American Society of Hematology have proposed guidelines for the management of neonatal and pediatric TE.[64] Supportive therapy is recommended for clinically silent thrombi, including catheter-associated events. As soon as practical, clotted catheters should be removed, and all documented thrombi should be followed by serial imaging. If venous access is required, one can either monitor the clot closely or provide anticoagulation therapy.

Vitamin K Antagonist Therapy

Warfarin is an oral anticoagulant used in the pediatric population primarily in the context of congenital heart disease and in older, reliable children who require long-term anticoagulation. It is a vitamin K antagonist that initially requires bridging with UFH or LMWH until therapeutic internationalized normalized ratio targets are achieved (see Chapter 143). Warfarin use is limited in children due to age-related changes in vitamin K dietary intake, lack of a liquid formulation, and differences in the levels of the vitamin K–dependent procoagulant proteins in childhood.

Heparin (Unfractionated Heparin or Low-Molecular-Weight Heparin)

UFH or LMWH is the mainstay of anticoagulant therapy in neonates and pediatrics. The UFH and LMWH dosing regimens are outlined in the box on Treatment of Neonatal and Pediatric Thromboembolism. Neonates require higher doses of heparin compared with adults due to lower AT levels, increased volume of distribution and faster clearance.[3,4,65,66] Anticoagulation is monitored using anti-FXa assays. Therapeutic anti-FXa levels are 0.3 to 0.7 units/mL for UFH and 0.5 to 1 unit/mL for LMWH. There are two types of anti-FXa assays: (1) with exogenous AT added and (2) without exogenous AT.[67] Studies comparing the two types of anti-FXa assays found a lack of correlation. Hence, it is necessary to know the type of anti-FXa assay conducted in a particular laboratory when interpreting results. The aPTT can also be used to monitor UFH; aPTT values that correlate with anti-FXa levels of 0.3 to 0.7 units/mL are considered therapeutic.

TREATMENT OF NEONATAL AND PEDIATRIC THROMBOEMBOLISM

Unfractionated Heparin (UFH)

Bolus: 75 units/kg over 10 min
Maintenance: neonatal: 28 units/kg/h; pediatric: 20 units/kg/h; therapeutic goal, anti-FXa of 0.3–0.7 units/mL
Prophylaxis: 10 units/kg/h
Follow platelet count to detect possible HIT (risk is low)

Enoxaparin

Neonatal: 1.5 mg/kg subcutaneously every 12 h; Pediatric ≥ 2 months: 1 mg/kg subcutaneously every 12 h (with normal renal function; round dose to nearest milligram; consider higher dose [see text][42])
Check anti-FXa level by peripheral venipuncture 4–6 h after second or third dose; therapeutic goal, anti-FXa of 0.5–1 unit/mL (0.4–0.6 unit/mL if concurrent thrombocytopenia or other bleeding risk factor)
Follow platelet count to detect possible HIT (risk is low)
Hold for 24 h before procedures

Prophylaxis With Enoxaparin

Neonatal: 0.75 mg/kg subcutaneously every 12 h; Pediatric ≥ 2 months: 0.5 mg/kg subcutaneously every 12 h
If checked, anti-FXa level 4–6 h after second or third dose should be less than 0.4 units/mL
Hold for 12 h before procedures

Purpura Fulminans

Concurrent heparin: UFH dose of 28 units/kg/h with target anti-FXa of 0.3–0.7 units/mL; LMWH dose of 1.0–1.5 mg/kg every 12 ho with therapeutic target anti-FXa range of 0.5–1 units/mL and replacement with FFP or protein C concentrate
FFP: 10–20 mL/kg every 6–12 h for purpura fulminans
Protein C concentrate for severe protein C deficiency: load with 100–120 units/kg, then 60–80 units/kg every 6 h × three doses (goal protein C activity 100%). Once therapeutic anticoagulation is achieved, maintenance therapy with 4–60 units/kg every 6–12 h (goal protein C activity >25%).

Warfarin

Loading dose day 1: 0.2 mg/kg (maximum 10 mg)
  Loading dose day 2–4 based on INR:
  1.1–1.3: repeat initial loading dose
  1.4–1.9: 50% of loading dose
  2.0–3.0: 50% of loading dose
  3.1–3.5: 25% of loading dose
  >3.5: hold dose until INR <3.5, then re-start at 50% of the previous dose
Target INR range is based on the warfarin indication

Antithrombin Repletion

AT (functional) should be maintained at >50% of normal levels for effective heparin-based anticoagulation
Dose in international units = (desired − current AT[a]) × weight (kg)

[a] Expressed as % normal level based on functional AT level.

AT, Antithrombin; *FFP*, fresh frozen plasma; *HIT*, heparin-induced thrombocytopenia; *LMWH*, low-molecular-weight heparin; *TE*, thromboembolism; *UFH*, unfractionated heparin.

Prophylactic UFH is recommended to maintain patency of umbilical arterial lines (0.25 to 1 unit/mL, 25 to 200 units/kg/day, the lowest dose possible) and for cardiac catheterization (bolus 100 to 150 units/kg with catheter insertion, repeat for prolonged procedures). In most other cases, LMWH has emerged as the anticoagulant of choice both for prophylaxis and treatment. The advantages of LMWH include better bioavailability, longer half-life allowing twice daily administration, dose-dependent clearance, predictable anticoagulant response, and a lower risk of heparin-induced thrombocytopenia (HIT) and osteoporosis compared with UFH (see Chapter 143). While the current American College for Chest Physicians guidelines recommend a dose of 1.5 mg/kg of LMWH for treatment of neonatal TE,[10] there is growing evidence to suggest that neonates may, in fact, require higher doses up to 1.7 and 2.0 mg/kg for term and preterm neonates, respectively, to obtain a therapeutic anti-FXa level.[68] Dosing for children greater than 1 year of age is similar to adult dosing of 1 mg/kg of LMWH.

HIT antibodies are found in up to 1.5% of neonates, particularly in those who have undergone cardiac surgery. A systematic review, conducted by Avila et al., reported an estimated incidence of 0.33% in older children, primarily associated with cardiopulmonary bypass surgery. Overt HIT with TE, however, is rare, but it can occur. Diagnostic criteria for HIT are described in detail in Chapter 131. Treatment includes cessation of heparin and anticoagulation with argatroban, danaparoid, or lepirudin.[69]

Direct Oral Anticoagulants

DOACs targeting thrombin or FXa show potential as an alternative to LMWH or warfarin for treatment of TE. Several pediatric clinical trials with the DOACs have been completed. Dabigatran, a direct oral thrombin inhibitor, showed non-inferiority with similar safety and efficacy compared with the standard of care in the DIVERSITY trial that enrolled children less than 18 years of age. Complete and partial thrombus resolution was demonstrated in 46% and 32%, respectively, of children treated.[32] Compared with the standard of care, rivaroxaban, a direct oral FXa inhibitor, shows promise showed safety and efficacy in neonates and children without a significant difference in the rate of recurrent VTE in the EINSTEIN-Jr trial.[70] Notably, both trials evaluated DOAC use after at least 5 days of parenteral anticoagulation.[32,70]

Thrombolytic Therapy

Guidelines for thrombolytic management of neonatal and pediatric TE are provided by the British Hemostasis and Thrombosis Task Force,[64] Scientific Subcommittee on Perinatal and Pediatric Thrombosis of the International Society of Thrombosis and Hemostasis,[71] and the American College of Chest Physicians.[10] All groups agree that thrombolysis should be considered for extensive TE associated with organ dysfunction or limb-threatening ischemia (e.g., PE with hypotension or shock, superior vena cava syndrome, large mobile right atrial thrombus, and congenital heart disease with shunt thrombosis). The choice of using systemic or catheter-directed thrombolysis depends on the bleeding risk, availability of correct catheter size, and expertise of the interventional radiologist, thrombosis team, and intensive care unit.

Low-dose thrombolysis is recommended to open occluded catheters. tPA is the drug that has been most widely studied in pediatric patients. Transfusion support for hypofibrinogenemia and thrombocytopenia should be provided to minimize the bleeding risk. Contraindications for thrombolytic therapy include active bleeding and major surgery or bleeding within the past 10 days. If UFH or LMWH is given concomitantly with tPA, it should be administered at prophylactic doses (0.75 mg/kg every 12 hours for LMWH or 10 units/kg/h for UFH; see the box on tPA Thrombolysis for Neonatal and Pediatric TE). Surgical thrombectomy is reserved for organ-, limb-, or life-threatening thrombosis when tPA administration is impractical or predicted to be ineffective.

TISSUE PLASMINOGEN ACTIVATOR THROMBOLYSIS FOR NEONATAL THROMBOEMBOLISM

Concurrent Heparin (UFH or Enoxaparin) Should Be Considered at Prophylactic Dosing; UFH Preferred

Low dose: 0.01–0.06 mg/kg/h (max 2 mg/h)
High dose: 0.1–0.5 mg/kg/h
Life- or limb-threatening thrombi: starting dose of 0.1–0.5 mg/kg/h for up to 6 h
If no response, consider increase by 0.1 mg/kg/h increments to maximum 0.5 mg/kg/h
Consider using FFP 10 mL/kg before thrombolytic therapy in neonates
Maintain fibrinogen greater than 100 mg/dL
Maintain platelet count above 100,000
Reversal of severe bleeding with aminocaproic acid at 100 mg/kg intravenously every 6 h

FFP, Fresh frozen plasma; *TE*, thromboembolism; *tPA*, tissue plasminogen activator; *UFH*, unfractionated heparin

Post-Thrombotic Syndrome

Post-thrombotic syndrome (PTS) is a common, long-term complication of pediatric patients with extremity DVT with a frequency of approximately 26% but ranging from 10% to 70%.[72] The diagnosis is made at least 12 months after acute DVT diagnosis, with a median time ranging between 2 and 10.5 years. PTS is a result of luminal narrowing from residual thrombosis and damage to the venous valves through inflammation and during vascular recanalization, ultimately leading to venous outflow obstruction and vascular hypertension.[73] Clinical symptoms of swelling, pain, heaviness, cramping, dilation of superficial veins, ulceration, and skin changes of the affected limb are secondary to capillary leakage of plasma proteins and inflammatory cells.[72] Symptoms may be intermittent or persistent and are frequently worsened by physical activity. PTS associated risk factors include extent of DVT, lack of resolution, the number of vessels involved, and CVL-associated thrombus.[73] Avila et al. also documented an association between sex and line-related thrombosis in the lower extremity. They found that males with line-related thrombosis had a higher risk of PTS compared with females. Additionally, females with non-line-related DVT were at higher risk for PTS than females with line-related thrombosis. The Manco-Johnson Instrument (MIJI), modified Villalta Scale (MVS), and CAPTSure score are PTS tools used in the pediatric population which have good reliability.[73] Management of PTS is predominantly supportive with the use of compression stockings to manage symptoms.[73]

COVID-19 and Thrombosis in Children

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) caused a worldwide pandemic in 2020 and 2021 (and is ongoing), impacting global morbidity and mortality. Children with Coronavirus Disease of 2019 (COVID-19) account for a small percentage of cases (2.1% to 7.8%) and generally present with milder clinical symptoms.[74] The cumulative incidence of COVID-19 associated hospitalizations in children and adolescents was 49.7/100,000 in the USA from March 1, 2020, to August 14, 2021.[75] Additionally, a discrete entity, COVID multisystem inflammatory syndrome in children (MIS-C), manifested in children. This condition is now well described in the literature and generally arises weeks after SARS-CoV-2 infection. Patients with MIS-C can present with mild to life-threatening symptoms and often have clinical Kawasaki-like symptoms.[74,76]

Although the pathophysiology of COVID-19 is still under investigation, its association with the development of thrombosis has been established predominantly in adults. The hypercytokinemia and immune dysregulation in COVID-19 is thought to contribute to the thromboembolic risk in patients. This is possibly instigated by binding of SARS-CoV-2 to the angiotensin-converting enzyme 2 (ACE-2), which is expressed on vascular endothelium, causing local injury.[77–79] Autopsies have reported that thromboembolic disease in patients with COVID have manifested in both macro- and microvasculature.[80]

In children, COVID-induced hemostatic imbalance and thrombosis is described in the literature. Abnormalities in coagulation parameters, such as prolongation of PT and aPTT, elevated D-dimer and fibrinogen, mild thrombocytopenia, or thrombocytosis, can be present during COVID-associated illnesses.[81] The thrombotic risk in pediatrics is still being established and current thromboprophylaxis guidelines are extrapolated from adult experience and expert opinion.[82] A multi-institutional retrospective cohort study conducted in the USA documented an incidence of VTE in hospitalized patients with COVID-19 and MIS-C of 2.1% and 6.5%, respectively.[82] Children with asymptomatic SARS-CoV-2 infection had an incidence of 0.7%. Thrombotic complications included DVT, PE, intracardiac thrombosis, CSVT, and AIS.[76,82] Prothrombotic risk factors included age ≥12, underlying cancer diagnosis, MIS-C, and elevated D-dimer.[82]

There are various approaches to thromboprophylaxis in COVID-related illness. Thromboprophylaxis guidelines suggested by the pediatric/neonatal hemostasis and thrombosis subcommittee leadership of the ISTH Scientific and Standardization Committee recommend prophylactic anticoagulation in children with additional clinical risk factors in the setting of COVID-associated illness or markedly elevated d-dimer levels, in the dearth of contraindication.[83] Choice of anticoagulant is either low-dose LMWH or UFH, with consideration of post-discharge anticoagulation in patients with persistent risk factors, including elevated d-dimer. Aspirin, an antithrombotic therapy, commonly used in Kawasaki disease, is suggested by the American College of Rheumatology in all patients with MIS-C until the platelet count normalizes, and echocardiography shows normal coronary arteries at least 4 weeks from diagnosis.[84] Despite utilizing thromboprophylaxis, current literature reports that approximately one-half to two-thirds of patients with COVID-related illness develop thrombosis, which establishes the need for further investigation.[81,82] At present, the safety and efficacy of low- versus high-dose anticoagulation has not been established in this population and is currently under investigation with the COVAC-TP clinical trial (NCT04354155). One can adopt what is currently accepted for thromboprophylaxis in adults with COVID-19 when the children are older than 16.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Bertamino M, Riccardi F, Banov L, et al. Hemophilia care in the pediatric age. *J Clin Med.* 2017;6(5):54.

Brandão LR, Simpson EA, Lau KK. Neonatal renal vein thrombosis. *Semin Fetal Neonatal Med.* 2011;16(6):323.

Jaffray J, Young G. Deep vein thrombosis in pediatric patients. *Pediatr Blood Cancer.* 2018;65:e26881.

Kenet G, Chan AK, Soucie JM, et al. Bleeding disorders in neonates. *Haemophilia.* 2010;16:168.

Monagle P, Chan AK, Goldenberg NA, et al. Antithrombotic therapy in neonates and children: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed. American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest.* 2012;141:e737S.

Neunert C, Noroozi N, Norman G, et al. Severe bleeding events in adults and children with primary immune thrombocytopenia: a systematic review. *J Thromb Haemost.* 2015;13(3):457.

Ramiz S, Rajpurkar M. Pulmonary embolism in children. *Pediatr Clin North Am.* 2018;65(3):495–507.

Williams S, Chan AK. Neonatal portal vein thrombosis: diagnosis and management. *Semin Fetal Neonatal Med.* 2011;16(6):329.

REFERENCES

1. Andrew M, Paes B, Milner R, et al. Development of the human coagulation system in the healthy premature infant. *Blood*. 1988;72(5):1651–1657.
2. Andrew M, Paes B, Milner R, et al. Development of the human coagulation system in the full-term infant. *Blood*. 1987;70(1):165–172.
3. Andrew M, Vegh P, Johnston M, Bowker J, Ofosu F, Mitchell L. Maturation of the hemostatic system during childhood. *Blood*. 1992;80(8):1998–2005.
4. Monagle P, Barnes C, Ignjatovic V, et al. Developmental haemostasis. Impact for clinical haemostasis laboratories. *Thromb Haemost*. 2006;95(2): 362–372. https://doi.org/10.1160/TH05-01-0047.
5. Attard C, van der Straaten T, Karlaftis V, Monagle P, Ignjatovic V. Developmental hemostasis: age-specific differences in the levels of hemostatic proteins. *J Thromb Haemost*. 2013;11(10):1850–1854. https://doi.org/10.1111/jth.12372.
6. Reverdiau-Moalic P, Delahousse B, Body G, Bardos P, Leroy J, Gruel Y. Evolution of blood coagulation activators and inhibitors in the healthy human fetus. *Blood*. 1996;88(3):900–906.
7. Tripodi A, Ramenghi LA, Chantarangkul V, et al. Normal thrombin generation in neonates in spite of prolonged conventional coagulation tests. *Haematologica*. 2008;93(8):1256–1259. https://doi.org/10.3324/haematol.12566.
8. Gassiot S, Ruiz-Llobet A, Suleman W, Sarrate E, Berrueco R. Thrombin generation in children using ThromboScreen reagent kit with ST Genesia—a pilot study. *Int J Lab Hematol*. 2021;43(6):1612–1619. https://doi.org/10.1111/ijlh.13668.
9. Parmar N, Albisetti M, Berry LR, Chan AKC. The fibrinolytic system in newborns and children. *Clin Lab*. 2006;52(3-4):115–124.
10. Monagle P, Chan AKC, Goldenberg NA, et al. Antithrombotic therapy in neonates and children: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest*. 2012;141(2 suppl):e737S–e801S. https://doi.org/10.1378/chest.11-2308.
11. Israels SJ, Rand ML, Michelson AD. Neonatal platelet function. *Semin Thromb Hemost*. 2003;29(4):363–372. https://doi.org/10.1055/s-2003-42587.
12. Bertamino M, Riccardi F, Banov L, Svahn J, Molinari AC. Hemophilia care in the pediatric age. *J Clin Med*. 2017;6(5). https://doi.org/10.3390/jcm6050054.
13. Blanchette VS, Rand ML. Platelet disorders in newborn infants: diagnosis and management. *Semin Perinatol*. 1997;21(1):53–62. https://doi.org/10.1016/s0146-0005(97)80020-1.
14. Kenet G. Bleeding disorders in neonates. *Thromb Res*. 2005;115(suppl 1) 41–44.
15. Roberts I, Stanworth S, Murray NA. Thrombocytopenia in the neonate. *Blood Rev*. 2008;22(4):173–186. https://doi.org/10.1016/j.blre.2008.03.004.
16. Blanchette VS, Johnson J, Rand M. The management of alloimmune neonatal thrombocytopenia. *Baillieres Best Pract Res Clin Haematol*. 2000;13 (3):365–390. https://doi.org/10.1053/beha.2000.0083.
17. D'Orazio JA, Neely J, Farhoudi N. ITP in children: pathophysiology and current treatment approaches. *J Pediatr Hematol Oncol*. 2013;35(1):1–13. https://doi.org/10.1097/MPH.0b013e318271f457.
18. Rodeghiero F, Stasi R, Gernsheimer T, et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. *Blood*. 2009;113(11):2386–2393. https://doi.org/10.1182/blood-2008-07-162503.
19. Neunert C, Noroozi N, Norman G, et al. Severe bleeding events in adults and children with primary immune thrombocytopenia: a systematic review. *J Thromb Haemost JTH*. 2015;13(3):457–464. https://doi.org/10.1111/jth.12813.
20. Brenner B, Kuperman AA, Watzka M, Oldenburg J. Vitamin K-dependent coagulation factors deficiency. *Semin Thromb Hemost*. 2009;35(4):439–446. https://doi.org/10.1055/s-0029-1225766.
21. Girolami A, Ruzzon E, Tezza F, Allemand E, Vettore S. Congenital combined defects of factor VII: a critical review. *Acta Haematol*. 2007; 117(1):51–56. https://doi.org/10.1159/000096789.
22. Kulkarni R, Lusher JM. Intracranial and extracranial hemorrhages in newborns with hemophilia: a review of the literature. *J Pediatr Hematol Oncol*. 1999;21(4):289–295. https://doi.org/10.1097/00043426-199907000-00009.
23. Federici AB. Diagnosis of inherited von Willebrand disease: a clinical perspective. *Semin Thromb Hemost*. 2006;32(6):555–565. https://doi.org/10.1055/s-2006-949661.
24. Hsieh L, Nugent D. Factor XIII deficiency. *Haemoph Off J World Fed Hemoph*. 2008;14(6):1190–1200. https://doi.org/10.1111/j.1365-2516.2008.01857.x.
25. Chalmers EA. Neonatal coagulation problems. *Arch Dis Child Fetal Neonatal Ed*. 2004;89(6):F475–F478. https://doi.org/10.1136/adc.2004.050096.
26. Saxonhouse MA, Manco-Johnson MJ. The evaluation and management of neonatal coagulation disorders. *Semin Perinatol*. 2009;33(1):52–65. https://doi.org/10.1053/j.semperi.2008.10.007.
27. Napolitano M, Mariani G, Lapecorella M. Hereditary combined deficiency of the vitamin K-dependent clotting factors. *Orphanet J Rare Dis*. 2010; 5:21. https://doi.org/10.1186/1750-1172-5-21.
28. Nowak-Göttl U, von Kries R, Göbel U. Neonatal symptomatic thromboembolism in Germany: two year survey. *Arch Dis Child Fetal Neonatal Ed*. 1997;76(3):F163–F167. https://doi.org/10.1136/fn.76.3.f163.
29. van Ommen CH, Heijboer H, Büller HR, Hirasing RA, Heijmans HS, Peters M. Venous thromboembolism in childhood: a prospective two-year registry in The Netherlands. *J Pediatr*. 2001;139(5):676–681. https://doi.org/10.1067/mpd.2001.118192.
30. El-Naggar W, Yoon EW, McMillan D, et al. Epidemiology of thrombosis in Canadian neonatal intensive care units. *J Perinatol Off J Calif Perinat Assoc*. 2020;40(7):1083–1090. https://doi.org/10.1038/s41372-020-0678-1.
31. Andrew M, David M, Adams M, et al. Venous thromboembolic complications (VTE) in children: first analyses of the Canadian Registry of VTE. *Blood*. 1994;83(5):1251–1257.
32. Halton J, Brandão LR, Luciani M, et al. Dabigatran etexilate for the treatment of acute venous thromboembolism in children (DIVERSITY): a randomised, controlled, open-label, phase 2b/3, non-inferiority trial. *Lancet Haematol*. 2021;8(1):e22–e33. https://doi.org/10.1016/S2352-3026(20)30368-9.
33. Raffini L, Huang YS, Witmer C, Feudtner C. Dramatic increase in venous thromboembolism in children's hospitals in the United States from 2001 to 2007. *Pediatrics*. 2009;124(4):1001–1008. https://doi.org/10.1542/peds.2009-0768.
34. Rajpurkar M, Biss T, Amankwah EK, et al. Pulmonary embolism and in situ pulmonary artery thrombosis in paediatrics. A systematic review. *Thromb Haemost*. 2017;117(6):1199–1207. https://doi.org/10.1160/TH16-07-0529.
35. Witmer C, Raffini L. Treatment of venous thromboembolism in pediatric patients. *Blood*. 2020;135(5):335–343. https://doi.org/10.1182/blood.2019001847.
36. Ruhle F, Stoll M. Advances in predicting venous thromboembolism risk in children. *Br J Haematol*. 2018;180(5):654–665. https://doi.org/10.1111/bjh.15060.
37. Goldenberg NA, Kittelson JM, Abshire TC, et al. Effect of anticoagulant therapy for 6 weeks vs 3 months on recurrence and bleeding events in patients younger than 21 years of age with provoked venous thromboembolism: the kids-DOTT randomized clinical trial. *JAMA*. 2022;327(2):129–137. https://doi.org/10.1001/jama.2021.23182.
38. Young G, Albisetti M, Bonduel M, et al. Impact of inherited thrombophilia on venous thromboembolism in children: a systematic review and meta-analysis of observational studies. *Circulation*. 2008;118(13):1373–1382. https://doi.org/10.1161/CIRCULATIONAHA.108.789008.
39. Jaffray J, Young G. Deep vein thrombosis in pediatric patients. *Pediatr Blood Cancer*. 2018;65(3). https://doi.org/10.1002/pbc.26881.
40. Park CK, Paes BA, Nagel K, Chan AK, Murthy P, Thrombosis and Hemostasis in Newborns (THiN) Group Neonatal central venous catheter thrombosis: diagnosis, management and outcome. *Blood Coagul Fibrinolysis Int J Haemost Thromb*. 2014;25(2):97–106. https://doi.org/10.1097/MBC.0b013e328364f9b0.
41. Schmidt B, Andrew M. Neonatal thrombosis: report of a prospective Canadian and international registry. *Pediatrics*. 1995;96(5 Pt 1): 939–943.
42. van Ommen CH, Nowak-Göttl U. Inherited thrombophilia in pediatric venous thromboembolic disease: Why and who to test? *Front Pediatr*. 2017;5:50. https://doi.org/10.3389/fped.2017.00050.
43. Manco-Johnson MJ, Grabowski EF, Hellgreen M, et al. Laboratory testing for thrombophilia in pediatric patients. On behalf of the Subcommittee for Perinatal and Pediatric Thrombosis of the Scientific and Standardization Committee of the International Society of Thrombosis and Haemostasis (ISTH). *Thromb Haemost*. 2002;88(1):155–156.
44. Ouellette AC, Darling EK, Sivapathasundaram B, et al. Incidence, risk factors, and outcomes of neonatal renal vein thrombosis in Ontario: population-based cohort study. *Kidney360*. 2020;1(7):640–647. https://doi.org/10.34067/KID.0000912019.
45. Lau KK, Stoffman JM, Williams S, et al. Neonatal renal vein thrombosis: review of the English-language literature between 1992 and 2006. *Pediatrics*. 2007;120(5):e1278–e1284. https://doi.org/10.1542/peds.2007-0510.
46. Williams S, Chan AKC. Neonatal portal vein thrombosis: diagnosis and management. *Semin Fetal Neonatal Med*. 2011;16(6):329–339. https://doi.org/10.1016/j.siny.2011.08.005.

47. Morag I, Epelman M, Daneman A, et al. Portal vein thrombosis in the neonate: risk factors, course, and outcome. *J Pediatr*. 2006;148(6):735–739. https://doi.org/10.1016/j.jpeds.2006.01.051.
48. Greenway A, Massicotte MP, Monagle P. Neonatal thrombosis and its treatment. *Blood Rev*. 2004;18(2):75–84. https://doi.org/10.1016/S0268-960X(03)00042-0.
49. deVeber G, Andrew M, Adams C, et al. Cerebral sinovenous thrombosis in children. *N Engl J Med*. 2001;345(6):417–423. https://doi.org/10.1056/NEJM200108093450604.
50. Kenet G, Kirkham F, Niederstadt T, et al. Risk factors for recurrent venous thromboembolism in the European collaborative paediatric database on cerebral venous thrombosis: a multicentre cohort study. *Lancet Neurol*. 2007;6(7):595–603. https://doi.org/10.1016/S1474-4422(07)70131-X.
51. Manco-Johnson MJ, Knapp-Clevenger R. Activated protein C concentrate reverses purpura fulminans in severe genetic protein C deficiency. *J Pediatr Hematol Oncol*. 2004;26(1):25–27. https://doi.org/10.1097/00043426-200401000-00008.
52. Chalmers EA. Perinatal stroke—risk factors and management. *Br J Haematol*. 2005;130(3):333–343. https://doi.org/10.1111/j.1365-2141.2005.05554.x.
53. Ramiz S, Rajpurkar M. Pulmonary embolism in children. *Pediatr Clin North Am*. 2018;65(3):495–507. https://doi.org/10.1016/j.pcl.2018.02.002.
54. Lee EY, Zurakowski D, Boiselle PM. Pulmonary embolism in pediatric patients survey of CT pulmonary angiography practices and policies. *Acad Radiol*. 2010;17(12):1543–1549. https://doi.org/10.1016/j.acra.2010.08.012.
55. Victoria T, Mong A, Altes T, et al. Evaluation of pulmonary embolism in a pediatric population with high clinical suspicion. *Pediatr Radiol*. 2009;39(1):35–41. https://doi.org/10.1007/s00247-008-1037-0.
56. Raffini L, Raybagkar D, Cahill AM, Kaye R, Blumenstein M, Manno C. May-Thurner syndrome (iliac vein compression) and thrombosis in adolescents. *Pediatr Blood Cancer*. 2006;47(6):834–838. https://doi.org/10.1002/pbc.20728.
57. Warad DM, Rao AN, Bjarnason H, Rodriguez V. Clinical outcomes of May-Thurner syndrome in pediatric patients: a single institutional experience. *TH Open Companion J Thromb Haemost*. 2020;4(3):e189–e196. https://doi.org/10.1055/s-0040-1714694.
58. White MH, Cramer BM, Gill AE, Hawkins CM, Patel KN. Outcomes and risk factors associated with thrombosis recurrence and post-thrombotic syndrome in children and adolescents with May-Thurner syndrome. *Blood*. 2017;130(suppl 1):3417 https://ashpublications.org/blood/article/130/Supplement%201/3417/114588/Outcomes-and-Risk-Factors-Associated-with. Accessed March 30, 2021.
59. Vercellio G, Baraldini V, Gatti C, Coletti M, Cipolat L. Thoracic outlet syndrome in paediatrics: clinical presentation, surgical treatment, and outcome in a series of eight children. *J Pediatr Surg*. 2003;38(1):58–61. https://doi.org/10.1053/jpsu.2003.50010.
60. Ohman JW, Thompson RW. Thoracic outlet syndrome in the overhead athlete: diagnosis and treatment recommendations. *Curr Rev Musculoskelet Med*. 2020;13(4):457–471. https://doi.org/10.1007/s12178-020-09643-x.
61. Trenor CC, Fisher JG, Khan FA, et al. Paget-Schroetter syndrome in 21 children: outcomes after multidisciplinary care. *J Pediatr*. 2015;166(6):1493–1497. https://doi.org/10.1016/j.jpeds.2015.03.030. e1.
62. Arthur LG, Teich S, Hogan M, Caniano DA, Smead W. Pediatric thoracic outlet syndrome: a disorder with serious vascular complications. *J Pediatr Surg*. 2008;43(6):1089–1094. https://doi.org/10.1016/j.jpedsurg.2008.02.038.
63. Matos JM, Gonzalez L, Kfoury E, Echeverria A, Bechara CF, Lin PH. Outcomes following operative management of thoracic outlet syndrome in the pediatric patients. *Vascular*. 2018;26(4):410–417. https://doi.org/10.1177/1708538117747628.
64. Williams MD, Chalmers EA, Gibson BES. Haemostasis and Thrombosis Task Force, British Committee for Standards in Haematology. The investigation and management of neonatal haemostasis and thrombosis. *Br J Haematol*. 2002;119(2):295–309. https://doi.org/10.1046/j.1365-2141.2002.03674.x.
65. Andrew M, Ofosu F, Schmidt B, Brooker L, Hirsh J, Buchanan MR. Heparin clearance and ex vivo recovery in newborn piglets and adult pigs. *Thromb Res*. 1988;52(6):517–527. https://doi.org/10.1016/0049-3848(88)90125-9.
66. McDonald MM, Jacobson LJ, Hay WW, Hathaway WE. Heparin clearance in the newborn. *Pediatr Res*. 1981;15(7):1015–1018. https://doi.org/10.1203/00006450-198107000-00007.
67. Newall F, Johnston L, Ignjatovic V, Monagle P. Unfractionated heparin therapy in infants and children. *Pediatrics*. 2009;123(3):e510–e518. https://doi.org/10.1542/peds.2008-2052.
68. Malowany JI, Monagle P, Knoppert DC, et al. Enoxaparin for neonatal thrombosis: a call for a higher dose for neonates. *Thromb Res*. 2008;122(6):826–830. https://doi.org/10.1016/j.thromres.2007.11.009.
69. Risch L, Huber AR, Schmugge M. Diagnosis and treatment of heparin-induced thrombocytopenia in neonates and children. *Thromb Res*. 2006;118(1):123–135. https://doi.org/10.1016/j.thromres.2004.12.020.
70. Young G, Lensing AWA, Monagle P, et al. Rivaroxaban for treatment of pediatric venous thromboembolism. An Einstein-Jr phase 3 dose-exposure-response evaluation. *J Thromb Haemost JTH*. 2020;18(7):1672–1685. https://doi.org/10.1111/jth.14813.
71. Manco-Johnson MJ, Grabowski EF, Hellgreen M, et al. Recommendations for tPA thrombolysis in children. On behalf of the Scientific Subcommittee on Perinatal and Pediatric Thrombosis of the Scientific and Standardization Committee of the International Society of Thrombosis and Haemostasis. *Thromb Haemost*. 2002;88(1):157–158.
72. Goldenberg NA, Donadini MP, Kahn SR, et al. Post-thrombotic syndrome in children: a systematic review of frequency of occurrence, validity of outcome measures, and prognostic factors. *Haematologica*. 2010;95(11):1952–1959. https://doi.org/10.3324/haematol.2010.026989.
73. Avila ML, Pullenayegum E, Williams S, Yue N, Krol P, Brandão LR. Postthrombotic syndrome and other outcomes of lower extremity deep vein thrombosis in children. *Blood*. 2016;128(14):1862–1869. https://doi.org/10.1182/blood-2016-03-704585.
74. Jiang L, Tang K, Levin M, et al. COVID-19 and multisystem inflammatory syndrome in children and adolescents. *Lancet Infect Dis*. 2020;20(11):e276–e288. https://doi.org/10.1016/S1473-3099(20)30651-4.
75. Delahoy MJ, Ujamaa D, Whitaker M, et al. Hospitalizations associated with COVID-19 among children and adolescents—COVID-NET, 14 States, March 1, 2020–August 14, 2021. *MMWR Morb Mortal Wkly Rep*. 2021;70(36):1255–1260. https://doi.org/10.15585/mmwr.mm7036e2.
76. Zaffanello M, Piacentini G, Nosetti L, Ganzarolli S, Franchini M. Thrombotic risk in children with COVID-19 infection: a systematic review of the literature. *Thromb Res*. 2021;205:92–98. https://doi.org/10.1016/j.thromres.2021.07.011.
77. Bhaskar S, Sinha A, Banach M, et al. Cytokine storm in COVID-19-immunopathological mechanisms, clinical considerations, and therapeutic approaches: The REPROGRAM consortium position paper. *Front Immunol*. 2020;11:1648. https://doi.org/10.3389/fimmu.2020.01648.
78. Chan N, Eikelboom J. Hypercoagulability and thrombosis in COVID-19: a modifiable cause for mortality? *Eur Heart J*. 2021;42(33):3143–3145. https://doi.org/10.1093/eurheartj/ehab417.
79. Malas MB, Naazie IN, Elsayed N, Mathlouthi A, Marmor R, Clary B. Thromboembolism risk of COVID-19 is high and associated with a higher risk of mortality: a systematic review and meta-analysis. *EClinicalMedicine*. 2020;29:100639. https://doi.org/10.1016/j.eclinm.2020.100639.
80. Wichmann D, Sperhake JP, Lütgehetmann M, et al. Autopsy findings and venous thromboembolism in patients with COVID-19: a prospective cohort study. *Ann Intern Med*. 2020;173(4):268–277. https://doi.org/10.7326/M20-2003.
81. Mitchell WB, Davila J, Keenan J, et al. Children and young adults hospitalized for severe COVID-19 exhibit thrombotic coagulopathy. *Pediatr Blood Cancer*. 2021;68(7):e28975. https://doi.org/10.1002/pbc.28975.
82. Whitworth H, Sartain SE, Kumar R, et al. Rate of thrombosis in children and adolescents hospitalized with COVID-19 or MIS-C. *Blood*. 2021;138(2):190–198. https://doi.org/10.1182/blood.2020010218.
83. Goldenberg NA, Sochet A, Albisetti M, et al. Consensus-based clinical recommendations and research priorities for anticoagulant thromboprophylaxis in children hospitalized for COVID-19-related illness. *J Thromb Haemost*. 2020;18(11):3099–3105. https://doi.org/10.1111/jth.15073.
84. ACR MIS-C and COVID-19-Related Hyperinflammation Task Force. *Clinical Guidance for Pediatric Patients with Multisystem Inflammatory Syndrome in Children (MIS-C) Associated with SARS-CoV-2 and Hyperinflammation in COVID-19 (Version 3)*; 2021. https://www.rheumatology.org/Portals/0/Files/ACR-COVID-19-Clinical-Guidance-Summary-MIS-C-Hyperinflammation.pdf.

PART XII CONSULTATIVE HEMATOLOGY

CHAPTER 149

HEMATOLOGIC CHANGES IN PREGNANCY

Arielle L. Langer, Michael Paidas, and Caroline Cromwell

Hematologic conditions are often seen during pregnancy as part of normal physiology and complications of varying severity. The primary physiologic, hematologic changes during pregnancy relate to the expansion of plasma volume and the prothrombotic state that develops as the pregnancy advances. They are thought to prepare the mother and fetus for eventual placental separation. Understanding the complex physiology of pregnancy and the interplay with hematology facilitates proper planning, anticipation, and discussion with the treating physicians and the patient. Preexisting hematologic conditions may affect pregnancy, and conversely, the effect of pregnancy on proceeding conditions should be considered. The evolving clinical picture as the pregnancy progresses must also be considered. Multidisciplinary planning and communication are essential, especially between obstetrics and hematology.

Common new onset hematologic conditions, such as anemia and thrombocytopenia, are seen for a variety of reasons during pregnancy and addressed in this chapter. The management of congenital and acquired cytopenias will also be covered. Management of hematologic malignancies presents a particular challenge. Inherited and acquired bleeding disorders affect pregnant women, and coagulation parameters must be monitored during pregnancy and delivery, including attention to the use of spinal anesthesia. Venous thromboembolism (VTE) during pregnancy remains a high-morbidity high-risk situation that can be challenging. These hematologic problems in management are discussed in this chapter.

ANEMIA IN PREGNANCY

Anemia in pregnancy affects approximately half of all pregnancies worldwide. It is more prevalent in low-income nations. Anemia in pregnancy is usually defined as a hemoglobin level below 11 g/dL. Common maternal signs of anemia include pallor, tachypnea, fatigue, and headache. Hemoglobin levels less than 6 g/dL in pregnant women can be associated with significant maternal and fetal complications, including high-output heart failure in the mother. Maternal anemia is associated with increased rates of both preterm (less than 37 weeks of gestation) and low-birthweight deliveries.[1,2] The etiology of anemia in pregnancy varies, and potential causes are discussed below.

Physiologic Anemia of Pregnancy

Physiologic anemia of pregnancy, also termed dilutional anemia of pregnancy, occurs because the plasma volume increases to a greater proportion than the red blood cell (RBC) mass, resulting in dilution. Generally, hemoglobin levels decrease throughout pregnancy and may increase modestly during the last month of pregnancy. Total circulatory volume increases by approximately 50% during pregnancy. Erythropoietin levels increase throughout pregnancy in response, but increased erythropoiesis does not fully compensate for the expanded plasma volume. Hemoglobin as low as 10 g/dL can be credibly attributed to physiologic anemia of pregnancy. However, this is a diagnosis of exclusion, and other causes should be considered before this attribution is made. Plasma volume and RBC mass return to baseline within approximately 6 weeks postpartum.

Iron Deficiency Anemia

Iron deficiency anemia, which is the most common cause of anemia in pregnancy, is a risk factor for preterm delivery and low birthweight. Iron requirements increase during pregnancy because of maternal and fetal erythropoiesis. Anemia and low ferritin levels are considered diagnostic of iron deficiency (see Chapter 37). However, ferritin levels may increase in the last several weeks of pregnancy because ferritin is an acute-phase reactant. Consequently, concurrent determination of the transferrin saturation is important in women with an ambiguous picture. The clinical symptoms of iron deficiency are the same as in other contexts, including fatigue, pallor, pica, tachycardia, and poor exercise tolerance. The diagnosis and treatment of iron deficiency anemia is generally like those in nonpregnant patients, except for a greater emphasis on the use of intravenous iron given time constraints.

Iron supplementation is routinely provided during pregnancy because the typical diet in the United States provides only 50% of daily iron requirements for pregnant women and is due to the relatively high prevalence of iron deficiency in women of childbearing age. Typically, supplementation with 15 to 30 mg/day of elemental iron is administered to prevent adverse outcomes from iron deficiency anemia. Supplementation should begin at the start of gestation and continue until several months postpartum. The side effects associated with iron therapy include constipation, diarrhea, and nausea. Such side effects may be particularly troublesome because constipation and nausea are common symptoms of pregnancy.

Multiple studies have shown that routine iron supplementation in pregnancy decreases the incidence of iron deficiency anemia. In a randomized double-blind study in which 275 iron-replete pregnant women at no more than 20 weeks' gestation received either a daily iron supplement or placebo from the time of enrollment to 28 weeks' gestation, the incidence of both low-birthweight and preterm low-birthweight infants was lower among women who received daily iron. [3] Research indicating that low fetal and infant iron stores predict worse cognitive function[4,5] provides further support for normalizing maternal iron stores and optimizing transfer of iron to the fetus. In addition to justifying routine iron supplementation in prenatal vitamins, this is the primary motivation for increased use of intravenous iron.

There are limits to the effectiveness of routine iron supplementation. Patients may develop iron deficiency despite supplementation or be intolerant of oral supplementation. As mentioned, the side effect of constipation can be particularly troublesome with oral iron because constipation and hemorrhoids are common complaints during pregnancy. Active inquiry about these side effects may uncover nonadherence to oral iron when iron deficiency anemia persists.

Intravenous iron is appropriate in certain circumstances: intolerance of oral iron therapy has not improved iron stores and anemia, perhaps even severe iron deficiency anemia, persists. Due to lack of safety data, intravenous iron is generally avoided in the first trimester of pregnancy. All intravenous formulations of iron deficiency are effective and safe in pregnancy. For convenience and expediency, especially given the time constraint of pregnancy, formulations that can be administered in one or two visits are preferred.

Indications for intravenous iron in pregnancy
- Intolerance of oral iron
- Failure to improve with oral iron
- Severe anemia

Folate and Vitamin B_{12} Deficiencies

Folate and vitamin B_{12} (cobalamin) deficiency also occur more commonly during pregnancy (see Chapter 40). Vitamin B_{12} and folate are critical for fetal growth because they are used to produce tetrahydrofolate, which is essential for DNA synthesis.

Folate deficiency accounted for most cases of megaloblastic anemias in pregnancy prior to routine supplementation. The folic acid requirement for nonpregnant women is 50 to 100 μg/day, but this increases to 150 μg in pregnancy because of the increase in maternal RBC mass and fetal demands for folate because of rapid cell proliferation. With fortification of the food supply with folate and the routine use of folate-containing supplements during pregnancy, folate deficiency is an uncommon cause of anemia in pregnancy but should be considered, particularly in women not adherent to folate supplements.

The diagnosis of folate deficiency is best based on RBC folate levels, although many laboratories only offer serum folate assays. An elevated homocysteine level also helps confirm the diagnosis. Pregnant women should receive a minimum of 400 μg of folic acid per day. There are harms associated with excess supplementation because folate, which is a water-soluble vitamin, is readily excreted in the urine if ingestion exceeds requirements.

Vitamin B_{12} deficiency can also occur during pregnancy. Serum vitamin B_{12} levels may be less reliable during pregnancy due to altered protein binding. Diagnosis of vitamin B_{12} deficiency can be confirmed by elevated levels of homocysteine and methylmalonic acid. If a woman is found to be deficient in vitamin B_{12} during pregnancy, vitamin B_{12} injections are usually preferred over oral supplementation for initial therapy. A vitamin B_{12} dose of 1000 mcg intramuscularly is given weekly for 4 weeks and then monthly thereafter. Alternatively, after initial repletion with parenteral vitamins or if anemia and deficiency are mild, supplementation can be administered with vitamin B_{12} 1000 mcg by mouth daily. For ongoing therapy, oral dosing has been shown to be equivalent to parenteral administration, even in individuals with malabsorption of vitamin B_{12}.

HEMOGLOBINOPATHIES AND PREGNANCY

Sickle Cell Disease

Every year, more than 300,000 children are born with either sickle cell disease or thalassemia (see Chapters 41 and 42). Prenatal counseling is now routine in many countries. In the United States, all newborns are screened for sickle cell disease (see Chapters 42 and 43). Management of pregnant patients with sickle cell disease requires coordination of care between the hematologist and obstetrician. Many women with sickle cell disease experience more frequent vaso-occlusive crises and other sickle cell–related complications during pregnancy. The increased frequency of vaso-occlusive crises, particularly during the latter half of pregnancy, results from heightened metabolic requirements in pregnancy, increased venous stasis, and the physiologic prothrombotic and inflammatory states associated with pregnancy. In addition, pathophysiologic changes in the renal and immune function of patients with sickle cell disease increase their susceptibility to urinary tract infections and pyelonephritis. By causing tissue hypoxia, sickling of RBCs within the placental vasculature may have deleterious effects on the fetus.

Additional complications in pregnant women with sickle cell disease and thalassemia include an increased risk of preeclampsia, thromboembolic events, placental abruption, intrauterine growth retardation, low birthweight, and postpartum infections. Studies have shown that women with sickle cell disease experience a higher incidence of stillbirths and perinatal mortality,[6] as well as an increased risk of preterm labor and premature rupture of membranes. [7] Consequently, women with sickle cell disease require close medical attention throughout the prenatal period, including counseling about intrauterine diagnosis of sickle cell disease, when appropriate. Pregnant women can undergo chorionic villi sampling as early as the 9th week of gestation or amniocentesis in the 15th to 16th weeks if fetal evaluation is desired (see Chapter 43). The reticulocyte count should be monitored to assess for bone marrow suppression or the development of nutritional deficiency. Urinalysis with urine culture is performed at least every trimester to monitor for asymptomatic bacteriuria, as this is more common in women with sickle cell disease than in those without and is treated, unlike in nonpregnant patients, because of its association with preterm birth and low birthweight infants. Finally, beginning at between 24 and 28 weeks' gestation, patients should have weekly clinic visits and begin serial ultrasonography to monitor fetal growth.

Pregnant women with sickle cell disease should receive 5 mg/day of supplemental folic acid to address the extra demands of chronic hemolysis and pregnancy. Hydroxyurea can be continued in women with sickle cell disease who wish to become pregnant provided that they track their menstrual cycles and stop taking the drug as soon as a pregnancy test is positive. Hydroxyurea should be withheld during pregnancy but may be restarted in the late second or third trimester in women with serious symptoms. Use of hydroxyurea in pregnancy may be associated with low birthweight and prematurity, but it is difficult to ascertain if it is causal because women with severe sickle cell disease are already at risk of these complications. Ultimately, the risks associated with hydroxyurea must be weighed against the negative effect of poorly controlled sickle cell disease.

Studies examining the benefit of prophylactic blood transfusions have yielded mixed results; the only randomized trial revealed a reduction in vasoocclusive crises with transfusion but no improvements in fetal or other maternal outcomes.[8] Given the risk of alloimmunization and iron overload, prophylactic simple or exchange transfusion is not appropriate for all pregnant women with sickle cell disease. However, individualized care is needed, and prophylactic transfusion may be appropriate for women with severe disease phenotypes.

Transfusion should be considered if the hemoglobin level falls below 6 g/dL if there are progressive complications related to sickle cell disease, or in the face of obstetrical complications. With these criteria, many women require transfusion during pregnancy, although at a lower volume than that needed for prophylactic transfusion. Women with a sickle cell crisis during pregnancy should receive analgesics, hydration, and oxygen while undergoing evaluation for infection or other precipitating causes.

At delivery, pregnant women with sickle cell disease are at risk of high-output heart failure. Supplemental oxygen and cautious hydration should be given to prevent sickling of RBCs and the associated complications. During the postpartum period, hemoglobin levels must be monitored, and VTE prophylaxis should be given unless there are contraindications to anticoagulation. VTE prophylaxis should be continued for 5 to 7 days after vaginal delivery and 2 to 6 weeks after a cesarean section (see Chapters 140 and 141).

Thalassemia

Pregnant women with thalassemia typically have β-thalassemia minor or α-thalassemia minor—conditions with relatively benign clinical phenotypes rather than β-thalassemia major, β-thalassemia intermedia, α-thalassemia major (also called hemoglobin Barts), or hemoglobin H disease (see Chapter 41). Pregnancy is a common setting for the diagnosis of thalassemia minor in previously asymptomatic women who are found to have anemia on routine laboratory evaluation. Dilutional anemia of pregnancy may be exacerbated in women with thalassemia minor, although this effect is variable and more common with β-thalassemia minor than α-thalassemia minor. Thalassemia minor does not adversely affect fetal development, fetal morbidity and mortality, or maternal morbidity and mortality.

Due to delayed pubertal growth or hypogonadism with associated anovulation, women with α- and β-thalassemia major have diminished fertility. Fertility is more variable with hemoglobin H and β -thalassemia intermedia. In the setting of widespread transfusion and iron chelation therapy, pregnancy has become more frequent in these women, and most have successful pregnancies without complication. [9] However, there is an increased risk of maternal complications, especially heart failure. Women with β-thalassemia major and, to a lesser extent, those with β-thalassemia intermedia are at risk of intrauterine growth restriction (IUGR) and low-birthweight babies. Women with β-thalassemia intermedia and hemoglobin H disease often require transfusion during pregnancy.

Iron chelators carry a risk of teratogenicity and must be stopped at least during the first trimester of pregnancy; use in the third trimester—or, even more rarely, in the second trimester—is reserved for women in whom it is unsafe to withhold therapy throughout pregnancy. Therefore, cardiac status and the presence and extent of iron overload must be assessed when counseling women with thalassemia about the risks of pregnancy. Every effort should be made to optimize control of iron overload prior to conception to minimize the risk of decompensation during pregnancy and facilitate tolerance to discontinuation of iron chelation therapy during pregnancy.

Like those with sickle cell disease, women with thalassemia require vigilant follow-up throughout pregnancy. This includes interval monitoring of maternal vital signs and fetal heart-rate, maternal hemoglobin levels, and fetal growth as assessed by ultrasonography beginning between 24 and 28 weeks' gestation. Prenatal genetic testing can be performed if desired, with results used for counseling and guidance of optimal care of the fetus. Women may choose to undergo testing after conception with the intention of terminating the pregnancy if the test is positive; in such cases, chorionic villus sampling may be indicated. Genetic testing is required for antepartum diagnosis of β-thalassemia because silencing of the beta globin gene until after birth results in a normal hemoglobin electrophoresis during pregnancy. Alpha thalassemia major can be diagnosed early by genetic testing, but it will also manifest as severe fetal anemia during the second trimester. When counseling about α- thalassemia major, it is important to note that intrauterine transfusion has rendered fetuses with this disorder viable, albeit still at high risk. Referral should be made to a maternal fetal medicine specialist trained in intrauterine transfusion if women with α-thalassemia major wish to continue their pregnancies.

Iron overload and pregnancy: Women at risk for iron overload due to chronic transfusion, sickle cell disease or thalassemia should have iron status investigated and optimized prior to pursuing pregnancy to reduce the risk of maternal complication, especially heart failure.

OTHER HEMOLYTIC ANEMIAS

Hereditary Spherocytosis

Hereditary spherocytosis is the most common inherited hemolytic anemia among people of northern European descent (see Chapter 46). Since some patients have low levels of hemolysis under normal conditions, the disease may not become clinically apparent until pregnancy. Pregnant women with hereditary spherocytosis are susceptible to pigment gallstones and folate deficiency related to the increased requirements of pregnancy superimposed on accelerated hemolysis. Pregnancy outcomes for women with hereditary spherocytosis are generally good, with better outcomes in those who have undergone splenectomy.[10] Care is primarily supportive with folate (5 mg/day) and transfusion as needed. Splenectomy should not be undertaken during pregnancy. Accelerated hemolysis during pregnancy abates after delivery, and the need for splenectomy can be determined at that time.

Glucose 6-Phosphate Dehydrogenase Deficiency

Glucose 6-phosphate dehydrogenase (G6PD) deficiency leads to hemolytic anemia in the face of oxidative stress (see Chapter 45). The condition may increase the risk of spontaneous abortion, low-birthweight fetuses, and neonatal jaundice if oxidative stressors are not avoided. Women with G6PD deficiency should be instructed to avoid medications with oxidative potential. Complications after the ingestion of oxidative drugs have been reported in carriers of the G6PD deficiency gene if a male fetus has inherited the disease, particularly with medications that cross the placenta.

Paroxysmal Nocturnal Hemoglobinuria

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare clonal disorder caused by somatic mutation in the membrane-anchoring protein PIGA (see Chapter 32). *PIGA* mutation leads to abnormal function of critical membrane proteins that in wild-type individuals are anchored by the gene product of *PIGA*. Features of the clinical phenotype include hemolysis, thrombosis, and bone marrow failure. Hemolysis results from deficiency in the membrane proteins CD55 and CD59, which renders affected individuals susceptible to complement-mediated intravascular hemolysis.

The clinical manifestations of PNH can have a devastating impact on pregnancy if left untreated. Prior to the availability of complement inhibitors, common complications included severe anemia, thrombocytopenia, thrombotic events, preterm delivery, low-birthweight infants, neonatal death, and maternal death, with a maternal mortality rate as high as 20%.[11] As with treatment of PNH in other contexts, eculizumab, a C5 inhibitor, has significantly improved pregnancy outcomes. Accelerated clearance of eculizumab can occur during pregnancy. As such, patients should be monitored for breakthrough hemolysis and eculizumab dosed more frequently if this is noted. A structurally similar C5 inhibitor with a longer half-life, ravulizumab, is likely also appropriate for treatment during pregnancy, but there is less experience with the use of this agent. Pegcetacoplan is a C3 inhibitor with proven efficacy for PNH, but its use in pregnancy should be avoided because animal studies have shown potential for harm, and human data are not yet available.

For women not already on therapeutic anticoagulation, prophylactic dose anticoagulation with low molecular weight heparin should be initiated and continued for 6 to 12 weeks postpartum because of the high risk of thrombosis. Women on therapeutic anticoagulation should have this continued with therapeutic dosing of low molecular weight heparin (further discussed in the section on VTE).

PNH in pregnancy: In addition to control of hemolysis with a C5 inhibitor, all women should receive at least prophylactic dose anticoagulation during pregnancy and the postpartum period.

Autoimmune Hemolytic Anemia

Autoimmune hemolytic anemia (AIHA; see Chapter 47) can occur de novo during pregnancy or be a flare of preexisting disease. Warm AIHA poses a greater risk than cold agglutinins because the relatively small size of immunoglobulin (Ig) G molecules allows them to cross the placenta and adversely affect the fetus. In contrast, the larger IgM antibodies in cold agglutinin disease do not cross the placenta. Patients with AIHA are treated with glucocorticoids and, less commonly, intravenous immunoglobulin (IVIg), both of which have an excellent safety record in pregnancy. Supportive transfusions can be administered when needed. For more severe cases, rituximab can be given during pregnancy and has not been clearly associated with birth defects. However, rituximab can cross the placenta and induce temporary B-cell lymphopenia in the neonate with a subsequent increased risk of infection. As such, rituximab administration during pregnancy is usually reserved for women with hemolysis that cannot be controlled with corticosteroids alone or who require a high dose of corticosteroids. For other women, rituximab is best deferred to the postpartum period. While rituximab is detectable in breastmilk, concentrations are low enough that this is unlikely to pose a risk of immunosuppression and should not be considered a contraindication while breastfeeding.

In rare instances, pregnancy can precipitate AIHA, likely due to the effect of pregnancy on immune regulation; management is the same as it is for flares of known disease or new onset disease.

THROMBOCYTOPENIA

Thrombocytopenia during pregnancy is common, occurring in 5% to 10% of pregnancies. Although there is not one clear value to define thrombocytopenia in pregnancy, generally, platelet counts less than 100,000/µL are a cause for concern. Gestational thrombocytopenia (GT) is the most common cause of thrombocytopenia in pregnancy followed by pre-eclampsia and immune thrombocytopenia (ITP). Much rarer causes include disseminated intravascular coagulation (DIC), HELLP (hemolysis, elevated liver enzymes, low platelet count) syndrome, thrombotic thrombocytopenic purpura (TTP), complement-mediated hemolytic uremic syndrome (CM-HUS), and medication induced. Although inconsequential for most women, the evaluation of thrombocytopenia is essential to rule out any systemic disorders that may affect pregnancy outcome. In addition to determining the etiology of thrombocytopenia, a key consideration is potential use of epidural anesthesia and spinal block or need for cesarean section. Most obstetrical anesthesiologists require a platelet count of approximately 75,000 for epidural anesthesia and, as with all surgery, a platelet count of at least 50,000 is preferred for cesarean section. Given that the need for cesarean section cannot always be predicted, a minimum platelet count of 50,000 should be the goal prior to delivery.

Gestational Thrombocytopenia

GT is quite common, accounting for most cases of thrombocytopenia in pregnancy. The platelet count nadir is usually greater than 100,000/µL, and a count lower than 60,000/µL effectively rules out this etiology. The nadir occurs in the third trimester and most women with GT have no or minimal thrombocytopenia in the first trimester. Patients are asymptomatic and have no history of thrombocytopenia outside of pregnancy. The platelet count normalizes within 6 to 12 weeks of delivery but usually within 1 week. The etiology of GT remains unclear, but may reflect dilution from increased plasma volume, increased platelet sequestration, including in the placenta, or an autoimmune phenomenon (i.e., mild and self-limited ITP triggered by pregnancy).

Monitoring is all that is required for management of GT. Typically, women with GT can receive epidural anesthesia because the platelet count remains sufficiently high to alleviate concerns about bleeding. If the platelet count falls below 75,000/µL alternative diagnoses should be considered, including ITP.

Immune Thrombocytopenia

ITP is responsible for pregnancy-associated thrombocytopenia in approximately 3% of cases (see Chapter 129). It is the most common cause of significant thrombocytopenia during the first two trimesters of pregnancy, a period when GT is usually mild or not yet present. ITP can recur in women with previously documented disease or can develop de novo during pregnancy.

Most pregnant patients with ITP have a long-standing history of thrombocytopenia. ITP may be difficult to distinguish from GT because patients with these conditions can present with similar clinical and laboratory findings, especially in otherwise well women who may not have prior platelet counts for comparison. It is important to distinguish between ITP and GT because ITP may require intervention during pregnancy and there is a small but significant risk of neonatal thrombocytopenia in the setting of ITP. Approximately one-quarter of neonates born to mothers with ITP have thrombocytopenia, although less than 10% have platelet counts less than 30,000/µL, and only 1% to 2% have bleeding.[12]

For diagnostic evaluation, all patients should be tested for human immunodeficiency virus (HIV), as is the case with any diagnosis of ITP. Depending on the clinical context, it may be appropriate to test for hepatitis B and C and to evaluate for systemic lupus erythematosus (SLE). Platelet antibody testing is not helpful due to poor sensitivity and specificity. The peripheral smear may reveal large platelets but should otherwise be normal, and the absence of giant platelets does not rule out ITP. Bone marrow biopsy and aspirate are not indicated unless there are other hematologic abnormalities present that suggest a different diagnosis.

Treatment options are generally similar to those for nonpregnant patients with ITP (Table 149.1). Glucocorticoids are considered first-line treatment, starting with either dexamethasone 40 mg daily for 4 days or prednisone 1 mg/kg daily followed by gradual tapering. Although dexamethasone is usually preferred over prednisone in nonpregnant patients because of a somewhat better response rate and side-effect profile,[13] the greater fetal exposure may favor prednisone, especially if the patient is not near term or at term. Side effects of corticosteroids should be discussed with the patient and include weight gain, bone loss, hypertension, and gestational diabetes. IVIg can also be used, but given its shorter duration of action, it is usually reserved for women who need a rapidly acting intervention shortly before delivery. Rituximab can be used for patients not responsive to steroids or IVIg, although there is a risk of neonatal B-cell leukopenia, as discussed above (see the section on AIHA). There is limited experience

TABLE 149.1 Safety of Medications for Immune Thrombocytopenia in Pregnant Patients

Treatment	Safety in Pregnancy[a]
Intravenous immunoglobulin (IVIg)	Safe
Dexamethasone	Safe, more fetal exposure than prednisone
Prednisone	Safe, less fetal exposure than dexamethasone
Rituximab	Not associated with fetal anomalies, can result in neonatal lymphopenia and associated immunocompromise
TPO mimetics (romiplostim, eltrombopag)	Limited data available support safety, case of neonatal thrombocytosis reported, use limited to refractory immune thrombocytopenia (ITP)

[a] The usual side effect profile of this medications still apply and should be considered in selecting agents.

with TPO mimetics, such as eltrombopag and romiplostim, and these agents should be reserved for patients refractory to IVIg, corticosteroids, and rituximab or those refractory to IVIG and corticosteroids whose delivery is imminent because the response to rituximab can be delayed for several weeks. In women with refractory ITP in pregnancy, a brief course of a TPO mimetic was not associated with thrombosis or adverse neonatal outcomes, although one neonate did have thrombocytosis.[14] Splenectomy is rarely indicated for refractory thrombocytopenia in pregnancy because other options are available. As with ITP in general, platelet transfusion should be avoided unless there is active bleeding because the response is transient.

As noted above, thresholds for intervention should be approached with nuance in pregnancy. In the absence of bleeding or other invasive interventions, a platelet count as low as 30,000/μL can be tolerated for most of the pregnancy. However, when nearing delivery, it is prudent to intervene to achieve a platelet count great than 50,000/μL for delivery or 75,000/μL if epidural anesthesia is desired by the patient. Most women will be able to achieve these platelet counts with corticosteroid administration in the late third trimester.

As discussed previously, maternal antiplatelet IgG can cross the placenta, and cause thrombocytopenia in fetuses. Percutaneous umbilical blood sampling is the most accurate means to obtain the fetal platelet count, but it is not recommended because the procedure is associated with a high complication rate and 1% fetal mortality. The maternal platelet count and antiplatelet antibody level do not accurately predict the fetal platelet count. The obstetrical and pediatrics team should be aware of the risk of neonatal thrombocytopenia, and cord blood platelet count should be checked. A repeat platelet count between days 2 and 5 of life is also recommended because the nadir platelet count usually occurs during this time.

Pre-eclampsia With Severe Features and HELLP Syndrome

Thrombocytopenia in pregnant women also occurs in association with pre-eclampsia and HELLP syndrome, potentially serious multisystem disorders associated with pregnancy. A platelet count below 100,000/μL is one criterion used to define severe pre-eclampsia and is associated with increased rates of maternal complications, including eclampsia, renal injury, pulmonary edema, myocardial infarction, placental abruption, and mortality. Preeclampsia with severe features also carries an increased risk of poor fetal outcomes including IUGR and stillbirth. Preeclampsia with severe features, regardless of whether the criterion for severe features is thrombocytopenia, may progress to HELLP syndrome, although HELLP syndrome may occur without preceding pre-eclampsia. Risk factors for preeclampsia include but are not limited to pre-eclampsia in a previous pregnancy, a family history of preeclampsia, chronic hypertension, obesity, multiple gestation, and autoimmune disease.

HELLP syndrome represents a severe variant of preeclampsia. Complement activation is present in some cases but its role in the pathogenesis of the disorder remains unclear. As with preeclampsia, most women present in the third trimester, but it can occur in the second trimester or within 48 hours of delivery. Diagnosis is made on the basis of laboratory studies and a blood smear because symptoms such as abdominal pain, nausea, and fatigue are non-specific, especially in pregnancy. Patients can develop severe right upper quadrant pain if intraparenchymal or subcapsular hepatic hemorrhage occurs, but this is a late finding in the evolution of HELLP syndrome.

As the acronym implies, laboratory studies will show thrombocytopenia, hemolytic anemia, and transaminitis (Table 149.2).[15] The blood smear usually reveals a schistocyte (3 or more per high power field) confirming that hemolysis is due to a microangiopathic process. If initial testing is ambiguous, particularly with respect to microangiopathic hemolytic anemia, tests should be repeated to avoid missing this serious diagnosis. If HELLP syndrome is suspected or identified, comprehensive evaluation for other impacts of severe pre-eclampsia should be made, including renal dysfunction and proteinuria, visual disturbances, weight gain or edema, and headache.

TABLE 149.2 **Diagnostic Criteria for HELLP Syndrome**

Hemolysis (at least 2 of 4)	• Schistocytes on peripheral blood smear • Serum bilirubin ≥1.2 mg/dL • Haptoglobin ≤25 mg/dL OR lactate dehydrogenase (LDH) ≥2× upper limit of normal • Anemia not due to bleeding
Transaminitis (required)	• Aspartate aminotransferase (AST) or alanine aminotransferase (ALT) ≥2× upper limit of normal
Thrombocytopenia (required)	• Platelets <100,000/μL

HELLP, Hemolysis, elevated liver enzymes, low platelet.

Pre-eclampsia has complex pathophysiology with both fetal and maternal contributions. Abnormal angiogenesis and remodeling of maternal spiral arteries, endothelial dysfunction, placental hypoperfusion and ischemia, and defective trophoblast differentiation are all thought to play a role. Endothelial dysfunction is the central mechanism that links pre-eclampsia with HELLP syndrome. Injury to the vascular endothelium results in fibrin deposition, platelet activation, and the release of vasoactive agents such as serotonin and thromboxane A_2. The thrombocytopenia in pre-eclampsia likely reflects this cascade of changes. Fibrin deposition in the hepatic sinusoids may be responsible for hepatocellular injury. There often is evidence of abnormal complement activation, but it is unclear whether this is a consequence or driver of endothelial damage. Coagulopathy may develop as a consequence of HELLP syndrome and liver injury, further complicating the risks of delivery. As such, laboratory evaluation should also include determination of the partial thromboplastin time, prothrombin time, and fibrinogen level.

HELLP syndrome may be further complicated by acute renal failure, pulmonary edema, shock, stroke, eclampsia, retinal detachment, diabetes insipidus, placental abruption, and death. With greater recognition of this condition, maternal mortality is rare, estimated at 1.1% in a large review.[16] Unfortunately, fetal demise and neonatal mortality remain as high as 20%.[17] IUGR and neonatal cytopenias can also occur.

HELLP syndrome may be difficult to distinguish from acute fatty liver of pregnancy (AFLP), which usually occurs in the third trimester. Although the origin of AFLP is not clear, microvesicular fatty infiltration of the central zone of the liver is observed. In some cases, fatty infiltration can be detected by ultrasonography or computed tomography (CT). Patients present with a variety of symptoms, including malaise, fatigue, right upper quadrant pain, dyspnea, and mental status changes. Laboratory findings include abnormal liver function tests consistent with cholestatic disease, elevated ammonia levels, low levels of fibrinogen and antithrombin, and a prolonged prothrombin time. These abnormalities may be the result of compromised liver synthetic function alone or hepatic injury leading to DIC (see Chapter 137). Patients with AFLP usually have little or no evidence of a microangiopathic, hemolytic anemia but have more severe liver injury and synthetic dysfunction than those with HELLP syndrome. Women may have overlapping features of AFLP and HELLP syndrome, and both conditions occur more commonly in women with preceding pre-eclampsia. AFLP and HELLP syndrome are both managed with delivery and supportive care. Therefore, distinguishing between them is not essential.

Balancing maternal and fetal risks remains the most difficult consideration in the management of HELLP syndrome, particularly in women who present early in pregnancy. The only well-established treatment is prompt delivery, which carries the attendant risks of prematurity. However, HELLP syndrome itself is associated with poor fetal and neonatal outcomes from complications such as placental abruption. Women with HELLP syndrome should be admitted to the hospital for maternal and fetal monitoring and assessed for stability. Collaboration between hematology and obstetrics is essential. If clinical features are not severe, delivery can be delayed for 48 hours and corticosteroids administered to promote fetal lung maturation. As with other preeclampsia syndromes, magnesium sulfate is administered for seizure prevention, and blood pressure must be controlled. Women at 34 to 35 weeks' gestation or more should be delivered without delay. Vaginal delivery can be attempted if the patient is stable, but monitoring of the platelet count and coagulation status is important. Epidural anesthesia can be considered if the platelet count is high enough. Volume expansion, vasodilators, and eculizumab can be used to attempt to delay delivery when HELLP syndrome is diagnosed early in pregnancy, but the efficacy and safety of these agents is uncertain.[18,19]

Women with HELLP syndrome and hypertensive syndromes of pregnancy should be counseled about the risks with future pregnancies. The risk of recurrence with future pregnancies is about 7% with HELLP syndrome and up to 36% with some hypertensive syndromes of pregnancy.[20] There is increasing evidence that aspirin (81 mg/day) may prevent preeclampsia. As such, all women with a history of HELLP syndrome or preeclampsia associated with thrombocytopenia should receive aspirin prophylaxis in future pregnancies.

Thrombotic Thrombocytopenic Purpura and Complement-Mediated Hemolytic Uremic Syndrome

TTP and CM-HUS (also called atypical HUS) are important considerations when evaluating pregnant women with thrombocytopenia (see Chapter 132). Although rare, the incidence of both disorders is increased in pregnancy. Like HELLP syndrome, TTP and CM-HUS are thrombotic microangiopathies that are associated with significant morbidity and mortality if appropriate therapy is not provided. Microvascular injury and platelet agglutination with resulting thrombocytopenia and microangiopathic hemolytic anemia are hallmarks of TTP and CM-HUS (Fig. 149.1). For TTP, pregnancy remains one of the most common triggers for flares with both congenital deficiency and autoimmune etiologies of ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) deficiency. As discussed elsewhere (see Chapter 132), lack of ADAMTS13, which is a protease that cleaves von Willebrand factor (vWF), leads to an excess of ultra large vWF multimers that cause inappropriate platelet activation and aggregation leading to microthrombus formation. In contrast, the levels of ADAMTS13 are normal in CM-HUS. Instead, abnormal complement activation is the driver of CM-HUS. Many patients with CM-HUS triggered by pregnancy or infection have an underlying mutation in a complement protein, but identification of a mutation is not required for the diagnosis. The potential contribution of complement activation to the pathogenesis of HELLP syndrome may explain why some women with this syndrome fail to improve postpartum, and develop a laboratory picture more consistent with CM-HUS. In such cases, endothelial damage and complement activation due to HELLP syndrome may be the trigger for CM-HUS. Alternatively, there may be an overlap in etiology from the start.

Both timing and laboratory parameters can be useful in distinguishing between TTP, CM-HUS, and HELLP syndrome. Renal injury in TTP is less severe than that in CM-HUS, and profound thrombocytopenia and neurologic symptoms are less common with CM-HUS than TTP. In contrast to HELLP syndrome, serious liver injury is unusual with CM-HUS or TTP. Although TTP can occur at any point in pregnancy, it is most common in the second and third trimesters. Complement levels increase gradually after 20 weeks' gestation. Therefore, CM-HUS most commonly occurs near term or after delivery, and it rarely occurs before the latter part of the second trimester. HELLP syndrome is most common in the third trimester and typically resolves with delivery. Persistence of HELLP syndrome beyond 48 hours after delivery should prompt consideration of CM-HUS.

TTP is treated with plasmapheresis and corticosteroids. Rituximab is generally recommended for autoimmune TTP but can be deferred to the postpartum period if remission is achieved with plasmapheresis and corticosteroids. Women with congenital deficiency of ADAMTS13 as the driver of TTP can be treated with plasma infusion alone.

While plasmapheresis has been used for CM-HUS, eculizumab is now well established as a superior treatment for CM-HUS and there is experience with its use in pregnancy (see the section on PNH). Ravulizumab, a C5 inhibitor with a longer half life, is likely to be at least as effective as eculizumab, but there is less information about its safety in pregnancy.

Delivery is not indicated for the management of maternal complications of TTP or CM-HUS, and treatment usually enables safe continuation of pregnancy. If there is fetal distress, however, delivery may be needed while maternal treatment is ongoing.

The risk of recurrent TTP or CM-HUS in future pregnancies is variable. For acquired TTP, hemolysis parameters, blood counts, and ADAMTS13 levels should be monitored during pregnancy. For congenital TTP, flares during pregnancy are so common that prophylactic plasma infusion (10 to 15 mL/kg every 2 weeks or more frequently if needed) is advisable. For CM-HUS, recurrence is unlikely during the first half of gestation because of the timing of pregnancy-related complement level changes. As such, monitoring of hemolysis parameters and blood counts can be deferred until after 20 weeks' gestation. Treatment for recurrent CM-HUS and acquired TTP can be safely withheld with close monitoring and patient participation in symptom monitoring.

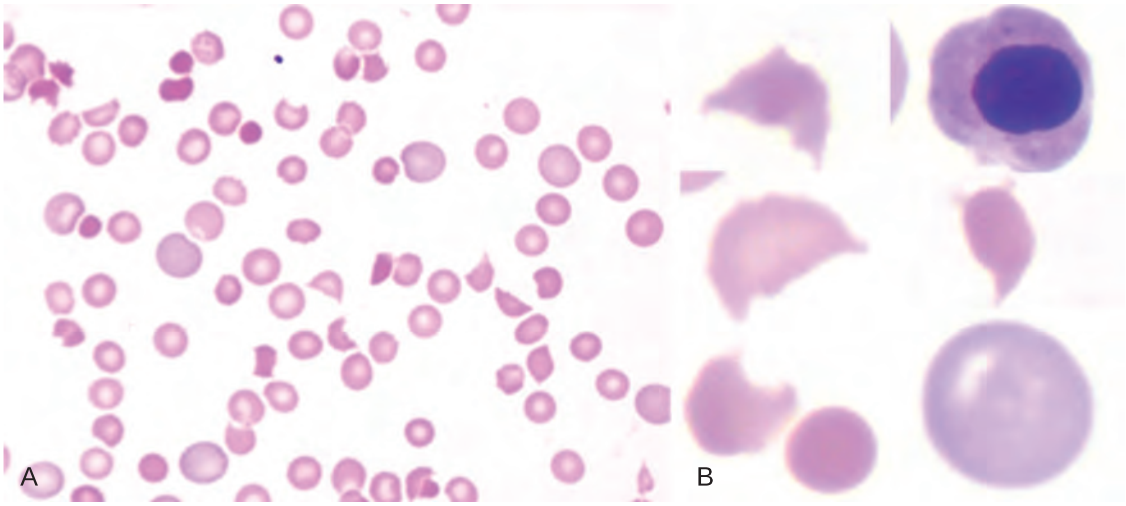


Figure 149.1 MICROANGIOPATHIC HEMOLYTIC ANEMIA . Microangiopathic hemolytic anemia in pregnancy, peripheral blood smear (A and B). Evidence of microangiopathy with the formation of schistocytes, fragmented forms and spherocytes, associated with polychromasia and nucleated red blood cells (A, B, detail).

The prominence of liver injury can be helpful in distinguishing HELLP syndrome from other thrombotic microangiopathies that can flare in pregnancy such as TTP or CM-HUS.

LEUKEMIA AND LYMPHOMA

The diagnosis of hematologic malignancy during pregnancy can be traumatic for the patient and her family and is a treatment challenge. Although significant advances in treatment have been made, there is limited information about treatment in pregnancy where the risk to the fetus must be considered. The risks of chemotherapy for the fetus depend on gestational age.

Diagnosis of hematologic malignancies in pregnancy can be difficult because many of the nonspecific symptoms and signs that accompany these disorders, such as fatigue, loss of appetite, weight loss, and anemia, are not uncommon in pregnancy. Diagnostic procedures such as bone marrow aspiration or lymph node biopsy can usually be performed safely during pregnancy. Because of the risk of radiation exposure to the fetus, ultrasonography and magnetic resonance imaging (MRI) are often the mainstay for imaging, even though CT scanning is associated with limited radiation exposure.

The risk of teratogenicity from chemotherapy is highest if treatment is administered during fetal organogenesis, which mainly occurs during weeks 5 to 10 of gestation. If given before 5 weeks of gestation, chemotherapy is unlikely to affect organogenesis but may be associated with spontaneous abortion. Chemotherapy administration in the second or third trimester has been associated with IUGR, low birthweight, prematurity, and stillbirth. Observational studies have not shown long-term effects of such treatment on cognitive function except for those attributable to prematurity or an increased rate of malignancy.[21,22] Chemotherapy at or near term is linked to neonatal cytopenias from myelosuppression. Chemotherapy dosing is usually based on prepregnancy weight. Radiation may be feasible for fields remote from the uterus such as the thorax or extremities.

Hodgkin Lymphoma

The relatively high incidence of hematologic malignancies during pregnancy compared with other cancers reflects the fact that Hodgkin lymphoma is common in women of childbearing age. The diagnosis of Hodgkin lymphoma in pregnancy rarely impacts pregnancy outcome. Rates of remission and 20-year overall survival in women diagnosed with Hodgkin disease during pregnancy are similar to those in nonpregnant women. If there is no urgent need for intervention, treatment can be deferred until after delivery. If urgent treatment is needed, ABVD (doxorubicin, bleomycin, vinblastine, and dacarbazine) is often used because this regimen does not appear to be associated with teratogenicity when given in the second or third trimester. Safety data with newer agents such as brentuximab vedotin are lacking.

Non-Hodgkin Lymphoma

Non-Hodgkin lymphoma (NHL) rarely occurs during pregnancy. There are case reports and case series of pregnant women with NHL who have been treated successfully with chemotherapy during the second and third trimesters of pregnancy. Treatment can be deferred until after delivery in women with indolent NHL. For women with aggressive NHL, treatment considerations depend on the risks of cytotoxic chemotherapy to the fetus.

Acute Leukemias

Treatment of acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) can rarely be delayed. Thrombocytopenia and the potential for DIC, especially with acute promyelocytic leukemia (APL), need to be considered. Women at or near term should undergo delivery prior to initiation of therapy, but treatment should be started promptly thereafter. Women in the first trimester should be counseled about the high risk of a poor outcome, and discussion about therapeutic abortion is warranted. For pregnancies in the second trimester or early in the third trimester, standard induction therapy should be given without delay.

For treatment of AML in pregnancy, daunorubicin is preferred over idarubicin, which has increased placental transfer. Amphotericin B is considered the antifungal of choice in pregnancy because it is not associated with teratogenicity. In general, pregnant women with AML have outcomes similar to those of nonpregnant women.

For treatment of APL in pregnancy, all-trans retinoic acid (ATRA) is not recommended in the first trimester, and arsenic trioxide is not recommended at any stage of pregnancy. The impact on outcome of withholding these agents should be disclosed when women and their families are considering termination of pregnancy.

There is limited safety information on the use of newer treatments for AML in pregnancy. Likewise, given the rarity of AML in pregnancy, the safety of agents such as peg-asparaginase in women of childbearing age is uncertain.

Chronic Myeloid Leukemia

The use of tyrosine kinase inhibitors (TKIs) in the first trimester of pregnancy is associated with a risk of spontaneous abortion and fetal malformations. Outcomes with the use of TKIs during the second and third trimester are less clear but appear to be more favorable. Nonetheless, it is advisable to discontinue TKIs prior to attempting conception.

Two approaches can be considered for women with CML planning for pregnancy. First, temporary or long-term cessation of TKIs should be considered if TKI therapy has been used for at least 3 years and resulted in a deep molecular response for at least 2 years. TKI treatment should be stopped prior to attempting to become pregnant, and women should be monitored for relapse. Second, women who do not qualify for discontinuation of TKI or who have relapsed with discontinuation should be counseled about the risks of withholding TKIs throughout pregnancy. If they wish to proceed, interferon alfa can be used to control blood counts during pregnancy. Interferon alfa is also the preferred agent for treatment of CML in pregnancy; patients can be switched to a TKI in the second or third trimester if an experienced hematologist considers this necessary. Observation alone may be sufficient for pregnant women with minimal blood count abnormalities or if CML is diagnosed late in pregnancy. Aspirin thromboprophylaxis can be considered for women with significantly elevated platelet counts.

TKIs pass into breast milk and are associated with growth retardation. Therefore, if TKIs are given after delivery, breastfeeding is discouraged.

Myeloproliferative Neoplasms: Essential Thrombocythemia, Polycythemia Vera, and Primary Myelofibrosis

Although myeloproliferative neoplasms (MPNs) are associated with spontaneous abortion, placental abruption, IUGR, low birthweight, and preeclampsia, the risk of these complications is low, as is the risk to the mother.

Essential thrombocythemia (ET) has a bimodal peak of distribution and is the most common MPN in women of childbearing age (see Chapter 71). Most women of childbearing age with ET do not require treatment apart from aspirin, which can be safely continued in pregnancy. Although aspirin may be considered optional in low-risk patients with ET, the predisposition to pre-eclampsia and increased risk of pregnancy loss and thrombosis render aspirin advisable in pregnancy. There is no role for prophylactic anticoagulation, but women should be informed about the symptoms and signs of VTE, and seek medical attention if these develop.[23]

The platelet count often decreases during pregnancy, even in women with ET. Women who require anticoagulation therapy during pregnancy should be managed with heparin and those who develop thrombosis during pregnancy may benefit from cytoreductive therapy with interferon alfa. Interferon improves maternal and fetal outcomes. [23] If interferon fails to lower the platelet count, hydroxyurea can be considered for women in the second or third trimester of pregnancy. When administered in the second or third trimester to women with sickle cell disease, hydroxyurea is associated with little risk, although it may predispose to lower birthweight and prematurity. There is limited experience with anagrelide in pregnancy, so it should be avoided.

Women who develop acquired von Willebrand syndrome (AVWS) in pregnancy secondary to ET should be considered for cytoreductive therapy to reduce the risk of postpartum hemorrhage (PPH). Unless there is bleeding, cytoreductive therapy can usually be deferred until the third trimester. However, treatment must be started early enough in the third trimester to reduce the risk of bleeding during labor. The platelet count target is less stringent with AVWS than it is with high-risk ET alone.

Primary myelofibrosis (PMF) is uncommon in women of childbearing age and most women with advanced disease have reduced fertility. If PMF is diagnosed during pregnancy, transfusion support is the primary treatment. Aspirin prophylaxis should be considered if the platelet count is above 50,000/μL. Medications such as ruxolitinib should be avoided because they may be teratogenic.

Of the MPNs, polycythemia vera requires the most active management. Aspirin should be continued in pregnancy. Phlebotomy may be needed to maintain the hematocrit below 45%, although it may be reasonable to aim for a lower target of 40% to 42%. Phlebotomy requirements may decrease during pregnancy due to dilutional anemia of pregnancy. If phlebotomy alone is insufficient for hematocrit control or women are at high risk because of a prior history of thrombosis, interferon alfa can be used. Interferon has a positive impact on maternal outcomes and reduces the risk of pregnancy complications associated with MPNs.[23] Women with a history of thrombosis should be managed with heparins, but prophylactic anticoagulation is rarely warranted. Some experts recommend anticoagulation therapy for 6 weeks postpartum, but data supporting this recommendation are lacking and postpartum hematocrit control is likely to be excellent because of peripartum blood loss.

CLINICAL VIGNETTE: ESSENTIAL THROMBOCYTHEMIA IN PREGNANCY

A 35-year-old G2P1 is noted to have a platelet count of 720,000/ μ L at her first prenatal visit and is referred to hematology. Iron studies are normal and there is no evidence of an inflammatory condition. Retrospective review of complete blood counts indicates that her platelet count was mildly elevated over the last several years with a highest count of 590,000/ μL 1 year ago. Although her platelet count ranged from 450,000/μL to 500,000/μL early in her pregnancy 2 years ago, her count was in the upper range of normal range in the third trimester. The pregnancy was uncomplicated, and she underwent spontaneous vaginal delivery at 39 weeks' gestation. She has no chronic medical conditions or prior thrombotic events. Her only medications are prenatal vitamins.

Gene sequencing reveals a calreticulin mutation and confirms the diagnosis of ET. She is at 13 weeks' gestation at the time of her first hematology clinic visit. She denies bleeding or easy bruising. She is started on aspirin (81 mg daily). Her platelet count decreases to 675,000/μL in the third trimester, and she has a normal spontaneous vaginal delivery at 40 weeks' gestation. The risks and benefits of continued aspirin in the context of low risk ET are discussed, and she opts to stop aspirin 6 weeks after delivery.

BLEEDING DISORDERS

All women with acquired or congenital bleeding disorders should be evaluated by a hematologist prior to delivery. Bleeding disorders increase the risk of PPH and are important to consider prior to neuraxial anesthesia or cesarean section. Some bleeding disorders can be associated with a risk of in the fetus or neonate.

Disseminated Intravascular Coagulation

DIC in pregnant women can occur in the settings of HELLP syndrome, placental abruption, amniotic fluid embolism, uterine rupture, intrauterine fetal demise, sepsis, and AFLP. This condition leads to a consumptive coagulopathy characterized by a low platelet count and reduced levels of fibrinogen (see Chapter 137). The platelet count should be maintained above 50,000/μL in women undergoing cesarean section with transfusion. Regardless of mode of delivery, cryoprecipitate should be given to maintain the fibrinogen level above 100 mg/dL or above 150 mg/dL if neuraxial anesthesia is planned. Plasma may also be needed, but it should be reserved for significant coagulation test prolongation.

When evaluating the etiology of DIC, amniotic fluid embolism, although rare, needs to be considered because it is associated with a mortality rate as high as 80%. Up to a fifth of women with an amniotic fluid embolism develop a coagulopathy, so amniotic fluid embolism should be considered in any pregnant or postpartum woman with DIC.

von Willebrand Disease

von Willebrand disease (VWD) is the most common inherited bleeding disorder (see Chapter 133). Management of VWD in pregnancy depends on the type. VWD does not impair fertility or increase the likelihood of miscarriage but is associated with an increased rate of PPH even when well managed.[24] Up to 75% of women with moderate to severe VWD experience significant peripartum bleeding.[25] Rates are lowest for type I VWD as a result of the physiologic increase in VWF and factor VIII levels starting in the second trimester and peaking near term. VWF levels peak around 35 weeks' gestation, whereas factor VIII levels peak early in the third trimester.[26] The level of VWF in the third trimester at 34 to 36 weeks' gestation informs treatment in patients with type I VWD. No treatment is needed for VWF levels over 80 IU/dL or perhaps even over 50 IU/dL. In patients with type 2 VWD, activity levels (VWF:GPIbM or VWF:RCo) less than 80 IU/dL should prompt intervention for delivery. With type 3 VWD, hemostatic levels are unlikely to be achieved and plans for delivery should not await third trimester values.

Desmopressin can be associated with blood pressure spikes, hyponatremia, thrombosis, and an oxytocin-like effect that may stimulate uterine contraction. Because desmopressin challenge is not recommended during pregnancy, responsiveness to desmopressin should be assessed prior to conception in women with type 1 or type 2 VWD. Desmopressin has been used safely to manage minor procedures during pregnancy in women with known responsiveness. To minimize risks, exposure should be limited.

If not administered earlier to facilitate epidural anesthesia, desmopressin should be given just after umbilical cord clamping to limit fetal exposure. When given, fluid administration should be used with caution because of the risk of hyponatremia. A second dose of desmopressin can be considered 8 to 24 hours later to coincide with removal of the epidural catheter. Women given desmopressin alone remain at risk for delayed PPH; to reduce this risk, concomitant tranexamic acid (1 g every 6 hours for up to 7 days) is often given.

Patients with type 3 VWD and those with type 1 or 2 VWD who have unknown responsiveness to desmopressin or are unresponsive to desmopressin require factor replacement. The goal of factor replacement is to achieve VWF levels of at least 100 IU/dL and ideally over 150 IU/dL. Factor replacement for moderate to severe VWD should continue for 5 to 7 days postpartum to minimize the risk of delayed PPH. Tranexamic acid should be used as an adjunct in women with ongoing heavy bleeding.

Patients without a documented bleeding disorder who have bleeding complications during pregnancy should be assessed for VWD. This assessment begins with a thorough bleeding history with particular attention to menses, and proceeds to VWD testing. If tests are normal, they should be repeated 12 weeks or more postpartum to exclude the influence of pregnancy on factor levels (see Chapter 133).

HEMOPHILIA A AND B

Hemophilia A and B are X-linked recessive diseases causing factor VIII and factor IX deficiency, respectively (see Chapter 134). As such, women are primarily carriers but can be rarely affected with a mild phenotype from asymmetric X-chromosome inactivation (lyonization) or compound heterozygosity as an offspring of an affected father and carrier mother. Female children of affected fathers are obligate carriers.

Women who are carriers should have factor levels measured and receive factor infusion peripartum if levels are below 50%. Like VWD, even when factor levels normalize during pregnancy, there still is a risk of PPH. Normalization of factor levels is more common in hemophilia A than hemophilia B carriers because of the substantial increase in FVIII during pregnancy; changes in FIX levels during pregnancy are more modest. If factor infusion is needed, a level of 100 to 150 IU/dL should be targeted and replacement continued for at least 3 days after vaginal delivery and for 5 to 7 days after cesarean delivery. Concomitant tranexamic acid may be of benefit and can be given for up to a week after delivery. However, tranexamic acid alone is insufficient treatment for women with reduced factor levels.

For women carrying a male fetus, there is a 50% chance of the offspring being affected. Women who would consider termination of an affected fetus may wish to pursue chorionic villus sampling. Due to the risk of hemorrhage, vaginal delivery may be pursued, but vacuum- and forceps-assisted delivery should be avoided, as should scalp electrodes, fetal blood sampling and intramuscular injections. Umbilical cord blood sampling may be used to determine if the child is affected. Up to 10% of affected neonates have bleeding complications in the perinatal period, including intracranial hemorrhage. Ideally, a pediatric hematologist with experience treating hemophilia should be involved in the management prior to delivery and postpartum.

Acquired Hemophilia A

Acquired hemophilia A is a rare autoimmune disease secondary to antibodies or inhibitors directed against FVIII (see Chapter 134). Achievement of hemostasis can be problematic in patients with this condition and eradication of the inhibitor prior to delivery, or more ideally conception, is recommended. Corticosteroids and rituximab are the treatments of choice during pregnancy. If there is serious bleeding, recombinant FVIIa (rFVIIa) or activated prothrombin complex concentrate (aPCC) may be required. Although emicizumab is highly effective in nonpregnant patients,[27] it should probably be avoided in pregnancy until more data are available because it crosses the placenta and has a long half-life (see Chapter 136).

Most inhibitors in hemophilia A are IgG antibodies. Therefore, the r antibodies can cross the placenta, and cause bleeding in the fetus or neonate. As with neonates at risk of congenital hemophilia, instrumentation during delivery should be avoided, umbilical cord blood evaluated, and a pediatric hematologist involved in the care of the neonate.

Factor XI Deficiency

FXI deficiency is heterogeneous in its inheritance and phenotype (see Chapter 135). Inheritance may be autosomal dominant or recessive and is more common in individuals of Ashkenazi Jewish heritage. This condition is increasingly included in genetic screening offered to women during preconception counseling or early in pregnancy. Women with FXI deficiency may or may not have a prior history of bleeding.

The FXI level should be quantified at 32 to 34 weeks' gestation to facilitate planning. Levels measured in the first or second trimester may be less useful because some studies suggest that levels decrease later in pregnancy.[28,29]

TABLE 149.3 Management of Factor XI Deficiency

Factor XI Level	>40%	20%–40%	<20%
Management	No intervention Consider TXA if significant bleeding history	Neuraxial anesthesia: FXI replacement No neuraxial anesthesia: TXA alone	Bleeding history present or neuraxial anesthesia: FXI replacement No bleeding history and no neuraxial anesthesia: TXA alone

FXI, Factor XI, *TXA*, tranexamic acid 1 g intravenously every 6 hours for 24 to 48 hours.
Lee CA, Chi C, Pavord SR, et al. The obstetric and gynaecological management of women with inherited bleeding disorders—review with guidelines produced by a taskforce of UK Haemophilia Centre Doctors' Organization. *Haemoph Off J World Fed Hemoph*. 2006;12(4):301–336.

The FXI level, the presence or absence of a history of bleeding and the intended mode of anesthesia determine the need for intervention. The bleeding history is central because there is poor correlation between FXI levels and the propensity for bleeding. No treatment is needed for women with a normal activated partial thromboplastin time (aPTT), an FXI level above 40%, and no bleeding history, even if neuraxial anesthesia is planned. An approach for women with lower FXI levels or a bleeding history is presented in Table 149.3.[30] Tranexamic acid is useful for patients not requiring FXI replacement; it is administered intravenously at a dose of 1 g every 6 hours for 24 to 48 hours. Women who require FXI replacement should be given plasma (15 to 20 mL/kg) prior to neuraxial anesthesia or active labor, and more should be given 24 hours later if labor is ongoing, or an epidural catheter remains in place (see Chapter 135).

Because of the mild bleeding diathesis associated with FXI deficiency, antepartum diagnosis of affected fetuses and elective termination are rare considerations. Umbilical cord blood sampling is usually limited to neonates of mothers with more severe deficiency (e.g., FXI levels below 20%, although there is no absolute cutoff) and may be a consideration prior to circumcision or other procedures. There is no need to avoid intramuscular injections (e.g., hepatitis B vaccination), even if the mother has a history of bleeding.

CLINICAL VIGNETTE: ASYMPTOMATIC FACTOR XI DEFICIENCY

A 28-year-old woman with no significant past medical history is referred to hematology because Factor XI deficiency was detected on genetic carrier screening. Her partner is not a carrier. She has no history of bleeding, including no heavy menstrual bleeding or excessive bleeding after wisdom tooth extraction or anterior cruciate ligament repair. Her factor XI level is 22%. Her aPTT is at the upper limit of normal. She would like epidural anesthesia during delivery. She is managed with a 48-h course of tranexamic acid with the first dose administered when she is in active labor. She delivers a healthy infant and does not have excessive bleeding.

Other Platelet Function Disorders

Disorders of platelet function other than VWD are rare and heterogenous (see Chapter 128). Disorders may be severe, such as Glanzmann thrombasthenia, or mild, such as granule deficiencies. The rise in VWF during pregnancy may partially compensate for milder platelet defects. Management for delivery should be in accordance with the standard practice for the specific platelet disorder in the setting of bleeding. The risks of neuraxial anesthesia are higher and necessitate more aggressive management. Unassisted delivery is preferred if vaginal delivery is pursued in disorders where there is a risk of an affected fetus.

VENOUS THROMBOEMBOLISM

VTE is a leading cause of maternal morbidity and mortality (see Chapter 141). The risk of VTE, which includes deep vein thrombosis (DVT) and pulmonary embolism (PE), increases two- to fourfold during pregnancy and the early postpartum period. In women with a previous VTE, pregnancy increases the risk of a recurrent event. Factors accounting for the prothrombotic state associated with pregnancy include altered levels of procoagulants and anticoagulants; increased estrogen levels, which lead to venous distention and subsequent venous stasis; increased plasma volume; and compression of the left iliac vein by the gravid uterus, which leads to decreased lower extremity blood flow velocity, particularly in the left leg (Table 149.4).[28,29] Along with these increases in procoagulant proteins, protein S, one of the most important anticoagulant proteins, decreases. Finally, fibrinolytic activity decreases during pregnancy as the levels of plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2) increase.

D-dimer testing has no role for risk stratification in pregnant women because no patient with symptoms of VTE should be considered at low risk during pregnancy, and the test has poor performance characteristics in this setting with estimated sensitivity and specificity of 73% and 15%, respectively.[31] Lower extremity compression ultrasound can be conducted in the normal manner in pregnant women. Lower extremity edema may decrease the sensitivity of lower extremity ultrasound for the diagnosis of distal DVT. In cases where the pretest probability is high, and the ultrasound is of poor quality, repeat assessment may be needed. A limitation of ultrasound is the inability to visualize the iliac veins. In such cases, MRI, particularly with direct thrombus imaging, may be useful. If DVT is diagnosed, evaluation for PE is often unnecessary because the treatment is the same.

In pregnant women with symptoms suggestive of PE, such as shortness of breath or pleuritic chest pain, imaging is needed. Options include ventilation/perfusion (V/Q) lung scan and CT pulmonary angiography (CTPA). The calculated radiation risks to the fetus with a V/Q scan and CTPA are 0.5 and 0.1 mGy, respectively, values well below the 50 mGy threshold associated with an increased risk of fetal health problems. However, the calculated minimum radiation dose to each breast for an average 60 kg woman is significantly higher with CTPA than with V/Q scanning, although the dose with CTPA can be reduced by approximately 20% with the use of bismuth breast shields. Therefore, V/Q scanning is often preferred over CTPA in some centers provided the chest X-ray is normal. In pregnant women with documented PE, the severity can be assessed by measuring biomarkers such as troponin and brain natriuretic peptide (BNP) levels. If these are elevated, a transthoracic echocardiogram is indicated for evaluation of right heart strain and admission to a hospital is warranted.

TABLE 149.4 **Hemostatic Changes in Pregnancy**

Prothrombin	↑
Protein C, antithrombin	=
Protein S	↓
Factor XI	↓/=
Factors V, VII, VIII, IX, X, XII	↑
Factor XIII	↑/↓
von Willebrand factor	↑
PAI-1, PAI-2	↑
Fibrinogen	↑
D-dimer	↑

VTE in pregnancy is treated with low-molecular-weight heparin (LMWH) at standard therapeutic doses (e.g., enoxaparin 1 mg/kg twice daily, dalteparin 100 U/kg twice daily, or 200 U/kg once daily). LMWH does not cross the placenta for hemodynamically unstable patients or those with an imminent procedure (e.g., possible cesarean section), unfractionated heparin (UFH) is preferable. The greater difficulty of administration, the requirement for IV access, and the higher rate of heparin-induced thrombocytopenia (HIT) motivate the preference for LMWH.[32] Care must be taken to adjust heparin dosing during pregnancy with accompanying expected weight gain, as well as reducing dosing postpartum, as appropriate. Patients with borderline renal function, extremes of body weight, or evidence of breakthrough thrombosis should have anti-Xa level measurements to ensure appropriate dosing.

VTE in pregnancy should be treated for at least 3 months or at least 6 weeks postpartum, whichever is longer. A course of 6 months of anticoagulation may be considered for women with more extensive or hemodynamically significant VTE. Breastfeeding is safe with LMWH.

Warfarin is contraindicated in pregnancy because it crosses the placenta and is a known teratogen. Women considering pregnancy who are on warfarin should transition to LMWH prior to conception because of this risk. While the risk of teratogenicity rapidly diminishes as organogenesis is complete, warfarin also poses a risk of fetal hemorrhage because it can inhibit fetal vitamin K-dependent clotting factor synthesis. As such, even though teratogenicity is no longer the primary consideration, warfarin should be avoided whenever possible in the second and third trimesters. Rare clinical scenarios, such as women with mechanical heart valves (discussed further in a subsequent section) or impaired renal function, are the primary setting in which warfarin during late pregnancy might be considered. For severe renal insufficiency, subcutaneous UFH can be used at a dose of 250 U/kg every 12 hours. Patients can be transitioned to warfarin in the postpartum period, which is safe while breastfeeding. Women whose course of anticoagulation therapy will stop 6 weeks after delivery may prefer to remain on heparin to avoid the monitoring and dose adjustments required with warfarin.

Although small amounts of fondaparinux may cross the placenta, it has not been associated with teratogenicity or fetal hemorrhage.[33] Fondaparinux is the preferred agent for use in pregnant women with a history of HIT or heparin allergy. Oral direct anticoagulants (dabigatran, apixaban, rivaroxaban, and edoxaban) are not recommended in pregnancy or during breast feeding because they cross the placenta and pass into the breast milk.

Anticoagulation should be discontinued at the time of parturition whenever possible. UFH and LMWH should, in most circumstances, be stopped 12 to 24 hours before delivery. If the risk of recurrent VTE is particularly high or a PE is quite recent, women receiving LMWH can be converted to intravenous UFH, which can be stopped 4 to 6 hours before delivery, thereby minimizing the time of anticoagulation. Neuraxial anesthesia can be safely administered when the aPTT is normal. If spontaneous labor occurs, neuraxial anesthesia should be delayed until 24 hours after the last dose of LMWH and 12 hours after the last dose of subcutaneous UFH. UFH or LMWH may be reinitiated 6 to 12 hours after delivery or removal of the epidural catheter.

For patients with impaired renal function precluding the use of low-molecular-weight heparins during pregnancy, higher doses of unfractionated heparin can be administered subcutaneously.

PROPHYLACTIC ANTICOAGULATION DURING PREGNANCY

Given the prothrombotic state associated with pregnancy, prophylactic anticoagulation should be considered in pregnant women with a history of VTE. Recurrence rates for VTE during pregnancy may be as high as 12% based on the results of retrospective studies, but more recent prospective studies have shown antepartum VTE recurrence rates as low as 2.4%.[34] This exceeds the rate of complications of prophylactic dose anticoagulant and argues in favor of antepartum prophylaxis. Women with prior pregnancy- or estrogen-associated VTE, as well as those with prior unprovoked VTE, are at highest risk of recurrence and should routinely receive prophylactic dose LMWH heparin during pregnancy with a transition to UFH at 36 to 37 weeks' gestation to facilitate delivery. Women with a single prior provoked VTE that was not in the context of pregnancy or exogenous estrogen use can forgo antepartum prophylactic anticoagulation, but postpartum prophylaxis for 6 weeks is recommended, as this is the highest risk period for VTE, particularly PE.

THROMBOPHILIAS AND PREGNANCY

Inherited thrombophilias often prompt a consideration for VTE thromboprophylaxis during pregnancy. Inherited thrombophilias include factor V Leiden, prothrombin gene mutation (G20210A), antithrombin deficiency, protein S deficiency, and protein C deficiency (see Chapter 138). Multiple studies have shown that these disorders have no influence on the risk of recurrent VTE. Therefore, their presence does not impact on the decision about antepartum thromboprophylaxis.

Women may be diagnosed with an inherited thrombophilia through family history or commercial genetic testing. In the absence of a personal history of VTE, the type of thrombophilia and the family history may help guide the need for prophylactic anticoagulation. Guidelines from the American Society of Hematology use a threshold risk of VTE of at least 2% antepartum and 1% postpartum to recommend prophylactic anticoagulation.[35] These cutoffs were determined by weighing the benefits of anticoagulation against the risk of bleeding. Following this guidance, most women with inherited thrombophilias do not require anticoagulation prophylaxis because the risks outweigh the benefit (Table 149.5).

Methylenetetrahydrofolate reductase (MTHFR) polymorphisms are not a risk factor for VTE. Women with MTHFR polymorphisms should be counseled about the need for folate supplementation, but do not require an increased dose.

Screening for inherited thrombophilia solely on the basis of recurrent pregnancy loss is controversial. Negative testing does not exclude the possibility that the patient has an inherited or acquired hypercoagulable state causing pregnancy loss; positive testing does not establish causality. The only tests that confer a clear management approach are antiphospholipid antibodies. Therefore, it may be more fruitful to discuss the risks and benefits of empirical treatment with prophylactic dose LMWH rather than pursue laboratory testing apart from antiphospholipid antibodies. A metanalysis of studies evaluating the use of aspirin and heparin in women with recurrent pregnancy loss failed to demonstrate an increase in live birthrate attributable to these medications.[36] This analysis included women with inherited thrombophilias and women who had tested negative. As such, there is no clear evidence for anticoagulation to support pregnancy carriage in women who do not have antiphospholipid antibodies. Guidance against testing and prophylactic anticoagulation in this setting is in accordance with American College of Obstetricians and Gynecologists (ACOG) guidelines.[37] These guidelines also recommend against thrombophilia testing and anticoagulation in women with prior placental abruption, preeclampsia, or IUGR.

TABLE 149.5 Prophylactic Anticoagulation for Inherited Thrombophilias

Inherited Thrombophilia	Prophylactic Anticoagulation Recommendation[35]
FVL or PGM heterozygous	No
FVL homozygous or FVL and PGM compound heterozygous	Antepartum and postpartum
PGM homozygous	Only postpartum
Protein S or Protein C deficiency	Only postpartum if positive family history, otherwise no
Antithrombin deficiency	Antepartum and postpartum if positive family history, otherwise no

FVL, Factor V Leiden; *PGM*, prothrombin gene mutation.

In a rare instance that placental infarction is documented, empirical prophylactic anticoagulation may be considered for future pregnancies. It is vital that a pathologist experienced in the examination of placentae makes this determination because some thrombosis is expected with placental separation. An accurate determination that infarction occurred antepartum and not during parturition is central to considering this as pathologic or evidence of a propensity for thrombosis.

Motivation to undertake the anticoagulation is often higher in women undergoing assisted reproduction due to its complexity and the expense involved. If LMWH is considered in this setting because of recurrent pregnancy loss, hematologists should acknowledge the lack of evidence supporting its use so that patients can make an informed decision.

CLINICAL VIGNETTE: FACTOR V LEIDEN DURING PREGNANCY

A 25-year-old woman wishes to become pregnant. Her mother was diagnosed with deep vein thrombosis at the age of 70 years and was found to be heterozygous for the factor V Leiden mutation. The patient has no history of VTE, even while taking an estrogen-containing oral contraceptive for 7 years. She is currently trying to become pregnant and had one spontaneous abortion at approximately 6 weeks' gestation. Her primary care physician tested her for factor V Leiden, and she was found to be heterozygous for the mutation. She is asking if she should be on anticoagulation therapy during her next pregnancy. She sees a hematologist and is counseled against prophylactic anticoagulation. She becomes pregnant again 3 months later and has an uneventful pregnancy with a normal spontaneous vaginal delivery at term.

Antiphospholipid Antibody Syndrome

Antiphospholipid antibody syndrome (APS) is the most common form of acquired thrombophilia and is a well-established cause of thrombosis and pregnancy loss (see Chapter 139). Antiphospholipid antibodies include the lupus anticoagulant and antibodies against cardiolipin and β_2-glycoprotein I. Antibodies can occur as a manifestation of various conditions, such as SLE and other rheumatic diseases, infection, and drug reactions. Since most antiphospholipid antibodies have no clinical impact, a distinction is made between individuals who are solely antibody positive and those who have APS. APS is defined by the combination of laboratory testing and either pregnancy loss or thrombotic events (Table 149.6). When pregnancy loss is the only clinical manifestation, the term obstetrical APS is used.

Antiphospholipid antibodies exert their prothrombotic effects through multiple mechanisms, including interacting with phospholipids on the surface of platelets, increasing platelet adhesiveness and production of von Willebrand multimers. In pregnant patients, antiphospholipid antibodies may cause pregnancy loss by inducing thrombosis in the placenta or interference with placental trophoblast function. This latter mechanism may explain why many women with

TABLE 149.6 Antiphospholipid Syndrome

Vascular Thrombosis
One or more episodes of arterial or venous thrombosis confirmed by imaging
Pregnancy morbidity
Three or more consecutive spontaneous abortions before 10 weeks of gestation
Death of a fetus beyond 10 weeks of gestation with normal fetal morphology
Premature birth before 34 weeks of gestation
Laboratory Criteria (all measured on two or more occasions at least 12 weeks apart)
Lupus anticoagulant
Anticardiolipin antibody IgG or IgM
Anti-B_2 glycoprotein IgG or IgM

obstetrical APS do not develop thrombotic manifestations despite persistent antibody positivity. It is therefore possible that their autoantibodies inhibit placental trophoblast function but are unable to activate coagulation. Other women have both obstetrical APS and thrombotic complications.

Any individual with a thrombotic event attributed to APS, whether venous or arterial, requires lifelong anticoagulation therapy, including during pregnancy. Patients with prior obstetric APS should be managed with prophylactic dose LMWH and aspirin in pregnancy and for 6 weeks after delivery; aspirin is added for prevention of preeclampsia. Many hematologists and obstetricians omit LMWH if the clinical criterion for APS is delivery before 34 weeks' gestation because the primary pathology can then be attributed to the preeclampsia spectrum and may be sufficiently mitigated by aspirin alone. If antibodies are identified without qualifying as APS (e.g., as part of serologic testing during the work-up of SLE), women are managed with aspirin alone due to the increased risk of preeclampsia. Women receiving aspirin alone antepartum are often given a concomitant 6-week course of LMWH prophylaxis after cesarean delivery because of the risk of thrombosis with this procedure.

For women with significant renal insufficiency, which is of relevance in APS because of its association with SLE, UFH can be used in place of LMWH. Direct oral anticoagulants and warfarin should not be used during pregnancy.

Mechanical Heart Valves

Coupled with the prothrombotic physiology of pregnancy, mechanical heart valves increase the risk of thrombosis. While this topic is complex and beyond the scope of this review, and management is usually left to cardiology, there are several points that are important for hematologists. Although warfarin is the standard of care for prevention of valve thrombosis, warfarin is usually contraindicated in pregnancy because it is teratogenic, and it increases the risk of fetal and neonatal bleeding. Options for management are (1) continuing warfarin throughout pregnancy, (2) substituting LMWH for the first trimester of pregnancy (the period of highest risk for teratogenicity) and then resuming warfarin during the second trimester, and (3) LMWH for the entirety of pregnancy and resuming warfarin postpartum. The decision must be individualized, but factors that raise the risk of substitution of LMWH include mechanical valves in the mitral and tricuspid positions, concurrent atrial fibrillation, multiple mechanical valves, and prior thrombosis of the valve. In these circumstances, the risk to maternal health of using LMWH is high enough that the recommendation for most, if not all, women will be to continue warfarin. The dose of warfarin correlates with thrombotic risk, so risks to the fetus are higher for women requiring higher doses to achieve a therapeutic INR. For patients who switch to LMWH during pregnancy, anti-Xa monitoring every 1 to 2 weeks is warranted and the dose of LMWH levels should be adjusted to achieve levels at the upper end of the therapeutic range. The frequency of anti-Xa monitoring is motivated by the dire consequence of valve thrombosis as well as the expected change in GFR during pregnancy. If renal insufficiency precludes the use of LMWH, UFH can be substituted. Collaboration between obstetrics, cardiology, and hematology through pregnancy is vital for these high-risk patients.

FUTURE DIRECTIONS

Management of the hematologic complications of pregnancy continues to be a challenge due to both maternal and fetal factors. Hematologic problems remain among the most common complications of pregnancy, and many chronic blood disorders interact with the physiology of pregnancy. The physicians and patient involved benefit from a multidisciplinary approach with hematology collaborating with obstetrics and obstetrical anesthesiology to deliver optimal care. In certain disorders, the management is clear and will likely remain unchanged in the future. In other disorders, treatment paradigms may shift as new treatments are discovered for nonpregnant patients or the safety of existing therapies are better characterized. In all instances, further well-designed studies will continue to advance evidence-based management of the pregnant patient.

REFERENCES

1. Lieberman E, Ryan KJ, Monson RR, Schoenbaum SC. Association of maternal hematocrit with premature labor. *Am J Obstet Gynecol*. 1988;159(1):107–114.
2. Zhou LM, Yang WW, Hua JZ, et al. Relation of hemoglobin measured at different times in pregnancy to preterm birth and low birth weight in Shanghai, China. *Am J Epidemiol*. 1998;148(10):998–1006.
3. Cogswell ME, Parvanta I, Ickes L, et al. Iron supplementation during pregnancy, anemia, and birth weight: a randomized controlled trial. *Am J Clin Nutr*. 2003;78(4):773–781.
4. Geng F, Mai X, Zhan J, et al. Impact of fetal-neonatal iron deficiency on recognition memory at 2 months of age. *J Pediatr*. 2015;167(6):1226–1232.
5. Congdon EL, Westerlund A, Algarin CR, et al. Iron deficiency in infancy is associated with altered neural correlates of recognition memory at 10 years. *J Pediatr*. 2012;160(6):1027–1033.
6. Koshy M, Burd L. Management of pregnancy in sickle cell syndromes. *Hematol Oncol Clin North Am*. 1991;5(3):585–596.
7. Sun PM, Wilburn W, Raynor BD, Jamieson D. Sickle cell disease in pregnancy: twenty years of experience at Grady Memorial Hospital, Atlanta, Georgia. *Am J Obstet Gynecol*. 2001;184(6):1127–1130.
8. Koshy M, Burd L, Wallace D, et al. Prophylactic red-cell transfusions in pregnant patients with sickle cell disease. A randomized cooperative study. *N Engl J Med*. 1988;319(22):1447–1452.
9. Cassinerio E, Baldini IM, Alameddine RS, et al. Pregnancy in patients with thalassemia major: a cohort study and conclusions for an adequate care management approach. *Ann Hematol*. 2017;96(6):1015–1021.
10. Maberry MC, Mason RA, Cunningham FG, Pritchard JA. Pregnancy complicated by hereditary spherocytosis. *Obstet Gynecol*. 1992;79(5 (Pt 1)):735–738.
11. Ray JG, Burows RF, Ginsberg JS, Burrows EA. Paroxysmal nocturnal hemoglobinuria and the risk of venous thrombosis: review and recommendations for management of the pregnant and nonpregnant patient. *Haemostasis*. 2000;30(3):103–117.
12. Guillet S, Loustau V, Zarour A, et al. Risk factors of neonatal immune thrombocytopenia in pregnant women previously diagnosed with ITP: results from a French Nationwide Prospective Study. *Blood*. 2020;136(Suppl 1):36–37.
13. Wei Y, Ji X, Wang Y, et al. High-dose dexamethasone vs. prednisone for treatment of adult immune thrombocytopenia: a prospective multicenter randomized trial. *Blood*. 2016;127(3):296–302. quiz 370.
14. Michel M, Ruggeri M, Gonzalez-Lopez TJ, et al. Use of thrombopoietin receptor agonists for immune thrombocytopenia in pregnancy: results from a multicenter study. *Blood*. 2020;136(26):3056–3061.
15. Hypertension in pregnancy Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. *Obstet Gynecol*. 2013;122(5):1122–1131.

16. Sibai BM, Ramadan MK, Usta I, Salama M, Mercer BM, Friedman SA. Maternal morbidity and mortality in 442 pregnancies with hemolysis, elevated liver enzymes, and low platelets (HELLP syndrome). *Am J Obstet Gynecol*. 1993;169(4):1000–1006.
17. van Pampus MG, Wolf H, Westenberg SM, et al. Maternal and perinatal outcome after expectant management of the HELLP syndrome compared with pre-eclampsia without HELLP syndrome. *Eur J Obstet Gynecol Reprod Biol*. 1998;76(1):31–36.
18. Burwick RM, Feinberg BB. Eculizumab for the treatment of preeclampsia/HELLP syndrome. *Placenta*. 2013;34(2):201–203.
19. Visser W, Wallenburg HC. Temporising management of severe pre-eclampsia with and without the HELLP syndrome. *Br J Obstet Gynaecol*. 1995;102(2):111–117.
20. van Oostwaard MF, Langenveld J, Schuit E, et al. Recurrence of hypertensive disorders of pregnancy: an individual patient data metaanalysis. *Am J Obstet Gynecol*. 2015;212(5). 624.e1-17.
21. Amant F, Van Calsteren K, Halaska MJ, et al. Long-term cognitive and cardiac outcomes after prenatal exposure to chemotherapy in children aged 18 months or older: an observational study. *Lancet Oncol*. 2012;13(3):256–264.
22. Avilés A, Neri N. Hematological malignancies and pregnancy: a final report of 84 children who received chemotherapy in utero. *Clin Lymphoma*. 2001;2(3):173–177.
23. Maze D, Kazi S, Gupta V, et al. Association of treatments for myeloproliferative neoplasms during pregnancy with birth rates and maternal outcomes: a systematic review and meta-analysis. *JAMA Netw Open*. 2019;2(10):e1912666.
24. Leebeek FWG, Duvekot J, Kruip MJHA. How I manage pregnancy in carriers of hemophilia and patients with von Willebrand disease. *Blood*. 2020;136(19):2143–2150.
25. Fausett B, Silver RM. Congenital disorders of platelet function. *Clin Obstet Gynecol*. 1999;42(2):390–405.
26. Chediak JR, Alban GM, Maxey B. von Willebrand's disease and pregnancy: management during delivery and outcome of offspring. *Am J Obstet Gynecol*. 1986;155(3):618–624.
27. Knoebl P, Thaler J, Jilma P, et al. Emicizumab for the treatment of acquired hemophilia A. *Blood*. 2021;137(3):410–419.
28. Cerneca F, Ricci G, Simeone R, et al. Coagulation and fibrinolysis changes in normal pregnancy. Increased levels of procoagulants and reduced levels of inhibitors during pregnancy induce a hypercoagulable state, combined with a reactive fibrinolysis. *Eur J Obstet Gynecol Reprod Biol*. 1997;73(1):31–36.
29. Hellgren M. Hemostasis during normal pregnancy and puerperium. *Semin Thromb Hemost*. 2003;29(2):125–130.
30. Lee CA, Chi C, Pavord SR, et al. The obstetric and gynaecological management of women with inherited bleeding disorders—review with guidelines produced by a taskforce of UK Haemophilia Centre Doctors' Organization. *Haemoph Off J World Fed Hemoph*. 2006;12(4):301–336.
31. Damodaram M, Kaladindi M, Luckit J, Yoong W. D-dimers as a screening test for venous thromboembolism in pregnancy: is it of any use? *J Obstet Gynaecol J Inst Obstet Gynaecol*. 2009;29(2):101–103.
32. Bates SM, Greer IA, Middeldorp S, et al. VTE, thrombophilia, antithrombotic therapy, and pregnancy: antithrombotic therapy and prevention of thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest*. 2012;141(2 Suppl):e691S–e736S.
33. De Carolis S, di Pasquo E, Rossi E, et al. Fondaparinux in pregnancy: could it be a safe option? A review of the literature. *Thromb Res*. 2015;135(6):1049–1051.
34. Brill-Edwards P, Ginsberg JS, Gent M, et al. Safety of withholding heparin in pregnant women with a history of venous thromboembolism. Recurrence of Clot in This Pregnancy Study Group. *N Engl J Med*. 2000;343(20):1439–1444.
35. Bates SM, Rajasekhar A, Middeldorp S, et al. American Society of Hematology 2018 guidelines for management of venous thromboembolism: venous thromboembolism in the context of pregnancy. *Blood Adv*. 2018;2(22):3317–3359.
36. de Jong PG, Kaandorp S, Di Nisio M, et al. Aspirin and/or heparin for women with unexplained recurrent miscarriage with or without inherited thrombophilia. *Cochrane Database Syst Rev*. 2014(7):CD004734.
37. American College of Obstetricians and Gynecologists' Committee on Practice Bulletins–Obstetrics ACOG Practice Bulletin No. 197: Inherited Thrombophilias in Pregnancy. *Obstet Gynecol*. 2018;132(1):e18–e34.

CHAPTER 150

HEMATOLOGIC MANIFESTATIONS OF END-ORGAN FAILURE

Marissa Laureano and Christopher Hillis

The hematologic system is intricately connected to other vital organs. There are multiple pathways that affect the red blood cells (RBCs), white blood cells, platelets, and hemostasis. This chapter will discuss the hematologic abnormalities encountered in liver disease, renal disease, splenic dysfunction, heart failure, respiratory disease, and endocrinopathies. For further details about the individual blood systems affected, the reader should refer to the specific chapters dealing with those components (e.g., anemia, coagulopathies).

HEMATOLOGIC MANIFESTATIONS OF LIVER DISEASE

The liver plays key roles in hematopoiesis and hemostasis. It produces the hematopoietic growth factors thrombopoietin and erythropoietin (10% of total production),[1] contributes to heme biosynthesis, and is a site of extramedullary hematopoiesis. It contributes to hemostasis through synthesis of coagulation factors, coagulation inhibitors, and fibrinolytic proteins.[1] It is also involved in the clearance of plasma proteins, including activated coagulation factors, proteolytic enzyme—inhibitor complexes, fibrin, and fibrinogen degradation products.[2]

Liver cirrhosis is associated with the development of portal hypertension, which leads to the formation of portal gastropathy and varices, which can result in gastrointestinal (GI) bleeding. Portal hypertension also causes hypersplenism decreasing the fraction of circulating platelets, leukocytes, and erythrocytes through splenic sequestration.[3]

RED BLOOD CELL ABNORMALITIES

Morphologic Abnormalities

In liver disease, the size and shape of the RBC membrane is affected by abnormal lipid metabolism. Excess cholesterol in the outer RBC membrane bilayer causes macrocytosis and target cells (Fig. 150.1).[4] These morphologic changes usually do not lead to significant anemia by themselves, but they are sometimes encountered in the setting of anemia due to other impairments of red cell homeostasis in liver disease (e.g., hypersplenism, GI bleeding). Excess membrane cholesterol also decreases RBC membrane fluidity due to impaired migration of integral membrane proteins and inability to repair peroxidized membrane lipids.[1] When these less deformable RBCs traverse the splenic microcirculation, cytoskeletal damage and permanent deformation can occur, resulting in the formation of acanthocytes (spur cells) and increased clearance in the reticuloendothelial system.[5]

Spur cell anemia is a hemolytic anemia associated with severe cirrhosis.[6] It is rare and associated with a poor prognosis. Treatments for spur cell anemia associated with liver disease are limited. The most effective therapy is liver transplantation. Clinical improvement using flunarizine, pentoxifylline, and cholestyramine has been reported.[7]

Anemia

Anemia in patients with liver disease occurs through hemodilution, sequestration, reduced production, and blood loss. The anemia may be related to the liver disease or the underlying cause of the liver disease itself. Fluid retention increases whole blood volume in patients with cirrhosis which can result in normal red cell mass but a subnormal hematocrit from hemodilution. Portal hypertension related to liver disease can result in hypersplenism with subsequent sequestration of erythrocytes.[8] Erythropoiesis is reduced by nutritional deficiencies in folate or vitamin B_{12} resulting from poor intake or malabsorption. Bone marrow suppression can occur from alcohol consumption, viral hepatitis, hepatitis-associated aplastic anemia, and anemia of chronic disease.[9] Patients with chronic liver disease or cirrhosis can also experience nonimmune hemolysis because of acquired alterations in the RBC membrane (e.g., spur cell anemia), Zieve syndrome (hemolytic anemia, hypertriglyceridemia, and jaundice), or treatment-related marrow suppression (e.g., ribavirin).[9]

The therapeutic approach to anemia in liver disease includes transfusion support for symptomatic disease, identification and treatment of GI bleeding, nutrient supplementation for deficiencies, discontinuation of bone marrow suppressive medications, alcohol cessation, and treatment for the primary cause of liver disease.

WHITE BLOOD CELL ABNORMALITIES

Leukopenia

Leukopenia, particularly neutropenia, has also been noted in patients with cirrhosis. Causes of leukopenia include splenic sequestration, medications, or bone marrow suppression mediated by primary infections or toxins (e.g., hepatitis B or C, alcohol).[3] Patients with cirrhosis may also be at higher risk for severe infections due to impairment in neutrophil recruitment and phagocytic function.[10] Granulocyte colony-stimulating factor (G-CSF) is thought to improve neutrophil transendothelial migration in patients with cirrhosis.[11] The use of G-CSF in patients with decompensated cirrhosis was shown to improve survival in a randomized trial[12]; however, the role of G-CSF in the treatment of patients with cirrhosis and the exact mechanisms through which it may ameliorate liver disease are still uncertain and warrant further research.

PLATELET ABNORMALITIES

Thrombocytopenia

Thrombocytopenia is the most common cytopenia associated with liver disease, occurring in up to 77% of patients with cirrhosis.[13] Severe thrombocytopenia ($<30 \times 10^9$/L) and spontaneous bleeding are uncommon.

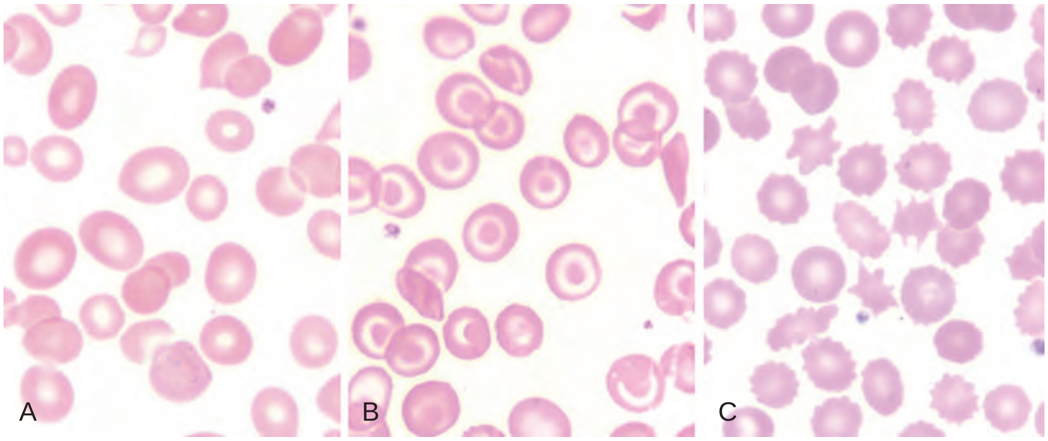


Figure 150.1 RED BLOOD CELL MORPHOLOGY IN LIVER DISEASE. (A) Macrocytes have a mean corpuscular volume greater than 100 fL and can be oval shaped. Commonly associated disorders include liver disease and vitamin B_{12} and folate deficiency. (B) Target cells are characterized by the bull's-eye appearance of the red blood cell (RBC). They are a result of an increased surface-to-volume ratio related to excess RBC membrane (e.g., liver disease) or disproportionate reduction of cytoplasmic content (e.g., hemoglobinopathies, iron deficiency). (C) Acanthocytes, or spur cells, typically appear as contracted RBCs lacking central pallor with multiple irregular membrane projections. The morphologic appearance reflects the irreversible cytoskeletal damage that has occurred because of passage of nondeformable RBCs through the reticuloendothelial system. They are most commonly seen in severe liver disease but can also be features of rare neuroacanthocytosis syndromes or lipoprotein disorders.

Many factors contribute to thrombocytopenia in liver disease. Decreased platelet counts may result from hypersplenism, impaired thrombopoiesis related to nutritional deficiencies (folate or vitamin B_{12}), direct toxicity of alcohol, viral hepatitis, or reduced hepatic synthesis of thrombopoietin.[14] Autoantibodies to platelet antigens have been demonstrated in patients with cirrhosis, suggesting that accelerated destruction of platelets may occur via immune-mediated mechanisms.[15] Hepatitis C is a secondary cause of immune thrombocytopenia. Thrombocytopenia in patients with cirrhosis, especially in combination with leukopenia, is associated with increased morbidity and mortality.[3]

Platelet Dysfunction

Intrinsic platelet defects leading to abnormal platelet aggregation or adhesion include impaired transmembrane signaling and thromboxane A2 synthesis, storage pool deficiency, or defects in platelet glycoprotein Ib or $\alpha_{IIb}\beta_3$ receptors.[16] Extrinsic defects resulting in platelet dysfunction include circulating fibrinogen degradation products, bile salts, abnormal high-density lipoproteins, and excess production of nitric oxide.[16]

The clinical significance of platelet dysfunction demonstrated in vitro is unclear. This discordance between laboratory findings and clinical bleeding may be explained by two observations. First, platelets studied under physiologic flow conditions show normal adhesion to fibrinogen and collagen even in cirrhosis.[17] Second, elevated levels of von Willebrand factor (vWF) are commonly found in patients with cirrhosis and may compensate for thrombocytopenia or platelet dysfunction.[18]

Platelet transfusions can be considered in thrombocytopenia associated with liver disease; however, they are generally not indicated unless the patient has severe thrombocytopenia ($<10 \times 10^9$/L) or platelets less than 50×10^9/L with bleeding. Platelet counts greater than 50×10^9/L are usually considered adequate for invasive procedures. Thrombopoietin (TPO) mimetic agents (e.g., eltrombopag, romiplostim) may offer potential treatment for these patients. Avatrombopag, a second generation TPO agonist, has been examined in patients with chronic liver disease in a pre-procedural setting. Compared to placebo, avatrombopag reduced the frequency of platelet transfusion or rescue therapy.[18] The 2020 American College of Gastroenterology guidelines suggest that TPO agonists can be considered for patients with cirrhosis and a platelet count $< 50 \times 10^9$/L in anticipation of an elective procedure (conditional recommendation, very low level of evidence).[19]

COAGULATION AND LIVER DISEASE

The manifestations of aberrant coagulation in liver disease reflect an interplay between procoagulant and anticoagulant mechanisms. Patients with cirrhosis are at an increased risk of both bleeding and thrombosis.

Coagulation factor and vitamin K deficiency, dysfibrinogenemia, and systemic fibrinolysis contribute to impaired hemostasis. Clinical manifestations range from asymptomatic laboratory abnormalities to life-threatening hemorrhage. However, most cases of spontaneous bleeding in chronic liver disease are due to variceal bleeding rather than abnormalities in coagulation.

Hepatic dysfunction leads to reduced synthesis of most coagulation factors. The number and degree of clotting factor deficiencies reflect the severity of liver damage. Factor VII, having the shortest half-life (6 hours) of the coagulation factors, often declines early and is reflected by prolongation of the prothrombin time (PT)/international normalized ratio (INR). Conversely, factor VIII and vWF levels may be normal or elevated in liver disease because of upregulated compensatory extrahepatic synthesis or impaired hepatic clearance.[2]

Reductions in factors II, VII, IX, and X in patients with liver disease may also result from vitamin K deficiency caused by malnutrition, malabsorption, use of antibiotics, or biliary tract obstruction. For these coagulation factors, vitamin K is required as a cofactor in γ-carboxylation, a process that converts inactive precursors to biologically active factors.

Defects and deficiencies in coagulation factors are suggested by prolonged PT/INR and partial thromboplastin time (PTT) measurements and confirmed by individual factor levels. The INR is incorporated in the Child-Pugh and Model of End-stage Liver Disease scores as a marker of synthetic dysfunction to estimate the severity of liver disease and stratify patients for transplant, respectively. Despite routine use in clinical practice, there are limitations to the PT/INR or PTT in the context of liver disease. First, the INR is not validated for patients with cirrhosis. Second, there is a paucity of evidence that correcting these abnormal values by treatment with plasma or procoagulant agents prevents bleeding.[20]

Several reasons account for the lack of correlation between PT/INR or PTT with bleeding. Liver disease results in deficiencies of procoagulant proteins, but also deficiencies in anticoagulant proteins (antithrombin, proteins C, and protein S). PT/INR and PTT assays only reflect procoagulant protein levels and do not reflect alterations in anticoagulant proteins, the endothelium, or platelet number and function. Tests of thrombin generation such as thromboelastography (TEG) have been studied in randomized control trials (RCTs) to guide transfusions in variceal[21] and nonvariceal bleeding[22] in patients with liver disease. Use of TEG was associated with a lower use of blood products without compromising hemostasis.

Fibrinogen synthesis is generally preserved unless liver disease is severe.[1] Acquired dysfibrinogenemia has been described in approximately 75% of patients with acute and chronic liver disease.[23] Aberrant polymerization of fibrin monomers may be related to excess sialic acid residues on fibrinogen, interfering with the activity of thrombin.[24] Laboratory findings of dysfibrinogenemia include elevated PT, PTT, or thrombin time, low or normal fibrinogen by immunologic assay, and reduced fibrinogen by functional assay (see box on Hemostatic Indices in Liver Disease).

The presence of hyperfibrinolysis in liver disease and its contribution to bleeding risk is controversial. Aside from tissue plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1), all fibrinolytic and antifibrinolytic proteins are synthesized by the liver. Increased fibrinolysis may involve release of t-PA in the setting of infection, surgery, ascitic fluid, and altered synthetic functions of the liver.[25] Laboratory tests can indicate increased fibrinolysis (see box on Hemostatic Indices in Liver Disease), but they cannot adequately assess the overall activity of profibrinolytic and antifibrinolytic components. Hyperfibrinolysis may theoretically aggravate bleeding through consumption of coagulation factors, inhibition of fibrin polymerization, and reduced platelet aggregation via degradation of vWF and glycoprotein Ib and $\alpha_{IIb}\beta_3$.[26]

HEMOSTATIC BALANCE IN LIVER DISEASE

	Promotes Thrombosis	Promotes Bleeding
Primary hemostasis	• Increased vWF • Decreased ADAMTS13	• Thrombocytopenia • Platelet dysfunction
Secondary hemostasis	• Increased factor VIII • Decreased protein C, protein S, antithrombin	• Factor deficiencies: II, V, VII, IX, XI • Vitamin K deficiency • Hypofibrinogenemia • Dysfibrinogenemia
Fibrinolysis	• Reduced plasminogen • Increased PAI-1	• Reduced α2-antiplasmin, TAFI, factor XIII • Increased t-PA

ADAMST13, A disintegrin and metalloproteinase with thrombospondin; PAI-1, plasminogen activator inhibitor-1; TAFI, thrombin activatable fibrinolysis inhibitor; t-PA, tissue plasminogen activator; vWF, von Willebrand factor.

TREATMENT OF LIVER DISEASE–RELATED BLEEDING

Correction of asymptomatic hemostatic abnormalities in patients with liver disease is not indicated and potentially harmful.

In bleeding patients, multiple blood products can be considered. Platelet transfusions can be considered to maintain a count greater than 50×10^9/L. RBC transfusions should maintain adequate hemoglobin or hematocrit levels and address symptomatic anemia. An RCT demonstrated that a restrictive RBC transfusion threshold (<7 g/dL) in patients with an acute upper GI bleed reduced rebleeding and increased survival.[27] Prothrombin complex concentrates (PCCs) have been shown to improve coagulation tests, but further studies are required to assess the effect on clinical outcomes.[28] Diverse effects of PCCs include thrombotic complications and allergic reactions. Frozen plasma (FP) can also be considered, but there is a high risk of volume overload with the amount of product required to improve coagulation parameters. For this reason, guidelines suggest that FP should not be used.[19]

A trial of oral vitamin K can be considered in patients with possible vitamin K deficiency. Vitamin K can be given intravenously for earlier onset of action but carries a small risk of anaphylaxis. Subcutaneous and intramuscular administrations should be avoided due to inconsistent absorption and risk of hematoma formation, respectively.

Recombinant factor VIIa (rFVIIa) has been shown to transiently normalize the PT,[29] yet correlation with improved clinical outcomes has been inconsistent. Randomized trials examining rFVIIa in active GI and variceal bleeding did not show any benefit over placebo.[30] rFVIIa appears to reduce blood loss and blood product requirements when used prophylactically in some invasive procedures[31,32]; however, the optimal dosing and settings in which to use rVIIa is still unclear. These potential benefits may be offset by an observed increase in arterial thromboembolic events.[33] The 2016 American Association for the Study of Liver Diseases (AASLD) guidance does not recommend FP or rFVIIa to normalize the INR in cirrhotic patients with portal hypertensive bleeding.[34]

HEMOSTATIC INDICES IN LIVER DISEASE

Laboratory Changes	PT	PTT	TCT	Fib	Clauss	Plt	Platelet Aggregation	FVII	DD	ELT
Thrombocytopenia	N	N	N	N	N	↓	N	N	N	N
Platelet dysfunction	N	N	N	N	N	N	Abnormal	N	N	N
Vitamin K deficiency[a]	↑	↑	N	N	N	N	N	↓	N	N
Factor deficiency	↑	↑	N	N	N	N	N	↓	N	N
Hypofibrinogenemia	N/↑	N/↑	↑	↓	↓	N	N	N	N	N
Dysfibrinogenemia	N/↑	N/↑	↑	N	↓	N	N	N	N	N
Hyperfibrinolysis	N/↑	N/↑	N/↑	N/↓	N/↓	N	N	↓	↑	↓
DIC	N/↑	N/↑	N/↑	↓	↓	↓	N	N/↓	↑	↓

[a] Differentiating between vitamin K deficiency and liver disease can be challenging with conventional laboratory tests. If available, performing a factor II assay with and without Echis venom (factor II biologic and factor II Echis) may be useful. Ecarin is derived from *Echis carinatus* snake venom and can activate prothrombin irrespective of γ-carboxylation. Factor II activity (biologic) is reduced in both vitamin K deficiency and liver disease. In contrast, the factor II Echis is reduced in liver disease but is normal in vitamin K deficiency.

Clauss, Clauss fibrinogen; *DD*, D-dimer; *DIC*, disseminated intravascular coagulation; *ELT*, euglobulin lysis time (measure of fibrinolysis); *Fib*, fibrinogen; *FVII*, factor VII functional assay; *N*, normal; *Plt*, platelet; *PT*, prothrombin time; *PTT*, partial thromboplastin time; *TCT*, thrombin clotting time.

Hyperfibrinolysis and dysfibrinogenemia may exacerbate bleeding. Administration of fibrinogen concentrate or cryoprecipitate may help control bleeding in those with low fibrinogen. Hyperfibrinolysis has been associated with conditions such as trauma and postpartum hemorrhage (PPH). Cochrane Systematic Reviews revealed that tranexamic acid, an antifibrinolytic agent, reduces postpartum blood loss[35] and decreases mortality in trauma patients.[36] Tranexamic acid has also been shown to reduce blood loss and the need for transfusion in liver transplantation.[37] Thrombotic risks must be considered before use (see box on Managing Bleeding due to Coagulopathy of Liver Disease).

HYPERCOAGULABILITY AND THROMBOSIS IN PATIENTS WITH LIVER DISEASE

Variable rates of venous thromboembolism (VTE) in patients with cirrhosis are reported; however, these patients are still at risk of VTE and are not "autoanticoagulated."[38] Reported incidences of deep venous thrombosis (DVT) or pulmonary embolism (PE) have ranged from 0.5% to 6.3%.[39] Thrombotic complications can involve portal, mesenteric, and hepatic veins. The prevalence of portal vein thrombosis (PVT) is approximately 0.6% to 15.8% of patients with cirrhosis, [40] and is associated with inferior prognosis in liver transplantation.[41]

Despite increased bleeding risks in liver disease, patients still benefit from prophylactic or therapeutic anticoagulation. PVT has been associated with poor prognosis and a higher risk of variceal bleeding.[42] Furthermore, microvascular thrombosis has been proposed to promote hepatic fibrosis and progression of cirrhosis.[43] Clinical decisions are limited by a paucity of studies establishing the optimal dose, duration, monitoring, or choice of anticoagulant and importantly, clear clinical benefit and safety. Use of vitamin K antagonists (VKAs) for anticoagulation in patients with liver disease is complicated by unreliable INR measurements.

There are currently no standard means of identifying patients with liver disease requiring thromboprophylaxis nor means to reconcile the perceived increased risk of bleeding in these patients. The 2020 American College of Gastroenterology (ACG) guidelines recommend pharmacologic VTE prophylaxis for inpatients with cirrhosis in the absence of bleeding or thrombocytopenia (platelets $<50 \times 10^9$/L).[19] Patients who may benefit from perioperative primary prophylaxis are those awaiting liver transplantation, because PVT worsens the long-term posttransplant prognosis.[41]

MANAGING BLEEDING DUE TO COAGULOPATHY OF LIVER DISEASE

- Basic coagulation tests should be ordered; these include CBC, PT (INR), PTT, thrombin clotting time, fibrinogen, D-Dimer, FDP, and mixing studies. The need for more specialized tests (e.g., specific factor levels) will be dictated by the clinical situation.
- It is important to identify any localized source of bleeding (e.g., varices) amenable to procedural intervention.
- A trial of 5–10 mg of vitamin K is reasonable with prolonged PT and PTT but should be used with other therapies.
- In patients with thrombocytopenia platelet transfusions can be used, targeting platelet counts greater than 50×10^9/L.
- In the setting of hypofibrinogenemia, replacement can be attempted with 10–20 units of cryoprecipitate or 4 g of fibrinogen concentrate initially while following laboratory results.
- PCC can be considered to improve coagulation parameters, but it must be used judiciously given the paucity of evidence around clinical outcomes and possible risks associated with its use.
- Factor rVIIa can be considered as a temporizing measure. Its use in liver disease is off label and the thromboembolic risk must be considered.

CBC, Complete blood count; *DIC*, disseminated intravascular coagulation; *FDP*, fibrin degradation product; *FP*, frozen plasma; *INR*, international normalized ratio; *PT*, prothrombin time; *PTT*, partial thromboplastin time; *rFVIIa*, recombinant factor VIIa.

There are several indications for therapeutic anticoagulation in the setting of cirrhosis such as DVT, PE, PVT, and Budd-Chiari syndrome. The comprehensive management of these disorders is beyond the scope of this chapter. The 2020 ACG guidelines suggest anticoagulation for acute main PVT, mesenteric vein thrombosis, or extension of PVT into the mesenteric veins in patients with cirrhosis.[19] However, the risks and benefits of anticoagulation must be carefully considered. The guidelines also advocate for prevention of variceal bleeding with β-blockers (or endoscopic therapy if β-blockers cannot be used safely) in cirrhotic patients requiring anticoagulation for acute or chronic PVT.[19] The value of anticoagulation for chronic PVT is less certain. It can be considered in the presence of a thrombophilia, progression of the thrombus, history of bowel ischemia due to thrombus extension into mesenteric veins, or in anticipation of a liver transplant.[19]

The use of direct oral anticoagulants (DOACs) is increasing, and they are approved in the setting of non-valvular atrial fibrillation, treatment of VTE, VTE prophylaxis after hip or knee replacement, and cancer associated VTE. However, most randomized control trials examining the use of DOACs excluded those with acute liver disease, cirrhosis, or thrombocytopenia. The risks and benefits of anticoagulation should be discussed, and management individualized to each patient's circumstances. The US Food and Drug Administration (FDA) recommends that all DOACs be avoided in patients with Child-Pugh C liver disease. Dabigatran and apixaban can be used cautiously in patients with Child-Pugh B liver disease.[44] All DOACs can likely be used in those with Child-Pugh A liver disease (Table 150.1).[44]

HEMATOLOGIC MANIFESTATIONS OF RENAL DISEASE

Renal disease and its causes are associated with numerous hematologic abnormalities including anemia, platelet dysfunction, and thrombosis. Morphologic abnormalities may include echinocytes, or abnormal RBC membranes (Table 150.2).

ANEMIA

Anemia in chronic kidney disease (CKD) is associated with poor outcomes, including reduced quality of life, increased cardiovascular disease, hospitalizations, cognitive impairment, and mortality. In a study of 5222 patients with CKD (serum creatinine >1.5 mg/dL and >2.0 mg/dL in females and males, respectively) the prevalence

TABLE 150.1 Primary Routes of Clearance of Various Anticoagulants[44]

	Target	Hepatic Clearance	Renal Clearance
Dabigatran	IIa	20%	80%
Apixaban	Xa	75%	25%
Edoxaban	Xa	50%	50%
Rivaroxaban	Xa	65%	35%
Warfarin	IX,X, II	100%	0%
Unfractionated heparin	II, Xa, IX, XIa, and XIIa (mainly II)	Primarily metabolized through the reticuloendothelial system and liver	-
Low molecular weight heparin	IIa and Xa (mainly Xa)	<10%[45]	Predominantly renal clearance

TABLE 150.2 Summary of Hematologic Manifestations of End-Organ Damage

	Red Blood Cells	White Blood Cells	Platelets	Morphology	Thrombosis/Hemorrhagic Risk
Liver disease	Anemia	Leukopenia, specifically neutropenia	Thrombocytopenia Platelet dysfunction	Macrocytosis Target cells	Increased risk of thrombosis and bleeding (alteration in hemostatic balance) Increased risk of varices
Renal failure	Anemia		Platelet dysfunction	Echinocytes (burr cells)	Increased risk of thrombosis in nephrotic syndrome
Anatomic/functional asplenia		Neutrophilia/lymphocytosis Increased risk of infection from encapsulated organisms	Thrombocytosis	Howell-Jolly bodies Target cells	Increased risk of thrombosis
Endocrine disorders	Anemia				
Respiratory disease	Secondary erythrocytosis		Thrombocytopenia		
Heart failure	Anemia				Increased risk of thrombosis

of anemia was 47.7%.[46] Anemia in CKD is usually normocytic and hypoproliferative. The underlying mechanisms of anemia are multifactorial and can be related to relative erythropoietin (EPO) deficiency, iron deficiency, and disordered iron homeostasis.[47,48]

Relative Erythropoietin Deficiency

EPO is a glycoprotein hormone mainly produced in the kidney by interstitial fibroblasts.[49] With normal kidney function, hypoxic conditions in the outer medulla trigger production of EPO, which binds to receptors on erythroid progenitors increasing RBC production.[50] Patients with CKD have insufficient EPO levels because of two proposed mechanisms: decreased production capacity caused by tissue damage as well as an altered hypoxic set point for EPO production.[51] As a result, anemic CKD patients have 10 to 100 times lower EPO levels compared with similarly anemic patients with normal renal function.[48]

Several RCTs have demonstrated clinical benefits with the use of erythropoiesis stimulating agents (ESAs) for patients with CKD and anemia.[52,53] However, targeting near normal hemoglobin (Hb) levels is associated with hospitalizations, graft/fistula thrombosis, stroke, and a trend towards increased mortality.[52] The 2017 Renal Association clinical practice guidelines recommend the use of ESAs to target a hemoglobin between 10 and 12 g/dL in adults.[54] Iron deficiency should also be addressed prior to initiation of ESAs.[54]

True Iron Deficiency

CKD patients, particularly those on hemodialysis, have increased iron losses because of chronic bleeding, phlebotomy, and blood loss in the hemodialysis apparatus.[55] ESAs also cause iron depletion through increased erythropoiesis. Therefore, optimization of iron status is required. The UK National Institute for Health and Care Excellence (NICE) recommends intravenous IV iron therapy for patients with CKD with iron deficiency.[56] Tests that can be used to diagnose iron deficiency include the percentage of hypochromic cells (>6%), reticulocyte hemoglobin content (<29 pg), or a combination of the transferrin saturation (<20%) and ferritin (<100 µg/L).

Disordered Iron Homeostasis

Patients with CKD often have both functional and true iron deficiency. Functional iron deficiency is related to an excess of hepcidin, a key hormone involved in iron homeostasis (see Chapter 36 and Chapter 37). Excess hepcidin in CKD patients is secondary to increased production caused by chronic inflammation as well as decreased renal clearance.

BLEEDING RISK

Patients with CKD have increased bleeding because of frequent use of antithrombotic medications, defects in platelet aggregation, and abnormal platelet-endothelial cell interaction. One of the major factors contributing to platelet dysfunction is a defect in glycoprotein IIb/IIIa.[57] Other factors include ineffective ADP response to stimulated aggregations and increased production of nitric oxide.[58,59] Anemia also contributes to uremic bleeding caused by altered flow of platelets with reduced proximity and interaction with endothelial cells.[60]

CKD is associated with uremic platelet dysfunction, a bleeding diathesis that is thought to be due to several factors such as vWF dysfunction, reduced thromboxane A2 and adenosine diphosphate (ADP), and accumulation of uremic toxins.[61] Treatment for uremic platelet dysfunction is indicated in patients with CKD who are actively bleeding or scheduled to undergo a procedure with a risk of bleeding. Desmopressin (DDAVP) can be considered for uremic bleeding. It acts by stimulating the release of vWF multimers from endothelial cells.[62] Another treatment option is conjugated estrogen, which achieves peak effect over 5 to 7 days.[63] The mode of action is not well understood but is thought to result from a reduction in nitric oxide.[64] Correction of anemia, whether through ESAs or RBC transfusions to a level greater than 10 g/dL, has also been shown to improve bleeding time in CKD patients.[63,65] Renal replacement therapy such as dialysis clears uremic toxins and can ameliorate uremic platelet dysfunction.[61]

THROMBOSIS

CKD is associated with an increased risk of thrombotic complications and conditions such as atrial fibrillation. The increased thrombotic risk with nephrotic syndrome has been well described and is associated with the degree of hypoalbuminemia as well as a type of nephrotic syndrome (membranous nephropathy being the highest risk).[64] Patients with nephrotic syndrome have increased platelet aggregation and adhesion because of elevated levels of vWF and unbound arachidonic acid (which is usually albumin bound).[67,68]

There is also increased activation of the coagulation cascade caused by an imbalance between synthesis and urinary loss of thrombotic and antithrombotic factors. Decreased fibrinolysis also occurs because of reduced plasminogen levels and reduced availability of albumin as a cofactor for plasminogen-fibrin interaction.[69,70]

The management of thromboembolic events (renal vein thrombosis, DVT, PE) associated with nephrotic syndrome is similar to conventional anticoagulation strategies for DVT or PE, with the use of heparin, low-molecular-weight heparin, or warfarin. There have been few published studies examining the use of DOACs in patients with nephrotic syndrome. DOACs have a variable degree of renal clearance, with dabigatran being the most and apixaban being the least renally cleared.[71] Prophylactic anticoagulation in patients who are high risk for VTE (membranous nephropathy, albumin <2 g/dL) with low to intermediate risk of bleeding can be considered.[72]

THROMBOTIC MICROANGIOPATHIES

Hemolytic uremic syndrome (HUS) is defined by concomitant microangiopathic hemolytic anemia (MAHA), thrombocytopenia, and acute kidney injury.[73] Pathology demonstrates microvascular platelet thrombi, vessel wall thickening, and detachment of endothelial cells from the basement membrane. Damage to microvascular glomerular endothelium is the inciting and sustaining event involved in platelet consumption and MAHA. HUS may occur secondary to Shiga toxin–producing bacteria, *Streptococcus pneumoniae* infection, drugs, or primary complement dysregulation (atypical HUS). In Shiga toxin–associated HUS, the toxin binds to the high affinity Gb3 receptor expressed on glomerular endothelial cells leading to cell damage. In atypical HUS, the fenestrated endothelium of the glomerulus is particularly susceptible to uncontrolled alternative complement pathway activation arising from impaired regulatory proteins or hyperactive components. The kidney plays a central role in all of these processes, as initial damage to glomerular endothelial cells leads to loss of thromboresistance and platelet activation and consumption, causing thrombocytopenia with microangiopathic hemolytic anemia.[74]

In adults with nondiarrheal or idiopathic HUS, initial management is similar to thrombotic thrombocytopenic purpura (TTP) with plasma exchange therapy. If plasma exchange is not immediately available, plasma infusion may be temporarily used. In patients with atypical or complement mediated HUS, eculizumab, a monoclonal antibody against C5, should be considered.[75] In adults with postdiarrheal HUS, supportive therapy is the mainstay of treatment with intravenous fluids, platelet and red blood cell transfusions, and hemodialysis as indicated for acute kidney injury (AKI).

HEMATOLOGIC MANIFESTATIONS OF OTHER END-ORGAN DISEASES

Functional and Anatomic Asplenia

The spleen has multiple functions which include filtration of erythrocytes and platelets, hematopoiesis, sequestration of RBCs and platelets, and infection prevention. The functional or anatomical loss of the spleen can have various hematologic manifestations.

Red Blood Cell Abnormalities

Multiple morphological abnormalities can manifest in the peripheral blood smears of patients with functional or anatomic asplenia. Howell-Jolly bodies, small basophilic inclusions found in RBCs, represent nuclear material that would typically be removed by the spleen (see Fig. 156.6). The spleen also removes deformed erythrocytes; therefore target cells, RBCs with a relative excess of membrane, may also be present.

Immunological Consequences

The spleen plays a key role in immunological function. It houses cells involved in innate and adaptive immunity and has the unique ability to target encapsulated organisms through IgM memory B cells which reside in the marginal zone of the spleen.[76] Asplenia or splenic dysfunction can subsequently lead to increased risk of severe infection, particularly infections with encapsulated organisms such as *Streptococcus pneumoniae*, *Haemophilus influenzae* type B, and *Neisseria meningitidis*.[77] Patient education and vaccinations against encapsulated organisms in patients with asplenia/splenic dysfunction is recommended.[78]

Thrombosis Risk in Patients With Splenic Dysfunction

Splenectomy is associated with an increased risk of thrombosis. A proposed mechanism is excess circulating procoagulant microparticles that are cleared by the spleen.[79] The underlying conditions that predispose patients to autosplenectomy (e.g., sickle cell disease) or require surgical splenectomy (autoimmune hemolytic anemia, other hemoglobinopathies, RBC enzymopathies and membranopathies) are also hypercoagulable states.[79]

PVT is well described as a potential postoperative complication of splenectomy occurring in approximately 3.3% of cases.[80] VTE risk is also high with a retrospective cohort study using USA census data examining 8149 patients that were admitted to Veteran Affairs hospitals who underwent splenectomy. The risk of being hospitalized for VTE was higher in patients who were splenectomized (relative risk of 2.2).[81]

RESPIRATORY DISEASE

Red Blood Cell Abnormalities

In response to hypoxia, renal erythropoietin production is upregulated.[82] Over time, chronic lung disease can result in secondary erythrocytosis.[83] Management of the underlying condition is key to improving secondary erythrocytosis. In contrast to polycythemia vera, the role of phlebotomy is less clear. There is no evidence to support a hematocrit target and phlebotomy is typically reserved for symptomatic individuals only.[84] It is also uncertain whether there is a connection between secondary erythrocytosis and VTE.[85]

HEART FAILURE

Red Blood Cell Abnormalities

The prevalence of anemia in patients with stable heart failure (HF) is approximately 30%[86] and even higher in hospitalized patients with HF. The underlying pathophysiology involves multiple contributing factors such as renal dysfunction, hemodilution, inflammation, and iron deficiency.[86] Anemia is important to address in this population as it is associated with an increased risk of mortality and hospitalization.[87]

The benefit of transfusion support is transient at best and should be avoided in patients with HF who are asymptomatic. The use of ESAs is controversial and the 2017 American College of Cardiology (ACC) guidelines advise that they should not be used due to lack of benefit demonstrated in randomized control trials (Class III, B-R).[88]

Iron deficiency affects up to 50% of patients with heart failure and it is associated with a poorer prognosis.[89] Randomized control trials demonstrate that IV ferric carboxymaltose may improve quality of life and exercise capacity.[90,91] The 2016 European Society of Cardiology guidelines recommend that IV ferric carboxymaltose be considered in patients with heart failure with reduced ejection fraction (HFrEF) and iron deficiency (Class IIa, Level A).[91] Iron deficiency was classified as a serum ferritin less than 100 μg/L or ferritin of 100 to 299 μg/L and transferrin saturation of less than 20%. The 2017 ACC guidelines also recommend that IV iron be considered to improve functional status and quality of life in patients with New York Heart Association (NYHA) Class II and III HF with iron deficiency (Class IIb, Level B-R).[88]

Thrombosis Risk in Patients With Heart Failure

Heart failure has been associated with a higher risk of VTE. A systematic review published in 2016 demonstrated that hospitalized patients with HF had an increased risk of VTE (relative risk of 1.5) that was independent of their reason for hospitalization.[92] Heart failure is thought to be a prothrombotic due to endothelial dysfunction, inflammation, impaired blood flow due to abnormal cardiac contractility, and a relative increase in procoagulant molecules.[91] Fibrinogen, von Willebrand factor, and plasma viscosity are increased in patients with HF. Anticoagulation for concomitant HF and atrial fibrillation is well accepted, but there is a paucity of evidence with regards to thromboprophylaxis in HF alone.

ENDOCRINOPATHIES

Anemia is the main hematologic manifestation of endocrine dysfunction and there are multiple pathways that affect the production of red blood cells.

Thyroid dysfunction such as hypothyroidism may affect erythropoietin production resulting in anemia.[94,95] The anemia is often normocytic, but it can be macrocytic if there are other concomitant autoimmune disorders, specifically pernicious anemia due to malabsorption of vitamin B_{12} or folic acid. Thyroid dysfunction can also lead to menorrhagia causing iron deficiency with microcytic anemia.[96]

Testosterone is hypothesized to affect red cell productions through multiple mechanisms which include colony forming units for erythrocytes (CFU-E), erythropoietin, hepcidin inhibition, and increases in insulin-like growth factor-1 (IGF-1).[97] A double-blinded placebo-controlled trial published in 2017 examined the use of testosterone gel in men over the age of 65 with low testosterone levels. Testosterone treatment increased the percentage of men whose hemoglobin increased by 1 g/dL over 12 months compared to placebo (54% vs. 15%).[98] This effect was consistent in men with unexplained anemia as well as men with a known cause of anemia.[98]

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Al-Sharefi A, Mohammed A, Abdalaziz A, Jayasena CN. Androgens and anemia: current trends and future prospects. *Front Endocrinol (Lausanne)*. 2019;10:754.

Babitt JL, Lin HY. Mechanisms of anemia in CKD. *J Am Soc Nephrol*. 2012;23(10):1631–1634.

Chong A-Y, Lip GYH. Viewpoint: the prothrombotic state in heart failure: a maladaptive inflammatory response? *Eur J Heart Fail*. 2007;9(2):124–128.

Crary SE, Buchanan GR. Vascular complications after splenectomy for hematologic disorders. *Blood*. 2009;114(14):2861–2868.

Ferro D, Celestini A, Violi F. Hyperfibrinolysis in liver disease. *Clin Liver Dis*. 2009;13(1):21–31.

Gangireddy VGR, Kanneganti PC, Sridhar S, et al. Management of thrombocytopenia in advanced liver disease. *Can J Gastroenterol Hepatol*. 2014;28(10):558–564.

Garcia-Tsao G, Abraldes JG, Berzigotti A, Bosch J. Portal hypertensive bleeding in cirrhosis: risk stratification, diagnosis, and management: 2016 practice guidance by the American Association for the Study of Liver Diseases. *Hepatology*. 2017;65(1):310–335.

Gonzalez-Casas R, Jones EA, Moreno-Otero R. Spectrum of anemia associated with chronic liver disease. *World J Gastroenterol*. 2009;15(37):4653–4658.

Grote Beverborg N, van Veldhuisen DJ, van der Meer P. Anemia in heart failure: still relevant? *JACC Hear Fail*. 2018;6(3):201–208.

Haas T, Fries D, Tanaka K, et al. Usefulness of standard plasma coagulation tests in the management of perioperative coagulopathic bleeding: is there any evidence? *Br J Anesth*. 2015;114(2):217–224.

Hedges SJ, Dehoney SB, Hooper JS, et al. Evidence-based treatment recommendations for uremic bleeding. *Nat Clin Pract Nephrol*. 2007;3(3):138–153.

Jain N, Reilly RF. Clinical pharmacology of oral anticoagulants in patients with kidney disease. *Clin J Am Soc Nephrol*. 2019;14(2):278–287.

Leung N. Hematologic manifestations of kidney disease. *Semin Hematol*. 2013;50(3):207–215.

Marks PW. Hematologic manifestations of liver disease. *Semin Hematol*. 2013;50(3):216–221.

Mikhail A, Brown C, Williams JA, et al. Renal association clinical practice guideline on anaemia of chronic kidney disease. *BMC Nephrol*. 2017;18(1).

Moore AH. Thrombocytopenia in cirrhosis: a review of pathophysiology and management options. *Clin Liver Dis*. 2019;14(5):183–186.

Northup PG, Caldwell SH. Coagulation in liver disease: a guide for the clinician. *Clin Gastroenterol Hepatol*. 2013;11(9):1064–1074.

Ponikowski P, Voors AA, Anker SD, et al. ESC guidelines for the diagnosis and treatment of acute and chronic heart failure: the Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur J Heart Fail*. 2016;18(8):891–975.

Qamar AA, Grace ND, Groszmann RJ, et al. Incidence, prevalence, and clinical significance of abnormal hematologic indices in compensated cirrhosis. *Clin Gastroenterol Hepatol*. 2009;7(6):689–695.

Qamar A, Vaduganathan M, Greenberger NJ, Giugliano RP. Oral anticoagulation in patients with liver disease. *J Am Coll Cardiol*. 2018;71(19):2162–2175.

Simonetto DA, Singal AK, Garcia-Tsao G, et al. ACG clinical guideline: disorders of the hepatic and mesenteric circulation. *Am J Gastroenterol*. 2020;115(1):18–40.

Rubin LG, Schaffner W. Care of the asplenic patient. *N Engl J Med*. 2014;371(4):349–356.

Singhal R, Brimble KS. Thromboembolic complications in the nephrotic syndrome: pathophysiology and clinical management. *Thromb Res*. 2006;118(3):397–407.

Tripodi A, Mannucci PM. The coagulopathy of chronic liver disease. *N Engl J Med*. 2011;365(2):147–156.

Yancy CW, Jessup M, Bozkurt B, et al. 2017 ACC/AHA/HFSA focused update of the 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice guidelines and the heart failure society of America. *J Am Coll Cardiol*. 2017;70(6):776–803.

Zanetto A, Senzolo M, Ferrarese A, et al. Assessment of bleeding risk in patients with cirrhosis. *Curr Hepat Rep*. 2015;14(1):9–18.

REFERENCES

1. Marks PW. Hematologic manifestations of liver disease. *Semin Hematol*. 2013;50(3):216–221. https://doi.org/10.1053/j.seminhematol.2013.06.003.
2. Tripodi A, Mannucci PM. The coagulopathy of chronic liver disease. *N Engl J Med*. 2011;365(2):147–156. https://doi.org/10.1056/NEJMra1011170.
3. Qamar AA, Grace ND, Groszmann RJ, et al. Incidence, prevalence, and clinical significance of abnormal hematologic indices in compensated cirrhosis. *Clin Gastroenterol Hepatol*. 2009;7(6):689–695. https://doi.org/10.1016/j.cgh.2009.02.021.
4. Morse EE. Mechanisms of hemolysis in liver disease. *Ann Clin Lab Sci*. 1990;20(3):169–174. http://www.ncbi.nlm.nih.gov/pubmed/2188563.
5. Biemer JJ. Acanthocytosis—biochemical and physiological considerations. *Ann Clin Lab Sci*. 1980;10(3):238–249.
6. Privitera G, Meli G. An unusual cause of anemia in cirrhosis: spur cell anemia, a case report with review of literature. *Gastroenterol Hepatol Bed Bench*. 2016;9(4):335–339. https://doi.org/10.22037/ghfbb.v0i0.880.
7. Aihara KI, Azuma H, Ikeda Y, et al. Successful combination therapy – flunarizine, pentoxifylline, and cholestyramine – for spur cell anemia. *Int J Hematol*. 2001;73(3):351–355. https://doi.org/10.1007/BF02981961.
8. Boyer TD, Habib S. Big spleens and hypersplenism: fix it or forget it? *Liver Int*. 2015;35(5):1492–1498. https://doi.org/10.1111/liv.12702.
9. Gonzalez-Casas R, Jones EA, Moreno-Otero R. Spectrum of anemia associated with chronic liver disease. *World J Gastroenterol*. 2009;15(37):4653–4658. https://doi.org/10.3748/wjg.15.4653.
10. Sipeki N, Antal-Szalmas P, Lakatos PL, Papp M. Immune dysfunction in cirrhosis. *World J Gastroenterol*. 2014;20(10):2564–2577. https://doi.org/10.3748/wjg.v20.i10.2564.
11. Fiuza C, Salcedo M, Clemente G, Tellado JM. Granulocyte colony-stimulating factor improves deficient in vitro neutrophil transendothelial migration in patients with advanced liver disease. *Clin Diagn Lab Immunol*. 2002;9(2):433–439. https://doi.org/10.1128/CDLI.9.2.433-439.2002.
12. Prajapati R, Arora A, Sharma P, Bansal N, Singla V, Kumar A. Granulocyte colony-stimulating factor improves survival of patients with decompensated cirrhosis: a randomized-controlled trial. *Eur J Gastroenterol Hepatol*. 2017;29(4):448–455. https://doi.org/10.1097/MEG.0000000000000801.
13. Zanetto A, Senzolo M, Ferrarese A, Simioni P, Burra P, Rodríguez-Castro KI. Assessment of bleeding risk in patients with cirrhosis. *Curr Hepat Rep*. 2015;14(1):9–18. https://doi.org/10.1007/s11901-015-0250-1.
14. Moore AH. Thrombocytopenia in cirrhosis: a review of pathophysiology and management options. *Clin Liver Dis*. 2019;14(5):183–186. https://doi.org/10.1002/cld.860.
15. Gangireddy VGR, Kanneganti PC, Sridhar S, Talla S, Coleman T. Management of thrombocytopenia in advanced liver disease. *Can J Gastroenterol Hepatol*. 2014;28(10):558–564. https://doi.org/10.1155/2014/532191.
16. Witters P, Freson K, Verslype C, et al. Review article: Blood platelet number and function in chronic liver disease and cirrhosis. *Aliment Pharmacol Ther*. 2008;27(11):1017–1029. https://doi.org/10.1111/j.1365-2036.2008.03674.x.
17. Lisman T, Bongers TN, Adelmeijer J, et al. Elevated levels of von Willebrand factor in cirrhosis support platelet adhesion despite reduced functional capacity. *Hepatology*. 2006;44(1):53–61. https://doi.org/10.1002/hep.21231.
18. Terrault N, Chen YC, Izumi N, et al. Avatrombopag before procedures reduces need for platelet transfusion in patients with chronic liver disease and thrombocytopenia. *Gastroenterology*. 2018;155(3):705–718. https://doi.org/10.1053/j.gastro.2018.05.025.
19. Simonetto DA, Singal AK, Garcia-Tsao G, Caldwell SH, Ahn J, Kamath PS. ACG Clinical Guideline: disorders of the hepatic and mesenteric circulation. *Am J Gastroenterol*. 2020;115(1):18–40. https://doi.org/10.14309/ajg.0000000000000486.
20. Haas T, Fries D, Tanaka K, Asmis L, Curry N, Schochl H. Usefulness of standard plasma coagulation tests in the management of perioperative coagulopathic bleeding: is there any evidence? *Br J Anesth*. 2015;114(2):217–224.
21. Rout G, Shalimar Gunjan D, et al. Thromboelastography-guided blood product transfusion in cirrhosis patients with variceal bleeding: a randomized controlled trial. *J Clin Gastroenterol*. 2020;54(3):255–262. https://doi.org/10.1097/MCG.0000000000001214.
22. Kumar M, Ahmad J, Maiwall R, et al. Thromboelastography-guided blood component use in patients with cirrhosis with nonvariceal bleeding: a randomized controlled trial. *Hepatology*. 2020;71(1):235–246. https://doi.org/10.1002/hep.30794.
23. Francis JL, Armstrong DJ. Acquired dysfibrinogenaemia in liver disease. *J Clin Pathol*. 1982;35(6):667–672. https://doi.org/10.1136/jcp.35.6.667.
24. Martinez J, MacDonald K, Palascak J. The role of sialic acid in the dysfibrinogenemia associated with liver disease: distribution of sialic acid on the constituent chains. *Blood*. 1983;61(6):1196–1202. https://doi.org/10.1182/blood.V61.6.1196.1196.
25. Saray A, Mesihovic R, Gornjakovic S, et al. Association between high d-dimer plasma levels and ascites in patients with liver cirrhosis. *Med Arh*. 2012;66(6):372–374. https://doi.org/10.5455/medarh.2012.66.372-374.
26. Ferro D, Celestini A, Violi F. Hyperfibrinolysis in liver disease. *Clin Liver Dis*. 2009;13(1):21–31. https://doi.org/10.1016/j.cld.2008.09.008.
27. Villanueva C, Colomo A, Bosch A, et al. Transfusion strategies for acute upper gastrointestinal bleeding. *N Engl J Med*. 2013;368(1):11–21. https://doi.org/10.1056/NEJMoa1211801.
28. Drebes A, de Vos M, Gill S, et al. Prothrombin complex concentrates for coagulopathy in liver disease: single-center, clinical experience in 105 patients. *Hepatol Commun*. 2019;3(4):513. https://doi.org/10.1002/hep4.1293.
29. Ejlersen E, Melsen T, Ingerslev J, Andreasen RB, Vilstrup H. Recombinant activated factor VII (rFVIIa) acutely normalizes prothrombin time in patients with cirrhosis during bleeding from oesophageal varices. *Scand J Gastroenterol*. 2001;36(10):1081–1085. https://doi.org/10.1080/003655201750422701.
30. Bosch J, Thabut D, Bendtsen F, et al. Recombinant factor VIIa for upper gastrointestinal bleeding in patients with cirrhosis: a randomized, double-blind trial. *Gastroenterology*. 2004;127(4):1123–1130. https://doi.org/10.1053/j.gastro.2004.07.015.
31. Friederich PW, Henny CP, Messelink EJ, et al. Effect of recombinant activated factor VII on perioperative blood loss in patients undergoing retropubic prostatectomy: a double-blind placebo-controlled randomised trial. *Lancet*. 2003;361(9353):201–205. https://doi.org/10.1016/S0140-6736(03)12268-4.
32. Warren O, Mandal K, Hadjianastassiou V, et al. Recombinant activated factor VII in cardiac surgery: a systematic review. *Ann Thorac Surg*. 2007;83(2):707–714. https://doi.org/10.1016/j.athoracsur.2006.10.033.
33. Levi M, Levy JH, Andersen HF, Truloff D. Safety of recombinant activated factor VII in randomized clinical trials. *N Engl J Med*. 2010;363(19):1791–1800. https://doi.org/10.1056/NEJMoa1006221.
34. Garcia-Tsao G, Abraldes JG, Berzigotti A, Bosch J. Portal hypertensive bleeding in cirrhosis: risk stratification, diagnosis, and management: 2016 practice guidance by the American Association for the Study of Liver Diseases. *Hepatology*. 2017;65(1):310–335. https://doi.org/10.1002/hep.28906.
35. Novikova N, Hofmeyr GJ, Cluver C. Tranexamic acid for preventing postpartum haemorrhage. *Cochrane Database Syst Rev*. 2015;2015(6):1–62. https://doi.org/10.1002/14651858.CD007872.pub3.
36. Ker K, Roberts I, Shakur H, Coats TJ. Antifibrinolytic drugs for acute traumatic injury. *Cochrane Database Syst Rev*. 2015;2015(5). https://doi.org/10.1002/14651858.CD004896.pub4.
37. Badenoch A, Sharma A, Gower S, et al. The effectiveness and safety of tranexamic acid in orthotopic liver transplantation clinical practice. *Transplantation*. 2017;101(7):1658–1665. https://doi.org/10.1097/TP.0000000000001682.
38. Northup PG, Caldwell SH. Coagulation in liver disease: a guide for the clinician. *Clin Gastroenterol Hepatol*. 2013;11(9):1064–1074. https://doi.org/10.1016/j.cgh.2013.02.026.
39. Buresi M, Hull R, Coffin C. Venous thromboembolism in cirrhosis: a review of the literature - PubMed. *Can J Gastroenterol*. 2012;26(12):905–908. https://pubmed.ncbi.nlm.nih.gov/23248793/.
40. Kinjo N, Kawanaka H, Akahoshi T, et al. Portal vein thrombosis in liver cirrhosis. *World J Hepatol*. 2014;6(2):64–71. https://doi.org/10.4254/wjh.v6.i2.64.
41. Conzen KD, Pomfret EA. Liver transplant in patients with portal vein thrombosis: medical and surgical requirements. *Liver Transplant*. 2017;23(S1):S59–S63. https://doi.org/10.1002/lt.24856.
42. Mantaka A, Augoustaki A, Kouroumalis EA, Samonakis DN. Portal vein thrombosis in cirrhosis: diagnosis, natural history, and therapeutic challenges. *Ann Gastroenterol*. 2018;31(3):315–329. https://doi.org/10.20524/aog.2018.0245.
43. Ponziani FR, Zocco MA, Garcovich M, D'Aversa F, Roccarina D, Gasbarrini A. What we should know about portal vein thrombosis in cirrhotic patients: a changing perspective. *World J Gastroenterol*. 2012;18(36):5014–5020. https://doi.org/10.3748/wjg.v18.i36.5014.
44. Qamar A, Vaduganathan M, Greenberger NJ, Giugliano RP. Oral anticoagulation in patients with liver disease. *J Am Coll Cardiol*. 2018;71(19):2162–2175. https://doi.org/10.1016/j.jacc.2018.03.023.
45. Johansen KB, Balchen T. Tinzaparin and other low-molecular-weight heparins: what is the evidence for differential dependence on renal clearance? *Exp Hematol Oncol*. 2013;2(1):21. https://doi.org/10.1186/2162-3619-2-21.
46. McClellan W, Aronoff SL, Bolton WK, et al. The prevalence of anemia in patients with chronic kidney disease. *Curr Med Res Opin*. 2004;20(9):1501–1510. https://doi.org/10.1185/030079904X2763.
47. Leung N. Hematologic manifestations of kidney disease. *Semin Hematol*. 2013;50(3):207–215. https://doi.org/10.1053/j.seminhematol.2013.06.002.

48. Babitt JL, Lin HY. Mechanisms of anemia in CKD. *J Am Soc Nephrol*. 2012;23(10):1631–1634. https://doi.org/10.1681/ASN.2011111078.
49. Maxwell PH, Osmond MK, Pugh CW, et al. Identification of the renal erythropoietin-producing cells using transgenic mice. *Kidney Int*. 1993; 44(5):1149–1162. https://doi.org/10.1038/ki.1993.362.
50. Mulcahy L. The erythropoietin receptor. *Semin Oncol*. 2001;28(2 Suppl 8): 19–23. https://doi.org/10.1016/S0093-7754(01)90208-8.
51. Fehr T, Ammann P, Garzoni D, et al. Interpretation of erythropoietin levels in patients with various degrees of renal insufficiency and anemia. *Kidney Int*. 2004;66(3):1206–1211. https://doi.org/10.1111/j.1523-1755.2004.00880.x.
52. Singh AK, Szczech L, Tang KL, et al. Correction of anemia with epoetin alfa in chronic kidney disease. *N Engl J Med*. 2006;355(20):2085–2098. https://doi.org/10.1056/NEJMoa065485.
53. Furuland H, Linde T, Ahlmen J, Christensson A, Strombom U, Danielson B. A randomized controlled trial of haemoglobin normalization with epoetin alfa in pre-dialysis and dialysis patients. *Nephrol Dial Transpl*. 2003;18(2):353–361. https://pubmed.ncbi.nlm.nih.gov/12543892/.
54. Mikhail A, Brown C, Williams JA, et al. Renal association clinical practice guideline on anaemia of chronic kidney disease. *BMC Nephrol*. 2017;18(1). https://doi.org/10.1186/s12882-017-0688-1.
55. Eschbach JW, Cook JD, Scribner BH, Finch CA. Iron balance in hemodialysis patients. *Ann Intern Med*. 1977;87(6):710–713. https://doi.org/10.7326/0003-4819-87-6-710.
56. National Institute for Health and Care Excellence (NICE). Chronic kidney disease: managing anaemia. Guideline NG8. 2015 https://www.nice.org.uk/guidance/ng8 [updated 2021, NG203].
57. Benigni A, Boccardo P, Galbusera M, et al. Reversible activation defect of the platelet glycoprotein IIb–IIIa complex in patients with uremia. *Am J Kidney Dis*. 1993;22(5):668–676. https://doi.org/10.1016/S0272-6386(12)80429-X.
58. Horowitz HI, Stein IM, Cohen BD, White JG. Further studies on the platelet-inhibitory effect of guanidinosuccinic acid and its role in uremic bleeding. *Am J Med*. 1970;49(3):336–345. https://doi.org/10.1016/S0002-9343(70)80025-0.
59. Remuzzi G, Perico N, Zoja C, Corna D, Macconi D, Viganó G. Role of endothelium-derived nitric oxide in the bleeding tendency of uremia. *J Clin Invest*. 1990;86(5):1768–1771. https://doi.org/10.1172/JCI114904.
60. Livio M, Marchesi D, Remuzzi G, Gotti E, Mecca G, De Gaetano G. Uraemic bleeding: role of anaemia and beneficial effect of red cell transfusionS. *Lancet*. 1982;320(8306):1013–1015. https://doi.org/10.1016/S0140-6736(82)90050-2.
61. Hedges SJ, Dehoney SB, Hooper JS, Amanzadeh J, Busti AJ. Evidence-based treatment recommendations for uremic bleeding. *Nat Clin Pract Nephrol*. 2007;3(3):138–153. https://doi.org/10.1038/ncpneph0421.
62. Zeigler ZR, Megaludis A, Fraley DS. Desmopressin (d-DAVP) effects on platelet rheology and von willebrand factor activities in uremia. *Am J Hematol*. 1992;39(2):90–95. https://doi.org/10.1002/ajh.2830390204.
63. Livio M, Mannucci PM, Viganò G, et al. Conjugated estrogens for the management of bleeding associated with renal failure. *N Engl J Med*. 1986;315(12):731–735. https://doi.org/10.1056/NEJM198609183151204.
64. Zoja C, Noris M, Corna D, et al. L-arginine, the precursor of nitric oxide, abolishes the effect of estrogens on bleeding time in experimental uremia. *Lab Invest*. 1991;65(4):479–483. http://www.ncbi.nlm.nih.gov/pubmed/1656142.
65. Cases A, Escolar G, Reverter JC, et al. Recombinant human erythropoietin treatment improves platelet function in uremic patients. *Kidney Int*. 1992;42(3): 668–672. https://doi.org/10.1038/ki.1992.333.
66. Barbour SJ, Greenwald A, Djurdjev O, et al. Disease-specific risk of venous thromboembolic events is increased in idiopathic glomerulonephritis. *Kidney Int*. 2012;81(2):190–195. https://doi.org/10.1038/ki.2011.312.
67. Zwaginga JJ, Koomans HA, Sixma JJ, Rabelink TJ. Thrombus formation and platelet-vessel wall interaction in the nephrotic syndrome under flow conditions. *J Clin Invest*. 1994;93(1):204–211. https://doi.org/10.1172/JCI116947.
68. Yoshida N, Aoki N. Release of arachidonic acid from human platelets. A key role for the potentiation of platelet aggregability in normal subjects as well as in those with nephrotic syndrome. *Blood*. 1978;52(5):969–977. https://pubmed.ncbi.nlm.nih.gov/698401/.
69. Singhal R, Brimble KS. Thromboembolic complications in the nephrotic syndrome: pathophysiology and clinical management. *Thromb Res*. 2006;118(3):397–407. https://doi.org/10.1016/j.thromres.2005.03.030.
70. Rabelink TJ, Zwaginga JJ, Koomans HA, Sixma JJ. Thrombosis and hemostasis in renal disease. *Kidney Int*. 1994;46(2):287–296. https://doi.org/10.1038/ki.1994.274.
71. Jain N, Reilly RF. Clinical pharmacology of oral anticoagulants in patients with kidney disease. *Clin J Am Soc Nephrol*. 2019;14(2):278–287. https://doi.org/10.2215/CJN.02170218.
72. Gordon-Cappitelli J, Choi MJ. Prophylactic anticoagulation in adult patients with nephrotic syndrome. *Clin J Am Soc Nephrol*. 2020;15(1): 123–125. https://doi.org/10.2215/CJN.05250419.
73. Barbour T, Johnson S, Cohney S, Hughes P. Thrombotic microangiopathy and associated renal disorders. *Nephrol Dial Transplant*. 2012;27(7):2673–2685.
74. Ruggenenti P, Noris M, Remuzzi G. Thrombotic microangiopathy, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura. *Kidney Int*. 2001;60(3):831–846. https://doi.org/10.1046/j.1523-1755.2001.060003831.x.
75. Cataland SR, Wu HM. How I treat: the clinical differentiation and initial treatment of adult patients with atypical hemolytic uremic syndrome. *Blood*. 2014;123(16):2478–2484. https://doi.org/10.1182/blood-2013-11-516237.
76. Weller S, Braun MC, Tan BK, et al. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood*. 2004;104(12):3647–3654. https://doi.org/10.1182/blood-2004-01-0346.
77. Leone G, Pizzigallo E. Bacterial infections following splenectomy for malignant and nonmalignant hematologic diseases. *Mediterr J Hematol Infect Dis*. 2015;7(1). https://doi.org/10.4084/MJHID.2015.057.
78. Rubin LG, Schaffner W. Care of the asplenic patient. *N Engl J Med*. 2014;371(4):349–356. https://doi.org/10.1056/NEJMcp1314291.
79. Crary SE, Buchanan GR. Vascular complications after splenectomy for hematologic disorders. *Blood*. 2009;114(14):2861–2868. https://doi.org/10.1182/blood-2009-04-210112.
80. Krauth MT, Lechner K, Neugebauer EAM, Pabinger I. The postoperative splenic/portal vein thrombosis after splenectomy and its prevention—an unresolved issue. *Haematologica*. 2008;93(8):1227–1232. https://doi.org/10.3324/haematol.12682.
81. Kristinsson SY, Gridley G, Hoover RN, Check D, Landgren O. Long-term risks after splenectomy among 8,149 cancer-free American veterans: a cohort study with up to 27 years follow-up. *Haematologica*. 2014;99(2): 392–398. https://doi.org/10.3324/haematol.2013.092460.
82. Jelkmann W. Regulation of erythropoietin production. *J Physiol*. 2011; 589(6):1251–1258. https://doi.org/10.1113/jphysiol.2010.195057.
83. Sagone AL, Balcerzak SP. Smoking as a cause of erythrocytosis. *Ann Intern Med*. 1975;82(4):512–515. https://doi.org/10.7326/0003-4819-82-4-512.
84. McMullin MFF, Mead AJ, Ali S, et al. A guideline for the management of specific situations in polycythaemia vera and secondary erythrocytosis: a British society for haematology guideline. *Br J Haematol*. 2019;184(2): 161–175. https://doi.org/10.1111/bjh.15647.
85. Bhatt VR. Secondary polycythemia and the risk of venous thromboembolism. *J Clin Med Res*. 2014;6(5):395. https://doi.org/10.14740/jocmr1916w.
86. Grote Beverborg N, van Veldhuisen DJ, van der Meer P. Anemia in heart failure: still relevant? *JACC Hear Fail*. 2018;6(3):201–208. https://doi.org/10.1016/j.jchf.2017.08.023.
87. Gupta K, Kalra R, Rajapreyar I, et al. Anemia, mortality, and hospitalizations in heart failure with a preserved ejection fraction (from the TOPCAT Trial). *Am J Cardiol*. 2020;125(9):1347–1354. https://doi.org/10.1016/j.amjcard.2020.01.046.
88. Yancy CW, Jessup M, Bozkurt B, et al. 2017 ACC/AHA/HFSA focused update of the 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology/American Heart Association Task Force on clinical practice guidelines and the Heart Failure Society of America. *J Am Coll Cardiol*. 2017;70(6):776–803. https://doi.org/10.1016/j.jacc.2017.04.025.
89. von Haehling S, Ebner N, Evertz R, Ponikowski P, Anker SD. Iron deficiency in heart failure: an overview. *JACC Hear Fail*. 2019;7(1):36–46. https://doi.org/10.1016/j.jchf.2018.07.015.
90. Anker SD, Comin Colet J, Filippatos G, et al. Ferric carboxymaltose in patients with heart failure and iron deficiency. *N Engl J Med*. 2009; 361(25):2436–2448. https://doi.org/10.1056/NEJMoa0908355.
91. Ponikowski P, Voors AA, Anker SD, et al. 2016 ESC guidelines for the diagnosis and treatment of acute and chronic heart failure: the task force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur J Heart Fail*. 2016;18(8):891–975. https://doi.org/10.1002/ejhf.592.
92. Tang L, Wu YY, Lip GYH, Yin P, Hu Y. Heart failure and risk of venous thromboembolism: a systematic review and meta-analysis. *Lancet Haematol*. 2016;3(1):e30–e44. https://doi.org/10.1016/S2352-3026(15)00228-8.
93. Chong A-Y, Lip GYH. Viewpoint: The prothrombotic state in heart failure: a maladaptive inflammatory response? *Eur J Heart Fail*. 2007;9(2):124–128. https://doi.org/10.1016/j.ejheart.2006.05.009.
94. Golde DW, Bersch N, Chopra IJ, Cline MJ. Thyroid hormones stimulate erythropoiesis in vitro. *Br J Haematol*. 1977;37(2):173–177. https://doi.org/10.1111/j.1365-2141.1977.tb06833.x.

95. Ma Y, Freitag P, Zhou J, Brüne B, Frede S, Fandrey J. Thyroid hormone induces erythropoietin gene expression through augmented accumulation of hypoxia-inducible factor-1. *Am J Physiol Integr Comp Physiol*. 2004; 287(3):R600–R607. https://doi.org/10.1152/ajpregu.00115.2004.
96. Krassas GE. Thyroid disease and female reproduction. *Fertil Steril*. 2000; 74(6):1063–1070. https://doi.org/10.1016/S0015-0282(00)01589-2.
97. Al-Sharefi A, Mohammed A, Abdalaziz A, Jayasena CN. Androgens and anemia: current trends and future prospects. *Front Endocrinol*. 2019;10:754. https://doi.org/10.3389/fendo.2019.00754.
98. Roy CN, Snyder PJ, Stephens-Shields AJ, et al. Association of testosterone levels with anemia in older men a controlled clinical trial. *JAMA Intern Med*. 2017;177(4):480–490. https://doi.org/10.1001/jamainternmed.2016.9540.

CHAPTER 151

HEMATOLOGIC MANIFESTATIONS OF SOLID TUMORS

Kathryn DeCarli, Peter Barth, Andrew M. Brunner, and Fred J. Schiffman

Hematologic abnormalities are commonly seen among patients with malignancy. These derangements range from the incidental to the life-threatening and may complicate management or prompt the initiation of additional or alternative therapies. Hematologic abnormalities can be seen as the initial manifestation of cancer, providing a crucial diagnostic clue. In addition, the hematologic aspects of cancer, and the therapies that we use to treat these irregularities, can provide insight into the biology of tumorigenesis. This chapter reviews some of the impact that malignancy can have on erythrocytes, leukocytes, platelets, and hemostasis.

ERYTHROCYTES

Anemia

The most common hematologic manifestation of cancer is anemia; anywhere from 30% to 90% of patients with cancer have documented anemia before the initiation of any treatment.[1] Anemia may be the first presentation of malignancy; for instance, anemia is frequently noted in patients with colon cancer and more frequent in advanced stage disease.[2] The pathogenesis of cancer-related anemia is multifactorial, and can arise from direct cancer tissue invasion with resultant blood loss, tumor involvement of the bone marrow, or hemolysis; it can be a direct effect of chemotherapy or radiation therapy, be associated with chronic renal dysfunction secondary to the malignancy or treatment, among other causes. These causes can be generally divided according to the pathogenesis of the anemia: that resulting from direct blood loss, anemia caused by erythrocyte destruction, and anemia caused by a hypoproliferative marrow state.

A number of cancer-specific mechanisms can be responsible for a hypoproliferative state, including nutritional deficiencies, decreased erythropoietin (EPO) production because of acute or chronic kidney injury, and injury to the bone marrow itself. The most common nutritional abnormality in cancer-related anemia is iron deficiency; roughly 40% of cancer patients may have iron-deficiency,[3] which can be secondary to poor oral intake, as well as from blood loss, commonly seen in gastrointestinal and gynecologic malignancies. Compounding any deficiency in total body iron stores are the myelosuppressive effects on erythropoiesis and iron homeostasis that may result from cancer-associated inflammation and cytokine activation.[4] Many cancers are associated with a systemic inflammatory state,[5] manifested by elevated levels of inflammatory cytokines such as IL-2 and IL-6, which can directly inhibit erythropoiesis and also suppress the amount of iron available for erythropoiesis.

Various systemic mediators of anemia are disrupted in malignancy. When serum EPO concentrations are measured among cancer patients, these levels are inappropriately low in comparison to patients without malignancy but who have the same degree of anemia caused by iron deficiency.[6] Cancer patients also often demonstrate increased levels of hepcidin, a protein critical to iron homeostasis, which acts by decreasing the binding to and breaking down of the iron transporter ferroportin.[7] This subsequently results in increased storage of iron in the form of ferritin, and at the same time decreases free iron that would be used in erythropoiesis. In contrast to iron deficiency, vitamin B_{12} deficiency is less frequently observed in patients with anemia and cancer, seen in only 5% to 10% of patients.

Many chemotherapeutic agents have hematologic sequelae; patients undergoing treatment may experience varying degrees of anemia resulting from chemotherapy-induced bone marrow suppression. The European Cancer Anaemia Survey prospectively studied over 15,000 patients with a variety of solid tumors undergoing treatment. Before therapy, 10% of patients had hemoglobin levels below 10 g/dL, and during therapy this increased to almost 40% of patients and was correlated with a worsening performance status.[8] In the same study, radiation therapy alone had less impact on the incidence of anemia, but the combination of chemotherapy and radiation led to a greater degree of anemia than chemotherapy alone. Anemia caused by chemotherapy is related to the cumulative dose, combination of chemotherapeutic agents, and dose schedule. For example, in the treatment of lung cancer with cisplatin and paclitaxel, anemia worsens with an increasing total dose of cisplatin, or if paclitaxel is given over a 24-hour period rather than shorter courses.[9]

Anemia in cancer patients can also occur in the setting of red cell destruction, such as is seen in autoimmune hemolytic anemia, or in the setting of microangiopathic hemolytic anemia (MAHA), which is associated with concurrent thrombocytopenia. Although the true incidence of MAHA among cancer patients is difficult to determine, it is a well-defined entity that has been described in a small (perhaps <10%) subset of cancers, particularly mucin-producing disseminated adenocarcinomas.[10] It is important to distinguish secondary thrombotic microangiopathies (TMAs), which can occur in patients with malignancy, from thrombotic thrombocytopenic purpura (TTP), given the marked differences in underlying pathophysiology. TTP is the result of congenital or acquired absence or inhibition of the von Willebrand cleaving enzyme called "A Disintegrin And Metalloproteinase with Thrombospondin Motifs-13" (ADAMTS13). In contrast, secondary TMAs may have a slight decrease in ADAMTS13 activity, but do not have the severe deficiency of ADAMTS13 activity, typically characterized by a level of less than 10%, that is seen in TTP.[11] Accordingly, patients with secondary TMAs typically have a poor response to therapeutic plasma exchange. Cancer-associated TMAs may arise from direct endothelial damage rather than diminished ADAMTS13 activity. Cancer patients may also develop a MAHA related to the specific medications used during treatment, including cyclosporine, mitomycin, and gemcitabine, and agents inhibiting vascular endothelial growth factor (anti-VEGF) signaling. (See box on Management of Cancer-Related Anemia.)

Erythrocytosis

Outside of patients with myeloproliferative neoplasms, and specifically polycythemia vera, erythrocytosis is an uncommon manifestation of cancer. Polycythemia vera, and other myeloproliferative neoplasms, are typified by acquisition of somatic mutations that upregulate the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway, most commonly via the

JAK2 V617F mutation, and phenotypically present as an expansion of myeloid-origin cells, with an elevated hemoglobin and suppressed EPO levels. In these cancers, erythrocytosis is caused by primary expansion of the malignant clone. For such patients, various therapeutic interventions may be considered, including therapeutic phlebotomy or hydroxyurea (see Chapter 70, The Polycythemias). In solid tumors, erythrocytosis is an uncommon paraneoplastic phenomenon. Paraneoplastic erythropoiesis has been described in several tumors, including renal cell carcinoma.[18] In some instances, there may be association with a common predisposing condition; for instance, patients with von Hippel-Lindau disease are at risk for renal and central nervous system tumors, and the mechanism of erythrocytosis is typically through paraneoplastic EPO expression. Nonetheless, elevated RBC counts are rare in patients with malignancy, particularly those receiving chemotherapy, and usually do not require intervention.

MANAGEMENT OF CANCER-RELATED ANEMIA

Hemoglobin levels typically decrease early in the course of chemotherapy treatment; more than half of patients experience a greater than 1 g/dL drop over the course of the first 9 weeks of therapy.[8] The treatment of anemia related to malignancy depends upon correct identification of the underlying etiology. As noted previously, iron-deficiency anemia is common in patients with malignancy. Among those patients with cancer who have an absolute iron deficiency (transferrin saturation <20%, ferritin <30 ng/mL), there is evidence that they may benefit from a short course of either oral or low-dose intravenous iron. In this setting, the addition of erythropoiesis-stimulating agents (ESAs) is not necessary.

For many patients, transfusion of blood products is an effective therapeutic intervention. Red cell transfusion provides rapid symptomatic relief and is also a source of iron; one unit of packed red blood cells (RBCs) contains roughly 200 mg of iron. Logistic limitations with RBC transfusion, and transfusion-related morbidities, particularly with a high cumulative transfusion burden, have spurred the incorporation of ESAs as alternative or adjunct agents for treating anemia in cancer patients. The use of ESAs during myelosuppressive treatment may increase the hemoglobin level and decrease transfusion requirements by up to 50%. However, ESA use is associated with an increased rate of cardiovascular and thrombotic events. This relationship between ESA use and thrombosis may be related to the target hemoglobin concentration during therapy, since higher hemoglobin targets are associated with increased rates of thrombotic events in cancer patients.

In addition to thrombotic events, ESA use has been associated with poorer overall survival or time to cancer progression in some settings.[12] Data regarding ESAs and progression of disease are conflicting; some studies in patients with breast cancer and patients with head and neck cancer suggested worsening progression-free survival or worsened local control of disease with ESA use.[13] A number of mechanisms have been proposed, including decreased chemosensitivity in the setting of ESA use, or alterations in tumor vascularity and oxygen supply.[14] Aberrant expression of the EPO receptor has been identified on the cell surface of certain tumor cells, while other putative mechanisms include augmentation of red cell mass and effects on tumor oxygenation.[15] Nonetheless, larger studies on the impact of ESA on progression suggest any effect may be small overall.

Related in part to the above concerns, ESA use is generally restricted to specific limited indications in patients with cancer; notably, such practice recommendations are generally limited to solid tumors with chemotherapy-induced anemia.[16] In specific hematologic malignancies, such as myelodysplastic syndromes (MDS), ESA therapies remain a backbone of the treatment of anemia.[17] For the treatment of anemia in patients with solid tumors, transfusion of blood products and, if indicated, iron therapy, remain the standard of care. There are other scenarios where ESAs may be useful, particularly among patients with moderate or severe chronic kidney disease, or in palliative settings, as well as for specific patient scenarios such as the treatment of patients who identify as Jehovah's Witnesses. In such situations, reversible causes of anemia should be ruled out before ESA use, and the minimal amount of ESA be used to avoid RBC transfusion.

PLATELETS

Thrombocytopenia

Chemotherapy and immunosuppressive agents are the most common causes of thrombocytopenia in the cancer patient. For instance, patients receiving cisplatin/gemcitabine for bladder cancer or carboplatin/gemcitabine for lung cancer have rates of clinically significant thrombocytopenia of 30% to 60%.[19] Among patients with hematologic malignancy, thrombocytopenia is even more common, particularly with induction or conditioning chemotherapy regimens. The typical mechanism by which chemotherapy causes thrombocytopenia is through marrow suppression; although, some agents are also associated with immune-mediated thrombocytopenia, and thrombocytopenia may also occur as a sequelae of other processes such as MAHA or TMA (above). Immune thrombocytopenia is typically characterized by a sudden and isolated drop in platelet count shortly after drug administration. Several chemotherapeutics, including gemtuzumab, trastuzumab, and oxaliplatin, have been associated with immune-mediated thrombocytopenia, potentially related to the presence of drug-dependent antibodies.[20]

Thrombocytopenia may also be a result of bone marrow infiltration by tumor cells, thrombotic microangiopathy, consumptive coagulopathy, or as an autoimmune manifestation of the malignancy itself. Bone marrow involvement, often occult, is more common in prostate, lung, and breast cancer; such patients typically have multiple cell lines involved, and may display a leukoerythroblastic appearance on the peripheral smear. Associated laboratory features of disseminated intravascular coagulation (DIC), including an elevated D-dimer and fibrinogen degradation products, can be seen in cancer patients, and are more common with advanced disease. (See box on Management of Cancer-Related Thrombocytopenia.)

Thrombocytosis

While thrombocytopenia may be more clinically apparent because of bleeding or delays in the administration of chemotherapy, the presence of thrombocytosis—typically reactive to the underlying inflammatory state—is more frequently seen in patients with solid tumors. The clinical impact of thrombocytosis is less clear; however, having an elevated platelet count at cancer diagnosis has been associated with a greater risk for venous thromboembolism (VTE).[24] Cancer-related thrombocytosis is thought to be mediated by inflammatory cytokines, and elevated levels of interleukin (IL)-6, IL-11, and TPO have been documented in cancer patients. Rarely, thrombocytosis may be the result of a paraneoplastic phenomenon, which has been described in breast and ovarian malignancies. In many tumor types, including breast, renal cell, gastric, and advanced stage non–small-cell lung cancer, elevated platelet counts seem to be associated with an adverse prognosis. The mechanism for this association is unclear.

Platelet Dysfunction Secondary to Malignancy

In addition to abnormalities in absolute platelet number, cancer patients may have alterations in platelet function, which have been hypothesized to contribute to tumor progression and coagulopathy. Platelets derived from cancer patients are in an activated state, with increased surface P-selectin expression and elevated serum levels of platelet factor IV and β-thromboglobulin.[25] Activated platelets may contribute to the thrombophilic state in cancer patients, and also interact with leukocytes, endothelial cells, and tumor cells, all of which may contribute to the early stages of tumor cell dissemination. Several platelet receptors may be involved in the platelet support of metastasis, including glycoprotein (GP) IIb/IIIa, adenosine diphosphate (ADP) receptors, P-selectin and thrombin receptors, and others, with likely multiple mechanisms underlying the platelet effects on malignancy.[26] Platelets stabilize otherwise short-lived tumor cells in the circulation. Platelet-tumor cell interaction enhances tumor cell adhesion to the vessel wall, and appears to alter vessel wall permeability, and in this way may facilitate tumor cell invasion. Platelets protect tumor cells from immune surveillance and destruction, in part by shielding tumor cells from natural killer cells, although other mechanisms are likely. Platelets may provide nutrient support (growth factors) and release proangiogenic factors. Platelets also contain numerous growth factors, coagulation factors and adhesive molecules, chemokines, and bioactive lipids that may enhance metastatic efficiency. Platelets are similarly enriched in angiogenic growth factors such as basic fibroblast growth factor and VEGF, as well as other mediators, and may play a role in angiogenesis. Selective P-selectin and thrombin receptor activation on platelets may release α-granules enriched in either VEGF or endostatin. Platelet-derived transforming growth factor-β and direct platelet–tumor cell interactions promote epithelial mesenchymal transformation and tumor metastases. Together, these suggest numerous putative roles for platelets in tumorigenesis and evolution itself.

LEUKOCYTES

Neutropenia

Neutropenia is common during treatment with chemotherapy, particularly with antimetabolite use.[27] Other etiologies of neutropenia in cancer patients include infiltration of the bone marrow space and occasionally, radiation therapy, particularly when sites of active hematopoiesis are included in the radiation field, such as with the pelvis or spine. In addition, particularly in the course of treatment, cancer patients may experience viral or antibiotic-associated neutropenia. (See box on Management of Cancer-Related Neutropenia.)

An important consideration during chemotherapy is to recognize that chronic benign neutropenia, as can be seen in those of African and Middle Eastern descent,[37] may be a contributing factor and does not require dose-adjustment of chemotherapy. Rather, such neutropenia may reflect a common single nucleotide polymorphism (SNP) variant in the *ACKR1* gene.[38]

Bone Marrow Metastases

Multiple lineage cytopenias in association with cancer warrants consideration of bone marrow metastasis (Fig. 151.1) or secondary/concurrent hematologic malignancy. Metastatic carcinoma can involve bone marrow and may lead to subsequent marrow fibrosis and failure. Bone marrow metastases are relatively infrequent, with early reports suggesting they are present in fewer than 10% of patients with metastatic disease, most frequently in metastatic sarcoma or melanoma, or prostate, breast, or lung cancer.[39] Patients with solid tumors who have bone marrow involvement are more likely to experience cytopenias from chemotherapy, and typically carry a worse prognosis. It has long been recognized that some patients will have metastatic disease involving the bone marrow that may even precede more overt metastatic burden. Patients with early marrow involvement of non-small-cell lung cancer are more likely to relapse compared to those without marrow involvement[40]; similar findings are reported in patients with breast cancer.[41,42] The precise mechanism for poor outcomes is likely multifactorial, potentially related to greater overall tumor burden, as well as to the specific biologic features that allow the tumor to grow in the bone marrow niche, potentially also rendering it more resistant to chemotherapy.[43] Such tumor cell evasion strategies could include changes in homing, adhesion, immune escape, and angiogenic potential. Indeed, there may be specific molecular signatures within tumor subclones that facilitate metastasis to the bone marrow.[44] The bone marrow niche itself may also play a role in promoting tumor survival and in decreased immune surveillance. One example is the occurrence of bone marrow micrometastases in women

MANAGEMENT OF CANCER-RELATED THROMBOCYTOPENIA

Treatment of thrombocytopenia in cancer patients is generally supportive. For patients with active bleeding, platelet transfusion and correction of other coagulopathies are the mainstays of therapy. The use of agents such as tranexamic acid in these patients may also be considered. In the absence of active bleeding, a threshold of $<10 \times 10^9$/L for prophylactic platelet transfusion is generally recommended. This is based on a study that randomized 600 patients with hematologic malignancies to prophylaxis or no prophylactic transfusions. Both were treated for bleeding events.[21] Rates of World Health Organization grade 2, 3, or 4 bleeding were high in both groups, but higher in the no-prophylaxis group (50%), compared with the prophylaxis group (43%). Prophylactic platelet transfusion has been used in patients with solid tumors, but without as much evidence-based support.

Other therapies, with or without platelet transfusion, may also have a role in chemotherapy-related thrombocytopenia. Thrombopoietin (TPO) receptor agonists are an area of increasing interest in the management of thrombocytopenia during chemotherapy, particularly as a means to maintain treatment schedules, which may be delayed by thrombocytopenia. For instance, a randomized phase II trial compared placebo to eltrombopag in patients receiving gemcitabine-based chemotherapy and found that fewer patients receiving eltrombopag required dose delays and/or reductions in chemotherapy compared with those receiving placebo.[22] Nonetheless, specific interactions with other chemotherapeutic agents will likely be important to understand; for instance, in a trial of patients with MDS randomized to receive azacitidine alone or in combination with eltrombopag, the patients receiving eltrombopag actually had worsened platelet transfusion requirements.[23]

MANAGEMENT OF CANCER-RELATED NEUTROPENIA

Patients who develop neutropenia during chemotherapy are at risk for neutropenic fever. A number of comorbid conditions are predictive of the development of neutropenic fever, including older age, poor performance status, comorbid medical conditions, low baseline white blood cell counts, low body mass index/BSA, advanced disease, and the use of myelosuppressive chemotherapy agents.[28,29] General treatment principles for neutropenic fever include the consideration of prophylactic antibiotics or myeloid growth factor support, such as granulocyte colony-stimulating factor (G-CSF).[30–32] Consideration should be given to the elevated risk of neutropenic fever associated with specific chemotherapeutic agents, as well as the specific malignancy, particularly in situations where dose-dense or intense chemotherapy regimens provide survival benefit. Depending on the regimen and disease, prophylactic G-CSF should be considered to reduce the duration of neutropenia, particularly when there is a greater than 20% overall risk of neutropenic fever. Once a patient develops neutropenic fever, G-CSF is typically reserved for situations where they are not responding to appropriate treatment and develop life-threatening conditions.[33]

The benefit of growth factor support for infection and to maintain treatment timing should be weighed with potential risks from such agents. Several tumors express G-CSF receptors; there is a theoretic concern that exogenous G-CSF may increase proliferation of these tumors. In addition, the use of myeloid growth factors during chemotherapy for solid tumors has been theorized to increase the risk of subsequent myeloid malignancy by acting as a survival signal to hematopoietic progenitor cells damaged by chemotherapy.[34] A meta-analysis of 25 randomized clinical trials found that there was an almost twofold increase in acute myeloid leukemia in those patients assigned to receive chemotherapy with growth factor support, compared with those who did not receive growth factor support, although the absolute rates of secondary leukemia were low in each group.[35,36]

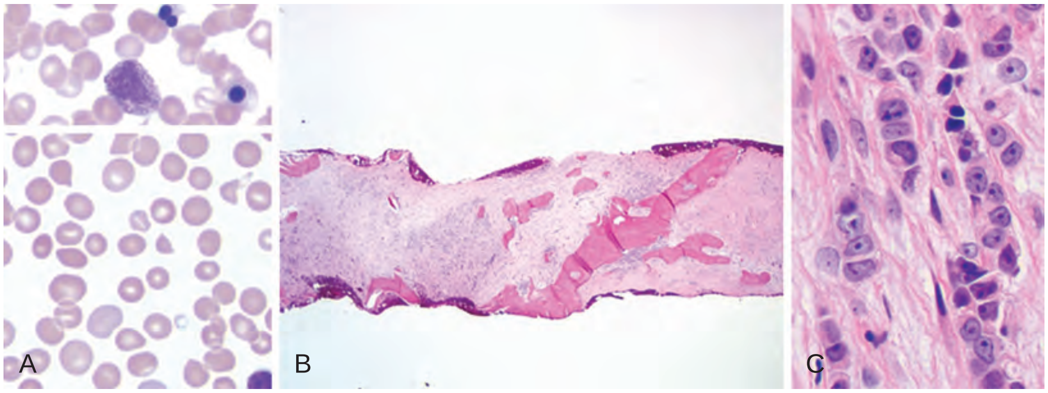


Figure 151.1 PERIPHERAL BLOOD AND BONE MARROW FROM A PATIENT WITH METASTATIC BREAST CANCER . The patient was a 77-year-old woman who presented with anemia and thrombocytopenia. On physical examination, she was found to have a breast mass. (A) The peripheral smear showed a leukoerythroblastosis *(top)* with nucleated red blood cells and immature granulocytic precursors. Platelets were reduced, and red blood cells exhibited anisopoikilocytosis *(bottom)* with occasional teardrop forms and rare schistocytes. (B) The bone marrow biopsy was fibrotic and had thickened bone with new bone formation. (C) The bone marrow cavity was replaced by tumor cells infiltrating through bands of fibrosis.

with early-stage breast cancer; approximately 30% of women with stage I to III breast cancer may have bone marrow micrometastases, which is associated with worse prognosis.[41]

The pathophysiology by which marrow involvement by cancer causes cytopenias is not fully understood. Total marrow replacement by tumor is rare, and only a small percentage of healthy marrow may be needed to support normal peripheral blood counts, as indicated by the observation that normal blood counts are often found in normal older adults with hypocellular marrows. Cancer may disrupt the normal bone marrow interactions required for healthy hematopoiesis, including by directly competing with hematopoietic progenitors in the niche.[45]

THROMBOSIS AND CANCER

The association of thrombosis and cancer is widely recognized, dating to the middle of the 19th century, and can be considered the earliest recognized paraneoplastic syndrome. Armand Trousseau was the first to associate thrombosis and malignancy, the first to suggest screening for malignancy in recurrent or idiopathic thromboembolic disease, the first to suggest that the pathophysiology was not mechanical obstruction, but a change in the character of the coagulation system itself, and the first to suggest that the association may be integral to the cancer growth itself. Particularly poignant was the fact that Trousseau predicted his own occult malignancy when he developed “phlebitis,” dying 6 months later of gastric cancer. More recently, some of the biologic mechanisms underlying the increased risk for thrombosis in patients with cancer have been better delineated.

The incidence of cancer-associated thrombosis has steadily increased in recent years,[46] possibly related to the increasing age of the population, with an associated increase in cancer prevalence, longer survival with active malignancy, and better detection tools. Patients with active malignancy have a higher risk for thrombosis than other medical patients: for instance, surgical oncology patients have a higher thrombosis rate than other surgical patients undergoing major procedures.[47,48] Although reports of thrombosis rates vary, in general approximately 10% to 15% of cancer patients develop VTE at some point during their illness[49]; conversely, approximately 20% of all VTE events occur in cancer patients.[50] Together, a cancer diagnosis may increase the relative risk of venous thrombosis by up to 15-fold.[51] In comparison, heterozygous carriers of the common hereditary thrombophilia, factor V Leiden, increases the risk for venous thrombosis by approximately five-fold.[52] Virtually all solid tumor types, as well as hematologic malignancies, carry this significantly increased risk for thrombosis. Certain tumor types, particularly mucinous adenocarcinomas, appear to have particularly high risk for thrombosis, including lung, gastrointestinal cancers—notably pancreatic adenocarcinoma—as well as primary brain tumors.[53]

Development of thrombosis in the cancer patient generally carries adverse prognostic impact. In patients with advanced solid tumors, those who have venous thromboembolic disease at the time of presentation have a 1-year survival nearing only 10%, compared with 1-year survival of closer to 40% among those without thrombosis at diagnosis.[54] It is less clear whether this thrombotic tendency is simply a marker of aggressive disease or whether the development of thrombosis itself is the cause of the increased mortality. Thrombosis is second only to disease progression as a cause of death among cancer patients, and accounts for nearly 10% of deaths among patients receiving outpatient chemotherapy.[55] This mortality risk appears to be independent of cancer stage at the time of thrombus identification, and therefore may reflect the underlying cancer biology. Mucinous adenocarcinomas, for example, may directly release tissue factor (TF) and circulating mucin molecules that activate the clotting cascade and platelets, thereby promoting thrombosis.

Biologic Mechanisms Underlying Thrombosis in Cancer

The etiology of thrombosis is typically described in the context of the classic Virchow triad: stasis or altered blood flow, injury to the vessel wall, and hypercoagulability intrinsic to the blood and circulating plasma. Cancer can promote thrombosis by all three of these processes. Mechanical compression and/or disruption of blood vessels by a tumor may cause disrupted flow or stasis. Upon interaction with cancer cells, monocytes or macrophages release cytokines, including tumor necrosis factor, IL-1, and IL-6, which create a procoagulant state within the vessel.[56] The damaged vessel surface has reduced expression of naturally occurring anticoagulants, such as thrombomodulin, heparan sulfate, CD39/ecto-ADPase, nitric oxide, and prostacyclin.[57,58] The damaged endothelium also has increased expression of procoagulant proteins, particularly TF; circulating blood is exposed to the procoagulant subendothelial matrix, which contributes to a hypercoagulable state.

Despite the contributions of stasis and vessel changes in cancer-associated thrombosis, the best-documented contributing factor to thrombosis in the cancer patient is elevated procoagulant plasma factors, many of which are generated by the tumor itself. TF plays a critical role in the initiation of the coagulation cascade, particularly via the extrinsic pathway, by binding to and activating factor VII.[59] TF is increased in many malignancies, including pancreatic cancer, breast cancer, and non–small cell lung cancer, compared with the non-transformed epithelium.[60] Low oxygen levels are often found in the tumor microenvironment, which result in the activation and upregulation of proteins encoded by oncogenes, such as *RAS*[61] or *MET*,[62] and inactivation of tumor suppressor genes such as *PTEN*.[63] The downstream effect of this is to directly induce TF expression, as well as other genes involved in hemostasis. Targeting the human *MET* oncogene to mouse liver using a lentiviral vector results in hepatocarcinoma, associated with a coagulopathy similar to that in malignancy, with striking thrombotic events, similar to Trousseau syndrome, the migratory thrombophlebitis associated with malignancy.[64] Elevated TF expression and activation of the coagulation system appear to correlate with both thrombotic tendency and disease progression.

In addition to TF expression in tumors, activated endothelium, and tumor-associated macrophages, TF is also increased in platelets and microparticles from cancer patients compared with healthy controls.[65] The generation of TF-expressing microparticles may be an important mechanism of cancer hypercoagulability. Microparticles are vesicular structures, generally between 100 and 1000 nm in diameter, that are released from cell membranes under a variety of situations, including activation, malignant transformation, stress, or death.[66] They can be detected in plasma in a wide range of disease states, including sepsis and cancer. Microparticles have been reported to arise from platelets, monocytes, endothelial cells, and tumor cells, and carry on their surfaces a range of proteins that are derived from the cell of origin, including TF.[67] Cancer cells, directly and indirectly, lead to the generation and circulation of TF-bearing microparticles, and the levels of circulating TF-bearing microparticles in cancer patients appear to correlate with the subsequent risk for venous thromboembolic disease. In one study, approximately a third of patients with detectable TF-bearing microparticles developed venous thrombosis, compared with no thrombotic events in those without detectable TF-bearing microparticles.[68]

Coagulation and Tumor Progression

The activation of coagulation pathways in cancer may also play a role in tumor progression. For example, TF expression levels often correlate with a more aggressive tumor phenotype, and both clotting-dependent and clotting-independent signaling mechanisms of TF may be important in regulating tumor metastasis.[69,70] TF has been described to induce angiogenesis via upregulation of vascular endothelial growth factor (VEGF).[71]

Thrombin is the key terminal enzyme of the coagulation cascade, resulting in fibrin deposition and platelet activation. It also serves as a growth factor for a number of cell types, including fibroblasts, endothelial cells, smooth muscle cells, and tumor cells. The cellular effects of thrombin are mediated through seven transmembrane-spanning G protein–coupled protease-activated receptors (PARs), principally PAR1.[72] PAR1 is activated by thrombin through cleavage of its N-terminus, which exposes a tethered ligand that then binds to the second transmembrane domain of the receptor. PARs are expressed on platelets and other cells, as well as on tumor cells.[73] Thrombin-activated tumor cells have PAR1-dependent enhanced expression of a number of cell surface integrins.[74] Thrombin may enhance tumor progression via several mechanisms, including enhancing tumor cell proliferation, activating tumor–platelet adhesion, tumor adhesion to the matrix or endothelium, tumor implantation, growth, and metastasis, and tumor angiogenesis.[75] Thrombin alters tumor cell gene expression with upregulation of angiogenesis-related genes such as *VEGF*, matrix metalloproteinase 2 (*MMP-2*),[76] angiopoietin 2 (*ANG2*),[77] and other genes and microribonucleic acids affecting tumor cell proliferation and invasion.

Diagnosis of Coagulopathies in Cancer Patients

Thrombotic events in patients with cancer are diagnosed similarly to those in patients without cancer. However, several subtleties exist. For example, thrombosis may be confused with either intravascular disease progression or other processes such as intravascular fungal infections. In many cases, the diagnosis of thrombosis, or an abnormality in coagulation, may be made in an asymptomatic patient, leading to a more complicated decision regarding the risks and benefits of therapy. The process of fibrin formation and degradation is continuous in the setting of many patients with malignancy, and may relate to elevated thrombin-antithrombin complexes and fibrin degradation products. Thus, cancer is thought by some to be a process of "chronic DIC."[78] The clinical spectrum of coagulopathy in cancer ranges from asymptomatic to life-threatening thrombohemorrhagic disease. Thrombotic manifestations of DIC include arterial and VTE, migratory thrombophlebitis, marantic endocarditis, and microvascular thrombosis with organ failure or skin necrosis. The consumptive phase of DIC may manifest as bleeding, treatment of which is supportive and patient specific. Some may respond favorably to low-dose heparin, and/or plasma therapy, while waiting for treatment of the underlying malignancy. In general, the treatment of cancer-associated DIC is centered on the treatment of the underlying tumor and adjunctive care for any coagulopathic sequelae.

With increased use of high-resolution computed tomographic imaging studies, pulmonary emboli (PEs) are increasingly being identified incidentally, in the absence of clinical suspicion. This has led to the question of how to manage incidentally discovered thrombosis. A retrospective cohort study of the period from 2004 to 2010 compared the rate of recurrent VTE and overall survival between those patients whose PEs were detected incidentally on imaging studies with those whose PEs were suspected by classic clinical criteria; there was no difference in recurrent VTE or overall survival when both groups were anticoagulated.[79] A separate prospective observational study enrolled consecutive cancer patients newly diagnosed with combined deep venous thrombosis and PE from 2006 to 2009; 60% of the incidental thromboses were PEs, whereas only 26% of the symptomatic events were PEs. There was also a lower risk for recurrent thrombosis among those patients with an incidental thrombus compared with those with a symptomatic thrombus, and no differences in major bleeding and overall survival between groups.[80] Taken together, these two studies support the routine anticoagulation treatment of an incidentally found thrombosis, and provide some insight into the risk of recurrent thrombosis according to the mechanism of presentation.

Intravenous Catheter Thrombosis in Malignancy

Many patients with cancer undergo placement of a central venous catheter (CVC), commonly in the form of an implantable "port-a-cath" or "port," for chemotherapy infusion. Presence of an indwelling central line is a risk factor for thrombosis; this is augmented in patients with underlying cancer. Nearly 4% of patients with cancer who undergo port placement may develop a CVC-associated thrombosis, and the risk of thrombosis is increased with age, previous VTE, anemia, or leukocytosis prior to initiation of chemotherapy[81]; risk also varies according to the type of catheter, being higher with a peripherally inserted central catheter compared with an implanted portal catheter. Because of the high risk of bleeding in cancer patients, routine VTE prophylaxis is not recommended among cancer patients with CVCs, in spite of the known thrombotic risk. For those patients who develop CVC-associated thrombosis, general treatment recommendations are for anticoagulation for 3 months. Low-molecular-weight heparin (LMWH), apixaban, edoxaban, or rivaroxaban are preferred over the vitamin K antagonist, warfarin, in this population. Anticoagulation is continued while the line is in place. (See box on Management of Thrombosis in Patients With Cancer.)

TABLE 151.1 Studies of Direct Oral Anticoagulants in Patients With Cancer

Name of RCT (Author, Year of Publication)	DOAC Investigated	Population	VTE Recurrence Rates	Bleeding Rates
Hokusai VTE cancer trial (Raskob, 2018)	Edoxaban	1046 cancer patients with VTE at 114 centers in 13 countries, randomized to receive edoxaban or dalteparin	At 12 months, VTE recurrence was 7.9% in the edoxaban group vs. 11.3% in the dalteparin group (HR 0.71; 95% CI 0.48–1.06)	At 12 months, the rate of major bleeding was 6.9% in the edoxaban group vs. 4.0% in the dalteparin group (HR 1.77; 95% CI 1.03–3.04)
SELECT-D trial (Young, 2018)	Rivaroxaban	406 cancer patients with DVT or PE in the United Kingdom, randomized to receive rivaroxaban or dalteparin	At 6 months, VTE recurrence was 11% in the dalteparin group vs. 4% in the rivaroxaban group (HR 0.43; 95% CI, 0.19–0.99)	At 6 months, the rate of major bleeding was 4% in the dalteparin group vs. 6% in the rivaroxaban group (HR 1.83; 95% CI, 0.68–4.96)
ADAM VTE trial (McBane, 2019)	Apixaban	300 cancer patients with VTE at 28 sites in the United States, randomized to receive apixaban or dalteparin	At 6 months, VTE recurrence was 0.7% in the apixaban group vs. 6.3% in the dalteparin group (HR 0.099, 95% CI, 0.013–0.780)	At 6 months, the rate of major bleeding was 0% in the apixaban group vs. 1.4% in the dalteparin group ($P = .138$; HR and 95% CI not estimable)
Caravaggio (Agnelli, 2020)	Apixaban	1155 cancer patients at 119 centers in 9 European countries, Israel, and USA, randomized to receive apixaban or dalteparin	At 6 months, VTE recurrence was 5.6% in the apixaban group vs. 7.9% in the dalteparin group (HR 0.63; 95% CI, 0.37–1.07)	At 6 months, the rate of major bleeding was 3.8% in the apixaban group vs. 4.0% in the dalteparin group (HR 0.82; 95% CI, 0.4–1.69)

MANAGEMENT OF THROMBOSIS IN PATIENTS WITH CANCER

Cancer patients with a VTE have a high rate of recurrent thrombosis and bleeding after anticoagulation when compared with the general population, and merit a tailored treatment approach. In one study, the 12-month cumulative incidence of recurrent thromboembolism among cancer patients was 20.7% versus 6.8% in patients without cancer, whereas the 12-month cumulative incidence of major bleeding was 12.4% in patients with cancer versus 4.9% in patients without cancer.[82]

A variety of anticoagulants have been studied in the cancer population, including unfractionated heparin, weight-adjusted low molecular weight heparin (LMWH), warfarin, and direct oral anticoagulants (DOACs). Warfarin poses many difficulties for the general population, including numerous and often unpredictable drug interactions and a long half-life. These challenges are amplified in cancer patients for several reasons: variable nutritional intake of vitamin K, an altered bioavailability of dietary vitamin K due to frequent use of antibiotics, and a frequent need to interrupt anticoagulation for procedures and inconsistent degrees of thrombocytopenia.

The long-term use of LMWH in cancer patients has been compared with warfarin. In the CLOT trial, patients with cancer and VTE received dalteparin 200 IU/kg subcutaneously once daily for 5–7 days, and were then randomized to either continued dalteparin 150 IU/kg subcutaneously once daily, or to dose-adjusted warfarin with a target international normalized ratio of 2–3. Patients treated with dalteparin had a lower hazard ratio for recurrent thromboembolism compared with the oral-anticoagulant group (0.48); rates of major (6% vs. 4%) and all bleeding (14% vs. 19%) were similar. In this study, there was no difference in overall survival.[83] A similar study that compared enoxaparin with warfarin for treatment of venous thrombosis in cancer also showed an approximately 50% reduction of combined outcome of major bleeding or recurrent VTE within 3 months with enoxaparin compared with warfarin (10.5% vs. 21.1%).[84] Until recently, LMWH was generally preferred for cancer patients with VTE.

Oral anticoagulation agents that directly inhibit factor Xa or thrombin are increasingly used in the treatment of VTE. Four randomized, controlled trials of the anti-Xa agents rivaroxaban, apixaban, and edoxaban have been performed to date, with major findings summarized in Table 151.1.[85–88] In general, DOACs have been associated with lower rates of VTE recurrence among cancer patients and higher rates of major bleeding and clinically-relevant, non-major bleeding (CRNMB). As of this writing, the ASCO Clinical Practice Guidelines support the use of apixaban, rivaroxaban, or LMWH for thromboprophylaxis in selected high-risk outpatients with cancer. Safety concerns regarding these agents include the possible interactions with chemotherapeutic agents as well as the inability to reverse them quickly in a population that has potential to rapidly develop cytopenias and bleeding.

FUTURE DIRECTIONS

Future studies on hematopoiesis and coagulation in cancer patients have the potential to provide insight into new treatment strategies for the many hematologic manifestations of cancer, as well as the treatment of the underlying malignancy. Historically, management of the hematologic manifestations of malignancy have centered on supportive care; however, hematologic abnormalities themselves may also play a role in tumor survival and growth. A deeper understanding of how growth factors may play a role in tumor growth could identify new therapeutic targets and strategies. Similarly, platelets appear to play increasingly intricate roles in tumor metastasis and survival. Given the prevalence of thrombosis in cancer patients, new anticoagulation strategies will also prove increasingly important in the management of this unique population.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Aapro M, Jelkmann W, Constantinescu SN, et al. Effects of erythropoietin receptors and erythropoiesis-stimulating agents on disease progression in cancer. *Br J Cancer*. 2012;106(7):1249–1258.

Adamson J. The anemia of inflammation/malignancy: mechanisms and management. *Hematology*. 2008;1:159–165.

Agnelli G, Becattini C, Meyer G, et al. Apixaban for the treatment of venous thromboembolism associated with cancer. *N Engl J Med*. 2020;382(17):1599–1607.

Becker PS, Griffiths EA, Alwan LM, et al. NCCN Guidelines Insights: hematopoietic growth factors, version 1.2020: featured updates to the NCCN guidelines. *J Natl Compr Canc Netw*. 2020;18(1):12–22.

Boccaccio C, Sabatino G, Medico E, et al. The MET oncogene drives a genetic programme linking cancer to haemostasis. *Nature*. 2005;434 (7031):396–400.

Carmona-Bayonas A, Gomez J, Gonzalez-Billalabeitia E, et al. Prognostic evaluation of febrile neutropenia in apparently stable adult cancer patients. *Br J Cancer*. 2011;105(5):612–617.

den Exter PL, Hooijer J, Dekkers OM, et al. Risk of recurrent venous thromboembolism and mortality in patients with cancer incidentally diagnosed with pulmonary embolism: a comparison with symptomatic patients. *J Clin Oncol*. 2011;29(17):2405–2409.

Franco AT, Corken A, Ware J. Platelets at the interface of thrombosis, inflammation, and cancer. *Blood*. 2015;126(5):582–588.

Gilreath JA, Rodgers GM. How I treat cancer-associated anemia. *Blood*. 2020;136(7):801–813.

Heit JA, Spencer FA, White RH. The epidemiology of venous thromboembolism. *J Thromb Thrombolysis*. 2016;41(1):3–14.

Hisada Y, Mackman N. Tissue factor and cancer: regulation, tumor growth, and metastasis. *Semin Thromb Hemost*. 2019;45(4):385–395.

Khorana AA, Dalal M, Lin J, et al. Incidence and predictors of venous thromboembolism (VTE) among ambulatory high-risk cancer patients undergoing chemotherapy in the United States. *Cancer*. 2013;119(3):648–655.

Khorana A, Francis CW, Culakova E, et al. Thromboembolism is a leading cause of death in cancer patients receiving outpatient chemotherapy. *J Thromb Haemost*. 2007;5(3):632–634.

Lechner K, Obermeier HL. Cancer-related microangiopathic hemolytic anemia: clinical and laboratory features in 168 reported cases. *Medicine*. 2012;91(4):195–205.

Ludwig H, Van Belle S, Barrett-Lee P, et al. The European Cancer Anaemia Survey (ECAS): a large, multinational, prospective survey defining the prevalence, indicence, and treatment of anaemia in cancer patients. *Eur J Cancer*. 2004;40(15):2293–2306.

Lyman GH, Yau L, Nakov R, et al. Overall survival and risk of second malignancies with cancer chemotherapy and G-CSF support. *Ann Oncol*. 2018;29(9):1903–1910.

McBane 2nd RD, Wysokinski WE, Le-Rademacher JG, et al. Apixaban and dalteparin in active malignancy-associated venous thromboembolism: the ADAM VTE trial. *J Thromb Haemost*. 2020;18(2):411–421.

Meads MB, Hazelhurst LA, Dalton WS. The bone marrow microenvironment as a tumor sanctuary and contributor to drug resistance. *Clin Cancer Res*. 2008;14(9):2519–2526.

Miller CB, Jones RJ, Piantadosi S, et al. Decreased erythropoietin response in patients with the anemia of cancer. *N Engl J Med*. 1990;322(24):1689–1692.

Prandoni P, Lensing AWA, Piccioli A, et al. Recurrent venous thromboembolism and bleeding complications during anticoagulant treatment in patients with cancer and venous thrombosis. *Blood*. 2002;100(10):3484–3488.

Rizzo JD, Brouwers M, Hurley P, et al. American Society of Hematology/American Society of Clinical Oncology clinical practice guideline update on the use of epoetin and darbepoetin in adult patients with cancer. *Blood*. 2010;116(20):4045–4059.

Sallah S, Wan JY, Nguyen NP. Venous thrombosis in patients with solid tumors: determination of frequency and characteristics. *Thromb Haemost*. 2002; 87(4):575–579.

Simanek R, Vormittag R, Ay C, et al. High platelet count associated with venous thromboembolism in cancer patients: results from the Vienna Cancer and Thrombosis Study (CATS). *J Thromb Haemost*. 2010;8(1):114–120.

Sørensen HT, Mellemkjaer L, Olsen JH, et al. Prognosis of cancers associated with venous thromboembolism. *N Engl J Med*. 2000;343(25): 1846–1850.

Stanworth SJ, Estcourt LJ, Powter G, et al. A no-prophylaxis platelet-transfusion strategy for hematologic cancers. *N Engl J Med*. 2013; 368(19):1771–1780.

Taplitz RA, Kennedy EB, Bow EJ, et al. Antimicrobial prophylaxis for adult patients with cancer-related immunosuppression: ASCO and IDSA Clinical Practice Guideline Update. *J Clin Oncol*. 2018;36(30):3043–3054.

Tsopanoglou NE, Maragoudakis ME. Role of thrombin in angiogenesis and tumor progression. *Semin Thromb Hemost*. 2004;30(1):63–69.

Walker AJ, Card TR, West J, et al. Incidence of venous thromboembolism in patients with cancer—a cohort study using linked United Kingdom databases. *Eur J Cancer*. 2013;49(6):1404–1413.

Young AM, Marshall A, Thirlwall J, et al. Comparison of an oral factor Xa inhibitor with low molecular weight heparin in patients with cancer with venous thromboembolism: results of a randomized trial (SELECT-D). *J Clin Oncol*. 2018;36(20):2017–2023.

Zwicker JI, Liebman HA, Neuberg D, et al. Tumor-derived tissue factor-bearing microparticles are associated with venous thromboembolic events in malignancy. *Clin Cancer Res*. 2009;15(22):6830–6840.

REFERENCES

1. Knight K, Wade S, Balducci L. Prevalence and outcomes of anemia in cancer: a systematic review of the literature. *Am J Med*. 2004;116(7):11–26. Supplement 1.
2. Väyrynen JP, Tuomisto A, Väyrynen SA, et al. Preopoerative anemia in colorectal cancer: relationships with tumor characteristics, systemic inflammation, and survival. *Sci Rep*. 2018;8(1):1126.
3. Ludwig H, Müldür E, Endler G, et al. Prevalence of iron deficiency across different tumors and its association with poor performance status, disease status and anemia. *Ann Oncol*. 2013;24(7):1886–1892.
4. Adamson J. The anemia of inflammation/malignancy: mechanisms and management. *Hematology*. 2008;1:159–165.
5. Lippitz B. Cytokine patterns in patients with cancer: a systematic review. *Lancet Oncol*. 2013;14(6):e218–e228.
6. Miller CB, Jones RJ, Piantadosi S, et al. Decreased erythropoietin response in patients with the anemia of cancer. *N Engl J Med*. 1990;322(24):1689–1692.
7. Vela D, Vela-Gaxha Z. Differential regulation of hepcidin in cancer and non-cancer tissues and its clinical implications. *Exp Mol Med*. 2018;50(2). e436-e436.
8. Ludwig H, Van Belle S, Barrett-Lee P, et al. The European Cancer Anaemia Survey (ECAS): a large, multinational, prospective survey defining the prevalence, incidence, and treatment of anaemia in cancer patients. *Eur J Cancer*. 2004;40(15):2293–2306.
9. Groopman JE, Itri LM. Chemotherapy-induced anemia in adults: incidence and treatment. *J Natl Cancer Inst*. 1999;91(19):1616–1634.
10. Lechner K, Obermeier HL. Cancer-related microangiopathic hemolytic anemia: clinical and laboratory features in 168 reported cases. *Medicine (Baltimore)*. 2012;91(4):195–205.
11. Bendapudi PK, Li A, Hamdan A, et al. Impact of severe ADAMTS13 deficiency on clinical presentation and outcomes in patients with thrombotic microangiopathies: the experience of the Harvard TMA Research Collaborative. *Br J Haematology*. 2015;171(5):836–844.
12. Rizzo JD, Brouwers M, Hurley P, et al. American Society of Hematology/American Society of Clinical Oncology clinical practice guideline update on the use of epoetin and darbepoetin in adult patients with cancer. *Blood*. 2010;116(20):4045–4059.
13. Aapro M, Moebus V, Nitz U, et al. Safety and efficacy outcomes with erythropoiesis-stimulating agents in patients with breast cancer: a meta-analysis. *Ann Oncol*. 2015;26(4):688–695.
14. Hedley BD, Allan AL, Xenocostas A. The role of erythropoietin and erythropoiesis-stimulating agents in tumor progression. *Clin Cancer Res*. 2011;17(20):6373–6380.
15. Aapro M, Jelkmann W, Constantinescu SN, et al. Effects of erythropoietin receptors and erythropoiesis-stimulating agents on disease progression in cancer. *Br J Cancer*. 2012;106(7):1249–1258.
16. Gilreath JA, Rodgers GM. How I treat cancer-associated anemia. *Blood*. 2020;136(7):801–813.
17. Giagounidis A. Current treatment algorithm for the management of lower-risk MDS. *Hematology*. 2017;2017(1):453–459.
18. Wiesener MS, Seyfarth M, Warnecke C, et al. Paraneoplastic erythrocytosis associated with an inactivating point mutation of the von Hippel-Lindau gene in a renal cell carcinoma. *Blood*. 2002;99(10):3562–3565.
19. Hitron A, Steinke D, Sutphin S, et al. Incidence and risk factors of clinically significant chemotherapy-induced thrombocytopenia in patients with solid tumors. *J Oncol Pharm Pract*. 2011;17(4):312–319.
20. Curtis BR, Kaliszewski J, Marques MB, et al. Immune-mediated thrombocytopenia resulting from sensitivity to oxaliplatin. *Am J Hematol*. 2006;81(3):199–201.
21. Stanworth SJ, Estcourt LJ, Powter G, et al. A no-prophylaxis platelet-transfusion strategy for hematologic cancers. *N Engl J Med*. 2013;368(19):1771–1780.
22. Winer ES, Safran H, Karaszewska B, et al. Eltrombopag for thrombocytopenia in patients with advanced solid tumors receiving gemcitabine-based chemotherapy: a randomized, placebo-controlled phase 2 study. *Int J Hematol*. 2017;106(6):765–776.
23. Dickinson M, Cherif H, Fenaux P, et al. Azacitidine with or without eltrombopag for first-line treatment of intermediate- or high-risk MDS with thrombocytopenia. *Blood*. 2018;132(25):2629–2638.
24. Simanek R, Vormittag R, Ay C, et al. High platelet count associated with venous thromboembolism in cancer patients: results from the Vienna Cancer and Thrombosis Study (CATS). *J Thrombosis Haemost*. 2010;8(1):114–120.
25. Franco AT, Corken A, Ware J. Platelets at the interface of thrombosis, inflammation, and cancer. *Blood*. 2015;126(5):582–588.
26. Nash GF, Turner L, Scully MF, et al. Platelets and cancer. *Lancet Oncol*. 2002;3(7):425–430.
27. Crawford J, Dale DC, Lyman GH. Chemotherapy-induced neutropenia: risks, consequences, and new directions for its management. *Cancer*. 2004;100(2):228–237.
28. Carmona-Bayonas A, Gómez J, González-Billalabeitia E, et al. Prognostic evaluation of febrile neutropenia in apparently stable adult cancer patients. *Br J Cancer*. 2011;105(5):612–617.
29. Klastersky J, Paesmans M, Rubenstein EB, et al. The multinational association for supportive care in cancer risk index: a multinational scoring system for identifying low-risk febrile neutropenic cancer patients. *J Clin Oncol*. 2000;18(16):3038–3051.
30. Aapro M, Bohlius J, Cameron DA, et al. 2010 Update of EORTC guidelines for the use of granulocyte-colony stimulating factor to reduce the incidence of chemotherapy-induced febrile neutropenia in adult patients with lymphoproliferative disorders and solid tumours. *Eur J Cancer*. 2011;47(1):8–32.
31. Crawford J, Becker PS, Armitage JO, et al. Myeloid growth factors, version 2.2017, NCCN Clinical Practice Guidelines in Oncology. *J Natl Compr Cance Netw*. 2017;15(12):1520–1541.
32. Taplitz RA, Kennedy EB, Bow EJ, et al. Antimicrobial prophylaxis for adult patients with cancer-related immunosuppression: ASCO and IDSA Clinical Practice Guideline Update. *J Clin Oncol*. 2018;36(30):3043–3054.
33. Becker PS, Griffiths EA, Alwan LM, et al. NCCN Guidelines Insights: hematopoietic growth factors, version 1.2020: featured updates to the NCCN guidelines. *J Natl Compr Canc Netw*. 2020;18(1):12–22.
34. Beekman R, Touw IP. G-CSF and its receptor in myeloid malignancy. *Blood*. 2010;115(25):5131–5136.
35. Lyman GH, Dale DC, Wolff DA, et al. Acute myeloid leukemia or myelodysplastic syndrome in randomized controlled clinical trials of cancer chemotherapy with granulocyte colony-stimulating factor: a systematic review. *Cent Rev Dissem (UK)*. 2010
36. Lyman GH, Yau L, Nakov R, et al. Overall survival and risk of second malignancies with cancer chemotherapy and G-CSF support. *Ann Oncol*. 2018;29(9):1903–1910.
37. Haddy TB, Rana SR, Castro O. Benign ethnic neutropenia: what is a normal absolute neutrophil count? *J Lab Clin Med*. 1999;133(1):15–22.
38. Rappoport N, SImon AJ, Amariglio N, et al. The Duffy antigen receptor for chemokines, ACKR1—"Jeanne DARC" of benign neutropenia. *Br J Haematol*. 2019;184(4):497–507.
39. Anner RM, Drewinko B. Frequency and significance of bone marrow involvement by metastatic solid tumors. *Cancer*. 1977;39(3):1337–1344.
40. Pantel K, Angstwurm M, Riethmüller G, et al. Frequency and prognostic significance of isolated tumour cells in bone marrow of patients with non-small-cell lung cancer without overt metastases. *Lancet*. 1996;247(9002):649–653.
41. Braun S, Pantel K, Müller P, et al. Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II, or III breast cancer. *N Engl J Med*. 2000;342(8):525–533.
42. Giuliano AE, Hawes D, Ballman KV, et al. Association of occult metastases in sentinel lymph nodes and bone marrow with survival among women with early-stage invasive breast cancer. *JAMA*. 2011;306(4):385–393.
43. Meads MB, Hazelhurst LA, Dalton WS. The bone marrow microenvironment as a tumor sanctuary and contributor to drug resistance. *Clin Cancer Res*. 2008;14(9):2519–2526.
44. Woelfle U, Cloos J, Sauter G, et al. Molecular signature associated with bone marrow micrometastasis in human breast cancer. *Cancer Res*. 2003;63(18):5679–5684.
45. Shiozawa Y, Pedersen EA, Havens AM, et al. Human prostate cancer metastases target the hematopoietic stem cell niche to establish footholds in mouse bone marrow. *J Clin Invest*. 2011;121(4):1298–1312.
46. Stein PD, Beemath A, Meyers FA, et al. Incidence of venous thromboembolism in patients hospitalized with cancer. *Am J Med*. 2006;119(1):60–68.
47. Martino MA, Borges E, Williamson E, et al. Pulmonary embolism after major abdominal surgery in gynecologic oncology. *Obstet Gynecol*. 2006;107(3):666–671.
48. Sallah S, Wan JY, Nguyen NP. Venous thrombosis in patients with solid tumors: determination of frequency and characteristics. *Thromb Haemost*. 2002;87(4):575–579.
49. Khorana AA, Dalal M, Lin J, et al. Incidence and predictors of venous thromboembolism (VTE) among ambulatory high-risk cancer patients undergoing chemotherapy in the United States. *Cancer*. 2013;119(3):648–655.
50. Laporte S, Mismetti P, Décousus H, et al. Clinical predictors for fatal pulmonary embolism in 15,520 patients with venous thromboembolism: findings from the Registro Informatizado de la Enfermedad TromboEmbolica venosa (RIETE) Registry. *Circulation*. 2008;117(13):1711–1716.
51. Heit JA, Spencer FA, White RH. The epidemiology of venous thromboembolism. *J Thromb Thrombolysis*. 2016;41(1):3–14.

52. Simone B, De Stefano V, Leoncini E, et al. Risk of venous thromboembolism associated with single and combined effects of Factor V Leiden, Prothrombin 20210A and Methylenetethraydrofolate reductase C677T: a meta-analysis involving over 11,000 cases and 21,000 controls. *Eur J Epidemiol*. 2013;28(8): 621–647.
53. Walker AJ, Card TR, West J, et al. Incidence of venous thromboembolism in patients with cancer—a cohort study using linked United Kingdom databases. *Eur J Cancer*. 2013;49(6):1404–1413.
54. Sørensen HT, Mellemkjaer L, Olsen JH, et al. Prognosis of cancers associated with venous thromboembolism. *N Engl J Med*. 2000;343(25): 1846–1850.
55. Khorana AA, Francis CW, Culakova E, et al. Thromboembolism is a leading cause of death in cancer patients receiving outpatient chemotherapy. *J Thromb Haemost*. 2007;5(3):632–634.
56. Bauer KA, ten Cate H, Barzegar S, et al. Tumor necrosis factor infusions have a procoagulant effect on the hemostatic mechanism of humans. *Blood*. 1989;74(1):165–172.
57. Blann AD. How a damaged blood vessel wall contributes to thrombosis and hypertension. *Pathophysiol Haemost Thromb*. 2003;33(5-6):445–448.
58. Kanthi YM, Sutton NR, Pinsky DJ. CD39: interface between vascular thrombosis and inflammation. *Curr Atheroscler Rep*. 2014;16(7):425.
59. Rondon AMR, Kroone C, Kapteijn MY, et al. Role of tissue factor in tumor progression and cancer-associated thrombosis. *Semin Thromb Hemost*. 2019;45(4):396–412.
60. Hisada Y, Mackman N. Tissue factor and cancer: regulation, tumor growth, and metastasis. *Semin Thromb Hemost*. 2019;45(4):385–395.
61. Zeng M, Kikuchi H, Pino MS, et al. Hypoxia activates the K-ras proto-oncogene to stimulate angiogenesis and inhibit apoptosis in colon cancer cells. *PLoS One*. 2010;5(6):e10966.
62. Pennacchietti S, Michieli P, Galluzzo M, et al. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. *Cancer Cell*. 2003;3(4):347–361.
63. Kohnoh T, Hashimoto N, Ando A, et al. Hypoxia-induced modulation of PTEN activity and EMT phenotypes in lung cancers. *Cancer Cell Int*. 2016;16:33.
64. Boccaccio C, Sabatino G, Medico E, et al. The MET oncogene drives a genetic programme linking cancer to haemostasis. *Nature*. 2005;434 (7031):396–400.
65. Tesselaar ME, Romijn FP, Van Der Linder IK, et al. Microparticle-associated tissue factor activity: a link between cancer and thrombosis? *J Thromb Haemost*. 2007;5(3):520–527.
66. Morel O, Toti F, Hugel B, et al. Cellular microparticles: a disseminated storage pool of bioactive vascular effectors. *Curr Opin Hematol*. 2004; 11(3):156–164.
67. Nomura S, Ozaki Y, Ikeda Y. Function and role of microparticles in various clinical settings. *Thromb Res*. 2008;123(1):8–23.
68. Zwicker JI, Liebman HA, Neuberg D, et al. Tumor-derived tissue factor-bearing microparticles are associated with venous thromboembolic events in malignancy. *Clin Cancer Res*. 2009;15(22):6830–6840.
69. Poon RT-P, Lau CP-Y, Ho W-YJ, et al. Tissue factor expression correlates with tumor angiogenesis and invasiveness in human hepatocellular carcinoma. *Clin Cancer Res*. 2003;9(14):5339–5345.
70. Bromberg ME, Konigsberg WH, Madison JF, et al. Tissue factor promotes melanoma metastasis by a pathway independent of blood coagulation. *Proc Natl Acad Sci USA*. 1995;92(18):8205–8209.
71. Ollivier V, Bentolila S, Chabbat J, et al. Tissue factor-dependent vascular endothelial growth factor production by human fibroblasts in response to activated factor VII. *Blood*. 1998;91(8):2698–2703.
72. Ayala YM, Cantwell AM, Rose T, et al. Molecular mapping of thrombin-receptor interactions. *Proteins*. 2001;45(2):107–116.
73. Boire A, Covic L, Agarwal A, et al. PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. *Cell*. 2005;120(3):303–313.
74. Even-Ram SC, Maoz M, Pokroy E, et al. Tumor cell invasion is promoted by activation of protease activated receptor-1 in cooperation with the alpha vbeta 5 integrin. *J Biol Chem*. 2001;276(14):10952–10962.
75. Tsopanoglou NE, Maragoudakis ME. Role of thrombin in angiogenesis and tumor progression. *Semin Thromb Hemost*. 2004;30(1):63–69.
76. Smiljanic K, Obradovic M, Jovanovic A, et al. Thrombin stimulates VSMC proliferation through an EGFR-dependent pathway: involvement of MMP-2. *Mol Cell Biochem*. 2014;396(1-2):147–160.
77. Caunt M, Huang YQ, Brooks PC, et al. Thrombin induces neoangiogenesis in the chick chorioallantoic membrane. *J Thromb Haemost*. 2003;1(10):2097–2102.
78. Levi M. Management of cancer-associated disseminated intravascular coagulation. *Thromb Res*. 2016;140(suppl 1):S66–S70.
79. den Exter PL, Hooijer J, Dekkers OM, et al. Risk of recurrent venous thromboembolism and mortality in patients with cancer incidentally diagnosed with pulmonary embolism: a comparison with symptomatic patients. *J Clin Oncol*. 2011;29(17):2405–2409.
80. Font C, Farrús B, Vidal L, et al. Incidental versus symptomatic venous thrombosis in cancer: a prospective observational study of 340 consecutive patients. *Ann Oncol*. 2011;22(9):2101–2106.
81. Decousus H, Bourmaud A, Fournel P, et al. Cancer-associated thrombosis in patients with implanted ports: a prospective multicenter French cohort study (ONCOCIP). *Blood*. 2018;132(7):707–716.
82. Prandoni P, Lensing AWA, Piccioli A, et al. Recurrent venous thromboembolism and bleeding complications during anticoagulant treatment in patients with cancer and venous thrombosis. *Blood*. 2002;100 (10):3484–3488.
83. Lee AYY, Levine MN, Baker RI, et al. Low-molecular-weight heparin versus a coumarin for the prevention of recurrent venous thromboembolism in patients with cancer. *N Engl J Med*. 2003;349(2):146–153.
84. Meyer G, Marjanovic Z, Valcke J, et al. Comparison of low-molecular-weight heparin and warfarin for the secondary prevention of venous thromboembolism in patients with cancer: a randomized controlled study. *Arch Intern Med*. 2002;162(15):1729–1735.
85. Raskob GE, van Es N, Verhamme P, et al. Edoxaban for the treatment of cancer-associated venous thromboembolism. *N Engl J Med*. 2018; 378(7):615–624.
86. Young AM, Marshall A, Thirlwall J, et al. Comparison of an oral factor Xa inhibitor with low molecular weight heparin in patients with cancer with venous thromboembolism: results of a randomized trial (SELECT-D). *J Clin Oncol*. 2018;36(20):2017–2023.
87. McBane 2nd RD, Wysokinski WE, Le-Rademacher JG, et al. Apixaban and dalteparin in active malignancy-associated venous thromboembolism: The ADAM VTE trial. *J Thromb Haemost*. 2020;18(2):411–421.
88. Agnelli G, Becattini C, Meyer G, et al. Apixaban for the treatment of venous thromboembolism associated with cancer. *N Engl J Med*. 2020;382(17):1599–1607.

CHAPTER 152

HEMATOLOGIC MANIFESTATIONS OF HIV/AIDS

Maryam Own and James B. Bussel

Human immunodeficiency virus (HIV) infection is associated with a number of hematologic complications throughout the course of the disease. To a certain extent, these complications depend on the stage of the disease and how well controlled HIV infection is but to a certain extent it does not. In untreated infection, the major hematologic complications were cytopenias, either isolated or multiple simultaneously. As combination antiretroviral therapy (ART) became standard of care in developed countries, cytopenias were observed far less frequently, and other complications were seen as a function of a longer life span. In particular, these are lymphomas and venous thromboembolic events; Kaposi sarcoma is not covered in this chapter because it is not a hematologic malignancy. Therefore, this review will focus on lymphoma (both Hodgkin and non-Hodgkin including acquired immunodeficiency syndrome [AIDS]-defining lymphomas) and on thromboembolic events with a section on cytopenias (Table 152.1). The introduction will be a state of the art description of HIV itself and will focus on adults.

INTRODUCTION TO HUMAN IMMUNODEFICIENCY VIRUS

An estimated 38 million people are living with HIV worldwide, of which approximately 25.4 million are on ART. Most cases currently come from sub-Saharan Africa, followed by the Caribbean. In 2019, there were 1.7 million new infections documented and 690,000 AIDS-related deaths recorded, with 23% attributed to men who have sex with men (MSM), 19% to clients of sex workers and their partners, 8% to sex workers themselves, 10% to drug use, 2% to transgender individuals, and the remaining 38% were unknown in the report (other factors include sexual assault of women and vertical transmission). Compared to 2010, 2019 witnessed a 23% reduction in the incidence of HIV and a 39% reduction in AIDS-related mortality. Furthermore, when looking at patients from 2015 to 2019, 59% of patients had an undetectable viral load. Despite substantial global strides in achieving control of HIV, there are fears expressed by the Joint United Nations Programme on HIV/AIDS (UNAIDS) driven by adverse consequences of COVID-19 on HIV, as a result of which 20% of individuals were unable to access therapy for longer than 6 months. As a result, UNAIDS projects an increase of 110,000 AIDS-related deaths in the upcoming 2 years.[1]

In the United States,[2] the incidence of HIV between 2014 and 2018 was 36,400 per year diagnosed with HIV due to any cause. Of the newly diagnosed cases, 66% were attributed to MSM, 24% to heterosexual contact, 7% to drug use, and 4% to a combination of MSM and drug use. When stratified by race/ethnicity, new HIV infections were found in 43% Black/African American, 26% Hispanic/Latino, 26% White, and 6% Other. This highlights a racial/ethnic disparity in healthcare, as Blacks/African Americans account for only 13% of the general population. Furthermore, there were evident disparities in geographical locations within the United States, where 52% of all the cases were in the South, 15% in the Northeast, 13% in the Midwest, and 20% in the West of the United States. The geographical disparity of cases in the US may be related to poverty, lack of access to health care, lack of appropriate sex education, or other (unknown) factors. These are all issues that pertain to the application of management strategies in the future.

AIDS is caused by two lentiviruses, HIV types 1 and 2 (HIV-1 and HIV-2) were first documented in the United States in 1981 when clusters of Kaposi sarcoma and pneumocystis jiroveci pneumonia in homosexual men were identified. HIV-1, a single-stranded RNA retrovirus with multiple names, was identified as the culprit. It is believed to have been zoonotically transmitted to humans from African primates of chimpanzee origin in central Africa, harboring simian immunodeficiency virus (SIV) (40% to 60% DNA homology to HIV-1).[3] Although there are no confirmed direct cases of such transmissions, HIV's cross-over to humans is attributed to the exposure of blood from hunting bushmeat, similar to other zoonoses (i.e., Ebolavirus and SARS-CoV-2). HIV-1 consists of four distinct lineages, termed groups M, N, O, and P, each of which resulted from independent cross-species transmission events. The majority of HIV infections are caused by the M group, which is further subdivided into 11 clades: A to K. Subtype B is the predominant type in the Western world, whereas subtype C is dominant in southern Africa. The lesser-known virus, HIV-2, also from the same family and often not accounted for when speaking about "HIV," is mostly found in West Africa and is theorized to have been zoonotically transmitted from sooty mangabey monkeys.[4] It has six phylogenetic lineages, A to F. HIV-2 is characterized by low virulence, lower viral loads (in the thousands rather than millions), and individuals that remain as "long term non-progressors" to AIDS. When individuals do progress, the process mimics HIV-1 in pathology.[5] One needs to be aware of HIV-2, especially when immunodeficiency is suspected in an individual from West Africa, as the fourth generation immunoassay for HIV detection in the United States only tests for HIV-1; the earlier tests identified both.

As mentioned above, most HIV is transmitted sexually. In the US, it is mostly found in MSM, whereas in sub-Saharan Africa, it is transmitted mostly via heterosexual penile-vaginal sex. Exposure to infected blood via injected drug use is another common way to acquire HIV. In the past, hemophiliacs and thalassemics also acquired HIV through contaminated blood transfusions in the 1970s and 1980s before the virus was identified, and other transfused individuals receiving multiple single donor products also were infected. Currently, screening of donors and viral eradication in plasma products has made this mode of viral acquisition very rare to essentially non-existent. Mothers can also transmit HIV either perinatally (maternal-fetal hemorrhage of blood or swallowed blood during delivery) or to neonates via breastmilk. Individuals are at an increased risk of contracting HIV if the exposure involves a high viral load (in the acute phase of the infection) or having damage to the mucosa thus providing access to the virus; this can be seen in co-infections with other sexually transmitted infections (STIs) or in unprotected receptive anal sex. Factors that are protective against acquiring HIV include the use of protection during intercourse and male circumcision since foreskin harbors a concentration of Langerhans cells and T cells that are HIV targets.[6] Protective factors preventing the host from transmitting HIV include achieving an undetectable viral load by the host through compliance with ART. This includes the use of ART during pregnancy and delivery to prevent vertical transmission to the neonate, reducing risk from 25.5% to 8.3%[7] in the first study, with further reductions seen in subsequent studies. Sexual partners of HIV-positive patients can also reduce their risk by taking pre-exposure prophylaxis (PrEP) consisting of tenofovir disoproxil with emtricitabine or lamivudine. If an individual is exposed to a high-risk sexual encounter or a needle-stick injury,

Downl
15, 202

TABLE 152.1 **Hematologic Complications of Human Immunodeficiency Virus Infection**

Cytopenias including human immunodeficiency virus (HIV)-immune thrombocytopenia, anemia, and other cytopenias
Lymphoma
Non-Hodgkin B cell lymphoma
Acquired immunodeficiency syndrome (AIDS)-defining lymphoma
Hodgkin lymphoma
T-cell lymphoma
Thromboembolic events
Venous
Arterial

that is, healthcare workers, then post-exposure prophylaxis (PEP) of 28 days duration with tenofovir + emtricitabine with raltegravir is indicated. The efficacy of PEP has been observed in retrospective studies, where a Cochrane systematic review concluded that HIV-positive individuals were less likely to have been taking PEP (OR 0.19, 95% CI 0.06 to 0.52).[8]

HIV is composed of a viral capsule constructed of glycoproteins gp120 and gp41 that form a lipid bilayer. These glycoproteins facilitate entry into cells by binding to the CD4 receptor and chemokine co-receptors CXCR4 and/or CCR5. Within the viral capsule, there are two positive-sense, single-stranded RNA strands that carry nine genes. Of those, three are integral to viral replication and survival: gag, pol, and env genes transcribe core proteins p24 and p17, reverse transcriptase and integrase, and the glycoproteins respectively. In the cytoplasm, the enzyme reverse transcriptase generates a single strand of DNA from which a double DNA strand (dsDNA) is transcribed. dsDNA enters the nucleus, and viral integrase integrates the viral DNA into the host cell's DNA. From then on, the virus uses the host's machinery to replicate. The viral protease enzyme is vital in the final step of cleaving gag and gag-pol precursor proteins into the functional units known as gp120 and gp41.[9]

HIV not only infects T cells and myeloid cells (monocyte/macrophages) but also glial cells in the central nervous system (CNS). Furthermore, there are resting memory CD4 T cells that contain HIV but do not contribute to viral replication, making up a "latent HIV reservoir" posing important challenges to the eradication of the virus despite the ability to achieve an undetectable viral load with ART.[10] This latter can be very important in that CNS virus and/or HIV in other extra-vascular locations may not be eradicated by highly active antiretroviral therapy (HAART) and contributes to viral resurgence and ongoing inflammation. Targeting viral entry as a therapeutic strategy gained traction when a CCR5-delta 32 mutation, found in populations of European descent, conferred resistance to acquiring HIV infection in homozygotes by inhibiting viral entry.[11] Entry inhibitors have been developed to target the CCR5. Further, this was proven in vivo in the only two cases cured of HIV after receiving hematopoietic stem cell transplantation selected for the CCr5-delta 32 mutation as part of the treatment for acute myeloid leukemia and Hodgkin lymphoma.[12] In 2018, a physicist in China used CRISPR-Cas9 gene-editing technology to genetically engineer twin girls with knockout CCR5 to prevent them from contracting HIV from their father.[13] In addition to the questionable clinical reasoning and the unethical nature of his actions, one could speculate that the twin girls may be at increased risk of autoimmune diseases, as *CCr5-delta 32* mutation has been associated with MS[14] and lupus nephritis.[15] Nonetheless, these cases demonstrate the utility of targeting viral entry as a therapeutic modality although once an infection has occurred, the previously described latent cells may vitiate this strategy.

In the United States, the 4th generation enzyme immunoassay is the standard HIV screening test used to detect HIV in the acute setting, where it is able to detect HIV IgM, IgG antibodies, and p24 antigen 15 to 20 days after exposure in comparison to the first-generation enzyme immunoassay, which only detected IgG after 35 to 45 days.[16] The gold standard confirmatory testing for HIV remains the western blot, which has a window of 45 to 60 days. The presence of neutralizing antibodies has raised the question of a possible vaccine, which to this day remains a very active but puzzlingly unsolved area of research. One of the first vaccines developed in 1998 targeted neutralizing antibodies against gp120. Despite achieving high titers in the serum, the vaccine did not work as the targeted region V3 was shielded within a trimeric structure rendering it inaccessible. Surprisingly, in vivo animal experiments demonstrated that neutralizing gp120 antibodies were able to prevent HIV infection in pig-tailed macaques receiving IgG from HIV-1 infected chimpanzees.[17] Other explanations for the lack of neutralizing antibodies in humans include the mutation rate of the HIV reverse transcriptase, giving HIV the ability to escape the host's immune system. Furthermore, there are many clades and subtypes of the virus, another factor making a universal vaccine even more challenging.[18]

The highest viral load is seen in the acute infection phase, accompanied by a drop in the CD4 count. The viral load starts to decline after approximately 4 weeks because of the cytotoxic T-cell immune response. The CD4 count then starts to increase but does not completely recover to what it was prior to the infection, referred to as an immunological set point. This is followed by the asymptomatic phase, which can last for a decade. Without anti-HIV treatment, the virus continues to replicate, and the CD4 T cell count continues to decline until an AIDS-defining opportunistic infection (OI) or a malignancy causes death. AIDS, by definition, occurs either when the CD4 T cell count is below 200 or by having one of the AIDS-defining illnesses irrespective of the CD4 T cell count. This "natural history" of untreated HIV infection is mitigated by starting antiretroviral therapy, where the CD4 T cell count starts to climb up and the HIV plasma viral load declines, potentially to undetectable levels using commercial assays with sensitivity typically down to 50 HIV viral copies/mL, thus reducing morbidity and mortality drastically (see Fig. 152.2).

Currently, HIV is treated with ART targeting the replication process at various stages from entry to maturity. There are nucleoside/nucleotide reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), integrase inhibitors (INSTI), and protease inhibitors (PI). Additional ART includes entry inhibitors that prevent the virus from entering the targeted cells. These include enfuvirtide (Fuzeon) and maraviroc (Selzentry). Most HIV-infected persons are on 2 NRTI and 1 INSTI. In the past, a person with HIV took 13 or more pills a day, which in addition to side effects, added challenges to medication compliance. Nowadays, patients with HIV take 1 to 4 pills/day depending on the presence of co-infections with hepatitis B or C and the genotypic profile of HIV. Newer medications also offer an improved side effect profile, particularly for cytopenic effects, and there are single pills formulated to contain all of the antiretroviral therapy (Table 152.2). Very recently, long-acting ART was approved, involving a monthly injection.

Antiretroviral therapy protects against opportunistic infections by restoring the CD4 T cell count and suppressing viral replication. The increase in CD4 T cell counts when starting ART can, in a minority of patients (~20%), sometimes lead to a paradoxical effect known as the immune reconstitution inflammatory syndrome (IRIS), especially if the individual had a low CD4 count or a high viral load. IRIS is a strong immune response characterized by cytokine release leading to an array of clinical syndromes. IRIS can resemble an opportunistic infection when the target is an occult infection that has now become unmasked in the setting of increased immune surveillance, that is, TB leading to constitutional symptoms or untreated hepatitis B leading to liver failure; or an autoimmune phenomenon, where the recovery of the immune system aggravates a pre-existing autoimmune disease, that is, thyroiditis, similar to the use of checkpoint inhibition. Depending on the cause, its management can target treatment of the inciting infection, which sometimes necessitates interrupting ART until treatment is completed if concurrent treatment is not possible. IRIS can resolve on its own if it arose from a previously recently adequately treated opportunistic infection, where the immune system precipitously recognizes remnants of it. To this end, IRIS remains

Downl
15, 202

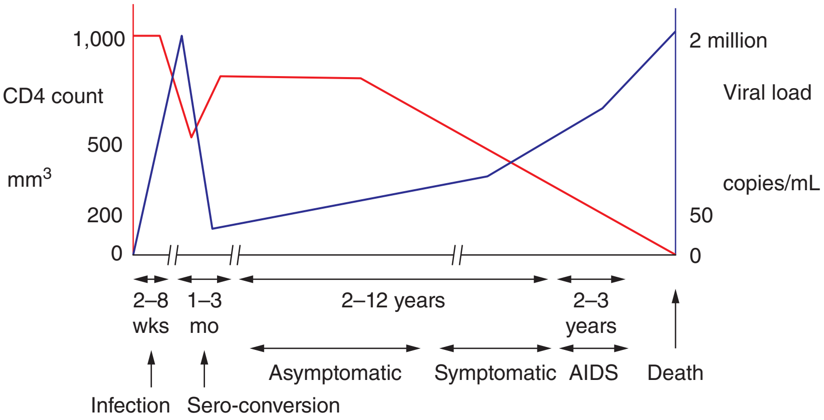


Figure 152.1 CD4 COUNT AND VIRAL LOAD WITHOUT ANTIRETROVIRAL THERAPY (ART). *(From HIV iBase, London, United Kingdom.)*

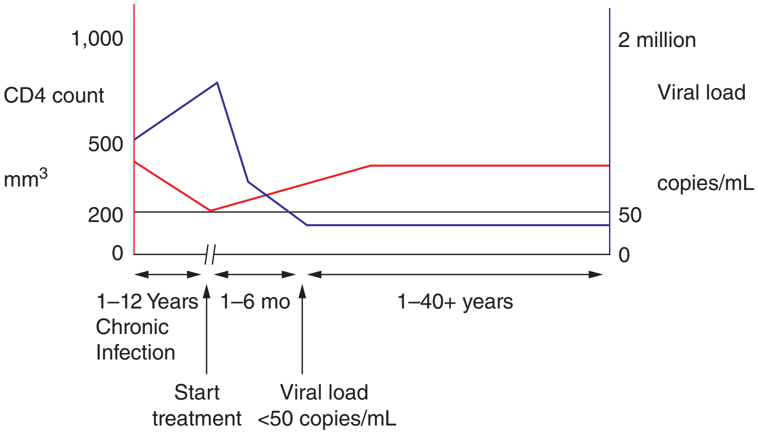


Figure 152.2 EFFECT OF ART ON CD4 COUNT AND VIRAL LOAD. *(From HIV iBase, London, United Kingdom.)*

a diagnosis of exclusion, with the main diagnostic clue being its timing.[19]

In assessing a patient, one should consider the following three factors: the absolute T-cell count (CD4 count), followed by whether they take antiretroviral therapy, and whether the peripheral blood viral load (VL) is undetectable. These three factors determine the risk of imminent mortality (Figs. 152.1 and 152.2). The classic analogy is to think of a train traveling on railroad tracks approaching the edge of a cliff. The speed of the train is the viral load, the distance to the cliff is the absolute T-cell count, and the cliff represents AIDS, where an individual is now at risk of an opportunistic infection or dying from complications of HIV.

Currently, in well-managed care settings, the great majority of HIV-infected patients are taking effective antiretroviral therapy that often involves 1 pill a day covering three agents. Testing for resistance is possible if a compliant patient nonetheless develops a high viral load, and the relatively large number of agents usually makes it possible to identify an effective combination. Isolated cytopenias are infrequent and generally seen in settings without available anti-HIV treatment. As will be discussed, thromboembolic events and malignancies persist, albeit at a lower rate.

HUMAN IMMUNODEFICIENCY VIRUS AND CYTOPENIAS

Cytopenias occur frequently in HIV infection. The effect of HIV on blood counts is pervasive and multifactorial. In the United States when HIV infections became prevalent and recognized (i.e., in the first half of the 1980s), it became clear that leukopenia, especially lymphopenia, anemia, and thrombocytopenia, all occurred but often for different reasons. At one end of the spectrum was uncontrolled HIV infection itself, causing low platelets for multiple reasons. This could be antiplatelet antibodies to amino acids 49 to 66 on glycoprotein IIIa as described in one seminal study by the Karpatkin group at New York University (NYU). Soon thereafter, infection of megakaryocytes by HIV was described as the creation of an inflammatory milieu of the bone marrow, all of which contributed to lower blood counts.

Confirmation of the role of active viral infection in the decrease in the platelet count has come from the use of antiretroviral treatments, which bring up the platelet count when the viral load goes to zero. Early on, there were several examples. First, when the initial randomized azidothymidine (AZT) study was performed, a subsequent letter showed that the patients who entered the study with counts between 100 and 150,000/μL (entry with counts below 100,000/μL was not allowed) showed that platelet counts increased on AZT therapy. This was in marked contrast to the hemoglobin and leukocyte count, both of which decreased in patients on AZT. Another study from Switzerland used a crossover design. Patients were randomized to either receive AZT or placebo, and then after a number of weeks they crossed over to the opposite arm. The results were astonishingly clear that the platelet counts went up dramatically on AZT and did not rise or fall back to baseline on placebo. Finally, Oksenhendler, in a non-randomized study, demonstrated the efficacy of not only AZT but also splenectomy in increasing the count in patients with HIV-related thrombocytopenia. These findings demonstrated that the thrombocytopenia at least resembled immune thrombocytopenia (ITP) in certain ways and furthermore that it was clearly linked to the presence of the virus. The bottom line was that thrombocytopenia was of sufficient importance that Holtzman et al. published a study in 1994 suggesting that at least 40% of patients with HIV

Downl
15, 202

would eventually develop thrombocytopenia and also suggested that worse thrombocytopenia was linked to greater mortality. However, this description is reflective of past history and applies only to a situation in which ART is not readily available.

Currently, thrombocytopenia is very infrequent in patients with HIV who are generally well controlled on ART. Furthermore, the viral load suddenly increasing either from resistance or non-compliance is not typically heralded by thrombocytopenia. In high-income countries, thrombocytopenia is generally not an issue in patients on ART, and neither is platelet dysfunction in general. In lower-income countries, which do not have a wide availability of testing or multiple up-to-date treatments of HIV, there is a substantial incidence of thrombocytopenia in these patients as there was initially in the United States. Thus there are still numerous reports from lower-income countries, for example, Ethiopia, in which thrombocytopenia is relatively frequent. Other causes could be co-infections. The best-described co-infection with HIV has been hepatitis C, in which thrombocytopenia, in particular, occurs relative to liver disease and hypersplenism. Liver disease can be a cause of unexplained thrombocytopenia, as seen in a study of non-HIV-infected ITP patients published by Rajan in 2006. Another co-infection that would cause cytopenias is CMV which is certainly not a rare infection in these or any group of hospital-bound patients.

Leukopenia is the least well-defined cytopenia in HIV-infected patients. Some of it is due to lymphopenia as the virus affects patients with infection, as described in the Introduction. Some of it may be related to other medications, inflammation, and/or infections.

Alternatively, anemia seems to be a result of low-grade inflammation in the marrow. It could also be from poor nutrition, but that is not very common and, if present, is typically very mild. Thrombocytopenia, leukopenia, and anemia, frequently present in the past, were often a result of an overt infection that was rampant and resulted in pancytopenia, which could be substantial and was often a sign of impending mortality. However, this fortunately is quite uncommon in the current climate. Similarly, true autoimmune disease is relatively uncommon in the current climate as well. This means that “ITP” and autoimmune hemolytic anemia are no longer commonly found in HIV-infected patients.

Much of anemia, leukopenia, and thrombocytopenia in the past involved not only viral loads but also the toxicity of medications for HIV such as AZT and medications used as prophylaxis or treatment of associated infections such as Bactrim for *Pneumocystis* pneumonia (PCP) or valganciclovir for cytomegalovirus (CMV). AZT compliance could be measured not so much by identifying a mild degree of anemia but rather by seeing that the mean corpuscular volume (MCV) had substantially increased. However, newer drugs have been designed so that they do not cause cytopenias and therefore these are not much of a problem in the setting of ART. Similarly, the use of Bactrim or trimethoprim sulfa 3 days a week in prophylaxis rarely causes anemia. In the differential diagnosis of anemia, all of the standard causes would need to be considered, for example, GI bleeding, iron deficiency, or B12 deficiency.

Treatments of cytopenias typically depend on the etiology. Management of an underlying disease, including but not limited to HIV itself, is almost always useful. For HIV-related ITP, steroids are generally useful and surprisingly safe if not administered for long periods of time. PCP is certainly a risk during discontinuation and fungal infection, especially candida, is probably the most common if prophylaxis is not given. Two studies from our center demonstrated the utility of a now-infrequently used ITP treatment, IV anti-D (Winrho SD), in the HIV-related ITP. Surprisingly, in this population, a small, randomized crossover study suggested that it was more effective than IVIG. Anemia was not a particular problem in these patients despite the hemolysis from anti-D and the predisposition to anemia. Oksenhendler demonstrated the utility of splenectomy. Other treatments now commonly used for classical ITP, such as rituximab, are either not used (the former) or supported by anecdotal reports (the latter). All of these treatments were developed in the pre-ART era and are now infrequently used.

In summary, in well-treated HIV infection, at the present time, cytopenias are relatively uncommon and have not been a serious problem. In patients that are not well treated either for compliance, resistance, or unavailability of ART medications, cytopenias continue to be seen as they were in the original cohorts in the 1980s before effective, nontoxic, antiretroviral treatments were developed. Again, the better shape a patient is in from the point of view of nutrition, undetectable viral load, inflammation, and not requiring large amounts of concomitant medications, the less likely they are to have cytopenias or other problems.

HUMAN IMMUNODEFICIENCY VIRUS AND LYMPHOMAS

Lymphomas of all types are increased in people living with HIV. This increased occurrence continues even in well-treated patients with low to undetectable viral cultures although the incidence is lower. The explanation for this is complicated and remains not fully worked out. Theories include: (1) failure of adequate immune surveillance resulting in failure to eradicate early lesions, (2) chronic inflammation characterizing HIV may stimulate lymphocytes such that they may then become malignant, and (3) increased co-infections, especially failure to regulate EBV. These explanations tend to be consistent with finding B cell non-Hodgkin lymphoma but do not explain equally well the increased risk of Hodgkin disease.

The link between immunosuppression and cancers has been well established, whether it is related to inherited immune deficiencies, medications (e.g., steroids or chemotherapy), or uncontrolled HIV with decreased immune surveillance. Polymorphic lymphoproliferative disorders are reported in both HIV-positive individuals and solid organ transplant recipients. This overlap is likely related to the state of immunosuppression. Also, in congenital immunodeficiencies, failure of immunoregulation may likely be abnormal and lead to uncontrolled proliferation including the development of lymphoma. Most lymphomas arise from B-lymphocytes; however, HIV does not directly infect B cells, and various theories have been proposed to explain this phenomenon.

The classic theory is that the state of immunosuppression leads to decreased immune surveillance, along with co-infection with oncogenic viruses: herpesviruses, Epstein-Barr virus (EBV), and Kaposi sarcoma-associated herpesvirus (KSHV), leading to a loss of immunoregulatory mechanisms. HIV infection is also often accompanied by a chronic pro-inflammatory state resulting in cytokine overproduction (i.e., IL-6 and IL-10). In turn, this leads to uncontrolled stimulation of B cells. Dysregulated B-cell stimulation aids in the emergence of monoclonal B cells; these can be seen without indicating the presence of malignancy. Certain genetic alterations may then take place, which leads to the emergence of lymphomas. Furthermore, there are unique characteristics of lymphomas that present within the HIV population.[20]

The introduction of HAART, through the suppression of the HIV viral load and normalizing the CD4 count leading to greater immunocompetence, played a significant role in the reduction of cases of NHL in the HIV population. However, non-Hodgkin lymphoma (NHL) still comprises the great majority of lymphoma cases in HIV-positive individuals. There are non-AIDS defining NHL subtypes, i.e., DLBCL and Burkitt lymphoma, that remain higher in prevalence in the HIV-positive population despite viral control with antiretroviral therapy. Some lymphomas, such as Hodgkin lymphoma, tend to present at later stages with extra nodal disease, are more aggressive, and are more likely to have EBV co-infection when compared to those seen in HIV-negative patients described in greater detail below.[21] Prior to HAART, most patients with HIV succumbed to opportunistic infections or one of the aforementioned AIDS-defining malignancies, including Kaposi sarcoma (see Table 152.2). HAART made the prognosis of HIV not dismal and restored the lifespan of patients to more normal. Other malignancies, beyond the AIDS-defining ones, have surfaced and have been likened in prevalence to those who underwent solid organ transplantation and are taking lifelong immunosuppressive therapy, where they were noted to occur at a higher rate when compared to the general population. Of all AIDS-defining cancers, lymphomas make up 50% of cases.

Downl
15, 202

TABLE 152.2 Antiretroviral Therapy

Single Tablet Regimens	Indication	Components	Side Effects/Contraindications	Mechanism of Action for Class/ List of Drugs
Biktarvy	1st line initial therapy for most people (2 NRTIs + INSTI)	Bictegravir Emtricitabine Tenofovir alafenamide	Nausea, headache, and diarrhea. **Cannot be taken with hepatitis B medication**	**INSTI: inhibits HIV integrase from integrating viral genome into the DNA of a host cell** Bictegravir Dolutegravir Elvitegravir **NRTI: competitive inhibition of HIV reverse transcriptase by incorporating into viral DNA and inducing chain termination** Abacavir Bictegravir Emtricitabine Lamivudine Tenofovir alafenamide Tenofovir disoproxil-fumarate **NNRTI: noncompetitive inhibition of HIV reverse transcriptase via binding of the enzyme's active site** Efavirenz Doravirine Rilpivirine **PI: preventing the cleavage of gag and gag-pol precursors by HIV protease, which is required for the formation of the viral capsid** Darunavir **Booster: Inhibits CYP3A, which is responsible for metabolizing darunavir or elvitegravir thus serving as a pharmacoenhancer** Cobicistat
Triumeq		Dolutegravir Abacavir Lamivudine	Insomnia, headache, and fatigue. **Cannot be used in HLA-B*5701 given hypersensitivity to abacavir**	
Dovato		Dolutegravir Lamivudine	Headache, nausea, diarrhea, fatigue. Reversible increase in serum Cr without affecting kidney function. **Cannot be used if VL > 50,000 or HBV co-infection**	
Atripla	1st line for people with certain clinical conditions (2 NRTIs AND NNRTI or INSTI + booster or PI + booster)	Efavirenz Emtricitabine Tenofovir disoproxil-fumarate	Suicidal ideation and QTc prolongation (efavirenz). **Should not be taken with medication treating hepatitis B. Efavirenz should be avoided in pregnancy due to increased risk of neural tube defects**	
Complera		Rilpivirine Emtricitabine Tenofovir disoproxil-fumarate	Insomnia, headache, and depressive disorders	
Delstrigo	1st line for people with certain clinical conditions (2NRTI AND NNRTI or INSTI + booster or PI + booster)	Doravirine Lamivudine Tenofovir disoproxil-fumarate	Dizziness, nausea, abnormal dreams, and headaches, depression, and suicidal ideation.	
Genvoya		Elvitegravir Cobicistat Emtricitabine Tenofovir alafenamide	Weight gain, nausea, and vomiting. Cobicistat can cause a reversible increase in serum creatinine without affecting actual kidney function	
Odefsey		Rilpivirine Emtricitabine Tenofovir alafenamide	Insomnia, headache, depression and suicidal ideation Rash, edema, and urticaria due to rilpivirine. Reversible increase in serum creatinine without affecting actual kidney function	
Stribild		Elvitegravir Cobicistat Emtricitabine Tenofovir disoproxil-fumarate	Nausea, diarrhea, abnormal dreams, weight gain, depression, suicidal ideation	
Symfi		Efavirenz Lamivudine Tenofovir disoproxil-fumarate	Headache, body pain, fever, diarrhea, depression, and rash	
Symtuza		Darunavir Cobicistat Emtricitabine Tenofovir alafenamide	**Contraindicated in patients taking steroids, direct oral anticoagulants, and high dose statin. Relative contraindication to co-administration with Colchicine if renal or hepatic impairment present**	
Triumeq		Dolutegravir Abacavir Lamivudine	Weight gain, suicidal ideation, reversible Cr increase that does not affect kidney function. **Cannot be used in HLA-B*5701 given hypersensitivity to abacavir**	

INSTI, Integrase strand transfer inhibitor; *NRTI*, nucleoside/nucleotide reverse transcriptase inhibitors; *NNRTI*, non-nucleoside reverse transcriptase inhibitor; *PI*, protease inhibitor.[100,101]

A metanalysis by Grulich et al. looked at the incidence of cancers in HIV/AIDS individuals and compared it to immunosuppressed transplant recipients; seven papers were included for the HIV group and six for the transplant group. The incidence of non-Hodgkin lymphoma was 70-fold more in HIV patients when compared to the general population before the introduction of ART. The metanalysis did not consider the CD4 count of the HIV patients and the degree of the immunosuppression in their findings and did not further stratify the results for HIV versus AIDS. Both groups in the metanalysis had an increased incidence of both Hodgkin lymphoma and non-Hodgkin

Downl
15, 202

lymphoma, with a substantially higher meta-analysis standardized incidence ratio (SIR) in the HIV population for both conditions. For Hodgkin lymphoma, the metanalysis SIR for HIV/AIDS was 11.03 (8.43 to 14.4, CI 95%) versus 3.89 (2.42 to 6.26, CI 95%) in transplant recipients. For non-Hodgkin lymphoma in HIV/AIDS, it was 76.67 (39.4 to 149, CI 95%) versus 8.07 (6.40 to 10.2, CI 95%) in transplant recipients.[22] Robbins et al. examined the incidence of cancers in the HIV population over time, obtaining data from US HIV AIDS Cancer Match Study, and included 275,975 HIV-positive patients. They found that the incidence of Hodgkin lymphoma declined by 4%, whereas the incidence of non-Hodgkin lymphoma declined by 15% over the first seven years, followed by a 5.5% decline subsequently.[23] Despite this decline after the introduction of ART, the HIV population still has a higher incidence of both non-Hodgkin lymphoma and Hodgkin lymphoma than the general population (10-fold and 11-fold more respectively).[24]

Non-Hodgkin lymphoma is characterized by multiple subtypes. Many but not all of these are increased in HIV infection, and the subtypes that are increased can be further distinguished between those that are related to the state of the HIV and those that are independent of it.

Diffuse Large B-Cell Lymphoma

Diffuse large B-cell lymphoma (DLBCL), an aggressive lymphoma, is the most common type of NHL in the HIV population, constituting 50% of lymphomas in HIV. Unlike Hodgkin lymphoma and Burkitt lymphoma, DLBCL is associated with low CD4 T cell counts. Traditionally, DLBCL had been further sub-classified cytologically into centroblasts (multilobulated vesicular nuclei with numerous peripheral nucleoli), immunoblasts (round vesicular nucleus with one central nucleoli), and anaplastic cells (bizarre pleomorphic nucleus). This classification is no longer used when thinking of DLBCL given the lack of both inter-observer and intra-observer reproducibility, advances in molecular genetics that aid in better prognostication of disease, and heterogeneity of DLBCL per se in the general population. However, this classification is not entirely useless in the HIV population, as cytology can still confer information, and the utility of the new World Health Organization (WHO) 2016 classification of DLCBL for HIV remains unclear. DLBCL with centroblastic features is 70% EBV-negative, whereas 80% to 90% of immunoblastic DLBCL are EBV-positive[25] and express latent membrane protein 1 (LMP-1), a viral oncoprotein resembling CD40 that activates nuclear factor kappa B (NF-κB) leading to unregulated B-cell proliferation.[21,26]

In patients with HIV, centroblastic DLBCL occurs in individuals with almost normal CD4 counts, whereas immunoblastic DLBCL occurs late in the course of HIV infection and in those who are immunosuppressed, not surprising given the role of EBV primarily in the latter. Diamond et al. examined data from 1988 to 2000 from the San Diego County AIDS and Cancer registries and found that immunoblastic DLBCL decreased from 26% to 7% in the population of patients with AIDS and cancer after the introduction of ART.[27] Despite increases in both overall CD4 T cell count and survival, centroblastic DLBCL increased from 21% to 44% in the same population.

As of 2016, the WHO classifies DLBCL by cell of origin derived from a micro-array gene expression profile (GEP). There are three categories: germinal center B-cell (GC), activated B-cell (ABC), and unclassified (accounting for 20% of DLBCL).[28] In the general population, this classification predicts response to R-CHOP (rituximab-cyclophosphamide-hydroxydaunorubicin-Oncovin-prednisone) therapy, to which GC has a more favorable 5-year survival response when compared to ABC (60% vs. 35%). The majority of GC are centroblastic, whereas the majority of ABC are immunoblastic.

Unfortunately, GEP is expensive and not readily available. So instead, the Hans algorithm using three immunohistochemical markers, CD10, Bcl-6, and MUM1, is used. With this algorithm, DLBCL is classified as GC if CD10 is positive or if MUM-1 is negative with a positive BCL-6. All other DLBCL cases are classified as non-GC.

HIV-negative individuals with GC DLBCL have a better prognosis when compared to non-GC DLBCL, but this does not apply to HIV-positive patients. A study by Chadburn et al. examined tissue from 81 DLBCL patients with AIDS enrolled in AIDS-malignancy consortium (AMC) trials comparing the effects of R-CHOP versus CHOP (AMC010) and concurrent R+EPOCH versus sequential EPOCH followed by Rituximab. Hans algorithm was used to classify the samples into GC and non-GC. No difference in overall survival between the two groups was observed ($P = 0.859$). The presence of EBV did not correlate with event-free survival. Also, there was no correlation between CD4 counts and the presence of EBV ($P = 0.47$). However, immunohistochemical staining with Ki-67, a marker of cell proliferation, predicted overall survival ($P = 0.05$), with a high proliferation index associated with a better clinical outcome, possibly providing a target for chemotherapeutic agents.[29]

Madan et al. examined differences between HIV-positive and HIV-negative individuals by describing 39 de novo DLBCL in 12 AIDS DLBCL and 27 non-AIDS DLBCL[30] Classically, GC is associated with CD10, BCL-6, Cyclin H, and non-GC with MUM1, BCL-2, CD38, PAL1, and CD44. Each of the immunohistochemical stains was designated a number on the basis of the positivity of the samples. Furthermore, the different markers for GC and non-GC were combined to give a score, which was plotted on a two-dimensional contour plot. For the HIV-negative DLBCL samples, the plot had two separate clusters delineating the two subtypes. When the HIV-positive samples were plotted, the two distinct clusters were not appreciated, instead, an intermediate state of both GC and non-GC was appreciated with overlap with both GC and non-GC from the HIV-negative samples. This alludes to a possibly different underlying mechanism for lymphomagenesis, which may imply a need to consider different targets for therapy.

Another adverse prognostic factor termed the "double-expressor" phenotype exists where MYC and BCL-2 are both overexpressed without genetic re-arrangement. This is distinct from "double-hit" or "triple-hit" lymphomas, a term designated for high-grade B-cell lymphoma (HGBL), which morphologically resemble DLBCL. They are considered a separate entity in the 2016 WHO classification, which denotes that re-arrangements in MYC and BCL2 and/or BCL6 are implicated in a worse prognosis.[28]

The "Double-Expresser" phenotype is also associated with poorer outcomes, where the 5-year overall survival is around 40% with R-CHOP therapy. Dodero et al. compared clinical outcomes in double-expresser DLBCL treated with R-CHOP versus dose-adjusted EPOCH-R (etoposide + prednisone + vincristine + cyclophosphamide + doxorubicin + rituximab). One hundred fourteen patients with the double-expresser DLBCL phenotype were identified in three Italian institutions. Fifty-one patients received dose-adjusted EPOCH-R between 2013 and 2017, and 63 patients received R-CHOP between 2009 and 2017. The two primary outcomes were overall survival (OS) and progress-free survival (PFS). In the dose-adjusted EPOCH-R group, dose-escalation to level 3 was possible in 73% of patients under the age of 65 compared to many fewer patients above 65, where the majority received dose-adjusted EPOCH-R at level 1 because of either comorbidities or therapy-associated toxicities. There were no statistical differences in OS and PFS in patients receiving R-CHOP versus dose-adjusted EPOCH-R; however, when adjusting for age, patients below the age of 65 exhibited favorable clinical outcomes in the dose-adjusted EPOCH-R group when compared to patients receiving R-CHOP, where PFS was 82% versus 43% ($P = 0.02$) and OS 90% vs. 63% ($P = 0.042$). The clinically significant difference after age adjustment likely emerged because younger patients were able to better tolerate higher levels of dose-adjusted EPOCH-R therapy.[31]

Other mutations have been observed in HIV-related DLBCL: over 90% of cases were noted to harbor somatic hypermutations in the immunoglobulin variable genes responsible for extracellular antigen binding. Specifically, there was overexpression of the IGHV4–34 gene in DLBCL, which is normally expressed on the surface of naïve B cells but not expressed by mature activated or memory B cells.[32] This aberrant overexpression in HIV-related DLBCL implies that

Downl
15, 202

there may be an antigen-driven process that indirectly stimulates B cells leading to lymphomagenesis. Similar to HIV-negative lymphomas, other mutations in proto-oncogenes PIM-1 and RhoH/TTF (signal transducers) and PAX-5 and c-MYC (transcription factors) have been observed in HIV-related DLBCL; these are typical features of aggressive lymphomas.[33] Furthermore, over 60% of HIV-related DLBCL harbor mutations in BCL-6,[34] a transcription repressor protein-encoding gene required for germinal center formation, where it normally suppresses p53, a tumor suppressor gene that regulates the cell cycle.[35]

Primary CNS lymphoma (PCNSL) is an AIDS-defining malignancy. It involves the brain, leptomeninges, or spinal cord, is considered a subtype of DLBCL in over 90% of cases and is classified under the immunoblastic variant in which almost all cases are EBV driven and express LMP-1. It is associated with CD4 counts below 50 and thus occurs late rather than early in the course of HIV. Post-transplant patients can also present with PCNSL, comprising around 0.9% of diagnosed lymphomas in these patients. Immunocompetent HIV-negative patients can also get PCNSL; however, it is exceedingly rare with an incidence of 7 per million in the United States, presents in much older individuals (median age of 67 vs. 37 in HIV vs. 52 in transplant patients), and is not EBV driven. In the early 1990s, over 60% of PCNSL cases were found in HIV-positive individuals. In 2007–2011, only 12% of PCNSL cases were attributed to HIV, which correlates with better immunocompetence in ART-treated HIV-positive individuals.[36]

Plasmablastic lymphoma (PBL), a subtype of DLBCL comprising 3% of lymphoma cases in HIV, is also considered an AIDS-defining lymphoma. Half of the cases involve the oral cavity and jaw, with the other half mostly involving the GI tract or any other site, i.e., skin, bones, or sinonasal cavity. This lymphoma is morphologically categorized into a monomorphic and a plasmacytic variant. The former resembles DLBCL, where it appears immunoblastic or centroblastic in morphology (described above), and the latter variant resembles plasma cells. More than half of the cases express EBV with LMP-1, and the remainder expresses HHV8 alone, similar to primary effusion lymphoma (described in greater detail below). The immunohistochemical profile stains $CD38^+$, $CD138^+$, IRF4/MUM1, CD79a, BLIMP-1 and is negative for mature B-cell markers such as CD19, CD20, and Pax 5. Some PBLs are weakly positive for CD45 which confers a positive prognostic factor along with EBV positive cases. It carries an extremely poor prognosis with a median survival between 3 and 5 months.[21,26,37,38,60]

Burkitt Lymphoma

Burkitt lymphoma (BL), also an aggressive NHL, histologically identified by the starry sky appearance is typically seen in children; however, it is the second most common lymphoma subtype in adults with HIV after DLBCL, representing 30% of NHL. BL is classified into three subtypes. Endemic BL typically involves the jaw and is EBV-driven in most cases. Geographically, it is found in tropical Africa and Papua New Guinea, correlating nicely with the incidence of malaria, where the malaria infection is thought to decrease one's resistance to EBV. Sporadic BL is not confined geographically nor has the predilection for EBV (20% of cases) and usually presents in the ileocecal region in the abdomen in children and adolescents in the United States and Western Europe with a predilection for persons of European descent.[39] The third form is immunodeficiency-associated BL, found mainly in HIV patients typically with CD4 counts above 200. It behaves in many ways like the sporadic type, where they both have similarities in staging and organ involvement. Furthermore, this subtype is also observed to a lesser extent in post-transplant patients and also in rare case reports of congenital immunodeficiencies, that is, Common variable immunodeficiency (CVID), Bloom syndrome, and Wiskott-Aldrich syndrome. All three subtypes have translocations leading to constitutive expression of the MYC proto-oncogene on chromosome 8. Eighty percent of BL have a t(8;14), where chromosome 14 encodes the heavy immunoglobulin chain. The remainder of BL translocations involve the immunoglobulin light chain, where 15% are with the kappa chain t(2;8) and lambda chain t(8;22). Immunohistochemically, BL stains for CD20, CD79a, CD10, and BCL-6 and is negative for BCL-2 and TdT.[40] Interestingly, EBV-associated BL does not express LMP-1; instead, it expresses EBNA1, a viral protein responsible for DNA replication in EBV's latent phase, which is thought to contribute to the survival of B cells by protecting them from apoptosis induced by the c-myc gene.[41] For unclear reasons, it was observed that BL increased from 4% to 9% after the introduction of ART in the San Diego County AIDS and Cancer registry from 1988 to 2000.[27]

Primary Effusion Lymphoma

Primary effusion lymphoma (PEL), a B-cell lymphoma, is almost exclusively associated with HHV8, also known as KSHV. Over 90% of cases are also associated with EBV and LMP-1 expression. PEL presents as malignant effusions, most commonly of the pleura. It can also be present in the peritoneum, pericardium, and to a lesser extent, joint spaces. It is associated with severe immunosuppression and represents around 1% to 3% of lymphomas found in HIV, often co-occurring with other HHV8- associated malignancies like Kaposi sarcoma or multi-centric Castleman disease (described below). HHV8 contributes to oncogenesis via at least three viral proteins: (1) Latency-associated nuclear antigen (LANA), which works on multiple pathways that include inhibiting tumor suppressors p53 and Rb, upregulating the WNT/β-catenin pathway by sequestering the negative regulator GSK-3β, thereby promoting oncogenesis; (2) homologue of FLICE inhibitory protein (vFLIP), which activates NFκB; and (3) viral IL-6, which induces VEGF and cell proliferation.[42]

PEL does not have a characteristic cytological appearance as it can look immunoblastic in morphology or even show Reed-Sternberg cells. It expresses CD45, CD30, CD38, CD138, and MUM1 and does not express other B-cell markers such as CD19 and CD20. Furthermore, a solid variant of PEL exists, where it is morphologically similar but lacks effusions and rather affects tissue such as lungs and lymph nodes instead of cavities. It can also differ immunohistochemically in that approximately a quarter of cases do not express CD45 and do express CD20 and CD3.[21]

Hodgkin Lymphoma

Compared to the general population, HIV-positive individuals are at a 5- to 15-fold increased risk of developing Hodgkin lymphoma (HL). Interestingly, unlike some of the subtypes of NHL, HL is not related to the degree of immunosuppression, with the median CD4 count usually ranging between 150 and 260 at the time of diagnosis. The presence of a low CD4 count still confers a poor prognostic factor, as it increases the risk of opportunistic infections during chemotherapy. The incidence of HL after the introduction of ART has not decreased, with multiple retrospective studies demonstrating an increase in the incidence of HL in the United States and some studies demonstrating the incidence is stable and unrelated to the introduction of HART.

Simard et al. examined records from the HIV/AIDS Cancer Match Study, where they looked at data from over 400,000 cases of cancers in patients with HIV or AIDS between 1980 and 2006, which included 15 states. The data were stratified into three periods: 1980–1989, prior to the introduction of antiretroviral therapy; 1990–1995, when monotherapy or dual therapy began; and 1996–2006, when HAART began. The 5-year cumulative incidence was 0.09 (0.07 to 0.11), 0.15% (0.14 to 0.17), and 0.19% (0.17 to 0.21), respectively, with 95% confidence intervals.[43] Similarly, a prospective cohort by Powles et al. followed 11,112 HIV-positive individuals from the Chelsea and Westminster HIV cohort in London and calculated standardized incidence ratios for various cancers before and after the introduction of HAART from 1983 to 2007. The data gathered included immunosuppression, whether AIDS was present, and the type of antiretroviral therapy. The data were grouped into three categories with the

Downl
15, 202

following SIRs for HL with a 95% confidence interval: 1983–1995 pre-HAART era, 4.45 (1.44 to 10.37);1996–2001 dubbed as the early HAART era, 11.06 (4.78 to 21.80); 2002–2007 established HAART era (i.e., widely available) 32.35 (4.78 to 21.80).[44]

Contrarily, a Swiss nested-control study by Clifford et al. failed to demonstrate the increase in HL incidence following the introduction of HAART. The Swiss HIV Cohort study used a total of 14,606 patients with HIV between the years 1984 and 2007 were included. A total of 47 cases of HL were identified in the analysis, and each of the cases were matched to 10 controls with similar age, calendar period, gender, mode of HIV transmission, and treating center. The risk of HL between 2001 and 2007 was similar to 1984 and 1995. The authors attributed the differences present in other studies to the bimodal age distribution of HL and the prolonged survival conferred by HAART, which lead to a falsely increased incidence.[45] Furthermore, a retrospective cohort study by Kowalkowski et al. reported that the incidence of HL decreased from 2.51 per 1000 to 0.06 in 1000 after 10 years of ART.[46] The authors examined 31,576 HIV patients on HAART from the Veterans Affairs HIV clinical Case Registry between 1985 and 2010, of which 211 cases of HL were identified. Data were collected on patients' age (mean age 45) and year of HIV diagnoses (whether it was pre-HAART prior to 1996, early ART 1996–2001, or late HAART 2002–2010; 71% of HIV diagnoses were made in the late HAART era), ethnicity (62% were non-white), and presence of comorbid conditions. Furthermore, CD4 count and HIV viral load were obtained throughout the study as well; 45% of the patients had a CD4 less than 200 prior to antiretroviral therapy. By the end of the study, 55% of patients had a CD4 count greater than 350, and only 25% of patients had a CD4 count less than 200. Half of the cohort had received antiretroviral therapy for at least an average of 5 years. Of note, patients diagnosed with HL were noted to have been on antiretroviral therapy for a shorter duration of time when compared to patients without a diagnosis of HL for both protease-inhibitor-based therapies and NNRTI based therapies ($P < 0.0001$).

HIV-associated HL is EBV-positive in more than 90% of cases when compared to HIV-negative HL, where only a third of cases are positive for EBV.[47] Patients present at later stages, i.e., stage III or IV, and extra-nodal involvement, where the bone marrow is the culprit in 50% of cases, followed by liver and spleen. HL is classified into nodular-sclerosing, mixed cellularity, lymphocyte-rich, and lymphocyte-deplete. The histological classification of HIV-associated HL does not differ from the standard classification. The predominant subtype between the two populations differs in that for HIV patients, the majority are mixed cellularity comprising between 25% and 55%, followed by lymphocyte-deplete, which comprises between 5% and 20% in comparison to immunocompetent HIV-negative individuals in whom nodular-sclerosing is the predominant subtype in the general population. The lymphocyte-depleted subtype is extremely rare in immunocompetent individuals representing around 1% of all HL cases, whereas 15% of cases are LD for those HIV-positive.[34] Furthermore, EBV appears to play a role in the pathogenesis of the LD HIV+, where it is present in 60% to 72% of patients with the expression of LMP-1, which aids in B-cell survival.[48] The immunohistochemical characteristics largely do not differ in HIV-positive individuals when compared to other HL cases with Reed-Sternberg (RS) stain $CD15^{+}$, $CD30^{+}$, faintly $PAX5^{+}$, $CD45^{-}$, and some RS $CD20^{+}$.[34] Interestingly, in HIV-positive cases, there is a reversal of the CD4:CD8 ratio observed with a predominance towards $CD8^{+}$ cells, which is expected given that HIV leads to $CD4^{+}$ depletion.[49]

Before the introduction of HAART, the prognosis of HL lymphoma in individuals with HIV was poor. This was mainly due to the poor tolerability of the chemotherapeutic agents in the setting of low CD4 counts, which made patients susceptible to opportunistic infections and their sequelae. HL in HIV-positive patients has a similar prognosis when compared to HL in the general population, and the treatment approach is similar. The treatment of choice for HL is dependent on staging, whether there is a recurrence of the HL or if it is refractory to the initial therapy, and, like all treatments, is tailored to the patient. Historically, HL was treated with high penetration X-rays and later with nitrogen mustard in 1946. High dose radiation leads to the development of secondary cancers in later years and direct toxic effects on the bone marrow leading to pancytopenias and/or thyroid, lung, and cardiovascular toxicities.[50] Currently, radiation therapy is still used but at much lower doses and in combination with chemotherapeutic agents. ABVD therapy ± radiation therapy is considered first-line given its efficacy and lower side effect profile. Other chemotherapeutic agents such as BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone), Stanford V (vinblastine, doxorubicin, vincristine, bleomycin, mustard, etoposide, and prednisone), and others are also used with varying results, depending on the staging and response of the patient. In HIV-positive patients, HAART is continued with therapy and typically not discontinued.[51]

T-Cell Lymphoma

Lymphomas derived from T lymphocytes are much less common than B-cell lymphomas and account for 7% of diagnosed lymphomas in the United States. Similarly, in HIV-infected patients, the diagnosis of T-cell lymphoma is relatively rare, with most published reports consisting of case reports or small case series.

Biggar et al. examined data from the AIDS and cancer registry between 1978 and 1996 in 11 states in the US. 10,592 patients with NHL and AIDS were identified. Only 96 cases were identified as T-cell lymphomas representing 1.4% of all HIV-related NHL. The authors used histological diagnoses to identify cases but were given incomplete immunohistochemical records in several cases, which might lead to the underrepresentation of T-cell lymphoma. The cases belonged to the following subtypes: 36 peripheral T-cell lymphomas (PTCL), 35 of mycosis fungoides (1 Sézary syndrome), 13 cutaneous lymphomas, 11 adult T-cell leukemia/lymphoma (ATLL), and 1 angiocentric T-cell lymphoma.[52] Similarly, in the general population, T cell lymphoma is most commonly PTCL followed by mycosis fungoides.[53,54]

A retrospective analysis performed by Arzoo et al. examined 429 HIV-positive lymphomas between 1982 and 2001 at Los Angeles County-University of Southern California Medical Center and the University of Southern California/Norris Comprehensive Cancer Center. 11 patients diagnosed with T-cell lymphomas were identified, and their characteristics were compared to HIV-positive patients diagnosed with B-cell lymphoma. The 11 cases of T-cell lymphoma were diverse: 4 had PTCL, 3 anaplastic large cell lymphoma (ALCL), 1 ATLL, 1 angioimmunoblastic, and 1 enteropathy-associated T-cell lymphoma.

There were similarities and differences between the T-cell and B-cell lymphomas. The median CD4 count at the time of diagnosis did not statistically differ between the two groups (101 for T-cell lymphoma vs. 72 for B-cell lymphoma [$P = 0.54$]), but T-cell lymphoma was more likely to have: (1) bone marrow infiltration (45% vs. 15%, $P = 0.019$), and (2) cutaneous involvement (36% vs. 2%, $P < 0.001$). Of those 11 T-cell lymphoma patients, seven died, six within a year, with a median survival of 10.6 months. Remission was only achieved in four patients: three ALCLs were treated with CHOP (one died after achieving CR for 3.6 months) and one patient received COPE; 1 PTCL was treated with radiation. The enteropathy-associated T-cell lymphoma patient relapsed, and his therapy was ongoing at the time of the study.[55] Clinical significance and conclusions from this study should be interpreted with great caution as the sample size was extremely small and not representative of what is known of the demographics for T-cell lymphoma in prior studies with larger sample-sized as demonstrated by Biggar et al.

Case Illustration

A 50-year-old man from West Africa who immigrated to the United States a decade prior with no known medical history presented to the Emergency Department with profound epistaxis mounting to 3

Downl
15, 202

cups of blood over a day. The patient described night sweats, weight loss, and a non-productive cough for the last month that coincided with the appearance of a right groin mass. As far as his social history, he was retired and married with children. He never smoked tobacco nor used any illicit drugs. His physical exam was notable for tachycardia to 106 bpm, hypotension to 90/60 mmHg, palatal petechiae, bilateral posterior and anterior cervical lymphadenopathy that was more appreciated posteriorly, bilateral submental lymphadenopathy, bilateral inguinal lymphadenopathy and a large right inguinal mass measuring 6 × 4 cm. The right groin mass was mobile, non-tender, non-reducible, and non-pulsatile. The nose-bleeding was well controlled with nasal packing. He received 2 L of lactated Ringer solution along with a unit of 1pRBC, to which he responded appropriately.

Initial Labs

His labs on admission were significant for a hemoglobin of 6.4 for which he was transfused. He also had an lactate dehydrogenase (LDH) of 3194 U/L with normal haptoglobin. His reticulocyte index was 0.64, indicative of hypo-proliferative bone marrow. His B12 and folate were within normal limits, his iron was low at 31 μg/dL with an iron saturation of 17.7%, His total iron binding capacity (TIBC) was low at 175, and his ferritin was over 16,500 ng/mL along with other inflammatory markers (erythrocyte sedimentation rate [ESR] 70 mm/h, C-reactive protein [CRP] 20.4 mg/dL) indicating a mixed picture of anemia due to bleeding and anemia of chronic disease.

Other labs were notable for a white blood cell (WBC) 10.2 with a decreased absolute lymphocyte count of 1.00. His globulin gap was elevated at 4.1 (protein of 6.4, albumin of 2.3). His triglycerides were 491 and AST 127. His HIV-1 came back positive with an initial viral load of 617,916 and a CD4 count of 275. He was also tested for HIV-2, which came back negative. He also tested positive for hepatitis B with a viral load of 1219. His Hep B surface and core antibodies were both negative.

The patient's initial presentation and labs were concerned for uncontrolled HIV with a concurrent opportunistic infection, malignancy such as lymphoma, and a systemic inflammatory syndrome-like hemophagocytic lymphohistiocytosis. (He has an H-score of 174, making the probability of a hemophagocytic syndrome around 54% to 70%.)

Further Diagnostic Laboratory Studies

The patient's EBV PCR was positive greater than 400,000, which raised concerns for lymphoma. Serology for toxoplasma was negative; serum cryptococcal antigen was also negative.

As part of an autoimmune workup, an ANA was negative.

Blood cultures on admission had no growth. The fungal cultures were also negative, as was the beta-D-glucan assay. Bronchoalveolar lavage was negative for both *Pneumocystis jiroveci* (PJP) and tuberculosis. His SARS-CoV-2 PCR was negative.

An ultrasound of his right groin was obtained and unexpectedly showed an incidental finding of an enlarged cystic structure measuring 6.1 × 4.4 × 4.8 cm without vascularity representing a seroma. Given extensive evidence of lymphadenopathy on physical examination, a CT chest, abdomen, and pelvis with IV contrast showed mediastinal, bilateral hilar, bilateral axillary lymphadenopathies, bilateral peri-lymphatic nodularity in the lungs, and a 9 mm right lower lobe nodule with multiple retroperitoneal and inguinal lymphadenopathy, and mild splenomegaly. The liver also had two punctate hypodensities in the posterior right hepatic lobe. A brain MR for staging was unremarkable. A positron emission tomography (PET) scan showed diffuse splenic uptake suspicious of lymphomatous involvement. There was also multifocal fluorodeoxyglucose (FDG) avid cervical, abdominopelvic, and extremity lymph nodes consistent with prior findings of lymphadenopathy described above.

The patient underwent an excisional biopsy of the right-inguino-femoral lymph node, which confirmed the diagnosis of diffuse large B-cell lymphoma, not otherwise specified; non-germinal center type, with a "double expressor" phenotype.

The lymph node's architecture was completely effaced and mainly consisted of an infiltrate of medium-large-sized lymphocytes. The lymphocytes stained positive for CD20, PAX5, MUM1 (>90%), BCL-2 (>90%), and c-myc (~60%). The immunohistochemical staining was negative for CD10, BCL-6 (~20%, p53, and Epstein-Barr virus-encoded RNA (EBERs). The sample had an elevated proliferation fraction Ki67 (>90%). Based on the Hans algorithm, which has high concordance with gene-expression profiling, the DLBCL-NOS is classified under the non-germinal center type, traditionally indicative of poor response to R-CHOP. Furthermore, the positive c-Myc and BCL-2 classify the lymphoma under the "double expressor" phenotype, also considered a poor prognostic factor.

The cytogenetic analysis showed t(14;18) with associated IGH-BL2 gene rearrangement, consistent with DLBCL. Furthermore, an unbalanced t(8;17) with loss of 8p and 17p (site of TP53) was also observed, another poor prognostic factor. The patient did not have a BCL6, MYC, or MYC-IGH rearrangement and no PAX5 gene rearrangement either.

Bone marrow biopsy confirmed extra nodal involvement with cellularity greater than 90% (expected cellularity for the patient's age is around 50%) with nodular aggregates of medium to large-sized lymphocytes with irregular nuclei and prominent nucleoli and abundant eosinophilic cytoplasm which were CD20$^+$, Pax5$^+$, CD79a$^+$ but negative for CD34, CD118. LMP1 stain was equivocal (EBV proto-oncogene), LANA (HHV8) negative. The cytogenetics were identical to the ones observed in the lymph node biopsy. The liver biopsy was negative for lymphomatous involvement and unremarkable. Biopsy of the terminal ileum, sigmoid, and rectum showed atypical lymphoid infiltration consistent with B-cell lymphoma at the terminal ileum. Immunostaining was positive for CMV and negative for EBERs.

Management

The patient was initiated on HAART starting with Biktarvy (bictegravir, emtricitabine, and tenofovir alafenamide) with entecavir to treat concomitant hepatitis B. Prior to the initiation of chemotherapy, the patient had a lumbar puncture, where there were no malignant cells in the CSF. He received intrathecal methotrexate prior to the initiation of his chemotherapy for CNS prophylaxis. Over the span of 2 months, his HIV viral load decreased from 617,916 to 344. His hepatitis B viral load became undetectable with treatment, but his CD4 count decreased from 275 to 100.

Given his HIV-1-related immunosuppression and further immunosuppression with lymphoma treatment, the patient was started on Bactrim for PJP prophylaxis, valacyclovir for EB viral prophylaxis, and fluconazole for fungal prophylaxis. The patient developed transaminitis requiring discontinuation of Bactrim and fluconazole and was started on atovaquone instead for PJP prophylaxis. His DLBCL was treated with prednisone which was complicated by CMV viremia to 9036; this was treated with a 3-week-course of ganciclovir. His repeat CMV viral load prior to discharge was 170.

He received two cycles of EPOCH as an inpatient for his DLBCL. This was complicated by neutropenia requiring G-CSF. His EBV viral load was undetectable prior to discharge. The patient was discharged with plans to follow up as an outpatient but unfortunately was lost to follow-up.

Systematic Approach to Lymphadenopathy

Given the example case before, lymphadenopathy is very common in HIV-infected patients and has a broad differential diagnosis in this population. Like TB, lymphomas are typically characterized by lymphadenopathy and constitutional symptoms: weight loss, night sweats, and fevers. Certain lymphomas within the HIV population are almost exclusively present in immunocompromised individuals, such as AIDS-defining primary CNS lymphoma, while other

Downl
15, 202

non-AIDS defining lymphomas such as Burkitt lymphoma are present irrespective of the CD4 count.

Development of lymphadenopathy could be part of HIV, a sign of an opportunistic infection, or even lymphoma. As with CVID patients, lymphadenopathy is relatively common and most likely *not* lymphoma. Even deciding when to biopsy can be very difficult. A more complete set of different etiologies to consider are infectious, including HIV itself, inflammatory (including autoimmune, reaction to drugs), and neoplastic. Lymphadenopathy from drugs can be confounding as drugs that are used in treating opportunistic infections in HIV can cause them. Examples are drugs in the sulfonamides class, such as sulfamethoxazole used in combination with trimethoprim for PJP prophylaxis and pyrimethamine used in the treatment of toxoplasmosis. Even though lymphadenopathy occurs commonly in non-HIV-infected people, there are special circumstances to certain etiologies within the HIV population.

HIV itself can cause lymphadenopathy during the acute viral illness or an exacerbation, and it typically manifests as posterior cervical lymphadenopathy that is symmetrical in nature, often accompanied by diarrhea, myalgias, night sweats, and fevers, all of which are very similar to the presentation of lymphoma. The clinical syndrome can also mimic infectious mononucleosis. Acute HIV is more likely to present with painful mucocutaneous ulcers, whereas infectious mononucleosis presents with exudative tonsillitis covered in a white pseudomembrane, which is usually absent in HIV. Chronic HIV can also cause "persistent generalized lymphadenopathy" in asymptomatic individuals with declining CD4 counts due to viral replication, which can be further classified histologically as either acute/early, intermediate/chronic stage, or advanced/burned out stage. Once the CD4 is less than 200, one can see a worrisome paradoxical regression of the lymphadenopathy.

Declining CD4 counts put individuals at risk of developing OI, malignancy, or lymphoma, and they all typically present with similar constitutional symptoms and further cloud the clinical picture. Prioritizing the differential will depend in part on geography. For example, an HIV-positive patient in the absence of risk factors in the United States is less likely to have *Mycobacterium tuberculosis* infection when compared to a patient in Uganda. A multi-centered retrospective cohort study by Bogoch et al. examined 107 peripheral lymph node biopsies in multiple centers in Boston, where malignancy comprised 42.9%, and tuberculosis (TB) was less than 3% of the cases.[56] Unfortunately, in low-income countries, this can lead to a misdiagnosis of TB and a delayed diagnosis of lymphoma. The delay in diagnosis could be attributed to the lack of resources, equipment, and skills needed for a diagnostic biopsy. Buyego et al. retrospectively reviewed 183 charts of HIV-positive patients diagnosed with lymphoma in the Uganda Cancer Institute, of which a third were initially started on TB treatment for a median of 3.5 months prior to the diagnosis of lymphoma.[57]

The most common opportunistic infection worldwide is scrofula presenting as painless cervical lymphadenopathy caused by TB, which can present with almost normal CD4 counts. HIV infection increases the risk of TB by 20-fold when compared to the general population.[58] This is primarily attributed to the decline in CD4 counts, which normally, upon activation, release cytokines such as interferon-gamma and IL-12 responsible for eliciting the immune response to eradicate intracellular pathogens such as TB.[59] The clinical importance of this response is demonstrated by Mendelian susceptibility to mycobacterial diseases (MSMD), rare immunodeficiency syndrome characterized by deficiencies in IL-12 or Interferon-gamma receptors leading to increased susceptibility to TB, and even mycobacteria of low virulence such as the ones contained in the BCG vaccine.[60] Other infections causing lymphadenopathy include Infectious mononucleosis, disseminated *Mycobacterium avium* complex, cytomegalovirus, secondary syphilis, toxoplasmosis, *Bartonella henslae*, leading to bacillary angiomatosis, and acute EBV. When CD4 counts start dropping below less than 200 one begins to think of *Candida*, *Aspergillus*, and, although rare to present with lymphadenopathy, *Pneumocystis* (PJP w/ PNA) can also have hilar and mediastinal lymphadenopathy. When the CD4 counts drop even lower, the differential broadens further to include *Histoplasma capsulatum* and *Cryptococcus neoformans*.

Rare causes of lymphadenopathy can also be non-infectious and non-neoplastic in etiology. These conditions can affect immunocompetent individuals and include Kimura disease presenting as painless lymphadenopathy in the head and neck with a predilection for Asian males in their late 20 s or early 30 s; Kikuchi necrotizing lymphadenitis, which predominantly affects young women, and Castleman disease, which in the HIV-population, is a lymphoproliferative disorder with two histological categories that encompass the hyaline-vascular type and plasma-cell type. The hyaline-vascular type has a lymphoid subtype variant identified by mantle zone hyperplasia (i.e., lymphocytes surrounding the germinal center), which is potentially mistaken for malignant follicular lymphoma or mantle cell lymphoma. The clinical classification of Castleman can be further subdivided into unicentric and multicentric. Unicentric: involves one lymph node most commonly located in the mediastinum, is less likely to present with constitutional symptoms, and in the majority of cases happens to be classified under hyaline-vascular histological subtype. It is curable with surgical excision. On the other hand, a multicentric form of Castleman disease (systemic subtype) carries a poor prognosis and presents with generalized lymphadenopathy and sometimes splenomegaly along with constitutional symptoms. Histologically, multicentric Castleman disease (MCD) falls under the plasma cell type, and ever so rarely under the hyaline-vascular histological subtype, hence the interchangeability of the terms "multicentric" and "plasma cell type." Unlike the unicentric subtype, it is managed by a combination of immunotherapy, steroids, and sometimes chemotherapy. MCD is further classified as HHV-8–positive and HHV-8–negative. HHV-8+ MCD is found at a higher prevalence in HIV-positive patients when compared to HIV-negative patients. Within the HIV population, MCD tends to almost always associate with HHV-8. Production of IL-6 by the HHV-8 virus is believed to lead to its pathogenesis. Some cases have concurrent Kaposi sarcoma, one of the AIDS-defining cancers where HHV-8 is integral for its oncogenesis.[61,62] These causes of lymphadenopathy remain relatively uncommon within the HIV population; however, they contribute to a comprehensive differential.

Although there are certain characteristics of imaging that may help guide the clinician in discerning the etiology of the lymphadenopathy, it should be taken into the clinical context of the patient, that is, whether well controlled on HAART with very low viral load, and adequate CD4 count. Most cases are often biopsied to reach a diagnosis. There are different imaging modalities one can use: ultrasound (US), magnetic resonance imaging (MRI), computed tomography (CT), and positron emission tomography (PET).

Normally the lymph node consists of an outer layer, the capsule which covers the cortex (B cells and follicular dendritic cells), followed by the paracortex (T-cells rich), then the most inner part, the medulla (lymph sinuses, arteries, and veins), and the hilum from where vessels enter and exit. On imaging, normal lymph nodes are less than 1 cm, homogenous, have well-defined borders, and have a smooth contour. Benign lymph nodes also have a central fatty hilum that can be appreciated on CT and MRI.

When assessing a lymph node, consider location, size (abnormal is typically greater than 1 cm), shape, consistency, adherence, and tenderness to help in the differentiation of benign and malignant. Normally tenderness signifies a rapid increase in size, causing the pain receptors in the capsule to be activated, which usually happens from inflammatory processes. However, tenderness does not exclude malignancy, given tenderness can be from necrosis or hemorrhage into the lymph node. If the lymph node is large and painless, then that raises the index of suspicion.

For lymph nodes that are on the surface, an ultrasound is an inexpensive test that can give information and help in guiding the biopsy. This is most applicable to the commonly present cervical lymphadenopathy. Lymph nodes of the head and neck are normally less than 0.8 cm in size. The outer cortex appears hypoechoic due to the presence of lymphoid follicles, whilst the medulla is hyperechoic. The long-to-short axis ratio of less than two is abnormal, meaning that a normal lymph node will become more round than oval. This happens during malignancy or in TB. An oval lymph node does not exclude malignancy, as early in the course the lymph node still retains

Downl
15, 202

its oval shape. There are also anatomical exceptions to this, as parotid lymph nodes are normally round. The presence of cortical thickening with asymmetry and ill-defined borders is pathological for malignancy. Color flow or power doppler is a mode in the United States that can help in evaluating the vascularity of the lymph node. One would expect the hilar to have flow based on the anatomy. Lack of flow in the hilum is pathologic in addition to the presence of peripheral vascularity, although this can be present in both malignant and inflammatory processes. The lymph node will look more heterogeneous than homogenous.

The lack of certain findings or presence of others is insufficient to distinguish lymphomas from reactive lymph nodes secondary to inflammatory etiologies such as Kikuchi disease, TB, and sarcoidosis from metastatic lymph nodes. Therefore, a biopsy is almost always indicated, and starting with an ultrasound (US)-guided fine-needle aspiration is helpful in excluding metastatic disease (to be discussed in further detail). However, fine needle aspiration is sub-optimal for diagnosing lymphomas, and a larger screwcut or excisional biopsy is needed if an excisional biopsy is not possible or appropriate.

The biggest limitation of US imaging is its inability to access cavitary and retroperitoneal lymph nodes, which are often present in HIV. These lymph nodes are best accessed using cross-sectional imaging modalities discussed below.

Years ago, prior to the introduction of cross-sectional imaging, conventional lymphangiography was used to assess lymph nodes, most commonly in Hodgkin disease. At the foot between the toes, an oil-based contrast is injected. Images are obtained after the procedure to visualize the lymphatic system and lymph nodes; the dye remains in the node for months-years and allows for follow-up imaging after treatment. The major complication is pulmonary oil embolism which can cause symptoms; cardiopulmonary disorders, which are contraindication. Advancements in MR, CT imaging, and positron emission tomography have rendered this obsolete.

The pathological findings of lymph nodes in MR and CT imaging overlap with US findings. The size, shape, borders, and absence or presence of central necrosis and calcification are some of the characteristics used in assessing the suspicious lymph node. Contrast is used with the CT scan to evaluate for enhancement, i.e., infectious lymphadenopathy tends to have peripheral enhancement with perinodal inflammation and central hypoattenuation compared to metastatic disease, which presents with central hypoattenuation alone in comparison.

In MR, the differences in T1 and T2 signaling aid in the characterization of lymph nodes. For example, in Castleman disease, lymph nodes are T1 hypointense compared to muscles and T2 hyperintense with a linear T2 hypo-intensity that correlates with fibrosis. The use of gadolinium-contrast characterizes flow kinetics, blood volume, enhancement kinetics, and microvascular permeability. Diffusion-weighted MRI can also be useful in differentiating between benign and malignant lymph nodes, where the diffusion coefficient is decreased in malignancy.

Ultimately, imaging alone is not enough to make the diagnosis but instead complements the findings of the biopsy. Imaging can help in providing the lymph node needed for the highest yield, usually the largest one. Inguinal and axillary lymph nodes are often not biopsied, as they are more often reactive and will not provide the diagnosis. The gold standard is an excisional biopsy with preserved architecture is preserved and allows for a diagnosis of lymphoma and its classification. Starting with a fine needle aspiration can diagnose metastatic carcinoma if this is suspected.

In half of the cases, a cytologic diagnosis of lymphoma can be made based on morphology alone. A core biopsy helps with architectural evaluation; however, it has "compression artifacts" meaning that cells may appear smaller and nuclei can appear darker when compared to the gold standard. This, along with sending the aspirate for flow cytometry immunophenotyping and immunohistochemical staining of the sample, helps in making the diagnosis. However, this may not be enough in a third of the cases, and an excisional biopsy is required. FNA may later be used for disease surveillance post-treatment.

The pattern of inflammatory etiologies can be divided into follicular/nodular versus interfollicular/paracortical. The former is not neoplastic and is where reactive follicular hyperplasia, Castleman disease, kimura, and rheumatoid lymphadenopathy fall. Interfollicular is further divided into three categories: diffuse, sinus, and mixed. Lymphoma can have an interfollicular or diffuse pattern, and so do viral etiologies like EBV, CMV, and drug sensitivity reactions. Infections that can present in a mixed pattern are toxoplasmosis and cat-scratch disease caused by *Bartonella henselae*. Granulomatous lymphadenitis also appears in a mixed pattern and is subdivided into infectious and non-infectious etiologies. Tuberculosis, tularemia, and later stages of *Bartonella henselae* can present in this manner. Non-infectious would be sarcoidosis or any sarcoid-like etiology, including CVID.

Also, in reactive follicular hyperplasia, the normal anatomy of the lymph node is preserved. The follicles are more prominent in the cortex with a lot of variation in size and shape. There should almost be no inflammatory cells present in the capsule or the fat covering the capsule. One can appreciate follicles with reaction centers. This is not the case in follicular lymphoma, where the normal architecture of the lymph node is effaced, no prominence of the follicles is appreciated, and they are equally distributed between the capsule and medulla without variation in size or shape and appear faded rather than reactive.

Summary

Lymphadenopathy in HIV is common, has a million etiologies, and not rarely can be malignant. Multiple studies (blood tests, imaging, and biopsies) are often needed to resolve the differential diagnosis. Considering the patient context is important to limit the differential at least initially.

HUMAN IMMUNODEFICIENCY VIRUS AND THROMBOSIS

There is an estimated 2- to 10-fold increased risk for venous thrombosis (VT)[63] and 2-fold increased risk for myocardial infarction in the HIV population[64] when compared to the general population. Traditional risk factors for both HIV-infected and uninfected populations are well studied for both types of thromboembolic events (TEE). Arterial thromboses commonly include coronary artery disease (CAD) and cerebrovascular accidents (CVAs), which are often related to hypertension, diabetes, and dyslipidemia. Other less common TEE include acute mesenteric arterial occlusion and embolism to the lower extremities. Venous thrombosis follows the Virchow triad: stasis of blood flow from immobilization due to surgery, hospitalization, or long flights; endothelial damage attributed to oxidative stress either from chronic inflammation or direct trauma such as the case in intravenous drug use or venous catheter placement; and hypercoagulability from inflammation related to active infection or infection, genetic predispositions such as factor V Leiden, or acquired causes in the cases of protein C and/or S deficiency. When it comes to the above-mentioned traditional risk factors, HIV patients are more likely to be hospitalized for either malignancy and/or infection when compared to the general population, given the chronic state of immunosuppression. Other risk factors for VTs in HIV infections stem from the ongoing chronic inflammation leading to endothelial damage from oxidative stress; acquired deficiencies for protein S, antithrombin, and heparin cofactor II; increases in pro-coagulant tissue factor, D-dimer, and von Willebrand factor; and production of antiphospholipid antibodies. As for arterial thrombosis, a combination of dyslipidemia from antiretroviral medication, ongoing inflammation, and endothelial damage increases the risk for cardiovascular disease in this population. This will be discussed in greater detail below.

Markers of inflammation and activation of coagulation are elevated in uncontrolled HIV and, to a lesser extent, even in well-controlled HIV (viral load undetectable) when compared to the

Downl
15, 202

general population. Those markers include interleukin-6 (IL-6), high-sensitivity C-reactive protein (where hsCRP differs from CRP in that it detects values below 0.3 mg/L, whereas CRP only detects up to 3 mg/L), D-dimer, and release of tissue factor from activated monocytes thereby conferring an increased risk of thrombosis. Furthermore, elevated D-dimer levels in particular, have been correlated with all-cause mortality in the HIV population, as described below.

The Strategies for Management of Antiretroviral Therapy (SMART) Study group published a landmark non-blinded randomized trial, $CD4^+$ Count–Guided Interruption of Antiretroviral Treatment, which demonstrated the importance of continuous ART in the management of HIV when compared to intermittent ART use. The rationale behind the study was to determine whether intermittent use increased the quality of life of patients by reducing adverse effects related to medication side effects and to try to decrease the division of multi-drug resistant HIV so that the wild type prevailed. SMART recruited a total of 5472 participants with a CD4 count above 350/mm^3; the study took place between January 2002 and January 2006. The participants were randomly assigned to a drug conservation (DC) group versus viral suppression (VS) group and approximately a quarter of the participants were followed for more than 2 years. In the DC, ART was started once the CD4 count was below 250 or HIV-related infections occurred, or the CD4 comprised less than 15% of the total lymphocyte count. ART was subsequently discontinued once the CD4 count reached 350 and resumed as above. Contrarily, the VS group remained on ART indefinitely. The primary outcome measure was the development of an opportunistic infection or death. Secondary outcomes were the occurrence of a major cardiovascular event and the development of renal or hepatic disease. The study was terminated by an independent data and safety monitoring board, given the lack of benefit in the DC group when compared to the superior outcomes of the VS group, in addition to the increased risk of death (84%, $P < 0.001$). Participants who were in the VS group were less likely to acquire an opportunistic infection and die from other causes, i.e., cancers, cardiovascular disease, and opportunistic infections. Furthermore, the risk of episodes of major events including cardiovascular, renal, and hepatic disease, was higher in the DC group, which is counterintuitive when thinking of the ART side effects profile. This was further demonstrated when accounting for CD4 count in the VS group, where individuals with higher CD4 count were at a lower risk for experiencing major events, thus shedding light on confounding factors such as degree of immunosuppression and ongoing viral replication that may have contributed to major events initially thought to solely be related to medication side effects.[65]

Kuller et al. launched a nested case-control study using stored blood samples from the SMART group study measuring biomarkers of inflammation and thrombosis correlating them to mortality in addition to a study comparing the differences in biomarkers between the DC and VS groups. They hypothesized that viral replication resulted in activation of coagulation and inflammation and that this was associated with an increased risk of all-cause mortality. For the nested case-control study, plasma samples were from 85 deceased participants, who were matched to 170 patients (1:2 matching). Levels of hsCRP, IL-6, and D-dimer at entry were significantly higher amongst the deceased when compared to matched controls. This difference remained statistically significant when looking at the latest obtained plasma levels prior to death as well. The association was strongest for IL-6 and D-dimer for all-cause mortality. For the comparison of inflammatory markers between the DC and VS groups, 499 participants were enrolled, 249 and 250 respectively. Similarly, baseline plasma levels were obtained, followed by levels at 1 month. At baseline, D-dimer was found to be higher in those not taking ART when compared to those with ART with either a viral load below or more than 400 copies/mL. At 1 month, those in the DC group had significantly higher IL-6 and D-dimer levels. Increases in IL-6 and D-dimer were positively correlated to HIV-RNA levels. hsCRP differences between DC and VS groups were not significant. The authors concluded that IL-6 and D-dimer were associated with all-cause mortality and that inflammatory markers were increased in those not taking ART and in participants with high viral replication.[66]

When compared to HIV-negative individuals, increases in inflammatory markers persisted irrespective of the presence of comorbid diseases in HIV-positive individuals. Armah et al. measured IL-6, D-dimer, soluble CD14 (sCD14, marker of monocyte activation) in 1525 HIV-positive and 843 HIV-negative individuals in plasma between 2005 and 2006 obtained from the Veteran Aging Cohort Study, a prospective observational cohort of HIV-positive veterans matched to HIV-negative veterans based age, race, and location. Within the cohort, HIV-positive veterans were more likely to be younger, male, have dyslipidemia, have a hepatitis C co-infection, and smoke. They were less likely to be using a lipid-lowering medication, have cardiovascular disease, renal disease, uncontrolled hypertension, diabetes, obesity, and have a self-reported drinking problem. When adjusting for age and race between the two groups, no significant differences between IL-6, D-dimer, and sCD14 were noted. However, when adjusting for viral copies above 500 copies/mL or CD4 counts below 200, i.e., uncontrolled HIV, IL-6 and D-dimer were significantly higher in the HIV-positive group. Furthermore, when only adjusting for CD4 counts below 200, a significantly higher sCD14 was found, signifying a higher monocyte activation in the immunosuppressed group. This correlation persisted when samples were selected for veterans without comorbid conditions, problematic drinking, or smoking, which emphasizes the role of ART in reducing the viral load and increasing the CD4 counts as a strategy to ameliorate the underlying inflammatory response, thus reducing mortality.[67]

In a randomized comparison, Baker et al. investigated the effects of initiating ART on IL-6, D-dimer, and hsCRP in a subgroup of participants from the SMART study who were either therapy naïve or off ART for 6 months or longer prior to randomization. This included 126 and 128 participants in the DC and VS groups, respectively. The VS group immediately started ART, whereas only 1.6% at 2 months and 10.3% of DC participants were placed on ART. Inflammatory markers were measured at baseline, 2 months, and 6 months. The reduction in D-dimer levels in the VS group compared to the DC were significantly lower, where the VS group had a reduction of 21.7% and 32.2% at 2 and 6 months, respectively. Furthermore, D-dimer levels in the VS group were significantly lower with time (0.40, 0.27, 0.24 µg/mL at 0, 2, and 6 months respectively), with the most substantial reduction observed in those achieving viral levels below 200 copies/mL (i.e., viral suppression). This was not observed with IL-6 and hsCRP, where the two groups were similar, and no statistically significant reductions were observed with the initiation of ART. Not all participants achieved viral suppression at 6 months, and perhaps a longer observation period would be needed to observe a significant change in IL-6 and hsCRP in response to ART.[68]

The chronic inflammation, CD4 depletion, and ongoing viral replication in untreated HIV or ongoing at low levels while on AART all contribute to a net pro-coagulant effect that directly involves the coagulation cascade, the endothelium, and aberrantly activated monocytes that release tissue factors. In addition to monocytes, other sources of tissue factor include endothelial cells, platelets, and microparticles. It is hypothesized that monocytes are activated by lipopolysaccharides (LPS) released by translocated gut bacteria. The activated monocyte then releases TF, which then activates the extrinsic clotting pathway leading to thrombin (and thrombus) formation. The early stage of HIV infection results in CD4 depletion, which occurs markedly in mucosal tissues that include the gastrointestinal tract. The reduction in antimicrobial barriers in mucosa leads to further damage in the GI mucosa, compromising the tight epithelial junctions that prevent the translocation of bacterial products, such as LPS, which is produced by gram-negative bacteria leading to monocyte activation.[69,70] Brenchley et al. published their proof of this through a series of collected data on HIV-infected and non-infected individuals and in vivo experiments on SIV-infected macaques. First, the authors confirmed that chronically HIV-infected individuals had elevated LPS when compared to participants without HIV ($P < 0.0001$) and also noted that newly infected HIV participants did not have this increased LPS—theorized to be from the immune system's ability to neutralize LPS at the initial stages of infection. Second, they demonstrated a similar increase in macaques, where LPS was measured at baseline prior to

Downl
15, 202

being inoculated with simian immunodeficiency virus and measured the levels at day 100 after infection. Third, to prove that the source of LPS was a result of gut translocation, the macaques underwent a 2-week antibiotic course to deplete the gut from bacteria. This was followed by subsequent LPS measurements in the serum along with measurement of bacterial fecal load, which showed a decrease in both parameters. At week 2, the antibiotics were no longer effective in suppressing the gut bacterial load, which was evident by the increase in the bacterial load in fecal samples and the concurrent increase in the measured LPS in the plasma. Next, an in vivo experiment was conducted to prove that LPS activated monocytes. Monocytes are known for secreting sCD14 and IL-1 in response to LPS released by bacteria in infections. Hence, plasma sCD14 levels were measured and correlated significantly with plasma LPS levels ($r = 0.3$, $P = 0.001$). Individuals with acute and chronic HIV, whether well-controlled or with AIDS, had higher levels of sCD14 when compared to HIV-negative individuals ($P < 0.0001$). To prove that this correlation with LPS was not confounding, a second marker LPS-binding protein was measured. LPS stimulates the release of LPS-binding protein from gastrointestinal epithelial cells and is released by gastrointestinal epithelial cells and hepatocytes. Similar to sCD14, LPS-binding protein was increased in those with HIV infections when compared to non-infected individuals ($P = 0.0099$), and similar to LPS, it correlated positively with sCD14 levels ($r = 0.44$, $P < 0.0001$).[70] LPS activation of monocytes leading to tissue factor (TF) production and coagulation activation was demonstrated in humans by Funderburg et al., where mononuclear cells were isolated using flow cytometry from blood collected from 19 non-infected donors (control) and 60 HIV patients[71] and incubated with LPS, flagellin, and IL-6. TF activity was then measured along with levels of D-dimer. Prior to incubation, when compared to non-infected donors, HIV-infected patients stratified by VL load of greater than 400 copies/mL and VL less than 400 had a higher median percentage of monocytes expressing surface TF (11%, 45.4%, 26.8%, respectively; $P < 0.001$). Exposure to bacterial LPS and flagellin showed an increase in surface expression of TF on monocyte, while IL-6 failed to show such an increase. D-dimer plasma levels were higher in HIV patients when compared to controls and correlated positively with monocytes expressing TF ($r = 0.37$, $P = 0.007$). However, correlation does not equal causation, as D-dimer is known to be elevated in pro-inflammatory states, and it is difficult to ascertain the applicability of the above-mentioned data in vivo without eliminating confounders.

Protein S deficiency is the most commonly found coagulation abnormality in individuals with HIV, where 65% of individuals are found to have varying degrees of deficiency. Protein S deficiency can be categorized into different types: Type I, a quantitative deficiency meaning a decrease in the total and free protein S antigen; Type II, a functional deficiency meaning the level of the total and free protein S antigen are normal, but the activity is decreased; and Type III, which is the most commonly encountered in HIV, where the total levels of the protein are normal, but the levels of the free active form are reduced thereby conferring a functional deficiency.[63] The levels also correlate with the CD4 count, where CD4 less than 200 correlates with lower levels of the active form. In healthy individuals, protein S is synthesized in the liver; 40% of it circulates free (not bound to C4b binding protein) in the plasma, whereas the remainder is inactive because it is bound to C4b-binding protein complex—a protein within the classical and lectin complement pathways that inhibits the complement system, and is an acute-phase reactant. Free protein S functions as a co-factor for protein C and forms an activated cell-surface complex that inactivates factors Va and VIIIa, thus limiting coagulation. Many factors could contribute to protein S deficiency. One is decreased synthesis by the liver, but the primary one is HIV infection in response to inflammation with the increased C4bBp reducing the amount of free protein S. There can also be concurrent malnutrition or hepatitis C co-infection, production of anti-protein S autoantibodies, ongoing consumptive coagulopathy in the setting of infection or malignancy, or binding to microparticles that are released during apoptosis of T cells, activation of monocytes, or any cell necrosis.[72,73]

Compared to protein S, the prevalence of protein C deficiency in HIV is not well established in published reports in which it ranges from 0% to 14%. One study found a prevalence of 7% for protein C deficiency compared to 32% for protein S deficiency in HIV-positive individuals with known VTE.[74] Proposed mechanisms are similar in that there is decreased synthesis, presence of inflammation, that is, opportunistic infections, and low-grade consumptive coagulopathy.[75]

Other less elucidated deficiencies that have been observed in HIV patients include antithrombin and heparin cofactor II deficiencies. Acquired antithrombin deficiency is thought to result from decreased synthesis by the liver (from malnutrition, nephrotic syndrome, or protein-losing enteropathy) or consumptive states, that is, DIC; antithrombin in protein C is made in endothelial cells and has a much longer half-life (20 hours) compared to that of protein S (4-hour serine protease inhibitor that irreversibly inactivates thrombin factor IIa), IXa, Xa, XIa, XIIa, and VIIa. Heparin cofactor II is a thrombin inhibitor and is seen primarily in HIV patients with CD4 counts below 200 and is thought to be related to degrees of immunosuppression.[72,73,76] This was measured by Toulon et al., where levels of heparin cofactor activity were measured in 96 HIV-positive patients and compared to 96 age-matched controls. 38.5% of HIV-positive samples were found to be deficient when compared to 2.1% in the age-matched controls.[77] Heparin cofactor II deficiency alone is insufficient for causing thromboembolic events based on case reports in homozygous and heterozygous heparin cofactor deficiencies and can be a normal variant in healthy subjects.[78]

Patients with HIV have been found to have increased levels of von Willebrand factor (vWF), a marker for endothelial damage likely the result of ongoing inflammation, oxidative damage, and increased T cell activation.[79] vWF is a glycoprotein made and released by the endothelium cells and stored in Weibel-Palade bodies and alpha-granules in platelets, where it (1) aids in platelet aggregation and adherence to the endothelium, (2) binds to factor VIII, thereby stabilizing it for clot formation. The highest levels of vWF are seen in HIV patients with lower CD4 counts and/or high viral loads, whereas HAART keeps vWF levels lower, as demonstrated by Aukrust et al., who examined 43 HIV patients in Norway. Plasma levels of vWF were negatively correlated with CD4+ counts ($r = -0.54$, $O < 0.001$) and correlated positively with viral load ($r = 0.52$, $P < 0.01$). Twenty of the 43 patients were followed for a median of 5 years. Of the participants, 50% developed AIDS and were classified as "disease progressors" with CD4 less than 50, and 50% had CD4 counts above 100 and were classified as "slow progressors". At baseline, both groups did not differ in CD4 count at the beginning of the study, nor did they differ in vWF levels; however, "disease progressors" showed a marked increase in vWF during the study period, whereas the "slow progressors" had stable levels of vWF throughout. Furthermore, levels of vWF were examined prior to and after the initiation of HAART in 15 patients at 12 and 26 weeks of therapy, where a significant decrease in plasma vWF in all the patients was observed and also correlated with a maximal decrease in viral load ($r = 0.67$, $P < 0.01$).[80] These findings of higher VWF are all consistent with more active, less well-controlled HIV disease.

Platelets in HIV-positive patients also have a role in the increased risk of thrombosis and plaque formation leading to cardiovascular events as they tend to show greater activation when compared to people without HIV. HIV-1 directly binds to fibronectin found on the surface of platelets via gp120.[81] Activated platelets express P-selectin, an alpha granule glycoprotein, which binds to its corresponding receptor P-selectin glycoprotein ligand-1 (PSGL-1) found on the endothelium and surfaces of leukocytes, thereby facilitating adhesion and migration for clot formation. Mayne et al. isolated platelets from 46 HIV-positive patients and 18 healthy controls and, using flow cytometry, measured the proportion of platelets and microparticles expressing surface P-selectin. Compared to controls, 17.3% of HIV-positive patients were found to express surface P-selectin compared to 1.1% of healthy controls ($P < 0.001$). Furthermore, patients with detectable viremia were more likely to express surface P-selectin compared to those with an undetectable viral load (23.3% vs. 12.5%, $P = 0.022$).[82]

Abacavir, a guanosine-analog NRTI associated with hypersensitivity in individuals with HLA-B*5701, has been also associated with an increased risk for myocardial infarctions for those who were recently exposed to the drug (RR, 1.70 [95% CI, 1.17–2.47]) related

Downl
15, 202

to increased platelet activation.[83] This was demonstrated in vitro by Satchell et al. in a prospective cross-sectional study in Ireland that included 120 patients. Forty of the patients were therapy naïve, forty were on antiretroviral therapy that included abacavir, and forty were on antiretroviral therapy without abacavir. Blood samples were collected and tested for other parameters used in matching that included low-density lipoprotein (LDL), high-density lipoprotein (HDL), total cholesterol, triglycerides, fasting glucose, and creatinine. Platelet reactivity was determined using four different agonists at different concentrations and included: adenosine diphosphate (ADP), collagen, epinephrine, and thrombin receptor-activating peptide (TRAP), where platelet aggregation was determined using light absorbance measured at 3-minute intervals over 21 minutes. Different light absorbances were then translated into percentage aggregation. There was no statistical difference in platelet aggregation appreciated when comparing the ART-naïve group to those who received ART as a whole. Interestingly, the abacavir-ART group had statistically significant greater platelet reactivity when compared to those receiving ART without abacavir (ADP, $P = 0.001$; collagen, $P = 0.008$; epinephrine, $P = 0.002$; and TRAP, $P = 0.078$). The mechanism underlying the observed increased risk of myocardial infarctions is hypothesized to be due to competitive inhibition of soluble guanylate cyclase (sGC)—normally activated by nitric oxide—by the active form of abacavir, carbovir triphosphate; where sGC is needed for the production of cGMP and activation of downstream intracellular protein kinases responsible for vasodilation and inhibition of platelet aggregation.[84]

OI have a weak association with an increased risk of venous thromboembolism (VTE) in patients with HIV when compared to HIV patients without opportunistic infections in a retrospective cross-control study, especially in those with cytomegalovirus (OR 18.89, 95% CI 2.074 to 172.013, $P = 0.003$), both *Mycobacterium tuberculosis* and *Mycobacterium avium-intracellulare* (OR 3.71, 95% CI 1.06 to 12.96, $P = 0.07$), and in those with more than one lifetime opportunistic infection (OR 3.927, 95% CI 1.447 to 10.657, $P = 0.008$).[85] CMV is known to directly infect endothelial cells possibly leading to the activation of the coagulation cascade.[75] Of note, the patients included in the study citing the weak associations above had a statistically significant difference in the Nadir CD4 count (40 in the VTE arm vs. 250 in the control arm, $P = 0.0003$). This raises the question of whether the presence of OIs is a confounding factor, as CD4 count is an established risk factor for the development of VTE and likely accounts for the wide confidence intervals presented by the data above.

Patients with AIDS have antiphospholipid antibodies in the great majority of cases, specifically lupus anticoagulant and anticardiolipin; whether this contributes to an increased risk of VTE and arterial thrombosis remains controversial, as do many studies of anti-phospholipid antibodies. Lupus anticoagulant is associated with thrombosis in systemic lupus erythematosus (SLE); its presence is conferred by a prolonged partial thromboplastin time, which is a result of the antibody targeting the phospholipids present on the clotting assay. Around 1% to 2% of the healthy population is seropositive for lupus anticoagulant.[86] In 1988, Perkocha et al. found that the incidence of lupus anticoagulant was 20% to 73% in asymptomatic AIDS patients with and without the presence of opportunistic infections, with the incidence between 20% to 73%.[87] Similar findings were noted in Chile in 2003 by Palomo et al., where they examined 63 HIV-positive patients for all three antiphospholipid antibodies. In their cohort, 0% of patients were positive for lupus anticoagulant, 12.7% positive for anticardiolipin antibodies, and 6.3% for anti-beta-2 glycoprotein 1 antibodies. The presence of the antiphospholipid antibodies did not correlate with the presence of thrombosis or thrombocytopenia.[88] Furthermore, the presence of antiphospholipid antibodies did not correlate with CD4 count, viral count, or presence of DVT.[73] PJP has also been associated with an increased risk for VTE, where 96% of infected patients have been found to have lupus anticoagulant antibodies.[72] Rarely, anticardiolipin has been reported in association with thrombosis in TIA and skin necrosis in case reports. It is hypothesized that antiphospholipid antibodies may be products of B-cell polyclonal stimulation.[72]

Pregnancy and postpartum are known risk factors for VTE in women, even more so in pregnant women with HIV. In the general population, the relative risk for development of VTE during pregnancy or in the post-partum period is 4.29 (95% CI, 3.49 to 5.33; $P < 0.001$). Moreover, women in the postpartum period had a higher annual incidence of VTE when compared to pregnant women (511.2 vs. 95.8 per 100,000).[89] The increased risk of VTE is attributed to an increased state of hypercoagulability resulting from increased production of factors VII, VIII, IX, X[90] and venous stasis secondary to decreased mobility during pregnancy and direct compression of the uterus on vessels thus decreasing venous capacitance. HIV-positive pregnant women are a 157-fold increase in VTE when compared to HIV-negative pregnant women,[75] and likely the result of additional risk factors relating to inflammation discussed above being present. Warfarin is avoided in the treatment of VTE in pregnancy given its teratogenic profile, and instead, heparin products are used such as low-molecular heparin, enoxaparin, or fondaparinux when heparin-induced thrombocytopenia with thrombosis (HITT) is of concern.

The treatment of venous thromboembolism does not differ in the HIV population; however, special consideration should be given as therapy can interfere with HAART resulting in either subtherapeutic or supratherapeutic anticoagulation. According to the CHEST guideline and Expert Panel Report, patients without cancer diagnosed with their first VTE should be initiated on anticoagulation for a duration of 3 months (Grade 1B). An extended duration (stopped at the clinicians' discretion) can be considered if the VTE is unprovoked and the bleeding risk for the patient is low (Grade 2B). Recurrent VTEs warrant lifetime anticoagulation if bleeding risk is low to moderate (Grade 1B). Novel oral anticoagulants (NOACs) such as apixaban, rivaroxaban, or dabigatran are the preferred agents as they do not require monitoring and have a lower bleeding risk when compared to vitamin K antagonist therapy with warfarin, which requires weekly INR monitoring. NOACs are contraindicated in patients with severe renal impairment. Oral warfarin is preferred over subcutaneously injected low-molecular-weight heparins (LMWH), such as enoxaparin and dalteparin, given the route of administration and price differences. In patients with cancer, extended anticoagulation is recommended over 3 months of therapy (Grade 1B),[91] where LMWH are the preferred agents given superior outcomes in preventing VTE recurrence based on the CLOT trial.[92]

When considering drug-drug interaction with HAART and anticoagulants, it can be helpful to classify drugs into ones that are metabolized by cytochrome P450 isozymes in the liver, where drugs can either lead to enzymatic inhibition or induction versus urine drug secretion mediated by P-glycoprotein protein, a transmembrane efflux pump, that participates in drug elimination in the proximal tubule in the kidney and has another role in drug absorption in epithelial cells in the small intestines.[93] Many drugs are both metabolized by CYP-450 isoenzymes and transported by P-glycoprotein. Inhibitors of CYP3A4 are typically also inhibitors of P-glycoprotein, and such is the case for rivaroxaban and apixaban (NOACs).

This becomes of importance in patients taking PIs as they are a good substrate for P-glycoprotein, inhibit CYP3A4, and are also metabolized by CYP3A4 (except for tipranavir). When PIs are taken with NOACs, the concentration of the PI increases as a result of the CYP3A4 inhibition by the NOAC, which in turn further inhibits the CYP3A4. For example, rivaroxaban is metabolized by CYP-450 (18% by CYP3A4 and 14% by CYP2J2), where further inhibition by PI of the CYP3A4 can lead to increased concentration of the NOAC–around 40% is renally secreted unchanged via P-glycoprotein as well.[94] Contrarily, Dabigatran is a NOAC that is mainly renally secreted via glomerular secretion and not affected by CYP-450 inhibition and is preferred with the use of PI. However, administration of dabigatran must be spaced by 2 hours from taking the PI, as the pro-drug is also a substrate for P-glycoprotein, where concurrent administration with ritonavir (PI) has been shown to increase dabigatran's bioavailability by 200% times[95] as it increases intestinal absorption via P-glycoprotein. Most NNRTIs are inducers of CYP3A4, which as described above affects rivaroxaban and apixaban leading to subtherapeutic anticoagulation.[95] Unlike PIs and NNRTIs, NRTIs do not have any effects on

Downl
15, 202

CYP-450 isozymes and therefore have lower drug-drug interactions with NOACs. Fortunately, NRTIs are considered first-line HAART therapy for HIV (see Table 152.2).

Warfarin metabolism is affected by PIs, NNRTIs, elvitegravir (an integrase inhibitor), and cobicistat (booster used in combination with elvitegravir). Warfarin is a racemic mixture with R and S stereoisomers that are metabolized by different CYP-450 isozymes. The R-warfarin stereoisomer is metabolized by CYP1A2 and CYP3A4, whereas the S-warfarin stereoisomer is metabolized by CYP2C9 and is 3–5 times more potent in inhibiting vitamin K epoxide reductase complex when compared to R-warfarin.[96] Protease inhibitors and NNRTIs exhibit variable effects on warfarin metabolism leading to either increased or decreased anticoagulation.[75] Elvitegravir is a CYP2C9 inducer, and is administered with cobicistat, a strong CYP3A4 inhibitor; as such the effects are difficult to predict as one drug would increase the levels of warfarin while the latter would increase it–literature is sparse, with a case report showing a 42-year-old man requiring a 60% dose increase.[97] In the general population, warfarin requires weekly INR monitoring in all patients and should be monitored more frequently in HIV patients whenever adjustments to their HAART regimen are made.

Finally, LMWH such as enoxaparin can be used with HAART without issues as it is mainly metabolized by the liver via desulfation and/or depolymerization to smaller fragments that are biologically less active.[98] Patients should be monitored for the occurrence of HITT, which typically results in thrombocytopenia 5 to 10 days after starting heparin products. HIV patients are at an increased risk for developing HITT when compared to HIV-negative patients (13% vs. 0.2%), according to a retrospective cohort study by Thomson et al.[99] and is hypothesized to be the result of antibody production from chronic polyclonal stimulation of B cells akin to the production of antiphospholipid antibodies. In cases of HITT, fondaparinux is used and is secreted unchanged in the urine.

SUMMARY

So, where are we with HIV-related hematologic disease at the time of writing this chapter? Many questions are answered, but many remain. ART has changed the face of HIV: HIV infection now does not automatically lead to AIDS, and HIV infection has become a chronic disease with an estimated survival of many years. This, of course, is achieved by dint of taking many medications during this time and monitoring many laboratory parameters. Nonetheless, despite highly active ART, there is an increased incidence of lymphoma (NHL and HL) and thrombosis (TEE) in infected patients who are taking ART, even if the viral load is undetectable. Both NHL and TEE occur more frequently than in non-HIV-infected individuals, although both complications are more common in uncontrolled HIV-infected patients as compared to those on ART.

There are AIDS-defining types of NHL, but these are rare, and most cases of NHL in HIV-infected patients are not AIDS-defining. However, even for the non-AIDS defining lymphomas, the types and the distribution within types (especially for DLBCL) are different than that seen in non-HIV-infected patients. Furthermore, many cases of NHL, but far from all, are EBV driven. While anti-viral treatments continue to evolve, a universal feature is that effective ART is widely available in developed countries.

TEE seems to be related to inflammation and is more commonly venous than arterial. It appears multifactorial, which may mean that a general predisposition to TEE is more likely to be activated if there is a second cause predisposing to TEE, e.g., antiphospholipid antibody and Factor V Leiden. TEE continues to be seen in HIV-infected patients on ART even though, like NHL, the increase compared to non-infected patients is less if the HIV is well-controlled.

Cytopenias were very frequent in the past in developed countries but now occur not at all frequently and only in those not on ART. For example, HIV-TP was seen in essentially half of the HIV-infected patients in the 1980s before the advent of universal ART. Current reports are frequent, but all come from areas in which ART is not in wide use.

Overall in the United States, HIV has become a very chronic disease, and the progress in developing effective but safe ART has been nothing short of remarkable, including finding ways to deliver multiple medications in one capsule a day or now, even one injection a month. The absence of an effective vaccine continues to be palpable, although pre- and post-exposure prophylaxis reduces transmission. Nonetheless, there are more than 30 million cases of HIV worldwide, and all too many of them are untreated, resulting in opportunistic infections as well as hematologic complications.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Armah KA, McGinnis K, Baker J, et al. HIV status, burden of comorbid disease, and biomarkers of inflammation, altered coagulation, and monocyte activation. *Clin Infect Dis*. 2012;55(1):126–136.

Aukrust P, Bjørnsen S, Lunden B, et al. Persistently elevated levels of von Willebrand factor antigen in HIV infection. *Thromb Haemost*. 2000; 84(08):183–187.

Baker JV. Chronic HIV disease and activation of the coagulation system. *Thromb Res*. 2013;132(5):495–499.

Baker JV, Neuhaus J, Duprez D, et al. Changes in inflammatory and coagulation biomarkers: a randomized comparison of immediate versus deferred antiretroviral therapy in patients with HIV infection. *J Acquir Immune Defic Syndr (1999)*. 2011;56(1):36.

Bibas M, Biava G, Antinori A. HIV-associated venous thromboembolism. *Mediterr J Hematol Infect Dis*. 2011;3(1).

Chadburn A, Chiu A, Lee JY, et al. Immunophenotypic analysis of AIDS-related diffuse large B-cell lymphoma and clinical implications in patients from AIDS Malignancies Consortium clinical trials 010 and 034. *J Clin Oncol*. 2009;27(30):5039.

Coyle TE. Hematologic complications of human immunodeficiency virus infection and the acquired immunodeficiency syndrome. *Med Clin North Am*. 1997;81(2):449–470.

Diamond C, Taylor TH, Aboumrad T, Anton-Culver H. Changes in acquired immunodeficiency syndrome-related non-Hodgkin lymphoma in the era of highly active antiretroviral therapy: incidence, presentation, treatment, and survival. *Cancer*. 2006;106(1):128–135.

Dodero A, Guidetti A, Tucci A, et al. Dose-adjusted EPOCH plus rituximab improves the clinical outcome of young patients affected by double expressor diffuse large B-cell lymphoma. *Leukemia*. 2019;33(4): 1047–1051.

Dunleavy K, Wilson WH. How I treat HIV-associated lymphoma. *Blood*. 2012;119(14):3245–3255.

Funderburg NT, Lederman MM. Coagulation and morbidity in treated HIV infection. *Thromb Res*. 2014;133:S21–S24.

Gloghini A, Dolcetti R, Carbone A. Lymphomas occurring specifically in HIV-infected patients: from pathogenesis to pathology. In. *Seminars in Cancer Biology*. New York: Academic Press; 2013, December:457–467.

Goebel FD. Immune reconstitution inflammatory syndrome (IRIS)—another new disease entity following treatment initiation of HIV infection. *Infection*. 2005;33(1):43–45.

Good BL, Gomes DC, Fulco PP. An unexpected interaction between warfarin and cobicistat-boosted elvitegravir. *AIDS*. 2015;29(8):985–986.

Klein SK, Slim EJ, De Kruif MD, et al. Is chronic HIV infection associated with venous thrombotic disease? A systematic review. *Neth J Med*. 2005; 63(4):129–136.

Kuller LH, Tracy R, Belloso W, et al. Inflammatory and coagulation biomarkers and mortality in patients with HIV infection. *PLoS Med*. 2008;5(10):e203.

Madan R, Gormley R, Dulau A, et al. AIDS and non-AIDS diffuse large B-cell lymphomas express different antigen profiles. *Mod Pathol*. 2006; 19(3):438–446.

Mayne E, Funderburg NT, Sieg SF, et al. Increased platelet and microparticle activation in HIV infection: upregulation of P-selectin and tissue factor expression. *J Acq Immune Defic Syndr (1999)*. 2012;59(4):340.

Musselwhite LW, Sheikh V, Norton TD, et al. Markers of endothelial dysfunction, coagulation and tissue fibrosis independently predict venous thromboembolism in HIV. *AIDS*. 2011;25(6):787.

Myerson M, Glesby MJ, eds. *Cardiovascular Care in Patients With HIV*. Springer; 2019.

Palomo I, Alarcon M, Sepulveda C, et al. Prevalence of antiphospholipid and antiplatelet antibodies in human immunodeficiency virus (HIV)-infected Chilean patients. *J Clin Labo Anal*. 2003;17(6):209–215.

Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the Use of Antiretroviral Agents in Adults and Adolescents with HIV.

Downl
15, 202

Department of Health and Human Services. Available at https://clinicalinfo.hiv.gov/sites/default/files/inline-files/AdultandAdolescentGL.pdf.
Re A, Cattaneo C, Rossi G. Hiv and lymphoma: from epidemiology to clinical management. *Mediterr J Hematol Infect Dis*. 2019;11(1).
Rubinstein PG, Aboulafia DM, Zloza A. Malignancies in HIV/AIDS: from epidemiology to therapeutic challenges. *AIDS*. 2014;28(4):453.
Saif MW, Greenberg B. HIV and thrombosis: a review. *AIDS Patient Care STDS*. 2001;15(1):15–24.
Saif MW, Bona R, Greenberg B. AIDS and thrombosis: retrospective study of 131 HIV-infected patients. *AIDS Patient Care STDS*. 2001;15(6):311–320.
Satchell CS, O'Halloran JA, Cotter AG, et al. Increased platelet reactivity in HIV-1–infected patients receiving abacavir-containing antiretroviral therapy. *J Infect Dis*. 2011;204(8):1202–1210.
Strategies for Management of Antiretroviral Therapy (SMART) Study Group CD4+ count–guided interruption of antiretroviral treatment. *N Engl J Med*. 2006;355(22):2283–2296.
Thompson GR, Lawrence VA, Crawford GE. HIV infection increases the risk of heparin-induced thrombocytopenia. *Clin Infect Dis*. 2007;45(10):1393–1396.
Worm SW, Sabin C, Weber R, et al. Risk of myocardial infarction in patients with HIV infection exposed to specific individual antiretroviral drugs from the 3 major drug classes: the data collection on adverse events of anti-HIV drugs (D: A: D) study. *J Infect Dis*. 2010;201(3):318–330.

Downl
15, 202

REFERENCES

1. Joint United Nations Programme on HIV/AIDS (UNAIDS). Global AIDS Update, 2020. https://www.unaids.org/en/resources/documents/2020/global-aids-report. Published July 2020.
2. Centers for Disease Control and Prevention. HIV Surveillance Report, 2017; vol. 29. http://www.cdc.gov/hiv/library/reports/hiv-surveillance.html. Published November 2018.
3. Stebbing J, Gazzard B, Douek DC. Where does HIV live? *N Engl J Med*. 2004;350(18):1872–1880.
4. Hahn BH, Shaw GM, De KM, Sharp PM. AIDS as a zoonosis: scientific and public health implications. *Science*. 2000;287(5453):607–614.
5. Nyamweya S, Hegedus A, Jaye A, et al. Comparing HIV-1 and HIV-2 infection: lessons for viral immunopathogenesis. *Rev Med Virol*. 2013; 23(4):221–240.
6. Siegfried N, Muller M, Deeks JJ, Volmink J. Male circumcision for prevention of heterosexual acquisition of HIV in men. *Cochrane Database Syst Rev*. 2009(2)).
7. Connor EM, Sperling RS, Gelber R, et al. Reduction of maternal-infant transmission of human immunodeficiency virus type 1 with zidovudine treatment. *N Engl J Med*. 1994;331(18):1173–1180.
8. Young T, Arens FJ, Kennedy GE, Laurie JW, Rutherford GW. Antiretroviral post-exposure prophylaxis (PEP) for occupational HIV exposure. *Cochrane Database Syst Rev*. 2007(1).
9. Calles NR, Evans D, Terlonge D. *Pathophysiology of the human immunodeficiency virus. HIV curriculum for the health professional*. Baylor Pediatrics International AIDS Initiative. Texas, USA: Baylor College of Medicine; 2010:7–14.
10. Sengupta S, Siliciano RF. Targeting the latent reservoir for HIV-1. *Immunity*. 2018;48(5):872–895.
11. Simmons G, Wilkinson D, Reeves JD, et al. Primary, syncytium-inducing human immunodeficiency virus type 1 isolates are dual-tropic and most can use either Lestr or CCR5 as coreceptors for virus entry. *J Virol*. 1996; 70(12):8355–8360.
12. Peterson CW, Kiem HP. Lessons from London and Berlin: designing a scalable gene therapy approach for HIV cure. *Cell Stem Cell*. 2019;24(5):685–687.
13. Knoppers BM, Kleiderman E. "CRISPR babies": What does this mean for science and Canada? *CMAJ*. 2019;191(4):E91–E92.
14. Ghorban K, Dadmanesh M, Hassanshahi G, et al. Is the CCR5 Δ 32 mutation associated with immune system-related diseases? *Inflammation*. 2013;36(3):633–642.
15. Cheng FJ, Zhou XJ, Zhao YF, Zhao MH, Zhang H. Chemokine receptor 5 (CCR5) delta 32 polymorphism in lupus nephritis: a large case-control study and meta-analysis. *Autoimmunity*. 2014;47(6):383–388.
16. Branson, B. M., & Stekler, J. D. (2012). Detection of acute HIV infection: we can't close the window. *J Infect Dis*. 2012;205(4):521-524.
17. Shibata R, Igarashi T, Haigwood N, et al. Neutralizing antibody directed against the HIV–1 envelope glycoprotein can completely block HIV–1/SIV chimeric virus infections of macaque monkeys. *Nature Med*. 1999;5(2):204–210.
18. Mascola JR, Haynes BF. HIV-1 neutralizing antibodies: understanding nature's pathways. *Immunol Rev*. 2013;254(1):225–244.
19. Goebel FD. Immune reconstitution inflammatory syndrome (IRIS)—another new disease entity following treatment initiation of HIV infection. *Infection*. 2005;33(1):43–45.
20. Dolcetti R, Gloghini A, Caruso A, Carbone A. A lymphomagenic role for HIV beyond immune suppression? Blood. *J Am Soc Hematol*. 2016;127 (11):1403–1409.
21. Linke-Serinsöz E, Fend F, Quintanilla-Martinez L. Human immunodeficiency virus (HIV) and Epstein-Barr virus (EBV) related lymphomas, pathology view point Philadelphia. *Semin Diagn Pathol*. 2017;34(4):352–363.
22. Grulich AE, Van Leeuwen MT, Falster MO, Vajdic CM. Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis. *Lancet*. 2007;370(9581):59–67.
23. Robbins HA, Shiels MS, Pfeiffer RM, Engels EA. Epidemiologic contributions to recent cancer trends among HIV-infected people in the United States. *AIDS*. 2014;28(6):881.
24. Grulich AE, Vajdic CM. The epidemiology of cancers in human immunodeficiency virus infection and after organ transplantation. *Semin Oncol*. 2015;42(2):247–257.
25. Rubinstein PG, Aboulafia DM, Zloza A. Malignancies in HIV/AIDS: from epidemiology to therapeutic challenges. *AIDS*. 2014;28(4):453.
26. Dunleavy K, Wilson WH. How I treat HIV-associated lymphoma. *Blood*. 2012;119(14):3245–3255.
27. Diamond C, Taylor TH, Aboumrad T, Anton-Culver H. Changes in acquired immunodeficiency syndrome-related non-Hodgkin lymphoma in the era of highly active antiretroviral therapy: incidence, presentation, treatment, and survival. *Cancer*. 2006;106(1):128–135.
28. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016; 127(20):2375–2390.
29. Chadburn A, Chiu A, Lee JY, et al. Immunophenotypic analysis of AIDS-related diffuse large B-cell lymphoma and clinical implications in patients from AIDS Malignancies Consortium clinical trials 010 and 034. *J Clin Oncol*. 2009;27(30):5039.
30. Madan R, Gormley R, Dulau A, et al. AIDS and non-AIDS diffuse large B-cell lymphomas express different antigen profiles. *Mod Pathol*. 2006;19(3):438–446.
31. Dodero A, Guidetti A, Tucci A, et al. Dose-adjusted EPOCH plus rituximab improves the clinical outcome of young patients affected by double expressor diffuse large B-cell lymphoma. *Leukemia*. 2019;33(4):1047–1051.
32. Capello D, Martini M, Gloghini A, et al. Molecular analysis of immunoglobulin variable genes in human immunodeficiency virus-related non-Hodgkin's lymphoma reveals implications for disease pathogenesis and histogenesis. *Haematologica*. 2008;93(8):1178–1185.
33. Gaidano G, Pasqualucci L, Capello D, et al. Aberrant somatic hypermutation in multiple subtypes of AIDS-associated non-Hodgkin lymphoma. *Blood*. 2003;102(5):1833–1841.
34. Chadburn A, Abdul-Nabi AM, Teruya BS, Lo AA. Lymphoid proliferations associated with human immunodeficiency virus infection. *Arch Pathol Lab Med*. 2013;137:360–370.
35. Abbas AK, Lichtman AH, Pober JS. B cell activation and antibody production. *Cell Mol Immunol*. 2005;190:191.
36. Shiels MS, Pfeiffer RM, Besson C, et al. Trends in primary central nervous system lymphoma incidence and survival in the US. *Br J Haematol*. 2016;174(3):417–424.
37. Bhatt R, Desai DS. Plasmablastic lymphoma. In: *StatPearls [Internet]*. Treasure Island (FL): StatPearls Publishing; 2022.
38. Meyers C, editor. *HIV/AIDS-Associated Viral Oncogenesis*. Cancer Treatment and Research 177. Cham: Springer; 2018.
39. Morton LM, Wang SS, Devesa SS, et al. Lymphoma incidence patterns by WHO subtype in the United States, 1992–2001. *Blood*. 2006;107(1): 265–276.
40. Saleh K, Michot JM, Camara-Clayette V, Vassetsky Y, Ribrag V. Burkitt and Burkitt-like lymphomas: a systematic review. *Curr Oncol Rep*. 2020; 22(4):1–9.
41. Carbone A, Cesarman E, Spina M, Gloghini A, Schulz TF. HIV-associated lymphomas and gamma-herpesviruses. *Blood*. 2009;113(6):1213–1224.
42. Gloghini A, Dolcetti R, Carbone A. Lymphomas occurring specifically in HIV-infected patients: from pathogenesis to pathology. *Semin Cancer Biol*. 2013:457–467.
43. Simard EP, Pfeiffer RM, Engels EA. Cumulative incidence of cancer among individuals with acquired immunodeficiency syndrome in the United States. *Cancer*. 2011;117(5):1089–1096.
44. Powles T, Robinson D, Stebbing J, et al. Highly active antiretroviral therapy and the incidence of non–AIDS-defining cancers in people with HIV infection. *J Clin Oncol*. 2009;27(6):884–890.
45. Clifford GM, Rickenbach M, Lise M, et al. Hodgkin lymphoma in the Swiss HIV cohort study. *Blood*. 2009;113(23):5737–5742.
46. Kowalkowski MA, Mims MA, Day RS, et al. Longer duration of combination antiretroviral therapy reduces the risk of Hodgkin lymphoma: a cohort study of HIV-infected male veterans. *Cancer Epidemiol*. 2014; 38(4):386–392.
47. Glaser SL, Clarke CA, Gulley ML, et al. Population-based patterns of human immunodeficiency virus-related Hodgkin lymphoma in the Greater San Francisco Bay Area, 1988–1998. *Cancer*, 2003;98(2):300–309.
48. Thida AM, Tun AM, Lymphocyte depleted Hodgkin lymphoma. In: *StatPearls [Internet]*. Treasure Island (FL): StatPearls Publishing; 2020.
49. Carbone A, Spina M, Gloghini A, Tirelli U. Classical Hodgkin's lymphoma arising in different host's conditions: pathobiology parameters, therapeutic options, and outcome. *Am J Hematol*. 2011;86(2):170–179.
50. Witkowska M, Majchrzak A, Smolewski P. The role of radiotherapy in Hodgkin's lymphoma: what has been achieved during the last 50 years? *BioMed Res Int*. 2015:2015.
51. Re A, Cattaneo C, Rossi G. HIV and lymphoma: from epidemiology to clinical management. *Med Hematol Infect Dis*. 2019;11:1.
52. Biggar RJ, Engels EA, Frisch M, Goedert JJ, AIDS Cancer Match Registry Study Group. Risk of T-cell lymphomas in persons with AIDS. *JAIDS*. 2001;26(4):371–376.
53. Phan A, Veldman R, Lechowicz MJ. T-cell lymphoma epidemiology: the known and unknown. *Curr Hematol Malign Rep*. 2016;11(6): 492–503.
54. Foss F, ed. *T-Cell Lymphomas*. New York: Springer Science & Business Media; 2012.

Downl
15, 202

55. Arzoo KK, Bu X, Espina BM, et al. T-cell lymphoma in HIV-infected patients. *JAIDS*. 2004;36(5):1020–1027.
56. Bogoch II, Andrews JR, Nagami EH, et al. Clinical predictors for the aetiology of peripheral lymphadenopathy in HIV-infected adults. *HIV Med*. 2013;14(3):182–186.
57. Buyego P, Nakiyingi L, Ddungu H, et al. Possible misdiagnosis of HIV associated lymphoma as tuberculosis among patients attending Uganda Cancer Institute. *AIDS Res Ther*. 2017;14(1):1–6.
58. Getahun H, Gunneberg C, Granich R, Nunn P. HIV infection–associated tuberculosis: the epidemiology and the response. *Clin Infect Dis*. 2010; 50(Supplement_3):S201–S207.
59. Bell LC, Noursadeghi M. Pathogenesis of HIV-1 and *Mycobacterium tuberculosis* co-infection. *Nature Rev Microbiol*. 2018;16(2):80–90.
60. Picard C, Fieschi C, Altare F, et al. Inherited interleukin-12 deficiency: IL12B genotype and clinical phenotype of 13 patients from six kindreds. *Am J Hum Genet*. 2002;70(2):336–348.
61. Goldblum JR, Lamps LW, McKenney JK, Myers JL, Ackerman LV. Lymph nodes. In: *Rosai and Ackerman's Surgical Pathology*. Philadelphia, PA: Elsevier; 2018:1503–1631.
62. Barrionuevo-Cornejo C, Dueñas-Hancco D. Lymphadenopathies in human immunodeficiency virus infection. *Semin Diagn Pathol*. 2018;35(1): 84–91.
63. Bibas M, Biava G, Antinori A. HIV-associated venous thromboembolism. *Medit J Hematol Infect Dis*. 2011;3(1).
64. Triant VA, Lee H, Hadigan C, Grinspoon SK. Increased acute myocardial infarction rates and cardiovascular risk factors among patients with human immunodeficiency virus disease. *J Clin Endocrinol Metab*. 2007;92: 2506–2512.
65. Strategies for Management of Antiretroviral Therapy (SMART) Study Group CD4+ count–guided interruption of antiretroviral treatment. *N Engl J Med*. 2006;355(22):2283–2296.
66. Kuller LH, Tracy R, Belloso W, et al. Inflammatory and coagulation biomarkers and mortality in patients with HIV infection. *PLoS Med*. 2008; 5(10):e203.
67. Armah KA, McGinnis K, Baker J, et al. HIV status, burden of comorbid disease, and biomarkers of inflammation, altered coagulation, and monocyte activation. *Clin Infect Dis*. 2012;55(1):126–136.
68. Baker JV, Neuhaus J, Duprez D, et al. Changes in inflammatory and coagulation biomarkers: a randomized comparison of immediate versus deferred antiretroviral therapy in patients with HIV infection. *J Acquir Immun Defic Syndr*. 2011;56(1):36.
69. Klatt NR, Funderburg NT, Brenchley JM. Microbial translocation, immune activation, and HIV disease. *Trends Microbiol*. 2013;21(1):6–13.
70. Brenchley JM, Price DA, Schacker TW, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nature Med*. 2006;12(12):1365–1371.
71. Funderburg NT, Mayne E, Sieg SF, et al. Increased tissue factor expression on circulating monocytes in chronic HIV infection: relationship to in vivo coagulation and immune activation. *Blood*. 2010;115(2):161–167.
72. Saif MW, Greenberg B. HIV and thrombosis: a review. *AIDS Patient Care STDS*. 2001;15(1):15–24.
73. Klein SK, Slim EJ, De Kruif MD, et al. Is chronic HIV infection associated with venous thrombotic disease? A systematic review. *Neth J Med*. 2005; 63(4):129–136.
74. Majluf-Cruz A, Silva-Estrada M, Sánchez-Barboza R, et al. Venous thrombosis among patients with AIDS. *Clin Appl Thromb Hemost*. 2004; 10(1):19–25.
75. Myerson M, Glesby MJ, eds. *Cardiovascular Care in Patients With HIV*. Cham: Springer; 2019.
76. Saif MW, Bona R, Greenberg B. AIDS and thrombosis: retrospective study of 131 HIV-infected patients. *AIDS Patient Care STDS*. 2001;15(6): 311–320.
77. Toulon P, Lamine M, Ledjev I, et al. Heparin cofactor II deficiency in patients infected with the human immunodeficiency virus. *Thromb Haemost*. 1993;69(05):730–735.
78. Villa P, Aznar J, Vaya A, et al. Hereditary homozygous heparin cofactor II deficiency and the risk of developing venous thrombosis. *Thromb Haemost*. 1999;82:1011–1014.
79. Funderburg NT, Lederman MM. Coagulation and morbidity in treated HIV infection. *Thrombosis Res*. 2014;133:S21–S24.
80. Aukrust P, Bjørnsen S, Lunden B, et al. Persistently elevated levels of von Willebrand factor antigen in HIV infection. *Thromb Haemost*. 2000;84(08):183–187.
81. Baker JV. Chronic HIV disease and activation of the coagulation system. *Thromb Res*. 2013;132(5):495–499.
82. Mayne E, Funderburg NT, Sieg SF, et al. Increased platelet and microparticle activation in HIV infection: upregulation of Pselectin and tissue factor expression. *J Acquir Immun Defic Syndr*. 2012;59(4):340.
83. Worm SW, Sabin C, Weber R, et al. Risk of myocardial infarction in patients with HIV infection exposed to specific individual antiretroviral drugs from the 3 major drug classes: the data collection on adverse events of anti-HIV drugs (D: A: D) study. *J Infect Dis*. 2010;201(3):318–330.
84. Satchell CS, O'Halloran JA, Cotter AG, et al. Increased platelet reactivity in HIV-1–infected patients receiving abacavir-containing antiretroviral therapy. *J Infect Dis*. 2011;204(8):1202–1210.
85. Musselwhite LW, Sheikh V, Norton TD, et al. Markers of endothelial dysfunction, coagulation and tissue fibrosis independently predict venous thromboembolism in HIV. *AIDS*. 2011;25(6):787.
86. Coyle TE. Hematologic complications of human immunodeficiency virus infection and the acquired immunodeficiency syndrome. *Med Clin*. 1997; 81(2):449–470.
87. Perkocha LA, Rodgers GM. Hematologic aspects of human immunodeficiency virus infection: laboratory and clinical considerations. *Am J Hematol*. 1988;29(2):94–105.
88. Palomo I, Alarcon M, Sepulveda C, et al. Prevalence of antiphospholipid and antiplatelet antibodies in human immunodeficiency virus (HIV) infected Chilean patients. *J Clin Lab Anal*. 2003;17(6):209–215.
89. Heit JA, Kobbervig CE, James AH, et al. Trends in the incidence of venous thromboembolism during pregnancy or postpartum: a 30-year population-based study. *Ann Intern Med*. 2005;143(10):697–706.
90. Bremme KA. Haemostatic changes in pregnancy. *Best Pract Res Clin Haematol*. 2003;16(2):153–168.
91. Kearon C, Akl EA, Ornelas J, et al. Antithrombotic therapy for VTE disease: CHEST guideline and expert panel report. *Chest*. 2016;149(2): 315–352.
92. Lee AY, Levine MN, Baker RI, et al. Low-molecular-weight heparin versus a coumarin for the prevention of recurrent venous thromboembolism in patients with cancer. *N Engl J Med*. 2003;349(2):146–153.
93. Finch A, Pillans P. P-glycoprotein and its role in drug-drug interactions. *Aust Prescr*. 2014;37(4):137–139.
94. Mueck W, Stampfuss J, Kubitza D, Becka M. Clinical pharmacokinetic and pharmacodynamic profile of rivaroxaban. *Clin Pharmacokinet*. 2014;53(1):1–16.
95. Egan G, Hughes CA, Ackman ML. Drug interactions between antiplatelet or novel oral anticoagulant medications and antiretroviral medications. *Ann Pharmacother*. 2014;48(6):734–740.
96. Choonara IA, Haynes BP, Cholerton S, Breckenridge AM, Park BK. Enantiomers of warfarin and vitamin K1 metabolism. *Br J Clin Pharmacol*. 1986;22(6):729–732.
97. Good BL, Gomes DC, Fulco PP. An unexpected interaction between warfarin and cobicistat-boosted elvitegravir. *AIDS*. 2015;29(8):985–986.
98. Nutescu EA, Burnett A, Fanikos J, Spinler S, Wittkowsky A. Pharmacology of anticoagulants used in the treatment of venous thromboembolism [published correction appears in *J Thromb Thrombolysis*. 2016;42(2): 296-311]. *J Thromb Thrombolysis*. 2016;41(1):15–31.
99. Thompson GR, Lawrence VA, Crawford GE. HIV infection increases the risk of heparin-induced thrombocytopenia. *Clin Infect Dis*. 2007;45(10):1393–1396.
100. Educo. (2020, May 04). Drug Guide. Retrieved August 14, 2020, from https://www.positivelyaware.com/drug-guides/.
101. Panel on Antiretroviral Guidelines for Adults and Adolescents. *Guidelines for the Use of Antiretroviral Agents in Adults and Adolescents With HIV*. Department of Health and Human Services. https://clinicalinfo.hiv.gov/sites/default/files/inline-files/AdultandAdolescentGL.pdf.

Downl
15, 202

CHAPTER 153

HEMATOLOGIC FINDINGS AND CONSEQUENCES OF NOVEL CORONAVIRUS (SARS-COV-2) INFECTION

Leonard Naymagon and Douglas Tremblay

At the end of 2019, a novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) lead to a cluster of infections in Wuhan. China. The disease caused by this virus was designated as coronavirus-disease-2019 (COVID-19) by the World Health Organization (WHO). COVID-19 spread globally and went on to infect millions of people on all continents except Antarctica, causing an unprecedented global impact. As of February 2021, over one hundred million COVID-19 cases have been reported globally, with well over two million deaths attributed to the virus. Updated figures can be found on the WHO website: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports. The true extent of the pandemic is likely not yet fully appreciated, as only a fraction of cases are evaluated by medical professionals, diagnosed, and reported. Using the prevalence of seropositivity in a population with reported positive cases, it is estimated that the number of infections may be 6 to 24 times higher than reported. The reported death toll is also likely a significant underestimate. A US-based study that assessed excess deaths in 2019–2020 indicated that as many a quarter of all COVID-19 related deaths may not be officially counted.

Clinical manifestations of COVID-19 are primarily respiratory, including dyspnea and hypoxemia, and in many cases can progress to acute respiratory distress syndrome (ARDS). However, manifestations may be varied and multisystemic, and presentations are heterogeneous and range from asymptomatic to fatal (Fig. 153.1). Although healthy patients at any age are susceptible to severe disease, advanced age and comorbidities are clear risk factors for poor outcomes in COVID-19. In a study of 17 million patients in the United Kingdom, the following risk factors were identified to be significantly associated with COVID-19 mortality: age >60; male sex; BMI $>30\,\mathrm{mg/kg}^2$; tobacco use; non-White ethnicity; asthma; diabetes; non-hematological cancer diagnosed within the last 5 years; hematologic cancer; reduced kidney function; liver disease; stroke or dementia; prior organ transplant; rheumatoid arthritis, lupus, or psoriasis; and other immunosuppressed states.[1] Patients with hematologic malignancies, in particular, may have perturbations in myeloid and lymphoid maturation that leave them especially susceptible to the exuberant cytokine storm associated with severe disease. Evidence suggests these patients have very poor outcomes if infected with COVID-19.

Although respiratory failure is likely the primary driver of morbidity and mortality in the majority of COVID-19 cases, mounting experience indicates that severe COVID-19 can be a multisystem syndrome capable of involving multiple organ systems. The hematologic complications of COVID-19 are particularly prominent and have drawn significant interest. Early in the pandemic, clinicians reported thromboembolic complications including pulmonary embolism, deep vein thrombosis, thrombosis of intravascular access catheters, and stroke. In addition, laboratory parameters including some coagulation assays (particularly D-dimer), blood counts (including the lymphocyte count among others), and inflammatory markers (such as ferritin) have been noted to be characteristically altered in COVID-19 (Table 153.1). This chapter reviews the hematologic manifestations of COVID-19 and discusses their significance.

THROMBOSIS IN COVID-19

Evidence for Thromboembolic Disease as a Pathophysiologic Factor in COVID-19

There has been evidence to suggest that, at least among some patients, a pathophysiologic component of severe COVID-19 disease may be related to a provoked thrombophilic state (Table 153.2). Critically ill patients with COVID-19 have demonstrated high rates of overt venous thromboembolism (VTE) in sizable cohorts. In a prospective cohort study of 150 patients with COVID-19 and ARDS, 64 thromboembolic complications were identified, most often pulmonary embolism (PE).[2] Notably, the rate of PE was significantly increased as compared with a propensity-matched non-COVID historical prospective ARDS cohort. A series of 184 consecutive ICU patients with COVID-19 on appropriate thromboprophylaxis yielded a cumulative VTE incidence of 27% (higher than typically seen in comparable non-COVID ICU populations).[3] In a retrospective study of 3334 hospitalized COVID-19 patients, the diagnosis of VTE was associated with an increased risk of mortality (adjusted hazard ratio [HR], 1.37; 95% CI 1.02 to 1.86).[4] Additionally, high rates of central venous catheter clotting have been reported among COVID-19 patients, particularly with regard to hemodialysis access.[2]

Beyond overt venous thromboembolic disease, there has been interest in the role of occult VTE, including microthrombosis, contributing to adverse outcomes. Autopsy studies of COVID-19 patients have revealed extensive micro and macro pulmonary emboli that had been undiagnosed prior to death. For instance, a post-mortem examination of 21 COVID-19 patients yielded 4 instances of overt PE, 5 of diffuse alveolar capillary microthrombosis, and 3 cases demonstrating stigmata of thrombotic microangiopathy in glomerular capillaries.[5] Another small autopsy study reported severe endothelial injury, microangiopathy, and microthrombosis in the lungs of 7 patients who died of COVID-19 (findings that were not present in controls who had died from influenza or other non-COVID causes).[6] Some mechanically ventilated COVID-19 patients have manifested pulmonary physiology more consistent with pulmonary vascular disease rather than pure ARDS, suggesting that a diffuse thrombotic process may contribute to respiratory decompensation among a subset of patients.[7] Interest has also arisen regarding D-dimer levels, which are frequently elevated among COVID-19 patients, and has been associated with poor outcomes and been posited as possible laboratory indicators of thrombotic microangiopathy or other forms of microthrombosis. Observational studies have also noted increased levels of factor VIII and von Willebrand factor among patients with severe COVID-19.

In addition to high rates of VTE, high rates of arterial thrombosis have been reported among COVID-19 cohorts. In a highly publicized single-institution series, 5 cases of acute ischemic stroke were identified among COVID-19 patients within a 2-week period (with all patients younger than 50 years of age, and with generally minimal vascular risk factors).[8] Similarly, 20 COVID-19 patients with acute limb ischemia were reported by a single institution over a

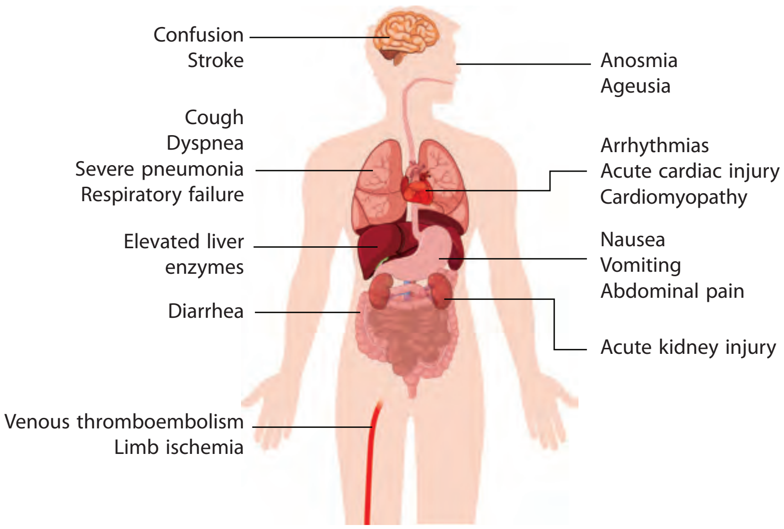


Figure 153.1 THE CLINICAL MANIFESTATIONS OF COVID-19 MAY AFFECT A WIDE RANGE OR ORGAN SYSTEMS.

3-month period.[9] A large retrospective study of over 3000 hospitalized COVID-19 patients reported myocardial infarction among 8.9%.[4]

Limitations to Current Evidence Regarding Thromboembolic Disease in COVID-19

The perception that acquired hypercoagulability and thromboembolic disease (both micro and macro) may play a role in the pathophysiology of COVID-19 has to date been based largely on anecdotal observations that are hampered by numerous limitations (Table 153.3). It is certainly well established that critical illness and prolonged hospitalization are major risk factors for VTE, and in this sense, the finding that VTE is common among severely ill COVID-19 patients is neither novel nor surprising. Although some of the aforementioned studies report VTE rates among critically ill COVID-19 patients that may be higher than historical non-COVID controls, several other COVID-19 studies report more moderate rates that are more in line with historical standards.[4] The high frequency of VTE encountered in some cohort studies may have been overestimated in the setting of inconsistently reported thromboprophylaxis practices and the extensive and nonsystematic use of diagnostic imaging studies. Systematic comparisons of VTE rates among COVID-19 and non-COVID-19 patients are not yet available, and it is presently difficult to assert that COVID-19 is significantly "more thrombophilic" than other comparatively severe respiratory illnesses.

Autopsy studies demonstrating occult pulmonary thromboembolic disease and thrombotic microangiopathy among COVID-19 patients remain few, with the number of confirmed reports paling in comparison to the number of known COVID-19 related fatalities. Further, there may be selection bias in these autopsy reports, with only the most idiosyncratic and otherwise inexplicable deaths preferentially selected for autopsy. Elevated D-dimer, though increasingly interpreted as a measure of occult thrombotic burden in COVID-19 at some centers, is a highly non-specific marker of VTE and may be a manifestation of inflammation rather than thrombosis. In a study of 449 COVID-19 patients, there was no difference in admission D-dimer levels as compared with 104 patients with a non-COVID pneumonia.[10] In a study of 1065 hospitalized COVID-19 patients, although D-dimer levels and trends were associated with poor outcomes, they proved poor predictors both when taken in isolation and when incorporated into multivariable models.[11] Similarly, elevations of factor VIII and von Willebrand factor are likely reflective of the highly proinflammatory nature of COVID-19 and not indicative of any unique prothrombotic process.

Series describing arterial thrombotic complications such as stroke and limb ischemia have been small and few, and it is difficult to form meaningful conclusions from the limited cases available for study. Reported rates of myocardial infarction (MI) have likely included large quantities of cases of type 2 MI (or "demand ischemia"), with cases of true acute thrombotic coronary obstruction likely rare.[4] Like many other purported manifestations of "COVID-19 hypercoagulability," such demand ischemia is a common feature of critical illness across etiologies. Reports of pulmonary physiology reminiscent of venous thromboembolic disease remain purely anecdotal with limited published data to date.

The Role of Anticoagulation in COVID-19

Some limited data suggest that anticoagulation may improve outcomes in COVID-19. In an often-cited study reported early in the course of the pandemic, the use of prophylactic heparin was associated with improvement in mortality in a very specific subgroup (those with a sepsis-induced coagulopathy score ≥4) of 449 patients with severe COVID-19. However, it was unclear why this specific subgroup was chosen, why the rate of prophylactic anticoagulation was so low (only 99 patients received it), and why prophylactic anticoagulation was offered in some cases and not others. In addition, the baseline characteristics of the two groups were not well described and not compared, and the analysis favoring anticoagulation was univariable.[12] In a large single-hospital system retrospective study, therapeutic dose anticoagulation was associated with improved in-hospital survival among intubated patients; however, some potentially significant clinical factors were not controlled for, and the benefit conferred by anticoagulation was not observed in non-intubated patients.[13] A follow-up study from the same group using a largely overlapping retrospective cohort found that prophylactic dose anticoagulation was associated with lower rates of intubation and in-hospital mortality than the group not receiving anticoagulation. However, given that prophylactic anticoagulation is already the established standard of care for the vast majority of hospitalized patients (unless contraindicated), the clinical utility of such a finding is not readily apparent. It should be observed that in such studies that those patients who do not receive prophylactic anticoagulation likely carry contraindications for prophylaxis (such as active bleeding, coagulopathy, or severe thrombocytopenia) which may themselves be associated with poor outcomes. Such deeply ingrained practices may be difficult to account for. Notably, in this study, therapeutic dose anticoagulation did not yield significantly improved outcomes relative to prophylactic anticoagulation.

TABLE 153.1 Common Hematologic Manifestations of COVID-19

	Prevalence (%)	Prognostic implications	Suspected Pathophysiology
Venous thrombosis	5–30 (in severe disease)	May predict mortality	Heightened inflammation, critical illness, prolonged hospitalization
Arterial thrombosis	Likely rare, not well described	May predict mortality	Poorly understood, perhaps vascular inflammation
Elevated D-dimer	50–80 (among hospitalized patients)	May be associated with though not strongly predictive of mortality	Inflammation-driven acute phase reactant Possibly increased fibrinogenesis/fibrinolysis in some instances
Hyperferritinemia	55–80	May predict severe disease	Inflammation-driven acute phase reactant
Lymphopenia	35–85	May predict mortality	Cytokine-driven apoptosis, direct infection of lymphocytes, or destruction of lymphatic organs
Thrombocytopenia	30–40	May predict morality	Infection of megakaryocytes, immune mediate destruction
Anemia	20–35	May predict severe disease and mortality	Decreased production

TABLE 153.2 Frequently Cited Evidence Supporting the Central Role of an Acquired Thrombophilic State in the Pathophysiology of COVID-19

High observed rates of venous thromboembolism (VTE) in many severe coronavirus-disease-2019 (COVID-19) cohorts.
Association between VTE incidence and mortality in some cohorts.
High observed rates of central venous catheter clotting.
Autopsy studies demonstrating evidence of occult pulmonary thrombosis, microthrombosis, and thrombotic angiopathy.
Pulmonary physiology reminiscent of pulmonary vascular disease among some mechanically ventilated patients.
Frequently elevated D-dimers and their association with outcomes.
Reports of otherwise unexplained arterial thrombotic events.

TABLE 153.3 Limitations to Available Evidence Supporting the Notion That an Acquired Thrombophilic State May Play a Major Role in the Pathophysiology of COVID-19

Rates of VTE may be similar to those in comparably severe illnesses of other etiologies.
Lack of systematic comparisons of VTE rates among COVID-19 and non-COVID-19 patients.
Observations of high rates of central venous catheter clotting remain anecdotal and not yet rigorously studied.
Autopsy studies implicating the role of occult thrombotic disease are very few in number and subject to selection bias.
Reports of pulmonary physiology similar to that of pulmonary vascular disease remain few, unsystematic, and speculative.
Elevated D-dimers, though often present, are non-specific and likely represent acute phase response to heightened inflammation.
Arterial thrombotic events have been reported in a small number of series, most ischemic cardiac events represent “demand ischemia.”

There is currently no truly rigorous data that offer convincing evidence that anticoagulation beyond what is typically recommended for generic (non-COVID) hospitalized patients is of benefit in COVID-19. For the time being, it remains unclear whether anticoagulation may truly have a protective effect on COVID-19 infections and if so whether any particular group of COVID-19 patients may benefit most. A propensity-matched analysis of 4343 COVID-19 patients demonstrated no outcome-benefit among those patients who were on anticoagulation for other indications prior to COVID-19 diagnosis.[14] Such finding suggest that if there is benefit to be derived from anticoagulation, it may be more likely later in the disease course and/or among sicker patients (such as those requiring hospitalization). However, some studies investigating intervention with anticoagulation among critically ill COVID-19 patients have demonstrated similarly negative results. For instance, in a multicenter target trial emulation among 2809 critically ill COVID-19 patients, those who received early therapeutic anticoagulation had a similar risk for death as those who did not.[15] A smaller observational study actually demonstrated worse survival outcomes among inpatients empirically treated with therapeutic dose anticoagulation compared to those treated with prophylactic dose anticoagulation.[16] More recently, three large-scale prospective international trials investigating preemptive use of therapeutic heparin among critically ill COVID patients were halted after their data safety monitoring boards determined that such interventions appeared futile and possibly unsafe in interim analyses (although the specific data prompting these decisions are not available at the time of this writing, concerns regarding safety were presumably based on excess bleeding risk detected in the treatment groups).

In spite of the lack of convincing evidence, many clinicians and centers have pursued and advocated for therapeutic (or sometimes intermediate dose) anticoagulation for critically ill patients with COVID-19 in the absence of documented thromboembolism. While it is certainly true that some critically ill patients with high clinical suspicion for VTE may be too unstable or otherwise unable to undergo confirmatory testing, and empiric therapeutic anticoagulation may be reasonable among such patients, routine use of therapeutic anticoagulation among COVID-19 patients with clearly negative VTE testing is not presently evidence based and should be pursued with caution outside the context of a clinical trial. It should also be noted that decision tools employed by some centers to identify those COVID-19 patients who may benefit from therapeutic anticoagulation frequently employ unfounded and seemingly arbitrary criteria such as specific D-dimer cutoffs or thresholds of respiratory support requirement, which have not been validated. Current guidelines for anticoagulation among COVID-19 patients from the International Society of Thrombosis and Hemostasis (ISTH) as well as the American Society of Hematology (ASH) are very similar to the established standards used among all hospitalized patients (Table 153.4). Specifically, they state that hospitalized COVID-19 patients should generally receive prophylactic dose anticoagulation unless contraindicated and that therapeutic dose anticoagulation

TABLE 153.4 Clinical Guidance on the Diagnosis, Prevention,and Treatment of Venous Thromboembolism in Hospitalized Patients With COVID-19 From the International Society of Thrombosis and Hemostasis

Diagnosis of VTE in hospitalized COVID-19 patients	Practitioners should use standard-of-care objective testing (i.e., computed tomography pulmonary angiogram [CTPA], ventilation/perfusion [V/Q] scan, magnetic resonance imaging [MRI] venography, Doppler ultrasonography) to diagnose venous thromboembolism (VTE) based on clinical index of suspicion. A pragmatic approach (i.e., point-of-care bedside ultrasonography or echocardiography) can also be combined with standard-of-care objective testing (50% of respondents).
	Routine screening for VTE using bedside Doppler ultrasonography of the lower extremities or based on elevated D-dimer levels is not recommended.
VTE prophylaxis in non-ICU hospitalized COVID-19 patients	A universal strategy of routine thromboprophylaxis with standard-dose UFH or LMWH should be used after careful assessment of bleed risk, with LMWH as the preferred agent. Intermediate-dose LMWH may also be considered (30% of respondents).
	VTE prophylaxis recommendations should be modified based on extremes of body weight, severe thrombocytopenia or deteriorating renal function.
VTE prophylaxis in sick ICU hospitalized COVID-19 patients	Routine thromboprophylaxis with prophylactic-dose UFH (unfractionated heparin) or LMWH (low molecular weight heparin) should be used after careful assessment of bleed risk. Intermediate-dose LMWH (50% of respondents) can also be considered in high risk patients. Patients with obesity as defined by actual body weight or BMI should be considered for a 50% increase in the dose of thromboprophylaxis. Treatment-dose heparin should not be considered for primary prevention until the results of randomized controlled trials are available.
	Multi-modal thromboprophylaxis with mechanical methods (i.e., intermittent pneumonic compression devices) should be considered (60% of respondents).
Duration of VTE prophylaxis for hospitalized COVID-19 patients	Either LMWH (30%) or a DOAC (direct oral anticoagulant) (i.e., rivaroxaban or betrixaban; 30% of respondents) can be used for extended-duration thromboprophylaxis.
	Extended post-discharge thromboprophylaxis should be considered for all hospitalized patients with COVID-19 that meet high VTE risk criteria. The duration of post-discharge thromboprophylaxis can be approximately 14 days at least (50% of respondents), and up to 30 days (20% of respondents).
VTE treatment in hospitalized COVID-19 patients	Established guidelines should be used to treat patients with confirmed VTE, with advantages of LMWH in the inpatient setting and DOACs in the post-hospital discharge setting. A change from treatment-dose DOAC or vitamin K antagonists (VKA) to in-hospital LMWH should be considered especially for patients in critical care settings or with relevant concomitant medications, and dependent on renal function and platelet counts. Anticoagulant regimens should not change based solely on D-dimer levels.
	A change of anticoagulant regimen (i.e., from prophylactic or intermediate-dose to treatment- dose regimen) can be considered in patients without established VTE but deteriorating pulmonary status or ARDS (50% of respondents).
	The duration of treatment should be at least 3 months (50% of respondents).

Adapted from Spyropoulos AC, Levy JH, Ageno W, et al. Scientific and standardization committee communication: clinical guidance on the diagnosis, prevention, and treatment of venous thromboembolism in hospitalized patients with COVID-19. *J Thromb Haemost*. 2020;18(8):1859–1865.

should be used among those with demonstrated thromboembolism unless contraindicated.[17] Notable differences from standard anticoagulation protocols acknowledged as reasonable in the ISTH guidelines include consideration of intermediate-dose low-molecular-weight (LMW) heparin prophylaxis among some "high-risk" hospitalized patients and consideration of extended VTE prophylaxis following hospital discharge (this likely based on reports of post-discharge VTE among some COVID-19 patients) (see Table 153.4). It should be noted however that these specialized prophylaxis considerations are not based on strong evidence and highlight current uncertainty in clinical practice.

COVID-19 ASSOCIATED COAGULOPATHY

The frequent observation of elevated D-dimer levels among COVID-19 patients early in the course of the pandemic raised concern that disseminated intravascular coagulation (DIC) may be a recurrent feature of the syndrome. However, this is not supported by other laboratory parameters common among COVID-19 patients. Indeed, COVID-19 patients often manifest elevated fibrinogen levels and rarely demonstrate significant prolongation of aPPT and/or prothrombin time (PT) and infrequently have severe thrombocytopenia (though relatively mild thrombocytopenia may be common) (Table 153.5). Further, the observation that D-dimer levels tend to correlate with inflammatory markers such as erythrocyte sedimentation rate (ESR) or ferritin suggests that they are likely more a manifestation of an inflammatory response rather than an indicator of coagulopathy among COVID-19 patients. Indeed, few COVID-19 patients (and likely only the most ill among them) are likely to meet ISTH criteria for DIC. The utility of trending laboratory markers such as D-dimer among COVID-19 patients is of uncertain benefit. Uptrending or downtrending D-dimers may be indicators

TABLE 153.5 Comparison of Coagulation Parameters and Markers of Fibrinolysis in Disseminated Intravascular Coagulation and COVID-19

	DIC	COVID-19
D-dimer	Increased	Increased
Fibrinogen	Decreased	Increased
Platelets	Decreased	Normal or slightly decreased
PT	Prolonged	Normal
PTT	Prolonged	Normal

DIC, Disseminated intravascular coagulation; *PT*, prothrombin time; *PTT*, partial thromboplastin time.

of worsening or improving clinical status, respectively (most likely because they are prone to track with inflammation); however, it is not clear that these trends provide any more information than what might be inferred based on clinical observation, or more limited laboratory monitoring.[11] Based on currently available evidence, monitoring of such parameters among COVID-19 patients need not necessarily exceed typical laboratory assessments among comparably ill patients due to other etiologies.

INFLAMMATORY MARKERS AND CYTOKINE STORM

A key pathophysiologic mediator of severe COVID-19 disease is an acute inflammatory syndrome. This clinical impression was widespread early in the pandemic by marked elevations in C-reactive protein (CRP), erythrocyte sedimentation rate, interleukin (IL)-6, and ferritin in severe COVID-19 cases. Elevations in these inflammatory markers were noted to be prognostically important based on several retrospective cohort studies. In particular, ferritin has been noted to be severely elevated in patients with COVID-19. Although ferritin is a known acute phase reactant elevated in many inflammatory conditions including infections, extreme elevations are also a clinical hallmark of hyperferritinemic syndromes such as macrophage activation syndrome (MAS), adult-onset Still's disease, and catastrophic antiphospholipid syndrome. In addition, extremely high ferritin is also reminiscent of secondary hemophagocytic lymphohistiocytosis (sHLH). However, patients with COVID-19 rarely develop organomegaly and other diagnostic evaluations including natural killer (NK) cell activity, soluble IL-2 receptor, and a bone marrow, liver, spleen, or lymph node biopsy are rarely obtained. In one small single-center study, only three patients out of 40 (7.5%) likely had sHLH based on a predictive scoring model (Hscore).

The prognostic value of ferritin levels in COVID-19 remains uncertain. In an early study from Wuhan, China, ferritin elevation was able to discern between mild and critical COVID-19, although the definitions of severity were poorly defined. In a large New York City based study, patients who died had a significantly higher ferritin level than those who did not. However, mortality was poorly predicted by both admission and maximum ferritin levels, with an area under the curve (AUC) for a receiver operator characteristic (ROC) curve of 0.677 and 0.638 respectively.[18] Interestingly, the AUC increased among younger age brackets. Further data are needed to clarify the prognostic role of ferritin and its integration into risk models to inform the use of COVID-19 therapies.

Parallels can also be drawn between severe COVID-19 infections and cytokine release syndrome (CRS) associated with chimeric antigen receptor (CAR) T-cell therapy. As a result, tocilizumab has been successfully employed for severe COVID-19 and continues to be evaluated. In addition, the JAK-STAT pathway has been identified as a potential target as many inflammatory cytokines are mediated by this intracellular signaling pathway. Dexamethasone has been among the first therapies to demonstrate a survival improvement in COVID-19, a benefit postulated to be due to mitigation of the inflammatory response.[19] Multiple therapeutic trials are evaluating agents that modulate the immune response to SARS-CoV-2 as a means to improve outcomes in COVID-19.

COVID-19 in children is generally mild; however, a small subset of patients develop a hyperinflammatory process similar to Kawasaki disease and toxic shock syndrome. This disease was termed multisystem inflammatory syndrome in children (MIS-C). Clinical manifestations are distinct from COVID-19 in adults and include fevers, rash, gastrointestinal complaints (vomiting, diarrhea, abdominal pain), conjunctival injection, and mucosal changes with a lack of prominent pulmonary symptoms. Hematologic abnormalities are common, with lymphopenia, anemia, and thrombocytopenia present in approximately 80%, 70%, and 50% of cases, respectively. Ferritin is elevated in approximately 75% of cases. The pathophysiology of MIS-C is not well understood but presumed to be related to an abnormal immune response to SARS-CoV-2 infection.

CHARACTERISTICS OF THE COMPLETE BLOOD COUNT AND WHITE BLOOD CELL DIFFERENTIAL IN COVID-19

Lymphopenia

Among the most common hematologic abnormalities in patients with COVID-19 is lymphopenia, present in 35% to 83% of patients based on initial reports from Wuhan, China. In a larger United States based study, a lymphocyte count less than 1.0×10^9/L was observed in 60% of patients.[20] The etiology behind this lymphopenia is not known, although several mechanisms have been postulated. Some studies have reported that lymphocytes express angiotensin-converting enzyme-2 (ACE-2), the receptor for SARS-CoV-2, thus suggesting direct infection with the virus. However, several studies have not confirmed this finding and have suggested a separate mechanism whereby SARS-CoV-2 infects and destroys lymphatic organs (though it should be noted that evidence of ACE2 expression in lymph nodes, thymus, or spleen is currently lacking). A more plausible explanation for lymphopenia may be that proinflammatory cytokines, such as TNFα and IL-6, lead to decreased production and increased apoptosis of lymphocytes. Functional studies have also demonstrated that SARS-CoV-2 may promote excessive activation of CD8+ T cells, possibly leading to exhaustion and impaired functioning of CD4+ T cells. It should be noted that many viral infections cause lymphopenia, such as respiratory syncytial virus, severe measles, and H1N1 influenza. Thus, the novelty of lymphopenia in COVID-19 compared with other viral infections may be somewhat overstated.

The degree and trend of lymphopenia appear to be at least somewhat prognostic, although its independent associations have not been fully evaluated. In a preliminary series from Wuhan, China, COVID-19 non-survivors developed more persistent and marked lymphopenia as compared with survivors. In another report from China, baseline lymphocyte count was significantly higher in survivors as compared with non-survivors (1.1×10^9/L vs. 0.6×10^9/L, $P < .0001$). However, it remains unclear if lymphopenia is independently associated with mortality. Further studies are needed to establish the mechanisms and risk associated with lymphopenia in COVID-19.

Thrombocytopenia

Thrombocytopenia is less prevalent than lymphopenia in acute COVID-19 infections. A large early series from China estimated the prevalence of thrombocytopenia to be less than 150×10^9/L at 36.2%, with a higher prevalence in severe disease (57.7%) versus non-severe disease (31.6%).[21] In a separate study, the platelet nadir was prognostically important, with an in-hospital mortality of 92.1% in patients with a platelet count of 0 to 50×10^9/L compared with 4.7% in patients with platelet count above 150×10^9/L. Several mechanisms of thrombocytopenia in COVID-19 have been proposed. SARS-CoV-2 has been postulated to directly infect CD13 positive cells in the bone marrow, as has been documented with other coronaviruses. The cytokine storm associated with COVID-19 infection may also decrease hematopoietic progenitor cell numbers. Immune-mediated thrombocytopenia with COVID-19 has also been reported, suggesting peripheral destruction of platelets. Finally, increased consumption due to systemic thrombosis and/or low level DIC may be occurring in a small subset of patients, although as above, the thrombotic state of COVID-19 is distinct from DIC.[22] It is likely that the mechanisms behind COVID-19 induced thrombocytopenia are multifactorial. Even with severe thrombocytopenia, bleeding does not appear to be a major feature of COVID-19.

Other Complete Blood Count Abnormalities

In contrast to thrombocytopenia and lymphopenia, anemia is not a prominent feature of COVID-19 infection. In preliminary studies from Wuhan, China, the median hemoglobin level was 13.4 g/dL.[21]

In addition, the percentage of patients requiring red blood cell (RBC) transfusions appears to be low, with one study estimating that only 1.6% of hospitalized COVID-19 patients required an RBC transfusion. There has been some reporting of COVID-19-associated hemolysis; however, this has been limited to a few small case series. There is some evidence from a single-center retrospective study that even mild anemia may be predictive for the need for mechanical ventilation and death; however, the clinical implications of this observation are unclear. An elevated red cell distribution width (RDW), even in nonanemic patients, has also been shown to be a negative predictor for mortality in COVID-19 patients, but as with many of the presented observations, rigorous data in this regard are limited.

VACCINATION

The United States Food and Drug Administration (FDA) granted an Emergency Use Authorization (EUA) to the first COVID-19 vaccine by Pfizer-BioNTech on December 11, 2020. One week later, a second vaccine produced by Moderna was granted an EUA. Both Moderna and Pfizer-BioNTech are messenger RNA (mRNA) vaccines, which deliver mRNA in a lipid nanoparticle that expresses the full length spike protein to elicit an immune response. Advantages of this technology include a completely in vitro manufacturing process that facilitates rapid production. Other vaccines utilizing a recombinant protein nanoparticle or adenovirus vector that express the spike protein are in development and will likely gain approval in the near future. The Pfizer-BioNTech and Moderna mRNA vaccines have demonstrated extraordinary effectiveness in preventing infection, with 95% and 94% efficacy respectively against symptomatic COVID-19. Even more impressive, the rates of severe COVID-19 were reduced to nearly zero in the large phase III clinical trials, which led to the approval of these vaccines.[23,24] Notable adverse events include myalgias at the site of injection, headaches, chills, and fatigue, particularly after the second dose. Anaphylaxis is extraordinarily rare, with a rate of 5 and 2.8 events per one million doses for the Pfizer-BioNTech and Moderna mRNA vaccines, respectively. Shortly before this writing (March 01, 2021) an adenoviral vector vaccine (Janssen COVID-19 vaccine, also referred to as the Johnson & Johnson vaccine) was granted EUA. This viral vector vaccine is based on an adenovirus modified to contain the gene for expression of the SARS-CoV-2 spike protein. Phase III trials have demonstrated that 28 days after a completed single vaccination, the vaccine had an efficacy of 66% in preventing symptomatic COVID-19, 85% efficacy in preventing severe COVID-19, and 100% efficacy in preventing COVID-19-related hospitalization or death.

Of note, clinical trials did not include immunocompromised subjects. In particular, patients with hematologic malignancies have been excluded from the clinical testing of COVID-19 vaccines. The immunogenicity of SARS-CoV-2 vaccines in patients with hematologic malignancies is therefore unknown. Many hematologic malignancies are characterized by perturbations in the bone marrow resident cells and microenvironment that may impact the generation and preservation of plasma cells necessary for durable immunity. The immune response to the COVID-19 vaccine will likely be a function of the nature and status of the underlying hematologic malignancy.[25] In addition, immune dysfunction as a result of cancer-directed therapy, such as B-cell depletion or hematopoietic stem cell transplantation, is also a concern. The European Hematology Association has released guidance that recommends COVID-19 vaccinations for all patients with hematologic malignancies. In patients who have received B-cell-depleting therapy or hematopoietic stem cell transplantation, an interval of 3 to 6 months prior to vaccination should be observed.[26] When vaccination is more widely available, efforts should be made to provide the vaccine at least 2 to 4 weeks prior to planned immunosuppressive therapy, transplant, or splenectomy.

In February of 2021, reports emerged of thrombocytopenia that developed after the administration of the COVID-19 vaccine.[27,28] The most extensive description of this phenomenon at the time of this writing comes from a case series of 20 patients collected from the Centers for Disease Control (CDC), US FDA, and Vaccine Adverse Events Reporting System (VAERS). Nine patients received the Pfizer-BioNTech and 11 received the Moderna vaccine. The median onset after vaccination was 5 days and the majority had a platelet count of less than 10×10^9/L. Of note, out of the 20 patients identified in this report, one had a known history of ITP in remission, another had baseline thrombocytopenia with positive anti-platelet antibodies, and a third had inherited thrombocytopenia with a baseline platelet count of around 40 to 60×10^9/L. Three other patients had autoimmune conditions. Fifteen of the 20 patients received ITP (immune thrombocytopenic purpura)-directed treatment including steroids in 14, intravenous immune globulin (IVIG) in 12, rituximab in 2, romiplastim in 1, vincristine in 1, and aminocaproic acid in 1. Eight patients received platelet transfusions. Improvement in platelet counts were described in 14 patients after treatment.[29]

The favorable response to ITP-directed treatment suggests that antibody-mediate platelet clearance is the mechanism in the majority of these cases. However, at the time of this report, over 20 million people had been vaccinated, making the case rate of ITP related to the vaccine as less than 1 case per million vaccinated persons. The authors extrapolated the number of cases reported during this monitoring period and found that it correlated with 80,000 cases per year. There are 50,000 cases of secondary ITP diagnosed annually; thus, the rate is just slightly above expected from the normal population, with an increase incidence perhaps explained by amplified surveillance post-vaccination for COVID-19. Other vaccines are known to lead to secondary ITP, such as the measles-mumps-rubella vaccine.

While it possible that the mRNA COVID-19 vaccine may have triggered a small number of cases of secondary ITP, this complication appears to be extraordinarily rare. Given the morbidity and mortality associated with COVID-19 infection, the benefits far outweigh the risks of vaccination. Clinicians should be mindful of these reports and check platelet counts in patients who are post-vaccination and complaining of excessive bleeding/bruising. In those who develop extreme thrombocytopenia, treatment for ITP should be rapidly implemented (see Chapter 129).

At the time of this writing, reports are emerging describing cases of thrombosis with thrombocytopenia following administration of the Johnson & Johnson COVID-19 vaccine and the AstraZeneca COVID-19 vaccine (another adenovirus vector vaccine presently available in Europe, though not in the United States). Some among these cases have demonstrated the presence of autoantibodies to the platelet factor 4 (PF4) antigen (similar to what is found in heparin-induced thrombocytopenia and thrombosis [HITT]). Although notable, such complications following vaccination remain profoundly rare, particularly following the Johnson & Johnson vaccine wherein (at the time of this writing) less than one case per one million vaccinated patients has been reported. The six reported cases following the Johnson & Johnson vaccine have presented as cerebral venous sinus thromboses (CVST), among females aged 18 to 48 years, with onset 6 to 13 days after vaccination. Such events have been noted somewhat more commonly following the AstraZeneca vaccine; however, because of the rarity of these events and the potential severity of COVID-19, the European Medicines Agency (EMA) continues to advise that the overall benefits of the vaccine outweigh the risk. A causal relationship between these adenovirus vector vaccines and these thrombotic/thrombocytopenic events has not yet been confirmed. Importantly, there does not appear to be any association between these vaccines (or any COVID-19 vaccines) and thromboembolic events overall. A history of thrombosis, thrombophilia, and/or HIT (heparin-induced thrombocytopenia) does not represent a contraindication to vaccination, and patients with such histories should continue to be encouraged to pursue vaccination.

MONOCLONAL ANTIBODIES

Another recent treatment advance for COVID-19 is the development of monoclonal neutralizing antibodies against the spike protein. Several such agents have been evaluated (largely in the outpatient setting), and two have obtained an EUA by the FDA. Bamlanivimab-etesevimab is a combination of two monoclonal antibodies derived from two separate patients convalescing from COVID-19 infection that bind two different epitopes of the spike protein. In a phase II/III clinical trial, the combination of bamlanivimab and etesevimab led to a small, statistically significant reduction in emergency department (ED) visits or hospitalization.[30] A phase III trial, which has not been published at the time of this writing, evaluated 1035 non-hospitalized patients with COVID-19 demonstrated the combined outcome of hospitalization and death was lower (2% vs. 7%) in the bamlanivimab-etesevimab as compared to placebo.[31] A second monoclonal antibody product, casirivimab-imdevimab, has also obtained EUA from the FDA based on a preliminary report showing that these agents reduce viral load, particularly in patients who were SARS-CoV-2 antibody–negative. Among these antibody-negative patients, fewer patients treated with casirivimab-imdevimab required a medical visit within the subsequent month compared with those receiving placebo (6% vs. 15%).[32] If these agents are employed in clinical practice, they should be given as a single infusion as soon as possible after the diagnosis is confirmed and no longer than 10 days after symptom onset.

REFERENCES

1. Williamson EJ, Walker AJ, Bhaskaran K, et al. Factors associated with COVID-19-related death using OpenSAFELY. *Nature*. 2020;584(7821):430–436.
2. Helms J, Tacquard C, Severac F, et al. High risk of thrombosis in patients with severe SARS-CoV-2 infection: a multicenter prospective cohort study. *Intensive Care Med*. 2020;46(6):1089–1098.
3. Klok FA, Kruip M, van der Meer NJM, et al. Incidence of thrombotic complications in critically ill ICU patients with COVID-19. *Thromb Res*. 2020;191:145–147.
4. Bilaloglu S, Aphinyanaphongs Y, Jones S, et al. Thrombosis in hospitalized patients with COVID-19 in a New York city health system. *JAMA*. 2020;324(8):799–801.
5. Menter T, Haslbauer JD, Nienhold R, et al. Postmortem examination of COVID-19 patients reveals diffuse alveolar damage with severe capillary congestion and variegated findings in lungs and other organs suggesting vascular dysfunction. *Histopathology*. 2020;77(2):198–209.
6. Ackermann M, Verleden SE, Kuehnel M, et al. Pulmonary vascular endothelialitis, thrombosis, and angiogenesis in Covid-19. *N Engl J Med*. 2020;383(2):120–128.
7. Poor HD, Ventetuolo CE, Tolbert T, et al. COVID-19 critical illness pathophysiology driven by diffuse pulmonary thrombi and pulmonary endothelial dysfunction responsive to thrombolysis. *Clin Transl Med*. 2020;10(2):e44.
8. Oxley TJ, Mocco J, Majidi S, et al. Large-vessel stroke as a presenting feature of Covid-19 in the young. *N Engl J Med*. 2020;382(20):e60.
9. Bellosta R, Luzzani L, Natalini G, et al. Acute limb ischemia in patients with COVID-19 pneumonia. *J Vasc Surg*. 2020;72(6):1864–1872.
10. Yin S, Huang M, Li D, Tang N. Difference of coagulation features between severe pneumonia induced by SARS-CoV2 and non-SARS-CoV2. *J Thromb Thrombolysis*. 2021;51(4):1107–1110.
11. Naymagon L, Zubizarreta N, Feld J, et al. Admission D-dimer levels, D-dimer trends, and outcomes in COVID-19. *Thromb Res*. 2020;196:99–105.
12. Tang N, Bai H, Chen X, et al. Anticoagulant treatment is associated with decreased mortality in severe coronavirus disease 2019 patients with coagulopathy. *J Thromb Haemost*. 2020;18(5):1094–1099.
13. Paranjpe I, Fuster V, Lala A, et al. Association of treatment dose anticoagulation with in-hospital survival among hospitalized patients with COVID-19. *J Am Coll Cardiol*. 2020;76(1):122–124.
14. Tremblay D, van Gerwen M, Alsen M, et al. Impact of anticoagulation prior to COVID-19 infection: a propensity score-matched cohort study. *Blood*. 2020;136(1):144–147.
15. Al-Samkari H, Gupta S, Leaf RK, et al. Thrombosis, bleeding, and the observational effect of early therapeutic anticoagulation on survival in critically ill patients with COVID-19. *Ann Intern Med*. 2021;174(6):888.
16. Motta JK, Ogunnaike RO, Shah R, et al. Clinical outcomes with the use of prophylactic versus therapeutic anticoagulation in coronavirus disease 2019. *Crit Care Explor*. 2020;2(12):e0309.
17. Spyropoulos AC, Levy JH, Ageno W, et al. Scientific and standardization committee communication: clinical guidance on the diagnosis, prevention, and treatment of venous thromboembolism in hospitalized patients with COVID-19. *J Thromb Haemost*. 2020;18(8):1859–1865.
18. Feld J, Tremblay D, Thibaud S, et al. Ferritin levels in patients with COVID-19: a poor predictor of mortality and hemophagocytic lymphohistiocytosis. *Int J Lab Hematol*. 2020;42(6):773–779.
19. Group RC, Horby P, Lim WS, et al. Dexamethasone in hospitalized patients with Covid-19. *N Engl J Med*. 2020;384(8):693–704.
20. Richardson S, Hirsch JS, Narasimhan M, et al. Presenting characteristics, comorbidities, and outcomes among 5700 patients hospitalized with COVID-19 in the New York city area. *JAMA*. 2020;323(20):2052–2059.
21. Guan W-J, Ni Z-Y, Hu Y, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med*. 2020;382(18):1708–1720.
22. Panigada M, Bottino N, Tagliabue P, et al. Hypercoagulability of COVID-19 patients in intensive care unit: a report of thromboelastography findings and other parameters of hemostasis. *J Thromb Haemost*. 2020;18(7):1738–1742.
23. Polack FP, Thomas SJ, Kitchin N, et al. Safety and efficacy of the BNT162b2 mRNA Covid-19 vaccine. *N Engl J Med*. 2020;383(27):2603–2615.
24. Baden LR, El Sahly HM, Essink B, et al. Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine. *N Engl J Med*. 2021;384(5):403–416.
25. Dhodapkar MV, Dhodapkar KM, Ahmed R. Viral immunity and vaccines in hematologic malignancies: implications for COVID-19. *Blood Cancer Discovery*. 2021;2(1):9–12.
26. Recommendations for COVID-19 vaccination in patients with hematologic cancer. [press release]. https://ehaweb.org/covid-19/eha-statement-on-covid-19-vaccines/recommendations-for-covid-19-vaccination-in-patients-with-hematologic-cancer/2021.
27. Toom S, Wolf B, Avula A, et al. Familial thrombocytopenia flare-up following the first dose of mRNA-1273 COVID-19 vaccine. *Am J Hematol*. 2021;96(5):E134–E135.
28. Tarawneh O, Tarawneh H. Immune thrombocytopenia in a 22-year-old post Covid-19 vaccine. *Am J Hematol*. 2021;96(5):E133–E134.
29. Lee E-J, Cines DB, Gernsheimer T, et al. Thrombocytopenia following Pfizer and Moderna SARS/CoV-2 vaccination. *Am J Hematol*. 2021;96(5):534–537.
30. Gottlieb RL, Nirula A, Chen P, et al. Effect of bamlanivimab as monotherapy or in combination with etesevimab on viral load in patients with mild to moderate COVID-19: a randomized clinical trial. *JAMA*. 2021;325(7):632–644.
31. Coronavirus (COVID-19) Update: FDA authorizes monoclonal antibodies for treatment of COVID-19 [press release]. https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-monoclonal-antibodies-treatment-covid-19-02021.
32. Weinreich DM, Sivapalasingam S, Norton T, et al. REGN-COV2, a neutralizing antibody cocktail, in outpatients with Covid-19. *N Engl J Med*. 2021;384(3):238–251.

CHAPTER 154

HEMATOLOGIC ASPECTS OF PARASITIC DISEASES

David J. Roberts

Parasitic diseases are not common in medical, let alone hematologic, practice in North America or Europe. However, much of the world's population is infected by and becomes symptomatic as a result of a plethora of parasites, and many of these infections represent global public health problems.

Although, some significant parasitic diseases are transmitted in temperate climates, the majority of parasites of significance to human health are endemic in the tropical world. This reflects not only socioeconomic circumstances but also the origin of our species in tropical Africa, where the human host, parasites, and also vectors have established complex relationships over evolutionary timescales.[1,2] Notwithstanding such geographic variation in the incidence of parasitic disease, both travelers and recent immigrants now present to hematology clinics and laboratories all over the world with increasing frequency. Even in those circumstances, there are marked variations in practice in North America as well as in Europe, where the United Kingdom reports more cases of imported malaria than the United States and indeed has a 10-fold greater incidence of malaria per capita, reflecting the increased frequency of travel to and from endemic areas compared with North American populations.

Patients with malaria, leishmaniasis, trypanosomiasis, and babesiosis may present directly or indirectly to hematologists. This chapter is concentrated on the biologic, clinical, and hematologic features of these infections and the hematologic aspects or complications of their treatment. Comprehensive accounts of the general medical aspects of these diseases are provided in many other recent textbooks.[3–5]

MALARIA

Malaria is a major public health problem in tropical areas, and it is estimated that it is responsible for approximately 400,000 deaths annually and 150 to 300 million infections.[5a] The vast majority of morbidity and mortality caused by malaria is caused by infection with *Plasmodium falciparum*, although *Plasmodium vivax, Plasmodium ovale*, and *Plasmodium malariae* are also responsible for human infections. A fifth species, *Plasmodium knowlesi*, has been shown to cause human infection in some parts of Southeast Asia (for review, see the article by Jeyaprakasam et al.[6]). In endemic areas, a significant proportion of the mortality and morbidity is from anemia. In Europe and North America, malaria is not infrequently a clinical problem in travelers or recent arrivals from malaria-endemic areas, and hematologists may be involved in the diagnosis and management of the disease. Moreover, in nonendemic areas, malaria may cause a fatal transfusion-transmitted infection, and detection of blood donors who may be carrying the disease represents a major challenge for blood services.

Epidemiology

Approximately 1 billion people live in areas of endemic or epidemic malaria. The global mortality and morbidity were revised to 350 million cases and 1 million deaths per year, respectively, following an evaluation of the prevalence of infection in Southeast Asia in 2005 (Fig. 154.1). There is, however, substantial evidence that the incidence of severe disease has fallen considerably across the world in the last 15 years, following the widespread introduction of artemisinin combination treatment, impregnated bed nets, and residual spraying because the resources available for malaria control have increased substantially through the Global Fund, the World Health Organization's Global Malaria Programme, and the Roll Back Malaria Partnership to End Malaria (https://endmalaria.org/). The current estimated annual death total from malaria in Africa has fallen from 700,000 in 2004 to 386,000 in 2019.[5]

The distribution of malaria is determined by features of host, vector, and parasite. In summary, the global distribution of autochthonous or endogenous malaria is limited by the lower temperature limits for development of the parasite in the mosquito (sporogony) of 20°C for *P. falciparum* and 15°C for other human malarias. Within these limits, transmission does not occur above 1500 m in arid regions or in the Central and South Pacific (because of the absence of suitable vectors). In addition, *P. vivax* malaria is rare in Africa, where the population frequency of the blood group Duffy negative (Fya^-Fyb^-) is high. *P. ovale* requires a lengthy period of sporogony and is confined to areas of Africa and Southeast Asia with a high density of susceptible *Anopheles* spp. *P. knowlesi* is transmitted from macaque monkeys in forest areas of Borneo, Malaysia, Thailand, and Vietnam.[6]

In some malarious areas the seasonal pattern of clinical malaria is determined by the increase in vector density after rainfall, leading to an increase in new infections as transmission increases. In naive individuals, parasites can cause chronic infection lasting many months.

The intensity of transmission determines the distribution of clinical symptoms in different age groups.[7] In general, in areas of high transmission, younger children experience severe disease. Where transmission is less intense, older children experience severe disease. Finally, if the rate of transmission is very low, few cases of malaria are seen in any age group, and such populations would have little natural immunity. In such areas, a sudden increase in vectorial capacity (through the accidental introduction of efficient vectors or higher density, biting, or survival of the resident vectors), more rapid parasite sporogony, or migration of infected or nonimmune populations can result in epidemics where large numbers fall ill in all age groups. The transition from high to low transmission has been classified by holoendemicity, hyperendemicity, mesoendemicity, and hypoendemicity. These categories can be related epidemiologically to age-specific rates of parasite prevalence or splenomegaly and theoretically to the reproductive ratio of malarial infection.[8]

Malaria exerts a substantial selection for human traits that protect from infection. About a third of the variability in the risk of severe and complicated malaria is now explained by additive host genetic effects.[9] Substantial protection from malaria is derived from sickle cell trait and thalassemia traits which are truly polymorphic characteristics in many parts of the world. Other red cell traits conferring resistance to severe disease include the ABO blood groups and polymorphisms of ATPas4 (the major calcium pump on red cells) and polymorphisms of the glycophorins, including the Dantu blood group antigen.[10] Understanding genetic epidemiology has provided the foundation of population genetics and has provided classic examples of principles of genetic selection in vivo—for example, balancing selection for sickle cell trait and negative epistasis for sickle cell trait and α-thalassemia. The homozygous forms of these characteristics cause significant clinical disease, such as sickle cell disease, β-thalassemia, and glucose-6-phosphate dehydrogenase (G6PDH) deficiency. In endemic areas these genetic diseases represent major public health problems (for review, see Williams[11] and Luzzatto et al.[12]).

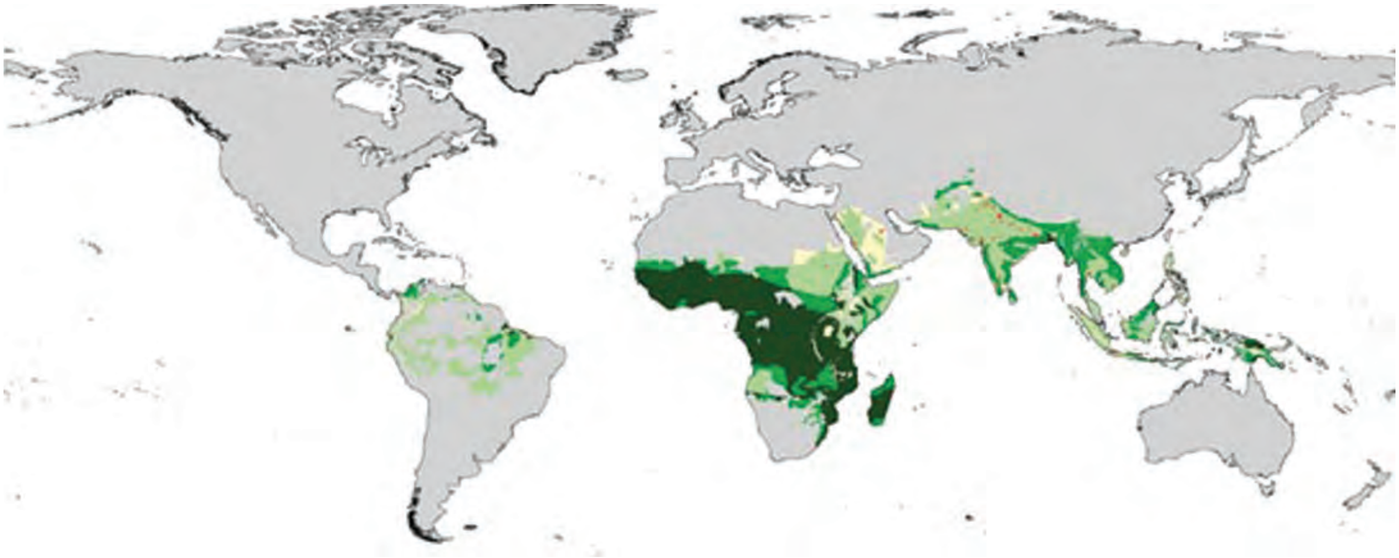


Figure 154.1 GLOBAL MALARIA ENDEMICITY. Areas are colored according to malaria endemicity (prevalence): *light green*, hypoendemic (areas in which childhood infection prevalence is less than 10%); *medium green*, mesoendemic (areas with infection prevalence between 11% and 50%); *dark green*, hyperendemic and holoendemic (areas with an infection prevalence of 50% or more); unclassified areas *(yellow)* represent only 6% of the global population at risk and are caused by discrepancies in recent data. *Gray* areas are a combined mask of areas outside the transmission limits and areas of population density less than 1 person/km^2. *(Data from Snow RW, Guerra CA, Noor AM, et al. The global distribution of clinical episodes of Plasmodium falciparum malaria. Nature. 2005;434:214.)*

Parasitology

In *P. falciparum* (see later for a discussion of the other human parasites) the infective sporozoite forms are inoculated into the bloodstream from the salivary glands of a female *Anopheles* mosquito (Fig. 154.2). These thin, needle-shaped cells, 10 to 12 µm in length, circulate briefly with a half-life of approximately 30 minutes before traversing macrophages and several hepatocytes and ultimately residing in a single hepatocyte.[13] Here rapid multiplication takes place over 5 to 8 days to produce a liver schizont, 80 µm in diameter and containing 30,000 ± 10,000 merozoites that are released into the bloodstream, where they infect erythrocytes.[13] When ready to leave hepatocytes, the parasite induces cell death in the hepatocytes and causes the release of merozoites in membrane-enclosed structures or merosomes that are extruded from the infected cell, thereby avoiding host cell defense mechanisms.[14]

The merozoites bind and then invade red blood cells (RBCs; for review of RBC and merozoite interactions, see Satchwell[15]). The host plasma membrane is invaginated to form the parasitophorous vacuole. For the first 10 hours the developing parasites appear as fine "ring forms." Between 10 and 15 hours the cytoplasm thickens, and 16 hours after invasion, granules of the black pigment hematin, the end product of hemoglobin (Hb) digestion, begin to appear. Ligands are expressed at the surface of the infected RBC that mediate adhesion to host receptors on venular endothelium. These trophozoites no longer circulate throughout the body but are sequestered in the peripheral circulation. Nuclear division begins, at approximately 30 hours, to form schizonts containing up to 32 merozoites. At 48 hours the RBC is ruptured to release the merozoites into the circulation to continue further cycles of asexual multiplication.

The erythrocytic cycle of schizogony may achieve a 10-fold increase in parasitemia in vivo and a patent or microscopically detectable infection 6 days after the liver stage is completed. After two or more cycles the infection becomes clinically apparent by the paroxysms of fever that accompany the release of merozoites. Cycles of schizogony continue until the rate of parasite multiplication is reduced by chemotherapy, specific or nonspecific defense mechanisms, or occasionally the demise of the host.

Some merozoites do not multiply but become committed during the previous erythrocytic cycle to form male or female gametocytes.[16]

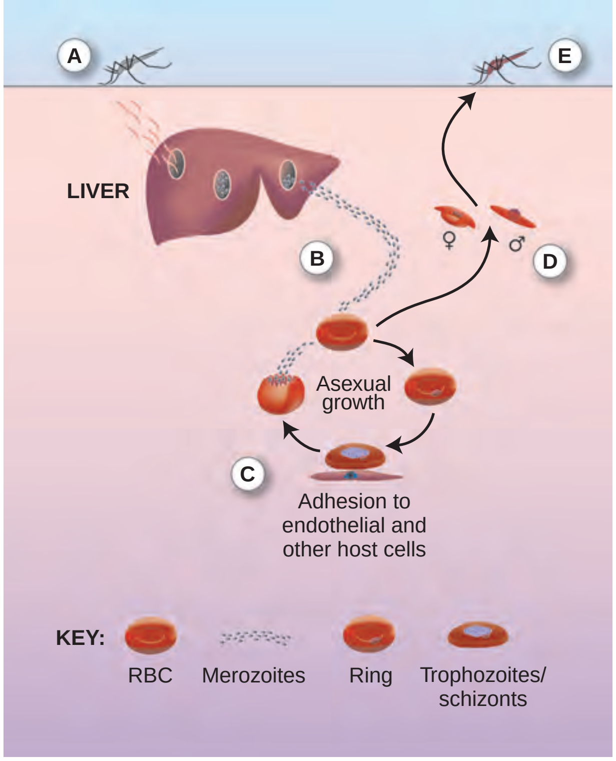


Figure 154.2 *PLASMODIUM* LIFE CYCLE. (A) The asexual life cycle begins when sporozoites from a female mosquito taking a blood meal enter the circulation and invade hepatocytes. (B) Up to 10,000 merozoites are formed. Following rupture of the hepatocyte, infective merozoites are released and invade erythrocytes (red blood cells [RBCs]). (C) Within RBCs, the parasite develops through the stages of rings, trophozoites, and schizonts. Mature schizonts burst to release erythrocytic merozoites that invade new RBCs. (D) A small proportion of merozoites in RBCs transform into male and female gametocytes that are ingested by the mosquito. (E) The male and female gametes fuse and transform into an oocyst, which divides asexually into many sporozoites that migrate to the salivary gland from where they are released during the next blood meal.

Gametocytes are distinguished by dispersed pigment in a single nucleus, in a fully grown parasite, and are sequestered for the first 5 days of their development in the peripheral circulation. Thus 8 to 10 days after the start of clinical infection, mature, crescent-shaped gametocytes appear in the blood.

The sexual phase (or sporogony) of the parasite life cycle begins after a male and female gametocyte are ingested by a feeding female *Anopheles* mosquito. In the midgut of the mosquito the gametocytes shed the RBC membrane. This change is apparently precipitated by the drop in temperature. A female gametocyte forms a single macrogamete, but male gametocytes undergo several rounds of nuclear division to produce flagellated microgametes. These microgametes are motile and migrate to fertilize a macrogamete. The resulting zygote enlarges to form a mobile ookinete and migrates through the epithelial wall of the mosquito midgut to rest finally on the external surface. The oocyst divides repeatedly to form up to 10,000 sporozoites, which travel up through the hemolymph to enter the acinar cells of the salivary glands. Once there, they are infective when injected into the host.

Major differences exist in the life cycles of other human *Plasmodium* spp. First, in *P. vivax* and *P. ovale* infections, some sporozoites entering the liver form dormant hypnozoites that begin to divide only after a variable period of some months to cause further blood-stage infections or relapses. Second, the cycle of erythrocytic development in *P. malariae* takes 72 hours and thus causes quartan fever (i.e., on days 1 and 4).

Pathophysiology of Malarial Anemia

Malaria gives ample reasons for both increased RBC destruction and reduced RBC production (see Fig. 154.3 for overview).

Loss of Infected Erythrocytes

Destruction of RBCs is inevitable as parasites complete their 48-hour growth cycle and lyse their temporary host cell. Some parasites may be removed from erythrocytes as immature ring forms by phagocytic cells, leaving the RBCs with residual parasite antigens to continue to circulate, albeit with reduced survival.[17] Infected erythrocytes may also be phagocytosed by macrophages following opsonization by immunoglobulins and/or complement components.[18,19] Other signals for recognition of infected erythrocytes by macrophages include abnormally rigid membranes and exposure of phosphatidylserine and other altered host antigens and, in animal models of malaria, antiphosphatidylserine antibodies.[20–23]

Loss of Uninfected Erythrocytes

The activity and the number of macrophages are increased in malarial infection, and increased removal of uninfected cells may occur. Moreover, the signals for recognition of uninfected erythrocytes for removal by macrophages are enhanced. Uninfected erythrocytes bind increased amounts of immunoglobulin and/or complement as detected in the direct antiglobulin test (DAT or Coombs test).[21] These antibodies do not have a particular specificity but are more likely to represent immune complexes absorbed onto the surface of RBCs.[21] Furthermore, hemozoin may activate complement directly and cause deposition of C3b on uninfected RBCs.[24] However, an association between positive DAT results and severe anemia has not been established.[25] More recent studies have shown not only that RBCs from patients with malaria were more susceptible to phagocytosis but also that RBCs from children with acute malaria had increased surface immunoglobulin G (IgG) and low levels of RBC CR1 and CD55 compared with control subjects.[26]

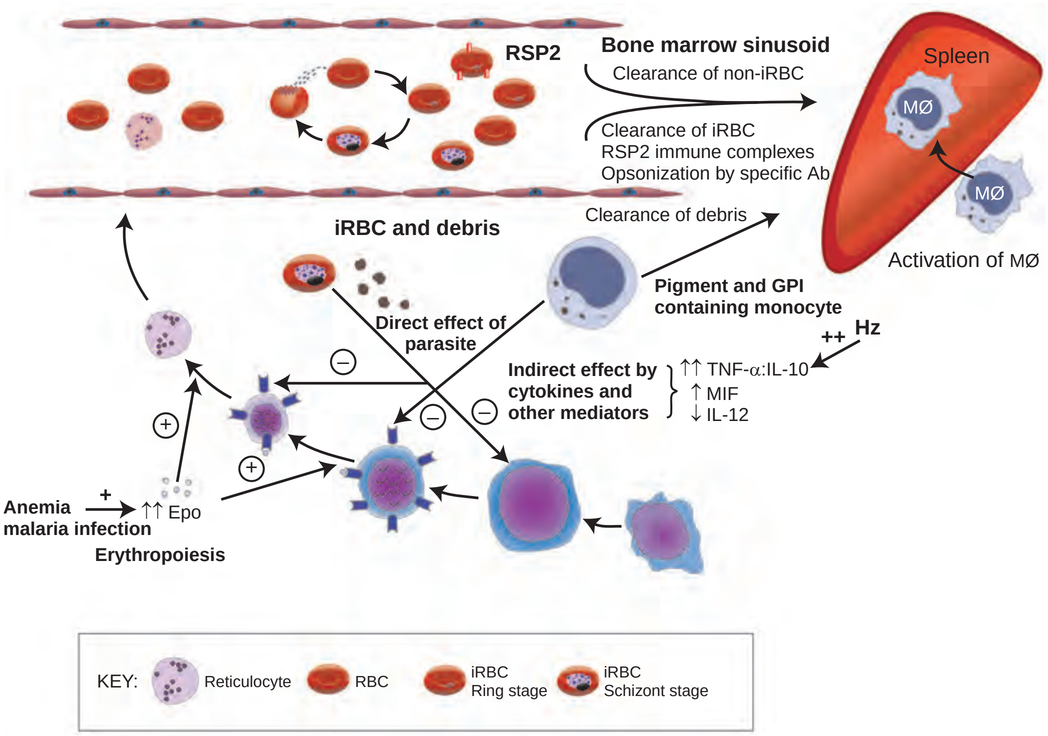


Figure 154.3 PATHOGENESIS OF MALARIAL ANEMIA. Severe malarial anemia is characterized by destruction of infected red blood cells (iRBCs) following schizogony and clearance of both iRBC and uninfected RBCs. During malarial infection, changes in membrane protein composition occur, and the resultant immune complexes of RBC, Ag, and immunoglobulin (e.g., RBC-RSP2-Ig) are cleared by MØ to the spleen, where they become activated. Pigment-containing MØ may release inflammatory cytokines and other biologically active mediators such as 4-hydroxynonenal. MIF may be released by MØ, or a plasmodial homologue may suppress erythropoiesis. Malarial pigment or other parasite products may have a direct inhibitory effect on erythropoiesis. Inhibition of erythropoiesis may occur at one or more sites in the growth and differentiation of hematopoietic progenitors. Both indirect and direct effects may cause suppression of the bone marrow and spleen, resulting in inadequate reticulocyte counts for the degree of anemia. *Ab*, Antibody; *Ag*, antigen; *Epo*, erythropoietin; *GPI*, glycosylphosphatidylinositol anchors of merozoite proteins; *Hz*, hemozoin; *Ig*, immunoglobulin; *IL*, interleukin; *MIF*, macrophage inhibitory factor; *MØ*, macrophages; *RSP2*, ring surface protein-2; *TNF*-α, tumor necrosis factor-α.

Role of the Spleen

Some degree of splenomegaly is a normal feature of malarial infection, and the prevalence of splenomegaly in regions of malarial transmission is used as a major indicator of the level of malarial endemicity. The importance of the spleen in host defense against malaria has been demonstrated in experimental systems, and individuals whose spleens have been surgically removed are thought to be more susceptible to severe infection. Indeed, the phenomenon of parasitic sequestration is thought to have evolved primarily as an immune evasion strategy so the mature parasite can avoid passing through the spleen.[27]

Active erythrophagocytosis is a conspicuous feature within the bone marrow during *P. vivax* and *P. falciparum* malaria,[28,29] and it is highly probable that this also occurs within the spleen. Cytokines may be responsible for activating macrophages during malarial infection. Children with acute *P. falciparum* malaria have high circulating levels of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α),[30] a synergistic combination of cytokines that activates macrophages.

Researchers in several studies have attempted to define the pathophysiologic changes in the spleen during acute malaria. In animal models, malaria is accompanied by increased intravascular clearance of infected or rigid, heat-treated cells by the spleen,[31] as well as alterations in the splenic microcirculation.[32] In studies of human malaria, it has been found that increased splenic clearance of heated RBCs occurs during acute attacks.[33] Ex vivo models of splenic function suggest that the spleen removes not only mature infected RBCs but also uninfected cells marked for clearance by low deformability, aggregated band 3, complement deposition, or phosphatidylserine exposure. Moreover, "pitting" of ring-stage infected cells may remove the parasite and leave the RBC marked with parasite antigen to return to the circulation (reviewed by White[34]).

Changes to the uninfected RBCs during infection also contribute to their own enhanced clearance by phagocytes. Uninfected RBCs in children and adults with severe disease are less deformable, and this is a significant predictor of the severity of anemia and indeed outcome, consistent with the notion that these cells are being removed by the spleen.[20] It has also been found that IgG-sensitized RBCs are rapidly removed from the circulation by the spleen and that unusually rapid clearance persists well into the convalescent phase.[35]

Thus, all the available evidence points to increased reticuloendothelial clearance in *P. falciparum* malaria, persisting long after recovery. These changes are presumably a host defense mechanism, maximizing the clearance of parasitized erythrocytes. Nevertheless, the contribution of clearance of uninfected erythrocytes to anemia during acute malaria is substantial and it has been estimated that loss of unparasitized erythrocytes accounts for approximately 90% of the drop in Hb resulting from a single infection. The severity and duration of anemia is determined not only by red cell clearance but also by dyserythropoiesis and reticulocytopenia.

Role of Ineffective Erythropoiesis in Severe Malarial Anemia

Reticulocytopenia has been confirmed in numerous clinical studies of malarial anemia.[36–38] The histopathologic study of the bone marrow of children with malarial anemia shows erythroid hyperplasia with increased numbers of erythroid precursors (Fig. 154.4). However, the maturation is abnormal by light and electron microscopy. Abdalla et al.[39,40] described the hallmark characteristics of such abnormal maturation, namely cytoplasmic and nuclear bridging and irregular nuclear outline. They later confirmed that the distribution of the erythroid progenitors through the cell cycle is abnormal in malarial anemia, with an increased proportion of cells in the G_2 phase compared with healthy control subjects.[41] Dörmer et al.[38] confirmed these findings using the same criteria as Abdalla et al.; they studied six patients with falciparum malaria before and after treatment.

Bone marrow dyserythropoiesis persists for many weeks following the start of malaria treatment in acute malaria and reticulocyte counts are therefore low when patients are symptomatic. In such settings, Hb concentration in acute falciparum malaria is lowest around 1 week after presentation with fever,[42] while in acute vivax malaria the nadir in Hb is a few days after presentation.[43] In acute uncomplicated malaria, anemia is worse in younger children and those with protracted infections. In high transmission areas, where previous infections may confer some protection from the effects of infection, Hb concentrations often increase soon after treatment.[44]

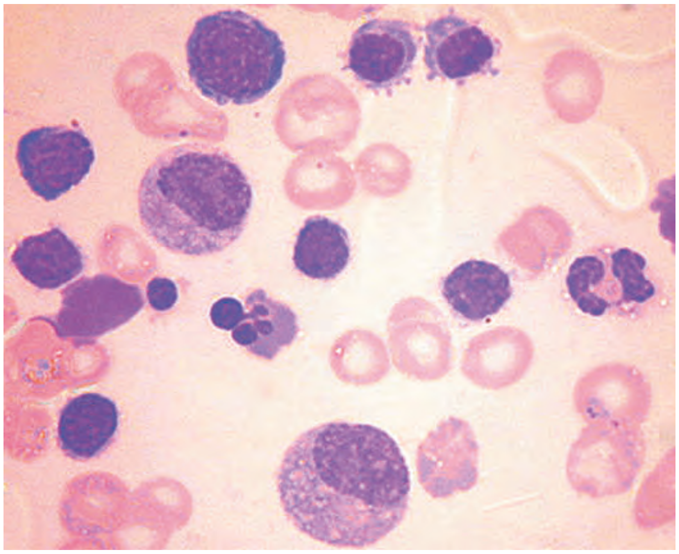


Figure 154.4 BONE MARROW ASPIRATE IN MALARIA. Although erythropoiesis is usually normoblastic in individuals with acute or chronic malaria, the examination of bone marrow frequently shows changes reflecting dyserythropoiesis, such as the irregular nuclei and cytoplasmic bridges in erythroblasts seen in this figure. Two young rings of *Plasmodium falciparum* can be seen in an erythrocyte. *(Courtesy Dr. Saad H. Abdalla.)*

Modulation of Erythropoiesis by Cytokines

Given the importance of erythropoietin (Epo) to erythropoiesis, attention has been focused on the levels of this crucial cytokine in malarial infection. Serum Epo was appropriately raised in studies of African children with malarial anemia, and high levels of this cytokine have been associated with better outcomes of disease.[45,46] African children with severe malaria, particularly young children, were shown to have supraphysiologic levels of Epo compared with age-matched community control subjects with nonmalarial anemia.[47,48] Evidence from some observational clinical studies suggested that these high levels of Epo in children with malarial anemia were associated with a cytoprotective effect and so reduce the risk for neurologic sequelae or death in children with cerebral malaria.[49] Other studies in children over 18 months old with cerebral malaria, showed that high Epo levels were associated with prolonged coma duration and increased mortality.[50] Moreover, clinical studies using Epo as an adjuvant in other neurological diseases have shown that although Epo was associated with some neurological benefits, treatment with Epo increased mortality from other causes. There have been no large-scale trials of recombinant Epo in malaria.

The prime candidates for the host factors mediating dyserythropoiesis are imbalances of TNF-α, IFN-γ, and interleukin-10 (IL-10). The concentrations of TNF-α and IFN-γ have been correlated with the severity of the disease.[51–53] Whereas low concentrations of TNF-α (<1 ng/mL) stimulate erythropoiesis, higher levels of TNF-α have been shown to suppress erythropoiesis.[53] Furthermore, it is possible that high levels of these inflammatory cytokines may contribute to reduced and abnormal production of erythrocytes and also to increased erythrophagocytosis.

Recent evidence has suggested that the release of hepcidin is associated with malarial infection and that high levels of hepcidin could contribute to the sequestration of iron and impair erythropoiesis.[54,55] The stimulus for hepcidin secretion may be proinflammatory cytokines such as IL-6, but malaria-infected erythrocytes may also enhance hepcidin production by peripheral blood mononuclear cells. Intriguingly, hepcidin released during blood-stage parasitemia may be a key regulator of *P. falciparum* liver-stage development.[56] These data now support suggestions from large-scale studies of iron supplementation that iron may be unhelpful and possibly even harmful when given during acute malarial infection. Indeed, there are both clinical and experimental evidence that iron deficiency may confer protection from severe malaria. Malaria parasites show reduced growth in RBCs from subjects with iron deficiency, and infection is augmented during the phase of iron supplementation after severe iron deficiency.[57]

High levels of the T-helper cell type 2 (Th2)-type cytokine, IL-10, might prevent the development of severe malarial anemia. Low levels of IL-10 have been described in African children with severe malarial anemia.[51] Similarly, defective IL-12 production has been shown experimentally to be associated with increased severity of malaria in a rodent model, and low IL-12 levels have been associated with severe malaria in African children.[58] The role of IL-12 in clinical malaria appears complex, but IL-12 and other cytokines may activate adaptive natural killer (NK) cells to reduce parasitemia by antibody-dependent cytotoxicity.[59]

Modulation of Erythropoiesis by Infected Erythrocytes

There is also substantial evidence that lysate of infected erythrocytes may directly modulate the function of host cells. During its blood stage, the malaria parasite proteolyzes host Hb in an acidic vacuole to obtain amino acids, releasing heme as a byproduct, which is auto-oxidized to potentially toxic hematin (aquaferriprotoporphyrin IX [$H_2O\text{–}Fe_{III}PPIX$]). β-Hematin forms as a crystalline cyclic dimer of $Fe_{III}PPIX$ and is complexed with protein and lipid products as malarial pigment or hemozoin. Schwarzer et al.[60,61] showed that the function of monocytes and of monocyte-derived macrophages is severely inhibited after ingestion of malaria pigment or hemozoin.[62,63]

The hemozoin polymer of heme moieties may be complexed with biologically active compounds. The oxidation of membrane lipids catalyzed by the ferric heme produces the lipoperoxides.[61,63] There is accumulating evidence that 4-hydroxynonenal (HNE) and other lipoperoxides, including 15-hydroxyarachidonic acid [15-(*R*,*S*)-HETE], may play a role in the pathophysiology of malaria. It has been shown that HNE and HETE are generated in parasitized erythrocytes and that HNE and endoperoxides produced in pigment-containing monocytes or macrophages may cause cell-cycle arrest and impair erythroid growth.[63,64] Hemozoin may also directly inhibit erythroid development in vitro and cause apoptosis of erythroid precursors.[65] Furthermore, increased levels of plasma hemozoin and pigment in monocytes have been associated with anemia.[49]

Modulation of Erythropoiesis by Infection

In children presenting with acute malaria, bacteremia is associated not only with anemia but also with excess mortality.[66] Malaria infection is particularly associated with non-typhi salmonella bacteriemia, due at least in part to impaired neutrophil function.[67]

Parvovirus B19 may cause a transient reticulocytopenia and thus severe and sudden anemia in those with a hemolytic anemia or fetal anemia and intrauterine death. Most children in Africa have serologic evidence of infection early in life. However, acute infection with this virus is uncommon in those presenting with severe malarial anemia,[46] and a recent parvovirus B19 infection and falciparum malaria appear to be independent risk factors for anemia in children in Africa.[68]

Prevalence and Etiology of Anemia in Low-Income Countries

In endemic areas the etiology of anemia is complex. Acute or chronic malarial infection is a major precipitating factor in children with severe anemia causing admission to hospital.[47]

Longitudinal studies from The Gambia have shown that whereas the mean Hb levels in children vary significantly through the year, anemia is much more common in the rainy season, when malaria transmission is at its highest.[69] However, the rains are also associated with an increase in waterborne diarrheal disease and poor food supplies. So the seasonal increase in anemia in malaria-endemic areas arises on a background of low or frankly deficient stores of hematinics and/or other micronutrients. Iron deficiency, which affects at least 20% of the world's population, is a major factor in the seasonal surge of anemia in the tropical rainy season. Low iron stores at birth and dietary iron deficiency may be exacerbated by hookworm or schistosomal infection. It is now also clear that malarial infection is associated with reduced uptake of available iron and reduced incorporation of iron into developing erythroid cells.[54,55] Folate deficiencies and/or increased requirements may occur in many populations, although frank folate deficiency is uncommon.

A low baseline Hb level at the start of the season for malaria transmission is a major risk factor for developing severe malarial anemia and has encouraged studies aimed at preventing the development of anemia by hematinic supplementation with or without antimalarial prophylaxis or surveillance.[70]

Quantifying the contribution of these individual factors to anemia reliably requires intervention studies. In Tanzania, Menendez et al.[71] and Schellenberg et al.[72] have shown that iron supplementation and antimalarial prophylaxis prevented 30% and 60%, respectively, of all cases of moderate anemia presenting during the malaria transmission season in children and in infants.

Translating the results of these studies from well-defined and carefully controlled study areas has not been easy. Considerable concerns about iron supplementation programs for children have been raised in sub-Saharan Africa and in areas of high malaria endemicity. One large trial of iron supplementation was stopped because of increased hospital admission and death in the group receiving iron. However, meta-analysis of iron supplementation in malaria-endemic areas has shown that iron alone or with antimalarial treatment does not increase the risk for clinical malaria or death when regular malaria surveillance and treatment services are provided.[73] It is increasingly clear that iron supplementation in these areas must be based on either defining iron-deficient children or combining iron administration with effective infection control and treatment strategies.[73,74]

Features of Malarial Anemia

Spectrum of Disease Caused by Malarial Infection

The signs and symptoms of malarial infection in humans are caused by the asexual blood stage of the parasite. Infection with blood-stage parasites may result in a wide range of outcomes and pathologic conditions. Indeed, the spectrum of severity ranges from asymptomatic infection to rapidly progressive, fatal illness. The clinical presentation of malarial infection is also wide and influenced by age, immune status, and pregnancy, as well as by the species, genotype, and perhaps the geographic origin of the parasite. In endemic areas, many infections present as an uncomplicated febrile illness. In more severe forms of the disease, children may present with prostration or inability to take oral fluids, or in younger children an inability to suckle. Alternatively, these children may exhibit a number of syndromes of severe disease, including anemia, coma, respiratory distress, and hypoglycemia and may also have a high rate of bacteremia.[66,75]

In most age groups, anemia is frequently accompanied by more than one syndrome of severe disease, and the already substantial case fatality rate of 15% to 20% for severe malaria in African children rises significantly when multiple syndromes of severe disease are present.[75] The age distribution of anemia and other syndromes of severe disease is a consistent but puzzling feature of the epidemiology of clinical malaria. Children born in endemic areas are protected from severe malaria in the first 6 months of life by the passive transfer of maternal immunoglobulins and by fetal Hb. Beyond infancy, the most common form of presentation of severe disease changes from anemia in children aged 1 to 3 years old, in areas of high transmission, to cerebral malaria in older children, in areas of lower transmission.[44,76] As transmission intensity declines further, severe malaria is most frequently found in older age groups.

Clinical Features of Malarial Anemia

The blood stage of falciparum malaria may cause life-threatening anemia; an Hb level of less than 5 g/dL is considered to represent severe disease. Children with anemia may present with malaise, fatigue, and dyspnea or respiratory distress, which usually represents metabolic acidosis, but in an ill child acute respiratory infection must be carefully excluded.[75,77]

Acidosis is due largely to excessive lactic acid and other anions. Salicylate toxicity and dehydration may also play a role. However, the majority of children presenting with respiratory distress are severely anemic, have a metabolic acidosis secondary to reduced oxygen-carrying capacity, and respond to rapid transfusion of fresh blood.[78]

A minority of those with respiratory distress do not respond to appropriate resuscitation. They probably represent a heterogeneous clinical group and may have renal failure, systemic bacterial infection, or a more profound syndrome of systemic disturbance caused by malarial parasites.

Resuscitation of children with anemia and/or severe metabolic problems must be conducted carefully. Fluid boluses given to African children with shock and life-threatening infections showed somewhat surprisingly that rapid infusion of crystalloids or albumin (20 to 40 mL/kg) significantly increased 48-hour mortality in these critically ill children with impaired perfusion.[79] The TRACT trial has recently provided additional evidence for management of severe anemia and shown that conservative management of uncomplicated severe anemia (Hb 40 to 60 g/L) is safe, if children are reviewed regularly. The trial showed that severely anemic children without fever can be safely transfused with 30 mL/kg of blood but a blood infusion of 20 mL/kg is needed for safe transfusion of febrile children with severe anemia (see box on Transfusion Management of Severe Anemia in African Children.).[80,81] Fluid resuscitation and blood transfusion must be carefully supervised, and it may be relevant that after transfusion in children with acute malarial anemia, BNP levels are high in children with severe malarial anemia suggesting that cardiac function may be compromised and should be carefully monitored during fluid and blood therapy for acute malaria.[82]

Hemolytic Syndromes, Including Blackwater Fever

The sudden appearance of Hb in the urine, indicating severe intravascular hemolysis leading to hemoglobinemia and hemoglobinuria or blackwater fever, received particular attention in early studies of anemia in expatriates living in endemic areas. There was an association between blackwater fever and the irregular use of quinine for chemoprophylaxis. This drug can act as a hapten and stimulate production of a drug-dependent, complement-fixing antibody. Recent studies of sudden intravascular hemolysis have shown that it is rare in Africa but more common in Southeast Asia and Papua New Guinea, where some cases are associated with G6PD deficiency and treatment with a variety of drugs, including quinine, mefloquine, and artesunate.[83] Treatment with artesunate frequently induces anemia without induction of antibodies. Post-artesunate delayed hemolysis (PADH) occurs in 40% of returning travelers with severe malaria treated with artesunate, occurring more commonly in patients of European than of African origin.[84] Hemolysis is largely, but not entirely, due to clearance by splenic macrophages of once-infected cells that may circulate for many days after removal of the ring-stage parasite from red cells. The once-infected cells retain several parasite proteins, including histidine-rich protein-2. Post-artesunate hemolysis is associated with increased levels of this protein, which can be detected by testing at 1:500 dilution of blood with the rapid diagnostic dipsticks for HRP-2 antigen.[85]

TRANSFUSION MANAGEMENT OF SEVERE ANEMIA IN AFRICAN CHILDREN

The Phase III Transfusion and Treatment of severe anemia in African Children Trial (TRACT) showed:

- conservative management of uncomplicated severe anemia [hemoglobin (Hb) 40–60 g/L] was safe
- transfusion volume (20 vs. 30 mL/kg whole blood equivalent) for children with severe anemia (Hb <60 g/L) had strong but opposing effects on mortality, depending on fever status (>37.5°C).

In 2020 a stakeholder meeting of pediatric and blood transfusion groups from Africa reviewed the results and additional analyses and concluded:

- the definition of severe anemia can be standardized as Hb <60 g/L
- children with uncomplicated severe anemia do not require immediate transfusion, but do require monitoring because ~50% will develop severe and life-threatening anemia requiring subsequent transfusion
- clinical and Hb monitoring reviews are needed in a child with severe anemia (both uncomplicated and complicated) to identify those needing transfusion
- the most effective and safest volume of blood for transfusion depends on the axillary temperature when a blood transfusion is ordered
  - if temperature is equal to or <37.5°C then transfuse 30 mL/kg whole blood or 15 mL/kg red cell concentrate
  - if temperature is >37.5°C then transfuse 20 mL/kg whole blood or 10 mL/kg red cell concentrate
- there is no need for separate recommendations for children with malaria (even those with high parasite burdens)
- optimal transfusion of SCD patients or of children with poor nutritional status may need further specific studies.

Hematologic Features of Malarial Infection

The anemia of falciparum malaria is typically normocytic and normochromic, with a notable absence of reticulocytes, although microcytosis and hypochromia may be present because of the very high frequency of α- and β-thalassemia traits and/or iron deficiency in many endemic areas.[47,69]

The anemia of malaria may be accompanied by changes in the white cell and platelet counts and in clotting parameters, but these changes are not in themselves diagnostic, nor do they guide management. Malaria is accompanied by a modest leukocytosis, although leukopenia may also occur. Occasionally, leukemoid reactions have been observed. Leukocytosis has been associated with severe disease.[86] A high neutrophil count may also suggest intercurrent bacterial infection. Monocytosis and increased numbers of circulating lymphocytes are also seen in acute infection, although the significance of these changes is not established. However, malarial pigment is often seen in neutrophils and in monocytes and has been associated with severe disease and unfavorable outcome.[87,88]

Thrombocytopenia is almost invariable in malaria and so may be helpful as a sensitive but nonspecific marker of active infection. However, severe thrombocytopenia (platelet count $<50 \times 10^9$/L) is rare. Increased removal of platelets may follow absorption of immune complexes or platelet activation, but there is no evidence for platelet-specific alloantibodies. Thrombocytopenia has not generally been associated with disease severity, although platelets have been shown to contribute to disease pathology in both animal and human malaria.[89] Moreover, in human infections, platelets may form clumps with infected erythrocytes.[90] One explanation of inconsistent relationships reported between the severity of malaria and the thrombocytopenia may be that findings of low levels of platelets might not only be a marker of parasite burden but also be protective from severe disease as parasite growth is inhibited by physiological concentrations of platelets.[91,92] Platelet factor 4 may bind to and enter malaria-infected RBCs and induce cell death of intra-erythrocytic parasites.[93]

Disordered coagulation and clinical evidence of bleeding are not infrequent in nonimmune adults contracting malaria and presenting with severe disease. Patients may present with bleeding at injection sites, gums, or epistaxis. Abnormal results of laboratory tests for hemostasis, suggesting activation of the coagulation cascade, occur in acute infection. However, histologic evidence of intravascular fibrin deposition is notably absent in adults dying as a result of severe malaria.[94] Factor XIII, normally responsible for cross-linking fibrin, is inactivated during malarial infection, and these data may explain low levels of fibrin deposition in the face of increased procoagulant activity.[95]

During acute disease the levels of a disintegrin and metalloproteinase with thrombospondin motif 13 (ADAMTS13) protease are moderately reduced, and the concentration of high-molecular-weight von Willebrand multimers is increased, but the role of this adhesive pathway in the etiology of severe and cerebral malaria has not been established. Infected RBCs from different isolates or clones may variously bind to a series of endothelial receptors including CD36 and ICAM-1. Adhesion of infected RBCs to the endothelial receptor for protein C is associated with cerebral malaria and may induce endothelial activation and brain swelling.[96]

The bone marrow is typically hypercellular. The most striking findings are of grossly abnormal development of erythroid precursors or dyserythropoiesis. The developing erythroid cells typically demonstrate cytoplasmic and nuclear bridging and irregular nuclear outline.[39,40] These changes are probably central to the pathophysiology of malarial anemia and are discussed in detail later. The proportion of abnormal erythroid precursors and the degree of dyserythropoiesis are markedly greater in chronic compared with acute infection, suggesting that the inhibition and abnormal maturation of erythroid precursors may have somewhat differing etiologies in acute and chronic infection.[39]

The role of hematinic deficiency in children presenting with malaria and anemia may be difficult to assess. The relative importance of absolute and functional iron deficiency have not been defined. Iron will not be absorbed during acute infection, because hepcidin levels are raised.[54,55] Nevertheless, many hospitals give a course of iron supplementation after an episode of acute malaria, although no general guidelines have been established. Chronic hemolysis may increase folate requirements, but frank deficiency is uncommon in children presenting with acute malaria, at least in East Africa.[47] Folate deficiency may be more common in West Africa, and protocols for folate supplementation after malaria must reflect local experience.

Malarial Anemia in Pregnancy

Pregnancy is accompanied by a series of physiologic changes that predispose not only to anemia but also to malaria. Hemodilution causes a physiologic decrease in Hb. Moreover, the demand for both iron and folate increases as the fetus grows and often precipitates frank folate or iron deficiency, particularly in multigravid women.

Occult malarial infection, often without fever, may cause anemia and placental dysfunction. This effect is greatest in primigravidas and has been attributed to the adhesion of parasitized erythrocytes to chondroitin sulfate A and hyaluronic acid in the placenta[97,98] (for review, see Rogerson et al.[99]). Fetal growth is impaired, and babies born to women with placental malaria are, on average, 100 g lighter than those born to women without malaria. The subsequent contribution of malaria to infant mortality is substantial.[100] Furthermore, the increase in hematopoiesis demanded by hemolysis during malarial infection may precipitate frank folate deficiency. Finally, women who are not immune to malaria are more likely to develop hypoglycemia and pulmonary edema during pregnancy. The increase in maternal and fetal morbidity and mortality secondary to malaria may be prevented by routine hematinic supplementation, implementation of insecticide-impregnated bed-nets, and by intermittent treatment with antimalarials during the second and third trimesters with sulfadoxine-pyrimethamine (Fansidar) where parasites remain sensitive to this combination, dihydroartemisinin-piperaquine in Africa and areas of drug resistance to sulphadoxine-primethamine.[101]

Hyperreactive Malarial Splenomegaly

Although splenomegaly secondary to malarial infection usually regresses as immunity is acquired, some people living in endemic areas develop progressive, massive splenomegaly.[102] The pathophysiology of such hyperreactive malarial splenomegaly (HMS) is poorly understood but certainly results in B-cell hyperplasia with high levels of polyclonal IgM, reaching a level greater than two standard deviations (SDs) above the local reference mean. The specific antimalarial antibody titer is high, although only a small proportion of the polyclonal IgM response is directed at malarial antigens. B-cell hyperplasia may provoke proliferation of both T cells and macrophages, and IgM levels may cause cryoglobulinemia and stimulate erythrophagocytosis.

Patients may present typically between 20 and 40 years old, with massive splenomegaly without lymphadenopathy, anemia that may be severe, neutropenia, and thrombocytopenia. The anemia may cause life-threatening hemolytic crises and/or neutropenia associated with severe bacterial infection. Thrombocytopenia is rarely symptomatic. The bone marrow shows hypercellularity, with a lymphocytosis in marrow and peripheral blood. The high IgM levels distinguish HMS from chronic lymphocytic leukemia or other lymphoproliferative disorders. However, the polyclonal proliferation of B cells may transform to a true clonal lymphoproliferative disorder. This appears to be derived from a naive B cell, although the exact classification of such lymphoproliferative disorders is poorly defined[103]; however, there is a single report of a high incidence of B prolymphocytic leukemia in one population.

Treatment for HMS is lifelong antimalarial prophylaxis. Signs and symptoms usually subside over 1 to 2 years, but relapse may occur if antimalarial therapy ceases. Splenectomy is contraindicated because it is technically difficult, with considerable intra-operative mortality, and it may predispose to severe infection (for review see Leoni et al.[104]).

Anemia and *P. vivax*, *P. ovale*, and *P. malariae* Infection

In *P. vivax* and *P. ovale* malaria, high parasitemias are rare because invasion of erythrocytes is limited to reticulocytes. However, there is an emerging consensus that *P. vivax* monoinfection may cause severe disease with cerebral malaria and/or anemia (for review, see Price et al.[105]). The vivax-infected RBC can adhere to host cells, but sequestration in the peripheral circulation and organ-specific syndromes of disease are much less common than in falciparum malaria.

P. vivax malaria has been associated with anemia during pregnancy and with low birth weight of children of affected mothers. Here cytokines or other inflammatory mediators appear to cause placental dysfunction.[99,105] *P. malariae* infection is also rarely fatal but is distinguished by the persistence of blood-stage parasites for up to 40 years. It can, however, cause a progressive and fatal nephrotic syndrome.[106]

Malaria Diagnosis

The diagnosis of malaria is based on the identification of circulating blood-stage parasites. The standard methods of preparing thick and thin films are straightforward and allow a simple method to diagnose infection (Table 154.1).[107–110] However, malaria diagnosis poses particular problems for inexperienced staff. The main biologic problem is that the level of circulating parasites is only weakly associated with the overall parasite burden because falciparum-infected erythrocytes

TABLE 154.1 Malaria Diagnosis

- Thick and thin films should be prepared for cases of suspected malaria.
- Immunochromatographic tests lack sensitivity to detect low levels of parasites that may be highly clinically significant.
- Routine Giemsa or May-Grünwald-Giemsa stains are unlikely to give satisfactory results because the pH is too low.
- Films can be stained with Giemsa or Leishman stain (thin films) or Field stain (thick films). (For details, see Bailey JW, Williams J, Bain BJ, et al. Guideline: the laboratory diagnosis of malaria. *Br J Haematol*. 2013;163[5]:573. doi: 10.1111/bjh.12572.)
- Two hundred high-power fields (×100 objectives) should be examined in a thick film.
- Asexual parasitemia should be reported after counting 1000 RBCs in a thin film. A graticule or grid may help counting.
- If parasitemia is less than 1:1000 RBCs, parasitemia may be counted in a thick film in relation to the number of WBCs.
- During active infection, a daily parasite count should be obtained.
- Counting and species determination of malaria parasites should be verified by a second observer.
- Reports on negative films despite a strong clinical suspicion of malaria should be qualified that negative films do not exclude a diagnosis of malaria. Repeat films should be requested if clinically appropriate. Thrombocytopenia may heighten suspicion of malaria.

RBC, Red blood cell; *WBC*, white blood cell.

are sequestered in capillary venules; thus the parasitemia is not a reliable guide to the severity of disease. Second, missing or delaying the diagnosis of falciparum malaria may result in serious morbidity and mortality. Finally, the most sensitive methods for diagnosis of infection, namely microscopy, require both skill and time.

In endemic areas, laboratory staff are skilled at the examination of thick films and routinely are able to detect 1 parasite in 100 high-power fields of a thick film, which corresponds to a sensitivity of approximately 5 to 50 parasites/μL.[107,111] Thin films are used for determining the species of the parasites, and the circulating asexual forms of the four main malaria species can be readily identified, whereas the sexual forms (gametocytes) of the species require some skill and regular practice (Figs. 154.5 and 154.6).

Nevertheless, diagnosis of malaria by microscopy in nonendemic countries has proven problematic. Routine laboratories may only achieve sensitivities of the order of 500 parasites/μL using thick films.[111,112] Quality assurance schemes show that performance of routine hematology laboratories in the recognition of and species determination of malaria parasites is poor.[109,112]

There has therefore been a strong drive to use nonmicroscopic methods for malaria diagnosis. Until recently, it has been the received wisdom that these methods are not sufficiently sensitive for clinical diagnosis but may have a role in detecting parasites of more than 500 parasites/μL when experienced staff are not available and/or as part of an out-of-hours service. However, advances in rapid antigen tests and in loop-mediated isothermal amplification (LAMP) amplification of deoxyribonucleic acid (DNA) have brought modern technology closer to an established role in the laboratory service and have enabled a significant change in practice where resources are limited.[113,114] Operationally, this means that hematology laboratories need to make the diagnosis of malaria and must maintain the skills needed for reliable examination of thick films.

A number of methods based on the fluorescent staining of parasite DNA and/or ribonucleic acid (RNA) and the concentration of parasites have been devised. However attractive these methods may appear, their sensitivity is limited by background staining of cellular debris, and the limit of their sensitivity is approximately 100 parasites/μL The time taken in preparing samples and the specialized equipment and skills needed to use these methods limit their effectiveness in routine practice (for review see Amir[115]).

Detection of circulating malarial antigens by rapid diagnostic tests (RDTs) is another potentially attractive alternative to the laborious method of screening blood films. There are over 200 different RDT brands that utilize three antigens, namely, Plasmodium histidine-rich protein 2 (PfHRP-2), Plasmodium lactate dehydrogenase (pLDH), and Plasmodium aldolase (pALDO). pfHRP-2 is produced exclusively by *P. falciparum* and the assay is very Pf sensitive, but an alternative antigen(s) is needed in areas where *P. vivax* is more common. The sensitivity of these immunochromatographic tests decreases with low parasitemia (<100 parasites/μL), genetic variability of the antigen(s) including deletion of PfHRP-2, and prozone effect when the parasitemia is over 4%.[113] The formulation of the tests using dipstick antigens allows rapid testing to be performed by laboratory staff. However, the sensitivity is variable and may range from 100 to 1000 parasites/μL, and this is comparable to the sensitivity achieved by inexperienced microscopists and may approach that achieved by experienced microscopists.

In lower- and middle-income countries, RDTs have allowed change in strategy from empiric treatment of a febrile child for malaria to parasitological diagnosis before therapy, with guidance to confirm RDT diagnoses with microscopy. WHO malaria treatment guidelines now recommend against routine presumptive treatment unless strong clinical suspicion of a severe disease without the opportunity for a timely laboratory diagnosis. The current recommendations for malaria diagnosis in the United Kingdom emphasize that the optimum diagnostic procedure is examination of thick and thin blood films by an expert to detect and speciate the malarial parasites. The sensitivity of RDTs is highest for *P. falciparum* and *P. vivax* malaria. However, RDTs for other *Plasmodium* species are not as reliable.[116]

Amplification of circulating parasite DNA using the repeated ribosomal RNA (rRNA) genes is an extremely sensitive method of malaria diagnosis.[117] The sensitivity may be as low as 0.005 parasites/μL or 5 parasites/mL.[118,119] Undoubtedly, this is a useful tool for reference laboratories and epidemiologic surveys. LAMP amplification of parasite DNA is more sensitive than RDTs but less sensitive than conventional polymerase chain reaction (PCR) at low-level parasitemia. LAMP tests require DNA extraction and some basic molecular techniques and so are unlikely to replace RDTs and microscopy in the point-of-care diagnosis for suspected cases of malaria but may be suitable for epidemiological surveys of asymptomatic infection.[114]

Management of Malaria

Malaria requires urgent effective chemotherapy to prevent progression of disease and may be the most crucial public health intervention to reduce global mortality from malaria. The drug treatment of malaria must take account of the expected pattern of drug resistance in the area where infection was contracted, the severity of clinical disease, and the species of parasite. The spread of drug-resistant parasites and the optimal use of affordable, effective drugs are of continual concern, and these have recently been reviewed (Table 154.2).[117,120,121]

Artemisinin-based combination treatments have been the mainstay of treatment for falciparum malaria in Southeast Asia for more than 15 years and are now recommended as first-line treatment throughout the rest of the world.[122] However, resistance to these drugs is emerging, and very young children (particularly those underweight for age), patients with high parasitemias, and patients in very low transmission intensity areas with emerging parasite resistance are at risk for treatment failure and should be monitored closely.[120,121] The hematologic side effects of antimalarial drugs in use today are few. Amodiaquine is associated with neutropenia and agranulocytosis. Artemisinin-based treatments may cause reticulocytopenia and hemolysis in approximately 10% to 15% of patients following intravenous artesunate treatment (see above).[84,85] High levels of circulating PfHRP-2 predict late hemolysis and memoglobin concentrations should be checked approximately 14 days following treatment in those treated with intravenous artemisinins.[85,117]

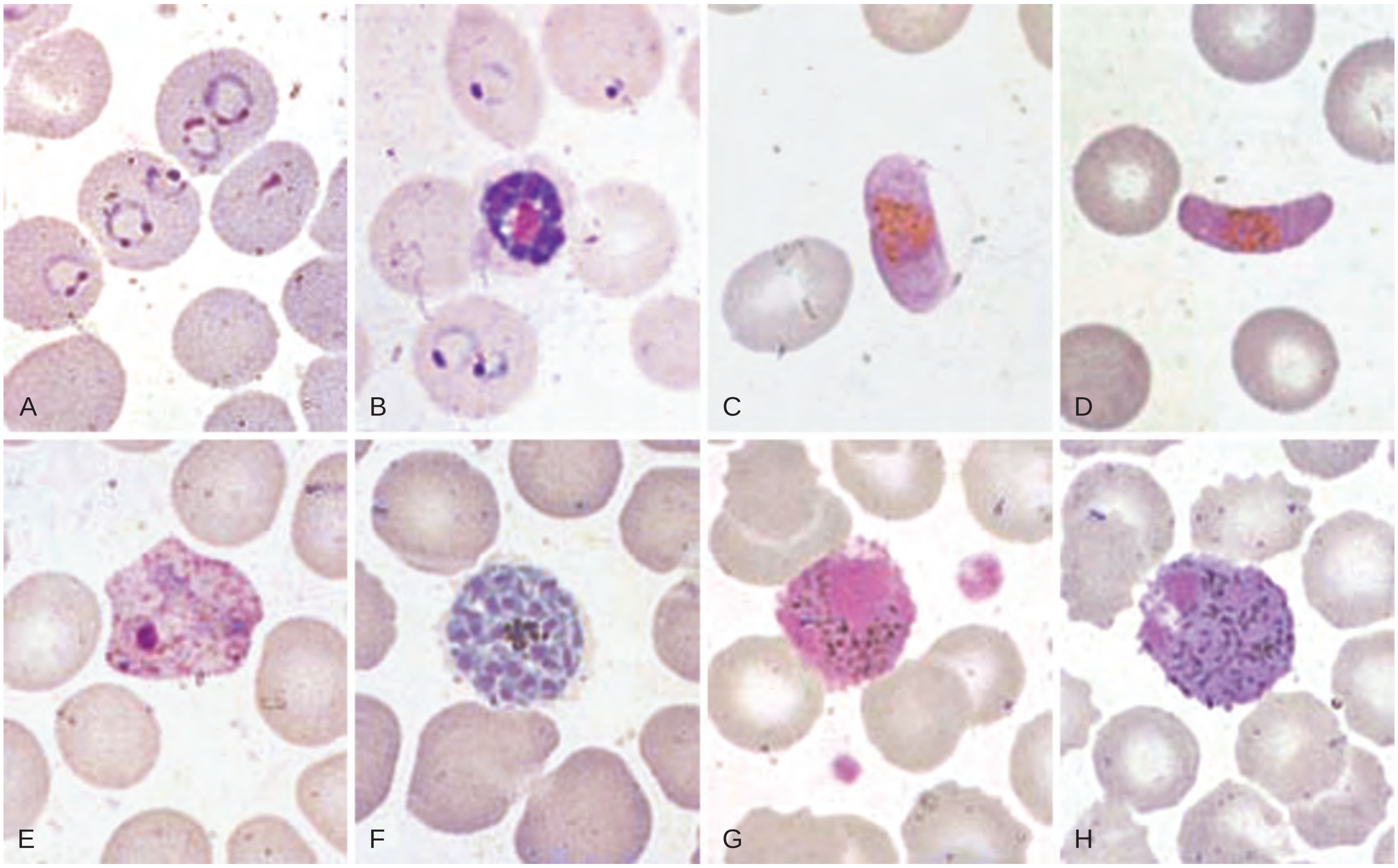


Figure 154.5 BLOOD-STAGE MALARIA (THIN BLOOD FILMS). *Plasmodium falciparum:* Fine rings (A) predominate, with mature trophozoites and schizonts (B) appearing uncommonly in the peripheral circulation because infected cells adhere to postcapillary venules. Host cells are not enlarged. Basophilic clefts and spots of irregular shape and size (Maurer clefts and dots) may be seen in erythrocytes containing more mature parasites. They are thought to be aggregates of parasite proteins that are being exported from the parasite to the surface of the red cell. Crescent-shaped male (C) and female (D) gametocytes are diagnostic. *Plasmodium vivax:* All stages of asexual parasites, from young trophozoites (E) to schizonts, appear in the peripheral circulation in vivax malaria together with gametocytes. The parasites are large and ameboid and produce schizonts with approximately 16 daughter cells (merozoites) (F). Pigment is well developed. Host red cells are enlarged and uniformly covered with fine eosinophilic stippling (Schüffner dots). Gametocytes are round, with the male (microgametocytes; G) being approximately 7 μm and the female (macrogametocytes; H) being 10 μm or more in diameter. *Continued*

In severely ill patients, good nursing care is vital. Monitoring and treatment of fits and hypoglycemia are essential, and antipyretics should be given. Certainly, blood transfusion is required for the treatment of severe malarial anemia,

A consensus has emerged following the TRACT trial (see box on Transfusion Management of Severe Anemia in African Children).[80,81] Children with uncomplicated severe anemia do not require immediate transfusion and should be monitored as half these children will develop severe and life-threatening anemia requiring subsequent transfusion. The most effective and safest volume of blood for transfusion for afebrile children is 30 mL/kg whole blood and for febrile children is 20 mL/kg whole blood. Longer-storage RBC units are not inferior to shorter-storage RBC units for tissue oxygenation as measured by reduction in blood lactate levels and improvement in cerebral tissue oxygen saturation among children with severe anemia.[82]

Whatever clinical guidelines emerge, in reality blood transfusion in the heartland of malaria-endemic areas is beset by many practical and theoretic problems. First, the absence of well-characterized donor panels (and thus systematic blood collection) frequently jeopardizes the supply of blood. Second, even when standard screening for human immunodeficiency virus (HIV) is in place, the residual risk for viral transmission in the serologic window of infectivity varies from country to country but recent estimates are that the risks remain for HIV at 1 in 3000 to 1 in 16,000, for hepatitis C 1:200 to 1:5000, and hepatitis B 1:400 to 1:2000.[123] At a practical level, positive indirect antiglobulin test results in the setting of acute infection may make the exclusion of alloantibodies difficult. Depending on the clinical urgency and transfusion history, the least serologically incompatible blood may have to be given.

One therapeutic option available in North America and in Europe for the urgent treatment of nonimmune patients with severe disease would be an exchange blood transfusion. This procedure removes non-sequestered, infected erythrocytes but there are no randomized trials supporting the use of exchange transfusion in malaria. Some experts have suggested that this treatment could be given for hyperparasitemia (>20%) in severely ill nonimmune patients, but there is doubt whether this rationale is still relevant when artesunate kills circulating, non-sequestered ring-stage infected erythrocytes.[44,124] The salient features that make this clinical problem a major public health concern are the very large numbers of children affected and the difficulty of satisfactory treatment by blood transfusion outside specialist centers.

Malaria as a Transfusion-Transmitted Infection

Malaria is undoubtedly the most common transfusion-transmitted infection in the world. In endemic areas a large proportion of adult donors will be parasitemic, perhaps 20% to 80%, depending on the rate of transmission. Here donor deferral is impractical, and treatment of recipients with a course of effective antimalarials is the most

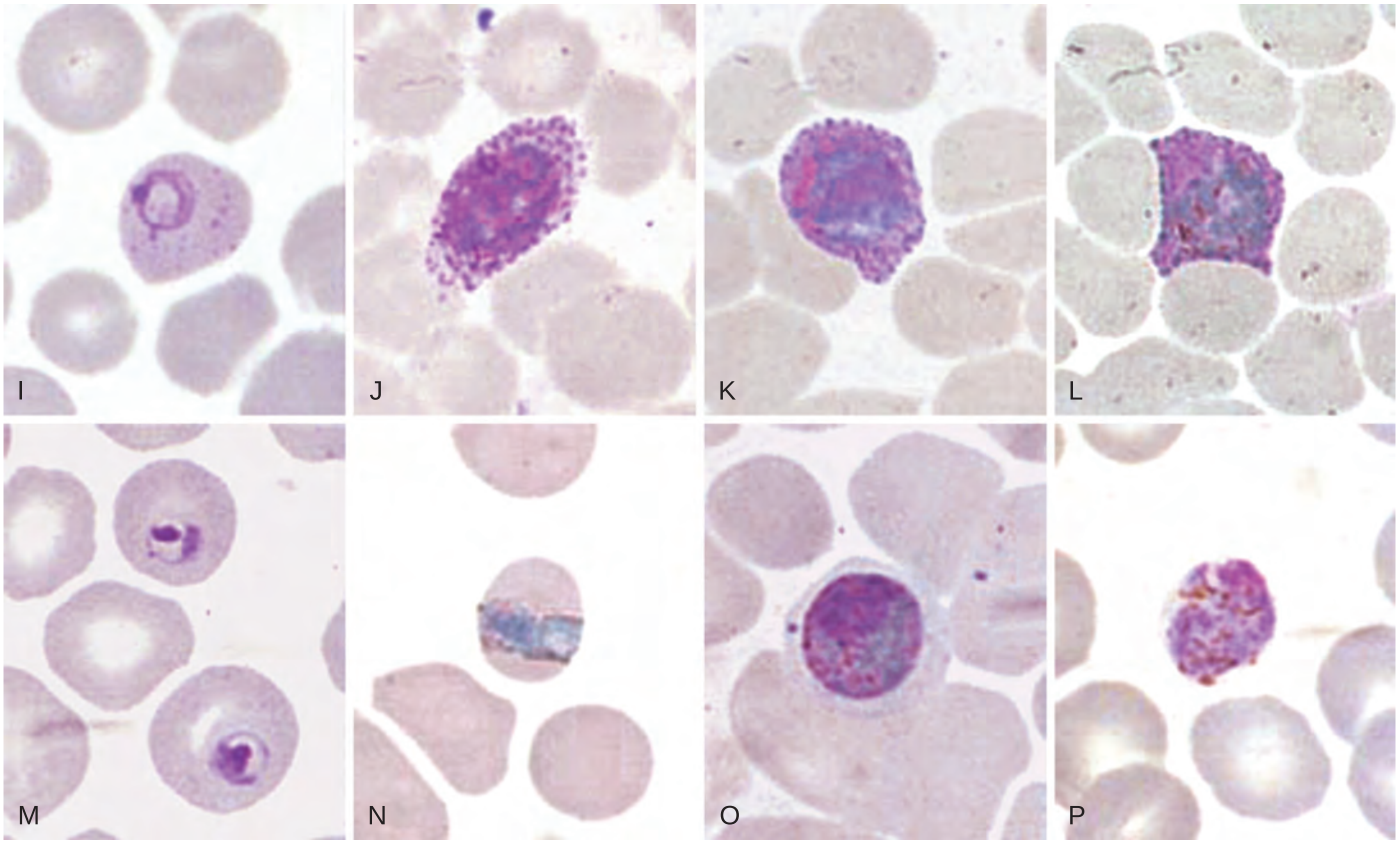


Figure 154.5 cont'd *Plasmodium ovale:* Intraerythrocytic ring forms (I) have a prominent nucleus. The older parasites (J) differ from *P. vivax* in being more compact and producing about eight merozoites at schizogony. Like *P. vivax*, the host red cells contain Schüffner dots and tend to be ovoid and fimbriated. Male (microgametocytes) and female (macrogametocytes) gametocytes (K and L) are smaller than those of *P. vivax. Plasmodium malariae:* All intraerythrocytic stages may appear in the peripheral circulation, from young trophozoites (M) to compact schizonts with eight merozoites. Band forms (N) stretching across the red cell are common. With special staining, a very fine stippling (Ziemann dots) is sometimes seen. Host red cells are not enlarged. Gametocytes, no larger than their host cells, are round and compact with distinct blackish pigment, being finer in the males (O), in which the nucleus is more diffuse and the cytoplasm somewhat mauvish, whereas the granules are fewer and larger in the female (P), which stains a bluer color.

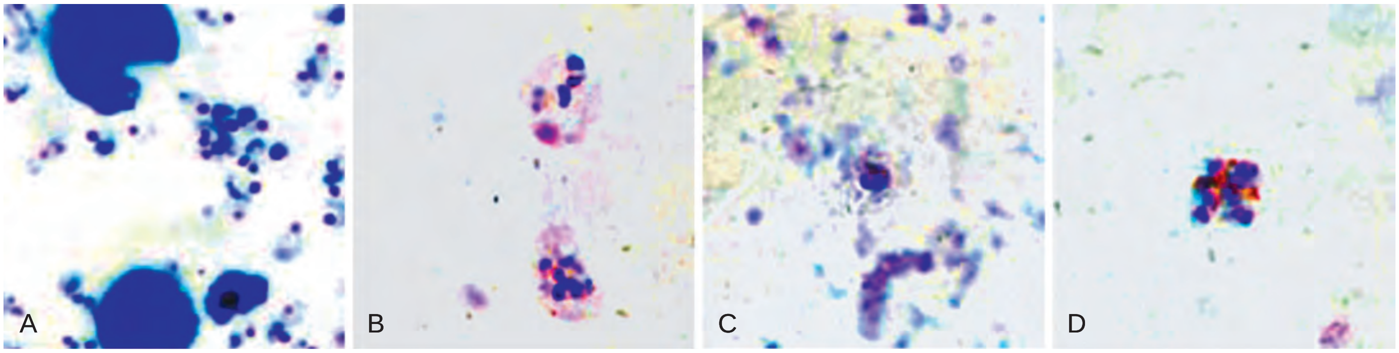


Figure 154.6 BLOOD-STAGE MALARIA (THICK BLOOD FILMS). *Plasmodium falciparum:* Usually only young rings (A) are seen in acute infections, although sometimes in very large numbers. *Plasmodium vivax:* All stages may be present; here two young trophozoites are seen (B), with Schüffner dots seen as "ghost cells" in the thinner parts of the film where the host cell has been hemolyzed. *Plasmodium ovale:* The Schüffner dots of *P. ovale* also may show up in a thick film as a ghost cell, but the parasite can be distinguished from *P. vivax* by the solid appearance and heavy pigment even of young trophozoites (C). *Plasmodium malariae:* Younger parasites can be recognized by their heavy pigment, but this may be so heavy that it obscures the other inner structures. Schizonts containing up to eight merozoites with a central mass of pigment (D) are characteristic. *P. malariae* is difficult to differentiate from *P. ovale* in thick films, in which the parasites are easily confused. However, unlike *P. malariae, P. ovale* may be seen in ghost cells (C).

practical alternative. In nonendemic areas, transmission of malaria is an occasional but potentially devastating complication of blood transfusion, and considerable thought and resources are required to combat the problem effectively.

The first case of transfusion-transmitted malaria (TTM) was in 1911.[125] Between 1911 and the mid-1970s the incidence of TTM rose to more than 140 cases per year, with *P. vivax* the most common species causing infection, although the proportion of cases from *P. falciparum* has steadily increased, perhaps reflecting the speed and destination of international travel. It is striking that the background problem, namely malaria in returned travelers, is much more common in the United Kingdom than in the United States, with the per

TABLE 154.2 **Commonly Used Antimalarial Drugs and Their Side Effects**

	Oral Dose	Parenteral Dose	Side Effects
Sulfadoxine-pyrimethamine	Sulfadoxine 25 mg/kg Pyrimethamine 1.25 mg/kg as a single dose (maximum 1500 mg sulfadoxine and 75 mg pyrimethamine)	For IM injection, doses as for oral	Use with caution in first trimester Causes kernicterus in neonates Skin rashes, fatal Stevens–Johnson syndrome
Artemether	3.2 mg/kg day 1 1.6 mg/kg days 2–7	3.2 mg/kg IM day 1, then 1.6 mg/kg for 3 days, then oral	Reticulocytopenia
Artesunate	4 mg/kg once daily for 7 days	2.4 mg/kg IM day 1, then 1.2 mg/kg for 3 days, then oral	Reticulocytopenia
Artemether/lumefantrine	Fixed-dose combination: artemether (20 mg) with lumefantrine (120 mg), adults give 4 tablets initially, followed by 5 further doses of 4 tablets each given at 8, 24, 36, 48, and 60 h (total 24 tablets over 60 h) If 5–15 kg, then 1 tablet initially followed by 5 further doses of 1 tablet each, given at 8, 24, 36, 48, and 60 h (total 6 tablets over 60 h); 15–25 kg, then 2 tablets initially, followed by 5 further doses of 2 tablets each given at 8, 24, 36, 48, and 60 h (total 12 tablets over 60 h); body weight 25–35 kg, 3 tablets initially, followed by 5 further doses of 3 tablets each given at 8, 24, 36, 48, and 60 h (total 18 tablets over 60 h)		
Quinine	10 mg/kg of salt (maximum 600 mg) every 8 h for 7 days, together with or followed by either doxycycline 200 mg once daily for 7 days or clindamycin 450 mg every 8 h for 7 days [unlicensed indication]	20 mg/kg in 10 mL/kg isotonic fluid over 4 h, then 10 mg/kg over 2 h given every 12 h in children and every 8 h in adults, together with or followed by either doxycycline 200 mg once daily for 7 days or clindamycin 450 mg every 8 h for 7 days [unlicensed indication]	Thrombocytopenia, intravascular hemolysis (blackwater fever) when used prophylactically, nausea, tinnitus, deafness

IM, Intramuscular.

capita incidence differing by nearly a factor of 10 and a higher proportion of cases from *P. falciparum* in Europe and the United Kingdom compared with the United States.

Recent experience in the United Kingdom and the United States has emphasized the seriousness of TTM. Two of the last five cases of malaria owing to blood transfusion in the United Kingdom were fatal.[126–128] In the United States, 14 cases of TTM were reported between 1990 and 1999, but only 5 cases were reported between 2000 and 2009.[129–131] Detecting these cases after transfusion is frequently delayed because malarial infection acquired in nonendemic countries rarely figures in immediate differential diagnosis and requires careful examination of the blood film.

The mainstay of preventing TTM in the United Kingdom is donor deferral backed up by detection of circulating antibodies to malaria antigens. The guidelines for donor deferral have recently been revised (Table 154.3).[132] These criteria recognize that malaria in the nonimmune patient is likely to present within four months of return from an endemic area and that significant immunity to falciparum malaria may be acquired by residence after 6 months in a malarious area.

The criteria also require that residents, as well as those having had malaria or an undiagnosed febrile illness, may be reinstated, after four months without malaria or a fever that could have been due to malaria or three years after treatment for an episode of malaria, if antimalarial antibodies cannot be detected. The importance of antimalarial antibody testing rests on the fact that it is a very sensitive method to detect chronic infection, whereas the identification of circulating malarial antigens or nucleic acids or microscopy would fail to detect a level of 1 parasite/mL, which would still give a highly infectious dose in a unit of blood.

TABLE 154.3 **UK Donor Selection Guidelines for Donors at Risk of Transmitting Malaria**

Donor Risk Category	Guidelines
Resident	Defined as having lived in sub-Saharan Africa (except South Africa) or Papua New Guinea for a continuous period of 6 months at any time of life Permanent deferral unless malaria antibody test results are negative at least 4 months after returning from a malarious area Any subsequent visits to any malarious area each require a 4-month deferral period and negative antibody test results before reinstatement
History of malaria	Permanent deferral unless malarial antibody test results are negative at least 3 years after cessation of treatment or last negative test results
Undiagnosed febrile illness	While abroad or within 4 weeks of return Deferral for 12 months or 4 months if malarial antibody test results are negative
All other risks	Deferral for 12 months or 4 months if malarial antibody test results are negative

The assays for antimalarial antibodies previously used indirect immunofluorescence antibody tests (IFATs) to detect reactivity to a crude parasite lysate as a target antigen. However, several enzyme-linked immunosorbent assays (ELISAs) using recombinant malarial antigens have proved to be a more practical if slightly less sensitive alternative to IFATs.[128,133,134] These tests detect antimalarial antibodies in less than 2% of donors who have visited endemic areas. It has been calculated that the return of 90% of malarial antibody–positive visitors to the donor pool releases an extra 50,000 units per year in the United Kingdom, and this is a highly cost-effective process to reduce the attrition of eligible blood donors. In the United States, over 200,000 donors per year are deferred after travel to malaria-endemic areas.

Donor deferral is based on the potential of a donor to carry malaria and is therefore based on the area of travel, length of stay or residence, elapsed time since leaving the endemic area, and history of malaria. It has been repeatedly shown that application of even the most thorough donor questionnaires allows some of those carrying malaria to give blood because guidelines are frequently incorrectly applied or questions are answered inaccurately in routine practice.[130]

In Canada donors reporting diagnosis or treatment of malaria defer permanently, and in the United States donors are deferred for 3 years after treatment.[130] These criteria and the respective regulations of other major international blood services will inevitably cause unnecessary deferral of those who never actually had malaria but also may permit some individuals with low-level chronic infection to donate because malaria may very occasionally present more than 3 years after travelers return from endemic areas.[129,130,135] The last case of malaria transmitted in the United Kingdom was by someone who had left a malarious area 8 years previously, and the longest recorded case of recrudescence of malarial infection is 44 years for *P. malariae*.

Permanent deferral of all those visiting malaria-endemic areas is unlikely to be a viable strategy to prevent TTM because donor bases are declining. Preventing malaria transmission through blood transfusion requires comprehensive, regularly reviewed, and effectively implemented guidelines for donor deferral and laboratory testing. Even the best strategy is a compromise, and medical laboratory staff should be aware of the rare but potentially serious possibility of fever after transfusion that could be caused by malaria.

VISCERAL LEISHMANIASIS

Leishmaniasis is a generic term for infection by 30 or so species of the obligate intracellular parasites from the genus *Leishmania*, and different species may cause systemic or visceral, mucocutaneous or cutaneous infection. Visceral leishmaniasis (VL), or kala-azar, presents with a wide spectrum of systemic and hematologic features.

Epidemiology

VL occurs in all countries bordering the Mediterranean Sea and across the Middle East, including Saudi Arabia and Yemen. Indian VL occurs in the eastern regions of India (particularly Assam, Bengal, Bihar, Uttar Pradesh, Tamil Nadu, and Sikkim) and in Nepal and Bangladesh. African kala-azar is endemic in Kenya, Ethiopia, and the Sudan and sporadically elsewhere in tropical Africa. In the Americas, VL occurs in foci across Mexico, Central America, Colombia, Venezuela, Guyana, Brazil, Bolivia, and northern Argentina (Fig. 154.7).

The total burden of disease is difficult to estimate but significant. Leishmaniasis burden is endemic in 88 countries, with 15,000 to 40,000 new cases of VL per year, with the vast majority occurring in India, Bangladesh, Nepal, Northern Sudan, and northeastern Brazil.[136] VL is associated with poverty and undernutrition in endemic areas, particularly in the hyperendemic foci of the southern part of the Sudan and the Ganges river basin. In the Mediterranean area, it is increasingly seen in association with HIV infection, where infection rates in HIV-infected people may be as high as 10%.

VL is caused by a number of species of the *Leishmania donovani* complex.[137,138] In the Mediterranean region and areas in the Middle East and Central Asia through to China, *Leishmania infantum* predominates, whereas *L. donovani* is more prevalent in India. *Leishmania tropica* is a less common cause of VL in these areas. Throughout their range in the Old World, parasites are transmitted by the female sandfly of the *Phlebotomus* genus. Leishmaniasis is caused by different

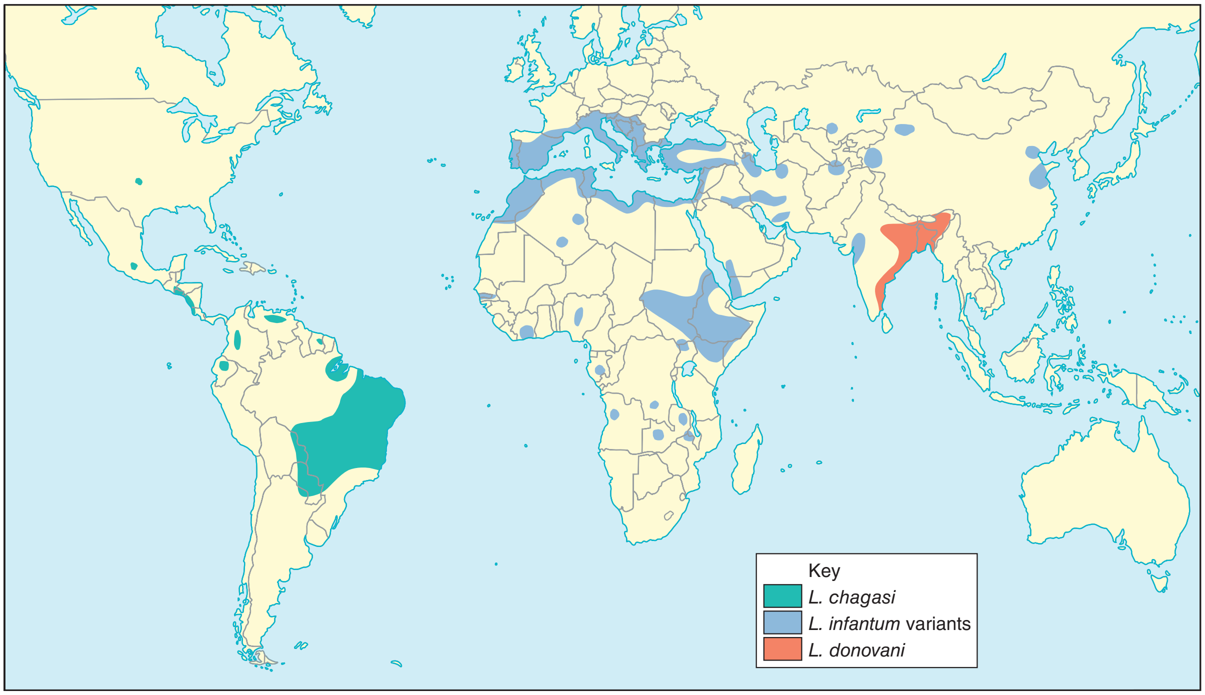


Figure 154.7 EPIDEMIOLOGY OF VISCERAL LEISHMANIASIS (VL). VL caused by parasites of the *Leishmania donovani–Leishmania infantum* complex occurs in the Mediterranean littorals, the Middle East, and adjacent parts of the former Soviet Union, the Sudan, East Africa, the Indian subcontinent and China, and South America *(Leishmania chagasi)*. An arid, warm environment provides ideal ecologic conditions for the breeding of many species of sandfly. Zoonotic kala-azar from *L. infantum* and *L. chagasi* is commonly associated with dry, rocky, hill country, where cases are typically scattered. In India, *L. donovani* is essentially an anthroponosis. This type of kala-azar may occur in severe epidemic fashion, as can kala-azar in the Sudan.

parasites and vectors in the New World, where *Leishmania chagasi* and *Leishmania amazonensis* are transmitted by the *Lutzomyia* genus of sandfly.

Leishmania organisms are present in blood, and so the disease can be transmitted by blood transfusion, as a sexually transmitted disease, as a congenital infection, by needle sharing for intravenous drug abuse, or within a laboratory by intradermal inoculation of *L. donovani* promastigotes.[139,140] Very few cases of leishmaniasis have occurred as transfusion-transmitted infections in Europe or North America, with under 10 cases in infants or immunocompromised patients reported over the past 50 years. In endemic areas, this problem represents a much greater but unquantifie risk.

Parasitology

Leishmania amastigotes live and multiply within macrophages by binary fission. They are round or ovoid bodies, approximately 2 to 3 µm in diameter. Occasional rupture of cells allows invasion of uninfected monocytes and macrophages by free forms. Sandflies ingest amastigotes within macrophages from blood or skin. In the insect's stomach, free amastigotes multiply and divide asexually, becoming elongated and developing flagella as metacyclic promastigotes. Within 2 weeks, such infective forms migrate through the lining of the stomach and enter the proboscis of the sandfly, allowing them to be inoculated in the human host while the sandfly takes a blood meal. Promastigotes are taken up by macrophages, where they become amastigotes by simple fission.

The large, elongated, fusiform promastigote measures 15 to 20 µm in length and 0.5 to 3.5 µm in width. Cultured promastigotes may also demonstrate rounded forms 4 to 5 µm in diameter. In Giemsa or other Romanowsky stains, a large nucleus and smaller, distinct rodlike kinetoplast are obvious. The organisms must be distinguished from *Histoplasma capsulatum*.

Pathology

Parasites spread within macrophages to local lymph nodes and then to the liver, spleen, and bone marrow. They are also present more widely, in particular in the gastrointestinal tract and epidermis. In the subclinical cases, a cellularly mediated immune response causes a granulomatous lesion and resolution of the infection. However, in clinical VL little if any inflammatory response to the rapidly extensive and expanding parasite-laden macrophages is seen. Where it shows, a granuloma develops at the site of the initial inoculation but may not be apparent at the time of presentation.

Features of Visceral Leishmaniasis

Clinical Features

The spectrum of clinical disease is wide, ranging from asymptomatic infection to acute or chronic illness. Perhaps only 1% to 3% of all infections are symptomatic, with an incubation period of 10 days to 10 years but typically between 3 and 6 months.

The high number of seropositive individuals in relation to clinical cases suggests that spontaneous cure without symptoms or with mild systemic symptoms and hepatosplenomegaly occurs in the majority of individuals. In a large series of children with VL in Brazil, the overall case fatality rate was 10%, and mucosal bleeding, jaundice, dyspnea, bacterial infections, and low neutrophil count of less than 500/mm^3 or low platelet count of less than 50,000/mm^3 were associated with a poor outcome.[141]

In endemic areas, a significant number of seropositive children, who do not develop classic VL, have a subclinical form of disease with malaise, fever, poor weight gain, intermittent cough, diarrhea, hepatomegaly, and variable splenomegaly. In these cases, leishmania were neither cultured nor seen in bone marrow aspirates.[142] There are also reports of VL presenting as mild, nonspecific illness with fever, fatigue, cough, and abdominal pain in army personnel returning from the Middle East. In these cases, *L. tropica* was isolated from bone marrow or lymph nodes.[143]

Patients with typical chronic VL often present with malaise and considerable fatigue and the slow onset of low-grade fever, anorexia, and weight loss. They usually have anemia, progressive and occasionally massive splenomegaly, hepatomegaly, lymphadenopathy, and hypergammaglobulinemia, with increasing skin pigmentation (hence the name *kala-azar* from the Hindi for "black sickness"). Patients may have jaundice, petechia, or purpura; heart murmurs; and edema. The size of the spleen is related to the length of infection and severity of the pancytopenia. In Africa, patients may have diffuse polymorphic papular lesions at presentation. Oral, nasal, nasopharyngeal, and laryngeal ulcers may occur during active disease or after treatment. The large spleen, often extending to the right iliac fossa, may be painful. Other features of acute disease include cough, epistaxis, and in some severe cases concurrent infection of the respiratory or gastrointestinal tracts and/or tuberculosis.

The typical features of VL may be preceded by bacteremia, bacterial infection, acute hepatitis, or Guillain-Barré syndrome. VL may be associated with hepatic necrosis, cholecystitis, or neuropathy. Occasionally, lymphadenopathy may occur without systemic features of the disease. Here histology shows noncaseating granulomas and amastigotes within macrophages. A more acute presentation in recent immigrants and visitors to endemic areas may include a high periodic fever similar to classic malaria, with malnutrition, bleeding, hepatitis, and/or acute renal failure (for review see Torres-Guerrero et al.[144]).

Immunocompromised Hosts

Coinfection with HIV and VL is now a well-recognized clinical entity in the Mediterranean region and undoubtedly occurs more widely[145–147] (for review, see van Griensven et al.[148] and Lindoso et al.[149]). VL occurs in the setting of late-stage acquired immunodeficiency syndrome (AIDS) with CD4$^+$ lymphocyte counts of less than 200×10^6/L. Patients present with fever, splenomegaly, and pancytopenia with frequent and sometimes atypical involvement of the gastrointestinal and respiratory systems and skin. The typical hematologic features of VL are sometimes absent, and serologic tests are often negative, although antibodies to 14- and 16-kDa *Leishmania* antigens may be detected by Western blot analysis.[150] However, organisms are plentiful in bone marrow and even in buffy coat preparations. Some patients may have few symptoms, and the diagnosis of VL in immunocompromised patients requires a high index of suspicion. Treatment is difficult, with poor responses to pentavalent antimony. Liposomal amphotericin is the drug of choice. However, relapses are common, and maintenance treatment is required.[148]

VL may also occur after transplant and after immunosuppressive treatments, including chemotherapy, rituximab, or other immunodeficiency states.[151] Although patients present with a typical combination of fever, pancytopenia, and splenomegaly, the diagnosis may be missed if not considered.

Hematologic Features

VL causes a moderate normocytic, normochromic anemia.[152] The pathogenesis of anemia is multifactorial and includes hemodilution, shortened RBC survival, and reduced erythropoiesis.[153,154] The plasma iron is low, with plentiful stored iron typical of the anemia of chronic disease.[155] One report has suggested that Epo levels are reduced compared with what would be expected for the degree of anemia.[156] Occasionally, folate deficiency secondary to malabsorption and/or increased cell turnover or coexistent iron deficiency are associated with macrocytic or microcytic anemia, respectively.[157]

The direct Coombs test results are usually positive for C3 components, and IgG and anti-I agglutinins may be present, but the presence and strength of the test is not correlated with the severity of the anemia. The positive direct Coombs test results appear to be caused

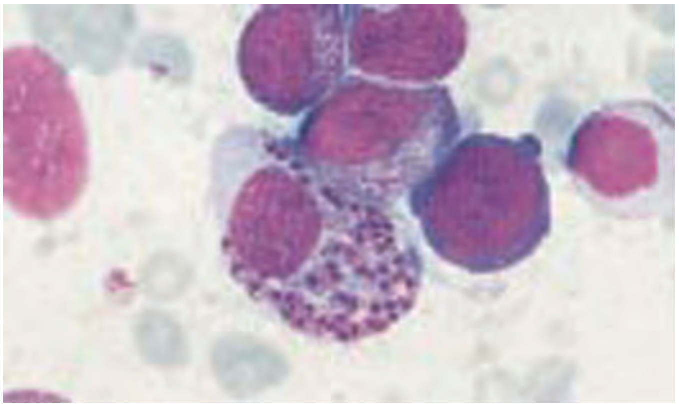


Figure 154.8 *LEISHMANIA INFANTUM* IN MACROPHAGE FROM BONE MARROW. Although typically found in macrophages as shown here, isolated extracellular amastigotes from disrupted host cells are commonly seen in such preparations.

by absorbed immune complexes onto erythrocytes.[158] The neutrophil survival is reduced, and the differential white blood cell count shows neutropenia, relative lymphocytosis, and low eosinophil levels, with a white blood cell count typically in the range 2 to 4 × 10^9 L.

Neutrophil function may be impaired.[159] Pancytopenia is more severe in those with concurrent HIV infection. A leukemoid reaction to infection has been reported.

Typically, the platelet count is reduced to 50 to 200 × 10^9/L as platelet survival is reduced. Mucosal bleeding may occur, but extensive purpura is uncommon.

The prothrombin time is usually mildly prolonged to 2 to 4 seconds longer than in control subjects, secondary to impaired liver function. Fibrinolytic activity is increased, and in advanced cases, fibrinogen levels may be reduced. Disseminated intravascular coagulation or vasculitis may occur.[152,159] Full coagulation screening is advisable if a splenic aspirate or bone marrow biopsy is planned.

The bone marrow is usually hypercellular, with increased erythroid, myeloid, and platelet precursors. Lymphocytes may be increased, and macrophages contain Leishman-Donovan bodies (Fig. 154.8).[160] If weight loss and malabsorption are extensive, the marrow may undergo gelatinous transformation.

The mechanisms of leukopenia and thrombocytopenia are multifactorial. There is evidence for reduced bone marrow production of granulocytes and platelets and also for their autoimmune destruction and/or increased splenic clearance. After treatment, hematologic recovery is slow, and full recovery may take many months.[155]

Laboratory Features

The epidemiologic context and clinical features can suggest a diagnosis of leishmaniasis. Hematologic and biochemical tests are nonspecific; biochemical tests may show mild elevation of bilirubin and transaminase levels and more commonly a raised level of alkaline phosphatase. Previously the nonspecific formol-gel test was used to indicate hypergammaglobulinemia. Diagnosis is confirmed by direct examination of parasites by microscopy, culture, or PCR (for detailed review, see Boelaert et al.[161] and Thakur et al.[162]).

Morphology

The pathognomonic amastigotes of *Leishmania* spp. can be found in a variety of sources. The diagnosis is often made by splenic puncture or by bone marrow aspiration where large numbers of amastigotes may be seen within macrophages. Amastigotes are stained using Giemsa or other Romanowsky stains and demonstrate a purple nucleus and the large anterior kinetoplast (see Fig. 154.8).

In non-immunocompromised patients with VL, splenic aspirates are positive in more than 95%, bone marrow aspirates positive in more than 85%, buffy coat macrophages positive in more than 70%, and lymph nodes positive in more than 60%. In practice, samples are taken from the least invasive sites first, although lymphadenopathy is not invariable.

Where coinfection of HIV and leishmania occurs, amastigotes are more reliably found in bone marrow than in peripheral blood and may also be demonstrated in biopsy specimens from a wide variety of sites. Biopsy material can be stained with polyclonal anti-*Leishmania* serum and indirect immunofluorescence. However, direct demonstration of parasites is less sensitive than culture or PCR.

Splenic Aspiration

Splenic aspiration is rarely performed in North America and Europe but is a common diagnostic procedure in endemic areas. Aspiration is contraindicated in the presence of a bleeding tendency, portal hypertension, or a splenic hydatid cyst. Splenic aspirates are obtained from the middle of the long axis of the spleen. After the skin is cleaned, a 21-gauge needle attached to a 5-mL syringe is inserted subcutaneously in line with the long axis of the spleen. The needle should be swiftly inserted to a depth of approximately 2 or 3 cm at an angle of roughly 45 degrees to the skin, applying negative pressure. The needle should be withdrawn immediately while maintaining negative pressure and taking only a few seconds for the entire procedure. The splenic tissue may be expelled from the needle using a small amount of air and by fixing the stain using standard procedures.

Culture

Amastigotes may be grown as motile promastigotes from aspirates on the classic Novy-MacNeal-Nicolle (NNN) medium or other suitable media at 22°C to 24°C, and populations double only every 2 to 3 days.[163,164] Inoculation of material into the susceptible golden hamster is no longer used to grow parasites.

Molecular Methods

The PCR may detect low levels of infection and has been used for diagnosis and monitoring of treatment as well as to determine the genotype of the parasites.[162,165] Blood from filter paper can be used to prepare DNA, but as for direct morphology, PCR from bone marrow samples is a more sensitive method to demonstrate parasites. Quantitative PCR may be particularly useful for monitoring treatment and relapse in HIV- and *Leishmania*-infected patients.[166,167] Isothermal DNA amplification methods are being applied to develop tests to be used where sophisticated equipment is not available.[168]

Serologic Tests

Serologic tests have to be interpreted in light of the clinical findings. The tests are useful to screen suspected cases or populations for VL. However, they are unreliable in immunocompromised patients, who frequently are seronegative in spite of patent infection. Conversely, those harboring a subclinical infection may be seropositive, but samples may fail to yield evidence of parasites. Finally, serologic findings are positive many years after treatment or self-cure.

An indirect IFAT or ELISA using freeze-dried *Leishmania* antigen has sensitivity and specificity greater than 95%. A DAT is also available with purified, freeze-dried antigen and is a highly sensitive and specific test.[169] The freeze-dried antigen is very stable even when stored in extreme conditions.

The ELISA and immunochromatographic tests using *Leishmania* recombinant K39 antigen are 100% sensitive and more than 98% specific in Asia.[169] The sensitivity and specificity of the DAT and K39 immunochromatographic test are broadly similar, although the K39 test was less sensitive in the Sudan than in Asia.[170,171] Alternative target antigens are being developed for rapid, point-of-care tests although PCR, qPCR, and LAMP may provide inexpensive and sensitive tests for the parasite.[167,172]

Treatment in patients without HIV coinfection may be monitored using urine antigen tests for detection of the K39 and K26 *Leishmania* antigens or by DNA-based methods.[167] These test results become negative 3 weeks after commencing treatment.

Management

General supportive care is important and includes correction of nutritional and hematinic deficiencies, blood transfusion in severe anemia, and antibiotics for secondary infections (for review of diagnosis, therapy, and management, see report from the World Health Organization[172]).

Specific chemotherapy for VL uses a number of potentially toxic drugs. Intercurrent infections must be identified and treated, and general measures include improvement of nutritional status (for review of treatment, see Copeland et al.[173] and Sundar et al.[174]). Use of pentavalent antimonials, including sodium stibogluconate (Pentostam) and meglumine antimonate (Glucantime), are now limited, especially in South Asia, by increasing drug resistance, but they remain effective in East Africa. There is concern that drug resistance will develop to the alternative therapies, and combination treatment aiming at short courses that also delay the development of drug resistance are in progress.

Amphotericin B is effective against *Leishmania* and is used in endemic areas where high levels of resistance to pentavalent antimonials are present. It may be given as a slow intravenous infusion in 5% dextrose over 4 to 6 hours, beginning at 250 μg/kg/day, increasing to 1 mg/kg/day until a total dose of 20 mg/kg/day has been given. Side effects include anaphylaxis, anemia, fever, bone chills, bone pain, and thrombophlebitis. Hypokalemia and hypomagnesemia are significant problems, and potassium loss and supplementation may be reduced by the concurrent use of amiloride to prevent renal tubular potassium leakage.[175]

Reduced toxicity from amphotericin is achieved using a liposomal amphotericin preparation (AmBisome), which has been made more widely available through preferential pricing in endemic areas. It is an effective and safe treatment and is used when affordable. A test dose of 1 mg should be given, and then the recommended dose is 1 to 3 mg/kg daily for 10 to 21 days to a cumulative dose of 21 to 30 mg/kg, alternatively 3 mg/kg for 5 consecutive days, followed by 3 mg/kg after 6 days for 1 dose. In patients with HIV, a dose of liposomal amphotericin at 4 mg/kg/day on days 1 to 5 followed by 4 mg/kg/day on days 10, 17, 24, 31, and 38 is recommended. The relapse rate is high, and close monitoring of patients is required.

Miltefosine was developed in India and is effective orally against VL in India and Africa.[176,177] The current regime is 2.5 mg/kg/day for adults and children older than 2 years for 4 weeks. Higher than 95% cure rates have been achieved. Gastrointestinal upset occurs frequently. Miltefosine is teratogenic and may reduce fertility. It has a long half-life and has a low therapeutic index, which may contribute to the development of resistance, and so combination chemotherapy is being investigated to combat development of resistance.

Intramuscular paromomycin has been used at a dose of 20 mg/kg/day for 21 days. It has been shown to be equivalent to amphotericin B for the treatment of VL in India.[178] Moreover, patients treated for VL in Africa with sodium stibogluconate and paromomycin have an improved outcome compared with therapy with sodium stibogluconate alone.[179]

Post–kala-azar dermal leishmaniasis may occur in up to 10% of patients after treatment for VL. It is seen mainly in India and is common in Africa. It occurs after treatment of VL and may appear up to 10 years after treatment. It presents as a maculopapular rash spreading around the mouth, trunk, and limbs. These nodules and papules contain amastigotes. Prolonged treatment may be required to eliminate infection.

Leishmaniasis as a Transfusion-Transmitted Infection

Leishmaniasis poses problems as a potential transfusion-transmitted infection in endemic areas. The disease can be transmitted by blood and platelet concentrates, although leukodepletion by filtration reduces organisms by many orders of magnitude and so probably also the risk for transmission. In Europe, given the low absolute risk for transmission, donor deferral is used to reduce the risk for donation by anyone who is parasitemic.[180] However, surveys of blood donors in areas where the parasite is transmitted suggest parasite DNA can be detected in 0.3% to 1.75% of donors. It has been noted that service personnel returning from the Eastern Mediterranean represent a group of potential donors who may have been exposed to infection.[181]

AFRICAN TRYPANOSOMIASIS

African trypanosomiasis is caused by the flagellate protozoa *Trypanosoma brucei* spp., named after David Bruce, a Scottish parasitologist who first demonstrated the parasite in the blood of cattle affected by nagana. In humans, Aldo Castellani demonstrated trypanosomes in the cerebrospinal fluid (CSF) of patients with sleeping sickness. Bruce was able to show the protozoa in blood and demonstrated that they were transmitted from antelopes to cattle by the tsetse fly and blood of patients with sleeping sickness.

Epidemiology

Trypanosomes are transmitted by some species of the large and distinctive tsetse flies (*Glossina* spp.) in a sporadic distribution south of the Sahara and north of the Zambezi River. Of the three subspecies, *Trypanosoma brucei brucei* infects animals, whereas *Trypanosoma brucei gambiense* causes infection and sleeping sickness in Central and West Africa, and *Trypanosoma brucei rhodesiense* causes disease in East and Central Africa.[182] *T. b. gambiense* is transmitted mainly between humans by the *Glossina palpalis* group (tsetse fly) in foci along watercourses in West and Central Africa. *T. b. rhodesiense* is transmitted by the *Glossina morsitans* groups widely distributed in the East African savannah. Here the disease is clearly a zoonosis, with antelopes and domestic cattle as reservoir hosts; it afflicts mainly hunters, guides, and game wardens and may occasionally occur in tourists on safari in the game parks of East Africa (Fig. 154.9).

Historically the disease has caused major epidemics, causing hundreds of thousands of deaths, and has prevented human settlement in large areas of Africa. Over 50 million people are now at risk for trypanosomiasis, and it has been estimated that the disease causes over 50,000 deaths each year. At present, over 90% of clinical cases in Africa are caused by *T. b. gambiense*.

Most cases are reported in the Democratic Republic of the Congo and northwestern Uganda, with a few hundred or fewer each year reported in neighboring East African countries.

Case-finding and control measures have substantially reduced the risk for epidemics. Epidemics of disease have been associated with a breakdown in health services, for example, in villages in the areas of Lake Victoria in Uganda and more recently in the Democratic Republic of the Congo, Angola, and southern Sudan (for review of current epidemiologic and clinical aspects of this disease, see Simarro et al.[182] and Kennedy and Rogers[183]).

Parasitology

The small, mobile trypomastigotes circulate and may be seen in peripheral blood. These flattened, fusiform organisms are pleomorphic but are typically of a size similar to an RBC, approximately 20 μm long, and have an undulating membrane, attached to the protruding flagellum, extending from the anterior pole of the elongated body

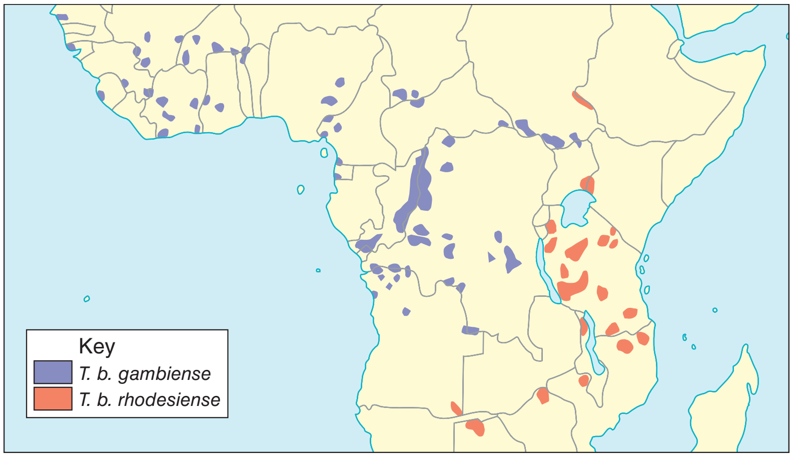


Figure 154.9 DISTRIBUTION OF *TRYPANOSOMA BRUCEI* INFECTION IN HUMANS. African trypanosomiasis is confined to equatorial Africa, with a patchy distribution depending on detailed topographic conditions. It is caused by two subspecies of *T. brucei*: *T. b. gambiense* infection is widespread in West and Central Africa, transmitted mainly by riverine species of tsetse fly *(Glossina)*, but *T. b. rhodesiense*, transmitted mostly by savannah species, is restricted to the eastern and east-central areas of Africa, with some overlap between the two. Although domestic pigs form an important reservoir for *T. b. gambiense* infection, various wild ruminants are the major sources for *T. b. rhodesiense*. The epidemic reemergence of African trypanosomiasis in recent years is exemplified by the death of at least 96 people in Angola in 2003, when 3115 cases were confirmed among a suspected 270,000 new cases in that country. In the same year it was estimated that some 500,000 people across Africa had trypanosomiasis, which was likely to have a mortality rate of approximately 80%. Recent reports indicate a spread of transmission from the northern toward the southern parts of Angola. *(Modified from World Health Organization Map No. 98005.)*

(Fig. 154.10). Parasites multiply by binary fission and may cause chronic parasitemia and evade humoral immune responses by clonal antigenic variations of the major surface glycoprotein.[184]

When trypomastigotes are taken up in a blood meal by the tsetse fly, they multiply in the midgut by simple fission. Later they penetrate the wall of the gut and migrate to the salivary glands. There, as morphologically distinct epimastigotes, they become infective (or metacyclic) trypomastigotes 15 to 30 days after first infecting the fly. In endemic areas, up to 1% to 5% of flies may be infected. Flies remain infective until they die, several months later.

Pathology

The lymphatic, cardiac, and central nervous systems (CNSs) are involved by the disease. Initially, proliferation of trypanosomes within lymph nodes and the spleen is accompanied by expansion of the lymphocytes, macrophages, and erythrophagocytosis. Polyclonal activation of lymphocytes results in high production of IgM and rheumatoid factor, and anti-DNA antibodies may appear. Later fibrosis and endarteritis supervene with proliferation of endothelial cells and perivascular infiltration of plasma cells and lymphocytes. The liver shows infiltration of the portal tracts and fatty degeneration. In *T. b. rhodesiense*, cardiac involvement may be extensive, with endocarditis, myocarditis, and pericarditis leading to extensive damage and death.

In the second stage of the disease, trypanosomes multiply within the CNS and cause a chronic meningoencephalitis. Edema, hemorrhages, granulomatous lesions, and thrombosis contribute to cerebral degeneration. Lymphocytosis and plasma cells with large eosinophilic inclusions (Mott cells) may be found in the CNS.

Intriguingly, nonpathogenic species of African trypanosomes may be killed by the human high-density lipoprotein particles astonishingly subverting a parasite pathway for heme uptake. The complex of haptoglobin-related protein and apolipoprotein L1 (ApoL1) are taken up into the parasite by a parasite glycoprotein receptor, which binds the haptoglobin–Hb complex.[185,186] *T. b. rhodesiense* is resistant to killing by human sera because the ApoL1, which induces parasite apoptosis, is neutralized in the lysosome by serum resistance–associated

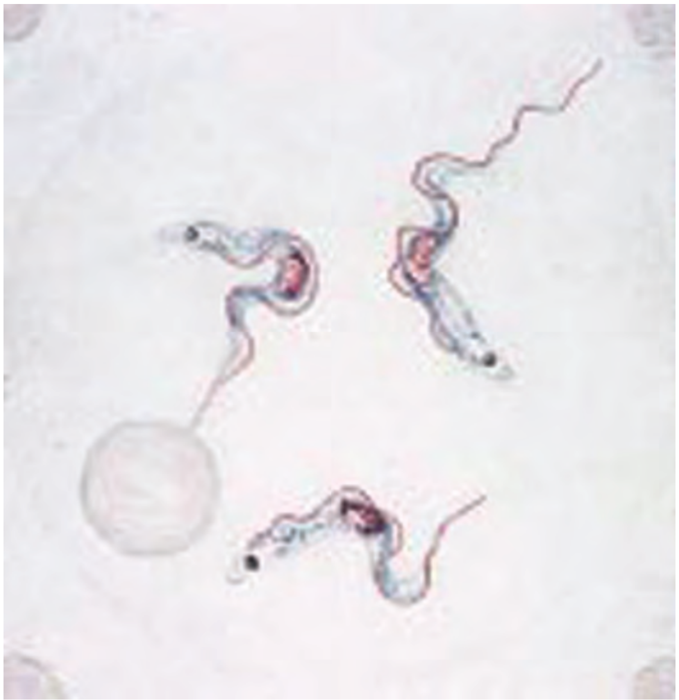


Figure 154.10 ORIGINAL MICRO-PHOTOGRAPH OF *TRYPANOSOMA (TRYPANOZOON) BRUCEI GAMBIENSE* IN HUMAN BLOOD BY J. EVERETT DUTTON. Polymorphic trypanosomes were first discovered more than a century ago, in 1895, by David Bruce in the blood of domestic cattle with the wasting disease nagana in South Africa. The first observation of these protozoa in humans was made by R. M. Forde, who noted, in 1902, "small worm-like, extremely active bodies" in the blood of a sick European seaman in The Gambia. The parasites seen here in a Romanowsky-stained thin blood film, which were described and named by Dutton in 1902, are responsible for sleeping sickness in West Africa. *(Reproduced by kind permission of the Director, Liverpool School of Tropical Medicine.)*

protein, which binds to a specific ApoL1 domain.[187] The selection of trypanolytic ApoL1 variants have a cost. In African Americans, focal segmental glomerulosclerosis and hypertension-attributed end-stage kidney disease are associated with two independent sequence variants in the *APOL1* gene.[188]

Features of African Trypanosomiasis

Clinical Features

Trypanosoma brucei gambiense

Local inflammation at the site of inoculation causes a distinctive chancre or painful, indurated ulcer appearing 2 to 3 days after an infective bite and lasting for up to 1 month. Trypanosomes multiply in the lymphatic system for 6 to 14 days before causing patent infection in the blood, characterized by waves of parasitemia and fever. Later, invasion of the CNS may occur by transit of organisms through the choroid plexus and/or endothelial cells.

In the blood stage of infection, fever, headache, and arthralgia are prominent. Lymphadenopathy is common, particularly in the posterior triangle of the neck (Winterbottom sign). There may be intermittent rashes (often circinate erythema), pruritus, or edema. Moderate splenomegaly occurs in 10% to 20% of patients. A few infected patients are asymptomatic.

This early stage of infection may last for up to 2 years or more before CNS involvement, although rapidly progressive disease may occur. The CNS phase of disease is marked by the progressive onset of headache; disinhibited behavior changes; and mood, thought, and sleep disturbances. Wasting is prominent. Signs of diffuse CNS damage in the last phases of the disease include dementia, extrapyramidal and cerebellar dysfunction, and hyperesthesia. Tendon reflexes are increased, and signs of upper motor neuron lesions are widespread. Death usually occurs by intercurrent infection.

Trypanosoma brucei rhodesiense

T. b. rhodesiense is more virulent, causing a more serious acute disease, with systemic features including serous effusions; hepatocellular jaundice; and a mild, normocytic, normochromic anemia. Hepatosplenomegaly and lymphadenopathy are common, but involvement of cervical lymph nodes is less typical. Myocarditis is rare but may cause death before CNS involvement. Here CNS disease occurs sooner and is more rapidly progressive than *T. b. gambiense* infection and is fatal within 1 to 3 months.

Infection with *T. b. gambiense* may resolve in the vast majority of cases. Where infection persists, there is typically a long asymptomatic phase. Numerous lymph nodes are enlarged to 1 to 2 cm in diameter and are soft, mobile, and painless.

The differential diagnosis of trypanosomiasis includes malaria, typhoid, fever, and viral hepatitis in the acute phase, whereas lymphadenopathy may suggest infectious mononucleosis or tuberculosis. In the cerebral phase of the disease, syphilis, tuberculosis, HIV-associated cryptococcal meningitis, or chronic viral encephalitis must be considered.

Children

Those affected in utero may be born with CNS abnormalities. In children the disease is more rapidly progressive, and epileptic seizures and psychomotor retardation are the principal features of the disease.

HIV Infection and Other Immunosuppressed Patients

The frequency of HIV seropositivity has been reported to show no significant differences between those presenting with trypanosomiasis and age-matched control subjects.[189] However, there are case reports of human African trypanosomiasis presenting in immunocompromised patients in America, many years after possible initial infection in Africa.[190,191] These cases suggest that a long phase of tolerance to trypanosomes, lasting over 20 years, may be followed by clinical relapse during immunosuppressive therapy or disease.

Hematologic and Laboratory Features

The main hematologic features are a normocytic, normochromic anemia and moderate thrombocytopenia.[192] The anemia of trypanosomiasis is multifactorial. Hemodilution, hemolysis, and dyserythropoiesis or ineffective erythropoiesis all contribute to the pathogenesis. Hemolysis may be caused directly by lytic factors released by trypanosomes or indirectly by deposition of immune complexes and subsequent clearance of coated erythrocytes. In vitro studies have suggested that the variant surface glycoprotein from trypanosomes may be shed and taken up onto the erythrocyte membrane, where antibody and complement deposition could contribute to hemolysis. Bone marrow response is inadequate, and although the nature of the defect is unclear, there is failure in incorporation of iron into erythroid precursors, as is seen in many forms of the anemia of chronic disease. In mice infected with human trypanosomiasis, TNF-α contributes to bone marrow suppression, but detailed studies of the pathogenesis of anemia in human infections have not been reported. Serum IgM levels may be elevated to four times normal and with raised IgG levels may cause an increased erythrocyte sedimentation rate.

The white blood cell count may be elevated with monocytosis, lymphocytosis, and circulating plasma cells, but eosinophilia is absent. Plasma cells with eosinophilic inclusion or Mott morular cells may occasionally be seen.

Petechia and purpura may be secondary to vascular injury, thrombocytopenia, and a complex but poorly defined coagulopathy. Abnormal liver function may cause prolonged clotting times. Functionally, both thrombosis and fibrinolysis are increased, and fibrin degradation products (D-dimers) are raised in acute disease; in *T. b. rhodesiense*, a frank disseminated intravascular coagulation may become evident.[193]

Examination of the bone marrow shows hypercellularity. Gelatinous degeneration may be seen in patients with wasting.

Mononuclear cells are present in the CSF late in the disease, and the cell count corresponds to the degree of neurologic involvement and may reach more than 300/μL in severely ill patients. The CSF protein levels are elevated (0.4 to 1.0 g/L).

As disease progresses, the circulating parasites become scarce. However, anemia and endocrine dysfunction, including amenorrhea, reduced libido, and impotence, becomes apparent.

Parasitologic Assays

Direct detection of the parasite is particularly important in light of the toxicity of the treatment. Trypanosomes may be detected in lymph node aspirates and in wet and thick blood smears (Fig. 154.11). An aspirate may be taken from enlarged nodes. Aspirated fluid can be examined under a coverslip, and motile trypanosomes are typically seen at the edges of the coverslip.

Trypanosomes can be seen in wet smears, where a drop of blood is placed on a slide and examined under a coverslip. Alternatively, thick or thin Giemsa-stained blood films are made as for malaria diagnosis. The sensitivity may be increased by microcentrifugation.[194]

More recent methods used to detect the low levels of parasitemia seen in *T. b. gambiense* include quantitative examination of the buffy coat using acridine orange[195] and small-scale ion-anion exchange chromatography.[196] Erythrocytes are retained in the column, whereas trypanosomes are eluted and are visible after concentration by centrifugation. The sensitivity of detection with these methods ranges from 10^4/mL for wet smears to 10^2/mL for ion-exchange columns and centrifugation. In *T. b. rhodesiense*, examination of the blood is more likely than aspiration of lymph nodes to yield a positive result (for a review of diagnostic methods using microscopy, see Chappuis et al.[197]).

Inoculation of the aspirate or samples into susceptible animals or in vitro culture has been used to detect low-level parasitemia. Mice and rats were used to detect *T. b. rhodesiense*, whereas the multimammate

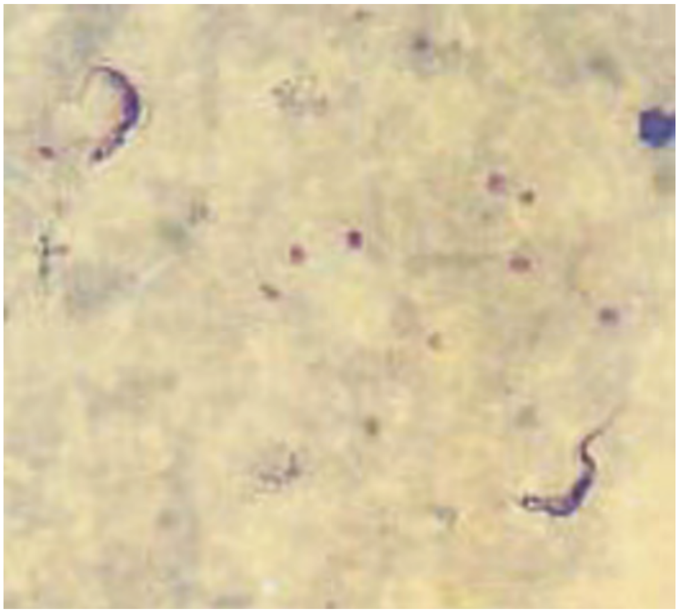


Figure 154.11 *TRYPANOSOMA BRUCEI RHODESIENSE* IN HUMAN BLOOD. *T. brucei brucei* parasitizes wild and domestic animals but does not infect humans. The different subspecies can be distinguished with certainty only by biochemical techniques, such as electrophoretic typing of their isoenzymes or by the use of DNA probes. *T. brucei gambiense* and *T. b. rhodesiense* (and *T. b. brucei* of animals) are virtually indistinguishable in blood films. Note the small kinetoplast and free flagellum. Both subspecies from humans will infect guinea pigs, but only *T. b. rhodesiense* is infective to rats, in which the parasites are polymorphic—that is, long, thin, intermediate, and short, stumpy forms of trypomastigotes may coincide.

rat *(Mastomys natalensis)* and guinea pigs were used for *T. b. gambiense*. PCR of the 18 S rRNA gene can detect parasites with sensitivity similar to that of parasitologic and serologic methods.[198,199]

Cerebrospinal Fluid

Trypanosomes may be found in the CSF as disease progresses, although a double-centrifugation technique may be required to demonstrate organisms. In this technique, 5 to 10 mL of CSF is centrifuged, and the sediment is taken up into a capillary tube and recentrifuged before the capillary tube is examined under a coverslip (Fig. 154.12).[200] Specific molecular markers for this stage of CNS disease are being sought by proteomic analysis of the CSF.

Serology

Serologic tests are used for passive population screening in control programs (for review, see Chappuis et al.[197]). A Card Agglutination Test for Trypanosomiasis (CATT) is robust and can be used without extensive laboratory facilities.[201,202] The test contains freeze-dried trypanosomes with the LiTat 1.3 variant antigens and can be obtained from the Institute of Tropical Medicine Antwerp (www.itg.be). It is quite sensitive (>95%), but it lacks specificity, owing to cross-reactivities with animal *Trypanosoma* spp. Although useful as a patient screening test in a hospital for suspected cases of trypanosomiasis, the predictive value for positive test results falls when screening populations for active cases. It has nevertheless been reported to double the number of active cases found. Some patients may have false-negative results for this test if they are infected by parasites that do not express this variant antigen. Further versions of this assay have been developed to use dried blood on filter paper, namely the micro-CATT and macro-CATT, but these are less sensitive than the whole-blood assay.

In *T. b. gambiense*, serologic tests are not widely available, and direct examination of blood and CSF for parasites is the first line of investigation. The card indirect agglutination trypanosomiasis test (*Tryp*Tect CIATT) can detect circulating antigens in *T. b. gambiense* and *T. b. rhodesiense* infection by latex agglutination. The sensitivities of the test are 95.8% for *T. b. gambiense* and 97.7% for *T. b. rhodesiense*, and therefore significantly higher than that of lymph node puncture, microhematocrit centrifugation, and CSF examination after single- and double-centrifugation.[203]

A rapid latex agglutination test (LATEX/*T. b. gambiense*) contains a mixture of three variable surface antigens of the bloodstream form of trypanosomes and has been used to detect antibodies in patients infected with *T. b. gambiense*. At 1:16 serum dilution, test specificity was 99%, whereas sensitivity ranged from 83.8% to 100%, depending on the geographic origin of the samples. The test sensitivity falls to 66% for CSF samples from second-stage patients.[204] A rapid latex agglutination test, LATEX/IgM, for the semiquantitative detection of IgM in CSF is available.[205] Finally, immunofluorescence assays and ELISA tests for antibodies using whole parasites are highly sensitive and specific, although they are less practical for mass screening. A series of new approaches is being evaluated for control and elimination of trypanosomiasis, including tiny targets for tsetse fly control, use of RDTs, and oral treatment with fexinidazole or oxaboroles.[206]

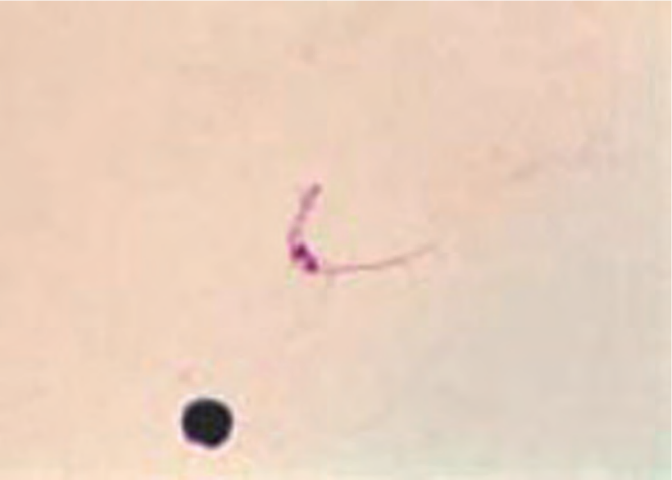


Figure 154.12 TRYPOMASTIGOTE OF *TRYPANOSOMA BRUCEI BRUCEI* IN CEREBROSPINAL FLUID. A single organism is seen in this sample taken from cerebrospinal fluid filtered in a “minicolumn.”

Management

Treatment of trypanosomiasis depends on the subspecies of trypanosome present. The drugs may be difficult to source and are toxic, so treatment requires expert help.

Early-stage (stage 1) *T. b. gambiense* may be treated with pentamidine for 7 days (more usually intramuscularly, 4 mg/kg/day). Hematologic side effects include neutropenia, and more general side effects include hypotension, hypercalcemia, hyperkalemia, renal failure, and hyperglycemia. Pentamidine will not cure CNS disease, and so examination of the CSF is required after initial treatment of the blood-stage disease.

Stage 2 *T. b. gambiense* is treated with a combination of intravenous eflornithine (α-difluoromethylornithine) and oral nifurtimox (NECT) given as 200 mg/kg of eflornithine as an intravenous infusion over 1 to 2 hours every 12 hours for 7 days combined with nifurtimox at 5 mg/kg orally every 8 hours for 10 days.[207] This combination caused less neutropenia, is less toxic, and has fewer relapses than monotherapy with eflornithine.

Melarsoprol is a trivalent organic arsenical compound and is more toxic but cheaper than eflornithine. It is used in Africa for the treatment of late-stage CNS trypanosomiasis. It is given by slow intravenous bolus injections at a dose of 2.2 mg/kg/ day for 10 days.[208]

Melarsoprol causes a secondary encephalopathy in 5% to 10% of treated cases and is fatal in half of these.[209] The incidence and severity of encephalopathy may be reduced by concurrent administration of prednisolone (1 mg/kg/day up to 40 mg) started 1 to 2 days before melarsoprol treatment.[210] When steroids are used, it is necessary to give patients antimalarial and antihelminthic therapy, especially if *Strongyloides stercoralis* is present.

Prior treatment of blood-stage disease with pentamidine may reduce antigen levels and possibly adverse events. Promethazine, anticonvulsants, and antiemetics are important adjunctive treatments before commencing CNS therapy.

Polyneuropathy occurs in 10% of cases treated with melarsoprol and may be severe, causing quadriplegia. If neuropathy is suspected, melarsoprol should be stopped immediately and thiamine (100 mg, three times daily) given until symptoms subside.

Patients require prolonged specialist follow-up over 2 years, including blood and CSF examination for parasites. A rising CSF leukocyte count is a good guide to CSF relapse even in the absence of a demonstration of parasites.[211]

Suramin is effective against the first stage of both forms of the disease. It is generally used for treatment of *T. b. rhodesiense* because it is active against onchocerciasis which is more prevalent in *gambiense* HAT endemic areas and it can cause severe immunological reactions in co-infected patients.[212] In treatment for early-stage *T. b. rhodesiense*, suramin is given as five intravenous injections (20 mg/kg, up to 1.5 g) on days 1, 3, 6, 14, and 21. Adverse effects include fever, proteinuria, paresthesia, pruritus, and urticaria. Hemolytic anemia, agranulocytosis, and thrombocytopenia have been reported as side effects (for review of treatment for trypanosomiasis, see WHO Expert Report).[213]

African Trypanosomiasis as a Transfusion-Transmitted Infection

Trypanosomiasis is a transfusion-transmitted infection. Asymptomatic or early-stage patients with trypanosomiasis are clearly a threat to the blood supply. Patients are excluded in the United Kingdom, Europe, and North America by the general donor queries relating to fever and constitutional symptoms. In endemic areas, exclusion of infected donors with early-stage *T. b. gambiense* is clearly a more complex problem because patients may be asymptomatic for long periods if the degree of risk is unknown and no specific screening procedures are in place. In summary, hematologic involvement in trypanosomiasis is peripheral to the main two features of the disease, but examination of lymph node aspirates, blood, and CSF is essential for diagnosis and management of the disease. Occasionally, patients may become severely ill and have complex hematologic abnormalities. In endemic areas, trypanosomiasis may pose a risk to the blood supply.

AMERICAN TRYPANOSOMIASIS

American trypanosomiasis (or *Chagas disease*, named after the Brazilian parasitologist Carlos Chagas) is caused by infection with *Trypanosoma cruzi*. This flagellated protozoan is transmitted by the triatomine insects, the reduviid bugs. The acute phase of infection is characterized by fever and high parasitemia, followed by a chronic phase with positive serologic results and low parasitemia but with end-organ damage to the heart, peripheral nervous system, and gastrointestinal tract, causing a chronic cardiomyopathy, neuropathy, megaesophagus, and megacolon.

Epidemiology

Chagas disease may occur in the Americas from the southern United States to Chile, but the highest prevalence is in Bolivia and Brazil (Fig. 154.13). There are approximately 10 million seropositive people in Latin America, and the number of people infected and the incidence of new infection are falling rapidly.[214] Infections are found not only in rural areas but also in recent immigrants to urban areas in North and South America and to Europe.[215] It is estimated that approximately 100,000 seropositive individuals are living in the United States. True, endogenous (autochthonous) infection in the United States is vanishingly rare, but human infections have been reported in Texas, California, Tennessee, and Louisiana. Visitors to endemic areas are only rarely infected, with only a handful of cases being recorded in intrepid travelers spending time in traditional housing in rural areas. The range of reservoir hosts and species of triatomine bugs transmitting infection is wide.

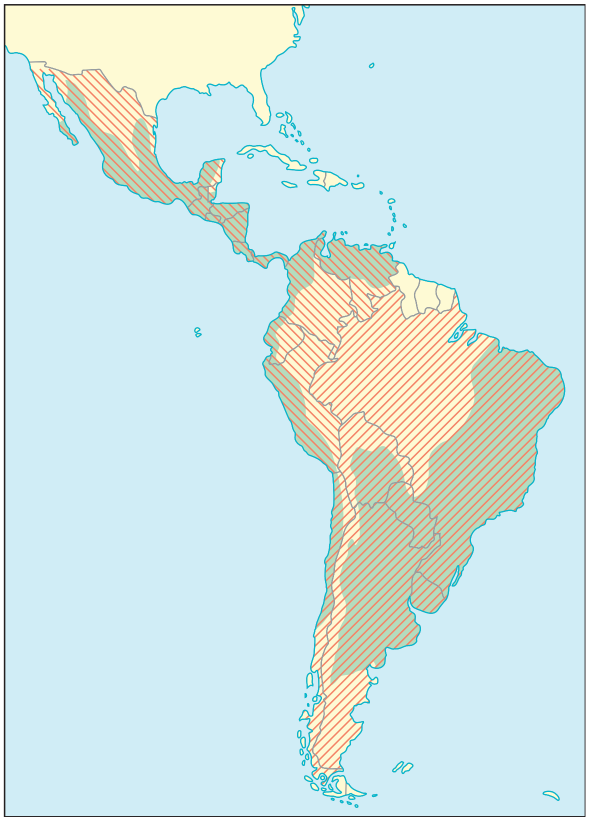


Figure 154.13 DISTRIBUTION OF CHAGAS DISEASE. Human infection is endemic in parts of Central and South America from the Andes to the Atlantic coast and as far south as the latitude of the River Plate (Río de la Plata), shown here in *green*. Two major intergovernmental programs were started in 1991 to eliminate domestic vectors by a combination of spraying residual insecticides in houses, the use of insecticidal paints, and the deployment of fumigant canisters. The countries covered in the two initiatives, the second of which started in 1997, are shown in the figure (hatched red). The latter program instituted universal blood screening to avoid transmission from infected blood donors. Remarkable progress has been made. Transmission (by the major vector *Triatoma infestans*) was eliminated in Uruguay by 1997 and in Chile by 1999. Major reductions in transmission but, to date, not complete control have also been reported in other endemic countries. (Southern Cone Initiative; Andean and American Initiative areas.)

Parasites may also be transmitted by blood transfusion (see later), vertically from mother to child by breastfeeding, organ transplant, and rarely by sexual transmission. Several outbreaks in Brazil have been reported after contamination of food by triatomine bugs and their feces. Laboratory infection by accidental ingestion or inoculation of parasites is well recorded.

Parasitology

T. cruzi parasites are from the order Kinetoplastida and family Trypanosomatidae, existing as infective trypomastigotes in the bloodstream of vertebrate hosts. These organisms are fusiform cells, 10 to 20 μm in length, with a distinctive large posterior kinetoplast containing mitochondrial DNA. They can enter phagocytes, muscle, and nerve cells, and a wide variety of other cell types and here transform to oval amastigotes 2 to 5 μm in diameter. They multiply by fission, and amastigotes develop into mature trypomastigotes released on rupture of the cell to begin a new cycle of invasion and multiplication.

Slender, highly motile and broader, less motile trypomastigotes have been distinguished, which may be relatively more infective for host cells and insects, respectively. The species of triatomine bugs that commonly transmits disease is able to cause extensive infestation of simple mud-and-wattle thatched houses in rural Latin America, where Chagas disease is a disease of poverty associated with poor housing. These bugs are infected by circulating trypomastigotes after taking a blood meal on sleeping victims. In the midgut, trypomastigotes transform into epimastigotes and multiply by fission before migrating to the hindgut, where they develop into metacyclic trypomastigotes, adherent to the epithelium of the rectum. These infective forms are excreted into the feces, and people are infected by rubbing this infected material from the bug into the skin or conjunctival membranes. In the human host, multiplication into macrophages is followed by rapid dissemination of trypomastigotes into the blood and hence to tissues. The genome sequence of *T. cruzi* has stimulated a plethora of comparative and genomic approaches to the study of the parasite.[216–218]

Trypanosoma rangeli Infections

The nonpathogenic trypanosomes can be transmitted directly to people by the bite of the triatomine bugs, and they exist only as circulating trypomastigotes. No tissue amastigotes exist. Circulating organisms are sparse, but sometimes diagnosis can be made by careful microscopic examination of the distinctive morphology of these organisms in blood smears. The anterior position of the nucleus and small kinetoplast distinguish it from *T. cruzi* (Fig. 154.14). The antibodies produced to *T. rangeli* cross-react with those from *T. cruzi* and hence cause false-positive serologic test results for *T. cruzi*. However, the species may be differentiated by xenodiagnosis or molecular genetics in specialized laboratories.[219] The importance of recognizing this nonpathogenic infection is to avoid unnecessary treatment for *T. cruzi*.

Pathology

There is a wide variation in the pathogenicity of isolates and some regional variation in the clinical spectrum of acute and chronic illness.

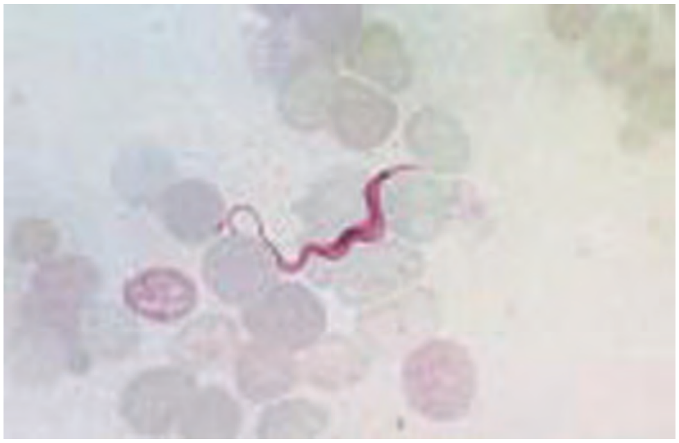


Figure 154.14 *TRYPANOSOMA RANGELI. T. rangeli* is a long, slender trypanosome also transmitted by reduviid bugs from wild animals to humans. It is readily distinguished by its shape from *Trypanosoma cruzi* in blood films and appears to be nonpathogenic to humans.

Molecular typing has demonstrated considerable heterogeneity of *T. cruzi* isolates, although the association between strains of parasite and the outcome of infection are unclear. Clearly the organism must have many mechanisms of the immune evasion to cause chronic infections in a high proportion of individuals. Some of these have been elegantly defined, including resistance to activation of the alternate pathway of complement, specific mechanisms of entering the host cells, and evasion of intracellular killing by the oxidative burst and lysozymes. Both CD4$^+$ and CD8$^+$ T cells are important for killing *T. cruzi*–infected cells, and IFN-γ has shown to be important to controlling disease, whereas transforming growth factor-β and IL-10 enhance parasite replication (for review, see Rios et al.[220]).

End-organ damage occurs after tissues have been infected by *T. cruzi* amastigotes, but the mechanisms leading to extensive cell damage are not clear. After multiplication, tissue amastigotes form pseudocysts in the heart with little or no inflammatory reaction, although occasionally acute myocarditis with focal hemorrhage and inflammation may lead to heart failure. As the disease progresses, the inflammatory response is increased and is associated with increased tissue damage. There may be an autoimmune component to this inflammatory response, but the precise pathogenesis is poorly understood.[221] In the heart, chronic myocarditis leads to a decline in cardiac function. In late-stage disease, amastigotes can be found in almost all organs. Acute myocarditis with focal hemorrhage and inflammation may also lead to heart failure. Involvement of the brain, meninges, liver, lymph nodes, and spleen is common. Damage to the muscle walls and intramural nerve plexus in the esophagus and colon leads to dilation of these structures in the later phases of the disease.

Features of American Trypanosomiasis

Clinical Features

In about half the patients, a granuloma (or chagoma) occurs where parasites have been inoculated. The tender erythematous papule becomes keratotic and later heals, forming a hyperpigmented scar. When the conjunctiva are inoculated, the extensive unilateral periorbital edema may be prolonged (Romaña sign).

The severity of the acute illness is variable and ranges from asymptomatic infection or a mild febrile illness to a severe, potentially fatal illness with cardiac failure and meningoencephalitis in a minority of cases. Myalgia, generalized lymphadenopathy, hepatosplenomegaly, headaches, facial or generalized edema, vomiting, diarrhea, and anorexia are common features of the acute disease. The disease may be worse in children. The acute illness typically resolves in 4 to 8 weeks. Chagas disease must be distinguished from typhoid fever, VL, brucellosis, toxoplasmosis, and malaria in cases of chronic, febrile illness in endemic areas.

Chronic Disease

The acute phase of *T. cruzi* infection is followed by a variable latent or indeterminate phase with no clinical symptoms. Indeed, patients may never present with signs of end-organ damage. However, up to one-third of clinically infected patients may develop cardiac involvement and show right bundle branch block, atrioventricular conduction abnormalities, and/or abnormal T and Q waves. The patient may experience palpitations, chest pain, edema, and dizziness or syncope or dyspnea. The heart is enlarged, and intramural thrombus may cause sudden death.

In a further minority of chronically infected patients, the gastrointestinal tract is infected with abnormal motility of the esophagus and colon, leading to dysphagia and/or severe constipation. Occasionally, other hollow organ systems may be affected.

Pregnancy and Congenital Infection

In pregnant women, Chagas disease can cause spontaneous abortion, premature birth, intrauterine growth retardation, and stillbirth.

Congenital infection occurs in 1% to 2% of women with chronic infection. Prompt diagnosis of circulating parasites in neonates at risk for congenital Chagas disease is essential to beginning early treatment. It is recognized that infection can also be transmitted by breastfeeding. Most children are asymptomatic, but 10% to 20% of children have a mild systemic illness with hepatosplenomegaly. More severely affected newborn children have pneumonitis, meningoencephalitis, and diffuse dermal granulomas. Petechiae, purpura, and a generalized bleeding tendency may occur.[222]

Immunocompromised Patients and Organ Transplantation

The disease may recrudesce after chemotherapy for malignant disease, immunosuppressive therapy, or after organ transplant. The clinical disease may be fulminating with obvious parasitemia. Patients may present with acute myocarditis and congested cardiac failure. Irregular erythematous indurated lesions have been described during *T. cruzi* recrudescence after solid-organ transplant. Here tissue amastigotes can be demonstrated by fine-needle aspiration.

In the United States, screening of solid-organ donors is based on the assessment of risk; donors born in or with significant periods of residence in Latin America, born of women from Latin America, and/or noted to have clinical findings such as cardiomegaly consistent with Chagas disease should be screened by serology. As screening of donors has become more frequent, most donor-derived infections have been detected by PCR testing before symptoms arise and treated quickly and effectively with anti-trypanosomal chemotherapy. Current recommendations suggest monitoring the recipient of an organ from an infected donor for at least 6 months, before decreasing surveillance.[223]

Human Immunodeficiency Virus

Coinfection with *T. cruzi* and HIV has been reported from urban centers in Brazil. *T. cruzi* develops in end-stage disease with CD4$^+$ T-cell counts of less than 400/µL. Patients may be severely ill, the majority with meningoencephalitis and often with a space-occupying lesion. Typically, the CSF shows a mild lymphocytosis (<100 cells/µL). Parasites may be seen in the blood and occasionally the CSF. Cardiac involvement and heart failure are common.

Hematologic and Laboratory Features

A mild, normocytic, normochromic anemia is typical of acute illness. The pathogenesis of the anemia and contributions of hemolysis and bone marrow depression have not been studied in humans, but in experimental infections in mice, uncontrolled infection and TNF-α can be shown to contribute to depressed hematopoiesis. A modest lymphocytosis and increases in liver and muscle enzymes may be present. Nonspecific electrocardiographic changes, first-degree heart block, and cardiomegaly suggest early myocardial involvement.

Hematologic abnormalities are not usually found in the late stage of disease, in which cardiomyopathy with cardiac failure, rhythm disturbance, and angina and systemic emboli from intramural thrombus may occur. Trials of systemic anticoagulation have not been conducted.

Diagnosis

Microscopy

During the acute phase of the disease, motile trypanosomes can be identified in wet preparations or buffy coat preparations using concentration methods and detailed morphologic examination, including Giemsa-stained, gently prepared thick and thin films to avoid damage to parasites (Fig. 154.15). Trypanosomes can be aspirated from the chagoma in the acute phase of disease and visualized in a wet preparation of needle aspirates. Parasites may occasionally be found in lachrymal fluid.

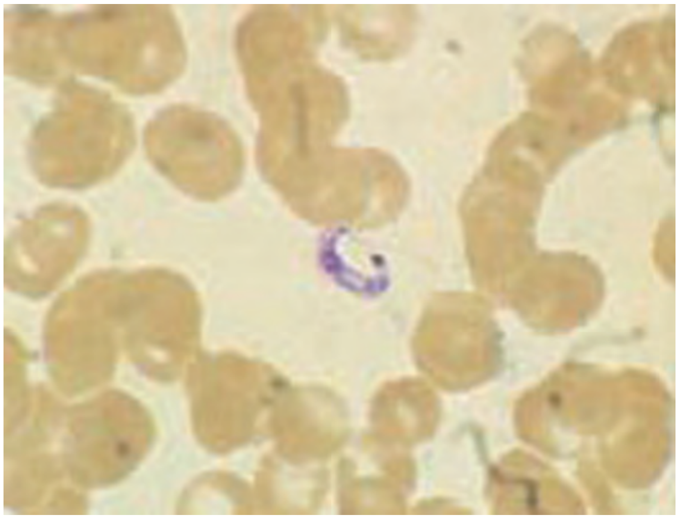


Figure 154.15 *TRYPANOSOMA CRUZI* IN HUMAN BLOOD FILM. The causative agent occurs in blood films, characteristically as short C-shaped or S-shaped trypomastigotes with a prominent kinetoplast. It is otherwise monomorphic.

Sensitivity is increased by the concentration methods described for *T. brucei* earlier. Organisms may also be sought in centrifuged serum after blood has clotted or by centrifugation after lysis of RBCs with 0.8% ammonium chloride. Trypomastigotes can also be detected in other specimens, including CSF, bone marrow, pericardiac fluid, and tissue biopsy specimens. Organisms must be distinguished from the morphologically similar nonpathogenic *T. rangeli* (see Fig. 154.14 and earlier).

After the acute phase, circulating parasites are not visible, although inoculation of blood into susceptible animals (xenodiagnosis), in vitro culture, or molecular methods may demonstrate patent infection. Parasitemia may be obvious in immunocompromised patients in the chronic phase of disease.

Xenodiagnosis

Infection of laboratory triatomine bugs by a seropositive patient is more sensitive than morphologic examination in chronically infected patients. It is also possible to infect susceptible laboratory animals. However, these techniques are available only in specialized centers in Latin America.

In Vitro Culture

Epimastigotes can be cultured using blood agar (NNN) medium or other media such as Schneider insect medium. After 4 weeks or more of culture at 26°C, epimastigotes may be detected.[224]

Serology

Serologic testing is a sensitive method of detecting infection after the acute phase. In Latin America, a number of commercial assays are available using crude epimastigote lysates in a variety of different formats. Serologic tests have used IFAT and ELISA using crude antigen prepared from in vitro cultures of epimastigotes. These tests must be carefully controlled with appropriate positive or negative sera. Chronically infected patients usually give a positive test result if titers are greater than 1:80. These have a sensitivity and specificity of more than 95%. These antibodies may cross-react with antibodies to malaria, leishmaniasis, syphilis, and some autoimmune conditions. ELISA should be used in conjunction with a confirmatory test such as Western blot using parasite lysate.[225]

In South and North America, a number of companies market ELISA-based assays using recombinant antigens, synthetic peptides, or a concentrated extract of excretory-secretory antigens from either Brazil or Tulahuen strain *T. cruzi* trypomastigotes (total trypomastigote excretory-secretory antigens [TESAs]). These assays may provide more rapidly available and cheaper tests without loss of sensitivity and specificity (see Trypanosomiasis as a Transfusion-Transmitted Infection section for further discussion).

Serologic testing may be useful in the evaluation of people who may have been exposed to Chagas disease, pregnant women, and patients about to undergo immunosuppressive treatment or to receive chemotherapy or who have been diagnosed with HIV or other immunosuppressive illness. Children born to seropositive women will have maternally derived IgG anti-*T. cruzi* antibodies but demonstrate IgM anti-*T. cruzi* antibodies if congenitally infected.[226]

Molecular Diagnosis

Amplification of *T. cruzi*-specific sequences by PCR is the most widely applicable, sensitive, and specific method for detecting parasites. A number of assays have been developed, based on the application of repetitive sequences in kinetoplast DNA. A meta-analysis has suggested that PCR is not more sensitive than ELISA for detection of *T. cruzi* in chagasic patients. The recommendation is to use two serologic tests, which may be supplemented by PCR-based assays in the immunocompromised patient, in whom seroconversion may not occur in patent infection.[227]

Treatment

The detailed treatment of Chagas disease is beyond the scope of this chapter. Treatment of the acute, intermediate, and chronic phases requires expert supervision because parasitologic cure is achieved in only half the cases and monitoring progress requires specialist testing and evaluation and where necessary treatment of end-organ damage. The current and potential future chemotherapy have recently been reviewed.[228–230]

Nifurtimox is a synthetic nitrofuran that inhibits pyruvic acid synthesis by inhibition of lactic dehydrogenase. It is given for 30 to 120 days at 3 to 5 mg/kg three times per day. The most serious side effects are peripheral neuropathy, mental disturbance (including psychosis), and hemolytic anemia associated with G6PD.

Benznidazole is widely available in Latin America and is given at 5 mg/kg of body weight per day for 60 days. Severe hematologic complications are common, and bone marrow depression is a serious side effect. Thrombocytopenia may be severe, and neutropenia may progress to agranulocytosis. Other serious complications include photosensitivity, neuropathy, and weight loss.[230]

Supportive therapy is required for heart failure and acute meningoencephalitis.[228] Surgery may be required for alleviation of esophageal and chronic dysfunction. In the absence of effective or simple therapy, control of triatomine bugs and an improvement in the housing stock are essential to prevent and control disease.

American Trypanosomiasis as a Transfusion-Transmitted Infection

Chagas disease may occur as a transfusion-transmitted infection and represents a real threat to the safety of the blood supply in endemic areas. In endemic areas, seropositivity is high, reaching 50% in some areas of Bolivia and 1% to 2% in major areas in Brazil. Control of blood transfusion–transmitted infection is part of the World Health Organization strategy for control of Chagas disease.[231] Outside Latin America, Chagas disease has been an occasional cause of transfusion-transmitted infection, mainly in immunosuppressed patients. It is possible that minor infections are not recognized in healthy individuals. Clearly the high rates of seropositivity in individuals from endemic areas suggest that recent immigrants may easily transmit the disease.

Before blood donor screening, seven transfusion-associated *T. cruzi* infections were documented in the United States between 1988 and 2006.[232,233] These infections were manifested in patients with malignancy and associated with platelet transfusions. In Europe, transfusion-transmitted Chagas disease has been less common, but has occurred after a red cell transfusion.[234] These cases have allowed an estimate of the risk of infection in a recipient associated with an infective platelet unit of 13.3% (95% confidence interval 5.6 to 25.7) but is likely to be less than 1% for RBCs and plasma components. Infection may cause severe acute disease in recipients.

Transmission of Chagas disease has been reduced by donor screening using the Ortho ELISA or the Abbott PRISM chemiluminescent immunoassay (ChLIA) as a primary screening test and the radioimmunoprecipitation assay (RIPA) or the Abbott enzyme strip assay (ESA) as the confirmatory test.[235]

All blood donors were tested between 2007 and 2017 and no incident cases representing local or autochthonous transmission were detected. After 2017, the FDA has recommended screening donors once. Widespread donor screening has shown an overall seroprevalence of 1:27,500, with the highest rates in Florida (1:3800), followed by California (1:8300). Of these 104 *T. cruzi*-infected donors 28% were born in Mexico, 26% in the United States, 16% in El Salvador, and 11% in Bolivia. The other 20 donors were born in nine other countries of Central and South America, illustrating widespread distribution of endogenous infection in South and Central America.[236]

The United Kingdom has implemented systematic screening of at-risk blood donations for *T. cruzi* infection since 1999, testing blood donors born in endemic areas, or donors born to mothers native of endemic areas, or recipients of blood transfusions in endemic areas,[237] and similar measures have been implemented by an increasing number of countries including Spain, France, and Switzerland. It is possible to treat blood with gentian violet (0.25 g/L for 24 hours at 4°C) or with methylene blue to kill circulating organisms. It has also been shown that amotosalen or riboflavin plus ultraviolet A photochemical inactivation technology is effective in inactivating *T. cruzi* in platelet concentrates and plasma.[238] In addition, prophylactic benznidazole can be given to immunosuppressed patients receiving blood products in endemic areas.

It is only to be hoped that sensitive screening tests are consistently applied to reduce transmission of Chagas disease by blood products in endemic and nonendemic countries. On a more general note, it is encouraging that the initiatives to control *T. cruzi* infection across Latin America are showing real evidence of success.[239,240]

BABESIOSIS

Babesiosis is an intraerythrocytic infection caused by parasites from the order Piroplasmida and the family Babesiidae. It was first observed as causing parasitic inclusions in erythrocytes of cattle in Romania by Victor Babes at the end of the 19th century.[241] The infection is zoonotic and is transmitted by hard-bodied ticks and causes fever and hemolytic anemia. Several distinct *Babesia* spp. cause disease in different geographic areas.[242,243]

There are two well-defined *Babesia* species that cause human infection. *Babesia microti* is a parasite of small rodents in the northeastern United States and is spread by nymphs, larvae, and adult forms of the hard-bodied ixodid ticks (*Ixodes dammini*). Infection of the white-tailed deer by ticks allows multiplication and spread of the infected ticks. Infections can be transferred to humans by all forms of the tick after prolonged feeding. The infection is therefore most common in people vacationing or working in forested areas of the northeastern United States. The northeastern US offshore islands of Nantucket, Martha's Vineyard, Block Island, and

Shelter Island are foci of the disease, but it also occurs in eastern states, particularly Connecticut, Rhode Island, Delaware, and New York State, as well as upper Midwestern states. Hundreds of cases have been recorded since the first identified case in 1969. Visitors and workers in endemic areas should avoid tick bites by using appropriate clothing and repellents.

In Europe, *Babesia divergens* and the morphologically indistinguishable parasite *Babesia bovis* are transmitted to humans by ticks (*Ixodes ricinus*) from cattle. Fewer than 50 cases have been recorded. European cases of *B. microti* and *Babesia canis* infection have also been reported.

A handful of cases of infections with *Babesia duncani* in the western United States have been reported. These organisms are morphologically indistinguishable from *B. microti* but are distinguished by molecular methods. The MO1-type piroplasm caused illness in an index case in Missouri in 1992, and the WA1-2 and CA1-5 piroplasms caused disease in eight cases reported in Washington State and California.[244,245] A further *B. microti*-like organism, *B. venatorum*, has caused infection in a small series of older men who had undergone splenectomy for a lymphoproliferative disease.[246] The same organism has been isolated in a much larger series of patients from China.[247]

B. microti and *B. duncani* have been transmitted by red cell and platelet components in over 260 documented cases of transfusion-transmitted infection.[248,249] Transplacental infection by *B. microti* has also been reported.

Parasitology

Parasites appear to be introduced into the bloodstream, where they invade erythrocytes. There they multiply by asexual fission to produce two to four merozoites. These infective forms are released after lysis of the erythrocyte and begin another cycle of invasion multiplication. Parasites are cleared by macrophages. The contribution of antibodies and cell-mediated immune response has not been defined, although *B. bovis* expresses clonally variant antigens on the surface of infected erythrocytes.[27]

Clinical Features

The North American *B. microti* infections have an incubation period of several weeks, and after infection by blood transfusion, clinical symptoms have taken from 17 days to many months to become manifest.[250–252] The spectrum of clinical disease is wide. *B. microti* may cause asymptomatic infection or present with a mild flulike illness in most cases in people with normal immune and splenic function.[253] The cardinal manifestations are fever and hemolytic anemia. Splenectomy, old age, and immunosuppression, including HIV, may increase the risk for more severe clinical disease.[254–256] Hematological features of infection may include not only mild anemia but also severe pancytopenia, splenic rupture, disseminated intravascular coagulopathy (DIC), or even hemophagocytic lymphohistiocytosis (HLH).[252] Patients who have had a splenectomy should avoid exposure to ticks in forested areas where the disease is transmitted.

B. divergens in Europe predominantly causes symptomatic infection in people who have had a splenectomy.[257,258] Here, the case fatality rate is on the order of 50%. However, symptomatic disease does also occur in immunocompetent individuals.[259]

Patients present with high fever, often with chills and sweats, jaundice, fatigue, malaise, headache, arthralgia, and myalgia. Gastrointestinal disturbances are common, and people may complain of dark urine secondary to hemoglobinuria. In some cases, the disease progresses with respiratory failure secondary to ARDS, disseminated intravascular coagulation, and renal failure requiring the appropriate supportive care.[260] Such severe cases resemble severe malaria in nonimmune persons, and it has been suggested that the pathophysiology, like in malaria, includes adhesion and sequestration of infected erythrocytes and the release of proinflammatory cytokines. These diseases may be confused with falciparum malaria, leptospirosis, or viral hepatitis. In pregnancy, acute babesiosis may mimic the HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count).[260]

Hematologic and Laboratory Features

The hematologic features of the disease are dominated by substantial intravascular hemolysis.[242] Physical examination shows pallor, jaundice, and mild hepatosplenomegaly.

The laboratory findings are those of a compensated intravascular hemolytic anemia and thus feature low Hb and haptoglobin levels, increased reticulocyte count and serum lactate dehydrogenase, and hemoglobinuria and proteinuria. Moderate thrombocytopenia is common.[251,252,261] Electron microscopy suggests uninfected erythrocytes are damaged during infection and so likely to be cleared more rapidly than normally.[262] The white blood cell count is usually decreased with atypical lymphocytosis and occasional evidence of hemophagocytosis.[252] However, leukocytosis may occur, particularly in *B. divergens* infections.

About 5% to 10% of patients with acute babesiosis develop warm-antibody autoimmune hemolytic anemia 2 to 4 weeks after diagnosis, and after treatment and clearance of the parasites.[262–264] Almost all the cases with AIHA after acute babesiosis were asplenic.

Diagnosis

The blood films stained with Giemsa or Romanowsky stain show ring-like intraerythrocytic parasites. Morphology is variable, and ring, rod, and ameboid forms of *Babesia* parasites may be seen (Fig. 154.16).[265–267] Occasionally, multiple intraerythrocytic forms can be seen, linked to form a tetrad or, colloquially, a “Maltese cross.” However, at low parasitemia, *Babesia* parasites may easily be mistaken for ring-stage forms of *P. falciparum*. Moreover, false-positive sightings of *Babesia* spp. may be caused by platelets, nonspecific stain deposit overlying erythrocytes, or indeed other intraerythrocytic inclusions.

Parasitemias are variable, and in *B. microti* infections may be low or transient.[253] In symptomatic infection, parasitemias typically range from less than 1% to 10% and rarely much higher, more than 70%, in severe infections. Low-level parasitemias may be detected by inoculation of the blood into susceptible animals, including the golden hamster (*Mesocricetus auratu*), but this form of testing is not routinely available.

An IFAT using crude antigen is available in the United States through the Division of Parasitic Diseases at the Centers for Disease Control and Prevention.[268] Titers greater than 1:64 are regarded as positive and have been reported to be 88% to 96% sensitive and 90% to 100% specific. However, the degree of cross-reactivity between Babesia species is variable. If the infection is contracted on the West Coast of America, samples should also be tested for antibodies to the *B. duncani*, because of the limited cross-reactivity with *B. microti*.[269]

PCR analysis is the most useful method of detecting or confirming low levels of parasitemia, and it can also be used to monitor treatment. Real-time PCR assays target the *Babesia* 18 S rRNA gene and, with use of a fluorescent probe, can distinguish *B. microti* from *B. duncani*.[270,271] PCR tests may be positive in the absence of positive serology in immunocompromised patients and/or those patients who have received rituximab, whereas serology for *Babesia* spp. may be positive in the absence of positive microscopy or positive PCR tests in asymptomatic individuals who were infected but who have cleared the infection spontaneously.

Treatment

Many *B. microti* infections are self-limiting, and therapy is used for moderate or severe disease.[272–274] The combination of atovaquone and azithromycin is the treatment of choice for mild to moderate illness, whereas clindamycin and quinine and exchange transfusion are indicated for severe disease.

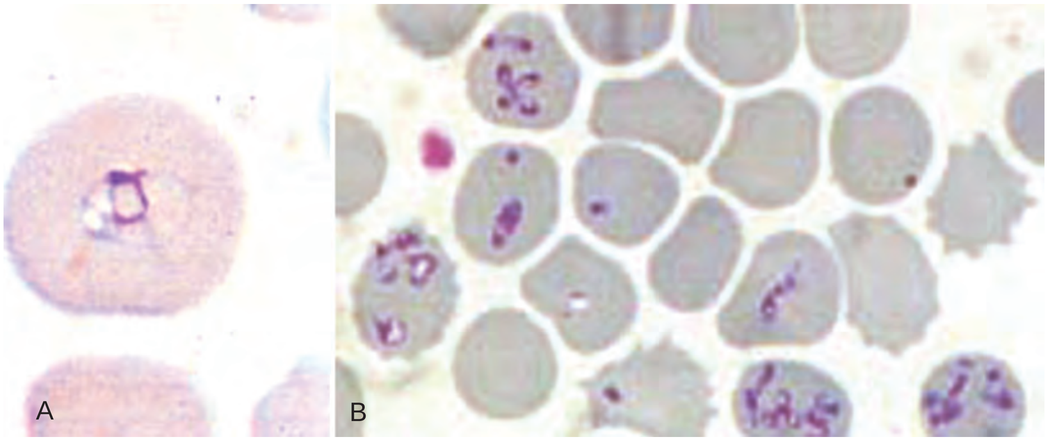


Figure 154.16 *BABESIA* PARASITES. Human infection with species of piroplasm transmitted by the bite of the tick *Ixodes ricinus* infected from cattle is a rare occurrence. Infection in normal people with this piroplasm may give rise to a self-limiting fever and parasitemia, as in the case of infection with the rodent parasite *Babesia microti* on the northeastern seaboard of the United States via the tick *Ixodes scapularis* (A). Heavy red-cell infection may develop, however, in splenectomized patients, leading to fatal hemolytic anemia. This patient died as a result of an infection acquired from the cattle parasite *Babesia divergens* in Scotland (B). Other species of *Babesia* that occasionally infect humans, for example, the WA1, CA1, and MO1 isolates from the United States, are distinguished by molecular means.

B. divergens infections are often fatal in splenectomized patients, and therapy is based on somewhat limited experience. Three cases have been treated successfully with large-volume exchange transfusions (2 to 3 blood volumes) and intravenous clindamycin and oral quinine.[275] This regimen of clindamycin and intravenous and oral quinine gives the best chance of success in the absence of randomized controlled trial evidence.

Exchange transfusion has been suggested as a useful adjunctive treatment if the parasitemia rises to greater than 10% and/or in severely ill patients. *B. divergens* infections can run a rapidly progressive course, and early exchange transfusion should be considered. A prolonged course of treatment may be required in immunocompromised patients.[276] The parasite and Hb levels should be regularly monitored during treatment.

Serologic testing and PCR testing of seropositive cases may be helpful in making a diagnosis. An indirect IFAT is available for *B. divergens* and *B. microti*. The IFAT titer is usually greater than 1:64 in acute infection. The reported sensitivity of the *B. microti* IFAT is 88% to 96%, and its specificity is 90% to 100%. Antimalarial antibodies may cross-react in this test.

Babesiosis as a Transfusion-Transmitted Infection

Babesia infection poses a substantial and increasing threat to the blood supply. Over 150 cases of transfusion-transmitted babesiosis were reported from 1979 to 2015, with 32 recorded deaths.[248,249] Preventing transfusion-transmitted infection by donor screening proved to be very difficult as the questions lacked sensitivity. An arrayed fluorescence immunoassay and PCR test were developed to test for donors with patent babesia infection. Of 89,000 donors from the Northeastern United States (Connecticut, Massachusetts, Minnesota, Wisconsin) 355 or 0.38% were positive by either test, and 69 of these 355 donors were positive by PCR, with 9/69 positive by PCR alone.[277] Infections were detected in donors throughout the year. In 2019, the US FDA issued "Recommendations for reducing the risk of transfusion-transmitted babesiosis" as applied to the collection of blood and blood components, except source plasma.[278] The document instructed blood operators to test each donation using a licensed Babesia NAT on donations collected in the following jurisdictions: Connecticut, Delaware, Maine, Maryland, Massachusetts, Minnesota, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, Vermont, Virginia, Wisconsin, and Washington, DC.[278] Subsequent screening of donors and follow-up of recipients suggested that infections in the window period, when the infections could not be detected by immunoassay or PCR, represented 2% of positive donations or 1:15,000 donations.[279] A serology assay(s) using recombinant antigens are under development that may improve the detection of infective donors.[280–283] In the United Kingdom, a conservative approach has been taken, and all donors who have visited the northeastern United States between May and October are excluded, in addition to the standard criteria of screening out febrile and/or anemic donors.[87]

EOSINOPHILIA

Eosinophilia may be caused by a wide variety of systemic diseases and parasitic infections. The association with parasitic disease is through part of the Th2 T-cell response stimulated by helminths (worms), filaria, and cestodes. In general, protozoan infections, such as malaria, amebiasis, and giardiasis, are not associated with eosinophilia. However, case reports do exist suggesting that isosporosis due to infection with *Isospora belli*, toxoplasmosis, and infection with *Dientamoeba fragilis* can cause eosinophilia.

The rise in absolute eosinophil count depends on the degree of tissue invasion and is therefore modest with tapeworms and adult roundworms resident in the bowel but much higher where invasion occurs, for example, with *Toxocara canis* or filaria. Some parasites, such as ascariasis (roundworms) and clonorchiasis, have migratory larval stages.

The differential diagnosis of eosinophilia in those who have lived in tropical areas is therefore wide. Evaluating the patients must begin by establishing the degree of eosinophilia (minimal, $<1 \times 10^9$/L; moderate, 1 to 3×10^9/L; high, $>3 \times 10^9$/L), the relation to travel (where necessary), and the presence of symptoms. A wide range of systemic diseases are associated with eosinophilia, and the eosinophil count may be high in drug allergy, pulmonary infiltrate with eosinophilia, and vasculitides.

Eosinophilia in Travelers

Evaluating the cause of eosinophilia in travelers to tropical areas where many parasitic diseases are endemic requires a systematic approach to narrow down likely possibilities by considering existing systemic diseases that may cause eosinophilia (particularly allergy, drug ingestion, autoimmune disease, vasculitis, or arthritis); the areas visited; duration of stay; and history of exposure to soil-transmitted nematodes, freshwater potentially infected with schistosomiasis, and rural areas where loiasis, onchocerciasis, and hydatid disease may be contracted.

Physical examination may show subcutaneous swellings associated with filaria or hepatosplenomegaly consistent with schistosomiasis, hydatid disease, or toxocara. Laboratory examination requires a stepwise approach, given the breadth of the differential diagnosis (Table 154.4). The details of specific parasitologic tests are beyond the scope of this chapter, and detailed investigation would certainly require consultation with colleagues in infectious diseases (see also further reading, CDC, Laboratory Identification of Parasites of Public Health Concern).

Some parasitic causes of eosinophilia may not be diagnosed during the incubation period, because larval stages of nematode worms may cause eosinophilic drug migration, but eggs will not be excreted in stools for many months. Moreover, filarial infections do not produce detectable parasites in blood or skin for many months after exposure.

In immunocompromised patients or those about to receive a diagnosis, eosinophilia may be crucial, given the risks of giving immunosuppressive therapy such as chemotherapy or hematopoietic stem cell or solid organ transplant to a patient with a chronic parasitologic infection. Patients with undiagnosed eosinophilia and possible exposure to *Strongyloides* should be given an empirical course of treatment.

TABLE 154.4 Approach to Investigation of Eosinophilia in a Returning Traveler

History	Allergy
	Drugs and vitamins (L-tryptophan)
	Regions, localities, and duration of exposure
Physical examination	Skin, subcutaneous tissues
	Liver/spleen
	Signs of other systemic disease
Initial investigations	Full blood count and differential white blood cell count
	Stool examination for ova and parasites (×3)
	Urine analysis
	Examination of midday urine for ova and parasites (×3) (in those who have traveled to Africa or the Middle East)
Further investigations as suggested by travel and exposure from history	As suggested by travel and exposure from history
	Strongyloides culture and serologic testing
	Duodenal aspirate (strongyloidiasis, hookworm)
	Serologic testing (schistosomiasis, filariasis)
	Day/night blood films (filariasis)
Further studies if suggested by history and physical examination	Skin snips (onchocerciasis)
	Chest X-ray examination (hydatid cyst, tropical pulmonary eosinophilia, paragonimiasis)
	Soft tissue X-ray examination (cysticercosis)
	Sputum examination for ova and parasites (paragonimiasis)
	Abdominal ultrasound examination (hydatid cyst)
	Cystoscopy with or without biopsy (schistosomiasis)
	Rectal snips (schistosomiasis)

OTHER PARASITIC DISEASES

Many parasitic diseases cause some minor hematologic disturbance. A few may present with distinct hematologic features or indeed syndromes.

Filariasis

Lymphatic filariasis is caused by *Wuchereria bancrofti* and *Brugia malayi*. Infection with *W. bancrofti* occurs throughout the tropics, but by far the majority of cases are in Asia. The distribution of *B. malayi* is more restricted to China, Southeast Asia, and Southern India. The male and female adult worms live in the lymphatics, and the female worm releases a vast number of microfilariae, each 250 to 300 μm in length. Microfilariae develop but do not appear to multiply in the mosquito.

Infection may present with lymphangitis; often recurrent and unlike bacterial infections, the inflammatory features may spread distally. Over time, lymphatic obstruction may cause hydrocele, lymphedema (if severe elephantiasis), chyluria, and tropical pulmonary eosinophilia. *B. malayi* causes neither hydrocele nor chyluria.

Filariasis is most easily diagnosed by finding microfilariae in peripheral blood in a wet preparation. Motile microfilariae can be seen under low power and may be concentrated by centrifugation or filtration using a 3-μm filter. They are speciated in thin or thick films by their nuclear distribution and sheath characteristics (Fig. 154.17), and the two pathogenic species must be distinguished from the nonpathogenic species *Mansonella perstans* and *Mansonella ozzardi*, which do not have sheaths. Circulating *W. bancrofti* antigens can also be detected in the circulation by ELISA or immunochromatographic methods. Adult worms can sometimes be imaged by ultrasound. Filarial DNA from all species can be detected by PCR. Serologic testing is unhelpful because many people become exposed without developing clinical symptoms.

The worms cause marked or severe eosinophilia (see later) with counts greater than 1×10^9/L. Migration of worms through the lungs may exacerbate the eosinophil count and cause minor respiratory symptoms and fluctuating radiologic signs. Other causes of tropical pulmonary eosinophilia are the worms (helminths) *Ascaris*, *Strongyloides*, *Schistosoma*, and *Toxocara*. Of these organisms causing pulmonary symptoms and signs, filariasis alone is responsive to diethylcarbamazine. Albendazole and ivermectin may also be used against filarial infection.

Toxoplasmosis

Toxoplasmosis may cause a mild illness or a more prolonged course with constitutional symptoms, atypical lymphocytes, and thrombocytopenia. Congenital toxoplasmosis as a result of infection acquired

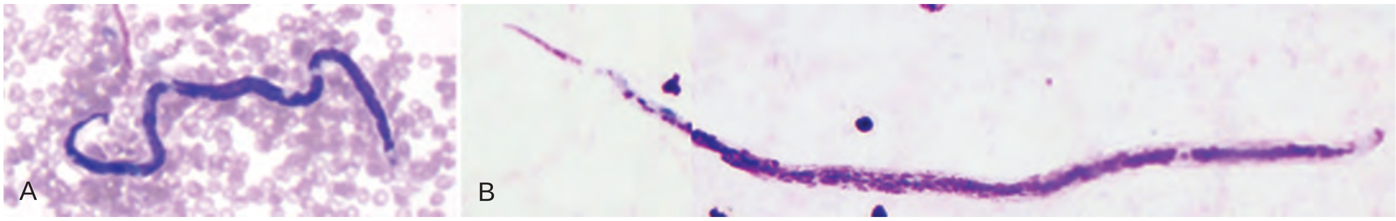


Figure 154.17 MICROFILARIA (*BRUGIA MALAYI*) ON THICK (A) AND THIN FILMS (B). The species is determined by the nuclear distribution and sheath characteristics.

during pregnancy is a cause of neonatal thrombocytopenia, where it may be accompanied by cerebral calcification, hepatitis, and pneumonitis. In immunocompromised patients, new or reactivated toxoplasmosis may cause severe disease, including encephalitis, pneumonitis, and hepatitis. Thrombocytopenia is frequently accompanied by anemia and leukopenia.

Amebiasis

Amebiasis causes hypochromic, microcytic anemia, both as a result of chronic blood loss and as an anemia of chronic disease where the disease progresses to formation of a liver abscess. Neutrophilia accompanies severe tissue damage caused by perforation of the bowel or a liver abscess, or it may be present in a secondary bacterial infection. Sometimes a leukemoid reaction with high white blood cell count and extreme left-shifted myeloid cells can be seen. Prolonged and/or extensive liver damage may cause prolongation of the prothrombin time.

Giardiasis

Acute giardiasis causes folate deficiency through malabsorption of folate in the small intestine. Chronic infection can cause vitamin B_{12} deficiency because ileal absorption of the vitamin is impaired.

Hookworm Infection

Adult hookworms attach themselves to the lining of the small bowel and take blood meals. The accumulated blood loss may be extensive because worms consume 2.0 mL (*Necator americanus*) or 0.5 mL (*Ancylostoma duodenale*) of blood each day. These infections are a common contributing factor to iron deficiency anemia in children, in whom infection is acquired by eating or walking barefoot on larva-infected soil. The disease is usually diagnosed by finding excreted eggs in stool samples, and treatment is with albendazole.

Tapeworm Infection

The fish tapeworm (*Diphyllobothrium latum*) is a rare cause of vitamin B_{12} deficiency. This tapeworm is transmitted in the Far East by eating raw or partially cooked fish. Infection has to be extensive to cause vitamin B_{12} deficiency; however, such cases are rare, even in endemic areas.

Schistosomiasis

Schistosoma haematobium causes blood loss in urine. Infection is acquired by swimming in freshwater, where cercariae from the infected snail host enter the skin and migrate to the blood vessels of the bladder. Chronic blood loss is a cause of iron deficiency anemia in children in endemic areas, but infection is likely to be diagnosed at an early stage in travelers because of the striking symptoms of painless hematuria. Treatment is with praziquantel.

FUTURE DIRECTIONS

Parasitic diseases present many problems for global heath. They cause a wide spectrum of hematologic abnormalities, and in endemic areas, a broad knowledge of the parasitic disease is vital for everyday practice. In nonendemic areas, there are a few situations where the diagnosis or management of these diseases may fall within the remit of hematologists and hematology laboratories, particularly malaria and the diagnosis of anemia, cytopenias, eosinophilia, and hepatosplenomegaly. Here, a high index of suspicion is often needed for the diagnosis to be made. A good travel history is crucial to establishing exposure to parasitic disease and to prompt the search for the appropriate organisms. Several parasitic diseases pose a threat for the safe supply of blood, and the problems of screening of these infections are far from solved. Beyond everyday practice, the pathophysiology and prevention of these diseases pose major challenges for biomedical research and public health.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Angheben A, Boix L, Buonfrate D, et al. Chagas disease and transfusion medicine: a perspective from non-endemic countries. *Blood Transfus*. 2015;13:540.

Akel T, Mobarakai N. Hematologic manifestations of babesiosis. *Ann Clin Microbiol Antimicrob*. 2017;16:6.

Ashley EA, Poespoprodjo JR. Treatment and prevention of malaria in children. *Lancet Child Adolesc Health*. 2020;4:775.

Babokhov P, Sanyaolu AO, Oyibo WA, et al. A current analysis of chemotherapy strategies for the treatment of human African trypanosomiasis. *Pathog Glob Health*. 2013;5:242.

Bailey JW, Williams J, Bain BJ, et al. Guideline: the laboratory diagnosis of malaria. General Haematology Task Force of the British Committee for Standards in Haematology. *Br J Haematol*. 2013;163:573.

Bennett JE, Dolin R, Blaser MJ. *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*. 9th ed. London: Churchill Livingstone; 2020.

Bern C, Messenger LA, Whitman JD, Maguire JH. Chagas disease in the United States: a public health approach. *Clin Microbiol Rev*. 2019;33: e00023–19.

Brun R, Blum J, Chappuis F, et al. Human African trypanosomiasis. *Lancet*. 2011;375:148.

Clark MA, Goheen MM, Cerami C. Influence of host iron status on *Plasmodium falciparum* infection. *Front Pharmacol*. 2014;5:84.

Centre for Disease Control. DPDx—Laboratory Identification of Parasites of Public Health Concern. https://www.cdc.gov/dpdx/index.html.

Copeland NK, Aronson NE. Leishmaniasis: treatment updates and clinical practice guidelines review. *Curr Opin Infect Dis*. 2015;28:426–437.

Coura JR, Borges-Pereira J. Chagas disease: 100 years after its discovery. A systemic review. *Acta Trop*. 2010;115:5.

Cox FE. History of human parasitology. *Clin Microbiol Rev*. 2002;15:595.

Desai M, Hill J, Fernandes S, et al. Prevention of malaria in pregnancy. *Lancet Infect Dis*. 2018;18:e119.

Drakesmith H, Prentice AM. Hepcidin and the iron-infection axis. *Science*. 2012;338:768–772.

Fang DC, McCullough J. Transfusion-transmitted *Babesia microti*. *Transfus Med Rev*. 2016;30:132.

Genovese G, Friedman DJ, Ross MD, et al. Association of trypanolytic ApoL1 variants with kidney disease in African Americans. *Science*. 2010;329:841.

Gething PW, Casey DC, Weiss DJ, et al. Mapping *Plasmodium falciparum* mortality in Africa between 1990 and 2015. *N Engl J Med*. 2016;375:2435.

Jimenez-Marco T, Fisa R, Girona-Llobera E, et al. Transfusion-transmitted leishmaniasis: a practical review. *Transfusion*. 2016;56(Suppl 1):S45.

Kariuki SN, Williams TN. Human genetics and malaria resistance. *Hum Genet*. 2020;139:801.

Kennedy PGE, Rodgers J. Clinical and neuropathogenetic aspects of human African trypanosomiasis. *Front Immunol*. 2019;10:39.

Kumari D, Perveen S, Sharma R, et al. Advancement in leishmaniasis diagnosis and therapeutics: an update. *Eur J Pharmacol*. 2021;910:174436.

Lalloo DG, Shingadia D, Bell DJ, et al. UK malaria treatment guidelines 2016. *J Infect*. 2016;72:635–649.

Langhorne J, Duffy PE. Expanding the antimalarial toolkit: targeting host-parasite interactions. *J Exp Med*. 2016;213:143–153.

Lindoso JA, Cunha MA, Queiroz IT, et al. Leishmaniasis-HIV coinfection: current challenges. *HIV AIDS (Auckl)*. 2016;8:147–156.

Maitland K, Olupot-Olupot P, Kiguli S, et al. Transfusion volume for children with severe anemia in Africa. *N Engl J Med*. 2019;381:420.

Marsh K, Forster D, Waruiru C, et al. Indicators of life-threatening malaria in African children. *N Engl J Med*. 1995;332:1399.

Millar SB, Cox-Singh J. Human infections with *Plasmodium knowlesi*—zoonotic malaria. *Clin Microbiol Infect*. 2015;21:640.

Morrell CN. Understanding platelets in malaria infection. *Curr Opin Hematol*. 2014;21:445–449.

Mungai M, Tegtmeier G, Chamberland M, et al. Transfusion-transmitted malaria in the United States from 1963 through 1999. *N Engl J Med*. 2001; 344:1973.

Price RN, Commons RJ, Battle KE, et al. *Plasmodium vivax* in the era of the shrinking *P. falciparum* map. *Trends Parasitol*. 2020;36:560.

Roussel C, Ndour PA, Kendjo E, et al. Intravenous artesunate for the treatment of severe imported malaria: implementation, efficacy, and safety in 1391 patients. *Clin Infect Dis*. 2021;73(10):1795–1804.

Sahu PK, Satpathi S, Behera PK, et al. Pathogenesis of cerebral malaria: new diagnostic tools, biomarkers, and therapeutic approaches. *Front Cell Infect Microbiol*. 2015;5:75.

Satchwell TJ. Erythrocyte invasion receptors for *Plasmodium falciparum*: new and old. *Transfus Med*. 2016;26:77–88.

Schwarz NG, Loderstaedt U, Hahn A, et al. Microbiological laboratory diagnostics of neglected zoonotic diseases (NZDs). *Acta Trop*. 2017;165:40.

Sharp PM, Plenderleith LJ, Hahn BH. Ape origins of human malaria. *Annu Rev Microbiol*. 2020;74:39.

Simarro PP, Cecchi G, Paone M, et al. The atlas of human African trypanosomiasis: a contribution to global mapping of neglected tropical diseases. *Int J Health Geogr*. 2010;9:57.

Soriano-Arandes A, Angheben A, Serre-Delcor N, et al. Control and management of congenital Chagas disease in Europe and other non-endemic countries: current policies and practices. *Trop Med Int Health*. 2016;5:590–596.

Steinmann P, Stone CM, Sutherland CS, et al. Contemporary and emerging strategies for eliminating human African trypanosomiasis due to *Trypanosoma brucei gambiense*: review. *Trop Med Int Health*. 2015;6: 707–718.

Sundar S, Singh A. Recent developments and future prospects in the treatment of visceral leishmaniasis. *Ther Adv Infect Dis*. 2016;3:98–109.

Vannier E, Krause PJ. Human babesiosis. *N Engl J Med*. 2012;366:2397.

Wendel S. Transfusion transmitted Chagas disease: is it really under control? *Acta Trop*. 2010;115:28.

White NJ. Malaria parasite clearance. *Malar J*. 2017;16:88.

White NJ. Anaemia and malaria. *Malar J*. 2018;17:371.

Woodrow CJ, White NJ. The clinical impact of artemisinin resistance in Southeast Asia and the potential for future spread. *FEMS Microbiol Rev*. 2017;41:34.

World Health Organization. Control of the leishmaniases. *World Health Organ Tech Rep Ser*. 2010;949:1.

World Health Organization. Control and surveillance of human African trypanosomiasis. *World Health Organ Tech Rep Ser*. 2013:1–237.

World Health Organization. World malaria report 2020: 20 years of global progress & challenges. https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2020.

REFERENCES

1. Cox FE. History of human parasitology. *Clin Microbiol Rev*. 2002;15:595.
2. Sharp PM, Plenderleith LJ, Hahn BH. Ape origins of human malaria. *Annu Rev Microbiol*. 2020;74:39–63.
3. Mabey G, Godfrey R, Parry E, et al. *Principles of Medicine in Africa*. Cambridge, UK: Cambridge University Press; 2013.
4. Bennett JE, Dolin R, Blaser MJ. *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*. 9th ed. London: Churchill Livingstone; 2019.
5. Farrar JF, Hotez P, Junghanss T, eds. *Manson's Tropical Diseases*. 23rd ed. London: Saunders; 2013.

5a. World Health Organization. World malaria report 2020: 20 years of global progress & challenges. https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2020.

6. Jeyaprakasam NK, et al. *Plasmodium knowlesi* infecting humans in Southeast Asia: What's next? *PLoS Neglected Tropical Diseases*. 31 Dec. 2020;14(12): e0008900. https://doi.org/10.1371/journal.pntd.0008900.
7. Smith DL, Dushoff J, Snow RW, et al. The entomological inoculation rate and *Plasmodium falciparum* infection in African children. *Nature*. 2005;438:492.
8. Smith DL, McKenzie FE, Snow RW, et al. Revisiting the basic reproductive number for malaria and its implications for malaria control. *PLoS Biol*. 2007;5:e42.
9. Kariuki SN, Williams TN. Human genetics and malaria resistance. *Hum Genet*. 2020;139:801.
10. Ndila CM, Uyoga S, Macharia AW, et al. Human candidate gene polymorphisms and risk of severe malaria in children in Kilifi, Kenya: a case-control association study *Lancet Haematol*. 52018e333.
11. Williams TN. Sickle cell disease in sub-Saharan Africa. *Hematol Oncol Clin North Am*. 2016;30:343.
12. Luzzatto L, Nannelli C, Notaro R. Glucose-6-phosphate dehydrogenase deficiency. *Hematol Oncol Clin North Am*. 2016;30:373–393.
13. Fairley NH. Sidelights on malaria in man obtained by subinoculation experiments. *Trans R Soc Trop Med Hyg*. 1947;40:621.
14. Sturm A, Amino R, van de Sand C, et al. Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science*. 2006;313:1287.
15. Satchwell TJ. Erythrocyte invasion receptors for *Plasmodium falciparum:* new and old. *Transfus Med*. 2016;26:77–88.
16. Bruce MC, Alano P, Duthie S, et al. Commitment of the malaria parasite *Plasmodium falciparum* to sexual and asexual development. *Parasitology*. 1990;100:191.
17. Angus BJ, Chotivanich K, Udomsangpetch R, et al. In vivo removal of malaria parasites from red blood cells without their destruction in acute falciparum malaria. *Blood*. 1997;90:2037.
18. Groux H, Gysin J. Opsonization as an effector mechanism in human protection against asexual blood stages of *Plasmodium falciparum:* functional role of IgG subclasses. *Res Immunol*. 1990;141:529.
19. Scholander C, Carlson J, Kremsner PG, et al. Extensive immunoglobulin binding of *Plasmodium falciparum*-infected erythrocytes in a group of children with moderate anemia. *Infect Immun*. 1998;66:361.
20. Ishioka H, Ghose A, Charunwatthana P, et al. Sequestration and red cell deformability as determinants of hyperlactatemia in falciparum malaria. *J Infect Dis*. 2016;213:788–793.
21. Waitumbi JN, Opollo MO, Muga RO, et al. Red cell surface changes and erythrophagocytosis in children with severe *Plasmodium falciparum* anemia. *Blood*. 2000;95:1481.
22. Fernandez-Arias C, Rivera-Correa J, Gallego-Delgado J, et al. Anti-self phosphatidylserine antibodies recognize uninfected erythrocytes promoting malarial anemia. *Cell Host Microbe*. 2016;19:194–203.
23. Fraser M, Jing W, Bröer S, Kurth F, Sander L-E, Matuschewski K, Maier AG. Breakdown in membrane asymmetry regulation leads to monocyte recognition of *P. falciparum*-infected red blood cells. *PLoS Pathog*. 2021;17 :e1009259.
24. Pawluczkowycz AW, Lindorfer MA, Waitumbi JN, et al. Hematin promotes complement alternative pathway-mediated deposition of C3 activation fragments on human erythrocytes: potential implications for the pathogenesis of anemia in malaria. *J Immunol*. 2007;179:5543.
25. Abdalla SH, Kasili FG, Weatherall DJ. The Coombs direct antiglobulin test in Kenyans. *Trans R Soc Trop Med Hyg*. 1983;77:99.
26. Stoute JA, Odindo AO, Owuor BO, et al. Loss of red blood cell-complement regulatory proteins and increased levels of circulating immune complexes are associated with severe malarial anemia. *J Infect Dis*. 2003; 187:522.
27. Allred DR. Immune evasion by *Babesia bovis* and *Plasmodium falciparum:* cliff-dwellers of the parasite world. *Parasitol Today*. 1995;11:100.
28. Wickramasinghe SN, Looareesuwan S, Nagachinta B, et al. Dyserythropoiesis and ineffective erythropoiesis in *Plasmodium vivax* malaria. *Br J Haematol*. 1989;72:91.
29. Wickramasinghe SN, Phillips RE, Looareesuwan S, et al. The bone marrow in human cerebral malaria: parasite sequestration within sinusoids. *Br J Haematol*. 1987;66:295.
30. Kwiatkowski D, Hill AV, Sambou I, et al. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. *Lancet*. 1990;336:1201.
31. Wyler DJ. The spleen in malaria. *Ciba Found Symp*. 1983;94:98.
32. Weiss L, Geduldig U, Weidanz W. Mechanisms of splenic control of murine malaria: reticular cell activation and the development of a blood-spleen barrier. *Am J Anat*. 1986;176:251.
33. Looareesuwan S, Ho M, Wattanagoon Y, et al. Dynamic alteration in splenic function during acute falciparum malaria. *N Engl J Med*. 1987; 317:675.
34. White NJ. Malaria parasite clearance. *Malar J*. 2017;16(1):88.
35. Lee SH, Looareesuwan S, Wattanagoon Y, et al. Antibody-dependent red cell removal during *P. falciparum* malaria: the clearance of red cells sensitized with an IgG anti-D. *Br J Haematol*. 1989;73:396.
36. Vryonis G. Observations on the parasitization of erythrocytes by *Plasmodium vivax*, with special reference to reticulocytes. *Am J Hyg*. 1939; 30:41.
37. Srichaikul T, Panikbutr N, Jeumtrakul P. Bone-marrow changes in human malaria. *Ann Trop Med Parasitol*. 1967;61:40.
38. Dörmer P, Dietrich M, Kern P, et al. Ineffective erythropoiesis in acute human *P. falciparum* malaria. *Blut*. 1983;46:279.
39. Abdalla S, Weatherall DJ, Wickramasinghe SN, et al. The anaemia of *P. falciparum* malaria. *Br J Haematol*. 1980;46:171.
40. Abdalla SH, Wickramasinghe SN. A study of erythroid progenitor cells in the bone marrow of Gambian children with falciparum malaria. *Clin Lab Haematol*. 1988;10:33.
41. Abdalla SH, Wickramasinghe SN, Weatherall DJ. The deoxyuridine suppression test in severe anaemia following *Plasmodium falciparum* malaria. *Trans R Soc Trop Med Hyg*. 1984;78:60.
42. Price RN, Simpson JA, Nosten F, et al. Factors contributing to anemia after uncomplicated falciparum malaria. *Am J Trop Med Hyg*. 2001;65:614.
43. Douglas NM, Anstey NM, Buffet PA, et al. The anaemia of *Plasmodium vivax* malaria. *Malar J*. 2012;11:135.
44. White NJ. Anaemia and malaria. *Malar J*. 2018;17:371.
45. Kurtzhals JA, Rodrigues O, Addae M, et al. Reversible suppression of bone marrow response to erythropoietin in *Plasmodium falciparum* malaria. *Br J Haematol*. 1997;97:169.
46. Newton CR, Warn PA, Winstanley PA, et al. Severe anaemia in children living in a malaria endemic area of Kenya. *Trop Med Int Health*. 1997;2:165.
47. Pascual C, Kai O, Cheung JO, et al. Suppression of erythropoiesis in malarial anemia is associated with hemozoin in vitro and in vivo. *Blood*. 2006;108:2569.
48. Abugri J, Tetteh JKA, Oseni LA, et al. Age-related pattern and monocyte-acquired haemozoin associated production of erythropoietin in children with severe malarial anaemia in Ghana. *BMC Res Notes*. 2014;7:551.
49. Casals-Pascual C, Idro R, Gicheru N, et al. High levels of erythropoietin are associated with protection against neurological sequelae in African children with cerebral malaria. *Proc Natl Acad Sci USA*. 2008;105:2634.
50. Shabani E, Opoka RO, Idro R, et al. High plasma erythropoietin levels are associated with prolonged coma duration and increased mortality in children with cerebral malaria. *Clin Infect Dis*. 2015;60:27.
51. Kurtzhals JA, Adabayeri V, Goka BQ, et al. Low plasma concentrations of interleukin 10 in severe malarial anaemia compared with cerebral and uncomplicated malaria. *Lancet*. 1998;351:1768.
52. Othoro C, Lal AA, Nahlen B, et al. A low interleukin-10 tumor necrosis factor-alpha ratio is associated with malaria anemia in children residing in a holoendemic malaria region in western Kenya. *J Infect Dis*. 1999;179:279.
53. Zamai L, Secchiero P, Pierpaoli S, et al. TNF-related apoptosis-inducing ligand (TRAIL) as a negative regulator of normal human erythropoiesis. *Blood*. 2000;95:3716.
54. de Mast Q, van Dongen-Lases EC, Swinkels DW, et al. Mild increases in serum hepcidin and interleukin-6 concentrations impair iron incorporation in haemoglobin during an experimental human malaria infection. *Br J Haematol*. 2009;145:657.
55. Howard CT, McKakpo US, Quakyi IA, et al. Relationship of hepcidin with parasitemia and anemia among patients with uncomplicated *Plasmodium falciparum* malaria in Ghana. *Am J Trop Med Hyg*. 2007;77:623.
56. Portugal S, Carret C, Recker M, et al. Host-mediated regulation of superinfection in malaria. *Nat Med*. 2011;17:732.
57. Clark MA, Goheen MM, Fulford A, et al. Host iron status and iron supplementation mediate susceptibility to erythrocytic stage *Plasmodium falciparum*. *Nat Commun*. 2014;5:4446.
58. Burrack KS, Hart GT, Hamilton SE. Contributions of natural killer cells to the immune response against *Plasmodium*. *Malar J*. 2019;18:321.

59. Askenase MH, Han S-J, Byrd AL, et al. Bone-marrow-resident NK cells prime monocytes for regulatory function during infection. *Immunity*. 2015;42:1130–1142.
60. Schwarzer E, Turrini F, Ulliers D, et al. Impairment of macrophage functions after ingestion of *Plasmodium falciparum*-infected erythrocytes or isolated malarial pigment. *J Exp Med*. 1992;176:1033.
61. Schwarzer E, Möller O, Arese P, et al. Increased levels of 4-hydroxynonenal in human monocytes fed with malarial pigment hemozoin. A possible clue for hemozoin toxicity. *FEBS Lett*. 1996;388:119.
62. Fiori PL, Rappelli P, Mirkarimi SN, et al. Reduced microbicidal and anti-tumour activities of human monocytes after ingestion of *Plasmodium falciparum*-infected red blood cells. *Parasite Immunol*. 1993;15:647.
63. Schwarzer E, Ludwig P, Valente E, et al. 15(S)-hydroxyeicosatetraenoic acid (15-HETE), a product of arachidonic acid peroxidation, is an active component of hemozoin toxicity to monocytes. *Parassitologia*. 1999; 41:199.
64. Skorokhod OA, Caione L, Marrocco T, et al. Inhibition of erythropoiesis in malaria anemia: role of hemozoin and hemozoin-generated 4-hydroxynonenal. *Blood*. 2010;116:4328.
65. Lamikanra AA, Merryweather-Clarke AT, Tipping AJ, et al. Distinct mechanisms of inadequate erythropoiesis induced by tumor necrosis factor alpha or malarial pigment. *PLoS One*. 2015;10:e0119836.
66. Berkley J, Mwarumba S, Bramham K, et al. Bacteraemia complicating severe malaria in children. *Trans R Soc Trop Med Hyg*. 1999;93:283.
67. Mooney JP, Galloway LJ, Riley EM. Malaria, anemia, and invasive bacterial disease: a neutrophil problem? *J Leukoc Biol*. 2019;105:645.
68. Herr W, Krumkamp R, Hogan B, et al. A cross-sectional study on risk factors for infection with Parvovirus B19 and the association with anaemia in a febrile paediatric population in Ghana. *Sci Rep*. 2020;10:15695.
69. Brewster DR, Greenwood BM. Seasonal variation of paediatric diseases in The Gambia, west Africa. *Ann Trop Paediatr*. 1993;13:133.
70. Dicko A, Klion AD, Thera MA, et al. The etiology of severe anemia in a village and a periurban area in Mali. *Blood*. 2004;104:1198.
71. Menendez C, Kahigwa E, Hirt R, et al. Randomised placebo-controlled trial of iron supplementation and malaria chemoprophylaxis for prevention of severe anaemia and malaria in Tanzanian infants. *Lancet*. 1997;350:844.
72. Schellenberg D, Menendez C, Kahigwa E, et al. Intermittent treatment for malaria and anaemia control at time of routine vaccinations in Tanzanian infants: a randomised, placebo-controlled trial. *Lancet*. 2001;357:1471.
73. Neuberger A, Okebe J, Yahav D, et al. Oral iron supplements for children in malaria-endemic areas. *Cochrane Database Syst Rev*. 2016;2:CD006589.
74. Drakesmith H, Prentice AM. Hepcidin and the iron-infection axis. *Science*. 2012;338:768–772.
75. Marsh K, Forster D, Waruiru C, et al. Indicators of life-threatening malaria in African children. *N Engl J Med*. 1995;332:1399.
76. Snow RW, Omumbo JA, Lowe B, et al. Relation between severe malaria morbidity in children and level of *Plasmodium falciparum* transmission in Africa. *Lancet*. 1997;349:1650.
77. Krishna S, Waller DW, ter Kuile F, et al. Lactic acidosis and hypoglycaemia in children with severe malaria: pathophysiological and prognostic significance. *Trans R Soc Trop Med Hyg*. 1994;88:67.
78. English M, Waruiru C, Marsh K. Transfusion for respiratory distress in life-threatening childhood malaria. *Am J Trop Med Hyg*. 1996;55:525.
79. Maitland K, Kiguli S, Opoka RO, et al. Mortality after fluid bolus in African children with severe infection. *N Engl J Med*. 2011;364:2483.
80. Maitland K, Olupot-Olupot P, Kiguli S, et al. Transfusion volume for children with severe anemia in Africa TRACT Group. *N Engl J Med*. 2019;381:420.
81. Maitland K, Kiguli S, Olupot-Olupot P, et al. Transfusion management of severe anaemia in African children: a consensus algorithm. *Br J Haematol*. 2021;193:1247.
82. Dhabangi A, Ainomugisha B, Cserti-Gazdewich C, et al. B-type natriuretic peptide and plasma hemoglobin levels following transfusion of shorter-storage versus longer-storage red blood cells: results from the TOTAL randomized trial. *Am Heart J*. 2017;183:129.
83. Price R, van Vugt M, Phaipun L, et al. Adverse effects in patients with acute falciparum malaria treated with artemisinin derivatives. *Am J Trop Med Hyg*. 1999;60:547.
84. Roussel C, Ndour PA, Kendjo E, et al. Intravenous artesunate for the treatment of severe imported malaria: implementation, efficacy, and safety in 1391 patients. *Clin Infect Dis*. 2021;73(10):1795–1804.
85. Ndour PA, Larréché S, Mouri O, et al. Measuring the *Plasmodium falciparum* HRP2 protein in blood from artesunate-treated malaria patients predicts post-artesunate delayed hemolysis. *Sci Transl Med*. 2017;9:eaaf9377.
86. Ladhani S, Lowe B, Cole AO, et al. Changes in white blood cells and platelets in children with falciparum malaria: relationship to disease outcome. *Br J Haematol*. 2002;119:839.
87. Amodu OK, Adeyemo AA, Olumese PE, et al. Intraleucocytic malaria pigment and clinical severity of malaria in children. *Trans R Soc Trop Med Hyg*. 1998;92:54.
88. Lyke KE, Diallo DA, Dicko A, et al. Association of intraleukocytic *Plasmodium falciparum* malaria pigment with disease severity, clinical manifestations, and prognosis in severe malaria. *Am J Trop Med Hyg*. 2003;69:253.
89. Grau GE, Mackenzie CD, Carr RA, et al. Platelet accumulation in brain microvessels in fatal pediatric cerebral malaria. *J Infect Dis*. 2003;187:461.
90. Pain A, Ferguson DJ, Kai O, et al. Platelet-mediated clumping of *Plasmodium falciparum*-infected erythrocytes is a common adhesive phenotype and is associated with severe malaria. *Proc Natl Acad Sci USA*. 2001;98:1805.
91. McMorran BJ, Wieczorski L, Drysdale KE, et al. Platelet factor 4 and Duffy antigen required for platelet killing of *Plasmodium falciparum*. *Science*. 2012;338:1348.
92. Jongruamklang P, Rebetz J, Kapur R, et al. Platelets inhibit erythrocyte invasion by *Plasmodium falciparum* at physiological platelet: erythrocyte ratios. *Transfus Med. Epub ahead of print*. 2021 https://doi.org/10.1111/tme.12791.
93. Love MS, Millholland MG, Mishra S, et al. Platelet factor 4 activity against P. falciparum and its translation to nonpeptidic mimics as antimalarials. *Cell Host Microbe*. 2012;12:815.
94. MacPherson GG, Warrell MJ, White NJ, et al. Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. *Am J Pathol*. 1985;119:385.
95. Holst FG, Hemmer CJ, Foth C, et al. Low levels of fibrin-stabilizing factor (factor XIII) in human *Plasmodium falciparum* malaria: correlation with clinical severity. *Am J Trop Med Hyg*. 1999;60:99.
96. Kessler A, Dankwa S, Bernabeu M, et al. Linking EPCR-binding PfEMP1 to brain swelling in pediatric cerebral malaria. *Cell Host Microbe*. 2017; 22:601.
97. Fried M, Duffy PE. Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. *Science*. 1996;272:1502.
98. Beeson JG, Rogerson SJ, Cooke BM, et al. Adhesion of *Plasmodium falciparum*-infected erythrocytes to hyaluronic acid in placental malaria. *Nat Med*. 2000;6:86.
99. Rogerson SJ, Desai M, Mayor A, et al. Burden, pathology, and costs of malaria in pregnancy: new developments for an old problem. *Lancet Infect Dis*. 2018;18:e107.
100. Guyatt HL, Snow RW. Malaria in pregnancy as an indirect cause of infant mortality in sub-Saharan Africa. *Trans R Soc Trop Med Hyg*. 2001;95:569.
101. Desai M, Hill J, Fernandes S, et al. Prevention of malaria in pregnancy. *Lancet Infect Dis*. 2018;18:e119.
102. Bedu-Addo G, Bates I. Causes of massive tropical splenomegaly in Ghana. *Lancet*. 2002;360:449.
103. Zhu D, Thompsett AR, Bedu-Addo G, et al. VH gene sequences from a novel tropical splenic lymphoma reveal a naive B cell as the cell of origin. *Br J Haematol*. 1999;107:114.
104. Leoni S, Buonfrate D, Angheben A, Gobbi F, Bisoffi Z. The hyper-reactive malarial splenomegaly: a systematic review of the literature. *Malar J*. 2015;14:185.
105. Price RN, Commons RJ, Battle KE, et al. *Plasmodium vivax* in the era of the shrinking *P. falciparum* map. *Trends Parasitol*. 2020;36(6):560.
106. Gilles HM, Hendrickse RG. Possible aetiological role of *Plasmodium malariae* in "nephrotic syndrome" in Nigerian children. *Lancet*. 1960;1:806.
107. Warhurst DC, Williams JE. ACP broadsheet no 148. July 1996. Laboratory diagnosis of malaria. *J Clin Pathol*. 1996;49:533.
108. Bain BJ, Bates I, Laffan M, Lewis SM, Dacie JV. *Dacie and Lewis Practical Haematology*. 12th ed. Edinburgh, UK: Churchill Livingstone; 2016.
109. Bailey JW, Williams J, Bain BJ, et al. Guideline: the laboratory diagnosis of malaria. General haematology task force of the British Committee for Standards in Haematology. *Br J Haematol*. 2013;163:573.
110. Miller CE, Bain BJ. The utility of blood and bone marrow films and trephine biopsy sections in the diagnosis of parasitic infections. *Mediterr J Hematol Infect Dis*. 2015;7:e2015039.
111. Moody A. Rapid diagnostic tests for malaria parasites. *Clin Microbiol Rev*. 2002;15:66.
112. Milne LM, Kyi MS, Chiodini PL, et al. Accuracy of routine laboratory diagnosis of malaria in the United Kingdom. *J Clin Pathol*. 1994; 47:740.
113. Kavanaugh MJ, Azzam SE, Rockabrand DM. Malaria rapid diagnostic tests: literary review and recommendation for a quality assurance, quality control algorithm *Diagnostics (Basel)*. 112021768.
114. Morris U, Aydin-Schmidt B. Performance and application of commercially available loop-mediated isothermal amplification (LAMP) kits in malaria endemic and non-endemic settings. *Diagnostics (Basel)*. 2021;11(2):336.

115. Amir A, Cheong FW, De Silva JR, Lau YL. Diagnostic tools in childhood malaria. *Parasit Vectors*. 2018;11:53.
116. Huong NM, Davis TME, Hewitt S, et al. Comparison of three antigen detection methods for diagnosis and therapeutic monitoring of malaria: a field study from southern Vietnam. *Trop Med Int Health*. 2002;7:304.
117. Lalloo DG, Shingadia D, Bell DJ, et al. UK malaria treatment guidelines 2016. *J Infect*. 2016;72:635–649.
118. McNamara DT, Thomson JM, Kasehagen LJ, et al. Development of a multiplex PCR-ligase detection reaction assay for diagnosis of infection by the four parasite species causing malaria in humans. *J Clin Microbiol*. 2004;42:2403.
119. Benito A, Rubio JM. Usefulness of seminested polymerase chain reaction for screening blood donors at risk for malaria in Spain. *Emerg Infect Dis*. 2001; 7:1068.
120. WorldWide Antimalarial Resistance Network (WWARN) Lumefantrine PK/PD Study Group. Artemether-lumefantrine treatment of uncomplicated *Plasmodium falciparum* malaria: a systematic review and meta-analysis of day 7 lumefantrine concentrations and therapeutic response using individual patient data. *BMC Med*. 2015;13:227.
121. Woodrow CJ, White NJ. The clinical impact of artemisinin resistance in Southeast Asia and the potential for future spread. *FEMS Microbiol Rev*. 2017;41:34.
122. Dondorp AM, Fanello CI, Hendriksen IC, et al. Artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT): an open-label, randomised trial. *Lancet*. 2010;376:1647.
123. Yooda AP, Sawadogo S, Soubeiga ST, et al. Residual risk of HIV, HCV, and HBV transmission by blood transfusion between 2015 and 2017 at the Regional Blood Transfusion Center of Ouagadougou, Burkina Faso. *J Blood Med*. 2019;10:53.
124. Tan KR, Wiegand RE, Arguin PM. Exchange transfusion for severe malaria: evidence base and literature review. *Clin Infect Dis*. 2013;57:923.
125. Bruce-Chwatt LJ. Transfusion malaria revisited. *Trop Dis Bull*. 1982;79:827.
126. De Silva M, Contreras M, Barbara J. Two cases of transfusion-transmitted malaria (TTM) in the UK. *Transfusion*. 1988;28:86.
127. Chiodini PL, Hartley S, Hewitt PE, et al. Evaluation of a malaria antibody ELISA and its value in reducing potential wastage of red cell donations from blood donors exposed to malaria, with a note on a case of transfusion-transmitted malaria. *Vox Sang*. 1997;73:143.
128. Kitchen A, Mijovic A, Hewitt P. Transfusion-transmitted malaria: current donor selection guidelines are not sufficient. *Vox Sang*. 2005;88:200.
129. Mungai M, Tegtmeier G, Chamberland M, et al. Transfusion-transmitted malaria in the United States from 1963 through 1999. *N Engl J Med*. 2001;344:1973.
130. Mali S, Tan KR, Arguin PM. Malaria surveillance—United States, 2009. *MMWR Surveill Summ*. 2011;60:1.
131. Kitchen AD, Barbara JAJ, Hewitt PE. Documented cases of post-transfusion malaria occurring in England: a review in relation to current and proposed donor-selection guidelines. *Vox Sang*. 2005;89:77.
132. Joint United Kingdom (UK) Blood Transfusion and Tissue Transplantation Services Professional Advisory Committee. Donor Selection Guidelines: Malaria www. https://www.transfusionguidelines.org/dsg/wb/guidelines/ma001-malaria.
133. Kitchen AD, Lowe PHJ, Lalloo K, et al. Evaluation of a malarial antibody assay for use in the screening of blood and tissue products for clinical use. *Vox Sang*. 2004;87:150.
134. Mangano VD, Perandin F, Tiberti N, et al. Risk of transfusion-transmitted malaria: evaluation of commercial ELISA kits for the detection of anti-*Plasmodium* antibodies in candidate blood donors. *Malar J*. 2019; 18:17.
135. O'Brien SF, Ward S, Gallian P, et al. Malaria blood safety policy in five non-endemic countries: a retrospective comparison through the lens of the ABO risk-based decision-making framework. *Blood Transfus*. 2017;17:94.
136. Alvar J, Vélez ID, Bern C, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS ONE*. 2012;7:e35671.
137. Ross R. Note on the bodies recently described by Leishman and Donovan. *Br Med J*. 1903;2:1261.
138. Garnham PC. The leishmanias, with special reference to the role of animal reservoirs. *Am Zool*. 1965;5:141.
139. Symmers WS. Leishmaniasis acquired by contagion: a case of marital infection in Britain. *Lancet*. 1960;1:127.
140. Campino L, Santos-Gomes G, Pratlong F, et al. The isolation of *Leishmania donovani* MON-18, from an AIDS patient in Portugal: possible needle transmission. *Parasite*. 1994;1:391.
141. Sampaio MJ, Cavalcanti NV, Alves JG, et al. Risk factors for death in children with visceral leishmaniasis. *PLoS Negl Trop Dis*. 2010;4:e877.
142. Badaro R, Jones TC, Carvalho EM, et al. New perspectives on a subclinical form of visceral leishmaniasis. *J Infect Dis*. 1986;154:1003.
143. Magill AJ, Grögl M, Gasser Jr RA, et al. Visceral infection caused by *Leishmania tropica* in veterans of Operation Desert Storm. *N Engl J Med*. 1993;328:1383.
144. Torres-Guerrero E, Quintanilla-Cedillo MR, Ruiz-Esmenjaud J, Arenas R. Leishmaniasis: a review. *F1000Res*. 2017;6:750.
145. Pratlong F, Dedet JP, Marty P, et al. Leishmania-human immunodeficiency virus coinfection in the Mediterranean basin: isoenzymatic characterization of 100 isolates of the *Leishmania infantum* complex. *J Infect Dis*. 1995; 172:323.
146. Alvar J, Cañavate C, Gutiérrez-Solar B, et al. Leishmania and human immunodeficiency virus coinfection: the first 10 years. *Clin Microbiol Rev*. 1997;10:298.
147. Ramos-Santos C, Hernandez-Montes O, Sanchez-Tejeda G, et al. Visceral leishmaniosis caused by *Leishmania (L.) mexicana* in a Mexican patient with human immunodeficiency virus infection. *Mem Inst Oswaldo Cruz*. 2000; 95:733.
148. van Griensven J, Carrillo E, López-Vélez R, Lynen L, Moreno J. Leishmaniasis in immunosuppressed individuals. *Clin Microbiol Infect*. 2014;20:286.
149. Lindoso JA, Cunha MA, Queiroz IT, et al. Leishmaniasis-HIV coinfection: current challenges. *HIV AIDS (Auckl)*. 2016;8:147–156.
150. Mary C, Lamouroux D, Dunan S, et al. Western blot analysis of antibodies to *Leishmania infantum* antigens: potential of the 14-kD and 16-kD antigens for diagnosis and epidemiologic purposes. *Am J Trop Med Hyg*. 1992;47:764.
151. Basset D, Faraut F, Marty P, et al. Visceral leishmaniasis in organ transplant recipients: 11 new cases and a review of the literature. *Microbes Infect*. 2005;7:1370.
152. al-Jurayyan NA, al-Nasser MN, al-Fawaz IM, et al. The haematological manifestations of visceral leishmaniasis in infancy and childhood. *J Trop Pediatr*. 1995;41:143.
153. Wickramasinghe SN, Abdalla SH, Kasili EG. Ultrastructure of bone marrow in patients with visceral leishmaniasis. *J Clin Pathol*. 1987;40:267.
154. Goto Y, Cheng J, Omachi S, Morimoto A. Prevalence, severity, and pathogeneses of anemia in visceral leishmaniasis. *Parasitol Res*. 2017;116: 457.
155. Pippard MJ, Moir D, Weatherall DJ, et al. Mechanism of anaemia in resistant visceral leishmaniasis. *Ann Trop Med Parasitol*. 1986;80:317.
156. Saeed AM, Khalil EA, Elhassan AM, et al. Serum erythropoietin concentration in anaemia of visceral leishmaniasis (kala-azar) before and during antimonial therapy. *Br J Haematol*. 1998;100:720.
157. Sinha AK, Rijal S, Karki P, et al. Incidence of megaloblastic anaemia and its correction in leishmaniasis—a prospective study at BPKIHS hospital, Nepal. *Indian J Pathol Microbiol*. 2006;49:528.
158. Vilela RB, Bordin JO, Chiba AK, et al. RBC-associated IgG in patients with visceral leishmaniasis (kala-azar): a prospective analysis. *Transfusion*. 2002; 42:1442.
159. Scatena P, Messina F, Gori S, et al. Visceral leishmaniasis in a patient treated for polyarteritis nodosa. *Clin Exp Rheumatol*. 2003;21(6 Suppl 32): S121.
160. Bhatia P, Haldar D, Varma N, et al. A case series highlighting the relative frequencies of the common, uncommon and atypical/unusual hematological findings on bone marrow examination in cases of visceral leishmaniasis. *Mediterr J Hematol Infect Dis*. 2011;3:e2011035.
161. Boelaert M, Verdonck K, Menten J, et al. Rapid tests for the diagnosis of visceral leishmaniasis in patients with suspected disease. *Cochrane Database Syst Rev*. 2014;6:CD009135.
162. Thakur S, Joshi J, Kaur S. Leishmaniasis diagnosis: an update on the use of parasitological, immunological and molecular methods. *J Parasit Dis*. 2020;44:1.
163. Nicolle C. Isolement et culture des corps de Leishman. *Arch Inst Pasteur Tunis*. 1908;2:55.
164. Evans DA. *Handbook on Isolation, Characterization and Cryopreservation of Leishmania*. Geneva: World Health Organization; 1989.
165. Srivastava P, Mehrotra S, Tiwary P, et al. Diagnosis of Indian visceral leishmaniasis by nucleic acid detection using PCR. *PLoS One*. 2011; 6:e19304.
166. Antinori S, Calattini S, Longhi E, et al. Clinical use of polymerase chain reaction performed on peripheral blood and bone marrow samples for the diagnosis and monitoring of visceral leishmaniasis in HIV-infected and HIV-uninfected patients: a single-center, 8-year experience in Italy and review of the literature. *Clin Infect Dis*. 2007;44:1602.
167. Sundar S, Singh OP. Molecular diagnosis of visceral leishmaniasis. *Mol Diagn Ther*. 2018;22:443.
168. Nzelu CO, Kato H, Peters NC. Loop-mediated isothermal amplification (LAMP): an advanced molecular point-of-care technique for the detection of *Leishmania* infection. *PLoS Negl Trop Dis*. 2019;13:e0007698.

169. Meredith SE, Kroon NC, Sondorp E, et al. Leish-KIT, a stable direct agglutination test based on freeze-dried antigen for serodiagnosis of visceral leishmaniasis. *J Clin Microbiol*. 1995;33:1742.
170. Singh S, Gilman-Sachs A, Chang KP, et al. Diagnostic and prognostic value of K39 recombinant antigen in Indian leishmaniasis. *J Parasitol*. 1995; 81:1000.
171. Chappuis F, Rijal S, Soto A, et al. A meta-analysis of the diagnostic performance of the direct agglutination test and rK39 dipstick for visceral leishmaniasis. *BMJ*. 2006;333:723.
172. World Health Organization Control of the leishmaniases. *World Health Organ Tech Rep Ser*. 2010;949:1.
173. Copeland NK, Aronson NE. Leishmaniasis: treatment updates and clinical practice guidelines review. *Curr Opin Infect Dis*. 2015;28:426.
174. Sundar S, Singh A. Chemotherapeutics of visceral leishmaniasis: present and future developments. *Parasitology*. 2018;145:481.
175. Smith SR, Galloway MJ, Reilly JT, et al. Amiloride prevents amphotericin B related hypokalaemia in neutropenic patients. *J Clin Pathol*. 1988;41:494.
176. Sundar S, Jha TK, Thakur CP, et al. Oral miltefosine for Indian visceral leishmaniasis. *N Engl J Med*. 2002;347:1739.
177. Ritmeijer K, Dejenie A, Assefa Y, et al. A comparison of miltefosine and sodium stibogluconate for treatment of visceral leishmaniasis in an Ethiopian population with high prevalence of HIV infection. *Clin Infect Dis*. 2006;43:357.
178. Sundar S, Jha TK, Thakur CP, et al. Injectable paromomycin for visceral leishmaniasis in India. *N Engl J Med*. 2007;356:2571.
179. Melaku Y, Collin SM, Keus K, et al. Treatment of kala-azar in southern Sudan using a 17-day regimen of sodium stibogluconate combined with paromomycin: a retrospective comparison with 30-day sodium stibogluconate monotherapy. *Am J Trop Med Hyg*. 2007;77:89.
180. Jimenez-Marco T, Fisa R, Girona-Llobera E, et al. Transfusion-transmitted leishmaniasis: a practical review. *Transfusion*. 2016;56:S45–S51.
181. Cardo LJ. Leishmania: risk to the blood supply. *Transfusion*. 2006;46:1641.
182. Simarro PP, Cecchi G, Paone M, et al. The atlas of human African trypanosomiasis: a contribution to global mapping of neglected tropical diseases. *Int J Health Geogr*. 2010;9:57.
183. Kennedy PGE, Rodgers J. Clinical and neuropathogenetic aspects of human African trypanosomiasis. *Front Immunol*. 2019;10:39.
184. Silva Pereira S, Jackson AP, Figueiredo LM. Evolution of the variant surface glycoprotein family in African trypanosomes. *Trends Parasitol*. 2021;S1471-4922(21). 00177-0017.
185. Smith AB, Esko JD, Hajduk SL. Killing of trypanosomes by the human haptoglobin-related protein. *Science*. 1995;268:284.
186. Vanhollebeke B, De Muylder G, Nielsen MJ, et al. A haptoglobin-hemoglobin receptor conveys innate immunity to *Trypanosoma brucei* in humans. *Science*. 2008;320:677.
187. Smith EE, Malik HS. The apolipoprotein L family of programmed cell death and immunity genes rapidly evolved in primates at discrete sites of host-pathogen interactions. *Genome Res*. 2009;19:850.
188. Genovese G, Friedman DJ, Ross MD, et al. Association of trypanolytic ApoL1 variants with kidney disease in African Americans. *Science*. 2010; 329:841.
189. Meda HA, Doua F, Laveissière C, et al. Human immunodeficiency virus infection and human African trypanosomiasis: a case-control study in Côte d'Ivoire. *Trans R Soc Trop Med Hyg*. 1995;89:639.
190. Sudarshi D, Lawrence S, Pickrell WO, et al. Human African trypanosomiasis presenting at least 29 years after infection—what can this teach us about the pathogenesis and control of this neglected tropical disease? *PLoS Negl Trop Dis*. 2014;8:e3349.
191. Boodman C, Libman M, Ndao M, Yansouni CP. Case report: *Trypanosoma brucei gambiense* human African trypanosomiasis as the cause of fever in an inpatient with multiple myeloma and HIV-1 coinfection. *Am J Trop Med Hyg*. 2019;101:123.
192. Chisi JE, Misiri H, Zverev Y, et al. Anaemia in human African trypanosomiasis caused by *Trypanosoma brucei rhodesiense*. *East Afr Med J*. 2004;81:505.
193. Moore AC, Ryan ET, Waldron MA. Case records of the Massachusetts General Hospital. Weekly clinicopathological exercises. Case 20-2002—a 37-year-old man with fever, hepatosplenomegaly, and a cutaneous foot lesion after a trip to Africa. *N Engl J Med*. 2002;346:2069.
194. Woo PT. The haematocrit centrifuge for the detection of trypanosomes in blood. *Can J Zool*. 1969;47:921.
195. Bailey JW, Smith DH. The use of the acridine orange QBC technique in the diagnosis of African trypanosomiasis. *Trans R Soc Trop Med Hyg*. 1992; 86:630.
196. Lumsden WH, Kimber CD, Evans DA, et al. *Trypanosoma brucei:* miniature anion-exchange centrifugation technique for detection of low parasitaemias: adaptation for field use. *Trans R Soc Trop Med Hyg*. 1979;73:312.
197. Chappuis F, Loutan L, Simarro P, Lejon V, Büscher P. Options for field diagnosis of human African trypanosomiasis. *Clin Microbiol Rev*. 2005; 18:133.
198. Deborggraeve S, Lejon V, Ekangu RA, et al. Diagnostic accuracy of PCR in gambiense sleeping sickness diagnosis, staging and post-treatment follow-up: a 2-year longitudinal study. *PLoS Negl Trop Dis*. 2011;5:e972.
199. Mugasa CM, Adams ER, Boer KR, et al. Diagnostic accuracy of molecular amplification tests for human African trypanosomiasis—systematic review. *PLoS Negl Trop Dis*. 2012;6:e1438.
200. Cattand P, Miezan BT, de Raadt P. Human African trypanosomiasis: use of double centrifugation of cerebrospinal fluid to detect trypanosomes. *Bull World Health Organ*. 1988;66:83.
201. Magnus E, Vervoort T, Van Meirvenne N. A card-agglutination test with stained trypanosomes (C.A.T.T.) for the serological diagnosis of *T. b. gambiense* trypanosomiasis. *Ann Soc Belg Med Trop*. 1978;58:169.
202. Pansaerts R, Van Meirvenne N, Magnus E, et al. Increased sensitivity of the card agglutination test CATT/*Trypanosoma brucei gambiense* by inhibition of complement. *Acta Trop*. 1998;70:349.
203. Nantulya VM. TrypTect CIATT—a card indirect agglutination trypanosomiasis test for diagnosis of *Trypanosoma brucei gambiense* and *T. b. rhodesiense* infections. *Trans R Soc Trop Med Hyg*. 1997;91:551.
204. Büscher P, Lejon V, Magnus E, et al. Improved latex agglutination test for detection of antibodies in serum and cerebrospinal fluid of *Trypanosoma brucei gambiense* infected patients. *Acta Trop*. 1999;73:11.
205. Lejon V, Büscher P, Sema NH, et al. Human African trypanosomiasis: a latex agglutination field test for quantifying IgM in cerebrospinal fluid. *Bull World Health Organ*. 1998;76:553.
206. Steinmann P, Stone CM, Sutherland CS, et al. Contemporary and emerging strategies for eliminating human African trypanosomiasis due to *Trypanosoma brucei gambiense:* review. *Trop Med Int Health*. 2015;6:707–718.
207. Priotto G, Kasparian S, Mutombo W, et al. Nifurtimox-eflornithine combination therapy for second-stage African *Trypanosoma brucei gambiense* trypanosomiasis: a multicentre, randomised, phase III, non-inferiority trial. *Lancet*. 2009;374:56.
208. Schmid C, Richer M, Bilenge CM, et al. Effectiveness of a 10-day melarsoprol schedule for the treatment of late-stage human African trypanosomiasis: confirmation from a multinational study (IMPAMEL II). *J Infect Dis*. 2005;191:1922.
209. Pépin J, Milord F, Khonde AN, et al. Risk factors for encephalopathy and mortality during melarsoprol treatment of *Trypanosoma brucei gambiense* sleeping sickness. *Trans R Soc Trop Med Hyg*. 1995;89:92.
210. Pépin J, Milord F, Guern C, et al. Trial of prednisolone for prevention of melarsoprol-induced encephalopathy in gambiense sleeping sickness. *Lancet*. 1989;1:1246.
211. Pépin J, Milord F, Khonde A, et al. Gambiense trypanosomiasis: frequency of, and risk factors for, failure of melarsoprol therapy. *Trans R Soc Trop Med Hyg*. 1994;88:447.
212. Babokhov P, Sanyaolu AO, Oyibo WA, et al. A current analysis of chemotherapy strategies for the treatment of human African trypanosomiasis. *Pathog Glob Health*. 2013;5:242–252.
213. World Health Organization. Control and surveillance of human African trypanosomiasis: report of a WHO expert committee. https://apps.who.int/iris/handle/10665/95732.
214. Coura JR, Borges-Pereira J. Chagas disease: 100 years after its discovery. A systemic review. *Acta Trop*. 2010;115:5.
215. Basile L, Jansá JM, Carlier Y. Chagas disease in European countries: the challenge of a surveillance system. *Euro Surveill*. 2011;16(37):19968.
216. El-Sayed NM, Myler PJ, Bartholomeu DC, et al. The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. *Science*. 2005; 309:409.
217. El-Sayed NM, Myler PJ, Blandin G, et al. Comparative genomics of trypanosomatid parasitic protozoa. *Science*. 2005;309:404.
218. Herreros-Cabello A, Callejas-Hernández F, Gironès N, Fresno M. *Trypanosoma cruzi* genome: organization, multi-gene families, transcription, and biological implications. *Genes*. 2020;11:1196.
219. Vallejo GA, Chiari E, Macedo AM, et al. A simple laboratory method for distinguishing between *Trypanosoma cruzi* and *Trypanosoma rangeli*. *Trans R Soc Trop Med Hyg*. 1993;87:165.
220. Rios LE, Vázquez-Chagoyán JC, Pacheco AO, et al. Immunity and vaccine development efforts against *Trypanosoma cruzi*. *Acta Trop*. 2019; 200:105168.
221. Bonney KM, Luthringer DJ, Kim SA, et al. Pathology and pathogenesis of Chagas heart disease. *Annu Rev Pathol*. 2019;14:421.
222. Rios L, Campos EE, Menon R, et al. Epidemiology and pathogenesis of maternal-fetal transmission of Trypanosoma cruzi and a case for vaccine development against congenital Chagas disease. *Biochim Biophys Acta Mol Basis Dis*. 2020;1866:165591.

223. Chin-Hong PV, Schwartz BS, Bern C, et al. Screening and treatment of Chagas disease in organ transplant recipients in the United States: recommendations from the chagas in transplant working group. *Am J Transplant*. 2011;11:672.
224. Luz ZM, Coutinho MG, Cancado JR, et al. Hemoculture: sensitive technique in the detection of *Trypanosoma cruzi* in chagasic patients in the chronic phase of Chagas disease. *Rev Soc Bras Med Trop*. 1994;27:143.
225. WHO Expert Committee *Control of Chagas Disease: second report of the WHO expert committee. WHO technical report series number 905*. Brasilia, Brazil: World Health Organization; 2002.
226. Russomando G, Sánchez Z, Meza G, et al. Shed acute-phase antigen protein in an ELISA system for unequivocal diagnosis of congenital Chagas disease. *Expert Rev Mol Diagn*. 2010;10:705.
227. Brasil PE, Castro R, Castro L. Commercial enzyme-linked immunosorbent assay versus polymerase chain reaction for the diagnosis of chronic Chagas disease: a systematic review and meta-analysis. *Mem Inst Oswaldo Cruz*. 2016;111:1–19.
228. Malik LH, Singh GD, Amsterdam EA. The epidemiology, clinical manifestations, and management of Chagas heart disease. *Clin Cardiol*. 2015;9:565.
229. Soriano-Arandes A, Angheben A, Serre-Delcor N, et al. Control and management of congenital Chagas disease in Europe and other non-endemic countries: current policies and practices. *Trop Med Int Health*. 2016;5:590–596.
230. Sales Jr PA, Molina I, Fonseca Murta SM, et al. Experimental and clinical treatment of Chagas disease: a review. *Am J Trop Med Hyg*. 2017;97:1289.
231. Guhl F, Lazdins-Helds JK, eds. *Reporte del grupo de trabajo científico sobre la enfermedad de Chagas*. Geneva: World Health Organization; 2007. http://www.who.int/tdr/publications/tdr-research-publications/reporte-enfermedad-chagas/en/index.html.
232. Benjamin RJ, Stramer SL, Leiby DA, Dodd RY, Fearon M, Castro E. *Trypanosoma cruzi* infection in North America and Spain: evidence in support of transfusion transmission. *Transfusion*. 2012;52:1913.
233. Kessler DA, Shi PA, Avecilla ST, Shaz BH. Results of lookback for Chagas disease since the inception of donor screening at New York Blood Center. *Transfusion*. 2013;53:1083.
234. Blumental S, Lambermont M, Heijmans C, et al. First documented transmission of *Trypanosoma cruzi* infection through blood transfusion in a child with sickle-cell disease in Belgium. *PLoS Negl Trop Dis*. 2015; 9:e0003986.
235. Bern C, Messenger LA, Whitman JD, Maguire JH. Chagas disease in the United States: a public health approach. *Clin Microbiol Rev*. 2019; 33:e00023–19.
236. Bern C, Montgomery SP, Katz L, Caglioti S, Stramer SL. Chagas disease and the US blood supply. *Curr Opin Infect Dis*. 2008;21:476.
237. Requena-Méndez A, Albajar-Viñas P, Angheben A, et al. Health policies to control Chagas disease transmission in European countries. *PLoS Negl Trop Dis*. 2014;8:e3245.
238. Van Voorhis WC, Barrett LK, Eastman RT, et al. *Trypanosoma cruzi* inactivation in human platelet concentrates and plasma by a psoralen (amotosalen HCl) and long-wavelength UV. *Antimicrob Agents Chemother*. 2003;47:475.
239. Schofield CJ, Jannin J, Salvatella R. The future of Chagas disease control. *Trends Parasitol*. 2006;22:583.
240. Lidani KCF, Andrade FA, Bavia L, et al. Chagas disease: from discovery to a worldwide health problem. *Front Public Health*. 2019;7:166.
241. Hunfeld K-P, Hildebrandt A, Gray JS. Babesiosis: recent insights into an ancient disease. *Int J Parasitol*. 2008;38:1219.
242. Vannier E, Krause PJ. Human babesiosis. *N Engl J Med*. 2012;366:2397.
243. Kakoma I, Mehlhorn H. *Babesia* of domestic animals vol 7. In: Kreier JP, ed. *Parasitic protozoa*. 2nd ed. San Diego: Academic Press; 1994:141.
244. Herwaldt B, Persing DH, Précigout EA, et al. A fatal case of babesiosis in Missouri: identification of another piroplasm that infects humans. *Ann Intern Med*. 1996;124:643.
245. Bloch EM, Herwaldt BL, Leiby DA, et al. The third described case of transfusion-transmitted *Babesia duncani*. *Transfusion*. 2012;52:1517.
246. Blum S, Gattringer R, Haschke E, et al. The case: hemolysis and acute renal failure. *Kidney Int*. 2011;80:681.
247. Jiang J-F, Zheng Y-C, Jiang R-R, et al. Epidemiological, clinical, and laboratory characteristics of 48 cases of "*Babesia venatorum*" infection in China: a descriptive study. *Lancet Infect Dis*. 2015;15:196.
248. Herwaldt BL, Linden JV, Bosserman E, Young C, Olkowska D, Wilson M. Transfusion-associated babesiosis in the United States: a description of cases. *Ann Intern Med*. 2011;155:509.
249. Fang DC, McCullough J. Transfusion-transmitted *Babesia microti*. *Transfus Med Rev*. 2016;30:132.
250. Meldrum SC, Birkhead GS, White DJ, et al. Human babesiosis in New York State: an epidemiological description of 136 cases. *Clin Infect Dis*. 1992;15:1019.
251. Hatcher JC, Greenberg PD, Antique J, et al. Severe babesiosis in Long Island: review of 34 cases and their complications. *Clin Infect Dis*. 2001; 32:1117.
252. Akel T, Mobarakai N. Hematologic manifestations of babesiosis. *Ann Clin Microbiol Antimicrob*. 2017;16:6.
253. Ruebush 2nd TK, Juranek DD, Chisholm ES, et al. Human babesiosis on Nantucket Island. Evidence for self-limited and subclinical infections. *N Engl J Med*. 1977;297:825.
254. Benach JL, Habicht GS. Clinical characteristics of human babesiosis. *J Infect Dis*. 1981;144:481.
255. Ong KR, Stavropoulos C, Inada Y. Babesiosis, asplenia, and AIDS. *Lancet*. 1990;336:112.
256. Falagas ME, Klempner MS. Babesiosis in patients with AIDS: a chronic infection presenting as fever of unknown origin. *Clin Infect Dis*. 1996; 22:809.
257. Berry A, Morassin B, Kamar N, et al. Clinical picture: human babesiosis. *Lancet*. 2001;357:341.
258. Kjemtrup AM, Conrad PA. Human babesiosis: an emerging tick-borne disease. *Int J Parasitol*. 2000;30:1323.
259. Martinot M, Zadeh MM, Hansmann Y, et al. Babesiosis in immunocompetent patients, Europe. *Emerg Infect Dis*. 2011;17:114.
260. Gulersen M, Brost BC, Bobrovnikov V, et al. Acute babesiosis in pregnancy: a novel imitator of hemolysis, elevated liver enzymes, and low platelet count syndrome. *Obstet Gynecol*. 2016;128:197.
261. Uhnoo I, Cars O, Christensson D, et al. First documented case of human babesiosis in Sweden. *Scand J Infect Dis*. 1992;24:541.
262. Woolley AE, Montgomery MW, Savage WJ, et al. Post-babesiosis warm autoimmune hemolytic anemia. *N Engl J Med*. 2017;376:939.
263. Santos MA, Tierney Jr LM, Manesh R. Babesiosis-associated warm autoimmune hemolytic anemia. *J Gen Intern Med*. 2020;35:928.
264. Rajapakse P, Bakirhan K. Autoimmune hemolytic anemia associated with human babesiosis. *J Hematol*. 2021;10:41.
265. Sun T, Tenenbaum MJ, Greenspan J, et al. Morphologic and clinical observations in human infection with *Babesia microti*. *J Infect Dis*. 1983; 148:239.
266. Chan WY, MacDonald C, Keenan A, Xu K, Bain BJ, Chiodini PL. Severe babesiosis due to *Babesia divergens* acquired in the United Kingdom. *Am J Hematol*. 2021;96:889.
267. Hoare CA. Comparative aspects of human babesiosis. *Trans R Soc Trop Med Hyg*. 1980;74:143.
268. Krause PJ, Telford 3rd SR, Ryan R, et al. Diagnosis of babesiosis: evaluation of a serologic test for the detection of *Babesia microti* antibody. *J Infect Dis*. 1994;169:923.
269. Centers for Disease Control and Prevention Babesiosis. Laboratory diagnosis. https://www.cdc.gov/dpdx/babesiosis/index.html.
270. Wang G, Villafuerte P, Zhuge J, et al. Comparison of a quantitative PCR assay with peripheral blood smear examination for detection and quantitation of *Babesia microti* infection in humans. *Diagn Microbiol Infect Dis*. 2015;82:109.
271. Rollend L, Bent SJ, Krause PJ, et al. Quantitative PCR for detection of *Babesia microti* in *Ixodes scapularis* ticks and in human blood. *Vector Borne Zoonotic Dis*. 2013;13:784.
272. Weiss LM, Wittner M, Tanowitz HB. The treatment of babesiosis. *N Engl J Med*. 2001;344:773.
273. Ranque S. The treatment of babesiosis. *N Engl J Med*. 2001;344:773.
274. Vannier EG, Diuk-Wasser MA, Ben Mamoun C, et al. Babesiosis. *Infect Dis Clin North Am*. 2015;29:357.
275. Brasseur P, Gorenflot A. Human babesiosis in Europe. *Mem Inst Oswaldo Cruz*. 1992;87:131.
276. Schwartz J, Padmanabhan A, Aqui N, et al. Guidelines on the use of therapeutic apheresis in clinical practice-evidence-based approach from the Writing Committee of the American Society for Apheresis. The seventh special issue. *J Clin Apher*. 2016;31:149.
277. Moritz ED, Winton CS, Tonnetti L, et al. Screening for *Babesia microti* in the U.S. Blood Supply. *N Engl J Med*. 2016;375:2236.
278. U.S. Department of Health and Human Services. Recommendations for reducing the risk of transfusion-transmitted babesiosis: guidance for industry. (https://www.fda.gov/media/114847/download).
279. Moritz ED, Winton CS, Johnson ST, et al. Investigational screening for *Babesia microti* in a large repository of blood donor samples from nonendemic and endemic areas of the United States. *Transfusion*. 2014; 54:2226–2236.

280. Lodes MJ, Houghton RL, Bruinsma ES, et al. Serological expression cloning of novel immunoreactive antigens of *Babesia microti*. *Infect Immun*. 2000; 68:2783.
281. Luo Y, Jia H, Terkawi MA, et al. Identification and characterization of a novel secreted antigen 1 of *Babesia microti* and evaluation of its potential use in enzyme-linked immunosorbent assay and immunochromatographic test. *Parasitol Int*. 2011;60:119.
282. Priest JW, Moss DM, Won K, et al. Multiplex assay detection of immunoglobulin G antibodies that recognize *Babesia microti* antigens. *Clin Vaccine Immunol*. 2012;19:1539.
283. Cheng K, Coller KE, Marohnic CC, et al. Performance evaluation of a prototype architect antibody assay for *Babesia microti*. *J Clin Microbiol*. 2018;56:e00460.

CHAPTER 155

HEMATOLOGIC PROBLEMS IN THE SURGICAL PATIENT: BLEEDING AND THROMBOSIS

Iqbal H. Jaffer and Jeffrey I. Weitz

The management of hematological issues in surgical patients can be challenging due to myriad factors. Bleeding and thrombotic issues may be present concurrently, and the impact of underlying pathological issues may be masked by the primary pathology warranting surgery. Nevertheless, the role of the hematologist in the management of these patients is to aid patients with a history of bleeding or thrombosis. This chapter will review: (1) the preoperative evaluation of bleeding risk; (2) strategies to promote intraoperative hemostasis; (3) the management of patients with hemostatic abnormalities or those taking anticoagulants or long-acting antiplatelet drugs; and (4) perioperative prevention of venous thromboembolism (VTE).

PREOPERATIVE EVALUATION OF HEMOSTATIC RISK

History

Preoperative evaluation of hemostatic risk begins with a carefully taken history (see Chapter 126). Particular attention should be directed at specific bleeding symptoms and any history of bleeding associated with surgical procedures, including circumcision, tonsillectomy, and dental extractions. For women, it is important to inquire about a history of menorrhagia or excessive bleeding associated with childbirth. Detailed family history and record of medication use, including non-prescription medications, should be obtained.

Obtaining an adequate bleeding history may be complicated by the fact that even in the absence of hemostatic defects, many people consider their bleeding to be excessive. In surveys, healthy individuals frequently report excessive nosebleeds (5% to 39%), gingival bleeding (7% to 51%), easy bruising (12% to 24%), menorrhagia (23% to 44%), postpartum bleeding (6% to 23%), and bleeding following dental extraction (up to 13%) and tonsillectomy (up to 11%).[1–3] The combination of objective evidence and a constellation of symptoms is more helpful than either alone in suggesting the presence and etiology of an underlying bleeding disorder.

Coagulation Testing

The need for routine coagulation testing before surgical or invasive procedures remains controversial. Those in favor of testing point to the asymptomatic nature of some hemostatic abnormalities that may cause surgical bleeding and the occasional failure to obtain a detailed history.[4–6] Given the variety of potential hemostatic defects; however, no simple screening system will identify all patients at increased risk for bleeding. Those against routine laboratory testing[7] point to retrospective studies indicating that they rarely detect unexpected bleeding disorders[8,9] and emphasize the problems associated with the evaluation of false-positive results. A retrospective review of the value of preoperative determination of the platelet count, prothrombin time (PT), and activated partial thromboplastin time (aPTT) in 828 patients undergoing major noncardiac surgery found that only 2% had abnormal results, and most were expected based on history and physical examination.[10] Furthermore, abnormal laboratory test results and intraoperative blood loss or postoperative bleeding complications were not related (see Chapter 126). A number of prospective studies have also concluded that routine laboratory screening tests in asymptomatic patients are not predictive of perioperative or postoperative bleeding.[11–15] Thus, in the absence of historical risk factors or physical examination findings suggestive of an underlying bleeding tendency, the likelihood of an unsuspected, clinically significant, congenital or acquired coagulopathy is low enough that routine laboratory screening is not warranted, particularly for those undergoing low-risk procedures.[16] The British Committee for Standards in Haematology issued guidelines reiterating this position: indiscriminate coagulation testing before surgical or invasive procedures is a poor predictor of bleeding risk, and is not recommended in the absence of a positive personal or family bleeding history.[17]

There are several reasons why routine coagulation tests such as the PT/international normalised ratio (INR) and aPTT may be poorly predictive of underlying coagulopathy and bleeding risk.[17] First, these tests were designed to measure time to clot formation in artificial in vitro assays and do not reliably depict global hemostasis in vivo (see Liver Disease section). More importantly, the PT/INR and aPTT may be insensitive to mild but clinically relevant bleeding disorders such as von Willebrand disease or mild hemophilia (see Chapters 133 and 134). Conversely, they may detect conditions such as factor XII deficiency or a lupus anticoagulant that do not increase the risk for bleeding.

Notwithstanding the controversy about the value of preoperative laboratory screening (see Chapter 127), it is reasonable to personalize the approach to preoperative evaluation depending on the hemostatic risk of the proposed surgery or invasive procedure (Tables 155.1 and 155.2). A hemostatic history should always be obtained, and no laboratory testing is required in patients undergoing procedures at low risk for bleeding. For procedures associated with a high risk of bleeding, screening could include a PT/INR, aPTT, and determination of the platelet count. In practice, hematologists are rarely consulted for routine screening because surgeons have adopted approaches based on their own training and local practice patterns. Instead, consultation is sought because of a history suggestive of a bleeding disorder or because an abnormal test result is found on screening. If a referral is requested because of an abnormal screening test, the abnormality needs to be identified using a focused history and physical exam, and appropriate laboratory testing to further evaluate the issue.

HEMOSTATIC AGENTS

A variety of hemostatic agents are available and may be useful for the prevention or treatment of bleeding in surgical patients. These agents work through a variety of mechanisms to facilitate hemostasis, including enhancement of primary hemostasis, enhancement of thrombin generation and fibrin formation, and inhibition of fibrinolysis.[18] However, it is important to note that there is a paucity of safety data

sevier on April
ights reserved.

TABLE 155.1 Risk for Bleeding with Surgical or Invasive Procedures

Risk	Type of Procedure	Examples
Low	Nonvital organs involved, exposed surgical site, limited dissection, percutaneous access	Lymph node biopsy, dental extraction, cataract extraction, most cutaneous surgery, laparoscopic procedures, coronary angiography
Moderate	Vital organs involved, deep or extensive dissection	Laparotomy, thoracotomy, mastectomy, major orthopedic surgery, pacemaker insertion
High	Bleeding likely to compromise surgical result, bleeding complications frequent	Neurosurgery, ophthalmic surgery, cardiopulmonary bypass, prostatectomy or bladder surgery, major vascular surgery, renal biopsy, bowel polypectomy

TABLE 155.2 Perioperative Management Strategies for Patients on Chronic Oral Anticoagulant Therapy

Clinical Situation	Suggested Anticoagulation Management
Low bleeding-risk surgery (dental, cataract, skin)	• Consider reducing dose of warfarin to achieve an INR ≤2.0 • Consider holding dose of DOAC until after the procedure
Low thrombotic risk Aortic valve prosthesis without other thrombotic risk factors[a] *or* AF with low stroke risk *or* VTE >3 months previously	• Stop warfarin 5 days prior to surgery, safe to operate when INR ≤1.5 • Hold DOACs for 5 half-lives—dabigatran for 2–3 days, rivaroxaban, apixaban, edoxaban for 2 days • Restart warfarin in evening of the day of surgery after hemostasis secured; restart DOACs on postoperative day 3 or 4 • Start prophylactic LMWH on the morning of the day after surgery and continue until INR >1.8 or full dose of DOACs resumed
Moderate thrombotic risk Mitral or multiple valve prostheses *or* Aortic prosthesis with risk factors for thrombosis *or* AF at high stroke risk *or* VTE within past 3 months	• Stop warfarin 5 days before surgery • Begin twice-daily LMWH in therapeutic doses starting 3 days before surgery with last dose at least 12 h prior to surgery • Hold DOACs for 5 half-lives—dabigatran for 3–4 days, rivaroxaban, apixaban, edoxaban for 2–3 days; no bridging • Restart warfarin in evening of the day of surgery after hemostasis secured; restart DOACs on postoperative day 3 or 4 • Start prophylactic LMWH on the morning of the day after surgery and continue until INR >1.8 or full dose of DOACs resumed

[a] Risk factors include caged-ball or single tilting-disk valve, AF, history of stroke/transient ischemic attack or other embolic event, left ventricular failure, underlying hypercoagulable state including cancer.
AF, Atrial fibrillation; *DOAC*, direct oral anticoagulant; *INR*, international normalized ratio; *LMWH*, low-molecular-weight heparin; *VTE*, venous thromboembolism.

involving hemostatic agents because most trials have been designed to assess therapeutic efficacy, rather than potential complications such as thrombosis.[18,19] The use of desmopressin, topical hemostatic agents, antifibrinolytic agents, and recombinant factor VIIa (rFVIIa) will be discussed here. Blood products (platelets, fresh-frozen plasma, and cryoprecipitate) and factor VIII and IX concentrates are discussed elsewhere (see Chapters 133–136).

Desmopressin

Desmopressin (1-deamino-8-D-arginine vasopressin, or DDAVP) is a synthetic analogue of the antidiuretic hormone arginine vasopressin. Intravenous, subcutaneous, or intranasal administration of DDAVP results in transient increases in plasma concentrations of factor VIII and von Willebrand factor as a result of their release from vascular endothelium.[20] Peak levels (typically two to four times baseline) are achieved 30 to 60 minutes after intravenous and 60 to 90 minutes after subcutaneous or intranasal administration (see Chapters 133 and 134).[21] Doses may be repeated at intervals of 12 to 24 hours, but tachyphylaxis may occur after three or four doses,[22] limiting further usefulness of DDAVP. The expression of glycoprotein Ib (GPIb) and GPIIb/IIIa on the platelet membrane is also enhanced after DDAVP administration.[23] DDAVP is the treatment of choice for patients with mild hemophilia A or type 1 von Willebrand disease who require low-risk surgical procedures (see Chapters 133 and 134). Moderate- or high-risk procedures usually require administration of clotting factor concentrates.[22] DDAVP may also be useful for patients with congenital or acquired platelet function disorders.[21,23,24]

DDAVP does not reduce blood loss or transfusion requirements after cardiopulmonary bypass surgery.[25,26] Worrisome also is the fact that a metanalysis shows a 2.4-fold increase in perioperative myocardial infarction in cardiac surgery patients treated with DDAVP.[26] Thus, the routine use of DDAVP in cardiac, orthopedic, or other elective surgical procedures is not recommended.[19,27] A more recent metanalysis of 38 randomized placebo-controlled trials that included nearly 2500 surgical patients found that DDAVP slightly reduced blood loss (by approximately 80 mL) and transfusion requirements (by approximately 0.3 units) but did not reduce the proportion of patients receiving transfusions.[28] Although the authors acknowledged that the clinical impact of the reduced transfusion requirement is questionable, they felt this could not be ignored because of the low cost of DDAVP. The incidence of thromboembolic events in the DDAVP and placebo groups was similar (5.4% and 4.6%, respectively), but they pointed out that identification of harm from DDAVP is limited by study design, and that the safety concerns remain unresolved.[28] Nonetheless, certain subgroups of patients, such as those with platelet dysfunction, may derive benefit.[23,27–29] Because of the small but important risk for myocardial infarction,[30] DDAVP should be used with caution in any surgical patient with a history of or risk factors for coronary artery disease.

Topical Hemostatic Agents

Topical hemostatic agents can be grouped into several categories: physical agents such as bone wax or alkylene oxide copolymers (Ostene), absorbable agents such as gelatin foams, oxidized cellulose, or microfibrillar collagen, biologic agents such as topical thrombin, fibrin sealants, or platelet gels, synthetic agents such as polyethylene glycol hydrogels, cyanoacrylates, glutaraldehyde or cross-linked albumin, and hemostatic dressings.[31] A thorough discussion of the currently available products, including mechanisms of action, specific advantages and disadvantages, and recommendations for their use is provided in a comprehensive review.[31] A brief overview of the different types of topical hemostatic agents follows.

Physical Agents

Bone wax and Ostene control hemorrhaging by occluding bleeding channels on cut bone surfaces and are often used in cardiac and orthopedic surgery. Theoretically, Ostene may be better than bone wax because it does not impede bone growth and is eventually absorbed. Both may increase the risk for local infection.[31]

Absorbable Agents

Gelatin foams are derived from animal products and provide a physical matrix upon which clotting occurs. These products expand to double their volume, an attractive feature for use in penetrating wounds, but potentially problematic if used near nerves or in confined spaces.[31]

Oxidized cellulose is derived from wood pulp. It provides a physical matrix for initiation of clotting and has excellent handling characteristics. By lowering surrounding pH, oxidized cellulose exerts an antimicrobial effect, but this property not only limits its use with biologic agents such as thrombin that are pH sensitive, but also can contribute to local inflammation.[31]

Microfibrillar collagen is derived from bovine components. It contributes to hemostasis by promoting platelet adherence and activation and is effective in controlling wide-spread parenchymal bleeding. Consequently, it can be useful even in the face of heparin therapy, although it is less effective in the setting of thrombocytopenia.[31]

Biologic Agents

Thrombin derived from bovine plasma was used for more than 40 years as a topical hemostatic agent in surgical patients. However, bovine thrombin can trigger the formation of antibodies that cross-react with human thrombin, leading to hemorrhagic complications.[32–34] Because of these issues, plasma-derived and recombinant forms of human thrombin were developed. A phase III randomized, double-blind trial found that the efficacy and safety of recombinant human thrombin and bovine thrombin were comparable, but there were fewer immunologic complications with recombinant human thrombin.[35]

Fibrin sealants are topical hemostatic agents composed of purified, virally inactivated human fibrinogen and human thrombin. Some products also add human factor XIII to induce fibrin cross-linking and antifibrinolytic agents to prevent clot breakdown.[36] The components of fibrin sealants are supplied in separate chambers of a dual-syringe delivery device that combines them at the time of administration. The final steps of the coagulation cascade are reproduced, resulting in the formation of a stable fibrin clot. Fibrin sealants are particularly effective for controlling oozing from raw surfaces.[31]

Platelet gel combines microfibrillar collagen and thrombin with patient-derived plasma that contains fibrinogen and platelets. Like fibrin sealants, the product is applied using a dual-chamber syringe device. The presence of platelets improves clot strength and provides growth factors, but the need for centrifugation and processing of patient blood before use is a disadvantage.[31]

Synthetic Agents

Cyanoacrylates are liquid monomers that rapidly polymerize in the presence of water and bind adjacent surfaces together. Octyl-2-cyanoacrylate is useful for closing small wounds or incisions, and provides good cosmetic results.[31]

Polyethylene glycol hydrogel can be sprayed onto tissues, where it rapidly forms a cross-linked polymer matrix and serves as a sealant that inhibits cell ingrowth and adhesion formation. It is useful for preventing pericardial adhesions and as a mechanical sealant for vascular reconstructions in situations where swelling and expansion are not a concern.[31]

Glutaraldehyde cross-linked bovine albumin is primarily used to seal sutures or staple lines in complex cardiovascular procedures. However, because it can restrict tissue growth, it should not be used circumferentially around developing structures, particularly in children.[31]

Hemostatic Dressings

Progress in the field of topical hemostatic agents over the last decade has expanded into the development of hemostatic dressings. Several products containing combinations of gauze, lyophilized fibrinogen, and thrombin, chitin, chitosan (polysaccharides found in arthropod skeletons and produced by fermenting algae), or mineral zeolite are available. In general, the use of hemostatic dressings is still under investigation, primarily by the military and emergency first responders.[31]

Antifibrinolytics

Antifibrinolytic agents include the synthetic lysine analogs 6-aminohexanoic acid (epsilon aminocaproic acid) and 4-(amino methyl) cyclohexane carboxylic acid (tranexamic acid), and the serine protease inhibitor, aprotinin. Although both types of antifibrinolytic agents have been used in managing surgical bleeding, the lysine analogs are available in oral forms that facilitate their use in other clinical situations as well.

Aminocaproic Acid and Tranexamic Acid

Both aminocaproic acid and tranexamic acid bind reversibly to the lysine binding sites on plasminogen, thereby attenuating its capacity to bind to fibrin, which is essential for its activation by plasminogen activators.[21] Although tranexamic acid is approximately 10 times more potent than aminocaproic acid and has a longer half-life, both drugs have similar hemostatic effects.[37]

Because the oral form of tranexamic acid was not commercially available in the United States for several years, aminocaproic acid became the lysine derivative of choice for oral delivery. Aminocaproic acid is commonly used to treat mucosal hemorrhage (menorrhagia, epistaxis, dental bleeding) in patients with congenital coagulopathies and is also effective for the prevention of oral bleeding in patients taking oral anticoagulants who require dental work (see Chapters 133–135). Both aminocaproic acid and tranexamic acid can be administered intravenously as well as topically.

Although aminocaproic acid is sometimes used to treat bleeding in patients with thrombocytopenia, randomized controlled trials are lacking. The use of antifibrinolytic drugs in patients with gastrointestinal bleeding is reasonable given the high concentration of fibrinolytic enzymes in the digestive tract, and a metanalysis found reductions in recurrent bleeding, need for surgery, and mortality.[38] However, improvements in the efficacy of other medical and endoscopic treatments have limited the use of these drugs in this setting, although they are still useful for some patients with underlying bleeding disorders.[21] The urinary tract is also rich in plasminogen activators, and some clinical trials comparing tranexamic acid or aminocaproic acid with placebo in patients undergoing prostatectomy have shown reduced blood loss, but not a reduced need for transfusion or decreased mortality.[21]

The largest experience with aminocaproic acid is in patients undergoing cardiac or orthopedic surgery. Two meta-analyses have shown that aminocaproic acid and tranexamic acid are effective in reducing surgical blood loss, but have yielded inconsistent results in their capacity to reduce transfusion requirements.[27,39] A wide variety of dosing schedules may partly explain these heterogeneous results.[27]

A meta-analysis showed that both agents were effective in reducing blood loss and transfusion requirements in those undergoing cardiac surgery.[40]

Several studies have demonstrated that antifibrinolytic agents reduce blood loss in orthopedic surgery. A meta-analysis of 43 randomized controlled trials in patients undergoing total hip or knee arthroplasty, spine fusion, surgery for musculoskeletal infection, or tumor surgery found that aprotinin and tranexamic acid significantly reduced the number of patients requiring transfusion, with a dose-effect relationship suggested for tranexamic acid.[41] A meta-analysis of 11 clinical trials involving patients undergoing total hip replacement found a significant reduction in intraoperative blood loss and transfusion requirements with tranexamic acid, without any increase in VTE or other complications.[42] Therefore, many centers routinely administer tranexamic acid to patients undergoing elective joint arthroplasty to reduce blood loss and decrease the need for transfusion.

Antifibrinolytics, particularly tranexamic acid, have also been studied for the treatment of trauma patients, in whom 30% of deaths are attributed to hemorrhage.[43] A landmark trial, Clinical Randomisation of an Antifibrinolytic in Significant Haemorrhage 2 (CRASH-2), evaluated the safety and efficacy of tranexamic acid in the setting of trauma.[44] This randomized, placebo-controlled, multinational trial that included more than 20,000 trauma patients demonstrated a significant reduction in all-cause mortality and death because of bleeding in the treatment group. More severely injured patients and those treated within 3 hours of injury derived the greatest benefit, and there was no difference in the rate of vascular occlusive events between the two groups.[44] Tranexamic acid is now incorporated into trauma clinical practice guidelines and treatment protocols.[45]

In the CRASH-3 trial, tranexamic acid was compared with placebo in 12,737 patients with traumatic brain injury, 72% of whom were treated within 3 hours of their injury.[46] With tranexamic acid, the risk of head injury-related death was reduced in patients with mild-to-moderate head injury but not in those with severe head injury.[46] Therefore, early administration of tranexamic acid may be of benefit in patients with mild to moderate traumatic brain injury.

Aminocaproic acid and tranexamic acid are not without risks. Although there are reports of thrombosis associated with these agents, no significant increase in thrombotic complications was observed when the drugs were used in patients undergoing cardiac surgery, liver transplantation, or orthopedic surgery.[22,24,41,47,48] However, when tranexamic acid is administered to patients undergoing cardiac surgery in doses exceeding 80 mg/kg, there is a risk of seizures.[49,50]

OFF-LABEL USE OF rFVIIA

A 56-year-old man has undergone aortic arch replacement for an aortic dissection. During the procedure he underwent circulatory arrest for 35 min, with a lowest recorded temperature of 18°C. His total bypass time was 225 min. After rewarming, there was considerable bleeding and he received 10 units of packed red blood cells, 8 units of fresh frozen plasma, 4 pooled units of platelets, and 20 units of cryoprecipitate. He was transferred from the operating room to the intensive care unit in stable condition.

A consultation is made to the hematologist 1 h later because of excessive drainage (>500 mL) from his chest tubes.

Important information to consider:

- likelihood of surgical bleeding versus coagulopathy
- current temperature
- evidence of clotting within the chest tubes
- current coagulation studies (if available)
- hemodynamic status

Upon discussion with the surgeon and intensive care unit team, it is noted that the temperature is 35.5°C, there is little clot in the chest tubes, and he requires hemodynamic support with epinephrine and norepinephrine. His recent coagulation studies reveal an INR of 1.8, an aPTT of 48 s, a thrombin time of 59 s, and a fibrinogen level of 2.5 g/L.

Possible treatments:

1. warming blanket
2. protamine sulfate 50 mg by slow intravenous infusion: for heparin rebound
3. calcium gluconate 1–2 g intravenous bolus: for excessive citrate (from blood products)
4. blood products (using a warming line)
5. consider rFVIIa: 40–80 μg/kg

Interpretation: The likelihood is that despite the extensive transfusion, the combination of a prolonged time on bypass and cooling to 18°C has resulted in a coagulopathy. This is further evidenced by the prolongation of the coagulation tests and the lack of visible clots within the chest tubes. Although items 1–3 are important as adjunctive therapies, they are unlikely to be sufficient on their own. The utility of further blood products in this situation cannot be understated, because in order for rFVIIa to work effectively, it relies on the presence of underlying substrate, which is effected by judicious use of blood products. Although the use of rFVIIa in this scenario is off-label, its use is justified when the surgeon feels that the likelihood that bleeding is too diffuse to be stopped by surgical intervention and is likely caused by coagulopathy.

aPTT, Activated partial thromboplastin time; *rFVIIa*, recombinant factor VIIa.

Recombinant Factor VIIa

rFVIIa is licensed in the United States only for the prevention and treatment of bleeding in hemophilia A or B patients with inhibitors, patients with acquired hemophilia, and in those with congenital factor VII deficiency (see Chapters 134 and 135). This drug is believed to induce hemostasis at local sites of tissue injury through enhancement of thrombin generation on the surface of thrombin-activated platelets.[51] rFVIIa also activates thrombin activatable fibrinolysis inhibitor, which in turn stabilizes the clot by inhibiting fibrinolysis.[52]

rFVIIa is often used for a variety of off-label indications, such as to control refractory bleeding after surgery or major trauma, and to prevent bleeding in surgeries where blood loss is expected to be excessive (see box on Off-Label Use of rFVIIa). A prospective, multicenter, randomized, controlled trial that included 143 patients with blunt trauma and 134 patients with penetrating trauma found that in the group with blunt trauma, three successive doses of rFVIIa significantly decreased red blood cell transfusion (mean reduction of 2.6 units) and decreased by approximately half the number of patients requiring massive transfusion (more than 20 units of red blood cells).[53] Although similar trends were observed in the patients with penetrating trauma, the differences were not statistically significant. Despite the reduction in the need for blood products, there was no survival benefit. After these initial encouraging reports of the use of rFVIIa in trauma patients, questions regarding optimal dosing and timing of administration remained. To address these issues, the Western Trauma Association Multi-Center Trials Group conducted a case registry of 380 adult trauma patients who received adjunctive rFVIIa for hemorrhage control.[54] This registry was unable to define a precise role for rFVIIa in traumatic bleeding. However, pH less than 7.2, platelet count less than 100,000/μL, and blood pressure less than or equal to 90 mmHg were each found to be predictors of poor response to rFVIIa, suggesting that shock, acidosis, and thrombocytopenia should be corrected before rFVIIa is given to trauma patients who are bleeding.[54] The CONTROL trial, which randomized 560 actively bleeding trauma patients to rFVIIa or placebo, found no differences in overall mortality, organ system failure, or adverse events in either group.[55] Thus, despite the encouraging initial reports, there is currently insufficient evidence to support the routine use of rFVIIa in the setting of trauma.

The use of rFVIIa in cardiac surgery is controversial. Because perioperative bleeding is a major cause of morbidity and mortality, there is off-label use of rFVIIa in this patient population.[56] Several case reports and uncontrolled case series in both adult and pediatric populations have suggested that rFVIIa is effective in decreasing blood loss and transfusion requirements in patients with intractable bleeding after cardiopulmonary bypass. However, in the absence of data from well-designed clinical trials demonstrating efficacy and with the current widespread use of tranexamic acid in such patients, rFVIIa is rarely used in this setting.

The primary safety concern with the off-label use of rFVIIa is thrombosis. In hemophilia, the risk for thrombosis is estimated to be less than 1%.[57,58] In contrast, the risk is much higher with off-label use. Both arterial and venous thromboembolic events have been reported, and thromboembolic events were the probable cause of death in most of the reported fatalities. Half of the thromboembolic events occurred within 24 hours of the last dose of rFVIIa, and many occurred within 2 hours.[59] Therefore, off-label use of rFVIIa should be restricted to cases where there are no alternatives. The results of a metanalysis that assessed the use of rFVIIa in randomized trials suggested that most the arterial complications occur in older patients (>65 years) and in those patients receiving rFVIIa doses in excess of 80 μg/kg.[60]

MANAGEMENT OF PATIENTS WITH HEMOSTATIC ABNORMALITIES

Patients with known hemostatic abnormalities are often referred before surgery for assessment of bleeding risk and recommendations regarding perioperative management. The approach to these patients should be according to the following considerations: (1) evaluation of the risk for bleeding associated with the specific surgery or procedure (see Table 155.1); (2) careful consideration of the need for surgery and its urgency; greater risks are warranted for correction of life-threatening conditions than for elective procedures; (3) recognition of the nature and severity of the patient's hemostatic abnormality and the ability to correct it; and (4) consideration of the duration of replacement that will be required, with potential bleeding that may be associated with events in the postoperative period such as removal of sutures and deep drains, and the need for postoperative rehabilitation. The following sections discuss perioperative management of some common coagulation abnormalities; the management of congenital factor deficiencies and von Willebrand disease in Chapters 133–136.

Thrombocytopenia

Thrombocytopenia is one of the most common acquired hemostatic abnormalities, and the availability of platelet transfusion makes consideration of both emergency and elective surgery reasonable even in patients with severe thrombocytopenia. The best index of bleeding risk in thrombocytopenic patients is the platelet count. In nonsurgical patients, a threshold platelet count of 10,000/μL is widely used for prophylactic transfusion, yet there is inadequate scientific evidence to determine the platelet count below, which increased the risk for surgical bleeding.[61,62] The American Society of Anesthesiologists Task Force on Perioperative Blood Management concluded that prophylactic platelet transfusion in surgical patients is usually indicated when the count is below 50,000/μL and is rarely indicated when the count is above 100,000/μL.[62] Clinical trials addressing this issue are still lacking.[63] For low-risk surgery, a single transfusion to increase the platelet count above 50,000/μL followed by close observation may suffice, whereas transfusion to maintain the platelet count at greater than 50,000/μL for moderate-risk surgery and greater than 100,000/μL for high-risk surgery is usually appropriate. The optimal duration of postoperative platelet support has not been carefully studied, but even for moderate or high-risk surgery, platelet transfusions may be needed for less than 1 week because they are principally required for primary hemostasis. The platelet count should be monitored closely during the postoperative period, with the expectation that platelet survival will be shortened by infection, fever, or bleeding. In addition, platelet transfusion may be indicated for surgical patients even on the face of a normal count if there is known or suspected platelet dysfunction, intake of long-acting antiplatelet drugs, or microvascular bleeding.

When thrombocytopenia is caused by increased platelet destruction (e.g., immune thrombocytopenic purpura), prophylactic platelet transfusion is largely ineffective and is indicated only for active, serious bleeding (see Chapters 128 and 129). In preparation for surgery, therapy with steroids and/or intravenous γ-globulin often will increase the platelet count to a satisfactory level so that transfusion is not needed. Rho(D) immunoglobulin (WinRho) may also be useful in this setting (see Chapter 128).

Platelet Dysfunction

Patients with platelet dysfunction represent a large group for whom preoperative consultation is sought, typically because of a history of abnormal bleeding or the discovery of a prolonged bleeding time, or abnormalities in other tests of platelet function despite a normal platelet count (see Chapters 126 and 127). Drugs are the most common cause of acquired platelet dysfunction (see Chapter 128). Many commonly used medications, including aspirin, nonsteroidal anti-inflammatory drugs, antibiotics, antidepressants (selective serotonin reuptake inhibitors), cardiovascular drugs, and newer antiplatelet agents, including ADP receptor, GPIIb/IIIa and protease-activated receptor-1 antagonists (see Chapter 143), can cause platelet dysfunction. Ethanol as well as certain foods and herbal supplements can also inhibit platelets (see Chapter 128); a careful history is therefore essential. Any drugs that interfere with platelet function should be reviewed before surgery and their need for ongoing use considered.

Several medical conditions can cause acquired platelet dysfunction (see Chapter 128). The etiology may be obvious in cases of renal or liver disease, myeloproliferative disorders, leukemia, myelodysplastic syndromes, or dysproteinemia, but consideration of undiagnosed intrinsic platelet defects (storage pool disease or platelet release defects) or von Willebrand disease may be necessary. Treatment of the underlying disease is the most effective approach, if possible. If not, platelet transfusion may be indicated, but the dose required to achieve hemostasis is difficult to predict and depends in part on the severity of the underlying platelet abnormality. As discussed earlier, treatment with DDAVP may be appropriate in selected patients. The exact mechanism of action of DDAVP in acquired platelet dysfunction is not well understood, but one study suggests that DDAVP interacts directly with platelets and exerts a priming effect on platelet aggregation stimulated by ADP or collagen.[64] Expression of GPIb and GPIIb/IIIa on the platelet surface is enhanced after DDAVP administration.[24]

Kidney Disease

Impaired hemostasis has long been recognized in patients with chronic kidney failure and is discussed in greater detail in Chapter 128. The pathogenesis is multifactorial but is due in large part to alterations in platelet function.[65] Anemia also contributes to platelet dysfunction in chronic kidney failure. Red blood cells release ADP, which in turn inactivates vascular prostacyclin, an inhibitor of platelet function.[66] Correction of anemia, now routinely accomplished using recombinant erythropoietin, also improves the rheologic factors that facilitate platelet interaction with the vessel wall. An increase in hematocrit, whether using erythropoietin or transfusion, is accompanied by significant shortening of the bleeding time and improvement of platelet adhesion.[67] In addition to platelet dysfunction and altered balance between mediators of normal endothelial function, the pathophysiology of uremic bleeding is complicated by the comorbidities in this patient population, such as vascular disease and hypertension, and the medical treatment of those conditions.[68]

Liver Disease

Hemostatic alterations in patients with acute or chronic liver disease (see Chapter 128) are complex and involve both procoagulant and anticoagulant pathways.[69,70] Although patients with liver disease are typically felt to have deficient hemostasis, this concept has been challenged in the recent literature.[71] These patients are not "autoanticoagulated," as is often assumed. In fact, they are not protected from and may even be at increased risk for thrombosis, particularly in the portal venous system.[72–74] The presence of genetic thrombophilic mutations may further increase this risk.[75] The procoagulant tendency associated with chronic liver disease[76,77] suggests that prophylaxis against VTE may be warranted in high-risk situations.[71] However, the perceived bleeding risk often limits the use of anticoagulant prophylaxis,[78] and appropriately designed pharmacologic clinical studies are clearly needed.[72] Mechanical thromboprophylaxis is a reasonable alternative for hospitalized patients with severe liver disease.

Patients with severe decompensated liver disease and markedly abnormal coagulation test results are at increased risk for bleeding, and surgery should be avoided except as a lifesaving measure. In evaluating hemostasis in patients with less severe disease, the PT/INR and aPTT may be good indicators of decreased synthesis of clotting factors and vitamin K deficiency, but they are poor predictors of bleeding risk. Several studies have shown the failure of the PT/INR and aPTT to predict bleeding after liver biopsy.[79,80] Similarly, preoperative hemostatic testing has generally not been shown to be useful for predicting bleeding during liver transplantation.[81] A study in patients with liver disease found that INR values in the range of 1.3 to 2.0 generally correspond to levels of factors II, V, and VII that are adequate for hemostasis.[82] A preoperative platelet count is needed to identify thrombocytopenia, and some assessment of platelet function may be useful to determine whether platelet function is abnormal in the setting of a normal or near-normal platelet count. The thrombin time or fibrinogen level should also be determined to evaluate for dysfibrinogenemia. Tests for fibrinogen/fibrin degradation products, D-dimer levels, euglobulin clot lysis time, or thromboelastography may be useful to evaluate for disseminated intravascular coagulation (DIC) or accelerated fibrinolysis (see Chapter 137).

In patients with mild liver disease and a mild-moderate prolongation of the INR (<2.0), serious surgical bleeding is unlikely in the absence of other hemostatic abnormalities, and prophylactic intervention is rarely required for low- or moderate-risk surgery. For high-risk surgery or for patients with markedly abnormal coagulation tests, transfusion of fresh-frozen plasma is the most frequently used approach for correcting the coagulation abnormality, although prospective studies demonstrating the value of fresh-frozen plasma administration in this setting are lacking. There are, however, guidelines that caution against the indiscriminate use of plasma before invasive procedures.[83] It should be noted that the PT of fresh-frozen plasma is approximately 15 seconds, which corresponds to an INR of 1.5, so complete correction of the PT/INR is unlikely to be achieved.[84] Administration of platelets should be considered for more severe degrees of thrombocytopenia, although recovery will be decreased in the presence of splenomegaly. Administration of DDAVP may be useful in some cases for correcting abnormal platelet function.[85] Intravenous administration of 5 to 10 mg of vitamin K will usually shorten the PT/INR if vitamin K deficiency is a contributory factor. Antifibrinolytic agents also may be useful for the reduction of perioperative hemorrhage in patients with liver disease.

INTRAOPERATIVE AND POSTOPERATIVE BLEEDING

Patients with excessive bleeding during or after surgery require rapid evaluation and treatment. The first step is to distinguish between "surgical" and "coagulopathic" causes of bleeding. Failure to surgically control bleeding vessels at the operative site is the most frequent cause of postoperative bleeding and is suggested by evidence of hemorrhage restricted to the operative site and manifesting as an expanding hematoma, excessive blood in surgical drains, or saturated wound dressings. Conversely, coagulopathic bleeding is suggested by oozing at the operative site in addition to evidence of bleeding outside the operative field that may manifest as petechiae, purpura, or bleeding at sites of venipuncture, urinary or vascular catheters, or nasogastric and endotracheal tubes.[86] In addition to careful physical examination, laboratory tests, including PT/INR, aPTT, and platelet count, are an essential part of the evaluation. Other useful tests may include a fibrinogen level, and a test for fibrin degradation products or D-dimer and thromboelastography to search for evidence of DIC or increased fibrinolysis (see Chapter 137). The peripheral blood smear should also be reviewed to examine platelet morphology and number, and to identify possible red blood cell fragmentation that may occur in DIC. The possibility of a pre-existing hemostatic abnormality that may have been missed before surgery should also be considered.

When evaluating coagulation test results in surgical patients, changes that normally occur in response to surgery must be considered. These vary depending on the extent of tissue dissection and the duration of the procedure. Consumption and hemodilution from crystalloid and blood product infusion can lead to acute reductions of coagulation factors and platelets during surgery and in the initial postoperative period. This is typically followed by changes resulting from the acute-phase response, including increases in fibrinogen and factor VIII levels and the platelet count during the first postoperative week.[87]

Alterations in coagulation factor levels that occur during surgery and in the initial postoperative period can limit the reliability of the PT/INR and aPTT when evaluating the bleeding surgical patient. Clinical practice guidelines have consistently recommended transfusion of fresh-frozen plasma if the PT/INR and aPTT results are prolonged more than 1.5-fold compared with the mean reference range.[88,89] However, there are differing opinions on the utility of coagulation studies to guide transfusion practices,[89,90] and prospective studies are needed.

Coagulopathy Associated With Massive Blood Loss/Transfusion

Massive bleeding (loss of one or more blood volumes in a 24-hour period) may occur in the setting of severe trauma or major surgery and requires aggressive fluid resuscitation and transfusion of blood products. The mechanisms involved in the coagulopathy that accompanies massive transfusion have not yet been fully elucidated, but are multifactorial in nature.[91] Similar to chronic liver disease, procoagulant, anticoagulant, profibrinolytic, and antifibrinolytic factors are all affected, resulting in a complex coagulopathy. Impaired thrombin generation is compensated in part by reductions in the activity of antithrombin and other protease inhibitors. Fibrinogen levels can fall rapidly and are proportional to the degree of hemodilution. Antifibrinolytic protein levels are decreased, rendering clots more susceptible to lysis.[92] In addition to a dilutional coagulopathy, other complications, including consumptive coagulopathy, hypothermia, electrolyte abnormalities (because of citrate intoxication), and acid-base disturbances, may also develop.[92]

In patients without a pre-existing coagulopathy, replacement of approximately 1.5 blood volumes is the threshold for the development of dilutional coagulopathy.[93] At that point, PT/INR, aPTT, fibrinogen level, and platelet count should be determined. If the PT/INR or aPTT is prolonged greater than 1.5 times the control, the fibrinogen level is below 100 mg/dL, or the platelet count is reduced to 50,000 to 70,000/μL, clinical coagulopathy is suspected and appropriate blood product administration is indicated, particularly if additional blood loss is expected. The same coagulation parameters should be measured again with the replacement of each additional half-blood volume (i.e., 5 to 6 units of red blood cells).[94]

Over the last several years, there has been an increased emphasis on more aggressive blood component therapy, driven by data from both military and civilian trauma populations.[95–98] These studies have demonstrated that higher ratios (approaching 1:1:1) of plasma and platelets to red blood cells are associated with improved outcomes in massively transfused patients.[99] Patients receiving less than massive transfusion may also benefit from higher plasma-to-red blood cell ratios.[100] Furthermore, maintaining an adequate fibrinogen level is essential for the successful management of dilutional coagulopathy.[92] Appropriate and timely management of these patients must be facilitated to avoid prolonged hypotension, acidosis, and tissue-level ischemia, all of which can predispose to the development of DIC (see Chapter 136).

Cardiopulmonary Bypass

Cardiopulmonary bypass is associated with unique hemostatic changes. Perfusion through the extracorporeal membrane oxygenator has profound effects on platelets and clotting factors: platelet count, hematocrit, and levels of coagulation and fibrinolytic factors are reduced to approximately 50% of baseline after starting bypass and remain reduced throughout the procedure, with the exception of factor V, which may be further reduced to less than 20%.[101–103] These changes may be caused in part by exposure to artificial surfaces and also by a tissue factor–dependent pathway related to surgical trauma.[104] Cardiopulmonary bypass results in significant platelet dysfunction[105] reflected by the release of α-granule contents, the generation of platelet microparticles, abnormal ex vivo platelet aggregation test results, and transient prolongation of the bleeding time (see Chapter 127).[102–106] In addition to quantitative and qualitative platelet function defects, cardiopulmonary bypass also results in platelet activation, which may trigger thrombotic and inflammatory complications.[106] Patients with acute coronary syndrome (who may be candidates for urgent cardiac surgery) are sometimes given GPIIb/IIIa inhibitors, which may further impair platelet function (see Chapter 145). If surgery cannot be delayed, consideration of prophylactic platelet transfusions may be required.[107] Inadequate neutralization of heparin with protamine sulfate after cardiopulmonary bypass may result in a prolonged aPTT and thrombin time with a normal reptilase time and is an indication for administration of additional protamine sulfate. The use of DDAVP, antifibrinolytic drugs, and rFVIIa to reduce the hemorrhagic complications of cardiac surgery was discussed earlier. Fibrinogen concentrate has also been shown to reduce blood loss and transfusion requirements during cardiac surgery.[108]

Orthotopic Liver Transplantation

In addition to the complex coagulopathy of end-stage liver disease, orthotopic liver transplantation is accompanied by major alterations in hemostasis. The surgical procedure itself can be divided into three stages, each with its own profile of coagulation abnormalities.[109] Bleeding during the pre-anhepatic stage, while the host liver is surgically isolated, is due primarily to the patient's pre-existing coagulopathy and is determined by the severity of the underlying liver disease. Excessive fibrinolysis may be encountered during this stage in 10% to 20% of those with cirrhosis. The anhepatic stage begins with surgical removal of the liver. Bleeding during this stage is primarily hemostatic because of DIC and excessive fibrinolysis.[110] As the donor liver is re-perfused, the post-anhepatic stage begins, and serious bleeding is often encountered because of a combination of hyperfibrinolysis, metabolic acidosis, hypothermia, electrolyte abnormalities, and sometimes impaired cardiac function.[110,111]

The multifactorial nature of the coagulopathy associated with orthotopic liver transplantation requires a combination of therapeutic interventions. In addition to transfusion of blood products, other hemostatic agents such as DDAVP and antifibrinolytics may be useful and were discussed earlier. There has also been much interest in the use of rFVIIa, and some randomized, placebo-controlled trials have been completed, but questions about optimal dosing, cost-effectiveness, and safety remain to be fully answered. Furthermore, it is still questionable whether rFVIIa should be used prophylactically in liver transplantation patients outside of a prospective clinical trial.[112]

PERIOPERATIVE ANTICOAGULATION MANAGEMENT

Hematologists are frequently consulted for advice on the perioperative management of patients who are taking oral anticoagulants. Important considerations to be considered include: (1) the bleeding risk associated with the surgery; (2) the underlying indication for anticoagulant therapy; (3), the timing of the most recent thrombotic event; (4) other comorbid conditions that may increase the risk for thrombosis and/or the potential consequences of thrombosis while oral anticoagulation is temporarily interrupted; and (5) the half-life of the anticoagulant agent. Regarding the first point, experience has accumulated in recent years suggesting that many relatively minor procedures can be carried out without any interruption of warfarin or direct oral anticoagulant (DOAC) therapy.[113] These include minor dental procedures (single or multiple extractions and simple endodontic procedures), cataract removal, minor dermatologic procedures, and low-risk endoscopy procedures. Audits suggest that discontinuation of anticoagulation occurs more frequently than is necessary in patients undergoing these minor procedures.[114] Apart from the risk for thromboembolism, the inappropriate use of bridging therapy also places patients at risk for bleeding complications. Suggested management strategies are summarized in Table 155.2.

Traditionally, patients on chronic oral anticoagulation therapy with warfarin were admitted to the hospital several days before surgery for "bridging" anticoagulation, at which time warfarin was discontinued and dose-adjusted unfractionated heparin (UFH) was administered by continuous infusion (see Chapter 143). The short half-life of UFH allows it to be safely discontinued approximately 4 to 6 hours ahead of the procedure. This was followed using subcutaneous low-molecular-weight heparin (LMWH) in place of UFH.[115–117] LMWH has the advantage that in most patients with normal renal function, no monitoring is required, so that therapy can be administered in the outpatient setting. In addition, the risk for heparin-induced thrombocytopenia is less with LMWH than with UFH.[118] However, the longer half-life (generally in the range of 4 to 6 hours) necessitates withdrawal of these agents at least 12 hours (for prophylactic doses) or 24 hours (for full therapeutic doses) preoperatively, although even then significant circulating anticoagulant activity may remain in the plasma.[119] A randomized trial suggests that bridging with LMWH does not reduce the risk of thromboembolic events and increases the risk of bleeding in patients with atrial fibrillation.[120] Although these results have changed practice, it is important to note that atrial fibrillation patients with prior stroke were underrepresented in the study, and patients with mechanical heart valves were excluded.

Temporary interruption of warfarin is often required for more major surgery. Even with a normal diet, 4 to 5 days should be allowed for full or near-full reversal of warfarin when it is being targeted to an INR of 2.0 to 3.0. More rapid reversal over 24 to 36 hours can be achieved, if necessary, by administration of a small oral dose of vitamin K1 (1.0 to 2.5 mg).[121,122] Large doses of vitamin K1 (>10 mg) are generally unnecessary for this purpose and will lead to prolonged refractoriness to warfarin after it is reinitiated. Although frequently used, subcutaneous administration of vitamin K1 is associated with highly variable absorption and should be avoided. Intravenous vitamin K1 has the advantage of more rapid INR reversal compared to the oral route,[123] but it should be administered slowly to avoid anaphylactoid reactions. Urgent reversal of warfarin before surgery should not be attempted using fresh-frozen plasma, because large volumes (15 to 20 mL/kg) are usually required to reverse the INR, and complete normalization cannot usually be achieved. Furthermore, the effect is relatively short-lived because of the short half-life (approximately 6 hours) of transfused factor VII. Therefore, concomitant vitamin K1 administration is required to ensure the maintenance of

TABLE 155.3 **Comparison of the Features of the Direct Oral Anticoagulants**

	Dabigatran	Rivaroxaban	Apixaban	Edoxaban
Target	Thrombin (IIa)	Factor Xa	Factor Xa	Factor Xa
Active drug	No	Yes	Yes	Yes
Onset time (h)	0.5–2	2–4	3–4	1–3
Half-life (h)	12–17	5–13	~12	10–14
Renal excretion (%)	80	33	27	50
Reversal agent	Idarucizumab	Andexanet alfa; four-factor PCC if andexanet unavailable		

IV, Intravenous; *PCC*, prothrombin complex concentrate.

adequate hemostasis. Four-factor prothrombin complex concentrate (PCC; 25 to 50 units/kg) is preferred over plasma for urgent warfarin reversal prior to surgery or to control hemorrhage in warfarin-treated patients.[122,124]

DOACs have replaced warfarin for the prevention of stroke and systemic embolism in most patients with atrial fibrillation and for prevention and treatment of VTE (see Chapters 140 and 147). Working knowledge of the elimination half-lives of these agents is important in deciding when and if to discontinue them before elective procedures (Table 155.3). In general, for all but minor procedures, the goal is to have negligible amounts of the drug (<10%) in the circulation at the time of surgery. This means stopping dabigatran 2 to 3 days before surgery depending on kidney function, and stopping rivaroxaban, apixaban, or edoxaban 1 to 2 days prior to surgery.[125–127] In the setting of urgent surgery, dabigatran can be reversed with a 5 g bolus of idarucizumab.[128–130] Although andexanet alfa can reverse the anticoagulant effects of rivaroxaban, apixaban and edoxaban, it has not been evaluated for urgent reversal prior to surgery. In such cases, four-factor PCC can be considered.[131,132]

Perioperative Thromboprophylaxis

VTE is the most common cause of preventable death in hospitalized patients. Thromboprophylaxis in medical patients is discussed in Chapters 140 and 141. In surgical patients, the risk of postoperative VTE depends on the type of surgery and patient factors. Patients undergoing non-orthopedic surgery have a variable risk of postoperative VTE, whereas those undergoing major orthopedic procedures or surgery for abdominal or pelvic cancer are at high risk. Therefore, the two groups are discussed separately.

Non-orthopedic Surgery

In patients undergoing non-orthopedic surgery, the risks of VTE are grouped into four categories: very low (<0.5%), low (~1.5%), moderate (~3%), and high (~6%).[133] In both the very low- and low-risk groups, guidelines recommend that patients be managed with early ambulation. In the moderate- and high-risk groups, guidelines recommend pharmacologic thromboprophylaxis with LMWH.[133] LMWH is preferred over low-dose UFH because its use is associated with a reduced risk of heparin-induced thrombocytopenia (see Chapter 131).[134] Although LMWH is usually given only while the patient is hospitalized, extended prophylaxis for at least 4 weeks is recommended for patients undergoing abdominal or pelvic surgery for cancer. If the risk of bleeding is deemed to be too high to enable pharmacologic thromboprophylaxis, such as in patients undergoing neurosurgery or spinal surgery, intermittent pneumatic compression devices should be used until the bleeding risk subsides.[133]

Orthopedic Surgery

Patients undergoing orthopedic surgery are at high risk for postoperative VTE because they are relatively immobile after surgery and because manipulation of the lower limb vascular structures may disrupt the vascular endothelium.[135] Therefore, patients undergoing hip or knee arthroplasty or surgery for hip fracture are all categorized as high risk. Accordingly, postoperative pharmacologic prophylaxis is recommended, and the choices in patients undergoing hip or knee arthroplasty include rivaroxaban, apixaban, LMWH, or dose-adjusted warfarin; aspirin is also included as an option in preference to no prophylaxis.[133] In patients undergoing knee arthroplasty, guidelines recommend that prophylaxis be continued for at least 10 to 14 days after surgery, and in those undergoing hip arthroplasty or surgery for hip fracture, prophylaxis should be continued for at least 35 days.[136] A recent trial that randomized patients undergoing hip or knee arthroplasty to extended thromboprophylaxis with rivaroxaban or aspirin after an initial 5-day course of rivaroxaban reported similar rates of postoperative VTE and bleeding.[137] The DOACs have not been extensively evaluated in patients undergoing surgery for hip fracture, and LMWH is often used for thromboprophylaxis in such patients although rivaroxaban is more convenient for out-of-hospital administration.[136] Most patients without a prior history of VTE do not require thromboprophylaxis after arthroscopic surgery.[136]

SUMMARY

The management of surgical or trauma patients in the perioperative period can be challenging because of the divergent procedures, patient-related and procedure-related risks, and the limitations of time and acuity of the situation. Prior bleeding or thrombotic events and the use of concomitant medications need to be considered when developing a management plan that incorporates judicious use of coagulation tests, hemostatic agents, and/or blood products. Collaboration with the surgical or trauma teams improves patient outcomes. Ongoing research into the management of surgical and trauma patients will likely aid clinical decision-making in the future.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Achneck HE, Sileshi B, Jamiolkowski RM, et al. A comprehensive review of topical hemostatic agents: efficacy and recommendations for use. *Ann Surg*. 2010;251(2):217–228.

Bolliger D, Görlinger K, Tanaka KA. Pathophysiology and treatment of coagulopathy in massive hemorrhage and hemodilution. *Anesthesiology*. 2010;113(5):1205–1219.

Brown JR, Birkmeyer NJO, O'Connor GT. Meta-analysis comparing the effectiveness and adverse outcomes of antifibrinolytic agents in cardiac surgery. *Circulation*. 2007;115(22):2801–2813.

CRASH-3 Trial Collaborators. Effects of tranexamic acid on death, disability, vascular occlusive events and other morbidities in patients with acute traumatic brain injury (CRASH-3): a randomised, placebo-controlled trial. *Lancet*. 2019;394(10210):1713–1723.

Douketis JD, Spyropoulos AC, Kaatz S, et al. Perioperative bridging anticoagulation in patients with atrial fibrillation. *N Engl J Med*. 2015;373(9):823–833.

Douketis JD, Spyropoulos AC, Spencer FA, et al. Perioperative management of antithrombotic therapy: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed. American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest*. 2012;141(2):e326S–e350S.

Falck-Ytter Y, Francis CW, Johanson NA, et al. Prevention of VTE in orthopedic surgery patients: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed. American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest*. 2012;141(2 Suppl):e278S–e2325.

Holbrook A, Schulman S, Witt DM, et al. Evidence-based management of anticoagulant therapy: Antithrombotic Therapy and Prevention of

Thrombosis, 9th ed. American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest*. 2012;141(2_suppl):e152S–e184S.

Levi M, Levy JH, Andersen HF, Truloff D. Safety of recombinant activated factor VII in randomized clinical trials. *N Engl J Med*. 2010;363(19): 1791–1800.

Milas BL, Jobes DR, Gorman RC. Management of bleeding and coagulopathy after heart surgery. *Semin Thorac Cardiovasc Surg*. 2000;12(4):326–336.

Practice Guidelines for Perioperative Blood Management: an updated report by the American Society of Anesthesiologists Task Force on Perioperative Blood Management. *Anesthesiology* 2015;122(2):241-275.

Tripodi A, Mannucci PM. The coagulopathy of chronic liver disease. *N Engl J Med*. 2011;365(2):147–156.

REFERENCES

1. Sramek A, Eikenboom JC, Briet E, et al. Usefulness of patient interview in bleeding disorders. *Arch Intern Med*. 1995;155(13):1409–1415.
2. Gabriel P, Mazoit X, Ecoffey C. Relationship between clinical history, coagulation tests, and perioperative bleeding during tonsillectomies in pediatrics. *J Clin Anesth*. 2000;12(4):288–291.
3. Mauser Bunschoten EP, van Houwelingen JC, Sjamsoedin Visser EJ, et al. Bleeding symptoms in carriers of hemophilia A and B. *Thromb Haemost*. 1988;59(3):349–352.
4. Bowie EJ, Owen Jr. CA. The significance of abnormal preoperative hemostatic tests. *Progr Hemost Thromb*. 1980;5:179–209.
5. Burk CD, Miller L, Handler SD, Cohen AR. Preoperative history and coagulation screening in children undergoing tonsillectomy. *Pediatrics*. 1992;89(4 Pt 2):691–695.
6. Rapaport SI. Preoperative hemostatic evaluation: which tests, if any? *Blood*. 1983;61(2):229–231.
7. Suchman AL, Griner PF. Diagnostic uses of the activated partial thromboplastin time and prothrombin time. *Ann Intern Med*. 1986;104(6):810–816.
8. Eisenberg JM, Clarke JR, Sussman SA. Prothrombin and partial thromboplastin times as preoperative screening tests. *Arch Surg. 1960*. 1982;117(1):48–51.
9. Kaplan EB, Sheiner LB, Boeckmann AJ, et al. The usefulness of preoperative laboratory screening. *JAMA*. 1985;253(24):3576–3581.
10. Ng KF, Lai KW, Tsang SF. Value of preoperative coagulation tests: reappraisal of major noncardiac surgery. *World J Surg*. 2002;26(5):515–520.
11. Close HL, Kryzer TC, Nowlin JH, Alving BM. Hemostatic assessment of patients before tonsillectomy: a prospective study. *Otolaryngol Head Neck Surg*. 1994;111(6):733–738.
12. Gravlee GP, Arora S, Lavender SW, et al. Predictive value of blood clotting tests in cardiac surgical patients. *Ann Thorac Surg*. 1994;58(1):216–221.
13. Houry S, Georgeac C, Hay JM, Fingerhut A, Boudet MJ. A prospective multicenter evaluation of preoperative hemostatic screening tests. The French Associations for Surgical Research. *Am J Surg*. 1995;170(1):19–23.
14. Macpherson CR, Jacobs P, Dent DM. Abnormal peri-operative haemorrhage in asymptomatic patients is not predicted by laboratory testing. *South Afr Med J*. 1993;83(2):106–108.
15. Myers ER, Clarke-Pearson DL, Olt GJ, Soper JT, Berchuck A. Preoperative coagulation testing on a gynecologic oncology service. *Obstet Gynecol*. 1994;83(3):438–444.
16. Eckman MH, Erban JK, Singh SK, Kao GS. Screening for the risk for bleeding or thrombosis. *Ann Intern Med*. 2003;138(3):W15–W24.
17. Chee YL, Crawford JC, Watson HG, Greaves M. Guidelines on the assessment of bleeding risk prior to surgery or invasive procedures. British Committee for Standards in Haematology. *Br J Haematol*. 2008;140(5):496–504.
18. Levy JH. Hemostatic agents. *Transfusion*. 2004;44(12 Suppl):58s–62s.
19. Mannucci PM, Levi M. Prevention and treatment of major blood loss. *N Engl J Med*. 2007;356(22):2301–2311.
20. Mannucci PM, Ghirardini A. Desmopressin: twenty years after. *Thromb Haemost*. 1997;78(2):958.
21. Mannucci PM. Hemostatic drugs. *N Engl J Med*. 1998;339(4):245–253.
22. Mannucci PM, Bettega D, Cattaneo M. Patterns of development of tachyphylaxis in patients with haemophilia and von Willebrand disease after repeated doses of desmopressin (DDAVP). *Br J Haematol*. 1992;82(1):87–93.
23. Porte RJ, Leebeek FW. Pharmacological strategies to decrease transfusion requirements in patients undergoing surgery. *Drugs*. 2002;62(15):2193–2211.
24. Norfolk DR, Seghatchian J. Pharmacological therapies to minimise platelet transfusion. *Transfusion Sci*. 2000;22(3):149–153.
25. Cattaneo M, Harris AS, Stromberg U, Mannucci PM. The effect of desmopressin on reducing blood loss in cardiac surgery – a meta-analysis of double-blind, placebo-controlled trials. *Thromb Haemost*. 1995;74(4):1064–1070.
26. Levi M, Cromheecke ME, de Jonge E, et al. Pharmacological strategies to decrease excessive blood loss in cardiac surgery: a meta-analysis of clinically relevant endpoints. *Lancet*. 1999;354(9194):1940–1947.
27. Ozier Y, Schlumberger S. Pharmacological approaches to reducing blood loss and transfusions in the surgical patient. *Canad J Anaesth*. 2006; 53(6 Suppl):S21–S29.
28. Crescenzi G, Landoni G, Biondi-Zoccai G, et al. Desmopressin reduces transfusion needs after surgery: a meta-analysis of randomized clinical trials. *Anesthesiology*. 2008;109(6):1063–1076.
29. Steinlechner B, Zeidler P, Base E, et al. Patients with severe aortic valve stenosis and impaired platelet function benefit from preoperative desmopressin infusion. *Ann Thorac Surg*. 2011;91(5):1420–1426.
30. Lowe GD. Desmopressin and myocardial infarction. *Lancet*. 1989;1(8643):895–896.
31. Achneck HE, Sileshi B, Jamiolkowski RM, Albala DM, Shapiro ML, Lawson JH. A comprehensive review of topical hemostatic agents: efficacy and recommendations for use. *Ann Surg*. 2010;251(2):217–228.
32. Streiff MB, Ness PM. Acquired FV inhibitors: a needless iatrogenic complication of bovine thrombin exposure. *Transfusion*. 2002;42(1):18–26.
33. Ortel TL, Mercer MC, Thames EH, Moore KD, Lawson JH. Immunologic impact and clinical outcomes after surgical exposure to bovine thrombin. *Ann Surg*. 2001;233(1):88–96.
34. Sarfati MR, Dilorenzo DJ, Kraiss LW, Galt SW. Severe coagulopathy following intraoperative use of topical thrombin. *Ann Vasc Surg*. 2004;18(3):349–351.
35. Chapman WC, Singla N, Genyk Y, et al. A phase 3, randomized, double-blind comparative study of the efficacy and safety of topical recombinant human thrombin and bovine thrombin in surgical hemostasis. *J Am Coll Surg*. 2007;205(2):256–265.
36. Albala DM, Lawson JH. Recent clinical and investigational applications of fibrin sealant in selected surgical specialties. *J Am Coll Surg*. 2006;202(4):685–697.
37. Verstraete M. Clinical application of inhibitors of fibrinolysis. *Drugs*. 1985;29(3):236–261.
38. Henry DA, O'Connell DL. Effects of fibrinolytic inhibitors on mortality from upper gastrointestinal haemorrhage. *BMJ Clin Res*. 1989;298(6681):1142–1146.
39. Henry DA, Moxey AJ, Carless PA, et al. Anti-fibrinolytic use for minimising perioperative allogeneic blood transfusion. *Cochrane Database Syst Rev*. 2001;1:Cd001886. https://doi.org/10.1002/14651858.cd001886.
40. Brown JR, Birkmeyer NJ, O'Connor GT. Meta-analysis comparing the effectiveness and adverse outcomes of antifibrinolytic agents in cardiac surgery. *Circulation*. 2007;115(22):2801–2813.
41. Zufferey P, Merquiol F, Laporte S, et al. Do antifibrinolytics reduce allogeneic blood transfusion in orthopedic surgery? *Anesthesiology*. 2006;105(5):1034–1046.
42. Sukeik M, Alshryda S, Haddad FS, Mason JM. Systematic review and meta-analysis of the use of tranexamic acid in total hip replacement. *J Bone Joint Surg Br*. 2011;93(1):39–46.
43. Kauvar DS, Lefering R, Wade CE. Impact of hemorrhage on trauma outcome: an overview of epidemiology, clinical presentations, and therapeutic considerations. *J Trauma*. 2006;60(6 Suppl):S3–S11.
44. Shakur H, Roberts I, Bautista R, et al. Effects of tranexamic acid on death, vascular occlusive events, and blood transfusion in trauma patients with significant haemorrhage (CRASH-2): a randomised, placebo-controlled trial. *Lancet*. 2010;376(9734):23–32.
45. Cap AP, Baer DG, Orman JA, Aden J, Ryan K, Blackbourne LH. Tranexamic acid for trauma patients: a critical review of the literature. *J Trauma*. 2011;71(1 Suppl):S9–S14.
46. CRASH-3 Trial Collaborators. Effects of tranexamic acid on death, disability, vascular occlusive events and other morbidities in patients with acute traumatic brain injury (CRASH-3): a randomised, placebo-controlled trial. *Lancet*. 2019;394(10210):1713-1723.
47. Crescenti A, Borghi G, Bignami E, et al. Intraoperative use of tranexamic acid to reduce transfusion rate in patients undergoing radical retropubic prostatectomy: double blind, randomised, placebo controlled trial. *BMJ Clin Res*. 2011;343:d5701.
48. Hardy JF. Pharmacological strategies for blood conservation in cardiac surgery: erythropoietin and antifibrinolytics. *Canad J Anaesth*. 2001;48(4 Suppl):S24–S31.
49. Sharma V, Katznelson R, Jerath A, et al. The association between tranexamic acid and convulsive seizures after cardiac surgery: a multivariate analysis in 11,529 patients. *Anaesthesia.* 2014;69(2):124–130.
50. Lecker I, Wang DS, Whissell PD, Avramescu S, Mazer CD, Orser BA. Tranexamic acid-associated seizures: causes and treatment. *Ann Neurol*. 2016;79(1):18–26.
51. Hedner U. Recombinant factor VIIa (Novoseven) as a hemostatic agent. *Semin Hematol*. 2001;38(4 Suppl 12):43–47.
52. Hedner U. Recombinant factor VIIa: its background, development and clinical use. *Curr Opin Hematol*. 2007;14(3):225–229.
53. Boffard KD, Riou B, Warren B, et al. Recombinant factor VIIa as adjunctive therapy for bleeding control in severely injured trauma patients: two parallel randomized, placebo-controlled, double-blind clinical trials. *J Trauma*. 2005;59(1):8–15. discussion 15-8.
54. Knudson MM, Cohen MJ, Reidy R, et al. Trauma, transfusions, and use of recombinant factor VIIa: a multicenter case registry report of 380 patients from the Western Trauma Association. *J Am Coll Surg*. 2011;212(1):87–95.

55. Dutton RP, Parr M, Tortella BJ, et al. Recombinant activated factor VII safety in trauma patients: results from the CONTROL trial. *J Trauma*. 2011;71(1):12–19.
56. Scarpelini S, Rizoli S. Recombinant factor VIIa and the surgical patient. *Curr Opin Crit Care*. 2006;12(4):351–356.
57. Roberts HR. Clinical experience with activated factor VII: focus on safety aspects. *Blood Coag Fibrinoly*. 1998;9:Suppl 1:S115–S118.
58. Roberts HR. Recombinant factor VIIa (Novoseven) and the safety of treatment. *Semin Hematol*. 2001;38(4 Suppl 12):48–50.
59. Thomas GO, Dutton RP, Hemlock B, et al. Thromboembolic complications associated with factor VIIa administration. *J Trauma*. 2007;62(3):564–569.
60. Levi M, Levy JH, Andersen HF, Truloff D. Safety of recombinant activated factor VII in randomized clinical trials. *N Engl J Med*. 2010;363(19): 1791–1800.
61. Rebulla P. Platelet transfusion trigger in difficult patients. *Transf Clin Biol*. 2001;8(3):249–254.
62. Practice Guidelines for Perioperative Blood Management: an updated report by the American Society of Anesthesiologists Task Force on Perioperative Blood Management. *Anesthesiology*. 2015;122(2):241-275.
63. Clark P, Mintz PD. Transfusion triggers for blood components. *Curr Opin Hematol*. 2001;8(6):387–391.
64. Balduini CL, Noris P, Belletti S, Spedini P, Gamba G. In vitro and in vivo effects of desmopressin on platelet function. *Haematologica*. 1999;84(10): 891–896.
65. Noris M, Remuzzi G. Uremic bleeding: closing the circle after 30 years of controversies? *Blood.* 1999;94(8):2569–2574.
66. Dember LM. Critical care issues in the patient with chronic renal failure. *Crit Care Clin*. 2002;18(2):421–440.
67. Moia M, Mannucci PM, Vizzotto L, Casati S, Cattaneo M, Ponticelli C. Improvement in the haemostatic defect of uraemia after treatment with recombinant human erythropoietin. *Lancet*. 1987;2(8570):1227–1229.
68. Sohal AS, Gangji AS, Crowther MA, Treleaven D. Uremic bleeding: pathophysiology and clinical risk factors. *Thromb Res*. 2006;118(3): 417–422.
69. Caldwell SH, Hoffman M, Lisman T, et al. Coagulation disorders and hemostasis in liver disease: pathophysiology and critical assessment of current management. *Hepatology*. 2006;44(4):1039–1046.
70. Lisman T, Leebeek FW. Hemostatic alterations in liver disease: a review on pathophysiology, clinical consequences, and treatment. *Digest Surg*. 2007;24(4):250–258.
71. Tripodi A, Mannucci PM. The coagulopathy of chronic liver disease. *N Engl J Med*. 2011;365(2):147–156.
72. Northup PG, McMahon MM, Ruhl AP, et al. Coagulopathy does not fully protect hospitalized cirrhosis patients from peripheral venous thromboembolism. *Am J Gastroenterol*. 2006;101(7):1524–1528. quiz 1680.
73. Dabbagh O, Oza A, Prakash S, Sunna R, Saettele TM. Coagulopathy does not protect against venous thromboembolism in hospitalized patients with chronic liver disease. *Chest*. 2010;137(5):1145–1149.
74. Sogaard KK, Horvath-Puho E, Gronbaek H, Jepsen P, Vilstrup H, Sorensen HT. Risk of venous thromboembolism in patients with liver disease: a nationwide population-based case-control study. *Am J Gastroenterol*. 2009;104(1):96–101.
75. Amitrano L, Brancaccio V, Guardascione MA, et al. Inherited coagulation disorders in cirrhotic patients with portal vein thrombosis. *Hepatology*. 2000;31(2):345–348.
76. Lisman T, Bakhtiari K, Pereboom IT, Hendriks HG, Meijers JC, Porte RJ. Normal to increased thrombin generation in patients undergoing liver transplantation despite prolonged conventional coagulation tests. *J Hepatol*. 2010;52(3):355–361.
77. Gatt A, Riddell A, Calvaruso V, Tuddenham EG, Makris M, Burroughs AK. Enhanced thrombin generation in patients with cirrhosis-induced coagulopathy. *J Thromb Haemost*. 2010;8(9):1994–2000.
78. Aldawood A, Arabi Y, Aljumah A, et al. The incidence of venous thromboembolism and practice of deep venous thrombosis prophylaxis in hospitalized cirrhotic patients. *Thromb J*. 2011;9(1):1.
79. Caturelli E, Squillante MM, Andriulli A, et al. Fine-needle liver biopsy in patients with severely impaired coagulation. *Liver.* 1993;13(5):270–273.
80. Dillon JF, Simpson KJ, Hayes PC. Liver biopsy bleeding time: an unpredictable event. *J Gastroenterol Hepatol*. 1994;9(3):269–271.
81. Ozier Y, Steib A, Ickx B, et al. Haemostatic disorders during liver transplantation. *Eur J Anaesthesiol*. 2001;18(4):208–218.
82. Deitcher SR. Interpretation of the international normalised ratio in patients with liver disease. *Lancet*. 2002;359(9300):47–48.
83. Rockey DC, Caldwell SH, Goodman ZD, Nelson RC, Smith AD. Liver biopsy. *Hepatology.* 2009;49(3):1017–1044.
84. Abdel-Wahab OI, Healy B, Dzik WH. Effect of fresh-frozen plasma transfusion on prothrombin time and bleeding in patients with mild coagulation abnormalities. *Transfusion*. 2006;46(8):1279–1285.
85. Mannucci PM, Vicente V, Vianello L, et al. Controlled trial of desmopressin in liver cirrhosis and other conditions associated with a prolonged bleeding time. *Blood*. 1986;67(4):1148–1153.
86. Adams GL, Manson RJ, Turner I, Sindram D, Lawson JH. The balance of thrombosis and hemorrhage in surgery. *Hematol Oncol Clin North Am*. 2007;21(1):13–24.
87. Slichter SJ, Funk DD, Leandoer LE, Harker LA. Kinetic evaluation of haemostasis during surgery and wound healing. *Br J Haematol*. 1974;27(1): 115–125.
88. Consensus Conference. Fresh-frozen plasma: indications and risks. *JAMA*. 1985;253(4):551–553.
89. O'Shaughnessy DF, Atterbury C, Bolton Maggs P, et al. Guidelines for the use of fresh-frozen plasma, cryoprecipitate and cryosupernatant. *Br J Haematol*. 2004;126(1):11–28.
90. Murray D, Pennell B, Olson J. Variability of prothrombin time and activated partial thromboplastin time in the diagnosis of increased surgical bleeding. *Transfusion*. 1999;39(1):56–62.
91. Bolliger D, Gorlinger K, Tanaka KA. Pathophysiology and treatment of coagulopathy in massive hemorrhage and hemodilution. *Anesthesiology.* 2010;113(5):1205–1219.
92. Levy JH. Massive transfusion coagulopathy. *Semin Hematol*. 2006; 43(1 Suppl 1):S59–S63.
93. Hiippala ST, Myllyla GJ, Vahtera EM. Hemostatic factors and replacement of major blood loss with plasma-poor red cell concentrates. *Anesth Aanalg*. 1995;81(2):360–3655.
94. Drummond JC, Petrovitch CT. The massively bleeding patient. *Anesthesiol Clin North Am*. 2001;19(4):633–649.
95. Borgman MA, Spinella PC, Perkins JG, et al. The ratio of blood products transfused affects mortality in patients receiving massive transfusions at a combat support hospital. *J Trauma*. 2007;63(4). 805-183.
96. Gunter Jr. OL, Au BK, Isbell JM, Mowery NT, Young PP, Cotton BA. Optimizing outcomes in damage control resuscitation: identifying blood product ratios associated with improved survival. *J Trauma*. 2008;65(3): 527–534.
97. Holcomb JB, Wade CE, Michalek JE, et al. Increased plasma and platelet to red blood cell ratios improves outcome in 466 massively transfused civilian trauma patients. *Ann Surg*. 2008;248(3):447–458.
98. Zink KA, Sambasivan CN, Holcomb JB, Chisholm G, Schreiber MA. A high ratio of plasma and platelets to packed red blood cells in the first 6 hours of massive transfusion improves outcomes in a large multicenter study. *Am J Surg*. 2009;197(5):565–750. discussion 570.
99. Holcomb JB, Tilley BC, Baraniuk S, et al. Transfusion of plasma, platelets, and red blood cells in a 1:1:1 vs a 1:1:2 ratio and mortality in patients with severe trauma. *JAMA*. 2015;313(5):471.
100. Wafaisade A, Maegele M, Lefering R, et al. High plasma to red blood cell ratios are associated with lower mortality rates in patients receiving multiple transfusion (4</=red blood cell units<10) during acute trauma resuscitation. *J Trauma*. 2011;70(1):81–88. discussion 88-89.
101. Colman RW. Hemostatic complications of cardiopulmonary bypass. *Am J Hematol*. 1995;48(4):267–272.
102. Gelb AB, Roth RI, Levin J, et al. Changes in blood coagulation during and following cardiopulmonary bypass: lack of correlation with clinical bleeding. *Am J Clin Pathol*. 1996;106(1):87–99.
103. Mammen EF, Koets MH, Washington BC, et al. Hemostasis changes during cardiopulmonary bypass surgery. *Semin Thromb Hemost*. 1985;11(3): 281–292.
104. Boisclair MD, Lane DA, Philippou H, et al. Mechanisms of thrombin generation during surgery and cardiopulmonary bypass. *Blood*. 1993;82(11): 3350–3357.
105. Zilla P, Fasol R, Groscurth P, Klepetko W, Reichenspurner H, Wolner E. Blood platelets in cardiopulmonary bypass operations. Recovery occurs after initial stimulation, rather than continual activation. *J Thorac Cardiovasc Surg*. 1989;97(3):379–388.
106. Weerasinghe A, Taylor KM. The platelet in cardiopulmonary bypass. *Ann Thorac Surg*. 1998;66(6):2145–2152.
107. Milas BL, Jobes DR, Gorman RC. Management of bleeding and coagulopathy after heart surgery. *Semin Thorac Cardiovasc Surg*. 2000; 12(4):326–336.
108. Karlsson M, Ternstrom L, Hyllner M, et al. Prophylactic fibrinogen infusion reduces bleeding after coronary artery bypass surgery. A prospective randomised pilot study. *Thromb Haemost*. 2009;102(1): 137–144.
109. Porte RJ. Coagulation and fibrinolysis in orthotopic liver transplantation: current views and insights. *Semin Thromb Hemost*. 1993;19(3):191–196.

110. Bakker CM, Metselaar HJ, Groenland TN, et al. Increased tissue-type plasminogen activator activity in orthotopic but not heterotopic liver transplantation: the role of the anhepatic period. *Hepatology*. 1992;16(2):404–408.
111. Dmitrewski J, Russell S, Vijeyasingham R, McMaster P. Hematologic problems and organ transplantation. *World J Surg*. 1996;20(9):1160–1165.
112. Porte RJ, Caldwell SH. The role of recombinant factor VIIa in liver transplantation. *Liver Transplant*. 2005;11(8):872–874.
113. Douketis JD, Spyropoulos AC, Spencer FA, et al. Perioperative management of antithrombotic therapy: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed. American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest*. 2012;141(2):e326S–e350S.
114. Skolarus LE, Morgenstern LB, Froehlich JB, Lisabeth LD, Brown DL. Guideline-discordant periprocedural interruptions in warfarin therapy. *Circ Cardiovasc Qual Outcomes*. 2011;4(2):206–210.
115. Kearon C, Hirsh J. Management of anticoagulation before and after elective surgery. *N Engl J Med*. 1997;336(21):1506–1511.
116. Spandorfer J. The management of anticoagulation before and after procedures. *Med Clin North Am*. 2001;85(5):1109–1116. v.
117. Spyropoulos AC. Bridging therapy and oral anticoagulation: current and future prospects. *Curr Opin Hematol*. 2010;17(5):444–449.
118. Walenga JM, Prechel M, Jeske WP, Bakhos M. Unfractionated heparin compared with low-molecular-weight heparin as related to heparin-induced thrombocytopenia. *Curr Opin Pulmon Med*. 2005;11(5):385–391.
119. O'Donnell MJ, Kearon C, Johnson J, et al. Brief communication: preoperative anticoagulant activity after bridging low-molecular-weight heparin for temporary interruption of warfarin. *Ann Intern Med*. 2007;146(3):184–187.
120. Douketis JD, Spyropoulos AC, Kaatz S, et al. Perioperative bridging anticoagulation in patients with atrial fibrillation. *N Engl J Med*. 2015;373(9):823–833.
121. Crowther MA, Douketis JD, Schnurr T, et al. Oral vitamin K lowers the international normalized ratio more rapidly than subcutaneous vitamin K in the treatment of warfarin-associated coagulopathy. A randomized, controlled trial. *Ann Intern Med*. 2002;137(4):251–254.
122. Holbrook A, Schulman S, Witt DM, et al. Evidence-based management of anticoagulant therapy: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed. American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest*. 2012;141(2_suppl):e152S–e184S.
123. Watson HG, Baglin T, Laidlaw SL, Makris M, Preston FE. A comparison of the efficacy and rate of response to oral and intravenous vitamin K in reversal of over-anticoagulation with warfarin. *Br J Haematol*. 2001;115(1):145–149.
124. Leissinger CA, Blatt PM, Hoots WK, Ewenstein B. Role of prothrombin complex concentrates in reversing warfarin anticoagulation: a review of the literature. *Am J Hematol*. 2008;83(2):137–143.
125. Levy JH, Key NS, Azran MS. Novel oral anticoagulants: implications in the perioperative setting. *Anesthesiology.* 2010;113(3):726–745.
126. Douketis JD. Perioperative management of antithrombotic therapy: lifting the fog. *J Thromb Haemost*. 2009;7(12):1979–1981.
127. Hankey GJ, Eikelboom JW. Dabigatran etexilate: a new oral thrombin inhibitor. *Circulation.* 2011;123(13):1436–1450.
128. Eikelboom JW, Quinlan DJ, van Ryn J, Weitz JI. Idarucizumab: the antidote for reversal of dabigatran. *Circulation*. 2015;132(25):2412–2422.
129. Pollack Jr. CV, Reilly PA, Bernstein R, et al. Design and rationale for RE-VERSE AD: A phase 3 study of idarucizumab, a specific reversal agent for dabigatran. *Thromb Haemost*. 2015;114(1):198–205.
130. Reilly PA, van Ryn J, Grottke O, Glund S, Stangier J. Idarucizumab, a specific reversal agent for dabigatran: mode of action, pharmacokinetics and pharmacodynamics, and safety and efficacy in phase 1 subjects. *Am J Emerg Med*. 2016;34(11, Supplement):26–32.
131. Pernod G, Albaladejo P, Godier A, et al. Management of major bleeding complications and emergency surgery in patients on long-term treatment with direct oral anticoagulants, thrombin or factor-Xa inhibitors: Proposals of the Working Group on Perioperative Haemostasis (GIHP) – March 2013. *Arch Cardiovasc Dis*. 2013;106(6–7):382–393.
132. Reiffel JA, Weitz JI, Reilly P, et al. NOAC monitoring, reversal agents, and post-approval safety and effectiveness evaluation: a cardiac safety research consortium think tank. *Am Heart J*. 2016;177:74–86.
133. Anderson DR, Morgano GP, Bennett C, et al. American Society of Hematology 2019 guidelines for management of venous thromboembolism: prevention of venous thromboembolism in surgical hospitalized patients. *Blood Adv*. 2019;3(23):3898–3944.
134. McGowan KE, Makari J, Diamantouros A, et al. Reducing the hospital burden of heparin-induced thrombocytopenia: impact of an avoid-heparin program. *Blood*. 2016;127(16):1954–1959.
135. Perka C. Preoperative versus postoperative initiation of thromboprophylaxis following major orthopedic surgery: safety and efficacy of postoperative administration supported by recent trials of new oral anticoagulants. *Thromb J*. 2011;9:17.
136. Falck-Ytter Y, Francis CW, Johanson NA, et al. Prevention of VTE in orthopedic surgery patients: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed. American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest*. 2012;141(2 Suppl):e278S–e325S.
137. Anderson DR, Dunbar M, Murnaghan J, et al. Aspirin or rivaroxaban for VTE prophylaxis after hip or knee arthroplasty. *N Engl J Med*. 2018;378(8):699–707.

CHAPTER 156

THE SPLEEN AND ITS DISORDERS

Thomas A. Ollila, Adam S. Zayac, and Fred J. Schiffman

Galen described the spleen as the "organ of mystery," with functions related to mood and good or ill humors. It was not until the 18th century that the spleen's relationship to the immune and hematologic systems was appreciated. The complexities and multiplicity of splenic function continue to be the focus of research and observation. Although many of its functions overlap with, or can be assumed by other organs, it is an important regulator of immune response and hematologic homeostasis. The spleen efficiently phagocytoses erythrocytes, recycles iron, recognizes and destroys pathogens, and induces adaptive immune responses. An appreciation for the subtleties of its anatomy and function is important for the physician evaluating patients with many hematologic, immunologic, hepatic, and infectious diseases.

NORMAL SPLENIC ANATOMY AND FUNCTION

Embryology

The spleen arises from the mesoderm and, by the ninth week of gestation, layers of the left dorsal mesogastrium condense with the appearance of blood vessels. Sheaths are formed around arterioles by reticular cells and fibers.[1,2] Macrophages are present and develop phagocytic function by the end of the first trimester. Lymphocytes appear during the fourth month, and a formal delineation between red and white pulp can be identified by the sixth month. Germinal centers do not develop during fetal development, but primitive inactive follicles are evident at birth. In mice, the homeobox gene *Tlx1* (formerly known as *Hox11*), which controls the genesis of the splanchnic mesodermal plate, is essential for development of the spleen.[3] Both the basic helix-loop-helix transcription factor capsulin and the Wilms tumor suppressor 1 (*WT1*) gene are necessary for formation of the spleen.[2] The genetic basis for development of the human spleen is less well understood. The role of the ribosome in splenic development has recently risen to the forefront with discovery of isolated congenital asplenia (ICA) with variable penetrance amongst patients with mutations in ribosomal protein SA, a component of the small subunit of the ribosome.[4,5] However, to date its exact role has not yet been elucidated. The spleen is capable of supporting hematopoiesis during fetal life and, in a variety of pathologic states, postnatally.[6] The circulation of primitive hematopoietic stem cells in peripheral blood during prenatal life through birth makes it difficult to distinguish hematopoiesis arising from stem cells in the spleen as opposed to the incidental presence of hematopoietic cells within the circulation.

Anatomy

The spleen is the body's largest filter of the blood.[1,2,7] Located directly below the diaphragm and adjacent to the stomach, it is covered by a fibrous capsule with blood vessels, lymphatics, and nerves coated by peritoneal mesothelium. The splenic artery arises from the celiac axis, enters the capsule at the hilum, and branches into trabecular arteries. The trabecular arteries then branch into central arteries and enter the white pulp. The periarterial lymphatic sheath consists of a cuff of T lymphocytes, plasma cells, and macrophages around the central arteries. As the arteries branch, the sheath narrows. B-lymphocyte clusters appear in follicles along the periarterial lymphatic sheath at arterial branch points (Fig. 156.1).

The components of the white pulp are connected by a reticular network and supporting stromal cells.[1] On the cut surface of the normal spleen, white pulp is visible as white nodules approximately 1 to 2 mm in diameter, although their size varies with age and antigenic stimulation. The nodules are fully developed at birth, increase in size during childhood (especially following immunizations and infections), peak during puberty, and involute in adulthood. Uninfected adults with an intact immune system usually do not show evidence of germinal centers. The secondary germinal center comprises a mantle zone of B lymphocytes surrounding the follicle. Antigen trapping and processing take place in the marginal zone of the white pulp.

The red pulp of the spleen consists of vascular sinuses, the cords of Billroth, and the terminal branches of the penicilliary arteries (Fig. 156.2).[1,2] Vascular sinuses are lined with $CD8^+$ littoral endothelial cells that resemble endothelial cells with long processes and a basement membrane with ring fibers that attach to macrophage-derived dendritic processes. No tight junctions or interdigitations connect the cytoplasmic processes. Intact leukocytes, erythrocytes, and platelets are able to squeeze through the potential spaces between these cells and between the ring fibers (Fig. 156.3). Processes of the reticular cells of the cords of Billroth are outside the sinus walls. Endothelial cells line pulp veins but not the cords.

The reticular structure of the spleen facilitates its immune response. Venous sinus endothelial cells contain a plasma membrane-associated network of stress fibers composed of actin and myosin-like filaments. These filaments may cross the plasma membrane and insert into the mesh-like basement membrane. As the fibers tense, they create fenestrations through which erythrocytes must pass if they hope to continue their journey. With age, erythrocytes lose the ability to change their shape and navigate the pathways created by these fibers and are unable to continue into the circulation. The limitation is similar for erythrocytes containing parasitic infections. This mechanism, combined with mannose receptors and Toll-like receptors, helps the infrastructure of the spleen play a role in the host's overall immune response.

Circulation of blood through red pulp lined with endothelial-like littoral cells represents a rapid and closed circulation. Circulation into the cords is slower and open, thereby permitting the macrophages lining the cord to remove damaged or aged cells.

Accessory spleens are present in up to a third of the population and result from failure of precursor cells to fuse during embryologic development. Usually, they receive blood flow from the splenic artery and are located near the spleen, but they can be distant and mistaken for a tumor when noted on imaging studies or physical examination. Accessory spleens may develop similar conditions as the spleen proper and should be considered within the differential diagnosis for patients who have continued abnormalities after splenectomy.

Functions

The functions of the spleen and their anatomic locations are summarized in Table 156.1. Correlation with anatomy and histology are shown in Fig. 156.4.

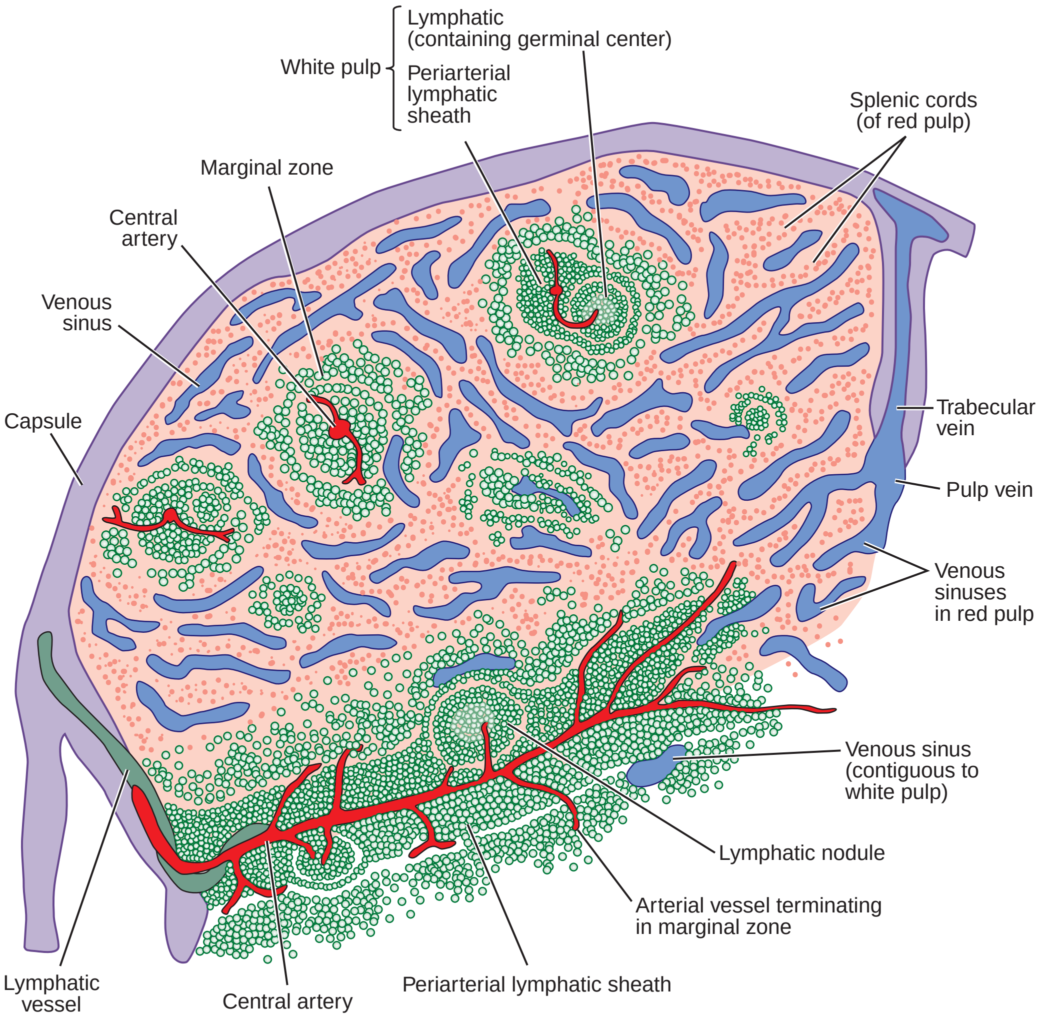

White pulp
Lymphatic (containing germinal center)
Periarterial lymphatic sheath
Splenic cords (of red pulp)
Marginal zone
Central artery
Venous sinus
Capsule
Trabecular vein
Pulp vein
Venous sinuses in red pulp
Venous sinus (contiguous to white pulp)
Lymphatic nodule
Arterial vessel terminating in marginal zone
Lymphatic vessel
Central artery
Periarterial lymphatic sheath

Figure 156.1 ORGANIZATION OF THE HUMAN SPLEEN PRESENTED SCHEMATICALLY. See text for description of blood flow and cell distribution. *(Used, with permission, from Emerson SG. Hematopoiesis: The development of blood cells. In: Schiffman FJ, ed. Hematologic Pathophysiology. Philadelphia, PA: Lippincott-Raven; 1998:10.)*

The Red Pulp

Splenic macrophages dominate the function of the red pulp and are responsible for filtering blood, removing bacteria, and recycling iron.[1,2] The macrophages in the spleen have distinct features, including distinct Fc-γ receptors, than those derived from blood monocytes.[8]

Removal of Damaged and Aged Formed Elements

The venous system of the red pulp enables it to filter whole blood, removing senescent erythrocytes and other blood cells.[2,9] Arterial blood is delivered to the cords of the red pulp through an open system of reticular fibers, fibroblasts, and macrophages without an endothelial lining. Blood then passes from the cords into the efferent venous sinuses, which are lined with endothelial-like littoral cells with a discontinuous structure. Stress fibers extend beneath the basal plasma membrane and run parallel to the axis of the littoral cells. These cords direct the blood into sinuses through slits modulated in size by the stress fibers. In many animals, the stress fibers and splenic capsule are contractile, giving the spleen the ability to serve as a reservoir of red cells while reducing blood viscosity at rest. In humans, however, there is no evidence that the spleen serves such a function or is capable of significant changes in volume with rest and exercise. To return to the circulation, cells must pass through the slits between venous sinus littoral cells (see Fig. 156.3), the size of which may be controlled in part by actin and myosin filaments within the stress fibers in the basal portion of endothelial cells. This surface is believed to be an important site for the culling and pitting of aged or damaged cells.

Compared with when they are young and have a healthy metabolic reserve, older erythrocytes and platelets are unable to tolerate the hostile splenic environment. The spleen has a pH between 6.8 and 7.2, is hypoxic (partial pressure of oxygen [PO_2]: 54 mm Hg), and hypoglycemic (glucose concentration approximately 60% of that in venous blood). With age, damaged enucleated cells undergo changes in complex membrane carbohydrates, which facilitate recognition by splenic macrophages and removal from the circulation. *Culling* describes the destruction of erythrocytes: the normal removal of aging cells or the removal of damaged cells in pathologic states. Most platelets and leukocytes are not removed by the spleen as they age but adhere to vessel walls and migrate into tissues where they die. *Pitting* refers to the removal of inclusions from within erythrocytes, which are then released back into the circulation. The erythrocyte membrane is in close apposition to the macrophage membrane so that aged or damaged glycoproteins, antibody, or complement on the surface of the cell are easily recognized and activate phagocytosis. Particulate matter—Howell-Jolly bodies (basophilic nuclear remnants), Heinz bodies (clumps of damaged hemoglobin), Pappenheimer bodies (abnormal iron granules), and malarial and other parasites—are removed and the cell is returned to the bloodstream. If membrane-containing adsorbed antibody is present, it is removed and the remainder of the cell is released with changes in shape and volume. Internal vesicles near the erythrocyte membrane appear as if they were on the surface of the cell and are termed *pits* or

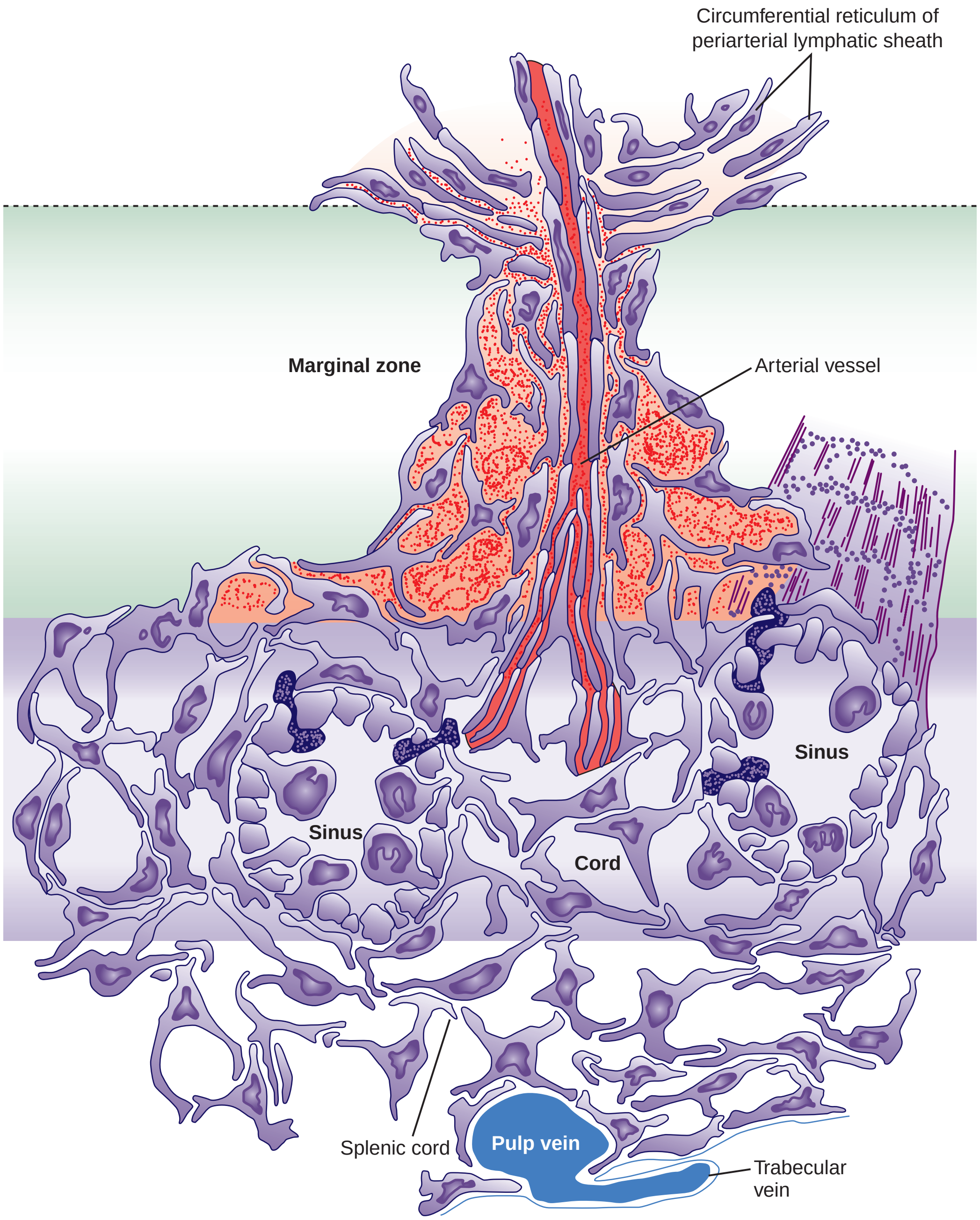


Figure 156.2 DIAGRAM OF THE SPLENIC ARTERY. Arterial blood pools in the splenic cords before entering the splenic sinuses and returning to systemic circulation. *(Used, with permission, from Emerson SG. Hematopoiesis: The development of blood cells. In: Schiffman FJ, ed. Hematologic Pathophysiology. Philadelphia, PA: Lippincott-Raven; 1998:12.)*

pocks. The spleen also normally removes these, with the polished erythrocyte returned to the circulation. The lack of this polishing function can be used to assess splenic dysfunction as the number of pits or pocks per erythrocyte is increased when the spleen fails to remove them.

Recycling of Iron

Macrophages in the liver and spleen are important for recycling iron as erythrocytes are phagocytized and hydrolyzed in the phagolysosome (Chapters 36 and 37).[2] Degradation of hemoglobin releases heme, which is catabolized into biliverdin, carbon monoxide, and ferrous iron (Fe^{2+}). Iron is then either released as a low-molecular-weight species for rapid reuse or stored as ferritin. As ferritin accumulates, it aggregates into hemosiderin. Iron-laden macrophages are a feature of iron overload states in both the liver and spleen. Erythrocytes that are destroyed intravascularly release hemoglobin that binds to haptoglobin. CD163 on the surface of splenic macrophages mediates endocytosis of circulating hemoglobin and haptoglobin-bound hemoglobin.

Iron is released from stores in splenic macrophages in response to the erythropoietic drive expressed by the bone marrow. The precise mechanisms are not well understood. Uptake of iron is mediated in most cells by a divalent cation transporter, the natural resistance-associated macrophage protein 2 (NRAMP2), in recycling endosomes that express the transferrin receptor. NRAMP2 transports ferritin into the cytoplasm across the endosomal membrane. In addition to the more widely expressed NRAMP2, macrophages and monocytes express NRAMP1, which was initially described as an iron-transporting protein affecting resistance to intracellular pathogens. A third molecule involved in iron metabolism in the spleen is lipocalin-2, which is secreted by macrophages and other myeloid cells when activated by exposure to bacterial metabolites. Lipocalin-2 binds to bacterial siderophores and sequesters iron from microorganisms. These and other related molecules tightly link the iron-recycling and host-resistance functions of the red pulp of the spleen.

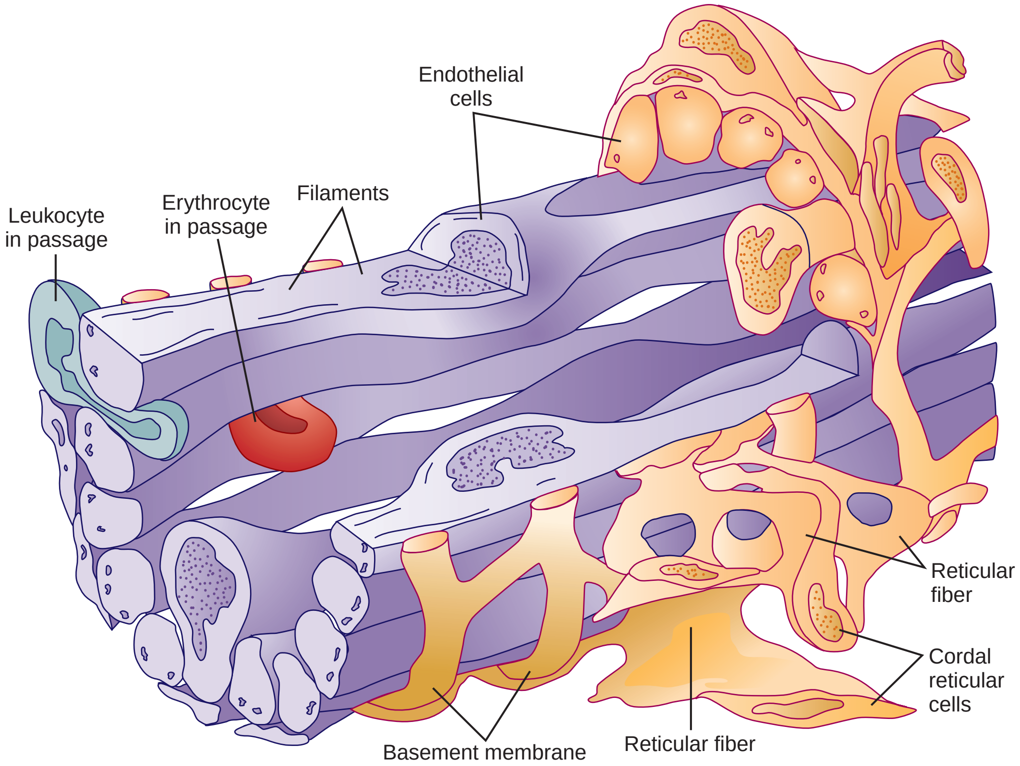


Figure 156.3 ORGANIZATION OF THE SPLENIC SINUSES. Erythrocytes and leukocytes are scrutinized as they squeeze through the reticular fibers of endothelial cells to enter the splenic sinuses. The fenestrae contract and relax according to sympathetic stimuli to regulate cell passage. *(Used, with permission, from Emerson SG. Hematopoiesis: The development of blood cells. In: Schiffman FJ, ed. Hematologic Pathophysiology. Philadelphia, PA: Lippincott-Raven; 1998:13.)*

TABLE 156.1 **Functions of the Spleen**

Category	Function	Effector Cells/Areas
Clearance/ Filtration	Antibody-mediated clearance Culling and pitting	Macrophages Reticular meshwork
Immune Response		
Marginal zone	Interaction of antigens with effector cells	Monocytes, lymphocytes
	Antigen processing	Macrophages
	Immune recognition	T cells (periarteriolar sheath)
White pulp	Immunoregulation, antibody production	B cells (lymphoid follicles)
	Antigen processing, preservation	Macrophages
Red pulp	Phagocytosis	Macrophages, monocytes, neutrophils
Hematologic	Hematopoiesis	Cords and sinuses
	Storage of erythrocytes, platelets, leukocytes	Reticular meshwork, red pulp
	Finishing/polishing of erythrocytes	Reticular meshwork, macrophages
Hemostasis	Production of factor VIII, von Willebrand factor	Endothelial cells

Antibody Production

After stimulation and differentiating in the follicles of the white pulp, antigen-specific plasmablasts and plasma cells lodge in the red pulp, where they are closer to macrophages.[2] A similar translocation occurs in lymph nodes, where plasmablasts migrate to the medullary cords. This translocation occurs after upregulation of expression of CXC-chemokine receptor 4 (CXCR4), which binds CXC-chemokine ligand 12 (CXCL12) expressed in red pulp.

The White Pulp

The lymphoid region of the spleen incorporates multiple components of the immune system that are organized to enhance recognition and/or response to pathogens.[10] The white pulp contains lymphoid sheaths around branching arterial vessels, similar to the structure of lymph nodes. Specific chemokines attract B and T cells to their appropriate domains. The periarteriolar lymphatic sheath contains T cells that interact with dendritic cells and circulating B cells. T cells and dendritic cells are attracted to the periarteriolar lymphatic sheath through CC-chemokine ligands 19 and 21. Clonal expansion of activated B cells occurs in follicles. B-cell migration to follicles requires CXCL13. Expression of all these homeostatic chemokines is reduced when signaling through the lymphotoxin receptor or tumor necrosis factor (TNF)-α receptor 1 is absent, which results in disorganization of the domains of the white pulp.

Marginal Zone

Similar to the migration of leukocytes across endothelial barriers in inflammation, leukocytes leave the bloodstream and enter the white pulp in the marginal zone of the spleen due to the function of

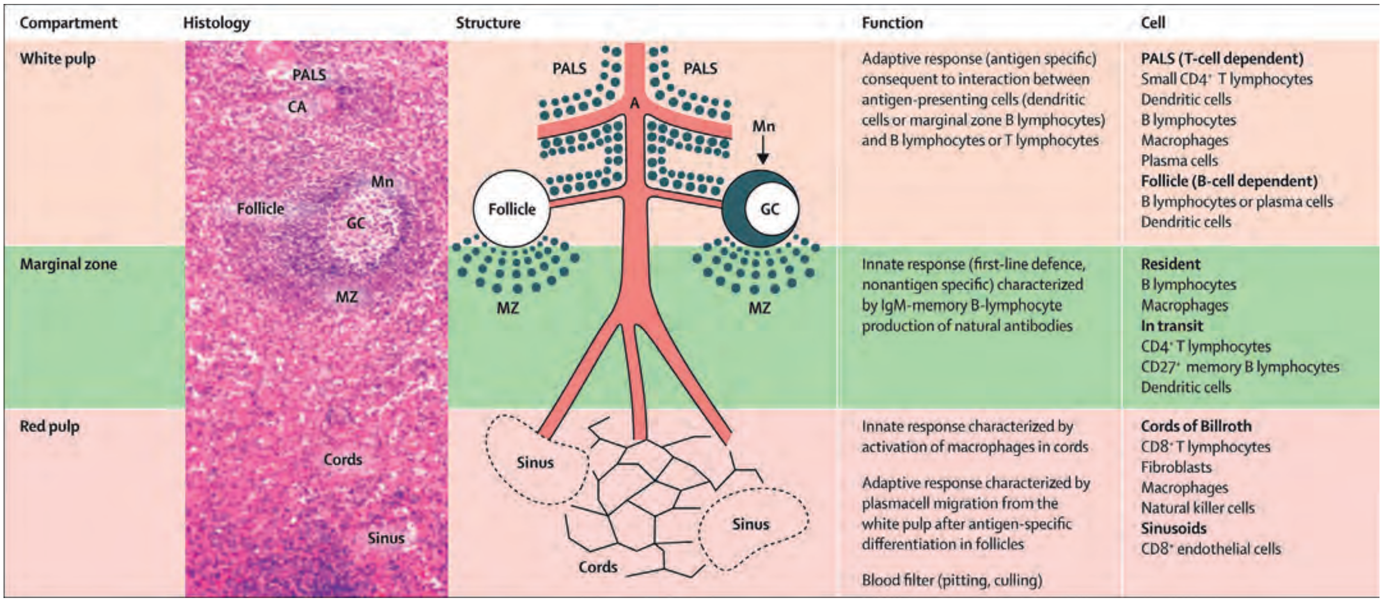

Compartment
Histology
Structure
Function
Cell
White pulp
PALS
CA
Mn
Follicle
GC
MZ
Cords
Sinus
PALS
PALS
A
Mn
Follicle
GC
MZ
MZ
Sinus
Sinus
Cords
Adaptive response (antigen specific) consequent to interaction between antigen-presenting cells (dendritic cells or marginal zone B lymphocytes) and B lymphocytes or T lymphocytes
PALS (T-cell dependent)
Small CD4⁺ T lymphocytes
Dendritic cells
B lymphocytes
Macrophages
Plasma cells
Follicle (B-cell dependent)
B lymphocytes or plasma cells
Dendritic cells
Marginal zone
Innate response (first-line defence, nonantigen specific) characterized by IgM-memory B-lymphocyte production of natural antibodies
Resident
B lymphocytes
Macrophages
In transit
CD4⁺ T lymphocytes
CD27⁺ memory B lymphocytes
Dendritic cells
Red pulp
Innate response characterized by activation of macrophages in cords
Adaptive response characterized by plasmacell migration from the white pulp after antigen-specific differentiation in follicles
Blood filter (pitting, culling)
Cords of Billroth
CD8⁺ T lymphocytes
Fibroblasts
Macrophages
Natural killer cells
Sinusoids
CD8⁺ endothelial cells

Figure 156.4 STRUCTURE, FUNCTION, AND CELL POPULATIONS OF THE THREE FUNCTIONAL COMPARTMENTS OF THE SPLEEN. The histology panel shows a hematoxylin- and eosin-stained section of normal spleen. *A*, Artery; *CA*, central arteriola; *GC*, germinal center; *Mn*, mantle zone; *MZ*, marginal zone; *PALS*, periarteriolar lymphoid sheath. *(Used, with permission, from Di Sabatino, A., Carsetti R, Corazza GR. Post-splenectomy and hyposplenic states. Lancet. 2011;378[9785]:86–97.)*

G protein-coupled receptors.[2,10] The marginal zone is an area for cells in transit and home to resident cells with complex interactions. Marginal zone metallophilic macrophages form an inner ring close to the white pulp, marginal zone macrophages form an outer ring, and dendritic cells and B cells are located between the two sets of macrophages. Both trafficking and retention of dendritic cells and B cells involve complex interactions with integrins and other adhesion molecules.

Innate Immunity

The spleen is important in the recognition of antigens, in the production of antibodies, and in the clearance of opsonized and nonopsonized particles from the bloodstream (see Table 156.1).[2] The spleen structure facilitates monitoring of the contents of the blood, with blood flowing through the marginal zone directly along the white pulp. The white pulp is involved in adaptive immunity, whereas the marginal zone is involved in both innate and adaptive immunity (Table 156.2).

Trapping and processing antigens is a major function of the marginal zone. Macrophages are abundant here, and the spleen captures more than 5% of the cardiac output (> 250 mL/min), which allows a large volume of blood to be immunologically scrutinized. Antigens penetrate the germinal center where T lymphocytes predominate. Processing carbohydrate antigens is a special function of splenic marginal zone lymphocytes. Marginal zone macrophages express lectin receptors and scavenger molecules.

Splenic macrophages are more sensitive to small amounts of opsonic antibody or complement present on the surface of particles than are macrophages in the liver, lung, or bone marrow. In the absence of a spleen, individuals may fail to remove bacteria with limited opsonic coating, and the production of antigen-specific immunoglobulin (Ig) M is impaired. An asplenic individual has defective recognition of carbohydrate antigens, defective production of IgM early during infection, and defective removal of lightly opsonized particles—all crucial components of response to an invasive infection with encapsulated organisms.

Marginal zone B lymphocytes are sensitive to detection of blood-borne pathogens and rapidly differentiate into either antigen-presenting cells (APCs) or IgM-producing plasma cells. After activation in the marginal zone, some B cells migrate into the white pulp, where they function to activate naive CD4$^+$ T cells.

Similarly, blood-borne dendritic cells activated in the marginal zone migrate into the white pulp. This process appears to be important in control of certain parasitic infections. For example, in *Leishmania donovani* infection, the spread of infection is inversely proportional to the upregulation or downregulation of CCR7 expression on activated dendritic cells and migration of APCs into the white pulp. In human immunodeficiency virus (HIV)-positive patients with low CD4 T-cell counts (less than 300 cells/μL), there is evidence that IgM memory B-cell depletion might be a risk factor for pneumococcal disease. Effective antiretroviral therapy appears to reverse this depletion, in turn decreasing the risk for pneumococcal infection.

Adaptive Immunity

Although the white pulp is both anatomically and functionally similar to lymph nodes, all cells enter the white pulp via the marginal zone rather than through high endothelial venules and afferent lymphatic vessels, exposing them to an environment highly sensitive to detection of blood-borne pathogens.[2,10] Circulating dendritic cells capture bacteria from the blood and transport them to the spleen, where they mediate the initial differentiation of B cells to plasmablasts or APCs. Activated APCs entering the white pulp activate T cells, resulting in changes in receptor expression that enable them to migrate to the edge of B-cell follicles. Contact with activated T cells induces an isotype switch in follicular B cells, which then migrate to the red pulp and marginal zones or remain in splenic germinal centers. The white pulp of the spleen is the largest mass of lymphoid tissue in the body. The B lymphocytes, which ultimately produce antibodies, predominate in the nearby germinal centers and mantle zones. These areas all increase in size and activity with immunization or infection. The complex interaction between the cells in close apposition, autocrine, and paracrine signaling, and migration of cells into, within, and ultimately out of the spleen, contributes to the extraordinary repertoire of innate and adaptive immunity within the organ. Changes in splenic architecture are quite dramatic with septicemia, the most marked being depletion of splenic B-cell areas that occurs during overwhelming enterococcemia. Bacterial virulence factors appear to contribute to depletion of B- and T-cell areas.

Hematopoiesis

Active hematopoiesis can be seen in the fetal spleen throughout the second trimester, decreasing during the third trimester.[7] Erythropoiesis and megakaryocytopoiesis predominate, with myelopoiesis present to a lesser extent. As hematopoiesis transitions from the hepatic phase

TABLE 156.2 **Innate Immune Pattern Recognition Receptors That Are Highly Expressed on Splenic Phagocytic Cells**[a]

Name	Abbreviation	Function	Splenic Cells Expressing	Recognizes or Affects	Pathogens Recognized
Toll-like receptor networks	TLR	Pattern recognition of pathogen-associated molecular patterns	Marginal zone macrophages Dendritic cells	Lipopolysaccharide Flagellin Double-stranded RNA	Multiple bacteria, especially gram negative
Natural resistance–associated macrophage protein-1	NRAMP1	Solute carrier family 11 (proton-coupled divalent metal ion transporters); phagolysosomal function	Macrophages	Lysosomal targeting	*Mycobacterium tuberculosis* *Mycobacterium avium* *Mycobacterium leprae*
Natural resistance–associated macrophage protein-2	NRAMP2	Proton-dependent cation transporter; iron absorption	Macrophages (apical duodenal cells, erythroid cells)	Impairs bacterial gene expression, protein synthesis	*Brucella* spp.
Sialic acid–binding Ig-like lectin-1; sialoadhesin	SIGLEC1, CD169	Immunoglobulin superfamily	Splenic and peritoneal macrophages; Many hematopoietic cells	Mannose	*Streptococcus pneumoniae*
Specific intercellular adhesion molecule 3–grabbing nonintegrin related 1	SIGNR1, CD209b	ICAM3-binding nonintegrin homologue, mannose receptor	Dendritic cells	Polysaccharide antigens, ManLAM	Fungi, other pathogens
Macrophage receptor with collagenous structure	MARCO	Type 1 scavenger	Marginal zone macrophages	Lipopolysaccharide	*Staphylococcus aureus* *Escherichia coli*

ICAM, Intercellular adhesion molecule; *ManLAM*, mannosylated lipoarabinomannan; *RNA*, ribonucleic acid.

[a] These receptors contribute to resistance to infection with pathogens in the absence of prior exposure, specific immune responses, or surface opsonic immunoglobulin or complement.

into the bone marrow, it becomes less evident within the fetal spleen. The spleen is not normally a site of hematopoiesis in postnatal life in humans, but it is a rich source of hematopoietic stem cells. In pathologic states associated with bone marrow failure, its role expands from that of a primarily lymphoid organ to that of a hematopoietic organ.[2,11,12] Extramedullary hematopoiesis is a significant cause of splenomegaly primarily in bone marrow disorders (e.g., myelofibrosis or osteopetrosis (Chapter 72) and chronic hemolytic anemias (Chapters 41, 44, and 47) (e.g., thalassemia). The stromal cells of the spleen appear to be capable of supporting hematopoiesis and may produce the C-KIT ligand, as do marrow stromal cells. When splenic hematopoiesis occurs, the erythrocytes and platelets that circulate tend to be less mature than when hematopoiesis occurs in the bone marrow, which suggests that egress from the spleen is easier than from the marrow. This may not always be a benign function; emerging evidence suggests that the myeloid progenitors in the spleen may offer a therapeutic target in hematologic malignancy.[14]

Stem Cells

Experiments from the 1950s first demonstrated the presence of splenic stem cells.[15] Either lead shielding around the spleen or the injection of spleen cells allowed for the reestablishment of hematopoiesis in lethally irradiated mice. Resident stem cells are capable of restoring hematopoietic and immunologic function after lethal irradiation.[13] The spleen has been shown to be a source of multipotent stem cells in humans and other animals. These stem cells have been shown to contribute to the regeneration of multiple types of tissue, including pancreatic islet cells, bone, cranial nerves, and salivary glands. Undifferentiated splenic monocytes assembled in clusters in the cords of the subcapsular red pulp have been shown to accumulate in injured myocardial tissue.[16,17] In murine models, splenic stem cells are in a G1 “pre-activated” phase, as opposed to the G0 phase of bone marrow, perhaps explaining how they are quickly deployed in response to acute inflammatory stress.[18] These stem cells are considered to have limited potential to undergo malignant transformation.

Storage of Cells

The normal adult human spleen contains 20 to 40 mL of blood and does not serve as a reservoir for blood or erythrocytes. However, in many conditions associated with splenomegaly, especially portal hypertension, the pulp cords widen to create an organ with more storage volume. Vascular pooling of blood and formed elements occurs, regardless of the underlying cause of the splenomegaly. Platelets and granulocytes, however, are normally stored in the red pulp of the spleen. As much as one-third of the total platelet mass may be stored in the spleen and released when cytokines affecting platelet adhesiveness are released. The lack of musculature in the human splenic capsule prevents the distention and contraction that occur in many animals, such as dogs, although the spleen appears to contract in divers during periods of breath-hold apnea. Indeed, the indigenous Bajau people of Southeast Asia with increased variants in the PDE10A gene have increased spleen size, possibly explaining their extraordinary diving ability as a result and an increased pool of oxygenated blood which can be expelled into circulation with splenic contraction, an action formerly not thought to occur in humans.[19] Platelet counts and granulocyte counts rise significantly after splenectomy, then fall.

The circulating masses of these cell pools are chronically increased post-splenectomy, which may contribute to an increased incidence of atherosclerosis years after splenectomy.

EXAMINATION OF THE SPLEEN

Maneuvers for examination of the spleen involve inspection, percussion, and palpation, but their sensitivity and specificity vary based on patient factors (such as body habitus) and operator skill.[20] The pretest probability of splenomegaly, based on associated historical and clinical data, influences the positive-predictive value of finding splenomegaly on physical examination. If the pretest probability of splenomegaly is less than 10%, then physical examination maneuvers are inadequate for determining the presence or absence of splenomegaly. For those patients with a greater than 10% pretest probability of splenomegaly (for instance, patients with suspected infectious mononucleosis), the examination begins with inspection and then percussion of Traube's space—defined by the left sixth rib superiorly, the left anterior axillary line, and the costal margin. For adequate examination of the spleen, the patient must be fully supine, relaxed, with arms adjacent to the trunk. The sensitivity and specificity of Traube's method is 62% and 72%, respectively, amongst all patients, and improves to 78% and 82%, respectively, amongst fasting, non-obese patients.[20] There are multiple methods for percussion to assess for splenomegaly, each with their own eponymous signs. In addition to percussion of Traube's space, Nixon's method is performed by percussing cranially, starting at the midpoint of the left costal margin with the patient in right lateral decubitus position. A positive Nixon's sign is denoted by dullness to percussion extending over eight centimeters above the costal margin perpendicularly, with a sensitivity of 59% and specificity of 94%.[20] Lastly Castell's method, felt to be the most sensitive physical examination technique, is elicited by percussion of the lowest intercostal space (either eighth or ninth rib) in the left axillary line at full inspiration and full expiration with the patient laying supine. A positive Castell's sign is characterized by resonance with percussion on full expiration and dullness upon full inspiration, with a sensitivity of 82% and specificity of 83%.[20] Dullness to percussion in Traube's space, or by any of the above percussive tests should be followed by palpation. Beginning in the right iliac fossa, the examiner's hand gently advances toward the left upper quadrant. This minimizes the likelihood that a grossly enlarged spleen will be missed, and the lower pole and medial border should be easily appreciated. If the spleen cannot be felt with this approach, the left hand of the examiner is placed on the left flank, lifting the lower part of the rib cage to displace the spleen medially toward the examiner's right hand. The splenic notch should be felt in the inferior medial border. Rotating the patient into the right lateral decubitus position while still recumbent may make it easier to palpate the spleen. The spleen should move with deep inspiration. The degree of enlargement is usually measured in centimeters below the costal margin. Depending on the position of the spleen within the abdomen, it may be difficult to appreciate even significant enlargement. Normal splenic size in an adult is up to 250 g and up to 13 cm in its long axis. Up to half of spleens weighing 600 to 750 g are not palpable. Greater degrees of splenomegaly are easier to appreciate on physical examination. In terms of sensitivity and specificity, there does not appear to be a difference between examination in the supine position and examination in the right lateral decubitus position, although both of these parameters are dependent on the experience of the examiner.

Up to 15% of normal children and 3% of young adults have palpable spleens without evidence of illness. The spleen involutes with age, and a spleen that is palpable in an older person is unlikely to be a normal variant and more likely to be associated with clinical disease.

Due to various abnormalities, the spleen may not be located in its usual anatomic position. It may migrate from its normal abdominal position to the left mid-abdomen or even the left lower quadrant.[21] While a rare finding, this highlights the importance of attentive examination of the lower abdomen when palpating for the spleen especially as a "wandering spleen" may be associated with other abnormalities.[22]

IMAGING OF THE SPLEEN

Radionuclide scintigraphy assesses anatomic and functional aspects of the spleen. The most common procedure is a liver-spleen scan in which technetium-99m (^{99m}Tc) sulfur colloid is injected intravenously and taken up by hepatic and splenic macrophages. It is the phagocytic activity of macrophages, rather than the presence of the spleen itself or any aspects of lymphoid function, that is assessed. A dynamic ^{99m}Tc scan can also assess the distribution of blood within the portal system and suggest the presence of portal hypertension. Infusion of ^{99m}Tc-labeled or Indium-111–labeled platelets with scintigraphy to determine their relative distribution in the liver and spleen has been used with considerable, but not absolute, success to predict the clinical efficacy of splenectomy in patients with immune thrombocytopenia purpura (ITP).

Ultrasonography readily shows the size, shape, and several aspects of splenic anatomy, including the presence of cysts and abscesses. The procedure is noninvasive, painless, of low cost, avoids radiation exposure for patients, and is a good screening study when the spleen is thought to be enlarged. In 95% of the normal population, the long axis of the spleen measures less than 12 cm on ultrasound examination. Accessory spleens tend not to be well-visualized on routine ultrasonography. In the hands of a skilled operator, endoscopic ultrasound-guided biopsy permits accurate diagnosis of lesions as small as a centimeter or less, which compares well with results obtained using computed tomography (CT). High-frequency sonography has significantly better resolution and can visualize both nodularity within the spleen, which occurs in childhood as immunity is acquired, and accessory spleens and splenosis after splenic rupture or trauma.

CT imaging has the advantage of showing the anatomy and some aspects of splenic function. Contrast material in the gastrointestinal tract helps delineate splenic tissue when it impinges on this system. Intravenous contrast material is required to delineate splenic lesions whose density is the same as normal splenic tissue. Abscesses have a rim of contrast agent enhancement.

CT can be used to estimate the volume of the spleen. Accessory spleens have the same attenuation as a normal spleen, which is somewhat less than that of liver. Accessory spleens are usually located in the gastrosplenic ligament near the hilum. Subcapsular and intrasplenic hematomas and splenic lacerations are clearly seen on CT. Leukemias and many inflammatory diseases produce diffuse splenomegaly, while granulomas and infarcted areas eventually calcify. Cysts, abscesses, and some malignancies may have a homogeneous pattern on CT scan. Although not replacing other methods of staging, splenic enlargement on CT may direct follow-up studies and indicate prognosis.

In addition to imaging the spleen, CT can be used to direct therapeutic interventions. Abscesses, hematomas, and cysts can be drained (see box on Management of Splenic Cysts). As long as portal pressures are normal and vascular lesions have been adequately excluded, thin-needle aspiration under CT guidance is generally a safe intervention. The safety and value of core needle biopsy has been better explored in recent years as in the past it was frequently avoided from perceived risk of bleeding. In a prospective series of 30 patients, there was only one bleeding complication successfully managed with conservative measures.[23] Two retrospective series of 52 and 97 patients along with a pooled analysis of 748 patients found low rates (1% to 4%) of major complications after ultrasound-guided splenic needle biopsy and high-diagnostic yield, with 18G-25G biopsy needles.[24,25]

MANAGEMENT OF SPLENIC CYSTS

A 37-year-old woman with no past medical history comes in for evaluation of early satiety and left shoulder pain. She reports that she feels full after only a few bites of any meal and sometimes becomes nauseated and vomits. Physical examination is remarkable for an enlarged, nontender spleen. Imaging shows a large splenic cyst.

- Many conditions can lead to cyst formation in the spleen, including parasitic infections, trauma, hemangiomas, and polycystic kidney disease.
- Asymptomatic nonparasitic cysts may be observed with careful attention, and one should plan for intervention should they become symptomatic, rupture, or become infected.
- Symptomatic cysts may require percutaneous drainage with radiologic guidance or sometimes surgical procedures, including partial or total splenectomy.
- Parasitic cysts should be treated in consultation with infectious disease specialists as the particular parasitic infection, radiologic appearance, and patient comorbidities will guide choice of therapies.

CT scanning of the abdomen, performed as part of the initial emergency department evaluation of patients with abdominal trauma, may avert hospital admission and prevent exploratory laparotomy.[26]

Magnetic resonance imaging (MRI) is useful for identifying vascular lesions, which would otherwise require angiography, and splenic infections. It is more difficult to image the contents of the upper abdomen with MRI than with CT because of respiratory motion. With more rapid imaging technology, MRI has gained considerable utility for imaging infectious and vascular lesions of the spleen. Hepatosplenic candidiasis and other infections to which immunocompromised patients are susceptible can be identified noninvasively with modern MRI technology.

Through the use of T2 and T2* relaxation sequences, MRI imaging of the spleen allows quantitative measurement of iron burden in organs that accumulate iron, although the liver and heart are more commonly imaged for this purpose.[27,28]

Positron emission tomography (PET) scanning using ^{18}F-2-deoxyglucose is used in the diagnosis, staging, and monitoring of both non-Hodgkin and Hodgkin lymphoma (Chapters 80 and 81). It is more sensitive than CT scanning for identifying involved lymph nodes and superior to both lymphangiography and CT scanning at imaging the spleen. The availability of more sensitive, specific, and less toxic approaches to imaging permits more precise staging of Hodgkin lymphoma without the complications of splenectomy (Fig. 156.5).[29,30]

The role of PET scanning is expanding in other disorders as whole-body PET scanners and appropriate small-molecule markers for individual diseases are more widely available. Imaging studies in small-animal models are very promising; splenic involvement can be identified with high sensitivity in mice.

TESTS OF SPLENIC FUNCTION

The peripheral blood smear may be the most sensitive tool for identification of functional or anatomic hyposplenia. The presence of Howell-Jolly bodies, which are nuclear remnants normally removed by the spleen, is an excellent indicator of hyposplenism (Fig. 156.6). These are rarely seen until the spleen is largely nonfunctional or overwhelmed by other phagocytic functions, such as extravascular hemolysis. Newborn infants commonly have visible Howell-Jolly bodies, and splenic function appears to be at least somewhat impaired in the first week of life. Pappenheimer bodies (siderotic granules normally removed by the spleen) are often seen in hyposplenic states, particularly when a component of hemolysis exists. Erythrocyte morphologic features reflect the lack of membrane polishing by the spleen, with the presence of acanthocytes and target cells. Granulocyte and platelet numbers are increased during asplenic states, including splenic infarction and surgical splenectomy.

To confirm suspected hyposplenism, the simplest test is a count of pitted or pocked erythrocytes. Fixation in 0.5% to 1.0% glutaraldehyde and examination under interference optics should reveal endocytic vesicles containing hemoglobin, ferritin, and remnants of mitochondria. These form in mature erythrocytes and are normally removed by the functioning spleen. The number of pitted cells (not the number of pits per cell) is inversely proportional to splenic function, with normal persons having less than 2% pitted cells.[31] The absence of Howell-Jolly bodies on a peripheral blood smear cannot be used as evidence of adequate splenic immune function.[32]

ASPLENIA AND HYPOSPLENIA

Congenital asplenia may be an isolated lesion or associated with severe cyanotic congenital heart disease and bilateral right-sidedness. Life-threatening cardiac lesions, including transposition of the great vessels, pulmonary artery atresia or stenosis, septal defects, anomalous venous drainage, and a single atrioventricular valve, are components of bilateral right-sidedness. The liver is central, and both lungs have three lobes. The peripheral blood smear shows Howell-Jolly bodies and other signs of hyposplenism. There is considerable variation in the anatomic and functional findings, and it is difficult to predict with accuracy the degree of splenic dysfunction on the basis of the anatomy alone. Children who survive the cardiac difficulties in the neonatal period have a significant incidence of sepsis secondary to a variety of organisms.

Polysplenia is associated with bilateral left-sidedness. Dextrocardia, bilateral superior venae cavae, septal defects, and anomalous pulmonary venous return are associated cardiac lesions. Both lungs have two lobes, the liver is midline, and bowel malrotation is common. The splenic tissue is divided into two to nine masses. The peripheral blood smear usually does not suggest hyposplenism, and no clear association with an increased risk for infection has been documented.

Asplenia occurring without heart disease is less likely to be detected before an infection develops than when associated cardiac lesions bring the patient to medical attention. Some of these patients also have *situs inversus*. Isolated cases have been reported of asplenia discovered after death of an otherwise healthy adult or child from overwhelming sepsis with encapsulated organisms. Familial instances of congenital asplenia are likely to be instances of genetic abnormalities (e.g., *HOX1/TLX1*), but these have not yet been documented. Examination of a blood smear for Howell-Jolly bodies is a simple procedure and may be lifesaving in the rare patients affected with these disorders.

If Howell-Jolly bodies are observed on the blood smear of an otherwise healthy person, imaging should be performed to assess for the presence of a spleen. Immunization with polysaccharide vaccines and early intervention with antibiotics for apparent infection may prove lifesaving for these individuals. Because approximately 20% of invasive pneumococcal infections in these patients are non-vaccine strains, a high degree of vigilance remains essential, even with more widespread immunization of healthy children.

Splenic Infarction

Although the classic teaching of splenic infarct is that of left upper quadrant or flank tenderness, many are noted incidentally on imaging with a third without any pain.[33] The causes are diverse with the most frequent etiologies atrial fibrillation or other cardioembolic and malignancy followed by hypercoagulability, hemoglobinopathy, infection including sepsis, trauma and complications of cirrhosis. Atherosclerosis of the splenic or celiac artery may be a risk factor. The choice of whether or not to anticoagulate is less clear and one needs to consider the cause. For example, atrial fibrillation or a thrombosis associated with malignancy would typically trigger anticoagulation, but a splenic infarct associated with sickle cell disease does not.

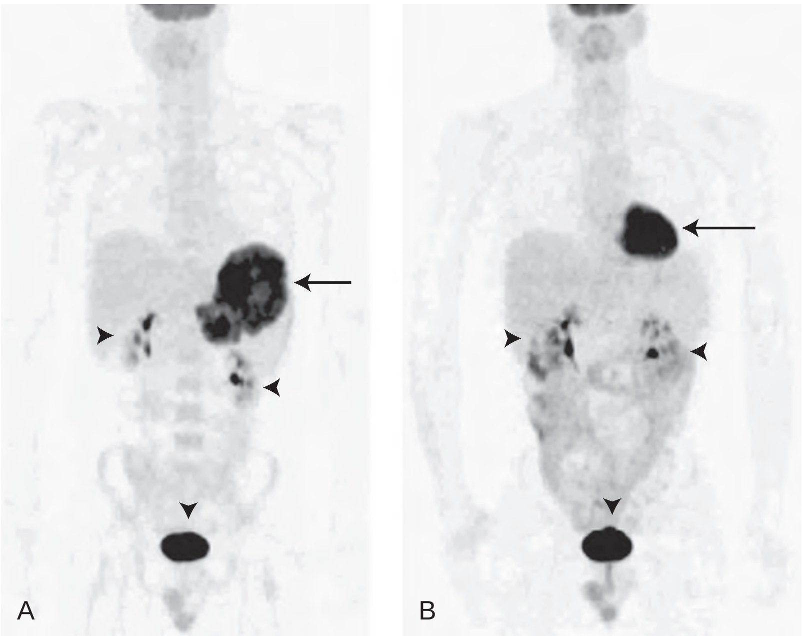


Figure 156.5 POSITRON EMISSION TOMOGRAPHY SCANNING TO IMAGE AREAS OF INVOLVEMENT IN HODGKIN DISEASE. (A) Pretreatment imaging showing the spleen *(large mass in left upper quadrant with arrow)* and activity in the kidney and bladder *(arrowheads)*. (B) The same patient after three cycles of chemotherapy. The *arrow* now points to normal activity in the myocardium not seen previously and *arrowheads* point to persistent urinary tract activity. The spleen is particularly well imaged on positron emission tomography scan. *(Used, with permission, from Friedberg JW, Chengazi V. PET scans in the staging of lymphoma: Current status. Oncologist. 2003;8:438.)*

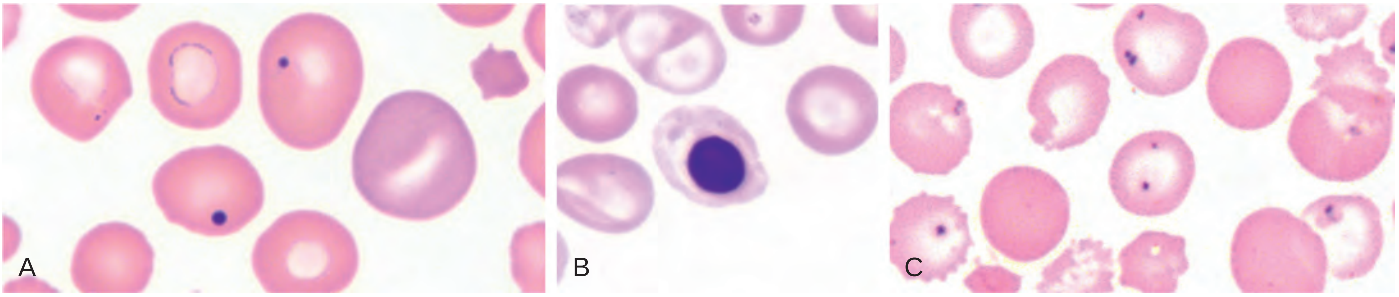


Figure 156.6 RED BLOOD CELL FINDINGS IN HYPOSPLENISM. (A) Red blood cells with Howell-Jolly bodies. (B) Nucleated red blood cell. (C) Cells with Pappenheimer bodies. Red blood cells with Howell-Jolly bodies are seen in patients with hyposplenism. The cytoplasmic inclusions (A) are nuclear remnants that are usually round and stain similar to the nucleus of a nucleated red blood cell (B). They occur normally during red cell maturation but are typically removed by a normal spleen. Their presence in the blood indicates less-than-normal splenic function. Pappenheimer bodies (C), which can also be seen in hyposplenism, are siderotic granules that are irregular in shape and frequently multiple.

ACQUIRED HYPOSPLENISM

Infarction in Sickle Cell Disease

The course of sickle cell disease is marked by progressive dysfunction of multiple organs over many years, and one of the earliest organs to be affected is the spleen. Serial measurements of the numbers of pitted erythrocytes in patients with sickle cell disease demonstrate that splenic dysfunction develops progressively over the first few years of life in the major sickle syndromes (Figs. 156.7 and 156.8). The hypoxic, acidotic, hypoglycemic environment of the spleen creates optimal conditions for tactoid formation and for sickling of the poorly deformable erythrocytes, which then block splenic blood vessels and infarcts the tissues. Splenic environmental conditions that enhance acidosis or hypoxia, or additional erythrocyte membrane or enzyme abnormalities that promote irreversible sickling, increase splenic infarction. The hyposplenism is reversible with transfusion for at least the first few years of life but becomes irreversible with progressive damage to blood vessels by 6 years of age in patients with sickle cell disease.

Splenic sequestration is a manifestation of the infarction process due to sickling in the spleen that extends to involve larger veins. Distensible splenic tissues result in pooling of a large amount of blood, whereas the venous drainage is occluded by sickled hypoxic erythrocytes. Patients with sickle cell disease who have not yet had multiple episodes of infarction and whose spleens have not undergone fibrosis are susceptible to this syndrome. Unlike the chronic process of smaller vessel infarction, the acute splenic sequestration crisis can be life threatening because a large amount of blood can collect in the highly distended spleen. The tendency for recurrence and the potential for a fatal outcome have resulted in the common recommendation for splenectomy in a patient who has had one severe splenic sequestration crisis, or more than one less severe crisis. Occlusion of venous drainage also occurs in the liver, but the less distensible capsule of the liver and the options for venous drainage through the portal system make this less likely to be life-threatening.

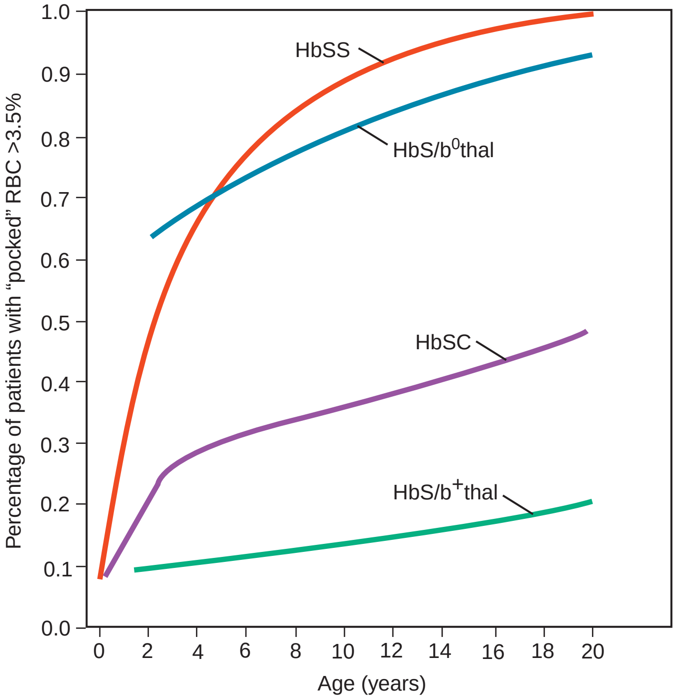


Figure 156.7 DEVELOPMENT OF FUNCTIONAL ASPLENIA IN SICKLE CELL DISORDERS. *B*$^{+}$*thal*, Beta^{+}-thalassemia; *b*^{0}*thal*, beta^{0}-thalassemia; *HbS*, hemoglobin S; *HbSC*, hemoglobin SC; *HbSS*, hemoglobin SS; *RBC*, red blood cell. *(Used, with permission, from Pearson HA, Gallagher D, Chilcote R, et al. Developmental pattern of splenic dysfunction in sickle cell disorders. Pediatrics. 1985;76:392.)*

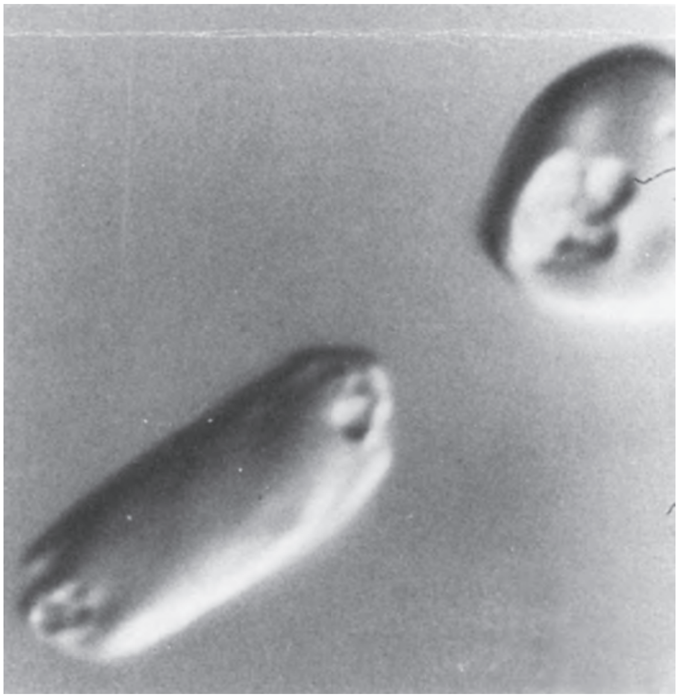


Figure 156.8 POCKED ERYTHROCYTES IN THE SICKLE HEMOGLOBINOPATHIES. *(Used, with permission, from Sills R, Oski FA. RBC surface pits in the sickle hemoglobinopathies. Am J Dis Child. 1979;133:526.)*

Immunologic and Autoimmune Diseases

Poor phagocytic function of the spleen is associated with impaired function of the Fc receptors on splenic macrophages in a variety of immunologic, rheumatic, and inflammatory disorders. Among those disorders in which hyposplenism has been clearly defined and associated with a risk for infection are systemic lupus erythematosus, rheumatoid arthritis, sarcoidosis, systemic vasculitis, ulcerative colitis, celiac disease, amyloidosis, chronic graft-versus-host disease, mastocytosis, and congenital and acquired immunodeficiency. The diseases themselves and the immunosuppressive therapies that are used in their management may contribute to the risk for infection in these conditions. Immunization with polysaccharide antigens and recognition of the risk of bacterial infection are important steps in reducing the risks of splenic hypofunction.

Splenomegaly and the production of autoantibodies such as antiplatelet or antiphospholipid antibodies, are features of the acquired immunodeficiency syndrome (AIDS). In the late stages of AIDS, atrophy of lymphoid follicles and depletion of T-cell-dependent areas are common. It is not clear that impairment of phagocytic function is a component of splenic atrophy in AIDS.

Therapy-Induced Splenic Hypofunction

Radiation therapy affects splenic function depending on the dose administered. In general, phagocytic cells are not affected by irradiation, but lymphoid cells are extremely sensitive. B-cell function is nearly ablated with as little as 500 cGy. T-cell lymphoblastogenesis is eliminated by administration of 3000 cGy. With doses of 2000 cGy, splenic hypofunction is usually transient because the macrophages and splenic stroma are not affected, and circulating B and T lymphocytes can repopulate the splenic follicles. Permanent splenic hypofunction may develop with doses of 4000 cGy or higher. The risk for infection is significant after such therapy.

Corticosteroid therapy impairs the affinity of the Fc receptors of splenic macrophages for opsonized IgG and decreases the adhesiveness of granulocytes and monocytes. This results in an acute pharmacologic splenectomy, even at commonly administered therapeutic dosages. The function of the Fc receptors on hepatic, pulmonary, and bone marrow macrophages is far less affected than that of splenic macrophages. The acute rise in platelet count or hemoglobin values seen with corticosteroid therapy in ITP or warm autoimmune hemolytic anemia is due to decreased clearance of sensitized cells. With prolonged therapy, the production of antibodies by splenic lymphocytes is also affected, and splenic function continues to be impaired.

Intravenous IgGs appear to decrease the phagocytic function of the spleen by binding to Fc receptors and impeding their recognition of opsonized particles. This is a transient effect because the Fc receptors are internalized and recycled, and opsonic function returns to normal within 2 to 3 weeks. Occupancy and impairment of function of Fc receptors is also seen with administration of anti-IgD to Rh-positive patients, which induces a transient hemolytic anemia. Reticuloendothelial blockade, or occupancy of these receptors, refers to the impaired ability of the spleen to recognize and remove other IgG-coated particles, including bacteria, in the presence of these agents.

SPLENOMEGALY AND HYPERSPLENISM

An enlarged spleen is not a disease state in itself but usually indicates some underlying pathologic state.[34] The processes run the gamut from minor to life threatening, from congenital disorders to those at the end of life. It is useful to approach the differential diagnosis of splenomegaly by considering the processes that may cause splenic enlargement and then to focus on the specific diagnoses within those categories. The spleen may be enlarged as a result of infiltration, hypertrophy of normal elements (macrophages and lymphoid components), extramedullary hematopoiesis, inflammatory or immunologic processes, and systemic or portal congestion (see box on Timing of Return to Contact Sports in Athletes Who Have Had Infectious Mononucleosis). The degree of splenomegaly correlates well with involvement of the spleen in many malignant disorders, such as Hodgkin disease. Rarely, anatomic abnormalities will cause splenomegaly. Acutely, the spleen can be enlarged with splenic sequestration crisis (Chapter 43).[35] Splenomegaly is important to investigate because it is frequently the finding of a serious disorder whose earlier recognition and treatment may prevent significant long-term morbidity and mortality. The diseases associated with splenomegaly are detailed in Table 156.3. Patients with splenomegaly do not necessarily have hypersplenism, and patients with hypersplenism may indeed have normal-sized spleens, such as those seen in ITP.

TIMING OF RETURN TO CONTACT SPORTS IN ATHLETES WHO HAVE HAD INFECTIOUS MONONUCLEOSIS

A 17-year-old male high school student is diagnosed by his primary care physician with infectious mononucleosis. The patient is concerned about the upcoming soccer season and wants to start training with the team in 4 weeks.

- More than half of patients with infectious mononucleosis develop splenomegaly within the first 14 days of illness.
- Most reports of splenic rupture in the setting of infectious mononucleosis occur in the first 21 days of illness.
- There is a paucity of data to support imaging the spleen to document resolution of splenomegaly before returning to contact sports. Although one study, by O'Connor, et al., evaluated 19 patients after infectious mononucleosis with ultrasonography, which showed normal spleen size (<12 cm) in 16 patients 1 month after diagnosis, with the remaining 3 patients having normalization of spleen size at 2 months.[36] However, there remain no consensus guidelines for return to play or integration of imaging into this decision-making process.
- Noncontact sports may be safely resumed after at least 21 days from the onset of initial symptoms, and contact sports should be safe in most cases after at least 28 days. Infectious mononucleosis-associated splenic rupture has been reported as far as 8 weeks after symptom onset.[37]
- The timing of return to sports and the risks should be discussed with the patient, especially if the patient may not have returned to baseline after prolonged fatigue.

Hypersplenism refers to nonimmune, indiscriminate destruction of the formed elements of the blood by a spleen that is usually enlarged, affected by portal hypertension, or both. The bone marrow is hyperplastic, and the peripheral blood cell counts are decreased because of destruction of mature formed elements. Splenectomy corrects the cytopenias. Any of the formed elements of the blood—erythrocytes, neutrophils, or platelets—can be affected alone or in combination. Splenic hypertrophy in cirrhosis is due to an increase in splenic macrophages and their activity. Because the normal function of splenic macrophages is to remove senescent cells, this is an exaggeration of a physiologic process.

Hypersplenism develops in patients who receive chronic transfusions, and there are several components that contribute to the development of hypersplenism. The antigenic load of allogeneic transfused cells stimulates the immune system. Additionally, transfused cells have shorter survival than normal red cells (mean of 60 instead of 120 days, unless specially prepared neocytes are used), which increases the work of splenic macrophages. Finally, iron overload causes hemosiderosis of both the spleen and the liver, so that portal hypertension may develop and further increase splenic pathology. Hypersplenism increases the transfusion requirement in patients who are already transfusion dependent.

Interestingly, spleen status at the time of allogeneic hematopoietic stem cell transplantation affects outcomes. Patients with splenomegaly have delayed engraftment while those with prior splenectomy have early engraftment and no difference in mortality.[38] The increased risk in infection may be mitigated by the high-intensity setting in which stem cell transplantation occurs.

TABLE 156.3 Causes of Splenomegaly

Primary Process	Pathogenesis	Examples
Anatomic	Developmental abnormalities	Cysts, pseudocysts, hamartomas, peliosis, hemangiomas
Hematologic	Hemolysis	Intrinsic (membrane, enzyme, hemoglobin disorders), extrinsic (immune)
	Extramedullary hematopoiesis	Myeloproliferative neoplasms/myelodysplasias, myelofibrosis, osteopetrosis
Infectious	Bacteria	Acute and chronic systemic infection, abscesses, subacute bacterial endocarditis
	Mycobacteria	Miliary tuberculosis
	Spirochetes	Syphilis, Lyme disease, leptospirosis
	Viruses	Epstein-Barr virus; cytomegalovirus; human immunodeficiency virus; hepatitis A, B, C
	Rickettsia	Rocky Mountain spotted fever, Q fever, typhus
	Fungi	Disseminated candidiasis, histoplasmosis, South American blastomycosis
	Parasites	Malaria, babesiosis, toxoplasmosis, *Toxocara canis*, *Toxocara cati*, leishmaniasis, schistosomiasis, trypanosomiasis
Immunologic	Collagen vascular diseases	Felty syndrome, systemic lupus erythematosus, mixed connective tissue disorder, systemic vasculitis, Sjögren syndrome, systemic mastocytosis
	Immunodeficiency	Common variable immunodeficiency
	Immune/inflammatory	Graft-versus-host disease, serum sickness, large granular lymphocyte lymphocytosis, Weber-Christian panniculitis
Neoplastic	Primary malignancies	Lymphomas, leukemias
	Metastatic malignancies	Breast, lung, skin, colon
Infiltrative	Storage diseases	Gaucher disease, Niemann-Pick disease, GM_1 gangliosidosis, glycogen storage disease type IV, Tangier disease, Wolman disease, mucopolysaccharidoses, hyperchylomicronemia types I and IV
Congestive	Portal hypertension	Intrahepatic cirrhosis, extrahepatic cirrhosis (Budd-Chiari syndrome)
	Systemic	Congestive heart failure
	Local	Splenic vein thrombosis

SPLENIC IRRADIATION

Indications

Splenic irradiation is considered as a palliative treatment for symptom relief related to splenomegaly, or hypersplenism, in patients who have either declined splenectomy or are high-risk surgical candidates. Symptoms related to splenomegaly that may be palliated with irradiation include left upper quadrant pain, early satiety, and mechanical discomfort related to splenomegaly. These most commonly occur in patients with myeloproliferative, or lymphoproliferative, neoplasms (Chapter 72). However, there are no prospective studies on the use of splenic irradiation to guide its use, or to aid in duration and intensity of treatment. Several retrospective studies have been performed across a variety of diseases with mixed results.

Complications

While splenic irradiation spares the patient from an invasive procedure (discussed in detail below), it carries with its own unique and potentially life-threatening complications.

SPLENECTOMY

Indications and Timing

Splenectomy should be performed for clinical indications rather than for specific diagnoses. In many instances, removal of the spleen will improve the condition of patients with hemolytic anemia due to intrinsic disorders of erythrocyte membranes and enzyme disorders, and of those with chronic conditions, such as storage diseases and portal hypertension. Specific clinical indices that require intervention should be identified, and parameters that can be used to identify clinical improvement (usually an increase in peripheral blood cell counts, growth, or energy level) should be determined before the procedure is performed. For patients with inherited erythrocyte membrane or enzyme disorders, such as hereditary spherocytosis or pyruvate kinase deficiency, marked reticulocytosis indicating significant metabolic energy required for erythropoiesis, somatic growth failure, or lack of exercise tolerance would be potential indications for splenectomy. Avoidance of formation of gallstones, formerly often considered an indication for splenectomy when a diagnosis of hereditary spherocytosis was made, is less important today because minimally invasive surgical procedures have improved the management of cholelithiasis. Patients with storage disorders such as Gaucher disease often develop splenomegaly and hypersplenism, and subsequent cytopenias require intervention. As new therapies are developed, such as eliglustat for Gaucher disease and ruxolitinib for primary myelofibrosis, these patients experience a significant reduction in spleen size and increase in quality of life, which may mitigate the need for splenectomy.[39,40] The clinical benefit to be obtained from splenectomy should at least balance, and preferably outweigh, the potential long-term risks of post-splenectomy septicemia, an increased risk for thrombosis, and the shift of storage cells from the spleen to other organs, such as the bone marrow or liver, where they may do more harm in the absence of the spleen. Not all patients with hemolytic anemias require splenectomy. If such patients develop an aplastic crisis due to parvovirus B19 infection while their spleens are intact, they may require a transfusion. Many patients with mild chronic hemolysis and well-compensated anemia may be better off with their spleens remaining intact. Patients with ITP should undergo surgical splenectomy when the risks of bleeding or of medical therapies (such as long-term corticosteroids or the anti-CD20 monoclonal antibody rituximab) are such that the benefits of splenectomy exceed the risks. The introduction of thrombopoietin mimetics for treatment of chronic ITP has further delayed or eliminated the need for splenectomy in many patients (Chapter 130). The indications for splenectomy are different in adults than in children, and are affected by the presence of underlying disorders, such as systemic lupus erythematosus and HIV. In an attempt to avoid immunologic consequences of a total splenectomy, several centers are performing partial splenectomies for hereditary spherocytosis, although in some cases a second procedure is later required.[41]

Splenectomy for sickle cell disease is usually performed for splenic sequestration that is severe, persistent, or recurrent. In some sickle syndromes, the spleen does not autoinfarct in childhood, and persistent splenomegaly may increase the degree of anemia. The risk for subsequent development of gallstones should be considered in patients with hemoglobinopathies and other hemolytic anemias so that cholecystectomy can be performed simultaneously, if deemed indicated.[35]

The timing of splenectomy (when it is to be performed) should again be chosen to minimize risks and maximize benefits. Immunity to carbohydrate antigens, such as those in the cell walls of encapsulated organisms such as *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* type b, develops over the first 2 to 3 years of life. When splenectomy can be delayed until the patient is at least 2 and preferably more than 5 years of age, specific immunity and response to administered polysaccharide vaccines will improve host defenses and lessen the risk for post-splenectomy sepsis. When patients have a disease, such as Gaucher disease, or hemolytic anemia predisposing to iron overload, such as thalassemia, the presence of the spleen as a preferential site for the storage of harmful cellular breakdown products may protect other organs from damage. Delaying splenectomy until a clear clinical indication is present will balance risks and benefits to an optimal degree. The recently developed oral substrate inhibitor, eliglustat, has shown promising success in reducing splenomegaly in patients with Gaucher disease (Chapter 54).[39] As this drug is studied further, it is possible that splenectomy may not be needed as often.

Surgical Options

When splenectomy is clearly indicated, acute complications are rarely a consideration in the decision to perform surgery. Nevertheless, advances in surgical procedures have minimized the short-term risks of the procedure itself and of postoperative complications such as intestinal obstruction from adhesions. Subtotal splenectomy can be performed when total splenectomy is not desirable, such as for the removal of a cyst, a pseudocyst, or tumors, after trauma, or for Gaucher disease. Wedge resection with mattress sutures and cyanoacrylate adhesives and microfibrillar collagen omental packs have greatly improved partial splenectomy procedures in the past decade. Laparotomy is required when extensive peritoneal adhesions are present and for removal of massively enlarged spleens. A sufficiently large incision to permit full visualization and mobilization is essential when the spleen is very large or when inspection is a major part of the surgical procedure. A retroperitoneal approach is useful when the spleen is not massively enlarged but needs to be fully removed, such as when cytopenias are the indication for the procedure. This approach shortens the postoperative recovery time and avoids induction of peritoneal adhesions.

Minimally invasive procedures have become standard for most splenectomies. Laparoscopy is now the procedure of choice for splenectomy. Although the operative time is significantly greater than for laparotomy, the postoperative recovery time, risk for damage to the pancreas, likelihood of developing a sub-phrenic abscess and peritoneal adhesions postoperatively, and nutritional and metabolic challenges to the patient are considerably reduced. Laparoscopic splenectomy can be performed even in thrombocytopenic patients. The outcome of laparoscopic splenectomy in ITP is affected by the experience and skill of the surgeon and by the patient's obesity. Prolonged pre-surgical use of corticosteroids may induce obesity and impair tissue healing, resulting in a higher risk for complications from laparoscopic splenectomy than when splenectomy is performed before adverse drug effects develop. In the case of massive splenomegaly, splenic morcellation (division and removal in small pieces) may be necessary prior to laparoscopic retrieval.

Appreciation of the risk for post-splenectomy septicemia and refinements in noninvasive, accurate radiologic monitoring techniques have led to more conservative approaches to splenic injury. Nonoperative management has increased over time and has an acceptable mortality and complication rate in selected patients, although early discharge may put patients at risk for the later complications.[42] Noninvasive imaging and minimally invasive surgical procedures have greatly affected these trends. After traumatic rupture, splenic tissue may regenerate as both micro- and macroscopic ectopic implants in the peritoneal cavity, a process termed *splenosis*. Although splenosis appears to be partially protective against overwhelming post-splenectomy infection in animals, its protective value in humans is not known and may depend on the adequacy of splenic tissue perfusion. If poorly vascularized, the ectopic splenic tissue may not provide adequate contact between macrophages and the antigens of the infecting organism. It is generally valuable to attempt preservation of splenic function when possible after traumatic rupture. Some surgeons attempt to induce splenosis when traumatic splenectomy is unavoidable, in the hope of minimizing late complications.

As the late complications of splenectomy are better appreciated, surgeons have become increasingly creative at performing procedures that preserve or restore splenic function. Even large cysts that were indications for splenectomy until recently have been managed with spleen-conserving procedures, such as partial or total cyst removal, which can often be performed laparoscopically.[43] Pancreatic surgery often requires sacrifice of the spleen; however, increasing numbers of procedures are being performed with salvage of the spleen.

Splenic transplantation is being used as a means of developing immune tolerance, as well as a means of reducing the risk for infection following organ transplantation.[44] Allogeneic spleens have been transplanted within multivisceral grafts with only minimal graft-versus-host disease. To date, there are more animal than human data, and it is unclear how large an impact this approach will have on visceral, especially small-bowel and pancreatic, allotransplantation.[45] If protection against infection is the main consideration, an intact spleen is superior to a repaired or autotransplanted spleen, whereas accessory spleens and splenosis may be only marginally better than asplenia.[32]

Complications After Splenectomy

Acute complications in the perioperative period include rupture of the spleen, the development of a sub-phrenic abscess, and injury to the pancreas during the operative procedure. In a healthy patient, the immediate risk of splenectomy is limited.[46] The degree of splenomegaly greatly affects the risk for rupture and pancreatic injury. The technical difficulty of performing a splenectomy is much greater when the spleen is massively enlarged than when the spleen is small. Once the arterial supply is ligated, rupture of the spleen is rarely a problem. The splenic hilum is retroperitoneal, so if the spleen is very large, mobilization to gain access to the splenic artery and vein can be difficult. After recovery from surgery, intestinal obstruction due to formation of peritoneal adhesions is a complication that, if it is to occur at all, usually occurs within the first few months.

Several late complications of splenectomy give cause for concern, including severe infection, increased risk for venous thromboembolism, coronary artery disease, and possibly malignancy (Fig. 156.9).[47] However, the precise risk is not known, as there have been conflicting data on the risk of malignancy, particularly in those with traumatic causes for splenectomy.[47,48] Age-appropriate cancer and cardiovascular screening with evaluation of symptoms are essential preventive interventions that are probably underused because patients may not be aware of the risks of splenectomy performed early in life.

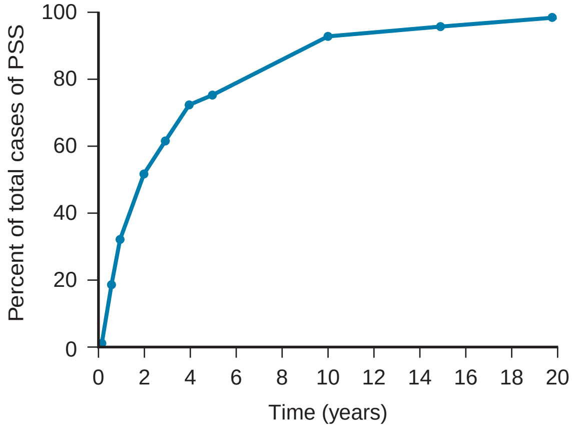


Figure 156.9 THE INTERVAL FROM SPLENECTOMY TO POST-SPLENECTOMY SEPSIS. Of the total (n = 288), 3.1% occurred more than 20 years after splenectomy. *PSS*, Post-splenectomy sepsis. *(Used, with permission, from Lutwick LI. Infections in asplenic patients. In: Mandell GL, Bennett JE, Dolin R, eds. Principles and Practice of Infectious Diseases. 7th ed. Philadelphia: Churchill Livingstone; 2009.)*

Post-splenectomy septicemia is rare but may be rapidly lethal.[49] In the absence of protective levels of opsonic IgG antibodies produced in the spleen, hepatic and pulmonary macrophages are unable to effectively clear organisms from the bloodstream. Organisms that enter the bloodstream and would ordinarily be removed by splenic macrophages are able to evade recognition by macrophages whose Fc and C3b receptors appear to be less avid than those of splenic macrophages. Circulation of the blood through the liver and lung is more rapid than through the spleen, and there is little opportunity for macrophages to recognize organisms with surfaces containing little IgG and only small amounts of C3bi. The important filtration function of the venous sinus endothelial cells is absent following splenectomy. The generation of cytokines, including TNF-α and bacterial endotoxins, leads to cardiovascular collapse and shock. It is difficult to rescue an asplenic patient once shock develops, even with effective antibiotic therapy. The risk that this will happen varies with the indication for splenectomy (Table 156.4) and the patient's medical condition. Factors that impair host defenses significantly increase the risk for infection. These include deficient opsonins (hypogammaglobulinemia and specific antibody-production deficiency), reticuloendothelial blockade related to increased phagocytic activity of macrophages in other organs, impaired antigen processing or recognition (AIDS, lymphoma, other malignancies, and some collagen vascular diseases), neutropenia, and high iron load (thalassemia). The tetrapeptide tuftsin, primarily produced in the spleen, enhances the phagocytic activity of monocytes and neutrophils; its absence in asplenic patients appears to contribute to depressed neutrophil function and the subsequent increased risk for infection.

Patients at the lowest risk for overwhelming post-splenectomy sepsis are those in whom splenectomy cures the underlying problem, such as isolated ITP, hereditary spherocytosis, and trauma. Patients undergoing splenectomy for trauma have a 50-fold greater risk for subsequent septic death than trauma patients with intact spleens, whereas the risk in patients with sickle cell disease is increased 350-fold over that of the general population, and other authors report the risk to be 600 times greater. Similar to using hemodialysis to attempt to replace the function of the kidney, work is ongoing to devise an artificial spleen as a mechanical way to filter pathogens from the blood.

An increased risk for vascular complications may result from splenectomy. Acute portal vein thrombosis occurs within 2 months of splenectomy in 5% to 37% of patients, which is probably the result of local surgical factors. The surgical approach seems to affect the rate of post-splenectomy portal and splenic vein thrombosis, with a laparoscopic approach and morcellation associated with a higher rate of thrombosis compared with open splenectomy (55% vs. 19%, respectively). Patients with thalassemia and prior splenectomy appear to also have an increased incidence of venous thromboembolism beyond the portal venous system. In addition, splenectomy appears to be a risk factor for the development of pulmonary hypertension.

TABLE 156.4 Incidence of Post-Splenectomy Sepsis

Indication for Splenectomy	Cumulative Incidence of Bacterial Sepsis (%)
Trauma	1.5
Hematologic disorders	3.4
Portal hypertension	8.2
Hodgkin disease	10
Sickle cell disease	15
Thalassemia	25

Data from Gorse GJ. The relationship of the spleen to infection. In: Bowlder AJ, ed. *The Spleen: Structure, Function, and Clinical Significance.* New York, NY: Van Nostrand Reinhold; 1990:269, with permission.

Vascular events after splenectomy are likely multifactorial in origin, being attributed to a combination of hypercoagulability, platelet activation, activation of endothelium due to the persistence of particulate matter, and damaged cells in the bloodstream.

Atherosclerosis that develops many years after splenectomy in patients with hereditary spherocytosis or hereditary stomatocytosis may be related to subsequent thrombocytosis that leads to enhancement of plaque formation. Statistically, the increased risk for atherosclerosis is not particularly high, but for individuals with other risk factors such as hypertension, diabetes, high levels of cholesterol or homocysteine, heterozygous protein C or S deficiency, or factor V Leiden deficiency, splenectomy may pose a more significant risk. Although no human data are currently available in this area, some studies have suggested that the spleen might be involved in lipid metabolism in both rats and rabbits.

Prevention of Complications

Post-splenectomy Septicemia

The major risk for post-splenectomy sepsis is infection with encapsulated organisms such as *Staphylococcus pneumoniae*, *H. influenzae* type b, and *N. meningitidis*, which require opsonization for effective phagocytosis. Polysaccharide vaccines are available for all three bacteria.[50,51] The highest risk period for children is in the first 2 years of life, when their ability to mount an antibody response to purified polysaccharides has not developed completely, so protein-conjugated vaccines are now in widespread use. This has significant potential benefit for patients when asplenia is not recognized, because they might then be immunized as part of their routine care. The antibody responses to vaccines, especially the conjugated vaccines, differ in IgG subclasses from those produced following natural infection. Overwhelming post-splenectomy sepsis and death from sepsis in functionally hyposplenic patients should be preventable.[49] It is recommended that immunization be done at least 14 days, but ideally 1 to 12 weeks, prior to the anticipated splenectomy to optimize antigen recognition and processing, and induce more effective immunity.[52] If emergency splenectomy is performed, it is recommended that vaccination be postponed until at least 14 days post-splenectomy to avoid the transient immune suppression often seen with general anesthesia and surgery (see box on Vaccination of a Patient Scheduled for Elective Versus Emergent Splenectomy). Adults with prior vaccination to *H. influenzae* type b are generally presumed to be immune and may receive the pneumococcal and meningococcal vaccines alone. Reimmunization for children is recommended 2 to 5 years after initial immunization. Splenectomized adults should be revaccinated with the PPSV23 once after 5 years, whereas meningococcal revaccination should occur every 5 years. The Advisory Committee on Immunization Practices recommends that adult patients who are vaccine naïve, receive the pneumococcal conjugate vaccine (PCV13) followed 8 weeks later by the pneumococcal polysaccharide vaccine (PPSV23). Because these recommendations change with experience and vaccine use, it is wise to consult current guidelines for individual patients. The US Centers for Disease Control and Prevention website should be consulted for the most up-to-date recommendations. Additional organisms to consider in post-splenectomy sepsis include *Escherichia coli*, *Pseudomonas aeruginosa*, and *Capnocytophaga canimorsus*.[53]

VACCINATION OF A PATIENT SCHEDULED FOR ELECTIVE VERSUS EMERGENT SPLENECTOMY

A 50-year-old man with refractory ITP is scheduled for splenectomy. Having heard there is a risk for different types of infection after splenectomy, he asks about what he can do to reduce his risk.

- Patients scheduled for elective splenectomy should receive the following at least 14 days, ideally 10–12 weeks, prior to the procedure:[52]
- *Streptococcus pneumoniae* vaccines
- PCV13 (Prevnar)
- PPSV23 (Pneumovax)-given AT LEAST 2 months after PCV13; revaccinated 5 years after initial dose and again at age 65 years
- *Haemophilus influenzae type b* (Hib) vaccine: Hib conjugate (Hiberix, Act-HIB), can be omitted for adults with known prior vaccination
- *Neisseria meningitidis* vaccines
- Meningococcal serotypes ACWY: MenACWY (Menactra or Menveo)
- Meningococcal serotype B: MenB (Bexsero or Trumenba)
- Annual seasonal influenza vaccine
- If the procedure is done emergently, wait at least until the 14th postoperative day.

There are fewer data on the efficacy of prophylactic antibiotics for asplenic, or hyposplenic, patients, except for young children with sickle cell disease, in whom antibiotics should be initiated as soon as the diagnosis is established. Present recommendations are that twice-daily oral penicillin V potassium or amoxicillin be continued in sickle cell patients until 5 years of age, or 3 years post-splenectomy. Beyond this, recommendations vary by international organization regarding antimicrobial prophylaxis.[54,55] High-risk patients with surgical or functional asplenia, including those with diagnoses of thalassemia, Hodgkin disease, other malignancies, immunodeficiency disease, or chronic graft-versus-host disease, should receive prophylactic antibiotics unless specific contraindications exist. Patients who have a history of pneumococcal sepsis post-splenectomy may be considered candidates for life-long daily prophylaxis as well. Whether or not such patients receive prophylactic antibiotics, and regardless of their immunization status, they may develop overwhelming infections with other organisms, including gram-negative organisms and *Staphylococcus aureus*. Some experts recommend providing a supply of emergency antibiotics to have "on-hand" (emphasizing that patients will need to check expiration dates regularly) and be immediately available at the time of febrile illness, prior to traveling to an emergency room for prompt evaluation. If the patient already takes a prophylactic antibiotic, the emergency agent should have a broader coverage. There are no randomized trials to guide therapy and experts have recommended the use of amoxicillin-clavulanate, cefuroxime axetil, macrolides, or extended-spectrum fluoroquinolones. Updated recommendations should be consulted for specific circumstances.

The single most important measure to prevent post-splenectomy septicemia is education of the patient, family, and physician. Recognition of the risk, the institution of appropriate preventive measures, and the rapid administration of antibiotics to a patient who is showing signs of infection (fever and chills) can be lifesaving.

Atherosclerosis

The magnitude of the risk for developing atherosclerosis is not clear.[21,56] Appropriate preventive measures include identifying and minimizing concurrent risk factors, such as obesity, dietary patterns, a sedentary lifestyle, and the presence of other hereditary factors that would predispose to thrombosis. Low-dose aspirin could be considered, but no data exist regarding its efficacy in this situation.

CONCLUSIONS AND FUTURE DIRECTIONS

Although very little novel information has emerged about clinical aspects of splenic function in recent years, appreciation of many subtleties of splenic function and advances in supportive care in several fields have had significant impacts on the management of disorders of the spleen. Better education and more effective immunizations have helped prevent serious complications related to splenic disorders. Advances in imaging and surgery have transformed the diagnosis and management of congenital and traumatic splenic disorders.

Enhanced understanding of the complexity of communication interaction between the cells of the immune and hematopoietic system has improved our appreciation of the molecular, cellular, system, and network basis for clinical observations about splenic function. The ability of splenic macrophages, in concert with dendritic cells and B cells, to recognize and ingest a variety of microorganisms—even in patients without previous exposure to the pathogens—provides great insight into the reasons that the spleen provides protection against a variety of very specific infections. More detailed understanding of the impact of the splenic microenvironment on cellular functions, the role of stromal infrastructure on the ability of cells to interact with and respond to invading pathogens, and the integration of components of the immune system has contributed to an appreciation of clinical observations about the role the spleen plays in normal biology. The intersection of hematopoiesis, recycling of aging cells, and iron metabolism is most clearly demonstrated in the spleen.

Although not necessary for life under baseline circumstances, the contribution of the spleen to homeostasis under stress is considerable. Ongoing efforts to preserve and manage splenic function will continue to benefit from enhanced appreciation of the underlying biology.

REFERENCES

1. Weiss L. *Cell and Tissue Biology: A Textbook of Histology*. Baltimore, MD: Urban & Schwarzenberg;; 1988.
2. Mebius RE, Kraal G. Structure and function of the spleen. *Nat Rev Immunol*. 2005;5(8):606–616.
3. Kanzler B, Dear TN. Hox11 acts cell autonomously in spleen development and its absence results in altered cell fate of mesenchymal spleen precursors. *Dev Biol*. 2001;234(1):231–243.
4. Bolze A, Mahlaoui N, Byun M, et al. Ribosomal protein SA haploinsufficiency in humans with isolated congenital asplenia. *Science*. 2013;340(6135):976–978.
5. Bolze A, Boisson B, Bosch B, et al. Incomplete penetrance for isolated congenital asplenia in humans with mutations in translated and untranslated RPSA exons. *Proc Natl Acad Sci USA*. 2018;115(34):E8007–E8016.
6. Golub R, Cumano A. Embryonic hematopoiesis. *Blood Cells Mol Dis*. 2013;51(4):226–231.
7. Brendolan A, Rosado MM, Carsetti R, et al. Development and function of the mammalian spleen. *Bioessays*. 2007;29(2):166–177.
8. Nagelkerke SQ, Bruggeman CW, den Haan JMM, et al. Red pulp macrophages in the human spleen are a distinct cell population with a unique expression of Fc-γ receptors. *Blood Adv*. 2018;2(8):941–953.
9. Lewis SM, Williams A, Eisenbarth SC. Structure and function of the immune system in the spleen. *Sci Immunol*. 2019;4(33). eaau6085.
10. Junt T, Scandella E, Ludewig B. Form follows function: lymphoid tissue microarchitecture in antimicrobial immune defence. *Nat Rev Immunol*. 2008;8(10):764–775.
11. Chiu SC, Liu HH, Chen CL, et al. Extramedullary hematopoiesis (EMH) in laboratory animals: offering an insight into stem cell research. *Cell Transplant*. 2015;24(3):349–366.
12. Oda A, Tezuka T, Ueno Y, et al. Niche-induced extramedullary hematopoiesis in the spleen is regulated by the transcription factor Tlx1. *Sci Rep*. 2018;8(1):8308.
13. Dor FJ, Ramirez ML, Parmar K, et al. Primitive hematopoietic cell populations reside in the spleen: Studies in the pig, baboon, and human. *Exp Hematol*. 2006;34(11):1573–1582.
14. Wu C, Hua Q, Zheng L. Generation of myeloid cells in cancer: the spleen matters. *Front Immunol*. 2020;11:1126.
15. Morrison SJ, Uchida N, Weissman IL. The biology of hematopoietic stem cells. *Annu Rev Cell Dev Biol*. 1995;11:35–71.
16. van der Laan AM, Ter Horst EN, Delewi R, et al. Monocyte subset accumulation in the human heart following acute myocardial infarction and the role of the spleen as monocyte reservoir. *Eur Heart J*. 2014;35(6):376–385.
17. Dutta P, Hoyer FF, Grigoryeva LS, et al. Macrophages retain hematopoietic stem cells in the spleen via VCAM-1. *J Exp Med*. 2015;212(4):497–512.
18. Coppin E, Florentin J, Vasamsetti SB, et al. Splenic hematopoietic stem cells display a pre-activated phenotype. *Immunol Cell Biol*. 2018. doi:10.1111.imcb.12035.
19. Ilardo MA, Moltke I, Korneliussen TS, et al. Physiological and Genetic Adaptations to Diving in Sea Nomads. *Cell*. 2018;173(3):569–580. e515.
20. Grover SA, Barkun AN, Sackett DL. The rational clinical examination. Does this patient have splenomegaly? *JAMA*. 1993;270(18):2218–2221.
21. Ahmed S, Horton KM, Fishman EK. Splenic incidentalomas. *Radiol Clin North Am*. 2011;49(2):323–347.
22. Varga I, Babala J, Kachlik D. Anatomic variations of the spleen: current state of terminology, classification, and embryological background. *Surg Radiol Anat*. 2018;40(1):21–29.
23. Hanlon K, Wilson MR, Kay D, et al. Safety and diagnostic yield of splenic core biopsy; a methodical approach using combined Haematology/Radiology assessment in a tertiary referral centre. *Br J Haematol*. 2019;186(2):371–373.
24. Olson MC, Atwell TD, Harmsen WS, et al. Safety and accuracy of percutaneous image-guided core biopsy of the spleen. *AJR Am J Roentgenol*. 2016;206(3):655–659.
25. Patel N, Dawe G, Tung K. Ultrasound-guided percutaneous splenic biopsy using an 18-G core biopsy needle: our experience with 52 cases. *Br J Radiol*. 2015;88(1055). 20150400.
26. Haan JM, Boswell S, Stein D, Scalea TM. Follow-up abdominal CT is not necessary in low-grade splenic injury. *Am Surg*. 2007;73(1):13–18.
27. Hackett S, Chua-anusorn W, Pootrakul St P, Pierre TG. The magnetic susceptibilities of iron deposits in thalassaemic spleen tissue. *Biochim Biophys Acta*. 2007;1772(3):330–337.
28. Labranche R, Gilbert G, Cerny M, et al. Liver iron quantification with MR imaging: a primer for radiologists. *Radiographics*. 2018;38(2):392–412.
29. Juweid ME, Stroobants S, Hoekstra OS, et al. Use of positron emission tomography for response assessment of lymphoma: consensus of the Imaging Subcommittee of International Harmonization Project in Lymphoma. *J Clin Oncol*. 2007;25(5):571–578.
30. Valette F, Querellou S, Oudoux A, et al. Comparison of positron emission tomography and lymphangiography in the diagnosis of infradiaphragmatic Hodgkin's disease. *Acta Radiol*. 2007;48(1):59–63.
31. Corazza GR, Ginaldi L, Zoli G, et al. Howell-Jolly body counting as a measure of splenic function. A reassessment. *Clin Lab Haematol*. 1990;12(3):269–275.
32. Connell NT, Brunner AM, Kerr CA, Schiffman FJ. Splenosis and sepsis: the born-again spleen provides poor protection. *Virulence*. 2011;2(1):4–11.
33. Brett AS, Azizzadeh N, Miller EM, et al. Assessment of clinical conditions associated with splenic infarction in adult patients. *JAMA Intern Med*. 2020;180(8):1125–1128.
34. Yongxiang W, Zongfang L, Guowei L, et al. Effects of splenomegaly and splenic macrophage activity in hypersplenism due to cirrhosis. *Am J Med*. 2002;113(5):428–431.
35. Al-Salem AH. Indications and complications of splenectomy for children with sickle cell disease. *J Pediatr Surg*. 2006;41(11):1909–1915.
36. O'Connor TE, Skinner LJ, Kiely P, Fenton JE. Return to contact sports following infectious mononucleosis: the role of serial ultrasonography. *Ear Nose Throat J*. 2011;90(8):E21–E24.
37. Ceraulo AS, Bytomski JR. Infectious mononucleosis management in athletes. *Clin Sports Med*. 2019;38(4):555–561.
38. Akpek G, Pasquini MC, Logan B, et al. Effects of spleen status on early outcomes after hematopoietic cell transplantation. *Bone Marrow Transplant*. 2013;48(6):825–831.
39. Mistry PK, Lukina E, Ben Turkia H, et al. Effect of oral eliglustat on splenomegaly in patients with Gaucher disease type 1: the ENGAGE randomized clinical trial. *JAMA*. 2015;313(7):695–706.

40. Vannucchi AM, Kiladjian JJ, Griesshammer M, et al. Ruxolitinib versus standard therapy for the treatment of polycythemia vera. *N Engl J Med*. 2015;372(5):426–435.
41. Buesing KL, Tracy ET, Kiernan C, et al. Partial splenectomy for hereditary spherocytosis: a multi-institutional review. *J Pediatr Surg*. 2011;46(1): 178–183.
42. Dodgion CM, Gosain A, Rogers A, et al. National trends in pediatric blunt spleen and liver injury management and potential benefits of an abbreviated bed rest protocol. *J Pediatr Surg*. 2014;49(6):1004–1008. discussion 1008.
43. Mattioli G, Pini Prato A, Cheli M, et al. Italian multicentric survey on laparoscopic spleen surgery in the pediatric population. *Surg Endosc*. 2007;21(4):527–531.
44. Wluka A, Olszewski WL. Innate and adaptive processes in the spleen. *Ann Transplant*. 2006;11(4):22–29.
45. Kato T, Tzakis AG, Selvaggi G, et al. Transplantation of the spleen: effect of splenic allograft in human multivisceral transplantation. *Ann Surg*. 2007;246(3):436–444. discussion 445-436.
46. Bickenbach KA, Gonen M, Labow DM, et al. Indications for and efficacy of splenectomy for haematological disorders. *Br J Surg*. 2013;100(6): 794–800.
47. Kristinsson SY, Gridley G, Hoover RN, et al. Long-term risks after splenectomy among 8,149 cancer-free American veterans: a cohort study with up to 27 years follow-up. *Haematologica*. 2014;99(2):392–398.
48. Mellemkjaer L, Olsen JH, Linet MS, et al. Cancer risk after splenectomy. *Ugeskr Laeger*. 1995;157(37):5097–5100.
49. Price VE, Blanchette VS, Ford-Jones EL. The prevention and management of infections in children with asplenia or hyposplenia. *Infect Dis Clin North Am*. 2007;21(3):697–710. viii-ix.
50. Grijalva CG, Nuorti JP, Arbogast PG, et al. Decline in pneumonia admissions after routine childhood immunisation with pneumococcal conjugate vaccine in the USA: a time-series analysis. *Lancet*. 2007;369(9568):1179–1186.
51. Stephens DS. Conquering the meningococcus. *FEMS Microbiol Rev*. 2007;31(1):3–14.
52. Robert Bona M. Elective (diagnostic or therapeutic) splenectomy. In: William C, Mentzer M, eds. *UpToDate*. Waltham, MA: UpToDate; 2020.
53. Di Sabatino A, Carsetti R, Corazza GR. Post-splenectomy and hyposplenic states. *Lancet*. 2011;378(9785):86–97.
54. Davies JM, Lewis MPN, Wimperis J, et al. Review of guidelines for the prevention and treatment of infection in patients with an absent or dysfunctional spleen: prepared on behalf of the British Committee for Standards in Haematology by a Working Party of the Haemato-Oncology Task Force. *Brit J Haematol*. 2011;155(3):308–317.
55. Kanhutu K, Jones P, Cheng AC, et al. Spleen Australia guidelines for the prevention of sepsis in patients with asplenia and hyposplenism in Australia and New Zealand. *Intern Med J*. 2017;47(8):848–855.
56. Troendle SB, Adix L, Crary SE, Buchanan GR. Laboratory markers of thrombosis risk in children with hereditary spherocytosis. *Pediatr Blood Cancer*. 2007;49(6):781–785.

CHAPTER 157

AGING AND HEMATOLOGIC DISORDERS

Kah Poh Loh, Mazie Tsang, Shakira J. Grant, Richard J. Lin, and Heidi D. Klepin

The population is aging, and it is estimated more than 23.4% of the population in the United States will be older than 65 years by 2060.[1] Many hematologic disorders, including anemia and hematologic malignancies such as myelodysplastic disorders, myeloproliferative neoplasms, chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), multiple myeloma, and certain types of lymphoma, are more common with aging. Compared with younger adults, older adults are more likely to have age-related vulnerabilities such as functional and cognitive impairment. Therefore there is a need to personalize management therapies based on age-related vulnerabilities, rather than relying on chronological age alone, to reduce unnecessary testing and to improve outcomes in this population. The consequences of aging on hematopoiesis are reviewed in Chapter 19. The effects of aging on blood stem and progenitor cells likely account for many of the hematological findings observed in the elderly.

ANEMIA

Anemia in older and younger adults is defined by the World Health Organization (WHO) as hemoglobin less than 13 g/dL for men and less than 12 g/dL for women.[2] For adults aged 65 years and older, the prevalence of anemia is approximately 10% to 11% and increases to 20% to 25% for those aged 85 years and older, and to 50% for those in nursing homes.[3,4] Common causes of anemia in the older age group include iron deficiency/bleeding, chronic disease/inflammation, renal insufficiency, and hematologic malignancies.[3,5] Approximately 30% to 40% of older adults with anemia lack a discernible cause despite a thorough investigation. Unexplained anemia has been associated with acquired somatic mutations in genes associated with myeloid malignancies as detected by whole-exome sequencing (see Chapter 19).[6]

The work-up of anemia should include history taking (symptoms and their effect on daily activities and duration), physical examination, and laboratory testing (complete blood count with differential, renal function, and peripheral smear; depending on the white blood cell differential other testing may include reticulocyte count, iron indices, folate, B_{12}, copper, and zinc levels, thyroid function studies, haptoglobin, liver function test and enzymes, fecal occult blood). If primary bone marrow disorders or anemia is unexplained (especially when patients are symptomatic requiring transfusion), a bone marrow aspirate/biopsy is recommended. It is important to consider age-related vulnerabilities in the work-up of anemia. If patients are frail and/or work-up likely will not change management, periodic monitoring may be considered as an alternative to an invasive bone marrow aspirate/biopsy.

GENERAL PRINCIPLES IN OLDER ADULTS WITH HEMATOLOGIC MALIGNANCIES

Chronological age alone does not determine underlying fitness due to heterogeneity in underlying health status. A geriatric assessment encompasses the use of validated tools to assess age-related vulnerabilities, including functional status and physical performance, cognition, nutrition, psychological health, comorbidities, social support, and medications (Table 157.1). Age-related vulnerabilities assessed as part of the geriatric assessment predict both morbidity and mortality in several hematologic malignancies.[7–10]

Geriatric assessment complements standard oncologic assessments and can be used (1) to uncover vulnerabilities in older adults that would not otherwise be captured during routine visits, (2) to predict treatment tolerance and prognosticate, (3) to assign treatment, and (4) to guide supportive care interventions.[11] Studies have shown that geriatric assessment-guided care improves patient and caregiver satisfaction, increases discussion of age-related vulnerabilities, reduces treatment toxicity without worsening survival, improves treatment tolerance, improves quality of life (QOL), and reduces healthcare utilization.[12–15]

Two specific tools based on the geriatric assessment have been developed to predict chemotherapy toxicity—the Chemotherapy Risk Assessment Scale for High-Age Patients (CRASH) and the Cancer Aging and Research Group (CARG) calculators. The CARG toxicity calculator was developed and validated among older patients with solid tumors, whereas the CRASH toxicity calculator was developed and validated in older patients with a variety of cancers, including hematological malignancies. Specific age-related vulnerabilities incorporated in the CRASH toxicity calculator included Instrumental Activities of Daily Living (IADL), Eastern Cooperative Group (ECOG) Performance Status, Mini-Mental State Examination, and Mini-Nutritional Assessment. The risk of both hematologic and nonhematologic toxicities can be estimated using this tool (see Table 157.2 for a case example).

Studies of the use of geriatric assessment for treatment assignment, although limited, have been done in several hematologic malignancies.[16] Geriatric assessment may prevent both undertreatment (providing less than recommended therapy, thereby leading to worse outcomes) and overtreatment (providing intensive treatment where the harms outweigh the benefits or providing intensive treatment for a cancer that is not expected to affect an older adult in his or her remaining lifetime).[17] Based on geriatric assessment, patients are generally classified as fit, vulnerable, or frail. Alternatively, they can be classified as fit or unfit. It is important to note that these classifications are context- and treatment-specific. For example, an older adult may be fit to receive lower-intensive treatment but unfit to receive high-intensity treatment.

Age-related vulnerabilities are dynamic, and an unfit patient may become fit during the disease trajectory or vice versa. Therefore geriatric assessment may guide supportive care interventions to reverse these vulnerabilities.[11] For example, functional impairment is associated with chemotherapy toxicity and poor survival. For these patients, geriatric assessment may inform the need for interventions such as exercise and physical therapy to improve functional status, therefore improving treatment tolerability and prognosis.

Older adults and their caregivers often overestimate chance of cure.[18,19] When appropriate, geriatric assessment may provide an opportunity for hematologists to guide older adults and caregivers toward end-of-life choices that are consistent with their values and preferences. Studies have shown that geriatric assessment-guided care increases discussion of patient goals, healthcare proxy, and advance directive wishes,[20,21] as well as improves completion of advances directives.[15] This may in turn encourage early referral to palliative care, which in the context of AML, has been shown to improve QOL, improve psychological health, and decrease use of chemotherapy in the last 30 days of life.[22]

In the next few sections, the use of geriatric assessment in lymphoma, AML, myelodysplastic syndrome (MDS), multiple myeloma, and hematopoietic stem cell transplantation (HSCT) is described.

TABLE 157.1 Components of Geriatric Assessment and Examples of Validated Tools

Components	
Functional status	• Activities of Daily Living (ADL) • Instrumental Activities of Daily Living (IADL)
Physical performance	• Short Physical Performance Battery (SPPB) • Timed Up and Go (TUG) • Gait speed
Cognition	• Mini-Cog • Mini-Mental Status Exam (MMSE) • Montreal Cognitive Assessment (MoCA)
Nutrition	• Self-reported weight loss in the last 6 months • Mini Nutritional Assessment (MNA)
Psychological health	• Geriatric Depression Scale-15 (GDS-15) • Generalized Anxiety Disorder-7 (GAD-7)
Comorbidities	• Older Americans Resources and Services (OARS) Comorbidity Scale • Cumulative Illness Rating Scale
Social support	• OARS Social Resources Scale
Medications	• Number of medications (for polypharmacy) • Beers Criteria (for high-risk medications)

LYMPHOMA IN OLDER ADULTS

Lymphomas primarily occur in older adults, with at least half of lymphoma cases occurring in patients who are 65 and older.[23] Choosing a treatment regimen for older patients can be challenging because many older patients may not be able to tolerate standard of care regimens due to their comorbidities, functional status, and increased risk for toxicities. This is especially relevant for older patients with aggressive non-Hodgkin lymphomas, which are often treated with intensive, curative anthracycline-containing regimens.[23] While there is no precise definition of frailty to identify those who are unfit for curative treatment, geriatric assessment can be an important guide.

For patients with aggressive lymphomas, a geriatric assessment may help with treatment assignment. In a randomized controlled trial of 224 older patients with diffuse large B-cell lymphoma (DLBCL), a geriatric assessment was used to assign treatment to either standard-dose rituximab, cyclophosphamide, doxorubicin hydrochloride, vincristine, and prednisone (R-CHOP) or the less-intensive rituximab plus cyclophosphamide, etoposide, vincristine, and prednisone (R-miniCEOP). The outcomes were similar between the two groups. However, patients who were older than 72 and had low-risk disease appeared to have a better outcome when treated with R-miniCEOP ($P = .011$).[16] In a systematic review on the use of the geriatric assessment for older patients with DLBCL, four prospective observational studies showed that independent of the treatment regimen, geriatric assessment could predict overall response rates, overall survival, and therapy-related toxicities. Choice of the regimen to be used was left to the discretion of the treating oncologist rather than based on a geriatric assessment.[7] Although there have been other studies investigating less-intensive chemotherapy regimens, geriatric assessment was not used for treatment assignment. When curative treatment is considered for aggressive lymphoma, older adults without impairment on geriatric assessment

TABLE 157.2 Case Example Using the Chemotherapy Risk Assessment Scale for High-Age Patients Tool to Estimate Hematologic and Nonhematologic Toxicities

Case Example

A 75-year-old male is diagnosed with diffuse large B-cell lymphoma and standard-dose R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone) is considered. He requires some help with getting to places, shopping, preparing meals, doing housework, doing handyman work, laundry, taking medications, and managing money. He can use the telephone independently. On Mini-Mental State Exam, he lost two points for recall and two points in attention and calculation. Based on the CRASH tool, his estimated risk of hematologic and nonhematologic toxicities is 23%–35% and 66%–67%, respectively.

For individual risk score and risk category, please refer to https://moffitt.org/media/4238/crash_scoring_analysis.pdf

	Case	**Score**
Hematologic risk factors		
Diastolic blood pressure (>72 = 1)	90	1
Instrumental Activities of Daily Living (<26 = 1)[a]	22	1
LDH (>459=2)	180	0
HEME SCORE		**2 (ranges from 0 to 5); translates to 23%–35% or intermediate-low risk**
Nonhematologic risk factors		
Eastern Cooperative Group Performance Status (1 – 2 = 1; 3 – 4 = 2)	1	1
Mini-Mental State Exam (<30 = 2)[b]	26	2
Mini Nutritional Assessment (<28 = 2)[c]	23.5	2
NONHEME SCORE		**5 (ranges from 0 to 6); translates to 66%–67% or intermediate high risk**
COMBINED SCORE		**7 (ranges from 0 to 11); translates to 58%–72% or intermediate high risk**

[a] Assesses ability to use the telephone, get to places beyond walking distance, go shopping for groceries, prepare meals, do housework, do handyman work, do laundry, take medications, and manage money; ranges from 10 to 28 (https://moffitt.org/media/4240/instrumental_activities_of_daily_living.pdf).
[b] Assesses orientation, registration, attention, calculation, recall, and language; ranges from 0 to 30 (https://moffitt.org/media/4241/mini_mental_state_examination.pdf).
[c] Ranges from 0 to 30 (https://moffitt.org/media/4243/mna_english-full_new.pdf).
CRASH, Chemotherapy Risk Assessment Scale for High-Age Patients.

should be strongly considered for standard treatments regardless of age. Older patients with vulnerabilities may benefit from modified treatment plans.

Components of a geriatric assessment can independently assist with prognostication. In older patients with CLL, physical performance was the best predictor of outcome when compared with comorbidities, cognition, and functional status. Seventy-five patients, out of 97 enrolled in a clinical trial of the German CLL Study Group, underwent a geriatric assessment before initiation of low-dose fludarabine. Components of a geriatric assessment in this study included the cumulative illness rating scale (CIRS) to measure comorbidities, timed up-and-go (TUG) to measure objective physical function also called "physical performance," the dementia detection (DEMTECT) test, and IADL for self-reported functional status. Although there was little correlation between the CIRS, TUG, DEMTECT, and IADL results with treatment toxicity, decreased performance in TUG (i.e., slower speed) or DEMTECT test was strongly associated with decreased survival. Overall survival was remarkably poor in patients who underperformed in either TUG or DEMTECT test (median overall survival 53.8 vs. 40.5 vs. 18.2 months for TUG test $\leq$10 s vs. 11 to 19 s vs. $\geq$20 s; median overall survival 53.8 vs. 57.8 vs. 17.0 months for DEMTECT test >12 vs. 9 to 12 vs. <9 points, respectively, $P < .05$). The differences were significant after adjusting for age and disease-specific factors that are known to be of use in determining prognosis.[8] These results are supported by a recent study in 448 patients with hematologic malignancies that showed that for every 0.1-m/s decrease in measured usual gait speed, there was an associated higher mortality, odds of unplanned hospitalizations, and emergency department visits.[24] Therefore gait speed should be routinely used to assess frailty in older adults with blood cancers.

In summary, geriatric assessment is an important tool in the prognostication and assignment of treatment for patients with lymphoma and CLL. Geriatric assessment is underutilized in clinical practice because of its perceived complexity. Additional prospective, randomized studies are warranted to validate the use of geriatric assessment in determining treatment approaches and uncovering vulnerabilities.

MYELODYSPLASTIC SYNDROMES

MDSs (see Chapter 61) are a heterogeneous group of clonal hematopoietic disorders that are characterized by ineffective hematopoiesis and peripheral blood cytopenias. Patients diagnosed with MDSs often experience morbidity associated with cytopenias and are at risk for progression to AML. MDS is associated with impairments in QOL, functional decline, and high healthcare use with a 5-year survival rate estimated at less than 50%. The majority of people diagnosed with MDS (proximately 80%) are older than 70 years of age. Diagnosis and risk stratification are described in Chapter 61. The disease can be indolent, having minimal effect on QOL and survival or aggressive with a high risk of progression to AML and posing morbidity risk due to anemia, neutropenia, and/or thrombocytopenia. The commonly used revised International Prognostic Scoring System (R-IPSS) is applicable to older adults and was developed in an older age cohort (median age 71 years). In the development cohort, age was a prognostic factor for survival but not for progression to AML, having more impact on lower-risk (indolent disease) versus higher-risk disease.

Treatment for MDS is guided by risk stratification and symptoms as described in Chapter 61. For older adults, particularly those older than the age of 75 years, treatment decisions must also take into account a patient's overall fitness and competing comorbid conditions. Most patients with MDS have additional comorbidities; a higher comorbidity burden affects non-MDS life expectancy as well as treatment tolerance. Comorbidity burden has been associated with shorter survival independent of age and disease risk.[25] Comorbidity is not static in this population, but comorbid conditions accumulate over time, requiring reassessment during ongoing management.[26]

In general, supportive care remains the mainstay of treatment for patients with low-risk disease regardless of fitness level and in many cases for those who are frail regardless of disease-risk stratification. Patients in the higher-risk IPSS categories are more likely to experience morbidity-related cytopenias and progress to acute leukemia in a shorter time interval from the time of diagnosis. For these patients, hypomethylating agents represent the mainstay of treatment. Randomized studies have shown improvements in survival, QOL, and longer time to progression to AML, including among those age 75 and older.[27] Challenges for older adults using hypomethylating therapy include myelosuppression, which often worsens for several months before a response is achieved, and the duration of therapy, with most clinical trials treating for more than 6 months for all patients and more than 12 months for responders.

Few older adults are candidates for high-intensity therapy with curative intent (see the section on Stem Cell Transplantation and Cellular Therapy).

Assessment of fitness is critical during initial treatment decision-making and during ongoing management to maximize the opportunity for benefit and to guide supportive care. Although data form large prospective trials remain limited, use of geriatric assessment can identify those who are fit (i.e., no impairments detected in geriatric domains) and who are most likely to tolerate guideline-based therapies. Several studies have specifically evaluated strategies for assessing frailty among patients with MDSs. Frailty is a state of decreased physiologic reserve associated with morbidity and mortality and commonly arising from decreased organ reserve, lack of activity, poor nutritional intake, stress, and/or physiologic changes of aging. The two most common methods for characterizing frailty are the phenotype method (weight loss, weakness, slow gait speed, low physical activity, and exhaustion)[28] or the cumulative deficit burden strategy.[29] Investigators leading the Canadian MDS registry have shown that frailty is independently associated with a worse overall survival using several assessment methods.[30] First, the Clinical Frailty Scale (nine-item descriptive scale assigned by physician judgement) was evaluated among 445 patients with MDS and shown to be independently prognostic of overall survival adjusting for R-IPSS and comorbidity.[30] This scale represents an efficient, practical screening tool to incorporate frailty screening into practice. The investigators further evaluated a deficit accumulation frailty index which included 42 variables inclusive of comorbid conditions, laboratory tests, symptoms, self-reported function, and physical performance tests. This MDS-specific frailty index was associated with survival adjusting for age, IPSS score, and transfusion dependence. It improved discrimination of survival by 37%.[31] To enhance usability of the index, a 15-item MDS-specific frailty index was developed using machine learning techniques. This index is weighted toward readily available laboratory data and includes three easy to obtain patient characteristics (fatigue score, assistance with food preparation, and 4-meter walk time). While validation of this tool is needed in other settings, it is a useful tool and an online calculator is available for predicting overall survival at the following link: https://qxcalc.app.link/mdsfrailty.

Finally, a prospective study investigating use of geriatric assessment among older adults with MDS demonstrated a high prevalence of vulnerabilities (78%) at the time of diagnosis and showed that those requiring assistance with IADL and with higher comorbidity burden had shorter survival independent of disease-related factors or treatment.[32] In addition, requiring assistance with IADL, impaired cognition, or mobility limitation were each associated with significantly fewer azacitidine cycles received compared with those without deficits.

In summary, assessing fitness or frailty is relevant to assist initial treatment decision-making, inform expected benefits of therapy, and identify vulnerabilities that can direct supportive care interventions such as referral for physical therapy. Highest yield assessments include frailty screening tools, self-reported function (i.e., activities of daily living [ADL], IADL), gait speed, cognition screening, and evaluation of comorbidity burden. Future studies will help move beyond prognostication and toward intervention on vulnerabilities to improve quality and extent of survivorship.

ACUTE MYELOID LEUKEMIA

There are approximately 20,000 new cases of AML diagnosed in the United States annually, and the majority of those are among adults older than age 65. Although outcomes for patients with AML have improved in recent years, a persistent age-related outcome disparity exists with 5-year survival rates less than 10% for those older than 65 years of age. Due to concerns regarding poor treatment outcomes, many older adults receive no therapy after AML diagnosis. Recently, as described in Chapter 60, treatment options, including isocitrate dehydrogenase (IDH) inhibitors (enasidenib and ivosidenib), FLT-3 inhibitors (midostaurin and gliteritinib), Hedgehog inhibitor (glasdegib), BCL-2 inhibitor (venetoclax), and others (gemtuzumab, CPX-351), have expanded for older and less fit adults. Current guideline recommendations emphasize treatment over best supportive care with more intensive treatment for those who are fit and likely to withstand the stress of treatment versus less-intensive strategies for those who are not considered "fit" to optimize the risk to benefit estimation.[33]

A key unresolved issue in these recommendations are the criteria for "fitness" for intensive therapy. To date, no validated set of criteria have been prospectively evaluated, and this decision is largely based on a provider's subjective estimation and rely on chronologic age. Recent drug approvals for "unfit" older adults with AML have used age 75 years or older as the most consistent criteria, followed by presence of comorbidities (i.e., cardiac and renal disease) and oncology performance status to define the population treated. Use of more refined strategies are needed to ensure fit patients, particularly those older than age 75 years, are appropriately identified.

Several nonrandomized studies have shown that geriatric assessment can inform estimation of fitness in the setting of therapy for AML. Geriatric assessment detects vulnerabilities not otherwise captured.[34,35] A prospective study assessing older patients with good ECOG performance status considered fit to receive intensive therapy identified the following impairments at the time of treatment initiation: cognitive impairment (24%), depression (26%), distress (50%), ADL dependence (34%), impaired objectively measured physical performance (31%), and comorbidity burden (40%).[35] In this cohort study (N=74) evaluating inpatient geriatric assessment prior to intensive chemotherapy, two measures were independently associated with survival: objectively measured physical performance (assessed using the short physical performance battery [SPPB]) and cognitive impairment.[36] The SPPB is a validated measure of lower extremity function that predicts future disability, hospitalizations, and mortality among older adults in varied settings. The SPPB is composed of a short walk (4 meters), repeated chair stands, and balance tests and is more sensitive to change in function than self-report measures.

Geriatric assessment has also been studied to a limited extent in the nonintensive treatment setting.[34,37] A multisite observational study evaluated pretreatment geriatric assessment among patients with MDS or AML who received best supportive care, hypomethylating agents, and intensive therapy per clinician discretion. Among those who received either best supportive care or low-intensity therapy dependence in ADL, high fatigue score and impaired oncology performance status were independently associated with inferior survival.[34] A frailty score was created from these variables, ranging from 0 (low risk), 1 to 2 (intermediate), and 3 impairments (high risk) that predicted survival. A multisite study validating this frailty score has been completed in the setting of nonintensive chemotherapy in Europe, and published results are pending.[38]

In addition to providing useful information at the time of initial treatment decision-making, geriatric assessment measures can provide important information during the course of treatment due to the dynamic nature of measured vulnerabilities. For example, in older adults receiving intensive therapy, short-term declines in physical function can be expected.[39,40] Symptom burden may also change during treatment. These acquired vulnerabilities can impact longer-term outcomes and candidacy for subsequent therapies such as stem cell transplantation. A recent analysis demonstrated that objectively measured physical function (SPPB score) and depressive symptoms measured at the time of postremission therapy evaluation are independently associated with survival among older adults treated intensively for AML.[41] These observations suggest that repeat geriatric assessment at key decision intervals during the course of treatment and survivorship can provide prognostic information and guide supportive care targeting those vulnerabilities to improve "fitness."

Another important consideration when evaluating older adults with AML is alignment of treatment choices to patient values and the importance of providing information on both disease control and QOL. Age-related differences in health-related QOL have been evaluated in an observational study comparing older and younger patients treated intensively for AML.[42] As expected, most patients report a significant symptom burden at diagnosis with similar improvements in QOL in both older and younger age groups over time for those who survive. Compared with younger patients, older adults had less recovery in measures of physical function than younger patients did. Several studies have evaluated differences in QOL between those treated with different regimens. In a randomized trial comparing azacitidine to conventional therapy (standard induction chemotherapy, low-dose ara-c, or supportive care only) for newly diagnosed older adults with AML, global QOL was similar between treatments, although comparisons were limited over time due to attrition.[43] Observational studies have also shown no clear difference in QOL between those treated with azacitidine or conventional therapy and no clear indication that QOL was associated with treatment allocation.[44,45] The inclusion of well-designed QOL objectives in randomized treatment trials will be important to inform patients on how they will feel and function during and after treatment to guide personalized decision-making as therapeutic options evolve.

In summary, most older adults with AML should be offered therapy. Initial treatment decision-making should be informed by both tumor biology and careful assessment of patient characteristics to inform "fitness." Although there is no gold standard definition of fitness, older adults with adequate physical performance (SPPB score $\geq$9), normal cognition, independence in ADL, and lack of significant comorbidity can be considered fit for intensive therapy. Use of geriatric assessment can inform treatment decision-making and management during the course of treatment with emphasis on identifying modifiable vulnerabilities (i.e., physical function, symptoms) that can guide supportive care.

MULTIPLE MYELOMA IN OLDER ADULTS

Multiple myeloma is a disease of older adults, with a median age at diagnosis of 70 years (Chapter 91). With approximately 32,000 new cases diagnosed annually in the United States, multiple myeloma is the second most common hematologic malignancy following leukemia.[46] As therapies for multiple myeloma continue to evolve rapidly to include novel agents and targeted therapies, improved survival has resulted in multiple myeloma becoming a chronic disease for some. This trend toward improved survival is less apparent for older adults (age $>$ 70 years) due to their increased risk of early mortality (death within 6 to 12 months following diagnosis).[47,48] The observed survival differences are partly due to the higher prevalence of cognitive and functional impairments and multimorbidity detected with advancing age.[49] Recognizing the heterogeneity of aging and the limitations associated with use of chronologic age and performance status as proxies for an older adult's fitness level, geriatric assessment has become an essential component of the care for older adults with cancer.

In the multiple myeloma setting, various tools are available to detect vulnerabilities and shape clinicians' decision-making around treatment intensity and treatment-related toxicity risk prediction. Palumbo and colleagues developed the International Myeloma Working Group's frailty tool, which incorporates age, performance status, functional status (IADL, and ADL), and comorbid burden (Charlson comorbidity index [CCI]).[9] In this pooled analysis of

869 newly diagnosed older adults with multiple myeloma enrolled in three prospective trials, patients were classified as fit, intermediate fit, and frail using an additive score; scores were predictive of death, survival, treatment discontinuation, and nonhematologic toxicities.[9] The Revised Myeloma Comorbidity Index (R-MCI) is an expansion of the previously described Freiburg Comorbidity index[50] that incorporates frailty status along with age, comorbid burden, disease cytogenetics, performance status, and the presence of lung or renal disease. In this prospective study of 552 patients, 13% (*n* = 74) were classified as frail using the R-MCI. Frailty was associated with an inferior overall survival.[51] Further attempts to streamline assessments of geriatric domains in adults with multiple myeloma have been retrospectively explored using data from the Frontline Investigation of Revlimid and Dexamethasone versus Standard Thalidomide (FIRST) (MM-020) trial.[52,53] Among 1618 newly diagnosed, transplant-ineligible multiple myeloma patients, frailty status was defined by age, presence of comorbidities (CCI), and ECOG Performance Status. Forty-nine percent of patients were found to be frail, with frail patients experiencing worse overall survival compared with nonfrail patients (median overall survival 42.1 vs. 70.1 months; $P < .0001$).[53]

Further attempts to describe the predictive and prognostic value of deficits in age-related vulnerabilities using administrative and claims data have been pursued. Using the Rockwood Accumulation of Deficits approach for the Medicare Health Outcomes Survey (MHOS), a frailty index was developed and applied to 305 patients with multiple myeloma.[54] Using the MHOS-based frailty index, 53% of patients were considered frail. Furthermore, each 10% increase in frailty index was associated with a 16% increased risk for a poor outcome.[54] Recently investigators applied an accumulation of deficits approach frailty index to a large (*n* = 3807) database of US veterans with multiple myeloma. Thirty percent of the cohort were considered frail, with higher frailty scores associated with worse overall survival, with hazard ratios of 1.33, 1.97, 2.86, and 3.22 for prefrail, mildly frail, moderately frail, and severely frail, respectively.[55]

Assessment of vulnerabilities using a geriatric assessment remains essential in the care of adults with multiple myeloma. However, whether personalization of therapy based on geriatric assessment–detected vulnerabilities could improve the outcomes of older adults remains unanswered. The planned phase 3 Myeloma XIV (FiTNEss) trial will address this question in transplant-ineligible older adults.[56] The results of this and other prospective trials could shape future treatment approaches, ultimately leading to improved outcomes for older adults with multiple myeloma.

HEMATOPOIETIC STEM CELL TRANSPLANTATION AND CELLULAR THERAPY IN OLDER ADULTS

HSCT is more frequently considered for older patients with hematologic malignancies, and studies from the Center for International Blood and Marrow Transplant Research (CIBMTR) have shown improved long-term outcomes in recipients receiving autologous and allogeneic grafts.[57] The increased use of HSCT is likely the result of advances in reduced-intensity conditioning regimens, broadened donor pools, improved supportive care including antimicrobials, and better selection of appropriate older candidates using validated geriatric assessment tools.[57]

Traditionally, the Hematopoietic Cell Transplant-Comorbidity Index (HCT-CI) is used in combination with chronologic age to risk stratify older adults prior to HSCT.[58] Recognizing increased geriatric vulnerabilities and heightened sensitivity to therapeutic toxicities among older adults, Muffly and colleagues conducted the first prospective study of geriatric assessment in older allogeneic HSCT patients.[10] They found that several geriatric deficits, including functional and physical limitation, mental health impairment, and multimorbidity, significantly reduced post-HSCT survival beyond chronologic age or performance status alone. These findings were subsequently validated in several small prospective and retrospective studies[57] and laid the foundation for the larger ongoing Blood and Marrow Transplant Clinical Trials Network (BMTCTN-1704) study: Composite Health Assessment Model for Older Adults: Applying Pre-transplant Comorbidity, Geriatric Assessment, and Biomarkers to Predict Non-Relapse Mortality after Allogeneic Transplantation (CHARM, NCT03992352).

The use of a geriatric assessment for autologous HSCT decision-making has shown promise, with initial data suggesting a role for the geriatric assessment in predicting the likelihood of receiving HSCT.[59] In a prospective study of 40 newly diagnosed older adults with multiple myeloma, the administration of a pretransplant geriatric assessment identified 62% with at least one measurable IADL deficit, 76.9% with polypharmacy, and 47.5% with one or greater comorbid conditions. Patients with fewer geriatric assessment–detected vulnerabilities were also more likely to undergo transplantation (*n* = 26). In addition, age and IADL dependence were strongly associated with receipt of HSCT.[59] Use of a pre- and post-stem cell transplantation geriatric assessment was also found to predict length of stay and event-free survival in multiple myeloma.[60]

Beyond risk stratification for HSCT outcomes, geriatric assessment may uncover additional geriatric vulnerabilities for targeted interventions. Derman and colleagues published results from a single institution pre–post implementation study of a transplant optimization program.[61] They found that a geriatric assessment–guided, multidisciplinary team clinic composed of interprofessional providers reliably provided targeted rehabilitation and optimization for older, vulnerable patients pre-HSCT. The geriatric assessment–selected, optimized postintervention cohort had fewer inpatient deaths, shorter length of stay, and lower 1-year nonrelapse mortality and higher overall survival compared with the preintervention cohort. These results provided support for geriatric assessment–guided interventions to improve HSCT and cellular therapy outcomes.

More recently, novel cellular immunotherapy such as chimeric antigen receptor T-cell (CAR-T) therapy is revolutionizing the treatment of hematologic malignancies. However, it remains unknown how older, vulnerable patients will tolerate these intensive therapies with unique toxicity profiles. In a recent single institution study with prospectively collected geriatric assessment data, Lin and colleagues showed that older lymphoma patients had similar outcomes after CAR-T therapy compared with younger patients, suggesting that chronologic age alone should not exclude older patients.[62] Moreover, CAR-T could be safely administrated to older patients with preexisting functional limitation and cognitive impairment. Larger studies with longer follow-up are needed to evaluate prognostically important geriatric assessment domains among older CAR-T patients.

In summary, geriatric assessment is playing an increasingly important role to risk stratify and to identify potential modifiable deficits for older patients undergoing HSCT and cellular therapies. It is likely that incorporating geriatric assessment tools to assess HSCT candidacy will become the standard of care based on the CHARM study. However, prospective, randomized, multicenter studies are needed to evaluate whether geriatric assessment tools can guide HSCT cellular therapy decision-making and treatment intensity; and to determine the impact of targeted geriatric interventions on HSCT and cellular therapy outcomes for older, vulnerable patients with hematologic malignancies.

CONCLUSION

With the growing number of older adults with cancer, a geriatric assessment provides a way to personalize management in this population. However, this approach is underutilized and not routinely incorporated in clinical practice due to various barriers such as perceived lack of time, resources, and evidence to support its use. Increasing numbers of studies on the utility of geriatric assessment in hematologic malignancies are emerging and, along with continued education of hematologists, will facilitate its dissemination and implementation.

ACKNOWLEDGMENTS

Dr. Loh is supported by the National Cancer Institute of the National Institutes of Health (K99CA237744) and Wilmot Research Fellowship Award. Dr. Klepin is supported by The National Institute on Aging (4R33AG059206-03). Dr. Grant is supported by the National Cancer Institute of the National Institutes of Health (5-K12-CA120780-13) and the National Institute on Aging at the National Institutes of Health (1 R03 AG074030-01).

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Artz AS, Thirman MJ. Unexplained anemia predominates despite an intensive evaluation in a racially diverse cohort of older adults from a referral anemia clinic. *J Gerontol A Biol Sci Med Sci*. 2011;66(8):925–932.

Benboubker L, Dimopoulos MA, Dispenzieri A, et al. Lenalidomide and dexamethasone in transplant-ineligible patients with myeloma. *N Engl J Med*. 2014;371(10):906–917.

Buckstein R, Wells RA, Zhu N, et al. Patient-related factors independently impact overall survival in patients with myelodysplastic syndromes: an MDS-CAN prospective study. *Br J Haematol*. 2016;174:88–101.

Della Porta MG, Malcovati L, Strupp C, et al. Risk stratification based on both disease status and extra-hematologic comorbidities in patients with myelodysplastic syndrome. *Haematologica*. 2011;96(3):441–449.

Derman BA, Kordas K, Ridgeway J, et al. Results from a multidisciplinary clinic guided by geriatric assessment before stem cell transplantation in older adults. *Blood Adv*. 2019;3(22):3488–3498.

Deschler B, Ihorst G, Platzbecker U, et al. Parameters detected by geriatric and quality of life assessment in 195 older patients with myelodysplastic syndromes and acute myeloid leukemia are highly predictive for outcome. *Haematologica*. 2013;98(2):208–216.

El-Jawahri A, Abel GA, Traeger L, et al. Quality of life and mood of older patients with acute myeloid leukemia (AML) receiving intensive and non-intensive chemotherapy. *Leukemia*. 2019;33:2393–2402.

El-Jawahri A, Nelson-Lowe M, VanDusen H, et al. Patient-clinician discordance in perceptions of treatment risks and benefits in older patients with acute myeloid leukemia. *The Oncologist*. 2019;24(2):247–254.

Engelhardt M, Domm A-S, Dold SM, et al. A concise revised Myeloma Comorbidity Index as a valid prognostic instrument in a large cohort of 801 multiple myeloma patients. *Haematologica*. 2017;102(5):910–921.

Facon T, Dimopoulos MA, Meuleman N, et al. A simplified frailty scale predicts outcomes in transplant-ineligible patients with newly diagnosed multiple myeloma treated in the FIRST (MM-020) trial. *Leukemia*. 2020;34(1):224–233.

Goede V, Bahlo J, Chataline V, et al. Evaluation of geriatric assessment in patients with chronic lymphocytic leukemia: results of the CLL9 trial of the German CLL study group. *Leuk Lymphoma*. 2016;57(4):789–796.

Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med*. 2014;371(26):2488–2498.

Kleber M, Ihorst G, Gross B, et al. Validation of the Freiburg comorbidity index in 466 multiple myeloma patients and combination with the international staging system are highly predictive for outcome. *Clin Lymphoma Myeloma Leuk*. 2013:541–551.

Klepin HD, Geiger AM, Tooze JA, et al. Geriatric assessment predicts survival for older adults receiving induction chemotherapy for acute myelogenous leukemia. *Blood*. 2013;121(21):4287–4294.

Klepin HD, Ritchie E, Major-Elechi B, et al. Geriatric assessment among older adults receiving intensive therapy for acute myeloid leukemia: report of CALGB 361006 (Alliance). *J Geriatr Oncol*. 2020;11(1):107–113.

Klepin HD, Tooze JA, Pardee TS, et al. Effect of intensive chemotherapy on physical, cognitive, and emotional health of older adults with acute myeloid leukemia. *J Am Geriatr Soc*. 2016;64(10):1988–1995.

Lin RJ, Behera M, Diefenbach CS, Flowers CR. Role of anthracycline and comprehensive geriatric assessment for elderly patients with diffuse large B-cell lymphoma. *Blood*. 2017;130(20):2180–2185.

Lin RJ, Lobaugh SM, Pennisi M, et al. Impact and safety of chimeric antigen receptor T-cell therapy in older, vulnerable patients with relapsed/refractory large B-cell lymphoma. *Haematologica*. 2021;106(1):255–258.

Liu MA, DuMontier C, Murillo A, et al. Gait speed, grip strength, and clinical outcomes in older patients with hematologic malignancies. *Blood*. 2019;134(4):374–382.

Merli F, Luminari S, Rossi G, et al. Cyclophosphamide, doxorubicin, vincristine, prednisone and rituximab versus epirubicin, cyclophosphamide, vinblastine, prednisone and rituximab for the initial treatment of elderly "fit" patients with diffuse large B-cell lymphoma: results from the ANZINTER3 trial of the Intergruppo Italiano Linfomi. *Leuk Lymphoma*. 2012;53(4):581–588.

Mohamedali H, Breunis H, Timilshina N, et al. Older age is associated with similar quality of life and physical function compared to younger age during intensive chemotherapy for acute myeloid leukemia. *Leuk Res*. 2012;36(10):1241–1248.

Molga A, Wall M, Chhetri R, et al. Comprehensive geriatric assessment predicts azacitidine treatment duration and survival in older patients with myelodysplastic syndromes. *J Geriatr Oncol*. 2020;11:114–120.

Muffly LS, Kocherginsky M, Stock W, et al. Geriatric assessment to predict survival in older allogeneic hematopoietic cell transplantation recipients. *Haematologica*. 2014;99(8):1373–1379.

Naqvi K, Garcia-Manero G, Sardesai S, et al. Association of comorbidities with overall survival in myelodysplastic syndrome: development of a prognostic model. *J Clin Oncol*. 2011;29(16):2240–2246.

Oliva EN, Nobile F, Alimena G, et al. Quality of life in elderly patients with acute myeloid leukemia: patients may be more accurate than physicians. *Haematologica*. 2011;96(5):696–702.

Palumbo A, Bringhen S, Mateos M-V, et al. Geriatric assessment predicts survival and toxicities in elderly myeloma patients: an International Myeloma Working Group report. *Blood*. 2015;125:2068–2074.

Rosko AE, Huang Y, Benson DM, et al. Use of a comprehensive frailty assessment to predict morbidity in patients with multiple myeloma undergoing transplant. *J Geriatr Oncol*. 2019;10(3):479–485.

Saad M, Loh KP, Tooze JA, et al. Geriatric assessment and survival among older adults receiving post-remission therapy for acute myeloid leukemia. *Blood*. 2020;136:2715–2719.

Sorror ML, Storb RF, Sandmaier BM, et al. Comorbidity-age index: a clinical measure of biologic age before allogeneic hematopoietic cell transplantation. *J Clin Oncol*. 2014;32(29):3249–3256.

Starkman R, Alibhai S, Wells RA, et al. An MDS-specific frailty index based on cumulative deficits adds independent prognostic information to clinical prognostic scoring. *Leukemia*. 2020;34(5):1394–1406.

REFERENCES

1. Schoch C, Kern W, Schnittger S, Büchner T, Hiddemann W, Haferlach T. The influence of age on prognosis of de novo acute myeloid leukemia differs according to cytogenetic subgroups. *Haematologica*. 2004;89(9):1082–1090.
2. Patel KV. Epidemiology of anemia in older adults. *Semin Hematol*. 2008;45(4):210–217.
3. Guralnik JM, Eisenstaedt RS, Ferrucci L, Klein HG, Woodman RC. Prevalence of anemia in persons 65 years and older in the United States: evidence for a high rate of unexplained anemia. *Blood*. 2004;104(8): 2263–2268.
4. Ferrucci L, Guralnik JM, Bandinelli S, et al. Unexplained anaemia in older persons is characterised by low erythropoietin and low levels of pro-inflammatory markers. *Br J Haematol*. 2007;136(6):849–855.
5. Artz AS, Thirman MJ. Unexplained anemia predominates despite an intensive evaluation in a racially diverse cohort of older adults from a referral anemia clinic. *J Gerontol A Biol Sci Med Sci*. 2011;66(8):925–932.
6. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med*. 2014;371(26):2488–2498.
7. Lin RJ, Behera M, Diefenbach CS, Flowers CR. Role of anthracycline and comprehensive geriatric assessment for elderly patients with diffuse large B-cell lymphoma. *Blood*. 2017;130(20):2180–2185.
8. Goede V, Bahlo J, Chataline V, et al. Evaluation of geriatric assessment in patients with chronic lymphocytic leukemia: results of the CLL9 trial of the German CLL study group. *Leuk Lymphoma*. 2016;57(4):789–796.
9. Palumbo A, Bringhen S, Mateos M-V, et al. Geriatric assessment predicts survival and toxicities in elderly myeloma patients: an International Myeloma Working Group report. *Blood*. 2015;125:2068–2074.
10. Muffly LS, Kocherginsky M, Stock W, et al. Geriatric assessment to predict survival in older allogeneic hematopoietic cell transplantation recipients. *Haematologica*. 2014;99(8):1373–1379.
11. Loh KP, Soto-Perez-de-Celis E, Hsu T, et al. What every oncologist should know about geriatric assessment for older patients with cancer: Young International Society of Geriatric Oncology position paper. *J Oncol Pract*. 2018;14(2):85–94.
12. Mohile SG, Epstein RM, Hurria A, et al. Communication with older patients with cancer using geriatric assessment: a cluster-randomized clinical trial from the National Cancer Institute Community Oncology Research Program. *JAMA Oncol*. 2019:1–9.
13. Soo W-K, King M, Pope A, Parente P, Darzins P, Davis ID. Integrated geriatric assessment and treatment (INTEGERATE) in older people with cancer planned for systemic anticancer therapy. *J Clin Oncol*. 2020;38(15_suppl). 12011–12011.
14. Mohile SG, Mohamed MR, Culakova E, et al. A geriatric assessment (GA) intervention to reduce treatment toxicity in older patients with advanced cancer: A University of Rochester Cancer Center NCI community oncology research program cluster randomized clinical trial (CRCT). *J Clin Oncol*. 2020;38(15_suppl). 12009–12009.
15. Li D, Sun C-L, Kim H, et al. Geriatric assessment-driven intervention (GAIN) on chemotherapy toxicity in older adults with cancer: a randomized controlled trial. *J Clin Oncol*. 2020;38(15_suppl). 12010–12010.
16. Merli F, Luminari S, Rossi G, et al. Cyclophosphamide, doxorubicin, vincristine, prednisone and rituximab versus epirubicin, cyclophosphamide, vinblastine, prednisone and rituximab for the initial treatment of elderly "fit" patients with diffuse large B-cell lymphoma: results from the ANZINTER3 trial of the Intergruppo Italiano Linfomi. *Leuk Lymphoma*. 2012;53(4):581–588.
17. DuMontier C, Loh KP, Bain PA, et al. Defining undertreatment and overtreatment in older adults with cancer: a scoping literature review. *J Clin Oncol*. 2020;38:2558–2569. https://doi.org/10.1200/JCO.19.02809.
18. El-Jawahri A, Nelson-Lowe M, VanDusen H, et al. Patient-clinician discordance in perceptions of treatment risks and benefits in older patients with acute myeloid leukemia. *The Oncologist*. 2019;24(2):247–254.
19. Loh KP, Mohile SG, Lund JL, et al. Beliefs about advanced cancer curability in older patients, their caregivers, and oncologists. *Oncologist*. 2019;24(6):e292–e302.
20. Flannery MA, Culakova E, Loh KP, et al. Improving person-centered communication of goals, proxy, and advance directives in older patients with advanced cancer: secondary analysis from a University of Rochester NCI Community Oncology Research Program (NCORP) cluster randomized controlled trial (CRCT). *J Clin Oncol*. 2019;37(15_suppl). 11523–11523.
21. Abel GA, Uno H, Tanasijevic AM, et al. Feasibility and impact of embedded geriatric consultation for frail older adults with blood cancer: a randomized controlled trial. *Blood*. 2019;134(Supplement_1). 67–67.
22. El-Jawahri A, LeBlanc TW, Kavanaugh A, et al. Multisite randomized trial of integrated palliative and oncology care for patients with acute myeloid leukemia (AML). *J Clin Oncol*. 2020;38(15_suppl). 12000–12000.
23. Thieblemont C, Coiffier B. Lymphoma in older patients. *J Clin Oncol*. 2007;25(14):1916–1923.
24. Liu MA, DuMontier C, Murillo A, et al. Gait speed, grip strength, and clinical outcomes in older patients with hematologic malignancies. *Blood*. 2019;134(4):374–382.
25. Naqvi K, Garcia-Manero G, Sardesai S, et al. Association of comorbidities with overall survival in myelodysplastic syndrome: development of a prognostic model. *J Clin Oncol*. 2011;29(16):2240–2246.
26. Della Porta MG, Malcovati L, Strupp C, et al. Risk stratification based on both disease status and extra-hematologic comorbidities in patients with myelodysplastic syndrome. *Haematologica*. 2011;96(3):441–449.
27. Xicoy B, Jiménez MJ, García O, et al. Results of treatment with azacitidine in patients aged ≥75 years included in the Spanish Registry of Myelodysplastic Syndromes. *Leuk Lymphoma*. 2014;55(6):1300–1303.
28. Fried LP, Tangen CM, Walston J, et al. Frailty in older adults: evidence for a phenotype. *J Gerontol A Biol Sci Med Sci*. 2001;56(3):M146–M156.
29. Rockwood K, Mitnitski A. Frailty in relation to the accumulation of deficits. *J Gerontol A Biol Sci Med Sci*. 2007;62(7):722–727.
30. Buckstein R, Wells RA, Zhu N, et al. Patient-related factors independently impact overall survival in patients with myelodysplastic syndromes: an MDS-CAN prospective study. *Br J Haematol*. 2016;174:88–101.
31. Starkman R, Alibhai S, Wells RA, et al. An MDS-specific frailty index based on cumulative deficits adds independent prognostic information to clinical prognostic scoring. *Leukemia*. 2020;34(5):1394–1406.
32. Molga A, Wall M, Chhetri R, et al. Comprehensive geriatric assessment predicts azacitidine treatment duration and survival in older patients with myelodysplastic syndromes. *J Geriatr Oncol*. 2020;11:114–120.
33. Sekeres MA, Guyatt G, Abel G, et al. American Society of Hematology 2020 guidelines for treating newly diagnosed acute myeloid leukemia in older adults. *Blood Adv*. 2020;4(15):3528–3549.
34. Deschler B, Ihorst G, Platzbecker U, et al. Parameters detected by geriatric and quality of life assessment in 195 older patients with myelodysplastic syndromes and acute myeloid leukemia are highly predictive for outcome. *Haematologica*. 2013;98(2):208–216.
35. Klepin HD, Geiger AM, Tooze JA, et al. The feasibility of inpatient geriatric assessment for older adults receiving induction chemotherapy for acute myelogenous leukemia. *J Am Geriatr Soc*. 2011;59(10):1837–1846.
36. Klepin HD, Geiger AM, Tooze JA, et al. Geriatric assessment predicts survival for older adults receiving induction chemotherapy for acute myelogenous leukemia. *Blood*. 2013;121(21):4287–4294.
37. Sherman AE, Motyckova G, Fega KR, et al. Geriatric assessment in older patients with acute myeloid leukemia: a retrospective study of associated treatment and outcomes. *Leuk Res*. 2013;37(9):998–1003.
38. Grishina O, Schmoor C, Döhner K, et al. DECIDER: prospective randomized multicenter phase II trial of low-dose decitabine (DAC) administered alone or in combination with the histone deacetylase inhibitor valproic acid (VPA) and all-trans retinoic acid (ATRA) in patients >60 years with acute myeloid leukemia who are ineligible for induction chemotherapy. *BMC Cancer*. 2015;15:430.
39. Klepin HD, Ritchie E, Major-Elechi B, et al. Geriatric assessment among older adults receiving intensive therapy for acute myeloid leukemia: report of CALGB 361006 (Alliance). *J Geriatr Oncol*. 2020;11(1):107–113.
40. Klepin HD, Tooze JA, Pardee TS, et al. Effect of intensive chemotherapy on physical, cognitive, and emotional health of older adults with acute myeloid leukemia. *J Am Geriatr Soc*. 2016;64(10):1988–1995.
41. Saad M, Loh KP, Tooze JA, et al. Geriatric assessment and survival among older adults receiving post-remission therapy for acute myeloid leukemia. *Blood*. 2020;136:2715–2719.
42. Mohamedali H, Breunis H, Timilshina N, et al. Older age is associated with similar quality of life and physical function compared to younger age during intensive chemotherapy for acute myeloid leukemia. *Leuk Res*. 2012;36(10):1241–1248.
43. Dombret H, Seymour JF, Butrym A, et al. International phase 3 study of azacitidine vs conventional care regimens in older patients with newly diagnosed AML with >30% blasts. *Blood*. 2015;126(3):291–299.
44. El-Jawahri A, Abel GA, Traeger L, et al. Quality of life and mood of older patients with acute myeloid leukemia (AML) receiving intensive and non-intensive chemotherapy. *Leukemia*. 2019;33:2393–2402.
45. Oliva EN, Nobile F, Alimena G, et al. Quality of life in elderly patients with acute myeloid leukemia: patients may be more accurate than physicians. *Haematologica*. 2011;96(5):696–702.
46. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin*. 2020;70:7–30.
47. Binder MNB, Gertz MA, et al. Mortality of patients with multiple myeloma after the introduction of novel therapies in the United States. *Blood*. 2019;134:72.
48. Kumar VAM, Biswas S, et al. Trends in early mortality from multiple myeloma (MM): a population-based analysis. *Blood*. 2019;134:71.

49. Vuyyala SCK, Fu P, et al. The prognostic factors for early fatal outcome in patients with multiple myeloma. *Blood*. 2019;134:270.
50. Kleber M, Ihorst G, Gross B, et al. Validation of the Freiburg comorbidity index in 466 multiple myeloma patients and combination with the international staging system are highly predictive for outcome. *Clin Lymphoma Myeloma Leuk*. 2013:541–551.
51. Engelhardt M, Domm A-S, Dold SM, et al. A concise revised Myeloma Comorbidity Index as a valid prognostic instrument in a large cohort of 801 multiple myeloma patients. *Haematologica*. 2017;102(5):910–921.
52. Benboubker L, Dimopoulos MA, Dispenzieri A, et al. Lenalidomide and dexamethasone in transplant-ineligible patients with myeloma. *N Engl J Med*. 2014;371(10):906–917.
53. Facon T, Dimopoulos MA, Meuleman N, et al. A simplified frailty scale predicts outcomes in transplant-ineligible patients with newly diagnosed multiple myeloma treated in the FIRST (MM-020) trial. *Leukemia*. 2020;34(1):224–233.
54. Mian H, Brouwers M, Kouroukis CT, Wildes TM. Comparison of frailty scores in newly diagnosed patients with multiple myeloma: a review. *J Frailty Aging*. 2019;8(4):215–221.
55. Patel BG, Luo S, Wildes TM, Sanfilippo KM. Frailty in older adults with multiple myeloma: a study of US veterans. *JCO Clin Cancer Inform*. 2020;4:117–127.
56. Schagen SB, Boogerd W, Muller MJ, et al. Cognitive complaints and cognitive impairment following BEP chemotherapy in patients with testicular cancer. *Acta Oncol*. 2008;47(1):63–70.
57. Jayani R, Rosko A, Olin R, Artz A. Use of geriatric assessment in hematopoietic cell transplant. *J Geriatr Oncol*. 2020;11(2):225–236.
58. Sorror ML, Storb RF, Sandmaier BM, et al. Comorbidity-age index: a clinical measure of biologic age before allogeneic hematopoietic cell transplantation. *J Clin Oncol*. 2014;32(29):3249–3256.
59. Wildes TM, Tuchman SA, Klepin HD, et al. Geriatric assessment in older adults with multiple myeloma. *J Am Geriatr Soc*. 2019;67(5):987–991.
60. Rosko AE, Huang Y, Benson DM, et al. Use of a comprehensive frailty assessment to predict morbidity in patients with multiple myeloma undergoing transplant. *J Geriatr Oncol*. 2019;10(3):479–485.
61. Derman BA, Kordas K, Ridgeway J, et al. Results from a multidisciplinary clinic guided by geriatric assessment before stem cell transplantation in older adults. *Blood Adv*. 2019;3(22):3488–3498.
62. Lin RJ, Lobaugh SM, Pennisi M, et al. Impact and safety of chimeric antigen receptor T-cell therapy in older, vulnerable patients with relapsed/refractory large B-cell lymphoma. *Haematologica*. 2021;106(1):255–258.

CHAPTER 158

ONCO-CARDIOLOGY: FOCUS ON CARDIAC COMPLICATIONS OF HEMATOLOGIC TREATMENTS

Andrea Gallardo-Grajedau and Gagan Sahni

Recent progress in chemotherapy and immunotherapy for hematological malignancies has improved cancer survivorship over the past few decades. However, cardiovascular disease is a prominent cause of death in many cancer survivors, with emerging cardiotoxicity from cancer therapies being a significant contributor to the morbidity and mortality of this population.

Onco-cardiology as a subspecialty of cardiology that seeks to identify, prevent, diagnose and manage the cardiovascular complications of cancer therapies, which may be related to both cancer itself and the adverse effects of cancer therapies. Among the myriad cardiovascular complications of cancer therapies, chemotherapy-induced left ventricular dysfunction or cancer therapy-related cardiac dysfunction (CTRCD) as per 2014 guidelines from the American Society of Echocardiography is among the commonest.[1] However, the spectrum of cardiotoxicity includes other cardiovascular conditions including and not limited to atrial fibrillation and other arrhythmias, hypertension, acute coronary syndromes (ACSs), venous thromboembolism, pericardial syndromes, and valvular disease.

This chapter aims at a discussion of cardiotoxicity due to chemotherapy, immunotherapy, radiation therapy, and bone marrow transplantation, all of which are commonly used therapies for hematological malignancies.

CARDIOTOXICITY FROM CHEMOTHERAPY USED IN HEMATOLOGICAL MALIGNANCIES (TABLE 158.1)

Anthracyclines

Oncological Indication

Anthracyclines are cytostatic antibiotics discovered in the 1960s and encompass several anthracyclines including doxorubicin, epirubicin, idarubicin, daunorubicin, and liposomal encapsulated doxorubicin, used for a broad range of solid tumors, sarcomas, and hematological malignancies.

Cardiotoxicity

Anthracycline-induced cardiotoxicity (AIC) can develop in an acute, subacute, or chronic manner. Injury to the myocyte may begin as early as the initial exposure, with some studies detecting a rise in troponin levels immediately after the first dose is administered.[2] This occurs in less than 1% of patients and manifests as an acute, transient decline in myocardial contractility, which is usually reversible.

The subacute form occurs in 1.6% to 2.1% of patients within the first year after treatment whereas the late-onset chronic form presents at least 1 year after completion of therapy in 1.6% to 5% of patients receiving lower doses. Both the subacute and chronic forms tend not to be reversible.[2–4]

AIC occurs in a dose-dependent manner, increasing with a cumulative dose. Studies evaluating cumulative probability of doxorubicin-induced heart failure have found rates in the range of 3% to 5% at doses less than 400 mg/m^2, 7% to 26% at 550 mg/m^2, and 18% to 48% at 700 mg/m^2.[5] However, subclinical cardiotoxicity defined by troponin rise, strain imaging, and MRI studies has been shown with doses less than 250 mg/m^2 (Fig. 158.1).

Mechanism of Cardiotoxicity

Previously, the most commonly accepted hypothesis for AIC was the generation of reactive oxygen species (ROS) by electron exchange between the anthracycline and oxygen molecules and the formation of complexes with iron that undergo redox cycling and generate oxygen radicals. Although in vivo and in vitro studies confirmed increased ROS production in cardiomyocytes after anthracycline therapy, neither antioxidants nor iron chelation have prevented the development of cardiomyopathy (Fig. 158.2).

More recent studies demonstrate topoisomerase (Top) 2b as the primary mediator of AIC. Top2b is present in all quiescent cells, including cardiomyocytes and is responsible for unwinding deoxyribonucleic acid (DNA) chains during normal DNA replication, transcription, or recombination. Anthracycline inhibition of Top2b results in double-stranded breaks in DNA, production of ROS, and mitochondrial dysfunction which leads to cardiomyocyte apoptosis.[6] Mice models have shown Top2b deletion from the heart protects cardiomyocytes from anthracycline-induced cardiomyopathy, which strongly implicates Top2b as the key mediator of AIC.[7]

Prevention and Treatment

Anthracycline cardiotoxicity should be mitigated without compromising oncological efficacy. The strategies may include dose limitation, altering infusions schedules, use of alternative molecules such as liposomal encapsulated doxorubicin, using less cardiotoxic derivatives (e.g., epirubicin or idarubicin), and cardio-protection using targeted cardioprotective agent (e.g., dexrazoxane) in conjunction with treatment.

Dexrazoxane is the only FDA-approved, targeted, cardioprotective agent for AIC. It changes Top2's configuration to a closed-clamp form through tight binding to Top2's ATP-binding sites, thus preventing anthracyclines from binding to the Top2 complex.[8] Even though in some pediatric studies it has shown a relative risk reduction of up to 79% in cardiotoxicity, there have been adult studies suggesting myelosuppression, secondary malignancy, and reduced anthracycline efficacy which has not been validated in any randomized controlled trials. However, currently dexrazoxane is only approved by the FDA for adults needing additional anthracyclines after an initial dose of greater than 300 mg/m^2.

Other cardioprotective agents including beta-blockers, angiotensin-converting enzyme inhibitors (ACEi), and angiotensin receptor blockers (ARBs) have been studied for both primary and secondary prevention in smaller studies.[9] Stringent control of concomitant risk factors such as diabetes, dyslipidemia, hypertension, and avoiding combination cardiotoxic chemo and concomitant radiotherapy to the mediastinum/left chest are other ways to mitigate AIC.[10]

TABLE 158.1 Cardiotoxicity From Therapies Used in Hematological Malignancies

Agent		Hematological Indications	Cardiovascular Toxicity	Prevention and Treatment
Anthracyclines	Doxorubicin Epirubicin Idarubicin Daunorubicin Liposomal encapsulated doxorubicin	Bone marrow transplant Leukemias Lymphomas	Acute and chronic heart failure [3%–26%]	• Cardioprotection with dexrazoxane [Top 2b inhibitor] • Dose limitation and avoiding combination radiation/cardiotoxic chemo • Altering infusions schedules • Use of alternative molecules such as liposomal encapsulated doxorubicin • Use of less cardiotoxic derivatives (e.g., epirubicin or idarubicin) • Role of ACE-I, ARBs and beta-blockers in primary prevention
Tyrosine kinase inhibitors		Chronic myelogenous leukemia (CML) Acute lymphoblastic leukemia (ALL)	Heart failure Arrhythmias/QT prolongation Hypertension Myocardial ischemia/ACS	
	Imatinib		Heart failure (0.5%–1.7%)	• Use of cardioprotective agents such as beta-blockers and ACE-I if indicated
	Dasatinib		Pulmonary hypertension (0.45–12.1%) Pleural effusion	• Screening for PAH with transthoracic echocardiogram • Combination therapy of vasodilators (phosphodiesterase-5 inhibitor, endothelin receptor antagonists, etc.)
	Nilotinib		QT prolongation Vascular events (PVD, CVD)	• Baseline ECG • Correct electrolyte abnormalities • Check drug-drug interactions for QT prolongation • Stop therapy or reduce dose if QTc >480 • Screening for PVD with ABI
	Ponatinib		Vascular events, venous and arterial thrombosis	• Controlling risk factors such as diabetes and hypertension • Use of ACE-I, statins, aspirin • Screening for PVD
	Ibrutinib	Refractory chronic lymphocytic leukemia (CLL) Waldenstrom macroglobulinemia Mantle cell lymphoma (MCL)	Atrial fibrillation	• Strategy of immediate rhythm control • Interactions with antiarrhythmic drugs: amiodarone, diltiazem, and verapamil, digoxin • Interactions with anticoagulants: apixaban and rivaroxaban, dabigatran • Close monitoring during anticoagulation • Alternatives left atrial appendage closure devices, vena cava filter
Arsenic trioxide		Relapsed or refractory acute promyelocytic leukemia (APL) Myelodysplastic syndrome Multiple myeloma Non-Hodgkin lymphoma	QT prolongation, Ventricular arrhythmias, torsades de pointes and sudden cardiac death	• ECG surveillance • Monitoring and supplementation of electrolytes • ROS scavengers and antioxidants (experimental role of (vit C, vit E, glutathione, carotenoids, flavonoids and antioxidants)
Immune checkpoint inhibitors		Plasma cell myeloma Myelodysplastic syndrome Acute myeloid leukemia	Myocarditis	• Measure hs-cTnT and perform periodic EKG especially in first 3 months • Transthoracic echocardiogram if clinical suspicion of myocarditis • Withhold therapy if ICI-myocarditis suspected and admit patient • CMR gold standard for diagnosis of ICI-myocarditis, if not, EMB • Treatment with steroids or aggressive immunosuppressive regimens as required
CAR-T therapy		Highly refractory and relapsing hematological malignancies	Sinus tachycardia, arrhythmias, QT prolongation, ST changes, LVSD, profound hypotension, shock during CRS	• Tocilizumab, steroids, pressors • Echocardiogram to assess LVSD during CRS • Ace-I / ARBs when blood pressure is stable • Hold anticoagulation and dual antiplatelet therapy prior to CAR-T therapy to avoid intracranial hemorrhage

Continued

TABLE 158.1 **Cardiotoxicity From Therapies Used in Hematological Malignancies—cont'd**

Agent	Hematological Indications	Cardiovascular Toxicity	Prevention and Treatment
Radiation therapy	Hodgkin and non-Hodgkin lymphoma	Pericarditis (acute and constrictive) CAD (late) Valvular dysfunction (late) Restrictive heart failure	• Minimizing radiation dose given to left chest and mediastinum • Use of charged particle (e.g., proton or carbon ion) therapy • Prior CT-guided field planning to minimize cardiac involvement • Prone patient position, breast boards, and having the patient hold a deep breath to decrease the involvement of the anterior heart

ABI, Ankle-brachial index; *ACE-I*, angiotensin-converting enzyme inhibitor; *ACS*, acute coronary syndrome; *ARB*, angiotensin receptor blocker; *BNP*, brain natriuretic peptide; *CAD*, coronary artery disease; *CRS*, cytokine release syndrome; *ECG*, electrocardiogram; *EMB*, endomyocardial biopsy; *hs-cTnT*, high-sensitivity cardiac troponin T, *PVD*, peripheral vascular disease; *Top2b*, topoisomerase 2b.

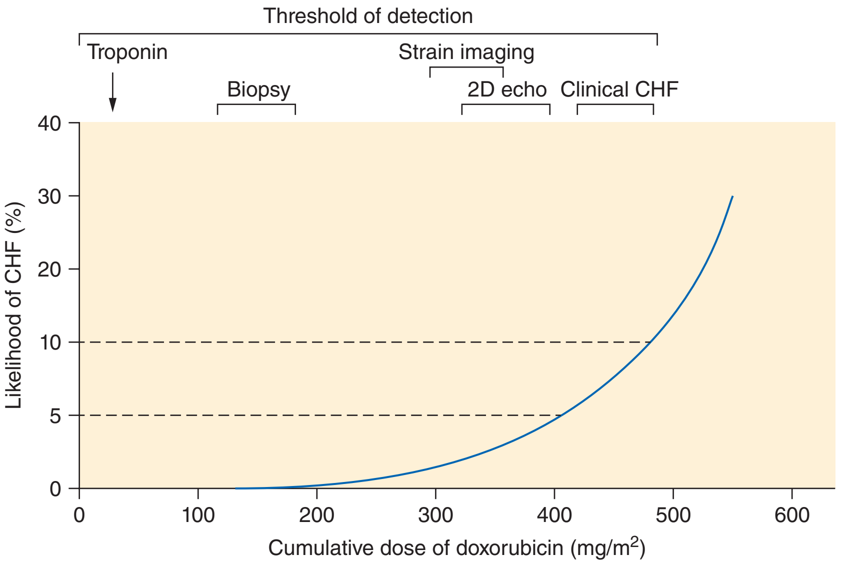


Figure 158.1 THE CORRELATION BETWEEN DOXORUBICIN DOSE AND THE INCIDENCE OF CONGESTIVE HEART DISEASE (CHF). Critical diagnostic points are approximated on the dose scale. *2D echo*, Two-dimensional echocardiography. *(From Ewer MS, Ewer SM. Cardiotoxicity of anticancer treatments. Nat Rev Cardiol. 2015;12:547–558.)*

Tyrosine Kinase Inhibitors

The vast majority of chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL) (Chapters 68 and 69) are treated with a spectrum of tyrosine kinase inhibitors (TKIs) which targets the Abl kinase portion of Bcr-Abl in these hematological malignancies.

Imatinib was the first tyrosine kinase inhibitor to be discovered with high specificity for Bcr-Abl protein; however, more than 30% of patients with CML are either unable to tolerate or will develop resistance to imatinib. Thereby, newer generation BCR-ABL kinase inhibitors—dasatinib, nilotinib, bosutinib, and ponatinib—have been developed as efficacious alternatives or second-line therapy. Of these, only ponatinib inhibits the "gatekeeper" T315I mutation, which is present in up to 20% of patients with resistance to other tyrosine kinase inhibitors. The various TKIs have distinct vascular safety profiles, due to each compound's different kinase inhibition profiles and non-kinase targets.[11]

The spectrum of cardiovascular toxicities associated with TKIs includes heart failure (HF), arrythmia, QT prolongation, hypertension, and ACS/myocardial ischemia. The individual cardiotoxicity of the most common TKIs and their mechanism of cardiac damage is discussed separately as below.

Imatinib

In patients treated with imatinib monotherapy, clinical heart failure is very rare, occurring in 0.5% to 1.7% of patients.[11] Initial studies that used serum B-type natriuretic peptide (BNP) or troponin T levels as a marker of cardiac dysfunction reported no cardiotoxicity with imatinib therapy[12]; however, some noninvasive imaging studies have demonstrated a decline in the left ventricular ejection fraction with this therapy.[13] Although pathological findings characteristic of toxin-induced myopathy have been demonstrated on biopsy in imatinib-treated patients, studies have failed to correlate with clinical heart failure. Which patients are most susceptible to developing cardiac dysfunction with imatinib therapy and are candidates for appropriate preventative and management interventions is currently unknown.

Vascular toxicity is rare with imatinib treatment; in fact, it may actually have beneficial roles on the vasculature, having shown attenuation of in-stent restenosis and diabetes-associated atherosclerosis in mouse models. Interestingly, in a large retrospective cohort, patients with CML on imatinib treatment had lower rates of peripheral vascular events compared with those treated initially with placebo. These favorable effects also extend to the pulmonary vasculature, where pre-clinical data suggesting reversal of pulmonary arterial hypertension (PAH).[14–17]

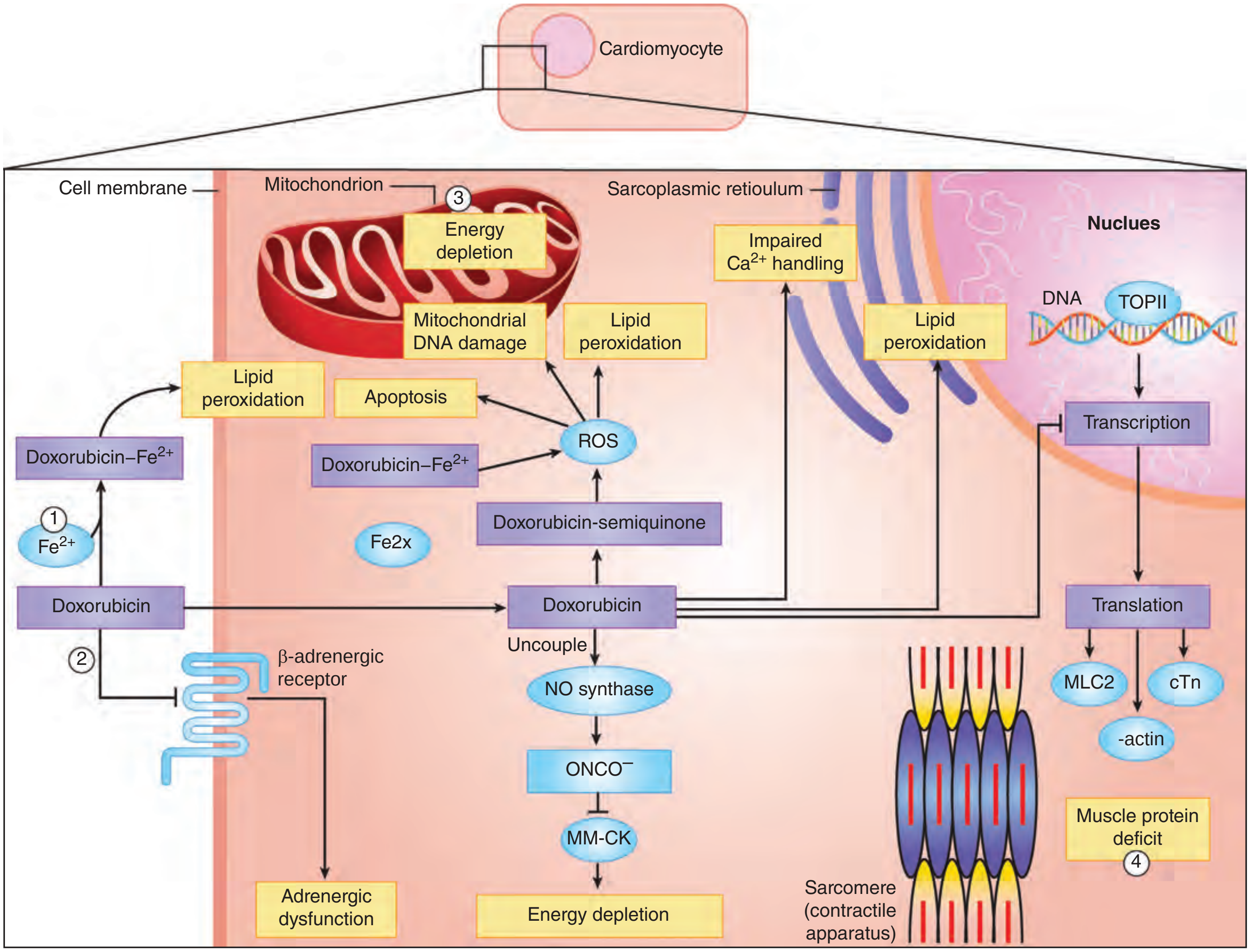

Cardiomyocyte
Cell membrane
Mitochondrion
③
Energy depletion
Sarcoplasmic retioulum
Impaired Ca^{2+} handling
Nuclues
DNA
TOPII
Mitochondrial DNA damage
Lipid peroxidation
Lipid peroxidation
Lipid peroxidation
Apoptosis
Transcription
Doxorubicin–Fe^{2+}
ROS
Doxorubicin–Fe^{2+}
①
Fe^{2+}
Fe2x
Doxorubicin-semiquinone
Doxorubicin
Doxorubicin
Translation
②
β-adrenergic receptor
Uncouple
NO synthase
MLC2
cTn
-actin
$ONCO^{-}$
MM-CK
Muscle protein deficit
④
Adrenergic dysfunction
Energy depletion
Sarcomere (contractile apparatus)

Figure 158.2 POTENTIAL OPPORTUNITIES FOR CARDIOPROTECTION. Doxorubicin chemotherapy has a range of effects on cardiomyocytes. It induces lipid peroxidation at the cell and mitochondrial membranes by way of complexing with Fe^{2+} and induces apoptosis, mitochondrial DNA damage, and energy depletion through its production of ROS. Furthermore, it impairs Ca^{2+} processing in the sarcoplasmic reticulum and inhibits the transcription of important muscle elements, weakening the heart muscle. It also downregulates adrenergic receptors and interrupts cell signaling. (1) Administration of dexrazoxane can prevent Fe^{2+} complex formation. (2) Intravenous immunoglobulin therapy can reduce inflammatory cytokines. (3) l-Carnitine can bolster mitochondrial function. (4) Anti-heart-failure therapies, such as angiotensin-converting enzyme inhibitors and beta-blockers, can prevent further damage. *cTn*, Cardiac troponin; *MLC2*, myosin light chain 2; *MM-CK*, myofibrillar isoform of the CK enzyme; *ROS*, reactive oxygen species; *TOPII*, topoisomerase 2. *(From Lipshultz SE, Sambatakos P, Maguire M, et al. Cardiotoxicity and cardioprotection in childhood cancer. Nat Rev Clin Oncol. 2013;10:697–710.)*

Dasatinib

Dasatinib frequently causes pulmonary hypertension (PAH), the incidence varying from 0.45% to 12.1% in different series.[18,19] One-third of these cases have irreversible PAH that persists even on discontinuation of the drug. The mechanism of dasatinib-associated PAH remains unclear.

A French PAH registry reported a series of 9 dasatinib-associated PAH cases, the median time between initiation of dasatinib therapy and PAH diagnosis being 8 to 48 months. At diagnosis, most patients had severe clinical, functional, and hemodynamic impairment with minimal acute vasodilator response, suggesting that isolated acute vasoconstriction is not the main mechanism of dasatinib-induced PAH, which seems to be a delayed and sometimes irreversible phenomenon.[18]

Interestingly, dasatinib is associated with a higher incidence of pleural effusion, from 14% to 35%. The mechanisms underlying development of pleural effusion are probably immune-mediated.[20]

Nilotinib

QT prolongation and vascular events are the main CV adverse events seen with nilotinib. Nilotinib prolongs the QT interval more commonly than other tyrosine kinase inhibitors with an incidence of 1% to 10%. The mechanism behind this action potential delay is an inhibition of phosphoinositide-3-kinase due to “on-target” effects of nilotinib.[21]

A baseline ECG should be obtained in all patients prior to starting TKI’s such as nilotinib. Electrolyte abnormalities must be corrected and drug-drug interactions that influence QTc interval (antibiotics, antiemetic, antipsychotic or antidepressant drugs) must be evaluated prior to beginning treatment. ECGs should be repeated a few days after initiation/modification of anticancer therapy. Strict monitoring and repletion of magnesium and electrolyte levels are warranted when using this agent and a corrected QT of greater than 480 ms is an indication to hold therapy or ΔQTc greater than 60 ms during monitoring.[11,21]

Vascular events have been reported in up to 6.15% of patients who received nilotinib, including severe peripheral atherosclerosis needing angioplasty and limb amputation.[22] In fact, a prospective study of 129 patients with CML screened for peripheral vascular disease (PVD) with ankle-brachial index (ABI) during therapy demonstrated PVD in 26% of patients treated with first-line nilotinib, and 35.7% with second-line nilotinib—far higher than previously reported.[23]

Nilotinib also affects coronary arteries causing vasospasm, direct antiangiogenic, and pro-atherogenic effects on endothelial cells, procoagulant effects, and acceleration of atherosclerotic processes in the coronary and peripheral vasculature. Therefore, it is necessary to have rigorous metabolic control, modification of cardiovascular risk factors, and is to get baseline screening for CAD with stress testing and PVD with ABI in high-risk patients.[22]

Besides VEGF inhibition, an accelerated metabolic dysregulation may be involved in accelerated vascular atherosclerosis, since nilotinib may cause increases in glucose, total cholesterol and LDL, insulin resistance, and compensatory hyperinsulinemia. Nilotinib treatment is also associated with hypothyroidism, which can affect lipid and glucose metabolism, which are all risk factors for developing atherosclerosis.[24,25]

Ponatinib

Ponatinib is highly effective in CML patients who are resistant to other TKIs. However, in a study with a median follow-up of 27.9 months, serious arterial thrombotic events occurred in 19% of ponatinib-treated patients, including CV events (10%), cerebrovascular events (7%), and peripheral vascular events (7%), with some patients having greater than 1 event. In addition, 5% of patients experienced VTE and at least five patients died from vascular events (VE) thought to be due to ponatinib.[26] Other studies have shown a high incidence of HTN in up to 26% of patients treated with ponatinib.[27] Subsequently, ponatinib now has a black box warning about increased risk of arterial and venous occlusive events which occur earlier and more frequently than even nilotinib.

The underlying mechanisms of ponatinib-induced VE are largely unknown. Ponatinib inhibits numerous tyrosine kinases such as SRC, FGFR, PDGFR, and VEGFR1–3 which result in nitrous oxide depletion, platelet aggregation, endothelial dysfunction, and vasospasm that eventually lead to a high incidence of vascular events.[28]

Controlling risk factors such as diabetes and hypertension, use of ACE-inhibitors, statins, aspirin, and screening for PVD are thereby essential in prevention and treatment of ponatinib and nilotinib induced cardiotoxicities.

Ibrutinib

Ibrutinib is an irreversible inhibitor of Bruton tyrosine kinase (BTK), which has become an important part of the therapeutic approach in patients with refractory chronic lymphocytic leukemia (CLL), Waldenstrom macroglobulinemia, and mantle cell lymphoma (MCL) (Chapters 76, 86, and 92). However, ibrutinib use is associated with an almost fourfold increase in the risk of atrial fibrillation (AF). The reported incidence of new-onset AF in patients treated with ibrutinib can be as high as 16%, most occurring in the first 2 years of its use. The mechanism of AF may be induced via inhibition of cardiac PI3K-Akt signaling by inhibition of cardioprotective BTK and TEC kinases by ibrutinib according to a preclinical mouse model theory.[29,30]

Due to the interactions between ibrutinib and both antiarrhythmic and anticoagulant drugs, the management of ibrutinib-related AF is particularly challenging. Amiodarone, diltiazem, and verapamil, which are inhibitors of CYP3A4, a primary site for ibrutinib metabolism, increase ibrutinib exposure and should not be co-administered, while digoxin interacts with ibrutinib through inhibition of P-glycoprotein. A strategy of immediate rhythm control with cardioversion followed by a short course of anticoagulation, during which ibrutinib can be administered at decreased doses, seems to be preferable over rate control in patients who present with new-onset AF.[31]

There are also drug interactions between ibrutinib and the oral anticoagulants, apixaban and rivaroxaban through CYP3A interaction, and dabigatran through P-glycoprotein interaction. Also, ibrutinib has significant intrinsic anticoagulant activity due to unclear mechanisms; because of this anticoagulant activity with the increased risk of bleeding, additional platelet inhibition is generally contraindicated during the ibrutinib therapy course.[31] Close monitoring is warranted during anticoagulation therapy. In case of failed cardioversion or recurrent AF with a high risk of bleeding, additional treatment alternatives could be the use of left atrial appendage closure devices or placement of a vena cava filter.

Therapy decisions need to be individualized, based on feasibility of rate or rhythm control, patients' risk factors for thromboembolism and bleeding, and the availability of effective alternative antineoplastic treatments for the underlying hematologic malignancy.

Arsenic Trioxide

Oncological Indication

Arsenic trioxide (ATO) has been recommended as the front-line agent for treatment of relapsed or refractory acute promyelocytic leukemia (APL). It has also been found to be effective in other hematologic malignancies, such as myelodysplastic syndrome, multiple myeloma, and non-Hodgkin lymphoma.

Cardiotoxicity

Chronic ATO use can result in cardiac toxicities including QT prolongation, ventricular arrhythmias, torsades de pointes, and sudden cardiac death. Lesser reported CV events include other EKG changes, myocarditis, pericarditis, myocardial infarction, and heart failure. Myocardial damage might be irreversible and sometimes even lethal.

Mechanism of Cardiotoxicity

Apoptosis induced by arsenic depends on cell type, time, and concentration of exposure. When arsenic concentrations reach 0.5 to 2.0 mol/L, apoptosis is triggered by different pathways: ROS-mitochondrial disruption, caspase activation (especially caspase 3), MAPK signaling and p53. Depletion of nitric oxide by increase in synthesis of inflammatory mediators like leukotriene E4 and prostacyclin, tumor necrosis factor alpha (TNF-α), and NF-κB in vascular endothelial cells is another mechanism for the other cardiac toxicities caused by ATO.

The mechanism underlying QT prolongation is prolongation of the cardiomyocyte action potential duration via (1) increasing cardiac Ca^{2+} currents with up-regulation of Ica and L channels (L type calcium current) and (2) reducing surface expression of the cardiac K+ current human related gene (hERG) that encodes for cardiomyocyte K+ channels causing a down-regulation of Ik1 (inward rectifier potassium current). Also, ATO induces cardiac fibrosis because it increases TGF-B1 secretion leading to adverse cardiac electrical events.[32,33]

Arsenic-induced atherosclerosis can be attributed to different mechanisms, such as increasing mRNA transcripts of growth factors such as GM-CSF, TGF beta and the inflammatory cytokines like tumor necrosis factor-alpha and interleukin-6. Importantly, arsenic exposure enhances the aggregation of platelets and increases plasminogen activator inhibitor type-1 (PAI-1) expression, thus leading to reduced fibrinolysis.

Prevention and Treatment

ROS scavengers and antioxidants (ascorbic acid [vitamin C]; alpha-tocopherol [vitamin E]; glutathione [GSH]; carotenoids, flavonoids, and antioxidant enzymes such as glutathione reductase, glutathione peroxidase, and glutathione S-transferase) could provide a possible approach to treat arsenic-mediated heart injuries.

In addition, some valuable medicinal plants have demonstrated cardio-protection effect against ATO-induced toxicity such as *Boerhavia diffusa*, *Corchorus olitorius*, *Salvia miltiorrhiza*, *Silibum marianum*, and *Terminalia arjuna* in smaller studies.[34]

Regular ECG surveillance, monitoring and supplementation of electrolytes and use of cardioprotective agents such as beta-blockers and ACE-I (if indicated) are strategies to detect and circumvent CV events caused by ATO.

CARDIOTOXICITY FROM IMMUNOTHERAPY USED IN HEMATOLOGICAL MALIGNANCIES

Immune Checkpoint Inhibitors

Oncological Indication

Immune checkpoint inhibitors (ICIs) improve survival outcomes in patients with non-hematologic malignancies including prostate, lung, urothelial, and renal cancers, as well as melanoma. However, these agents have shown the ability to reestablish immune function in patients with certain hematologic malignancies, including multiple Hodgkin disease and B-cell lymphomas.

Cardiac Toxicity

The remarkable efficacy of ICIs as antineoplastic therapies is shadowed by their potential for inducing autoimmune or inflammatory side effects, termed immune-related adverse events (irAEs). Cardiovascular irAEs, particularly myocarditis, are increasingly recognized with a prevalence reported from 0.06% to 2.4%. Other manifestations include pericardial disease, vasculitis, Takotsubo syndrome, destabilization of atherosclerotic lesions, venous thromboembolism, or conduction abnormalities.[35]

Mechanism of Cardiotoxicity

The therapeutic mechanisms of the most commonly used ICIs are based on blocking either the cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) or programmed cell death protein-1 (PD-1) pathways. Cardiac irAEs appear more frequently in patients treated with CTLA-4 antagonists as compared with PD-1 inhibitors, and the risk increases with combination therapy, sometimes even leading to ICI's discontinuation in up to 50% of patients.[36,37]

The mechanism of ICI-induced myocarditis has been hypothesized to be due to a loss of the inhibitors of T-cell inactivation, leading to an autoimmune T-cell infiltration of the myocardium causing autoimmune myocarditis, release of biomarkers such as troponin signaling cardiac injury and eventually dilated cardiomyopathy and death due to heart failure. There is a suggestion that patients with autoimmune diseases, diabetes mellitus, pre-existing CV disease, and those who did not receive a flu vaccine in the preceding 6 months are at highest risk.[38]

The onset of ICI-associated myocarditis has been primarily reported within 3 months of treatment initiation in 81% of cases in registry data with a median time of 17 to 65 days after the first dose of the ICI. Symptoms are non-specific and may include dyspnea, chest pain, fatigue, myalgia, palpitations, syncope, dizziness, or altered mental status. Subclinical myocarditis has also been described, posing management challenges.

Prevention and Treatment

Baseline and serial measurements of serum high-sensitivity cardiac troponin T (hs-cTnT) and the performance of EKGs is reasonable in patients undergoing ICI therapy, especially during the first 3 months. A transthoracic echocardiogram and cardiac MRI (cMR) should be performed in patients suspected of ICI-induced cardiotoxicity, with cardiac biopsy reserved for cases with equivocal imaging findings or presentation. ICI therapy should be withheld in patients presenting with overt heart failure, significant myocardial injury with high levels of troponin, and those presenting with malignant arrhythmias such as heart block and ventricular tachyarrhythmias, according to the practice guideline for the management of ICI-associated myocarditis by the American Society of Clinical Oncology.[39]

Almost all patients (94%) will have increased serum troponin levels, which should immediately prompt suspicion of ICI-associated myocarditis. The ECG is frequently abnormal, ranging from nonspecific changes to ACSs in up to 89% of patients.[40] Echocardiography is useful for determining cardiac function, but the gold standard is cardiac magnetic resonance imaging (cMR). However, cMR changes demonstrating myocardial inflammation or necrosis through late gadolinium enhancement (LGE) may not be present in the early phases of immune mediated myocarditis. Registry data has shown that presence of LGE varied with the timing of cMR being present in only 21% patients if done before 4 days from admission and in up to 72% patients if performed after day 4 of admission for suspected immune mediated myocarditis.[41]

In symptomatic but clinically stable cases with abnormal tests, treatment should be initiated promptly with high dose prednisone or methylprednisolone. Decompensated cases require more aggressive and early immunosuppressive regimens, such as 2 mg/kg prednisone + mycophenolate, anti-thymocyte globulin, or infliximab (to be avoided in cases of heart failure); these cases may also require advanced heart failure support (Fig. 158.3).[42]

CAR-T Therapy

Oncological Indication

Chimeric antigen receptor T cells (CAR-T) are genetically engineered T cells that express artificial receptors that can bind to tumor associated antigens and augment T-cell-mediated killing of target cancer cells. CAR-T-cell therapies expressing CD19 receptors have shown remarkable early success in highly refractory and relapsing hematological malignancies such as ALL in children and young adults, large B-cell lymphoma, and multiple myeloma.[43]

Cardiac Toxicity

Cytokine release syndrome (CRS) is a frequent adverse effect of CAR-T-cell therapy, which may result in cardiotoxicity in the form of cardiomyopathy, heart failure, capillary leak syndrome, myocardial injury with troponin elevations and arrhythmias.[44]

Though CRS usually develops earlier in the course of CAR-T therapy (median time 2.2 days), the cardiomyopathy usually develops after a median duration of 12.5 days (2 to 24 days range). The reported incidence of cardiomyopathy has been variable in several retrospective studies but was as high as 10.3% in some studies where

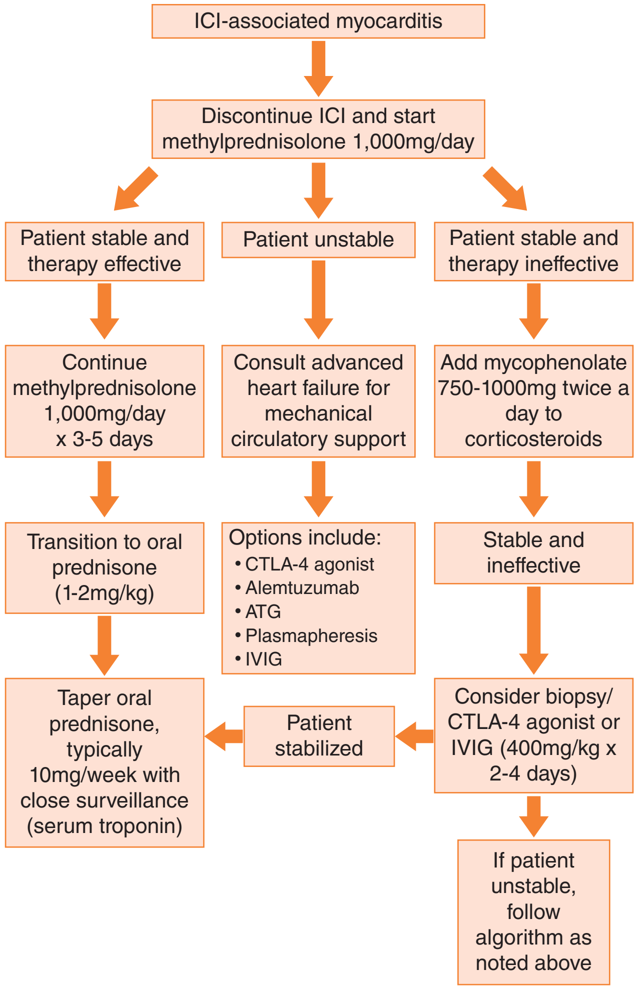

ICI-associated myocarditis
Discontinue ICI and start methylprednisolone 1,000mg/day
Patient stable and therapy effective
Patient unstable
Patient stable and therapy ineffective
Continue methylprednisolone 1,000mg/day x 3-5 days
Consult advanced heart failure for mechanical circulatory support
Add mycophenolate 750-1000mg twice a day to corticosteroids
Transition to oral prednisone (1-2mg/kg)
Options include:
• CTLA-4 agonist
• Alemtuzumab
• ATG
• Plasmapheresis
• IVIG
Stable and ineffective
Taper oral prednisone, typically 10mg/week with close surveillance (serum troponin)
Patient stabilized
Consider biopsy/ CTLA-4 agonist or IVIG (400mg/kg x 2-4 days)
If patient unstable, follow algorithm as noted above

Figure 158.3 PROPOSED TREATMENT ALGORITHM FOR IMMUNE CHECKPOINT INHIBITOR-ASSOCIATED MYOCARDITIS. *(From Zhang L, Reynolds KL, Lyon AR, et al. The evolving immunotherapy landscape and the epidemiology, diagnosis, and management of cardiotoxicity: JACC: CardioOncology Primer. JACC CardioOncol. 2021;3(1):35–47.)*

serial echocardiograms were performed at baseline and after development of grade greater than 2 CRS.[45]

Mechanism of Cardiac Toxicity

The pathophysiology of cardiac dysfunction during CRS is unclear but is likely mediated by interleukin-6 (IL-6), which is known to cause myocardial depression in infectious and inflammatory states. The refractory hypotension and vascular leak syndrome seen in CRS could contribute to the LV dysfunction,[46–49] as could a non-specific stress induced or Takotsubo cardiomyopathy not directly caused by the CAR-T cells.[50] Another plausible theory is direct cardiotoxicity through an autoimmune cross-reactivity of CAR-T cells against Titin, a striated muscle protein.[51]

Prevention and Treatment

Prompt recognition of CRS is crucial since the vast majority of cardiotoxicity occurs in patients with grade 2 CRS. Patients should have a baseline echocardiogram prior to CAR-T and inflammatory markers such as IL-6, CRP, and ferritin should be routinely checked during CAR-T therapy. If grade greater than 2 CRS does occur, patients should have a repeat echocardiogram.

Supportive hemodynamic care with judicious use of intravenous fluids and vasopressors, targeted anti-IL-6 therapy, as well as possibly broader immunosuppression with corticosteroids, are the cornerstones of the management.

Anti IL-6 therapy with tocilizumab is first line therapy approved by FDA for management of CRS related toxicity, especially in the setting of hemodynamic compromise, unstable arrhythmias, myocardial damage with troponin elevation or new cardiomyopathy with LVEF less than 40%. Siltuximab is another monoclonal antibody that blocks IL-6 with a higher affinity, not yet FDA approved for CRS, but may be considered in patients not responding to tocilizumab and corticosteroids.[52]

Corticosteroids are also effective in the treatment of CRS. However, given concerns of reduced CAR-T cell efficacy with their use, though not yet proven, corticosteroids are considered a second-line therapy for the management of CRS refractory to tocilizumab. If neurotoxicity occurs as part of the CRS, dual antiplatelet therapy and anticoagulation should be held to avoid intracranial bleeding with a friable blood brain barrier, though there are no established guidelines regarding this.

CARDIOTOXICITY OF RADIATION TREATMENT

Radiotherapy used to treat carcinomas, especially thoracic cancers such as Hodgkin lymphoma or lung and breast cancer, carries a high risk of developing cardiovascular side effects. Major cardiac side effects include pericarditis, coronary artery diseases, cardiomyopathy, valvular dysfunction, and heart failure (Table 158.2).

In general, radiation-related cardiovascular disease is a chronic concern, with a median time to diagnosis of 19 years (10 to 40 years range). The heart is affected by radiation in a dose-dependent manner. Cardiomyocyte toxicity and cardiomyopathy are most common in radiation doses greater than 30 Gy. However, damaging effects can also be seen even after using doses as low as 2 Gy.

Risk factors associated with radiation-induced heart disease include an earlier age of treatment (such as young children with mediastinal Hodgkin lymphoma), concomitant therapy with trastuzumab (which binds to the extracellular segment of the HER2/neu receptor) or anthracyclines, irradiation of the left side of the heart, prior coronary artery disease or other heart diseases, or the presence of other cardiovascular risk factors.

Radiation-induced cardiac (RIC) effects are mediated primarily by the generation of free radicals, pro-inflammatory cytokines that cause DNA strand disruption, resulting in a spectrum of structural changes in the heart; these include pericardial inflammation, chronic development of fibrosis in the myocardium, valvular heart diseases, fibrosis of conduction system, and endothelial damage in the coronary vessels. This eventually leads to clinical manifestations such as constrictive pericarditis, pericardial effusion, restrictive cardiomyopathy, valvular stenosis and regurgitation, and accelerated coronary atherosclerosis.

Pericardial manifestations occur the earliest in 8% to 30% of patients, radiation-induced coronary vasculopathy occurs in up to 85% of patients typically affecting the ostia or proximal segments of the coronary arteries, and valvular disease occurs in 7% to 39% of patients at 10 years and 12% to 60% of patients at 20 years, with mitral and aortic valves the most affected.[53]

Prevention of cardiac damage during radiation therapy involves minimizing the radiation dose given, use of charged-particle (e.g., proton or carbon ion) therapy, and prior CT-guided field planning to minimize cardiac involvement. The use of prone patient position, breast boards, and having the patient hold a deep breath to decrease the involvement of the anterior heart have also been suggested to minimize cardiac damage.

To date, no treatment is available to reverse or treat the effects of RIC. The focus of treatment paradigms has been to optimize medical management of other cardiac risk factors, such as hypertension and diabetes, and prevent disease through education toward a smoke-free

TABLE 158.2 **RACD Is a Spectrum of Deleterious Effects, Ranging From Preclinical Findings to Symptomatic Clinical Disease**

Component of the Heart	Effects of Radiation Therapy	Examples of Clinical Sequelae
Myocardium	Myocardial edema, myocardial inflammation, myocardial fibrosis	Cardiomyopathy, systolic dysfunction, diastolic dysfunction, congestive heart failure
Heart valves	Valvular, subvalvular, annular, and aortic-mitral curtain thickening and/or calcification restricted leaflet mobility, reduced size of valve orifice	Valve regurgitation, valve stenosis, volume, or pressure overload
Pericardium	Pericardial thickening, pericardial calcification, pericardial constriction	Pericarditis (acute or chronic), pericardial effusion, chronic pericardial disease, cardiac tamponade
Coronary arteries	Myofibroblast replacement and enhanced platelet deposition, plaque rupture	Coronary artery disease, accelerated atherosclerosis, coronary stenosis, angina pectoris, ischemic heart disease, myocardial infarction, resting and inducible regional wall abnormalities
Vascular	Narrowing of capillary lumens, thrombosis rupture of vessel walls, damage to endothelial cell membranes, reduced capillary density	Reduced collateral flow, reduced vascular reserve, microvascular disease, myocardial infarction, systemic embolization
Conduction system	Fibrosis of conduction pathways, compression from adjacent calcification	Conduction defects including arrhythmias, heart block, and sick sinus syndrome; autonomic dysfunction

From Desai MY, Jellis CL, Kotecha R, et al. Radiation-associated cardiac disease: a practical approach to diagnosis and management. *JACC Cardiovasc Imaging.* 2018;11(8):1132–1149.

and heart-healthy lifestyle. Smoking cessation and counseling have played critical roles in reducing risk. It is also important to identify prior risk factors of heart disease in cancer patients before administering thoracic radiation therapy. This intervention includes smoking cessation and treatment of hypertension, hyperlipidemia, and diabetes.[54]

CARDIAC CONSIDERATIONS DURING HEMATOPOIETIC STEM CELL TRANSPLANTATION

Hematopoietic stem cell transplantation (HSCT) is a potential cure for various hematologic malignancies with the number of survivors having undergone HSCT projected to reach 500,000 patients by the year 2030. It involves administration of myelosuppressive chemotherapy with or without total body irradiation (TBI) followed by infusion of a hematopoietic stem cell graft that induces immunologic response on malignant cells. The preparative regimen and the immune response are the major sources of treatment-related toxicity.[55]

Cardiovascular disease and cardiovascular complications are one of the most common complications associated with HSCT, reported in 0.9% to 43% of patients. These complications can occur acutely within the first 100 days or several years after the initial transplantation period. These cardiotoxicities include cardiomyopathy, arrhythmias, ischemic events, CHF, pericarditis, tamponade, and cardiac arrest. Many factors, including cyclophosphamide, anthracyclines, TBI, prior mediastinal radiotherapy, and transfusion-associated iron overload, increase the risk of post-HSCT cardiac complications.

Acute Cardiac Complications

Studies have shown that the overall incidence of acute cardiotoxicity during HCT is moderate (0.9% to 8.9% range) depending on the study.

A median of 5.2% (4% to 9.4% range) of persons receiving HSCT develop arrhythmias, including AF, atrial flutter, and supraventricular tachycardia. Risk factors are advanced age, obesity, prior anthracycline use, lower ejection fraction at baseline, prior history of arrhythmias (especially AF), renal dysfunction, chronic CAD, and acute weight gain of greater than 7% over baseline due to fluid overload during HSCT. Ventricular arrhythmias are rare, occurring in less than 1% of recipients. Developing post-transplant arrhythmias is also predictive of longer periods of hospitalization, increased likelihood of needing intensive care, and an increased risk of death (OR 3.5) at 1-year post-transplant.[56]

Other cardiac complications include congestive heart failure. Most preparative regimens for allogeneic HSCT use cyclophosphamide (CY). High-dose CY can induce myocardial necrosis which clinically presents with dyspnea, tachycardia, hypotension, decreased QRS voltage on electrocardiogram, and pericardial effusion within 10 days of drug administration. The histology shows a unique pattern of fibrin microthrombi in capillaries, fibrin strands in the interstitium, and fibrin strands in myocytes with higher rates of heart failure in those receiving doses $\geq 170\ \mathrm{mg/m^2}$. However, with reduced CY dose employed in contemporary regimens, heart failure appears less frequent (<2%).

Reduced intensity conditioning (RIC) regimens that spare some of the toxicity of a myeloablative preparative regimen have been developed in recent years. Melphalan and fludarabine are agents used in some RIC regimens and have been reported to cause cardiovascular complications. Melphalan has been associated with AF, atrial flutter, and supraventricular tachycardia in up to 9% of cases. The combination of melphalan and fludarabine has been reported in some case series to cause heart failure ranging in severity from minor and reversible systolic dysfunction to fatal heart failure.

The development of RIC made HSCT a treatment option for patients with comorbidities and who are older. Now patients with a cardiac comorbidity, defined as the presence of coronary artery disease, congestive heart failure, myocardial infarction, or an ejection fraction greater than 50% are considered as having a low-risk comorbidity. Furthermore, recent data suggests that those patients with an ejection fraction less than 50% are still eligible for HSCT and patients with borderline left ventricular systolic dysfunction can safely undergo HSCT without alterations in overall survival or treatment-related complications.[57]

Long-Term Cardiac Complications

Individuals who survive the first 100 days following HSCT remain at an increased risk for cardiovascular complications many years after transplantation. Ten-year cumulative incidence of hypertension is 37.7%, diabetes (18.1%), hyperlipidemia (46.7%), and having more than one of these cardiac risk factors at 31.4%. Most of these risk factors are seen in allogeneic HSCT survivors with the risk being highest in older and obese patients and those with a history of acute graft-versus-host disease. The risk of cardiovascular anthracycline-induced cardiomyopathy complications in autologous HSCT survivors, however, also remains of concern with the cumulative incidence of HF at 4.8% 5 years after HSCT and increasing to 9.1% at 15 years. Overall, this cardiovascular risk is 4.5-fold higher than that seen in the general population. Prior anthracycline use and chest radiation also increases this risk.[58]

Evaluation of cardiac systolic function by multiple gated acquisition scan (MUGA), echocardiogram, or cMR is a recommended part of a pre-transplantation evaluation. Attention to any signs of left ventricular dysfunction or premature supraventricular complexes on screening electrocardiogram should be reviewed carefully; consideration of preventative therapy with beta-blockers and angiotensin-converting enzyme (ACE) inhibitors should be considered in this population, given their higher risk for arrhythmias during transplantation. Referral to a cardio-oncology clinic, for those with any cardiovascular risk factors or prior cardiac disease or arrhythmias, is recommended.

Preventive and therapeutic strategies for cardiovascular complications in the HSCT population are poorly studied. To our knowledge there has only been one randomized controlled trial (OVERCOME trial) evaluating the benefit of cardioprotective strategies in patients undergoing HCT; however, this study only looked at autologous HCT patients with a short-term follow-up of 6 months. This study reported that ACE inhibitors and beta-blockers might prevent against LVEF reduction in patients with hematologic malignancies receiving intensive chemotherapy.[59]

Lifestyle modifications should be encouraged in patients who are undergoing HSCT, including abstinence from tobacco use, low fat diet, regular exercise, control of diabetes, baseline testing of lipids, electrocardiogram screening, and imaging with transthoracic echocardiography.

CONCLUSION

Contemporary anticancer therapies, particularly stem cell transplantation and immunotherapy, including ICI and chimeric antigen receptor (CAR) T-cell therapy, have changed the landscape of treatment for patients with hematological malignancies. However, each of these emerging interventions along with traditional chemotherapeutics, including anthracyclines and the spectrum of tyrosine kinase inhibitors, are associated with serious cardiovascular-adverse effects. The prevention and treatment of cardiotoxicities resulting from each of these therapies is variable, and studies validating these are still lacking robust data. Regardless, the identification of high-risk patients with preexisting cardiovascular risk factors and disease is important, and pre-treatment control of these risk factors and emerging imaging and biomarker modalities are the cornerstone of reduction of cardiac complications of hematological treatments.

SUGGESTED READINGS

The full Reference list is available at Elsevier eBooks for Practicing Clinicians.

Alvi RM, Frigault MJ, Fradley MG, et al. Cardiovascular events among adults treated with chimeric antigen receptor T-cells (CAR-T). *J Am Coll Cardiol*. 2019;74(25):3099–3108.

Asnani A, Manning A, Mansour M, et al. Management of atrial fibrillation in patients taking targeted cancer therapies. *Cardiooncology*. 2017;3:2.

Bosch X, Rovira M, Sitges M, et al. Enalapril and carvedilol for preventing chemotherapy-induced left ventricular systolic dysfunction in patients with malignant hemopathies: the OVERCOME trial (preventiOn of left Ventricular dysfunction with Enalapril and caRvedilol in patients submitted to intensive ChemOtherapy for the treatment of Malignant hEmopathies). *J Am Coll Cardiol*. 2013;61(23):2355–2362.

Brahmer JR, Lacchetti C, Schneider BJ, et al. Management of immune-related adverse events in patients treated with immune checkpoint inhibitor therapy: American Society of Clinical Oncology Clinical Practice Guideline. *J Clin Oncol*. 2018;36(17):1714–1768.

Bronte E, et al. Cardiotoxicity mechanisms of the combination of BRAF-inhibitors and MEK-inhibitors. *Pharmacol Ther*. 2018;192:65–73.

Curigliano G, et al. Management of cardiac disease in cancer patients throughout oncological treatment:ESMO consensus recommendations. *Ann Oncol*. 2020;31(2):171–190.

Desai MY, Jellis CL, Kotecha R, et al. Radiation-associated cardiac disease: a practical approach to diagnosis and management. *JACC Cardiovasc Imaging*. 2018;11(8):1132–1149.

Ganatra S, Carver JR, Hayek SS, et al. Chimeric antigen receptor T-cell therapy for cancer and heart: JACC Council Perspectives. *J Am Coll Cardiol*. 2019;74(25):3153–3163.

Herrmann J. Common vascular toxicities of cancer therapies. *Cardiol Clin*. 2019;37(4):365–384.

Jaiswal S, et al. Clonal hematopoiesis and risk of atherosclerotic cardiovascular disease. *N Engl J Med*. 2017;377(2):111–121.

Li W, et al. Vascular and metabolic implications of novel targeted cancer therapies: focus on kinaseminhibitors. *J Am Coll Cardiol*. 2015; 66(10):1160–1178.

Libby P, et al. Clonal hematopoiesis: crossroads of aging, cardiovascular disease, and cancer: JACC review topic of the week. *J Am Coll Cardiol*. 2019;74(4):567–577.

Mincu RI, et al. Cardiovascular adverse events associated with BRAF and MEK inhibitors: a systematicn review and meta-analysis. *JAMA Netw Open*. 2019; 2(8):e198890.

Plana JC, et al. Multi-modality imaging in the assessment of cardiovascular toxicity in the cáncer patient. *JACC Cardiovasc Imaging*. 2018;11(8): 1173–1186.

Plana JC, Galderisi M, Barac A, et al. Expert consensus for multimodality imaging evaluation of adult patients during and after cancer therapy: a report from the American Society of Echocardiography and the European Association of Cardiovascular Imaging. *J Am Soc Echocardiogr*. 2014;27(9): 911–939.

Postow MA, Sidlow R, Hellmann MD. Immune-related adverse events associated with immune checkpoint blockade. *N Engl J Med*. 2018;378(2):158–168.

Tonorezos ES, Stillwell EE, Calloway JJ, et al. Arrhythmias in the setting of hematopoietic cell transplants. *Bone Marrow Transplant*. 2015;50(9): 1212–1216.

Touyz RM, et al. Vascular toxicities with VEGF inhibitor therapies-focus on hypertension and arterial thrombotic events. *J Am Soc Hypertens*. 2018;12(6): 409–425.

Tuzovic M, Mead M, Young PA, et al. Cardiac complications in the adult bone marrow transplant patient. *Curr Oncol Rep*. 2019;21(3):28.

Vineetha VP, Raghu KG. An overview on arsenic trioxide-induced cardiotoxicity. *Cardiovasc Toxicol*. 2019;19(2):105–119.

Zhang L, Zlotoff DA, Awadalla M, et al. Major adverse cardiovascular events and the timing and dose of corticosteroids in immune checkpoint inhibitor-associated myocarditis. *Circulation*. 2020;141(24):2031–2034.

REFERENCES

1. Plana JC, Galderisi M, Barac A, et al. Expert consensus for multimodality imaging evaluation of adult patients during and after cancer therapy: a report from the American Society of Echocardiography and the European Association of Cardiovascular Imaging. *J Am Soc Echocardiogr*. 2014;27(9): 911–939. https://doi.org/10.1016/j.echo.2014.07.012.
2. Grenier MA, Lipshultz SE. Epidemiology of anthracycline cardiotoxicity in children and adults. *Semin Oncol*. 1998;25(4 Suppl 10):72–85.
3. Lipshultz SE, Alvarez JA, Scully RE. Anthracycline associated cardiotoxicity in survivors of childhood cancer. *Heart*. 2008;94(4):525–533. https://doi.org/10.1136/hrt.2007.136093.
4. Wouters KA, Kremer LC, Miller TL, Herman EH, Lipshultz SE. Protecting against anthracycline-induced myocardial damage: a review of the most promising strategies. *Br J Haematol*. 2005;131:561–578.
5. Curigliano G, Mayer EL, Burstein HJ, Winer EP, Goldhirsch A. Cardiac toxicity from systemic cancer therapy: a comprehensive review. *Prog Cardiovasc Dis*. 2010;53(2):94–104. https://doi.org/10.1016/j.pcad.2010.05.006.
6. Zhang S, Liu X, Bawa-Khalfe T, et al. Identification of the molecular basis of doxorubicin-induced cardiotoxicity. *Nat Med*. 2012;18(11):1639–1642. https://doi.org/10.1038/nm.2919.
7. Vejpongsa P, Yeh ET. Topoisomerase 2β: a promising molecular target for primary prevention of anthracycline-induced cardiotoxicity. *Clin Pharmacol Ther*. 2014;95(1):45–52. https://doi.org/10.1038/clpt.2013.201.
8. Lyu YL, Kerrigan JE, Lin CP, et al. Topoisomerase II beta mediated DNA double-strand breaks: implications in doxorubicin cardiotoxicity and prevention by dexrazoxane. *Cancer Res*. 2007;67(18):8839–8846. https://doi.org/10.1158/0008-5472.CAN-07-1649.
9. Elghazawy H, Venkatesulu BP, Verma V, et al. The role of cardio-protective agents in cardio-preservation in breast cancer patients receiving anthracyclines ± trastuzumab: a meta-analysis of clinical studies. *Crit Rev Oncol Hematol*. 2020;153:103006. https://doi.org/10.1016/j.critrevonc.2020.103006.
10. Valcovici M, Andrica F, Serban C, Dragan S. Cardiotoxicity of anthracycline therapy: current perspectives. *Arch Med Sci*. 2016;12(2):428–435. https://doi.org/10.5114/aoms.2016.59270.
11. Li W, Croce K, Steensma DP, McDermott DF, Ben-Yehuda O, Moslehi J. Vascular and metabolic implications of novel targeted cancer therapies: focus on kinase inhibitors. *J Am Coll Cardiol*. 2015;66(10):1160–1178. https://doi.org/10.1016/j.jacc.2015.07.025.
12. Perik PJ, Rikhof B, de Jong FA, Verweij J, Gietema JA, van der Graaf WT. Results of plasma N-terminal pro B-type natriuretic peptide and cardiac troponin monitoring in GIST patients do not support the existence of imatinib-induced cardiotoxicity. *Ann Oncol*. 2008;19(2):359–361. https://doi.org/10.1093/annonc/mdm468.
13. Kerkelä R, Grazette L, Yacobi R, et al. Cardiotoxicity of the cancer therapeutic agent imatinib mesylate. *Nat Med*. 2006;12(8):908–916. https://doi.org/10.1038/nm1446.
14. Masuda S, Nakano K, Funakoshi K, et al. Imatinib mesylate-incorporated nanoparticle-eluting stent attenuates in-stent neointimal formation in porcine coronary arteries. *J Atheroscler Thromb*. 2011;18(12):1043–1053. https://doi.org/10.5551/jat.8730.
15. Lassila M, Allen TJ, Cao Z, et al. Imatinib attenuates diabetes-associated atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2004;24(5):935–942. https://doi.org/10.1161/01.ATV.0000124105.39900.db.
16. Giles FJ, Mauro MJ, Hong F, et al. Rates of peripheral arterial occlusive disease in patients with chronic myeloid leukemia in the chronic phase treated with imatinib, nilotinib, or non-tyrosine kinase therapy: a retrospective cohort analysis. *Leukemia*. 2013;27(6):1310–1315. https://doi.org/10.1038/leu.2013.69.
17. Ghofrani HA, Seeger W, Grimminger F. Imatinib for the treatment of pulmonary arterial hypertension. *N Engl J Med*. 2005;353(13):1412–1413. https://doi.org/10.1056/NEJMc051946.
18. Montani D, Bergot E, Günther S, et al. Pulmonary arterial hypertension in patients treated by dasatinib. *Circulation*. 2012;125(17):2128–2137.
19. Young-Woo Jeon S-EL, Soo-Hyun Kim, et al. Six-year follow-up of dasatinib-related pulmonary arterial hypertension (PAH) for chronic myeloid leukemia in single center. Paper presented at: 55th ASH Annual Meeting and Exposition; December 7– 10, 2013; New Orleans, LA.
20. de Lavallade H, Punnialingam S, Milojkovic D, et al. Pleural effusions in patients with chronic myeloid leukaemia treated with dasatinib may have an immune-mediated pathogenesis. *Br J Haematol*. 2008;141(5):745–747. https://doi.org/10.1111/j.1365-2141.2008.07108.x.
21. Lu Z, Wu CY, Jiang YP, et al. Suppression of phosphoinositide 3-kinase signaling and alteration of multiple ion currents in drug-induced long QT syndrome. *Sci Transl Med*. 2012;4(131):131ra50. https://doi.org/10.1126/scitranslmed.3003623.
22. Le Coutre P, Rea D, Abruzzese E, et al. Severe peripheral arterial disease during nilotinib therapy. *J Natl Cancer Inst*. 2011;103(17):1347–1348. https://doi.org/10.1093/jnci/djr292.
23. Kim TD, Rea D, Schwarz M, et al. Peripheral artery occlusive disease in chronic phase chronic myeloid leukemia patients treated with nilotinib or imatinib. *Leukemia*. 2013;27(6):1316–1321. https://doi.org/10.1038/leu.2013.70.
24. Racil Z, Razga F, Drapalova J, et al. Mechanism of impaired glucose metabolism during nilotinib therapy in patients with chronic myelogenous leukemia. *Haematologica*. 2013;98(10):e124–e126. https://doi.org/10.3324/haematol.2013.086355.
25. Yoshizato T, Nannya Y, Yoshiki Y, et al. Nilotinib-induced hypothyroidism in a patient with chronic myeloid leukemia. *Int J Hematol*. 2011;93(3): 400–402. https://doi.org/10.1007/s12185-011-0790-2.
26. Hagop M, Kantarjian D-WK, Pinilla-Ibarz J, et al. Ponatinib (PON) in patients (pts) with Philadelphia chromosome-positive (Phþ) leukemias resistant or intolerant to dasatinib or niloti-nib, or with the T315I mutation: longer-term follow up of the PACE trial. Paper presented at: 50th ASCO Annual Meeting; May 30 to June 3, 2014; Chicago, IL.
27. Price KE, Saleem N, Lee G, Steinberg M. Potential of ponatinib to treat chronic myeloid leukemia and acute lymphoblastic leukemia. *Onco Targets Ther*. 2013;6:1111–1118. https://doi.org/10.2147/OTT.S36980. Published 2013 Aug 20.
28. Loren CP, Aslan JE, Rigg RA, et al. The BCR-ABL inhibitor ponatinib inhibits platelet immunoreceptor tyrosine-based activation motif (ITAM) signaling, platelet activation and aggregate formation under shear. *Thromb Res*. 2015;135(1):155–160. https://doi.org/10.1016/j.thromres.2014.11.009.
29. Kapelios CJ, Bonou MS, Diamantopoulos P, et al. Ibrutinib-related atrial fibrillation: therapeutic challenges. *J Oncol Pharm Pract*. 2019;25(5): 1258–1260. https://doi.org/10.1177/1078155218785983.
30. Leong DP, Caron F, Hillis C, et al. The risk of atrial fibrillation with ibrutinib use: a systematic review and meta-analysis. *Blood*. 2016;128(1): 138–140. https://doi.org/10.1182/blood-2016-05-712828.
31. Asnani A, Manning A, Mansour M, Ruskin J, Hochberg EP, Ptaszek LM. Management of atrial fibrillation in patients taking targeted cancer therapies. *Cardiooncology*. 2017;3:2 https://doi.org/10.1186/s40959-017-0021-y. Published 2017 Mar 9.
32. Drolet B, Simard C, Roden DM. Unusual effects of a QT-prolonging drug, arsenic trioxide, on cardiac potassium currents. *Circulation*. 2004;109(1): 26–29. https://doi.org/10.1161/01.CIR.0000109484.00668.CE.
33. Mumford JL, Wu K, Xia Y, et al. Chronic arsenic exposure and cardiac repolarization abnormalities with QT interval prolongation in a population-based study. *Environ Health Perspect*. 2007;115(5):690–694. https://doi.org/10.1289/ehp.9686.
34. Vineetha VP, Raghu KG. An overview on arsenic trioxide-induced cardiotoxicity. *Cardiovasc Toxicol*. 2019;19(2):105–119. https://doi.org/10.1007/s12012-018-09504-7.
35. Balanescu DV, Donisan T, Palaskas N, et al. Immunomodulatory treatment of immune checkpoint inhibitor-induced myocarditis: pathway toward precision-based therapy. *Cardiovasc Pathol*. 2020;47:107211. https://doi.org/10.1016/j.carpath.2020.107211.
36. Tawbi HA, Forsyth PA, Algazi A, et al. Combined nivolumab and ipilimumab in melanoma metastatic to the brain. *N Engl J Med*. 2018;379(8): 722–730. https://doi.org/10.1056/NEJMoa1805453.
37. Larkin J, Chiarion-Sileni V, Gonzalez R, et al. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *N Engl J Med*. 2015;373(1):23–34. https://doi.org/10.1056/NEJMoa1504030. [published correction appears in N Engl J Med. 2018 Nov 29;379(22):2185].
38. Postow MA, Sidlow R, Hellmann MD. Immune-related adverse events associated with immune checkpoint blockade. *N Engl J Med*. 2018;378(2): 158–168. https://doi.org/10.1056/NEJMra1703481.
39. Brahmer JR, Lacchetti C, Schneider BJ, et al. Management of immune-related adverse events in patients treated with immune checkpoint inhibitor therapy: American Society of Clinical Oncology Clinical Practice Guideline. *J Clin Oncol*. 2018;36(17):1714–1768. https://doi.org/10.1200/JCO.2017.77.6385.
40. Mir H, Alhussein M, Alrashidi S, et al. Cardiac complications associated with checkpoint inhibition: a systematic review of the literature in an important emerging area. *Can J Cardiol*. 2018;34(8):1059–1068. https://doi.org/10.1016/j.cjca.2018.03.012.
41. Zhang L, Awadalla M, Mahmood SS, et al. Cardiovascular magnetic resonance in immune checkpoint inhibitor-associated myocarditis. *Eur Heart J*. 2020;41(18):1733–1743. https://doi.org/10.1093/eurheartj/ehaa051.

42. Zhang L, Zlotoff DA, Awadalla M, et al. Major Adverse cardiovascular events and the timing and dose of corticosteroids in immune checkpoint inhibitor-associated myocarditis. *Circulation*. 2020;141(24):2031–2034. https://doi.org/10.1161/CIRCULATIONAHA.119.044703.
43. Sadelain M, Brentjens R, Rivière I. The basic principles of chimeric antigen receptor design. *Cancer Discov*. 2013;3(4):388–398. https://doi.org/10.1158/2159-8290.CD-12-0548.
44. Ganatra S, Carver JR, Hayek SS, et al. Chimeric antigen receptor T-cell therapy for cancer and heart: JACC Council Perspectives. *J Am Coll Cardiol*. 2019;74(25):3153–3163. https://doi.org/10.1016/j.jacc.2019.10.049.
45. Ganatra S, Redd R, Hayek SS, et al. Chimeric antigen receptor T-cell therapy-associated cardiomyopathy in patients with refractory or relapsed non-Hodgkin lymphoma. *Circulation*. 2020;142(17):1687–1690. https://doi.org/10.1161/CIRCULATIONAHA.120.048100.
46. Fitzgerald JC, Weiss SL, Maude SL, et al. Cytokine release syndrome after chimeric antigen receptor T cell therapy for acute lymphoblastic leukemia. *Crit Care Med*. 2017;45(2):e124–e131. https://doi.org/10.1097/CCM.0000000000002053.
47. Alvi RM, Frigault MJ, Fradley MG, et al. Cardiovascular events among adults treated with chimeric antigen receptor T-cells (CAR-T). *J Am Coll Cardiol*. 2019;74(25):3099–3108. https://doi.org/10.1016/j.jacc.2019.10.038.
48. Asnani A. Cardiotoxicity of immunotherapy: incidence, diagnosis, and management. *Curr Oncol Rep*. 2018;20(6):44 https://doi.org/10.1007/s11912-018-0690-1. Published 2018 Apr 11.
49. Porter DL, Hwang WT, Frey NV, et al. Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. *Sci Transl Med*. 2015;7(303):303ra139. https://doi.org/10.1126/scitranslmed.aac5415.
50. Lee DW, Gardner R, Porter DL, et al. Current concepts in the diagnosis and management of cytokine release syndrome. *Blood*. 2015 Aug 20;126(8): 1048 https://doi.org/10.1182/blood-2014-05-552729. Dosage error in article text] [published correction appears in *Blood*. 2016 Sep 15;128(11):1533]. *Blood*. 2014;124(2):188-195.
51. Cameron BJ, Gerry AB, Dukes J, et al. Identification of a Titin-derived HLA-A1-presented peptide as a cross-reactive target for engineered MAGE A3-directed T cells. *Sci Transl Med*. 2013;5(197):197ra103. https://doi.org/10.1126/scitranslmed.3006034.
52. Riegler LL, Jones GP, Lee DW. Current approaches in the grading and management of cytokine release syndrome after chimeric antigen receptor T-cell therapy. *Ther Clin Risk Manag*. 2019;15:323–335. https://doi.org/10.2147/TCRM.S150524. Published 2019 Feb 28.
53. Desai MY, Jellis CL, Kotecha R, Johnston DR, Griffin BP. Radiation-associated cardiac disease: a practical approach to diagnosis and management. *JACC Cardiovasc Imaging*. 2018;11(8):1132–1149. https://doi.org/10.1016/j.jcmg.2018.04.028.
54. Hufnagle JJ, Goyal A. *Radiation therapy induced cardiac toxicity*. StatPearls. Treasure Island (FL): StatPearls Publishing; May 16, 2020.
55. Tuzovic M, Mead M, Young PA, Schiller G, Yang EH. Cardiac complications in the adult bone marrow transplant patient. *Curr Oncol Rep*. 2019;21(3):28 https://doi.org/10.1007/s11912-019-0774-6. Published 2019 Mar 2.
56. Tonorezos ES, Stillwell EE, Calloway JJ, et al. Arrhythmias in the setting of hematopoietic cell transplants. *Bone Marrow Transplant*. 2015;50(9): 1212–1216. https://doi.org/10.1038/bmt.2015.127.
57. Hurley P, Konety S, Cao Q, Weisdorf D, Blaes A. Hematopoietic stem cell transplantation in patients with systolic dysfunction: can it be done? *Biol Blood Marrow Transplant*. 2015;21(2):300–304. https://doi.org/10.1016/j.bbmt.2014.10.011.
58. Chow EJ, Mueller BA, Baker KS, et al. Cardiovascular hospitalizations and mortality among recipients of hematopoietic stem cell transplantation. *Ann Intern Med*. 2011;155(1):21–32. https://doi.org/10.7326/0003-4819-155-1-201107050-00004.
59. Bosch X, Rovira M, Sitges M, et al. Enalapril and carvedilol for preventing chemotherapy-induced left ventricular systolic dysfunction in patients with malignant hemopathies: the OVERCOME trial (preventiOn of left Ventricular dysfunction with Enalapril and caRvedilol in patients submitted to intensive ChemOtherapy for the treatment of Malignant hEmopathies). *J Am Coll Cardiol*. 2013;61(23):2355–2362. https://doi.org/10.1016/j.jacc.2013.02.072.

CHAPTER 159

RESOURCES FOR THE HEMATOLOGIST: INTERPRETIVE COMMENTS AND SELECTED REFERENCE VALUES FOR NEONATAL, PEDIATRIC, AND ADULT POPULATIONS

Andrea N. Marcogliese and Lisa Hensch[a]

APPENDIX CONTENTS

Red Blood Cell Testing

- Estimated Blood Volumes (Plasma Volume and Red Blood Cell Mass)
- Complete Blood Count (Red Blood Cell) Parameters
- Red Blood Cell Distribution Width
- Reticulocyte Count (Absolute and Percentage)
- Immature Reticulocyte Fraction
- Reticulocyte Hemoglobin Equivalent
- Haptoglobin
- Plasma Hemoglobin
- Hemoglobin F and Hemoglobin A2
- Sickle Cell Screen (Hemoglobin S Solubility Test)
- Ferritin
- Total Iron-Binding Capacity, Transferrin Saturation
- Iron
- Erythropoietin
- Folate (RBC or Serum/Plasma)
- Vitamin B_{12} (Cobalamin)
- Homocysteine
- Methylmalonic Acid
- Osmotic Fragility
- Red Cell Glycolytic Intermediate Metabolites in Normal Adults, Term Infants, and Premature Infants

White Blood Cell and Immunologic Testing

- Leukocyte Counts (Absolute and Percentage)
- Lymphocyte Subsets
- Immunoglobulin A
- Immunoglobulin E
- Immunoglobulin G
- Immunoglobulin G Subclasses
- Immunoglobulin M
- Immunoglobulin Light Chains
- Complement Fractions C3 and C4

Platelet and Coagulation Testing

- Platelet Count and Mean Platelet Volume
- Immature Platelet Fraction
- Prothrombin Time/International Normalized Ratio (PT/INR)
- Activated Partial Thromboplastin Time (aPTT)
- Fibrinogen
- Mixing Studies
- Lupus Anticoagulant
- Coagulation Factor Levels (II, V, VII, VIII, IX, X, XI, XII, Contact Pathway Factors)
- Bethesda Assay/Nijmegen-Bethesda Assay
- von Willebrand Antigen/Activity
- Natural Anticoagulants (Antithrombin, Protein C, Protein S, Tissue Factor Pathway Inhibitor)
- D-dimer
- Anti-Xa Assay
- PFA-100
- Platelet Aggregometry

This appendix is intended to provide useful supplemental information to clinicians in selecting, obtaining, and interpreting certain key laboratory tests relevant to hematology. Reference values are given in Tables and interpretive comments appear in associated Boxes. The most appropriate reference range for a particular test is dependent on the specific testing methods employed (including reagents and instrumentation), patient age and gender, testing conditions, preanalytical variables, and other concurrent medical conditions and is determined and validated by the laboratory performing the test. The listed reference ranges thus provide only a rough guide to differentiating normal from abnormal results. Clinicians are encouraged to seek out the most applicable and appropriate reference range for a particular test, preferably using reference data that are specific to the performing laboratory and the patient population being tested. Although the tables herein provide some age-specific information, caution is particularly emphasized in interpreting results obtained from testing infants and children.

RED BLOOD CELL TESTING

Estimated Blood Volumes (Includes Plasma Volume, Red Blood Cell Mass)

The red cell volume, plasma volume, and total blood volume are calculated from hematocrit (packed cell volume) and isotope dilution after reinjection of red cells labeled with a radioactive isotope. Performance of a red cell mass determination is occasionally indicated in the investigation of polycythemia to differentiate primary or secondary polycythemia (elevated red cell mass) from relative polycythemia (normal red cell mass). Table 159.1 provides typical reference ranges.

Total blood volume can be roughly estimated using the following:
Preterm Neonates: 100 mL/kg
Term Neonates: 85 mL/kg
Infants: 80 mL/kg
Adults: 70 mL/kg

Total blood volume can also be estimated by calculation[b]:
Total Blood Volume for Males = $[0.3669 \times (\text{Height in Meters})^3] + [0.03219 \times (\text{Weight in Kilograms})] + 0.6041$;
Total Blood Volume for Females = $[0.3561 \times (\text{Height in Meters})^3] + [0.03308 \times (\text{Weight in Kilograms})] + 0.1833$.

---

[a] Previously collated by Donald L. Yee, Catherine M. Bollard, and Sharon M. Geaghan.

[b] From Nadler SB, Hidalgo JU, Bloch T. Prediction of blood volume in normal human adults. *Surgery*. 1962;51:224,.

TABLE 159.1 Estimated Blood Volumes

			Total Blood Volume (mL/kg)	
Age	**Plasma Volume (mL/kg)**	**Red Blood Cell Mass[a] (mL/kg)**	**From Plasma Volume**	**From Red Blood Cell Mass**
Newborn	41.3 M 46.0 F	43.1 M	82.1 M 78.0 F	86.1M 84.7 F
1–7 days	51–54	37.9	82–86	77.8
1–12 months	46.1	25.5	78.1	72.8
1–3 years	44.4 M 47.2 F	24.9	73.8 M 81.8 F	69.1
4–6 years	48.5 M 49.6 F	25.5	80.0 M 85.6 F	67.5
7–9 years	52.2 M 49.0 F	24.3	87.6 M 86.1 F	67.5
10–12 years M	51.9 M 46.2 F	26.3	87.6 M 83.2 F	67.4
13–15 years	51.2		88.3	
16–18 years	50.1		90.2	
Adults	39–50	20–35	68–88	55–75

[a] Red cell volume is approximately 5 mL/kg less in females compared with males. Increases in plasma volume, red cell volume, and overall blood volume are seen in residents living at high altitude compared to those living at sea level. Red cell volume may be increased up to 80% in residents living at high altitudes (4500 meters) compared to those living at sea level.*

Reprinted by permission of the Society of Nuclear Medicine from Price DC, Ries C. Appendix. In: Handmaker H, Lowenstein JM, eds. *Nuclear Medicine in Clinical Pediatrics*. New York: Society of Nuclear Medicine; 1975:279. Red cell volume reference from International Committee for Standardization in Haematology: recommended methods for measurement of red-cell and plasma volume. *J Nucl Med.* 1980;21:793.

*Heinicke K, Prommer N, Cajigal J, et al. Long-term exposure to intermittent hypoxia results in increased hemoglobin mass, reduced plasma volume, and elevated erythropoietin plasma levels in man. *Eur J Appl Physiol.* 2003;88(6):535–543. F, Female; M, male.

COMPLETE BLOOD COUNT (RED BLOOD CELL) PARAMETERS

Summary points	• Used to identify anemia or polycythemia (RBC count, hemoglobin, hematocrit) • RBC indices (MCV, MCH, MCHC, RDW) can characterize anemia according to RBC size and degrees of hemoglobinization and anisopoikilocytosis
Methodology	Automated hematology analyzer—flow cytometric or impedance methods
Specimen requirements	Whole blood: EDTA
Indications	Evaluation of anemia or polycythemia
Reference range	Tables 159.2, 159.2A and 159.2B

Interpretation	• Anemia (low hemoglobin, hematocrit, RBC count)
	  • Microcytic, hypochromic: iron deficiency (often with high RDW), thalassemia (typically normal RDW but can be high, high RBC count), sideroblastic anemia (may be dimorphic RBCs on peripheral smear), some cases of anemia of chronic disease, hyperthyroidism
	  • Normocytic, normochromic: aplastic anemia, anemia of chronic disease, renal disease, hemolysis, bone marrow infiltration, drugs, lead poisoning, myelodysplastic syndrome
	  • Macrocytic: vitamin B_{12} or folate deficiency, liver disease, antimetabolite drugs, inherited bone marrow failure, some cases of aplastic anemia, myelodysplastic syndrome, hemolysis with high reticulocyte count, hypothyroidism, alcohol
	• Polycythemia (high hemoglobin, hematocrit)
	  • Primary: polycythemia vera, altered erythropoietin receptor
	  • Secondary: pulmonary disease, high-affinity hemoglobin, tumor related

Related tests	• Peripheral smear review, reticulocyte count, bone marrow aspiration/biopsy, tests of renal and hepatic function, hemoglobin fractionation/electrophoresis • Microcytic, hypochromic anemias: iron studies, lead level (children), hemoglobin fractionation/electrophoresis • Macrocytic anemias: vitamin B_{12}, folate, homocysteine, methylmalonic acid • Normocytic anemias: markers of hemolysis (reticulocyte count, haptoglobin, indirect bilirubin, LDH) • Polycythemia: family history, *JAK2* mutation testing, erythropoietin level
Interfering substances	Clotted or hemolyzed sample, cold agglutinin, lipemia, marked turbidity

EDTA, Ethylenediaminetetraacetic acid; *LDH*, lactate dehydrogenase; *MCH*, mean corpuscular hemoglobin; *MCHC*, mean corpuscular hemoglobin concentration; *MCV*, mean corpuscular volume; *RBC*, red blood cell; *RDW*, red blood cell distribution width.

TABLE 159.2 Reference Hematologic Values in Children and Adults

	Hemoglobin (g/dL)		Hematocrit (%)		Red Blood Cell Count (10^{12}/L)		MCV (fL)		MCH (pg)		MCHC (g/dL)	
Age	**Mean**	**−2 SD**	**Mean**	**−2 SD**	**Mean**	**−2 SD**	**Mean**	**−2 SD**	**Mean**	**−2 SD**	**Mean**	**−2 SD**
Birth (cord blood)	16.5	13.5	51	42	4.7	3.9	108	98	34	31	33	30
1–3 days (capillary)	18.5	14.5	56	45	5.3	4.0	108	95	34	31	33	29
1 weeks	17.5	13.5	54	42	5.1	3.9	107	88	34	28	33	28
2 weeks	16.5	12.5	51	39	4.9	3.6	105	86	34	28	33	28
1 months	14.0	10.0	43	31	4.2	3.0	104	85	34	28	33	29
2 months	11.5	9.0	35	28	3.8	2.7	96	77	30	26	33	29
3–6 months	11.5	9.5	35	29	3.8	3.1	91	74	30	25	33	30
0.5–2 years	12.0	10.5	36	33	4.5	3.7	78	70	27	23	33	30
2–6 years	12.5	11.5	37	34	4.6	3.9	81	75	27	24	34	31
6–12 years	13.5	11.5	40	35	4.6	4.0	86	77	29	25	34	31
12–18 years												
Female	14.0	12.0	41	36	4.6	4.1	90	78	30	25	34	31
Male	14.5	13.0	43	37	4.9	4.5	88	78	30	25	34	31
18–49 years												
Female	14.0	12.0	41	36	4.6	4.0	90	80	30	26	34	31
Male	15.5	13.5	47	41	5.2	4.5	90	80	30	26	34	31

These data have been compiled from several sources. Emphasis is given to studies employing electronic counters and to the selection of populations that are likely to exclude individuals with iron deficiency. The mean ±2 SDs can be expected to include 95% of the observations in a normal population. Note: Values for RBC, Hemoglobin, and Hematocrit are increased in residents living at higher altitudes. Values for hemoglobin are 11% higher for residents living at high altitudes, with corresponding increases in RBC and Hematocrit.*

MCH, Mean corpuscular hemoglobin; *MCHC*, mean corpuscular hemoglobin concentration; *MCV*, mean corpuscular volume; *SD*, standard deviation.

Data from Dallman PR. Blood-forming tissues. In: Rudolph A, ed. *Pediatrics.* 16th ed. New York: Appleton-Century-Crofts; 1977:1111.

*Heinicke K, Prommer N, Cajigal J, et al. Long-term exposure to intermittent hypoxia results in increased hemoglobin mass, reduced plasma volume, and elevated erythropoietin plasma levels in man. *Eur J Appl Physiol.* 2003;88(6):535–543

TABLE 159.2A Reference Hemoglobin Values (g/dL) in Children by Altitude

Altitude (meters)	**Age (months)**			
	6–11.9	**12–23.9**	**24–35.9**	**36–59.9**
0–999	8.53–13.43	8.66–13.66	9.08–14.04	9.34–14.18
1000–1999	9.21–13.73	9.37–13.85	9.85–14.01	10.19–14.19
2000–2999	9.58–14.5	9.82–14.66	10.29–14.93	10.62–15.14
3000–3999	9.78–15.62	10.04–15.76	10.58–15.94	10.92–16.12
≥4000	10.15–16.95	10.38–17.06	10.95–17.11	11.36–17.40

m, Hemoglobin was measured using a HemoCue H6201 DM Analyzer.

Data adapted from Ocas-Córdova S, Tapia V, Gonzales GF. Hemoglobin concentration in children at different altitudes in Peru: proposal for [Hb] correction for altitude to diagnose anemia and polycythemia. *High Alt Med Biol.* 2018;19(4):398–403

TABLE 159.2B Reference Hemoglobin and Hematocrit Values in Adults at High Altitude (4000 meters)

	Male	**Female**
Hemoglobin (g/dL)	13–21	12–19
Hematocrit (%)	45–61	41–56

Data from Vásquez R, Villena M. Normal hematological values for healthy persons living at 4000 meters in Bolivia. *High Alt Med Biol.* 2001;2(3):361–367

RED BLOOD CELL DISTRIBUTION WIDTH

Summary points	• Measures variability of red blood cell size (anisocytosis) • Reported as either CV, SD, or both
Methodology	Automated hematology analyzer
Specimen requirements	Whole blood: EDTA
Indications	Evaluation of anemia
Reference range	RDW-CV: 11%–15% (based on adults)[a]

Interpretation	• Elevated with increased variability in red blood cell size and shape; normal with more uniform, red blood cell size
	• Hypochromic microcytic anemia
	  • Usually elevated with iron deficiency, hereditary sideroblastic anemia, thalassemia intermedia and major, and red blood cell fragmentation
	  • Normal with anemia of chronic disease and thalassemia minor
	• Normocytic or macrocytic anemia
	  • Usually elevated in folate or vitamin B_{12} deficiency, mixed iron and folate or vitamin B_{12} deficiency, some hemoglobinopathies, myelofibrosis, acquired sideroblastic anemia, immune hemolytic anemia, and cold agglutinins
	  • Usually normal in anemia of chronic disease, some hemoglobinopathies, hemorrhage, hereditary spherocytosis, and bone marrow failure
	• RDW-CV = (SD of red cell volume/MCV)(100) reported more commonly than the RDW-SD
	• RDW-SD is less influenced by MCV and may be a more accurate assessment of variability in red blood cell size
	• Reference range will vary with the instrument used—check with laboratory

Related tests	• RBC, Hgb, Hct, MCV, MCH, MCHC, peripheral blood smear
Interfering substances	• Hemolyzed or clotted specimen
References	• Briggs C. Quality counts: new parameters in blood cell counting. *Int J Lab Hematol*. 2009;31:277. • [a]Evans TC, Jehle D. The red blood cell distribution width. *J Emerg Med*. 1991;9:71.

CV, Coefficient of variation; *EDTA*, ethylenediaminetetraacetic acid; *Hgb*, hemoglobin; *Hct*, hematocrit; *MCH*, mean corpuscular hemoglobin; *MCHC*, mean corpuscular hemoglobin concentration; *MCV*, mean corpuscular volume; *RBC*, red blood cell count; *RDW*, red blood cell distribution width; *SD*, standard deviation.

RETICULOCYTE COUNT (ABSOLUTE AND PERCENTAGE)

Summary points	• Used to assess erythroid production • In the setting of anemia, can aid in distinguishing between destructive process (hemolysis) and marrow production problem
Methodology	Automated hematology analyzer, flow cytometry
Specimen requirements	Whole blood: EDTA
Indications	Differential diagnosis of anemia (destruction versus marrow production problem); evaluate marrow response to anemia; monitor response to therapy (treatment of nutritional anemias, post immunosuppressive therapy for aplastic anemia, post growth factor therapy, post chemotherapy, or bone marrow transplant)
Reference range	Table 159.3
Interpretation	• Elevated with marrow response to anemia, implying decreased RBC life span (hemolysis), marrow recovery (post injury, chemotherapy, or bone marrow transplant), or response to treatment (immunosuppressive therapy in aplastic anemia, nutritional replacement in iron, B_{12}, or folate deficiency) • Decreased (or inadequately elevated) in nutritional anemias, marrow suppression (infections, toxic exposures, malignancy), marrow failure • Corrected reticulocyte count (absolute reticulocyte count/reticulocyte maturation time) and RPI [reticulocyte percentage × (hematocrit/45) × (1/reticulocyte maturation time)] adjust for the degree of anemia and account for reticulocyte maturation time in assessing the appropriateness of the marrow responseAbsolute count is more meaningful than the percentage in some settings (aplastic anemia)
Related tests	Complete blood count, reticulocyte hemoglobin, immature reticulocyte fraction, mean reticulocyte volume, corrected reticulocyte count, RPI, direct antiglobulin test, markers of hemolysis
Interfering substances	Clotted or hemolyzed sample

EDTA, Ethylenediaminetetraacetic acid; *RBC*, red blood cell; *RPI*, reticulocyte production index.

TABLE 159.3 Reticulocyte Counts in Infants and Children by Flow Cytometry

Age	Number	Percentage	Absolute Count (10^9/L)
0–14 days[a]	21	5.33 + 1.99	318.44 + 50.04
		(1.61/8.28)	(238.9/404.1)
14 days–1 year[a]	32	2.03 + 0.52	89.00 + 22.94
		(1.09/3.00)	(45.8/137.8)
1–3 years[a]	63	2.01 + 0.72	88.38 + 20.49
		(1.11/5.00)	(50.2/141.0)
3–8 years[a]	60	2.19 + 0.66	98.83 + 27.97
		(0.95/4.03)	(45.4/163.1)
8–12 years[a]	18	2.07 + 0.78	90.20 + 27.16
		(1.18/3.78)	(54.8/154.4)
12–adult[a]	14	2.25 + 0.99	91.62 + 24.80
		(1.32/4.91)	(58.6/146.2)
Age	**Number**	**Percentage**	**Absolute Count (10^4/µL)**
Term newborns[b]	150	2.44 + 1.15	7.42 + 2.50
		(1.1/5.5)	(4.9/15.0)
Preterm newborns[b]	30	2.55 + 1.01	7.18 + 2.88
		(0.9/6.5)	(4.0/17.1)
1 week–16 years[b]	750	1.16 + 0.39	5.87 + 1.23
		(0.6/1.9)	(5.0/6.5)

Note: Flow cytometry may yield results 0.5%–1.0% higher than microscopy following supravital staining.

[a] Data from 188 pediatric outpatients (0–18 years of age) for whom all hematologic parameters are within age-appropriate reference ranges. Unpublished data from Lucile Packard Children's Hospital at Stanford. Analysis was performed on the Becton Dickinson FACSCalibur flow cytometer. Results are expressed as mean +1 SD (min/max).

[b] Data from Herkner KR, Bock A, Muhl A, et al. Reticulocyte counting and maturity grading in pediatrics using the Sysmex R-1000 automated reticulocyte analyzer. *Sysmex Int J.* 1992;2:4.

IMMATURE RETICULOCYTE FRACTION

Summary points	• Indicator of marrow response to anemia • May be useful to follow engraftment after bone marrow transplant
Methodology	Automated hematology analyzer—flow cytometric method
Specimen requirements	Whole blood: EDTA
Indications	Suspected anemia, differential diagnosis of anemia, assess marrow response to anemia, after marrow injury, chemotherapy, growth factor treatment, or bone marrow transplant
Reference range	Table 159.4
Interpretation	• Decreased in marrow failure or suppression states, increased with marrow response to anemia • Increased levels after bone marrow transplant appear to correlate with engraftment and precede other cell counts
Related tests	Complete blood count, reticulocyte count, reticulocyte hemoglobin, mean corpuscular volume reticulocyte
Interfering substances	Clotted or hemolyzed sample
Reference	Piva E, Brugnara C, Chiandetti L, et al. Automated reticulocyte counting: state of the art and clinical applications in the evaluation of erythropoiesis. *Clin Chem Lab Med.* 2010;48:1369.

EDTA, Ethylenediaminetetraacetic acid.

TABLE 159.4 Immature Reticulocyte Fraction Reference Values (95% Intervals)

N	Analyzer	Range
73	Beckman Coulter LH 750	0.19–0.42[a]
50	Sysmex XE 2100	0.01–0.18[b]
175 F, 142 M	Abbott CELL-DYN 4000	0.14–0.35[c]
225	Abbott CELL-DYN 4000	0.15–0.35[d]

[a] Banfi G, Mauri C, Morelli B, et al. Reticulocyte count, mean reticulocyte volume, immature reticulocyte fraction, and mean sphered cell volume in elite athletes: reference values and comparison with the general population. *Clin Chem Lab Med.* 2006;44:616.

[b] Noronha JFA, Grotto HZW. Measurement of reticulocyte and red blood cell indices in patients with iron deficiency anemia and beta-thalassemia minor. *Clin Chem Lab Med.* 2005;43:195.

[c] Van den Bossche J, Devreese K, Malfait R, et al. Reference intervals for a complete blood count determined on different automated haematology analysers: Abx Pentra 120 Retic, Coulter Gen-S, Sysmex SE9500, Abbott Cell Dyn 4000 and Bayer Advia 120. *Clin Chem Lab Med.* 2002;40:69.

[d] Buttarello M, Bulian P, Farina G, et al. Five fully automated methods for performing immature reticulocyte fraction: comparison in diagnosis of bone marrow aplasia. *Am J Clin Pathol.* 2002;117:871.

F, Female; *M*, male.

RETICULOCYTE HEMOGLOBIN EQUIVALENT (RET-HE)

Summary points	• May be useful in the early detection of iron deficiency • CHr is a similar but not identical parameter; whether Ret-He or CHr is reported depends on the hematology analyzer used Can be used to monitor response to therapy
Methodology	Automated hematology analyzer—flow cytometric method
Specimen requirements	Whole blood: EDTA
Indications	Suspected anemia, assessment of iron status, differential diagnosis of anemia, assess response to growth factor therapy
Reference range	Table 159.5
Interpretation	• Decreased in iron deficiency and may be decreased in thalassemia • Decreased levels in renal failure may suggest functional iron deficiency
Related tests	Complete blood count, reticulocyte count, immature reticulocyte fraction, mean corpuscular volume reticulocyte, iron studies
Interfering substances	Hemolyzed or clotted specimen
References	Brugnara C, Mohandas N. Red cell indices in classification and treatment of anemias: from M.M. Wintrobe's original 1934 classification to the third millennium. *Curr Opin Hematol.* 2013;20:222. Piva E, Brugnara C, Chiandetti L, et al. Automated reticulocyte counting: state of the art and clinical applications in the evaluation of erythropoiesis, *Clin Chem Lab Med.* 2010;48:1369.

CHr, Reticulocyte hemoglobin content; *EDTA*, ethylenediaminetetraacetic acid; *Ret-He*, reticulocyte hemoglobin equivalent.

TABLE 159.5 Reticulocyte Hemoglobin Equivalent and Reticulocyte Hemoglobin Content Reference Values (95% Intervals)

N	Parameter	Range (pg)
55	Ret-He	30.5–35.5[a]
196	Ret-He	30.2–36.7[b]
55	CHr	29.2–33.6[a]
57	CHr	32.2–32.8[c]

[a] Garzia M, Di Mario A, Ferraro E, et al. Reticulocyte hemoglobin equivalent: an indicator of reduced iron availability in chronic kidney diseases during erythropoietin therapy. *Lab Hematol*. 2007;13:6.
[b] Canals C, Remacha AF, Sardá MP, et al. Clinical utility of the new Sysmex XE 2100 parameter—reticulocyte hemoglobin equivalent—in the diagnosis of anemia. *Haematologica*. 2005;90:1133.
[c] Bovy C, Gothot A, Krzesinski JM, et al. Mature erythrocyte indices: new markers of iron availability. *Haematologica*. 2005;90:549.
CHr, Reticulocyte hemoglobin content; *Ret-He*, reticulocyte hemoglobin equivalent.

HAPTOGLOBIN

Summary points	• Binds free hemoglobin in plasma • Haptoglobin-hemoglobin complex removed by the reticuloendothelial system • Often used to evaluate for intravascular hemolysis but has low sensitivity and specificity • Acute-phase reactant
Methodology	Immunoassay
Specimen requirements	Lithium heparin, EDTA, or plain tube
Indications	Evaluation of hemolysis, especially intravascular
Reference range	Table 159.6
Interpretation	• Levels low in neonates and young infants, severe extravascular hemolysis, liver disease, massive tissue hemorrhage, and congenital deficiency states • High levels occur with pregnancy, steroids, estrogen, and acute-phase responses Preexisting high levels may mask hemolysis
Related tests	Plasma-free hemoglobin concentration, LDH, bilirubin, reticulocyte count
Interfering substances	Lipemia
Reference	Owen JA, Better FC, Hoban J. A simple method for the determination of serum haptoglobins. *J Clin Pathol*. 1960;13:163.

EDTA, Ethylenediaminetetraacetic acid; *LDH*, lactate dehydrogenase.

TABLE 159.6 Reference Values for Serum Haptoglobin by Age

Age	Hemoglobin-Binding Capacity (mg/dL)	
	Mean	Range (95%)
Cord blood	0	0
1–7 days	10	0–41
1–4 weeks	28	0–45
1–3 months	59	41–95
3–6 months	91	64–134
6–12 months	115	43–160
1–5 years	109	51–160
5–10 years	107	62–186
>10 years	110	41–165

From Kalil M, Badr-El-Din MK, Kassem AS. Haptoglobin level in normal infants and children. *Alex Med J*. 1967;13:1.

PLASMA HEMOGLOBIN

Summary points	• Disruption of red cells will cause elevations in plasma hemoglobin • Useful to assess for intravascular hemolysis and for following patients on ECMO or with a VAD • Sensitive to traumatic blood draws and artifactual red cell disruption and may cause false positives • Also known as plasma-free hemoglobin
Methodology	Colorimetric or quantitative spectrophotometry
Specimen requirements	Whole blood: EDTA, heparin (sodium or lithium)
Indications	• Evaluation of hemolysis, especially acute intravascular (transfusion reactions, mechanical destruction of red cells) • Evaluation of patients on ECMO or VAD to assess pump function and disruption of red cells from the pump
Reference range	< 30 mg/dL (Institutional reference range may vary)
Interpretation	• Elevated with intravascular red cell destruction • Increasing levels over baseline for patients on ECMO or VAD may indicate a problem with pump function and may herald renal injury
Related tests	LDH, bilirubin, reticulocyte count, haptoglobin, creatinine
Interfering substances	• Lipemia, hyperbilirubinemia, other causes of turbidity • Artifactually hemolyzed specimen • Delay in the separation of plasma from red cells (> 2 h from specimen collection)
References	• Dufour N, Radjou A, Thuong M. Hemolysis and plasma free hemoglobin during extracorporeal membrane oxygenation support: from clinical implications to laboratory details. *ASAIO J*. 2020;66(3):239-246 • Omar HR, Mirsaeidi M, Socias S, et al. Plasma free hemoglobin is an independent predictor of mortality among patients on extracorporeal membrane oxygenation support. *PLoS One*. 2015;10(4):e0124034 • Barbhuiya MA, Pederson EC, Straub ML, et al.: Automated measurement of plasma cell-free hemoglobin using the hemolysis index check function. *J Appl Lab Med*. 2020;5(2):281–289

EDTA, Ethylenediaminetetraacetic acid; *LDH*, lactate dehydrogenase; *ECMO*, extracorporeal membrane oxygenation; *VAD*, ventricular assist device.

HEMOGLOBIN F AND HEMOGLOBIN A_2

Summary points	• Hemoglobin F is composed of two α and two γ chains—the dominant hemoglobin during gestation and early infancy • Hemoglobin A_2 is composed of two α and two δ chains—a normal minor hemoglobin in adulthood • Hemoglobin A_2 present at very low levels at birth, gradually increasing to adult levels over the first 1–2 years of life
Methodology	Gel electrophoresis, capillary electrophoresis, or high-performance liquid chromatography
Specimen requirements	Whole blood: EDTA
Indications	• Assess for β-thalassemia (including trait) or other hemoglobinopathy • Evaluate for bone marrow failure syndromes or juvenile myelomonocytic leukemia (elevated hemoglobin F); evaluate patients being treated with hydroxyurea for hemoglobin F response (e.g., sickle cell anemia)
Reference range	Tables 159.7 and 159.8
Interpretation	• Hemoglobin F is elevated in β-thalassemia, δβ-thalassemia, hereditary persistence of fetal hemoglobin, sickle cell anemia, other hemoglobin disorders, some bone marrow failure syndromes, and juvenile myelomonocytic leukemia • Hemoglobin A_2 is elevated in β-thalassemia, unstable β-chain variants, megaloblastic anemia, thyrotoxicosis • Hemoglobin A_2 is decreased in δβ-thalassemia and at times in iron deficiency, lead poisoning, anemia of chronic disease, sideroblastic anemia, and hypothyroidism • Infants with suspected β-thalassemia disorders may require repeat testing at 6 months to 1 year of age to reliably evaluate hemoglobin A_2 levels
Related tests	Complete blood count, peripheral smear review, isoelectric focusing, molecular diagnostic testing for β-chain mutations

EDTA, Ethylenediaminetetraacetic acid.

TABLE 159.7 Reference Values for Hemoglobin F and Hemoglobin A_2 in the First 2 Years of Life

	Hemoglobin F (%)[a]		Hemoglobin A_2 (%)[b]	
Age	**Mean**	**±2 SD**	**Mean**	**±2 SD**
1–7 days	75	61–80	–	–
2 weeks	75	66–81	–	–
1 months	60	46–67	0.8	0.4–1.3
2 months	46	29–61	1.3	0.4–1.9
3 months	27	15–56	2.2	1.0–3.0
4 months	18	9.4–29	2.4	2.0–2.8
5 months	10	2.3–22	2.5	2.1–3.1
6 months	7	2.7–13	2.5	2.1–3.1
8 months	5	2.3–12	2.7	1.9–3.5
10 months	2.1	1.5–3.5	2.7	2.0–3.3
12 months	2.0	1.3–5.0	2.7	2.0–3.3
13–16 months	0.6	0.2–1.0	2.6	1.6–3.3
17–20 months	0.6	0.2–1.0	2.9	2.1–3.5
21–24 months	0.6	0.2–1.0	2.8	2.1–3.5

[a] Data from Schröter W, Nafz C. Diagnostic significance of hemoglobin F and A_2 levels in homo- and heterozygous β-thalassemia during infancy. *Helv Paediatr Acta.* 1981;36:519.

[b] Data from Metaxotou-Mavromati AD, Antonopoulou HK, Laskari SS, et al. Developmental changes in hemoglobin F levels during the first two years of life in normal and heterozygous β-thalassemia infants. *Pediatrics.* 1982;69:738.

SD, Standard deviation.

TABLE 159.8 Hemoglobin A_2 Levels (%) in Normal and Heterozygous β-Thalassemia Infants During the First 2 Years of Life

	Hemoglobin A_2 (%)	
Age (Months)	**Normal Mean ± SD**	**β-Thalassemia Heterozygotes Mean ± SD**
1	0.8 ± 0.4	–
2	1.3 ± 0.5	1.8 ± 0.3
3	2.2 ± 0.6	3.8 ± 0.9
4	2.4 ± 0.4	4.0
5–6	2.5 ± 0.3	5.2 ± 0.7
7–9	2.7 ± 0.4	5.3 ± 0.7
10–12	2.7 ± 0.4	5.0 ± 0.6
13–16	2.6 ± 0.5	5.4 ± 0.8
17–20	2.9 ± 0.4	5.5 ± 0.6
21–24	2.8 ± 0.4	5.6 ± 0.9

SD, Standard deviation.

Data from Metaxotou-Mavromati AD, Antonopoulou HK, Laskari SS, et al. Developmental changes in hemoglobin F levels during the first two years of life in normal and heterozygous β-thalassemia infants. *Pediatrics.* 1982;69:736; Steinberg MH, Adams JG. Hemoglobin A2: Origin, evolution, and aftermath. *Blood.* 1991;78:2165.

SICKLE CELL SCREEN (HEMOGLOBIN S SOLUBILITY TEST)

Summary points	• Rapid test to evaluate for the presence of hemoglobin S >30% • Also known as a *sickle prep* or by the brand name of a commonly used test kit—*Sickledex*
Methodology	Differential solubility
Specimen requirements	Whole blood: EDTA, some laboratories accept heparin or ACD anticoagulants
Indications	Suspected hemoglobin S disorder
Reference range	Negative
Interpretation	• Positive when hemoglobin S >30%, negative when hemoglobin S <20% • Hemoglobin S between 20% and 30% may be positive or negative • False positive with cryoglobulinemia, multiple myeloma, dysglobulinemia, certain rare hemoglobinopathies (C Harlem or C Georgetown); false negative with severe anemia, high hemoglobin F, infants under 6 months • Recent transfusion may give false-positive or false-negative results • Does not distinguish between sickle cell trait and sickle cell disease or compound heterozygotes for hemoglobin S and another hemoglobinopathy • Positive results should be confirmed by another method
Related tests	Hemoglobin fractionation/electrophoresis
Interfering substances	Hemolyzed sample
References	Lange RD, Minnich V, Moore CV. Effect of oxygen tension and of pH on the sickling and mechanical fragility of erythrocytes from patients with sickle cell anemia and the sickle cell trait. *J Lab Clin Med.* 1951;37:789. Nalbandian RM, Nichols BM, Camp FR Jr, et al. Dithionite tube test—a rapid, inexpensive technique for the detection of hemoglobin S and non-S sickling hemoglobin. *Clin Chem.* 1971;17:1028.

ACD, Acid citrate dextrose; *EDTA*, ethylenediaminetetraacetic acid.

FERRITIN

Summary points	• Ferritin concentration reflects body iron stores; decreased in iron deficiency, increased in iron overload • Useful in distinguishing between iron deficiency anemia and anemia of chronic disease, thalassemia minor • Assessment of iron deficiency may be confounded by infection, inflammation, malignancy, and liver disease, all of which raise ferritin concentration • More sensitive and reliable than serum iron, transferrin (total iron-binding capacity), and transferrin saturation for detecting iron deficiency • Is an acute-phase reactant
Methodology	Immunoassay
Specimen requirements	Lithium heparin or plain tube
Indications	Assess suspected iron deficiency or iron overload
Reference range	Table 159.9
Interpretation	• Levels are age and gender dependent. Levels in adult females are generally lower than in adult males, but following menopause, the levels progressively approach those of males • Levels elevated with iron overload, Gaucher disease, acute hepatitis, malignancies, hemophagocytic lymphohistiocytosis, other highly inflammatory states
Related tests	Iron, transferrin (total iron-binding capacity), transferrin saturation, soluble transferrin receptor
Interfering substances	Anti-animal antigen antibodies

TABLE 159.9 Serum Ferritin in Children and Adults

	Male Subjects		Female Subjects[a]	
Age	ng/mL	µg/L	ng/mL	µg/L
1–30 days[b]	6–400	6–400	6–515	6–515
1–6 months[b]	6–410	6–410	6–340	6–340
7–12 months[b]	6–80	6–80	6–45	6–45
1–5 years[c,d]	6–24	6–60	6–24	6–60
6–9 years[c,d]	10–55	10–55	10–55	10–55
10–14 years[d]	23–70	23–70	6–40	6–40
14–19 years[d]	23–70	23–70	6–40	6–40

[a] Data from Brugnara C. Iron deficiency and erythropoiesis: new diagnostic approaches. *Clin Chem* 2003;49:1573; Cook JD. The measurement of serum transferrin receptor. *Am J Med Sci.* 1999;318:269.

[b] From Soldin SJ, Morales A, Albalos F, et al. Pediatric reference ranges on the Abbott IMx for FSH, LH, prolactin, TSH, T4, T3, free T4, free T3, T-uptake, IgE, and ferritin. *Clin Biochem.* 1995;28:603. Study was based on hospitalized patients. Values represent 2.5th–97.5th percentiles.

[c] From Lockitch G, Halstead AC, Wadsworth L, et al. Age- and sex-specific pediatric reference intervals and correlations for zinc, copper, selenium, iron, vitamins A and E, and related proteins. *Clin Chem.* 1988;34:1625. Study was based on healthy children; children with anemia, microcytosis, and thalassemia syndromes were excluded. Values represent the 0.025th–0.975th fractiles.

[d] No significant differences between males and females; range derived from combined data.

SOLUBLE TRANSFERRIN RECEPTOR

Summary points	• Transferrin receptor derived mainly from erythroid marrow cells • Reflects iron status independent of concurrent inflammation
Methodology	Immunoassay
Specimen requirements	Lithium heparin or plain tube
Indications	Assess for iron deficiency, especially in the setting of inflammation
Reference range	Table 159.10
Interpretation	• Levels decreased with erythroid hypoplasia states, increased with increased iron demand (erythroid hyperplasia [hemolysis, recent blood loss, thalassemia], iron deficiency) • Unaffected by inflammation or liver disease • African Americans and individuals at high altitude may have slightly higher values
Related tests	Iron, transferrin (total iron-binding capacity), transferrin saturation, ferritin
Interfering substances	Lipemia

TABLE 159.10 Soluble Transferrin Receptor Levels in Infants, Children, and Adults

Soluble Transferrin Receptor Levels in Infants and Children[a]

Age (Years)	Number	sTfR (mg/L)
0.05–0.5	13	1.26–3.17
0.5–1	17	1.12–2.91
1–1.5	16	1.37–2.52
1.5–2	20	1.33–2.93
2–3	46	0.98–2.91
3–4	38	1.08–2.55
4–6	76	1.10–2.74
6–9	64	0.93–2.63
9–12	44	0.81–2.67
12–18	85	0.91–1.91

Soluble Transferrin Receptor Levels in Adults[b]

Age (Years)	sTfR (mg/L)
13–17	1.75 ± 0.39
18–30	1.65 ± 0.34
31–40	1.60 ± 0.39
41–50	1.54 ± 0.39
51–65	1.59 ± 0.34

sTfR, Soluble transferrin receptor.
From Ooi CL, Lepage N, Nieuwenhuys E, et al. Pediatric reference intervals for soluble transferrin receptor and transferrin receptor-ferritin index. *World J Pediatr.* 2009;5:122.
SD, Standard deviation; *sTfR*, soluble transferrin receptor.
From von Schmiesing A, Schmidt SA, Kratzer W, et al. Determination of reference values for the soluble transferrin receptor (sTfR) on a cross sectional population sample. *Clin Lab.* 2009;55:193.
[a] Values are expressed in mg/L. Data were obtained from a total of 436 apparently healthy children. Values are 2.5th–97.5th percentiles.
[b] Values are expressed as means ± SD based on testing of 2050 adults (1060 males).

TOTAL IRON-BINDING CAPACITY, TRANSFERRIN SATURATION

Summary points	• Transferrin—the major iron transporter protein in plasma • TIBC—often measured as transferrin concentration, especially in pediatrics, because of the smaller specimen volume required for children • One molecule of transferrin can bind two molecules of iron • TIBC and transferrin concentration—typically elevated in iron deficiency due to increased transferrin gene expression • Transferrin saturation—calculated as serum iron concentration divided by the iron-binding capacity; low in iron deficiency, elevated in iron overload
Methodology	Immunoassay and calculation
Specimen requirements	Lithium heparin or plain tube
Indications	Assess suspected iron deficiency, iron overload, and acute iron poisoning (transferrin saturation elevated in the latter)
Reference range	Table 159.11
Interpretation	• Pregnancy and oral contraceptives increase transferrin concentration, leading to low transferrin saturation • Acute-phase response can decrease transferrin and TIBC • Spuriously high TIBC can occur with acute hepatitis and after iron dextran injection
Related tests	Iron, ferritin, soluble transferrin receptor
Interfering substances	Lipemia

TIBC, Total iron-binding capacity.

TABLE 159.11 Serum Iron-Binding Capacity, Transferrin, and Transferrin Saturation Reference Values

Iron-Binding Capacity[a]

	Male Subjects		Female Subjects	
Age	**µg/dL**	**µmol/L**	**µg/dL**	**µmol/L**
1–30 days	94–232	16.8–41.5	94–236	16.8–42.2
31–182 days	116–322	20.8–57.6	89–311	15.9–55.7
182–365 days	176–384	31.5–68.7	138–365	24.7–65.3
1–3 years	204–382	36.5–68.3	184–377	32.9–67.5
4–6 years	180–390	32.2–69.8	162–352	29.0–63.0
7–9 years	183–369	32.8–66.1	167–336	29.9–60.1
10–12 years	173–356	31.0–63.7	198–383	35.4–68.6
13–15 years	193–377	34.5–67.5	169–358	30.3–64.1
16–18 years	174–351	31.1–62.8	194–372	34.7–66.6

Transferrin[a]

Age	Male Subjects (g/L)	Female Subjects (g/L)
1–30 days	0.97–2.05	0.92–2.08
31–182 days	1.06–3.25	1.28–3.09
182–365 days	1.78–3.57	1.46–3.64
1–3 years	1.96–3.65	1.49–3.82
4–6 years	2.02–3.50	1.74–3.99
7–9 years	1.49–3.53	1.86–3.68
10–12 years	1.73–3.80	1.85–3.77
13–15 years	1.71–3.74	1.93–3.91
16–18 years	1.94–3.48	1.81–4.16

Transferrin Saturation[b,c,d]

Age	Male Subjects (ng/mL)	Female Subjects (ng/mL)
1–5 years	0.07–0.44	0.07–0.44
6–9 years	0.17–0.42	0.17–0.42
10–14 years	0.11–0.36	0.02–0.40
14–19 years	0.06–0.33	0.06–0.33

[a] From Soldin SJ, Brugnara C, Gunter KC, et al. *Pediatric Reference Ranges*. 2nd ed. Washington, DC: AACC Press; 1997:100, 101, 143.

[b] From Lockitch G, Halstead AC, Wadsworth L, et al. Age- and sex-specific pediatric reference intervals and correlations for zinc, copper, selenium, iron, vitamins A and E, and related proteins. *Clin Chem* 34:1625, 1988. Study was based on healthy children; children with anemia, microcytosis, and thalassemia syndromes were excluded. Values represent the 0.025th–0.975th fractiles.

[c] No significant differences between males and females; range derived from combined data.

[d] Transferrin saturation calculated from iron (µmol/L)/total iron-binding capacity. Transferrin, transferrin saturation, and total iron-binding capacity results may be misleading in patients receiving iron therapy. Note that the lower reference limits for serum iron and transferrin saturation in this study are below the limits used to define acceptable levels for nutritional health for these two analytes (see O'Neal RM, Johnson OC, Schaefer AE: Guidelines for classification and interpretation of group blood and urine data collected as part of the National Nutrition Survey. *Pediatr Res.* 1970;4:103).

IRON

Summary points	- Reflects recent iron ingestion and absorption - Undergoes circadian variation - Less reliable than ferritin for assessing iron deficiency
Methodology	Spectrophotometry
Specimen requirements	Lithium heparin or plain tube
Indications	Assess suspected iron deficiency, iron overload, acute iron poisoning, iron absorption (iron challenge test)
Reference range	Table 159.12
Interpretation	- Acute-phase response, malignancy, and ascorbate deficiency can decrease iron concentration, whereas liver disease, ineffective erythropoiesis, and aplastic and sideroblastic anemias can increase it - For oral iron challenge testing, an increase of at least 100 μg/dL 1–4 h after ingestion of 65 mg elemental iron is thought to indicate adequate absorption
Related tests	Ferritin, transferrin (total iron-binding capacity), transferrin saturation, soluble transferrin receptor
Reference	Cook JD. Diagnosis and management of iron-deficiency anaemia. *Best Pract Res Clin Haematol.* 18:319, 2005.

TABLE 159.12 Serum Iron Reference Values

	Male Subjects		Female Subjects	
Age	**μg/dL**	**μmol/L**	**μg/dL**	**μmol/L**
1–30 days	32–112	5.7–20.0	29–127	5.2–22.7
31–365 days	27–109	4.8–19.5	25–126	4.5–22.6
1–3 years	29–91	5.2–16.3	25–101	4.5–18.1
4–6 years	25–115	4.5–20.6	28–93	5.0–16.7
7–9 years	27–96	4.8–17.2	30–104	5.4–18.6
10–12 years	28–112	5.0–20.0	32–104	5.7–18.6
13–15 years	26–110	4.7–19.7	30–109	5.4–19.5
16–18 years	27–138	4.8–24.7	33–102	5.9–18.3

From Soldin SJ, Brugnara C, Gunter KC, et al. *Pediatric Reference Ranges.* 2nd ed. Washington, DC: AACC Press; 1997:100, 101, 143.

ERYTHROPOIETIN

Summary points	- Glycoprotein hormone secreted mostly by the kidney but also by the liver - Level varies inversely with hematocrit, stimulated by hypoxia - May be secreted by certain tumors, leading to polycythemia - Results obtained with one method may differ significantly from those obtained with another—any serial testing over time should be performed using the same test method
Methodology	Immunoassay
Specimen requirements	Lithium heparin, EDTA, or plain tube
Indications	Can differentiate primary (decreased concentration) from secondary erythrocytosis (elevated), but testing may be problematic given the broad overlap between the two groups. Levels are usually increased in anemia except in chronic renal failure patients, in whom levels may be used to monitor erythropoietin therapy
Reference range	Adult values approximately 3–16 milli–International Unit/mL; Table 159.13
Interpretation	Normal or low levels with primary polycythemia; elevated levels with most forms of secondary polycythemia
Interfering substances	Lipemia
Reference	Cotes PM, Doré CJ, Yin JA, et al. Determination of serum immunoreactive erythropoietin in the investigation of erythrocytosis. *N Engl J Med.* 1986;315:283.

EDTA, Ethylenediaminetetraacetic acid.

TABLE 159.13 Erythropoietin Reference Values in Infants and Children

Serum Erythropoietin Levels in Term Infants During the First Year of Life[a]

Days After Birth	Erythropoietin (Milli–International Unit/mL)	Hemoglobin (g/dL)	RBC ($\times 10^6/\mu L$)
0–6	33.0 ± 31.4 (11)	15.6 ± 2.2 (11)	4.51 ± 0.74 (11)
7–50	11.7 ± 3.6 (7)	12.8 ± 1.1 (5)	3.92 ± 0.35 (5)
51–100	21.1 ± 5.5 (13)	11.4 ± 1.0 (10)	4.09 ± 0.51 (10)
101–150	15.1 ± 3.9 (5)	11.2 ± 1.1 (3)	4.21 ± 0.31 (3)
151–200	17.8 ± 6.3 (6)	–	–
>201	23.1 ± 9.7 (10)	11.8 ± 0.8 (9)	4.57 ± 0.24 (9)

Plasma Erythropoietin Reference Ranges in Children[b]

Age (Years)	Number	Male Subjects	Number	Female Subjects
1–3	122	1.7–17.9	97	2.1–15.9
4–6	89	3.5–21.9	76	2.9–8.5
7–9	79	1.0–13.5	80	2.1–8.2
10–12	98	1.0–14.0	90	1.1–9.1
13–15	100	2.2–14.4	148	3.8–20.5
16–18	66	1.5–15.2	77	2.0–14.2

[a] Values are expressed as means ± SD; parentheses enclose the number of specimens. Patients were not transfused and weighed 2500 g or more.

[b] Values are expressed in milli–International Unit/mL. Data were obtained from a total of 1122 hospitalized and outpatient children ages 1–18 years, using a commercially available enzyme-linked immunosorbent assay methodology. Values are 2.5th–97.5th percentiles.

From Yamashita H, Kukita J, Ohga S, et al. Serum erythropoietin levels in term and preterm infants during the first year of life. *Am J Pediatr Hematol Oncol.* 1994;16:213.

From Krafte-Jacobs B, Williams J, Soldin SJ. Plasma erythropoietin reference ranges in children. *J Pediatr.* 1995;126:601.

FOLATE (RBC OR SERUM/PLASMA)

Summary points	• Folate—commonly assessed in the setting of macrocytic anemia along with vitamin B_{12} (should be done concurrently) • RBC folate less sensitive to short-term dietary changes than serum folate; more reflective of tissue folate stores, more specific for folate deficiency • Folate deficiency usually nutritional in origin or due to inadequate intake in the setting of increased demand (pregnancy, hemolytic anemias, liver disease)
Methodology	Immunoassay
Specimen requirements	RBC: whole blood: EDTA; protect from light for RBC Serum: plain or serum separator tube (fasting state preferred)
Indications	Macrocytic or megaloblastic anemia, workup of possible nutritional deficiencies or myelodysplastic syndrome, unexplained neurologic symptoms, or dementia
Reference range	Table 159.14

Interpretation	• Decreased in nutritional deficiencies (pregnancy, alcoholism, older adults), antimetabolite therapy (methotrexate, for example), rarely due to inherited metabolic disorders
	• May be decreased in vitamin B_{12} (cobalamin) deficiency
	• Additional testing for pathway intermediates homocysteine and MMA can help distinguish between folate and vitamin B_{12} deficiency in patients who have borderline levels of either
	  ◦ Homocysteine—elevated in both folate and vitamin B_{12} deficiency
	  ◦ MMA—elevated in vitamin B_{12} but not folate deficiency

Related tests	Vitamin B_{12}, homocysteine, MMA, complete blood count
Interfering substances	Excessive light exposure before testing, hemolysis, lipemia
Reference	Kaferle J, Strzoda CE. Evaluation of macrocytosis. *Am Fam Physician*. 2009;79:203.

EDTA, Ethylenediaminetetraacetic acid; *MMA*, methylmalonic acid; *RBC*, red blood cell.

TABLE 159.14 Folate Reference Values

RBC Folate Thresholds[a]		
<305 nmol/L (140 ng/mL)	Folate deficiency	
305–340 nmol/L	Subclinical deficiency (with elevated homocysteine)	
>900 nmol/L (400 ng/mL)	Reduced risk for neural tube defect in pregnancy	
Plasma Levels of Folic Acid in Children[b]		
Age	**Males (nmol/L)**	**Females (nmol/L)**
0–1 year	16.3–50.8	14.3–51.5
2–3 years	5.7–34.0	3.9–35.6
4–6 years	1.1–29.4	6.1–31.9
7–9 years	5.2–27.0	5.4–30.4
10–12 years	3.4–24.5	2.3–23.1
13–18 years	2.7–19.9	2.7–16.3

[a] Data from Green R. Indicators for assessing folate and vitamin B-12 status and for monitoring the efficacy of intervention strategies. *Am J Clin Nutr*. 2011;94:666S.
[b] From Hicks JM, Cook J, Godwin ID, et al. Vitamin B12 and folate. Pediatric reference ranges. *Arch Pathol Lab Med*. 1993;117:704. Data were collected from hospitalized patients; 2.5th–97.5th percentile values adapted from the Hoffman technique. Specimens from more than 100 patients in each age-group were analyzed by radioimmunoassay.

VITAMIN B_{12} (COBALAMIN)

Summary points	• Commonly assessed in the setting of macrocytic anemia along with folate (should be done concurrently to avoid undiagnosed vitamin B_{12} deficiency) • Deficiency often due to inadequate gastric absorption (pernicious anemia, gastric bypass) or lack of small bowel reabsorption (malabsorption, Crohn disease, small intestinal surgery) • Nutritional deficiency is uncommon, but strict vegans are at risk (vitamin B_{12} available only from animal sources); also seen in alcoholics, older adults, malnutrition • Elevated vitamin B_{12} may accompany severe disease states and warrants consideration of additional evaluation
Methodology	Immunoassay
Specimen requirements	Plain or serum separator tube
Indications	Macrocytic or megaloblastic anemia, pancytopenia, workup of possible nutritional deficiencies or myelodysplastic syndrome, unexplained neurologic symptoms or dementia, hypersegmented neutrophils on peripheral smear
Reference range	Table 159.15

Interpretation	• Low levels diagnostic of deficiency; pregnancy lowers vitamin B_{12} level
	• Follow-up testing for low levels to diagnose pernicious anemia: parietal cell antibodies, anti-intrinsic factor antibodies
	• Additional testing for pathway intermediates homocysteine and MMA can help distinguish between folate and vitamin B_{12} deficiency in patients with borderline levels of either
	  • Homocysteine—elevated in both folate and vitamin B_{12} deficiency
	  • MMA—elevated in vitamin B_{12} but not folate deficiency
	• High vitamin B_{12} may accompany solid neoplasms, hematologic malignancy, liver or kidney disease, functional B_{12} deficiency, and autoimmune lymphoproliferative syndrome

Related tests	Folate, homocysteine, MMA, complete blood count, parietal cell antibodies, anti-intrinsic factor antibodies, serum gastrin
Interfering substances	Excessive light exposure before testing, hemolyzed sample
References	Andrès E, Serraj K, Zhu J, et al. The pathophysiology of elevated vitamin B_{12} in clinical practice. *Q J Med*. 2013;106:505. Kaferle J, Strzoda CE. Evaluation of macrocytosis. *Am Fam Physician*. 2009;79:203.

MMA, Methylmalonic acid.

TABLE 159.15 Plasma Levels of Vitamin B_{12} in Children

Age	Males (pmol/L)	Females (pmol/L)
0–1 year	216–891	168–1117
2–3 years	195–897	307–892
4–6 years	181–795	231–1038
7–9 years	200–863	182–866
10–12 years	135–803	145–752
13–18 years	158–638	134–605

From Hicks JM, Cook J, Godwin ID, et al. Vitamin B12 and folate. Pediatric reference ranges. *Arch Pathol Lab Med*. 1993;117:704. Data were collected from hospitalized patients; 2.5th–97.5th percentile values adapted from the Hoffman technique. Specimens from more than 100 patients in each age-group were analyzed by radioimmunoassay.

HOMOCYSTEINE

Summary points	- Used in differentiating vitamin B_{12} from folate deficiency, evaluating hereditary metabolic disorders, and assessing cardiovascular risk
Methodology	Liquid chromatography—tandem mass spectrometry, stable isotope dilution analysis, quantitative enzymatic
Specimen requirements	Plain, EDTA, heparin, or serum separator tube (fasting state preferred)
Indications	Suspected B_{12} or folate deficiency with equivocal initial testing (folate, vitamin B_{12} level), disorder of methionine metabolism, assess cardiovascular or prothrombotic risk
Reference range	Table 159.16

Interpretation	- Testing for pathway intermediates homocysteine and MMA can help distinguish between folate and vitamin B_{12} deficiency in patients with borderline levels of either
	  - Homocysteine—elevated in both folate and vitamin B_{12} deficiency
	  - MMA—elevated in vitamin B_{12} but not folate deficiency
	- Levels decrease with vitamin supplementation
	- May be elevated due to age, medications, smoking, renal disease, poor nutrition, thyroid disease, MTHFR 677 C→T polymorphism
	- Serum/plasma should be separated quickly from red blood cells to prevent false elevations
	- Recent consumption of high-protein meal may falsely elevate the level

Related tests	Folate, vitamin B_{12}, MMA, complete blood count, methionine, plasma amino acids, urine organic acids
Reference	Refsum H, Smith AD, Ueland PM, et al. Facts and recommendations about total homocysteine determinations: an expert opinion. *Clin Chem*. 2004;50(1):3.

EDTA, Ethylenediaminetetraacetic acid; *MMA*, methylmalonic acid.

TABLE 159.16 **Homocysteine Reference Values in Infants and Children**

Age	Median (µmol/L)	Range (µmol/L)
Newborn[a]	5.2	0.8–10.9
4 days[b]	6.22	5.0–7.48
6 weeks–6 months[b]	7.47	6.1–9.22
1–10 years[b]	5.24	4.68–5.97
10.5–15 years[b]	6.52	5.7–7.75
15.5–19 years[b]	7.75	6.61–9.12

[a] Data from blood spots from 200 normal newborns using tandem mass spectrometry. Data from Turgeon CT, Magera MJ, Cuthbert CD, et al. Determination of total homocysteine, methylmalonic acid, and 2-methylcitric acid in dried blood spots by tandem mass spectrometry. *Clin Chem*. 2010;56:1686.
[b] Data from cross-sectional study of 700 children; range in interquartile. Data from Bjørke Monsen AL, Refsum H, Markestad T, et al. Cobalamin status and its biochemical markers methylmalonic acid and homocysteine in different age groups from 4 days to 19 years. *Clin Chem*. 2003;49:2067.

METHYLMALONIC ACID

Summary points	• Used in differentiating vitamin B_{12} from folate deficiency and evaluating hereditary metabolic disorders
Methodology	Liquid chromatography—tandem mass spectrometry
Specimen requirements	Plain, EDTA, heparin, or serum separator tube; urine levels may also be measured
Indications	Suspected vitamin B_{12} or folate deficiency with equivocal initial testing (folate, vitamin B_{12} level), metabolic disorder (methylmalonic acidemia)
Reference range	Table 159.17

Interpretation	• Testing for pathway intermediates homocysteine and MMA can help distinguish between folate and vitamin B_{12} deficiency in patients with borderline levels of either
	  • Homocysteine—elevated in both folate and vitamin B_{12} deficiency
	  • MMA—elevated in vitamin B_{12} but not folate deficiency
	• Elevated in vitamin B_{12} deficiency, renal failure, thyroid disease, bacterial overgrowth of small bowel
	• Markedly elevated levels in a child suggest hereditary metabolic disorder but must be confirmed

Related tests	Folate, vitamin B_{12}, homocysteine, complete blood count
Interfering substances	Hemolyzed sample, lipemia

EDTA, Ethylenediaminetetraacetic acid; *MMA*, methylmalonic acid.

TABLE 159.17 Reference Values for Methylmalonic Acid

Age	Median (Range) µmol/L
Newborn[a]	0.6 (0.2–2.0)
4 days[b]	0.29 (0.24–0.39)
6 weeks–6 months[b]	0.78 (0.36–1.51)
1–10 years[b]	0.13 (0.11–0.17)
10.5–15 years[b]	0.17 (0.13–0.22)
15.5–19 years[b]	0.14 (0.12–0.18)
Adult[c]	<0.4

[a] Data from blood spots from 200 normal newborns using tandem mass spectrometry, median value, and range given. Data from Turgeon CT, Magera MJ, Cuthbert CD, et al. Determination of total homocysteine, methylmalonic acid, and 2-methylcitric acid in dried blood spots by tandem mass spectrometry. *Clin Chem*. 2010;56:1686.

[b] Data from cross-sectional study of 700 children; range in interquartile. Data from Bjørke Monsen AL, Refsum H, Markestad T, et al. Cobalamin status and its biochemical markers methylmalonic acid and homocysteine in different age groups from 4 days to 19 years. *Clin Chem*. 2003;49:2067.

[c] Data from Bjørke Monsen AL, Ueland PM. Homocysteine and methylmalonic acid in diagnosis and risk assessment from infancy to adolescence. *Am J Clin Nutr*. 2003;78:7.

OSMOTIC FRAGILITY

Summary points	• Used in the differential diagnosis of hemolytic anemia • Usually increased osmotic fragility with hereditary spherocytosis • Increased osmotic fragility can be seen in other conditions with increased spherocytes and other hematologic disorders
Methodology	Lysis of erythrocytes in a gradient of hypoosmolar solutions; degree of hemolysis commonly measured by spectrophotometry
Specimen requirements	Whole blood: EDTA or heparin
Indications	Hemolytic anemia, suspected hereditary spherocytosis
Reference range	Normal curves generated by individual laboratories; patient results are plotted against a normal curve
Interpretation	• Patients with hereditary spherocytosis will show increased lysis of red blood cells in hypoosmolar solutions compared with normal controls • Increased osmotic fragility also seen in other causes of spherocytosis (autoimmune hemolytic anemia, posttransfusion), other hematologic disorders (pyruvate kinase deficiency, G6PD deficiency, hereditary stomatocytosis, hereditary pyropoikilocytosis), Rh-null phenotype • Decreased osmotic fragility seen in sickle cell disease, thalassemia, iron deficiency • Normal test does not exclude hereditary spherocytosis (the most fragile cells may have already lysed)—repeat testing at a time when the patient is at baseline state and hematologically stable may be useful as may perform the test with incubation overnight (increases sensitivity) • Measurements taken either immediately or after incubation for 24 h at 37°C enhance assay sensitivity • Results may vary depending on patient age, gender, medication history, and presence or absence of active hemolysis • Flow cytometry for band 3 using EMA is more sensitive and specific for hereditary spherocytosis and related hereditary disorders than osmotic fragility
Related tests	Flow cytometry for band 3 using EMA, peripheral smear, direct antiglobulin test, complete blood count, red cell glycolytic intermediate metabolites (Table 159.18)
Interfering substances	Hemolyzed specimen, specimen age may falsely increase background hemolysis (laboratory may require submitting a concurrent sample from normal nonrelative to control for this)
References	King MJ, Zanella A. Hereditary red cell membrane disorders and laboratory diagnostic testing. *Int J Lab Hematol*. 2013;35:237. Park SH, Park CJ, Lee, BR, et al. Comparison study of the eosin-5′-maleimide binding test, flow cytometric osmotic fragility test, and cryohemolysis test in the diagnosis of hereditary spherocytosis. *Am J Clin Pathol*. 2014;142:474.

EDTA, Ethylenediaminetetraacetic acid; *EMA*, eosin-5′-maleimide; *G6PD*, glucose-6-phosphate dehydrogenase.

TABLE 159.18 Red Cell Glycolytic Intermediate Metabolites in Normal Adults, Term Infants, and Premature Infants[a]

Metabolite	Normal Adults (n =10)	Term Infants (n =10)	Premature Infants (n =11)
Glucose-6-phosphate	24.8 ± 9.8	45.2 ± 8.7	66.8 ± 34.8
Fructose-6-phosphate	5.4 ± 1.0	9.9 ± 2.3	20.5 ± 8.9
Fructose-1,6-diphosphate	4.6 ± 1.0	3.8 ± 0.7	3.6 ± 0.8
Dihydroxyacetone phosphate	4.9 ± 3.5	11.9 ± 5.0	18.6 ± 10.7
Glyceraldehyde-3-phosphate	2.6 ± 0.7	1.9 ± 1.6	6.5 ± 3.2
3-Phosphoglycerate	61.6 ± 12.4	58.2 ± 14.4	47.5 ± 14.2
2-Phosphoglycerate	4.3 ± 1.8	4.9 ± 1.6	4.4 ± 2.5
Phosphoenolpyruvate	8.8 ± 2.6	7.6 ± 2.9	7.4 ± 3.0
Pyruvate	73.5 ± 33.1	70.4 ± 32.3	78.4 ± 4.15
2,3-Diphosphoglycerate	4423 ± 1907	3609 ± 800	3152 ± 2133

Units per 100 mL of red blood cells.

[a] Premature infants had birth weight below 2200 g and gestational age of less than 37 weeks and were clinically healthy at the time of investigation.

Reproduced with permission from Oski FA. Red cell metabolism in the newborn infant. V. Glycolytic intermediates and glycolytic enzymes. *Pediatrics*. 1969;44:84; Beutler E. G6PD deficiency. *Blood*. 1994;84:3613.

WHITE BLOOD CELL AND IMMUNOLOGIC TESTING

LEUKOCYTE COUNTS (ABSOLUTE AND PERCENTAGE)

Summary points	• Also called *WBC count and differential* • Part of complete blood count
Methodology	Automated hematology analyzer—flow cytometric or impedance methods, may use myeloperoxidase staining for granulocytes, differential may be performed by automated or manual methods
Specimen requirements	Whole blood: EDTA
Indications	Suspected infection, hematologic disorder, or immune deficiency
Reference range	Table 159.19

Interpretation	• Results can vary with time of day, hormonal status, ethnicity (e.g., lower neutrophil counts in persons of African descent)
	• With infection, total white blood cell (leukocyte) count is often elevated, but may be normal or low
	  • Predominance of neutrophils with left shift (immature myeloid precursors) suggests bacterial infection or sepsis but nonspecific; also seen with physical or emotional stress, growth factor treatment
	  • Predominance of lymphocytes and/or monocytes suggests a viral or atypical bacterial infection
	  • Eosinophilia suggests allergy, drug effect, or invasive parasitic infection
	• Abnormal cells suggest leukemia, lymphoma, or myelodysplastic syndrome but can also be seen after growth factor treatment
	• Low counts suggest marrow suppression due to infection, medication, autoimmunity, toxic exposure, metabolic disorder, bone marrow failure, myelodysplastic syndrome, leukemia, or immune deficiency
	• Generally, automated differentials are more accurate, but a manual differential may be indicated if abnormal or immature cells are present

Related tests	Manual differential, peripheral smear, lymphocyte subsets
Interfering substances	Clotted or hemolyzed sample

EDTA, Ethylenediaminetetraacetic acid; WBC, white blood cell.

LYMPHOCYTE SUBSETS

Summary points	• B-, T-, and NK-cell subsets used to diagnose and characterize immunodeficiency along with other lymphocyte function testing
Methodology	Flow cytometry
Specimen requirements	Whole blood: EDTA or heparin
Indications	Suspected or known immune deficiency; monitor status of HIV disease or immunomodulatory/immunosuppressive therapy
Reference range	Table 159.20
Interpretation	• Absolute declines in lymphocyte subsets can aid diagnosis and characterization of immune deficiencies: primarily B cell, T cell, or combined • T- and B-cell subsets may decline with immunomodulatory or immunosuppressive therapy • Absolute CD4 counts—monitored in patients with HIV; may define therapy thresholds or risks for specific pathogens • Results may vary with diurnal rhythm
Related tests	Complete blood count, lymphocyte mitogen testing, lymphocyte antigen response testing, antigen-specific IgG/IgM titers after vaccination, PPD testing for the delayed type of hypersensitivity response
Interfering substances	Hemolyzed sample, lipemia

EDTA, Ethylenediaminetetraacetic acid; *HIV*, human immunodeficiency virus; *Ig*, immunoglobulin; *NK*, natural killer; *PPD*, purified protein derivative.

TABLE 159.19 Reference Ranges for Leukocyte Counts in Children and Adults[a]

Age	Total Leukocytes		Neutrophils[b]			Lymphocytes			Monocytes		Eosinophils	
	Mean	Range	Mean	Range	%	Mean	Range	%	Mean	%	Mean	%
Birth	18.1	9.0–30.0	11.0	6.0–26.0	61	5.5	2.0–11.0	31	1.1	6	0.4	2
12 h	22.8	13.0–38.0	15.5	6.0–28.0	68	5.5	2.0–11.0	24	1.2	5	0.5	2
24 h	18.9	9.4–34.0	11.5	5.0–21.0	61	5.8	2.0–11.5	31	1.1	6	0.5	2
1 week	12.2	5.0–21.0	5.5	1.5–10.0	45	5.0	2.0–17.0	41	1.1	9	0.5	4
2 weeks	11.4	5.0–20.0	4.5	1.0–9.5	40	5.5	2.0–17.0	48	1.0	9	0.4	3
1 month	10.8	5.0–19.5	3.8	1.0–9.0	35	6.0	2.5–16.5	56	0.7	7	0.3	3
6 months	11.9	6.0–17.5	3.8	1.0–8.5	32	7.3	4.0–13.5	61	0.6	5	0.3	3
1 year	11.4	6.0–17.5	3.5	1.5–8.5	31	7.0	4.0–10.5	61	0.6	5	0.3	3
2 years	10.6	6.0–17.0	3.5	1.5–8.5	33	6.3	3.0–9.5	59	0.5	5	0.3	3
4 years	9.1	5.5–15.5	3.8	1.5–8.5	42	4.5	2.0–8.0	50	0.5	5	0.3	3
6 years	8.5	5.0–14.5	4.3	1.5–8.0	51	3.5	1.5–7.0	42	0.4	5	0.2	3
8 years	8.3	4.5–13.5	4.4	1.5–8.0	53	3.3	1.5–6.8	39	0.4	4	0.2	2
10 years	8.1	4.5–13.5	4.4	1.8–8.0	54	3.1	1.5–6.5	38	0.4	4	0.2	2
16 years	7.8	4.5–13.0	4.4	1.8–8.0	57	2.8	1.2–5.2	35	0.4	5	0.2	3
21 years	7.4	4.5–11.0	4.4	1.8–7.7	59	2.5	1.0–4.8	34	0.3	4	0.2	3

Basophils[c]	
Absolute range	3–100/mm^3
Percentage range	0.10%–1.47%

[a] Numbers of leukocytes are in thousands per cubic millimeter, ranges are estimates of 95% confidence limits, and percentages refer to differential counts. Neutrophils include band cells at all ages and a small number of metamyelocytes and myelocytes in the first few days of life.

[b] Normal ranges vary slightly depending on automated analyzer used. The total leukocyte count is used to convert percentage values to absolute counts. Some automated hematology analyzers provide a full or limited differential. aValues are expressed in mg/L. Data were obtained from a total of 436 apparently healthy children. Values are 2.5th–97.5th percentiles.

[c] Data based on 668 normal individuals ages 5–74 years; range reflects 2.5th–97.5th percentiles; counts obtained using a Hemalog D cytochemical automated counter. From Simmons A, Leaverton P, Elbert G. Normal laboratory values for differential white cell counts established by manual and automated cytochemical methods (Hemalog D™). *J Clin Pathol.* 1974;27:55.

sTfR, Soluble transferrin receptor.

From Ooi CL, Lepage N, Nieuwenhuys E, et al. Pediatric reference intervals for soluble transferrin receptor and transferrin receptor-ferritin index. *World J Pediatr.* 2009;5:122. Dallman PR. Blood-forming tissues. In: Rudolph AM, ed. *Pediatrics*. 16th ed. New York: Appleton-Century-Crofts; 1977:1178.

TABLE 159.20 Reference Ranges for Pediatric Lymphocyte Subsets at Different Ages[a]

Age		Total Lymphocytes	CD4	CD8	CD2	CD3	CD19	Helper/Suppressor Ratio
Kotylo et al. (1993)								
0–6 months	%	0.62–0.72	0.50–0.57	0.08–0.31	0.55–0.88	0.55–0.82	0.11–0.45	–
(*n* = 10)	A	5.4–7.2	2.8–3.9	0.35–2.5	3.9–5.3	3.5–5.0	0.43–3.3	6.22
6–12 months	%	0.60–0.69	0.49–0.55	0.08–0.31	0.55–0.88	0.55–0.82	0.11–0.45	–
(*n* = 9)	A	5.3–6.7	2.6–3.5	0.35–2.5	3.8–4.9	3.4–4.6	0.43–3.3	6.22
12–18 months	%	0.56–0.63	0.46–0.51	0.08–0.31	0.55–0.88	0.55–0.82	0.11–0.45	–
(*n* = 9)	A	4.9–5.9	2.3–2.9	0.35–2.5	3.5–4.2	3.2–3.9	0.43–3.3	6.22
18–24 months	%	0.52–0.59	0.42–0.48	0.08–0.31	0.55–0.88	0.55–0.82	0.11–0.45	–
(*n* = 10)	A	4.4–5.5	1.9–2.5	0.35–2.5	3.1–3.9	2.8–3.5	0.43–3.3	6.22
24–30 months	%	0.45–0.57	0.38–0.46	0.08–0.31	0.55–0.88	0.55–0.82	0.11–0.45	–
(*n* = 9)	A	3.9–5.2	1.5–2.2	0.35–2.5	2.6–3.6	2.3–3.3	0.43–3.3	6.22
30–36 months	%	0.39–0.53	0.33–0.44	0.08–0.31	0.55–0.88	0.55–0.82	0.11–0.45	–
(*n* = 10)	A	3.3–5.1	1.2–2.0	0.35–2.5	2.2–3.5	1.9–3.1	0.43–3.3	6.22
3 years	%	0.22–0.69	0.27–0.57	0.14–0.34	0.65–0.84	0.55–0.82	0.09–0.29	–
(*n* = 73)	A	1.6–5.4	0.56–2.7	0.33–1.4	1.2–4.1	1.0–3.9	0.20–1.3	3.24

Age[b]		Total Lymphocytes	CD3	CD4	CD8	CD16/56	CD19
Shearer et al. (2003)[b]							
0–3 months	%		73 (53–84)	52 (35–64)	18 (12–28)	8 (4–18)	15 (6–32)
	A	5.4 (3.4–7.6)	3.68 (2.5–5.5)	2.61 (1.6–4.0)	0.98 (0.56–1.7)	0.42 (0.17–1.10)	0.73 (0.3–2.0)
3–6 months	%		66 (51–77)	46 (35–56)	16 (12–23)	6 (3–14)	25 (11–41)
	A	6.3 (3.9–9.0)	3.93 (2.5–5.6)	2.85 (1.8–4.0)	1.05 (0.59–1.6)	0.42 (0.17–0.83)	1.55 (0.43–3.0)
6–12 months	%		65 (49–76)	46 (31–56)	17 (12–24)	7 (3–15)	24 (14–37)
	A	5.9 (3.4–9.0)	3.93 (1.9–5.9)	2.67 (1.4–4.3)	1.04 (0.5–1.7)	0.40 (0.16–0.95)	1.52 (0.61–2.6)
1–2 years	%		65 (53–75)	41 (32–51)	20 (14–30)	7 (3–15)	25 (16–35)
	A	5.5 (3.6–8.9)	3.55 (2.1–6.2)	2.16 (1.3–3.4)	1.04 (0.62–2.0)	0.36 (0.18–0.92)	1.31 (0.72–2.6)
2–6 years	%		66 (56–75)	38 (28–47)	23 (16–30)	9 (4–17)	21 (14–33)
	A	3.6 (2.3–5.4)	2.39 (1.4–3.7)	1.38 (0.7–2.2)	0.84 (0.49–1.3)	0.30 (0.13–0.72)	0.75 (0.39–1.4)
6–12 years	%		69 (60–76)	37 (31–47)	25 (18–35)	9 (4–17)	18 (13–27)
	A	2.7 (1.9–3.7)	1.82 (1.2–2.6)	0.98 (0.65–1.5)	0.68 (0.37–1.1)	0.23 (0.10–0.48)	0.48 (0.27–0.86)
12–18 years	%		73 (56–84)	41 (31–52)	26 (18–35)	9 (3–22)	14 (6–23)
	A	2.2 (1.4–3.3)	1.48 (1.0–2.2)	0.84 (0.53–1.3)	0.53 (0.33–0.92)	0.19 (0.07–0.48)	0.3 (0.11–0.57)

Samples analyzed by Epics Profile (Coulter) flow cytometer following whole blood lysis.
%, Relative percentage; A, absolute cell count × 10^9/L.
Data from Kotylo PK, Fineberg NS, Freeman KS, et al. Reference ranges for lymphocyte subsets in pediatric patients. *Am J Clin Pathol.* 1993;100:111. See also Denny T, Yogev R, Gelman R, et al. Lymphocyte subsets in healthy children during the first 5 years of life. *JAMA.* 1992;267:1484. Copyright 1992, American Medical Association.
[a] Values are expressed as 95th percentile reference range.
[b] Data represent median value, 10th-90th percentiles; based on studies from healthy human immunodeficiency virus (HIV)-negative HIV-nonexposed children using flow cytometry at multiple core laboratories. Approximately 700 children per age group.
[c] Modified from Shearer WT, Rosenblatt HM, Gelman RS, et al. Lymphocyte subsets in healthy children from birth through 18 years of age: The Pediatric AIDS Clinical Trials Group P1009 study. *J Allergy Clin Immunol.* 2003;112:973.

IMMUNOGLOBULIN A

Summary points	• Important component of mucosal immunity—dimeric form in mucosal secretions, monomeric form in serum • IgA deficiency may occur asymptomatically and may or may not confer increased infection risk • Elevated levels may be seen in monoclonal gammopathies or in the setting of chronic inflammation or infection (liver disease, collagen vascular disease, autoimmune disease, chronic infections) leading to polyclonal gammopathy
Methodology	Nephelometry
Specimen requirements	Plain or serum separator tube
Indications	Suspected immune deficiency (recurrent infections), monoclonal gammopathy, isolated IgA deficiency, anaphylactic transfusion reaction to plasma
Reference range	Table 159.21
Interpretation	• Decreased with immune deficiency, celiac disease, some autoimmune diseases, medications, protein loss (nephrotic syndrome, protein-losing enteropathy), non-IgA monoclonal gammopathy • Increased in polyclonal hypergammaglobulinemia, monoclonal gammopathies, infections (tuberculosis, sepsis), chronic liver disease, collagen vascular disease, autoimmune disease, IgA nephropathy • Reference intervals may vary with methodology and patient ethnicity
Related tests	IgG, IgM, serum protein electrophoresis with immunofixation
Interfering substances	Lipemia, other causes of severe specimen turbidity, microbial contamination
Reference	Woof JM, Kerr MA. The function of immunoglobulin A in immunity. *J Pathol*. 2006;208:270.

Ig, Immunoglobulin.

TABLE 159.21 Plasma Levels of Immunoglobulin A in Males and Females, by Age

	Male			Female		
Age	**Number**	**mg/dL**	**g/L**	**Number**	**mg/dL**	**g/L**
Soldin et al. (1996)[a]						
1–30 days	61	1–20	0.01–0.20	56	1–19	0.01–0.19
31–182 days	52	7–56	0.07–0.56	60	1–59	0.01–0.59
183–365 days	37	9–107	0.09–1.07	23	15–90	0.15–0.90
1–3 years	105	18–171	0.18–1.71	127	25–141	0.25–1.41
4–6 years	138	60–231	0.60–2.31	135	47–206	0.47–2.06
7–9 years	111	77–252	0.77–2.52	105	41–218	0.41–2.18
10–12 years	104	61–269	0.61–2.69	115	73–239	0.73–2.39
13–15 years	104	42–304	0.42–3.04	118	82–296	0.82–2.96
16–18 years	112	89–314	0.89–3.14	100	90–322	0.90–3.22

	Male/Female	
Age	**mg/dL**	**g/L**
Lockitch et al. (1988)[b]		
0–12 months	0–100	0.00–1.00
1–3 years	24–121	0.24–1.21
4–6 years	33–235	0.33–2.35
7–9 years	41–368	0.41–3.68
10–11 years	64–246	0.64–2.46
12–13 years	70–432	0.70–4.32
14–15 years	57–300	0.57–3.00
16–19 years	74–419	0.74–4.19

[a] From Soldin SJ, Bailey J, Beatey J, et al. Pediatric reference ranges for immunoglobulins G, A, and M on the Behring nephelometer. *Clin Chem.* 1996;42:S308.

[b] From Lockitch G, Halstead AC, Quigley G, et al. Age- and sex-specific pediatric reference intervals: study design and methods illustrated by measurement of serum proteins with the Behring LN nephelometer. *Clin Chem*. 1988;34:1618. Results are 0.025th–0.975th fractiles.

IMMUNOGLOBULIN E

Summary points	• Important in immune response to deep parasitic (nematode) infections • Important mediator of allergic response; level often elevated in patients with bronchial asthma or atopic dermatitis
Methodology	Nephelometry, immunoassay
Specimen requirements	Plain or serum separator tube
Indications	Suspected allergic disease; monitoring allergic bronchopulmonary aspergillosis
Reference range	Table 159.22
Interpretation	• Low levels in hypogammaglobulinemia and selective IgE deficiency • Elevated levels in allergic diseases, deep parasitic infections, malignancy, drug allergy, immune deficiency, some infections, and inflammatory disorders; rarely monoclonal IgE gammopathies • IgE measurement has limited value in diagnosing atopy given the large overlap between atopic and nonatopic individuals • Normal levels do not exclude allergy
Related tests	IgA, IgG
Interfering substances	Lipemia, other causes of extreme turbidity, microbial contamination
References	Bacharier LB, Geha RS. Regulation of IgE synthesis: the molecular basis and implications for clinical modulation. *Allergy Asthma Proc*. 1999;20:1. Hamilton RG, Adkinson NF Jr. Clinical laboratory assessment of IgE-dependent hypersensitivity. *J Allergy Clin Immunol*. 2003;111:S687.

Ig, Immunoglobulin.

TABLE 159.22 Immunoglobulin E in Males and Females, by Age

Age	Females	Males
Soldin et al. (1995)[a]		
0–12 months	<8 KIU/L	<12 KIU/L
1–3 years	<28 KIU/L	<90 KIU/L
4–10 years	<137 KIU/L	<163 KIU/L
11–18 years	<398 KIU/L	<179 KIU/L
Soldin et al. (1995)[b]		
0–12 months	0–20 KIU/L	2–24 KIU/L
1–3 years	2–55 KIU/L	2–149 KIU/L
4–10 years	8–279 KIU/L	4–249 KIU/L
11–15 years	5–295 KIU/L	7–280 KIU/L
16–18 years	7–698 KIU/L	5–268 KIU/L
Ownby et al. (1991)[c]		
Cord blood	0.02–2.08 KIU/L	
Lindberg and Arroyave (1996)[d]		
<1 year	0–6.6 IU/mL	
1–2 years	0–20.0 IU/mL	
2–3 years	0.1–15.8 IU/mL	
3–4 years	0–29.2 IU/mL	
4–5 years	0.3–25.0 IU/mL	
5–6 years	0.2–17.6 IU/mL	
6–7 years	0.2–13.1 IU/mL	
7–8 years	0.3–46.1 IU/mL	
8–9 years	1.8–60.1 IU/mL	
9–10 years	3.6–81.0 IU/mL	
10–11 years	8.0–95.0 IU/mL	
11–12 years	1.5–99.7 IU/mL	
12–13 years	3.9–83.5 IU/mL	
13–16 years	3.3–188.0 IU/mL	

[a] From Soldin SJ, Lenherr S, Kumar A. Pediatric reference ranges for IgE. *Clin Chem*. 1995;41:S92.
[b] From Soldin SJ, Morales A, Albalos F, et al. Pediatric reference ranges on the Abbott IMx for FSH, LH, prolactin, TSH, T4, T3, free T4, free T3, T-uptake, IgE, and ferritin. *Clin Biochem*. 1995;28:603. Study was based on hospitalized patients. Values represent 2.5th–97.5th percentiles.
[c] From Ownby DR, Johnson CC, Peterson EL. Maternal smoking does not influence cord serum IgE or IgD concentrations. *J Allergy Clin Immunol*. 1991;88:555. Values constitute 95% of the measured samples.
[d] From Lindberg RE, Arroyave C. Levels of IgE in serum from normal children and allergic children as measured by an enzyme immunoassay. *J Allergy Clin Immunol*. 1986;78:614. Three hundred forty-six nonatopic children were selected for study by enzyme-linked immunosorbent assay methodology.

IMMUNOGLOBULIN G

Summary points	• Largest fraction of serum immunoglobulins; important component of secondary humoral immunity • Elevation seen in monoclonal gammopathies and many polyclonal infectious or inflammatory conditions
Methodology	Nephelometry
Specimen requirements	Plain or serum separator tube
Indications	Suspected immune deficiency (primary or secondary) or monoclonal gammopathy, assess response to immunization or recent or previous infection when antigen-specific IgM and IgG are tested
Reference range	Table 159.23
Interpretation	• Polyclonal elevations seen in hypergammaglobulinemia, chronic liver disease, collagen vascular diseases, autoimmune diseases, some infections • Monoclonal elevations seen in monoclonal gammopathies (plasma cell dyscrasias); confirmed by serum protein electrophoresis with immunofixation • Decreased in immune deficiencies, hypogammaglobulinemia, states of protein loss (protein-losing enteropathy, nephrotic syndrome), some malignancies, medications • Normal IgG levels do not exclude humoral immunodeficiency—further functional studies (assessment of antibody response post vaccine administration), and/or IgG subclasses may be required if high degree of clinical suspicion • Reference intervals may vary with methodology and patient ethnicity
Related tests	IgM, IgA, lymphocyte subsets, serum protein electrophoresis with immunofixation
Interfering substances	Lipemia, other causes of severe turbidity, microbial contamination

Ig, Immunoglobulin.

TABLE 159.23 Plasma Levels of Immunoglobulin G in Males and Females, by Age

	Male			Female		
Age	**Number**	**mg/dL**	**g/L**	**Number**	**mg/dL**	**g/L**
Soldin et al. (1996)[a]						
1–30 days	61	260–986	2.60–9.86	56	221–1031	2.21–10.31
31–182 days	52	195–643	1.95–6.43	60	390–794	3.90–7.94
183–365 days	37	184–974	1.84–9.74	23	407–774	4.07–7.74
1–3 years	105	507–1305	5.07–13.05	127	550–1407	5.50–14.07
4–6 years	138	571–1550	5.71–15.50	135	675–1540	6.75–15.40
7–9 years	111	700–1680	7.00–16.80	105	589–1717	5.89–17.17
10–12 years	104	818–1885	8.18–18.85	115	705–1871	7.05–18.71
13–15 years	104	709–1861	7.09–18.61	118	891–1907	8.91–19.07
16–18 years	112	632–1979	6.32–19.79	100	953–2108	9.53–21.08

	Male/Female	
Age	**mg/dL**	**g/L**
Lockitch et al. (1988)[b]		
0–12 months	273–1660	2.73–16.60
1–3 years	533–1078	5.33–10.78
4–6 years	593–1723	5.93–17.23
7–9 years	673–1734	6.73–17.34
10–11 years	821–1835	8.21–18.35
12–13 years	893–1823	8.93–18.23
14–15 years	842–2013	8.42–20.13
16–19 years	646–1864	6.46–18.64

[a] Soldin SJ, Bailey J, Beatey J, et al. Pediatric reference ranges for immunoglobulins G, A, and M on the Behring nephelometer. *Clin Chem*. 1996;42:S308.

[b] Lockitch G, Halstead AC, Quigley G, et al. Age- and sex-specific pediatric reference intervals: study design and methods illustrated by measurement of serum proteins with the Behring LN nephelometer. *Clin Chem*. 1988;34:1618. Results are 0.025th–0.975th fractiles.

IMMUNOGLOBULIN G SUBCLASSES

Summary points	• IgG subclass deficiencies are uncommon but may be associated with recurrent bacterial infections • Total serum IgG may be normal, even with a subclass deficiency
Methodology	Nephelometry
Specimen requirements	Plain or serum separator tube
Indications	Suspected humoral or combined immune deficiency with normal total serum IgG levels, recurrent respiratory, or sinus infections. Testing IgG subclasses has a limited role in the assessment of antibody-mediated immune function in patients with recurrent sinusitis and respiratory infections
Reference range	Table 159.24
Interpretation	• Decreased levels in hypogammaglobulinemia • Most frequent abnormality: IgG2 deficiency or combined IgG2–IgG4 deficiency. IgG2 deficiency may be associated with IgA deficiency in patients with chronic respiratory infection • Decreased IgG3 levels may be seen in primary immunodeficiencies (Wiskott–Aldrich syndrome) and type 1 diabetes mellitus • 70% of serum IgG is IgG1—selective IgG1 deficiency is rare • Significance of decreased IgG4 in absence of IgG2 deficiency uncertain
Related tests	IgG, IgM, IgA
Interfering substances	Lipemia, other causes of severe turbidity, microbial contamination
Reference	Maguire G. Are there any clinical indications for measuring IgG subclasses? *Ann Clin Biochem*. 2002;39:374.

Ig, Immunoglobulin.

TABLE 159.24 Immunoglobulin G Subclasses[a]

Age	IgG1	IgG2	IgG3	IgG4
Cord blood, preterm	3.4–9.7	0.7–1.7	0.2–0.5	0.2–0.7
Cord blood, term	5.8–13.7	0.6–5.2	0.2–1.2	0.2–1.0
5 years	5.6–12.7	0.4–4.4	0.3–1.0	0.1–0.8
6 years	6.2–11.3	0.5–4.0	0.3–0.8	0.2–0.9
7 years	5.4–10.5	0.9–3.5	0.3–1.1	0.2–1.1
8 years	5.6–10.5	0.7–4.5	0.2–1.1	0.1–0.8
9 years	3.9–11.4	0.7–4.7	0.4–1.2	0.2–1.0
10 years	4.4–10.8	0.6–4.0	0.3–1.2	0.1–0.9
11 years	6.4–10.9	0.9–4.3	0.3–0.9	0.2–1.0
12 years	6.0–11.5	0.9–4.8	0.4–1.0	0.2–0.9
13 years	6.1–11.5	0.9–7.9	0.2–1.1	0.1–0.8
Adults	4.8–9.5	1.1–6.9	0.3–0.8	0.2–1.1

[a] Values for males and females, expressed in g/L. Twenty schoolchildren from each age-group were tested. Only samples with C-reactive protein within the reference range were included. Enzyme-linked immunosorbent assay methodology was used on serum.

From Miles J, Riches P. The determination of IgG subclass concentrations in serum by enzyme-linked immunosorbent assay: establishment of age-related reference ranges for cord blood samples, children aged 5–13 years and adults. *Ann Clin Biochem*. 1994;31:245.

IMMUNOGLOBULIN M

Summary points	• Important component of the adaptive humoral immune response as initial antibody type produced after antigen exposure • Measurement of antigen-specific IgM and IgG can be useful in the diagnosis of recent or previous infection • Most common monoclonal immunoglobulin subtype elevated in Waldenström macroglobulinemia; associated with hyperviscosity syndrome
Methodology	Nephelometry
Specimen requirements	Plain or serum separator tube
Indications	Suspected immune deficiency, monoclonal gammopathy, assess response to immunization or recent or previous infection when antigen-specific IgM and IgG are tested
Reference range	Table 159.25
Interpretation	• Elevated in polyclonal hypergammaglobulinemia, chronic liver disease, collagen vascular disease, autoimmune disease, some infections, some immune deficiencies, monoclonal gammopathy (lymphoplasmacytic lymphoma and Waldenström macroglobulinemia), cryoglobulinemia • Decreases in immune deficiencies, hypogammaglobulinemia, chronic protein loss (protein-losing enteropathy, malnutrition), some malignancies • Reference intervals may vary with methodology and patient ethnicity
Related tests	IgG, IgA, lymphocyte subsets, serum protein electrophoresis with immunofixation
Interfering substances	Lipemia, other causes of severe turbidity, microbial contamination
Ig, Immunoglobulin.	

TABLE 159.25 Immunoglobulin M in Males and Females, by Age

	Male			Female		
Age	**Number**	**mg/dL**	**g/L**	**Number**	**mg/dL**	**g/L**
Soldin et al. (1996)[a]						
1–30 days	61	12–117	0.12–1.17	56	19–104	0.19–1.04
31–182 days	52	27–147	0.27–1.47	60	9–212	0.09–2.12
183–365 days	37	41–197	0.41–1.97	23	4–216	0.04–2.16
1–3 years	105	63–240	0.63–2.40	127	70–298	0.70–2.98
4–6 years	138	64–248	0.64–2.48	135	81–298	0.81–2.98
7–9 years	111	49–231	0.49–2.31	105	62–270	0.62–2.70
10–12 years	104	58–249	0.58–2.49	115	81–340	0.81–3.40
13–15 years	104	57–298	0.57–2.98	118	69–361	0.69–3.61
16–18 years	112	59–291	0.59–2.91	100	86–360	0.86–3.60

	Male/Female	
Age	**mg/dL**	**g/L**
Lockitch et al. (1988)[b]		
0–12 months	0–216	0.0–2.16
1–3 years	28–218	0.28–2.18
4–6 years	36–314	0.36–3.14
7–9 years	47–311	0.47–3.11
10–11 years	46–268	0.46–2.68
12–13 years	52–357	0.52–3.57
14–15 years	23–281	0.23–2.81
16–19 years	35–387	0.35–3.87

[a] From Soldin SJ, Bailey J, Beatey J, et al. Pediatric reference ranges for immunoglobulins G, A, and M on the Behring nephelometer. *Clin Chem.* 1996;42:S308. Plasma or serum.

[b] From Lockitch G, Halstead AC, Quigley G, et al. Age- and sex-specific pediatric reference intervals: study design and methods illustrated by measurement of serum proteins with the Behring LN nephelometer. *Clin Chem.* 1988;34:1618. Results are 0.025th–0.975th fractiles. Plasma or serum.

IMMUNOGLOBULIN LIGHT CHAINS

Summary points	• Measurement of free light chains is done for diagnosis and monitoring of amyloid, nonsecretory myeloma, and light chain myeloma • Kappa/lambda ratio is critical—excess of kappa versus lambda or excess of lambda versus kappa light chains is consistent with a plasma cell dyscrasia
Methodology	Nephelometry
Specimen requirements	Plain or serum separator tube
Indications	Suspected monoclonal gammopathy, amyloidosis, other plasma cell dyscrasia
Reference range	Table 159.26
Interpretation	Elevated free light chain with either kappa or lambda light chain restriction consistent with amyloidosis or another plasma cell dyscrasia
Related tests	Urine free light chains, urine protein electrophoresis with immunofixation, serum protein electrophoresis with immunofixation, serum immunoglobulin levels (IgG, IgA, IgM)
Interfering substances	Lipemia, other causes of severe turbidity, microbial contamination
Comments	May be more sensitive than serum protein electrophoresis with immunofixation for diagnosis of light chain disease

Ig, Immunoglobulin.

COMPLEMENT FRACTIONS C3 AND C4

Summary points	• Used to measure complement activation or deficiency • C3 and C4, measured together, examine both classical and alternative complement pathways
Methodology	Nephelometry, quantitative immunoturbidimetric, radioimmunodiffusion, electroimmunodiffusion
Specimen requirements	Plain or serum separator tube
Indications	Measure activity in collagen vascular diseases (systemic lupus erythematosus), complement deficiency, immune complex disease, glomerulonephritis, hereditary angioedema
Reference range	Table 159.27
Interpretation	• Reduced C4 with or without reduced C3 implies classical pathway activation as seen in lupus, immune complex disease, hereditary angioedema due to C1 inhibitor deficiency, chronic liver disease • Reduced C3 with normal C4 implies activation by the alternative pathway (e.g., sepsis) • Elevated C3 and/or C4 implies an acute-phase response • Primary deficiency is rare and requires functional assay
Related tests	Total complement CH50, C1q inhibitor
Interfering substances	Lipemia, microbial contamination
Reference	Wen L, Atkinson JP, Giclas PC. Clinical and laboratory evaluation of complement deficiency. *J Allergy Clin Immunol.* 113:585, 2004.

TABLE 159.26 Serum Levels of Immunoglobulin Light Chains in Males and Females[a]

Age	Kappa (g/L)	Lambda (g/L)	Kappa/Lambda Ratio
Newborn	8.10 (7.7–8.7)	4.70 (4.5–5.0)	1.74 (1.7–1.9)
Premature	4.29 (3.1–4.9)	2.91 (2.6–3.1)	1.70 (1.6–1.9)
1 month	4.46 (3.6–4.8)	2.32 (1.9–3.5)	1.80 (1.6–2.0)
2 months	2.58 (2.4–2.7)	1.88 (1.8–2.0)	1.66 (1.6–1.7)
3 months	2.07 (1.7–2.5)	2.25 (2.0–2.3)	1.09 (1.0–1.3)
4 months	2.09 (1.9–2.4)	2.40 (1.6–2.6)	0.98 (0.8–1.0)
5 months	2.36 (2.0–3.2)	2.54 (2.4–2.9)	1.02 (1.0–1.1)
1 year	4.46 (3.0–5.3)	3.91 (2.7–4.8)	0.99 (0.9–1.2)
2 years	5.32 (3.6–6.4)	3.82 (2.8–4.0)	1.42 (1.3–1.5)
3 years	4.98 (4.1–5.4)	3.67 (3.1–4.8)	1.31 (1.1–1.4)
4 years	6.78 (4.8–8.3)	4.87 (4.0–5.5)	1.51 (1.2–1.7)
5 years	6.58 (5.4–7.1)	4.01 (3.8–4.7)	1.55 (1.3–1.7)
6 years	8.02 (6.0–8.5)	4.01 (3.0–6.0)	1.61 (1.6–1.7)
7 years	5.90 (4.6–7.5)	4.08 (3.9–4.5)	1.64 (1.4–1.8)
8 years	8.34 (7.3–9.1)	4.64 (5.1–6.4)	1.50 (1.4–1.7)
9 years	8.26 (7.1–9.6)	4.95 (3.3–5.5)	1.69 (1.5–1.8)
10 years	9.03 (8.2–9.6)	5.32 (4.9–5.7)	1.80 (1.3–1.9)
11 years	7.18 (6.0–8.3)	4.10 (3.8–4.8)	1.67 (1.4–1.8)
12 years	9.17 (8.2–9.6)	4.90 (4.1–6.0)	1.82 (1.7–2.0)
13 years	9.59 (8.2–10.5)	5.86 (5.3–6.1)	1.64 (1.4–1.7)
14 years	9.45 (8.0–10.8)	5.50 (4.9–5.8)	2.09 (1.9–2.2)
15 years	10.18 (7.2–11.0)	4.75 (4.1–5.0)	2.25 (1.7–2.3)
16 years	9.37 (7.0–11.2)	5.09 (4.6–5.6)	1.89 (1.5–2.2)

[a] Median and ranges given as 10th–90th percentiles.

From Bradwell AR, Carr-Smith HD, Mead GP, et al. Serum test for assessment of patients with Bence Jones myeloma. *Lancet.* 2003;361:489; Herkner KR, Salzer H, Böck A, et al. Pediatric and perinatal reference intervals for immunoglobin light chains kappa and lambda. *Clin Chem.* 1992;38:548.

TABLE 159.27 Complement Fractions C3 and C4 in Males and Females, by Age

Study	Age	g/L
C3		
Zillow et al. (1993)[a]	Healthy neonates	0.30–0.98
Lockitch et al. (1988)[b]	0–5 days	0.26–1.04
	1–19 years	0.51–0.95
	Adult	0.45–1.8
C4		
Lockitch et al. (1988)[b]	0–5 days	0.06–0.37
	1–19 years	0.08–0.44
	Adult	0.11–0.5

[a] From Zillow G, Zillow EP, Burger R, et al. Complement activation in newborn infants with early onset infection. *Pediatr Res.* 1993;34:199. Results are 0–100th percentiles. Thirty-two healthy full-term newborns, birth weights between 2500 and 4020 g, were tested. All mothers (vagina, rectum) and infants (ear, gastric) were culture negative.

[b] Lockitch G, Halstead AC, Quigley G, et al. Age- and sex-specific pediatric reference intervals: study design and methods illustrated by measurement of serum proteins with the Behring LN nephelometer. *Clin Chem.* 1988;34:1618. Results are 0.025th–0.975th fractiles.

PLATELET AND COAGULATION TESTING

PLATELET COUNT AND MEAN PLATELET VOLUME

Summary points	• Used to assess the integrity of hematopoietic and hemostatic systems
Methodology[a]	Automated hematology analyzer—flow cytometric or impedance methods
Specimen requirements	Whole blood: EDTA
Indications	Suspected thrombocytopenia or thrombocytosis
Reference range	Table 159.28

Interpretation	• Thrombocytopenia
	  • Primary marrow process: infiltration by tumor or fibrosis, bone marrow failure, marrow suppression (drugs, toxins, infections), myelodysplasia
	  • Secondary consumptive or destructive process: immunologic destruction, hypersplenism, microangiopathy, disseminated intravascular coagulopathy
	• Thrombocytosis
	  • Primary: essential thrombocythemia, other MPNs
	  • Secondary: acute-phase reactant, splenectomy, iron deficiency
	• MPV may increase in destructive processes when larger, immature platelets prevail
	• MPV is elevated in hereditary macrothrombocytopenia (Bernard-Soulier syndrome, MYH9 disorders) and ITP
	• MPV is low in Wiskott-Aldrich syndrome

Related tests	Peripheral smear, complete blood count, platelet aggregometry
Interfering substances	Clotted or hemolyzed sample; difficult or prolonged collections may activate platelets and artifactually decrease the count

[a] Platelet count may also be estimated by peripheral smear review (count platelets in five fields using a 100× oil-immersion lens, take the average count, and multiply by 15,000) or manually counted using phase contrast microscopy and a hemocytometer. Automated methods using a hematology analyzer are usually more accurate and preferred.

EDTA, Ethylenediaminetetraacetic acid; *ITP*, immune thrombocytopenic purpura; *MPV*, mean platelet volume; *MYH9*, myosin heavy chain 9; MPNs, myeloproliferative neoplasms.

TABLE 159.28 **Platelet Counts and Mean Platelet Volumes in Children and Adults**

Age (Years)	Platelet Count (10^9/L)	Mean Platelet Volume (fL)
1–10	220–422	8.3–10.9
10–18	165–396	8.6–12.1
18–45	159–376	8.5–12.8
45–65 (males)	156–300	8.6–12.0
45–65 (females)	156–351	9.0–12.9
>65	139–363	8.8–12.4

Data from Giacomini A, Legovini P, Gessoni G, et al. Platelet count and parameters determined by the Bayer ADVIA 120 in reference subjects and patients. *Clin Lab Haematol.* 2001;23:181. Fifty male and 50 female subjects per age range were tested. Range represents 95% confidence limits.

IMMATURE PLATELET FRACTION

Summary points	• Similar to reticulated platelet count by flow cytometry, but available on automated hematology analyzers • Analogous to reticulocyte count for erythropoiesis, IPF assesses the rate of thrombopoiesis, can help distinguish decreased marrow production versus peripheral destruction as likely cause of thrombocytopenia
Methodology	Automated hematology analyzer—flow cytometric method using light scatter and fluorescence dyes that stain RNA
Specimen requirements	Whole blood: EDTA
Indications	Aid in the differential diagnosis of thrombocytopenia, assess marrow response after marrow injury, chemotherapy, or bone marrow transplant
Reference range	Table 159.29
Interpretation	• Increased with increased platelet production as seen in thrombocytopenia due to peripheral destruction (ITP, TTP; assuming normally functioning marrow) • Rise in IPF occurs with marrow engraftment posttransplant; precedes a rise in platelet count by a couple of days • Normal or decreased with impaired thrombopoiesis (bone marrow failure, suppression) • Increased in patients with macrothrombocytopenia • May be artifactually elevated with platelet clumping
Related tests	Platelet count, mean platelet volume, complete blood count
Interfering substances	Clotted or hemolyzed specimen
References	Briggs C. Quality counts: new parameters in blood cell counting. *Int J Lab Hematol.* 2009;31:277. Miyazaki K, Koike Y, Kunishima S, et al. Immature platelet fraction measurement is influenced by platelet size and is a useful parameter for discrimination of macrothrombocytopenia. *Hematology.* 2015;20:587.

EDTA, Ethylenediaminetetraacetic acid; *IPF*, immature platelet fraction; *ITP*, immune thrombocytopenic purpura; *RNA*, ribonucleic acid; *TTP*, thrombotic thrombocytopenic purpura.

TABLE 159.29 **Immature Platelet Fraction Reference Values in Healthy Adults**

N	Mean (%)	Range (%)
50	3.4	1.1–6.1[a]
142	1.7	0.4–5.4[b]
129	3.3	1.0–10.3[c]

[a] Briggs C, Kunka S, Hart D, et al. Assessment of an immature platelet fraction (IPF) in peripheral thrombocytopenia. *Br J Haematol.* 2004;126:93.

[b] Cho YG, Lee JH, Kim DS, et al. Clinical usefulness of the simple technique to diagnose thrombocytopenia using immature platelet fraction. *Korean J Lab Med.* 2007;27(1):1.

[c] Abe Y, Wada H, Tomatsu H, et al. A simple technique to determine thrombopoiesis level using immature platelet fraction (IPF). *Thromb Res.* 2006;118:463.

Coagulation Screening Tests

PROTHROMBIN TIME (PT) AND INTERNATIONAL NORMALIZED RATIO (INR)

Summary points	• PT is used to screen for extrinsic or common clotting factor deficiencies INR is used as a universal standard to monitor warfarin therapy
Methodology	Clot detection
Specimen requirements	Citrate
Indications	Suspected congenital or acquired coagulopathy involving the extrinsic and/or common pathways, monitor anticoagulation
Reference range	Table 159.30
Interpretation	• Reference ranges dependent on reagents and instrumentation, requiring laboratory-specific and population-specific ascertainment • Prolonged PT suggests a deficiency of one or more factors (II, V, VII, X) or the presence of a coagulation inhibitor • A prolonged PT with normal aPTT could suggest vitamin K deficiency, early liver failure, early disseminated intravascular coagulation, or FVII deficiency • PT can also be prolonged in the setting of markedly low fibrinogen • Mild common pathway factor deficiencies may appear with a prolonged PT and normal aPTT depending on the sensitivity of each assay • INR is intended to normalize PT reagents so that PT measured at any center worldwide yields comparable INR values • INR is a calculated value determined by the International Sensitivity Index (ISI) which is specific to the instrument and reagent used • Calculation of INR: $\mathrm{INR} = \left\{\frac{\mathrm{PT}^{\mathrm{Patient}}}{\mathrm{PT}^{\mathrm{Mean}}}\right\}^{\mathrm{ISI}}$ $\mathrm{PT}^{\mathrm{Patient}} = \text{Patient's PT}$ $\mathrm{PT}^{\mathrm{Patient}} = \text{Mean PT of Normal Range}$ $\mathrm{ISI} = \text{International Sensitivity Index}$ • The INR was only intended to be used to monitor therapy for patients taking Vitamin K (VKA) antagonists, though it is frequently employed outside of this setting, particularly in liver disease
Related tests	aPTT, thrombin time, coagulation factor levels, fibrinogen, factors II, V, VII, VIII, IX, X, XI, other contact pathway factors, lupus anticoagulant, mixing studies
Interfering substances	High hematocrit (>55%) can lead to false prolongation of the PT Direct oral anticoagulants can have variable effects on PT Clotted or hemolyzed sample
References	Ng VL. Prothrombin time and partial thromboplastin time assay considerations. *Clin Lab Med.* 2009;29(2):253–263. Ten Cate H, Henskens YM, Lancé MD. Practical guidance on the use of laboratory testing in the management of bleeding in patients receiving direct oral anticoagulants. *Vasc Health Risk Manag.* 2017;13:457–467.

aPTT, Activated partial thromboplastin time; *FVII*, factor VII; *INR*, international normalized ratio; *ISI*, international sensitivity index; *PT*, prothrombin time.

TABLE 159.30 Reference Values for Coagulation Tests in Infants, Children, and Adults

Coagulation Test	Day 1	Day 3	1 Month–1 Year	1–5 Years	6–10 Years	11–16 Years	Adults
PT (s)	15.6[a]	14.9[a]	13.1	13.3[a]	13.4[a]	13.8[a]	13
	14.4–16.4	13.5–16.4	11.5–15.3	12.1–14.5	11.7–15.1	12.7–16.1	11.5–14.5
INR	1.26[a]	1.20[a]	1	1.03[a]	1.04[a]	1.08[a]	1
	1.15–1.35	1.05–1.35	0.86–1.22	0.92–1.14	0.87–1.20	0.97–1.30	0.80–1.20
aPTT (s)	38.7[a]	36.3[a]	39.3[a]	37.7[a]	37.3[a]	39.5[a]	33.2
	34.3–44.8	29.5–42.2	35.1–46.3	33.6–43.8	31.8–43.7	33.9–46.1	28.6–38.2
Fibrinogen (g/L)	2.8	3.3	2.42[a]	2.82[a]	3.04	3.15	3.1
	1.92–3.74	2.83–4.01	0.82–3.83	1.62–4.01	1.99–4.09	2.12–4.33	1.9–4.3
II	54[a]	62[a]	90[a]	89[a]	89[a]	90[a]	110
	41–69	50–73	62–103	70–109	67–110	61–107	78–138
V	81[a]	122	113	97[a]	99[a]	89[a]	118
	64–103	92–154	94–141	67–127	56–141	67–141	78–152
VII	70[a]	86[a]	128	111[a]	113[a]	118	129
	52–88	67–107	83–160	72–150	70–156	69–200	61–199
VIII	182	159	94[a]	110[a]	117[a]	120[a]	160
	105–329	83–274	54–145	36–185	52–182	59–200	52–290
vWF[b]	N/A	N/A	N/A	82	95	100	92
				60–120	44–144	46–153	50–158
IX	48[a]	72[a]	71[a]	85[a]	96[a]	111[a]	130
	35–56	44–97	43–121	44–127	48–145	64–216	59–254
X	55[a]	60[a]	95[a]	98[a]	97[a]	91[a]	124
	46–67	46–75	77–122	72–125	68–125	53–122	96–171
XI	30[a]	57[a]	89[a]	113	113	11	112
	7–41	24–79	62–125	65–162	65–162	65–139	67–196
XII	58[a]	53[a]	79[a]	85[a]	81[a]	75[a]	115
	43–80	14–80	20–135	36–135	26–137	14–117	35–207
Prekallikrein[b]	N/A	N/A	N/A	95	99	99	112
				65–130	66–131	53–145	62–162
HMWK[b]	N/A	N/A	N/A	98	93	91	92
				64–132	60–130	63–119	50–136
XIIIa[b]	N/A	N/A	N/A	108[a]	109[a]	99	105
				72–143	65–151	57–140	55–155
XIIIs[b]	N/A	N/A	N/A	113[a]	116[a]	102	97
				69–156	77–154	60–143	57–137

aPTT, Activated partial thromboplastin time; *HMWK*, high-molecular-weight kininogen; *INR*, international normalized ratio; *PT*, prothrombin time; *vWF*, von Willebrand factor. For each assay mean and boundaries including 95% of the population are shown. All factor levels except fibrinogen are expressed as percentages where 100% = 1.0 unit/mL.

[a] Denotes values that are significantly different from adult values ($p < .05$). Data from Monagle P, Barnes C, Ignjatovic V, et al. Developmental haemostasis. Impact for clinical haemostasis laboratories. *Thromb Haemost.* 2006;95:362.

[b] From Andrew M, Vegh P, Johnston M, et al. Maturation of the hemostatic system during childhood. *Blood.* 1992;80:1998.

ACTIVATED PARTIAL THROMBOPLASTIN TIME

Summary points	• aPTT is primarily used to screen for intrinsic clotting factor deficiencies, but also picks up common pathway deficiencies and may be prolonged in von Willebrand disease • aPTT is one method used to monitor unfractionated heparin therapy
Methodology	Clot detection
Specimen requirements	Citrate
Indications	• Suspected congenital or acquired coagulopathy involving the intrinsic and/or common pathways, monitor anticoagulation
Reference range	See Table 159.30
Interpretation	• Reference ranges dependent on reagents and instrumentation, requiring laboratory-specific and population-specific ascertainment (e.g., aPTT is normally prolonged during the neonatal period) • Normal aPTT does not exclude mild, but clinically significant, factor deficiency (especially hemophilia B and von Willebrand disease) • Prolonged aPTT with normal PT suggests a deficiency of one or more factors (VIII, IX, XI, or other contact pathway factors) or the presence of inhibitors such as lupus anticoagulant or heparin (consider heparin contamination in specimen drawn from a heparinized line) • Prolonged PT and aPTT suggests moderate to severe vitamin K deficiency, liver disease, disseminated intravascular coagulation, common pathway factor deficiency • Markedly low fibrinogen can prolong the aPTT • Factor XII, HMWK, and prekallikrein deficiencies can prolong the aPTT but are not associated with clinical bleeding • Lupus anticoagulant can prolong the aPTT but is associated with risk for thrombosis rather than bleeding • High circulating factor VIII and/or thrombin can shorten the aPTT and may be useful for assessing thrombotic risk
Related tests	PT, thrombin time, coagulation factor levels, fibrinogen, factors II, V, VII, VIII, IX, X, XI, other contact pathway factors, lupus anticoagulant, mixing studies
Interfering substances	High hematocrit (>55%) can lead to false prolongation of the aPTT Direct oral anticoagulants can have variable effects on aPTT Contamination from lines containing heparin or direct thrombin inhibitors Elevated C-reactive protein leads to false prolongation Clotted or hemolyzed sample
References	Ng VL. Prothrombin time and partial thromboplastin time assay considerations. *Clin Lab Med.* 2009;29(2):253–263. Ten Cate H, Henskens YM, Lancé MD. Practical guidance on the use of laboratory testing in the management of bleeding in patients receiving direct oral anticoagulants. *Vasc Health Risk Manag.* 2017;13:457–467. Levy JH, Szlam F, Wolberg AS, Winkler A. Clinical use of the activated partial thromboplastin time and prothrombin time for screening: a review of the literature and current guidelines for testing. *Clin Lab Med.* 2014;34(3):453–477.

aPTT, Activated partial thromboplastin time; *HMWK*, high-molecular-weight kininogen; *PT*, prothrombin time

FIBRINOGEN

Summary points	• Used to detect acquired or congenital afibrinogenemia, hypofibrinogenemia, dysfibrinogenemia • Can be used for the detection and assessment of both hemorrhagic and thrombotic diatheses • Fibrinogen should be carefully monitored in fibrinolytic therapy and DIC
Methodology	Clot detection (functional, Clauss and PT-derived), Immunoassay (antigenic)
Specimen requirements	Citrate
Indications	Investigation of bleeding due to suspected decrease or dysfunction of fibrinogen Assessment of thrombotic risk (hyperfibrinogenemia) Monitoring during thrombolytic therapy
Reference range	Tables 159.30 and 159.32
Interpretation	• Fibrinogen is decreased in the setting of congenital afibrinogenemia, congenital hypofibrinogenemia, hemodilution, disseminated intravascular coagulation, fibrinolysis, and liver disease. • Dysfibrinogenemia (abnormal fibrinogen function) can be detected by discrepancies between functional and antigenic levels of fibrinogen • Acquired dysfibrinogenemia can occur in the setting of liver disease • Systemic thrombolytic therapy can rapidly reduce fibrinogen levels, monitoring fibrinogen levels during therapy is recommended • Fibrinogen is an acute-phase reactant, and levels can become increased in response to a variety of physiologic stimuli including: inflammation, pregnancy/oral contraceptives, and disseminated malignancy • Elevated fibrinogen is a risk factor for thrombosis
Related tests	PT, aPTT, thrombin time, reptilase time, thromboelastography/thromboelastogram
Interfering substances	High levels of fibrin degradation products or paraproteins can inhibit fibrin polymerization High levels of heparin (if surpass the heparin neutralization limits of the assay) Icteric or hemolyzed specimens (optical methods), turbid or lipemic specimens (mechanical clot detection)
References	Mackie IJ, Kitchen S, Machin SJ, Lowe GD, Haematology, Haemostasis and Thrombosis Task Force of the British Committee for Standards in Haematology. Guidelines on fibrinogen assays. *Br J Haematol.* 2003;121(3):396–404.

aPTT, Activated partial thromboplastin time; *PT*, prothrombin time

TABLE 159.31 Natural Anticoagulant Reference Values in Infants, Children, and Adults

Assay	Day 1	Day 3	1 month–1 year	1–5 years	6–10 years	11–16 Years	Adults
AT	76[a]	74[a]	109[a]	116[a]	114[a]	111[a]	96
	58–90	60–89	72–134	101–131	95–134	96–126	66–124
	18	22	41	49	59	26	43
PC clotting	32[a]	33[a]	77[a]	94[a]	94[a]	88[a]	103
	24–40	24–51	28–124	50–134	64–125	59–112	54–166
	20	22	24	39	50	20	44
PS clotting	36[a]	49[a]	102[a]	101[a]	109[a]	103[a]	75
	28–47	33–67	29–162	67–136	64–154	65–140	54–103
	22	24	41	49	59	27	44
TFPI free (ng/mL)			7.13[a]	10.16	6.69[a]	7.66[a]	10.7
			5.63–8.44	5.06–9.05	4.29–9.31	5.15–8.74	6.12–12.34
			13	21	20	15	20
TFPI total (ng/mL)			77.49	76.33	73.99[a]	74.09[a]	87.49
			69.42–85.58	61.27–89.8	59.13–88.02	61.63–87.36	63.64–104.38

[a]Denotes values that are significantly different from adult values ($p < .05$).

AT, Antithrombin; *PC*, protein C; *PS*, protein S; *TFPI*, tissue factor pathway inhibitor.

Data from Monagle P, Barnes C, Ignjatovic V, et al. Developmental haemostasis. Impact for clinical haemostasis laboratories. *Thromb Haemost.* 2006;95:362.

For each assay the first two rows show mean and boundaries including 95% of the population. The next row shows the number of individual samples.

TABLE 159.32 Comparison of Selected Coagulation Factor Values in Newborns[a]

Study	Fibrinogen (mg/dL)	Factor II (units/mL)	Factor VIII (units/mL)	Factor IX (units/mL)	Factor XII (units/mL)	Antithrombin (units/mL)	Protein C (units/mL)
Term							
Hathaway and Bonnar (1987) and Manco-Johnson et al. (1988)[b]	240 (150)	0.52 (0.25)	1.5 (0.55)	0.35 (0.15)	0.44 (0.16)	0.56 (0.32)	0.32 (0.16)
Andrews et al. (1987, 1988)[c]	283 (177)	0.48 (0.26)	1.0 (0.50)	0.53 (0.25)	0.53 (0.20)	0.63 (0.25)	0.35 (0.17)
Corrigan (1992)[d]	246 (150)	0.45 (0.22)	1.68 (0.50)	0.40 (0.20)	0.44 (0.16)	0.52 (0.20)	0.31 (0.17)
Preterm							
Hathaway and Bonnar (1987) and Manco-Johnson et al. (1988)[b]	300 (120)	0.45 (0.26)	0.93 (0.54)	0.41 (0.20)	0.33 (0.23)	0.40 (0.25)	0.24 (0.18)
Andrews et al. (1987, 1988)[c]	243 (150)	0.45 (0.20)	1.1 (0.50)	0.35 (0.19)	0.38 (0.10)	0.38 (0.14)	0.28 (0.12)
Corrigan (1992)[d]	240 (150)	0.35 (0.21)	1.36 (0.21)	0.35 (0.10)	0.22 (0.09)	0.35 (0.10)	0.28 (0.12)

Note: In summary, plasma levels of the vitamin K–dependent factors (including proteins C and S), contact factors, and antithrombin are decreased at birth.

[a]Data are expressed as mean and lower limit of normal (in parentheses). Preterm, 30–36 weeks' gestational age.

[b]Data from Hathaway WE, Bonnar J. *Hemostatic Disorders of the Pregnant Woman and Newborn Infant.* New York: Elsevier Science; 1987; Manco-Johnson M, Marlar R, Jacobson L, et al. Severe protein C deficiency in newborn infants. *J Pediatr.* 1988;113:359.

[c]Data from Andrews M, Paes B, Milner R, et al. Development of the human coagulation system in the full-term infant. *Blood.* 1987;70:165; Andrews M, Paes B, Milner R, et al. Development of the human coagulation system in the healthy premature infant. *Blood.* 1988;72:1651.

[d]Data from Corrigan JJ Jr. Normal hemostasis in fetus and newborn: coagulation. In: Polin RA, Fox WW, eds. *Fetal and Neonatal Physiology*. Philadelphia: WB Saunders; 1992:1368–1371; From Hathaway W, Corrigan J. Report of scientific and standardization subcommittee on neonatal hemostasis. *Thromb Haemost.* 1991;65:323.

MIXING STUDIES

Summary points	• Performed by mixing 1:1 normal pooled plasma (NPP) with patient sample with PT and/or aPTT measured immediately and after incubation for 1–2 h • Used to determine if PT or aPTT is prolonged due to factor deficiency or inhibitor
Methodology	Clot detection
Specimen requirements	Citrate
Indications	Suspected congenital or acquired factor deficiency or inhibitor involving the intrinsic, extrinsic, and/or common pathways Evaluation for lupus anticoagulant
Reference range	Institutional reference range for PT and/or aPTT

Interpretation	• PT mixing study:
	  • Corrected immediately: FVII deficiency, common factor pathway deficiency (factors II, V, X) if aPTT also prolonged. Corrected PT mixing study most frequently suggests multiple factor deficiency
	  • Incompletely corrected: Lupus anticoagulant or extrinsic/common factor pathway inhibitor
	• aPTT mixing study:
	  • Corrected immediately: Intrinsic pathway factor deficiency (factors VIII, IX, XI, XII) if only aPTT prolonged, common pathway factor deficiency (factors II, V, X) if PT also prolonged
	  • Corrected immediately, incompletely corrected after incubation: FVIII inhibitor
	  • Incompletely corrected: Lupus anticoagulant or intrinsic/common pathway factor inhibitor
	• Mixing studies are difficult to standardize, consultation with the performing lab is recommended when only partial correction occurs
	• Weak inhibitors may be completely corrected by mixing study
	• Generally should not be performed on patients with known anticoagulant therapy and can be of some utility when the type of anticoagulant is unknown
	• When clinical suspicion is high for the deficiency of a particular factor, performing directed factor studies may be more efficient

Related tests	Lupus anticoagulant, factor studies, PT, aPTT
Interfering substances	High hematocrit (>55%) can lead to false prolongation Anticoagulant therapy (heparin, direct thrombin inhibitors, direct oral anticoagulants) Clot or hemolysis
References	Favaloro EJ. Coagulation mixing studies: utility, algorithmic strategies and limitations for lupus anticoagulant testing or follow up of abnormal coagulation tests. *Am J Hematol.* 2020;95(1):117–128. doi:10.1002/ajh.25669

aPTT, Activated partial thromboplastin time; *FVII*, factor VII; *FVIII*, factor VIII; *NPP*, normal pooled plasma; *PT*, prothrombin time

LUPUS ANTICOAGULANT

Summary points	• A variety of different assays can be utilized for the detection of lupus anticoagulant (LA) and are based on identifying phospholipid-dependent inhibition • Guidelines recommend two tests be performed; the dRVVT and a sensitive aPTT are generally used • Repeat testing should be performed >12 weeks after initial testing for confirmation of lupus anticoagulant
Methodology	Clot detection
Specimen requirements	Citrate
Indications	Performed for patients suspected of having antiphospholipid antibody syndrome or those found to have unexpectedly prolonged aPTT on routine coagulation testing
Reference range	Cut-off determined by each institution and should be >99th percentile of the distribution of healthy donors. A dRVVT ratio of >1.2 is generally considered to be positive
Interpretation	• LA is a misnomer. While the presence of LA can prolong routine coagulation tests, particularly the aPTT, it is a risk factor for thrombosis rather than bleeding • dRVVT is the first test considered, while the second test is commonly a sensitive aPTT-based assay (low phospholipid content using a silica activator) • A screening test is performed first and is determined to be positive when the clotting time is longer than the institutional reference range • The confirmatory test is performed by increasing the concentration of phospholipids in the assay. Demonstration of correction (greater than the local cut-off) following the addition of excess phospholipids confirms the presence of LA • LA is determined to be present when at least one of the two assays performed is positive, though this must be confirmed with repeat testing >12 weeks after initial testing • Samples should be obtained in the absence of anticoagulation therapy whenever possible, as the various anticoagulants have variable effects on lupus anticoagulant testing. Notably, the dRVVT is particularly sensitive to anti-Xa therapies
Related tests	aPTT, mixing studies, antiphospholipid assays
Interfering substances	Anticoagulants: heparin, direct thrombin inhibitors, direct oral anticoagulants, VKA Clotted or hemolyzed specimens
References	Pengo V, Tripodi A, Reber G, et al. Update of the guidelines for lupus anticoagulant detection. Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis. *J Thromb Haemost.* 2009;7(10):1737–1740. doi:10.1111/j.1538-7836.2009.03555.x Favaloro EJ. The Russell viper venom time (RVVT) test for investigation of lupus anticoagulant (LA). *Am J Hematol.* 2019;94(11):1290–1296. doi:10.1002/ajh.25606

aPTT, Activated; *dRVVT*, dilute Russell Viper Venom time; *LA*, lupus anticoagulant; *VKA*, vitamin K antagonist

COAGULATION FACTOR LEVELS (II, V, VII, VIII, IX, X, XI, XII, CONTACT PATHWAY FACTORS)

Summary points	• Choice of which specific factor(s) to measure driven by clinical scenario and screening test results (e.g., isolated prolonged PT suggests FVII deficiency, isolated preoperative aPTT suggests measurement of at least FVIII, FIX, and FXI, to assess bleeding risk)
Methodology	Clot detection, chromogenic (factors VIII and IX)
Specimen requirements	Citrate
Indications	Suspected coagulation factor deficiency, especially as suggested by prolonged coagulation screening test and subsequent mixing study that corrects, assessment of risk factors for thrombosis (e.g., high FVIII), monitoring of factor-replacement therapy in patients with coagulopathy
Reference range	Tables 159.30 and 159.32
Interpretation	• No definitive threshold for what constitutes a hemostatically adequate factor level, but the criteria for diagnosing mild factor deficiency are often set at 40%–50% • FVIII and von Willebrand factor are acute-phase reactants • Factor deficiency may be due to reduced production (hereditary factor deficiency, liver disease, vitamin K deficiency), increased consumption (disseminated intravascular coagulation), or specific inhibitor (FVIII) • Acquired deficiency of multiple factors occurs with liver disease, vitamin K deficiency (including vitamin K–antagonist therapy or toxicity), disseminated intravascular coagulation—FV, FVII, and FVIII may aid in distinguishing between these • Rare inherited combined factor deficiencies exist (combined FV/FVIII, vitamin K–dependent factors) • With exception of FXI, contact factor deficiencies (FXII, high-molecular-weight kininogen, prekallikrein) may cause prolonged aPTT but are not associated with increased bleeding risk • Vitamin K–dependent and contact factors are physiologically low in early infancy • FV and FVIII are particularly labile, with potential for spuriously low levels—carefully consider abnormally low levels in clinical and laboratory context and assess the need for repeat/confirmatory testing • One-stage (aPTT-based) assays are affected by the presence of lupus anticoagulant and various anticoagulants, chromogenic assays may be preferred in these settings • Chromogenic assays should be considered in the evaluation of mild hemophilia A and are preferred for monitoring particular types of factor VIII and IX replacement therapies
Related tests	Specific factor inhibitor testing, lupus anticoagulant, mixing studies
References:	Marlar RA, Strandberg K, Shima M, Adcock DM. Clinical utility and impact of the use of the chromogenic vs one-stage factor activity assays in haemophilia A and B. *Eur J Haematol.* 2020;104(1):3–14. https://doi.org/10.1111/ejh.13339

aPTT, Activated partial thromboplastin time; *FIX*, factor IX; *FV*, factor V; *FVII*, factor VII; *FVIII*, factor VIII; *FXI*, factor XI; *FXII*, factor XII; *PT*, prothrombin time.

BETHESDA ASSAY/NIJMEGEN-BETHESDA ASSAY

Summary points	• Bethesda unit (BU) = the amount of inhibitor that will neutralize 50% factor VIII activity after 2-h incubation • Assay generally used for the identification of factor VIII inhibitors, but the methodology can be used for other factor inhibitors • Heat inactivation of residual factor activity can be used for the assessment of patients on replacement therapy
Methodology	Clot-based or chromogenic
Specimen requirements	Citrate
Indications	Assessment of patients with suspected factor inhibitors, particularly those with hemophilia A receiving factor-replacement therapy
Reference range	0 BU/mL
Interpretation	• Testing is usually performed for patients with less than the usual response to replacement therapy • Identification of inhibitor suggests patients will require increased factor-replacement or change-in-factor therapy • Recent factor therapy may affect test results depending on institutional methodology (use of heat inactivation) • This assay will detect inhibitors that affect invitro activity, inhibitors causing increased clearance of factors will not be detected by this method • While traditionally used for factor VIII inhibitors (the most commonly identified factor inhibitor), testing for other factor inhibitors can be performed using the same methodology
Related tests	Factor activity assays, aPTT, PT
Interfering substances	Recent replacement therapy may lead to invalid results Lupus anticoagulant and heparin therapy can cause false positives Hemolyzed or clotted samples
References	Duncan E, Collecutt M, Street A. Nijmegen-Bethesda Assay to measure factor VIII inhibitors. In: Monagle P, ed. *Haemostasis. Methods in Molecular Biology (Methods and Protocols).* Vol 992. Totowa, NJ: Humana Press; 2013

aPTT, Activated partial thromboplastin time; *BU*, Bethesda unit; *PT*, prothrombin time.

VON WILLEBRAND ANTIGEN/ACTIVITY

Summary points	• von Willebrand disease (vWD) is the most common inherited bleeding disorder and is caused by abnormalities in the vW protein – these may be quantitative or qualitative abnormalities • Values are affected by a variety of clinical conditions, and repeated testing may be required for diagnosis
Methodology	Immunoturbidometric/Immunoassay
Specimen requirements	Citrate
Indications	For patients with suspected vWD, family history of vWD, bleeding of unknown etiology Evaluation of patients with suspected acquired von Willebrand syndrome due to ECMO, VAD, Wilms tumor, and other settings
Reference range	50%–150%
Interpretation	• von Willebrand antigen/activity are affected by a variety of clinical conditions and may be falsely elevated in acute-phase reactions, traumatic venipuncture, menstruation, estrogen medications or oral contraceptives, stress, and pregnancy • Type 1: Quantitative deficiency of vWF (<30%), normal or mildly decreased factor VIII, normal multimers, normal activity:antigen ratio • Type 1C: Shortened half-life of vWF, level <30%, normal or mildly decreased factor VIII, normal multimers, normal activity:antigen ratio • Type 2A: Qualitative defect of vWF with low or low-normal vW antigen, low vW activity, normal or mildly decreased factor VIII, loss of high-molecular-weight multimers, low activity:antigen ratio • Type 2B: Qualitative defect of vWF with normal or low vW antigen, low vW activity, normal or mildly decreased factor VIII, loss of high-molecular-weight multimers, often with low activity:antigen ratio, thrombocytopenia, increased aggregation with low-dose ristocetin • Type 2M: Qualitative defect of vWF with normal or low vW antigen, low vW activity, normal or mildly decreased factor VIII, normal multimers, low activity:antigen ratio • Type 2N: Qualitative defect (decreased binding affinity for factor VIII) of vW with normal or low vWF antigen, normal or low vW activity, decreased factor VIII, normal multimers, normal activity:antigen ratio • Type 3: Quantitative defect of vWF with undetectable vW antigen/activity, low factor 8, and absent multimers • Acquired von Willebrand syndrome may or may not present with a low ratio, multimer analysis should be performed if there is clinical suspicion and shows loss of high-molecular-weight multimers • Additional testing may need to be performed for appropriate typing of vWD
Related tests	aPTT, factor VIII, Exon 28 sequencing, vW collagen binding assay, ristocetin-induced platelet aggregation
Interfering substances	Lipemic plasmas: may underestimate vWF Rheumatoid factor: may lead to an overestimation of vWF Anti-bovine and/or anti-rabbit antibodies: may lead to an overestimation of vWF
References	Nichols WL, Hultin MB, James AH, et al. von Willebrand disease (VWD): evidence-based diagnosis and management guidelines, the National Heart, Lung, and Blood Institute (NHLBI) Expert Panel report (USA). *Haemophilia*. 2008;14(2):171–232. doi:10.1111/j.1365-2516.2007.01643.x

aPTT, Activated partial thromboplastin time; *ECMO*, extracorporeal membrane oxygenation; *VAD*, ventricular assist device; *vWD*, von Willebrand disease; *vWF*, von Willebrand factor.

NATURAL ANTICOAGULANTS (ANTITHROMBIN, PROTEIN C, PROTEIN S, TISSUE FACTOR PATHWAY INHIBITOR)

Summary points	• Severe deficiency associated with severe thrombotic complications or lethality • Acquired deficiencies more common than inherited • Therapeutic repletion may be indicated in some deficiency states
Methodology	Clot-based (functional protein C and S), chromogenic (functional), or immunoassay (antigenic)
Specimen requirements	Citrate
Indications	Assessment of thrombotic risk, diagnosis of severe anticoagulant deficiency states, anticoagulant therapy monitoring (antithrombin status with heparin therapy), assessment of repletion need with concentrate (AT, PC)
Reference range	Tables 159.31 and 159.32
Interpretation	• Levels lower in early infancy and may be reduced in acute thrombosis, consumptive coagulopathy, liver disease • Estrogen, heparin, and asparaginase therapy, nephrotic syndrome associated with decreased AT • PC, PS vitamin K dependent—levels reduced with deficiency and vitamin K antagonist exposure • PS decreased in the setting of inflammation, coexisting activated PC resistance (factor V Leiden), pregnancy, estrogen therapy, sickle cell anemia, nephrotic syndrome • No definitive threshold for what levels constitute adequate levels protective against thrombosis, but levels below approximately 70% are often considered abnormal
Related tests	Thrombophilia evaluation, anticoagulation monitoring tests
Interfering substances	Hemolyzed sample or lipemia

AT, Antithrombin; *PC*, protein C; *PS*, protein S.

D-DIMER

Summary points	- Fibrin degradation byproduct of the fibrinolytic process after cross-linking by FXIII - Used as a biomarker in thrombosis, disseminated intravascular coagulation - Varied assay methods without clear standardization—reference values specific to method and population critical to interpretation
Methodology	Immunoassay
Specimen requirements	Citrate or EDTA
Indications	Assessment of possible venous thromboembolism, risk stratification for recurrent thrombosis, evaluation of disseminated intravascular coagulation
Reference range	Table 159.33
Interpretation	- Elevated with recent fibrin formation and subsequent fibrinolysis (assuming normal fibrinolytic function) - Elevated with inflammation, malignancy, surgery, recent trauma, bleeding, liver disease, pregnancy - Varied assay methods without clear standardization—reference values specific to method and population critical to interpretation
Related tests	Thrombophilia evaluation, anticoagulation monitoring tests
Interfering substances	Hemolyzed sample or lipemia
Reference	Adam SS, Key NS, Greenberg CS. D-dimer antigen: current concepts and future prospects. *Blood*. 2009;113:2878.

EDTA, Ethylenediaminetetraacetic acid; *FXIII*, factor XIII.

TABLE 159.33 D-Dimer Reference Values in Infants, Children, and Adults

	Day 1	Day 3	1 Month–1 Year	1–5 Years	6–10 Years	11–16 Years	Adults
D-dimer	1.47[a]	1.34[a]	0.22	0.25[a]	0.26[a]	0.27[a]	0.18
	0.41–2.47	0.58–2.74	0.11–0.42	0.09–0.53	0.1–0.56	0.16–0.39	0.05–0.42
	20	23	20	40	39	21	32

Data from Monagle P, Barnes C, Ignjatovic V, et al. Developmental haemostasis: impact for clinical haemostasis laboratories. *Thromb Haemost.* 2006;95:362. First two rows show mean and boundaries including 95% of the population. The next row shows the number of individual samples.

[a] Denotes values that are significantly different from adult values ($P < .05$).

ANTI-XA ASSAY

Summary points	• Anti-Xa assay provides a more direct method of measuring heparin effect and has less variability than the aPTT which can be affected by several interfering substances (ex. lupus anticoagulant, high factor VIII) • Assay depends on availability of antithrombin; assays that do not have an exogenous source of antithrombin may correlate better with in vivo heparin activity • May be used to exclude presence of apixaban or rivaroxaban prior to urgent surgical interventions, but not currently recommended for monitoring of these drugs
Methodology	Chromogenic
Specimen requirements	Citrate
Indications	• Used to monitor anticoagulant effect in patients receiving UFH or LMWH therapy • Can be used to assess for oral anti-Xa therapy with rivaroxaban or apixaban
Reference range	Range depends on clinical target and anticoagulant 0.35–0.7 U/mL for UFH 0.2–0.4 U/mL for prophylactic LMWH 0.5–1.0 U/mL for treatment LMWH
Interpretation	• Anti-Xa assays can be used to titrate UFH or LMWH therapy to clinical targets • When also monitoring UFH therapy with aPTT, discordant results are not uncommon • Discordant results between the aPTT and anti-Xa assays can be seen in the setting of increased factor VIII or decreased factor II • A supratherapeutic aPTT with a therapeutic anti-Xa level may be associated with increased risk for bleeding • Anti-Xa assays calibrated for LMWH can be used to assess for residual rivaroxaban or apixaban effects, particularly if urgent intervention is needed
Related tests	aPTT, thrombin time
Interfering substances	Hemolysis, high bilirubin, and release of PF4 from platelets if not appropriately processed all cause falsely decreased values
References	Guervil DJ, Rosenberg AF, Winterstein AG, Harris NS, Johns TE, Zumberg MS. Activated partial thromboplastin time versus antifactor Xa heparin assay in monitoring unfractionated heparin by continuous intravenous infusion. *Ann Pharmacother*. 2011;45(7–8):861–868. doi:10.1345/aph.1Q161 Takemoto CM, Streiff MB, Shermock KM, et al. Activated partial thromboplastin time and anti-xa measurements in heparin monitoring: biochemical basis for discordance. *Am J Clin Pathol*. 2013;139(4):450–456. doi:10.1309/AJCPS6OW6DYNOGNH Amiral J, Dunois C, Amiral C, Seghatchian J. Anti-Xa bioassays for the laboratory measurement of direct Factor Xa inhibitors in plasma, in selected patients. *Transfus Apher Sci*. 2016;55(2):249–261. doi:10.1016/j.transci.2016.09.003

aPTT, activated partial thromboplastin time; *LMWH*, low molecular weight heparin; *UFH*, unfractionated heparin.

PFA-100

Summary points	• Widely available method measuring time to platelet plug formation under high shear and in presence of platelet agonists (epinephrine and ADP) • Suboptimal sensitivity and specificity for platelet function disorders and von Willebrand disease
Methodology	Automated aspiration of whole blood
Specimen requirements	Citrate
Indications	Assessment of primary hemostasis
Reference range	Table 159.34
Interpretation	• Determination of site-specific reference ranges recommended—longer closure times have been reported • Prolonged closure times with more severe von Willebrand disease and platelet dysfunction but also with anemia, thrombocytopenia, and antiplatelet therapy • Specimen agitation and delayed time to assay may give spurious results
Related tests	Platelet aggregometry, von Willebrand disease testing
Interfering substances	Antiplatelet therapy and other drugs

ADP, Adenosine diphosphate.

TABLE 159.34 PFA-100 Closure Times in Healthy Neonates, Children, and Adults

	Collagen/Epinephrine (s)	Collagen/ADP (s)
Term neonates (*n* = 17)	81 (61–108)	56 (48–65)
Children (*n* = 57)	117 (83–163)	91 (72–111)
Adults (*n* = 31)	106 (82–142)	85 (67–111)

ADP, Adenosine diphosphate.
From Carcao MD, Blanchette VS, Dean JA, et al. The Platelet Function Analyzer (PFA-100): a novel in-vitro system for evaluation of primary haemostasis in children. *Br J Haematol.* 1998;101:70. Mean and ranges given as 5th–95th percentiles.

PLATELET AGGREGOMETRY

Summary points	• "Gold standard" for measuring platelet function • Poor interlaboratory standardization of sample collection methods, agonists, agonist concentrations, parameters of interest and other methodologic aspects can limit interpretability • Test availability often limited to specialized hemostasis laboratories due to significant technical and quality control requirements
Methodology	Turbidometric or impedance
Specimen requirements	Citrate
Indications	Assessment of platelet function
Reference range	Laboratory dependent; control specimen often required
Interpretation	• Common agonists include ADP, arachidonic acid, collagen, epinephrine, ristocetin, thrombin; pattern of aggregation and presence or absence of primary or secondary waves help to define certain rare, inherited but well-characterized platelet function disorders (e.g., Glanzmann thrombasthenia, Bernard-Soulier syndrome) • Other less specific abnormalities seen in acquired disorders: uremia, myeloproliferative neoplasms, myelodysplastic syndromes, dysproteinemia, liver disease, cardiopulmonary bypass, drug effect
Related tests	PFA-100, VerifyNow assay
Interfering substances	Antiplatelet therapy and other drugs

ADP, Adenosine diphosphate.